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Importin Beta: Conducting a Much Larger Cellular Symphony

Review

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Importin β, once thought to be exclusively a nuclear transport receptor, is emerging as a global regulator of diverse cellular functions. Importin β acts positively in multiple interphase roles: in nuclear import, as a chaperone for highly charged nuclear proteins, and as a potential motor adaptor for movement along microtubules. In contrast, importin β plays a negative regulatory role in mitotic spindle assembly, centrosome dynamics, nuclear membrane formation, and nuclear pore assembly. In most of these, importin β is counteracted by its regulator, Ran-GTP. In light of this, the recent discovery of Ran's involvement in spindle checkpoint control suggested a potential new arena for importin β action, although it is also possible that one of importin β's relatives, the karyopherin family of proteins, manages this checkpoint. Lastly, importin β plays a role in transducing damage signals from the axons of injured neurons back to the cell body.

When scientists coined the name "importin" for the first nuclear transport receptor (Gorlich et al., 1994), some readers were put off by a name which sounded a bit too "important." Now it appears a prescient choice, as new roles for the prototype nuclear import receptor α and β emerge.

Nuclear Import: What Did They Know and When Did They Know It?

To set the stage for understanding importin β's new roles, a review of its central role in nuclear import helps greatly. Virtually all communication between the nucleus and cytoplasm occurs through the massive macromolecular structure perforating the double nuclear membranes, the nuclear pore. With the advent of in vitro nuclear import assays, three essential proteins were discovered to mediate nuclear import: importin α, which recognizes cargo proteins with a nuclear localization signal (NLS); importin β, which binds and ferries the complex into the nucleus; and the small regulatory GTPase Ran. Specifically, when the α/β/NLS cargo complex reaches the far side of the pore, nuclear Ran-GTP binds to importin β and dissociates the complex, completing import (Figure 1A). Genetic studies in yeast were instrumental in identifying homologous transport receptors that act in an identical manner. Mechanistically speaking, the importin α/β/NLS cargo complex passes through the pore by binding to successive key nucleoporins containing phenylalanine-glycine or FG repeats (Adam and Adam, 1994; Radu et al., 1995; Gorlich et al., 1995a; Hurt, 1996; Wozniak et al., 1998; Gorlich and Kutay, 1999; Damelin and Silver, 2000; Siebrasse and Peters, 2002; Suntharalingam and Wente, 2003; Fahrenkrog et al., 2004).

The comforting thought that nuclear import was always the result of importin α recognizing a short positively charged or "classical" NLS, importin β mediating interaction with the pore, and Ran-GTP completing import, was soon dispelled by the finding of a new type of NLS and another receptor in both vertebrates and yeast. In vertebrates, the new NLS, found in hnRNP A1, was longer, glycine-rich, and not at all positively charged. Its receptor, termed "transportin" by authors emboldened by the previous receptor, turned out to be a relative of importin β but, surprisingly, required no importin α-like adaptor subunit (Pollard et al., 1996; Aitchison et al., 1996). Furthermore, it was discovered that even importin β can bind certain cargoes on its own without an adaptor (Gorlich and Kutay, 1999). Already the rules were being broken.

Before long, importin β had more relatives and adaptors than could be accommodated by even the most creative wordsmith (snurportin being the possible highlight of this effort) (Gorlich et al., 1997; Huber et al., 1998). The importin β superfamily of nuclear transport receptors, also termed the "karyopherin" family, now includes 14 members in yeast and more than 20 in humans (Table 1) (for reviews, see Gorlich and Kutay, 1999; Macara, 2001; Fried and Kutay, 2003; Damelin et al., 2002; Mosammaparast and Pemberton, 2004; elegant yeast studies are covered in more detail in the latter two reviews). All have in common an N-terminal Ran binding domain. Many direct the import of various cargo but, surprisingly, others direct nuclear export.

The first identified export receptor, exportin 1 or Crm1, recognized the now canonical leucine-rich nuclear export signal (NES) found in many proteins from yeast to vertebrates (Fornerod et al., 1997a; Stade et al., 1997). These include everything from shuttling transcription factors to the viral proteins HIV Rev and HTLV Rex (Gorlich and Kutay, 1999; Mosammaparast and Pemberton, 2004). Indeed, the general rule appears to be that a protein that shuttles between nucleus and cytoplasm usually contains an NLS that binds to an importin and an NES that binds to an exportin. Other receptor assignments abound: exportin-t ferries newly synthesized tRNA out of the nucleus, while exportin 6 banishes profilin/actin complexes from the nucleus (Table 1) (Fried and Kutay, 2003; Stuven et al., 2003).

There are exceptions to this importin β family-oriented program. Messenger RNA appears to use an entirely different dimer of proteins to exit the nucleus (TAP/NXF1/Mex67 and p15/NXT/ Mtr2; Cole, 2001; Reed and Hurt, 2002; Cullen, 2003; Stutz and Izaurralde, 2003; Weis, 2003). Even individual proteins can break the rules: for example, β catenin and HIV Vpr interact directly with the nuclear pore during their passage (Gorlich and Ku-

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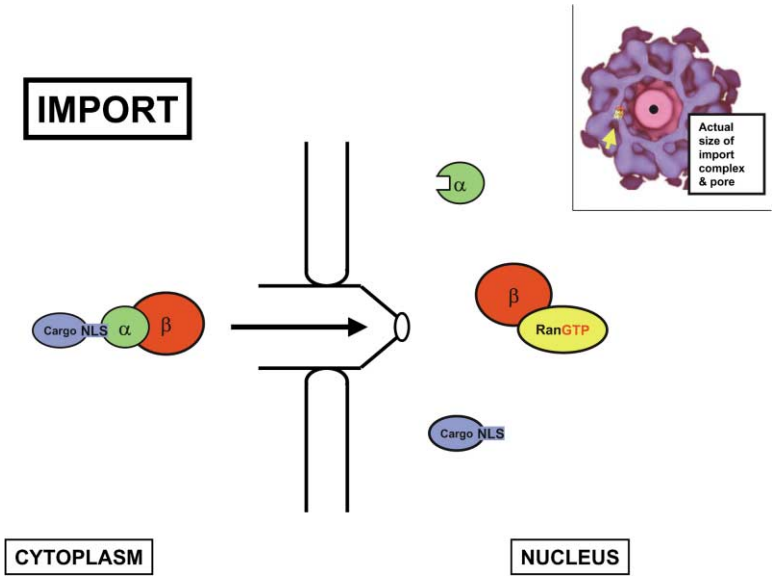

Figure 1. Nuclear Import

In the first discovered NLS import pathway, a "classical" positively charged NLS present on a protein cargo is recognized by importin α, which in turn binds to importin β. This import complex translocates through the nuclear pore (not drawn to scale; see text for models). Upon reaching the nucleoplasmic side of the pore, Ran GTP binds to importin β, causing β to dissociate from importin α, which in turn dissociates from the NLS cargo. The inset shows the actual scale of the import complex (α/β/NLS cargo; marked by yellow arrowhead) compared to the nuclear pore. The pore structure is taken from Yang et al. (1998). The blue portion represents the scaffold of the nuclear pore, whereas the pink represents the central transporter region. For comparison, a black circle has been inserted by us to indicate the approximate size of the diffusional channel in the nuclear pore (90 Å), if centrally located. However, the pore can expand its functional capacity for translocation up to ~390 Å when a cargo is bound to an importin β family member receptor.

tay, 1999; Yokoya et al., 1999; Mosammaparast and Pemberton, 2004). It is safe to say, however, that almost all known nuclear import and export pathways are mediated by importin β family members.

How can importins and exportins carry cargo in different directions? The answer lies in the existence of a Ran-GTP/GDP gradient, which acts to establish a clear distinction between the nuclear (RanGTP) and cytoplasmic (RanGDP) compartments (Melchior et al., 1993; Moore and Blobel, 1993; for review, see Macara, 2001; Damelin et al., 2002; Dasso, 2002; Smith et al., 2002; Steggerda and Paschal, 2002; Gorlich et al., 2003; Weis, 2003). This gradient, which may actually resemble a steep step, can be visualized by fluorescent sensors (Kalab et al., 2002). The gradient results from the following: the Ran GTP-exchange factor, RCC1, is almost exclusively localized to chromatin (Figure 2). Equally important, RanGAP, the RanGTPase-activating protein, is localized to the external face of the pore and the cytoplasm.

As described above, import receptor/cargo complexes are disrupted by RanGTP. In contrast, export receptor/cargo complexes are stabilized by RanGTP and do not generally form in Ran's absence. Once exportin/cargo/RanGTP complexes reach the cytoplasm, hydrolysis of GTP to GDP leads to complex disassembly and the completion of export (Figure 3). The satisfying simplicity of this world view, with importins and exportins moving between nucleus and cytoplasm in a carefully Ran-choreographed ballet, explains the basics of nuclear transport. Modifications of this basic plan add interest and evolutionary diversity (Gorlich and Kutay, 1999; Fried and Kutay, 2003), but the majority of transport follows these simple rules.

Importin β Structure: The Key to Its Versatility

In nuclear import, the original importin β continues to be the prima ballerina of the group, perhaps not surprising at an estimated concentration of 3 μM in the cell (Kutay et al., 1997). Importin β consorts with one of up

Table 1. Examples of the Importin β Family Members[a]

Vertebrate Transport Receptor	Function/Cargo	Yeast Homologs
Importin β	Import of ribosomal proteins, HIV Rev, HIV Tat, HTLV Rex, SREBP-2, PTHrP, cyclin B1, Smad proteins, T-cell protein tyrosine phosphatase	Kap95
Importin β/Adaptor Complexes		
Importin β/Importin α	Import of classical NLS-containing proteins	Kap95/Kap60
Importin β/snurportin 1	Import of m_3G capped U snRNPs	
Importin β/XRIPα	Import of Replication Protein A	
Importin β/Importin 7	Import of histone H1, suppression of aggregation	
Transportin 1	Import of hnRNP-A1, -A2, -F, ribosomal proteins, TAP, histones	Kap104
Transportin SR1 and SR2	Import of proteins with SR domains	Mtr10 (Kap111)
Importin 5	Import of ribosomal proteins, histones	Pse1 (Kap121)
Importin 7 (RanBP7)	Import of ribosomal proteins	Nmd5 (Kap119)
Crm1	Export of NESs-containing proteins, m_7G capped U snRNAs, snurportin1	Crm1 (Kap124, Xpo1)
Exportin-t	Export of tRNA	Los1 (Kap127)
CAS	Export of importin α	Cse1 (Kap109)
Exportin 5	Export of precursors of miRNA, tRNA, and others	Msn5 (Kap142)
Exportin 6	Export of profilin/actin	

[a] Derived from Gorlich and Kutay (1999), Strom and Weis (2001), Chook and Blobel (2001), Kim (2004), Lund et al (2004), Mosammaparast and Pemberton (2004), and references in text.

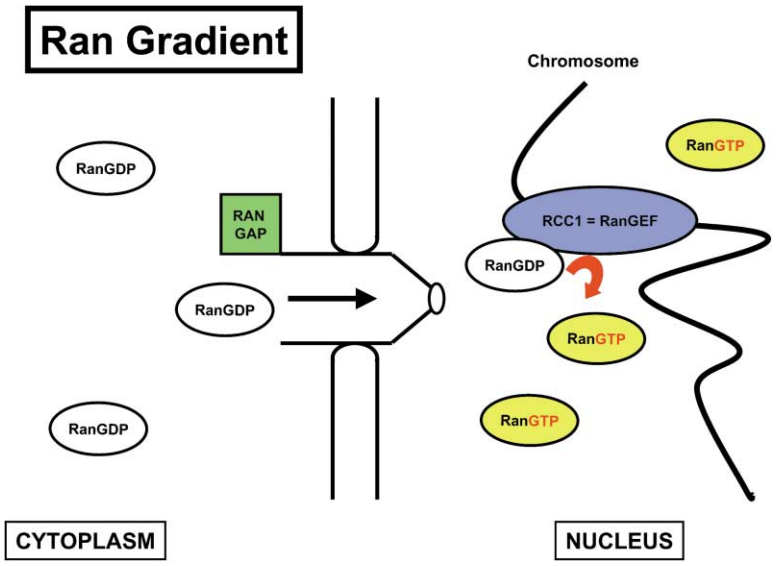

Figure 2. The Nuclear/Cytoplasmic Ran Gradient in Interphase

Ran is present in both the nucleus and cytoplasm. However, RCC1, the Ran GTP exchange factor (GEF) is a chromatin bound protein and therefore found only in the nucleus. RanGDP entering the nucleus is quickly converted to RanGTP. When RanGTP exits the nucleus, presumably in export complexes (see Figure 3), the Ran GTPase activating protein, RanGAP, found both on the cytoplasmic filaments of the pore and in the cytoplasm, causes Ran to hydrolyze its GTP to GDP. In consequence, in interphase nuclear Ran is in the GTP state and cytoplasmic Ran is in the GDP state. While this is widely accepted, on a cautionary note, it has been argued from computer modeling that the RanGTP/GDP gradient observed around nuclei and spindles in *Xenopus* extracts may not be as easy to produce in a smaller somatic cell environment (Gorlich et al., 2003).

to 11 different partners to ferry distinct cargoes (Jakel et al., 2002; Fried and Kutay, 2003). These partners include adaptor proteins, ranging from its original importin α costar to snurportin or, alternatively, other importin β-like receptors. Each heterodimer achieves new cargo specificity. What allows importin β to be so productively promiscuous in its interactions? Early deletion and mutational analysis led to a simple three-domain view of importin β: an N-terminal RanGTP binding domain, a middle zone that interacts with the nuclear pore, and a C-terminal importin α binding domain (Figure 4) (Chi and Adam, 1997). With the advent of crystal structures, a more detailed view emerged. Importin β consists entirely of 19 HEAT repeats (Figure 4). Each HEAT repeat contains two α helices, A and B, joined by a loop. The overall protein coils into a short superhelix, with extensive interaction surfaces both on the inside and the outside of the superhelix (Figure 4). Five proteins or protein fragments have been cocrystallized with β: a fragment of importin α (Cingolani et al., 1999), RanGTP (Vetter et al., 1999), a fragment of the transcription factor cargo protein SREBP-2 (Lee et al., 2003), the small Parathyroid hormone-related cargo protein PTHrP (Cingolani et al., 2002), and a short run of FG repeats from the yeast nucleoporin Nsp1 (Bayliss et al., 2000) (see Conti, 2002; Bednenko et al., 2003a; Stewart, 2003, for excellent reviews). Remarkably, structural analyses show that each protein uses a different binding site on importin β and a different mode of interaction. In some cases, several importin β HEAT repeats open up to accommodate the bound protein, while in other cases regions of the superhelix wrap around the chosen partner. It appears that the spring-like superhelical importin β has an inherently large degree of flexibility (Stewart, 2003), which it uses to recognize its many partners.

Models for Nuclear Transport

This is a good place to discuss the molecular mechanism at the heart of the nuclear pore. Indeed, we do not know how the pore allows receptors through what is a

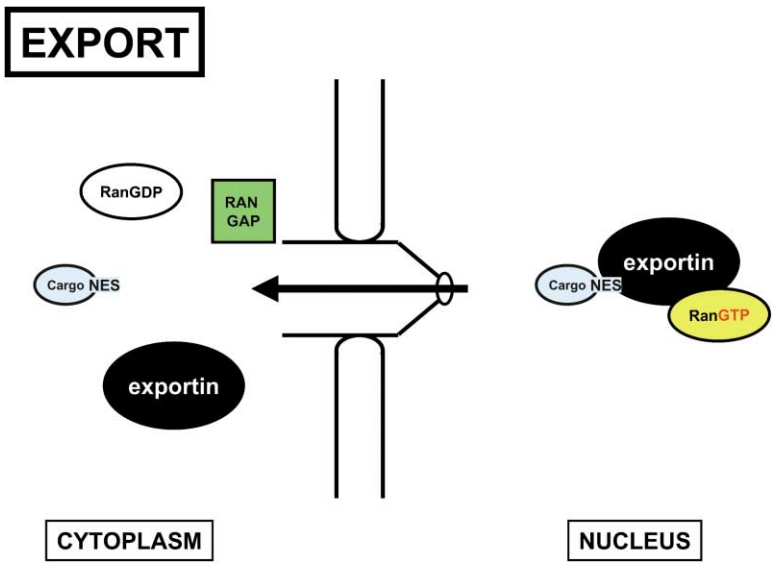

Figure 3. Nuclear Export

The export receptor Crm1/exportin1 binds its leucine-rich NES-bearing cargo protein only in the presence of RanGTP. The trimeric export complex then translocates through the nuclear pore. Upon reaching the cytoplasmic side of the pore, Ran-GAP causes hydrolysis of RanGTP to RanGDP, which causes disassembly of the export complex.

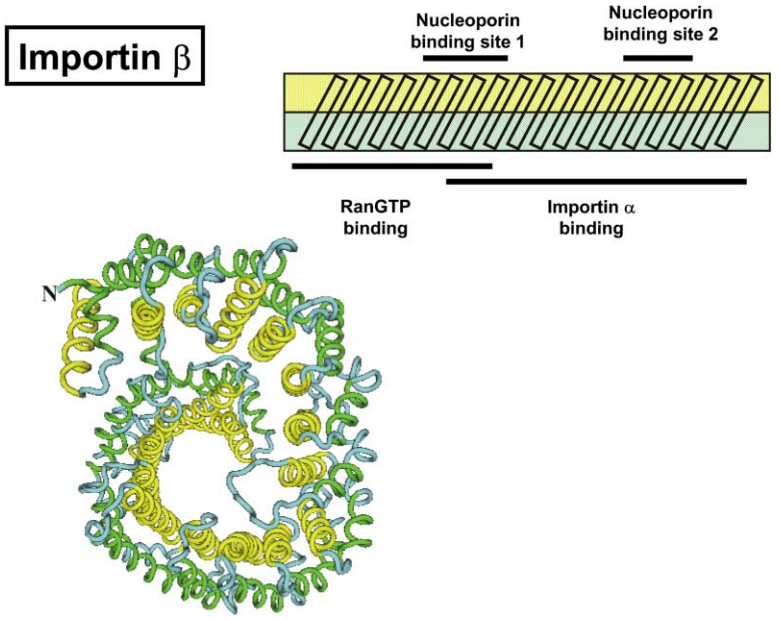

Figure 4. The Structure of Importin β

This model of human importin β was taken from the NCBI Entrez Structure database (MMDB Id 10316), where it can be manipulated in 3D by the viewer with Cn3D 4.1 software. The crystal structure was obtained from a cocrystal with importin α's β binding domain (α is omitted in this figure; Cingolani et al., 1999). Importin β consists of 19 tandem HEAT repeats. Each HEAT repeat contains two α helices, A and B, which we have colored green and yellow, respectively, after Cingolani et al. (1999). The B helices (yellow) line the internal face of importin β's overall spiral structure. The A helices (green) comprise the external face. Cargoes, Nups, receptors, and regulatory targets bind to varying regions of importin β and induce diverse structural changes.

very large hole lined at its core by FG (phenylalanine-glycine) repeat nucleoporins while simultaneously excluding cytosolic proteins ≥40 kd. All models argue that importin β family members carrying their cargo can bind to the unstructured FG repeat filaments proposed to fill the central pore channel. In one model these filaments perhaps wave like strands of seaweed, excluding all other proteins from entry by creating an entropic barrier (Rout et al., 2000; Denning et al., 2003). Another nuclear-pore wordsmith has referred to these proposed FG strands as "oily spaghetti" (Macara, 2001). A competing model argues that the FG regions of nucleoporins interact to form a hydrophobic meshwork across the pore opening (Ribbeck and Gorlich, 2001, 2002). In this model, importin β family members specifically bind and "melt" through the meshwork. Of note, recent work in yeast (Strawn et al., 2004) has used successive mutations to pare down the number of total FG Nups, as well as certain specific FG Nups, to create a "minimal" functional yeast pore. Whatever the exact mechanism of nuclear import, energy hydrolysis at the pore is not absolutely required for translocation; it is needed, however, for large cargo import and, indirectly, to maintain the RanGTP/GDP gradient that fuels nuclear transport.

The above models focus on the general propensity of β-family receptors to bind to FG Nups. Other data indicate that importin β encounters an increasing gradient of affinity on its way into the nucleus, binding most tightly to the last FG Nup on the pore, from which it is released by RanGTP (Gorlich et al., 1995b; Shah et al., 1998; Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003). Indeed, electron microscopy shows importin β locked onto the nuclear face of the pore when RanGTP is not present (Gorlich et al., 1995b). Export receptors on their way out bind most tightly to the FG Nup closest to the cytoplasm (Fornerod et al., 1997b). A challenge for the next generation of translocation models will be to integrate this apparent affinity gradient with the more generalized structural impediment models—of filament or mesh.

An interesting recent finding by Bednenko et al. (2003b) reveals that importin β possesses two distinct FG repeat binding sites (Figure 4). The authors propose that this could allow importin β to bind to two nuclear pore proteins at once, binding one and releasing the other as part of a multistep translocation through the nuclear pore. Put more simply, by having two different nucleoporin binding "arms," importin β may swing through the long FG corridor of the nuclear pore like Tarzan venturing into the nuclear interior.

Nuclear Import Links to Disease

Through its involvement in multiple nuclear import pathways, importin β literally impacts thousands of proteins and many aspects of cellular physiology. In a recent compelling example, mutations in the master regulator of mammalian sex determination, the SRY transcription factor (*s*ex-determining *r*egion of the *Y* chromosome), cause affected individuals with XY chromosomes to have female organs. With respect to transport, a newly discovered subset of SRY mutations acts by impairing the ability of SRY to accumulate in the nucleus. Work by Harley et al. (2003) has identified two separate NLSs in SRY, the C-terminal of which interacts with importin β and is critical for nuclear entry. The authors suggest a threshold effect, requiring sufficient levels of SRY in the nucleus during a specific "developmental window" in order to promote male development. This link between a defect in nuclear import and a human syndrome is likely to be just the tip of the iceberg for such connections (Kau et al., 2004).

Importin β Family Members as Cytoplasmic Chaperones

Ribosomal proteins, histones, and many other nuclear proteins are extremely basic. Upon translation, they face an immediate peril of aggregation with polyanions in the cytoplasm. An intriguing function that has been pro-

posed for import receptors is to act as cytoplasmic chaperones, shielding the basic domains of these cargo proteins and thus preventing aggregation. The requirements for suppression of aggregation can be quite stringent, i.e., fulfilled by only one of the abundant cargo's possible import receptors. Jakel et al. (2002) suggest that this might be one reason for the large diversity in import receptors, including the 11 different importin β heterodimers (see Table 1 for a selected list). The impβ/imp7 heterodimer, for example, is the only combination capable of suppressing the aggregation of the most basic cargoes, such as histone H1. Thus, when partnered with importin 7, importin β serves two roles: it envelops its cargo, shielding its basic charges and preventing aggregation, then ferries the cargo safely into the nucleus.

Importin β Family Members as Microtubule Motor Adaptors?

Many viruses, including Herpes simplex, HIV, and adenovirus, use transport along microtubules to deliver their genomes to the nuclear periphery (reviewed by Campbell and Hope, 2003). This visualized viral trafficking suggested that some cellular import substrates may also use the microtubule fast track to the nucleus. Indeed, the small protein PTHrP, a cargo that binds directly to importin β, requires microtubules for its import (Lam et al., 2002). In addition, the tumor suppressor p53 associates with tubulin and is prevented from entering the nucleus by anti-dynein antibodies (Giannakakou et al., 2000). Lastly, in plants importin α localizes to microtubules and actin microfilaments (Smith and Raikhel, 1998). While few in number and still somewhat indirect, these studies suggest that import complexes, like viruses, may travel along microtubules toward the nucleus.

Mitotic Roles and Revelations: Early Links to Mitosis

Up to this point, we have considered importin β's cellular roles exclusively during interphase. However, importin β, α, Ran, and Ran's effectors do not remain idle in mitosis but, instead, may rule the structural roost. Together, they appear to control mitotic functions ranging from spindle assembly to nuclear envelope and pore assembly. Centrosome dynamics, as well as the spindle checkpoint, are also emerging as targets of Ran control.

In many ways, this story can be viewed as one with Ran as the protagonist. The plot line of this scientific story started murky. Early clues connecting Ran to mitosis were numerous, but obscure. For example, in yeast, mutants of Ran and the Ran effectors had notably pleiotropic phenotypes. Many involve some form of mitotic arrest (for review, see Sazer and Dasso, 2000). In vertebrates, the Ran GEF RCC1 was discovered to be localized on chromosomes, and when mutated caused defects in the cell cycle, both in human cells and in *Xenopus* egg extracts (Nishitani et al., 1991; Dasso et al., 1994; Kornbluth et al., 1994; Ren et al., 1994). Indeed, Ran was referred to as "the cell cycle regulatory protein Ran/TC4" long before its involvement with nuclear import was known (Coutavas et al., 1993; Ren et al., 1994; Clarke et al., 1995). But in the ebullient "transport only" years these mitotic connections were forgotten.

Importins and Mitotic Spindle Assembly

As the plot twisted and turned, extracts of *Xenopus* eggs were once again used to revisit the connection between Ran and mitosis. Normally, chromosomes added to a mitotic *Xenopus* extract can initiate the formation of a bipolar mitotic spindle. A burst of publications described how the removal of Ran, either through depletion or mutation, severely inhibited spindle formation, whereas high levels of RanGTP promoted spindle assembly (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). It began to appear that RanGTP was the determining factor for where spindles form. A model emerged: normally chromosomes, as by far the major source of RCC1, produce a cloud of RanGTP. RanGTP then promotes spindle assembly. In consequence, at mitosis microtubules form only around the chromosomes and become organized into a mitotic spindle. This cloud of RanGTP has been visualized by using FRET-based biosensors (Kalab et al., 2002).

How was Ran functioning? A set of elegant studies revealed the answer. Spindle assembly involves tubulin polymerization into microtubules, followed by organization into spindles. Microtubule accessory proteins, including TPX2, NuMA, and the kinesin XCTK2, act as Spindle Assembly Factors (SAFs) (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001; Du et al., 2002; Schatz et al., 2003; Ems-McClung et al., 2004). The abundant transport receptors importin α and β act as negative regulators of spindle formation in mitotic extracts by sequestering TPX2, NuMA, and XCTK2. Importin α and β bind to and inhibit the spindle assembly factors everywhere in mitotic cytosol except in the vicinity of chromosomes. There, the high concentrations of RanGTP relieve importin α/β inhibition and allow localized spindle assembly (Figure 5).

A potential fail-safe modification for this scheme might be that during interphase importins α and β would import the spindle assembly factors into the nucleus and thus prevent their interference with interphase microtubules (Kahana and Cleveland, 1999; Dasso, 2002). Indeed, many spindle-associated proteins, including TPX2 and NuMA, are exclusively nuclear throughout interphase.

In vivo support for this model has been obtained. RanGTP is indeed observed localized around the spindles of *Drosophila* embryos (Trieselmann and Wilde, 2002) and is needed for spindle assembly in *C. elegans* (Askjaer et al., 2002; Geles et al., 2002). Inhibition of spindle assembly by importin β was demonstrated in vivo by microinjection of importin β into mitotic mammalian cells (Nachury et al., 2001; see also Guarguaglini et, 2000). Since then, several new aspects of regulation have emerged: RanGTP also promotes microtubule polymerization by increasing microtubule rescue frequency (Wilde et al., 2001). In addition, these events have been linked by phosphorylation to cell cycle regulatory circuits by Aurora A kinase (Trieselmann et al., 2003; Tsai et al., 2003:, Kufer et al., 2003). In sum, it is now generally accepted that Ran acts as a positive regulator of spindle assembly, by counteracting importin α and β.

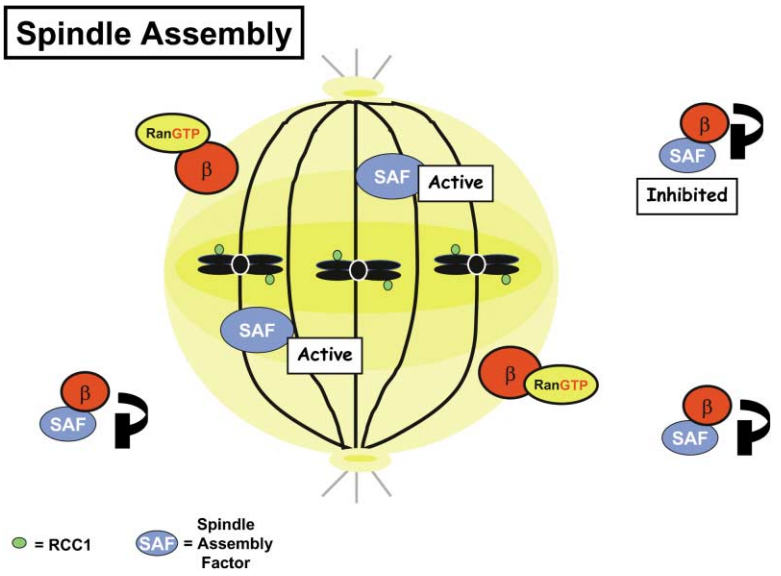

Figure 5. Spindle Assembly Is Regulated by Importin α and β

At mitosis, microtubules are observed only in the spindle and in short asters extending from the spindle. Recent work finds that importin α and β regulate spindle assembly by binding to key spindle assembly factors (SAFs), such as TPX2, NuMA and the kinesin XCTK2, inhibiting them except in areas where RanGTP is present. RanGTP is produced only in the region of the mitotic chromosomes (yellow circle and yellow zone around chromosomes), because the RanGEF RCC1 (small circle on chromosomes) remains chromosome-bound at mitosis. Thus, near the mitotic chromosomes, RanGTP releases importin α and β from the SAF spindle assembly factors, allowing them to promote spindle formation. RanGTP is also shown at the centrosomal poles.

What Are Other Key Ran-Regulated Mitotic Events?

Because of its role in nuclear import and now spindle assembly, Ran has been proposed to act as a positional marker for chromosomes throughout the cell cycle. In a more serious vein, Ran has been called a "master regulator" of cellular processes that occur at or around the chromatin. These processes include spindle assembly, centrosome/spindle-pole dynamics, nuclear membrane assembly, and nuclear pore assembly.

Clearly, the complexity of these phenomena precludes a simplistic model in which a cloud of RanGTP emanating from the chromosomes releases importin α/β from a multitude of targets controlling different mitotic events. If so, why wouldn't all targets be released at once? Instead, sequential release must be orchestrated, likely by the more celebrated parallel mitotic kinase/phosphatase events that move the cell cycle from one phase to the next.

Kinetochores and Centrosomes: New Arenas for Ran

The spindle assembly checkpoint ensures that all duplicated chromosomes have attached to both poles of the mitotic spindle before anaphase can commence (Peters, 2002). To do this, checkpoint regulatory proteins bind to the kinetochores. Once the full complement of chromosomes has attached, the checkpoint proteins exit, mitotic cyclin is destroyed, and sister chromatids separate. An involvement of Ran in the checkpoint was first hinted at when a dramatic increase in RCC1 was observed on chromosomes at the beginning of anaphase in cycling *Xenopus* extracts, the exact time of spindle checkpoint release (Arnaoutov and Dasso, 2003). Moreover, when an excess of RCC1 was added to the extract, resulting in high RanGTP, the spindle checkpoint was bypassed and checkpoint regulators were ejected from the kinetochores. In contrast, when excess RanGAP1 or RanBP1 was added, resulting in low RanGTP, the spindle checkpoint regulators remained attached to kinetochores. Overall, it has been hypothesized that achieving high RanGTP at the kinetochores may be the molecular key to turning off the spindle checkpoint once chromosomes are aligned on the metaphase plate (Arnaoutov and Dasso, 2003; for review see Li et al., 2003). Importin β has not been seen to have an effect in this work. One wonders if another β family member may be involved.

The centrosome is now emerging as another new arena for Ran. The centrosome, consisting of paired centrioles surrounded by a matrix containing γ tubulin ring complexes, is the major microtubule organizing center (MTOC) in mammalian cells. Duplication of this MTOC to give two pairs of centrioles is essential for establishing spindle bipolarity. Surprisingly, in the highly regulated cell cycle world, there appears to be no checkpoint that monitors the number of centrosomes in the cell—with unfortunate results. If centrosome number is incorrect, aneuploidy and a cancerous phenotype can result. Recent in vivo studies now find a subset of cellular Ran tightly associated with the centrosome throughout the cell cycle. Ran is anchored within the centrosomal matrix by the large centrosomal scaffolding protein, AKAP450 (Keryer et al., 2003). Electron microscopy confirms this localization and biochemical studies confirm the strong interaction: only 8 M urea removes Ran. Centrosomal Ran appears to have important functional roles: when RanGTP is decreased by RanBP1 overexpression, centrosomal cohesion is lost (Di Fiore et al., 2003). Each pair of centrioles split in two to give supernumerary spindle poles. Dramatic multipolar mitosis spindles result. In more recent work, importin β overexpression also causes supernumerary spindle poles, presumably by overriding centrosomal RanGTP function in a different manner (Ciciarello et al., in press; see also Powers and Dasso, 2004). The current model is that importin β arrives carrying a necessary microtubule assembly/cohesion factor, likely traveling along microtubules to reach the centrosome. The centrosomal RanGTP disrupts the importin β-inhibited assembly complexes, freeing the factor such that proper centrosome cohesion is maintained and only two spindle poles form (Keryer et al., 2003; Di Fiore et al., 2004). To put it more simply, importin β and Ran are postulated to work

closely together in the molecular birth control of centrosomes.

The above centrosome and kinetochore revelations may well lead to a change in our worldview. Instead of a simple model with RanGTP high on the chromosomes and low in the surrounding cytoplasm, a more architecturally elaborate scheme has been suggested (Di Fiore et al., 2003). In this scheme, Ran and its effectors are arranged in local networks in or around specific substructures of the mitotic apparatus, i.e., the chromosomes, kinetochores, and spindle poles (reviewed in Di Fiore et al., 2004). At mitosis, there would be at least three tips to the RanGTP iceberg. We might predict that other RanGTP-"producing" structures in the cell will be discovered in the future. A detailed visual search for Ran or RCC1 on cellular substructures would be likely starting points for such discoveries. A cautionary note, however, is that RanGTP was recognized in the above by a single RanGTP-specific antibody; the development of alternate tools should speed the search along.

Building the Nucleus with Ran and Importin β

Having found connections to so many mitotic structures, what of the nucleus—the elephant in the drawing room of telophase? At the M-to-G1 transition in higher eukaryotic cells, nuclear membranes must reform and nuclear pores reassemble to form a nuclear envelope around each daughter genome. Given the complexity of manipulating large cellular structures in vivo with any certainty of target, researchers again turned to *Xenopus* extracts to search for potential roles for Ran and importins in nuclear assembly.

It had long been known that nuclear structure can be reconstituted in vitro when chromatin is added to interphase *Xenopus* extracts. This is possible because of the large stockpile of disassembled nuclear components stored in eggs for later development. In *Xenopus* eggs or extracts thereof, nuclear envelopes replete with functional nuclear pores form around added chromatin, free DNA, or even DNA-coated beads, indicating the robustness of the nuclear assembly process (see Gant and Wilson, 1997; Vasu and Forbes, 2001, for reviews; Forbes et al., 1983; Lohka and Masui, 1984; Blow and Laskey, 1986; Newport, 1987; Heald et al., 1996). Early studies found that the addition of mutant Ran to *Xenopus* extracts prevented correct nuclear assembly, but the mechanism was not known (Kornbluth et al., 1994; Dasso et al., 1994; Pu and Dasso, 1997). Interestingly, when RCC1- or RanGTP-coated beads were added to *Xenopus* extracts, correct nuclear envelope assembly also formed around these DNA-free beads (Zhang and Clark, 2000). This striking result brought Ran back into the arena of nuclear assembly for another look. Nuclear assembly around natural chromatin substrates in vitro involves three sequential steps: (1) recruitment of membrane vesicles to the surface of chromatin, (2) vesicle-vesicle fusion to form double nuclear membranes, and (3) assembly of nuclear pore complexes into the double membranes. Careful experimentation showed that RanGTP was definitely required for the membrane fusion step: in Ran-depleted extracts, membrane vesicles didn't fuse (Hetzer et al., 2000, 2001; Zhang and Clark, 2000, 2001). In contrast, in the presence of extra RanQ69L, a mutant permanently locked in the GTP bound state, excessive nuclear membrane fusion, replete with nuclear pores, was observed (Harel et al., 2003). Thus, Ran plays a positive role in nuclear membrane assembly.

What about importin β? Early hints for its involvement came from bead experiments (Zhang et al., 2002). Recent work using natural chromatin templates reveals that importin β indeed negatively regulates two separate steps of nuclear assembly (Figure 6) (Harel et al., 2003; Walther et al., 2003). In the presence of an excess of importin β, either human or *Xenopus*, membrane vesicles are correctly recruited to chromatin but fail to fuse to one another to form nuclear membranes (Harel et al., 2003; R. Chan, C. Lau, and D.J.F., unpublished data). *Xenopus* importin α has a weaker but distinctive effect on fusion (Hachet et al., 2004). Thus, the action of importin α and β on nuclear membrane fusion parallel their inhibitory action on spindle assembly.

The importin β inhibition of membrane fusion can be reversed by added RanGTP (Harel et al., 2003). Indeed, it appears that the ratio of importin β to Ran is a critical factor in achieving a correct nuclear envelope. Too much importin β and no fusion occurs; too much RanGTP and hyperabundant, invaginated nuclear membrane can form. Importin β and Ran thus act as dueling regulators to promote correct nuclear membrane fusion (Figure 6B).

Importin β also negatively regulates nuclear pore assembly (Harel et al., 2003; Walther et al., 2003). To test this, nuclei with closed double membranes but no nuclear pores were assembled using BAPTA, a known chemical inhibitor of pore assembly. Such nuclei can be easily rescued for pore assembly by dilution into fresh cytosol (Macaulay and Forbes, 1996). However, when importin β is added, no pore rescue occurred. This inhibition of pore assembly by importin β was similarly observed with annulate lamellae, a cytoplasmic mimic of the nuclear envelope (Walther et al., 2003). The targets of importin β inhibition are likely a subset of both FG and non-FG nucleoporins (see Walther et al., 2003; Harel et al., 2003, for discussion).

One would always prefer to have in vivo confirmation of in vitro studies. A number of in vivo studies bear on this point. In *S. pombe*, a Ran GEF mutation (i.e., low RanGTP) results in unusual nuclear envelope fragmentation (Demeter et al., 1995). Moreover, mutations of Ran cycle proteins and of importins disrupt nuclear envelope assembly in *C. elegans*, as well as in *Drosophila* (Askjaer et al., 2002; Bamba et al., 2002; Timinszky et al., 2002). Importantly, an unbiased screen for yeast mutants in nuclear pore and envelope assembly yielded a distinct set of Ran cycle mutants (Ryan et al., 2003).

In conclusion, the ratio of importin β to RanGTP is critical to nuclear assembly. It regulates membrane fusion such that a nuclear membrane forms in a spatially correct manner, i.e., on the surface of chromatin and not in the nuclear interior or cytoplasm, and in a proportionately correct manner, with equivalent amounts of inner and outer nuclear membranes. The next challenge will be to identify the specific targets for importin β's negative regulation for both the membrane fusion and nuclear pore assembly steps.

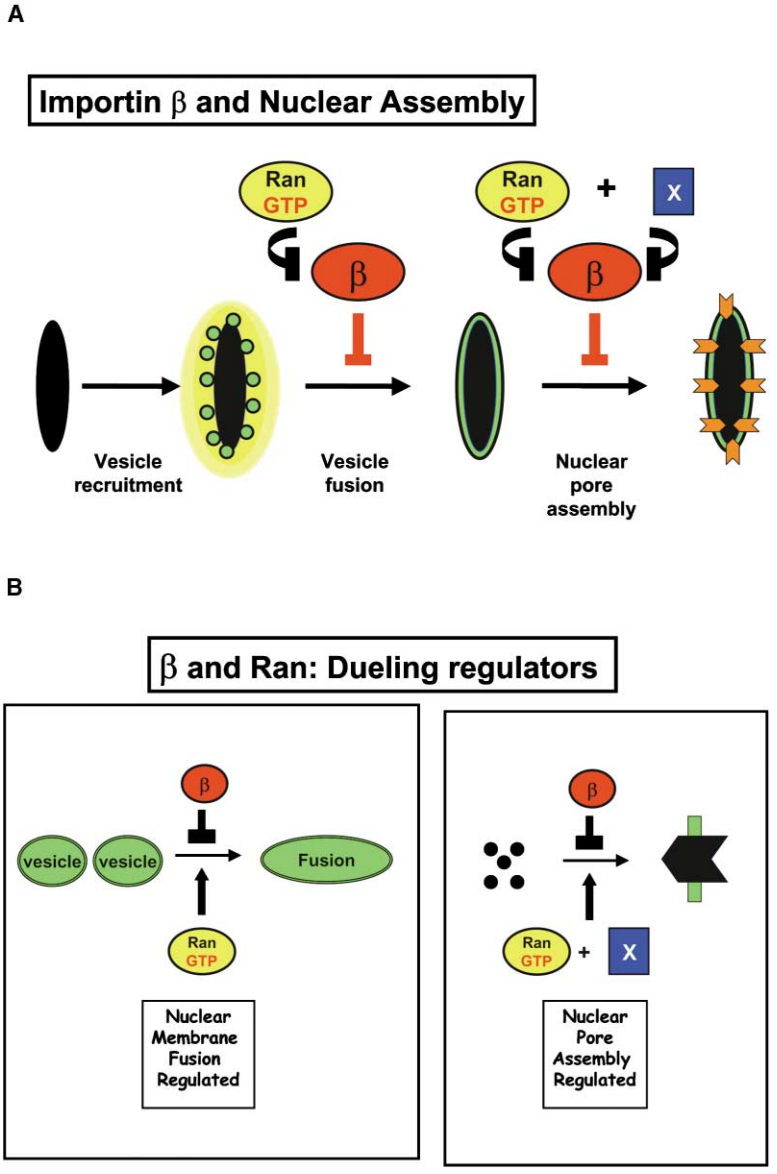

Figure 6. Importin β and Ran Dual Regulation of Nuclear Assembly

(A and B) Sperm chromatin (black oval) added to *Xenopus* egg extract recruits membrane vesicles (green circles). These vesicles fuse side to side to form double nuclear membranes. Nuclear pore complex assembly then ensues in a step that requires fusion between the outer and inner nuclear membranes and recruitment of soluble nucleoporins to the fusion pore to form a complete nuclear pore complex (orange chevrons). Importin β (red circle) negatively regulates two of the steps in nuclear formation: the vesicle fusion step and the nuclear pore formation step. (The latter was assayed on pore-free, membrane-enclosed nuclear intermediates; Harel et al., 2003). RanQ69L-GTP (yellow circle) counteracts importin β's negative regulation of membrane fusion, but not its negative regulation of pore assembly. Importin β has been found bound to disassembled nucleoporins in *Xenopus* extracts (Walther et al., 2003); a subset of these Nup/importin β interactions can be reversed by RanGTP but, importantly, others cannot. It is hypothesized that in addition to Ran, a second factor (blue square) is required to antagonize importin β's negative regulation of nuclear pore assembly (Harel et al., 2003).

The Long Reach of Importins in Neurons

A striking example of an unconventional role for importins has recently been reported in injured neurons. Neuronal regeneration is known to occur in response to axonal injury, even when that injury lies dozens of centimeters away from the neuronal cell body. An immediate burst of injury-induced action potentials is observed, followed by a second wave of signals hours or days later. This second wave has been hypothesized to involve retrograde transport of macromolecules along the axon from the site of injury. Hanz et al. (2003) now show that importin α and β play a role in the second wave, reporting the injury to the cell (see also Guzik and Goldstein, 2004). Upon injury, they observe an immediate increase in translation of localized importin β transcripts in the distant axon. Importin α, apparently already present in sufficient levels, then binds to importin β together with as yet unidentified NLS-bearing "signaling" cargoes. These complexes travel along the axonal microtubules via dynein, moving toward the cell nucleus. Hanz et al. (2003) actually demonstrate retrograde transport of labeled NLS-peptide in the injured neurons. Importantly, an excess of synthetic NLS-peptide introduced into the axon competes with the signal response and clearly results in a significant delay in regenerative outgrowth. This use of localized importin β mRNA in axons and de novo synthesis of importin β to trigger the long-range response is a novel signaling mechanism that may extend to systems beyond neuronal injury.

Concluding Remarks

The past few years have revealed importin β and Ran to have unexpectedly broad roles in multiple arenas within the eukaryotic cell (Figure 7). The roles for importin β in interphase are active ones. In contrast, in mitosis importin β to date acts as a negative regulator.

Importin β, acting either on its own or through the adaptor importin α, provides the specificity of these

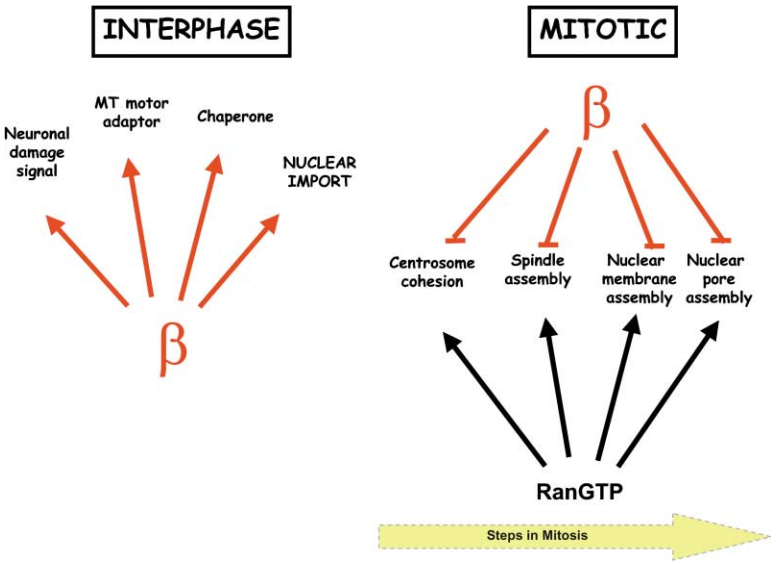

Figure 7. Summary of Importin β Functions

In interphase, importin β to date acts primarily in positive or active roles (red arrows): as a transport receptor in nuclear import; as a chaperone for very positively charged, newly synthesized nuclear proteins; as a potential adaptor for carrying certain cargoes such as viruses along microtubules to the nucleus; and in a recently described signaling pathway conveying axonal damage to the neuronal cell body. In contrast, in mitosis importin β, released upon nuclear breakdown, serves at least to date in different negative regulatory roles. It acts to regulate mitotic events such as centrosome cohesion, spindle assembly, nuclear membrane assembly, and nuclear pore assembly. Importin β is counteracted by RanGTP, which is produced and/or localized around both the centrosomes and the chromatin (see Legend to Figure 6 for an exception). The interplay of importin β's negative regulation and Ran's positive regulation assure that specific structures (spindles, membranes) form in the correct location and in the correct amount. As importin β's repertoire expands in the future, negative roles in interphase and positive roles in mitosis may be found.

interactions. Ran, on the other hand, ends importin β's reign in almost all arenas in the same manner, by luring importin β away from its specific target. In this yin/yang model of regulatory interplay, importin β's structural versatility in binding allows it to provide the specificity for each control circuit. Ran's distinctive spatial localizations provide the regulatory yin to importin β's yang.

In the future, we may find other regulators of importin β. Hypothetically, these could be novel small GTPase alternatives to Ran or a different type of regulatory partner altogether, i.e., a kinase or protease. We might also expect to see other abundant relatives of importin β, for example, exportin1/Crm or transportin, playing their own unique regulatory roles within the cell outside the arena of nuclear transport. The adaptor importin α will likely also expand its regulatory roles (Goldfarb et al., 2004); already it negatively regulates the spindle assembly factor TPX2, as well aspects of nuclear membrane fusion. Other abundant adaptors may join the regulatory fray.

New cytoplasmic locations of Ran and its effectors may also be revealed, foretelling new areas of control. In one such example, Keryer et al. (2003) find a subset of Ran localized in polarized pillar cells to the apical tips of a specialized population of microtubules, far from the centrosome. Similarly, RCC1, long designated the sole GDP-GTP exchange factor for Ran and found to date only on chromosomes, may be joined by other Ran GEFs. No RCC1 has been detected on centrosomes, yet RanGTP is apparently localized there. Its source should prove highly interesting.

Lastly, yeast importin β family members have been found specifically bound in the nucleus to sets of transcriptionally active genes, whereas Ran cycle enzymes are found on sets of inactive genes (Casolari et al., 2004). These findings imply hitherto unsuspected roles in genome regulation.

The global view is that the abundant cellular protein importin β does not rest on its laurels after nuclear import, but pitches in—in multiple interphase roles and in regulating multiple cell cycle events. It is counteracted by RanGTP. This pair thus joins kinase/phosphatase and ubiquitin ligase/protease paradigms in a new and versatile regulatory archetype. What's more, importin β may soon be coming to a cellular theater near you.

Acknowledgments

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