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The N-Terminal Domain of ERK1 Accounts for the Functional Differences with ERK2

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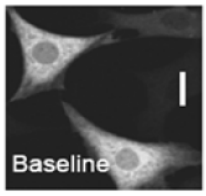
1

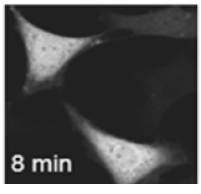
Control condition: Cells transfected with ERK1-GFP

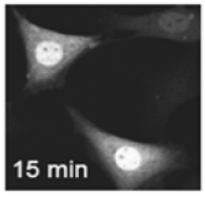
Cellular localisation of ERK1-GFP fusion protein was verified.
In non stimulated cells the level of ectopic expression is crucial, since cells with very bright fluorescence invariably showed pronounced nuclear translocation, independently of ERK activation.

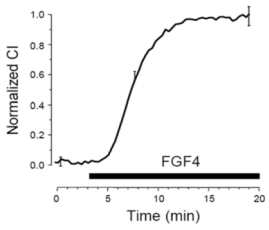


ERK1-GFP translocates in the nucleus of NIH-3T3 cells after stimulation.

Cells transfected with ERK1-GFP after treatment with 80 ng/ml of FGF4.



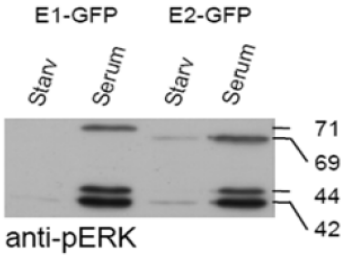




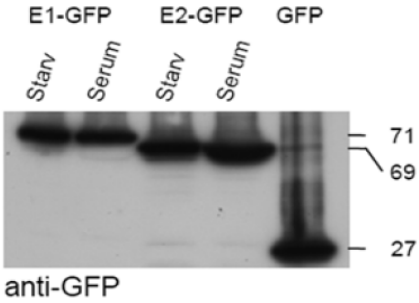
Time course of the normalized Concentration Index of cells stimulated with FGF4 (n = 18).

2

ERK-GFP fusion proteins are phosphorylated following serum stimulation, as demonstrated by western blot with a phospho-specific ERK antibody.



The fusion proteins have the correct molecular weight also when assayed with an anti-GFP antibody.

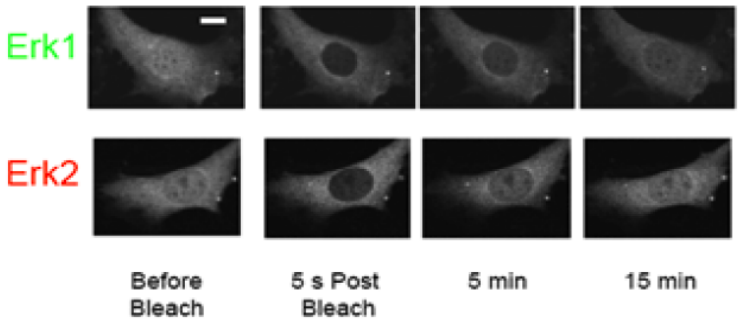


No degradation products of the fusion proteins at molecular weight lower than 69/71 kDa. Identical results were obtained employing both the N-and C-terminal fusion proteins. Control cells transfected with the GFP reporter only did not display any distribution change after stimulation (data not shown).

3

Comparison of the nucleo-cytoplasmic shuttling of ERK1 and ERK2.

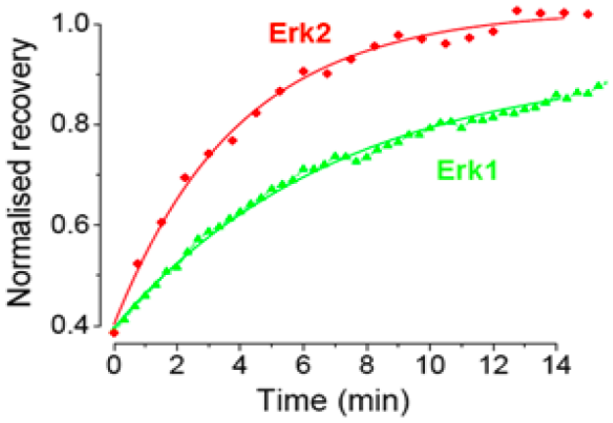
ERK2 shuttles between the nuclear and cytosolic compartments with a speed that depends on its activation state. We verified whether a similar process occurs with ERK1 by photobleaching the nucleus and imaging the recovery of fluorescence.
Since photobleaching is irreversible, the recovery in the nucleus is due to the exchange of protein between cytoplasmatic and nuclear compartments.



After photobleaching of the nucleus of starved NIH 3T3 cells, the ERK1 fluorescence recovered more slowly than ERK2, indicating a slower turnover of ERK1 across the nuclear membrane.

4

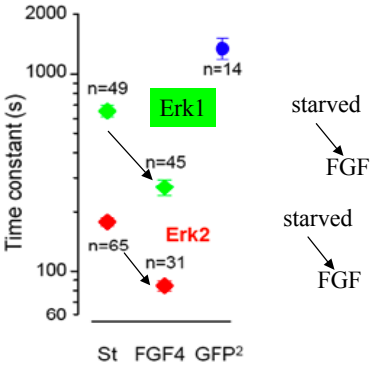
ERK1-GFP and ERK2-GFP shuttle across the nuclear membrane with different kinetics.



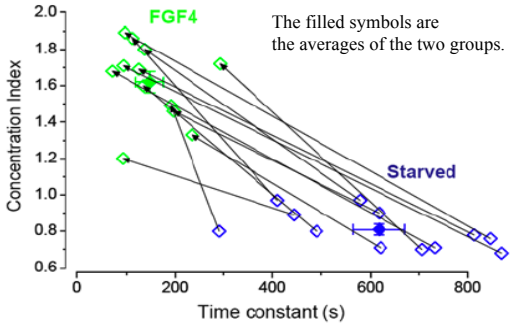
Time course of the recovery

5

The turnover of ERK1 is slower both in starved cells and after stimulation with FGF.



In ERK1 starved cells the turnover is 3.7 time slower (653 s) than ERK2 (178 s) and in the stimulated cells ERK1 turnover (266 s) is 3.1 time slower than ERK2 (84 s). The blue symbol shows the turnover for a GFP dimer. This molecule is smaller than ERK-GFP but it crosses the nuclear membrane more slowly than ERK1, indicating that also for ERK1 is operating a mechanism of facilitated diffusion.



Scatter diagram showing the recovery and Concentration Index of all paired measurements. Lines join observations relative to the same cell before and after stimulation.

6

Alignment of the amino acid sequences of rat ERK1 and ERK2.
The N-terminus is shown with a larger font. The 20 aa present only in ERK1 are displayed in bold.

Erk1 MAAAAAAPG**GGGGEPRGTAGVVPVVPGEV**EVVKGQPFDVGPRYTQLQYIG
Erk2 MAAAAAAGP EMVRGQVFDVGPRYTNLSYIG

EGAYGMVSSAYDHVRKTRVAIKKISPFEHQTYCQRTLREIQILLGFRHENVIGIRDILRAPTLEAMRDVYIVQDLMETDLYKLL
EGAYGMVCSAYDNLNKVRVAIKKISPFEHQTYCQRTLREIKILLRFRHENIIGINDIIRAPTIEQMKDVYIVQDLMETDLYKLL

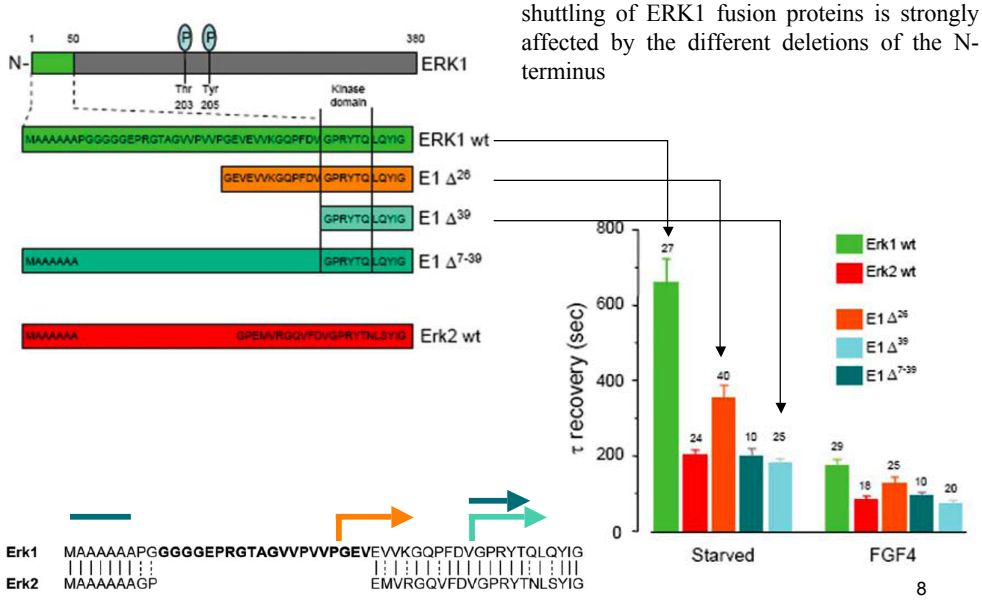
KSQQLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLINTTCDLKICDFGLARIADPEHDHTGFLTEYVATRWYRAPEIML
KTQHLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNTTCDLKICDFGLARVADPDHDHTGFLTEYVATRWYRAPEIML

NSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKSDS
NSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINLKARNYLLSLPHKNKVPWNRFLPNADS

KALDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDEPVAEEPFTFDDSKALDLLDRMLTFNPNKRITVEEALAHPYLEQYYD
KALDLLDKMLTFNPHKRIEVEQALAHPYLEQYYDPSDEPIAEAPFKFDDSKALDLLDKMLTFNPHKRIEVEQALAHPYLEQYYD

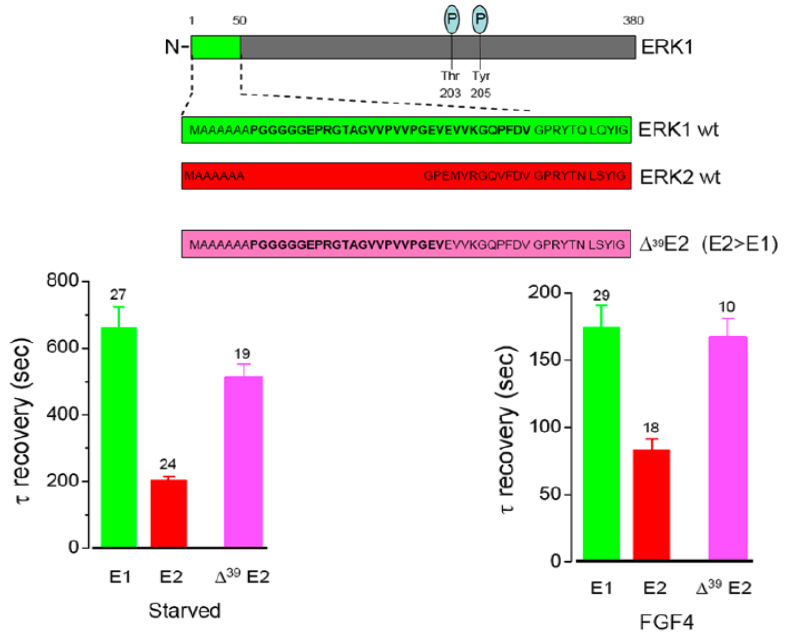
PTDEPVAEEPFTFDMELDDLPKERLKELIFQETARFQPGAPEAP
PSDEPIAEAPFKFDMELDDLPKEKLKELIFEETARFQPGYRS

ERK1 deletants:



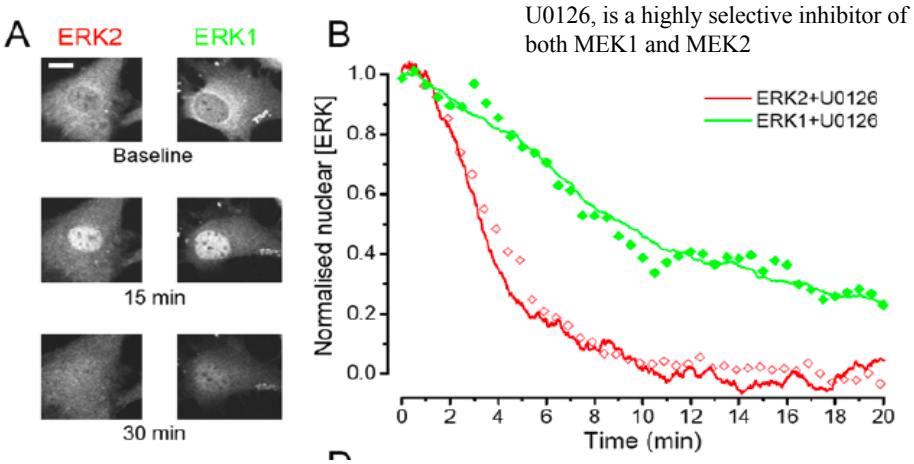
The time constant of the nucleo-cytoplasmic shuttling of ERK1 fusion proteins is strongly affected by the different deletions of the N-terminus

Fusion of ERK2-GFP with the N-terminus of ERK1 (D39 E2/E.E1).



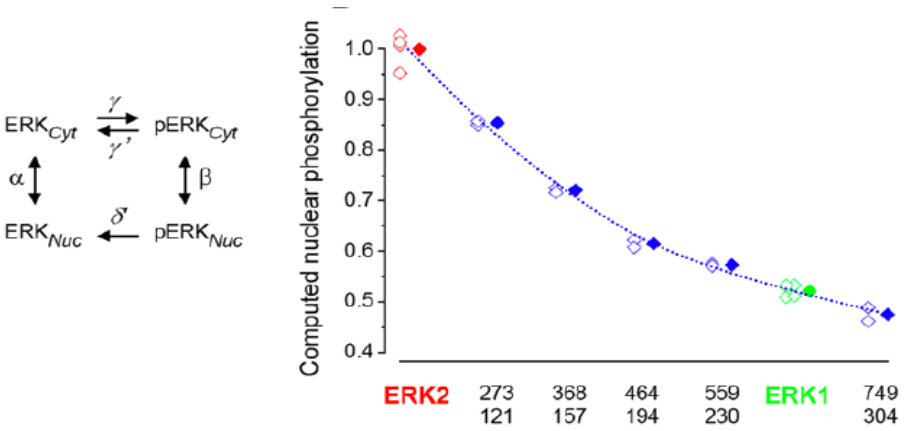
9

Cells transfected with ERK1-GFP and ERK2-GFP were treated with FGF4 for 15 min to allow complete nuclear translocation and then with the ERK blocker U0126. The inactivation of the ERK pathway caused the immediate loss of nuclear accumulation of ERK-GFP unmasking the action of nuclear dephosphorylation.



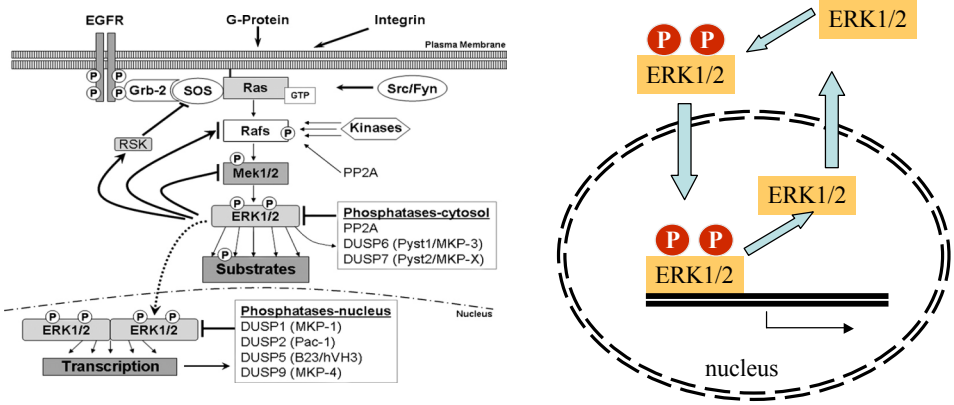
10

Phosphorylation in the nucleus as a function of the shuttling speed



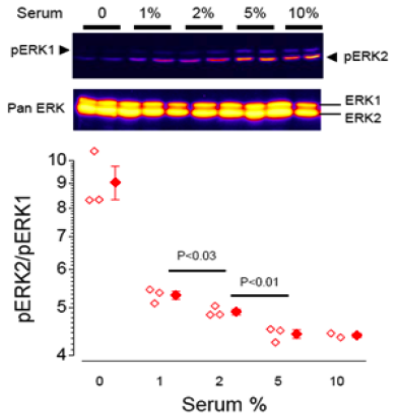
The total level of phosphorylation of ERK1 is only about half of ERK2.

11

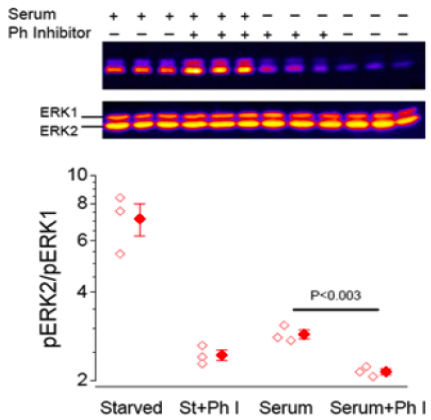


12

Comparison between the activation of ERK1 and 2



NIH 3T3 cells have been starved for 24 hr before treatment for 15 min with increasing concentrations of serum as indicated. The densitometric analysis of the gel blotted with the phospho-specific antibody has been performed with a linear imager to quantify the intensity of the phospho-ERK1 and 2 bands. From each experiment we computed the ratio pERK2/pERK1 which is a measure of the relative activation of the two kinases. The graphs shows that ERK1 activation lags behind ERK2.



Effects of phosphatase inhibition on the relative activations of ERK1 and 2. Cells have been starved for 24 hr before a 30 min treatment with serum 10% and/or a cocktail of phosphatase inhibitors. Inhibition of phosphatases in presence of serum caused a further increase of phosphorylation compared to serum only. This increase was larger for ERK1, indicating a stronger dependence of ERK1 on de-phosphorylation. Notice that there is no vertical correspondence between the western blot and the quantification.

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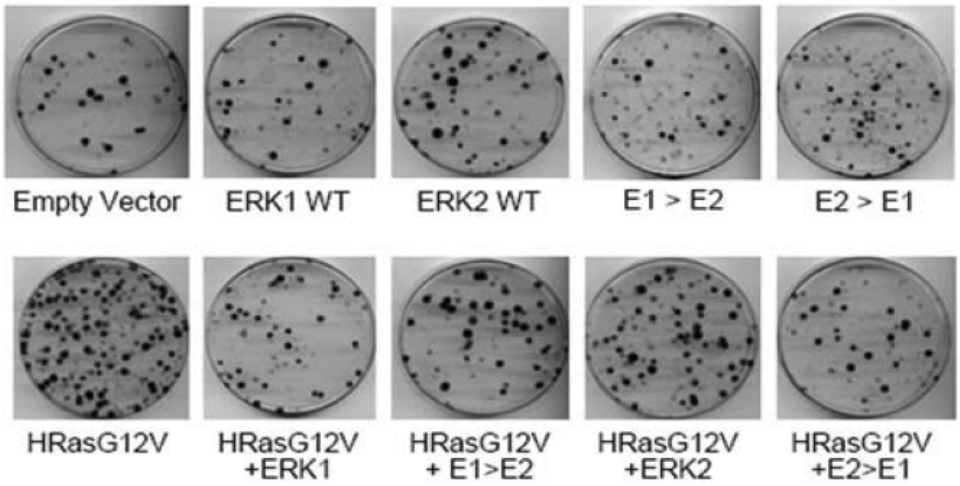
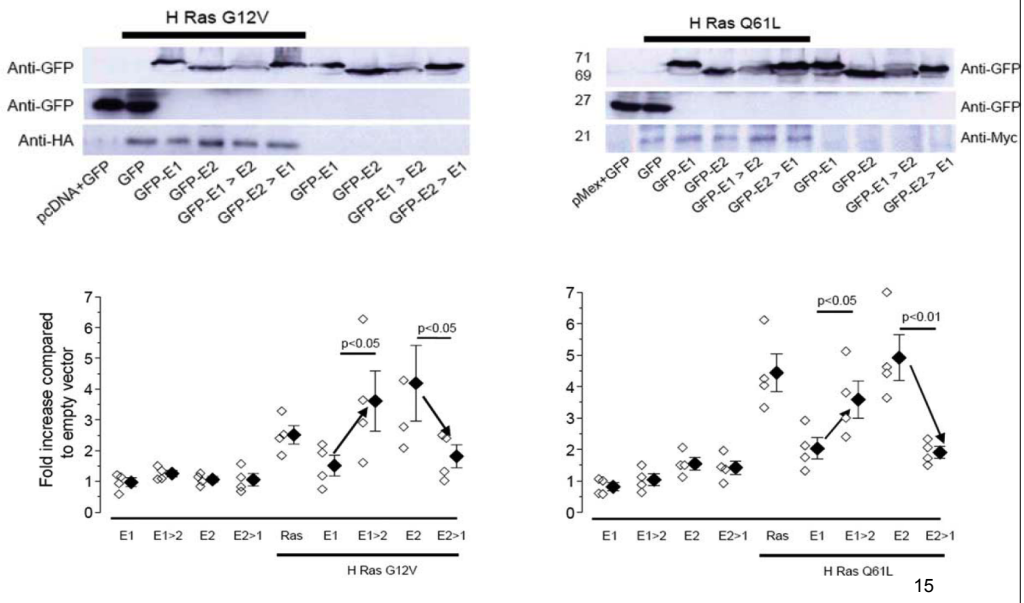


14

Functional consequences of N-terminus mutations of ERK1 and ERK2



15

Effects of the overexpression of N-terminus mutations of ERK1/2 on the nuclear target MSK

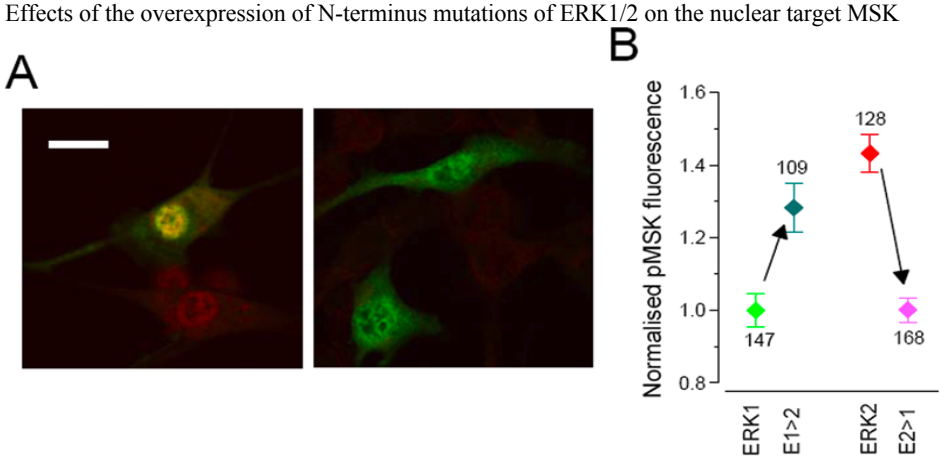
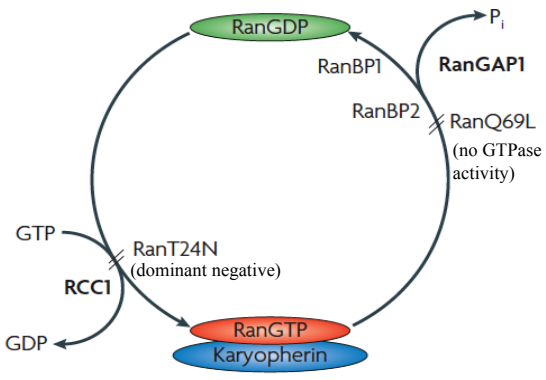


Figure 9. Effects of the overexpression of N-terminus mutations of ERK1/2 on the nuclear target MSK. A) Representative fields of fibroblast treated for 24 hr with 10% serum. In this example, cells have been transfected with ERK2-GFP and were stained with the pMSK specific antibody (red). The left panel show that the treatment caused nuclear translocation of ERK2-GFP and activation of MSK, which is well visible both in the transfected cell (the nucleus is yellow) and in nearby non-transfected cells. The inhibitor U0126 (25 μM) was administrated 6 hr before fixation. In these conditions ERK did not accumulate in the nucleus and there was not detectable phosphorylation of MSK, indicating that, MSK requires ERK activity to be phosphorylated after serum treatment. Bar 20 μm. B) Intensity of the pMSK signal measured in cells transfected with ERK1, ERK2, E1Δ39 (indicated as E1>E2) and Δ39E2 (indicated as E2>E1). The experiment was repeated in triplicate and the fluorescence of each cell has been normalised to the mean fluorescence of the ERK1 group to allow the comparison of the different experiments. Number of cells indicated nearby each symbol. U0126 suppressed almost completely the pMSK signal (mean = 0.48±0.04, n = 76; not shown). Since cell fluorescence was not normally distributed, significativity was assessed with the Kolmogorov-Smirnoff test. ERK1≠ERK2 ($p \leq 0.0005$); E1>E2≠E2>E1 ($p \leq 0.0005$). E1 and E2>E1 are not significantly different ($p \leq 0.2$). E1>E2 is larger that ERK1 ($p \leq 0.0005$) but it is also slightly but significantly smaller than ERK2 ($p \leq 0.01$).
doi:10.1371/journal.pone.0003873.g009

16

Ran function in nucleocytoplasmic transport, spindle assembly and DNA replication.

The GTP–GDP cycle of Ran.
Ran is loaded with GTP by the guanine nucleotide-exchange factor RCC1. RanGTP adopts a distinct conformation that allows it to interact with a transport factor from the importin-β superfamily, also known as the karyopherins. Hydrolysis of GTP to GDP by Ran requires the interaction of a Ran GTPase-activating protein, RanGAP1, and is stimulated by Ran-binding protein-1 (RanBP1) or RanBP2. RanGDP has a different conformation that does not interact strongly with karyopherin and can be considered inactive. Mutants of Ran block the GTP–GDP cycle: RanT24N has a reduced affinity for nucleotides and forms a stable complex with RCC1, thereby blocking RanGTP formation, whereas RanQ69L cannot hydrolyse GTP and is locked in the GTP-bound conformation.



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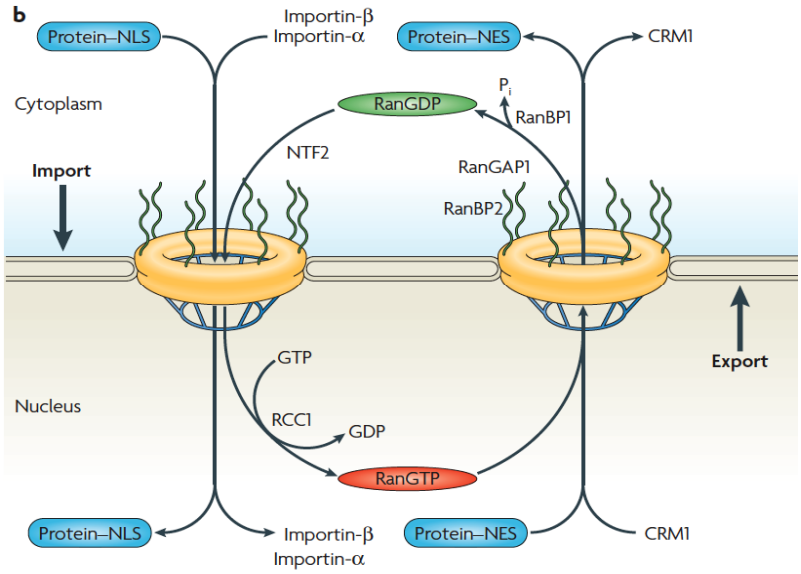
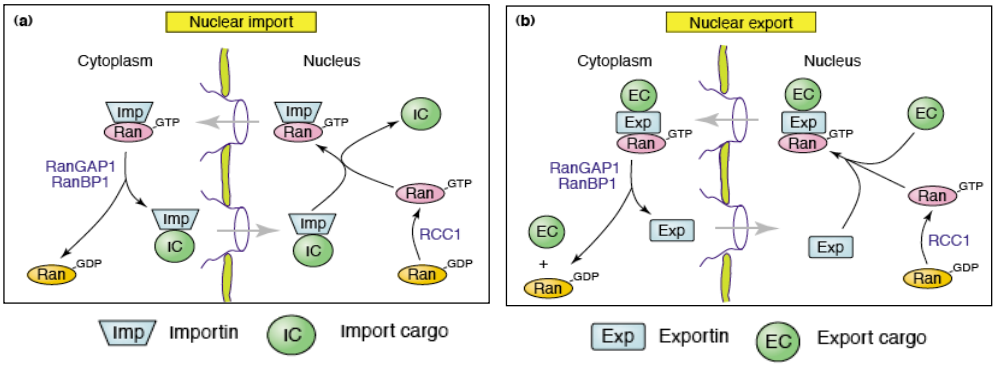
Ran directs nucleocytoplasmic transport



Ran-regulated nuclear import (a) and export (b).
In the **import cycle (a),** import receptors, such as importin b and its adaptor importin a, bind to their cargo in the cytoplasm and transit into the nucleus. Nuclear RanGTP binds to importin and stimulates cargo release. Importin–RanGTP returns to the cytoplasm. Hydrolysis of RanGTP to RanGDP (stimulated by RanGAP1 and RanBP1) releases importin from Ran, facilitating the recycling of importin.
In the export cycle **(b), export** receptors exportin, such as Crm1, bind to their cargo and RanGTP in the nucleus and transit into the cytoplasm. In the cytoplasm, RanGTP hydrolysis leads to the release of cargo and the recycling of exportin.



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In addition to its characterized roles in nucleocytoplasmic transport Ran, a small GTPase of the Ras superfamily, has functions in:

-DNA reduplication inhibition
-centrosome duplication and microtubule dynamics
-kinetochore attachment of microtubules and chromosome alignment
-nuclear-envelope dynamics and NPC formation

Structure, dynamics and function of nuclear pore complexes

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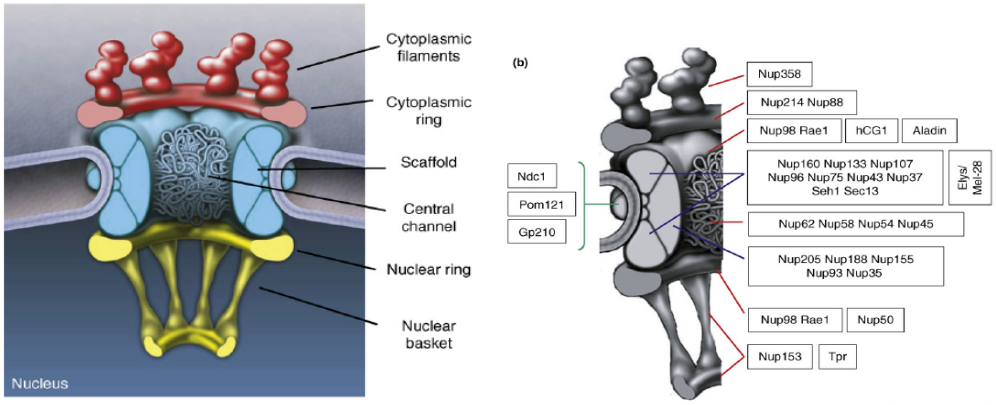
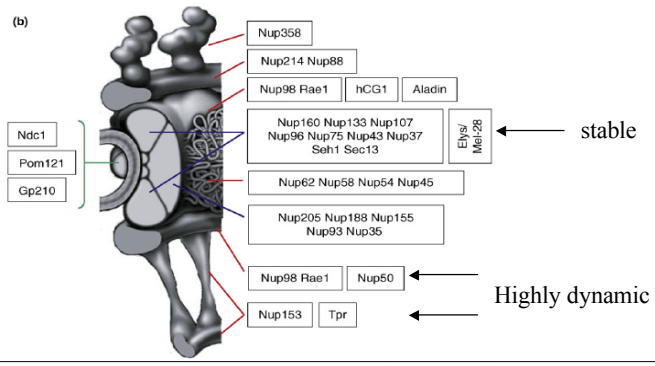
Molecular and Cell Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA 92037, USA



Trends in Cell Biology Vol.18 No.10 2008

Dynamic organization of NPCs

A remarkable, yet largely uncharacterized, feature of the NPCs is their dynamic molecular organization. Using a systematic approach, in which 19 GFP-tagged nucleoporins were studied by fluorescence recovery after photobleaching (FRAP), it was shown that the residence times of different nups at the NPC varied from a few seconds to >70 h depending on their location or function

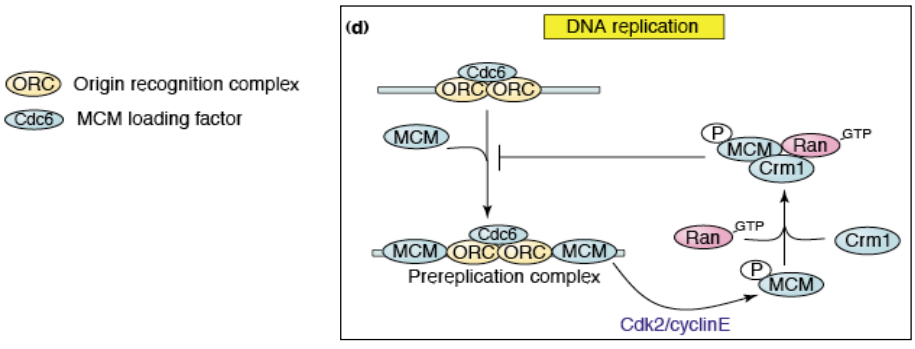


The functional importance of 'dynamic nucleoporins' is still unclear.

•Mobile nucleoporins could help to deliver cargo to the NPC: RNA-binding nucleoporins could assist newly transcribed RNAs to reach the pore and be exported to the cytoplasm
•Pore-independent functions
•Changes in protein composition in response to altered transport requirements.
•The presence of tissue and developmental-specific nucleoporins has been reported; however, there is no evidence that NPCs of different composition coexist in the same cell.

DNA reduplication inhibition

(d) **RanGTP inhibits DNA reduplication in the same cell cycle**. During S phase, high cyclin-dependent kinase (Cdk) activity phosphorylates MCM helicase. This promotes the interaction of MCM with both RanGTP and Crm1 in the nucleus, thereby preventing MCM from participating in DNA reduplication.



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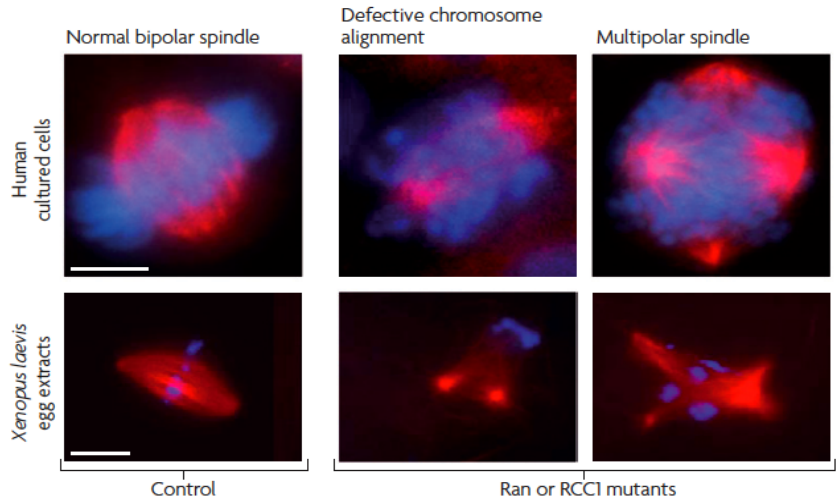
RAN in mitosis?

Disruption of the GTP–GDP cycle of Ran causes aberrant mitotic spindle assembly.

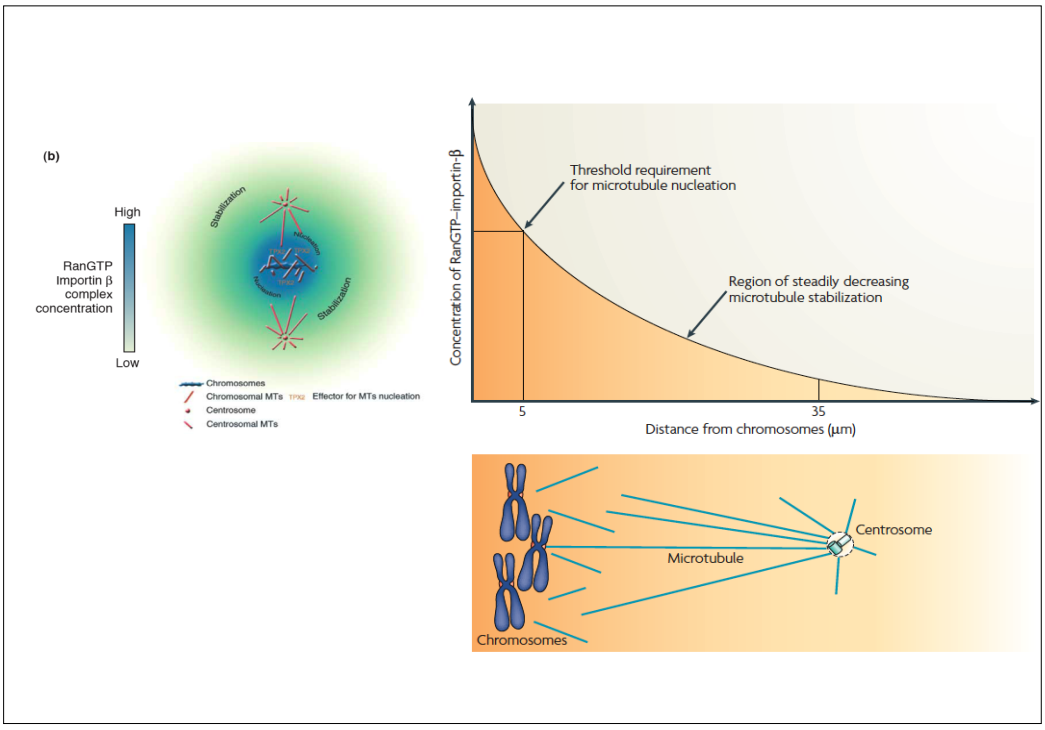


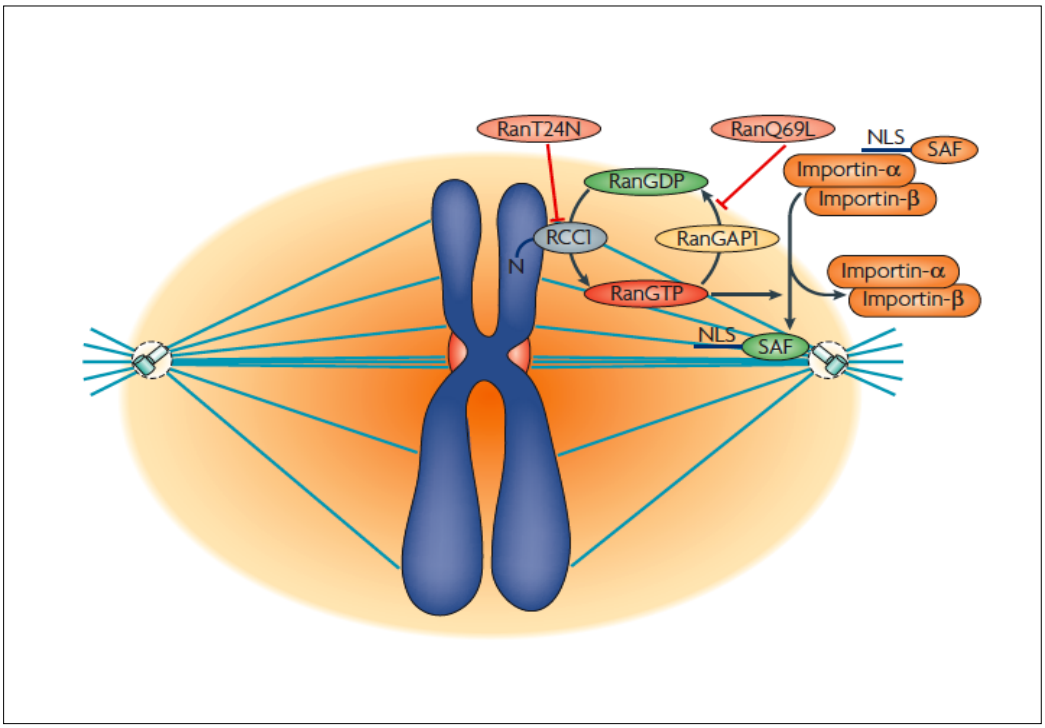
Expression of Ran mutants or mislocalization of the guanine nucleotide exchange factor RCC1 from the chromosomes produces defects in chromosome alignment at metaphase or multipolar spindles. These defects are similar to those produced in *Xenopus laevis* egg extracts by the addition of Ran mutants, and indicate that the proper spatial generation of RanGTP is necessary for correct spindle assembly. Scale bars, 10 μm.

Table 1 | **Mitotic targets of RanGTP in spindle assembly**

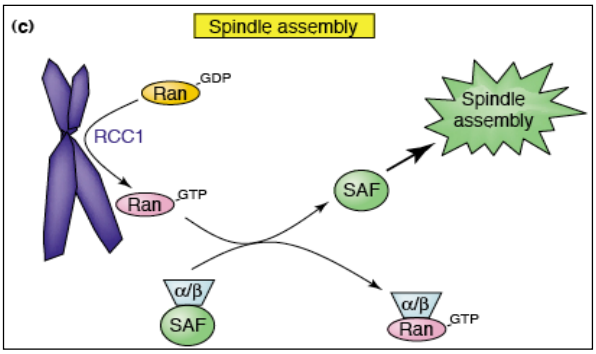
Spindle assembly factor	Mitotic functions	Experimental system	Karyopherin	Refs
Direct targets				
TPX2	MAP, MT bundling	XEE, CMCs	Importin-α	75, 99, 100
NuMA	MAP	XEE	Importin-β	95, 96
Kid	Chromokinesin	HCE, CMCs	Importin-α, -β	99, 109
XCTK2	Kinesin, MAP	XEE	Importin-α, -β	162
RAE1	MAP, RNA binding	XEE	Importin-β	152
NuSAP	MAP, MT stabilization and bundling	XEE, CMCs	Importin-α, -β, -7	118
HURP	MAP, k-fibre stabilization and tension	XEE, CMCs	Importin-β	101, 102
Lamin B	Mitotic matrix formation	XEE	Unknown	107
CDK11	Microtubule stabilization	XEE	Importin-α, -β	106
CRB3-CLPI	Polarized membrane targeting	Epithelial cells	Importin-β	163
Xnf7*	Microtubule bundling	XEE	Importin-β	164
Indirect targets				
TACC/Maskin/Alp7‡	MT-stabilizing factor	XEE, *S. pombe*	Importin-α?	165, 166
XMAP215	MAP	XEE	Unknown	64, 101
Aurora A	Mitotic kinase	XEE, HCE	Importin-α, -β	99, 100
XRHAMM	MT nucleation	XEE	Unknown	104
BRCA1–BARD1	Mitotic spindle assembly	XEE, CMCs	Unknown	105
CRM1-dependent pathway				
RanBP2	K-fibre formation	CMCs	CRM1	82
RanGAP1	K-fibre formation	CMCs	CRM1	82
CPC	K-fibre attachment, checkpoint signalling	CMCs	CRM1	115

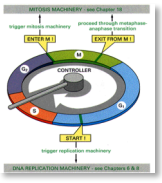
*The table lists spindle assembly factors that have been reported to be regulated by RanGTP, except for Xnf7, the microtubule-bundling activity of which does not appear to be regulated by RanGTP[164]. ‡In *Schizosaccharomyces pombe*, Alp7 may be directly regulated through dissociation of karyopherins by RanGTP[166]. Where regulation is described, the relevant karyopherin is named. Experiments in mitotic *Xenopus laevis* egg extracts (XEE), human cell extract (HCE), cultured mammalian cells (CMCs) or *S. pombe* are indicated accordingly. BARD1, BRCA1-associated RING domain protein; BRCA1, breast cancer type-1 susceptibility protein; CDK11, cyclin-dependent kinase-11; CPC, chromosome passenger complex; CRM1, chromosome-region maintenance protein-1; k-fibre, kinetochore fibre; MAP, microtubule-associated protein; MT, microtubule; NuSAP, nucleolar and spindle-associated protein; RanBP2, Ran-binding protein-2; RanGAP1, Ran GTPase-activating protein-1; TACC, transforming acidic coiled-coil-containing protein 1; Xnf7, *X. laevis* nuclear factor-7.





(c) **RanGTP stimulates spindle assembly during mitosis**. A high RanGTP concentration is generated on mitotic chromosomes by chromosome-bound RCC1. This RanGTP locally stimulates the release of spindle assembly factors (SAF) from the inhibitory binding of importin a/b to promote spindle assembly towards chromosomes.





α/β Importin α/β

SAF Spindle assembly factors

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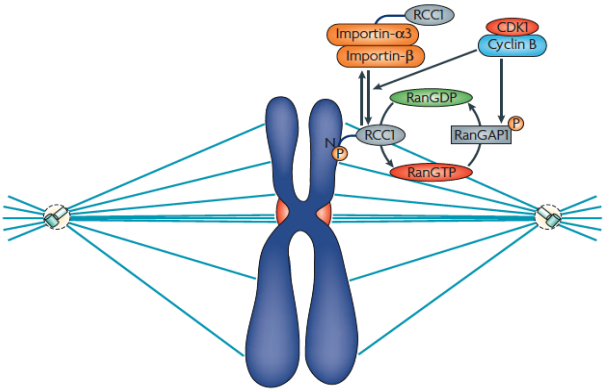
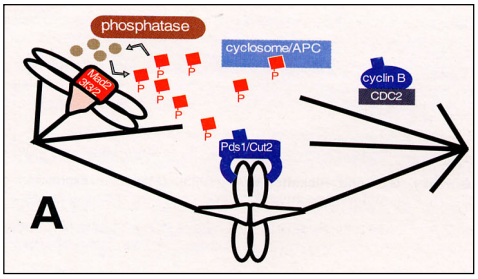
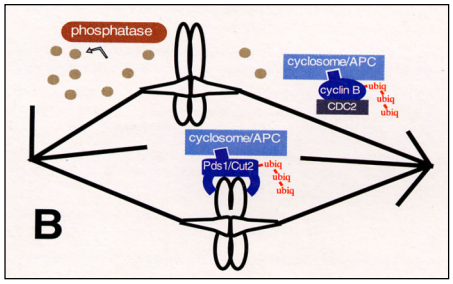
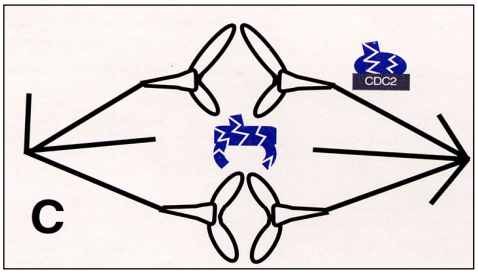


Regulation of RanGTP generation during prometaphase and metaphase by phosphorylation. Cyclin-dependent kinase-1 (CDK1)–cyclin B phosphorylates the guanine nucleotide-exchange factor RCC1 at sites in the N-terminal region.
Phosphorylation is antagonistic to the interaction of an importin-α3–importin-β dimer with this region, which also functions as a nuclear localization sequence. The N-terminal region is important for the interaction of RCC1 with chromatin, and phosphorylation thereby activates RCC1 to produce RanGTP at the surface of chromatin. The Ran GTPase activating protein RanGAP1, which opposes RCC1 by stimulating the hydrolysis of GTP by Ran, is also a substrate for CDK1–cyclin B and other mitotic kinases, although the effect on its function is unknown.

Metaphase:Inhibition of Anaphase Promoting Complex (APC) by kinase activity of non-aligned chromosomes.



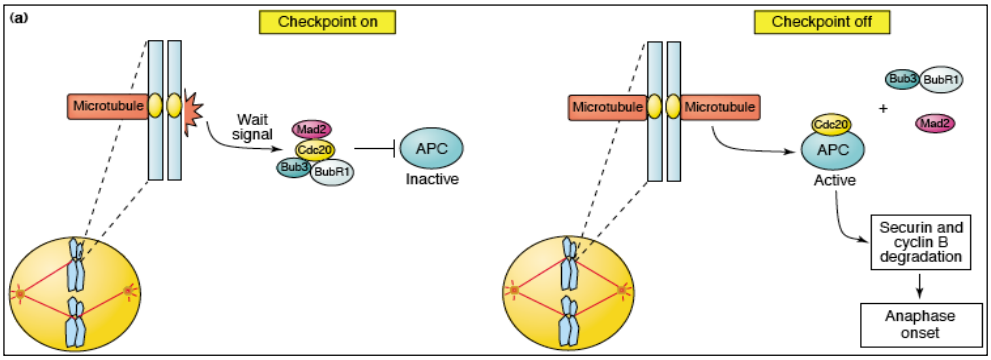




Tension: inhibition of the kinase activity responsible for APC inhibition

Anaphase: synchronization of chromatid separation

Activation and silencing of spindle checkpoint during mitosis.



(a) An unattached kinetochore sends out a 'wait' signal to halt cell-cycle progression at the metaphase–anaphase transition. This wait signal is generated by dynamic association of the spindle-checkpoint components with the unattached kinetochore, which leads to the inhibition of Cdc20/FZY by the checkpoint components. As a result, anaphase-promoting complex (APC) is inactivated and the cells are arrested at metaphase. Upon proper attachment of microtubules to all kinetochores, checkpoint components dissociate from Cdc20/FZY, allowing the activation of APC by Cdc20/FZY. Ubiquitination of securin and cyclin B by APC leads to their destruction, thereby triggering chromosome segregation and anaphase onset.

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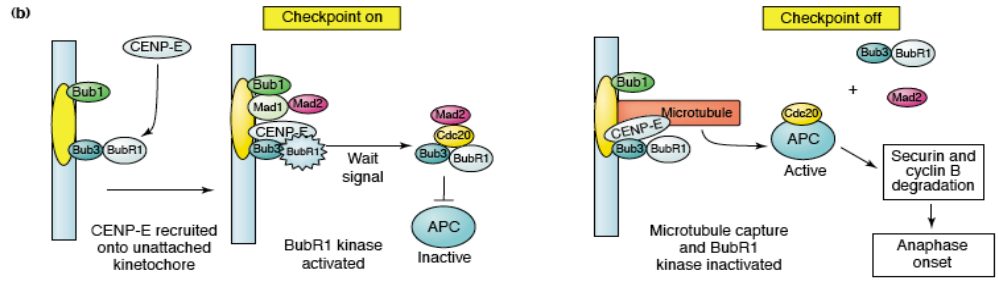

(b). CENP-E (kinetochore-associated kinesin)-dependent BubR1 kinase is required for turning on and off of the spindle-checkpoint signal. BubR1 is required for recruiting CENP-E to the unattached kinetochore. Direct association of CENP-E and BubR1 activates BubR1 kinase activity, which is essential for mitotic checkpoint signaling. The spindle checkpoint is silenced by CENP-E dependent capturing of microtubule, which alters or disrupts the interaction of CENP-E with BubR1 and inactivates BubR1 kinase.

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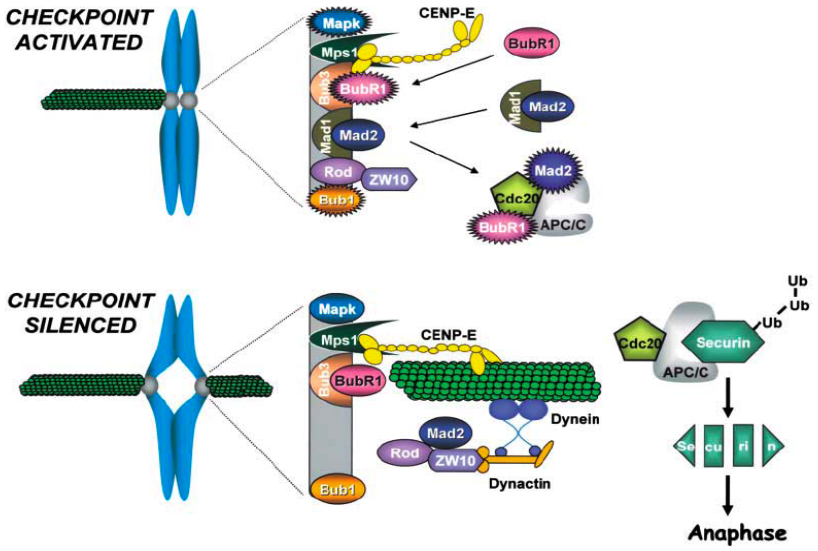


nuclear-envelope dynamics and NPC formation

NPC life cycle Assembly

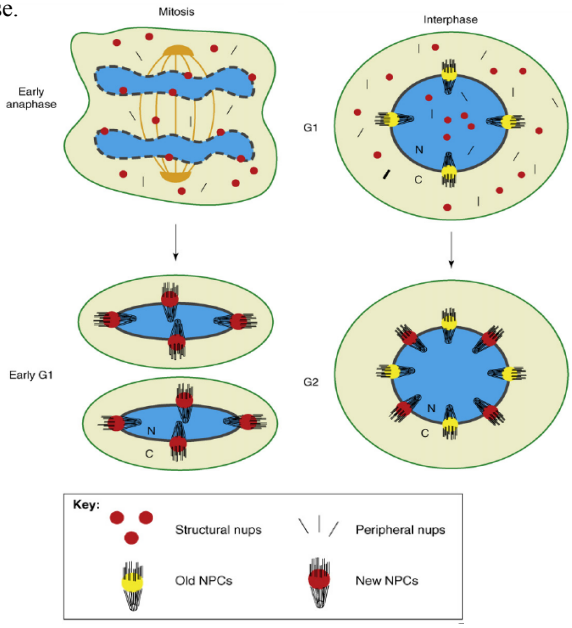
The biogenesis of nuclear pores is essential for cell survival and proliferation. There are two phases during the metazoan cell cycle in which NPCs need to be assembled: first, at the end of mitosis, when theNEreforms around the segregated chromosomes and second, during interphase in which the cells double their number of pores in preparation for the next round of division.

Even though both processes lead to the same final structure, they occur under very different conditions. Mitotic-NPC assembly takes place concomitantly with reformation of the nuclear membrane around segregated chromosomes. At this time, NPCs are rebuilt from disassembled subcomplexes that were dispersed into the cytoplasm during breakdown of the NE. By contrast, NPC assembly during interphase occurs in an intactNEusing newly synthesized nups and in a cellular environment in which the nucleus and the cytoplasm are physically separated.

Interphase assembly is the only existing mechanism in organisms, such as yeasts, that undergo closed mitosis (i.e. their NE does not break down during cell division). Although accumulating evidence indicates that both types of pore assembly might occur through similar processes, owing to their very different environments it is likely that differences will be uncovered.

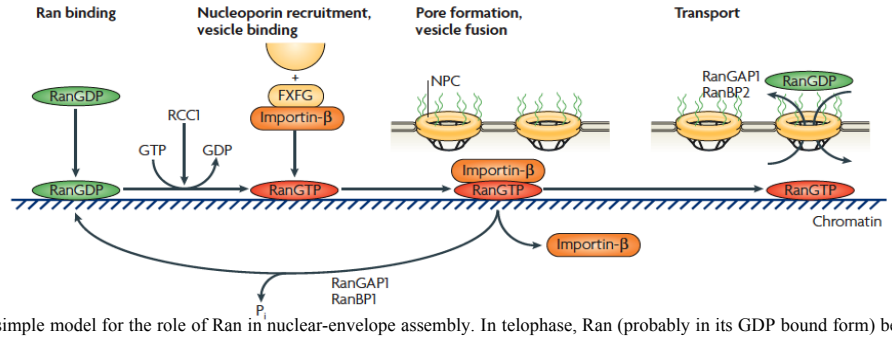
NPC assembly during mitosis and interphase.

Mitotic-NPC assembly occurs concomitantly with the formation of new nuclear envelopes (NEs) around chromatin. During this time, NPCs assemble by recycling subcomplexes that were dispersed into the cytoplasm during NPC and NE breakdown. Note that, during mitosis, the cytoplasmic and nuclear contents are mixed together. Mitotic assembly is a step-wise process that begins with the recruitment of structural nups to chromatin during early anaphase. By contrast, during interphase, NPCs assemble into an intact NE when the nucleus and cytoplasm are physically separated. During this process, NPCs use newly synthesized nucleoporins present on both sides of the nuclear envelope.



Concentration of Ran on chromatin induces decondensation, nuclear envelope formation and nuclear pore complex assembly

European Journal of Cell Biology **81**, 623–633 (2002, November)

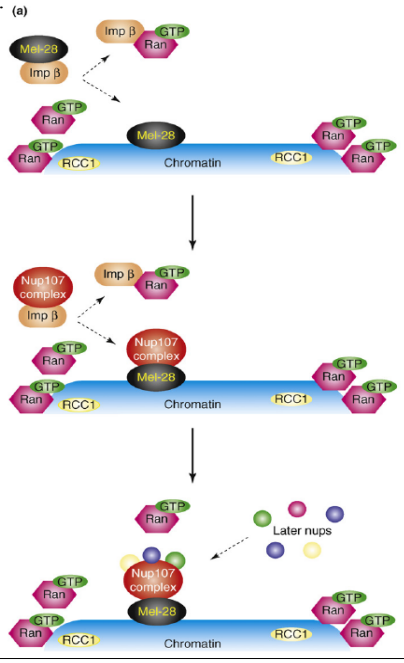


A simple model for the role of Ran in nuclear-envelope assembly. In telophase, Ran (probably in its GDP bound form) becomes recruited and concentrated on the surface of chromatin. Localized generation of RanGTP by the guanine nucleotide-exchange factor RCC1 recruits nucleoporins and vesicles through importin-β, which acts as a localization factor. The interaction of RanGTP with importin-β complexes causes their local release and assembly into nuclear pore complexes (NPCs), the fusion of vesicles to form a double membrane and the restarting of nuclear transport. Importin-β is released from Ran by the action of the Ran GTPase-activating proteins RanGAP1 and RanBP1 (Ran binding protein-1) or RanBP2, which stimulate GTP hydrolysis by Ran. FXFG represents a Phe-Gly (FG) repeat sequence.

Importin b and RanGTPase regulation of NPC assembly.

Interphase assembly

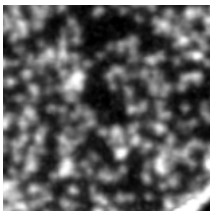
During mitosis, importin β binds and sequesters the Elys/Mel-28 nucleoporin (Mel-28), preventing its interaction with chromatin. When the importin-β–Mel-28 complexes are in the proximity of DNA, where there is a high concentration of RanGTP owing to the chromatin association of the Ran GDP–GTP exchange factor [regulator of chromosome condensation (RCC1)], RanGTP binds to the transport receptor, thereby releasing Mel-28 and enabling it to bind to chromatin. Following the same mechanism, the importin- β-bound Nup107–160 complex is released by RanGTP in the proximity of DNA and recruited to chromatin through Mel-28. The chromatin-bound Nup107–160 complex can then recruit other nucleoporins in a step-wise manner.

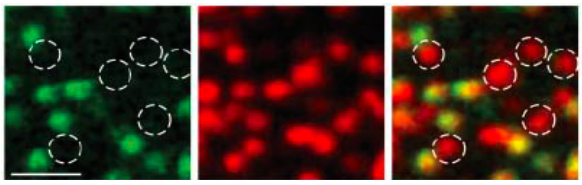


Nuclear Pores Form de Novo from Both Sides of the Nuclear Envelope

Maximiliano A. D'Angelo,* Daniel J. Anderson,* Erin Richard, Martin W. Hetzer†

Nuclear pore complexes are multiprotein channels that span the double lipid bilayer of the nuclear envelope. How new pores are inserted into the intact nuclear envelope of proliferating and differentiating eukaryotic cells is unknown. We found that the Nup107-160 complex was incorporated into assembly sites in the nuclear envelope from both the nucleoplasmic and the cytoplasmic sides. Nuclear pore insertion required the generation of Ran guanosine triphosphate in the nuclear and cytoplasmic compartments. Newly formed nuclear pore complexes did not contain structural components of preexisting pores, suggesting that they can form de novo.





Experiments using in vitro-assembled nuclei have indicated that NPCs can form de novo, without using subunits from pre-existing pores

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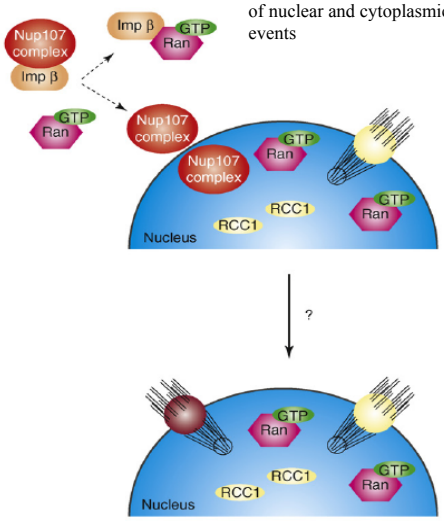
Importin b and RanGTPase regulation of NPC assembly.

Interphase assembly: During interphase, the number of pores doubles to prepare the cells for re-entering mitosis.
Previously, it was believed that NPC doubling was restricted to S-phase; however, recent studies have indicated that NPCs are assembled continuously from G1 to G2 phases.
NPC assembly during interphase requires the RanGTP-dependent release of the Nup107–160 complex from importin β on the cytoplasmic and nuclear side of the NE. How the released complexes coordinate the formation of a functional NPC from both sides of the nuclear envelope is still unclear.

interphase NPC assembly requires the coordination of nuclear and cytoplasmic events



Interphase NPC assembly form independently of pre-existing pores and from both sides of the nuclear envelope, following a mechanism that is at least partially conserved with mitotic-NPC assembly: This raises the question of whether the chromatin-bound intermediates observed during mitosis indeed represent that part of the pore that is inserted into the NE from the nucleoplasmic side during interphase.
If so, the mitotic- and interphase-assembly mechanisms would involve a coordinated interaction between chromatin- bound subcomplexes, cytoplasmic subcomplexes and transmembrane NPC components to assemble the multiprotein NPC into the double membrane of the NE.

Disassembly

NPC disassembly has only been described during mitosis and there is no evidence of pores being dismantled during interphase.

Similar to the assembly process, NPC disassembly takes place through an ordered process.

nucleocytoplasmic transport