Role of the Kinases NEK6, NEK7 and NEK9 in the Regulation of the Centrosome Cycle

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Memoria presentada per Sara Sdelci
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Director: Joan Roig Amorós

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A Babbo, Mamma, Lilo e Giammy

Un amigo muy sabio un día que estaba bastante desesperada me dijo:

<<Tu no te preocupes, ya veras como ese tío te va a dar trabajo y de aquí a un año te estarás riendo de todas las movidas que has pasado; todo pasa y simplemente nos hace mas fuertes.>>

Pues, no se si fue por los muchos mojitos que llevaba encima o por cualquier otra razón, pero le creí y de hecho fue verdad.

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To grasp the full gravity of the situation it is necessary to bear in mind the following consideration. In times of crisis people are generally blind to everything outside their immediate necessities. For work which is directly productive of material wealth they will pay. But science, if it is to flourish, must have no practical end in view. As a general rule, the knowledge and the methods which it creates only subserve practical ends indirectly and, in many cases, not till after the lapse of several generations. Neglect of science leads to a subsequent dearth of intellectual workers able, in virtue of their independent outlook and judgment, to blaze new trails for industry or adapt themselves to new situations. Where scientific enquiry is stunted the intellectual life of the nation dries up, which means the withering of many possibilities of future development. This is what we have to prevent.

de “THE WORLD AS I SEE IT”
(Paragraph “The Plight of Science”)
Albert Einstein

“[…]Considerate la vostra semenza:
Fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza.”

de “DIVINA COMEDIA”
Inferno; Canto XXVII, vv 118-120
Dante Alighieri

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RESUM

Aquest projecte de tesi es centra en l'estudi del mòdul de senyalització format per les NIMA *related proteins*, Nek6, Nek7 i Nek9 i la seva funció durant les primeres fases de la mitosi, amb particular interès en la separació del centrosoma i la seva maduració.

Nek9 va ser identificat pel Dr. Joan Roig fa deu anys (Roig *et al*, 2002); aquesta proteïna és una quinasa de 120 kDa, formada per un domini quinasa en el seu extrem N-terminal, un domini homòleg de RCC1 que actua com auto-inhibidor i una cua per la qual la proteïna dimeritza (C-terminal). Nek9 s'expressa en totes les línies cel·lulars i teixits estudiats i la seva expressió és constant en tot el cicle cel·lular. Aquesta quinasa està inactiva durant la interfase, com a resultat de l'efecte inhibidor exercit pel seu domini RCC1.

L'activació d'aquesta proteïna ocorre durant la mitosi a través d'un mecanisme complex els detalls del qual no es coneixien bé fins fa poc. Aquest mecanisme implica la fosforilació per diferents quinases (Cdk1 i Plk1), la supressió de l'auto-inhibició i l'auto-fosforilació. El nostre grup ha descrit recentment com l'activació de Nek9 és dirigida per la fosforilació de Plk1; Plk1 és capaç d'unir-se a Nek9 durant la mitosi a través del domini PBD (*Polo-Box Domain*) i posteriorment fosforilar Nek9 en el domini d'activació (Thr210) (Bertran *et al*, 2011).

Durant la mitosi, Nek6 i Nek7 (proteïnes molt similars, compostes principalment pel domini quinasa i diferents en el seu petit domini N-terminal) són capaços d'unirse a la regió C-terminal de Nek9. Un cop activat, Nek9 pot fosforilar Nek6 i Nek7 directament i activar-les (Belham *et al*, 2003).

Quan Nek9 està activa localitza en el centrosoma, el que suggereix que el mòdul Nek9/Nek6/Nek7 té funcions importants en l'organització dels centrosomes i dels microtúbuls del fus mitòtic, durant la divisió cel·lular. Confirmant aquesta idea, s'ha demostrat que la microinjecció d'anticossos anti-Nek9 indueix la parada del cicle cel·lular a prometafase amb un fus mitòtic desorganitzat i cromosomes no alineats, conduint a una mitosi anormal que podria provocar aneuploïdia (Roig *et*

al, 2002). D'altra banda, l'inhibició de XNek9, l'homòleg de Nek9 en *Xenopus*, interfereix amb la formació del fus mitòtic (Roig *et al*, 2005). En el mateix sentit, l'inhibició de la funció de Nek7 o Nek6 condueix a una mitosi anormal deguda a una formació defectuosa del fus mitòtic (O'Regan & Fry, 2009)

SEPARACIÓ DEL CENTROSOMA

La simetria del fus és una característica intrínseca d' aquesta estructura i garanteix la separació correcta dels cromosomes duplicats en dos grups iguals, per ser distribuïts entre les dues cèl·lules filles. Un dels factors que asseguren la bipolaritat correcta del fus i per tant la seva simetria és la separació del centrosoma. La separació del centrosoma està impulsat per molts factors diferents que originen, com a mínim, dues formes de separació del centrosoma: la via de la profase i la via de la prometafase. Durant la profase (via de la profase) la separació del centrosoma està dirigida principalment per les forces generades per proteïnes motores (principalment Eg5 i Dynein). Per una altra banda, en la via de la prometafase la separació del centrosoma està impulsada per la interacció entre els dos asters centrosomals (que comencen a formar-se en la profase tardana) i també per la interacció dels microtúbuls del aster amb microtúbuls originats a partir dels cinetocors. Aquestes interaccions creen una xarxa de forces (forces de tracció i empenta) que finalment separen el centrosoma (Tanenbaum & Medema, 2010; O'Connell & Khodjakov, 2007) i provoquen la formació de un fus bipolar.
La via de la profase i de la prometafase generalment estan presents i cooperen per garantir la correcta separació del centrosoma i la bipolaritat del fus. No obstant això, s'ha demostrat una major importancia de la via de la profase respecte a la de la prometafase jaque el fracàs de la via de la profase condueix a una separació incorrecta dels cromosomes (Silkworth *et al*, 2012), evidenciant la importància de la separació del centrosoma durant la profase.
La separació del centrosoma durant la profase es controlada per l'activitat de Plk1 i Eg5, però el mecanisme pel qual això ocorre no es coneix encara del tot (Mardin *et al*, 2010; Smith *et al*, 2011). En particular, es sap que Polo esta relacionada amb la separació del

centrosoma des de 1991 en *Drosophila* (Llamazares *et al*, 1991) i des de 1996 en mamífers (Lane & Nigg, 1996): s'ha descrit que les cèl·lules amb Polo/Plk1 inhibit no poden separar el centrosoma amb la conseqüent formació del fus monopolar. A més, la separació del centrosoma durant la profase està dirigida principalment per l'activitat de proteïnes motores com Eg5 (Whitehead & Rattner, 1998; Tanenbaum *et al*, 2008; Woodcock *et al*, 2010). Eg5 és fosforilada per Cdk1 (Thr926) i aquesta fosforilació determina la seva associació amb els microtúbuls (Blangy *et al*, 1995).

Els detalls moleculars sobre les funcions que realitza el mòdul Nek9/Nek6/Nek7 durant la formació del fus es desconeixen, encara que el nostre grup va identificar la quinesina mitòtica Eg5 com a substrat d'aquest mòdul, com Nek6 fosforila Eg5 [S1033] (Rapley *et al*, 2008).

En aquesta tesi doctoral es descriu com el mòdul Nek9/Nek6/Nek7 podria proporcionar un vincle entre Plk1 i Eg5 en el context de la separació del centrosoma en profase (Bertran *et al*, 2011).

Per això, primer es van analitzar els efectes de la inhibició de Plk1, Eg5, Nek9, Nek6 Nek7 mitjançant siRNA en la separació dels centrosomes en les cèl·lules en profase, observant com aquesta inhibició afectava la separació del centrosoma durant la profase. A continuació es va tractar de determinar si l'activació de Nek9 o Nek6 podria induir la separació del centrosoma. Les cèl·lules es van transfectar amb la forma activa d'aquestes dues quinases; una quantitat considerable de cèl·lules que estaven en la interfase tenien el centrosoma separat, demostrant que Nek9 i Nek6 actives són suficients per induir-ne la separació. Per provar si Nek9 i Nek6 actives exerceixen el seu efecte a través de la regulació de Eg5, les cèl·lules simultàniament van ser transfectades també amb siRNA per Eg5, perdent del tot la separació del centrosoma, indicant que Nek9 i Nek6 actives no poden rescatar el fenotip provocat mitjançant la inhibició de Eg5.

La hipòtesi principal d'aquest treball és que el reclutament de Eg5 en el centrosoma és clau per a la seva correcta separació; la inhibició de les quinases Plk1, Nek6, Nek7 o Nek9 dóna lloc a cèl·lules en profase amb els centrosomes no separats, només perquè Eg5 no és reclutat

correctament als centrosomes. Tot això ha estat confirmat mitjançant immunofluorescències; també hem demostrat com les formes actives de Nek9 o Nek6 son capaces de rescatar la localització Eg5 als centrosomes, permetent-ne la separació.

Per provar si la fosforilació en la Ser1033 controla l'acumulació de Eg5 als centrosomes i la seva separació durant la profase, es van transfectar les cèl·lules amb siRNA contra EG5 per després introduir Eg5 WT o Eg5[S1033A]. Eg5 WT podia localitzar-se perfectament en el centrosoma i rescatar el fenotip normal; contràriament, Eg5[S1033A] no podia rescatar el fenotip normal, provocant un retard en la finalització de la mitosi. Així, hem establert que la fosforilació de Eg5 en el residu ser1033 és un pas clau per a l'organització del fus mitòtic i la correcta progressió de la mitosi.

MADURACIÓ DEL CENTROSOMA

La maduració del centrosoma garanteix el nombre correcte de microtúbuls que són necessàris per la formació del fus i assegurar les forces per separar les cromàtides germanes. La maduració del centrosoma requereix l'acumulació de proteïnes del PCM (*PeriCentriolar Material*), esdeveniment que comença a finals de la fase G2 i assoleix els seus nivells màxims en prometafase/metafase (Piehl *et al*, 2004). L'acumulació de les proteïnes del PCM determina un increment de cinc vegades en la mida del centrosoma (Piehl *et al*, 2004) i està controlada principalment per l'activitat de Plk1 i CDK1 (Zhang *et al*, 2009; Haren *et al*, 2009).

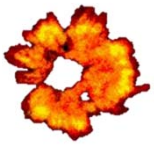
El γ-TuRC, un complex format per diverses proteïnes incloent γ-tubulin a, és el principal responsable de la nucleació de microtúbuls des del centrosoma (Job *et al*, 2003). La localització centrosomal del γ-TuRC està dirigida per Nedd1 (Haren *et al*, 2006; Luders *et al*, 2006), que també es part d'aquest complex, depenent d'alguna manera de Cdk1 i Plk1 (Zhang *et al*, 2009; Haren *et al*, 2009). Fins i tot si el reclutament de Nedd1, i en conseqüència el de γ-TuRC, al centrosoma depèn de la activitat de CDK1 i Plk1, malgrat no sigui per fosforilació directa, ens indica que hi ha alguns passos que falten per comprendre exhaustivament el procés.

Els nostres experiments mostren la importància de Nek9 en la regulació de la maduració del centrosoma dirigida per Plk1. La inhibició de Nek9 mitjançant siRNA, però no la de Nek6 o Nek7, determina una disminució en l'acumulació de γ-tubulin a i de Nedd1 en el centrosoma durant la prometafase; això ho hem demostrat amb la quantificació de la senyal de la flourescencia dels anticossos contra les proteïnes γ-tubulin a i Nedd1 en les imatges d'immunofluorescència. A més es va investigar el paper de Plk1 *dowstream* Nek9; inhibint Plk1 i transféctant les cèl·lules amb la forma activa de Nek9 es veia que aquestes cèl·lules eren capaces de rescatar el fenotip normal (centrosomes madurs) indicant que almenys alguns dels esdeveniments de maduració controlats per Plk1 són a través de Nek9.

Hem trobat també que Nek9 pot interactuar directament amb Nedd1 durant la mitosi i que aquesta interacció determina una fosforilació directa de Nedd1 per Nek9 (Ser377) i la seva acumulació en el centrosoma durant la mitosi. La importància d'aquest lloc de fosforilació queda clar mutant aquest residu a un que imita la forma fosforilada de la proteïna (S377D). A les cèl·lules on Nek9 es inhibit amb siRNA els centrosomes no maduren correctament; la transfecció de Nedd1[S377D] parcialment (però significativament) rescata el fenotip normal, indicant la importància d'aquest lloc per l'acumulació de Nedd1, i en conseqüència γ-tubulin a, en el centrosoma durant la mitosi. Hem provat també l'efecte del mutant no-fosforilable (S377A); substituint Nedd1 endogen amb Nedd1[S377A] trobem que no és capaç d'acumular-se en el centrosoma i que no suporta l'acumulació de la γ-tubulin a allà, determinant també un retard de les cèl·lules en superar la prometafase.

Els nostres resultats mostren que Nek9 sembla ser l'enllaç entre l'activitat de Plk1 i el reclutament de Nedd1 en el centrosoma i que la via formada per Plk1/Nek9/Nedd1 pot ser un element clau en el control de la maduració del centrosoma.

INTRODUCTION



CELL CYCLE

The cell cycle is the life cycle of a dividing cell. In eukaryotic cells it can be divided into interphase (that includes the G1, S and G2 phases), mitosis (M phase) and cytokinesis.

During interphase (the longest period of the cell cycle), the cell is going to grow (during G1 or gap1 and G2 or gap2 phases) and duplicate its DNA and centrosome (S, or synthesis, phase) (Salaün *et al*, 2008; Johnson & Walker, 1999).

Mitosis is the process by which cell separates chromosomes in its nucleus into two identical sets in two nuclei. Mitosis is followed immediately by cytokinesis, which divides physically the mother cell in two daughters cells (Balasubramanian *et al*, 2000).

MITOSIS

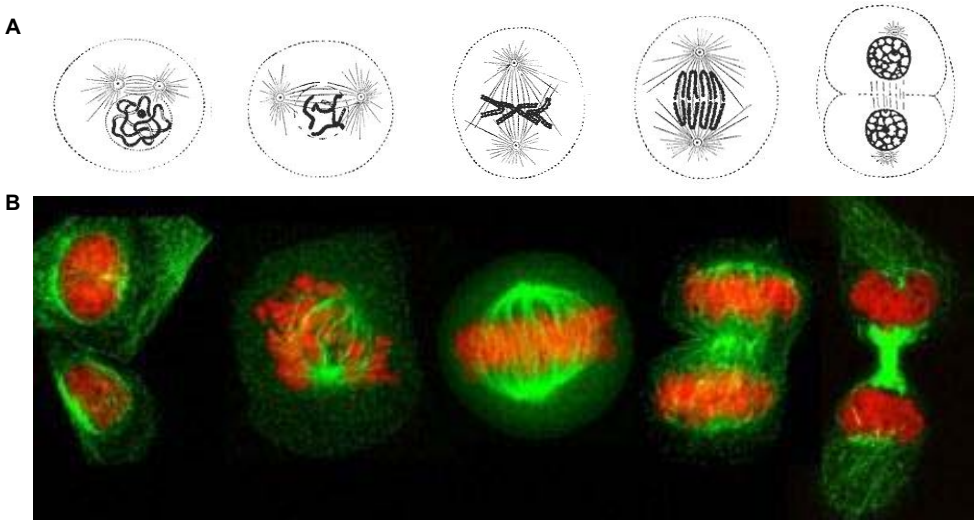


Figure I. (A) Representations of mitotic phases From left to right: prophase, prometaphase, metaphase, anaphase and telophase. Adapted from "*Anatomy of the Human Body"*. **(B) Immunofluorescence of mitotic cells** Cells immunostained for tubulin (green) and nuclei (red). From left to right prophase (upper cell), prometaphase, metaphase, anaphase (late) and telophase. Image taken from http://exploreable.wordpress.com

Fully described by Walter Flemming in 1882, mitosis is the most spectacular and sophisticated event of the cell cycle (Fig. I).

Mitosis can be divided in five different phases called prophase, prometaphase, metaphase, anaphase and telophase (Robbins *et al*, 1964).

During prophase, changes occur both in the cytoplasm and the nucleus of the cell. This is the first phase of mitosis in which chromatin starts to condense into chromosomes, the nucleoli disappear and the mitotic spindle is starting to form in the cytoplasm around the centrosomes.

The second mitotic phase is prometaphase; the nuclear envelop (NE) fragments (NEB = Nuclear Envelope Breakdown) and chromosomes, now well condensed, start their movements to organize themselves into the metaphase plate. During this phase kinetochores complete their assembly at the centromeric region of chromosomes and, in late prometaphase, the nucleation of new microtubules to intensify the spindle starts from mature kinetochores.

Metaphase is the discrete time in which chromosomes are perfectly aligned at the metaphase plate. Centrosomes localize perpendicularly respect to the metaphase plate and specularly between each other. For each chromosome, the kinetochores of the sister chromatids are anchored to microtubules coming from the two different centrosomes.

In anaphase sister chromatids separate and move toward the opposite poles in two equivalent groups, anchored and stretched by means of microtubules.

Finally, during telophase two daughter nuclei are re-formed and the NE re-appears, as well as the nucleoli; chromosomes decondense and the cell incurs in cytokinesis.

REGULATION OF MITOSIS

Mitosis is an extremely complex and finely regulated process; it comprises many steps that in most of the cells must be ended in about one hour. Regulation of mitotic progression is mainly orchestrated by irreversible ubiquitinilation-dependent protein degradation through the anaphase-promoting complex/cyclosome (APC/C) (Acquaviva & Pines, 2006) and reversible protein phosphorylation/dephosphorylation catalyzed by specialized protein kinases and phosphatases (Nigg, 2001; Torres-rosell, 2005; Jensen & Johnston, 2002; Rieder, 2011; Doree & Galas, 1994).

MITOTIC KINASES

In mammalians, principally four different protein kinase families are involved in the regulation of the mitotic process: they are the Cyclin-dependent kinase

family (Cdks), the Polo-like kinase family (Plks), the Aurora kinase family and the NIMA-related kinase family (Neks).

CDK FAMILY

The Cyclin-dependent kinase family is a large family of proteins that contains several Cdks able to regulate different processes of the cell cycle (Table 1) and the activation of these kinases depends on the association with a cyclin or a cyclin-like regulatory subunit (Johnson & Walker, 1999). The alteration of the activity of some members of this family leads to uncontrolled proliferation, as well as genomic and chromosomal instability (Malumbres & Barbacid, 2009).

Symbol	Other Symbols†	Cyclin-binding element	Cyclin and Cyclin-like partners	Human locus	Hs.aa	Hs.Ensembl	Hs. Acc..	Mm.Acc.	Cellular process	Mutations
CDK family										
CDK1	Cdc2	PSTAIRE	A1, A2, B1, B2, (B3), D- E-	10q21.2	297	ENSG00000170312	P06493	P11440	Cell cycle: mitosis (S-phase)	N.D.
CDK2		PSTAIRE	A1, A2, B1, B3, D, E1, E2, Cables1, SpdYA, SpdYC	12q13.2	298	ENSG00000123374	P24941	P97377	Cell cycle: S, G2, meiosis	P45L glioblastoma multiforme
CDK3		PSTAIRE	A1, A2, E1, E2, C, Cables1	17q25.1	305	ENSG00000108504	Q00526	Q80YP0	Cell cycle: G1/S unclear	S106N glioma
CDK4	PSK-J3	PISTVRE	D1, D2, D3	12q14.1	303	ENSG00000135446	P11802	P30285	Cell cycle: G1	R24C, R24H, N41S melanoma
CDK5	TPKII	PSSALRE	p35, p39 (D-, E and G-type cyclins), Cables1	7q36.1	292	ENSG00000164885	Q00535	P49615	Neuron biology; Cell Cycle?	N.D.
CDK6	PLSTIRE	PLSTIRE	D1, D2, D3	7q21.2	326	ENSG00000105810	Q00534	Q64261	Cell Cycle: G1	P199L melanoma
CDK7	CAK, MO15, STK1	NRTALRE	H	5q13.2	346	ENSG00000134058	P50613	Q03147	CDK activating kinase; transcription	N.D.
CDK8	K35	SMSACRE	C, (K)	13q12.13	464	ENSG00000132964	P49336	Q8R3L8	Transcription	R424C intestine tumor; D189N lung tumor
CDK9	PITALRE, CDC2L4, C-2K	PITALRE	K, T1, T2	9q34.11	372	ENSG00000136807	P50750	Q99J95	Transcription	N.D.
CDK10	PISSLRE	PISSLRE		16q24.3	360	ENSG00000185324	Q15131	Q3UMM4	Transcription, Cell Cycle: G2/M	N.D.
CDK11A	CDC2L2, CDC2L3, PITSLREB, p58GTA, CDK11	PITSLRE	D3 (p58 isoform); L1, L2 (p110 isoform)	1p36.33	780 (p110 isoform)	ENSG00000008128	Q9UQ88	P24788	Transcription, splicing (p119 isoform); Cell cycle: centriole biology and cytokinesis (p58 isoform)	N.D.

Table 1 Cdk family members.
adapted from (Malumbres *et al*, 2010)

CDK1

Cdk1 is considered “the Master of Mitosis” for its role in cell cycle transition. In yeast, the activity of Cdk1 is necessary for both, G1/S and G2/M transitions (Fisher & Nurse, 1996) while in mammalian it is “only” responsible for the G2/M progression (Draetta & Beach, 1988).

Cdk1 associates with both, Cyclin A that, at the end of interphase, facilitates the onset of mitosis in the nucleus. and Cyclin B. The Cdk1-Cyclin B complex appears slightly latter that Cdk1-Cyclin A (that is degraded at NEB), and is

responsible for driving cells through mitosis (Malumbres & Barbacid, 2005). Once the complex Cdk1-Cyclin B is formed it is immediately inactivated by Wee1 kinase; this inhibitory phosphorylation is antagonized by the Cdc25 phosphatase which activity promotes the activation of the Cdk1-Cyclin B complex and allows entry into mitosis (Lindqvist *et al*, 2009). The Cdk1-Cyclin B complex phosphorylates many substrates, thus orchestrating mitosis entry and progression. Indeed, Cdk1-Cyclin B activity is required for NEB; nuclear envelop destabilization is in fact mediated by Cdk1-dependent lamina phosphorylation (Peter *et al*, 1990). Among other processes, Cdk1 is also involved in centrosome separation and spindle formation through Eg5 phosphorylation ((Blangy *et al*, 1995), see Eg5 paragraph below) as well chromosome condensation (Kimura, 1998). Finally, after metaphase, Cdk1 promotes the APC/C activation, thus assuring the ubiquitination of substrates that must be degraded during the metaphase/anaphase transition (Rudner & Murray, 2000), Cyclin B included.

PLK FAMILY

The Polo-Like Kinase family comprises five different proteins (Plk1, Plk2, Plk3, Plk4 and Plk5). Different studies revealed that this kinase family is well

	YEAST		PLANTS	INVERTEBRATES			VERTEBRATES		
	S. pombe	*S. cerevisae*	*A. thaliana*	*C. elegans*	*Drosophila*	*S. purpuratus*	*D. rerio*	*Xenopus*	*H. sapiens*
PLK1	plo1	Cdc5	-	plk-1 plk-2 plk-3	polo	PLK1	plk1	Plx1	PLK1
PLK2	-	-	-	-	-	PLK2	plk2b plk3	Plx2 Plx3 Plx5	PLK2 PLK3 PLK5
SAK	-	-	-	zyg -1	sak	SAK	plk4	Plx4	PLK4

Subfamilies

Table 2 Plk Families across Evolution.
Adapted from (de Cárcer *et al*, 2011b)

conserved through evolution and various homologues of its members have been found in different species, from budding yeast (Cdc5) to *Drosophila* (Polo), *Xenopus* (Plxs) and mammals (Plks) (Table 2). They all share a small conserved domain called polo-box (polo-box domain, PBD) required for the localization of the protein throughout cell cycle (Lee *et al*, 1998; de Cárcer *et*

al, 2011b) and which constitutes part of an auto-regulatory domain (Nigg, 1998).

Plk2, **Plk3** and **Plk4** are predominantly active during interphase, regulating the G1/S transition (Plk2 and Plk3) (Warnke *et al*, 2004; Zimmerman & Erikson, 2007), centriole duplication (Plk2 and Plk4) (Warnke *et al*, 2004; Bettencourt-Dias *et al*, 2005; Habedanck *et al*, 2005), the DNA-Damage response (Plk3) (Sang *et al*, 2009) and apoptosis (Jiang *et al*, 2006; Sang *et al*, 2009) (Fig. II). Moreover, some studies indicate the possible involvement of some of these kinases in the regulation of mitotic progression (Plk2) (Cizmecioglu *et al*, 2008) and cytokinesis (Plk3) (Jiang *et al*, 2006).

NAME	PROTEIN STRUCTURE	LOCALIZATION	KINASE	PB	PBind	FUNCTION
PLK1	1 — 603 (K82, T210, W414, H538/K540)	Centrosomes Kinetochores Midbody	Yes	2	No	Centrosome, mitosis, cytokinesis
PLK2	1 — 686 (K111, T239, W503, H629/K631)	Centrosomes	Yes	2	Yes	Centrosome, Genotoxic stress, Neuron differentiation
PLK3	1 — 646 (K91, T219, W463, H590/K592)	Nucleus Nucleolus	Yes	2	Yes	DNA replication, genotoxic stress
PLK5	1 — 336 (W207)	Cytoplasm	No	2	Yes(?)	Neuron differentiation
PLK4	1 — 970 (K41, T169)	Centrosomes	Yes	1	No	Centriole biogenesis

Kinase Domain PBind PBD

Table 3 Plks: Structure, Localization and Function.
adapted from (de Cárcer *et al*, 2011b)

Plk5 is the member of the family more recently described and is only expressed in a few non-proliferative tissues like the central nervous system (mainly in the cortical brain neurons and glia cells) and in the granular layer of the cerebellum. Overexpression of this protein results in a G0/G1 arrest and, moreover, the kinase activity is not needed to determine this effect (De Cárcer *et al*, 2011b; de Cárcer *et al*, 2011a) (Fig. II).

PLK1

The founding member of the family, Plk1, is one of the key regulators of cell division.

Plk1 is the functional homolog of *Drosophila* Polo kinase (Nigg, 1998); its structure comprises an N-terminal Ser/Thr kinase domain and a C-terminal

regulatory domain with two polo box domains (PBD) necessary for the specific recognition and binding to the phosphorylated motive S[S/T]P on Plk1 targets (Elia *et al*, 2003a; Park *et al*, 2010) (Table 3). Indeed, the PBD is able to recognize and bind phosphorylated peptides previously primed by another kinase, which generally is Cdk1.

Plk1 protein levels begin to increase during S phase, reaching a maximum during the G2/M transition and decreasing after mitosis (Golsteyn *et al*, 1995). Plk1 is activated through phosphorylation at the G2/M boundary.

The activation of Plk1 is accomplished by phosphorylation of a specific residue in its activation loop, the Thr210. Bora, co-activator of Aurora A, accumulates in G2 and interacts with Plk1 controlling the availability of its activation loop for the phosphorylation at Thr210 by Aurora A, with consequently Plk1 activation (Seki *et al*, 2008; Macůrek *et al*, 2008). In mitosis the activity of Plk1 is enhanced by phosphorylation at Ser137 as well as by substrate binding (Park *et al*, 2010; Seki *et al*, 2008; Macůrek *et al*, 2008; Jang *et al*, 2002).

Plk1 is one of the key regulators of mitotic progression (Fig. II), and its inhibition provokes a delay in the entry into mitosis and prometaphase arrest. Plk1 inhibited cells show in fact multiples problems to nucleate astral microtubules in prophase and polymerization of these microtubules can only initiate after NEB; cells form monopolar spindles incapable to attach kinetochores (Lénárt *et al*, 2007; Petronczki *et al*, 2008). Plk1 activity is required for multiples steps during mitosis, including activation of Cdk1-Cyclin B complexes, centrosome disjunction (Mardin *et al*, 2011), centrosome maturation (Haren *et al*, 2009), centrosome separation (Smith *et al*, 2011), the regulation of microtubule dynamics, cohesin release/cleavage during sister chromatid separation (Sumara *et al*, 2004; Arnaud *et al*, 1998), APC activation (Descombes & Nigg, 1998; Nigg, 1998; Glover *et al*, 1998) and cytokinesis (Descombes & Nigg, 1998; Nigg, 1998; Glover *et al*, 1998; Seong *et al*, 2002; Petronczki *et al*, 2007).

Plk1 associates with different subcellular structures and this allows it to properly accomplish all its functions. Specifically, Plk1 associates with

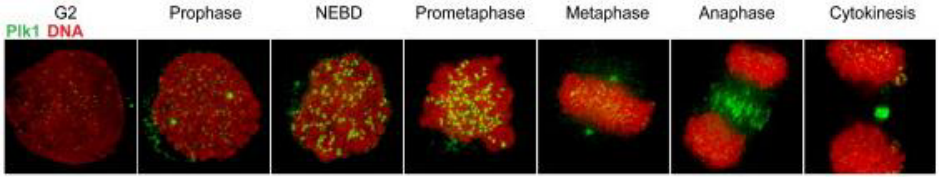


Figure III Plk1 subcellular localization during mitosis
adapted from (Petronczki *et al*, 2008)

centrosomes from prophase up to metaphase, with kinetochores from prometaphase to anaphase, with central spindle during anaphase and with midbody from telophase to cytokinesis (Fig. III). This accurate relocalization explains how this kinase can interact with a large number of substrates localized in several structures throughout mitosis and is, at least in part, determined by the PBD, as this domain determines the interaction between Plk1 and substrates only when these are phosphorylated at specific sites (priming) (Elia *et al*, 2003b).

AURORA FAMILY

The Aurora kinase Family was first described in *S.cerevisiae* and *Drosophila* (Chan & Botstein, 1993; Glover *et al*, 1995). In mammalian cells, three member of this family were identified, called Aurora A, Aurora B and Aurora C. These three kinases comprise a conserved catalytic domain (C-terminal region) and a non-catalytic domain (N-terminal region) different in size and sequence. Evolutionarily, A and B type Auroras derive from a common ancestor, while C type evolved from the B type (Brown *et al*, 2004) and this reason could explain why Aurora A has distinct functions while Aurora B and Aurora C share similar functions; nevertheless, all three kinases are involved in the control of processes required for mitotic progression.

Aurora A is activated by binding to different co-activator/substrates (as for instance Bora), mechanism that guarantees a localized activation of this kinase (Tsai *et al*, 2003). Active Aurora A localizes to the centrosome, mainly around the PCM (PeriCentriolar Material) (Roghi *et al*, 1998), and spindle

(localization that is TPX2-mediated) (Bayliss *et al*, 2003; Sardon *et al*, 2008) and is required for G2/M transition.

One of the substrates of Aurora A is Plk1. Indeed, when Aurora A forms a complex with its substrate and co-activator Bora is able to phosphorylate Plk1 at Thr210, determining the activation of this kinase just before mitotic entry (late G2 phase) (Seki *et al*, 2008; Macůrek *et al*, 2008).

Aurora A also phosphorylates Cdc25B and this phosphorylation cooperates in the regulation of the G2/M transition; however it is not strictly necessary for entry into mitosis (Dutertre *et al*, 2004).

Moreover, Aurora A is clearly implicated in the control of centrosome separation (Glover et al. 1995; Barr and Gergely 2007) and maturation (Barr and Gergely 2007), astral microtubule nucleation (Giet et al. 2002) and spindle microtubules stabilization (Sardon et al. 2008), functions that have been described in several animal cells (*C.elegans, Drosophila, Xenopus* and Human).

Aurora B localizes at different sites throughout mitosis. Specifically, during prophase the kinase accumulates at chromosomes, in prometaphase and metaphase at kinetochores, during anaphase at the central spindle and finally in telophase at the midbody (Adams *et al*, 2001a). The correct localization of Aurora B is mediated by the formation of a "chromosome passenger" complex (CPC) which comprises INCENP (inner centromere protein), survivin and borealin/DASRA (Honda *et al*, 2003; Adams *et al*, 2000, 2001b). Studies in mammalian cells have shown that the interaction between Aurora B and kinetochores is highly dynamic and in fact the centromeric pool of Aurora B is constantly exchanging with the cytoplasmic pool (Murata-hori *et al*, 2002). The recruitment of Aurora B to kinetochores is mediated by Aurora A through phosphorylation of CENP-A (kinetochore-specific histone-H3 variant centromere protein A) at Ser7 and consequent Aurora B recruitment at the inner plate of the kinetochore (Kunitoku *et al*, 2003).

Aurora B is involved in the regulation of chromosome biorentation; downregulation of the expression of this kinase using siRNAs impairs chromosomes alignment at the metaphase plate (Adams *et al*, 2001b) and similar results are obtained inhibiting the kinase with microinjection of specific antibodies (Kallio *et al*, 2002). Aurora B inhibition provokes an increase of

syntelic attachments of sister chromatids to spindle poles (Hauf *et al*, 2003); Aurora B activity in fact promotes microtubule release from the kinetochores This kinase is required to phosphorylate different proteins, such as MCAK (Mitotic Centromere-Associated Kinesin), involved in the spindle assembly checkpoint (SAC), by correcting the non-amphitelic attachments of microtubules to the kinetochores (Ohi *et al*, 2004). Furthermore, Aurora B is implicated in the regulation of chromosome condensation by phosphorylating histone H3 at Ser10 (modification conserved from yeast to mammalian) (Giet & Glover, 2001; Hsu *et al*, 2000; Hirota *et al*, 2005) and determining the recruitment of condensin to chromosomes (Giet & Glover, 2001). In late mitosis Aurora B is also required for the proper release of the cohesion between sister chromatids (metaphase/anaphase transition) (Rogers *et al*, 2002). Finally, overexpression of a catalytically inactive Aurora B in various cell types prevents cytokinesis indicating that Aurora B has a role also controlling this process (Terada *et al*, 1998). Indeed, during cytokinesis, active Aurora B is implicated in the formation of the microtubule midzone and cytokinesis completion (Carmena *et al*, 2009); however a more recent study has demonstrated that the kinase activity is not required during the formation of the cleavage furrow (Smurnyy *et al*, 2011).

Aurora C is normally expressed only in testis (Hu *et al*, 2000) and has been first described as an anaphase centrosome protein (Kimura *et al*, 1999). However, when overexpressed, Aurora C behaves like Aurora B localizing to kinetochores during mitosis, interacting with chromosome passenger proteins (such as survivin and INCENP) and regulating processes like chromosome condensation and cytokinesis (Sasai *et al*, 2004). Additionally, overexpressed Aurora C not only mimics Aurora B but can also rescue its depletion (Sasai *et al*, 2004).

THE NIMA FAMILY

NIMA (Never In Mitosis, gene A) was first described in the 70s by Ronald Morris after a genetic screen for cell cycle mutants in the multicellular filamentous *fungus Aspergillus nidulans* (Morris, 1975).

Morris screened several temperature sensitive cell cycle mutants and classified them as *bim* mutants, for those blocked in mitosis with condensed chromosomes and formed mitotic spindles, or *nim* mutants, for those that were never in mitosis due to an interphase arrest. The *nim* mutant group included a gene that was called *nim*A. Mutants of *nim*A provoked an arrest in late G2 with replicated spindle pole bodies (the functional equivalent of centrosomes in *fungi*). Cloning of *nim*A showed that this gene encodes a Ser/Thr protein kinase which received the name of NIMA (Osmani *et al*, 1987); the structure of this protein comprises an N-terminal catalytic domain (conserved throughout evolution) and a C-terminal regulatory domain (Fry & Nigg, 1995; O'Connell *et al*, 2003).

This kinase was described to perform several function during mitosis in different models. NIMA has a fundamental role in the G2/M transition, mitotic progression and mitotic exit. In particular this kinase regulates Cdc2/Cyclin B entry into the nucleus, NE organization, chromosome condensation, bipolar spindle formation and finally cytokinesis (O'regan *et al*, 2007; O'Connell *et al*, 2003). The activity of this kinase is finely regulated by different mechanisms like phosphorylation, stabilization through oligomerization and subcellular localization (Fry & Nigg, 1995; Osmani & Ye, 1996; O'Connell *et al*, 2003), suggesting the importance of its activity during normal mitotic progression.

NIMA homologues are expressed in all eukaryotes but, contrary to NIMA in *Aspergillus nidulans*, they are not essential for cell cycle progression (Osmani & Ye, 1996). The complexity of the family increases going up in the scale of evolution. Regarding that, yeast only has one NIMA related protein called Fin while ciliated organisms have generally more than one, possibly to regulate the behavior of microtubule structures such as the centrosome/basal body; the number of homologues seems in fact to be proportional to the complexity of the cellular ciliary structure (Parker *et al*, 2007; Quarmby & Mahjoub, 2005).

NEKS (NIMA-RELATED KINASES)

In mammals eleven different NIMA related kinases (Neks) have been described (Nek1 to 11, plus the Nek2 A, B and C isoforms and the Nek11 L and S isoforms) (O'Connell *et al*, 2003). They share 40-45% of homology with NIMA (and 40-85% between each other) in the catalytic domain, generally

localized in the N-terminal domain (except for Nek10, see Fig. IV) while differing from NIMA and other NIMA related proteins in the C-terminal domain, suggesting that each kinase might have a distinct function (O'Connell *et al*, 2003) (Fig. IV).

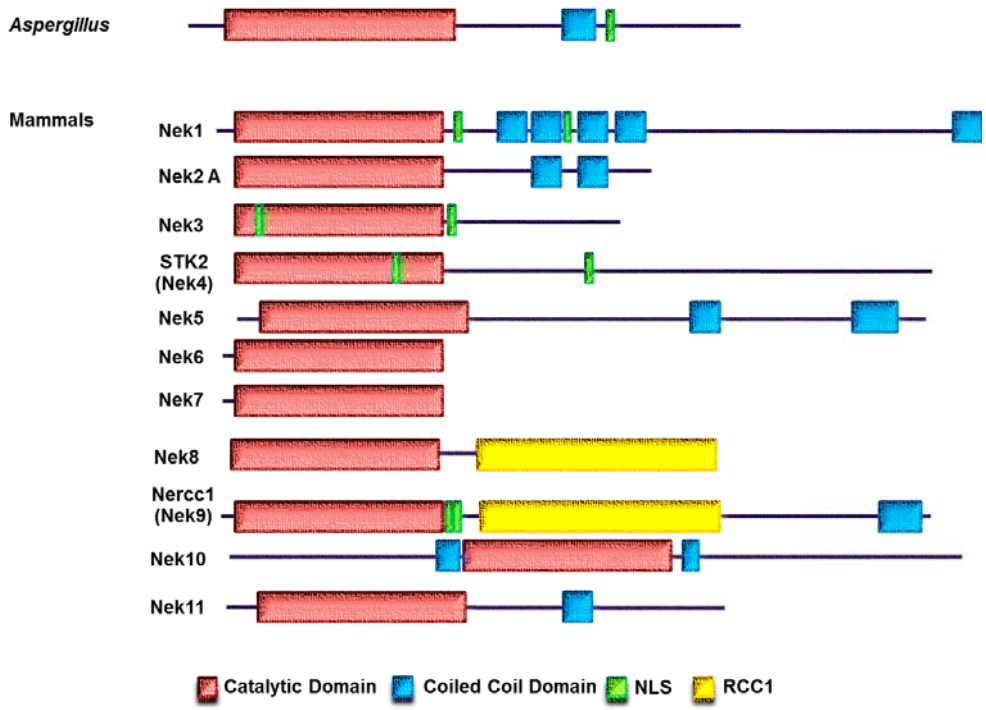


Figure IV NIMA (*Aspergillus*) and mammalian Neks structures.
Adapted from (O'Connell *et al*, 2003)

The first mammalian family members described (in the early 90s) were Nek1, Nek2 and Nek3 (Nigg, 1993; Letwinl *et al*, 1992) but the sequencing of the human (and mouse) genome revealed the presence of 11 genes encoding for Neks in mammalians (Forrest *et al*, 2003). At least, four of the eleven mammalian Neks have been described to be involved in the regulation of mitosis; they are Nek2, Nek6, Nek7 and Nek9. The remained seven kinases are involved in different process predominantly related with the physiology of cilia structure.

Nek1 regulates the formation of the primary cilium and centrosome stability (Shalom *et al*, 2008; Lanza *et al*, 2010); possibly as a result, Nek1 is important for neural development and mutations of this kinase result in polycystic kidney disease (PKD) (Surpili *et al*, 2003; Lanza *et al*, 2010).

Furthermore, Nek1 is implicated in the regulation of DNA-Damage response; double-strand breaks induce expression and activation of the kinase and promote its localization at the sites of the break, where Nek1 takes part in DNA repair (Polci *et al*, 2004; Chen *et al*, 2009; Pelegrini *et al*, 2010).

Nek3 is implicated in the regulation of cell polarity and morphology, having a role in the reorganization of cytoskeletal α-tubulin (Tanaka & Nigg, 1999). Recently Nek3 has been related to the reorganization of the neural cytoskeleton by altering the levels of acetylated tubulin, linking this kinase to neural disorder (Chang *et al*, 2009).
Moreover, a correlation between Nek3 overexpression and cancer has been found. In a yeast-two hybrid analysis Nek3 was described directly interacting with the vav protein family (guanine exchange factors) in response to prolactin receptor signaling. In tumor cells, prolactin signaling is involved in cytoskeletal reorganization during proliferation, migration, and invasion processes; Nek3 overexpression results in an increase ability of the cells to migrate and invade, as well as in an higher proliferation rate (Miller *et al*, 2005, 2007).

Nek4, as well as Nek3, controls cellular morphology and polarity. Nek4 downegulation promotes that neoplastic cells develop resistance to microtubules drugs used in cancer treatment (Doles & Hemann, 2010).
Nek4 has also been related to ciliary dysfunction as a component of both, the RPGRIP1 (ciliopathy-associated protein homologs, RPGR interacting protein 1) and RPGRIP1L (RPGRIP1-like) associated protein complex. RPGRIP1 and RPGRIP1L can act as cilium specific scaffolds to recruit Nek4 to the complex, where the kinase contributes to the stability of the primary cilium. Downregulation of Nek4 in ciliated cells determines a significant decrease in cilium assembly (Coene *et al*, 2011).

Nek5 has been described as a nuclear protein during interphase, a cilium associated protein in G0-non cycling cells and as a centrosomal protein during mitosis.
This kinase seems to be implicated in the control of centrosome splitting and maturation. The downregulation of the expression of Nek5 might cause premature centrosome splitting, impaired γ-tubulin accumulation to the

centrosome during mitosis and delay in early mitotic progression (Sahota, 2010-Published Doctoral Thesis), indicating that possibly Nek5 is a mitotic Nek.

Nek8 is a cilia related protein described to be implicated, as Nek1, in PKD. This kinase localizes at the base of the cilium and is required for maintaining cilia functionality (Quarmby & Mahjoub, 2005; Zalli *et al*, 2012; Shiba *et al*, 2010). Dowregulation of Nek8 promotes cytoskeletal disorganization with a diminished number of actin fibers (Liu, 2002), probably as a result of a direct influence on actin expression (Bowers & Boylan, 2004).
Nek8 is implicated, at least, in two human cystic diseases, the autosomal dominant polycystic kidney desease (ADPKD) and nephronophthesis (NPHP) (Cai & Somlo, 2008; Otto *et al*, 2008). Indeed Nek8 results mutated and misslocalized in jck mouse model (juvenile cystic disease), as it is found along the entire length of the cilia instead only at the basis. This abnormal Nek8 localization seems to be directly related with PC1 and PC2 misslocalization (polocystin1 and polycistin2), two membrane proteins that cooperate in the formation of calcium channels in the plasma membrane, ER and cilia (Nauli *et al*, 2003) and that resulted mutated in almost all APKD cases, suggesting that Nek8 interacts with the signal transduction pathway of these two proteins (Sohara *et al*, 2008). Recently the localization of Nek8 at the base of primary cilia has been confirmed and better characterized. Nek8 in fact has been described as localized at the inv compartment sited at the base of primary cilia where inv, protein which names the compartement, functions as anchor for Nek8 assuring its correct localization (Shiba *et al*, 2010).

Nek10 is involved in the G2/M arrest DNA-damage mediated. After UV irradiation, Nek10 promotes an increase in the activation of the MAPKs Erk1 and Erk2, and of its upstream kinase Mek1 (MAPKK), which are required for G2/M arrest. According to that, inhibition of Nek10 results in an impaired Mek/Erk activation after UV irradiation (but not after mitogenic stimulation) (Moniz & Stambolic, 2011).

Nek11 is as well implicated in the DNA-damage response and its role in this process is better characterized. Nek11 is in fact activated by Chk1 (checkpoint

kinase 1) during the DNA-damage response. Once active, Nek11 is able to phosphorylate Cdc25A determining its ubiquitin-mediated degradation, thus inducing G2/M arrest (Melixetian *et al*, 2009).

At least two distinct isoforms of this kinase exist, Nek11L (long isoform) and Nek11S (short isoform), even though the S isoform is rarely detected in somatic cells (Noguchi *et al*, 2002). The expression and the localization of the L isoform are cell cycle dependent; protein levels are low during G1 phase and increase from S to M (Noguchi *et al*, 2002). During interphase the kinase is nuclear, precisely nucleolar. In the nucleolus Nek11 (in particular the L isoform) can interact with Nek2A; Nek2A phosphorylates and activates Nek11 in G1/S arrested cells, suggesting a novel possible nucleolar role for the Nek family (Noguchi *et al*, 2004). After NEB Nek11 localizes to polar microtubules and finally colocalizes with the DNA from anaphase until the end of mitosis (Noguchi *et al*, 2004).

MITOTIC NEKS

NEK2

Among all Neks, Nek2 is the most closely related kinase to *Aspergillus nidulans* NIMA; they share about 44% sequence identity in the catalytic domain and for this reason Nek2 has been the most studied Nek until now. Vertebrate Nek2 proteins have been described in several species from *Xenopus laevis* to pig, mouse and human. Proteins homologous to Nek2 have also been described in *Drosophila*, *Dictyostelium discoideum* (Gräf, 2002) and Budding Yeast (Grallert & Hagan, 2002; Schweitzer & Philippsen, 1992).

In Humans (as in *Xenopus Leavis* and other vertebrates) Nek2 exists predominantly in two different splice variants. The longer one is called Nek2A and its mRNA comprises 8 exons. The smaller one, Nek2B, results from alternative splicing at the end of the intron 7 that originates an mRNA which comprises exons 1 to 7. The two variants have a molecular weight respectively of 48 and 44 kDa and are expressed in all adult human cell lines studied, although the protein levels of Nek2A are higher than Nek2B (Fry *et al*, 1998a). Studies in *Xenopus leavis* show that Nek2B is expressed also in oocytes, eggs and preneurular embryos while Nek2A protein levels can detected only after gastrula-neurula transition (Uto *et al*, 1999).

Both of Nek2 splice variants have a catalytic domain in their N-terminal region and a C-terminal domain that acts as a regulatory domain. The C-terminal domain of Nek2 comprises two coiled-coil regions; the one positioned nearest to the N-terminal domain has a leucin zipper (LZ) motif (six heptad-spaced leucin residues) through which Nek2 can homodimerize; the homodimeriazation promotes trans-phosphorylation and kinase activation (Fry *et al*, 1999) (Fig. V).

More recently a third isoform of the protein, Nek2C, has been described; this kinase results from an eight aminoacids depletion respect to the isoform A, in the proximity of the alternative splicing site (Wu *et al*, 2007) (Fig. V).

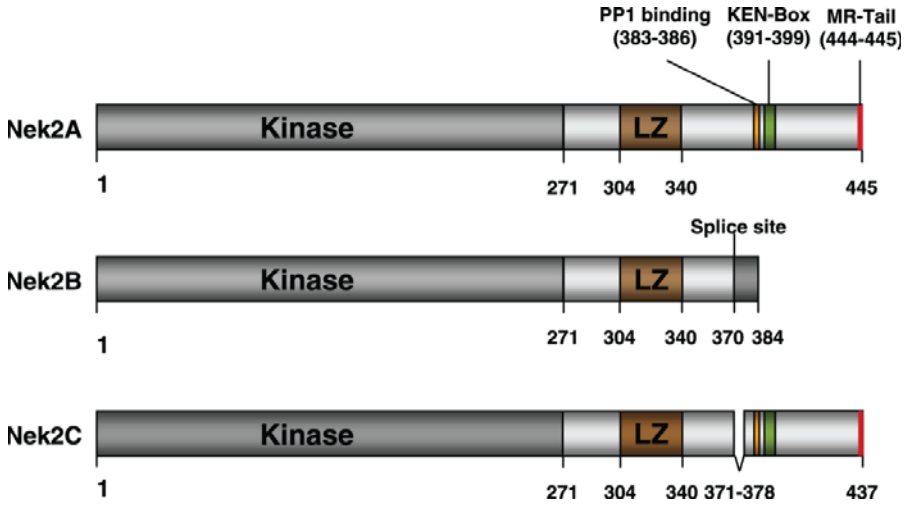


Figure V Nek2A, Nek2B and Nek2C structures. Schematic representation of the Nek2A, Nek2B, and Nek2C proteins highlighting the positions of the catalytic domain (Kinase), leucine zipper motif (LZ), PP1 binding site, KEN-box, and MR-tail.
Adapted from (Wu *et al*, 2007)

NEK2A

Nek2A expression and activation are cell cycle dependent; both start at the G1/S transition and reach maximum levels during the G2/M transition, while in prometaphase the protein is degraded by APC/C-mediated proteasomal degradation (Hayes *et al*, 2006) through its APC/C-dependent degradation motifs, the KEN box (amino acids 391– 399), and the MR-tail (amino acids 444–445) (Hames & Fry, 2002; Wu *et al*, 2007) (Fig. 5). The mechanism of Nek2A activation has been recently described and requires the activity of several proteins: the Mst2 kinase (mammalian STE20-like protein kinase 2, an

Hippo pathway component), hSav1 (protein salvador homolog 1, a scaffold protein), PP1γ (Ser/Thr-protein phosphatase PP1-gamma catalytic subunit) and Plk1. Before the G2/M transition Nek2 is in a complex with Mst2 and PP1γ. The activation of Nek2 requires a specific phosphorylation by Mst2, constantly inhibited in the complex by PP1γ. The liberation of Mst2/Nek2 from the complex happens during G2/M transition when Plk1 is active and can phosphorylate Mst2. Once phosphorylated, Mst2 disengages PP1γ and now can phosphorylate Nek2A which reaches the maximum of its activation (Mardin *et al*, 2011) (Fig. VIA). Moreover, this phosphorylation allows the translocation of Nek2 to the pre-mitotic centrosomes where the complex Mst2/Nek2A is stabilized by hSav1 (Mardin *et al*, 2010).

Once at the centrosome Nek2A can accomplish its most studied function, controlling centrosome splitting. The role of Nek2A in centrosome splitting depends on the association of this kinase with C-Nap (centrosomal Nek2A-associated protein 1) (Fry *et al*, 1998b), and Rootletin (Bahe *et al*, 2005; Yang *et al*, 2006). These two proteins are part of the centrosomal link; in particular C-Nap localizes to the proximal ends of centrioles (Mayor *et al*, 2000), where it acts as a docking site for Rootletin (Bahe *et al*, 2005). Nek2A phosphorylates both, C-Nap and Rootletin, displacing them from pre-mitotic centrosome and determining the dissolution of the centrosomal link (late G2) (Fry *et al*, 1998b; Bahe *et al*, 2005) (Fig. VIB). Centrosome splitting is inhibited by irradiation and DNA-damage, suggesting a possible role of Nek2A in the control of DNA-damage response (Fletcher *et al*, 2004).

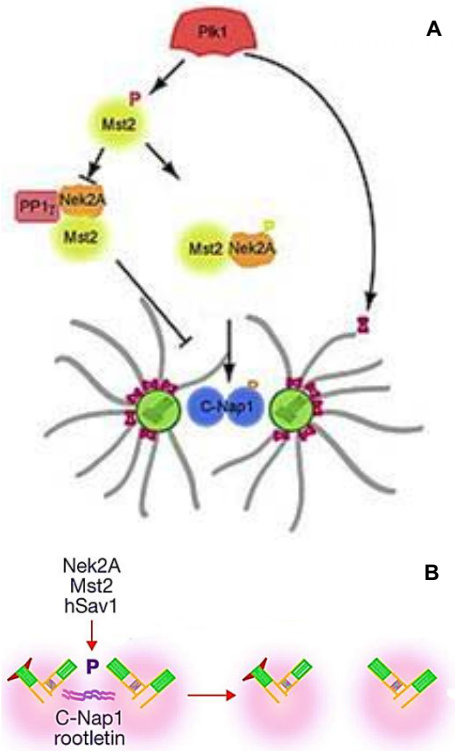


Figure VI (A) Mechanism of Nek2A activation. Plk1 phosphorylates Mst2 that disengages PP1γ and phosphorylate Nek2A determining its translocation to the centrosome. Adapted from (Mardin *et al*, 2011). **(B) Role of Nek2A in centrosome splitting.** Nek2A phosphorylation of C-Nap and Rootletin provokes dissolution of the centrosomal link. Adapted from (Mardin *et al*, 2010)

Nek2A has also been described to be involved in the regulation of chromosome segregation in early mouse embryos; dowregulation of the expression of Nek2A determines micronuclei and breakage-fusion-bridges formation, as well as abnormal nuclear morphology, indicating that this kinase can control the correct segregation of chromosomes (Sonn *et al*, 2004). Recently the function of Nek2A in the control of chromosome segregation has been confirmed, explaining the role that Nek2A covers in the regulation of the SAC. Nek2A is able to phosphorylate Hec-1 (also called Ndc80) at Ser165; this protein is a conserved mitotic regulator dedicated to ensure faithful chromosome segregation and genome integrity through its function at the kinetochore. Hec-1 phosphorylation on Ser165 mainly happens at kinetochore of misaligned chromosomes and determines the accumulation of Mad1 and Mad2 there, activating the SAC. Loss of this phosphorylation provokes the displacement of Mad1 and Mad2, that are no longer recruited at kinetochores of misaligned chromosomes, allowing the abnormal progression of mitosis with breakage-fusion-bridges formation and micronuclei accumulation (Wei *et al*, 2011).

Another non-centrosomal function for Nek2A has been recently described related to the stabilization of cytoskeletal microtubules. Nek2A can interact with the cytoplasmic pool of Nip2 (Centrobin) (Jeong *et al*, 2007) which generally forms homo-aggregates. Nip2 is a specific daughter-centriole protein, also found to bind stable microtubules. The interaction between Nek2A and Nip2 determines the phosphorylation of Nip2 by Nek2A. This phosphorylation allows the disassembly of Nip2 aggregates and mediates the binding of Nip2 to the cytoskeletal microtubules, ensuring their stabilization (Jeong *et al*, 2007). Recently, a possible meiotic role of the interaction between Nek2A and Nip2 has also been described, as the loss of this interaction provokes abnormal meiotic spindle formation (Sonn *et al*, 2011).

Nek2A has also been described as involved in the regulation of cilium disassembly during mitotic entry. Nek2A in fact localizes to the distal portion of the basal body and its depletion prevents the disassembly of the cilium while the overexpression of the active form of Nek2A results in a reduction of ciliation and cilium length (Spalluto *et al*, 2012).

NEK2B

Nek2B has been less studied than Nek2A. The expression of this Nek2 variant is also cell cycle dependent but different from the expression of the A variant. In fact during G1, S and G2 the amount of the protein is low and increases from the G2/M transition until anaphase.

Nek2B is not involved in the regulation of centrosome splitting, nor separation, but seems to be implicated in correct mitotic progression, as besides having high levels in M, the dowregulation of this kinase provokes an increase in the mitotic index as well in the rate of multinucleated cell (Fletcher *et al*, 2005).

Nek2B was better characterized in *Xenopus leavis*, model in which this kinase was found to be involved in the assembly and maintenance of the centrosome. Depletion of XNek2B in meiotic oocytes does not affect mitotic spindle formation probably because there are not centrosome in this phase of *Xenopus leavis* maturation; in contrast depletion of XNek2B in early embryos drastically impairs centrosome assembly (Uto & Sagata, 2000). Moreover, the incubation of sperm cells in XNek2B-depleted CSF (cytostatic factor) egg extracts determines an important delay in centrosome maturation and astral microtubules assembly (Fry *et al*, 2000).

NEK2C

Due to its high similarity with Nek2A this kinase shares many properties with this isoform including kinase activity, dimerization, PP1γ interaction and centrosome localization. The non centrosomal pool however localizes completely differently from the isoform A (or B), into the nucleus. This localization suggests a possible role of Nek2C in the reorganization of the chromatin during mitosis, but nothing in this direction has been described (Wu *et al*, 2007).

NEK9

Nek9, also previously called Nercc1 (Roig *et al*, 2002) and (erroneously) Nek8 (Holland *et al*, 2002), was discovered in 2002 as a protein interacting with Nek6 (one of the smallest members of the NIMA related kinase family; see Nek6 and Nek7 paragraph below) (Roig *et al*, 2002). The mRNA of this kinase results from the transcription of 23 exons of a gene residing in the chromosome 14 (14q24.3). The sequence of Nek9 is highly conserved in mammalians, birds and amphibians (about 62% of identity) and relatively

conserved in fishes and invertebrate animals (about 20-50% of identity). The Nek9 protein has a molecular weight of 120 kDa, is expressed in all mammalian cell lines and tissues studied and comprises three domains: the catalytic domain (52-308), the RCC1 domain (347-726) and the C-terminal domain (891-940) (Roig *et al*, 2002; Holland *et al*, 2002) (Fig. VII).

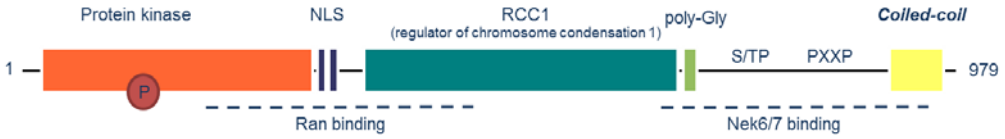


Figure VII Nek9 structure. Schematic representation of the Nek9 protein highlighting the positions of the catalytic domain (Protein Kinase), Nuclear Localization Sequence (NLS) RCC1-like domain (RCC1) and the Coiled-coil domain (Coiled-coil).

The catalytic domain is situated in the N-terminal domain, as in most NIMA family members, and it is a typical Ser/Thr protein kinase sequence. Mutation in the ATP binding site present in this domain (Nek9[K81M]) results in an inactive kinase, as well as mutation of the proton acceptor residue in the catalytic site (Nek9[D176A]) (Roig *et al*, 2005).

The RCC1 protein is the guanine nucleotide exchange factor (GEF) for the nuclear GTP binding protein Ran (Renault *et al*, 2001), first described as Regulator of Chromatin Condensation (RCC) (Ohtsubo *et al*, 1987). Since then, RCC1-like domains have been described in several proteins, participating in protein localization and/or protein-protein interaction. The RCC1-like domain of Nek9, as the RCC1 protein, comprises seven RCC sequences and can bind the small GTP binding protein Ran, although for Nek9 the meaning of this interaction remains unknown as Nek9 lacks the residues necessary for the exchange activity of RCC1 (Roig *et al*, 2002). The Nek9 RCC1-like domain has an important role in the regulation of Nek9 activity; indeed, during the inactive state of the protein this domain interacts with the catalytic domain preventing the activation of the kinase (Roig *et al*, 2002).

The C-terminal domain contains a coiled coils motif which determines the homodimerization of the protein (Roig *et al*, 2002). However, the high molecular weight (about 600 kDa) observed in gel filtration assays for Nek9 cannot be explained with solely the homodimerization of the kinase,

suggesting the association of Nek9 dimer with other proteins or the formation of higher order oligomers (Roig *et al*, 2002).

The Nek9 coiled coil domain associates with LC8 (LC8) both in exponential and mitotic cells (Regué *et al*, 2011). LC8 is a small protein originally described as a component of the dynein/dynactin complex and found to bind several proteins, probably functioning as a dimerization hub, facilitating the organization of partial disorganized proteins. Thanks to the (K/R)XTQT Nek9 motif (adjacent to the C-terminal coiled coil), LC8 interacts with the kinase, promoting Nek9 multimerization and influencing Nek9 quaternary structure (Regué *et al*, 2011), thus explaining, at least in part, the high molecular weight found in gel filtration experiments. The binding between LC8 and Nek9 is regulated by Nek9 autophosphorylation of Ser944, a residue immediately N-terminal to the (K/R)XTQT motif, as this phosphorylation results in the disruption of LC8 interaction. Moreover, the binding of LC8 to Nek9 interferes with the interaction of Nek9 with Nek6 (and possibly Nek7) and impedes Nek6 activation, that depends on Nek6 binding to Nek9 (Belham *et al*, 2003), suggesting that LC8 is a controller of signal transduction through the Nek9/Nek6 module (Regué *et al*, 2011) (see also Nek6 and Nek7 paragraph below).

Other motifs are also present along the sequence of Nek9; between the catalytic domain and the RCC1 domain there are two putative nuclear localization sequences (NLS; 306-313 and 325-330); however, these motifs are not functional in this location. Interestingly, Thr333, situated close to the NLS, is phosphorylated *in vivo* and this modification may contribute to regulate the functionality of the NLS motifs (Roig *et al*, 2002; Holland *et al*, 2002).

Between Nek9 RCC1 domain and the C-terminal domain there is a poly-glycine motif with nine glycine residues (752-760) which probably serves as a flexible hinge. Finally, also in the C-terminal portion of the protein, there is a PEST region (Polypeptide sequence enriched in proline (P), glutamate (E), serine (S), and threonine (T); 734-779) and two putative SH3-domain-binding motifs PXXP (823-830 and 881-888) possible implicated in protein binding (Roig *et al*, 2002; Holland *et al*, 2002).

The expression of Nek9 is not cell cycle dependent, in contrast to its activation. During the cell cycle Nek9 levels are stable; in interphase the kinase is inactive and localizes diffusely in the cytoplasm (Roig *et al*, 2005).

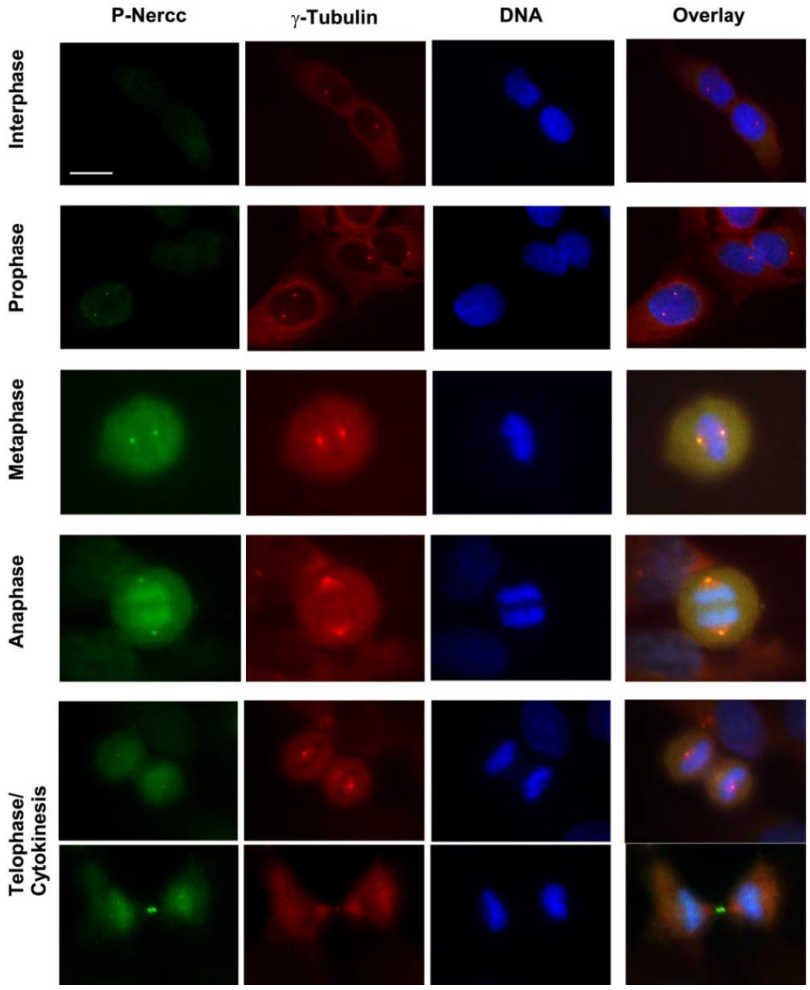


Figure VIII P-Nek9 Localization. Cells immunostained for P-Nek9 (Thr 210 P-specific antibody–green), γ-tubulin (red) and DNA (DAPI-blue).
Adapted from (Roig *et al*, 2005)

In vitro, (in presence of subcellular concentration of ATP) inactive Nek9, purified from exponentially growing cells, can autophosphorilate and activate (Roig *et al*, 2002, 2005). Nek9 dimerization and the binding between the RCC1 like domain and the catalytic domain are important factors in the regulation of Nek9 activation, since Nek9 constructs lacking the coiled coil motif are not able to be activated *in vitro* while recombinant Nek9 lacking the RCC1 like domain (ΔRCC1) is constitutively active. *In vitro* Nek9 dimerization promotes the activation of the protein, possibly through trans-phosphorilation

of the activation loop (Roig *et al*, 2002), a process facilitated by the binding between Nek9 and LC8 (Regué *et al*, 2011). *In vivo*, the activation of Nek9 starts at the beginning of mitosis, although during interphase ATP levels are on the order of mM (sufficient for *in vitro* activation), suggesting that mechanisms of inhibition exist and keep Nek9 inactive. Mitotic Nek9 activation depends on sequential phosphorylations started by Cdk1 (priming). Cdk1 phosphorylation happens at multiple sites and results in an electrophoretic mobility shift (Roig *et al*, 2002). This hyper-phosphorylated status however does not directly determine the activation of Nek9, since Cdk1 is not able to phosphorylate the activation loop of Nek9, that in vivo only occurs in the 5% of total amount of the protein (Roig *et al*, 2005). Our group has recently described Plk1 as a Nek9 activator, as Plk1 is able to bind Nek9 and phosphorylate the kinase at Thr210 thus activating it (Bertran *et al*, 2011). Cdk1 controls the binding of Plk1 to Nek9, since Cdk1 phosphorylation at Ser869 of Nek9 results in the interaction between Plk1 and Nek9, suggesting a two-step activation mechanism that involves Nek9 sequential phosphorylation by Cdk1 and Plk1 (Bertran *et al*, 2011) (see Article 1 below).
Once active, Nek9 localizes to the mitotic centrosomes (prophase to telophase), mitotic spindle (metaphase to anaphase) and midbody (cytokinesis) (Fig. VIII).

Nek9 activity is crucial for normal mitotic progression as overexpression of a Nek9 kinase-deficient form (Nek9[K81M]) provokes cell cycle arrest and apoptosis (Roig *et al*, 2002). According to that, microinjection of anti-Nek9-antibodies in prophase cells provokes several mitotic abnormalities. For instance, microinjected cells fail to build a correct mitotic spindle and arrest in prometaphase or, when showing a spindle, fail to align the chromosomes resulting in a defective chromosome segregation and aneuploidy (Roig *et al*, 2002).
The fundamental role of Nek9 in the regulation of the mitotic process has also been confirmed in the *Xenopus* system, as mitotic *Xenopus* egg extracts immunodepleted of XNek9 fail to form normal bipolar spindles (Roig *et al*, 2005) (Fig.IX).

The molecular basis of these observation were unknown at the start of this work, although they might be in part related with the capacity of Nek9 to control the phosphorylation of the important mitotic kinesin Eg5, through the

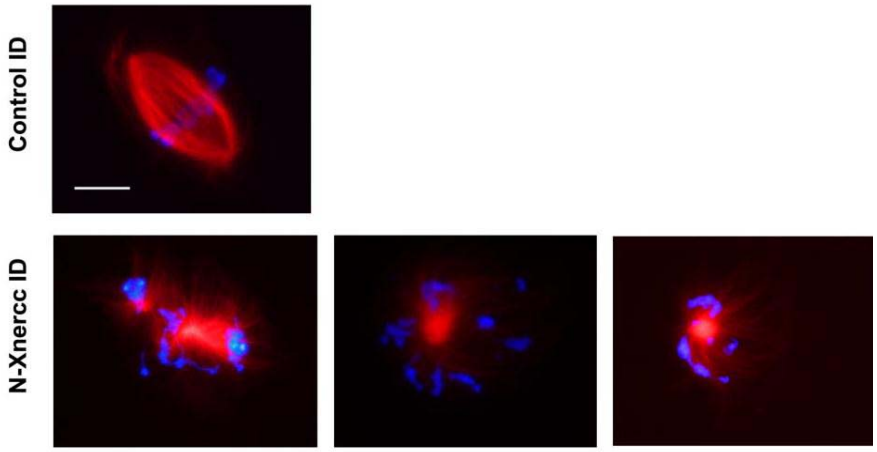


Figure IX Effect of X-Nek9 immunodepletion on the bipolar spindle formation in *Xenopus* eggs extract. Cell immunostained for tubulin (red) and DNA (DAPI – blue) Adapted from (Roig *et al*, 2005)

activation of its substrates Nek6 and Nek7 (Belham et al. 2003) which directly phosphorylate Eg5 at the Ser1033 (see Nek6 and Nek7 paragraph below) (Rapley *et al*, 2008). Additionally, Nek9 is able to immunoprecipitate with several components of the γ-TuRC (γ-tubulin Ring Complex), protein complex essential for microtubule nucleation during mitosis (see γ-tubulin paragraph below) (Roig *et al*, 2005), suggesting some possible role of Nek9 in the regulation of microtubule nucleation, an hypothesis that has been investigated in this work.

A non-directly mitotic role of Nek9 has been recently described and associates the kinase with autophagy. Indeed, cells depleted for Nek9 cannot recruit cargo to vescicles or regulate vescicle trafficking (Behrends *et al*, 2011).

NEK6 AND NEK7

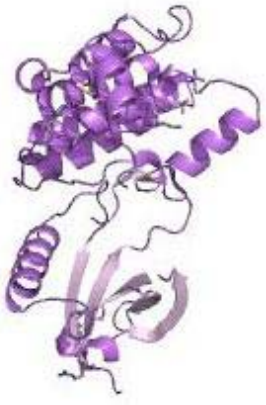


Figure X Representation of Nek6/7 Tertiary Structure. Adapted from www.ebi.ac.uk

Nek6 and Nek7 are the smallest member of the NIMA family and the structure of these two kinases only comprises an N-terminal domain containing the catalytic domain typical of NIMA, while lacks the typical C-terminal regulatory domain of Neks. Nek6 and Nek7 are 86% identical and differ only for a small portion located in the N-terminal domain, before the catalytic domain. The high homology of these two kinases might suggest they can be functional equivalents, although the small N-terminal portion through which they differ may be responsible for substrate specificity (Shigeru *et al*, 2002). The molecular weight of these two kinases is about 35 kDa, since that Nek6 comprises 313 residues and Nek7 302 residues (Hashimoto *et al*, 2002; Kandli *et al*, 2000). Both kinases show a similar tertiary structure which comprises the globular kinase domain and a short N-terminal disordered region (Richards *et al*, 2009; Meirelles *et al*, 2011) (Fig. X).

The expression of this two kinases during cell cycle is distinct; in fact Nek7 protein levels do not change while Nek6 levels increase entering in mitosis and start to decrease from metaphase (Belham *et al*, 2003). During mouse embryogenesis Nek6 and Nek7 show different, but complementary, patterns of expression and moreover in adult mice they localize in distinct tissue, as Nek6 is mainly expressed in intestine and placenta while Nek7 in kidney (Feige & Motro, 2002; Kandli *et al*, 2000). In mice, the absence of Nek7 is lethal and animals cannot finish embryogenesis or die in early post-natal stages with severe growth retardation (Salem *et al*, 2010).

The activation of Nek6 and Nek7 depends on their binding to Nek9 C-terminal domain. During early mitosis Nek9 binds Nek6 and Nek7 and phosphorylates Nek6[S206] and Nek7[S195], directly activating the kinases (Belham *et al*, 2003). Once activated by Nek9, Nek6 and Nek7 can autophosphorylate, increasing their catalytic activity, respectively Nek6 at Thr202 and Ser37 and Nek7 at Thr191 (Belham *et al*, 2003; O'Regan & Fry, 2009). Mutations at the kinase ATP-binding pocket (Nek6[K75M] and Nek7[K64M]) result in partially

inactive kinases; nevertheless consecutive mutations (Nek6[K75M/K76M] and Nek7[K64M/K65M]) completely abolish the catalytic activity of the protein (Belham *et al*, 2003; O'Regan & Fry, 2009). A novel mechanism of inhibition of Nek6 (and supposedly Nek7) activation has been recently described and depends on the binding of LC8 to the C-terminal domain of Nek9. As described above, LC8 binds to Nek9 interfering with its interaction with Nek6 (and possibly Nek7). Nek9 activation at the onset of M phase promotes autophosphorilation at Ser944 with consequent loss of Nek9/LC8 interaction, allowing the binding of Nek6 to Nek9. An active form of Nek9 constitutively bound to LC8 (ΔRCC1[S944A]) is not able to bind and activate Nek6, indicating that the interaction between Nek9 and LC8 controls the activation of Nek6 (Regué *et al*, 2011).

Structural studies have demonstrated that the binding between Nek9 and Nek7 results in the release of the autoinhibitory Nek7[Y97] from the active site, modifying the conformation of the kinases and allowing its activation (Richards *et al*, 2009), a mechanism that may also function for Nek6[Y108] and may synergize with phosphorylation by Nek9 during the activation of Nek6/7. Finally, increasing levels of Nek9 C-terminal domain inhibit active Nek6, indicating the presence of further mechanisms of regulation of Nek6 (and probably Nek7) activity yet to be understood (Belham et al. 2003).

The localization of Nek6/7 depends on their activation state. During interphase Nek6 and Nek7 localize diffuse in the cytoplasm, with a small amount of Nek7 at centrosomes (Yassachar *et al.* 2006, Kim *et al.* 2007, O'Regan and Fry 2009). When active, during mitosis, Nek6 partially localizes at centrosomes (prophase to metaphase), at central spindle (anaphase and telophase) and, in cytokinesis, at midbody (O'Regan and Fry 2009), while active Nek7 localizes at centrosomes throughout mitosis (O'Regan & Fry, 2009; Yissachar *et al*, 2006).

There are several evidences about the important of these two kinases in the control of mitotic processes; for instance our group have described Nek6 and Nek7 as able to phosphorylate Eg5 (Kinesin 5; see Eg5 paragraph below) at Ser1033. Eg5 is an essential motor protein involved in the formation of the bipolar spindle. Its activity consists in bind to antiparallel microtubules and slide them apart, determining the separation of the centrosome during mitosis (Blangy *et al*, 1995). The phosphorylation of the Ser1033 happens in mitosis in

a small pool of the kinesin (3% of the total amount of Eg5) and is necessary for the translocation of a pool of Eg5 to the centrosome and its vicinity, contributing to the formation of normal bipolar spindle (Rapley *et al*, 2008). Furthermore, human cell lines depleted for Nek6 or Nek7 show fragile mitotic spindle, prometaphase delay (Yissachar *et al*, 2006; O'Regan & Fry, 2009) and incorrect cytokinesis progression (O'Regan & Fry, 2009; Yissachar *et al*, 2006), while MEFs extracted from Nek7 KO mice do not delay in prometaphase but show several problems in chromosome segregation and cytokinesis (Salem *et al*, 2010).

Moreover, In human cell lines the depletion of Nek7 has been described to impair centrosomal γ-tubulin accumulation, which determines a reduced ability of the cell to nucleate microtubules and organize the mitotic spindle (Kim *et al*, 2007). PCM accumulation results impaired also in G1 phase cells and seems to determine an aberrant centriole duplication during S phase (Kim & Rhee, 2011); some our data do not agrees with these results (see below) as we have not seen any PCM content variation after depletion of Nek7 (nor Nek6) neither in interphase or mitosis cells (Sdelci *et al*, 2012).

Finally, Nek6 has also been described to be involved in DNA-damage induced cell cycle arrest (Yun, 2008). The activation of Nek6 during mitosis seems to be completely abrogated after UV or IR irradiation; this inactivation has been proposed to be the result of a Chk1 phosphorylation in the N-terminal domain of Nek6 (near to the Nek9 phosphorylation site), after Chk1 activation upon DNA-damage; Nek6 overexpression overrides the G2/M block and cells can proceed into mitosis (Yun, 2008).

THE CENTROSOME

First described as a “special organ of cell division” by Theodor Boveri in 1888, the major role of the centrosome is the organization of microtubules both in the cytoskeleton of interphase cells and in the spindle of mitotic cells. Normally, during G1 only one centrosome is present in the cell and it derives from the mother cell; during S phase the centrosome duplicates and thus in G2, when the process ends, cells have a pair of centrosomes.

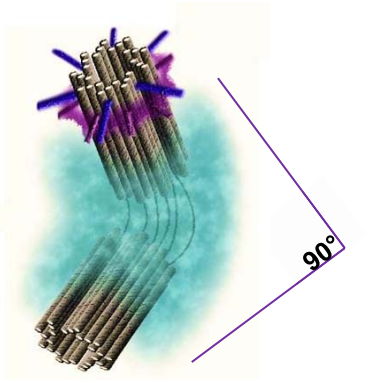


Figure XI Centrosome Representation. The two centrioles orthogonally distributed (two green structures formed with 9 triplets of microtubules – grey) are immersed in the PCM (light blue) and connected through filamentous proteins. Adapted from (Anderhub *et al*, 2012)

The centrosome comprises a pair of orthogonally distributed centrioles, immersed in an electron-dense amorphous mass of protein called Peri-Centriolar Material (PCM). The two centrosomes are connected by means of proteinaceus filaments mainly composed by two proteins: Rootletin and C-Nap (Bahe *et al*, 2005; Yang *et al*, 2006) (Fig. XI), although recently the proteins Cep68 and Cdk5Rap2 (Cep215) have also been described as part of the link. In particular, Cep68 localizes between the two centrioles while Cdk5Rap2 surrounds them and the depletion of both proteins results in premature centrosome splitting, indicating a possible role of Cep68 and Cdk5Rap2 in centrosome cohesion (Graser *et al*, 2007).

Additionally, the maintenance of centrosomal integrity involves the activity of β-catenin, protein better known for its involvement in the Wnt signaling pathway (Dierick & Bejsovec, 1999). β-catenin can localize to the proximal and distal centriole ends and, moreover, between centrosomes acting like a docking protein for the recruitment of Rootletin and C-Nap (Bahmanyar *et al*, 2008).

THE CENTRIOLE

The centriole, discovered by Edouard van Beneden in 1883, is the structural core of the centrosome and is present in many eukaryotic cells while being absent in higher plants and most *fungi* (Quarmby & Parker, 2005; Bornens & Azimzadeh, 2007).

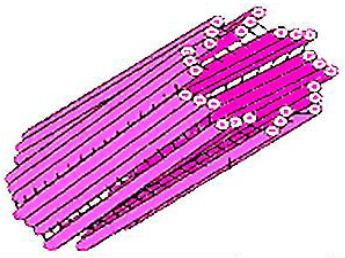


Figure XII Centriole Structure.
Nine microtubules triplets connected between each other and "clock wise" distributed.
Adapted from http://kids.britannica.com/comptons/art-53097

The centriole is a cylindrically-shaped cell structure of ~100-150 nm in diameter and 100-400 nm in length, usually composed of nine triplets of microtubules (Fig. XII).

Each centrosome comprises two orthogonal centrioles, and thus in G1 only two centrioles are present in the cell. During S phase a new centriole (daughter centriole) starts to grow out of the side of each parent centriole (mother centriole) as part of centrosome duplication. Finally, in G2 the elongation of the daughter centrioles finishes, resulting in a cell containing a pair of connected centrosomes with two centrioles in each of them.

The centriole is important for centrosome behavior and results to be involved in the formation of mitotic spindle (although it is known that they are not essential for this (La Terra *et al*, 2005)), cilia and flagella (the cellular position of which is determined by the mother centriole (Feldman *et al*, 2007)) (Quarmby & Mahjoub, 2005), cytoskeletal microtubules and cell polarity preservation (Feldman *et al*, 2007) and finally for cytokinesis (Salisbury *et al*, 2002).

PERICENTRIOLAR MATERIAL

The PCM is an electron-dense amorphous mass that surrounds the centrosome and contains proteins involved in microtubule nucleation and anchoring, such as γ-tubulin, γ-tubulin Complex Proteins (GCPs), Ninein (Delgehyr *et al*, 2005) pericentrin (PCNT, also called kendrin) (Zimmerman *et al*, 2004) and Cdk5Rap2 (Fong *et al*, 2008). The size of the centrosome depends on the accumulation of PCM components during the different phases of cell cycle. PCM recruitment is mediated by key regulators of mitosis such Plk1, and happens just at the onset of mitosis, a process known as

centrosome maturation (see Centrosome Maturation paragraph below) (Blagden & Glover, 2003).

A relation between PCM and centrioles exists, indicating that this two parts of the centrosome are not completely independent. Reduction in the normal amount of PCM results in fact in impaired centriole duplication (Kim & Rhee, 2011; Keryer *et al*, 2003) and conversely, centrioles destruction can provoke the dissolution of the PCM (Bobinnec *et al*, 1998).

THE CENTROSOME CYCLE

The centrosome cycle is the process by which centrosomes duplicate, separate, maturate and are distributed between the two daughter cells (Fig. XIII).

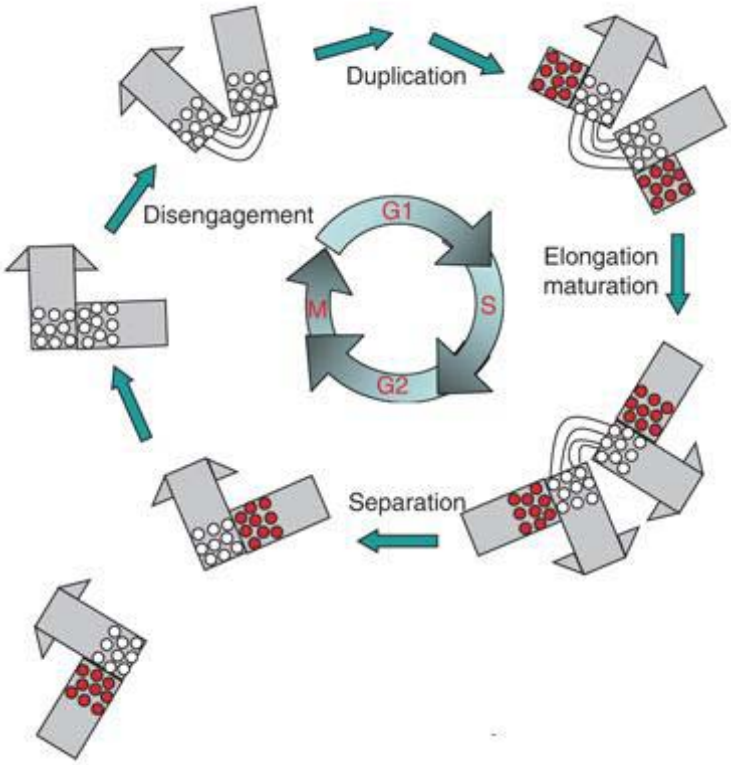


Figure XIII Centrosome Cycle representation. White dots for mother (parental) centriole, red dots for daughter centriole. Adapted from (Chang *et al*, 2010)

CENTROSOME DISJUNCTION

The first phase of the centrosome cycle is centrosome disjunction (or disengagement) that consists in the disorientation of the two centrioles, resulting in the loss of their orthogonal arrangement. This phase is crucial for the licensing of centrosome duplication, as the orthogonal configuration of duplicated centrioles seems to prevent centrosome duplication (Tsou & Stearns, 2006).

Previously, centrosome disjunction was though to happen during the G1/S transition, although a more recent work has shown that this process begins in early M phase and depends on Plk1 and separase activities (M.-F. B. Tsou et al. 2009). Centriole disjoining is suggested to be controlled by two "centriole licensing pathways" respectively in early mitosis (Plk1-mediated) and in anaphase/telophase transition (Plk1 and separase-mediated). At the onset of anaphase, Plk1 regulates the activation of separase, a cysteine protease that triggers sister chromatid separation (Nasmyth *et al*, 2000). By analogy to sister chromatid cohesion, Plk1 might promote a separase-independent removal of an hypothetical centriolar ''glue'' protein (ideally responsible for cohesion) in early mitosis, while in anaphase might recruit separase and mediate an anaphase-specific separase cleavage of this ''glue'' protein. In agreement with that, Plk1 inhibition before anaphase onset, as well as separase inhibition, determines a 50% of decrease of centrosome disjunction while the combination of both inhibitions results in the complete abrogation of this process, as well as of centrosome duplication (M.-F. B. Tsou et al. 2009). One of the possible substrate of separase might be PCNT (that would be the "glue protein"). PCNT localizes at the PCM and can be cleaved in a consensus site by separase determining centrosome disjunction; a mutant form of PCNT lacking this cleavage site (non-cleavable PCNT) results in fact in loss of centrosome disjunction with consequent loss of centrosome duplication (Matsuo *et al*, 2012).

CENTROSOME DUPLICATION

The duplication of the centrosome is a process indispensable to guarantee the correct number of centrosomes *per* cell. Centrosomes duplicates once and only once during the cell cycle and, although centrosome disjunction is completed at the anaphase/telophase transition, the first sign of centrosome

duplication can be appreciated only in S phase. During S phase a short structure can be seen near the proximal end of both preexisting centrioles, named mother (or parental) centrioles. These new structures are called daughter centrioles and originate from the parental centrioles (Alvey, 1985). Centrosome duplication depends on Cdk2 activity and, in particular, on the interaction between Cdk2 and cyclin E and/or A, that is also required for G1/S transition and DNA replication (Hinchcliffe & Sluder, 2001).
The elongation of the daughter centriole is completed in G2 and will not be described here in detail (Nigg & Stearns, 2011).

CENTROSOME SPLITTING

The splitting of the centrosomes determines the physical disconnection between the two centrosome. This process requires the breakage of the proteinaceus filaments that connect the two centrosomes and it is controlled by Nek2A phosphorylation of Rootletin and C-Nap (Fry *et al*, 1998b, 1998a; Mardin *et al*, 2010, 2011) in a Plk1 dependent manner (Mardin *et al*, 2011) (see Nek2A paragraph above).

CENTROSOME SEPARATION

Centrosome separation is the process by which centrosomes start to move apart to finally localize at the opposite poles of the cell, ensuring the bipolarity of the forming mitotic spindle. This process is regulated by different factors such as motor proteins (in particular Eg5 and dynein), cytoskeletal actin, kinetochores and the NE (Tanenbaum & Medema, 2010).
The separation of the centrosome starts early in mitosis, before NEB (prophase) and continues until the end of prometaphase. The factors involved during centrosome separation in prophase are different from the factors that promote this process during prometaphase, originating two pathways of centrosome separation respectively called prophase pathway and prometaphase pathway. The redundancy of these pathways attests to the importance of centrosome separation for the proper formation of a bipolar spindle during mitosis.

PROPHASE PATHWAY

First, during prophase, centrosomes move apart in opposite direction slipping on the surface of the NE.

The sliding of the centrosomes depends principally on the kinesin Eg5. Eg5 is a plus-end directed motor protein which starts to localize to astral microtubules and centrosomes from prophase (Blangy *et al*, 1995). Eg5 is indispensable for the formation of bipolar spindle (Tanenbaum *et al*, 2008; Woodcock *et al*, 2010; Whitehead & Rattner, 1998; Smith *et al*, 2011), as the inhibition of this kinesin results in monopolar spindles formation (Kapoor *et al*, 2000) (see Eg5 paragraph below).

Another important motor protein in the regulation of centrosome separation and spindle formation is dynein. Dynein is a minus-end directed motor protein (in contrast to Eg5) that localizes to different subcellular compartments. Especially through its localization to the NE and cortex (Kardon & Vale, 2009), dynein contributes to prophase centrosome separation.
In particular, dynein is recruited to the NE during G2, prior to centrosome separation, indicating a possible role in the preparation of this process. Moreover, it is known that NE associated dynein can generate forces pulling on microtubules, but is still not clear the mechanism by which dynein can act in this context (Salina et al. 2002; Joel et al. 2002; Reinsch and Karsenti 1997). The pool of the protein that localizes to the cortex also contributes to prophase centrosome separation. Dynein in fact, when attached to the cortex can bind microtubules growing from the centrosomes, stretching them toward the cortex and thus determining centrosome separation (Sharp et al. 2000). However, some points remain unclear; for instance is not known how astral microtubules can interact with the cortex so early in mitosis. Moreover, centrosomes could move only when the pulling force from one side is higher than the one from another side (Tanenbaum and Medema 2010).

The localization of Dynein to the cortex is, at least in *Drosophila*, actin mediated. This process does not involve the actomyosin counterpart, reveling a specific role of actin in the control of prophase centrosome separation (Cao *et al*, 2010). A second role of actin in the regulation of early centrosome separation has been described. In fact, actin-dependent Eg5-opposing forces impede centrosome separation in G2 while actin depolymerization, as well as destabilization of interphase microtubules (MTs), is enough to take out this

obstruction and to speed up the Plk1/Eg5-dependent centrosome separation from prophase (Smith *et al*, 2011).

Eg5 and dynein can compensate each other and in fact the formation of monopolar spindles due to the lack of Eg5 is rescued with dynein depletion, or depletion of the dynein binding protein Lis1, indicating that dynein and Lis1 produce an inward force that counteracts the Eg5-dependent outward force (Tanenbaum *et al*, 2008). This interplay between Eg5 and dynein can contribute to explain the elasticity of the metaphase spindle (Shimamoto *et al*, 2011) and could also be involved in the early separation of centrosomes.

Finally, it has been proposed that the polymerization of microtubules from centrosomes can promote centrosome separation by the mechanical forces originated from the new growing astral microtubules, in particular early, when centrosome are close to each other and the corresponding astral microtubules can interact easily (Dogterom *et al*, 2005).

PROMETAPHASE PATHWAY

The mechanisms of centrosome separation after prophase are prevalently dependent on microtubule pushing forces and chromokinesins.
After NEB, microtubules start to grow also from kinetochores (forming the K-fibers) and in the vicinity of DNA (see Microtubule Nucleation paragraph below) and these microtubules can contribute to centrosome separation, determining an acceleration of this process (Toso et al. 2009; Silk, Holland, and Cleveland 2009). Kinetochores can in fact promote the separation of the centrosome using poleward microtubule flux and a reduced number of stable k-fibers delays centrosome separation, caused by the decrease of the net force generated by microtubule flux (Toso *et al*, 2009).

Chromokinesins also contribute to the correct bipolarity of the spindle, participating in the regulation of centrosome separation. These proteins are kinesins that associate with mitotic chromosomes and belong to the Kinesin-4 and Kinesin-10 families, which both have plus-end directed motility (Mazumdar & Misteli, 2005). Chromokinesins bind chromosome arms and can walk along microtubules growing from centrosomes, generating a pushing

force that turns the chromosome away from spindle poles (Antonio *et al*, 2000; Karsenti & Vernos, 2001).

Finally, actin bundles have also been shown to be able to promote the separation of the centrosome in Hela cells (Whitehead *et al*, 1996). Moreover some other studies in mammals have demonstrated that actin mediated centrosome separation after NEB needs the myosin counterpart, unlike that of before NEB (Rosenblatt *et al*, 2004).

NEB is not coordinated with centrosome separation which can be completed before (prophase pathway) or after (prometaphase pathway) it. Even though the last effect of the prophase or prometaphase pahtway results to be the same (both lead to centrosome separation), a recent study has shown that the prophase pathway is more effective to ensure correct chromosome segregation (Silkworth *et al*, 2012), emphasizing the importance of centrosome separation before the NEB.

Eg5

Kinesins are molecular motors that use energy from ATP hydrolysis to transport cargoes along microtubule tracks. There are, at least, 14 families of kinesins and, depending on their structural organisation, each kinesin is suited for different functions. Some are involved in transporting vesicles and organelles in cells, others are essential for axonal transport in neurons or in intraflagellar transport in cilia. Finally, a group of kinesins contributes in different steps of mitosis. Eg5 (Kinesin 5, also called Kif11) belongs to this last kinesin group.

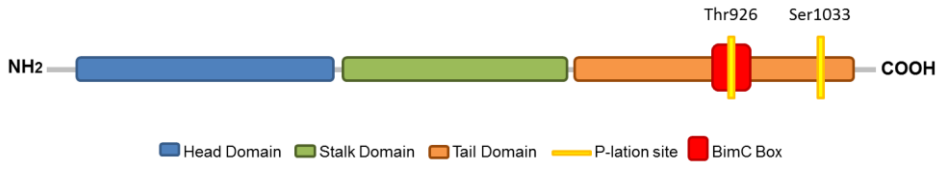


Figure XIV Eg5 structure.

The sequence of Eg5 comprises 1056 residues, resulting in an approximate weight of 125 kDa. This kinesin is a plus-end motor protein that, as a result of

its tetrameric structure, is able to crosslink antiparallel microtubules and slide them apart, exerciting its well known fuction in separating centrosomes and thus contributing to bipolar spindle organization (Tanenbaum *et al*, 2008; Blangy *et al*, 1995; Walczak & Heald, 2008; Ferenz *et al*, 2009; Gaglio *et al*, 1997).

The structure of Eg5 comprises a motor domain (head) a coiled coil domain (stalk) and a tail domain containing a conserved motif called BimC Box (Kashina *et al*, 1996) (Fig. XIV). The motor domain is the functional domain by which Eg5 can bind microtubules. This domain comprises the ATP binding site; Eg5, as well as the majority of the kinesins, binds microtubules and walks on them hydrolyzing ATP.

Eg5 quaternary structure is an homotetramer formed by four Eg5 subunits (a pair of anti-parallel dimers) (Fig. XVA). In solution Eg5 is mainly present as a dimer; the first association with microtubules after the ATP hydrolization determines an increase of affinity for a second Eg5 dimer resulting in the

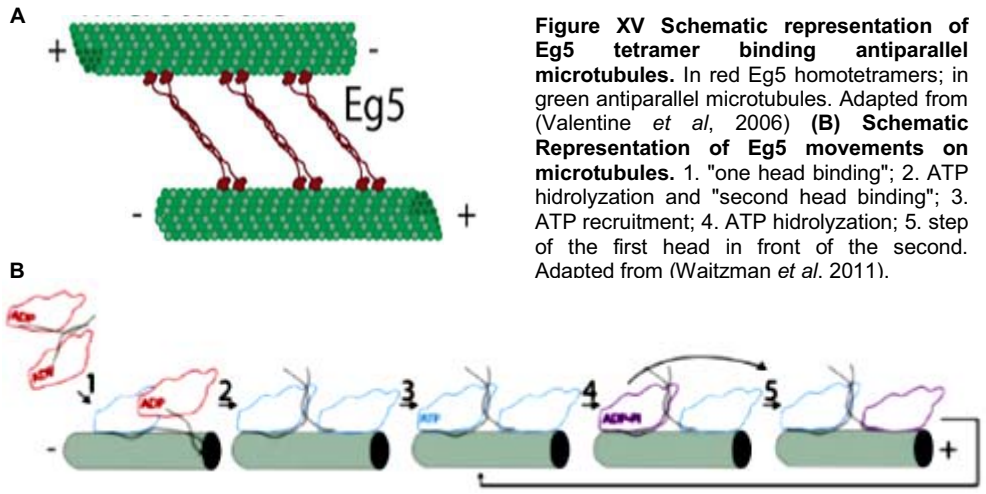


Figure XV Schematic representation of Eg5 tetramer binding antiparallel microtubules. In red Eg5 homotetramers; in green antiparallel microtubules. Adapted from (Valentine *et al*, 2006) **(B) Schematic Representation of Eg5 movements on microtubules.** 1. "one head binding"; 2. ATP hidrolyzation and "second head binding"; 3. ATP recruitment; 4. ATP hidrolyzation; 5. step of the first head in front of the second. Adapted from (Waitzman *et al*, 2011).

association between the two dimers, process that requires a second ATP hydrolysis (slow process). Since Eg5 has a tetrameric structure, the heads (two up and two down) start to bind ATP alternatively, associating with microtubules and move one before the other; the hydrolysis of ATP permits in fact that the first heads can unhook microtubules and rebind them in front of the second heads (Waitzman *et al*, 2011) (Fig. XV B).

The localization of this kinesin during its inactive state (interphase) is diffuse in the cytosol while during mitosis Eg5 localizes first at centrosomes and the small centrosomal asters (prophase and prometaphase), next at spindle

(metaphase to anaphase) and finally at midbody (telophase to cytokinesis) (Fig. XVI). This localization is assured by the binding with microtubules and is regulated by Cdk1 Eg5[T926] (a residue sited in the conserved BimC box motif) phosphorylation,; mutation of this site determines the complete dissociation of Eg5 from microtubules and the collapse of the spindle (Blangy *et al*, 1995).

Our group has described that during mitosis Eg5 can be phosphorylated by Nek6 at Ser1033 and this phosphorylation, in cooperation with the Cdk1 phosphorylation, determines the translocation of Eg5 to the centrosome and its vicinity (Rapley et al. 2008). Even if this phosphorylation happens only in a small pool of Eg5 (~3% of the total amount of the kinesin), it is important for correct mitotic spindle formation, since overexpression of a mutant that cannot be phosphorylated at that site (Eg5[S1033A]) results in defective bipolar spindle formation (Rapley et al. 2008).

Centrosomal Eg5 localization is difficult to rationalize, as Eg5 would be expected to bind antiparallel microtubules, predominantly present in the middle of the spindle, but not near the centrosome. In addition, Eg5 is a plus end directed motor, a feature that implicates that Eg5 would move in the

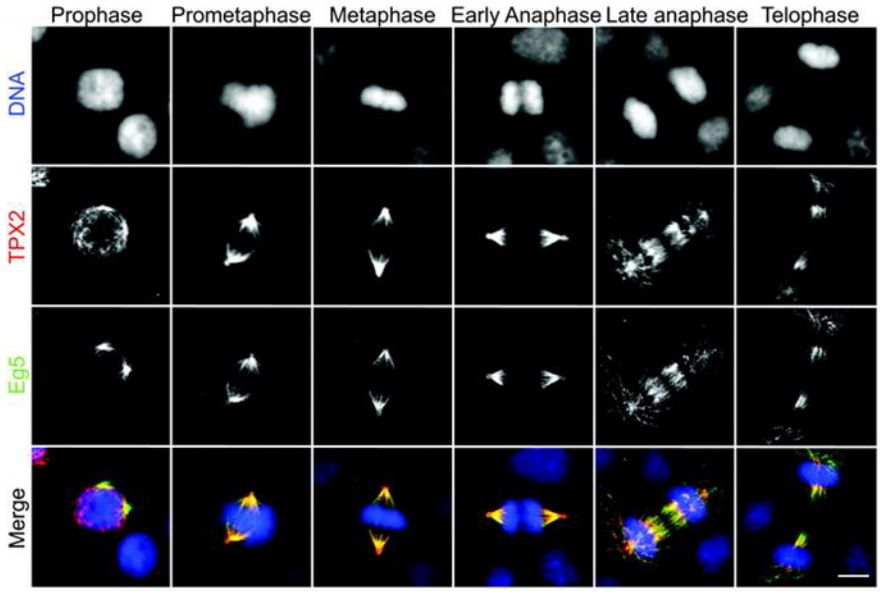


Figure XVI Eg5 and Tpx2 Localization during mitosis. Immunofluorescence images with antibody for TPX2 (red), Eg5 (green) and DAPI (blue). Adapted from (Ma *et al*, 2011).

opposite direction of the centrosome, where microtubule minus ends reside. Until now, there are no valid explanations about the mechanism that determines Eg5 centrosomal localization, besides that it depends on Nek6 and Cdk1 phosphorilations.

Nevertheless, the interaction of Eg5 with other proteins, such as dynein or the dynein/dynactin complex or the spindle assembly factor TPX2 (Fig. XXIV), has been described. Indeed, it is known that p150Glued (the largest dynactin subunit) can interact with the tail of Eg5 during mitosis (Blangy *et al*, 1997). The interaction between Eg5 and the dynein/dynactin complex was also found in *Xenopus*, where the interplay between this two motors seems to mediate the dynamic interaction between Eg5 and spindle microtubules, excluding at the spindle center and at the spindle poles, where Eg5 is more static (Uteng *et al*, 2008).

The association of Eg5 to the spindle depends also on its interaction with the spindle assembly factor TPX2 (Ma *et al*, 2011). This association reduces the velocity of Eg5 on microtubules, inhibiting microtubule sliding and determining the accumulation of Eg5 at the spindle (Eckerdt *et al*, 2008; Ma *et al*, 2010, 2011).

Taken together, these findings explain how dynein/dynactin complex and TPX2 may contribute to the localization of Eg5 in the mitotic spindle, however they do not explain its localization to the centrosome. These interactions may suggest the presence of some other similar mechanisms involved in the regulation of this specific localization.

Finally, although Eg5 is required for early centrosome separation and correct bipolar spindle formation, this kinesin results to be dispensable for maintaining of the spindle bipolarity during metaphase (Kapoor *et al*, 2000), suggesting the implication of other mechanisms to keep bipolarity after the initial construction of the spindle.

CENTROSOME MATURATION

Centrosome maturation is a fundamental process that guarantees the nucleation of the correct amount of new microtubules from centrosomes, ensuring the correct microtubule density in the spindle. This process starts early in mitosis (prophase), when centrosome becomes 3/4 fold bigger than in

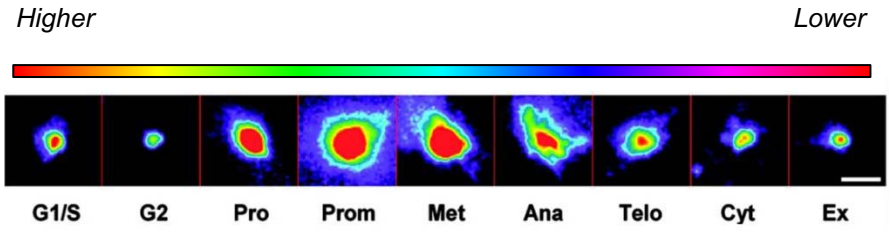


Figure XVIII Centrosome magnification during the different phases of cell cycle. Magnification of centrosome from cells immunostained for γ-tubulin (Spectrum LUT coded). LUT scale indicates the increase of the saturation of the signal intensity. Pro is prophase, Prom is prometaphase, Met is metaphase, Ana is Anaphase, Telo is Telophase and Cyt is Cytokinesis (Scale Bar 3 μm). Adapted from (Piehl *et al*, 2004).

G2, and reaches maximum levels between prometaphase and metaphase (5 fold bigger; Fig. XVIII) (Piehl *et al*, 2004).

The maturation of the centrosome depends on the accumulation of PCM proteins recruited from the cytosol. Several components of the PCM have been identified but not all of them are directly involved in microtubules nucleation.

γ-TUBULIN

First discovered in *Aspergillus nidulans* (Oakley & Oakley, 1989), γ-tubulin has been described to be one of the PCM members directly involved in microtubule polymerization, forming the scaffold upon which the nucleation of α/β-tubulin dimers can start. *In vitro* microtubule nucleation happens spontaneously while *in vivo* a "nucleator" is needed to stabilize the forming microtubule and γ-tubulin is the best characterized microtubules nucleator. This protein associates with all already described MTOCs (microtubule organizing center) and results essential for their function. In the cytosol γ-tubulin interacts with other proteins to associate as a complex to the minus-end of the growing microtubule. This complex, named γ-TuRC, comprises a different number of protein subunits according to the species (Wiese & Zheng, 2006). How γ-tubulin can promote nucleation of microtubules has been discussed for a long time and γ-tubulin (and consequently the γ-TuRC) has been suggested to act as a seed onto which microtubule assembly can happen. In particular two putative mechanism of nucleation have been proposed: the template nucleation model and the protofilament model. By the template model, the γ-TuRC essentially mimics the end of a microtubule

allowing the assembly of a new microtubules through the binding between γ-tubulin and α/β tubulin dimers. Conversely, in the protofilament model α/β tubulin dimers bind to the γ-TuRC creating a sheet, which grows and coils to form a microtubule. For its matching with the symmetry of a microtubules, the template model is preferred respect to the protofilament model; moreover structural data also support the theory of the template model (Guillet *et al*, 2011).

The recruitment of γ-tubulin (and consequently the γ-TuRC) to the centrosome depends on an adaptor protein, also part of the γ-TuRC, called Nedd1 (Neural precursor cell Expressed, Developmentally Downregulated-1; also named GCP-WD) (Zhang *et al*, 2009; Haren *et al*, 2006; Luders *et al*, 2006).

NEDD1/GCP-WD

Nedd1 was first described as a developmentally regulated gene during the growth of the mouse central nervous system. Nedd1 is a 72 kDa protein that in human exist at least in four splice variants (a, b, c, d) that differ between each other in a small portion of the N-terminal region, due to a downstream localization of the translation initiation codon respect to the variant “a” (first described, it is the longest one).

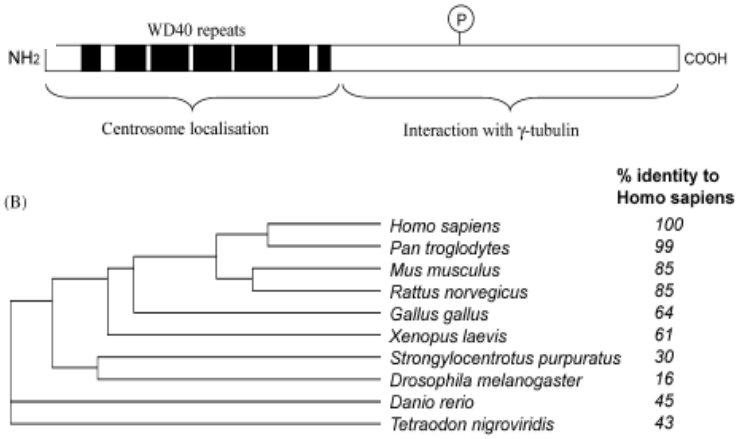


Figure XIX Human Nedd1 structure and identity rate between different species.
Adapted from (Manning & Kumar, 2007).

Nedd1 in human is expressed in all tissues, however at low concentration; homologues of this protein exist in several species and the sequence is highly conserved in a region of WD40 repeats located at the N-terminal half of the protein (Fig. XIX).

This protein was classified as a grow suppressor gene because its overexpression in neurons determined the arrest of cell cycle progression (Kumar *et al*, 1994). More recently Nedd1 has been described to localized at centrosomes, having a role in mitotic regulation (Haren *et al*, 2006; Luders *et al*, 2006). Nedd1 in fact localizes to the centrosome both in interphase and in mitosis (Haren *et al*, 2006; Luders *et al*, 2006), however the increase in the amount of centrosomal Nedd1 during mitosis is about 4 fold; additionally, during mitosis Nedd1 also concentrates to the spindle (Manning & Kumar, 2007).

The capacity of Nedd1 to bind γ-tubulin depends on its C-terminal region (Manning *et al*, 2010), while through its N-terminal region Nedd1 binds to the centrosome. Nedd1 is the responsible for the accumulation of γ-tubulin to the centrosome during mitosis (Haren et al., 2006; Luders et al., 2006), but also control the levels of γ-tubulin to the centrosome in interphase (Manning *et al*, 2010) and on top of spindle microtubules (Uehara *et al*, 2009). The ability of Nedd1 to recruit γ-tubulin to the centrosome during mitosis is regulated by Plk1 and Cdk1 phosphorylation (Zhang *et al*, 2009; Haren *et al*, 2009). Cdk1 phosphorylates Nedd1 at Thr550 and this phosphorylation serves as a priming for Plk1 phosphorylation of Nedd1 at several sites (Thr382, Ser397, Ser637 and Ser426). Plk1 Nedd1[S426] phosphorylation is responsible for the localization of Nedd1 to the spindle and the consequent γ-tubulin recruitment there. Mutants that do not permit this phosphorylation show loss of Nedd1 and γ-tubulin recruitment to the mitotic spindle (Haren *et al*, 2009). Moreover, the dowregulation of Plk1 by siRNA or its chemical inhibition determine an important decrease of Nedd1 and γ-tubulin to the spindle and mitotic centrosome (Zhang *et al*, 2009; Haren *et al*, 2009), indicating that Plk1 is indispensable for the recruitment of Nedd1 at both structures. The mechanism by which Plk1 controls the centrosomal accumulation of Nedd1 was not described at the beginning of this work. This process does not seem to be controlled by a direct Plk1 phosphorylation (Haren *et al*, 2009; Zhang *et al*, 2009), indicating a possible missing intermediate player between Plk1 and

Nedd1 in the recruitment of γ-TuRC to the mitotic centrosome, hypothesis investigated in this thesis (see ARTICLE 2 below)

REGULATION OF MICROTUBULE NUCLEATION

Microtubule nucleation is regulated by many factors and can start from several sites in the mitotic cell (microtubule organizing centers or MTOCs).

Centrosome are the best described MTOC and polymerization of new microtubules from them depends on the recruitment of γ-tubulin and the anchoring of this protein (as part of the γ-TuRC) on the surface of the centrosome. This recruitment is Nedd1 dependent (Haren et al. 2006; Luders, Patel, and Stearns 2006; Zhang et al. 2009) while the anchoring is mediated by several proteins such as Cdk5/Rap2 (Fong *et al*, 2008) or pericentrin (Zimmerman *et al*, 2004; Haren *et al*, 2006).

Although centrosomes are the most prevalent MTOCs in cells that have these orgnalelles, it is known that higher plants (Shimamura et al. 2004) as well as some animals during their meiotic division, like mouse (Calarco-Gillam et al. 1983), *Xenopus* (Heald et al. 1996) and *Drosophila* (Matthies et al. 1996) do not have centrosomes. Moreover laser ablation of both centrosomes during prophase cells does not disrupt bipolar spindle (Khodjakov et al. 2000), indicating the involvement of other non-centrosomal mechanisms of microtubule nucleation.

Non-centrosomal sites of microtubule nucleation are kinetochores (KMTs), the chromatin and preexisting microtubules; from there, nucleation of microtubule is generated in a centrosome-independent manner (McGill and Brinkley 1975). Chromatin-directed microtubule assembly is driven by the small GTPase Ran (Kalab, Pu, and Dasso 1999) and the localization of its guanine nucleotide exchange factor, RCC1 (Moore, Zhang, and Clarke 2002), that is bound to chromatin through the cell cycle and start to produce a cytoplasmatic gradient of Ran-GTP after NEB. The Ran-GTP gradient promotes the liberation from importins of cargo proteins as spindle assembly factors (like TPX2), necessary for the target of essential mitotic proteins like Eg5 (Wittmann et al. 2000) and HURP (Wong and Fang 2006) to the minus end of microtubules. TPX2 is also able to interact with Aurora A and contributes to its activation. Aurora A recruits a complex formed by Msps/XMAP215, Eg5 and HURP to the microtubules to stabilize them (Koffa et al. 2006), even though HURP has

been shown to be able to do that in an Aurora A independent manner (Ran-GTP dependent) (Casanova et al. 2008).

Moreover, the CPC (consisting of Aurora B, INCENP, survivin and borealin/DRASDA) is able to bind microtubules and stabilize them, localizing to the chromosome arms during early prometaphase. Aurora B is in fact able to hyperphosphorylates Op18/Stathmin, an inhibitor of microtubule polymerization, and blocks its activity reducing microtubules dynamics, contributing to the formation of a bipolar spindle (Andersen et al. 1997). CPC has also a direct role in microtubule nucleation because this complex can promote microtubules polymerization without the involvement of the Ran-GTP gradient (Kelly et al. 2007), while the contrary is not true (Maresca et al. 2009).

Additionally to these mechanisms, the HAUS complex can start nucleation of microtubules directly from the growing spindle. This complex is formed by 8-subunit proteins that share homology with augmin in *Drosophila*. The HAUS complex can interact directly with the γ-TuRC complex, interaction that is mediated by Nedd1 (Texido-Travesa et al. 2010), targets γ-TuRC to the microtubules of the spindle and promotes nucleation of other microtubules, resulting in a denser and stronger spindle (Goshima et al. 2008; Uehara et al. 2009).

Finally, every structure able to bind “microtubules nucleators” can act as nucleation site. For istance, NE contains nucleoporin proteins that are shown to be able to interact with γ-tubulin (Rebollo et al. 2004).

REGULATION OF SPINDLE ASSEMBLY

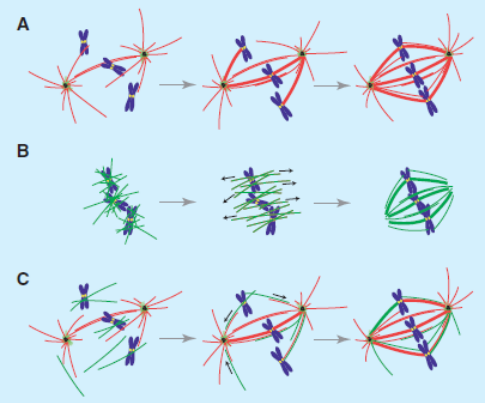


Figure XVII Models of spindle assembly. **(A)** 'Search-and-Capture': microtubules nucleate from centrosomes and contact chromosomes and kinetochores by chance. **(B)** 'Self-Organization': randomly oriented microtubules nucle- ated in the absence of centrosomes are organized into a bipolar array. **(C)** Combined model: peripheral microtubules or those emanating from chromosomes are captured and incorpo- rated into the centrosome-nucleated array to generate the spindle. Microtubules nucleated by the centrosome are labeled in red, microtubules that are not, are labeled in green.
Adapted from (Gadde & Heald, 2004).

In most cells, different mechanisms of spindle formation cooperate between them and are needed to assure the correct segregation of the sister chromatids and guarantee a correct division of the genetic information between the two daughter cells. In animal cells the main characters of the spindle are centrosomes, chromosomes and microtubules and the collaboration between forces produced by them results in the formation of the bipolar spindle.

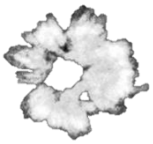
The first hypothesis of spindle formation has been called search-and-capture hypothesis. As its name says, the search-and-capture hypothesis states that microtubules growing from centrosome elongate until capture chromosomes, interacting with their kinetochores, to align them at the metaphase plate (Hayden *et al*, 1990; Alexander & Rieder, 1991). Although theoretically this hypothesis could explain how the spindle is build, in reality it shows many limitations, as spindle microtubules, moving randomly, would need several hours to attach all kinetochores and assembly the metaphase plate (Wollman *et al*, 2005), indicating that other mechanism are needed to optimize the timing of the process.

Indeed, a second hypothesis of spindle formation involves forces generated by microtubules nucleated at kinetochores or near chromatin. This model explains, at least in part, the building of mitotic spindle in cells where centrosomes are absent (Khodjakov *et al*, 2000; Basto *et al*, 2006). Nucleation of *de novo* microtubules at DNA depends on the activity of the small GTPase Ran (Carazo-Salas *et al*, 2001). This mechanism proceeds in two ways, first

stimulating microtubule nucleation by chromatin (Karsenti & Vernos, 2001) and second concentrating microtubules stabilizing factor (such as TPX2) to the growing spindle (Bastiaens *et al*, 2006). The importance of this pathway results evident because changes in the Ran-GTP gradient during the first steps of spindle formation provoke disruption of the premature spindle; even though these mechanisms are dispensable for the maintenance of the mitotic bipolar spindle (Petr *et al*, 2006).

Formation of microtubules in the vicinity of chromatin and from kinetochores also improves the search-and-capture model, since kinetochores result easier to capture. Indeed, DNA-nucleated microtubules can be captured and incorporated into the centrosome-nucleated array to generate the spindle, indicating a kind of cooperation between different mechanism to assure the correct formation of the mitotic spindle (Wadsworth & Khodjakov, 2004; Gadde & Heald, 2004) (Fig. XVII).

OBJECTIVES



The objective of this doctoral thesis is to describe the possible roles of the NIMA related kinases Nek6, Nek7 and Nek9 in the regulation of the centrosome cycle, and especially in those phases of this cycle (centrosome separation and maturation) which take place during early mitosis.

MATERIALES AND METHODS

Relative to Additional Results

CELL CULTURE

HeLa and HTC116 cells were cultured in a 5% CO2 atmosphere and 37ºC in DMEM (Dubelco's modified Eagle's medium) suplemented with 10% FBS (Foetal Bovine Serum), L-glutamine (2mM), penicillin streptomycin (100 IU/ml and 100 µg/ml, respectively).

TRANSFECTION

Cells were transfected with siRNA using Lipofectamine™ 2000 (Invitrogene) according to the manufacturer instructions and (in the case of) arrested in G2/M transition after 24 hours post transfection using the small Cdk1 inhibitor RO-3306 (ENZO Biotechnology).

The sequence of the siRNA duplex for targeting Nek2 was: 5'-GAAAGGCAATACTTAGATGdTdT-3'.

The sequence of the siRNA duplex for targeting Nek9 was: 5'-AAUAGCAGCUGUGUGAGUCUUGCCU -3'.

CELL EXTRACTS AND WESTERN BLOT ANALYSIS

Cells were lysed with lysis buffer that contained 50mM de Tris (pH 7.5), 100mM NaCl, 50mM NaF, 1mM DTT, 1mM EDTA, 1mM EGTA, 10 mM β-glycerophosphate, 2 mM Na3VO4 , 25 nM calyculin A, 1% TX100, 0.5mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin. The cytosolic fraction was obtained by centrifugation at 13200 rpm for 10 minutes. Protein concentrations were determined using the Bradford reagent (BioRad).

Membranes were probed with anti-Nek9 antibody (Belham *et al*, 2003) and anti-Nek2 antibody. Secondary antibodies were from Jackson Immuno Research Laboratories (West Grove, PA, USA) and were detected by ECL chemiluminiscence (Thermo Scientific).

CELL CYCLE ANALYSIS

Cells were washed twice with PBS, treated with trypsin and resuspended in 5 ml of PBS. Cell pellets retrieved after centrifugation (200 g) were mixed with 0,5ml of PBS and 4,5 ml of ethanol 70% and fixed for 2 h at -20ºC. Subsequent centrifugation of the samples was followed by a wash in PBS and

staining with a PBS solution containing 10% triton X-100 (sigma), 20 ug/ml propidium iodide (sigma) and 2 mg/ml RNAsa A (DNAsa free- sigma) at 37ºC for 15 min. Cells were analysed using a CouLter XL (Beckman CouLter) analyser.

IMMUNOCYTOCHEMISTRY

Cells were grown on coverslips and fixed and permeabilized as described earlier (Rapley *et al*, 2008). Primary antibodies used in this study were mouse anti-γ -tubulin (1:500) (Sigma), mouse anti-Nedd1 (1:500) (Abcam), rabbit anti-PCNT (1:2000) (Abcam), rabbit anti-centrin (1:2000), rabbit anti-Cdk5Rap2 (1:100) (Bethyl Laboratories), rabbit anti-PHistone3 phospho-Ser10 (Cell Signalling), rabbit anti-CenpF(1:5000) (Abcam). Primary antibodies were detected with Alexa Fluor 488 goat anti-rabbit IgG (1:250) and alexa Fluor 555 goat anti-mouse IgG (1:250). DNA was stained with DAPI (0,01 mg/ml).

TREATMENT

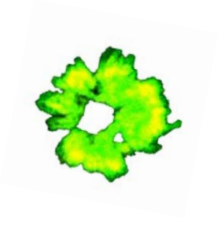
HeLa cells were arrested at G2/M transition with the Cdk1 inhibitor RO-3306 for 20h (9 μM). Arrested cells were released in fresh and pre-warmed DMEM for the indicated times.

FISH ASSAY

Cells were grown on coverslips, trasfected with siRNAs for 48h and then incubated in denaturing solution (Isothermal Denaturing Solution-Cellay). Coverslips where dehydratated with ethanol (85% to 100%) and than incubated with probes during 5' at 37°C.

Probes detected Chr7 centromere, locus D7Z1 (Red-DY590) and Chr8 centromere, locus D8Z2 (Green-DY490) (Cellay).

RESULTS



ARTICLE 1

(Bertran *et al*, 2011)

Thesis author contribution:

Figures 4 to 9

Supplementary Figures S4 to S9

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THE EMBO JOURNAL

Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5

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The NIMA-family kinases Nek9/Nercc1, Nek6 and Nek7 form a signalling module required for mitotic spindle assembly. Nek9, the upstream kinase, is activated during prophase at centrosomes although the details of this have remained elusive. We now identify Plk1 as Nek9 direct activator and propose a two-step activation mechanism that involves Nek9 sequential phosphorylation by CDK1 and Plk1. Furthermore, we show that Plk1 controls prophase centrosome separation through the activation of Nek9 and ultimately the phosphorylation of the mitotic kinesin Eg5 at Ser1033, a Nek6/7 site that together with the CDK1 site Thr926 we establish contributes to the accumulation of Eg5 at centrosomes and is necessary for subsequent centrosome separation and timely mitosis. Our results provide a basis to understand signalling downstream of Plk1 and shed light on the role of Eg5, Plk1 and the NIMA-family kinases in the control of centrosome separation and normal mitotic progression.

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Introduction

The cyclin-dependent kinase CDK1 orchestrates the onset of mitosis through the regulation of multiple proteins either directly or in collaboration with a number of helper kinases (Nigg, 2001), among them the Polo-like kinase Plk1 (Petronczki *et al*, 2008; Archambault and Glover, 2009) and different members of the NIMA family (O'Connell *et al*, 2003). Plk1 is involved in the complex mechanism that culminates in CDK1 activation during mitotic entry and is crucial for different mitotic events including the formation of the spindle. The molecular basis for some of Plk1 functions is only beginning to be understood and relies on the recognition of previously phosphorylated proteins by the Polo-box domain (PBD) of Plk1, which also targets the kinase to different sites of action such as the centrosomes and centromeres (Elia *et al*, 2003). The functions of the various members of the NIMA family of protein kinases (Nek1-11 in mammalian cells) are not so well known as these of Plk1. *Aspergillus nidulans* NIMA, the founding member of the family, is necessary for entry into mitosis and has several roles during mitotic progression, including the regulation of chromosome condensation and spindle formation, although it is not clear whether all these functions are shared with its mammalian counterparts. Different Neks have been implicated in the control of the centrosome and microtubule cytoskeleton (Quarmby and Mahjoub, 2005). Among them Nek2, active in S through G2, regulates premitotic centrosome disjunction, while Nek9 (also known as Nercc1) and the ~80% identical Nek6 and Nek7, active during mitosis, are involved in the control of spindle structure and function (O'Regan *et al*, 2007).

Nek9 is activated at centrosomes during early mitosis, interacts with both Nek6 and Nek7 and directly phosphorylates and activates them (Roig *et al*, 2002, 2005; Belham *et al*, 2003). Microinjection of anti-Nek9 antibodies into prophase cells induces prometaphase arrest and in some cases aberrant chromosome segregation, resulting in mitotic catastrophes or aneuploidy (Roig *et al*, 2002), while Nek9 depletion from *Xenopus* meiotic egg extracts results in delayed spindle assembly, reduced number of bipolar spindles and appearance of aberrant microtubule structures (Roig *et al*, 2005). Downregulation of either Nek6 or Nek7 by RNAi delays cells at metaphase with fragile mitotic spindles (O'Regan and Fry, 2009) and for Nek7 has been shown to result in an increased incidence of multipolar spindle phenotypes (Yissachar *et al*, 2006). Mice lacking Nek7 die during late embryogenesis or at early postnatal stages, and Nek7 (−/−) cells show increased tendency for chromosomal lagging as well as abnormalities in primary cilia number (Salem *et al*, 2010). Nek6-deficient mice die early during embryogenesis (our unpublished results). Thus, Nek9 together with Nek6/7 form a mitotically activated module with key roles during mitotic progression and more specifically spindle organization (Nek6 and Nek7 seem to be functionally equivalent in most instances, thus when adequate the two kinases will be collectively referred to as Nek6/7). Nevertheless, to this date a clear picture of the module activation mechanism, integration with other mitotic signalling systems and precise functions during mitosis has been missing.

We have previously suggested that Nek9 could be controlling spindle organization in part through the action of Nek6/7 and their ability to phosphorylate the kinesin Eg5 at a site necessary for normal mitotic progression (Rapley *et al*, 2008). Here, we present data showing that Plk1, in conjunction with CDK1, activates Nek9 early in mitosis, and that downstream of Plk1, Nek9 and Nek6/7 are responsible for centrosome separation during prophase through the control of Eg5

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recruitment to centrosomes. Our results emphasize Nek9, Nek6 and Nek7 importance in mitotic signalling and describe the molecular mechanism controlling the separation of the centrosomes during prophase.

Results

Nek9 mitotic phosphorylation sites

Nek9 is phosphorylated at unknown sites during mitosis resulting in a change in electrophoretical mobility (Roig *et al*, 2002). This does not directly result in Nek9 activation, a process that occurs at centrosomes during prophase, involves only a small (<5%) fraction of Nek9 and requires further phosphorylation of Nek9 activation loop (Roig *et al*, 2005). To better understand this two-step activation mechanism and identify the protein kinases responsible for the described modifications, we immunoprecipitated endogenous Nek9 from exponentially growing and mitotic HeLa cells and identified the sites of phosphorylation present by mass spectrometry (MS) analysis (Supplementary Figure S1). Approximately 80% of sequence coverage was obtained in each case, leading to the identification of four Nek9 phosphosites from exponentially growing cells and six from mitotic cells. None of these sites corresponded to known Nek9 activation loop or autophosphorylation sites (Roig *et al*, 2005), thus indicating that the analysed sample contained mostly inactive Nek9. All sites modified in exponential cells (Ser29, Thr333, Ser750 and Ser869) were also present in mitotic cells, although a higher phosphorylated/unphosphorylated peptide ratio indicated that the corresponding phosphosites were more abundant in mitotic cells (see Supplementary Figure S1). Additionally, phosphorylated Ser827 and Thr885 were only detected in Nek9 from mitotic cells. All identified sites but Thr333 conform to a [ST]P sequence, and thus are putative phosphorylation sites for CDK1, a protein kinase that we have shown is able to readily phosphorylate Nek9 *in vitro* (Roig *et al*, 2002 and also see below).

Plk1 interacts with Nek9 through the PBD

Three of the Nek9 phosphorylation sites identified, Ser29, Ser750 and Ser869, conform to a S[S/T]P sequence, a motif that when phosphorylated at the serine/threonine immediately preceding the proline (usually by proline-directed protein kinases such as CDK1) can be recognized by Plk1 PBD (Elia *et al*, 2003). Thus, we tested whether Plk1 could interact with Nek9 in exponentially growing and mitotic cells. Figure 1A shows that Plk1 specifically coimmunoprecipitates with Nek9 in mitosis in HeLa cells (similar results were obtained with mouse embryo fibroblasts, see Supple-

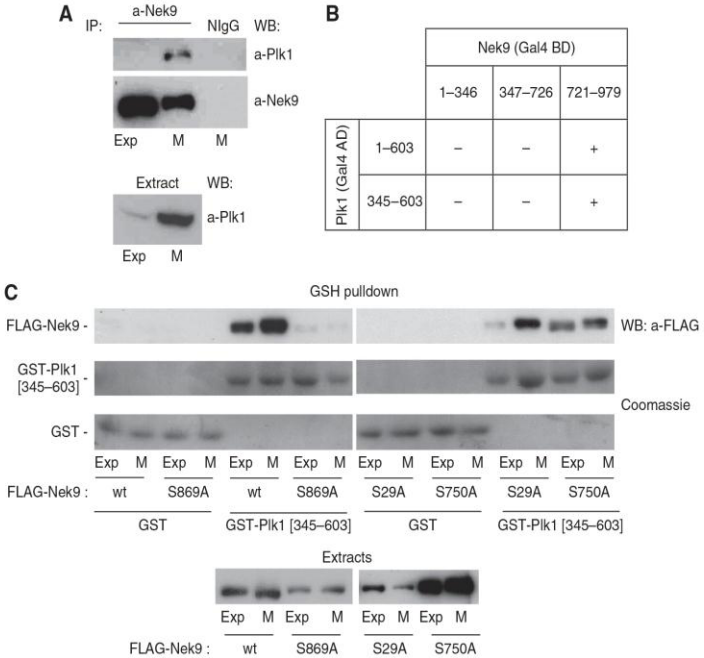


Figure 1 Plk1 interacts with Nek9 through the PBD. (**A**) a-Nek9 or normal IgG (NIgG) immunoprecipitates from exponentially growing (Exp) or nocodazole-arrested mitotic (M) HeLa cell extracts were analysed by western blot (WB) using the indicated antibodies. Plk1 in the corresponding extracts is shown in the lower panel. (**B**) The ability of the full-length Plk1 (1–603) or Plk1 PBD (345–603) to interact with the different domains of Nek9 (kinase domain: 1–346; RCC1 domain: 347–726; C-terminal tail:721–979) was assessed using the two-hybrid assay (see Supplementary Figure S2B). Gal4 AD/BD, Gal4 activation/binding domains. (**C**) *In vitro* binding of different Nek9 forms to GST-Plk1 PBD. Extracts of exponentially growing (Exp) or nocodazole-arrested mitotic (M) HeLa cells expressing the indicated FLAG-tagged forms of Nek9 were incubated with GST or GST-PBD (*GST-Plk1[345–603]*) bound to GSH beads. After repeated washes, bound Nek9 was detected by WB with a-FLAG antibody, and GST-fusion proteins by Coomassie staining. FLAG-Nek9 in the corresponding extracts is shown in the lower panel.

mentary Figure S2A). This interaction could also be detected using the yeast two-hybrid system (Figure 1B; Supplementary Figure S2B), allowing us to map the Nek9–Plk1 interaction to the C-terminal tail of Nek9 (where two of the three Nek9 putative PBD-binding sites reside), and the PBD domain of Plk1. To confirm Nek9 binding to the PBD and to identify the Nek9 S[S/T]P phosphorylation sites responsible for interaction, we tested whether bacterially expressed Plk1 PBD fused to GST (GST-Plk1[345–603]) could bind different recombinant forms of FLAG-tagged Nek9 from cell extracts. Figure 1C shows that FLAG-Nek9 wild type was able to interact with GST-Plk1 PBD but not GST beads. The Nek9–PBD interaction was increased in mitotic extracts and was totally abrogated by mutation of Nek9 Ser869 to the non-phosphorylable residue alanine. Mutation of Nek9 Ser29 to alanine did not have any effect (consistently with our two-hybrid results), while mutation of Ser750 had only a minor effect on the binding. We concluded that Nek9 specifically binds Plk1 during mitosis through an interaction between phosphorylated Nek9[Ser869] and Plk1 PBD. This is further supported by additional experiments showing that in contrast to wild-type Nek9, Nek9[S869] does not interact with endogenous Plk1 in mitosis (see Supplementary Figure S2C).

Plk1 phosphorylates and activates Nek9 in vitro

We next tested whether Plk1, alone or in combination with CDK1, could phosphorylate Nek9. For this we used purified kinase-deficient FLAG-Nek9[K81M] (Roig *et al*, 2002). As expected from our previous results (Roig *et al*, 2002), FLAG-Nek9[K81M] was phosphorylated by purified CDK1/cyclin B complexes (Figure 2A). Purified Plk1 readily phosphorylated FLAG-Nek9[K81M] to a similar extent (up to ~6 mol of phosphate/mol of protein), and in the *in vitro* conditions used showed only slight or no synergy with CDK1. Phosphorylation of Nek9 at Thr210 in the kinase activation loop is required for Nek9 activation (Roig *et al*, 2005); using a phosphospecific antibody that recognizes Nek9[Thr210-P] (Roig *et al*, 2005), we determined that Plk1 was able to modify this site *in vitro* (Figure 2B; Supplementary Figure S3). Under identical conditions, CDK1/cyclin B and the non-relevant kinase JNK1 were not able to phosphorylate

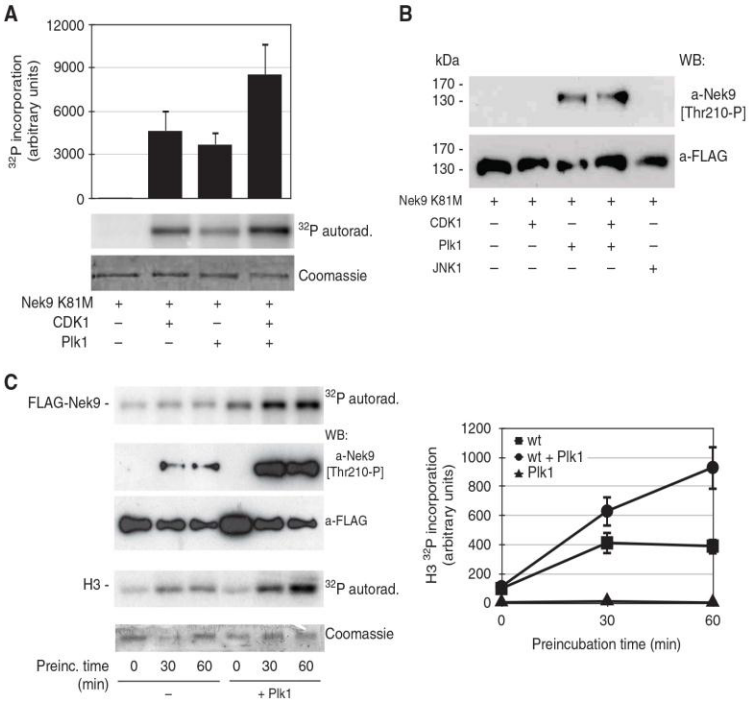


Figure 2 Plk1 phosphorylates and activates Nek9. (**A**) Kinase-defective FLAG-Nek9[K81M] was expressed and purified from 293T cells and incubated with the indicated kinases for 30 min at 30°C in the presence of [γ-^{32}P]ATP/Mg^{2+}. After SDS–PAGE, Nek9 was visualized by Coomassie staining, and ^{32}P incorporation was visualized by autoradiograph (lower and middle panels) and quantified by PhosphorImager (upper graph, mean ± s.e.m. of three independent experiments). *CDK1*, CDK1/cyclin B. (**B**) FLAG-Nek9[K81M] obtained as in (**A**) was incubated with the indicated kinases for 60 min at 30°C in the presence of ATP/Mg^{2+} and analysed by western blot (WB) using the indicated antibodies. *CDK1*, CDK1/cyclin B. (**C**) FLAG-Nek9 was expressed and purified from 293T cells and incubated with or without purified Plk1 in the presence of ATP/Mg^{2+} for the indicated times at 25°C. After incubation, [γ-^{32}P]ATP/Mg^{2+} and histone H3 were added to the reactions and further incubated for 10 min. ^{32}P incorporation into Nek9 and H3 was visualized by autoradiograph. In parallel, total Nek9 and Nek9[Thr210-P] were visualized by WB using the indicated antibodies (left, lower panels), and H3 was visualized by Coomassie staining (left). ^{32}P incorporation into H3 was quantified by PhosphorImager (right graph, mean ± s.e.m. of three independent experiments).

Nek9[Thr210]. To determine whether, as expected from previous data, Nek9[Thr210] phosphorylation by Plk1 resulted in Nek9 activation, we incubated purified FLAG-Nek9 without or with Plk1 in the presence of [γ-^{32}P]ATP/Mg^{2+}. After different times, the model substrate histone H3 was added, and ^{32}P incorporation into H3 quantified (Figure 2C). The ATP/Mg^{2+} concentration used (100 μM) supports Plk1 phosphorylation of Nek9, but also Nek9 autoactivation through autophosphorylation (Roig *et al*, 2005); thus, in the absence of Plk1, Nek9 phosphorylation and activity towards histone H3 increased with time. Nevertheless, Plk1 induced a further increase in Nek9 phosphorylation when present, including Nek9 phosphorylation in the activation loop at Thr210, and a concomitant increase of activity towards histone H3 (that it is not a substrate of Plk1). We therefore concluded that *in vitro* Plk1 is able to directly activate Nek9.

CDK1 and Plk1 are necessary for Nek9 activation in vivo during mitosis

We next determined whether CDK1 and Plk1 activities were necessary for Nek9 activation *in vivo* during mitosis. We arrested cells in metaphase with the proteasome inhibitor MG132. As expected from previous data (Roig *et al*, 2005), this induced a shift in Nek9 electrophoretical mobility (a result of Nek9 mitotic phosphorylation) as well as Nek9[Thr210] phosphorylation (Figure 3). The APC/C subunit Cdc27 was used as readout for CDK1 and Plk1 activities, as it changes its apparent MW in response to changes in phosphorylation by both kinases (van Vugt *et al*, 2004). When under these conditions CDK1 was inhibited with Purvalanol A (Gray *et al*, 1998), neither Nek9 reduced electrophoretical mobility nor phosphorylation at Thr210 could be observed. Inhibition of Plk1 with BI2536 (Lénárt *et al*, 2007) although not affecting Nek9 reduced electrophoretical mobility completely abrogated the Nek9[Thr210-P] signal. Thus, while CDK1 activity is necessary for Nek9 phosphorylation in mitosis and the resulting change in electrophoretical mobility, Nek9 Thr210 phosphorylation and mitotic activation requires both CDK1 and Plk1.

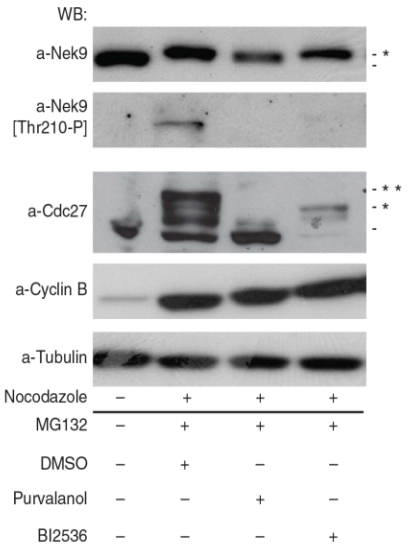


Figure 3 CDK1 and Plk1 are necessary for Nek9 activation during mitosis. HeLa cells were arrested in mitosis with nocodazole. Mitotic cells were collected, washed and released in media containing MG132 (20 μM) plus DMSO, Purvalanol A (20 μM) or Bi2536 (100 nM) for 2 h, and cell extracts were analysed by western blot (WB) using the indicated antibodies. Untreated cells are shown in the first lane as a control. Asterisks mark protein bands with altered mobility due to phosphorylation.

Plk1, Nek9, Nek6, Nek7 and the mitotic kinesin Eg5 are necessary for normal centrosome separation during prophase

One of the most conspicuous functions of Plk1 is the control of centrosome separation during early mitosis (Lane and Nigg, 1996), although how the kinase performs this function remains unknown. In mammalian cells (Whitehead and Rattner, 1998; Tanenbaum *et al*, 2008; Woodcock *et al*, 2010), prophase centrosome separation depends on the activity of Eg5 (kinesin-5), a BimC-family kinesin that is like Plk1 involved in the assembly and maintenance of a bipolar spindle during mitosis by sliding anti-parallel microtubules apart (Sawin *et al*, 1992; Blangy *et al*, 1995; Kapitein *et al*, 2005). Eg5 is a substrate for Nek6 (Rapley *et al*, 2008) and Nek7 (our unpublished data), and therefore we sought to determine whether the Nek9/Nek6/7 module could provide a link connecting Plk1 and Eg5 in the context of centrosome separation. For this we analysed the effects of Plk1, Eg5, Nek9, Nek6 or Nek7 downregulation by RNAi on the extent of separation of duplicated centrosomes in prophase cells (Figure 4).

Our results confirmed the requirement for Eg5 and Plk1 for mitotic centrosome separation prior to nuclear envelope breakdown in HeLa cells. While prophase cells transfected with control siRNA mainly contained centrosomes that were separated >2 μm (and frequently located at opposite sides of the nucleus), in cells with reduced amounts of Eg5 and Plk1 centrosomes were for the most part overlapping or separated <2 μm. Similarly, Nek6, Nek7 or Nek9 depletion, as well as combined Nek6 and Nek7 or Nek6, Nek7 and Nek9 depletion, resulted in a diminished number of prophase cells with centrosomes separated >2 μm and in the appearance of a significant number of prophase cells with either unseparated centrosomes or centrosomes separated but closer than 2 μm (Figure 4A and C). Furthermore, even when centrosomes were separated >2 μm, intercentrosomal distances were greatly diminished when compared to these of control cells, and in almost no cases reached 9 μm, the control median centrosomal separation in prophase (Figure 4A, box plot). Downregulation of Nek6, Nek7 or Nek9 resulted in some cases in the appearance of cells that contained more than two centrosomes. Costaining with anti-centrin antibody (Supplementary Figure S4A) confirmed that this was not the result of PCM fragmentation and suggested that supernumerary centrosomes could be the result of abortive mitosis, a hypothesis that is supported by the frequent observation of multiple nuclei associated to the existence of more than two centrosomes.

Our observations could be repeated with alternative siRNAs against Nek6 and Nek7 (Supplementary Figure S4B) as well as against Nek9 (Figure 4B), thus confirming the specificity of our results. Furthermore, the use of siRNAs

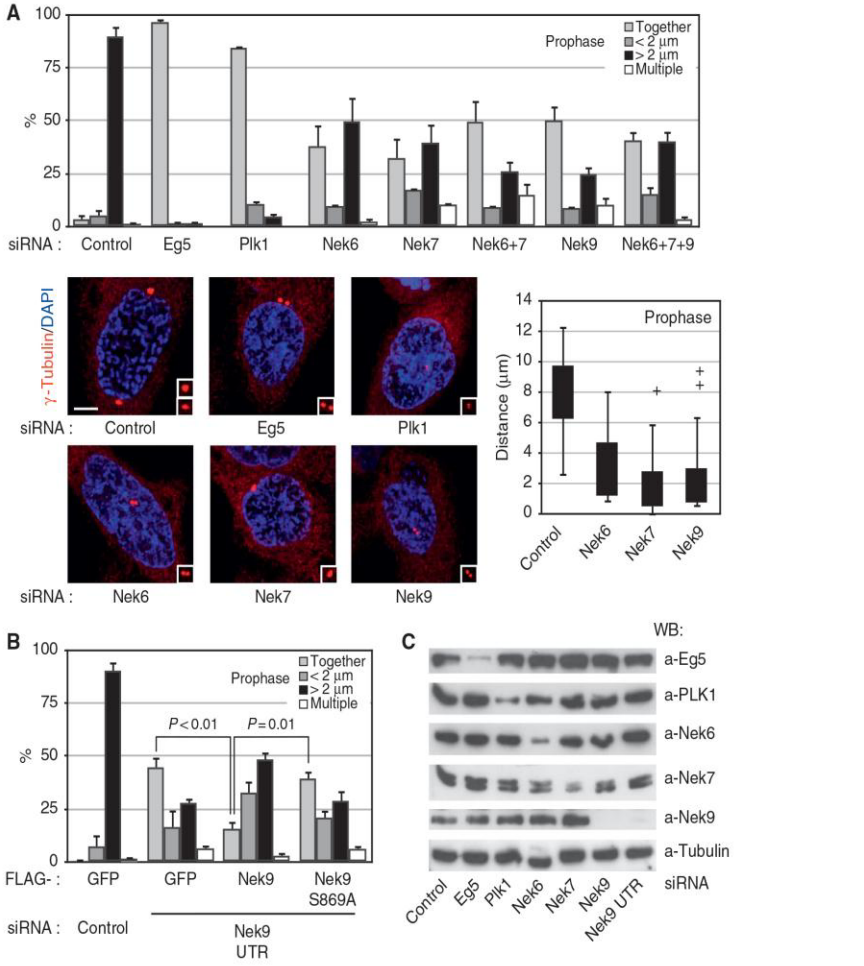
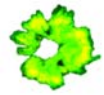


Figure 4 Plk1, Nek9, Nek6, Nek7 and Eg5 are necessary for normal centrosome separation in prophase. (**A**) HeLa cells were transfected with the indicated siRNAs, and after 24 (Eg5, Plk1) or 48 (control, Nek6, Nek7, Nek9) hours, fixed and stained with antibodies against γ-tubulin (red) and DAPI (blue). Cells showing condensed chromosomes and intact nuclei (assessed by the shape of the DNA and a γ-tubulin exclusion from the nucleus) were scored as in prophase (these cells were 100% positive for histone H3[Ser10] phosphorylation, thus confirming the cell-cycle phase assignation, data not shown). The percentage of prophase cells showing two unseparated centrosomes (together), two centrosomes separated <2 µm (<2 µm), fully separated centrosomes (>2 µm) or more than two centrosomes (multiple centrosomes) is shown in the upper graphic (mean ± s.e.m. of three independent experiments; ~50 cells counted in each experiment). Additionally, the distribution of distances from the centre of the duplicated centrosomes in each case is shown as a box plot (boxes show the first and third quartiles, whiskers mark minimum and maximum values unless these exceed 1.5 × interquartile range and crosses correspond to outliers; 20 cells counted for each experimental condition). Representative examples of the observed phenotypes are shown (bar, 5 µm). In each case, insets show magnified centrosomes. (**B**) HeLa cells were cotransfected with either control or Nek9 3′ UTR siRNAs plus expression plasmids for the indicated FLAG-tagged proteins, and 48 h latter processed and FLAG-positive cells scored as in (**A**) (mean ± s.e.m. of three independent experiments; ~40 cells counted in each experiment; statistical significance was determined using the standard Student's *t*-test). Levels of endogenous and recombinant Nek9 as determined by western blot are shown in Supplementary Figure S4C. (**C**) Efficiency of the different RNAi treatments used in (**A**) or (**B**) as determined by western blot of total cell extracts.

directed against Nek9 mRNA 3′ UTR allowed us to down-regulate the levels of endogenous kinase without affecting our ability to express different recombinant forms of Nek9 (Figure 4B; Supplementary Figure S4C). Cotransfection of Nek9 wild type partially rescued the effect of the Nek9 UTR siRNAs, significantly reducing the number of cells with unseparated centrosomes while increasing the percentage of cells with fully separated centrosomes. In contrast, Nek9[Ser869Ala], which is unable to bind Plk1 PBD (see Figure 1C and Supplementary Figure S2C), although expressed at similar levels that wild-type Nek9, was not able to significantly rescue the observed effects of endogenous Nek9 downregulation, thus further stressing the relationship between Plk1 and Nek9 functions.



Active Nek9 and Nek6 induce centrosome separation in an Eg5-dependent manner

We next sought to determine whether the activation of the Nek9/Nek6/7 module could be sufficient to induce centrosome separation. For this, we artificially increased the cellular activity of either Nek9 or Nek6 by expressing Nek9[Δ346–732], a constitutively active form of the kinase that lacks the autoinhibitory RCC1 domain (Roig *et al*, 2002), or wild-type Nek6 (partially active when expressed above endogenous levels; Belham *et al*, 2003). To test whether active Nek9 and Nek6 exerted their effect through the regulation of Eg5, we simultaneously transfected the cells with control or Eg5 siRNAs. Figure 5A shows that expression of Nek6 or Nek9[Δ346–732] significantly increased the number of cells with separated centrosomes. The effect was cell-cycle independent, as the expression of Nek6 or Nek9[Δ346–732] did not change the cell-cycle profile of the cells (as assessed by FACS; Supplementary Figure S5) and consequently most of the transfected cells that contained separated centrosomes were in interphase (Figure 5A, see example cells). Centrosome separation was not induced by

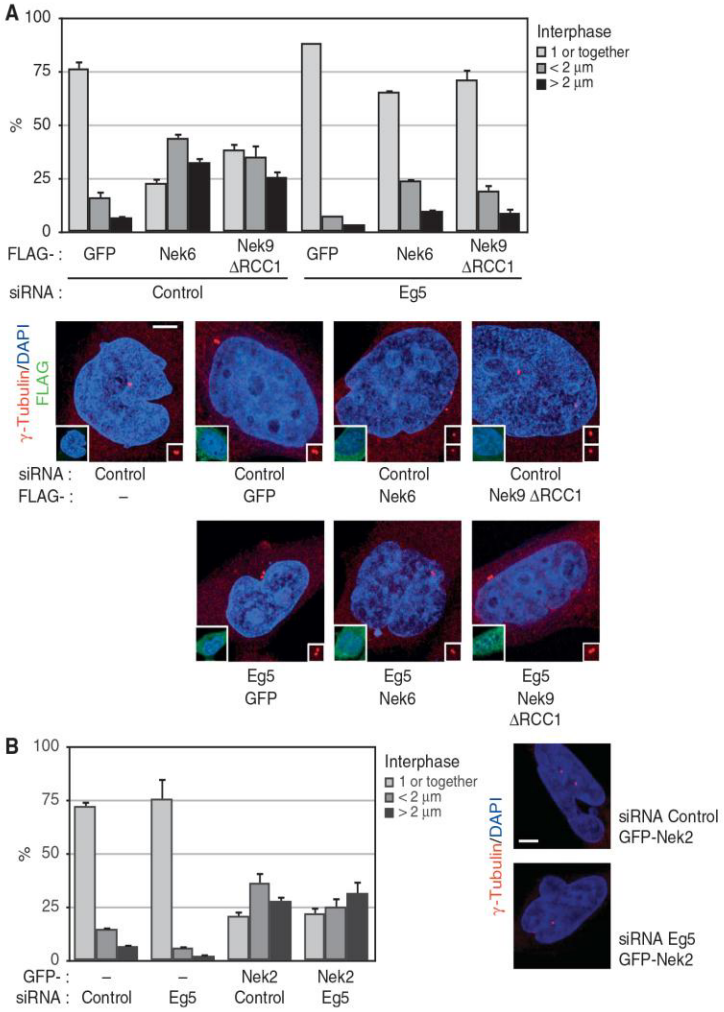


Figure 5 Active Nek9 and Nek6 induce centrosome separation in an Eg5-dependent manner. (**A**) HeLa cells were transfected with either control or Eg5 siRNAs, after 16 h retransfected with expression plasmids for the indicated FLAG-tagged proteins (*Nek9ΔRCC1*, Nek9[Δ346–732]) and 24 h latter fixed and stained with anti-γ-tubulin (red) and anti-FLAG (green) antibodies plus DAPI (blue). The percentage of FLAG-positive cells showing 1 or 2 unseparated centrosomes (1 or together), two centrosomes separated <2 μm (<2 μm) or fully separated centrosomes (>2 μm) is shown in the upper graphic (mean ± s.e.m. of three independent experiments; ~50 cells counted in each experiment). Representative examples of the observed phenotypes (anti-γ-tubulin plus DAPI) are shown below (bar, 5 μm). Insets show the same field stained with anti-FLAG plus DAPI. The effect of the different treatments on the levels of Eg5 can be seen in Figure 6, upper right panel. (**B**) As in (**A**), cells transfected with either control or Eg5 siRNAs and expression plasmids for GFP or GFP-Nek2 (mean ± s.e.m. of three independent experiments; ~50 cells counted in each experiment).

wild-type Nek9 or Nek7, which are not active under the expression conditions used, by neither kinase-deficient Nek9 [K81M,Δ346–732] or Nek6[K74,75M] (data not shown).

Remarkably, downregulation of Eg5 almost totally abrogated Nek6 or Nek9-induced centrosome separation, suggesting that Nek6 and Nek9 induce centrosome separation through the regulation of the kinesin. The dependence on Eg5 additionally distinguished Nek9/Nek6-induced centrosome separation from Nek2-induced centrosome disjunction, the dissolution of the physical link that keeps together duplicated centrosomes, precedes separation and is controlled by this NIMA-family kinase (Fry *et al*, 1998; Faragher and Fry, 2003). Nek2 effects on centrosomes were totally independent of Eg5 (Figure 5B) and can be attributed to non-directed drifting of the disjointed centrosomes after Nek2 ectopic activation in interphase.

Active Nek9 and Nek6 can rescue Plk1 but not Eg5 downregulation in prophase centrosome separation

We inquired whether expression of active Nek9 or Nek6 could compensate for either Eg5 or Plk1 downregulation during prophase centrosome separation. For this we transfected cells with control, Eg5 and Plk1 siRNAs in combination with expression plasmids for GFP (control), active Nek9[Δ346–732] or Nek6 and determined the distance between centrosomes in transfected prophase cells (Figure 6). As expected, most of control cells contained fully separated centrosomes; expression of either Nek9[Δ346–732] or Nek6 did not change this significantly (not shown). In accordance with our previous results, depletion of Eg5 abrogated prophase centrosome separation and active Nek9 or Nek6 expression was not able to rescue this effect. In cells depleted of Plk1 and expressing GFP as a control protein, centrosome separation in prophase was almost completely abolished, but in contrast, expression of active Nek9[Δ346–732] or Nek6 in Plk1-depleted cells was able to restore the percentage of cells with separated centrosomes to levels similar to control cells (see also box plot indicating the distribution of intercentrosomal distances in individual cells).

Our results show that Nek9, Nek6 and Nek7 act downstream of Plk1 and upstream of Eg5 during early centrosome separation and suggest that Plk1 inhibition precludes centrosome separation as a result of the failure to activate the Nek9/Nek6/7 module.

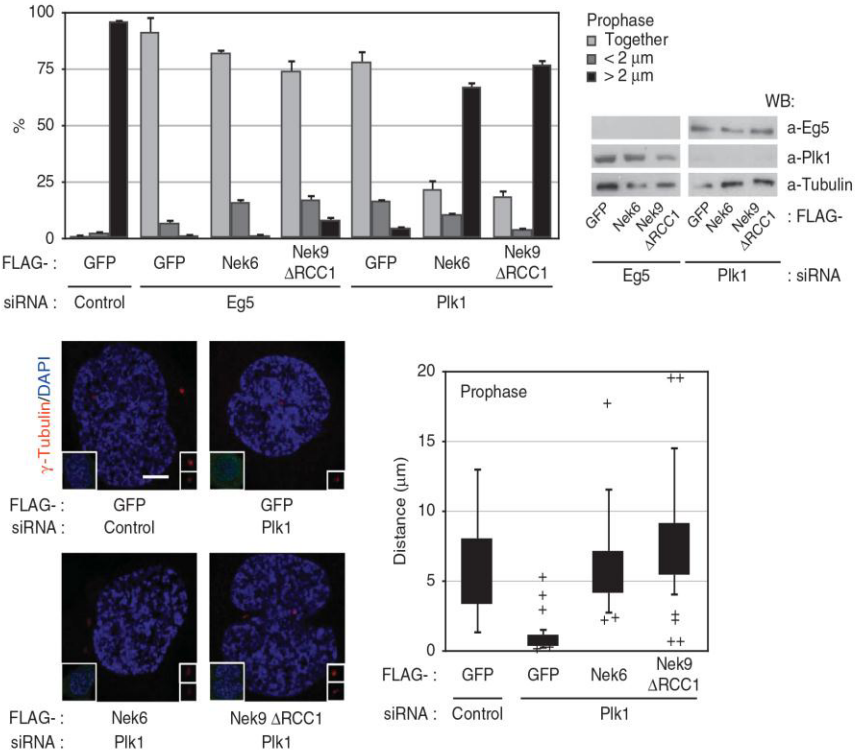


Figure 6 Active Nek9 and Nek6 can rescue Plk1 but not Eg5 downregulation in prophase centrosome separation. HeLa cells were transfected with control, Eg5 or Plk1 siRNAs plus the indicated plasmids and processed as in Figure 5 (*Nek9ΔRCC1*, Nek9[Δ346–732]). The percentage of FLAG-positive prophase cells showing two unseparated centrosomes (together), two centrosomes separated <2 μm (<2 μm) or fully separated centrosomes (>2 μm) is shown in the upper graphic (mean ± s.e.m. of three independent experiments; ~40 cells counted in each experiment). The effect of the different transfections on the levels of Plk1 and Eg5 is shown (right). Lower panels show representative examples of the observed phenotypes (anti-γ-tubulin plus DAPI staining, bar, 5 μm; insets show the same field stained with anti-FLAG plus DAPI) and a box plot of the distribution of distances from the centre of the duplicated centrosomes in FLAG-positive cells (as in Figure 4; 30 cells counted for each experimental condition).

Plk1 controls Eg5 phosphorylation at the Nek6 site Ser1033, that together with the CDK1 site Thr926 is necessary for prophase centrosome separation and Eg5 recruitment

Eg5 is phosphorylated during mitosis at Ser1033, a site that we have previously shown is modified by Nek6/7 (Rapley *et al*, 2008). Eg5[Ser1033-P] accumulates at centrosomes in prophase, and we therefore speculated that Plk1 and Nek9 might control prophase centrosome separation through Nek6/7 phosphorylation of this residue. Using an antibody that specifically recognizes Eg5[Ser1033-P] (Rapley *et al*, 2008), we first confirmed by RNAi that mitotic levels of Eg5[Ser1033-P] depend on Nek6, but also Nek7 and their upstream kinase Nek9 (Supplementary Figure S6A). Next, we sought to determine whether Eg5[Ser1033] phosphorylation also depends on Plk1. For this, we arrested cells in mitosis with nocodazole or by depleting either Plk1 or Eg5 by RNAi. Mitotic arrest was confirmed by FACS (not shown) and the phosphorylation state of Cdc27, and the levels of Eg5[Ser1033-P] were compared with those present in exponentially growing cells. Figure 7A shows that Eg5[Ser1033-P] was detected in nocodazole-arrested cells but not in exponentially growing cells. Plk1 downregulation by RNAi resulted in the abrogation of Eg5[Ser1033-P] from mitotic cells. Additionally, Eg5 depletion had a similar effect, thus confirming the specificity of the antibody. Similar results were obtained by using the Plk1 inhibitor BI2536 that resulted in mitotic cells without any observable Eg5[Ser1033-P] accumulation (Supplementary Figure S6B).

We have previously shown that Eg5[Ser1033] phosphorylation is necessary for normal mitotic progression (Rapley *et al*, 2008). We now tested whether phosphorylation of Ser1033

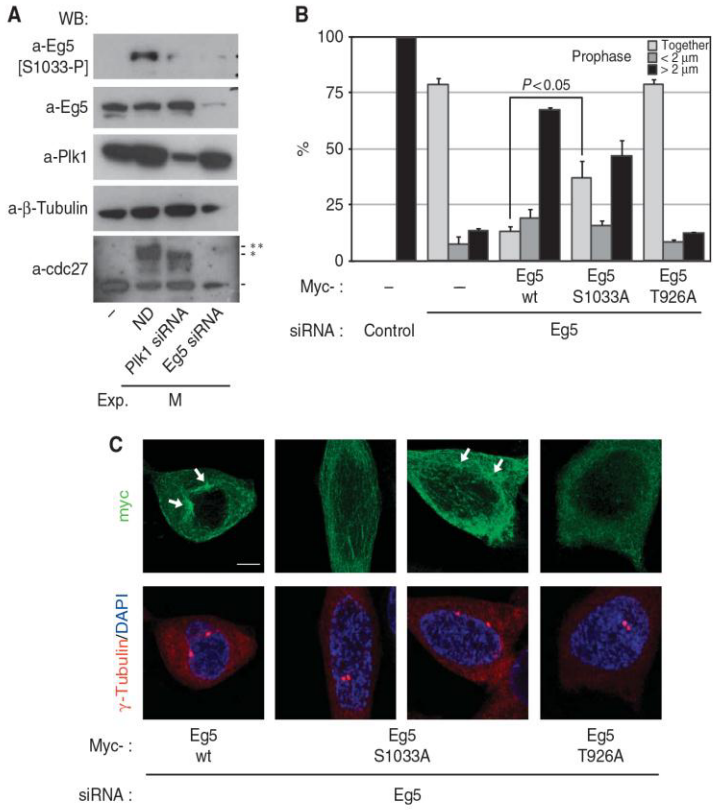


Figure 7 Plk1 controls Eg5 phosphorylation at the Nek6 site Ser1033. Both Ser1033 and the CDK1 site Thr926 phosphorylation are necessary for prophase centrosome separation and Eg5 recruitment. (**A**) HeLa cells were arrested in mitosis by either nocodazole (ND) treatment or RNAi against Plk1 or Eg5 (24 h transfection), collected after mitotic shake off and cell extracts were analysed by western blot (WB) using the indicated antibodies. Mitotic arrest was confirmed by FACS (not shown) and the phosphorylatin state of Cdc27. Untreated cells (Exp) are shown in the first lane as a control. Asterisks mark protein bands with altered mobility due to phosphorylation. (**B**) HeLa cells were transfected with either control or Eg5 siRNAs, after 16 h retransfected with expression plasmids for the indicated Myc-tagged proteins (cDNAs rendered resistant to the siRNA by several silent point mutations), fixed and stained with antibodies against Myc, γ-tubulin and DAPI. The percentage of Myc-positive prophase cells showing two unseparated centrosomes (together), two centrosomes separated $<2\,\mu m$ ($<2\,\mu m$) or fully separated centrosomes ($>2\,\mu m$) is shown in the upper graphic (mean ± s.e.m. of three independent experiments; ~40 cells counted in each experiment). Levels of endogenous and recombinant Eg5 as determined by WB are shown in Supplementary Figure S6C. (**C**) Cells transfected and processed as in (**B**). Representative examples of the observed phenotypes (Myc–Eg5, green) are shown below (bar, 5 μm). Insets show the same field stained with γ-tubulin (red) plus DAPI (blue). Centrosomal accumulation of Eg5 is noted with arrows.

was necessary for centrosome separation during prophase. We depleted endogenous Eg5 by RNAi and concomitantly expressed different Myc-tagged Eg5 variants, which were rendered resistant to the siRNA by several silent point mutations and were all expressed at similar levels (Supplementary Figure S6C). Figure 7B shows that in cells depleted of endogenous Eg5, wild-type Eg5 (but not the CDK1 site mutant Eg5[Thr926Ala]) was capable of supporting centrosome separation in prophase cells. Remarkably, Eg5[Ser1033Ala] was substantially less effective in rescuing endogenous Eg5 depletion. An additional mutant, Eg5[Ser1033Asp], showed a tendency to be more efficient in sustaining centrosome separation that Myc–Eg5[Ser1033Ala], although the differences between these two forms were statistically not significant, leading us to conclude that Eg5[Ser1033Asp] only partially mimicked Eg5[Ser1033-P] (data not shown).

During mitosis, Eg5 binding to centrosomes and microtubules depends on CDK1 phosphorylation of Thr926 (Blangy *et al*, 1995). We explored the possibility that Ser1033 phosphorylation could be in addition necessary for Eg5 centrosomal recruitment during prophase, thus explaining the requirement of this site for normal centrosome separation during early mitosis. Prophase localization of different Eg5 recombinant forms in transfected cells is shown in Figure 7C. As expected (Blangy *et al*, 1995; Sawin and Mitchison, 1995), in prophase cells, wild-type Eg5 (but not Eg5[Thr926Ala]) accumulated on centrosomes and the proximal ends of microtubules. Strikingly, Eg5[Ser1033Ala] was not present at prophase centrosomes in cells that had failed to separate them. Reduced centrosomal amounts of this mutant were observed in cells with separated centrosomes. In addition to these observations, it is worth noting that except Eg5[Thr926Ala], all Eg5 forms showed a cytoplasmatic distribution that was compatible with that of microtubules.

Thus, our results show that, like Nek9 and Nek6/7, Plk1 is necessary for mitotic Eg5[Ser1033] phosphorylation, and suggest that this modification together with Thr926 phosphorylation by CDK1, is required for normal Eg5 recruitment to centrosomes and subsequent centrosome separation during prophase.

Plk1, Nek9, Nek6, Nek7 are necessary for centrosome recruitment of Eg5 during prophase; active Nek9 and Nek6 are able to rescue Plk1 downregulation in prophase Eg5 recruitment

We reasoned that Plk1, Nek9, Nek6 and Nek7 should be necessary for Eg5 centrosomal recruitment, and that cells that failed to separate the centrosomes during prophase as a result of interfering with the different protein kinases should present diminished centrosomal levels of Eg5. Downregulation of the different kinases by RNAi confirmed this (Figure 8A). While prophase cells transfected with control siRNA showed separated centrosomes with an evident accumulation of Eg5 in the vicinity of centrosomes, transfection with Plk1, Nek6, Nek7 or Nek9 siRNAs resulted in prophase cells with unseparated centrosomes and without any apparent recruitment of Eg5 to these organelles. We next sought to determine whether the observed ability of active Nek9 and Nek6 to rescue Plk1 downregulation during centrosome separation is concomitant with a recovery in the amount of centrosomal Eg5. Figure 8B shows that active Nek9[Δ346–732] or Nek6 cotransfection at least partially restores Eg5 pericentrosomal accumulation in cells transfected with Plk1 siRNA (see Supplementary Figure S7 for additional examples of Eg5 localization under these conditions).

Additional experiments showed that in fact, transfection of active Nek9[Δ346–732] or Nek6 is able to induce ectopic Eg5 accumulation around centrosomes in parallel to centrosome separation even in interphase (Supplementary Figure S8A) and that this is accompanied with Eg5[S1033] phosphorylation (Supplementary Figure S8B; Rapley *et al*, 2008). Thus, a physiological correlation exists between Eg5 recruitment and centrosome separation in prophase cells, and activation of the Nek9/Nek6 module is both necessary and sufficient to induce both phenomena in a cell-cycle-independent manner.

Failure to phosphorylate Eg5[Ser1033] results in a delay in prometaphase

We finally wished to determine how mitotic progression would be affected by substituting endogenous Eg5 by the non-phosphorylable form Eg5[Ser1033Ala] (and thus by interfering with normal centrosome separation during prophase). For this, HeLa cells were transfected with control siRNA plus a control protein (FLAG-GFP) or Eg5 siRNA plus either a FLAG-GFP, Eg5 wild type or Eg5[Ser1033Ala], and arrested at the G2/M border using the Cdk1 inhibitor RO-3306. Cells were released by repeated washes, fixed at different time points and mitotic cells categorized according to mitotic phase. Centrosome separation was assessed in prometaphase cells by γ-tubulin staining (Figure 9). Note that, similarly to other synchronization methods (Gavet and Pines, 2010), RO-3306 treatment results in premature centrosome splitting/separation in a significative amount of G2 cells (see Supplementary Figure S9); this effect is Eg5 independent and may result in an underestimation of the effects on centrosome separation of the different forms of Eg5 used. In all conditions, ~50% of cells immediately entered mitosis upon removal of the CDK1 inhibitor, thus confirming both synchronization and the reversibility of the treatment. Of these, most were in prometaphase 30 min postrelease. Control cells and cells transfected with Eg5 siRNAs plus wild-type Eg5 progressed into metaphase (~60% of cells at 60 min) and then into telophase/cytokinesis (~80% at 240 min). As expected, most of the cells transfected with Eg5 siRNAs plus a control protein remained in prometaphase with unseparated centrosomes for the length of the experiment (76% at 240 min). Cells transfected with Eg5 siRNAs plus Eg5[Ser1033Ala] entered prometaphase at a similar rate than control cells (63% at 30 min), but only a small percentage of them progressed into metaphase and later into telophase. Remarkably, at 60 min, >57% of the cells expressing Eg5[Ser1033Ala] were still in prometaphase (as compared with 25% for cells expressing wild-type Eg5) and a significative part of the cells showed unseparated centrosomes (36 ± 2% of prometaphase cells with centrosomes separated <2 μm, as compared with 8 ± 1% in cells expressing wild-type Eg5). This percentage slowly diminished with time in parallel with the apparition of metaphase and telophase/cytokinetic cells at longer time points. After 240 min, 35% of cells expressing Eg5[Ser1033Ala] remained in prometaphase, while 45% of cells having progressed to telophase/cytokinesis. We conclude that failure to phosphorylate Eg5[Ser1033], and thus to recruit the kinesin to centrosomes and properly separate these organelles during early mitosis

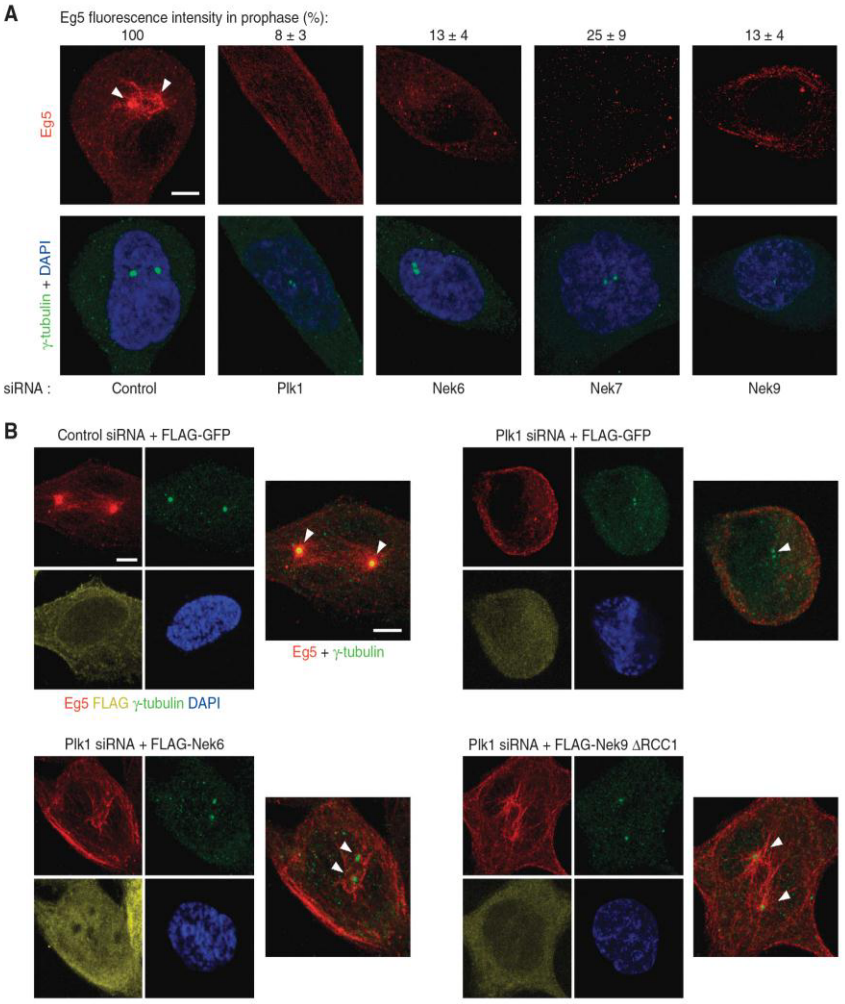


Figure 8 Plk1, Nek9, Nek6, Nek7 are necessary for centrosome recruitment of Eg5 during prophase. Active Nek9 and Nek6 can rescue Plk1 downregulation in prophase Eg5 recruitment. (**A**) HeLa cells were transfected with the indicated siRNAs, and after 24 (Plk1) or 48 (control, Nek6, Nek7, Nek9) hours, fixed and stained with antibodies against Eg5, γ-tubulin and DAPI. Representative examples of Eg5 (red) distribution in prophase cells are shown. Insets show the same field stained with γ-tubulin (green) and DAPI (blue). Centrosomal accumulation of Eg5 is noted with arrowheads. Bar, 5 μm. The efficiency of the different RNAi treatments can be seen in Figure 4C. Centrosomal Eg5 fluorescence intensity was quantified with ImageJ software on images acquired under constant exposure, using a circular area of 2 μm diameter surrounding a single centrosome (identified by γ-tubulin staining; an adjacent area of the same dimensions within each cell was quantified and subtracted as background). Results are expressed as a percentage of the intensities measured in control cells ± s.e.m. (three independent experiments; >20 centrosomes counted in each experiment). (**B**) HeLa cells were cotransfected with either control or Plk1 siRNAs and expression plasmids for the indicated proteins and after 24 h fixed and stained with antibodies against Eg5, γ-tubulin and DAPI (*Nek9ΔRCC1*, Nek9[Δ346–732]). After incubation with labelled secondary antibodies, FLAG was detected with Fab-prelabelled anti-FLAG (see Materials and methods). Representative examples of Eg5 distribution in prophase cells are shown. Images show the same field stained with Eg5 (red), γ-tubulin (green), FLAG (yellow) and DAPI (blue), and a composite of Eg5 (red) plus γ-tubulin (green). Centrosomal accumulation of Eg5 is noted with arrowheads. Bar, 5 μm.

results in prometaphase delay (and in some cases possibly in prometaphase arrest). This highlights the importance for normal mitotic progression of the mechanism responsible for the phosphorylation of Eg5[Ser1033].

Altogether, our results show that Plk1 controls Eg5[Ser1033] phosphorylation through the activation of Nek9, Nek6 and Nek7, and that this modification is required for the recruitment of Eg5 to centrosomes, early centrosome separation and normal progression through mitosis.

Discussion

The NIMA-family kinases Nek9, Nek6 and Nek7 form a signalling module required for normal spindle assembly

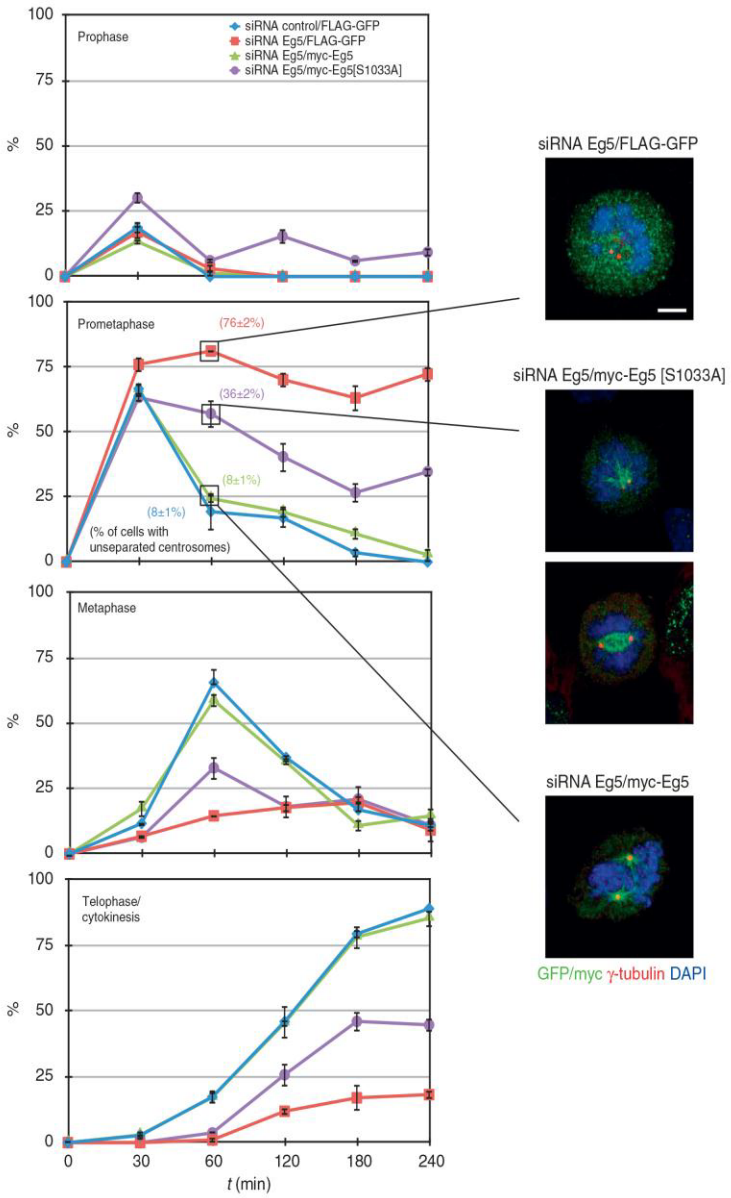


Figure 9 Effects of Eg5[Ser1033Ala] on cell-cycle progression. HeLa cells were transfected with either control or Eg5 siRNAs, after 16 h retransfected with expression plasmids for the indicated Myc-tagged proteins (cDNAs rendered resistant to the siRNA by several silent point mutations). After 24 h, cells were incubated 20 h with 9 μM RO-3306. Synchronization in G2 was confirmed by FACS. Cells were released in fresh media after repeated washes, and at the indicated times fixed and stained with antibodies against myc or GFP, γ-tubulin and DAPI. Mitotic myc- or GFP-positive cells were categorized according to mitotic phase (mean ± s.e.m. of three independent experiments; ~40 mitotic cells counted in each experiment). Representative examples of prometaphase cells as well as the percentage of cells in this phase of the cell cycle with unseparated centrosomes (distance <2 μm) at 60 min postrelease is shown, see Supplementary Figure S9 (mean ± s.e.m. of three independent experiments; ~30 cells counted in each experiment; bar, 5 μm).

and function during mitosis (Roig *et al*, 2002, 2005; Belham *et al*, 2003; O'Regan and Fry, 2009). While Nek6 and Nek7 are both directly activated by Nek9, the mechanism of activation of Nek9 has remained elusive. Based on the observations presented in this work, we put forward CDK1 and Plk1 as Nek9 physiologic activators. We propose a two-step activation mechanism for this NIMA-family kinase in which CDK1 (together with cyclin B1 and perhaps cyclin A) phosphory-

lates Nek9 at Ser869, inducing Plk1 binding and subsequent Plk1 phosphorylation and activation of Nek9. Nek9 activation could directly result from Plk1 phosphorylation of Nek9[Thr210], although conceivably Plk1 phosphorylation of additional sites outside the Nek9 activation loop may also contribute to activation by releasing Nek9 autoinhibition thus triggering Thr210 autophosphorylation (Roig *et al*, 2005). Our model provides a basis to understand the temporal and spatial pattern of Nek9 activation, occurring at prophase centrosomes, where it colocalizes with active CDK1 and Plk1. Additionally, it integrates signalling through the NIMA family with that of CDK1 and Plk1, suggesting new ways through which these two major mitotic kinases could control the organization and function of the mitotic machinery.

Regarding the conservation of the proposed Nek9 activation mechanism, an S[S/T]P site in a similar position to that of human Ser869 is lacking in mouse and rat Nek9, although in these and other organisms Ser750 is conserved. We thus suggest that this residue (that in human cells is also phosphorylated *in vivo* but only marginally affects Plk1 binding) could act as the main Plk1-binding site when the homologue of Ser869 is not present. A relationship between the NIMA, CDK and Polo families may have been long conserved through evolution and may even be bidirectional, as *Aspergillus* NIMA is activated in mitosis through a mechanism that involves NIMXCDC2 (Ye *et al*, 1995) and *Schizosaccharomyces pombe* Fin1, like NIMA and Nek9 involved in the regulation of spindle formation, has been described to be necessary for Polo (Plo1) association to the spindle pole body (Grallert and Hagan, 2002).

Early functional reports of *Drosophila* Polo (Llamazares *et al*, 1991) or mammalian Plk1 (Lane and Nigg, 1996) described the failure to separate centrosomes in mitosis (associated to the appearance of monopolar spindles) as one of the major results of interfering with these kinases. Since then it has been well established that Plk1 has diverse functions during early, mid and late mitosis (Petronczki *et al*, 2008; Archambault and Glover, 2009), among them the regulation of centrosome separation and maturation, two Plk1 roles the molecular basis of which still remains to be fully understood. We herein propose that Plk1 controls centrosome separation in prophase through Nek9 and Nek6/7 signalling to the kinesin Eg5. Accordingly, and without discarding the existence of additional Nek9/Nek6/7-independent roles of Plk1 during centrosome separation, we suggest that the main cause for the failure of this process in cells with diminished Plk1 activity is the absence of activation of the Nek9/Nek6/7 module. This results in lack of Eg5 phosphorylation at Ser1033, a previously described modification (Rapley *et al*, 2008) that we now show is necessary for Eg5 recruitment to centrosomes and prophase centrosome separation.

Previous studies have suggested that vertebrate cells can separate centrosomes through two distinct and partially redundant pathways: a prophase pathway that relies on microtubule-based motors, the nuclear membrane and possibly microtubule pushing forces, and a prometaphase pathway that is based in interactions between the two microtubule asters, astral microtubule pulling forces and kinetochore-generated pushing forces (see Rosenblatt, 2005 and Tanenbaum and Medema, 2010 for reviews). The prophase pathway is, at least in mammalian cells, strongly dependent on Eg5 (our data and Whitehead and Rattner, 1998; Tanenbaum *et al*, 2008; Woodcock *et al*, 2010) and our results indicate that phosphorylation of Eg5[Ser1033] controls this pathway by allowing the recruitment of the kinesin to the vicinity of the centrosomes during prophase. Attesting to the redundancy of the two centrosome separation pathways and the robustness of the mechanisms that results in spindle bipolarity, cells that fail to phosphorylate Eg5[Ser1033] remain longer in prometaphase, but for the most part reach metaphase (although with a marked delay) and progress to later mitotic phases. Thus, Ser1033 phosphorylation is not necessary for prometaphase centrosome separation or the non-prophase functions of Eg5 during spindle assembly (a process that depends on Eg5 unless it initiates with well-separated centrosomes; Ferenz *et al*, 2009). Nevertheless, our results suggest that Eg5[Ser1033] phosphorylation and thus prophase centrosome separation promote and accelerate the building of the bipolar spindle, probably allowing prometaphase mechanisms to work more efficiently on already separated centrosomes. It remains to be determined whether, in addition of the timely formation of a bipolar spindle, Eg5[Ser1033] phosphorylation influence the accuracy of chromosome segregation as well.

How does Ser1033 phosphorylation induce Eg5 pericentrosomal localization and control prophase centrosome separation? Mutation of this residue to a non-phosphorylable alanine results in a form of the kinesin that has a greatly impaired centrosomal localization but is still able to bind microtubules and to localize to the metaphase spindle (Rapley *et al*, 2008). Conversely, mutation of the CDK1 phosphorylation site Thr926 (required for Eg5 microtubule binding during mitosis; Blangy *et al*, 1995; Sawin and Mitchison, 1995) results in abrogation of Eg5 recruitment to centrosomes and of centrosome separation. Consequently, Ser1033 phosphorylation does not control the ability of Eg5 to bind microtubules but it relies on it to concentrate the kinesin at the vicinity of centrosomes and separate them (Thr926 phosphorylation may not be required in conditions in which high Nek9/Nek6/7 activity results in a significant increase in the levels of Eg5[Ser1033-P], see Figure 5 and Supplementary Figure S8). We can hypothesize that phosphorylation of Ser1033 allows a pool of Eg5 to preferentially bind microtubules proximal or even anchored to the centrosome, either directly or through the interaction with a yet to be identified centrosomal protein (in turn this may be directly modulated by Plk1 or other yet to be described Plk1 targets, accounting for the only partial ability of Nek9 and Nek6 to rescue Eg5 recruitment to the centrosomes in cells with diminished levels of Plk1, see Figure 8 and Supplementary Figure S7). Accumulation of enough Eg5 at the vicinity of a prophase centrosome will allow separation from the opposing centrosome by exerting forces on the relatively few microtubules emanating from it. Centrosomal localization of Eg5 may not be necessary during prometaphase, as in this latter stage of mitosis the increased number and length of microtubules (with Eg5 bound through their lengths) would ensure sufficient overlap as to produce force in collaboration with other separation mechanisms and without the need of concentrating Eg5 at centrosomes. Whether this hypothesis is correct, as well as how it is related to novel functions of Eg5 C-terminal domain (Weinger *et al*, 2011), the action of other motor systems like dynein/dynactin (Blangy *et al*, 1997; Uteng

et al, 2008; Ferenz *et al*, 2009) or the positioning of centrosomes in respect to the nucleus (Splinter *et al*, 2010) remains to be determined.

In summary, our data identify two major mitotic regulators, Plk1 and CDK1 as upstream activators of the Nek9/Nek6/7 module, firmly positioning the NIMA-family kinases Nek9, Nek6 and Nek7 at the centre of mitotic signalling. A first example of the roles that these kinases can perform downstream of CDK1 and Plk1 is described, shedding light on one of the most conspicuous but less understood roles of Plk1 during early mitosis, centrosome separation, and defining the elements that in mammalian cells control this process as well as its importance during mitotic progression. It is now clear that NIMA-family kinases control different but consecutive steps of the centrosomal cycle, namely centrosome disjunction (regulated by Nek2 in an Eg5-independent manner and essential when Eg5 function is partially compromised, see Mardin *et al*, 2010) and separation (regulated by Nek9 and Nek6/7 and executed by Eg5). Whether this is the result of sharing the diverse functions of an ancestral NIMA and whether Nek9, directly or through Nek6 and Nek7, is responsible for additional mitotic roles downstream of Plk1 as the phenotypes that result from interfering with these kinases suggest (Roig *et al*, 2002, 2005; O'Regan and Fry, 2009), will surely be the subject of future investigations.

Materials and methods

Plasmids and reagents

Different Nek9 and Nek6 expression plasmids have been described elsewhere (Roig *et al*, 2002; Belham *et al*, 2003). Additional Nek9 mutants were constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions, using specific primers (S869A 5′-CAAGTAGAAGCCT CGGCACCTCGGCTGAATCCTGC-3′, S29A 5′-GGTTGCGGGGACTCG GCTCCGGGGCCTAGCGCC-3′, S750A 5′-ACTGTGTTTCAGAGCTCTG CCCCGGGAGGAGGCGGCGG-3′- with the appropriate reverse complements). pCMV5-FLAG-GFP was constructed by cloning eGFP into pCMV5-FLAG. For construction of PGEX-Plk1[345–603], a PCR fragment corresponding to Plk1 PBD was cloned into a modified PGEX vector (Pharmacia Biotech). All constructs were sequenced after generation. RNAi-resistant forms of Eg5 have been described in Rapley *et al* (2008).

FLAG-Nek9 and FLAG-Nek9[K81M] were expressed in 293T cells and purified by immunoprecipitation with anti-FLAG antibody (Sigma), followed by repeated washes and elution using FLAG peptide (Sigma). Purified Plk1 and CDK1/cyclin B were purchased from Invitrogen. Histone H3 was from Roche. Nocodazole, MG132 and Purvalanol A were from Sigma. Bi2536 was from Axon Medchem. RO-3306 was from Enzo Life Sciences.

Cell culture and transfection

HeLa, U2OS and HEK 293T cells were cultured as described (Roig *et al*, 2002). Cells in mitosis were obtained by mitotic shake off of nocodazole-arrested (0.25 mM, 16 h) cultures. HEK 293T cells were transfected using different expression plasmids with Lipofectamine (Invitrogen) according to the manufacturer's instructions. HeLa cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNAs were transfected using siPORT NeoFX Transfection Agent (Ambion) according to the manufacturer's instructions. siRNA and DNA cotransfection was performed using Lipofectamine 2000.

siRNA duplexes were as following: Eg5, 5′-CUGAAGACCUGAA GACAAUdTdT-3′ (Ambion) (Weil *et al*, 2002); Plk1, 5′-CGAGCTGCT TAATGACGAGTT-3′ (Dharmacon) (Oshimori *et al*, 2006); Nek6, 5′-AAUAGCAGCUGUGUGAGUCUUGCCU-3′ (Ambion) (O'Regan and Fry, 2009); Nek7, 5′-AAUAGUGAUCUGAAGGAAGAGGUGG-3′ (Invitrogen); Nek9, 5′-AAUAGCAGCUGUGUGAGUCUUGCCU-3′ (Invitrogen); Nek9 UTR, 5′-GCTGCCTTGGGAATTCAGTdTdT-3′ and 5′-GCAGCCAAACTTTGATTAAdTdT-3′ (Ambion).

Immunoprecipitation and western blot analysis

Immunoprecipitations and western blotting were performed as described in Roig *et al* (2002). Anti-Nek9, anti-Nek9[Thr210-P], anti-Nek6 and anti-Eg5[Ser1033-P] polyclonal antibodies have been described in Roig *et al* (2002, 2005); Belham *et al* (2003) and Rapley *et al* (2008). Other antibodies used are anti-Nek7 (Cell Signaling), anti-cdc27, anti-cyclin B1 (Santa Cruz Biotechnology), anti-Plk1 (Calbiochem), anti-Eg5 (BD Bioscience), anti-GFP (Roche and Invitrogen), anti-FLAG and anti-β-tubulin (Sigma). Secondary antibodies were from Jackson ImmunoResearch Laboratories and were detected by ECL chemiluminescence (Thermo Scientific).

MS analysis

For phosphopeptide identification, Coomassie-stained protein bands were excised and *in situ* digested with trypsin and LC/MS/MS analysis of phosphorylation sites was performed at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA) as described previously (Roig *et al*, 2005).

Two-hybrid analysis

cDNAs coding for the human Plk1 and Nek9 fragments indicated in Figure 1 was subcloned into pGBKT7 and pGADT7, respectively, and yeast two-hybrid analysis was performed as described in Rapley *et al* (2008).

Kinase assays

Protein kinase assays were carried out as described previously (Roig *et al*, 2002) using 100 μM ATP.

Immunofluorescence

Cells were grown on coverslips fixed with methanol and permeabilized as described earlier (Rapley *et al*, 2008). Primary antibodies used were mouse anti-γ-tubulin (Sigma), mouse anti-FLAG (Sigma), mouse anti-GFP (Invitrogen), rabbit anti-centrin (Groen *et al*, 2004), rabbit anti-histone H3[Ser10-P] (Cell Signaling), rabbit anti-Myc (Sigma) and mouse anti-Eg5 (BD). Primary antibodies were detected with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG (Invitrogen). When needed, anti-FLAG antibodies were detected with Alexa Fluor 647-Fab fragments using the Zenon mouse IgG labelling kit (Invitrogen). DNA was stained with DAPI (Sigma).

Images were taken using a Leica TCS SPE confocal system with a DM2500 CSQ upright microscopy and a ×63 1.30 ACS Apo lens, and edited using Leica LAS AF software (Leica Microsystems) and Photoshop (Adobe).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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Author contributions: MTB designed and performed the experiments in Figures 1–3 and 7, and Supplementary Figures S1–S3 and S6; SS designed and performed the experiments in Figures 4–9 and Supplementary Figures S4–S9; LR performed the experiments in Figure 1 and Supplementary Figure S2; JA was instrumental for the initial stages of the study including the reported MS analysis; CC was instrumental for the realization of the study, discussed the data and commented and contributed to the paper; JR conceived the study, designed experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Information

Supplementary information consist of nine supporting figures.

Figure S1.

(A) Coomassie staining of the Nek9 immunoprecipitates used in the LC/MS/MS analysis of phosphopeptides. *Exp*., exponentially growing cells; *M*, mitotic cells. *NIgG*, normal IgG.

(B) Phosphopeptides and their corresponding unphosphorylated counterparts identified in the LC/MS/MS analysis of Nek9. The most probable phosphorylation site is marked in each case with and asterisk (*). Peak intensities for each peptide and sample are shown and used to infer the percentage of total peptide that is phosphorylated in each case (*phosphopeptide, % of total*), as well as the fold increase of this percentage in mitotic cells as compared to exponential cells (*Fold increase in M*).

Figure S2.

(A) normal IgG (*NIgG*) or a-Nek9 immunoprecipitates from exponentially growing (*Exp*.) or nocodazole-arrested mitotic (*M*) embryonic mouse fibroblasts extracts were analyzed by western blot (*WB*) using the indicated antibodies. Plk1 in the corresponding extracts is shown in the lower panel.

(B) The ability of full length Plk1 or Plk1 PBD (Plk1[345-603]) to interact with the different domains of Nek9 (kinase domain: Nek9[1-346]; RCC1 domain: Nek9[347-726]; C-terminal tail:Nek9[721-979]) was assessed using two hybrid by histidine and adenine prototrophy plus expression of α-galactosidase activity (right plates). *AD*, Gal4 activation domain; *BD*, Gal4 DNA binding domains; *C+*, positive control (BD-p53 and AD-SV40); *C-*, negative control (BD-lamin and either AD-Plk1 or AD-Plk1[345-603].

(C) HeLa cells were transfected with empty FLAG vector (-), FLAG-Nek9 wild type or FLAG-Nek9[S869A]. anti-FLAG immunoprecipitates were obtained from exponentially growing (*Exp*) or

nocodazole-arrested mitotic (M) cells and immunobloted with the indicated antibodies. Plk1 in the corresponding extracts is shown in the lower panel.

Figure S3.

Kinase-defective FLAG-Nek9[D176A] was expressed and purified from 293T cells and incubated for 60 minutes at 30 °C with [γ-^{32}P]ATP/Mg^{2+} in presence or absence of Plk1. After SDS-PAGE, Nek9 was visualized by Coomassie staining, and ^{32}P incorporation was visualized by autoradiograph (upper panel). Identical samples were analyzed by western blot (*WB*) using the indicated antibodies (lower panel).

Figure S4.

(A) Representative HeLa cell with multiple centrosomes. The cell has been transfected with Nek9 siRNA, and after 48 hours, fixed and stained with antibodies against γ-tubulin (red), centrin (green) and DAPI (blue). Insets show magnified centrosomes (γ-tubulin, centrin and overlap).

(B) HeLa cells were transfected with control siRNA or alternative siRNAs directed against Nek6 (Nek6_2, 5'-AGAGGCAUCCCAACACGCUGUCUUU-3', Invitrogen) or Nek7 (Nek7_2, 5'-AUAAACUUCACUAAAUUGUCCGCGA-3', Invitrogen), and after 48 hours fixed, stained and scored as in Figure 4. with antibodies against γ-tubulin (red) and DAPI (blue). The percentage of prophase cells showing 2 unseparated centrosomes (*together*), 2 centrosomes separated less than 2 μm (< *2μm*), or fully separated centrosomes (> *2μm*) is shown in the upper graphic (mean ± SEM of 3 independent experiments; ~50 cells counted in each experiment). The efficiency of the different RNAi treatments used as determined by western blot of total cell extracts is shown.

(C) Levels of endogenous and recombinant Nek9 as determined by western blot for the different conditions used in Figure 4C. An asterisk marks an unspecific band.

Figure S5

DNA content of cells transfected with the indicated FLAG-tagged proteins (Nek9ΔRCC1, Nek9 [Δ346-732]). 24h after transfection, the DNA content of FLAG-positive cells was analyzed by FACS. There is no significant difference in cell-cycle distribution between different conditions. Similar results were obtained when control siRNA was cotransfected.

Figure S6.

(A) HeLa cells were transfected with the indicated siRNAs, and after 48 hours, Eg5 was immunoprecipitated from the corresponding lysates. Eg5[Ser1033-P] and total Eg5 were detected by western blot (*WB*) of the immunoprecipitates. The efficiency of the different RNAi treatments used was determined by western blot of total cell extracts. *Exp*., exponentially growing cells; *I*, interphase cells (2mM thymidine, 16h); *M*, mitotic cells (0.25 mM nocodazole, 16h).

(B) HeLa cells where incubated with nocodazole (*ND*, 0.25 mM) or BI2536 (100 nM) for 16 hours. Cells in mitosis (*M*) were collected after mitotic shake off, and cell extracts were analyzed by western blot (*WB*) using the indicated antibodies. Mitotic arrest was confirmed by FACS (*not shown*) and the phosphorylation state of Cdc27. Untreated cells (*Exp*.) are shown in the first lane as a control. Asterisks mark protein bands with altered mobility due to phosphorylation.

(C) Levels of endogenous and recombinant Eg5 as determined by western blot for the different conditions used in Figure 7B.

Figure S7.

HeLa cells were transfected, fixed and stained as in Figure 8B. Representative examples of the observed phenotypes in prophase cells are shown (Eg5, red; γ-tubulin, green; FLAG, yellow; DAPI, blue). Centrosomes are noted with arrowheads. Bar, 5 μm.

Figure S8.

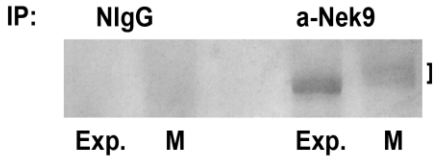
(A) HeLa cells were transfected with the indicated expression plasmids, and after 24h fixed and stained as in Figure 8B. Representative examples of Eg5 distribution in interphase cells are shown. Images show the same field stained with Eg5 (red), γ-tubulin (green), FLAG (yellow) and DAPI (blue), and a composite of Eg5 (red) plus γ-tubulin (green). Centrosomal accumulation of Eg5 is noted with arrowheads. Bar, 5 μm. (B) Levels of Eg5[S1033-P], Eg5 and FLAG-tagged proteins as determined by western blot of cell extracts for the different conditions used in (A). First two lanes show untransfected cell extracts from untreated (*Exp*.) and nocodazole-arrested mitotic cells (*M*). Note that FLAG-transfected cells are growing exponentially.

Figure S9.

As in Figure 9. Percentage of cells with separated and unseparated centrosomes (distance < 2μm) in RO-3306 arrested cells (*left*) or prometaphase cells (60 min postrelease from RO-3306 arrest, *right*). Mean ± SEM of 3 independent experiments (~30 cells counted in each experiment) is shown.

Figure S1

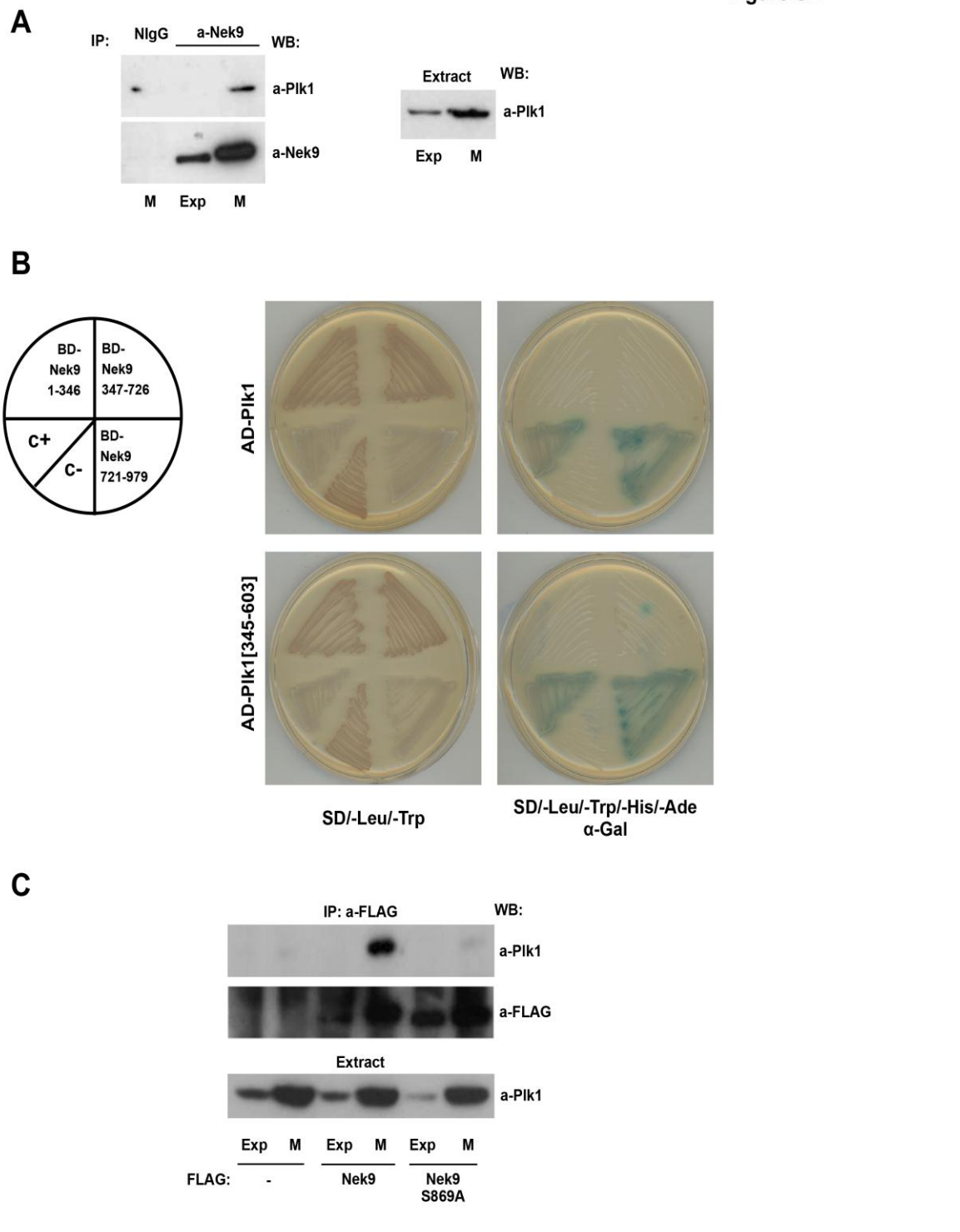
A



B

Site	Identified peptide	Peak intensity		phosphopeptide (% of total)		Fold increase in M
		Exp.	M	Exp.	M	
Ser29	$_{10}$HCDSINSDFGSESGGCGDSSPGP SASQGPR$_{39}$	7.16 E5	1.80 E5	2.4	67.3	28 x
	$_{10}$HCDSINSDFGSESGGCGDSS*PG PSASQGPR$_{39}$	1.74 E4	3.70 E5			
Thr333	$_{331}$SSTVTEAPIAVVTSR$_{345}$	1.43 E6	6.21 E5	2.1	18.7	9 x
	$_{331}$SST*VTEAPIAVVTSR$_{345}$	3.12 E4	1.43 E5			
Ser750	$_{735}$SNSSGLSIGTVFQSSSPGGGGGG GGGEEEDSQQESETPDPSGGFR$_{779}$	4.14 E5	4.35 E4	3.4	56.3	17 x
	$_{735}$SNSSGLSIGTVFQSSSPGGGGGG GGGEEEDSQQES*ETPDPSGGFR$_{779}$	1.46 E4	5.60 E4			
Ser827	-	-	-	-	-	-
	$_{815}$ELENAEFIPM#PDS*PSPLSAAF SESEKDTLPYEELQGLK$_{852}$					
Ser869	853VASEAPLEHKPQVEASSPR$_{871}$	1.01 E6	1.52 E5	12.4	82.7	7 x
	$_{853}$VASEAPLEHKPQVEAS*SPR$_{871}$	1.43 E5	7.29 E5			
Thr885	-	-	-	-	-	-
	$_{882}$GTPLT*PPACACSSLQVEVER$_{901}$					

Figure S2


A
IP:
NIgG
a-Nek9
WB:
a-Plk1
a-Nek9
M
Exp
M
Extract
WB:
a-Plk1
Exp
M
B
BD-
Nek9
1-346
BD-
Nek9
347-726
C+
C-
BD-
Nek9
721-979
AD-Plk1
AD-Plk1[345-603]
SD/-Leu/-Trp
SD/-Leu/-Trp/-His/-Ade
α-Gal
C
IP: a-FLAG
WB:
a-Plk1
a-FLAG
Extract
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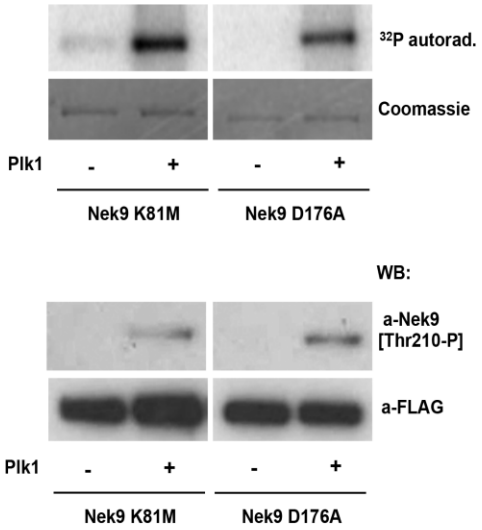
Exp
M
Exp
M
Exp
M
FLAG:
-
Nek9
Nek9
S869A

Figure S3


32P autorad.
Coomassie
Plk1 - + - +
Nek9 K81M
Nek9 D176A
WB:
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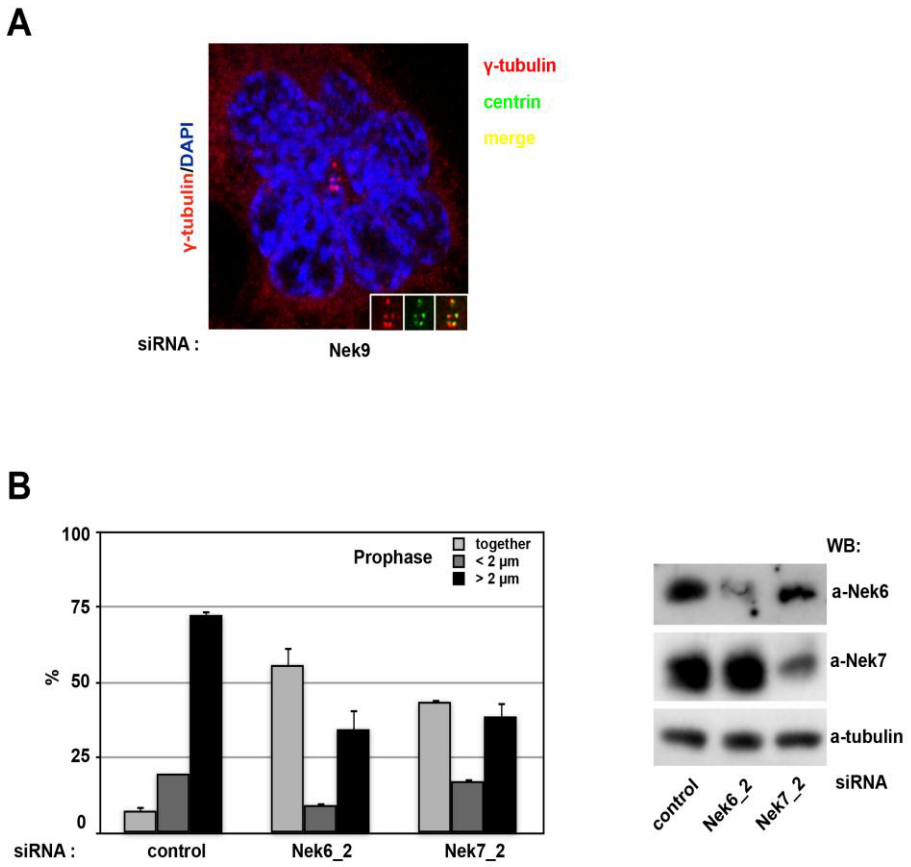
a-FLAG
Plk1 - + - +
Nek9 K81M
Nek9 D176A

Figure S4


A
γ-tubulin
centrin
merge
γ-tubulin/DAPI
siRNA :
Nek9
B
100
75
50
25
0
%
Prophase
together
< 2 µm
> 2 µm
siRNA :
control
Nek6_2
Nek7_2
WB:
a-Nek6
a-Nek7
a-tubulin
control
Nek6_2
Nek7_2
siRNA
C
WB:
*-
Nek9-
a-Nek9
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FLAG- :
GFP
GFP
Nek9
Nek9 S869A
siRNA :
Control
Nek9 UTR

A

Figure S5

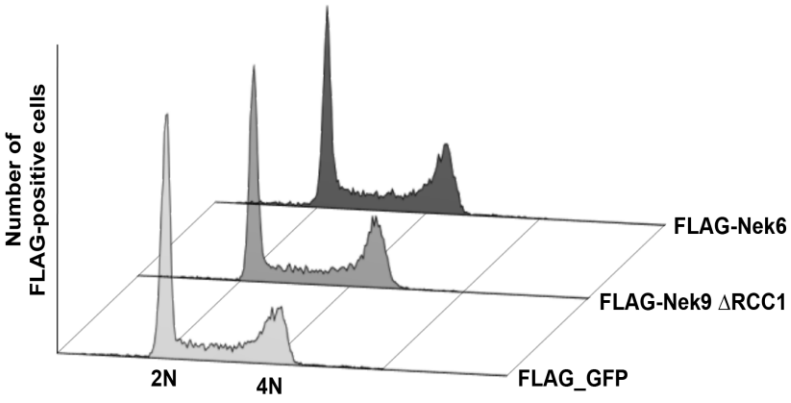

Number of
FLAG-positive cells
FLAG-Nek6
FLAG-Nek9 ΔRCC1
FLAG_GFP
2N
4N

Figure S6

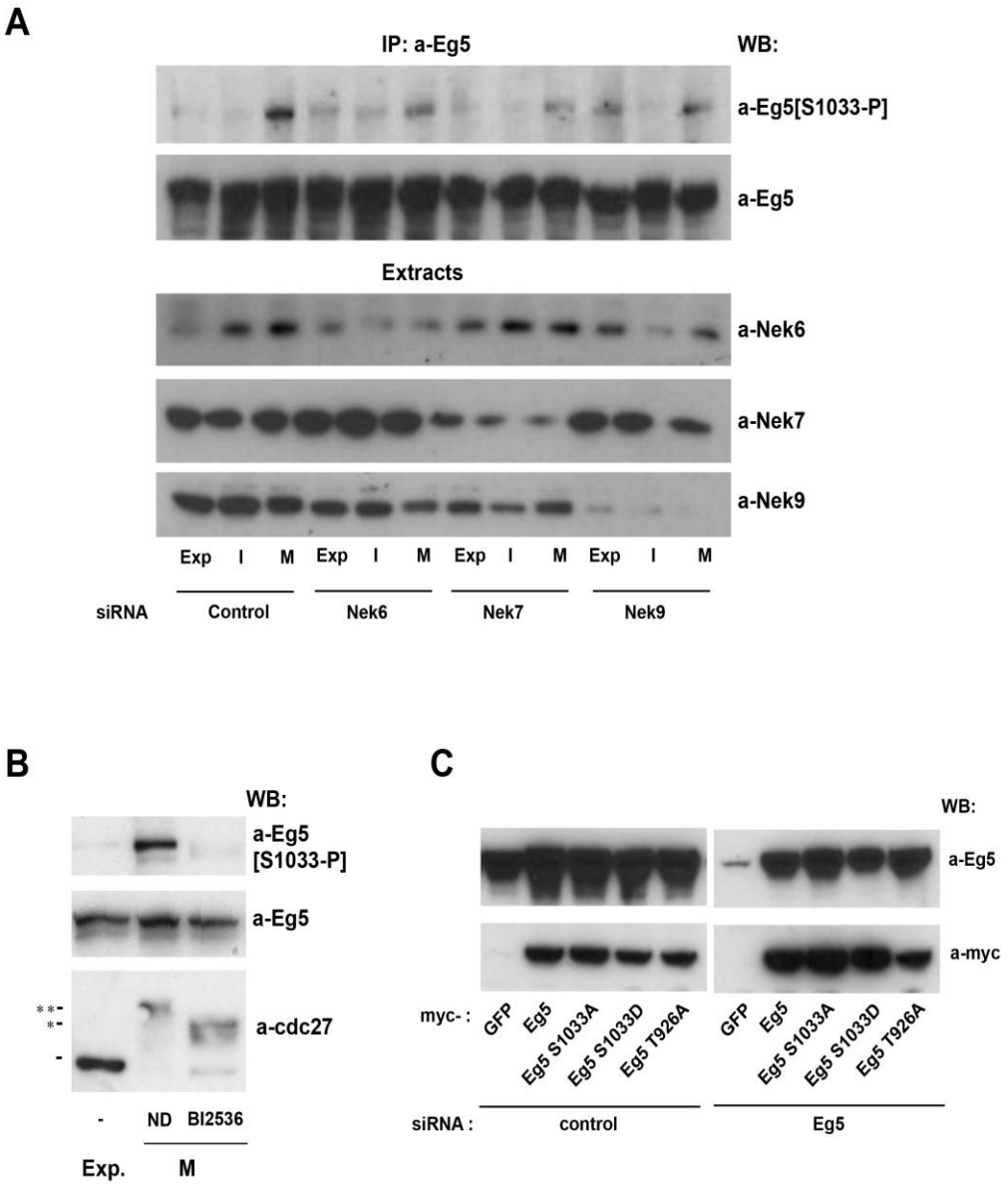

A
IP: a-Eg5
WB:
a-Eg5[S1033-P]
a-Eg5
Extracts
a-Nek6
a-Nek7
a-Nek9
Exp I M Exp I M Exp I M Exp I M
siRNA
Control
Nek6
Nek7
Nek9
B
WB:
a-Eg5 [S1033-P]
a-Eg5
a-cdc27
**-
*-
-
- ND BI2536
Exp.
M
C
WB:
a-Eg5
a-myc
myc- :
GFP
Eg5
Eg5 S1033A
Eg5 S1033D
Eg5 T926A
siRNA :
control
Eg5

Figure S7

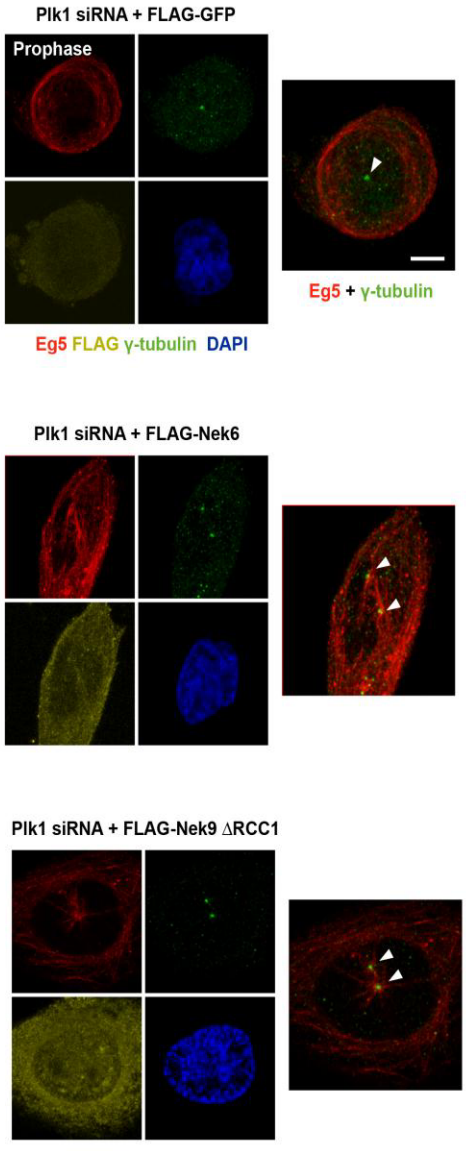

Plk1 siRNA + FLAG-GFP
Prophase
Eg5 + γ-tubulin
Eg5 FLAG γ-tubulin DAPI
Plk1 siRNA + FLAG-Nek6
Plk1 siRNA + FLAG-Nek9 ΔRCC1

Figure S8

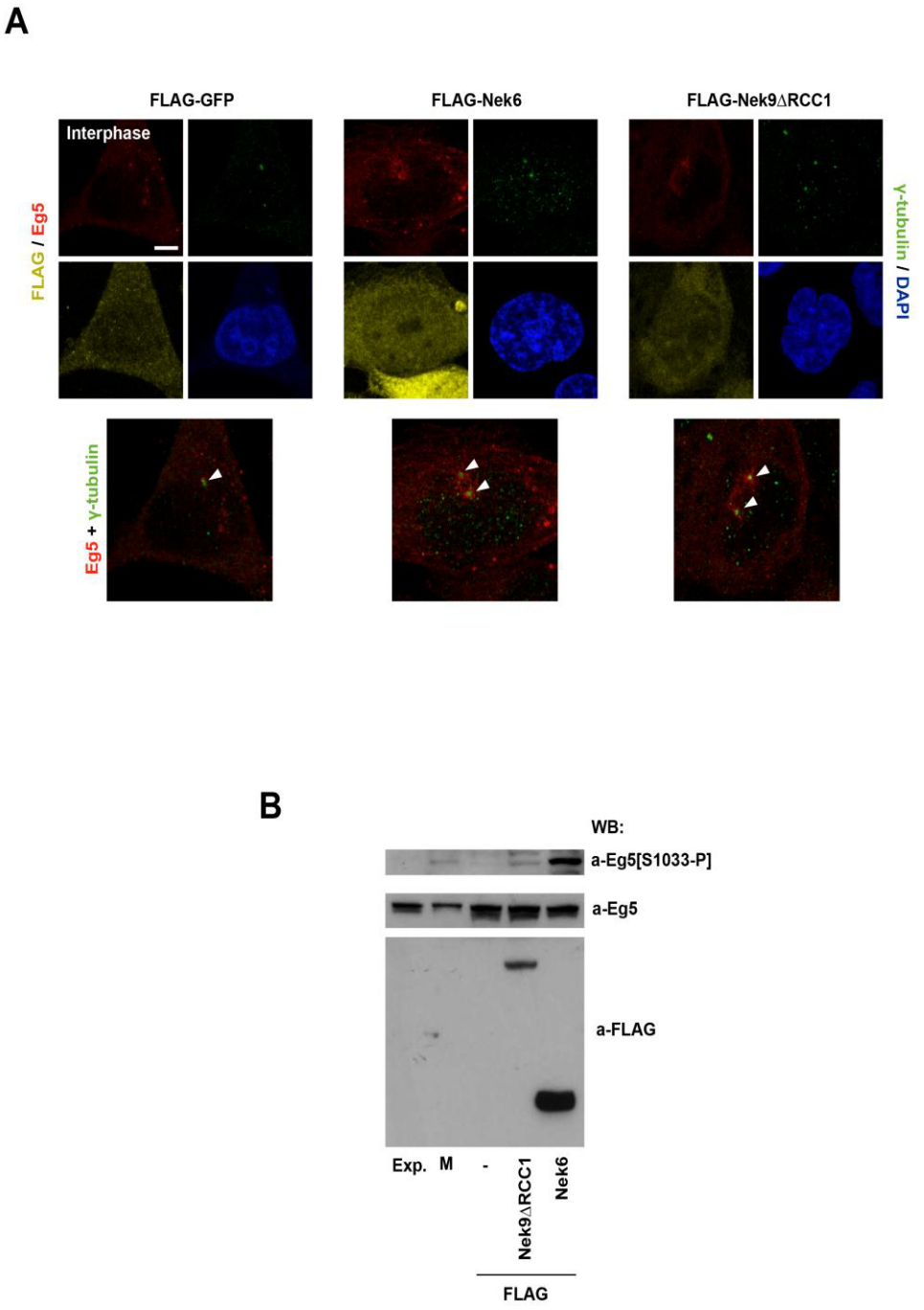
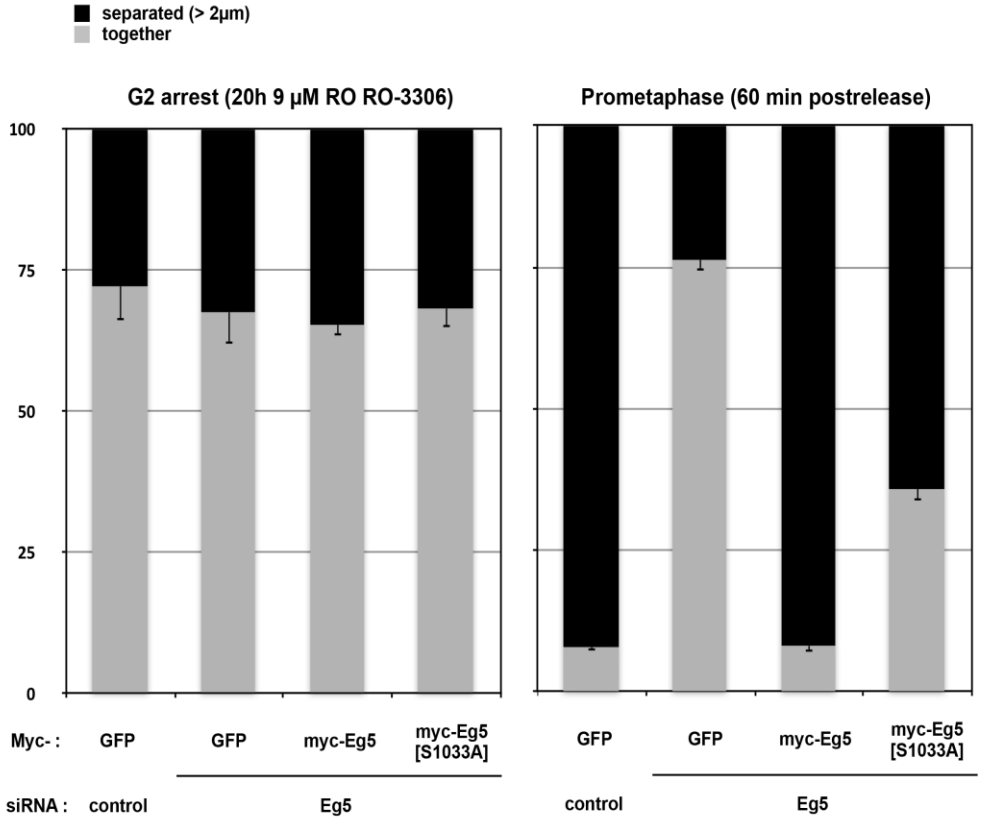

A
FLAG-GFP
FLAG-Nek6
FLAG-Nek9ΔRCC1
Interphase
FLAG / Eg5
γ-tubulin / DAPI
Eg5 + γ-tubulin
B
WB:
a-Eg5[S1033-P]
a-Eg5
a-FLAG
Exp.
M
-
Nek9ΔRCC1
Nek6
FLAG

Figure S9


separated (> 2µm)
together
G2 arrest (20h 9 µM RO RO-3306)
Prometaphase (60 min postrelease)
100
75
50
25
0
Myc- :
GFP
GFP
myc-Eg5
myc-Eg5 [S1033A]
GFP
GFP
myc-Eg5
myc-Eg5 [S1033A]
siRNA :
control
Eg5
control
Eg5

ARTICLE 2

(Sdelci *et al*, 2012)

Thesis author contribution:

Figures 2 to 4

Supplementary Figures S2 and S4

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Report

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

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Summary

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

Results

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

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

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

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

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

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

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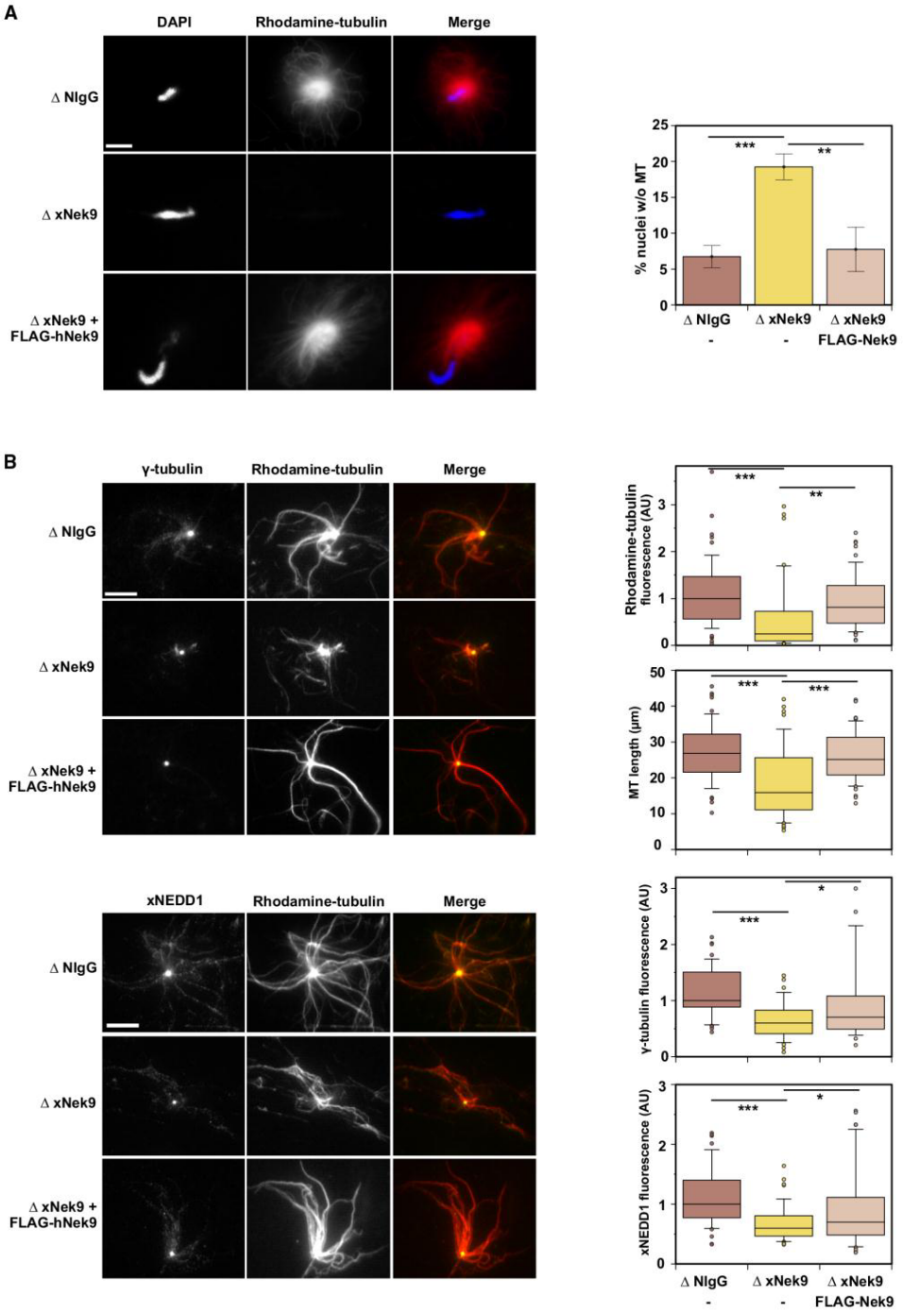

Figure 1. xNek9 Depletion Impairs Normal Aster Formation in *Xenopus* Mitotic Egg Extracts by Reducing MT Density as Well as Centrosomal γ-Tubulin and xNEDD1

(A) Aster formation induced by addition of sperm nuclei to control, xNek9-depleted and xNek9-depleted extract containing 10 nM purified FLAG-Nek9. Scale bar represents 10 μm. Graph shows mean ± SEM (n = 3 experiments, 30 structures counted for each experimental condition; statistical

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NEDD1 accumulation at prometaphase centrosomes (Figures 2A and 2B; Figure S2B) without altering the content of the core centriolar protein centrin, or the pericentriolar material (PCM) protein pericentrin indicating that general centrosome structure was intact (Figure S2C). Moreover, Nek9 silencing had no observable effect on the amount of γ-tubulin and NEDD1 at interphase centrosomes (Figures 2A and 2B; Figure S2B). The specificity of Nek9 requirement was demonstrated by the full rescue of γ-tubulin recruitment to prometaphase centrosomes in Nek9-silenced cells expressing recombinant wild-type Nek9 (Figure S2B).

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

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

We have previously shown that Nek9[S869A] does not bind to the Nek9 activator Plk1 [13]. To test whether this interaction is required for Nek9 function in centrosomal γ-tubulin recruitment, we examined whether Nek9[S869A] could rescue the defects observed upon Nek9 silencing. In contrast to the wild-type form, Nek9[S869A] was unable to promote the rescue (Figure S2B). We then examined the ability of a constitutively active form of Nek9 (Nek9[Δ346–732] [11], to compensate for the downregulation of Plk1 during this process. Figure 3A shows that expression of FLAG-Nek9[Δ346–732] had no effect on the amount of centrosomal γ-tubulin in interphase but slightly increased the pool of centrosomal γ-tubulin in mitotic cells, an effect abrogated by NEDD1 silencing. Remarkably, it was Plk1-independent, as FLAG-Nek9[Δ346–732] was capable of rescuing the centrosomal levels of γ-tubulin in Plk1-depleted mitotic cells (in contrast, recombinant wild-type Nek9 slightly increased γ-tubulin centrosomal levels in Plk1-depleted cells but not to control levels; data not shown).

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

NEDD1 Interacts with and Is a Substrate of Nek9

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

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

Nek9 Regulates Centrosomal γ-TuRC Recruitment by Phosphorylating NEDD1

We then examined the functional consequences of NEDD1 phosphorylation by Nek9. As we could not express a functional recombinant full-length xNEDD1 for rescue experiments in *Xenopus* egg extracts, we used HeLa cells. We first generated a form of NEDD1 in which Ser377 was replaced with a phosphomimetic residue. FLAG-NEDD1[S377D] or NEDD1[S377E] (but not wild-type FLAG-NEDD1 expressed at similar levels) rescued the mitotic recruitment of γ-tubulin in Nek9-silenced cells (Figure 3B; however, NEDD1[S377D] was not able to rescue Plk1 downregulation, data not shown). These results strongly suggested that Nek9 controls the centrosomal recruitment of γ-tubulin mainly through the phosphorylation of NEDD1 at Ser377.

We next generated a form of NEDD1 that could not be phosphorylated, NEDD1[S377A] and tested its ability to support γ-tubulin recruitment to the mitotic centrosome in NEDD1-silenced cells. Expression of NEDD1 wild-type in NEDD1-silenced cells restored γ-tubulin recruitment to the prometaphase centrosomes to levels similar to control cells. By contrast, expression of NEDD1[S377A] under similar conditions did not (Figure 4A). Furthermore, it did not restore normal progression through mitosis (Figure 4B): although cells entered into mitosis and progressed into prometaphase with a timing similar to that of control or FLAG-NEDD1-expressing cells, they failed to progress further and accumulated in prometaphase with diminished MT asters. Substitution of endogenous NEDD1 by the NEDD1[S377A] in nonsynchronized cells similarly increased the mitotic index (Figure S4D).

Because NEDD1[S377A] interacted with γ-tubulin in a similar manner as wild-type NEDD1 (Figure S4E), we examined whether Ser377 phosphorylation could regulate the mitotic recruitment of NEDD1 to centrosomes. In contrast to wild-type NEDD1, NEDD1[S377A] failed to accumulate at centrosomes in prometaphase cells (Figure 4C). We conclude that the phosphorylation of NEDD1 on Ser377 by Nek9 controls NEDD1

analysis, t test). Levels of endogenous and recombinant xNek9 in the extracts are shown in Figure S1B. In this and subsequent figures *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Aster formation induced by addition of purified centrosomes. Scale bar represents 10 μm.

The distribution of MT length and MT fluorescence, γ-tubulin and NEDD1 fluorescence intensities are shown as box plots (50 structures counted for each experimental condition; statistical analysis, t test). AU, arbitrary units.

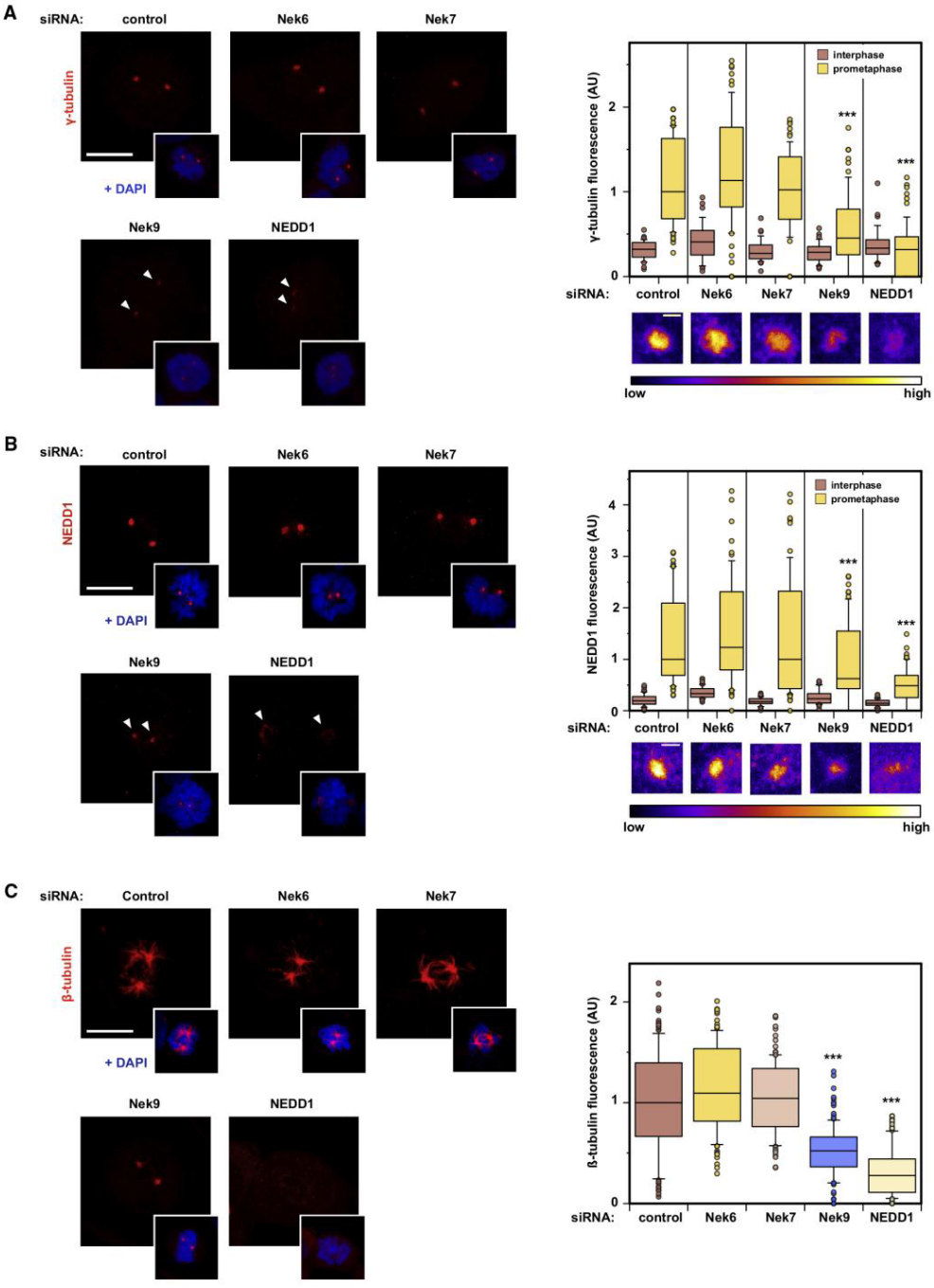

Figure 2. Nek9 Is Necessary for Normal Centrosomal MT Nucleation as Well as for γ-Tubulin and NEDD1 Recruitment in Mitosis Independently of Nek6/7 (A and B) Effect of different siRNA transfections on γ-tubulin (A) and NEDD1 (B) contents in interphase and prometaphase HeLa cells. Representative examples of the observed phenotypes in prometaphase are shown (scale bar represents 10 μm). The distribution of intensities is shown in each case as a box plot (three independent experiments, 20 cells counted for each experimental condition of each experiment). Asterisks indicate a statistically significant difference with the corresponding controls in prometaphase. For each condition, a representative prometaphase centrosome was LUT coded to represent staining intensity (scale bar represents 1 μm).

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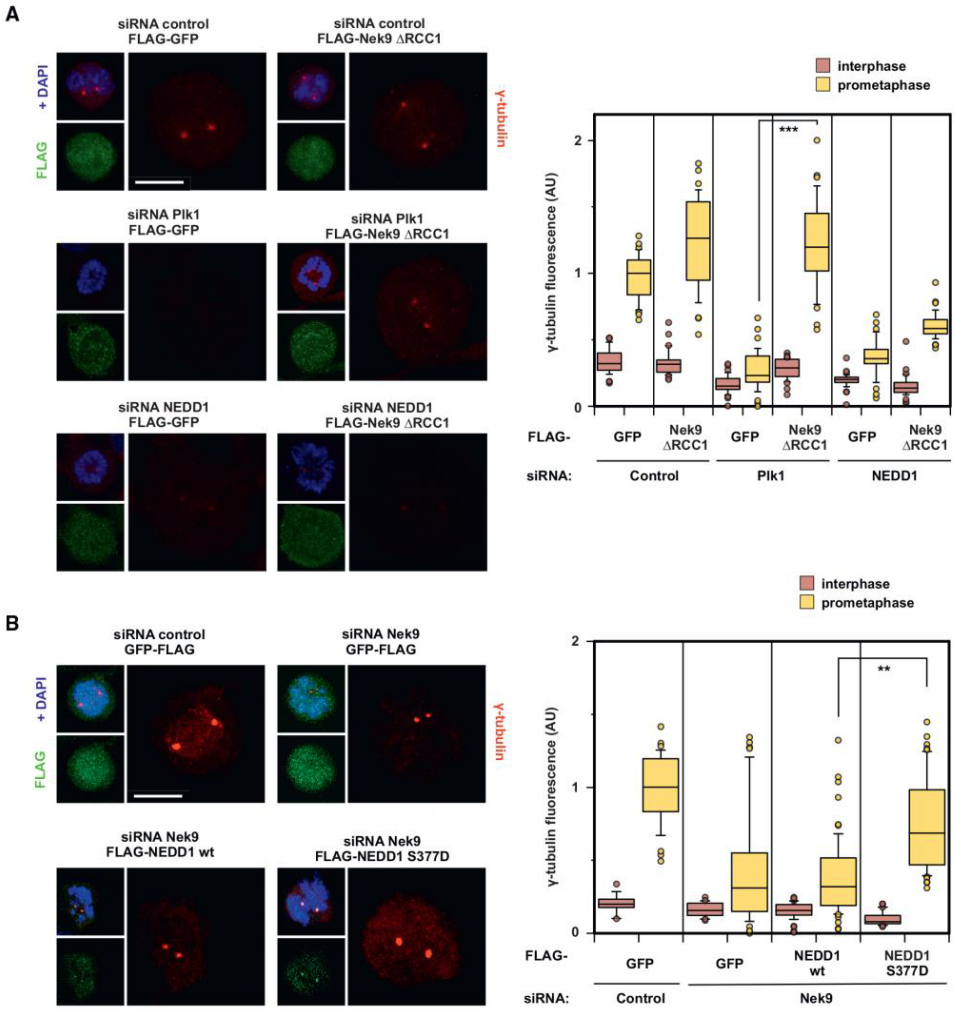

Figure 3. Nek9 Is Downstream of Plk1 and Upstream of NEDD1 in the Control of γ-Tubulin Recruitment to the Mitotic Centrosomes

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

recruitment and thereby γ-tubulin recruitment to the centrosome in the early phases of mitosis.

Discussion

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

(C) Effect of different siRNA transfections on centrosomal MT nucleating activity. Twenty-four (NEDD1) or 48 hr after transfection HeLa cells were cold-treated to depolymerize MTs and following incubation for 20 s in warm medium were fixed and stained. Representative examples of the observed phenotypes in prometaphase are shown (scale bar represents 10 μm). The distribution of intensities is shown as a box plot (three independent experiments, 50 cells counted for each experimental condition of each experiment). Statistical analysis was performed with the Mann-Whitney U-test. Protein levels after the different siRNA transfections are shown in Figure S2A.

Nek9 Controls NEDD1 Recruitment Downstream of Plk1
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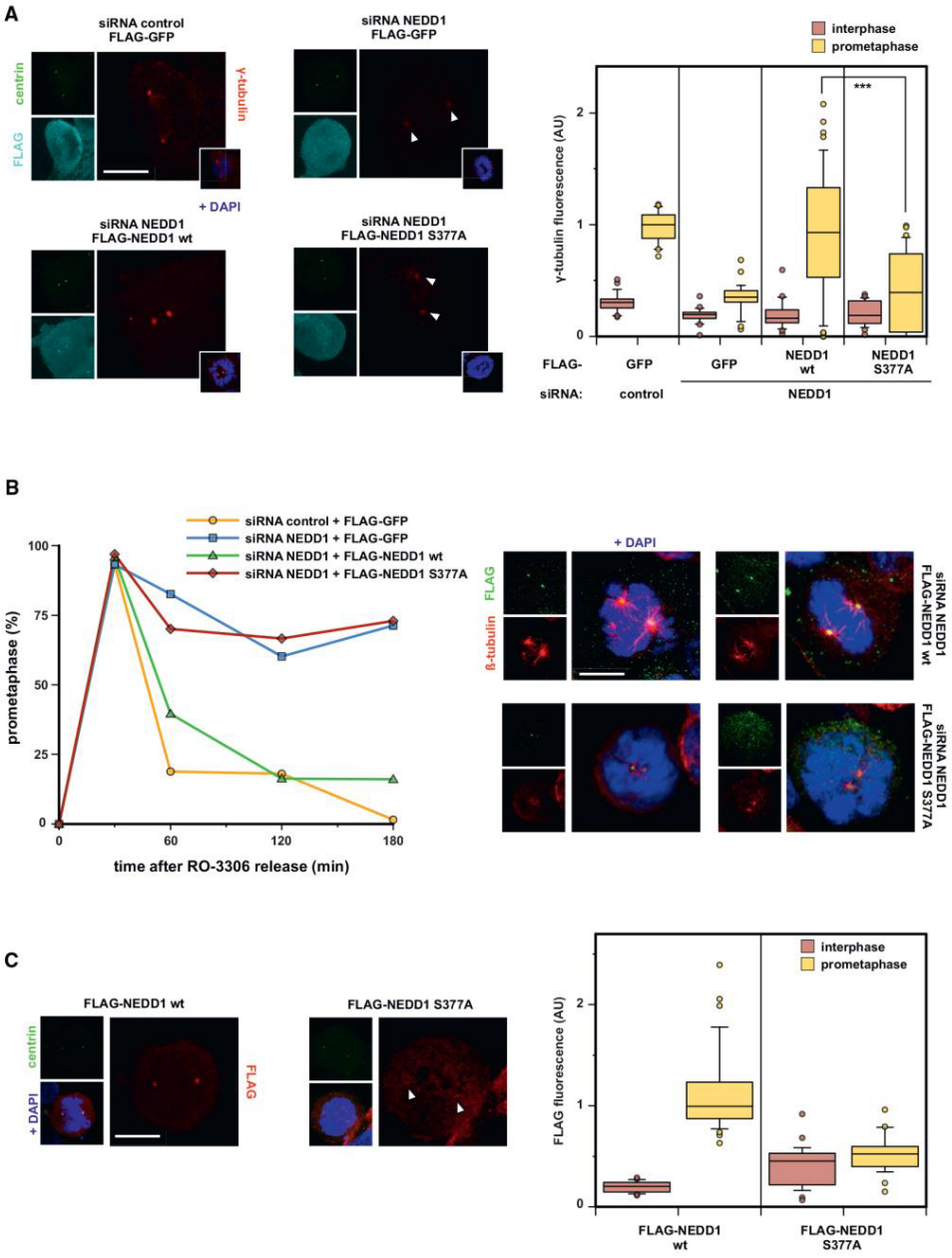

Figure 4. NEDD1[S377] Is Necessary for γ-Tubulin Recruitment to the Centrosome and Normal Mitotic Progression and Controls NEDD1 Recruitment to the Centrosome during Mitosis

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

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

(C) Importance of NEDD1 Ser377 for NEDD1 centrosomal localization as assessed by transfection in HeLa cells. Representative examples of the observed phenotypes are shown (scale bar represents 10 μm). The distribution of centrosomal FLAG intensities in FLAG-positive cells is shown as a box plot (three independent experiments, 20 cells counted for each experimental condition of each experiment).

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accumulation of NEDD1 and γ-TuRC during mitosis has not yet been completely elucidated [8, 19]. We now show that Nek9, a Plk1-activated kinase [13], controls the mitotic levels of NEDD1 and γ-tubulin at the centrosome. We also show that Nek9 phosphorylates NEDD1 at a single residue and thereby controls its recruitment to the centrosome downstream of Plk1.

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

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

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

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

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

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

Supplemental Information

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

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Supplemental Information

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

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Supplemental Inventory

1. Supplemental Figures

Figure S1, related to Figure 1

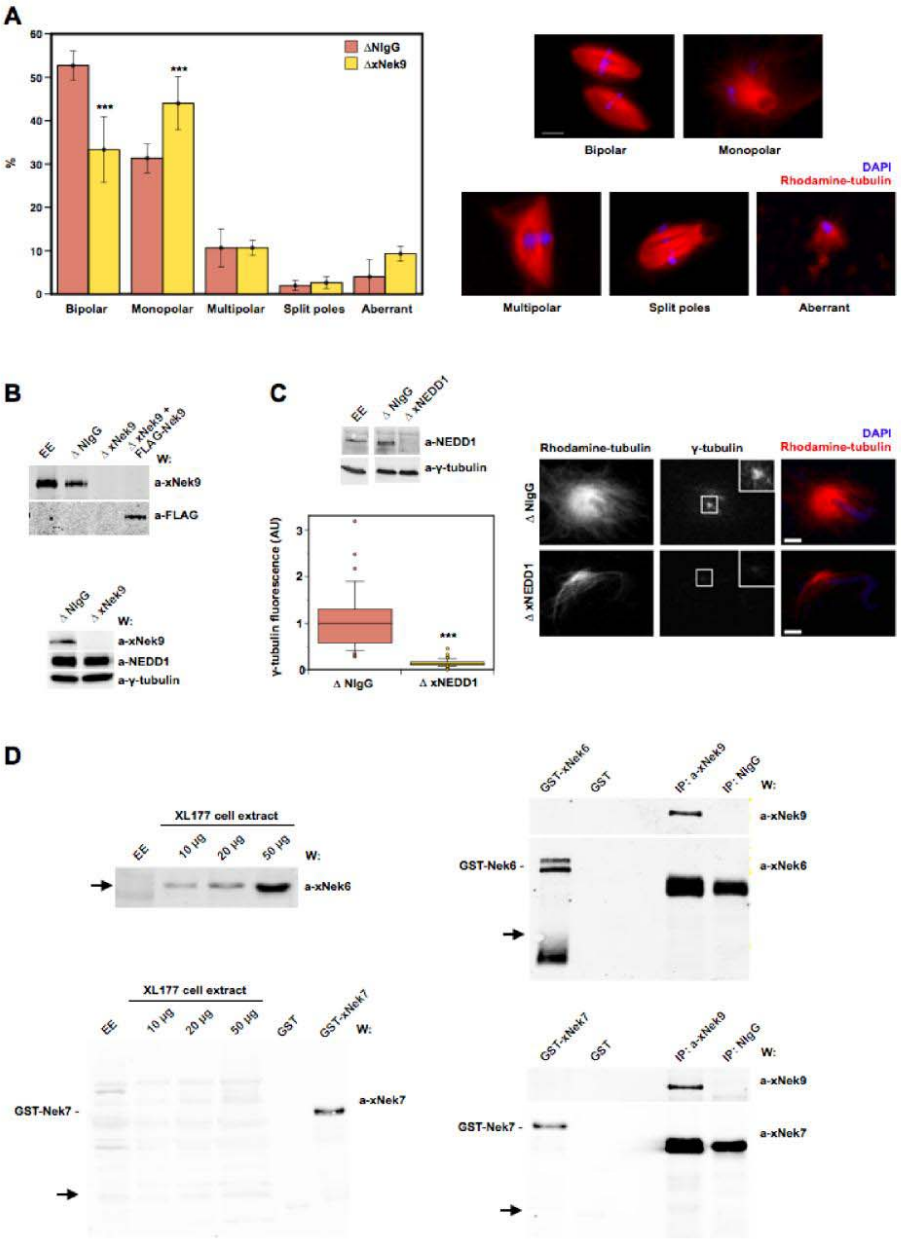
Figure S2, related to Figure 2

Figure S3

Figure S4, related to Figures 3 and 4

2. Supplemental Experimental Procedures

3. Supplemental References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ΔNIgG
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Multipolar
Split poles
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DAPI
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W:
a-xNek9
a-FLAG
a-NEDD1
a-γ-tubulin
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Δ xNEDD1
Rhodamine-tubulin
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XL177 cell extract
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20 µg
50 µg
a-xNek6
GST-Nek6 -
GST-xNek6
GST
IP: a-xNek9
IP: NIgG
GST-Nek7 -
GST-xNek7
a-xNek7

Figure S1. xNek9 Depletion Impairs Normal Spindle Formation in *Xenopus* Mitotic Egg Extracts, Related to Figure 1

Centrosomal **γ**-tubulin levels depend on NEDD1 in *Xenopus* egg extracts. Egg extracts do not contain detectable amounts of xNek6 and xNek7

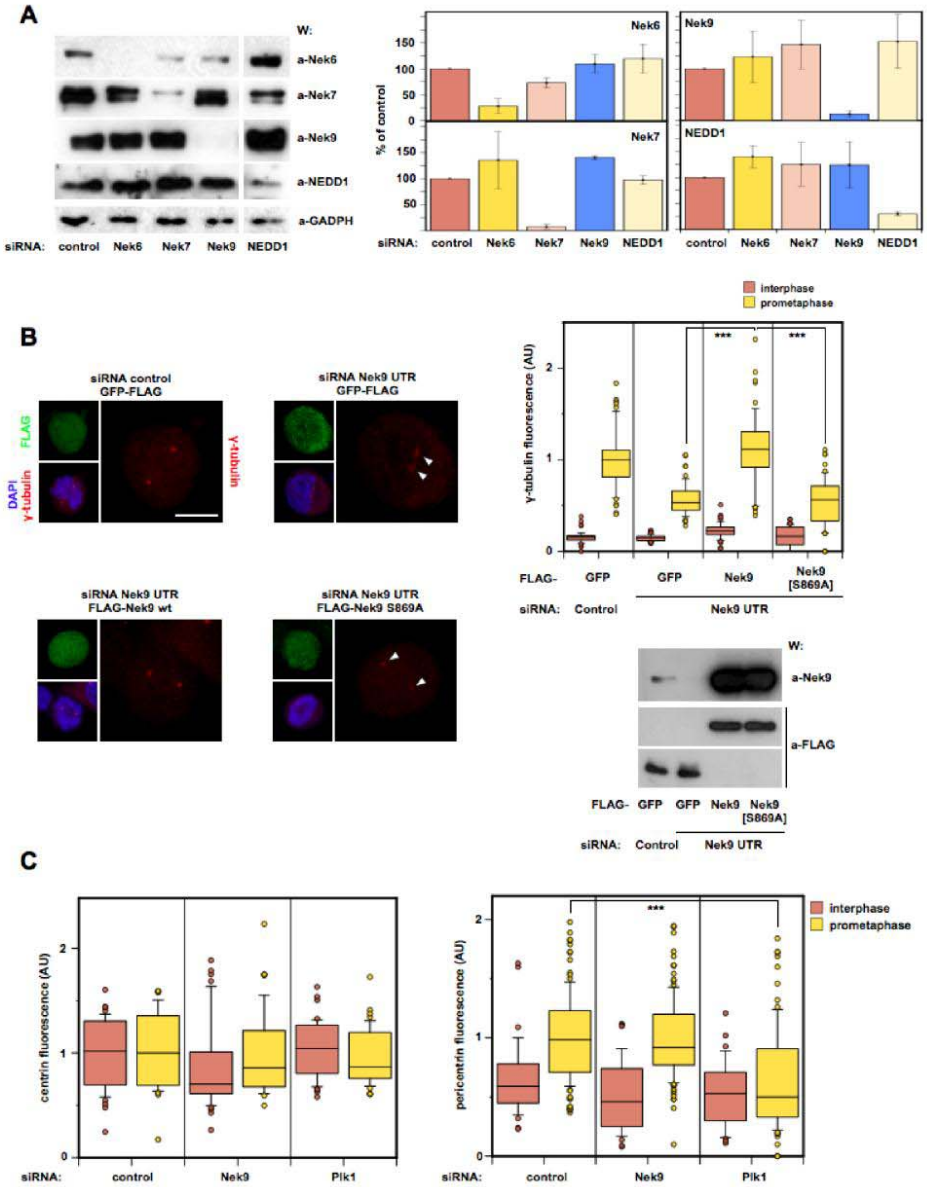
(A) Bipolar spindle assembly was assessed in control depleted (ΔIgG) and xNek9 depleted (ΔxNek9) cycled *Xenopus* egg extract. Results are represented in a graph showing the different mitotic structures found in the extracts and their average appearance in percent (mean ± SEM from 3 independent experiments; 50 structures were analyzed in each experiment). In this and subsequent figures * $p<0.05$ ** $p<0.01$ *** $p<0.001$.

Representative examples of the observed phenotypes are shown (bar, 10 μm).

(B) Representative western blot showing levels of endogenous and recombinant xNek9 in the extracts used in Figure 1 and S1A. Note that the anti-xNek9 antibody does not recognize human FLAG-Nek9 (*data not shown*). Levels of xNek9, γ-tubulin and xNEDD1 in control and xNek9-immunodepleted extracts are shown in the lower panel.

(C) Aster formation induced by addition of sperm nuclei to control- and xNEDD1-depleted extracts. The box plot shows the distribution of γ-tubulin intensities in centrosomes (representative example of 3 independent experiments, n>30 measurements in each case). Western blots show xNEDD1 and γ-tubulin levels; note that global γ-tubulin levels are not affected by xNEDD1 depletion. Representative examples of the observed phenotypes are shown (bar, 5 μm).

(D) Mitotic egg extract, a *Xenopus* XL177 cells lysate and xNek9 immunoprecipitates were tested for the presence of xNek6 and xNek7. Left panels: 1μl egg extract (*EE*) and increasing amounts of XL177 cell lysate, immunoblotted with anti-xNek6 or anti-xNek7 antibodies. 50ng GST plus GST-xNek6 or GST-xNek7 were loaded as control for antibody specificity. Right panels: control IgG and xNek9 immunoprecipitation samples were immunoblotted with anti-xNek6 or anti-xNek7 antibodies. 50ng GST plus GST-xNek6 or GST-xNek7 were loaded as control for antibody specificity. Arrows mark the expected size of xNek6 and xNek7 respectively. Note that the anti-XNek7 antibody, while capable of recognizing recombinant GST-XNek7, fails to unmistakably react with any endogenous band in the different cellular systems used. Both antibodies were tested against GST-xNek6 and GST-xNek7 recombinant protein to confirm specificity and rule out any cross reaction (*data not shown*).


A
W:
a-Nek6
a-Nek7
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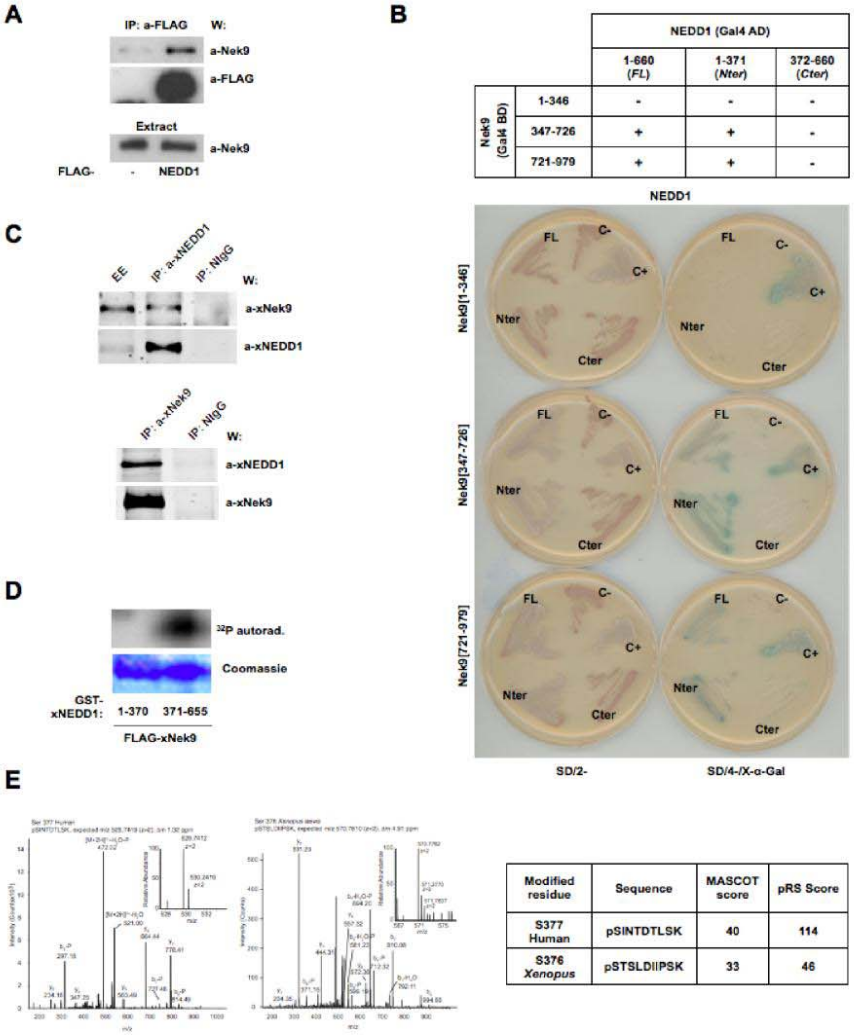
a-NEDD1
a-GADPH
siRNA: control Nek6 Nek7 Nek9 NEDD1
% of control
Nek6
Nek9
Nek7
NEDD1
B
siRNA control
GFP-FLAG
siRNA Nek9 UTR
GFP-FLAG
FLAG
DAPI
γ-tubulin
siRNA Nek9 UTR
FLAG-Nek9 wt
siRNA Nek9 UTR
FLAG-Nek9 S869A
interphase
prometaphase
γ-tubulin fluorescence (AU)
***
FLAG- GFP GFP Nek9 Nek9 [S869A]
siRNA: Control Nek9 UTR
W:
a-Nek9
a-FLAG
C
centrin fluorescence (AU)
pericentrin fluorescence (AU)
siRNA: control Nek9 Plk1

Figure S2. Different Controls to Figure 2, Related to Figure 2

(A) Representative western blots showing the levels of the indicated proteins after the different siRNA transfections in Figure 2. In each case bands were quantified and the average +/- SEM of the obtained results in three independent experiments are shown as a bar graph.

(B) HeLa cells were cotransfected with either control or Nek9 3′ UTR siRNAs plus expression plasmids for the indicated FLAG-tagged proteins and 48 h latter fixed and stained with antibodies against γ-tubulin (red), FLAG (green) and DAPI (blue). Representative examples of the observed phenotypes are shown (bar, 10 μm). FLAG-positive cells were scored and the intensity of γ-tubulin was quantified and represented as in Figure 2 (three independent experiments, 20 cells counted for each experimental condition of each experiment). Levels of endogenous and recombinant Nek9 were determined by western blot using the indicated antibodies.

(C) HeLa cells were transfected with the indicated siRNAs and after 24 (Plk1) or 48 (control, Nek9) hours, fixed and stained with antibodies against centrin or pericentrin and DAPI. Centrin and pericentrin intensity in interphase and prometaphase cells were quantified and represented as in Figure 2 (three independent experiments, 15 -centrin- or 30 -pericentrin-cells counted for each experimental condition of each experiment).



Modified residue	Sequence	MASCOT score	pRS Score
S377 Human	pSINTDTLSK	40	114
S376 *Xenopus*	pSTSLDIIPSK	33	46

Figure S3. Nek9 Binds to and Phosphorylates NEDD1

(A) anti-FLAG immunoprecipitates from HEK293T cells transfected with FLAG- or FLAG-NEDD1 were immunoblotted with anti-Nek9 and anti-FLAG. Nek9 in the corresponding extracts is shown in the lower panel.

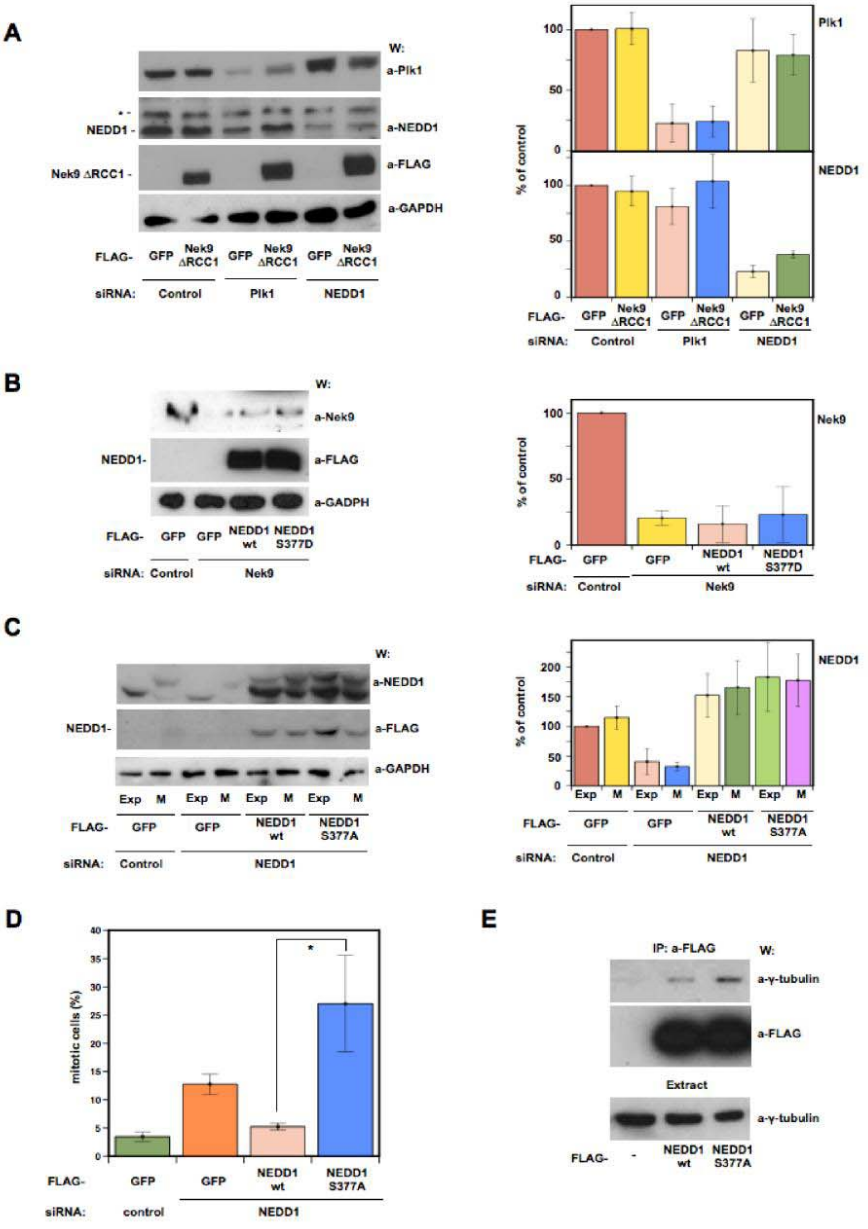
(B) The ability of the full-length NEDD1 (1–660), NEDD1 N-terminal (1-371) or C-terminal domain (372-660) fused to the Gal4 activation domain (*Gal4 AD*) to interact with the different domains of Nek9 (kinase domain: 1–346; RCC1 domain: 347–726; C-terminal tail: 721–979) fused to Gal4 binding domain (Gal4 BD) was assessed using yeast two hybrid by histidine and adenine prototrophy plus expression of α-galactosidase activity. Right plates, *SD/4-/X-α-Gal*, SD/-Leu/-Trp/-His/-Ade/α-Gal. Left plates show cells selected for the presence of the two corresponding plasmids (SD/2-, SD/-Leu/-Trp).). C+, positive control (BD-p53 and AD-SV40); C-, negative control (AD-SV40 and corresponding BD-Nek9 domains). Gal4 AD/BD, Gal4 activation/binding domains.

(C) Normal IgG, anti-xNEDD1 and anti-xNek9 immunoprecipitates from mitotic extracts were immunoblotted with anti-NEDD1 and anti-xNek9 antibodies. *EE*, extracts.

(D) Bacterially expressed GST-fusion proteins of xNEDD1 N-terminal domain (residues*1-370*) and C-terminal domain (*371-655*) were incubated with preactivated xNek9 kinase in the presence of [γ-^{32}P]ATP/Mg^{2+}. After SDS–PAGE, xNEDD1 polypeptides were visualized by Coomassie staining (bottom) and ^{32}P incorporation was visualized by autoradiograph (top).

(E) Representative MS/MS spectra of the identified phosphorylated peptides containing both human NEDD1[Ser377] and *X. laevis* NEDD1[Ser376]. Peptide sequences, Mascot score and PhosphoRS score of identified phosphosites are shown as a table. Note that for the *Xenopus* peptide phosphorylation assignment is ambiguous between Ser376, Thr377 and Ser378.

(F) An alignment of the protein sequence surrounding the identified Nek9 phosphorylation site in several NEDD1 orthologues. Numbering refers to human NEDD1 (top sequence). The asterisks mark hNEDD1 Ser377 and xNEDD1 Ser376.


A
W:
a-Plk1
NEDD1 -
a-NEDD1
Nek9 ΔRCC1 -
a-FLAG
a-GAPDH
FLAG- GFP Nek9 ΔRCC1 GFP Nek9 ΔRCC1 GFP Nek9 ΔRCC1
siRNA: Control Plk1 NEDD1
% of control
Plk1
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a-Nek9
NEDD1-
a-FLAG
a-GADPH
FLAG- GFP GFP NEDD1 wt NEDD1 S377D
siRNA: Control Nek9
Nek9
C
W:
a-NEDD1
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a-FLAG
a-GAPDH
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FLAG- GFP GFP NEDD1 wt NEDD1 S377A
siRNA: Control NEDD1
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mitotic cells (%)
FLAG- GFP GFP NEDD1 wt NEDD1 S377A
siRNA: control NEDD1
E
IP: a-FLAG
W:
a-γ-tubulin
a-FLAG
Extract
a-γ-tubulin
FLAG- - NEDD1 wt NEDD1 S377A

Figure S4. Different Controls and Supplementary Material to Figures 3 and 4, Related to Figures 3 and 4

(A–C) Representative western blots showing the levels of the indicated proteins after the different siRNA and plasmid transfections in Figures 3A, 3B and 4A. In each case bands were quantified and the average +/- SEM of the obtained results in three independent experiments are shown as a bar graph. In (A) the asterisk indicates an unespecific band recognized by anti-NEDD1 antibodies.

(D) HeLa cells were cotransfected with either control or NEDD1 siRNAs plus expression plasmids for the indicated FLAG-tagged proteins, and 24 h latter fixed and stained with antibodies against H3[Ser10]-P and FLAG. The mitotic index (defined as the percentage of positive H3[Ser10]-P cells) of FLAG-positive cells is shown. Results are represented as an histogram (mean ± SEM; four independent experiments, 60 cells counted for each experimental condition of each experiment). Statistical analysis was performed with the t-test.

(E) Mutation of Ser377 to an alanine does not affect the binding between NEDD1 and γ-tubulin. anti-FLAG immunoprecipitates from HEK293T cells transfected with FLAG-, FLAG-NEDD1 wt or FLAG-NEDD1[S377A] were immunoblotted with anti-γ-tubulin and anti-FLAG. γ-tubulin in the corresponding extracts is shown in the lower panel.

Supplemental Experimental Procedures

Plasmids

Nek9 expression plasmids have been described elsewhere [1-5]. xNek9 Cter (residues 707-944) was amplified by PCR from pCMV5 FLAG-xNek9 xNek6 and xNek7 were amplified by PCR from an oocyte cDNA library (a gift from J. Maller, [3]) and subcloned into PGEX-KG. The cDNA of xNEDD1 was amplified via PCR from a *Xenopus laevis* embryo (stage 26) cDNA library using the primers 5'-CGGTCGACTTCAAAAATTGGCCC-3' and 5'-GCGAATTCATGCAGGATAACATCAGAC-3'. xNEDD1 Cter (residues 371-655), xNEDD1 Nter (1-370), were amplified by PCR and subcloned into pGEX-4T1. The cDNA of hNEDD1 was obtained from the German Science Centre for Genome Research (RZPD). Full-length hNEDD1 (1-660) was cloned into pFLAG-CMV-2 (Sigma) using the primers 5'-CCATAGATCTGATGCAGGAAAACCTC-3' and 5'-AAGTCGACTCAAAAGTGGGCCCGTAAT-3'. NEDD1 RNAi-resistant NEDD1 was generated by introducing several silent mutations: 5'-GG(G/T)CA(A/G)AA(G/A)CA(G/A) AC(A/G)TG(T/C)GTCAA(T/C) TTA-3' by subsequent rounds of PCR. Additional mutants were constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions, using specific primers (NEDD1[S377A]: 5'-AAAGCAGGTTTGCCTCGAGCCATAAACACAGACACTTTAT-3'; NEDD1 [S377D]: 5'-GAAAAAGCAGGTTTGCCTCGAGACATAAACACAGACACTTTAT-3'; NEDD1 [S377E]: 5'-GAAAAAGCAGGTTTGCCTCGAGAGATAAACACAGACACTTTAT-3'; with the appropriate reverse complements). cDNAs coding for the indicated human NEDD1 regions were subcloned into pGADT7 using the following primers: pGADT7 NEDD1: 5'-AGATCATGCAGGAAAACCTCAGATTTGC-3' and 5'-GTCGACTCAAAAGTGGGCCCGTAATC-3'; pGADT7 NEDD1[1-371]: 5'-AGATCATGCAGGAAAACCTCAGATTTGC-3' and 5'-GTCGACAGGCAAACCTGCTTTTTCTTGA-3'; pGADT7[372-660] NEDD1: 5'-AGATCTTTGCAGGTTTGCCTCGAAG-3' and 5'-GTCGACTCAAAAGTGGGCCCGTAATC-3'. CMV5-FLAG-GFP was constructed by cloning eGFP into pCMV5-FLAG. All constructs were fully sequenced.

Cycled Spindle Assembly and Other Egg Extract Experiments

Spindle assembly in cycled mitotic egg extracts was done as described before [6-8]. Centrosome aster experiments were described in [9]. For Nek9 depletion, protein A-conjugated Dynabeads (Dynal) coupled to anti-xNek9 antibodies were incubated in 1 volume of mitotic extract for 20 minutes at 4ºC. The depletion efficiency was assayed by Western blot. FLAG-Nek9 was used to restore endogenous concentration (as estimated by Western blot analysis) in rescue experiments. xNEDD1 depletions were done coupling anti-NEDD1 antibodies to protein A-conjugated Dynabeads and performing two rounds of depletion at 4ºC, each of 30 minutes.

Antibodies

Anti-Nek9 and anti-Nek6 antibodies have been described elsewhere [1,2,4]. Specific antibodies against xNek9, xNEDD1, xNek6 and xNek7 were raised by injection of GST-fusion protein of xNek9[707-944], xNedd[371-655], xNek6 or xNek7 respectively into rabbits and purified via affinity chromatography. Other antibodies used are anti-Nek7 (Cell Signaling), anti-NEDD1 (Abnova), anti-Plk1 (Calbiochem)

and anti-FLAG (Sigma). Secondary antibodies were from Jackson ImmunoResearch Laboratories or from Invitrogen and were detected by ECL chemiluminescence (Thermo Scientific) or the Odyssey system (Li-cor).

For immunofluorescence, primary antibodies used were mouse anti-γ-tubulin (GTU-88), mouse anti-FLAG, mouse anti-β-tubulin (Sigma), rabbit anti-centrin [10] pericentrin (generated at the CRG), rabbit anti-histone H3[Ser10-P] (Cell Signaling), and mouse anti-NEDD1 (Abnova). Primary antibodies were detected with Alexa Fluor 488 or 555 goat anti-rabbit IgG and Alexa Fluor 488 or 555 goat anti-mouse IgG (Invitrogen). When needed, anti-FLAG antibodies were detected with Alexa Fluor 647-Fab fragments using the Zenon mouse IgG labelling kit (Invitrogen). DNA was stained with DAPI (Sigma). Cells showing nuclear envelope breakdown and condensed but not aligned chromosomes were scored as in prometaphase (these cells were 100% positive for histone H3[Ser10] phosphorylation, thus confirming the cell-cycle phase assignation).

Images of microtubule structures in egg extracts were taken using a Leica LAS AF 6000 inverted light microscope system and a 63.0 x 1.40 HCX PL APO CS lens, and edited using Leica LAS AF software (Leica Microsystems), Fiji (ImageJ) and Photoshop (Adobe).

Images of mammalian cells were taken using a Leica TCS SPE confocal system with a DM2500 CSQ upright microscopy and a × 63 1.30 ACS Apo lens, and edited using Leica LAS AF software (Leica Microsystems), Fiji (ImageJ) and Photoshop (Adobe).

Cell Culture and Transfection

Xenopus XL177 cells [11] were cultured and extracted as described previously [12]. HeLa cells were cultured as described [1]. Expression plasmids were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNAs were transfected using siPORT NeoFX Transfection Agent (Ambion) according to the manufacturer's instructions. siRNA and DNA cotransfection was performed using Lipofectamine 2000. siRNA duplexes were as in [5] plus siRNA NEDD1: 5'-GGGCAAAAGCAGACATGTG-3' (Dharmacon; [13]. G2 synchronization was accomplished with RO-3306 (9 μm for 20h; Enzo Life Science) and confirmed by FACS analysis.

Immunological Techniques

Immunoprecipitations, western blotting and cell immunofluorescence were performed as described in [1,4,9].

Immunofluorescence Quantification

Average microtubule length and microtubule fluorescence in egg extracts intensity were measured after fixation using a custom made macro running on Matlab software (Math Works) as previously described [14]. y-tubulin or xNEDD1 staining intensity in asters assembled in egg extracts under the different conditions were quantified on non-saturated images with ImageJ software after single image thresholding according to the centrosome size.

Quantification of fluorescence intensities in mammalian cells was performed on non-saturated images acquired under constant exposure with ImageJ software,

using a circular area surrounding a single centrosome (an adjacent area of the same dimensions within each cell was quantified and subtracted as background).

Statistical Methods

Boxes in box plots correspond to the 25th and 75th percentiles (first and third quartiles), whiskers mark from the 10th to 90th percentile. Points outside these are outliers and are shown individually. Additionally, the median (50th percentile) is marked as a line. Statistical analysis was performed with the indicated tests; * $p<0.05$ ** $p<0.01$ *** $p<0.001$.

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed as described in [4].

***In vitro* Kinase Assays**

In vitro kinase assays were performed as described in [1]. GST fusion proteins of xNEDD1[1-370] and xNEDD1[371-655] were purified from bacteria and incubated with FLAG-xNek9 immunoprecipitated from HEK293T cells and pre-activated with incubation with 100 µM ATP.

Mass Spectrometry Analysis

To identify phosphopeptides liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed. For that, purified NEDD1 proteins were resolved in SDS-PAGE, stained with Coomassie Blue, bands excised and samples reduced, alkylated, and digested in-gel with trypsin (Promega). For each sample, 20% was analyzed without enrichment while the reminder of the sample was subjected to a titanium dioxide (TiO) phosphopeptide-enrichment strategy. For LC-MS/MS analysis, peptides were either analyzed on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) or in a LTQ Orbitrap Velos Pro (Thermo Fisher Scientific). For each MS scan, the 10 to 20 most intense ions where selected for fragmentation in the LTQ linear ion trap. MS/MS fragmentation was performed using phosphopeptide-focused multistage activation. MS/MS data were queried against Xenopus laevis NCBI database or IPI_Human database using Mascot v2.3 (Matrix Science).

Supplemental References

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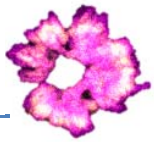
DISCUSSION



ARTICLE 1

EMBO J. 2011

Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5.

Bertran MT, **Sdelci S**, Regué L, Avruch J, Caelles C, Roig J.

Thesis author contribution:

Figures 4 to 9

Supplementary Figures S4 to S9

NEK9 IS A PLK1-ACTIVATED KINASE THAT CONTROLS EARLY CENTROSOME SEPARATION THROUGH NEK6/7 AND EG5

The symmetry of the spindle is an intrinsic characteristic of this mitotic structure and guarantees the correct separation of the duplicated chromosomes in two identical groups that will be distributed into the two daughter cells. One of the factors that ensure the bipolarity of the spindle is the separation of the centrosome, in animal cells the main MTOC.

Attesting to the importance of the formation of a bipolar spindle during early mitosis, centrosome separation is driven by many different factors that can be grouped in the prophase pathway and the prometaphase pathway. During prophase the separation of the centrosome is mainly mediated by forces generated by motor proteins (mainly Eg5 and Dynein). Instead, the prometaphase pathway is driven by the interaction between the two centrosomal asters and the interaction of astral microtubules with k-fibres. These interactions create a network of forces (pulling and pushing forces) that finally separate centrosomes (Tanenbaum & Medema, 2010; O'Connell & Khodjakov, 2007) leading to the formation of the bipolar spindle. The prophase and prometaphase pathways are both present in most cell types and cooperate to guarantee the correct separation of the centrosome, ensuring the bipolarity of the spindle. Despite the apparent redundancy of these pathways, it has been shown that failure of the prophase pathway leads to an incorrect separation of the chromosomes (Silkworth *et al*, 2012) revealing that the prometaphase pathway is not enough to guarantee the correct segregation of chromosomes and emphasizing the outright importance of centrosome separation during prophase.

Prophase centrosome separation is controlled by Plk1 (Llamazares *et al*, 1991) (Lane & Nigg, 1996) and depends on the activity of Eg5, although the molecular details of the involved mechanism are not well understood (Mardin *et al*, 2010; Smith *et al*, 2011).

During mitosis Eg5 is phosphorylated by Cdk1 at Thr926, a phosphorylation that permits its association with microtubules (Blangy *et al*, 1995). Our group described a second phosphorylation site of Eg5 that occurs at Ser1033. Nek6

and Nek7, once activated by Nek9, are able to phosphorylate Eg5[S1033] and this phosphorylation contributes to the localization of Eg5 to the centrosome and is needed for the correct building of the spindle (Rapley *et al*, 2008).

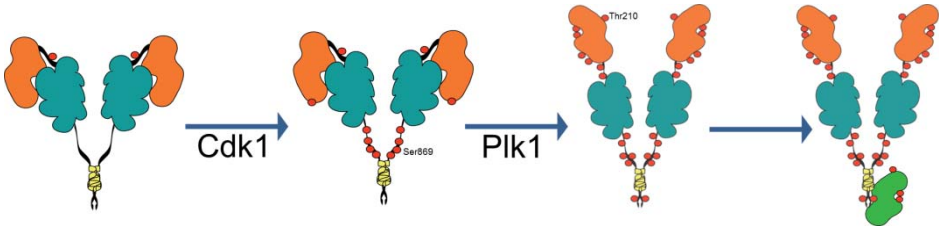


Figure A Mechanism of Nek9 activation. Cdk1 phosphorylates Nek9 at Ser869 (mitosis entry). This phosphorylation is a priming for Plk1 binding and phosphorylation at Thr210. Nek9[T210] phosphorylation results in the activation of Nek9 and in the consequent binding and activation of Nek6/Nek7 (green-shape).

In this article (Article 1) we propose a mechanism that explains how Plk1 can promote centrosome separation during early mitosis (prophase pathway) through Nek9 and subsequent Nek6 and Nek7 activation and Eg5[S1033] phosphorylation. The NIMA related kinases Nek6, Nek7 and Nek9 form a signalling module necessary for bipolar spindle formation (Roig *et al*, 2005) and mitotic progression (Roig *et al*, 2002). The mechanism of activation of Nek6 and Nek7 has been described to depend on Nek9 binding and phosphorylation (Belham *et al*, 2003; Regué *et al*, 2011). In contrast, the activation of Nek9 has remained unsolved until our work described Cdk1 and Plk1 as Nek9 physiologic activators. Thus in this article we propose a two-step activation mechanism for this NIMA-family kinase in which Cdk1 phosphorylates Nek9[S869] (among others), inducing Plk1 binding and subsequent Plk1 Nek9[T210] phosphorylation that results in the activation of Nek9 (Fig. A).

The work presented herein shows that, downstream of Plk1, Nek9, Nek6 and Nek7 regulate prophase centrosome separation. Like Plk1, Nek9, Nek6 and Nek7 are required for this process (Article 1, Fig. 4A), as prophase cells depleted for these kinases show a significantly reduced ability to separate centrosomes.

To demonstrate that Plk1 was fundamental to this process we substituted endogenous Nek9 with one form that is not able to bind to Plk1 (Nek9[S869A]). Nek9[S869A] does not recover the loss of prophase

centrosome separation caused by Nek9 depletion, indicating that the binding to Plk1 is required to accomplish this process (Article 1, Fig. 4B). This result explains at least in part the mechanism by which Plk1 drives centrosome separation during early mitosis, showing how this process is dependent on the activation of the Nek9/Nek6/Nek7 signalling pathway.

Nek9 and Nek6 (and supposedly Nek7) are not only necessary for centrosome separation, but also sufficient. The overexpression of active Nek6 or Nek9 determines in fact the separation of the centrosome even in interphase when under normal physiological conditions centrosomes stay together (Article 1, Fig. 5A), demonstrating the robustness of this mechanism of control of centrosome separation. The mechanism is shown to depend on Eg5 because the depletion of this kinesin completely abolishes this effect, defining Eg5 as the dowsntream effector of the module. Furthermore, we confirm this result in Figure 6 (Article 1), in which we show that active Nek9 and Nek6 are able to rescue the loss of prophase centrosome separation induced by Plk1 depletion, but not that induced by Eg5 depletion.

The requirement of Eg5 also discriminates between centrosome separation mediated by Plk1 and Nek9/Nek6/7 and Nek2-induced centrosome splitting. Nek2 (specifically Nek2A) is the NIMA-related kinase responsible for the dissolution of the link between centrosomes (centrosome splitting), and the overexpression of Nek2 results in centrosome separation (Fry *et al*, 1998a) probably due to the uncontrolled movements of the two split centrosomes. We establish here that the separation of the centrosome provoked by Nek2 overexpression is Eg5-independent (Article 1, Fig. 5B), thus indicating that the Nek2 and Nek9/Nek6/Nek7 pathways are independent. The relative importance of Nek2 for centrosome separation and its relationship to Eg5-driven separation has been studied by Mardin and colleagues; these authors showed that the chemical inhibition of Eg5 with monastrol did not reduce the ability of the cells to form a normal bipolar spindle if centrosomes have been split by Nek2 and then separated (Mardin *et al*, 2010). Conversely, the inhibition of Nek2 does not provoke reduced centrosome separation in cells with normal levels of Eg5 (Fry *et al*, 1998a; Mardin *et al*, 2010), a result that we have confirmed (Article 1, Fig. 5B).

The localization of Eg5 to the centrosome was previously shown to be Plk1 dependent (Mardin *et al*, 2010; Smith *et al*, 2011), an observation compatible

with our results, and supported by Eg5[S1033] phosphorylation (Rapley *et al*, 2008). Now we show that Eg5[S1033] phosphorylation not only is necessary for the recruitment of Eg5 to centrosomes but also for prophase centrosome separation. Although Eg5[S1033A] is able to bind to microtubules (indicating that non centrosomal functions of the kinesin are not affected), it cannot accumulate around the centrosome; as a result, cells carrying this mutant do not separate centrosomes in prophase, strongly supporting the role of the Nek9/Nek6/Nek7 signalling pathway in the control of centrosome separation (Article 1, Fig. 7). This result is confirmed by the fact that depletion of Nek6, Nek7 or Nek9, as well as the depletion of Plk1, not only causes loss of centrosome separation but also the disappearance of Eg5 from the vicinity of centrosome (Article 1, Fig. 8A). Moreover, the robustness of this mechanism is demonstrated by the fact that overexpression of active Nek6 or Nek9 after Plk1 depletion not only results in centrosome separation, but also in Eg5 centrosomal recruitment (Article 1, Fig. 8B). Overexpression of active Nek6 or Nek9 also induces the recruitment of Eg5 at interphase centrosome (Article 1, Fig. S8), explaining how that overexpression can trigger the aforesaid premature non-mitotic centrosome separation outside mitosis (Article 1, Fig. 5A).

Regarding the importance of the described mechanism during mitotic progression, the substitution of endogenous Eg5 with Eg5[S1033A] causes a significant delay in mitotic progression after G2 phase synchronization. Cells that fail to phosphorylate Eg5[Ser1033] remain longer in prometaphase, even if most of them arrives at metaphase and can finish mitosis (Article 1, Fig. 9 and Fig. S9), probably because of the partial redundancy of the prophase and prometaphase centrosome separation pathways. We would like to note that the completion of these mitosis not necessarily results in the proper accomplishment of this process and the large time that these cells spend to finish mitosis might indicate that mitosis is not proceeding properly. In fact, a recent work describes how the separation of the centrosomes during prophase results necessary for the correct segregation of the chromosomes (Silkworth *et al*, 2012). We can thus speculate that cells carrying the Eg5[S1033A] mutant although able to separate centrosomes in prometaphase instead than in prophase and thus finish mitosis, may be segregating improperly the chromosomes. Further experiments quantifying aneuploidy rates will be

necessary to determine if that is actually the case as in fact our experiments depleting cells from Nek9 suggest (see Additional Results, Fig. 7).

The molecular mechanism responsible for the localization of Eg5 to the centrosome is not easy to explain due to the low number of antiparallel microtubules there and the orientation of the microtubules, with plus ends moving away from centrosomes. Having in mind the results presented here we can hypothesize that the phosphorylation of the Ser1033 determines the recruitment of a small amount of Eg5 to the vicinity of the centrosome (3% of the total Eg5 amount (Rapley *et al*, 2008)), (I) (to interact with an unknown centrosomal or pericentrosomal protein (in a microtubules independent manner) or (II) determining the binding of Eg5 to some yet to be described protein (e.g. a motor) able to localize/stabilize Eg5 to the minus end of microtubules and thus the vicinity of the centrosome (microtubule dependent mechanism). It is known that Eg5 centrosomal localization is microtubule dependent ((Mardin *et al*, 2010) and our unpublished results), suggesting that probably the second hypothesis is the most likely (but not totally discarding the other). We thus can hypothesize the presence of a second motor protein able to transport Eg5 on microtubule tracks or a protein able to stabilize Eg5 on microtubules in the vicinity of centrosome. The dynein/dynactin complex and Eg5 have already been described to interact (Blangy *et al*, 1997; Uteng *et al*, 2008; Tanenbaum *et al*, 2008). These interaction seems to be involved in the dynamicity of Eg5 on the spindle, excluding the very central part of the spindle and the centrosome or centrosome vicinity (Uteng *et al*, 2008), thus explaining, at least in part, the elasticity of the spindle. Besides, the interaction between Eg5 and central spindle microtubules is promoted by TPX2, a spindle assembly factor (Eckerdt *et al*, 2008; Ma *et al*, 2011); taken together these findings reveal that the localization of Eg5 on the mitotic spindle is the result of the interplay between dynein/dynactin complex and TPX2 (Gable *et al*, 2012). Although these interactions do not explain the recruitment of Eg5 to the centrosome, they suggest the presence of a possible similar mechanism for that. In particular dynein, for its minus-end directed movement, might result to be the best candidate to transport Eg5 poleward (opposite to its usual plus-end direction), in a microtubules dependent manner. Ongoing work aimed to identify Eg5-interacting factors may determine the validity of this hypothesis. In this regard it is worth noting that the recruitment of Eg5 to the centrosome

mediated by Plk1 cannot be explained exclusively through the activation of the Nek9/Nek6/Nek7 signalling module. Figure 8 (Article 1) and Figure S7 (Article 1) show that active Nek6 or Nek9 can only partially recover the diminished amount of centrosomal Eg5, after Plk1 depletion, suggesting that the recruitment of Eg5 to the centrosome can be controlled by Plk1 (or other not yet described Plk1 targets) through the mechanism that we describe in collaboration to other yet to be described alternative mechanisms, maybe related to the centrosomal accumulation of additional proteins.

Taken together, these considerations might indicate that Eg5-mediated prophase centrosome separation facilitates the building of the spindle during early mitosis, giving a head start for those mechanism that operate during the prometaphase pathway, rendering the construction of the bipolar spindle a remarkably robust mechanism able to assure the formation of two distinct and symmetrical asters capable to capture and separate chromosomes in two identical groups.

ARTICLE 2

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Nek9 phosphorylation of NEDD1/GCP-WD contributes to Plk1 control of γ-tubulin recruitment to the mitotic centrosome.

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Thesis author contribution:
Figures 2 to 4
Supplementary Figures S2 and S4

NEK9 PHOSPHORYLATION OF NEDD1/GCP-WD CONTRIBUTES TO PLK1 CONTROL OF Γ-TUBULIN RECRUITMENT TO THE MITOTIC CENTROSOME

The process of centrosome maturation guarantees that the centrosome has an adequate level of microtubule nucleation activity resulting in the production of the correct number of microtubules to organize a normal mitotic spindle, thus conferring to the spindle the ability to capture chromosomes and segregate them into the two daughter cells.

Centrosome maturation requires the accumulation of different PCM proteins, a process that starts in late G2 and reaches maximum levels during the prometaphase/metaphase transition, resulting in a five-fold increase of the centrosome size (Piehl *et al*, 2004). One of the most important protein complexes recruited to the centrosome during maturation is the γ-TuRC. The recruitment of this complex to the mitotic centrosome is essential to trigger the nucleation of new spindle microtubules. The recruitment of γ-TuRC, and thus γ-tubulin, to the centrosome is mediated by the adaptor protein Nedd1 (Luders *et al*, 2006; Manning *et al*, 2010; Haren *et al*, 2006). The accumulation of Nedd1 to the mitotic centrosome, and consequently the localization of γ-TuRC there, is mediated by phosphorylations, mainly driven by the key mitotic regulators Plk1 and Cdk1. Cdk1 and Plk1 activity are required for Nedd1 centrosomal localization; however, direct Cdk1/Plk1 phosphorylations are not sufficient to accomplish this process (Haren *et al*, 2009; Zhang *et al*, 2009), suggesting the presence of Cdk1/Plk1 substrate(s), upstream Nedd1 and downstream of these mitotic kinases.

Our work shows how Nek9 directly controls the accumulation of Nedd1 to the mitotic centrosome (and thus of γ-TuRC) both in Hela cells and *Xenopus* egg extracts, through a Nedd1 single-site phosphorylation.

In experiments done by our collaborators, mitotic *Xenopus* egg extracts depleted for XNek9 showed a reduced ability to support microtubules nucleation. In addition, the accumulation of γ-tubulin and Nedd1 to the mitotic centrosome was diminished.

The evidence that Nek9 was directly implicated in centrosome maturation and microtubule nucleation in *Xenopus* system encouraged us to investigate the effect of Nek9 depletion also in human cells (specifically in Hela cells). We found that cells depleted for Nek9 were not able to maturate centrosome because they were unable to recruit γ-tubulin and Nedd1 to the organelle (Article 2, Fig. 2A/B). The loss of centrosomal accumulation of γ-tubulin and Nedd1 results in a clear disruption of the ability of Nek9 depleted cells to nucleate normal levels of microtubules during mitosis (Article 2, Fig. 2C), in agreement with the results obtained with *Xenopus* egg extracts. In this regard it is important to observe that the depletion of Nek9 does not impair centrosome integrity; in fact, as shown in Figure S2C (Article 2), the centrosomal levels of centrin (centrosome core structure) and PCNT (marker of PCM integrity) result unaltered both in interphase and mitosis (prometaphase) in cells depleted of Nek9.

Nek9 functions can be accomplished through the activation of its downstream kinases Nek6 and Nek7; a clear example of that is the regulation of centrosome separation described in Article 1 (Bertran *et al*, 2011). The absence of these two kinases in *Xenopus* eggs indicates a possible independent Nek9 role in the regulation of centrosome maturation. Depletion of Nek6 and Nek7 has been previously proposed to results in fragile spindle formation (O'Regan & Fry, 2009) and depletion of Nek7 in impaired centrosome maturation (Kim *et al*, 2007; Kim & Rhee, 2011). While we did not investigate here (neither in the Article 1) the formation of fragile spindle after Nek9, Nek6 or Nek7 depletion, surprisingly, in our hands, the depletion of Nek7 or of Nek6 does not induce any change in the structure of the interphase or mitotic PCM (as measured by Nedd1, γ-tubulin and PCNT levels) in interphase or in mitosis (Article 2, Fig 2A and S2C). Accordingly, after dowregulation of Nek6 or Nek7 microtubule nucleation activity does not show any significant variation (Article 2, Fig. 2C), suggesting that the spindle defects reported in other works depend on other not yet described Nek6/Nek7 function, maybe stabilizing preformed microtubules.

Incidentally, the lack of Nek6 and Nek7 expression in *Xenopus* eggs as well as in *Xenopus* early embryos indicates that centrosome separation in this model might be regulated different than in human cells, since here it is controlled by Nek6/Nek7 Eg5[S1033] phosphorylation and Eg5 peri-centrosomal localization (Article 1; (Bertran *et al*, 2011)). Eg5 is normally expressed in *Xenopus* eggs and embryos and during mitosis it localizes at spindle, with significant accumulation at spindle poles (Houliston *et al*, 1994) and, as in human cells, its activity is required for bipolar spindle formation (Guellec *et al*, 1991). These data suggest the presence of another mechanism of regulation of Eg5 spindle poles localization and centrosome separation function in this system, possibly related to the Nek-independent prometaphase pathway of centrosome separation.

Our results show that Nek9[S869A], a mutant unable to bind Plk1 (Article 1; (Bertran *et al*, 2011)), cannot recover the loss of centrosomal γ-tubulin accumulation provoked by Nek9 depletion during mitosis, contrary to the wild type form (Article 2, Fig. S2B). Moreover an active form of Nek9 completely rescues the lack of γ-tubulin recruitment to the mitotic centrosome after Plk1 depletion (Article 2, Fig. 3A). Our results confirm the central role of Plk1 in the recruitment of γ-tubulin to the mitotic centrosome, and reveal that it relies (at least in part) on Nek9 activation. On the other hand, the loss of γ-tubulin to the mitotic centrosome determined by Nedd1 depletion is not restored overexpressing the active form of Nek9, suggesting the downstream position of Nedd1 respect to Nek9 (Article 2, Fig.3A).

Nek9 was previously shown to interact with γ-tubulin and other components of the γ-TuRC (Roig *et al*, 2005). In agreement with those results, our results show that Nek9 interacts with and directly phosphorylate Nedd1 in mitosis (Ser377) (Article 2, Fig. S3). Nedd1 is highly phosphorylated in mitosis but until now the mechanism of Nedd1 accumulation to the mitotic centrosome was not clearly understood; here we shown how a single phosphorylation site, Ser377, results fundamental to this process. Remarkably, Nedd1[S377] was previously found phosphorylated in mitosis in a Plk1-dependent manner, although it is not a Plk1 direct phosphorilation site (Santamaria *et al*, 2011). We

confirm this site as phosphorylated in mitosis (Article 2, Fig S3D/E) and moreover, we show Nek9 as the best candidate to accomplish this phosphorylation. Nedd1 Ser377 falls in the classical NIMA consensus motif and is conserved across different species (Article 2, Fig. S3F).

Nedd1[S377D], a phosphomimetic mutant, is able to recover the decrease of the accumulation of γ-tubulin to the mitotic centrosome provoked by Nek9 depletion, while the overexpression of the wild type form of Nedd1 is not (Article 2, Fig.3B), suggesting that Nek9 is the kinase responsible to accomplish this phosphorylation. Nedd1[S377A] fails to accumulate to the mitotic centrosome (Article 2, Fig. 3C), and as well fails to efficiently support the centrosomal recruitment of γ-tubulin (Article 2, Fig. 3A), suggesting that this phosphorylation is required for the accumulation of endogenous Nedd1, and thus γ-tubulin, to the mitotic centrosome.

The importance of this single phosphorylation site is confirmed by the fact that Nedd1[S377A], unlike the wild type form, does not guarantee the correct mitotic progression after G2 phase synchronization (Article 2, Fig. 4B); result that explains the higher mitotic index found in cell transfected with this mutant (Article 2, Fig S4D) and is most probably related to the phenotype resulting from Nek9 downregulation (see below).

It has been shown that centrosome laser-ablation does not impair the building of the mitotic spindle and cell can finish mitosis in the same time window than cell with centrosomes (Khodjakov *et al*, 2000). By the same principle, cells naturally without centrosome can form a normal bipolar spindle and divide without any problem (Shimamura *et al*, 2004; Heald *et al*, 1996; Matthies *et al*, 1996). It remains thus to be elucidated why these cells are not able to build a spindle nucleating microtubules from non-centrosomal MTOCs, at least not in the same time window than control cells. Here we show that the loss of centrosome maturation strongly impaired mitotic progression, resulting in an important prometaphase delay. Apparently, our results are in disagreement with the aforesaid findings. However, we are showing here a model where two centrosomes are present in the system, although non-functional due

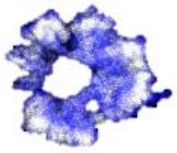
to their partially immature state. This may explain the differences that we observe from models where centrosomes are disrupted (Khodjakov *et al*, 2000) or are naturally absent (Shimamura *et al*, 2004; Heald *et al*, 1996; Matthies *et al*, 1996). This observation can indicate that the physical presence of the centrosome might induce a signal to preferentially nucleate microtubules from centrosome instead of from non-centrosomal MTOCs. Alternatively centrosomes, although immature, may compete for components needed to nucleate microtubules, thus disturbing no-centrosomal microtubule nucleation. These hypothesis might explain the prometaphase delay we see in mitotic cells that fail to phosphorylate Nedd1[S377], revealing the fundamental role of the centrosome to guarantee correct mitotic progression.

It would be also interesting to investigate possible alternative roles of Nek9 in microtubules nucleation from non-centrosomal MTOCs. The involvement of Nek9 in the regulation of non-centrosomal nucleation pathways might contribute to explain better the strong phenotype we observe dowregulating this kinase (see below).

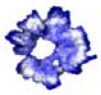
The role of Plk1 in the control of centrosome maturation not only concerns the recruitment of Nedd1 and γ-tubulin to the centrosome but also implicates the recruitment of other PCM component such as PCNT, Cep-192 and Cdk5Rap2 (Haren *et al*, 2009; Santamaria *et al*, 2011). PCNT and Cdk5Rap2 are known to be implicated in the anchor of γ-tubulin to the centrosome, providing an interaction point for the γ-TuRC complex. Plk1-mediated centrosome maturation depends thus in Nedd1, PCNT and Cdk5Rap2 centrosomal recruitment, and possibly in a complex network of interactions and a multiple steps mechanism that ensures the proper maturation of the centrosome (Haren *et al*, 2009). Here we show that overexpression of the active form of Nek9 completely rescues the loss of maturation induced by Plk1 dowregulation. This observation might indicate that Nek9 has diverse roles in the control of PCM accumulation during centrosome maturation, since that the overexpression of the active form of this kinase would be expected to only partially recover Plk1 dowregulation. A second hypothesis to explain this remarkable rescue might be that overexpressed active Nek9 in a background of diminished (but existent) Plk1 activity may be enough

to induce centrosome maturation, since the low amount of Plk1 might likewise promote some PCNT and/or Cdk5Rap2 centrosomal recruitment, while active Nek9 would be the responsible for Nedd1 and γ -tubulin accumulation to the mitotic centrosome. In this regard, it may be interesting to see if active Nek9 is able to rescue Plk1 inhibition during centrosome maturation by a chemical Plk1 inhibitor, generally more efficient than siRNA downregulation.

CONCLUSIONS



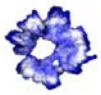
ARTICLE 1



NEK9 IS A PLK1-ACTIVATED KINASE THAT CONTROLS EARLY CENTROSOME SEPARATION THROUGH NEK6/7 AND EG5

1. Nek9/Nek6/Nek7 signalling pathway (as well as Plk1 and the mitotic kinesin Eg5) is necessary for the correct separation of the centrosome during prophase.
2. The active forms of the kinase Nek9 or Nek6 induce centrosome separation in interphase cells, in an Eg5-dependent manner.
3. Active Nek9 and Nek6 moreover can rescue the lack of centrosome separation induced by Plk1 downregulation in prophase cells. Conversely the effects on centrosome separation provoked by Eg5 dowregulation could not overturned by active Nek9 or Nek6.
4. Plk1 controls Eg5 phosphorylation at the Nek6 site Ser1033 and allows the correct separation of the centrosome trough Eg5 centrosomal recruitment.
5. Plk1, Nek9, Nek6, Nek7 are necessary for centrosome recruitment of Eg5 during prophase.
6. Active Nek9 and Nek6 can rescue the lack of Eg5 recruitment to the centrosome induced by Plk1 downregulation.
7. Failure to phosphorylate Eg5[Ser1033] results in a delay in prometaphase

ARTICLE 2



NEK9 PHOSPHORYLATION OF NEDD1/GCP-WD CONTRIBUTES TO PLK1 CONTROL OF Γ-TUBULIN RECRUITMENT TO THE MITOTIC CENTROSOME.

1. Nek9 plays a direct role in Nedd1 and γ-tubulin recruitment to the centrosome in human cells during mitosis (prometaphase)
2. Nek9 acts downstream of Plk1 and upstream of Nedd1 for the recruitment of γ-tubulin to the centrosome. Active Nek9 can in fact rescue the lack of centrosomal γ-tubulin recruitment induced by Plk1 dowregulation but not the one Nedd1-induced.
3. Nedd1 can interacts during mitosis with Nek9 and moreover Nedd1 is a substrate of this kinase.
4. Nek9 regulates centrosomal γ-Turc recruitment phosphorylating Nedd1
5. Failure to phosphorylate Nedd1[Ser377] results in a impaired centrosomal Nedd1 and γ-tubulin accumulation during prometaphase.
6. The lack of Nedd1[Ser377] phosphorylation provokes delay in prometaphase.

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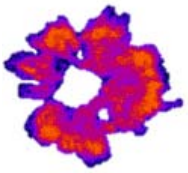

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