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The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation

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Summary

Nek6 and Nercc1 (also known as Nek9) belong to the NIMA family of protein kinases. Nercc1 is activated in mitosis, whereupon it binds, phosphorylates and activates Nek6. Interference with Nek6 or Nercc1 in mammalian cells causes prometaphase-metaphase arrest, and depletion of Nercc1 from *Xenopus* egg extracts prevents normal spindle assembly. Herein we show that Nek6 is constitutively associated with Eg5 (also known as Kinesin-5 and Kif11), a kinesin that is necessary for spindle bipolarity. Nek6 phosphorylated Eg5 at several sites in vitro and one of these sites, Ser1033, is phosphorylated in vivo during mitosis. Whereas CDK1 phosphorylates nearly all Eg5 at Thr926 during mitosis, Nek6 phosphorylates ~3% of Eg5, primarily at the spindle poles. Eg5 depletion caused mitotic arrest, resulting in cells with a monopolar spindle. This arrest

could be rescued by wild-type Eg5 but not by Eg5[Thr926Ala]. Despite substantial overexpression, Eg5[Ser1033Ala] rescued 50% of cells compared with wild-type Eg5, whereas an Eg5[Ser1033Asp] mutant was nearly as effective as wild type. Thus, during mitosis Nek6 phosphorylates a subset of Eg5 polypeptides at a conserved site, the phosphorylation of which is crucial for the mitotic function of Eg5.

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Key words: NIMA, Nek, Nercc1, Nek9, Nek6, Kinesin, Eg5, Kinesin-5, Spindle, Mitosis

Introduction

The NIMA family of protein kinases are named after NIMA (never in mitosis A), a kinase in *Aspergillus nidulans* that participates in a broad array of mitotic processes (O'Connell et al., 2003). The human genome encodes 11 protein kinases, the catalytic domain of which is evolutionarily related to that of NIMA (O'Connell et al., 2003; Roig and Avruch, 2006). Available evidence indicates that the NIMA-family kinases regulate aspects of microtubule function, including those related to cilia and the mitotic spindle (Quarmby and Mahjoub, 2005). Thus, Nek2 has a central role in centrosome maturation and disjunction, whereas Nek1 and Nek8 are proposed to contribute to ciliary function (Quarmby and Mahjoub, 2005). Nek6, Nek7 (Kandli et al., 2000) and Nercc1 (also known as Nek9) (Roig et al., 2002; Holland et al., 2002) are involved in the control of mitotic spindle formation. Except for Nek2 (Hayward and Fry, 2006), few substrates of NIMA-family kinases have been described.

Nercc1 is activated in mitosis and microinjection of anti-Nercc1 antibodies in prophase results in prometaphase arrest or in abnormalities in chromosome segregation (Roig et al., 2002). In *Xenopus* mitotic egg extracts, depletion of Nercc1 impairs both spindle assembly and Ran-GTP-induced aster formation (Roig et al., 2005). In mammalian cells, Nercc1 is able to bind, phosphorylate and activate Nek6 and Nek7, and Nek6 activity increases in mitosis concomitant with Nercc1 activation (Belham et al., 2003). Induced

overexpression of kinase-dead Nek6 in HeLa cells results in prometaphase-metaphase arrest (Yin et al., 2003). Similarly, interference with Nek7 (approximately 80% identical to Nek6) results in an increase in multinuclear cells, and in mitotic cells with a multipolar spindle (Yissachar et al., 2006). Thus, the Nercc1, Nek6 and Nek7 kinases represent a mitotic signaling cassette; the identification of the targets of this module will be necessary for understanding its function at a molecular level.

Eg5 (also known as Kinesin-5 and Kif11) is a plus-end-directed kinesin of the BimC family (Le Guellec et al., 1991) that forms bipolar tetramers that are capable of moving towards the plus ends of two microtubules simultaneously, thus bundling, sorting, and enabling anti-parallel microtubule movement through sliding (Sawin et al., 1992; Kapitein et al., 2005). Eg5 is necessary for premitotic centrosome separation, spindle-pole formation and separation, poleward translocation of microtubules, and postmitotic centrosome movement, and is thus one of the motors required for proper spindle organization and function (Walczak et al., 1998). Inhibition or depletion of Eg5 results in mitotic arrest with monopolar microtubule structures and condensed chromosomes surrounding the two unseparated centrosomes (Blangy et al., 1995). The regulation of Eg5 activity is less well defined than its several functions; nevertheless, one clear-cut mode of Eg5 regulation is through the control of its binding to microtubules, which requires

CDK1 (also known as CDC2) phosphorylation of a single residue (Thr926 in human Eg5) in the conserved C-terminal tail (Blangy et al., 1995; Sawin and Mitchison, 1995). The mechanism by which CDK1 phosphorylation of some BimC-family members induces microtubule binding is unknown; moreover, not all family members have a conserved CDK1 site motif, and *Schizosaccharomyces pombe* Cut7 does not require phosphorylation of the corresponding motif in order to associate with the mitotic spindle (Drummond and Hagan, 1998). Aurora-family kinases have been shown to phosphorylate Eg5 orthologs, modifying the kinesin in the stalk domain in *Xenopus laevis* (Giet et al., 1999) or in the C-terminal tail domain in *Caenorhabditis elegans* (Bishop et al., 2005). Although the function of stalk phosphorylation is not clear, in *C. elegans* the absence of the Aurora AIR-2 affects kinesin localization to the spindle (Bishop et al., 2005).

Herein we show that the kinase Nek6 binds to and phosphorylates Eg5 in vitro and in vivo during mitosis at a unique C-terminal site. This phosphorylation occurs on a minority of the spindle-associated Eg5 and does not alter spindle association, but is required for normal Eg5 function in enabling spindle bipolarity. These results uncover a new mode of Eg5 regulation and identify the first physiological substrate of the Nercc1-Nek6 signaling module.

Results

Nek6 binds to the tail of Eg5

Overexpressed Nek6 retrieves a single ~120-kDa polypeptide band that was identified, by LC/MS/MS of tryptic peptides, as containing the Nercc1 protein kinase (Roig et al., 2002); further analysis of these tryptic digests indicated the additional presence of the kinesin Eg5. Using extracts from both exponentially growing and mitotic cells we found that endogenous Eg5 specifically co-precipitates with endogenous Nek6 (Fig. 1A). In addition, small amounts of Eg5 co-precipitated with Nercc1, but only from extracts of mitotic cells (Fig. 1A). The Nercc1-Nek6 interaction also occurred predominantly in mitosis, concomitant with Nercc1 activation (Fig. 1B). In a yeast two-hybrid analysis, both Nek6 and Nercc1 exhibited the ability to bind directly to the Eg5 C-terminal tail (residues 762-1057) but not to the motor-head (1-361) or stalk (362-761) domains (Table 1; supplementary material Fig. S1). Inasmuch as Eg5 is associated with Nek6 in both interphase and mitosis, but is

associated with Nercc1 only in mitosis when the Nercc1-Nek6 interaction is maximal, it is likely that the Eg5-Nercc1 interaction in vivo occurs through the Nercc1-Nek6 interaction.

Nek6 phosphorylates Eg5 in vitro and in vivo during mitosis

Recombinant FLAG-Nek6 (Belham et al., 2001; Belham et al., 2003) readily phosphorylates GST-HsEg5 (human) or His₆-XIEg5 (*X. laevis*) (Fig. 2A) (but not GST alone, data not shown). Although Nercc1 phosphorylates myelin basic protein (MBP) and GST-Nek7, it is not able to detectably phosphorylate GST-HsEg5 in parallel (data not shown). Maltose-binding protein (MalBP) fusions of the Eg5 head (1-361), stalk (362-761) and tail (762-1057) were incubated with active FLAG-Nek6 or FLAG-Nercc1 plus [γ -³²P]ATP/Mg²⁺ (Fig. 2B). Whereas the Eg5 stalk is hardly phosphorylated, FLAG-Nek6 phosphorylates the Eg5 head and tail polypeptides, incorporating up to 0.25 and 0.90 moles ³²P (³²PO₄) per mole of protein substrate over a 2-hour reaction, respectively. No ³²P incorporation occurred with kinase-deficient Nek6[Lys74Met Lys75Met], and mutation of the described CDK1 phosphorylation site in the Eg5 tail (Eg5 762-1057[Thr926Ala]) did not affect the observed phosphorylation (data not shown). As with full-length Eg5, Nercc1, although capable of phosphorylating GST-Nek7 or MBP, is unable to phosphorylate the MalBP-Eg5 fusion polypeptides. The identity of the Nek6-catalyzed phosphorylation sites was determined by LC/MS/MS analysis of tryptic peptides derived from MalBP-Eg5[1-361] and MalBP-Eg5[762-1057] phosphorylated to approximately 0.1 and 0.3 moles PO₄/mole protein, respectively. In the head, a single phosphorylation at Ser269 was detected whereas, in the tail, phosphorylation was present at Ser776, Thr901 and Ser1033. Notably, each of these sites except Ser776 conforms to the Lxx[S/T] motif previously defined (Lizcano et al., 2002) as the preferred Nek6 phosphorylation site.

We next immunoprecipitated endogenous Eg5 from exponentially growing and nocodazole-arrested mitotic U2OS cells, and analyzed Eg5 tryptic peptides by LC/MS/MS; approximately 80% coverage of Eg5 sequence was obtained for both samples. Eg5 from exponentially growing cells exhibited only a single phosphorylation site at Thr926; the detection of this CDK1-catalyzed phosphorylation site probably reflects the presence of mitotic cells in the sample. Eg5 that was isolated from mitotic cells exhibited

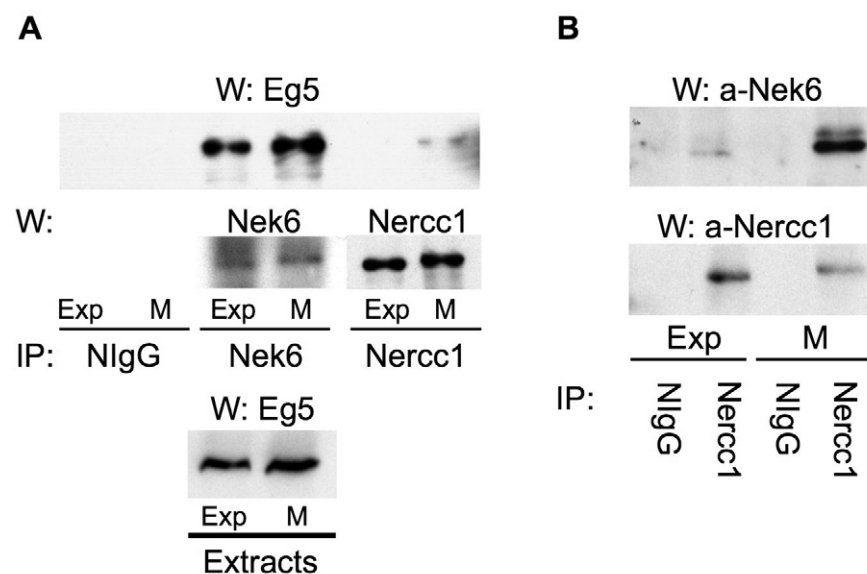


Fig. 1. Eg5 interacts with Nek6 and Nercc1.

(A) Immunoprecipitates (IP) were prepared from exponentially growing (Exp) or nocodazole-arrested mitotic (M) U2OS cell extracts using either normal rabbit IgG (NlgG), anti-Nek6 or anti-Nercc1 antibodies and analyzed by immunoblot (W) with the indicated antibodies. Eg5 in the corresponding extracts is shown in the lower panel.

(B) Immunoprecipitates were prepared from exponentially growing or nocodazole-arrested mitotic U2OS cells using NlgG or anti-Nercc1 antibody, and immunoblotted for Nek6 and Nercc1.

Table 1. Eg5 interacts with Nek6 and Nerccl

Gal4 BD	Eg5 (Gal4 AD)		
	1-361	362-761	762-1057
Nerccl	–	–	+
Nek6	–	–	+

The ability of the Eg5 head (Eg5 1-361), stalk (Eg5 362-761) and tail (Eg5 762-1057) domains to interact with Nek6 or Nerccl was assessed using the two-hybrid assay (see supplementary material Fig. S1). Gal4 AD/BD, Gal4 activation/binding domains.

two phosphorylation sites, at Thr926 and Ser1033; the latter corresponds to one of the sites of Eg5 that was phosphorylated by Nek6 in vitro. Inasmuch as Nek6, similar to CDK1, is specifically activated during mitosis (Belham et al., 2003), these results indicate that Eg5 is phosphorylated both by CDK1 and by Nek6 during mitosis.

A Nek6 motif corresponding to human Eg5[Ser1033] is conserved in the BimC family of kinesins

Alignment of the C-terminal amino acid sequences of several BimC kinesins (Fig. 2C) reveals that most, but not all, show a short conserved region at the tail domain surrounding the CDK1 site (in human Eg5, Thr926); this region is named the 'BimC box' (Heck et al., 1993; Sawin and Mitchison, 1995). Notably, all but two of the BimC kinesins (murine Eg5 and *Saccharomyces* KIP1) have a site containing the Lxx[S/T] motif that matches the location of human Eg5[Ser1033]. The nonessential *Saccharomyces cerevisiae* kinesin KIP1 lacks such a site entirely, as well as a BimC box/CDK1 site; however, the other *S. cerevisiae* BimC kinesin, Cin8, does contain the Lxx[S/T] site. Rodent Eg5 proteins exhibit a Lxx[S/T] site that is 11 (mouse) or 20 (rat) residues C-terminal to the location of human Eg5[Ser1033]. Interestingly, *Drosophila melanogaster* KLP61F and *S. pombe* Cut7 have two adjacent or overlapping Lxx[S/T] sites, whereas *C. elegans* BMK-1 has three, with two overlapping. Altogether, it is clear that a residue equivalent to human Eg5[Ser1033] is conserved in the BimC family, indicating that a Nek6 site motif in this region of Eg5 is likely to have an important physiological role, probably related to its phosphorylation by a protein kinase with site specificity that is similar to Nek6.

Phosphopeptide maps of mitotic Eg5 confirm Ser1033 as a relevant mitotic phosphorylation site

We performed two-dimensional tryptic ^{32}P -peptide maps of endogenous Eg5 from ^{32}P -labelled mitotic U2OS cells, to estimate the relative extent of phosphorylation at the two Eg5 sites detected and to determine whether other phosphorylation sites exist. Eg5 retrieved from ^{32}P -labelled cycling cells showed no detectable ^{32}P incorporation, whereas Eg5 retrieved from nocodazole-arrested U2OS cells exhibited substantial labeling (Fig. 2D). Two-dimensional tryptic ^{32}P -peptide maps (Fig. 2E, left; Fig. 2F, top left) of mitotic Eg5 showed five reproducible ^{32}P -peptides, four of which correspond to ^{32}P spots present in tryptic digests derived from Eg5 phosphorylated by CDK1 in vitro (Fig. 2E, right), whereas the most anodally migrating ^{32}P -peptide in the digest (Fig. 2E, black arrow) is absent in the maps of Eg5 phosphorylated by CDK1 in vitro.

^{32}P -Eg5 generated by phosphorylation with Nek6 in vitro yields a complex ^{32}P -peptide map (Fig. 2F, upper right), as anticipated from the multiple phosphorylation sites detected on LC/MS/MS of similar digests. Notably, the map of Eg5[Ser1033Ala]

phosphorylated by Nek6 in vitro is essentially identical (Fig. 2F, lower right) except for the absence of a single ^{32}P spot (indicated by the arrow, Fig. 2F). Mixing of the digests of mitotic Eg5 with Nek6-phosphorylated Eg5 confirmed that the most anodally migrating ^{32}P -peptide in both digests co-migrate (Fig. 2F, lower left). These results confirm that Eg5[Ser1033] is phosphorylated in vivo during mitosis and indicate that the relative incorporation of $^{32}\text{PO}_4$ into Ser1033 in nocodazole-arrested U2OS cells is approximately 3% of that incorporated into Thr926. Moreover, they strongly support the view that, in mitotic U2OS cells, phosphorylation of Eg5 at sites other than Thr926 and Ser1033, if present, contribute much less than 1% to overall Eg5 phosphorylation.

Nek6 overexpression and activation induces Eg5[Ser1033] phosphorylation in vivo

We generated phosphospecific antibodies directed against sequences surrounding human Eg5[Ser1033-P], as well as those surrounding the orthologous site on *X. laevis* Eg5 (Ser1046). The resulting antibodies were totally specific for the Nek6-phosphorylated forms of Eg5 (supplementary material Fig. S2), and were used to study the effects of Nek6 overexpression and its resulting activation (Belham et al., 2003) on the phosphorylation of Eg5[Ser1033]. Fig. 3A shows that coexpression of Nek6 with Eg5 is accompanied by a substantial increase in Eg5[Ser1033-P] immunoreactivity, which is not seen with kinase-dead Nek6 or when Nek6 is coexpressed with Eg5[Ser1033Ala]. Thus, active Nek6 can phosphorylate Ser1033 in vivo as well as in vitro {our attempts to study the effects of Nek6 downregulation on the endogenous levels of Eg5[Ser1033-P] were hampered by the incomplete depletion of the kinase obtained using different siRNAs and multiple experimental conditions; in these experiments we did not detect any changes on the levels of active Nek6 (Nek6[Ser206-P]) or Eg5[Ser1033-P], neither did we observe any effect on mitotic progression, data not shown}.

Determination of Eg5[Ser-P] levels during mitosis

Endogenous Eg5 was immunoprecipitated from freely cycling and nocodazole-arrested U2OS cells, as well as from cells harvested at intervals after nocodazole washout (Fig. 3B); no Ser1033-P immunoreactivity was detected in Eg5 from freely cycling U2OS cells, whereas a strong signal, which faded over the first 2 hours after nocodazole washout, was evident in mitotic (nocodazole arrested) cells. The pattern of Eg5[Ser1033-P] immunoreactivity parallels that of Nerccl phosphorylation at the activating site on the T-loop. Surprisingly, whereas Nek6 T-loop phosphorylation increases in parallel with Eg5[Ser1033-P] immunoreactivity during mitotic arrest, it exhibits a further increase at 2-4 hours after nocodazole washout; the mechanism of this late-mitotic and/or early-G1 phosphorylation of Nek6 is not known and its timing suggests a previously unappreciated function of Nek6 in mitotic exit or early G1. Nevertheless, these results further confirm that the mitotic phosphorylation of Eg5[Ser1033] coincides with Nerccl activation and is probably mediated by the Nerccl-Nek6 complex.

Subcellular localization of Nek6-phosphorylated Eg5

We sought next to define the subcellular localization of Nek6-phosphorylated Eg5. Our anti-HsEg5[Ser1033-P] antibodies recognize several bands of lower molecular weight on immunoblots (identity unknown), rendering them unsuitable for immunocytochemistry. We therefore employed the anti-

and along the length of the metaphase spindle, somewhat more prominently near the poles but extending to the spindle midzone without covering it; XIEg5 was not detectable on astral microtubules. As expected, anti-XIEg5[Ser1046-*P*] antibodies labeled only mitotic XL177 cells (Fig. 4), with a maximal signal detectable in metaphase (Fig. 3D; Fig. 4). Eg5[Ser1046-*P*]

A

B

C

BimC box

HsEg5/1-1056 880 FLDQM T I D E D K L I A Q N L E L N E T I K I G T K L N - C F L E Q D K L D I P T G T T P Q R K S V L Y P S T L V R T E P R E H L L D Q L K R K Q P E L L M L N C S E N N K 969
MmEg5/1-1064 891 F V A Q M T S D E E S C K A G S L E L D K T I K T G T K L N - C F L Q D K L D I P T G T T P E R K K V L Y P T T L V R T E P R E Q L L D Q L K Q K Q P - P M L N S E A S K 978
XlEg5/1-1067 887 L V E Q V Q D D K E M E G E Q R L E N E Q V Q S G E N K V H - V Y K E E L R N D V P T G T T P Q R K D V V P S L I K T K P R O V L L E Q F R Q Q Q E Y L E S I S V I S E A 980
KLP61/1-1066 887 A T D L V G S N R F S E H A E H Q R Q Q I C Q E V - R Q Q S E K T Y A P E T T S K N D F V P R T L V A T S R H Q I V R R Y Q E L D W S D L O T A T I D E C 976
BimC/1-1184 810 L K S R F S A T S E K R C V T G E L V S D C T K K A T D E L S - S L D E F T K S T I V V - T K E A A V V N S V V L V H V D S V N V P Q R I L P S F P L V S E L N Q P V P E V R 897
BimC/1-1184 959 F Q Q E A T H H H A T E E S I A P L S N D V R K P T D L S Q E Q N R S E E Y V A T G T T K R K Y D W I S V P S T E S H E V L K S R L R T T K E M E V L F N S D D Q L S 1049
KIP1/1-1211 932 L T D Y D E N I M Q I S K T Q D E V L S E H C E K Q S K I L G M D I F T A H S L E K P L H E I T P E A S V I K A L P L D Y P K Q F I Y R D A E N K S K D O T S N R T C I 1022
CUT7/1-1085 964 I T D H V L K G T T S A N H T N E L L G L G S C N M E T T I E D T S W K L I T E Q E S K E L P A T P S W T R D S L I K E T T N E N L D S K - - - - - 1042
Cin8p/1-1000 877 L K N N - T K F N D Q F E Q L I N K H M L K D N I K N S I T S H S H I T N V D D I Y N T I E N I M K N Y G N K E N A T K D E M I E I - - - - - 945

HsEg5/1-1056 970 E E T I P O V D - - - - - V E E A V L G Q Y T E E P L S Q E P S V D A G V D C S S I G C V P F F Q R K S H G K D K E N K G - I N T E R S E V E E T T E H L Y T K S R L P L A Q 1053
MmEg5/1-1064 979 - E T S Q M D - - - - - E E R A L E Q C T E L V S P E T T E H P S A D C S S R C L P F F Q R K P H C K D K E N R G - L N P V E Y V E E A S D S I S L Y T K S R L P L H T S 1061
XlEg5/1-1067 981 V E P P P Q E D S - - - - - L E D E P P V A N D S V I E K S C I D L S M T C Q E K G V P F F Q Q K A L R K E K E N R G N A T L E R S K I M D E V E S L P K S K L P L R M Q 1066
KLP61/1-1066 977 S E G E H D V S M H S V Q L S E T E T I M N S T P I E P V D G V T V R K G G T T R N S N S N A L K P P V A T G G K R S S L S R S T P S K I T S P R G S P A F V R H N K E N V A - 1066
BimC/1-1184 898 L L G F G E C N - - - - - - - - - - D D F D T D E L A T K R R V T S F V R R D S L L E T T N F P S P S I I K R R E Q A I E E E D F E N - - 958
BimC/1-1184 1050 G P S S P G C G S P - - - - - S K G P Y N D V E D E G T A P T V T N V N S N T G L R E V D A N V A R P V Y T G E K S T D Q D G S P V S D S A T 1124
KIP1/1-1211 1023 P N L S T N E N F P - - - - - L S Q F S P K T P V P P D Q L P K V L I P K S I N S A K S N R S K T L P N T E C T G E S O N N K R R F T E P I L K G E E T N D I L Q N 1106
CUT7/1-1085 1043 - - - - - - - - - - - - - - - - K F V R E T Y T S S N Q T E P D V D Y K P S N S R S T L S R S R S A Y S K M K R - - - - - 1085
Cin8p/1-1000 946 - 980

HsEg5/1-1056 1054 I N L - - - - -
MmEg5/1-1064 1062 I N L - - - - -
XlEg5/1-1067 1067 N - - - - -
KLP61/1-1066 - - - - -
BimC/1-1184 - - - - -
BimC/1-1184 1125 E A E G M N - G P S K R R R S N S V A D T K L P N K M L A R R M A G M M E G R E N V P P P G I S N G R R L G R P S P
KIP1/1-1211 1107 K L H Q - - - - -
CUT7/1-1085 - - - - -
Cin8p/1-1000 981 E N K S E N V D N E G S R M K L I E - - - - -

LXXS*

D

E

F

25. (F) Top left, two-dimensional phosphopeptide map of endogenous Eg5. Two-dimensional maps of recombinant GST-Eg5 wild type (wt; top right) and mutant (mut; bottom right) are shown. The absence of the most anodally migrating ^{32}P -peptide in the lower right map indicates the absence of the phosphorylation site. The top right map shows comparable amounts of the digests of mitotic ^{32}P -Eg5 and FLAG-Nek6. The bottom right map shows the digest of mitotic ^{32}P -Eg5, which contains Ser1033. In the digests of mitotic ^{32}P -Eg5 (E, left; F,

immunoreactivity is highly concentrated at spindle poles, as can be clearly seen by co-staining with γ -tubulin (Fig. 3D, second row). A minor amount of XIEg5[Ser1046-P] was detectable on spindle microtubules; however, no signal was observed at astral microtubules. Interestingly, whereas XIEg5[Ser1046-P] reactivity was evident almost exclusively at the spindle poles from prophase through to anaphase (Fig. 4), a clear signal was seen abutting the cytokinetic furrow at telophase. The highly localized concentration of XIEg5[Ser1046-P] at the spindle poles is consistent with the view that Ser1046 phosphorylation occurs on a minor subset of CDK1-phosphorylated Eg5; this in turn is in agreement with our phosphopeptide maps, which show that the extent of Eg5 phosphorylation by Nek6 is much less than that catalyzed by CDK1. Moreover, the Nercc1-Nek6 module is also activated at spindle poles (Roig et al., 2005), suggesting that Eg5 phosphorylation by Nek6 occurs at poles.

Eg5[Ser1033Ala] disrupts normal Eg5 function but not mitotic localization

Different Eg5 forms (wild type, Thr926Ala, Ser1033Ala, Ser1033Asp) were purified from baculovirus-infected insect cells and characterized in vitro. On gel filtration, the oligomeric state of the wild-type and mutant Eg5 polypeptides were indistinguishable. By adding equal amounts of these Eg5 variants to assembled microtubules, followed by pelleting, we assessed their ability to bind microtubules. If the amount of wild-type Eg5 in the pellet is set at 100%, then Eg5[Thr926Ala] is recovered at approximately 28%, the Eg5[Ser1033Ala] mutants at approximately 63% and Eg5[Ser1033Asp] at 42% (supplementary material Fig. S3). Despite these differences in microtubule binding, each Eg5[Ser1033] variant exhibited rapid movement on microtubules, performing indistinguishably by visual inspection on microtubule gliding assays. The ability of these variants to bind to the mitotic spindle

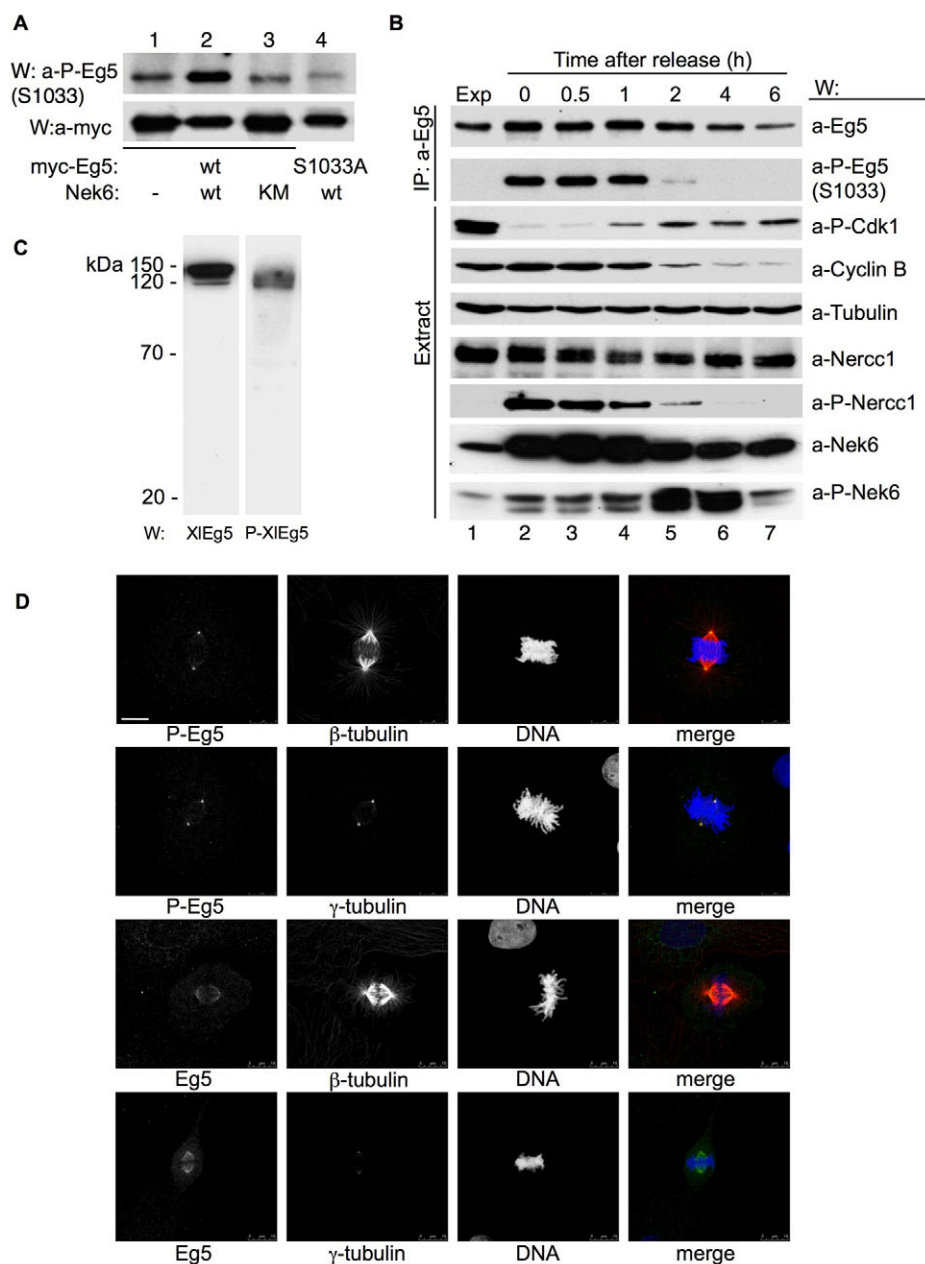


Fig. 3. Eg5[Ser1033] phosphorylation in vivo. (A) U2OS cells were co-transfected with either myc-Eg5 wild type (lanes 1-3) or myc-Eg5[Ser1033Ala] (lane 4) and empty plasmid (lane 1), FLAG-Nek6 wild type (lanes 2 and 4) or FLAG-Nek6 [Lys74Met Lys75Met] (lane 3). 24 hours later, anti-Myc immunoprecipitates were subjected to immunoblot with anti-Myc (lower panel) and anti-Eg5[Ser1033-P] (top panel) antibodies. (B) U2OS cells growing exponentially were either untreated (first lane, Exp) or arrested with nocodazole (0.25 mM overnight; lane 2); arrested cells were allowed to exit mitosis in nocodazole-free medium and extracted at the times indicated (lanes 3-7). Eg5 immunoprecipitates (top two rows) and cell extracts (bottom seven rows) were analyzed by immunoblotting with the indicated antibodies. a-P-Eg5(S1033), anti-Eg5[Ser1033-P]; a-P-CDK1, anti-CDK1[Tyr15-P]; a-P-Nercc1, anti-Nercc1[Thr210-P]; a-P-Nek6, anti-Nek6[Ser206-P]. (C) Extracts from XL177 *X. laevis* cells were resolved by SDS-PAGE and immunoblotted with either anti-XIEg5 (left) or anti-XIEg5[Ser1046-P] (right). (D) XL177 cells growing exponentially were fixed and stained with antibodies to XIEg5 (lower two rows), XIEg5[Ser1046-P] (upper two rows) and either β - or γ -tubulin as indicated. DNA is stained with DAPI. Representative cells in metaphase are shown. Scale bar: 10 μ m.

in vivo was evaluated during transient expression (Fig. 5A). Myc-Eg5 wild type binds along the length of the mitotic spindle (Fig. 5A, left), whereas mitotic cells expressing Myc-Eg5[Thr926Ala] invariably showed a diffuse localization, usually being associated with a monopolar spindle (Fig. 5A, middle). The Myc-Eg5[Ser1033Ala] mutant showed a diffuse cytoplasmic localization in interphase and a strong association with a normal-appearing spindle (Fig. 5A, right), similar to Eg5 wild type. Thus, at these levels of expression, Myc-Eg5[Ser1033Ala] binds normally to the spindle and does not appear to alter mitotic progression.

Nevertheless, a higher level of expression of Myc-Eg5[Ser1033Ala] did interfere with normal spindle assembly, in comparison with a similar amount of Myc-Eg5 wild type (not shown). To evaluate the functional efficacy of this mutant in a systematic and quantitative manner, we used siRNAs that were capable of depleting endogenous Eg5 (Weil et al., 2002) and concomitantly introduced the Myc-bound Eg5-variant cDNAs, which were rendered resistant to the siRNA by several silent point mutations, achieving comparable levels of expression of each Eg5 polypeptide (Fig. 5B). GFP was used as a negative control.

Transfected cells were identified by Myc positivity. Fig. 5C shows that, as expected, *Eg5* siRNA that was co-transfected with GFP resulted in approximately 80% of transfected cells exhibiting a mitotic arrest with condensed chromosomes arranged around a monopolar spindle focused on two non-separated centrosomes (black columns). Cells expressing Myc-Eg5 wild type were predominantly in interphase (Fig. 5C, gray columns) and the fraction of transfected cells exhibiting a monopolar spindle was greatly reduced as compared with the GFP-transfected cells; by contrast, Myc-Eg5[Thr926Ala], despite an expression level that was comparable to wild-type Myc-Eg5, did not ameliorate (and may increase further) the siRNA-induced mitotic arrest. Notably, Myc-Eg5[Ser1033Ala] was substantially less effective in rescue than Myc-Eg5 wild type. Whereas Myc-Eg5 wild type reduced the fraction of cells with monopolar spindles from ~80% to ~10%, Myc-Eg5[Ser1033Ala] reduced this proportion to ~40%, which is significantly less than that achieved by Eg5 wild type. Note that both Myc-Eg5[Ser1033Ala] and Myc-Eg5 wild type were considerably overexpressed as compared with endogenous Eg5, so it is possible that overexpression of the mutant enables some rescue

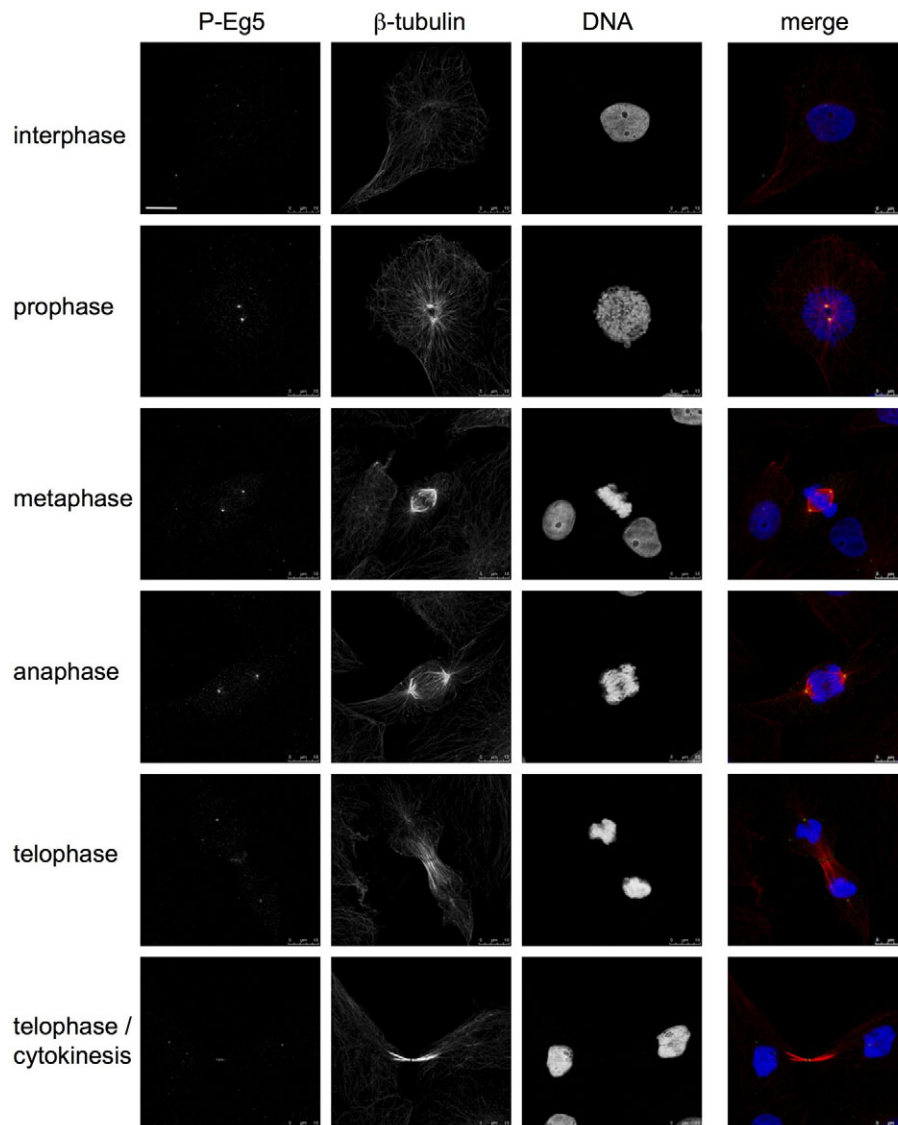


Fig. 4. Phosphoserine 1046 immunolocalization in XL177 cells in interphase and different phases of mitosis. As in Fig. 3D. Scale bar: 10 μ m.

that would not be evident at endogenous levels of expression; consequently, these data probably represent an overestimate of the efficacy of Eg5[Ser1033Ala]. In comparison to Myc-Eg5[Ser1033Ala], the Myc-Eg5[Ser1033Asp] mutant was more efficacious, reducing the fraction of cells with monopolar spindles to ~20%, significantly better than Myc-Eg5[Ser1033Ala] and quite close to Myc-Eg5 wild type.

Thus, although the Ser1033Ala mutation does not interfere with the ability of Eg5 to bind to the mitotic spindle, it does impair one or more of the several functions of Eg5 that are necessary for normal mitotic progression.

Discussion

The results presented herein demonstrate that, during mitosis, the human kinesin Eg5 is phosphorylated at both the CDK1 site Thr926 (Blangy et al., 1995) and at the newly identified site Ser1033. Whereas Thr926 phosphorylation is necessary for spindle association of Eg5, elimination of Ser1033 does not detectably affect Eg5-spindle binding; nevertheless, Eg5[Ser1033Ala] is clearly impaired for one or more functions that are required for normal spindle assembly, which can be better supplied by an Eg5[Ser1033Asp] mutant. Thus, Eg5 physiological activity is not only regulated through the control of its microtubule interaction by Thr926 phosphorylation, but also through the regulation of other yet-to-be-defined functional parameters by Ser1033 phosphorylation. Comparison of peak intensities in our MS analysis of Eg5 indicates that ~90% of mitotic Eg5 is phosphorylated at Thr926, whereas comparison of the ^{32}P content of peptides on our two-dimensional maps indicates that ^{32}P incorporation at Eg5[Ser1033] is approximately 3% that of Thr926. Additionally, our results indicate that Ser1033 (Ser1046 in *Xenopus* Eg5) phosphorylation is characteristic of the subset of kinesin localized at or near the spindle poles, thus showing that cells have differentially

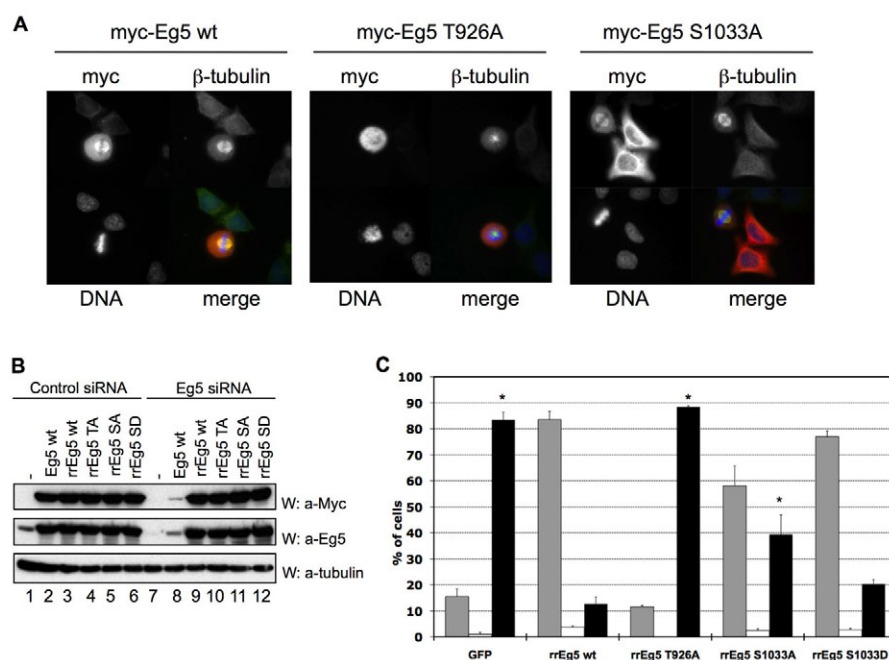
modified and spatially localized pools of Eg5. This might help to explain how the kinesin can perform the multiple cellular roles that have been assigned to it (including centrosome separation, bundling and sorting of microtubules during spindle formation, exertion of anti-parallel forces in the mid-spindle, control of centrosome structure during mitosis, control of microtubule flux, and postmitotic centrosome movement).

The evidence in support of Nek6 being the kinase responsible for the mitotic phosphorylation of Eg5[Ser1033] is strong. Nek6 is constitutively associated with Eg5 throughout the cell cycle and is activated in M-phase, when it becomes associated with the mitotically activated Nercc1. At that time, a ternary complex of Nercc1-Nek6 and Eg5 is detectable, coincident with the appearance of Eg5[Ser1033] phosphorylation. Eg5[Ser1033], which is situated in the Lxx[S/T] motif shown to be preferred by Nek6, is avidly phosphorylated by Nek6 in vitro (along with other sites), whereas Nercc1, despite the considerable similarity of its catalytic domain to Nek6, is unable to phosphorylate it. Furthermore, Nek6 was able to induce Ser1033 phosphorylation in vivo when overexpressed. Although others have reported that depletion of Nek6 promotes metaphase arrest (Yin et al., 2003), we did not observe either mitotic arrest or inhibition of Eg5[Ser1033] phosphorylation despite RNAi-induced depletion of endogenous Nek6 by ~80%. Furthermore, in our experiments we did not detect any changes on the levels of active Nek6 (Nek6[Ser206-P]), suggesting that much more severe Nek6 depletion is required. Thus, although we cannot formally rule out the possibility that one or more unknown kinases phosphorylate the site, the present data strongly suggest that Nek6, which is targeted to Eg5, is the kinase responsible for the mitotic phosphorylation of Eg5[Ser1033].

NIMA-family kinases (Neks in mammals), together with Polo- and Aurora-family members and the mitotic checkpoints kinases, are

Fig. 5. Eg5[Ser1033] phosphorylation is required for normal Eg5 function in mitotic spindle assembly.

(A) U2OS cells transiently expressing Myc-Eg5 wild type, Myc-Eg5[Thr926Ala] or Myc-Eg5[Ser1033Ala] were stained for Myc, β -tubulin and DNA. A representative transfected cell in mitosis is shown in each case, except for Myc-Eg5[Ser1033Ala], for which a mitotic cell plus two transfected interphase cells is shown. (B) HeLa cells were co-transfected with either control oligonucleotides (lanes 1-6) or siRNA oligonucleotides directed against human Eg5 (lanes 7-12), together with empty plasmid (lanes 1 and 7) or plasmids encoding Myc-Eg5 wild type (wt) that was unmodified (lanes 2 and 8) or Myc-Eg5 variants that were rendered RNAi resistant (rrEg5 wt, Eg5 wild type; rrEg5 TA, Eg5[Thr926Ala]; rrEg5 SA, Eg5[Ser1033Ala]; rrEg5 SD, Eg5[Ser1033Asp]). Cell lysates were immunoblotted with anti-Myc (upper panel) or anti-Eg5 (middle panel) to determine recombinant and total Eg5 expression, respectively, and with anti- α -tubulin antibodies to evaluate loading (bottom panel). (C) HeLa cells were co-transfected with siRNA oligonucleotides directed against human Eg5 and with plasmids encoding Myc-GFP (control) or several RNAi-resistant Myc-Eg5 (rrEg5) variants. At 48 hours after transfection, cells were fixed and stained with anti-Myc and anti- β -tubulin antibodies, and with DAPI. Myc-positive cells were scored as being in interphase (gray columns), in normal mitosis (white columns) or in an abnormal mitosis (mostly monopolar spindles; black columns). The figure represents the mean of three independent experiments, wherein >100 cells were scored for each point in each of the experiments; error bars indicate s.e.m. The fraction of abnormal mitoses in cells expressing rrEg5 wild type was compared with each of the other conditions by Student's *t*-test; **P*<0.05. The comparison of Ser1033Ala with Ser1033Asp (S1033D) gave a *P* value of 0.068.



proposed to orchestrate mitosis under the direction of CDK1 (Nigg, 2001; O'regan et al., 2007). Nek2 regulates premitotic centrosome separation, and phosphorylates the centrosomal proteins C-Nap1 (Fry et al., 1998), Nlp (Rapley et al., 2005), rootletin (Bahe et al., 2005), centrobins (Jeong et al., 2007) and β -catenin (Bahmanyar et al., 2008). Nercc1 is activated in mitosis, phosphorylates and activates Nek6 and Nek7, and is necessary for correct spindle formation (Roig et al., 2002; Belham et al., 2003; Roig et al., 2005). Apart from these findings, little is known concerning the physiological substrates of the 11 Nek proteins. The deficiency of Nek1 and Nek8 in mice is accompanied by polycystic kidney disease, a syndrome that is generally associated with defective ciliary function; consequently, it has been suggested that Neks in general might control different aspects of the centrosome and/or microtubule cellular systems (Quarmby and Mahjoub, 2005). Herein we show that Eg5, a microtubule-based motor with a central role in the construction of the mitotic spindle, is a Nek6 substrate and provide evidence that Nek6 phosphorylation of Eg5 is crucial for Eg5 function during mitosis, providing a plausible mechanism for at least part of the reported phenotypes resulting from interference with Nek6 (Yin et al., 2003), its activator kinase Nercc1 (Roig et al., 2002; Roig et al., 2005) and possibly Nek7 (Yissachar et al., 2006) (note that Nek7 is highly similar – 84% identical – to Nek6; it is not clear whether both kinases have redundant roles in different cell types). Although this is the first example of regulation of a kinesin by a NIMA-family kinase, it should be noted that Nek1 has been reported to interact with KIF3A (Surpili et al., 2003).

With regards to the physiological role of the mitotic phosphorylation of Eg5[Ser1033], we show that, in mitotic cells, this modification occurs on a minority of the Eg5 polypeptides, in contrast to the almost universal phosphorylation of Eg5[Ser926]. As to the localization of this subset of Ser1033-phosphorylated Eg5 polypeptides, in mitotic *Xenopus* cells, Eg5 phosphorylated at the homologous site (Ser1046) accumulates at centrosomes during the beginning of mitosis, and subsequently is evident at the spindle poles and on the microtubules proximal to the pole; during mitotic exit, Eg5[Ser1046-P] concentrates at the cytokinetic bridge. This distribution matches precisely the pattern of activated Nek6 (J.R. and J.A., unpublished results). Our data indicate that Eg5[Ser1033] phosphorylation is not necessary for Eg5 binding to microtubules, a function that is most probably controlled exclusively by Thr926 phosphorylation (see Fig. 5A) (in contrast to Eg5[Thr926Ala], both Eg5[Ser1033Ala] and Eg5[Ser1033Asp] localized to microtubules in cells depleted of endogenous Eg5, eliminating the possibility that Eg5[Ser1033] mutants localize to the spindle as a result of oligomerization with endogenous kinesin) (see supplementary material Fig. S4). Finally, we show that Eg5[Ser1033] phosphorylation is necessary for normal bipolar mitotic spindle formation. On the basis of these observations, we propose that Ser1033 phosphorylation controls a small centrosomal pool of Eg5, the function of which is related to centrosome separation before and/or during spindle formation; if so, this would place NIMA-family kinases, specifically Nek2 and the Nercc1-Nek6 module, in charge of controlling both centrosome disjunction and separation, and consequently bipolar spindle formation and maintenance. Additional functions might be performed by Eg5[Ser1033-P] during cytokinesis. Others have observed the localization of some Eg5 at the cytokinetic furrow (Blangy et al., 1995), and have suggested that it serves an important role in the late phases of mitosis (Whitehead and Rattner, 1998). Our finding of *X. laevis* Eg5[Ser1046-P] at the cytokinetic bridge suggests that this Eg5 subset is the probable mediator of this putative function;

nevertheless, our experiments thus far with Eg5[Ser1033] mutants (as in Fig. 5) have not uncovered phenotypes (e.g. polyploidy) that indicate a role for Eg5 in cytokinesis.

How might Ser1033 phosphorylation control Eg5 activity? Conceivably, phosphorylation could affect motor activity through tail-head interactions or, less likely, global alteration of the tertiary structure of the kinesin; nevertheless, the baculoviral Eg5[Ser1033] mutants did not differ dramatically from wild type in microtubule binding or motility on microtubule monolayers, suggesting that major effects on motor function are not likely. Phosphorylation could also regulate Eg5 binding to unknown proteins or cellular structures, helping to localize in a timed manner a fraction of the kinesin to concrete subcellular regions (e.g. the centrosome) to exert forces in different fashions. Additionally, Eg5 modification could affect Eg5 stability [Eg5 polypeptide levels are cell-cycle regulated (see Fig. 3B) and, in mammals and *Xenopus*, the residue that is orthologous to Ser1033 is adjacent to a conserved putative KEN box]. Several of these possibilities are currently under study.

Regarding the control of Eg5 function by phosphorylation events other than those catalyzed by CDK1 or Nek6, the Aurora-family member pEg2 (Aurora-A) has been reported to modify the kinesin (Giet et al., 1999); interphase Eg5 phosphorylation by an unidentified kinase has also been reported (Blangy et al., 1995). Our results show that, at least in human U2OS cells, the action of CDK1 and Nek6 is sufficient to account for all mitotic Eg5 phosphorylation, and that, in interphase, Eg5 is not appreciably phosphorylated. Our MS analysis covers 80% and 75% of the Eg5 protein sequence (from exponential and mitotic cells, respectively), and exclusively identifies the Thr926 and Ser1033 phosphosites (this is further supported by our phosphopeptide mapping experiments). Despite coverage of the corresponding residues in our MS/MS analyses, we did not detect other reported phosphosites, such as Ser931 (Nousiainen et al., 2006) or Thr485, although the latter has been described to be a DNA-damage-induced phosphorylation event (Matsuoka et al., 2007), or any stalk-domain phosphorylation (previously reported in *X. laevis* Eg5 and attributed to the Aurora kinase pEg2) (Giet et al., 1999); similarly, we did not detect phosphorylation at the sites that correspond to the residues phosphorylated in the *C. elegans* Eg5 ortholog BMK-1 by the Aurora kinase AIR-2 (Bishop et al., 2005). Perhaps Aurora-family phosphorylation of Eg5 is cell-type specific and the observed interphase phosphorylation of Eg5 reported by others reflects modifications engendered by cell stress or during extraction.

In summary, we show that the Nercc1-Nek6 module binds to Eg5 and that Nek6 can phosphorylate the BimC-family kinesin Eg5 at Ser1033 in vitro and induce its phosphorylation in vivo. We also show that Ser1033 is phosphorylated in mitosis, when Nek6 is active, and that phosphorylated Eg5 accumulates at centrosomes and spindle poles. Although not necessary for Eg5 spindle localization, Ser1033 phosphorylation is required for normal Eg5 function during spindle formation. We propose that Nercc1-Nek6 activation at centrosomes and spindle poles results in Eg5 phosphorylation and, thus, that modified Eg5 performs a specific, crucial function around the poles that is necessary for normal bipolar spindle organization, perhaps related to centrosome separation. Nek6 thus joins CDK1 (and possibly the Aurora-family kinases) as an enzyme capable of phosphorylating and regulating Eg5 kinesins. The conservation of at least one LxxS motif C-terminal to the BimC box in practically all the members of the BimC family strongly points to an evolutionarily conserved relationship between NimA kinases and BimC kinesins, two families of proteins originally

identified and shown to be necessary for the completion of mitosis in the mold *A. nidulans* (Osmani et al., 1988; Enos and Morris, 1990).

Materials and Methods

Plasmid constructs

MalBP fusions of Eg5 fragments and *X. laevis* Eg5[773-1067] were generated by PCR cloning into pMAL-c2 $\times$ (New England Biolabs). GST-Eg5 was generated by PCR cloning full-length Eg5 into pEBG. To generate Eg5 mutants, site-directed mutagenesis was performed according to the manufacturer's instructions (Stratagene) using specific primers (T927A 5'-ATCCCAACAGGTACGGCTCCACAGAGGAAAAGT-3', S1033A 5'-AACACACTGGAGAGGGCTAAAGTGAAGAAACT-3', S1033D 5'-AACACACTGGAGAGGGATAAAGTGAAGAAACT-3' with the appropriate reverse complement), whereas RNAi-resistant Eg5 primers encoded silent mutations (5'-GGAAACCTAACTGAAGATTAAAGACAATAAAGCAGACC-3' with reverse complement). All constructs were sequenced after generation.

Cell culture and transfection

HEK293T, U2OS and HeLa cells were grown at 37°C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 50 IU of penicillin per ml and 50 mg of streptomycin per ml (Invitrogen). XL177 cells were grown in 70% Leibovitz-15 media supplemented with L-glutamine, 10% heat-inactivated FCS and 50 IU of penicillin per ml and 50 mg of streptomycin per ml at 25°C. HEK293T and HeLa cells were transfected with the indicated expression plasmids using Lipofectamine and Lipofectamine 2000, respectively, according to the manufacturer's instructions (Invitrogen). siRNA and DNA co-transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions.

Antibodies and immunotechniques

Polyclonal anti-Nek6, anti-Nercc1, anti-P-Nek6/7 and anti-phospho-Nercc1 antibodies were produced as described previously (Belham et al., 2003; Roig et al., 2002; Roig et al., 2005). Anti-XIEg5 antibodies were produced as described (Miyamoto et al., 2004). Monoclonal anti-Eg5 antibodies and anti-cyclin-B antibodies were purchased from BD Biosciences (San Jose, CA), anti-CDK1 Y15 from Cell Signaling (Danvers, MA), normal IgG from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-myc, anti- β -tubulin, anti- α -tubulin and anti- γ -tubulin from Sigma (St Louis, MO). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell lysis, immunoprecipitation and immunoblotting methods have been described previously (Roig et al., 2002) unless stated.

Two-hybrid analysis

cDNAs coding for the human Eg5 fragments indicated in Table 1 were subcloned into pGBKT7 and transformed into *S. cerevisiae* AH109 strain by the lithium acetate method. *Nercc1* and *Nek6* cDNAs were subcloned into pGADT7 and transformed into *S. cerevisiae* Y187 strain. Mating was carried out according to the manufacturer's procedures (Clontech Laboratories, Paol Alto, CA) in 2 $\times$ YPD medium for 16 hours. Selection was carried out in synthetic defined (SD)/-Leu/-Trp/-His/-Ade and interactions were verified using α -Gal.

Protein expression and purification

MalBP-Eg5 fusion-protein expression in DH5 α was induced with 0.4 mM IPTG for 5 hours at 37°C and purified using amylose resin according to the manufacturer's instructions (New England Biolabs, Ipswich, MA). GST-Eg5 was expressed in HEK293T cells by transient transfection. At 48 hours later, cells were lysed in buffer A [150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 mM Tris pH 8.0, protease inhibitor tablet (Roche Applied Science, Penzberg, Germany)], adsorbed to glutathione sepharose (Amersham Biosciences, Buckinghamshire, UK) and eluted with 25 mM reduced glutathione after washing. FLAG-Nek6 wild type, FLAG-Nek6[Lys74/75Met] and FLAG-Nercc1 were transiently expressed in HEK293T cells. 48 hours after transfection cells were lysed in buffer B [150 mM NaCl, 1% NP-40, 50 mM Tris pH 8.0, protease inhibitor tablet (Roche)], the recombinant polypeptides adsorbed to FLAG-agarose and eluted with FLAG peptide (0.1 μ g/ μ l, Sigma) after washing. His6-Eg5 polypeptides were generated in SF9 cells using recombinant baculovirus according to the manufacturer's instructions (Invitrogen). His6-Eg5 polypeptides were extracted and purified using Nickel agarose according to the manufacturer's instructions (Qiagen, Hilden, Germany) followed by FPLC gel filtration.

In vitro kinase assays

MalBP, MalBP-Eg5 fusion proteins and MBP (2 μ g each) were phosphorylated by FLAG-Nek6 wild type or were preactivated (with 100 μ M ATP for 30 minutes at 30°C) FLAG-Nercc1 wild type in kinase buffer (50 mM MOPS pH 7.4, 10 mM MgCl₂, 2 mM EGTA, 20 mM β -glycerophosphate, 5 μ M ATP, 0.83 pmol [γ -³²P] ATP) in a total volume of 50 μ l for 30 minutes at 30°C. GST-Eg5 and His6-XIEg5 (2 μ g each) were phosphorylated as above except that kinase buffer was supplemented

with 20 μ M ATP. Reactions were stopped by the addition of 20 μ l 5 $\times$ Laemmli buffer. Samples were separated by SDS-PAGE, Coomassie-blue stained, exposed to a PhosphorImager and sometimes quantitated by liquid scintillation counting.

Mass spectrometry (MS)

Endogenous Eg5 was immunoprecipitated from extracts of cycling or nocodazole-arrested (0.5 μ g/ml, 24 hours) U2OS cells. The lysis buffer contained 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 10 mM β -glycerophosphate, 1 mM Na₃VO₄, 100 nM calyculin, 0.1 M NaF and 50 mM Tris pH 8.0. Immunoprecipitation used 2 mg extract protein and 8 μ g anti-Eg5 (BD Biosciences) conjugated to 120 μ l protein G Dynal beads (Invitrogen) in lysis buffer, incubated with rotation for 1 hour at 4°C. After three washes in lysis buffer, bound proteins were eluted into 70 μ l 3 $\times$ Laemmli buffer. Recombinant MalBP-Eg5[1-361] and MalBP-Eg5[762-1057] were phosphorylated by FLAG-Nek6 as described above using 20 μ M ATP; parallel assays were performed with [γ -³²P] ATP to define the extent of phosphorylation. After SDS-PAGE, fixation and staining, in situ tryptic digestion of gel slices and LC/MS/MS analysis of phosphorylation sites was performed at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA) as described previously (Roig et al., 2005). Note that the human Eg5 sequence used here, published in the NCBI database as accession number P52732 and confirmed by our MS analysis, differs by one residue from that used previously (Blangy et al., 1995; Blangy et al., 1997; Nousiainen et al., 2006); we found human Eg5 residues 674 and 675 to be EL, whereas the latter authors report these as RNS, resulting in one additional residue.

In vivo labeling and two-dimensional tryptic phosphopeptide mapping

U2OS cells either growing exponentially or treated with nocodazole (250 ng/ml) for 16 hours were washed with TBS and resuspended in phosphate-free Dulbecco's modified Eagle's medium containing 10% PBS-dialyzed FCS, without or with nocodazole. After 30 minutes at 37°C, ³²P (1 mCi/ml) was added for an additional 90 minutes. The ³²P-labeled cells were extracted and Eg5 immunoprecipitates were subjected to SDS-PAGE, fixation and staining. The gel slices containing Eg5 were equilibrated in 50 mM ammonium bicarbonate (pH 8), homogenized and subjected to several rounds of tryptic digestion. The dried, salt-free digest was separated by thin-layer electrophoresis at pH 1.9 followed by thin-layer chromatography (TLC) as described previously (Belham et al., 2003). ³²P was visualized and quantified using a PhosphorImager.

Phosphospecific antibodies

Rabbits were immunized with synthetic peptides corresponding to HsEg5[1028-1038] or XIEg5[1041-1051] containing a phosphorylated serine at position 1033 and 1046, respectively, coupled to KLH through an N-terminal cysteine. Phosphopeptide-specific antibodies were affinity purified from sera depleted of antibodies reactive with the non-phosphorylated peptides.

Immunohistochemistry

XL177 cells grown on coverslips were rinsed with 70% phosphate-buffered saline (PBS), fixed by immersion in methanol at -20°C for 5 minutes, rinsed with PBS, immersed in solution A (3% bovine serum albumin in PBS plus 0.1% Triton X-100 and 0.02% azide) for 30 minutes and subsequently in solution A containing specific antibodies for (final concentration): rabbit anti-phospho-XIEg5 (1:100); rabbit anti-Eg5 (1:2000); mouse anti- β -tubulin (1:1000); anti- γ -tubulin (1:400). Primary antibodies were visualized with either Cy2-conjugated donkey anti-rabbit or rhodamine-X-conjugated donkey anti-mouse. DNA was visualized with 4,6-diamidino-2-phenylindole (DAPI, 0.01 mg/ml). Rinsed coverslips were mounted on a microscope slide. Images were taken using a Leica TCS SPE confocal system with a DM2500 CSQ upright microscopy and a 63 $\times$ 1.30 ACS Apo lens, and edited using Leica LAS AF 1.6.3 software (Leica Microsystems, Mannheim, Germany).

Motor assays

Microtubule pelleting and gliding assays were performed as previously described (Vale et al., 1985; Saxton, 1994; Lockhart and Cross, 1996).

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References

- Bahe, S., Stierhof, Y. D., Wilkinson, C. J., Leiss, F. and Nigg, E. A. (2005). Rootletin forms centriole-associated filaments and functions in centrosome cohesion. *J. Cell Biol.* **171**, 27-33.
- Bahmanyar, S., Kaplan, D. D., Deluca, J. G., Giddings, T. H., O'Toole, E. T., Winey, M., Salmon, E. D., Casey, P. J., Nelson, W. J. and Barth, A. I. (2008). beta-Catenin is a Nek2 substrate involved in centrosome separation. *Genes Dev.* **22**, 91-105.
- Belham, C., Comb, M. J. and Avruch, J. (2001). Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase. *Curr. Biol.* **11**, 1155-1167.
- Belham, C., Roig, J., Caldwell, J. A., Aoyama, Y., Kemp, B. E., Comb, M. and Avruch, J. (2003). A mitotic cascade of NIMA family kinases. Nerec1/Nek9 activates the Nek6 and Nek7 kinases. *J. Biol. Chem.* **278**, 34897-34909.
- Bishop, J. D., Han, Z. and Schumacher, J. M. (2005). The *Caenorhabditis elegans* Aurora B kinase AIR-2 phosphorylates and is required for the localization of a BimC kinesin to meiotic and mitotic spindles. *Mol. Biol. Cell* **16**, 742-756.
- Blangy, A., Lane, H. A., d'Hérin, P., Harper, M., Kress, M. and Nigg, E. A. (1995). Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation *in vivo*. *Cell* **83**, 1159-1169.
- Blangy, A., Arnaud, L. and Nigg, E. A. (1997). Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. *J. Biol. Chem.* **272**, 19418-19424.
- Drummond, D. R. and Hagan, I. M. (1998). Mutations in the bimC box of Cut7 indicate divergence of regulation within the bimC family of kinesin related proteins. *J. Cell Sci.* **111**, 853-865.
- Enos, A. P. and Morris, N. R. (1990). Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in *A. nidulans*. *Cell* **60**, 1019-1027.
- Fry, A. M., Mayor, T., Meraldi, P., Stierhof, Y. D., Tanaka, K. and Nigg, E. A. (1998). C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J. Cell Biol.* **141**, 1563-1574.
- Giet, R., Uzbekov, R., Cubizolles, F., Le Guellec, K. and Prigent, C. (1999). The *Xenopus laevis* aurora-related protein kinase pEg2 associates with and phosphorylates the kinesin-related protein XIEg5. *J. Biol. Chem.* **274**, 15005-15013.
- Hayward, D. G. and Fry, A. M. (2006). Nek2 kinase in chromosome instability and cancer. *Cancer Lett.* **237**, 155-166.
- Heck, M. M., Pereira, A., Pesavento, P., Yannoni, Y., Spradling, A. C. and Goldstein, L. S. (1993). The kinesin-like protein KLP61F is essential for mitosis in *Drosophila*. *J. Cell Biol.* **123**, 665-679.
- Holland, P. M., Milne, A., Garka, K., Johnson, R. S., Willis, C., Sims, J. E., Rauch, C. T., Bird, T. A. and Virca, G. D. (2002). Purification, cloning, and characterization of Nek8, a novel NIMA-related kinase, and its candidate substrate Bicd2. *J. Biol. Chem.* **277**, 16229-16240.
- Jeong, Y., Lee, J., Kim, K., Yoo, J. C. and Rhee, K. (2007). Characterization of NIP2/centrobin, a novel substrate of Nek2, and its potential role in microtubule stabilization. *J. Cell Sci.* **120**, 2106-2116.
- Kandli, M., Feige, E., Chen, A., Kilfin, G. and Motro, B. (2000). Isolation and characterization of two evolutionarily conserved murine kinases (Nek6 and nek7) related to the fungal mitotic regulator, NIMA. *Genomics* **68**, 187-196.
- Kapitein, L. C., Peterman, E. J., Kwok, B. H., Kim, J. H., Kapoor, T. M. and Schmidt, C. F. (2005). The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* **435**, 114-118.
- Le Guellec, R., Paris, J., Couturier, A., Roghi, C. and Philippe, M. (1991). Cloning by differential screening of a *Xenopus* cDNA that encodes a kinesin-related protein. *Mol. Cell. Biol.* **11**, 3395-3398.
- Lizcano, J. M., Deak, M., Morrice, N., Kieloch, A., Hastie, C. J., Dong, L., Schutkowski, M., Reimer, U. and Alessi, D. R. (2002). Molecular basis for the substrate specificity of NIMA-related kinase-6 (NEK6). Evidence that NEK6 does not phosphorylate the hydrophobic motif of ribosomal S6 protein kinase and serum- and glucocorticoid-induced protein kinase *in vivo*. *J. Biol. Chem.* **277**, 27839-27849.
- Lockhart, A. and Cross, R. A. (1996). Kinetics and motility of the Eg5 microtubule motor. *Biochemistry* **35**, 2365-2373.
- Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R., Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, Z., Solimini, N., Lerenthal, Y. et al. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**, 1160-1166.
- Miyamoto, D. T., Perlman, Z. E., Burbank, K. S., Groen, A. C. and Mitchison, T. J. (2004). The kinesin Eg5 drives poleward microtubule flux in *Xenopus laevis* egg extract spindles. *J. Cell Biol.* **167**, 813-818.
- Nigg, E. A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. *Nat. Rev. Mol. Cell. Biol.* **2**, 21-32.
- Nousiainen, M., Silljé, H. H., Sauer, G., Nigg, E. A. and Körner, R. (2006). Phosphoproteome analysis of the human mitotic spindle. *Proc. Natl. Acad. Sci. USA* **103**, 5391-5396.
- O'Connell, M. J., Krien, M. J. and Hunter, T. (2003). Never say never: the NIMA-related protein kinases in mitotic control. *Trends Cell Biol.* **13**, 221-228.
- O'regan, L., Blot, J. and Fry, A. M. (2007). Mitotic regulation by NIMA-related kinases. *Cell Div.* **2**, 25.
- Osmani, S. A., Pu, R. T. and Morris, N. R. (1988). Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. *Cell* **53**, 237-244.
- Quarmby, L. M. and Mahjoub, M. R. (2005). Caught Nek-ing: cilia and centrioles. *J. Cell Sci.* **118**, 5161-5169.
- Rapley, J., Baxter, J. E., Blot, J., Wattam, S. L., Casenghi, M., Meraldi, P., Nigg, E. A. and Fry, A. M. (2005). Coordinate regulation of the mother centriole component nlp by nek2 and plk1 protein kinases. *Mol. Cell. Biol.* **25**, 1309-1324.
- Roig, J. and Avruch, J. (2006). Nek protein kinases. In *SIGMA-RBI Handbook of Receptor Classification and Signal Transduction*, 5th Edn (ed. K. J. Watling), pp. 286-290. Natick: Sigma-RBI.
- Roig, J., Mikhailov, A., Belham, C. and Avruch, J. (2002). Nerec1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. *Genes Dev.* **16**, 1640-1658.
- Roig, J., Groen, A., Caldwell, J. and Avruch, J. (2005). Active Nerec1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. *Mol. Biol. Cell* **16**, 4827-4840.
- Sawin, K. E. and Mitchison, T. J. (1995). Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. *Proc. Natl. Acad. Sci. USA* **92**, 4289-4293.
- Sawin, K. E., LeGuellec, K., Philippe, M. and Mitchison, T. J. (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* **359**, 540-543.
- Saxton, W. M. (1994). Isolation and analysis of microtubule motor proteins. *Methods Cell Biol.* **44**, 279-288.
- Surpili, M. J., Delben, T. M. and Kobarg, J. (2003). Identification of proteins that interact with the central coiled-coil region of the human protein kinase NEK1. *Biochemistry* **42**, 15369-15376.
- Vale, R. D., Reese, T. S. and Sheetz, M. P. (1985). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* **42**, 39-50.
- Walczak, C. E., Vernos, I., Mitchison, T. J., Karsenti, E. and Heald, R. (1998). A model for the proposed roles of different microtubule-based motor proteins in establishing spindle bipolarity. *Curr. Biol.* **8**, 903-913.
- Weil, D., Garçon, L., Harper, M., Duménil, D., Dautry, F. and Kress, M. (2002). Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. *Biotechniques* **33**, 1244-1248.
- Whitehead, C. M. and Rattner, J. B. (1998). Expanding the role of HsEg5 within the mitotic and post-mitotic phases of the cell cycle. *J. Cell Sci.* **111**, 2551-2561.
- Yin, M. J., Shao, L., Voehringer, D., Smeal, T. and Jallat, B. (2003). The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis. *J. Biol. Chem.* **278**, 52454-52460.
- Yissachar, N., Salem, H., Tennenbaum, T. and Motro, B. (2006). Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. *FEBS Lett.* **580**, 6489-6495.