

Ran on tracks – cytoplasmic roles for a nuclear regulator

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Summary

The GTPase Ran is best known for its crucial roles in the regulation of nucleocytoplasmic transport in interphase cells and in the organization of the spindle apparatus during mitosis. A flurry of recent reports has now implicated Ran in diverse cytoplasmic events, including trafficking of an ephrin receptor homolog in nematode oocytes, control of neurite outgrowth in *Drosophila* and mammalian neurons, and retrograde signaling in nerve axons after injury. Striking findings suggest that the guanine-nucleotide state of Ran can be regulated by local translation of the Ran-binding protein RanBP1 in axons, and

that an additional Ran-binding protein, RanBP10, can act as a microtubule-binding cytoplasmic guanine-nucleotide exchange factor for Ran (RanGEF) in megakaryocytes. Thus, the Ran GTPase system can act as a spatial regulator of importin-dependent transport and signaling in distal cytoplasm, and as a regulator of cytoskeletal dynamics at sites that are distant from the nucleus.

Key words: Ran, Axonal transport, Nucleocytoplasmic transport

Introduction

The GTPase Ran has fundamental roles in the regulation of transport through the nuclear pore, and in assembly and function of the mitotic spindle. The functions of Ran are strongly dependent on its guanine-nucleotide state, which is regulated by the activity of guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (which catalyze the formation of RanGTP and RanGDP, respectively) and by several Ran-binding proteins (RanBPs). Ran regulates nuclear transport in interphase cells by acting as a molecular switch for the importin (karyopherin) family of cargo transporters. RCC1, which is thought to be the only RanGEF, is restricted to the nucleus in interphase because of its high affinity for chromatin, which ensures that the GTP-bound form of Ran (RanGTP) is prevalent in the nucleus. RanGTP interacts directly with importin- β and indirectly, via CAS (the protein product of the cellular apoptosis susceptibility gene, originally implicated in apoptosis and cell proliferation; also known as exportin-2), with importin- α . By contrast, the GDP-bound form of Ran, which is prevalent in the cytoplasm, does not bind to importins, which are exported from the nucleus in association with RanGTP. In the cytosol, competitive binding of RanBP1 releases RanGTP from importins, and rebinding is prevented by RanGAP-mediated hydrolysis of Ran to the GDP-bound state. Thus, the asymmetric distribution of Ran effectors and consequently of GTP- and GDP-bound Ran provides directionality to nuclear transport (Fig. 1) (reviewed by Kalab and Heald, 2008; Poon and Jans, 2005; Rensen et al., 2008).

In addition to its role in nuclear transport, Ran is also important in mitotic-spindle assembly in cycling cells. Visualization of RanGTP using fluorescence resonance energy transfer (FRET) shows that it is highly concentrated on and around mitotic chromosomes in animal cells (Kalab et al., 2002; Kalab et al., 2006). The affinity of RCC1 for chromatin is thought to enable the formation of RanGTP-RanGDP gradients even in mitotic cells, which lack the geographical subdivision of nucleus and cytoplasm. Current models for mitotic-spindle assembly postulate that the Ran

gradient has a global role in spatial organization of the mitotic apparatus, and that RanGTP also acts locally to regulate the activity of spindle-assembly factors and other proteins (Clarke and Zhang, 2008).

Although the RanGTP gradient can potentially be modulated throughout the cell in mitosis, sequestration of RCC1 within the nuclear envelope in interphase and postmitotic cells would seem to require that processes that are dependent on active regulation of Ran be restricted to regions in close proximity to the nucleus. However, a number of recent papers have now shaken this view. In this Commentary, we review new findings on cytoplasmic roles for Ran and on new mechanisms for Ran regulation in cytoplasmic locations that are distant from the nucleus, including neuronal axons and cytoplasmic protrusions in megakaryocytes.

Cytoplasmic roles for Ran in interphase and postmitotic cells

Most of the work that has been carried out on Ran and its targets has utilized yeast, *Xenopus* oocytes or a limited range of mammalian cell lines. Neurons, in which Ran has only been studied more recently, differ from these model systems in that they are postmitotic and, in most cases, are larger and have lengthy axonal and dendritic processes that are not present in commonly used cell lines. A genome-wide RNAi screen in primary *Drosophila* neurons recently identified Ran as an important regulator of embryonic neuronal morphology (Sepp et al., 2008). Neurons from *Drosophila* embryos were incubated for 1 week in culture with dsRNAs and then inspected for different morphological phenotypes, including excessive branching, defasciculation (separation of axons from a bundle of axons known as a fascicle), axon blebbing, cell loss and reduced outgrowth. Cultures in which *RAN* was knocked down by RNAi revealed both excessive branching and defasciculation. This observation was recapitulated in mouse embryonic cortical neurons (Sepp et al., 2008), in which RNAi of *RAN* caused defects in neurite outgrowth that were indicated by the presence of processes with

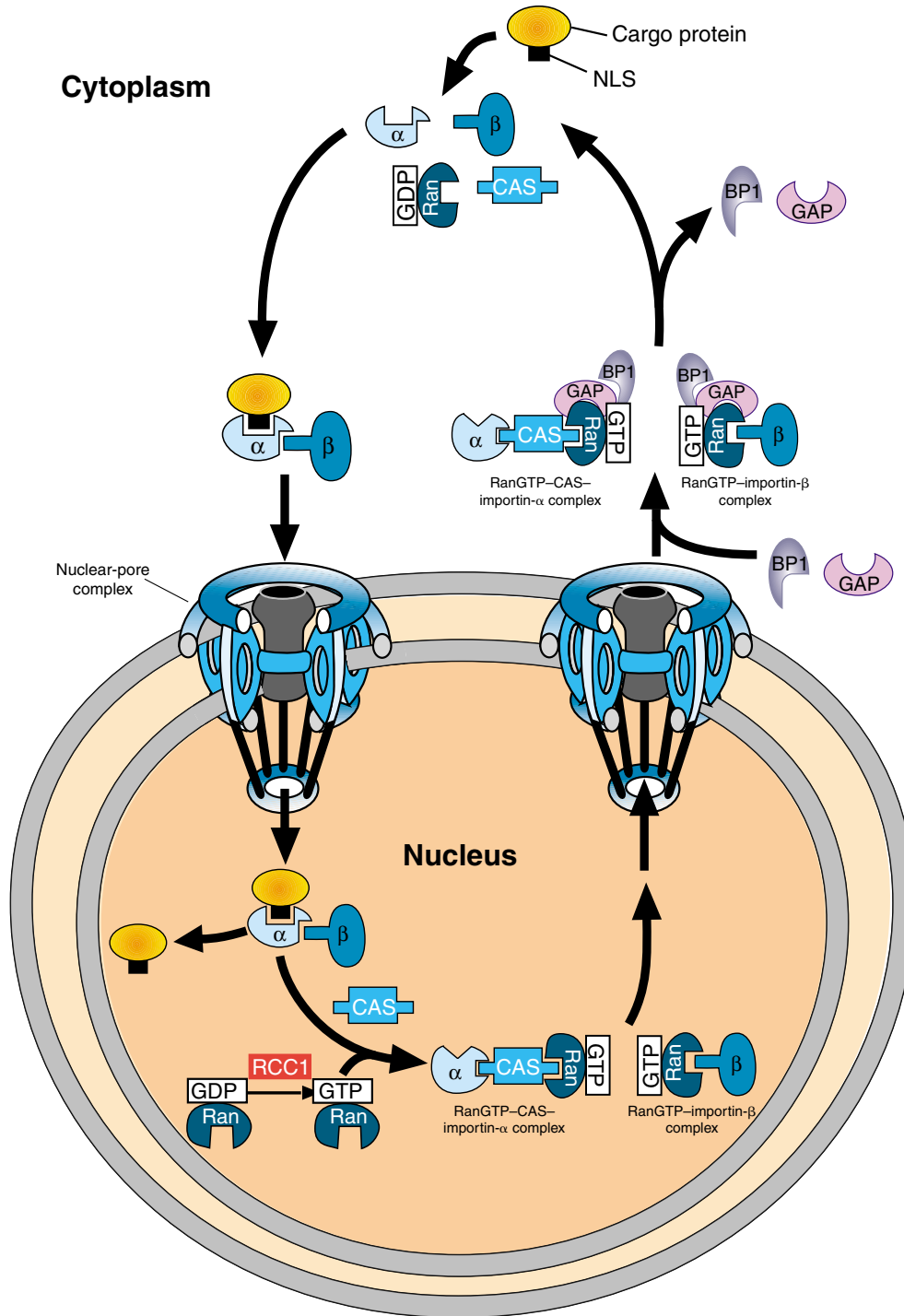


Fig. 1. Ran-regulated nucleocytoplasmic transport. The model depicts Ran-dependent regulation of nuclear import by importins. A gradient of high RanGTP in the nucleus versus high RanGDP in the cytoplasm is established by nuclear localization of the RanGEF RCC1, and by cytoplasmic localization of RanBP1 (BP1) and RanGAP (GAP), which facilitate displacement and hydrolysis of RanGTP. RanGTP exits the nucleus in a complex with either importin- β (β) or CAS and importin- α (α). In the cytoplasm, RanGTP encounters RanBP1 and RanGAP, which catalyze its dissociation from the importin complex and hydrolysis to the GDP-bound form. The importins are then free to associate with each other to form a high-affinity carrier for the import of nuclear localization signal (NLS)-containing cargo proteins to the nucleus.

abnormal blebbing (Sepp et al., 2008). In addition to the blebbing phenotype, Ran-deficient neurons showed an increase in branch arborization. The branching and blebbing phenotypes were also shown to occur *in vivo* (in explant slices). Notably, immunostaining for Ran in dissociated cortical cultures found high levels in cell nuclei, as expected, but also in processes. These results suggest an evolutionarily conserved role for Ran in the regulation of neurite extension during embryonic development (Sepp et al., 2008).

Other studies have implicated the Ran-binding protein RanBP9 (also known as RanBPM) and, by extension, Ran in cytoplasmic

signaling systems in neuronal processes, and in the regulation of neuronal outgrowth. Yeast two-hybrid screens identified RanBP9 as an interactor of the cytoplasmic domains of the neural cell-adhesion molecule L1 (Cheng et al., 2005) and the axon guidance receptor plexin A1 (Togashi et al., 2006). Overexpression of RanBP9 in mouse primary cerebellar neurons reduced neurite outgrowth that was regulated by either the L1 or plexin signaling pathways (Cheng et al., 2005; Togashi et al., 2006). Truncation or suppression of RanBP9 reduced responses to the plexin-A1 ligand semaphorin 3A (Togashi et al., 2006).

Additional examples of Ran's involvement in cytoplasmic signaling pathways were recently reported in the nematode *Caenorhabditis elegans* and in the protozoan parasite *Toxoplasma gondii*. In *C. elegans*, major sperm protein (MSP) acts as a signaling protein for oocyte meiotic maturation, and the MSP signal is, in part, transduced by the ephrin receptor homolog VAB1, which functions as a negative regulator of meiotic maturation unless activated by MSP. Endocytic transport of VAB1 is modulated by direct binding of Ran to the VAB1 intracellular domain (Cheng et al., 2008). In *T. gondii*, Ran is distributed throughout the parasite cytoplasm, rather than concentrating in and around the nucleus (Frankel and Knoll, 2008). Thus, Ran and Ran-binding proteins are directly implicated in diverse cytoplasmic signaling or trafficking events in a range of cell types, from protozoans to mammals.

Localized regulation of axonal Ran in injured neurons

The studies outlined above provide evidence that Ran has cytoplasmic functions in neurons, although they do not identify a mechanism for the specific effects of Ran on neuron growth. Other studies, however, have shown that the principal targets of Ran regulation in nuclear transport are also found in neuronal processes. In particular, importins function in neuronal processes throughout the development, functioning and repair of neuronal systems, with roles in axon guidance during development in *Drosophila* (Ting et al., 2007), in acute signaling from synapses to cell bodies in *Aplysia californica* neurons and in rodent hippocampi (Brilli et al., 2009; Lai et al., 2008; Thompson et al., 2004), and in the injury response in lesioned peripheral sensory neurons (Hanz and Fainzilber, 2006; Hanz et al., 2003; Perlson et al., 2005). Work from our laboratory demonstrated that importins are found throughout axons and dendrites at significant distances from the cell body, and that levels of importin- β are increased through local translation of axonal importin- β -encoding mRNA after lesion (Hanz et al., 2003). This leads to the formation of a complex between importin- α and importin- β that binds to nuclear-localization signal (NLS)-containing cargo proteins with high affinity; the increased level of importin- β also provides additional binding sites for proteins that interact directly with importin- β (Perlson et al., 2005). The complex is transported retrogradely from axons to cell bodies via an interaction of importin- α with the microtubule-associated motor protein dynein. The dual role of importins in retrograde transport in neuronal processes and nuclear import in cell bodies establishes this protein family as fundamental integrators and regulators of intracellular communication in neurons.

The essential roles of importins in the axonal transport of signaling cargos led us to examine the possibility that Ran regulates this process. We found Ran in both its GTP- and GDP-bound forms in axons of rat sciatic nerve at distances of 4–6 cm from neuronal cell bodies (Yudin et al., 2008). In non-injured axons, RanGTP was observed in a complex with CAS, importin- α and dynein. RanBP1 and RanGAP, which facilitate the hydrolysis of RanGTP to RanGDP (see Introduction) (Fig. 1), were expressed at low levels in uninjured axons, and their concentration in axonal cytoplasm was markedly increased upon nerve injury. This upregulation occurred through local translation of axonal *Ranbp1* mRNA, and through an as-yet-uncharacterized mechanism for RanGAP. The newly synthesized RanBP1, together with RanGAP, facilitated dissociation of Ran from the importin- α -dynein complex in axons and the hydrolysis of RanGTP to GDP (Yudin et al., 2008), allowing binding of newly translated importin- β to importin- α on dynein and thus creating a retrograde injury-signaling complex that is ready to bind to cargo.

In contrast to the classical nuclear transport model, this mechanism might require local production of RanGTP in axonal cytoplasm, perhaps by an as-yet-unidentified axonal RanGEF (Fig. 2) (see also below). In vivo perturbation of the system by introducing a dominant-negative Ran mutant or by adding blocking antibodies against Ran or RanBP1 inhibited injury responses in the targeted neuronal population. These findings establish a new role for Ran in the regulation of retrograde injury signaling in peripheral sensory neurons, and unveil a new function for Ran in the regulation of transport in the cytoplasm. Moreover, although we originally envisaged that Ran might have a role as a 'safety catch' to regulate the binding of cargo to importins on dynein (Yudin et al., 2008), it is tempting to speculate that, in other circumstances, Ran might itself act as a cargo adaptor. For example, the Ran-mediated endocytic trafficking described by Cheng et al. (Cheng et al., 2008) in nematodes might occur through interactions of Ran both with dynein complexes and endosomes.

The mechanism described above for the regulation of axonal Ran is based on the hypothesis that there is minimal hydrolysis of importin-bound RanGTP under normal conditions, when the level of axonal RanBP1 is low. RanBP1 influences Ran-RanGAP interactions by increasing the rate of association between the proteins, thus influencing the rate of hydrolysis of RanGTP to RanGDP (Seewald et al., 2003). Strict control of axonal RanBP1 levels might therefore allow localized changes in RanGTP hydrolysis. Maintaining latent stores of RanBP1 in the form of locally concentrated mRNA, and the activation of such stores by localized translation when required, provides an efficient mechanism for enabling spatially restricted changes in the nucleotide-bound state of Ran. Localization of mRNA might be more 'cost-efficient' than protein transport, as a single mRNA can be translated into protein multiple times to initiate or maintain protein activities at spatially restricted sites within a cell. Transport and localization of specific mRNAs have been described in a variety of cells and organisms, from yeast to mammalian neurons (Gerst, 2008; Wang et al., 2007).

Only a fraction of the mRNA transcripts that are expressed in neurons are targeted into dendrites or axons (Willis et al., 2005; Willis and Twiss, 2006). Such targeting is usually dependent on localization motifs in the 3' untranslated region (UTR) of specific transcripts, which are recognized by RNA-binding proteins that mediate mRNA transport. mRNA-associated complexes are shuttled as granules on microtubules or actin filaments by motor proteins of the kinesin, dynein or myosin families (Bullock, 2007). Upon arrival at the target site, the RNA granules may dissociate to allow translation or be maintained as a localized storage point of transcript. A few examples of primary sequence motifs for RNA localization have been described in the literature (e.g. Zhang et al., 2001), but localization signals are more likely to function through secondary and tertiary structural elements. For instance, cis-acting RNA elements might encode stable secondary structures that allow recognition and docking of trans-acting RNA-binding proteins. Such structural localization motifs are very heterogeneous in their nature and complexity, and, hence, are difficult to predict. Indeed, the 3' UTR region that contains axonal localization information in RanBP1 does not contain any previously characterized localization motifs at the level of the primary sequence (Yudin et al., 2008). Intriguingly, local axonal translation of both RanBP1 and importin- β is Ca²⁺-sensitive (Yudin et al., 2008), which might indicate co-regulation of these and other transcripts that are required for the injury response.

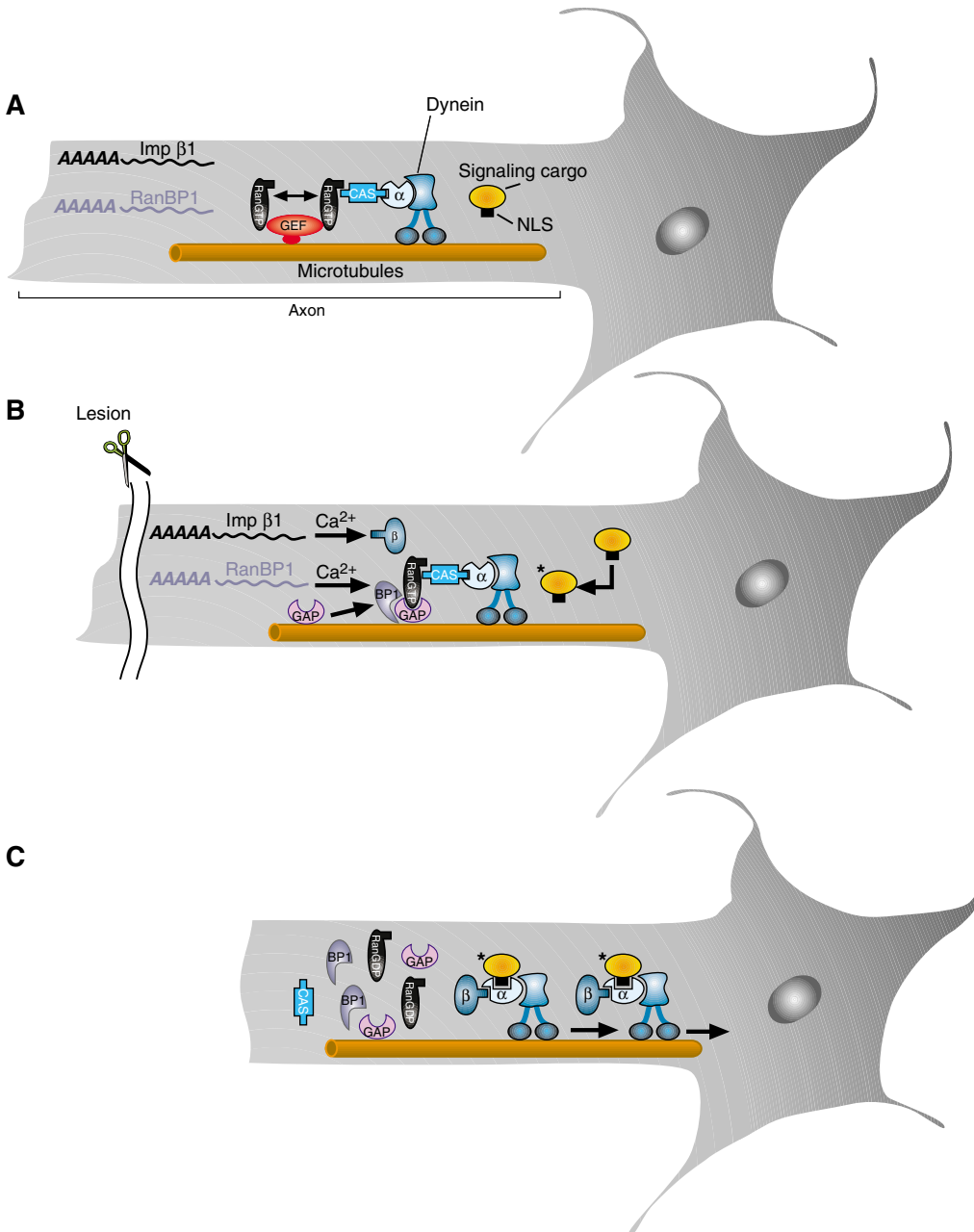


Fig. 2. Temporal or transient regulation of the GTP-bound state of Ran controls axonal retrograde signaling in response to injury. (A) Under normal conditions, RanGTP might be generated by a microtubule-bound RanGEF (GEF; see text) and, when bound to axonal CAS and importins, might prevent interaction between importin- α and importin- β , as well as the binding of cargo proteins to importins. mRNAs encoding importin β 1 (Imp β 1) and RanBP1 are found in the axon. (B) Following lesion, Ca^{2+} -dependent localized translation of these mRNAs leads to increased levels of the corresponding proteins, which is thought to occur concomitantly with activation (*) of signaling cargos containing NLS. (C) The newly synthesized RanBP1 (BP1) stimulates both dissociation of RanGTP from the CAS–importin- α –dynein complex and RanGAP (GAP)-synergized hydrolysis of RanGTP, thus allowing formation of a cargo-binding complex of importin- α with de-novo-synthesized importin- β . Updated and modified from Yudin et al. (Yudin et al., 2008), and reprinted with permission.

Regulation of Ran state and cytoskeleton dynamics by a novel cytoplasmic RanGEF

The presence of RanGTP in the axonal cytoplasm in the sciatic nerve, together with the fact that the sole known RanGEF, RCC1, is thought to be restricted to the nucleus, suggests that axons might contain a hitherto unrecognized RanGEF to activate Ran. A number of cytoplasmic proteins that contain RCC1-like domains have been described, such as the multidomain GEF protein alsin (ALS2) (Hadano et al., 2007), and there is some evidence that splice variants of RCC1 can be found at low levels in the cytoplasm under certain conditions (Hood and Clarke, 2007). So far, RanGEF activity has not been established for any of these cytoplasmic RCC1-related molecules. Strikingly, however, a cytoplasmic RanGEF candidate, RanBP10, was recently identified on the basis of its homology to RhoGEFs, rather than to RCC1 (Schulze et al., 2008).

Schulze et al. (Schulze et al., 2008) set out to study the dynamics of microtubules in megakaryocytes, which are large polyploid blood cells that – upon maturation – generate nascent blood platelets within microtubule-based cytoplasmic extensions. This process requires significant mobilization of microtubules that are enriched in β 1-tubulin, the form that is required for efficient thrombopoiesis in the cell periphery. RanBP10 was implicated as a β 1-tubulin interactor in a yeast two-hybrid screen, and this was confirmed biochemically using both megakaryocyte lysates and purified proteins *in vitro*. Furthermore, RanBP10 was demonstrated to be capable of binding to both endogenous Ran and β -tubulin simultaneously in HEK 293 cells. Finally, immunocytochemistry showed that RanBP10 associated with microtubules in megakaryocytes (Fig. 3).

Close examination revealed similarity between a region of the RanBP10 amino-acid sequence and a consensus RhoGEF domain

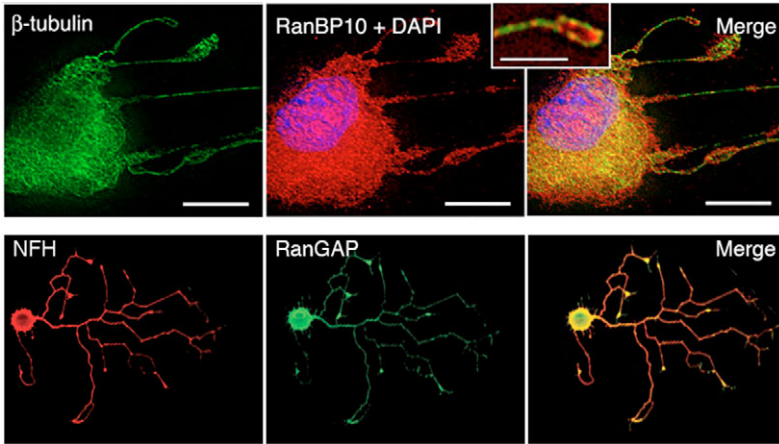


Fig. 3. Subcellular localization of cytoplasmic RanGEF and RanGAP. The upper panels show localization of the putative GEF RanBP10 on microtubules in megakaryocytes [reprinted with permission from Schulze et al. (Schulze et al., 2008)], and the lower panels show localization and concentration of RanGAP at the tips of growing sensory-neuron axons [reprinted with permission from Yudin et al. (Yudin et al., 2008)]. The juxtaposition of these images (albeit from different cell types) suggests that, if both molecules (or their close homologs) were coexpressed, it might be possible to create localized Ran gradients in the cytoplasm of morphologically complex cells by restricting the amount of GEF to microtubules and that of GAP to other subcellular compartments. NFH, neurofilament heavy chain (a neuronal marker). Scale bars: 15 μm (upper panels; note that the lower panels are not on the same scale).

(Fig. 4). A recombinant N-terminal domain of RanBP10 exhibited Ran nucleotide-exchange activity, albeit at a rate that was an order of magnitude lower than that observed for RCC1 in the same assay. A point mutation in the candidate GEF domain of RanBP10 abolished nucleotide-exchange activity and reduced binding to Ran. Finally, shRNA-induced depletion of RanBP10 in megakaryocytes caused disruption of the microtubule cytoskeleton. Thus, the results of Schulze et al. (Schulze et al., 2008) suggest that spatiotemporally restricted generation of RanGTP by RanBP10 on the cytoplasmic microtubule cytoskeleton might influence microtubule organization and cytoskeletal dynamics. Notably, we observed concentrates of RanGAP at the growing axon tip in sensory neurons in culture (Yudin et al., 2008). It is tempting to compare these different observations from the megakaryocyte and axonal models (Fig. 3),

and to speculate that the RanGEF activity of microtubule-bound RanBP10 or related molecules might antagonize RanGAP activity in other cytoplasmic microdomains, creating localized variation in RanGTP levels and thereby modulating cytoskeleton growth.

RanBP10 is closely related to RanBP9, which – as discussed above – has been implicated in cytoplasmic signaling through a neural cell-adhesion molecule and an axon-guidance receptor in neurites (Cheng et al., 2005; Togashi et al., 2006). The candidate GEF domains of RanBP9 and RanBP10 are highly conserved (Fig. 4), which suggests that RanBP9 might also harbor RanGEF activity. Moreover, the fact that RanBP9 is linked to axon-guidance receptors on the one hand (Togashi et al., 2006) and to microtubules on the other (Nakamura et al., 1998) suggests that both of these Ran-binding molecules might directly transduce guidance signaling to cause cytoskeletal responses.

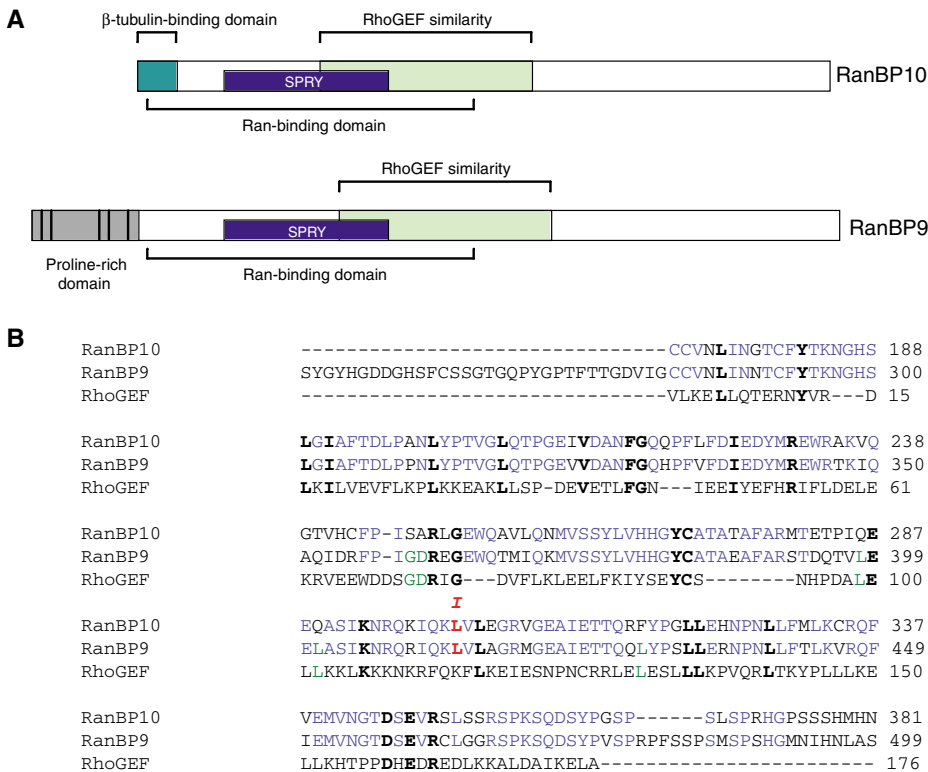


Fig. 4. Is RanBP10 the only cytoplasmic RanGEF? Schematic domain comparisons (A) and sequence alignment (B) of the putative GEF domains suggest that RanBP9 is also a likely RanGEF candidate. In panel B, residues in bold black font are identical in both Ran-binding proteins and a consensus RhoGEF sequence, and residues in blue are identical in RanBP10 and RanBP9. Mutation of the leucine residue shown in red to isoleucine was shown by Schulze et al. (Schulze et al., 2008) to attenuate RanGEF activity of RanBP10.

Conclusions and perspectives

The Ran field has been dominated by the notion of a spatial gradient of nuclear RanGTP versus cytoplasmic RanGDP, which is maintained by separation of the GEF and GAP activities that regulate Ran between nuclear (or, in mitotic cells, chromatin-bound) and cytoplasmic compartments, respectively (Kalab and Heald, 2008; Rensen et al., 2008). The identification of neuronal-process-localized mRNA transcripts encoding importin- β 1 and RanBP1 (Hanz et al., 2003; Yudin et al., 2008) indicates a potential new mechanism for regulating the GTP state of Ran in a temporal or transient manner. Local translation of axonal and dendritic mRNAs is proving to be of increasing importance for rapid and specific modulation of many aspects of neuronal physiology (Wang et al., 2007). Indeed, subcellular targeting and localized translation of mRNA is emerging as a widespread phenomenon in eukaryotic cells in general (Lecuyer et al., 2007). It will therefore be crucial to identify and characterize the localization motifs in mRNA transcripts encoding RanBP1 and importin- β 1, and to characterize the motor and adaptor proteins that are involved in their transport. The identification of localization motifs will open the door to specific knockdown or perturbation of the localized transcripts, and will potentially allow the examination of cytoplasmic roles of the Ran system without perturbing nuclear or mitotic mechanisms.

Some of the most intriguing questions arising from the new data are whether additional RanGEFs apart from RanBP10 remain to be discovered, and how significant (quantitatively and physiologically) these putative RanGEFs are likely to be. Before addressing these issues, however, it will first be important to confirm the GEF activity of RanBP10 in additional assays and biological systems. Schulze et al. (Schulze et al., 2008) used an *in vitro* FRET assay to monitor nucleotide-exchange activity of recombinant RanBP10, and it would be useful to see this activity quantified in the direct GEF assays that are commonly used for RCC1 (Bischoff and Ponstingl, 1995). Moreover, the level of Ran nucleotide-exchange activity observed for RanBP10 was an order of magnitude less than that reported for RCC1 (Schulze et al., 2008); it will be interesting to determine whether this is because RanBP10 activity is dependent on specific conditions or cofactors. Does RanBP9 also harbor exchange activity for Ran, as is implied by its sequence similarity to RanBP10 (Fig. 4)? Are additional RhoGEF-related molecules likely to do 'double duty' as RanGEFs? Given the diversity and promiscuity of RhoGEF-mediated signaling (Bos et al., 2007; Schiller, 2006), a positive answer to the latter question might shift the Ran field to an entirely new level of complexity. Clearly, it will be important to characterize in detail the expression of RanBP10 and other candidate RanGEFs in different cell types and tissues, and to follow up on such studies with transgenic and knockdown approaches to determine the physiological significance of the putative new RanGEFs relative to RCC1.

Finally, we should note that the findings highlighted above have emerged from highly specialized cell types – neurons and megakaryocytes. Neurons are among the largest and most morphologically complex cells known, and megakaryocytes have a unique life cycle that culminates in an elaborate wave of proplatelet formation concomitant with compression and eventual degradation of the nucleus (Patel et al., 2005). Cytoplasmic Ran regulation in these cell types might reflect an increased need for specialized regulatory or transport systems in large or specialized cells, as reported by Kaltschmidt and colleagues for NF κ B transport in neurons versus non-neuronal cells (Mikenberg et al., 2006; Mikenberg et al., 2007). Alternatively, the findings of our group

(Yudin et al., 2008) and of Schulze et al. (Schulze et al., 2008) might simply reflect the fact that it is easier to make such observations in cells that extend cytoplasmic protrusions a large distance from the nucleus. Nonetheless, the original observations on the cytoplasmic roles of importins in neuronal axons were quickly followed by reports that similar mechanisms are at work in HeLa cells and *Xenopus* oocytes (Mesika et al., 2005; Salman et al., 2005). We therefore expect that regulation of the guanine-nucleotide state of Ran in the cytoplasm will prove to be important in many eukaryotic cell types and tissues, and look forward to future work that will address these issues.

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