Chromosoma
DOI 10.1007/s00412-010-0286-5

RESEARCH ARTICLE

Nuclear reformation after mitosis requires downregulation of the Ran GTPase effector RanBP1 in mammalian cells

Marilena Ciciarello · Emanuele Roscioli · Barbara Di Fiore · Laura Di Francesco · Fabrizia Sobrero · Delphine Bernard · Rosamaria Mangiacasale · Amnon Harel · Maria Eugenia Schininà · Patrizia Lavia

Received: 14 May 2010 / Revised: 28 June 2010 / Accepted: 30 June 2010

Abstract The GTPase Ran regulates nucleocytoplasmic transport in interphase and spindle organisation in mitosis via effectors of the importin beta superfamily. Ran-binding protein 1 (RanBP1) regulates guanine nucleotide turnover on Ran, as well as its interactions with effectors. Unlike other Ran network members that are steadily expressed, RanBP1 abundance is modulated during the mammalian cell cycle, peaking in mitosis and declining at mitotic exit. Here, we show that RanBP1 downregulation takes place in mid to late telophase, concomitant with the reformation of nuclei. Mild RanBP1 overexpression in murine cells causes RanBP1 to persist in late mitosis and hinders a set of events underlying the telophase to interphase transition, including chromatin decondensation, nuclear expansion and nuclear lamina reorganisation. Moreover, the reorganisation of nuclear pores fails associated with defective nuclear relocalisation of NLS cargoes. Co-expression of importin beta, together with RanBP1, however mitigates these defects. Thus, RanBP1 downregulation is required for nuclear reorganisation pathways operated by importin beta after mitosis.

Communicated by K. Weis

Electronic supplementary material The online version of this article (doi:10.1007/s00412-010-0286-5) contains supplementary material, which is available to authorized users.

M. Ciciarello · E. Roscioli · F. Sobrero · R. Mangiacasale · P. Lavia (✉)
CNR National Research Council,
Institute of Molecular Biology and Pathology,
c/o Sapienza University of Rome,
Rome 00185, Italy
e-mail: patrizia.lavia@uniroma1.it

B. Di Fiore
Wellcome Trust/Cancer Research UK, Gurdon Institute,
University of Cambridge,
Cambridge, UK

L. Di Francesco · M. E. Schininà
Department of Biochemical Sciences,
Sapienza University of Rome,
00185 Rome, Italy

D. Bernard
Department of Plant Sciences, University of Cambridge,
Cambridge, UK

A. Harel
Department of Biology, Technion-Israel Institute of Technology,
Haifa, Israel

Introduction

Exit from mitosis is a crucial window of the cell cycle, in which the nucleus is reorganised and the compartmentalization of macromolecules between the nucleus and the cytoplasm is re-established. In higher eukaryotes, three essential features must be reset after mitosis: (1) chromatin must return to a decondensed, transcriptionally competent state, (2) the nuclear envelope (NE) must reassemble around chromatin and reorganise nuclear pores, and (3) import and export of macromolecules through reassembled nuclear pore complexes (NPCs) must be resumed. These events require the GTPase Ran (RAs-related nuclear protein) and the regulators of its nucleotide-bound state (collectively called the Ran network), i.e. the chromatin-bound exchange factor regulator of chromosome condensation 1 (RCC1), which generates Ran guanosine triphosphate (RanGTP) in the nucleus, and the cytoplasmic factors RanGTPase-activating protein 1 (RanGAP1) and Ran-binding protein 1 (RanBP1), which cooperate to hydrolyse RanGTP and generate Ran guanosine diphosphate (RanGDP) in the cytoplasm.

Published online: 24 July 2010

In vitro nuclear reconstitution assays with cell-free extract first indicated that blocking the Ran cycle —by imbalancing the RCC1:RanBP1 ratio—impaired chromatin decondensation and reformation of functional nuclear membranes (Pu and Dasso 1997; Nicolas et al. 1997). Indeed, Ran binding to chromatin and RanGTP generation by RCC1 are required for chromatin decondensation and recruitment of NE precursor vesicles to the chromatin surface in vitro (Zhang and Clarke 2000, 2001; Hetzer et al. 2000). Neither GTP-locked (non-hydrolysable), nor non-exchangeable Ran mutants operate properly in these processes, indicating that dynamic cycles of RanGDP and RanGTP formation are required. The Ran cycle is also required for the formation of NPCs (Zhang et al. 2002a; Walther et al. 2003; D'Angelo et al. 2006).

Ran operates in all downstream processes via effectors of the importin beta family of shuttling transport receptors, with which Ran interacts in the GTP-bound form. Importin beta is a major vector of nuclear protein import in interphase. In nuclear import cycles, RanGTP generated in the nucleus binds importin beta in incoming import complexes, thereby dissociating the complexes and releasing free, biologically active cargoes in the nucleus. RanGTP and importin beta then exit the nucleus as a complex that is dissociated in the cytoplasm in the presence of RanBP1 to restart a novel import cycle. This same mechanism continues to operate after NE breakdown, when nuclear transport ceases, and determines at this time RanGTP-dependent release of factors acting in mitotic spindle organisation from interactions with importin beta. Thus, there is continuity in the mechanism with which RanGTP disassembles importin beta complexes and releases free, functional cargoes, both in interphase and in mitosis. That has led RanGTP and importin beta to be regarded as complementary, and yet, antagonistic components of a global regulatory system exerting opposite effects on downstream targets (reviewed by Weis 2003; Harel and Forbes 2004).

After mitosis, importin beta roles are probably more diversified. Importin beta excess inhibits the recruitment of NE precursor vesicles onto the chromatin surface in a RanGTP-reversible manner (Zhang et al. 2002b; Harel et al. 2003). This antagonism is in keeping with the general idea that RanGTP binding to importin beta releases free molecules from importin beta-containing complexes. Importin beta also has roles in regulating nucleoporins (NUPs) during NPC assembly, but these roles are not necessarily counteracted by RanGTP (Harel et al. 2003; Delmar et al. 2008; Rotem et al. 2009). The differential sensitivity of importin beta to RanGTP in these processes likely reflects the nature of its interactions with relevant partners in this cell cycle window, which engage specific structural domains. RanGTP binding destabilises only some of these interactions, while others (e.g., those involving phenylalanine-glycine (FG)-repeat containing NUPs) remain unperturbed. This suggests that nuclear reorganisation steps at the exit from mitosis have differential requirements for Ran and importin beta functions, which operate with greater regulatory specificity than predicted on the basis of the nuclear import paradigm.

In vivo studies with diverse model systems further hint at the specificity of control of the mitosis to interphase transition by Ran/importin beta. *Schizosaccharomyces pombe* strains defective for Ran network components that generate Ran cycle imbalance display abnormal nuclei and NEs after mitosis (Matynia et al. 1996; He et al. 1998; Salus et al. 2002; Gonzalez et al. 2009). *S.pombe* undergoes closed mitosis, but regulated NE growth occurs in late mitosis. A requirement for the Ran/importin beta system in nuclear, NE and NPC reorganisation was also revealed in *Saccharomyces cerevisiae* mutant strains (Ryan et al. 2003; Ryan et al. 2007), in *Caenorhabditis elegans* after RNA interference (RNAi) targeting importin beta and/or single Ran network members (Bamba et al. 2002; Askjaer et al. 2002), and in *Drosophila* embryos carrying a mutant importin beta allele (Timinszky et al. 2002; Tirian et al. 2003). Neither *C. elegans* nor *Drososphila* have a RanBP1 homologous protein, but some of RanBP1 functions are taken over by RanBP2/NUP358, a nucleoporin harbouring homologous Ran-binding domains. On the one hand, these studies highlight a conserved requirement for regulated activity of the Ran/importin beta system at the mitosis to interphase transition; on the other hand, intriguing elements of species-specificity emerge in modes of Ran control of nuclear and NE reorganisation. Information on how the system operates in this highly complex transition in mammalian cells is still fragmentary.

RanBP1 is the only component of the Ran network to be expressed with cell cycle-regulated abundance in mammalian cells: it increases during S phase due to transcriptional up-regulation (Di Fiore et al. 1999), continues to increase steadily in abundance until reaching peak levels in mitotic cells and declines thereafter (Guarguaglini et al. 2000). This quantitative regulation is intriguing, given that RanBP1 has roles both in regulating nucleotide turnover on Ran and in modulating its interactions with importin beta. We have now characterised RanBP1 downregulation in depth during late mitosis and have mapped it to the mid-to-late telophase window, concomitant with NE reformation. We show that this downregulation is critical for chromatin decondensation, nuclear expansion and NPC reorganisation as the NE reforms. Indeed, failure to downregulate RanBP1 generates multiple defects in nuclear reorganisation after mitosis, albeit not affecting the organisation of nuclei that have already formed. Co-expression of importin beta, however,

mitigates these defects; thus, RanBP1 persistence after mitosis renders importin beta limiting in pathways, regulating chromatin and NE reorganisation at interphase re-entry.

Materials and methods

Cell cultures and synchronisation

Cell lines were grown in D-MEM with 10% foetal calf serum (plus 500 μg/ml G418 for L929 centrin1-green fluorescent protein (GFP), a kind gift from Michel Bornens, Institut Curie, Paris) at 37°C, 5% CO_2. Cells were synchronised by culturing in 2.5 mM thymidine to arrest the cell cycle at the G1/S transition, then releasing in fresh medium containing 30 μM deoxycytidine. Cells were harvested at regular intervals after release, and progression to mitosis was monitored by coupled fluorescence-activated cell sorter (FACS; DNA content) and microscopy analysis (gamma-tubulin to monitor the centrosome status, alpha-tubulin and lamin B1 for assembly and disassembly of the mitotic apparatus and the nuclear lamina, respectively). Murine NIH3T3 cells were analysed 5–7 (G2/M enrichment) and 8–10 h (enrichment in G1 after M completion) after release from G1/S arrest in most experiments. In some experiments, mitotic cells from human osteosarcoma (U20S), human cervical carcinoma (HeLa) and rat Fisher cell lines were isolated at mitotic round-up by shake-off (usually 10 h after release from thymidine arrest), then replated to complete mitosis and harvested as indicated. Where indicated, 100 μM MG132 or 0.1% dimethyl sulfoxide (DMSO) was added 100 min after replating and cells were fixed after 40 min. In some experiments, thymidine synchronisation and release was followed by nocodazole (NOC, 100 ng/ml) addition; after 4 h, NOC was washed out, and progression until anaphase onset was monitored under an inverted microscope before adding MG132. Cell cycle phases were analysed in all experiments using a Coulter Epics XL flow cytometer (Beckman Coulter).

Transfection experiments

Mammalian expression constructs include CMV enhancer/promoter-driven plasmids expressing untagged, red fluorescent protein (RFP, dSRed)- or GFP-tagged RanBP1 (described in Guarguaglini et al. 2000 and Di Fiore et al. 2003; all used 5 μg DNA per 1×10^6 cells, unless indicated otherwise), H2B-GFP (0.1–0.5 μg), nuclear localization signal (NLS)-GFP import reporter (1 μg), GFP-tagged importin beta (2-4 μg; described in Ciciarello et al. 2004). Construct GFP-importin-beta$^{45\text{-}876}$ was generated from the 45-876 region of human importin beta (Kutay et al 1997; kindly provided by Dirk Gorlich, University of Heidelberg) via PCR amplification and ligation into pEGFP-N1 (Clontech). Empty vectors included pBluescript, pEGFP-N1 and pDsRed1-N1 (Clontech). Lipofectamine 2000 (Invitrogen) or Metafectene Easy (Biontex) were used as transfection reagents. The increase in RanBP1 and importin beta concentrations in transfected cells was assessed by (1) immunofluorescence (IF) densitometry of RanBP1- (or importin beta)-specific fluorescence at the single-cell level in transfected versus non-transfected cells, and (2) quantification of western blot signal in transfected versus non-transfected samples, normalised to actin and corrected for the transfection efficiency scored on parallel slides in the same experiment. Transfected cells expressed a three to fivefold increase in RanBP1 concentration relative to the average concentration in interphase cells, and a two to threefold increase for importin beta.

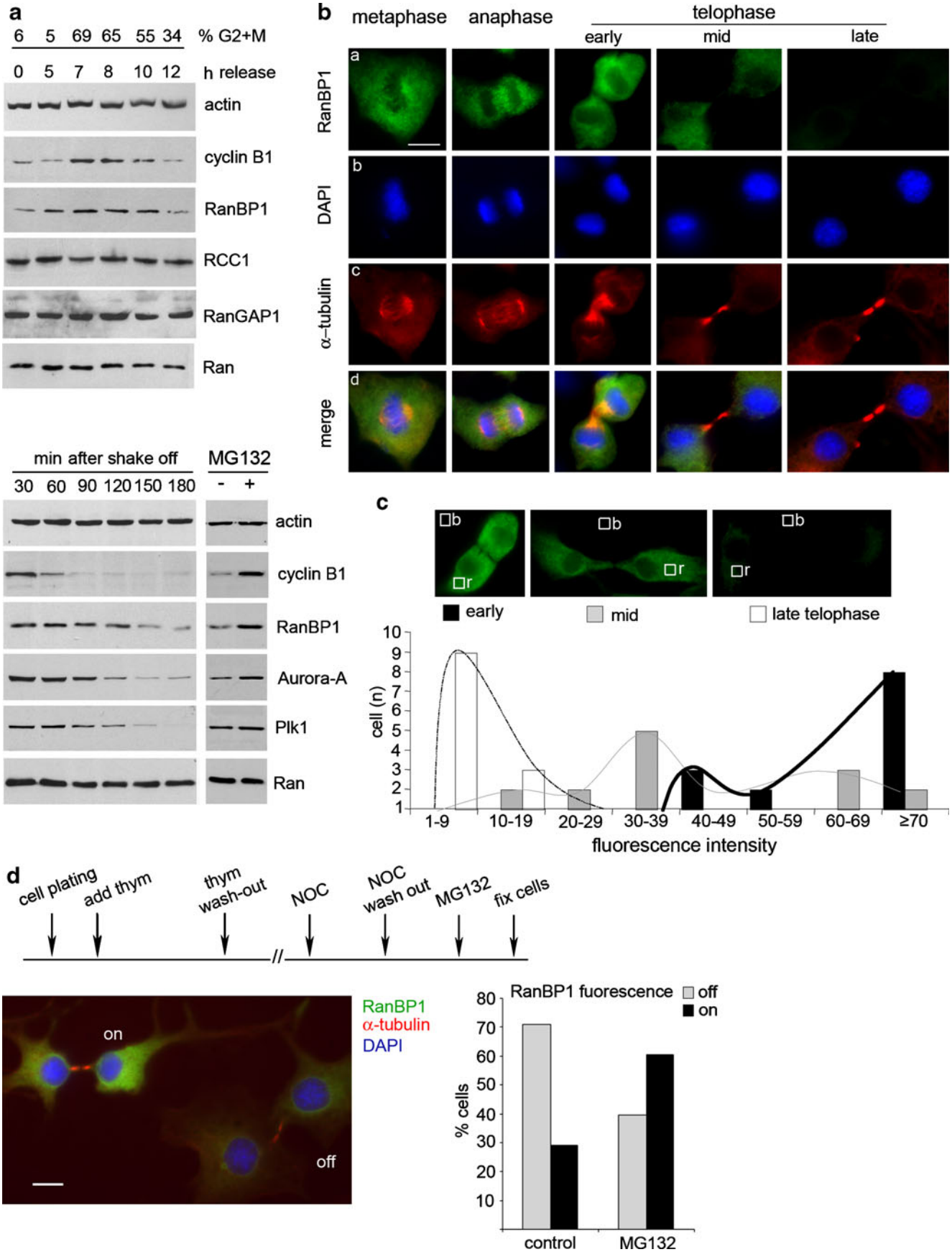
Time-lapse recording in vivo

Mitotic exit was recorded in synchronised NIH3T3 cells co-transfected with H2B-GFP and either RanBP1-RFP or pDsRed1-N1 vector. Cells were recorded from prometaphase. Bright field images were taken every 2 min and fluorescent images every 30 min under a Nikon Eclipse 300 inverted microscope (60×0.7 objective) equipped with a Digital Camera DXM 1200 and the ACT-1 software. Centrin1-GFP L929 cells were transiently transfected with RanBP1-RFP or pDsRed1-N1 vector and video-recorded

Table 1 Timing of mitotic exit in NIH3T3 cells transfected with vector or RanBP1-RFP

	pDsRed1-N1 vector			RanBP1-RFP			
Overall duration (min)	Mean	Min	Max	Mean	Min	Max	Student's *t* test
Anaphase	11.60	6	18	9.73	6	16	ns
Telophase	39.69	16	46	63.06	20	96	$p<0.05$
Recorded cells	19			16			

ns non significant


a
6 5 69 65 55 34 % G2+M
0 5 7 8 10 12 h release
actin
cyclin B1
RanBP1
RCC1
RanGAP1
Ran
min after shake off
MG132
30 60 90 120 150 180 - +
actin
cyclin B1
RanBP1
Aurora-A
Plk1
Ran
b
metaphase
anaphase
telophase
early
mid
late
RanBP1
DAPI
α-tubulin
merge
c
early
mid
late telophase
cell (n)
1-9
10-19
20-29
30-39
40-49
50-59
60-69
≥70
fluorescence intensity
d
cell plating
add thym
thym wash-out
NOC
NOC wash out
MG132
fix cells
on
off
RanBP1
α-tubulin
DAPI
RanBP1 fuorescence
off
on
% cells
control
MG132

◀ **Fig. 1** RanBP1 downregulation in mammalian late telophase cells. **a** Western blot analysis of Ran network members during the cell cycle. The *upper panel* shows whole cell extract from thymidine-arrested and released human U20S cultures; the proportion of (*G2+M*) cells was estimated by FACS at the indicated time points (h after thymidine release). The *lower panel* shows extracts from U20S cells collected at round-up and replated for the indicated times after mitotic shake-off; the timing of RanBP1 downregulation is compared to that of other mitotic regulators; in the *two rightmost lanes*, cells were treated (40 min) with MG132 (+) or DMSO (−) 100 min after shake-off. **b** RanBP1 IF patterns in NIH3T3 mitotic cells: note RanBP1 decrease (*row a*) concomitant with the onset of chromatin decondensation (*row b*) and midbody elongation (*row c*). *Bar* 10 μm. **c** Quantification of RanBP1-specific signal in NIH3T3 telophase cells. RanBP1 fluorescence intensity (*section r*) was measured by densitometry (*n*, 30 randomly selected cells per stage; examples are shown above); *section b* background fluorescence; values were corrected for the number of pixels to compare cells of different size. Cells are grouped in classes of fluorescence intensity (arbitrary units) and plotted relative to mitotic exit stages. **d** The schematics at the *top* show the combined thymidine/NOC synchronisation protocol used to enrich NIH3T3 cultures in mitotic cells; MG132 (or DMSO) were added after cells reached the metaphase to anaphase transition; after 40 min, samples were fixed and stained for RanBP1. The *left panel* shows an exemplifying field from NIH3T3 control cultures stained for tubulin (*red*), RanBP1 (*green*) and DAPI (*blue*); *histograms* represent the frequency of telophase cells negative (*light histograms*) or positive (*dark histograms*) for RanBP1 signal. *Bar* 10 μm

from metaphase for 36 or 50 h on an inverted Leica DMIRBE fluorescence microscope (10× objective) controlled by the Metamorph software. Transmission images were taken every 10 min and fluorescent images every 12 h.

Immunofluorescence microscopy

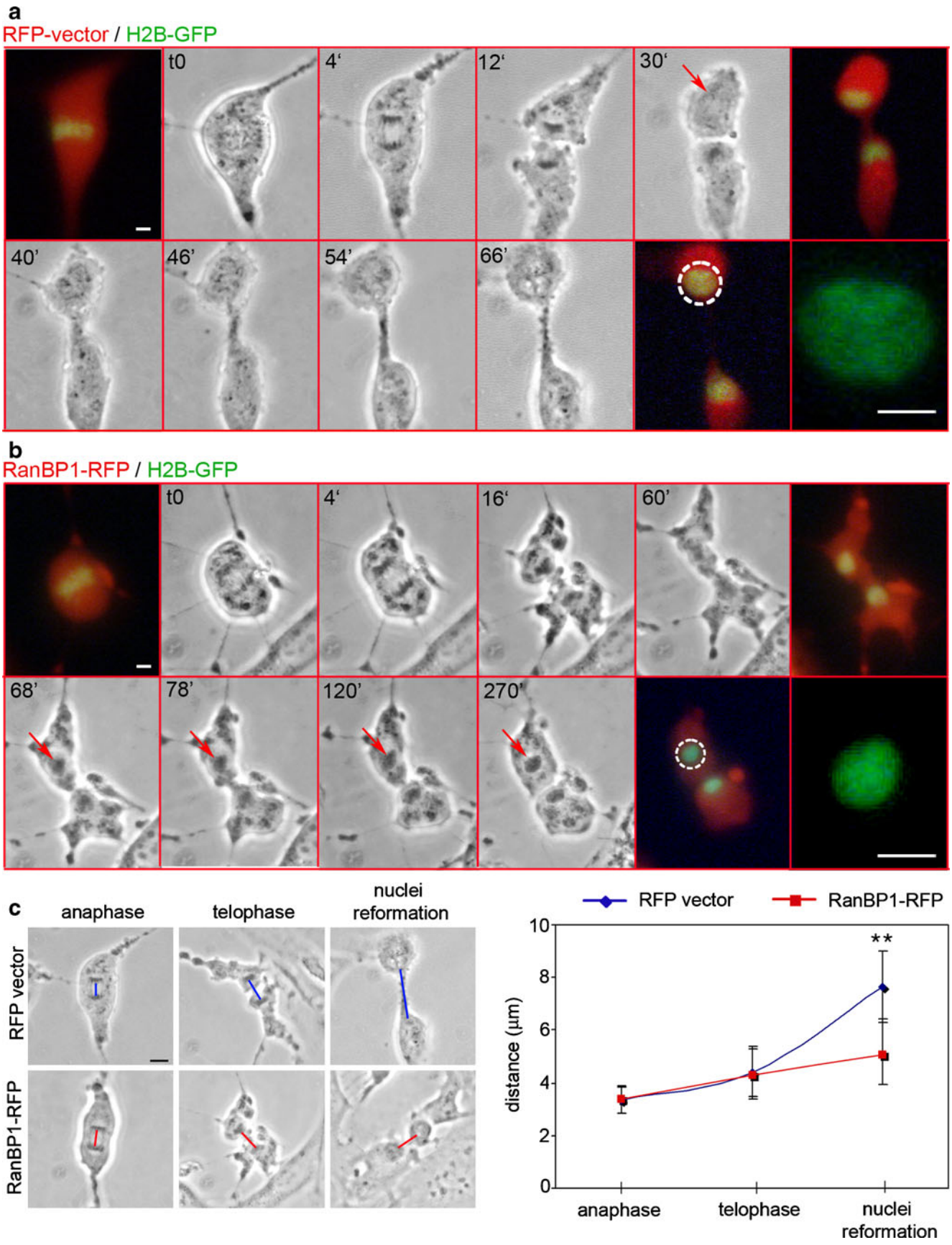
Cells grown on sterile coverslips were either fixed directly in 3.7% paraformaldehyde (PFA)/30 mM sucrose (to visualise the entire content of the protein under examination, e.g. NLS-GFP import reporter) or pre-incubated in 1% Triton X-100 in PHEM (45 mM HEPES pH 6.9; 45 mM PIPES pH 6.9; 10 mM ethylene glycol tetraacetic acid (EGTA); 5 mM $MgCl_2$ and 1 mM phenylmethanesulfonylfluoride (PMSF)) prior to fixing in 3.7% PFA/30 mM sucrose, to remove excess soluble proteins and visualise their association with particular structures (e.g. nuclear pores). In some experiments, 0.05–0.1% digitonin in transport buffer (110 mM KOAc; 2 mM $Mg(OAc)_2$; 0.5 mM EGTA; 20 mM Hepes) was used prior to fixation instead of Triton X-100 to obtain higher resolution of structure-tethered proteins; in these cases, traces of H2B-GFP were previously included in the transfection mix, as incorporation in chromatin resists digitonin permeabilisation and enables unambiguous identification of transfected cells. Primary antibodies were: alpha-tubulin, either unconjugated (B5-1-2) or fluorescein isothiocynate (FITC)-conjugated (DM1A), and gamma-tubulin (T3359), both from Sigma-Aldrich; phospho-H3 (ser10, 06-570, Upstate); RanBP1 (M-19, M-45 or C-19, Santa Cruz Biotechnology); Ran (clone 20, Transduction Laboratories, or C-20, Santa Cruz Biotechnology); RCC1 (C-20, Santa Cruz Biotechnology); lamin B1 (Zymed clone L-5 or Abcam ab16048 and ab8982), Mab414 (ab24609) and NUP153 (ab24700) from Abcam. Secondary antibodies were conjugated to FITC, Cy3 or 7-amino-4-methylcoumarin-3-acetic (AMCA) acid (Jackson Immunoresearch Laboratories), rhodamine (Santa Cruz Biotechnology), or Texas Red (Vector). DNA was stained with 4′6-diamidino-2-phenylindole (DAPI), and coverslips were mounted in Vectashield (Vector). Cells were examined under an epifluorescence Olympus AX70 microscope with a CCD camera (Photometrics or Diagnostic Instruments Inc.), or Leica DMR with a CoolSnap (Photometrics), or Nikon Eclipse 90i with a Qicam Fast 1394. Images were acquired with Nis-elements AR 3.1. Z-stack images were taken (0.2–0.3 μm distance) using the IAS2000 software (Delta Sistemi) and Autovisualise plus Autodeblur 9.3 deconvolution functions (AutoQuant Imaging, Inc).

Western immunoblotting

Protein extracts were separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filters, blocked and incubated with antibodies as described (Di Fiore et al. 2003), using antibodies to cyclin B1 (GNS1), actin (I-19), GFP (B-2), Ran (C-20), RanGAP1 (N-19), RCC1 (C-20), RanBP1 (M-45 and C-19) all from Santa Cruz Biotechnology; RanBP1 (clone 35), Aurora-A (clone 4) Ran (clone 20) and importin beta/karyopherin beta (clone 23) from BD Transduction Laboratories; Plk1 (ab14210), NUP153 (ab24700), cyclin B1 (ab72) and importin beta (ab2811) from Abcam. HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were revealed using ECL or ECL plus (GE Healthcare).

Co-immunoprecipitation (Co-IP)

HeLa cells were lysed in 1D buffer (1% NP40; 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EGTA, 1 mM EDTA) containing protease and phosphatase inhibitors (aprotinin 1 μg/ml; leupeptin 1 μg/ml; pepstatin 1 μg/ml; 1 mM PMSF; 50 mM NaF; 2 mM Na_3VO_4). Lysates were precleared with Protein G Sepharose 4 Fast Flow (GE Healthcare) or Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) at 4°C for 1 h. The precleared supernatant was collected and incubated with antibody against importin beta (ab2811, Abcam), or GFP (ab290 and ab6556, Abcam), or rhodamine conjugated anti-mouse IgG (Cappel)


a
RFP-vector / H2B-GFP
t0
4'
12'
30'
40'
46'
54'
66'
b
RanBP1-RFP / H2B-GFP
t0
4'
16'
60'
68'
78'
120'
270'
c
anaphase
telophase
nuclei reformation
RFP vector
RanBP1-RFP
RFP vector
RanBP1-RFP
distance (µm)
0
2
4
6
8
10
**
anaphase
telophase
nuclei reformation

◀ **Fig. 2** RanBP1 persistence during late mitosis hinders chromatin decondensation and nuclear expansion. **a** Still images from a control NIH3T3 cell co-transfected with pDsRed1-N1 vector (expresssing RFP) and H2B-GFP to identify metaphase alignment (*upper left panel*, taken as time 0). Chromatin begins to decondense about 30 min after alignment (*red arrow*), and is fully decondensed by 45 min. **b** Still images of a video-recorded NIH3T3 cell co-transfected with RanBP1-RFP and H2B-GFP: chromatin decondensation begins 60 min after alignment, but then fails to progress (*arrowed*); nuclei are still condensed after 270 min and fail to distance. Reforming nuclei, circled by *white dashed lines* (66 min after metaphase in **a** and 270 min in **b**), are enlarged ×4 in the *rightmost panels*. *Bars* 5 μm. **c** Distancing between reforming nuclei. *Lines* represent the distance between separating chromosome sets (anaphase) until nuclear reformation (distance between the outer nuclear edge) in still images of vector- (*n*, 19, *blue lines*) and RanBP1-RFP-transfected cells (*n*, 16, *red lines*). *Asterisks* indicate a highly significant difference in nuclear migration between cells transfected with pDsRed1-N1 vector (indicated as RFP) and with RanBP1-RFP (Student's *t* test)

for control, on a stirring wheel at 4°C overnight. The antibody/lysate complexes were then mixed with beads for 3 h at 4°C. At the end of the incubation, the resin was washed five times in 1D buffer, and protein complexes were eluted three times using loading buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 100 mM DTT), heated at 95°C for 5 min and centrifuged 5 min at 3000 rpm at 4°C to sediment the beads. Proteins in the supernatant were separated through SDS-PAGE and analysed by western blot. In some co-IP experiments, a cross-linking step was introduced: importin beta antibody (I2534, Sigma) or rhodamine-conjugated anti rabbit IgG (sc-2091, Santa Cruz Biotechnology) was mixed with Protein G Sepharose 4 Fast Flow or Protein A/G PLUS-Agarose (Santa Cruz Biotechnology), and incubated on a rotatory wheel at 4°C overnight. Unbound antibody was washed away, and protein G-bound antibody was washed twice with 200 mM sodium borate (pH 9), then cross-linked to beads by adding 20 mM dimethyl pimelimidate (DMP, Sigma-Aldrich) in 200 mM sodium borate (pH 9) for 1 h at room temperature. Unreacted amino groups were quenched by adding 200 mM ethanolamine for 2 h at room temperature. Free antibody was removed by washing the beads with 100 mM glycine (pH 2.5). After PBS washing, antibody cross-linked to beads was incubated with the precleared lysate (overnight, 4°C with rotation). At the end of incubation, the immunoprecipitated complexes were processed as above.

Statistical analysis

All experiments were repeated several times, and at least 200 cells were examined in each single experiment. Mean values and standard deviations were calculated from independent experiments; data were pooled and statistically analysed using either the $\chi 2$ test for frequencies of patterns (e.g. abnormal versus normal), or the Student's *t* test when comparing quantitative data (e.g. distances in Fig. 2, times in Table 1).

Results

RanBP1 downregulation occurs in a restricted mid to late telophase window

To map the timing of RanBP1 downregulation, we used human cells, in which the order of downregulation of mitotic factors is well characterised (reviewed by Pines 2006). Western analysis of protein extract from U20S cells synchronised by thymidine block, and release revealed highest RANBP1 abundance in G2 and M phases, followed by a decrease during progression to the next G1 phase, whereas other RAN network components remained unchanged (Fig. 1a, upper panel). This window was refined in a time-course analysis of thymidine-synchronised and released U20S cells that progressed synchronously until round-up (prometaphase), then were collected by shake-off, replated to terminate mitosis, and harvested at regular intervals after replating. Progression through mitosis was monitored by microscopy on parallel slides fixed at each time point (shown in Supplementary fig. 1). In western assays, cyclin B1 disappeared 1 h after shake-off; RanBP1 levels began to decline between 120 and 150 min after shake-off (Fig. 1a, lower panel), as cells transit from early to late anaphase (Supplementary fig. 1), similar to Aurora-A and PLK-1. Addition of the proteasome inhibitors MG132 (rightmost lanes), or lactacysteine, or ALLN (data not shown) to replated cell samples stabilised RanBP1 levels.

To define the timing of RanBP1 downregulation more precisely in the global reorganisation that takes place at the end of mitosis, we used murine NIH3T3 cells, in which RanBP1 downregulation was first detected (Guarguaglini et al. 2000). NIH3T3 populations enriched in mitotic cells after thymidine release were stained for RanBP1, in combination with markers serving as temporal landmarks for mitotic exit events, i.e. alpha-tubulin and MPM2-reactive mitotic phosphoepitopes (to monitor microtubule [MT] reorganisation in the central spindle and midbody), DAPI and phosphorylated H3 histone (to follow chromosome segregation and decondensation), and lamin B1 to depict nuclear reformation (Fig. 1b and data not shown). Quantification of the RanBP1 signal intensity revealed that late mitotic cells clustered in discrete classes of RanBP1 fluorescence (Fig. 1c): early telophases (defined by a thick central spindle, condensed chromatin, phosphorylated histone H3, and MPM2 reactivity) showed intense RanBP1 signals, comparable to those typically seen in metaphase cells. In late telophase (decondensed chromatin, loss of

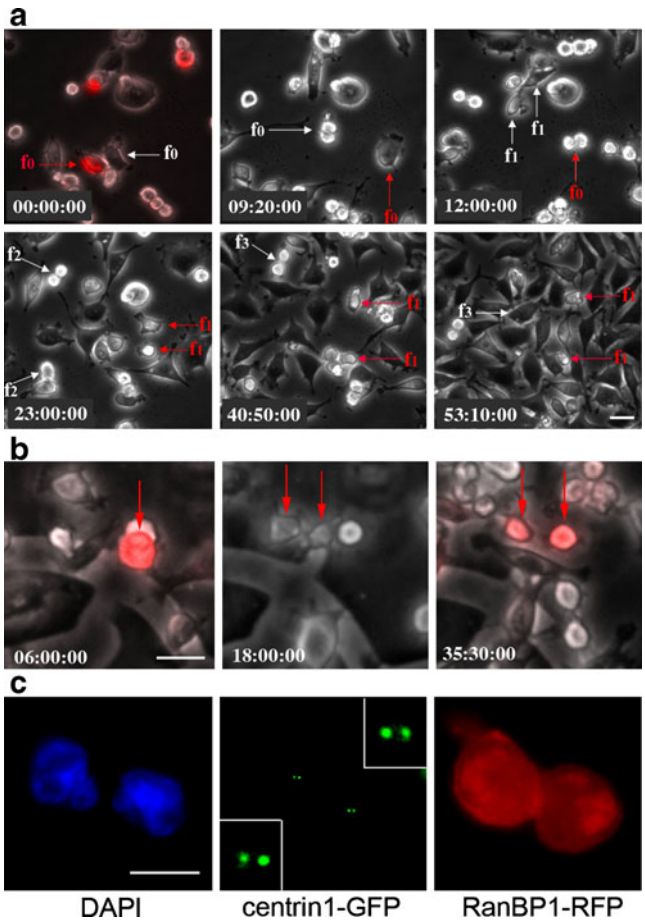


Fig. 3 RanBP1-overexpressing cells arrest after one mitosis with abnormal nuclei. **a** Representative frames from video recording of cell generations in centrin1-GFP L929 cultures transfected with RanBP1-RFP. The non-transfected cell (*white arrow*) divides three times in 50 h: *f0* indicates cells at recording start, *f1* to *f3* indicate subsequent cell division rounds. The only division undergone by the RanBP1-RFP-transfected cell (*red arrow*) is evident at 12 h. *Bar* 20 μm. **b** Representative frames from video recording of centrin1-GFP L929 cells transfected with RanBP1-RFP and seeded on a grid; the arrowed cell undergoes anaphase 6 h after the onset of recording; daughter cells form (*arrowed*), but do not divide again over the next 36 h. *Bar* 20 μm. Note that in **a** and **b** the fluorescence image is overlapped only on some of the trasmission images to identify RanBP1-RFP-transfected cells. **c** RanBP1-RFP-transfected cells in **b** were fixed at the end of recording and stained with DAPI: nuclei remain condensed and centrosomes fail to duplicate, as revealed by the status of centrioles depicted by centrin1-GFP (*blow-up in insets*). *Bar* 10 μm

histone H3 phosphorylation and of MPM2 reactivity, and nuclear expansion), RanBP1 signals were absent or barely detectable. Mid-telophase cells (decondensing chromatin, elongating midbody) were spread in classes of intermediate intensity, indicating that RanBP1 downregulation begins in this window. To further confirm this timing, we followed thymidine-synchronised and released NIH3T3 cells by time-lapse microscopy during mitotic progression. When

Table 2 Effect of RanBP1 overexpression on cell division (50-h video recording)

	pDsRed1-N1		RanBP1-RFP	
	n	%	*n*	%
>2 mitotic rounds	16	45.72	9	9.65
1 mitosis only	2	5.71	23	24.75
Never divide	2	5.71	22	23.65
Dead cells	15	42.86	39	41.95
Total recorded cells	35		93	

Table 3 Abnormal nuclei in RanBP1-RFP-transfected cells fixed after video recording (36 h)

	RanBP1-RFP		
	Normal nuclei	Aberrant nuclei	Total
Normal division timing	17	3	20
1 mitosis only	7	8	15
Never divide	10	0	10
Dead cells			3
Total scored cells	34	11	48

the cells passed the metaphase/anaphase transition, MG132 was added to the cultures; cells were then fixed and stained for RanBP1. Indeed, RanBP1 remained abundant in late telophases from MG132-treated cultures, while being barely detectable in untreated cultures at the same stage (Fig. 1d). This rules out the possibility that RanBP1 abundance observed in MG132-treated samples by western blotting (Fig. 1a) reflected a block in mitotic progression in the absence of proteasome function. Together, these experiments place RanBP1 downregulation in mid to late telophase and implicate the proteasome activity in this downregulation.

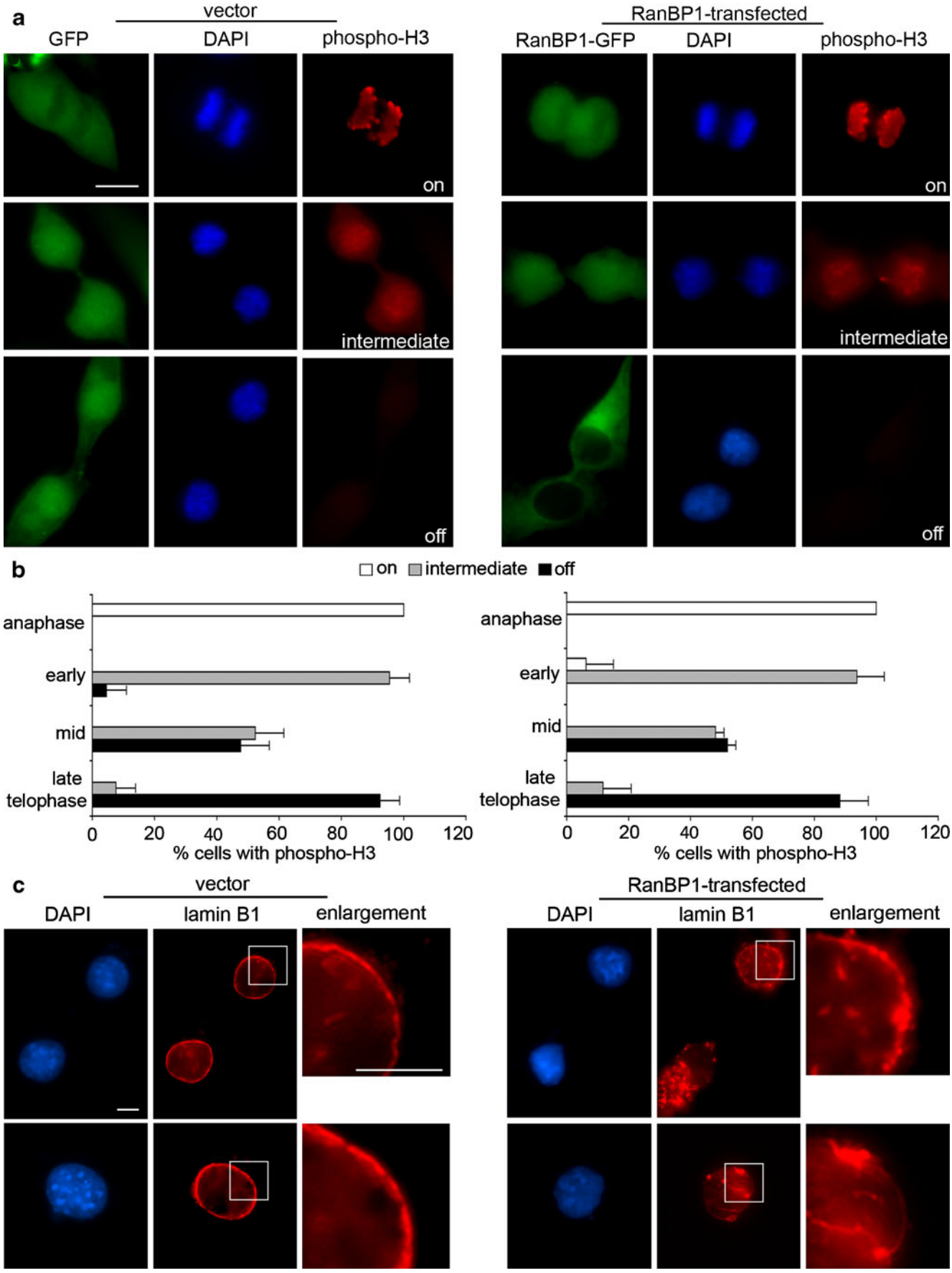
RanBP1 persistence in late mitotic stages impairs the reformation of normal interphase nuclei

To assess the relevance of RanBP1 downregulation in telophase, we decided to record mitotic exit in cells, in which this downregulation was prevented. We took advantage of the observation that RanBP1 plasmid transfection in NIH3T3 cells yields a three to fourfold increase in RanBP1 abundance compared to non-transfected cultures. This level of overexpression caused substantial RanBP1 amounts to persist in the late mitotic window, in which the endogenous protein becomes undetectable (Supplementary fig. 2), suggesting that the system responsible for endogenous RanBP1 downregulation is overridden.

In previous work, mild RanBP1 overexpression induced two prevalent abnormalities in asynchronously cycling NIH3T3 cells cultures: multipolar mitotic spindles and interphase cells with small nuclei (Guarguaglini et al. 2000; Di Fiore et al. 2003). To distinguish specific effects generated by RanBP1 persistence at mitotic exit from effects caused by increased RanBP1 levels throughout the cell cycle, we employed time-lapse microscopy and recorded cells released from thymidine arrest during mitotic progression and exit. RanBP1- and vector-transfected cells showed nuclei of similar size and shape prior to mitotic entry. After NE breakdown, all cells progressed through mitosis with a similar timing until telophase onset. Thereafter, control cells achieved chromatin decondensation, nuclear expansion and migration in less than 40 min (Table 1; representative panels are shown in Fig. 2a). In RanBP1-transfected cells, chromatin also began to decondense in early telophase, but nuclear expansion failed to progress, chromatin remained heterogeneously condensed, and the reforming nuclei remained of small size (panels in Fig. 2b and Supplementary video 1), associated with lengthened telophase duration (Table 1). The recording data indicate that small nuclei do not represent abnormal products of multipolar division, as they formed even in RanBP1-transfected cells that showed no obvious abnormalities in previous metaphase alignment and in which chromosomes segregated along a normal bipolar axis. In early telophase, nuclear migration began in a comparable manner in RanBP1 and vector-transfected cells, but then virtually stopped in RanBP1-overexpressing cells (Supplementary video 1), whereas it continued over almost twice the distance in control telophases (Fig. 2c).

We wondered whether the post-mitotic abnormalities depicted in Fig. 2 affected the subsequent fate of RanBP1-transfected cells and sought to follow their progression into a novel cell cycle. To that aim, we transfected RFP-tagged RanBP1 in a murine L929-derived cell line stably expressing centrin 1-GFP (Piel et al. 2000) so as to distinguish cell cycle stages from the centrosome status, then recorded the cells over the next 50 h. Non-transfected cells divided 2–3 times during the recording (i.e., one cell division every 15–18 h) (Supplementary video 2, representative panels are shown in Fig. 3a). Many RanBP1-transfected cells, instead, underwent the first round of mitosis, but never divided again thereafter (Table 2). To characterise RanBP1-transfected cells that never undergo a second mitosis, cells were seeded on a grid slide and recorded for 36 h (Supplementary video 3; representative panels in Fig. 3b). At the end of recording, the cells were fixed, stained and traced back onto the grid: these experiments showed that over 50% of RanBP1-transfected cells that had divided only once, yet could have had enough time to divide again (Table 3), displayed small nuclei with heterogeneous chromatin decondensation and an unbudded centriole pair, typical of the G1 phase (Fig. 3c). In summary, extending the time-lapse recording beyond the mitotic exit window indicates that cells exiting mitosis with persisting RanBP1 reform abnormal nuclei and cannot undergo a further round of cell division.

To pinpoint specific mitotic exit steps sensitive to RanBP1 levels, we examined RanBP1-transfected NIH3T3 cells fixed at regular intervals while they were completing mitosis. Mitotic chromatin decondensation begins in late anaphase, concomitant with the loss of histone phosphorylation, and is complete by telophase (Sugimoto et al. 2002). Lamin B1 begins to reassociate with chromatin in telophase and reorganises a regular supporting layer by early G1 (Moir et al. 2000). These experiments showed that RanBP1 transfection did not significantly affect the timing or pattern of histone H3 dephosphorylation compared to vector-transfected cultures (Fig. 4a, quantified in Fig. 4b). By contrast, lamin B1 displayed conspicuous abnormalities in RanBP1-expressing telophase cells: irregular aggregates formed, which persisted in the next G1 and prevented the assembly of a regular layer around chromatin (Fig. 4c). Thus, RanBP1 persistence induces the formation of abnormal nuclei without affecting chromatin dephosphorylation after


a
vector
GFP
DAPI
phospho-H3
on
intermediate
off
RanBP1-transfected
RanBP1-GFP
DAPI
phospho-H3
on
intermediate
off
b
on
intermediate
off
anaphase
early
mid
late telophase
0
20
40
60
80
100
120
% cells with phospho-H3
anaphase
early
mid
late telophase
0
20
40
60
80
100
120
% cells with phospho-H3
c
vector
DAPI
lamin B1
enlargement
RanBP1-transfected
DAPI
lamin B1
enlargement

◀ **Fig. 4** RanBP1 persistence in late mitosis does not affect histone H3 dephosphorylation, but prevents lamin B1 reorganisation. **a** Phospho-H3 patterns (classified as *on*, *intermediate* and *off*) in GFP- and RanBP1-GFP-transfected NIH3T3 cells in late mitotic stages. *Bar*, 10 μm. **b** The *histograms* show similar frequencies (shown as *%*) in H3 dephosphorylation patterns in late mitotic cells transfected with either GFP vector or with RanBP1-GFP. *n*, 300 counted mitotic cells in three experiments. **c** INM reorganisation, as revealed by lamin B1 staining, during mitotic exit (*upper row*) and at interphase re-entry (*lower row*). Note the homogeneous lamin B1 perinuclear rim in vector-transfected and the aggregates in RanBP1-transfected cells. Enlargements show ×4 magnification. *Bars* 5 μm

mitosis, but inhibits nuclear expansion and reformation of the inner nuclear membrane (INM).

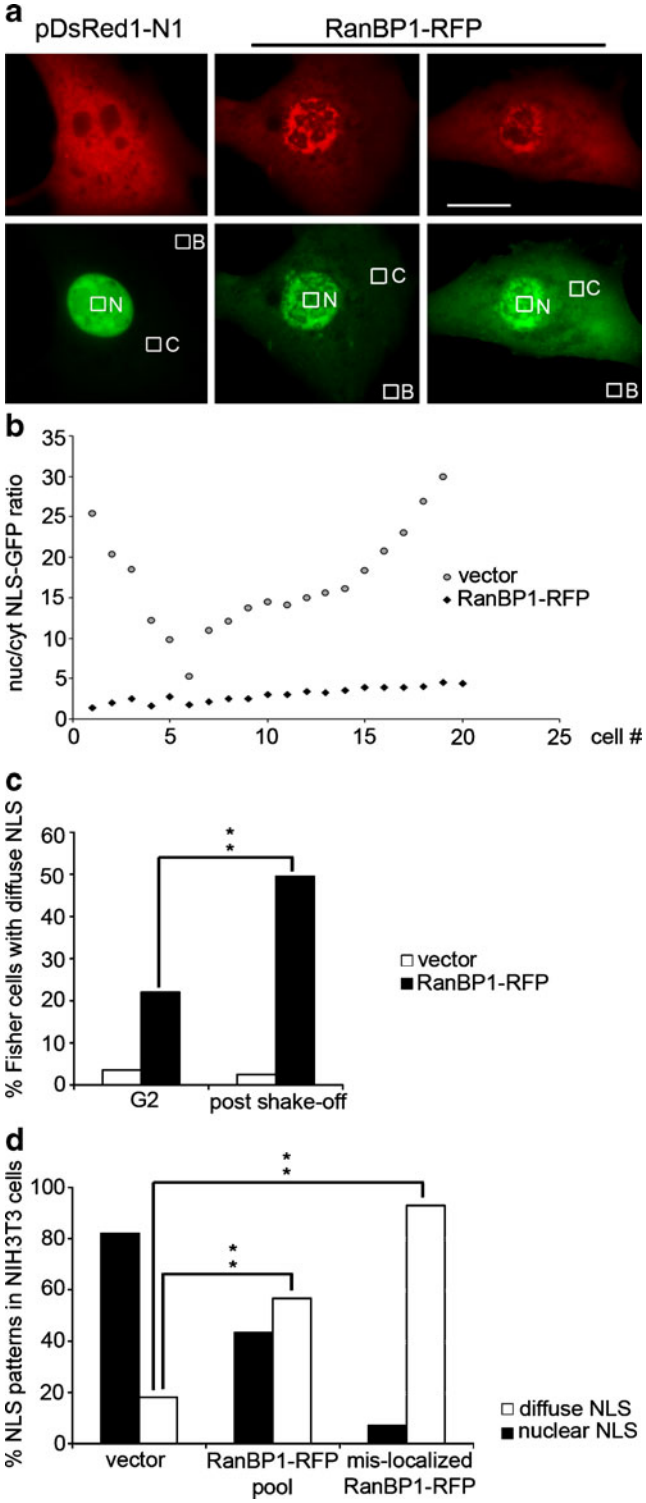
Persisting RanBP1 prevents the nuclear relocalisation of NLS cargo in late mitosis

In the time window in which endogenous RanBP1 is downregulated, the NE reassembles, NPCs are rebuilt, and nuclear import is resumed. Given that persisting RanBP1 inhibited nuclear expansion and INM reorganisation, we wondered whether the relocalisation of an NLS-GFP import reporter was also affected after mitosis. In mammalian cells, nuclear import resumption begins in telophase (Dultz et al. 2008), and an effective permeability barrier across the NPCs is fully re-established within about 2 h after mitosis (Dultz et al. 2009). We therefore harvested NIH3T3 cells co-transfected with NLS-GFP and with RanBP1-RFP (or pDsRed1-N1 vector in controls) in a time course after thymidine synchronisation and release, and we quantified the nuclear to cytoplasmic GFP signal ratio as a measure of the subcellular distribution of NLS-GFP construct at steady-state. 3 h after the mitotic peak (early G1 cells), NLS-GFP was completely nuclear in vector-transfected cultures (Fig. 5a, left panel): over 90% of cells had a nuclear to cytoplasmic NLS-GFP fluorescence ratio above 12, with an average value of 16.97 (Fig. 5b). RanBP1-transfected cells showed a significantly lower proportion of NLS-GFP molecules in reforming nuclei (two representative cells in Fig. 5a, mid and right panels, exemplify different degrees of defective NLS compartmentalisation in nuclei). The nuclear to cytoplasmic ratio dropped <5 in virtually all RanBP1-transfected cells (Fig. 5b). To examine with more certainty the effect of passage through mitosis on localisation of nuclear cargoes, we transfected RanBP1-RFP (or vector) into rat Fisher epithelial cells; unlike NIH3T3, these cells detach at mitotic round-up and can be isolated by shake-off, then replated to complete mitosis until the next G1. That enabled us to analyse NLS-GFP in distinct RanBP1-transfected cell populations collected prior to (G2) and after (next G1) mitosis. Results are quantified (Fig. 5c). We found that less than 20% of G2 cells showed incomplete NLS-GFP localisation in nuclei, with faint GFP fluorescence in the cytoplasm (i.e. comparable to the mislocalisation pattern exemplified in Fig. 5a, mid panel); defects were significantly more frequent and more severe after mitosis: over 50% of G1 cells showed substantial mislocalisation, with a significant fraction of the cargo remaining in the cytoplasm (comparable to Fig. 5a, rightmost panel). Thus, the passage through mitosis with persisting RanBP1 significantly hinders the passage of import cargo in reforming nuclei.

RanBP1 enters nuclei in an importin beta-aided but Ran-independent manner and is effectively exported out by CRM1 (Plafker and Macara 2000), such that the RanBP1 bulk is predominantly cytoplasmic at all times of the cell cycle (Guarguaglini et al. 2000). We noticed that RanBP1 was mislocalised in nuclei, with significant accumulation at the NE, in about one third of transfected cells examined at G1 re-entry. At time points preceding mitosis, RanBP1 was normally cytoplasmic, indicating that the level of over-expression induced in our experiments does not generally saturate CRM1-dependent export, but yields mislocalisation in a proportion of reforming nuclei after mitosis. We found that virtually all cells, in which persisting RanBP1 was mislocalised after mitosis, showed inefficient nuclear relocalisation of the NLS reporter (Fig. 5d), suggesting that one or more of the factor(s) implicated in transport through NPCs becomes limiting under these conditions.

RanBP1 persistence in late mitosis inhibits the reorganisation of NPCs after mitosis

The inefficient passage of NLS cargo in the nuclei of RanBP1-transfected cells has several possible underlying causes. The dependence on passage through mitosis raised the possibility that NPC reorganisation was also abnormal. To test this, we examined the distribution of NUP153, the most nuclear of NPC components, with active roles in NPC assembly (reviewed by Ball and Ullman 2005). NUP153 recruitment to the nuclear periphery precedes import resumption (Haraguchi et al. 2000). We also used Mab414 antibody, which recognises a group of FG-repeat containing NUPs, including NUP153 and the prominent Mab414 antigen NUP62 (Davis and Blobel 1987). NUP62 is recruited to the NPC after NUP153 (Bodoor et al. 1999), and Mab414 redistribution is therefore regarded as a marker of complete NPC reassembly. We found that both NUP153 and Mab414 were abnormally distributed in RanBP1-transfected telophase cells, forming irregular foci within chromatin; these abnormalities persisted as cells reentered interphase after mitosis, with a scattered and disorganised NUP distribution and a substantial failure to reorganise in a perinuclear rim (examples in Fig. 6a, quantified in Fig. 6b). In some



◀ **Fig. 5** Incomplete nuclear relocalisation of NLS-GFP in RanBP1-RFP-transfected cells after mitosis. **a** Exemplifying patterns of NLS-GFP import reporter in NIH3T3 cells examined 3 h after mitosis. Note the fully nuclear NLS-GFP compartmentalisation in the control cell co-transfected with pDsRed1-N1 vector (*left panel*). Examples of mild (*mid panel*) and severe (*left panel*) mislocalisation of NLS-GFP in RanBP1-RFP-transfected cells are shown. Note that nuclei are of small size and RanBP1-RFP is cytoplasmic with strong enrichment around the NE. **b** NLS-GFP localisation was evaluated by measuring the GFP intensity in the nucleus (*N*) and in the cytoplasm (*C*), both normalised for the background signal (*B*); the *graph* shows N/C ratios measured in individual cells in a sample of vector- (*n*, 20) and RanBP1-transfected (*n*, 19) cells. **c** Abnormal NLS-GFP localization in rat Fisher cells examined during G2 prior to mitotic shake-off (*n*, 190 vector- and 170 RanBP1-transfected cells) and at G1 re-entry after replating from mitotic shake-off (*n*, 40 vector- and 110 RanBP1-transfected cells). **d** NLS-GFP localization patterns in NIH3T3 cells examined at G1 re-entry (320 vector- and 300 RanBP1-RFP-transfected counted cells). *Asterisks* indicate highly significant differences ($p<0.001$, $\chi 2$ test)

experiments, we stained centrosomes for gamma-tubulin to unambiguously distinguish cells that reached the next G1 (one centrosome) from late G2 cells (duplicated centrosomes) that may still be present in thymidine pre-synchronised cultures (data not shown, but see Fig. 7c): this confirmed that persisting RanBP1 did not disrupt the organisation of NUP153 or Mab414-reactive NUPs in assembled NPCs in G2 cells, but selectively prevented their reorganisation after mitosis.

Importin beta co-expression suppresses the nuclear abnormalities caused by RanBP1 persistence at mitotic exit

The experiments at this point indicate that RanBP1 perturbs NUP relocalisation and NPC reorganisation. Importin beta has roles in localising NUPs around chromatin. We wondered whether RanBP1 persistence in telophase hindered importin beta function in orchestrating NUP relocalisation and NPC assembly when the nucleus reforms. If so, overexpressing importin beta, together with RanBP1, should restore importin beta activity and rescue RanBP1-dependent abnormalities. To test this, we examined NPC reassembly (using Mab414), INM reorganisation (probed by the lamin B1 antibody), and chromatin decondensation (by DAPI staining), in RanBP1-transfected cells with or without exogenously co-expressed importin beta. We first ascertained that GFP-tagged importin beta faithfully reproduces the binding pattern of the endogenous protein, as this construct has not been extensively characterised after transfection in mammalian cells. Indeed, we found that it was able to co-immunoprecipitate Ran, RanBP1 and NUP153 (Fig. 7a); these experiments were carried out in human HeLa cells for ease of reagents availability, and similar results were also obtained in rat Fisher and murine NIH3T3 cell lines (data not shown). Expression of importin beta construct alone generated abnormalities in mitotic chromosome alignment and spindle structure (data not shown; but see Nachury et al. 2001; Ciciarello et al. 2004), yet did not disrupt nuclear reformation after mitosis in either HeLa or NIH3T3 cells (histogram in Fig. 7b). When importin beta was co-transfected together with RanBP1, however, it reduced the occurrence of abnormal-

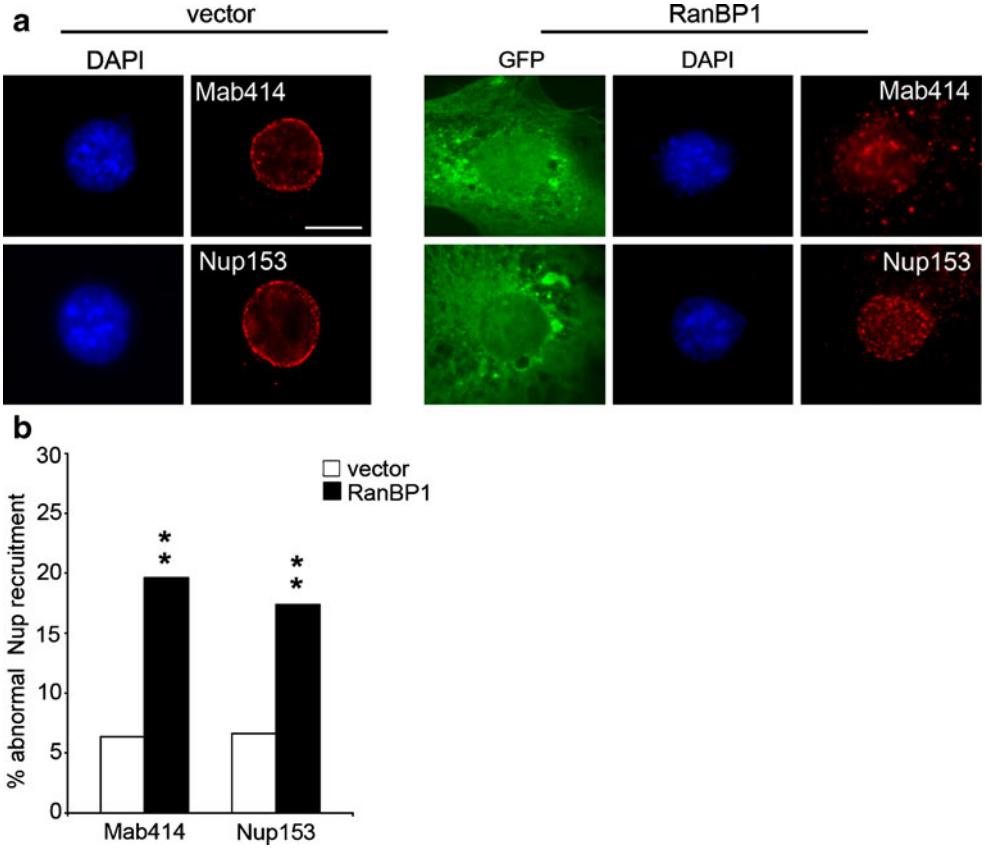


Fig. 6 RanBP1 excess impairs NUP reorganisation after mitosis. Mab414 (*upper row*) and NUP153 (*lower row*) in NIH3T3 cells transfected with GFP vector (*left panels*, GFP fluorescence not depicted) and with RanBP1-GFP (*right panels*). **b** Frequency of cells with abnormal Mab414 and NUP153 patterns in GFP vector- and RanBP1-GFP-transfected samples at time points corresponding to G1 re-entry after mitosis (at least 600 counted cells per sample). *Asterisks* indicate highly significant differences ($p<0.001$, $\chi 2$ test)

ities in Mab414 recruitment and chromatin reorganisation induced by RanBP1 alone: examples of phenotypically 'rescued' NIH3T3 cells co-transfected with RanBP1 and importin beta are shown in Fig. 7c. This rescue was dependent on the ratio of importin beta to co-expressed RanBP1 (Fig. 7b). In these experiments, RanBP1-transfected NIH3T3 cells were examined after release from thymidine arrest as described above: abnormalities were again found to be rare at time points preceding the mitotic peak, and importin beta induced no significant variations at this stage. By contrast, both RanBP1-dependent abnormalities and importin beta-dependent rescue were clear in cells re-entering G1 after mitosis, directly identified by co-staining centrosomes with gamma-tubulin (Fig. 7c). We also tested the effectiveness of importin beta$^{45\text{-}876}$: this mutant carries an N-terminal deletion that impairs the interaction with RanGTP, but retains the sites of interaction for NUPs, as indicated by its unaltered ability to bind NUP153 when transfected in mammalian cells (Fig. 7a). We found that importin beta$^{45\text{-}876}$ indeed mitigates the induction of nuclei with abnormal morphology and Mab414 misrecruitment caused by RanBP1 persistence in late mitosis (Fig. 7d). Thus, high RanBP1 levels render importin beta, and particularly the NUP-binding region, limiting at mitotic exit. This suggests that nuclear abnormalities induced by RanBP1 persistence in late mitosis reflect an impairment of importin beta functions, which is relieved by simultaneously raising the concentration of importin beta.

Discussion

Mammalian cells take little more than 1 h from metaphase to mitotic completion, during which profound changes take place. Here, we show that RanBP1 levels become almost undetectable in mammalian cells in mid to late telophase. This suggests that the overall RanBP1 abundance results from a dynamic balance between continuous increase taking place from S phase to anaphase, largely determined by transcriptional up-regulation (Di Fiore et al. 1999), and protein downregulation becoming predominant at telophase. Other Ran regulators are cyclically phosphorylated in mitosis and dephosphorylated as cells re-enter interphase (Swaminathan et al. 2004; Hutchins et al. 2004; Li and Zheng 2004), converging to suggest the idea that the Ran network is globally 'reset' at each cell cycle re-entry. The decline in RanBP1 abundance represents the most significant quantitative variation within the Ran network in this cell cycle window, suggesting that RanBP1 activity is differentially required in different stages of the mammalian cell cycle.

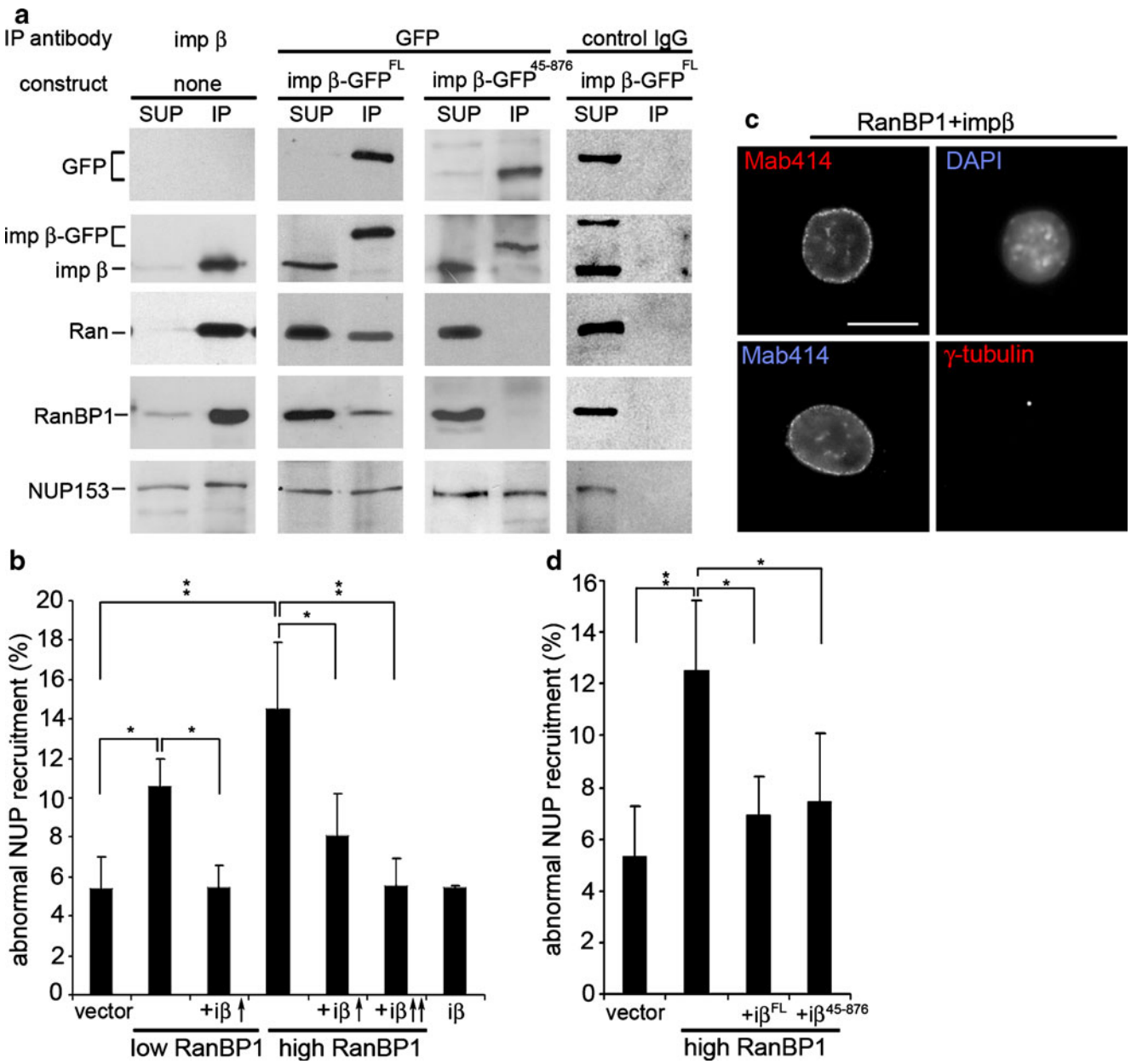


Fig. 7 Importin beta co-expression rescues RanBP1-dependent abnormalities. **a** Western blot analysis of the indicated proteins in co-IP of endogenous importin beta, full length GFP-importin beta$^{1\text{-}876}$ (indicated as *FL*) and GFP-importin beta$^{45\text{-}876}$. The *control panel* shows whole cell extract from GFP-importin beta$^{1\text{-}876}$-transfected cells precipitated with non-specific IgG. **b** Frequency of early G1 cells with abnormal Mab414 patterns in cultures transfected with the indicated constructs (low RanBP1, 3 μg; high RanBP1, 5 μg; *one arrow* indicates 2 μg and *two arrows* 4 μg of importin beta; at least 500 cells per sample were counted in three experiments). Differences in the frequency of abnormalities in low RanBP1- and high RanBP1- versus vector-transfected cells are significant ($p<0.01$, χ^2 test) and highly significant ($p<0.001$, χ^2 test), respectively; rescue by co-expressed importin beta is highly significant ($p<0.001$, χ^2 test) compared to RanBP1 alone; furthermore, the frequency of abnormalities is not statistically significant in control (*vector*), co-transfected (RanBP1+importin beta) or importin beta alone samples. **c** Mab414 recruitment and chromatin organisation (DAPI) are restored in cells co-transfected with RanBP1 (5 μg, as used throughout this paper) and importin beta (2 μg) examined at G1 re-entry after mitosis as verified by centrosome counting (*γ-tubulin panel*). **d** Frequency of cells with abnormal Mab414 patterns at G1 re-entry after mitosis in cultures transfected with the indicated constructs (700 to 800 counted cells per sample in four experiments). Co-expressed importin beta$^{1\text{-}876}$ and importin beta$^{45\text{-}876}$ (2 μg) induce a significant rescue compared to RanBP1 alone (5 μg) ($p<0.01$, χ^2 test)

The reorganisation of a nucleus surrounded by a functional NE is highly complex in eukaryotic cells and, despite remarkable progress in recent times, remains incompletely understood (see reviews by Hetzer et al. 2005; Güttinger et al. 2009; Hetzer and Wente 2009). High-resolution imaging methods have revealed a highly regulated temporal schedule for NE reassembly around the decondensing chromatin, sequential recruitment of NPC

components, resumption of nuclear import and re-establishment of an effective permeability barrier in the NPC channels (Bodoor et al. 1999; Moir et al. 2000; Haraguchi et al. 2000; Daigle et al. 2001; Rabut et al. 2004; Dultz et al. 2008; Dultz et al. 2009).

This work implicates RanBP1 downregulation in this process and depicts evident abnormalities caused by RanBP1 persistence at mitotic exit. NUPs fail to relocalise properly around chromatin after mitosis. The resulting NPC misassembly suggests a plausible cause for the defective nuclear localisation of NLS cargo. RanBP1 itself fails to shuttle normally between importin beta-aided import and CRM1-dependent export and accumulates around the NE or remains partly trapped in the nucleus in a fraction of cells. Concomitant with this, reforming nuclei fail to expand and INM reformation is abnormal; these defects, in turn, likely contribute to further hamper chromatin decondensation and nuclear growth (reviewed by Dechat et al. 2008). NE and NPC reorganisation are linked to chromatin architecture, strengthening the idea that the defects caused by RanBP1 persistence are interconnected. Nuclear migration is also abortive, reminiscent of defects recorded in cells in which RNAi to individual NE components prevented the NE reorganisation (Anderson et al. 2009). These effects of RanBP1 are specifically exerted at the mitosis to interphase transition and do not reflect a general disruption of nuclear organisation caused by RanBP1 excess, because: (1) defects are specifically induced when nuclei reform after mitosis, but not in the preceding interphase, in which nuclei are already formed; (2) slightly earlier events occurring in anaphase/early telophase, when endogenous RanBP1 is still abundant, e.g. histone H3 dephosphorylation, are not affected. Thus, misexpression of RanBP1 in late mitosis impairs nuclear reorganisation steps that are scheduled to take place when the endogenous protein is due to disappear. This is the first time that a specific change within the Ran network is shown to be required at the mitosis to interphase transition in the complex cell cycle of mammalian cells.

RanBP1 regulates the guanine nucleotide turnover on Ran, as well as its interactions with nuclear transport receptors in vitro. In intact cells, the topological separation between sites where interactions occur, disassemble and eventually release single molecules, is a key feature of the system. Due to this complexity, understanding how RanBP1 operates in cells at mitotic exit is an open challenge and can only be tentatively surmised. The results presented here can help to delineate possible scenarios.

First, RanBP1 co-activates RanGTP hydrolysis by RanGAP1 and inhibits RCC1-mediated GTP exchange (Bischoff et al. 1995). In that light, the reformation of abnormal nuclei in the presence of persisting RanBP1 might reflect an unscheduled inhibition of RCC1, with an ensuing drop in RanGTP levels below a critical threshold after mitosis: RanGTP would thus become insufficient to release importin beta partners required for nuclear reformation. The present findings ,however, do not favour this interpretation, because the reorganisation of nuclei in late mitosis, though being sensitive to RanBP1 persistence, is neither affected by overexpressing RCC1—which directly increases free RanGTP (data not shown), nor by importin beta—which binds RanGTP and reduces its availability (this study). Moreover, if RanBP1 decrease at mitotic exit was exclusively required to allow sufficient RanGTP accumulation when nuclei reform, then co-expression of importin beta, the antagonistic effector of RanGTP, should exacerbate RanBP1-dependent phenotypes, whereas that is in fact the opposite of what we find. Nuclear abnormalities produced by RanBP1 persistence are therefore unlikely to be solely caused through direct alterations in RanGTP levels.

These observations draw attention to another established function of RanBP1, i.e. its ability to regulate RanGTP interactions with importin beta (Chi et al. 1996; Bischoff and Görlich 1997; Lounsbury and Macara 1997; Floer et al. 1997; Gorlich et al. 2003; Lonhienne et al. 2009). RanBP1 facilitates the dissociation of RanGTP/importin beta complexes; physiologically, this is thought to provide a critical switch after termination of nuclear import, when RanGTP exits the nucleus with either importin beta or with importin alpha and CAS (the export factor for importin alpha). RanGTP is resistant to RanGAP1-mediated hydrolysis in "nuclear exit" complexes, but RanBP1 binding triggers the complex dissociation, thus permitting the recycling of individual components for a novel import cycle. A priori, therefore, RanBP1 downregulation at mitotic exit may serve to reduce RanGTP dissociation from importin beta, with higher RanGTP/importin beta complexes and lower free Ran available for the RanGTP/RanGDP cycle. In that scenario, persisting RanBP1 would abnormally increase the dissociation of RanGTP from importin beta, producing more free Ran available for new RanGTP production. RanBP1 excess would therefore reproduce the inhibitory effect of RanGTP on NE envelope formation (Hetzer et al. 2000) and the phenotypic rescue operated by importin beta would indicate a reversal of this inhibitory effect.

A variation of this idea may be suggested by the finding that RanBP1 is part of complexes containing RanGDP and importin beta, which have discrete abundance in the cytoplasm of interphase cells, as depicted in FRET experiments (Plafker and Macara 2002). These complexes are hypothesised to represent intermediate transport products, which could limit the amount of Ran exiting the nucleus that becomes immediately available for nuclear re-entry and RanGTP reformation, thus, helping to modulate the 'steepness' of the gradient between nuclear RanGTP and cytoplasmic RanGDP under certain circumstances (for

example, related to changes in the nuclear volume during the cell cycle). In mitosis, when the compartmentalization between the nucleus and the cytoplasm is lost, fractions of RanBP1, Ran and importin beta co-localise at mitotic microtubules (Trieselmann and Wilde 2002; Di Fiore et al. 2003; Ciciarello et al. 2004; Tedeschi et al. 2007; Hutchins et al. 2009). The co-localisation data suggest that mitotic MTs can provide a physical platform where these components interact dynamically, thus, determining the free or bound status of downstream factors. Indeed, RanBP1 silencing yields an excessive release of the MT-stabilising factor HURP (Tedeschi et al. 2007), an importin beta direct interactor (Koffa et al. 2006; Sillje et al. 2006) associated with the formation of hyperstable mitotic spindles (Li et al. 2007; Tedeschi et al. 2007). Thus, RanBP1 levels in early mitosis have direct relevance to regulate the distribution of a subset of importin beta partners in the mitotic apparatus.

The present work suggests that downregulation of RanBP1 at mitotic completion is important to reset importin beta interactions with factors acting in nuclear and NE reorganisation. RanBP1 persistence prevents the relocalisation of NUPs with which importin beta interacts directly, but raising the concentration of importin beta restores their localisation. Moreover, importin beta$^{45\text{-}876}$, which retains the NUP-binding domain, also operates a phenotypic rescue, albeit being defective for Ran binding. These data suggest that importin beta, and particularly its ability to bind NUPs, becomes limiting when RanBP1 persists in late mitosis. The finding that importin beta$^{45\text{-}876}$ restores the localisation of NUPs, in conditions in which persisting RanBP1 hinders the endogenous importin beta function, suggests that this relocalising function is largely independent of Ran, extending to mammalian cells the existence of a mechanism that has only been described in vitro thus far (Harel et al. 2003; Delmar et al. 2008; Rotem et al. 2009).

In summary, the present results indicate that the failure to downregulate RanBP1 in late mitosis impinges on importin beta functions in nuclear reformation pathways at interphase re-entry. Importin beta operates on cargoes in distinct mechanistic manners during mitosis. It binds a set of factors and keeps them in a functionally inhibited form until required. It acts as a localising device for factors that relocalise from the mitotic apparatus to the chromatin periphery at mitotic completion (Harel et al. 2003; D'Angelo et al. 2006; Rotem et al. 2009; Lau et al. 2009). A chaperone role has also been proposed, which would prevent abnormal contacts among NUPs during the build-up of NPCs (Frey and Görlich 2007). In order to exert this variety of functions, importin beta must establish differential interactions with distinct targets in space and time, requiring specificity and fine-tuning. We suggest that variations in RanBP1 abundance at the end of mitosis contribute to this fine-tuning and shift the balance among complexes containing importin beta. In summary, RanBP1 downregulation marks an important regulatory switch in the nuclear reformation programme that involves the re-establishment of importin beta-dependent functions after mitosis in mammalian cells.

Acknowledgements We are extremely grateful to Antonio Tedeschi (IMP, Vienna) for contributing to early stages of this work and to Giulia Guarguaglini (IBPM, Rome) for insightful comments on this manuscript. This work was supported by MIUR - Italian Ministry of University and Research (FIRB grant RBIN04T7MT and PRIN grant 200879X9N9-004), Associazione Italiana per la Ricerca sul Cancro (AIRC) and by funds from Assicurazioni Generali and Fondazione Roma Terzo Settore.

References

Anderson DJ, Vargas JD, Hsiao JP, Hetzer MW (2009) Recruitment of functionally distinct membrane proteins to chromatin mediates nuclear envelope formation in vivo. J Cell Biol 186:183–191

Askjaer P, Galy V, Hannak E, Mattaj IW (2002) Ran GTPase cycle and importins alpha and beta are essential for spindle formation and nuclear envelope assembly in living Caenorhabditis elegans embryos. Mol Biol Cell 13:4355–4370

Ball JR, Ullman KS (2005) Versatility at the nuclear pore complex: lessons learned from the nucleoporin Nup153. Chromosoma 114:319–330

Bamba C, Bobinnec Y, Fukuda M, Nishida E (2002) The GTPase Ran regulates chromosome positioning and nuclear envelope assembly in vivo. Curr Biol 12:503–507

Bischoff FR, Görlich D (1997) RanBP1 is crucial for the release of RanGTP from importin beta-related nuclear transport factors. FEBS Lett 419:249–254

Bischoff FR, Krebber H, Smirnova E, Dong W, Ponstingl H (1995) Co-activation of Ran GTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. EMBO J 14:705–715

Bodoor K, Shaikh S, Salina D, Raharjo WH, Bastos R, Lohka M, Burke B (1999) Sequential recruitment of NPC proteins to the nuclear periphery at the end of mitosis. J Cell Sci 112:2253–2264

Chi NC, Adam EJ, Visser GD, Adam SA (1996) RanBP1 stabilizes the interaction of Ran with p97 nuclear protein import. J Cell Biol 135:559–569

Ciciarello M, Mangiacasale R, Thibier C, Guarguaglini G, Marchetti E, Di Fiore B, Lavia P (2004) Importin beta is transported to spindle poles during mitosis and regulates Ran-dependent spindle assembly factors in mammalian cells. J Cell Sci 117:6511–6522

Daigle N, Beaudouin J, Hartnell L, Imreh G, Hallberg E, Lippincott-Schwartz J, Ellenberg J (2001) Nuclear pore complexes form immobile networks and have a very low turnover in live mammalian cells. J Cell Biol 154:71–84

D'Angelo MA, Anderson DJ, Richard E, Hetzer MW (2006) Nuclear pores form de novo from both sides of the nuclear envelope. Science 312:440–443

Davis LI, Blobel G (1987) Nuclear pore complex contains a family of glycoproteins that includes p62 glycosylation through a previously unidentified cellular pathway. PNAS 84:7552–7556

Dechat T, Pfleghaar K, Sengupta K, Shimi T, Shumaker DK, Solimando L, Goldman RD (2008) Nuclear lamins major factors in the structural organization and function of the nucleus and chromatin. Genes Dev 22:832–853

Delmar VA, Chan RC, Forbes DJ (2008) Xenopus importin beta validates human importin beta as a cell cycle negative regulator. BMC Cell Biol 9:14–25

Di Fiore B, Guarguaglini G, Palena A, Kerkhoven RM, Bernards R, 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 Fiore B, Ciciarello M, Mangiacasale R, Palena A, Tassin AM, Cundari E, Lavia P (2003) Mammalian RanBP1 regulates centrosome cohesion during mitosis. J Cell Sci 116:3399–3411

Dultz E, Zanin E, Wurzenberger C, Braun M, Rabut G, Sironi L, Ellenberg J (2008) Systematic kinetic analysis of mitotic dis- and reassembly of the nuclear pore in living cells. J Cell Biol 180:857–865

Dultz E, Huet S, Ellenberg J (2009) Formation of the nuclear envelope permeability barrier studied by sequential photoswitching and flux analysis. Biophys J 97:1891–1897

Floer M, Blobel G, Rexach M (1997) Disassembly of RanGTP-karyopherin beta complex, an intermediate in nuclear protein import. J Biol Chem 272:19538–19546

Frey S, Görlich D (2007) A saturated FG-repeat hydrogel can reproduce the permeability properties of nuclear pore complexes. Cell 130:512–523

Gonzalez Y, Meerbrey K, Chong J, Torii Y, Padte NN, Sazer S (2009) Nuclear shape, growth and integrity in the closed mitosis of fission yeast depend on the Ran-GTPase system, the spindle pole body and the endoplasmic reticulum. J Cell Sci 122:2464–2472

Gorlich D, Seewald MJ, Ribbeck K (2003) Characterization of Ran-driven cargo transport and the Ran GTPase system by kinetic measurements and computer simulation. EMBO J 22:1088–1100

Guarguaglini G, Renzi L, D'Ottavio F, Di Fiore B, Casenghi M, Cundari E, 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

Güttinger S, Laurell E, Kutay U (2009) Orchestrating nuclear envelope disassembly and reassembly during mitosis. Nat Rev Mol Cell Biol 10:178–191

Haraguchi T, Koujin T, Hayakawa T, Kaneda T, Tsutsumi C, Imamoto N, Akazawa C, Sukegawa J, Yoneda Y, Hiraoka Y (2000) Live fluorescence imaging reveals early recruitment of emerin LBR RanBP2 and Nup153 to reforming functional nuclear envelopes. J Cell Sci 113:779–794

Harel A, Forbes DJ (2004) Importin beta conducting a much larger cellular symphony. Mol Cell 16:319–330

Harel A, Chan RC, Lachish-Zalait A, Zimmerman E, Elbaum M, Forbes DJ (2003) Importin beta negatively regulates nuclear membrane fusion and nuclear pore complex assembly. Mol Biol Cell 14:4387–4396

He X, Hayashi N, Walcott NG, Azuma Y, Patterson TE, Bischoff FR, Nishimoto T, Sazer S (1998) The identification of cDNAs that affect the mitosis-to-interphase transition in Schizosaccharomyces pombe including sbp1 which encodes a spi1p-GTP-binding protein. Genetics 148:645–656

Hetzer MW, Wente SR (2009) Border control at the nucleus: biogenesis and organization of the nuclear membrane and pore complexes. Dev Cell 17:606–616

Hetzer MW, Bilbao-Cortes D, Walther TC, Gruss OJ, Mattaj IW (2000) GTP hydrolysis by Ran is required for nuclear envelope assembly. Mol Cell 5:1013–1024

Hetzer MW, Walther TC, Mattaj IW (2005) Pushing the envelope structure function and dynamics of the nuclear periphery. Annu Rev Cell Dev Biol 21:347–380

Hutchins JR, Moore WJ, Hood FE, Wilson JS, Andrews PD, Swedlow JR, Clarke PR (2004) Phosphorylation regulates the dynamic interaction of RCC1 with chromosomes during mitosis. Curr Biol 14:1099–1104

Hutchins JR, Moore WJ, Clarke PR (2009) Dynamic localisation of Ran GTPase during the cell cycle. BMC Cell Biol 10:66

Koffa MD, Casanova CM, Santarella R, Kocher T, Wilm M, Mattaj IW (2006) HURP is part of a Ran-dependent complex involved in spindle formation. Curr Biol 16:743–754

Kutay U, Izaurralde E, Bischoff FR, Mattaj IW, Görlich D (1997) Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. EMBO J 16:1153–1163

Lau CK, Delmar VA, Chan RC, Phung Q, Bernis C, Fichtman B, Rasala BA, Forbes DJ (2009) Transportin regulates major mitotic assembly events: from spindle to nuclear pore assembly. Mol Biol Cell 20:4043–4058

Li HY, Zheng Y (2004) Phosphorylation of RCC1 in mitosis is essential for producing a high RanGTP concentration on chromosomes and for spindle assembly in mammalian cells. Genes Dev 18:512–527

Li HY, Ng WP, Wong CH, Iglesias PA, Zheng Y (2007) Coordination of chromosome alignment and mitotic progression by the chromosome-based Ran signal. Cell Cycle 6:1886–1895

Lonhienne TG, Forwood JK, Marfori M, Robin G, Kobe B, Carroll BJ (2009) Importin-beta is a GDP-to-GTP exchange factor of Ran: implications for the mechanism of nuclear import. J Biol Chem 284:22549–22558

Lounsbury KM, Macara IG (1997) Ran-binding protein 1 (RanBP1) forms a ternary complex with Ran and karyopherin beta and reduces Ran GTPase-activating protein (RanGAP) inhibition by karyopherin beta. J Biol Chem 272:551–555

Matynia A, Dimitrov K, Mueller U, He X, Sazer S (1996) Perturbations in the spi1p GTPase cycle of Schizosaccharomyces pombe through its GTPase-activating protein and guanine nucleotide exchange factor components result in similar phenotypic consequences. Mol Cell Biol 16:6352–6362

Moir RD, Yoon M, Khuon S, Goldman RD (2000) Nuclear lamins A and B1: different pathways of assembly during nuclear envelope formation in living cells. J Cell Biol 151:1155–1168

Nachury MV, Maresca TJ, Salmon WC, Waterman-Storer CM, Heald R, Weis K (2001) Importin beta is a mitotic target of the small GTPase Ran in spindle assembly. Cell 104:95–106

Nicolas FJ, Zhang C, Hughes M, Goldberg MW, Watton SJ, Clarke PR (1997) Xenopus Ran-binding protein 1 molecular interactions and effects on nuclear assembly in Xenopus egg extracts. J Cell Sci 110:3019–3030

Piel M, Meyer P, Khodjakov A, Rieder CL, 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

Pines J (2006) Mitosis: a matter of getting rid of the right protein at the right time. Trends Cell Biol 16:55–63

Plafker K, Macara IG (2000) Facilitated nucleocytoplasmic shuttling of the Ran binding protein RanBP1. Mol Cell Biol 20:3510–3521

Plafker K, Macara IG (2002) Fluorescence resonance energy transfer biosensors that detect Ran conformational changes and a Ran·GDP-importin-b-RanBP1 complex in vitro and in intact cells. J Biol Chem 277:30121–30127

Pu RT, Dasso M (1997) The balance of RanBP1 and RCC1 is critical for nuclear assembly and nuclear transport. Mol Biol Cell 8:1955–1970

Rabut G, Doye V, Ellenberg J (2004) Mapping the dynamic organization of the nuclear pore complex inside single living cells. Nat Cell Biol 6:1114–1121

Rotem A, Gruber R, Shorer H, Shaulov L, Klein E, Harel A (2009) Importin beta regulates the seeding of chromatin with initiation sites for nuclear pore assembly. Mol Biol Cell 20:4031–4042

Ryan KJ, McCaffery JM, Wente SR (2003) The Ran GTPase cycle is required for yeast nuclear pore complex assembly. J Cell Biol 160:1041–1053

Ryan KJ, Zhou Y, Wente SR (2007) The karyopherin Kap95 regulates nuclear pore complex assembly into intact nuclear envelopes in vivo. Mol Biol Cell 18:886–898

Salus SS, Demeter J, Sazer S (2002) The Ran GTPase system in fission yeast affects microtubules and cytokinesis in cells that are competent for nucleocytoplasmic protein transport. Mol Cell Biol 22:8491–8505

Sillje HH, Nagel S, Korner R, Nigg EA (2006) HURP is a Ran-importin b-regulated protein that stabilizes kinetochore microtubules in the vicinity of chromosomes. Curr Biol 16:731–742

Sugimoto K, Urano T, Zushi H, Inoue K, Tasaka H, Tachibana M, Dotsu M (2002) Molecular dynamics of Aurora-A kinase in living mitotic cells simultaneously visualized with histone H3 and nuclear membrane protein importin alpha. Cell Struct Funct 27:457–467

Swaminathan S, Kiendl F, Körner R, Lupetti R, Hengst L, Melchior F (2004) RanGAP1*SUMO1 is phosphorylated at the onset of mitosis and remains associated with RanBP2 upon NPC disassembly. J Cell Biol 164:965–971

Tedeschi A, Ciciarello M, Mangiacasale R, Roscioli E, Rensen WM, Lavia P (2007) RANBP1 localizes a subset of mitotic regulatory factors on spindle microtubules and regulates chromosome segregation in human cells. J Cell Sci 120:3748–3761

Timinszky G, Tirian L, Nagy FT, Toth G, Perczel A, Kiss-Laszlo Z, Boros I, Clarke PR, Szabad J (2002) The importin-beta P446L dominant-negative mutant protein loses RanGTP binding ability and blocks the formation of intact nuclear envelope. J Cell Sci 115:1675–1687

Tirian L, Timinszky G, Szabad J (2003) P446L-importin-beta inhibits nuclear envelope assembly by sequestering nuclear envelope assembly factors to the microtubules. EJCB 82:351–359

Trieselmann N, Wilde A (2002) Ran localizes around the microtubule spindle in vivo during mitosis in Drosophila embryos. Curr Biol 12:1124–1129

Walther TC, Askjaer P, Gentzel M, Habermann A, Griffiths G, Wilm M, Mattaj IW, Hetzer MW (2003) RanGTP mediates nuclear pore complex assembly. Nature 424:689–694

Weis K (2003) Regulating access to the genome nucleocytoplasmic transport throughout the cell cycle. Cell 112:441–451

Zhang C, Clarke PR (2000) Chromatin-independent nuclear envelope assembly induced by Ran GTPase in Xenopus egg extracts. Science 288:429–432

Zhang C, Clarke PR (2001) Roles of Ran-GTP and Ran-GDP in precursor vesicle recruitment and fusion during nuclear envelope assembly in a human cell-free system. Curr Biol 11:208–812

Zhang C, Goldberg MW, Moore WJ, Allen TD, Clarke PR (2002a) Concentration of Ran on chromatin induces decondensation, nuclear envelope formation and nuclear pore complex assembly. EJCB 81:623–633

Zhang C, Hutchins JR, Muhlhausser P, Kutay U, Clarke PR (2002b) Role of importin-beta in the control of nuclear envelope assembly by Ran. Curr Biol 12:498–502