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NOVEL INSIGHTS INTO THE MECHANISMS OF MITOTIC SPINDLE ASSEMBLY BY NEK KINASES

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Abstract

The mitotic spindle is the apparatus upon which chromosomes are segregated during cell division. We have discovered new roles for two members of the NIMA-Related Kinase (NEK) family in different molecular processes of spindle assembly. Moreover, loss of these proteins leads to segregation errors that drive cancer progression.

Spindle assembly is a complex multi-step process that begins upon mitotic entry. In prophase, the two centrosomes separate and move around the nucleus to form opposing spindle poles. This requires dissolution of the inter-centrosomal linker that holds interphase centrosomes together and the cross-linking and anti-parallel sliding of microtubules nucleated from opposing poles. In prometaphase, the nuclear envelope breaks down giving microtubules access to the condensed chromosomes where they attach to the kinetochores of the sister chromatids. These microtubules become bundled into K (kinetochore)-fibres by inter-microtubule K-fibre bridging proteins, including transforming acidic coiled-coil protein 3 (TACC3) and cytoskeleton-associated protein 5 (CKAP5, also known as ch-TOG). These contribute to the robust kinetochore attachments required for chromosome congression. At metaphase, all chromosomes become aligned at the cell centre with bi-orientated K-fibres. Only once this has been achieved is the spindle assembly checkpoint (SAC) inactivated and cells allowed to enter anaphase (Fig. 1A).

The NIMA-Related Kinases (NEKs) are a family of 11 serine/threonine kinases in humans.[1] They are related to the *Aspergillus* NIMA (Never In Mitosis) kinase, which is essential for mitotic entry in this filamentous fungus. Several human NEKs contribute to mitotic progression suggesting conservation of function. Yet compared to other highly conserved mitotic kinases, such as the Cyclin-dependent kinases (CDKs), Polo-like kinases (PLKs) and Aurora kinases, the functions of the NEKs remain poorly understood. In two studies published recently in the Journal of Cell Biology, we made exciting discoveries into how two members of this family, NEK5 and NEK6, contribute to the timing and vigour of spindle assembly.[2,3]

The first study on NEK5 revealed that cells depleted of the kinase by RNAi showed defective chromosome segregation as a result of delayed centrosome separation in prophase.[2] The reason that centrosome separation was delayed in these cells was two-fold. Firstly, dissolution of the inter-centrosomal linker was impaired. Linker disassembly is achieved through NEK2 phosphorylating linker components, including Centrosomal NEK2-Associated Protein 1 (C-NAP1) and ciliary rootlet coiled-coil rootletin (CROCC, more commonly known as rootletin).[4] However, in the absence of NEK5 there was less NEK2 at prophase centrosomes, which resulted in the retention of these linker proteins. Secondly, as cells enter mitosis, centrosome maturation normally takes place, during which time centrosomes double in size through recruitment of proteins, such as γ-tubulin, centrosomal protein 192kDa (CEP192), CDK5 regulatory subunit associated protein 2 (CDK5RAP2) and pericentrin.[5] This

dramatically increases the microtubule nucleating capacity of the centrosome facilitating the cross-linking and sliding of microtubules that drive centrosomes apart. In cells depleted of NEK5, less γ-tubulin, CEP192, CDK5RAP2 and pericentrin were recruited to mitotic centrosomes leading to a reduced rate of microtubule nucleation. As a consequence, there would be less capacity to generate the microtubule-dependent forces required to separate centrosomes (Fig. 1B).

It remains to be seen what substrates NEK5 phosphorylates to promote centrosome separation and maturation. The mitotic consequences of NEK5 depletion may result from loss of centrosome integrity in interphase, as cells depleted of NEK5 had reduced levels of C-NAP1, NEK2, γ-tubulin, CEP192, CDK5RAP2 and pericentrin. NEK5 may therefore promote the recruitment of these centrosome components throughout the cell cycle. Conversely, loss of NEK5 resulted in accumulation of excess rootletin and a moderate increase in inter-centrosomal distance. This may be a consequence of less centrosomal NEK2 being present to regulate linker organization via basal phosphorylation,[4] or a compensatory response to resist centrosome splitting due to reduced levels of C-NAP1. Whatever the cause, it will be of great interest to identify the substrates through which NEK5 regulates the centrosomal levels of these proteins, and whether NEK2 and NEK5 directly cooperate to achieve disassembly of the centrosome linker.

For NEK6, it was already known that RNAi-mediated depletion or expression of kinase-inactive mutants led to fragile spindles incapable of satisfying the SAC.[6,7] However, NEK6 does not localize strongly to the spindle apparatus and its only reported substrate was kinesin family member 11 (KIF11). NEK6 phosphorylates KIF11 on S1033, thereby targeting it the centrosome to promote centrosome separation.[8] An S1033A phosphonull mutant resulted in some mitotic delay, but did not lead to monopolar spindles. Therefore, KIF11 is unlikely to be the only mitotic substrate of NEK6. To identify additional NEK6 substrates, phosphorylation and interaction-based screens were performed. As described in the second report, these led to identification of the cytoplasmic chaperone Heat Shock 70kDa Protein 1A (HSPA1, also known as HSP72) as a novel mitotic binding partner and substrate for NEK6.[3]

If HSP72 is a physiological substrate of NEK6, it would be expected to have a similar role in mitotic progression. Consistent with this, cells in which HSP72 function was blocked, either by RNAi depletion or chemical inhibition, exhibited poorly organized

spindles, chromosome congression defects and delayed anaphase onset. Detailed analysis revealed weakened K-fibres with substantially reduced levels of TACC3 and ch-TOG. Alongside a strongly active SAC and an inability to maintain metaphase chromosome alignment, this points to a failure to generate stable kinetochore-MT attachments and thus build robust K-fibers. Co-precipitation experiments revealed that the HSP72 inhibitor reduced interaction of TACC3 with ch-TOG, implying that HSP72 may specifically facilitate their assembly into a complex and recruitment to K-fibres (Fig. 1C).

How then does NEK6 regulate HSP72 function? HSP72 is phosphorylated by NEK6 on T66 within the nucleotide-binding domain and this phosphorylation, and interaction with Nek6, is mitosis-specific. Importantly, either NEK6 depletion or expression of a HSP72-T66A phosphonull mutant led to reduced K-fibres, whilst a HSP72-T66E phosphomimetic mutant could rescue the K-fibre defects that arose from NEK6 depletion. These data provide evidence that a major function of NEK6 is to promote K-fibre stabilization through phosphorylation of HSP72. However, whilst this can be explained in part by facilitating ch-TOG/TACC3 complex assembly, our data suggest an additional mechanism. Total HSP72 localises to spindle poles and spindle fibres similarly to ch-TOG and TACC3. However, Nek6-phosphorylated HSP72 is clearly detected not only at spindle poles but also at sites of kinetochore-microtubule attachment. This argues for a direct role for phosphorylated HSP72 in promoting plus-end attachment of microtubules to kinetochores. Furthermore, loss of HSP72 function led to reduced inter-polar distances that, together with reduced astral microtubules and misoriented spindles, suggests a defect in cortical attachment. Thus, HSP72 may well have dual functions in supporting spindle assembly: firstly, through promoting K-fibre stability via ch-TOG/TACC3 and, secondly, through ensuring microtubule plus ends make robust attachments to kinetochores and the cell cortex.

Chromosome segregation errors drive cancer progression and promote tumour heterogeneity. The assembly of a robust mitotic spindle to which the chromosomes attach in a stable and timely manner is therefore fundamental to preventing such errors. The studies on NEK5 and NEK6 demonstrate that both kinases are crucial to this process. In the absence of NEK5, centrosome separation is delayed in prophase and instead occurs via the prometaphase pathway that uses kinetochore microtubules and motor proteins to drive spindle pole separation. However, this promotes merotelic chromosome attachments that lead to a high frequency of

segregation errors, including chromosome fragmentation, lagging chromosomes and unresolved sister chromatids.[9] Significantly, merotely does not trigger SAC-dependent arrest so contributes directly to segregation errors as cells progress through mitosis unchecked.[10] Through targeting HSP72 to kinetochores, NEK6 contributes to the formation of stable kinetochore-microtubule attachments that are essential for chromosome congression and thus mitotic progression. In the absence of NEK6, fragile spindles are formed, chromosome congression fails and the SAC remains active. Inhibition of Nek6 in cells with a robust SAC offers a therapeutic avenue to block spindle assembly and thus cell division. Furthermore, inhibition of NEK6 in tumours that have a weakened SAC could lead to mitotic catastrophe and selective death of cancer cells.

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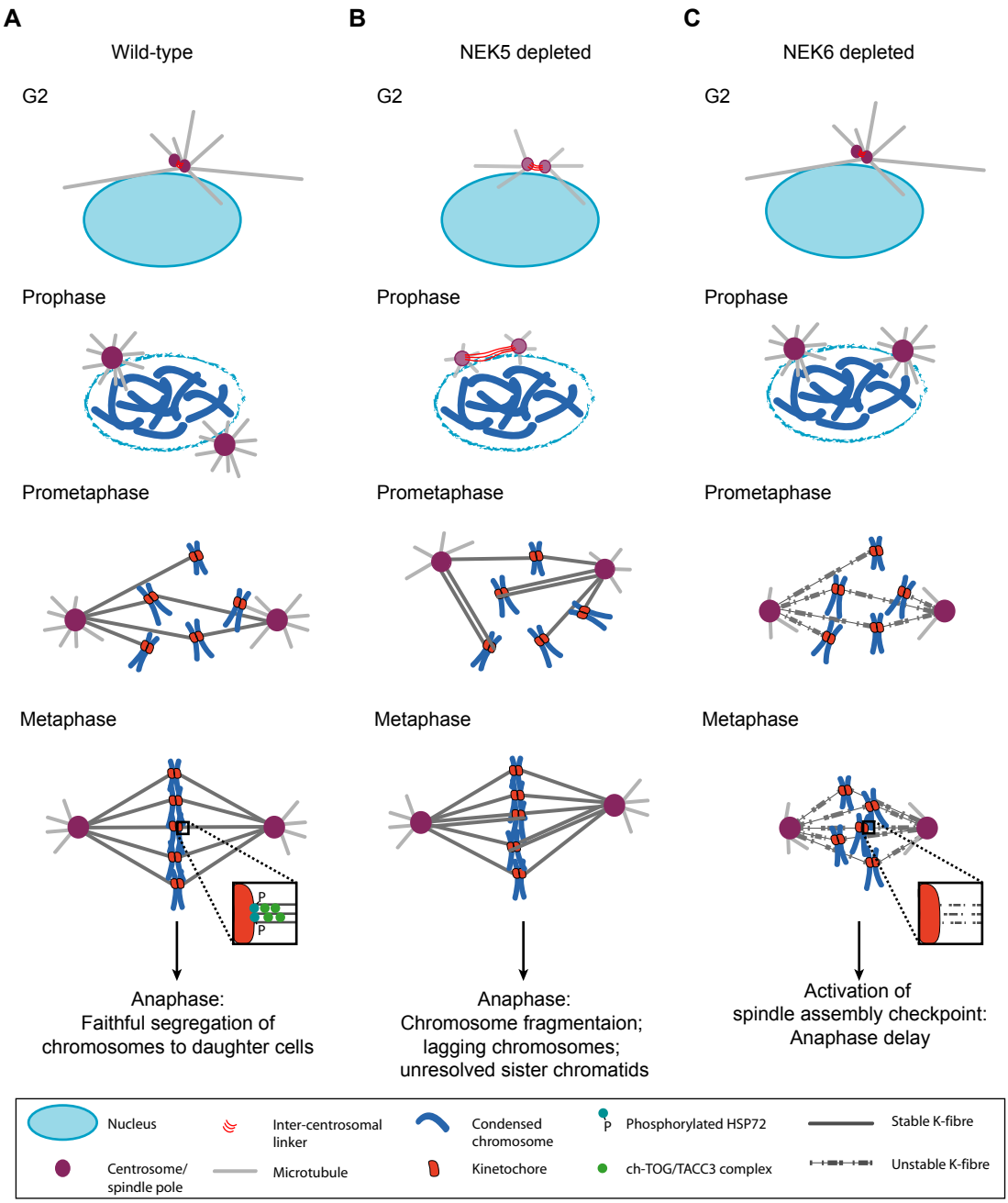
FIGURE LEGEND

Figure 1. NEK5 and NEK6 are required for efficient mitotic spindle assembly

A. G2 cells contain two centrosomes joined by a proteinaceous linker. As cells enter prophase this linker dissociates allowing microtubule-dependent motors to drive the centrosomes apart and establish the spindle poles. In prometaphase, nuclear envelope breakdown allows the microtubules to attach to the kinetochores of the sister chromatids. Heat Shock 70kDa Protein 1A (HSPA1, also known as HSP72) is phosphorylated by Never in Mitosis Gene A (NIMA)-Related Kinase (NEK) 6, which drives its recruitment to spindle microtubules and the kinetochores. In turn, HSP72 recruits the bridging proteins, transforming acidic coiled-coil protein 3 (TACC3) and cytoskeleton-associated protein 5 (CKAP5, also known as ch-TOG), which stabilise K (kinetochore)-fibres. Once all chromosomes have congressed to the spindle equator with bi-orientated K-fibres, the spindle assembly checkpoint (SAC) is satisfied and cells progress to anaphase and chromosome segregation.

B. In the absence of NEK5, centrosomes have reduced levels of NEK2 and reduced microtubule nucleation capacity. Consequently, when these cells enter prophase centrosome separation is delayed due to the retention of linker proteins and weakened microtubule-dependent sliding forces. Instead, centrosome separation occurs via the prometaphase pathway whereby attachment of microtubules to kinetochores generates an alternative force to separate centrosomes to opposite poles. However, this promotes the formation of, first, syntelic attachments (both sister chromatids attached to a single pole) and, subsequently, merotelic attachments (single sister chromatid attached to both poles). Merotelic attachments do not prevent chromosomes congression and satisfaction of the SAC, but segregation errors result.

C. In the absence of NEK6, centrosome separation may be delayed by reduced targeting of kinesin family member 11 (KIF11) to the centrosome in prophase. However, the more profound consequence is loss of HSP72, and thus ch-TOG/TACC3, from the K-fibres and kinetochores. Stable K-fibres, able to resist the forces exerted by chromosomal movements, are unable to form and chromosomes fail to congress to the spindle equator. The SAC cannot be satisfied and anaphase is delayed.


A
Wild-type
G2
Prophase
Prometaphase
Metaphase
P
P
Anaphase:
Faithful segregation of
chromosomes to daughter cells
B
NEK5 depleted
G2
Prophase
Prometaphase
Metaphase
Anaphase:
Chromosome fragmentaion;
lagging chromosomes;
unresolved sister chromatids
C
NEK6 depleted
G2
Prophase
Prometaphase
Metaphase
Activation of
spindle assembly checkpoint:
Anaphase delay
Nucleus
Centrosome/
spindle pole
Inter-centrosomal
linker
Microtubule
Condensed
chromosome
Kinetochore
P
Phosphorylated HSP72
ch-TOG/TACC3 complex
Stable K-fibre
Unstable K-fibre