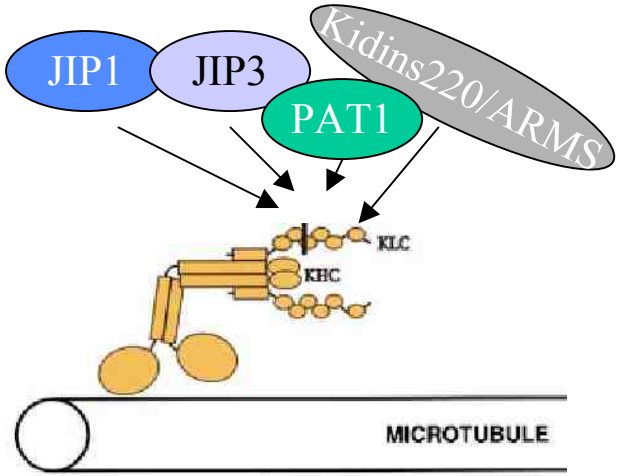
Do kinesin-1 cargoes that bind through KLC compete with each other for transport?

A Does overexpression of Kidins and truncated kidins inhibit JIP1 or JIP3 tip localization?

	JIP1	JIP3
GFP-kidins		
GFP-truncated kidins		



B Does overexpression of JIP1 or JIP3 inhibit kidins tip localization?

	kidins
JIP1	

	kidins
JIP3	

C Does overexpression of PAT1 inhibit JIP1 or JIP3 tip localization?

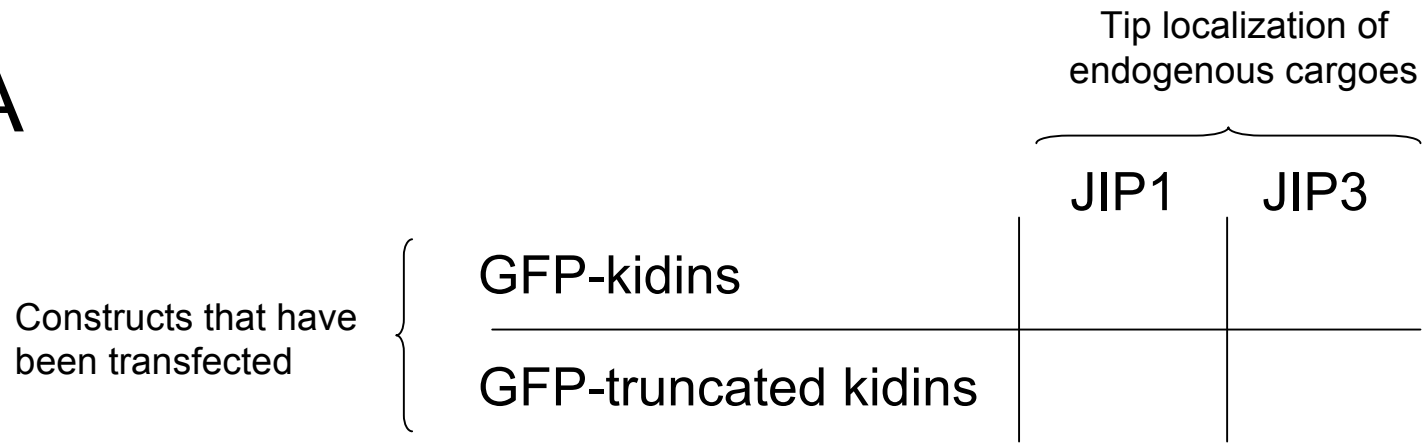
	JIP1
PAT1	

	JIP3
PAT1	

A

Does overexpression of Kidins and truncated kidins inhibit JIP1 or JIP3 tip localization?

A



A

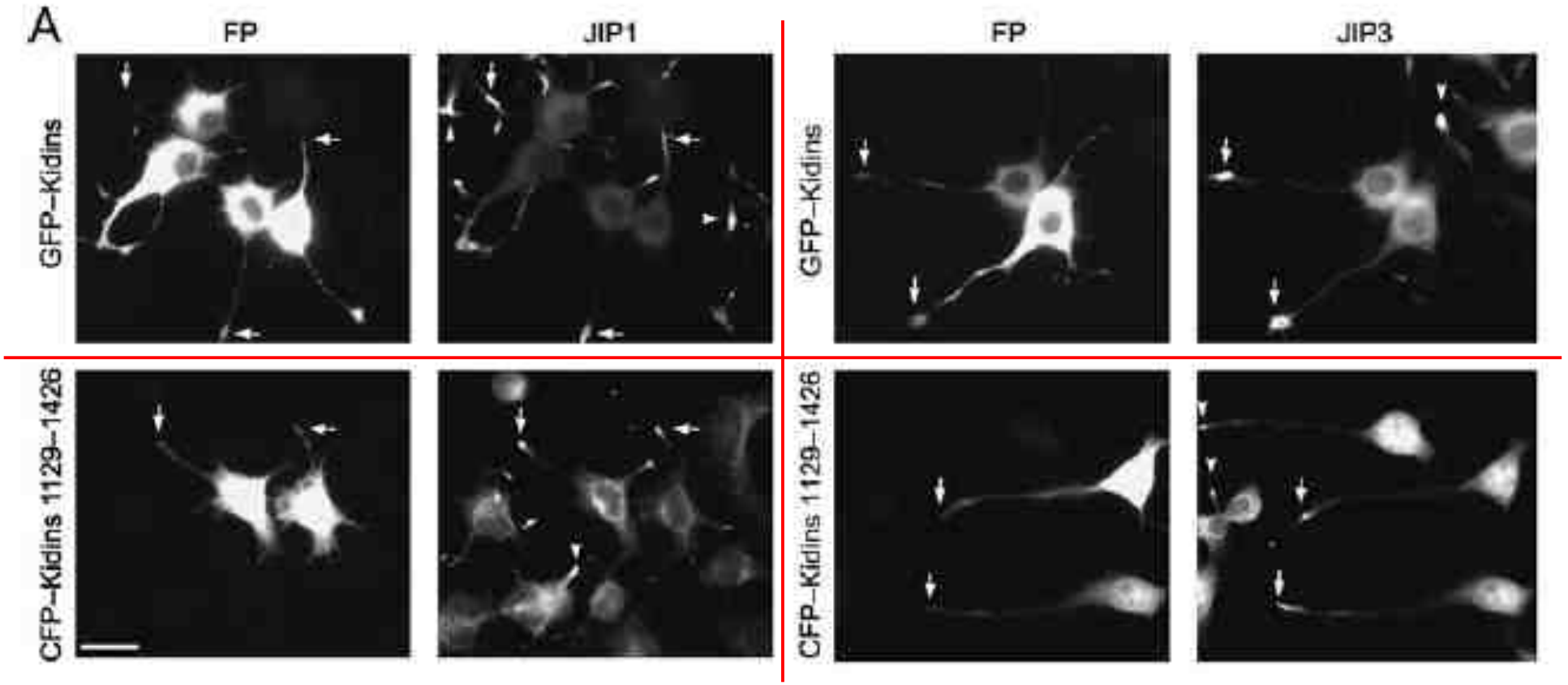
Tip localization of endogenous cargoes

JIP1 JIP3

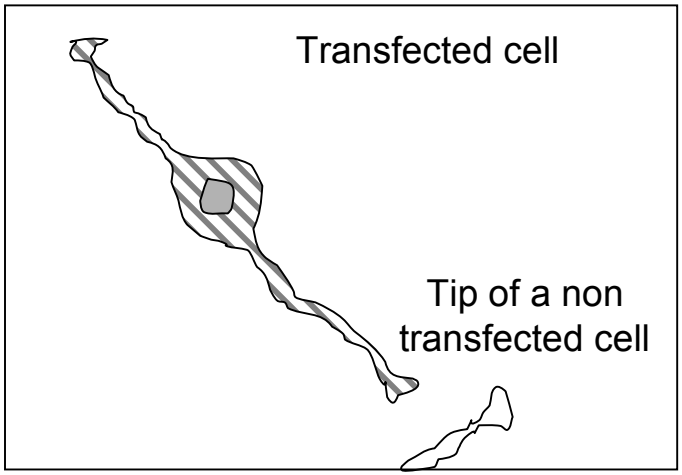
Constructs that have been transfected

GFP-kidins

GFP-truncated kidins

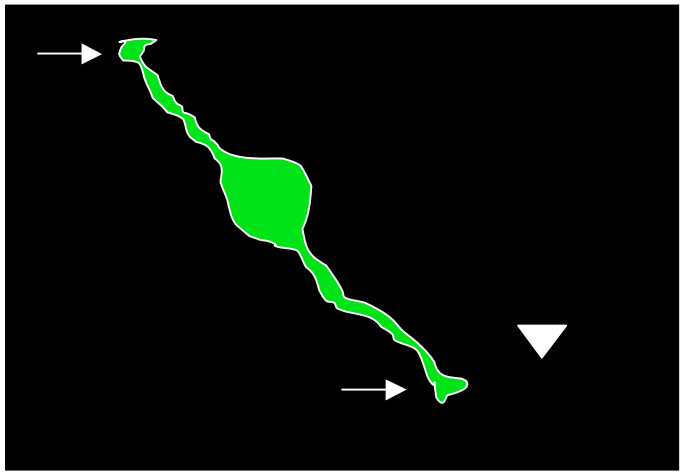


Transfection of GFP-Kidins220/ARMS constructs & IHC for endogenous JIP-1



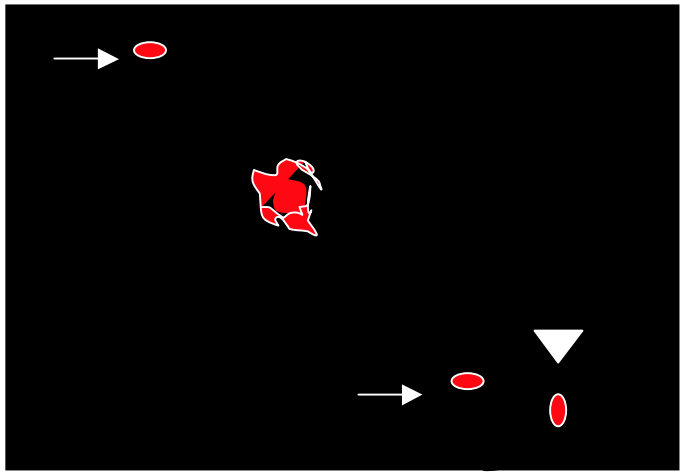
	JIP1	JIP3
GFP-kidins	?	
GFP-truncated kidins		

GFP–Kidins220/ARMS

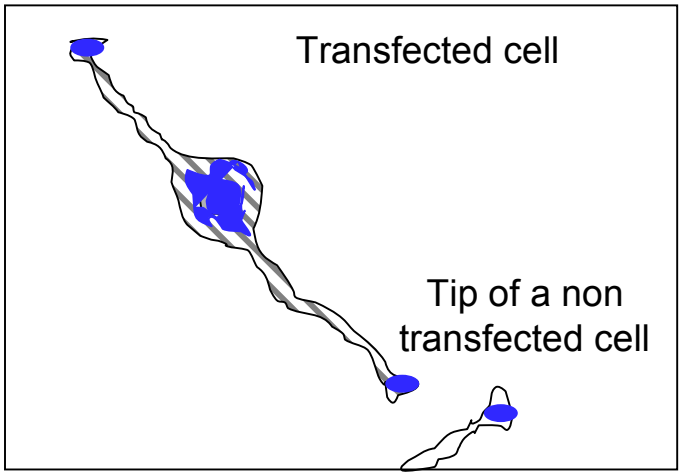


Anti-JIP-1
2° FITC

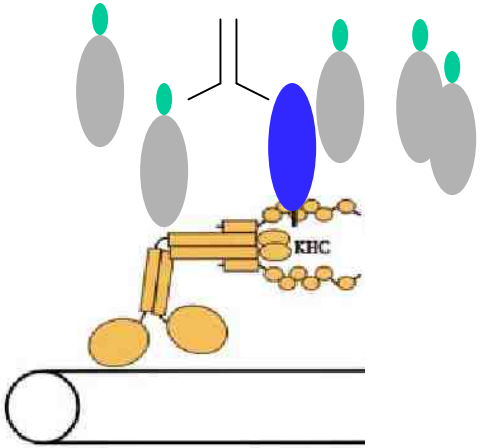
Anti-JIP1



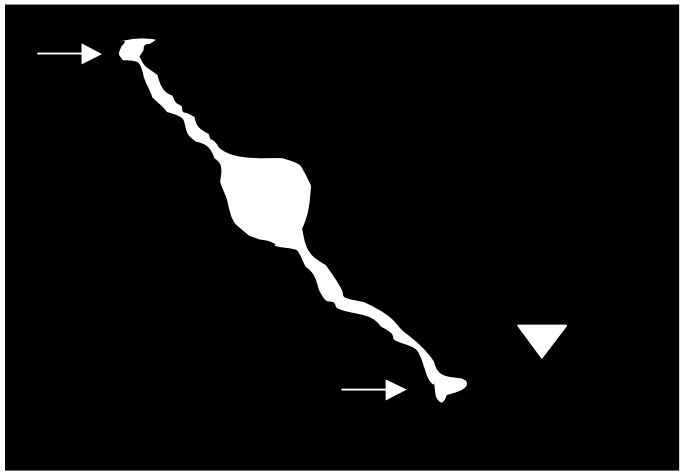
Transfection of truncated KLC-binding constructs & IHC for endogenous JIP-1



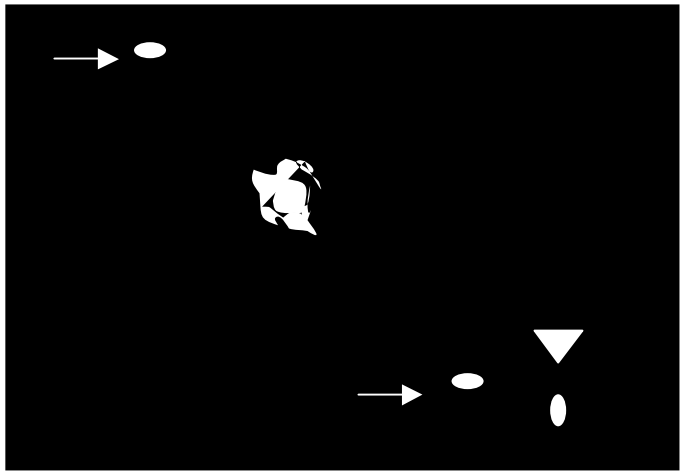
	JIP1	JIP3
GFP-kidins	+	
GFP-truncated kidins		



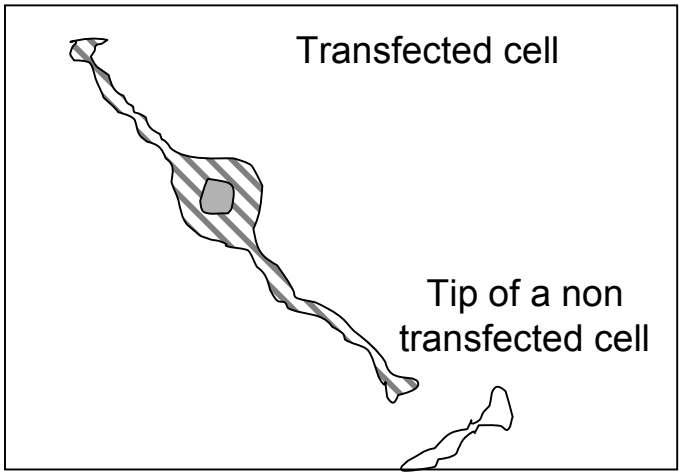
GFP–Kidins220/ARMS



JIP1

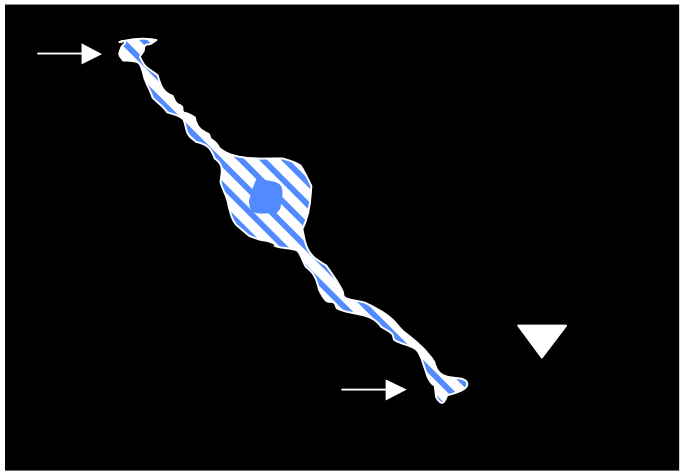


Transfection of truncated KLC-binding constructs & IHC for Endogenous JIP-1



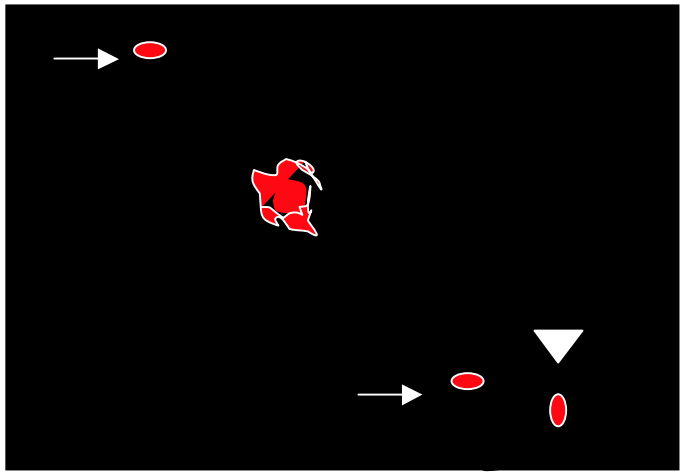
	JIP1	JIP3
GFP-kidins		
GFP-truncated kidins	?	

CFP–Kidins220/ARMS (1129–1426)

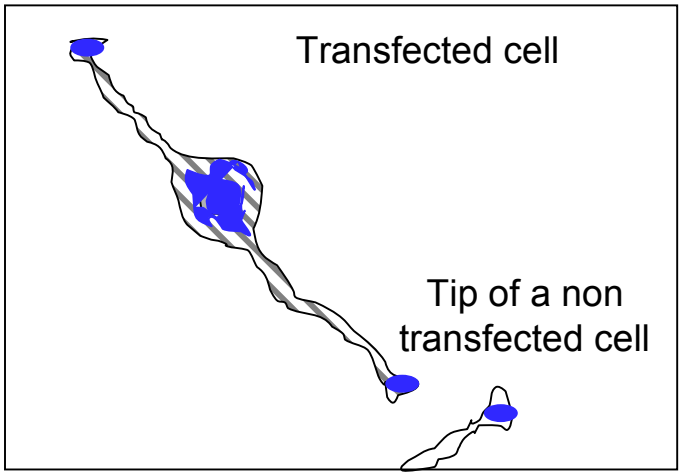


Anti-JIP-1
2° FITC

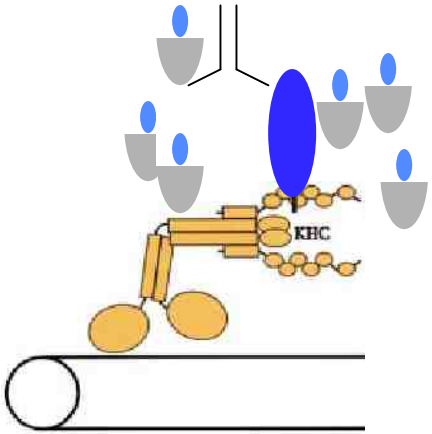
Anti-JIP1



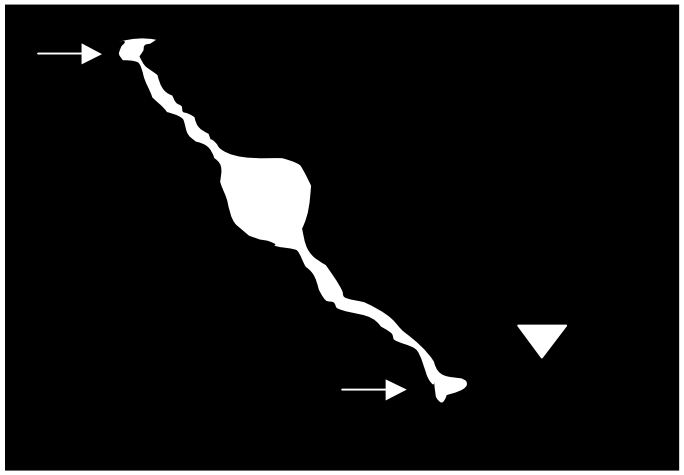
Transfection of truncated KLC-binding constructs & IHC for Endogenous JIP-1



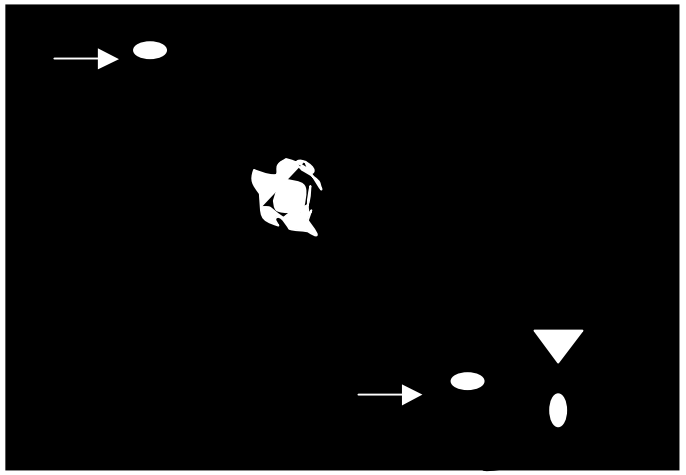
	JIP1	JIP3
GFP-kidins		
GFP-truncated kidins	+	



CFP–Kidins220/ARMS (1129–1426)



JIP1

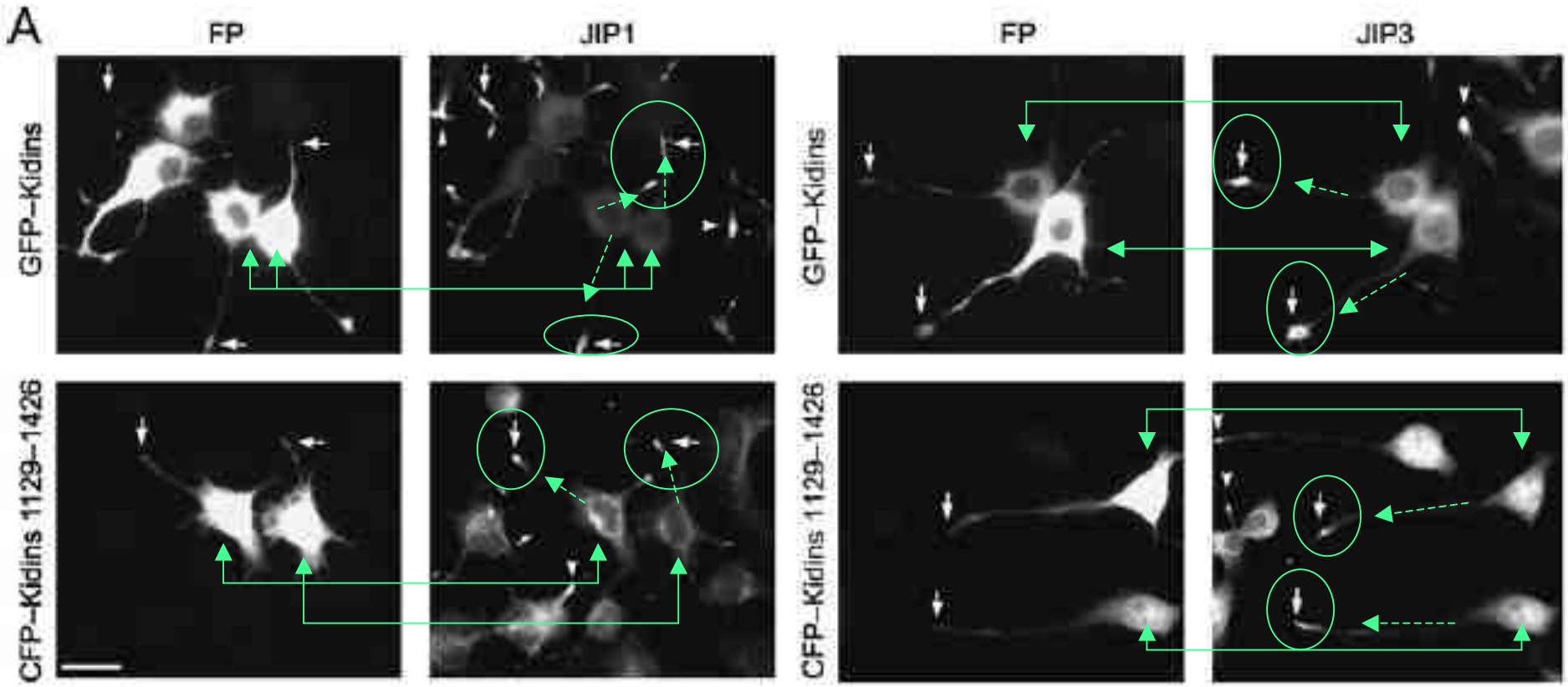


A

Overexpression of Kidins and truncated kidins does not inhibit JIP1 or JIP3 tip localization

Constructs that have been transfected	Tip localization of endogenous cargoes: JIP1	JIP3
GFP-kidins	+	+
GFP-truncated kidins	+	+

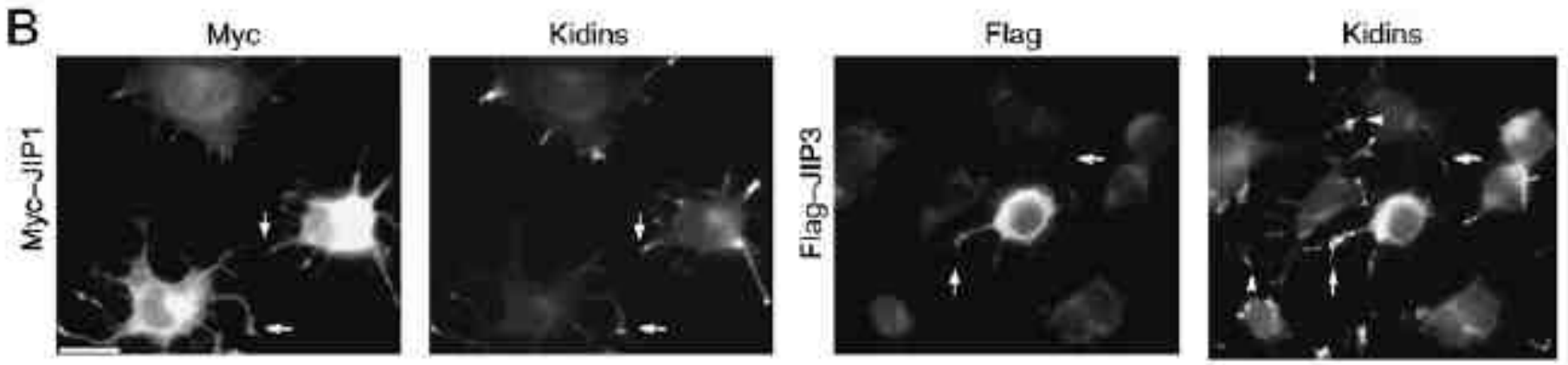
….



B

	kidins
JIP1	

	kidins
JIP3	



C

	JIP1
PAT1	

	JIP3
PAT1	

Conclusion: Kinesin-1 cargoes that bind through KLC do not compete with each other for transport.

Cooperative or independent transport?

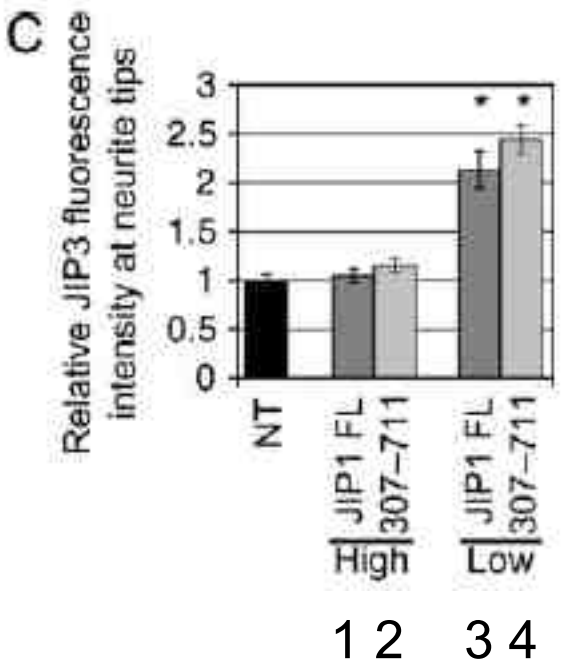
Differentiated CAD cells were transfected with plasmids encoding Myc-tagged full-length JIP1 and relative JIP3 fluorescence intensity at neurite tips was measured.

1- In cells expressing high levels of Myc–JIP1, the amount of endogenous JIP3 localized at neurite tips was similar to that in untransfected cells (NT), suggesting that there is no competition between JIP1 and JIP3 for Kinesin-1-mediated transport.

2- Similar results were obtained upon expression of a truncated version of JIP1 that binds both JIP3 and KLC but not JNK [Myc–JIP1 (307–711).

3- Surprisingly, in differentiated CAD cells expressing Myc–JIP1 at levels similar to the endogenous JIP1 protein (based on localization of the Myc-tagged protein to the neurite tip), there is a twofold increase in the amount of JIP3 at the tips of neurites.

4- Similar results were obtained upon low expression of a truncated version of JIP1 that binds both JIP3 and KLC but not JNK [Myc–JIP1 (307–711).

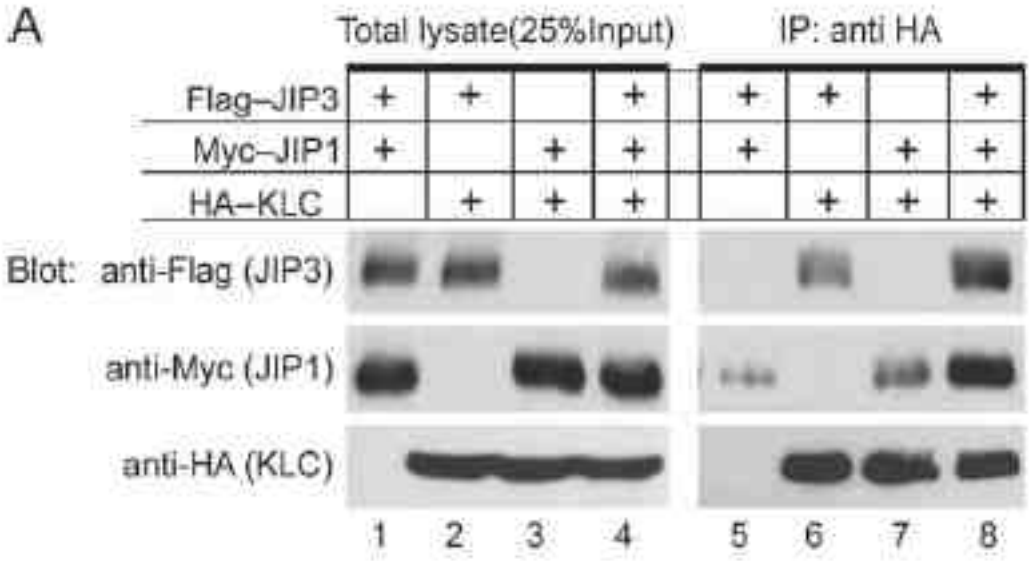


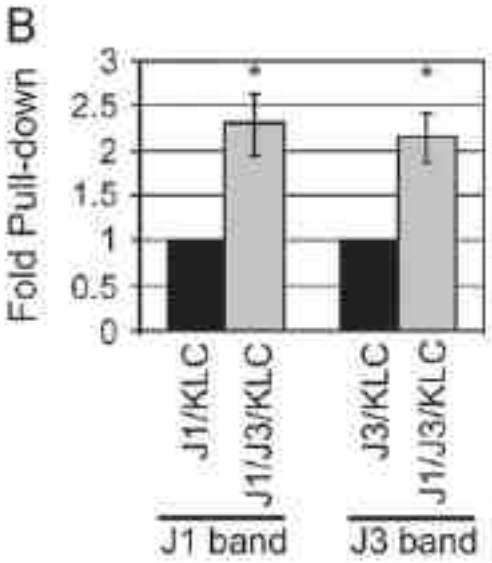
These results suggest that JIP1 facilitates transport of JIP3.

Cooperative binding of JIP1 and JIP3 to KLC

Lysates of COS cells expressing Flag–JIP3, Myc–JIP1 or HA–KLC were combined and analyzed by Western blot either directly (total lysate) or after immunoprecipitation with an anti-HA antibody

Quantification from six independent experiments of the fold increase in JIP1 or JIP3 pull-down in the absence and presence of the other JIP.
*p < 0.01

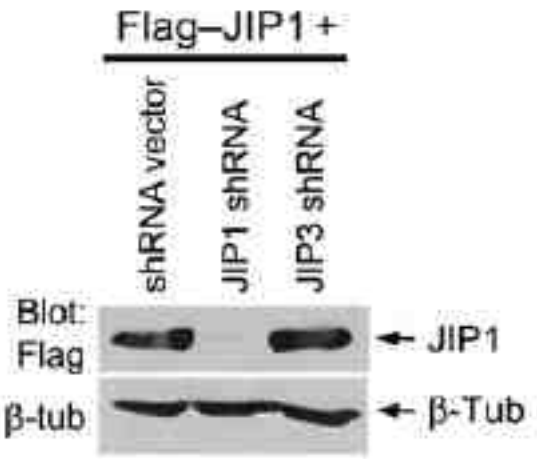




knockdown

Specificity of RNAi knockdown.

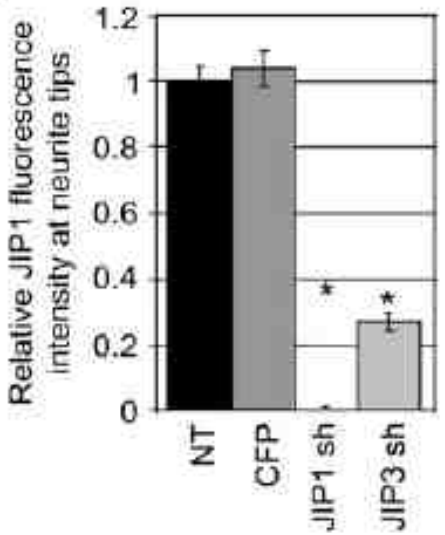
A



(A) COS cells were cotransfected with Flag–JIP1 and either an empty shRNA vector or shRNA plasmids targeting JIP1. The levels of remaining JIP1 were determined by immunoblotting total cell lysates with an anti-Flag antibody. Equal loading of total protein is indicated by blotting with an anti-b-tubulin antibody.

Knockdown of JIP1 abrogates JIP3 transport.

E

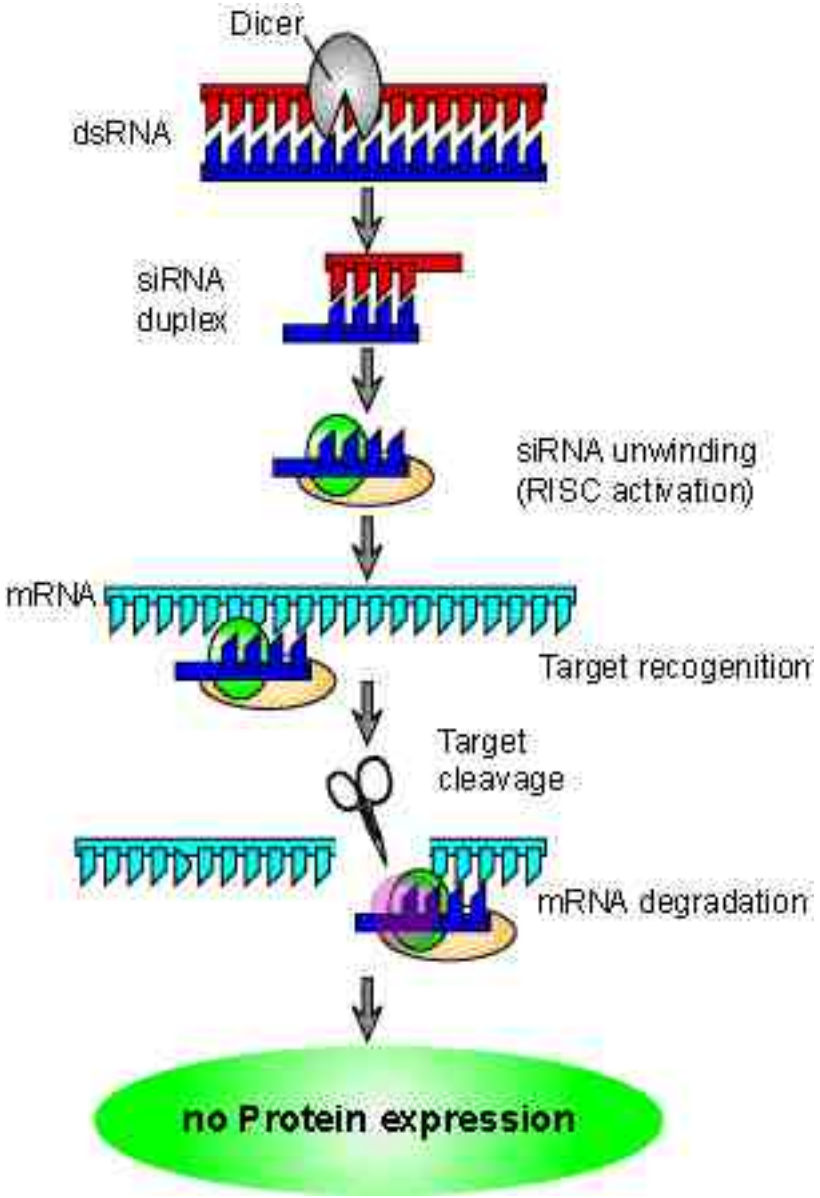


(E) Quantification of the relative JIP1 fluorescence intensity at neurite tips in transfected cells compared with NT cells.
n > 160 neurites for each construct. Error bars ¼ SEM. *p < 0.01. SEM

RNA Interference

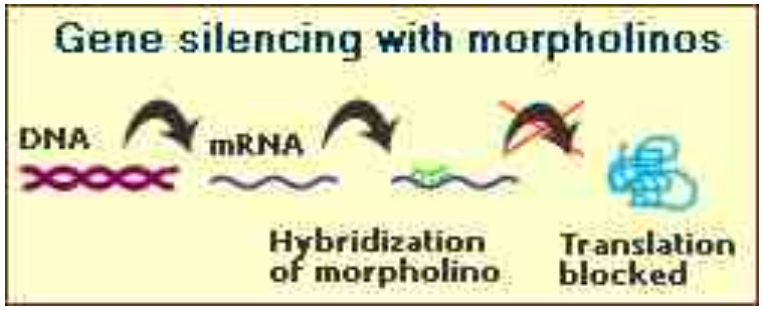
RNAi is a post-transcriptional process triggered by the introduction of double-stranded RNA (dsRNA) which leads to gene silencing in a sequence-specific manner.

RNA interference (RNAi) is one of the most exciting discoveries of the past decade in functional genomics. RNAi is rapidly becoming an important method for analyzing gene functions in eukaryotes and holds promise for the development of therapeutic gene silencing.

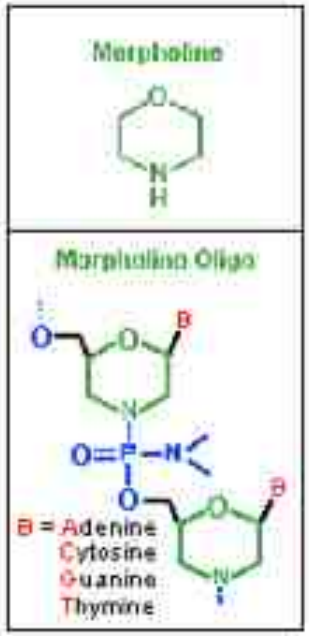


Other mRNA silencing technique

Morpholino antisense oligos



Morpholino oligos are short chains of about 25 Morpholino subunits. Each subunit is comprised of a nucleic acid base, a morpholine ring and a non-ionic phosphoro-diamidate intersubunit linkage. **Morpholinos do not degrade their RNA targets**, but instead act via an RNAse H-independent steric blocking mechanism. With their requirement for greater complementarity with their target RNAs, Morpholinos are free of the widespread off-target expression modulation typical of knockdowns which rely on RISC or RNase-H activity. They are completely stable in cells and do not induce immune responses.

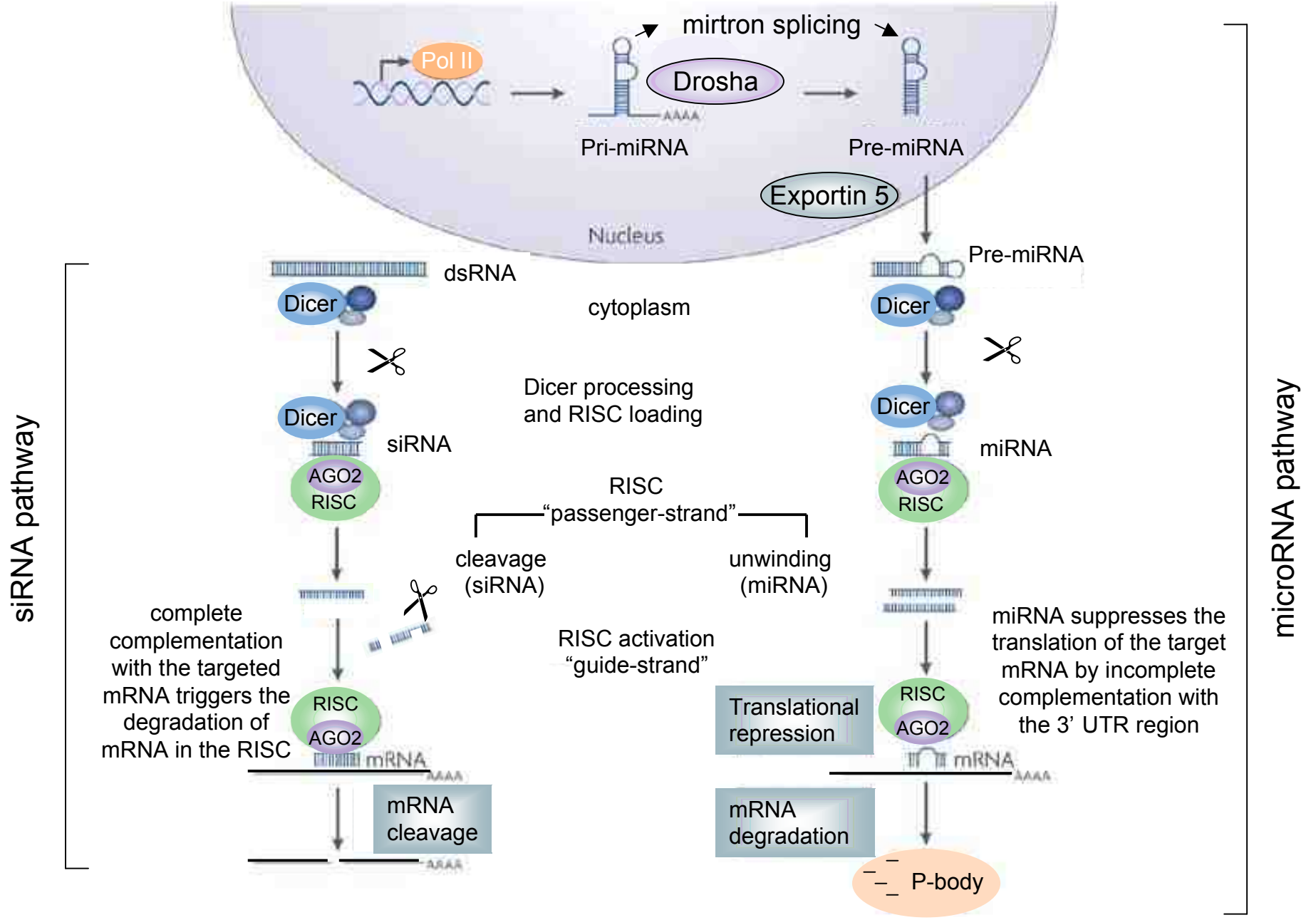


siRNA/miRNA

RNAi has been reported to naturally occur in organisms as diverse as **nematodes, trypanosomes, plants and fungi**. It most likely serves to protect organisms from viruses, modulate transposon activity and eliminate aberrant transcription products.

The first evidence that dsRNA could achieve efficient gene silencing through RNAi came from studies on the nematode Caenorhabditis elegans. Further analyses in the fruit fly Drosophila melanogaster have contributed greatly toward understanding the biochemical nature of the RNAi pathway.

Endogenously expressed **siRNAs** have **not been found in mammals**. However, many micro RNAs (**miRNAs**) have been identified in various organisms and cell types. These miRNAs are produced by **Dicer cleavage** of longer (~70 nt) endogenous precursors with imperfect hairpin RNA structures. The miRNAs bind to sites that have **partial sequence complementarity** in the 3' untranslated region of the target mRNA, causing **repression of translation and inhibition of protein synthesis**.



Nature Reviews Drug Discovery 6, 443-453 (June 2007)

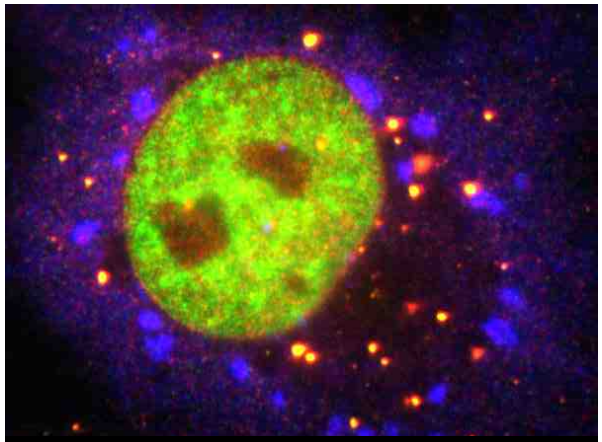
RNA interference (RNAi) pathways are guided by small RNAs that include small interfering RNA (siRNA) and microRNAs (miRNAs).

The siRNA pathway begins with cleavage of long double-stranded RNA (dsRNA) by the Dicer enzyme complex into siRNA. These siRNAs are incorporated into Argonaute 2 (AGO2) and the RNAi-induced silencing complex (RISC).

If the RNA duplex loaded onto RISC has perfect sequence complementarity, AGO2 cleaves the passenger (sense) strand so that active RISC containing the guide (antisense) strand is produced. The siRNA guide strand recognizes target sites to direct mRNA cleavage (carried out by the catalytic domain of AGO2). RNAi therapeutics developed to harness the siRNA pathway typically involve the delivery of synthetic siRNA into the cell cytoplasm. The microRNA pathway begins with endogenously encoded primary microRNA transcripts (pri-miRNAs) that are transcribed by RNA polymerase II (Pol II) and are processed by the Drosha enzyme complex to yield precursor miRNAs (pre-miRNAs). These precursors are then exported to the cytoplasm by exportin 5 and subsequently bind to the Dicer enzyme complex, which processes the pre-miRNA for loading onto the AGO2–RISC complex. When the RNA duplex loaded onto RISC has imperfect sequence complementarity, the passenger (sense) strand is unwound leaving a mature miRNA bound to active RISC. The mature miRNA recognizes target sites (typically in the 3'-UTR) in the mRNA, leading to direct translational inhibition. Binding of miRNA to target mRNA may also lead to mRNA target degradation in processing (P)-bodies.

Citoplasmic RNA granules: posttranscriptional regulation of gene expression.

RNA granules contain various ribosomal subunits, translation factors, decay enzymes, helicases, scaffold proteins, and RNA-binding proteins, they control the localization, stability, and translation of their RNA cargo (lack a limiting membrane and visible using light microscopy).



Cytoplasmic mRNA is routed to "stress granules" (blue) after translational silencing, and delivered to "processig bodies" (yellow) for degradation.

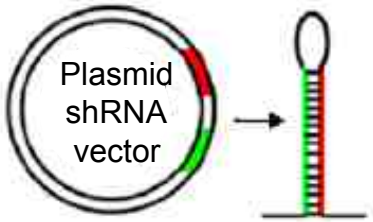
• P-bodies • Stress granules	Storage/degradation
• Neuronal granules	Transport from cell body to dendritic synapses for local translation

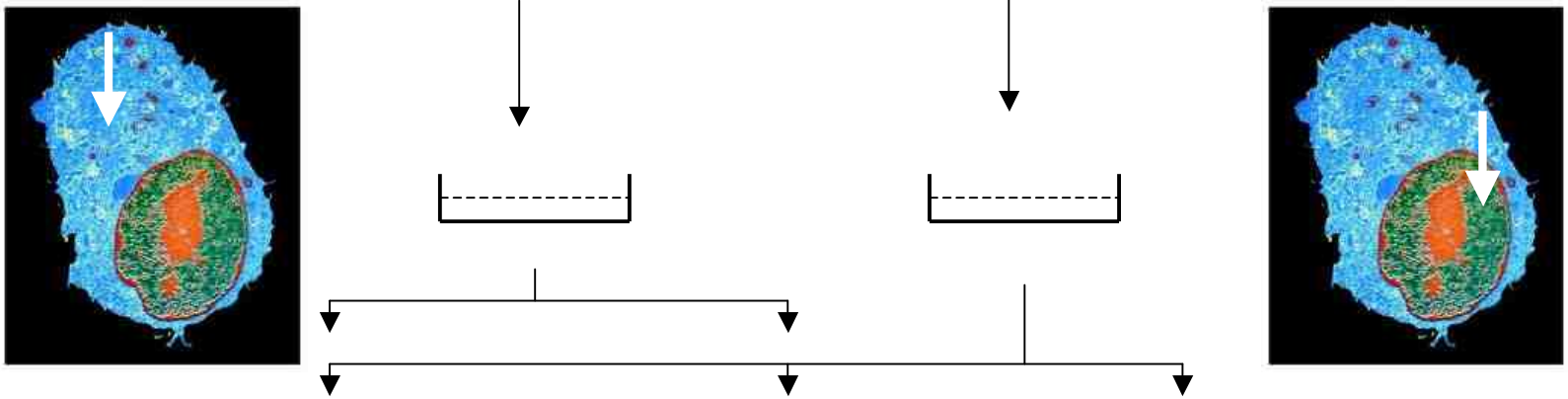
siRNA strategies:

Direct delivery
siRNA duplex

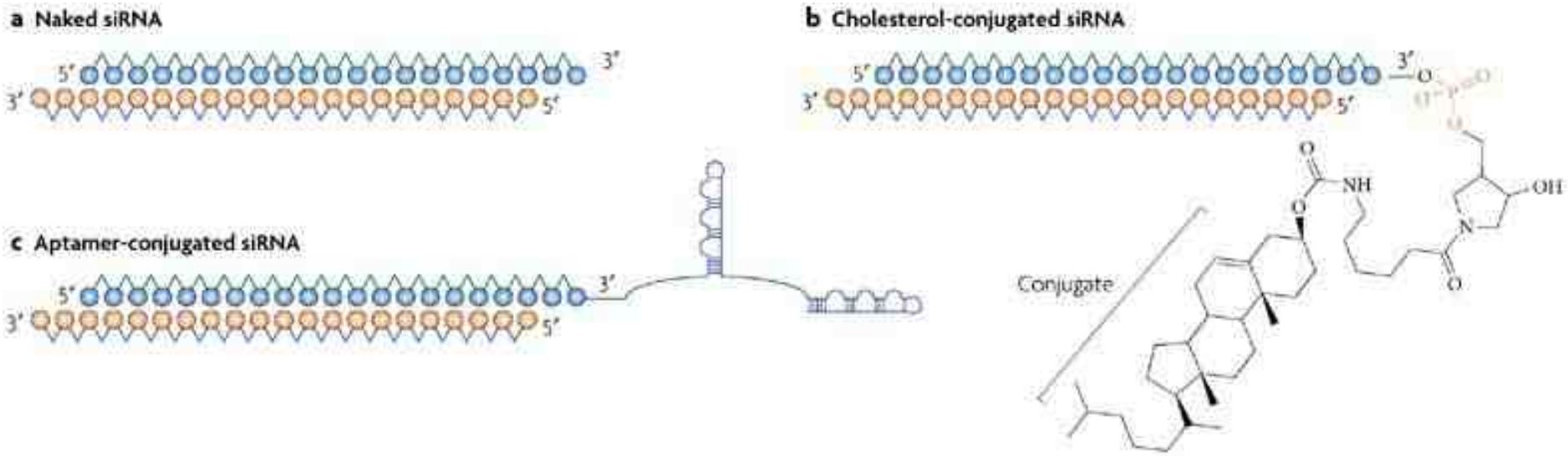
siRNA duplex

Integration of plasmids
shRNAs vector





Different strategies have been used to deliver and achieve RNAi-mediated silencing in vivo.



a | **Direct injection** of naked siRNA (unmodified or chemically modified) has proven efficacious in multiple contexts of ocular, respiratory and central nervous system disease.

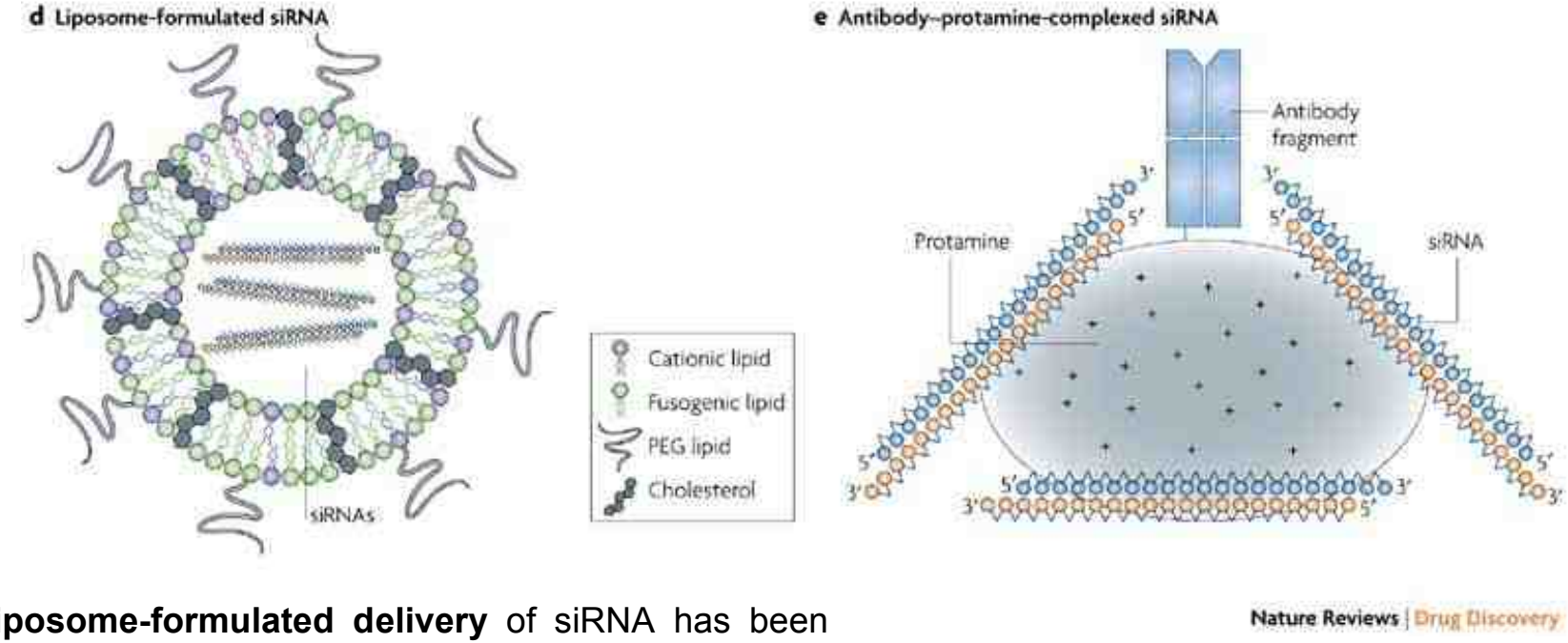
c | **Aptamer–siRNA** conjugates silence target genes in tumour cells following local injection in a tumour xenograft model.

b | **Direct conjugation** of siRNA to a natural ligand such as cholesterol has demonstrated in vivo silencing in hepatocytes following systemic administration.

Note: The passenger strand and the guide strand are represented in blue and orange, respectively.

Nature Reviews Drug Discovery 6, 443-453 (June 2007)

Different strategies have been used to deliver and achieve RNAi-mediated silencing in vivo.



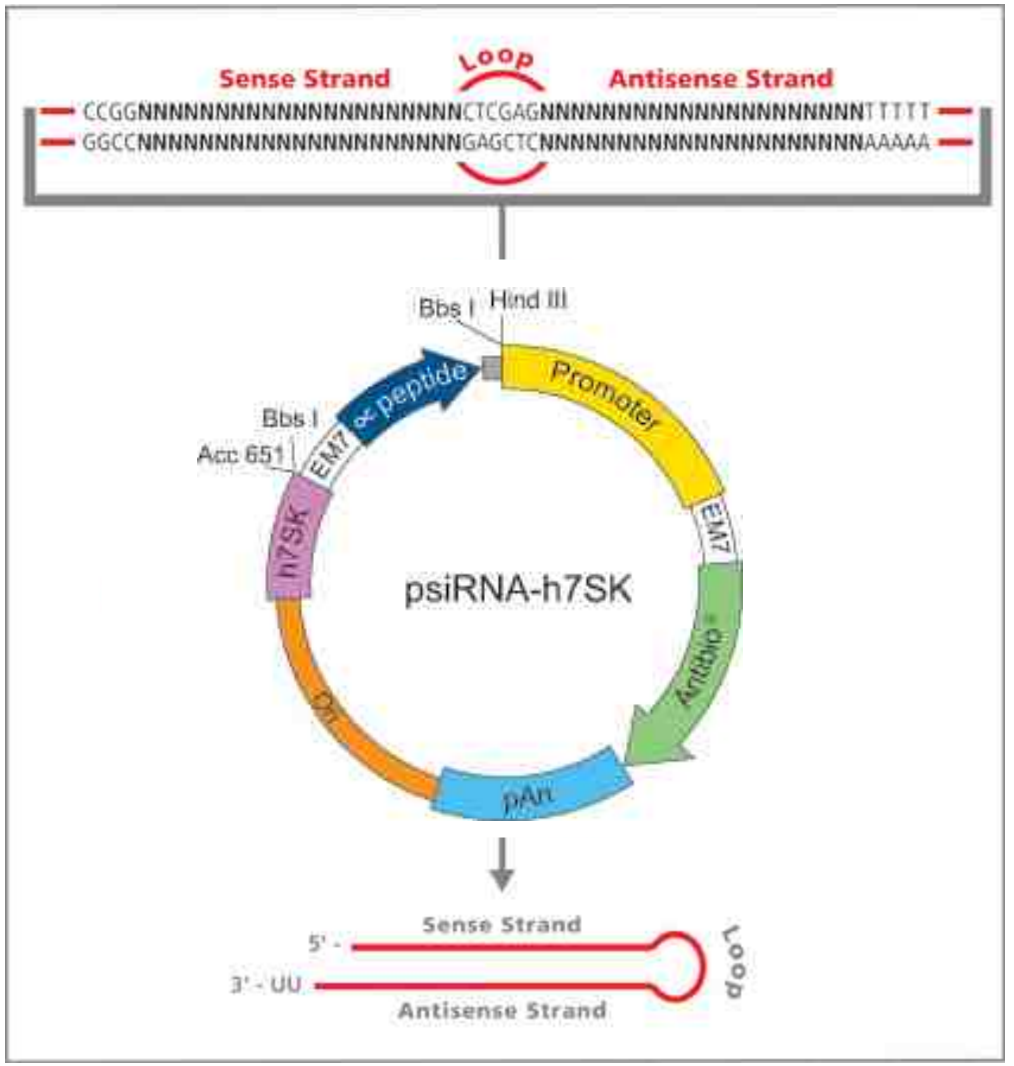
d | **Liposome-formulated delivery** of siRNA has been used to silence multiple targets following systemic administration. The composition of a stable nucleic acid–lipid particle (SNALP) is shown. Cationic lipids aid formulation, cellular uptake and endosomal release. Fusogenic lipids also function in endosomal release. Polyethylene glycosylated (PEG) lipids stabilize the formulation, regulate fusogenicity and shield surface charges. Cholesterol also helps to stabilize the formulation. Other cationic liposome formulations exist which may or may not contain a fusogenic or PEG lipid. The ratios of different components can vary between formulations.

e | **Antibody–protamine fusion proteins** have been used to non-covalently bind siRNAs through charge interactions and to deliver siRNA specifically to cells that express the surface receptor that is recognized by the antibody.

Nature Reviews Drug Discovery 6, 443-453 (June 2007)

Plasmid siRNA: psiRNA is an RNA polymerase III-based plasmid that produces short hairpin RNAs.

psiRNA is used to insert a DNA fragment of approximately 50 mer designed in such a way that after transcription from the human 7SK RNA polymerase III promoter it will generate short RNAs with a hairpin structure (shRNAs). shRNAs are more stable than synthetic siRNAs and since they are continuously expressed within the cells, this method permits long-lasting silencing of your gene of interest.



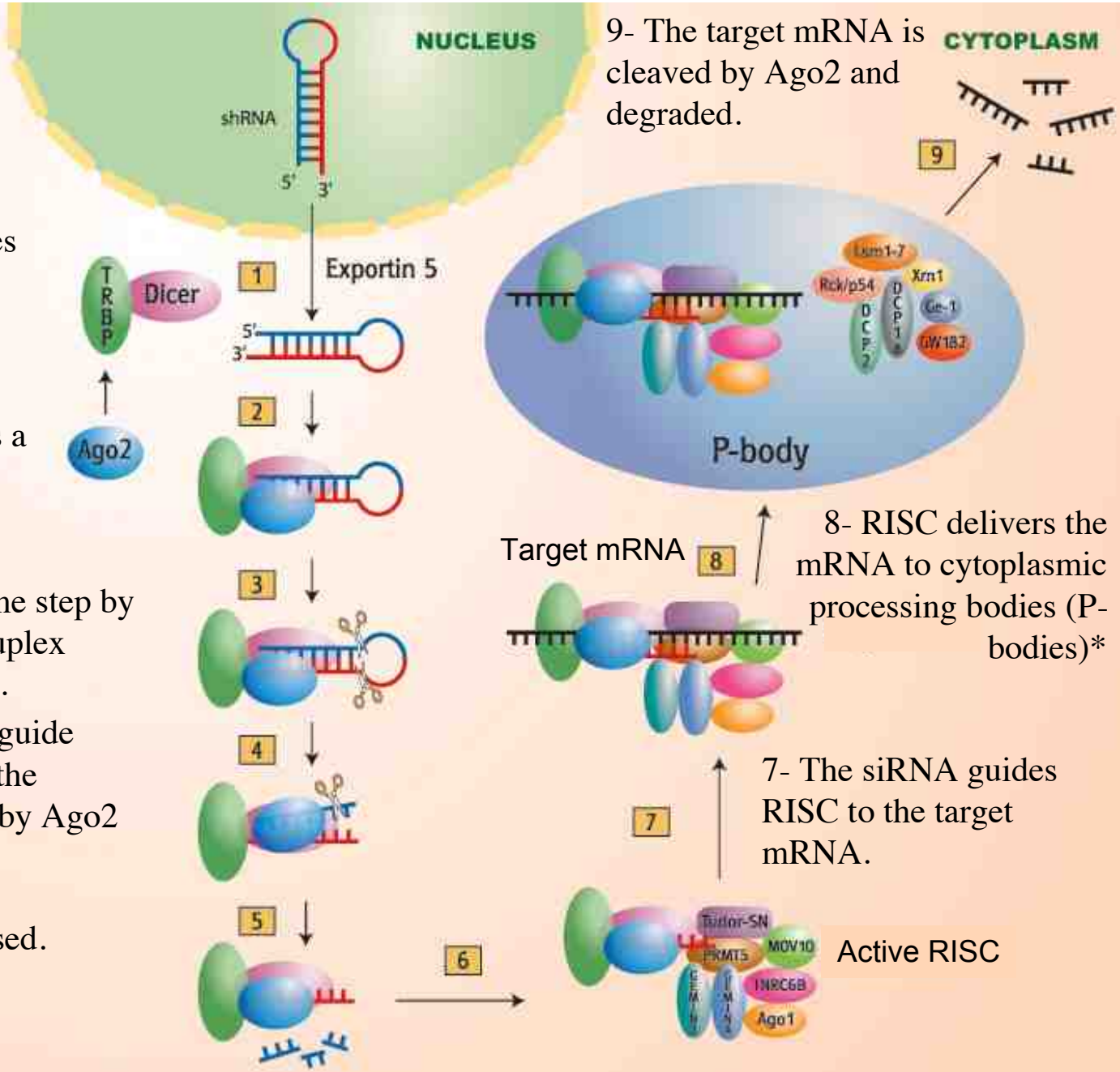
1- Plasmid-expressed short hairpin RNA (shRNA) requires the activity of endogenous Exportin 5 for nuclear export

2- Ago2 (Argonaute 2) is recruited by TRBP, that forms a dimer with Dicer, and then receives the shRNA

3- The shRNA is cleaved in one step by Dicer generating a 19-23 nt duplex siRNA with 2 nt 3' overhangs.

4- After identification of the "guide strand" in the siRNA duplex, the "passenger strand" is cleaved by Ago2

5- The "guide strand" is released.

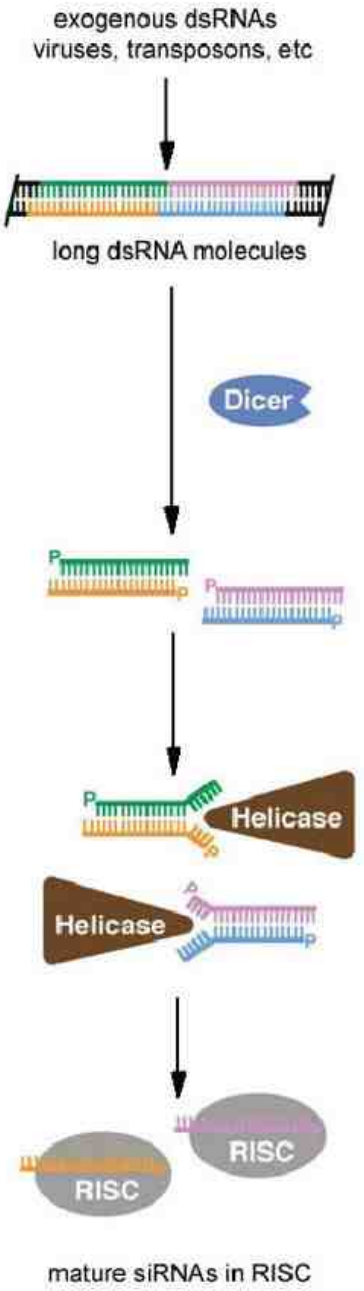


6- The "guide strand" is integrated in the active RISC that contains different Ago and Ago-associated proteins

*or GW-bodies

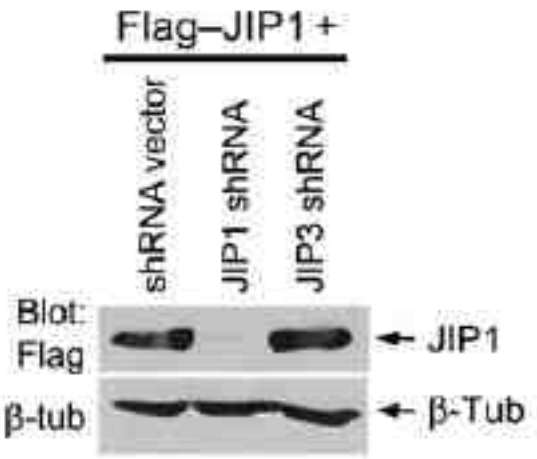
General Guidelines for siRNA target sequence design

- siRNA targeted sequence is usually 21 nt in length.
- Avoid regions within 50-100 bp of the start codon and the termination codon
- Avoid intron regions
- Avoid stretches of 4 or more bases such as AAAA, CCCC
- Avoid regions with GC content <30% or > 60%.
- Avoid repeats and low complex sequence
- Avoid single nucleotide polymorphism (SNP) sites
- Perform BLAST homology search to avoid off-target effects on other genes or sequences
- Always design negative controls by scrambling targeted siRNA sequence. The control RNA should have the same length and nucleotide composition as the siRNA but have at least 4-5 bases mismatched to the siRNA. Make sure the scrambling will not create new homology to other genes.



Specificity of RNAi knockdown.

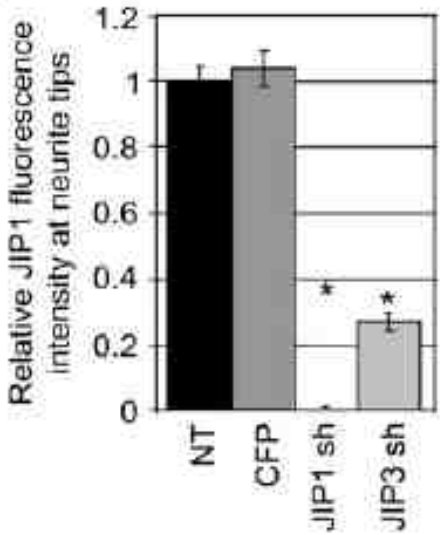
A



(A) COS cells were cotransfected with Flag–JIP1 and either an empty shRNA vector or shRNA plasmids targeting JIP1. The levels of remaining JIP1 were determined by immunoblotting total cell lysates with an anti-Flag antibody. Equal loading of total protein is indicated by blotting with an anti-b-tubulin antibody.

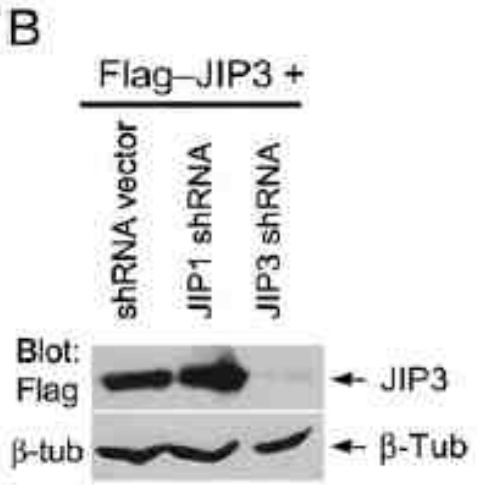
Knockdown of JIP1 abrogates JIP3 transport.

E



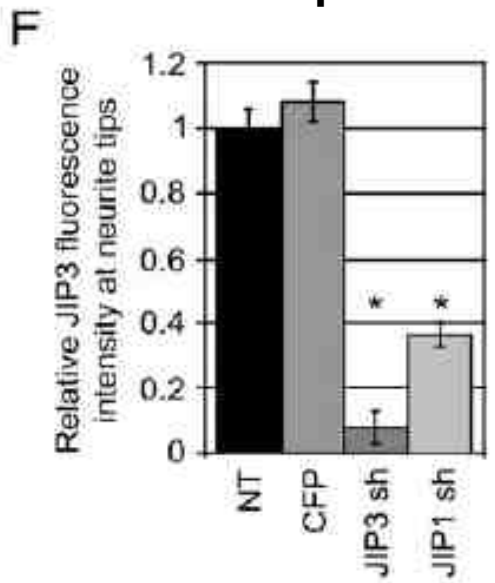
(E) Quantification of the relative JIP1 fluorescence intensity at neurite tips in transfected cells compared with NT cells.
n > 160 neurites for each construct. Error bars ¼ SEM. *p < 0.01. SEM

Specificity of RNAi knockdown.



(B) COS cells were cotransfected with Flag–JIP3 and either an empty shRNA vector or shRNA plasmids targeting JIP3. The levels of remaining JIP3 were determined by immunoblotting total cell lysates with an anti-Flag antibody. Equal loading of total protein is indicated by blotting with an anti-b-tubulin antibody.

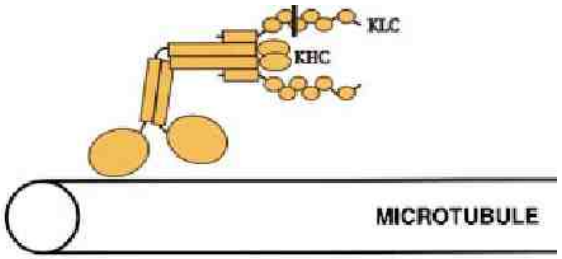
Knockdown of JIP3 abrogates JIP1 transport.

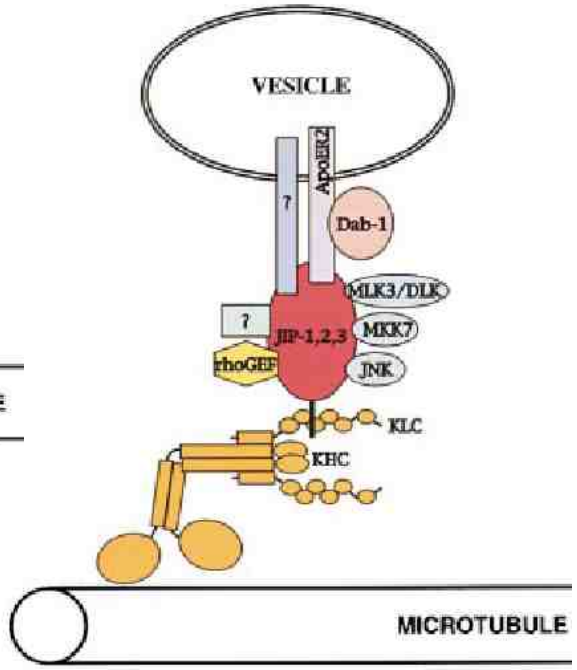


(F) Quantification of the relative or JIP3 fluorescence intensity at neurite tips in transfected cells compared with NT cells. n > 160 neurites for each construct. Error bars ¼ SEM. *p < 0.01. SEM

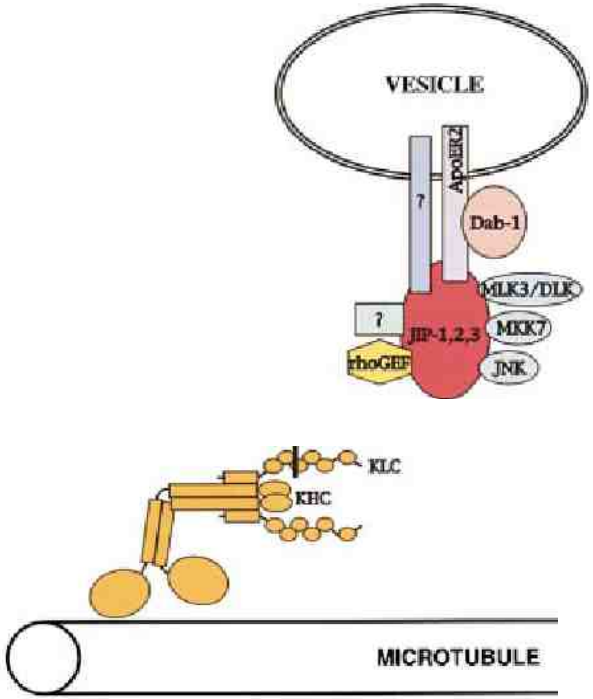
Are the JNK pathway kinases simply passive hitchhikers on the kinesin-1/JIP/vesicle complex, or can they actively regulate its transport?

Without cargoes, kinesins are running along microtubules?





Co-operative or competitive transport of different cargoes?



Signal for cargoes to detach?

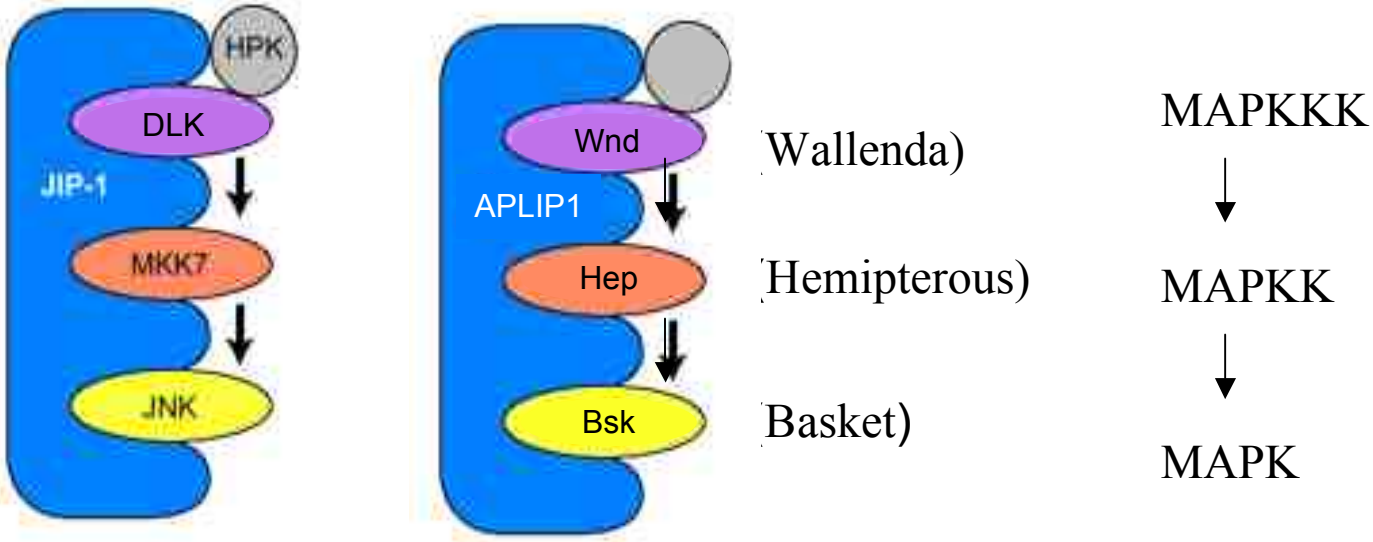
Current Biology 17, 1313–1317, August 7, 2007

Control of a Kinesin-Cargo Linkage Mechanism by JNK Pathway Kinases

Dai Horiuchi,[1,3] Catherine A. Collins,[2,3] Pavan Bhat,[2]
Rosemarie V. Barkus,[1] Aaron DiAntonio,[2]
and William M. Saxton[1,*]

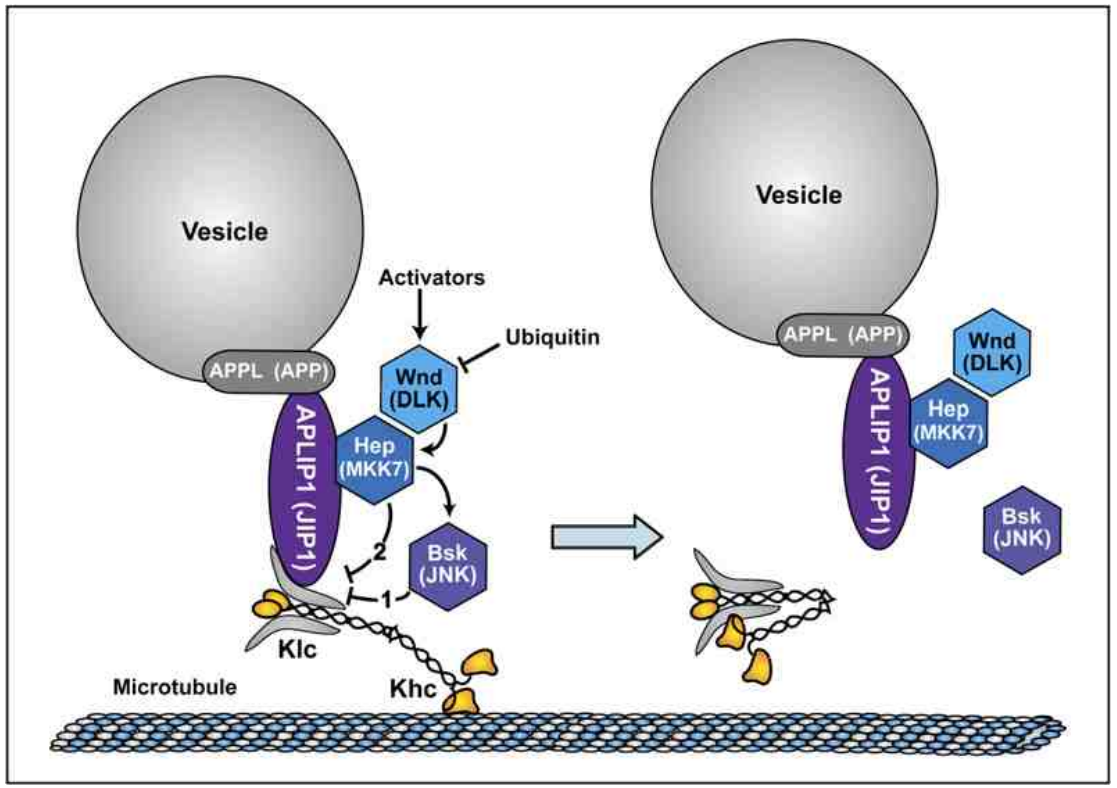
Drosophila genetic screen for factors that control kinesin-JIP linkage during axonal transport

Drosophila homologs

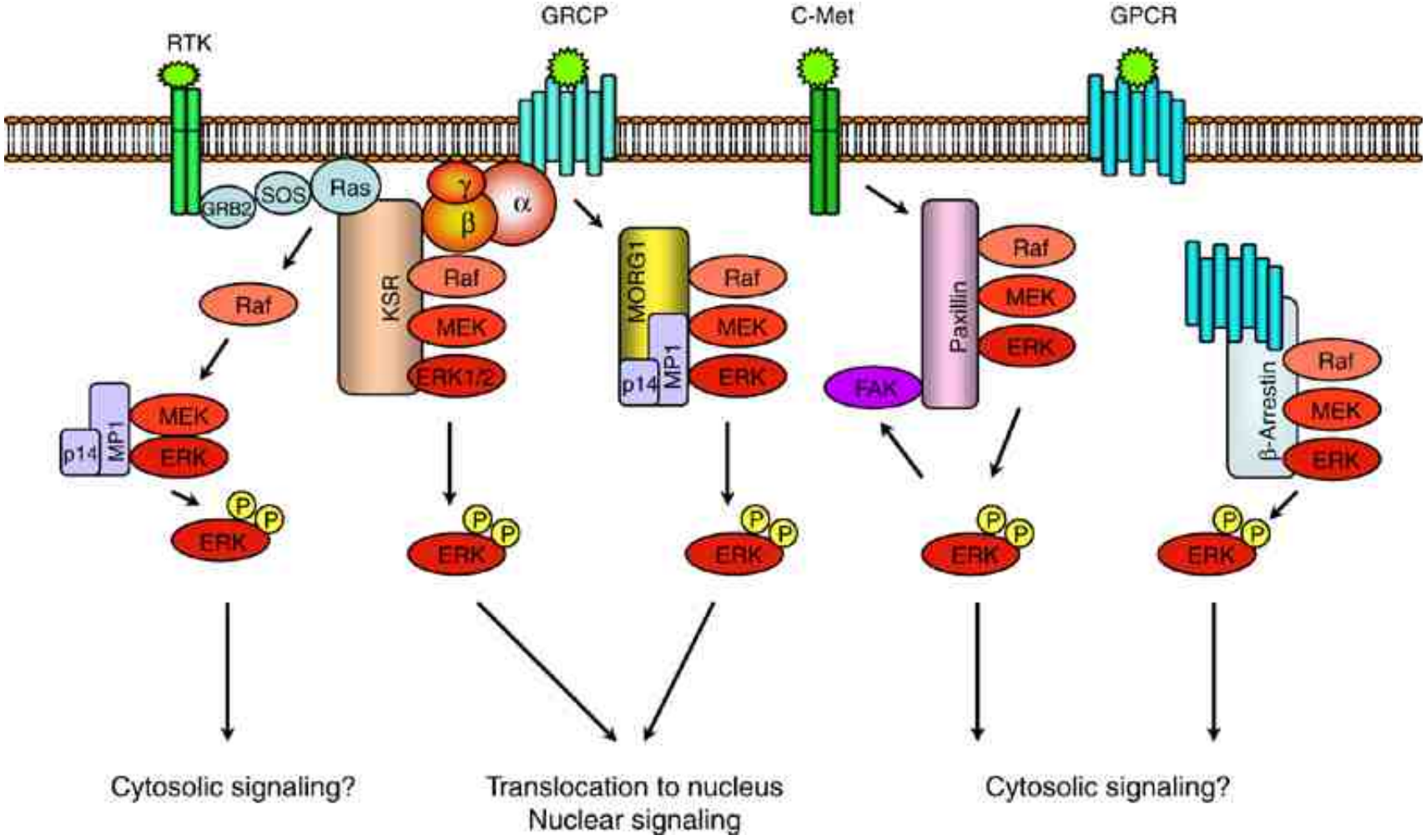


A Model for Wnd/MAPKKK Pathway Control of APLIP1/JIP1-Linked Kinesin-1 Cargo Transport

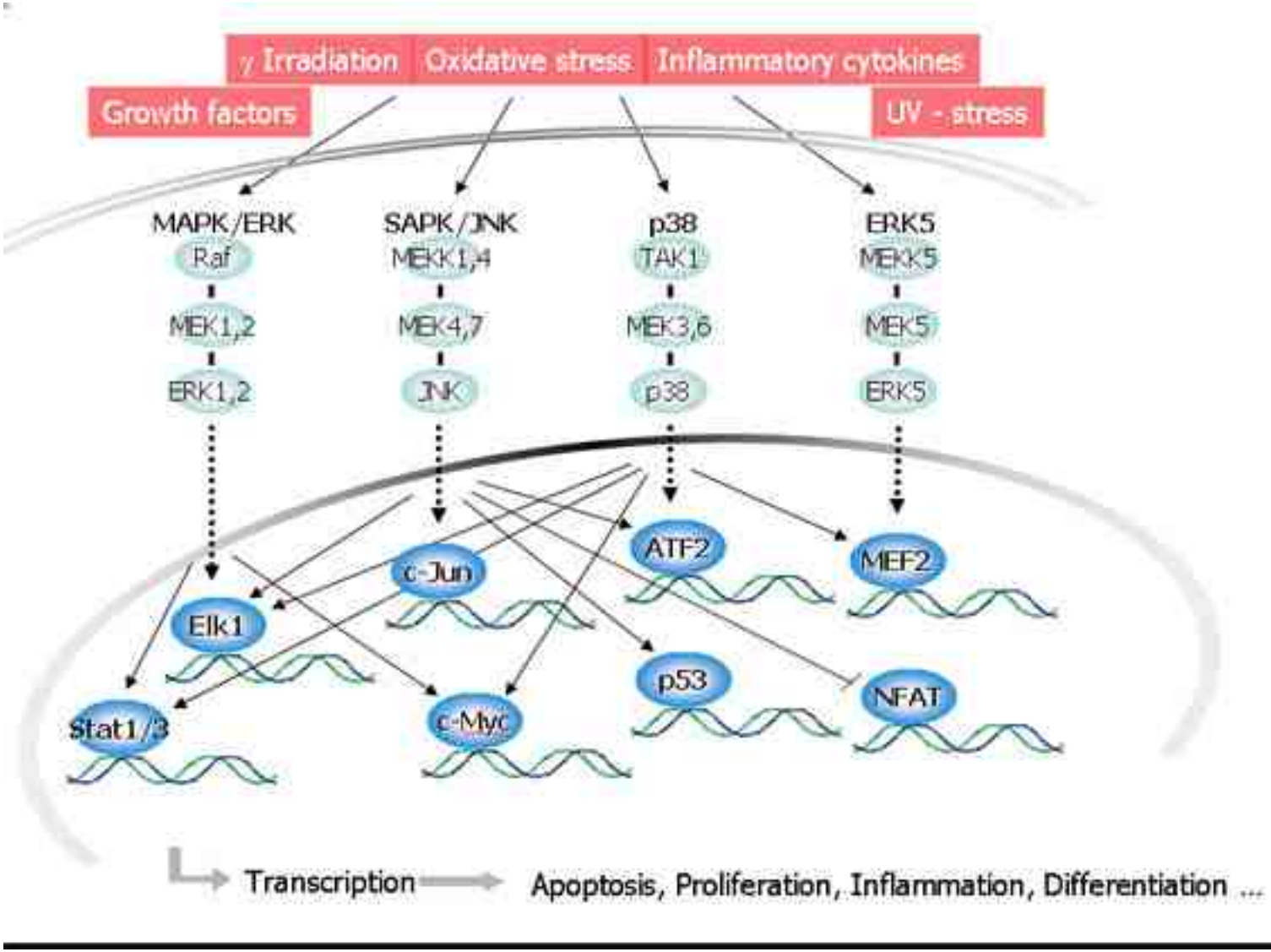
Wnd (MAPKKK), whose levels can be modulated by ubiquitination, is activated by unknown upstream signals. Wnd activates Hep (MAPKK) by phosphorylation, and activated Hep (MAPKK) then causes dissociation of APLIP1 (JIP1) from Klc, probably by phosphoactivation of Bsk (JNK), which then directly or indirectly modifies the linkage complex (pathway 1). It is also possible that phosphorylation of Hep (MAPKK) causes in the linkage complex a conformational change that inhibits APLIP1 (JIP1)-Klc binding independently of Bsk (pathway 2). Disruption of the APLIP1 (JIP1)-Klc linkage may allow kinesin to adopt an inactive, folded conformation that does not bind to microtubules.



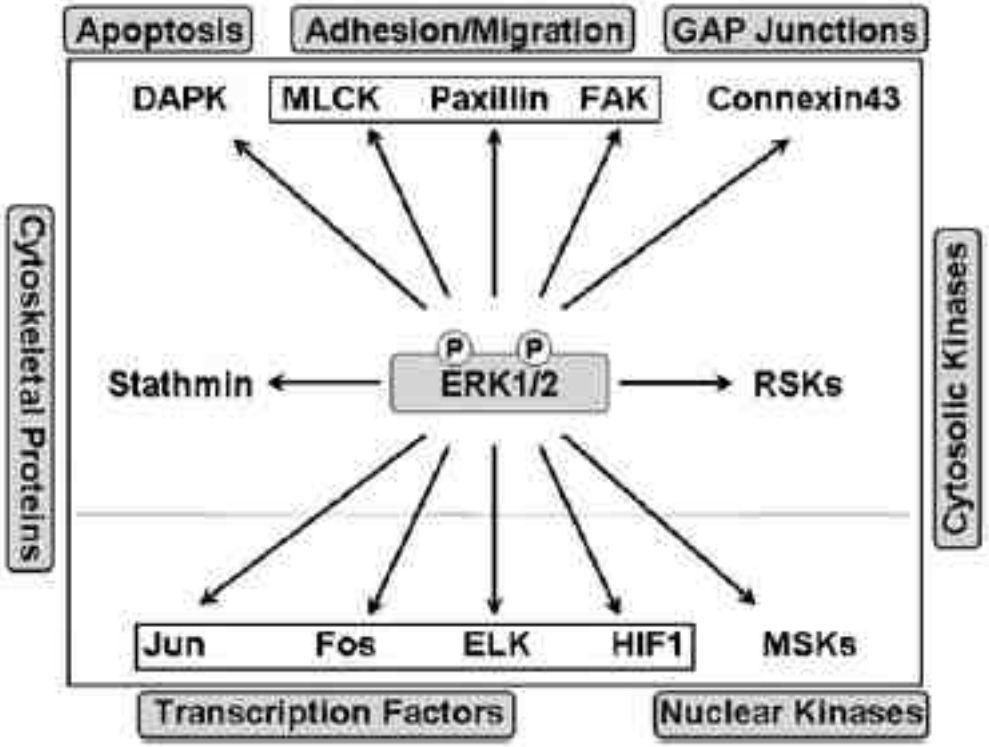
Components are labeled with Drosophila names and parenthetically with names of vertebrate homologs. Lines with arrowheads indicate activation influences, and lines with crossbars indicate inhibition influences.



Oncogene 26, 3185-3202 (2007)


γ Irradiation
Oxidative stress
Inflammatory cytokines
Growth factors
UV - stress
MAPK/ERK
Raf
MEK1,2
ERK1,2
SAPK/JNK
MEKK1,4
MEK4,7
JNK
p38
TAK1
MEK3,6
p38
ERK5
MEKK5
MEK5
ERK5
ATF2
MEF2
c-Jun
Elk1
p53
NFAT
Stat1/3
c-Myc
Transcription
Apoptosis, Proliferation, Inflammation, Differentiation ...

ERK has more than 100 known substrates. The diversity of these substrates is indicated by showing examples of substrates with divergent functions. ERK targets both transcription factors and kinases in the nucleus. It can also phosphorylate various kinases and structural proteins in the cytosol, while at the plasma membrane it targets proteins that regulate cell adhesion, cell–cell communication, and cell survival. The outcome of activation of the ERK pathway in a given cell will therefore be determined in part by where the active ERK is targeted in the cell and which substrates it has access to at those locations.



J.W. Ramos / The International Journal of Biochemistry & Cell Biology 40 (2008) 2707–2719

ERK1-deficient mice are viable, fertile, with normal size, but manifest a deficit in thymocyte maturation. Moreover, these mice exhibit an elevated synaptic plasticity in the striatum, which could be a result of a stimulus-dependent elevation in ERK2 phosphorylation, which was observed in neurons as well as fibroblasts of these mice.

ERK2-deficient mice die early in development, showing that ERK1 can't compensate for ERK2 in the embryo

Erk1

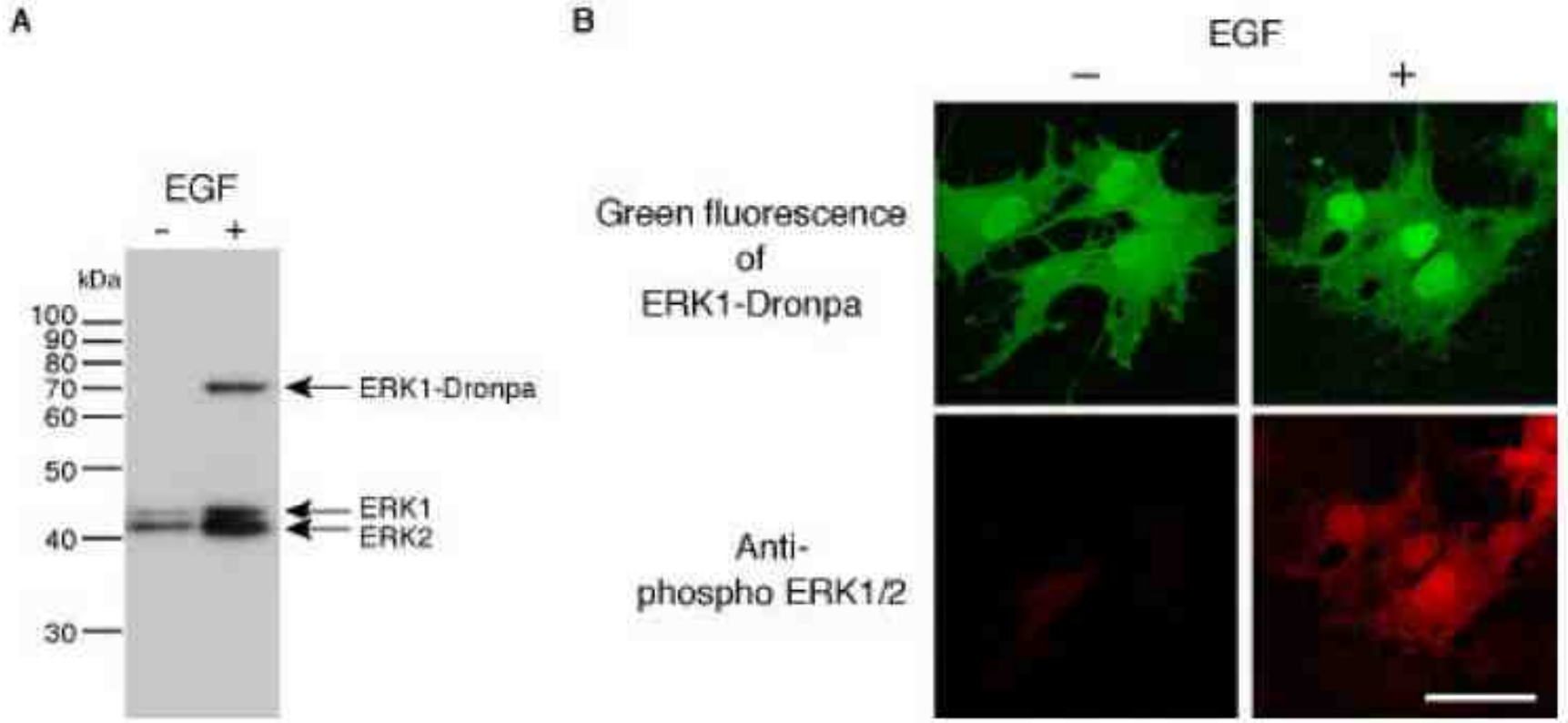
Regulated Fast Nucleocytoplasmic Shuttling Observed by Reversible Protein Highlighting

Ryoko Ando, Hideaki Mizuno, Atsushi Miyawaki*

Science **306**, 1370 (2004)

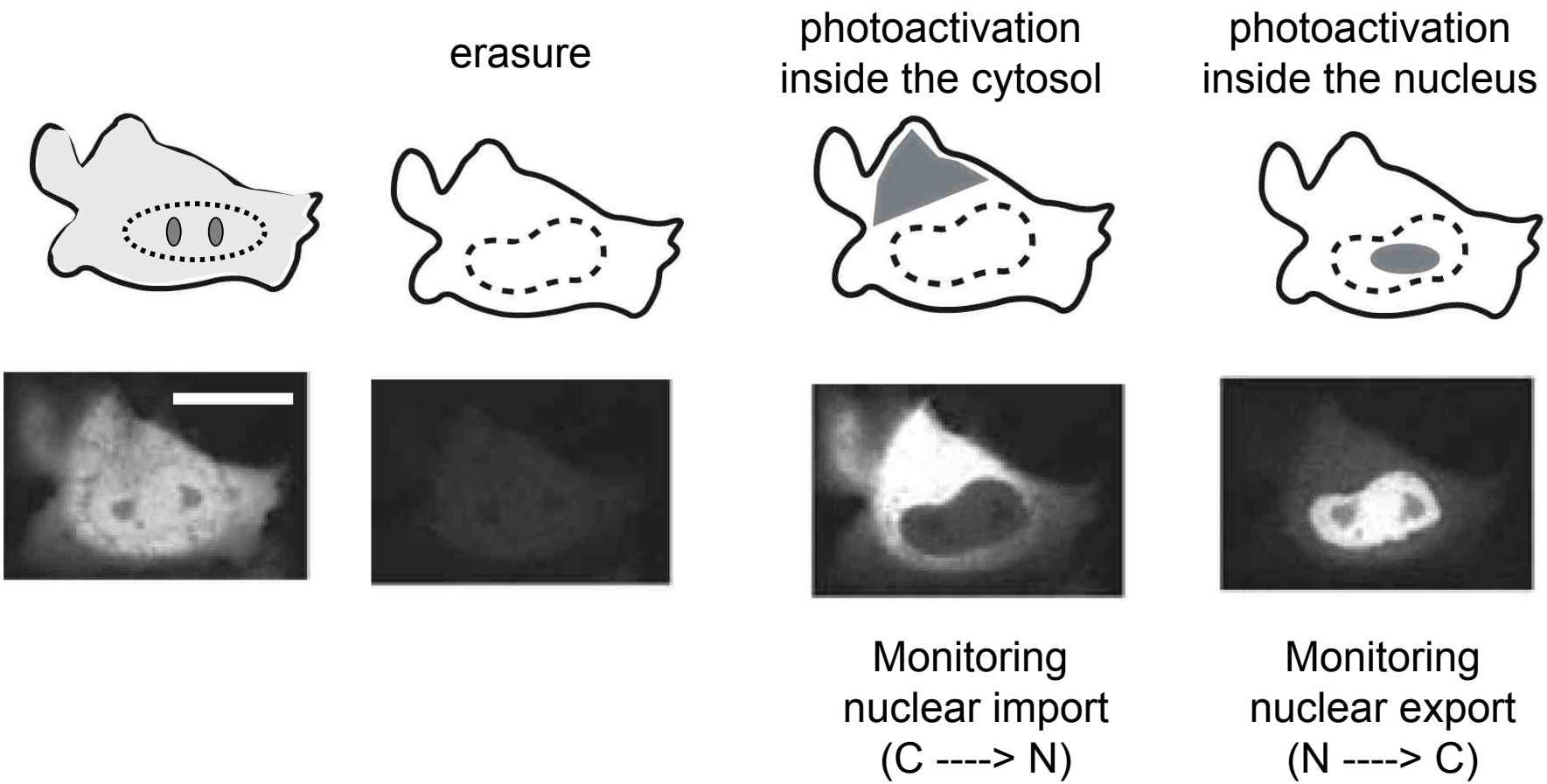
EGF-dependent phosphorylation and nuclear accumulation of ERK1-Dronpa in COS7 cells.

Dronpa is a GFP variant

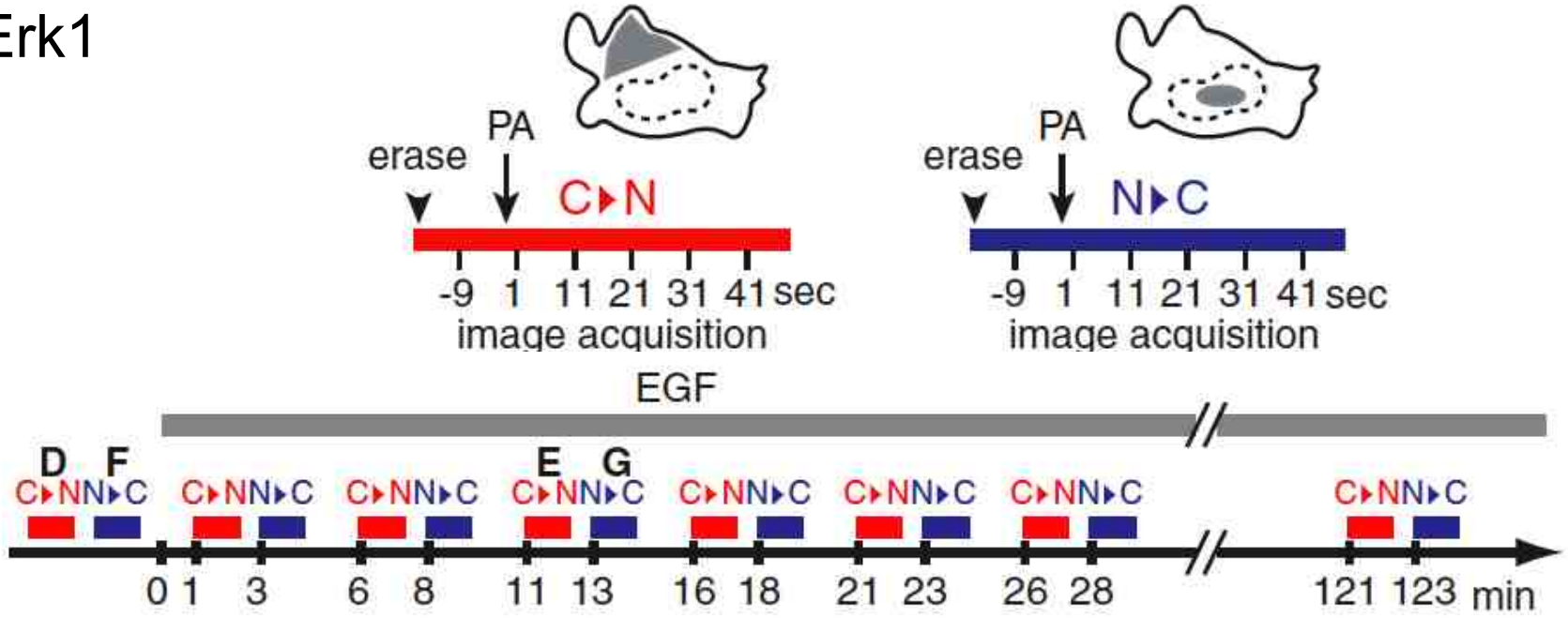


Erk1

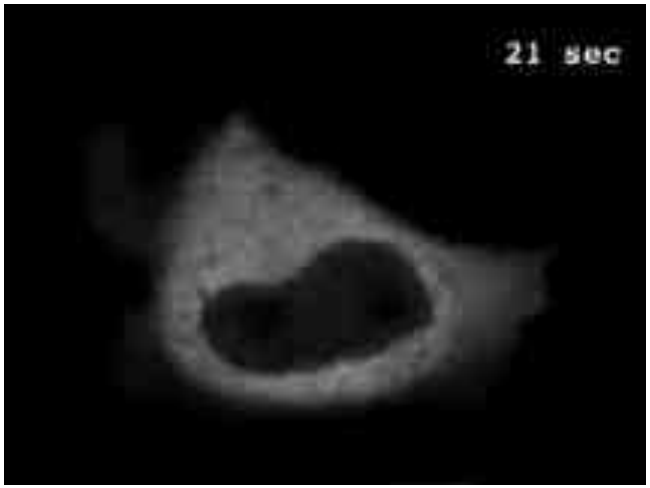
Each experimental period consisted of erasure, photoactivation, and acquisition of a series of confocal images.



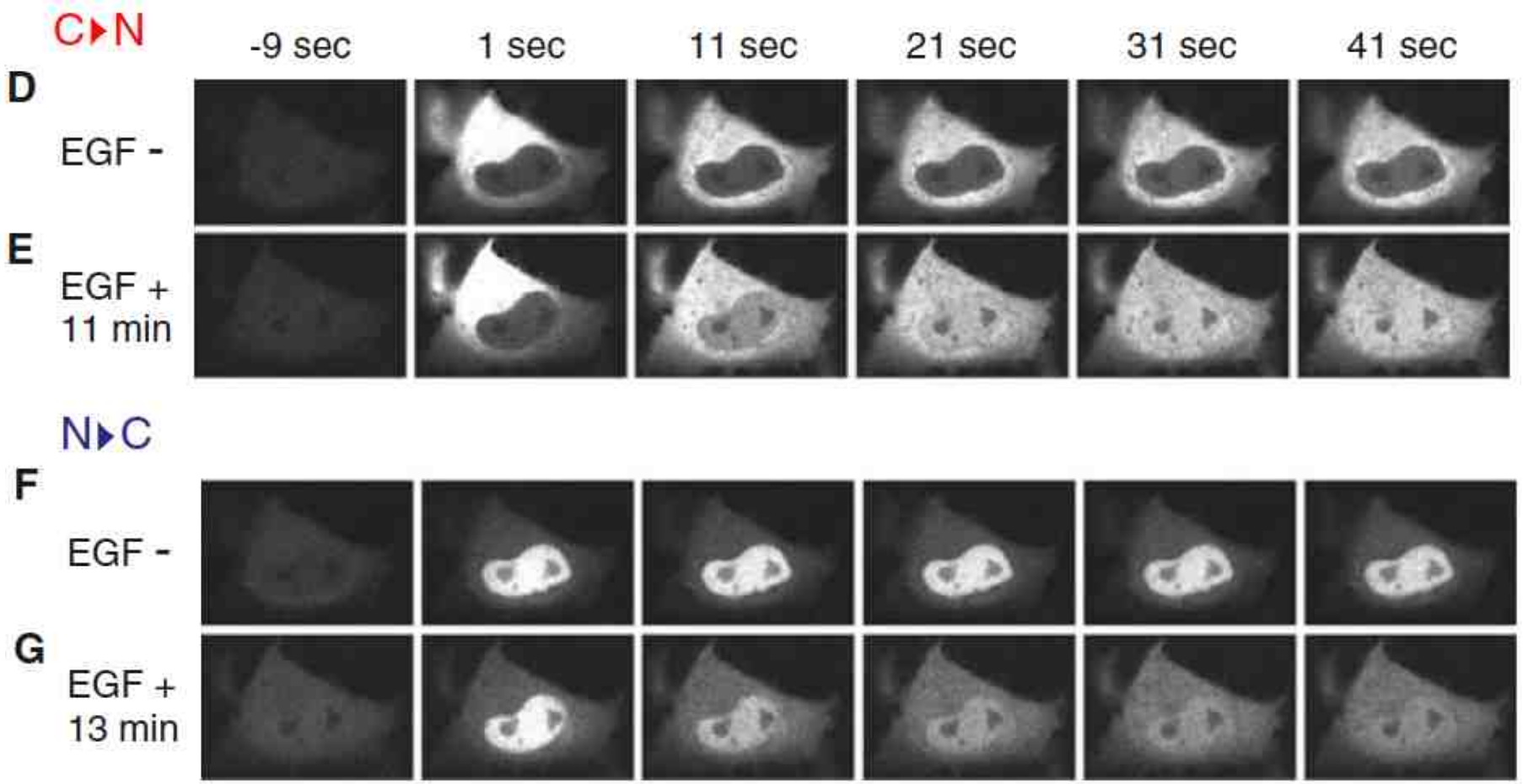
Erk1



This movie shows the nuclear import and export of ERK1-Dronpa in COS7 cells stimulated with 100 ng/ml EGF. Images of two representative monitoring experiments (t = 11 and 13 min)

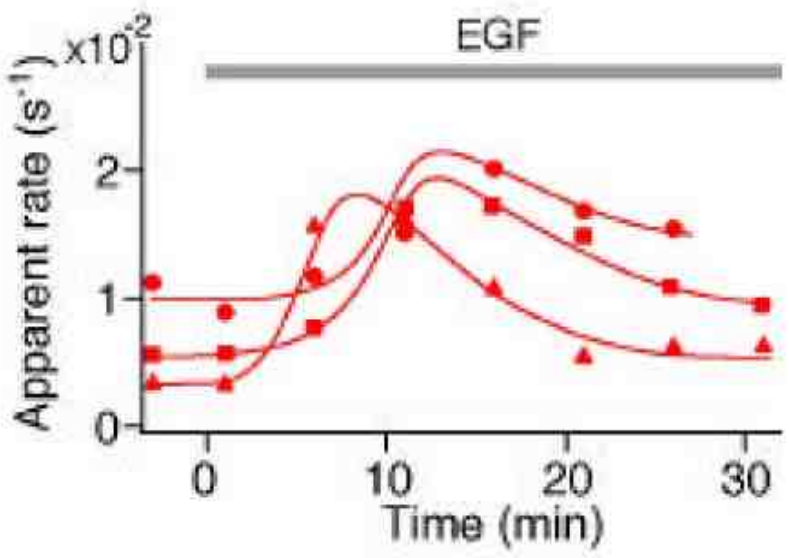


Erk1


C▸N
-9 sec
1 sec
11 sec
21 sec
31 sec
41 sec
D
EGF -
E
EGF +
11 min
N▸C
F
EGF -
G
EGF +
13 min

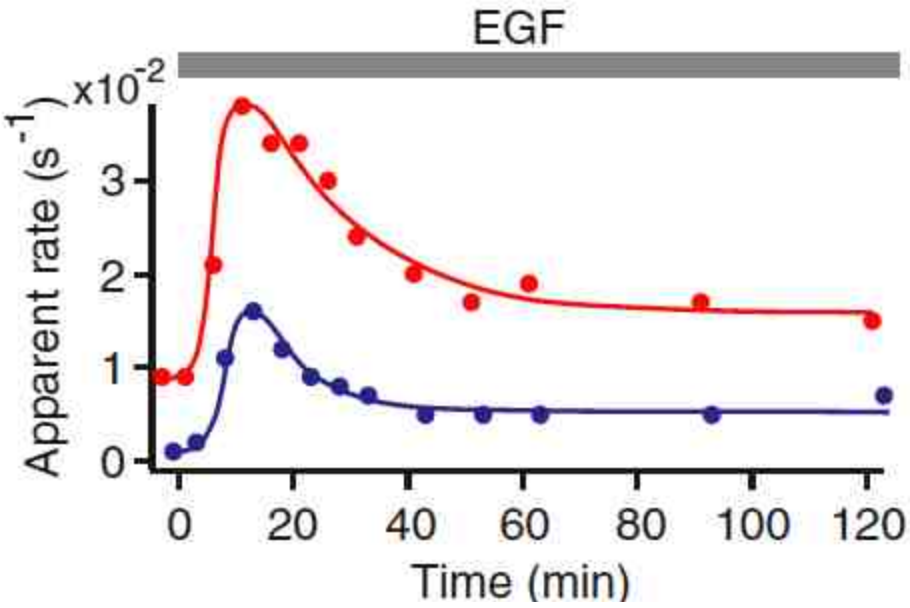
Notably, variation in the initial shuttling rate between different cells was observed. Thus, any changes in movement must be assessed using data from a single cell, because measurements are affected by the geometry of the cells and marked regions.

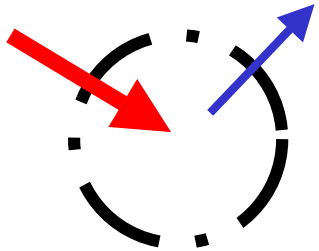
Time courses of the nuclear influx (red) rates of ERK1-Dronpa during stimulation with 10 ng/ml EGF obtained from different three COS7 cells (circles, squares, and triangles).



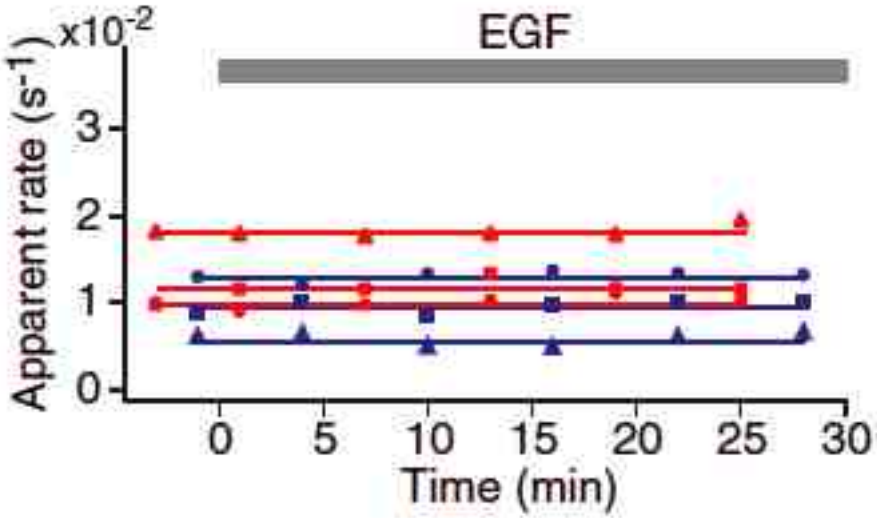
Time courses of the nuclear influx (red) and efflux (blue) rates of ERK1-Dronpa and importin-dronpa during during stimulation with 100 ng/ml EGF.

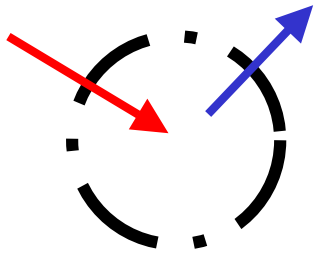
ERK-1





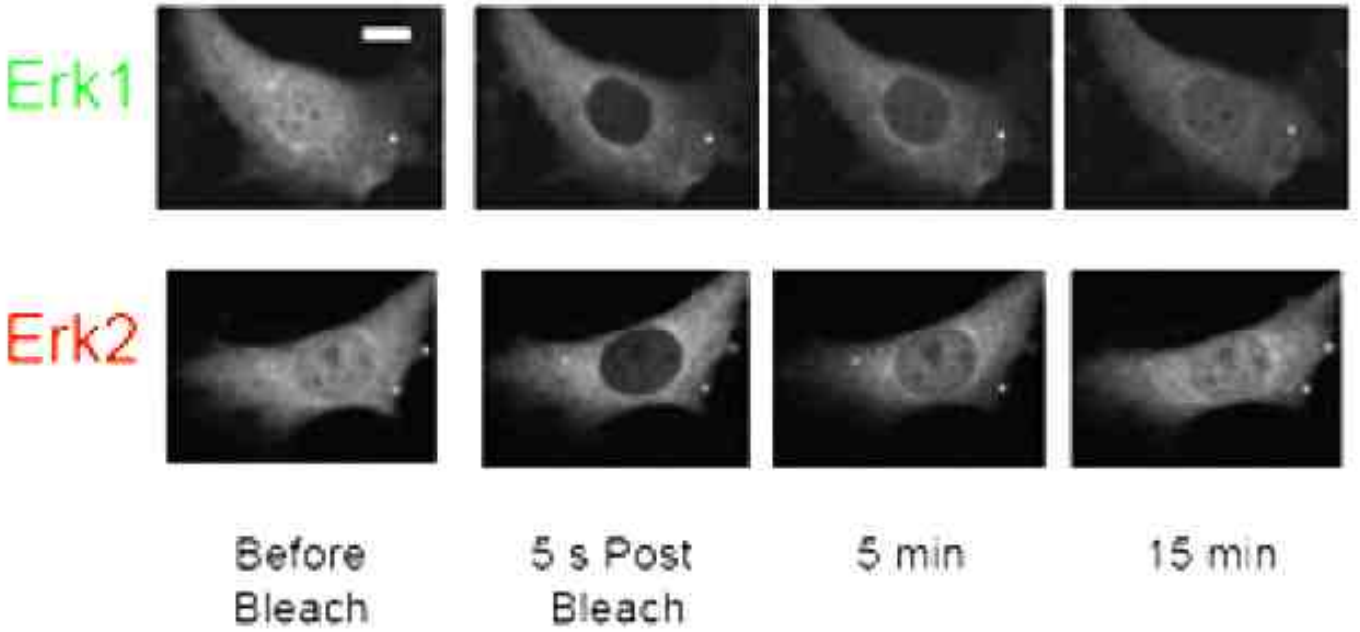
importin-dronpa





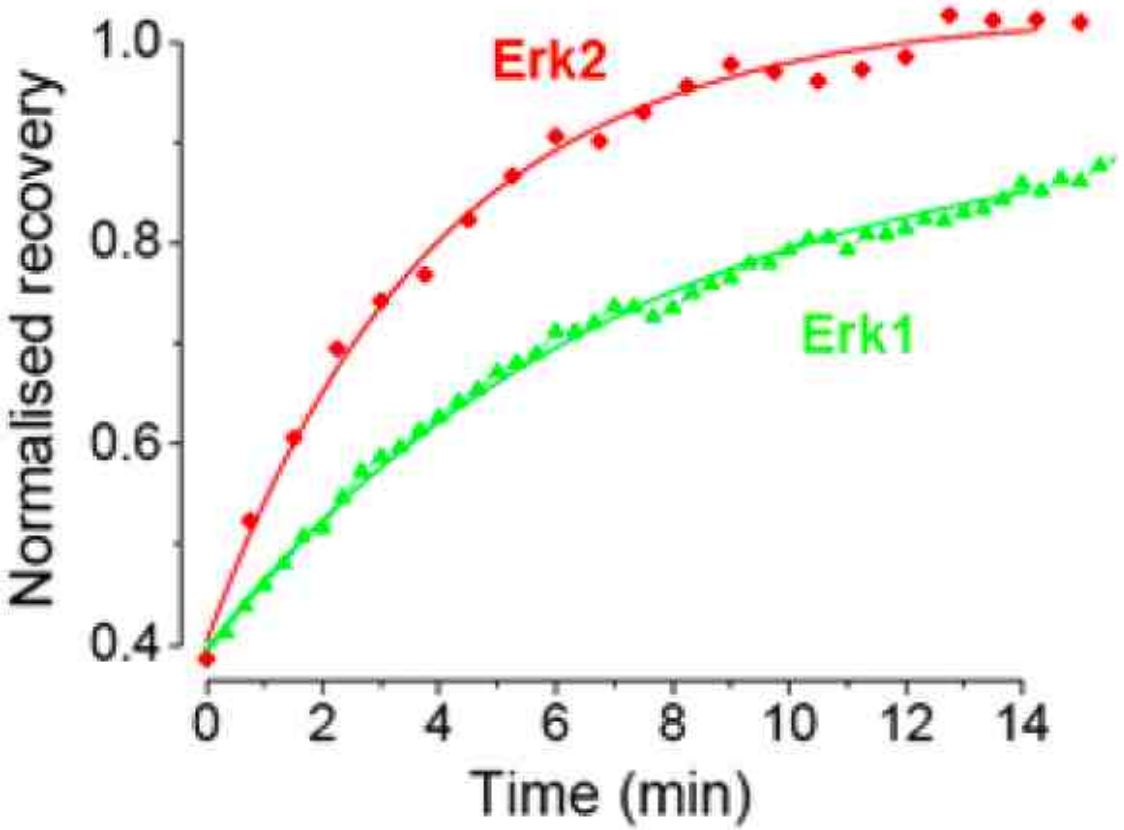
Comparison of the nucleo-cytoplasmic shuttling of ERK1 and ERK2.

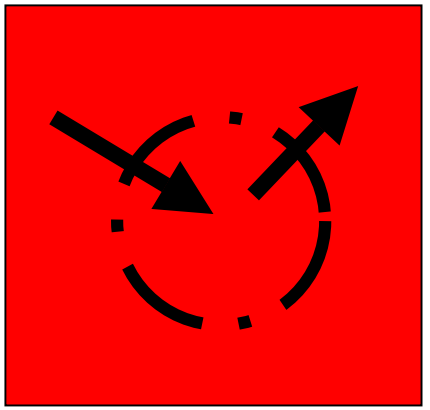
ERK2 shuttles between the nuclear and cytosolic compartments with a speed that depends on its activation state. We verified whether a similar process occurs with ERK1 by photobleaching the nucleus and imaging the recovery of fluorescence. Since photobleaching is irreversible, the recovery in the nucleus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

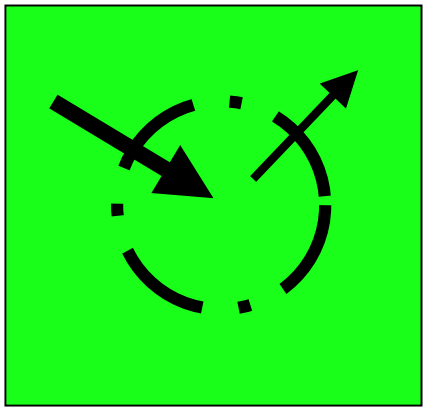


After photobleaching of the nucleus of starved NIH 3T3 cells, the ERK1 fluorescence recovered more slowly than ERK2, indicating a slower turnover of ERK1 across the nuclear membrane.

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







Time course of the recovery

December 2008 | Volume 3 | Issue 12 | e3873

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PLoS one

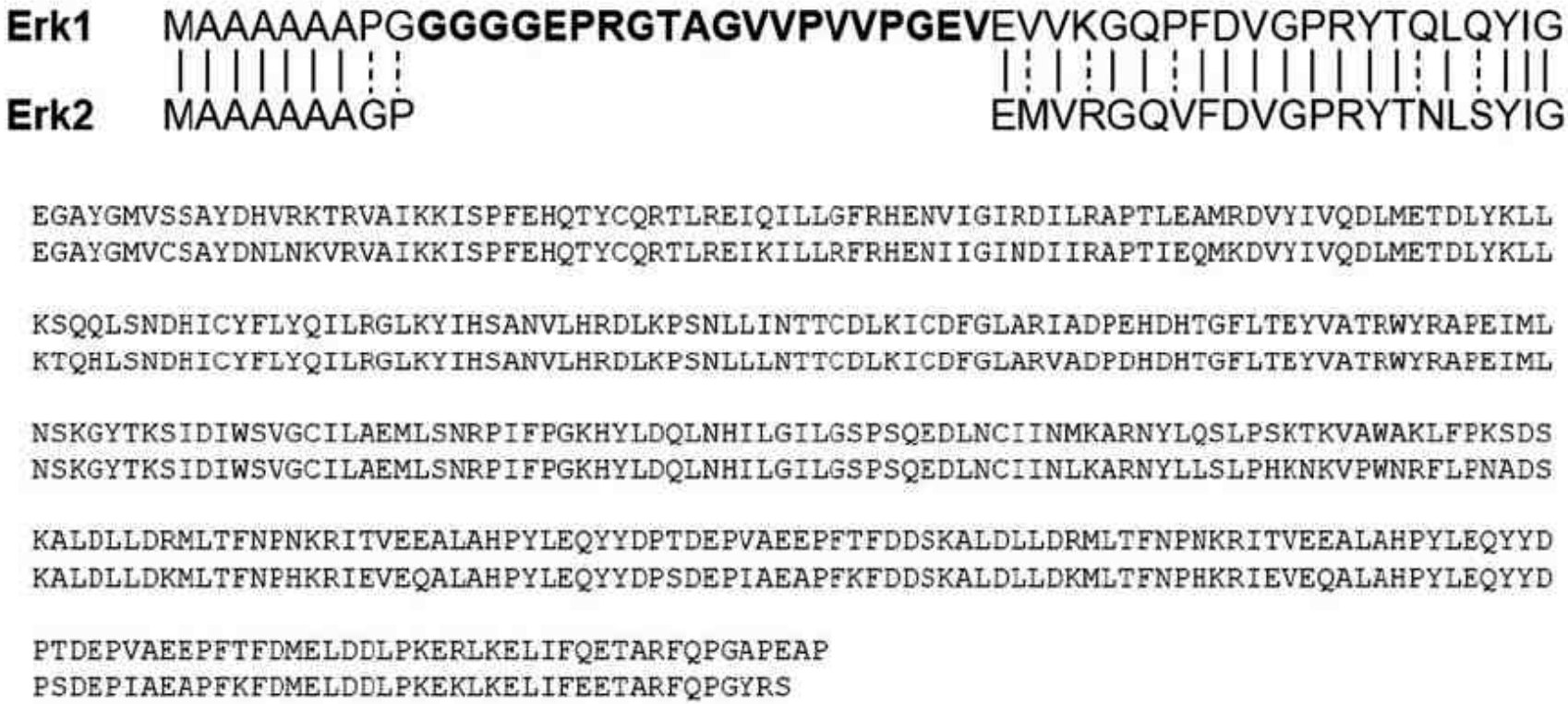
The N-Terminal Domain of ERK1 Accounts for the Functional Differences with ERK2

Matilde Marchi[2,5], Angela D'Antoni[3,6], Ivan Formentini[3], Riccardo Parra[4], Riccardo Brambilla[3], Gian Michele Ratto[1,2]*, Mario Costa[1]*

1 Institute of Neuroscience CNR, Pisa, Italy, **2** NEST-INFM, Scuola Normale Superiore, Pisa, Italy, **3** San Raffele Scientific Institute, Milano, Italy, **4** Scuola Normale Superiore, Pisa, Italy, **5** Italian Institute of Technology (IIT), Pisa, Italy, **6** Università degli Studi di Milano, Milano, Italy

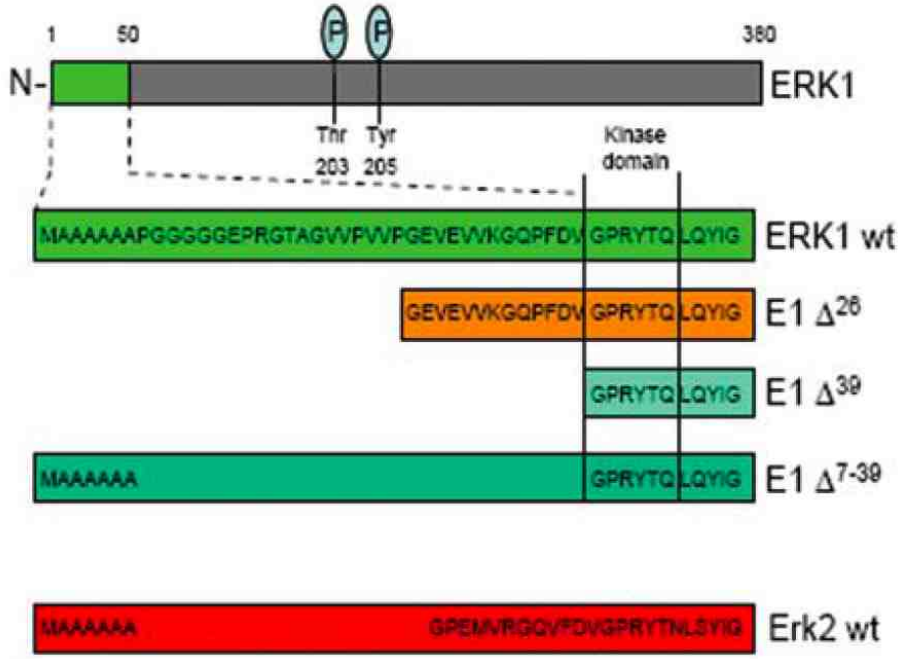
Alignment of the amino acid sequences of rat ERK1 and ERK2.

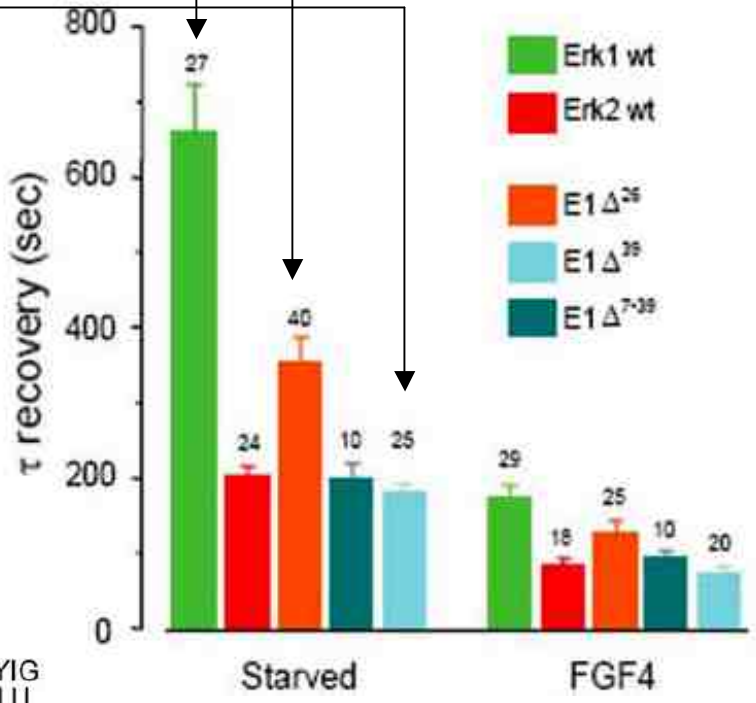
The N-terminus is shown with a larger font. The 20 aa present only in ERK1 are displayed in bold.



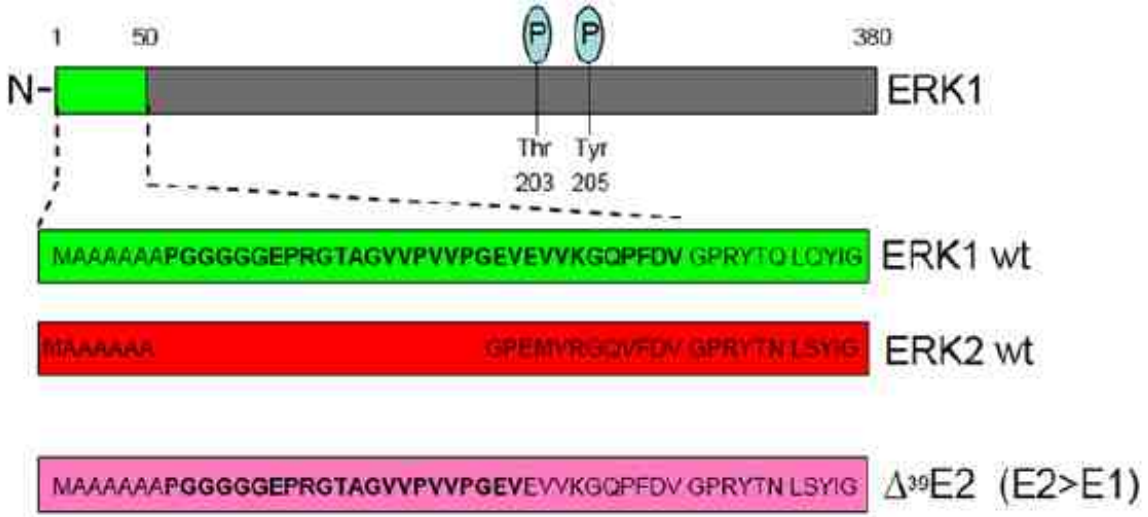
ERK1 deletants:

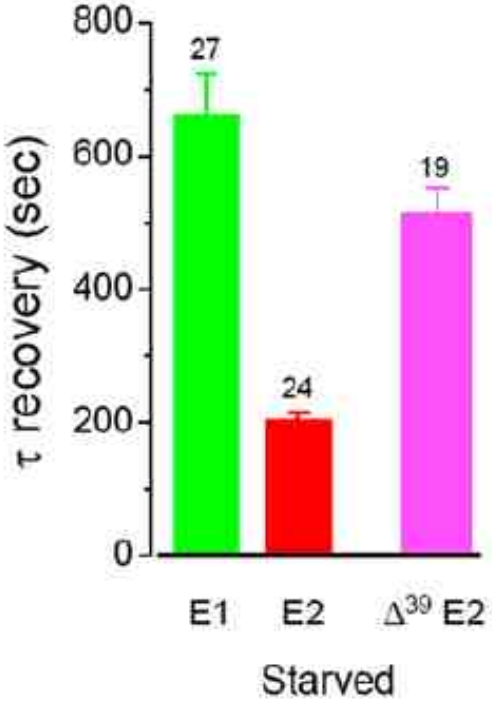
The time constant of the nucleo-cytoplasmic shuttling of ERK1 fusion proteins is strongly affected by the different deletions of the N-terminus

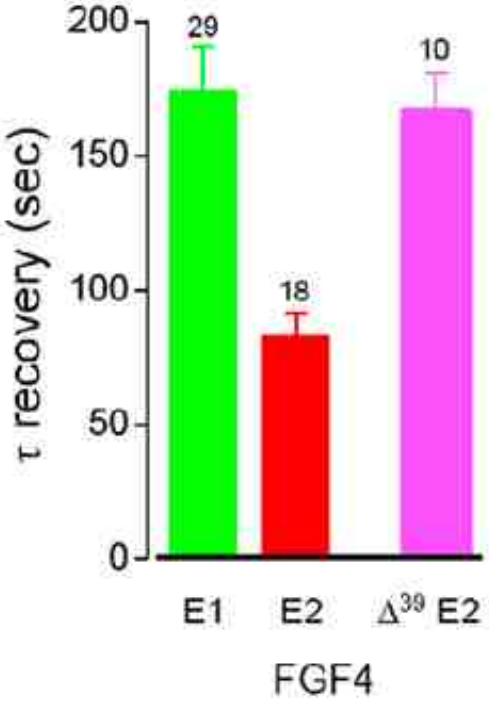




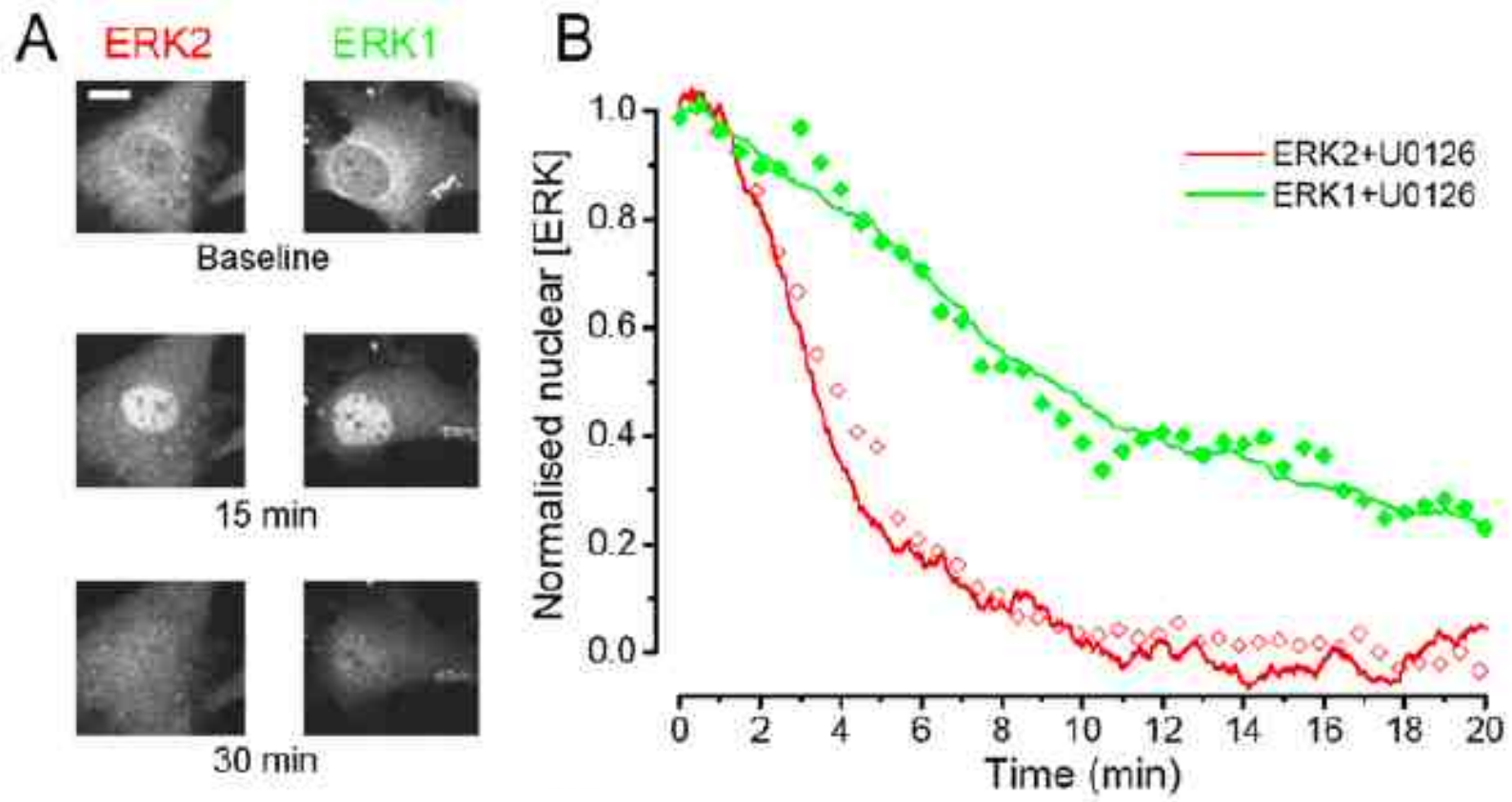
Fusion of ERK2-GFP with the N-terminus of ERK1 (Δ39 E2/E.E1).



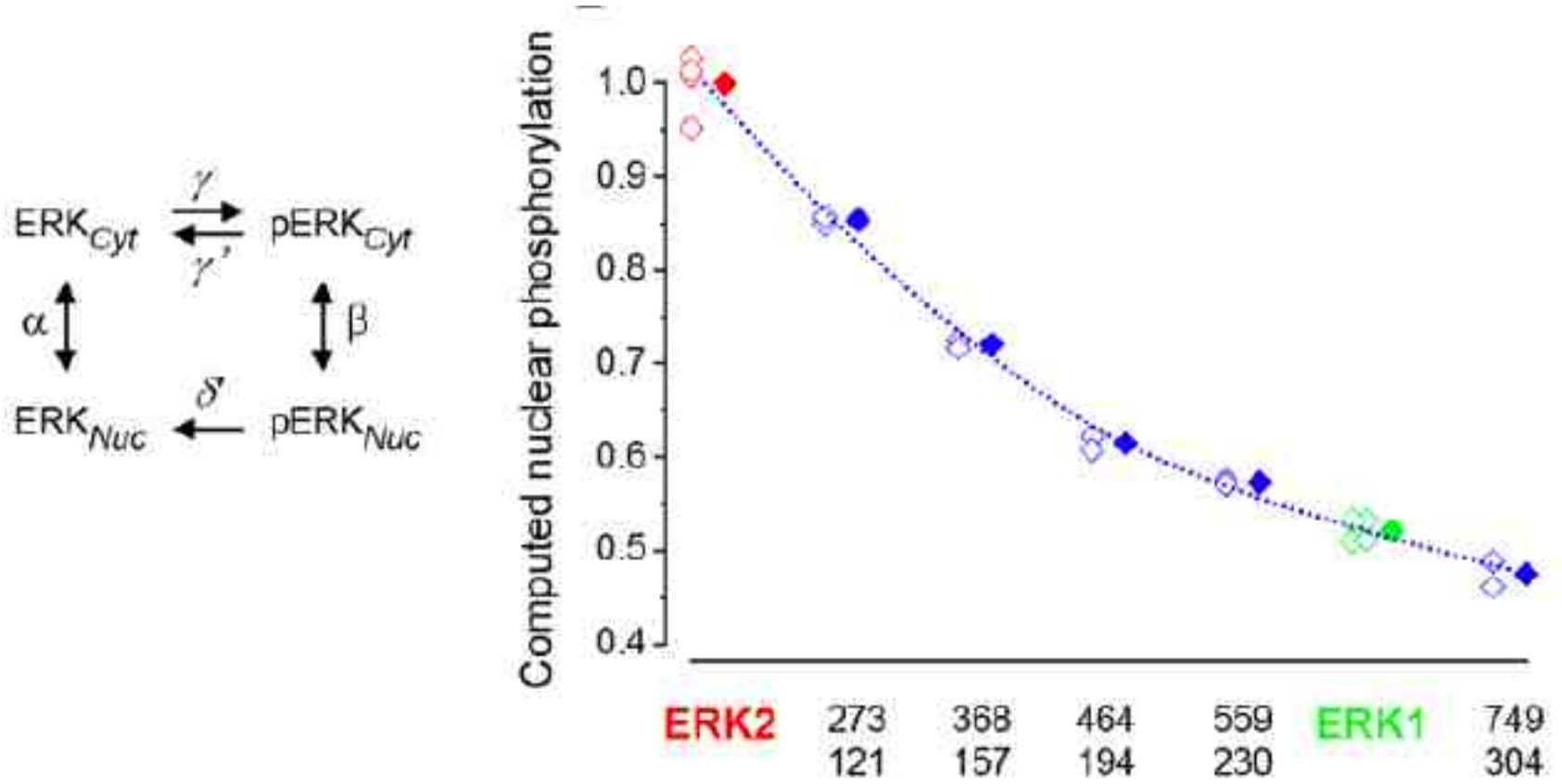




Cells transfected with ERK1-GFP and ERK2-GFP were treated with FGF4 for 15 min to allow complete nuclear translocation and then with the ERK blocker U0126. The inactivation of the ERK pathway caused the immediate loss of nuclear accumulation of ERK-GFP unmasking the action of nuclear dephosphorylation.

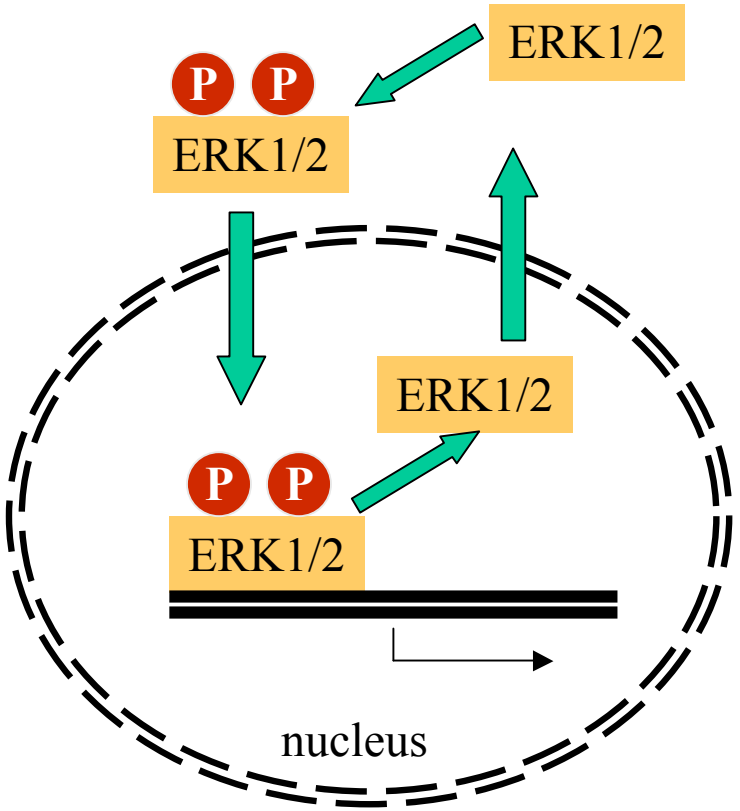


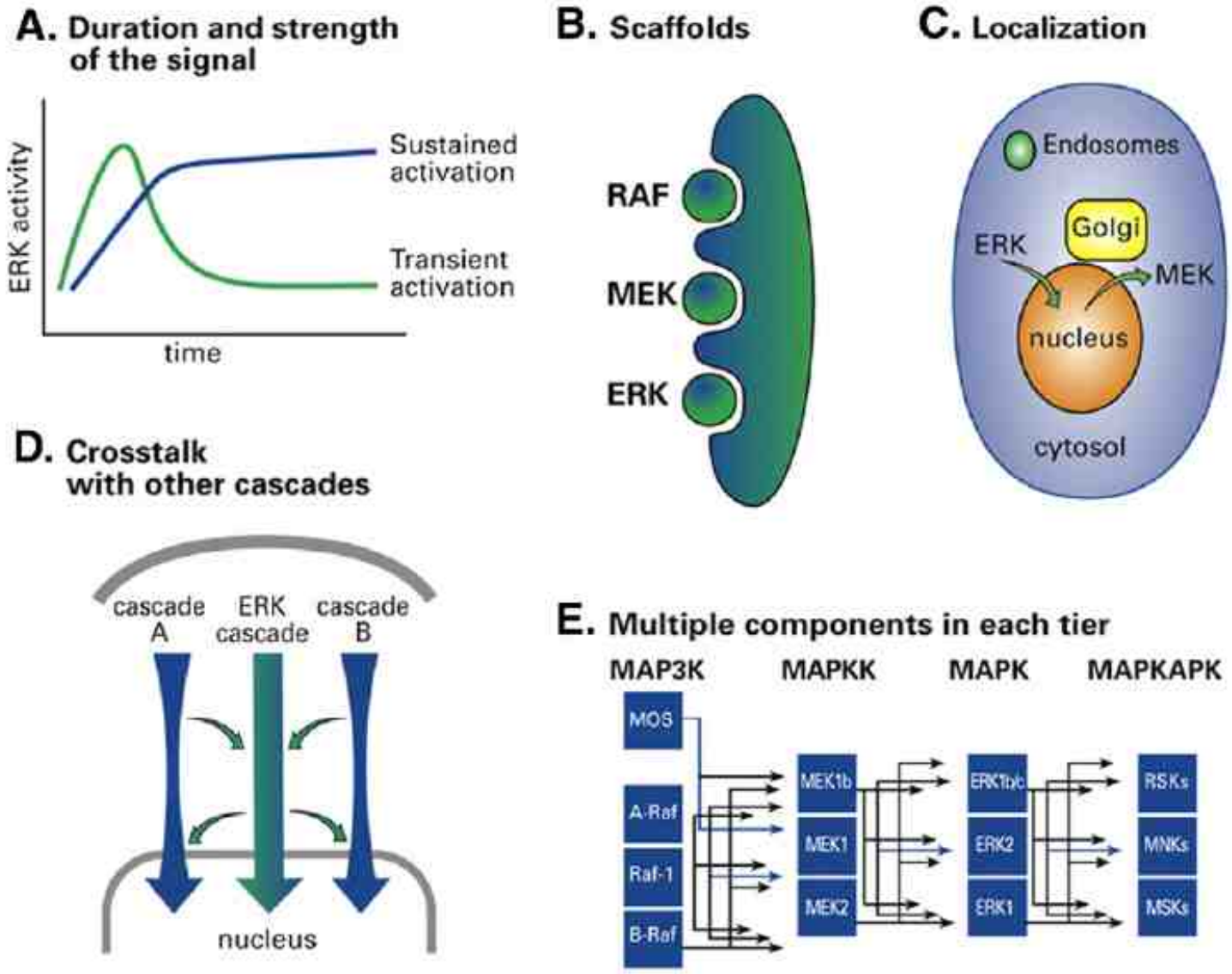
Phosphorylation in the nucleus as a function of the shuttling speed



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

Assuming that ERK principally undergoes inactivation within the nucleus, fast circulation across the nuclear envelope is predicted to more effectively increase gene expression than does simple nuclear retention.





Biochimica et Biophysica Acta 1773 (2007) 1213–1226