JCS ePress online publication date 2 July 2003

Mammalian RanBP1 regulates centrosome cohesion during mitosis

Barbara Di Fiore[1], Marilena Ciciarello[1], Rosamaria Mangiacasale[1], Antonella Palena[1], Anne-Marie Tassin[2], Enrico Cundari[1] and Patrizia Lavia[1,*]

[1]CNR Institute of Molecular Biology and Pathology, Section of Genetics, c/o University 'La Sapienza', 00185 Rome, Italy
[2]Institut Curie, Section Recherche, UMR144-CNRS, 75248 Paris Cedex, France
*Author for correspondence (e-mail: patrizia.lavia@uniroma1.it)

Accepted 16 April 2003
Journal of Cell Science 116, 3399-3411 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00624

Summary

The Ran GTPase plays a central function in control of nucleo-cytoplasmic transport in interphase. Mitotic roles of Ran have also been firmly established in *Xenopus* oocyte extracts. In this system, Ran-GTP, or the RCC1 exchange factor for Ran, drive spindle assembly by regulating the availability of 'aster-promoting activities'. In previous studies to assess whether the Ran network also influences mitosis in mammalian cells, we found that overexpression of Ran-binding protein 1 (RanBP1), a major effector of Ran, induces multipolar spindles. We now show that these abnormal spindles are generated through loss of cohesion in mitotic centrosomes. Specifically, RanBP1 excess induces splitting of mother and daughter centrioles at spindle poles; the resulting split centrioles can individually organize functional microtubule arrays, giving rise to functional spindle poles. RanBP1-dependent centrosome splitting is specifically induced in mitosis and requires microtubule integrity and Eg5 activity. In addition, we have identified a fraction of RanBP1 at the centrosome. These data indicate that overexpressed RanBP1 interferes with crucial factor(s) that control structural and dynamic features of centrosomes during mitosis and contribute to uncover novel mitotic functions downstream of the Ran network.

Key words: RanBP1, Ran GTPase, Mitosis, Spindle pole, Centriole, Centrosome

Introduction

Mitosis is the critical time of the cell cycle, during which the genetic material is faithfully distributed among daughter cells. Errors during the mitotic division result in the uneven segregation of chromosomes, yielding aneuploid or polyploid cells. Such genomic imbalances are among the most common hallmarks of cancer and are regarded as crucial in tumor progression (Lengauer et al., 1998; Pihan and Doxsey, 1999). Correct assembly and function of the mitotic apparatus are therefore essential to ensure the balanced transmission of genetic information through cell division.

The Ran GTPase network has attracted increasing interest during the past 10 years as the major regulator of nucleo-cytoplasmic transport in interphase cells. The directionality of transport in and out of the nucleus has been shown to rely on the different distributions of nucleotide-bound forms of Ran in specific subcellular compartments: Ran-GTP is generated essentially in the nucleus, where the RCC1 nucleotide exchange factor resides, whereas factors activating GTP hydrolysis (RanGAP1 and RanBP1) are largely cytoplasmic (Clarke and Zhang, 2001; Hetzer et al., 2002; Dasso, 2002). Nuclear RanGTP promotes the dissociation of import complexes – and hence the release of nuclear proteins in the nucleoplasm – as well as the assembly of export complexes, which, conversely, mediate transport of cytoplasmic proteins and RNAs to the cytoplasm.

More recent evidence also indicate that the Ran system carries out mitotic regulatory functions after nuclear envelope breakdown (NEB). In *Xenopus*-oocyte-extract-based in vitro systems, RanGTP and RCC1 are required for the assembly of mitotic microtubule (MT) arrays in spindle-like structures (Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Carazo-Salas et al., 1999). This is largely due to the ability of GTP-bound Ran to regulate the release of active 'aster-promoting activities' (APAs), including NuMA and TPX2 (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). In the presence of low concentrations of RanGTP, APAs are sequestered in inactivating complexes with importin α and β; APAs need be released in the free form in the presence of RanGTP to promote spindle assembly. Thus, the functional role of Ran in nucleo-cytoplasmic transport and in spindle formation relies essentially on one same mechanism – the ability of RanGTP locally to dissociate macromolecular complexes formed by import vectors and their partners (Melchior, 2001; Dasso, 2002). In this framework, the ability of released NuMA and TPX2 to orchestrate spindle assembly is essentially determined by the redistribution of nuclear and cytoplasmic components after NEB. The underlying biochemical basis of RanGTP activity in transport and in mitosis is otherwise identical except for the different localization of molecules that act as downstream targets of the Ran system before and after NEB. Because RCC1 remains largely chromatin-bound throughout mitosis in *Xenopus* extract (Carazo-Salas et al., 1999) and in somatic cells (Guarguaglini et al., 2000; Moore et al., 2002), GTP exchange on Ran during mitosis is expected to take place near

chromosomes. Indeed, visual evidence for the bulk of RanGTP being concentrated near mitotic chromosomes has recently been provided (Kalab et al., 2002).

Different mechanisms underlie spindle assembly in mammalian somatic cells and in meiotic *Xenopus* extracts, despite of the high conservation of molecular components (Merdes and Cleveland, 1997). One obvious difference in spindle organization lies in the role played by centrosomes in somatic cells but not in meiotic extract. Centrosomes act as the major organizing centers for MT nucleation in somatic cells, and hence their function is intimately connected with the organization of spindle poles. Thus, specific aspects of Ran-controlled processes during mitosis might differ in these systems.

Evidence from living cells, albeit still fragmentary, clearly implicate the Ran network in control of spindle organization and function. Injection of anti-RanBP1 antibody in mitosis perturbs MT dynamics to the point of impairing complete chromosome segregation (Guarguaglini et al., 2000). Microinjection of a deleted importin β protein, lacking the Ran-binding domain, causes misassembly of the spindle and chromosome misalignment (Nachury et al., 2001). Aberrant chromosome alignment is also seen in cells overexpressing RanBP1, associated with the formation of multipolar spindles (Guarguaglini et al., 2000). Consistent with the absence of a specific checkpoint that would detect multipolar spindles (Sluder et al., 1997), these cells do not arrest at metaphase but progress to ana-telophase and segregate uneven groups of chromosomes. Similar defects have been reported following expression of a RCC1 mutant that mislocalizes to the mitotic cytoplasm (Moore et al., 2002). Thus, the Ran network, as well as regulating spindle assembly in the proximity of chromatin in the *Xenopus* system, also controls aspects of spindle function in mammalian cells, in which spindle pole formation and mitotic MT nucleation are directed from centrosomes. To achieve these functions, components of the Ran network might locally act at crucial mitotic locations in animal cells.

Here, we focus on mitotic functions of the RanBP1 protein in mammalian cells. Expression of the mammalian *RanBP1* gene varies during the cell cycle (Di Matteo et al., 1995; Di Fiore et al., 1999), with highest protein levels in G2 and M phases, and an abrupt decline in late telophase (Guarguaglini et al., 2000). As recalled above, RanBP1 overexpression yields abnormal mitotic spindles with multiple poles (Guarguaglini et al., 2000). To date, this is one of the clearest phenotypes visualized during the mammalian mitosis under alteration of Ran network components. We have now sought to identify the underlying defects of multipolar spindle formation.

Correct reproduction and structural organization of centrosomes are crucial for the establishment of the spindle bipolarity. Multipolar spindles that direct chromosome mis-segregation often form in consequence of centrosome overduplication during cell transformation (Lingle and Salisbury, 2000; Brinkley, 2001; Doxsey, 2001). Here, we report that RanBP1 does not influence the centrosome duplication cycle but instead induces a specific and distinct aberration (unscheduled splitting between mother and daughter centrioles at spindle poles). This process is specifically induced after NEB in a MT- and Eg5-dependent manner. Split centrioles retain the ability to anchor functional MT arrays and give rise to multipolar spindles that direct abnormal chromosome segregation. We also show that a RanBP1 fraction localizes to centrosomes. These results uncover a novel aspect of mitotic centrosome cohesion, the maintenance of which is important to ensure proper chromosome segregation, and indicate that this function is sensitive to RanBP1 levels.

Materials and Methods

Plasmid construction

The murine RanBP1 ORF was amplified by PCR from the pCMV-RanBP1 construct (Battistoni et al., 1997) using the following oligonucleotide sets: (i) CCGGAATTCATGGCTGCGCAGGG-AGAG and CGCGGATCCCAGGTCATCATCCTCATCCG, for ligation to the pEGFP-N1 and pDsRed1-N1 *Eco*RI/*Bam*HI-digested vectors (both from Clontech); and (ii) AGAATTCGTCGCGC-GCGCCCCCATGGCGGCCGCCAA and CGCCTCGAGCTAA-GCGTAGTCTGGGACGTCGTATGGGTATTGTTTCTCCTCAGAC-TTCTC, encoding the hemagglutinin (HA) epitope, for ligation to the basic pCMV vector (previously named pX) (Battistoni et al., 1997) after *Eco*RI/*Xho*I digestion. Ligation of the amplified products yielded the pRanBP1-GFP, pRanBP1-RFP and pRanBP1-HA expression constructs, carrying the chimaeric tags at the C-terminus of the RanBP1 sequence. By densitometric analysis of western blots, the tagged and untagged RanBP1 expression plasmids yield a similar increase (over fourfold) in levels of total RanBP1 protein compared to non-transfected or vector-transfected cells.

Cell culture and synchronization

Murine NIH/3T3 embryo fibroblasts (ATCC CRL-1658), murine L929 lung epithelial cells (ATCC CCL-1) and derived cell lines stably transfected with centrin 1-GFP (Piel et al., 2000) (kindly given by M. Bornens, Institut Curie, Paris), human HeLa epithelial cells (ATCC CCL-2), were all grown in DMEM (Dulbecco's Modified Eagle Medium, Euroclone) supplemented with 10% fetal calf serum (FCS; Gibco BRL) at 37°C in the presence of 5% CO_2. Centrin 1-GFP L929 cell lines were cultured with G418 (500 μg ml^{-1}, Gibco BRL). For cell cycle synchronization experiments, cell cultures were maintained in low FCS (0.5%) for at least 48 hours to induce quiescence, and subsequently stimulated to synchronously re-enter the cell cycle by raising the FCS concentration to 15%. Cells were collected 9 hours, 15 hours and 22 hours after stimulation. To analyse G1-S progression to mitosis, NIH/3T3 and L929 cell cultures were blocked in the presence of thymidine (Sigma Aldrich, 2 mM for NIH/3T3 and 5 mM for L929 cells) for 24 hours, then released in complete DMEM supplemented with 30 μM deoxycytidine (Sigma Aldrich) and harvested 6-8 hours after release from thymidine arrest, when the cell population was mostly in G2-M by fluorescence-activated cell sorting (FACS) analysis and the mitotic index was highest by microscope scoring. Where indicated, cell cultures were released from thymidine arrest for 4-6 hours and subsequently exposed to 0.1 μg ml^{-1} nocodazole (NOC; Sigma Aldrich) or 100 μM monastrol (MA; Tocris) for 4 hours before harvesting. Cells were then fixed, or released in drug-free medium for 45 minutes (NOC) or 30 minutes (MA). For localization experiments, thymidine-arrested and released cultures were exposed to 1 μM Taxol (Sigma Aldrich) for 4 hours. In all cases cell cycle phase synchronization was analysed by FACS (Beckton Dickinson) as described (Battistoni et al., 1997).

Transfection experiments

NIH/3T3 cells were seeded in 60 mm Petri dishes onto sterile glass coverslips and transfected using Fugene (Roche Diagnostic, 3 μl $μg^{-1}$ DNA). L929 cells were transfected by electroporation (950 μF, 310 V) and reseeded onto sterile glass coverslips. Six hours after transfection, the medium was replaced with fresh medium. Cells were

routinely collected 36-48 hours after transfection (asynchronous cell cultures). Where indicated, transfected cell cultures were submitted to synchronization protocols starting 6-10 hours after transfection; the overall duration of thymidine arrest and release, with or without mitosis-arresting drugs, covered 36-42 hours of culture after transfection (see above). Cells were then harvested and processed for parallel FACS and indirect immunofluorescence (IF) assays.

Antibodies

Goat polyclonal anti-RanBP1 (M-19 for murine cells and C-19 for human cells) antibodies were from Santa Cruz Biotechnology and were used 0.5 μg ml^{-1} in western blotting and 2 μg ml^{-1} in IF experiments. Anti-HA (Y-11; Santa Cruz Biotechnology) antibody was used at 1:100 dilution. Monoclonal Ran antibody (clone 20; Transduction Laboratories) was used at 0.25 μg ml^{-1}. Goat polyclonal anti-RCC1 (C-20) and anti-RanGAP1 (N-19) antibodies (Santa Cruz Biotechnology) were used at 1 μg ml^{-1} and 2 μg ml^{-1}, respectively. Monoclonal α-tubulin (clone B-5-1-2; Sigma Aldrich) antibody was used at 1:1000 dilution. Monoclonal (GTU-88) and rabbit polyclonal anti-γ-tubulin antibodies (both from Sigma Aldrich) were used at 1:5000 dilution for western blotting and 1:1000 for IF assays. Monoclonal anti-GT335 antibody (used 1:3000) was kindly provided by P. Denoulet (Université Pierre et Marie Curie, Paris); rabbit polyclonal anti-centrin 2 antibody (used at 1:2000 dilution) was from M. Bornens (Institut Curie, Paris). The monoclonal antibody CTR453 (IgG_{2b}) was generated in M. Bornens's laboratory and has previously been characterized as specific for the centrosome (Bailly et al., 1989). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology. Secondary antibodies conjugated to fluorescein-, AMCA- (Jackson ImmunoResearch Laboratories), rhodamine (Santa Cruz Biotechnology), Texas Red (Vector) and Cy-3 (Amersham) were chosen depending on the basis of species specificity and used as recommended by the suppliers.

Immunofluorescence microscopy

Cells were grown on sterile glass coverslips, washed in PBS and fixed in methanol for 6 minutes at –20°C or in 3% PFA, 30 mM sucrose for 10 minutes at room temperature. Where indicated, cells were permeabilized for 30 seconds in 0.5% Triton X-100 in PHEM (45 mM PIPES pH 6.9, 45 mM HEPES pH 6.9, 10 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF) before fixation. Incubation with primary antibodies was carried out for 1 hour at 37°C. Secondary antibodies were incubated for 45 minutes. DNA was counterstained with DAPI (0.1 μg ml^{-1}). Coverslips were then mounted in Vectashield (Vector). IF was also performed as above using purified centrosomes from the KE37 cell line (see below), after sedimentation onto coverslips (at 20,000 ***g***, 15 minutes, 4°C) and fixation in methanol for 6 minutes at –20°C.

Fixed cell preparations were examined under an upright Olympus AX70 microscope equipped for epifluorescence and images were taken (100× objective) using either a CoolSnap FX, or a Photometrics CCD camera. Where indicated, fluorescence intensity was quantified in arbitrary units using Adobe Photoshop software on CCD images of single cells acquired under identical exposure and gain setting within each experiment. Video recording of living mitotic cells was carried out on an inverted fluorescence microscope (Leica DMIRBE) controlled by Metamorph software; cells transfected with pRanBP1-RFP were identified on the red channel and images were taken every 10 minutes (10× objective). Confocal images were taken (60× objective) using a TCS-SP2 confocal microscope (Leica) with a 488 nm laser excitation line.

Statistical analysis

To assess the statistical significance of the results, each experiment was repeated at least three times; means and standard deviations were calculated to compare the same category in different experiments. This procedure consistently gave extremely low, statistically insignificant deviations within each experimental condition. Data from different experiments were therefore pooled and *P* values were calculated on pooled data using the χ^2 test.

Protein extraction from the centrosomal fraction and immunoblotting analysis

Centrosomes were isolated from the KE37 cell line as described by Moudjou and Bornens (Moudjou and Bornens, 1994). Pelleted centrosomes were incubated for 1 hour at 4°C in extraction buffer (20 mM Tris-HCl pH 7.4, 2 mM EDTA) alone or in the presence of: (i) 0.5% NP40 (1D buffer); (ii) 0.5% NP40 and 0.5% deoxycholate (DOC, 2D buffer); (iii) 0.5% NP40, 0.5% DOC and 0.1% SDS (3D buffer); (iv) 8 M urea. Centrosome-associated and non-associated proteins were recovered in the pellet and supernatant fractions, respectively, by centrifugation at 10,000 ***g*** for 15 minutes. Proteins were separated through SDS-PAGE and transferred onto nitrocellulose filters (Schleicher & Schuell). Filters were saturated in 5% milk in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 0.1% Tween 20, for 1 hour at 37°C. Primary and secondary antibodies were incubated for 1 hour or 45 minutes, respectively, at room temperature. HRP-conjugated secondary antibodies were revealed with ECL plus (Amersham-Pharmacia).

Results

Spindle pole defects are induced by RanBP1 overexpression

Overexpression of RanBP1 in asynchronously cycling NIH/3T3 cell cultures was previously found to induce multipolar spindles (Guarguaglini et al., 2000). Such supernumerary poles might be originated through different mechanisms involving abnormal centrosome duplication, disruption of the centrosomal structure or centrosome mis-segregation to daughter cells at cytokinesis. As a first step to identify process(es) targeted by RanBP1 overexpression, we transfected murine NIH/3T3 cell cultures with RanBP1 expression construct and analysed the pattern of centrosomes in transfected cells. In a first set of experiments, asynchronous cell cultures were transfected with pRanBP1-HA for 36 hours, then fixed and processed for double IF to visualize centrosomal markers in HA-expressing cells. We used antibodies against centrin-2, a protein localized in the lumen of individual centrioles; γ-tubulin, the major pericentriolar matrix (PCM) protein required for MT nucleation; or GT335, an antibody recognizing glutamylated tubulin, a typical modification of centriole microtubules (Wolff et al., 1992). Many RanBP1-overexpressing mitosis showed supernumerary spots reactive to antibodies against centrosomal components: for example, Fig. 1Aa shows a cell expressing high levels of HA-tagged RanBP1 protein with four GT335-reactive spots at four distinct locations instead of the two paired spots expected for a bipolar spindle, strongly suggesting that the organization of centrioles was affected. We next used combinations of antibodies to detect pairs of centrosomal proteins in RanBP1-overexpressing cells – GT335 and anti-centrin 2 (Fig. 1Ab) and/or GT335 and anti-γ-tubulin (Fig. 1Ac). In this set of experiments, the spindle was not stained but DAPI staining revealed a high frequency of chromosome misalignment, consistent with the assembly of abnormal spindles (compare, for example, DAPI images in rows b and d). In abnormal mitoses, all analysed combinations

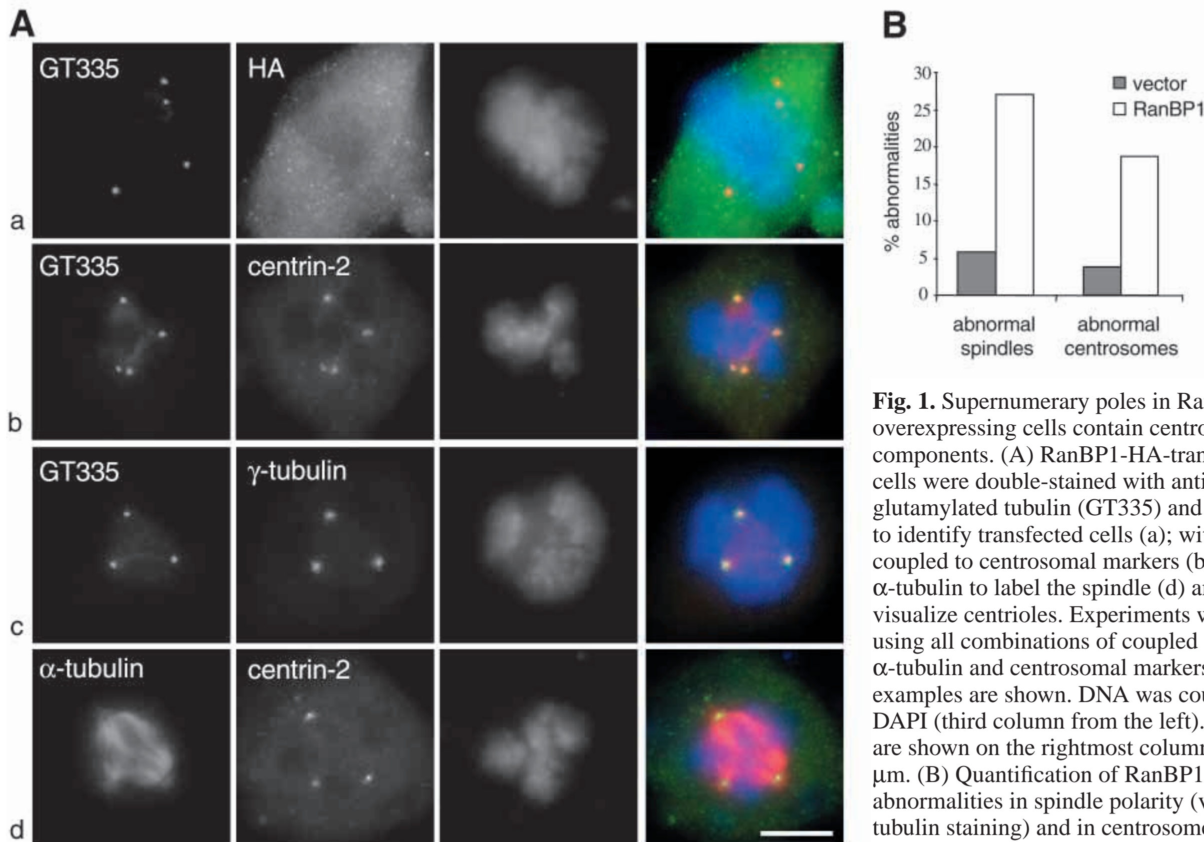

Fig. 1. Supernumerary poles in RanBP1-overexpressing cells contain centrosomal components. (A) RanBP1-HA-transfected NIH/3T3 cells were double-stained with antibodies to glutamylated tubulin (GT335) and anti-HA-antibody to identify transfected cells (a); with antibodies coupled to centrosomal markers (b,c); and with anti-α-tubulin to label the spindle (d) and anti-centrin 2 to visualize centrioles. Experiments were carried out using all combinations of coupled antibodies to HA, α-tubulin and centrosomal markers, and selected examples are shown. DNA was counterstained with DAPI (third column from the left). Merged pictures are shown on the rightmost column. Scale bar, 10 μm. (B) Quantification of RanBP1-induced abnormalities in spindle polarity (visualized by α-tubulin staining) and in centrosomes (shown as either >4 or abnormally separated centrin spots). Data from three independent experiments were pooled and 100 mitoses per group were scored in each experiment. Histograms show the proportion of cells with abnormalities in vector-transfected (gray) and RanBP1-transfected (white) cultures. *P* values calculated using the χ^2 test were highly significant.

of markers simultaneously labeled supernumerary poles (Fig. 1Ab,c), indicating that induction of multipolar spindles in RanBP1-overexpressing mitoses involves the entire centriolar structure rather than an abnormal recruitment of specific components to the poles. To further assess the involvement of centrioles in the altered structure of spindle poles, parallel RanBP1-transfected cultures were processed for anti-α-tubulin and anti-centrin 2, or anti-α-tubulin and anti-γ-tubulin combinations, to simultaneously reveal the spindle structure together with centrioles or with nucleating centers. Although we could not stain RanBP1 in these experiments, we recorded a high frequency of multipolar spindles in RanBP1- but not in vector-transfected cultures. Each pole in those abnormal spindles contains material reactive to anti-centrin 2 (for example, see Fig. 1Ad) or anti-γ-tubulin (data not shown) antibodies. We next wanted to establish whether centrosomal abnormalities induced by RanBP1 overexpression consistently gave rise to multipolar spindles, or whether part of them are irrelevant to mitotic spindle organization. NIH/3T3 cell cultures transfected with RanBP1-HA were seeded on twin slides within the same culture dish, fixed and processed in parallel IF assays to quantify mitotic cells that displayed either abnormal spindles (by α-tubulin staining) or abnormal centrosomes (revealed by anti-centrin 2 antibody) among RanBP1-overexpressing cells, recognized by HA staining. We considered as abnormal all cells that displayed abnormalities in either centrosome number or arrangement. Histograms in Fig. 1B show that RanBP1 transfection in NIH/3T3 cells yielded a fivefold increase in multipolar spindles compared with vector-transfected cells, and a 4.7-times increase in mitotic cells displaying abnormal centrin spots compared with controls. Thus, the induction of multipolar spindles parallels that of centrosomal abnormalities in RanBP1-overexpressing cells.

Centrosomal abnormalities in RanBP1-overexpressing cells are induced during mitosis

Normal cells undergo only one round of centrosome duplication, during which each of the two centrioles composing the centrosome duplicates in a semiconservative manner. Each centrosome eventually segregates to a daughter cell at cytokinesis and becomes 'licensed' to undergo a novel round of duplication in the next cell cycle. Loss of the spindle bipolarity is often related to abnormal centrosome duplication (Lingle and Salisbury, 2000; Brinkley, 2001; Doxsey, 2001). The influence of specific factors on centrosome duplication can be assessed after prolonged treatment of CHO cells with hydroxyurea, which blocks DNA synthesis but not centrosome duplication (Balczon et al., 1995). Ectopic expression of the *cyclinA* and *cdk2* genes in this system induces centrosome overduplication, whereas *pRb* and *p16* inhibit it, and *E2F-1* overexpression rescues the inhibition (Meraldi et al., 1999). Instead, overexpression of RanBP1 showed no additional effect

Table 1. RanBP1-dependent centrosomal abnormalities during cell cycle progression

	Interphase			Mitosis		
	% Abnormal	*N*	*P*	% Abnormal	*N*	*P*
A						
Vector	18.38	540	ns	15.73	200	<0.02
pRanBP1	17.84	610	ns	24.05	210	<0.02
B						
Vector	10.49	160	ns	6.36	170	<0.001
pRanBP1	6.04	150	ns	24.47	100	<0.001

A, serum-starved (G0/G1) and restimulated cells harvested 9, 15 and 22 h after cell cycle entry.

B, thymidine-arrested (G1/S) and released cells harvested 6, 7 and 8 h after S phase resumption.

P values were calculated using the χ^2 test; ns, not significant.

Table 2. RanBP1 levels in transfected mitoses with normal or abnormal phenotypes

Relative intensity[a]	Normal mitoses		Centrosomal abnormalities		
	N	%	*N*	%	*P*
1–2	36	59	9	23.1	<0.001
2–3	23	37.7	17	43.6	ns
3–4	2	3.3	7	17.9	<0.02
>4	0	0	6	15.4	<0.01
Total	61	100	39	100	

[a]Fluorescence intensities were measured on CCD images (see Materials and Methods) and are expressed relative to the faintest intensity in the lowest-expressing cell, taken as=1.

P values were calculated using the χ^2 test; ns, not significant.

on centrosome duplication compared with hydroxyurea alone, nor did it overcome the block of centrosome duplication imposed by pRb (P. Meraldi, personal communication). Thus, RanBP1 has no direct effect on centrosome duplication.

We next sought to restrict the cell cycle window in which centrosomal abnormalities are generated. RanBP1-transfected cell populations were synchronized and centrosomal components were analysed during synchronous progression through the cell cycle phases. We first brought NIH/3T3 cell cultures to G0/G1 arrest by serum starvation, then stimulated cell cycle re-entry with high serum. Cell samples were fixed 9 hours, 15 hours and 22 hours after cell cycle re-stimulation to obtain G1, S and G2/M phase enrichment, respectively, as indicated by FACS analysis (data not shown). Centrosomal abnormalities, revealed by staining centrioles with anti-centrin-2 antibody, were quantified as for Fig. 1B: cells with one or two paired dots (corresponding to one or two centrosomes, respectively) were taken as normal, whereas cells with more than two pairs of dots or with scattered dots were assumed to reflect overduplication and abnormal splitting of centrosomes, respectively, and considered to be abnormal. Serum re-stimulation of quiescent cells induces per se a high frequency of centrosome splitting in vector-transfected cells (Table 1), in line with previous reports (Sherline and Mascardo, 1982; Schliwa et al., 1982; Schliwa et al., 1983). RanBP1 overexpression had no additional effect on serum-induced centrosomal abnormalities in interphase; however a significant increase was recorded in RanBP1-overexpressing mitotic cells compared to control cultures (Table 1). To analyse S-to-M progression more accurately, cells were arrested at the G1/S transition with thymidine, then released in thymidine-free medium and centrosomes were analysed in cells that were allowed to progress towards mitosis. Again, no difference between vector- and RanBP1-transfected cells were observed in S or G2 interphase cells, whereas a high proportion of centrosomal abnormalities was recorded in mitoses from RanBP1-transfected compared to vector-transfected cultures (Table 1). Thus, centrosomal abnormalities induced by RanBP1 overexpression are specifically generated in mitosis.

Quantification of RanBP1-associated fluorescence in CCD images of single cells transfected with expression construct, or with vector alone, indicated that the RanBP1 signal increased by over fourfold, on average, in overexpressing cells: most transfected cells (~55%) displayed a three- to fivefold increase, and ~30% showed a five- to sevenfold increase in RanBP1 signal intensity compared to control cells. To assess whether the induction of centrosomal abnormalities did correlate with the level of exogenous RanBP1, we examined 100 mitotic cells from cultures transfected with pRanBP1-HA, then processed with anti-HA/FITC to visualize transfected cells, and GT335/rhodamine to visualize centrioles. Cells were analysed for the presence or absence of centrosomal abnormalities on the red channel, and the intensity of the FITC signal, quantified on the green channel. Among RanBP1-transfected mitoses that displayed a normal phenotype (*n*=61), the mean fluorescence scored 1.9 (±0.5), taking the faintest signals in the lowest-expressing cells as 1; ~60% of them displayed relative intensities below 2, and the remaining 40% fell between 2 and 3. Among RanBP1-transfected mitoses that developed centrosomal abnormalities (*n*=39), the mean relative fluorescence rose to 2.9 (±1.2); a minority (~23%) of these abnormal mitoses displayed a fluorescence intensity below 2, comparable to normal mitoses; all other cells had relative intensities above 2, with a discrete cell population (~15%) showing more than a fourfold increase in RanBP1 signal intensity (Table 2). Thus, RanBP1-transfected cells that develop mitotic centrosomal abnormalities tend to express the highest levels of exogenous RanBP1.

RanBP1 overexpression disrupts cohesion of sister centrioles in mitotic diplosomes

To resolve accurately the type of centrosomal abnormality induced by RanBP1 overexpression, we made use of L929-derived cell cultures stably transfected with centrin-1/GFP chimera (Piel et al., 2000). The incorporation of GFP-chimerized centrin in individual centrioles allows a higher resolution of centrosomes than indirect immunofluorescence techniques. This cell model therefore provides a particularly useful tool to analyse the effects of RanBP1.

We initially characterized centrin-1/GFP L929 cells from non-synchronized cultures and noticed that they spontaneously develop a somewhat higher level of centrosomal abnormalities (26.5% in 170 scored mitoses) compared with NIH/3T3 fibroblasts (10.7% in 400 mitoses). Of all centrosomal abnormalities detected among L929 mitotic cells, nearly half (12.3% of all mitoses) were represented by supernumerary, structurally integral centrosomes (arrangement II in Fig. 2B). The remaining abnormal mitoses showed diplosome splitting,

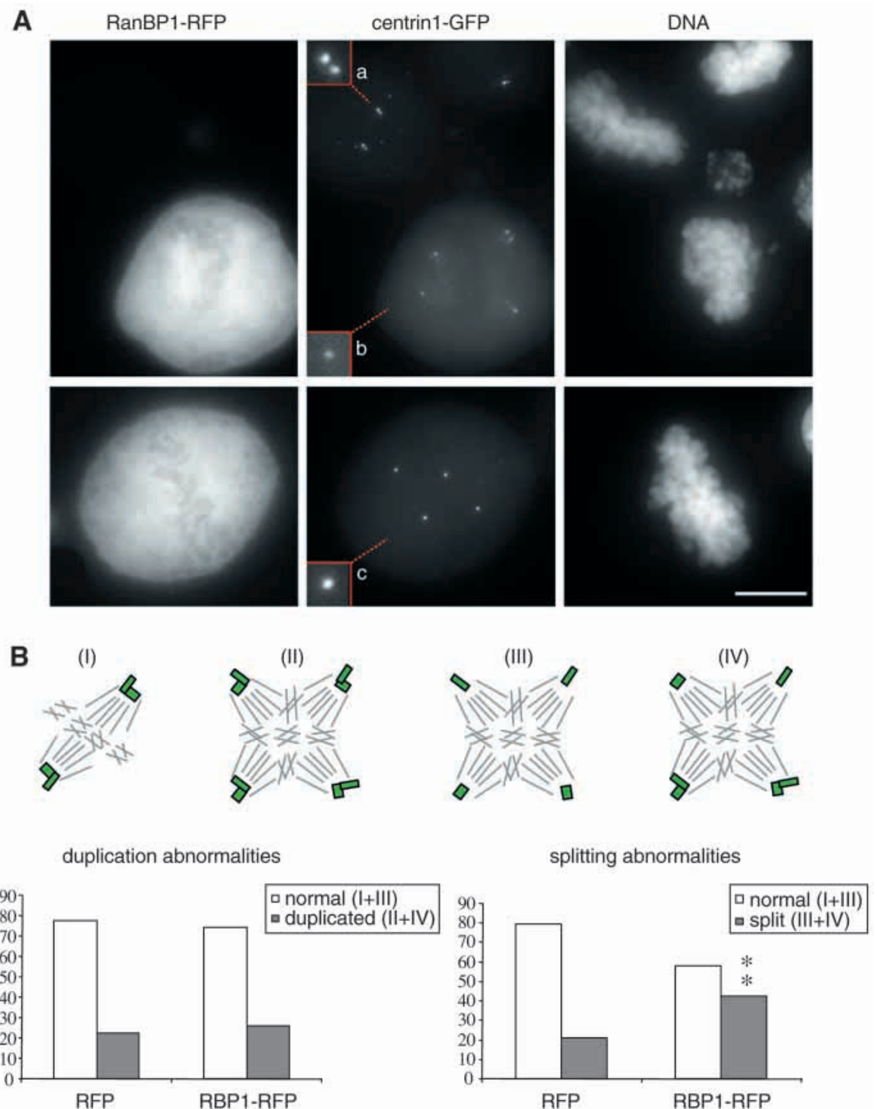

Fig. 2. RanBP1 overexpression induces centriole splitting in mitosis. (A) L929 cell cultures stably expressing a centrin 1-GFP chimera (Piel et al., 2000) were transfected with RanBP1-RFP, synchronized as described in the text, and mitotic cells recovered by 'shake-off' were analysed. In the upper row, the non-transfected cell (upper left corner, negative for RFP emission) shows correctly aligned chromosomes (DNA panel) and centriole pairs in each centrosome, as shown in the magnified insert (a). In RanBP1-transfected cells (positive for RFP emission), single split centrioles are visible: two examples are shown, magnified in inserts b and c. Scale bar, 10 μm. (B) Quantification of centrosome defects induced by RanBP1 overexpression. Possible distributions of centrioles in mitosis are: I, normal arrangement; II, overduplicated centrosomes; III, split centrioles; IV, overduplicated and split centrosomes. Only tetrapolar spindles are represented, for simplicity. Histograms in the left panel show the frequency of centrosome overduplication (gray), calculated by grouping patterns II and IV (i.e. all cells with more than four centrioles) as abnormal. The same samples were re-analysed for the frequency of centriole splitting (histograms in the right panel), calculated by grouping patterns III and IV as abnormal (i.e. all cells showing single centrioles, regardless of total centriole number). 200 mitotic cells from vector- and RanBP1-RFP-transfected cultures were scored. The asterisks mark a highly significant difference (P<0.001).

either associated with a normal number of centrioles (i.e. four centrin dots, arrangement III in Fig. 2B) or concomitant with supernumerary centrosomes (i.e. more than four centrin dots, arrangement IV in Fig. 2B). For comparison, the corresponding phenotypes among NIH/3T3 mitoses scored 3.8% (centrosomes overduplication) and 6.9% (diplosome splitting).

We next assessed the effect of transfected RanBP1-RFP chimeras in L929-derived cell cultures stably expressing centrin-1/GFP. Cells that reached mitosis after thymidine synchronization and release were collected by the 'shake off' method, then immediately re-seeded on microscope slides, and mitotic cells with supernumerary integral centrosomes or with split diplosomes were examined by analysing the arrangement of centrin-1/GFP centrioles (see scheme in Fig. 2B). In normal mitoses, chromosomes were correctly aligned and centrioles were arranged in typical diplosomes at each pole (Fig. 2A, left corner in upper row, see magnification in a). RanBP1 overexpression did not significantly affect centrosome duplication (Fig. 2B), consistent with results obtained in NIH/3T3 cell lines (see above), but specifically induced sister centrioles from single diplosomes to move apart from one another (Fig. 2A, magnification in b and c). As shown in Fig. 2B, ~45% of RanBP1-overexpressing mitotic cells showed split diplosome, compared with 20% in vector-transfected cells (P<0.001). We also analysed cultures that remained adherent during shaking off and were enriched in G2-phase cells: RanBP1 overexpression in these cultures failed to increase the frequency of abnormal centrosome numbers or splitting (data not shown), as previously observed in NIH/3T3 cultures, thereby confirming that RanBP1 specifically induces diplosome splitting during mitosis.

Splitting of centrioles during mitosis was previously reported to occur under induction of mitotic arrest (Sluder and Rieder, 1985; Gallant and Nigg, 1992). RanBP1 overexpression actually causes some increase in the mitotic index, as previously observed (Guarguaglini et al., 2000). However, the extent of the induced delay in our experiments was in the upper limit of the physiological range or just above it (Table 3), different from that induced by MT drugs or failure of cyclin-B degradation. Video recordings of cells transfected with pRFP vector or pRanBP1-RFP depicted no dramatic delay in the timing from prophase/prometaphase – indicated by

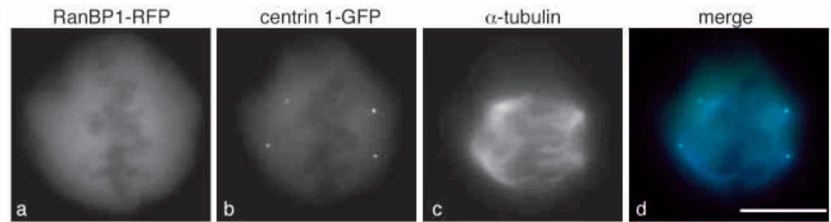

Fig. 3. Split centrioles organize functional spindle poles. (a) A RanBP1-RFP-transfected mitosis from centrin 1-GFP stably expressing L929 cell cultures. Centrin-1/GFP allows the visualization of centrioles (b); the spindle is stained with anti-α-tubulin, revealed with an AMCA-conjugated secondary antibody (c). Merging of b and c produces d, which depicts single split centrioles (green) at each spindle pole (AMCA-stained MTs, in blue). Scale bar, 10 μm.

Table 3. Effect of RanBP1 overexpression on mitotic progression

	Mitotic index		Time from prometa to anaphase (in vivo)[b]		% Mitoses in ana-telophase (fixed cultures)
	Asynchronous cultures	Thymidine release	Mean (min)	Mode (min)	
Vector	6.4 (*N*=800)	22.1 (*N*=560)	30.3 (*N*=33)	20–30 (76%)	54.2 (*N*=140)
pRanBP1[a]	10.6 (*N*=700)	28.4 (*N*=700)	45.4 (*N*=23)	30–40 (65%)	68.7 (*N*=260)

[a]Similar results were obtained with pRanBP1 untagged, pRanBP1-RFP and pRanBP1-HA.
[b]The timing of early mitosis was calculated from video-recorded images taken with 10-min intervals.

rounding-up of the cells – to anaphase in vivo: all video-recorded control cells reached anaphase within 40 minutes from mitosis onset, and most of them took 20-30 minutes. RanBP1-transfected cells underwent some delay, with most of them taking 30-40 minutes to execute the same stages. Thus, RanBP1-dependent delay in early mitosis is well below that induced by MT drugs or non-degradable cyclin B, which is in the order of hours. Furthermore, progression through mitotic substages was analysed in transfected cultures after IF to α-tubulin: this revealed a higher proportion of ana/telophases among RanBP1-overexpressing mitoses compared with controls. Thus, the induction of mitotic delay by RanBP1 overexpression is essentially caused by prolonged duration of ana/telophase stages, possibly reflecting hindrance in M exit (Battistoni et al., 1997; Guarguaglini et al., 2000), whereas earlier mitotic stages are not significantly affected. RanBP1 induction of centriole splitting is instead already visible in prometaphase. Thus, RanBP1-dependent centriole splitting is a specific phenomenon, not attributable to prolonged duration of mitosis.

We next wished to ascertain whether single split centrioles were able to assemble functional spindle poles. Centrin-1/GFP expressing L929 cultures were transfected with the RanBP1-RFP chimera and spindle MTs were labeled with anti-α-tubulin antibody, revealed with an AMCA-conjugated secondary antibody. As shown in Fig. 3, microtubule arrays nucleating from single centrioles are focused to form separate poles, hence forming a multipolar spindle.

Diplosome splitting by RanBP1 requires integrity of mitotic microtubules

Cohesion between parental centrioles requires MT integrity (Jean et al., 1999) and MT disruption by nocodazole favors the separation of parental centrosomes (Mayor et al., 2000). We wondered whether MTs are implicated in RanBP1-induced splitting between centrioles. NIH/3T3 cultures were transfected with RanBP1-HA or RanBP1-GFP and subsequently synchronized in G2/M phases by thymidine block and release as described above. Cells were then exposed to nocodazole (NOC) and either collected after 4 hours, with most cells arrested in prometaphase without spindle MTs, or allowed to resume mitosis by removing NOC and fixed 45 minutes after release. Both FACS analysis and microscope counting of mitotic cells (data not shown) were used to monitor synchronization. Mitotic centrosomes were analysed using either GT335 or anti-centrin-2 antibodies. In cultures exposed to NOC, the overall centrosomal organization was altered in interphase cells, with centrosomes being typically distanced and displaced from the juxtanuclear region (data not shown), consistent with the established role of MTs in anchoring centrosomal structures to each other and to their subcellular site (Jean et al., 1999). Fig. 4 shows the results obtained in RanBP1-overexpressing cultures. In cells that underwent mitosis after release from thymidine arrest, RanBP1 overexpression caused a highly significant increase in diplosome splitting compared with cultures transfected with vector. When NOC was added to G2 cultures to inhibit MT polymerization, the effects of RanBP1 overexpression were prevented, and the frequency of mitoses with split centrioles was comparable in RanBP1-overexpressing and in vector-transfected cultures. Thus, NOC per se does not affect the organization of sister centrioles within diplosomes, in contrast to its ability to induce separation of parental centrioles, yet counteracts the disruptive effect caused by RanBP1 excess, indicating that MT integrity is required for induction of diplosome splitting. The specificity of this requirement was further demonstrated by removing NOC from the culture medium and allowing the cells to reform MTs in vivo: upon resumption of mitosis, RanBP1-overexpressing mitoses again underwent diplosome splitting (Fig. 4).

We previously reported that multipolar spindles are similarly induced by wild-type RanBP1 and by the RanBP1$^{L186A/V188A}$ mutant, which carries inactivating mutations in the nuclear export signal (NES) and hence is retained in nuclei throughout interphase (Richards et al., 1996; Guarguaglini et al., 2000). If multipolar spindles are generated through loss of diplosome cohesion as a truly mitotic phenomenon, then similar effects to

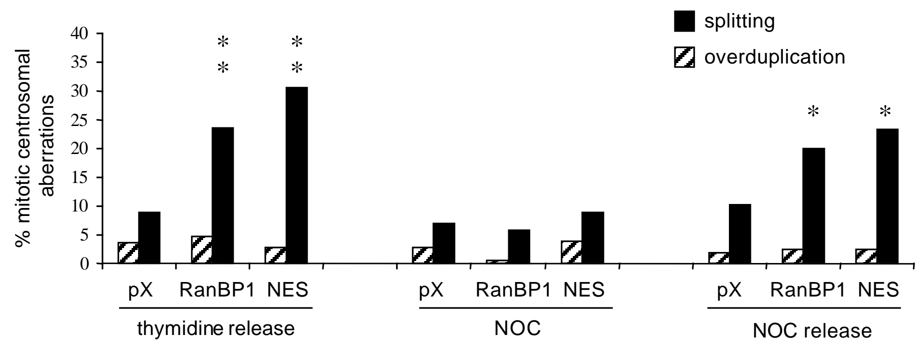

Fig. 4. Centriole splitting requires microtubule integrity. Centrosomal abnormalities [i.e. overduplication (hatched columns) and splitting (black columns)] were recorded in cultures transfected with vector, wild-type RanBP1 or RanBP1$^{L186A/V188A}$ mutant (indicated as NES) during mitosis following thymidine release, or after treatment with nocodazole (NOC), or released after NOC arrest. For each condition, three to five experiments were carried out with wild-type RanBP1 and at least two with the RanBP1$^{L186A/V188A}$ mutant. Data were pooled and analysed using the χ^2 test. *, $P<0.05$; **, $P<0.001$.

those reported thus far are expected in cells overexpressing the RanBP1$^{L186A/V188A}$ mutant, regardless of its abnormal localization during interphase. Indeed, the NES-defective RanBP1 mutant induced a highly significant increase in mitotic diplosome splitting in NIH/3T3 cultures released from thymidine arrest, similar to wild-type RanBP1 (Fig. 4). Parallel analysis of L929 centrin-1/GFP cultures enabled us to establish that the type of mitotic diplosome splitting induced by mutant and wild-type RanBP1 was indistinguishable (data not shown). The RanBP1$^{L186A/V188A}$ mutant failed instead to induce diplosome splitting in nocodazole-exposed cells, similar to wild-type RanBP1 (Fig. 4). Thus, the comparable ability of export-defective and wild-type RanBP1 to disrupt centriole cohesion in a MT-dependent manner further confirms that diplosome splitting takes place after NEB.

Diplosome splitting by RanBP1 requires Eg5 activity

The Eg5 kinesin controls the establishment of the spindle bipolarity by causing parental centrosome separation at the onset of mitosis (Walczak et al., 1998) and Ran can modulate Eg5 mobility on MTs (Wilde et al., 2001). Thus, we wondered whether Eg5 activity influenced RanBP1-induced splitting within mitotic diplosomes. Inhibition of Eg5 activity by monastrol (MA) prevents centrosome separation, yielding mitotic cells that typically arrest with monoastral spindles (Kapoor et al., 2000). In our experiments, RanBP1- or vector-transfected NIH/3T3 cell cultures were synchronized by thymidine block and release as above, and, when in G2 as judged by FACS analysis, MA was added for 4 hours. Cells were then fixed and centrosomes were analysed. By γ-tubulin staining of centrosomes and DAPI staining of chromosomes, monoastral mitoses with unseparated centrosomes were indistinguishable in RanBP1- and vector-transfected cells (Fig. 5A). Centrosome structure was more closely inspected using anti-centrin-2 antibody. Although all mitoses had a monoastral spindle, different arrangements could be appreciated at the centrosome level: monoastral mitoses in which two sets of paired centrioles were visible at the center of the spindle were defined ‘normal’ (Fig. 5Ba); mitoses showing more than two paired centrin spots (Fig. 5Bb) were assumed to reflect overduplication, whereas clearly distanced centrioles in at least one diplosome (Fig. 5Bc) were recorded as abnormal splitting. By these criteria, centriole splitting occurred with similar frequency in vector-transfected and RanBP1-overexpressing monoastral mitoses (Fig. 5C). Eg5 inhibition by MA is reversible and so cells released in MA-free medium readily re-establish bipolarity. Under these conditions, diplosome splitting was again appreciated in RanBP1-overexpressing cells that progressed through mitosis 30 minutes after MA removal (Fig. 5C). Thus, Eg5 function is required for induction of diplosome splitting by overexpressed RanBP1.

Centrosomal localization of RanBP1

Because RanBP1 overexpression affects centriole cohesion, we re-examined its localization relative to centrosomes. In NIH/3T3 interphase cells fixed with paraformaldehyde (PFA), RanBP1 is almost completely cytoplasmic; some enrichment at the spindle can be appreciated in mitotic cells (Guarguaglini et al., 2000). If a fraction of RanBP1 localizes at centrosomes, such a fraction might be masked by the abundant soluble pool and difficult to resolve. Indeed, partial permeabilization of NIH/3T3 cells with Triton X-100 prior to methanol or PFA fixation revealed a fraction of insoluble RanBP1 protein at the centrosome, revealed by γ-tubulin, in both interphase (Fig. 6Aa) and mitotic cells (Fig. 6Ab,c). A small centrosomal fraction of RanBP1 was also visualized in mouse L929 (Fig. 6Ad) and human HeLa cells (Fig. 6Ae) using independent antibodies. The co-localization of RanBP1 signals with γ-tubulin was confirmed by scanning NIH/3T3 cell spreads under confocal microscopy (Fig. 6B).

To extend these results, we analysed preparations of purified centrosomes isolated from the human lymphoblastic cell line KE37. RanBP1 was retained on isolated centrosomes analysed by IF (Fig. 6C) and showed a very similar labeling pattern to that revealed using the CTR453 antibody, which specifically recognizes the AKAP450 centrosomal matrix protein (Bailly et al., 1989). Western immunoblotting was then used to assess the strength of the interaction of RanBP1 with the KE37-derived centrosomal fraction (Fig. 6D). Purified centrosome preparations were treated with solubilizing detergents of increasing strength, and the soluble (supernatant) and insoluble (pellet) fractions were analysed with anti-RanBP1 antibody. As shown in Fig. 6D, the association of a RanBP1 fraction with centrosomes was resistant to strong solubilizing conditions: centrosomal RanBP1 was not solubilized by NP40 alone (1D buffer), nor by NP40 combined with DOC (2D buffer), nor with DOC and SDS simultaneously (3D buffer). Treatment of centrosomes with 8 M urea eventually solubilized centrosomal

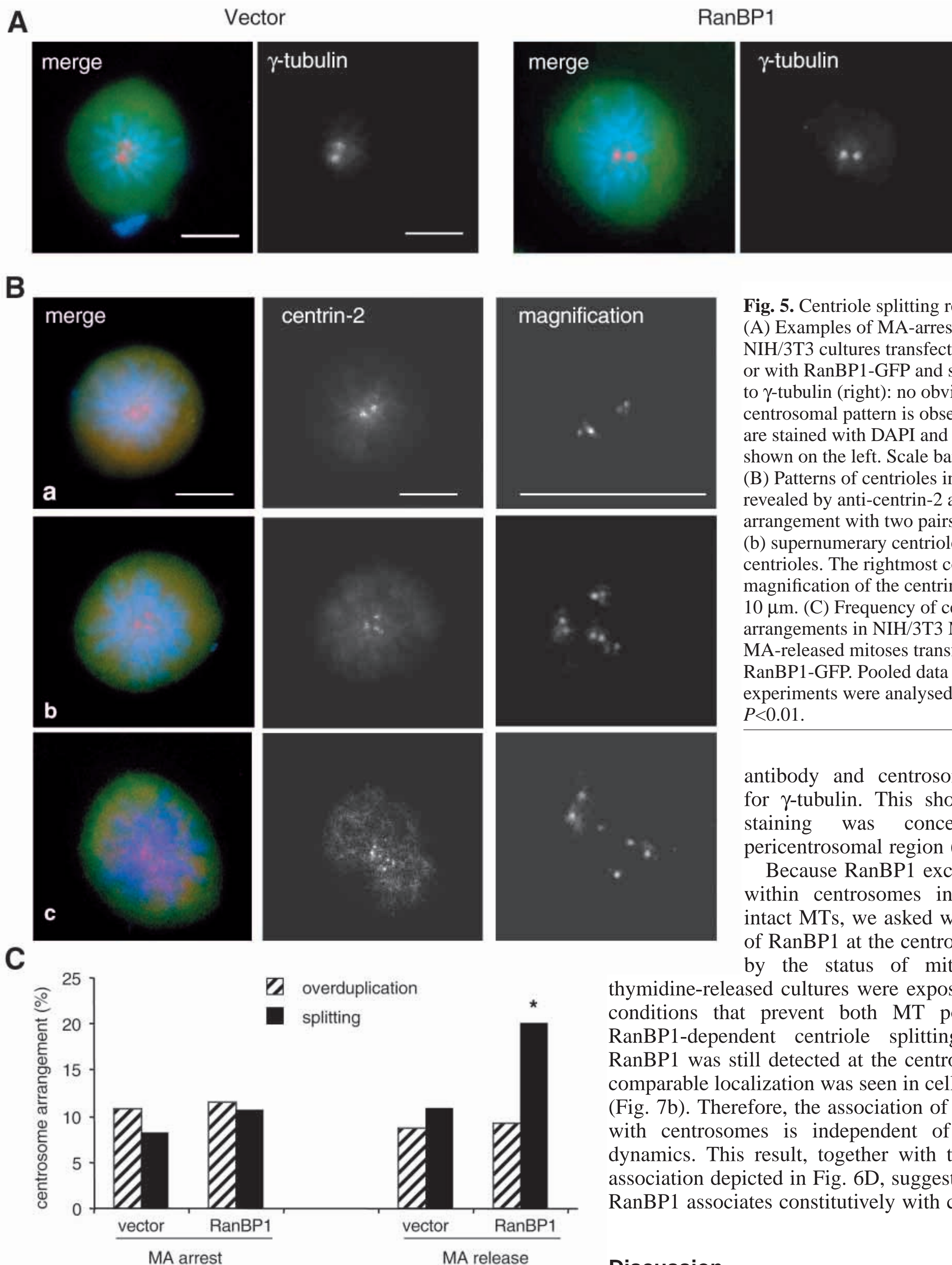

Fig. 5. Centriole splitting requires Eg5 activity. (A) Examples of MA-arrested mitoses from NIH/3T3 cultures transfected with GFP vector or with RanBP1-GFP and stained with antibody to γ-tubulin (right): no obvious difference in the centrosomal pattern is observed. Chromosomes are stained with DAPI and merged pictures are shown on the left. Scale bar, 10 μm. (B) Patterns of centrioles in monoastral mitoses revealed by anti-centrin-2 antibody: (a) normal arrangement with two pairs of centrioles; (b) supernumerary centriole pairs; (c) split centrioles. The rightmost column shows a magnification of the centrin 2 panels. Scale bar, 10 μm. (C) Frequency of centrosomal arrangements in NIH/3T3 MA-arrested and MA-released mitoses transfected with vector or RanBP1-GFP. Pooled data from four experiments were analysed using the χ^2 test. *, P<0.01.

RanBP1. Thus, a RanBP1 fraction is actually involved in a stable interaction with centrosomes.

To ascertain whether exogenously expressed RanBP1 also reached centrosomes, IF experiments were performed in cultures transfected with pRanBP1-HA. After solubilization and fixation, exogenous RanBP1 was revealed by anti-HA antibody and centrosomes were stained for γ-tubulin. This showed that anti-HA staining was concentrated in the pericentrosomal region (Fig. 6E).

Because RanBP1 excess alters cohesion within centrosomes in the presence of intact MTs, we asked whether localization of RanBP1 at the centrosome is influenced by the status of mitotic MTs. When thymidine-released cultures were exposed to NOC, under conditions that prevent both MT polymerization and RanBP1-dependent centriole splitting, a fraction of RanBP1 was still detected at the centrosome (Fig. 7a). A comparable localization was seen in cells exposed to Taxol (Fig. 7b). Therefore, the association of a RanBP1 fraction with centrosomes is independent of MT integrity or dynamics. This result, together with the strength of the association depicted in Fig. 6D, suggests that a fraction of RanBP1 associates constitutively with centrosomes.

Discussion

The formation of multipolar spindles predisposes mitotic cells to undergo chromosome mis-segregation. Frequent causes of multipolar spindle assembly include errors in centrosome duplication or segregation to daughter cells, which can lead to genomic imbalance and favor cell transformation and tumor progression (Lingle and Salisbury, 2000; Brinkley, 2001; Doxsey, 2001). Here, we have followed up previous indications

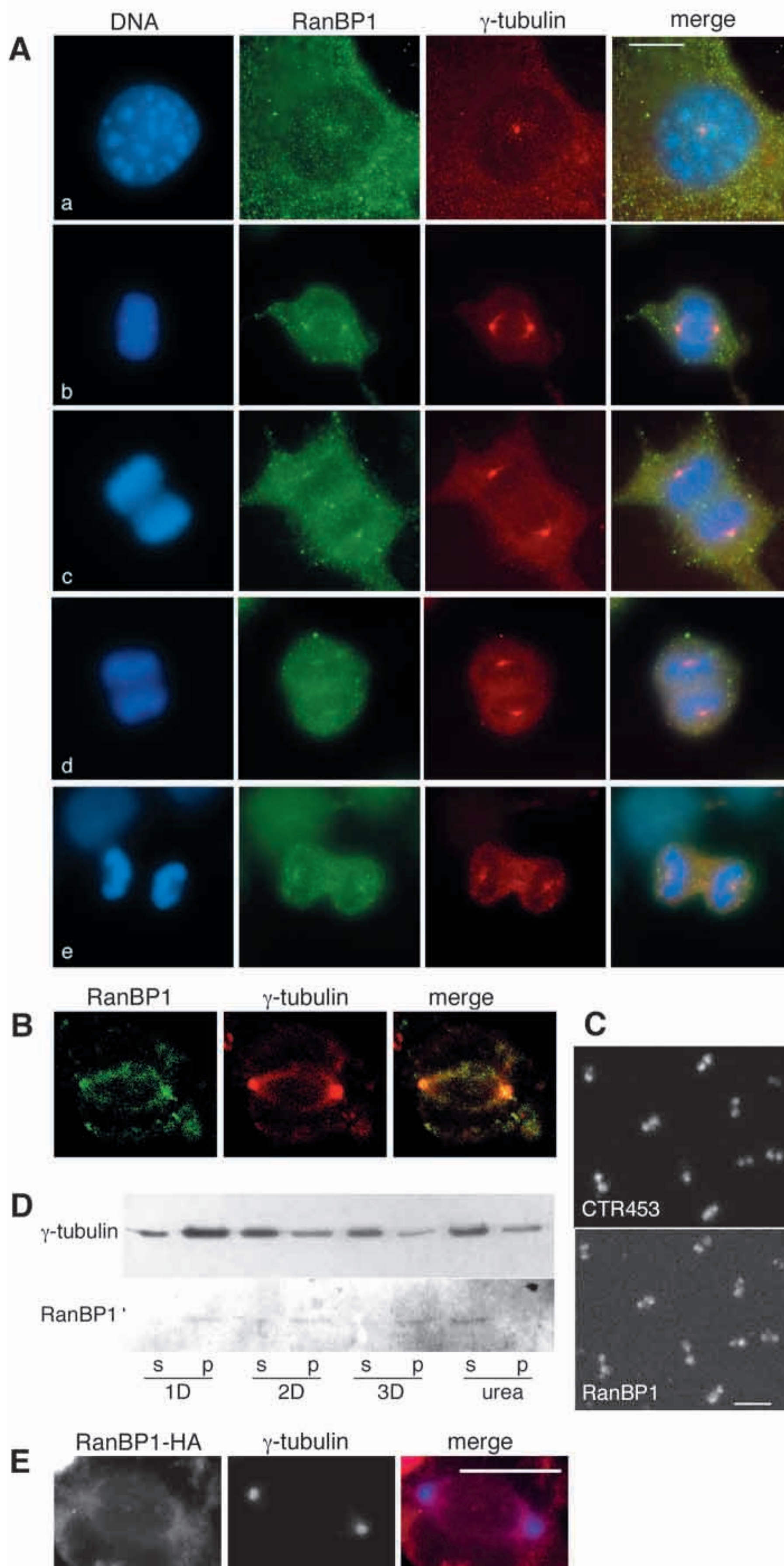

Fig. 6. A fraction of RanBP1 localizes at the centrosome. (A) Centrosomal RanBP1 in interphase (a), metaphase (b) and anaphase (c) NIH/3T3 cells. Examples of L929 (d) and HeLa (e) mitotic cells are also shown. Endogenous RanBP1 (second column) and γ-tubulin (third column) were revealed with FITC- and rhodamine-conjugated secondary antibodies, respectively. DNA was counterstained with DAPI (first column on the left). Signals are merged in the rightmost column. Scale bar, 10 μm. (B) Confocal signals for RanBP1 (FITC) (left) and γ-tubulin (rhodamine, middle) in a typical NIH/3T3 metaphase. Merged images are shown on the right. 15 focal planes of 2.28 μm thickness were scanned. (C) Anti-RanBP1 antibody (bottom) labels isolated KE37 centrosomes, stained by CTR453 (top). Scale bar, 50 μM. (D) RanBP1 is tightly associated with the centrosome fraction. Isolated centrosomes were extracted with buffers of increasing strength and analysed by western immunoblotting with the indicated antibodies. Abbreviations: p, pellet containing centrosome-associated proteins; s, supernatant containing solubilized proteins. The interaction of RanBP1 with centrosomes (bottom) is more resistant to detergents than that of γ-tubulin, a major PCM-recruited component (top). (E) Overexpressed RanBP1 localizes at spindle poles. An example of NIH/3T3 metaphase is shown. Anti-HA antibody, directed against exogenous RanBP1, is revealed with a rhodamine-conjugated secondary antibody. Centrosomes are stained with anti-γ-tubulin antibody revealed with an AMCA-conjugated secondary antibody. The merged image is shown in the right panel. Scale bar, 10 μm.

that RanBP1 overexpression yields multipolar spindles, and have sought to pinpoint the underlying defect. This is relevant in view of the fact that the *RanBP1* gene is a regulatory target of E2F- and retinoblastoma-related factors (Di Matteo et al., 1995; Di Fiore et al., 1999; Ishida et al., 2001), and hence can be expected to be deregulated in tumors in which this pathway is disrupted. Actually, both *RanBP1* and *RCC1* were recently identified as downregulated target genes of a novel anticancer drug (Damm et al., 2001), suggesting that either or both of these genes can actually be deregulated in transformed cells.

Ran is an abundant GTPase (10^7 molecules cell^{-1} in HeLa cells) (Bischoff and Ponstingl, 1991), and is estimated to be present in a 25-fold excess over endogenous RCC1 and fivefold excess over endogenous RanBP1 (Bischoff et al., 1995). In our transfection experiments, we

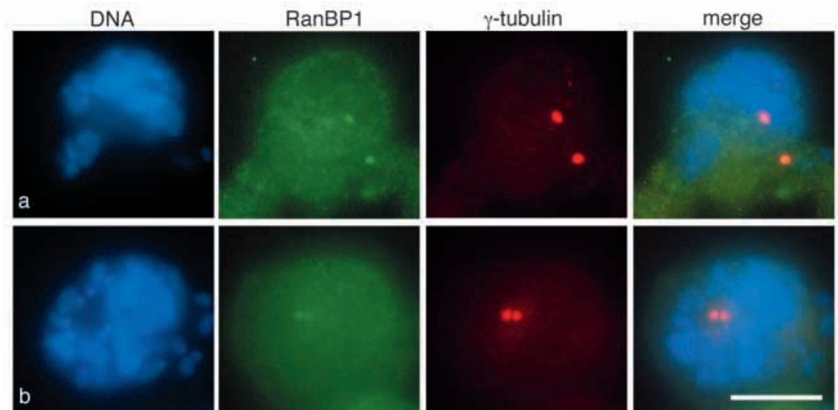

Fig. 7. RanBP1 retains its centrosomal localization after exposure to either nocodazole (a) or Taxol (b). Endogenous RanBP1 (second column) and γ-tubulin (third column) were revealed with FITC- and rhodamine-conjugated secondary antibodies, respectively. DNA was counterstained with DAPI (first column on the left). The rightmost column shows the merged signals of RanBP1, γ-tubulin and DAPI staining. Scale bar, 10 μm.

recorded an average fourfold increase in RanBP1 levels; furthermore, RanBP1-transfected cells that displayed mitotic centrosomal abnormalities typically showed higher than average levels of overexpression. That range of increase is expected to produce a significant shift in the balance of nucleotide hydrolysis and exchange on Ran. We previously reported that RanBP1 overexpression induces cell cycle abnormalities (Battistoni et al., 1997; Guarguaglini et al., 2000) comparable to those observed in the presence of Ran mutants (Ren et al., 1993; Ren et al., 1994; Moore et al., 2002), supporting the idea that RanBP1 acts by altering the Ran network. In addition, we have now sought to quantify the intracellular RanGTP levels using an antibody (AR12, a kind gift from I. Macara) that preferentially – although not exclusively – recognizes the GTP-bound conformation of Ran (Richards et al., 1995). Although these experiments do not allow us to draw a precise quantitative estimate, they do indicate that RanGTP levels are lowered in RanBP1-overexpressing compared with normal cells (data not shown).

Induction of multipolar spindles by RanBP1 excess reflects the aberrant splitting of single centrioles within diplosomes in mitosis. None of duplication of centrosomes, recruitment of γ-tubulin or glutamylation of centriole MTs are affected instead. Furthermore, no defects were recorded in focusing of MT arrays to the poles. Split centrioles retain their functional integrity and can organize polarized MT arrays, thereby giving rise to spindles with multiple poles. This is a novel finding and begins to identify aspects of centrosome organization and function that are influenced by members of the Ran network.

Cohesion and dynamics of centrosomes are highly regulated processes. After duplication, centrosomes remain tethered together throughout most of interphase, then separate in late G2 and eventually migrate to form the spindle poles. MTs contribute to the link between centrosomes (Jean et al., 1999). Cohesion in G2 and separation in mitosis are also regulated by a network of specific factors (Meraldi and Nigg, 2001), including the centrosomal C-Nap1 protein (Mayor et al., 2000), its upstream kinase Nek2 (Meraldi and Nigg, 2001) and the Inh2 regulator of Nek2 (Eto et al., 2002). Deregulated activity of these factors induces unscheduled centrosome separation but the integrity within centrosomes is not affected and so neither spindle assembly nor the mitotic division are necessarily perturbed (Mayor et al., 2002). RanBP1 overexpression influences neither the timing nor the extent of parental centrosome separation in interphase, but selectively perturbs cohesion of centrioles within diplosomes in mitosis.

Physiologically, centrioles undergo splitting during telophase, accompanied by extensive motility and repositioning of the mother centriole to the mid-body in preparation of cytokinesis (Piel et al., 2001). In early interphase, split centrioles act as duplication templates. Under abnormal circumstances, however, centrioles can split during mitosis, as observed during (for example) mitotic arrest induced by non-degradable cyclin B (Gallant and Nigg, 1992). Indeed, induction of mitotic delay by mercaptoethanol or colcemid was used as an experimental tool to study the functional relationship between centrioles and spindle poles (Sluder and Rieder, 1985). In RanBP1-overexpressing cultures, we recorded some increase in the mitotic index, but this was essentially due to prolonged of telophase. The timing of early mitotic progression was instead not dramatically perturbed in RanBP1-overexpressing cells, whereas centriole splitting could already be detected in prometaphase, as soon as the nuclear envelope disappeared – a stage that was not prolonged by RanBP1 overexpression. These observations support the conclusion that RanBP1-induced centrosomal abnormalities are not a consequence of abnormally prolonged mitosis.

The $RanBP1^{L186A/V188A}$ construct, which has a different localization from wild-type RanBP1 throughout interphase, has similar disruptive effects than wild-type on mitotic centrosomes. This is paralleled by the ability of this mutant to induce multipolar spindles as effectively as wild-type RanBP1 (Guarguaglini et al., 2000). These data are consistent with the view that overexpressed RanBP1 interferes with crucial factor(s) implicated in centrosome organization specifically during mitosis. Such factor(s) might be activated, and/or be capable of establishing crucial interactions at the centrosomal level, specifically after NEB in a manner that is similarly sensitive to NES-defective and wild-type RanBP1. The mitotic nature of the splitting phenomenon induced by RanBP1 excess was further evidenced in cells that resumed mitotic progression after NOC-induced arrest. NOC prevented the disruptive effect of RanBP1 excess, yet centrosome splitting was again appreciated after as little as 45 minutes after NOC removal and resumption of MT reconstitution in vivo. This experiment further strengthens the conclusion that centriole cohesion is sensitive to RanBP1 levels during mitosis and, furthermore, implicates MTs. Induction of diplosome splitting by high RanBP1 is also dependent on Eg5 activity, suggesting that

either centrosome separation is required or that some timely regulated interaction that is physiologically dependent upon Eg5 is required. It is noteworthy that RanBP1 was detected at centrosomes even in NOC-exposed cells. This observation and the ability of a RanBP1 fraction to localize at centrosomes already in interphase and to interact with centrosomes in a stable, detergent-resistant manner, converge to suggest that a small RanBP1 fraction constitutively associates with centrosomes. It has recently been found that a fraction of Ran also localizes at centrosomes, in the presence or absence of NOC (Keryer et al., 2003). Thus, the suppressive effect of NOC is not due to failure of RanBP1 or Ran to localize at centrosomes. Rather, MTs themselves or motor proteins might play a role in cohesion within diplosomes in a manner that is sensitive to high RanBP1 levels. We previously found that inactivation of mitotic RanBP1 by antibody microinjection impairs dynamics of the spindle MTs. RanBP1 excess might influence MT dynamics at spindle poles, and altered dynamics might in turn favor the aberrant separation of mother and daughter centrioles. An alternative – but not necessarily mutually exclusive – possibility is that one or more factor(s) that regulate the organization and/or the intrinsic dynamic features of mitotic centrosomes is transported to spindle poles in a MT-dependent manner after NEB and, once there, is sensitive to elevated levels of RanBP1. In the presence of NOC, the hypothetical protein(s) would not be transported to poles and so would not be in a position to modulate the behavior of centrioles, regardless of RanBP1 levels. Based on these observations, the mitotic role of Ran network components might be critically dependent on their ability to associate with specific mitotic structures. In mitosis, Ran members might reorganize in 'local factories' at specific locations and act on local downstream targets in the mitotic apparatus. The recent observation that spindle pole defects and chromosome misalignment are caused by a RCC1 mutant that mislocalizes to the cytoplasm but not by wild-type RCC1 (Moore et al., 2002) is consistent with this view.

Interestingly, while this work was in progress, disruption of spindle pole organization was observed in mammalian cells under interfering RNA-mediated inactivation of an important Ran target, TPX2 (Garrett et al., 2002). Although, in other studies, the major outcome of TPX2 inactivation was failure of MT connections between spindle poles, probably because of differences in the experimental conditions that yielded partial inactivation of TPX2 (Gruss et al., 2002), in the study by Garrett et al. (Garrett et al., 2002) multipolar spindles formed as a consequence of spindle pole fragmentation. Remarkably, these abnormalities are MT and Eg5 dependent, similar to those reported here under RanBP1 excess. The authors suggest that multipolar spindles induced in their conditions might reflect an imbalance between TPX2-dependent structural support and motor-driven force: when TPX2 is inactivated, the force would be exerted freely and cause spindle pole disruption. By analogy of reasoning, it is tempting to speculate that defective RanGTP formation caused by RanBP1 excess causes insufficient release of factor(s) that provide structural support to sister centrioles during spindle assembly.

In summary, a fraction of the RanBP1 protein is present at centrosomes throughout the cell cycle, where it can interact with Ran. At this location, RanBP1 can act on factor(s) that reach the centrosomes after NEB to contribute to the organization of mitotic centrosomes. The presence of excess RanBP1 favors the aberrant separation of individual centrioles in mitosis, giving rise to multipolar spindles. Further understanding the mechanisms through which Ran network components act locally in mitosis and control downstream targets in the assembly of mitotic structures will be a major field to disentangle in the near future.

We are indebted with M. Bornens, in whose laboratory some of the experiments reported here were performed. We are grateful to P. Denoulet and I. Macara for the gift of antibodies, to C. Celati for providing isolated centrosomes, and to E. Marchetti for help with confocal images. We also thank P. Meraldi for communicating unpublished results and G. Guarguaglini and M. Casenghi for helpful comments on this manuscript. This work was supported by grant ARC599 to AMT and by grants from Consiglio Nazionale delle Ricerche (CNR), Associazione Italiana per la Ricerca sul Cancro (AIRC) and Agenzia Spaziale Italiana (ASI) to PL. BDF and MC were supported by MIUR/CNR Doctoral fellowships, and RM was supported by a CNR research fellowship.

References

Bailly, E., Dorée, M., Nurse, P. and Bornens, M. (1989). p34^{cdc2} is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. *EMBO J.* **8**, 3985-3995.

Balczon, R., Bao, L., Zimmer, W. E., Brown, K., Zinkowski, R. P. and Brinkley, B. R. (1995). Dissociation of centrosome replication events from cycles of DNA synthesis and mitotic division in hydroxyurea-arrested Chinese hamster ovary cells. *J. Cell Biol.* **130**, 105-115.

Battistoni, A., Guarguaglini, G., Degrassi, F., Pittoggi, C., Palena, A., di Matteo, G., Pisano, C., Cundari, E. and Lavia, P. (1997). Deregulated expression of the *RanBP1* gene alters cell cycle progression in murine fibroblasts. *J. Cell Sci.* **110**, 2345-2357.

Bischoff, F. R. and Ponstigl, H. (1991). Mitotic regulator protein RCC1 is complexed with a nuclear Ras-related polypeptide. *Proc. Natl. Acad. Sci. USA* **88**, 10830-10834.

Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W. and Ponstingl, H. (1995). Co-activation of RanGTPase and inhibition of GTP dissociation by RanGTP binding protein RanBP1. *EMBO J.* **14**, 705-715.

Brinkley, B. R. (2001). Managing the centrosome numbers game: from chaos to stability in cancer cell division. *Trends Cell Biol.* **11**, 18-21.

Carazo-Salas, R. E., Guarguaglini, G., Gruss, O. J., Segref, A., Karsenti, E. and Mattaj, I. W. (1999). Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. *Nature* **400**, 178-181.

Clarke, P. R. and Zhang, C. (2001). Ran GTPase: a master regulator of nuclear structure and function during the eukaryotic cell division cycle? *Trends Cell Biol.* **11**, 366-371.

Damm, K., Hemmann, U., Garin-Chesa, P., Hauel, N., Kauffmann, I., Priepke, H., Niestroj, C., Daiber, C., Enenkel, B., Guilliard, B. et al. (2001). A highly selective telomerase inhibitor limiting human cancer cell proliferation. *EMBO J.* **20**, 6958-6968.

Dasso, M. (2002). The Ran GTPase: theme and variations. *Curr. Biol.* **12**, R502-R508.

Di Fiore, B., Guarguaglini, G., Palena, A., Kerkhoven, R. M., Bernards, R. and Lavia, P. (1999). Two E2F sites control growth-regulated and cell cycle-regulated transcription of the *Htf9-a/RanBP1* gene through functionally distinct mechanisms. *J. Biol. Chem.* **274**, 10339-10348.

Di Matteo, G., Fuschi, P., Zerfass, K., Moretti, S., Ricordy, R., Cenciarelli, C., Tripodi, M., Jansen-Durr, P. and Lavia, P. (1995). Transcriptional control of the *Htf9-a/RanBP-1* gene during the cell cycle. *Cell Growth Differ.* **6**, 1213-1224.

Doxsey, S. J. (2001). Centrosomes as command centres for cellular control. *Nat. Cell Biol.* **3**, E105-E108

Eto, M., Elliott, E., Prickett, T. D. and Brautigan, D. L. (2002). Inhibitor-2 regulates protein phosphatase-1 complexed with NimA-related kinase to induce centrosome separation. *J. Biol. Chem.* **277**, 44013-44020

Gallant, P. and Nigg, E. A. (1992). Cyclin B2 undergoes cell cycle-dependent nuclear translocation and, when expressed as a non-destructible mutant, causes mitotic arrest in HeLa cells. *J. Cell Biol.* **117**, 213-224.

Garrett, S., Auer, K., Compton, D. A. and Kapoor, T. M. (2002). hTPX2 is

required for normal spindle morphology and centrosome integrity during vertebrate cell division. *Curr. Biol.* **12**, 2055-2059

Gruss, O. J., Carazo-Salas, R. E., Schatz, C. A., Guarguaglini, G., Kast, J., Wilm, M., le Bot, N., Vernos, I., Karsenti, E. and Mattaj, I. W. (2001). Ran induces spindle assembly by reversing the inhibitory effect of importin alpha on TPX2 activity. *Cell* **104**, 83-93.

Gruss, O. J., Wittmann, M., Yokoyama, Hm, Pepperkok, R., Kufer, T., Sillje, H., Karsenti, E., Mattaj, I. W. and Vernos, I. (2002). Chromosome-induced microtubule assembly mediated by TPX2 is required for spindle formation in HeLa cells. *Nat. Cell Biol.* **4**, 871-879.

Guarguaglini, G., Renzi, L., D'Ottavio, F., di Fiore, B., Casenghi, M., Cundari, E. and Lavia, P. (2000). Regulated Ran-binding protein 1 activity is required for organization and function of the mitotic spindle in mammalian cells in vivo. *Cell Growth Differ.* **11**, 455-465.

Hetzer, M., Gruss, O. J. and Mattaj, I. W. (2002). The Ran GTPase as a marker of chromosome position in spindle formation and nuclear envelope assembly. *Nat. Cell Biol.* **4**, E177-E184.

Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M. and Nevins, J. R. (2001). Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. *Mol. Cell. Biol.* **21**, 4684-4699.

Jean, C., Tollon, Y., Raynaud-Messina, B. and Wright, M. (1999). The mammalian interphase centrosome: two independent units maintained together by the dynamics of the microtubule cytoskeleton. *Eur. J. Cell Biol.* **78**, 549-560.

Kalab, P., Pu, R. T. and Dasso, M. (1999). The Ran GTPase regulates mitotic spindle assembly. *Curr. Biol.* **9**, 481-484.

Kalab, P., Weis, K. and Heald. R. (2002). Visualization of a RanGTP gradient in interphase and mitotic *Xenopus* egg extracts. *Science* **295**, 2452-2456.

Kapoor, T. M., Mayer, T. U., Coughlin, M. L. and Mitchison, T. J. (2000). Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. *J. Cell Biol.* **150**, 975-988.

Keryer, G., Di Fiore, B., Celati, C., Lechtreck, K. F., Mogensen, M., Delouvée, A., Lavia, P., Bornens, M. and Tassin, A. M. (2003). Part of Ran is associated with AKAP450 at the centrosome. Involvement in microtubule organizing activity. *Mol. Biol. Cell* (in press).

Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature* **396**, 643-649.

Lingle, W. L. and Salisbury, J. L. (2000). The role of the centrosome in the development of malignant tumors. *Curr. Top. Dev. Biol.* **49**, 313-329.

Mayor, T., Stierhof, Y. D., Tanaka, K., Fry, A. M. and Nigg, E. A. (2000). The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. *J. Cell Biol.* **151**, 837-846.

Mayor, T., Hacker, U., Stierhof, Y. D. and Nigg, E. A. (2002). The mechanism regulating the dissociation of the centrosomal protein C-Nap1 from mitotic spindle poles. *J. Cell Sci.* **115**, 3275-3284.

Melchior, F. (2001). Ran GTPase cycle: one mechanism – two functions. *Curr. Biol.* **11**, R257-R260.

Meraldi, P. and Nigg, E. A. (2001). Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. *J. Cell Sci.* **114**, 3749-3757.

Meraldi, P., Lukas, J., Fry, A. M., Bartek, J. and Nigg, E. A. (1999). Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. *Nat. Cell Biol.* **1**, 88-93.

Merdes, A. and Cleveland, D. W. (1997). Pathways of spindle pole formation: different mechanisms; conserved components. *J. Cell Biol.* **138**, 953-956.

Moore, W., Zhang, C. and Clarke, P. R. (2002). Targeting of RCC1 to chromosomes is required for proper mitotic spindle assembly in human cells. *Curr. Biol.* **12**, 1442-1447.

Moudjou, M. and Bornens, M. (1994). Isolation of centrosomes from cultured animal cells. In *Cell Biology: A Laboratory Handbook* (ed. J. E. Celis), pp. 595-604. Academic Press.

Nachury, M. V., Maresca, T. J., Salmon, W. C., Waterman-Storer, C. M., Heald, R. and Weis, K. (2001). Importin beta is a mitotic target of the small GTPase Ran in spindle assembly. *Cell* **104**, 95-106.

Ohba, T., Nakamura, M., Nishitani, H. and Nishimoto, T. (1999). Self-organization of microtubule asters induced in *Xenopus* egg extracts by GTP-bound Ran. *Science* **284**, 1356-1358.

Piel, M., Meyer, P., Khodjakov, A., Rieder, C. L. and Bornens, M. (2000). The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. *J. Cell Biol.* **149**, 317-330.

Piel, M., Nordberg, J., Euteneuer, U. and Bornens, M. (2001). Centrosome-dependent exit of cytokinesis in animal cells. *Science* **291**, 1550-1553.

Pihan, G. A. and Doxsey, S. J. (1999). The mitotic machinery as a source of genetic instability in cancer. *Semin. Cancer Biol*. **9**, 289-302.

Ren, M., Drivas, G., D'Eustachio, P. and Rush, M. G. (1993). Ran/TC4: a small nuclear GTP-binding protein that regulates DNA synthesis. *J. Cell Biol*. **120**, 313-323.

Ren, M., Coutavas, E., D'Eustachio, P. and Rush, M. G. (1994). Effects of mutant Ran/TC4 proteins on cell cycle progression. *Mol. Cell. Biol*. **14**, 4216-4224.

Richards, S. A., Lounsbury, K. M. and Macara, I. G. (1995). The C terminus of the nuclear RAN/TC4 GTPase stabilizes the GDP-bound state and mediates interactions with RCC1, RAN-GAP, and HTF9A/RANBP1. *J. Biol. Chem.* **270**, 14405-14411.

Richards, S. A., Lounsbury, K. M., Carey, K. L. and Macara, I. G. (1996). A nuclear export signal is essential for the cytosolic localization of the Ran binding protein, RanBP1. *J. Cell Biol.* **134**, 1157-1168.

Schliwa, M., Pryzwansky, K. B. and Euteneuer, U. (1982). Centrosome splitting in neutrophils: an unusual phenomenon related to cell activation and motility. *Cell* **31**, 705-717.

Schliwa, M., Pryzwansky, K. B. and Borisy, G. G. (1983). Tumor promoter-induced centrosome splitting in human polymorphonuclear leukocytes. *Eur. J. Cell Biol.* **32**, 75-85.

Sherline, P. and Mascardo, R. N. (1982). Epidermal growth factor induces rapid centrosomal separation in HeLa and 3T3 cells. *J. Cell Biol.* **93**, 507-512.

Sluder, G. and Rieder, C. L. (1985). Centriole number and the reproductive capacity of spindle poles. *J. Cell Biol.* **100**, 887-896.

Sluder, G., Thompson, E. A., Miller, F. J., Hayes, J. and Rieder, C. L. (1997). The checkpoint control for anaphase onset does not monitor excess numbers of spindle poles or bipolar spindle symmetry. *J. Cell Sci.* **110**, 421-429.

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.

Wiese, C., Wilde, A., Moore, M. S., Adam, S. A., Merdes, A. and Zheng, Y. (2001). Role of importin-beta in coupling Ran to downstream targets in microtubule assembly. *Science* **291**, 653-656.

Wilde, A. and Zheng, Y. (1999). Stimulation of microtubule aster formation and spindle assembly by the small GTPase Ran. *Science* **284**, 1359-1362.

Wilde, A., Lizarraga, S. B., Zhang, L., Wiese, C., Gliksman, N. R., Walczak, C. E. and Zheng, Y. (2001). Ran stimulates spindle assembly by altering microtubule dynamics and the balance of motor activities. *Nat. Cell Biol.* **3**, 221-227.

Wolff, A., de Nechaud, B., Chillet, D., Mazarguil, H., Desbruyeres, E., Audebert, S., Edde, B., Gros, F. and Denoulet, P. (1992). Distribution of glutamylated alpha and beta-tubulin in mouse tissues using a specific monoclonal antibody, GT335. *Eur. J. Cell Biol.* **59**, 425-432.