UNIVERSITAT DE BARCELONA

Functional study of the NIMA protein kinases Nek9, Nek6 and Nek7 at the onset of mitosis. Control of the kinesin Eg5 and prophase centrosome separation

Susana Eibes González

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FACULTAT DE FARMÀCIA

Departament de Bioquímica i Biologia Molecular

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CONTROL OF THE KINESIN EG5 AND PROPHASE CENTROSOME SEPARATION

Susana Eibes González, 2016

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CONTROL OF THE KINESIN EG5 AND PROPHASE CENTROSOME SEPARATION

Memòria presentada per Susana Eibes González per optar al títol de doctor per la Universitat de Barcelona

Dr. Joan Roig Amorós

Dr. Carme Caelles Franch

Susana Eibes González

A Gemma

A mis padres

segunda costela flotante

miramos
até onde nos vira o vento

Samuel L. París

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RESUMEN

La mitosis es un proceso altamente regulado cuyo objetivo es asegurar la correcta distribución de los cromosomas entre las dos células nuevamente generadas. Diferentes proteínas quinasas han sido definidas como esenciales en este proceso pero el objetivo de esta tesis es caracterizar una de las rutas de señalización menos estudiada, la cual la componen las NIMA quinasas Nek9, Nek6 y Nek7.

Nek9 es activada al inicio de mitosis por un doble mecanismo mediado por CDK1 y Plk1. Una vez activada, se puede unir a Nek6 y Nek7 y fosforilarlas, promoviendo su activación. Finalmente, Nek6 y Nek7 son responsables de la fosforilación de la quinesina Eg5, promoviendo la acumulación de Eg5 en los centrosomas, y en consecuencia, la separación de los mismos en profase.

Aquí describimos las condiciones necesarias para la acumulación de Eg5 en los centrosomas después de la fosforilación en la Ser1033. Durante el desarrollo de este trabajo hemos explorado las circunstancias esenciales para una correcta localización de Eg5 en las células. Usando técnicas de interacción proteína-proteína y técnicas de silenciamiento proteico de candidatos con shRNA hemos determinado que otra proteína motora, dineína, junto con el adaptador BicD2 y la proteína TPX2, son responsables de la acumulación de Eg5 alrededor de los centrosomas. Además, hemos propuesto a TPX2 como un nuevo substrato regulado por Nek9 y hemos investigado el papel de esta fosforilación, la cual afecta la localización de TPX2 durante profase, antes de la rotura de la membrana nuclear.

Con esta tesis presentamos un modelo para la acumulación de Eg5 y la separación de los centrosomas en profase que puede ser resumido el los siguientes puntos:

- El complejo de dineína transporta Eg5 hacia el centrosoma independientemente de la fosforilación en la Ser1033. El adaptador BicD2 media esta interacción uniéndose directamente al dominio C-terminal de Eg5.
- TPX2 inhibe la movilidad de Eg5 en respuesta a la fosforilación en la Ser1033.
- La presencia de TPX2 en los centrosomas es necesaria para la localización de Eg5. La fosforilación de TPX2 por Nek9 promueve la localización de TPX2 en los centrosomas durante profase.

ABSTRACT

Mitosis is a tightly regulated process that aims to ensure the correct distribution of the chromosomes between the two newly generated cells. Many protein kinases have been defined as essential for this process: cyclin-dependent kinases, Aurora family and Polo family kinases are some of the most relevant players. The objective of this thesis is to characterize one of the less studied kinase pathways involved in this process, which is constituted by the NIMA-related kinases Nek9, Nek6 and Nek7.

Nek9 is activated at the onset of mitosis by a double step mechanism mediated by CDK1 and Plk1. Once Nek9 is activated it can bind to Nek6 and Nek7 and phosphorylate them, promoting their activation. Finally, Nek6 and Nek7 are responsible for the phosphorylation of the kinesin Eg5, promoting Eg5 accumulation at centrosome, and consequently, centrosome separation. The kinesin Eg5 is considered as one of the major players for centrosome separation and formation of the bipolar spindle. The tetramer configuration allows Eg5 to bind antiparallel MTs and slide them apart, exerting a force that promotes centrosome separation and the maintenance of the bipolar spindle.

Centrosome separation, however, is a highly intricate process that involves several pathways, including Eg5 activity. Dynein presents a directed activity towards the minus ends of MTs, which has a redundant role to Eg5 in centrosome separation. Dynein accumulation at the cell cortex and the nuclear membrane, through its adaptor BicD2, is also involved in centrosome tethering at the nuclear envelope, a necessary step prior to separation. Furthermore, dynein can control the position of Eg5 at the spindle via TPX2, an event that could also happen before nuclear envelope breakdown (NEB).

Here we describe the conditions required for Eg5 accumulation at the centrosomes after Ser1033 phosphorylation. During the development of this project we have explored the essential circumstances for correct Eg5 localization in cells. By using protein-protein interaction techniques and shRNA depletion of protein candidates we have determined that another motor protein, dynein, together with the adaptor BicD2 and the protein TPX2 are responsible for Eg5 accumulation around centrosomes. Additionally, we proposed TPX2 as a novel Nek9 substrate and we have investigated the role of this phosphorylation, which affects TPX2 localization during prophase, before NEB.

We present with this thesis a model for Eg5 accumulation at MT minus ends and centrosome separation during prophase summarized in the following points:

- **Dynein complex transports Eg5 towards the centrosome.**

Dynein interacts with Eg5 independently of the Ser1033 phosphorylation. The adaptor BicD2, which interacts directly with Eg5 tail domain, mediates the interaction.

Dynein motility towards MT minus ends and the presence of BicD2 on the complex are required for Eg5 localization at centrosomes. Thus, the dynein complex is required for Eg5 transport to the centrosomes during G2-M transition.

- TPX2 inhibits Eg5 motility in response to Ser1033 phosphorylation.

TPX2 is necessary for the correct localization of Eg5 at centrosomes during prophase. TPX2 mislocalization at centrosomes without altering its overall levels leads to failure in Eg5 localization, therefore the presence of TPX2 at centrosomes during prophase is required for Eg5 localization.

TPX2 interacts with Eg5 during mitosis and the interaction is abolished when the Ser1033 can't be phosphorylated. Thus, TPX2 is able to respond to Eg5 Ser1033 phosphorylation, which we propose is promoting the interaction between these two proteins, and consequently inhibiting Eg5 motility at centrosomal levels.

- TPX2 phosphorylation by Nek9 promotes its centrosomal localization.

Nek9 phosphorylation of TPX2 is responsible for TPX2 localization at the spindle poles during prophase. Nek9 phosphorylates TPX2 at residues that are proximal to a NLS, making TPX2 localization more cytoplasmic and promoting its accumulation to the area where Nek9 is more active, the centrosome.

LIST OF ABBREVIATIONS

3AT	3-Amino-1,2,4-triazole
AD	Activation Domain
APC	Anaphase Promoting Complex
ARHGDIB	Rho GDP-dissociation inhibitor 2
BD	Binding Domain
CAK	CDKs Activating Kinases
CDK1	Cyclin Dependent Kinase 1
CPC	Chromosomal Passenger Complex
D-Box	Degradation-Box
DDR	DNA Damage Response
DIC	Dynein Intermediate Chain
DLC	Dynein Light Chain
DMEM	Dulbecco's Modified Eagle's Medium
EPB 42	Erythrocyte membrane Protein Band 4.2
FBS	Fetal Bovine Serum
FLNA	Filamin A alpha
GSK-3	Glycogen Synthase Kinase-3
IR	Ionizing Radiation
JNK	Jun N-terminal protein kinases
LC/MS/MS	Liquid Chromatography/Mass Spectrometry
LiAc	Lithium Acetate
MAP	Microtubule Associate Protein
MEF	Embryonic Mice Fibroblast
MIEN1	Migration and Enhancer 1
MT	Microtubule
MTOC	Microtubule Organizing Center
NE	Nuclear Envelope
NEB	Nuclear Envelope Breakdown
NIMA	Never in Mitosis A
NLS	Nuclear Localization Signal
P-loop	Phosphate binding loop
PB	Polo-Box
PBD	Polo-Box Domain
PCM	Pericentriolar Material
PEG	Polyethylene Glycol
PEI	Polyethyleneimine
PKD	Polycystic Kidney Disease
Plk1	Polo like kinase 1
RHAMM	Receptor for Hyaluronan Mediated Motility
SAC	Spindle Assembly Checkpoint
SD	Synthetic Dropout
SNX1	Sortin nexin-1
T loop	Activation loop
TNS1	Tensin 1
WB	Western Blot
WT	*Wild Type*
X-α-Gal	5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside,
γ-TuRC	γ-Tubulin Ring Complex

TABLE OF CONTENTS

TABLE OF CONTENTS

INTRODUCTION

The cell cycle and Mitosis

One of basic principles of biology is that all cells arise from a pre-existing cell, being cell reproduction an essential process for the sustainment of life. The cell cycle comprises a series of coordinated cellular events that lead to DNA duplication and cell division with the aim of generating two identical daughter cells. It can be conceptually divided into two different periods, namely interphase (I) and M phase.

Interphase refers to the duration between two consecutive M phases. It is the time that a cell requires to grow and prepare for the next process of division. Interphase can be divided in three stages: G1, S and G2. **G1** starts just right after cell division and is considered the first growth phase. The **S** phase is the period comprising DNA synthesis and during this stage cells duplicate the DNA and the centrosomes. **G2** is the second stage of cell growth and also the moment when cells prepare for division.

The actual period of cell division is called **M** phase. It is divided in two stages: mitosis and cytokinesis. **Mitosis** is the process of chromosome segregation and nuclear division, while **cytokinesis** refers to the physical division of the cell in two daughter cells.

Early embryos of *Drosophila* exemplify the difference between mitosis and cell division. They proceed to nuclear division several times without cytokinesis, resulting in multinucleated cells (a syncytium). Only after 13 nuclear divisions each nucleus becomes surrounded by a separated cytoplasm.

The length of the different phases of the cell cycle varies between species, being interphase the longest period in somatic human cells, where mitosis takes about one hour and cytokinesis starts at the end of M phase, lasting just a few minutes (Morgan 2007).

Walther Flemming described **mitosis** in the late 1870's (Flemming 1878). Flemming defined how a structure colored by basophile dyes, the chromatin,

is condensed in threadlike structures and later separated in two identical half parts by a structure that he called the spindle. This process was followed by the reverse decondensation of the mentioned structures (Paweletz 2001). Nowadays we still use the terminology that Flemming coined to distinguish all the mitotic stages, as mitotic phases are designed by reference to the changes in chromosome and spindle behavior observable through microcopy. Thus, mitosis begins with **prophase**, where DNA starts to condensate and centrosomes get separated; this is followed by **prometaphase**, which starts with a sudden dissolution of the nuclear envelope (NE) in vesicles and continues with spindle assembly; **metaphase**, where the chromosomes are aligned at the equator of the cell and the spindle acquires its characteristic bipolar symmetry; **anaphase**, in which the chromosomes start to separate towards the spindle poles, and **telophase**, where the chromosomes decondense and the NE is reformed (Figure I).

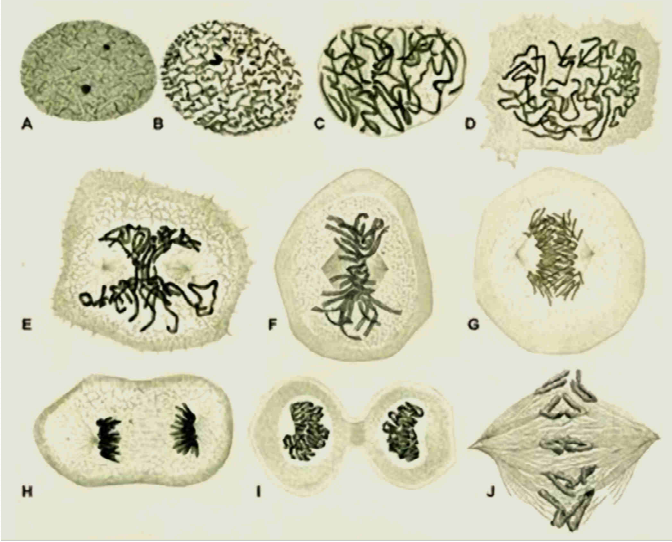

Figure I. Whalter Flemming drawings describing different stages during mitosis.

A-C: prophase. D-E: prometaphase. F: metaphase. G-H: anaphase. I: Telophase. J. Detailed metaphase. *Adapted from "Zellsubstanz, Kern und Zelltheilung" Whalter Flemming, 1878.*

Regulation of mitosis

Although the phases of mitosis have been morphologically well characterized since the XIX century, this morphological characterization presents ambiguities in its terminology, especially when comparing different organisms. For instance, some cells do not condense DNA or al least condensation is not detectable by microscopy while in other cases DNA condensation starts in interphase. Furthermore, in protists, ciliates, algae and fungi the NE is not disassembled and in other organisms, as *C. elegans*, it remains until anaphase. For this reason, C. Rieder and J. Pines proposed a new formula to define mitotic stages based in the molecular pathways that are key to every phase (Pines and Rieder 2001).

- **Transition one** or **antephase***:* This step is defined as a reversible period for preparation for mitosis. It is characterized by the activity of CDK/cyclin A, as well as other kinases such Plk1 and Aurora A. During this period different processes occur, such as chromatin condensation and centrosome maturation, needed for proper microtubule (MT) nucleation. Progress into mitosis can be arrested in this phase by the DNA damage checkpoint.

- **Transition two** or **entry into mitosis**: This stage is defined by the activation of CDK1 and nuclear translocation of cyclin B. It is defined as the non-return point; once cyclin B is accumulated at the nucleus cells cannot revert mitosis and return to the G2 stage.

- **Transition three** or **preparation for exit**: It is characterized by the presence of activated CDK1/cyclin B and Anaphase Promoting Complex (APC). In this stage the APC, an E3 ubiquitin ligase, can promote degradation of cylin A or other substrates (such as Nek2, see below) but not of cyclin B or securin.

- **Transition four** or **mitotic exit**: During this period APC-Cdc20 is activated and in consequence cyclin B and securin are degraded, initiating what has classically been defined as anaphase.

- **Transition five** or **return to interphase**: In this phase Cdc20 is degraded and replaced by Cdh1, amplifying the range of APC substrates (to include among others Cdc20). Cdh1 is finally phosphorylated and degraded before DNA replication.

Mitotic kinases

It can thus be seen that there are two essential molecular mechanisms that guide mitosis: phosphorylation and ubiquitin-mediated protein degradation.

The addition of several units of ubiquitin to a given protein targets it for degradation at the proteasome. Based on this fact, ubiquitination is used during mitosis as a way to regulate the amount of key proteins at different stages of the mitotic process.

Covalent incorporation of phosphate groups to proteins by protein kinases and the posterior elimination of these groups by protein phosphatases is another basic mechanism to regulate protein activity and localization during mitosis. Phosphate groups can be added in one of the three hydroxylated amino acids (Serine, Threonine and Tyrosine) through ATP hydrolysis. Serine and threonine, both aliphatic amino acids, are phosphorylated by the action of **Ser/Thr kinases** and dephosphorylated by **Ser/Thr phosphatases**. Phosphorylation of tyrosine, which contains an aromatic ring, requires enzymes with a larger catalytic site, which are the **Tyr kinase**s and **Tyr phosphatases**. Only in a few cases enzymes present dual reactivity and can (de)phosphorylate both serine/threonine and tyrosine residues.

Under physiological conditions, the incorporation of a phosphate group introduces ~ two negative charges to a protein. This modification can promote conformational changes in local areas of the protein by repulsion with acidic amino acids or attraction to basic residues located nearby. Additionally, phosphate incorporation can inhibit or promote interaction with other proteins.

Almost all protein kinases are evolutionarily related forming part of the eukaryotic protein kinase superfamily (Hanks and Hunter 1995). As a result,

the structure of the catalytic domain is highly conserved among protein kinases. It is characterized by a bilobed structure, comprising an N-terminal and a C-terminal lobe with the substrate binding pocket lying between them. The N-lobe is in charge of ATP coordination and proper positioning at the substrate-binding pocket. This domain includes the phosphate-binding loop (P-loop), a flexible glycine-rich area. The C-lobe contains the activation loop (T-loop), which has several residues that play a critical role in ATP binding, and the catalytic loop, which encloses an acidic amino acid (proton acceptor) that incorporates the leftover proton from the attacking substrate (Lim et al. 2014) (Figure II). Activation after phosphorylation by other kinases (or autophosphorylation) is quite common among these enzymes. Usually, phosphorylation within the activation loop promotes either the exposure of the peptide binding site or the proper positioning of the catalytic residues.

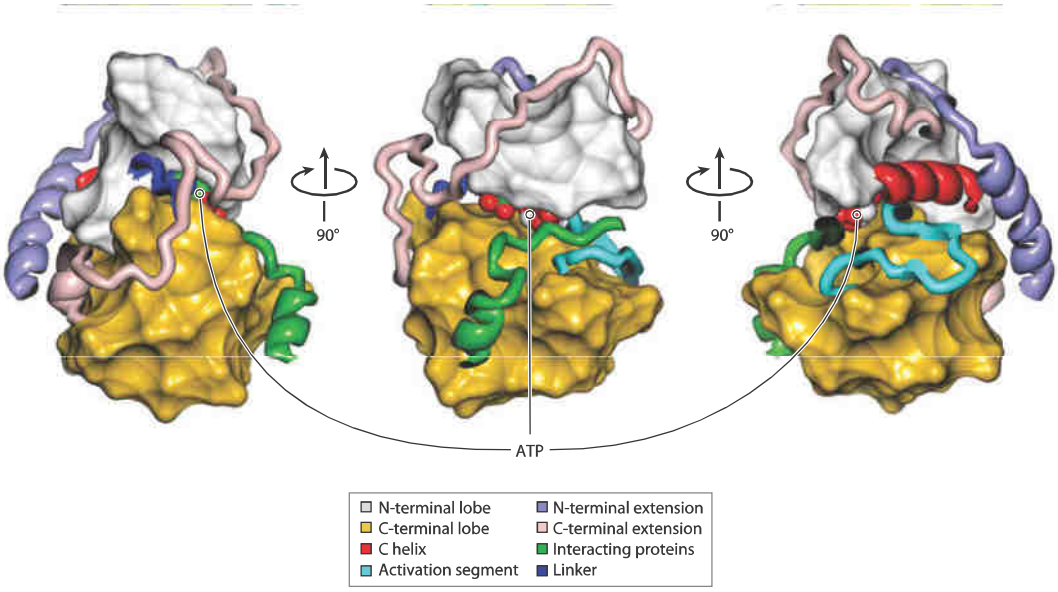

Figure II. Structure of protein kinase catalytic domain. Adapted from (Endicott et al. 2012).

In mammals, four families of kinases are mainly involved in mitosis: Cyclin Dependent Kinases (CDKs), Polo like kinases (Plks), Aurora kinases and NIMA kinases.

The CDK family

CDKs are Ser/Thr kinases that require a cyclin subunit for their activity, as the catalytic pocket of CDKs is only accessible after cyclin binding. Cyclins were named due to the cyclical variation of their concentration during cell cycle. Activity of CDKs is controlled through phosphorylation and either degradation or expression of different cyclins. CDKs preferentially modify serine and threonine residues directly followed by a proline (S/T**P**X) (Malumbres et al. 2009).

Mammals contain 20 CDKs (CDK1 to 20) and 29 cyclin subunits, which perform different functions mainly related with the control of cell cycle progression and transcription. Currently new functions have been proposed for CDKs, such as the control of different aspects of neuronal function, stem cell self-renewal, metabolism, DNA damage repair and epigenetic regulation (Lim and Kaldis 2013).

Although several CDKs are involved in the regulation of cell cycle progression, CDK1 is the only essential for proliferation (Malumbres and Barbacid 2009) (Figure III).

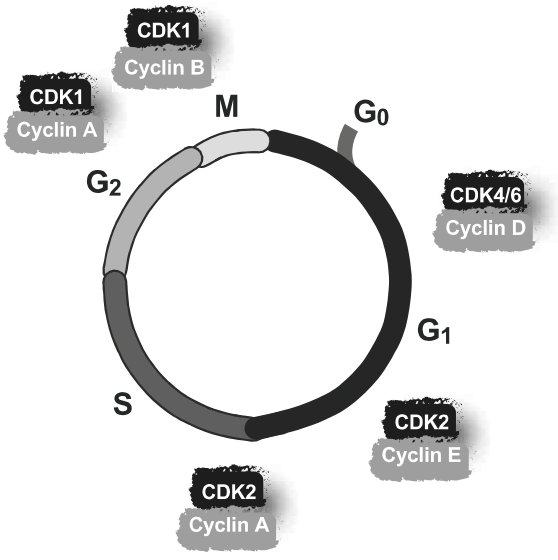

Figure III. CDKs and respective cyclins involved in cell cycle.

As noted above, the levels of CDK1 activity define some of the most important functional transitions that occur during mitosis.

Like for other CDKs, in addition to binding to the cyclin subunit, phosphorylation and dephosphorylation of CDK1 play a role in the control of kinase activity. Thus, phosphorylation at residues T14 and Y15 by Wee1 and Myt1 inhibits CDK1 activity whereas phosphorylation at T161 by CDKs Activating Kinases (CAKs) promotes the activation. At the same time dephosphorylation by Cdc25 of inhibitory residues rescues CDK1 activity (M Malumbres 2014).

During mitotic entry, Cdc25 phosphatase eliminates inhibitory phosphates from CDK1, promoting positive feedback loops of activation that are essential for M phase development (see below). Once CDK1 is active, cyclin B is phosphorylated hiding the nuclear export signal and enhancing the nuclear import signal, resulting in CDK1/cyclin shuttling to the nucleus. This translocation of the complex is required to accomplish mitotic entry (Lindqvist et al. 2009).

CDK1 bound to cyclin A (late G2) and cyclin B (M phase) phosphorylates more than 70 substrates and is the key regulator of the different processes occurring during G2, the G2/M transition and mitosis, such as centrosome separation and centrosome maturation, chromosome condensation, Golgi dynamics and nuclear envelope breakdown (NEB). At metaphase CDK1 activity is shut down, due to cyclin B degradation by the APC complex, and its substrates begin to be dephosphorylated to allow chromosome segregation, chromosome decondensation, re-assembly of the NE and cytokinesis (Malumbres 2014).

The Polo-like kinase family

Mammalian Polo-like kinases (Plks) are named after the *Drosophila* protein Polo, whose absence results in mitotic problems and abnormal spindle formation (Llamazares et al. 1991).

Plks belong to Ser/Thr kinase group and present two domains that define them. The kinase domain is located at the N-terminal side of the protein whereas the C-terminal contains the Polo-Box Domain (PBD), which is composed usually of two Polo-Box (PB) motifs that mediate protein interactions and regulate the phospho-selectivity of the enzyme. Mammalian cells contain five polo family kinases named Plk1 to 5. Plk4 is the most divergent kinase in the family. Whereas the rest of the members of the family are characterized by two consecutives PB in their C-terminal PBDs, Plk4 presents only one PB and two additional cryptic PB forming the so-called Cryptic PBD. On the other hand, Plk5 is a pseudokinase involved in neuronal differentiation and tumor suppression (de Cárcer et al. 2011).

Plks recognize, through their PBDs, sequence motifs of phosphorylated serine or threonine followed by a proline [pS/pT]PX with some preference for S[pS/pT]PX (Elia et al. 2003). This motif corresponds to optimal phosphorylation sequence of CDKs, MAP kinases, and Glycogen Synthase Kinase-3 (GSK-3). Thus, Plks may act sequentially to these kinases, whose phosphorylation generates new PBD-binding motifs that will guide interaction with substrates and importantly control subcellular localization.

The structure of Plks provides two non-exclusive models of signaling. One is the processive model, in which the Polo kinase recognizes a PBD-binding motif (phosphorylated by, for example, CDK1/cyclin B) in the same protein that later phosphorylates. The second one is the distributive model, in which the Plk recognizes a phosphorylated scaffold protein that allows posterior phosphorylation of a protein in the vicinity (Lowery et al. 2005).

The functions of Plks vary between the members of the family. Plk4 and Plk2 are involved in centriole biogenesis; in fact, Plk4 is only present in organisms with centrioles. Plk3 is involved in DNA replication and in G1/S and G2/M phase transition. Plk5 is an inactive kinase and also lacks some important residues in the PBD. Its function, as mentioned before, may be related to neuronal differentiation (Zitouni et al. 2014).

Plk1 functions are central to the control of cell cycle progression. This kinase controls centrosome maturation, mitotic entry, spindle assembly,

correct MT attachment to kinetochores, the regulation of the APC/C, cytokinesis and centrosome disengagement (Zitouni et al. 2014).

Plk1 activation requires phosphorylation at Thr210, which is located in the catalytic domain of the protein. Aurora A, another mitotic kinase, can modify this site (see below) although whether this phosphorylation is due to Plk1 autophosphorylation or Aurora A activity remains unclear (Paschal et al. 2012). Plk1 preferentially phosphorylates substrates with [D/E]X[S/T] motif, followed by an hydrophobic amino acid (Nakojima et al. 2003).

Moreover, Plk1 is overexpressed in many human tumors and has been proposed to be a marker for metastatic risk. For this reason, several molecules have been reported as Plk1 inhibitors. Interestingly, despite Plk1 known role during mitotic entry, these inhibitors arrest cells in prometaphase but not in G2, showing the kinase is dispensable for the G2/M transition (Strebhardt and Ullrich 2006).

The Aurora family

This family of kinases was first identified in *Drosophila* after a genomic screening with the aim to identify novel genes involved in bipolar spindle assembly. (Glover et al. 1995).

Three kinases belong to the Aurora family in mammals, Aurora A, B and C. Aurora kinases contain a regulatory domain at the N-terminus and a catalytic Ser/Thr kinase domain at the C-terminus. The different Auroras present a 75% of similarity between their catalytic domains and mainly differ in their regulatory domains or N-terminus.

Aurora A and B control different processes during mitosis in cycling cells whereas Aurora C, which is differentially expressed in testis, has an important role in meiotic cells (Vader and Lens 2008).

Activation of Aurora kinases is regulated, in a similar way to the kinases described above, by phosphorylation in the T-loop (Thr288 in human Aurora A and Thr232 in human Aurora B) and the action of different proteins that

facilitate the kinase autophosphorylation at this motif, see below (Carmena et al. 2009).

Aurora A

Aurora A activation is regulated by different cofactors during the G2/M transition and mitosis. At centrosomes, two proteins bind Aurora A and facilitate its activation. **Ajuba** is a MAP protein that binds to the regulatory domain of Aurora A and promotes autophosphorylation of the kinase (Bai et al. 2014) and is also required for Aurora centrosomal localization (Sabino et al. 2011). **Bora** is another partner of this kinase; its interaction with Aurora is required for phosphorylation of some substrates (such as Plk1) at the onset of mitosis (Hutterer et al. 2006). Bora is nuclear during interphase, but during the G2/M transition is translocated to the cytoplasm in a CDK1 dependent manner. This protein is essential for Plk1 activation in G2 and early mitosis (Bruinsma et al. 2014), it binds to Plk1 and allows its Thr210 phosphorylation by Aurora A. Once the cell enters in mitosis, Bora is degraded in a Plk1 dependent manner (Chan et al. 2008).

Moreover, **TPX2** (see below) is another important cofactor that has been shown to promote Aurora A activation and targeting to the spindle after NEB. This MAP binds to Aurora A, changes its conformation and protects it from dephosphorylation of the T-loop by PP1 (Bayliss et al. 2003).

Aurora A localizes at centrosomes during interphase and is present at the poles and spindle during mitosis. This kinase has several mitotic functions that may vary in importance in different organisms. Initially it regulates mitotic entry through phosphorylation and activation of Cdc25, the phosphatase that reverts CDK1/cyclin B inactivation by phosphorylation. Additionally, Aurora activity is required for proper activation of Plk1 at the onset of mitosis (Hochegger et al. 2013). Moreover, Aurora regulates centrosome maturation promoting recruitment of different proteins to spindle poles (Joukov et al. 2014), centrosome separation and spindle dynamics by promoting MCAK depolymerase localization at spindle poles (Zhang et al. 2008).

This kinase preferentially phosphorylates residues contained in the following motif: RRX[S/T] (Kettenbach et al. 2011).

Aurora B

As Plk1 and Aurora A, Aurora B localizes in a very specific manner during mitosis. It is present at centromeres during prometaphase and metaphase. During anaphase changes its localization to the spindle midzone and finally, during cytokinesis accumulates at the midbody (Keen and Taylor 2004). Together with INCENP, Surivivn and Borealin, which bind to Aurora B promoting its activation and recruitment to the centromeres, the kinase forms the so-called Chromosomal Passenger Complex (CPC).

The CPC plays essential roles during mitosis, which are directly related with Aurora B localization at the different stages of mitosis: correction of chromosome attachment to MTs errors, activation of the Spindle Assembly Checkpoint (SAC), and formation and regulation of the contractile apparatus that drives cytokinesis (Carmena et al. 2009).

The mitotic entry network

Cells decide to enter or not into mitosis in late G2. Mechanisms as the G2 checkpoint ensure that the cells are ready to start mitosis without damage. The final objective of the control pathways that compose the G2 checkpoint is to interrupt the molecular machinery involved in mitotic entry and, in case of DNA damage, gain some time to solve the damage before cell division. Mitotic entry is defined by an increased activity of CDK1/cyclin B and therefore, this protein complex is the final target of G2 checkpoint pathways (Rieder 2011).

CDK1 activation is supported by the action of Aurora A and Plk1. Plk1 promotes the degradation of the CDK1 inhibitor Wee1 and the activation of the CDK1 activating phosphatase Cdc25C. At the same time Plk1 activity induces Aurora A accumulation at centrosomes at late G2. On the other hand, Aurora A activates Plk1 by phosphorylation at Thr210 and promotes the

activation of Cdc25B phosphatase and cyclin B recruitment. Finally, Aurora A activation is also affected by CDK1 activity.

Thus, mitotic entry is conformed by a network of events in which every kinase supports the activation of the other ones (Figure IV). However, Aurora A and Plk1 are dispensable for mitotic entry in normal conditions, but they are more relevant for mitotic entry after G2 checkpoint arrest induced by DNA damage ((Lens et al. 2010) and references therein).

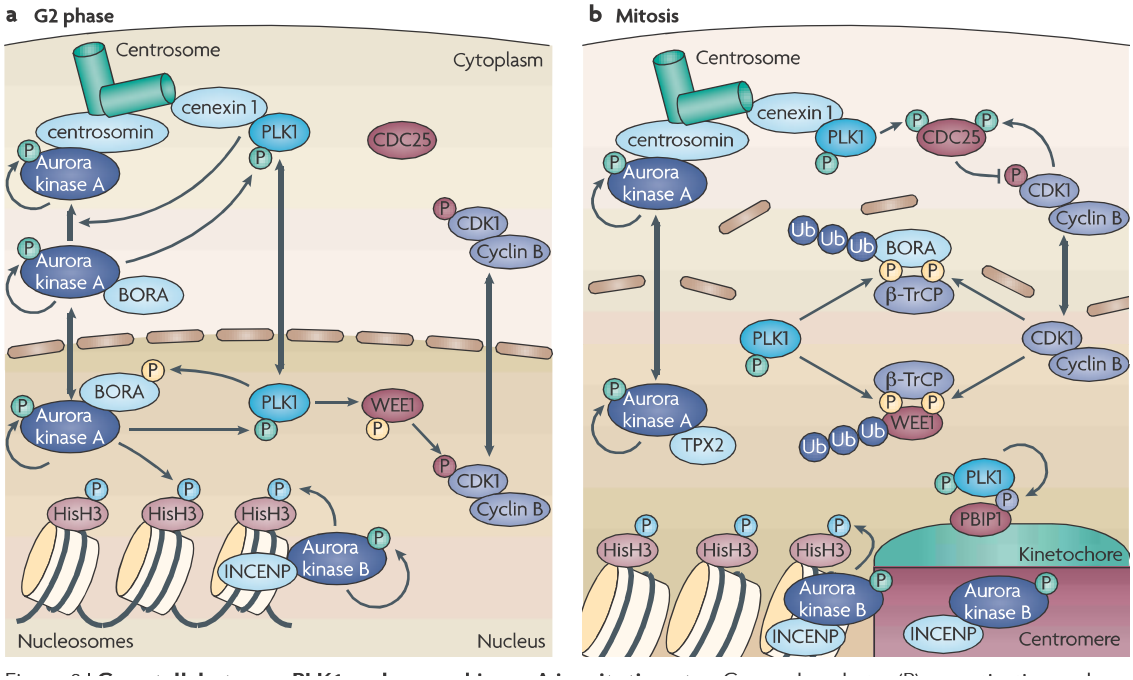

Figure IV. Summary of interplay between mitotic kinases in G2 (a) and mitosis (b). Adapted from (Lens et al. 2010).

The NIMA family

The NIMA family is named after NIMA (Never in Mitosis A), a protein kinase described in the filamentous fungi *Aspergillus nidulans*. In this organism, depletion of NIMA causes G2 arrest while its overexpression leads to premature mitosis. Thus, NIMA was characterized as an essential protein for mitotic entry and progression in *Aspergillus* (Oakley and Morris 1983).

NIMA phosphorylates Cdc2/cyclin B (the homologs of CDK1/cyclin B) promoting its nuclear localization at the onset of mitosis (Wu et al. 1998). NIMA also interacts with the nuclear pore complex protein Nup98, which supports the involvement of NIMA in Cdc2 nuclear import (Wu at al. 1998). Moreover, this kinase is involved in chromatin condensation through H3 phosphorylation at Ser10 (De Souza et al. 2000) and regulation of MT nucleation through its interaction with the protein TINA (O'Regan et al. 2007).

Sequence analysis of the human genome revealed 11 NIMA-related genes. These genes encode for kinases that share a similar catalytic domain with NIMA (Lu and Hunter 1995). They are named **Nek kinases** presenting diverse functions with possibly only four of them, Nek2, nek6, Nek7 and Nek9, directly related with mitotic regulation (Figure V).

Human Neks are structured in a N-terminal catalytic domain homologous to NIMA and a C-terminal regulatory domain, which is the most divergent part among them. The function of this family varies depending on the specific Nek. The non-mitotic Nek kinases are mainly involved either in cilia assembly or DNA damage response, although the function of some of the Neks still remains unclear.

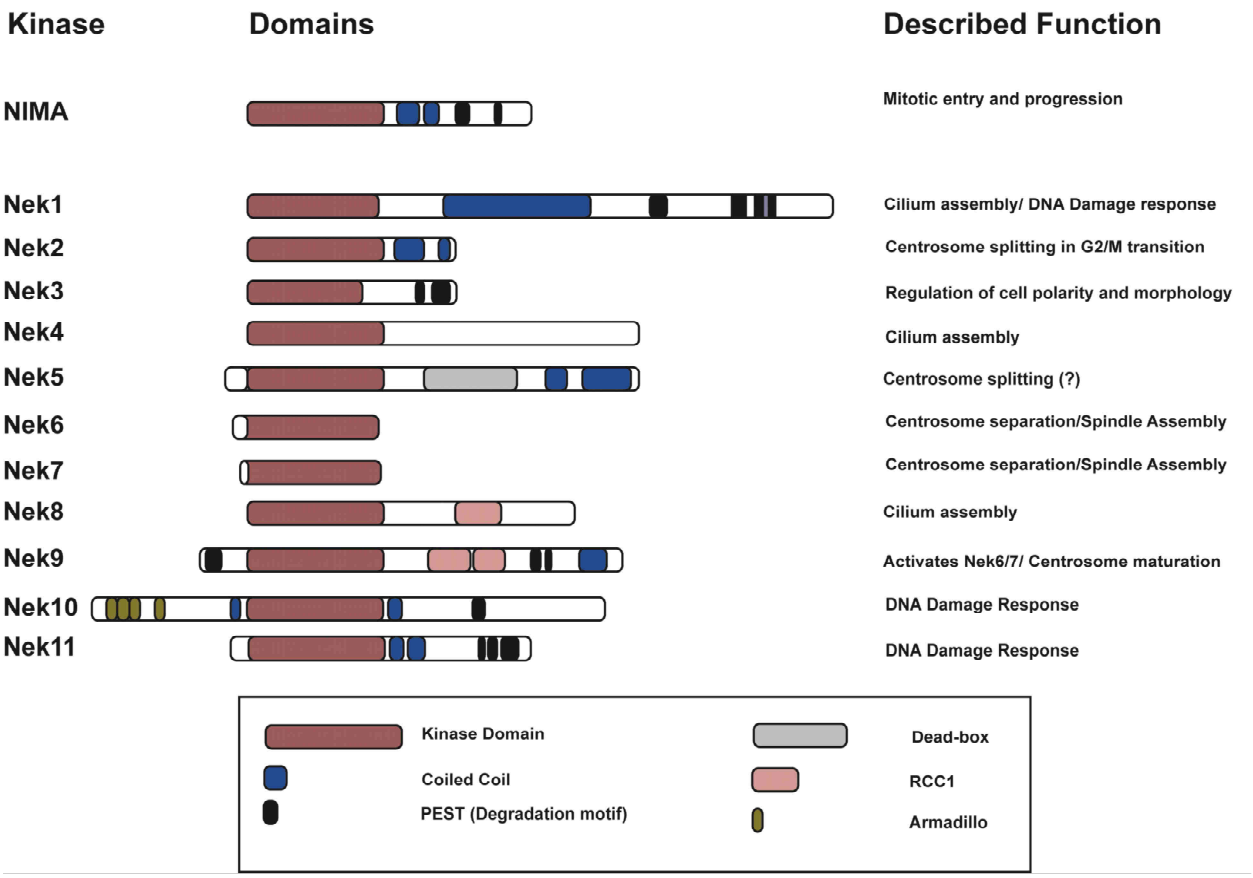

Figure V. Human NIMA related kinases or Neks.

Neks and ciliogenesis

At least two NIMA kinases are directly related to primary cilium assembly in mammals. Cilia are MT-based organelles formed in interphase from the mother centriole and involved in coordination of signaling pathways during development and tissue homeostasis, in which some of them are coordinated with cell cycle events (Quarmby and Parker 2005). Defects in primary cilia assembly leads to a group of diseases named ciliopathies, including retinal degeneration, polycystic kidney disease (PKD), liver disease and *situs inversus* (mirrored visceral organs) among others.

Nek1 KO mice present PKD and other defects like dwarfism and facial dysmorphism (Upadhya et al. 2000). Nek1 localizes at the cilium basal body and its depletion causes defects in cilia assembly (Shalom et al. 2008), which possibly explains the mice phenotype.

Nek8, in a similar way to Nek1, is also localizing at centrosomes and basal body of primary cilia and defects in its function or localization result in several ciliopathies, including kidney disorders (Otto et al. 2008) and *situs inversus* in mice (Manning et al. 2013).

A relationship of the Neks with cilia physiology is supported by the observation that this family of kinases is expanded in organisms with ciliated cells, specially these with different types of cilia (Quarmby and Parker 2005).

Neks and the DNA Damage Response (DDR)

After DNA damage, cells induce arrest in the cell cycle in order to repair the damage before continuing either with cell division or DNA replication. Checkpoints could arrest the cell in the G1/S transition, inter-S phase or G2/M transition, and are triggered by the ATM/ATR pathway, which phosphorylates and activates their effectors kinases Chk1/2 promoting a signaling pathway that eventually ends with the inactivation of the CDKs (Rieder 2011).

Nek11 is the best characterized Nek with a function in the DDR. It participates in the G2 arrest through the phosphorylation of Cdc25, promoting its ubiquitination and consequent degradation (Melixetian et al. 2009).

Nek1 may also have a role in this process, since after exposure to ionizing radiation its levels increase and switch from cytoplasm to nucleus (Polci et al. 2004).

Finally, Nek10 has also been associated with the DDR, as this kinase is required to induce the G2/M checkpoint after UV irradiation (Moniz and Stambolic 2011).

Other Neks

Nek3, Nek4 and Nek5 are the less studied proteins in the family. Nek3 function has been linked to neuronal morphogenesis and polarity development, probably trough MT acetylation in neurons (Chang et al. 2009).

Nek5, which is characterized by a dead-box domain in its amino acid sequence, was recently proposed as a Nek2 cofactor, being required for centrosome disjunction in mitosis (Prosser et al. 2015). Finally, Nek4 function remains elusive, but a recent work on the Nek4 interactome indicates that this kinase could also be associated with the DDR or ciliagenesis (Basei et al. 2015).

Mitotic NIMAs

Nek2

Nek2 is the most studied protein kinase in the family and is the closest to NIMA by kinase domain similarity. It is a 48 kDa protein which preferentially phosphorylates Ser/Thr placed in a basic context (Fry et al. 1995). Nek2 homodimerizes through two coiled coil domains located at the C-terminal part of the protein. The dimerization allows autophosphorylation, which is required for Nek2 activity (Fry et al. 1999).

Two different Nek2 isoforms were identified in *Xenopus* (Uto et al. 1999) and humans (Hames and Fry 2002), Nek2A and Nek2B. Nek2A is expressed from G1/S phase with a peak of expression in G2/M transition until prometaphase, where is rapidly degraded. In contrast, Nek2 protein levels during G1/S are low, increase during G2/M and last until anaphase (Hames and Fry 2002). This different regulation is due to the presence in Nek2A but not in Nek2B of a Degradation-Box (D-Box) sequence in its C-terminal domain, which promotes protein degradation mediated by APC/C-Cdc20 (Hames et al. 2002).

Additionally, a third Nek2 isoform has been identified in vertebrates. Nek2C is the result of an alternative splicing of Nek2A mRNA. Nek2C shares many properties with Nek2A, such kinase activity, PP1 interaction, centrosomal localization, MT binding, mitotic degradation and dimerization. This version differs in its localization during interphase, which is mainly nuclear. The function of Nek2C remains unclear, but due to its localization it has been

suggested that it may have a role in chromatin condensation during mitosis (Wu et al. 2007), similar to NIMA in *Aspergilus* (De Souza et al. 2000).

Nek2B is better characterized in *Xenopus leavis* embryos, where it is involved in maintenance of centrosome structure and spindle assembly. Depletion of Nek2B in these embryos drastically impairs centrosome assembly (Uto and Sagata 2000). In addition, Nek2B depletion in egg extracts results in a delay in centrosome maturation and MT aster formation (Fry et al. 2000).

Nek2A is activated at late G2 by the action of components of the Hippo pathway. The kinase is inactivated during interphase by a complex composed by Mst2 and PP1-γ. During G2/M transition Mst2 is phosphorylated by Plk1, which liberates Mst2/Nek2A from the inhibitory effect of PP1-γ. Once liberated, Mst2 phosphorylates Nek2A promoting its activation. Finally, this phosphorylation allows Nek2 translocation to the centrosomes, where Mst2/Nek2A complex is stabilized by hSav1 (Mardin et al. 2010). The Nek2A-Mst2-hSav1 complex is activated by the EGFR pathway after EGF induction (Mardin et al. 2013).

Once it is activated at late G2, Nek2 phosphorylates proteins that constitute the intercentrosomal linker promoting their physical dissociation from the complex they form. Nek2 phosphorylation promotes the timely dissolution of the centrosome linker during centrosome disjunction preceding the physical separation of the centrosomes. Linker proteins that are Nek2 substrates are C-Nap1 (Fry et al. 1998), Rootletin, Centlein, Cep68 (Fang et al. 2014) and ß-Catenin (Bahmanyar et al. 2008).

Nek9

Nek9 was first identified in 2002 by two independent works, and initially named Nek8 (erroneously) (Holland et al. 2002) and **Nercc1** (Roig et al. 2002).

Nek9 is a 120 kDa protein composed by three different domains: the catalytic domain (52-308), the RCC1 domain (347-726) and the C-terminal domain (891-940).

The N-terminal domain of Nek9 comprises the **catalytic domain**, as most of the mammalian Neks. Two different mutations in this domain result in an inactive kinase: K81M in the ATP binding site and D176A in the proton acceptor residue (Roig et al. 2005).

The **RCC1-like domain** in Nek9 comprises seven RCC1 sequences that allow RanGTP binding but lack the residues required for nucleotide exchange activity towards this small GTPase. However, this domain is responsible for maintaining Nek9 autoinhibitory conformation, since it interacts directly with the activation domain and prevents Nek9 autoactivation (Roig et al. 2002).

The **C-terminal domain or tail** presents a coiled coil motif, which allows Nek9 dimerization (Roig et al. 2002). This domain associates with LC8, a component of the dynein complex, which also binds several proteins possibly facilitating the organization of the protein and promoting dimerization. Nek9 interaction with LC8 is disrupted through phosphorylation of Nek9 Ser944, next to the motif that LC8 recognizes in Nek9. The C-terminal of Nek9 interacts with Nek6 and Nek7 and LC8 binding impedes this interaction and consequently, Nek6/7 activation (see below) (Regué et al. 2011).

Nek9 is expressed in all cell lines and tissues studied, but it predominates in kidney, testis, heart, skeletal muscle and brain. It is continuously expressed during the cell cycle and it is inactive in interphase. Nek9 activity requires the phosphorylation of a residue within its T-loop, Thr210. Nek9 is able to auto activate *in vitro* through the phosphorylation of this residue (Roig et al. 2005). *In vivo* Nek9 is activated at the onset of mitosis by a double step mechanism. First, CDK1 phosphorylates Nek9 creating a recognizable motif for Plk1 PBD and subsequently Plk1 phosphorylates Nek9 at Thr210 promoting its activation (Bertran et al. 2011). Initial activation is most probably amplified by autophosphorylation. Active Nek9 (P-Thr210) localizes at centrosomes during early mitosis and in later stages is detectable at chromosomes and at the cytokinetic furrow (Roig et al. 2005) (Figure VI).

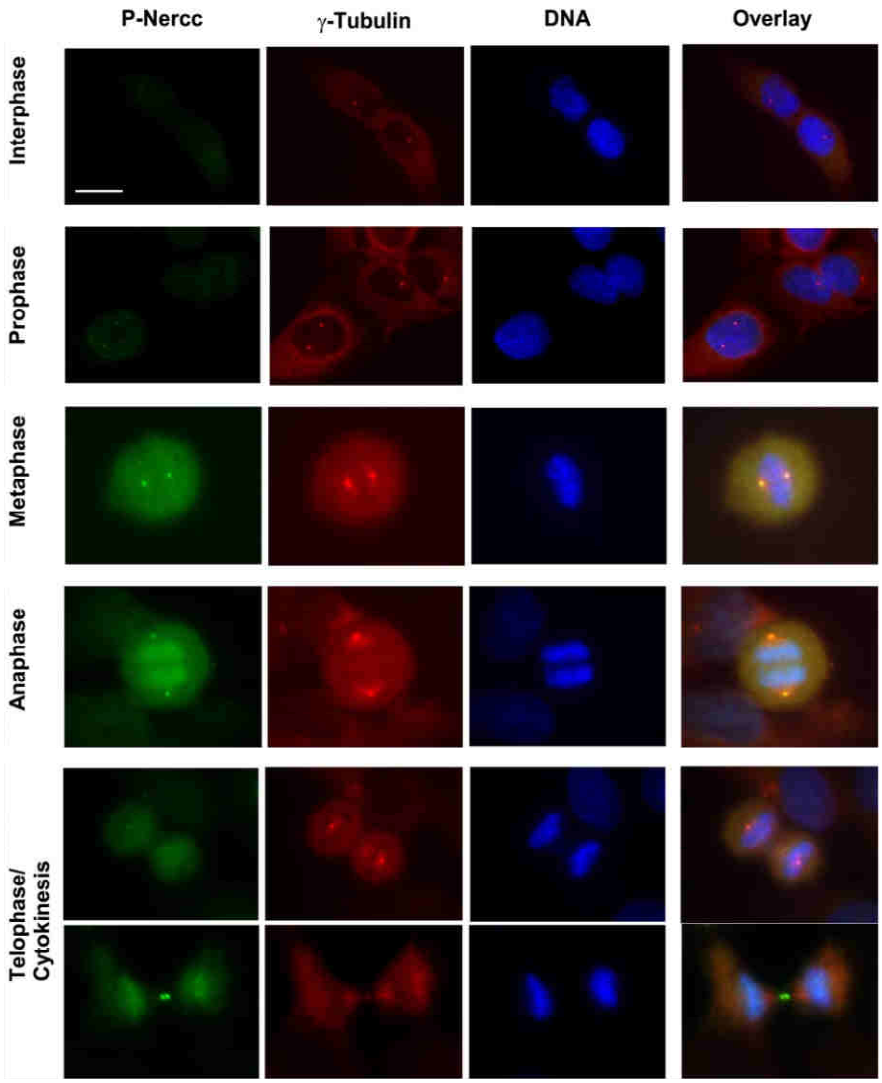

Figure VI. Localization of active Nek9 during cell cycle and different stages of mitosis.

Nek9 localizes at centrosomes from prophase to metaphase. The kinase migrates to chromosomes and cytokinetic furrow during anaphase and telophase/cytokinesis. Adapted from (Roig et al. 2005).

Regarding function, injection of anti-Nek9 antibodies in prophase cells leads to prometaphase arrest and spindles with misoriented chromosomes, which suggest a role in mitosis of this protein (Roig et al. 2002). Different studies have demonstrated specific roles for his kinase in mitosis, although multiple novel substrates and functions may be still unidentified. First, Nek9 is involved in centrosome maturation through the phosphorylation of the γ-tubulin adaptor protein, Nedd1. This phosphorylation is required for Nedd1 accumulation at

centrosomes and in consequence, γ-tubulin recruitment, a necessary step for sufficient MT nucleation at mitotic centrosomes (Sdelci et al. 2012).

Nek9 also binds to Nek6 and Nek7 and phosphorylates them (Ser206 in Nek6 and Ser195 in Nek7) promoting their activation (Belham et al. 2003). Nek6 and Nek7 kinases are required for centrosome separation in prophase; as a result, Nek9 is directly involved in this process (Figure VII and see Nek6 and Nek7 below).

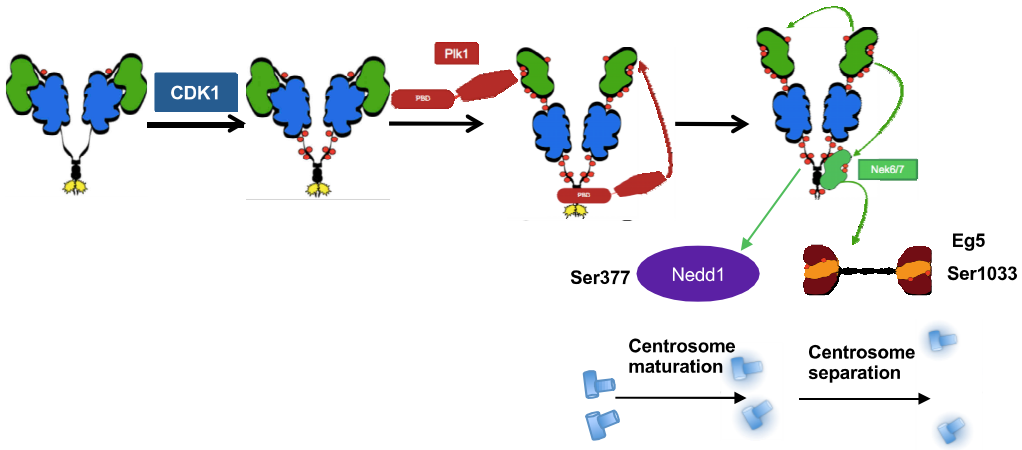

Figure VII. Mechanism of Nek9 activation at the onset of mitosis and the processes derived from its activation (Teresa Bertrán).

In addition to the mitotic functions, Nek9 was identified as a NIMA related human kinase that binds and phosphorylates the dynein adaptor protein BicD2 (Holland et al. 2002). However, the function of this phosphorylation is not known.

Finally, Nek9 was linked with cargo recruitment and vesicle trafficking regulation in a screening for human autophagy system regulation (Behrends et al. 2010).

Nek9 KO mice are not viable and die at very early stages of embryonic development (our unpublished data). In humans, different mutations in the kinase sequence were associated with diverse disorders. Nek9 mutation resulting in the expression of a shorter version of the protein is lethal in human embryos. This mutation causes lethal skeletal dysplasia in the

analyzed fetus with delay in mitosis and defects in cilia assembly (Casey et al. 2016). In addition, it was recently shown that somatic point mutations in Nek9 cause *Nevus comedunicos.* This rare disease is characterized by the presence of skin injuries similar to acne (Levinsohn et al. 2016).

Nek6 and Nek7

Nek6 and Nek7 are very similar with a 76% identical sequence (87% in the kinase domain) and differ mainly in a small amino acid sequence of the N-terminal domain, just before the catalytic domain. They were identified first in mice (Kandli et al. 2000) as NIMA-related kinases that lack the regulatory C-terminal part. Later, they were described in humans as ribosomal protein kinase p70 S6 kinases (Belham et al. 2001) although the physiological relevance of this has been contested (Lizcano et al. 2002).

Nek9 binds and activates Nek6 and Nek7 at the onset of mitosis through phosphorylation on residues in their T-loop (Belham et al. 2003). Additionally, it has been recently shown that Nek9 dimerization promotes allosteric activation of Nek7 through establishment of a back-to-back conformation of Nek7 that releases autoinhibition through Tyr97 (Haq et al. 2015).

In a similar way to active Nek9, Nek6 and Nek7 localize at spindle poles during early mitosis but only Nek6 is detected at mitotic spindle in later stages and to the midbody during cytokinesis. Moreover, these kinases are required for mitotic progression and spindle formation, and depletion of Nek6 and Nek7 can cause cell arrest in prometaphase (O'Regan & Fry 2009). Despite Nek6 and Nek7 mitotic role in cell culture, mice lacking the expression of Nek6 develop normally (our data) whereas Nek7 depletion causes lethality in early development (Salem et al. 2010), which suggests differentiated roles for both kinases.

Nek6 and Nek7 are able to phosphorylate the mitotic kinesin Eg5 at Ser1033 both *in vivo* and *in vitro* (Rapley et al. 2008); see below for an introduction to Eg5. Phosphorylated Eg5 concentrates mainly at spindle poles and at the midbody during cytokinesis. Ser1033 phosphorylation is required

for Eg5 accumulation at the centrosomes in prophase and the accumulation of this motor protein is indispensable for centrosome separation in early mitosis. Cells that express a non phosphorylable form of Eg5 fail to localize Eg5 around centrosomes at the onset of mitosis, and in consequence, centrosome separation is impaired (Bertran et al. 2011) (Figure VIII).

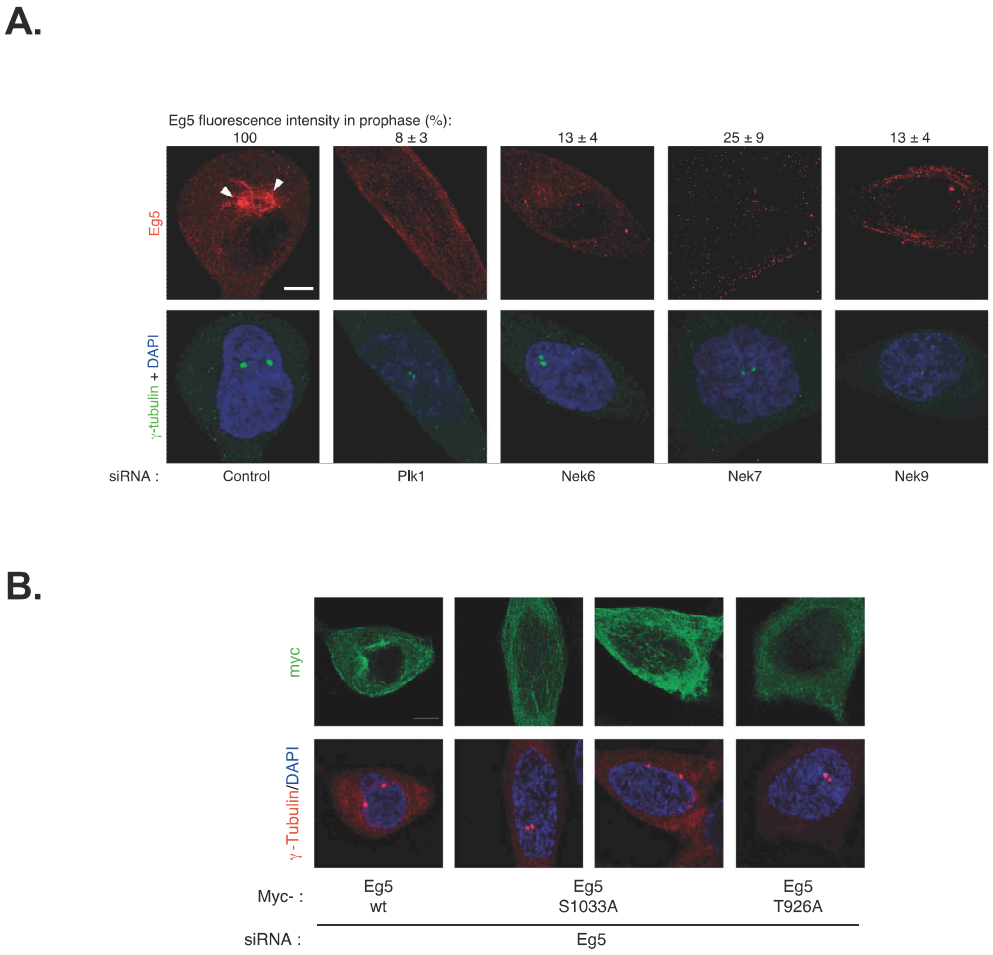

Figure VIII. Eg5 accumulation at centrosomes depends on Nek6/7 phosphorylation.

(A) Action of the kinases Plk1, Nek9, Nek6 and Nek7 is required for Eg5 localization at centrosomes during prophase. (B) Recombinant Eg5 with an alanine at the Nek6/7 phosphorylation site (Ser1033) or the CDK1 site (Thr926) are not able to accumulate at centrosomes. Adapted from (Bertran et al. 2011).

The Centrosome cycle

So far, the described functions of mitotic Neks are all directly connected with the centrosome cycle during the G2/M transition.

Centrosomes are the major MT Organizing Center (MTOC) of animal somatic cells. They are composed, depending on cell cycle stage, by either one or two centrioles, which are cylindrical structures formed by nine MT triplets organized in a clock-wise barrel shape, surrounded by an amorphous mass of proteins named Pericentriolar Material (PCM). The PCM is organized in concentric toroids of proteins such as CDK5RAP2, CEP192, CEP120, CPAP and the MT nucleator γ-Tubulin Ring Complex (γ-TuRC), which emerge from the mother centriole. Additional proteins organize radially, such as pericentrin (Lüders 2012). Before mitosis cells present two centrosomes containing two centrioles each, arranged perpendicularly. In each centrosome the older centriole is called mother centriole while the younger is the daughter centriole.

Centrioles are the scaffold for arrangement of several proteins that compose the PCM, where MT nucleation takes place. Thus, the PCM is the responsible for the MTOC activity of the centrosome. During mitosis, the PCM increases its size intensely in a process termed centrosome maturation, which also leads to an intensification in MT nucleation activity at the centrosome (Piehl et al. 2004).

Despite the importance of centrosomes in MT nucleation, this organelle is not essential for cell division in some organisms, like *Drosophila*. However, in organisms in which rapid and accurate segregation of chromosomes is required, centrosome become an essential organelle (Sir et al. 2013).

Centrosomes need to divide once every cell cycle, in order to organize (and be segregated as part of) the spindle poles (Nigg & Stearns 2011 and references therein). Thus, the centrosome cycle occurs simultaneously to cell cycle and it can be divided in the following stages (Figure IX): disengagement, duplication, maturation, disjunction and separation.

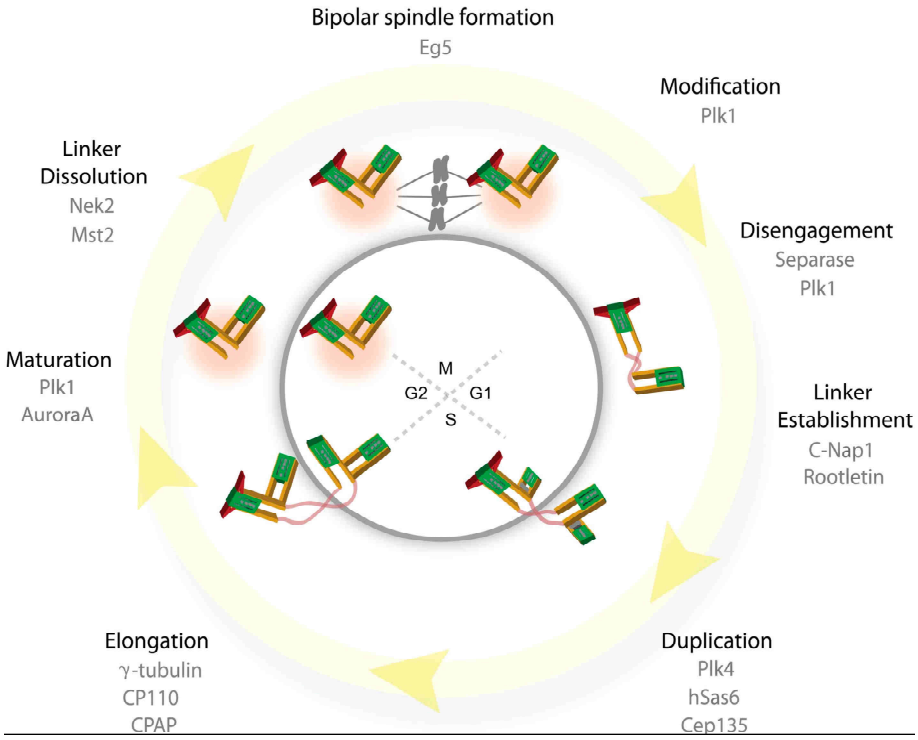

Figure IX. The centrosome cycle.

The centrosome cycle is separated in different stages coinciding with cell cycle and regulated by similar pathways. Taken from (Mardin and Schiebel 2012).

Centrosome disengagement

After mitosis cells receive a single centrosome with two perpendicularly attached centrioles. During telophase/early G1 mother and daughter centrioles loose their perpendicular organization and separate in a process that is called disengagement. Disengagement is the licensing step for posterior centriole duplication in S-phase and is controlled during M (Agircan et al. 2014). Both chromosome segregation and centriole disengagement are mediated by the activity of separase. After correct attachment of MTs to kinetochores in metaphase, the APC/C activates separase, inducing cohesin degradation and consequent chromosome separation. In centrioles, separase

induces centriole separation through the cleavage of cohesin and pericentrin. During this stage, Plk1 phosphorylates pericentrin promoting separase cleavage (Kim et al. 2015). In addition, it is known that Plk1 is required for centrosomal localization of sSgo, that inhibits centriole separation, and also for CEP68 degradation (Pagan et al. 2015). This suggests a dual pathway in which during early mitosis the kinase promotes the stabilization of cohesion through sSgo targeting and during late mitosis phosphorylates factors that induce disengagement.

In parallel to disengagement, a physical linker forms between the separated mother and the daughter centriole. This linker is composed by C-Nap1, which localizes at the basal of centrioles and rootletin, a filamentous protein that connects both centrioles. CDK5RAP2, CEP68, CEP135, LRRC45 and LGALS3BP are also involved in establishment of this linker (Agircan et al. 2014).

Centrosome duplication

Centriole duplication starts during late G1 and early S with the formation of a protocentriole attached to each existing centriole, and is triggered by the activity of the Plk4 kinase. Plk4 activity is required for stabilization of the coiled coil protein SAS-6. This protein is essential for the formation of the cartwheel-structure, which already presents ninefold symmetry. How this process is regulated in a way that only one centriole arises from the previous one in every cell cycle is an unsolved question.

Daughter centrioles start to elongate in late S phase and increase their length until the start of the next cell cycle. This step is executed by the addition of tubulin subunits with a much lower ratio than regular MT polymerization. Thus, elongation must to be precisely controlled, but the mechanisms that trigger this regulation are poorly understood. It has been proposed that the size of the cartwheel is actually controlling the extension of the centriole (Fırat-karalar and Stearns 2014).

Finally, capping the plus end of the daughter centriole with CP110 stops elongation. The lack of this protein in *Drosophila* results in longer centrioles, which, surprisingly, don't affect cell viability and division (Franz et al. 2013).

Centrosome maturation

During the G2/M transition centrosomes increase their size dramatically in a process called centrosome maturation, which is required for consequent MT nucleation and bipolar spindle formation in mitosis. This size increase is a consequence of accumulation of proteins that form the PCM, such pericentrin, CEP192 and CDK5RAP2. CEP192 acts as a scaffold for Aurora A recruitment at centrosomes and activation (Joukov et al. 2010). This step promotes activation of Plk1, which phosphorylates CEP192 triggering γ-TuRC binding and MT nucleation (Joukov et al. 2014). Thus, the Aurora A and Plk1 kinases are essential for maturation, although their importance may vary between organisms. The adapter protein Nedd1 mainly directs γ-tubulin accumulation at spindle poles. Nedd1 phosphorylation at Ser377 by Nek9 downstream of Plk1 promotes its recruitment at centrosomes and in consequence drives γ-tubulin accumulation in prophase (Sdelci et al. 2012).

Centrosome positioning

Centrosomal positioning is regulated during interphase with different objectives. First, centrosomes control the distribution of the Golgi apparatus. During mitosis the Golgi is dismembered and during interphase is organized around centrosomes due to the action of MTs emerging from centrosomes. This position was shown to be required for normal cell migration (Hurtado et al. 2011). Second, before cells enter into mitosis, centrosomes are tethered to the NE. This step is required for proper centrosome separation and bipolar spindle formation and is a direct result of dynein accumulation at the NE during the G2/M transition, through a mechanism dependent on the nuclear pores. First RanBP2 binds to the dynein-dynactin adaptor BicD2 and promotes dynein localization at NE. Dynein interaction with MTs that emerge

from centrosomes, and its movement towards minus ends results in centrosome anchoring to the nucleus (Splinter et al. 2010). Additionally another nucleoporin cooperates in this stage. Nup133 interacts with CENP-F during the G2/M transition. This interaction promotes the accumulation at NE of NuDE and NuDEL, which are proteins that interact with cytoplasmic dynein (Bolhy et al. 2011).

Centrosome splitting

As mentioned above, after centriole duplication and disengagement several proteins are positioned between the mother and daughter centrioles (that will become in turn both mothers). These proteins, thus, effectively form a linker that maintains both resulting centrosomes together. During mitosis centrosomes must separate to form the bipolar spindle; therefore, the intercentrosomal linker should be dissolved. This process is controlled by the activity of protein kinase Nek2 (see the Nek2 section, above). Downstream of components of the hippo pathway, Nek2 phosphorylates among others C-Nap1 and rootletin, part of the centrosome linker (Fry et al. 1998) (Hardy et al. 2014). The phosphorylation of these proteins promotes linker disassembly. However, depletion of Nek2 does not impede centrosome separation, which means that centrosome disjunction is not indispensable for centrosome separation.

Centrosome separation

Directed centrosome separation is a regulated process whose major players are protein motors and MTs. There are two different pathways according the timing of centrosome separation; the prophase pathway, where centrosomes separate before NEB and the prometaphase pathway, where centrosomes complete separation after dissociation of the nuclear membrane.

The Prophase pathway of centrosome separation

The main player in this pathway is the MT motor Eg5. Eg5 is a plus-end-directed member of the kinesin 5 family (see below) that is able to bind antiparallel MTs emerging from both centrosomes and slide them apart. Inhibition of Eg5 activity causes failure of centrosome separation, which results in mitotic cells with monopolar spindles.

Eg5 loading at centrosomes is a required step for separation. This localization is regulated by the action of different protein kinases. CDK1 phosphorylates Eg5 at Thr926 promoting Eg5 binding to MTs (Blangy et al. 1995). At the same time CDK1 and Plk1 drive the activation of the Nek9/Nek6/7 pathway and Nek6 and Nek7 phosphorylate Eg5 at Ser1033 promoting its accumulation at centrosomes by an unknown mechanism (Bertran et al. 2011).

Cells treated with small concentrations of Eg5 inhibitors are able to separate centrosome independently of this kinesin (van Heesbeen et al. 2013). Thus, this observation suggests that other redundant pathways exist promoting separation in absence of Eg5. One of them is based on dynein associated at NE, which during G2 is able to promote centrosome separation in absence of Eg5. Dynein is a minus end directed protein that pulls centrosomes along the NE during prophase (see below). In the presence of Eg5, both pathways cooperate to drive centrosome separation in prophase (Raaijmakers et al. 2012).

The prometaphase pathway of centrosome separation

In some cases centrosomes remain together at the moment of NEB, thereby separation depends on mechanisms acting during the prometaphase pathway. This pathway is characterized by its complexity, since several mechanisms are simultaneously involved.

First, kinetochores pushing-forces have been proposed to have a role for centrosome separation at this stage (Toso et al. 2009). These forces emerge

from k-fibers, which are bundles of MTs that connect centrosomes to kinetochores.

KIF15 (also known as Kinesin-12 and HKLP2) localizes predominantly between parallel MTs. Binding of this protein to non-K-fiber MT, promotes centrosome separation (Sturgill and Ohi 2013). It has recently been shown that this kinesin is able to drive centrosome separation in absence of Eg5 activity during the prometaphase stage (Sturgill et al. 2016).

Actin cytoskeleton also plays a role in this phase. Inhibition of myosin II blocks completion of centrosome separation in prometaphase, suggesting that this process depends on connections between the astral MTs with the cell cortex (Rosenblatt et al. 2004).

In addition to the pathways described above, other mechanisms could be regulating the efficiency of centrosome separation. As mentioned above, EGF induces premature centrosome separation during S phase through the activation of the Mst2-hSav2-Nek2A complex. The premature dissolution of the intercentrosomal linker possibly reduces the requirement of forces generated by Eg5, which means that in this case the prometaphase pathway could be enough to drive separation (Mardin et al. 2013).

Timing for centrosome separation

This dual mechanism for centrosome separation ensures the correct formation of the bipolar spindle and chromosome segregation. The different pathways may be more or less prominent depending on the cell type. Around 80% of HeLa cells present centrosomes separated during prophase, which means that in this cells 20% of the cells proceed through the prometaphase pathway (Woodcock et al. 2010).

Centrosome separation before NEB provides an advantage to cells, since cells that separate centrosomes through the prophase pathway present a smaller ratio of aneuploidy due to a faster and more accurate mitosis (Silkworth et al. 2012) (Kaseda et al. 2011). Regarding the timing of events, it has been shown that Eg5 loading at centrosomes should be performed in a

time window of 10 minutes to proceed with separation before NEB. More time for loading would allow to complete centrosome separation at this stage, but it would also result in longer mitosis (Kaseda et al. 2011). Thus, the prometaphase pathway possibilities faster mitosis (but at the risk of mitotic errors) in cells that failed to timely separate centrosomes in prophase (Figure X).

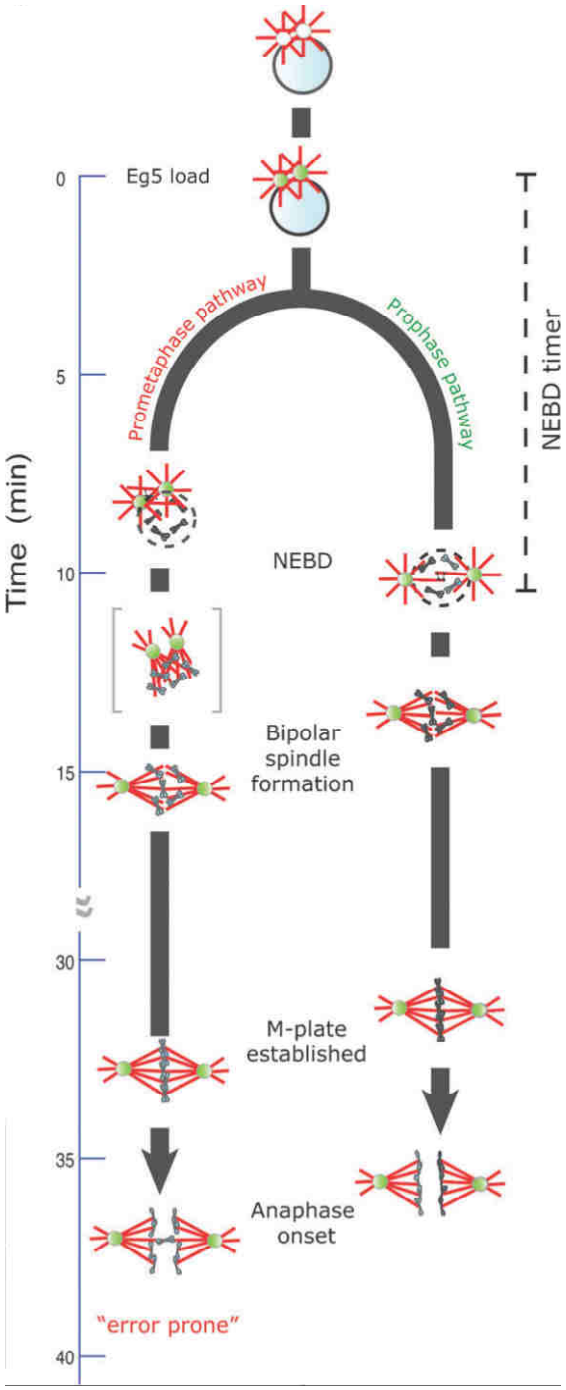

Figure X. Timing comparison between centrosome separation in prophase and prometaphase.

Prometaphase pathway leads to an increased ratio of aneuploidy and slower mitosis. Adapted from (Kaseda et al. 2012).

Spindle formation

MTs are formed by α and β tubulin heterodimers, which arrange linearly in polarized protofilaments. Thirteen parallel protofilaments form the cylindrical structure of a MT. MTs present two fundamental characteristics. First, they can grow or shrink in the presence of GTP by the gain or loss of tubulin subunits at both ends. Thus, alternation of both processes confers to the MT the property of dynamic stability, which is defined by switching states from growth to shrink (catastrophe) and shrink to growth (rescue) (Mitchison and Kirschner 1984).

The second important property of MTs is their polarity. Due to the asymmetry of the tubulin dimer subunits, the minus ends and the plus ends of MTs have different dynamics. The MT plus ends (with ß-tubulin exposed at their extremes) are highly dynamic, alternating states of shrinkage and growth. These ends are usually oriented towards the surface of the cell. On the other hand, minus ends of MT (exposing α-tubulin monomers) are less dynamic, and although they can grow they do it slower than plus ends. In cells, minus ends can be stabilized due to their association with the centrosome or other MTOCs (Akhmanova and Steinmetz 2015) (Figure XI).

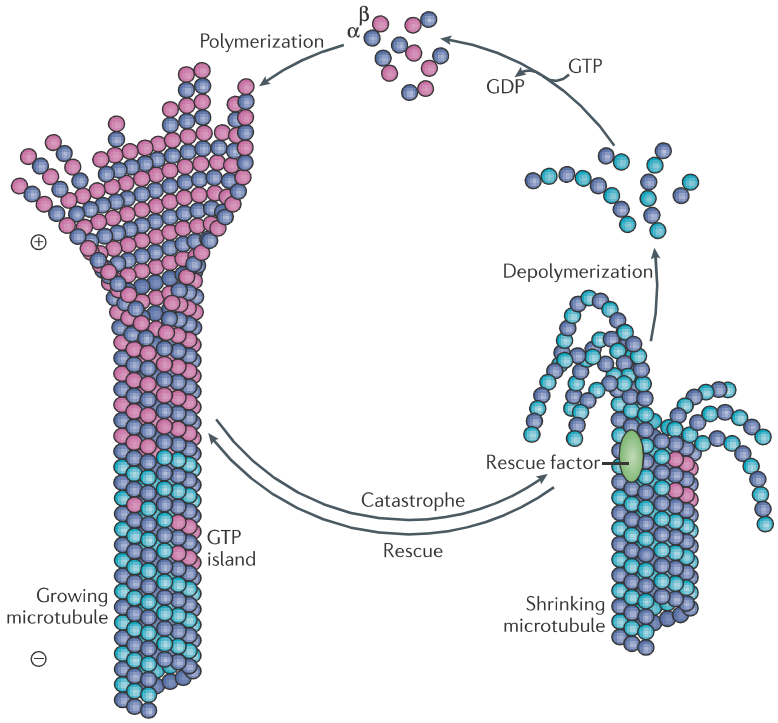

Figure XI. Representation of the dynamic stability of MT or MT assembly and disassembly cycle. Adapted from (Akhmanova and Steinmetz 2015).

Entry into mitosis starts with higher MT nucleation activity, dynamics and activation of different pathways that lead to a reorganization of MTs, promoting the formation of the mitotic spindle. Initially, due to the dynamic stability properties of MTs, Mitchison and Kirschner proposed **the "search and capture" model** for spindle assembly (Kirschner and Mitchison 1986). In this model, centrosomes nucleate MTs and chromosomes capture and stabilize their plus ends, resulting in the formation of the bipolar spindle. Through interaction with MTs from both poles chromosomes get bioriented at the equator of the cell (Duncan and Wakefield 2011).

Another pathway for spindle assembly is **the augmin pathway**. The augmin hetero-complex is able to interact simultaneously with MTs and the γ-TuRC, promoting MT nucleation from pre-existing MT along the spindle (Goshima et al. 2008).

Finally, the chromatin and the kinetochores also contributes to MT generation and spindle assembly (Gruss et al. 2001). The kinetochores stabilize MTs originated in the vicinity, which finally will be included in the centrosome-driven spindle, through a dynein and astral MT-mediated process (Rieder 2005). **The small Ran GTPase** mediates this pathway, though the localization of its guanine nucleotide exchange factor RCC1, which bids to chromatin. Ran(GTP) is more abundant around chromatin, inside the nuclei in interphase and forming a gradient around mitotic chromosomes after NEB. Ran was first described as a regulation factor involved in nuclear-cytoplasm transport during interphase (Melchior et al. 1993). During mitosis Ran also liberates cargos from importin β binding. These cargos are MT assembly factors, whose liberation either favor their activity in areas nearby chromosomes or activate proteins that function in spindle assembly (Figure XII).

One of the cargoes released from importin after Ran action is TPX2 (see below).

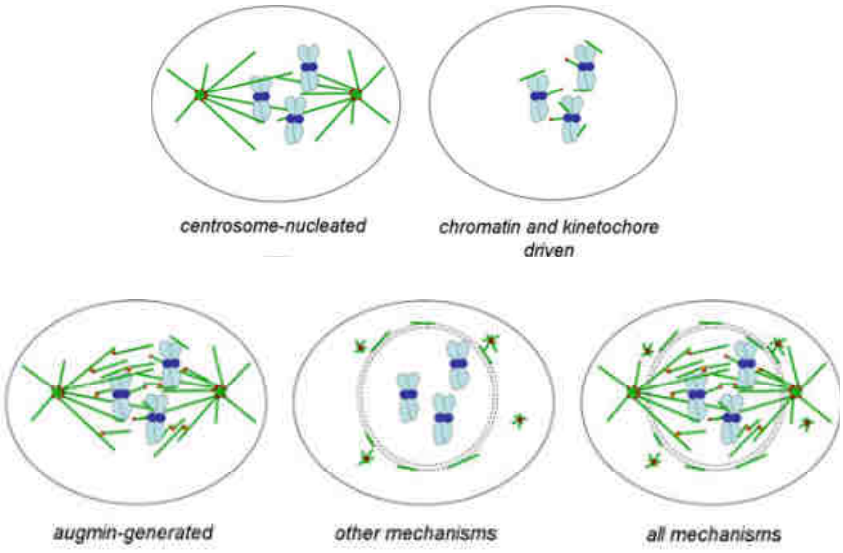

Figure XII. Mechanisms for bipolar spindle assembly. Adapted from (Duncan and Wakefield 2011).

TPX2

TPX2 is an 85 kDa MT Associate Protein (MAP) regulated by the RanGTP pathway that has several functions, including the stabilization of MTs and the activation of Aurora A. TPX2 has a dynamic localization along the cell cycle. During interphase it localizes at the nucleus while after NEB it localizes mainly at spindle poles. TPX2 localization at spindle poles depends on dynein activity (Wittmann et al. 2000), Eg5 and MT flux (Ma et al. 2010) and requires TPX2 C-terminal domain.

TPX2 primary sequence is conserved among vertebrates; for instance, mouse and *Xenopus* TPX2 share 78% and a 53% of identity with their human orthologous, respectively. Putative homologous proteins sharing less identity but with only some of TPX2 functions have been described in plants (Vos et al. 2008), worms (Özlü et al. 2005) and *Drosophila* (Goshima 2011).

Human TPX2 presents in its N-terminal part an activator domain for Aurora A that comprises 43 amino acids (Bayliss et al. 2003), whereas the main

function of the C-terminal domain is MT binding. The last 37 amino acids were described as essential for TPX2 interaction with the motor protein Eg5 (Eckerdt et al. 2008), see below. Nuclear localization during interphase could be explained by the presence of two nuclear localization signals (NLS). The primary NLS described corresponds to amino acids 284-287 in *Xenopus* TPX2, (313-315 in humans) (Giesecke and Stewart 2010). This non-classical NLS is recognized by importin-α, promoting TPX2 binding to the minor site of the importin, an atypical interaction that is also present other proteins, such phospholipid scramblase 4 (Lott et al. 2011). However, this is not the unique NLS present in human TPX2. A minimal basic sequence situated in amino acids 158-159 also drives importin binding and nuclear localization (Kahn et al. 2015) (our data, see below).

Finally, TPX2 presents a KEN box motif, which promotes its degradation at mitotic exit by APC/C Cdh1 complex to prevent TPX2 activity during the next cell cycle (Fang et al. 2014) (Figure XIII).

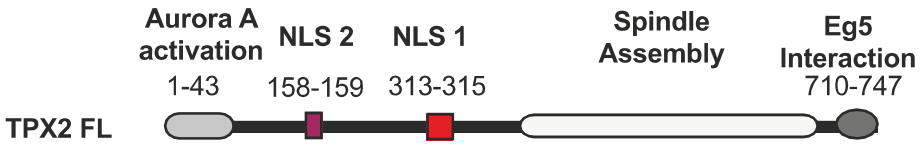

Figure XIII. Scheme representing the described domains in human TPX2 protein.

Described functions of TPX2

Despite its nuclear localization all TPX2 functions described until now are cytoplasmic and exclusive to mitosis, with the only possible exception of a role for TPX2 in the DDR during interphase (Neumayer et al. 2012).

This is based on the fact that TPX2 is bound to importin-α, which sequesters it to the nucleus during interphase and interferes with its different activities in the cytoplasm. After NEB, RanGTP releases TPX2 from importin around the chromosomes. This allows TPX2 to activate Aurora A, recruit

different proteins and stabilize MTs, which leads to the activation of MT polymerization dependent on RanGTP in M phase (Gruss et al. 2001). (Figure XIV).

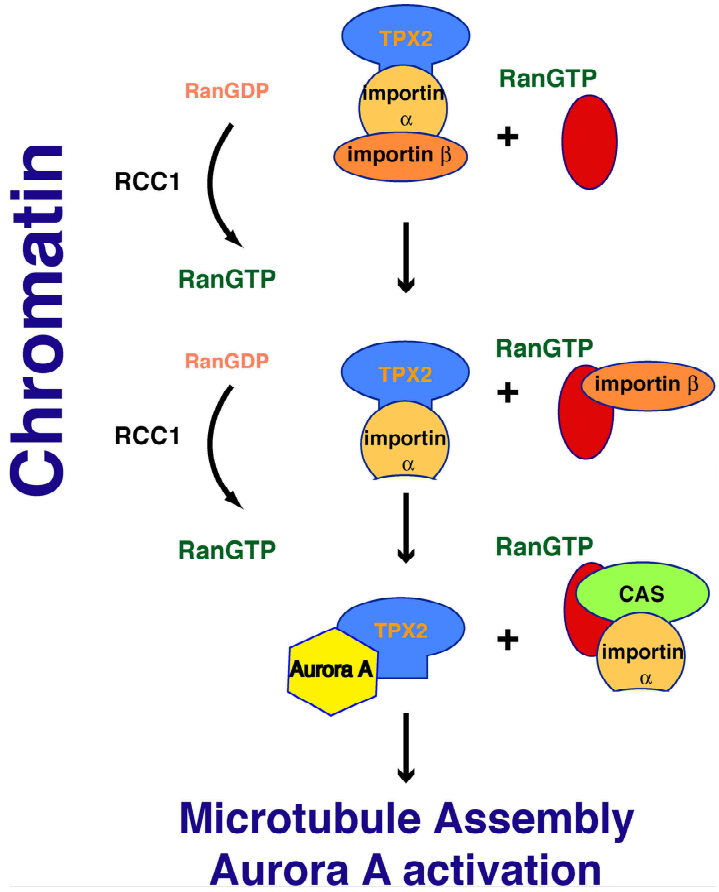

Figure XIV. TPX2 release from importin after RanGTP action (Gruss and Vernos 2004)

As mentioned, TPX2 promotes Aurora A activation (Trieselmann et al. 2003) (Tsai et al. 2003), and also controls Aurora A localization at the spindle (Kufer et al. 2002). The mechanism of Aurora A activation by TPX2 is based on the prevention of dephosphorylation by PP1 phosphatases of the crucial Aurora A Thr288. This interaction is mediated by the first 43 amino acids in TPX2, since expression of TPX2 lacking this domain prevents Aurora A interaction and induces Thr288 dephosphorylation (Bayliss et al. 2003).

Another TPX2 function is directly associated with MT nucleation and bundling, but how this function is performed still remains unclear. It has been has proposed that MT nucleation mediated by the RanGTP pathway is the consequence of the establishment of the XRHAMM-TPX2-γ-TuRC complex and Nedd1 phosphorylation by Aurora A (Scrofani et al. 2015). In addition, it has recently been suggested that TPX2 acts suppressing tubulin off-rates during MT assembly, leading to a reduced MT-shortening rate (Reid et al. 2016).

Work in mice has confirmed the central role of TPX2 in mitotic progression (Aguirre-Portoles et al. 2012). Null TPX2 embryos are not able to form a correct mitotic spindle and proceed with normal chromosome segregation. Additionally, it has been shown that TPX2 malfunction can result in cancer. Thus, haploinsuficient mice present a higher rate of lymphomas and lung tumors (Aguirre-Portoles et al. 2012). In fact, the connection of TPX2 with cancer has been described in several studies. Overexpression of TPX2 is observed in multiple cancer types, as colon, breast or lung cancer, and in many cases is also correlated with a bad prognosis. This has been proposed to be the result of aneuploidy onset after TPX2 malfunction (Pérez de Castro and Malumbres 2012).

TPX2 interaction with mitotic kinesins

TPX2 was first described in *Xenopus* egg extracts as a targeting factor for the motor protein Xklp2 to the spindle poles (Wittmann et al. 1998). Xklp2 is a homodimeric kinesin that moves towards the plus ends of MT and it was proposed to be involved in centrosome separation in a redundant manner to the kinesin Eg5 (Boleti et al. 1996). However, depletion of Xklp2 does not lead to monopolar spindles as in the case of Eg5 inhibition (Wittmann et al. 2000).

TPX2 interacts indirectly with Xklp2 trough the kinesin like protein C-terminal domain and this interaction is necessary for Xklp2 binding to MT and localization at centrosomes. Although this binding is necessary for proper kinesin targeting to centrosomes, it is not sufficient, as the dynein complex is

also required to bring Xklp2 to the minus ends of centrosomes (Boleti et al. 1996).

TPX2 also interacts with Eg5 trough its C-terminal domain, and this interaction is dependent of the last 35 amino acids in TPX2, originally defined in *Xenopus*. It has been shown that overexpression of the C-terminal domain of TPX2 in *Xenopus* S2 cells causes spindle pole separation failure and abnormal bipolar spindle formation. This phenotype is rescued expressing a form of the C-terminal domain of TPX2 that lacks the last 35 amino acids and thus it cannot bind Eg5 (Eckerdt et al. 2008).

The function of the observed Eg5-TPX2 binding seems to be to modulate the localization of the kinesin along the spindle. Eg5 during early mitosis moves towards the poles whereas its movement turns to plus ends directed during anaphase. While inhibition of dynein activity blocks Eg5 movement in astral MT, blocking Eg5 interaction with TPX2 results in an increase of the movement of Eg5 towards plus ends of MT (Gable et al. 2012).

It has recently been demonstrated that addition of TPX2 in mammalian cell extracts expressing fluorescent tagged Eg5 inhibits Eg5 movement along MTs *in vitro* and that this depends on TPX2 interaction with Eg5 (Balchand et al. 2015).

TPX2 is highly phosphorylated

Numerous studies described TPX2 phosphorylation in mitosis, but the function of these post-translational modifications remains unclear (Santamaria et al. 2011) as only a few residues have been studied in detail.

Xenopus TPX2 has been proposed to be a substrate for Plk1. Phosphorylation at Ser204 by this kinase promotes interaction with Aurora A, and in consequence, its activation. However, this site is not conserved in mice or humans (Eckerdt et al. 2009).

Aurora A phosphorylates *Xenopus* TPX2 in 3 different sites located at TPX2 N-terminal (Eyers and Maller 2004) without affecting their interaction. In humans, the kinase has been shown to phosphorylate residues Ser121 and

Ser125 (Ser90 and Ser94 in *Xenopus*) promoting interaction with CLASP1 (Fu et al. 2015). This interaction has been proposed to control spindle length regulating MT flux at plus ends of MTs.

CDK1 also phosphorylates human TPX2 at Thr72. This phosphorylation regulates TPX2 association at MTs, promoting TPX2 unbinding to the spindle. Thus, phosphonull mutant expression for this specific residue causes an increase in Aurora A activity and elongated spindle which could be related with an abnormal activity of the kinesin Eg5 (Shim et al. 2015).

RHAMM is a partner of TPX2

RHAMM (Receptor for Hyaluronan Mediated Motility) was first described as a soluble protein that altered cell migration and was able to bind hyaluronic acid. Later, it was proposed as a novel MAP, which interacts with MTs and actin filaments through its N-terminal domain (Assmann et al. 1999). Furthermore, RHAMM C-terminal domain is responsible for centrosomal targeting of this protein and RHAMM localization at centrosomes results from interaction with the dynein complex. This centrosomal localization could be related with a function of RHAMM on maintenance of spindle pole integrity (Maxwell et al. 2003).

TPX2 interacts during mitosis with RHAMM. This interaction was first described in *Xenopus* egg extracts. Without RHAMM TPX2-dependent MT nucleation was impaired and TPX2 did not properly localize at spindle poles (Groen et al. 2004). This interaction was later confirmed in human somatic cells. In human cells RHAMM is required for proper localization of TPX2 at centrosomes during mitosis, and this interaction is mediated through C-terminal domain of RHAMM and residues 40-319 in TPX2. Mislocalization of TPX2 after RHAMM depletion directly affects Aurora A Thr288-P levels at centrosomes (Chen et al. 2014).

RHAMM function is attenuated by the activity of the ubiquitin ligase BRCA1/BARD1 E3. This complex was described as necessary for proper mitotic spindle pole assembly and also TPX2 accumulation at spindle poles in *Xenopus* egg extracts (Joukov et al. 2006).

Motor proteins

Motor proteins are indispensable players in cell division. Centrosome separation, as exposed above, but also mitotic spindle organization require the function of these force generators. Motor proteins involved in spindle assembly and maintenance belong to two groups: the dynein family, which moves to the minus ends of MTs, and the more diverse kinesin family, which has members that move along the MTs in both directions and can also play a role destabilizing them.

Kinesins

Kinesins are a family of evolutionarily related molecular motors, which through ATP hydrolysis present different MT-based activities, such force ejection, motility or MT depolymerization. Mostly, kinesins present plus end directed motility when their motor domain is situated at the N-terminal of the protein and minus end directed motility when the motor is at the C-terminal domain. However, there are exceptions to this and this observation cannot be considered as a rule. Some kinesins such as these belonging to the kinesin-13 family are not motile and their function focus on the regulation of MT dynamics (Cross and McAinsh 2014).

Kinesins play different roles during the different stages of mitosis. In prophase these molecular motors are involved in centrosome separation sliding antiparallel MTs (Eg5). In prometaphase, kinesin 12/HKLP2 plays a redundant role to Eg5, promoting centrosome separation. As mentioned, this plus end directed kinesin could compensate the loss of Eg5 in the prometaphase pathway for centrosome separation (Tanenbaum et al. 2009). Kinesin 14 or HSET is a minus end directed motor involved in spindle pole focusing during prometaphase and MT sliding in metaphase. In later stages CENP-E controls kinetochore sliding on the MT lattice and kinetochore pulling and pushing contributing to the correct orientation of chromosomes. Other kinesins such as KID, Kinesin 4, MCAK and MKLP1/2 are also involved in

polar ejection forces, poleward MT flux, kinetochore pulling and central spindle sliding in anaphase (Cross and McAinsh 2014) (Verhey and Hammond 2009) (Figure XV).

Eg5

KIF11, **Kinesin 5** or **Eg5** is a plus end directed kinesin, which is structured in an N-terminal motor domain, a central coiled coil domain called stalk and a globular C-terminal tail domain. The coiled coil domain permits the homotetramerization of this protein with a bipolar arrangement that presents a pair of motor domains on either end.

The motor domain is composed by 350 residues and has all the elements that characterize a kinesin. The loop 5 is implicated in ADP release and interconnects changes in nucleotide state to other regions of the motor domain. This specific area is targeted for the best known Eg5 inhibitors, STLC and monastrol (Waitzman and Rice 2014).

The C-terminal or tail domain is the regulatory part of Eg5. This globular domain is phosphorylated at Thr926 by CDK1 during G2/M transition, promoting Eg5 binding to MTs (A Blangy et al. 1995). It was proposed that this binding is actually modulated by the interaction of Eg5 with the dynactin subunit p150 (Blangy et al. 1997). Additionally, this part, which also binds to MT, has been described as required for filament crosslinking and sliding. Basically, this part aids motor domain to persistently crosslink MTs (Weinger et al. 2011).

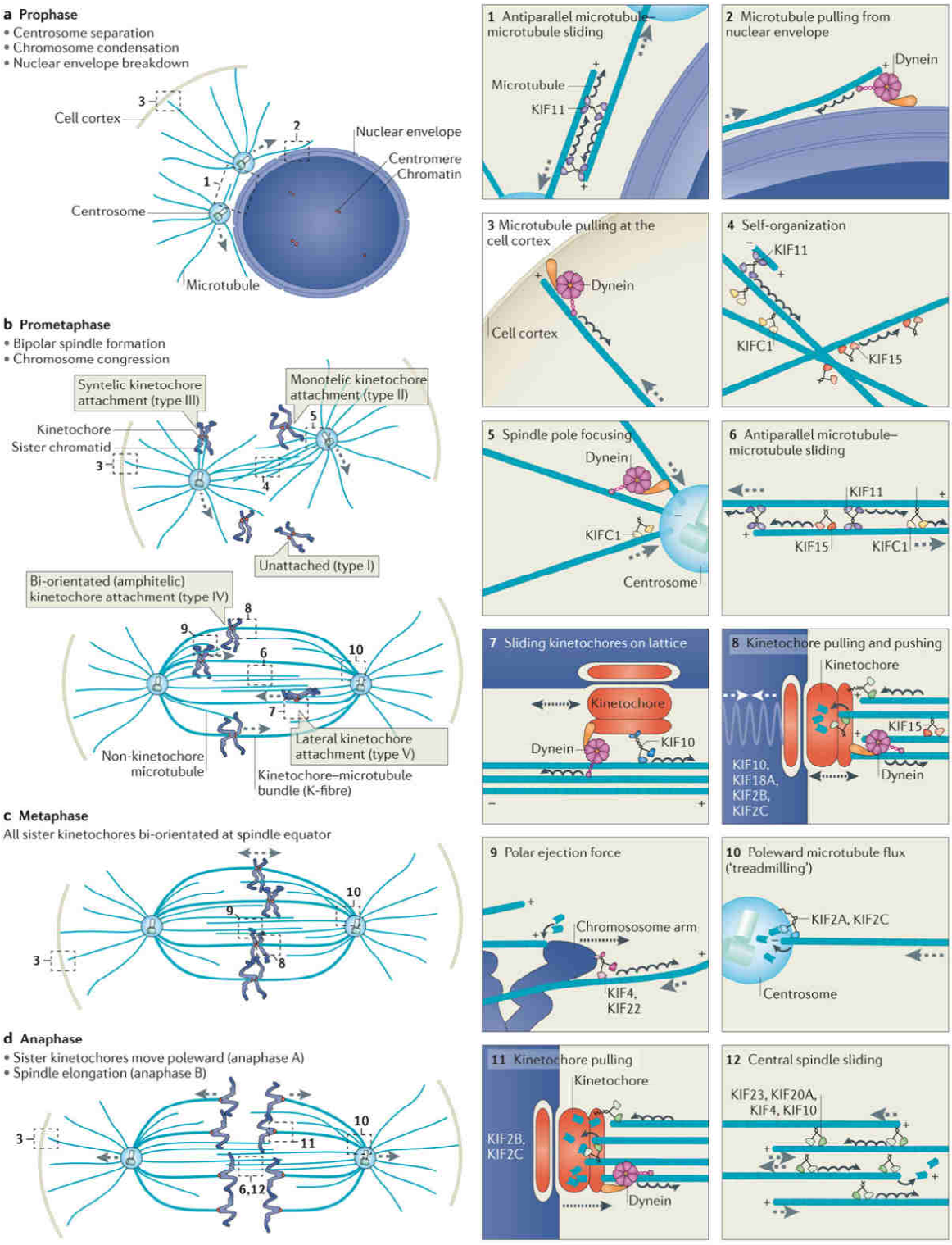

Figure XV. Schematic representation of all motor protein involved in mitotic events and their function at every stage (Cross and McAinsh 2014).

The tetrameric structure of Eg5 allows binding to two antiparallel MTs at the same time, and movement towards the plus ends on both, promotes the ejection of a force that slides MTs apart (Kapitein et al. 2005). This capability is the responsible Eg5 functions in mitosis, centrosome separation and bipolar spindle maintenance, through antiparallel MT sliding at the equator of the spindle (Ferenz et al. 2010). Thus, inhibition of Eg5 motor activity leads to monopolar spindles and failed chromosome segregation, suggesting that the kinesin could be a putative target for effective antimitotic drugs.

Eg5 localizes at spindle during mitosis with an important predominance at spindle poles. It has been suggested that Eg5 localization along the spindle is controlled by TPX2 (see above) and dynein. In *Xenopus* extracts, dynein transports Eg5 towards the spindle poles. This movement occurs in the entire spindle with the exception of spindle center and spindle poles, where Eg5 remains stationary (Uteng et al. 2008).

Additionally Eg5 interacts with **NuMA** during mitosis. NuMA localizes at the nucleus during interphase and in mitosis migrates to centrosomes. This protein associates with the dynein complex at spindle poles and is involved in the organization and stabilization of minus ends MT at spindle poles during mitosis. NuMA depends on Eg5 for its localization at centrosomes, and in consequence spindle MTs focus to the poles. In contrast, Eg5 localization at poles is not affected by NuMA (Iwakiri et al. 2013).

The dynein complex

Dyneins are multimeric MT-based motor complexes, with a motor domain belonging to the family of AAA+ proteins. These ATPases are associated with diverse activities, such protein unfolding, DNA and RNA duplex disassembly and prying apart macro-molecular complexes (Roberts et al. 2013). Mammals have two differentiated dynein families, cytoplasmic and axonemal dynein. Axonemal dynein is present in cilia and flagella whereas cytoplasmic dynein has roles on intracellular trafficking and mitosis.

Two heavy motor chains and several minor subunits compose human **cytoplasmic dynein**. The dynein intermediate chain (DIC) is a major binding platform for multiple dynein adaptor proteins whereas dynein light chains (DLC) are required for all the mitotic functions of dynein (Raaijmakers et al. 2013).

This molecular motor presents minus end directed motility, which enables several functions during cell cycle. Dynein transports cargo along MTs, which includes components of the centrosome, transcription factors and cytoskeletal filaments among others (Roberts et al. 2013). Dynein is also involved in centrosome separation and spindle pole focusing (Blagden and Glover 2003). Dynein can also exert tension on cellular structures. For instance, it can produce pulling forces on the MT network that is at cell cortex, controlling spindle positioning during cell division. Also, this protein is required for positioning of Golgi apparatus in perinuclear areas in interphase (Corthesy-Theulaz et al. 1992). During mitosis dynein functions include centrosome separation, centrosome tethering to the nuclear envelope and nuclear envelope breakdown by pulling nuclear membranes and associated proteins in a poleward manner along astral MTs (Salina et al. 2002).

While yeast dynein is processive *in vitro,* mammalian dynein requires different factors for its motility. Several adaptors proteins have been shown recently to regulate dynein function together with dynactin forming a protein complex with minus end processivity:.

Dynactin is another multi subunit complex composed by at least seven different components classified by their size (from 22 to 150 kDa). From all the subunits the largest one, p150 glued, interacts with DIC and is required for dynactin binding to MT (Karki and Holzbaur 1999). Dynactin was described as the essential co activator for dynein motility. Overexpression of dynactin subunits has been used to inhibit dynein function *in vivo*. For instance, expression of subunit p50 (dynamitin) disassembles the dynactin complex whereas overexpression of p150Glued results in disruption of dynein and dynactin interaction.

The **LIS1-NudE complex** also interacts with dynein. LIS1 was first described as a gene linked to lissencephaly, a brain cortex disease characterized by problems in neuronal migration. This complex promotes a persistent–force dynein state increasing dynein binding to MTs (McKenney et al. 2010).

Different protein cargo adaptors have been identified for dynein. All of them are coiled coil proteins whose major function is to connect dynein whit the cargo. Additionally, these proteins were described recently as required for activation of dynein processivity (McKenney et al. 2014). For instance, Hook3 was identified as an adaptor on early endosomes, Spindly on kinetochores, Rab11-FIP3 on Rab11 positive endosomes and BicD on either Rab6 positive vesicles or NE.

BICD2

BicD was first identified in *Drosophila*. BicD depletion in this organism is characterized by a bicaudal phenotype, which means that flies present two posterior segments (two abdomens). Later the function of this protein was related to embryo development through control of mRNA transport (Suter et al. 1989).

BicD interaction with dynein was first described in mammalian cells (Hoogenraad et al. 2001). Mammals present four proteins with homology to BicD: BicD1 and BicD2, directly related, and the more distantly related are BicDR1 and BicDR2. All of them bind to dynein through their N-terminal domain. The function of these proteins is related to transport but each one seems to have a distinct role. The function of BicDR2 still remains unknown.

BicD1 regulates endosomal sorting of neutrophin receptors in motor neuronal cells and is also involved in MT organization. It was shown that BicD1 interaction with dynein is required for focusing and anchoring of MTs to the centrosome (Fumoto et al. 2006).

BicD2 is the best studied of the human BicD homologs. It directly interacts through its C-terminal part with dynactin subunit dynamitin and associates

with the dynein complex through the N-terminal domain (Hoogenraad et al. 2001). This N-terminal domain promotes a strong interaction between dynein and dynactin. Overexpression of the N-terminal domain suppresses dynein function, probably acting as a dominant negative avoiding dynein anchoring to the cargo and resulting in dynein detachment from MT (Splinter et al. 2012). Tethering this BicD2 segment to mitochondria and peroxisomes induces transport of these organelles to the minus ends of MTs, which means that N-terminal part of BicD2 is sufficient to interact with dynein (Hoogenraad et al. 2003).

BicD2 N-terminal domain is not only an adaptor, it is also required for dynein activation or motility (McKenney et al. 2014). How this protein operates as an activator remains unclear, but it was proposed that it could induce a conformational change on dynein releasing the dynein motor from an inhibitory conformation (Bhabha et al. 2014).

BicD2 C-terminal part, apart from its interaction with dynamitin, interacts with Rab6 (Matanis et al. 2002). Rab6 is a small GTPase present at Golgi and vesicles derived from it, which acts as a receptor for vesicle transport. Rab6 interaction with BicD2 promotes BicD2 localization at Golgi during interphase (Hoogenraad et al. 2001). This BicD2 fragment also interacts with RanBP2 allowing its localization at NE during G2/M transition and is important for centrosome tethering to the NE (see above).

Additionally, the BiCD2 C-terminal domain is able to interact directly with the N-terminal domain forming a closed or inhibitory conformation of the protein that could control its interaction with dynein. It has been proposed that BicD2 binding to the cargo promotes a conformational change that releases the N-terminal part of BicD2. Once this domain is available it can associate with dynein, promoting minus-end transport of the cargo (Hoogenraad et al. 2001) (Figure XVI). This idea is supported by the observation that mutations in C-terminal domain of BicD2 reduce its interaction with the dynein-dynactin complex (Liu et al. 2013).

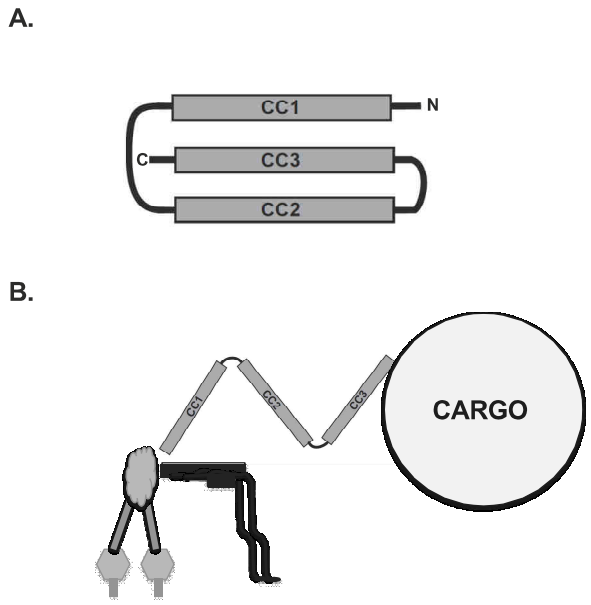

Figure XVI. Inhibitory and active conformation of BicD2.

(A) When BicD2 is not bound to cargo the C-terminal domain interacts with the N-terminal domain creating a closed and inhibitory conformation of the protein. (B) When BicD2 binds to the cargo the conformation of the protein changes to "opened" allowing interaction with both, dynein complex and cargo.

BicD2 regulation during cell cycle

BicD2 interactions are cell cycle dependent, whereas in G1 and S phase localizes at Golgi due to the binding to Rab6 positive vesicles, during G2 and G2/M transition BicD2 preferentially binds to the nuclear pore component RanBP2. This association promotes dynein recruitment to the NE in G2, a process that control centrosome tethering to the nucleus before mitotic entry. Thus, elimination of dynein function at this stage, promotes centrosome detachment from NE. Furthermore, BicD2 directly interacts through residues 336-595 with kinesin 1, a motor that can also interact with Rab6. Therefore, kinesin 1 and dynein form a complex with BicD2 and cargo, in which during G1 kinesin 1 activity predominates whereas during G2 dynein activity predominates (Splinter et al. 2010).

BicD2 association with RanBP2 in G2 is not only involved in centrosome tethering and separation, it was also proposed as required for apical nuclear migration and mitotic entry in neuronal progenitor cells (Hu et al. 2013).

BicD2 has been found phosphorylated during mitosis but no function for the phosphorylation was described so far in this protein. Interestingly, BicD2 was proposed as a substrate for Nek9 protein kinase (Holland et al. 2002), but the effect of Nek9 over BicD2 has never been studied.

BicD2 and disease

BicD2 mutations were recently associated with spinal muscular atrophy. This disease is characterized by loss in control of muscle movement triggered by damage of motor neurons. This phenotype is probably caused by deficiency in transport in motor neurons (Hoogenraad and Akhmanova 2016). These patients present single amino acid mutations spread along BicD2 gene sequence. Some of them were related to disruptions in binding with Rab6 GTPase or increased association with dynein-dynactin complex (Oates et al. 2013). Additionally, mutations on dynein heavy chain cause similar phenotypes (Hafezparast et al. 2003).

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OBJECTIVES

The objective of this thesis is to define how the NIMA-related kinases Nek9, Nek6 and Nek7 control centrosome separation through phosphorylation of the kinesin Eg5, which takes place at the onset of mitosis.

Detailed objectives:

- Identify the molecular requirements for normal Eg5 prophase localization.
- Describe the function of Eg5 Ser1033 phosphorylation in relationship to the proteins found to be instrumental for the localization of the kinesin.
- Integrate all the obtained data and propose a mechanism that could explain Eg5 accumulation at MT minus ends during prophase and thus, centrosome separation.

EXPERIMENTAL PROCEDURES

Reagents

All reagents were obtained from Sigma-Aldrich unless otherwise indicated.

Plasmids

Different Nek9 and Nek6 expression plasmids were previously described (Roig et al. 2002) (Belham et al. 2003). Myc-Eg5 plasmids were described in (Rapley et al. 2008). pEGFP C3 hTPX2 and pEQ70 zz-hTPX2 plasmids were a gift from Isabelle Vernos (CRG). pEGFP C2 mBicD2 was a gift from Anna Akhmanova (Utrech University). pCS2 3xFLAG hEg5 plasmids were obtained from Jens Lüders (IRB Barcelona). GFP-Emerald TPX2 constructs were obtained from Addgene: TPX2 [1-747] (mEmerald-TPX2-N-10 #54285), TPX2 [367-747] (mEmerald-TPX2T1-C-18 #54286) TPX2 [367-712] (mEmerald-TPX2T2-C-18 #54287) and TPX2 [713-747] (mEmerald-TPX2T3-C-18 #54288).

Different cDNAs were amplified by PCR from existing plasmids (table S1). In some cases the restricted fragment was introduced in an intermediate pCR 2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer's protocol. The cDNAs were digested from the vector and introduced in the desired plasmids.

Site-directed mutagenesis was performed using the appropriate primers (table S2) and either commercial QuickChange lighting Site-Directed Mutagenesis kit (Agilent Technologies) or alternatively through the use of Pfu Ultra High-Fidelity DNA Polymerase (Agilent Technologies) and DpnI (New England Biolabs).

Primers were synthesized by Sigma-Aldrich. Constructs were sequenced at Macrogen Inc.

DNA was purified using Macherey-Nagel NucleoSpin Plasmid kit according to the manufacturer's instructions.

Protein expression in bacteria

Proteins were expressed either in *E. coli* RosettaTM 2 or E. coli BL21 pRep4 and induced with 0.1 mM IPTG during 3 hours at 30 ºC or overnight at 25 ºC.

PolyHis fusion proteins were purified with Ni-NTA beads (Qiagen) and eluted with Imidazole following standard protocols.

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

Protein expression, size and purity were assessed by SDS-PAGE and Coomassie blue staining.

Cell culture and synchronization

HeLa, HEK 293T cells and Embryonic Mice Fibroblast (MEF) cells were cultured in a 5% CO2 atmosphere and 37 C 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).

When indicated, cells were synchronized in mitosis using 2 mM thymidine during 16 hours, washed and transfected with siRNA or plasmid. After 8 hours of transfection cell were treated again with media containing 2 mM thymidine during 16 hours, washed and released during 8 hours and finally supplemented with nocodazole 50 ng/ml during another 3 hours.

For G2 synchronization cells were treated with RO-3306 9 µM (Enzo) during 16-20 hours.

Transfection

HEK 293T cells were transfected using different expression plasmids using Polyethyleneimine (PEI, Polyscience, Inc) (Boussif et al. 1995). HeLa cells were transfected with LipofectamineTM 2000 according to the manufacturer's instructions (Invitrogen). siRNA transfection was performed using Lipofectamine siRNA-MAX (Invitrogen) according to manufacturer's instructions.

The sequences of the siRNA duplex for targeting the different proteins were as follows: TPX2 5'-GAAUGGAACUGGAGGGCUUUU-3' (Dharmacon) (Gruss et al. 2002), Nek6, 5'-AAUAGCAGCUGUGUGAGUCUUGCCU-3' (Ambion) (O'Regan and Fry 2009); Nek7, 5'-AAUAGUGAUCUGAAGGAAGAGGUGG-3' (Invitrogen); Nek9, 5'-AAUAGCAGCUGUGUGAGUCUUGCCU-3' (Invitrogen).

Lentiviral infection

shRNA was delivered through lentiviral infection. HEK 293 cells were transfected with PEI using the plasmids for lentiviral assembly REV, RRE and VSVG, together with the corresponding pLKO.1-shRNA plasmids (Sigma MISSION shRNA Library). Empty pLKO.1 vector was used as a control. 24 hours after transfection, HEK cells were incubated at 30 ºC during 16 hours stimulating virus assembly and continuously medium was collected and filtered. HeLa cells were treated with the resulting media for two consecutives days and then selected with puromycin (1µg/ml) during 3 days.

The shRNA clones used for targeting the different proteins were: hBICD2 TRCN0000005269 5'-CCGGGCTGCCAGGAGGACTTGGCCACTCGAGTGG

CCAAGTCCTCCTGGCAGCTTTTT-3' and TRCN0000005270 5'-CCGGCCTTTGGACAAGCACACACAACTCGAGTTGTGTGTGCTTGTCCAA

AGGTTTTT-3'; RHAMM TRCN0000061553 5'-CCGGGCCAACTCAAATCGGAAGTATCTCGAGATACTTCCGATTTGAGTT

GGCTTTTTG-3' and TRCN0000061555 5'-CCGGGCCAACTCAAATCGGAAGTATCTCGAGATACTTCCGATTTGAGTT

GGCTTTTTG-3'; Nek9 TRCN0000000929 5'-CCGGCCGAGGAATGGAAGGTTTAATCTCGAGATTAAACCTTCCATTCCTCGGTTTTT-3' and TRCN0000000930 5'-CCGGCCAAAGGAACTCAGACAGCAACTCGAGTTGCTGTCTGAGTTCCTTTGGTTTTT-3'.

Cell extracts, immunoprecipitation and western blotting analysis

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

Immunoprecipitations were carried out with the indicated antibodies pre-bound to protein G coupled to dynabeads (Invitrogen). Lysates were incubated with the beads for 90 minutes at 4 ^{o}C and washed three times with lysis buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Immobilon-P Transfer Membrane, Millipore) before proceeding with immunoblotting.

Membranes were probed with the following antibodies at 1 µg/ml: anti-Nek9, anti- Nek9 [Thr210-P] polyclonal antibodies, produced as described (Roig et al. 2002) and (Roig et al. 2005). Polyclonal anti-Eg5 [Ser1033-P], produced as described (Rapley et al. 2008). Anti-Nek6 (Abcam), anti-Nek7 (Cell Signaling and Epitomics), anti-BicD2 (Abcam), anti-myc (unpurified mouse hybridome produced in house), anti HA (mouse hybridome), anti-GADPH (Santa Cruz), anti-dynein IC (Santa Cruz), anti-Eg5 (BD Bioscience), anti-GFP (Torrey Pines), anti-FLAG (Sigma), anti-TPX2 (Thermo), anti-Eg5 [Thr926-P] (Abcam) and anti-β-tubulin (Sigma) were also used. Anti-HsAurora A (0.5 µg/ml), anti-TPX2 polyclonal, anti-TPX2 monoclonal and anti-RHAMM were a gift from Isabelle Vernos (CRG). Secondary antibodies were from Jackson Immuno

Research Laboratories and were detected by ECL Chemiluminiscence (Thermo Scientific).

Protein kinase assays

In vitro phosphorylation analysis of purified hTPX2 FL and mBicD2 fragments were done by incubation of purified hNek9 (200 ng) in phosphorylation buffer containing 50 mM MOPS pH 7.4, 5 mM $MgCl_2$, 10 mM β-glicerophosphate, 1 mM EGTA and 1 mM EGTA plus 100 μM [γ-^{32}P] ATP at 25 C during different points of time.

Reactions were terminated by addition of 5X Laemli sample buffer and boiling, and proteins were resolved by SDS-PAGE. Coomassie staining was used to visualize proteins and ^{32}P incorporation was measured with a PhosphorImager system (Molecular Dynamics).

Mass Spectrometry

2 μg of TPX2 or BiCD2 purified proteins were incubated with phosphorylation buffer and 100 μM ATP in the presence or absence of 200 ng of purified Nek9 during 15 minutes at 25 ^{o}C. Reactions were stopped using Laemli sample buffer and boiling. Proteins were resolved by SDS-PAGE at 7% and visualized using Coomassie staining. TPX2 changed its electrophoretical mobility after phosphorylation, and thus two bands corresponding to TPX2 were collected for each sample (level 1, unshifted, and level 2, shifted) corresponding with the upper and lower part of the bands respectively. Samples were in-gel digested with trypsin at the Parc Científic Proteomic's Platform. After digestion samples were dried in a Speed-Vac and resuspended in 50 μl of 1% formic acid for LC/MS/MS analysis at the Mass Spectrometry core facility at IRB Barcelona.

Immunocytochemistry

HeLa cells were grown on coverslips, rinsed with PBS and fixed with methanol at -20 ºC for 15 minutes. After rinsing with PBS, cells were incubated with PBS containing 3% bovine serum albumin, 0.1% Triton-X and 0.02% azide. Primary antibodies used were: mouse anti-γ-tubulin 1:500 (Sigma), rabbit anti-Nek9 [Thr210-P] 1:200, anti-pericentrin 1:5000 (Abcam), anti-lamin 1:1000 (Abcam), anti-H3-P 1:200 (Cell Signaling), anti-Eg5[Ser1033-P] 1:200, anti-Eg5 1:1000 (BD Bioscience and GeneTex), anti-CENP-F 1:5000 (Abcam), anti-GFP 1:500 (Torrey Pines and Invitrogene), anti-FLAG 1:1000 (Sigma), anti-β-tubulin 1:1000 (Sigma), anti phospho-Aurora A 1:200 (Cell Signaling), Anti-HsAurora A 1:500, anti-TPX2 polyclonal 1:1000, anti-TPX2 monoclonal 1:1000, anti-RHAMM 1:500 (from Isabelle Vernos, CRG). Anti-BicD2 (from Anna Akhmanova (Utrech University). Primary antibodies were detected with Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG and Alexa Fluor 555 goat anti-rabbit or anti-mouse IgG 1:500 (Invitrogen). DNA was stained with DAPI (0,01 mg/ml).

Images were acquired with an Orca AG camera (Hamamatsu) on a Leica DMI6000B microscope equipped with 1.4 NA 63x and 100x oil immersion objectives. AF6000 software (Leica) was used for image acquisition and edited using Fiji (Image J). Confocal images were obtained using a Leica DM2500 spectral confocal microscope.

Quantification of fluorescence intensities at centrosomes was performed with Fiji on non-saturated images acquired with constant exposure. We measured a circular area around centrosome and an adjacent area with the same dimension to subtract the background. All the values were normalized to the median of each control.

Quantification of cytoplasmic and nuclear intensity was performed by drawing cell surfaces with FIJI polygon tool and measuring nucleus intensity using DAPI as reference.

Statistical Methods

Boxes in box plots correspond to first quartile (lower) and third quartile (upper). The band inside the box represents the median. Whiskers correspond with he lowest value still within 1.5 IQR of the lower quartile, and the highest value still within 1.5 IQR of the upper quartile. Outliers were determined using Rout (Q=1) method. Statistical analysis was performed using the Mann-Whitney t-test. *p<0.05; **p<0.01; ***p<0.001.

Drug treatments

HeLa cells were treated for the indicated times with the following inhibitors: nocodazole 200 ng/ml; monastrol 100 μM; STLC 5 μM; taxol 10 μM, EHNA 10 μg/ml (VWR International); cytochalasin 1 mM; blebbistatin 20 μM; MLN-8237 500 nM (Sellek) and BI-2536 100 nM (Axon Medchem).

Mouse Embryonic Fibroblast (MEFs) culture and Adenoviral infection

TPX2 lox/lox MEFs were a generous gift from Marcos Malumbres, CNIO, and their production was previously described in (Aguirre-Portolés et al. 2012). MEFs were cultured following standard protocols. Adenoviruses expressing Flp or Cre were amplified using HEK 293 cells. Subsequently, MEFs were infected with adenoviruses in confluence and in low serum conditions. Three days after infection cells were split into plates containing 10% FBS medium, and 24 hours later, harvested for analysis.

Primers described in (Aguirre-Portolés et al. 2012) were used for genotyping by PCR and anti-mouse TPX2 antibody, a gift from Alex Bird (MPI Dortmund), were used for determination of protein level expression.

Tetracycline-inducible GFP-TPX2 cell line production

To establish tetracycline-inducible cell lines that express siRNA resistant GFP-TPX2, HeLa cells were transfected using the Flip-In System (Invitrogen) following the manufacturer's instructions. FRT cells were co-transfected with

a vector expressing Flp recombinase (pOG44) and an expression vector containing a FRT site linked to hygromycin resistance gene and GFP TPX2 WT under the control of a CMV promoter (pCDN5-FRT/TO GFT-TPX2) in a ratio 1:9..

Homologous recombination between the FRT sites in the host cells and the pCDNA5 vector was catalyzed by the expression of Flp recombinase from pOG44 vector. These cells were selected using hygromycin 0.6 mg/ml, resistance that is acquired after the integration of the pCDNA5/FRT into the FRT sites. After selection, single colonies were grown and tested for GFP TPX2 expression.

Yeast culture

Yeast *Saccharomyces cerevisiae* strains Y187 (matα) and AH109 (mata) were maintained in YPDA rich medium. Transformed yeast strains were maintained in minimal selective medium lacking tryptophan for AH109 [pGBKT7], leucine for Y187 [pGADT7] or tryptophan and leucine for diploids. Protein interactions were grown in minimal synthetic dropout (SD) selective medium lacking tryptophan, leucine, histidine and adenine. Mel1 activity of yeast strains with positive protein interactions was assayed adding the substrate X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (Clontech Laboratories) directly onto the plates.

Yeast transformation

Yeast transformation was performed using the lithium acetate (LiAc) method as described in the Yeast Protocol Handbook (Clontech Laboratories). In summary, yeast competent cells were prepared and suspended with the plasmid and an excess carrier DNA in a LiAc solution with polyethylene glycol (PEG), and incubated at 30 ^{o}C to prepare cells. After incubation, DMSO was added and the cells were heat shocked. Yeast was then plated on the appropriate SD medium to select for transformants.

Preparation of yeast protein extracts

Yeast extracts were obtained using the standard Urea/SDS protocol as described in the Yeast Protocol Handbook (Clontech Laboratories). Cell walls were disrupted by a combination of chemical (Urea and SDS) and physical (vortex with 5 mm glass beads) in a buffer containing urea 8 M, SDS 5% w/v, Tris-HCl [pH 6.8] 40 mM, EDTA 0,1 mM, bromophenol blue 0,4 mg/ml and a protease inhibitor solution with pepstatin, aprotinin, benzamide and leupeptin (see Clontech Protocol Handbook). Debris and unbroken cells were eliminated by centrifugation.

Mapping protein interactions using the yeast two-hybrid assay

The Matchmaker two-hybrid system was used according to the manufacturer's protocol (Clontech Laboratories) to map the interaction of BicD2 and TPX2 to prey proteins. The cDNA encoding different BicD2 domains and full length TPX2 were cloned into the yeast GAL4 DNA-binding domain vector pGBKT7, and the cDNA encoding the prey proteins were cloned into de GAL4 activation domain vector pGADT7. The yeast *S. cerevisiae* stains AH109 and Y187 were transformed by the LiAc method with the GAL4 DNA- binding domain or the GAL4 activation domain plasmids and selected by tryptophan or leucine prototrophy, respectively. Yeast mating was performed in 2X YPDA medium for 16 hours-20 hours, and diploids were selected by leucine and tryptophan prototrophy. Protein interactions were identified selecting for histidine prototrophy.

Yeast two-hybrid library screening

Yeast two-hybrid screening was performed using the Matchmaker GAL4 system 3 (Clontech Laboratories) according to manufacturer's instructions in a similar manner to that described above. *S. cerevisiae* strain AH109 expressing the fusion protein cloned into a pGBKT7-BD plasmid was mated with *S. cerevisiae* strain Y187 pretransformed with a human bone marrow

cDNA library constructed in pGADT7-AD (Clontech Laboratories) and plated on selective medium lacking tryptophan, leucine, histidine and adenine. DNA was isolated from positive colonies and sequenced to identify interacting preys.

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RESULTS

1. Eg5 accumulation at centrosomes depends on dynamic MT and eg5 motor activity

In order to investigate the necessary conditions for proper Eg5 localization during prophase we have used a set of drugs that affect either microtubule (MT) dynamics or MT-based motor activity (table 1). Different drugs were added during 1 to 2 hours to HeLa cell cultures. After treatment, cells were fixed and stained for Eg5 and a centrosomal marker. Prophases were identified using DAPI staining, by assessing chromosome condensation and the presence of apparently intact nuclei.

Treatment	Function	Concentration
Nocodazole	MT depolymerizing agent	200 ng/ml
Taxol	Inhibitor of MT dynamics.	10 μM
S-Trityl-L-cysteine (STLC)	Eg5 Inhibitor. Binds to the L5 loop of the motor domain. Leads to aberrant binding to MT and inhibits Eg5 motor activity.	5 μM
Monastrol	Eg5 inhibitor. Binds to the L5 loop of the motor domain. Leads to aberrant binding to MT and inhibits Eg5 motor activity.	100 μM
Erythro-9-[3-2-(hydroxynonyl)]adenine (EHNA)	Dynein motor inhibitor	10 μg/ml
Blebbistatin	Inhibitor of Myosin II. Described to delay centrosome separation (Rosenblatt et al. 2004)	20 μM
Cytochalasin	Inhibitor of actin cytoskeleton dynamics.	1 mM
MLN 8237	Aurora A kinase inhibitor	500 nM
Bi 2536	Plk1 kinase inhibitor	100 nM

Table 1. Concentration and function of the different drugs tested.

The fact that Eg5 appears to surround the centrosomes in prophase (see for example Figure 1A, control conditions) suggests that this kinesin may be actually bound at the MT that emerge from the centrosomes. Using **nocodazole** at a concentration when it acts as a MT depolymerization agent, we have found that Eg5 was no longer localized around centrosomes; therefore, Eg5 localization is indeed MT dependent (Figure 1).

Taxol is a drug that is extracted from *Taxus brevifolia* bark and is currently used as a chemotherapeutic agent. MTs are also the target of this drug but in this case taxol acts stabilizing the MT and protecting them from depolymerization. Our results with taxol, showing that the drug also interferes with Eg5 localization, suggest that not only Eg5 accumulation around centrosomes depends on the presence of MT, but also that is dependent on dynamic MTs. Taxol is able to induce MT aster formation in mitosis (Verde et al. 1991) and to block the organization capacity of centrosomes and kinetochores (De Brabander et al. 1981). We have observed that Eg5 can localize to the MT asters induced by taxol (Figure 1).

Under the two above conditions, in which MT are disrupted in different ways, centrosomes were separated, even with an abnormal Eg5 localization. This observation suggest that the MT control centrosome position during mitosis and, after linker dissolution by Nek2, the organelles can diffuse along the cytoplasm, something that has been previously observed also using nocodazole (Jean et al. 1999) (Meraldi and Nigg 2001).

STLC and **Monastrol** are two different compounds with a similar effect on Eg5. Both inhibit Eg5 activity, and thus interfere with bipolar aster formation, by impeding ADP release from the ATP binding pocket. Kinesins present different conformational states while they are processively walking along MT. States of binding and unbinding to MT are necessary for their movement. During the APO (or the nucleotide free) state, the protein is strongly bound to MT, once ATP is incorporated the kinesin releases MT, changes its conformation after ATP hydrolysis and is anew bound to the MT once ADP is released. Thus, since STLC and Monastrol are preventing ADP release, they are inhibiting Eg5 activity but also altering Eg5 capacity to bind MT. Using these two drugs we have found that, as expected, centrosomes cannot be

separated during prophase in the absence of normal Eg5 motor activity, and importantly, that Eg5 is not able to properly localize to centrosomes. But due to the double effect of the inhibitors we cannot conclude whether Eg5 no longer localizes properly as a result of its motility inhibition or because of its impeded MT binding.

As we have described during the introduction, the dynein-dynactin complex moves towards the minus ends of MT, in an opposite way to that of Eg5. Dynein activity is required for the accumulation of different PCM proteins during centrosome maturation (Blagden and Glover 2003) and influences Eg5 localization on the mitotic spindle (Gable et al. 2012). In order to investigate whether the dynein-dynactin complex is also transporting Eg5 to the centrosomes in prophase we have treated cells with a drug that inhibits cytoplasmic dynein activity, **EHNA** (Penningroth et al. 1982). We tested the efficiency of this drug measuring the amount of cells that presented a disorganized Golgi apparatus (Thyberg and Moskalewski 1985). In the conditions used, after 2 hours of treatment the presence of a disorganized Golgi increased only a 20% compared to the control (data not shown). Despite EHNA's low efficiency, Eg5 accumulation at centrosomes was significantly reduced in prophase cells treated with this drug. The distance between centrosomes was not significantly affected compared to the control but a slight tendency to decrease centrosome separation was observed (Figure 1).

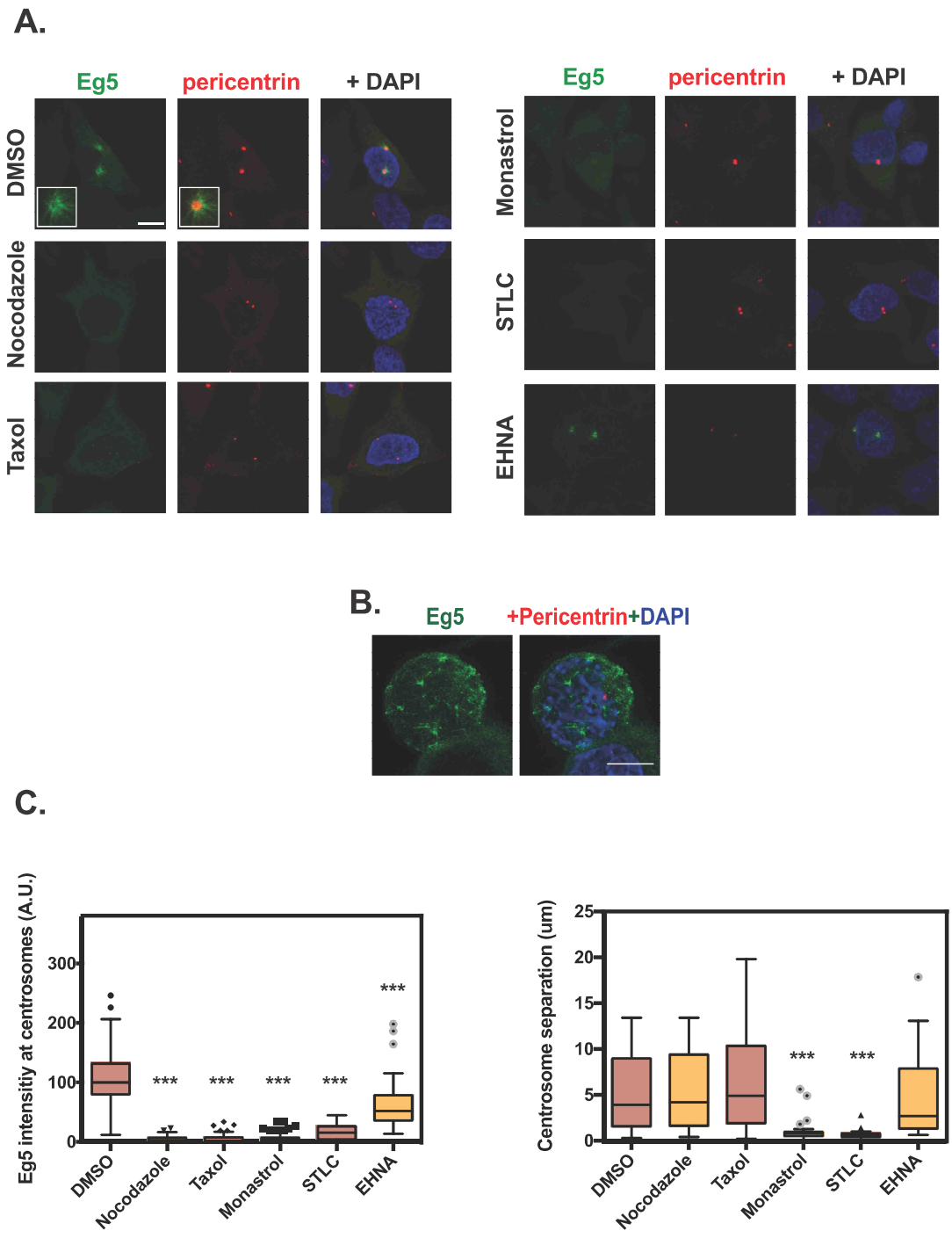

Figure 1. Effect of MT and Eg5 inhibitors on Eg5 accumulation in HeLa cells. (A) Immunofluorescence of prophase treated cells. Scale bar corresponds to 10 μm (B) Aster-like disposition of Eg5 in HeLa cells treated with taxol (C) Quantification of Eg5 intensity and centrosome separation n=30 prophase cells, 3 different experiments. Statistics were done with Mann-Whitney t-test.

Additionally, we investigated the role of the actin cytoskeleton in Eg5 localization as it has been previously related to centrosome separation (Cao et al. 2010). For this, we have performed cyto-immunochemistry to cells treated with **cytochalasin**, a drug that inhibits actin dynamics, and **blebbistatin,** a compound that inhibits the activity of the actin-associated motor protein, myosin. Both of them affected Eg5 centrosomal accumulation without altering centrosome separation compared to the control. We suggest that centrosome separation not only depends on Eg5 directed activity, it also depends on centrosome positioning (in part controlled by actin cytoskeleton) and other redundant pathways previously described (see the Discussion).

We also treated HeLa cells with two inhibitors of protein kinases that been described to have major roles in the control of the centrosome cycle, Aurora A (MLN-8237) and Plk1 (BI-2536). After 2 hours of treatment, cells that were starting mitosis failed completely to accumulate Eg5 and to separate centrosomes (Figure 2). Centrosome maturation as an essential step for Eg5 accumulation was recently described (Joukov et al. 2014) and both Plk1 and Aurora A are instrumental for this process. However, we cannot discard a direct role of these two kinases in Eg5 accumulation. For instance, Aurora A phosphorylates *in vitro* XEg5 but the function of the phosphorylation remains unclear (Cahu et al. 2008). Last but not least, Nek9 is activated by Plk1 at the onset of mitosis (Bertran et al. 2011) promoting the activation of Nek6/7 leading to the phosphorylation of the Ser1033 in Eg5 and Eg5 accumulation at centrosomes.

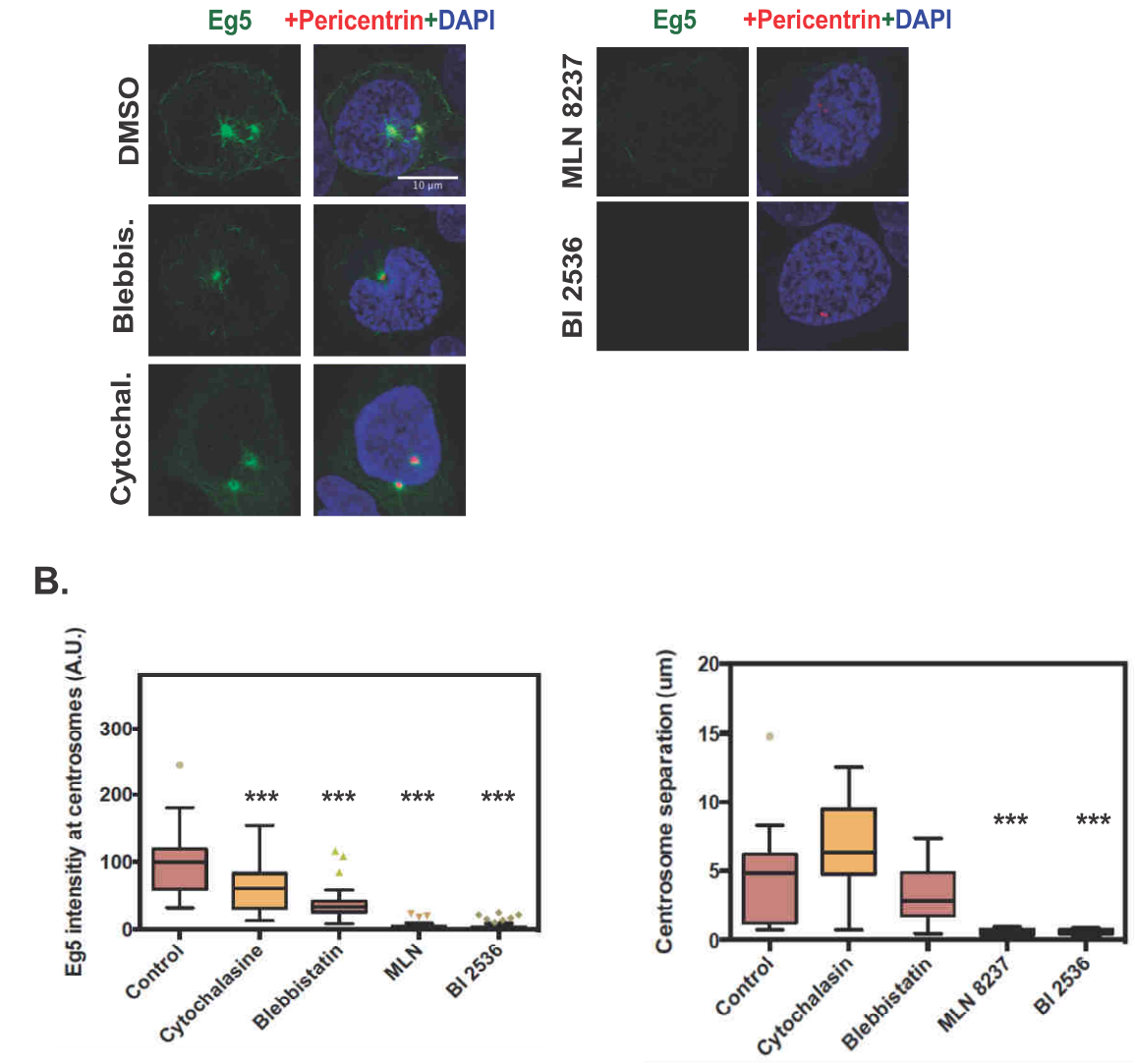

Figure 2. Effect of actin-related drugs and kinase inhibitors on Eg5 accumulation. (A) Immunofluorescence of prophase treated cells. Scale bar corresponds to 10 µm (B) Quantification of Eg5 intensity and centrosome separation n=10 cells. Statistics were done with Mann-Whitney t-test.

2. Eg5 interacts with dynein through the adaptor BicD2

Dynein interacts with Eg5 in a phosphorylation-independent manner

After detecting that Eg5 accumulation was affected by cytoplasmic dynein motility inhibition, we performed endogenous dynein immunoprecipitation in order to detect a possible interaction between these two proteins. Using an antibody that recognizes the two isophorms of dynein intermediate chain (DIC), we immunoprecipitated the dynein complex from HeLa cells and observed that it coprecipitated with endogenous Eg5 (Figure 3).

We next wanted to test whether this interaction was controlled by Eg5 Ser1033 phosphorylation. For this, we transfected HeLa cells with recombinant myc-tagged Eg5 with mutations in Ser1033 that either mimic phosphorylation (Ser1033Asp) or impedes phosphorylation (Ser1033Ala) and immunoprecipitated endogenous DIC. Additionally, we included the non-phosphorylable mutant for Eg5 Thr926 to detect a possible effect on Eg5-dynein interaction in the absence of CDK1 site phosphorylation (as has been suggested by (Blangy et al. 1997). We observed that all myc-Eg5 mutants co-precipitate in similar levels with dynein (Figure 3) and conclude that the dynein-Eg5 interaction is not strongly regulated by phosphorylation on neither the Nek6/7 (Ser1033) or CDK1 (Thr926) sites. However, endogenous Eg5 is able to tetramerize with the recombinant protein, and as a consequence, small variations on the strength of the interaction may be hard to be detected. For this, to prove that the Nek9/Nek6/7 kinase pathway indeed has no influence on Eg5-Dynein interaction, we infected HeLa cells with lentivirus expressing Nek9 shRNA and precipitated DIC. Nek9 depletion did not alter Eg5 association with dynein, thus strongly suggesting that Nek6/7 phosphorylation of Eg5 is not necessary for this association.

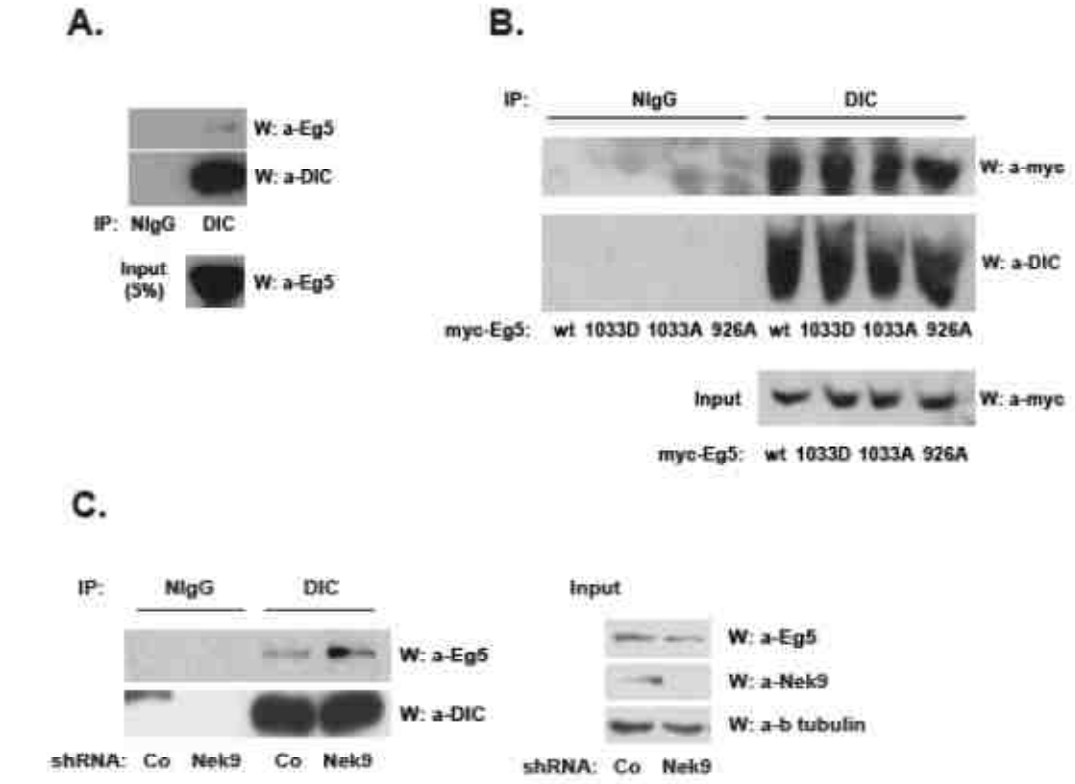

Figure 3. Dynein interacts with Eg5 in HeLa cells. (A) Co-precipitation of endogenous dynein with endogenous Eg5. Endogenous dynein was immunoprecipitated using an antibody against Dynein Intermediate Chain (DIC) (B) Co-precipitation of dynein with Eg5 mutants for phosphorylation on Ser1033 and Thr926. HeLa cells were transfected with phosphonull and phosphomimetic plasmid for Ser1033 and Thr926. (C) Co-precipitation of dynein with endogenous Eg5 in cells depleted for Nek9. Input reveals Nek9 depletion. This last figure was done with the collaboration of Núria Gallisà.

An alternative manner to detect whether phosphorylation is modulating the studied interaction is to induce phosphorylation with the expression of active forms of the kinases. Constitutive activation of Nek9 was the result of eliminating the inhibitory domain RCC1, resulting in the construct called Nek9 ΔRCC1 (Roig et al. 2002). Additionally, overexpression of Nek6 is enough to induce activation of this kinase (Belham et al. 2001). Endogenous dynein was purified from HeLa cells expressing empty FLAG as control, FLAG ΔRCC1 and FLAG Nek6. Our results show that Eg5 is associated with dynein independently of the expression of active kinases (Figure 4). Eg5 in cells

transfected with the active kinases presents an electrophoretic mobility shift due to a change in the pattern of phosphorylation and reacts with an anti-Eg5[S1033-P] antibody in the Eg5-dynein immunoprecipitates. RO-3306 was used as a control, since prolonged G2 arrest induces Eg5 phosphorylation and accumulation at centrosomes, possibly as a result of Plk1 (and thus Nek9) overactivation (see additional results). Thus, from our results we conclude that Eg5 phosphorylation neither favors nor interferes with dynein binding. This result suggests that Eg5 may be phosphorylated at Ser1033 once is bound to the complex. In fact, Nek6 can be detected coinmunoprecipitating with DIC, possibly as a result of its described interaction with Eg5 (Rapley et al. 2008) (Figure 4).

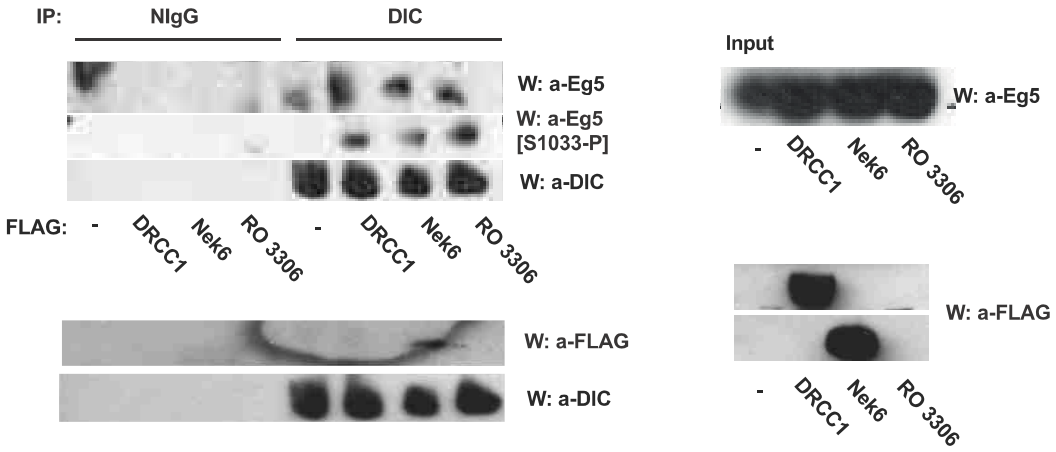

Figure 4. Eg5 interaction with dynein is independent of Eg5 phosphorylation. Dynein co-precipitation with endogenous Eg5 and S1033-P Eg5 in cells expressing active Nek9 (ΔRCC1) and active Nek6 (upper panel). FLAG Nek6 is co-precipitating with endogenous dynein (lower panel).

Dynein interacts with Eg5 through the adaptor BicD2

One of the main differences between yeast and mammalian dynein is that while yeast dynein is able to move along pre-assembled MTs in vitro, mammalian dynein requires the interaction and formation of a complex with other proteins that regulate dynein motility (McKenney et al. 2014), namely the dynactin complex (Gill et al. 1991), that interacts directly with dynein and MTs and different proteins that are bound to the dynein-dynactin motor complex and act as adaptors for the cargo. One of these adaptors is BicD2, the protein the responsible for dynein accumulation at nuclear envelope during G2-M transition, and indispensable for centrosome tethering at nuclear envelope just before mitosis and dynein-mediated centrosome separation (Splinter et al. 2012). Furthermore, BicD2 was described as the first Nek9 substrate (called Nek8 in the report), although the function of that phosphorylation remains unclear (Holland et al. 2002).

Since our hypothesis is that dynein complex is transporting Eg5 towards the centrosomes, we wanted to determine whether Eg5 was interacting with this complex as a cargo protein through BiCD2. In order to address this we downregulated BicD2 from HeLa cells using shRNA. Next, we immunoprecipitated endogenous dynein from the BicD2-depleted extracts and compare it to control cell extracts. We found that the dynein-eg5 interaction is greatly diminished in cells that were depleted of BicD2 (Figure 5). We thus conclude that the adaptor BicD2 mediates the dynein-Eg5 interaction.

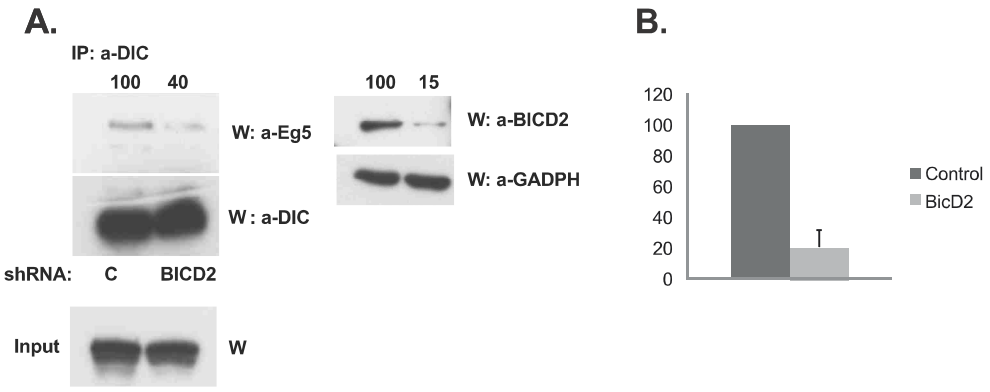

Figure 5. Eg5 interaction with dynein is dependent of BicD2. WB for dynein precipitation in HeLa cells depleted for BicD2. Eg5 interaction with dynein is diminished in absence of BicD2. A reduction of the 85% of BicD2 expression leads to a reduction of the 60% of immunoprecipitated Eg5 (B) Quantification of Eg5 immunoprecipitated with DIC in control and BicD2 depleted HeLa cells. Values obtained from n=3 experiments.

Eg5 tail domain interacts with BicD2 [487-575]

We next sought to determine whether Eg5 and BicD2 interacted and, if so, to map this interaction. HeLa cells were transfected with GFP-tagged mBicD2 and the recombinant protein was immunoprecipitated using anti-GFP antibodies. We found that BicD2 associates with endogenous Eg5 (Figure 6). Our results with dynein suggest that the interaction of Eg5 with BiCD2 would be independent of phosphorylation. To prove this, HEK293 cells were co-transfected with GFP-BicD2 and phospho-null and phospho-mimetic forms of Eg5 residues Ser1033 and Thr926, tagged either with myc or FLAG (Figure 6). We determined that both myc- or FLAG-Eg5 phosphonull and phosphomimetic mutants are able to associate with GFP BicD2 and that this association of Eg5 mutants is comparable to Eg5 WT. We also studied whether the interaction of BicD2 with Eg5 would be affected by active Nek9 and Nek6 and we co-transfected HeLa cells with the active kinases and recombinant BicD2. After BicD2 precipitation we determined that Eg5 associates with BicD2 independently of the presence of active Nek9 or Nek6. All these results are coherent with our previous results studying the interaction of Eg5 with dynein.

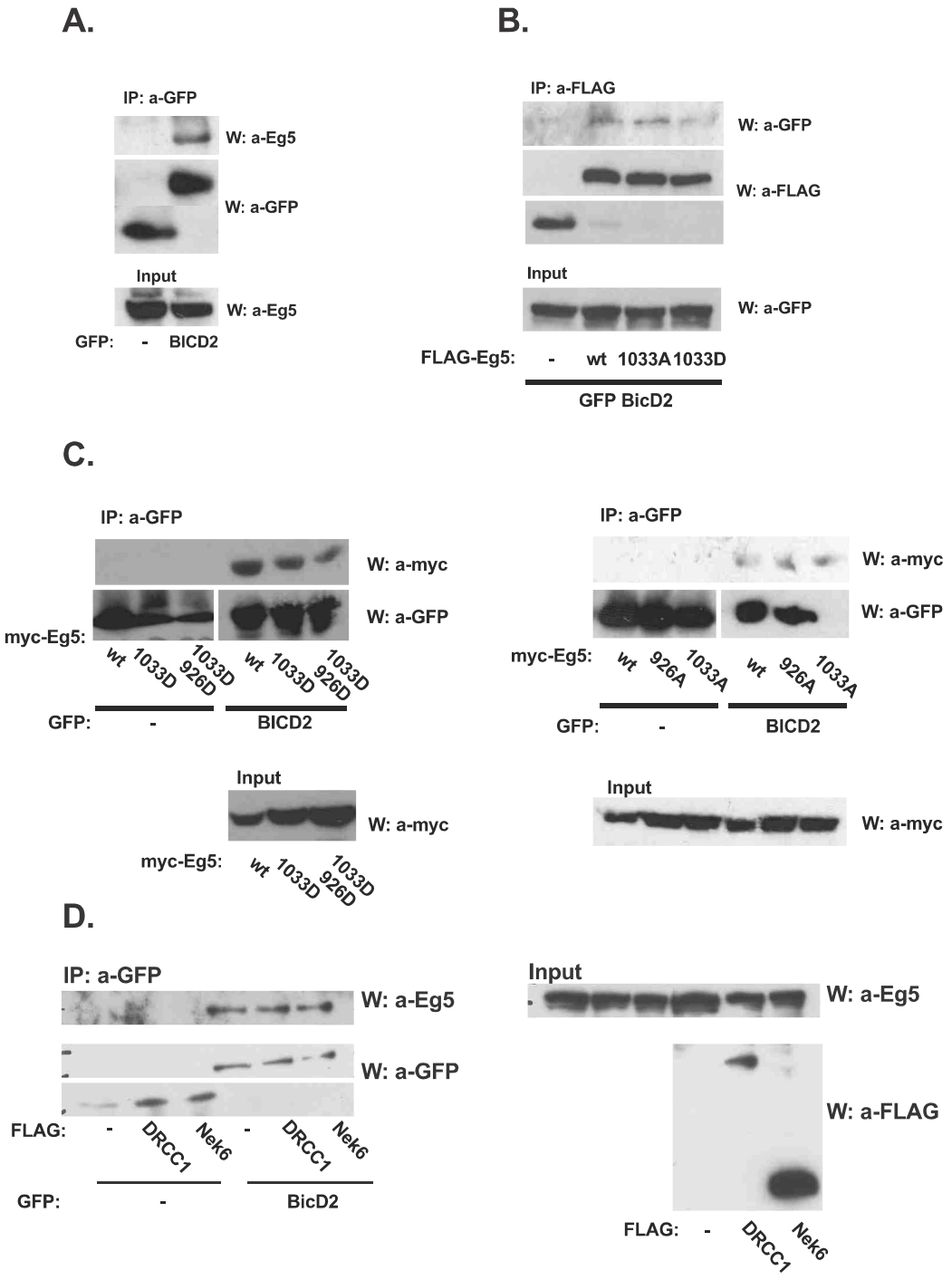

Figure 6. BicD2 interacts with Eg5 in a phosphorylation independent manner. (A) Endogenous Eg5 co-precipitates with GFP-BicD2. (B) Precipitation of recombinant FLAG Eg5. All Eg5 mutants bring out GFP BicD2. (C) Precipitation of GFP BicD2 brings out all the phosphomimetic mutants (left) and phoshonull mutants (right) for Ser1033 and Thr926 sites. (D) BicD2 associates to Eg5 in cells expressing active Nek9 (ΔRCC1) and Nek6. This last Figure was done with the collaboration of Núria Gallisà.

BicD2 presents three coiled coil regions. The first region (residues 20 to 270) has been shown to interact with the dynein-dynactin complex, the second region (340 to 579) was described as the Kinesin-1 interaction domain and the third region (662 to 804) is able to bind to RanBP2 and to cargo receptors such as Rab6 (Hoogenraad et al. 2001) (Matanis et al. 2002) (Hoogenraad et al. 2003).

To map the interaction between BicD2 and Eg5 we transfected HEK 293 cells with a series of different constructs expressing GFP BicD2 [1-575], GFP BicD2 [487-820] and GFP BicD2 [575-820] (Figure 7). We detected that BicD2 FL, BicD2 [1-575], BicD2 [487-820] but not shorter C-terminal BicD2 [575-820] associates with FLAG Eg5 FL. Thus, the amino acids required for interaction are between sites 487 and 575. We next built a construct expressing only this region. We found that BicD2 [487-575] is sufficient to interact with Eg5 and thus concluded that the kinesin (in contrast to classical cargo) binds BicD2 through this specific region.

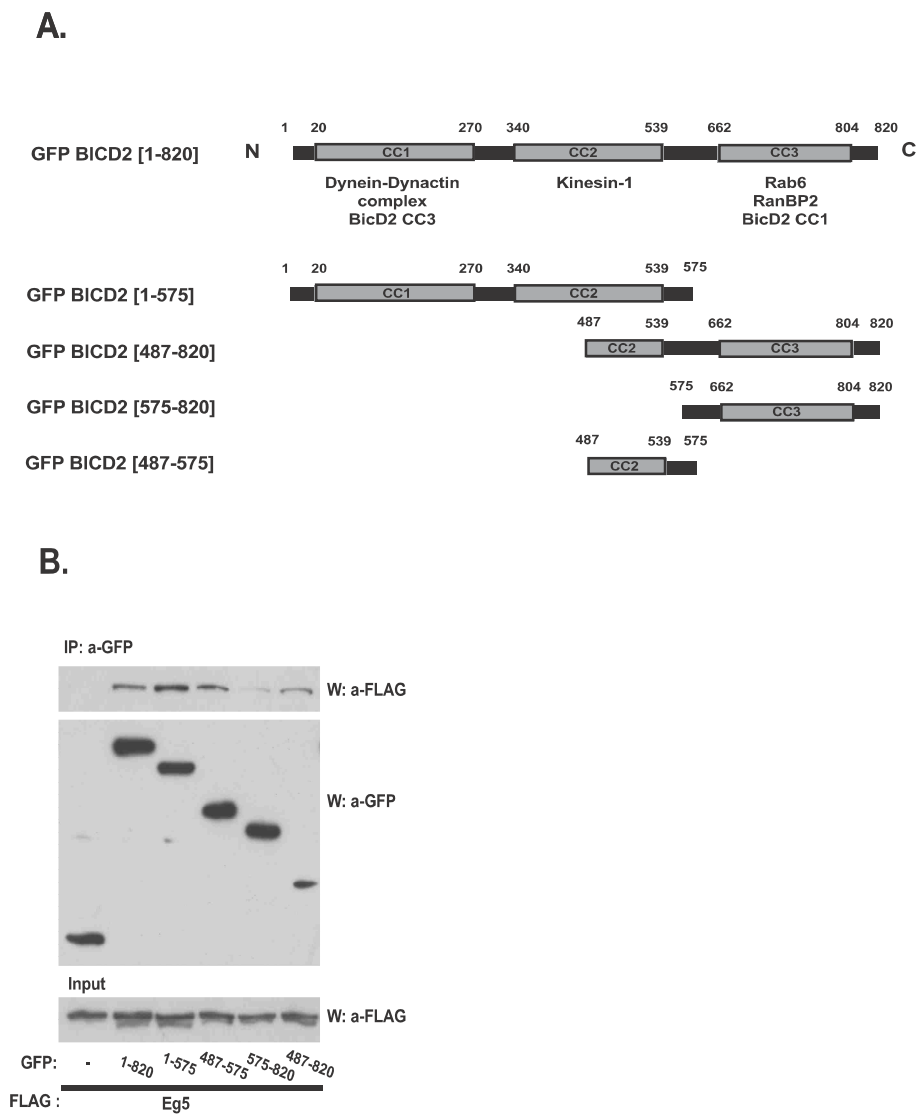

Figure 7. Eg5 interacts with BicD2 through [487-575] region. (A) Representation of the different regions of BicD2 analyzed. (B) Precipitation of BicD2 constructs represented in A. FLAG-Eg5 interaction with BicD2 is reduced when BicD2 lacks [487-575] amino acids.

To determine whether BicD2 directly interacts with Eg5, and to map the region of the kinesin responsible for that interaction, we performed Y2H analysis using pGBKT7-BD FL, N-terminal (1 to 575) and C-terminal (487 to 820) BicD2 and the three domains of Eg5 (motor, stalk and tail) expressed in pGADT-AD plasmids. We first tested the expression of the different constructs of BicD2 and their toxicity. We were able to detect expression by WB of the different fragments of BicD2 and determined that yeast expressing the pGBKT7-BD plasmids grow normally, which indicate that the constructs are

not toxic. Second, we analyzed a possible auto activation of the constructs, considering that coiled coil proteins increase the probability of this (see additional results for a discussion on this). We observed that BicD2 N-terminal presents the ability to activate the Y2H system and grow in media lacking histidine. Thus for this construct we use the histidine synthesis pathway inhibitor 3-Amino-1,2,4-triazole (3AT), that partially eliminated the background growth that results from the expression of this specific polypeptide (Figure 8).

After mating with yeast expressing the different Eg5 domains, we found that BicD2 FL is not able to interact with any of the Eg5 fragments. In contrast, using the BicD2 C-terminal construct (that contains the 487 to 820 identified above as one of the interacting regions) we detected interaction with the Eg5 tail domain. In the case of BicD2 N-terminal domain, due to auto activation, the results are difficult to interpret, but in media with 10 μM 3AT we detected that yeast co-expressing BicD2 N-terminal and Eg5 tail domain grow faster than the control and the other analyzed combinations. Thus, observing these results we propose that BicD2 would directly bind to Eg5 C-terminal tail domain only in its open conformation.

To assess in an independent manner whether there was any subtle effect of Ser1033 phosphorylation on the interaction, we compared the interaction between BicD2 C-terminal and Eg5 tail domain phospho-null and phospho-mimetic mutants. We performed a series of dilutions with different plasmid combinations (BicD2-C + Eg5 tail WT, BicD2-C + Eg5 tail 1033A and BicD2-C + Eg5 tail 1033D). Diploids expressing all combinations are able to grow in selection media, but in the case of BicD2 C-terminal and Eg5 tail phospho-mimetic, a slight decrease in yeast growth is detected, suggesting that phosphorylation may negatively effect the interaction between BicD2 and Eg5 (Figure 8).

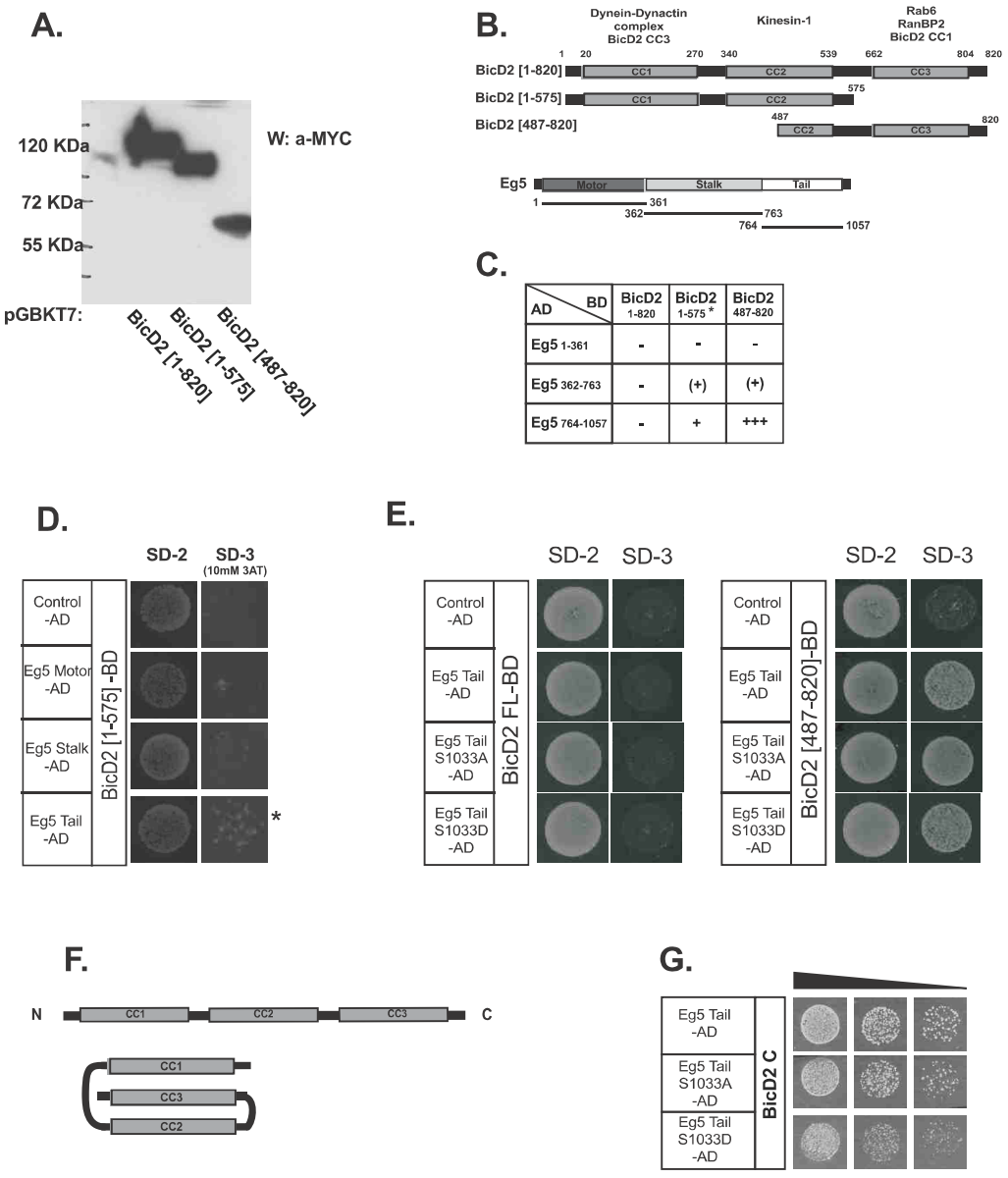

Figure 8. BicD2 c-terminus interacts directly with Eg5 tail domain. (A) Expression of BicD2 constructs in yeast. (B) Representation of the Eg5 and BicD2 constructs used for Y2H. (C) Determined interaction after mating. Parenthesis indicate slight growth detected but not conclusive. (D) Mating between BicD2 N-terminus and Eg5 domains. Asterisk marks a slight growth detected after 3AT addition. (E) Mating between BicD2 FL and C-terminus with Eg5 tail domain mutants. (F) Representation of BicD2 opened and closed conformation. (G) Dilutions for BicD2 c-terminus with Eg5 tail mutants.

BicD2 co-localizes with Eg5 in prophase and regulates Eg5 accumulation at centrosomes

We have so far described that Eg5 centrosomal localization depends on dynein and that Eg5 is able to interact with the dynein complex through direct binding to the adaptor BicD2. Since our results suggest that BicD2 is essential for the interaction between dynein and Eg5, we now sought to determine whether the presence of BicD2 is required for proper Eg5 centrosomal localization.

First, to elucidate the localization of BicD2 during prophase we transfected HeLa cells with GFP-tagged BicD2 and detected the protein through immunofluorescence. In transfected prophase cells GFP-BicD2 is localizing at the Nuclear Envelope (NE) as it was previously described by other groups (Splinter et al. 2010) and at MTs that emerge from centrosomes, in a similar way to Eg5. Thus, these two proteins are co-localizing at centrosomes during prophase (Figure 9A).

Next, to investigate whether BicD2 is necessary for Eg5 accumulation at centrosomes, we infected HeLa cells with lentivirus expressing BicD2 shRNA. We observed that in cells depleted for BicD2, Eg5 fails to localize at centrosomes during prophase. This was observed using two different shRNAs, (Figure 9B). Since one of the shRNAs is depleting BicD2 less efficiently, we continued the rest of experiments with the strongest one. The direct consequence of Eg5 failure to accumulate at centrosomes is a significant decrease on centrosome separation in prophase cells downregulated for BicD2 (Figure 9E and 9F).

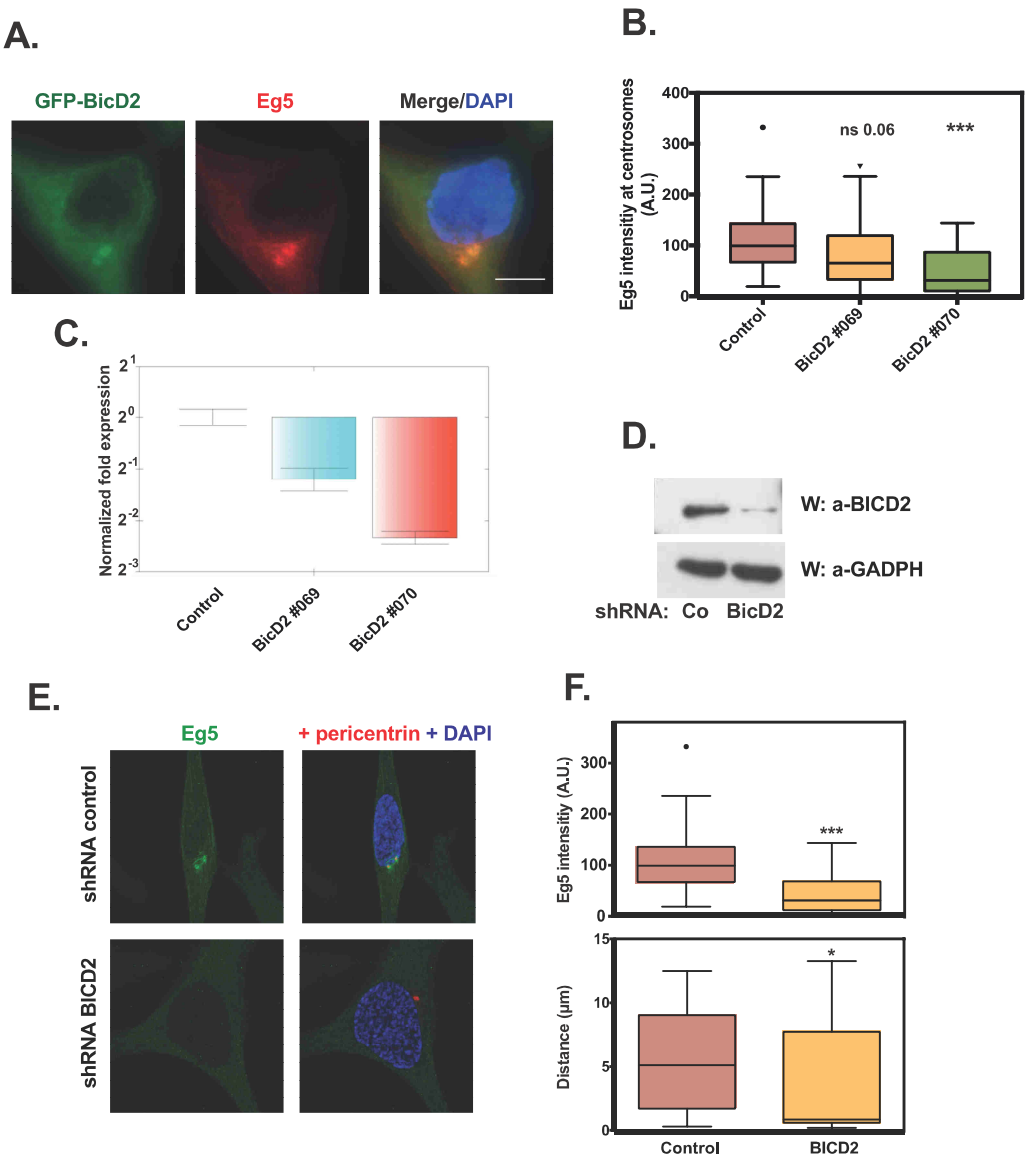

Figure 9. BicD2 co-localizes with Eg5 and is necessary for Eg5 centrosomal accumulation. (A) GFP BicD2 (green) co-localizes with Eg5 around centrosomes during prophase. Scale bar 10 µm (B) Eg5 intensity at centrosomes in prophase using two different shRNA. BicD2 shRNA #069 presents a tendency to reduced Eg5 at centrosomes but is not significantly different (p=0.06). (C) Quantitative PCR to detect mRNA levels of BicD2 after depletion with both shRNA. (D) WB for BicD2 depletion using shRNA #070. This clone was selected to continue with the analysis. (E) Immunofluorescence in prophase HeLa cells to detect Eg5 intensity at centrosomes in cells depleted for BicD2. (F) Quantification of Eg5 intensity at centrosomes and centrosome separation in control cells and BicD2 depleted. 3 independent experiments, 30 prophase cells.

As an alternative approach to investigate the role of BicD2 on Eg5 localization, we overexpressed different fragments of BicD2. To increase the number of detectable transfected prophases, we synchronized HeLa cells using a thymidine double block protocol. In parallel we established the level of expression of the different constructs by WB. Although expression is definitely different between fragments, we only analyzed GFP positive prophase cells with similar GFP signal levels. The BicD2 N-terminus (dynein interaction domain, residue 1-575) does not localize at either the nuclear envelope or the centrosome during prophase. However, the BicD2 C-terminus (RanBP2 interaction domain, residue 487-820) fragments localize to the nuclear envelope, even during interphase, and at centrosomes during prophase, in a similar way to the full-length construct. Since BicD2 [575-820] is not interacting with Eg5, our results show that centrosomal localization of BicD2 is not Eg5 dependent. The BicD2 [487-575] fragment is diffused along the cytoplasm, something expected, since this construct lacks the RanBP2 domain that targets it to the nuclear envelope, and proving that Eg5 is sufficient to localize to nuclear envelope or centrosomes.

Next, we measured the amount of Eg5 localizing at centrosomes in prophase cells transfected with the mentioned constructs. We saw a slight decrease on Eg5 accumulation in cells expressing both full length BicD2 and BicD2 [487-820], and interestingly BicD2 [487-575], described as the minimal region for Eg5-BicD2 interaction, strongly impaired Eg5 centrosomal accumulation during prophase, thus effectively acting as dominant negative (Figure 10).

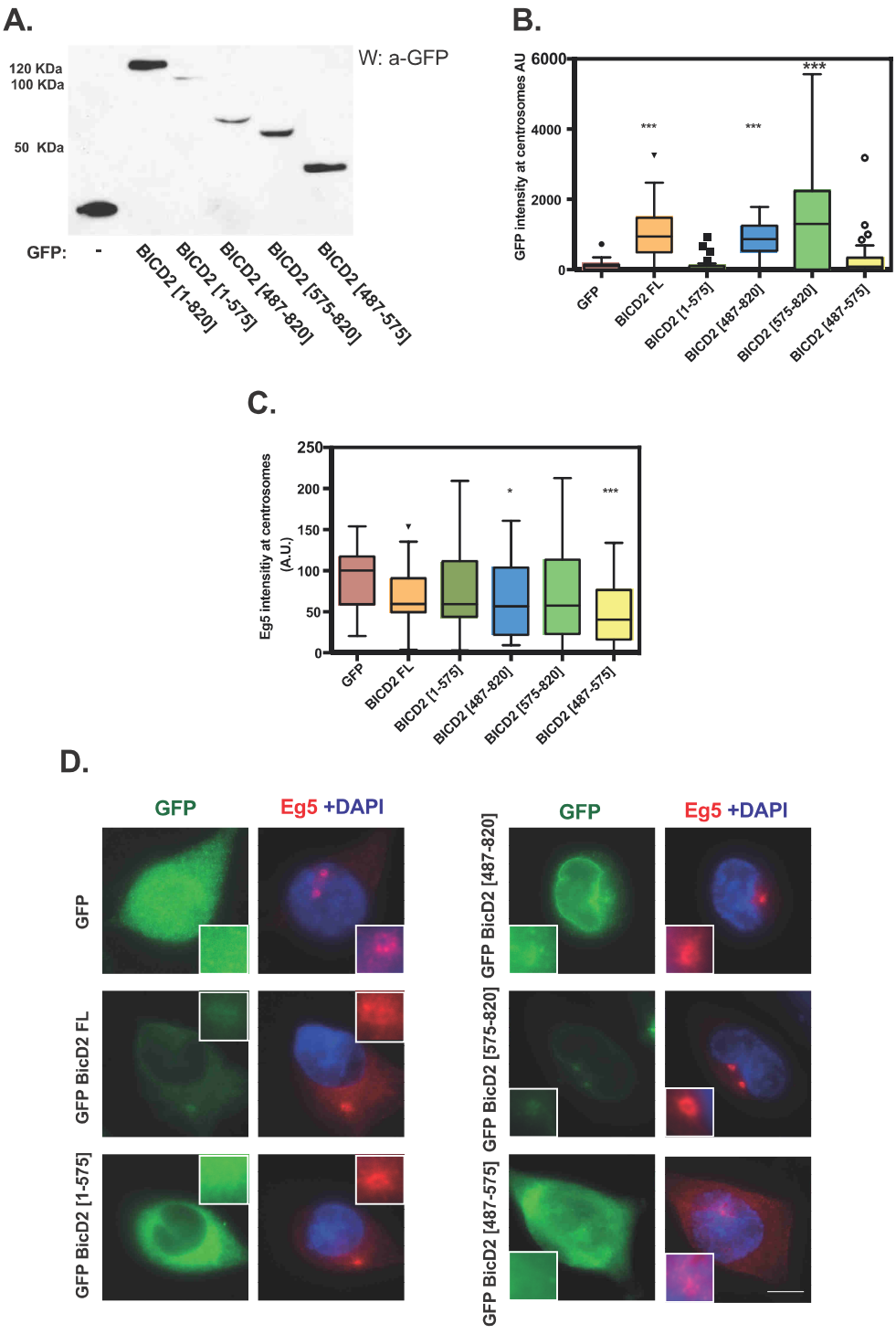

Figure 10. BicD2 [487-575] fragment acts as a dominant negative for Eg5 accumulation at centrosomes. (A) WB for expression of different BicD2 domains. (B) GFP intensity at centrosomes. Only BicD2 FL and C-terminus are able to localize at centrosomes during prophase. (C) Eg5 intensity at centrosomes in GFP positive prophase cells. N=30 prophase cells, 2 independent experiments. (D) Immunofluorescence in prophase HeLa cells, examples for (B) and (C)

3. TPX2 interacts with Eg5 in a phospho-dependent manner and is required for Eg5 pericentrosomal localization in prophase

TPX2 regulates Eg5 localization in prophase

TPX2 is a multifunctional protein with different roles during mitosis such as MT nucleation and bundling and Aurora A activation (Gruss et al. 2002) (Wittmann et al. 2000) (Bayliss et al. 2003). TPX2 is also required for dynamic reorganization of Eg5 localization along the mitotic spindle in metaphase (Ma et al. 2011) (Gable et al. 2012) (Balchand et al. 2015). Depletion of TPX2 leads to an increase of Eg5 plus end directed movement in astral MTs (Balchand et al. 2015). To determine whether TPX2 is also required for Eg5 localization at the minus ends of MTs during prophase we transfected HeLa cells with TPX2 siRNA (Figure 11). In cells depleted for TPX2, Eg5 fails to accumulate at centrosomes during prophase. In parallel, intercentrosome distance is slightly decreased.

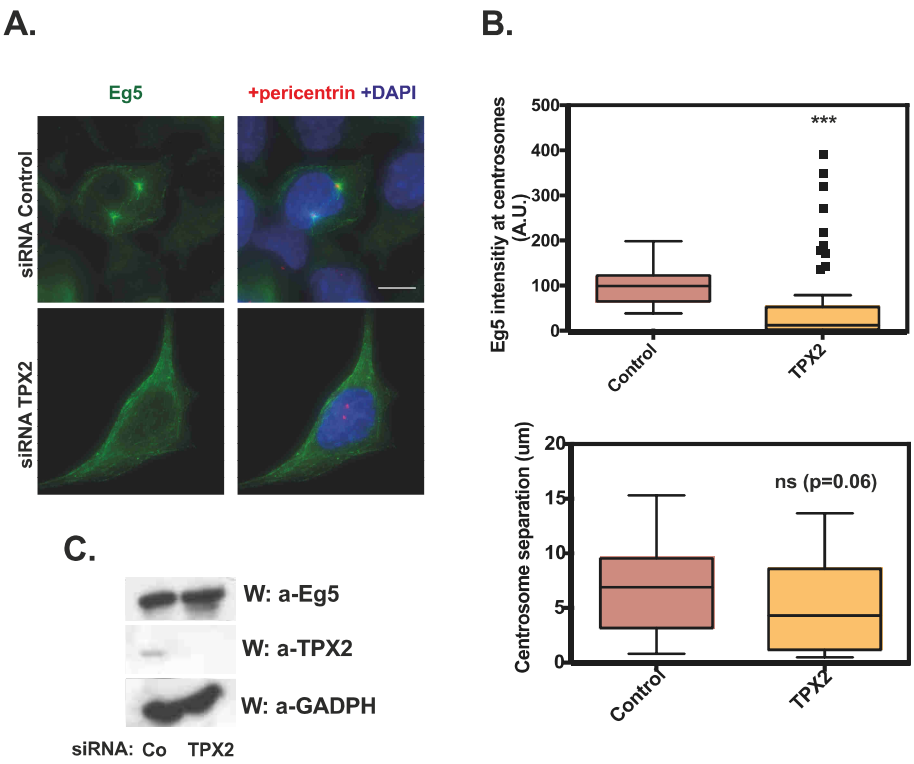

Figure 11. TPX2 controls Eg5 localization in prophase. (A) Prophase control and TPX2 depleted cells stained for Eg5. Scale bar 10 µm. (B) Quantification of cells from Figure A. Eg5 accumulation at centrosomes (upper panel) and centrosome separation in µm (lower panel). n=26 cells, 2 independent experiments. (C) WB against TPX2 and Eg5 in cells transfected with TPX2 siRNA. Statistics were done with Mann-Whitney t-test.

It has previously been shown that since TPX2 is a multifunctional protein spindle are not properly organized in TPX2 siRNA treated cells, and multipolar spindles are commonly detected (Wittmann et al. 2000). In our hands, centrosome structure (visualized with pericentrin) is grossly affected after TPX2 depletion. Thus, we cannot separate a direct effect of TPX2 depletion on Eg5 localization from a possible indirect effect due to centrosome disorganization. As we detect TPX2 at prophase centrosomes (see Figures 12 and 13, and a more extensive study of this below) we sought to mislocalize TPX2 from these organelles without altering TPX2 total levels and the structure of the centrosome. RHAMM is a protein that interacts with TPX2 during mitosis (Groen et al. 2004). This interaction promotes TPX2 stabilization and targeting to centrosomes (Chen et al. 2014). Upon RHAMM dowregulation with shRNA we observed that TPX2 intensity at prophase centrosomes is significantly reduced while pericentrin intensity remains unaltered (suggesting that centrosome structure is intact). Importantly, in these cells Eg5 fails to accumulate at centrosomes and centrosome separation is impaired (Figure 12). These results were observed with two different shRNAs (Figure 12F). Altogether our observations strongly suggest that Eg5 accumulation is dependent on TPX2 localization at centrosomes.

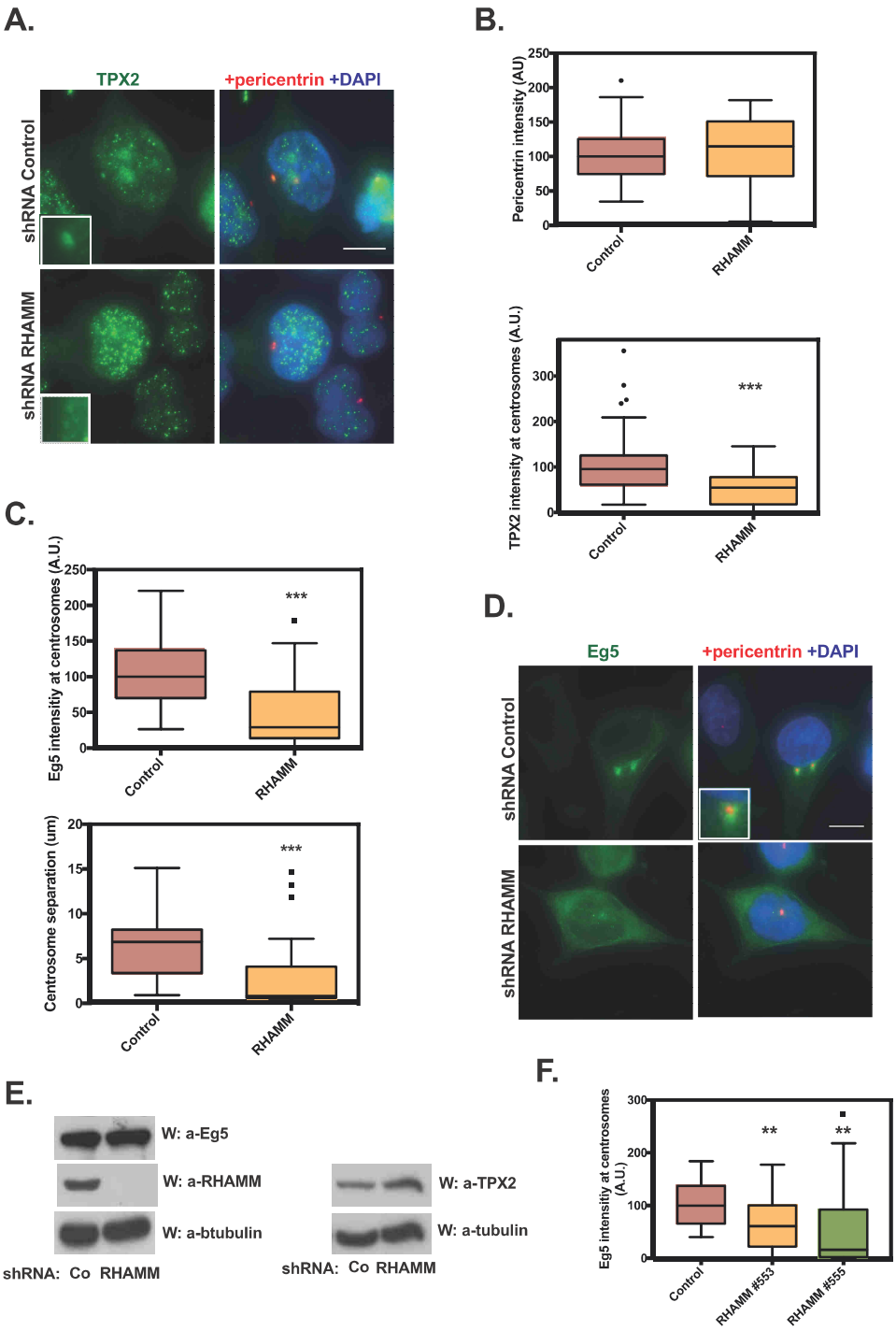

Figure 12. TPX2 centrosomal localization is required for Eg5 accumulation in prophase. (A) Prophase cells stained for TPX2 in control and RHAMM depleted cells. (B) Quantification of pericentrin intensity (up) and TPX2 intensity (down) at centrosomes. n=30 prophase cells, 3 independent experiments. (C) Quantification of Eg5 intensity at centrosomes and centrosome separation. N=30 prophase cells, 3 independent experiments. (D) Representative immunofluorescence of quantification in D. (E) WB showing protein levels of Eg5, TPX2 and RHAMM after RHAMM depletion. (E) Quantification of Eg5 intensity at centrosomes with two different shRNA. N=10 prophase cells. Statistics were done with Mann-Whitney t-test.

To further support our observation that TPX2 is necessary for Eg5 proper prophase localization, we used conditional TPX2 -/- mouse embryonic fibroblasts (MEFs) (a gift from M. Malumbres (CNIO)); experiments done in collaboration with Paula Martínez). MEFS were infected with Adenovirus expressing Flp recombinase as control and Adenovirus expressing Cre recombinase to induce TPX2 depletion. We observed that in MEFs TPX2 can also be detected at centrosomes in prophase, and that after Cre infection, TPX2 disappears from these organelles, confirming the specificity of the centrosomal staining by the antibody. More importantly, we also observed that in TPX2 -/- cells Eg5 intensity at centrosomes is significantly reduced compared to the control (Figure 13).

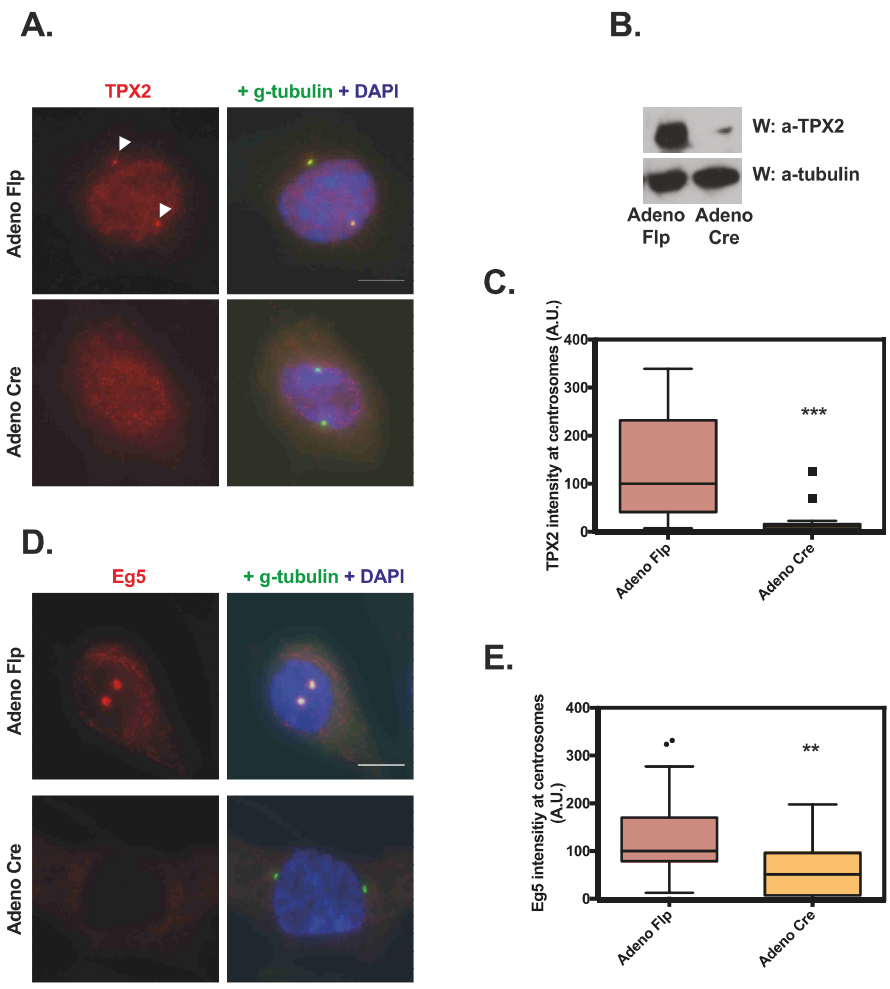

Figure 13. cTPX2 MEFs fail to localize Eg5 at centrosomes in prophase. (A) Prophase cells infected with Adeno Flp and Adeno Cre stained with TPX2. (B) WB for endogenous

TPX2 depletion after adeno Cre infection. (C) Quantification of TPX2 intensity at centrosomes. N=13-15 prophase cells, 2 independent experiments. (D) Endogenous Eg5 immunofluorescence. (E) Quantification of Eg5 intensity at centrosomes in MEFs after infection. N=13-15 N=13 prophase cells, 2 independent experiments. This part was dome with the collaboration of Paula Martínez.

TPX2 interacts with Eg5 in a phosphorylation dependent manner

Since we detected a function of TPX2 regulating Eg5 localization in prophase, we wanted to corroborate whether these two proteins are able to interact as it has been described (Fu et al. 2015). Despite all our efforts we could not reliably detect an interaction between TPX2 and Eg5 in asynchronous cells by immunoprecipitation. Reasoning that TPX2 and Eg5 may interact more abundantly in mitosis we synchronized cells using a thymidine double block protocol, and recombinant TPX2 was precipitated from cell extracts enriched in mitotic cells. We transfected HeLa cells with plasmids expressing GFP-TPX2 FL, the TPX2 C-terminus [367-747], the TPX2 C-terminus lacking the last 33 amino acids [367-710] and the shortest fragment corresponds to the described TPX2-Eg5 interaction domain [713-747] (Eckerdt et al. 2008).

Our results show that recombinant GFP-TPX2 associates to endogenous Eg5 in mitotic enriched extracts. In contrast, we could not detect association between any of the TPX2 C-terminus constructs and endogenous Eg5 (Figure 14). Thus, the TPX2 C-terminus is not sufficient to associate with Eg5.

On the other hand, we transfected HeLa cells with GFP TPX2 FL and TPX2 [1-710], a construct that lacks the proposed Eg5 interaction domain (Eckerdt et al. 2008). We detected that endogenous Eg5 associates with FL TPX2 but this interaction is disrupted when TPX2 lacks the last 37 amino acids from its C-terminal part. Thus, TPX2 [710-747] is necessary but not sufficient to associate with Eg5.

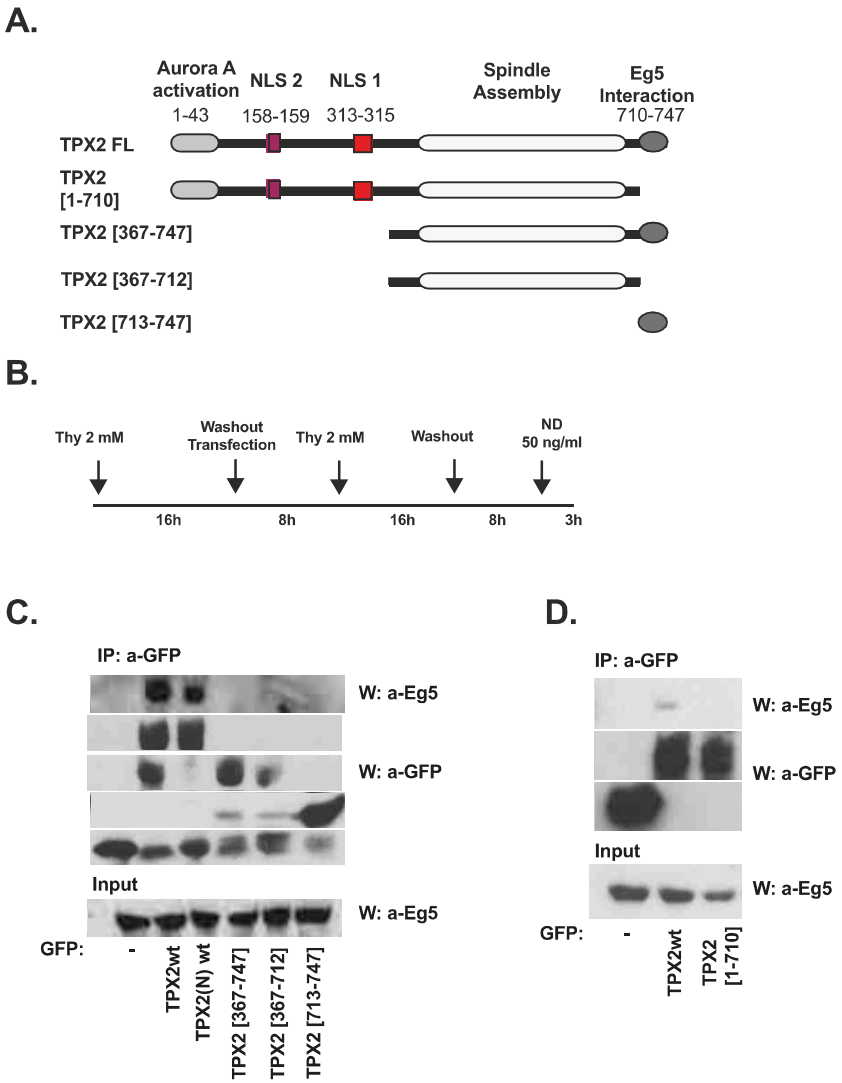

Figure 14. Endogenous Eg5 associates with TPX2 in mitosis. (A) Schematic representation of all the TPX2 constructs used and their domains. (B) Detailed protocol to obtain transfected and synchronized cells. (C) Immunoprecipitation of all the constructs represented in A. WB anti-Eg5 reveals association between TPX2 FL and Eg5. (D) Precipitation of TPX2 FL and TPX2 [1-710]. TPX2 association with Eg5 requires TPX2 the last 37 amino acids.

We next wanted to investigate whether the observed interaction was dependent on Ser1033 phosphorylation. To achieve this purpose we co-transfected HeLa cells with GFP-TPX2 FL and FLAG-Eg5 WT, the phosphonull Ser1033Ala, and the phosphomimetics Ser1033Asp, Ser1033AspAsp and Ser1033Glu. As expected from the experiments

described above, we detected an interaction between recombinant GFP-TPX2 and recombinant FLAG-Eg5. Strikingly, we discovered that association between Eg5 Ser1033Ala mutant and TPX2 is visibly reduced. In contrast, and although the results with these mutants are quite variable, we detected in several assays that the association presents a tendency to increase between Eg5 phosphomimetics and TPX2 (Figure 15). To confirm this result we performed the opposite analysis. HeLa cells were transfected with recombinant FLAG Eg5 WT, Ser1033 phosphonull and phosphomimetic and precipitated with FLAG antibody. We detected by WB that endogenous TPX2 is able to associate with recombinant Eg5 WT, and that this association is decreased for Ser1033Asp and abrogated with Ser1033Ala, confirming previous results.

We conclude that the interaction between TPX2 and Eg5 is dependent on Ser1033 phosphorylation, although mutant forms of Eg5 [Ser1033] are possibly not mimicking well the effect of phosphorylation. Thus, to further support this hypothesis, we depleted Nek9, thus abrogating Ser1033 phosphorylation (Bertran et al. 2011). HeLa cells were infected with Nek9 shRNA to depleted endogenous protein and transfected with recombinant TPX2. We detected that TPX2 loose the ability to associate with Eg5 in absence of Nek9 (FIG 15 D).

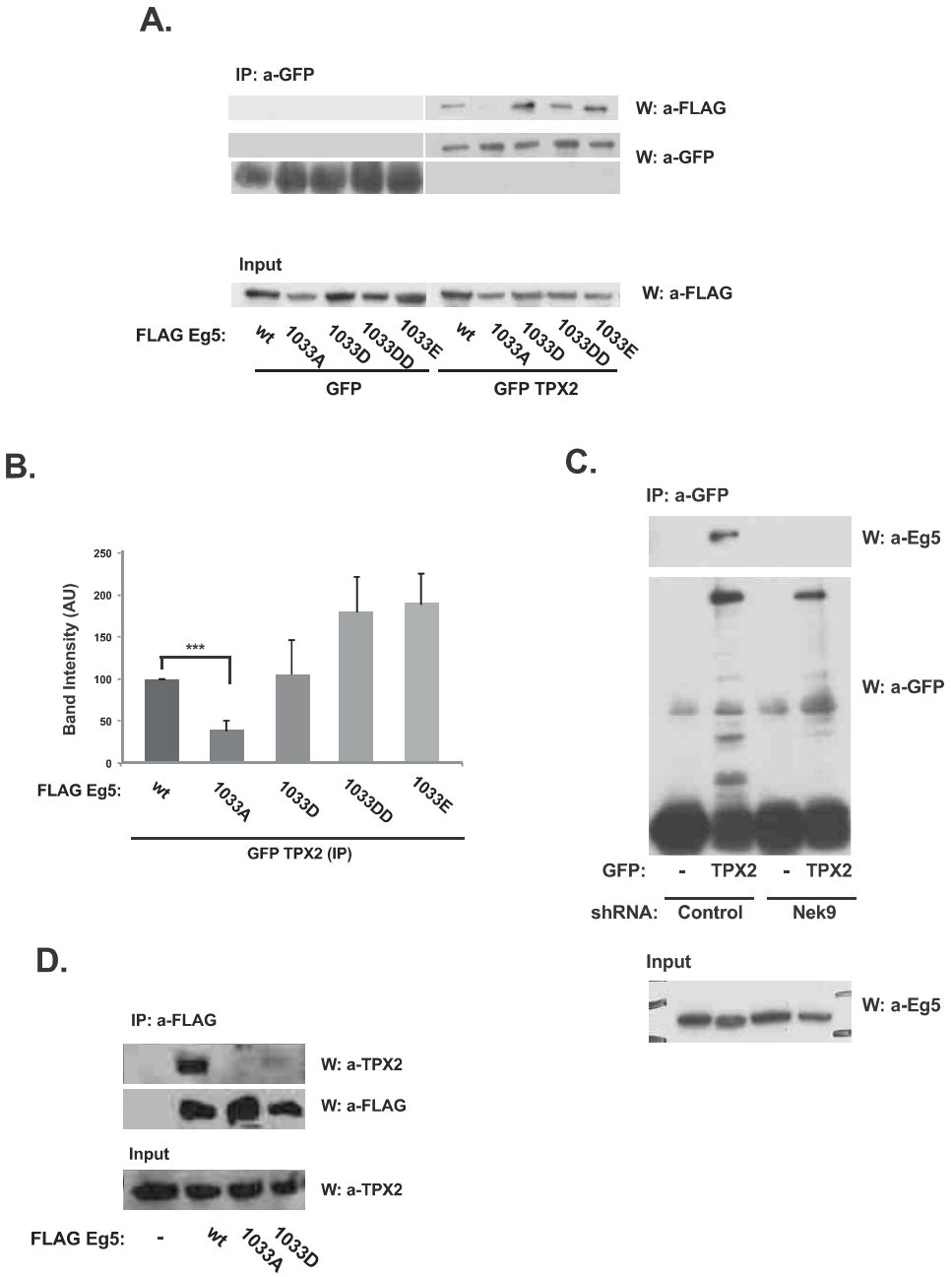

Figure 15. Ser1033-P is required for TPX2 association. (A) Precipitation of GFP-TPX2 in mitotic cells co expressing FLAG Eg5 mutants (1033DD corresponds with double Asp at that position). (B) Average intensity from all the repeated experiments of the WB band of Eg5 mutants associated to TPX2. All of them normalized to Eg5 WT band (C) Precipitation of GFP-TPX2 in cells Nek9 depleted and control. This last Figure was done with the collaboration of Núria Gallisà. (D) Precipitation of FLAG Eg5 mutants and WB revealed for endogenous TPX2.

Subsequently, since we were able to detect an interaction between Eg5 and TPX2 in mitosis, we wanted to determine whether this interaction was direct using Y2H analysis. We transformed yeast expressing BD-TPX2 FL and BD-TPX2 C-terminal [681-747] domain. We tested for toxicity and protein expression of these two constructs. We found that growth in yeast transformed with TPX2 FL is drastically reduced, which suggest that TPX2 expression is toxic. In contrast, yeast transformed with TPX2 C-terminal domain grows in a similar way to the control. Additionally, TPX2 C-terminal [681-747] domain is easily detectable by WB, but we were not able to detect TPX2 FL protein expression by WB. Next, we tested for auto activation of these proteins and unfortunately, TPX2 C-terminal domain yeast are able to grow in medium lacking adenine, as well as degrade X-gal turning the cells blue. Thus, this fact impedes to continue with Y2H analysis using this construct. In contrast, TPX2 FL is not activating the gene reporter system; therefore, we decided to continue with this construct despite its toxicity and poor protein expression.

We performed mating between yeast expressing BD-TPX2 and AD-Eg5 motor, Eg5-stalk, Eg5 tail, Eg5 tail Ser1033A and Eg5 tail Ser1033Asp. We selected diploids using SD-2 medium (without TRP and LEU) and interactors using SD-3 medium (without TRP, LEU and HIS). Diploids co-expressing TPX2 FL and Eg5 tail domain present a higher rate of growth in selection media, which suggest that TPX2 is interacting with the Eg5 tail domain (Figure 16). In this system, mutations of Ser1033 seem to negatively affect the interaction, again supporting the importance of this residue for the binding of Eg5 to TPX2. However, due to the toxicity and slight background growth of TPX2, additional techniques might be needed to confirm this result.

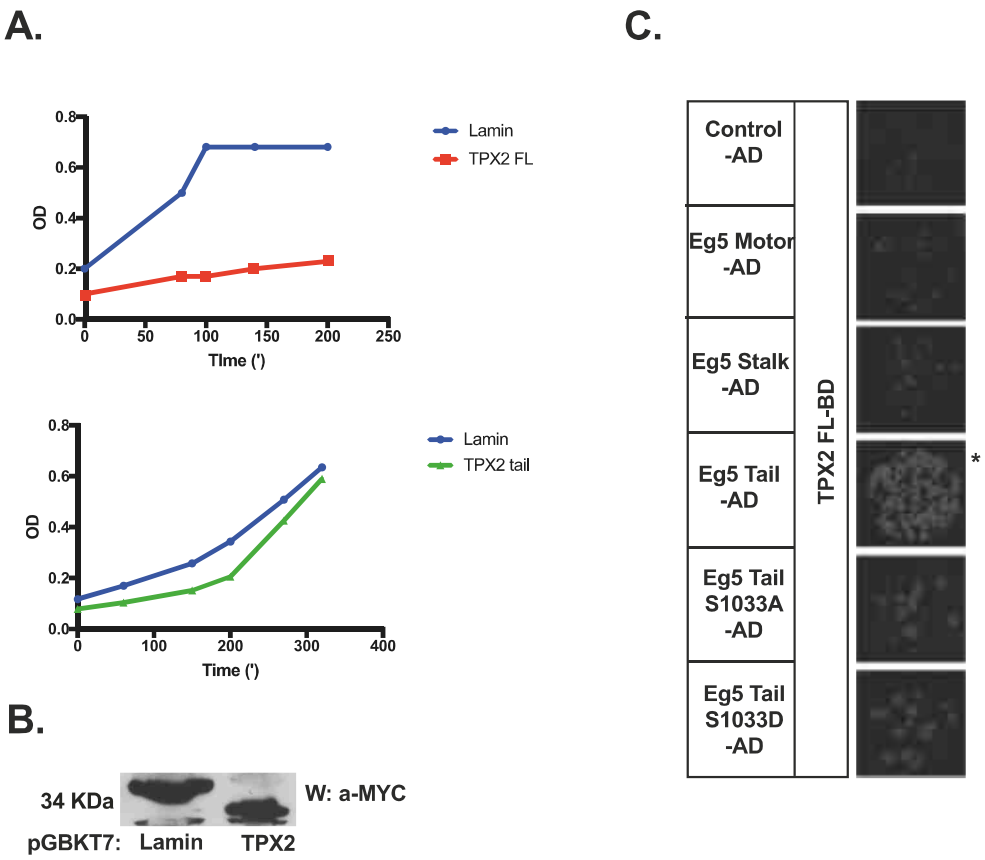

Figure 16. Y2H analysis for TPX2 and Eg5 fragments. (A) Growth curves for TPX2 FL (up) and TPX2 [641-747] (down). (B) WB detecting expression in yeast of TPX2 [641-747]. (C) Yeast expressing TPX2 FL and Eg5 domains growing in selection media. Asterisk indicates condition with faster growth

TPX2 localizes at centrosomes during prophase

It has been described that TPX2 localizes at the nucleus during interphase. Later, in mitosis, after NEB, localizes to the spindle due to the action of RanGTP, which releases TPX2 from importin binding (Wadsworth 2015). We now show that TPX2 localizes at centrosomes during prophase both in human and mouse cells (Figures 12 and 13). Furthermore, RHAMM depletion interferes with this localization (Figure 12) as it has been shown to do latter in mitosis (Chen et al. 2014). Endogenous TPX2 is detectable at centrosomes in prophase but not in interphase using monoclonal TPX2 antibody (Figure 17A),

which agrees with the existence of specific functions for TPX2 before NEB. To prove that we were visualizing mitotic cells with intact NE, and not early prometaphase, we co-stained HeLa cells with TPX2 and lamin B antibody. TPX2 is present at centrosomes while lamin staining marks a complete nucleus, thus indicating that the NE remains intact (Figure 17A). Additionally, we used G2 arrested cells by RO 3306 to determine whether TPX2 localizes at centrosomes in these non-mitotic cells that are able to accumulate Eg5 at centrosomes (see additional results). We detected by WB that TPX2 protein expression increases in RO treated cells, which also occurs in mitotic cells. Furthermore, TPX2 staining was positive at centrosomes in G2 arrested cells and Roscovitine and Bi2536 significantly reduce this signal after inhibition of CDKs and Plk1 (and consequentially Nek9). This result supports that this protein has centrosomal localization before nuclear envelope breakdown and this localization is dependent on Plk1 and CDK activity (Figure 17C-D).

Finally, to determine whether this stain was unspecific, we depleted TPX2 from cells using siRNA and found that TPX2 intensity at centrosomes was reduced.

To investigate whether TPX2 localization at centrosomes depends on Eg5, something that was previously described in later stages of mitosis (Ma et al. 2010), we treated HeLa cells with Eg5 inhibitor STLC and measured TPX2 intensity at centrosomes during prophase. We found that TPX2 centrosomal localization decrease after inhibiting Eg5 activity. Thus, TPX2 localization at centrosomes depends in part on Eg5 activity (Figure 17F).

In contrast to endogenous TPX2, we could not detect recombinant TPX2 in prophase centrosomes. We reasoned that this may be due to TPX2 toxicity, as cells entering mitosis with abnormal levels of the protein show disrupted centrosomes. Thus, to determine whether recombinant TPX2 is present at centrosomes during prophase we synchronized HeLa cells in mitosis using thymidine double block protocol. We set up the protocol in order to observe cells that were going into their fist mitosis after transfection, and also to obtain the higher possible number of cells in prophase. We detected that 9 hours after second thymidine washout between 5 and 10% of the total cells are in prophase. Using this protocol, we found that recombinant TPX2 localizes at

centrosomes during prophase using two different tags GFP and FLAG (Figure 17C).

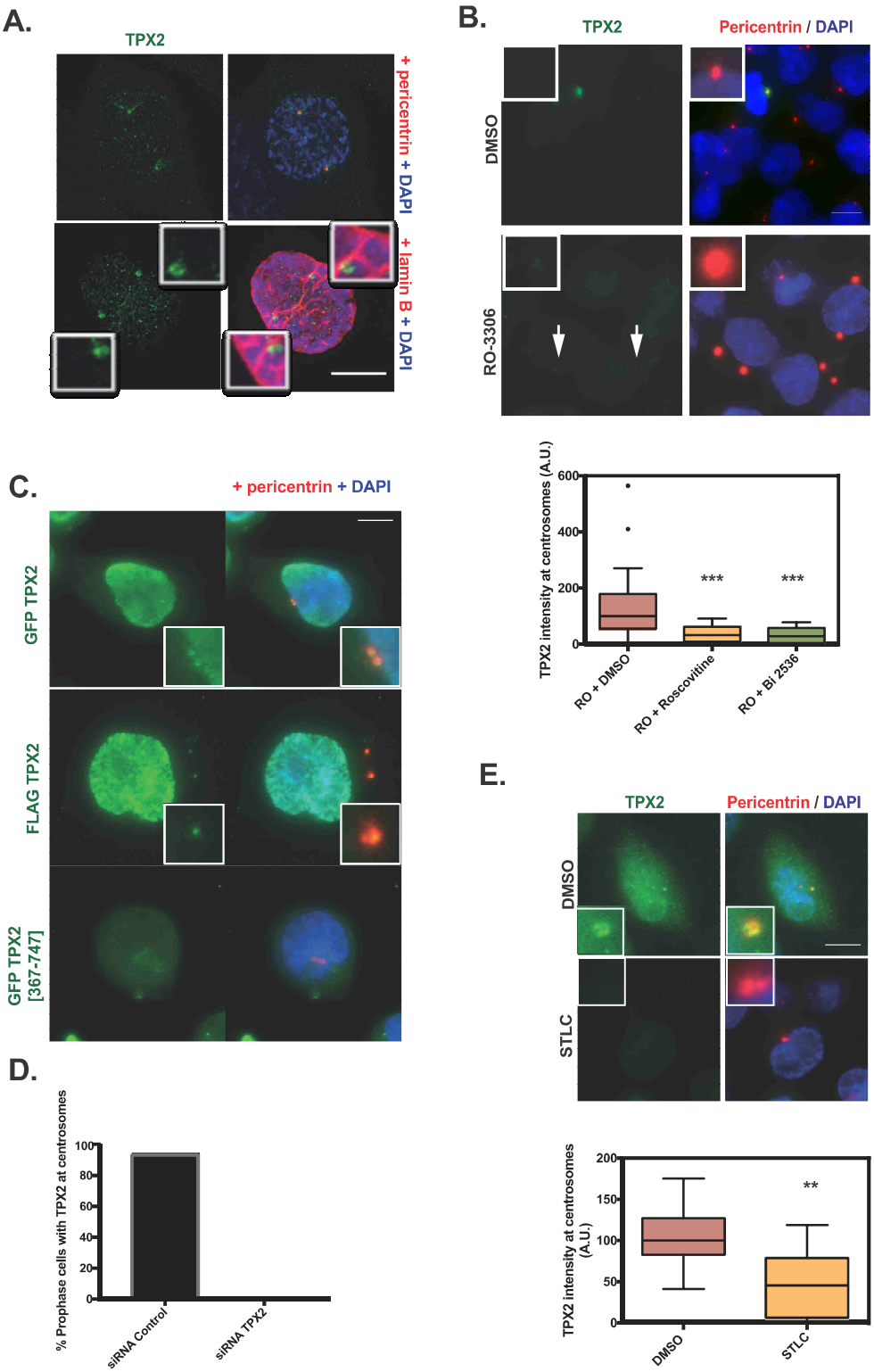

Figure 17. TPX2 localizes at centrosomes during prophase. (A) Endogenous TPX2 staining in prophase cells. Cells were co-stained with pericentrin as a centrosomal marker

and lamin B as a NE marker. (B) GFP and FLAG staining of prophase cells expressing FLAG-TPX2, GFP-TPX2 and GFP-TPX2 [367-747]. (C) Endogenous TPX2 staining in asynchronous and RO 3306 treated cells. Arrows indicates TPX2 signal at centrosomes. (D) TPX2 intensity at centrosomes in RO treated cells after addition of Roscovitine and Bi 2536. (E) Percentage of prophase cells that presented visible positive staining for TPX2 at centrosomes N=10 prophase cells. (F) TPX2 intensity at centrosomes in prophase cells treated with STLC during 1 to 2 hours. Scale bar: 10 μm.

Additionally to the localization of full length TPX2 in prophase we investigated the localization of TPX2 fragments. TPX2 C-terminal domain lacks the NLS, thus, as expected, these fragments are detectable at cytoplasm in interphase. During prophase, C-terminal TPX2 localizes in proximal areas of centrosomes, although forming a cloud over them, in a different arrangement than FL TPX2 (Figure 18). Remarkably, overexpression of TPX2 [367-747] causes a reduction in centrosome separation during prophase (Figure 18). To examine the cause of centrosome separation failure after TPX2 C-terminal expression, we measured the intensity of Eg5 at centrosomes in prophases that were positive for GFP staining. We found that Eg5 mislocalizes in prophase cells expressing TPX2 [367-747]. In these cells Eg5 is co-localizing with TPX2 C-terminal fragment, forming a cloud over the centrosomes, which suggest that c-terminal domain is acting as a dominant negative. Eg5 accumulation is rescued when the TPX2 C-terminal domain lacks the last 37 amino acids; region that our results and these of others (Eckerdt et al. 2008) (Balchand et al. 2015) prove is necessary for Eg5 interaction (Figure 18).

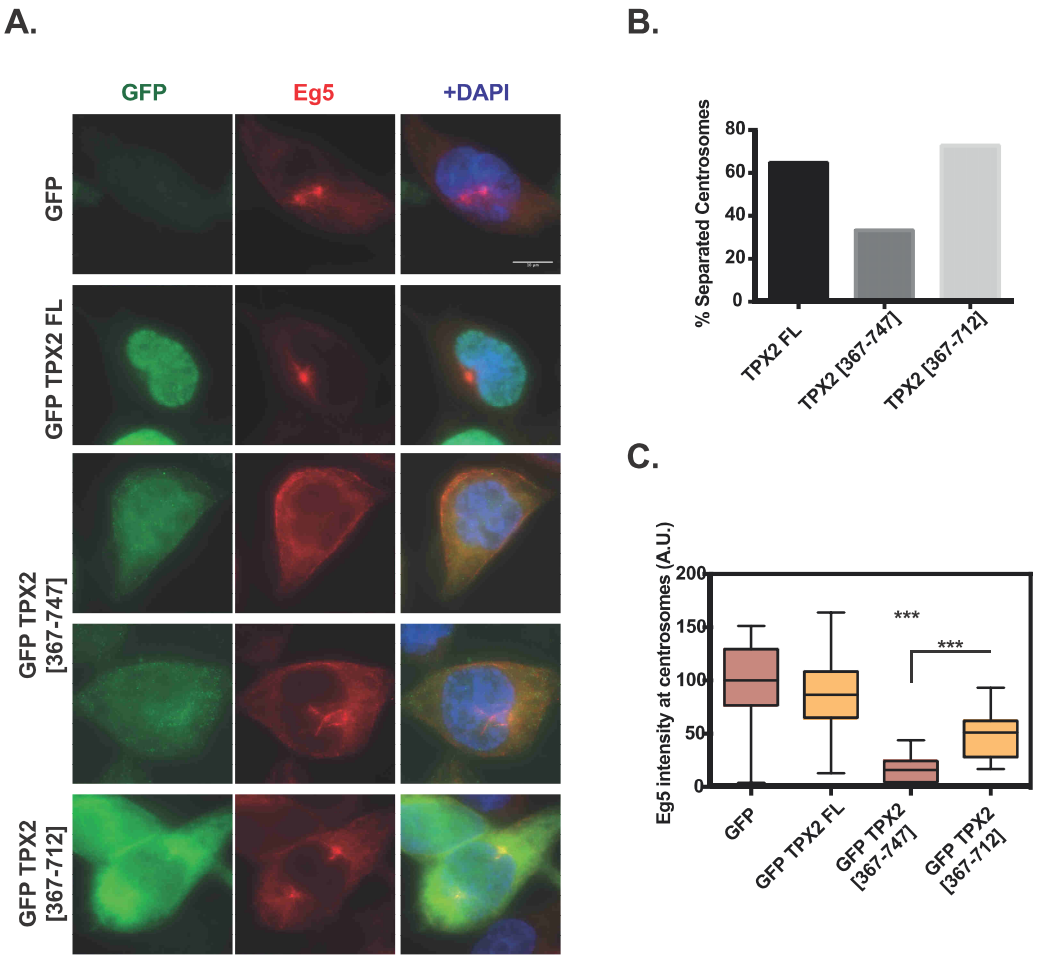

Figure 18. Overexpression of TPX2 C-terminus impairs Eg5 accumulation at centrosomes. (A) Immunofluorescence of prophase cells expressing TPX2 fragments. Eg5 distributes abnormally with TPX2 [367-747] expression. (B) Percentage of centrosomes separated in prophase cells expressing the indicated TPX2 fragments. (C) Eg5 intensity at centrosomes in prophase cells expressing TPX2 constructs.

TPX2 is a novel Nek9 substrate

Different high throughput Mass Spectrometry analysis concluded that TPX2 is highly phosphorylated *in vivo* during mitosis (for instance (Santamaria et al. 2011)). TPX2 presents several sites whit a [LF]XX[S/T] motif, which has been shown to be preferred by members of the NIMA kinase family (Lizcano et al. 2002) (Alexander et al. 2011) and our group results, and are therefore putative sites for Nek9. To investigate whether TPX2 is a substrate for Nek9 we purified TPX2 expressed in bacteria and proceed to incubate it with purified Nek9 and Mg^{2+}/ATP. We observed that TPX2 is highly phosphorylated *in vitro* by Nek9 (Figure 19). Notably, this phosphorylation promotes an electrophoretic mobility shift in hTPX2.

To determine the specific sites phosphorylated in TPX2 by Nek9 we performed LC/MS/MS analysis of hTPX2 incubated with Mg^{2+}/ATP and Nek9. TPX2 incubated without the kinase was used as a control. We found a total of 14 sites phosphorylated in the protein that was incubated with Nek9 but none in the control (table S4). Two of the sites detected were located in areas nearby one of the described (Giesecke and Stewart 2010) (Holvey et al. 2015) nuclear localization signals (NLS) of TPX2, Ser310 and Thr320.

Two NLS have been described in TPX2: NLS1 [313-315] and NLS2 [158-159]. To determine whether phosphorylation of these residues nearby the NLS is altering TPX2 interaction with importin we produced several mutants. *TPX2 NLS 1 mut* corresponds to mutations on the first NLS [313-KKR-315] to [313-MMQ-315]; *TPX2 NLS 2 mut* corresponds to mutations on the NLS [158-KK-159] to [158-MM-159] and *TPX2 NLS 1 and 2 mut* includes both NLS mutated. Additionally Ser310 and Thr320, together with the nearby Ser322 and Thr323 were also mutated to the corresponding phosphomimetic and phosphonull mutants (TPX2 4D or TPX2 4A). We detected that mutating only one NLS is not enough to promote cytoplasmic localization of TPX2; both NLS must be mutated at the same time to promote TPX2 release from the nucleus. Interestingly, TPX2 4D mutant increases its cytoplasmic localization when NLS 2 is mutated, in a similar ratio to NLS 1 and 2 mutant. Thus, TPX2 phosphomimetic mutant is able to prevent importin binding to NLS 1 (Figure 19).

This phenomenon is also observed with Thr316 and the NLS 2 mutation. Thr316, unlike Ser310 and Thr320, does not present a putative motif for Nek9 phosphorylation and it was not identified after MS of *in vitro* phosphorylated TPX2. We decided to add this threonine to the analysis because it appears phosphorylated during mitosis, is proximal to NLS1 and is a putative site for Aurora A.

Since we detected that phosphorylation on the analyzed sites alters TPX2 importin interaction, we next wanted to investigate the effect in TPX2 of active Nek9 expression in cells. To identify whether Nek9 and Nek6 activity were inducing TPX2 cytoplasmic localization we transfected HeLa cells with FLAG ΔRCC1 and FLAG Nek6 (Figure 20). Cells expressing active Nek9 present a higher cytoplasmic/nuclear ratio for endogenous TPX2. Something similar occurs with FLAG Nek6 cells. Thus, activity of Nek9 and Nek6 during interphase is affecting TPX2 subcellular localization. Furthermore, we also noticed, that TPX2 detectable levels by WB increase when active Nek9 is expressed in asynchronous cells.

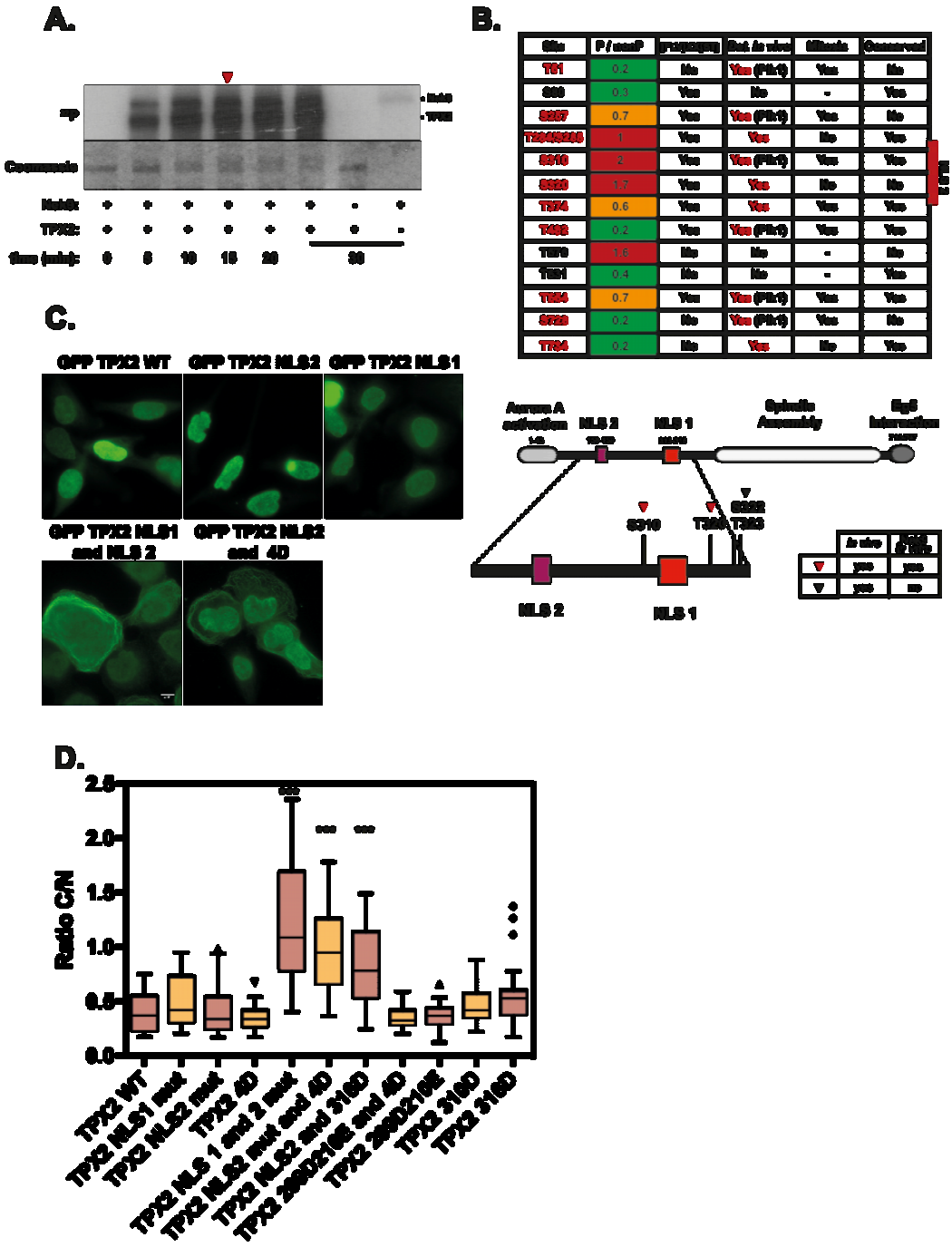

Figure 19. Nek9 phosphorylates TPX2. (A) In vitro phosphorylation of TPX2 by Nek9 at different time points. Red arrow indicates the sample analysed by MS. (B) TPX2 sites phosphorylated after Nek9 incubutation. Classified by their ratio of appearence (up). Representation of TPX2 domains and the location of the mutated sites (down). (C) Representative cells for TPX2 mutants localization interphase. (D) Quantification of ratio cytoplasm/nucleus for the distribution of the different mutants. N=30, 3 independent experiments.

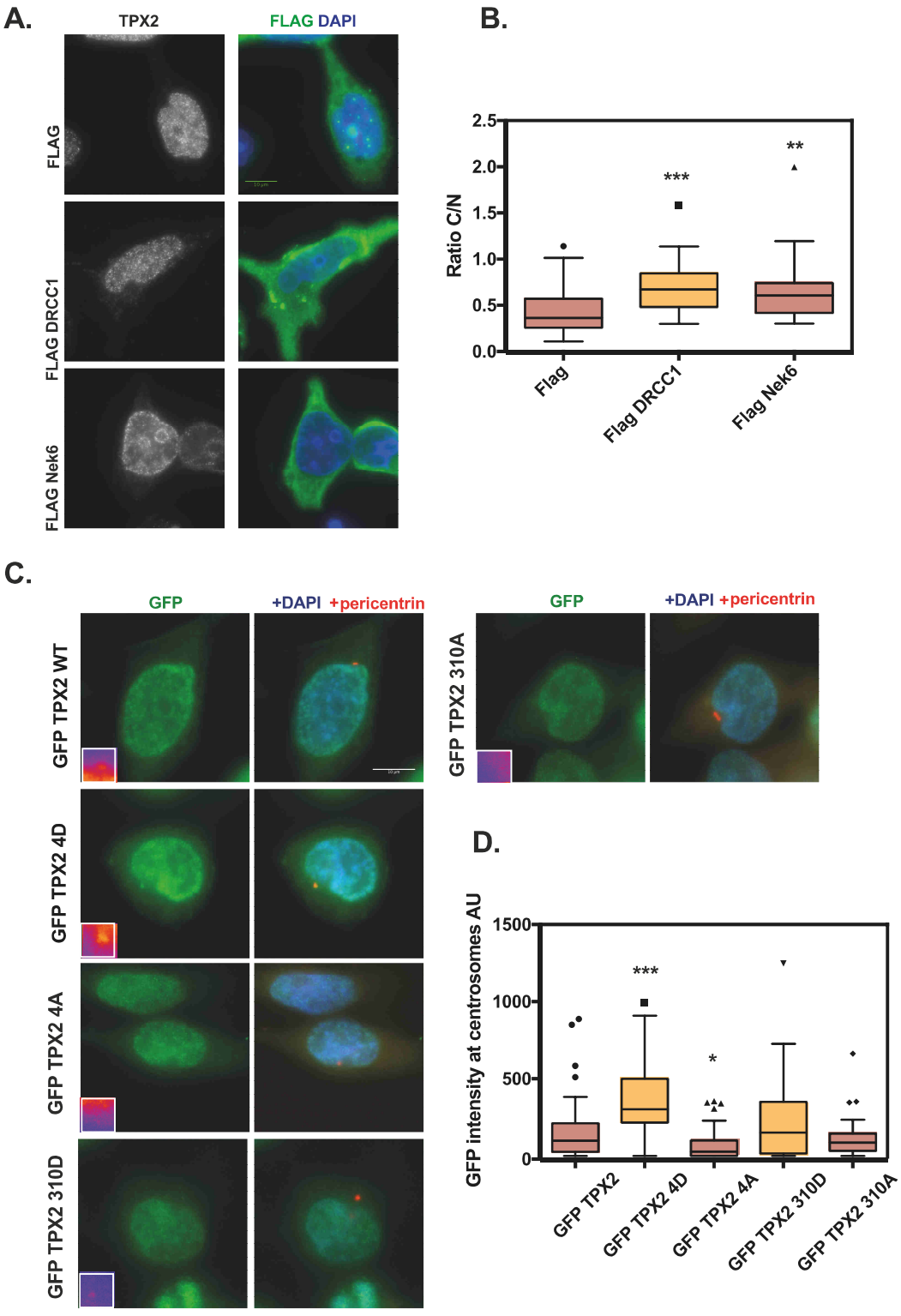

Figure 20. Nek9 regulates TPX2 localization. (A) Representative cells expressing active Nek9 and Nek6 and stained for endogenous TPX2 in interphase. (B) Quantification of cytoplasm/nucleus ratio of TPX2 in interphase cells transfected with active Nek9 and Nek6. N=30 cells, 3 independent experiments. (C) Representative cells expressing GFP TPX2

mutants in prophase. (D) Intensity at centrosomes of the different phosphonull and phosphomimetic mutants during prophase. N=40 prophase cells, 2 independent experiments.

As previously mentioned, Nek9 is activated at centrosomes during G2-M transition (Roig et al. 2005). We thus wanted to examine whether phosphorylation of Nek9 sites could be actually promoting TPX2 localization at centrosomes during prophase. We transfected HeLa cells with TPX2 mutants for the described sites. Cells were synchronized with thymidine double block, as described in figure 14, to detect recombinant TPX2 at the centrosomes in prophase. We found that the intensity of phosphomimetic mutant TPX2 4D at centrosomes during prophase is significantly increased and in the other hand, that phosphonull mutant 4A loses its centrosomal localization in prophase. Thus, the analyzed sites for phosphorylation govern TPX2 centrosomal localization.

To further test whether Nek9 may actually be controlling TPX2 centrosomal localization in prophase downregulated Nek9 expression by RNAi and checked TPX2 ability to localize at centrosomes. Thus, we transfected cells with siRNA to deplete Nek9 and measured TPX2 intensity at centrosomes during prophase. We saw that after Nek9 depletion TPX2 fail to localize to centrosomes. We did not detect any difference in TPX2 nucleus-cytoplasmic distribution, which suggest that endogenous Nek9 is regulating TPX2 distribution only at centrosome level, possibly through modification of a small pool of TPX2 locally (Figure 21).

As previously described, TPX2 centrosomal localization is also mediated by the protein RHAMM. To test whether Nek9 was actually affecting RHAMM position at centrosomes during prophase, and TPX2 mislocalization was only a consequence of this, we measured RHAMM intensity at centrosomes in prophase of control and Nek9 depleted cells. We found that RHAMM can localize properly at centrosome even with the absence of Nek9. Thus, Nek9 is regulating TPX2 in a direct manner, and not through RHAMM (Figure 21).

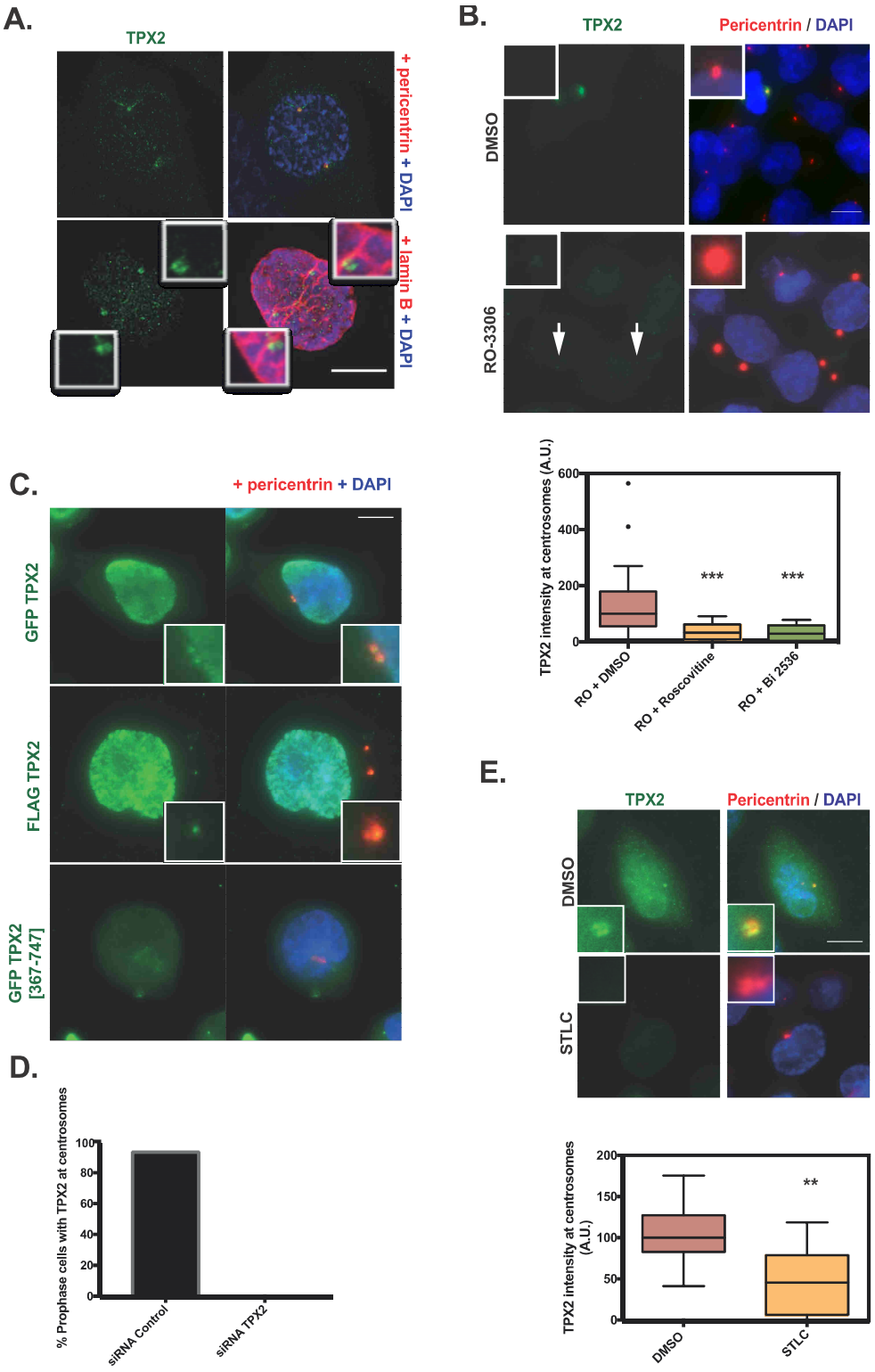

Figure 21. Nek9 controls TPX2 centrosomal localization in prophase. (A) Representative pictures of prophase cells transfected with siRNA control and siRNA Nek9. White arrows indicate TPX2 accumulation at centrosomes. Scale bar 10μm. (B) Quantification of

endogenous TPX2 at centrosomes during prophase. (C) Ratio cytoplasm/nucleus in prophase cells Nek9 depleted and control. (D) RHAMM intensity at centrosomes in prophase in cells Nek9 depleted and control. N=30, 3 independent experiments. (E) WB for endogenous Nek9 and TPX2 after Nek9 depletion.

4. Similarity between Eg5 and TPX2 interacting regions may define a putative motif for Eg5 interaction

As we detected that TPX2 and BicD2 are able to interact with Eg5, we next studied the similarity between these two proteins in order to determine whether a common amino acid sequence existed. We found that human TPX2 and human BicD2 present a significant degree of similarity in a small amino acidic region. This region corresponds to amino acids 494 to 506 in human BicD2 (496-508 in mouse BicD2) and amino acids 699 to 711 in human TPX2. Interestingly, as mentioned before, the minimal region of TPX2 that is able to interact with Eg5 was proposed to be located between amino acids 710-747 and we discovered that BicD2 interacts with Eg5 through amino acids 486-575. Our first hypothesis was that this motif is necessary for these two proteins to interact with a common Eg5 region at the C-terminus of the kinesin. To test this we mutated two conserved leucines (L501, L504 in mBicD2 and L702, L704 in TPX2) of the sequence to alanines and precipitated the WT and the mutant to detect Eg5 association. We found that Eg5 association with the leucine mutant (BicD2 L501A-L504A and TPX2 L702A-L704A) is slightly reduced compared to the WT. Although this is an encouraging result, we failed to detect any effect of a similar mutation in BicD2 using Y2H. Mutant forms of BicD2 C-terminus (L501A-L504A) were able to interact in a similar way to WT with Eg5 C-t. Further analysis, possibly using additional mutants and mapping the exact region in Eg5 that interacts with TPX2 and BicD2, will be necessary to finally conclude the existence of a common Eg5-binding motif.

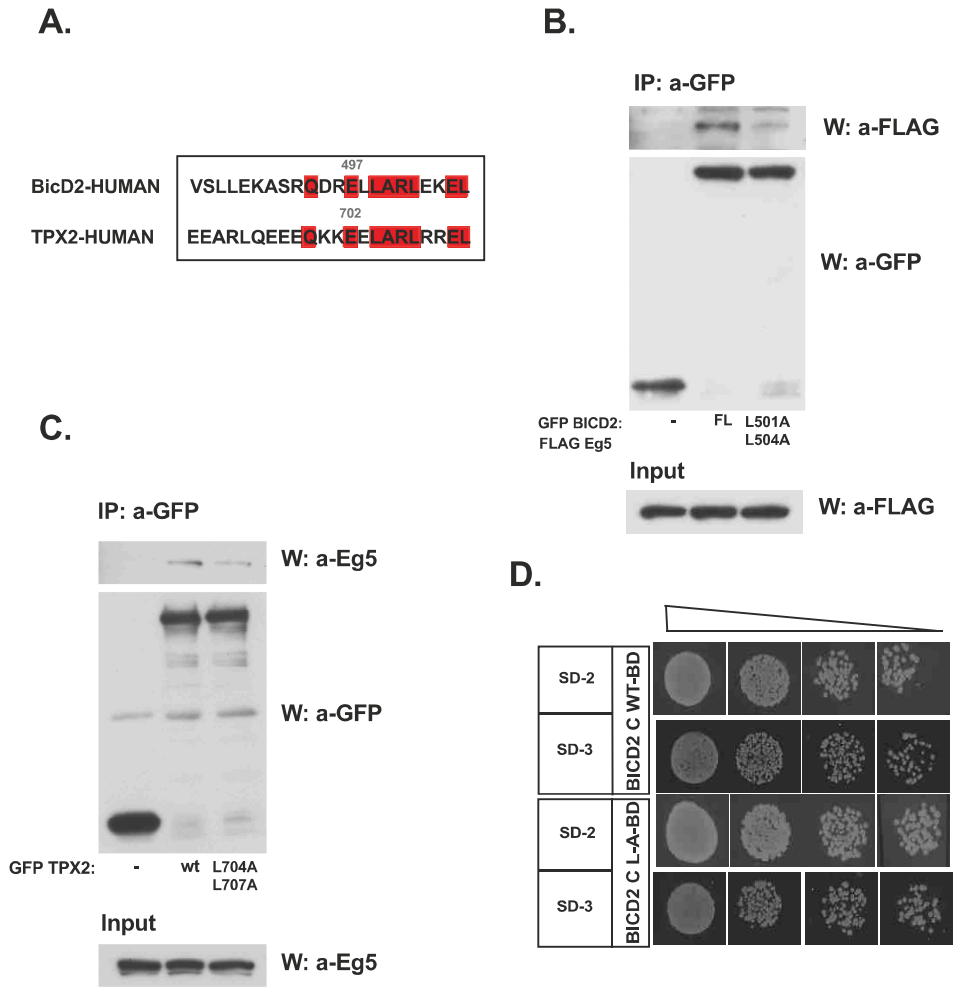

Figure 22. Determination of a common Eg5 interaction domain. (A) Representation of the shared sequence between TPX2 and BicD2. (B) Precipitation of recombinant BicD2 WT and leucine mutant to detect Eg5 association. (C) Precipitation of recombinant TPX2 WT and leucine mutant to detect Eg5 association. (D) Y2H dilutions for BicD2 C-terminal and Eg5 tail diploids. SD-2 is control media and SD-3 is selection media. Leucine mutant is represented as BicD2 C L-A.

DISCUSSION

Eg5 localizes at MT emerging from centrosomes

In this work, we show that Eg5 pericentrosomal localization in prophase depends on the presence of microtubules, Eg5 and dynein activity and the multifunctional protein TPX2.

We demonstrate that Eg5 localizes during prophase at MT emerging from centrosomes and, as expected from this observation, that Eg5 localization is MT dependent. Accordingly, treating cells with MT depolymerizing drugs results in Eg5 mislocalization during prophase. Treating cells with a MT stabilizing factor such as taxol induces a similar phenotype, suggesting that MTs need to be dynamic for Eg5 targeting at centrosomes. However, taxol has other effects in addition to MT stabilization. For instance, under taxol treatment, centrosomes loose their nucleation capability (De Brabander et al. 1981) complicating the interpretation of our results with this drug. Taxol also induces MT aster formation in mitosis but not in interphase (Verde et al. 1991). We observed that, in some cases, in prophase cells treated with taxol Eg5 localizes to aster-like structures, which probably correspond with tubulin asters. Tubulin asters occur as a result of MT clustering due to dynein activity towards minus ends of MT, which also promotes the migration of centrosomal material to the center of these asters (Blagden and Glover 2003). Moreover, in *Xenopus,* aster formation also requires homologous CDK1/cyclin B activity (Verde et al. 1991). Thus, taking into account all our results, aster-like localization of Eg5 in taxol-treated cells may be regulated by dynein activity and the presence of phosphorylated centrosomal proteins at the center of asters.

Prophase cells treated either with MT depolymerizing or stabilizing drugs present separated centrosomes independently of Eg5 localization around centrosomes, which we previously showed is necessary for separation. We interpret this observation as a consequence of a double role of MT in centrosome positioning. Thus, MTs will keep centrosomes in place and also be necessary for their motor-driven separation. In accordance with this, it has been reported that in prophase cells (after centrosome disjunction by Nek2)

without centrosomal nucleation activity, centrosomes disperse moving around the cytoplasm (Jean et al. 1999) (Meraldi and Nigg 2001).

Although we propose that Eg5 is accumulated close to the minus ends of the microtubules we also show that Eg5 (plus end-directed) motor activity is needed for normal localization of the kinesin in prophase. STLC and monastrol, as was mentioned above, impede ADP release from the Eg5 ATP pocket, interfering with the capability of Eg5 to bind to MT. In this case, centrosomes remain together during mitosis, as was previously described (Kapoor et al. 2000) and both mitotic progression and spindle formation is disrupted. Centrosome separation under the presence of inhibitors could be altered due to either the requirement of Eg5 MT binding or of a force balance between Eg5 and opposite-directed motor proteins such as dynein. With the aim of discarding the first option, cells could be treated with FCPT, a small permeable drug that also inhibits Eg5 activity but that it does so by impeding ATP entry to ATP pocket, thus, leaving Eg5 in a state capable of binding MTs (Groen et al. 2008).

Actin and centrosome separation

Disturbing the actin cytoskeleton dynamics we observed a significant reduction of Eg5 loading at centrosomes without affecting centrosome separation.

The actin cytoskeleton rearranges during mitosis to give rounded shape to the cell and increase cortex rigidity. Later, actin migrates to the cleavage furrow being part of the contractile ring during cytokinesis (Heng and Koh 2010). Additionally, actin has been proposed to be involved in spindle positioning and centrosome separation during cell division. During prophase, after induction of either depolymerization or stabilization of actin, centrosomes fail to separate in *Drosophila* embryos (Cao et al. 2010). However, in mammalian cells, in contrast to *Drosophila* cells, actin cytoskeleton has been proposed to be required for centrosome separation in prometaphase but not in prophase. Depolymerization of actin filaments does not impair centrosome separation in prophase mouse cells but causes a delay on the process. This

delay could be explained due to actin association with the centrosomes during G2/M transition (Wang et al. 2008). In the same context, it was proposed that actin controls centrosome directionality, guiding centrosome movement at this stage (Whitehead et al. 1996). In contrast to prophase, during prometaphase, inhibition of myosin II (an actin dependent motor protein) and consequently, inhibition of actin contractility on astral MT, disrupts centrosome separation (Rosenblatt et al. 2004).

Using two different drugs that affect actin cytoskeleton we could not detect any effect on centrosome separation in prophase cells (as previously reported) but surprisingly we found a significant decrease in the levels of Eg5 accumulation at centrosomes. Cytochalasin D acts binding to the caps of actin microfilaments avoiding either the incorporation or the disassembly of monomers. Thereby, it is a stabilizing drug, which main effects in mitosis are mitotic delay, aberrant spindle formation and accelerated chromosome segregation in anaphase (Heng and Koh 2010). Blebbistatin inhibits myosin motility interfering with the ADP release in the motor domain. Inhibition of myosin causes, as mentioned above, centrosome separation impairment in prometaphase and problems in correct spindle assembly (Heng and Koh 2010). Our results with these two drugs are difficult to interpret and more work will be needed to be understand them. They may indicate that actin has a yet to be understood role in recruiting or maintaining Eg5 around the centrosomes and that these organelles are able to separate even lacking half of the Eg5 amount surrounding them. Thus, one hypothesis is that centrosomes must load a minimal amount of Eg5 for separation, and once this quantity is achieved, the kinesin can drive separation, possibly with the assistance of redundant pathways. How could the actin cytoskeleton be controlling Eg5 loading at centrosomes? It was already described that the loss of cortical actin function has a similar phenotype that the loss of dynein, which suggest a role for actin in dynein recruitment at the cell cortex ((Tanenbaum and Medema 2010) and references therein). Another function of actin and also cortical dynein (Laan et al. 2012) is to stabilize astral MT, maintaining the connection between cell cortex and MT (Buttrick et al. 2008) and regulating astral MT length and dynamics. Thus, Eg5 localization at centrosomes could be partially

depended on astral MT assembly and stabilization and dynein recruitment at cell cortex.

In addition to the phenotype observed in prophase cells after actin cytoskeleton disruption, after performing Y2H and MS screenings we have found several actin-related proteins as novel interactors of the Eg5 tail domain although the significance of this observation is unknown (see additional results for discussion).

Kinases controlling Eg5 localization

We also proved during this work that, besides Nek9 and Nek6/7, Eg5 localization at centrosomes and centrosome separation is dependent in the activity of the mitotic kinases Plk1 and Aurora A.

Aurora A is required for accumulation of centrosomal proteins during late G2, which are essential for MT nucleation at centrosomes (Joukov et al. 2010) (Joukov et al. 2014). Furthermore this kinase is involved in the regulation of entry into mitosis and Plk1 activation (Luca et al. 2006). Plk1 functions are also central for centrosome maturation (Lee and Rhee 2011) (Smith et al. 2011) (Kim et al. 2015) and mitotic entry. Thus, both kinases contribute to centrosome configuration and centrosomal MT nucleation during the G2/M transition, which could affect Eg5 localization at spindle poles during prophase. Nevertheless, this affirmation does not discard a more directed function of the kinases controlling Eg5 localization. For instance, the homologous protein of Aurora A in *Xenopus* phosphorylates Eg5 at the stalk domain (Giet et al. 1999), which could regulate Eg5 multimerization, although our group has failed to detect any phosphoresidue in mitotic Eg5 besides these modified by CDK1 and Nek6/7 (Rapley et al. 2008). Additionally, Plk1 activity is required for activation of the Nek9/Nek6/7 pathway, which we have shown is central for centrosome separation at different levels, among them the phosphorylation of Eg5 at Ser1033. Thus, Aurora A and Plk1 may actually be involved in controlling several aspects of Eg5 localization and activity, not only through the promotion of centrosome maturation, but also the regulation of the Nek9/Nek6/7 pathway.

Dynein activity regulates Eg5 localization in prophase

As mentioned, Eg5 normally moves towards the plus ends of MT, but during mitosis it accumulates at spindle poles, where MT minus ends concentrate.

The homolog of Eg5 in budding yeast, Cin8, presents double directionality *in vitro*. Movement to minus or plus ends of MT varies depending on salt concentration, which actually regulates motor density at MT. In single MT where Cin8 density is low, the motor moves towards the minus ends. However, increasing protein density at MT, directionality switches to the plus ends (Roostalu et al. 2011). This type of behavior has never been observed for mammalian Eg5 *in vitro*. This implies that directionality of Eg5 is regulated in a more complex manner in mammalian cells and suggests the involvement of minus end directed motors.

Using the permeable small molecule EHNA to inhibit cytoplasmic dynein we detected that Eg5 accumulation at poles is decreased. Additionally, distance between centrosomes decreases, although it is not statistically significantly different to the control. The requirement of dynein activity for Eg5 localization at spindle poles was previously proposed in later stages of mitosis in *Xenopus* egg extracts (Uteng et al. 2008) and in mammalian cell lines (Gable et al. 2012) but has never been reported in prophase.

Highlighting the importance of dynein in the control of Eg5 localization, we have found that both motor proteins interact in asynchronous cell cultures altogether suggesting that dynein transports the kinesin towards the minus ends of MTs in the vicinity of the centrosome. In our experiments, the Dynein-Eg5 interaction is independent on Eg5 phosphorylation at Ser1033 by Nek6/7 and at Thr926 by CDK1. This latter observation, obtained with phosphonull Thr926 mutants, seem to be in conflict with published data that indicates that Eg5 phosphorylation by CDK1 regulates not only motor binding to MT but the interaction with dynactin (Blangy et al. 1997), although we have never directly tested whether interference with Thr926 phosphorylation affects dynactin binding independently of the interaction with dynein, and we cannot totally exclude the possibility that recombinant Eg5 forms complexes with endogenous Eg5 thus affecting the result of our experiments. Simultaneous

depletion of endogenous Eg5 could probably result in a more conclusive answer.

Regarding the interaction of Eg5 with dynein, we describe that the adaptor and activator BicD2 mediates this association. BicD2 associates with the C-terminal tail of Eg5 through its second coiled coil (CC2) domain, specifically with residues 487 to 575. When this small BicD2 fragment is overexpressed it acts as a dominant negative, impairing Eg5 accumulation at centrosomes. We propose that, as the BicD2 polypeptide binds to the C-terminal tail of Eg5 but is not able to interact with the dynein complex, its overexpression saturates the C-terminal tail of Eg5 avoiding its binding with endogenous BicD2 and consequently preventing the association of the kinesin to dynein complex.

Using Y2H we show that the interaction of Eg5 with BicD2 is direct. Surprisingly, using this system only the C-terminal domain of BicD2 is able to bind to Eg5 but not the full-length protein, although both contain the 487-575 region. This observation could be explained by the different conformations adopted by BicD2. This protein remains closed through the interaction of its C-terminal domain with the N-terminal domain. Once it binds to the cargo at the C-terminal CC3 domain, it switches to the open conformation allowing the interaction with the dynein complex (Hoogenraad et al. 2001). Eg5 binds BiCD2 at a different domain that the classical cargo binding domain, and we suggest that this cannot open the adaptor directly thus precluding full length BiCD2 binding to Eg5 in yeast. The fact that in mammalian cells this interaction is detectable suggests that additional signaling cues (possibly phosphorylation of BicD2 by a kinase such as Nek9 (Holland et al. 2002)) can control BicD2 change in conformation and binding to Eg5.

BicD2 CC2 domain, besides interacting with Eg5, is also required for interaction with another motor protein, kinesin-1. BicD2 modulates the interaction between the opposite directed motors kinesin-1 and dynein. Depending on cell cycle stage, activity of one of the motors overcomes that of the other one. Thus, during interphase kinesin-1 activity predominates and during late G2 dynein activity overcomes that of the kinesin, promoting centrosome tethering to the nucleus (Splinter et al. 2010) (Figure 23). We propose that Eg5 is being carried towards the poles by dynein during G2, in a

similar way to kinesin-1. It would be interesting to elucidate how this movement is regulated in late G2, its relationship to the binding of kinesin-1 and whether Eg5 directionality towards plus ends is compensating dynein activity during other phases of cell cycle.

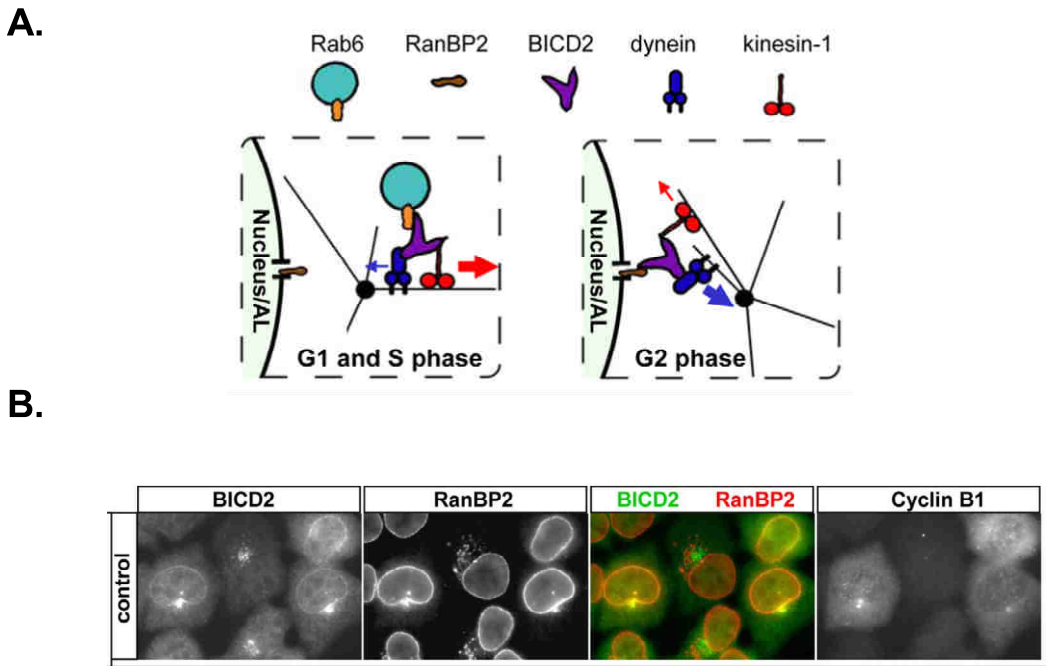

Figure 23. (A) Model for BicD2 interaction with dynein complex, kinesin 1 and RanBP2. Directionality of the motor proteins varies during cell cycle, predominating minus end motility during late G2, which regulates centrosome tethering at NE. (B) RanBP2 localizes at centrosomes and nuclear envelope at late G2, in an analogue way to BicD2. G2 stage is confirmed by cyclin B staining (Splinter et al. 2010).

Our work shows that BicD2 [1-575] localizes at the cytoplasm. This N-terminal fragment, although contains the dynein-binding domain and has been probed to be sufficient to activate dynein motility (Hoogenraad et al. 2001) (Splinter, et al. 2010) (Splinter et al. 2012) (McKenney et al. 2014), it is not able to accumulate at centrosomes and NE during prophase, in contrast to full length BicD2. However, the C-terminal fragment of BicD2 [487-820] is present

at centrosomes and NE during the G2/M transition. This fragment lacks the dynein-binding domain and it has been shown not to immunoprecipitate with the dynactin-dynein complex (Splinter, et al. 2010), although this localization could be explain as a result of its interaction with dynamitin, one of the dynactin subunits (Hoogenraad et al. 2001). In any case, it interacts with Eg5 and kinesin-1 and contain the RanBP2 binding domain (Splinter et al. 2012). BicD2 [575-820], containing the RanBP2-binding region but not the Eg5-binding is also present at centrosomes and NE. And finally BicD2 [487-575], binding exclusively to Eg5, has a diffuse localization. We conclude that BicD2 perinuclear and centrosomal localization during prophase depends on RanBP2 (as suggested previously, (Splinter et al. 2010)) and that Eg5 binding to BicD2 is not necessary for BicD2 centrosomal localization, suggesting that additional interactions of BicD2 C-terminus (possibly with the dynein machinery) are needed for this.

In contrast, BicD2 is instrumental for Eg5 localization. During prophase Eg5 localizes at centrosomes in a similar way to BicD2 and also RanBP2 (figure 23B). This observation, together with the requirement of BicD2 for Eg5 correct localization at centrosomes, indicates that BicD2 controls Eg5 positioning during late G2 and mitosis onset.

Regarding phosphorylation, although we propose that the interaction of Eg5 with dynein (and also with BicD2) is independent of Ser1033 phosphorylation our results suggest that Eg5 is phosphorylated once is bound to dynein complex. Thus, we propose a model in which first dynein binds to Eg5 and this interaction promotes Eg5 movement towards the minus ends of MT. Once Eg5 arrives to the centrosomes, active Nek9, which concentrates there (Roig et al. 2005) , through the action of Nek7 and Nek6 could promote Eg5 phosphorylation (Figure 24). We would like to note that a slight decrease in interaction with BiCD2 is observable for Eg5 phosphomimetic mutants in Y2H assays, for this reason we suggest that a competitive mechanism may exist in which once Eg5 is phosphorylated presents less affinity for BiCD2 and may be handed over to other interacting proteins (such as TPX2, see below).

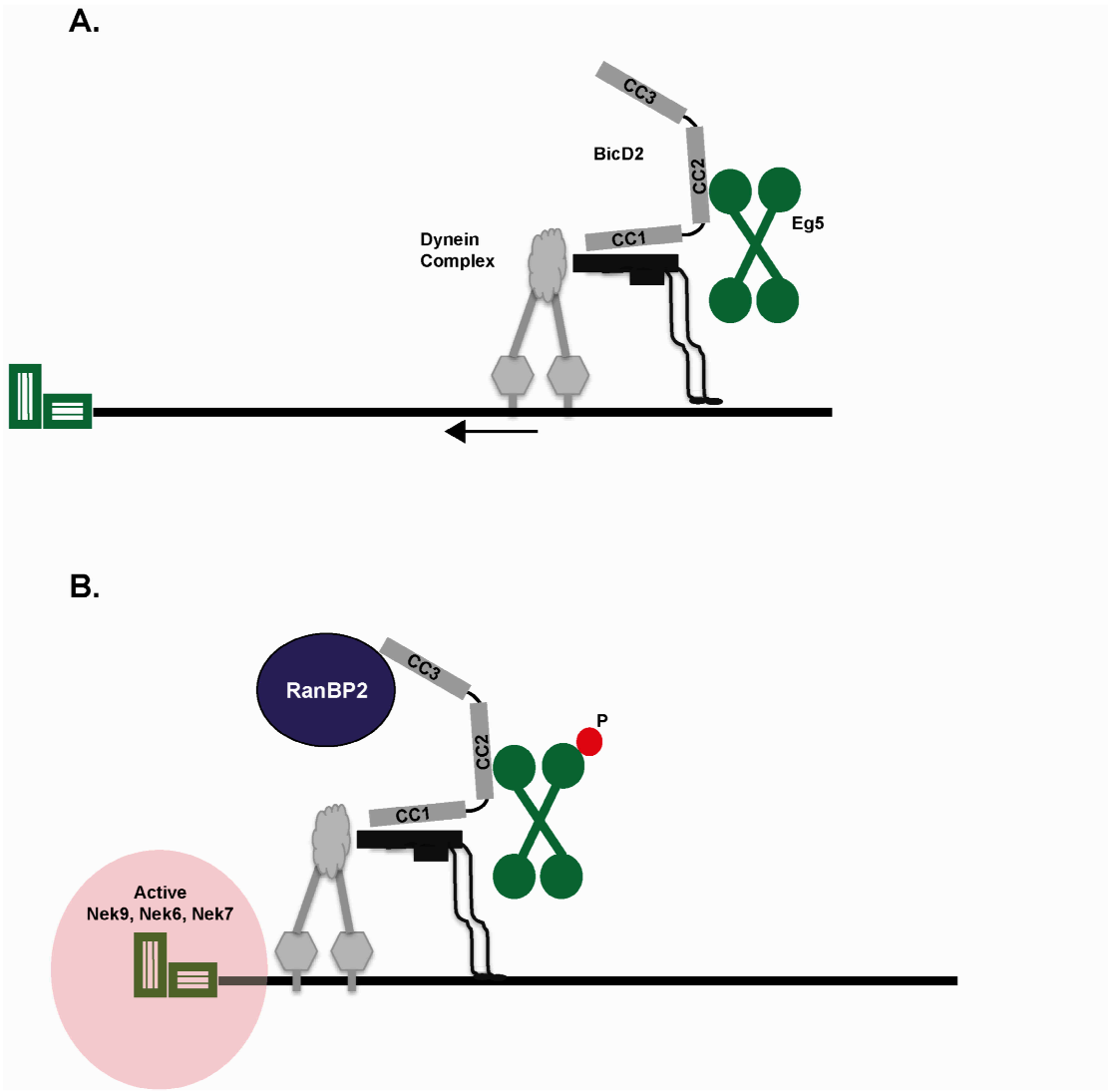

Figure 24. Model for Eg5 localization at spindle poles through dynein complex action. (A) BicD2 connects dynein complex (BicD2 CC1 domain) with Eg5 (BicD2 CC2 domain). This interaction promotes Eg5 movement towards minus ends of MT. (B) BicD2 is bound to RanBP2 at the poles and at NE. Once Eg5 reaches the spindle poles active Nek6 and Nek7 phosphorylate it at Ser1033.

Eg5 centrosomal accumulation depends on TPX2 localization at centrosomes

TPX2 is required for Eg5 accumulation

During the writing of this thesis the van Deursen lab has shown that Eg5 loading at centrosomes requires the interaction with the protein Dlg1, which is accumulated at centrosomes through the action of the PTEN-PDZ binding domain. They found that phosphorylated Eg5 binds to Dlg1 when is localizing at centrosomes (van Ree et al. 2016). However, this interaction is not dependent on phosphorylation of Eg5 Ser1033, since a phosphonull mutant is able to interact with Dlg1 with the same efficiency than the *wild type*. Eg5 phosphorylation is also not necessary to interact with the dynein machinery. Which is then the role of Eg5 Ser1033 phosphorylation and why this modification is required for proper Eg5 localization in prophase? The answer to this question has come through the study of the multifunctional MAP TPX2.

We have found that Eg5 centrosomal localization depends on TPX2 expression in mammalian HeLa cells and in mouse embryonic fibroblasts as Eg5 localization is impaired in cells with low TPX2 levels. As the structure of the centrosome is strongly affected in cells depleted for TPX2, we also used RHAMM as a tool to investigate the function of TPX2 without affecting these organelles. Depletion of RHAMM leads to a decrease of TPX2 levels at centrosomes without affecting their structure significantly, and strongly alters normal localization of Eg5 during prophase. Thus, we conclude that TPX2 localization at centrosomes is required for Eg5 accumulation around these organelles.

We also proved that Eg5 interacts with TPX2 in mitotic cell extracts. Thus, Eg5 interaction with TPX2 is visible in cells synchronized in mitosis using thymidine double block protocols and this result is not reproducible in asynchronous cell extracts or, importantly, in mitotic cell extracts from cells treated with high levels (200 ng/ml) of nocodazole. Therefore, we propose that the observed interaction occurs exclusively in mitosis and is MT-dependent.

TPX2 requires its C-terminal residues (713-747) to interact with Eg5 but this domain alone is not sufficient to promote this interaction. Thus, we think that although TPX2 interacts with Eg5 through its last 35 amino acids, this interaction requires additional parts of the protein. This could also suggest a possible requirement for the multimerization of TPX2. Both options are compatible with our failure to detect Eg5 association with the TPX2 C-terminal (residues 367-747) domain using standard immunoprecipitation protocols while detecting an effect on Eg5 localization after expressing this TPX2 fragment.

Y2H shows that TPX2 may interact directly with the C-terminal tail of Eg5. Unless there are additional regions of Eg5 that bind TPX2 this observation may be difficult to reconcile with published observations showing that TPX2 alters the movement of Eg5 monomers and dimers lacking the tail domain (Balchand et al. 2015), and due to the toxicity of TPX2 expression in yeast requires further confirmation by a different technique, but is consistent with a role of Ser1033 phosphorylation (in the C-t domain) in the regulation of Eg5 binding to TPX2.

In this regard, our results indicate that mutation of Eg5 Ser1033 strongly interferes with TPX2 interaction with the kinesin. Thus, TPX2 preferentially associates with Eg5 wt and different phosphomimetic mutants (Ser1033AspAsp and Ser1033Glu). This suggests an enhanced binding of TPX2 to Eg5 once the kinesin is phosphorylated. Puzzlingly, we could not detect a repetitive increased interaction with the Ser1033Asp mutant, which suggests that this substitution may not be phosphomimetic. Supporting this, expression of Eg5 Ser1033Asp mutant is not completely rescuing Eg5 depletion in mitotic cells (Rapley et al. 2008). Using Ser1033AspAsp we detected an amplified interaction between TPX2 and Eg5, suggesting that this mutant mimics better Ser1033 phosphorylation (that would result in an increase of ~2 negative charges) and this specific phosphorylation favors TPX2 interaction. Ser1033E would behave in an intermediate manner.

Considering the results mentioned above, we propose a model in which, after dynein (together with BicD2) has accumulated Eg5 at the vicinity of the centrosome, and Nek6/7 have modified Eg5 Ser1033, TPX2 recognizes phosphorylated Eg5 and strongly binds to it, acting as a clutch, decreasing Eg5 movement towards the plus ends of MT and possibly providing extra friction force or drag to Eg5 facilitating the action of the kinesin on MTs emanating from opposite centrosomes (Figure 25).

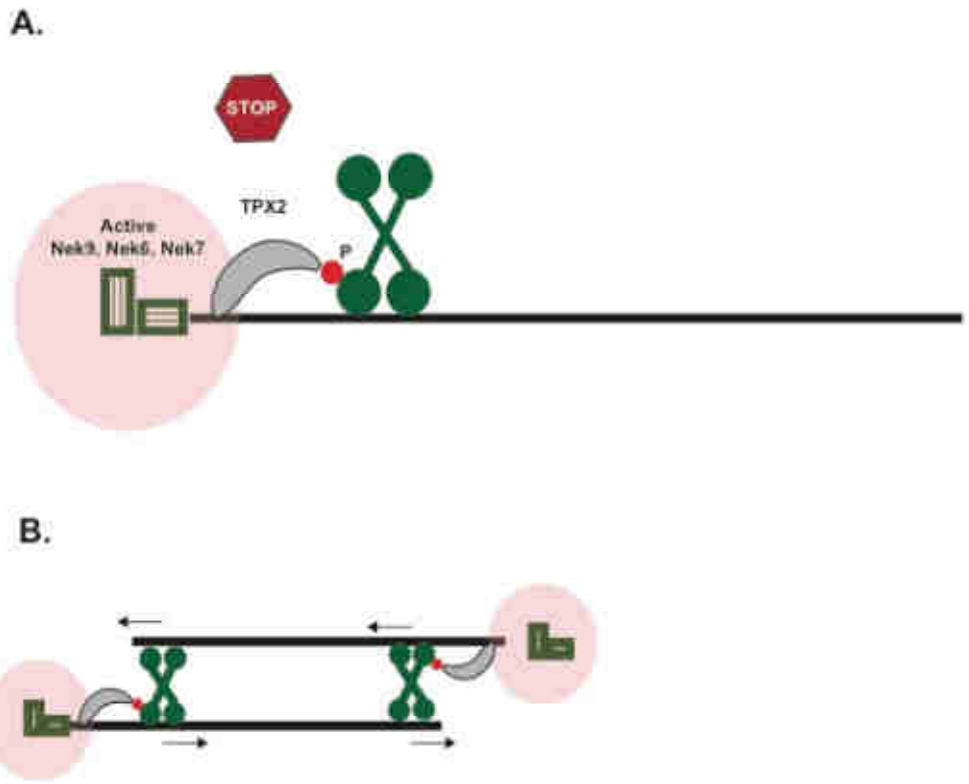

Figure 25. Model for TPX2 action on Eg5 at centrosomes. (A) Active Nek6 and Nek7 phosphorylate Eg5 at Ser1033 during G2/M transition. This phosphorylation is recognized by TPX2, which inhibits Eg5 motor activity at centrosomal levels. This effect promotes accumulation of Eg5 at minus ends of MT. (B) Accumulated Eg5 at centrosomes is required for force ejection between antiparallel MT emerging from both centrosomes. Centrosome separation is the consequence of MT sliding due to Eg5 activity.

TPX2 localizes at centrosomes before NEB

Our results identify a new role for TPX2 in prophase, before NEB. TPX2 localization during interphase has been proposed to be nuclear as a result of importin binding, and only after NEB to become cytoplasmic and be regulated by the RanGTP pathway, which releases TPX2 from importin. Most of the functions of TPX2 described until now occur after nuclear membrane dissolution, and we are proposing a cytoplasmic activity of this protein before the mentioned event. Our hypothesis is that before NEB TPX2 localization could be regulated independently of the importin/Ran system. This is supported by data showing that a pool of TPX2 is actively exported to the cytoplasm before NEB in plants. This event is required for proper spindle assembly afterward (Vos et al. 2008).

Indeed, we discovered that cytoplasmic TPX2 accumulates at centrosomes during prophase. This localization occurs before nuclear membrane disaggregation and can be induced with prolonged G2 arrest using the CDK1 inhibitor RO 3306. Interestingly, in G2-arrested cells TPX2 disappears from centrosomes when Plk1 and CDK's are inhibited. As we confirmed that under this conditions the Nek9 pathway is also inhibited (Bertran et al. 2011), we suggest that TPX2 centrosomal localization is dependent on the Plk1/Nek9 axis.

We show that Nek9 very efficiently phosphorylates TPX2 *in vitro* in at least 14 different residues. We have analyzed the function of the most relevant ones, but there are still some interesting residues that should be studied. Of these we would like to note that Ser654 is phosphorylated *in vivo* during mitosis and this phosphorylation depends on Plk1 activity (which activates Nek9) (Santamaria et al. 2011). This residue is located nearby the putative domain for Eg5 binding suggesting the exciting possibility is that Nek9 is controlling Eg5 interaction with TPX2 not only by Eg5 phosphorylation but also by TPX2 phosphorylation. This is in the line with the disruption of Eg5 interaction with TPX2 after Nek9 depletion.

Another residue of interest that we have not studied is Thr374, which is phosphorylated *in vivo* but has not been detected in mitosis. However the

modification of this residue has been shown by our group to depend on Nek9 by SILAC analysis of Nek9 *null* MEFs (Paula Martínez, unpublished results).

Using phosphomimetic mutants of residues Ser310, Thr320, Ser322 and Thr323 and disrupting the NLS [158-159] we detected a cytoplasmic localization of TPX2, something not detectable mutating only one of the NLS (either NLS [158-159] or NLS [313-315]). In the same direction, we identified an increased cytoplasmic signal of TPX2 in interphase cells that express active Nek9. Since Nek9 is mostly active at centrosomes during early mitosis (Roig et al. 2005), using mutants for the sites described above, we found a decreased localization at centrosomes of non-phosphorylable TPX2 (TPX2 [Ser310Ala;Thr320Ala;Ser322Ala;Thr323Ala]) and in contrast, a higher presence at prophase centrosomes of the phosphomimetic mutant (TPX2 [Ser310Asp;Thr320Glu;Ser322Asp;Thr323Glu]) compared to the *wild type*. Thus, we suggest that Nek9 phosphorylation at these residues promotes TPX2 localization at centrosomes during prophase without affecting RHAMM localization and also increases TPX2 cytoplasmic levels during interphase. Conversely, we show that Nek9 is necessary for TPX2 interaction with Eg5. For this reason we propose that TPX2 phosphorylation by Nek9 regulates TPX2 localization, most probably by directly regulating its interaction with importin and thus interfering with nuclear import (Figure 26).

Surprisingly, RHAMM interacts with TPX2 through a domain that comprises TPX2 NLS1 (Chen et al. 2014). Thus, RHAMM could be competing with importin α for TPX2 association. In this scenario TPX2 released from importing, may directly associate with RHAMM, which would promote its stabilization and centrosome localization. Since Nek9 is promoting TPX2 localization at centrosomes during prophase, we suggest that actually, Nek9 is promoting TPX2 release from importing and interaction with RHAMM. In this case, phosphomimetic mutants would present a higher affinity for RHAMM instead of importin α.

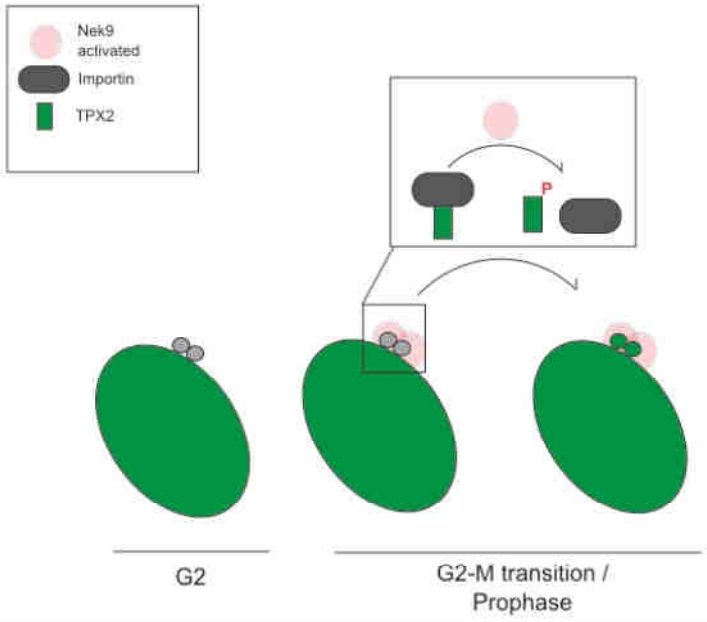

Figure 26. Model for TPX2 localization at centrosomes during prophase. Nek9 activates at centrosomes during G2/M transition. This kinase phosphorylates TPX2 affecting its binding to importin and promoting TPX2 localization at centrosomes.

We thus propose an alternative mechanism of control of TPX2 and other spindle assembly factors (SAFs) (Meunier and Vernos 2012), that would act in the vicinity of the centrosomes, where the Ran/GTP pathway is less effective, promoting importin release through phosphorylation of the NLS. The RanGTP pathway, the more general mechanism of control would predominate in the vicinity of chromatin, while NLS phosphorylation could modulate spindle assembly factors punctually at different areas, in collaboration with other mechanisms. For example MT nucleation from Golgi during mitosis is regulated through competition with importin binding. GM130 protein preferentially binds to importin during mitosis, promoting TPX2 release and action in the Golgi vicinity (Wei et al. 2015)

Therefore, we propose that at least three different pathways regulate spindle assembly factors activity during mitosis, depending on the area of localization: RanGTP at chromosomes, GM130 at Golgi and phosphorylation at centrosomes. Most of the active mitotic kinases accumulate at these

organelles, thus it would be of interest to determine the involvement of other kinases besides Nek9 in this putative mechanism controlling SAF function.

BicD2 and TPX2 present sequence similarity in the region required for interaction with Eg5

Analyzing BicD2 and TPX2 we found an amino acidic region in both with high similarity. This region corresponds to amino acids 494 to 506 in human BicD2 (496-508 in mouse BicD2) and amino acids 699 to 711 in human TPX2. Thus, this similar sequence is located within the minimal area for Eg5 interaction in TPX2 (Eckerdt et al. 2008) and also within the domain we described for Eg5 interaction in BicD2 (residues 486-575, see results).

It should be mentioned that the analyzed sequence is located in a coiled coil region in BicD2. Coiled coils are characterized by a pattern of hydrophobic and polar residues and this repetitive pattern often leads to false positives in predictions of similarity between large coiled coil regions (Rackham et al. 2010). However, although we were analyzing a short sequence located in a coiled coil region, we were only able to find similarity between BicD2, TPX2 and Rootletin, a centrosomal protein and also a substrate for Nek2. Furthermore, this region coincides with the domain of Eg5 interaction in both cases.

Studying the effect on Eg5 association we determined that mutations in this region decrease the ability of both, TPX2 and BicD2 to interact with Eg5 *in vivo.* Surprisingly, we did not detect any negative effect performing similar experiments with BicD2 *in vitro*, using Y2H.

The possibility of a shared sequence for Eg5 interaction in both proteins would suggest a competition between BicD2 and TPX2 for Eg5 association. This option would fit with the proposed model, in which Eg5 is carried towards the centrosomes by dynein complex trough BicD2 association, and once is at the poles, Eg5 preferentially associates with TPX2 promoting the inhibition and accumulation of the motor protein.

Concluding remarks

In this work, we have described two processes that contribute significantly to the understanding of mitosis and possibly suggest novel forms of regulation of non-mitotic processes.

We describe a new layer of regulation in centrosome separation and Eg5 function. Phosphorylation in the C-terminal domain of this motor protein contributes to its localization at spindle poles at a moment during mitosis when high amounts of the protein are required at minus ends of MTs to promote centrosome separation. Phosphorylation is one of the major mitotic signaling cues and motor proteins are important players to accomplish the correct segregation of the chromosomes. However, the effect of phosphorylation on this family of proteins is poorly studied, although most of them present at least one phosphorylated site during mitosis. Eg5 inhibitors have failed clinical trials for cancer treatment. Cancer cells are able to divide even in the absence of Eg5 activity. This scenario is in part due to the existence of redundant pathways to Eg5 in centrosome separation and bipolar spindle formation, but we think that a deeper knowledge in motor protein regulation by phosphorylation could be the key for understanding resistance to drug treatments and for the development of new combinatorial therapies that include kinase and motor inhibitors.

We also define what could be a new general regulatory pathway for spindle assembly factors. We propose a novel mechanism in which phosphorylation induces importin release of these factors in the centrosome/spindle pole vicinity, thus effectively making the SAFs independent of the RanGTP gradient and activating them locally. The RanGTP gradient acts predominantly near chromosomes but most of the proteins regulated by this pathway are also localizing at spindle poles during mitosis and their action in this area is indispensable for correct mitotic progression.

Finally, it is important to note that the regulatory mechanism that we described here could be also being recycled in non-mitotic or differentiated cells. For instance, Eg5 and TPX2 have function on neuronal development, through dendrites and axon formation (Lin et al. 2012) (Kahn et al. 2015).

Thus, phosphorylation of Eg5 and TPX2 by different kinases and the control of Eg5 interaction with TPX2 could also be of paramount importance in that context.

CONCLUSIONS

The dynein complex controls Eg5 localization at centrosomes.

1.1. Dynein activity is required for Eg5 accumulation at centrosomes.
1.2. Dynein interacts with Eg5 and this interaction is independent of Eg5 Ser1033 phosphorylation.
1.3. The adaptor BicD2 mediates the interaction of Eg5 with dynein.
1.4. BicD2 interacts directly with Eg5. This interaction involves the CC2 domain of BicD2 and the tail domain of Eg5.
1.5. BicD2 is required for Eg5 localization at centrosomes and centrosome separation in prophase.

TPX2 is required for Eg5 accumulation at centrosomes.

2.1. TPX2 is required for Eg5 accumulation at centrosomes.
2.2. RHAMM depletion affects TPX2 localization at centrosomes and consequently, Eg5 localization in prophase.
2.3. Eg5 does not properly localize at centrosomes in TPX2 -/- MEFs.
2.4. TPX2 interacts in mitosis with Eg5 and Eg5 Ser1033 phosphonull mutants abolish this interaction.
2.5. Nek9 controls the interaction between Eg5 and TPX2.

Nek9 promotes TPX2 localization at centrosomes during prophase.

3.1. Nek9 phosphorylates several TPX2 residues located proximal to the NLS *in vitro.*
3.2. Nek9 phosphorylation of TPX2 increases its cytoplasmic localization and centrosomal accumulation.
3.3. Nek9 is required for TPX2 localization at centrosomes in prophase. In the same way, TPX2 phosphomimetic mutants increase its presence at centrosomes during prophase.

ADDITIONAL RESULTS AND DISCUSSION

1. Yeast two hybrid screening for Eg5 tail domain interactors

Yeast Two Hybrid (Y2H) is a technique used to determine novel protein-protein interactions in which reporter genes are activated upon protein interaction allowing the expression of proteins that are essential for yeast growth in a selection media. The proteins of interest are expressed fused to two different parts of GAL4 transcription factor. The *bai*t protein is expressed bound to the DNA Binding Domain (BD), which will bind to the promoter of the reporter genes, and the *prey* protein is bound to the Activation Domain (AD), which recruits the transcriptional machinery. In case of prey-bait protein interaction, the GAL4 binding domain and activation domain will be close enough to support the transcription of the mentioned genes.

We have used this technique in order to try to identify novel interactors of the Eg5 C-terminal [764-1057] domain (*tail* domain) that could explain Eg5 localization at centrosomes and its dependence on Ser1033 phosphorylation. This domain of the motor protein is required for control of Eg5 binding to MT and localization through phosphorylation by CDK1 and Nek6/7 (Blangy et al., 1995) (Rapley et al. 2008). Novel interactions would allow us to understand the specific role of the C-terminal tail of Eg5 domain in the general function of the kinesin, and more specifically in the control of its localization during the different phases of mitosis. We initially sought to express the whole Eg5 tail domain bound to the BD, which includes the described CDK1 and Nek6/7 phosphorylation sites (Blangy et al., 1995) (Rapley et al. 2008), and perform a screening with a library of cDNAs expressed as AD-fusion proteins. We wanted to analyze the differences in the interaction pattern of a *wild type* (WT) peptide and a phosphomimetic peptide Ser1033Asp/Glu with a negative charged amino acid instead of the serine of interest, which could simulate the phosphate group, incorporated after phosphorylation.

We thus built pGBKT7-BD plasmids expressing Eg5 [764-1057] wild type, Ser1033Asp and Ser1033Glu mutants and transformed them into yeast (strain

AH109). We proved the expression of these constructs by western blot (WB) and assessed the toxicity in yeast associated to the expression of the different proteins (Figure 27). We saw that all the constructs are similarly expressed and their effect on growth is comparable to controls. Thus, we could not detect any toxicity caused by the plasmids. We then proceeded with the assessment of a possible auto activation of the system due to the expression of the protein fragments. It has been previously described that a 5% of the proteins promote auto activation of the Y2H system by acting as an AD of the transcription factor, and stimulating the transcription of the reporter genes (Van Criekinge and Beyaert 1999). Our results show that yeast expressing Eg5 [764-1057] is able to grow in selection media, and we conclude that the Eg5 tail domain is activating the reporter genes. Consequently this protein domain was discarded for the screening. A similar result was subsequently reported in (Iwakiri et al. 2013).

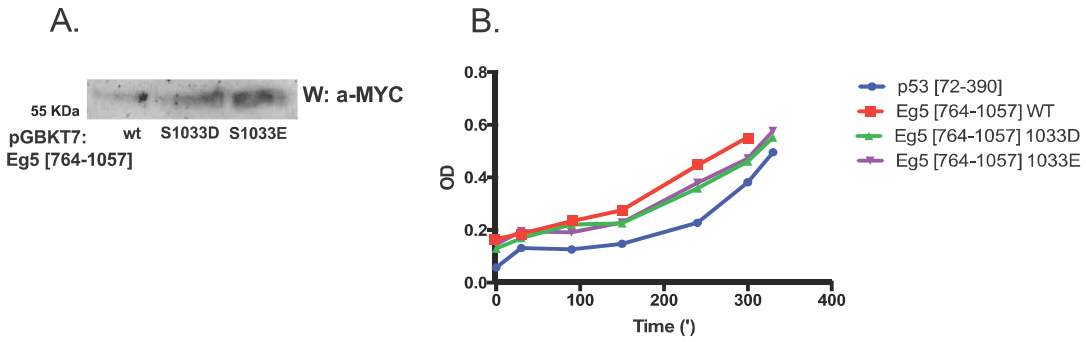

Figure 27. Expression and toxicity of the constructs pGBKT7-BD Eg5 [764-1057]. (A) Yeast strain AH109 was transfected with the plasmids mentioned before (using the lithium acetate protocol). Yeast that incorporated the plasmid was selected with a -Trp medium. All pGBKT7 plasmids are myc-tagged. (B) Yeast growth curve measuring yeast OD at 600 nm wavelength. The control yeast is expressing the murine p53 [72-390] fragment (Clontech).

It was described that, in the Y2H system, when a protein is acting as an activator can be due either to presence of coiled coil domains or an acidic region, which can induce the recruitment of the transcriptional machinery towards the promoter (Ruden 1992). For this reason we decided to eliminate the predicted coiled coil segments from our construct of interest, obtaining a new fragment of the Eg5 tail domain that comprise the residues between the positions 968 and 1057, which includes the Nek6/7 (our principal target) site but not the CDK1 phosphorylation site (Figure 28A). However, we also considered that the small size of this fragment, lacking essential residues for Eg5 regulation, could provide important limitations for the development of this assay.

Again, we checked for toxicity, expression and auto activation. Yeast transformed with Eg5 [968-1057] grows as the control, the expression of Eg5 [968-1057] is detectable by WB and it does not induce the auto-activation of the system. Thus, we finally proceed with the library screening (Figure 28).

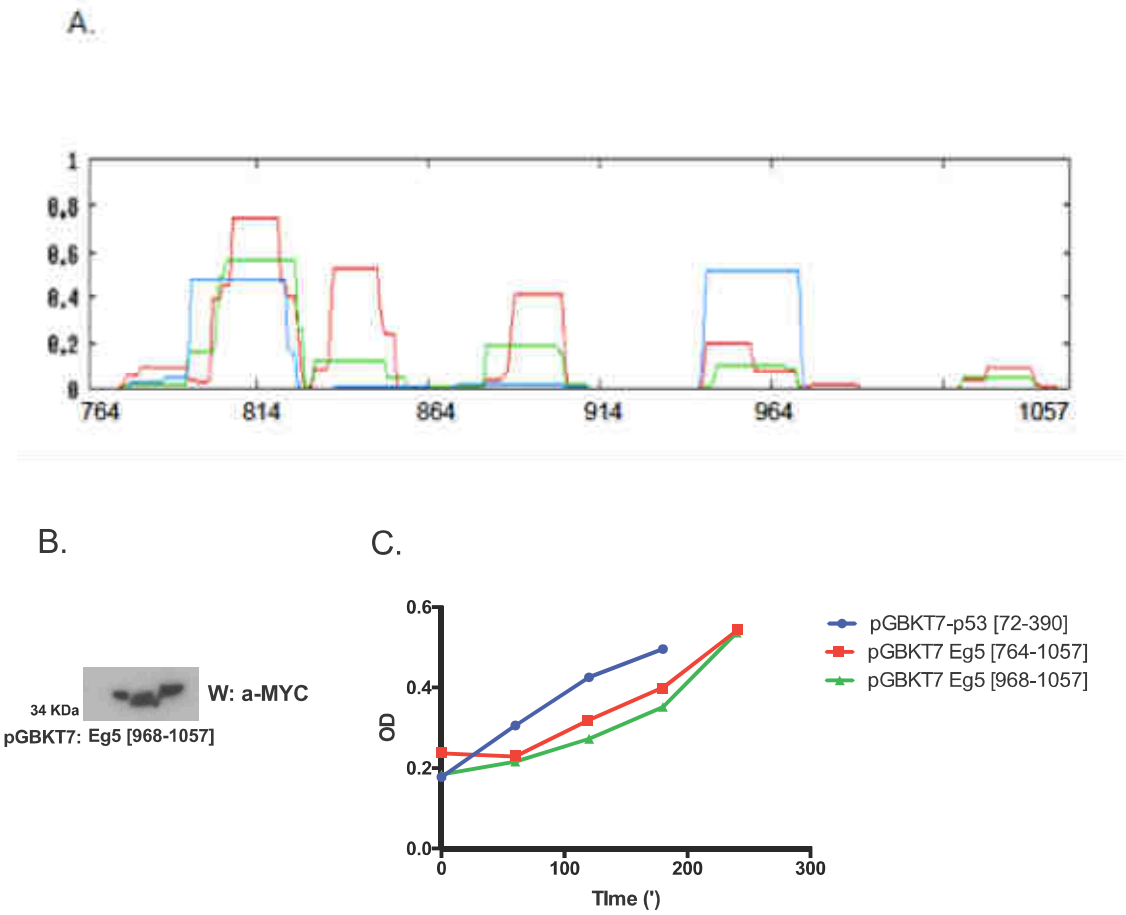

Figure 28. Expression and toxicity of pGBKT7-BD Eg5 [968-1057] construct. (A) Predicted coiled coils for Eg5 tail domain, identified using NPS@: Network Protein Sequence Analysis. (B) Eg5 [968-1057] expression as detected by western blot. (C) Yeast growth curve for Eg5 [968-1057] comparing it with p53 [72-390] fragment and Eg5 [764-1057].

We performed two different screenings with the Eg5 [968-1057] construct obtaining similar results (table S3). We detected over 100 positives colonies that were subsequently subcultured for DNA extraction. A 10% of the total positives were eliminated due to failure in DNA extraction, failure to identify a human cDNA, or presence of an empty plasmid. DNA sequences corresponding to non-coding mRNA regions, that were not in frame with the AD region or that do not encode proteins were discarded for analysis. Furthermore, we obtained several sequences that encode proteins classified

as usual false positives in this system (DNA transcription machinery, splicing factors…) although some of them were recently related with alternative functions of Eg5 (Bartoli et al. 2011).

The final list contains a total of 88 sequences characterized and classified according to their abundance and relevance, (see Supplementary table S3), and it does not contain an obvious candidate that could explain centrosomal localization of Eg5.

However, this list of candidates (grouped by function below) may be useful for future study new functions of kinesin Eg5 during other moments of cell cycle.

Transport: Sorting Nexin 1

Soting nexin 1 belongs to a family of membrane deforming proteins, which function in protein transport to the plasma membrane or the trans Golgi network transport (Royle 2013). Dynein activity is important for transport of vesicles containing Sorting nexin (Granger et al. 2014). In fact, this transport is also regulated by the action of opposite forces, minus end and plus end MT directed, such kinesin 1-dynein complex interaction. Kinesin 1 interaction with dynein complex is mediated by the coiled coil protein BicD2 (Splinter et al. 2010) and we showed in our results that dynein complex interacts with Eg5 through the same region of BicD2. Thus, this positive candidate may indicate a role for Eg5 in vesicle transport. In fact, it has been previously demonstrated that kinesin Eg5 is required for transport from Golgi to cell surface in Drosophila cells (Wakana et al. 2013).

Cell morphology: MIEN1, EPB4.2, Tensin1, Filamin A, RhoGDP dissociation inhibitor.

MIEN1 function remains elusive nowadays. This protein is overexpressed in breast cancer and is used as a marker of bad prognosis in cancer. It was described that MIEN1 induces cell migration by increasing surface expression

of annexin A2, a protein involved in signal transduction pathways (Kpetemey et al. 2015).

EPB4.2 is another protein with unknown function. Mutations in this gene are linked to spherocytosis disease, which is characterized by a destabilized cytoskeletal scaffold in erythrocytes (Washington et al. 2009).

Tensin1 is a protein localized in focal adhesion, which are structures that connect actin cytoskeleton with the extracellular matrix (Davis et al. 1991) .

We identified a **Filamin A** clone in the Y2H screening, although the cDNA was not in frame with the AD, but we decided to consider it as relevant due to the number of appearance as a positive interaction. The function of this huge protein is to crosslink acting filaments (F-actin), and it is involved in several processes where actin filaments branching is required, such ciliogenesis (Adams et al. 2012) or cytokinesis (Samwer et al. 2013).

RhoGDP dissociation inhibitor regulates actin dynamics and myosin activity (Hall and Hall 2013). There are also evidences that this protein affects MT organization and intermediate filaments, being this related with a function in cell migration (Ridley 2001).

It was recently proposed that the interplay between MT and actin filaments could be regulated by a kinesin of KIF14 family in *Xenopus* oocytes. This connection between MT and actin filaments is essential for proper development of cytokinesis (Samwer et al. 2013). This correlates with the observation of Eg5 (and Eg5 P-Ser1033) localization at cytokinetic furrow during cytokinesis (Rapley et al. 2008).

Altogether, our results suggest a possible role for Eg5 either in vesicle trafficking or actin-MT organization dynamics.

Despite using a pre-transformed human cDNA library we could not detect known interactors of the C-terminal tail of Eg5, as Nek6 and Nek7. Also, we cannot exclude that other true interactors were not detected by this assay. This could be attributed to some of the following reasons:

- Some proteins are not able to fold correctly when they are expressed in yeast. Some of the posttranslational modifications required for their final structure are not present in this organism.

- Localization at nucleus of the recombinant protein is required for activation of the reporter genes. However, some proteins present strong target signals, which promote their localization outside the nucleus.

- Some proteins are toxic when they are overexpressed in yeast. For instance, CDK/cyclin localization at nucleus increases toxicity and other proteins could promote degradation or activation of essential proteins for the survival of this organism.

- Finally, the used fragment is probably too short to identify interacting proteins. This fragment lacks the CDK1 phosphorylation site, which promotes Eg5 binding to MTs (Blangy et al. 1995), the motor domain, which binds to MTs and the stalk domain, which is required for Eg5 tetramerization.

2. Mass Spectrometry analysis to identify new Eg5 tail domain interactors

Protease digestion combined with liquid chromatography/mass spectrometry (LC/MS/MS) analysis is another valuable method to detect novel protein-protein interactions. Protein digestion with trypsin, followed by LC/MS/MS, results in the identification of the resulting peptides, comparing their mass with these predicted in a pre-existing database.

We have used this technique seeking to identify novel protein-protein interactions mediated by the regulatory domain of Eg5, the tail domain. To address this question we built three different CMV5 FLAG vectors expressing Eg5 [764-1057] WT, Ser1033Ala and Ser1033Asp. We first tested the expression and localization of these constructs. Expression levels of the Eg5 tail polypeptides were very poor, possibly due to toxicity. These Eg5 fragments were not able to localize neither at the spindle or the centrosome. It was shown previously by the Kapoor group that the Eg5 C-terminal domain is able to interact with MT providing to Eg5 tetramers permanent crosslinking ability to MTs (Weinger et al. 2011). However, our study of the C-terminal domain (residues [764-1057]) by immunofluorescence in human cultured cells suggests that in mitosis that this fragment is not able to bind to MTs by itself, since is not localizing at spindle or centrosomes (Figure 29). Therefore, this may indicate that the tail domain of Eg5 requires the presence of the motor domain for MT binding. This mislocalization of the fragment could be the reason for not detecting some possible interactions, especially if the interaction is MT dependent. Moreover, since the localization of Eg5 is dependent of phosphorylation (Rapley et al. 2008) and this phosphorylation could occur mainly at around centrosomes, where active Nek9 concentrates (Roig et al. 2005), most of the interacting proteins could be detectable only in the small percentage of mitotic cells.

However, we decided to proceed with the mass spectrometry analysis of the WT form.

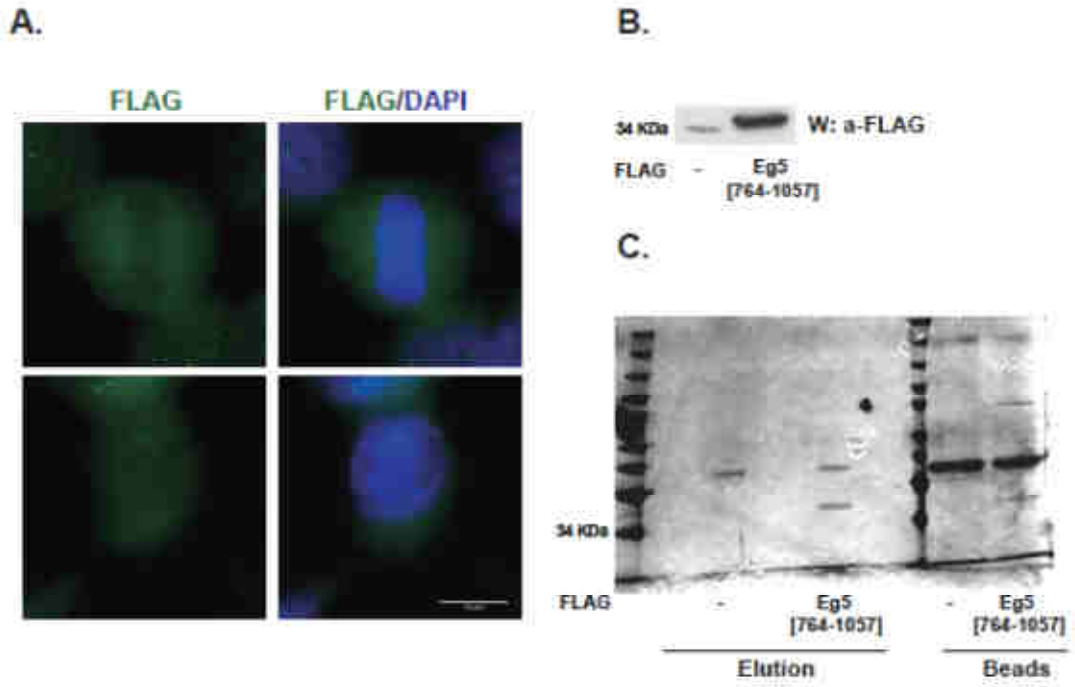

Figure 29. (A) Localization of Eg5 [764-1057] in a metaphase cell (up) and a prophase cell (down) Scale bar 10 µm. (B) Expression of the construct by WB. (C) Silver staining of Eg5 tail domain elution. A reproduction of this elution was sent to MS.

We expressed the FLAG Eg5 [764-1057] tail construct in HEK 293 cells and 48 hours later immunopurified it using magnetic beads coupled to M2 FLAG antibody (Figure 29). After elution during 1 hour at 4 ^{o}C with FLAG peptide (0,5 µg/ml, Sigma) the samples were charged in a SDS-PAGE gel and ran during 10 minutes in order to obtain a unique band for each sample. The bands were cut and in-gel digested with trypsin at the Parc Científic Proteomic's Platform. After digestion the samples were dried in a Speed-Vac and resuspended in µl of 1% formic acid for LC/MS/MS analysis, obtaining the results summarized in table 2. A total of 42 proteins were identified in the FLAG Eg5 tail elution but not in a control elution, and a total of 15 proteins were identified with at least two times the protein score (which correlates with the probability of correct identification of the protein based on the identified peptides that compose the sample) in the Eg5 tail elution compared to the control. Around 20% of the proteins that were exclusively present in the Eg5 tail sample were related with the cytoskeleton and surprisingly around a 40% belonged to proteins with mitochondrial localization. In the case of proteins

present in both samples, control and Eg5 tail domain, whit a higher protein score in the latter a 30% were cytoskeletal proteins, such as different subunits of alpha and beta tubulin. Additionally we were able to identify the kinase Nek9 and its interactor dynein light chain 1 (Regué et al. 2011) with the Eg5 tail.

A.

Accession	Description	Score Control	Score FLAG Eg5 tail
P54886-2	Isoform Short of Delta-1-pyrroline-5-carboxylate synthase		272.96
SE1673-1	FLAG TAIL		165.98
Q9UJS0	Calcium-binding mitochondrial carrier protein Aralar2		74.96
Q02978	Mitochondrial 2-oxoglutarate/malate carrier protein		37.87
P47756-2	Isoform 2 of F-actin-capping protein subunit beta		35.26
O75746	Calcium-binding mitochondrial carrier protein Aralar1		29.08
Q9NVI7-3	Isoform 3 of ATPase family AAA domain-containing protein 3A		25.51
Q16891-3	Isoform 3 of Mitochondrial inner membrane protein		21.46
P31146	Coronin-1A		19.29
Q00325-2	Isoform B of Phosphate carrier protein, mitochondrial		16.03
P62805	Histone H4		8.73
P25705-2	Isoform 2 of ATP synthase subunit alpha, mitochondrial		8.00
O95831-3	Isoform 3 of Apoptosis-inducing factor 1, mitochondrial		7.00
P15311	Ezrin		6.82
Q9H1K4	Mitochondrial glutamate carrier 2		6.69
Q9Y512	Sorting and assembly machinery component 50 homolog		6.64
P12273	Prolactin-inducible protein		5.75
Q8WVX9	Fatty acyl-CoA reductase 1		5.66
Q9Y230	RuvB-like 2		5.38
P53007	Tricarboxylate transport protein, mitochondrial		4.90
P04637-4	Isoform 4 of Cellular tumor antigen p53		4.70
Q53H12	Acylglycerol kinase, mitochondrial		4.39
P01876	Ig alpha-1 chain C region		4.38
P01859	Ig gamma-2 chain C region		4.34
P50402	Emerin OS=Homo sapiens		4.12
A8MT79	Putative zinc-alpha-2-glycoprotein-like 1		3.38
O43175	D-3-phosphoglycerate dehydrogenase		3.10
O60762	Dolichol-phosphate mannosyltransferase		3.00
O43242	26S proteasome non-ATPase regulatory subunit 3		2.92
P06576	ATP synthase subunit beta, mitochondrial		2.85
P81605	Dermcidin		2.83
Q9Y265-2	**Isoform 2 of RuvB-like 1**		2.67
Q9NX63	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial		2.55
Q15286-2	Isoform 2 of Ras-related protein Rab-35		2.49
P19474-2	Isoform 2 of E3 ubiquitin-protein ligase TRIM21		2.35
Q15431	Synaptonemal complex protein 1		2.26
P09493-2	Isoform 2 of Tropomyosin alpha-1 chain		2.15
P35610-3	Isoform 3 of Sterol O-acyltransferase 1		1.99
Q9NXG0	**Centlein**		1.98
P31947-2	Isoform 2 of 14-3-3 protein sigma		1.90
Q9UBX3	Mitochondrial dicarboxylate carrier		1.81
P15090	Fatty acid-binding protein, adipocyte		1.68
P07195	L-lactate dehydrogenase B chain	0.00	2.80

B.

Accession	Description	Score control	Score FLAG Eg5 tail
P11142-2	Isoform 2 of Heat shock cognate 71 kDa protei	38.37	103.27
P08107	Heat shock 70 kDa protein 1A/1B	37.93	81.20
P07437	Tubulin beta chain	6.53	64.46
P10275	Androgen receptor	26.70	59.27
P68363	Tubulin alpha-1B chain	7.16	55.09
P68371	Tubulin beta-4B chain	3.57	53.71
P05141	ADP/ATP translocase 2	8.30	48.38
P12236	ADP/ATP translocase 3	4.78	39.58
Q8TD19	Serine/threonine-protein kinase Nek9	14.17	27.72
P63167	Dynein light chain 1, cytoplasmic	7.75	24.54
P10809	60 kDa heat shock protein, mitochondrial	2.37	16.27
P08238	Heat shock protein HSP 90-beta	8.61	16.53
Q8N1N4-2	Isoform 2 of Keratin, type II cytoskeletal 78	4.65	15.33
P07900	Heat shock protein HSP 90-alpha	5.97	14.38
P05067-10	Isoform APP639 of Amyloid beta A4 protein	3.76	7.35
P0CG48	Polyubiquitin-C	3.46	6.26

Table 2. MS results for Eg5 tail elution. (A) Proteins eluted with FLAG Eg5 tail but not with the control. (B) Proteins with a two-fold higher score in Eg5 tail elution than the control. Mitochondrial proteins are marked in green. Cytoskeleton proteins are marked in orange.

In any case, just as it happened with Y2H, MS results for the C-terminal tail of Eg5 domain could give us some clues about possible roles of this kinesin outside of centrosome separation.

Mitochondrial transport

Surprisingly, 40% of the characterized peptides eluted with the C-terminal of Eg5 belong to mitochondrial proteins. Moreover, most of these proteins are located at the mitochondrial inner membrane. The C-terminal tail of Eg5 domain does not present any mitochondrial targeting signal in its peptide sequence, thus these interactions cannot easily be explained by Eg5 C-terminal fragment intake to mitochondria.

Mitochondrial transport is well described in neuronal axons. This organelle is transported mainly by MT but it can anchor to actin filaments (Hollenbeck and Saxton 2005). For MT mediated transport, kinesin 1 family of motor proteins is able to interact indirectly with mitochondria through some unknown adaptors, carrying mitochondria to the plus ends of MT. One of the linker proteins essential for transport is the protein Milton (Hollenbeck and Saxton 2005), which curiously, does not appear between the eluted proteins.

During mitosis, the mitochondrial network disaggregates in a process called fission, which is mediated by CDK1/cylin B (Taguchi et al. 2007). These fragments of mitochondria are accumulated at the cleavage furrow during cytokinesis in a MT dependent mechanism (Lawrence and Mandato 2013) to ensure correct segregation between the two daughter cells. Interestingly, Eg5 and Eg5 [P-1033] also localize at cleave furrow at this stage, which might imply a possible role for Eg5 in mitochondrial localization and segregation during mitosis.

Cell morphology, migration and actin cytoskeleton

We already discussed before the appearance of actin cytoskeleton related proteins. However, we want to emphasize the role of some of these proteins, which could be of interest for further investigations of Eg5 functions.

Ezrin is a protein in charge of regulating the actin cytoskeleton dynamics at cell cortex or membrane. Besides its actin-related function this protein is able to interact directly with MT and this interaction is necessary for proper positioning of aster spindle MT during mitosis (Solinet et al. 2013). Interestingly, this protein has been also linked to centrosome positioning and centrosome clustering during mitosis (Hebert et al. 2012).

Emerin is another protein first related to actin cytoskeleton but it was also proposed as a MAP, due to its binding to MT. Emerin localizes at inner nuclear membrane and its ability to bind MT is required for anchor centrosomes to the NE in fibroblasts. Depletion of emerin causes centrosome detachment from the nucleus in human dermal fibroblasts (Salpingidou et al. 2007) in a similar way to BicD2 depletion. Centrosome movement away from

nucleus in BicD2 depleted cells was attributed to Eg5 activity (Splinter et al. 2010). However, it is proposed that emerin is not required for centrosome positioning in other types of cell culture, such HeLa cells (Bolhy et al. 2011).

Centlein localizes at centrosomes and promotes the interaction between C-Nap and Cep68, thus being part of centrosomal linker in interphase (Fang et al. 2014). The centrosomal linker is dissolved before centrosome separation through the action of kinase Nek2 but this process is not essential for centrosome separation and formation of the bipolar spindle (Fletcher et al. 2005). Dissolution of the linker becomes essential for centrosome separation when Eg5 activity is reduced (Mardin et al. 2010).

RuvBL1 is a multifunctional protein, which is located at spindle and centrosomes during mitosis. RuvBL1 depletion leads to an increase on monopolar spindles (Fielding et al. 2008), which may indicate a direct or indirect role of this protein in centrosome separation. Furthermore, this protein is mainly localized at nucleus during interphase and at centrosome and spindle during mitosis, which convert it in a plausible mitotic factor regulated by RanGTP pathway.

However, any hypothesis involving the discovered interactions is just speculation. Further studies must be performed to confirm the connection of Eg5 with these proteins.

3. The Cdk1 inhibitor RO-3306 induces a prophase-like phenotype in HeLa cells.

RO-3306 is a selective CDK1/cyclin B and CDK1/cyclin A inhibitor. Treatment of cells with RO-3306 leads to a reversible and efficient cell arrest at the G2-M border (95% of efficiency (Vassilev 2006). After washing out the inhibitor the cells enter the cell cycle during the following 20 minutes, resulting in a high percentage of cells in prophase or starting prometaphase. We contemplated the use of RO-3306 to study prophase centrosome separation, but we decided to discard it due to RO-3306 resulting in premature centrosome separation in G2, in parallel with an abnormal Eg5 accumulation at centrosomes (Smith et al. 2011) and the accumulation of abnormal amounts of PCM proteins, such as pericentrin, as well as Plk1 overactivation.

Using this drug we have nevertheless gained some insights about Nek9 and the centrosome. Arresting cells in G2 with 9 μM RO-3306 during 16 to 20 hours we have found that, even with the absence of CDK1 activity, some mitotic proteins are accumulated and activated at the centrosome. For the proteins studied in this work, the resulting state mimics the characteristics of a prophase: centrosome maturation, accumulation of Eg5 and centrosome separation (Figure 30). We have determined the presence in these cells of activated Aurora A, activated Nek9 and phosphorylated Eg5 [Ser1033] (Figure 31). This is in agreement with our previously results claiming that Eg5 must be phosphorylated for its accumulation.

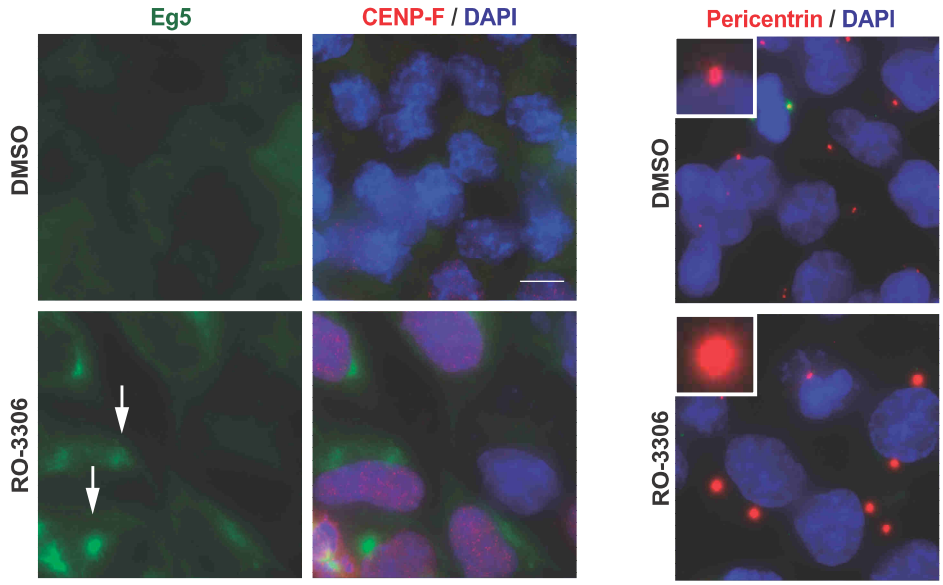

Figure 30. Eg5 accumulates at centrosomes in RO 3306 treated cells. White arrows indicate Eg5 accumulation (left). CENP-F was used to mark G2 cells (middle). Pericentrin levels increase dramatically after treatment (right).

It has been proposed that CDK1 inhibition is compensated by the activity of another CDK, for instance CDK2, and normal G2 condition can be rescued by inhibiting kinase Plk1 (Smith et al. 2011). In order to determine whether the phosphorylation of Nek9 and Aurora A was due to a compensatory activity of another CDK1 and an overactivation of Plk1, we have used Roscovitine, a general inhibitor of CDKs, and BI-3626, a Plk1 inhibitor, and measured the effect on centrosome maturation, Nek9 and Aurora A activation and Eg5 phosphorylation by immunofluorescence. We observed that inhibition of CDK's in general and Plk1 recovers HeLa cells to an interphase phenotype, with reduced pericentrin, γ-tubulin, Eg5 [Ser1033-P] and Nek9 [Thr210-P] centrosomal levels (Figure 27). In summary, we have defined here a new system to induce Eg5 centrosomal localization and Nek9 activation that with its caveats can be used in future analysis.

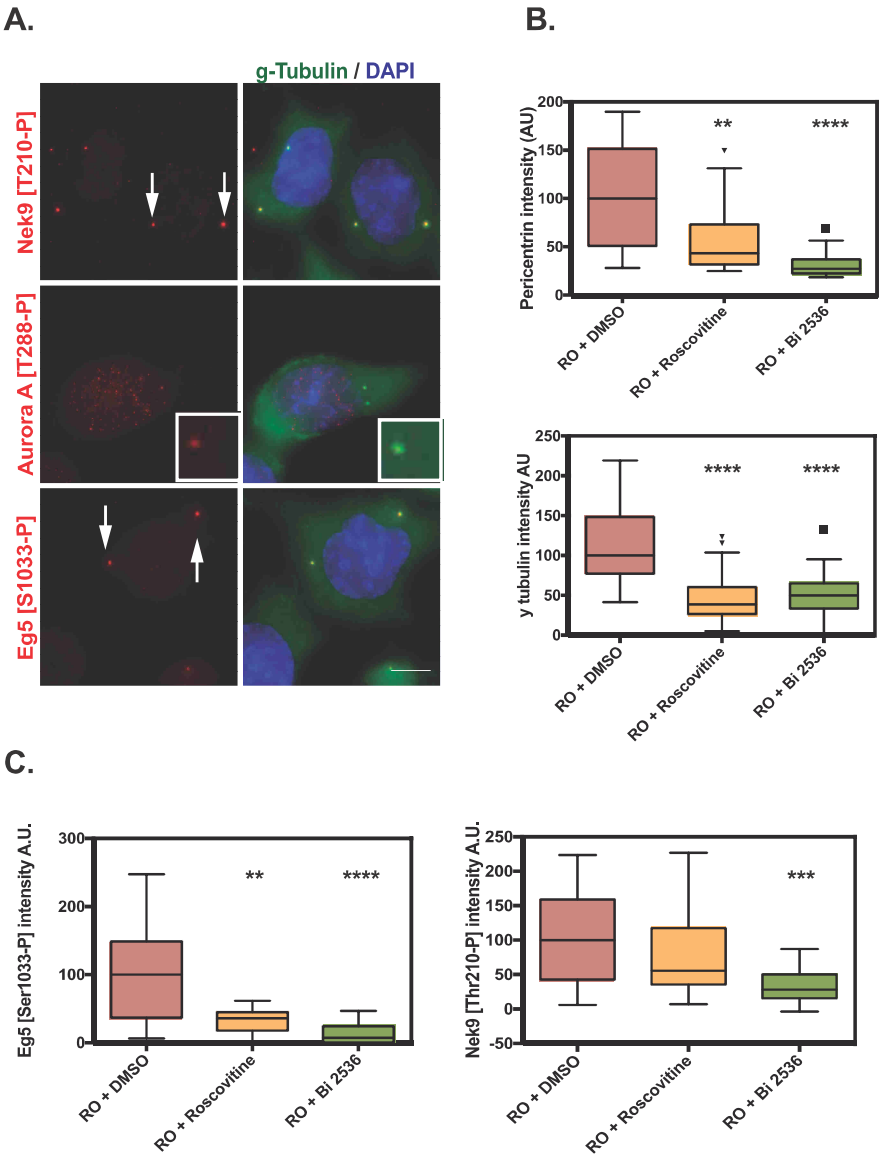

Figure 31. CDK and Plk1 activity in RO treated cells. (A) Phosphorylated Nek9, Aurora A and Eg5 localizes at centrosomes in RO treated cells. (B) Pericentrin and γ-tubulin intensity decreases after CDK and Plk1 inhibition. (C) Ser1033-P Eg5 and activated Nek9 intensity at centrosomes decreases after CDK and Plk1 inhibition. For G2 synchronization cells were treated with RO-3306 9 μM (Enzo) during 16-20 hours followed by the addition of DMSO; Roscovitine 50 μM (Calbiochem) or BI-2536 100 nM (Axon Medchem) during 2 hours. N=15-20 cells. Statistics were done with Mann-Whitney t-test.

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SUPPLEMENTARY TABLES

Vector	Insert	5' Restriction site	3' Restriction site	Primer Forward	Primer Reverse
CMV5-FLAG	Eg5 [764-1057]	EcoRI	BamHI	GAATTCAAACAAAATGACTTTTC	GGATCCTTAAAGGTTGATCTGGGCTCG
CMV5-FLAG	TPX2	BamHI	SalI	GGATCCATGTCACAAGTTAAAAG	GTCGACTTAGCAGTGGAATCGAG
pEGFP C2	BICD2 [487-820]	EcoRI	BamHI	CCCGAATTCTCTCTGCTGGAG	CTTGGATCCCCGCGGCCCTCT
pGEX T1.4	BICD2 [1-350]	BamHI	EcoRI	GGATCCATGTCCGCGCCGTC	GAATTCCAGCTTCTGGATCTCAG
pGEX T1.4	BICD2 [350-820]	BamHI	EcoRI	GGATCCAAACAGCAGCTGGTG	GAATTCCTACAGGCTCGGTGAGG
pEBG	BICD2 [487-820]	BamHI	Kpn	GGATCCTCTCTGCTGGAGAAGGCTAGC	GGTACCCTACAGGCTCGGTGAGGCTGGC
pCDNA5/FRT/TO	GFP MCS (pEGFPC3)	AflII	EcoRV	CTTAAGCCATGGTGAGCAAGGGCG	GATATCTCAGTTATCTAGATCCGGTG
pCDNA5/FRT/TO	GFP TPX2	HindIII	BamHI	-	GGATCCTTAGCAGTGGAATCGAGTGG
pGBKT7	TPX2	SmaI	BamHI	CCCGGGAGTAGAAGCCCAGAAAGCC	GGATCCTTAGCAGTGGAATCGAGTGG
pGBKT7	TPX2 [681-747]	SmaI	SalI	CCCGGGAGTAGAAGCCCAGAAAGCC	GTCGACTTAGCAGTGGAATCGAG
pGBKT7	BICD2	NdeI	BamHI	CATATGATGTCCGCGCCGTCGG	CTTGGATCCCCGCGGCCCTCT
pGBKT7	BICD2 [1-575]	NdeI	BamHI	CATATGATGTCCGCGCCGTCGG	GGATCCCCCGCGGCCCTCTG
pGBKT7	BICD2 [487-820]	NdeI	BamHI	CATATGTCTCTGCTGGAGAAGGC	CTTGGATCCCCGCGGCCCTCT

Table S1. Sequences of primers for cloning

pCS2 FLAG hEg5

Mutation	Primer 5'-3'
S1033A	AGAGGCATTAACACACTGGAGAGGGCTAAAGTGGAAG
S1033D	AGAGGCATTAACACACTGGAGAGGGATAAAGTGGAAG
S1033E	AGAGGCATTAACACACTGGAGAGGGAAAAAGTGGAAG
S1033DD	CACACTGGAGAGGGATGACAAAGTGGAAG
T926A	
T926E	GGATATCCCAACAGGTACGGAACCACAGAGGAAAAG

pGBKT7 hEg5

Mutation	Primer 5'-3'
[968-1057] (deletion)	GAGGACCTGCATATGAAAGAAGAGACAATTCCG

pEGFP C3 hTPX2

Mutation	Primer 5'-3'
S310A	GCCTTTCAACCTGGCCCAAGGAAAGAAAAGAACATTTG
S310D	GCCTTTCAACCTGGACCAAGGAAAGAAAAGAACATTTG
S310E	GCCTTTCAACCTGGAACAAGGAAAGAAAAGAACATTTG
T316A	CAACCTGTCCCAAGGAAAGAAAAGAACATTTGATGAAACAG
T316D	CAACCTGTCCCAAGGAAAGAAAAGAACATTTGATGAAACAG
T320A	GATGAAGCAGTTTCTACATATGTGCCCCTTG
T320E	GATGAAGAAGTTTCTACATATGTGCCCCTTG
S310A T320A S322A T323A	CATTTGATGAAGCAGTTGCTGCATATGTGCC
S310D T320E S322D T323E	CATTTGATGAAACAGTTTCTACATATGTGCCCCTTGCACAGCAAG
T338A	GAAGACTTCCATAAACGAGCCCCTAACAGATATC
T338E	GAAGACTTCCATAAACGAGAGCCTAACAGATATC
S356A	GATATTAACCTGTTACCCGCCAAATCTTCTGTGACCAAG
S356D	GATATTAACCTGTTACCCGACAAATCTTCTGTGACCAAG
S654A	GGGCCTTTCTGGTTCGCTAGTTCAGGAACCTTTTCAGC
S654D	GGGCCTTTCTGGTTCGATAGTTCAGGAACCTTTTCAGC
L704A L707A	GCAGAAAAAAGAGGAGCTGGCCAGGCTACGGAGAGAACTGGTGC
S738A	GCCTCTGACTGTGCCTGTATCTCCCAAATTCTCCACTCG
S738D	GCCTCTGACTGTGCCTGTATCTCCCAAATTCTCCACTCG
NLS 313-315 KKR-MMQ	GCCTTTCAACCTGTCCCAAGGAATGATGCAAACATTTGATGAAACAGTTTC
NLS 157-158 KK-MM	CTTCTTTTTGTTGTTAGAAACTTTCATCATCATAGAGGGTAGAATTTCATCGATGATTACAG
S209D T210E	CTCCAGCTCTTGCTCCTCCTCATCTTTTAGAAACTTCTGCTTTGCAGGTGGCATAC
T157E	TTGTTGTTAGAAACTTTCATTTTCTTATCGGGTAGAATTTCATCGATGATTACAGG
[1-710] (STOP CODON)	GGCCAGGCTACGGAGAGAATAGGTGCATAAGGCAAATCC
siRNA Resistant	CTGGGGAAGAACGGGACCGGGGGATTATTTCAGGG
xTPX2 S281A	CCGAAACCCTTCAACCTGGCCAAGGGCAAACGTAAGC
xTPX2 S281D	CCGAAACCCTTCAACCTGGACAAGGGCAAACGTAAGC

pEGFP C2 mBICD2

Mutation	Primer 5'-3'
S286A	GGCCTCAAGTTCGCTGATGATACTGTCACCGC
S286D	GGCCTCAAGTTCGATGATGATACTGTCACCGC
S345A	CTGCTCAGTGAGCTCCACATATCTGAGATCCAGAAGC
S345D	CTGCTCAGTGAGCTCCACATATCTGAGATCCAGAAGC
L501A-L504A	GACCGAGAGCTGCTGGCCCATCTGGAAAAGGAGCTG
[1-575] STOP CODON	GAGGGCCGCGGGTGACGGTCACCTGTC
[487-820] (deletion)	GATCTCGAGAGAATTCGCCACCTCTCTGCTGGAGAAGGCTAGCC
[575-820] (deletion)	GATCTCGAGAGAATTCGCCACCCGCCGGTCACCTGTCCTCTTG

Table S2. Sequences of primers for mutagenesis

1.

Library titer (cfu/ml)	8.1x10^6
Bait concentration (cfu/ml)	9.9x10^7
Mating efficiency	27%
Diploid concentration	2.2X10^6

2.

Library titer (cfu/ml)	5.6x10^7
Bait concentration (cfu/ml)	7.8x10^7
Mating efficiency	48%
Diploid concentration	2.6x10^7

Table S3. Y2H library titer for screening number 1 (1) and 2 (2).

Number of colonies	Protein name	NCBI number acces	Order of appereance	Nucleotide Range	Residues range	Frame	Function
6	CMRF35-like molecule 8	NM_007261.3	2, 5, 19, 21, 22, 27	757 to 1514	64 to 176	Yes	Cytolytic activity in natural killer cells
5	Integrin alpha-M isoform 2 precursor	NP_000623.2	11, 17, 25, 33, 61*	2206 to 2605	771 to 902	Yes	Uptake of complement-coated particles
4	A kinase (PRKA) interacting protein 1 (AKIP1)	NR_045418.1	39, 55, 43, 17*	21 to 382, 385 to 1225	1 to 156, 104 to 183	Yes	NF-kappa-B activation cascade
4	Sorting nexin-3	NG_023278.1	10, 16, 31, 46	297 to 503	1 to 55	Yes	Protein trafficking
4	Fused in sarcoma (FUS) transcription factor	NM_001170634.1	31*, 37*, 51*, 52*	744 to 1841	403 to 525	ND	RNA-binding protein
3	Migration and invasion enhancer 1 (MIEN1)	XM_005257736.2	4, 32, 60, 33*, 55*	1 to 839	1 to 97	Yes	Cell migration. Apoptosis.
3	Serine/arginine-rich splicing factor 2 (SRSF2)	NM_001195427.1	18, 3*, 56*, 47*	243 to 653, 649 to 1718	79 to 160	Yes	Splicing factor
2	M-phase phosphoprotein 8	BC003542.1	9, 20	2558 to 3335	428 to 627	Yes	Methylated histone binding
2	ZNF237 protein	AJ133355.1	29, 34	144 to 983	1 to 213	Yes	Transcription factor
2	Heterogeneous nuclear ribonucleoprotein A/B (HNRNPAB)	NM_031266.2	22*, 43*	915 to 1796	267 to 285	ND	RNA binding protein
1	Immunoglobulin kappa light chain	Y14736.1	56	68 to 938	1 to 214	yes	Antigen binding
1	Erythrocyte membrane protein band 4.2 (EPB42)	NM_001114134.1	12	1295 to 2068	422 to 676	Yes	Cell morphogenesis
1	WD repeat domain 48 (WDR48)	NM_020839.3	24	1535 to 2434	416 to 595	Yes	Regulator of deubiquitinating complexes
1	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) (HSPA5)	NM_005347.4	28	1548 to 2573	235 to 459	Yes	Chaperone
1	Cytochrome b5 reductase 4 (CYB5R4)	NM_016230.3	30	14 to 873	85 to 204	ND	Endoplasmic reticulum stress response
1	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 (ATP2A2)	NG_007097.2	36	366 to 469	49 to 84	Yes	Calcium-transporting ATPase
1	Nuclear receptor coactivator 4 (NCOA4)	XM_005277658.1	38	1899 to 3309	542 to 576	Yes	Androgen receptor binding
1	Zinc finger protein 7 (ZNF7)	NM_001282795.1	47	144 to 983	58 to 500	Yes	Transcription factor
1	Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB)	NM_001175.4	21*	379 to 1201	141 to 201	ND	Rho GDP-dissociation inhibitor
1	Bisphosphoglycerate mutase (BPGM)	NM_001724.4	25*	705 to 1800	213-261	ND	Carbohydrate metabolic process

Number of colonies	Protein name	NCBI number acces	Order of appereance	Nucleotide Range	Residues range	Frame	Function
1	Pyridine nucleotide-disulphide oxidoreductase domain 1 (PYROXD1)	NM_024854.3	39*	357 to 1399	173-421	ND	Oxidoreductase
1	Ribosomal L24 domain containing 1 (RSL24D1)	NM_016304.2	41*	509 to 1487	139-163	ND	Structural constituent of ribosome
1	Charcot-Leyden crystal protein (CLC)	NM_001828.5	53*	179 to 649	35 to 142	ND	Recognition of cell-surface glycans
1	Copper metabolism domain containing 1 (COMMD1)	NM_152516.2	16*	1 to 701	1 to 190	ND	Scaffold protein
1	NEDD4 binding protein 2-like 2 (N4BP2L2)	NM_033111.4	48*	516 to 1567	172-483	ND	Transcription factor
1	Metallothionein 2A (MT2A)	NM_005953.3	40*	22 to 446	1 to 62	ND	Methal binding
1	Niemann-Pick disease, type C2 (NPC2)	NM_006432.3	18*	197 to 921	30 to 151	ND	-
2	Filamin A, alpha (FLNA)	NM_001456.3	1, 24*	5355 to 6656	1705 to 2108	No	Actin filament binding
2	Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	BC111051.1	8, 35	924 to 1745	77 to 221	No	Translation factor
1	Golgin A4 (GOLGA4)	NM_001172713.1	41	68 to 823	42 to 260	No	Vesicle-mediated transport
1	Small nuclear ribonucleoprotein polypeptide G	NM_003096.2	6	46 to 483	1 to 76	No	Splicing factor
1	NLR family, apoptosis inhibitory protein (NAIP)	NM_004536.2	51	1741 to 1823	341 to 368	No	Anti-apoptotic protein
1	Immunoglobulin lambda like polypeptide 5 (IGLL5)	NM_001178126.1	57	578 to 1074	32 to 233	No	Antigen binding
1	Immunoglobulin heavy constant alpha 1,	BC092449.1	50*	783 to 1584	111 to 271	No	Antigen binding
1	Homo sapiens musculin (MSC)	NM_005098.3	2*	874 to 2027	5' UTR	-	Transcription factor
1	Integral membrane protein 2C (ITM2C)	NM_030926.4	12*	1112 to 2114	3'UTR	-	Apoptosis. Neural differenciation.
1	High mobility group AT-hook 2 (HMGA2)	NM_003483.4	38*	2150 to 2991	3'UTR	-	Transcription factor
1	Solute carrier family 4, sodium bicarbonate cotransporter, member 7 (SLC4A7)	XM_005265603.1	7	3671 to 3956	3'UTR	-	Anion transmembrane transport
1	Tensin 1 (TNS1)	NM_022648.4	3	8277 to 9413	3' UTR	-	Cell migration
1	Serpin peptidase inhibitor, clade B (ovalbumin) (SERPINB1) (PREDICTED)	NR_073112.1	29*	-	-	-	Protease inhibitor
2	Hepatitis B virus receptor binding protein (PREDICTED)	AY570731.1	11*, 40	320 to 1226	-	-	Immunity

Number of colonies	Protein name	NCBI number acces	Order of appereance	Nucleotide Range	Residues range	Frame	Function
2	Cytochrome P450, family 4, subfamily F, polypeptide 3 (CYP4F3) (PREDICTED)	AY792513.1	48,49	22371 to 23759	-	-	Oxidoreductase
3	Chromosome 9 open reading frame 3 (C9orf3)	NG_027833.1	1*, 15*, 49*	338861 to 340054	-	-	-
1	Chromosome 1, alternate assembly CHM1_1.1	NC_018912.2	57*	22524095 to 22524856	-	-	-
1	Chromosome 14 DNA sequence BAC R-661D19 of library RPCI-11	AL132801.5	45	156103 to 157323	-	-	-
1	Chromosome 12, alternate assembly CHM1_1.1	NC_018923.2	7*	57464453 to 57465909	-	-	-
1	RP3-391O22 on chromosome 6p21.2-21.31	AL031577.1	8*	50975 to 52053	-	-	-
1	LL0XNC01-136G2 on chromosome X	BX000483.7	4*	9360 to 9567	-	-	-
1	RP5-998C11 on chromosome 20q13.13-13.2	AL035106.28	10*	108104 to 108956	-	-	-
1	RP3-377F16 on chromosome 22	Z93783.6	23*	71021 to 71735	-	-	-
1	BAC RP11-414c23	AC006157.2	26*	107071 to 108226	-	-	-
1	Chromosome 17, alternate assembly CHM1_1.1	NC_018928.2	27*	-	-	-	-
1	Chromosome 16 clone CTD-3088G3	AC099489.2	35*	128896 to 129542	-	-	-
1	BAC clone RP11-313P13 from 7	AC005488.2	42*	55464 to 56340	-	-	-
1	Chromosome 8, clone RP11-281N10	AC104012.11	59*	121950 to 122564	-	-	-
1	BAC clone RP11-420C9 from 2	AC007318.4	60*	171400 to 172337	-	-	-
1	12 BAC RP11-756H6	AC126614.8	20*	7396 to 7764	-	-	-

Table S3 (B). Positive colonies in both Y2H screenings.
Sorted by number of appearance and frame regarding the HA tag.

p-site	Ratio phospho/No phospho			*In vivo*
	3_Prot_phospho band1	4_Prot_phospho band2	SUM	
51	0.500	0.000	0.500	Yes
80	0.500	0.167	0.667	No
257	1.000	0.500	1.500	Yes
284	1.000	0.333	1.333	No
285	0.000	0.333	0.333	Yes
310	0.000	1.000	1.000	Yes
320	3.000	1.000	4.000	Yes
374	0.750	0.500	1.250	Yes
482	0.333	0.000	0.333	Yes
576	2.000	1.333	3.333	No
631	0.667	0.167	0.833	No
654	1.000	0.500	1.500	Yes
728	0.250	0.000	0.250	Yes
734	0.250	0.000	0.250	Yes

Table S4. TPX2 phosphorylation sites after incubation with Nek9 *in vitro.*

Site	[FLV]XX[ST]	Phosphorylated in MS analysis	Coverage	SILAC	In vivo	Mitosis	Domain	Function
T51	No	Yes (only the first)	Yes		Yes	Yes	-	-
T59	No	No	Yes		Yes	Yes	-	-
T72	No	No	Yes	Yes	Yes	Yes	-	Regulates spindle association of TPX2
S110	No	No	Yes		Yes	-	-	-
T113	No	No	Yes		Yes	-	-	-
S121	No	No	Yes		Yes	Yes	-	Induces interaction with CLASP1
S125	Yes	Yes(c) (99.6)	Yes		Yes	Yes	-	Induces interaction with CLASP2
T147	No	No	Yes		Yes	Yes		-
S157	Yes	Yes (100)	Yes		Yes	Yes	NLS	-
S163	No	No	No		Yes	Yes	-	-
S174	No	No	Yes		Yes	Yes	-	-
T179	No	No	Yes		Yes	Yes	-	-
S185	No	No	Yes		Yes	Yes	-	-
S186	No	No	Yes		Yes	Yes	-	-
S209	Yes	No (33.03)	Yes		Yes	Yes	-	-
S218	Yes	Yes (c) (100)	Yes		Yes	Yes	-	-
S252	No	No	No		Yes	-	-	-
S257	No	Yes* (100)	Yes		Yes	Yes	-	-
T264	No	No	Yes		Yes	-	-	-
S285	No	Yes* (99.08)	Yes		Yes	-	-	-
S292	No	No	No		Yes	Yes	-	-
S293	No	No	No		Yes	Yes	-	-
S310	Yes	Yes* (100)	Yes		Yes	Yes	NLS	-
T316	No	No (89.6)	Yes		Yes	-	NLS	-
T338	No	No	Yes		Yes	Yes	Spindle assembly	-
S346	No	Yes (c) (100)	Yes		Yes	-	Spindle assembly	-
S356	Yes	Yes (100)	Yes		Yes	Yes	Spindle assembly	-
S359	No	No (83.6)	Yes		Yes	Yes	Spindle assembly	-
T369	No	No	Yes	Yes	Yes	Yes	Spindle assembly	-
T374	No	Yes* (100)	Yes	Yes	Yes	-	Spindle assembly	-
T382	No	No	Yes		Yes	-	Spindle assembly	-
T386	No	No	Yes		Yes	-	Spindle assembly	-
T482	Yes	Yes (only the first)	Yes		Yes	-	Spindle assembly	-
S486	No	No	Yes	Yes	Yes	Yes	Spindle assembly	-
T499	No	No	Yes		Yes	Yes	Spindle assembly	-
S539	No	Yes (c) (99.9)	Yes		Yes	Yes	Spindle assembly	-
S542	No	No	Yes		Yes	Yes	Spindle assembly	-
S634	No	No	Yes		Yes	-	Spindle assembly	-
S646	No	No (95.4)	Yes	Yes	Yes	Yes	Spindle assembly	-
S652	No	Yes (100)	Yes		Yes	Yes	Spindle assembly	-
S654	Yes	Yes* (100)	Yes		Yes	Yes	Spindle assembly	-
S728	No	Yes* (84.4)	Yes		Yes	-	Eg5 interaction domain	-
T734	No	Yes* (100)	Yes		Yes	-	Eg5 interaction domain	-
S738	No	No	Yes	Yes	Yes	Yes	Eg5 interaction domain	-

*** appear in both analysis**

(c) not coveraged the first time

Site	Aurora A Dependent	Plk1 Dependent	Reference	Comments
T51	ND	Yes	1,2,3	
T59	No	No	2,4	
T72	No	?	1,2,3,4,5,6,7,8,9	Phosphorylated by CDK1/2
S110	-	-	-	
T113	-	Yes?	3	
S121	Yes	-	1,3,4,6,10,11,12	
S125	Yes	-	1,3,4,6,10,11,13	
T147	Yes	No	1,2,3,13	
S157	ND	ND	2,8	
S163	No	-	3,14	
S174	No	?	3	
T179	No	-	3	
S185	-	-	12,15	
S186	-	-	1,2	
S209	Yes	Yes	2,3	
S218	Yes	Yes	2	
S252	No	No	-	
S257	No	No	2,3,4,14,	
T264	-	-	-	
S285	-	-	-	
S292	-	-	3,6,15	
S293			1,3,12,15,16	
S310	Yes	Yes	2,3,4,15	
T316	-	-	-	
T338	-	-	1,2,3,4	
S346	-	-	2, 3	
S356	No	?	-	
S359	No	?	1, 17	
T369	No	Yes	1, 2, 3, 7, 8, 11, 13, 17	Detected in many cancer cell lines
T374			1	
T382			-	
T386			3, 7	
T482			2	
S486	Yes	Yes	1, 2, 3, 4, 6, 7, 12, 15, 17	Detected in many cancer cell lines
T499	Yes	-	2, 3, 7, 17	Detected in many cancer cell lines
S539	Yes	Yes	2, 3	
S542	No	No	1, 2, 3	
S634	-	-	2	
S646	-	?	2, 6	
S652	No	Yes	1, 2, 3	
S654	No	Yes	1, 2, 3	
S728			2	
T734			-	
S738	-	-	1, 2, 3, 4, 7, 8, 11, 12, 13, 14, 15, 16, 17	Possible site of CDK1\ Detected in many cancer cell lines

1 Nousiainen M, et al. (2006)
2 Kettenbach AN, et al. (2011)
3 Santamaria A, et al. (2011)
4 Dephoure N, et al. (2008)
5 Shim SY, et al. (2015)
6 Olsen JV, et al. (2010)
7 Malik R, et al. (2009
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9 Chi Y, et al. (2008)
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12 Daub H, et al. (2008)
13 Grosstessner-Hain K, et al. (2011)
14 Franz-Wachtel M, et al. (2012)
15 Dulla K, et al. (2010)
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Table S5. Classification of all TPX2 phosphorylation sites *in vivo.*