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Review

Optical measurement of receptor tyrosine kinase oligomerization on live cells☆

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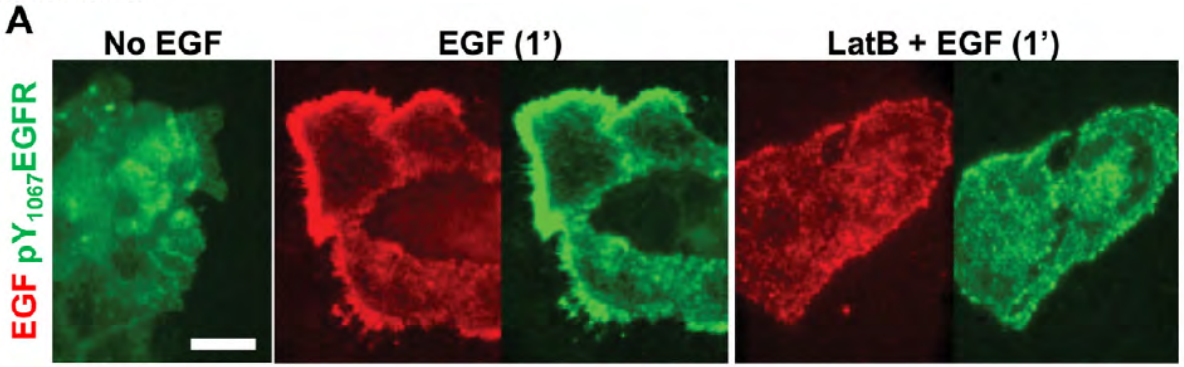

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ARTICLE

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EGFR oligomerization organizes kinase-activ dimers into competent signalling platforms

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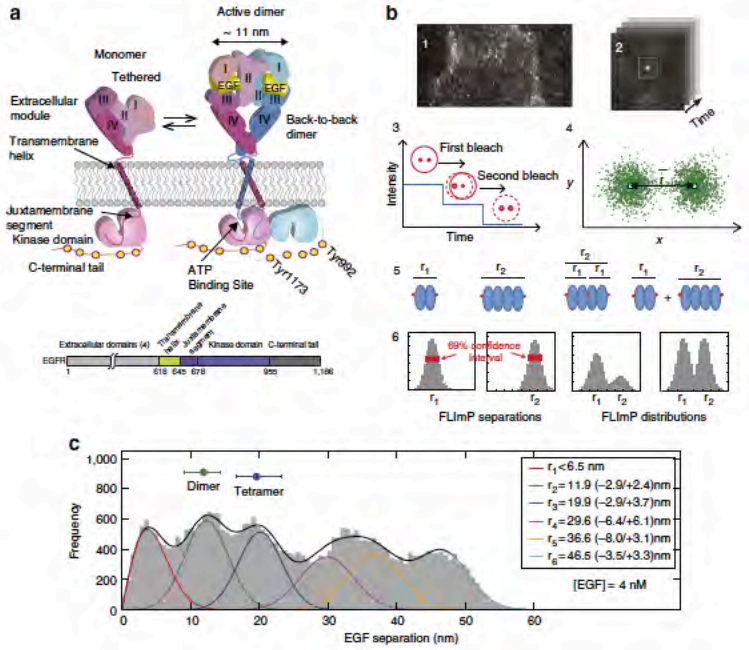
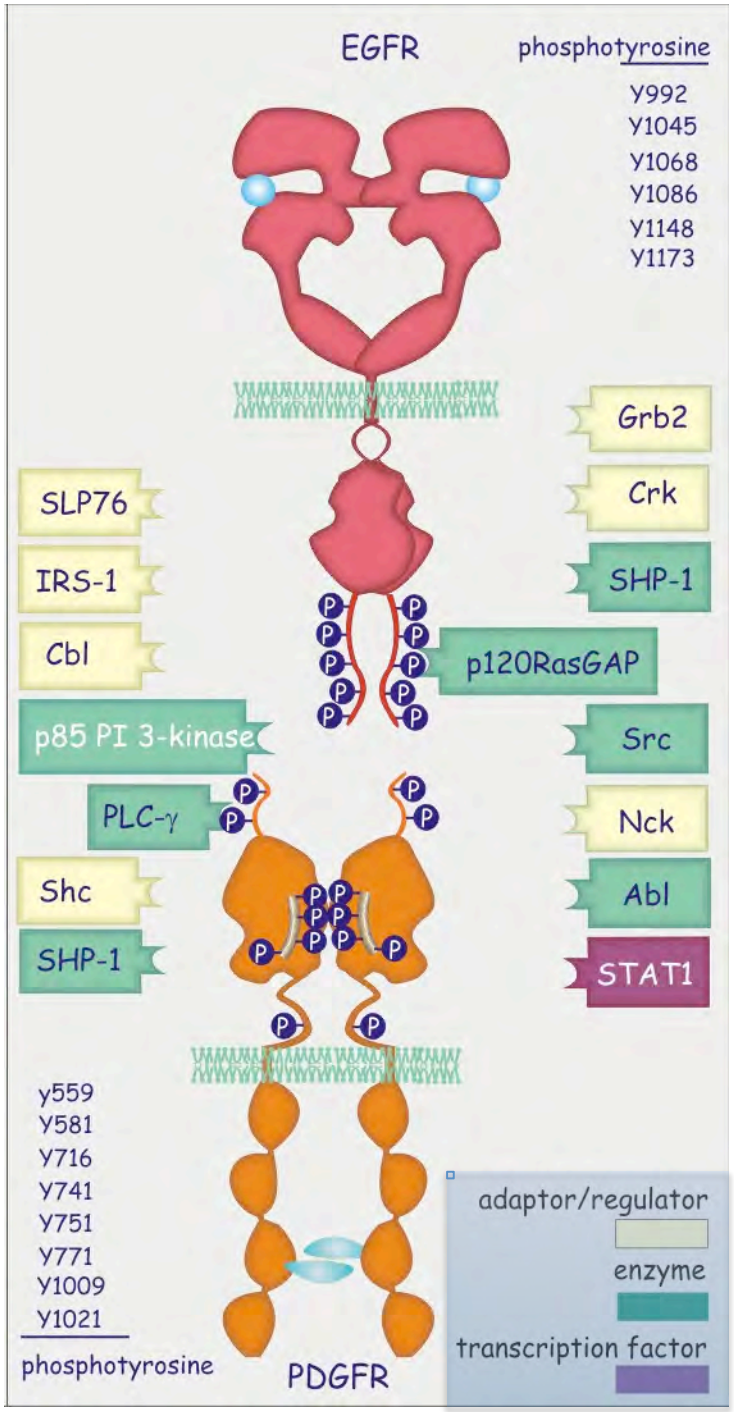
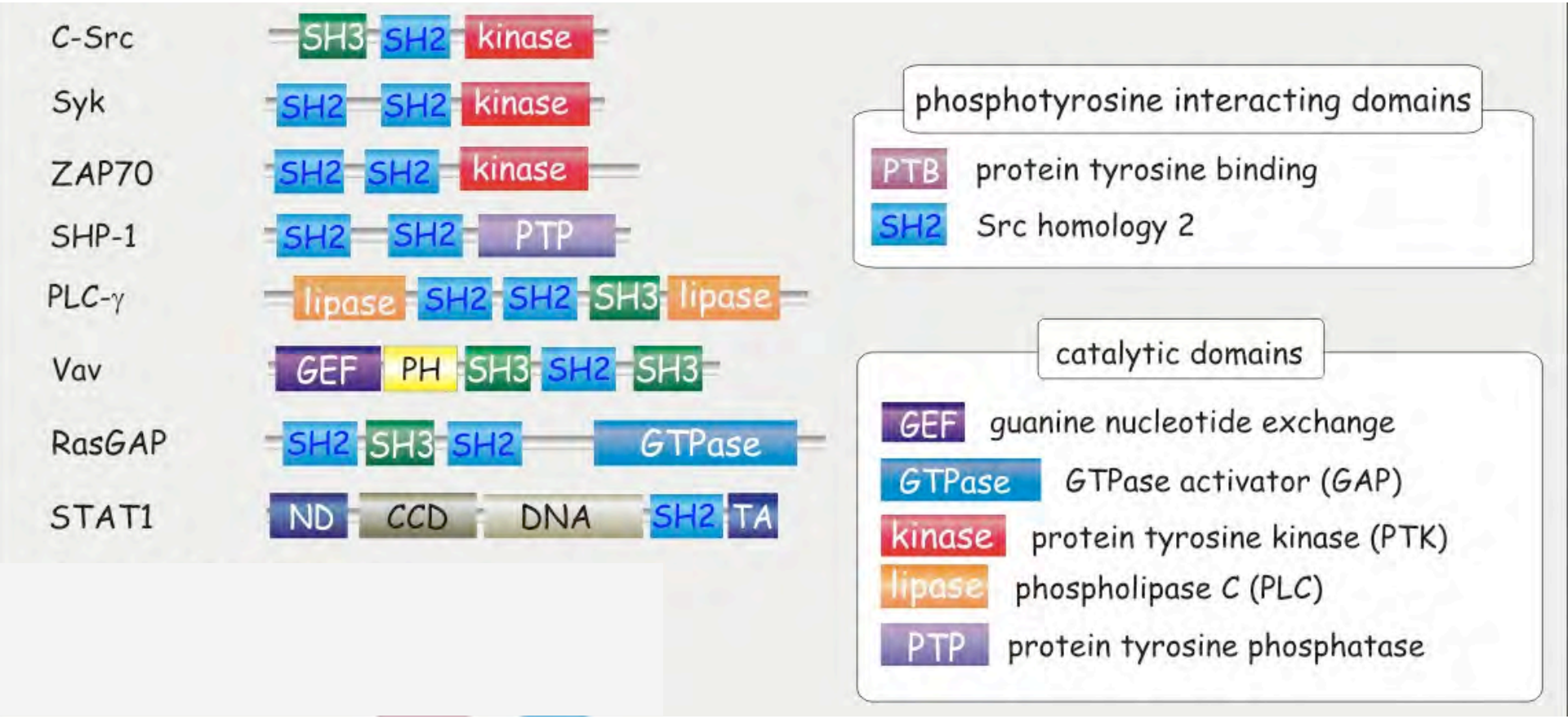
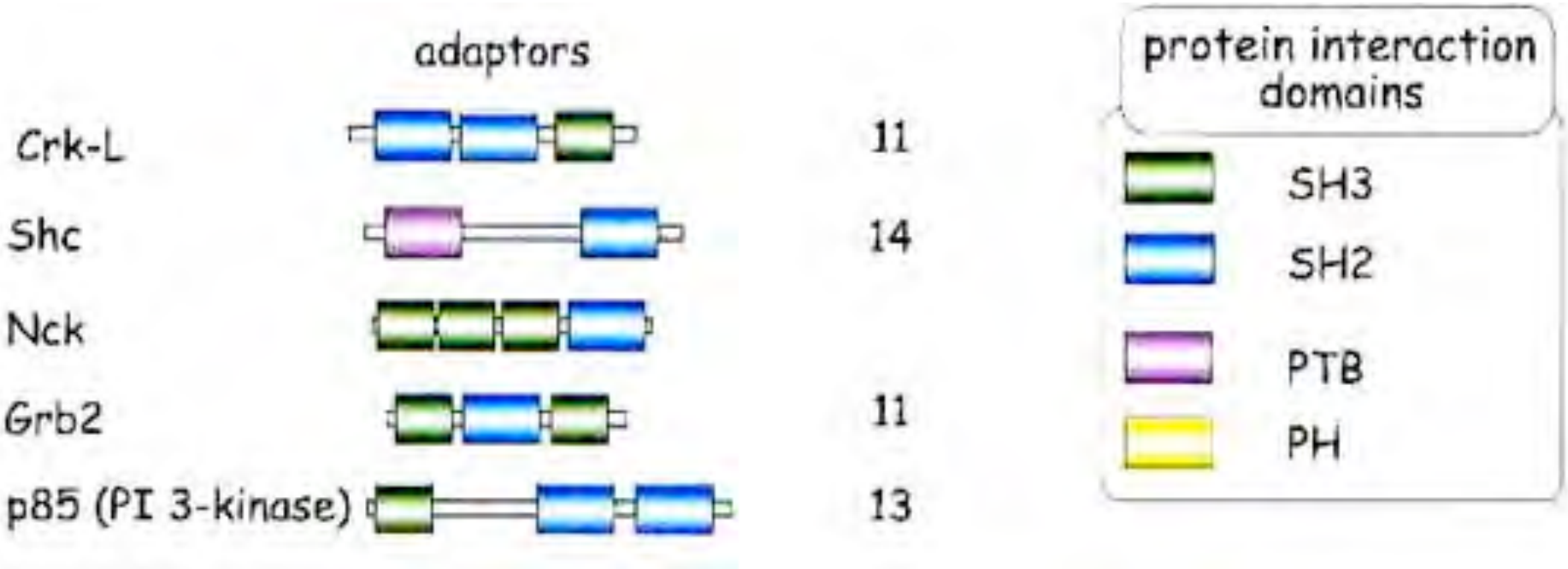


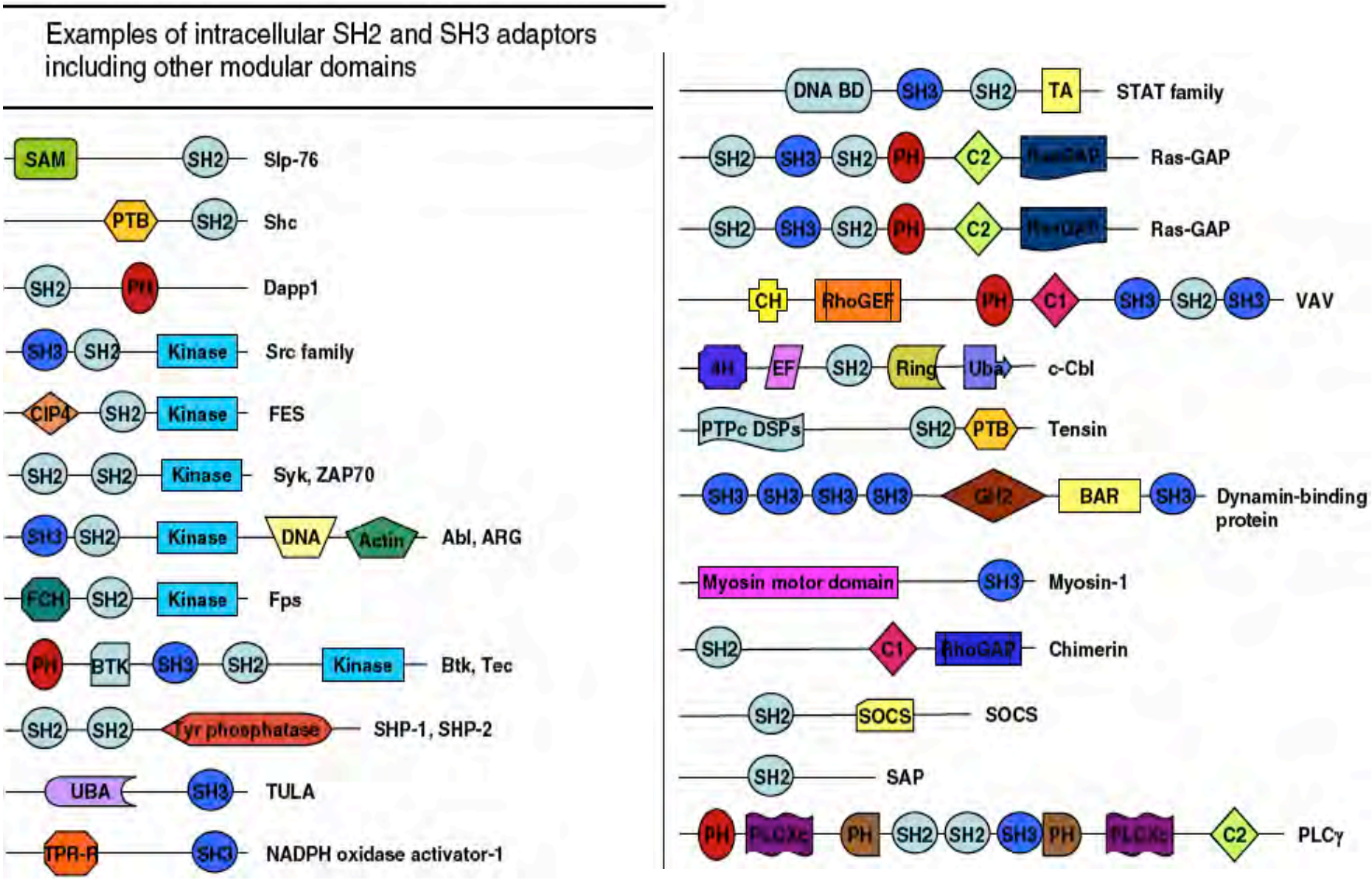

EGFR
phosphotyrosine
Y992
Y1045
Y1068
Y1086
Y1148
Y1173
Grb2
Crk
SHP-1
p120RasGAP
Src
Nck
Abl
STAT1
SLP76
IRS-1
Cbl
p85 PI 3-kinase
PLC-γ
Shc
SHP-1
y559
Y581
Y716
Y741
Y751
Y771
Y1009
Y1021
phosphotyrosine
PDGFR
adaptor/regulator
enzyme
transcription factor

1) Enzymes/transcription factors



2) ***Adaptors*** lack intrinsic catalytic activity, but link phosphorylated receptors with other effector proteins.





Intracellular adaptor proteins containing SH2 and/or SH3 domains in addition to other modular domains. These domains are critical in cellular and biological functions.

Signaling Efficiency: Scaffolding Proteins and Signaling Complexes

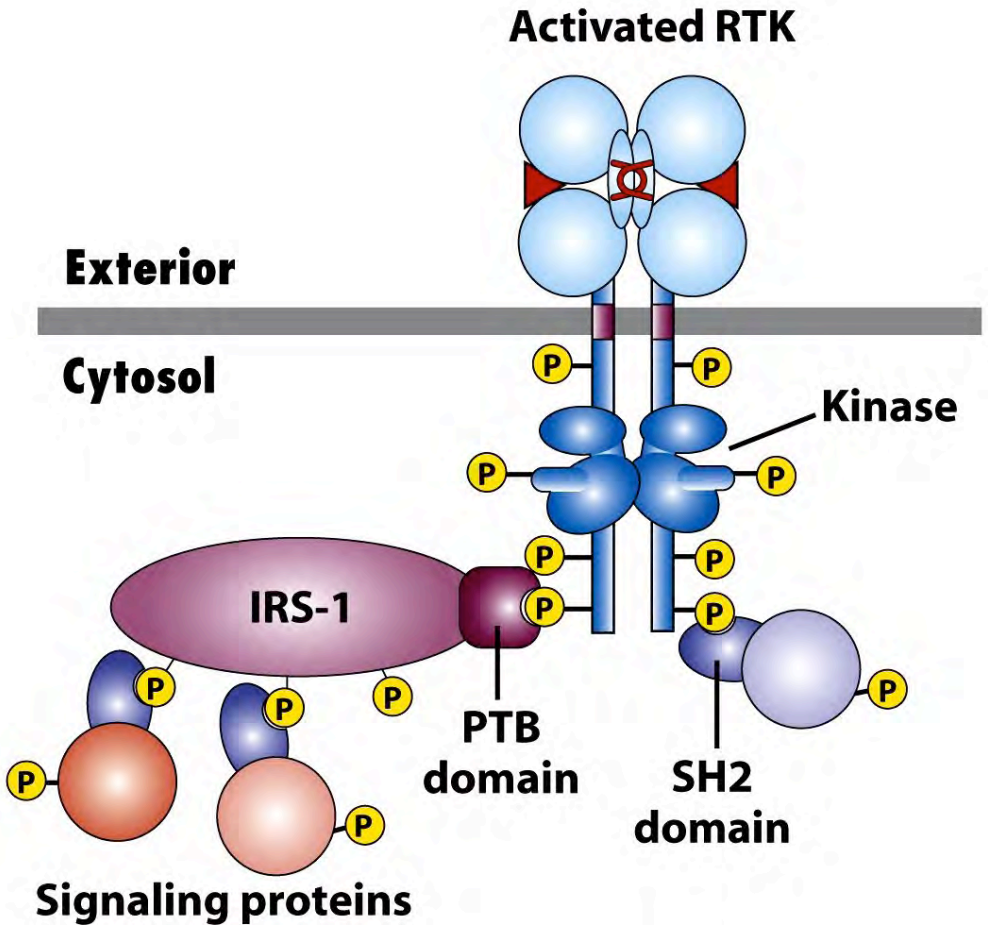
- **Scaffolding proteins** are large relay proteins to which other relay proteins are attached
- Scaffolding proteins can increase the signal transduction efficiency by grouping together different proteins involved in the same pathway
- In some cases, scaffolding proteins may also help activate some of the relay proteins

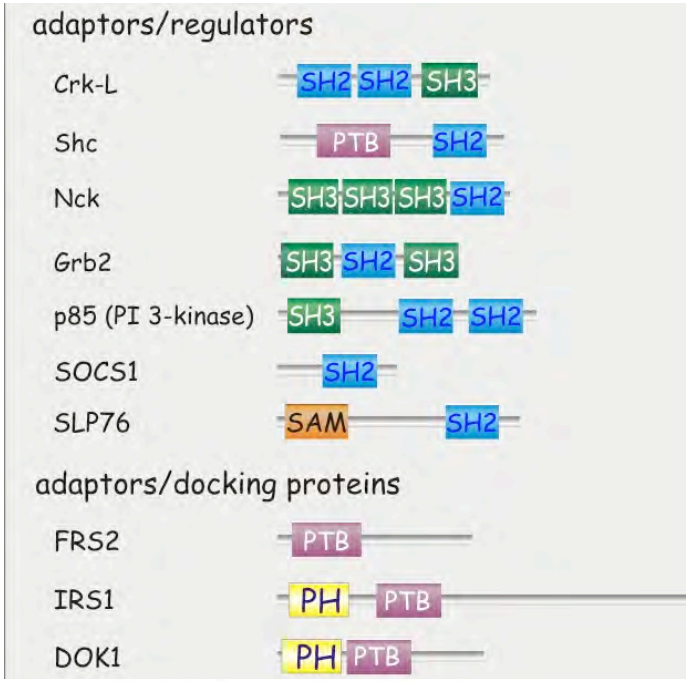
Recruitment of Signal Transduction Proteins to Activated Receptors

RTKs downstream effectors interact with phosphorylated RTKs via phosphotyrosine binding domains.

Two main binding domains PTB and SH2 are involved in the recruitment of adaptors such as the multi-docking protein known as the insulin receptor substrate-1 (IRS-1).

The binding of signaling proteins allows them to be phosphorylated by the receptor.

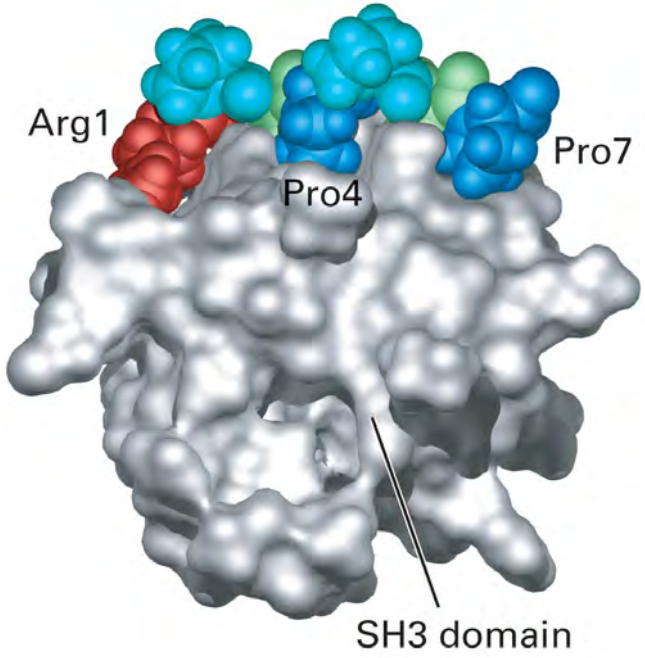


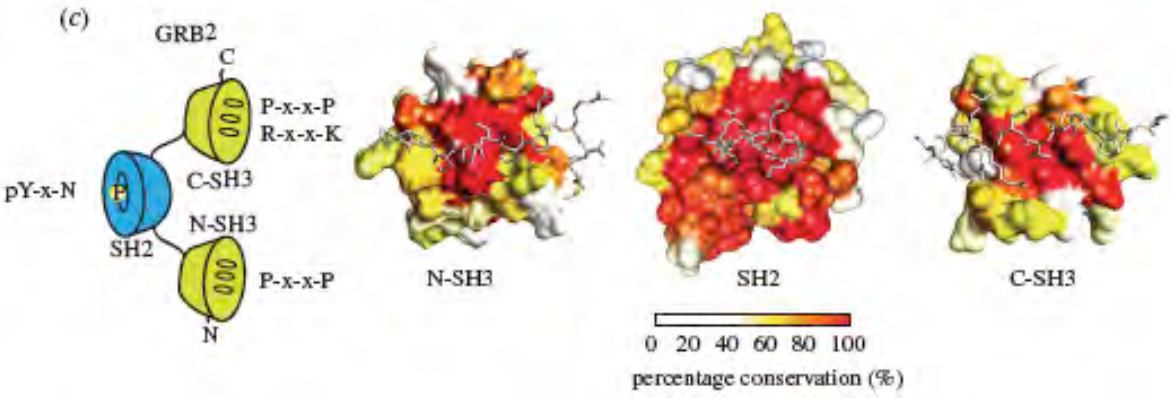


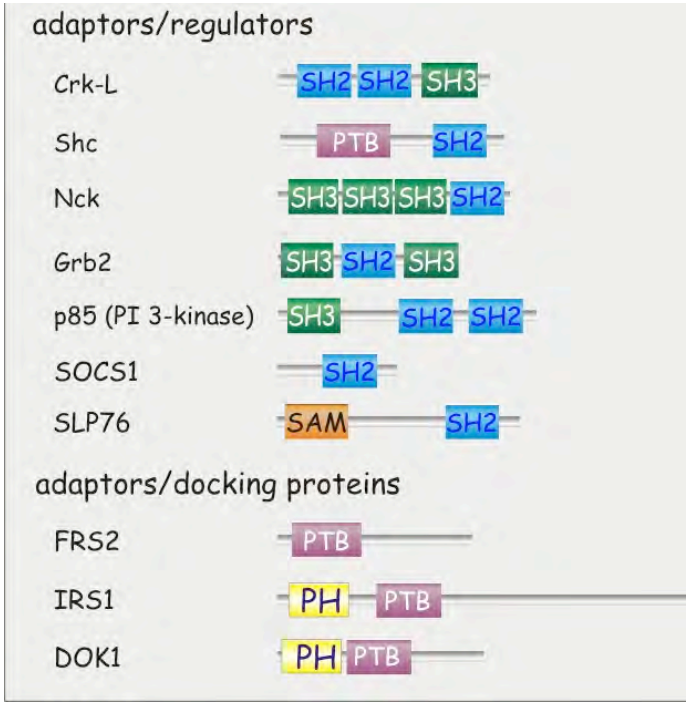
ADAPTORS PROTEINS: GRB-2

Protein domains of GRB2

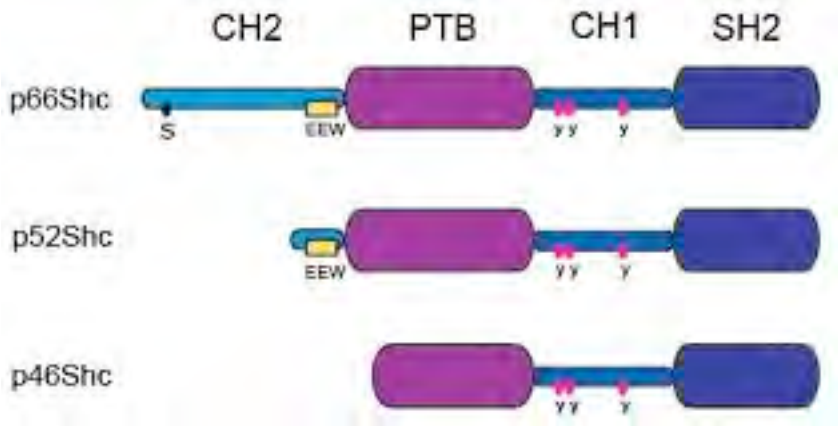
SH2: binds to phosphotyrosine residues
SH3: binds to proline rich sequences

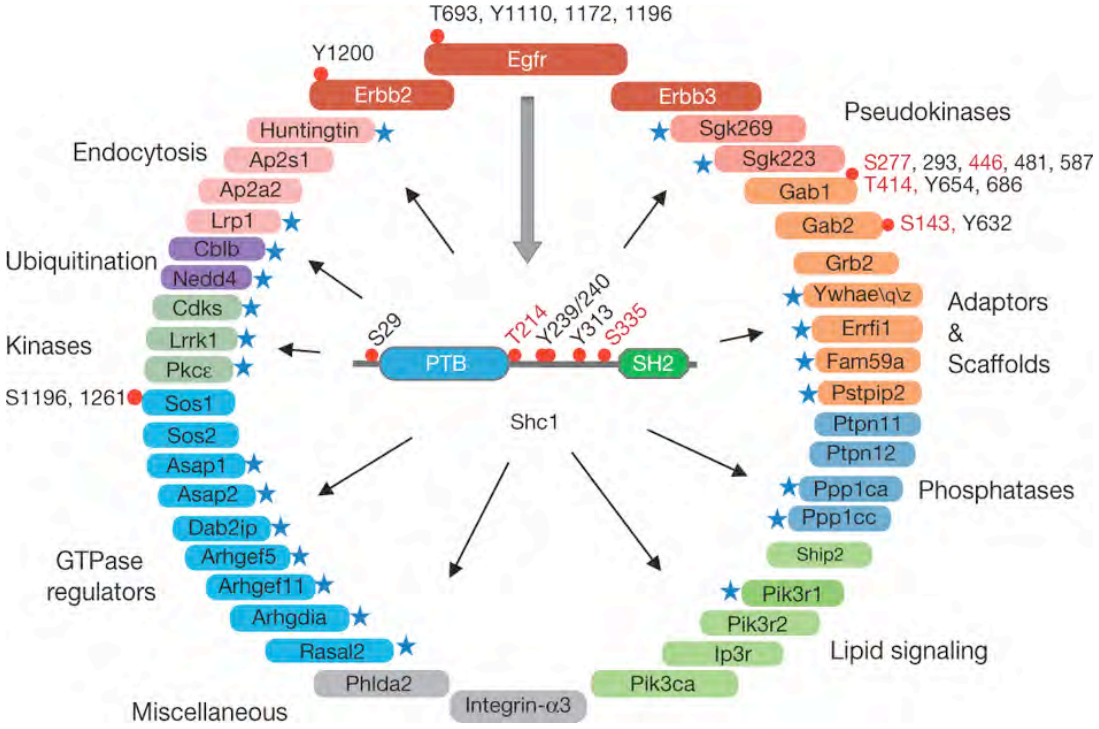






ADAPTORS PROTEINS: Shc

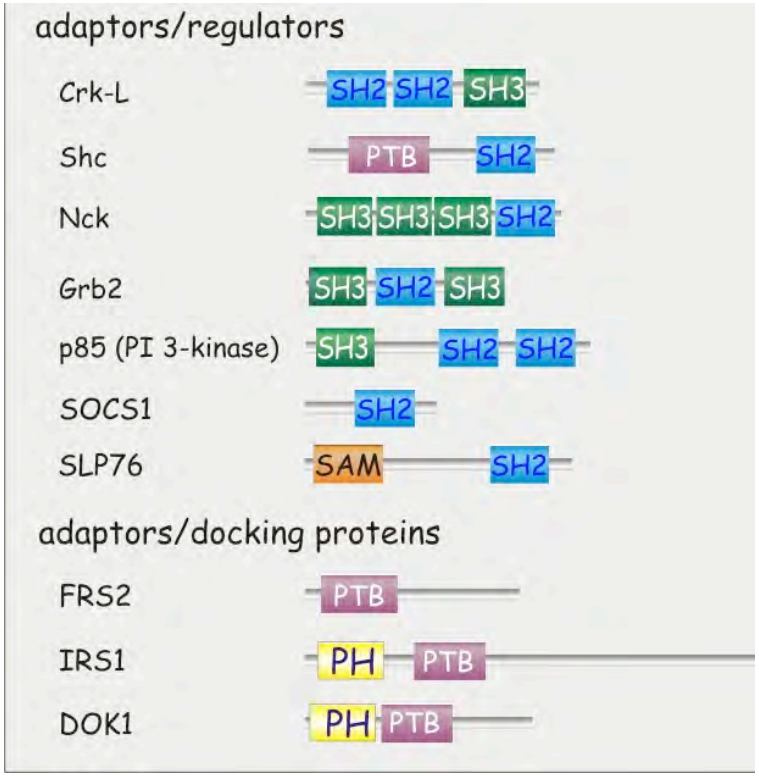




Protein domains of Shc

SH2: binds to phosphotyrosine residues

PTB: binds to phosphotyrosine rich sequences

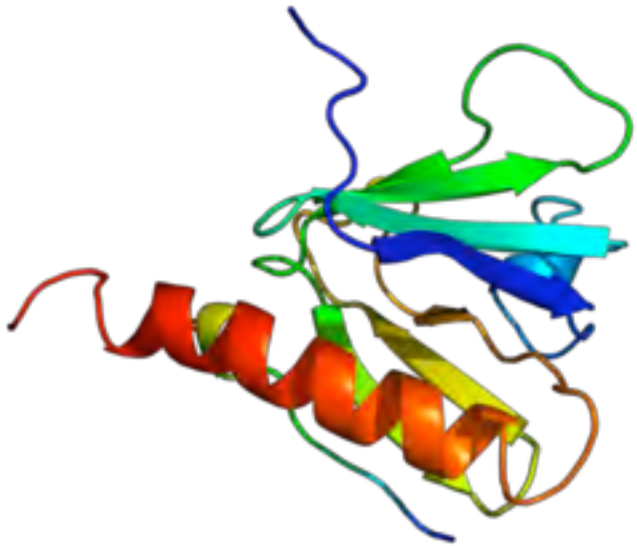


ADAPTORS PROTEINS: IRS-1

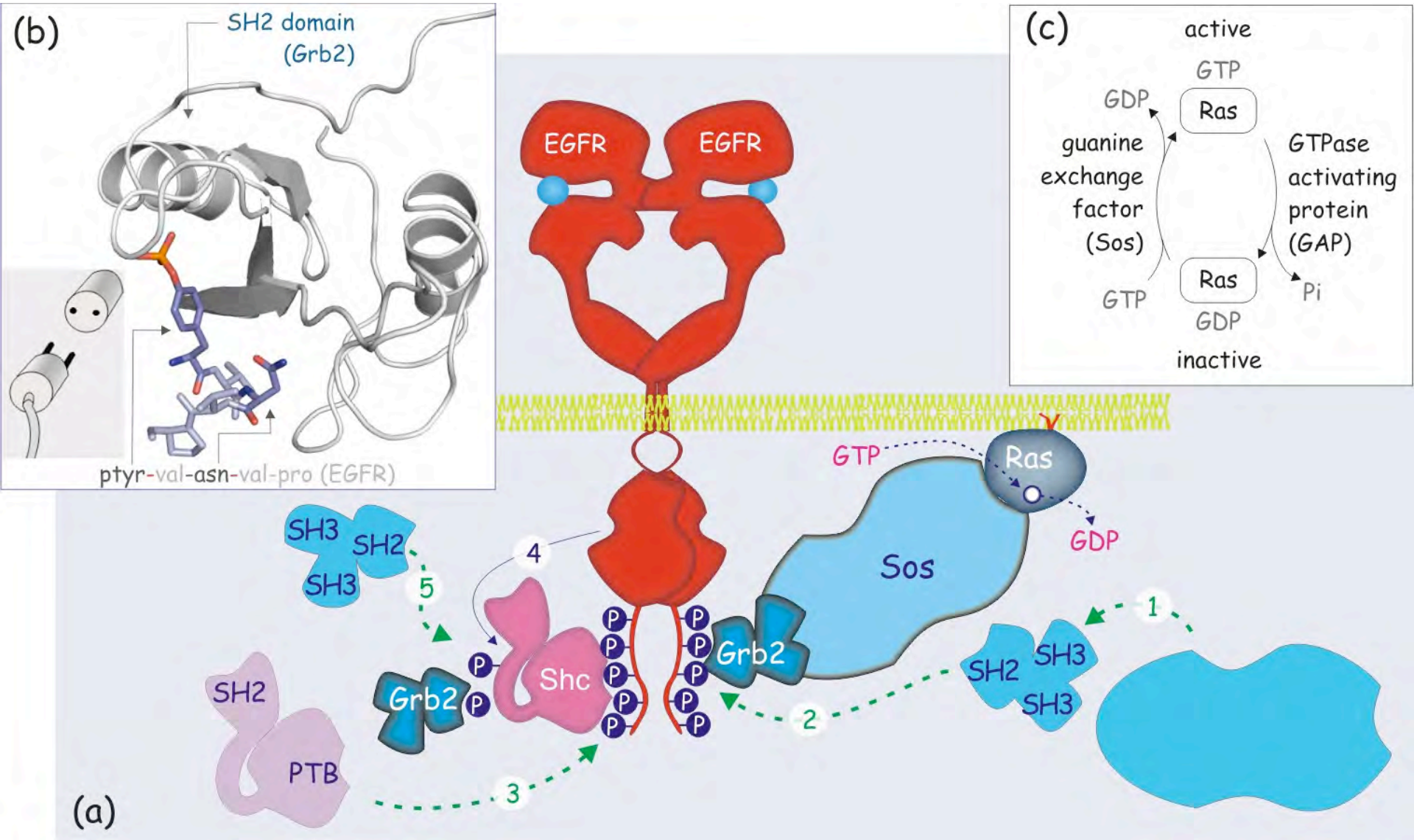
Protein domains of IRS-1:

PH: binds to bind PI lipids within biological membranes (PIP2-PIP3) and proteins such as the βγ-subunits of G proteins and PKc

PTB: bind to phosphotyrosine residues



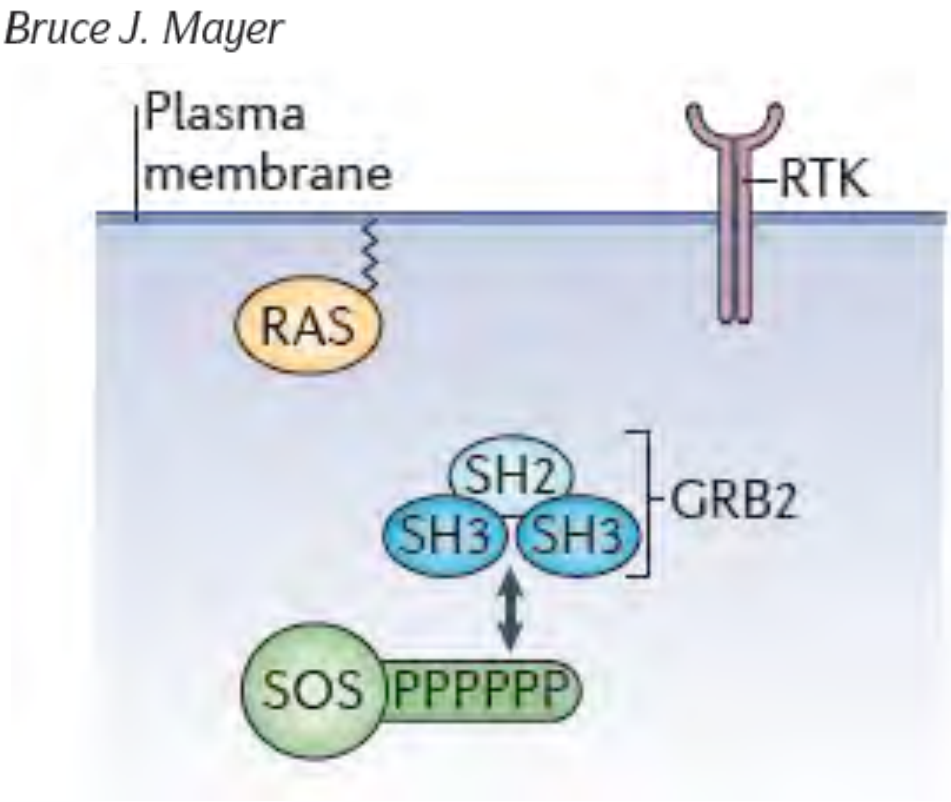
An example of a signaling complex – a protein relay system using phosphorylation as a signal

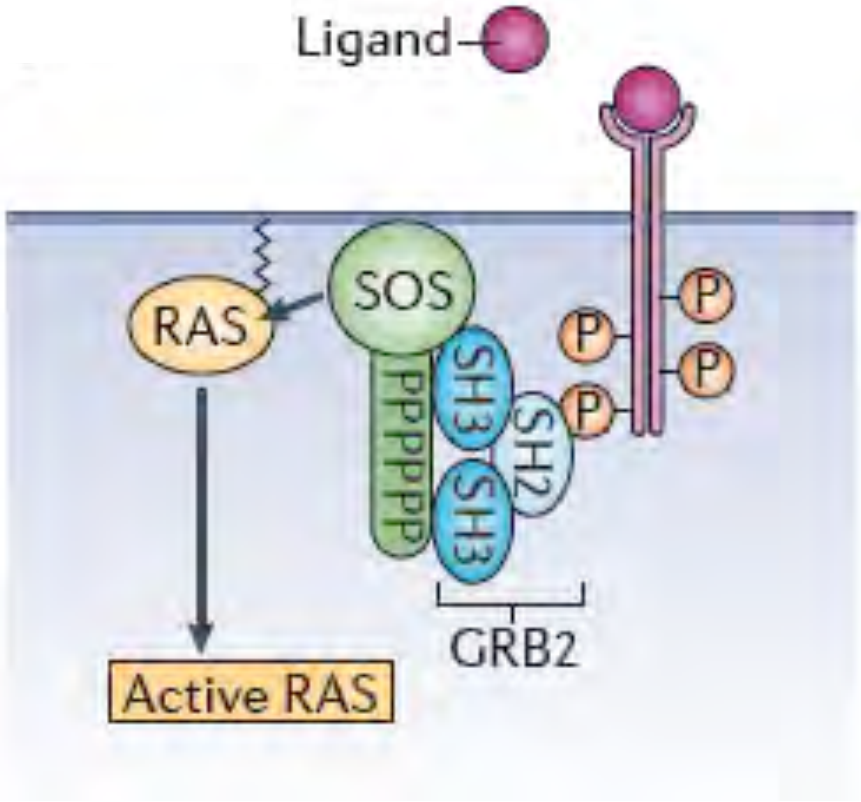


Nature Reviews Molecular Cell Biology | AOP, published online 30 September 2015; doi:10.1038/nrm4068

The discovery of modular binding domains: building blocks of cell signalling

Bruce J. Mayer

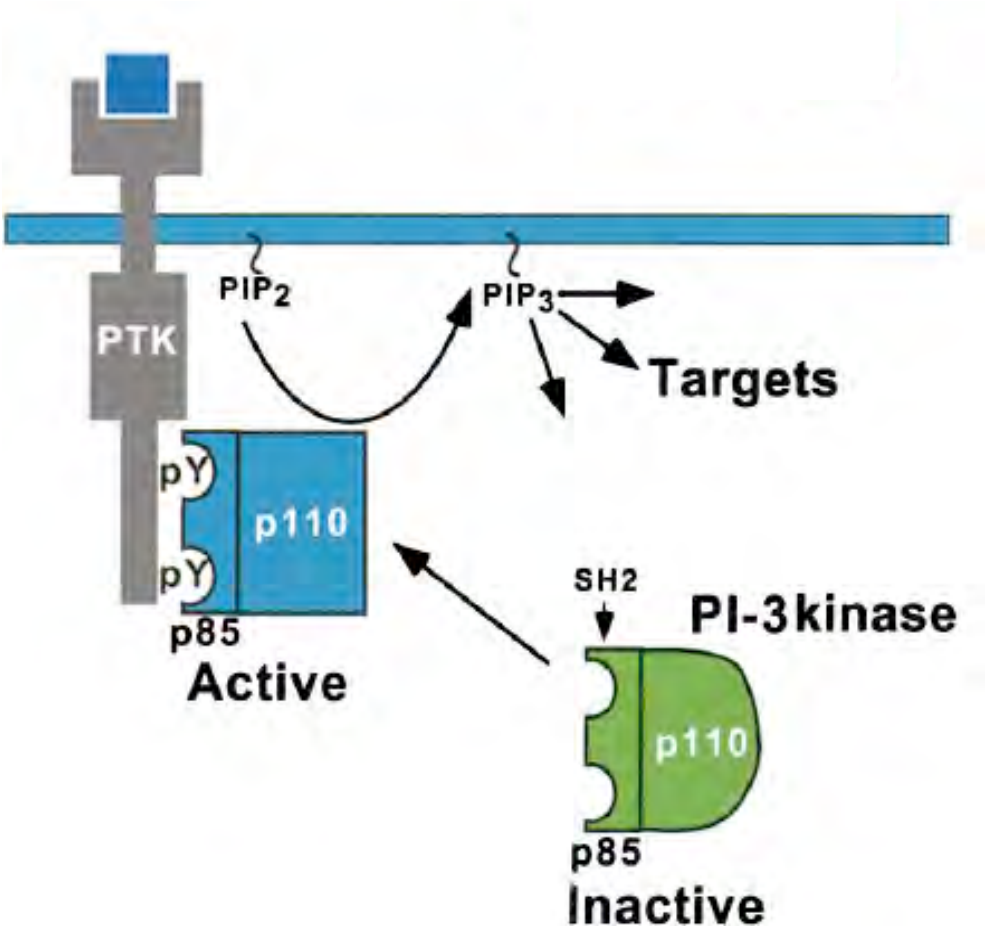




Paradigms for activation of RTK signaling cascade:

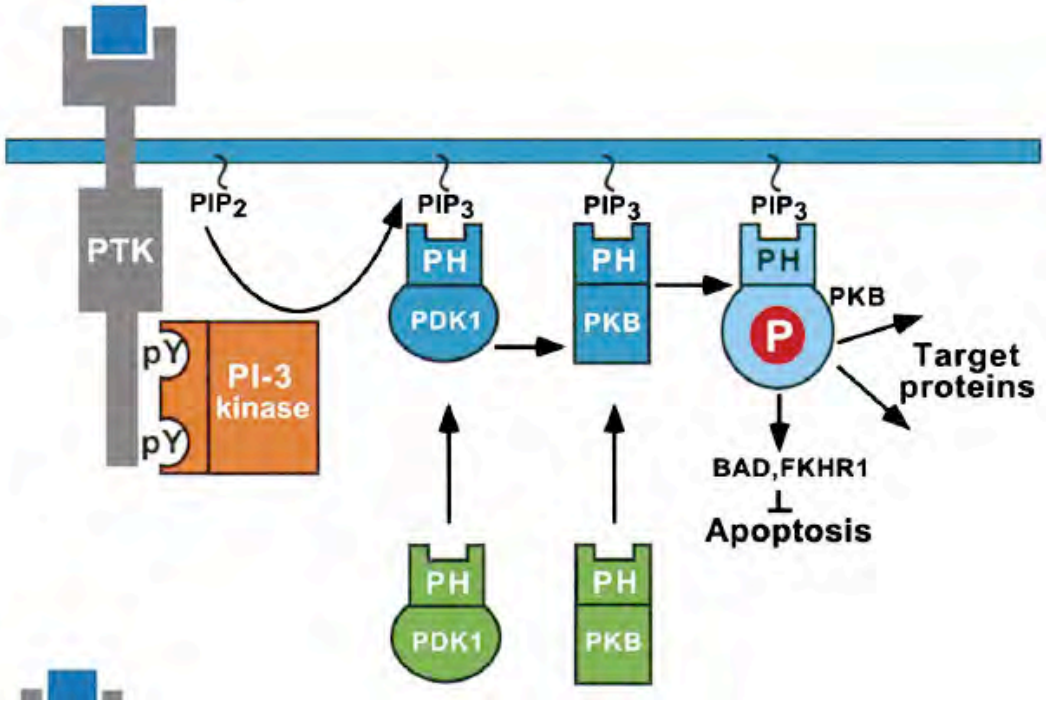
Translocation to the plasmalemma is essential for activation of most effector proteins

1- Activation by a conformational change



Binding of the SH2 domain of p85, the regulatory subunit of PI3-kinase to p-Tyr sites on activated receptors releases an autoinhibitory constraint that stimulates the catalytic domain (p110).

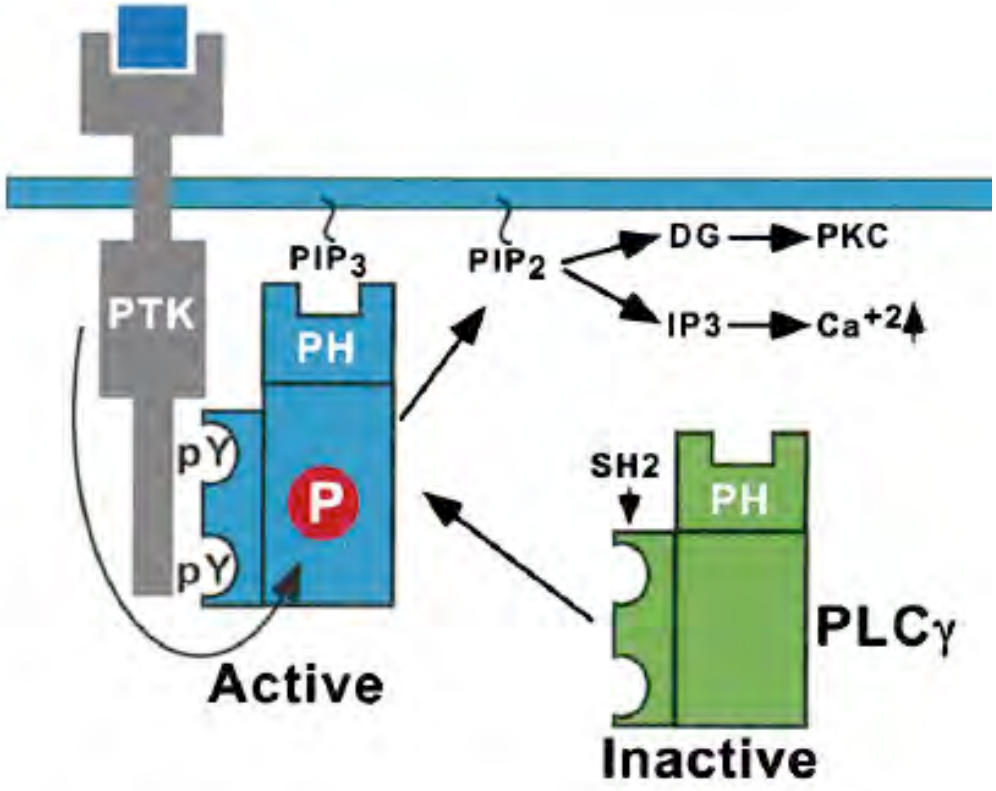
2- Activation by membrane translocation



PtdIns (3,4,5)P_3 generated in response to growth factor stimulation serves as a binding site for the PH domains of PDK1 and PKB.

Membrane translocation is accompanied by a release of an autoinhibition leading to activation of PDK1 and PKB kinase activities.

3- Activation by tyrosine phosphorylation

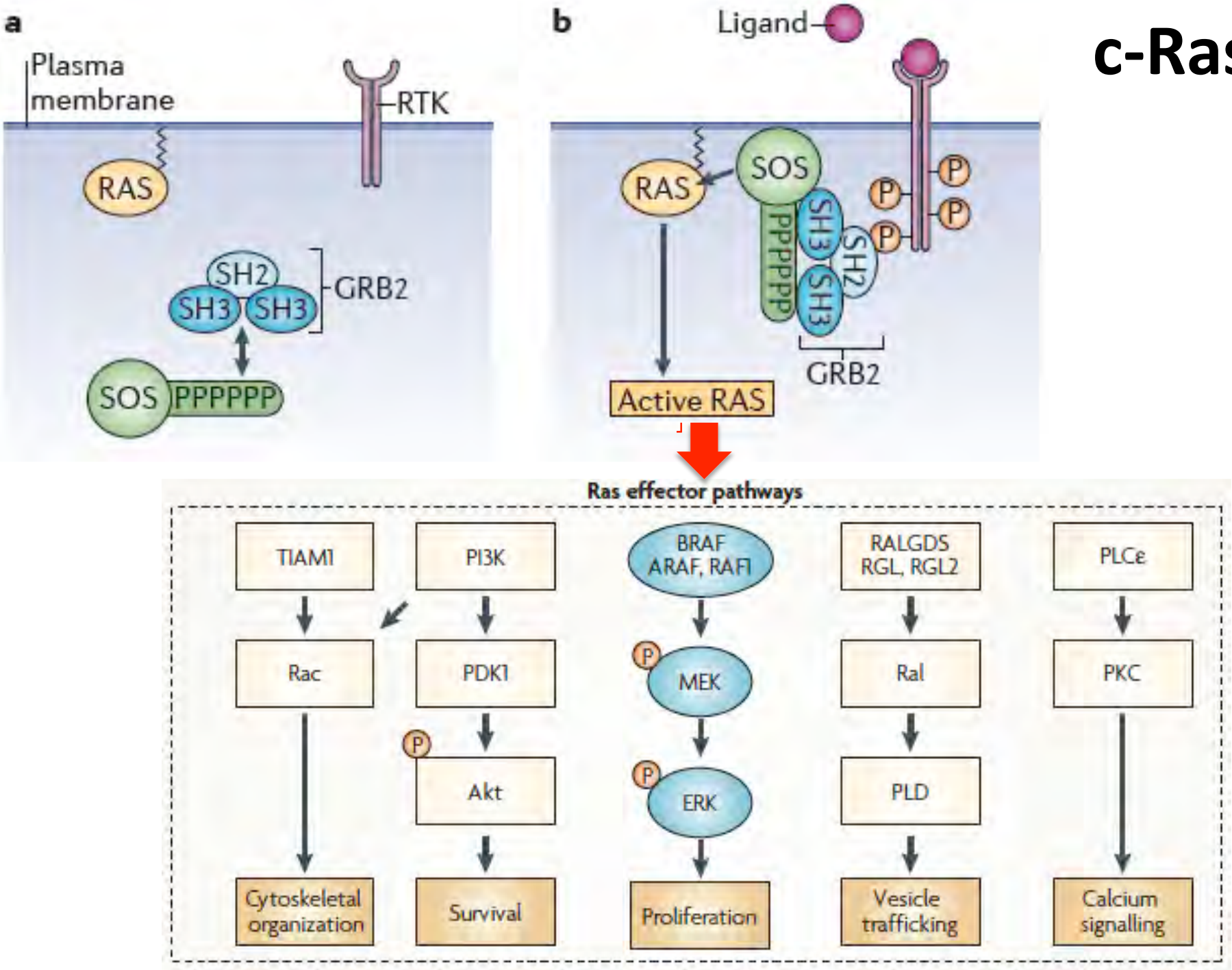


Binding of the SH2 domain of PLC-γ to P-Tyr sites in activated receptors facilitates tyrosine phosphorylation of PLC-γ, as well as membrane translocation. Tyrosine phosphorylation is essential for PLC-γ activation.

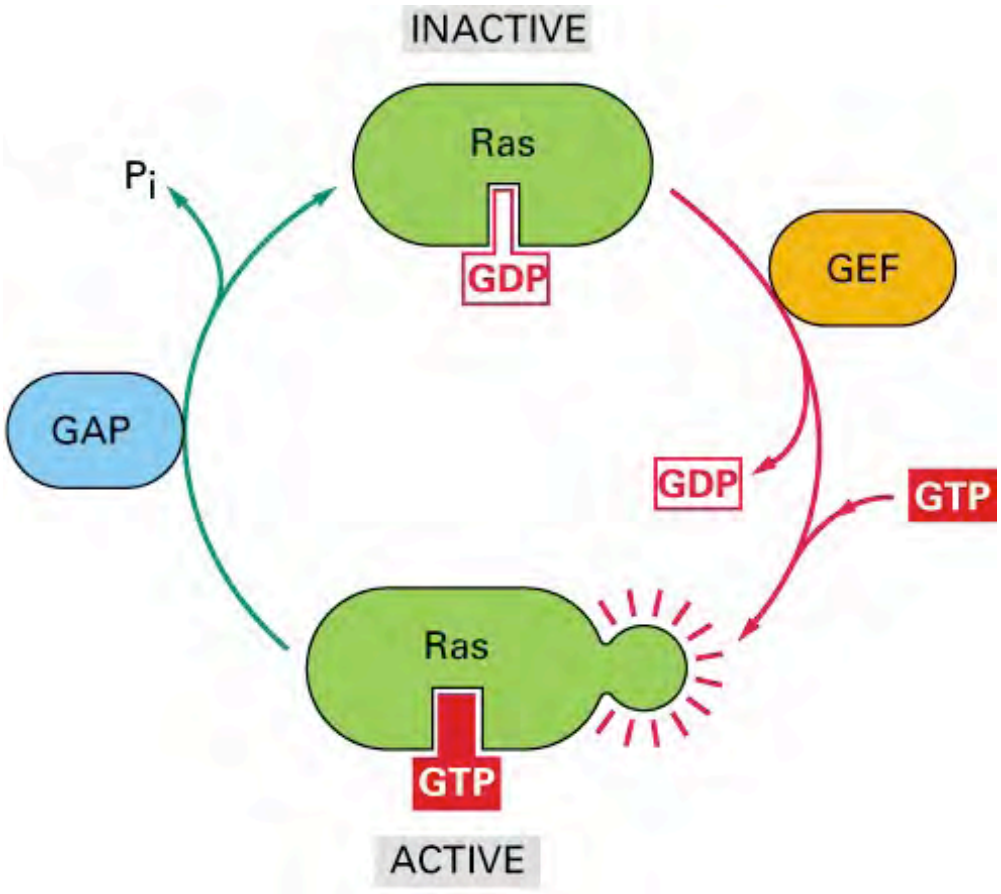
Ras (RAT-sarcoma) **is a monomeric GTPase**

- Ras is a G protein (guanosine-nucleotide-binding protein), a small GTPase
- The first two *ras* genes, *HRAS* and *KRAS*, were first identified from the Harvey sarcoma virus and Kirsten sarcoma virus, by Scolnick and colleagues at (NIH) in 1982. In 1982, activated and transforming human *ras* genes were discovered in human cancer cells. A third *ras* gene was subsequently discovered and named *NRAS*, for its initial identification in human neuroblastoma cells.
- Anchored at the plasma membrane owing to its prenylation and palmitoylation (HRAS and NRAS) or the combination of prenylation and a polybasic sequence adjacent to the prenylation site (KRAS).
- The C-terminal region of Ras first gets farnesylated at its Cys residue in the cytosol, allowing Ras to loosely insert into the membrane of the endoplasmatic reticulum and other cellular membranes.
- The three human *ras* genes encode extremely similar proteins made up of chains of 188 to 189 amino acids, designated H-Ras, N-Ras and K-Ras4A and K-Ras4B (from alternative splicing).

c-Ras


a
Plasma membrane
RTK
RAS
SH2
SH3
SH3
GRB2
SOS
PPPPPP
b
Ligand
RAS
SOS
PPPPPP
SH3
SH2
SH3
P
GRB2
Active RAS
Ras effector pathways
TIAM1
PI3K
BRAF ARAF, RAF1
RALGDS RGL, RGL2
PLCε
Rac
PDK1
MEK
Ral
PKC
Akt
ERK
PLD
Cytoskeletal organization
Survival
Proliferation
Vesicle trafficking
Calcium signalling

Ras is a monomeric GTPase

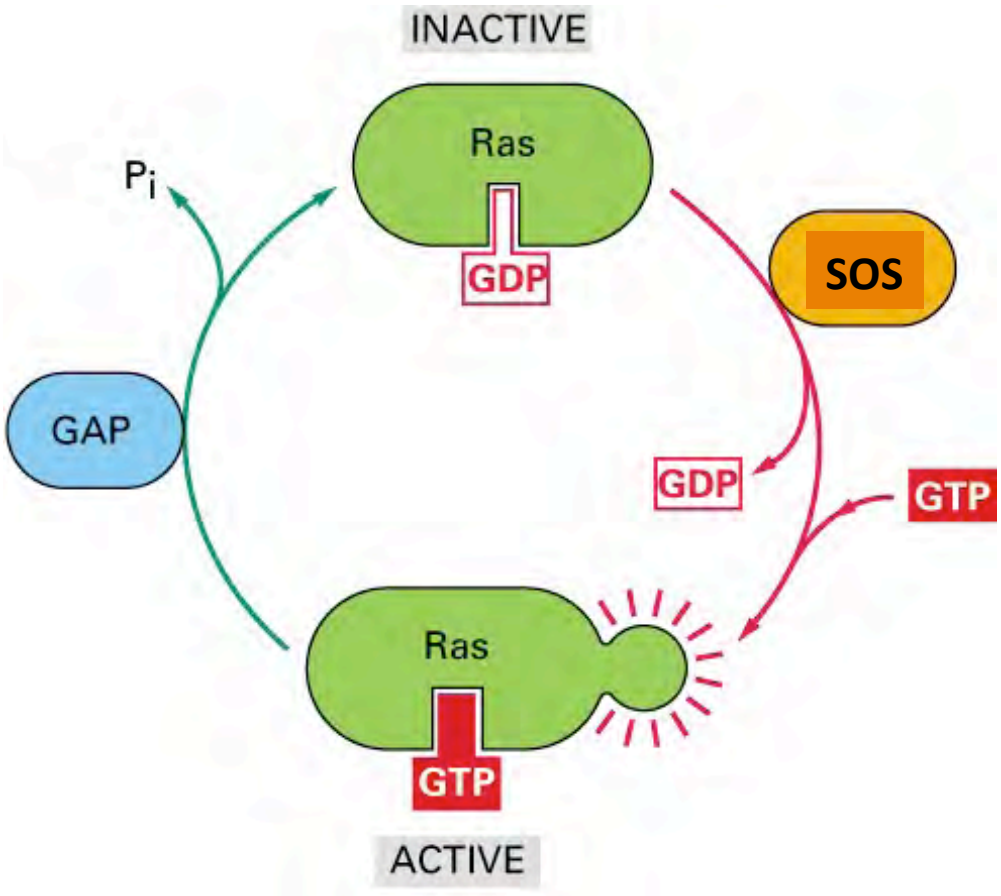


Ras has an intrinsic GTPase activity: the protein on its own will hydrolyze a bound GTP molecule into GDP. However this process is too slow for efficient function, and hence the GAP for Ras, *RasGAP*, may bind to and stabilize the catalytic machinery of Ras.

GEFs catalyze a "push and pull" reaction which releases GDP from Ras. Because intracellular GTP is abundant relative to GDP (approximately 10 fold more) GTP predominantly re-enters the nucleotide binding pocket of Ras and reloads the spring. Thus GEFs facilitate Ras activation.

The balance between GEF and GAP activity determines the guanine nucleotide status of Ras, thereby regulating Ras activity.

Ras is a monomeric GTPase



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The activation of Ras by RTKs

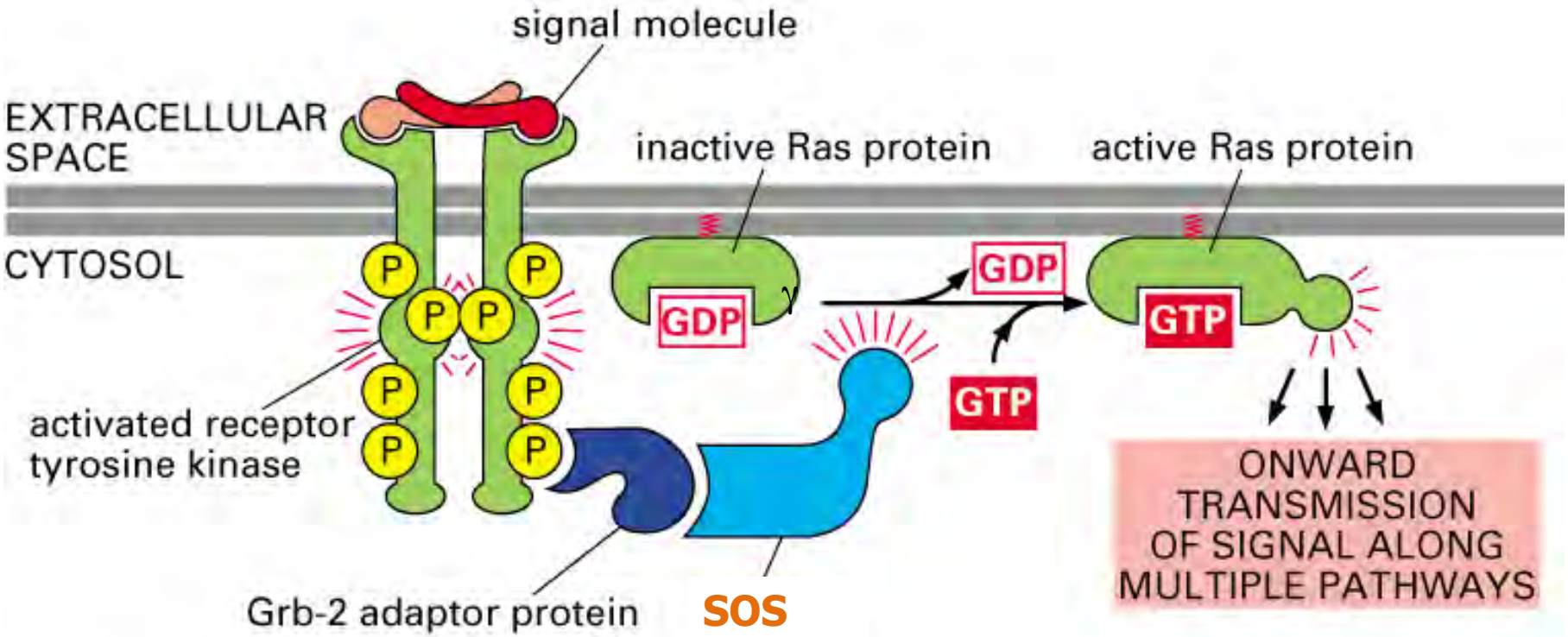


Figure 15–55. Molecular Biology of the Cell, 4th Edition.

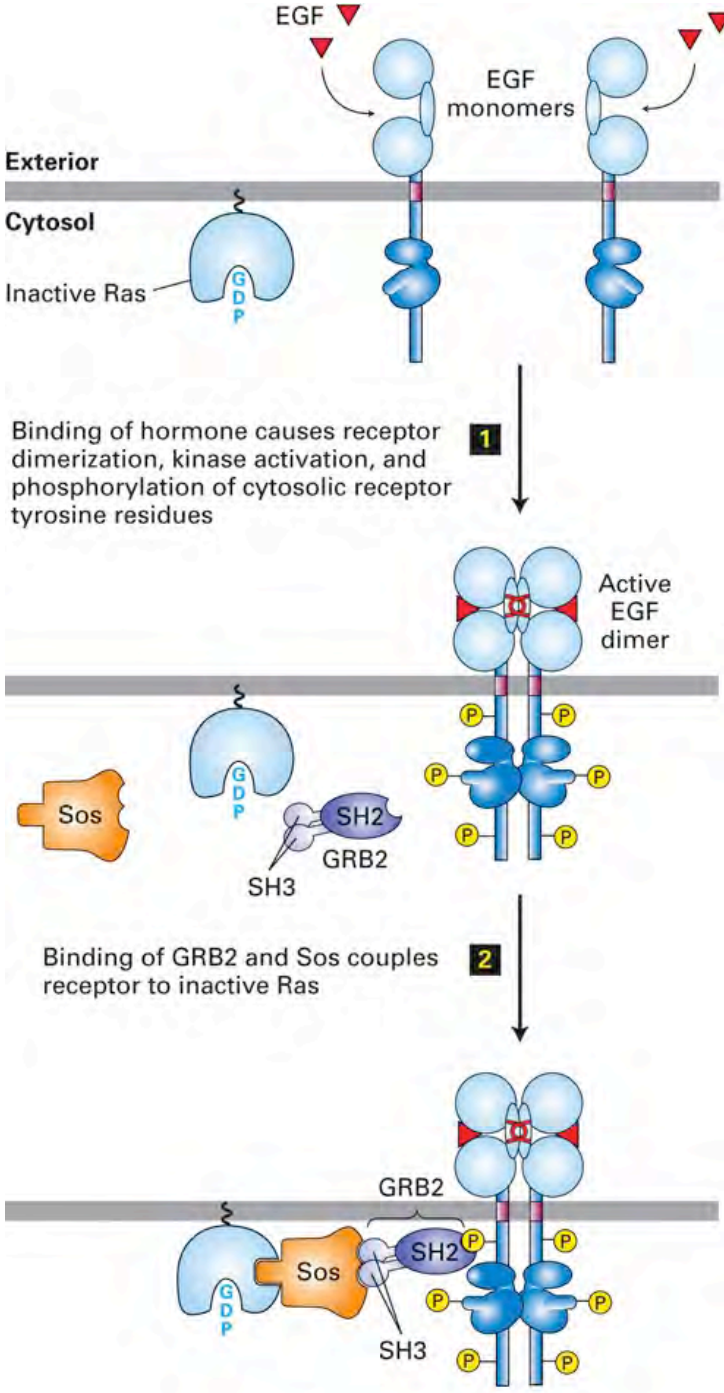
RTK Activation of Ras

EGF binding causes receptor clusterization and autophosphorylation on cytosolic tyrosines.

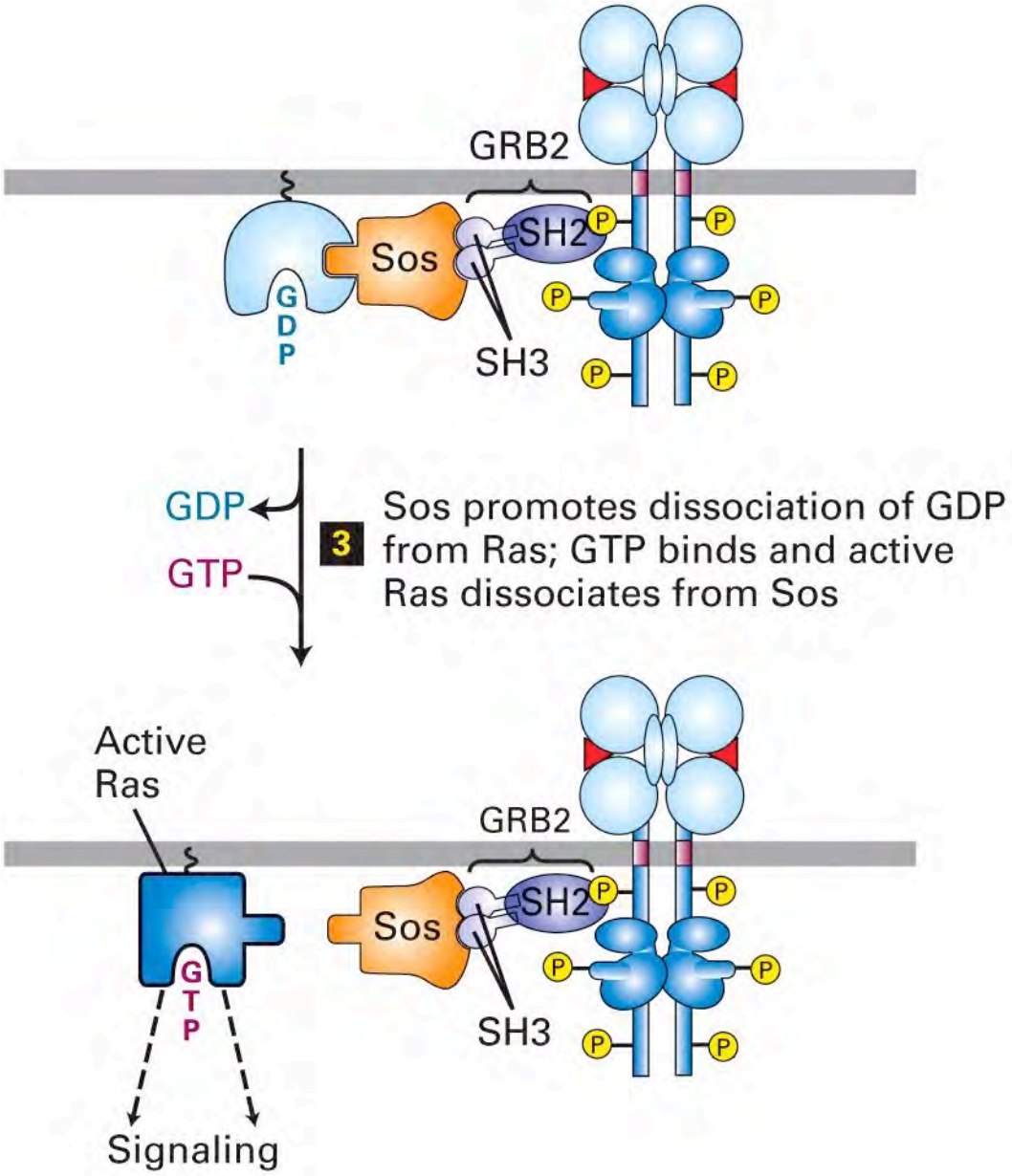
In Step 2, the adaptor protein GRB2 binds receptor phosphotyrosine residues via its SH2 domain. GRB2 contains SH3 domains that allow the GEF protein known as Sos to bind to the membrane complex.

The C-terminus of Sos inhibits its nucleotide exchange activity; binding of GRB2 relieves this inhibition

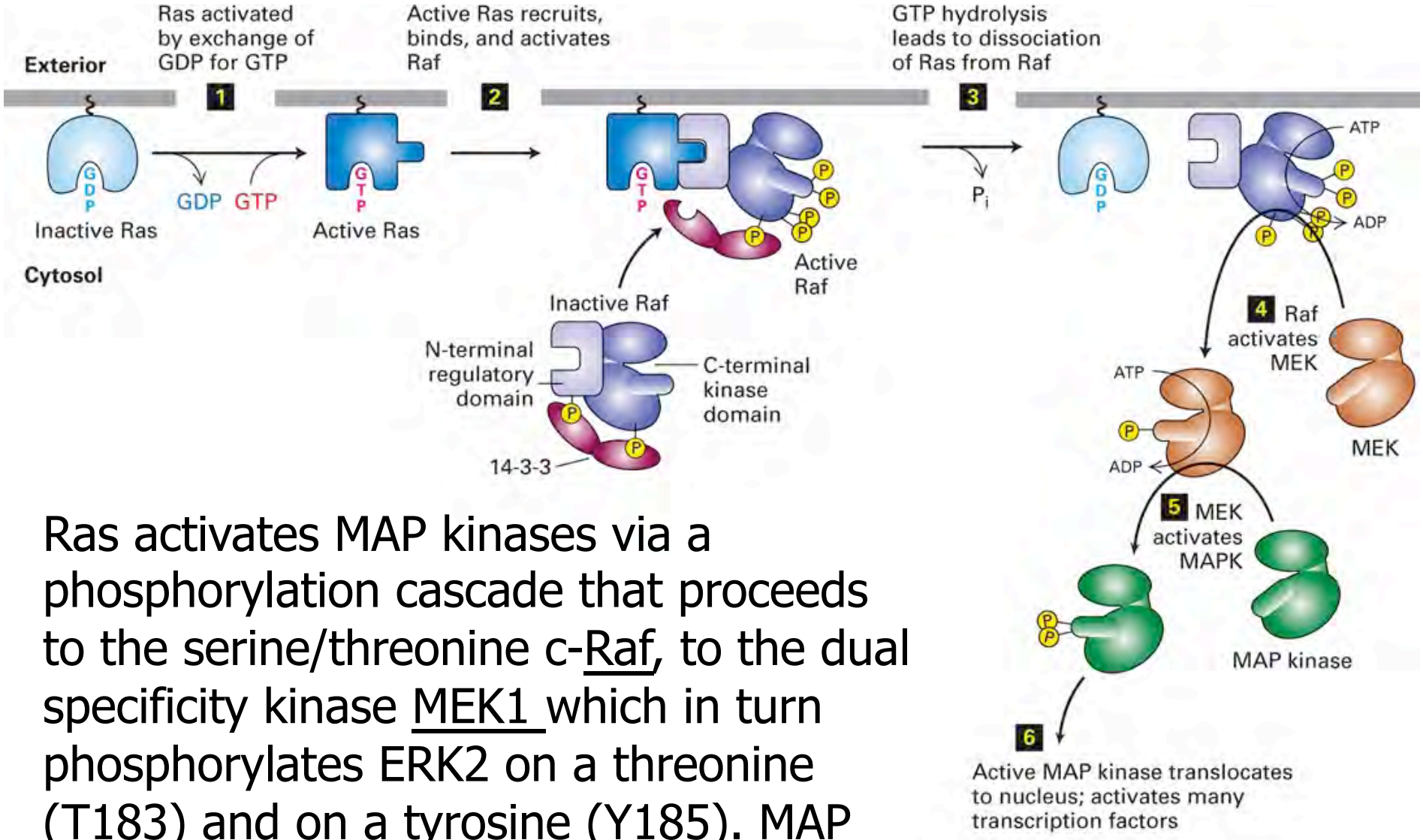
Sos converts inactive GDP-ras into active GTP-ras.



The activated Ras-GTP complex then dissociates from Sos, but remains tethered to the inner leaflet of the cytoplasmic membrane via a lipid anchor sequence. The active form of Ras then activates the MAP kinase portion of the signaling pathway.



Ras Activation of MAP Kinase

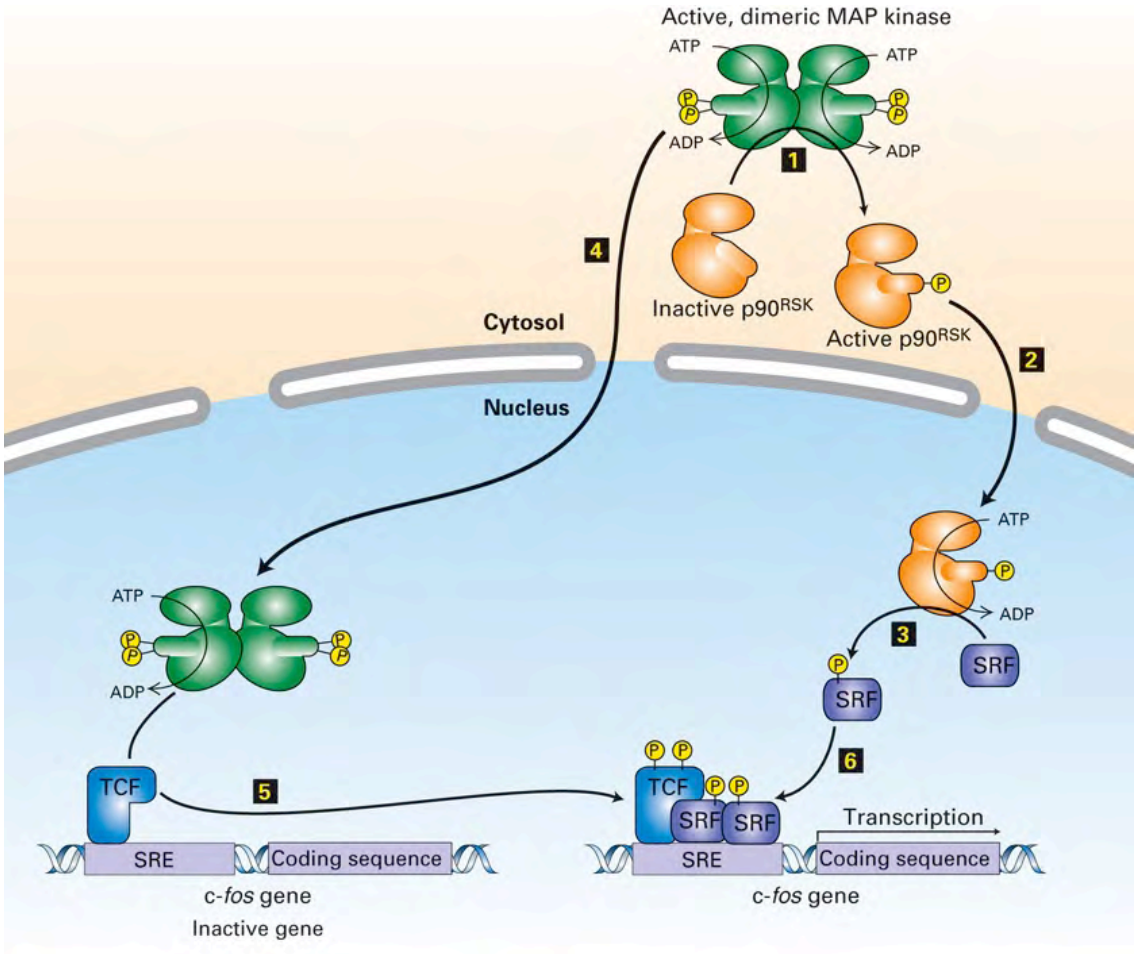


Ras activates MAP kinases via a phosphorylation cascade that proceeds to the serine/threonine c-Raf, to the dual specificity kinase MEK1 which in turn phosphorylates ERK2 on a threonine (T183) and on a tyrosine (Y185). MAP kinase then dimerizes and enters the nucleus.

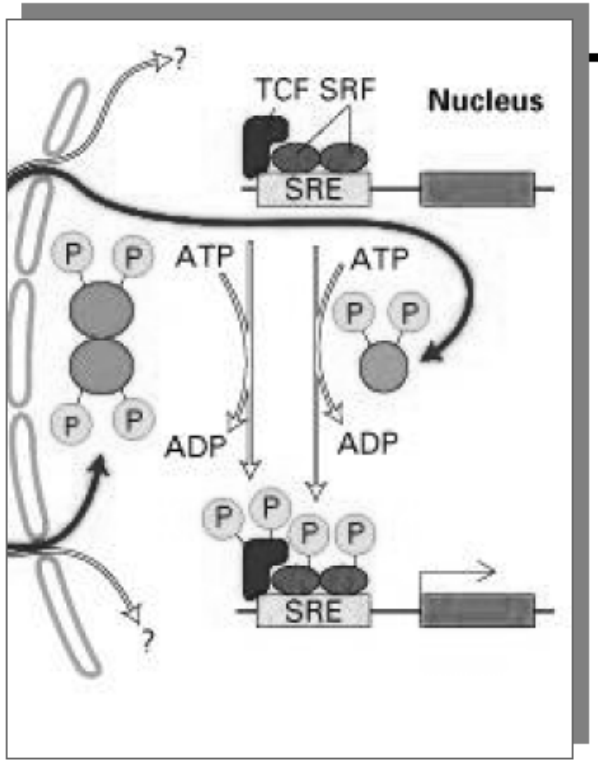
MAP Kinase Activation of Transcription

In the final steps of RTK-Ras/MAP kinase signaling, MAP kinase phosphorylates and activates the $p90^{RSK}$ kinase in the cytoplasm. Both kinases enter the nucleus where they phosphorylate ternary complex factor (TCF) and serum response factor (SRF), respectively.

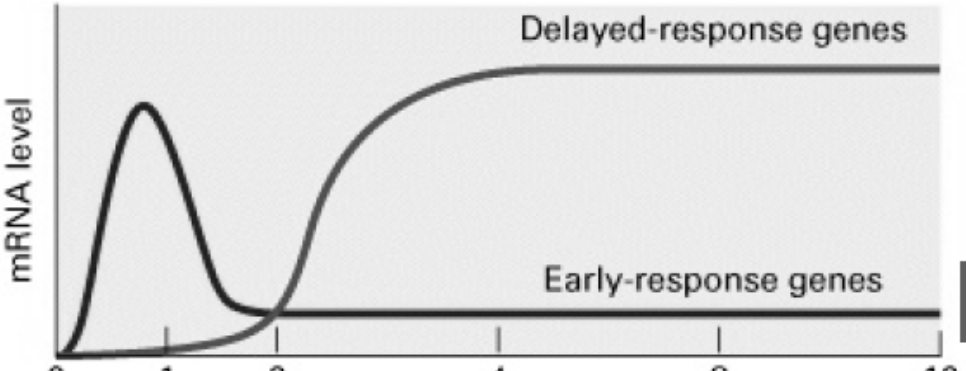
The phosphorylated forms of these TFs bind to serum response element (SRE) enhancer sequences that control genes regulated by growth factors present in serum (such as c-fos) and propel cells through the cell cycle.



Genes regulated by RTK/Ras pathway include early response genes.



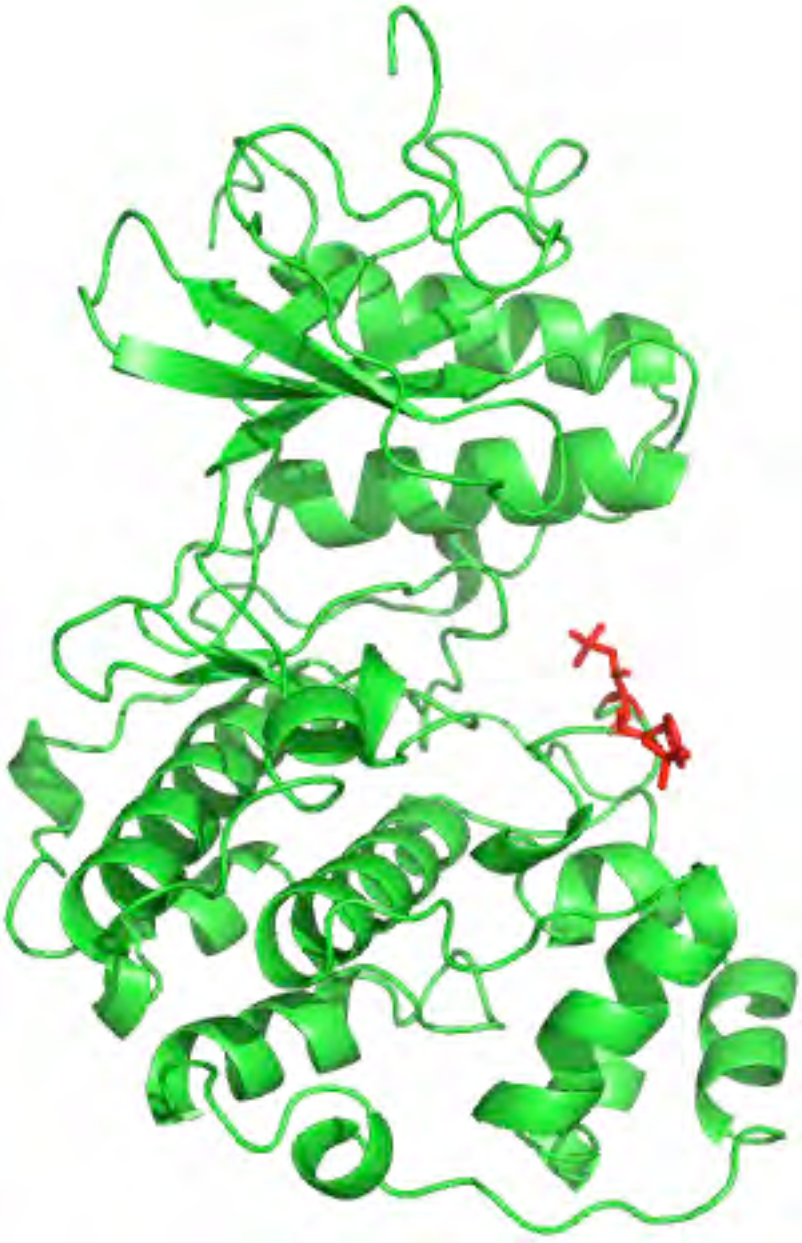
serum response elements;
-allows for activated transcription following growth factor (mitogen) stimulation
-found in genes involved in cellular proliferation.
Ex: c-fos gene
-c-fos is an early response gene
-required for the induction of delayed response genes including cyclin D.



D-type cyclins

C-fos transcription factor

MAP Kinases



- **Mitogen-activated protein kinases** are serine/threonine-protein kinases. They regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.

MAP kinases are found in eukaryotes only.

"Classical" MAPKs activation requires **two phosphorylation events**, both threonine and tyrosine residues, in order to lock the kinase domain in a catalytically competent conformation.

Inactivation of MAPKs is performed by a very conserved family of dedicated phosphatases is the so-called MAP kinase phosphatases (MKPs), dual-specificity phosphatases (DUSPs). They hydrolyze the phosphate from both phosphotyrosine and the phosphothreonine residues.

- Once activated, Ras propagates signaling further inside the cell via a kinase cascade that culminates in the activation of members of the MAP kinase family.
- MAP kinases phosphorylate TFs that regulate genes involved in the cell cycle, survival and in differentiation.

- As a result, mutations in *ras* genes can cause unintended and overactive signalling inside the cell and ultimately to cancer
- Ras is the most common oncogene in human cancer - mutations that permanently activate Ras are found in 20-25% of all human tumors and up to 90% in certain types of cancer (pancreatic cancer).

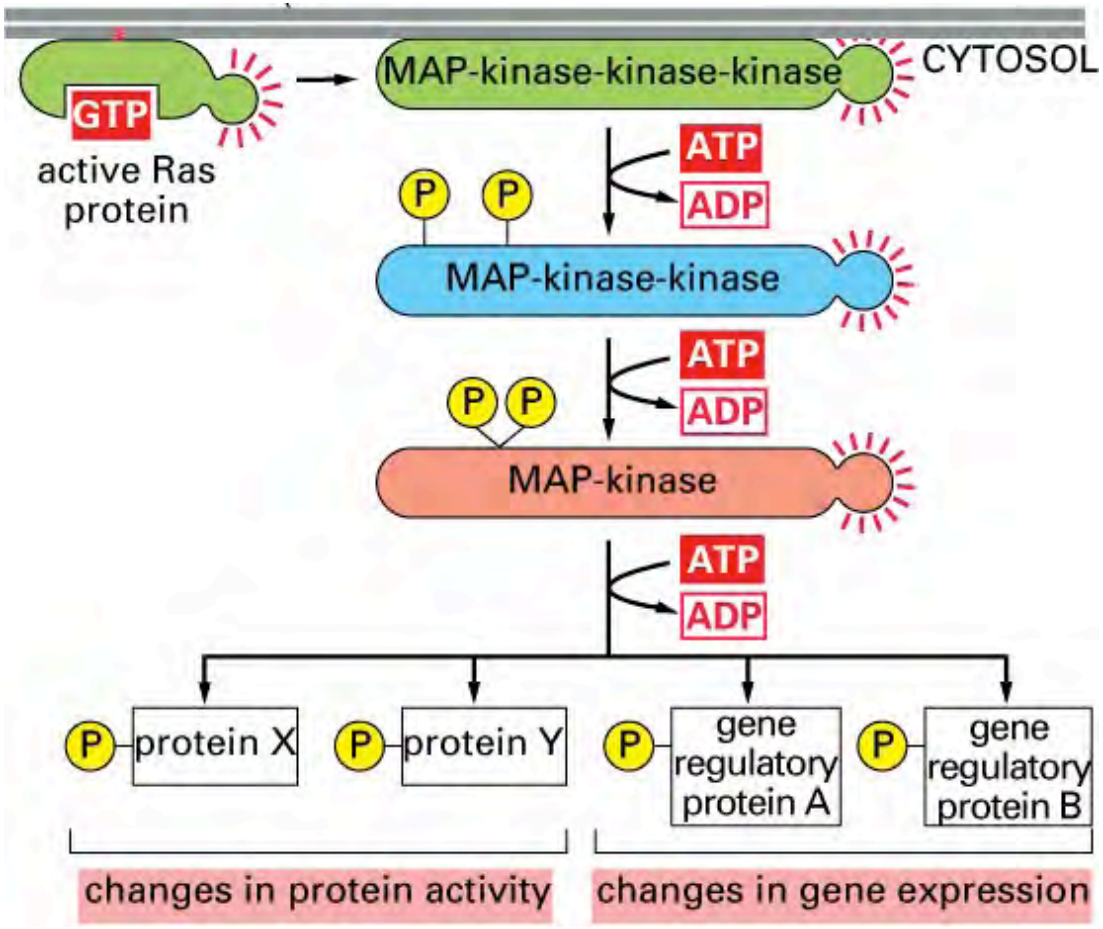


Table 2 | ***HRAS*, *KRAS*, *NRAS* and *BRAF* mutations in human cancer**

Cancer type	*HRAS*	*KRAS*	*NRAS*	*BRAF*
Biliary tract	0%	33%	1%	14%
Bladder	11%	4%	3%	0%
Breast	0%	4%	0%	2%
Cervix	9%	9%	1%	0%
Colon	0%	32%	3%	14%
Endometrial	1%	15%	0%	1%
Kidney	0%	1%	0%	0%
Liver	0%	8%	10%	3%
Lung	1%	19%	1%	2%
Melanoma	6%	2%	18%	43%
Myeloid leukaemia	0%	5%	14%	1%
Ovarian	0%	17%	4%	15%
Pancreas	0%	60%	2%	3%
Thyroid	5%	4%	7%	27%

The mutation data was obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer web site[148].

- Diversi tipi di cancro sembrano essere associati alla mutazione di una specifica isoforma RAS. Solitamente i carcinomi (in particolare quelli del colon e del pancreas) presentano mutazioni di KRAS, i tumori della vescica hanno mutazioni di HRAS e i tumori emopoietici presentano mutazioni di NRAS.

Oncogenes vs proto-oncogenes

- An **oncogene** is a gene that has the potential to cause cancer.
- In tumor cells, they are often mutated or expressed at high levels.
- The first confirmed oncogene was discovered in 1970 and was termed src. Src was in fact first discovered as an oncogene in a chicken retrovirus.
- In 1976 Dominique Stehelin, J. Michael Bishop and Harold E. Varmus demonstrated that oncogenes were activated proto-oncogenes, found in many organisms including humans (for this discovery Bishop and Varmus were awarded the Nobel Prize in Physiology or Medicine in 1989).

- A **proto-oncogene** is a normal gene that becomes an oncogene due to mutations or increased expression.
- Proto-oncogenes code for proteins that regulate cell growth and differentiation. Proto-oncogenes are often involved in signal transduction and execution of mitogenic signals.
- Upon *activation*, a proto-oncogene becomes a tumor-inducing agent, an oncogene.

CELLULAR ONCOGENES

- Present in cancer cells
- Contains introns characteristic of eukaryotic cells
- Encodes proteins triggering transformation of normal cells

VIRAL ONCOGENES

- Present in viruses
- Host cell origin
- Do not possesss introns
- Also called 'cancer genes'
- Encodes proteins triggering transformation of normal cells into cancer cells

VIRAL ONCOGENE	HUMAN ONCOGENE	ORIGIN	NATURE
V-src	C-src	Chicken	Sarcoma
V-ras	C-ras	Rat	Sarcoma
V-myc	C-myc	Chicken	Leukemia
V-fes	C-fes	Feline	Sarcoma
V-sis	C-sis	Simian	Sarcoma
V-mos	C-mos	Mouse	Sarcoma

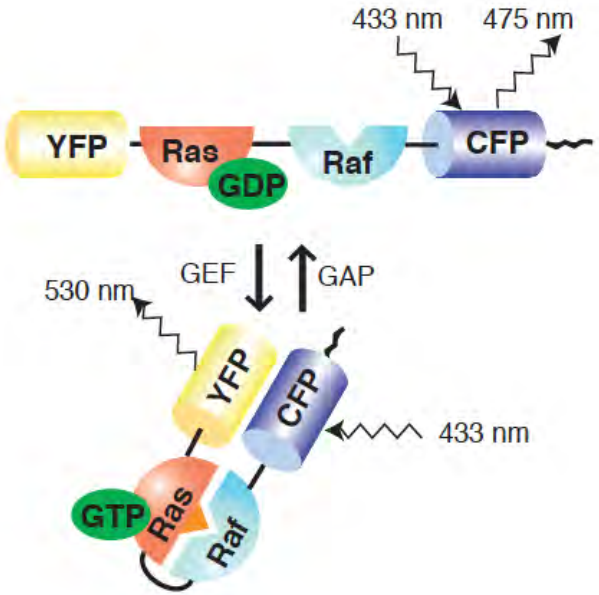
Visualizing Ras signalling in real-time

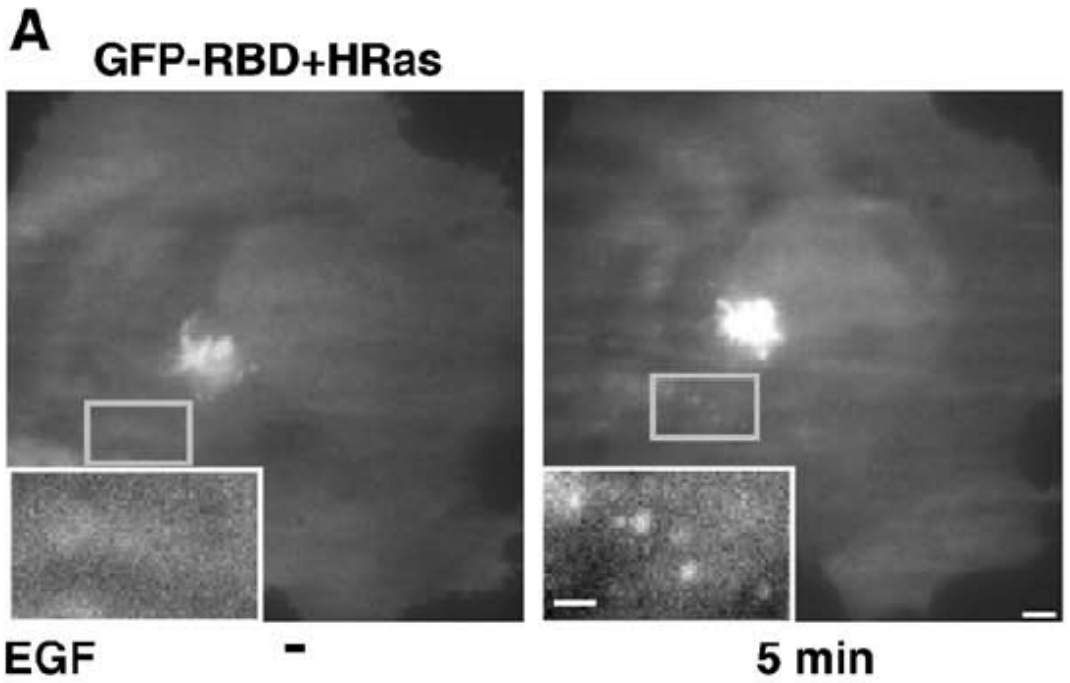
Simon A. Walker and Peter J. Lockyer*

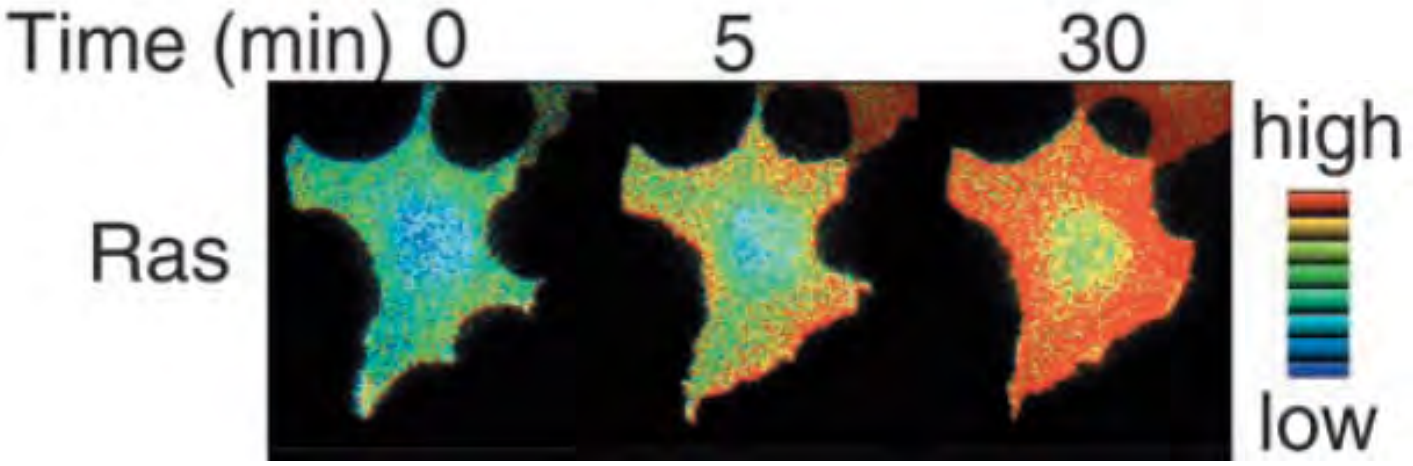
Laboratory of Molecular Signalling, The Babraham Institute, Babraham Research Campus

*Author for correspondence (e-mail: peter.lockyer@bbsrc.ac.uk)

Journal of Cell Science 117, 2879-2886 Published by The Company of Biologists 2004
doi:10.1242/jcs.01285







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Rasosomes spread Ras signals from plasma membrane 'hotspots'

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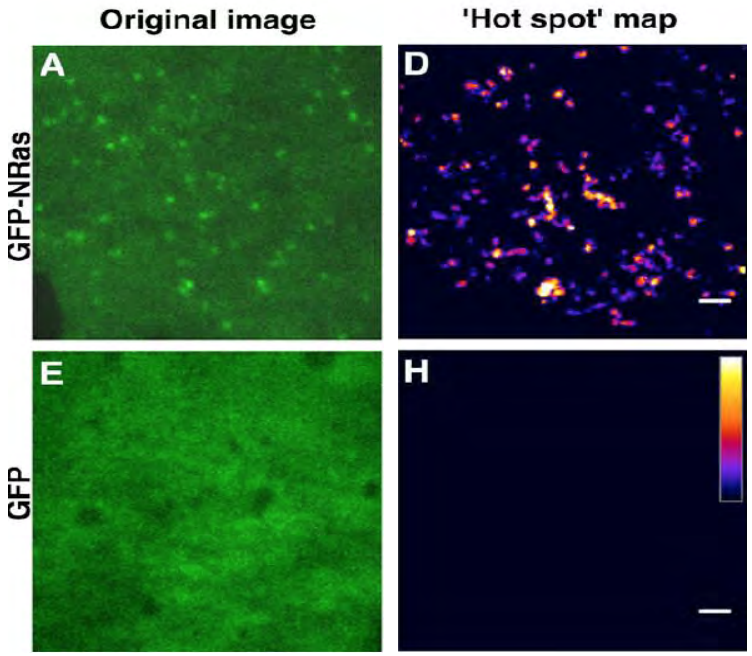
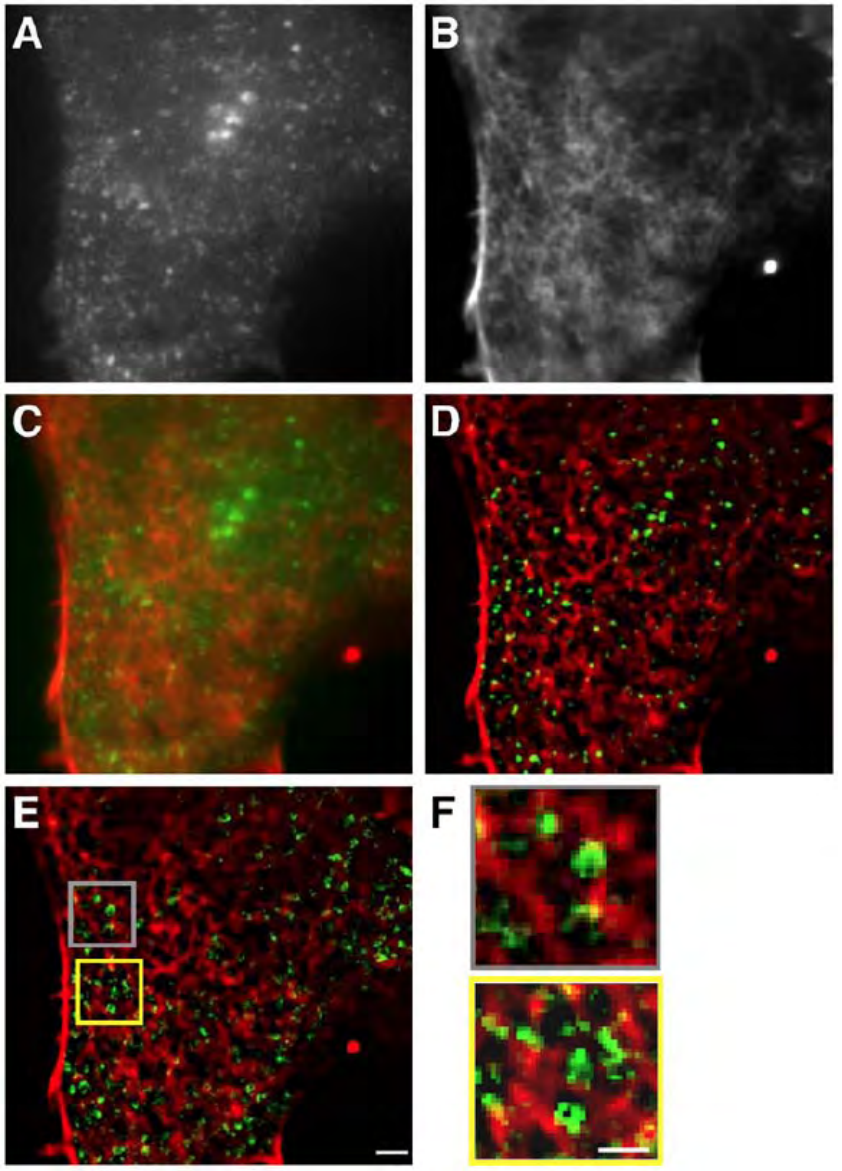
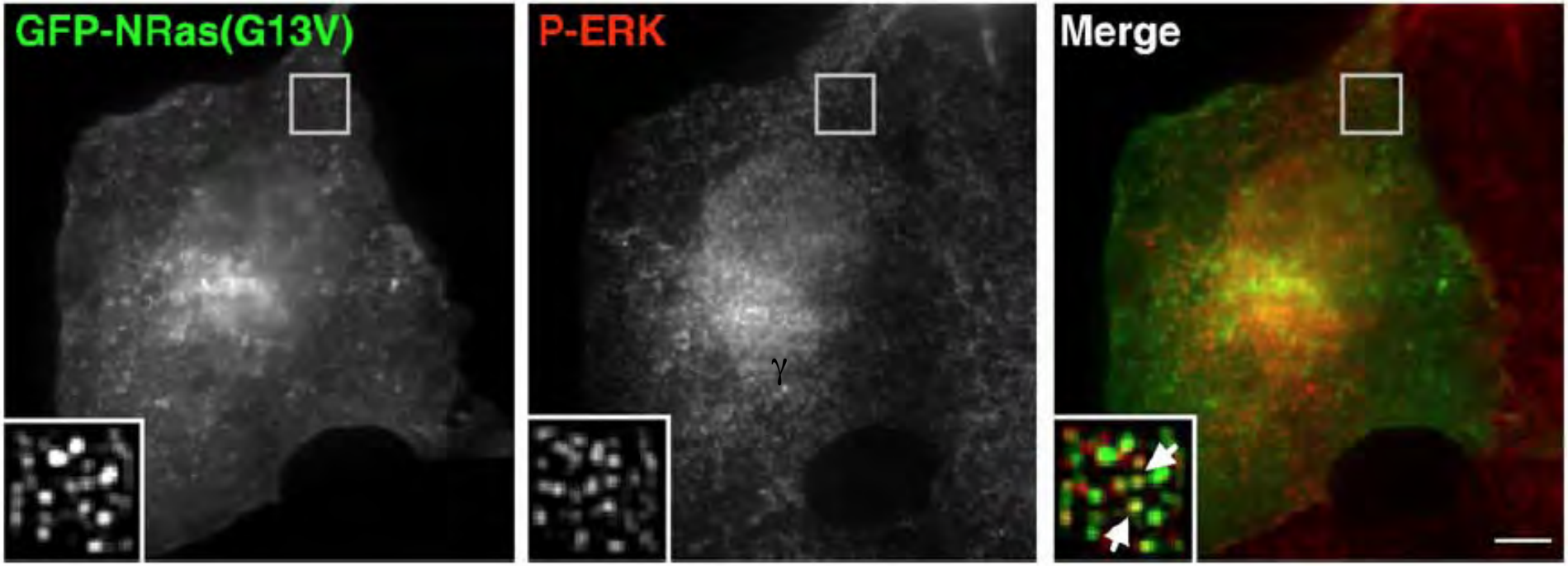


Ras-osomes move within distinct areas, rasosomal 'hotspots', near the PM.



Rasosomes move within cortical actin cages.



GFP-NRas expressing cells were labeled with anti-phosphorylated-ERK Abs. Insets show filtered images of the boxed regions with arrows that indicate phospho-ERK positive GFP-NRas rasosomes