Cell. Mol. Life Sci. 64 (2007) 1891 – 1914
1420-682X/07/151891-24
DOI 10.1007/s00018-007-6568-2

Cellular and Molecular Life Sciences

Review

Spatial control of mitosis by the GTPase Ran

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Received 29 December 2006; received after revision 13 March 2007; accepted 29 March 2007
Online First 5 May 2007

Abstract. Mitosis is the most potentially dangerous event in the life of a cell, during which the cell genetic identity is transmitted to daughters; errors at this stage may yield aneuploid cells that can initiate a genetically unstable clone. The small GTPase Ran is the central element of a conserved signaling network that has a prominent role in mitotic regulation. Pioneering studies with amphibian oocytes indicated that Ran, in the GTP-bound form, activates factors that regulate spindle assembly and dynamics. An increasing body of data indicate higher specificity and complexity in mitotic control operated by Ran in somatic cells. Newly identified target factors of Ran operate with different specificity, and it is emerging that mitotic progression requires the precise positioning of Ran network components and effectors at specific sites of the mitotic apparatus according to a highly regulated schedule in space and time. In this review we summarize our current understanding of Ran control of mitosis and highlight the specificity of mechanisms operating in mammalian somatic cells.

Keywords. Ran, RCC1, RanGAP1, RanBP1, mitotic spindle, microtubules, centrosomes, spindle checkpoint.

Introduction

Ran is an atypical member of the Ras GTPase superfamily [1] that regulates intracellular events, rather than connecting inner and outer events in the cell. Growing evidence indicate a central role of Ran as a regulator of mitosis – the most recently discovered of Ran functions. Given the importance of mitotic control to transmission of the cell genetic identity, and the weight of mitotic errors in the generation of aneuploidy, many efforts are being devoted to understand how Ran signaling operates in the process and novel results are being produced with astonishing rapidity. Here we review available data on mitotic control by Ran, with a focus on the specificity of regulatory mechanisms that operate in mammalian cells. As an introductory step, we briefly recapitulate how our conceptual paradigms for Ran functions have built up.

Nucleo-cytoplasmic transport was the first process in which a role of Ran was clearly characterized and rendered amenable to experimental testing in a variety of systems, opening up an era of exciting research that is still very productive. Ran at that point was defined as an essential regulator of intracellular transport, an eminently "housekeeping" function taking place in resting and highly proliferating cells. Roles of Ran in regulating cell cycle transitions also emerged, because mutant versions of Ran network members caused premature progression in the next cell cycle phase, but it was unclear whether those effects reflected a direct Ran function or were secondary to regulation of nucleo-cytoplasmic transport of particular factors (see [2–4] for reviews). In recent years, however, mitotic roles of Ran have

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emerged that are clearly independent of transport and are no longer the object of controversy. Prior to describing these roles, it is useful to recall the basic mechanisms of the Ran GTPase cycle.

The Ran network in interphase

As for all GTPases, the nucleotide-bound state of Ran is crucial to regulated processes downstream. Several proteins contain Ran-binding domains and can potentially interact with Ran, but only two catalytic factors are essential to regulate the Ran GTPase cycle: the GTP hydrolysis-activating protein RanGAP1, which hydrolyzes GTP on Ran and generates RanGDP [5], and the GTP nucleotide exchange factor (GEF) RCC1 (regulator of chromosome condensation 1), which loads GTP on Ran [6]. RanBP1 (Ran-binding protein 1) is a non-catalytic factor with a potential dual role: in isolation, it stabilizes RanGTP [7]; in the presence of regulators, it modulates nucleotide turnover on Ran, by both increasing the hydrolyzing activity of RanGAP1 and by inhibiting the GEF activity of RCC1 [8].

The interphase Ran GTPase cycle: a topological view. Nucleo-cytoplasmic transport depends on the subcellular localization of factors that regulate the nucleotide-bound state of Ran. RCC1 localizes in nuclei [9] and binds chromatin through an interaction with histones H2A and H2B [10]. This binding causes a modest increase in its exchange activity, and is important, more than for its catalytic effect, because it restricts the site of nucleotide exchange on Ran to chromatin (also see [11, 12]). RanGAP1 is cytoplasmic, with a significant fraction anchored at the cytoplasmic face of the nuclear envelope (NE) [13, 14], indicating that the cytoplasmic NE face is the preferred site of GTP hydrolysis on Ran. RanBP1 is also cytoplasmic [15, 16]. The high (though not exclusive, see below) concentration of Ran regulators on either side of the NE generates high concentrations of RanGTP and RanGDP in the nucleus and the cytoplasm, respectively (Fig. 1). The compartmentalization of Ran network components, however, is probably neither as strict, nor as absolute, as represented by simple models.

RCC1 localization may not be totally uniform, because antibodies raised to different RCC1 epitopes depicted heterogeneous fractions, some of low abundance, that have different subcellular localization, in some cases not fully overlapping with that of chromatin [17].

RanGAP1, though being largely cytoplasmic, has functional links to heterochromatin. The presence of

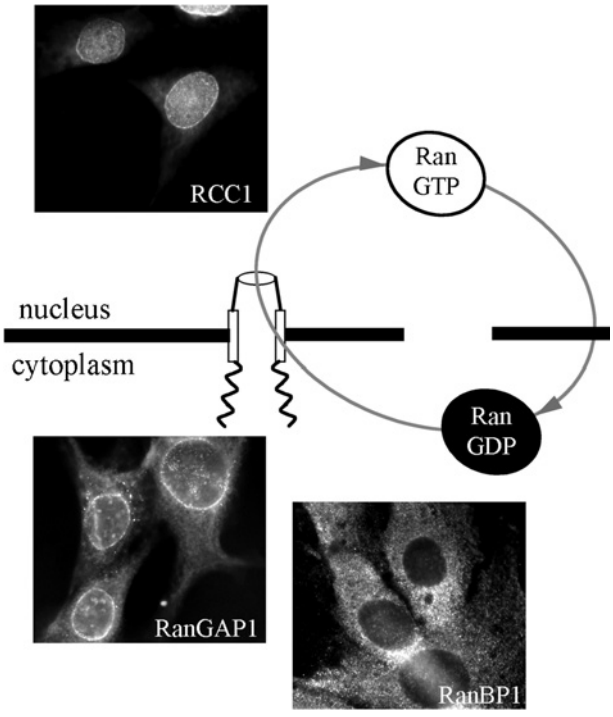

Figure 1. Interphase localization of Ran network members. The immunofluorescence panels show that the nucleotide exchange (RCC1) and hydrolyzing (RanGAP1, stimulated by RanBP1) factors for Ran largely reside on opposite sides of the NE: consequently, RanGTP and RanGDP production are compartmentalized in the nucleus and in the cytoplasm, respectively. This asymmetric distribution regulates the assembly and disassembly of transport complexes in different subcellular compartments.

nuclear export signals (NESs) in RanGAP1 implies that it can enter the nucleus but is effectively exported out. A mutant version of RanGAP1 in *Drosophila* causes the segregation distorter (SD) phenotype [18], a well-known meiotic drive system associated with failure of chromatin condensation in sperm heads. The SD phenotype manifestation depends on the simultaneous presence of a specific heterochromatic allele, called *responder* (*Rsp*). Interestingly, the SD phenotype can be rescued by increasing the amount of heterochromatin in the *Rsp* locus [19], or by overexpressing Ran or RCC1 [20]: thus, RanGAP1 acts in pathways regulating chromatin condensation (reviewed in [21]). Furthermore, in *Schizosaccharomyces pombe,* a fraction of RanGAP1 associates with both histone H3 and the H3-lysine 9 methyltransferase, which contributes to heterochromatin formation [22]. Consistently, RanGAP1 mutants show inefficient silencing of centromeric heterochromatin genes [23]. RanBP1 also harbors a NES that ensures its predominant cytoplasmic localization, but can transit through the nucleus [15, 24]. The RanBP1 fraction that is in the nucleus at any time may dynamically modulate RCC1 activity [8]. Some RanBP1 enrichment is seen around

the NE, but most of it is cytosolic and may therefore serve additional functions, independent of RanGAP1 activation at NE. Indeed, in a simulation analysis of the Ran system, Gorlich et al. [25] found that omitting RanBP1 affected only minimally the distribution of nucleotide-bound forms of Ran. This may seem to conflict with the established activity of RanBP1 in stimulating GTP hydrolysis on Ran; Gorlich et al. [25], however, pointed out that the calculated stimulation of the RanGAP1 reaction by RanBP1 applies to a preformed RanGTP/RanBP1 complex [8], within which RanBP1 exposes RanGTP to RanGAP1-mediated hydrolysis [26], whereas the RanBP1/RanGTP complex forms in fact at a slower rate than the direct RanGTP turnover by RanGAP1 [27]. Therefore, it is likely that, at any given time, RanGAP1 statistically operates on RanGTP without significant co-stimulation by RanBP1, rather than on the RanBP1/RanGTP complex. Thus, certain RanBP1 functions (*i.e.*, increasing RanGTP hydrolysis) may be overestimated, while others (*e.g.*, modulating RCC1 activity, or Ran interactions with transport factors) may not be weighted adequately.

Several more proteins can interact with Ran directly (*e.g.*, RanBP1 to RanBP16, RanBPM, Mog1, NeRCC1) and provide specific modulation in particular processes, though having no catalytic role in the Ran cycle. Among those, RanBP2 is a large protein containing four Ran-binding domains and endowed with an E3 SUMO-conjugating activity; RanBP2 is found at nuclear pore complexes (NPCs) in interphase, and its ability to SUMO-late RanGAP1 is essential to target RanGAP1 to key sites where GTP hydrolysis on Ran is required, *i.e.*, the NPCs in interphase and kinetochores (KTs) after nuclear envelope breakdown (NEB) (reviewed in [28, 29]).

RanBP3 favors the association of CRM1 (chromosome region maintenance 1), the export vector for proteins, with NES sequences in proteins that must be exported to the cytoplasm, an association that is stabilized by RanGTP (reviewed in [30]). By extension, RanBP3 may act as a co-factor in mitotic processes involving CRM1 (see below) by providing some subtle modulation of export complexes.

Nucleo-cytoplasmic transport: the founding paradigm. Given that much of our understanding of Ran comes from studies of nucleo-cytoplasmic transport (extensively reviewed in [31, 32]), here we briefly recall features that are essential to describe how Ran operates. Fundamentally, Ran regulates the assembly and disassembly of its effectors (*i.e.*, proteins interacting with RanGTP) with their partners. RanGTP effectors include transport vectors, *e.g.*, importin beta and several exportin members. In the simplest schematization, RanGTP regulates import and export complexes in opposite manners:

– RanGTP has a high affinity for importin beta, the major vector of nuclear import (also called karyopherin beta). Proteins to be transported into the nucleus (import "cargoes") are marked by nuclear localization signals (NLS). Such signals are specifically recognized by members of the importin alpha (karyopherin alpha) family, also defined as NLS receptors in some studies. Importin beta binds in turn importin alpha and stabilizes the trimeric import complex. Some NLS proteins, however, can bind importin beta directly, with no mediation by importin alpha subtypes [33]. Once the import complex enters the nucleus, RanGTP binds importin beta: this initiates the dissociation of the import complex and hence determines the release of the free NLS-protein in the nucleus. Thus, importin beta provides the RanGTP-regulatable moiety of the import complex.

– Conversely, RanGTP stabilizes export complexes, formed by different RNA classes with their specific export vectors (exportins), or by proteins carrying a NES signal with CRM1. Stabilization by RanGTP in the nucleus is required to translocate the export complex to the cytoplasm.

The ability of RanGTP to regulate cargo association with, or release from, transport vectors, underlies the localization of proteins and RNAs in the nucleus or the cytoplasm, where they can exert their productive activity. After NEB, transport factors are functionally "recycled" to perform mitotic functions, as discussed below. Studies of Ran functions often make use of Ran mutants that are either resistant to hydrolysis and mimic RanGTP (RanQ69L, RanL43E, RanG19V) or are, on the contrary, non-exchangeable (RanT24N) and therefore mimic RanGDP, or lack the domain of interaction with proteins (Ran delta 211–216). These mutants cause a vast array of phenotypic effects in different systems [2, 4]. Modeling studies [34] and yeast genetics [35] converge to indicate that Ran-dependent processes have a different scale of sensitivity to alterations in the Ran system, with interphase transport being the most "robust". Mitotic spindle assembly, instead, is highly sensitive to variations in the level or the localization of Ran regulators.

The "topology" of Ran function in mitosis: the gradient model

Data suggestive of mitotic roles of Ran have been available for some time, largely based on the observation that mutations in Ran network members, or alterations in their level of expression, synergized with tubulin mutations [36] or with the effects of micro-

tubule (MT)-targeting drugs, both in yeast and in mammalian cells [16, 37, 38].

A turning point was reached in 1999, when a role of Ran in aster assembly was demonstrated using M phase-arrested *Xenopus* egg-derived extract as a reconstitution system [39–43]: that unambiguously showed that RanGTP-mimicking mutants induced the assembly of MT asters and spindles, while RanT24N mutant was inhibitory. The process occurs in the absence of NE, and hence of nucleo-cytoplasmic transport. Two Ran-dependent factors in spindle assembly were initially identified: Gruss et al. [44] showed that RanGTP releases active TPX2 from importin alpha-containing complexes. TPX2 is an NLS-containing factor and was previously known to localize the kinesin Klp2 to poles, thereby regulating spindle pole formation [45, 46]. Nachury et al. [47] and Wiese et al. [48] showed a similar mechanism, but focused on the ability of RanGTP to release NuMA, also an NLS-containing factor and a well-known spindle pole organizer (reviewed in [49, 50]), from importin alpha/beta complexes, hence rendering NuMA competent for productive roles in spindle organization. As discussed in more detail below, the nature of NuMA as a direct regulatory target of Ran and importins was subsequently questioned [51], but these early studies established a paradigm for mitotic roles of Ran and importin molecules: they demonstrated for the first time that the same interplay between importin beta and RanGTP that ensures protein import in interphase nuclei also operates in mitosis. Prior to mitotic onset, importin alpha and beta 'sequester' NLS-containing factors required for aster assembly (collectively indicated as aster promoting activity, APA, or spindle activating factor, SAF) in interactions that render them biologically ineffective: hence, importin alpha and beta formally act as negative regulators of aster/spindle assembly. At mitotic onset, NLS-containing factors released from nuclei are engaged in inhibitory interactions with importin alpha/beta dimers, or with importin beta alone, away from RanGTP. Importin-dependent inhibition is reversed where RanGTP concentrates: by binding importin beta, RanGTP removes the inhibition over APA/SAF and initiates aster assembly. Since RCC1 binds chromatin throughout mitosis, RanGTP is generated therein. Indeed, RanGTP or RCC1 alone can substitute for chromatin in inducing aster assembly and are therefore essential elements in the process. Based on these findings, a model was formalized (schematically represented in Fig. 2), according to which RanGTP emanating from chromosomes and diluting away from them generates a gradient that acts as a positional marker at which aster assembly is initiated (see [30, 52, 53] for reviews).

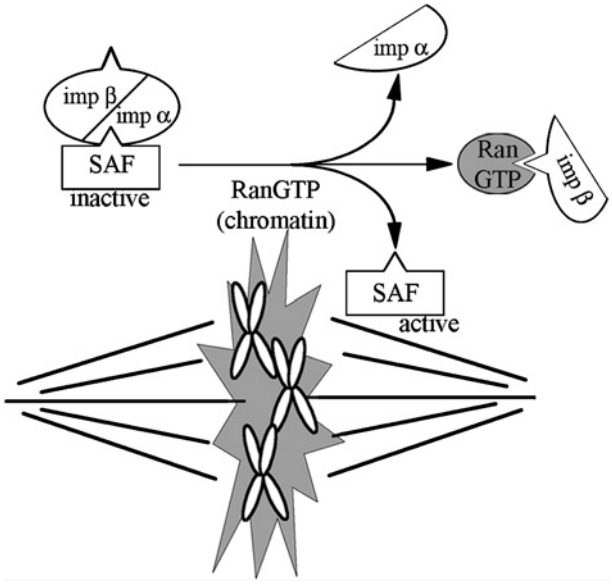

Figure 2. Schematic representation of the RanGTP-dependent spindle assembly model in amphibian oocyte systems. Distal to chromatin, importin alpha/beta dimers sequester spindle assembly factors (SAFs) in an inactive form. RanGTP concentrates around chromosomes and binds importin beta therein, hence releasing active SAFs that induce spindle assembly around chromosomes.

These observations led to the establishment of a now widely used experimental system, in which purified Ran is used with M-phase extract, instead of chromatin, to test the role of putative SAFs: this type of assay is defined the Ran-dependent, *versus* chromatin-dependent, aster assembly assay. The mitotic aster assembly model of importin-dependent repression and RanGTP-dependent reversal of repression is well suited to fulfill the requirement of a self-assembling structure. Its extension to somatic cells would serve a fundamental biological requirement, *i.e.*, the need to compartmentalize spindle activating factors away from cytoplasmic nucleation centers (centrosomes) before NEB: an attractive feature of the model, therefore, is that endowing spindle assembly factors with NLSs is sufficient to direct them in interphase nuclei and prevent premature spindle assembly. The model, however, leaves several unresolved questions for Ran-dependent mitotic control in mammalian cells:

a) Chromatin-driven spindle assembly operates in cells of the female germline, but is secondary to the centrosome-driven pathway in somatic cells (though the former can be activated under particular circumstances; see [54]). Growing evidence indicate that the Ran network operates differently in the two pathways: Ran partners, RCC1 and RanBP1, respectively induce and inhibit aster assembly in the chromatin-driven pathway, whereas they are not limiting in the centrosome-driven pathway, but modulate the spindle

morphology and functions, as seen below. In addition, targets of Ran regulation that have been extensively investigated in different systems, (*e.g.*, TPX2 and HURP) control specific, not entirely overlapping phenotypes in the two spindle assembly pathways (see below).

b) Besides spindle assembly, Ran regulates a vast array of mitotic functions (see below), which need subtle signals to operate. A "fixed" gradient with RCC1/RanGTP anchored at chromosomes would be unlikely to regulate such diversified effects if no dynamic elements were introduced in the system.

c) A thought-provoking modeling study identified size constraints for the mitotic Ran gradient to be effective [25]. The dynamics of the system was simulated based on the concentrations of Ran network components (all of which are experimentally determined in HeLa cells), describing a steep gradient of nuclear RanGTP with hydrolyzing factors in the cytoplasm. The simulation was also performed in conditions mimicking mitosis, *i.e.*, with RanGTP enrichment around chromatin under RCC1 activity, and in the absence of a NE capable of keeping RanGTP compartmentalized within the nucleus. Under these conditions the ratio of chromatin to cytoplasm size was found to be critical: a RanGTP gradient was effectively established in large cells in which chromatin represents a minor fraction of the total volume, but not in small cells such as those of somatic cell lineages. That study formalizes mathematically the intuitive notion that the RanGTP gradient operates with different efficacy in small somatic cells compared to cells that have a large cytoplasm, *i.e.*, eggs, oocytes and early embryonic cells.

d) Hydrolyzing factors do not simply diffuse homogenously in the cytoplasm, but interact with specific mitotic structures, giving rise to local enrichment and local discontinuities in the gradient (reviewed in [55]).

The Ran network in spatial and temporal control of mitosis in mammalian cells

Ran is now known to act in several temporal windows of mitosis. Thus, the initial representation of Ran mitotic control as being solely or mainly operated by antagonizing importin-dependent SAF inhibition is oversimplified to some extent, and diversification is emerging in regulatory pathways downstream of Ran control. Below we briefly recapitulate relevant mitotic processes that depend on Ran (an overview of which is schematically represented in Fig. 3) and highlight paradigmatic mechanisms through which Ran operates.

Control of centrosomal functions. Some centrosomal functions are regulated by Ran in interphase, but their consequences affect the assembly of the mitotic

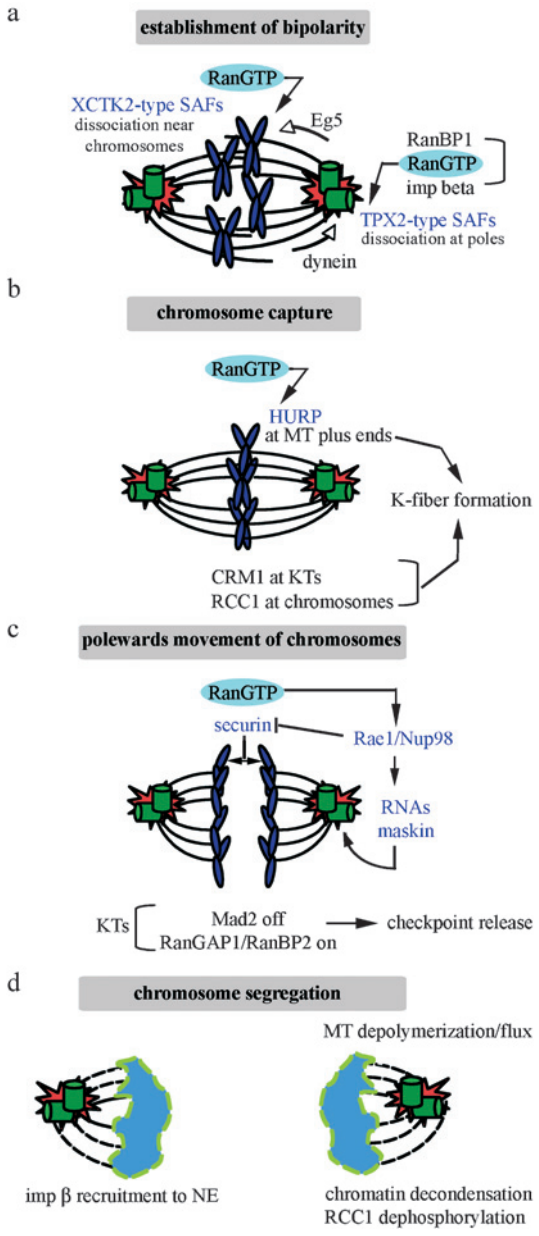

Figure 3. An overview of mitotic roles of Ran. Only some paradigmatic target factors of Ran are shown (see text for details). (*a*) In prometaphase, Ran regulates the release of SAFs at spindle poles, and of MT-stabilizing factors near chromosomes: thus MT arrays organize into a bipolar spindle. (*b*) Metaphase: chromosomal RanGTP facilitates chromosome capture by MTs. Concomitantly, RanGTP-dependent concentration of HURP at MT plus ends, RCC1 at chromosomes and localization of CRM1-dependent complexes at KTs regulate the formation of stable K fibers. RCC1 and CRM1 activities also set the schedule of checkpoint factors residency at KTs. (*c*) At the meta-to-anaphase transition, the binding of MTs to KTs triggers the release of spindle checkpoint factors and the recruitment of RanGAP1/RanBP2 at KTs: the spindle checkpoint is inactivated. Ran also regulates securin degradation through Rae1/Nup98: chromosomes are pulled polewards. Rae1 also contributes to pole maintenance via RNAs and RNA-binding factors. (*d*) In late anaphase, importins are recruited back around decondensing chromatin; RCC1 dephosphorylation may restore its ability to interact with importins and facilitate repositioning of the latter around reforming nuclei. MTs are in black; chromosomes in blue; centrosomes in green; PCM: pericentriol material, in red.

apparatus. Among those, correct centrosome duplication occurring during interphase is of crucial importance to the formation of bipolar spindles. Two independent lines of evidence implicate Ran in control of centrosome duplication: (a) CRM1 contributes to localize nucleophosmin (NPM) to centrosomes; NPM is frequently altered in human cancers [56], and one way through which it is thought to contribute to genomic stability in normal conditions is its ability to associate with newly duplicated centrosomes and prevent their reduplication within the same cell cycle. NPM harbors a NES signal and RanGTP stabilizes its interaction with CRM1: in other words, the association of NPM with centrosomes is RanGTP-dependent ([57]; for in-depth discussion see reviews in [56, 58, 59]); (b) the E1A oncoprotein disrupts control of centrosome duplication; this activity requires a physical interaction with Ran and inhibition of RCC1-mediated nucleotide exchange on Ran [60]; indeed, E1A fails to induce centrosome overduplication in the presence of mutations that abolish its interaction with Ran, or in RCC1-defective cells (reviewed in [61]).
Ran also contributes to anchor MTs to centrosomes in a complex with AKAP450, a large coiled-coil protein that acts as a scaffold for several centrosomal factors (*e.g.*, PKA, ninein, gamma-tubulin and others [62, 63]. Forced delocalization of the Ran/AKAP450 complex from centrosomes impairs the structural organization of asters [64]. Given the plethora of possible targets contacted by the complex, the identification of specific effectors has been elusive so far, but centrin is a possible target in Ran control of centrosomal organization [64].

Mitotic aster organization. As anticipated above, RanGTP-dependent aster formation was one of the earliest identified mitotic roles of Ran in the *Xenopus* extract system [39–43]. In that system, TPX2 plays a basic role in activating aster assembly ([44]; reviewed in [65]). Indeed, importin alpha can modulate MT nucleation through TPX2 [66]. TPX2 induces aster but not spindle formation, implying that other RanGTP-dependent factors are required for spindle organization in the extract system; the Aurora-A kinase has been identified as one of such necessary factors [67, 68]. In intact cells Aurora-A is a key regulator of centrosome maturation and spindle assembly (reviewed in [69]). In M phase-arrested *Xenopus* egg extract, Aurora-A-coated beads (but not beads coated with an inactive Aurora-A mutant) act as MT nucleation centers in a manner that requires RanGTP, TPX2 and gamma-tubulin [67]. Structural studies indicate that TPX2 directly activates Aurora-A function through a physical interaction that holds Aurora-A in an active conformation [70]. Molecular evidence is consistent with the structural models: indeed, the association of TPX2 with Aurora-A is increased by RanGTP in a manner that is abolished by importin alpha/beta, both in *Xenopus* egg- [71] and mitotic HeLa cell- [72] derived systems. Furthermore, importin alpha/beta inhibit TPX2-dependent phosphorylation of Aurora-A in a manner that is reversed by RanGTP [71]. A model emerges from these studies, in which RanGTP activation of spindle assembly via TPX2 requires Aurora-A, which in turn recruits and activates MT nucleation factors (given that Aurora-A-coated beads cannot assemble asters when incubated with purified tubulin [67]).
In somatic cells, TPX2 can also regulate chromatin-mediated spindle assembly under conditions in which centrosomal MTs are disassembled in the presence of nocodazole (NOC) [54]. When MTs are allowed to regrow after NOC washout, they begin their regrowth from chromatin. This process is prevented by RNA interference (RNAi) to TPX2, indicating therefore that TPX2 contributes to MT nucleation in somatic cells when the centrosome-driven pathway is destabilized and the chromatin-driven pathway is induced [54].
Under ordinary conditions in which centrosomal MT nucleation is normal, instead, TPX2 rather acts in mitotic spindle architecture. Human cells lacking TPX2 activity following RNAi do form asters, but then assemble spindles with fragmented poles [73–75]: thus, TPX2 is required for spindle pole integrity in somatic cells. A similar phenotype is caused by excess importin beta, which can be reversed by overexpression of TPX2 or of nonspecific NLS sequences [76]; this suggests that the antagonism between inhibitory interactions with importins, on the one hand, and RanGTP-dependent release of free TPX2, on the other hand, is basically conserved, but serves a different scope in spindle organization and is orchestrated according to specific spatial cues, in somatic cells and in the extract system.
In intact cells TPX2 regulates not only the functional activity of Aurora-A, but also its localization to the spindle MTs [77]. Interestingly, at least a set of mitotic abnormalities caused by excess importin beta in mitotic cells, *i.e.*, the appearance of small extrapoles, mirror those induced by lack of Aurora-A function [75]. These data are consistent with the finding that importins inhibit TPX2-dependent activation of Aurora-A function.

Control of spindle assembly factors tethered to a spindle matrix. Human lamin B1 (B3 in *Xenopus*) is a basic constituent of the nuclear lamina in the inner NE and interacts with HP1 and several chromatin-associated proteins to provide structural support to the

nucleus. Recently, RanGTP has been found to regulate a newly discovered function of lamin B1: after NEB, the latter forms a spindle-shaped "matrix" to which some spindle regulatory factors (*e.g.*, NuMA, Eg5, XMAP215) are tethered and hence recruited to MTs in a RanGTP-dependent manner [78]. Thus, one function of mitotic RanGTP is to modulate the delivery of lamin B1 matrix-bound factors that ultimately regulate MT organization and function. Lamin B1 has itself a NLS and its own organization in mitosis is dependent on RanGTP, which removes importin beta inhibition [78].

Structural organization of spindle poles and establishment of bipolarity. RanGTP increases the frequency of MT rescue (*i.e.*, the transition from MT shrinkage to MT growth) in the extract system, while neither growth nor shrinkage rates are affected [79, 80]. Given that RanGTP has no effect on purified tubulin, these experiments suggest that its ability to increase the frequency of MT rescue underlies the stabilizing effect of chromatin on MTs. RanGTP effects in the process are not restricted to MT stabilization *per se*, because MT-stabilizing drugs (*i.e.*, taxol and DMSO) activate the formation of asters but not that of spindles, whereas RanGTP addition induces spindles from taxol-stabilized asters [79]. In a study to investigate the underlying mechanisms of RanGTP-dependent spindle formation from asters, fluorescently labeled MT seeds were used [79]: the authors found that 80 % of seeds move towards minus ends of aster MTs, indicating a high prevalence of minus-end directed motor activities in the extract. When RanGTP was added, the fraction of seeds that moved towards MT plus ends doubled, indicating that RanGTP alters the balance of motors and increases plus end-directed movement. Eg5 inhibition blocked this increase, indicating that Eg5 is involved in plus end-directed movement activated by RanGTP. Ran does not regulate Eg5 directly, but experiments with labeled anti-Eg5 antibody suggest that RanGTP increases the amount of Eg5 that moves towards MT plus ends. No *in vivo* experiments have directly assessed this model. Interestingly, however, injection of RanT24N (RanGDP-like mutant) in *Drosophila* embryos results in the delocalization of KLP61F, the *Drosophila* Eg5 homologous kinesin, from the spindle MTs [81]. Together these data support the idea that RanGTP stabilizes MTs and promotes plus end-directed movement, at least in part, through Eg5. This contributes to establish spindles from asters.

Concomitant with this, factors acting at MT minus ends (*e.g.*, TPX2 and others) provide support to spindle poles, as explained above. Proteins that directly interact with Ran at spindle poles, *i.e.*, RanBP1 [82] and NeRCC1 (a spindle pole-associated protein harboring RCC1-homologous domains [83]), also contribute to the structural integrity of poles and, when expressed in deregulated manner, cause the spindle poles to fragment, often accompanied by loss of cohesion between sister centrioles in mitotic centrosomes: this gives rise to multipolar spindles. A bipolar spindle forms as a result of Ran-dependent pole organization, through Ran-interacting proteins and SAFs acting at MT minus ends, and Ran-dependent activation of plus end-directed movement through Eg5 (Fig. 4).

Regulation of MT dynamics. At the G2/M transition, cdc2-dependent phosphorylation resets the dynamic properties of MTs; most evident changes involve modulating the frequency of rescue and increasing that of catastrophe (the transition between MT growth and shrinkage). The Ran/importin system exerts direct effects on mitotic MT structure and function. As mentioned above, Ran mutants mimicking RanGTP increase the frequency of MT rescue *in vitro*, thereby modulating the stability of mitotic MTs and their ability to form a functional spindle [79, 80]. *In vivo* inhibition of RanBP1 activity by injection of specific antibody impairs MT dynamics and confers resistance to MT-depolymerizing drugs [24]. Furthermore, several factors are now identified that regulate MT stability in a Ran-dependent manner (Table 1), which are examined in more detail below.

Control of chromosome "search-and-capture" by MTs. Chromosomal RanGTP may facilitate capture of chromosomes by dynamic MTs that grow from asters. Mathematical modeling studies actually predict that the search-and-capture phase, if based only on random probing of the cytoplasm by growing MTs, would have an excessively long duration, inconsistent with the actual duration of mitosis *in vivo* [84]: this implies that positional cues aid directing MT growth towards chromosomes. Given the role of RanGTP in MT stabilization, chromosome-associated RanGTP comes to mind as the most likely candidate to provide such cues. In agreement with this prediction, injection of RanT24N impairs preferential MT growth towards chromosomes and enhances their projection in random directions in the cytoplasm in *Drosophila* syncytial embryos [81]. Although syncytial embryos represent a very specific biological situation (one large cytoplasm harboring many synchronously dividing sets of chromosomes), within which the Ran network may have evolved specific adjustments, not necessarily of general paradigmatic value, these experiments provides experimental support to the prediction that chromosomal RanGTP facilitates chromosome capture by growing MTs.

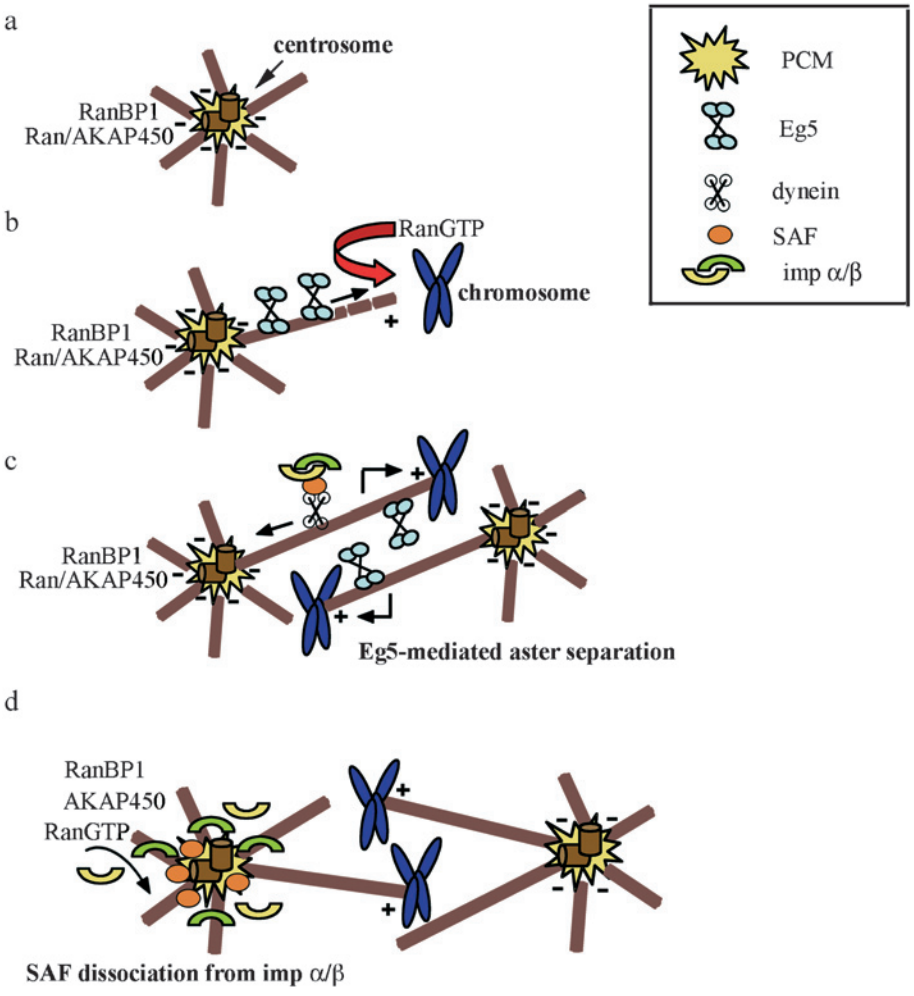

Figure 4. A model for the establishment of spindle bipolarity regulated by Ran. (*a*) Before mitosis, minus end-directed MT motors move towards the center of asters and asters show a radial symmetry. (*b*) After NEB, RanGTP increases plus end-directed movement in an Eg5-dependent manner: therefore, MTs grow preferentially towards chromosomes, where RanGTP concentrates. (*c*) During asymmetric MT growth from asters, Eg5 determines the sliding of parallel MTs and the separation of asters. Concomitantly, importins and SAFs (*e.g.*, TPX2) reach poles via the minus end-directed motor dynein. (*d*) SAFs are released from importin complexes by pole-associated RanGTP and confer structural support to spindle poles. Ran-interacting factors (*e.g.*, AKAP450, RanBP1, NeRCC1) also contribute to spindle pole integrity.

Spindle checkpoint schedule. Ran regulates the timing of residency of spindle checkpoint factors on KTs. During mitosis, the association of RCC1 with chromatin increases until anaphase onset, when the spindle checkpoint is eventually released. In addition, RCC1 overexpression – though not visibly affecting the spindle itself [85] – causes a checkpoint bypass in the presence of spindle damage [86]; this suggests that elevated RCC1 levels inactivate the checkpoint. The mechanism through which RanGTP controls checkpoint function and release is not completely clear. On the one hand, cells lacking functional RCC1, or treated with the CRM1 inhibitor leptomycin B, assemble unstable K fibers that fail to generate adequate tension on KTs [87]: this indicates that CRM1 and RanGTP (probably in complex with NES cargoes) are required for the assembly of functional K fibers capable of triggering the checkpoint release upon interaction with KTs. On the other hand, RanGAP1 and RanBP2 (both of which contain NESs, and may therefore be targeted by KT-associated CRM1/RanGTP complexes) are recruited at KTs [87–90] after MT attachment and release of checkpoint effectors (*e.g.*, Mad2, Bub1). At that point, GTP hydrolysis on Ran may interrupt locally, at the KTs level, the RCC1-dependent concentration of RanGTP on chromosomes. Thus, a self-regulatory Ran loop sets the spindle checkpoint schedule, introducing an inherent dynamic element in checkpoint control (reviewed in [29]).

M exit, nuclear reconstitution and G1 re-entry. Ran may have a role in cytokinesis, because a non-exchangeable Ran mutant impairs midbody organization and recruitment of kinesins KLP61F (homologous to Eg5) and KLP3A (a member of the kinesin-4 family) therein, resulting in defective cytokinesis [81]. The Ran network also has established roles in mitotic exit. Fission yeast mutants lacking Ran regulators develop defects at the M-to-G1 transition and eventually form small, condensed nuclei wrapped in abnormal NEs [91–93]. The NE does not disassemble during mitosis in *S. pombe*, indicating that nucleotide turn-over on Ran is critical for resetting the interphase

Table 1. Mitotic regulatory factors under direct or indirect Ran control.

SAF type	Direct mitotic targets	References	Indirectly regulated factors	References
NLS/α/β ('sequestering' mechanism)	TPX2	[44; 66]	Aurora-A	[67; 70–72; 77]
			Eg5	[74, 79]
			Aurora-A/maskin	[135]
			XRHAMM	[146]
			Xklp2	[45, 46]
			BRCA1/BARD1	[148]
	NuMA	[47; 48; 51]		
NLS/α/β (functional inhibition)	XCTK2	[133]		
	Kid	[72]		
Direct β binding	Rae1/Nup 98	[134]	NuMA	[143]
			maskin, RNAs	[134]
			APC/cdh1	[137]
	maskin	[136]		
	HURP	[68; 138–139]	XMAP215/TOG1	[68]
			Eg5	
Multiple importin-dependent inhibitory mechanisms	NuSAP	[142]		
Direct Ran interactors	NeRCC1	[82; 114]	γ-tubulin	[114]
			GCP2/Xgrip109	
			GCP3/Xgrip110	
			GCP6/Xgrip210	
	AKAP450	[64]		
CRM1 interactors	NPM	[57]		

state *in vivo*, even when NEB does not occur. Defects in nuclear reassembly after mitosis were also identified in, *C. elegans* following RNAi to components of the Ran/importin system [94, 95]. Extensive investigation of the role of Ran in nuclear and NE reformation (reviewed in [52, 96, 97]) suggest that Ran binding to chromatin is the most upstream event in nuclear reassembly and is required to promote chromatin decondensation [98, 99]. NE precursor vesicles are then recruited around chromatin. A full Ran cycle is thought to be required, because both GTP- and GDP- locked mutants behave improperly in the process [100, 101]. These results are consistent with the finding that alterations among Ran regulators that imbalance the GTPase cycle impair chromatin decondensation and nuclear reassembly *in vitro* [102], and *in vivo* in murine cells [16, 24].

NE assembly also requires importin beta [103, 104]. The mechanism of importin beta in the process is illustrated by studies of a *Drosophila* importin beta mutant, *ketel,* which is not regulatable by RanGTP. The ketel protein does not affect spindle assembly or function in syncytial embryos, but impairs NE reformation after mitosis [105, 106]. Interestingly, ketel sequesters to MTs factors required for NE reformation [107]. An analogy with the classical mechanism regulating nuclear import remains, to the extent to which a functional antagonism between RanGTP and importin beta operates; at this stage, however, RanGTP seems to act by relocalizing importin beta cargoes around reforming nuclei, rather than activating them from inhibitory interactions. Importin beta regulates NE vesicle recruitment in a manner that is reversible by RanGTP [104]. Later steps of NE reformation, *i.e.*, NPC reassembly, are still sensitive to negative regulation by importin beta, but are not reversed by RanGTP [104], implying that in this step importin beta regulates a different set of substrates in a RanGTP-insensitive manner; possibly, the interaction with nucleoporins (NUPs), basic constituents of NPCs that harbor importin beta-interacting motifs, acquires a predominant role at that stage. In synthesis, nuclear reassembly *in vitro* is initiated by Ran and progresses through steps that are differentially regulated by importin beta. We do not yet fully understand how the Ran network resets the interphase state after mitosis *in vivo.* A specific feature of the network in mammalian cells is that RanBP1 is down-regulated during telophase [16, 24]: this possibly represents the most obvious change in the Ran network and may act as a regulatory switch at mitotic exit. In addition, M-specific modifications of Ran network components and effectors must be erased to reset the interphase profiles (see below).

Imaging the Ran network in space and time: colors and complexity

Modeling and imaging studies have significantly advanced our understanding of how the Ran network operates in mitosis. The interpretation of the results,

however, is highly dependent on the type of reporter constructs that are used (see Fig. 5) and has sometimes been controversial.

Kalab et al. [108] first attempted to image the distribution of nucleotide-bound forms of Ran, mostly in the *Xenopus* system, but some living cells were also imaged for the first time. Two complementary chimeras were used in fluorescence resonance energy transfer (FRET) studies:

– Yellow/Ran-binding domain (RBD)-Cyano (YRC). The RBD is expected to interact with RanGTP, but not with RanGDP: the reporter should therefore undergo intramolecular FRET where RanGTP is low; loss of FRET monitors instead high RanGTP.

– Yellow-IBB (importin beta-binding domain from importin alpha)-Cyano (YIC): loses FRET in the presence of free importin beta, hence marking the absence of RanGTP; conversely, high FRET values are expected in the presence of RanGTP.

Experimentally, YRC shows low FRET, and YIC high FRET, around chromosomes (Fig. 5a). These results were taken to indicate an effective concentration of RanGTP around chromosomes, indicative of a steep "gradient" that pre-exists the activation of spindle assembly.

The homogeneity of that distribution, however, was called into question in subsequent work using a different set of reporter constructs. This new set of experiments was carried out in interphase cells [109], but the conclusions underscore mechanisms of general significance, including to mitotic processes, and are therefore worth recalling here. The reporter used in this study, Ran-GFP-Alexa (RGA), undergoes intramolecular FRET when Ran (either GTP- or GDP-bound) is free from interactions. Ran binding (by either RanBP1, or importin beta, or CRM1) causes loss of FRET. Indeed, high FRET occurs in the nucleus, indicating enrichment in free RanGTP. The reporter showed instead loss of FRET in the cytoplasm. Experiments using a "bipartite" sensor composed of chimeras containing Ran and RanBP1 (Fig. 5b), and characterization of the interactions, revealed significant amounts of a cytoplasmic trimeric complex containing RanGDP/RanBP1/importin beta, representing perhaps an intermediate in the recycling of import factors [110]. That interpretation suggests that diversified signaling complexes can integrate the simple functional antagonism between free RanGTP (activatory) *versus* free importin beta (inhibitory), in regulating NLS factors.

The release of NLS proteins from importins by RanGTP has recently been modeled and the concept of RanGTP-dependent spatial cues has been refined. Caudron et al. [111] represented the Ran system in a reaction-diffusion model within a spherical space. In the model, two spherical regions, corresponding to the chromatin and the cytoplasm are defined: at steady state, a steep gradient of free RanGTP is generated at chromatin. Considering that gradients extending over a long range are relevant to physiological processes, the authors sought to establish the reach of the gradient of RanGTP-importin beta interaction: the latter will determine where NLS factors are free, and hence is more important to spindle assembly than the free RanGTP gradient. The gradient of interaction extends up to the region in which RanBP1 is abundant: therein, RanBP1 binds RanGTP and stimulates hydrolysis, thus generating a further gradient (RanBP1-RanGTP-importin) that extends further away from chromatin. The model therefore predicts three concentric regions in which the availability of NLS factors is differently modulated. Two chimeras are used to image the gradients, Ran-Alexa 488 and importin beta-Cy3 (Fig. 5c). Measuring fluorescence lifetime imaging microscopy (FLIM) for Ran-Alexa 488 provides a spatial map of the lifetime of Ran, therefore allowing the actual imaging of the sites at which the interactions with importin beta occur. Indeed, maximal interactions (minimal FLIM value, high FRET) occur in the region around chromosomes, with a symmetrical radial decrease away from chromatin. Given that MT nucleation and stabilization occur at different distances from chromatin, the long-range gradient can differentially regulate these processes. Indeed, by quantifying MT nucleation and stabilization as a function of the RanGTP concentration, and relating the result to the width spanned by the RanGTP-importin beta gradient, it can be seen that MT nucleation requires high RanGTP concentration and can only occur in a restricted area around chromatin, whereas stabilization can take place in a larger surrounding area. To assess the model, sperm nuclei (carrying centrosomes) were incubated with extract devoid of TPX2 to eliminate chromosome-induced nucleation. Asters grew asymmetrically towards chromatin and eventually formed bipolar spindles capable of "capturing" sperm nuclei, though being somewhat aberrant in shape. Addition of RanGAP1/RanBP1 caused RanGTP hydrolysis and reduced the span of the RanGTP/importin beta gradient: hence, importin beta bound NLSs in a correspondingly larger volume, resulting in the loss of asymmetry of MTs growing from asters and loss of connection to chromatin. Thus, in this model, the long-range gradient of RanGTP-importin beta interaction is responsible for chromosome capture by MTs. RCC1 addition also attenuated the RanGTP/importin beta gradient, such that NLSs were released in a widespread manner in the cell, yielding spindles with severely altered structure. This study indicates that factors with specific MT-regula-

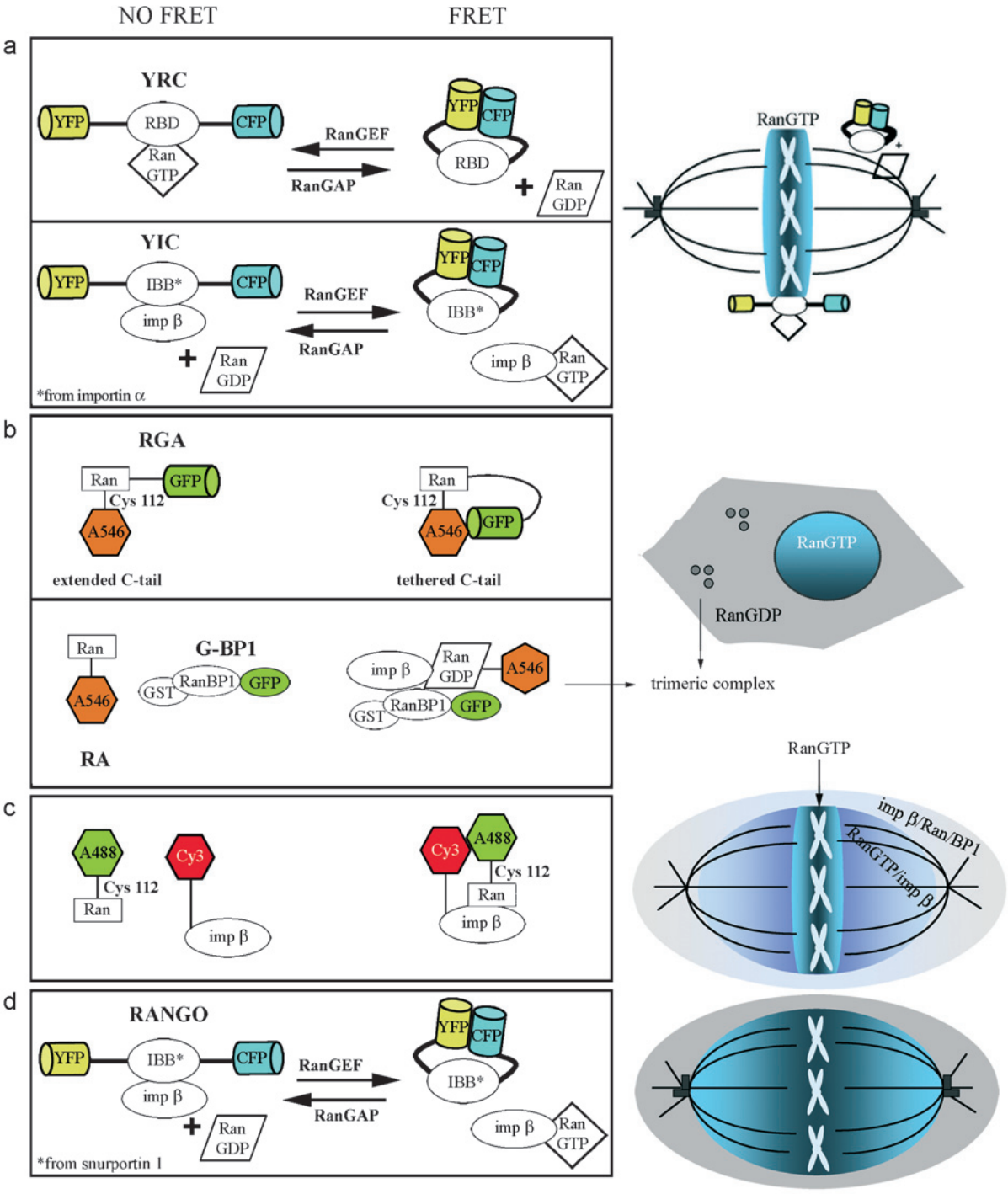

Figure 5. Imaging studies of Ran network. On the left, reporter constructs used in FRET experiments are shown; the interpretation of the results are schematized on the right (see text for details). (*a*) RanGTP enrichment (blue) around chromosomes in somatic cells [108]. (*b*) In addition to nuclear RanGTP (blue) and cytoplasmic RanGDP (gray), complex Ran interactions ("trimeric complex") can also be imaged in the cytoplasm of somatic cells [109]. (*c*) In amphibian egg-derived systems, gradients of increasing span can be imaged: free RanGTP concentrates around chromosomes (blue); RanGTP/importin beta interactions (sky blue) define a longer-range gradient; RanBP1 interaction with Ran/importin beta generates a third gradient that spans further outwards (pale blue): this defines regions in which mitotic targets will differentially operate depending on the concentration of Ran [111]. (*d*) The RanGTP gradient emanating from chromosomes and regulated by effectors extends as far as poles, and defines a "complementary" gradient of NLS factors that are differentially sensitive to the concentration of available RanGTP [112].

tory functions are activated in a defined spatial range to regulate spindle formation and shape. The model stresses the importance of the spatial distribution of free NLS proteins, rather than that of RanGTP *per se*, in determining the sites at which MTs are nucleated and stabilized, respectively. Although this may seem a subtle difference, it advances the conventional view of the Ran gradient by introducing the notion of threshold concentration; it remains to be seen to which extent the situation described by the model can realistically represent somatic cells.

A critical parameter affecting the effectiveness of any gradient is the size over which it extends. To compare Ran distribution in M phase extract and in somatic cells, Kalab et al. [112] synthesized a novel reporter, RANGO, carrying the snurportin-derived IBB (to avoid complications deriving from the dynamics of import complexes). RANGO undergoes intramolecular interactions, revealed by FRET, in the free form, *i.e.*, where importin beta is either absent or bound to RanGTP (Fig. 5d). In mitotic cells, high FRET values, indicative of free RANGO, were observed around chromosomes, as expected, and decreased towards the cell periphery. Unexpectedly, some FRET was also seen in the cytoplasm, extending as far as poles, suggesting that regions of either high RanGTP, or limiting importin beta, exist in the mitotic cytoplasm. An implication of these results is that RanGTP is not only limited to mitotic chromosomes; thus, significantly high concentrations of free importin beta cargoes exist in the cytoplasm.

The "free-RANGO" gradient was less steep (dropping over a larger distance from chromatin) in *Xenopus* extract compared to HeLa cells, though reaching spindle poles in both cases. This suggests therefore that the gradient operates differently in the two systems, consistent with mathematical modeling predictions [25]. Injecting RanGTP in increasing concentration in *Xenopus* oocytes induced an increase in free RANGO and a parallel increase in the aster number and volume; the former (free RANGO), however, plateaus at a lower RanGTP concentration compared to the induction of asters, suggesting that activities implicated in aster formation require higher RanGTP concentrations compared to RANGO-type cargoes. This again highlights the notion that distinct mitotic regulators can biologically operate under different RanGTP concentrations. Ran and importin beta mutants were then injected in pro- and metaphase cells to alter the mitotic gradient. RanQ69L induced ectopic MT asters, whereas full-length importin beta induced multipolar spindles, due to split spindle poles; both of these results were expected from published data. Interestingly, injecting importin beta mutants not regulatable by Ran, or not capable of binding cargoes, into prophase cells induced prometa/metaphase delay, associated with monopolar spindles; when metaphase cells were injected, instead, no significant effect was seen. This indicates that the free cargo gradient is necessary for the transition from radial asters to bipolar spindles in prophase, but is dispensable at later stages. These data indicate that the RanGTP gradient confers a kinetic advantage in early stages of spindle assembly in cells in which the centrosome-driven pathway is dominant. This implies that a certain degree of redundancy between spindle regulatory pathways exists in mitotic somatic cells. Another important conclusion from this study is that it formalizes the notion – intuitive up to this point – that the centrosome-driven (somatic cells) and the chromatin-driven (acentrosomal, *e.g.*, female meiotic cells) pathways differentially use the Ran/importin beta system to promote spindle assembly.

The localization of Ran network components in mitosis in mammalian cells

The results summarized above underscore the importance of imaging techniques in advancing our understanding and providing mechanistic insight into mitotic control by Ran. The imaging approach, however, has its intrinsic limitations [113]. In addition, fluorescently tagged molecules, though providing precise dynamics information, necessarily involve protein overexpression that imbalances the Ran network, hence profoundly altering mitotic progression. Furthermore, specific populations of molecules that associate with particular mitotic structures may not be revealed using this approach. Thus, it is important to back up imaging studies with the molecular characterization of endogenous Ran network members in mitotic cells. A growing body of data actually indicates that, in somatic cells, Ran operates through local "assemblies" of factors and effectors at crucial mitotic sites, embedded within the overall gradient, so as to emanate signals on local targets (spatial regulation) and produce regulatory "loops" that introduce temporal modulation.

Ran. Much of Ran associates with chromatin, partly through RCC1 and partly through direct association with histones H3 and H4 [11]. A fraction co-localizes with centrosomes throughout the cell cycle with an accumulation at spindle poles and MTs in mitosis [64]. Ran localizes to centrosomes independently of MT integrity, but depending on the interaction with AKAP450. At least part of the centrosomal Ran is GTP bound [64, 76]. Do cycles of nucleotide hydrolysis and exchange on Ran take place at centrosomes and spindle MTs? Neither RanGAP1 nor RCC1 are

found at spindle poles, but factors such as NeRCC1 [83, 114], or the centrosome-resident fraction of RanBP1 (see below) may regulate or stabilize nucleotide-bound Ran at poles. A recent study also suggests that Ran is phosphorylated by Polo-like kinase 1 (Plk1) [115]. Ran can only be exposed to Plk1 at centrosomes and at KTs: thus, Plk1-mediated phosphorylation may distinguish mitotic Ran fractions with specific functions and may open up novel perspectives in studies of mitotic roles of Ran.

RCC1. The association of RCC1 with chromatin increases during mitosis until anaphase onset [86]. During this window, RCC1 is subjected to phosphorylation by cdK1, which increases its affinity for chromatin and decreases its affinity for importins [116, 117]. By analogy with other cdK1 substrates, RCC1 likely returns to the non-phosphorylated state at anaphase, concomitant with pulling of chromosomes polewards: dephosphorylation might render RCC1 newly available to interact with importins and help recruiting the latter back around the separating chromosome sets. Interestingly, importins detach from the mitotic apparatus, and begin to accumulate around the edge of segregating chromatin, precisely at this stage [76, 118].

RanGAP1. A SUMO-lated fraction of RanGAP1 associates with MTs [13, 14] and KTs, together with RanBP2 [88]. At KTs, the RanBP2/RanGAP1 complex contributes to regulate the spindle checkpoint schedule [89, 90], though mechanistically the role of the complex is not entirely clarified. SUMO-lated RanGAP1 associates with KTs after spindle checkpoint factors are released and hydrolyzes GTP on Ran, giving rise to a Ran regulatory loop at KTs (reviewed in [29]; see below). RanGAP1 is also phosphorylated in mitosis [119]; the significance of this modification is elusive, but may confer specificity to mitotic functions of RanGAP1, because it is specifically operated by cyclin B1/cdk1, at least *in vivo*, and is reversed with a specific timing during mitotic exit. In addition to the RanGAP1/RanBP2 complex, several proteins associated with the interphase NE are also targeted to mitotic KTs after NEB (*e.g.*, [120]). At mitotic exit, these factors are recruited back at the NE with a specific temporal order [121, 122]. Entire NPC "blocks" that localized to mitotic KTs after NEB are now released and inserted back into the reforming NE [123]. Understanding the relationship between the NE and mitotic regulatory factors is currently evolving into a challenging and exciting task.

RanBP1. Unlike other Ran network components, RanBP1 is the only network member that is cell cycle-regulated in abundance, with increased levels in the G2 and M phases until telophase [16, 24]. Much of RanBP1 is found in the mitotic cytoplasm, but a fraction localizes at centrosomes throughout the cell cycle in a MT-independent manner, with an accumulation at spindle poles in mitosis, similar to Ran [82]. The abundance of RanBP1 in mitosis is critical to the structural integrity of spindle poles, because overexpression causes mitotic centrosomes to split and spindle poles to fragment; this gives rise to multipolar spindles [24, 82]. RanBP1 is also implicated in direct recruitment of certain proteins to mitotic centrosomes [124]. At mitotic exit, RanBP1 down-regulation is required for the reconstitution of interphase nuclei: forced RanBP1 expression during this window impairs the normal decondensation of mitotic chromatin [16, 24].

CRM1. After NEB, fractions of CRM1 redistribute at spindle poles and at KTs, as recently reviewed [29, 58, 59]. CRM1 acts in concert with RanGTP to regulate specific NES substrates implicated in control of centrosome duplication and in the spindle checkpoint schedule (see above).

Importin alpha and beta. After NEB, fractions of importin beta and alpha associate with mitotic MTs and accumulate at spindle poles [76]. Co-sedimentation assays indicate that the association with MTs is dynein dependent [76, 125]. Both importin beta and alpha are phosphorylated in mitosis [126]. At least for importin alpha, phosphorylation negatively modulates the association with nuclear membrane components [127]. In mitosis, the differential stability of complexes containing phospho-importin, the differential affinity of importin alpha subtypes for NLS sequences [128, 129], the relative abundance of importin alpha subtypes, and their phosphorylation state, may all contribute to confer specificity to the selection of individual SAFs that can be positively regulated by RanGTP, and perhaps of the mitotic sites at which their regulation takes place. Dephosphorylation of importins at mitotic exit may help to re-establish their interaction with NE components; in addition, down-regulation [73, 130] or modification [131] of at least some SAFs may also contribute to release importins from mitotic complexes and facilitate their recruitment around reforming nuclei.

Ran regulates mitotic factors in more than one way

The localization of Ran network components and effectors summarized above is consistent with the rapidly advancing identification of Ran-responsive factors that act at specific mitotic structures (Table 1).

Based on the sites at which they act, several functional classes can be distinguished:
– "APAs" or "SAFs" activating aster and spindle assembly;
– MAPs and kinesins regulating MT stabilization and dynamics;
– KT-associated spindle checkpoint factors.
The ability of RanGTP to dissociate importin beta from factors that interact with it through importin alpha, provides a simple and flexible way to regulate nucleo-cytoplasmic transport, as well as cell cycle transitions, and has been equated to the "yin-yang" symbol of antagonism and yet mutual dependence (reviewed in [96]). After NEB, this antagonism still operates but can follow specific, more diversified pathways. Mitotic factors can actually be grouped in 'mechanistic' classes, according to the pathway with which RanGTP regulates them:
(i) RanGTP can regulate the release of NLS-containing mitotic factors from typical importin alpha/beta complexes;
(ii) RanGTP can counteract the inhibition exerted by importin beta on mitotic factors that bind to it directly;
(iii) RanGTP can regulate, in concert with CRM1, mitotic factors that are not dependent on importin activity.
These different modes of regulation may be linked to the nature of the processes under Ran control and are discussed in some detail below.

"Yin/yang" model of importin beta and RanGTP control of NLS-containing spindle regulatory factors. As explained above, the first group of Ran-dependent factors is controlled by RanGTP removal of importin alpha/beta-dependent inhibition. Even within that group, importin beta can operate with different spatial specificity, as documented by the analysis of functional domains in Ran-responsive factors that have been dissected in detail, TPX2 [72, 132], XCTK2 [133] and kid [72].
TPX2 binds MTs and reaches spindle poles in two complementary ways: through an autonomous MT-binding domain [46] and through dynein [72, 132]. Importins alpha/beta also localize at spindle poles in a dynein-dependent manner (schematized in Fig. 6a, b); in addition, RNAi-mediated TPX2 inactivation prevents importin alpha/beta localization along MTs and accumulation at poles [76]. Thus, in mammalian cells, at least part of TPX2/importin complexes move along MTs towards poles instead of dissociating near chromosomes (Fig. 6c), suggesting that importin beta and alpha are transported along MTs via the TPX2 cargo that they negatively regulate; once there, pole-associated RanGTP binds importin beta and relieves the repression. To rationalize these observations, it should be considered that TPX2 is endowed with significant MT cross-linking activity (see [65] for review): in somatic cells it may be important, immediately after NEB, to limit TPX2 release and activity near MT plus ends and direct it towards minus ends, where its pole-organizing activity is required [73–75]. TPX2 transport in complexes with importins, and dissociation of the complexes by spindle pole-associated RanGTP, would allow that modulation. In more general terms, eluding disassembly near chromosomes enables transport of TPX2-like factors in a "neutral" state to their site of action in the mitotic apparatus, *e.g.*, poles in the case of TPX2; there, RanGTP concentrates over a restricted structure, such that dissociation of incoming importin-containing complexes now occurs. In systems that organize the spindle from chromatin, both the MT-nucleating and cross-linking activity of TPX2 may instead be immediately required to organize MT arrays. Thus, importin beta and RanGTP regulate TPX2 using different spatial mechanisms in different systems.
XCTK2 is a kinesin that binds MTs through both a motor and a non-motor (NM) domain, the latter harboring a functional NLS signal. The NM contributes to the MT-cross-linking activity of XCTK2. Given that the NM domain contains both the MT-binding and the NLS motifs, it binds either importin, or MTs, in a mutually exclusive manner [133]. RanGTP displaces importins alpha/beta from XCTK2, hence restoring MT binding ability to the NM domain. *In vitro* studies indicate that tubulin binds the NM domain with a 1:1 stoichiometry, whereas importins need be in large excess over XCTK2 to compete for MT binding [133]. These findings have important biological implications, because they indicate that the full MT-binding and cross-linking activity of XCTK2, which requires both the motor and the NM domain, is prevented where a large excess of importin beta/alpha are free from RanGTP interaction. Such inhibition, therefore, cannot operate near chromosomes, where RanGTP concentrates: therefore, complexes formed by this type of SAF are dissociated in the immediate proximity of chromosomes, *i.e.*, near MT plus ends. A similar mechanism applies to kid, a kinesin that contributes to chromosome motion to the metaphase plate: binding of kid by importin alpha/beta is mutually exclusive with MT binding and the latter is restored by RanGTP [72].
Most results summarized above were obtained using purified protein fragments and have had no real assessment in living cells, but they are important in demonstrating that RanGTP and importins regulate NLS factors in more than one way: XCTK2 and kid are regulated by RanGTP through a classical activation mechanism, *i.e.*, the displacement of a repressor

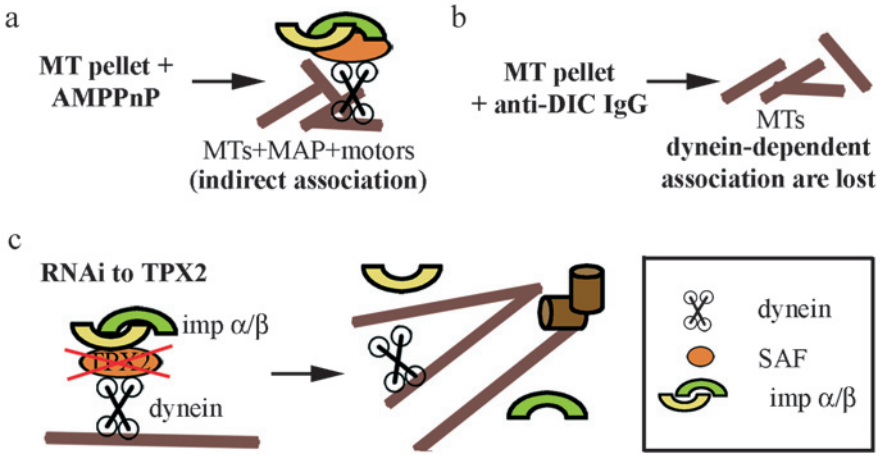

Figure 6. Proposed model for importin beta localization at poles: TPX2 carries its own inhibitor along MTs. (*a*) Schematic representation of MT co-sedimentation assays: importin alpha and beta co-sediment with polymerized MT in a manner that requires AMP-PnP (a non-hydrolyzable ATP analogue), indicating that their association with MTs is indirect. (*b*) The association is lost in the presence of anti-dynein intermediate chain (DIC) antibody, indicating that dynein activity mediates the association. (*c*) TPX2 inactivation by RNAi in living cells prevents importin localization at poles and causes spindle pole fragmentation. The data support a model in which importin beta/importin alpha/TPX2 complexes move along MTs via dynein and accumulate at poles, where RanGTP concentrates and regulates disassembly of the complex. MTs are in brown.

from a functional site, somewhat reminiscent of the operon model. For factors of TPX2 type, the antagonism between importin beta and Ran regulates spatially their availability.

Direct regulation of mitotic factors by importin beta. Some mitotic factors bind importin beta directly, with no mediation of importin alpha. Importin alpha-independent complexes regulated by importin beta may involve protein factors that share motifs with NUPs, by analogy with NE reconstitution at mitotic exit. In such complexes, novel layers of regulation may be operated by Ran partners; for example, RanBP1, besides its function as a modulator of GTP hydrolysis on Ran, may have a direct regulatory role through its ability to form intermediate complexes with importin beta and Ran [25, 109, 110].

Rae 1, a Ran-dependent factor with RNA partners and a possible dual function. Among importin alpha-independent targets of Ran, the Rae1 protein acts in several aspects of mitosis depending on the partners that bind to it. Rae1 is an mRNA export factor that was re-isolated in a search for putative spindle assembly factors under direct negative control by importin beta [134]. RNAi to Rae1 hinders mitotic progression, in HeLa cells, yielding defective spindles and misaligned/missegregating chromosomes. Rae1 binds importin beta in the absence of alpha, but does so more efficiently when complexed to the nucleoporin Nup98. Rae1 also associates with several RNA-binding proteins; among these, the translation factor maskin, notably, is also a downstream target of the TPX2/Aurora-A pathway [135, 136]. Rae1 also interacts with several RNA species. RNAs can indeed be detected around MTs and, interestingly, the ability of Rae1 to stabilize MTs in the presence of RanGTP is lost upon RNase treatment, yielding long and unfocussed MTs but no aster [134]. These findings suggest that Rae1 acts as a RanGTP-dependent vector of RNAs that have a role in aster and spindle formation. Assays in *Xenopus* oocytes, in which no transcription or translation occur, suggest that Rae1-interacting RNAs have structural roles in spindle organization, independent of their translation into proteins. Interestingly, in a *Drosophila* genome-wide RNAi screening for genes with potential mitotic roles, a large proportion turned out to encode RNA-binding or -processing factors (P. Somma and, M. Gatti, personal communication). The role of RNA in mitosis is a matter of speculation at this stage, but the finding that importin beta and Ran regulate (negatively and positively, respectively) their delivery at spindles through Rae1/Nup98 discloses a novel perspective in studies of spindle structure and function.

Rae1 has some sequence and structure homology to the checkpoint factor Bub3. In addition, a motif in Bub1 and Bub1R (called GLEBS) binds Bub3, and a homologous motif in Nup98 binds Rae1. These observations raised the possibility that Rae1/Nup98 complexes, in addition to spindle organization, also act in the spindle checkpoint. Indeed, the offspring of $Rae1^{+/-}$ mice crossed to $Nup98^{+/-}$ mice show a higher degree of aneuploidy compared to single mutants, with decreased levels of securin [137]. Therefore, Rae1/Nup98 complexes normally inhibit securin deg-

radation, and deletion of both factors overrides the spindle checkpoint. In the search for Rae1/Nup98 targets in this particular pathway, the cdh1 regulatory component of the anaphase-promoting complex (APC) was found to co-immunoprecipitate with Rae1/Nup98; furthermore, elevated levels of Rae1/Nup98 prevented APC^{cdh1}-dependent ubiquitination of securin [137]. Although it is not clear whether this function elicits a Rae1/Nup98-dependent pathway different from that associated with RNA and implicated in MT-focusing and stabilization, these results reveal novel mitotic functions downstream of Ran and exemplify a novel pathway of mitotic control by Ran that is not exerted via regulation of classical import complexes.

HURP regulates K fiber stability under direct control by importin beta and Ran. HURP was originally identified as a hepatoma up-regulated protein and was recently shown to regulate the formation of stable KT MTs by three independent groups: one group identified HURP in a search for Ran-dependent factors involved in spindle bipolarity in the *Xenopus* system [68]; another group identified HURP in a screening for G2/M-induced genes that co-vary with known mitotic genes, *e.g.*, *Plk1*, *CENPA*, *Nek2* [138]; and the third group identified HURP in a proteomic survey of human spindle-associated proteins [139]. All three groups found that HURP interacts with *in vitro* polymerized MTs and induces their bundling and stabilization; consistent with this, HURP-overexpressing cells are more resistant to MT depolymerization by NOC *in vivo*, whereas HURP-depleted cells are more sensitive [138].

In contrast to many RanGTP-dependent SAFs that are nuclear in interphase, HURP is cytoplasmic (having both NLS and NES sequences) and enters nuclei just before NEB. In mitosis, HURP co-localizes with the spindle, concentrating near chromosomes, and stains KT-associated MTs in metaphase. Immunoprecipitation (IP) assays from mitotic HeLa cells indicate that HURP interacts with importin beta directly; RanGTP-like mutant detaches HURP from importin beta, indicating therefore that the generation of free HURP is RanGTP dependent [139]. Furthermore, importin beta addition to MTs prevents their bundling by HURP: thus, the MT-bundling functions of HURP are under negative control by importin beta. All three studies converge to indicate failure of MT attachment to chromosomes and incomplete tension on KTs following RNAi-mediated HURP inactivation. These defects caused mitotic delay, but no complete arrest, because HURP-depleted cells eventually progressed to anaphase with incomplete chromosome alignment, suggesting that the spindle checkpoint was bypassed, eventually yielding chromosome missegregation. Thus, the main function of HURP is to bind and stabilize MTs in order to promote K fiber formation.

The association of HURP with MTs decreases in cells that overexpress importin beta or RanT24N (RanGDP-like) mutant, as well as in tsBN2 cells, carrying an inactive RCC1 allele. In RanQ69L (RanGTP-like) -overexpressing cells, in contrast, HURP localization along MTs is more extended, reaching to spindle poles: thus, RanGTP levels regulate HURP localization to MTs *in vivo* [139]. This suggests that chromosomal RanGTP, perhaps in cooperation with other chromatin-associated factors, regulates HURP concentration at MT plus ends towards chromosomes. In the *Xenopus* extract system, HURP is part of a large complex required for the transition from asters to spindles and including TPX2, XMAP215, Eg5 and Aurora-A [68]. Elimination of Aurora-A induced the most severely defective phenotypes in spindle formation, indicating that the complex formation and function is dependent on Aurora-A activity. HURP is a known target of phosphorylation by Aurora-A [140]. It is intriguing that Ran and importin beta regulate HURP directly, in addition to regulation through the TPX2/Aurora-A pathway. This suggests that, in addition to Aurora-A-dependent phosphorylation that may modulate protein-protein interactions, Ran has a specific and critical role in regulating the accumulation of HURP at MT plus ends and hence its MT-stabilizing function required for chromosome capture.

NuSAP, a target of many importins. NuSAP (nucleolar spindle-associated protein) exemplifies a novel paradigm in spatial regulation of mitotic processes by Ran. Some functional proprieties of NuSAP are reminiscent of those of HURP: NuSAP localizes to MTs and is enriched near chromatin, both in mitotic HeLa cells [141] and in *Xenopus* oocyte spindles [142]. Its overexpression in HeLa cells induces MT bundling and confers resistance to depolymerization by NOC, whereas RNAi-mediated inactivation causes abnormal spindle formation, with low MT density around chromatin and aberrations in all stages of mitosis. Chromosome capture and alignment are defective, but some cells proceed to anaphase with highly disorganized spindles that display defects in the midzone MTs and aberrant segregation. Cytokinesis is also aberrant and gives rise to binucleate cells; hence centrosomes missegregate, further originating multipolar cells that eventually loose viability over time [141].

Anti-NuSAP antibody addition to spindle assays *in vitro* reproduces some of the defects observed *in vivo* after RNAi. Conversely, NuSAP addition in both

'conventional' and RanGTP-dependent spindle assays induced strongly bundled MTs, indicating that NuSAP stabilizes and efficiently cross-links polymerized MTs.

NuSAP is regulated with extreme specificity: importin 7, importin beta and importin alpha can interact with it and differentially inhibit the outcome of NuSAP activity [142]. Indeed, NuSAP addition to purified tubulin produced two morphologically distinct structures: (i) aster-like structures, and (ii) MT fibers and tubulin sheets; importin beta inhibited aster, but not fiber formation; importin 7 inhibited MT fiber, but not aster formation; and importin alpha inhibited both. Thus, individual importin types suppress distinct NuSAP functions, suggesting that either distinct NuSAP domains, or the same domain in a monomeric and multimerized form, mediate the MT-stabilizing and the cross-linking activities, respectively. Inhibition of NuSAP functions was more effective when importin beta and importin 7 were simultaneously present, and even more effective when importin alpha was added, suggesting that different importin types act in additive manner in inhibiting NuSAP functions. Moreover, RanGTP differentially affects importin types: RanQ69L reversed the inhibition by importin beta effectively and that by importin 7 partially, whereas inhibition by importin alpha was not reversed. The finding that RanGTP does not reverse inhibition by all importin types equally efficiently suggests that specific interactions generate differentially stable complexes. These complexes may be sensitive to different RanGTP concentrations in the cell. If so, NUSAP would provide the first example of a single mitotic factor ensuring different temporal/spatial mitotic functions depending on the local concentration of RanGTP, consistent with theoretical predictions [111, 112].

NuMA. NuMA was one of the two earliest reported factors to be regulated by RanGTP and importin alpha/beta [47, 48] in what was thought to be the simplest version of the yin/yang paradigm. Subsequent work [51], however, suggested that NuMA regulation is more subtle than anticipated from the simple antagonism between RanGTP and importin beta. NuMA-derived fragments containing the MT-binding domain were found to bind MTs whether or not they did contain the NLS motif of interaction with importin alpha. Actually, the protein LGN (leucine-glycine-asparagine repeat-enriched protein), homologous to the *Drosophila* polarity factor PINS (partner of inscuteable), was identified as a major repressor of NuMA in aster assembly. These results were partly discrepant with those reported earlier [47, 48]. Most of the experiments in the three studies made use of purified NuMA fragments in (a) protein-protein interaction assays *in vitro*, and (b) aster assembly assays, using frog egg extract supplemented with, or depleted of, Ran or Ran-interacting proteins. The discrepancies between the three sets of data may reflect differences in the conditions and/or molar ratios between the partners tested in the *in vitro* assays: the ability of isolated NuMA fragments to organize asters and to respond to repression by negatively acting factors may vary depending on the mutual concentration of the components involved. It should be noted, however, that only Du et al. [51] designed specific deletion mutants to challenge the role of the NLS motif; they found that isolated NuMA fragments containing the MT-binding domain conserve the ability to stabilize MTs in a LGN-sensitive manner, even when they had lost the ability to bind importin alpha/beta and hence the responsiveness to Ran regulation; the ability to assemble proper asters, however, was impaired in these mutants. In the other two studies, the assayed NuMA fragments simultaneously contained the NLS and the MT-binding domain, and no deletion of the NLS motif was assayed to unambiguously establish whether it was essential. Formally, it is possible that the NLS-containing portion of NuMA cooperates with the MT-binding domain to reinforce the MT-organizing function of NuMA and, through its ability to bind importins and hence Ran, permits a further level of regulation in addition to that exerted by LGN. Another possible explanation for the discrepancy is that the experiments by Nachury et al. [47] and Wiese et al. [48], both of whom employed a depletion strategy to remove components from the extract to identify Ran-dependent APA or SAFs, depicted an ability of NuMA to induce asters in cooperation with other factors subjected to importin beta- and Ran-dependent regulation. Indeed, NuMA has now been shown to interact with Rae I [143], a genuine target of Ran regulation. In the latter work, both RNAi-dependent inactivation and overexpression experiments were carried out in somatic cells: altered amounts of NuMA were found to cause detrimental effects on spindle pole integrity, which were mitigated by simultaneous manipulation of Rae I levels [143]. Together, the data suggest that NuMA can regulate asters and spindle pole formation through multiple mechanisms, involving direct MT binding as well as interacting factors that modulate its activity; at least a subset of these functions appears to be sensitive to the concentration of RanGTP and importin alpha/beta.

RHAMM, BRCA1 and the cancer connection. Ran can interact with E1A and E7 viral oncogenes [60], suggesting that it may be targeted in processes of cell

transformation [61]. In addition, factors implicated in cancer are direct (HURP) or indirect (Aurora-A, through TPX2) Ran targets in mitotic control, as discussed above. A novel unexpected link between Ran signaling and cancer is now emerging, through the finding that BRCA1, a major tumor suppressor frequently altered in breast cancer, acts in cooperation with established Ran targets and is itself regulated by Ran.

RHAMM (receptor for hyaluronic acid-mediated motility) is a protein functionally related to TACC (transforming acidic coiled-coil) family members, with roles in cell transformation, migration and metastasis when overexpressed (reviewed in [144]). It has now been shown that RHAMM acts in Ran-dependent mitotic regulation, possibly in connection with BRCA1. RHAMM localizes to centrosomes and MTs in interphase, and to mitotic spindle MTs, with enrichment at poles, in human and *Xenopus* tissue culture cells, as well as in egg extract-induced spindles [145]. The centrosome- and spindle pole-targeting domain of RHAMM maps to a C-terminal region sharing homologies to domains present in the klp kinesin family; given the established role of TPX2 in localization of kinesin Xklp2 to poles [45], it was suggested that RHAMM, like Xklp2, is targeted to MT minus ends in association with TPX2; dynein is also required, because blocking dynein/dynactin activity prevented RHAMM localization to (unfocused) spindle poles, yielding a uniform distribution on spindle MTs [146]: thus, RHAMM can interact with MTs both directly and indirectly, through dynein, which localizes it to poles. Anti-RHAMM antibodies injection in HeLa cells yielded multipolar spindles, indicating that RHAMM contributes to maintain spindle pole integrity [145]. XRHAMM depletion blocked spindle assembly in metaphase-arrested *Xenopus* egg extract lacking centrosomes [146], and impaired pole formation when incubated with RanQ69L. XRHAMM add-back rescued spindle assembly largely yet incompletely, suggesting that other proteins operate in concert with XRHAMM. Both TPX2 and gamma-tubulin were identified as co-immunoprecipitating proteins with XRHAMM; whether these interactions are direct remains to be established, but these findings open up the interesting possibility that XRHAMM is part of a MT-nucleation complex containing gamma-TURC components and TPX2. XRHAMM depletion, though not affecting gamma-tubulin recruitment, caused TPX2 mislocalization all over MT length rather than at spindle poles. These findings together indicate a RanGTP-, TPX2- and gamma-tubulin-dependent role of XRHAMM in MT nucleation in the chromatin-driven pathway; in addition, RHAMM regulates spindle pole integrity through TPX2 localization, both in human cells and in the *Xenopus* system [145, 146].

Similar mitotic defects to those just described above are induced in the absence of BRCA1/BARD1, a heterodimeric complex with E3-ubiquitin ligase activity that acts in homologous recombination-mediated double strand break (DSB) repair, thereby contributing to maintaining genetic stability. At least a fraction of BRCA1 localizes to centrosomes [147], and a growing body of data also indicates high levels of aneuploidy and mitotic defects in BRCA1- and BARD1-deficient cells and tumors. Joukov et al. [148] have recently found that simultaneous RNAi to BRCA1/BARD1 in HeLa cells yields multipolar and disorganized spindles, lagging chromosomes and micronuclei formation. Furthermore, BRCA1/BARD1 depletion yielded unfocussed asters and spindles, in both mitotic extract and in RanGTP-dependent assembly assays. Defects were partially rescued by adding back BRCA1/BARD1 complex, but not ubiquitin-ligase defective mutants: thus, the E3 ubiquitin ligase activity of BRCA1/BARD1 contributes to RanGTP-dependent aster assembly. Interestingly, TPX2 failed to localize to the center of asters, but distributed all over MT length, in both chromatin-induced spindles with BRCA1/BARD1-depleted extract and in interfered HeLa cells. Add-back of wild-type BRCA1/BARD1, but not E3-defective mutant, rescued TPX2 localization. Many of these defects parallel those induced by RHAMM depletion. Indeed, BRCA1/BARD1 co-immunoprecipitate with XRHAMM and NuMA; BRCA1 was also found in a reciprocal IP with TPX2, suggesting that XRHAMM, TPX2, BRCA1/BARD1, and possibly NuMA, cooperate in aster assembly and spindle pole formation under Ran control. In investigating this possible cooperation, Joukov et al. [148] unexpectedly found that anti-XRHAMM antibody rescued aster defects caused by BRCA1/BARD1 depletion. This may suggest that XRHAMM is hyperactive in the absence of BRCA1/BARD1 activity, and that BRCA1/BARD1 contributes to spindle assembly by 'attenuating' excess activity of XRHAMM. Since the central and common defect in asters lacking RHAMM, or BRCA1/BARD1, is TPX2 mislocalization along MTs, a functional link emerges between the BRCA1/BARD1 and the XRHAMM/TPX2 pathways. It is possible that BRCA1/BARD1 ubiquitinates TPX2 or XRHAMM, in ways that are important to localize TPX2 at spindle poles; alternatively, BRCA1/BARD1 may ubiquitinate signaling partners that then regulate and localize TPX2/XRHAMM. Future studies will no doubt bring exciting novelties in this newly emerging aspect of mitotic control by Ran, with possible implications for breast cancer.

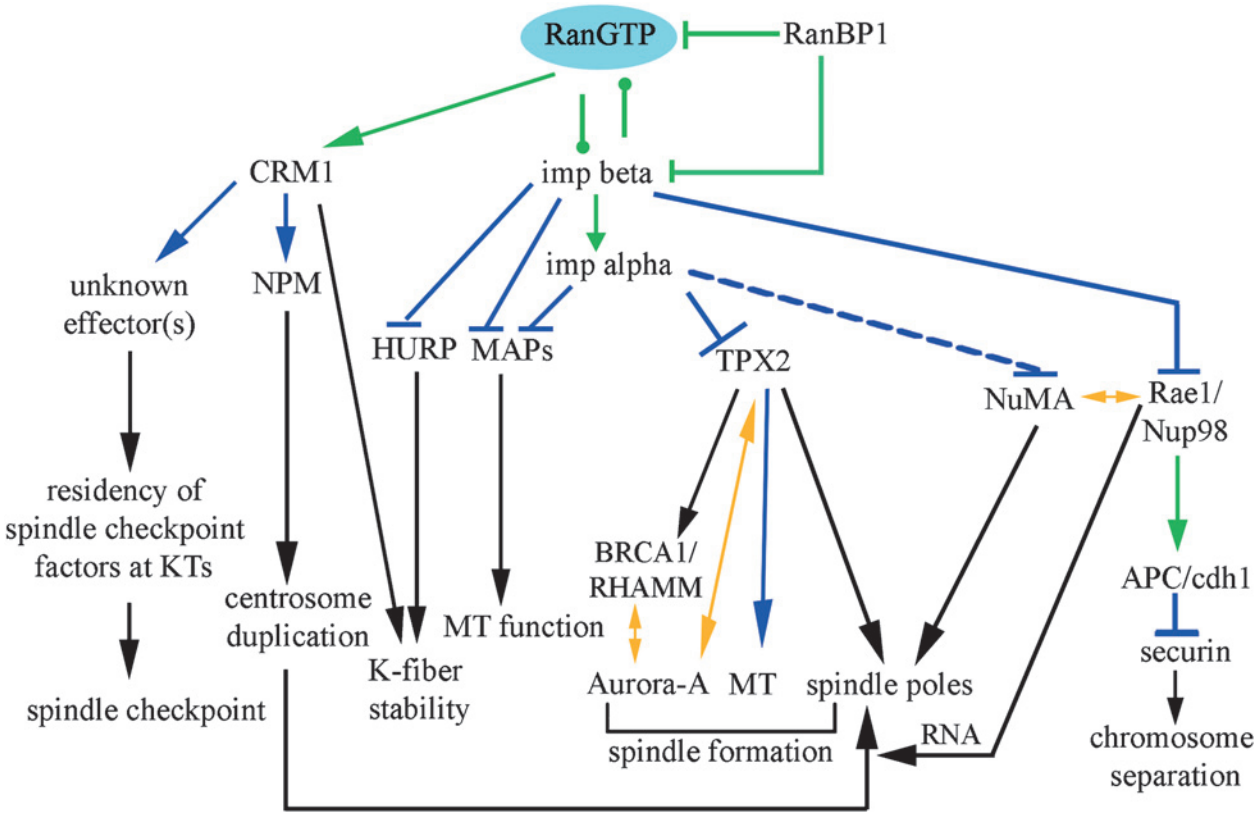

Figure 7. Ran-dependent "branched" pathways of mitotic control. RanGTP is represented upstream of mitotic processes. Green arrows indicate effectors that interact directly with RanGTP; direct targets of these effectors are indicated by blue arrows; yellow arrows indicate functionally interacting or cooperating factors; processes dependent on Ran control are arrowed in black. MAPs collectively indicate NuSAP and MT-interacting factors in general. Bars indicate repression and arrowheads activation; round-headed lines indicate the functional antagonism between importin beta and RanGTP.

Conclusions

In synthesis, mitosis is proving an arena in which RanGTP plays a mayor role. While the historical primacy of the *Xenopus* system has helped to dissect the basic biochemical mechanism(s) through which Ran operates, models derived from that system only are insufficient to account for the higher complexity and specificity emerging in mammalian cells:

a) Multiple stages of mitosis, besides spindle assembly, require spatial cues directed by Ran-dependent signals.

b) In somatic cells, the general idea that mitotic RanGTP is enriched at chromosomes and generates a gradient radiating outwards must be adjusted to accommodate the evidence that RanGTP operates under specific space constraints and follows more than one pathway. The underlying mechanisms of this control cannot be always anticipated from work with the *Xenopus* system, for both topological and functional reasons (large cells in which chromatin occupies a small volume and harbors MT-nucleation sites in the *Xenopus* system, *versus* somatic cells that are small and possess centrosomes as dominant sites of MT nucleation).

c) Besides RanGTP enrichment at chromosomes, Ran, its partners and effectors concentrate at key mitotic structures, some in a specifically modified form. This gives rise to a sophisticated picture, in which local Ran-dependent signals ensure a fine-tuning of the activity of downstream targets during mitotic progression at centrosomes, KTs and MTs.

d) The paradigm that RanGTP is activatory and RanGDP is inhibitory, originally established in studies of aster formation in the extract system, applies to cells only when considering any fixed moment in isolation; in mitotic progression, however, the introduction of elements that allow dynamic regulation in space and time is crucial. RNAi experiments in *C. elegans* indicate that the loss of either GTP exchange, or hydrolysis, on Ran cause comparable chromosome misalignment and missegregation phenotypes, whereas the lack of these activities give opposite outcomes on spindle formation in the *Xenopus* model. This is only apparently paradoxical, but indicates in fact that loading and unloading Ran at key sites is essential to ensure the dynamic control of mitotic processes in somatic

cells. A well-understood example in mammalian cells is the Ran GTPase auto-regulatory loop at KTs that regulates the spindle checkpoint schedule.

e) A hierarchy in the requirement for Ran regulators in mitosis is emerging; in, *C. elegans,* for example, RanGAP1 inactivation has stronger effects than RCC1 [95]. In mammalian mitotic cells, RanBP1 overexpression also yields more evident effects than excess RCC1 in spindle organization [55]: RanBP1 excess disrupts the spindle bipolarity, whereas RCC1 *per se* produces little effect when overexpressed, but impairs the spindle checkpoint response to MT damage. These observations are consistent with the idea that not only the availability of RanGTP, but also the dynamic modulation of its interaction with effectors in space and time, is important to regulate mitotic progression.

f) Ran regulates more factors than those harboring an NLS signal, with more complex determinants of specificity than was initially expected: (i) in NLS recognition by different importin members; (ii) in mechanisms of RanGTP control of distinct importin types; and (iii) in the sensitivity of processes to RanGTP concentrations.

g) The multiple mechanisms through which RanGTP can regulate downstream targets, and the interactions of targets with one another and with effectors, gives rise to "branched" pathways of regulation of specific mitotic steps (tentatively schematized in Fig. 7), which are only partly unraveled at this point. The integration of these pathways under Ran control ensures ordered mitotic progression.

In conclusion, local assemblies of Ran, its partners and effectors concentrate at specific sites of the mitotic apparatus, embedded in the overall gradient. These local assemblies fulfill dynamic functions as the mitotic program unfolds. Further research in the field will no doubt shed more light on diversified and subtle ways in which Ran orchestrates mitosis and on dysfunctions of the system that contribute to segregation errors and aneuploidy in the process of cell transformation. The astonishing versatility of Ran has not yet disclosed all of its features and more exciting results are still to be awaited.

Acknowledgements. Research in our laboratory is supported by the Italian Association for Cancer Research (AIRC) and MIUR-FIRB (Ministry of University and Research – Integrated funds for basic research) (grants RBIN04T7MT and RBAU01CH2M). M.C. is supported by a FIRC fellowship (Italian Foundation for Cancer Research) and, R.M. by a MIUR-FIRB research contract. We are extremely grateful to our colleagues Francesca Degrassi, Barbara Di Fiore, Patrizio Dimitri, Michael Elbaum, Maurizio Gatti, Giulia Guarguaglini, Andrea Musacchio and Patrizia Somma for many helpful discussions, comments and sharing of unpublished data.

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