Study of the phosphorylation and activation of the protein kinase NEK9 during mitosis

M. Teresa Bertran Domingo

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

Departament de Bioquímica i Biologia Molecular

STUDY OF THE PHOSPHORYLATION AND ACTIVATION OF THE PROTEIN KINASE NEK9 DURING MITOSIS

M. Teresa Bertran Domingo, 2012

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

Departament de Bioquímica i Biologia Molecular

Programa de Doctorat en Biomedicina

STUDY OF THE PHOSPHORYLATION AND ACTIVATION OF THE PROTEIN KINASE NEK9 DURING MITOSIS

Memòria presentada per M. Teresa Bertran Domingo per optar al títol de doctor per la Universitat de Barcelona

Dr. Joan Roig Amorós

M. Teresa Bertran Domingo

Als meus pares

I, a vegades, contra tot pronòstic una gran bestiesa capgira allò que crèiem lògic,
Tot fent evident,
Que per un moment,
Ens en sortim.

Manel

Quan surts per fer
el viatge cap a Ítaca,
has de pregar que el camí sigui llarg,
ple d'aventures, ple de coneixences.
Has de pregar que el camí sigui llarg,
que siguin moltes les matinades
que entraràs en un port
que els teus ulls ignoraven,
i vagis a ciutats per aprendre dels que saben.

Tingues sempre al cor la idea d'Ítaca.
Has d'arribar-hi, és el teu destí,
però no forcis gens la travessia.
És preferible que duri molts anys,
que siguis vell quan fondegis l'illa,
ric de tot el que hauràs guanyat fent el camí,
sense esperar que et doni més riqueses.

Ítaca t'ha donat el bell viatge,
sense ella no hauries sortit.
I si la trobes pobra, no és que Ítaca t'hagi enganyat.
Savi, com bé t'has fet,
sabràs el que volen dir les Itaques.

Konstandinos P. Kavafis - Carles Riba - Lluís Llach

Agraïments

Al Joan, per haver confiat en mi des del primer moment. Per tota la formació, consells i entusiasme, i sobretot per haver aconseguit que una química es converteixi en tota una biòloga cel·lular.

A la Carme, gràcies per tot el suport, consells i suggeriments donats al llarg d'aquests anys. I als membres del grup de farmàcia, pels diferents punts de vista rebuts en els seminaris dels dimarts. Als membres de la Comissió de seguiment, gràcies pels suggeriments a l'hora de seguir amb l'elaboració d'aquesta tesis.

A Sara, por todo tu entusiasmo y energia, sin ti parte de esta tesis no hubiera sido possible! Por ser una gran companyera y amiga dentro y sobre todo fuera del laboratorio. Grazie mille! A la Neus, moltes gràcies per tots els consells científics i no científics, i escoltar-me en els meus moments d'indecisió, que no han estat pocs. A la Cris, per haver fet que l'elaboració de la tesis hagi estat més fàcil i per haver estat una gran profe i ensenyar-me en temps rècord com funciona la selva de l'estabulari del PCB. Per tots els cafès, les bombes, les sortides i soparets fets al llarg d'aquests anys, moltes gràcies. Al Jordi per haver fet desaparèixer l'emo que vivia dins meu i per ser el monitor d'esquí més pacient que he vist mai! Gràcies per totes les hores compartides dins i fora del lab. Johan, el hombre más culto del mundo! Merci por compartir todas tus aventuras, fiestas y amistad! To Kader for all the moments we shared during these years, dinners, parties, skiing... Teşekkürler!! A Susana. Llegaste al lab en un año de locos. Grazas por aguantarnos y ayudarme en todo. Giuseppe, grazie por tu tranquilidad i todos los momentos compartidos. Durant aquests anys hem estat com una família amb molt bons moments, però entre tots també ens hem aguantat en moments més difícils. Gràcies a tots per ser com sou. Us trobaré a faltar! No cambieu mai!

Als que ja no estan al lab. A la Laura gràcies per ser la meva mestra, sempre estaves allà quan necessitava qualsevol cosa i per totes les coses compartides fora de la poiata. Al Rodrigo, per aportar el toc xilè al laboratori. Als antics membres del laboratori, Mabel, Marta, Julie, Salva. Em vau ensenyar com

treballar en un laboratori i el més important de tot em vau acollir com una més des del primer dia que vaig aterrar al lab. Als guinos i ex-guinos, especialment al Jordi, Carlos, Susanna i Carles, per aquells dinars de club del tupper i tots els sopars i cerveses plens de batalletes que n'han derivat.

També voldria agrair als membres del l'Advanced Microscopy facility, per totes les hores que m'heu estat ajudant a programar microscopis i tots els dubtes resolts a l'hora de l'enregistrament i manipulació de les imatges.

Gràcies també a tota la gent de l'IRB que heu compartit alguna cosa més que feina amb mi. Sense vosaltres i els cool offs, sopars, sortides i festes, això hagués estat molt feixuc.

No voldria deixar d'agrair a tota la gent que comparteix la meva vida fora la feina. Gràcies a les riudomenques, per ser-hi des de sempre i aportar-me una visió del món fora de la ciència. Especialment a les que vam formar un gueto riudomenc a la city i vam compartir moltes vivències. Gràcies també per preguntar per les meves cèl·lules i ratolinets, encara que us pogués sonar tot a xinès. Als químics, ja que tot i que no ens veiem gaire sovint, continuem sent grans amics. Gràcies per tots els viatges, sopars, casaments i naixements que hem viscut durant aquests anys. Als catalins i catalines, perquè qualsevol excusa és bona per reunir-nos al voltant d'una taula amb bon menjar i sobretot bons vins.

Als meus avis, per sempre preocupar-se per la seva neta i interessar-se en tot moment per mi. Al Francesc, gràcies per totes les coses que hem compartit sobretot en els últims anys, per fer de germà "gran" quan em posava molt pesada, per la teva tranquil·litat i per fer-me veure que les coses no són tan complicades.

Gràcies als meus pares. Heu cregut sempre més en mi que jo mateixa i m'heu ajudat a tirar endavant en els bons i mals moment. Moltes gràcies per tots els esforços que heu fet perquè hagi pogut arribar fins aquí.

INTRODUCCIÓ

El cicle cel·lular està bàsicament regulat per dos mecanismes postraduccionals que es troben íntimament connectats: la fosforilació de proteïnes i la proteòlisis. Cdk1 és una de les proteïnes que juga un paper més important en la mitosi, tot i que en els últims anys proteïnes de les famílies de les Aurora, Polo i NIMA també participen en processos importants durant l'entrada i la sortida de mitosis, la regulació del *checkpoint*, etc.

Les *Polo like kinases* (Plks) són reguladores essencials de la mitosi, la meiosi i la citocinesi. En mamífers s'han descrit cinc membres d'aquesta família, sent Plk1 el més important. A més de tenir un paper fonamental en l'entrada de la mitosi, com a part del complex que indueix l'activació de CDK1, Plk1 és crucial per diferents esdeveniments mitòtics com la separació i maduració dels centrosomes i la formació del fus mitòtic, entre d'altres. La base molecular d'algunes de les seves funcions comença a ser desxifrada i es basa en el reconeixement per part del domini Polo-box (PBD) de substrats que han estat prèviament fosforilats, cosa que també localitza la quinasa en diferents llocs com ara els centrosomes o els centròmers.

La família de les NIMA proteïna quinases (Neks) reben el nom per NIMA, una quinasa d'*Aspergilus nidulans* que participa en un ampli ventall de processos mitòtics. En mamífers s'han descrit onze proteïnes homologues a NIMA. D'entre totes elles, Nek2, Nek6, Nek7 i Nek9 estan implicades en el control del cicle centrosomal i la formació del fus mitòtic. Nek9 és fosforilada en mitosis i una part petita de la quinasa (5%) és activada als centrosomes. La microinjecció d'anticossos contra Nek9 en cèl·lules en profase indueix un arrest prometafàsic amb fusos aberrants i aneuploïdia. A més a més, la depleció de XNek9 en extractes mitòtics de *Xenopus laevis* presenta un retard en l'ensamblatge del fus, menys fusos bipolars i l'aparició d'estructures de microtúbuls aberrants.

Nek6 i Nek7 s'uneixen a la cua C-terminal de Nek9 i són fosforilades a la Ser206 (Ser195 en Nek7) i activades per Nek9, formant d'aquesta manera un

casset de senyalització de quinases mitòtiques. La *downregulació* tant de Nek6 com de Nek7 mitjançant RNAi, arresta les cèl·lules en metafase amb fusos mitòtics fràgils i per Nek7 s'ha demostrat que resulta en un increment en la incidència de fusos multipolars. Possiblement explicant part d'aquests fenotips està el fet que s'ha descrit que la quinesina Eg5 pot ser fosforilada per Nek6 i Nek7 en un lloc que és necessari per una normal progressió de la mitosi.

RESULTATS

Nek9 és activada per Plk1

Nek9 es troba hiperfosforilada en mitosi. Utilitzant l'espectometria de masses hem identificat diferents residus fosforilats a Nek9 en cèl·lules creixent exponencialment i cèl·lules arrestades en mitosi. Tots els llocs fosforilats, excepte la Thr333 són possibles llocs de fosforilació de Cdk1. A més a més, tres dels llocs fosforilats són possibles llocs d'unió per Plk1, que s'activa en mitosi a les mateixes localitzacions subcel·lulars que Nek9.

Posteriorment vam confirmar que Nek9 i Plk1 coimmunoprecipitaven exclusivament en mitosi tan en cèl·lules humanes (HeLa) com en fibroblasts d'embrió de ratolí. El mapatge de la interacció entre les dues proteïnes ens va revelar que Nek9 s'uneix exclusivament en mitosi al PBD de Plk1 mitjançant la Ser869.

També vam mirar si Cdk1 i/o Plk1 eren capaces de fosforilar Nek9. Vam confirmar que les dues quinases podien fosforilar *in vitro* FLAG-Nek9[K81M], una forma inactiva de la quinasa, i que hi havia una lleugera sinergia quan FLAG-[K81M] era incubada amb ambdues quinases. La fosforilació del llaç d'activació de Nek9 és un pas crucial per a l'activació de la quinasa. Utilitzant un anticòs que reconeix específicament Nek9[Thr210-P] vam determinar que Plk1 (però no Cdk1) era capaç de fosforilar, a més d'altres llocs, al llaç d'activació de la quinasa. Els mateixos resultats es van obtenir utilitzant una altra forma inactiva de la quinasa, FLAG-Nek9[D176A], en la qual l'àcid aspàrtic catalític està mutat a un residu no fosforilable.

Per saber si la fosforilació del residu Thr210 per part de Plk1 causava l'activació de la quinasa es va fer un assaig quinasa incubant la forma nativa de Nek9 en presència o absència de Plk1. El resultat ens va mostrar que quan Nek9 és incubada amb Plk1 aquesta s'activa més ràpidament que quan s'incuba solament amb ATP, per això vàrem poder concloure Plk1 pot activar Nek9 *in vitro*. El mateix resultat va ser confirmat utilitzant l'anticòs anti-Nek9[Thr210-P].

Utilitzant Purvalanol A, un inhibidor de Cdk1, o BI2536, un inhibidor de Plk1, vàrem detectar que la fosforilació a la Thr210 de Nek9 estava compromesa quan aquestes dues quinases estaven inhibides, suggerint d'aquesta manera que tan Cdk1 com Plk1 són necessàries per l'activació de Nek9 in vivo.

Una de les funcions més importants de Plk1 és el control de la separació dels centrosomes durant les primeres fases de la mitosi. En les cèl·lules de mamífers la separació dels centrosomes durant la profase depèn de l'activitat d'Eg5 (kinesin-5), una proteïna de la família de les quinesines BimC que està, com Plk1, implicada en l'ensamblatge i manteniment d'un fus bipolar durant la mitosi, mitjançant el lliscament entre microtúbuls antiparal·lels. Fins al moment no es coneixia com Plk1 controlava la separació dels centrosomes abans del trencament de l'envoltori nuclear (NEB) i si hi havia un enllaç entre la senyalització de Plk1 i d'Eg5. Anteriorment hem demostrat que Eg5 és un substrat de Nek6 i Nek7 i per això voldríem determinar si el mòdul Nek9/Nek6/7 podria ser la connexió entre Plk1 i Eg5 durant la profase.

Resultats del nostre grup demostren que Plk1, Nek9, Nek6, Nek7 i la quinesina mitòtica Eg5 són necessàries per una separació normal dels centrosomes en profase. A més a més, hem demostrat que Nek9 activa i Nek6 poden induir la separació dels centrosomes de manera Eg5 depenent i també que NEk9 activa i Nek6 poden rescatar la separació dels centrosomes en la *downregulació* de Plk1 però no d'Eg5

Per aquesta raó ens vam disposar a determinar si la *downregulació* de Plk1 tenia cap efecte sobre la fosforilació d'Eg5 a la Ser1033, el lloc de fosforilació descrit per Nek6/7. Utilitzant l'inhibidor específic de Plk1 BI2536, o bé l'RNAi de Plk1 vam demostrar que la fosforilació d'Eg5[Ser1033] es veia afectada quan Plk1 estava inhibida. A més a més, sabem que juntament amb el lloc de fosforilació per Cdk1, la Thr926, la fosforilació al lloc Ser1033 per part de Nek6 és necessària per a la separació dels centrosomes en profase i el reclutamentd'Eg5 als centrosomes.

Finalment, per a poder determinar quina era la conseqüència biològica d'aquesta mutació en el cicle cel·lular, vam estudiar la progressió de la mitosis en cèl·lules on la quinesina Eg5 endògena va ser reemplaçada per diferents formes mutants. D'aquests experiments vam poder concloure que en cèl·lules on Eg5 va ser substituïda per una forma amb la Ser1033 mutada a un residu no fosforilable (Eg5[Ser1033A]) presentaven un retard en prometafase, indicant que la fosforilació d'aquest residu és important per a una correcta progressió de la mitosi.

Per tal de continuar el nostre estudi de Nek9 *downstream* de Plk1 i en col·laboració amb el grup de la Dra. Judit Villén (University of Washington) vam determinar per espectrometria de masses diferents llocs a Nek9 fosforilats per Plk1. Posteriorment vàrem voler saber si alguns d'aquests llocs tenien algun paper en l'activació de la quinasa. Utilitzant l'anticòs Nek9[Thr210-P] vam comprovar que alguns dels llocs localitzats al domini RCC1 de Nek9 eren necessaris per a la fosforilació de Nek9 al llaç d'activació. Aquests resultats suggereixen un model en el qual el domini RCC1 podria actuar com un domini autoinhibidor i que quan alguns d'aquests llocs són fosforilats per Plk1 el domini quinasa és alliberat i Plk1 pot fosforilar directament Nek9 al llaç d'activació provocant d'aquesta manera l'activació de la quinasa.

Quan Plk1 s'inhibeix ja sigui utilitzant un RNAi o bé amb l'inhibidor específic BI2536 les cèl·lules es queden arrestades en prometafase. Per tal de buscar si algunes funcions de Plk1 són executades a través de Nek9, vam inhibir Plk1 i

vam expressar la forma activa de Nek9. Els nostres resultats preliminars indiquen que Nek9 activa pot rescatar parcialment el fenotip provocat per la inhibició de Plk1.

En els darrers anys, Plk1 ha esdevingut una diana anticancerígena, i també ha estat descrit que Nek9 es troba desregulada en alguns tipus de cèl·lules tumorals. Per aquesta raó creiem que els nostres resultats, tot i ser molt preliminars, podrien ser importants en casos en els quals Plk1 es troba *downregulada*, mentre que Nek9 està hiperactivada, ja que tot i inhibir Plk1, les cèl·lules podrien escapar del bloqueig i continuar proliferant.

Aurora A fosforila Nek9

Durant el nostre estudi de la fosforilació mitòtica de Nek9 ens vam preguntar si altres quinases mitòtiques podien fosforilar Nek9. Vam comprovar que la proteïna quinasa Aurora A es pot unir i fosforilar Nek9. El mapatge de la interacció ens va demostrar que Nek9 i Aurora A s'uneixen a través de la cua de Nek9. A més a més hem pogut demostrar que Aurora A recombinant produïda en bacteris pot fosforilar tant FLAG-Nek9 (immunopurificada a partir del tag FLAG de cèl·lules humanes) o bé GST-Nek9[317-750] i GST-Nek9[759-979] (dominis RCC1 i cua de Nek9, respectivament, produïts en bacteris). Hem mapat la fosforilació d'Aurora A a la Ser978, un lloc amb el domini de fosoforilació d'Aurora A que havia estat prèviament identificat *in vivo* utilitzant l'espectrometria de masses. En paral·lel també vàrem determinar per espectrometria de masses que la Thr333 situada en el domini RCC1 de Nek9 també era un lloc de fosforilació d'Aurora A.

També hem demostrat que la fosforilació d'Aurora A no és necessària per la fosforilació de Nek9 a la Thr210 i per tant tampoc per a l'activació. De totes maneres, quan s'inhibeix Aurora A, Nek9 activa no localitza als centrosomes, suggerint que la funció de la fosforilació per part d'Aurora A podria ser la correcta localitza de Nek9 activa durant la mitosi.

Chemical genetic approach

Inhibir quinases ha estat sempre una tasca difícil, tots els dominis quinasa de les proteïnes són estructuralment molt semblants i la utilització d'inhibidors comporta molta inespecificitat. Durant els últims anys hem inhibit Nek9 utilitzant diferents aproximacions, però per tal de tenir una inhibició aguda de la quinasa vàrem decidir utilitzar un enfocament químic genètic. Aquest mètode va ser desenvolupat pel laboratori del Dr Shokat (UCSF) i consisteix en fer mutacions funcionalment silencioses a la butxaca d'unió de l'ATP (gatekeeper residue) de la quinasa diana que la fan sensibles a la inhibició per un anàleg d'ATP que no inhibeix les quinases salvatges.

L'aliniament de les seqüències d'aminoàcids de Nek9 humana amb altres quinases a les quals s'havia fet d'una forma satisfactòria un mutant anàleg sensitiu, va revelar que la leucina 128 era el residu *gatekeeper* de Nek9. Un cop identificat, es va mutar el residu a alanina i a glicina i es va comprovar la seva activitat. Tot i que el mutant era actiu, es va decidir fer una mutació compensatòria per tal d'incrementar aquesta activitat. Per tal de fer això es va fer una alineament amb altres quinases i es va identificar la posició 67 com un bon candidat a ser mutat per a fer les mutacions compensatòries.

Després de comprovar que el mutant Nek9[L128G G67W] presentava activitat i a més es podia inhibir utilitzar un anàleg d'ATP, vam poder confirmar que aquest mutant es comporta com un anàleg sensitiu *in vitro*.

INDEX

INTRODUCTION

The Cell Cycle

Mitosis is the process responsible for the division of the replicated nuclear material, which, in parallel with the division of the cytoplasm during cytokinesis, results in one cell giving rise to two cells that are exact genetic copies. Ever since Rudolf Virchow proclaimed his famous "*Omnis Cellula E Cellula*" and Walther Flemming originally coined the term mitosis, the challenge has been to understand how cells divide and how they faithfully transmit genetic information from one cell generation to the next ((Maiato, 2010; Nigg, 2001; Mitchison & Salmon, 2001) and references herein).

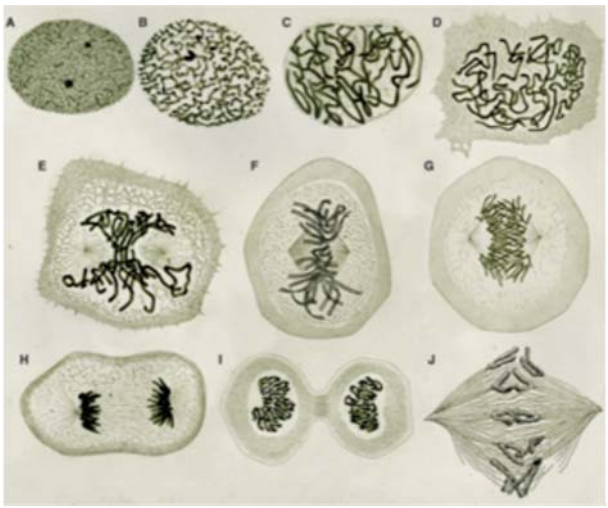


Figure 1. Drawings of mitosis in newt cells found in Flemming's (1) book. (A to J) Different mitotic phases B and C correspond to prophase, D and E to prometaphase, F to metaphase, G to anaphase and H to telophase. Adapted from Rieder & Kjodjakov 2001.

The main goal of mitosis is to segregate sister chromatids into two nascent cells, but also to properly separate centrosomes and the appropriate complements of cytoplasm and organelles. Traditionally, mitosis has been divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase according to the morphological changes of the cell (Figure 1). During prophase chromosomes condense, when nuclear envelope breaks down at prometaphase microtubules of the spindle capture the chromosomes, which then become aligned at the metaphase plate. During anaphase, the sister chromatids separate and are pulled to each pole of the spindle. In telophase, the chromosomes decondense and become enclosed by the nuclear envelope.

Nevertheless, some years ago, an alternative division of mitosis was proposed. Pines and Rieder argued that mitosis could be subdivided into five

transitional phases characterized by the activity of defined cell-cycle regulators, in particular the mitotic cyclin-dependent kinases and the anaphase-promoting complex/cyclosome (Pines & Rieder, 2001) (Figure 2). They propose a division of mitosis in five different transitions. The first one is characterized by the activation of Cdk/CyclinA, which is followed by the activation of CyclinB/Cdk1 being this activation the main event of the second transition. The third transition would be the equivalent to the traditional prometaphase. It is characterized by the presence of active CyclinB/Cdk1 and the Anaphase Promoting Complex (APC), though the latter being modulated by the kinetochore-attachment checkpoint. In the fourth transition the APC^{Cdc20} is fully active and CyclinB-Cdk1 activity starts to decrease due to the degradation of CyclinB. After Cdk1/CyclinB inactivation a new nuclear envelope is formed. In this final transition, Cdc20 is degraded and replaced in the APC by Cdh1. APC^{Cdh1} helps to coordinate late mitotic events.

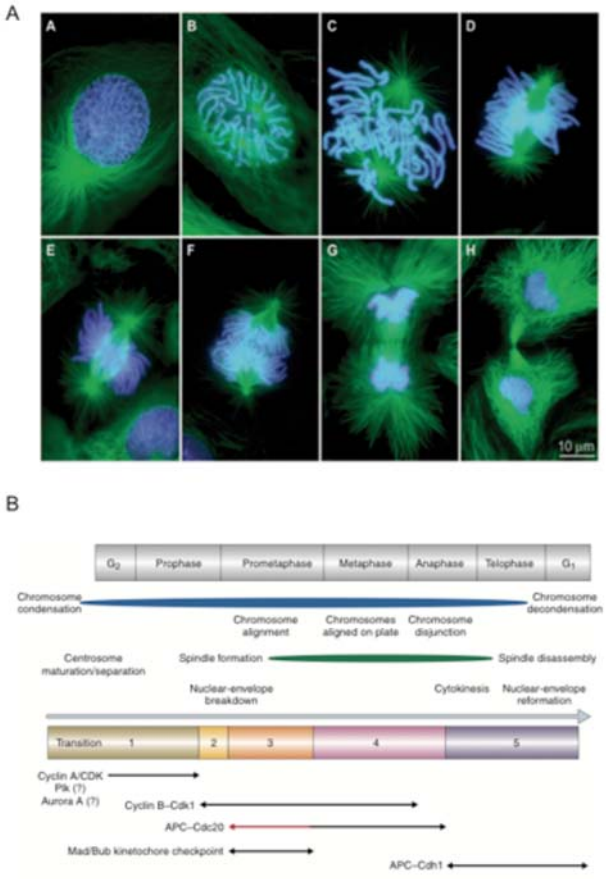


Figure 2. A. Fluorescence micrographs of mitosis in fixed newt lung cells stained with antibodies to reveal the microtubules (green), and with a dye (Hoechst 33342) to reveal the chromosomes (blue). Compare with Fig. 1. (Rieder & Khodjakov, 2003) Adapted from. **B.** Comparison of the traditional phases of mitosis (top) and our proposed transitions (bottom). Adapted from (Pines & Rieder, 2001).

Mitotic kinases

As stated above, the coordination of the progression through mitosis is mainly regulated by protein phosphorylation by several serine/threonine kinases, dephosphorylation of their substrates and protein degradation controlled by the APC. The protein kinase Cdk1 has historically been considered the major regulator of mitosis, but in the last decades proteins from the Plk, Aurora and Nek family of protein kinases have also shown to play important roles in the process.

Cdk family

Cyclin-dependent kinases are a large family of serine/threonine kinase whose best-characterized members are involved in the regulation of the cell cycle. In humans, there have been described 13 members of the Cdk family that can interact, at least, with 29 cyclins.

The most important member of the family is Cdk1. Cdk1 knock out mice are the only Cdk KO mice that die at the morula stage, meaning that these mice cannot undergo mitosis. Moreover, it has been described that in the absence of other interphase Cdks, Cdk1 can execute all the events required to drive cell division (Santamaría *et al*, 2007).

Cdk1 can be associated either with Cyclin-A, where it is involved in the S to G2 transition, or with Cyclin-B. The complex CyclinB1-Cdk1 is a mitotic inducer and its activity is carefully regulated. It has been shown that CyclinB1-Cdk1 activation initiates prophase, and that increasing levels of CyclinB1-Cdk1 activity triggers different mitotic events (Gavet & Pines, 2010). Once a B-type Cyclin binds to Cdk1, the complex is inactivated by Wee1 kinase. Subsequently, the pool of inactive CyclinB-Cdk1 is activated by the Cdc25 phosphatase and the active kinase phosphorylates a large number of substrates to reorganize the architecture of the cell for mitosis. More than 70 targets have been validated in yeast and mammals (Malumbres & Barbacid, 2005), though this number is likely to be an underestimation because proteomic studies have identified more than

300 Cdk1 potential targets (Holt *et al*, 2009). One of these substrates is the kinesin Eg5 (see below), that is phosphorylated by Cdk1 at Ser926 and this phosphorylation is crucial for the localization of the kinesin to microtubules (Blangy *et al*, 1995; Sawin & Mitchison, 1995). Finally, inactivation of the complex CyclinB-Cdk1 is necessary for a proper mitotic exit. This inactivation goes via degradation of the Cyclin-B through the proteolytic pathway controlled by the anaphase promoting complex (APC) ubiquitin ligase (Harper *et al*, 2002).

Other members of the Cdk family involved in cell cycle control are Cdk2, Cdk4, Cdk6, and their regulators (Cyclin-A, Cyclin-D or Cyclin-E) that regulate progression through G1. Cdk4 and Cdk6 phosphorylate members of the Retinoblastoma protein, and Cdk2, activated by Cyclin-E, is required for a proper progression of G1 phase (Malumbres & Barbacid, 2005). Studies in mice show that Cdk4, Cdk2 and Cdk6 are dispensable for mammalian cell division (Malumbres *et al*, 2004; Barrière *et al*, 2007). Cdk3 is highly similar to Cdk2 and its function remains to be clearly established (Malumbres, 2011).

Cdk10 is thought to be involved in the control of the G2/M phases though the exact function of the kinase remains elusive (Malumbres & Barbacid, 2005). Regarding Cdk11, it is essential for mice development (Li *et al*, 2004) and has been involved in centrosome maturation (Hu *et al*, 2007), spindle formation (Yokoyama *et al*, 2008), sister chromatid cohesion (Hu *et al*, 2007) and cytokinesis (Wilker *et al*, 2007).

Plk family

The Plk family of protein kinases is a family of five serine/threonine kinases found in all vertebrate lineages, except in plants, with very important roles in mitosis. The family can be divided into three different subfamilies. Plk1 subfamily which contains the mammalian Plk1 and *Drosophila polo*, the Plk2 subfamily containing Plk2, Plk3 and Plk5 and finally the SAK subfamily that contains *Drosophila* SAK and mammalian Plk4. All members of the Plk family share a conserved domain, named polo box domain (PBD), important for their localization

during the cell cycle (de Cárcer *et al*, 2011b; Weerdt & Medema, 2006) (Figure 3).

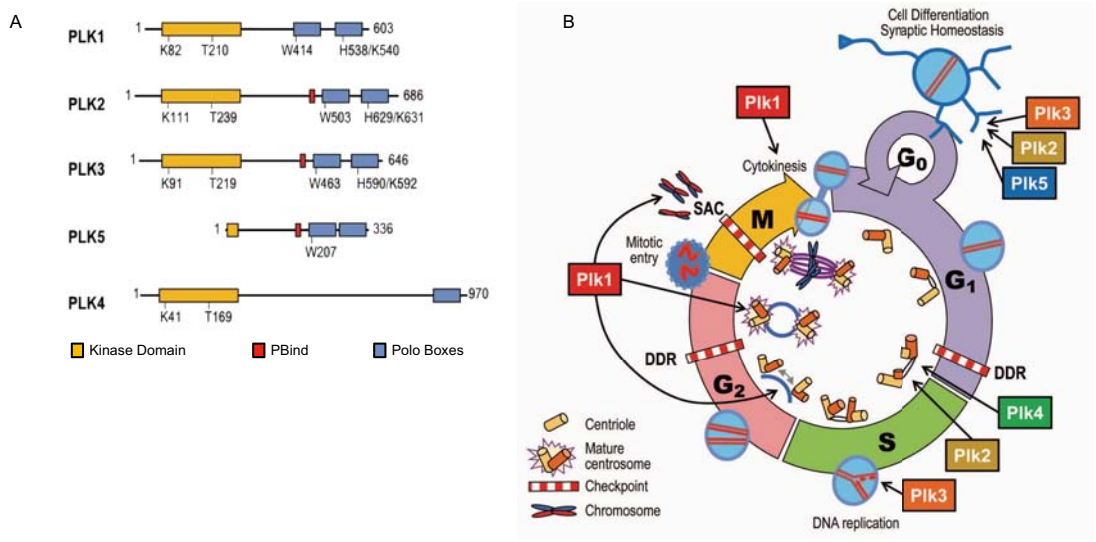


Figure 3. A. Cartoon representation of the different members of the Plk family. **B.** Schematic representation of the functions of the Plk family members along the cell cycle. Adapted from (de Cárcer *et al*, 2011b).

Plk1

Polo in *Drosophila* and Plk1 in vertebrates is the founding member of the Plk family. It was identified in screens for mutants with defects in cell division (Sunkel & Glover, 1988) and cloning of the protein identified it as a serine/threonine kinase (Llamazares *et al*, 1991). Plk1 is a key regulator of mitotic and meiotic division in all models analysed.

Plk1 is a 603 residues polypeptide that contains a N-terminal kinase domain and a C-terminal non-catalytic region composed of two homologous 70-80 residues segments termed Polo Boxes. Using a proteomic screen it has been showed that the entire C-terminal domain of Plk1, the PBD (Polo Box Domain) is a phophoserine/threonine binding domain which targets Plk1 to mitotic structures and substrates (Elia *et al*, 2003a).

Activation

Plk1 is activated at the G2/M transition and reaches peak levels in mitosis (van Vugt & Medema, 2005). The final activation of the kinase ends with the phosphorylation of the Thr210 in its activation loop. The activation mechanism of Plk1 has remained elusive for a long time and different models have been proposed, though the relative importance of them is still unclear.

It was first suggested that the Polo Box domain of the protein played a central role in the activation. Binding of the PBD to phosphorylated residues would cause conformational changes that released the inhibitory effect of the PBD toward the kinase domain and the protein would activate by autophosphorylation (Elia *et al*, 2003b).

Another mechanism of activation that has been proposed is that Plk1 is activated at G2 by Aurora A with the help of the protein Bora (Macůrek *et al*, 2008; Seki *et al*, 2008b). Bora has been proposed to play a dual role in Plk1 activation. In G2, it acts as an activator by binding to the PBD domain and allowing the access of Aurora A to the Thr210. Then, in mitosis, Bora by direct binding to Plk1 interferes with the further activation of the kinase. The inhibitory effect of Bora is relieved by its mitotic degradation, mediated through a ubiquitin-proteasome pathway ubiquitin ligase in a Plk1-dependent manner (Seki *et al*, 2008a). Thus, Bora activates Plk1 at the G2-M transition, and active Plk1 then phosphorylates Bora and promotes its degradation in mitosis. Phosphorylation of mitotic Plk1 on Thr210 appears to be maintained by Aurora A (Figure 4A).

Moreover, Ser137 seems to be an alternative site of activation in the kinase domain of Plk1 (Jang *et al*, 2002). It is briefly phosphorylated at late mitosis and may contribute to regulate the activity of the spindle assembly checkpoint (Weerdt *et al*, 2005).

Finally an additional activation mechanism of Polo at the kinetochores has been described. INCENP and Aurora B are required for Polo activation by Thr210

phosphorylation in centromere and kinetochores during prometaphase (Carmena *et al*, 2012).

Regulation of the activity

Plk1 can be regulated by phosphorylation and protein degradation, but the most striking feature of Plk1 is its changing localization to various cellular structures during mitotic progression. Plk1 accumulates at centrosomes during prophase, at kinetochores in prometaphase and metaphase, is recruited to the central spindle in anaphase and accumulates at midbody during telophase (Petronczki *et al*, 2008) (Figure 4B). The PBD contributes to substrate specificity and to the changes in subcellular localization of Plk1.

The PBD of Plk1 binds to phosphorylated peptides which had been "primed" by other kinases, especially Cdk1. The PBD recognizes substrates that contain an SpS/TP domain (where pS is phophoserine). It has been shown that mutation of the W414, H538 and K540 of the PBD to alanine, completely abrogates the binding of Plk1 to its substrates. The PBD of Plk1 is sufficient for its localization and it has been described that both Polo boxes are necessary for this (Elia *et al*, 2003b, 2003a; Seong *et al*, 2002).

Two models for how PBD might function to direct Plk1 kinase activity have been described. In the first one, called "processive phosphorylation model", the PBD binds to one extreme of the protein and Plk1 phosphorylates at another site of the same protein. On the other hand, in the "distributive phosphorylation model" the PBD binds to a protein that acts as scaffold and then Plk1 phosphorylates a different protein (Lowery *et al*, 2004, 2005).

Plk1 is degraded at the start of anaphase after ubiquitination by the anaphase promoting complex (APC) (Lindon & Pines, 2004).

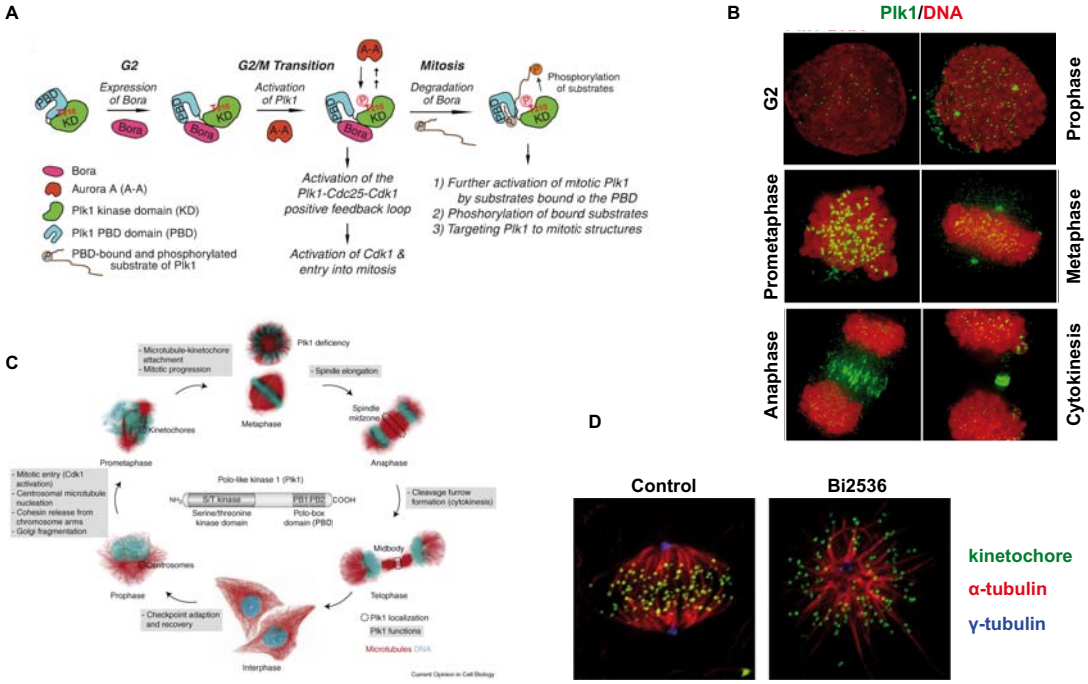


Figure 4. A. Plk1 activation mechanism. Adapted from (Seki *et al*, 2008a). B. Plk1 subcellular localization along the cell cycle. Modified from (Petronczki *et al*, 2008). C. Plk1 functions. Adapted from (Takaki *et al*, 2008). D. Cells treated with the Plk1 inhibitor BI2536. A standard prometaphase cell (left) and a prometaphase arrested cell by Plk1 inhibition (right) are shown with kinetochores (green), α-tubulin (red) and γ-tubulin (blue). Modified from (Petronczki *et al*, 2008).

Functions

Plk1 is involved in all mitotic processes, where it phosphorylates very different substrates (Petronczki *et al*, 2008). It promotes mitotic entry by phosphorylating Cdc25C (Toyoshima-Morimoto *et al*, 2002), Cyclin B (Toyoshima-Morimoto *et al*, 2001) or Wee1 (Watanabe *et al*, 2004). Afterwards it is required for centrosome maturation controlling the recruitment of γ-tubulin and other PCM proteins, such as Nedd1, Pericentrin, Cep192, Kizuna or CDK5RAP2 to the centrosomes (Haren *et al*, 2009; Oshimori *et al*, 2006). Binding of Plk1 to PBIP1 is crucial for localizing Plk1 to the kinetochores where it can phosphorylate BubR1, thus stabilizing kinetochore-microtubule interactions, or PICH, required for the spindle assembly checkpoint (Kang *et al*, 2006; Elowe *et al*, 2007; Baumann *et al*, 2007). Some cytokinetic substrates of Plk1 have been described, Rock2 or Mlkp2 for instance, highlighting the importance of Plk1 also in this mitotic phase (Lowery *et al*, 2007; Neef *et al*, 2003) (Figure 4C).

When Plk1 is inhibited, either by using RNAi (Sumara *et al*, 2004) or with chemical inhibitors as BI2536 (Lénárt *et al*, 2007) or ZK-thiazolidinone (Santamaria *et al*, 2007), cells enter mitosis after a short delay, get arrested in a prometaphase state and subsequently often die by apoptosis (Figure 4D). Plk1 -/- embryos fail to survive after eight-cell stage because embryos cannot enter mitosis. Plk1 +/- are healthy at birth but they present high incidence of tumours at the adult stage (Lu *et al*, 2008).

Plk2

Plk2 also known as SNK has a very similar structure to Plk1. It contains a kinase domain and two polo-boxes in the polo-box domain. It only differs in the Pbind domain, a domain that it uses to bind to some of its substrates in non-proliferative tissues where the priming kinases may not be present (de Cárcer *et al*, 2011b).

Plk2 is broadly expressed in different tissues, proliferating and non-proliferating, such as post-mitotic neurons. Its expression increases in G1 and early S phase, where it acts as a regulator (Simmons *et al*, 1992; Ma *et al*, 2003).

It concentrates at centrosomes, is required for centriole duplication in S phase (Warnke *et al*, 2004), and has been described that either overexpression or depletion of the protein leads to an aberrant number of centrosomes (Chang *et al*, 2010; Cizmecioglu *et al*, 2008).

Plk2 is a non-essential gene. Plk2 -/- knock out mice are viable (Ma *et al*, 2003) and it is not required for a proper cell cycle progression in HeLa or U2OS cells (Burns *et al*, 2003). Nevertheless, it plays some roles in cell cycle as knockouts for this protein present retarded growth and skeletal development. Plk2 has recently been involved in the control of neuronal physiology, some substrates have been identified and several functions of the protein in this environment have been described. As an example, Plk2 is upregulated during

neuronal activity and its activation influences the modulation of synapses in both proximal and distal dendrites (Seeburg *et al*, 2008; Ang *et al*, 2008).

Plk3

Plk3 (FNK/Prk) is structurally very similar to Plk2. The mRNA levels increase at G1, but protein levels remain constant through the cell cycle (Donohue *et al*, 1995; Bahassi *et al*, 2002). It is mainly expressed in human respiratory organs. Plk3 regulates entry intro S phase by promoting the accumulation of Cyclin E and Cdc25A activation favouring DNA replication (Zimmerman & Erikson, 2007a; Bahassi *et al*, 2004). Plk3 depleted cells progress normally in mitosis but fail to enter the cell cycle again (Zimmerman & Erikson, 2007b). Plk3 also functions in the cellular response to DNA damage where it becomes phosphorylated and activated (Xie *et al*, 2001). It also might sense genotoxic stress leading to cell cycle arrest and apoptosis (Bahassi *et al*, 2002). Plk3 deficient mice are viable although prone to develop tumours (Yang *et al*, 2008).

Plk4

Plk4 structurally differs from the other members of the Plk family, because it does not have the canonical two polo-boxes conforming a polo-box domain. Instead, it contains a single polo-box and a cryptic polo-box domain. Nevertheless, the cryptic polo-box domain has the same functions as the polo-box domain of Plk1 (Leung *et al*, 2002).

Plk4 plays an essential role in centrosome duplication (Bettencourt-Dias *et al*, 2005; Habedanck *et al*, 2005) and biogenesis (Kleylein-Sohn *et al*, 2007; Rodrigues-Martins *et al*, 2007). Overexpression of Plk4 leads to an excessive formation of centrioles, and the kinase is required, in cooperation with Cdk2, CP110 and Hs-SAS6, for precise reproduction of centrioles (and thus centrosomes) during the cell cycle (Habedanck *et al*, 2005). Depletion of Plk4 by RNAi prevents centriole formation causing mitotic defects (Bettencourt-Dias *et al*, 2005). It has been shown that Cep152 (Asterless in *Drosphila*) interacts with Plk4 acting as a scaffold for the kinase and other components of the centriole

duplication machinery and is required for Plk4-induced centriole overduplication (Cizmecioglu *et al*, 2010; Hatch *et al*, 2010). Plk4 -/- mice die at E7.5 showing Plk4 as essential for post-gastrulative embryonic development (Hudson *et al*, 2001)

Plk4 abundance in centrosome fluctuates during the cell cycle. In human cells, Plk4 levels are low at G1, increasing incrementally from S, when it becomes active, and reaching a maximum in mitosis (Sillibourne *et al*, 2010). Plk4 is a short life protein. It is ubiquitinylated via the SCFslimb/βTRCP pathway and degraded through the proteasome (Cunha-Ferreira *et al*, 2009; Rogers *et al*, 2009). Plk4 promotes its own degradation by catalizing βTRCP binding through trans autophosphorylation within homodimers (Guderian *et al*, 2010).

Plk5

Plk5 is the member of the Plk family that has been described more recently. It is predicted to be present in almost all vertebrates. All Plk5 orthologs contain a mutation in its autophosphorylation site and have changed the kinase motif DFG to AGL. As a consequence of these changes, Plk5 lacks kinase activity (Andrysik *et al*, 2010). Plk5 is only expressed in a few non-proliferative tissues, as in the central nervous system. It is mostly transcribed in cerebellum where is functionally important (de Cárcer *et al*, 2011a). It has been shown that Plk5 is a DNA damage induced gene not dependent of p53, and its overexpression induces G1 arrest followed by apoptotic cell death independently of its kinase activity (Andrysik *et al*, 2010; de Cárcer *et al*, 2011a).

Aurora family

The mammalian genomes contain three genes encoding Aurora kinases called Aurora A, B and C. The three Aurora kinases are serine/threonine kinases and have a N-terminal domain, a kinase domain and a C-terminal domain. Aurora A and B share 71% identity in the C-term catalytic domain. (Carmena & Earnshaw, 2003)

Aurora A

Aurora A was discovered in a screen of *Drosophila* mutations affecting the poles of the mitotic spindle (Glover *et al*, 1995).

Aurora A expression and localization is consistent with its function as a mitotic centrosomal kinase. During G1/S phase Aurora A levels are low, but in G2 phase the levels of Aurora A mRNA, protein levels and kinase activity rise rapidly reaching a peak in early mitosis (Barr & Gergely, 2007). Aurora A localizes to centrosomes in G2 and mitosis, and is also present in mitotic spindle during mitosis (Vader & Lens, 2008).

Even though Aurora A kinase can self-activate by autophosphorylation, several mitotic proteins have been reported to function as its activators. The best-characterized cofactor is TPX2, a MT-binding protein, which binds to the N-terminal domain of Aurora A and targets it to the spindle but not to centrosomes. The binding leads to the autophosphorylation at T288 (Kufer *et al*, 2002; Bayliss *et al*, 2003). Another co-activator of Aurora A is Ajuba which induces autophosphorylation and activation of Aurora A. Depletion of Ajuba prevents Aurora A activation at centrosomes at late G2 (Hirota *et al*, 2003). Moreover, it has been described that activation of Cdk1 initiates the release of Bora from the nucleus which binds to Aurora A and leads to its activation in a Plk1 dependent manner, as Plk1 phosphorylates Bora inducing its degradation and allowing Aurora A activation (Hutterer *et al*, 2006; Chan *et al*, 2008). Finally, it has recently been described that Nucleophosmin (NMP) activates Aurora A in G2 at centrosomes not through the classical phosphorylation at the Thr288 but through the Ser89 (Reboutier *et al*, 2012).

Aurora A participates in several crucial mitotic processes, such as the mitotic entry controlling centrosomal activation of Cdk1 and Plk1, but is also necessary for proper centrosome maturation and separation. Aurora A also has some roles in microtubule organization and it coordinates centrosome independent and

chromatin dependent spindle assembly (Barr & Gergely, 2007; Vader & Lens, 2008).

Aurora B

Aurora B is the catalytic subunit of the Chromosomal Passenger Complex (CPC). It is activated by autophosphorylation of Thr232 with the help of INCENP, Survivin and Borealin, the three regulatory subunits of the CPC. It transfers from the inner centromere in early mitosis to the spindle midzone, equatorial cortex and midbody in late mitosis and cytokinesis. The functions of Aurora B include regulation of chromosome-microtubule interaction, cohesion, spindle stability and cytokinesis (Carmena *et al*, 2009; Vader & Lens, 2008)

Aurora C

Aurora C is a chromosomal passenger protein localizing first to centromeres and then to the midzone of mitotic cells that cooperates with Aurora B to regulate mitotic chromosome segregation and cytokinesis in mammalian cells (Bolanos-Garcia, 2005).

NIMA family

The gene *nimA* (Never In Mitosis gene A) was identified in *Aspergillus nidulans* by Ron Morris in the 1970s with a genetic screen to find mutants that were sensitive to temperature (Morris, 1975). *NimA* mutants arrested in late G2 with duplicated spindle pole bodies.

By the middle 1980s nimA was cloned and shown to encode a serine/threonine kinase designated NIMA. NIMA is a 79 KDa protein with a N-terminal kinase domain and a C-terminal domain that contains some coiled coils and PEST sequence domains (Fry & Nigg, 1995). Protein levels of NIMA are regulated through the cell cycle. They are low during G1 and S, increasing in G2 and reaching a maximum at late G2 and early mitosis (Osmani & Ye, 1996).

Overexpression of NIMA induced a pseudo-mitotic state with condensed chromosomes and aberrant spindles. NIMA is required for mitotic entry in *Aspergillus* despite cdc2 activity (O'Connell *et al*, 2003), and it also participates in chromatin condensation, spindle formation and cytokinesis (O'Regan *et al*, 2007).

Homologs of NIMA have been characterized in all eukaryotes. Fungi and yeast have only one member of the NIMA related kinase (Neks), but the family is expanded in ciliated organisms where it has been suggested to coordinate cilia with cell cycle. Examination of different genomes shows that there is a correlation between number of Neks in organisms and whether or not it has ciliated cells that divide(Quarmby & Mahjoub, 2005).

In mammals, 11 proteins evolutionarily related to NIMA have been described. All these proteins share a kinase domain with high identity (40-50%) with the kinase domain of NIMA (Figure 5).

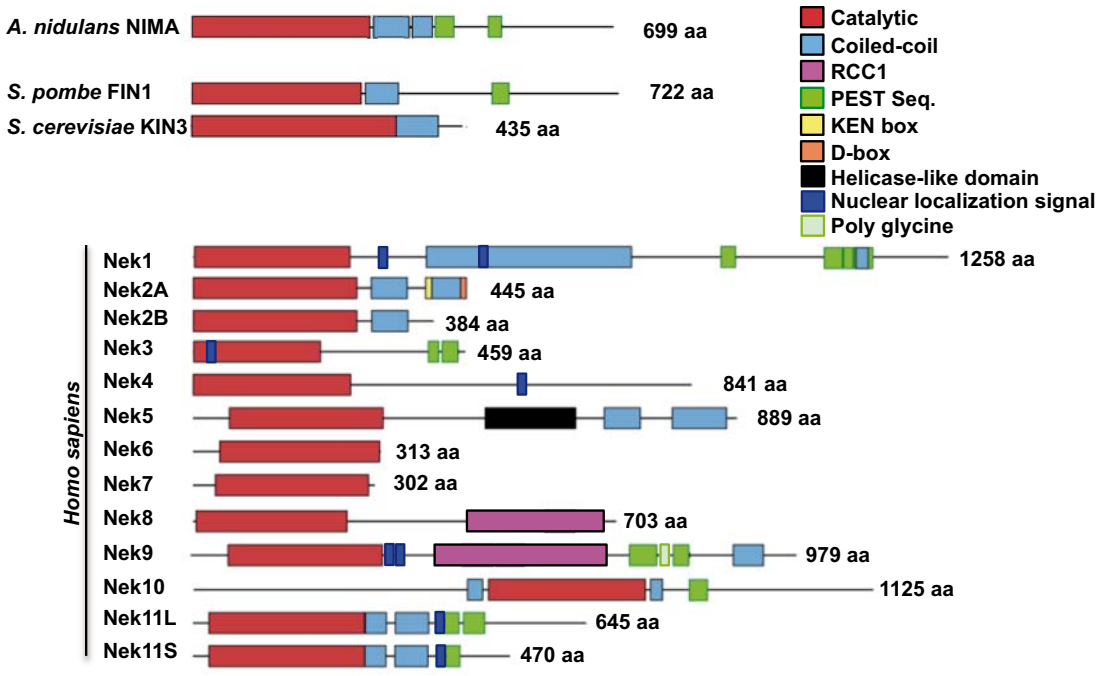


Figure 5. The NIMA-related kinases family. Modified from (O'Connell *et al*, 2003)

Nek1

Nek1 plays a role in centrosome integrity, affecting both ciliogenesis and centrosome stability (White & Quarmby, 2008). Nek1 is associated to centrosomes in interphase and with the mitotic spindle in mitosis (Mahjoub *et al*, 2005). Moreover, it has two functional nuclear localization signals and it is capable of carrying signals between the primary cilium and the nucleus (Hilton *et al*, 2009). Nek1 deficient mice are used as models for polycystic kidney disease (Upadhya *et al*, 2000).

Nek1 is involved in the DNA damage response after ionizing radiation, is necessary for efficient DNA damage checkpoint control and for proper DNA damage repair (Chen *et al*, 2008) and its overexpression leads to abnormal chromatin condensation (Feige *et al*, 2006).

Nek3

Nek3 is expressed in multiple tissues and cell lines and its expression does not change during the cell cycle progression (Tanaka & Nigg, 1999; Kimura & Okano, 2001). Nek3 depletion in breast cancer attenuates cytoskeletal re-organization as well as cell migration and invasion (Miller *et al*, 2007). It has also been shown that it is highly expressed in neurons and it may have some role in disorders where axonal degeneration is an important component (Chang *et al*, 2009).

Nek4

Nek4 (STK2) is ubiquitously expressed, and mRNA levels are constant through the cell cycle (Levedakou *et al*, 1994). Nek4 has a role in microtubule regulation and altered expression of the protein not only affects chemotherapeutic response but also confers differential sensitivity to select microtubule-disrupting drugs. Nek4 is frequently deleted in lung cancers and its

levels in several human cell lines correlate with differentially sensitivity to microtubule poisons (Doles & Hemann, 2010).

Nek8

Mouse Nek8 localizes to the proximal region of the primary cilium and is not observed in dividing cells. Nek8 RNAi does not affect ciliary assembly (Mahjoub *et al*, 2005). In vitro expression of Nek8 causes enlarged multinucleated cells with abnormal actin cytoskeleton (Liu *et al*, 2002). Localization of Nek8 to centrosomes and cilia is dependent on the catalytic activity but also on the C-terminal RCC1 domain of the kinase. The kinase domain of Nek8 is by itself active, but does not localize properly. On the contrary, the RCC1 domain localizes to the centrosomes and can be phosphorylated by the kinase domain. Therefore, a model has been proposed in which RCC1-like domain would contain a centrosome/ciliary targeting sequence, while the kinase domain would be required to autophosphorylate the RCC1 domain potentially causing a conformational change that would reveal the centrosome-targeting site in the full-length protein (Zalli *et al*, 2012).

Nek8 is mutated in polycystic kidney disease (PKD) mouse models (Liu *et al*, 2002), and mutations in Nek8 also cause nephronophthisis, an autosomal recessive kidney disease (Otto *et al*, 2008). It is also overexpressed in human breast cancers (Bowers & Boylan, 2004).

Nek10

Nek10 is the only Nek kinase with a centrally located kinase domain. It has been shown to promote MEK/ERK activation and G2/M arrest in response to UV radiation (Moniz & Stambolic, 2011).

Nek11

Nek11 has two isoforms, Nek11S and Nek11L, which stands for Long and Short isoform. The expression of Nek11L is higher than the expression of Nek11S in somatic cells. Nek11L is predominantly expressed in S/G2/M phase

(Noguchi *et al*, 2002). Nek11L interacts with active Nek2A and it has been described that Nek11 is activated by direct phosphorylation of Nek2A at the C-terminal domain (Noguchi *et al*, 2004).

Nek11 activity increases after IR-induced DNA damage. It has been shown that under DNA damage, Nek11 is activated by direct Chk1 phosphorylation and when active it phosphorylates Cdc25a and triggers it for degradation (Melixetian *et al*, 2009), thus preventing mitosis entry. Nek11 is overexpressed in a high percentage of human adenomas and carcinomas, and its expression has been inversely correlated with the tumour grade (Sørensen *et al*, 2010)

Mitotic Neks

Nek2

Nek2 is the closest relative to NIMA (Schultz *et al*, 1994). Three splice variants termed Nek2A, Nek2B and Nek2C have been described. All of them are ubiquitously expressed and localize to centrosomes. Besides, Nek2C can localize to the nucleus (Fry *et al*, 1998b, 1999) (Wu *et al*, 2007).

The expression of Nek2 is cell cycle regulated. Nek2 expression is undetectable during G1 phase, increases in G1/S transition and remains increased throughout S and G2. At the onset of mitosis Nek2A levels decrease rapidly whereas Nek2B levels are equal as in G2. During mitosis Nek2A remains absent while Nek2B only begins to decline upon re-entry in the next G1 phase (Fry, 2002).

Nek2A activates by autophosphorylation in G2, and it has been described that homodimerization of the protein is essential for this autophosphorylation and subsequent activation of the kinase (Fry *et al*, 1999). PP1 dephosphorylates Nek2A and it is only completely active when PP1 is inactivated at the onset of mitosis. This dephosphorylation is crucial for centrosome cohesion during the cell cycle (Meraldi & Nigg, 2001). It has recently been proposed that hSav and Mst2 directly interact with Nek2A. Mst2 phosphorylates Nek2A thereby recruiting

Nek2A to the centrosomes and promoting the phosphorylation and displacement of the linker proteins (Mardin *et al*, 2010). Moreover, Plk1 phosphorylates Mst2 and negatively regulates the binding of PP1γ to Nek2 (Mardin *et al*, 2011).

Nek2A is essentially a centrosomal protein and is in this organelle where it mainly exerts its functions. In late G2, Nek2 phosphorylates c-Nap1 (Fry *et al*, 1998a) and Rootletin (Bahe *et al*, 2005), two proteins that form the linkage between the two centrosomes. It has also been proposed that Nek2A binds to and phosphorylates β-catenin, which is in complex with Rootletin (Bahmanyar *et al*, 2008). The phosphorylation of these proteins initiates their displacement from the centrosomes, which then results in the splitting of the centrosomes.

In addition, Centrobin/NIP2 has also been identified as a Nek2 substrate. It has been described that phosphorylation of NIP2 by Nek2 stimulates the movement of NIP2 to unstable microtubules, where NIP2 can stabilize them (Jeong *et al*, 2007). In addition NIP2 phosphorylation by Nek2 is required for meiotic and mitotic spindle assembly in the early mouse embryo (Sonn *et al*, 2009, 2011).

Besides its functions in centrosomes, Nek2A is associated to the kinetochores and is necessary for faithful chromosome segregation. Nek2A phosphorylates Hec1 and this phosphorylation is essential for microtubule kinetochore attachments (Du *et al*, 2008) and for spindle assembly checkpoint (Wei *et al*, 2011).

Regarding Nek2B, it is not involved in the regulation of centrosome splitting prior to mitotic entry. Its downregulation results in mitotic delay that can lead to mitotic defects as formation of multinucleated cells (Fletcher *et al*, 2005). It has also been described that in *Xenopus laevis*, maternal Nek2B acts to promote assembly of a functional zygotic centrosome in a kinase-independent manner (Twomey *et al*, 2004).

Nek2C levels are also cell cycle regulated. They are low in G1 and increase during S and G2. The protein is degraded after mitotic entry as a result of APC/C-mediated ubiquitylation. It has not been established a clear function for this isoform, but it is believed that, due to its localization, is important for the phosphorylation of nuclear substrates (Wu *et al*, 2007).

Nek5

Nek5 localizes in the nucleus but also in centrosomes, spindle poles and basal bodies in a cell cycle dependent manner. Its mRNA levels are also cell cycle regulated, showing a peak of expression in the G2/M transition. It is overexpressed in cancer cell lines and Nek5 depletion causes centrosome splitting, loss of γ-tubulin from centrosomes and a delay in prometaphase (Sahota & Fry, 2010).

Nek9

Nek9 (originally named Nercc1) was identified as a Nek6 binding protein (Roig *et al*, 2002; Holland *et al*, 2002)(Belham *et al*, 2003).

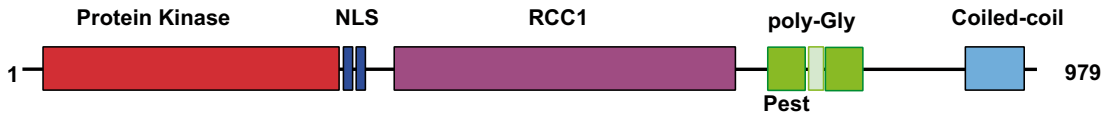


Figure 6. Nek9 cartoon representation

Structure

Nek9 is a 120 KDa protein with a 979 amino acids sequence (Figure 6). It is highly conserved in mammals, birds and amphibians (62% identical and 82% similar). Nek9 homologues in fish and invertebrate animals exist but are shorter and only 20 to 50% similar to human Nek9 (Parker *et al*, 2007).

Nek9 has three main domains, an N-terminal kinase domain (residues 52-308), a RCC1 domain (residues 347-726) and a C-terminal domain (761-979) containing different motifs and features, including a putative coiled coil. The kinase domain shows all features of a serine/threonine kinase domain and has

approximately 40% of identity with the kinase domain of NIMA. The kinase can be inactivated by the mutation of the ATP binding site at Nek9[K81M].

This kinase domain is followed by a functional NLS with two classical nuclear localization motifs ($_{306}$PLLRKRRR$_{313}$ and $_{325}$PTKRPR$_{330}$) although Nek9 is cytoplasmic, see below. Thereafter there is a domain containing seven consecutive RCC1 (Regulator of Chromosome Condensation) repeats of 52-68 residues that, by homology to other proteins, would possibly fold into a seven-blade propeller. The RCC1 domain acts as an autoinhibitory domain (Roig *et al*, 2002).

Following the RCC1 domain there are nine consecutive glycine residues that possibly conform a flexible hinge, encompassed within a PEST sequence. An acidic serine/threonine/proline-rich segment (761–890) follows next, which includes two motifs that conform to the SH3-domain-binding sequence PXXP ($_{823}$PXPXXPXP$_{830}$ and $_{881}$KXXPXXPP$_{888}$), and seven SP and TP sites motifs, putative Cdk1 phosphorylation sites (four overlapping the PXXP motifs) (Roig *et al*, 2002; Holland *et al*, 2002).

The C-terminal putative coiled-coil (891-940) is necessary for the oligomerization of Nek9 (Roig *et al*, 2002).

Localization

Nek9 is expressed in all human cell lines and mouse tissues tested, but it is more abundant in kidney, heart, testis, skeletal muscle, liver and brain. Protein levels remain constant during the cell cycle (Roig *et al*, 2002).

Although containing a functional NLS capable of directing other proteins to the nucleus (Roig *et al,* 2002), total Nek9 has a diffuse cytoplasmic localization. *Xenopus laevis* Nek9 has been shown to localize to spindle poles. (Roig *et al*, 2005)

A small amount of Nek9 (5%) is activated in prophase and active Nek9 localizes at centrosomes and spindle poles during mitosis. Interestingly, a significant fraction of active Nek9 is associated with chromosomes and the midbody after metaphase-anaphase transition, suggesting possible roles of Nek9 after metaphase (Figure 7).

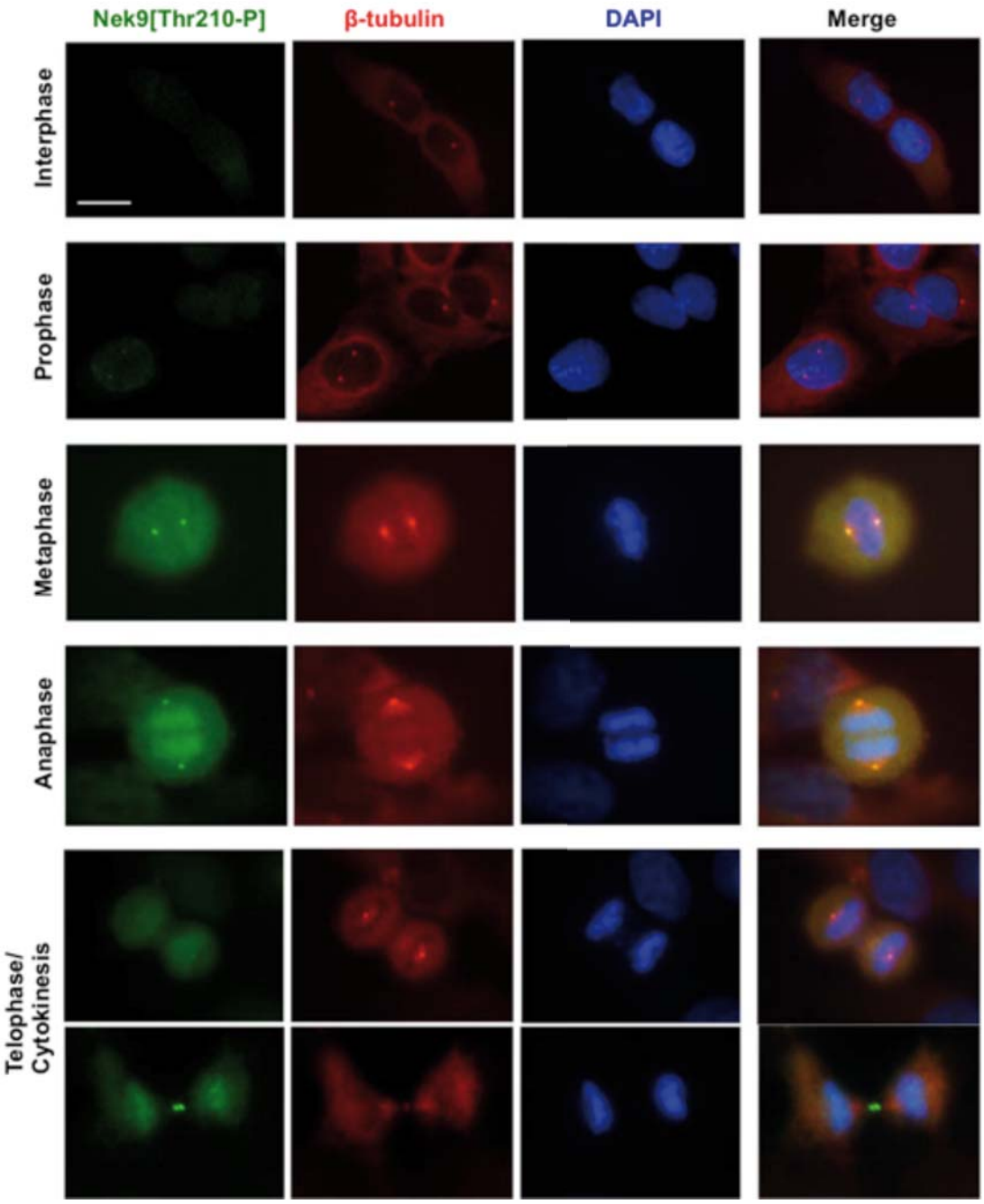


Figure 7. Active Nek9 localization in U2OS cells during mitotic progression. Nek9[Thr210-P] (green), γ-tubulin (red) and DNA (blue). Adapted from Roig et. al, 2005.

Activation

The activation mechanism of Nek9 has remained elusive until recently (see Results). The phosphorylation of Nek9 at Thr210 is necessary for the activation of the protein (Roig *et al*, 2005). Nek9 can be autoactivated *in vitro* when incubated at subcellular concentrations of ATP (100 μM) and this activation is regulated by homodimerization (the coiled coil domain is necessary for the activation) and also by the RCC1 domain, which acts as an autoinhibitory domain. Nek9 can be phosphorylated by Cdk1 *in vitro* (Roig *et al*, 2002). *In vivo*, Nek9 is not active during interphase, and is phosphorylated and activated in mitosis. Mitotic Nek9 has a slower electrophoretic mobility than the interphase kinase, though this difference in mobility does not lead to the activation of the kinase.

Nek9 interacts with the dynein light chain LC8 through a KXTQT motif at the C-terminal domain of Nek9. This binding modulates Nek9 oligodimerization and autophosphorylation. In response to Nek9 activation and autophosphorylation, LC8 binding to Nek9 is disrupted, thus allowing Nek9 to interact with Nek6/7 and activate these two related kinases (Regué *et al*, 2011).

Functions

Overexpression of inactive Nek9 (Nek9[K81M]) is toxic for cells. They do not divide and can undergo apoptosis (Roig *et al*, 2002). Moreover, Nek9 antibody microinjection in prophase cells causes different mitotic abnormalities such as prometaphase arrest or abnormalities in chromosomal segregation, resulting in aneuploidy or mitotic catastrophe (Roig *et al*, 2002) (Figure 8A). In parallel, immunodepletions of endogenous XNercc in *Xenopus lavevis* meiotic extracts interfere with both the formation of mitotic spindle and normal chromosome alignment (Roig *et al*, 2005) (Figure 8B).

In mitosis, Nek9 is able to bind and activate two other members of the Nek family. When Nek9 is active, it binds, phosphorylates and activates both Nek6 and Nek7 (see below) (Belham *et al*, 2003). We have also shown that this activation is important for the subsequent phosphorylation of the kinesin Eg5, and

that this phosphorylation is important for mitotic progression (See below and Results).

Moreover, Nek9 binds to γ-tubulin in mammalian cells and in *Xenopus* egg extracts (Roig *et al*, 2005), and also to Bicd2, though the biological relevance of these interactions is not known (Holland *et al*, 2002).

Recently it has been described a role of Nek9 in autophagy as depletion of the kinase impairs this process inhibiting the cargo recruitment to vesicles, vesicle trafficking and/or regulation (Behrends *et al*, 2010) .

During the course of this work we have described that Nek9 has a key role in prophase centrosome separation (Bertran *et al*, 2011) and prometaphase centrosome maturation (Sdelci *et al*, 2012) (See below).

Nek6/7

Nek6 and Nek7 are the smallest members of the NIMA family and both of them bind and are activated by Nek9 (Belham *et al*, 2003).

Structure

Nek6/7 have a N-terminal domain homolog to the kinase domain of NIMA, but lack the regulatory C-terminal domains typical of other Neks. The kinase domain of both kinases have an identity of 87% and differ in the N-terminal region of the protein, just before the kinase domain (Kandli *et al*, 2000).

Nek6 and Nek7 are two proteins of 317 and 302 aminoacids respectively with a molecular weight of 35KDa (Kandli *et al*, 2000). They show different expression patterns during embryogenesis (Feige & Motro, 2002) and they present complementary tissue distribution in adult mice (Kandli *et al*, 2000). Nek6 is highly expressed in intestine and placenta, while Nek7 expression is very high in kidney. Intermediate levels of both kinases are found in ovary and brain.

Nek6 low resolution and Nek7 high resolution structures have been solved. The structures show that both kinases are formed by a globular kinase domain and a disordered N-terminal domain (Richards *et al*, 2009b; Meirelles *et al*, 2011).

Activation

Nek6 expression increases in mitosis, when the kinase is phosphorylated and activated (Yin *et al*, 2003; Belham *et al*, 2003).

Nek9 binds to, phosphorylates and activates Nek6/7 by direct phosphorylation at Ser206 and Ser195 respectively. Once active, Nek6 autophosphorylates at Ser137 and Ser202 causing a higher activation of the kinase (Belham *et al*, 2003).

In parallel it has been described that Nek6 and Nek7 are also activated upon binding to the C-terminal domain of Nek9. It has been shown that Nek7 binding to the C-terminal domain of Nek9 releases the autoinhibitory residue Tyr97, thus causing a conformational change and allowing the activation of the kinase. It has also been proposed that this mechanism may exist in Nek6 with the analogous Tyr108 (Richards *et al*, 2009a).

Cellular localization

Nek6 is diffusely distributed throughout the cell cycle but a significant amount of it associates with different microtubule-based structures during mitosis. In metaphase, a fraction of it is associated with microtubules of the mitotic spindle, in anaphase it localizes in the central spindle and it is found in the midbody when cells undergo cytokinesis. On the contrary, Nek7 is localized to centrosomes either in interphase or mitosis (O'Regan & Fry, 2009; Yissachar *et al*, 2006) (Figure 9A and 9B).

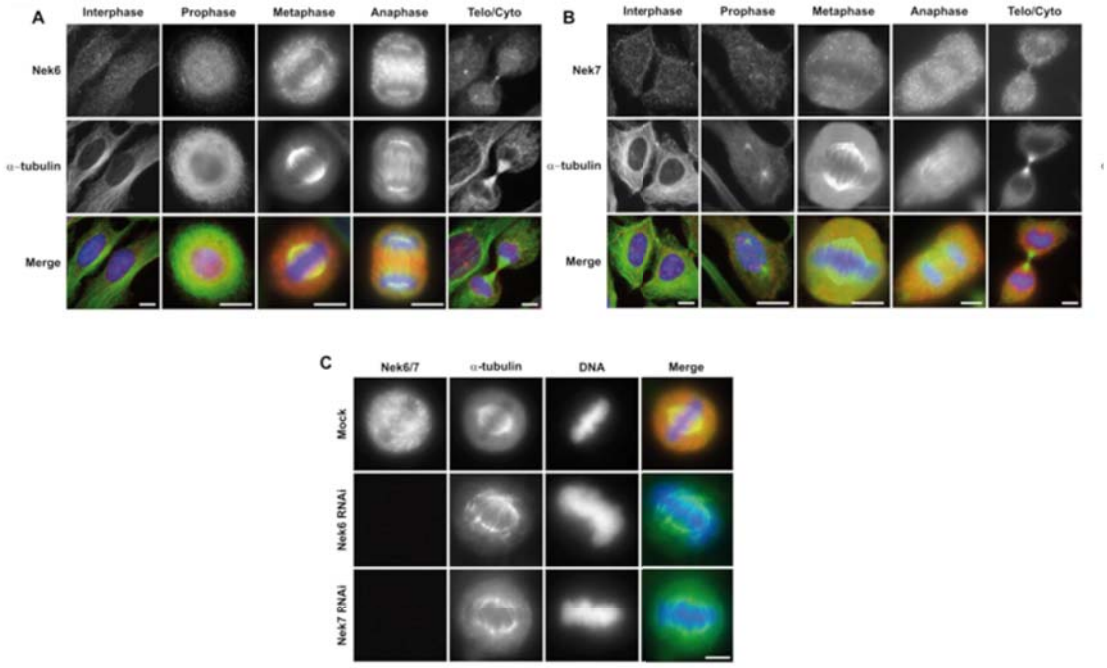


Figure 9. A. Nek6 localization along cell cycle in HeLa cells. **B.** Nek7 localization in HeLa cells. **C.** Mitotic defects after Nek6 and Nek7 depletion in HeLa cells. Adapted from O'Regan et. al, 2009.

Function

Expression of kinase inactive mutants Nek6[K74M][K75M] or Nek7[K63M][K64M] leads to apoptosis (O'Regan & Fry, 2009).

An important percentage of HeLa cells transfected with Nek6 or Nek7 RNAi present mitotic delay and undergo apoptosis. Loss of Nek6 is not rescued by Nek7 and vice versa, thus suggesting that both proteins may have different functions in these cells (O'Regan & Fry, 2009). These cells also show multipolar spindles with abnormal spindle assembly and chromosome alignment in metaphase. Besides, asymmetric chromosome segregation has also been shown. Moreover, Nek6/7 depleted cells have fragile spindles with less distinct microtubules and a reduction in overall spindle microtubule intensity (O'Regan & Fry, 2009; Yissachar *et al*, 2006) (Laurell *et al*, 2011) (Figure 9C).

It has also been described that Nek7 is necessary for centriole duplication in interphase (Kim & Rhee, 2011) and important for centrosome maturation in

interphase (Kim *et al*, 2007), although both things are controversial (Sdelci *et al*, 2012).

Absence of Nek7 leads to lethality at late embryogenesis or early post-natal stages with a severe retardation in weight. Mouse embryonic fibroblasts present various mitotic defects such as a high percentage of binuclear cells, presence of tetraploidy, chromosomal instability, micronuclei and also differences in frequency of primary cilia (Salem *et al*, 2010).

Different substrates of the kinases have been described *in vitro* and *in vivo*, and it has been shown that their N-terminal domain is important to mediate interactions with partners, thus suggesting the existence of different substrates for Nek6 and Nek7 (Belham *et al*, 2001; Meirelles *et al*, 2010; Rapley *et al*, 2008). Among all substrates, the best characterized is the kinesin Eg5. Once active, Nek6 associates to Eg5 and phosphorylates a small pool of Eg5 (3%) at Ser1033 *in vivo*. This phosphorylation is necessary for mitotic spindle formation (Rapley *et al*, 2008).

In addition, Chk1 and Chk2 directly phosphorylate Nek6 *in vitro*, and it has been described that Nek6 activation is abolished by IR and UV radiation and that inhibition of Nek6 activity is required for proper cell cycle arrest in the G2/M phase upon DNA damage (Lee *et al*, 2008).

Eg5

Eg5/Kif11 is a member of the bimC subfamily of kinesin-like microtubule motor proteins. It is a plus end directed motor composed by a N-terminal catalytic motor domain, an internal stalk domain capable of forming coiled coils, and a C-terminal tail domain (Figure 10A). Eg5 is a homotetramer. As such, it can crosslink two adjacent microtubules such that each dimeric motor interacts with a single protofilament on each microtubule (Kapitein *et al*, 2005). Eg5 needs the C-terminal non-catalytic domain of the protein for crosslinking and relative sliding of two microtubules (Weinger *et al*, 2011) and drives sliding of antiparallel microtubules hydrolyzing one ATP per 8nm step (Valentine *et al*, 2006) (Figure 10B).

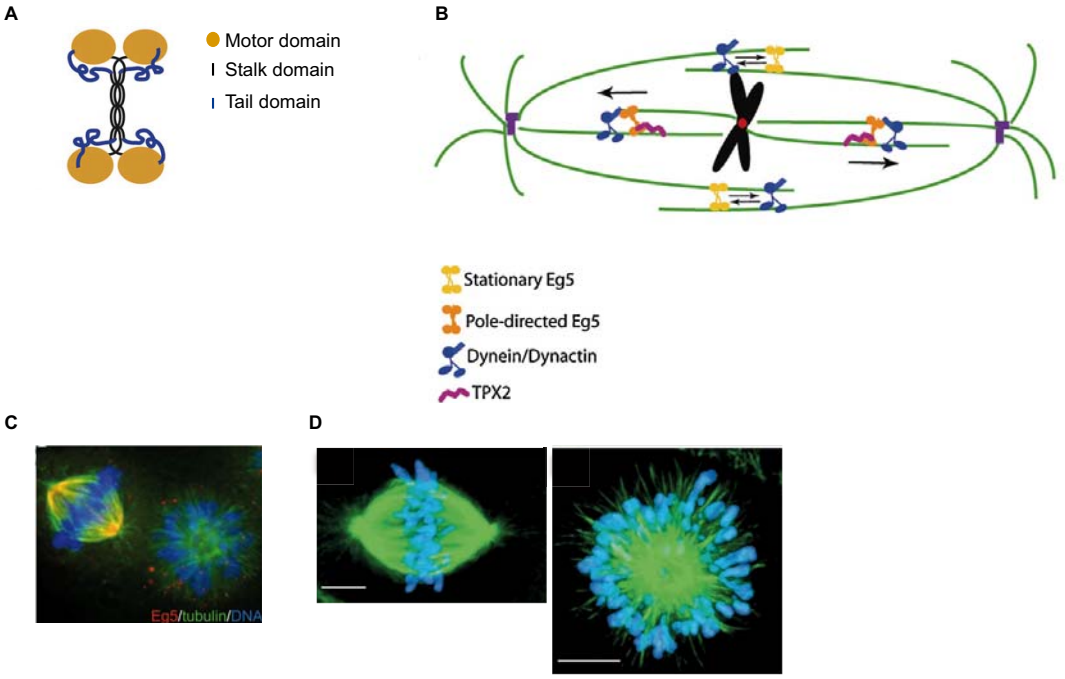


Figure 10. A. Eg5 domains. Adapted from (Weinger *et al*, 2011) **B.** Eg5 binds to antiparallel microtubules and slides them apart to form a bipolar spindle. Adapted from Ferrenz et al, 2010 **C.** Monopolar spindles observed after Eg5 RNAi. Adapted from (Stout *et al*, 2006) **D.** Treatment with Eg5 inhibitors lead to monopolar spindles. Adapted from (Mayer, 1999).

Eg5 localizes to spindle microtubules in mitosis with enrichment at centrosomes or spindle poles (Sawin & Mitchison, 1995) and is required for bipolar spindle formation in all model organisms but *C. elegans* (Hoyt, 1994). It has been described that forces for spindle assembly are generated by the dynamic instability of mitroctubules and by the action of dynein- and kinesin-related motor proteins (Ferenz *et al*, 2009, 2010). Inhibition of Eg5 either by RNAi (Weil *et al*, 2002) or with chemical inhibitors leads to monopolar spindle formation (Mayer, 1999; Maliga *et al*, 2002; Skoufias *et al*, 2006) (Figure 10C and 10D).

Spindle localization is consistent with its mitotic function, though the concentration at spindle poles is unexpected. This observation is consistent with the possibility that Eg5 can function in both parallel and antiparallel microtubules (Kapitein *et al*, 2005; Wildenberg *et al*, 2009).

Cdk1 phosphorylates Eg5 at Thr926. This phosphorylation is required for its spindle localization and binding to microtubules (Sawin & Mitchison, 1995; Cahu *et al*, 2008; Blangy *et al*, 1995). Moreover, a small amount of Eg5 (aprox 3%) is phosphorylated at Ser1033 by Nek6. This phosphorylation is necessary for correct mitotic progression (Rapley *et al*, 2008).

Eg5 binds to the C-terminal domain of TPX2. The interaction between both proteins is necessary for spindle microtubule organization and contributes to the localization of Eg5 to spindle microtubules but not spindle poles. The binding between Eg5 and TPX2 also regulates motor activity and microtubule targeting (Ma *et al*, 2010, 2011).

Brief introduction to the centrosome cycle and spindle formation

The centrosome and its cycle

Centrosomes are primary microtubule-organizing centres in animal cells. They regulate cell motility, adhesion and polarity in interphase and facilitate the organization of spindle poles during mitosis.

The centrosome is composed of two centrioles surrounded by the pericentriolar material (PCM), which consists of a matrix of fibrous proteins that nucleate and anchor microtubules. In a typical cell, the centrioles are perpendicularly oriented and are composed by nine microtubule triplets arranged radially and with a very precise symmetry (Figure 11).

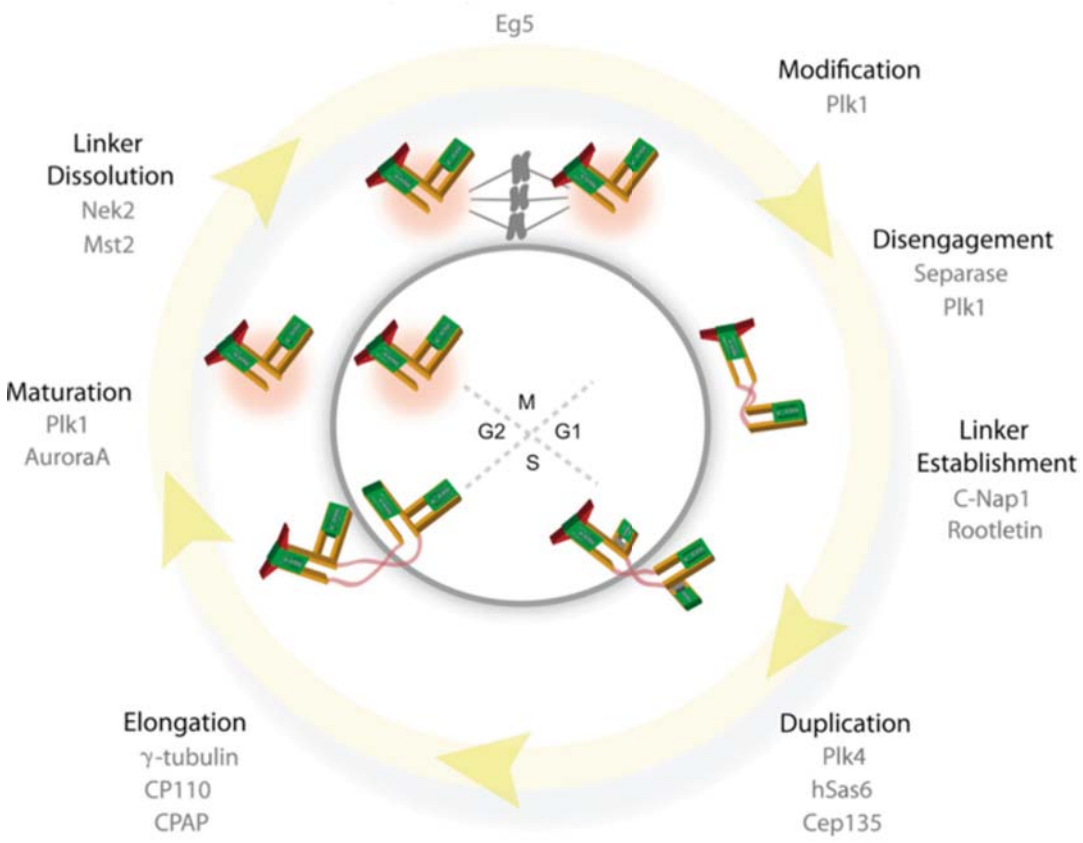


Figure 11. The centrosome cycle. Adapted from Mardin *et al* 2012.

At the beginning of G1 phase, cells contain a single centrosome with two perpendicularly aligned centrioles. At this stage the two centrioles are different: the mother, which was assembled at least two cycles ago (centriole with red cap in Figure 11), and the daughter centriole, which was originated in the previous cycle.

During G1 phase, centrioles disengage with the help of separase and Plk1 (Tsou & Stearns, 2006; Tsou *et al*, 2009), but they remain connected by a fibrous structure formed by C-Nap1, rootletin and β-catenin, among others. This is a crucial "licensing" step that will allow centriole duplication in S phase, simultaneously with DNA replication. Duplication involves the assembly of two new centrioles perpendicular to the existing centrioles.

In G2/M phase the flexible linker that holds the centrosomes together is lost due to the action of Nek2 that phosphorylates the different components of the linkage, and centrioles accumulate PCM (centrosome maturation). Centrosomes then separate and move to the opposite side of the nucleus forming a bipolar spindle.

The events that lead to centrosome separation and bipolar spindle assembly can be separated into two distinct temporal phases: prophase and prometaphase. During prophase, microtubule motors, such as Eg5 and dynein play an essential role in separating the centrosomes that are starting to nucleate microtubules and will become the spindle poles. Moreover, it has been shown that actin and microtubule pushing forces are also important for prophase centrosome separation (Tanenbaum & Medema, 2010).

While prophase centrosome separation occurs in almost all cells, the extent to which it occurs is often variable, and cells frequently enter mitosis with only partially separated centrosomes Thus, a robust mechanisms must exist in prometaphase to drive subsequent centrosome separation and bipolar spindle assembly. Eg5 and other motors as Hklp2 are essential for bipolar spindle

assembly, but microtubule pulling forces and microtubule-kinetochores pushing forces are also required (Tanenbaum & Medema, 2010).

Spindle assembly

In cells that have centrosomes, centrosome-based microtubule nucleation is the dominant mechanism for the production of new microtubules. γ-tubulin nucleates polymerization of microtubules when it forms a complex with members of the gamma complex proteins (GCP's) that is known as γ-TURC (Lüders & Stearns, 2007). The first model proposed for bipolar spindle assembly is the "search and capture" model that is the predominant mechanism by which chromosomes become properly aligned on the spindle. Centrosome-microtubules nucleate till they are captured and stabilized by one of the sister kinetochores. At this stage chromosomes are mono-oriented and remain oscillating until they find a microtubule from the other pole and become bioriented (Walczak & Heald, 2008).

Alternative mechanisms of spindle assembly have been proposed. The first of them is the Ran-dependent, chromatin-directed microtubule generation, which is dependent of Ran-GTPase via localization of RCC1. This process takes place in absence of centrosomes, resulting in spindles that take much longer to form and pass different intermediate structures not seen in presence of centrosomes (Duncan & Wakefield, 2011).

Another mechanism is the kinetochore-driven microtubule generation that exists in normal mitotic cells and is a dynein dependent process. There are also evidences that the CPC (Chromosome Passenger Complex) can nucleate microtubules during spindle formation. Another mechanism of microtubule nucleation is the microtubule-based mechanism dependent of the HAUS complex (Homolog to Augmin subunits). It has been proposed a model in which Augmin binds to existing spindle microtubules during prometaphase and targets active γ-tubulin, facilitating intra-spindle microtubule nucleation (Duncan & Wakefield,

2011). In most cells all the mentioned mechanisms cooperate resulting in the robust formation of the mitotic spindle.

OBJECTIVES

The objective of the thesis is the study of the phosphorylation and activation of the mitotic kinase Nek9.

Detailed objectives:

1. Description of the activation mechanism of Nek9 in the context of Nek9 biological roles.

2. Study of the phosphorylation of Nek9 by Aurora A.

3. Production of a Nek9 analog sensitive mutant to study the functions of Nek9 downstream of Plk1.

MATERIALS AND METHODS

Cell culture

HeLa, U2OS, HEK 293T cells and Embryonic Mice Fibroblast cells were cultured in a 5% CO_2 atmosphere and 37ºC in DMEM (Dubelco's modified Eagle's medium) supplemented with 10% FBS (Foetal Bovine Serum), L-glutamine (2mM), penicillin and streptomycin (100 IU/ml and 100 μg/ml,respectively)

Transfection

HEK 293T cells were transfected using different expression plasmids with Lipofectamine™ according to the manufacturer instructions (Invitrogen) or with Polyethyleneimine (PEI) (Polyscience, Inc) (Boussif *et al*, 1995). HeLa cells were transfected with Lipofectamine™ 2000 according to the manufacturer instructions (Invitrogen) and arrested in mitosis with Nocodazole (0,25mM, Sigma) 48 hours post transfection. siRNA and DNA co-transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions.

The sequences of the siRNA duplex for targeting the different proteins were: Plk1 5'-CGAGCUGCUUAAUGACGAGTT-3' (Oshimori *et al*, 2006), Aurora A 5'-AUGCCCUGUCUUACUGUCATT-3' (Kufer *et al*, 2002). Eg5 was depleted using established siRNA targeting published sequence, 5'-CUGAAGACCUGAAGACAAUTT-3' (Weil *et al*, 2002). Nek9 was depleted using Nek9 UTR, 5-GCUGCCUUGGGAAUUCAGUTT-3 and Nek9 UTR, 5-GCAGCCAAACUUUGAUUAATT-3 (all siRNAs were from Ambion).

Mutations

To generate Nek9 mutants, site-directed mutagenesis was performed according to the manufacturer's instructions (Stratagene) using specific primers (Table 1) with the appropriate reverse complement and the expression vector CMV5 FLAG Nek9 wild type (Roig *et al*, 2002) as the template. All constructs were sequenced after generation.

Mutation	Primer 5'-3'
S869A	CAAGTAGAAGCCTCGGCACCTCGGCTGAATCCTGC
S29A	GGTTGCGGGGACTCGGCTCCGGGGCCTAGCGCC
S750A	ACTGTGTTTCAGAGCTCTGCCCCGGGAGGAGGCGGCGG
T67E	GCCTTCGGGGAAGCCGAGCTGTACCGCCGCAC
S76D	GCCTTCGGGGAAGCCGAGCTGTACCGCCGCAC
S206D	CTTAATTCTGAGTATGCCATGGCTGAGACGCTTGTGGG
T438E	GTGTCATGTGGTGATGATTTCGAGGTCTGTGTGACTGATGAGG
T544E	GTGGCTGTGATGGGGAATTTCTGTTGACCCAGTCAG
T662E	GTCTCCTGCGGTGATGAGTTTGAGATTGCTGCCACTGATG
S978A	CCTGTAGACCCGCCCTCTAGTCTCCTGAGCCTATAGAGC
S978D	CCTGTAGACCCGACCTCTAGTCTCCTGAGCCTATAGAGC
L128A	CCACGCTGCTGATTGAGGCGGAATATTGTAATGGAGGG
L128G	CCACGCTGCTGATTGAGGGGGAATATTGTAATGGAGGG
L128A V66A	GCCTTCGGGGAAGTCACGCTGTACCGCCGCACC
L128G V66A	GCCTTCGGGGAAGTCACGCTGTACCGCCGCACC
L128A G67W	GCCTTCGGGGAAGCCTGGCTGTACCGCCGCACCG
L128G G67W	GCCTTCGGGGAAGCCTGGCTGTACCGCCGCACCG
L128A V78A	GGATGACTCACTGGTTGaGTGGAAGGAAGTCG
L128G V78A	GGATGACTCACTGGTTGAGTGGAAGGAAGTCG
D176A	CATAAAGCTGGAATCCTTCATAGAGCTATAAAGACATTAAATATT TTTCT

Table 1. Primers used to perform the different mutagenesis

GFP constructs were cloned using pEGFP C2 (Invitrogen) and the CMV5 FLAG mutants.

For construction of PGEX-Plk1 PBD [345-603], a PCR fragment was generated using pcDNA3.1 His6/Myc Plk1 as template and the fragment was cloned in a PGEX-KG vector.

Cell extracts, immunoprecipitation and western blotting 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, 2mM Na_3VO_4, 25nM calyculin A, 1% TX100, 0.5mM PMSF, 1µg/ml leupeptin, 1µg/ml aprotinin. Cytosolic fraction was obtained by centrifugation at 13200 rpm for 10 minutes. Protein concentrations were determined using the Bradford reagent (BioRad).

Immunoprecipitations were carried out with the indicated antibodies pre-bound to protein A/G dynabeads (Invitrogen). 1mg of lysate was incubated for 1h at 4ºC and washed three times with lysis buffer. Immunoblotting was carried out after separation of proteins by SDS-PAGE and transfer to PVDF membranes (Immobilon-P Transfer Membrane, Millipore).

Membranes were probed with the following antibodies: anti-Nek9, anti-Nek9[Thr210-P], anti-Nek6 and anti-Nek6[Ser206-P] polyclonal antibodies were produced as described (Belham *et al*, 2003) (Roig *et al*, 2002) and (Roig *et al*, 2005). Polyclonal anti-Eg5[Ser1033-P] was produced as described (Rapley *et al*, 2008). Anti-CyclinB1 (1:1000), anti-cdc27 (1:500), anti-GST (1:200) (Santa Cruz Biotechnoloy), anti-Plk1 (1:1000) (Calbiochem), anti-Eg5 (1:1000) (BD Bioscience), anti-GFP (1:1000) (Roche) (Invitrogen), anti-FLAG (1:1000) and anti-βtubulin (1:1000) (Sigma) were also used. Anti-HsAurora A (0.5 µg/ml) was a generous gift from Isabelle Vernos (CRG). Secondary antibodies were from Jackson Immuno Research Laboratories and were detected by ECL Chemiluminiscence (Thermo Scientific).

Protein expression in bacteria

Fusion proteins were expressed in *E. coli* RosettaTM 2 (DE3) or *E. coli* BL21 (DE3) induced with isopropil-β-D-thiogalctopyranoside (IPTG) for 16h at 18ºC or 3h at 30ºC.

GST fusion proteins were purified with glutathione-sepharose (GE Healthcare) following standard protocols, and were eluted with 25 mM reduced glutathione.

All purified proteins were resolved in SDS-PAGE acrylamide gels and stained with Coomassie blue (Sigma) to check protein presence, size and purity.

Protein purification from 293T cells

293T cells were transfected with either lipofectamine (Invitrogen) or Polyethyleneimine (PEI) (Polyscience, Inc) (Boussif *et al*, 1995) with FLAG recombinant Nek9. 24 hours post transfection cells were lysed with lysis buffer. 5 µg of antibody were bound to protein-A dynabeads (Invitrogen) for 30 minutes at 4ºC. After one wash, cell lysate was added and incubated for 2 hours at 4ºC. Beads were washed three times with lysis buffer and once with phosphorylation buffer (50mM MOPS at pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM β-glycerophosphate) and proteins were eluted in 100 µl of phosphorylation buffer with FLAG-peptide (Sigma) at 0.5 µg/ml for 30 minutes at 4ºC. SDS-PAGE of a small amount (10%) of the elution followed by Coomassie staining was performed to check the expression of the proteins.

GST pull down assays

2µg of GST-PBD or GST bound to GSH beads were incubated with 1mg of 293T extracts transfected with CMV5 FLAG Nek9 wild type or different Nek9 mutants for 1h at 4ºC and washed 3 times with lysis buffer. Immunoblotting was carried out after SDS-PAGE and transfer to PVDF membranes. Membranes were probed with anti-FLAG antibody (Sigma).

GST tagged Nek9 fragments were expressed in 293T. 1 mg of lysate was incubated was incubated with GSH beads for 1h at 4ºC and washed three times with lysis buffer. Proteins were resolved with SDS-PAGE and detected by western blot with the indicated antibodies.

Mass Spectrometry analysis

To determine Nek9 *in vivo* phosphorylation sites we used exponentially growing or mitotic arrested HeLa cells. Cells were lysed with buffer that contained 50mM of Tris (pH 7.5), 100mM NaCl, 50mM NaF, 1mM DTT, 1mM EDTA, 1mM EGTA, 10 mM β-glycerophosphate, 2mM Na_3VO_4, 25nM calyculin A, 1% TX100, 0.5mM PMSF and protease inhibitors (EDTA-free tables, Roche). Cytosolic fraction was obtained by ultracentrifugation (100000g for 1h).

To immunoprecipitate endogenous Nek9, 4µg of a-Nek9 antibody (Roig *et al,* 2002) prebound to protein A dynabeads (Fluka) were incubated with 30 mg of lisate for 2h at 4ºC. Immunocomplexes were washed in lysis buffer and proteins were separated by SDS-PAGE. Coomassie stained bands corresponding to Nek9 were excised and analysed by LC/MS/MS in the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA) to determine the phosphorylation sites.

For the determination of the phosphorylated sites in FLAG Nek9 [K81M], innactive FLAG Nek9 was purified from transfected HEK 293T cells. The protein was incubated with the indicated kinases in presence of 100 µM ATP for 30 mintues at 30ºC. Proteins were resolved by SDS-PAGE followed by Coomassie staining. Bands corresponding to Nek9 KM were excised and analysed by LC/MS/MS by the group of Dr Judit Villén at University of Washington (Seattle).

Kinase assays

Nek9 KM or GST-Nek9 domains phosphorylation assays were done by incubation of purified Nek9 in phosphorylation buffer plus 100 µM ATP and [γ-

^{32}P]ATP at 30ºC for 30 minutes in presence or absence of Cdk1/Cyclin-B (Invitrogen), Plk1 (Invitrogen) and Aurora A. Nek9 activation assays were carried out by incubation of eluted recombinant Nek9 in phosphorylation buffer plus 100 μM ATP and [γ-^{32}P]ATP at 25ºC for the indicated times in presence or absence of Plk1, plus a 10 minutes incubation at 25ºC with an exogenous substrate as H3. For the chemical genetics activation assays, FLAG Nek9 was immunoprecipitated from 293T cells. Immobilized Nek9 was activated at 25ºC for different times with 100 μM ATP. After activation Nek9 was washed with phosphorylation buffer and activity was measured by another incubation in presence of 10 μM ATP and [γ-^{32}P]ATP at 30ºC for 10 minutes with the exogenous substrate H3. Reactions were terminated by addition of 5X Laemli buffer and boiling, and proteins were resolved by SDS-PAGE. Coomassie staining was used to visualize proteins and kinase activity was measured with a PhosphorImager system or with the specific a-Nek9[Thr210-P] antibody as indicated.

Time lapse microscopy

For time lapse microscopy, HeLa cells were transfected with H2B-mCherry (Addgene), pEGFP empty vector (Invitrogen) or pEGFP Nek9[Δ346–732] and the indicted SiRNAs 24 hours before image acquisition. Automated Wide-field Olympus IX81 microscope equipped with temperature and CO2 incubation chamber was used to acquire time lapse imaging of cell cultures using the 20x 0.45 phase contrast objective lens every 5 minutes for 24 hours with the CellR software (Olympus Life Science Europe). Software autofocus was used to adjust the z-focus with transmission channel. Cy3, GFP (with filter cubes and excitation filters from AHF Analysentechnik) and transmission acquisition was performed on single plane 12bits-images with an ORCA camera (Hamamatsu Photonics). Subsequent analysis was performed with ImageJ (IJ) 1.43 (Rasband WS, 1997).

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 µg/ml propidium iodide (Sigma) and 2 mg/ml RNAse A (DNAsa free- Sigma) at 37ºC for 15 min. Cells were analysed using a CouLter XL analyser8 (Beckman CouLter).

Immunocytochemistry

HeLa cells were grown on coverslips, rinsed with PBS and fixed with methanol at -20 for 15 minutes. After PBS rinsed cells were incubated with PBS containing 3% bovine serum albumin, 0.1% Triton-X and 0.02% azide. Primary antibodies used in this study were: mouse anti-γ-tubulin (1:500) (Sigma), rabbit anti-Nek9[Thr210-P] and anti-Eg5[Ser1033-P]. Primary antibodies were detected with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG (Invitrogen). DNA was stained with DAPI (0,01 mg/ml). Images were obtained using Nikon E1000 upright epifluorescence microscope with ×60/1.4 Plan APO lens and edited using Fiji (Image J).

Inhibitors

HeLa cells were arrested in mitosis with nocodazole overnight (0,25mM). Mitotic cells were washed from the nocodazole arrest and incubated with DMSO, Purvalanol A (Sigma) (20µM) (Gray, 1998), Bi2536 (100nm) (Axon Medchem) (Lénárt *et al*, 2007) in the presence of the proteasome inhibitor MG132 (20 µM) (Calbiochem) for 2h, or MLN 8237 (SElleck) (Sardon *et al*, 2010) for 1h.

RESULTS

Nek9 activation mechanism

Nek9 mitotic phosphorylation sites

Nek9 is phosphorylated at unknown sites during mitosis resulting in a change in electrophoretical mobility (Roig *et al*, 2002) (Figure 12). 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).

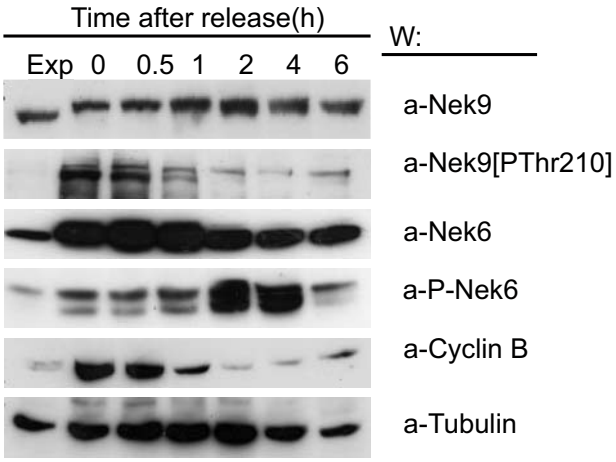
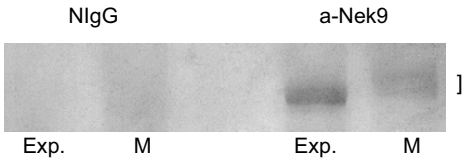


Figure 12. Nek9 has a different electrophoretic mobility in mitotic arrested cells. U2OS cells growing exponentially were either untreated (first lane, Exp) or arrested with nocodazole (0.25 mM overnight; lane 2); arrested cells were allowed to exit mitosis in nocodazole-free medium and extracted at the indicated times (lanes 3-7).

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 (LC/MS/MS) analysis (Figure 13). 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 exponentially growing cells (Ser29, Thr333, Ser750 and Ser869) were also present in mitotic cells, although with a much higher abundance in these last samples (higher phosphorylated/unphosphorylated peptide ratio as assessed from their peak intensities in the MS analysis). 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) (see below).

A



B

Site	Identified peptide	Peak intensity		phosphopeptide (% of total)		Fold increase in M
		Exp.	M	Exp.	M	
Ser29	$_{10}$HCDSINSDFGSESGGCGDSSPGPSASQGPR$_{39}$	7.16 E5	1.80 E5	2.4	67.3	28 x
	$_{10}$HCDSINSDFGSESGGCGDSS#PGPSASQGPR$_{39}$	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	$_{735}$SNSSGLSIGTVFQSSSPGGGGGGGGGEEEDSQQESETPDPSGGFR$_{779}$	4.14 E5	4.35 E4	3.4	56.3	17 x
	$_{735}$SNSSGLSIGTVFQSSSPGGGGGGGGGEEEDSQQES#ETPDPSGGFR$_{779}$	1.46 E4	5.60 E4			
Ser827	–	-	-	-	-	-
	$_{815}$ELENAEFIPMPDS#PSPLSAAFSESEKDTLPYEELQGLK$_{852}$					
Ser869	$_{853}$VASEAPLEHKPQVEASSPR$_{871}$	1.01 E6	1.52 E5	12.4	82.7	7 x
	$_{853}$VASEAPLEHKPQVEASS#PR$_{871}$	1.43 E5	7.29 E5			
Thr885	–	-	-	-	-	-
	$_{882}$GTPLT#PPACACSSLQVEVER$_{901}$					

Figure 13. 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 a # sign. 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*).

The Polo-box domain of Plk1 interacts with the Ser869 of Nek9

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 Polo-Box domain (PBD) (Elia *et al*, 2003a). Thus, we tested whether Plk1 could interact with Nek9 in exponentially growing and mitotic arrested cells. Figure 14 shows that Plk1 specifically coimmunoprecipitates with Nek9 in mitosis in HeLa cells and in mouse embryo fibroblasts.

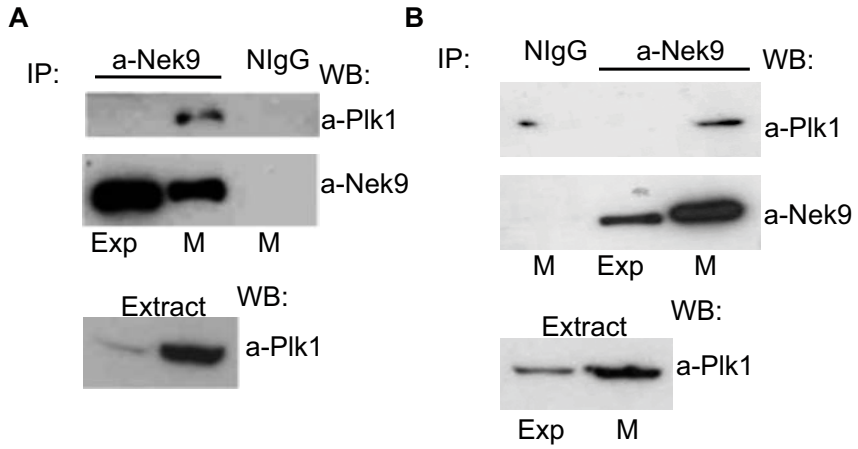


Figure 14. Nek9 binds to Plk1 exclusively in mitosis. **A.** a-Nek9 or normal IgG (*NIgG*) immunoprecipitates from exponentially growing (*Exp.*) or nocodazole-arrested mitotic (*M*) HeLa cell extracts were analyzed by western blot (*WB*) using the indicated antibodies. Plk1 in the corresponding extracts is shown in the lower panel. **B.** 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.

To confirm Nek9 binding to the PBD and to identify the Nek9 S[S/T]P phosphorylation sites responsible for the 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 15A 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 in HeLa cells showing that in contrast to wild type Nek9, Nek9[Ser869] does not interact with endogenous Plk1 in mitosis (see Figure 15B).

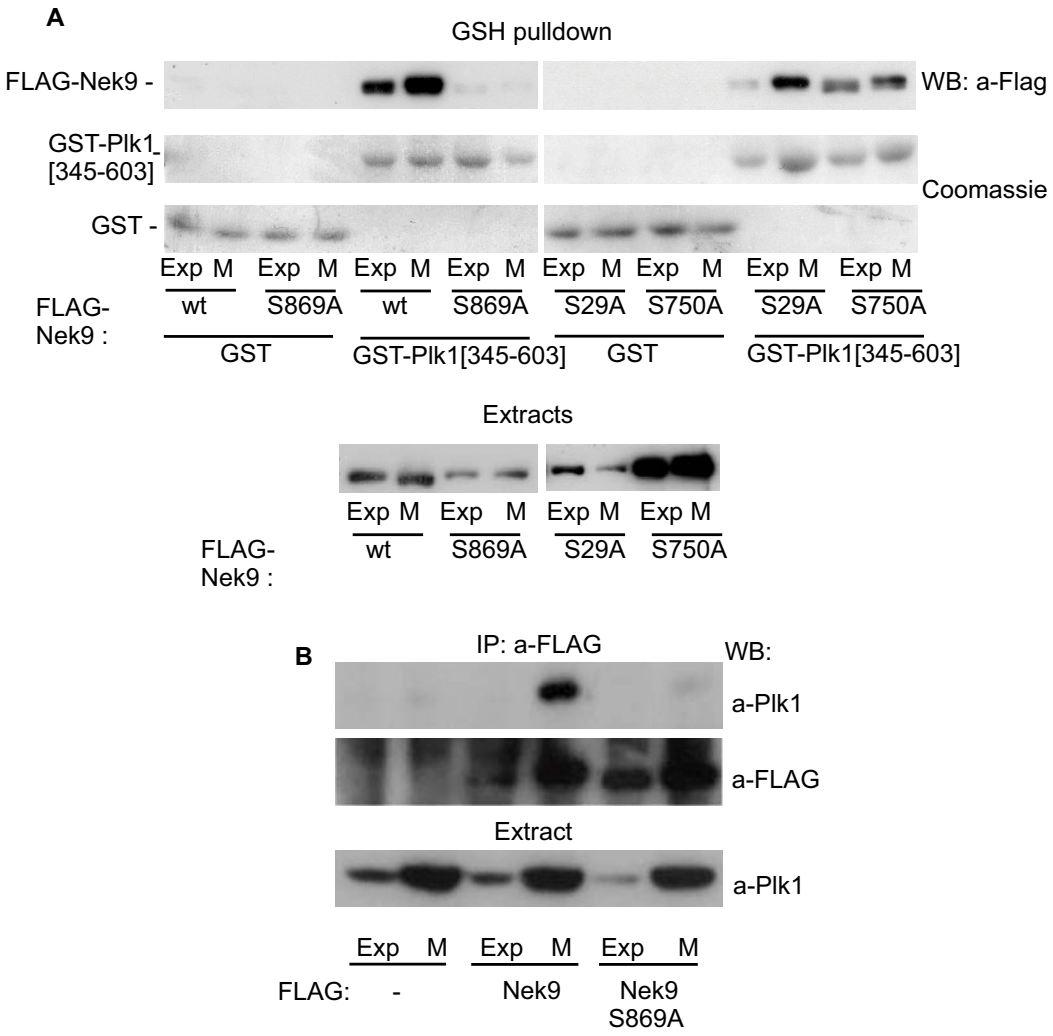


Figure 15. The PBD of Plk1 binds to the Ser869 of Nek9. **A.** *In vitro* binding of different Nek9 forms to GST-Plk1 PBD. Extracts of exponentially growing (*Exp.*) or nocodazole-arrested mitotic (*M*) HeLa cells expressing the indicated FLAG-tagged forms of Nek9 were incubated with GST or GST-PBD (*GST-Plk1[345-603]*) bound to GSH beads. After repeated washes, bound Nek9 was detected by western blot (*WB*) with a-FLAG antibody, and GST-fusion proteins by Coomassie staining. FLAG-Nek9 in the corresponding extracts is shown in the lower panel. **B.** 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.

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 16A). Purified Plk1 readily phosphorylated FLAG-Nek9[K81M] to a similar extent (up to ~6 moles of phosphate/mol of protein) (Figure 16B), and in the *in vitro* conditions used showed only slight or no synergy with Cdk1.

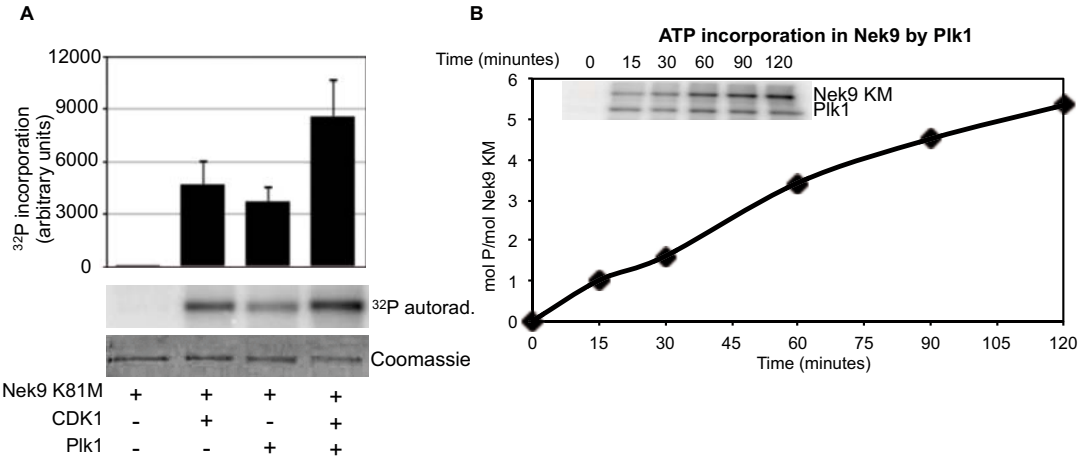


Figure 16. Nek9 is phosphorylated *in vitro* by Plk1. **A.** Kinase-defective FLAG-Nek9[K81M] was expressed and purified from 293T cells and incubated with the indicated kinases for 30 minutes at 30 °C in presence of [γ-^{32}P]ATP/Mg2+. After SDS-PAGE, Nek9 was visualized by Coomassie staining, and ^{32}P incorporation was visualized by autoradiograph (lower and middle panels) and quantified by PhosphorImager (mean ± SEM of 3 independent experiments). *Cdk1*, Cdk1/Cyclin B. **B.** Plk1 is able to incorporate until 6 molP/molNek9 into Nek9 KM. Nek9 KM expressed and purified from HEK293T cells was incubated with 100 μM of [γ^{32}P]ATP for the indicated times. After SDS-PAGE, Nek9 KM phosphorylation was quantified using a PhosphorImager.

Phosphorylation of Nek9 at Thr210 in the kinase activation loop is required for Nek9 activation (Roig *et al*, 2005). To determine whether Plk1 could be directly phosphorylating this activation loop, we incubated purified FLAG-Nek9[K81M] in presence of ATP/Mg^{2+} and using a phosphospecific antibody that specifically recognizes Nek9[Thr210-P] (Roig *et al, 2005)*, we determined that Plk1 was able to modify this site *in vitro* (Figure 17A). Under identical conditions, Cdk1/Cyclin B and the non-relevant kinase JNK1 were not able to phosphorylate Nek9[Thr210]. As FLAG-Nek9[K81M] could have some residual activity, we verified these

results using a mutant with the catalytic aspartic mutated to alanine FLAG-Nek9[D176A] (Figure 17B).

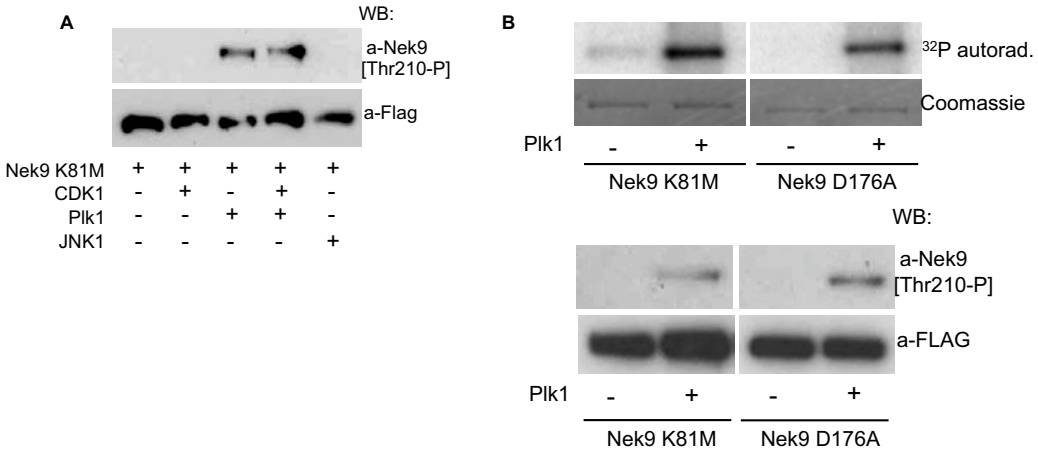


Figure 17. Nek9 is phosphorylated by Cdk1 and Plk1 at Thr210. **A.** FLAG-Nek9[K81M] expressed and purified from 293T cells was incubated with the indicated kinases for 60 minutes at 30 °C in presence of ATP/Mg^{2+} and analyzed by western blot (*WB*) using the indicated antibodies. **B.** Kinase-defective FLAG-Nek9[D176A] was expressed and purified from 293T cells and incubated for 60 minutes at 30 °C with [γ-^{32}P]ATP/Mg2+ 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).

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 18). 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.

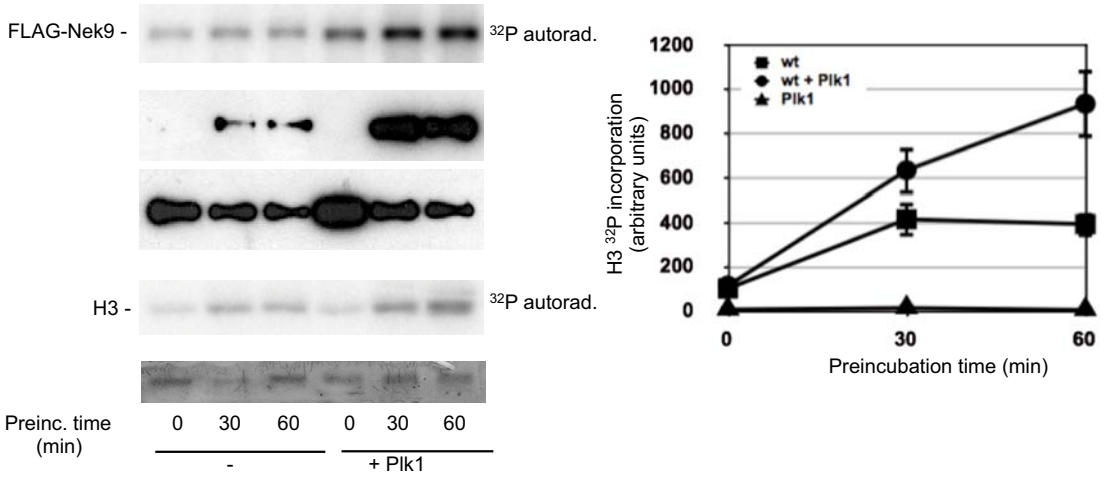


Figure 18. Nek9 is activated by Plk1 *in vitro*. FLAG-Nek9 was expressed and purified from 293T cells and incubated with or without purified Plk1 in the presence of ATP/Mg^{2+} for the indicated times at 25°C. After incubation, [γ-^{32}P]ATP/Mg^{2+} and histone H3 were added to the reactions and further 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 western blot (*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 ± SEM of 3 independent experiments).

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 19A). 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, (e.g. (van Vugt *et al*, 2004)). When under these conditions Cdk1 was inhibited with Purvalanol A (Gray, 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.

To support these results endogenous Nek9 was immunoprecipitated from exponentially growing or mitotic arrested HeLa cells incubated with or without Purvalanol A and the proteasome inhibitor MG132, and its activity was assayed by the incubation of the kinase with H3 at 10 μM ATP. Nek9 from exponentially growing cells cannot autoactivate at 10 μM ATP, but it is activated when cells are arrested in mitosis (Roig *et al*, 2002). As expected from the previous results, when mitotic cells are treated with Purvalanol A Nek9 activation is impaired (Figure 19B).

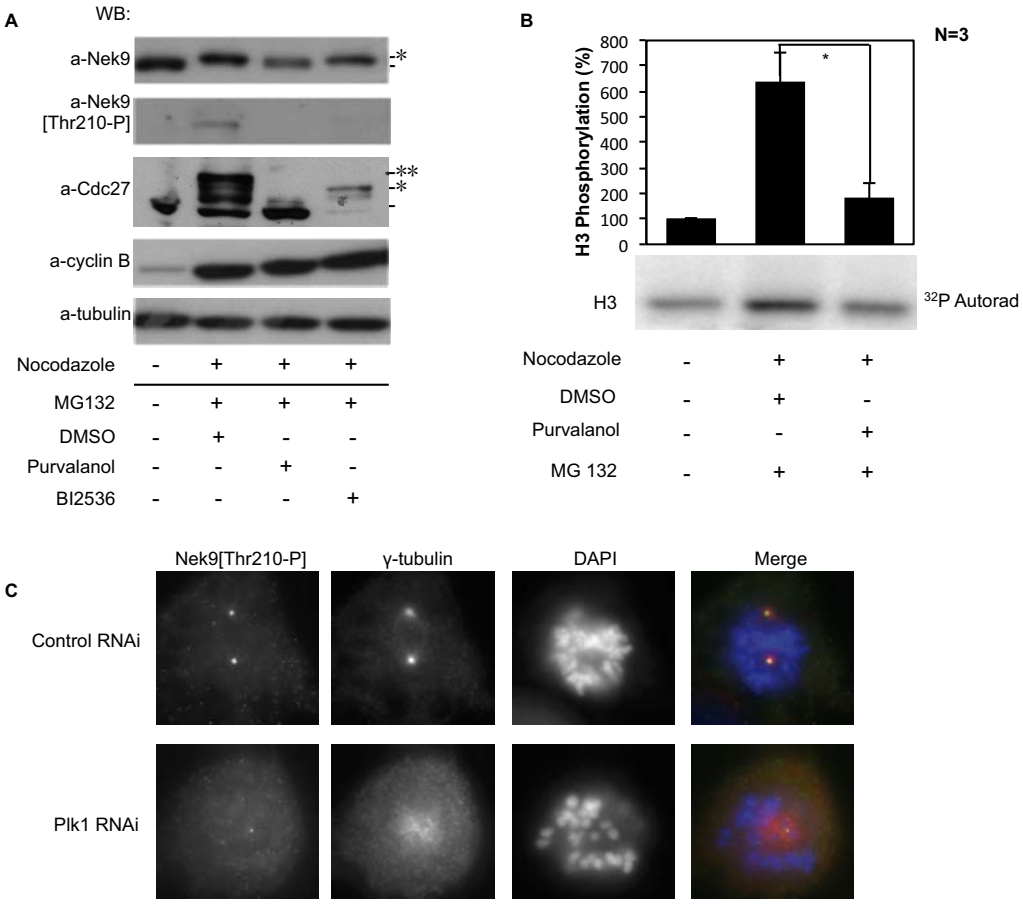


Figure 19. Cdk1 and Plk1 are necessary for Nek9 phosphorylation at Thr210 *in vivo*. **A.** HeLa cells where arrested in mitosis with nocodazole. Mitotic cells were collected, washed and released in media containing MG132 (20 μM) plus DMSO, Purvalanol A (20 μM) or BI2536 (100 nM) for 2h, and cell extracts were analyzed 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. **B.** Endogenous Nek9 from HeLa cells treated as in (A) was immunoprecipitated with a-Nek9 antibody. Immunoprecipitates were incubated 15 minutes at 25ºC in presence of [γ-^{32}P]ATP and H3 as a substrate. ^{32}P incorporation into Nek9 and H3 was visualized by autoradiograph (mean ± SEM of 3 independent experiments). **C.** Hela cells were transfected with the indicated RNAi. 24 hours post transfection cells were fixed and stained with the indicated antibodies.

Active Nek9 localizes at centrosomes in mitosis. In order to further confirm the importance of Plk1 for the activation of Nek9 at centrosomes we downregulated Plk1 using RNAi and checked for the presence of active Nek9 at centrosomes using the Nek9[Thr210-P] antibody. Our preliminary results show that when Plk1 is not present, active Nek9 is not localized at centrosomes, further showing that activation of Nek9 by Plk1 is necessary *in vivo* (Figure 19C).

Plk1 controls Eg5 phosphorylation at the Nek6 site Ser1033

Eg5 is phosphorylated during mitosis at Ser1033, a site that we have previously shown is modified by the Nek9-activated 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 (Bertran *et al,* 2011, Fig S6). 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 Plk1 by RNAi. Mitotic arrest was confirmed by FACS (*not shown*) and the levels of Eg5[Ser1033-P] were compared with these present in exponentially growing cells. Cdc27 was used as a control. Figure 20A shows that Eg5[Ser1033-P] was detected in nocodazole-arrested cells but not in exponentially growing cells both by western and immunofluorescence. 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 (Figure 20B).

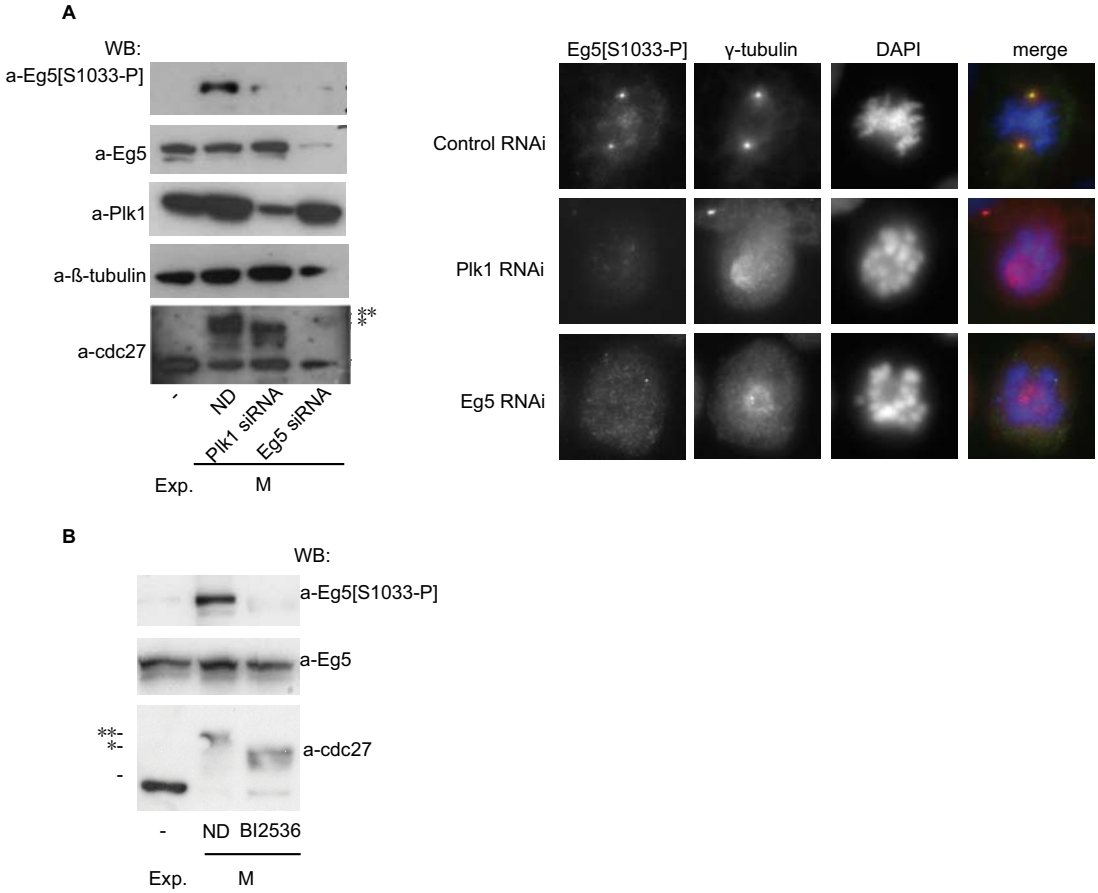


Figure 20. Plk1 controls Eg5 phosphorylation at Ser1033. **A.** HeLa cells where arrested in mitosis by either nocodazole (*ND*) treatment or RNAi against Plk1 or Eg5 (24 hours transfection), collected after mitotic shake off and cell extracts were analyzed by western blot (*WB*) or immunocytochemistry 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. In parallel, cells were fixed in methanol, ans stained with anti-γ-tubulin, anti-Eg5[Ser1033-P] and DAPI. **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.

Active Nek9 and Nek6 are able to induce Eg5 phosphorylation at Ser1033

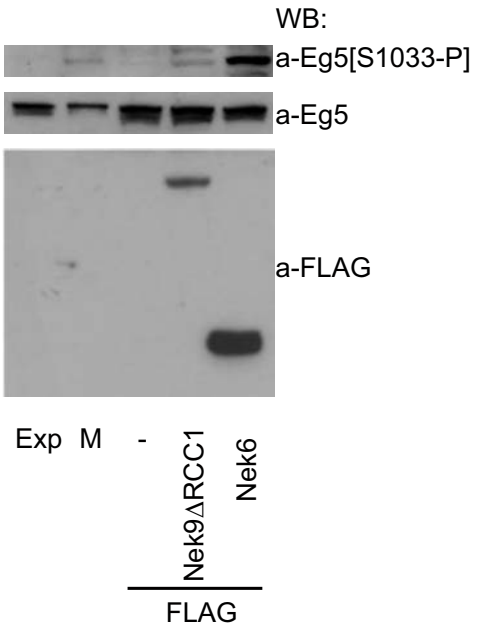


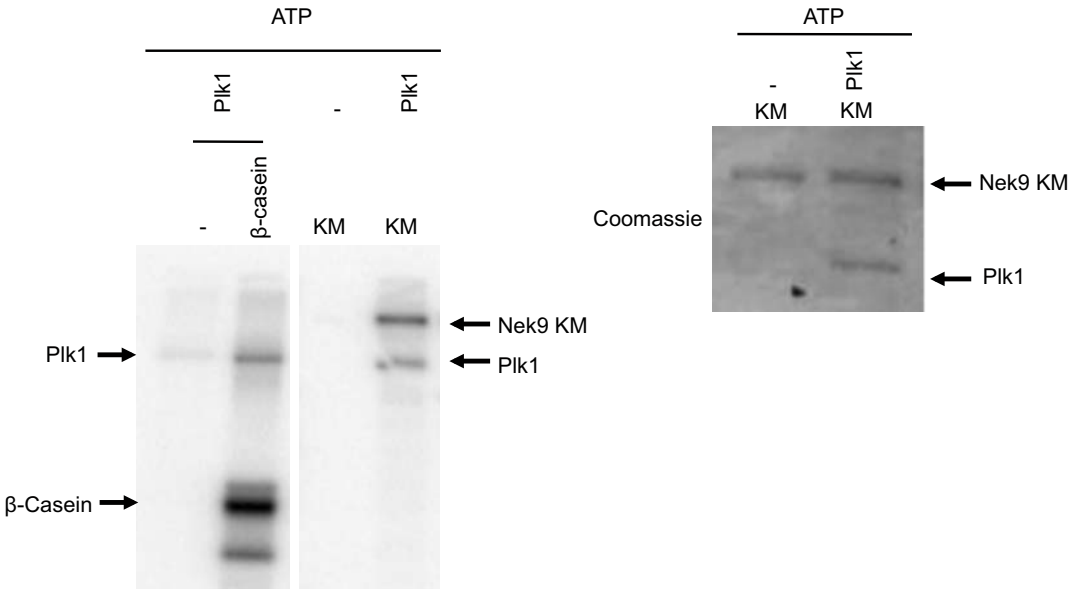
Figure 21. Active Nek9 and Nek6 are able to induce Eg5 phosphorylation at Ser1033. Levels of Eg5[Ser1033-P], Eg5 and FLAG-tagged proteins as determined by western blot of cell extracts from cells transfected with the indicated plasmids. First two lanes show untransfected cell extracts from untreated (*Exp.*) and nocodazole-arrested mitotic cells (*M*). Note that FLAG- transfected cells are growing exponentially.

Additional experiments showed that in fact, transfection of either active Nek9[Δ346–732] or Nek6 induced ectopic Eg5 accumulation around centrosomes in parallel to centrosome separation even in interphase (Bertran *et al*, 2011) and that this is accompanied with Eg5[Ser1033] phosphorylation (Figure 21; 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.

Identification of the sites phosphorylated by Plk1 in Nek9

In order to continue with our study of Nek9 activation downstream of Plk1 and in collaboration with the group of Dr. Judit Villén (University of Washington, Seattle) we determined by mass spectrometry the different Nek9 sites phosphorylated *in vitro* by Plk1.

For that we incubated the inactive form of Nek9, Nek9[K81M], with or without Plk1 plus ATP/Mg^{2+} and analysed the corresponding bands from an SDS-PAGE gel by mass spectrometry (LC/MS/MS). With this analysis we could identify different sites that were putative Plk1 phosphorylation sites (Figure 22). Several phosphosites were present in Nek9[K81M], that was purified from exponentially growing cells. Some of these sites such as Thr392 were much higher phosphorylated in the sample phosphorylated by Plk1, but did not conform to a putative phosphorylation site for Plk1. We also found phosphorylated two sites (Thr67 and Thr662) that conform to a canonical Plk1 phosphorylation sequence [DE]X[ST]ϕX[DE] (Nakajima *et al*, 2003) or [DNE]X[ST][Fϕ: noP][ϕX] (Alexander *et al*, 2011), where ϕ indicates hydrophobic residue and X indicates any residue. Moreover, we also found phosphorylated Ser944. This site has been described as an autophosphorylation site of Nek9 (Daub *et al*, 2008)(J. Avruch an J. Roig, unpublished results) which negatively regulates the binding with LC8 (Regué *et al*, 2011).


ATP
Plk1
-
Plk1
-
β-casein
KM
KM
Plk1
β-Casein
Nek9 KM
Plk1
ATP
-
Plk1
KM
KM
Coomassie
Nek9 KM
Plk1

Nek9 [K81M]			Nek9 [K81M] with Plk1			Fold increase with Plk1
Sites	**Peptide**	**Non-phos/ Phos Area**	**Sites**	**Peptide**	**Non-phos/ Phos Area**	
Ser-Ser-Thr 331-333	$_{331}$SSTVTEAPIAVVTSR$_{345}$	3.98E-02	Ser-Ser-Thr 331-333	$_{331}$SSTVTEAPIAVVTSR$_{345}$	5.05E-02	1.27 x
Thr 392	$_{388}$ELYT#WVNMQGGTK$_{401}$	1.55E-03	Thr 392	$_{388}$ELYT#WVNMQGGTK$_{401}$	2.73E-01	176 x
Ser 516	$_{512}$LGLDS#EEDYYTPQK$_{525}$	1.75E-03	Ser 516	$_{512}$LGLDS#EEDYYTPQK$_{525}$	3.65E-03	2.09 x
One P in blue region	$_{815}$ELENAEFIPMPDSPSPLSAAFSESEK$_{840}$	1.41E-03	One P in blue region	$_{815}$ELENAEFIPMPDSPSPLSAAFSESEK$_{840}$	1.54E-01	109 x
Ser 869	$_{853}$VASEAPLEHKPQVEASS#PR$_{871}$	9.97E-02	Ser 869	$_{853}$VASEAPLEHKPQVEASS#PR$_{871}$	1.45E-01	1.45 x
Thr 720	$_{717}$GWHT#ILIVEK$_{726}$	1.00E-01				
			Ser 2	VPLEIYAAMS#VLGEYER$_{9}$	5.54E-02	
			Thr 67	$_{61}$GAFGEAT#LYR$_{70}$	1.51E-02	
			Thr 320	$_{319}$VT#LLNAPTK$_{327}$	2.85E-02	
			Thr 326	$_{319}$VTLLNAPT#KRPR$_{330}$	2.47E-02	
			Tyr 358	$_{354}$TSEVY#IWGGGK$_{364}$	3.13E-02	
			Thr 662	$_{662}$T#IAATDDNHIF$_{672}$	9.23E-04	
			Thr 842	$_{836}$SESEKDT#LPY$_{845}$	1.81E-01	
			Thr 842	$_{841}$DT#LPYEELQGLK$_{852}$	5.72E-03	
			Ser 855	$_{853}$VAS#EAPLEHKPQVEASSPR$_{871}$	2.18E-03	
			Ser 855	$_{853}$VAS#EAPLEHKPQVEASSPR$_{871}$	2.77E-04	
			Ser 944	$_{933}$KLEGGQQVGMHS#K$_{945}$	3.87E-03	

Figure 22. Plk1 phosphorylates other Nek9 sites besides Thr210. Kinase defective FLAG-NEK9[K81M] was expressed and purified from HEK 293T cells and incubated with the indicated kinases for 30 minutes at 30ºC in presence of ATP/Mg^{2+}. After SDS-PAGE, Nek9 was visualized by Coomassie staining. Nek9 KM bands were excised and analysed by Mass Spectrometry. Phosphorylated sites found in the analysis. The table shows the different peptides identified and the relation between the phosphorylated and unphosphorylated area. The # sign indicates the putative phosphorylation site (previous residue). Underlining indicates canonical Plk1 phophorylation site.

To determine whether the phosphorylation of Thr67 and Thr662, the sites that could be phosphorylated by Plk1 *in vitro* and conformed to a Plk1 canonical phosphorylation site were important for the activation of Nek9 and thus the phosphorylation of Thr210, we mutated these residues to an acidic phosphomimetic residue, and checked for the activation state of these mutants using the phosphospecific Nek9[Thr210-P] antibody. We additionally mutated all the other residues that also conformed to a Plk1 canonical phosphorylation. Figure 23 shows that when putative Plk1 phosphorylation sites located at the RCC1 domain (Thr438, Thr544 and Thr662) are mutated to an acidic residue, Nek9 may be phosphorylated at Thr210 in interphase indicating that the phosphorylation of these sites could be important for the activation of the kinase. A similar effect was observed with the putative Plk1 site present in Nek9 activation loop, Ser206.

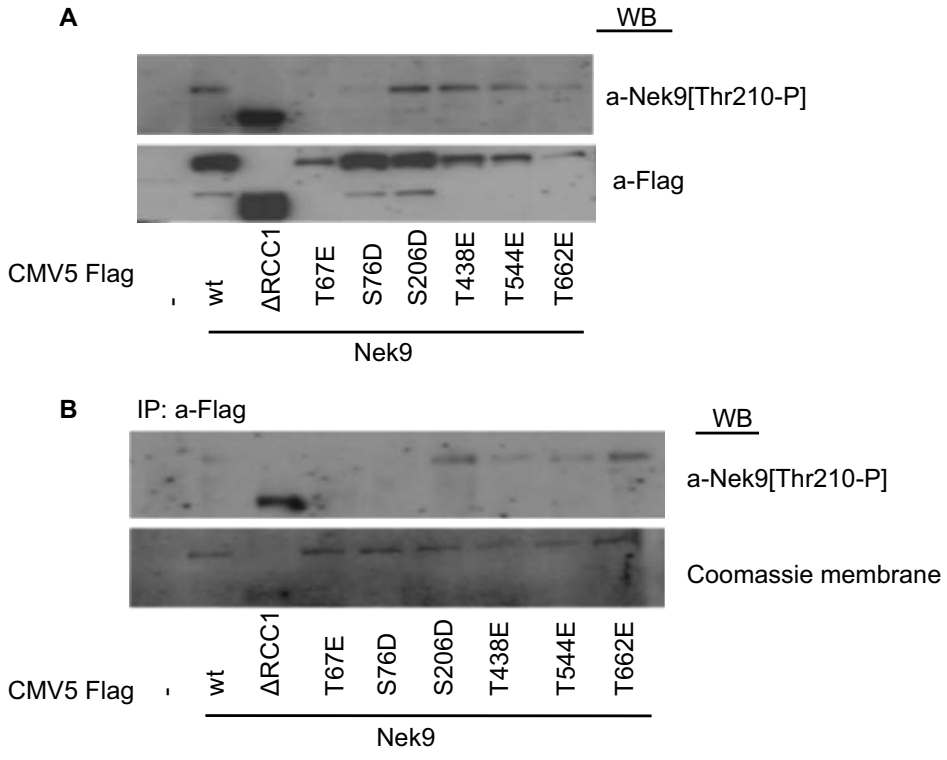


Figure 23. Plk1 putative phosphorylation sites localized at the RCC1 domain are important for Nek9 activation. 293T cells were transfected with the indicated plasmids. **A.** 24 hours post-transfection cells lysates were analysed by Western Blots of the cell lysates with the indicated antibodies. **B.** Anti-FLAG immunoprecipitates from cells treated as in A were immunoblotted with the indicated antibodies.

Nevertheless, in our experiments we saw some activation of the wild type form of Nek9 and we could not see activation of these mutants with a kinase assay (data not shown), altogether indicating that more work needs to be done to establish the importance of these sites for the activation mechanism of Nek9.

Active Nek9 can partially overcome the Plk1 downregulation phenotype

It is very well described that when Plk1 is inhibited using RNAi (Sumara *et al*, 2004) or with the specific inhibitor BI2536 (Lénárt *et al*, 2007) cells get arrested in prometaphase with unseparated and immature centrosomes. Our group has shown that some of the functions of Plk1 during these phases are performed by Nek9 (Bertran *et al*, 2011; Sdelci *et al*, 2012). Thus we sought to study whether active Nek9 could rescue Plk1 inhibition during cell cycle progression.

For this, we transfected HeLa cells with FLAG-Nek9[Δ346-732] and downregulated Plk1 activity by using RNAi or the chemical inhibitor BI2536, and determined the cell cycle profile of the treated cells with FACS. As expected, in cells where Plk1 was downregulated with RNAi the number of mitotic cells was higher compared to cells where a control RNAi was transfected. When active Nek9 was transfected in these cells, we could recover a normal cell cycle profile (Figure 24). A similar (although more subtle) result was observed using the chemical inhibitor BI2536. Figure 25 shows that when Plk1 was inhibited, cells were completely blocked in G2/M, while in cells where active Nek9 was transfected, a significant number of cells could scape from the mitotic block (5.2% +/- 1.06 vs 1.5% +/- 0.6 in mock treated cells). FLAG Nek9 [Δ346-732 K81M]

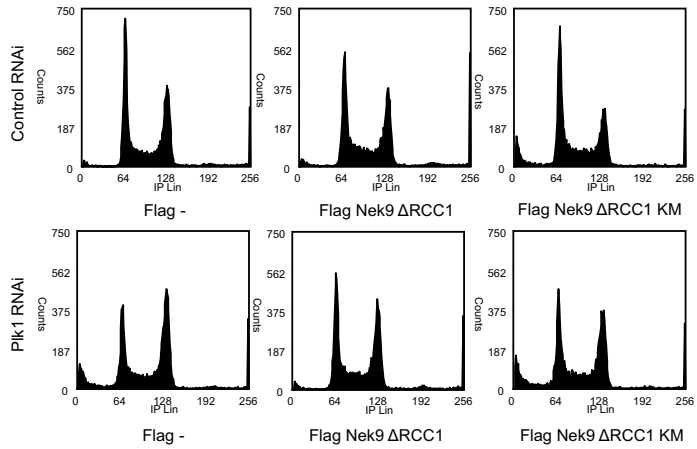


Figure 24. Active Nek9 can partially overcome the phenotype of downregulating Plk1. DNA content of HeLa cells transfected with control or Plk1 RNAi and the indicated plasmids. 24 hours post transfection DNA content of all cells was analysed by PI staining and FACS.

(FLAG Nek9 ΔRCC1 KM) did not result in the observed phenotypes, thus indicating that Nek9 activity is necessary to overcome Plk1 inhibition phenotype.

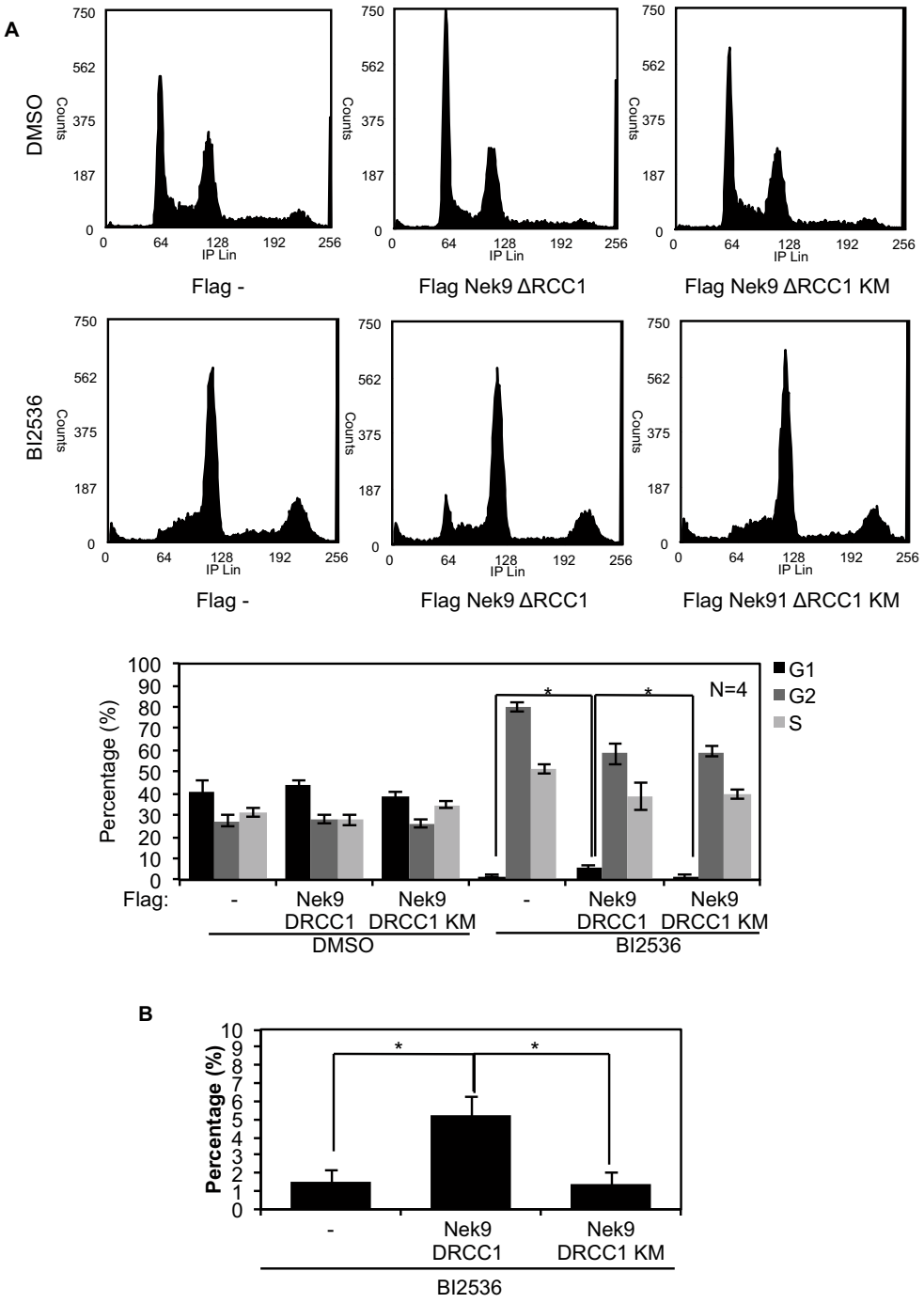


Figure 25. A. Active Nek9 can partially overcome Plk1 inhibition phenotype. DNA content of Hela cells transfected with the indicated plasmids. 24 hours post transfection cells were treated with DMSO or BI2536 (100nM) for 8 hours. Afterwards, the DNA content of all cells was analyzed by PI staining and FACS. (mean± SEM of four independent experiments). **B.** Magnification of the chart of cells in G1 treated with BI2536.

In order to know whether cells that were escaping from the mitotic block that resulted from interfering with Plk1 were able to finish mitosis or whether alternatively these cells were not entering mitosis altogether, we performed time-lapse microscopy experiments. For that, we cotransfected Plk1 RNAi, with or without the active form of Nek9, Nek9[Δ346–732], and mCherry-H2B in HeLa cells and followed the cells for 20 hours. As expected, cells where Plk1 RNAi was transfected were arrested in prometaphase and did not finish mitosis (Figure 26A). However, a significant number of cells where in addition to Plk1 RNAi, GFP-Nek9[Δ346-732] was transfected, entered mitosis, were able to escape from the blocking and finished mitosis (Figure 26B and C).

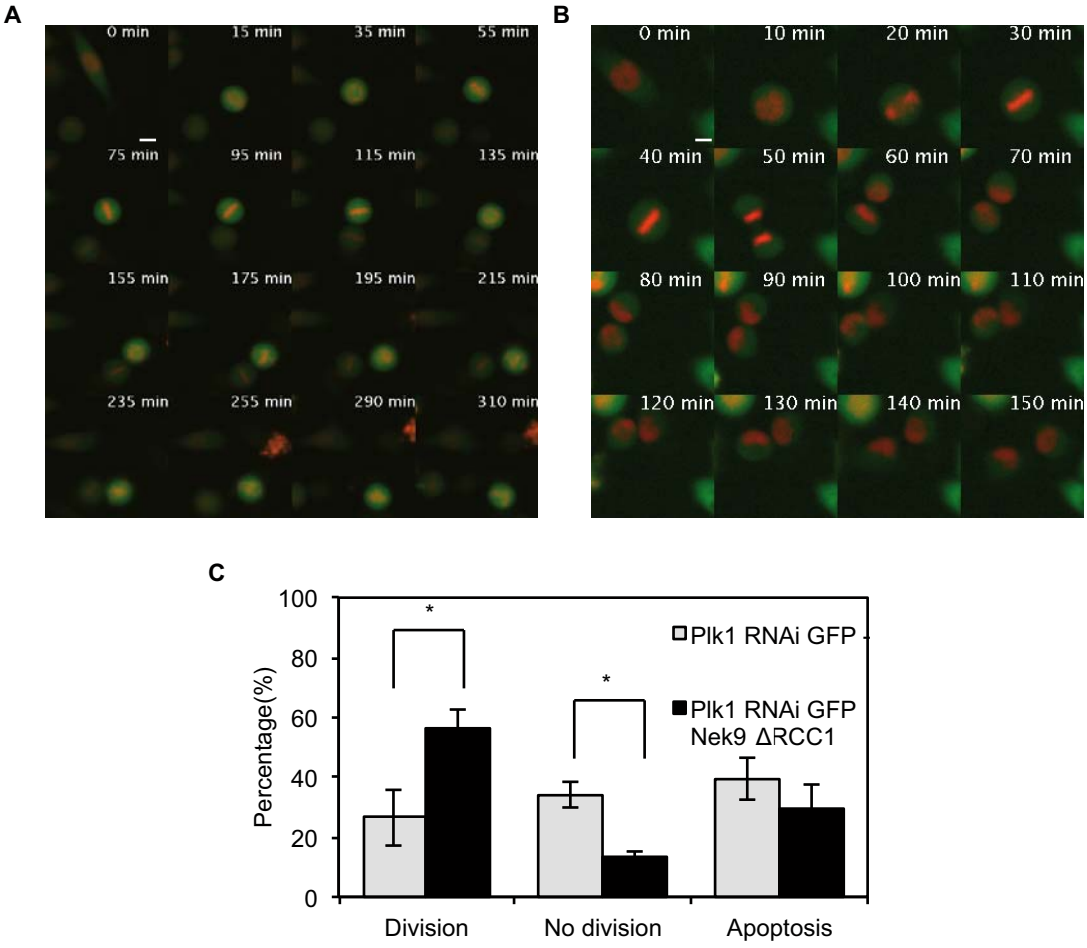


Figure 26. Cells transfected with active Nek9 can overcome Plk1 interference phenotype, and finish mitosis. HeLa cells were transfected with Plk1 RNAi and the indicated plasmids. 24 hours post transfection cells were imaged using a Automated Wide-field Olympus IX81 microscope. Images were acquired every 5 minutes during 20 hours. **A.** Stills from cells transfected with Plk1 RNAi and pEGFP. **B.** Stills from cells transfected with Plk1 RNAi and pEGFP Nek9 ΔRCC1 (GFP Nek9 [Δ346-732]) . Scale bars represent 10 μm. **C.** Quantification of three independent experiments (percentage of cells in every film ± SEM, 30 cells in each experiment).

Nek9 is an Aurora A substrate

During our study of the phosphorylation of Nek9 by Plk1, we decided to test whether another major mitotic protein kinase present at centrosomes, Aurora A, was involved in the mechanism of Nek9 activation. We found that Aurora A was also able to phosphorylate Nek9 and we decided to study this phosphorylation.

Nek9 is phosphorylated by Aurora A

To confirm the phosphorylation of Nek9 by Aurora A we purified kinase-deficient FLAG-Nek9[K81M] from 293T cells and incubated it with purified Aurora A. Figure 27A shows that Aurora A phosphorylates Nek9 *in vitro*. We next sough to map this phosphorylation. For this we incubated Nek9[1-317], Nek9[305-754] and Nek9[750-979] expressed and purified from bacteria, with [$\gamma-^{32}$P]ATP/Mg^{2+} in presence or absence of Aurora A. The results revealed that Aurora A is able to phosphorylate the C-terminal domain, Nek9[750-979], and the RCC1 domain, Nek9[305-754], of Nek9 (Figure 27B). In both cases, Cdk1/Cyclin B and Plk1 were used as positive controls.

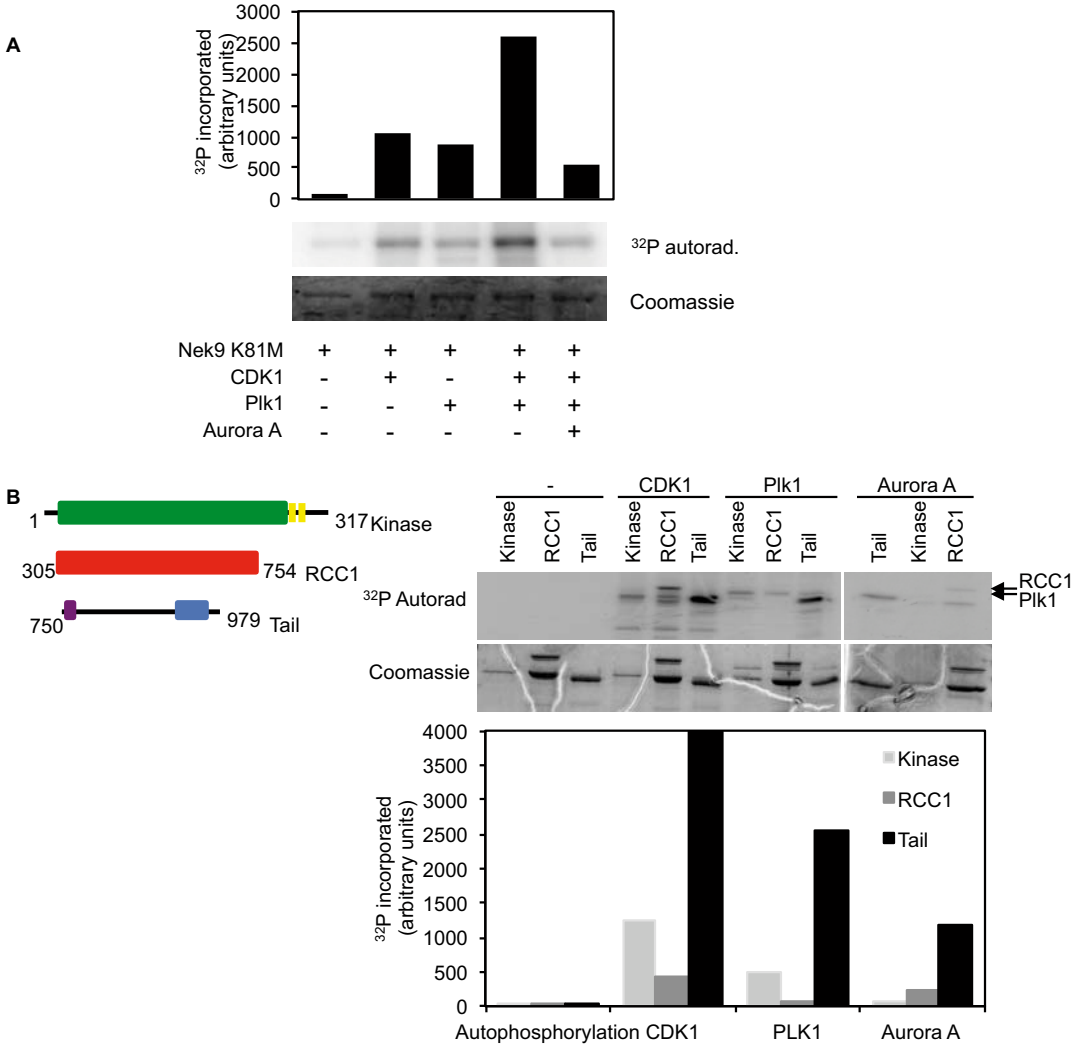


Figure 27. Aurora A phosphorylates the tail domain of Nek9. **A.** Nek9 KM purified from 293T was incubated with the indicated kinases for 30 minutes at 30ºC in presence of [γ-^{32}P]ATP/Mg^{2+}. After SDS-PAGE, ^{32}P incoroporation was visualized and quantified by PhosphorImager. **B.** Nek9[1-317], Nek9[305-750] and Nek9[754-979] domains were expressed and purified from bacteria using the standard protocol. 1µg of Nek9 domains were incubated with or without purified Cdk1, Plk1 or Aurora A. ^{32}P incorporation was visualized and quantified by autoradiography.

Aurora A phosphorylates the tail domain of Nek9 at the Serine 978 and the Thr333 of the RCC1 domain

To map more accurately the phosphorylation motif of Aurora A, we expressed different sub-motifs of Nek9 C-terminal domain in bacteria (Figure 28A) and checked for the phosphorylation of them *in vitro*. For that, we incubated each domain of Nek9 in presence of [γ-^{32}P]ATP/Mg^{2+} with or without Aurora A. Myelin basic protein (MBP) was used as a positive control of Aurora A phosphorylation. We confirmed that Aurora A was phosphorylating the C-terminal domain of Nek9 and that this phosphorylation was restricted to the last forty residues of the protein (Figure 28B).

With *in silico* analysis we identified Ser978 as a putative phosphorylation site for Aurora A, R[RK]X[ST][F;noP] (Ferrari *et al*, 2005; Alexander *et al*, 2011). Moreover, this site, had already been found phosphorylated in some mass spectrometry analysis performed by us and others (Daub *et al*, 2008) (Oppermann *et al*, 2009). We mutated this residue to an alanine and to an aspartic acid, and checked *in vitro* whether this mutation interfered with the phosphorylation of Aurora A as in Figure 28B. Figure 28C shows that when Ser978 was mutated, phosphorylation of Nek9[940-979] was totally abrogated confirming Ser978 of Nek9 as the unique Aurora A phosphorylation site in Nek9 C-terminal region.

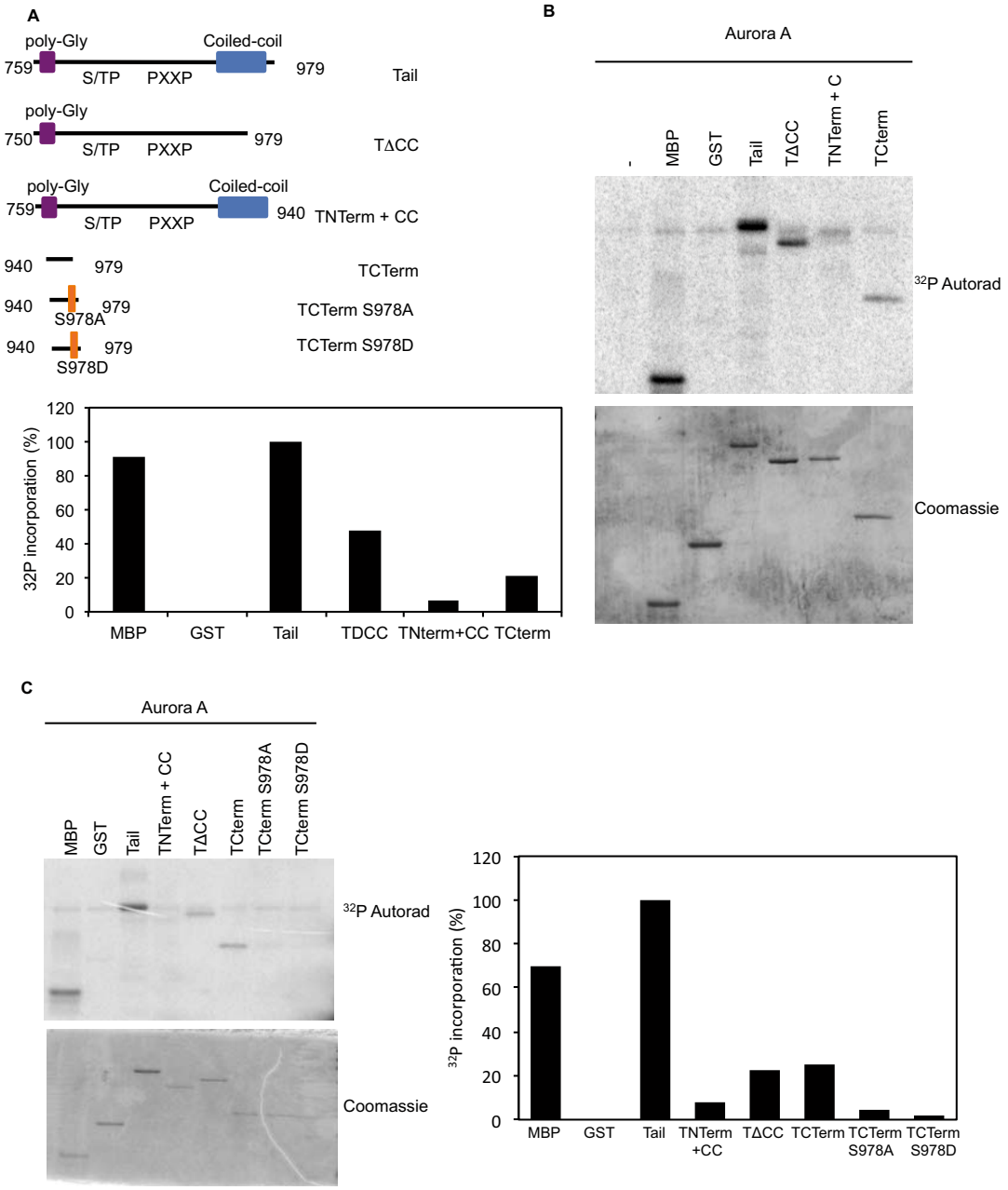
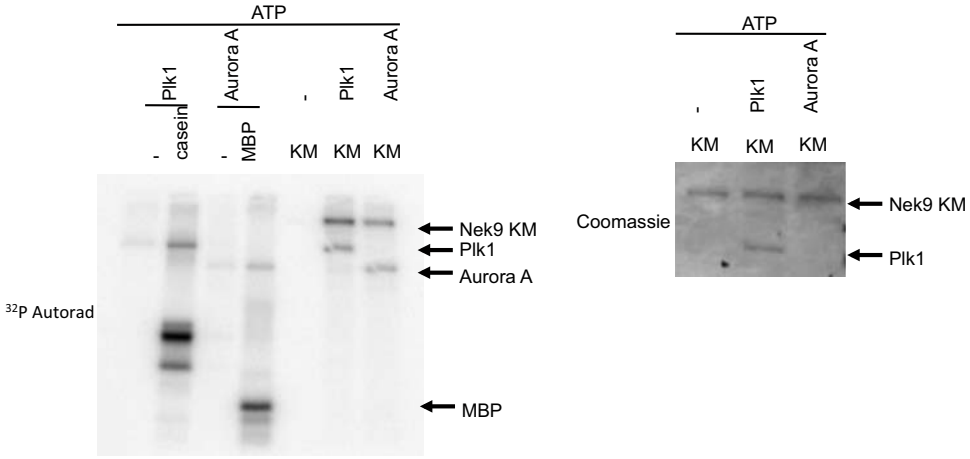


Figure 28. Aurora A phosphorylates the C terminal domain of Nek9 at Ser978. **A.** Different domains of Nek9 used. **B.** Nek9 fragments were expressed and purified in bacteria using the standard protocols. 1 µg of Nek9 fragments were incubated in presence of 0.2 µg Aurora A and 100 µM [γ-^{32}P]ATP for 30 minutes at 30ºC. The reaction was stopped by addition of SDS sample buffer, followed by SDS-PAGE and Coomassie staining. ^{32}P incorporation was quantified by PhosphorImager. ^{32}P incorporation into tail domain was set to 100%. **C.** Ser978 was mutated and the TCTerm domain was expressed and purified as in (B). Kinase assay was performed as in (B).

In parallel, we performed a mass spectrometry analysis of the inactive form of Nek9 (Nek9[K81M]) phosphorylated by Aurora A. For this we incubated purified Nek9[K81M] with ATP/Mg^{2+} in presence or absence of Aurora A. The bands corresponding to FLAG-Nek9[K81M] were excised from an SDS-PAGE gel and analysed by mass spectrometry (LC/MS/MS). The results revealed the presence of different sites phosphorylated although none of them conformed to a canonical Aurora A site. Interestingly, one of the phosphopeptides detected contained the residues 331-333. Thr333 does not exactly conform to a putative Aurora A phosphorylation site, R[RK]X[ST][F;noP] (Alexander *et al*, 2011), but it only differs in one residue, RSST#V and when comparing the phosphorylation of this peptide in the sample phosphorylated by Aurora A with the one that had not been phosphorylated, the phosphorylation rate significantly increases (43-fold) (Figure 29). Additionally the residues 331-3 had previously been found phosphorylated by us in our mitotic analysis (Figure 13) where it was found to be increased 9-fold in mitotic cells vs. exponentially growing cells, and our analysis of Plk1 phosphorylation of Nek9 (Figure 22) were although present in cells, was not increased upon incubation with Plk1. Additionally, others have described the presence of this phosphosite *in vivo* (Olsen *et al*, 2010; Wissing *et al*, 2007). All this leads us to propose Ser333 as a site phosphorylated by Aurora A *in vitro* and possibly *in vivo*. More work will be needed to confirm the phosphorylation of these sites and also to identify which is the biological significance of these phosphorylations.



Nek9 [K81M]			Nek9 [K81M] with Aurora A			Fold increase with Aurora A
Sites	**Peptide**	**Non-phos/ Phos Area**	**Sites**	**Peptide**	**Non-phos/ Phos Area**	
Ser-Ser-Thr 331-333	$_{331}$SSTVTEAPIAVVTSR$_{345}$	3.98E-02	Ser-Ser-Thr 331-333	$_{331}$SSTVTEAPIAVVTSR$_{345}$	1.73E+00	43.5 x
One P in blue region	$_{815}$ELENAEFIPMPDSPSPLSAAFSESEK$_{840}$	9.97E-02	One P in blue region	$_{815}$ELENAEFIPMPDSPSPLSAAFSESEK$_{840}$	4.23E-02	0.424 x
Thr 392	$_{388}$ELYT#WVNMQGGTK$_{401}$	1.55E-03				
Ser 516	$_{512}$LGLDS#EEDYYTPQK$_{525}$	1.75E-03				
Thr 720	$_{717}$GWHT#ILIVEK$_{726}$	1.41E-03				
Ser 869	$_{853}$VASEAPLEHKPQVEASS#PR$_{871}$	1.00E-01				
			Thr 326	$_{319}$VTLLNAPT#KRPR$_{330}$	1.65E-02	
			Tyr 350	$_{346}$TSEVY#IWGGGK$_{356}$	1.18E-03	
			Thr 355	$_{351}$SSTVT#EAPIAVVTSR$_{365}$	4.96E-02	
			Ser 855	$_{853}$VAS#EAPLEHKPQVEASSPR$_{871}$	6.83E-02	

Figure 29. Kinase defective FLAG-NEK9[K81M] was expressed and purified from HEK 293T cells and incubated with the indicated kinases for 30 minutes at 30ºC in presence of ATP/Mg2+. After SDS-PAGE, Nek9 was visualized by Coomassie staining. Nek9 KM bands were excised and analysed by Mass Spectrometry. Phosphorylated sites found in the analysis. The table shows the different peptides identified and the relation between the phosphorylated and unphosphorylated area.

Aurora A binds to the tail domain of Nek9

We next sought to determine whether Aurora A and Nek9 were interacting. For that we performed GST pull down assays of different Nek9 domains expressed in 293T cells with endogenous Aurora A. Figure 30 shows that Aurora A binds to Nek9[759-979] domain but not to Nek9[1-316] and Nek9[317-758] domains. Unfortunately, we were not able to detect endogenous binding between both proteins (not shown).

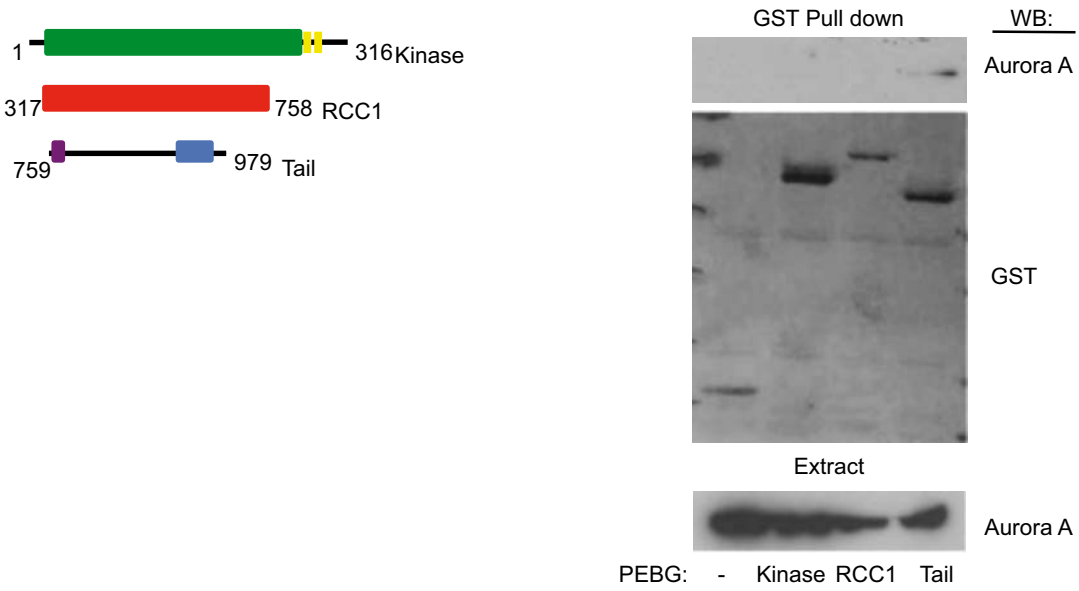


Figure 30. Aurora A binds to the tail domain of Nek9. GST pull-downs from HEK293T cells transfected with the indicated GST-Nek9 fragments were immunobloted with Aurora A antibody. A cartoon of the Nek9 fragments used is shown

Aurora A activity is not required for the phosphorylation of Thr210 but localization of active Nek9 at centrosomes is impaired when Aurora A is inhibited.

Aurora A has been shown to localize to centrosomes during mitosis, and it has several roles in bipolar spindle formation (Giet, 1999; Sardon *et al*, 2008) and centrosome maturation (Abe *et al*, 2006) (Barr & Gergely, 2007; Carmena *et al*, 2009). Besides, active Nek9 is also localized at centrosomes in mitosis (Roig *et al,* 2005), and we have published that it is important for centrosome separation in

prophase (Bertran *et al*, 2011) and also for centrosome maturation through Nedd1 (Sdelci *et al*, 2012).

Due to the similarity in either localization or function of both proteins, we wanted to find if Aurora A was necessary for the phosphorylation of Nek9 at Thr210 in mitosis. For that, we arrested cells in prometaphase with the proteasome inhibitor MG132. As expected, that leads to the phosphorylation of the activation loop of Nek9. As a positive control of the inhibition of the phosphorylation at Thr210 the treatment with Purvalanol A was used. Nevertheless, when we incubated cells with the inhibitor of Aurora A MLN8237 (Sardon *et al*, 2010) we could still detect phosphorylation of the Thr210, strongly suggesting that Aurora A activity is not required for the activation of Nek9 (Figure 31A).

To see if active Nek9 was localizing at centrosomes despite the absence of Aurora A, we transfected cells with control or Aurora A RNAi and checked for the phosphorylation of the Thr210 with immunofluorescence using the Nek9[Thr210-P] antibody. Figure 31B shows that, as expected, Nek9[Thr210-P] localizes at centrosomes in control cells during mitosis. Our results, although somehow preliminar, show that when Aurora A is downregulated, active Nek9 is not detectable at the centrosomes.

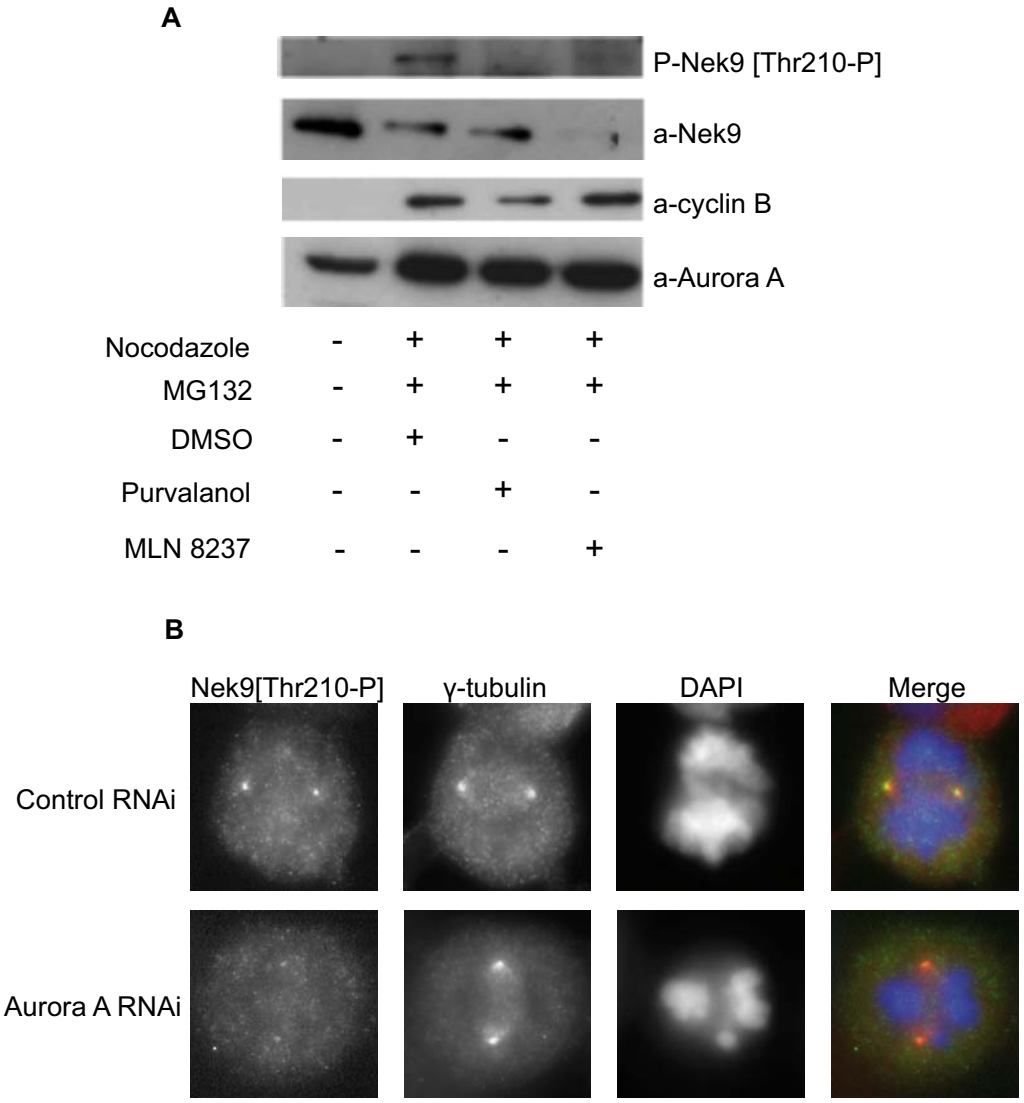


Figure 31. Aurora A is not necessary for the phosphorylation of the Thr210, but is required for the localization of active Nek9 at centrosomes. **A.** HeLa cells were arrested in mitosis with nocodazole. Mitotic cells were collected, washed and released in media containing MG132 (20 μM) plus DMSO, Purvalanol A (20 μM) or MLN 8237 (500 nM) for 1h, and cell extracts were analyzed by western blot (*WB*) using the indicated antibodies. Untreated cells are shown in the first lane as a control. **B.** Hela cells were transfected with control RNAi or Aurora A RNAi. 24 hours post transfection cells were fixed and stained with Nek9[Thr210-P] (green), γ-tubulin (red) and DAPI (blue).

Chemical Genetic Approach

Introduction

Inhibiting kinases has always been a difficult task to tackle (Cohen, 2002; Dar & Shokat, 2011), because even though there is a large number of kinases with very different functions, all kinase catalytic domains are structurally very similar and the use of inhibitors leads to a lot of unespecificity (Hunter, 2000). During the last years we have inhibited Nek9 using different approaches, but in order to have an acute and highly penetrant inhibition of the kinase we sought to inhibit it chemically. The lack of a suitable specific Nek9 inhibitor lead us to take a chemical genetic approach (Table 2).

A chemical genetic method for the generation of target-specific protein kinase inhibitors was developed some years ago in Dr Shokat laboratory (UCSF) (Bishop *et al*, 1998). As a general overview, the strategy consists on doing a functionally silent mutation in the ATP binding pocket (gatekeeper residue) of the target kinase that sensitizes it to inhibition by a bulky ATP analog and that does not inhibit wild type kinases.

The design strategy of the kinase-sensitization approach utilizes the engineering of a 'hole' in the ATP-binding site of the target kinase and a corresponding 'bump' on the small molecule inhibitor. Site-directed mutagenesis of a large amino acid side chain to a small side chain (alanine or glycine) generates the unique hole in the kinase of interest. The necessary criteria for the sensitizing mutation are similar for any target kinase (Bishop *et al*, 2001).

Mutagenesis of this large amino acid side chain to a small side chain (alanine or glycine) generates the unique hole in the kinase of interest. The criteria for the mutation site are:

- The wild-type residue should be large enough such that site-directed mutagenesis to glycine or alanine would create a novel binding pocket.

- The corresponding residue should not be occupied by an alanine or glycine in other protein kinases.
- Mutation of the residue should be functionally silent.
- Ideally, the residue identified for drug-sensitization would be generalizable to other protein kinases. This eliminates the need to redesign the kinase–inhibitor interface for each target kinase.

Method	Onset	Penetrance	Advantages	Disadvantages	Observed phenotype with Nek9
Antibody microinjection	Semi-acute	Variable	Semi-acute	Single cell, effect may depend on epitope and antibody	Ptk2 cells microinjected in interphase never enter mitosis. When injected during prophase they present prometaphase arrest, aberrant spindles and aneuploidy (Roig et al. 2002)
RNAi	48h	Depends on threshold and knockdown efficiency	Immediate results	Incomplete knockdown, slow onset of action, off-target effects	No centrosome separation (Bertran et al. 2011) No centrosome maturation (Sdelci et al. 2012) Delay in mitosis (Bertran et al. 2011)
Pharmacologi-cal inhibitor	Acute (min)	Complete with high concentration	Rapid onset, portable to different cell lines	Off-target effect, time-consuming drug discovery effort	Non-specific results (Unpublished results)
Chemical genetics	Acute	Complete with high concentration	Rapid onset, easy to distinguish on-target vs off-target effects	Time consuming, mutant constructions might no rescue gene function, less portable than pharmacological inhibitor	????

Table 2. Different approaches used to inhibit Nek9 and observed phenotype.

Leucine 128 identified as the putative gatekeeper residue of Nek9

In order to identify the gate-keeper residue of Nek9 we did a sequence alignment of Nek9 with other kinase domains of the kinome using the Protein Kinase Database (http://sequoia.ucsf.edu/ksd/) developed in Dr. Shokat's laboratory (Buzko & Shokat, 2002)(Gregan *et al*, 2007).

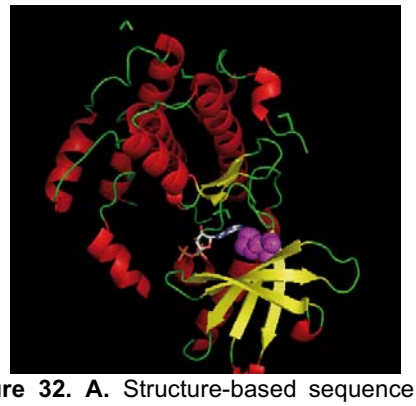
The alignment revealed that the putative Nek9 gatekeeper residue was Leucine 128 (Figure 32A).

To determine if this residue was localized in the ATP binding pocket, we took advantage of the structures of Nek2 (Westwood *et al*, 2009) (Figure 32B) and Nek7 (Richards *et al*, 2009a) (Figure 32C) which had already been solved. The homologous Met86 for Nek2 and Leu111 for Nek7 both localize at their ATP binding pocket, and due to the high similarity between the kinase domains of these kinases we assumed that Leu128 of Nek9 was also localizing at the ATP binding pocket.

A

```
Nek6          EDN------ELNIVLELADAGDLSQMIKYFKKQKRLIPERTVWKYFVQLCSAVEHMHSRR 167
Nek7          EDN------ELNIVLELADAGDLSRMIKHFKKQKRLIPERTVWKYFVQLCSALEHMHSRR 156
Nek9          DNT------TLLIELEYCNGGNLYDKILRQKD--KLFEEEMVVWYLFQIVSAVSCIHKAG 171
PKA           DNS------NLYMVMEYVPGGEMFSHLRRIGR----FSEPHARFYAAQIVLTFEYLHSLD 119
CAMKI         SGG------HLYLIMQLVSGGELFDRIVEKGG----YTERDRSRLIFQVLDAVKYLHDLG 117
JNK2          PQKTLEEFQDVYLVMELMDANLCQVIHMELDHER-------MSYLLYQMLCGIKHLHSAG 121
CDK2          TEN------KLYLVFEFLHQDLKKFMDASALTGIP---LPLIKSYLFQLLQGLAFCHSHR 119
Nek2          DRTN----TTLYIVMEYCEGGDLASVITKGTKERQYLDEEFVLRVMTQLTLALKECHRRS 131
c-src         EEP-------IYIVTEYMSKGSLLDFLKGETG--KYLRLPQLVDMAAQIASGMAYVERMN 115
                        : :  :    .                        *:   .   .
```

B



C

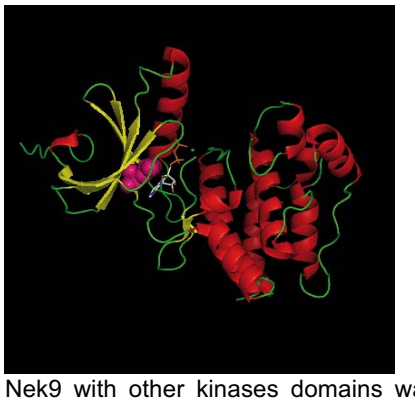


Figure 32. A. Structure-based sequence alignment of Nek9 with other kinases domains was performed using the Kinase Sequence Database (Buzko & Shokat, 2002). In yellow the gate-keeper residue. **B.** Nek2 structure with its gatekeeper residue shown in pink modified from pbd structuture 2w5a. **C.** Nek7 structure with its gatekeeper residue shown in pink modified from the pbd structure 2wqn.

Once we had identified Leucine 128 as the putative gatekeeper residue, we mutated it to a glycine and to an alanine.

We then tested the activity of Nek9 wild type, Nek9[L128A] and Nek9[L128G]. For that, we pre-activated FLAG tagged proteins immunoprecipitated from 293T cells at different times in presence of 100 μM ATP/Mg^{2+}. After washes, H3 was added and Nek9 kinase activity was determined at 10 μM [γ-^{32}P]ATP in presence of Mg^{2+}. As published, under these conditions Nek9 was activated (Roig *et al*, 2002), and the negative control used (Nek9[Ser206A][Thr210A]) had no activity. Regarding Nek9[L128A] and Nek9[L128G], they have catalytic activity and can phosphorylate H3, but in a lesser extend than the wild type (Figure 33). Similar behaviours have been already described in other analog sensitive kinases such as Plk1 (Burkard *et al*, 2007).

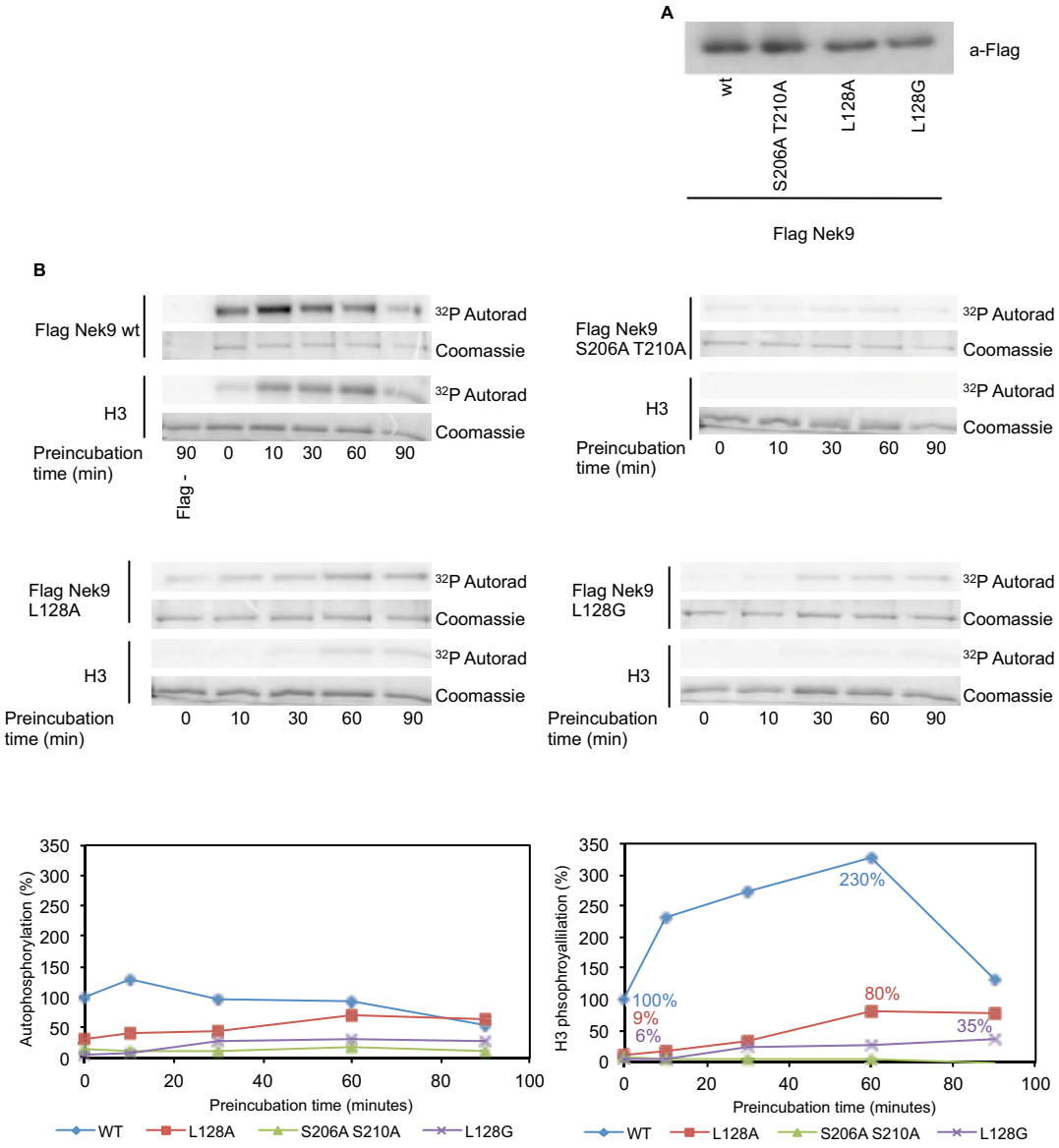


Figure 33. A. CMV5 FLAG mutants were transfected in HEK 293T cells. Expression was detected with an antibody against the FLAG tag. **B.** Different mutants were expressed as in A. After immunoprecipitation Nek9 was activated at different times at 25ºC in phosphorylation buffer in presence of 100 µM ATP/Mg^{2+}. After washes, Nek9 activity was measured by the addition of 10 µM [γ^{32}P] ATP and H3 during 10 minutes at 30ºC. ^{32}P incorporation was stopped by addition of SDS sample buffer followed by SDS-PAGE. Nek9 and H3 were visualized by Coomassie staining, and ^{32}P incorporation was visualized by autoradiograph and quantified with PhosphorImager. Phosphorylation of Nek9 at time 0 minutes was set to 100%. Basal and maximal activity indicated in the chart.

Compensatory mutations

Despite the broad applicability of the chemical genetic approach, a considerable number of kinases, roughly 30% of those tested to date, do not tolerate the mutation of the gatekeeper residue to glycine or alanine. These intolerant kinases have a severe loss of activity when the gatekeeper residue is mutated.

Zang and co-workers described a general method to rescue the activity of these intolerant kinases that consists on doing a compensatory mutation in the amino-terminal lobe of the ATP-binding pocket (Zhang *et al*, 2005). Doing a genetic selection with APH(3')-IIIa, which does not tolerate the mutation of the gatekeeper residue, they identified some residues (*sogg*) in the antiparallel β-sheet of the kinase N-terminal subdomain that were important for the activity of the mutated kinase. Then they performed structure-based sequence alignment with other intolerant kinases and identified these residues. This method can be extended to other kinases that do not tolerate the single space-creating gatekeeper mutation (Figure 34A).

In order to identify the *sogg* residues in Nek9, we performed structure-based sequence analysis using c-src (a tolerant kinase used as a model) with T-Coffee (Di Tommaso *et al*, 2011). The alignment revealed us the existence of some residues that could be good candidates to mutate (V66, G67, V78) (Figure 34B).

A

Secondary structure		β2			β3					β5			
Position in c-Src		283	284	285	295	296	297	298	299	338	339	340	341
Tolerant kinases	c-Src	E	V	W	V	A	I	K	T	Y	I	V	T
	Fyn	E	V	W	V	A	I	K	T	Y	I	V	T
	v-erbB	T	V	Y	V	A	I	K	E	Q	L	I	T
	CDK2	V	V	Y	V	A	L	K	K	Y	L	V	F
	Cdc28	V	V	Y	V	A	L	K	K	Y	L	V	F
	KIN28	V	V	Y	I	A	I	K	E	N	L	V	I
	CaMKIIα	V	V	R	Y	A	A	K	I	Y	L	V	F
	Fus3	V	V	C	V	A	I	K	K	Y	I	I	Q
Intolerant kinases	Cdc5	R	C	F	F	A	A	K	T	Y	I	L	L
	MEKK1	S	C	Y	M	A	V	K	Q	N	L	F	I
	GRK2	E	V	Y	Y	A	M	K	Q	S	E	I	L
	Pto	K	V	Y	V	A	L	K	R	I	L	I	Y
	APH(3')-IIIa	K	V	Y	L	A	L	K	K	N	L	L	M

B

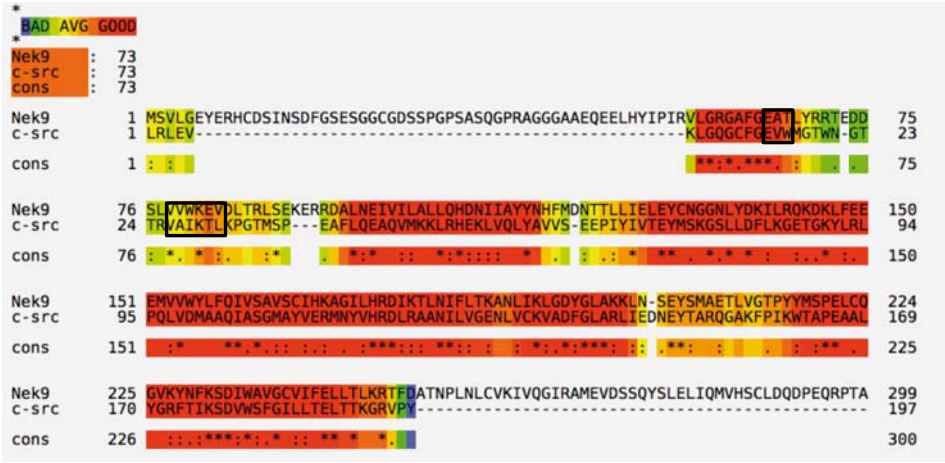


Figure 34. A. Table with the suggested sogg mutations in different kinases. Adapted from Zang et. al, 2005. **B.** Alignment of Nek9 and c-src to find the putative sogg mutations.

We mutated these residues individually, checked for the expression of the mutants (Figure 35A) and performed the same activity assay that has already been described (with an incubation time of 30 minutes).

Figure 35B shows that when we mutate Val78 either in Nek9[L128A] or Nek9[L128G] the activity of the mutant is completely abrogated. Regarding the residue Val66, the mutant Nek9[L128G V66A] is completely inactive, but when this residue is mutated in Nek9[L128A] it has an activity comparable to Nek9[L128A]. Moreover, both mutants Nek9[L128A G67W] and Nek9[L128G G67W] could phosphorylated H3 at a similar extent as Nek9[L128A].

Even though we did not increase the activity of Nek9[L128A] with any of the mutations, we decided to continue our study with the four mutants that showed some activity in this assay, Nek9[L128A], Nek9[L128A V66A], Nek9[L128A G67W], Nek9[L128G G67W].

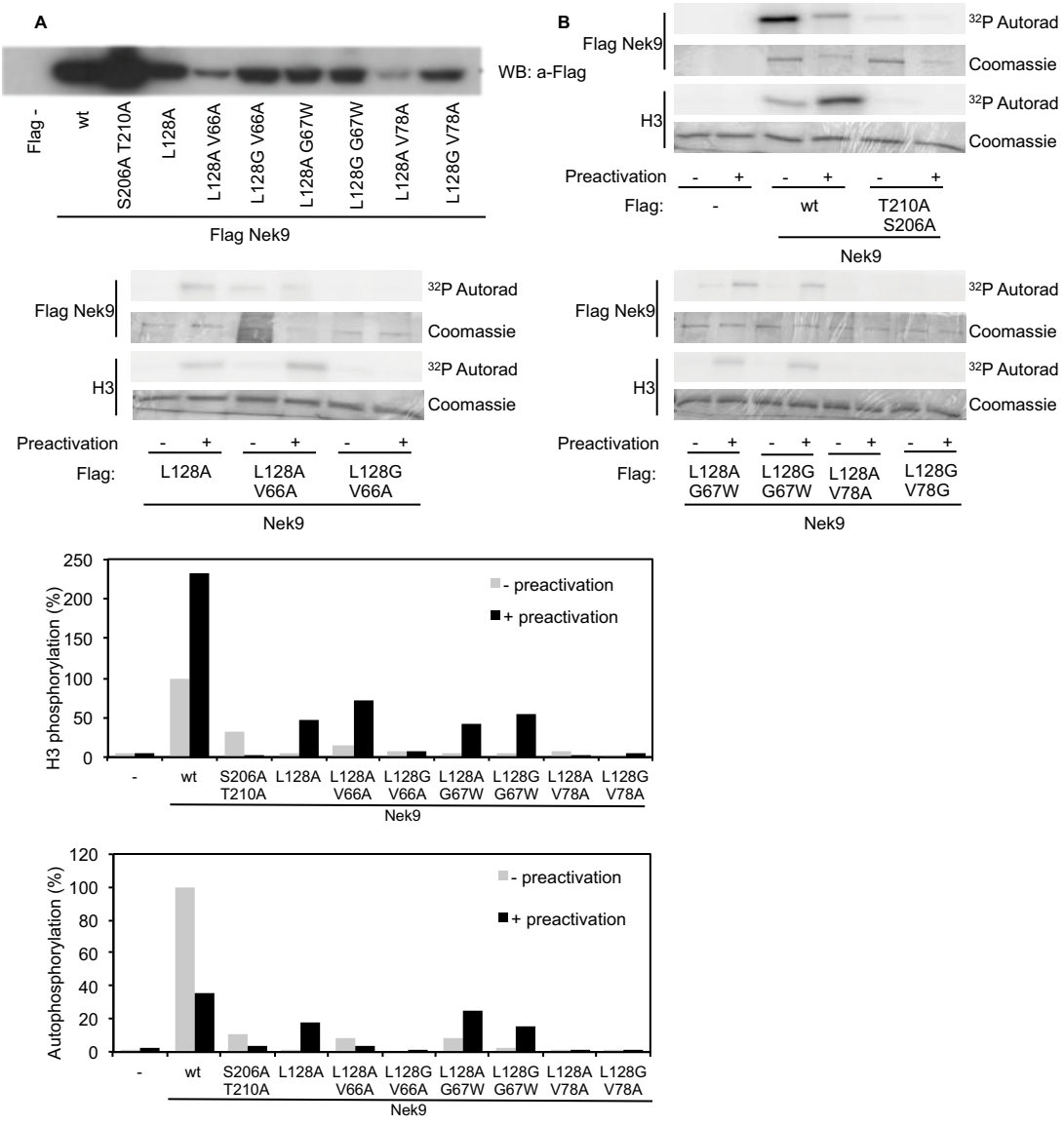


Figure 35. Compensatory mutation activation assays **A.** CMV5 FLAG mutants were transfected in HEK 293T cells. Expression was detected with an antibody against the FLAG tag. **B.** Different mutants were expressed as in (A). After immunoprecipitation Nek9 was activated for 40 minutes at 25ºC in phosphorylation buffer in presence of 100 μM ATP/Mg^{2+}. After washes, Nek9 activity was measured by the addition of 10 μM [γ^{32}P] ATP and H3 during 10 minutes at 30ºC. ^{32}P incorporation was stopped by addition of SDS sample buffer followed by SDS-PAGE. Nek9 and H3 were visualized by Coomassie staining, and ^{32}P incorporation was visualized by autoradiograph and quantified with PhosphorImager. Phosphorylation of Nek9 wt without preacitvation was set to 100%.

Inhibition of the analog sensitive mutant

The chemical molecules used as inhibitors are N6-substituted adenosine molecules (Shah *et al*, 1997) based on the PP1 series of threonine selective kinase inhibitors (Bishop *et al*, 1999), and bind only to kinases with an engineered gatekeeper, and not to the wild type kinases (Figure 36).

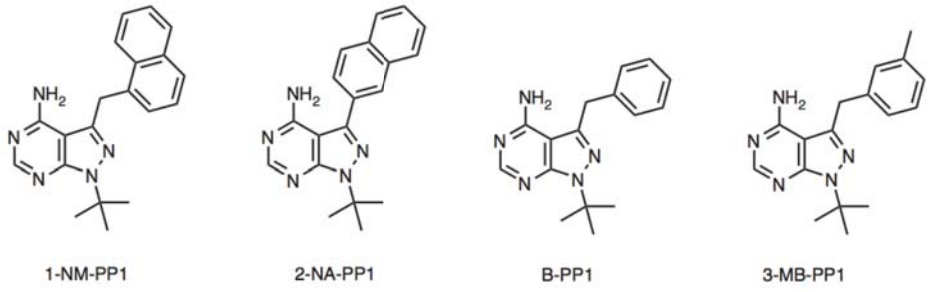


Figure 36. Strutures of the molecules typically used as ATP analog sensitives

We performed Nek9 activation assays with Nek9 wild type and the four mutants that were active, in presence of NM-PP1 to find if some of them could be inhibited by this ATP analog. For that we pre-activated FLAG-Nek9 wild type and the four mutants that showed activity with 100 μM ATP/Mg^{2+} and in presence or absence of NM-PP1 (10 μM). Nek9 activity was measured by the addition 10 μM [γ-^{32}P] ATP and presence or absence of NM-PP1 (10 μM). As expected Nek9 wild type was not inhibited in presence of NM-PP1. Even though three of the mutants (Nek9[L128A], Nek9[L128A V66A], Nek9[L128A G67W]) did not show inhibition in presence of the inhibitor, we were able to inhibit both Nek9[L128G G67W] autophosphorylation and activity towards histone H3 with the ATP analog NM-PP1 (Figure 37).

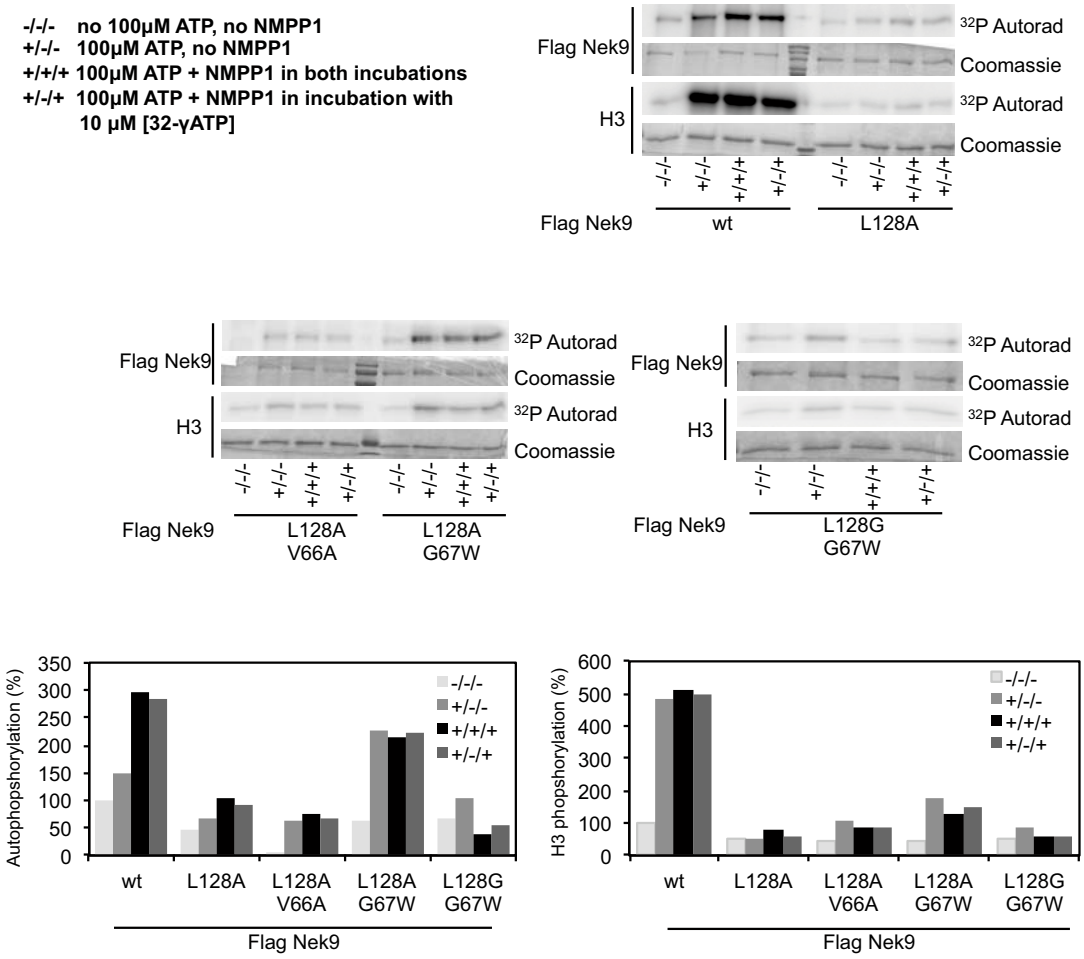


Figure 37. CMV5 FLAG mutants were transfected in HEK 293T cells. After immunoprecipitation Nek9 was activated for 40 minutes at 25ºC in phosphorylation buffer in presence of 100 µM ATP/Mg^{2+} and in presence or absence of NM-PP1 (10µM). After washes, Nek9 activity was measured by the addition of 10 µM [γ-^{32}P] ATP and H3 during 10 minutes at 30ºC with NM-PP1 (10µM). ^{32}P incorporation was stopped by addition of SDS sample buffer followed by SDS-PAGE. Nek9 and H3 were visualized by Coomassie staining, and ^{32}P incorporation was visualized by autoradiograph and quantified with PhosphorImager. Phosphorylation of Nek9 wt without preactivation and with no inhibitor was set to 100%.

Nowadays, different ATP analogs are commercially available, so we wondered if we could have some better inhibition of the mutant Nek9[L128G G67W] using a different inhibitor. For that, we performed Nek9 activation assays (See above for conditions) with different concentrations of two other inhibitors, NA-PP1 and 3MB-PP1, apart from NM-PP1. Figure 38 shows that the three inhibitors do not have any effect on Nek9 wild type activity, but that all of them inhibit the putative analog sensitive mutant. Even though the three inhibitors were able to inhibit the mutant Nek9[L128G G67W], the inhibitor NM-PP1, performed a

stronger inhibition of the kinase, so we decided to use this molecule as inhibitor for the rest of the experiments.

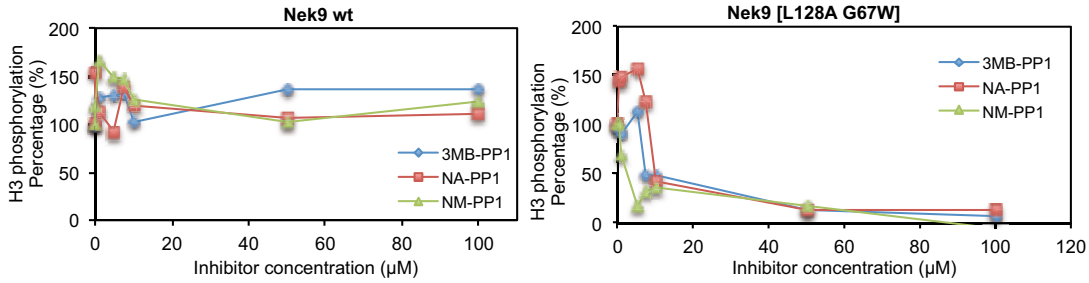


Figure 38. Nek9[L128G G67W] is inhibited with different ATP analogs. CMV5 FLAG mutants were transfected in HEK 293T cells. After immunoprecipitation Nek9 was activated for 30 minutes at 25ºC in phosphorylation buffer in presence of 100 μM ATP/Mg^{2+} and in presence of different concentrations of 3MB-PP1, NA-PP1 or NM-PP1 (0, 0.1, 1,5, 7.5, 10, 50, 100 μM). After washes, Nek9 activity was measured by the addition of 10 μM [γ-^{32}P] ATP and H3 during 10 minutes at 30ºC with the different inhibitors at the same concentration as in the preactivation step. Phosphorylation with 0 μM of inhibitor was set to 100%. ^{32}P incorporation was stopped by addition of SDS sample buffer followed by SDS-PAGE. ^{32}P incorporation was quantified with PhosphorImager.

After doing some more activation analysis of Nek9[L128G G67W] in presence of the ATP analog NM-PP1 to confirm the significant inhibition of the mutant (Figure 39), we could confirm that Nek9[L128G G67W] behaves as an analog sensitive mutant *in vitro*.

A similar project was initiated with Nek6 and Nek7 but we were not successful in getting a mutant that behaved as an analog sensitive mutant.

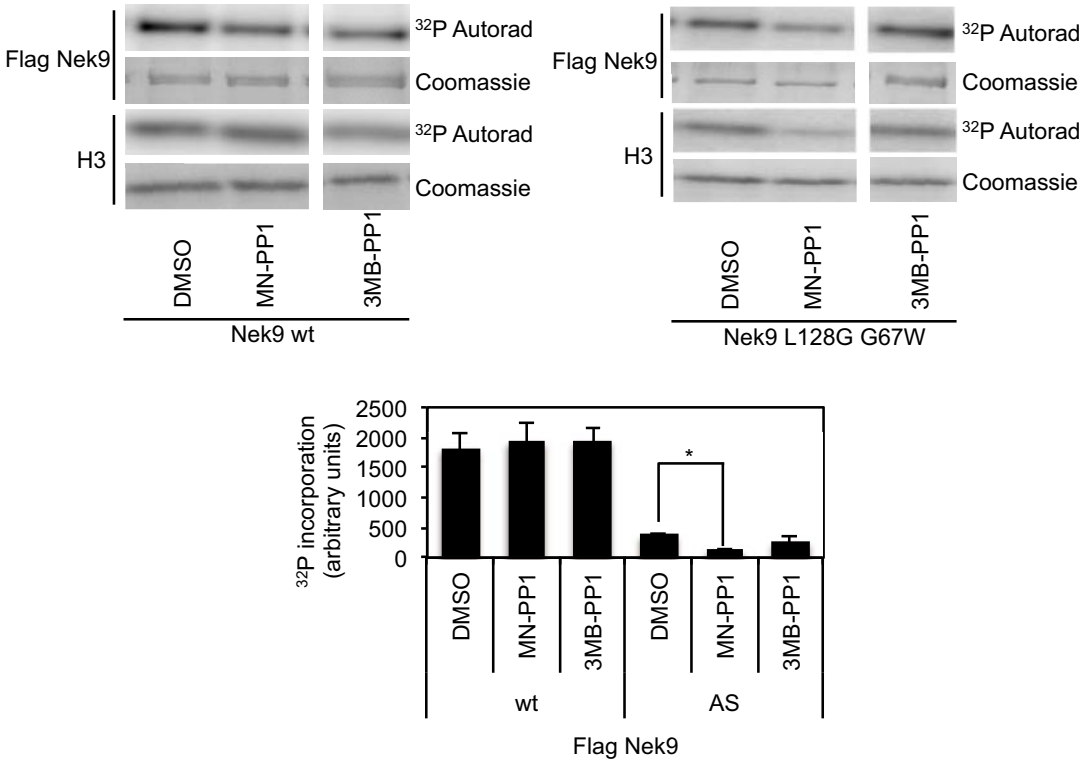


Figure 39. Nek9[L128G G67W] behaves as an analog sensitive mutant *in vitro*. CMV5 FLAG mutants were transfected in Hek 293T cells. After immunoprecipitation Nek9 was activated for 30 minutes at 25ºC in phosphorylation buffer in presence of 100 µM ATP/Mg^{2+} and in presence of different concentrations of 3MB-PP1 or NM-PP1 at 10 µM. After washes, Nek9 activity was measured by the addition of 10 µM [γ-^{32}P] ATP and H3 during 10 minutes at 30ºC with NM-PP1 (10µM) or 3-MB-PP1 (10µM). ^{32}P incorporation was stopped by addition of SDS sample buffer followed by SDS-PAGE. ^{32}P incorporation was quantified with PhosphoImage.

DISCUSSION

Nek9 is activated by Plk1 and this activation is necessary for prophase centrosome separation (See appendix 2, Bertran et. al, 2011)

The NIMA-kinases Nek9, Nek6 and Nek7 form a signalling cassette required for mitotic progression (Belham *et al*, 2003; Roig *et al*, 2002, 2005; O'Regan *et al*, 2007). It has been described that Nek9 is phosphorylated in mitosis and that only a small amount of it (5%) is activated at centrosomes, thus suggesting the existence of distinct and differentially phosphorylated pools of Nek9. Once active, Nek9 phosphorylates and activates Nek6 and Nek7. Nevertheless, the molecular mechanism that drives the activation of Nek9 has remained elusive until the beginning of this work.

By using a mass spectrometry approach we have identified different *in vivo* Nek9 mitotic phosphorylated sites. All the phosphorylated sites found in this analysis had already been reported in different high-throughput phosphorylation assays, and our work confirms their existence *in vivo* (see appendix 1). Apart from three putative Cdk1 phosphorylation sites (Ser29, Ser750, Ser869, all of them with a Pro residue at position +1), we found Thr333 phosphorylated, a site that has been found modified in all the MS analysis performed until now and that we propose is an Aurora A phosphorylation site (see below). We also found two sites phosphorylated, Ser827 (Oppermann *et al*, 2009; Daub *et al*, 2008) and Thr885 (Oppermann *et al*, 2009; Hegemann *et al*, 2011; Daub *et al*, 2008) that are only present in mitosis and that overlap a PXXP domain, which is a putative SH3 domain binding motif (Mayer, 2001), suggesting these sites as possible regulators of the interaction of Nek9 with other yet to be identified proteins. It has to be noted that we did not find phosphorylated Thr210, a residue described to be essential for the activation of Nek9, thus suggesting that the mitotic sample analysed is predominantly a phosphorylated but not active form of the kinase.

The nature of Ser29, Ser750 and Ser869 suggested that Plk1 could bind to Nek9, as most of them conform to a Cdk1 phosphorylation sites but also to a

Polo-Box domain binding site. Indeed, we found that Nek9 and Plk1 interacted in a mitotic specific manner. We were able to confirm this interaction both *in vitro* and *in vivo* mapping it to Plk1 PBD domain, and we could also show that the binding between Nek9 and Plk1 is conserved in mice. In addition, through mutation of the putative binding sites to non phosphorylable residues we showed that the interaction was through the Ser869 of Nek9. Moreover, our group also performed yeast two-hybrid assays, confirming our data and mapping the interaction between the tail domain of Nek9 and the PBD of Plk1 (Bertran *et al,* 2011). Last, during the progress of our work, Nek9 was found as a Plk1 interacting protein in a mass spectrometry assay designed to find Plk1 interactors (Kettenbach *et al*, 2011).

When looking for the conservation of the binding site among species, we found that Ser869 is only conserved in primates, but it is not found in mouse. Ser750, another putative Plk1 binding site, does not play an essential role in human but when it is mutated into a non-phosphorylable residue, the binding between Plk1 and Nek9 loses the mitotic specificity, indicating that it may help in the binding between the two proteins. As Ser750 is conserved in mouse and rat we suggest that this site could be the binding site between Nek9 and Plk1 in rodents (Figure 40).

```
Rat             FTIAATDDNHIFAWGNGGNGRLAMTPTERPHGSDICTSWPRPIFGSLHHVPDLSCRGWHT 718
Mouse           FTIAATDDNHIFAWGNGGNGRLAMTPTERPHGSDICTSWPRPIFGSLHHVPDLSCRGWHT 720
Human           FTIAATDDNHIFAWGNGGNGRLAMTPTERPHGSDICTSWPRPIFGSLHHVPDLSCRGWHT 720
                ************************************************************

Rat             ILIVEKVLNSKTIRSNSSGLSIGTVVQSSSPGGRIGGGGG-------EEEDSQQESETPD 771
Mouse           ILIVEKVLNSKTIRSNSSGLSIGTVVQSSSPGGGIGGGGGGGGGGGGEEEDSQQESETPD 780
Human           ILIVEKVLNSKTIRSNSSGLSIGTVFQSSSPGGGGGGGGG-------EEEDSQQESETPD 773
                ************************.*******  *****       *************

Rat             PSGGFRGTMEADRGMEGLISPTEAVGNSCGASSSCPGWLRKELENAEFIPMPDSPTPLSA 831
Mouse           PSGGFRGTMEADRGMEGLISPTEAVGNSCGASSSCPGWLRKELENAEFIPMPDSPAPLSA 840
Human           PSGGFRGTMEADRGMEGLISPTEAMGNSNGASSSCPGWLRKELENAEFIPMPDSPSPLSA 833
                ************************:*** ************************:****

Rat             AFSQSEKDTLPYEELQGLKVASEVPPEHQPAVGAWPPRLNPAVPCVGK--ALTSPACACS 889
Mouse           AFSQSEKDTLPYEELQGLKVASEVPPEPQRAAGAWPPRLDPAVPCVGK--ALTSAACACS 898
Human           AFSESEKDTLPYEELQGLKVASEAPLEHKPQVEASSPRLNPAVTCAGKGTPLTPPACACS 893
                ***:*******************.* * :  . * .***:***.*.**  .**..*****

Rat             ALQVEVERLQGLVLKCLDEQQKLQQENLQIVTQLQKLNKRLEGGQQVGMHSRGTQTAKED 949
Mouse           ALQVEVDRLQALVLKCLEEQQKLQQENLQMFTQLQKLNKKLEGGQQVGMHSRGTQTAKEE 958
Human           SLQVEVERLQGLVLKCLAEQQKLQQENLQIFTQLQKLNKKLEGGQQVGMHSKGTQTAKEE 953
                :*****:***.****** ***********:.********:***********:*******:

Rat             MEMDPKPDLDSDSWCLLGTDSCRPSL 975
Mouse           MEMDPKPDLDSESWCLLGTDSCRPSL 984
Human           MEMDPKPDLDSDSWCLLGTDSCRPSL 979
                ***********:**************
```

Figure 40. Alignment between human, rat and mouse Nek9. It is shown in red human Ser869 and in green Ser750

The binding between Nek9 and Plk1 takes place exclusively in mitosis between the Polo-Box domain of Plk1 and the Ser869 of Nek9. Ser869 is a putative Cdk1 phosphorylation site, and it is phosphorylated in mitosis, so the binding between the two proteins shows Nek9 as a classical Plk1 binding partner, which we propose is first phosphorylated by Cdk1, allowing this phosphorylation the binding to Plk1 (Lowery *et al*, 2004, 2005). Binding of both kinases through the Ser869 of Nek9 may be important for normal cell growth, because it has been found that Pro870 is mutated to a serine in lung neuroendocrine carcinoma (Greenman *et al*, 2007). With this mutation Cdk1 would not be able to recognize the site as a phosphorylation site, so the binding between Nek9 and Plk1 may be impaired in these tumours.

Regarding the activation mechanism of Nek9, it has been described that phosphorylation of the Thr210 is a crucial step for the activation of Nek9 and that only a small amount (5%) of the kinase is activated at centrosomes during mitosis (Roig *et al*, 2005). In addition, Nek9 can autophosphorylate *in vitro* at subcellular

ATP concentration leading to the autophosphorylation of the Thr210 and further activation of the kinase (Roig *et al*, 2002). Nek9 is thus inactive in interphase in concentrations of ATP that would support its activation *in vitro*, suggesting that mechanisms are in place that either inactivate the kinase (i.e. by dephosphorylation) or impede its activation (i.e. binding of the activation loop to the autoinhibitory RCC1 domain). It has remained elusive until now how these mechanisms are overturned in mitosis, and whether some other kinase could be also responsible of the phosphorylation of Thr210. With the use of two different inactive mutants (Nek9[K81M] and Nek9[D176A]) we have shown that Plk1 is able to directly phosphorylate Nek9 at its activation loop at Thr210. Moreover, we propose a mechanism in which Nek9 is first phosphorylated by Cdk1 at the Ser869 thus allowing the binding and subsequent phosphorylation of Plk1 directly to the activation loop (Thr210), connecting three of the main mitotic kinase families and suggesting a complete mechanism of activation of Nek9 that could be complemented by Nek9 autophosphorylation in order to increase kinase activation locally.

Surprisingly, Nek9 does not need to be primed by Cdk1 to be phosphorylated by Plk1 *in vitro*. Nevertheless, both kinases are necessary for its activation *in* vivo. This behaviour has already been observed in other Plk1 substrates such as TCTP (Yarm, 2002), Myt1 (Nakajima, 2003), NudC (Zhou *et al*, 2003) or Hbo1 (Wu & Liu, 2008), suggesting that priming phosphorylation is not absolutely required under conditions of high substrate and enzyme concentration.

Nek9 is phosphorylared *in vivo* in a Plk1 dependent manner (Apendix 1) (Santamaria *et al*, 2011; Kettenbach *et al*, 2012, 2011), and besides Thr210, *in silico* analysis of the sequence of Nek9 showed that it contains additional sites with the putative Plk1 phosphorylation motif [DE]X[ST]ϕX[DE] (Nakajima *et al*, 2003), [DNE]X[ST][Fϕ: noP][ϕX] (Alexander *et al*, 2011), where ϕ indicates hydrophobic residue and X indicates any residue. In order to find whether these sites were phosphorylated by Plk1 we performed a MS analysis of inactive form of Nek9 (Nek9[K81M]), incubated with or without Plk1 in presence of ATP/Mg^{2+}. Our mass spectrometry assay had a coverage of 53.45% of the protein and some

of the putative Plk1 phosphorylation sites were not covered (Thr438, Thr544, Thr662), so we could not conclude if they were phosphorylated by Plk1. Nevertheless, we found phosphorylated two putative Plk1 phosphorylation sites (Thr67 and Thr662), among other sites with unknown significance and that do not conform to a putative Plk1 phosphorylation site.

Thorness and Koshland demonstrated that substituting a phosphorylated residue by an acidic one is a very useful tool to mimic phosphorylation of a particular site (Thorness & Koshland, 1987). This is based on the fact that when phosphorylable residues (Ser, Thr and Tyr) are phosphorylated they have very similar chemical properties to negatively charged aminoacids (Asp and Glu). Nevertheless, sometimes more mutations are needed and even in some cases it has been shown that the phosphomimetic mutant behaves as the unphosphorylated form (Pearlman *et al*, 2011). To find if the phosphorylation of some of the candidate Plk1 phosphorylation sites were responsible of the activation of Nek9 we mutated each residue into an acidic residue and checked for the phosphorylation at the Thr210 with the anti-Nek9[Thr210-P] antibody.

Some of the *in silico* identified sites had already been found in phosphoproteomic analysis, such as the Thr76 (Daub *et al*, 2008), which was described as a Plk1 phosphorylation site (Appendix 1). In spite of that, we do not see any effect of its mutation to a phosphomimetic residue in the activation state of Nek9. After immunoprecipitation of phosphomimetic mutants and further western blot with the a-Nek9[Thr210-P] antibody we showed that Nek9[T438E], Nek9[T544E] and Nek9[T662E] may lead to the phosphorylation of the Thr210. These putative Plk1 phosphorylation sites are located in the RCC1 domain, which acts as an autoinhibitory domain. This result, although preliminary, suggest a mechanism in which Plk1 would phosphorylate some of these residues in the RCC1 domain, and as a consequence of some or a combination of these modifications the kinase domain would be released thus allowing the phosphorylation of the Thr210 and activation by Plk1 (Figure 41). Nevertheless, additional experiments will be needed to settle the molecular details responsible for Nek9 activation.

Our group is presently trying to determine Nek9 tridimensional structure of Nek9, so to unravel the structure of the protein will be crucial to confirm the importance of these and other residues in the binding between the kinase and the RCC1 domain.

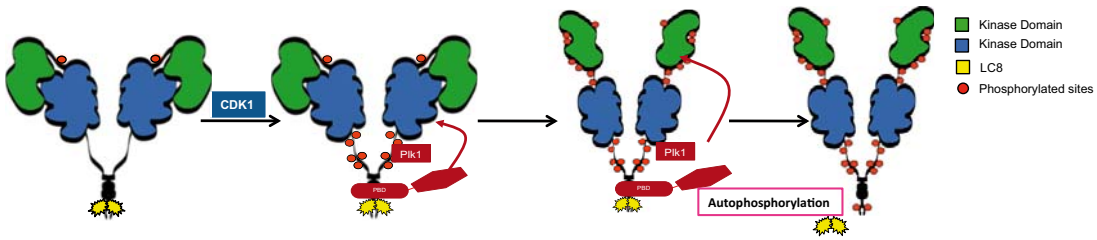


Figure 41. Proposed mechanism for Nek9 activation by Plk1

In summary, we propose a Nek9 activation mechanism that consists on a first phosphorylation by Cdk1 at Ser869 which would allow the binding of the PBD of Plk1. Once bound, Plk1 would phosphorylate Nek9 at the RCC1 domain, thus releasing the kinase domain. Then, the Thr210 would be accessible, would be phosphorylated by Plk1 and Nek9 would be activated. Once active, Nek9 may be able to autophosphorylate what would cause an amplification of the activation.

Concerning Nek9 functions, it has been described that antibody microinjection of Nek9 in Ptk2 cells causes prometaphase arrest and abnormalities in chromosome segregation (Roig *et al*, 2002). In addition, we have demonstrated that when Nek9 is downregulated by RNAi in HeLa cells prophase centrosome separation is impaired (Bertran *et al*, 2011) (Figure 42A).

Plk1 is implicated in spindle assembly (Petronczki *et al*, 2008), contributes to accumulation of γ-tubulin at centrosomes (Lane & Nigg, 1996), and its inhibition leads to monopolar spindle formation (Sumara *et al*, 2004) with unseparated and immature centrosomes, though the molecular mechanism that links Plk1 with the separation or maturation of centrosomes remained elusive. Our group could show that active Nek9 and Nek6 can overcome prophase centrosome separation when Plk1 is inhibited, demonstrating that Nek9 activation by Plk1 is necessary

for prophase centrosome separation, and thus shedding light on the relationship between Plk1 and centrosome separation (Figure 42B).

Centrosome separation is mainly driven by the action of mitotic motors. Our group and Smith and co-workers described that Eg5 and Plk1 are necessary for centrosome separation in G2 phase in a Cdk1 independent manner (Bertran *et al,*2011;Smith *et al*, 2011). Nevertheless, when Cdk1 is inhibited in synchronised cells centrosome separation is delayed (Smith *et al*, 2011). Cdk1 phosphorylates Eg5 at the Thr926, and this phosphorylation is necessary for binding of the kinesin to microtubules (Cahu *et al*, 2008; Blangy *et al*, 1998). Both, Plk1 and Cdk1 contribute to the enrichment of Eg5 at centrosomes but Plk1 does not directly phosphorylate Eg5 (Mardin *et al*, 2011), thus the link between Plk1 and Eg5 remained elusive.

We showed that Nek9 activation is necessary for prophase centrosome separation and we have already published that when Nek9 is activated, it activates Nek6 that in turn phosphorylates the kinesin Eg5 at Ser1033. The phosphorylation of this site is important for mitotic progression (Rapley *et al*, 2008).

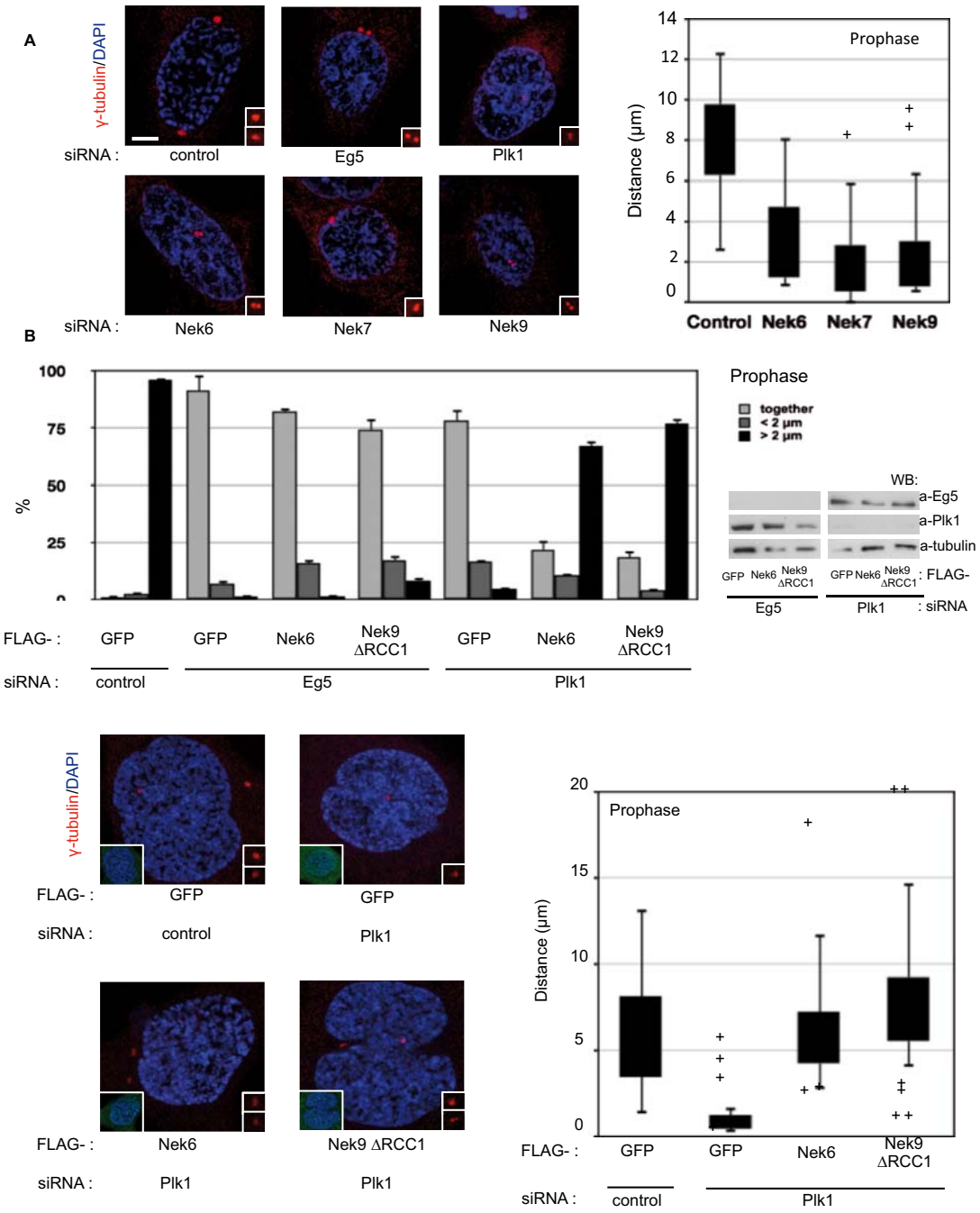


Figure 42. A. Nek9 is necessary for centrosome separation in prophase. Hela cells were transfected with the indicated RNAis. Fixed and stained with the indicated antibodies. The distribution of distances from the centre of the duplicated centrosomes in each case is shown as a box plot. Representative examples of the observed phenotypes are shown (bar, 5 μm). In each case, insets show magnified centrosomes. **B.** Active Nek9 and Nek6 can rescue Plk1 but not Eg5 downregulation in prophase centrosome separation. HeLa cells were transfected with the indicated RNAi and plasmids. The percentage of FLAG-positive prophase cells showing two unseparated centrosomes (together), two centrosomes separated < 2 μm is shown in the upper graphic (mean±s.e.m. of three independent experiments;B 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) and a box plot of the distribution of distances from the centre of the duplicated centrosomes in FLAG-positive cells. Modified from Bertran et al, 2011.

Taking all these data together with the results presented in this work, our group asked whether the link between Plk1, Eg5 and centrosome separation could be Nek9 and we demonstrate that Plk1 phosphorylation and activation of Nek9 through the phosphorylation of Thr210 is necessary for the phosphorylation of Eg5 at Ser1033. Moreover, results in the laboratory show that this phosphorylation is required for Eg5 centrosomal localization and prophase centrosome separation (Bertran *et al,* 2011). So with these experiments, we could show that the protein that links Plk1, Eg5 and centrosome separation is the mitotic kinase Nek9. The existence of two different centrosome separation pathways (prophase and prometaphase centrosome separation) was thought to be a part of the high redundancy that exists in spindle formation. However, it is important to highlight that it has been described that incomplete centrosome separation at NEB is associated with elevated frequencies of anaphase lagging chromosomes and merotelic kinetochore attachment, thus suggesting that both pathways may not be redundant and that prometaphase pathway could be a kind of backup system to ensure progression through mitosis in spite of the potential risk (Silkworth *et al*, 2011). Indeed, Nek9 has shown to be involved in the control of prophase centrosome separation, so these results indicate that an aberrant activation of Nek9 might lead to a similar phenotype as the one described in Slikworth *et al.* and consequently chromosomes would not segregate properly and cells may present aneuploidy.

Besides controlling the separation of centrosomes, Plk1 is also involved in the regulation of centrosome maturation, where it is important for the recruitment of γ-tubulin in a manner dependent of the adaptor protein Nedd1 (Lüders *et al*, 2006) (Haren *et al*, 2009). Results from our group have now established that this recruitment is dependent on Nek9 activation. We have shown that the activation of Nek9 by Plk1 is a crucial step for the recruitment of γ-tubulin and Nedd1 to the centrosomes in prometaphase (Sdelci *et al*, 2012), thus presenting another Plk1 function that goes through the activity of the NIMA kinase Nek9 (Figure 43).

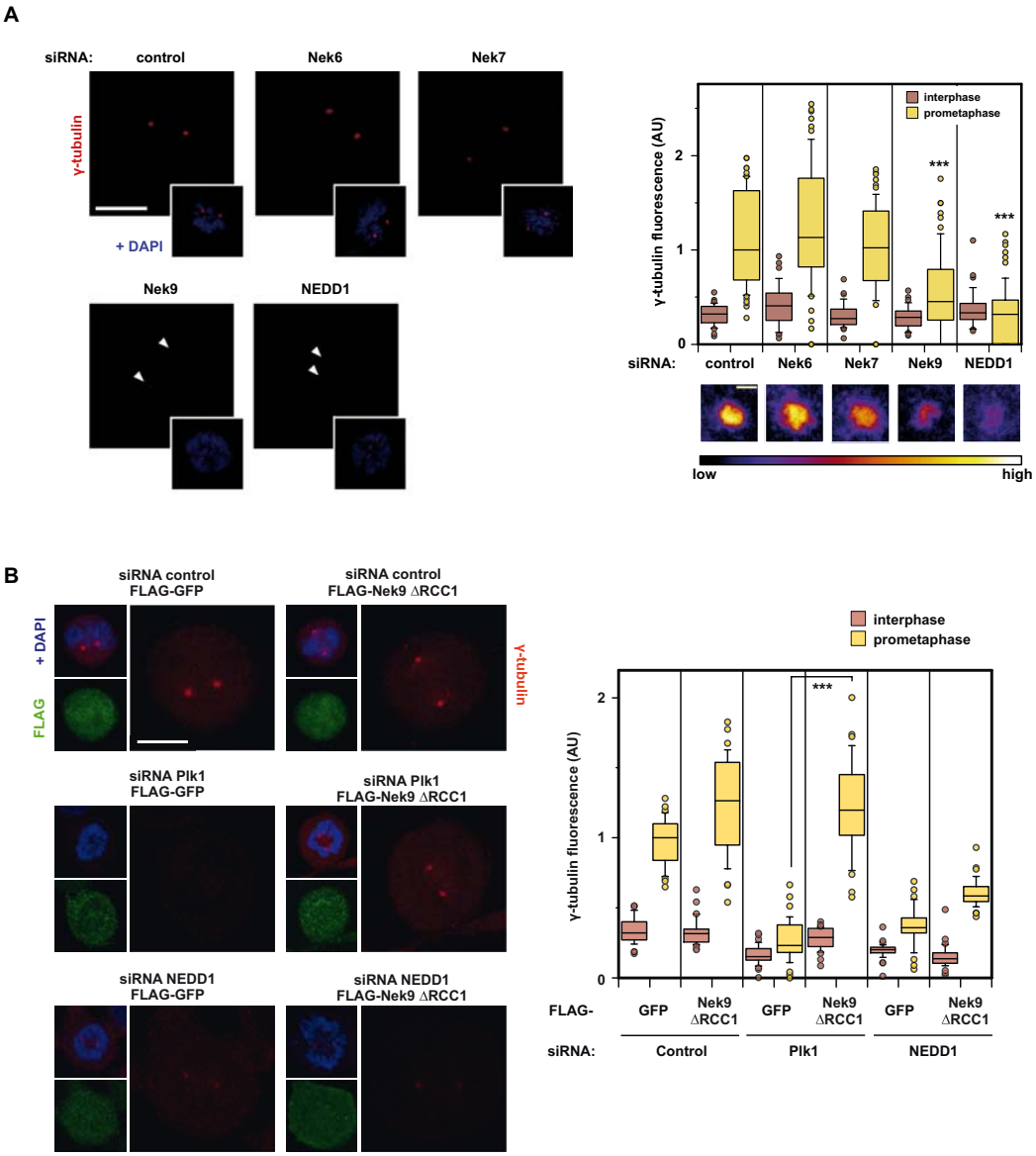


Figure 43. A. Nek9 downstream of Plk1 and upstream of Nedd1 is necessary for centrosome maturation in prometaphase. Effect of different siRNA transfections on μ-tubulin (A) contents in interphase and prometaphase HeLa cells. Representative examples of the observed phenotypes in prometaphase are shown (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 mm). **B.** Nek9 Is Downstream of Plk1 and Upstream of NEDD1 in the Control of γ-Tubulin Recruitment to the Mitotic Centrosomes. 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 g-tubulin intensities is shown as a box plot (three independent experiments, 20 cells counted for each experimental condition of each experiment). Modified from Sdelci et al 2012.

To summarise, our work describing that Nek9 is activated by a two-step mechanism involving Cdk1 and Plk1has allowed to functionally relate Nek9 with Cdk1 and Plk1, and suggested that Nek9 could perform some of the mitotic functions of Plk1, highlighting Nek9 as a key player for mitotic progression.

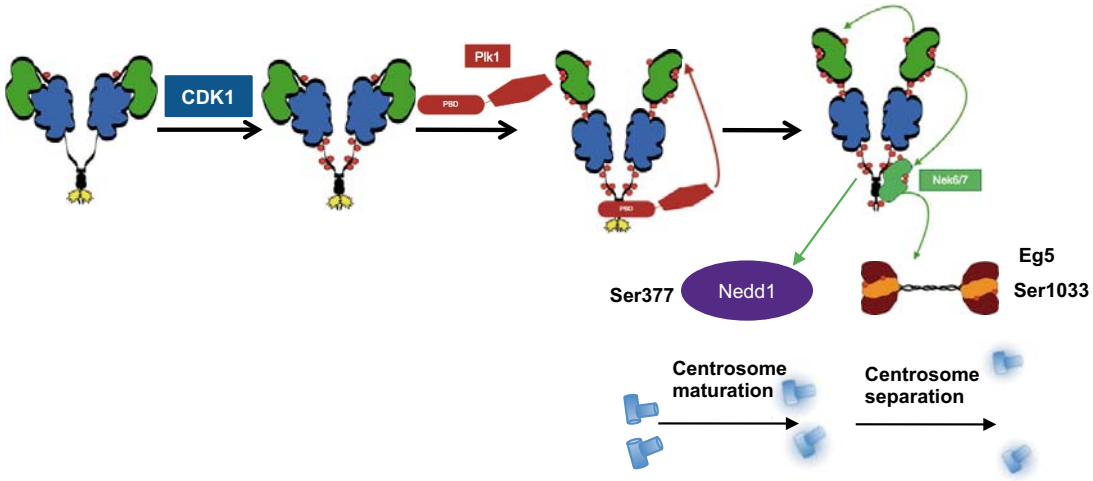


Figure 44. Schematic representation of the two steps Nek9 activation mechanism and the biological consequences of this activation.

Active Nek9 can partially overcome the downregulation of Plk1

Plk1 is overexpressed in a wide array of tumours (Eckerdt *et al*, 2005), and it appears to be of prognosis value in some of them (McInnes & Wyatt, 2011). During the last years, Plk1 has become a potential target for cancer treatment, and different inhibitors of this kinase have been used in clinical trials (Lens *et al*, 2010).

Moreover, Nek9 is also overexpressed in some tumours and has been reported as a putative potential therapeutic target for head and neck squamous cell carcinoma (Wu *et al*, 2011).

After situating Nek9 downstream of Plk1, our group has studied whether some of Plk1 functions could be performed by Nek9, by overexpressing the active form of Nek9, (FLAG-Nek9[Δ346–732]) in cells were we previously depleted Plk1, showing that this is the case for both centrosome separation (Bertran *et al*, 2011) and γ-tubulin centrosomal recruitment (Sdelci *et al*, 2012). We thus decided to inquire whether active overexpressed Nek9 could also compensate for Plk1 in other functions during mitotic progression. Our preliminary results using FACS show that some cells with overexpressed active Nek9 can escape from the mitotic arrest caused by the inhibition of Plk1. These results could be confirmed either by using Plk1 RNAi or the specific inhibitor BI2536. As can be seen in the cell cycle profiles, overexpression of Nek9[Δ346–732] alone does not have any effect in these profiles. Nevertheless, when it is expressed in cells with inhibited Plk1, a significant percentage of these cells do not get arrested in mitosis. These results have been confirmed with our results using time-lapse microscopy, where we could corroborate that cells with inhibited Plk1 but active Nek9 enter mitosis and can finish it.

These results, although somehow preliminary, suggest that, in agreement with our published work (Bertran *et al,* 2011, Sdelci *et al*, 2012) some of the functions done by Plk1 are implemented through Nek9, and that overexpressed active

Nek9 can partially overcome the mitotic arrest produced by the inhibition of Plk1. Nevertheless, even though cells with downregulated Plk1 and active Nek9 divide, we could observe that the time spent in mitosis is significantly longer than the average (60 minutes aprox) (not shown) (Brito & Rieder, 2009). In addition, we have to take into account how these mitosis take place. As shown in Figure 45, our preliminary results show that some of the cells with inhibited Plk1 but with active Nek9 divide, but in an aberrant manner. We can observe the presence of micronuclei in some cells, which can mean that the two daughter cells may have an aberrant number of chromosomes, and thus be aneuploid. In order to confirm this hypothesis, further experiments need to be performed, such as FISH experiments to show whether daughter cells present aneuploidy.

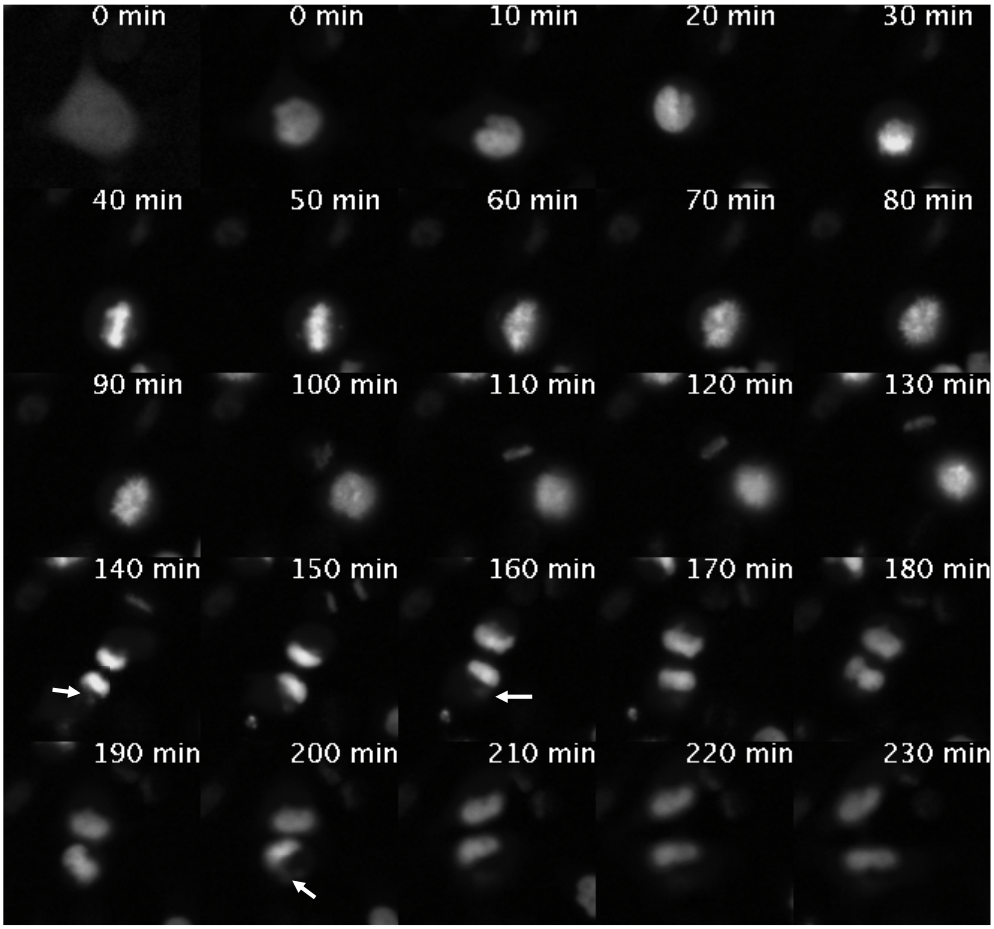


Figure 45. Stills from HeLa cells transfected with Plk1 RNAi, GFP-Nek9ΔRCC1 and mCherry-H2B. The first frame shows that cell was transfected with the indicated green plasmid. Other frames show the DNA of the cell.

Plk1 has different functions thorough the cell cycle (Petronczki *et al*, 2008) an it has been reported that its inhibition results in a wide array of defects ending in a prometaphase arrest due to the non-satisfaction of the SAC (Spindle Assembly Checkpoint) (Sumara *et al*, 2004, Lénárt *et al*, 2007). Here we report that active Nek9 can somehow overcome this arrest and cells can finish mitosis. Due to the high number of substrates described for Plk1 in all mitotic phases and the different phosphorylation motifs described for either Plk1 or NIMA (nor Nek9 phosphorylation consensus motif has been described) it is difficult to rationalize how Nek9 could be phosphorylating all the described substrates of Plk1 or how it could be doing all the described functions of Plk1. It is more plausible that some of the functions described for Plk1 are done through Nek9, while others, although necessary for a normal mitosis, could be dispensable in some cases for the completion of this process.

Regarding the individual functions of Plk1 and the consequences of its inhibition, several reports suggest that Plk1 activity is not fully necessary for the entrance of mitosis, even thought cells with inhibited Plk1 had a delay in the entrance of mitosis as a result of its role during Cdk1 activation (Sumara *et al,* 2004). Once cells enter mitosis, Plk1 is required for centrosome separation in prophase and centrosome maturation and we have already shown that Plk1 performs these functions through the activation of Nek9. During latter stages of mitosis, Plk1 localizes at kinetochores and Nek9 at the chromosomes. Nevertheless, none of the Nek9 function has been described at these stages of mitosis. Plk1 at kinetochores phosphorylates different substrates essential for the microtubule-kinetochore interactions and when it is inhibited, and cannot phosphorylate these substrates, attachments are not correctly formed, there is not an aligned metaphase plate and as a consequence aneuploidy can take place. As can be seen in Figure 45, we could not see a correct metaphase plate and we also see the formation of micronuclei suggesting an incorrect segregation of the chromosomes, thus suggesting that the mentioned Plk1 functions at kinetochores may not rely on Nek9 and that cells that go through mitosis without Plk1 activity as a result may need to overcome SAC, align somehow

chromosomes, and segregate them, which due to the inefficacy of the process could result in aneuploidy.

Finally, both proteins localize at the midbody in cytokinesis, so we also wondered whether some cytokinetic functions of Plk1 could be done through Nek9. Our preliminary results suggest that cytokinesis takes place properly in cells with depleted Plk1 and overexpressed active Nek9, indicating that cytokinetic functions of Plk1 could be done at least in part through Nek9. In order to confirm these possible functions of Nek9 additional experiments need to be performed. We hope that our newly described analogue sensitive Nek9 forms will help us in unravelling the putative roles of Nek9 at this mitotic phase.

We also have tot take into account that these results come from experiments with overexpressed levels of Nek9, and probably some of the effects shown may be related to this higher protein and activity levels. To corroborate the veracity of the results we present, experiments with transfected endogenous levels of active protein will need to be performed.

On the whole, these results could be of therapeutic interest as, in a tumour where both proteins are overexpressed, a treatment to inhibit Plk1 would not be sufficient for tumour growth suppression. We thus believe it is worth continuing this research.

Nek9 is an Aurora A substrate

Aurora A localizes to centrosomes and is required for centrosome maturation and spindle formation in *C. elegans* (Hannak *et al*, 2001) *D. melanogaster* (Glover *et al*, 1995; Berdnik & Knoblich, 2002) and humans (Terada *et al*, 2003) and its inhibition leads to the formation of monopolar spindles .

Moreover, there is a high percentage of cells with monopolar spindles when Aurora A is inhibited either with RNAi (Marumoto *et al*, 2003) or chemical inhibitors (Girdler *et al*, 2006) in human cells. Nevertheless, the phenotype is not always so severe as some studies made in *Xenopus laevis* or *C. elegans* show that cells do present bipolar disorganized spindles (Peset *et al*, 2005; Hannak *et al*, 2001). This results are parallel with the fact that *C. elegans* Aurora A is not required for centrosome separation but is necessary to prevent spindle collapse after NEBD, suggesting a role of Aurora A in centrosome separation only after NEBD in this system (Hannak *et al*, 2001).

We demonstrate that Aurora A phosphorylates Nek9 at the RCC1 domain and at the C-terminal tail domain. To map these phosphorylations we used two different approaches, the phosphorylation of different Nek9 fragments expressed in bacteria and the phosphorylation of full length Nek9 and further identification of phosphorylated sites by mass spectrometry. Regarding the first approach, it allowed us to identify Ser978, located at the tail domain of Nek9 as an Aurora A phosphorylation site. Ser978 conforms to a putative Aurora A phosphorylation motif R[RK]X[ST][F;noP] (Alexander *et al*, 2011), had already been found phosphorylated in two phosphoproteomic analysis, and Daub and co-workers found it as a site phosphorylated by Aurora A in mitosis (Apendix 1) (Oppermann *et al*, 2009; Daub *et al*, 2008).

Regarding the in vitro mass spectrometry approach of Nek9 phosphorylated by Aurora A presented here, we had a coverage of 50% in the analysis, and did not find phosphorylated the Ser978, even though the peptide was covered,

suggesting that its phosphorylation by Aurora may depend on additional factors or be infrequent in the conditions used. Nevertheless, we identified Thr333 as an Aurora A phosphorylation site. This residue has been found phosphorylated in all mass spectrometry analysis that have been performed either in exponentially growing or in mitosis arrested cells, but its modification is more abundant in mitosis (9-fold), indicating that the phosphorylation of this site is also regulated in a cell cycle manner. Actually, these results indicate again the putative existence of different pools of Nek9, which could be differently phosphorylated depending on whether it has to be activated or not.

In addition, phosphorylation of both sites was shown to be sensitive to treatment with Aurora A inhibitors (Kettenbach *et al*, 2011). Altogether all this data suggests that Ser978 and Thr333 are *bona fide* Aurora A site in Nek9.

Now, it remains to be clarified which are the biological functions of these phosphorylations. In order to clarify this we could downregulate Nek9 with RNAi (or alternatively take advantage of the Nek9 KO mice we are producing in the laboratory) and reconstitute these cells either with wild type Nek9 or with the mutated forms that are phosphorylated by Aurora A and check whether some of the functions described for Aurora A, such as centrosome maturation or separation, are impaired.

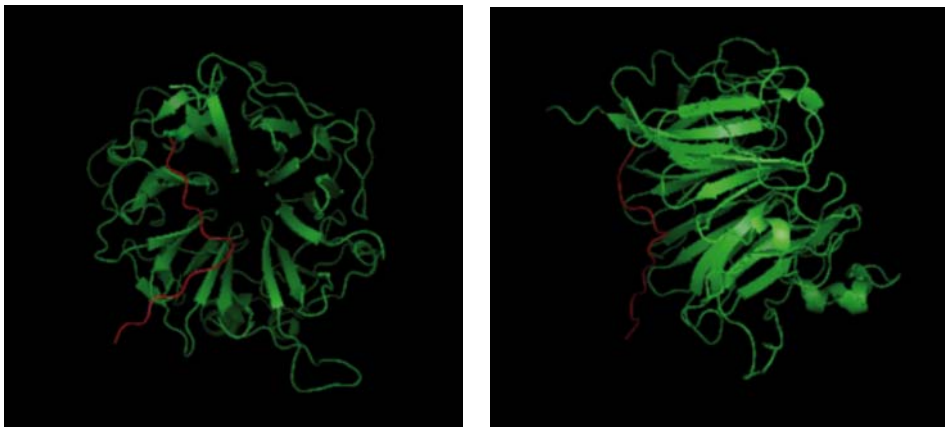
Aurora A is also necessary for the localization of some of its substrates to the centrosome. One of these described substrates is Ndel1, which is not localized to centrosomes when Aurora A cannot phosphorylate it (Mori *et al*, 2007). Here we describe that the phosphorylation of Aurora A is necessary for the localization of Nek9[Thr210-P] to the centrosomes, suggesting a parallel role as the described for Ndel1. However, it remains to be explained whether it is really required for the localization or if it is a localized activation. To find whether Nek9 is first activated and then localized to centrosomes or if it is located to the centrosomes before activation, we could produce phospho-antibodies against Aurora A phosphorylation sites and check whether this sites are phosphorylated before or after the phosphorylation of Thr210

Moreover, in order to know whether Aurora A is the responsible for the centrosomal localization of active Nek9, we could substitute endogenous Nek9 with non phosphorylable mutants of Aurora A phosphorylation sites and check, using the anti-Nek9[Thr210-P] antibody, if when these sites are mutated active Nek9 localizes properly at the centrosomes.

A possible molecular mechanism of how these phosphorylations occur and the possible consequences would be the following. At G2, Aurora A is activated and it is known that it activates Plk1 (Seki *et al*, 2008, Macurek *et al*, 2008). In parallel, Aurora A would also phosphorylate Nek9 and this phosphorylation would cause the recruitment of a pool of Nek9 to centrosomes, where it would be phosphorylated by Cdk1 and activated by Plk1, both active at these organelles. Thus our results suggest the existence of a molecular network with the four main families of mitotic kinases. The fact that Nek9 would need such intricate activation mechanism with the collaboration of the three most important mitotic kinases indicates that Nek9 is a key player of mitosis and that its activation is crucial for its correct progress.

Alternatively (or complimentarily), we can propose another putative function of the phosphorylation of Aurora A to the Thr333 of Nek9. Thr333 is located in the hinge between the kinase and the RCC1 domain of Nek9. This fragment of Nek9 could structurally resemble the N-terminal domain of human RCC1 that is folded on the top of the β-propeller (Figure 46). Moreover, alignment of Nek9 RCC1 domain with human RCC1 shows that some of the residues in this protein fragment are conserved between the two proteins strengthening this hypothesis. This suggests that the inhibitory interaction between the kinase and the RCC1 domain of Nek9 could be through this hinge between both domains. In mitosis, this link could be phosphorylated by Aurora A at Thr333 that would cause a conformational change that would help in the release of the kinase domain thus allowing further phosphorylation and activation by Plk1.

A



B

```
Nek9            ELLDRPLLRKRRREMEEKVTLLNAPTKRPRSSTVTEAPIAVVTSRTSEVYVWGGGKSTPQ 360
RCC1            --MSPKRIAKRRSPPADAIPKSKKVKVSHRSHSTEPGLVLTLGQGDVGQLGLGENVMERK 58
                  :.   : ***    : :.  :  .   ** :.  . : .: .        * .    :

Nek9            KLDVIKSGCSARQVCAGNTHFAVVTVEKELYTWVNMQGGTKLHGQLGHGDKASYRQPKHV 420
RCC1            KPALVSIPEDVVQAEAGGMHTVCLSKSGQVYSFG-----CNDEGALGRDTSVEGSEMVPG 113
                *  ::.   .. *. **. * . :: . ::*::       : .* **:. ...  :

Nek9            EKLQGKAIRQVSCGDDFTVCVTDEGQLYAFGSDYYGCMGVDKVAGPEVLEPMQLNFFLSN 480
RCC1            KVELQEKVVQVSAGDSHTAALTDDGRVFLWG-SFRDNNGVIGLLEPMKKSMVPVQVQLDV 172
                :    : : ***.**..*..:**:*::: :* .: .  **  :  *   . : ::. *.

Nek9            PVEQVSCGDNHVVVLTRNKEVYSWGCGEYGRLGLDSEEDYYTPQKVDVPKALIIVAVQCG 540
RCC1            PVVKVASGNDHLVMLTADGDLYTLGCGEQGQLGRVPELFANRGGRQGLERLLVPKCVMLK 232
                ** :*:.*::*:*:** : ::*: **** *:**  .*       : .: : *:  .*

Nek9            CDGTFLLTQSGKVLACGLNEFNKLGLNQCMSGIINHEAYHEVPYTTSFTLAKQLSFYKIR 600
RCC1            SRGSRGHVRFQDAFCGAYFTFAISHEGHVYGFGLSNYHQLGTPGTESCFIPQNLTSFKNS 292
                . *:   .:  ..:. .   *     .:  .  :.:     .* * *  :.::*: :*

Nek9            T-----IAPGKTHTAAIDERGRLLTFGCNKCGQLGVGNYKKRLGINLLGGPLGGKQVIRV 655
RCC1            TKSWVGFSGGQHHTVCMDSEGKAYSLGRAEYGRLGLGEGAEEKSIPTLISRLP--AVSSV 350
                *     :: *: **..:*..*:  ::*  : *:**:*:  :. .*  * . *    *  *

Nek9            SCGDEFTIAATDDNHIFAWGNGGNGRLAMTPTERPHGSDICTSWPRPIFGSLHHVPDLSC 715
RCC1            ACGASVGYAVTKDGRVFAWGMGTNYQLGTGQDE--------------------------- 383
                :** ..  *.*.*.::**** * * :*.    *

Nek9            RGWHTILIVEKVLNSKTIRSNSSGLSIGTVFQSSSPGGGGGGGGGEEEDSQQESETPDPS 775
RCC1            DAWSPVEMMGKQLENRVVLSVSSGGQHTVLLVKDKEQS---------------------- 421
                 .* .: :: * *:.:.: * *** .  .:: ...  .
```

Figure 48. A. Structure of human RCC1. In green the β-propeller of RCC1 and in red the N-terminal domain of RCC1. Modified from pbd strucuture 1a12. **B.** Alignment of human Nek9 with human RCC1. In red the N-terminal domain of RCC1

Chemical genetic approach

As a first step to study the effects of downregulating Nek9 in an acute manner to unravel further functions in more advanced stages of mitosis, we decided to create an analog sensitive mutant using a chemical genetic approach.

Following the method described by Dr. Shokat's laboratory we have been able to construct a mutant of Nek9, Nek9[L128G G67W] that acts as an analog sensitive mutant *in vitro*.

As can be seen in all experiments, the analog sensitive Nek9 mutant has a lower kinase activity than the wild type form of the protein. Nevertheless, this behaviour has already been described in other kinases that have been successfully studied with a similar approach (Banko *et al*, 2011; Burkard *et al*, 2007).

Our preliminary experiments show that Nek9[L128G G67W] functionally behaves *in vivo* as the wild type form in absence of the ATP analog (Figure 47). Now further characterization of the mutant will be needed to confirm *in* vivo that it acts as an analog sensitive mutant.

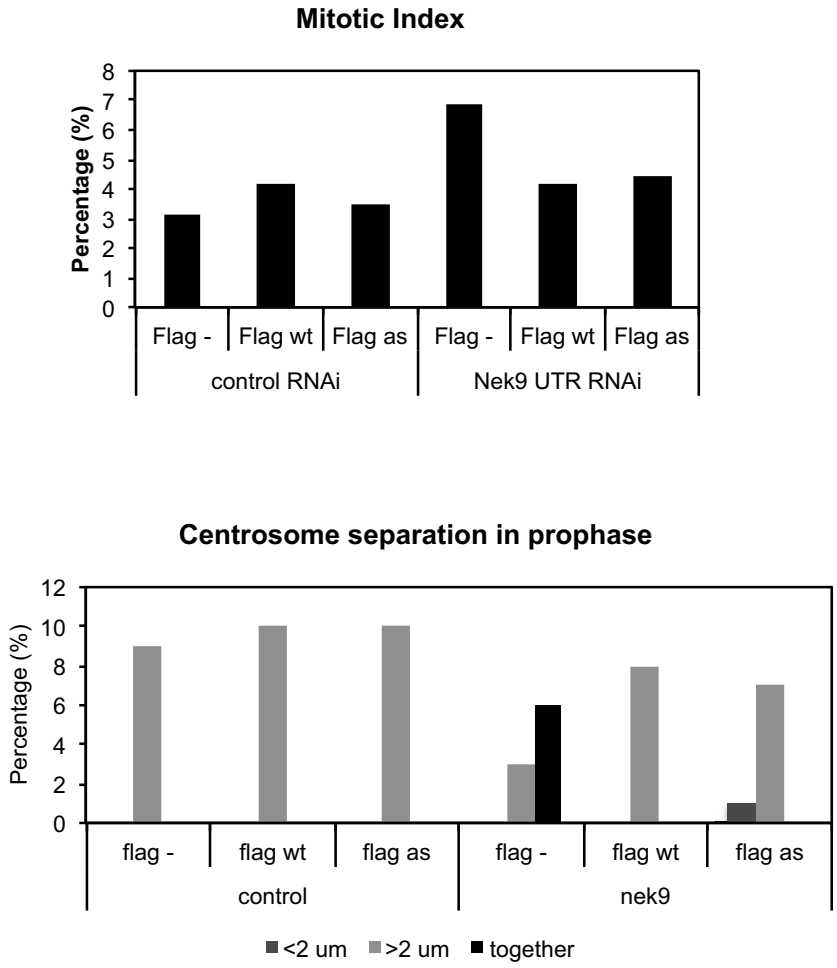


Figure 47. Nek9[L128G G67W] behaves as wild type form in absence of NM-PP1.

Once identified that Nek9[L128G G67W] acts as an analog sensitive *in vivo*, this mutant could be used to study other functions of Nek9 in the different phases of mitosis. Until now we have characterized the importance of Nek9 in centrosome separation in prophase and centrosome maturation in prometaphase. As cells where Nek9 is inhibited get arrested in prometaphase and present different defects in the early stages of mitosis ((Roig *et al*, 2005; Bertran *et al*, 2011; Sdelci *et al*, 2012) and our unpublished results) we have never been able to determine whether Nek9 has other functions in the later stages of mitosis. Nevertheless, its cellular localization and some mitotic defects present upon interference with Nek9, as the presence of micronuclei and

cytokinetic bridges (our unpublished results) (Figure 48), seem to indicate that the kinase might have roles in more advanced stages of mitosis.

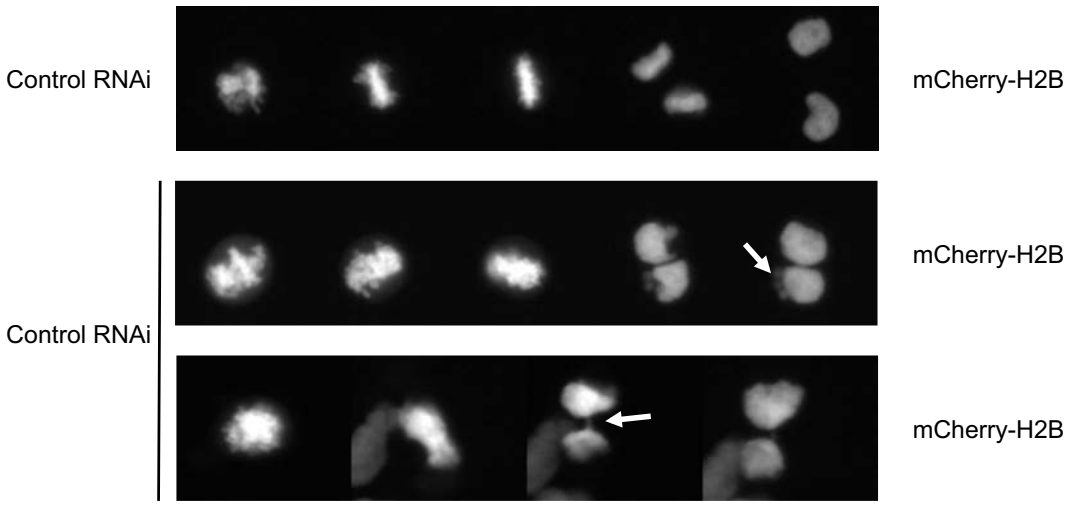


Figure 48. Different phenotypes shown in HeLa cells transfected with control RNAi or Nek9 RNAi. (unpublished results)

In order to identify this putative functions of Nek9 we could take advantage of the knock out mouse we are producing in the laboratory. Once we will be able to obtain KO MEFS from these mice we could use these cells, reconstitute them with either the wild type form or the analog sensitive form of Nek9 and afterwards inhibit the kinase with the ATP analog in an acute manner along the cell cycle.

In addition, this analog sensitive mutant could be useful to find different substrates for Nek9. Until now, only Nek6 and the recently described Nedd1 have been described as Nek9 substrates (Belham *et al*, 2003; Sdelci *et al*, 2012). The analog sensitive we construct should be able to use N^6-substituted ATP, that would allow us to identify using mass spectrometry which are the substrates that have been phosphorylated by Nek9[L128G G67W] (Banko *et al*, 2011)

CONCLUSIONS

Nek9 is activated by Plk1

1. Ser29 Ser750 Ser869 Ser827 and Ser885 are Nek9 mitotic phosphorylated sites *in vivo*.
2. Nek9 binds to Plk1 exclusively in mitosis and the binding is between the PBD domain of Plk1 and the Ser869 of Nek9.
3. Nek9 is phosphorylated *in vitro* by Cdk1 and Plk1
4. Plk1 phosphorylates Nek9 directly at the activation loop (Thr210) and this phosphorylation leads to the activation of the kinase *in vitro*.
5. Cdk1 and Plk1 are necessary for Nek9 activation *in vivo* during mitosis.
6. Plk1 controls Eg5 phosphorylation at Nek6 site Ser1033 through Nek9

Determination of Plk1 phosphorylation sites

1. Plk1 phosphorylates other Nek9 sites, besides Thr210
2. Phosphorylation of the putative Plk1 phosphorylation sites, Thr438, Thr544 and Thr662, at the RCC1 domain may contribute to the activation of Nek9.

Nek9 downstream of Plk1

1. Active Nek9 is able to partially overcome Plk1 inhibition prometaphase mitotic arrest

Nek9 is an Aurora A substrate

1. Aurora A phosphorylates Nek9 at Ser978 and Thr333
2. Aurora binds to the C-terminal domain of Nek9
3. Aurora A is not necessary for the phosphorylation of Thr210
4. Aurora A phosphorylation may be required for the localization of active Nek9 at centrosomes

Chemical genetic approach

1. Nek9[L128G][G67W] behaves as an analog sensitive mutant *in vitro*.

REFERENCES

Abe Y, Ohsugi M, Haraguchi K, Fujimoto J & Yamamoto T (2006) LATS2-Ajuba complex regulates gamma-tubulin recruitment to centrosomes and spindle organization during mitosis. FEBS letters 580: 782–8

Alexander J, Lim D, Joughin B a, Hegemann B, Hutchins JR a, Ehrenberger T, Ivins F, Sessa F, Hudecz O, Nigg E a, Fry AM, Musacchio A, Stukenberg PT, Mechtler K, Peters J-M, Smerdon SJ & Yaffe MB (2011) Spatial exclusivity combined with positive and negative selection of phosphorylation motifs is the basis for context-dependent mitotic signaling. *Science signaling* **4:** ra42

Andrysik Z, Bernstein WZ, Deng L, Myer DL, Li Y-Q, Tischfield J a, Stambrook PJ & Bahassi EM (2010) The novel mouse Polo-like kinase 5 responds to DNA damage and localizes in the nucleolus. *Nucleic acids research* **38:** 2931–43

Ang XL, Seeburg DP, Sheng M & Harper JW (2008) Regulation of postsynaptic RapGAP SPAR by Polo-like kinase 2 and the SCFbeta-TRCP ubiquitin ligase in hippocampal neurons. *The Journal of biological chemistry* **283:** 29424–32

Bahassi EM, Conn CW, Myer DL, Hennigan RF, McGowan CH, Sanchez Y & Stambrook PJ (2002) Mammalian Polo-like kinase 3 (Plk3) is a multifunctional protein involved in stress response pathways. *Oncogene* **21:** 6633–40

Bahassi EM, Hennigan RF, Myer DL & Stambrook PJ (2004) Cdc25C phosphorylation on serine 191 by Plk3 promotes its nuclear translocation. *Oncogene* **23:** 2658–63

Bahe S, Stierhof Y-D, Wilkinson CJ, Leiss F & Nigg E a (2005) Rootletin forms centriole-associated filaments and functions in centrosome cohesion. *The Journal of cell biology* **171:** 27–33

Bahmanyar S, Kaplan DD, Deluca JG, Giddings TH, O'Toole ET, Winey M, Salmon ED, Casey PJ, Nelson WJ & Barth AIM (2008) beta-Catenin is a Nek2 substrate involved in centrosome separation. *Genes & development* **22:** 91–105

Banko MR, Allen JJ, Schaffer BE, Wilker EW, Tsou P, White JL, Villén J, Wang B, Kim SR, Sakamoto K, Gygi SP, Cantley LC, Yaffe MB, Shokat KM & Brunet A (2011) Chemical genetic screen for AMPKα2 substrates uncovers a network of proteins involved in mitosis. *Molecular cell* **44:** 878–92

Barr AR & Gergely F (2007) Aurora-A: the maker and breaker of spindle poles. *Journal of cell science* **120:** 2987–96

Barrière C, Santamaría D, Cerqueira A, Galán J, Martín A, Ortega S, Malumbres M, Dubus P & Barbacid M (2007) Mice thrive without Cdk4 and Cdk2. *Molecular oncology* **1:** 72–83

Baumann C, Roman K, Hofmann K & Nigg EA (2007) PICH, a Centromere-Associated SNF2 Family ATPase , Is Regulated by Plk1 and Required for the Spindle Checkpoint. *Cell* **1:** 101–114

Bayliss R, Sardon T, Vernos I & Conti E (2003) Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. *Molecular cell* **12:** 851–62

Beausoleil S a, Villén J, Gerber S a, Rush J & Gygi SP (2006) A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nature biotechnology* **24:** 1285–92

Behrends C, Sowa ME, Gygi SP & Harper JW (2010) Network organization of the human autophagy system. *Nature* **466:** 68–76

Belham C, Comb MJ & Avruch J (2001) Identification of the NIMA family kinases NEK6 / 7 as regulators of the p70 ribosomal S6 kinase. *Current Biology* **11:** 1155–1167

Belham C, Roig J, Caldwell J a, Aoyama Y, Kemp BE, Comb M & Avruch J (2003) A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. *The Journal of biological chemistry* **278:** 34897–909

Berdnik D & Knoblich J a (2002) Drosophila Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. *Current biology : CB* **12:** 640–7

Bertran MT, Sdelci S, Regué L, Avruch J, Caelles C & Roig J (2011) Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. *The EMBO journal* **30:** 2634–47

Bettencourt-Dias M, Rodrigues-Martins a, Carpenter L, Riparbelli M, Lehmann L, Gatt MK, Carmo N, Balloux F, Callaini G & Glover DM (2005) SAK/PLK4 is required for centriole duplication and flagella development. *Current biology : CB* **15:** 2199–207

Bishop a C, Buzko O & Shokat KM (2001) Magic bullets for protein kinases. *Trends in cell biology* **11:** 167–72

Bishop a C, Shah K, Liu Y, Witucki L, Kung C & Shokat KM (1998) Design of allele-specific inhibitors to probe protein kinase signaling. *Current biology : CB* **8:** 257–66

Bishop AC, Kung C, Shah K, Witucki L, Shokat KM & Liu Y (1999) Generation of Monospecific Nanomolar Tyrosine Kinase Inhibitors via a Chemical Genetic Approach. *Enzyme***:** 627–631

Blangy A, Lane H a, D'Hérin P, Harper M, Kress M & Nigg E a (1995) Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell* **83:** 1159–69

Bolanos-Garcia VM (2005) Aurora kinases. *The international journal of biochemistry & cell biology* **37:** 1572–7

Boussif O, Lezoualc'Ht, Zanta MA, Mergnyt MD, Schermant D, Demeneixt B & Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine, *Proceedings of the National Academy of Sciences of the United States of America* 92:7297-7301

Bowers AJ & Boylan JF (2004) Nek8, a NIMA family kinase member, is overexpressed in primary human breast tumors. *Gene* **328:** 135–42

Brito D a & Rieder CL (2009) The ability to survive mitosis in the presence of microtubule poisons differs significantly between human nontransformed (RPE-1) and cancer (U2OS, HeLa) cells. *Cell motility and the cytoskeleton* **66:** 437–47

Burkard ME, Randall CL, Larochelle S, Zhang C, Shokat KM, Fisher RP & Jallepalli PV (2007) Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 4383–8

Burns TF, Fei P, Scata KA, David T, El-deiry WS & Dicker DT (2003) Silencing of the Novel p53 Target Gene Snk / Plk2 Leads to Mitotic Catastrophe in Silencing of the Novel p53 Target Gene Snk / Plk2 Leads to Mitotic Catastrophe in Paclitaxel (Taxol) -Exposed Cells. *Molecular and cellular biology* **10:** 5556–55571

Buzko O & Shokat KM (2002) A kinase sequence database : sequence alignments and family assignment. *Bioinformatics* **18:** 1274–1275

Cahu J, Olichon A, Hentrich C, Schek H, Drinjakovic J, Zhang C, Doherty-Kirby A, Lajoie G & Surrey T (2008) Phosphorylation by Cdk1 increases the binding of Eg5 to microtubules in vitro and in Xenopus egg extract spindles. *PloS one* **3:** e3936

Carmena M & Earnshaw WC (2003) The cellular geography of aurora kinases. *Nature reviews. Molecular cell biology* **4:** 842–54

Carmena M, Pinson X, Platani M, Salloum Z, Xu Z, Clark A, Macisaac F, Ogawa H, Eggert U, Glover DM, Archambault V & Earnshaw WC (2012) The chromosomal passenger complex activates Polo kinase at centromeres. *PLoS biology* **10:** e1001250

Carmena M, Ruchaud S & Earnshaw WC (2009) Making the Auroras glow: regulation of Aurora A and B kinase function by interacting proteins. *Current opinion in cell biology* **21:** 796–805

Chan EHY, Santamaria A, Silljé HHW & Nigg E a (2008) Plk1 regulates mitotic Aurora A function through betaTrCP-dependent degradation of hBora. *Chromosoma* **117:** 457–69

Chang J, Baloh RH & Milbrandt J (2009) The NIMA-family kinase Nek3 regulates microtubule acetylation in neurons. *Journal of cell science* **122:** 2274–82

Chang J, Cizmecioglu O, Hoffmann I & Rhee K (2010) PLK2 phosphorylation is critical for CPAP function in procentriole formation during the centrosome cycle. *The EMBO journal* **29:** 2395–406

Chen Y, Chen P, Chen C, Jiang X & Riley DJ (2008) Never-in-mitosis related kinase 1 funcitons in DNA damage response ad checkpoint control. *Cell Cycle* **7:** 3194–3201

Cizmecioglu O, Arnold M, Bahtz R, Settele F, Ehret L, Haselmann-Weiss U, Antony C & Hoffmann I (2010) Cep152 acts as a scaffold for recruitment of Plk4 and CPAP to the centrosome. *The Journal of cell biology* **191:** 731–9

Cizmecioglu O, Warnke S, Arnold M, Duensing S & Hoffmann I (2008) Plk2 regulated centriole duplication is dependent on its localization to the centrioles and a functional polo-box domain. *Cell cycle (Georgetown, Tex.)* **7:** 3548–55

Cohen P (2002) Protein kinases--the major drug targets of the twenty-first century? *Nature reviews. Drug discovery* **1:** 309–15

Cunha-Ferreira I, Rodrigues-Martins A, Bento I, Riparbelli M, Zhang W, Laue E, Callaini G, Glover DM & Bettencourt-Dias M (2009) The SCF/Slimb ubiquitin ligase limits centrosome amplification through degradation of SAK/PLK4. *Current biology : CB* **19:** 43–9

de Cárcer G, Escobar B, Higuero AM, García L, Ansón A, Pérez G, Mollejo M, Manning G, Meléndez B, Abad-Rodríguez J & Malumbres M (2011a) Plk5, a

polo box domain-only protein with specific roles in neuron differentiation and glioblastoma suppression. *Molecular and cellular biology* **31:** 1225–39

de Cárcer G, Manning G & Malumbres M (2011b) From Plk1 to Plk5: Functional evolution of polo-like kinases. *Cell Cycle* **10:** 2255–2262

Dar AC & Shokat KM (2011) The evolution of protein kinase inhibitors from antagonists to agonists of cellular signaling. *Annual review of biochemistry* **80:** 769–95

Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, Körner R, Greff Z, Kéri G, Stemmann O & Mann M (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Molecular cell* **31:** 438–48

Dephoure N, Zhou C, Villén J, Beausoleil S a, Bakalarski CE, Elledge SJ & Gygi SP (2008) A quantitative atlas of mitotic phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 10762–7

Doles J & Hemann MT (2010) Nek4 status differentially alters sensitivity to distinct microtubule poisons. *Cancer research* **70:** 1033–41

Donohue PJ, Alberts GF, Guo Y & Winkles JA (1995) Identification by Targeted Differential Display of an Immediate Early Gene Encoding a Putative Serine/Threonine Kinase. *The Journal of biological chemistry* **270(17):** 10351–10357

Du J, Cai X, Yao J, Ding X, Wu Q, Pei S, Jiang K, Zhang Y, Wang W, Shi Y, Lai Y, Shen J, Teng M, Huang H, Fei Q, Reddy ES, Zhu J, Jin C & Yao X (2008) The mitotic checkpoint kinase NEK2A regulates kinetochore microtubule attachment stability. *Oncogene* **27:** 4107–14

Duncan T & Wakefield JG (2011) 50 Ways To Build a Spindle: the Complexity of Microtubule Generation During Mitosis. *Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology* **19:** 321–33

Eckerdt F, Yuan J & Strebhardt K (2005) Polo-like kinases and oncogenesis. *Oncogene* **24:** 267–76

Elia AEH, Cantley LC & Yaffe MB (2003a) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science (New York, N.Y.)* **299:** 1228–31

Elia AEH, Rellos P, Haire LF, Chao JW, Ivins FJ, Hoepker K, Mohammad D, Cantley LC, Smerdon SJ & Yaffe MB (2003b) The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell* **115:** 83–95

Elowe S, Hümmer S, Uldschmid A, Li X & Nigg E a (2007) Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions. *Genes & development* **21:** 2205–19

Feige E & Motro B (2002) The related murine kinases , Nek6 and Nek7 , display distinct patterns of expression. *Mechanisms of Development* **110:** 219–223

Feige E, Shalom O, Tsuriel S, Yissachar N & Motro B (2006) Nek1 shares structural and functional similarities with NIMA kinase. *Biochimica et biophysica acta* **1763:** 272–81

Ferenz NP, Gable A & Wadsworth P (2010) Mitotic functions of kinesin-5. *Seminars in cell & developmental biology* **21:** 255–9

Ferenz NP, Paul R, Fagerstrom C, Mogilner A & Wadsworth P (2009) Dynein antagonizes eg5 by crosslinking and sliding antiparallel microtubules. *Current biology : CB* **19:** 1833–8

Ferrari S, Marin O, Pagano M a, Meggio F, Hess D, El-Shemerly M, Krystyniak A & Pinna L a (2005) Aurora-A site specificity: a study with synthetic peptide substrates. *The Biochemical journal* **390:** 293–302

Fletcher L, Cerniglia GJ, Yen TJ & Muschel RJ (2005) Live cell imaging reveals distinct roles in cell cycle regulation for Nek2A and Nek2B. *Biochimica et biophysica acta* **1744:** 89–92

Fry a M, Arnaud L & Nigg E a (1999) Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. *The Journal of biological chemistry* **274:** 16304–10

Fry a M, Mayor T, Meraldi P, Stierhof YD, Tanaka K & Nigg E a (1998a) C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *The Journal of cell biology* **141:** 1563–74

Fry a M, Meraldi P & Nigg E a (1998b) A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *The EMBO journal* **17:** 470–81

Fry AM (2002) The Nek2 protein kinase: a novel regulator of centrosome structure. *Oncogene* **21:** 6184–94

Fry AM & Nigg EA (1995) The NIMA kinase joins forces with Cdc2. *Cell Cycle* **5:** 9–12

Gavet O & Pines J (2010) Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. *Developmental cell* **18:** 533–43

Giet R (1999) The Xenopus laevis Aurora-related Protein Kinase pEg2 Associates with and Phosphorylates the Kinesin-related Protein XlEg5. *Journal of Biological Chemistry* **274:** 15005–15013

Girdler F, Gascoigne KE, Eyers P a, Hartmuth S, Crafter C, Foote KM, Keen NJ & Taylor SS (2006) Validating Aurora B as an anti-cancer drug target. *Journal of cell science* **119:** 3664–75

Glover DM, Leibowitz MH, McLean D a & Parry H (1995) Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell* **81:** 95–105

Gray NS (1998) Exploiting Chemical Libraries, Structure, and Genomics in the Search for Kinase Inhibitors. *Science* **281:** 533–538

Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, *et al* (2007) Patterns of somatic mutation in human cancer genomes. *Nature* **446:** 153–8

Gregan J, Zhang C, Rumpf C, Cipak L, Li Z, Uluocak P, Nasmyth K & Shokat KM (2007) Construction of conditional analog-sensitive kinase alleles in the fission yeast Schizosaccharomyces pombe. *Nature protocols* **2:** 2996–3000

Grosstessner-Hain K, Hegemann B, Novatchkova M, Rameseder J, Joughin B a, Hudecz O, Roitinger E, Pichler P, Kraut N, Yaffe MB, Peters J-M & Mechtler K (2011) Quantitative phospho-proteomics to investigate the polo-like kinase 1-dependent phospho-proteome. *Molecular & cellular proteomics : MCP* **10:** M111.008540

Guderian G, Westendorf J, Uldschmid A & Nigg E a (2010) Plk4 trans-autophosphorylation regulates centriole number by controlling betaTrCP-mediated degradation. *Journal of cell science* **123:** 2163–9

Habedanck R, Stierhof Y-D, Wilkinson CJ & Nigg E a (2005) The Polo kinase Plk4 functions in centriole duplication. *Nature cell biology* **7:** 1140–6

Han G, Ye M, Liu H, Song C, Sun D, Wu Y, Jiang X, Chen R, Wang C, Wang L & Zou H (2010) Phosphoproteome analysis of human liver tissue by long-

gradient nanoflow LC coupled with multiple stage MS analysis. *Electrophoresis* **31:** 1080–9

Hannak E, Kirkham M, Hyman a a & Oegema K (2001) Aurora-A kinase is required for centrosome maturation in Caenorhabditis elegans. *The Journal of cell biology* **155:** 1109–16

Haren L, Stearns T & Lüders J (2009) Plk1-dependent recruitment of gamma-tubulin complexes to mitotic centrosomes involves multiple PCM components. *PloS one* **4:** e5976

Harper JW, Burton JL & Solomon MJ (2002) The anaphase-promoting complex: it's not just for mitosis any more. *Genes & development* **16:** 2179–206

Hatch EM, Kulukian A, Holland AJ, Cleveland DW & Stearns T (2010) Cep152 interacts with Plk4 and is required for centriole duplication. *The Journal of cell biology* **191:** 721–9

Hegemann B, Hutchins JR a, Hudecz O, Novatchkova M, Rameseder J, Sykora MM, Liu S, Mazanek M, Lénárt P, Hériché J-K, Poser I, Kraut N, Hyman A a, Yaffe MB, Mechtler K & Peters J-M (2011) Systematic phosphorylation analysis of human mitotic protein complexes. *Science signaling* **4:** rs12

Hilton LK, White MC & Quarmby LM (2009) The NIMA-related kinase NEK1 cycles through the nucleus. *Biochemical and biophysical research communications* **389:** 52–6

Hirota T, Kunitoku N, Sasayama T, Marumoto T, Zhang D, Nitta M, Hatakeyama K & Saya H (2003) Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell* **114:** 585–98

Holland PM, Milne A, Garka K, Johnson RS, Willis C, Sims JE, Rauch CT, Bird T a & Virca GD (2002) Purification, cloning, and characterization of Nek8, a novel NIMA-related kinase, and its candidate substrate Bicd2. *The Journal of biological chemistry* **277:** 16229–40

Holt LJ, Tuch BB, Villén J, Johnson AD, Gygi SP & Morgan DO (2009) Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science (New York, N.Y.)* **325:** 1682–6

Hoyt M a (1994) Cellular roles of kinesin and related proteins. *Current opinion in cell biology* **6:** 63–8

Hu D, Valentine M, Kidd VJ & Lahti JM (2007) CDK11(p58) is required for the maintenance of sister chromatid cohesion. *Journal of cell science* **120:** 2424–34

Hudson JW, Kozarova a, Cheung P, Macmillan JC, Swallow CJ, Cross JC & Dennis JW (2001) Late mitotic failure in mice lacking Sak, a polo-like kinase. *Current biology : CB* **11:** 441–6

Hunter T (2000) Signaling--2000 and beyond. *Cell* **100:** 113–27

Hutterer A, Berdnik D, Wirtz-Peitz F, Zigman M, Schleiffer A & Knoblich J a (2006) Mitotic activation of the kinase Aurora-A requires its binding partner Bora. *Developmental cell* **11:** 147–57

Iliuk AB, Martin V a, Alicie BM, Geahlen RL & Tao WA (2010) In-depth analyses of kinase-dependent tyrosine phosphoproteomes based on metal ion-functionalized soluble nanopolymers. *Molecular & cellular proteomics : MCP* **9:** 2162–72

Jang Y-J, Ma S, Terada Y & Erikson RL (2002) Phosphorylation of threonine 210 and the role of serine 137 in the regulation of mammalian polo-like kinase. *The Journal of biological chemistry* **277:** 44115–20

Jeong Y, Lee J, Kim K, Yoo JC & Rhee K (2007) Characterization of NIP2/centrobin, a novel substrate of Nek2, and its potential role in microtubule stabilization. *Journal of cell science* **120:** 2106–16

Kandli M, Feige E, Chen a, Kilfin G & Motro B (2000) Isolation and characterization of two evolutionarily conserved murine kinases (Nek6 and nek7) related to the fungal mitotic regulator, NIMA. *Genomics* **68:** 187–96

Kang YH, Park J-E, Yu L-R, Soung N-K, Yun S-M, Bang JK, Seong Y-S, Yu H, Garfield S, Veenstra TD & Lee KS (2006) Self-regulated Plk1 recruitment to kinetochores by the Plk1-PBIP1 interaction is critical for proper chromosome segregation. *Molecular cell* **24:** 409–22

Kapitein LC, Peterman EJG & Kwok BH (2005) The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* **435:**

Kettenbach AN, Schweppe DK, Faherty BK, Pechenick D, Pletnev A a & Gerber S a (2011) Quantitative phosphoproteomics identifies substrates and functional modules of Aurora and Polo-like kinase activities in mitotic cells. *Science signaling* **4:** rs5

Kettenbach AN, Wang T, Faherty BK, Madden DR, Knapp S, Bailey-Kellogg C & Gerber S a (2012) Rapid determination of multiple linear kinase substrate motifs by mass spectrometry. *Chemistry & biology* **19:** 608–18

Kim S, Lee K & Rhee K (2007) NEK7 is a centrosomal kinase critical for microtubule nucleation. *Biochemical and biophysical research communications* **360:** 56–62

Kim S & Rhee K (2011) NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells. *Journal of Cell Science* **124:** 4126–4126

Kimura M & Okano Y (2001) Molecular cloning and characterization of the human NIMA-related protein kinase 3 gene (NEK3). *Cytogenetics and cell genetics* **95:** 177–82

Kleylein-Sohn J, Westendorf J, Le Clech M, Habedanck R, Stierhof Y-D & Nigg E a (2007) Plk4-induced centriole biogenesis in human cells. *Developmental cell* **13:** 190–202

Kufer T a, Silljé HHW, Körner R, Gruss OJ, Meraldi P & Nigg E a (2002) Human TPX2 is required for targeting Aurora-A kinase to the spindle. *The Journal of cell biology* **158:** 617–23

Lane H a & Nigg E a (1996) Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *The Journal of cell biology* **135:** 1701–13

Laurell E, Beck K, Krupina K, Theerthagiri G, Bodenmiller B, Horvath P, Aebersold R, Antonin W & Kutay U (2011) Phosphorylation of Nup98 by multiple kinases is crucial for NPC disassembly during mitotic entry. *Cell* **144:** 539–50

Lee M-Y, Kim H, Kim M-A, Jee HJ, Kim AJ, Bae Y-S, Park J-I, Chung JH & Yun J (2008) Nek6 is involved in G2/M phase cell cycle arrest through DNA damage-induced phosphorylation. *Cell Cycle* **7:17, 2705:** 2705–2709

Lens SM a, Voest EE & Medema RH (2010) Shared and separate functions of polo-like kinases and aurora kinases in cancer. *Nature reviews. Cancer* **10:** 825–41

Leung GC, Hudson JW, Kozarova A, Davidson A, Dennis JW & Sicheri F (2002) The Sak polo-box comprises a structural domain sufficient for mitotic subcellular localization. *Nature structural biology* **9:** 719–24

Levedakou EN, He M, Baptist EW, Craven RJ, Cance WG, Welcsh PL, Simmons a, Naylor SL, Leach RJ & Lewis TB (1994) Two novel human serine/threonine kinases with homologies to the cell cycle regulating Xenopus MO15, and NIMA kinases: cloning and characterization of their expression pattern. *Oncogene* **9:** 1977–88

Li T, Inoue A, Lahti JM & Kidd VJ (2004) Failure To Proliferate and Mitotic Arrest of CDK11 p110 / p58 -Null Mutant Mice at the Blastocyst Stage of Embryonic Cell Development Failure To Proliferate and Mitotic Arrest of CDK11 p110 / p58 -Null Mutant Mice at the Blastocyst Stage of Embryonic Cell. *Molecular and cellular biology* **24:** 3188

Lindon C & Pines J (2004) Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. *The Journal of cell biology* **164:** 233–41

Liu S, Lu W, Obara T, Kuida S, Lehoczky J, Dewar K, Drummornd IA & Beier DR (2002) A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in zebrafish. *Development* **129:** 5839–5846

Llamazares S, Moreira a, Tavares a, Girdham C, Spruce B a, Gonzalez C, Karess RE, Glover DM & Sunkel CE (1991) polo encodes a protein kinase homolog required for mitosis in Drosophila. *Genes & Development* **5:** 2153–2165

Lowery DM, Clauser KR, Hjerrild M, Lim D, Alexander J, Kishi K, Ong S-E, Gammeltoft S, Carr S a & Yaffe MB (2007) Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. *The EMBO journal* **26:** 2262–73

Lowery DM, Lim D & Yaffe MB (2005) Structure and function of Polo-like kinases. *Oncogene* **24:** 248–59

Lowery DM, Mohammad DH, Elia AEH & Yaffe MB (2004) The Polo-Box Domain A molecular Integrator of Mitotic Kinase Cascades and Polo-like Kinase Function. *Cell Cycle* **3:** 128–131

Lu L-Y, Wood JL, Minter-Dykhouse K, Ye L, Saunders TL, Yu X & Chen J (2008) Polo-like kinase 1 is essential for early embryonic development and tumor suppression. *Molecular and cellular biology* **28:** 6870–6

Lénárt P, Petronczki M, Steegmaier M, Di Fiore B, Lipp JJ, Hoffmann M, Rettig WJ, Kraut N & Peters J-M (2007) The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Current Biology* **17:** 304–15

Lüders J, Patel UK & Stearns T (2006) GCP-WD is a gamma-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. *Nature cell biology* **8:** 137–47

Lüders J & Stearns T (2007) Microtubule-organizing centres: a re-evaluation. *Nature reviews. Molecular cell biology* **8:** 161–7

Ma N, Titus J, Gable A, Ross JL & Wadsworth P (2011) TPX2 regulates the localization and activity of Eg5 in the mammalian mitotic spindle. *The Journal of cell biology* **195:** 87–98

Ma N, Tulu US, Ferenz NP, Fagerstrom C, Wilde A & Wadsworth P (2010) Poleward Transport of TPX2 in the Mammalian Mitotic Spindle Requires Dynein , Eg5 , and Microtubule Flux. *Molecular Biology of the Cell* **21:** 979–988

Ma S, Charron J & Erikson RL (2003) Role of Plk2 (Snk) in Mouse Development and Cell Proliferation Role of Plk2 (Snk) in Mouse Development and Cell Proliferation. *Molecular and cellular biology* **23:** 6936–6943

Macůrek L, Lindqvist A, Lim D, Lampson M a, Klompmaker R, Freire R, Clouin C, Taylor SS, Yaffe MB & Medema RH (2008) Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. *Nature* **455:** 119–23

Mahjoub MR, Trapp ML & Quarmby LM (2005) NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. *Journal of the American Society of Nephrology : JASN* **16:** 3485–9

Maiato H (2010) Mitosis: wisdom, knowledge, and information. *Cellular and molecular life sciences : CMLS* **67:** 2141–3

Maliga Z, Kapoor TM & Mitchison TJ (2002) Evidence that Monastrol Is an Allosteric Inhibitor of the Mitotic Kinesin Eg5. *Chemistry & biology* **9:** 989–996

Malumbres M (2011) Physiological relevance of cell cycle kinases. *Physiological reviews* **91:** 973–1007

Malumbres M & Barbacid M (2005) Mammalian cyclin-dependent kinases. *Trends in biochemical sciences* **30:** 630–41

Malumbres M, Cerezo A, Gala J, Ortega S, Dubus P & Barbacid M (2004) Mammalian Cells Cycle without the D-Type Cyclin-Dependent Kinases Cdk4 and Cdk6. *Cell* **118:** 493–504

Mardin BR, Agircan FG, Lange C & Schiebel E (2011) Plk1 controls the Nek2A-PP1γ antagonism in centrosome disjunction. *Current biology : CB* **21:** 1145–51

Mardin BR, Lange C, Baxter JE, Hardy T, Scholz SR, Fry AM & Schiebel E (2010) Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. *Nature cell biology* **12:** 1166–76

Marumoto T, Honda S, Hara T, Nitta M, Hirota T, Kohmura E & Saya H (2003) Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. *The Journal of biological chemistry* **278:** 51786–95

Matsuoka S, Ballif B a, Smogorzewska A, McDonald ER, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP & Elledge SJ (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science (New York, N.Y.)* **316:** 1160–6

Mayer BJ (2001) SH3 domains: complexity in moderation. *Journal of cell science* **114:** 1253–63

Mayer TU (1999) Small Molecule Inhibitor of Mitotic Spindle Bipolarity Identified in a Phenotype-Based Screen. *Science* **286:** 971–974

McInnes C & Wyatt MD (2011) PLK1 as an oncology target: current status and future potential. *Drug discovery today* **16:** 619–25

Meirelles GV, Carlos D, Lanza F, Bernachi S, Franco A & Leme P (2010) Characterization of hNek6 Interactome Reveals an Important Role for Its Short N-Terminal Domain and Colocalization with Proteins at the Centrosome research articles. *Journal of Proteome Research* **9:** 6298–6316

Meirelles GV, Silva JC, Mendonça YD a, Ramos CH, Torriani IL & Kobarg J (2011) Human Nek6 is a monomeric mostly globular kinase with an unfolded short N-terminal domain. *BMC structural biology* **11:** 12

Melixetian M, Klein DK, Sørensen CS & Helin K (2009) NEK11 regulates CDC25A degradation and the IR-induced G2/M checkpoint. *Nature cell biology* **11:** 1247–53

Meraldi P & Nigg E a (2001) Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. *Journal of cell science* **114:** 3749–57

Miller SL, Antico G, Raghunath PN, Tomaszewski JE & Clevenger CV (2007) Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. *Oncogene* **26:** 4668–78

Mitchison TJ & Salmon ED (2001) Mitosis: a history of division. *Nature cell biology* **3:** E17–21

Moniz LS & Stambolic V (2011) Nek10 mediates G2/M cell cycle arrest and MEK autoactivation in response to UV irradiation. *Molecular and cellular biology* **31:** 30–42

Mori D, Yano Y, Toyo-oka K, Yoshida N, Yamada M, Muramatsu M, Zhang D, Saya H, Toyoshima YY, Kinoshita K, Wynshaw-Boris A & Hirotsune S (2007) NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment. *Molecular and cellular biology* **27:** 352–67

Moritz A, Li Y, Guo A, Villén J, Wang Y, MacNeill J, Kornhauser J, Sprott K, Zhou J, Possemato A, Ren JM, Hornbeck P, Cantley LC, Gygi SP, Rush J & Comb MJ (2010) Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. *Science signaling* **3:** ra64

Morris NR (1975) Mitotic mutants of Aspergillus nidulans. *Genetical research* **26:** 237–54

Nakajima H (2003) Identification of a Consensus Motif for Plk (Polo-like Kinase) Phosphorylation Reveals Myt1 as a Plk1 Substrate. *Journal of Biological Chemistry* **278:** 25277–25280

Nakajima H, Toyoshima-morimoto F, Taniguchi E & Nishida E (2003) Identification of a Consensus Motif for Plk (Polo-like Kinase) Phosphorylation Reveals Myt1 as a Plk1 Substrate. *The Journal of biological chemistry* **278:** 25277–25280

Neef R, Preisinger C, Sutcliffe J, Kopajtich R, Nigg E a, Mayer TU & Barr F a (2003) Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. *The Journal of cell biology* **162:** 863–75

Nigg E a (2001) Mitotic kinases as regulators of cell division and its checkpoints. *Nature reviews. Molecular cell biology* **2:** 21–32

Noguchi K, Fukazawa H, Murakami Y & Uehara Y (2002) Nek11, a new member of the NIMA family of kinases, involved in DNA replication and genotoxic stress responses. *The Journal of biological chemistry* **277:** 39655–65

Noguchi K, Fukazawa H, Murakami Y & Uehara Y (2004) Nucleolar Nek11 is a novel target of Nek2A in G1/S-arrested cells. *The Journal of biological chemistry* **279:** 32716–27

Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, Jensen LJ, Gnad F, Cox J, Jensen TS, Nigg E a, Brunak S & Mann M (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Science signaling* **3:** ra3

Oppermann FS, Gnad F, Olsen JV, Hornberger R, Greff Z, Kéri G, Mann M & Daub H (2009) Large-scale proteomics analysis of the human kinome. *Molecular & cellular proteomics : MCP* **8:** 1751–64

Oppermann FS, Grundner-Culemann K, Kumar C, Gruss OJ, Jallepalli PV & Daub H (2012) Combination of chemical genetics and phosphoproteomics for kinase signaling analysis enables confident identification of cellular downstream targets. *Molecular & cellular proteomics : MCP* **11:** O111.012351

Oshimori N, Ohsugi M & Yamamoto T (2006) The Plk1 target Kizuna stabilizes mitotic centrosomes to ensure spindle bipolarity. *Nature cell biology* **8:** 1095–101

Osmani S a & Ye XS (1996) Cell cycle regulation in Aspergillus by two protein kinases. *The Biochemical journal* **317 (Pt 3:** 633–41

Otto E a, Trapp ML, Schultheiss UT, Helou J, Quarmby LM & Hildebrandt F (2008) NEK8 mutations affect ciliary and centrosomal localization and may cause nephronophthisis. *Journal of the American Society of Nephrology : JASN* **19:** 587–92

O'Connell MJ, Krien MJE & Hunter T (2003) Never say never. The NIMA-related protein kinases in mitotic control. *Trends in Cell Biology* **13:** 221–228

O'Regan L, Blot J & Fry AM (2007) Mitotic regulation by NIMA-related kinases. *Cell division* **2:** 25

O'Regan L & Fry AM (2009) The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. *Molecular and cellular biology* **29:** 3975–90

Parker JDK, Bradley B a, Mooers AO & Quarmby LM (2007) Phylogenetic analysis of the Neks reveals early diversification of ciliary-cell cycle kinases. *PloS one* **2:** e1076

Pearlman SM, Serber Z & Ferrell JE (2011) A mechanism for the evolution of phosphorylation sites. *Cell* **147:** 934–46

Peset I, Seiler J, Sardon T, Bejarano L a, Rybina S & Vernos I (2005) Function and regulation of Maskin, a TACC family protein, in microtubule growth during mitosis. *The Journal of cell biology* **170:** 1057–66

Petronczki M, Lénárt P & Peters J-M (2008) Polo on the Rise-from Mitotic Entry to Cytokinesis with Plk1. *Developmental cell* **14:** 646–59

Pines J & Rieder CL (2001) Re-staging mitosis: a contemporary view of mitotic progression. *Nature cell biology* **3:** E3–6

Quarmby LM & Mahjoub MR (2005) Caught Nek-ing: cilia and centrioles. *Journal of cell science* **118:** 5161–9

Rapley J, Nicolàs M, Groen A, Regué L, Bertran MT, Caelles C, Avruch J & Roig J (2008) The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation. *Journal of cell science* **121:** 3912–21

Reboutier D, Troadec M-B, Cremet J-Y, Fukasawa K & Prigent C (2012) Nucleophosmin/B23 activates Aurora A at the centrosome through phosphorylation of serine 89. *The Journal of cell biology* **197:** 19–26

Regué L, Sdelci S, Bertran MT, Caelles C, Reverter D & Roig J (2011) DYNLL/LC8 protein controls signal transduction through the Nek9/Nek6 signaling module by regulating Nek6 binding to Nek9. *The Journal of biological chemistry* **286:** 18118–29

Richards MW, O'Regan L, Mas-Droux C, Blot JMY, Cheung J, Hoelder S, Fry AM & Bayliss R (2009a) An autoinhibitory tyrosine motif in the cell-cycle-regulated Nek7 kinase is released through binding of Nek9. *Molecular cell* **36:** 560–70

Richards MW, Regan LO, Mas-droux C, Blot JMY, Hoelder S, Fry AM & Bayliss R (2009b) An Autoinhibitory Tyrosin Motif in the Cell-Cyvle-Regulated Nek7 Kinase Is Released through Binding of Nek9. *Molecular Cell* **36:** 560–570

Rodrigues-Martins a, Riparbelli M, Callaini G, Glover DM & Bettencourt-Dias M (2007) Revisiting the role of the mother centriole in centriole biogenesis. *Science* **316:** 1046–50

Rogers GC, Rusan NM, Roberts DM, Peifer M & Rogers SL (2009) The SCF Slimb ubiquitin ligase regulates Plk4/Sak levels to block centriole reduplication. *The Journal of cell biology* **184:** 225–39

Roig J, Groen A, Caldwell J & Avruch J (2005) Active Nercc1 Protein Kinase Concentrates at Centrosomes Early in Mitosis and Is Necessary for Proper Spindle Assembly. *Molecular Biology of the Cell* **16:** 4827–4840

Roig J, Mikhailov A, Belham C & Avruch J (2002) Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. *Genes & development* **16:** 1640–58

Sahota NK & Fry A Cell Cycle Studies on Human Nek3, Nek5 and Nek11 Protein Kinases. University of Liecester, 2010

Salem H, Rachmin I, Yissachar N, Cohen S, Amiel A, Haffner R, Lavi L & Motro B (2010) Nek7 kinase targeting leads to early mortality, cytokinesis disturbance and polyploidy. *Oncogene* **29:** 4046–57

Santamaria A, Eberspa U, Eis K, Husemann M, Mumberg D, Prechtl S, Schulze V, Siemeister G, Wortmann L, Barr FA & Nigg EA (2007) Use of the Novel Plk1 Inhibitor ZK-Thiazolidinone to Elucidate Functions of Plk1 in Early and Late Stages of Mitosis. *Molecular Biology of the Cell* **18:** 4024–4036

Santamaria A, Wang B, Elowe S, Malik R, Zhang F, Bauer M, Schmidt A, Silljé HHW, Körner R & Nigg E a (2011) The Plk1-dependent phosphoproteome of the early mitotic spindle. *Molecular & cellular proteomics : MCP* **10:** M110.004457

Santamaría D, Barrière C, Cerqueira A, Hunt S, Tardy C, Newton K, Cáceres JF, Dubus P, Malumbres M & Barbacid M (2007) Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* **448:** 811–5

Sardon T, Pache R a, Stein A, Molina H, Vernos I & Aloy P (2010) Uncovering new substrates for Aurora A kinase. *EMBO reports* **11:** 977–84

Sardon T, Peset I, Petrova B & Vernos I (2008) Dissecting the role of Aurora A during spindle assembly. *The EMBO journal* **27:** 2567–79

Sawin KE & Mitchison TJ (1995) Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. *Proceedings of the National Academy of Sciences of the United States of America* **92:** 4289–4293

Schultz SJ, Fry a M, Sütterlin C, Ried T & Nigg E a (1994) Cell cycle-dependent expression of Nek2, a novel human protein kinase related to the NIMA mitotic regulator of Aspergillus nidulans. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **5:** 625–35

Sdelci S, Schütz M, Pinyol R, Bertran MT, Regué L, Caelles C, Vernos I & Roig J (2012) Nek9 Phosphorylation of NEDD1/GCP-WD Contributes to Plk1 Control of γ-Tubulin Recruitment to the Mitotic Centrosome. *Current biology : CB* **22:** 1516–1523

Seeburg DP, Feliu-Mojer M, Gaiottino J, Pak DTS & Sheng M (2008) Critical Role of CDK5 and Polo-like Kinase 2 in Homeostatic Synaptic Plasticity during Elevated Activity. *Neuron* **58:** 571–583

Seki A, Coppinger J a, Du H, Jang C-Y, Yates JR & Fang G (2008a) Plk1- and beta-TrCP-dependent degradation of Bora controls mitotic progression. *The Journal of cell biology* **181:** 65–78

Seki A, Coppinger J a, Jang C-Y, Yates JR & Fang G (2008b) Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. *Science (New York, N.Y.)* **320:** 1655–8

Seong Y-S, Kamijo K, Lee J-S, Fernandez E, Kuriyama R, Miki T & Lee KS (2002) A spindle checkpoint arrest and a cytokinesis failure by the dominant-negative polo-box domain of Plk1 in U-2 OS cells. *The Journal of biological chemistry* **277:** 32282–93

Shah K, Liu Y, Deirmengian C & Shokat KM (1997) Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 3565–70

Silkworth WT, Nardi IK, Paul R, Mogilner A & Cimini D (2012) Timing of centrosome separation is important for accurate chromosome segregation. Molecular biology of the cell 23: 401–11

Sillibourne JE, Tack F, Vloemans N, Boeckx A, Thambirajah S, Bonnet P, Ramaekers FCS, Bornens M & Grand-perret T (2010) Autophosphorylation of Polo-like Kinase 4 and Its Role in Centriole Duplication. **21:** 547–561

Simmons DL, Neel BG, Stevens R, Evett G & Erikson RL (1992) Identification of an Early-Growth-Response Gene Encoding Novel Putative Protein Kinase. *Molecular and cellular biology* **12:** 4164

Skoufias D a, DeBonis S, Saoudi Y, Lebeau L, Crevel I, Cross R, Wade RH, Hackney D & Kozielski F (2006) S-trityl-L-cysteine is a reversible, tight binding inhibitor of the human kinesin Eg5 that specifically blocks mitotic progression. *The Journal of biological chemistry* **281:** 17559–69

Smith E, Hégarat N, Vesely C, Roseboom I, Larch C, Streicher H, Straatman K, Flynn H, Skehel M, Hirota T, Kuriyama R & Hochegger H (2011) Differential control of Eg5-dependent centrosome separation by Plk1 and Cdk1. *The EMBO journal* **30:** 2233–45

Sonn S, Jeong Y & Rhee K (2009) Nip2/centrobin may be a substrate of Nek2 that is required for proper spindle assembly during mitosis in early mouse embryos. *Molecular reproduction and development* **76:** 587–92

Sonn S, Oh GT & Rhee K (2011) Nek2 and its substrate, centrobin/Nip2, are required for proper meiotic spindle formation of the mouse oocytes. *Zygote (Cambridge, England)* **19:** 15–20

Sumara I, Gime JF, Gerlich D, Hirota T, Kraft C, Torre CD, Ellenberg J & Peters J (2004) Roles of Polo-like Kinase 1 in the Assembly of Functional Mitotic Spindles. *Current Biology* **14:** 1712–1722

Sunkel CE & Glover DM (1988) polo, a mitotic mutant of Drosophila displaying abnormal spindle poles. *Journal of cell science* **89 (Pt 1):** 25–38

Sørensen CS, Melixetian M, Klein DK & Helin K (2010) NEK11−Linking CHK1 and CDC25A in DNA damage checkpoint signaling. *Cell Cycle* **9:** 450–455

Tanaka K & Nigg E a (1999) Cloning and characterization of the murine Nek3 protein kinase, a novel member of the NIMA family of putative cell cycle regulators. *The Journal of biological chemistry* **274:** 13491–7

Tanenbaum ME & Medema RH (2010) Mechanisms of centrosome separation and bipolar spindle assembly. *Developmental cell* **19:** 797–806

Terada Y, Uetake Y, Kuriyama R (2003) Interaction of Aurora A and centrosomin at the microtubule-nucleating site in *Drosophila* and mammalian cells. J Cell Biol 162(5):757-63

Thorsness PE & Koshland DE (1987) Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. The Journal of biological chemistry 262: 10422–5

Di Tommaso P, Moretti S, Xenarios I, Orobitg M, Montanyola A, Chang J-M, Taly J-F & Notredame C (2011) T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic acids research* **39:** W13–7

Toyoshima-Morimoto F, Taniguchi E & Nishida E (2002) Plk1 promotes nuclear translocation of human Cdc25C during prophase. *EMBO reports* **3:** 341–8

Toyoshima-Morimoto F, Taniguchi E & Shinya N (2001) Labelling of purified sGC cyclin B1 and targets it to the nucleus during prophase. *Nature* **410:** 215–220

Tsou M-FB & Stearns T (2006) Mechanism limiting centrosome duplication to once per cell cycle. *Nature* **442:** 947–51

Tsou M-FB, Wang W-J, George K a, Uryu K, Stearns T & Jallepalli PV (2009) Polo kinase and separase regulate the mitotic licensing of centriole duplication in human cells. *Developmental cell* **17:** 344–54

Twomey C, Wattam SL, Pillai MR, Rapley J, Baxter JE & Fry AM (2004) Nek2B stimulates zygotic centrosome assembly in Xenopus laevis in a kinase-independent manner. *Developmental Biology* **265:** 384–398

Upadhya P, Birkenmeier EH, Birkenmeier CS & Barker JE (2000) Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 217–21

Vader G & Lens SM a (2008) The Aurora kinase family in cell division and cancer. *Biochimica et biophysica acta* **1786:** 60–72

Valentine MT, Fordyce PM, Krzysiak TC, Gilbert SP & Block SM (2006) Individual dimers of the mitotic kinesin motor Eg5 step processively and support substantial loads in vitro. *Nature cell biology* **8:** 470–6

van Vugt M a TM & Medema RH (2005) Getting in and out of mitosis with Polo-like kinase-1. *Oncogene* **24:** 2844–59

van Vugt M a TM, van de Weerdt BCM, Vader G, Janssen H, Calafat J, Klompmaker R, Wolthuis RMF & Medema RH (2004) Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. *The Journal of biological chemistry* **279:** 36841–54

Walczak CE & Heald R (2008) Mechanisms of mitotic spindle assembly and function. *International review of cytology* **265:** 111–58

Warnke S, Kemmler S, Hames RS, Tsai H, Hoffmann-rohrer U, Fry AM, Hoffmann I, Feld IN & Le L (2004) Polo-like Kinase-2 Is Required for Centriole Duplication in Mammalian Cells. **14:** 1200–1207

Watanabe N, Arai H, Nishihara Y, Taniguchi M, Watanabe N, Hunter T & Osada H (2004) M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 4419–24

Weerdt BC van de & Medema RH (2006) Polo-Like Kinases A Team in Control of the Division. *Cell Cycle***:** 853–864

Weerdt BCMVD, Vugt MATMV, Lindon C, Kauw JJW, Rozendaal MJ, Klompmaker R, Wolthuis RMF & Medema RH (2005) Uncoupling Anaphase-Promoting Complex/Cyclosome Activity from Spindle Assembly Checkpoint Control by Deregulating Polo-Like Kinase 1. *Molecular and cellular biology* **25:** 2031–2044

Wei R, Ngo B, Wu G & Lee W-H (2011) Phosphorylation of the Ndc80 complex protein, HEC1, by Nek2 kinase modulates chromosome alignment and signaling of the spindle assembly checkpoint. *Molecular biology of the cell* **22:** 3584–94

Weil D, Garçon L, Duménil D, Dauntry F & Kress M (2002) Targeting the Kinesin Eg5 to Monitor siRNA transfection in Mammalian Cells. *Biotechniques***:** 5–9

Weinger JS, Qiu M, Yang G & Kapoor TM (2011) A nonmotor microtubule binding site in kinesin-5 is required for filament crosslinking and sliding. *Current biology : CB* **21:** 154–60

Westwood I, Cheary D, Baxter JE, Richards MW, Rob LM, Montfort V, Fry AM & Bayliss R (2009) Insights into the Conformational Variability and Regulation of Human Nek2 Kinase. *Journal of Molecular Biology* **386:** 476–485

White MC & Quarmby LM (2008) The NIMA-family kinase, Nek1 affects the stability of centrosomes and ciliogenesis. *BMC cell biology* **9:** 29

Wildenberg SMJLVD, Tao L, Kapitein LC & Schmidt CF (2009) homotetrameri kinesin-5, klp61f, preferentially crosslinks microtubules into antiparallel orientations. *Current Biology* **18:** 1860–1864

Wilker EW, van Vugt M a TM, Artim S a, Huang PH, Petersen CP, Reinhardt HC, Feng Y, Sharp P a, Sonenberg N, White FM & Yaffe MB (2007) 14-3-3Sigma Controls Mitotic Translation To Facilitate Cytokinesis. *Nature* **446:** 329–32

Wissing J, Jänsch L, Nimtz M, Dieterich G, Hornberger R, Kéri G, Wehland J & Daub H (2007) Proteomics analysis of protein kinases by target class-selective prefractionation and tandem mass spectrometry. *Molecular & cellular proteomics : MCP* **6:** 537–47

Wu W, Baxter JE, Wattam SL, Hayward DG, Fardilha M, Knebel A, Ford EM, da Cruz e Silva EF & Fry AM (2007) Alternative splicing controls nuclear translocation of the cell cycle-regulated Nek2 kinase. *The Journal of biological chemistry* **282:** 26431–40

Wu Z, Doondeea JB, Gholami AM, Janning MC, Lemeer S, Kramer K, Eccles S a, Gollin SM, Grenman R, Walch A, Feller SM & Kuster B (2011) Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. *Molecular & cellular proteomics : MCP* **10:** M111.011635

Wu Z-Q & Liu X (2008) Role for Plk1 phosphorylation of Hbo1 in regulation of replication licensing. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 1919–24

Xie S, Wu H, Wang Q, Cogswell JP, Husain I, Conn C, Stambrook P, Jhanwar-Uniyal M & Dai W (2001) Plk3 functionally links DNA damage to cell cycle arrest and apoptosis at least in part via the p53 pathway. *The Journal of biological chemistry* **276:** 43305–12

Yang Y, Bai J, Shen R, Brown S a N, Komissarova E, Huang Y, Jiang N, Alberts GF, Costa M, Lu L, Winkles J a & Dai W (2008) Polo-like kinase 3 functions as a tumor suppressor and is a negative regulator of hypoxia-inducible factor-1 alpha under hypoxic conditions. *Cancer research* **68:** 4077–85

Yarm FR (2002) Plk Phosphorylation Regulates the Microtubule-Stabilizing Protein TCTP Plk Phosphorylation Regulates the Microtubule-Stabilizing Protein TCTP. **22:**

Yin M-J, Shao L, Voehringer D, Smeal T & Jallal B (2003) The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis. *The Journal of biological chemistry* **278:** 52454–60

Yissachar N, Salem H, Tennenbaum T & Motro B (2006) Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. *FEBS letters* **580:** 6489–95

Yokoyama H, Gruss OJ, Rybina S, Caudron M, Schelder M, Wilm M, Mattaj IW & Karsenti E (2008) Cdk11 is a RanGTP-dependent microtubule stabilization factor that regulates spindle assembly rate. *The Journal of cell biology* **180:** 867–75

Zalli D, Bayliss R & Fry AM (2012) The Nek8 protein kinase, mutated in the human cystic kidney disease nephronophthisis, is both activated and degraded during ciliogenesis. *Human molecular genetics* **21:** 1155–71

Zhang C, Kenski DM, Paulson JL, Bonshtien A, Sessa G, Cross JV, Templeton DJ & Shokat KM (2005) A second-site suppressor strategy for chemical genetic analysis of diverse protein kinases. *Nature Methods* **2:** 435–441

Zhou T, Aumais JP, Liu X, Yu-Lee L-Y & Erikson RL (2003) A role for Plk1 phosphorylation of NudC in cytokinesis. *Developmental cell* **5:** 127–38

Zimmerman WC & Erikson RL (2007a) Finding Plk3. *Cell Cycle* **6:** 1314–1318

Zimmerman WC & Erikson RL (2007b) Polo-like kinase 3 is required for entry into S phase. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 1847–52

APPENDIXES

-Nek9 in vivo phosphorylation sites

- Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. The EMBO Journal, 2011

Site	References
Ser2	(Oppermann *et al*, 2009; Daub *et al*, 2008)
Ser13	(Oppermann *et al*, 2009; Daub *et al*, 2008) (Kettenbach *et al*, 2012)
Ser16	(Oppermann *et al*, 2009; Daub *et al*, 2008)
Ser20	(Oppermann *et al*, 2009; Daub *et al*, 2008)
Ser28	(Oppermann *et al*, 2009) (Kettenbach *et al*, 2012)
Ser29	(Daub *et al*, 2008; Wissing *et al*, 2007; Grosstessner-Hain *et al*, 2011)(Hegemann *et al*, 2011; Bertran *et al*, 2011; Iliuk *et al*, 2010) (Matsuoka *et al*, 2007) (Kettenbach *et al*, 2011) (Kettenbach *et al*, 2012)
Ser35	(Matsuoka *et al*, 2007)
Tyr52	(Oppermann *et al*, 2009)
Ser76	(Hegemann *et al*, 2011; Daub *et al*, 2008; Oppermann *et al*, 2009)
Lys199	Kim W, et al. (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell 44, 325-40
Thr210	(Bertran *et al*, 2011; Holland *et al*, 2002; Roig *et al*, 2005)
Thr254	(Hegemann *et al*, 2011; Daub *et al*, 2008; Oppermann *et al*, 2009)
Ser331	(Han *et al*, 2010) (Kettenbach *et al*, 2011) (Kettenbach *et al*, 2012)
Ser332	(Iliuk *et al*, 2010; Daub *et al*, 2008; Wissing *et al*, 2007; Dephoure *et al*, 2008; Grosstessner-Hain *et al*, 2011) (Moritz *et al*, 2010) (Kettenbach *et al*, 2011)
Thr333	(Hegemann *et al*, 2011; Bertran *et al*, 2011; Iliuk *et al*, 2010; Olsen *et al*, 2010; Oppermann *et al*, 2009; Dephoure *et al*, 2008; Wissing *et al*, 2007; Beausoleil *et al*, 2006; Oppermann *et al*, 2012) (Moritz *et al*, 2010) (Kettenbach *et al*, 2011) (Kettenbach *et al*, 2011)
Thr335	(Oppermann *et al*, 2009; Wissing *et al*, 2007) (Kettenbach *et al*, 2011)
Ser357	(Kettenbach *et al*, 2011)
Thr358	(Oppermann *et al*, 2009; Wissing *et al*, 2007)
Ser413	(Oppermann *et al*, 2009)
Tyr520	(Iliuk *et al*, 2010)
Ser750	(Bertran *et al*, 2011; Daub *et al*, 2008)
Ser741	(Daub *et al*, 2008)
Ser735	(Wissing *et al*, 2007)
Ser801	(Oppermann *et al*, 2009)
Ser827	(Daub *et al*, 2008; Oppermann *et al*, 2009) (Bertran et al., 2011)
Ser829	(Daub *et al*, 2008; Oppermann *et al*, 2009)
Ser832	(Oppermann *et al*, 2009; Daub *et al*, 2008)
Ser836	(Daub *et al*, 2008)

Ser838	(Oppermann *et al*, 2009)
Ser868	(Grosstessner-Hain *et al*, 2011; Oppermann *et al*, 2009; Daub *et al*, 2008; Wissing *et al*, 2007) (Kettenbach *et al*, 2011) (Kettenbach *et al*, 2012)
Ser869	(Hegemann *et al*, 2011; Bertran *et al*, 2011; Olsen *et al*, 2010; Oppermann *et al*, 2009; Grosstessner-Hain *et al*, 2011) (Kettenbach *et al*, 2011) (Kettenbach *et al*, 2012)
Ser883	(Daub *et al*, 2008)(Kettenbach *et al*, 2011)
Thr886	(Oppermann *et al*, 2009; Daub *et al*, 2008; Hegemann *et al*, 2011) (Kettenbach *et al*, 2011) (Bertran et al., 2011)
Ser944	(Hegemann *et al*, 2011; Regué *et al*, 2011; Oppermann *et al*, 2009; Daub *et al*, 2008)
Ser978	(Oppermann *et al*, 2009; Daub *et al*, 2008) (Kettenbach *et al*, 2011)

Proteomics Analysis of Protein Kinases by Target Class-selective Prefractionation and Tandem Mass Spectrometry
Wissing *et al,* 2007

Peptide	Phosphosites	Cell line
HCDSINSDFGSESGGCGD*SS*PGPSASQG PR	Ser28 or Ser29	Jurkat
HCDSINSDFGSESGGCGD*SS*PGPSASQG PR	Ser28 or Ser29	HCT-116
*S*N*SS*GLSIGTVFQSSSPGGGGGGGGGEE EDSQQESETPDPSGGFR	Ser735 or Ser737 or Ser738	Jurkat
S*S*#TVTEAPIAVVTSR	Ser332	HCT-116
SST#VTEAPIAVVTSR	Ser332/Thr333	Jurkat
S*T*#PQKLDVIK	Thr358	Jurkat
VASEAPLEHKPQVEA*S*#SPR	Ser868	HCT-116
VASEAPLEHKPQVEA*S*#SPR	Ser868	A549
VASEAPLEHKPQVEA*S*#SPR	Ser868	Jurkat

A quantitative atlas of mitotic phosphorylation
Dephoure *et al,* 2008

Assyncronous or mitotic arrested HeLa cells

Peptide	Phosphosite
SST#VTEAPIAVVTSR	Thr333

A probability-based approach for high-throughput protein phosphorylation analysis and site localization
Beausoleil 2008
Nocodazole arrested HeLa cells

Peptide	Phosphosite
SS#TVTEAPIAVVTSR	Ser332

Kinase-Selective Enrichment Enables Quantitative Phosphoproteomics of the Kinome across the Cell Cycle Daub *et al,* 2008
S growing and mitotic arrested human cancer cells. Nek9 more phosphorylated in mitosis than in S phase

Phosphopeptides	Phosphosite	
HCDS#INS#DFGS#ESGGCGDSS#P	Ser13 Ser16	

GPSASQGPR	Ser20 Ser29	
RPRS#S#TVTEAPIAVVTSR	Ser331 Ser332	
SNSSGLSIGTVFQSSS#PGGGGGGG GGEEEDSQQESETPDPSGGFR	Ser750	
RTEDDS#LVVWK	Ser76	more than 4 folds phosphorylated by Plk1
GMEGLIS#PTEAMGNSNGASSSCPG WLRK	Ser793	
ELENAEFIPMPDS#PSPLSAAFSESE KDTLPYEELQGLK	Ser827	
ELENAEFIPMPDS#PS#PLSAAFSES EK	Ser827 Ser829	
ELENAEFIPMPDS#PS#PLS#AAFS#E SEK	Ser827 Ser829 Ser832 Ser836	
VASEAPLEHKPQVEAS#SPR	Ser868	
EEMEMDPKPDLDSDSWCLLGTDSC RPS#L	Ser978	more than 4 folds phosphorylated by Aurora A
GTPLT#PPACACSSLQVEVER	Thr886	
LNPAVTCAGKGT#PLT#PPACACSSL QVEVER	Thr883 Thr886	

Large-scale Proteomics Analysis of the Human Kinome Oppermann *et al,* 2009

Three different cell lines HTC111, MV4-11 and MDA-MB-435S. Nek9 was found phosphorylated in the three cell lines

Phosphopeptide	**Phosphosite**
KLEGGQQVGMHS#K	Ser944
ELENAEFIPMPDS#PS#PLS#AAFSESEKDTLPYEELQG LK	Ser827 Ser829 Ser832
ELENAEFIPMPDS#PSPLSAAFSESEKDTLPYEELQGLK	Ser827
EEMEMDPKPDLDSDSWCLLGTDSCRPS#L	Ser978
KELENAEFIPMPDS#PSPLSAAFSESEKDTLPYEELQGL K	Ser827
EEMEMDPKPDLDSDSWCLLGTDSCRPS#L	Ser978
EEMEMDPKPDLDSDSWCLLGTDS#CRPSL	Ser974
ELENAEFIPMPDS#PS#PLSAAFSESEKDTLPYEELQGL K	Ser827 Ser829

RTFDAT#NPLNLCVK	Thr254
GTMEADRGMEGLISPT#EAMGNSNGASSSCPGWLR	Thr795
GMEGLISPTEAMGNS#NGASSSCPGWLR	Ser801
ELENAEFIPMPDS#P#SPLS#AAFSESEKDTLPYEELQG LK	Ser827 Ser829 Ser832
RPRS#S#TVTEAPIAVVTSR	Ser331 Ser332
S#NSSGLSIGTVFQSSSPGGGGGGGGGEEEDSQQESE TPDPSGGFR	Ser735
TSEVYVWGGGKST#PQKLDVIK	Thr358
AGGGAAEQEELHY#IPIR	Tyr52
HCDS#INS#DFGS#ESGGCGDS#SPGPSASQGPR	Ser13 Ser16 Ser20 Ser28
VASEAPLEHKPQVEAS#SPR	Ser868
KELENAEFIPMPDSPS#PLSAAFSESEKDTLPYEELQGL K	Ser829
KLNSEY#SMAETLVGTPYYMSPELCQGVK	Tyr205
TEDDS#LVVWK	Ser76
S#VLGEYER	Ser2
GTPLT#PPACACSSLQVEVER	Thr886
SST#VT#EAPIAVVTSR	Thr333 Thr335
GTMEADRGMEGLIS#PTEAMGNSNGASSSCPGWLR	Ser793
KLNS#EYSMAETLVGTPYYMSPELCQGVK	Ser203
TFDAT#NPLNLCVK	Thr254
S#STVTEAPIAVVTSR	Ser331
HCDSINSDFGS#ESGGCGDSSPGPSASQGPR	Ser20
HCDS#INS#DFGSESGGCGDSSPGPSASQGPR	Ser13 Ser16
ELENAEFIPMPDS#PSPLSAAFSESEKDTLPYEELQGLK	Ser827
S#VLGEYER	Ser2
HCDS#INS#DFGS#ESGGCGDSSPGPSASQGPR	Ser13 Ser16 Ser20
ELENAEFIPMPDS#PSPLS#AAFSESEKDTLPYEELQGL K	Ser827 Ser832

Phosphoproteome analysis of human liver tissue by long-gradient nanoflow LC coupled with multiple stage MS analysis Han *et al,* 2009

Peptide	Phosphosite
S#STVTEAPIAVVTSR	Ser331

Quantitative Phosphoproteomics Reveals Widespread Full Phosphorylation Site Occupancy During Mitosis. Olsen *et al,* 2010

HeLa S3 cells. They applied high-resolution mass spectrometry–based proteomics to investigate the proteome and phosphoproteome of the human cell cycle on a global scale and quantified 6027 proteins and 20,443 unique phosphorylation sites and their dynamics.

Peptide	Phosphosite	Kinase motif
PTKRPRS#STVTEA	Ser331	CAMK2;PKA
KRPRSST#VTEAPI	Thr333	CAMK2;CK2;PKA/AKT
KPQVEAS#SPRLNP	Ser868	
PQVEASS#PRLNPA	Ser869	Cdk1;Polo box

Quantitative Phospho-proteomics to Investigate the Polo-like Kinase 1-Dependent Phospho-proteome. Grosstender *et al,* 2011

Phosphoproteome in HeLa cells treated with or without Plk1 inhibitor BI4834

Peptide	Site	BI4834
SS#TVTEAPIAVVTSR	Ser332	Not responding
VASEAPLEHKPQVEASS#PR	Ser869	Not responding
VASEAPLEHKPQVEAS#SPR	Ser868	Not responding
HCDSINSDFGSESGGCGDSS#PGPSASQGPR	Ser29	Not responding

Systematic phosphorylation analysis of human mitotic protein complexes Hegermann 2011

Peptide	Phosphosite
YRRTEDDS#LVVWKEV	Ser76
LKRTFDAT#NPLNLCV	Thr254
TKRPRSST#VTEAPIA	Thr333
KPQVEASS#PRLNPAV	Ser869
AGKGTPLT#PPACACS	Thr886
GQQVGMHS#KGTQTAK	Ser944

Quantitative **Phosphoproteomics Identifies Substrates and Functional Modules of Aurora and Polo-Like Kinase Activities in Mitotic Cells** Kettenback *et al,* 2011
HeLa cells treated with Nocodazol, Taxol, BI2536, MLN8054 or AZD1152

								Asynch/Taxol SILAC > 0.15?				
									Nocodazole/Taxol SILAC > 0.3?			
										Unperturbed mitosis/Taxol SILAC > 0.3?		
ModSite	**TAXOL**	**MLN025**	**MLN1**	**MLN5**	**AZDZM**	**BIENTRY**	**BIMITOSIS**	**Asyn**	**Noc**	**Mitosis**	**P-Site**	**Representative Peptide**
Ser28 Ser29	-0.12	0.59	0.29	0.27	-0.04	0.35	0.23	No	Yes	Yes	Ser28	R.HCDSINSDFGSESGGCGDS#SPGPSASQGPR.A
											Ser29	R.HCDSINSDFGSESGGCGDSS#PGPSASQGPR.A
Ser331 Ser332 Thr333 Thr335	0.00	-0.02	-0.41	-0.76	-0.88	0.03	0.21	No	Yes	Yes	Thr333	R.SST#VTEAPIAVVTSR.T
											Thr335	R.SSTVT#EAPIAVVTSR.T
											Ser331	K.RPRS#ST#VTEAPIAVVTSR.T
											Ser332	R.SS#TVTEAPIAVVTSR.T
Ser357 Thr358	N/D	3.78	4.13	1.09	N/D	N/D	N/D	No	N/A	N/A	Thr358	R.TSEVYVWGGGKST#PQKLDVIK.S
											Ser357	R.TSEVYVWGGGKS#TPQKLDVIK.S
Ser868 Ser869	-0.03	-0.03	0.00	-0.08	-0.09	-0.16	-0.10	No	Yes	Yes	Ser869	K.VASEAPLEHKPQVEASS#PR.L
											Ser868	K.VASEAPLEHKPQVEAS#SPR.L
Ser978	-0.12	N/D	-0.53	N/D	N/D	N/D	N/D	Yes	N/A	N/A	Ser978	K.EEMEMDPKPDLDSDSWCLLGTDSCRPS#L.-
Ser13 Ser28 Ser29	N/D	-0.50	0.04	N/D	-0.23	N/D	-2.24	No	N/A	N/A	Ser13 Ser28	R.HCDS#INSDFGSESGGCGDS#SPGPSASQGPR.A

											Ser13 Ser29	R.HCDS#INSDFGSESGGCGDSS#PGPSASQGPR.A
Ser20 Ser29	N/D	N/D	N/D	N/D	N/D	N/D	0.49	N/A	N/A	N/A	Ser20 Ser29	R.HCDSINSDFGS#ESGGCGDSS#PGPSASQGPR.A
Ser331 Ser332 Ser332 Thr333	N/D	N/D	-1.98	N/D	-1.37	N/D	N/D	N/A	N/A	N/A	Ser331 Ser332	K.RPRS#S#TVTEAPIAVVTSR.T
											Ser332 Thr333	K.RPRSS#T#VTEAPIAVVTSR.T
											Ser331 Thr333	K.RPRS#ST#VTEAPIAVVTSR.T
Thr883 Thr886	0.06	N/D	-0.03	-0.33	-0.05	-0.16	N/D	No	N/A	N/A	Thr883 Thr886	R.LNPAVTCAGKGT#PLT#PPACACSSLQVEVER.L

Rapid Determination of Multiple Linear Kinase Substrate Motifs by Mass Spectrometry Kettenbach *et al,* 2012
HeLa cells treated with nocodazole or taxol in presence or absence of BI2536

Nocodazole treated		
pPeptide Sequence	**Phosphosites**	**BI2536/DMSO**
K.RPRS#ST#VTEAPIAVVTSR.T	Ser331 Thr333	0.55
K.VASEAPLEHKPQVEAS#SPR.L	Ser868	0.95
K.VASEAPLEHKPQVEASS#PR.L	Ser869	0.96
R.HCDS#INSDFGSESGGCGDSS#PGPSASQGPRA	Ser13 Ser29	0.22
R.HCDSINSDFGSESGGCGDSS#PGPSASQGPRA	Ser29	1.22
R.SS#TVTEAPIAVVTSR.T	Ser332	1.09
R.SS#TVTEAPIAVVTSR.T	Ser332	0.95
R.SST#VTEAPIAVVTSR.T	Thr333	0.95

Taxol treated		
pPeptide Sequence	**Phosphosites**	**BI2536/DMSO**
K.VASEAPLEHKPQVEASS#PRL	Ser869	0.98
K.VASEAPLEHK^PQVEASS#PRL	Ser869	0.90
K.VASEAPLEHKPQVEASS#PRL	Ser869	0.98
K.VASEAPLEHKPQVEASS#PRL	Ser869	0.90
R.HCDS#INSDFGSESGGCGDSS#PGPSASQGPRA	Ser13 Ser29	0.21
R.HCDSINSDFGS#ESGGCGDSS#PGPSASQGPRA	Ser20 Ser29	1.40
R.HCDSINSDFGSESGGCGDSS#PGPSASQGPRA	Ser29	1.01
R.HCDSINSDFGSESGGCGDSS#PGPSASQGPRA	Ser29	1.19
R.S#STVTEAPIAVVTSRT	Ser331	1.11
R.S#STVTEAPIAVVTSRT	Ser332	1.16
R.SS#TVTEAPIAVVTSRT	Ser332	1.20
R.SST#VTEAPIAVVTSRT	Ser333	1.17

Autophosphorylation sites (Joan Roig and Joseph Avruch, Unpublished Results)

FLAG Nek9 wild type expressed and purified from HEK293T cells

Peptide	Phosphosites
GAFGEAT#LYRRTEDDSLVVWKEVDLTR	Thr67
KLNSEYS#MAET#LVGTPYYMSPELCQGVK	Ser206 Thr210
T#FDAT#NPLNLCVK	Thr250 Thr254
SST#VTEAPIAVVTS#RTSEVYIWGGGK	Thr333 Ser344
GWHTILIVEKVLNSKT#IR	Thr720
VAS*EAPLEHKPQVEAS#SPRLNPAVT#CAGK	Ser855 Ser868 Ser877
KLEGGQQVGMHS#K	Ser944

Systematic and Quantitative Assessment of the Ubiquitin-Modified Proteome Woong *et al,* 2011
Human ubiquitin-modified proteome (ubiquitinome) in HEK 293T and HCT116 cells.

Ubiquitinated peptide	Site
GDYGLAKKLNSEY	Lys199
DYYTPQKVDVPKA	Lys525

The Plk1-dependent Phosphoproteome of the Early Mitotic Spindle Santamaria *et al,* 2010

Gene Name	Phosphorylation ratio (TAL/Control)
Nek9	0.67

The EMBO Journal (2011), 1–14 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
www.embojournal.org

THE EMBO JOURNAL

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

M Teresa Bertran[1,4], Sara Sdelci[1,4], Laura Regué[1], Joseph Avruch[2], Carme Caelles[1,3] and Joan Roig[1,*]

[1]Cell Signalling Research Group, Molecular Medicine Program, Institute for Research in Biomedicine (IRB Barcelona), Barcelona, Spain, [2]Department of Molecular Biology and Medical Services, Massachusetts General Hospital and Department of Medicine, Harvard Medical School, Boston, MA, USA and [3]Department of Biochemistry and Molecular Biology (Pharmacy), Universitat de Barcelona, Barcelona, Spain

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.

The EMBO Journal advance online publication, 3 June 2011; doi:10.1038/emboj.2011.179
Subject Categories: signal transduction; cell cycle
Keywords: centrosome; Eg5; kinase; Nek; Plk1

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

*Corresponding author. Cell Signalling Research Group, Molecular Medicine Program, Institute for Research in Biomedicine (IRB Barcelona), Parc Cientific de Barcelona, c/Baldiri i Reixac, 10-12, 08028 Barcelona, Spain. Tel.: +34 63 829 7563; Fax: +34 93 403 7114; E-mail: joan.roig@irbbarcelona.org
[4]These authors contributed equally to this work

Received: 22 December 2010; accepted: 9 May 2011

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-

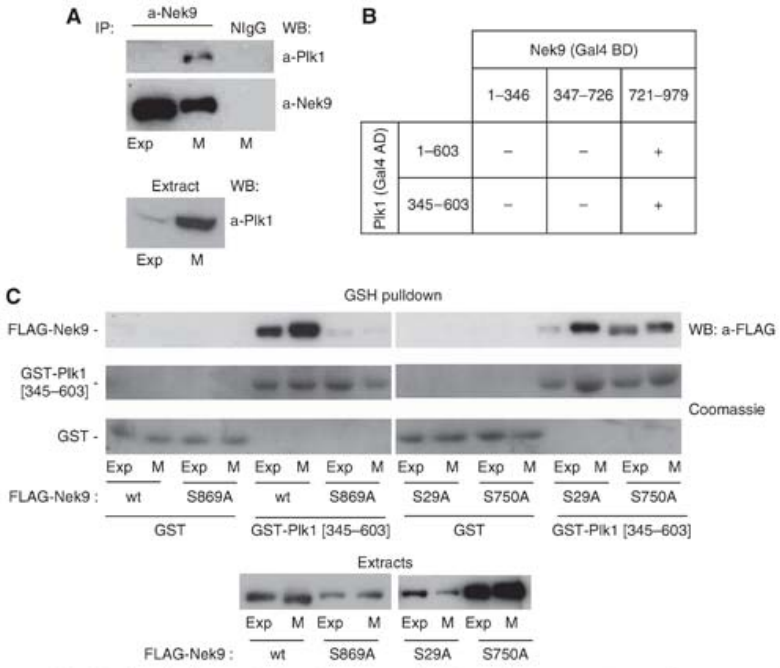


Figure 1 Plk1 interacts with Nek9 through the PBD. (**A**) a-Nek9 or normal IgG (NIgG) immunoprecipitates from exponentially growing (Exp) or nocodazole-arrested mitotic (M) HeLa cell extracts were analysed by western blot (WB) using the indicated antibodies. Plk1 in the corresponding extracts is shown 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

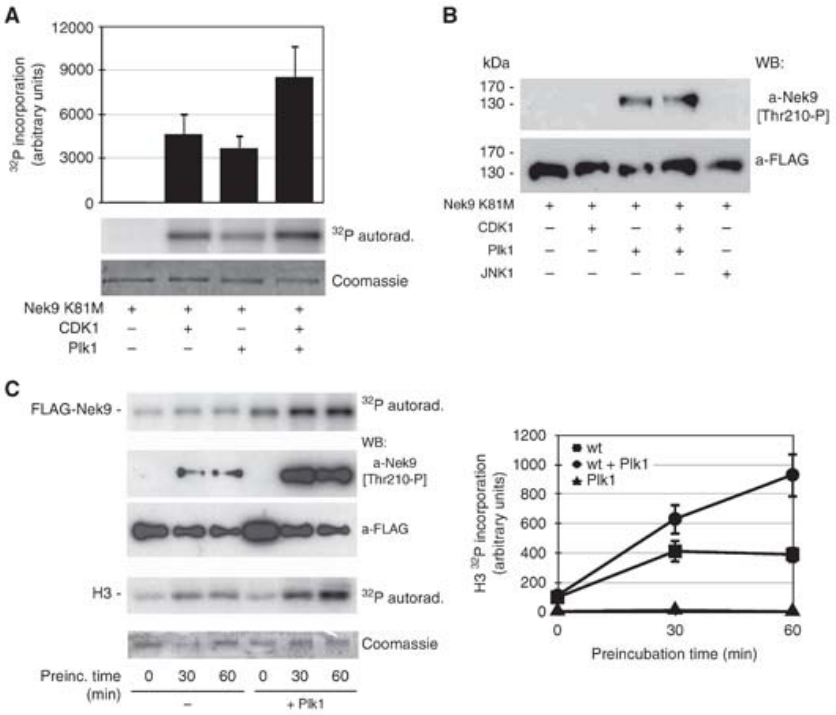


Figure 2 Plk1 phosphorylates and activates Nek9. (**A**) Kinase-defective FLAG-Nek9[K81M] was expressed and purified from 293T cells and incubated with the indicated kinases for 30 min at 30°C in the presence of [γ-^{32}P]ATP/Mg^{2+}. After SDS–PAGE, Nek9 was visualized by Coomassie staining, and ^{32}P 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

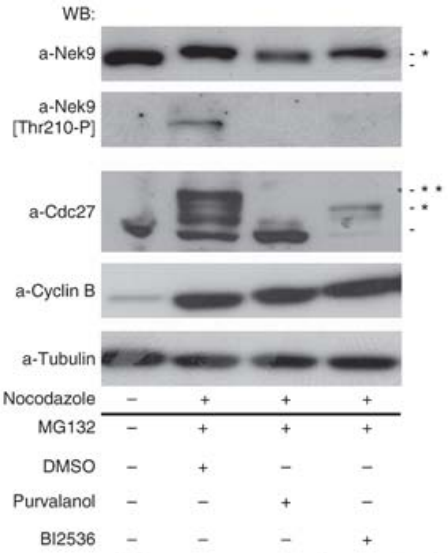


Figure 3 CDK1 and Plk1 are necessary for Nek9 activation during mitosis. HeLa cells were arrested in mitosis with nocodazole. Mitotic cells were collected, washed and released in media containing MG132 (20 μM) plus DMSO, Purvalanol A (20 μM) or Bi2536 (100 nM) for 2 h, and cell extracts were analysed 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.

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.

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

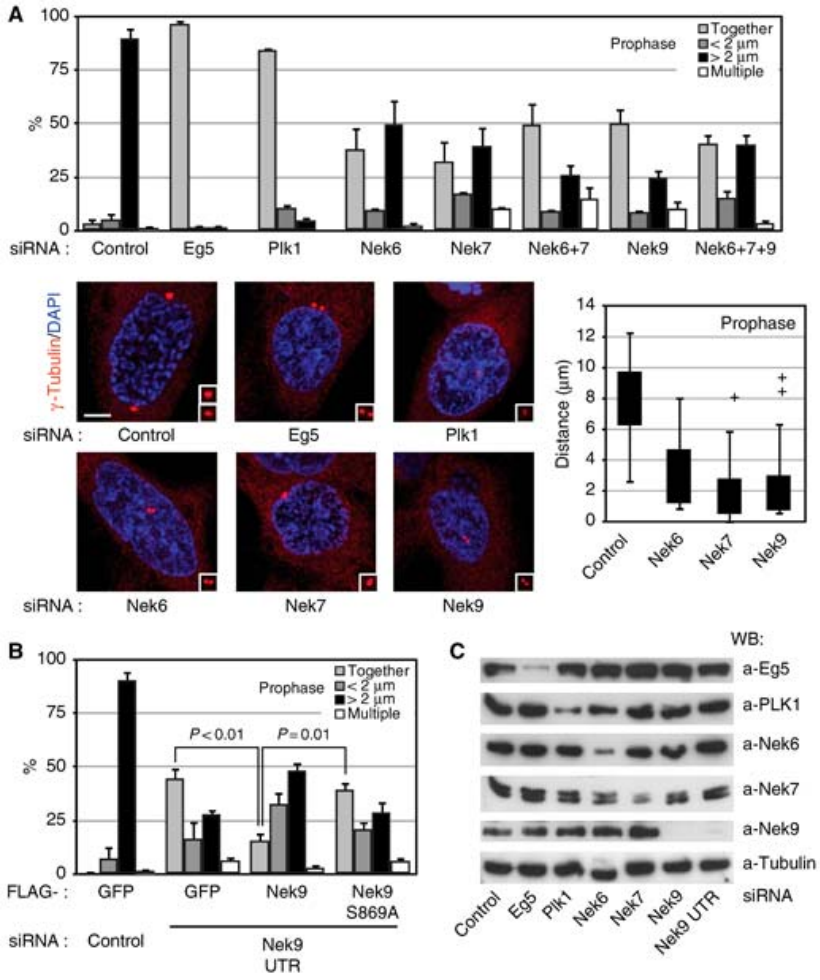


Figure 4 Plk1, Nek9, Nek6, Nek7 and Eg5 are necessary for normal centrosome separation in prophase. (**A**) HeLa cells were transfected with the indicated siRNAs, and after 24 (Eg5, Plk1) or 48 (control, Nek6, Nek7, Nek9) hours, fixed and stained with antibodies against γ-tubulin (red) and DAPI (blue). 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

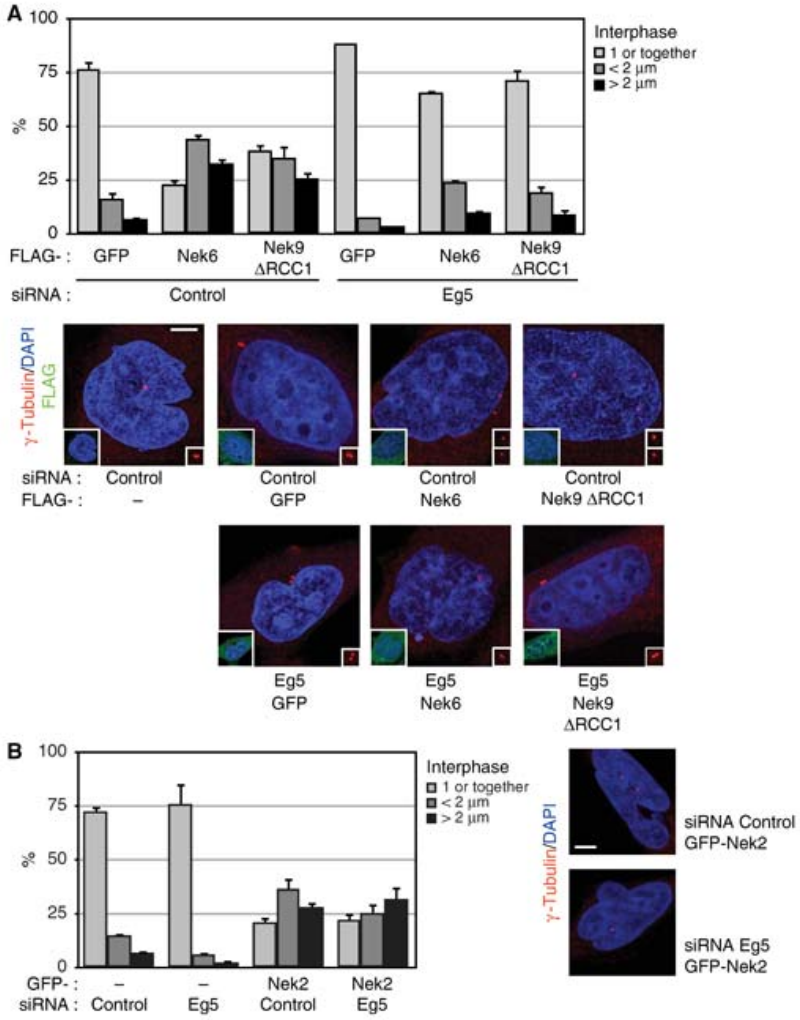


Figure 5 Active Nek9 and Nek6 induce centrosome separation in an Eg5-dependent manner. (**A**) HeLa cells were transfected with either control or Eg5 siRNAs, after 16 h retransfected with expression plasmids for the indicated FLAG-tagged proteins (*Nek9ΔRCC1*, Nek9[Δ346–732]) and 24 h latter fixed and 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.

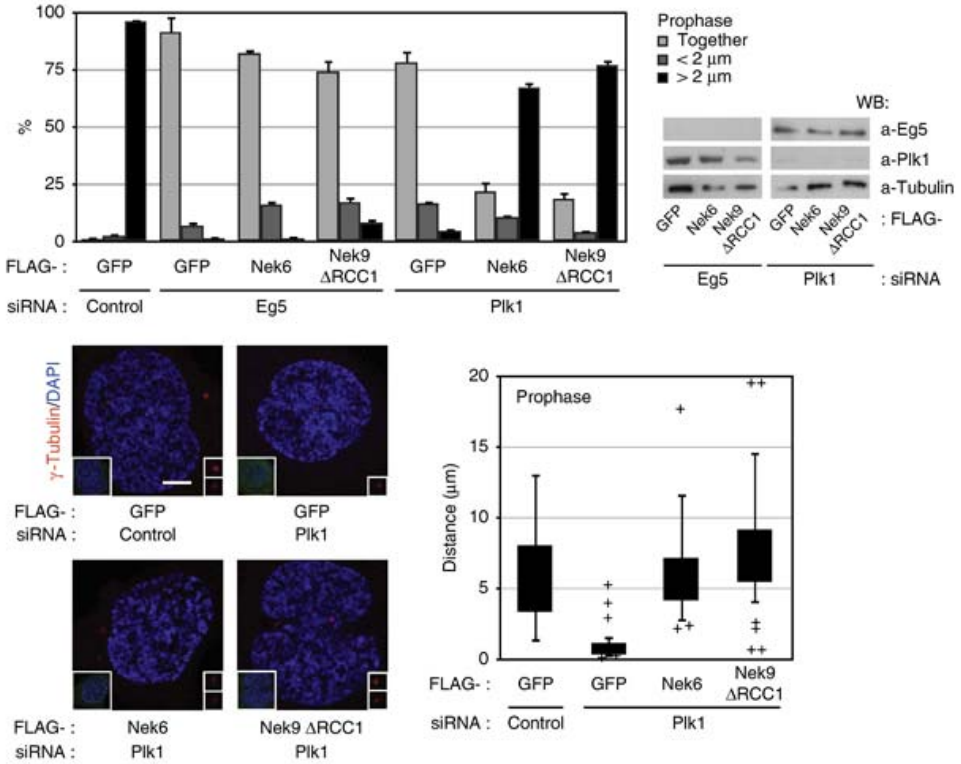


Figure 6 Active Nek9 and Nek6 can rescue Plk1 but not Eg5 downregulation in prophase centrosome separation. HeLa cells were transfected with control, Eg5 or Plk1 siRNAs plus the indicated plasmids and processed as in Figure 5 (*Nek9ΔRCC1*, Nek9[Δ346–732]). The percentage of FLAG-positive prophase cells 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

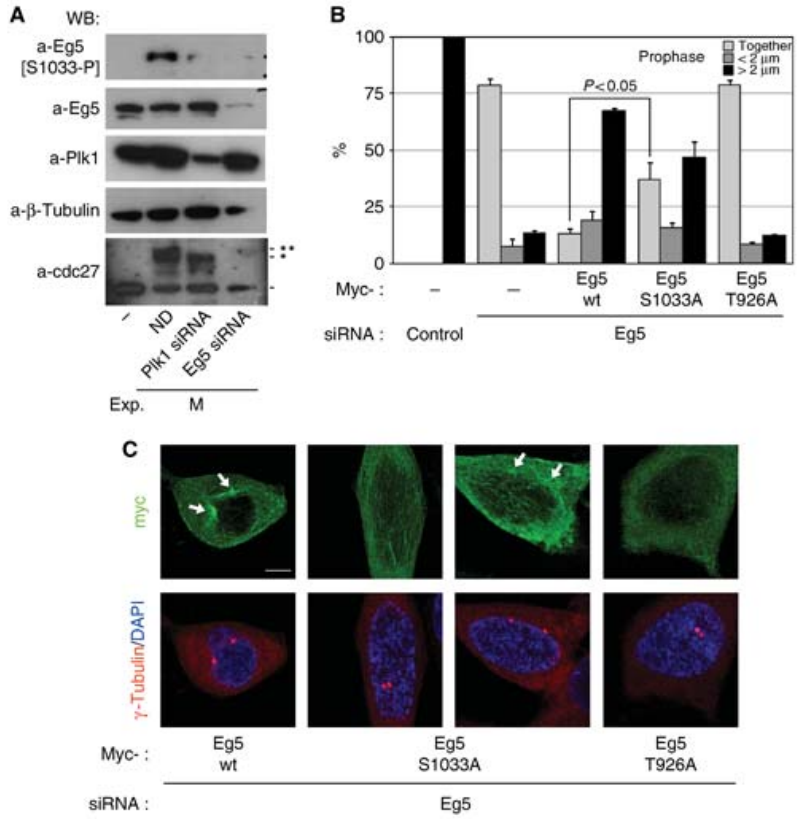


Figure 7 Plk1 controls Eg5 phosphorylation at the Nek6 site Ser1033. Both Ser1033 and the CDK1 site Thr926 phosphorylation are necessary for prophase centrosome separation and Eg5 recruitment. (**A**) HeLa cells were arrested in mitosis by either nocodazole (ND) treatment or RNAi against Plk1 or Eg5 (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 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). 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

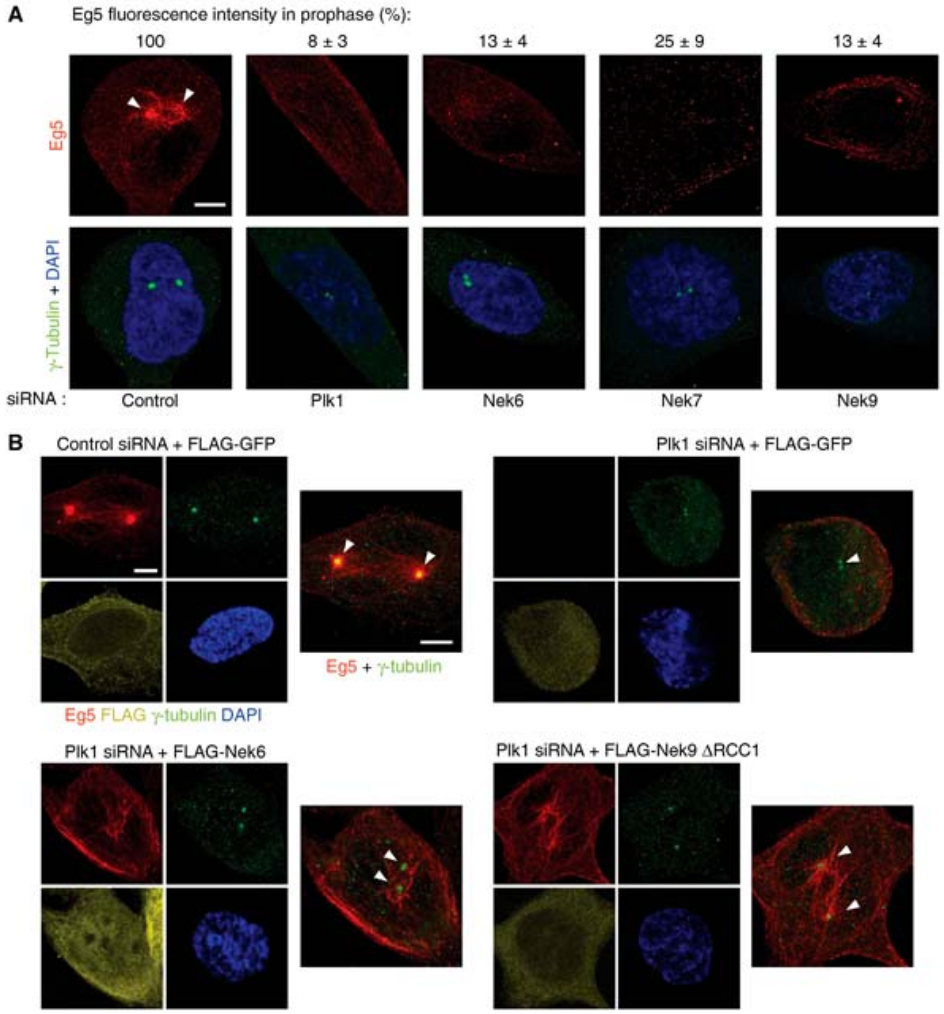


Figure 8 Plk1, Nek9, Nek6, Nek7 are necessary for centrosome recruitment of Eg5 during prophase. Active Nek9 and Nek6 can rescue Plk1 downregulation in prophase Eg5 recruitment. (**A**) HeLa cells were transfected with the indicated siRNAs, and after 24 (Plk1) or 48 (control, Nek6, Nek7, Nek9) hours, 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

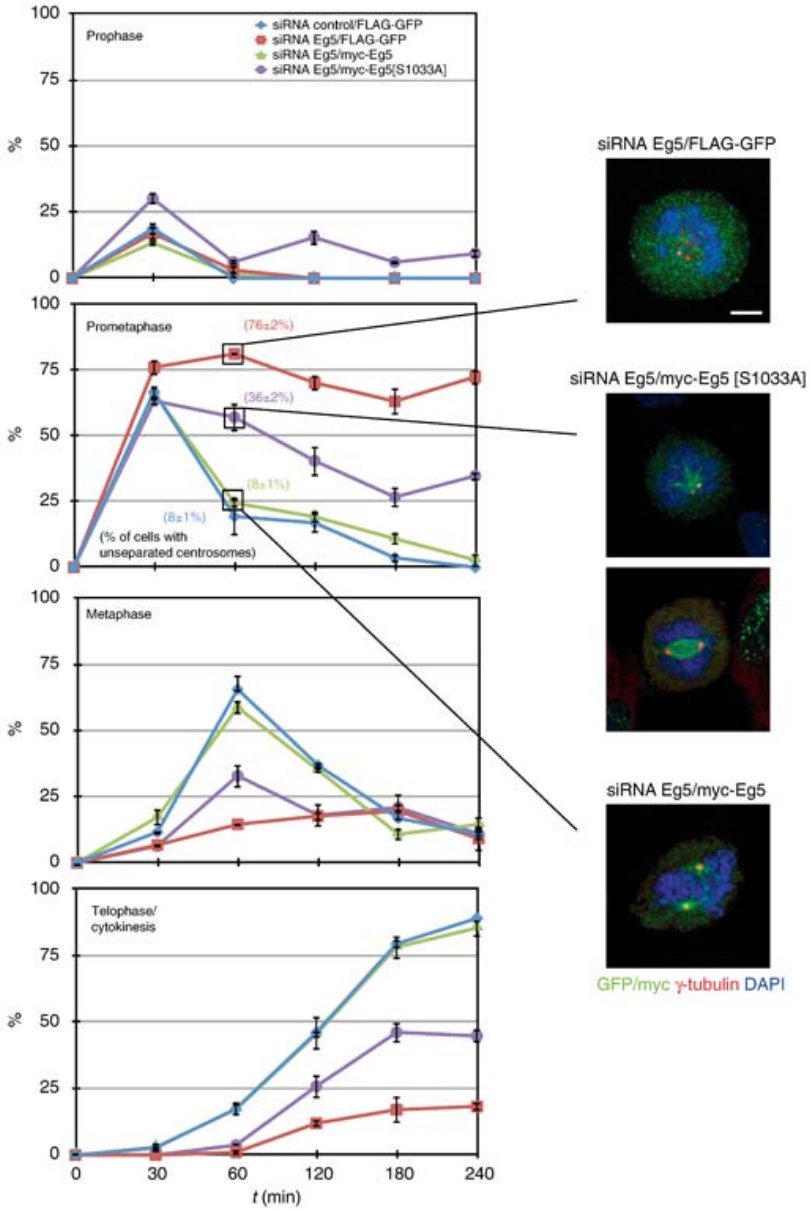


Figure 9 Effects of Eg5[Ser1033Ala] on cell-cycle progression. HeLa cells were transfected with either control or Eg5 siRNAs, after 16 h retransfected with expression plasmids for the indicated Myc-tagged proteins (cDNAs rendered resistant to the siRNA by several silent point mutations). After 24 h, 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

We thank C Vila and other members of the extended Cell Signaling group as well as the staff of the IRB Barcelona Advanced Digital Microscopy Core Facility for their help. We also thank I Vernos (Centre de Regulació Genòmica, Barcelona, Spain) and J Lüders (IRB Barcelona) for comments, A Fry (University of Leicester, Leicester, UK) for GFP-Nek2 and comments and A Groen, R Ohi and the Mitchison Laboratory (Harvard University, Boston, USA) for anti-centrin antibodies. JA acknowledges support from NIH grant DK17776 and institutional funds. JR acknowledges support from the Ramón y Cajal Program and the Plan Nacional I+D Grant BFU2008-03441/BMC (MICINN, Spain), and institutional funds.

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.

References

Archambault V, Glover DM (2009) Polo-like kinases: conservation and divergence in their functions and regulation. *Nat Rev Mol Cell Biol* **10:** 265–275

Belham C, Roig J, Caldwell JA, Aoyama Y, Kemp BE, Comb M, Avruch J (2003) A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. *J Biol Chem* **278:** 34897–34909

Blangy A, Arnaud L, Nigg EA (1997) Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. *J Biol Chem* **272:** 19418–19424

Blangy A, Lane HA, d'Hérin P, Harper M, Kress M, Nigg EA (1995) Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation *in vivo*. *Cell* **83:** 1159–1169

Elia AE, Cantley LC, Yaffe MB (2003) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science* **299:** 1228–1231

Faragher AJ, Fry AM (2003) Nek2A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. *Mol Biol Cell* **14:** 2876–2889

Ferenz NP, Paul R, Fagerstrom C, Mogilner A, Wadsworth P (2009) Dynein antagonizes Eg5 by crosslinking and sliding antiparallel microtubules. *Curr Biol* **19:** 1833–1838

Fry AM, Meraldi P, Nigg EA (1998) A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *EMBO J* **17:** 470–481

Gavet O, Pines J (2010) Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. *Dev Cell* **18:** 533–543

Grallert A, Hagan IM (2002) Schizosaccharomyces pombe NIMA-related kinase, Fin1, regulates spindle formation and an affinity of Polo for the SPB. *EMBO J* **21:** 3096–3107

Gray NS, Wodicka L, Thunnissen AM, Norman TC, Kwon S, Espinoza FH, Morgan DO, Barnes G, LeClerc S, Meijer L, Kim SH, Lockhart DJ, Schultz PG (1998) Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. *Science* **281:** 533–538

Groen AC, Cameron LA, Coughlin M, Miyamoto DT, Mitchison TJ, Ohi R (2004) XRHAMM functions in ran-dependent microtubule nucleation and pole formation during anastral spindle assembly. *Curr Biol* **14:** 1801–1811

Kapitein LC, Peterman EJ, Kwok BH, Kim JH, Kapoor TM, Schmidt CF (2005) The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* **435:** 114–118

Lane HA, Nigg EA (1996) Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J Cell Biol* **135:** 1701–1713

Lénárt P, Petronczki M, Steegmaier M, Di Fiore B, Lipp JJ, Hoffmann M, Rettig WJ, Kraut N, Peters JM (2007) The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr Biol* **17:** 304–315

Llamazares S, Moreira A, Tavares A, Girdham C, Spruce BA, Gonzalez C, Karess RE, Glover DM, Sunkel CE (1991) polo encodes a protein kinase homolog required for mitosis in Drosophila. *Genes Dev* **5:** 2153–2165

Mardin BR, Lange C, Baxter JE, Hardy T, Scholz SR, Fry AM, Schiebel E (2010) Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. *Nat Cell Biol* **12:** 1166–1176

Nigg EA (2001) Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol* **2:** 21–32

O'Connell MJ, Krien MJ, Hunter T (2003) Never say never. The NIMA-related protein kinases in mitotic control. *Trends Cell Biol* **13:** 221–228

O'Regan L, Blot J, Fry AM (2007) Mitotic regulation by NIMA-related kinases. *Cell Div* **2:** 25

O'Regan L, Fry AM (2009) The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. *Mol Cell Biol* **29:** 3975–3990

Oshimori N, Ohsugi M, Yamamoto T (2006) The Plk1 target Kizuna stabilizes mitotic centrosomes to ensure spindle bipolarity. *Nat Cell Biol* **8:** 1095–1101

Petronczki M, Lénárt P, Peters JM (2008) Polo on the rise-from mitotic entry to cytokinesis with Plk1. *Dev Cell* **14:** 646–659

Quarmby LM, Mahjoub MR (2005) Caught Nek-ing: cilia and centrioles. *J Cell Sci* **118:** 5161–5169

Rapley J, Nicolàs M, Groen A, Regué L, Bertran MT, Caelles C, Avruch J, Roig J (2008) The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation. *J Cell Sci* **121:** 3912–3921

Roig J, Groen A, Caldwell J, Avruch J (2005) Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. *Mol Biol Cell* **16:** 4827–4840

Roig J, Mikhailov A, Belham C, Avruch J (2002) Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. *Genes Dev* **16:** 1640–1658

Rosenblatt J (2005) Spindle assembly: asters part their separate ways. *Nat Cell Biol* **7:** 219–222

Salem H, Rachmin I, Yissachar N, Cohen S, Amiel A, Haffner R, Lavi L, Motro B (2010) Nek7 kinase targeting leads to early mortality, cytokinesis disturbance and polyploidy. *Oncogene* **29:** 4046–4057

Sawin KE, LeGuellec K, Philippe M, Mitchison TJ (1992) Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* **359:** 540–543

Sawin KE, Mitchison TJ (1995) Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. *Proc Natl Acad Sci USA* **92:** 4289–4293

Splinter D, Tanenbaum ME, Lindqvist A, Jaarsma D, Flotho A, Yu KL, Grigoriev I, Engelsma D, Haasdijk ED, Keijzer N, Demmers J, Fornerod M, Melchior F, Hoogenraad CC, Medema RH, Akhmanova A (2010) Bicaudal D2, dynein, and kinesin-1 associate with nuclear pore complexes and regulate centrosome and nuclear positioning during mitotic entry. *PLoS Biol* **8:** e1000350

Tanenbaum ME, Macûrek L, Galjart N, Medema RH (2008) Dynein, Lis1 and CLIP-170 counteract Eg5-dependent centrosome separation during bipolar spindle assembly. *EMBO J* **27:** 3235–3245

Tanenbaum ME, Medema RH (2010) Mechanisms of centrosome separation and bipolar spindle assembly. *Dev Cell* **19:** 797–806

Uteng M, Hentrich C, Miura K, Bieling P, Surrey T (2008) Poleward transport of Eg5 by dynein-dynactin in Xenopus laevis egg extract spindles. *J Cell Biol* **182:** 715–726

van Vugt MA, van de Weerdt BC, Vader G, Janssen H, Calafat J, Klompmaker R, Wolthuis RM, Medema RH (2004) Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. *J Biol Chem* **279:** 36841–36854

Weil D, Garçon L, Harper M, Duménil D, Dautry F, Kress M (2002) Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. *Biotechniques* **33:** 1244–1248

Weinger JS, Qiu M, Yang G, Kapoor TM (2011) A nonmotor microtubule binding site in Kinesin-5 is required for filament crosslinking and sliding. *Curr Biol* **21:** 154–160

Whitehead CM, Rattner JB (1998) Expanding the role of HsEg5 within the mitotic and post-mitotic phases of the cell cycle. *J Cell Sci* 111(Part 17)**:** 2551–2561

Woodcock SA, Rushton HJ, Castañeda-Saucedo E, Myant K, White GR, Blyth K, Sansom OJ, Malliri A (2010) Tiam1-Rac signaling counteracts Eg5 during bipolar spindle assembly to facilitate chromosome congression. *Curr Biol* **20:** 669–675

Ye XS, Xu G, Pu RT, Fincher RR, McGuire SL, Osmani AH, Osmani SA (1995) The NIMA protein kinase is hyperphosphorylated and activated downstream of p34cdc2/cyclin B: coordination of two mitosis promoting kinases. *EMBO J* **14:** 986–994

Yissachar N, Salem H, Tennenbaum T, Motro B (2006) Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. *FEBS Lett* **580:** 6489–6495

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.

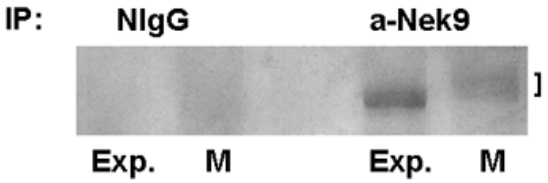
(A) HeLa cells were transfected with the indicated expression plasmids, and after 24h fixed and stained as in Figure 8B. Representative examples of Eg5 distribution in interphase cells are shown. Images show the same field stained with Eg5 (red), γ-tubulin (green), FLAG (yellow) and DAPI (blue), and 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

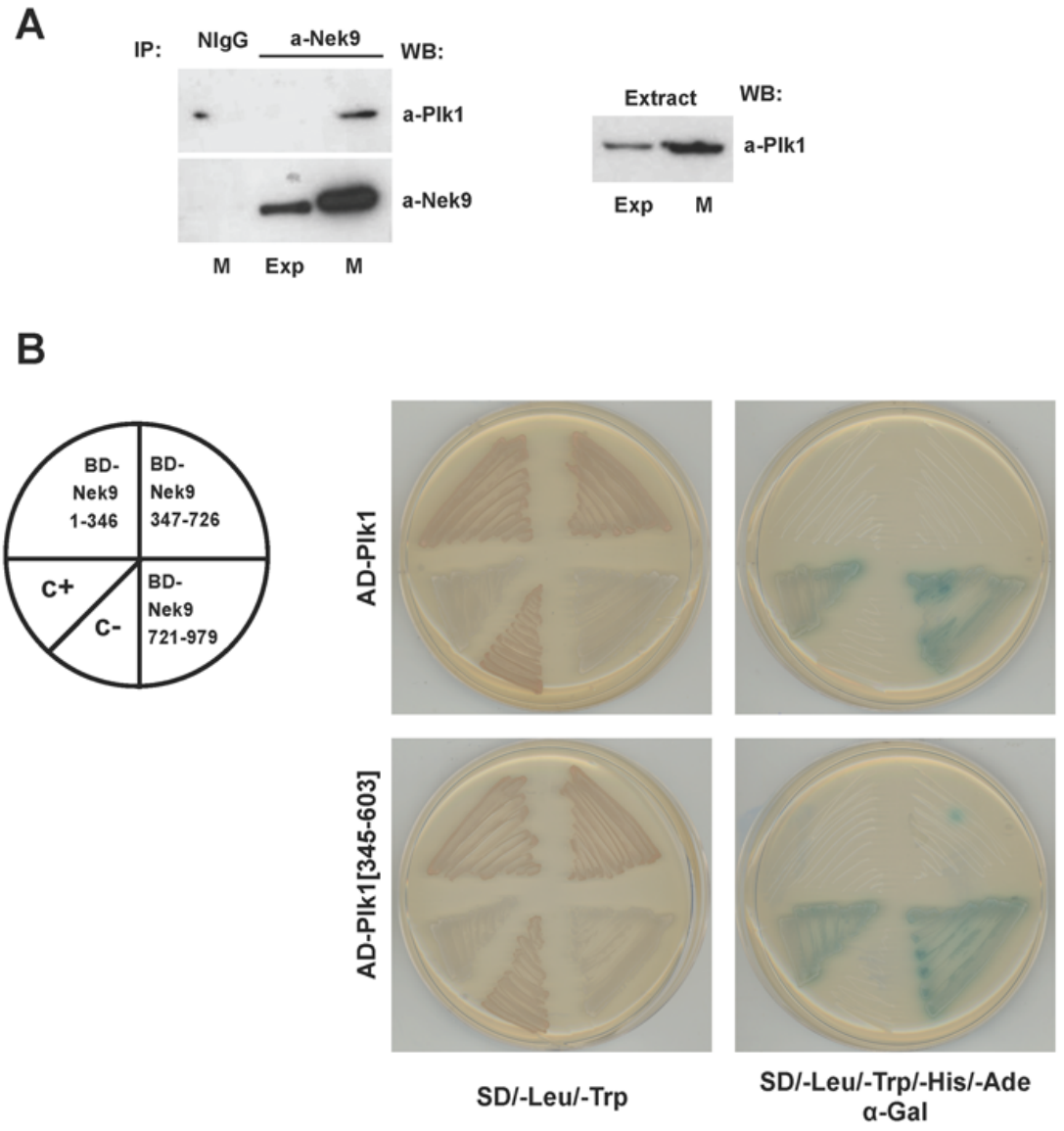

A
IP:
NIgG
a-Nek9
WB:
a-Plk1
a-Nek9
M
Exp
M
Extract
WB:
a-Plk1
Exp
M
B
BD-Nek9 1-346
BD-Nek9 347-726
C+
C-
BD-Nek9 721-979
AD-Plk1
AD-Plk1[345-603]
SD/-Leu/-Trp
SD/-Leu/-Trp/-His/-Ade α-Gal
C
IP: a-FLAG
WB:
a-Plk1
a-FLAG
Extract
a-Plk1
Exp
M
Exp
M
Exp
M
FLAG:
-
Nek9
Nek9 S869A

Figure S3

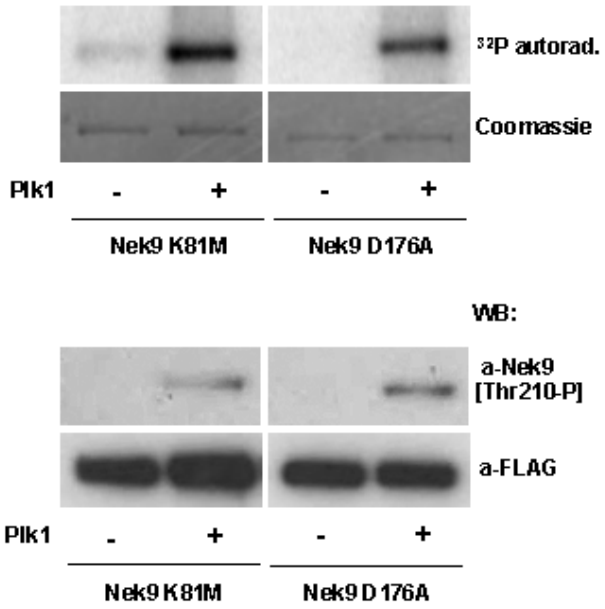
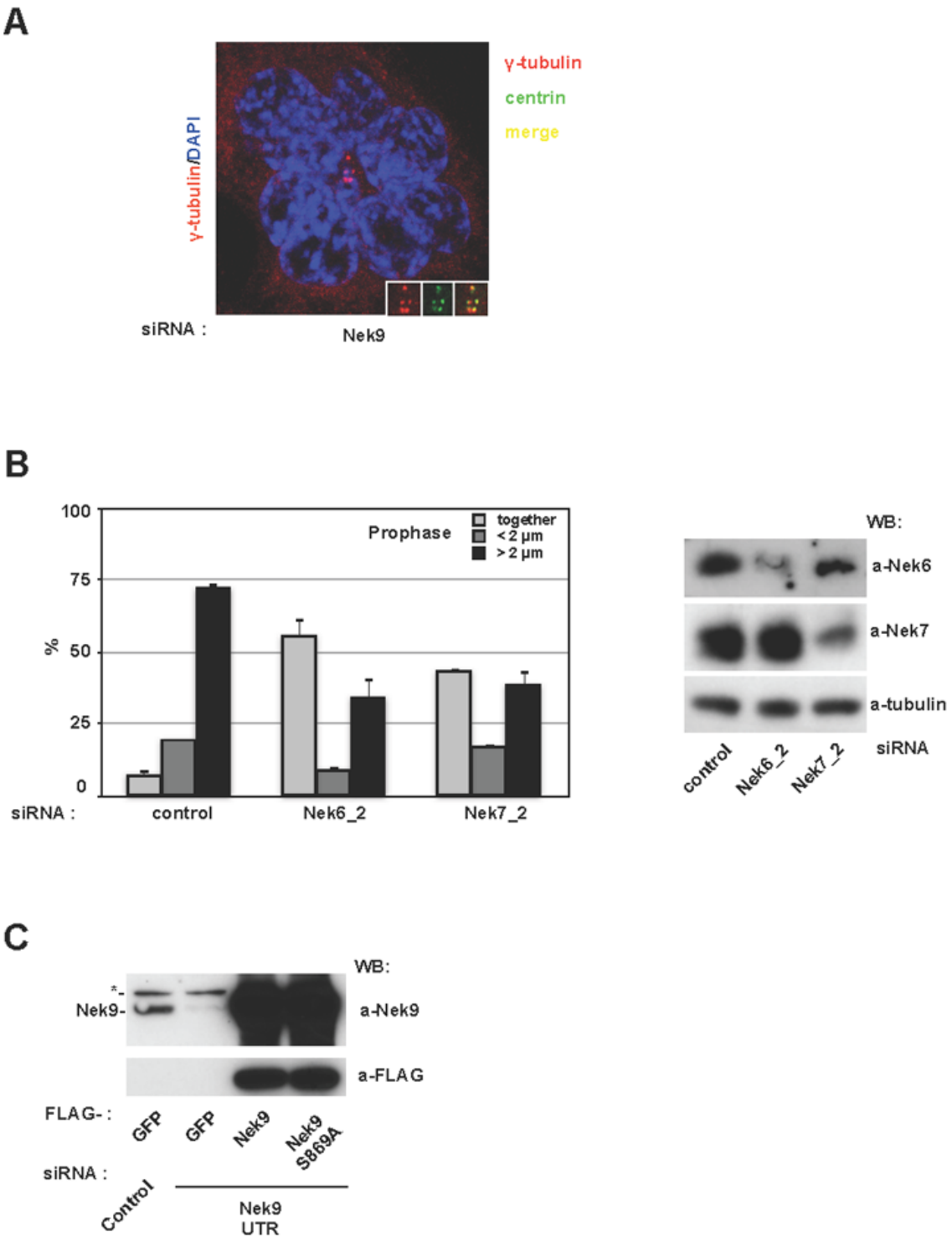

32P autorad.
Coomassie
Plk1 - + - +
Nek9 K81M
Nek9 D176A
WB:
a-Nek9 [Thr210-P]
a-FLAG
Plk1 - + - +
Nek9 K81M
Nek9 D176A

Figure S4


A
γ-tubulin/DAPI
γ-tubulin
centrin
merge
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
siRNA
control
Nek6_2
Nek7_2
C
WB:
*-
Nek9-
a-Nek9
a-FLAG
FLAG- :
GFP
GFP
Nek9
Nek9 S869A
siRNA :
Control
Nek9 UTR

A

Figure S5

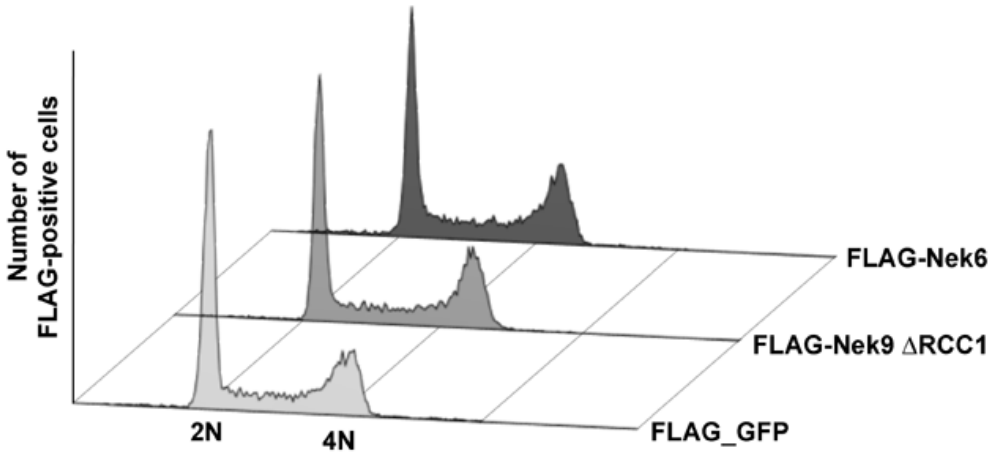

Number of
FLAG-positive cells
2N
4N
FLAG-Nek6
FLAG-Nek9 ΔRCC1
FLAG_GFP

Figure S6

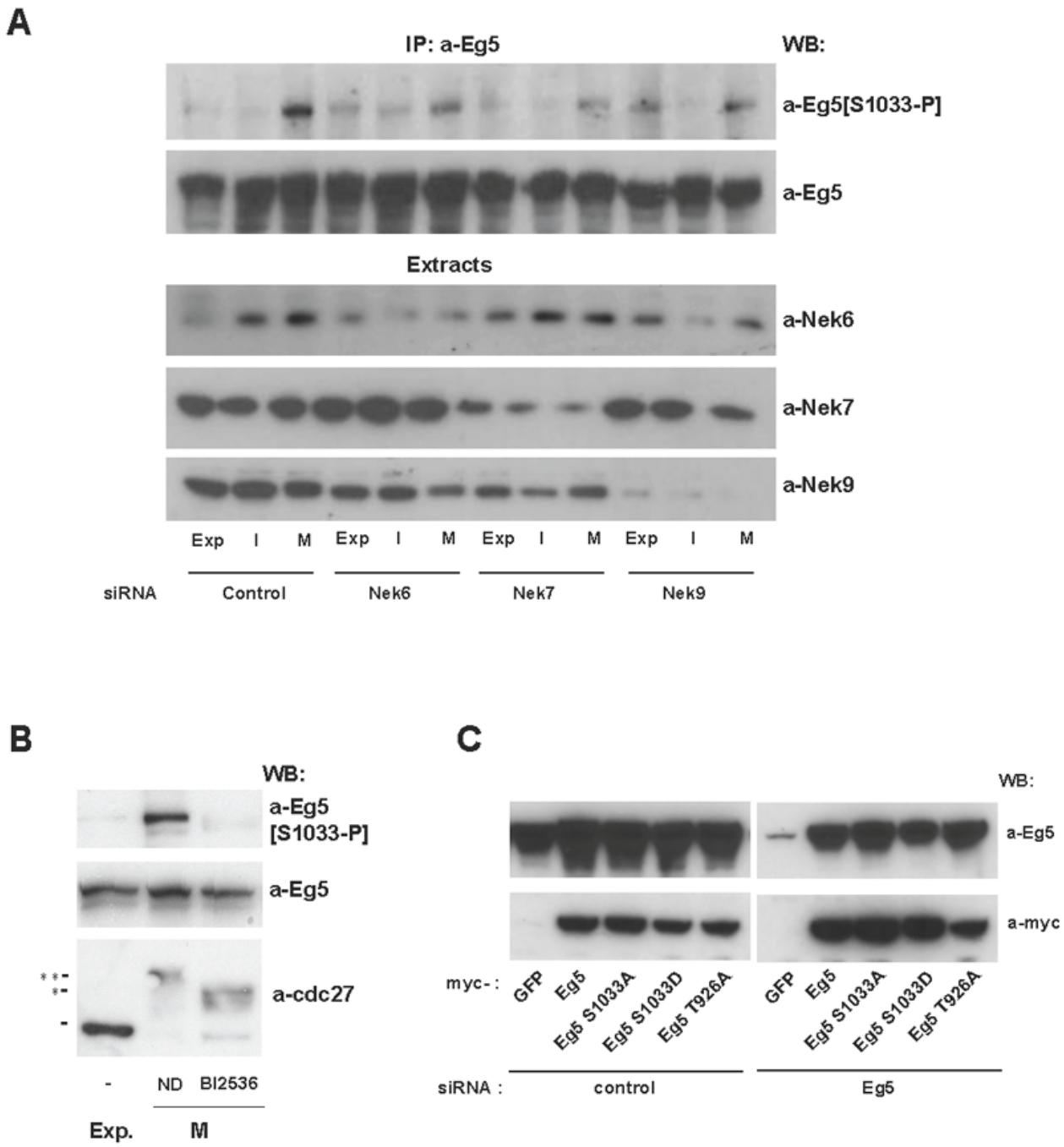

A
IP: a-Eg5
WB:
a-Eg5[S1033-P]
a-Eg5
Extracts
a-Nek6
a-Nek7
a-Nek9
Exp I M Exp I M Exp I M Exp I M
siRNA
Control
Nek6
Nek7
Nek9
B
WB:
a-Eg5 [S1033-P]
a-Eg5
a-cdc27
- ND BI2536
Exp. M
C
WB:
a-Eg5
a-myc
myc- :
GFP
Eg5
Eg5 S1033A
Eg5 S1033D
Eg5 T926A
siRNA :
control
Eg5

Figure S7

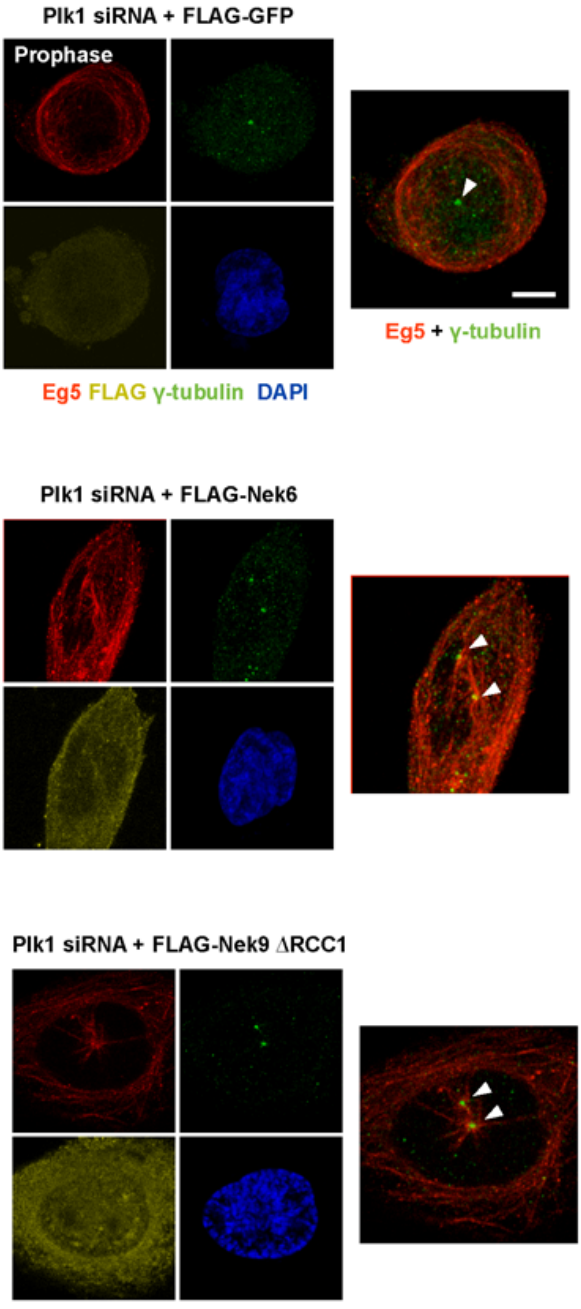

Plk1 siRNA + FLAG-GFP
Prophase
Eg5 + γ-tubulin
Eg5 FLAG γ-tubulin DAPI
Plk1 siRNA + FLAG-Nek6
Plk1 siRNA + FLAG-Nek9 ΔRCC1

Figure S8

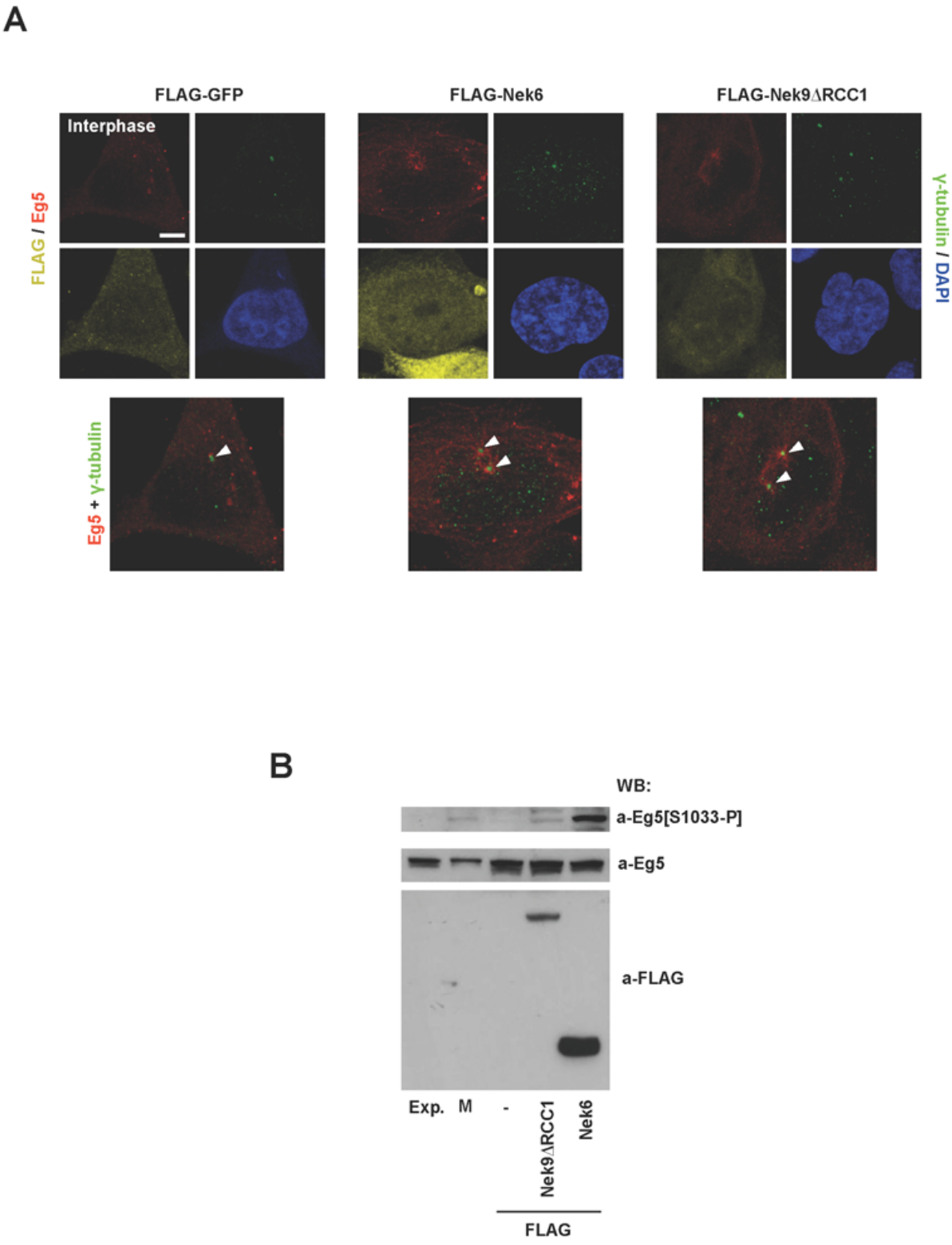
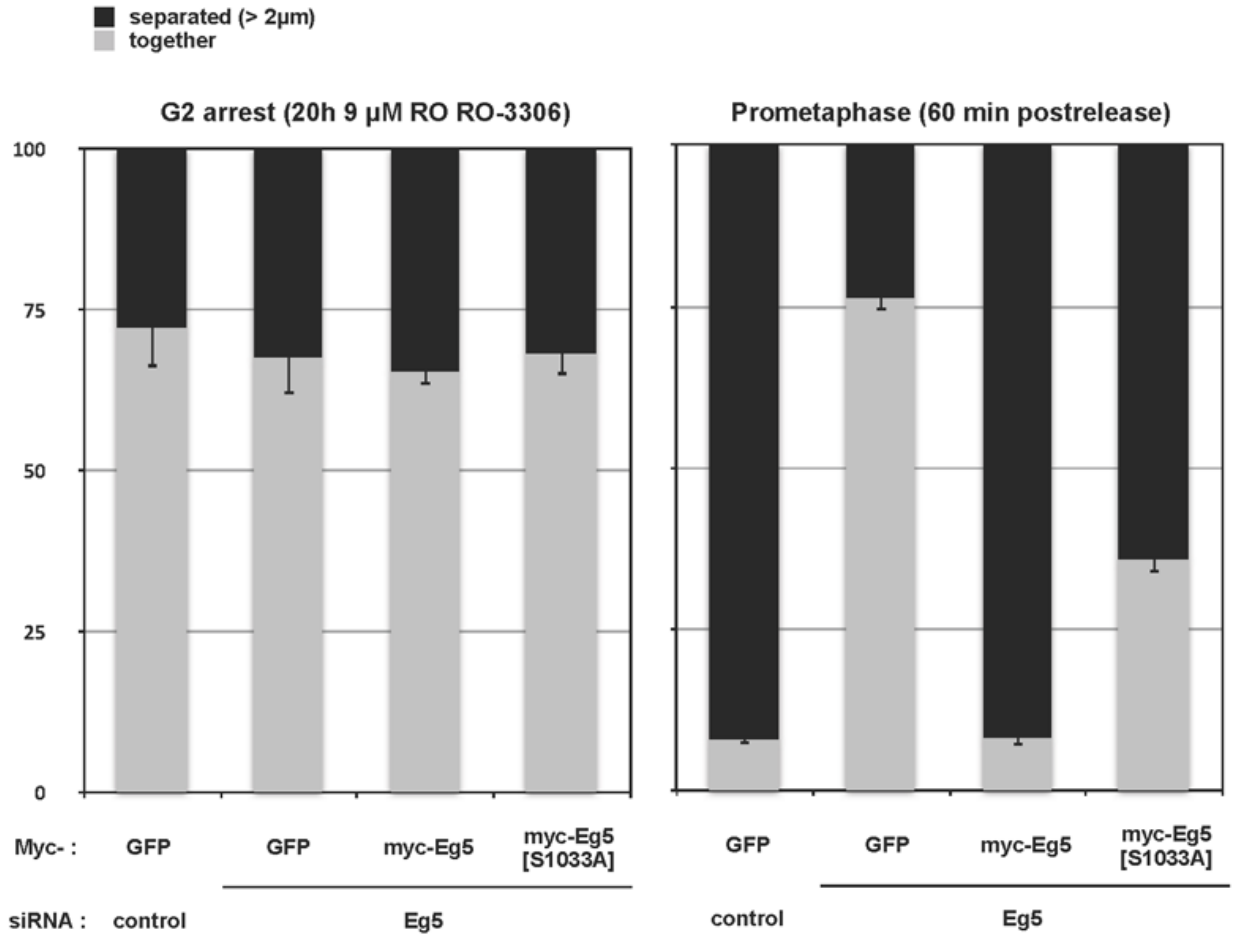

A
FLAG-GFP
FLAG-Nek6
FLAG-Nek9ΔRCC1
Interphase
FLAG / Eg5
γ-tubulin / DAPI
Eg5 + γ-tubulin
B
WB:
a-Eg5[S1033-P]
a-Eg5
a-FLAG
Exp.
M
-
Nek9ΔRCC1
Nek6
FLAG

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