Importin β is transported to spindle poles during mitosis and regulates Ran-dependent spindle assembly factors in mammalian cells

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Summary

Spatial control is a key issue in cell division. The Ran GTPase regulates several fundamental processes for cell life, largely acting through importin molecules. The best understood of these is protein import through the nuclear envelope in interphase, but roles in mitotic spindle assembly are also established. In mammalian cells, in which centrosomes are major spindle organizers, a link is emerging between the Ran network, centrosomes and spindle poles. Here, we show that, after nuclear envelope breakdown, importin β is transported to the spindle poles in mammalian cells. This localization is temporally regulated from prometaphase until anaphase, when importin β dissociates from poles and is recruited back around reforming nuclei. Importin β sediments with mitotic microtubules in vitro and its accumulation at poles requires microtubule integrity and dynamics in vivo. Furthermore, RNA interference-dependent inactivation of TPX2, the major Ran-dependent spindle organizer, abolishes importin β accumulation at poles. Importin β has a functional role in spindle pole organization, because overexpression yields mitotic spindles with abnormal, fragmented poles. Coexpression of TPX2 with importin β mitigates these abnormalities. Together, these results indicate that the balance between importins and spindle regulators of the TPX2 type is crucial for spindle formation. Targeting of TPX2/importin-β complexes to poles is a key aspect in Ran-dependent control of the mitotic apparatus in mammalian cells.

Key words: Importin β, Mitosis, Spindle poles, GTPase Ran, Microtubules, TPX2

Introduction

The GTPase Ran regulates fundamental processes during the cell cycle, including nucleocytoplasmic transport, assembly of the nucleus and nuclear envelope (NE), and mitotic spindle organization. Ran acts in these diverse processes through a conserved mechanism: depending on its nucleotide-bound state, Ran regulates the assembly or disassembly of multimeric complexes containing Ran effectors. In nuclear protein import, Ran effectors belonging to the importin family associate with their 'cargo' molecules in the cytoplasm and, after translocation through the nuclear pores, dissociate in the nucleus.

It is well established that nucleotide-bound Ran forms are compartmentalized. RanGTP is produced by the nuclear exchange factor RCC1, whereas GTP hydrolysis is catalyzed by RanGAP1 in cooperation with RanBP1, both of which are predominantly cytoplasmic. In interphase, this ensures that RanGTP is hydrolyzed in the cytoplasm and regenerated in the nucleus. RanGTP binds importin β with high affinity; this binding takes place in the nucleus and triggers the dissociation of import cargoes from their vectors (e.g. importin-α/importin-β dimers) (for reviews, see Hetzer et al., 2002; Weis, 2003; Quimby and Dasso, 2003). In other words, import complexes respond to nuclear RanGTP by releasing protein cargoes that can then productively operate in processes downstream.

Mitotic functions of Ran are also established. In *Xenopus* egg extracts, RanGTP activates spindle-activating factors (SAFs) that have crucial roles in mitotic spindle organization, including TPX2 (Gruss et al., 2001), NuMA (Nachury et al., 2001; Wiese et al., 2001) and the kinesin XCTK2 (Ems-Mc Clung et al., 2004). In somatic cells, the Ran network regulates the bipolar organization of the spindle (Guarguaglini et al., 2000; Moore et al., 2002; Di Fiore et al., 2003), microtubule (MT) anchorage at centrosomes (Keryer et al., 2003), MT dynamics (Guarguaglini et al., 2000) and the spindle checkpoint (Arnaoutov and Dasso, 2003; Salina et al., 2003; Joseph et al., 2004). Among Ran regulatory targets, a prominent role is played by TPX2, which regulates spindle pole structure (Wittmann et al., 2000; Garrett et al., 2002; Gruss et al., 2002), spindle assembly from MTs (Schatz et al., 2003) and activation and localization of the mitotic regulator Aurora A (Kufer et al., 2002; Trieselmann et al., 2003; Tsai et

al., 2003). Surprisingly at first – given that spindle assembly occurs in the absence of transport – Ran also regulates these mitotic processes via importins. SAFs carry nuclear localization sequences (NLSs) recognized by importins, from which they are released in a RanGTP-dependent manner. In interphase, the compartmentalization of RanGTP ensures that SAFs dissociate from importins only in nuclei, away from MT-organizing centres, thus preventing unscheduled spindle assembly until the NE dissolves. Because importin-bound SAFs are inactive until RanGTP dissociates the complexes, importins α and β formally act as negative regulators of the mitotic spindle.

RanGTP is produced by chromatin-bound RCC1, therefore the bulk remains concentrated around chromosomes in mitosis (Kalab et al., 2002; Smith et al., 2002). In *Xenopus*-oocyte-derived model systems for spindle assembly, this causes the release of SAFs from importin β to be restricted around chromatin. In somatic cells, in which centrosomes are crucial organelles in spindle organization, spindle functions orchestrated by Ran cannot rely exclusively on RanGTP-generated signals around chromosomes. Indeed, recent work has depicted subtle associations of Ran network members with specific mitotic structures, e.g. kinetochores (Joseph et al., 2002; Arnaoutov and Dasso, 2003; Joseph et al., 2004) and centrosomes. In particular, fractions of Ran (Keryer et al., 2003) and RanBP1 (Di Fiore et al., 2003) associate constitutively with centrosomes, where Ran is recruited through the AKAP450 protein (Keryer et al., 2003). These associations are crucial for mitotic progression: the delocalization of Ran from centrosomes, expression of Ran mutants unable to switch nucleotide-bound states, RanBP1 overexpression and TPX2 inactivation all cause the disorganization of asters, centrosome splitting or fragmentation, and multipolar spindles, implicating Ran-dependent signals in control of centrosomes and spindle poles (for a review, see Di Fiore et al., 2004).

It has not yet been clarified whether spatial control of the mammalian mitotic spindle proceeds through the local regulation of importin complexes by RanGTP. Investigating the spatial distribution of these factors during mitotic progression is relevant if we are to understand the temporal and spatial cues for Ran-dependent spindle control in somatic cells. As a step towards addressing this here, we have investigated the fate of importin β during the mammalian mitosis.

Materials and Methods

Plasmids

Human importin β (kindly provided by Dirk Gœrlich, University of Heidelberg, Germany) was amplified using the polymerase chain reaction (PCR) and ligated to pEGFP-N1 vector (Clontech) (pIB-GFP construct). The mammalian expression construct for TPX2 was a kind gift from Isabelle Vernos (EMBL, Heidelberg, Germany). The NLS construct, kindly given by Xin Wei Wang (NIH-NCI, Bethesda, MD), carries an SV40-derived NLS in frame with green fluorescent protein (GFP). The renilla luciferase vector was from Clontech.

Cell culture and synchronization

Human HeLa and HeLa-derived HC1 cells expressing centrin1-GFP (kindly given by Michel Bornens, Institut Curie, Paris, France), human MRC5 fibroblasts, murine NIH-3T3 embryo fibroblasts and murine L929 subcutaneous fibroblasts were cultured with 10% foetal calf serum (and 500 μg ml^{-1} G418 in the case of HC1 cultures). To enrich cultures in mitotic cells, 2 mM thymidine was added for 16-24 hours, then washed out and replaced with fresh medium containing 30 μM deoxycytidine for 7-10 hours. In some experiments, 10 μg ml^{-1} cycloheximide was added to inhibit protein synthesis. Where indicated, after release, 0.2 μg ml^{-1} nocodazole (NOC) or 1 μM Taxol (TAX) was added for the last 4-6 hours of culture. Cell cycle profiles were analysed in a FACStar (Beckton Dickinson). For MT regrowth assays, cells were incubated at 0°C for 1 hour and then at 37°C for 5-20 minutes.

RNA interference

For RNA interference (RNAi), small interfering RNA (siRNA) oligonucleotides (Dharmacon Research) targeting the TPX2 sequence 144-GAA UGG AAC UGG AGG GCU U-162 were annealed and transfected in HeLa cells at a final concentration of 80 nM following the standard protocol from Dharmacon Research. Oligofectamine (Invitrogen) was used as transfection reagent. For control, we used a 21-mer duplex targeting the luciferase sequence (GL-2). Cells were harvested 40 hours after transfection.

Transfection experiments

Cells were transfected using FUGENE (Roche Diagnostic; 3 ml per mg DNA) or Lipofectamine 2000 (Invitrogen; 1 ml per mg DNA). Tested doses of importin β construct ranged from 2 μg DNA per 1×10^6 cells to 6 μg DNA per 1×10^6 cells; 2 μg were used in most experiments. pCMV-based constructs expressing TPX2, NLS, GFP or luciferase were co-transfected in the amount indicated in the text and the total amount DNA in the transfection mixture was made 8 μg with pCMV empty vector. Cells were harvested 30 hours after transfection.

MT polymerization and sedimentation

Lyophilized tubulin from bovine brain (Cytoskeleton) was diluted to 1 mg ml^{-1} in cold 10% glycerol BRB80 buffer (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8) supplemented with 1 mM MgGTP. MTs were polymerized by gradually adding TAX to 10 μM and warming at 37°C for 30 minutes. Mixtures were centrifuged through a 40% glycerol cushion in BRB80 (300,000 ***g***, 37°C, 10 minutes). The MT pellet was resuspended in BRB80 containing 1 mM dithiothreitol and 20 μM TAX. Mitotic extracts were prepared from thymidine arrested/released cultures in BRB80 buffer containing protease and phosphatase inhibitors, and 20 μg ml^{-1} cytochalasin B, and homogenized in a loose-fitting Dounce homogenizer. The crude extract was centrifuged (20,000 ***g***, 30 minutes, 4°C) and the supernatant was incubated with 0.5 mM MgGTP, 3 mM $MgCl_2$, 20 μM TAX and either 5 mM AMP-PnP (Mg salt) or 2.5 mM MgATP (30 minutes, 33°C). Extract aliquots were incubated with or without exogenous polymerized MTs (0.3 mg ml^{-1} final tubulin concentration). Samples were centrifuged through a 1 M sucrose cushion in BRB80 with protease and phosphatase inhibitors, 10 μM TAX, and 0.5 mM ATP (Mg salt) (150,000 ***g***, 30 minutes, 22°C). In some experiments, extract aliquots (0.7-1.0 mg) were incubated (1 hour on a rotating wheel at 4°C) with 10 μg m70.1 antibody to dynein intermediate chain (DIC) (mouse IgM isotype, Sigma-Aldrich D5167), 10 μg non-specific antibody (goat anti-mouse IgM; Santa Cruz Biotechnology), or an equal volume of BRB80 (no-antibody control). MT polymerization and centrifugation were then carried out as above.

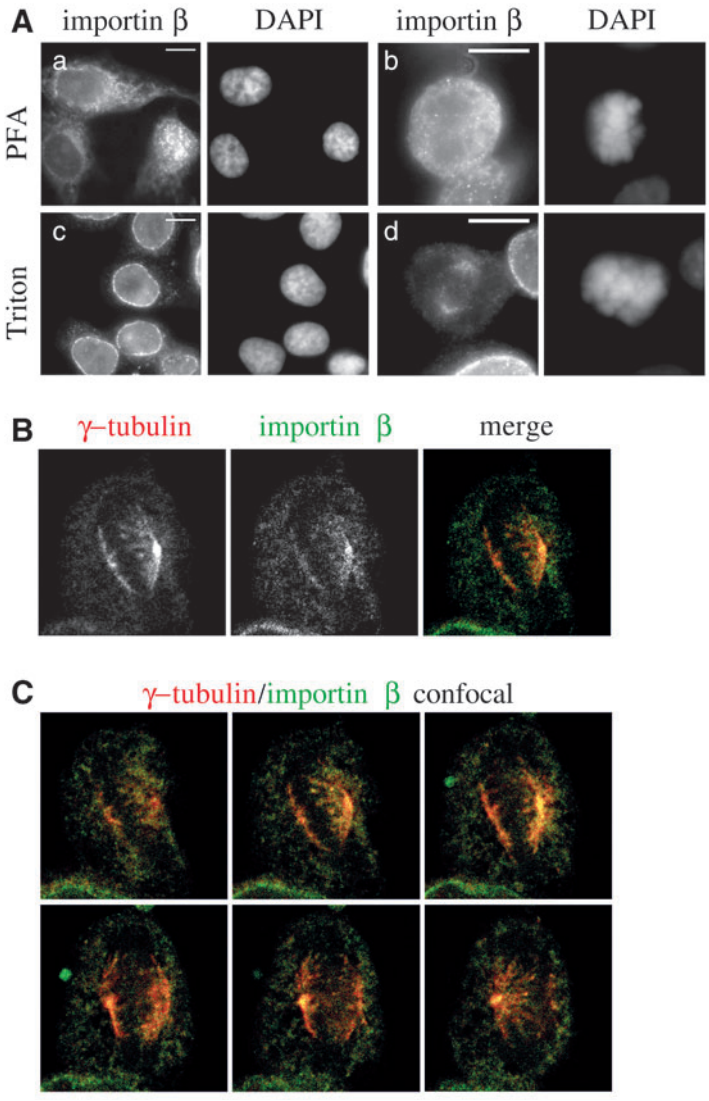
Western blotting

Protein extracts were separated using sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) on a 10% gel,

transferred on membranes and incubated with primary antibodies against importin β (clone 23, 1 μg ml^{-1}), Ran (clone 20, 0.25 μg ml^{-1}) and p150^{Glued} (clone 1, 610473, 1 μg ml^{-1}) from Transduction Laboratories, against α-tubulin (clone B-5-1-2, 3.35 μg ml^{-1}) and DIC (m70.1, mouse ascite fluid, 1:2000) from Sigma-Aldrich, against actin (clone I19, 0.5 μg ml^{-1}), p34/cdc2 (clone 17, 0.5 μg ml^{-1}), cyclin B1 (GNS1, 0.5 μg ml^{-1}), Ran (C-20, 0.5 μg ml^{-1}), and importins α1 and α2 (C-20 clones sc6918 and sc6917, respectively; both 1.5 μg ml^{-1}) from Santa Cruz Biotechnology. Reactive proteins were revealed using horseradish-peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology).

Immunofluorescence

Cells grown on coverslips were either fixed in 3% paraformaldehyde (PFA), 30 mM sucrose for 10 minutes or treated with 1% Triton X-100 in PHEM (45 mM HEPES pH 6.9, 45 mM PIPES pH 6.9, 10 mM EGTA, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride) for 4 minutes, washed and then fixed in 3% PFA, 30 mM sucrose for 10 minutes followed by 2 minutes in methanol. Primary antibodies were against importin β (see above, 1:50), γ-tubulin (T3559, Sigma-Aldrich, 1:1000), α-tubulin (clone B-5-1-2, Sigma-Aldrich, 1:2000), IAK1/Aurora-A (clone 4, Transduction Laboratories, 1:500), TPX2 (kindly provided by Ian Mattaj, EMBL, Heidelberg; 1:5000), centrin (mouse monoclonal 20H5, kindly given by Jeffrey Salisbury, Mayo Clinic Foundation, Rochester, MN; 1:5000), centrin 2 (rabbit polyclonal, kindly provided by M. Bornens, 1:1000) and RanGTP [AR-12 (Richards et al., 1995), kindly provided by Ian Macara, University of Virginia, Charlottesville, VA; 1:50], NuMA (Ab-2, Oncogene, 1:50). Secondary antibodies were conjugated to fluorescein isothiocyanate (FITC), Cy3 or 7-amino-4-methylcoumarin-3-acetic acid (AMCA) (Jackson Immunoresearch Laboratories), rhodamine (Santa Cruz Biotechnology), or Texas Red (Vector). Cells were examined under an epifluorescence Olympus AX70 microscope with a CCD camera (Photometrics) or a Leica DMR with a CoolSnap (Photometrics). Confocal images were taken under TCS-SP2 (Leica) or LSM 510 META (Zeiss) microscopes.



Results

Importin β localizes to the spindle poles after NE breakdown

Given that regulatory functions of Ran are mostly mediated via dissociation of importin β complexes, we decided to analyse the temporal and spatial distribution of importin β in mitotic cells. In HeLa cultures fixed with PFA, the importin β signal was mostly concentrated around the NE in interphase (Fig. 1Aa). After NE breakdown, importin β diffused throughout the mitotic cytoplasm with no discernible association with specific structures (Fig. 1Ab). When cells were subjected to mild extraction with Triton X-100 to eliminate soluble proteins before fixation, the anti-importin-β antibody decorated the nuclear periphery of interphase cells with a punctate pattern (Fig. 1Ac). This indicates that most importin β was solubilized, essentially leaving the nuclear pore complex (NPC) associated pool. Under these conditions, a discrete fraction of importin β was visible at spindle poles in mitotic cells (Fig. 1Ad). The specificity of the importin β signal at poles was confirmed using an independent Texas red-labelled antibody to anti-importin-β in the HeLa-derived cell line HC1, which expresses a centrin-1/GFP fusion protein that labels centrioles (Piel et al., 2000). When these cells reach mitosis, importin-β signals surrounding centriole pairs were visible at each pole. Confocal microscopy showed that importin β largely co-localizes with mitotic spindle poles, revealed by γ-tubulin staining (Fig. 1B), through all scanned planes (Fig. 1C). Signals were also visible, to a lesser extent, along the spindle MTs. The same localization of importin β at spindle poles was detected in murine NIH-3T3 and L929 cells (data not shown).

Accumulation of importin β at mitotic spindle poles is temporally regulated

The solubilization and fixation conditions described above were used thereafter to follow up importin β during mitotic

Fig. 1. A detergent-resistant fraction of importin β in HeLa cells. (A) IF analysis of importin β in interphase (a,c) or mitotic (b,d) cells after PFA fixation (a,b) or TritonX-100 pre-extraction (c,d); in each series, importin β is depicted on the left and DAPI staining on the right. Scale bars, 10 μm. (B) Confocal co-localization of importin β (FITC, green) and γ-tubulin (rhodamine, red) at spindle poles of a metaphase HeLa cell. (C) Merged signals colocalize to poles throughout all sections of the metaphase; representative focal planes (0.37 μm sections) are shown.

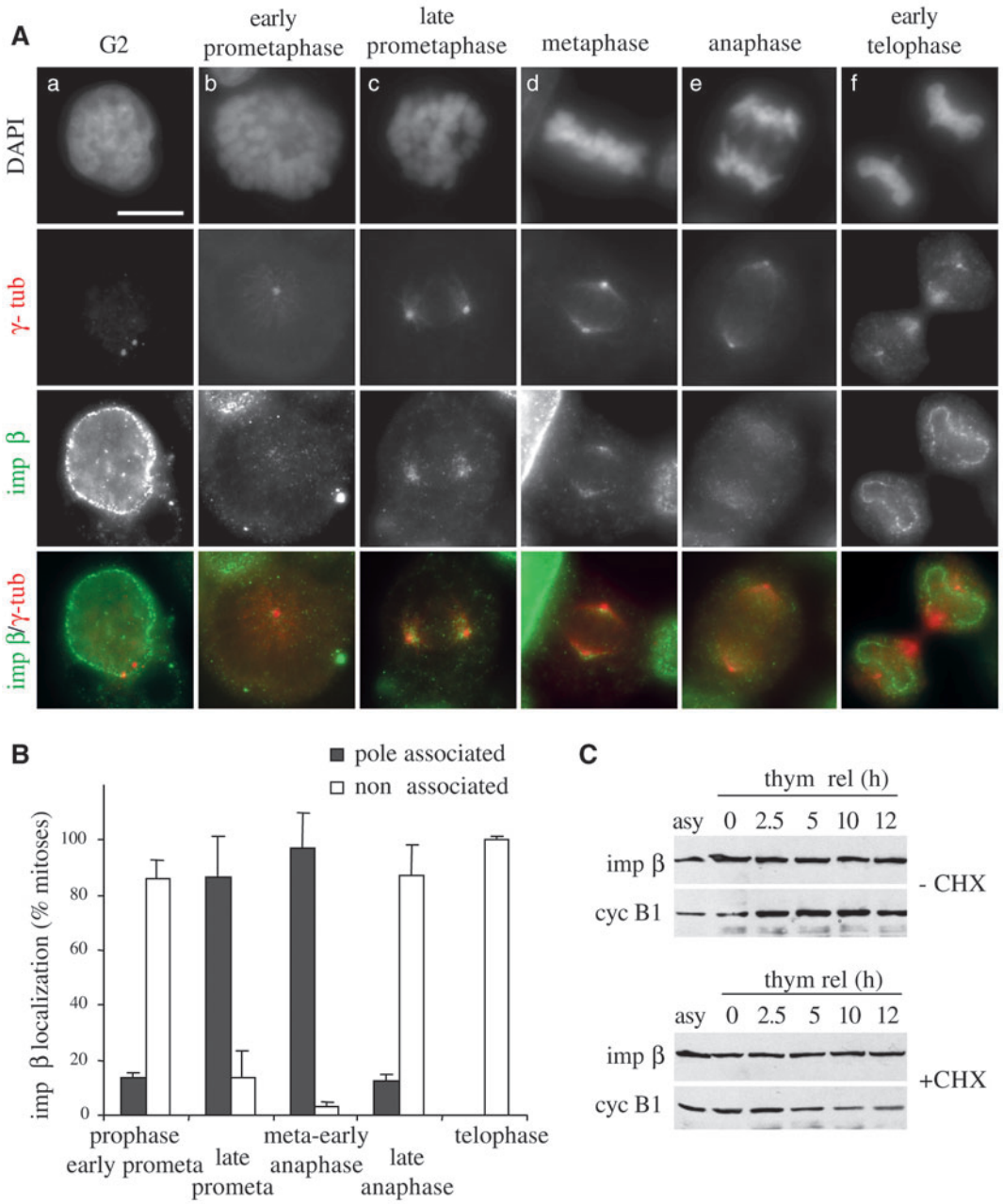


Fig. 2. Importin-β localization to spindle poles is temporally regulated during mitosis. (A) Progression from G2, in which the NE is still intact (a) through mitosis (b-f) is revealed by DAPI (top). Centrosomes are visualized by γ-tubulin staining (red); importin β is depicted in green. Scale bar, 10 μm. (B) Frequency of mitotic cells with pole-associated importin-β signals. At least 100 mitotic figures per stage were analysed in three experiments. (C) Western blot of importin β in extracts from asynchronous cultures (asy) in thymidine-arrested cells (time 0), and at the indicated times after thymidine wash-out, with or without inhibition of protein synthesis by cycloheximide (CHX). For control, cyclin B1 levels peaked 5-10 hours after the block release (G_2- to M-phase progression) in a protein-synthesis-dependent manner.

progression. In Fig. 2Aa, the typical pattern of importin β (dotted around nuclei) is visible in a G_2-phase cell identified by complete centrosome duplication: detergent-resistant signals associated with the NE are abundant at that stage. From G_2 to prometaphase, as the NE gradually dissolves, weak importin-β signals were visible in only a small proportion of extracted cells, whereas most cells failed to show any detectable importin-β signal (Fig. 2B); where they were visible, faint signals localized to the forming asters (Fig. 2Ab). However, PFA-fixed cells at the same stage displayed intense signals (not shown). Furthermore, by western-blot analysis of staged extracts after release from thymidine-induced G_1/S-phase arrest in the presence or absence of CHX, we found that importin-β levels remained stable in the absence of new protein synthesis for over 12 hours after S-phase onset, ruling out any stage-specific downregulation (Fig. 2C). Thus, the loss of signal from G_2 phase to early prometaphase must reflect a massive solubilization of importin β, suggesting that importin

β is transiently released from NPCs as the NE progressively disperses and does not interact tightly with cellular structures, so that most of it is removed by detergent extraction. After prometaphase, discrete importin-β signals were again clearly visible and were concentrated at spindle poles, as revealed by γ-tubulin staining (Fig. 2Ac-d). This suggests that a detergent-resistant association with spindle poles is established from prometaphase to anaphase. In anaphase and early telophase, when chromatin is still condensed, importin β dissociates from the poles and redistributes around the edge of the reforming nuclei (Fig. 2Ae-f).

A proportion of Ran associates with centrosomes throughout the cell cycle and GTP-bound Ran is found in the purified centrosome fraction from asynchronous cells (Keryer et al., 2003). Given that the interaction of RanGTP with importin β is expected to be crucial for mitotic activation of NLS-containing SAFs, it was important to establish whether RanGTP and importin β actually co-localize within mitotic cells. To ask this question, we used a conformational antibody (AR-12) that preferentially recognizes GTP-bound Ran (Richards et al., 1995). Mitotic HeLa cells were collected after release from thymidine arrest, permeabilized, incubated with AR-12 antibody in native conditions and then fixed and processed for immunofluorescence (IF). Two different combinations of secondary antibodies were used to reveal importin β and RanGTP antibodies (Fig. 3Aa,b). Under these conditions, a large proportion of RanGTP is extracted and the proportion that is still visible concentrates around poles and, to a minor extent, along MTs. From prometaphase to anaphase, importin β and RanGTP clearly colocalize to spindle poles.

In addition to the colocalization of RanGTP with importin β, activation of NLS-containing SAFs would obviously require that importin α, the direct 'receptor' of NLS sequences, localizes to the same sites. Importin-α isoforms with subtle preference for different NLS subsets are differently expressed in mammalian cells. We analysed importins α1 (Fig. 3B) and α2 (not shown) in HeLa cells. After prophase, PFA fixation reveals colocalization with the spindle, whereas detergent extraction of the soluble pool before fixation leaves a small fraction associated with the poles. In anaphase, importin α is recruited back to re-forming nuclei. Thus, importins α and β localize with RanGTP to spindle poles from prometaphase to anaphase.

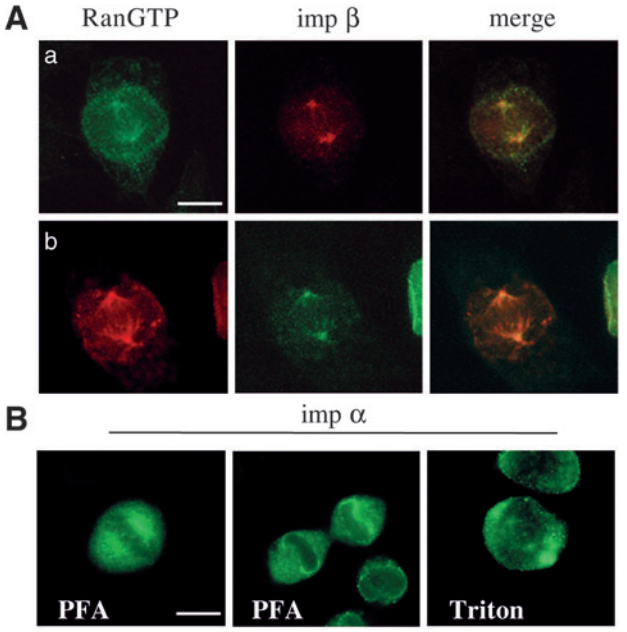


Fig. 3. RanGTP and importins α and β colocalize to spindle poles. (A) The AR-12 antibody was used in unfixed cells to label native RanGTP, followed by FITC- (a) or rhodamine- (b) conjugated secondary antibody. Importin β was revealed using Texas-Red- (a) or FITC- (b) conjugated secondary antibody. Scale bar, 10 μm. (B) Importin α in PFA-fixed metaphase (left) and anaphase (middle) cells; after Triton X-100 extraction (right), a pole-associated fraction is evident at spindle poles. Scale bars, 10 μm.

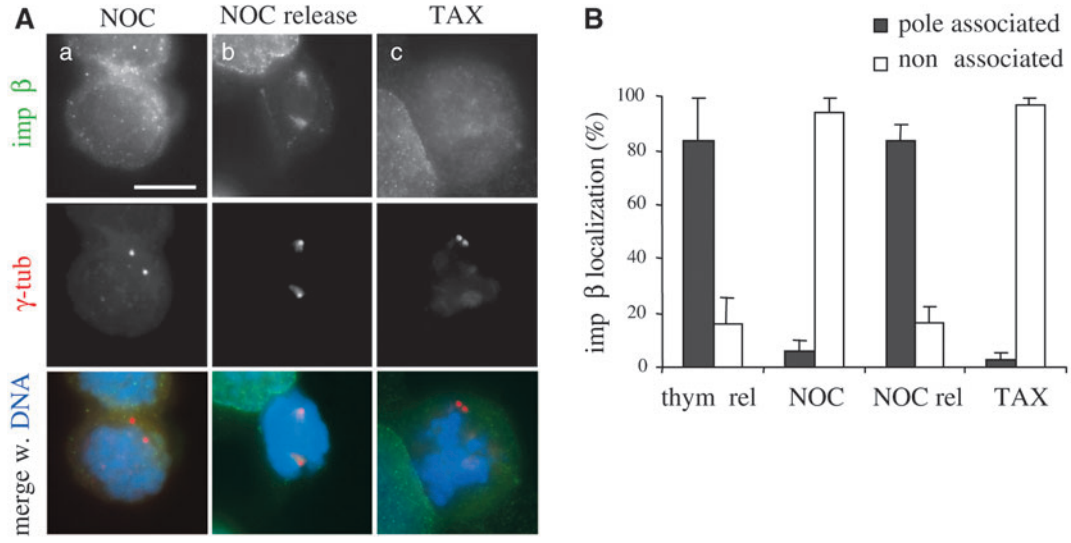


Fig. 4. Importin-β localization to asters/spindle poles depends on MT integrity and dynamics. (A) IF analysis of importin-β/FITC and γ-tubulin/rhodamine in mitotic cells released from thymidine arrest and exposed to NOC or TAX, or after NOC release. (B) Frequency of mitotic figures in which importin β did or did not localize to asters/spindle poles in the presence of MT-directed drugs. 200 mitoses per condition were analyzed in four experiments.

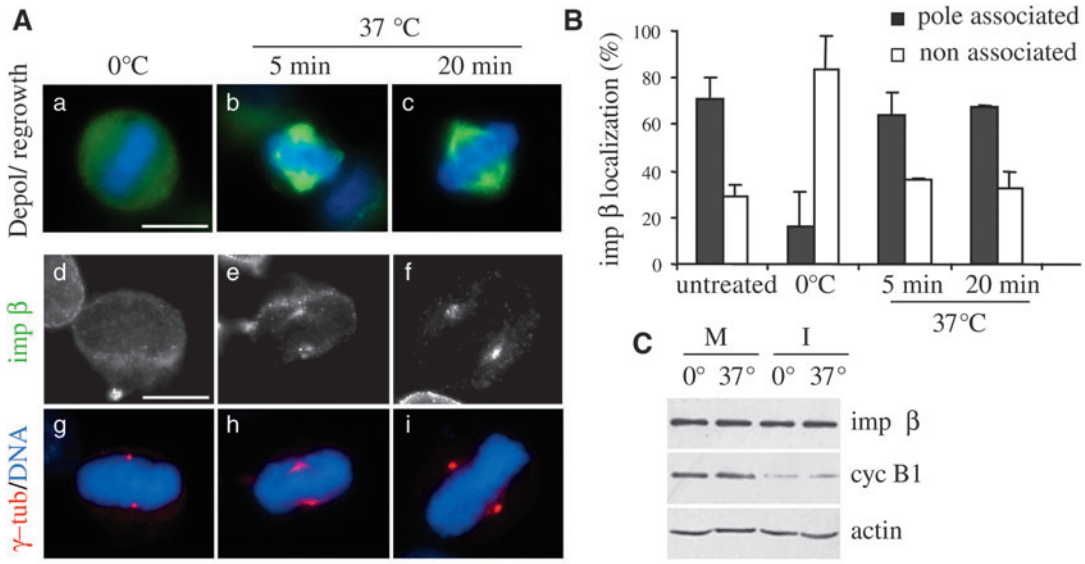


Fig. 5. The mitotic localization of importin β is sensitive to temperature-dependent MT depolymerization and regrowth. (A) Monitoring of MT depolymerization and regrowth by staining with α-tubulin/FITC antibody (a-c). In parallel slides, importin β was visualized by FITC (d-f) and γ-tubulin by rhodamine (visualized together with DAPI in g-i). Scale bar, 10 μm. (B) Frequency of mitoses in which importin β did or did not localize at spindle poles during the assay. At least 100 mitoses per condition were analysed (two experiments). (C) Western blot of importin β in interphase (I) and mitosis (M), with (37°C) or without (0°C) polymerized MTs.

Importin-β localization to spindle poles requires integrity of mitotic MTs

We next asked whether the accumulation of importin β at poles is dependent on MTs. HeLa cell populations were enriched in mitotic cells by thymidine presynchronization at the G_1/S-phase boundary, then releasing the block and exposing cells cultures to NOC to inhibit the polymerization of MTs during progression towards mitosis. Parallel slides from these cultures were stained for α-tubulin to control the effect of the drugs on the spindle MTs (not shown). After Triton X-100 extraction, importin-β signals were faint in NOC-treated mitotic cells and residual signals had no discrete localization (Fig. 4Aa). When NOC was washed out and MTs were allowed to reassemble for 30 minutes, importin β again localized to the poles of the re-forming spindles (Fig. 4Ab), implicating MT integrity in importin-β localization to spindle poles. To assess whether MT dynamics was also required, thymidine-arrested and -released cells were exposed to TAX during the last 4-6 hours of culture. Under these conditions, mitotic MTs were heavily reorganized in bundles and their dynamics was blocked (not shown). Importin β again failed to colocalize with γ-tubulin and showed a diffuse staining throughout the cell (Fig. 4Ac). Thus, drugs that target MT integrity or dynamics inhibit the localization of importin β to asters and spindle poles (Fig. 4B).

Because MT-targeting drugs arrest cells in early mitotic stages, we wanted to substantiate these findings further in cells in which MT function was impaired after the spindle was assembled. To achieve this, HeLa cells were incubated at 0°C to induce MT depolymerization in preassembled spindles (Fig. 5Aa). A metaphase with normal centrosomes, in which MTs were depolymerized after chromosome alignment was achieved, is shown in Fig. 5Ag: importin β diffused out in the cytoplasm with no discrete localization (Fig. 5Ad). When cells were reincubated at 37°C, MT polymerization was resumed within minutes (Fig. 5Ab,c), accompanied by the rapid relocalization of importin β to the poles (Fig. 5Ae,f). Results are quantified in Fig. 5B. We also analysed extracts from mitotic cells collected by 'shake-off' and from interphases that remained adherent during the shake-off procedure, with or without incubation at 0°C. No variation in importin-β levels was observed (Fig. 5C), so the weak, diffuse signals after cold-induced depolymerization reflect facilitated loss during the solubilization procedure in the absence of MTs, whereas protein levels remain stable. Together these findings suggest that importin-β molecules reach the spindle poles in a MT-dependent manner.

Importin β associates with MTs in the presence of motors

To test whether importin β interacts with mitotic MTs to reach the poles, we carried out cosedimentation assays with in-vitro-polymerized MTs. These were prepared from mitotic cell extracts treated with TAX and either AMP-PnP (a non-hydrolysable ATP analogue) to facilitate the association of MTs with motor proteins, or with ATP concentrations that destabilize the association. We found that some importin β did cosediment with MTs in the presence of AMP-PnP (Fig. 6A), with a comparable enrichment in the MT/motor pellet to that of the p34/cdc2 mitotic kinase, analysed for comparison. RCC1 was instead essentially found in the supernatant (not shown). Addition of exogenous tubulin did not increase the amount of cosedimenting importin β: this indicates that binding sites on endogenous MTs are not limiting, suggesting that other factors mediate the association of importin β with mitotic MTs. Importins α1 (data not shown) and α2 (Fig. 6A) revealed the same cosedimentation pattern with the motor-enriched, but not the tubulin-supplemented, MT pellet. To investigate whether importin β reaches spindle poles through active transport, we next used the m70.1 antibody against the intermediate chain of dynein, a major minus-end-directed motor. Consistent with other

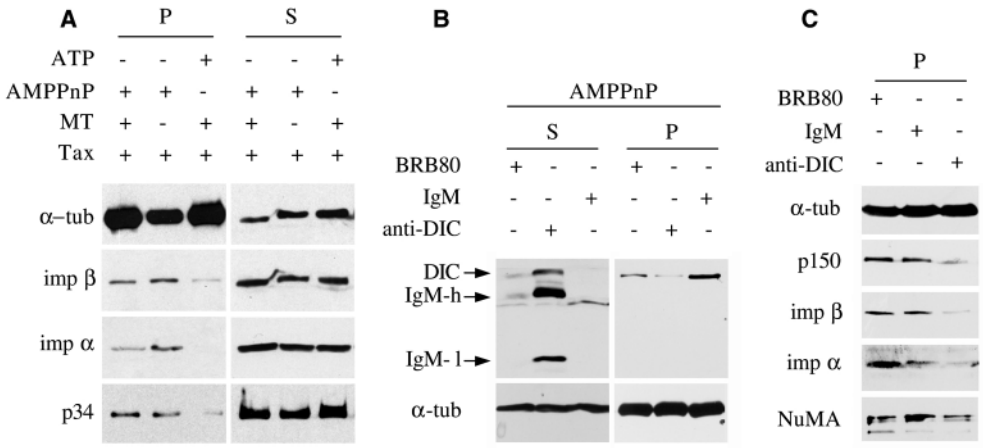


Fig. 6. Importins α and β sediment with polymerized mitotic MTs. (A) Fractions of importin α and β sediment with the MT pellet (P) after sucrose centrifugation of mitotic HeLa extracts treated with Taxol, and are enriched in the presence of AMP-PnP, but not with the addition of exogenous MTs. S, supernatant. (B) The incubation of mitotic extract with anti-DIC antibody prevents dynein association with the P fraction. Arrows indicate the position of dynein intermediate chain (DIC), antibody heavy (IgM-h) and light (IgM-l) chains in the S fraction. (C) Western-blot analysis of the indicated proteins in the MT pellet pre-incubated with anti-dynein antibody (anti-DIC lane), non-specific antibody (IgM lane) or buffer alone (BRB80 lane).

reports (Wittmann et al., 1998; Merdes et al., 2000), incubation of mitotic extract with this antibody substantially inhibited the association of dynein with polymerized MTs (Fig. 6B). As expected, under these conditions, the MT pellet contained less p150/dynactin (the activating partner of dynein) and NuMA, which is largely though not exclusively transported to poles by dynein-dynactin complexes (Merdes et al., 2000). The amount of cosedimenting importins α and β was also reduced compared with extract incubated with non-specific antibody or with buffer alone (Fig. 6C). These results therefore implicate dynein in transport of importins α and β along MTs.

Overexpressed importin β localizes to poles and induces mitotic spindle abnormalities

Evidence for a mitotic role of importin β in cells thus far rest upon injection experiments using a mutant protein lacking the Ran-interacting region in mitotic Ptk1 cells (Nachury et al., 2001). This caused disorganized spindles and chromosome misalignment, first suggesting that spindle assembly requires RanGTP-dependent dissociation of mitotic factors from importin β. The present finding that, after NE breakdown, importin β redistributes to the spindle poles, where a RanGTP-bound fraction resides, lead us to surmise that deregulated levels of importin β would affect spindle organization even when the full-length form is overexpressed in cells. To test this, we generated a GFP-tagged importin β mammalian expression vector (pIB-GFP). The chimaeric protein encoded by this plasmid localizes to the NE in interphase and to poles in mitosis (Fig. 7A), similar to the endogenous protein. For control, Triton X-100 did not affect the localization of GFP-tagged histone H_2B, which was entirely retained in nuclei and mitotic chromosomes, whereas GFP alone was fully washed out under these conditions (not shown).

In initial transfection experiments in HeLa cultures, high doses of importin-β/GFP yielded a low mitotic index, paralleled by an increase in interphase cells reactive to anti-cyclin-B1 antibody (and therefore in G_2 phase) compared with control cultures, suggesting that mitotic entry was inhibited. When lower importin-β doses were used, mitotic entry was not inhibited. Densitometric analysis of protein levels in western-blot experiments and quantification of GFP signals from the importin chimaeras in microscope images indicate that cells expressing approximately a 1:1 ratio of exogenous to endogenous protein underwent mitotic entry normally. Under these conditions, mitotic abnormalities were revealed by α-tubulin staining in HeLa cells, MRC5 human fibroblasts and NIH-3T3 murine fibroblasts. Most spindles had more than two poles (Fig. 7B); chromosome alignment failed in metaphase and lagging chromatids were frequent in anaphase. These abnormalities were accompanied by an increased mitotic index in all three cell lines (Fig. 7C), although no specific lengthening of a particular mitotic substage was recorded, suggesting that the spindle defects cause a general delay in mitotic progression. To characterize spindle abnormalities more accurately in importin-β-overexpressing cells, we analysed different combinations of markers for centrosomes and spindle poles. Using anti-centrin antibody, we consistently counted two or four centrioles in interphase, corresponding to the G_1- and G_2-phase configurations, respectively, indicating that centriole duplication was normal. However, centrin foci were present at all poles of abnormal spindles (not shown), which also recruited γ-tubulin (Fig. 7Da) and Ran-dependent SAFs (i.e. TPX2 and NuMA) (Fig. 7Db,c). All poles also recruit Aurora-A, a major regulatory target of TPX2 in spindle organization (not shown). These results indicate that MT arrays are anchored to functional poles or pole-derived structures, arguing against ectopic nucleation or focusing of MTs. In many abnormal mitoses, supernumerary poles were uneven in size, often with two major poles that defined a major spindle axis, and a small ‘extrapole’, suggesting uneven fragmentation of one pole. Centriole splitting, with an even partitioning of MT arrays and chromosomes distributed at all poles, was also detected in a proportion of abnormal mitoses (~39%), although pole fragmentation is the predominant outcome (48%) in importin-β-dependent induction of multipolar spindles.

Importin-β-induced abnormalities are mitigated by coexpression of TPX2 and NLSs

TPX2 is directly inhibited by importin α/β in vitro (Schatz et al., 2003; Tsai et al., 2003). We therefore asked whether co-expression of TPX2 would correct spindle abnormalities caused by importin β excess in mitotic human cells. Exogenous TPX2 localizes to spindle poles and induces spindle abnormalities, consistent with previous reports (Fig. 8A). Excess importin β, which alone generates spindle abnormalities, shows a lower effect when TPX2 is co-expressed (Fig. 8B). Both the induction of abnormalities caused by importin β or TPX2 alone and the reduction when both were simultaneously co-expressed were statistically significant.

As a further step, we reasoned that, if the effects caused by exogenous importin β reflect an altered activation of endogenous SAFs during mitosis, co-expression of any NLS might mitigate these abnormalities, because exogenously expressed NLS should engage a proportion of the importin α/β complexes and thus relieve importin-β inhibition of endogenous SAFs. As an experimental tool to test this, we used a construct containing the SV40 large-T-antigen-derived NLS, which is not expressed in uninfected cells, and expressed it in equimolar, twofold and threefold molar excess over the importin-β construct in co-transfection experiments. Co-expression of the NLS construct alleviated chromosome misalignment and spindle-pole fragmentation induced by excess importin β. This functional rescue was dose dependent (Fig. 8C). This effect is specific, because expression of the luciferase gene under the same conditions had no effect on importin-β-dependent spindle abnormalities.

Inactivation of TPX2 prevents importin-β accumulation at spindle poles

TPX2 moves to poles in a dynein-dependent manner (Wittmann et al., 1998; Wittmann et al., 2000). Together with the evidence reported thus far, this suggests the possibility that importins and TPX2 are transported along MTs in complexes with dynein to reach the poles. If this is the case, then eliminating TPX2 should affect the localization of importin β at poles. To test this idea, we investigated the localization of importin β after RNAi-mediated silencing of TPX2. After 40 hours of RNAi to TPX2, protein levels were barely detectable (Fig. 9A,B). Consistent with previous reports (Garret et al., 2002; Gruss et al., 2002), TPX2-interfered mitoses showed spindle abnormalities and failure of metaphase alignment (Fig. 9B, bottom). IF staining revealed that importin β was completely delocalized from the mitotic apparatus in cells lacking TPX2 (Fig. 9A, quantified in Fig. 9C, left). By contrast, NuMA is largely transported through dynein and is normally recruited in its typical crescent-shaped organization at poles of both normal and abnormal spindles, regardless of TPX2 function (Fig. 9B, Fig. 9C, right), indicating that dynein-dependent transport generally continues in the absence of TPX2.

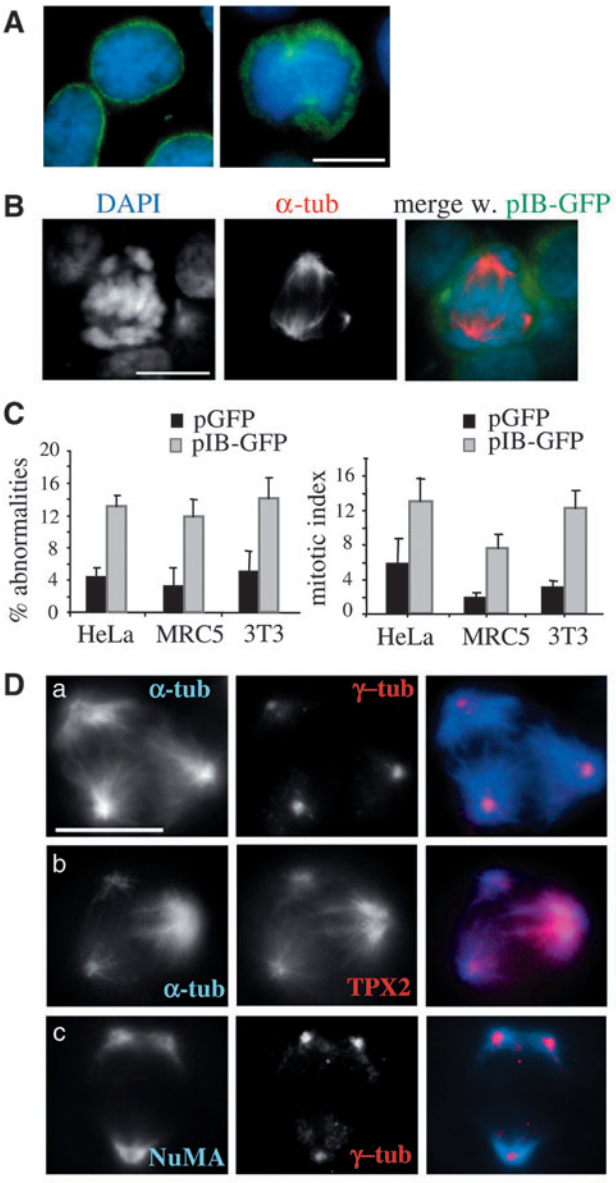


Fig. 7. Importin β overexpression induces spindle abnormalities. (A) Localization of exogenous importin-β/GFP (green) in transfected HeLa cells, showing stable association with the NE in interphase (left) and with spindle poles in mitosis (right). Merged signals with DAPI (blue) are shown. Scale bar, 10 μm. (B) A typical abnormal spindle in a mitotic cell expressing importin-β/GFP: notice the chromosome misalignment (DAPI, left) and a small 'extrapole' in addition to the two main poles (α-tubulin, middle). Scale bar, 10 μm. (C) Induction of mitotic abnormalities (left) and delay (right) in importin-β-expressing human and murine cell lines. Abnormalities were counted among 500 mitoses in HeLa cultures, 250 in MRC5 and 400 in NIH-3T3 (two independent experiments) and their frequency in importin-β- (pIB-GFP) and vector- (pGFP) transfected populations was statistically evaluated using the χ^2 test ($P<<0.001$ in HeLa cells, $P<0.02$ in MRC5 cells and $P<0.001$ in NIH-3T3 cells). The mitotic index was calculated in 1000 transfected cells in HeLa cultures, 800 in MRC5 cultures and 1200 in NIH-3T3 cultures (three experiments per cell line) and analysed using the χ^2 test ($P<<0.001$ in all cell lines). (D) Localization of mitotic regulators to poles of importin-β-induced abnormal spindles (GFP emission not depicted). Markers in the leftmost column are revealed by AMCA-conjugated secondary antibody (blue) and in the central column by a red-emitting secondary antibody (Cy3 or Texas Red). Merged images are shown on the right. Scale bar, 10 μm.

TPX2-lacking cells can hardly progress beyond prometaphase or misaligned metaphase. To rule out the possibility that the results in Fig. 9A reflect differences in importin-β localization in mitotic substances, we repeated the RNAi experiment, stained γ-tubulin to visualize the mitotic apparatus and compared the localization of importin β only in prometaphase cells from both control and TPX2-interfered cultures. Although TPX2 expression could not be directly

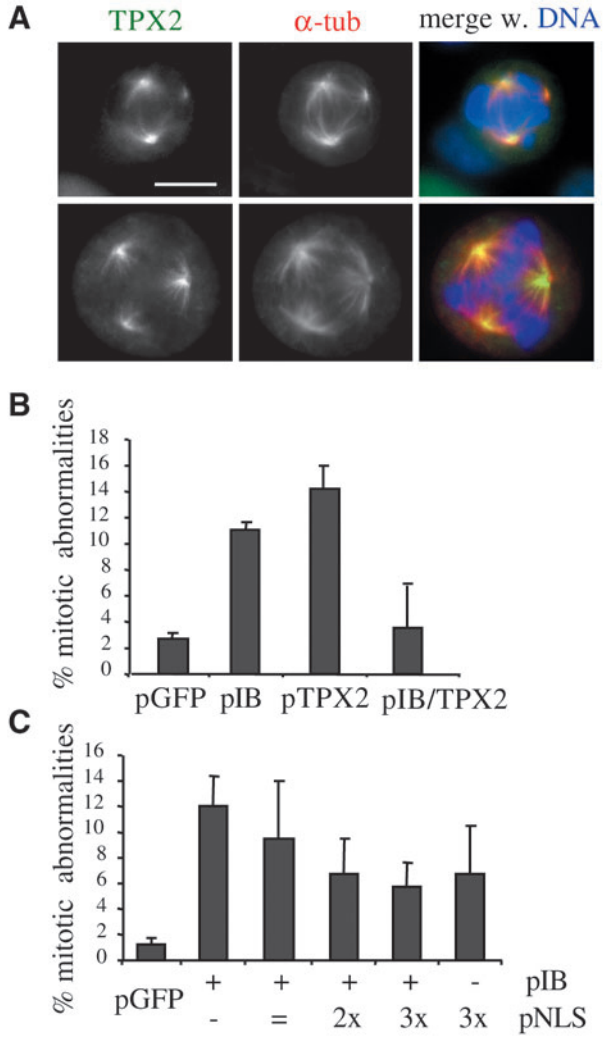


Fig. 8. Rescue of importin-β-dependent mitotic abnormalities by TPX2- and NLS-expressing constructs. (A) Examples of mitotic abnormalities induced by exogenous TPX2 in HeLa cells. Scale bar, 10 μm. (B) Frequency of mitotic abnormalities in HeLa cells transfected with importin β (pIB) or TPX2 constructs, alone or together (between 150 and 250 mitoses per group were counted in two experiments). Using pGFP vector transfection as the reference group, $P<0.01$ for TPX2 or pIB alone, and non-significant for TPX2 plus pIB. Using pIB transfection as the reference group, P was non-significant for TPX2 alone and $P<0.02$ for TPX2 plus pIB. (C) Frequency of mitotic abnormalities in HeLa cells transfected with importin β (+) or NLS constructs; symbols indicate the molar ratio of NLS to importin-β sequence used in transfection (between 170 and 260 mitoses per group were counted in two experiments). Using pGFP as the reference group, $P<0.001$ for pIB; $P<0.01$ for pIB coexpression with equimolar NLS; $P<0.05$ or at the limit of significance with higher NLS amount.

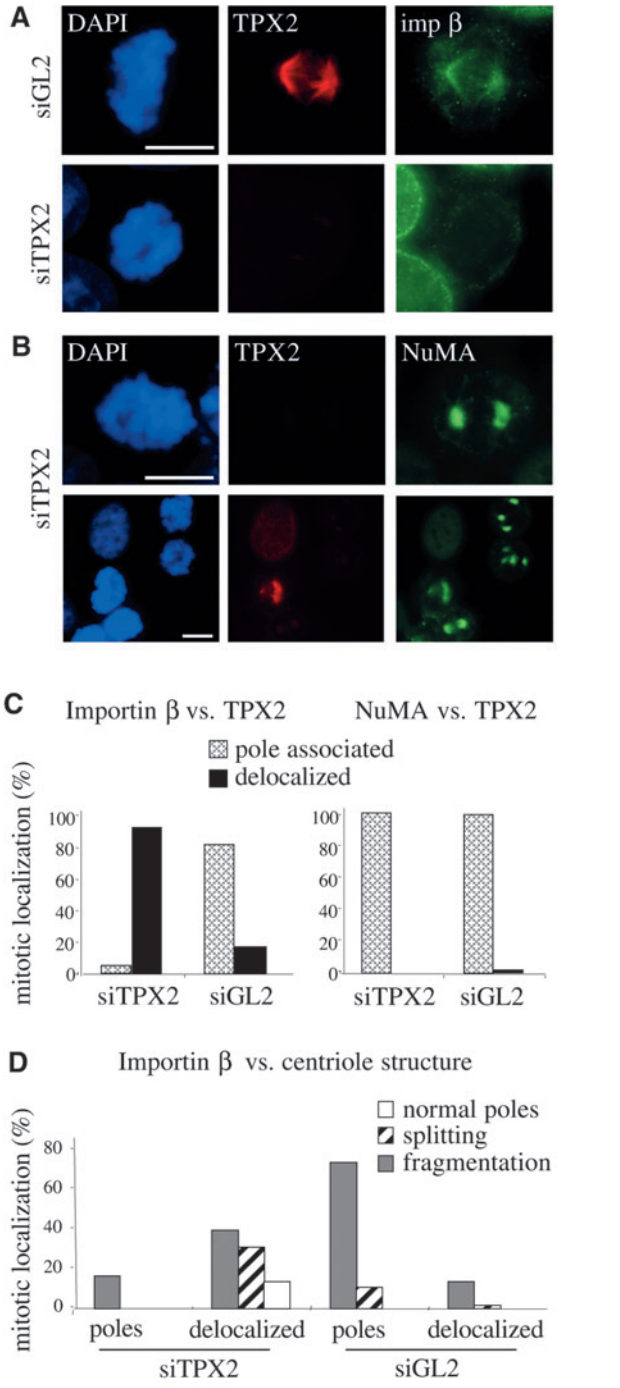


Fig. 9. Targeting of importin β at spindle poles is TPX2 dependent. (A) IF to importin β (green) in mitotic cells (revealed by DAPI, blue) from cultures treated with siRNA oligonucleotides targeting luciferase (GL2) (top) or TPX2 (bottom); TPX2 is stained in red and images are taken under identical exposure conditions. Scale bar, 10 μm. (B) NuMA recruitment (green) at spindle poles (top) in TPX2-interfered cultures (red); in the lower-magnification field (bottom), NuMA is correctly localized to poles of normal and abnormal spindles. Scale bars, 10 μm. (C) Frequency of mitotic cells showing importin β (left) or NuMA (right) signals at spindle poles in TPX2- or GL2-interfered cultures (n=150 mitoses per group). (D) Frequency of prometaphases (identified by DAPI staining) showing pole-associated or delocalized importin-β signals in spindles with normal or abnormal centrosomes, visualized by centrin-2 staining (n=100 mitoses per group).

visualized in these experiments, we scored all prometaphase figures, taking into account the fact that ~70% of cells fail to express TPX2 under our interference conditions. We found that importin β was delocalized in mitoses with abnormal phenotype – presumably caused by the lack of TPX2 – but accumulated at spindle poles in cells at the same stage treated with control siRNA oligonucleotides (not shown). Most spindle poles are disrupted in cells lacking TPX2. Using an antibody against centrin 2, we separately analysed mitotic cells presenting a normal centrosome configuration (i.e. two pairs of centrioles) and mitoses with split or fragmented poles. In TPX2-interfered cultures, we found that importin β consistently failed to accumulate at spindle poles, whether normal or abnormal in structure (Fig. 9D). These results argue against the possibility that importin β delocalization is merely a consequence of pole disruption and indicate that it is a specific feature of cells lacking TPX2.

Discussion

The conservation of Ran-dependent mechanisms in spindle control and in interphase transport is a striking example of the cell's ability to reuse regulatory components in diverse biological processes. Biochemical studies have delineated a mechanism whereby RanGTP regulates the activation of mitotic factors that contain NLS motifs, recognized by importin α/β, by triggering the dissociation of the complexes via a mechanism identical to that operating in RanGTP-dependent delivery of nuclear proteins in interphase. The present results provide the first evidence that spindle organization in mitotic cells relies on the mutual dependence between importin β and TPX2: TPX2 is required for importin-β transport to the spindle poles, where importin β acts as the RanGTP-regulatable moiety of the complex.

A central aspect emerging from the present study is the spatial and temporal regulation of importin-β localization during mitosis. During prophase, importin-β dissociates from the dissolving NE and becomes largely soluble. From prometaphase to early anaphase, importin β colocalizes to spindle poles with Ran, which is GTP bound at least in part, and with RanBP1. In anaphase/telophase, importin β detaches from the poles and is recruited back around the segregating chromatin. The regulated localization of importin β has implications for the mitotic functions of the Ran network. Several dynamic interactions might be established, with different functional roles (e.g. importin-β/RanGTP, RanGTP/RanBP1 and RanGDP/RanBP1/importin-β). A trimeric complex RanGDP/importin-β/RanBP1 of unknown function (but presumably representing an intermediate complex in dynamic interactions of Ran with effectors) has been identified in somatic cells (Plafker and Macara, 2002); it will be important to assess the existence and function of such a complex at spindle poles. By contrast, the interaction with RanBP1 alone is expected to stabilize RanGTP (Bischoff et al., 1995); this might be important to preserve GTP-bound Ran at asters/spindle poles until prometaphase, when importin-β complexes reach the asters. From that stage until metaphase, the colocalization of RanGTP and importin β provides the physical ground for SAF activation and spindle-pole organization. After transition to anaphase, importin-β relocalization around the reforming nuclei precedes chromatin decondensation, RanBP1 downregulation and Ran re-entry in nuclei (Guarguaglini et al., 2000) (M.C. and P.L., unpublished), and hence represents an early event in mitotic exit. Importin β plays crucial roles in NE formation (Zhang et al., 2002; Harel et al., 2003; Tirian et al., 2003). Recent work indicates that importin α/β lose the ability to bind RCC1 during mitosis (Li and Zheng, 2004) as the latter becomes phosphorylated by p34/cdc2 and interacts more tightly with mitotic chromatin (Li and Zheng, 2004; Hutchins et al., 2004). Reasonably, RCC1 returns to a dephosphorylated state at mitotic exit, which probably restores its affinity for importins and might facilitate the repositioning of importin β around chromatin in preparation of NE reformation. Importins α and β are also phosphorylated in mitosis (Yasuhara et al., 2004), and it will be important to assess how this modulates relevant interactions with mitotic components.

In investigating the mitotic localization of importin β at spindle poles, several novel features have emerged. First, MT integrity and dynamics are required, because importin β diffuses throughout the cell when the spindle MTs are disassembled, but relocalizes rapidly to poles when repolymerization occurs. That raised the questions of whether motors were involved. Importin α was previously found to interact with dynein in neuronal axons, in which a specialized system of retrograde transport has evolved. In this system, importin β further associates with the complex in an importin-α-dependent manner (Hanz et al., 2003). We have now found that dynein is also implicated in the association of importin α/β with the spindle MTs. TPX2 (Wittmann et al., 2000) and NuMA (Merdes et al., 2000) move to poles through dynein-dependent transport, although fractions of both factors can associate directly with MTs (Wittmann et al., 2000; Haren and Merdes, 2002). The implication of dynein in the interaction of importin α/β with MTs raised the possibility that importin β moves to MT minus ends in complexes with SAFs. The RNAi experiments reported here indicate that functional TPX2 is indeed required for the accumulation of importin β at mitotic spindle poles. Studies of interphase transport suggest that importin β stabilizes the binding of importin α members to NLS cargoes by preventing self-folding and auto-inhibition of importin α (for a review, see Conti, 2002). Mitotic SAFs can be grouped in two broad classes: the TPX2 type is functionally inhibited in complexes with importin α/β but can still bind MTs while in the complexes (Trieselmann et al., 2003; Tsai et al., 2003); by contrast, XCTK2 (Ems-Mc Clung et al., 2004) and Kid (Trieselmann et al., 2003) cannot bind MTs when complexed with importin α/β. The present results support a model in which importin β stabilizes mitotic complexes formed by importin α and TPX2 (or SAFs of similar biochemical type) while interacting with motors and MTs; at the poles, centrosomal RanGTP dissociates the complexes. By conferring responsiveness to RanGTP, importin β provides the regulatable complex component.

In this scenario, both the spatial organization and the quantitative balance of Ran and importin β are crucial to mitotic spindle function. Indeed, expression of exogenous importin β at levels that roughly doubled its intracellular amount perturbed the spindle organization, yielding pole fragmentation, chromosome misalignment and delayed mitotic progression. These abnormalities can be mitigated by co-expression of TPX2 and, more generally, of NLSs. The

functional rescue by exogenous NLSs suggest that they compete with endogenous SAFs for importin binding, consistent with the idea that importin α, the NLS receptor, acts as a bridging molecule between NLS-containing SAFs and importin β in RanGTP-dependent spindle assembly.

Importin β is growingly emerging as a negative regulator in several aspects of cell life. In *Caenorhabditis elegans*, RNAi experiments indicate a requirement for importin β in both spindle assembly and NE reformation (Askjaer et al., 2002). How might dynamic importin complexes regulate these different aspects? In *Drosophila* (in which there is no TPX2 homologue), a mutant allele of importin β that cannot respond to RanGTP regulation, *ketel* (Timinszky et al., 2002), does not apparently affect spindle assembly in syncytial embryos but impairs NE reformation after mitosis. The mutant importin β sequesters factors required for NE rather than for spindle assembly; interestingly, such NE factors are sequestered to the MTs (Tirian et al., 2003). The present results indicate that, in vertebrate cells, importin β regulates spindle function by ensuring the regulated delivery of TPX2 at asters/poles. Other fractions of the importin β pool might also participate in transient 'storage' of NE factors in an inactive form until mitosis is completed and the NE reorganization initiates. Together, these lines of evidence converge to suggest that importin β interacting with mitotic MTs performs diverse cellular functions depending on its cargo partners.

In summary, the ability of importin β to localize to spindle poles in a MT-, motor- and TPX2-dependent manner is a key feature in regulation of the mitotic spindle. In mammalian somatic cells, both the molecular balance and the regulated localization of importin β and SAFs of the TPX2 type are crucial requirements for this process.

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