Oncogene
February 2013, Volume 32, Pages 910-919
http://dx.doi.org/10.1038/onc.2012.98

P21-activated kinase 4 (PAK4) is required for metaphase spindle positioning and anchoring

G. Bompard[1, 2, a, *], G. Rabeharivelo[1, 2], J. Cau[1, 3], A. Abrieu[1, 2], C. Delsert[1, 2, 4] and N. Morin[1, 2]

[1] Universités Montpellier 2 et 1, Montpellier, France
[2] CRBM, CNRS, UMR 5237, Montpellier, France
[3] MRI, IGH, CNRS UPR1142, Montpellier, France
[4] FREMER, LGP, la Tremblade, France.

[a] Current address: IGH, CNRS UPR1142, 141, rue de la Cardonille, 34396 Montpellier, France.

*: Corresponding author : Nathalie Morin, email address : nathalie.morin@crbm.cnrs.fr

Abstract:

The oncogenic kinase PAK4 was recently found to be involved in the regulation of the G1 phase and the G2/M transition of the cell cycle. We have also identified that PAK4 regulates Ran GTPase activity during mitosis. Here, we show that after entering mitosis, PAK4-depleted cells maintain a prolonged metaphase-like state. In these cells, chromosome congression to the metaphase plate occurs with normal kinetics but is followed by an extended period during which membrane blebbing and spindle rotation are observed. These bipolar PAK4-depleted metaphase-like spindles have a defective astral microtubule (MT) network and are not centered in the cell but are in close contact with the cell cortex. As the metaphase-like state persists, centrosome fragmentation occurs, chromosomes scatter from the metaphase plate and move toward the spindle poles with an active spindle assembly checkpoint, a phenotype that is reminiscent of cohesion fatigue. PAK4 also regulates the acto-myosin cytoskeleton and we report that PAK4 depletion results in the induction of cortical membrane blebbing during prometaphase arrest. However, we show that membrane blebs, which are strongly enriched in phospho-cofilin, are not responsible for the poor anchoring of the spindle. As PAK4 depletion interferes with the localization of components of the dynein/dynactin complexes at the kinetochores and on the astral MTs, we propose that loss of PAK4 could induce a change in the activities of motor proteins.

Keywords: p21-activated kinase 4 ; metaphase ; spindle orientation ; spindle assembly checkpoint

1. Introduction

At the onset of mitosis, cells undergo massive cytoskeleton rearrangements that allow subsequent assembly of a bipolar mitotic spindle, accurate segregation of chromosomes and completion of cytokinesis. Both the actin and the microtubule (MT) networks must coordinate to ensure proper division and partitioning of the two daughter cells. The assembly of the mitotic spindle requires centrosome duplication and an increase in both the nucleation and dynamics of MTs. Progression

from prometaphase to metaphase is signaled by chromosome congression to the metaphase plate and formation of the bipolar spindle, that is anchored to the cell cortex through astral MTs. A surveillance mechanism, the mitotic checkpoint, is active until all kinetochores are correctly attached to the MTs emanating from spindle poles. Mispositioning of the spindle inside the cell body induces spindle rotation and delays anaphase onset. Once attachment and tension are properly achieved, the spindle assembly checkpoint (SAC) is silenced and chromosome segregation starts with the onset of anaphase.

PAKs are serine/threonine kinases that were first described as effectors of the small GTPases Rac and Cdc42. PAKs are divided in two subgroups with similar organization but different activation mechanisms, and are believed to have different substrate specificity in vivo. PAKs regulate many cellular functions, including cell survival, morphology, adhesion and motility, in part through the control of both actin and MT networks. During mitosis, PAK1 (subgroup I) regulates the G2/M transition and phosphorylates histone H3, Aurora-A and Plk1 kinases.[1 – 3] Furthermore, overexpression of PAK1 in breast cancer cells is linked to the formation of abnormal spindles. In the subgroup II, PAK4 is highly oncogenic, promotes anchorage-independent growth[4] and dominant-negative PAK4 can inhibit transformation induced by Ras.[5] Consistent with these effects, PAK4 is overexpressed in many tumor cells and promotes tumorigenesis in mammals.[6]

PAK4 regulates the G1 phase[7] and G2/M transitions.[8] We report here that PAK4 is also required for efficient mitotic progression. We show that PAK4 regulates spindle positioning in the cell during mitosis. PAK4 depletion does not interfere with chromosome congression to the metaphase plate. Loss of PAK4 results in a long metaphase-like arrest characterized by spindle rotation. Depending upon the state of adhesion of the cells, chromosome segregation and normal anaphase will eventually resume. Alternatively, centrosome fragmentation will occur while a subset of chromosomes detach from the metaphase plate, inducing a scattered chromosome phenotype with active SAC. PAK4 depletion induces extensive membrane blebbing, but phospho-cofilinmediated regulation of cortical actin, which is required for spindle anchoring, is not affected by PAK4 depletion, and blebbing inhibition does not prevent PAK4-induced mitotic spindle defects. In PAK4-depleted cells, bipolar metaphase spindles are not properly centered in the cells. This probably results from the poor anchoring of the spindle owing to defective astral MTs. Even though EB1 shows normal staining the MT plus-end tips, the $p150^{Glued}$ subunit of dynactin does not. Furthermore, dynein does not efficiently localize at kinetochores, and MT attachment to kinetochores is also defective. We propose that mislocalization of components of the dynein/dynactin complex on the spindle mediates mitotic phenotypes induced by PAK4 depletion.

RESULTS

PAK4 depletion lengthens mitosis

We have previously shown that upon release of HeLa cells synchronized at the G2/M transition by Cdk1 inhibitor RO3306, PAK4 depletion induces a G2/M block.[8] We further addressed here the functions of PAK4 later in mitosis by analyzing Luciferase- (Luc, control) or PAK4-depleted nonsynchronized HeLa–H2B–GFP by time lapse videomicroscopy. Once cells enter mitosis, chromosome alignment on the metaphase plate occurs with similar kinetics in Luc- or PAK4-depleted cells (Supplementary Figure 1A-B and Supplementary Movies 1–2). After completion of chromosome alignment, most of the Luc-depleted cells entered anaphase within 20–30 min (Figure 1a and Supplementary Movie 3). In contrast, PAK4-depleted cells remained for extended time in a metaphase-like state. These cells displayed strong cortical activity characterized by the formation of very dynamic membrane blebs (arrowhead) and by the rotation of the spindles visualized by movements of H2B bound chromosomes on the metaphase plate (arrows). Eventually, cells entered anaphase, with delays that were over 60 min for more than 50% of the cells, and completed mitosis (Figures 1a and b and Supplementary Movie 4). In addition, we noticed that 72 h post small interfering RNA (siRNA) transfection many PAK4-depleted cells detached from the plate. As PAK4 regulates both adhesion and apoptosis,[9,10] we performed an MTT viability test on Luc- and PAK4-depleted cells and found eight times more viable cells present in the supernatant of PAK4-depleted compared with control cells (Supplementary Figure 1C). These non-adhering cells are mostly defective mitotic cells with scattered chromosomes (data not shown). Plating PAK4-depleted cells on fibronectin significantly reduced the number of cells in suspension (Supplementary Figure 1C), but did not change the behavior of Luc-depleted cells (Figure 1a and Supplementary Movie 5). Under these conditions, a similar metaphase to anaphase delay accompanied by cell blebbing was observed in PAK4-depleted cells (Figures 1a and b). In addition, in up to 20% of the cells, the prolonged metaphase state resulted in one or several chromosomes breaking loose from the established metaphase plates and scattering on the spindle (Figure 1a (arrowhead), Supplementary Movie 6 and Table 1).

To examine whether PAK4 depletion-induced mitotic lengthening agreed with biochemical analyses, PAK4 and Luc-interfered cells were synchronized in prometaphase with nocodazole (NZ) and harvested at different time points upon release of NZ (Figure 1c). The efficiency of the PAK4 siRNA described earlier[8] was at least 95% (Figure 1c). In prometaphase (0 min), an electrophoretic mobility retardation corresponding to hyperphosphorylation of the Cdc27 subunit of the anaphase promoting complex and phospho-histone H3 signals (H3 Ser10P) were similar in Luc- and PAK4-depleted cells. Cdc27 and histone H3 dephosphorylation indicated that the metaphase to anaphase transition had started by 45 min and was completed by 90 min in control cells. In contrast, in PAK4-depleted cells, significant levels of phospho-Cdc27 and phospho-histone H3 were still visible after 90 and even 180 min NZ release. This delay in mitotic exit provoked by the loss of PAK4 was further confirmed by the expression of survivin that usually declines in G1. Survivin level declined by 180 min in Luc-depleted cells, whereas it remained stable upon PAK4 depletion. The prolonged mitotic state was confirmed by a strong phospho-histone H3 signal that was detected in PAK4-depleted but not control depleted asynchronous cells (Figure 1d).

Thus, in addition to the G2/M delay induced in PAK4-depleted cells that we reported earlier, we show here that PAK4 is required for efficient mitotic progression.

Characterization of defective PAK4-depleted metaphase spindles

A number of PAK4 substrates are involved in the regulation of MT and actin cytoskeletons.[11–13] The Xenopus PAK4 ortholog also monitors the dynamics of actin and MTs in interphase cells[14] and of MTs in mitotic egg extracts.[8] We thus analyzed the tubulin and actin cytoskeleton in Luc- and PAK4-depleted cells (Figure 2a). Although bipolar metaphase MT spindles were observed in PAK4-depleted cells, their astral MT array was very poorly organized and appeared not to make as many effective contacts with the cell cortex when compared with the control. Spindle poles appeared to be flattened, more diffuse and, as observed by live imaging, spindles were often off-centered, being in close contact with the actin cell cortex, and were not oriented parallel to the substratum (Figure 2a and Table 1).

To better analyze these phenotypes, we costained Luc- and PAK4-depleted cells with several components of the MT spindle. Spindle poles stained for γ-tubulin are shown as serial pictures of a Z-stack (Figure 2b). The two γ-tubulin spots were focused in the same sections of a serial stack in controls, but were often separated by more than 8 μ in different Z sections in PAK4-depleted cells (Figures 2b and Table 1). In these cells the angle between the substrate plane and the long spindle axis varied from 0.4 to 54 degrees compared with 0 to 4.5 degrees in control cells (see also Table 1) further confirming that PAK4 expression is required for the correct and stable orientation of the spindle parallel to the substratum. Finally, we noticed that in contrast to Luc-depleted cells, γ-tubulin staining was very diffuse in PAK4-depleted cells and resolved in several dots at one or both poles as seen on projection of the stack images (Figure 2b, arrowheads and Table 1) indicating that PAK4 may be required for centrosome cohesion and integrity.

As correct positioning of the MT spindle depends upon interaction of astral MTs with specific areas of the cell cortex, we analyzed the localization of the plus-end tracking MT protein EB1 that promotes MT growth and association with the cell cortex.[15] In control cells, EB1 is associated with the mitotic spindle and decorates the tips of long astral MTs that interact with the cell cortex (Figure 2c and inset for higher magnification (arrowhead)). In PAK4-depleted cells, EB1 is correctly loaded on MTs and tracks MT plus-end tips, but the astral MTs mostly remained short or even absent on spindles located near the cell cortex (Figure 2c and inset, arrowhead). Membrane blebs are usually devoid of astral MTs (Figure 2c, arrow), although occasionally some MTs could be seen spanning the entire diameter of the cell cytoplasm (data not shown).

In conclusion, PAK4-depleted metaphase cells are characterized by one or several of the following defects: blebbing, spindle rotation, chromosome lagging and loss of centrosome integrity (Table 1).

Cotransfection of PAK4 siRNA with HA–GST or HA–PAK4wt shows that PAK4wt largely rescued the different defective mitotic phenotypes (see normal metaphases in Table 2). In the remaining defective spindles, the number of defects was also reduced, demonstrating that the loss of PAK4 is responsible for all of the above described mitotic phenotypes (Table 2). Rescue was also confirmed by the detection of a strong phospho-histone H3 signal in PAK4-depleted cells expressing HA–GST, while reexpression of wt PAK4 reduced the signal to a basal level (Figure 2d).

The prolonged metaphase-like state upon PAK4 depletion results in chromosome scattering with persistant active checkpoint

In agreement with live observations, elongated spindles reminiscent of the anaphase spindles but lacking the MT midzone were observed in fixed PAK4-depleted cells. Instead of the two sets of separating chromosomes seen in control anaphase, chromosomes were often organized as three independant groups, one in the center of the spindle and the two others at the opposite poles of the spindle and in very close contact with the cortical region (Supplementary Figure 2, chromosome scattering in Table 1). EB1 staining was again properly localized on spindle MTs and to the short astral MTs (Supplementary Figure 2A, arrowheads).

NPG.ONC.ONC201298

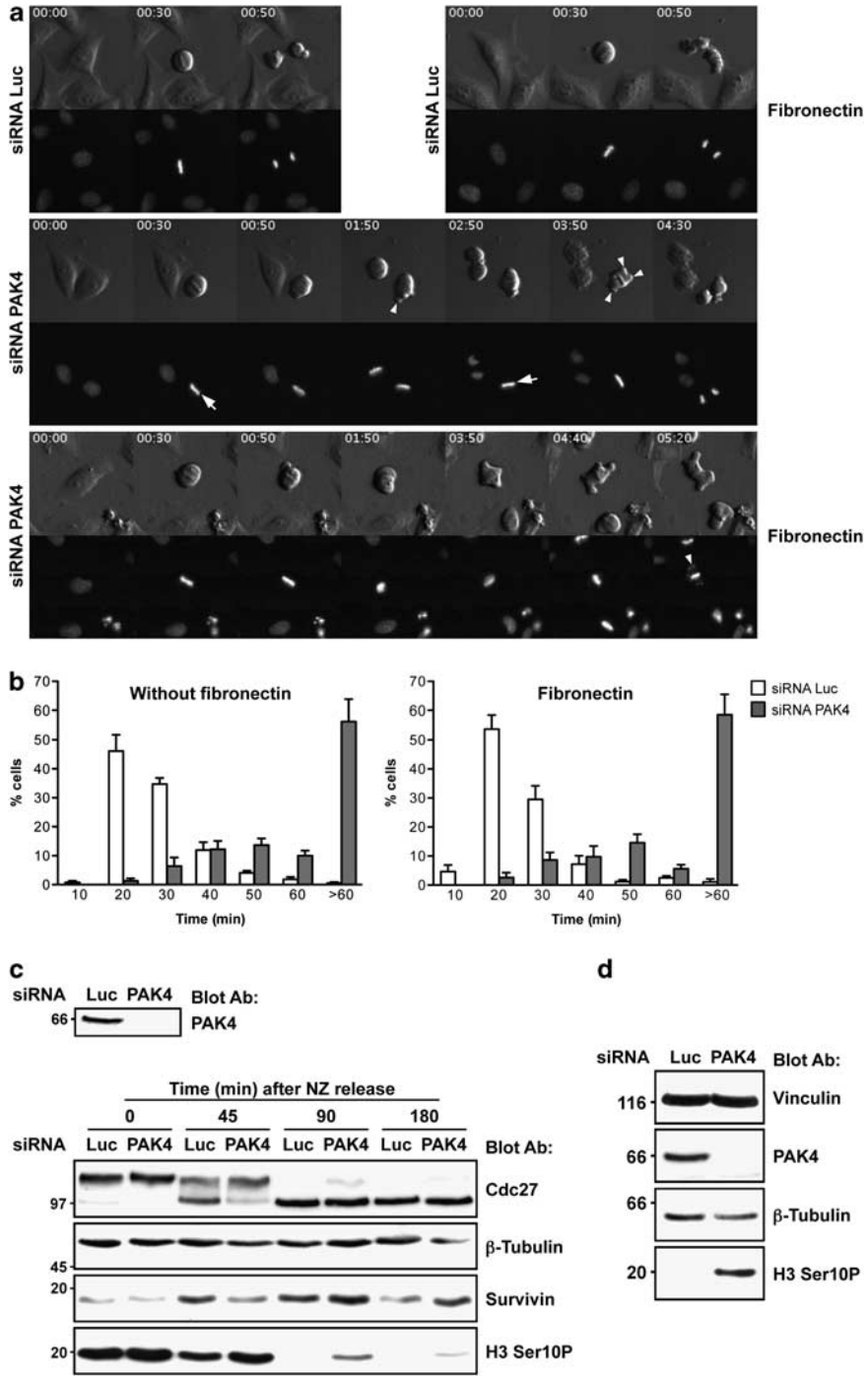

Figure 1. PAK4 depletion lengthens the mitotic state and induces chromosome scattering. (**a**) Luc- and PAK4-depleted Hela-H2B GFP cells plated on uncoated surface or on fibronectin were analyzed 72 h post siRNA transfection. Cells were monitored using cell light (LMC, top) and cell fluorescence (bottom) imaging, taking images every 10 min as indicated. Arrows indicate, (PAK4-depleted Hela-H2B GFP), rotating metaphase plate. Arrowheads indicate, (PAK4-depleted Hela-H2B GFP cells), membrane blebbing. Arrowheads indicate, (PAK4-depleted Hela-H2B GFP cells plated on fibronectin), chromosomes scattering. (**b**) Timing of metaphase to anaphase transition, in Luc- and PAK4-depleted cells, plated on fibronectin-coated or uncoated dishes before imaging, was determined by visual inspection of H2B signals and classified by time length intervals as cells percentage. Error bars are s.e.m. (**c**) Representative immunoblot analyses of protein extracts at different time points after NZ release of Luc- and PAK4-depleted HeLa cells using indicated antibodies, β-tubulin was used as a loading control. Protein extracts at the 0 time point were further analyzed by immunoblot for PAK4 depletion following Luc or PAK4 siRNA transfection. (**d**) Representative immunoblot analyses of protein extracts from asynchronous Luc- and PAK4-depleted Hela cells were analyzed as in (**b**).

In addition, the majority of these spindles presented several poles, as shown by γ-tubulin staining (Supplementary Figure 2B, arrowheads), but only two dots were stained by centrin (data not shown) indicating that the prolonged metaphase-like delay exacerbated the centrosome fragmentation process rather than causing centriole splitting.

To verify the mitotic status of these spindles with scattered chromosomes, Luc- and PAK4-depleted cells were costained for

NPG.ONC.ONC201298

Q2

Table 1

siRNA	*Luc*	*PAK4*
Blebbing mitotic cells[a,b] $P = 0.0022$	3.2 ± 0.54 ($n = 592$)	63.4 ± 5.58 ($n = 471$)
Off-centered spindles[c] $P = 0.0037$	16.8 ± 0.68 ($n = 71$)	71.9 ± 0.22 ($n = 114$)
Multipolar spindles[c,d] $P = 0.0011$	1.6 ± 0.64 ($n = 739$)	23.6 ± 2.85 ($n = 1195$)
Chromosome scattering[c] $P < 0.001$	4.4 ± 0.79 ($n = 739$)	23.8 ± 1.63 ($n = 1195$)
Spindle angle[e] $P < 0.001$	$1.1^\circ \pm 0.94$ ($n = 31$)	$12^\circ \pm 8.05$ ($n = 49$)

Abbreviations: Luc, Luciferase; siRNA, small interfering RNA. Numbers are expressed as a percentage of total metaphase/metaphase-like fixed cells plated on fibronectin. Numbers are means of at least three-independent experiments ± s.e.m. n = total cells counted. Student's t-test was performed to determine P values. [a]Expressed as a percentage of total mitotic (pre-anaphase) cells. [b]Determined from live cell imaging. [c]Expressed as a percentage of total metaphase like cells. [d]Spindles with more than 2 g-Tubulin foci. [e]Average spindle angles to the substratum.

spindle checkpoint proteins and chromosomal passenger complex proteins. In control cells, the staining with the centromere antibody CREST remains associated with chromosomes in metaphase and anaphase (Supplementary Figure 2C). Spindle checkpoint proteins Mad2 and BubR1 localize to the kinetochore of metaphase chromosomes and their staining is lost following perfect bipolar attachment of all kinetochores and checkpoint release (Figure 3a and Supplementary Figure 2C, arrowheads). Aurora-B kinase, which belongs to the chromosome passenger complex, relocalizes from the metaphase plate to the central spindle at anaphase in control cells (Figure 3b). In contrast, Mad2, BubR1 and Aurora-B remained bound to the scattered chromosomes of the PAK4-depleted spindle, confirming that they did not undertake the metaphase/anaphase transition (Figures 3a and b and Supplementary Figure 2C, arrowheads). Thus, despite their elongated morphology, PAK4-depleted spindles have the characteristics of prometaphase spindles with an active checkpoint.

To better visualize MT/kinetochore interactions, siRNA-transfected cells were cooled at 4 °C for 15 min to induce depolymerization of astral and polar MTs. MT-kinetochore interactions were evaluated on the cold stable kinetochore fibers by CREST and β-tubulin costaining. In Luc-depleted cells, cold treatment revealed stable kinetochore MT bundles, and bipolar attachment of the chromosomes to the spindle was easily seen on the serial images of the Z stack (Figure 3c and inset of slice 6, arrows). In contrast in PAK4-depleted metaphase-like (not shown) and elongated spindles, monopolar and abnormal attachment of kinetochores are seen (Figure 3c and inset of slice 16, arrow). These results demonstrate that in PAK4-depleted cells, proper bi-oriented attachment of chromosomes was not complete, resulting in prolonged SAC signaling.

As PAK4 depletion induces both defective astral MT/cortex and MT/KT interactions, we next analyzed the localization of the MT minus-end directed motor dynein and dynactin complexes that are important for MT attachment to the cortex and kinetochores and for poleward transport of checkpoint proteins from the kinetochores at the metaphase/anaphase transition.

PAK4 depletion modulates dynein/dynactin complex localization

Depletion/disruption of several members of the dynein/dynactin complexes or of interacting partners were shown to induce prometaphase arrest and spindle rotation, similar to PAK4 depletion.[16–20]

In control prometaphase cells, cytoplasmic dynein distributes to the spindle and to dot-like structures that are probably kinetochores (Figure 4a, arrowhead).[15,21] Kinetochore staining is lost at metaphase, as chromosomes become properly bioriented, and dynein/dynactin complexes migrate (Figure 4a, arrowheads) together with checkpoint proteins (data not shown),[22,23] along MTs towards the poles and the cortex. In anaphase, dynein staining becomes diffuse and associates with the central spindle (Figure 4a, arrowhead). In PAK4-depleted prometaphase cells, kinetochore staining was conserved (Figure 4a, arrowhead), but a strong increase of spindle pole staining was also observed (arrow). In metaphase-like cells, very punctate dynein signal accumulated around the spindle pole region (arrowhead) and was enriched at specific foci (arrow). In addition, membrane blebs were from time to time decorated by not only dynein but also γ-tubulin (data not shown). In spindles with lagging chromosomes, dynein localized along the MTs and to the spindle pole region, partially colocalizing with scattered γ-tubulin signals, and to some extent to fibrillous structures, in close contact with chromosomes and the cell cortex (Figures 4a, arrowhead).

As dynein staining is enriched at spindle poles in PAK4-depleted prometaphase cells, we asked whether its recruitment at the kinetochore was efficient. Prometaphase kinetochore-bound dynein interacts with Spindly and the RZZ complex through the ZW10 subunit.[24] The Zwilch subunit of the RZZ complex and Spindly localize to PAK4-depleted prometaphase kinetochores and remain bound to scattered chromosomes demonstrating that proteins involved in regulating dynein recruitment to the kinetochores are not affected by PAK4 depletion (Supplementary Figures 3A and B).

p150^{Glued} is part of the dynactin complex that links dynein to its cargoes, and is essential for most dynein functions, except its recruitment to the KT. In addition, p150^{Glued} with other CapGly plus-end MTs regulate astral MT interactions with the cell cortex[25,26] and positioning of cortical dynein/dynactin complex to the cell cortex generates forces on astral MTs to position the mitotic spindle.[27–29] In control and PAK4-depleted metaphase cells, p150^{Glued} localization to the spindle pole region was similar, but a fraction of p150^{Glued} also accumulated in PAK4-induced membrane blebs (Figure 4b, arrow). In addition, we found that p150^{Glued} only poorly decorated astral MTs and their plus-end tips in PAK4-depleted cells (Figure 4c). Western blot analyses show that mislocalization of p150^{Glued} and intermediate chain of dynein does not reflect a defective accumulation of these components in PAK4-depleted cells (Supplementary Figure 3C).

Taken together, our results indicate that PAK4 depletion does not prevent the initial recruitment of proteins of the RZZ complex, nor of Spindly that are required for dynein localization at kinetochores. Both dynein and dynactin mislocalizations during mitosis could be the priming events for the observed loss of spindle anchorage to the cortex and loss of proper kinetochore bioriented attachment to MT in PAK4-depleted cells.

Phospho-cofilin (Ser3) rich membrane blebs in PAK4-depleted metaphase-like cells are not responsible for the defective mitotic phenotype

Membrane blebs in metaphase-like PAK4-depleted cells are usually devoid of astral MTs. We thus wondered whether blebbing could initiate the PAK4 depletion-induced mitotic phenotype by disrupting the astral MT/cortex interaction, and cause spindle delocalization, rotation and loss of spindle tension.

Membrane blebs can be initiated by a rupture of the cortical actin network and often results from an overactivation of myosin II and actomyosin contraction (reviewed in Charras and Paluch[30]). During mitosis, stabilization of cortical actin depends upon Lim Kinase 1 phosphorylation of the actin severing factor cofilin.[31] As PAK4 mediates cofilin phosphorylation (Ser3) during

NPG.ONC.ONC201298

interphase,[32,33] we analyzed its putative function on cofilin phosphorylation in mitosis.

Actin and phospho-cofilin localization were analyzed on serial images from Z stacks. Actin filopodia decorate the plasma membrane of control cells (Figure 5a, arrowhead). In contrast, faint cortical actin staining decorated the blebs sprouting from the cell membrane as well as the base of the blebs in PAK4-depleted cells (Figure 5a, arrowhead). Phospho-cofilin relocated throughout the cytoplasm of control metaphase cells (Figure 5a).[31] Unexpectedly, phospho-cofilin was not lost but rather very enriched in the membrane blebs of PAK4-depleted cells (Figure 5A, arrow). This was confirmed by a slight increase of total cofilin phosphorylation in PAK4-depleted and prometaphase enriched HeLa cells (Figure 5b). The stabilization of phospho-cofilin, confirmed up to 180 min after NZ release, mimicked phospho-histone H3 and probably reflects the lengthened mitotic state induced by PAK4 depletion (Figure 5b). Thus, unlike in interphase, cofilin phosphorylation is not affected by PAK4 depletion during mitosis. As PAK4 also regulates MRLC phosphorylation,[34] we treated the cells with blebbistatin, a myosin II inhibitor previously used to inhibit blebbing.[35,36] The blebbing phenotype in PAK4-depleted cells was decreased from 80% ($n = 67$) in mock-treated cells to 20% ($n = 40$) following blebbistatin treatment. Phospho-cofilin was again found diffuse in the cytoplasm (compare with and without

Table 2

siRNA PAK4	*+ HA-GST*	*+ HA-PAK4 wt*
Normal metaphases $P = 0.0013$	31.7 ± 0.66	57.9 ± 0.66
Off-centered spindles	51.5 ± 7.1	19.8 ± 1.3
Blebbing mitotic cells	44.9 ± 5.1	20 ± 4.6
Spindles not parallel to the substratum	40.6 ± 5.5	15 ± 2.1
Multipolar spindles and/or chromosome scattering	43.6 ± 4.3	23.5 ± 2.1

Abbreviation: siRNA, small interfering RNA. Numbers are expressed as a percentage of total metaphase/metaphase like fixed cells plated on fibronectin. Numbers are means of three-independent experiments ± s.e.m. Total cells counted were $n = 148$ for rescue with HA-GST and $n = 162$ for rescue with HA-PAK4 wt.

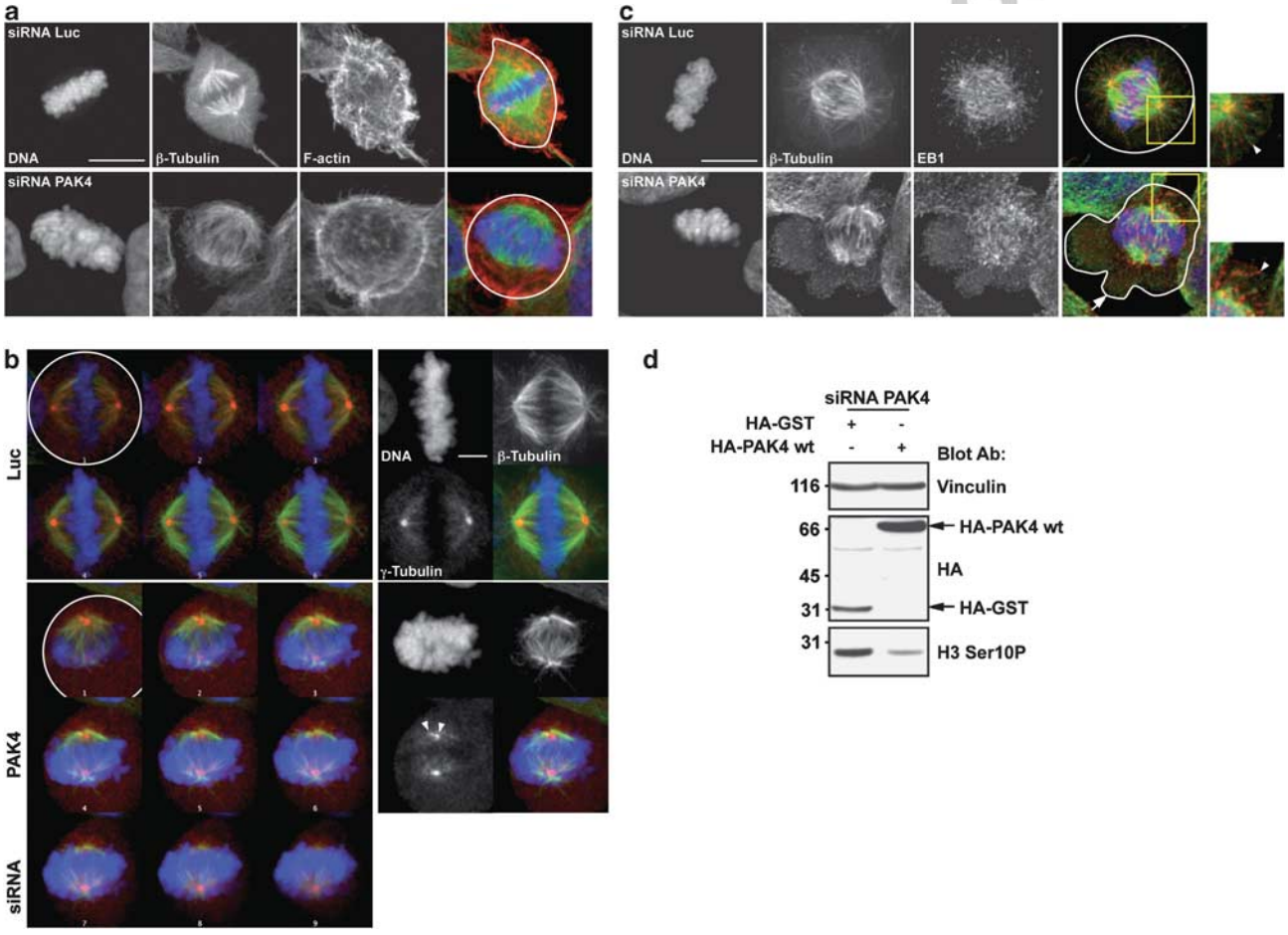

Figure 2. Depletion of PAK4 in HeLa cells results in the formation of defective metaphase spindles. Luc- and PAK4-depleted Hela cells were fixed 72 h post siRNA transfection, stained and representative metaphase cells were imaged. White outlines (**a**–**c**) highlight the cell cortex shape (**a**) Maximum intensity projections (MIP) of metaphase cells stained for DNA (blue), β-tubulin (green), F-actin (red) and merge of the three colors are shown. (**b**) Serial images (every 0.24 μm) of a Z-stack of a Luc- and PAK4-depleted metaphase cells stained for DNA (blue), β-tubulin (green), γ-tubulin (red) and merge of the three colors are shown to visualize spindle angle in PAK4-depleted metaphase-like cell. MIP images are on the right. Arrowheads show fragmented spindle poles. (**c**) MIP of metaphase cells stained for DNA (blue), β-tubulin (green), EB1 (red) and merge of the three colors are shown. Insets, on the merge images on the right (yellow squares) show magnified views of EB1 localizing at plus end tips of astral MTs (arrowheads). (**d**) Representative immunoblot analyses of protein extracts of HeLa cells cotransfected with siRNA PAK4 and either HA-GST or HA-PAK4 wt using HA-antibody to HA tag to verify expression of exogenous HA-tagged proteins, H3 Ser10P antibodies as a marker of PAK4 depletion rescue by HA-PAK4 wt and vinculin antibodies to demonstrate equal loading. Scale bars are 10 μm (**a**, **c**); 5 μm (**b**).

NPG.ONC.ONC201298

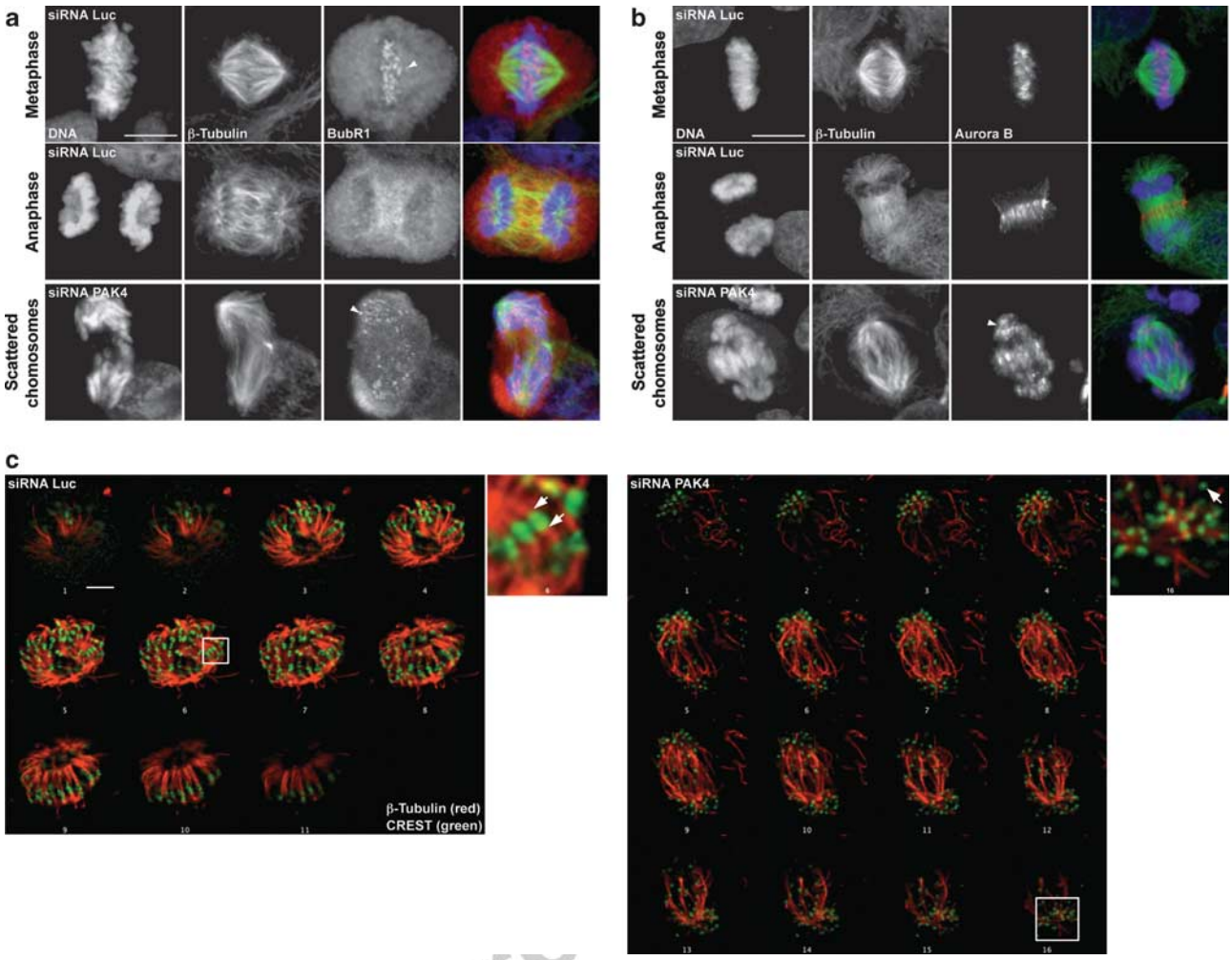

Figure 3. PAK4 depletion results in chromosome scattering, improper kinetochore/MT attachment and persistence of an active checkpoint. (**a**, **b**) Luc- and PAK4-depleted Hela cells were fixed 72 h post siRNA transfection, and stained using BubR1 antibodies to look at SAC (**a**) and Aurora B antibodies to look at chromosome passenger proteins (**b**). Metaphase and anaphase (siRNA Luc) and elongated spindles (siRNA PAK4) were imaged. Maximum intensity projection (MIP) of representative images is presented. Scale bars are 10 μm. (**c**) MT-kinetochore interactions were evaluated on the cold stable kinetochore fibers by centromeric CREST and β-tubulin costaining of Luc-depleted metaphase cell and PAK4-depleted elongated spindle cell. Serial images (every 0.24 μm) of a Z-stack with merge signals are shown. Insets of Luc-slice 6 and PAK4-slice 16 cells are shown to better visualize correct bioriented kinetochore attachment to MTs in Luc-depleted cell (arrows, inset) and monooriented abnormal kinetochore to MT attachment in PAK4-depleted cell (arrow, inset). Scale bar is 5 μm.

blebbistatin, Supplementary Figure 4). However, under these conditions, the lengthened mitosis (data not shown) and defective spindles (Supplementary Figure 4) still occurred. These results indicate that PAK4 regulates the actomyosin contraction pathway in mitosis, but that PAK4 depletion-dependent membrane blebbing is not responsible for PAK4 depletion-induced spindle defects.

DISCUSSION

Astral MTs and dynein/dynactin-dependent cortical pulling forces are major factors of an active surveillance mechanism that constantly regulates the correct positioning of the spindle in the center of mammalian cells.[29] Interfering with these pathways or micromanipulating the spindle position in the cell results in spindle rotation. In addition, bipolar attachment of kinetochores to MTs is required for the congression of chromosomes to the metaphase plate and normal transition to anaphase (reviewed in Musacchio and Salmon.[37]) Taken together, these studies show that the tension the metaphase spindle must attain for proper chromatid separation depends upon both cortical forces on astral MTs and on kinetochore-mediated forces acting on spindle poles.

In this report, we show that PAK4 is required for the assembly of the metaphase spindle. Depletion of PAK4 leads to a metaphase-like long arrest that is characterized by spindle rotation and extensive blebbing. Ultimately, fragmentation of spindle poles and chromosome scattering occur with an active SAC. Interestingly, while chromosome congression appeared to be normal, PAK4-depleted metaphase-like spindles start to rotate, a phenomenon accompanied by extensive blebbing.

We asked whether blebbing could mechanically disrupt the interaction between astral MTs and the cell cortex, preventing the establishement of a stable astral MT array and inducing the observed spindle defects. The cortical actin network that is required for astral MTs anchoring is regulated by Lim kinase 1-mediated inactivation of the severing factor cofilin. In interphase, Lim kinase 1 is a PAK4 substrate[32,33] and its depletion results in mitotic spindle rotation and spindle mispositioning.[31] However, we show that PAK4 does not regulate the phospho-cofilin level during mitosis nor the cortical actin network, as membrane blebs

NPG.ONC.ONC201298

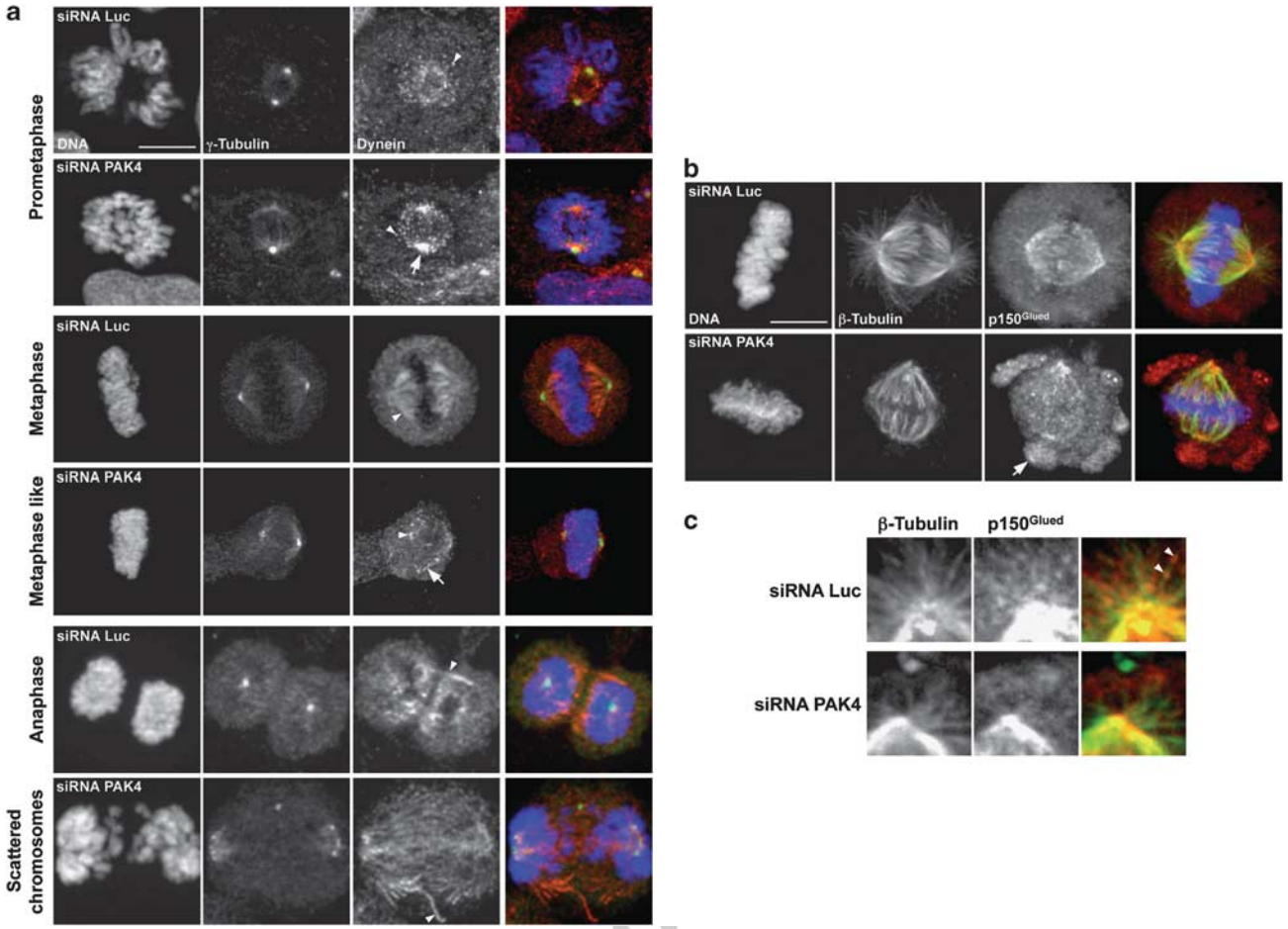

Figure 4. Membrane blebbing in PAK4-depleted metaphase-like cells is not responsible for PAK4-induced defective mitotic phenotype. (**a**) Luc- and PAK4-depleted Hela cells were fixed 72 h post siRNA transfection, and stained using cofilin Ser3P antibodies and phalloidin to visualize F-actin. Serial images (every 0.24 μm) of a Z-stack with merge signals are shown. Images on the right show DNA and β-tubulin staining of the first and last slices of the Z stack of the same cells help to visualize spindle angle. Scale bar is 5 μm. (**b**) Representative immunoblot analyses of protein extracts at different time points after NZ release of Luc- and PAK4-depleted HeLa cells using indicated antibodies, β-tubulin and total cofilin demonstrate equal loading. Cofilin Ser3P signals behave like histone H3 Ser10P in PAK4-depleted cells. Protein extracts at the 0 time point were further analyzed by immunoblot for PAK4 depletion following Luc or PAK4 siRNA transfection.

induced by the loss of PAK4 are rich in phospho-cofilin and present cortical actin at their base.

Blebbing often results from an overactivation of myosin II. Subgroup I PAK kinases may have opposite effects on myosin phosphorylation, as PAK1 can directly phosphorylate MRLC,[38–40] but also can impede MRLC phosphorylation via the phosphorylation of MLCK.[41,42]

Much less is known about subgroup II PAKs, but PAK4 was recently shown to increase myosin contractility during FcγR-mediated phagocytosis via phosphorylation of MRLC.[34] Here, we show that during mitosis, blebbistatin inhibits blebbing induced by the loss of PAK4, indicating that, as a subgroup I PAK, PAK4 can also negatively regulate myosin contractility. However, blebbing inhibition did not rescue PAK4 depletion-induced spindle defects, indicating that PAK4 targets several independent pathways during mitotic progression. We assume that membrane blebbing in PAK4-depleted metaphase-like cells is unlikely to be an early apoptotic event, as apoptotic membrane blebs are enriched in active cofilin and devoid of phospho-cofilin.[43] Instead, we propose that during metaphase, PAK4 may function in preventing premature blebbing, that normally occurs after anaphase onset to promote the generation of cortex/membrane required for cytokinesis.[44] Thus, blebbing is not the trigger that induces the spindle defects observed in PAK4-depleted cells.

Spindle positioning parallel to the substratum is dependent upon the integrin/Cdc42/PI3-kinase adhesion-mediated pathway, and PAK4 is involved in integrin-mediated cell adhesion and migration.[10,11] However, we hypothesize that the kinase is unlikely to regulate mitotic cell adhesion, as plating PAK4-depleted cells on fibronectin considerably reduced the levels of detached cells. In addition, disruption of the integrin pathway did not induce a persistent checkpoint signaling,[45,46] as we observed after PAK4 depletion.

Instead, the localization of active PAK4 to the centrosome and to the metaphase plate and its function in the regulation of MT dynamics in Xenopus egg extracts[8] led us to analyze MT behavior in PAK4-depleted mitotic cells.

Our data show that both astral MT and MT/kinetochore attachment are defective in PAK4-depleted spindles. Although EB1 correctly tracked the plus-end of defective astral MTs, $p150^{Glued}$ dynactin was strongly reduced at the MT plus-end tips. In addition, Dynein intermediate chain staining increased around the spindle pole region close to the cortex and decreased at the kinetochores of PAK4-depleted metaphase-like cells.

One of the consequences of the partial loss of proper localization of dynein and dynactin complexes in PAK4-depleted cells could be a reduced accumulation of centrosomal components that are involved in centrosome maturation, cohesion and MT anchoring in G2[47–49] and could explain the poor astral MT

NPG.ONC.ONC201298

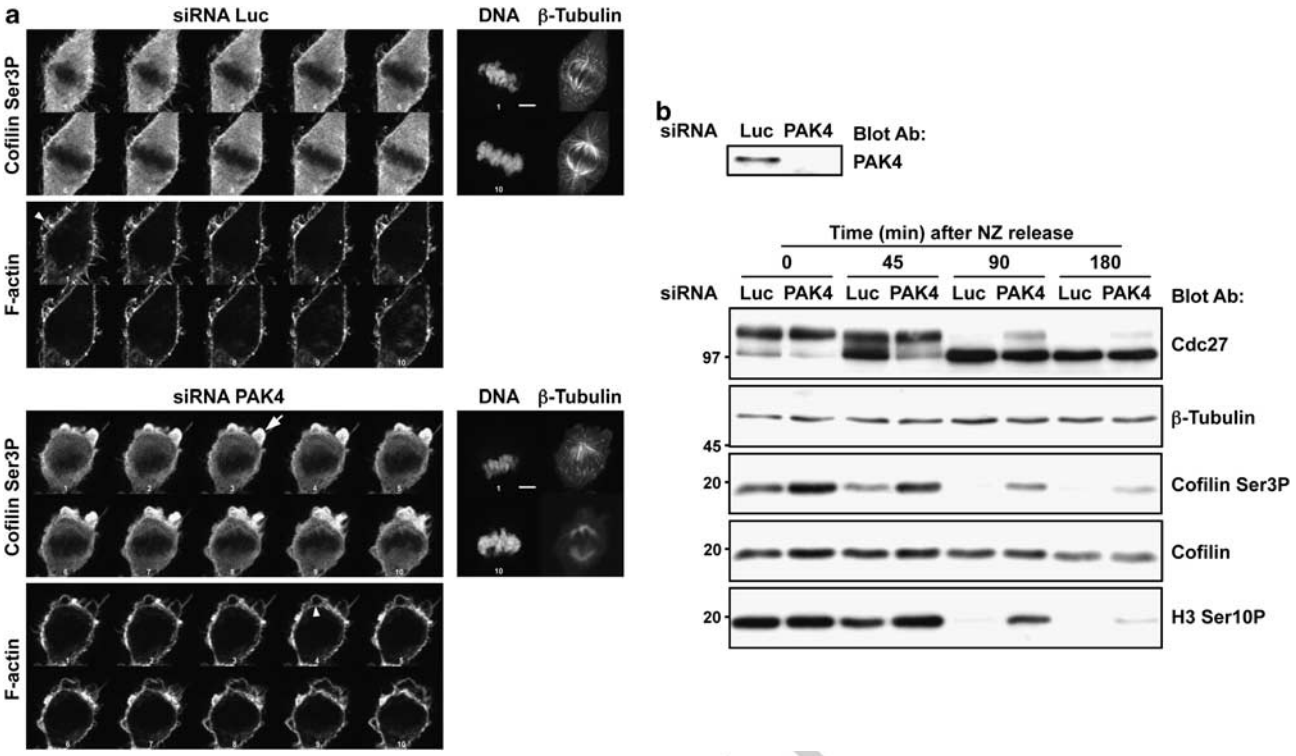

Figure 5. Components of dynein and dynactin complexes are not properly localized following PAK4 depletion. Luc- and PAK4-depleted Hela cells were fixed 72 h post siRNA transfection, stained and representative cells were imaged. Scale bars are 10 μm. (**a**) Maximum intensity projections (MIP) of prometaphase (siRNA Luc and PAK4), metaphase (siRNA Luc) and metaphase-like (siRNA PAK4), anaphase (siRNA Luc) and elongated spindle (siRNA PAK4) cells that were stained for DNA (blue), γ-tubulin (green), dynein intermediate chain (red) and merge of the three colors are shown. (**b**) MIP of metaphase (siRNA Luc) and metaphase-like siRNA PAK4 that were stained for DNA (blue), β-tubulin (green) $p150^{Glued}$ (red) and merge of the three colors are shown. Arrow and arrowhead in PAK4-depleted cell point to $p150^{Glued}$ enrichment, respectively, to membrane bleb and to spindle pole. (**c**) Higher magnification image of astral MTs from metaphase (siRNA Luc) and metaphase-like (siRNA PAK4) stained for β-tubulin (green) $p150^{Glued}$ (red). The merge image shows that $p150^{Glued}$ efficiently binds (arrowheads) to astral MTs in Luc- but not PAK4-depleted cell.

network that we observe in PAK4-depleted mitotic cells. In this context, forces generated by the spindle could mechanically stress the PAK4-depleted centrosomes with insufficient cohesion and induce fragmentation of spindle poles.

Spindly is required for dynein recruitment at kinetochores and expression of spindly motif mutants also induces metaphase-like arrest[19] similar to PAK4 loss. This phenotype, that ultimately results in a scattering phenotype with active checkpoint, was characterized by the uncoordinated loss of chromatid cohesion or cohesion fatigue, that results from the pulling forces of dynamic MTs on bioriented kinetochores.[50,51] Nonetheless, we show in this report that neither the kinetochore localization of Spindly nor of Zwilch subunit of the RZZ complex that is also required to anchor dynein at kinetochores are affected by PAK4 depletion.

In conclusion, our data indicate that during mitosis PAK4 regulates membrane blebbing, astral MTs, MT/kinetochore attachment and proper spindle tension. It is interesting to hypothesize that PAK4 could directly regulate dynein/dynactin complex activity by phosphorylation of one or several components of the complex. Dynamitin could be a potential candidate as this subunit of the dynactin complex is a substrate of drosophila PAK4 homolog MBT.[52] Dynamitin overexpression has been widely used to interfere with dynein activity, and moderate overexpression of dynamitin induces metaphase delays or arrest[16,24] similar to PAK4-induced mitotic defects. Yet another possibility would be that PAK4 acts on both the MT nucleation activity/integrity of centrosomes and/or MT/KT attachment via its known substrate Ran. We previously showed that active PAK4 is located together with phosphorylated Ran at centrosomes and that PAK4 regulates MT nucleation activity induced by active Ran GTPase in Xenopus egg extracts.[8] Indeed, interfering with components of the Ran network can induce loss of centrosome cohesion and appearance of multipolar spindles.[53–58] Thus, improper kinetochore attachment of MT in PAK4-depleted cells could result from reduced astral MTs, fragmentation of spindle pole and/or defective tension of the spindle. Alternatively, as active PAK4 is located on metaphase chromosomes[8] together with its substrate Ran, which is involved in SAC regulation[59] and stabilization of KT/MTs through HURP,[60,61] a PAK4/Ran pathway could regulate MT/kinetochore attachment.

In summary, we show that the oncogenic kinase PAK4 regulates mitotic progression and that this phenotype is not the consequence of defective cortical actin network. PAK4 depletion-induced mislocalization of components of dynein and dynactin complexes on the spindle could be a priming event for the loss of spindle anchorage to the cortex and proper kinetochore bioriented attachment to MT. Further investigations to identify the molecular targets of PAK4 on the spindle are ongoing and will greatly improve our understanding of spindle establishment and functions of PAK4 in tumorigenesis.

MATERIALS AND METHODS

Reagents and antibodies

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Mouse monoclonal antibodies against EB1, HA (12CA5), Mad2 (Santa Cruz), β-Tubulin, the intermediate chain (IC) of dynein, vinculin (Sigma-Aldrich), $p150^{Glued}$ Aurora B (AIM-1), BubR1 (BD Biosciences) and rabbit antibodies

NPG.ONC.ONC201298

directed against phospho-histone H3 (Ser10, Millipore), Survivin (Cell Signalling), Cofilin (Cystoskeleton), phospho-Cofilin (Ser3, Santa Cruz) were used. Human anti-nuclear-Centromere (CREST) antibodies were purchased from Europa Bioproducts. Spindly and Zwilch antibodies were kind gifts of, respectively, Dr A Desai and Dr A Musacchio. Rabbit polyclonal anti-PAK4 antibodies were described previously.[14] Rabbit polyclonal antibodies against Cdc27[62] and γ-Tubulin were, respectively, gifts from T Lorca and V Baldin (Montpellier, France). Polyclonal antibodies against β-Tubulin were a generous gift from Jose M Andreu (Centro de Investigaciones Biolgicas, Madrid, Spain).

DNA constructs

Coding sequences for GST and PAK4 wt were cloned into pRK5-HA, a gift from LM Machesky (Glasgow, UK), by a PCR approach using, respectively, pGEX-4T1 and pEGFP-C1 PAK4 wt as templates.[8]

Cell lines, immunofluorescence and synchronization

HeLa and HeLa H2B-GFP were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics.

Immunofluorescence experiments were performed as described.[8] Briefly, cells were fixed in methanol for 5 min at −20 °C or pre-extracted in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM $MgCl_2$, pH 6.9) buffer containing 0.2% Triton-X100 for 30 s prior methanol fixation for dynein staining using antibody directed against the intermediate chain. Secondary antibodies conjugated to Alexa 488 and 555 (Invitrogen) or fluorescein isothiocyanate (FITC, Sigma-Aldrich) were used. F-actin and DNA were, respectively, stained with phalloidin-Atto 647 and 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Cells were mounted in mowiol-containing ascorbic acid.

HeLa cells were synchronized in prometaphase using NZ as previously described.[8]

siRNA and rescue

siRNA were transfected into HeLa cells using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Proteins were targeted with the following sequences: PAK4 with 5′-GGUGAACAUGUAU GAGUGU-3′ and luciferase with 5′- CGUACGCGGAAUACUUCGA-3′. The PAK4 targeting sequence is a predesigned sequence recommended by Ambion's company and is located 344 nucleotides after PAK4 stop codon, absent from pRK5 HA-PAK4 wt construct. Consequently, the expressed mRNA is not sensitive to the designed siRNA. To evaluate mitotic progression in absence of PAK4, 24 h after siRNA transfection, cells were first incubated in medium containing 2.5 mM thymidine for 24 h and then released for 16 h in medium containing 40 ng/ml NZ. Prometaphase-arrested cells were collected by shake-off and lysed in RIPA derived lysis buffer (10 mM NaH_2PO_4, 100 mM NaCl, 5 mM EDTA, 1% Triton-X100, 0.5% NP-40, 80 mM β-Glycerophosphate, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail).

MTT cell proliferation assay

Supernatants from siRNA transfected HeLa cells cultured on uncoated dishes were collected 72 h after transfection, transfered to fibronectin coated dish and left to adhere 2 h at 37 °C. Vybrant® MTT reagent (Invitrogen) was added into the medium for 5 h following manufacturer's instructions. Cell viability was then evaluated by reading absorbance at 570 nm using an EL 808 absorbance microplate reader (Biotek).

Time-lapse microscopy

Time-lapse microscopy of HeLa H2B-GFP, grown on fibronectin or not coated surface, after 72 h siRNA transfection was performed using Leica DMIRE2 microscope with a LMC 20 × 0.4 lens and appropriate filters (Leica). Images were recorded with a cooled CCD Micromax camera (Princeton Instruments) driven by MetaMorph (Molecular Devices).

Fixed cells microscopy

Fixed cells and MT structures were viewed using a Zeiss Axioimager Z1 with Zeiss × 63 plan-apochromat 1.4 oil lenses. Micrographs were either collected using a Coolsnap HQ2 CCD camera (Roper scientific) driven by Metamorph 7.1 software (MT structures) or using an Axiocam Mrm camera with a structured illumination model (apotome) driven by Axiovision software.

Spindle angle measurements

Spindle angles measures derive from the measures of spindle poles positions that were taken from fixed and immunostained adherent cells. Poorly adherent cells were always lost at the washes and fixation steps unless they were centrifuged on the coverslip prior fixation.

To determine spindle angles, z-stack images were acquired from 0.24 μm thick sections of metaphase (Luc-) and metaphase-like (PAK4-) those were immunostained with anti γ-tubulin and anti β-tubulin antibodies. An imageJ software macro was developed to measure, the linear and vertical distances between the two poles of the spindles and the resulting spindle angle by inverse trigonometric function.

ABBREVIATIONS

PAK4, p21-activated kinase 4; MT, microtubules; KT, kinetochore; SAC, spindle assembly checkpoint.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Drs Lorca, Castro, Merdes for the gift of reagents. We are indebted to Dr A Desai, Dr K Vaughan and Dr A Musacchio for the kind gifts of reagents. We want to especially thank Dr Dan Fisher (IGMM, UMR5535 CNRS, Montpellier, France) for critical reading and editing of the manuscript. We thank the Montpellier RIO imaging facility. GB is supported by a grant from 'Fondation de la Recherche Médicale'. This work was supported by a grant MEGAPAK to NM from the ANR (Agence Nationale pour la Recherche) GENOPAT.

REFERENCES

1 Li F, Adam L, Vadlamudi RK, Zhou H, Sen S, Chernoff J *et al.* p21-activated kinase 1 interacts with and phosphorylates histone H3 in breast cancer cells. *EMBO Rep* 2002; **3**: 767-773.
2 Zhao ZS, Lim JP, Ng YW, Lim L, Manser E. The GIT-associated kinase PAK targets to the centrosome and regulates Aurora-A. *Mol Cell* 2005; **20**: 237-249.
3 Maroto B, Ye MB, von Lohneysen K, Schnelzer A, Knaus UG. P21-activated kinase is required for mitotic progression and regulates Plk1. *Oncogene* 2008; **27**: 4900-4908.
4 Whale A, Hashim FN, Fram S, Jones GE, Wells CM. Signalling to cancer cell invasion through PAK family kinases. *Front Biosci [Review]* 2011; **16**: 849-864.
5 Callow MG, Clairvoyant F, Zhu S, Schryver B, Whyte DB, Bischoff JR *et al.* Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines. *J Biol Chem* 2002; **277**: 550-558.
6 Liu Y, Xiao H, Tian Y, Nekrasova T, Hao X, Lee HJ *et al.* The pak4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice. *Mol Cancer Res* 2008; **6**: 1215-1224.
7 Nekrasova T, Minden A. PAK4 is required for regulation of the cell-cycle regulatory protein p21, and for control of cell-cycle progression. *J Cell Biochem* 2011; **112**: 1795-1806.
8 Bompard G, Rabeharivelo G, Frank M, Cau J, Delsert C, Morin N. Subgroup II PAK-mediated phosphorylation regulates Ran activity during mitosis. *J Cell Biol* 2010; **190**: 807-822.
9 Qu J, Cammarano MS, Shi Q, Ha KC, de Lanerolle P, Minden A. Activated PAK4 regulates cell adhesion and anchorage-independent growth. *Mol Cell Biol* 2001; **21**: 3523-3533.
10 Li Z, Lock JG, Olofsson H, Kowalewski JM, Teller S, Liu Y *et al.* Integrin-mediated cell attachment induces a PAK4-dependent feedback loop regulating cell adhesion through modified integrin alpha v beta 5 clustering and turnover. *Mol Biol Cell* 2010; **21**: 3317-3329.

NPG.ONC.ONC201298

11 Li Z, Zhang H, Lundin L, Thullberg M, Liu Y, Wang Y *et al.* p21-activated kinase 4 phosphorylation of integrin beta5 Ser-759 and Ser-762 regulates cell migration. *J Biol Chem* 2010; **285**: 23699 - 23710.
12 Wong LE, Reynolds AB, Dissanayaka NT, Minden A. p120-catenin is a binding partner and substrate for Group B Pak kinases. *J Cell Biochem* 2010; **110**: 1244 - 1254.
13 Ahmed T, Shea K, Masters JR, Jones GE, Wells CM. A PAK4-LIMK1 pathway drives prostate cancer cell migration downstream of HGF. *Cell Signal* 2008; **20**: 1320 - 1328.
14 Cau J, Faure S, Comps M, Delsert C, Morin N. A novel p21-activated kinase binds the actin and microtubule networks and induces microtubule stabilization. *J Cell Biol* 2001; **155**: 1029 - 1042.
15 Kobayashi T, Murayama T. Cell cycle-dependent microtubule-based dynamic transport of cytoplasmic dynein in mammalian cells. *PLoS One* 2009; **4**: e7827.
16 Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol* 1996; **132**: 617 - 633.
17 Zhou T, Zimmerman W, Liu X, Erikson RL. A mammalian NudC-like protein essential for dynein stability and cell viability. *Proc Natl Acad Sci USA* 2006; **103**: 9039 - 9044.
18 Ozaki Y, Matsui H, Nagamachi A, Asou H, Aki D, Inaba T. The dynactin complex maintains the integrity of metaphasic centrosomes to ensure transition to anaphase. *J Biol Chem* 2011; **286**: 5589 - 5598.
19 Gassmann R, Holland AJ, Varma D, Wan X, Civril F, Cleveland DW *et al.* Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells. *Genes Dev* 2010; **24**: 957 - 971.
20 Famulski JK, Vos LJ, Rattner JB, Chan GK. Dynein/Dynactin-mediated transport of kinetochore components off kinetochores and onto spindle poles induced by nordihydroguaiaretic acid. *PloS One* 2011; **6**: e16494.
21 Gehmlich K, Haren L, Merdes A. Cyclin B degradation leads to NuMA release from dynein/dynactin and from spindle poles. *EMBO Rep* 2004; **5**: 97 - 103.
22 Sharp DJ, Rogers GC, Scholey JM. Cytoplasmic dynein is required for poleward chromosome movement during mitosis in Drosophila embryos. *Nat Cell Biol* 2000; **2**: 922 - 930.
23 Yang Z, Tulu US, Wadsworth P, Rieder CL. Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *Curr Biol* 2007; **17**: 973 - 980.
24 Whyte J, Bader JR, Tauhata SB, Raycroft M, Hornick J, Pfister KK *et al.* Phosphorylation regulates targeting of cytoplasmic dynein to kinetochores during mitosis. *J Cell Biol* 2008; **183**: 819 - 834.
25 Gundersen GG, Gomes ER, Wen Y. Cortical control of microtubule stability and polarization [review]. *Curr Opin Cell Biol* 2004; **16**: 106 - 112.
26 Pearson CG, Bloom K. Dynamic microtubules lead the way for spindle positioning. *Nat Rev Mol Cell Biol* 2004; **5**: 481 - 492.
27 Carminati JL, Stearns T. Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. *J Cell Biol* 1997; **138**: 629 - 641.
28 Busson S, Dujardin D, Moreau A, Dompierre J, De Mey JR. Dynein and dynactin are localized to astral microtubules and at cortical sites in mitotic epithelial cells. *Curr Biol* 1998; **8**: 541 - 544.
29 O'Connell CB, Wang YL. Mammalian spindle orientation and position respond to changes in cell shape in a dynein-dependent fashion. *Mol Biol Cell* 2000; **11**: 1765 - 1774.
30 Charras G, Paluch E. Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* 2008; **9**: 730 - 736.
31 Kaji N, Muramoto A, Mizuno K. LIM kinase-mediated cofilin phosphorylation during mitosis is required for precise spindle positioning. *J Biol Chem* 2008; **283**: 4983 - 4992.
32 Dan C, Kelly A, Bernard O, Minden A. Cytoskeletal changes regulated by the PAK4 serine/threonine kinase are mediated by LIM kinase 1 and cofilin. *J Biol Chem* 2001; **276**: 32115 - 32121.
33 Soosairajah J, Maiti S, Wiggan O, Sarmiere P, Moussi N, Sarcevic B *et al.* Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin. *EMBO J* 2005; **24**: 473 - 486.
34 Bright MD, Frankel G. PAK4 phosphorylates myosin regulatory light chain and contributes to Fcgamma receptor-mediated phagocytosis. *Int J Biochem Cell Biol* 2011; **43**: 1776 - 1781.
35 Yanase Y, Carvou N, Frohman MA, Cockcroft S. Reversible bleb formation in mast cells stimulated with antigen is Ca2+/calmodulin-dependent and bleb size is regulated by ARF6. *Biochem J* 2010; **425**: 179 - 193.
36 Norman L, Sengupta K, Aranda-Espinoza H. Blebbing dynamics during endothelial cell spreading. *Eur J Cell Biol* 2011; **90**: 37 - 48.
37 Musacchio A, Salmon ED. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* 2007; **8**: 379 - 393.
38 Chew TL, Masaracchia RA, Goeckeler ZM, Wysolmerski RB. Phosphorylation of non-muscle myosin II regulatory light chain by p21-activated kinase (gamma-PAK). *J Muscle Res Cell Motil* 1998; **19**: 839 - 854.
39 Kiosses WB, Daniels RH, Otey C, Bokoch GM, Schwartz MA. A role for p21-activated kinase in endothelial cell migration. *J Cell Biol* 1999; **147**: 831 - 844.
40 Brzeska H, Szczepanowska J, Matsumura F, Korn ED. Rac-induced increase of phosphorylation of myosin regulatory light chain in HeLa cells. *Cell Motil Cytoskeleton* 2004; **58**: 186 - 199.
41 Sanders LC, Matsumura F, Bokoch GM, de Lanerolle P. Inhibition of myosin light chain kinase by p21-activated kinase. *Science* 1999; **283**: 2083 - 2085.
42 Goeckeler ZM, Masaracchia RA, Zeng Q, Chew TL, Gallagher P, Wysolmerski RB. Phosphorylation of myosin light chain kinase by p21-activated kinase PAK2. *J Biol Chem* 2000; **275**: 18366 - 18374.
43 Mannherz HG, Gonsior SM, Gremm D, Wu X, Pope BJ, Weeds AG. Activated cofilin colocalises with Arp2/3 complex in apoptotic blebs during programmed cell death. *Eur J Cell Biol* 2005; **84**: 503 - 515.
44 Charras GT, Hu CK, Coughlin M, Mitchison TJ. Reassembly of contractile actin cortex in cell blebs. *J Cell Biol* 2006; **175**: 477 - 490.
45 Toyoshima F, Matsumura S, Morimoto H, Mitsushima M, Nishida E. Ptdlns(3,4,5)P3 regulates spindle orientation in adherent cells. *Dev Cell* 2007; **13**: 796 - 811.
46 Mitsushima M, Toyoshima F, Nishida E. Dual role of Cdc42 in spindle orientation control of adherent cells. *Mol Cell Biol* 2009; **29**: 2816 - 2827.
47 Zimmerman W, Doxsey SJ. Construction of centrosomes and spindle poles by molecular motor-driven assembly of protein particles. *Traffic* 2000; **1**: 927 - 934.
48 Dammermann A, Merdes A. Assembly of centrosomal proteins and microtubule organization depends on PCM-1. *J Cell Biol* 2002; **159**: 255 - 266.
49 Goshima G, Nedelec F, Vale RD. Mechanisms for focusing mitotic spindle poles by minus end-directed motor proteins. *J Cell Biol* 2005; **171**: 229 - 240.
50 Stevens D, Gassmann R, Oegema K, Desai A. Uncoordinated loss of chromatid cohesion is a common outcome of extended metaphase arrest. *PloS One* 2011; **6**: e22969.
51 Daum JR, Potapova TA, Sivakumar S, Daniel JJ, Flynn JN, Rankin S *et al.* Cohesion fatigue induces chromatid separation in cells delayed at metaphase. *Curr Biol* 2011; **21**: 1018 - 1024.
52 Menzel N, Schneeberger D, Raabe T. The Drosophila p21 activated kinase Mbt regulates the actin cytoskeleton and adherens junctions to control photoreceptor cell morphogenesis. *Mech Dev* 2007; **124**: 78 - 90.
53 Nachury MV, Maresca TJ, Salmon WC, Waterman-Storer CM, Heald R, Weis K. Importin beta is a mitotic target of the small GTPase Ran in spindle assembly. *Cell* 2001; **104**: 95 - 106.
54 Di Fiore B, Ciciarello M, Mangiacasale R, Palena A, Tassin AM, Cundari E *et al.* Mammalian RanBP1 regulates centrosome cohesion during mitosis. *J Cell Sci* 2003; **116** (Part 16): 3399 - 3411.
55 Ciciarello M, Mangiacasale R, Thibier C, Guarguaglini G, Marchetti E, Di Fiore B *et al.* Importin beta is transported to spindle poles during mitosis and regulates Ran-dependent spindle assembly factors in mammalian cells. *J Cell Sci* 2004; **117** (Part 26): 6511 - 6522.
56 Wang W, Budhu A, Forgues M, Wang XW. Temporal and spatial control of nucleophosmin by the Ran-Crm1 complex in centrosome duplication. *Nat Cell Biol* 2005; **7**: 823 - 830.
57 Kalab P, Pralle A, Isacoff EY, Heald R, Weis K. Analysis of a RanGTP-regulated gradient in mitotic somatic cells. *Nature* 2006; **440**: 697 - 701.
58 Fan S, Fogg V, Wang Q, Chen XW, Liu CJ, Margolis B. A novel Crumbs3 isoform regulates cell division and ciliogenesis via importin beta interactions. *J Cell Biol* 2007; **178**: 387 - 398.
59 Dasso M. Ran at kinetochores. *Biochem Soc Trans* 2006; **34** (Part 5): 711 - 715.
60 Wong J, Fang G. HURP controls spindle dynamics to promote proper interkinetochore tension and efficient kinetochore capture. *J Cell Biol* 2006; **173**: 879 - 891.
61 Sillje HH, Nagel S, Korner R, Nigg EA. HURP is a Ran-importin beta-regulated protein that stabilizes kinetochore microtubules in the vicinity of chromosomes. *Curr Biol* 2006; **16**: 731 - 742.
62 Vigneron S, Brioudes E, Burgess A, Labbe JC, Lorca T, Castro A. Greatwall maintains mitosis through regulation of PP2A. *EMBO J* 2009; **28**: 2786 - 2793.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

NPG.ONC.ONC201298