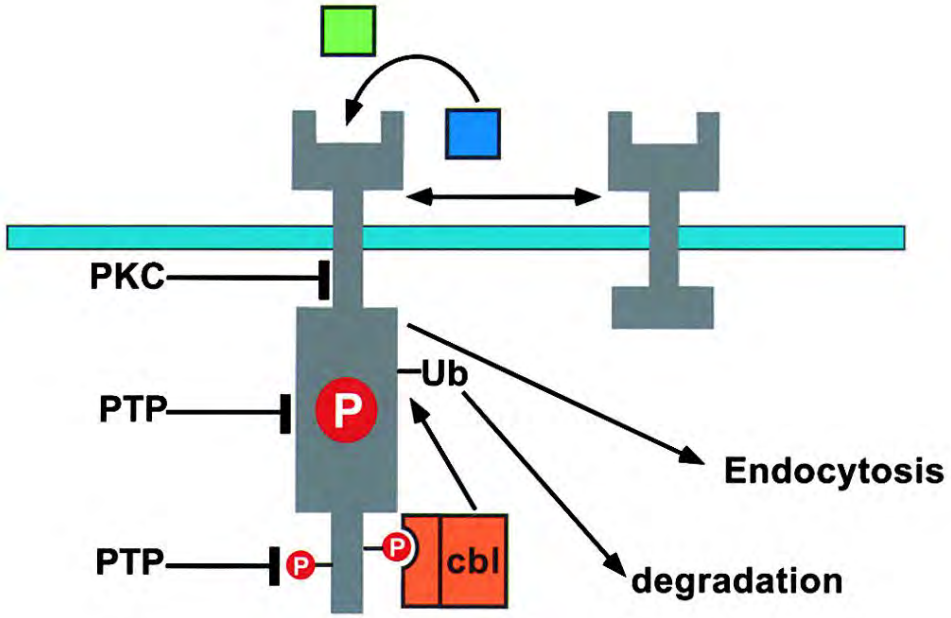
Negative Signalling

SIGNALLING NEGATIVO

- definitivo (irreversibile): termina i segnali rimuovendo le proteine attivate e genera un periodo refrattario nella cellula
- transitorio (reversibile): interferisce con l'intensita' e la durata del segnale in una definita finestra temporale
  ➔ modulazione fine

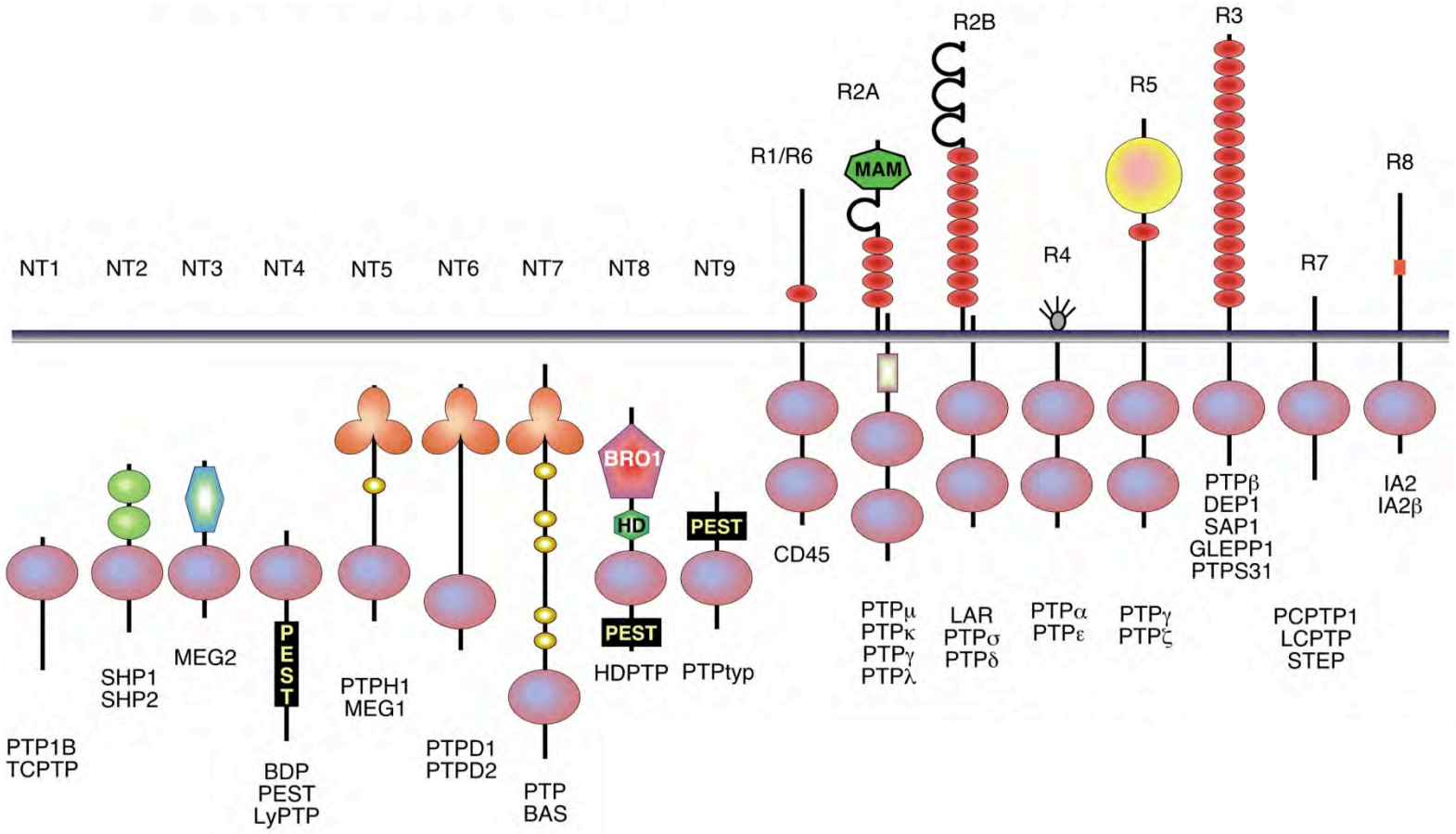
Mechanisms for Attenuation & Termination of RTK Activation



TRANSITORIO
1) Ligand antagonists
2) Receptor antagonists
3) Phosphorylation and dephosphorylation

DEFINITIVO
4) Receptor endocytosis
5) Receptor degradation by the ubiquitin-proteosome pathway

Classification of Protein Tyrosine Phosphatases



Andersen et al., Mol Cell Biol, 21, 7117, 2001

Functional Diversity Through Targeting and Regulatory Domains

PTP1B
TCPTP

C-terminal
- ER targeting
- Proteolytic cleavage

Proline rich segment
- SH3 binding sites

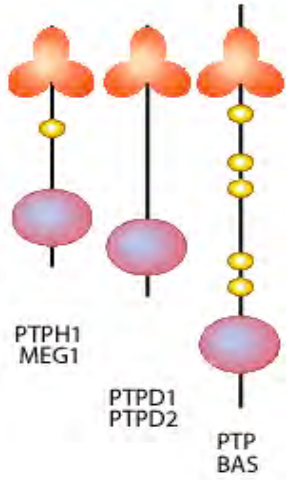
Alternative splicing
- Nucleus vs Cytoplasmic

SHP1
SHP2

SH2 domains
- Plasma membrane signaling complexes
- Auto-inhibition

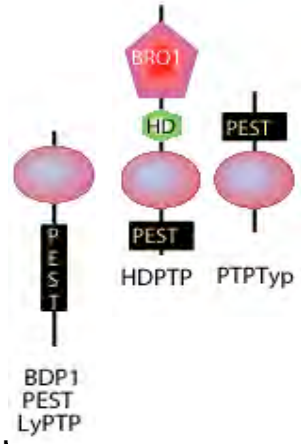
MEG2

Cellular retinaldehyde binding protein-like
- Golgi targeting
- Secretory vesicles
- Putative lipid-binding domain



FERM domain
- Subcellular targeting (e.g. cytoskeletal proteins)

PDZ domain(s)
- Protein-Protein interactions



PEST domain
- Protein-Protein Interactions

BRO1 domain
- Functionally uncharacterised; (Found in a number of signal transduction proteins)
- Vesicle associated

His-domain
- Functionally uncharacterised

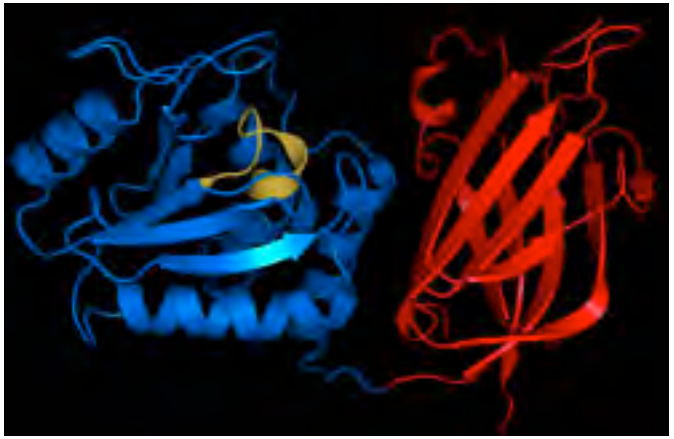
PTPs and Cancer

PTEN	Tumor Suppressor	Mutated in various human cancers. Cowden disease
DEP1	Tumor suppressor	Colon cancer susceptibility locus Scc1 (QTL in mice)
PTPκ	Tumor Suppressor	Primary CNS lymphomas
SHP2	Noonan Syndrome	Developmental disorder affecting 1:2500 newborn
	Stomach Ulcers	Target of *Helicobacter pylori*
Cdc25	Cell Cycle Control	Target of Myc and overexpressed in primary breast cancer
PRL-3	Metastasis	Upregulated in metastases of colon cancer
FAP-1	Apoptosis	Upregulated in cancers, inhibits CD95-mediated apoptosis

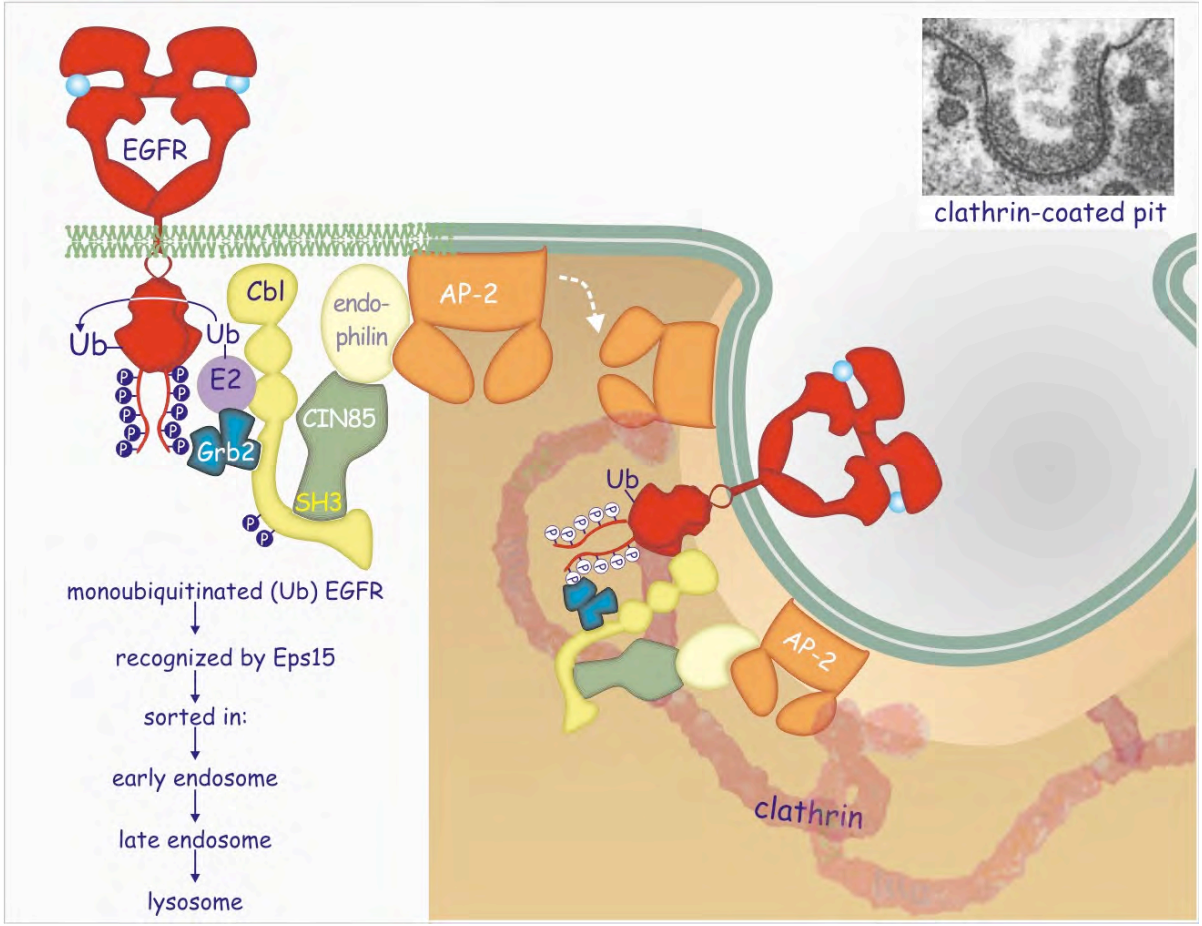
PTEN

Phosphatase and tensin homolog (**PTEN**) is a tumor suppressor gene. This phosphatase is involved in the regulation of the cell cycle, preventing cells from growing and dividing too rapidly.
The protein encoded by this gene is a phosphatidylinositol-3,4,5 trisphosphate 3-phosphatase.
Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of PIP3 in cells which functions as a tumor suppressor by negatively regulating Akt/PKB signaling pathway.

The structure of PTEN reveals that it consists of a phosphatase domain (Blue), and a C2 domain (red) which binds the phospholipid membrane. Thus PTEN binds the membrane through its C2 domain, bringing the active site to the membrane-bound PIP_3 to de-phosphorylate it.



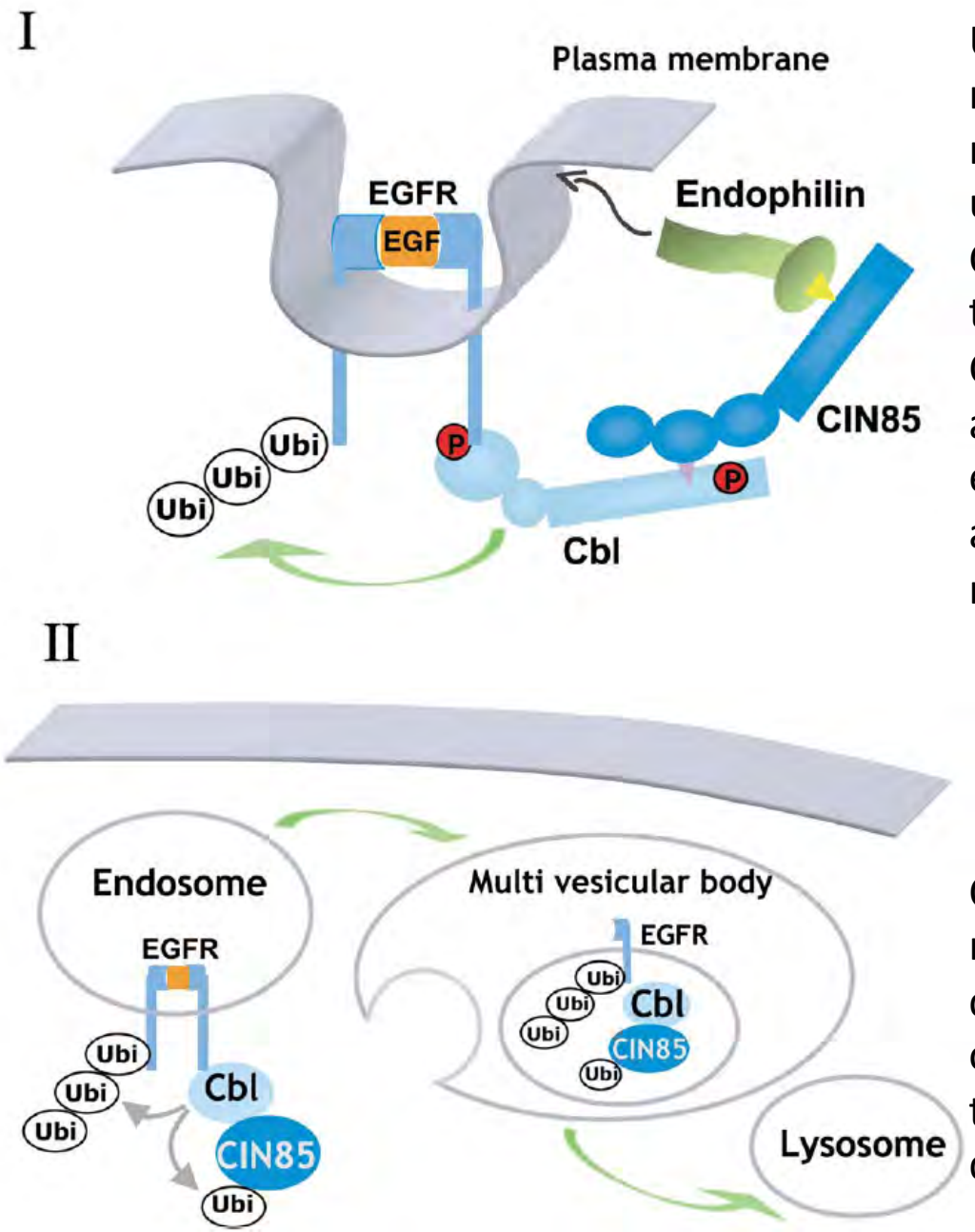
TERMINATION OF THE SIGNAL



- Activated EGF receptors are recognized by **Cbl** which either binds directly through a phosphotyrosine-binding motif or by interaction with the SH3 domain of Grb2. Cbl causes mono-ubiquitylation of the EGFR and this acts as a sorting signal directing the receptor into the lysosomal pathway for degradation.

- The receptor-Cbl complex is recognized by CIN85 and endophilin which couple the receptor to a complex of proteins that includes the key endocytic adaptor AP-2.

The complex then recruits clathrin monomers. As a result, active EGFRs accumulate in clathrin-coated membrane pits which then pinch off from the plasma membrane as endocytic vesicles. Within the intracellular network of vesicular transport pathways, the receptors are sorted into a pathway that takes them via the early and late endosomes towards the lysosome. They are thus destroyed.



Upon ligand-induced activation of EGF receptors, Cbl binds to phosphorylated receptors and promotes receptor ubiquitination (Ubi).
Cbl is also tyrosine-phosphorylated in this complex leading to translocation of CIN85/endophilin in the vicinity of active EGF receptors, whereby endophilins in concert with dynamin and amphiphysin regulate clathrin-mediated internalization.

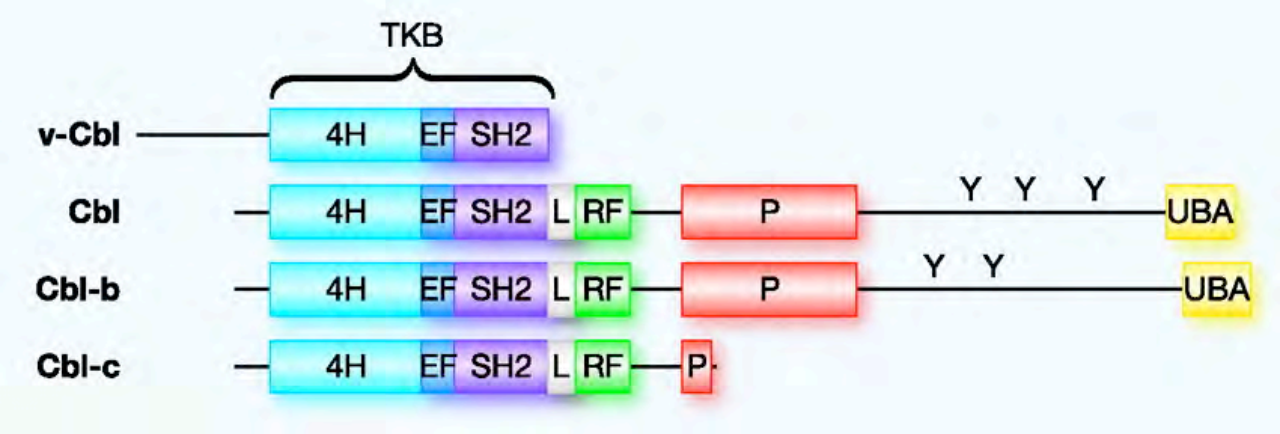
CIN85 and CMS are also monoubiquitinated by Cbl in these complexes and are implicated in sorting of EGF receptors along
the endocytic pathway for lysosomal degradation.

c-Cbl

c-CBL (**C**asitas **B**-lineage **L**ymphoma) is an E3 ubiquitin-protein ligase involved in cell signalling and protein ubiquitination.

c-Cbl has several regions encoding for functionally distinct domains:

- N-terminal **tyrosine kinase binding domain** (TKB domain): determines the protein which it can bind to
- **RING finger domain** : recruits enzymes involved in ubiquitination
- **Proline-rich region**: the site of interaction between Cbl and cytosolic proteins involved in Cbl's adaptor functions
- C-terminal **ubiquitin-associated domain** (UBA domain): the site of ubiquitin binding. This domain structure and the tyrosine and serine-rich content of the protein product is typical of an "adaptor molecule" used in cell signalling pathways



FEBS Letters 529 (2002) 110–115 FEBS 26431

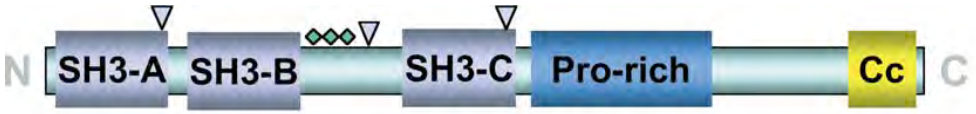
CIN85

CIN85 (Cbl-interacting protein of 85 kDa) is an adaptor protein.

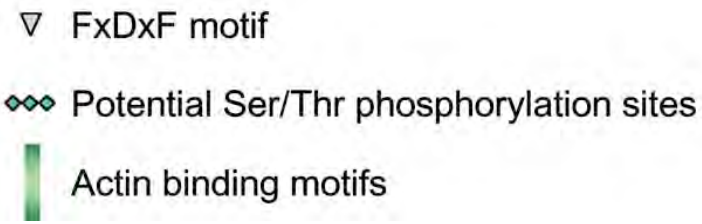
Minireview

CIN85/CMS family of adaptor molecules

Ivan Dikic*



CIN85
(Ruk, SETA, SH3KBP1)



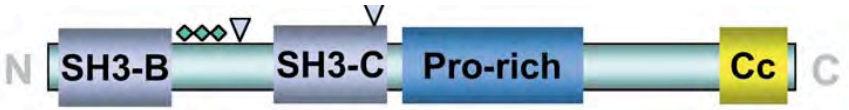
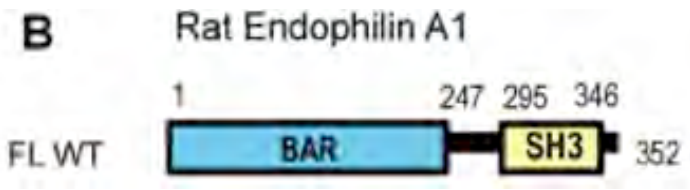
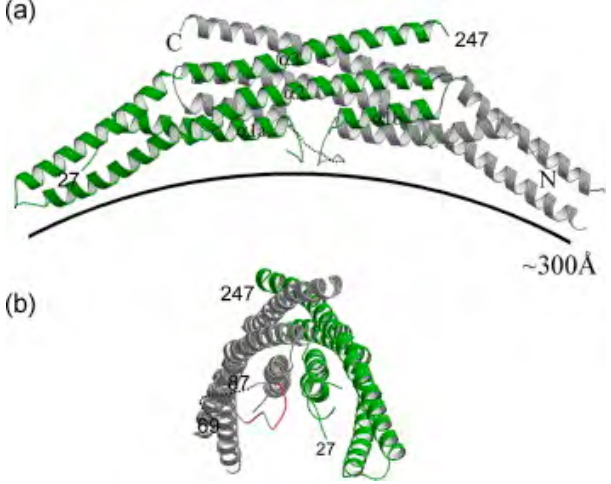


Table 1
CIN85/CMS binding partners

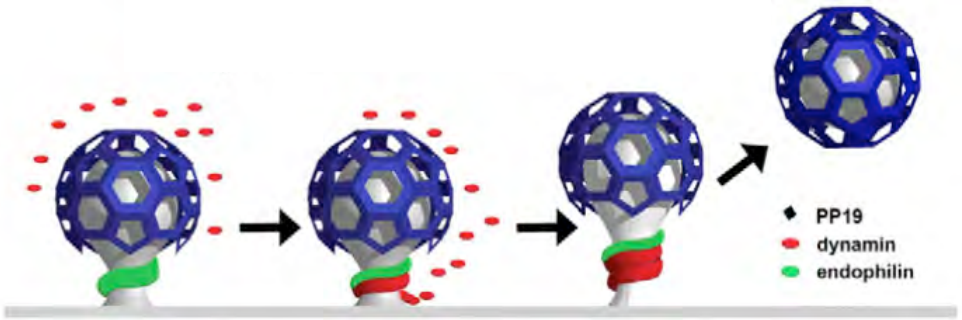
Protein	Interacts with	Function
c-Cbl	SH3-ABC of CIN85/CMS	Downregulation of RTKs
Cbl-b	SH3-ABC of CIN85	Downregulation of RTKs
BLNK: B-cell linker protein	SH3-ABC of CIN85	B-cell receptor signaling
SB1 (similar to NY-REN-45)	SH3-ABC of CIN85	Not yet defined
CD2	SH3-B of CD2AP and CIN85	T-cell receptor clustering. T-cell polarization
AIP1/Alix	SH3-B of CIN85	Apoptosis in glial cells
p85 subunit of PI-3 kinase	Proline-rich region of CIN85	Negative regulation of PI-3 kinase. Induction of apoptosis in neuronal cells
Grb2	Proline-rich region of CIN85	Regulation of RTK signaling
p130Cas	Proline-rich region of CMS/CIN85	Regulation of the actin cytoskeleton
Fyn, Src, Yes,	Proline-rich region of CMS	Regulation of Src family kinases
Endophilins A1, A2 and A3	Proline-rich region of CIN85	Regulation of RTK internalization
Nephrin	CD2AP C-terminus	Structural organization of kidney podocytes
Polycystin-2	CMS/CD2AP C-terminus	Maintenance of renal tubular structure
Podocin	CD2AP	Kidney glomerular architecture
CIN85/CMS	Coiled-coil region of CIN85/CMS	Homodimerization of CIN85/CMS
α-ear of AP2	FxDxF region of CIN85/CMS	Regulation of clathrin-mediated endocytosis

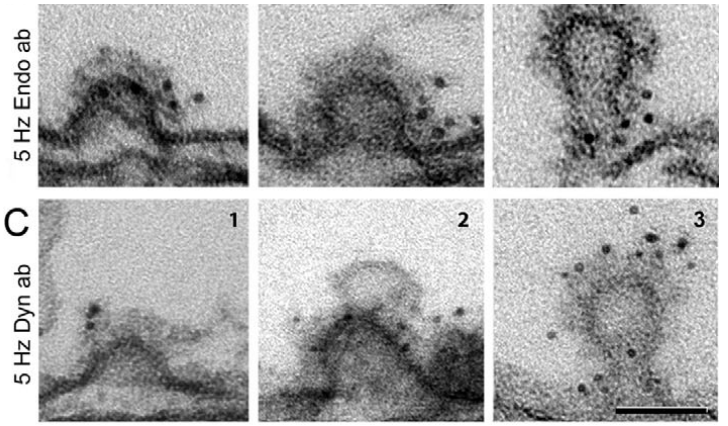
Endophilins



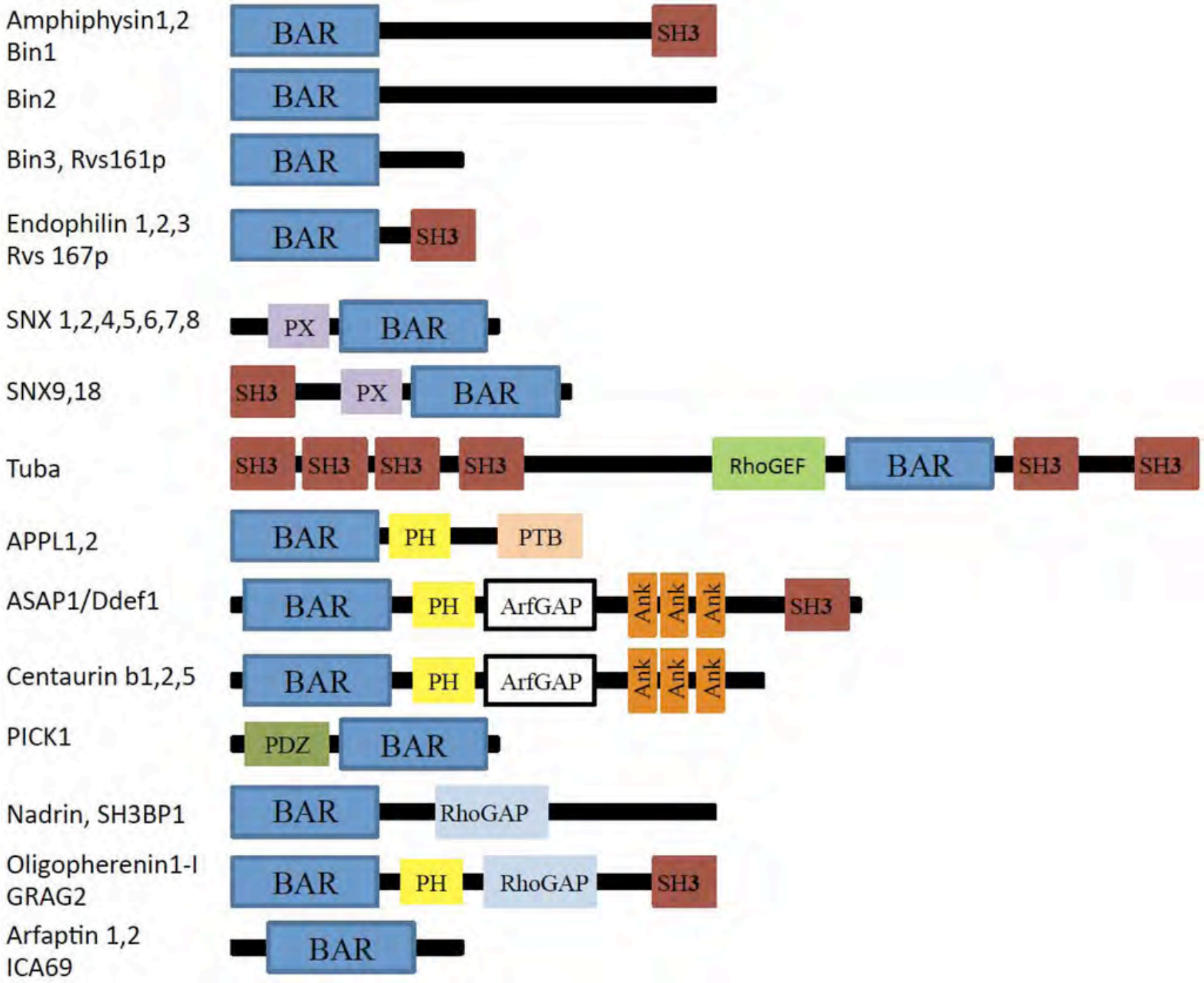


- Endophilin localizes in the vesicle pool at rest and in spirals at the necks of clathrin-coated pits (CCPs). Endophilin and dynamin colocalize at the base of the clathrin coat.
- Tubulation efficiency and the amount of dynamin recruited to lipid tubes are dramatically increased in the presence of endophilin.
- Blocking the interactions of the endophilin SH3 domain in situ reduces dynamin accumulation at the neck and prevents the formation of elongated necks observed in the presence of GTPγS.
- Endophilin recruits dynamin to a restricted part of the CCP neck, forming a complex, which promotes budding of new synaptic vesicles.



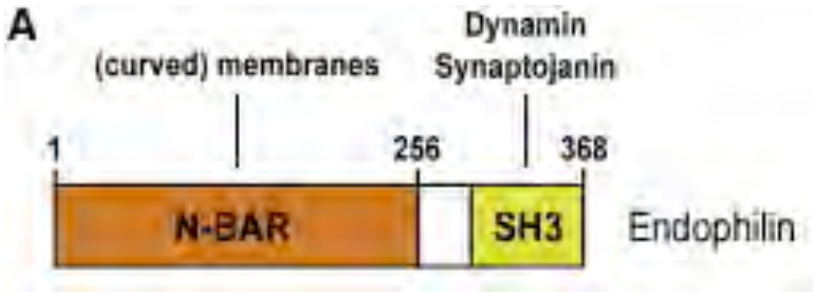


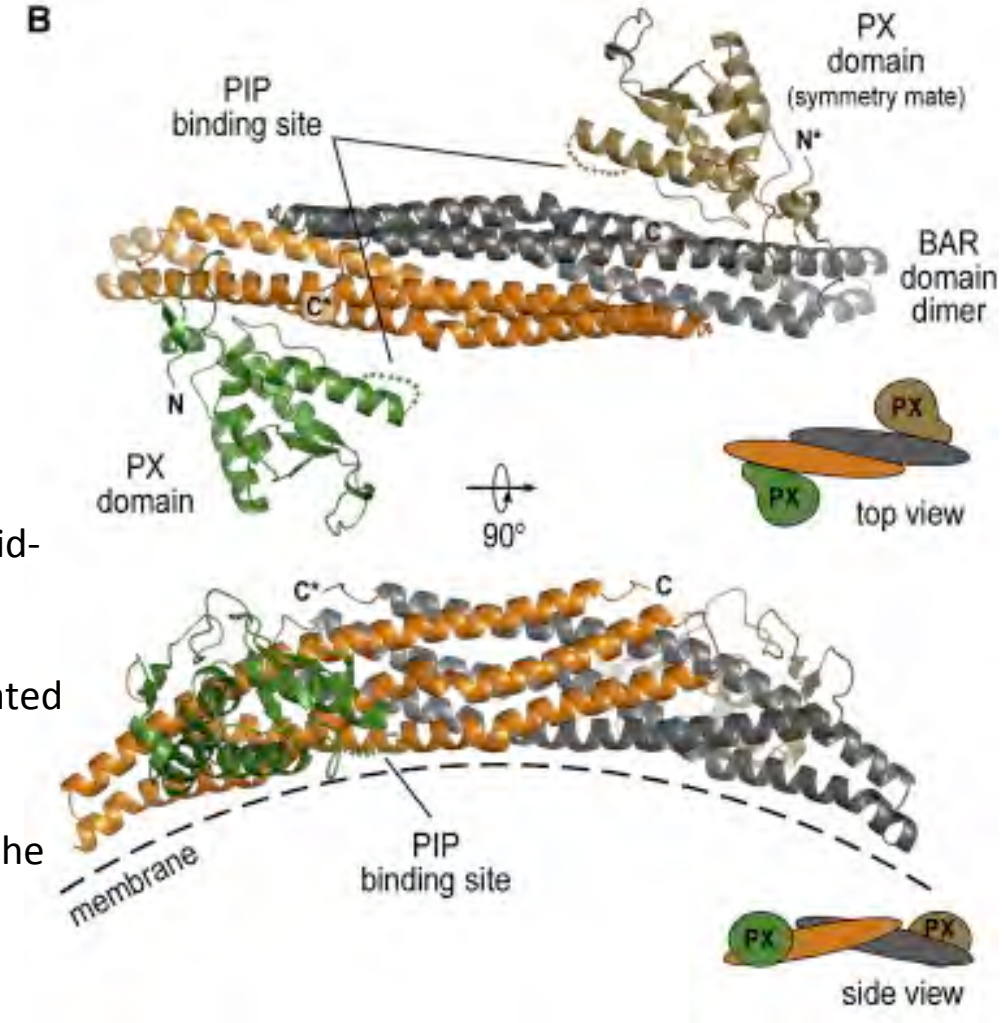
Clathrin-coated intermediates labeled with antibodies against endophilin (B) and dynamin (C)


Amphiphysin1,2
Bin1
BAR
SH3
Bin2
BAR
Bin3, Rvs161p
BAR
Endophilin 1,2,3
Rvs 167p
BAR
SH3
SNX 1,2,4,5,6,7,8
PX
BAR
SNX9,18
SH3
PX
BAR
Tuba
SH3
SH3
SH3
SH3
RhoGEF
BAR
SH3
SH3
APPL1,2
BAR
PH
PTB
ASAP1/Ddef1
BAR
PH
ArfGAP
Ank
Ank
Ank
SH3
Centaurin b1,2,5
BAR
PH
ArfGAP
Ank
Ank
Ank
PICK1
PDZ
BAR
Nadrin, SH3BP1
BAR
RhoGAP
Oligopherenin1-I
GRAG2
BAR
PH
RhoGAP
SH3
Arfaptin 1,2
ICA69
BAR

Structure and Plasticity of Endophilin and Sorting Nexin 9

Qi Wang,[1,2] Hung Yi Kristal Kaan,[1,2] Reshma Noordin Hooda,[1] Shih Lin Goh,[1] and Holger Sondermann[1,*]
[1]Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853
[2]These authors contributed equally to this work
*Correspondence: hs293@cornell.edu
DOI 10.1016/j.str.2008.07.016





- BAR domains form dimeric, crescent-shaped units that occur N- or C-terminally to other lipid-binding, adaptor, or catalytic modules.
- In crystal structures, Endophilin appears to be rigid in solution, and the SH3 domains are located at the distal tips of a BAR domain dimer with fixed curvature.
- We observed tip-to-tip interactions between the BAR domains in a trigonal crystal form, reminiscent of functionally important interactions described for F-BAR domain

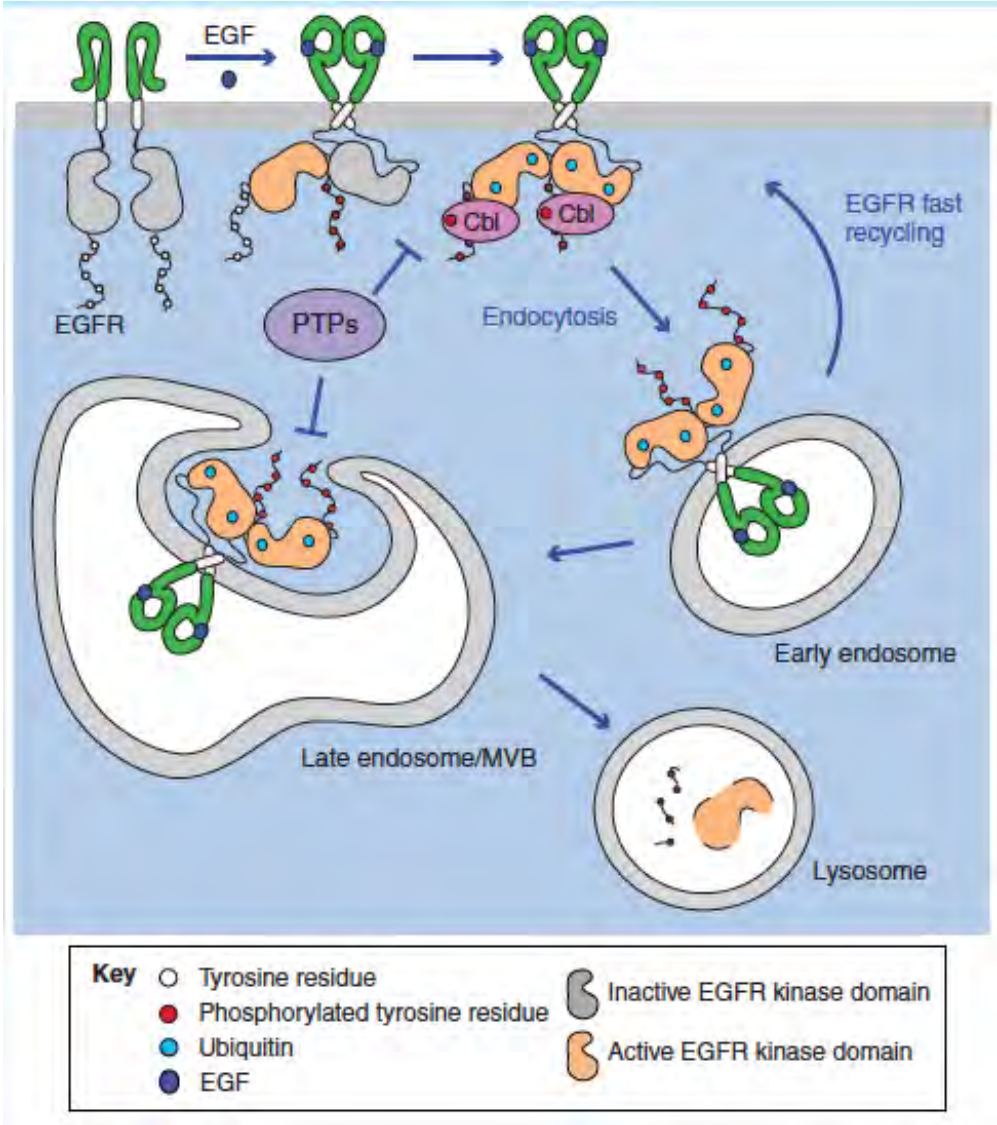
Regulation of epidermal growth factor receptor signalling by inducible feedback inhibitors

Oreste Segatto[1,*], Sergio Anastasi[1] and Stefano Alemà[2]

[1]Department of Experimental Oncology, Regina Elena Cancer Institute, 00158 Rome, Italy
[2]Institute of Cell Biology, CNR, 00016 Monterotondo, Italy
*Author for correspondence (segatto@ifo.it)

Journal of Cell Science 124, 1785-1793

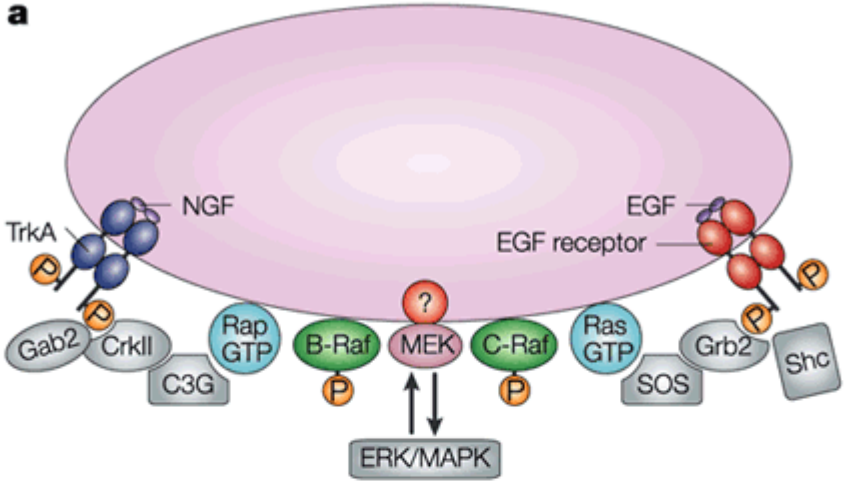
doi:10.1242/jcs.083303



- Tyrosine phosphatases (PTPs) reverse EGFR tyrosine phosphorylation at the cell membrane, as well as during endocytic trafficking.
- Activated EGFRs undergo rapid endocytosis.
- Internalised EGFRs reach early endosomes, from where receptors are rapidly recycled to the cell surface unless tagged robustly with ubiquitin.
- Ubiquitylated EGFRs are sorted into MVBs, a step that segregates the EGFR kinase activity from the cytosol and effectively terminates signalling.
- The MVBs/late endosomes fuse with lysosomes, where EGF and EGFR undergo proteolysis.
- Ubiquitylation directed by the EGFR-bound CBL E3 ligase is therefore crucial for the regulation of EGFR endocytosis and its role in signal attenuation.

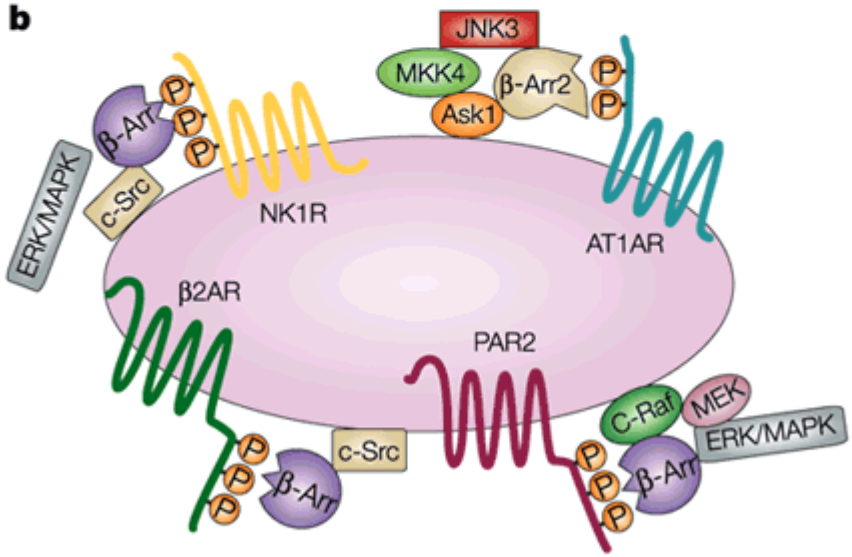
Hypothetical model of signaling complexes in endosomes

Adaptor proteins, Grb2 and Crk, exchange Factors (SOS) and small GTPases (Ras and Rap1) can translocate to endosomes with activated epidermal growth factor (EGF) and TrkA receptors.



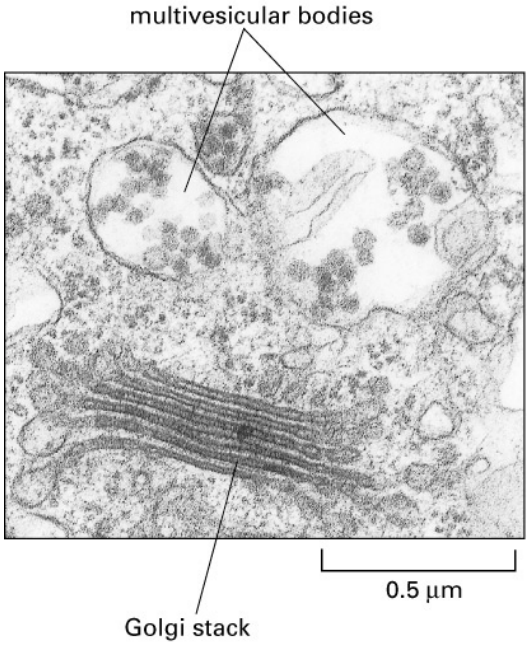
Binding of Raf to GTP-loaded Ras contributes to the recruitment of Raf to endosomes. Docking of MAPK and MEK to the endosomal membrane might involve an unidentified anchoring protein.

β-arrestins bound to phosphorylated GPCRs can interact with c-Src, as well as with other Src-family kinases. β-arrestins can also interact with various components of MAPK modules.

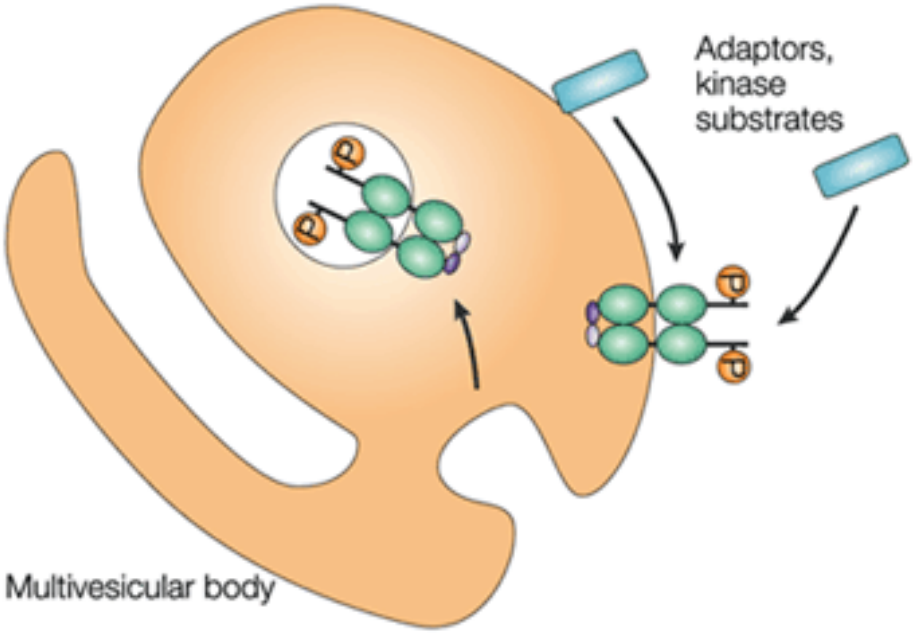


Termination of receptor-tyrosine-kinase signaling in multivesicular bodies

During the passage through the endosomal compartments, receptor tyrosine kinases (RTKs) become increasingly concentrated in the internal membranes of multivesicular bodies (MVBs). These internal structures might represent isolated vesicles or deep invaginations of the limiting endosomal membrane.

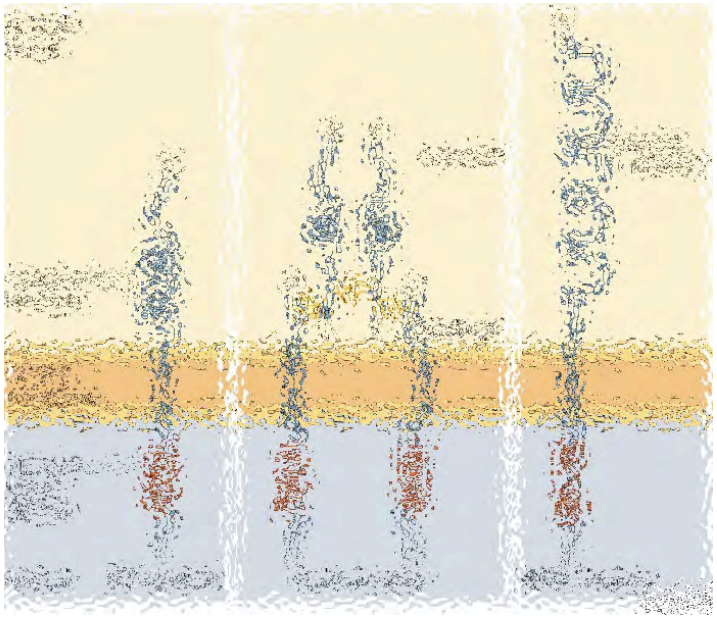


Receptors incorporated into vesicles are not accessible for interactions with adaptor proteins and cannot phosphorylate cytoplasmic signalling proteins.
So, sequestration of active receptors inside MVBs might terminate RTK signalling before their degradation in lysosomes.

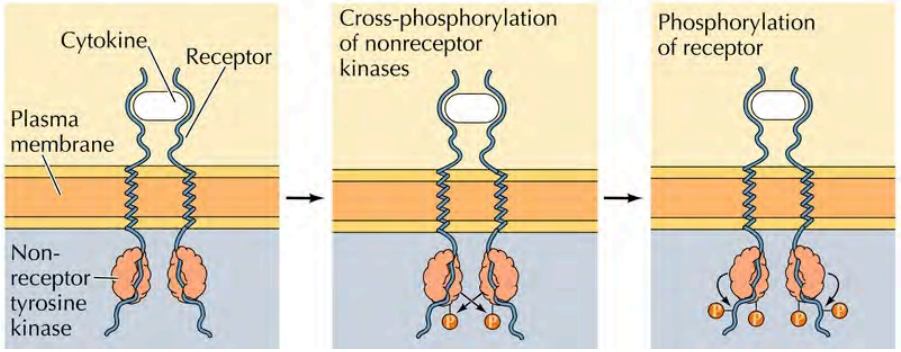


Enzyme-linked receptors fall into 3 categories:

- **Tyrosine Kinase Receptors**
  - Not only a receptor
  - Also an enzyme: Tyrosine kinase



- **Cytokine superfamily receptors**
  - No catalytic domain
  - Interact with non receptor protein-tyrosine kinases
    - Src family
    - JAK family



- **TGF-β receptors**

What is Cytokine?

- Secreted polypeptide or low molecular weight protein involved in *cell-to-cell signaling*.

- Acts in paracrine or autocrine fashion *through specific cellular receptors*.

- Can be produced by cells of any tissue and act on many *cells involved in immune and inflammatory response*.

Receptors Classification

RECEPTOR FAMILY	LIGANDS	
Class I cytokine receptors (hematopoietin)	IL-2	IL-21
	IL-3	IL-23
	IL-4	IL-27
	IL-5	GM-CSF
	IL-6	G-CSF
	IL-7	OSM
	IL-9	LIF
	IL-11	CNTF
	IL-12	Growth hormone
	IL-13	Prolactin
	IL-15	

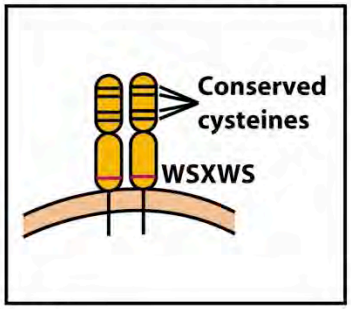


Figure 12-6b
Kuby IMMUNOLOGY, Sixth Edition

RECEPTOR FAMILY	LIGANDS
TNF receptors	TNF-α
	TNF-β
	CD27L
	CD30L
	CD40L
	Nerve growth factor (NGF)
	FAS

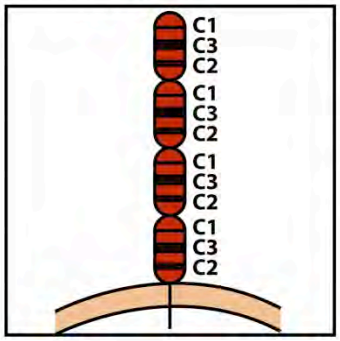


Figure 12-6d
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RECEPTOR FAMILY	LIGANDS
Class II cytokine receptors (interferon)	IFN-α
	IFN-β
	IFN-γ
	IL-10
	IL-19
	IL-20
	IL-22
	IL-24
	IL-26
	IL-28
	IL-29

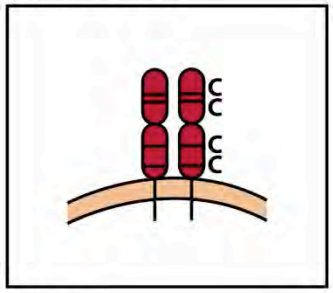

Figure 12-6c
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RECEPTOR FAMILY	LIGANDS
Immunoglobulin superfamily receptors	IL-1
	M-CSF
	C-Kit
	IL-18

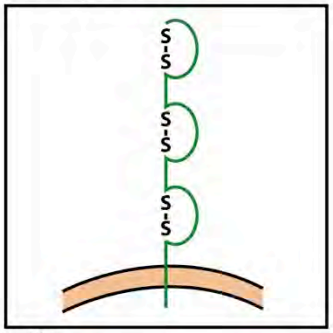

Figure 12-6a
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RECEPTOR FAMILY	LIGANDS
Chemokine receptors	IL-8
	RANTES
	MIP-1
	PF4
	MCAF
	NAP-2

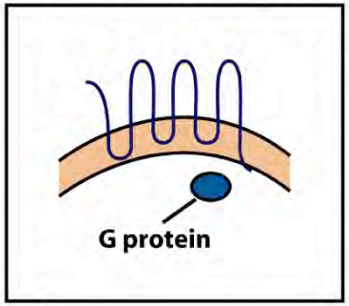

Figure 12-6e
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Cytokine receptors belong to families of receptor proteins, each with a distinctive structure.

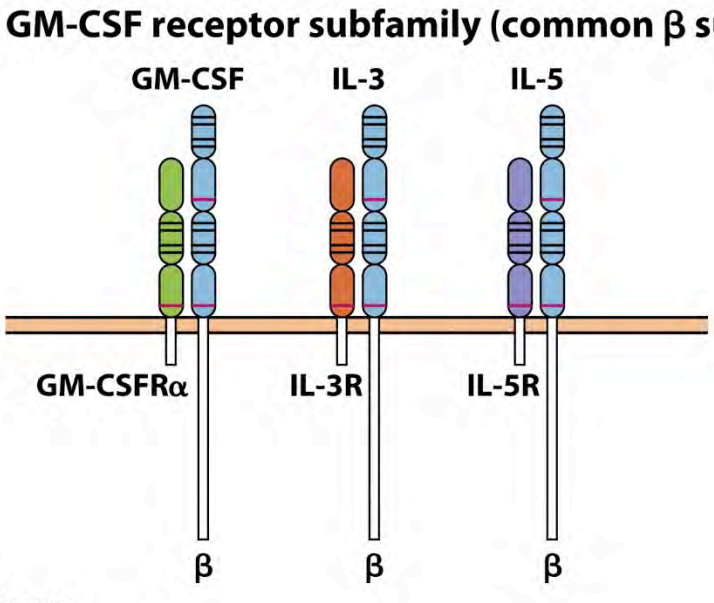

Figure 12-7a
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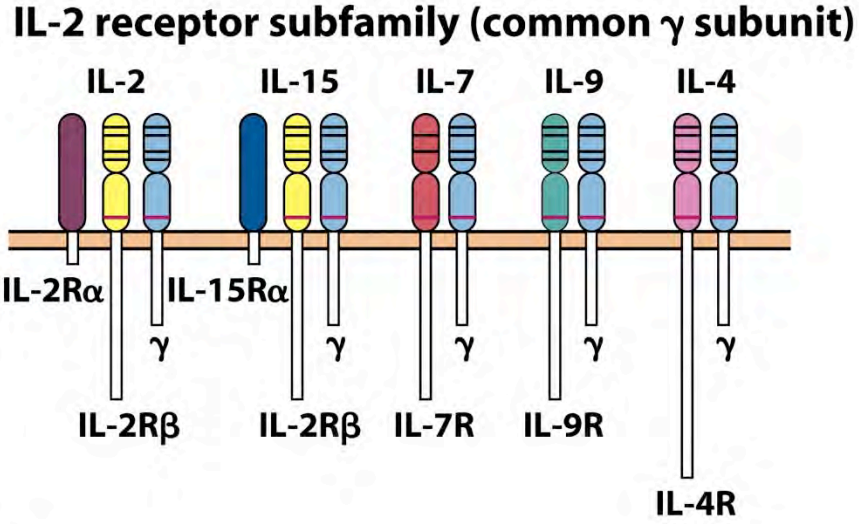

Figure 12-7c
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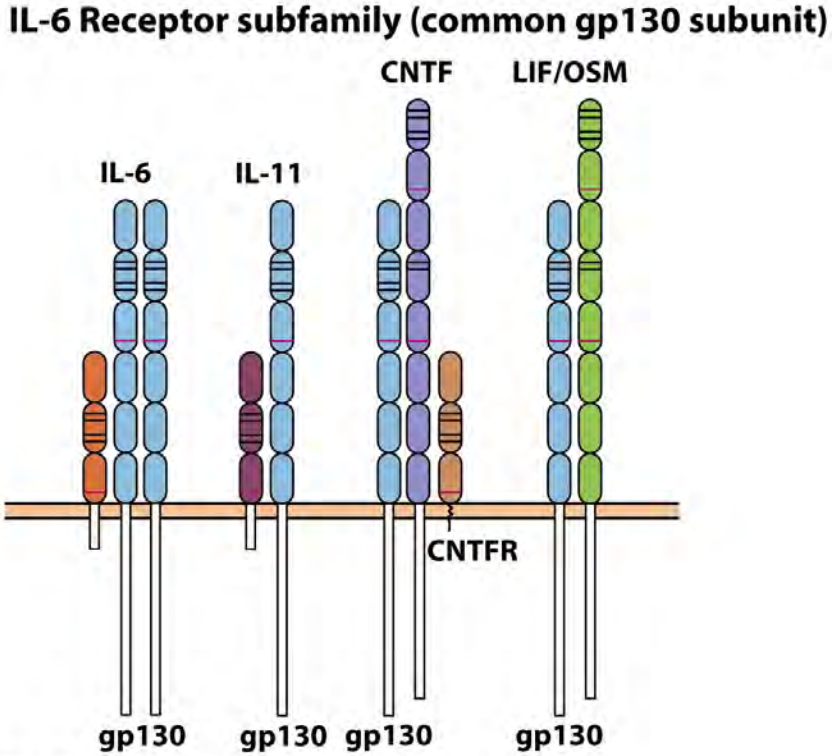

Figure 12-7b
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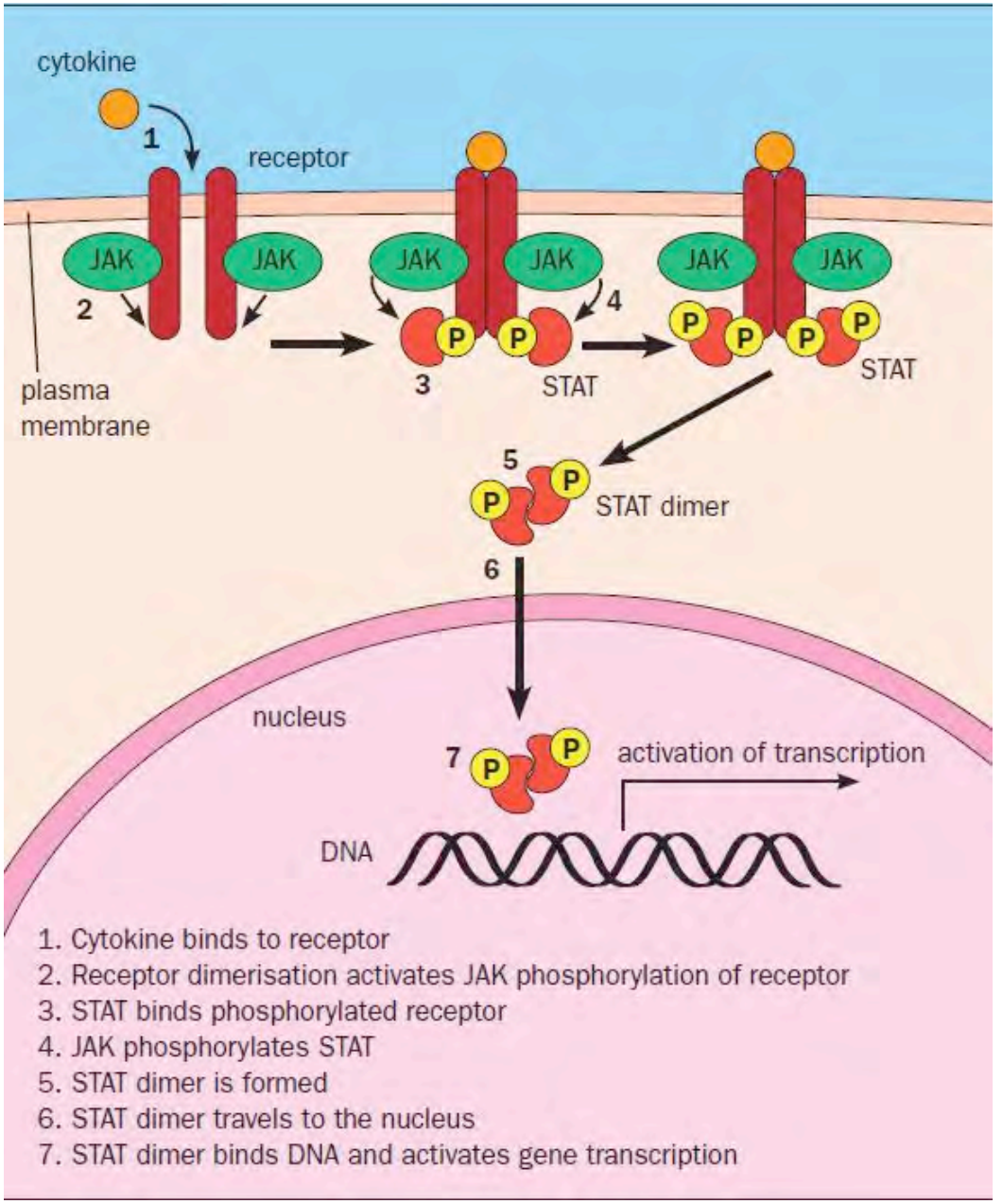
Cytokine receptors subfamilies have shared signaling subunits

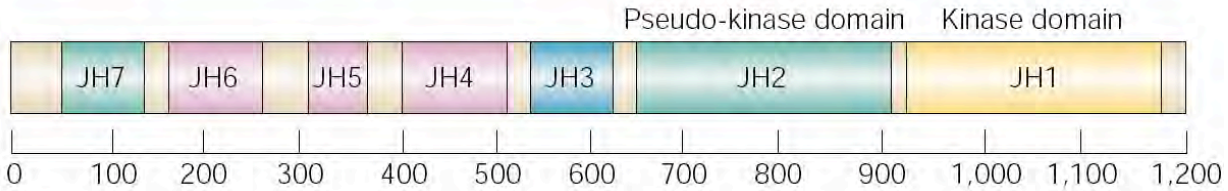
Cytokine Receptor Signaling

- Similar to Receptor Tyrosine Kinase signaling
- Receptor clustering
- **Cytokine receptors do NOT have any enzymatic activity, but bind cytosolic kinases**
- Phosporylation and activation of JAK kinases
- Binding of STAT to p-Receptor via SH2 domain
- Phosphorylation of STAT by JAK kinase
- Translocation of p-STAT into nucleus
- Activation of transcription
- Feedback regulation: SHP1 and SOCS



The JAK-family of tyrosine kinases

- Family members
  - JAK1 (135 kDa)
  - JAK2 (130 kDa)
  - JAK3 (120 kDa)
  - Tyk2 (140 kDa)



- Common feature
  - C-terminal kinase + pseudokinase
  - ≠ RTK by lacking transmembrane domains and SH2, SH3, PTB, PH
  - several regions homologous between JAK-members
  - Associated with cytokine receptors (type in and II)
- Function
  - Associated with cytokine receptors in non-stimulated cells in an inactive form

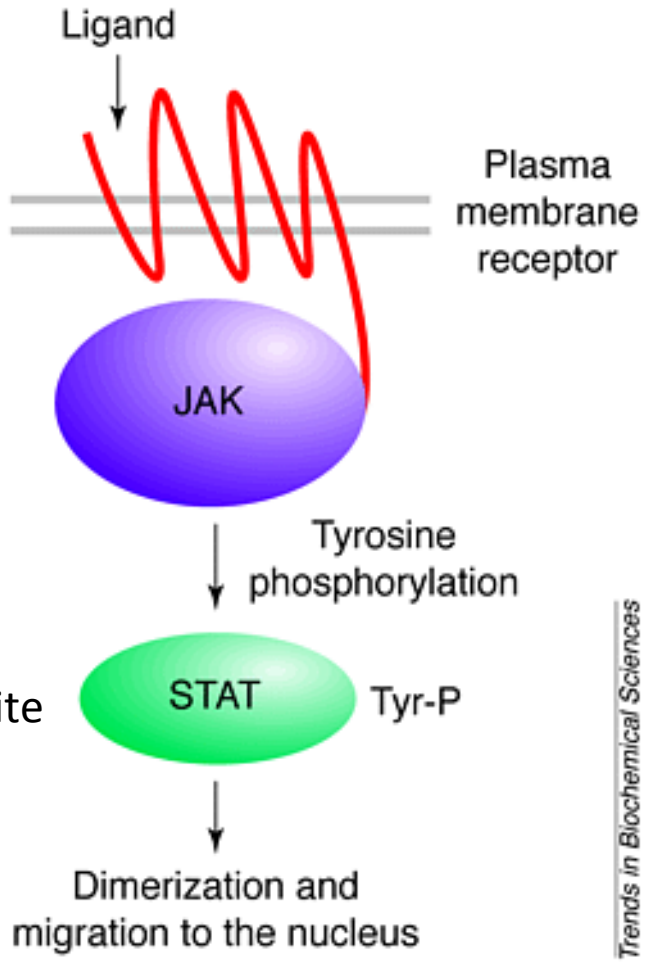
STAT proteins

STATs: Signal Transducers and Activators of Transcription

1. Transducers for signals from many cytokines
   Broad spectrum of biological effects

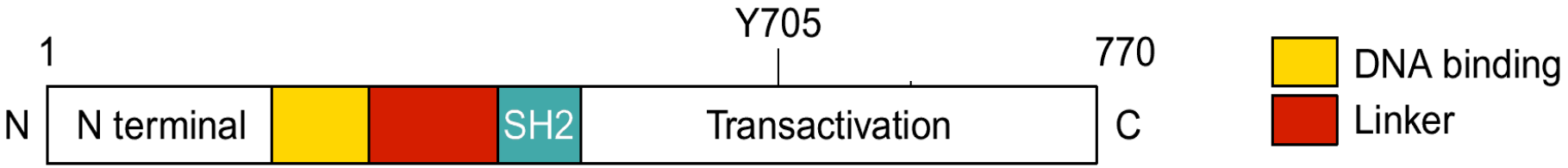
2. Transcriptional activators
   activation at the cell membrane, response in the nucleus

Rapid signal response
   The activation/deactivation cycle of STAT molecules is quite short, about 15 min for an individual molecule.

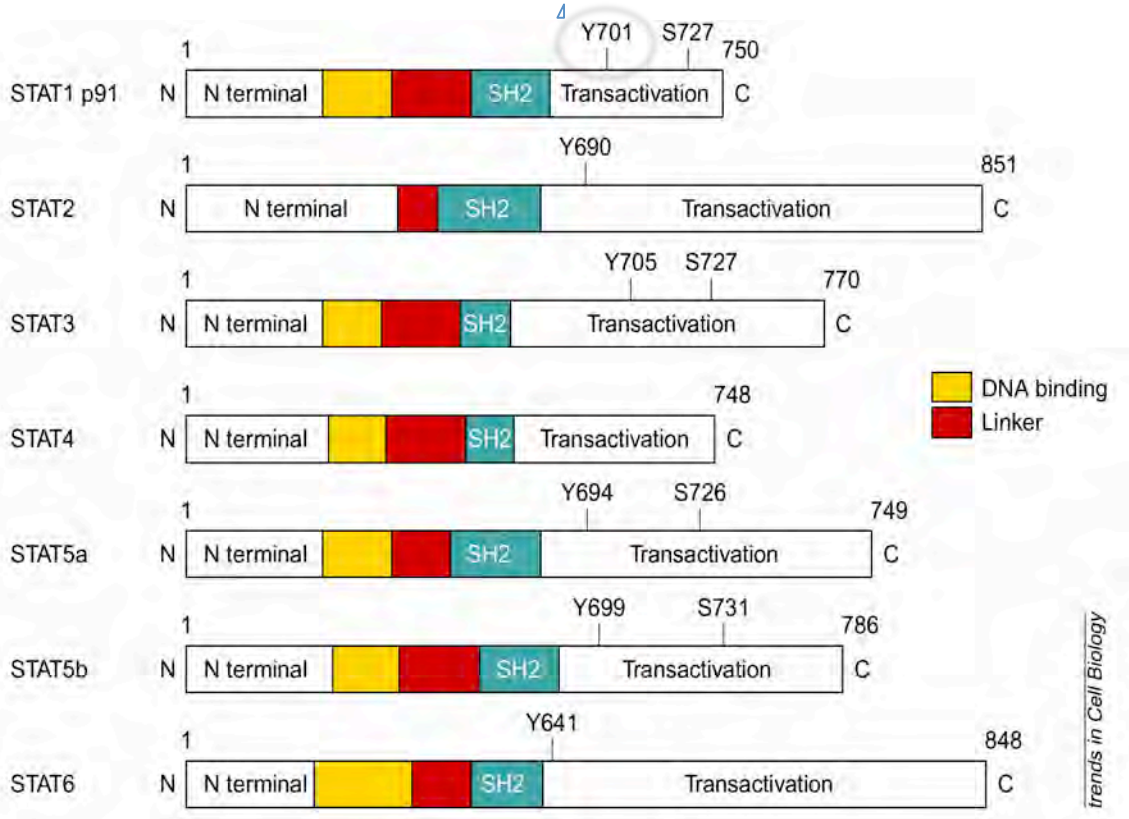


STAT proteins

- All STAT proteins contain an N-terminal SH2 domain that binds to phosphotyrosine in the receptor's cytosolic domain, a central DNA binding domain and a C-terminal domain with a critical tyrosine residue



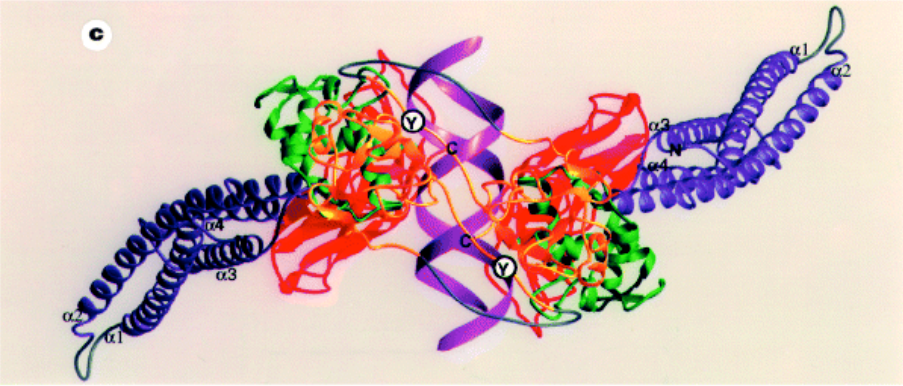
STAT-family members



- STAT1 - involved in IFNα/β- and IFNγ-response
- STAT2 - involved in IFNα/β-response. Mainly acting as partner for STAT1/p48
- STAT3 - involved in response to several cytokines including IL6. It activates several genes involved in acute phase response
  - **Important in growth regulation, embryonic development & organogenesis**
  - **Activation of STAT3 correlated with cell growth, link to cancer, binds c-Jun**
- STAT4 - involved in IL12-response
- STAT5a & 5b - involved in response to several cytokines including prolactin, IL-2, and regulates expression of milk proteins in breast tissue in response to prolactin
- STAT6 - involved in IL4-response
- non-mammalian family members (e.g. Drosophila)

STATs - structure and function

- **SH2-domain**
  - Three important functions in STATs:
    - important for recruitment of STAT to receptor
    - important for interaction with the JAK kinase
    - important for dimerization of STATs to an active DNA-binding form
- **Tyr-701**
  - conserved key Tyr residue located just C-terminal to SH2
  - essential for dimerization to an active DNA-binding form
  - function: Tyr^P binding for SH2 in partner

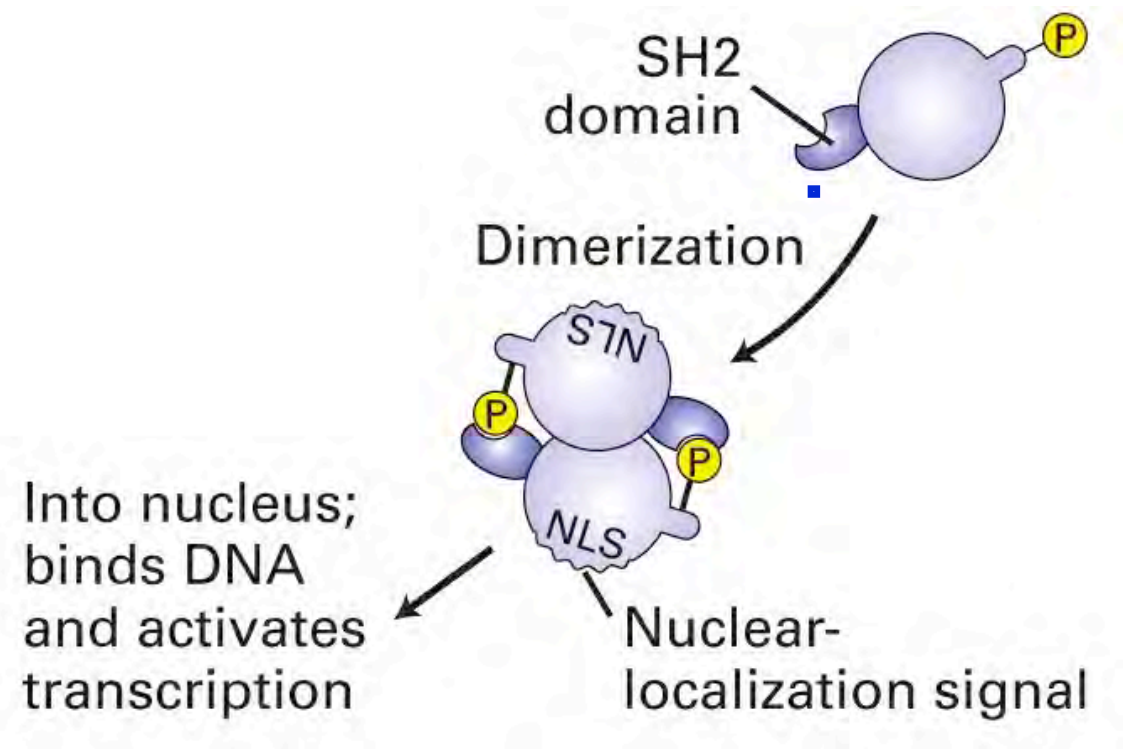


- **DNA-binding domain**

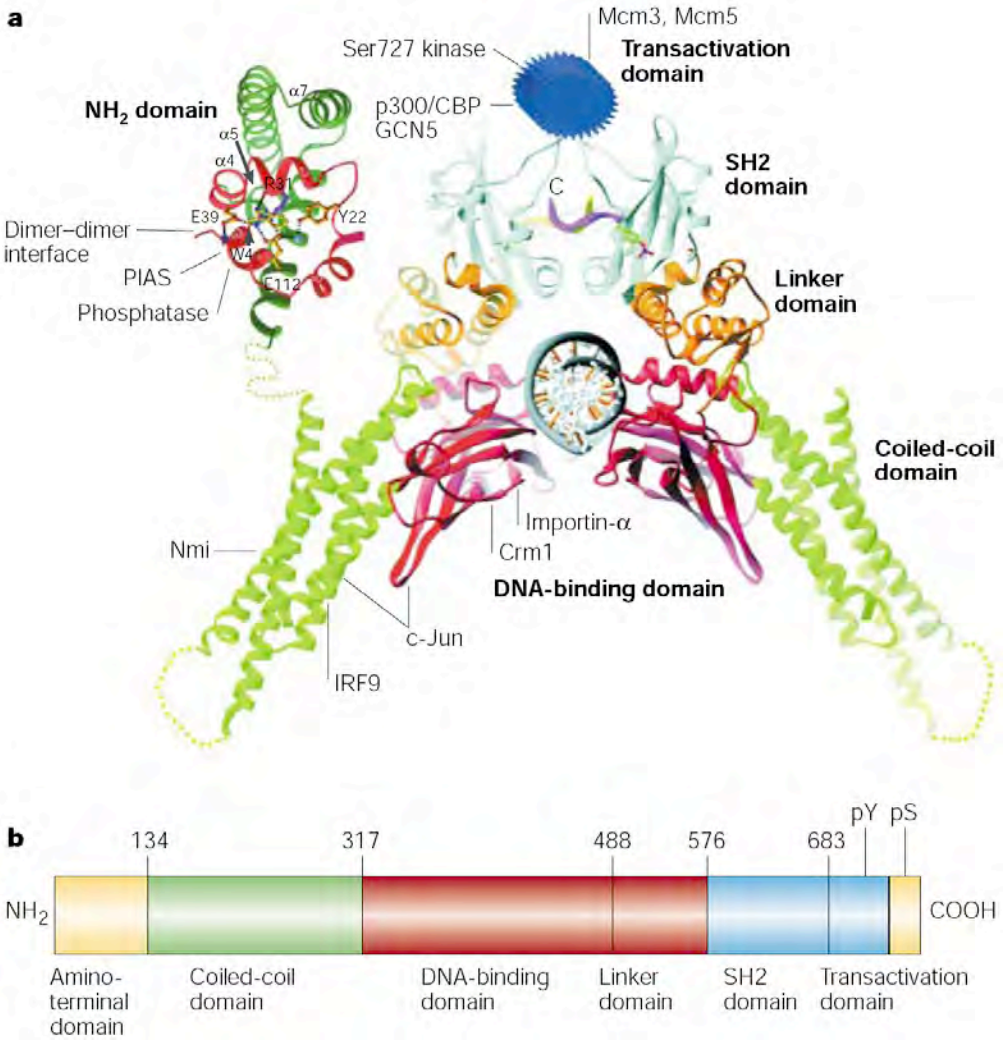
DBD located in the middle of the protein
Unique motif - All DBDs bind similar motifs in DNA symmetric inverted half sites.

STAT proteins

- Once the STAT is bound to the receptor, the C-terminal tyrosine is phosphorylated by an associated JAK kinase
- The phosphorylated STAT dissociates from the receptor, and two activated STATs form a dimer and then enters the nucleus



STAT-DBD structure



- Symmetry-axis through DNA, each monomer contacts a separate half site
- The dimer forms a C-shaped ”clamp” around DNA.
- The dimer is kept together by reciprocal SH2- Tyr^P interactions between the SH2 domain in one monomer and the phosphorylated Tyr in the other.
- The SH2 domain in each monomer is closely linked to the core DBD and is itself close to DNA, and is assumed also to contribute to DNA-binding.
- N-terminal coiled-coil region not close to DNA, probably involved in prot-prot interaction with flexible position

Specificity in response

- each cytokine activates a subgroup STAT
- some cytokines activate only one specific STAT

What does mediate specificity?

1. the SH2 - receptor interaction specific for certain combinations swaps-experiments of SH2 between STATs change specificity affinity of the SH2-receptor interaction is affected by the sequence context of the Tyr

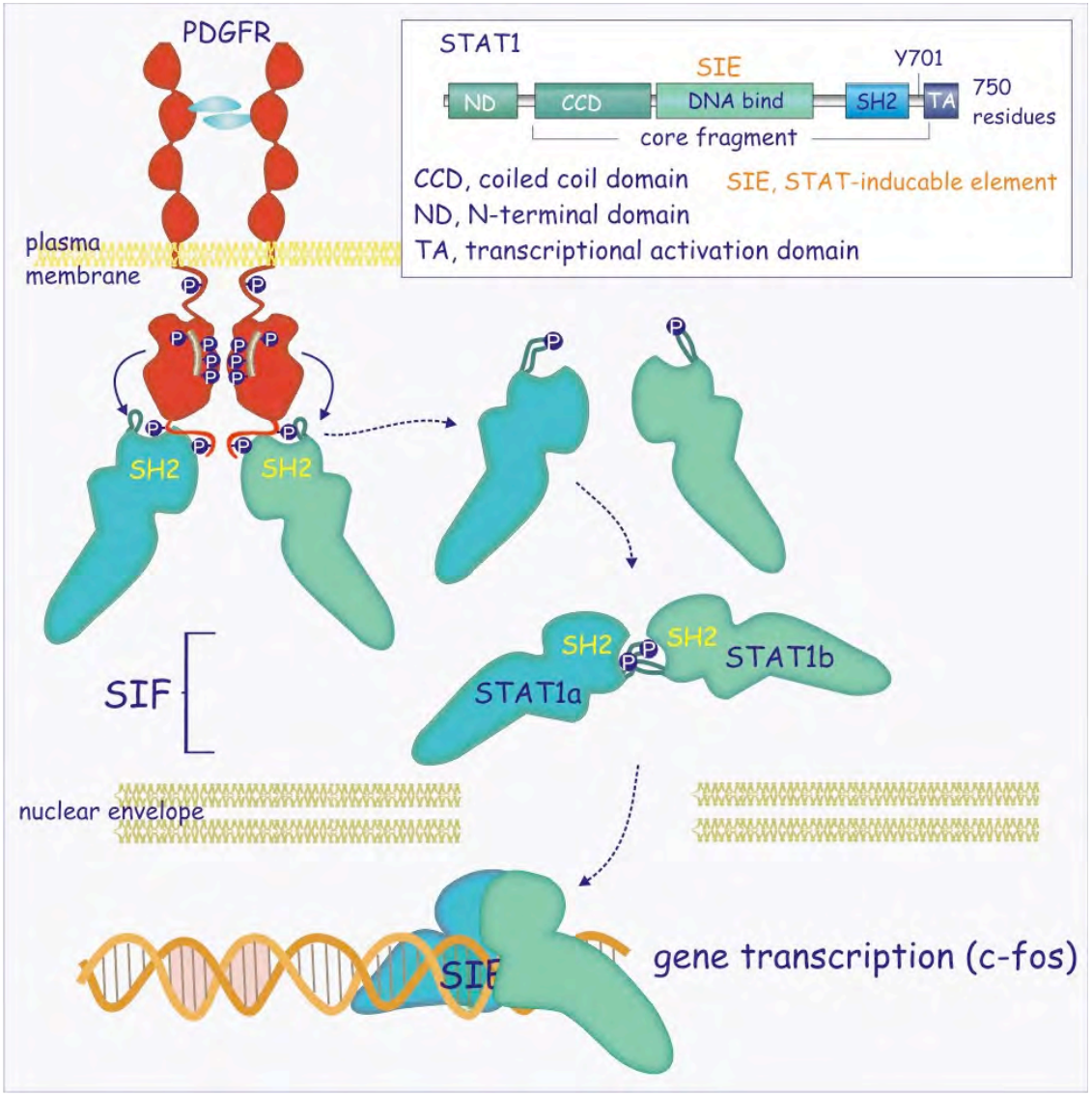
2. different STAT-dimers bind different response elements in the genome and turn on different genes

STAT1 knock-out mice illustrate biological specificity: $STAT1^{-/-}$
phenotype: total lack of IFN-response → highly sensitive to virus-infection

Several signalling pathways linked

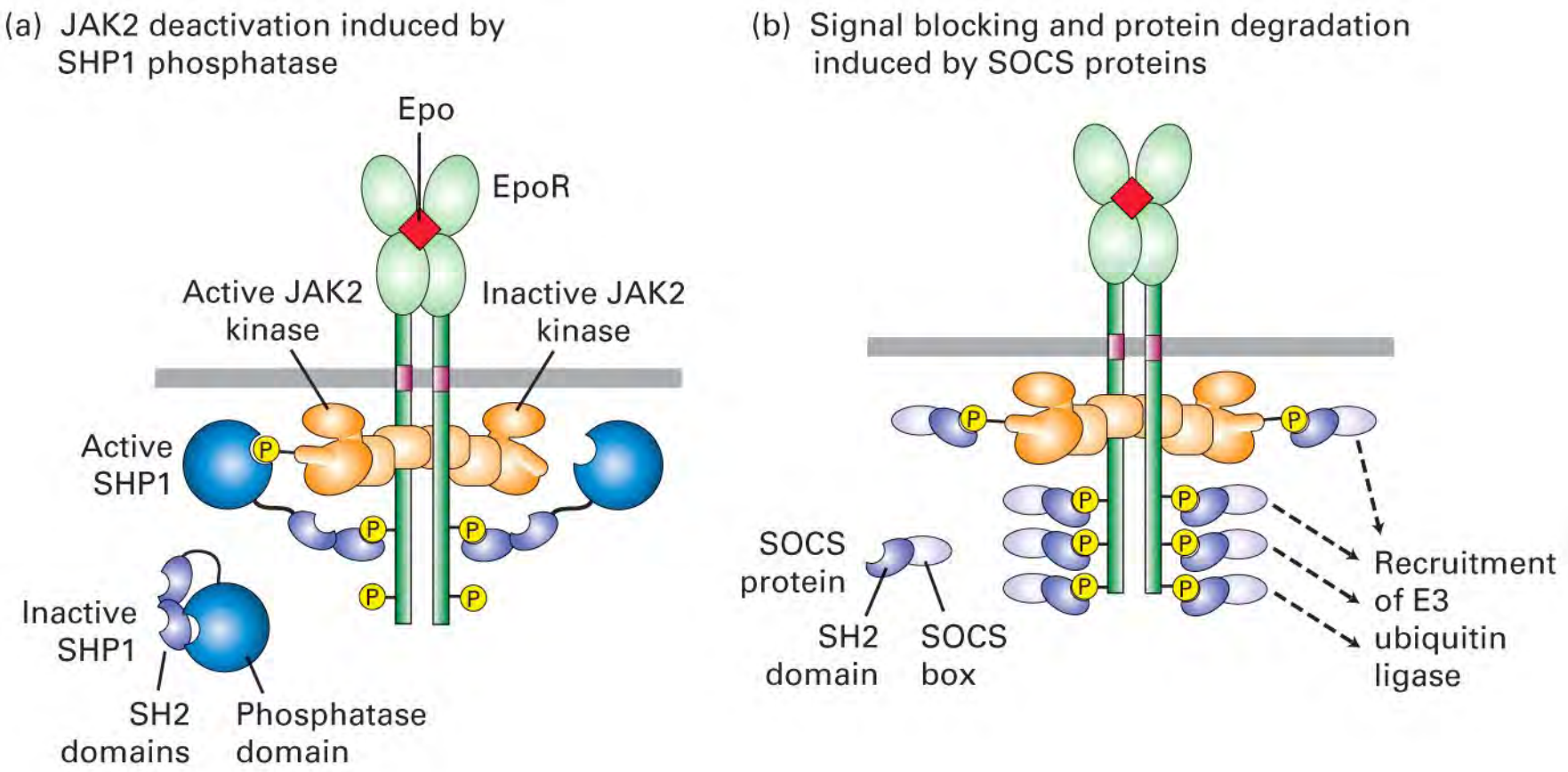
- STATs may also be Tyr-phosphorylated and hence activated by other receptor families
  - receptor tyrosine kinases (RTKs) may phosphorylate STATs
    - EGF stimulation → activation of STAT1, STAT3
  - non-receptor tyrosine kinases such as Src and Abl may also phosphorylate STATs
  - G-protein coupled 7TMS receptors such as angiotensine receptor (?)
- STAT may also be modified by Ser-phosphorylation
  - DNA-binding reduced (STAT3)
- JAKs may activate other signalling pathways than STATs
  - Tyr^P will recruit several protein-substrates and lead to phosphorylation and activation of other signalling pathways
    - e.g. JAK activation → activation of MAP-kinases
    - e.g. substrates: IRS-1, SHC, Grb2, HCP, Syp, Vav

Direct phosphorylation of STAT transcription factors.



Through their SH2 domains, STAT1a and STAT1b bind to the tyrosine-phosphorylated receptor and become phosphorylated. They then form a dimer, (called a Sis-inducible factor, SIF) which translocates to the nucleus, where it binds to a Sis-inducible element (SIE) within the fos promoter.

Negative Regulation of the JAK-STAT pathway

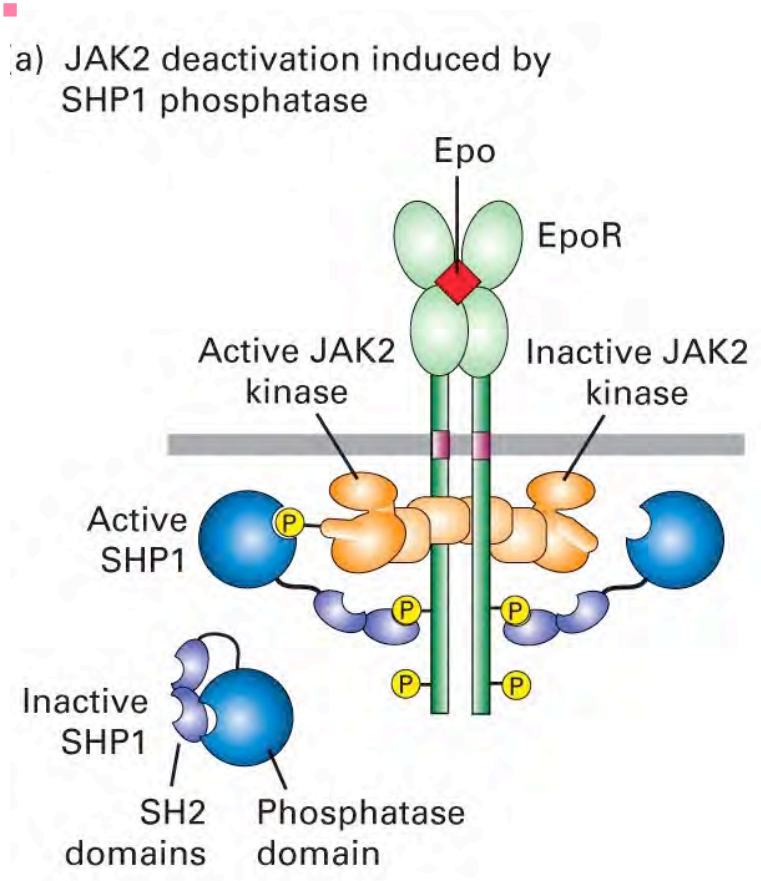


- Signal-induced transcription of target genes can not last for too long and needs de-sensitized
- Signaling from cytokine receptor is usually dampened by two classes of proteins: short term regulation by SHP1 phosphatase and long term regulation by SOCS proteins

Signaling from Cytokine Receptors Is Modulated by Negative Signals

SHP1 Phosphatase

- Mutant mice lacking SHP1 phosphatase die because of producing excess amount of erythrocytes and other blood cells. SHP1 negatively regulates signaling from several types of cytokine receptors in several types of progenitor cells
- Binding of an SH2 domain SHP1 to a particular phospho-tyrosine in the activated receptor unmasks its phosphatase catalytic site and brings it near the phosphrylated tyrosine in the lip region of JAK2
- Removal of the phosphate from this tyrosine inactivates the JAK kinase

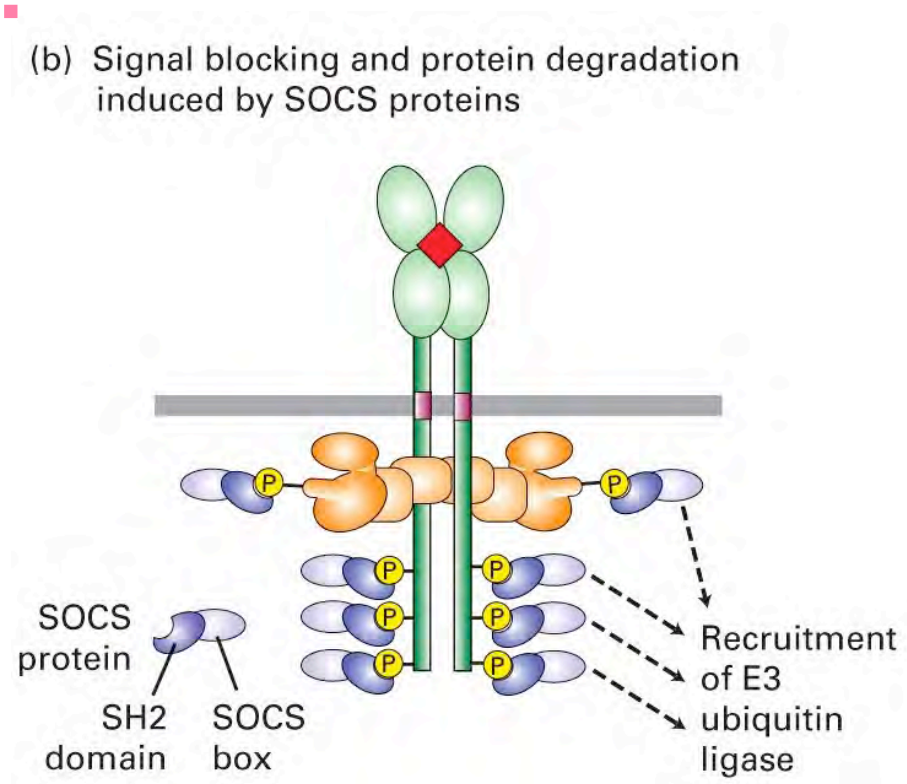


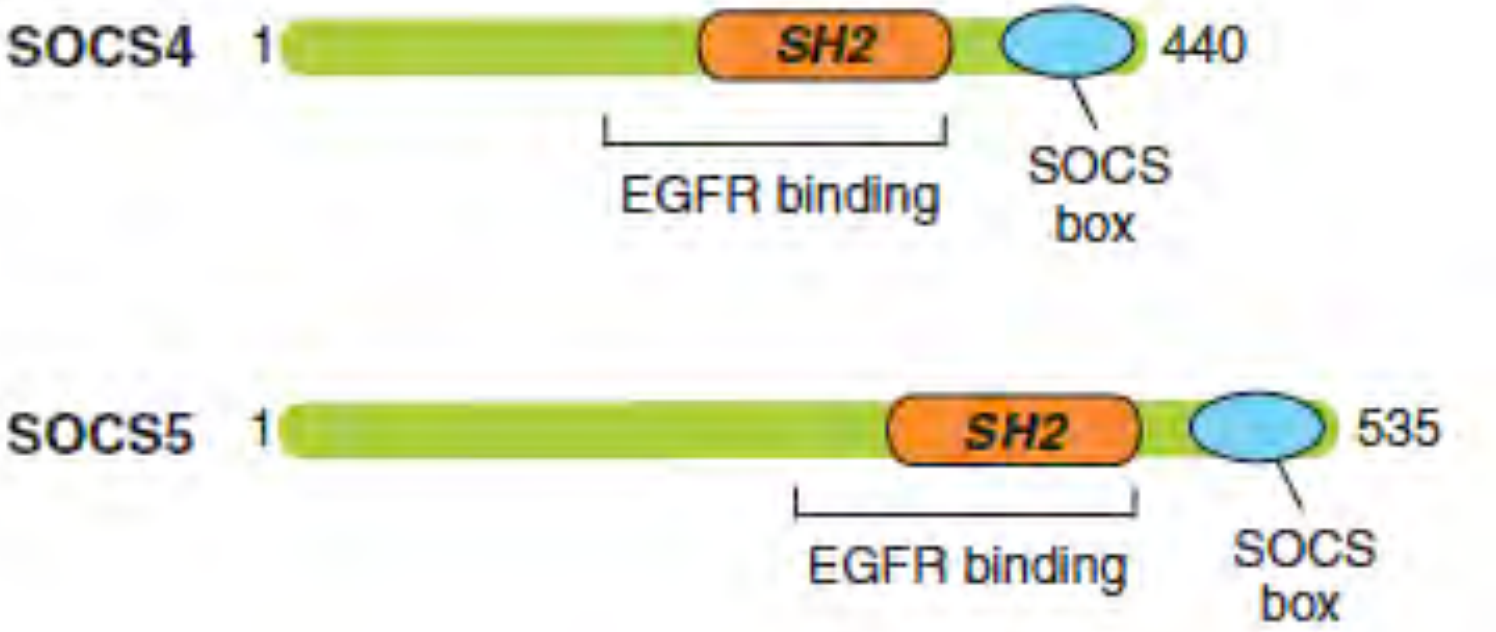
Signaling from Cytokine Receptors Is Modulated by Negative Signals

STAT proteins induce a class of small proteins termed SOCS proteins. These negative regulators are also known as CIS proteins

CIS proteins act in two ways to negatively regulate cytokine receptor stimulated signaling:

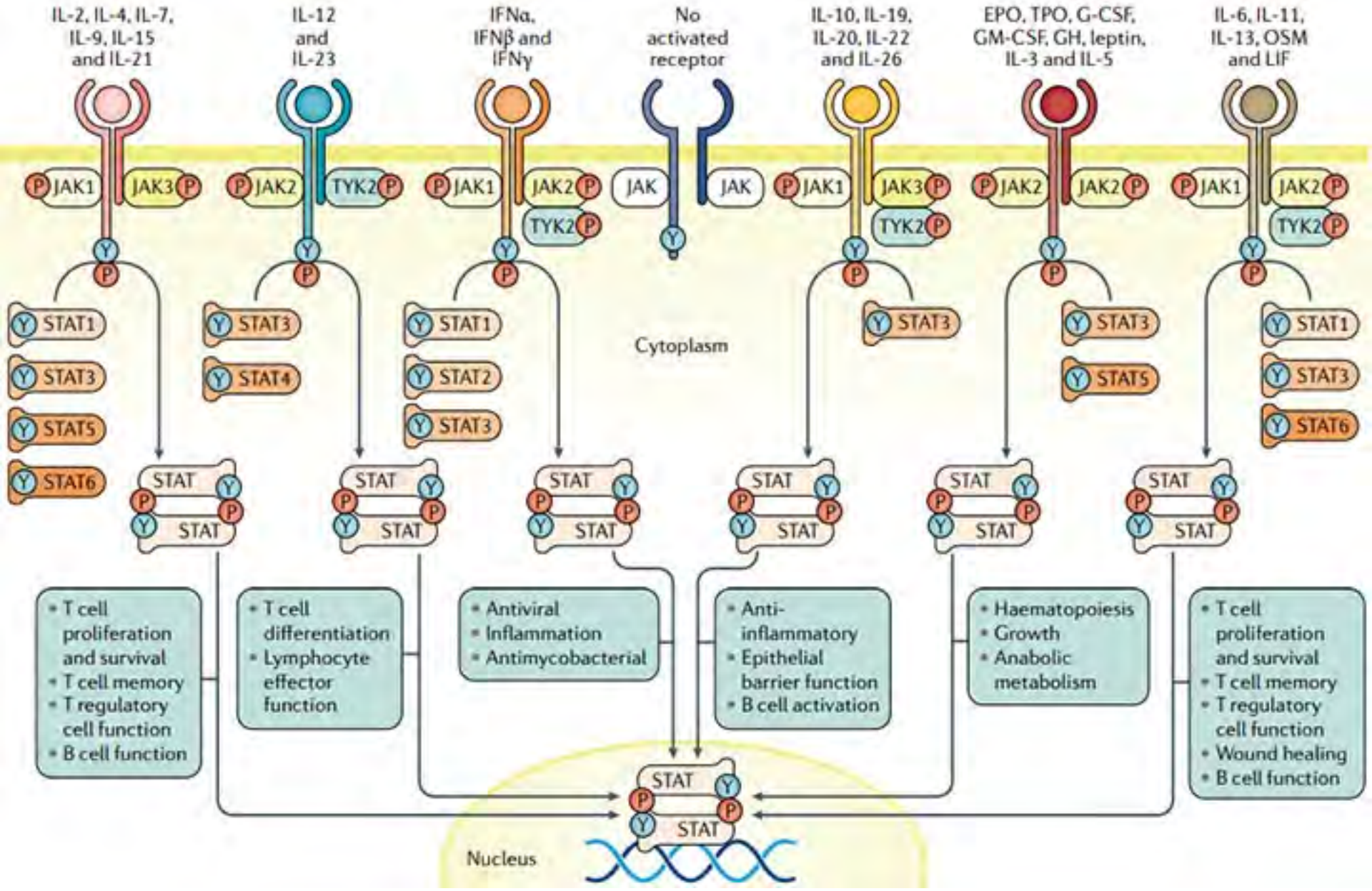
- The SH2 domain in several SOCS proteins bind to phosphotyrosines on an activated receptor, preventing binding of other SH2-containing signaling proteins and thus inhibiting receptor signaling
- SOCS-1 can bind to critical phosphotyrosine in the activation lip of activated JAK2 kinase thereby inhibiting its catalytic activity
- All SOCS proteins contain a SOCS box that recruits components of E3 ubiquitin ligases. As a result of SOCS-1 binding, JAK2 becomes polyubiquitinated and then degraded in proteasomes and thus terminate the signaling permanently



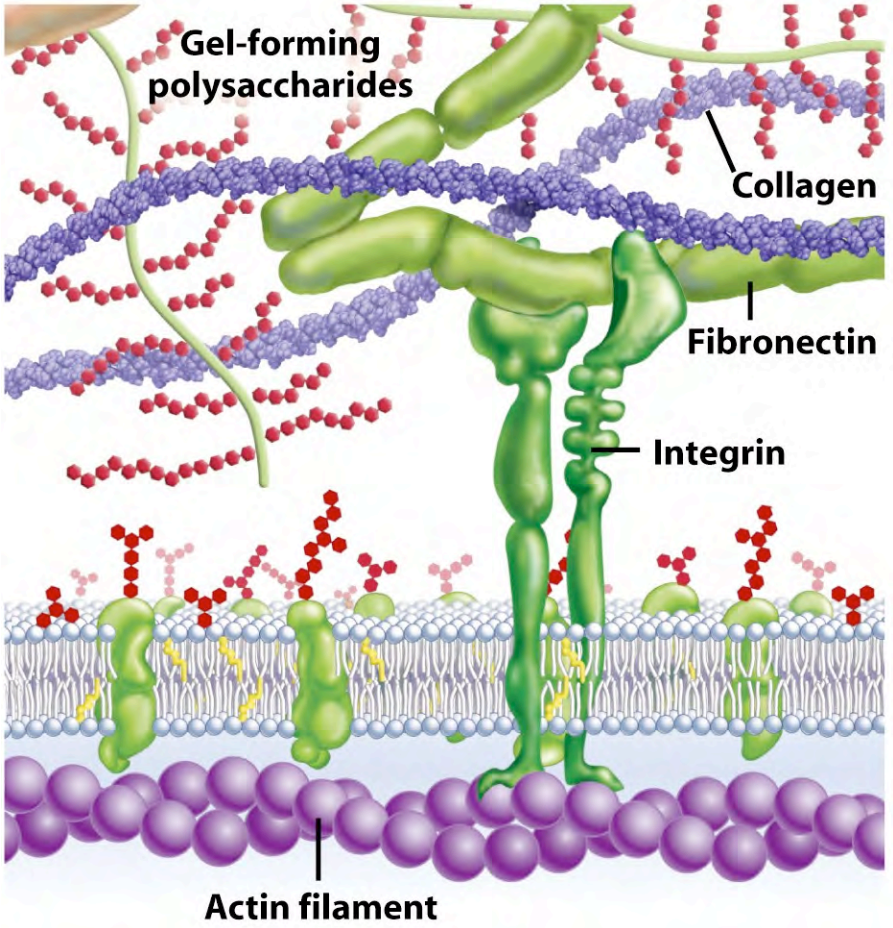


SOCS4 and SOCS5 bind to the EGFR through their respective SH2 domains, which share 87% sequence homology and a poorly defined Nterminal region.

The SOCS box recruits an E3 ligase and thereby leads to EGFR ubiquitylation.


IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21
IL-12 and IL-23
IFNα, IFNβ and IFNγ
No activated receptor
IL-10, IL-19, IL-20, IL-22 and IL-26
EPO, TPO, G-CSF, GM-CSF, GH, leptin, IL-3 and IL-5
IL-6, IL-11, IL-13, OSM and LIF
JAK1
JAK3
JAK2
TYK2
JAK
STAT1
STAT3
STAT5
STAT6
STAT4
STAT2
Cytoplasm
STAT
• T cell proliferation and survival
• T cell memory
• T regulatory cell function
• B cell function
• T cell differentiation
• Lymphocyte effector function
• Antiviral
• Inflammation
• Antimycobacterial
• Anti-inflammatory
• Epithelial barrier function
• B cell activation
• Haematopoiesis
• Growth
• Anabolic metabolism
• T cell proliferation and survival
• T cell memory
• T regulatory cell function
• Wound healing
• B cell function
Nucleus

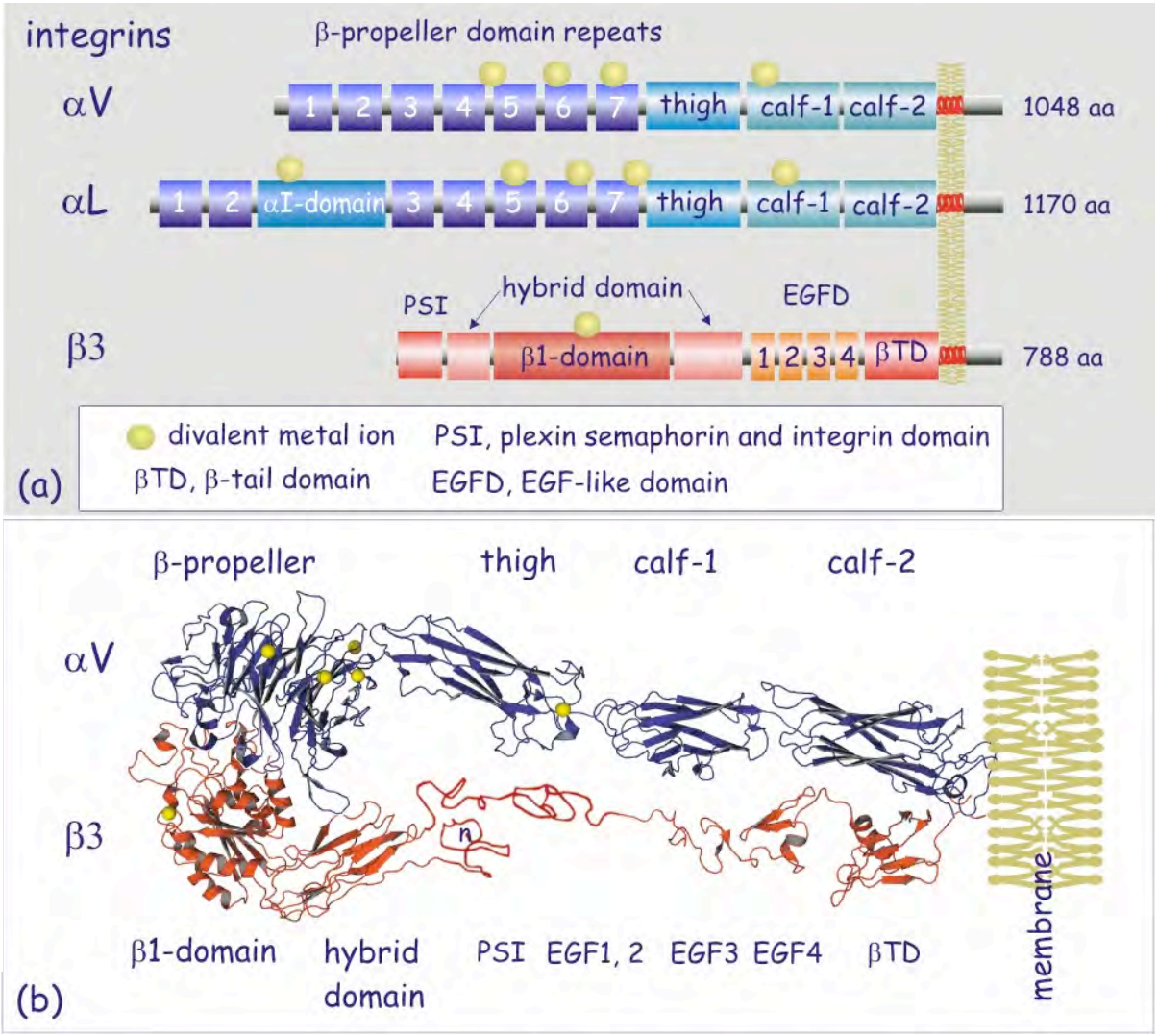
Integrins



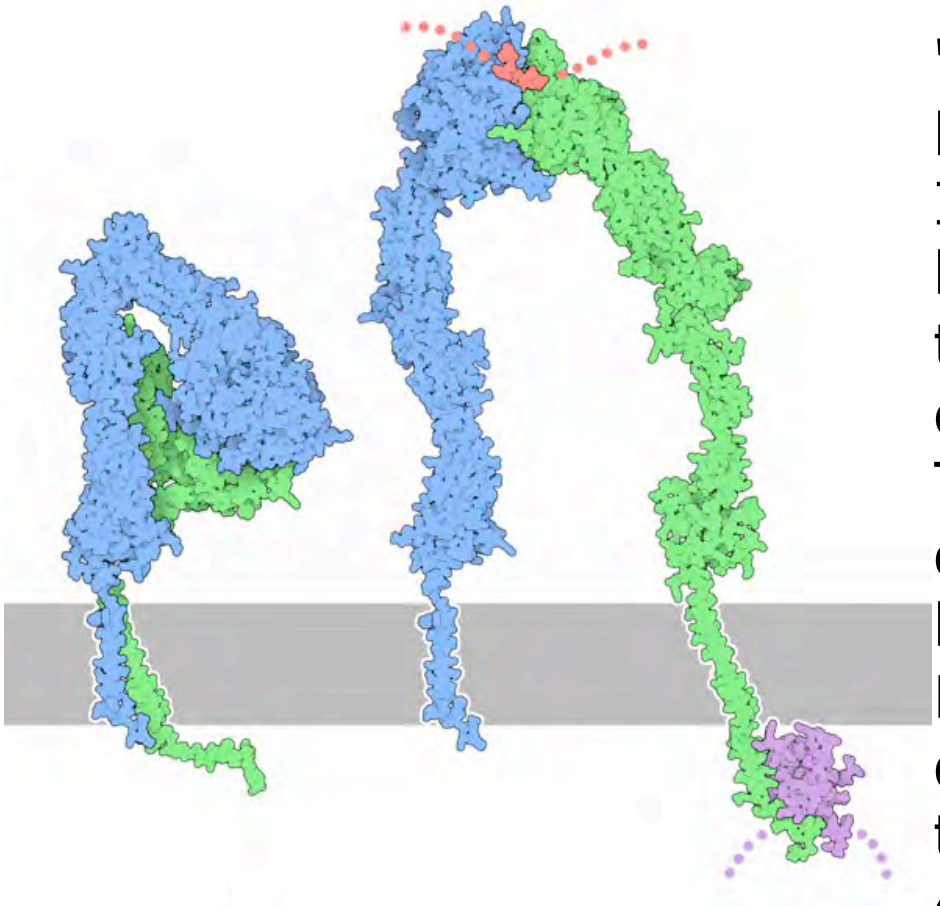
Integrins are transmembrane receptors that mediate the attachment between a cell and other cells or the extracellular matrix (ECM) components such as fibronectin, vitronectin, collagen, and laminin. In addition to transmitting mechanical forces across otherwise vulnerable membranes, they are involved in cell signaling and the regulation of cell cycle, shape and motility.

Domain architecture of integrins

Integrins are heterodimers containing two distinct chains, called the α (alpha) and β (beta) subunits.
In mammals, 18α and 8β subunits have been characterized.
The α and β subunits each penetrate the plasma membrane and possess small cytoplasmic domains.



Integrins activation



Integrin dimers are in a "bent" conformation which prevents them from interacting with their ligands. Therefore, integrin dimers must be 'unbent' in order to allow their binding to the ECM.

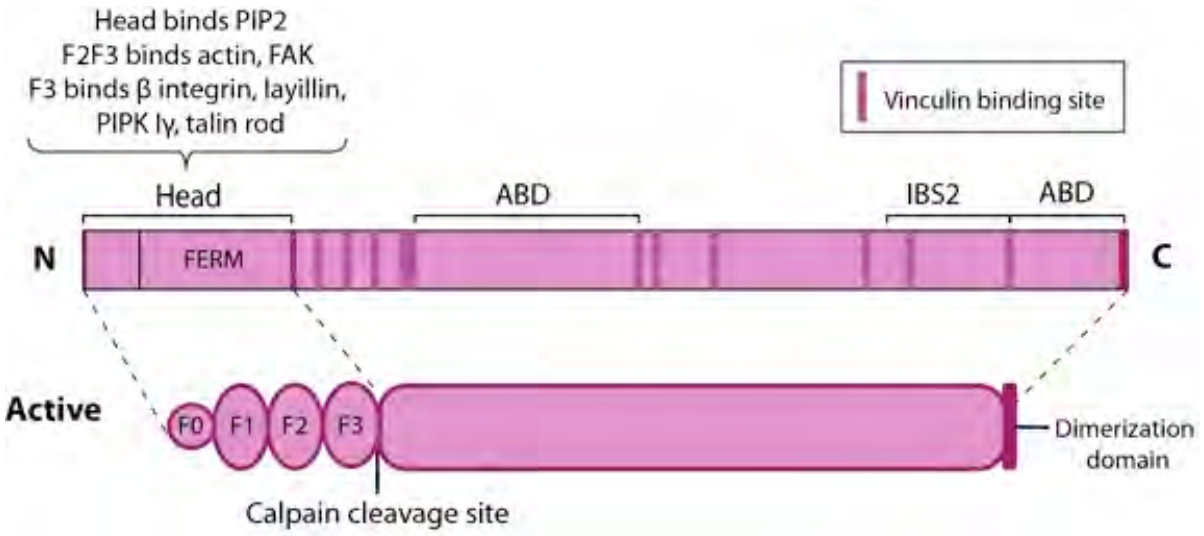
In cells, the priming is accomplished by **talin**, which binds to the β tail of the integrin dimer and changes its conformation.

Talin binding alters the angle of tilt of the β3 chain transmembrane helix which primes integrins.

Moreover, talin proteins are able to dimerize and thus are thought to trigger the clustering of integrin dimers which leads to the formation of a focal adhesion.

Talin

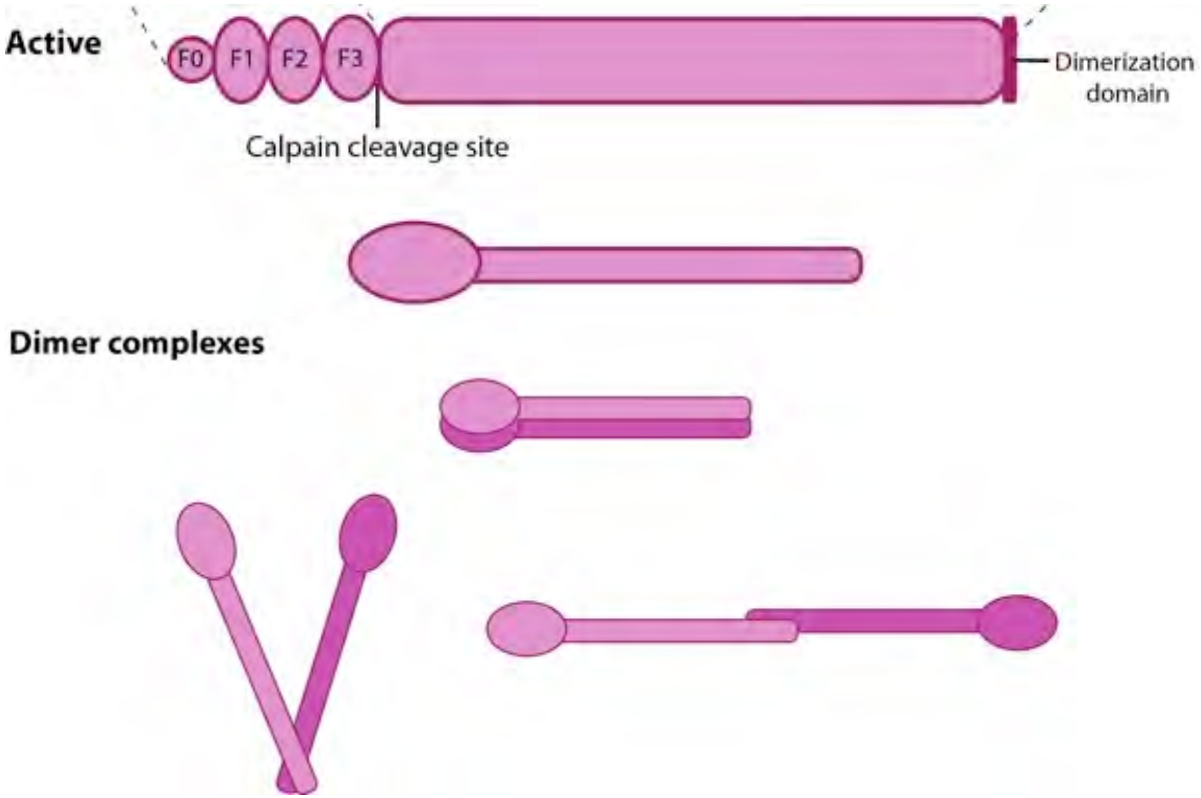
Talin is a 270kDa cytoskeletal protein concentrated at regions of cell–substratum contact and, in lymphocytes, at cell–cell contacts. It is a structural platform that is required for the initial linkage between the contractile cytoskeleton and sites of integrin/fibronectin adhesion



Integrin tail binding occurs via the F3 phosphotyrosine binding (PTB) domain via a unique interaction with the integrin membrane proximal region, which is sufficient for integrin activation. The basic patches on all subdomains can dock onto the plasma membrane and further enhance integrin activation. Specific interactions through basic residues on F3 are also essential for integrin clustering.

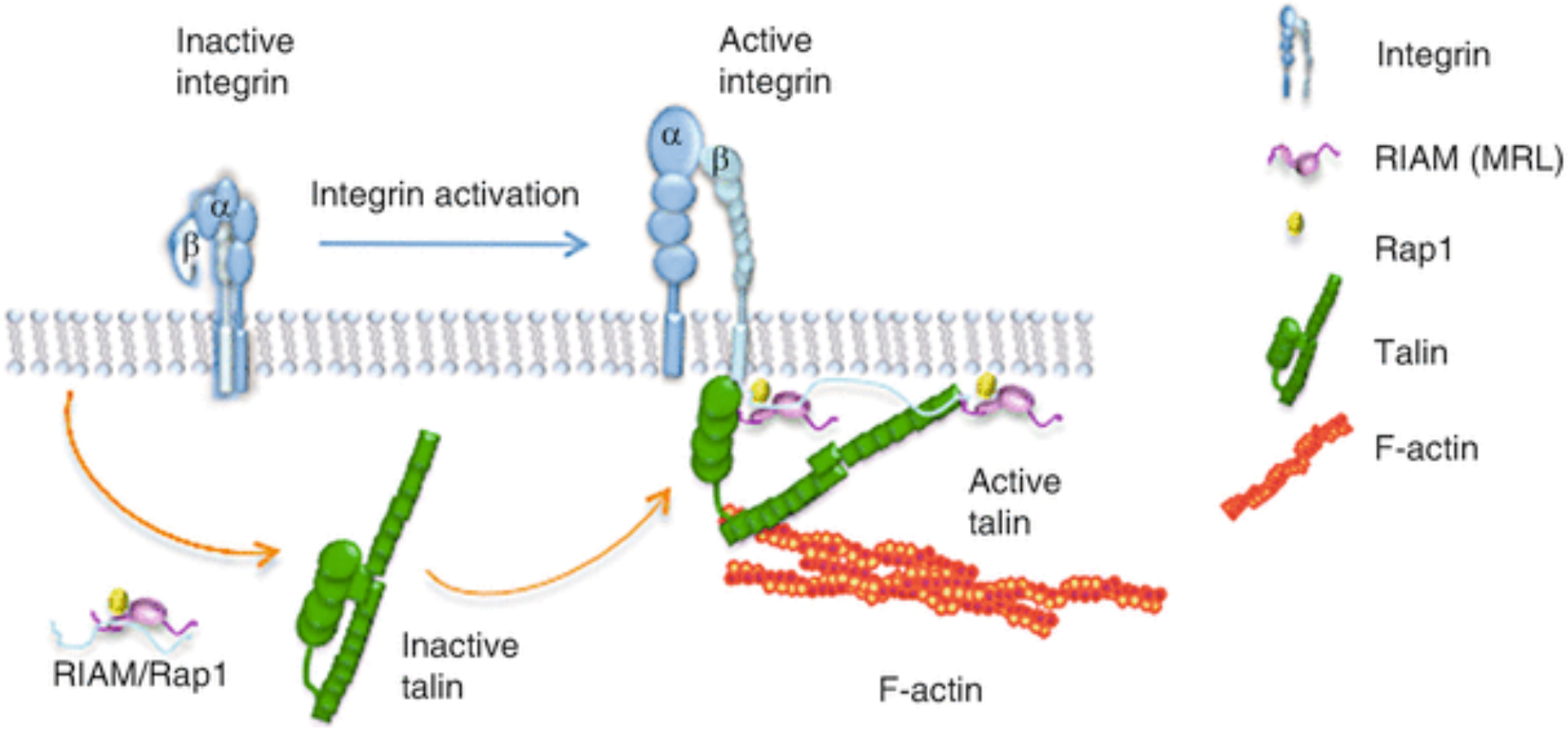
The rod contains an additional integrin-binding site (IBS2), two actin-binding sites (ABD) and several vinculin-binding sites that are shown to be exposed by stretch in response to force. Talin also contains numerous potential phosphorylation sites which are suggested to directly or indirectly regulate the association of talin with other factors

Talin activation and membrane recruitment



Talin is in an autoinhibited form in the cytosol due to the intermolecular association between the F3 subdomain and a helical bundle in the rod region. This not only blocks integrin binding site on F3 but also F2 and F3 binding to membrane. Activation likely involves binding to membrane phospholipids such as phosphatidylinositol 4,5-bis-phosphate (PIP2), vinculin and F-actin or calpain cleavage. This enhances talin's affinity for the β-integrin subunit by revealing binding sites.

Talin membrane localization and activation by RIAM

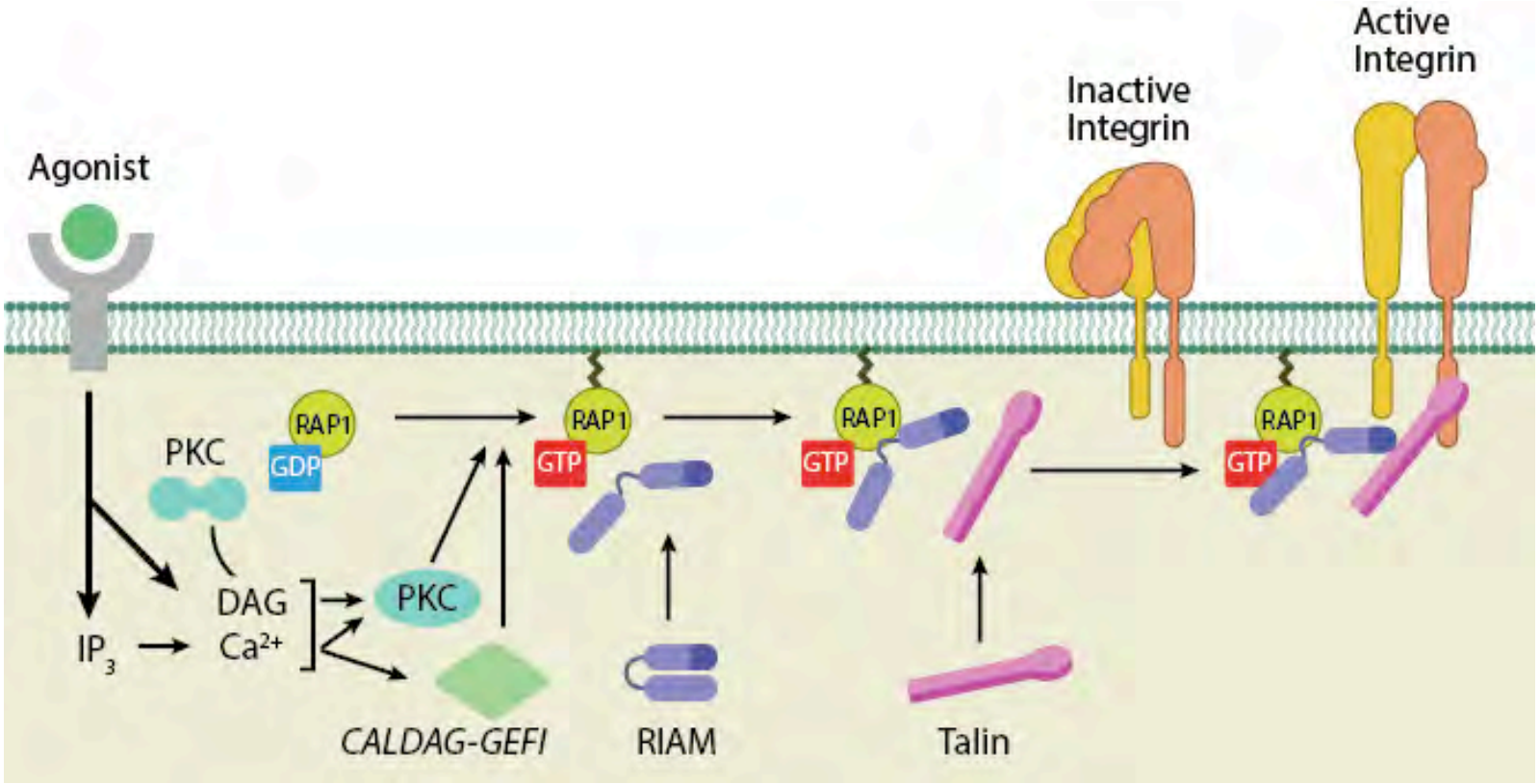


In resting cells, most integrins are kept inactive, possibly owing to conformational constraints in the cytoplasmic tails. A small proportion of the integrin dimers display the thermodynamically unfavourable, active conformation and can bind their ligand.

Upon agonist stimulation, Rap1 is transiently converted to the active GTP-bound form and directly or indirectly brings talin to the integrin cytoplasmic tail, maintaining them in their active conformation.

Rap1 activity is therefore required for ligand binding and outside-in signalling to take place, by the anchoring of the ligand-bound integrin to the actin cytoskeleton.

Talin activation and membrane recruitment



Ligand occupancy in certain cell-surface receptors (agonists) causes phospholipid hydrolysis releasing diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 increases cytosolic levels of calcium ions; DAG and Ca2+ can promote GTP-loading and membrane translocation of Rap1 either by activating Ca2+ and DAG-regulated GEF (CALDAG-GEF or Rap-GEF) or protein kinase C (PKC). Activated Rap1 in turn, recruits Rap1-GTP-interacting adaptor molecule (RIAM) along with its binding partner, talin to the plasma membrane.

Rap-1

(Ras-proximate-1 or Ras-related protein 1)

It is a small GTPase which belongs to Ras-related protein family; there are two isoforms of the Rap1 protein, each encoded by a separate gene, RAP1A and RAP1B.

Rap1 plays a unique, Ras-independent role in eukaryotic cells. Activated by virtually all receptor types and second messengers, Rap1 controls adhesion-related functions such as phagocytosis,cell-cell contacts and functional activation of integrins through inside-out signalling.

Cellular functions of the Rap1 GTP-binding protein: a pattern emerges

Emmanuelle Caron

Centre for Molecular Microbiology and Infection and Department of Biological Sciences, The Flowers Building, Room 2:41, Armstrong Road, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK

(e-mail: e.caron@ic.ac.uk)

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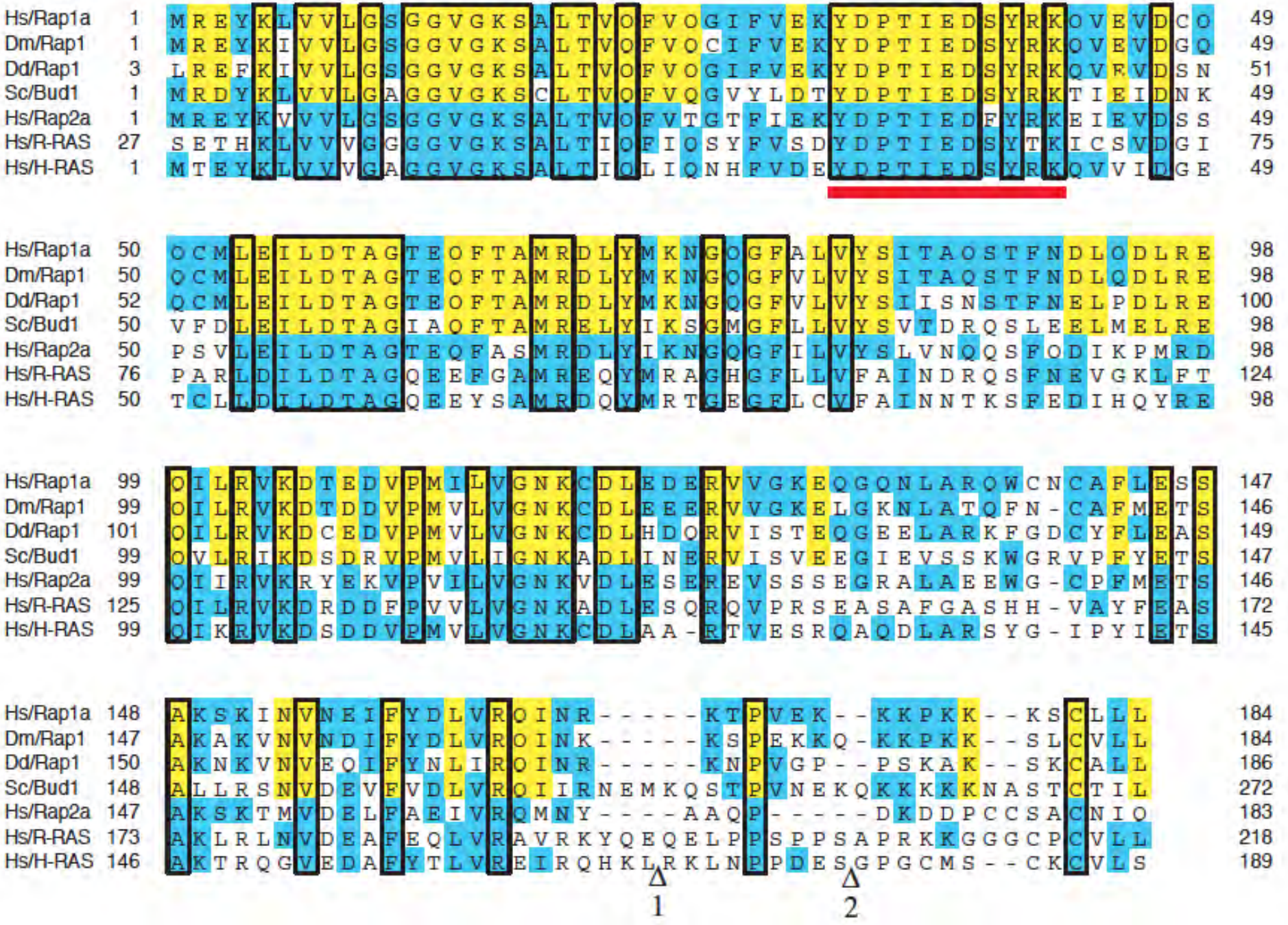
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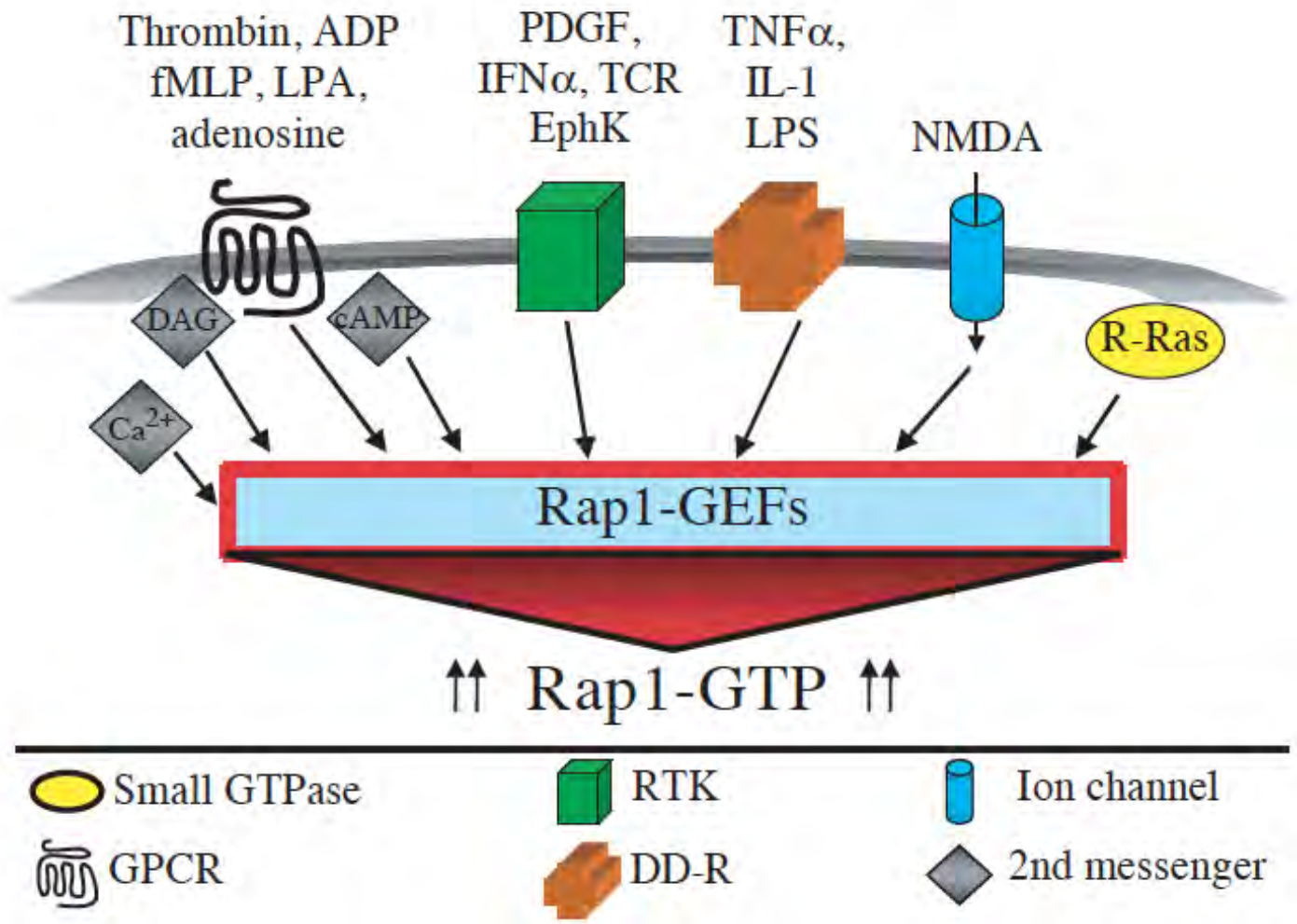
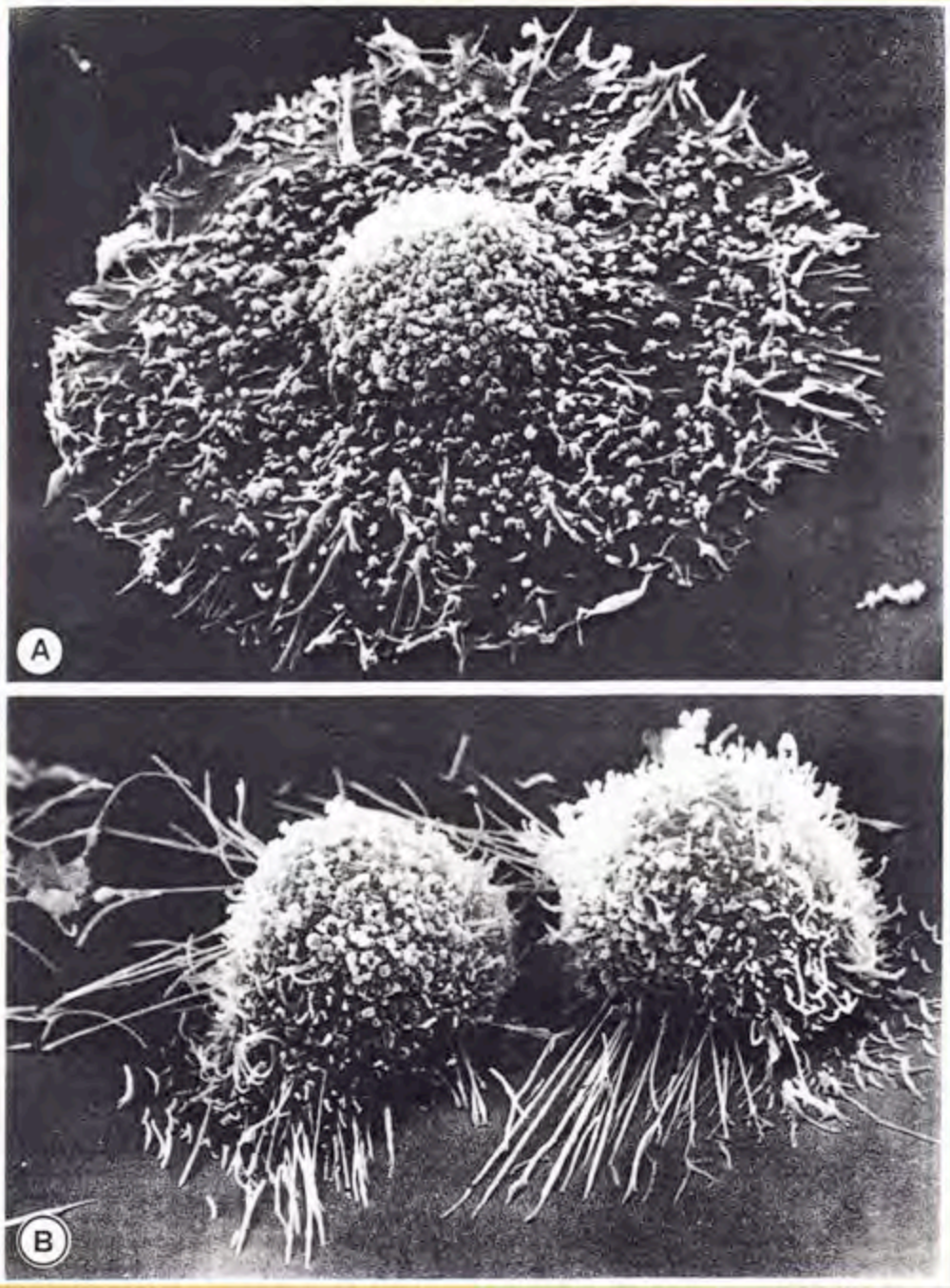
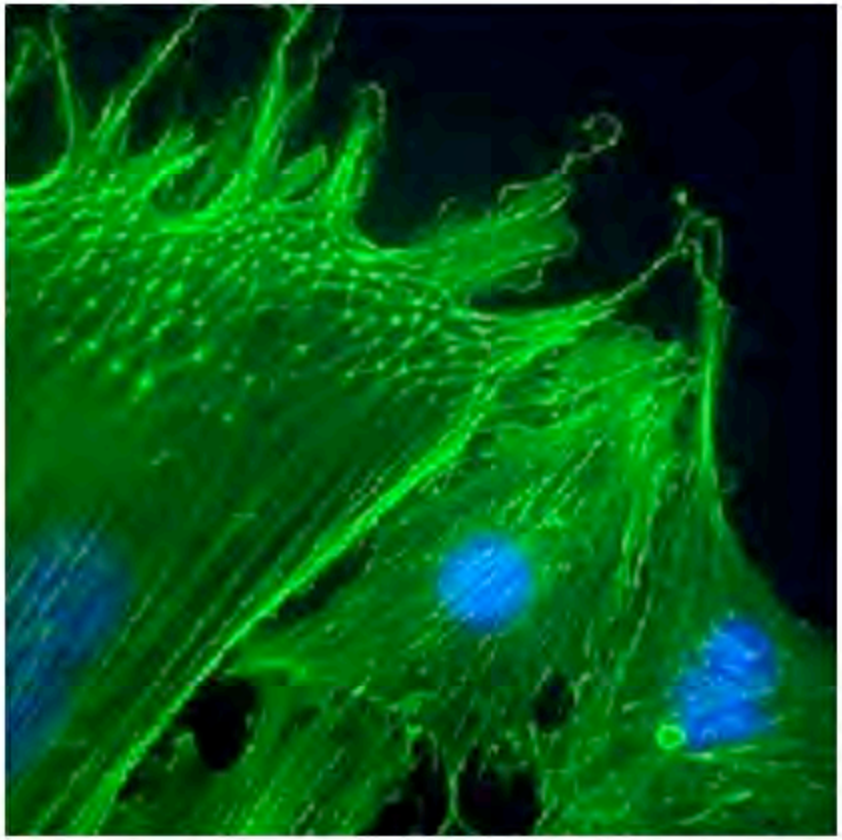
Dm/Rap1 1 MREYKIVVLGSGGVGKSALTVQFVQCIFVEKYDPTIEDSYRKQVEVDGQ 49
Dd/Rap1 3 LREFKIVVLGSGGVGKSALTVQFVQGIFVEKYDPTIEDSYRKQVEVDSN 51
Sc/Bud1 1 MRDYKLVVLGAGGVGKSCLTVQFVQGVYLDTYDPTIEDSYRKTIEIDNK 49
Hs/Rap2a 1 MREYKVVVLGSGGVGKSALTVQFVTGTFIEKYDPTIEDFYRKEIEVDSS 49
Hs/R-RAS 27 SETHKLVVVGGGGVGKSALTIQFIQSYFVSDYDPTIEDSYTKICSVDGI 75
Hs/H-RAS 1 MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGE 49
Hs/Rap1a 50 QCMLEILDTAGTEQFTAMRDLYMKNGQGFALVYSITAQSTFNDLQDLRE 98
Dm/Rap1 50 QCMLEILDTAGTEQFTAMRDLYMKNGQGFVLVYSITAQSTFNDLQDLRE 98
Dd/Rap1 52 QCMLEILDTAGTEQFTAMRDLYMKNGQGFVLVYSIISNSTFNELPDLRE 100
Sc/Bud1 50 VFDLEILDTAGIAQFTAMRELYIKSGMGFLLVYSVTDRQSLEELMELRE 98
Hs/Rap2a 50 PSVLEILDTAGTEQFASMRDLYIKNGQGFILVYSLVNQQSFQDIKPMRD 98
Hs/R-RAS 76 PARLDILDTAGQEEFGAMREQYMRAGHGFLLVFAINDRQSFNEVGKLFT 124
Hs/H-RAS 50 TCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHQYRE 98
Hs/Rap1a 99 QILRVKDTEDVPMILVGNKCDLEDERVVGKEQGQNLARQWCNCAFLESS 147
Dm/Rap1 99 QILRVKDTDDVPMVLVGNKCDLEEERVVGKELGKNLATQFN-CAFMETS 146
Dd/Rap1 101 QILRVKDCEDVPMVLVGNKCDLHDQRVISTEQGEELARKFGDCYFLEAS 149
Sc/Bud1 99 QVLRIKDSDRVPMVLIGNKADLINERVISVEEGIEVSSKWGRVPFYETS 147
Hs/Rap2a 99 QIIRVKRYEKVPVILVGNKVDLESERDVSSSEGRALAEEWG-CPFMETS 146
Hs/R-RAS 125 QILRVKDRDDFPVVLVGNKADLESQRQVPRSEASAFGASHH-VAYFEAS 172
Hs/H-RAS 99 QIKRVKDSDDVPMVLVGNKCDLAA-RTVESRQAQDLARSYG-IPYIETS 145
Hs/Rap1a 148 AKSKINVNEIFYDLVRQINR-----KTPVEK--KKPKK--KSCLLL 184
Dm/Rap1 147 AKAKVNVNDIFYDLVRQINK-----KSPEKKQ-KKPKK--SLCVLL 184
Dd/Rap1 150 AKNKVNVEQIFYNLIRQINR-----KNPVGP--PSKAK--SKCALL 186
Sc/Bud1 148 ALLRSNVDEVFVDLVRQIIRNEMKQSTPVNEKQKKKKKNASTCTIL 272
Hs/Rap2a 147 AKSKTMVDELFAEIVRQMNY----AAQP-----DKDDPCCSACNIQ 183
Hs/R-RAS 173 AKLRLNVDEAFEQLVRAVRKYQEQELPPSPPSAPRKKGGGCPCVLL 218
Hs/H-RAS 146 AKTRQGVEDAFYTLVREIRQHKLRKLNPPDESGPGCMS--CKCVLS 189
Δ 1
Δ 2

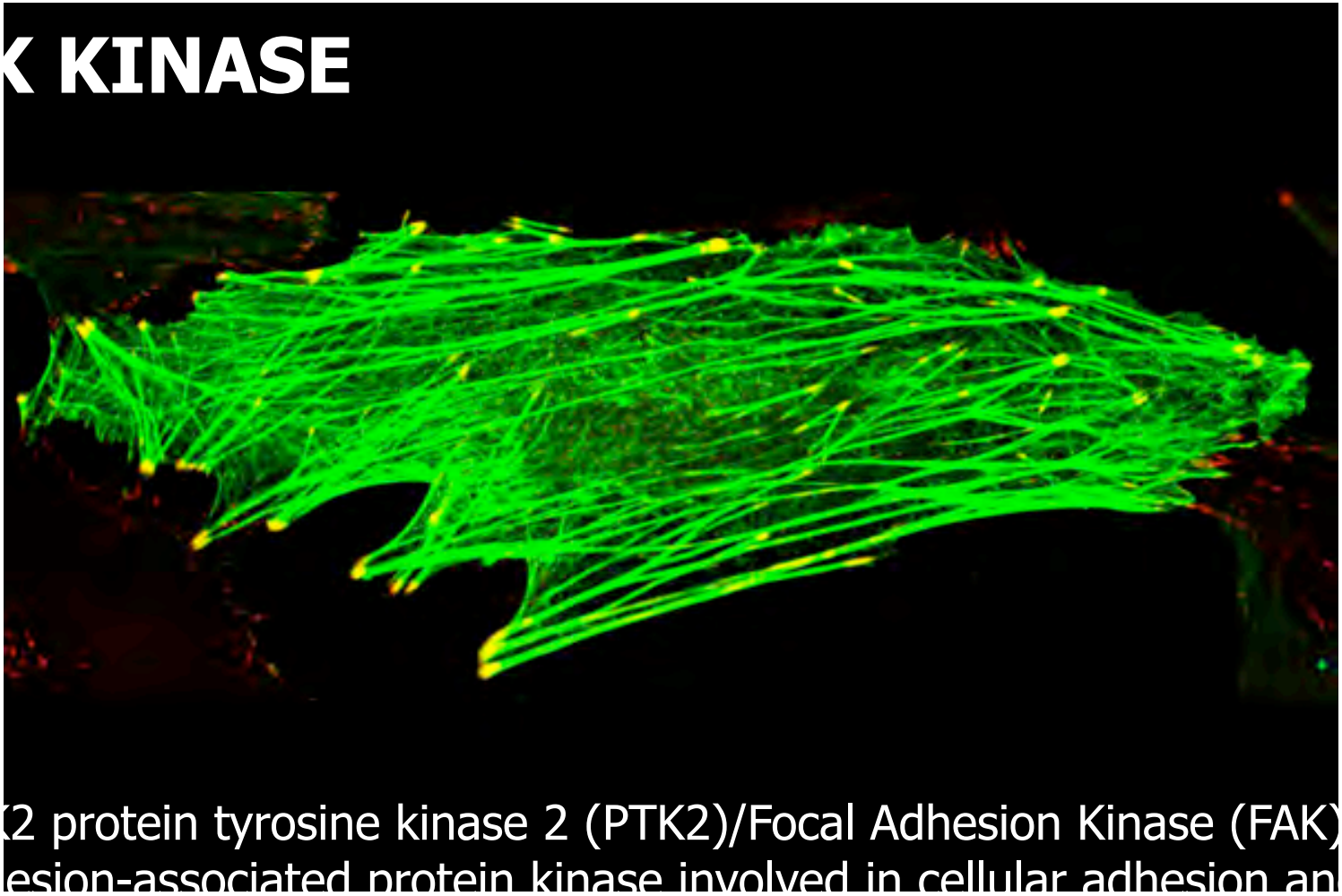


Many receptors and second messengers are coupled to the activation of Rap1 guanine nucleotide exchange factors (Rap1GEFs), and an increase in the cellular levels of active, GTP-bound Rap1.


A
B



K KINASE



2 protein tyrosine kinase 2 (PTK2)/Focal Adhesion Kinase (FAK)
esion-associated protein kinase involved in cellular adhesion an

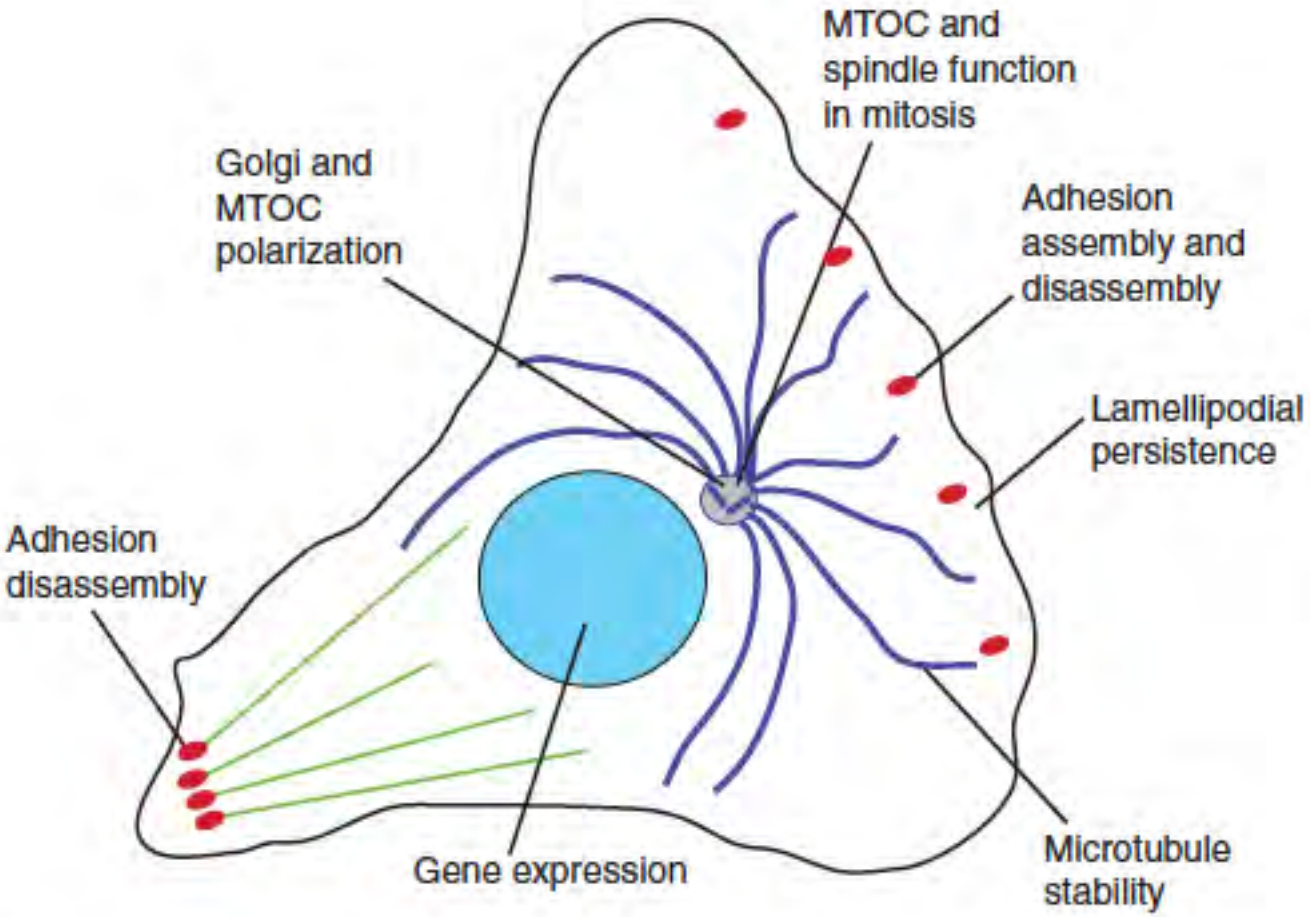


Fig. 2. Major cellular functions of FAK. A migrating cell with the leading edge (right) and trailing edge (left) is shown. Cell-ECM adhesions (red), stress fibers (green), microtubules (dark blue), the MTOC (grey) and nucleus (blue) are illustrated. Black lines denote cellular targets of FAK signaling.

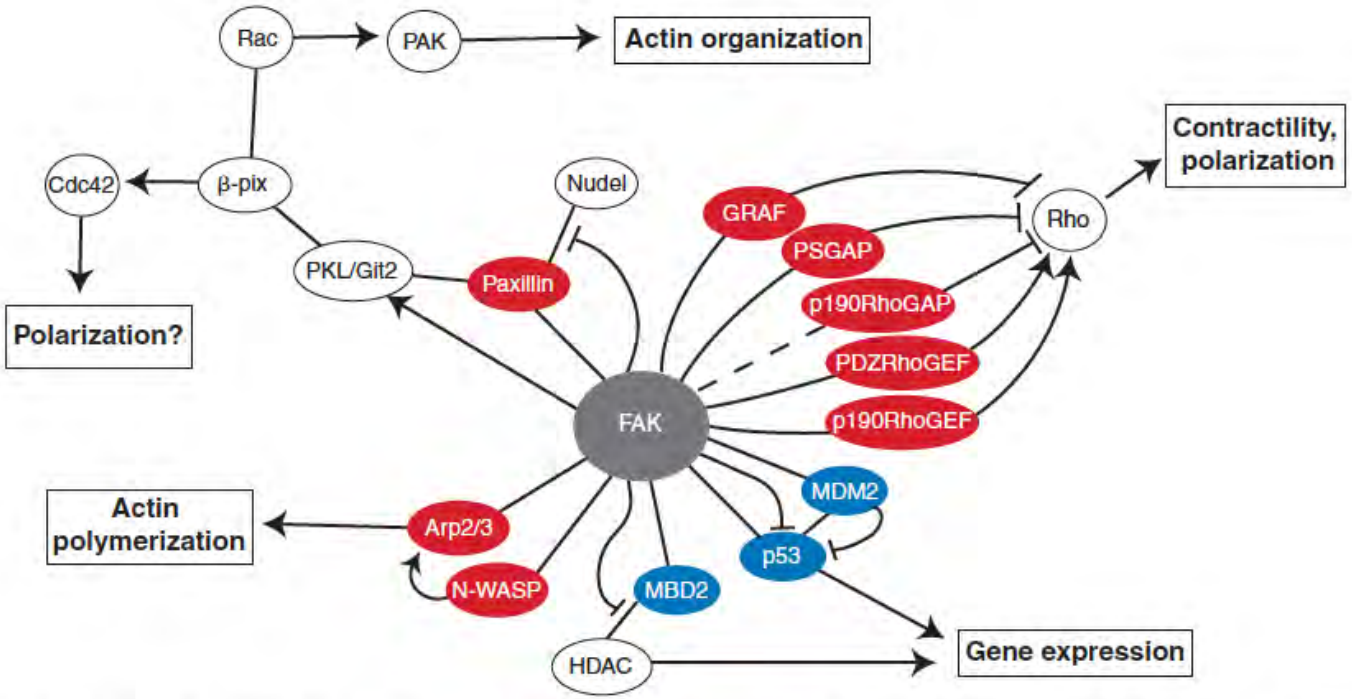


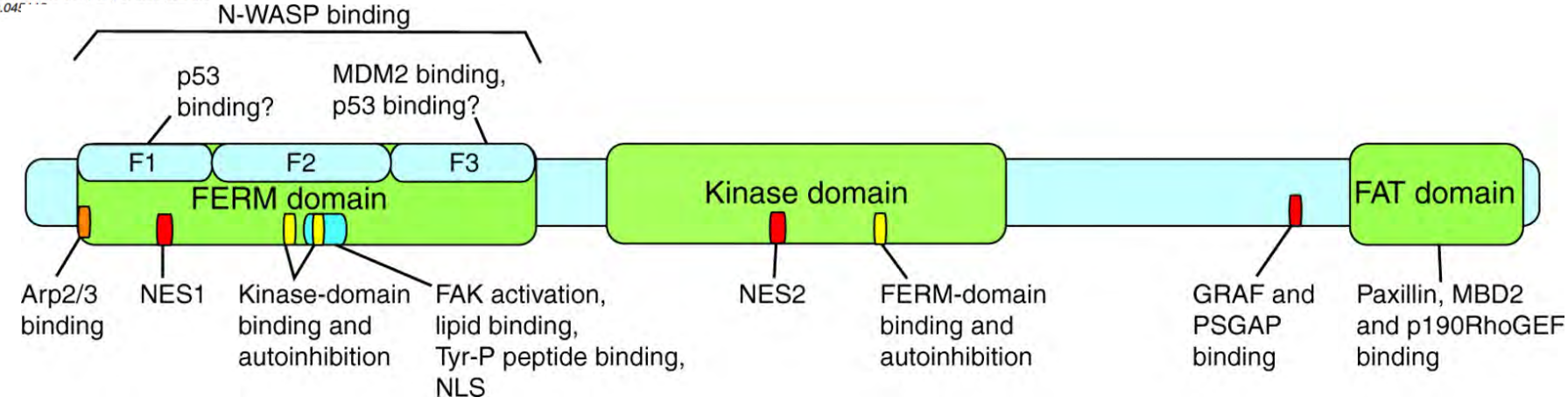
Fig. 3. Networks of FAK signaling that control the actin cytoskeleton and gene expression. Solid lines link proteins that physically interact and dashed lines denote indirect protein-protein interactions. Red proteins are FAK binding partners that are implicated in the regulation of the actin cytoskeleton and blue proteins are FAK binding partners that are implicated in regulating gene expression. Positive (arrows) and negative (bars) regulation of downstream proteins and/or events is indicated.

Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions

Michael D. Schaller
1 Medical Center Drive, Department of Biochemistry, Mary Babb Randolph Cancer Center and Center for Cardiovascular and Respiratory Sciences, West Virginia University School of Medicine, PO Box 9142, Morgantown, WV 26506, USA
mschaller@hsc.wvu.edu

Journal of Cell Science 123, 1007-1013

doi:10.1242/jcs.045



Sequence and structural analysis reveals 4 distinct domains:
① an N-terminal FERM domain;
② a centrally located catalytic tyrosine kinase domain;
③ a C-terminal focal-adhesion targeting (FAT) domain (a four-helix bundle);
④ and an unstructured proline-rich region between the catalytic and FAT domains

The FERM domain docks with the catalytic domain to autoinhibit kinase activity, but also interacts with other molecules to control FAK signaling. The FAT domain and proline-rich region are also docking sites for binding partners that function in localization and downstream signaling.

MOLECULAR AND CELLULAR BIOLOGY, Feb. 2002, p. 1203–1217 Vol. 22, No. 4
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.4.1203–1217.2002

Focal Adhesions Require Catalytic Activity of Src Family Kinases To Mediate Integrin-Matrix Adhesion

Leiming Li,[1,2] Masaya Okura,[1]† and Akira Imamoto[1,2,3]*

The Ben May Institute for Cancer Research and Center for Molecular Oncology,[1] Committee on Cell Physiology,[2] and Committee on Cancer Biology,[3] The University of Chicago, Chicago, Illinois 60637

Received 9 July 2001/Returned for modification 17 August 2001/Accepted 20 November 2001

Cytokine Receptors Activate Multiple Cytoplasmic PTK Families

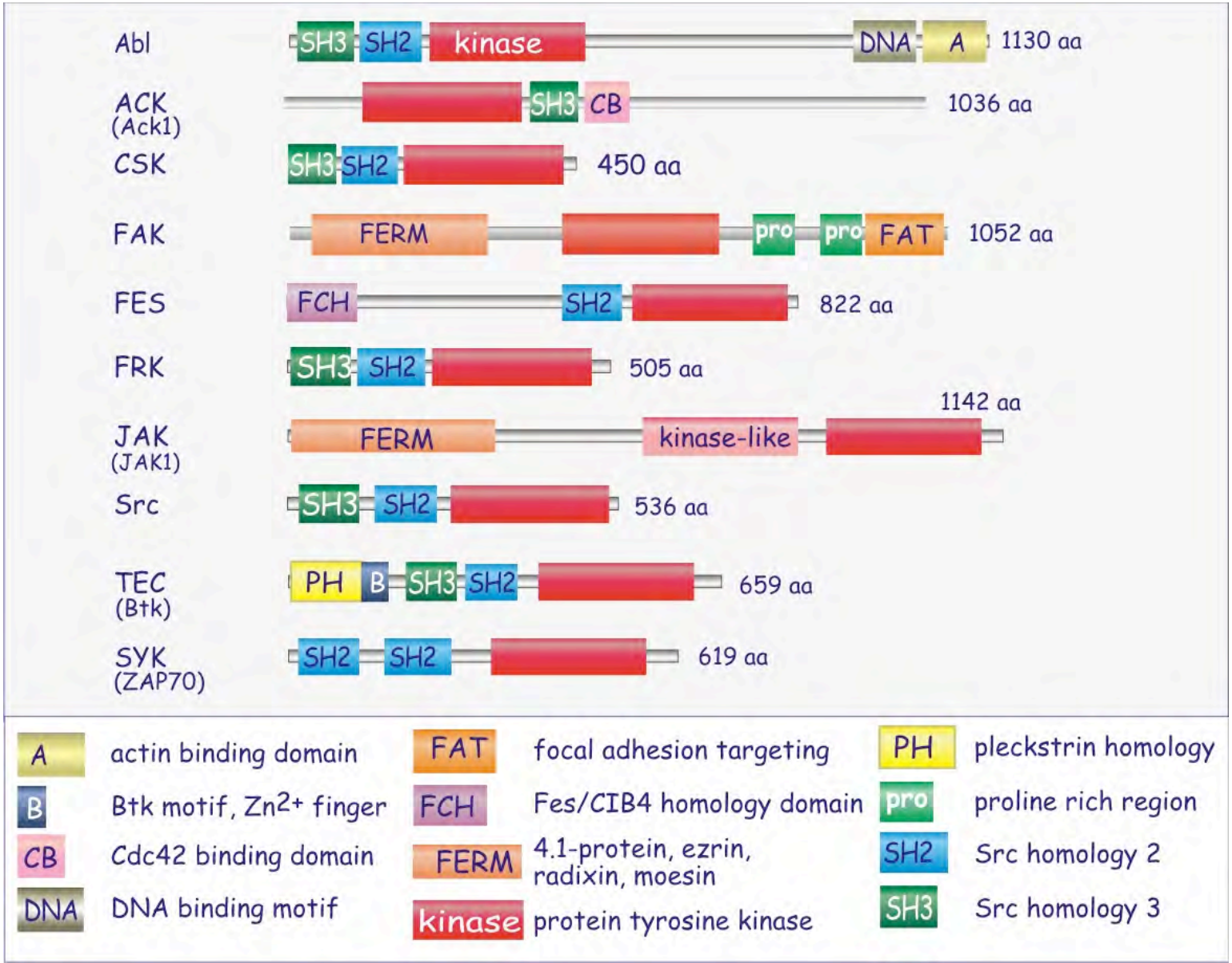


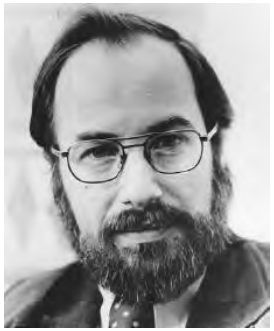
Table 1 Characteristics of Src family kinases

Src family member[a]	*Pattern of expression*	*Isoforms*	*Oncogenic forms*[b]
Blk	B cells		
Fgr	Myeloid cells, B cells		Oncogenic fusion with gag sequences in feline sarcoma virus; overexpressed in some leukemias and lymphomas
Fyn	Ubiquitous	T-cell-specific isoform (Fyn T)	
Hck	Myeloid cells	Two different translational starts	
Lck	T cells, NK cells, brain		Overexpressed in T-cell acute lymphocytic leukemias
Lyn	Brain, B cells, myeloid cells	Two alternatively spliced forms	
Src	Ubiquitous	Neuron-specific isoforms	Mutated and truncated in retroviruses; truncated in colon cancer; overexpressed in mammary, pancreatic and other cancers
Yes	Ubiquitous		Oncogenic fusion with gag sequences in avian sarcoma viruses; highly expressed in colon, malignant melanoma and other cancers
Yrk	Ubiquitous		

[a]Thomas and Brugge (1997). [b]Blume-Jensen and Hunter (2001)

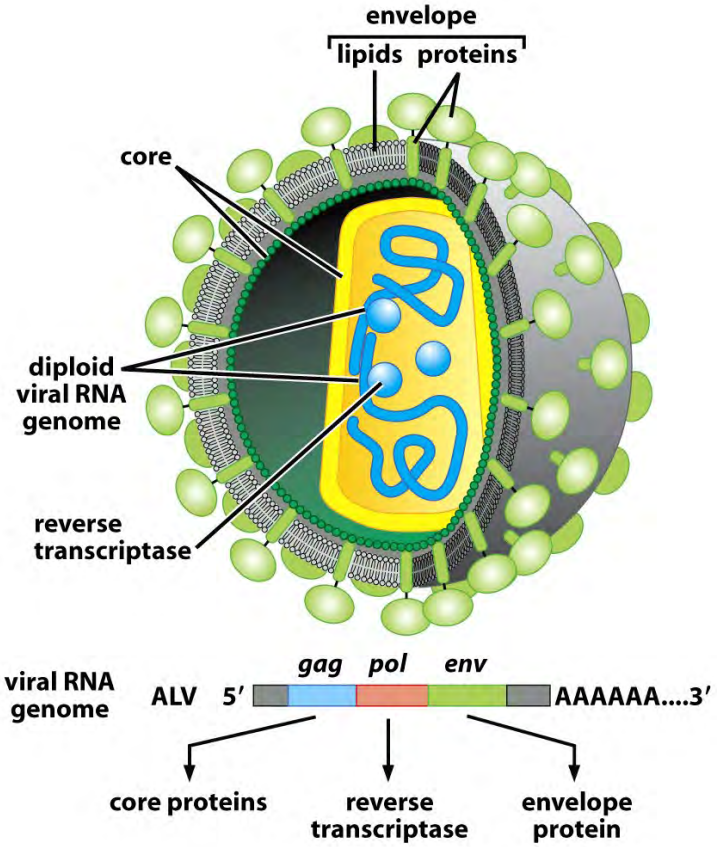
The story of Src

What Viruses and Nobel Laureates Taught Us About Cancer



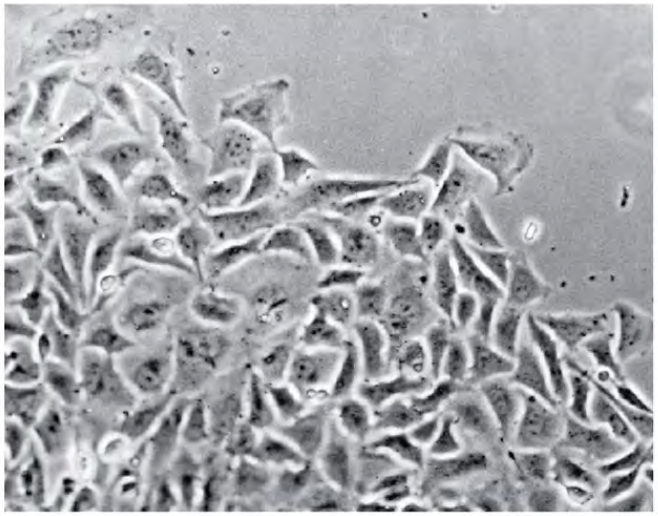
Howard Temin and David Baltimore

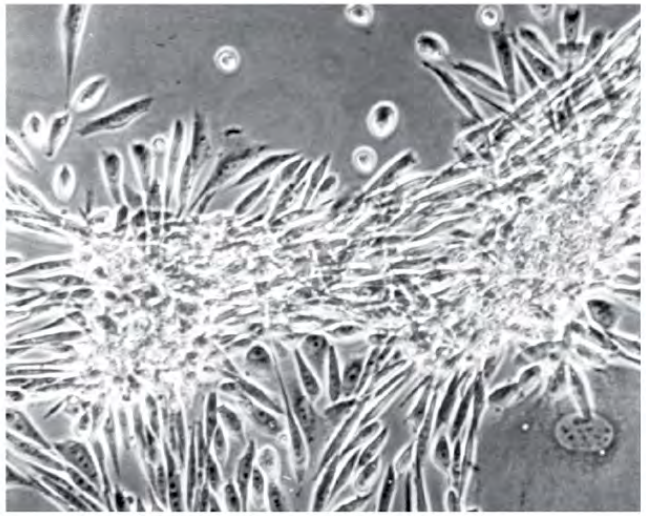
Nobel Prize in Physiology and Medicine 1975



No contact inhibition of cell division

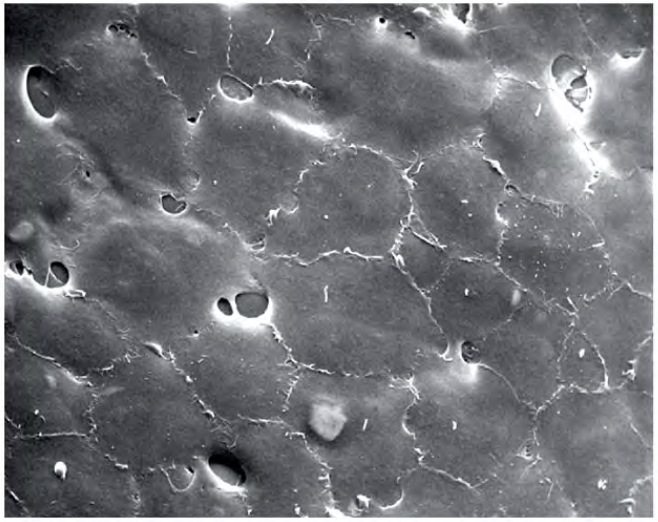
Normal

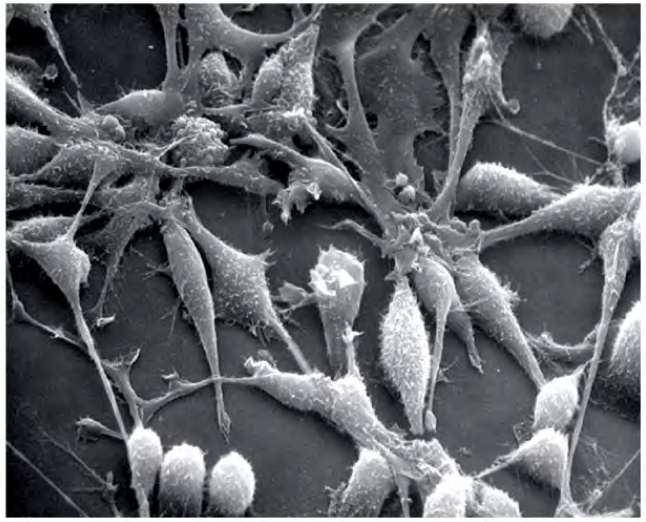




Cancer

Normal

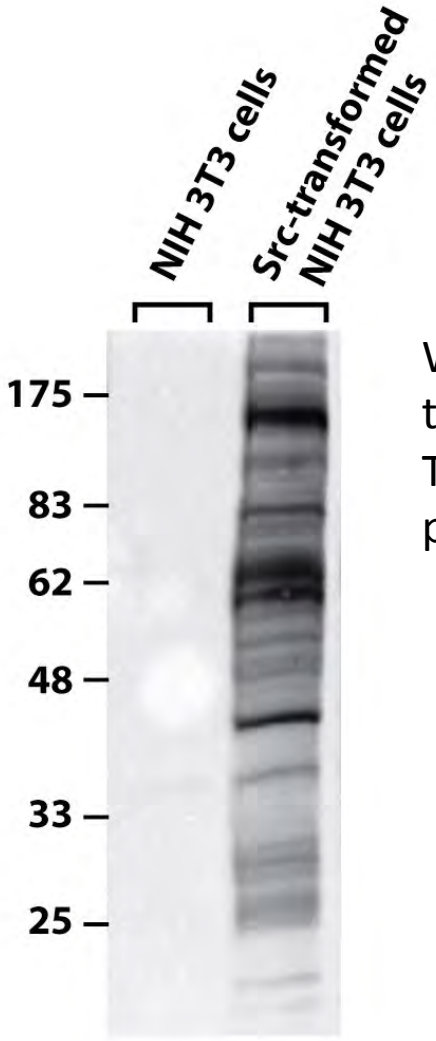




Cancer

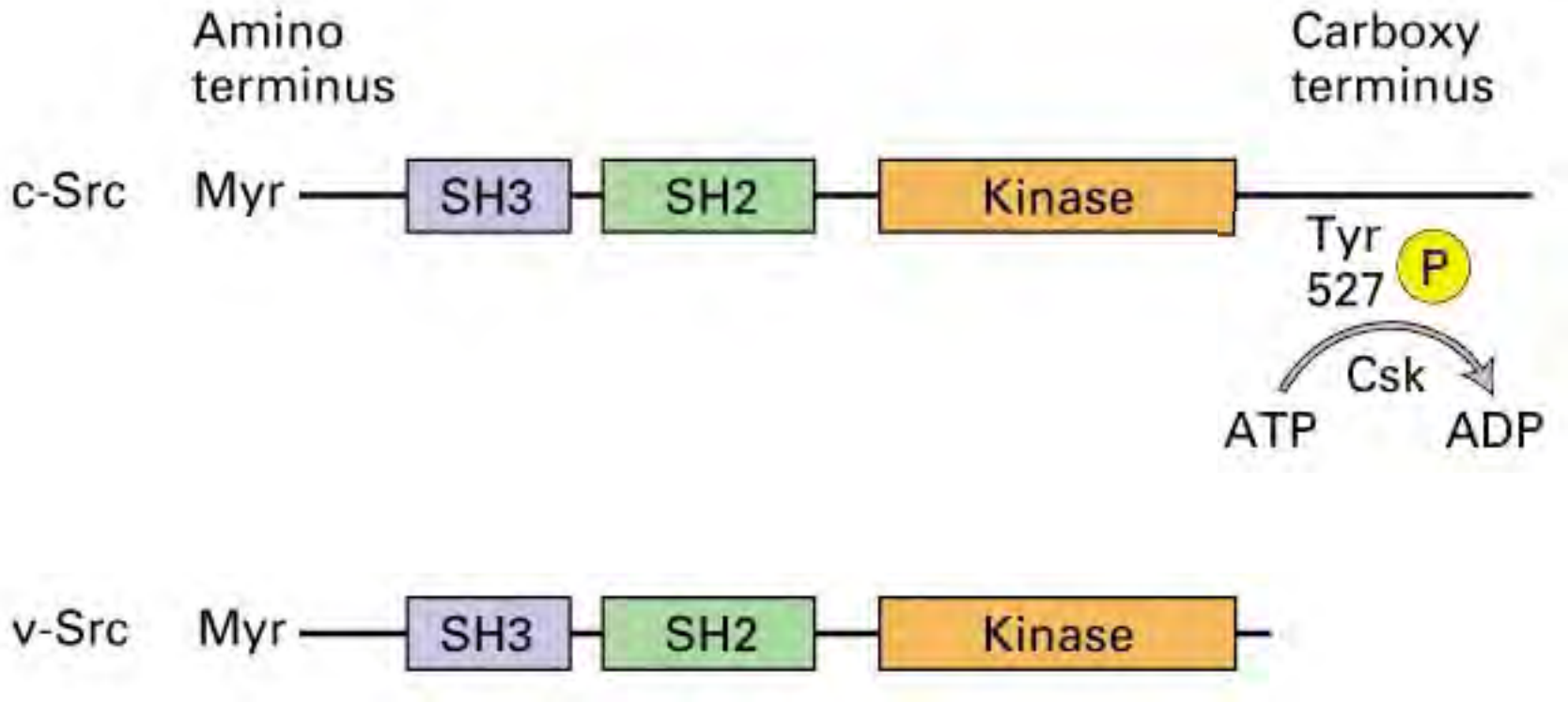
Normally, Src kinase intrinsic activity is low

What makes Src so active in transformed cells?



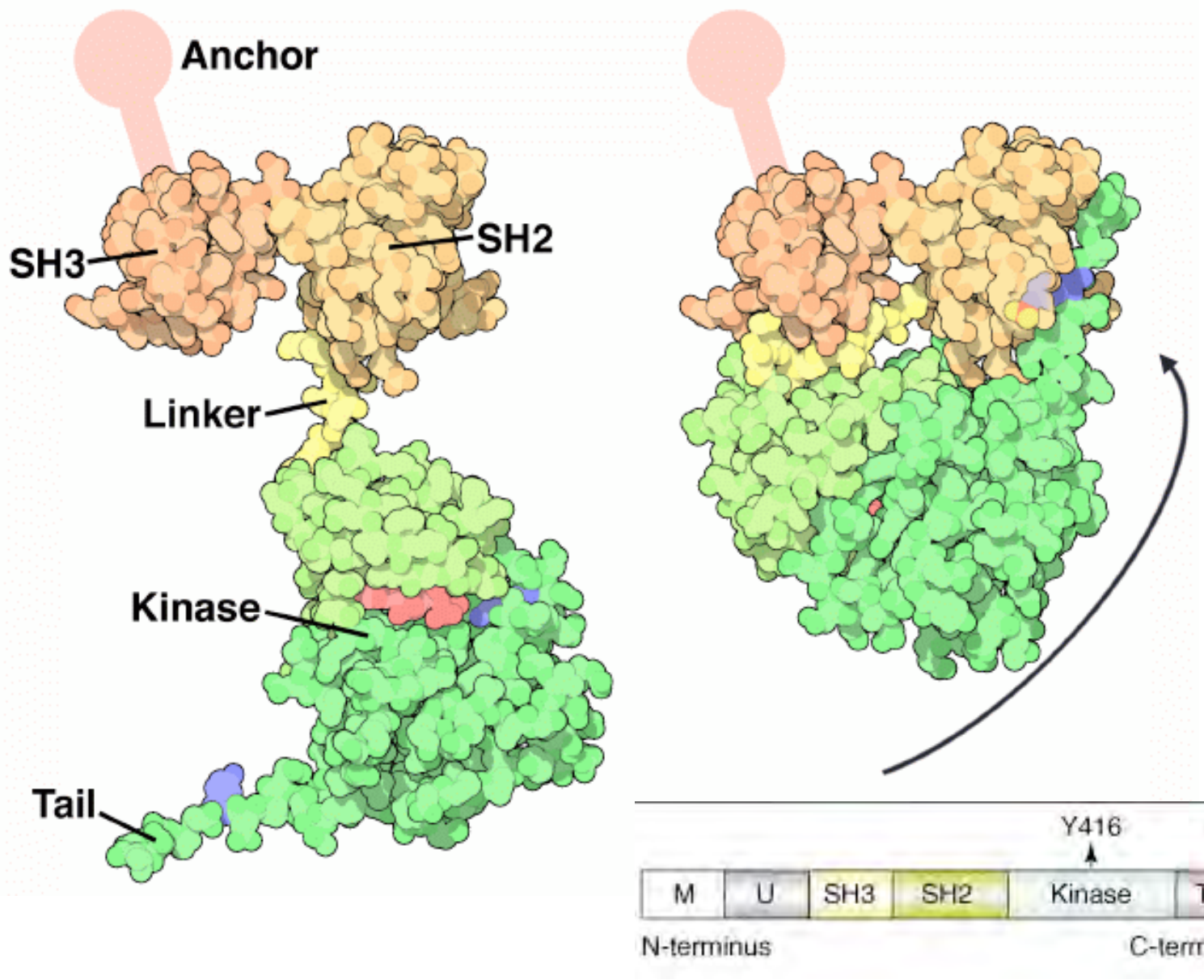
Western Blot with antibody that recognizes Tyr phosphorylated proteins

The structures of c-src and v-src provided an important clue!

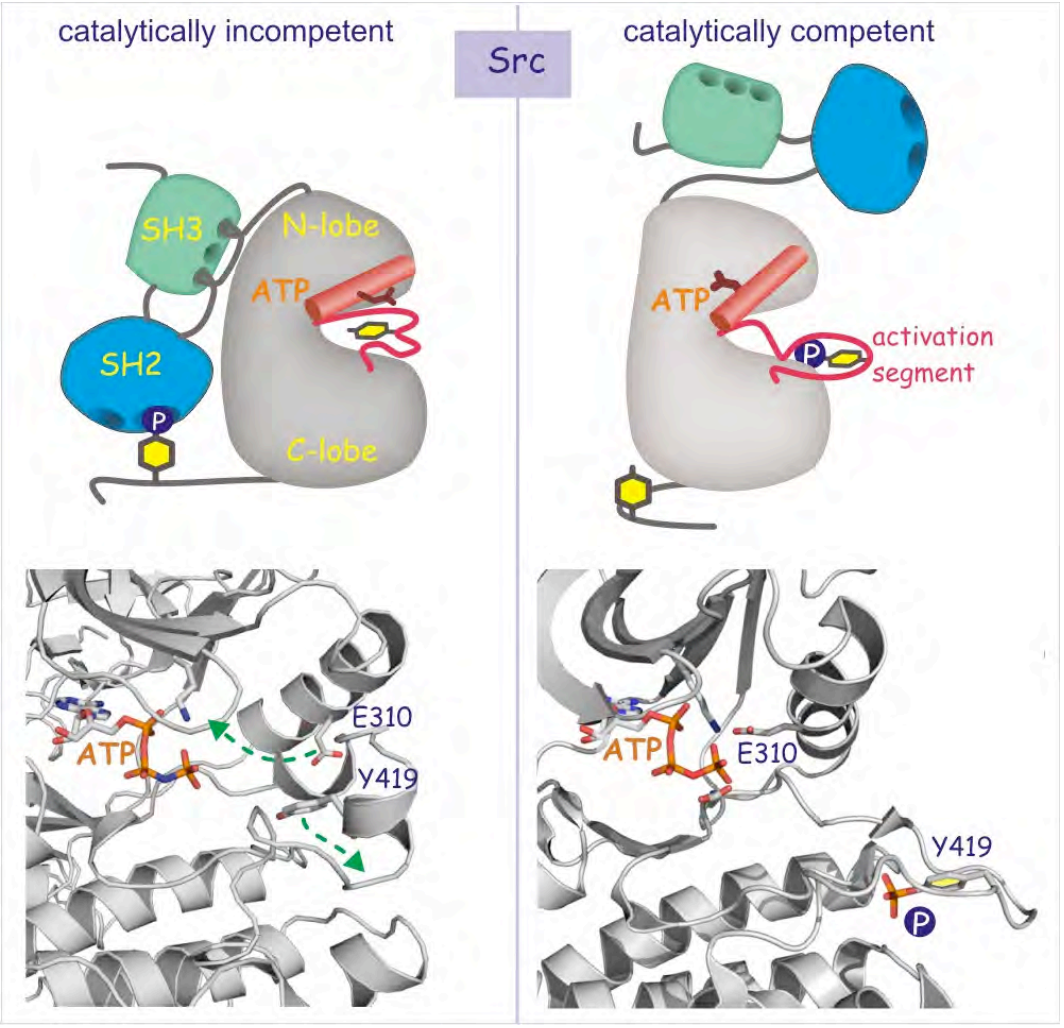


Lodish et al. Fig. 24-17

Tyrosine phosphorylation of the C-terminus creates an intramolecular and inhibitory interaction



Regulation of Src kinase activity

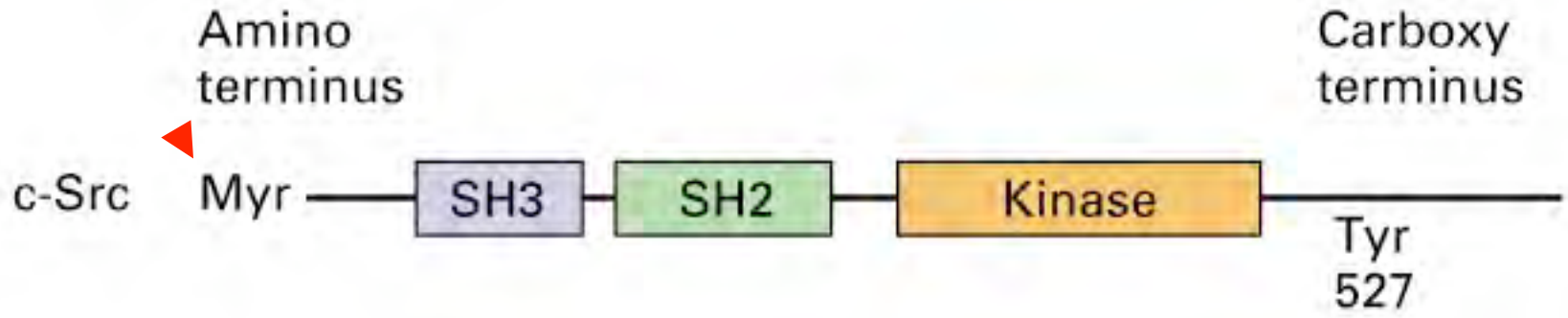


Phosphorylation of the C-terminal tyrosine of Src causes binding of its own SH2 domain. This event places the SH3 domain adjacent to the N-terminal lobe of the kinase domain which affects the coordination of ATP (orange). Detachment of the SH2 domain, through dephosphorhylation of the carboxy-terminal tyrosine (or through binding of the SH2 domain to tyrosine phosphates of other proteins) removes this restraint.

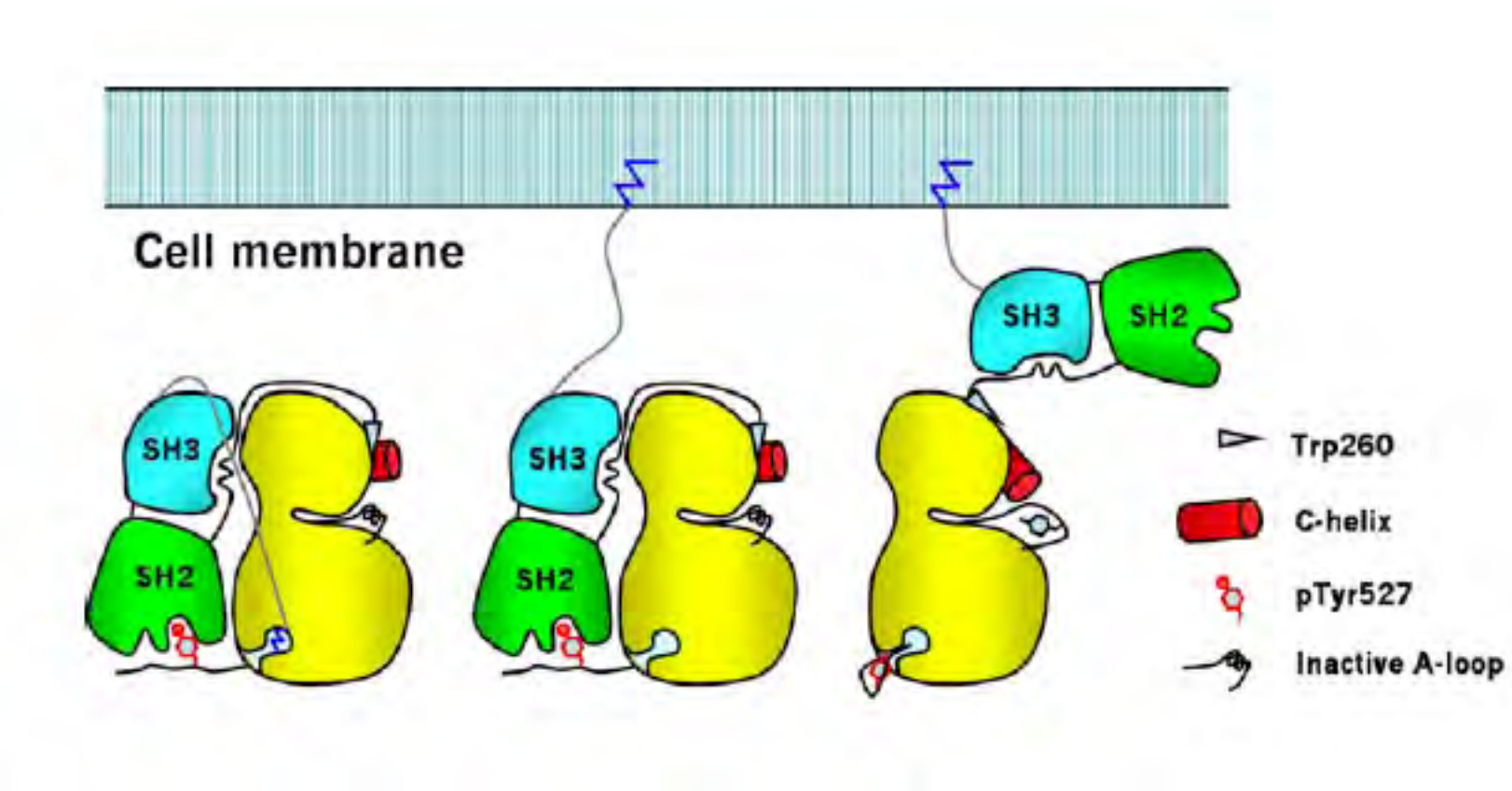
Subsequent phosphorylation of tyrosine-419 in the activation segment liberates the entry path for substrate; the protein kinase is now catalytically competent.

Where is Src within cells?

This is a covalently attached lipid: what might that mean?

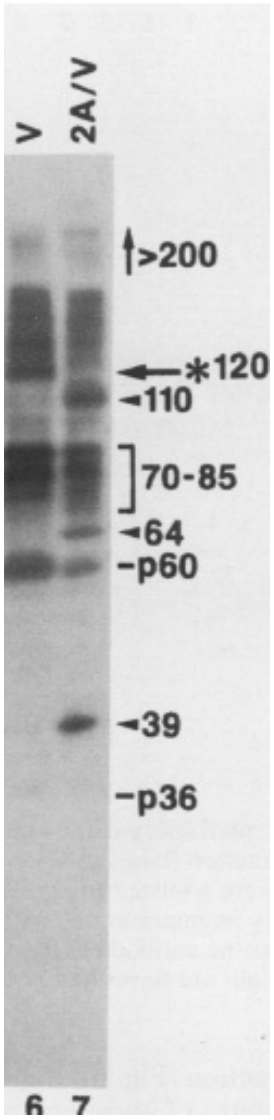


A more detailed model of Src activation



Cowen-Jacob et al. Structure 13, 861-871 (2005)

Identifying The Targets of Src: 1989…



Western blotting with anti-phosphotyrosine antibodies

V = v-Src transfected cells

2A/V = non-myristylated v-Src transfected cells

p120 catenin: modulates cell-cell adhesion

Reynolds et al. MCB (1989)

MOLECULAR AND CELLULAR BIOLOGY, Feb. 2002, p. 1203–1217 Vol. 22, No. 4
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.4.1203–1217.2002

Focal Adhesions Require Catalytic Activity of Src Family Kinases To Mediate Integrin-Matrix Adhesion

Leiming Li,[1,2] Masaya Okura,[1]† and Akira Imamoto[1,2,3]*

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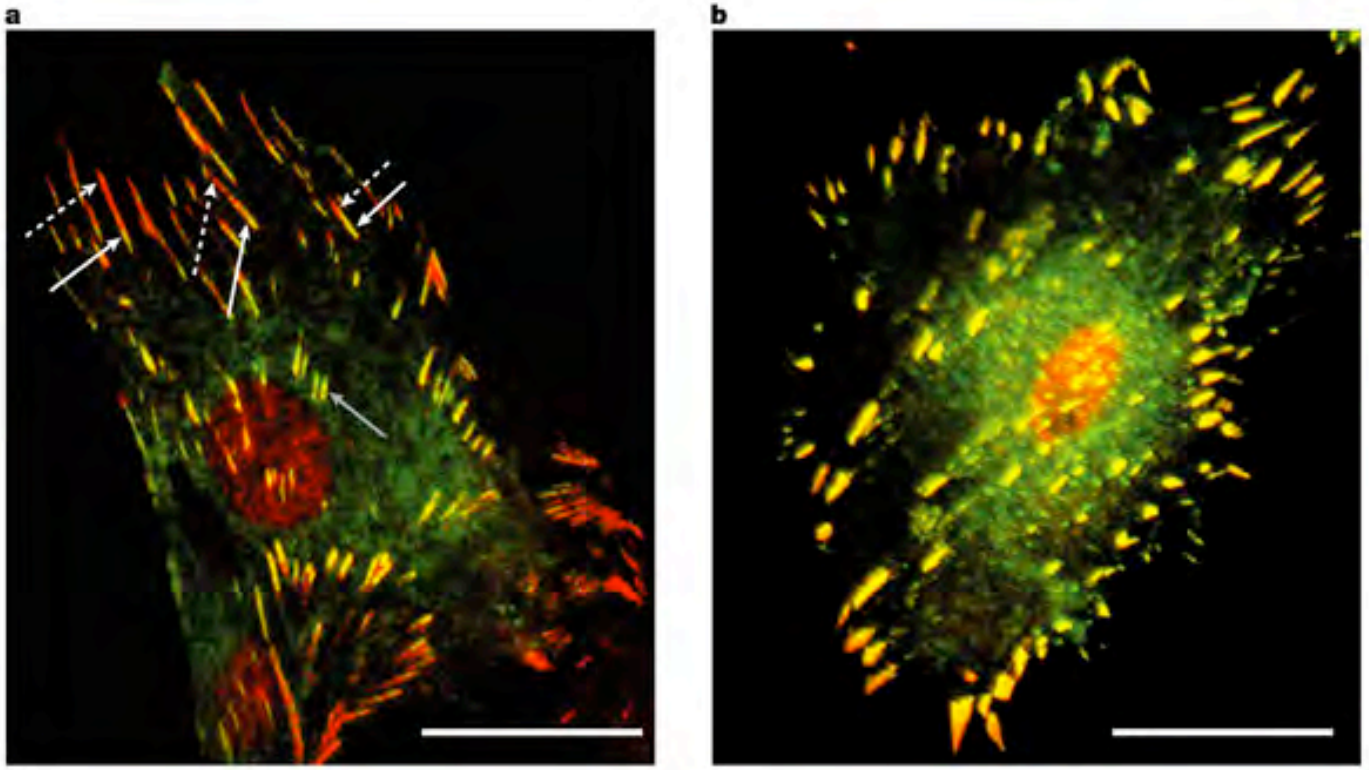
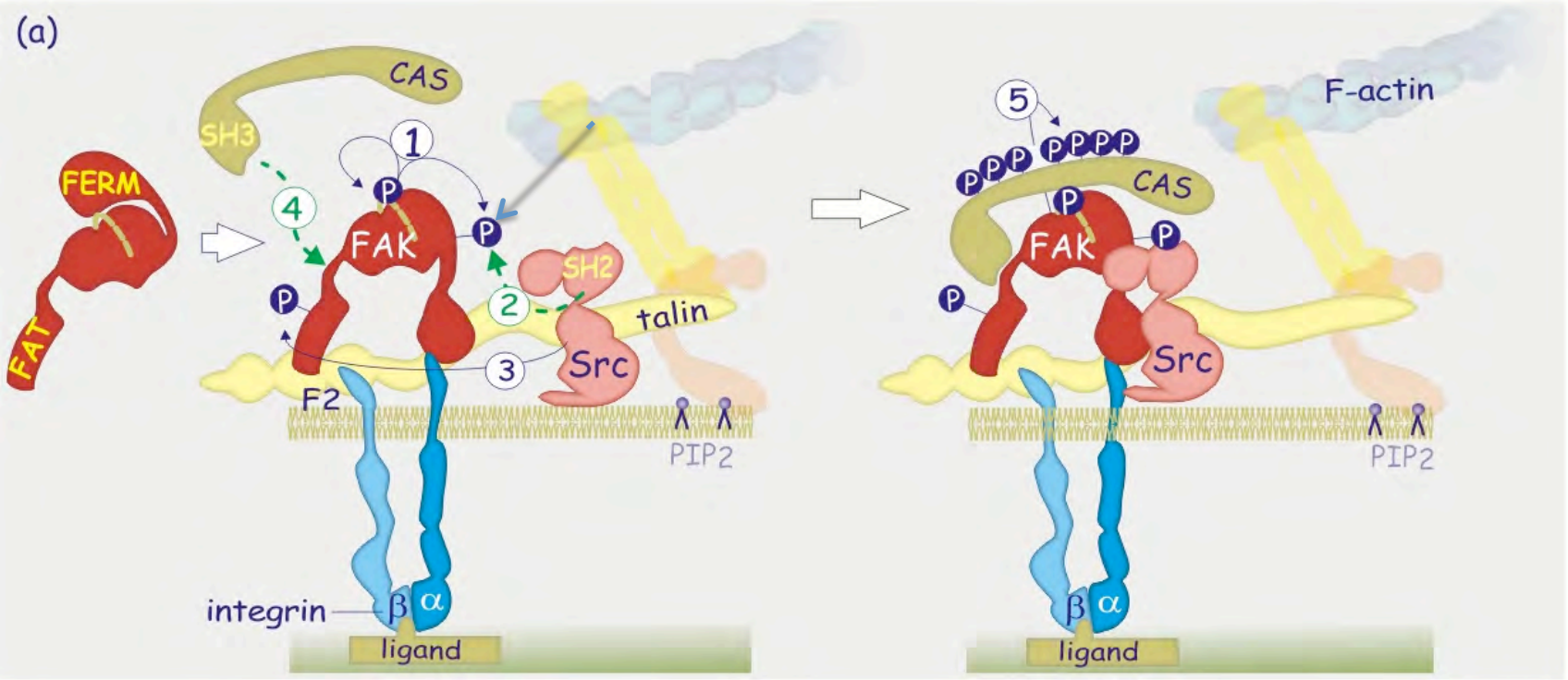
(A) In the case od inactive src, the focal adhesions are enlarged (at the termini of stabilized actin cables).
FAK is already at the membrane proximal region (broken arrow) and FAK–v-Src-KD is localized at the membrane-distal region.



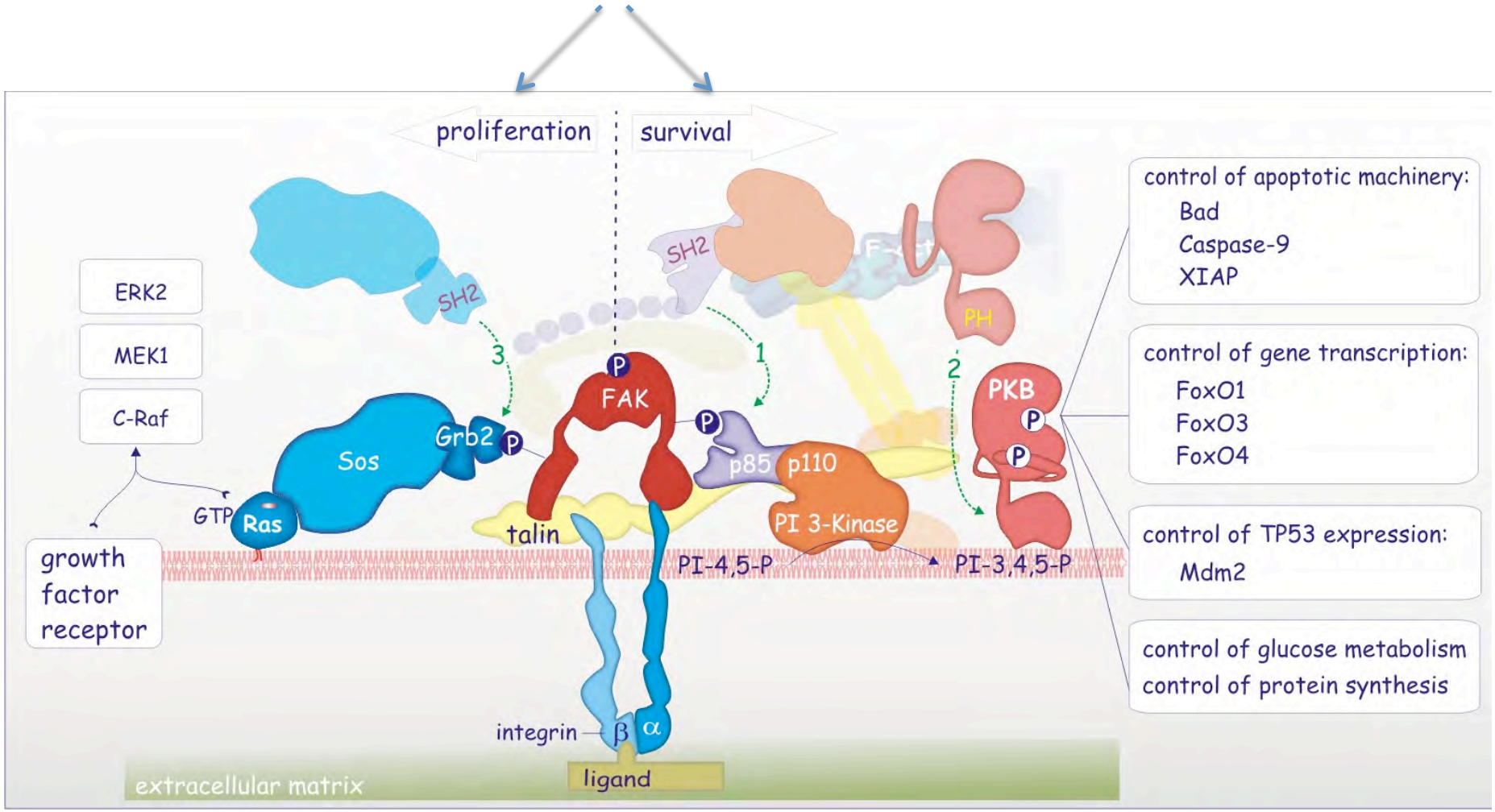
(B) FAK (red) and v-Src (green) co-localize in smaller adhesion structures at the cell periphery when v-Src is active, and focal adhesions (and the associated actin filaments) are dynamically regulated. These adhesion characteristics indicate that v-Src-KD impairs adhesion turnover and that focal-adhesion size is linked to Src-dependent dynamic regulation

Integrin signaling complex

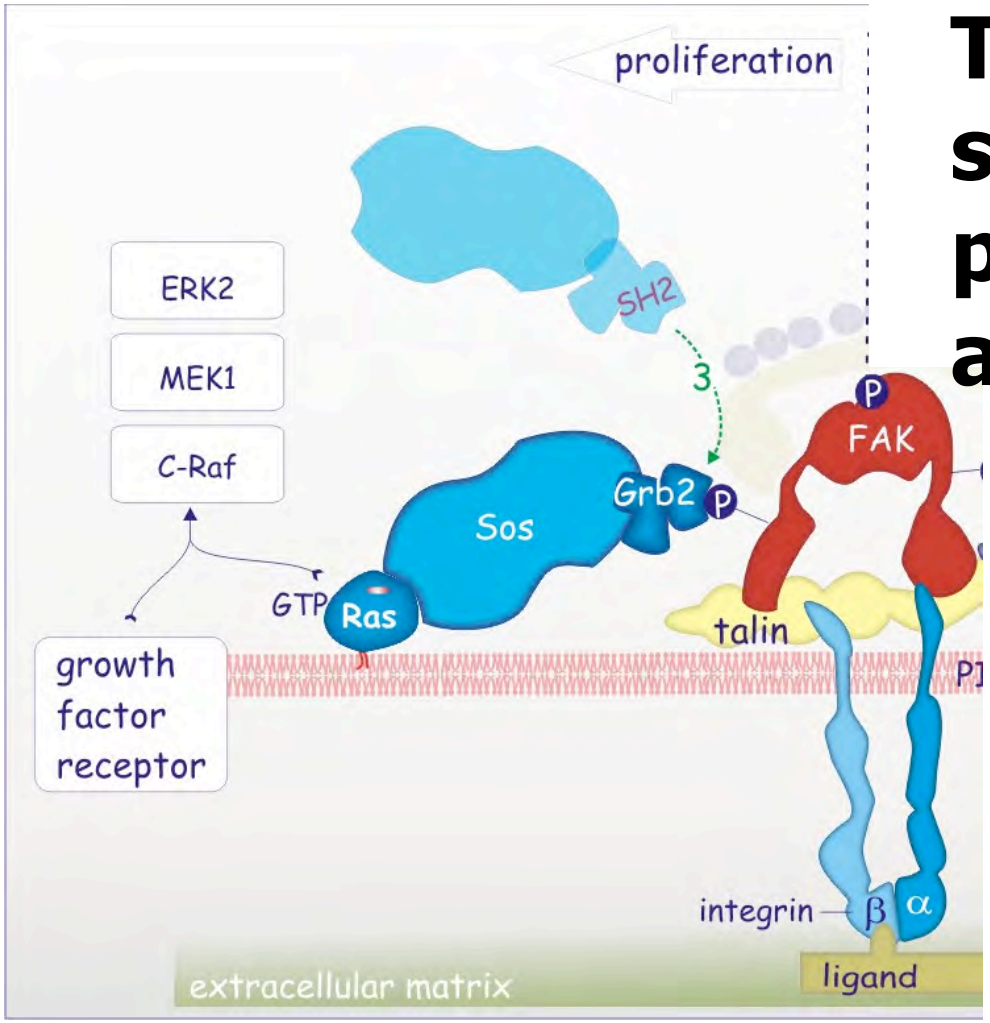


The focal adhesion kinase FAK associates with talin. Autophosphorylation of FAK then generates a docking site for the SH2 domain of Src which phosphorylates FAK at Y925. Src and FAK next phosphorylate the FAK-associated docking protein CAS at multiple sites.
An integrin-signalling complex is formed that acts in a manner similar to growth factor-receptor signaling complexes, i.e. attachment of adaptors and effectors and tyrosine phosphorylation substrates.

Integrin signaling



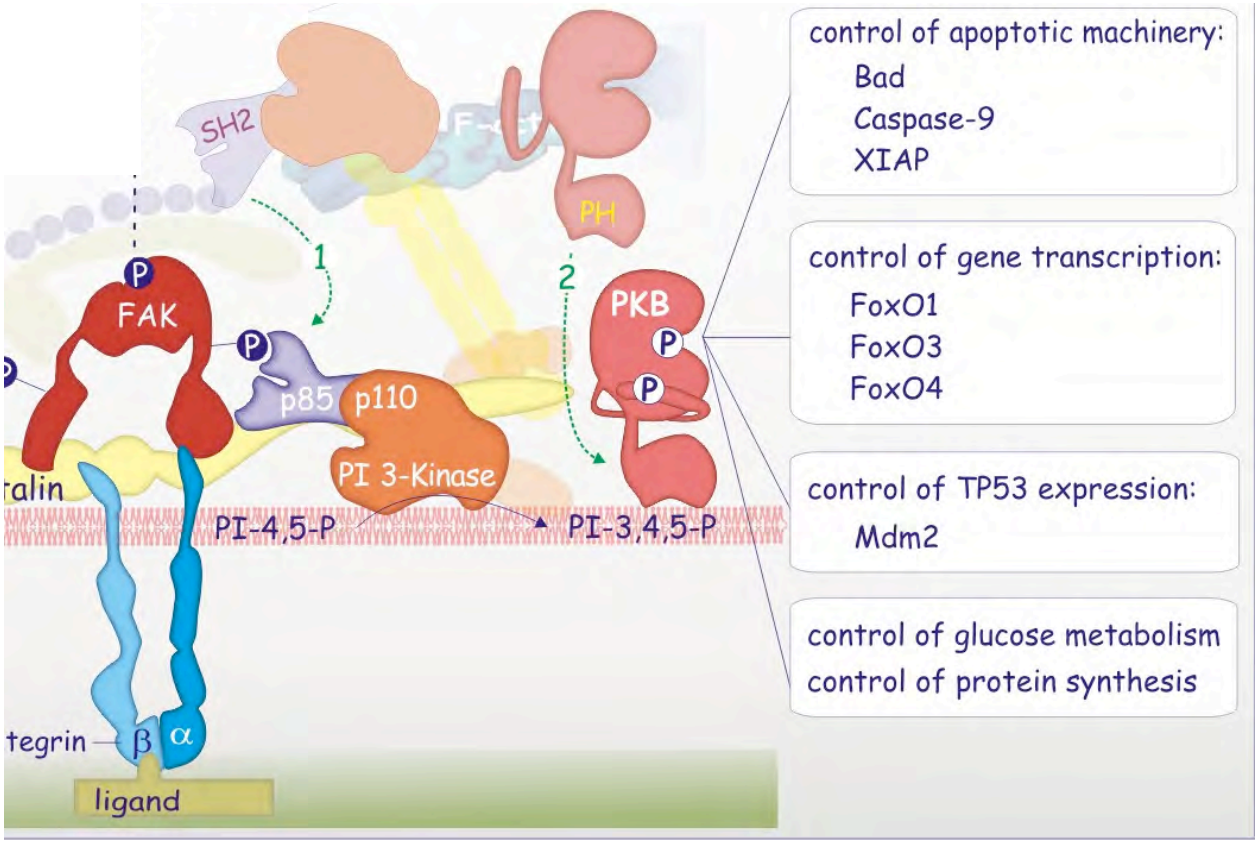
The focal adhesion site promotes cell proliferation through activation of Ras



Phosphorylated focal adhesion kinase (FAK) is a binding site for Grb2. This interaction recruits the Ras guanine exchange factor Sos, leading to activation of Ras.

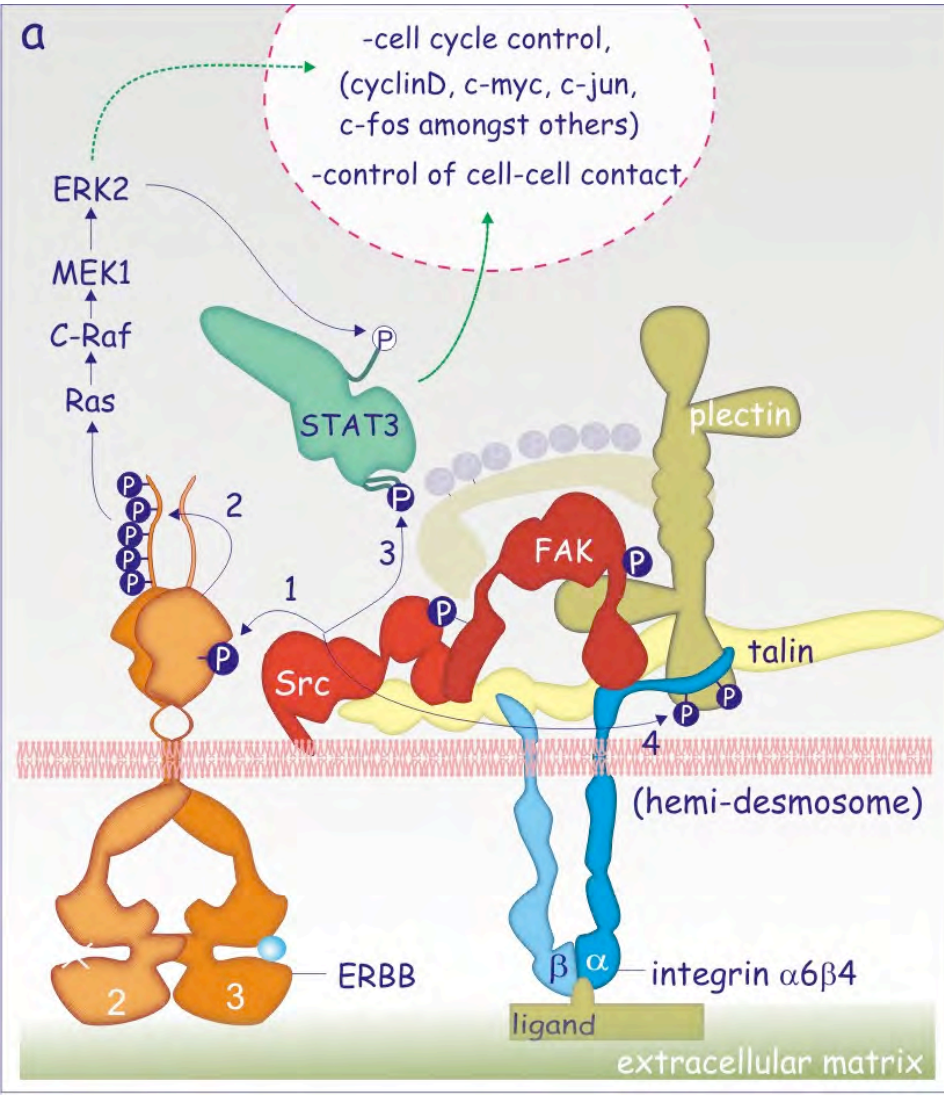
Ras-GTP initiates the activation of the Raf-ERK pathway, necessary for initiation of the cell cycle.

The focal adhesion site promotes cell survival through activation of PKB



Phosphorylated focal adhesion kinase (FAK) binds the SH2 domain of the regulatory subunit (p85) of PI 3-kinase. Subsequent production of PIP3 provides a binding site for PKB (and PDK1). After its activation PKB phosphorylates a large number of proteins that directly or indirectly deal with cell death.

Adhesion-mediated cell cycle control



In epithelial cells, integrin α6β4, forms a special adhesion complex named *hemi-desmosome*. These complexes are linked to intermediate filaments via **plectin**.

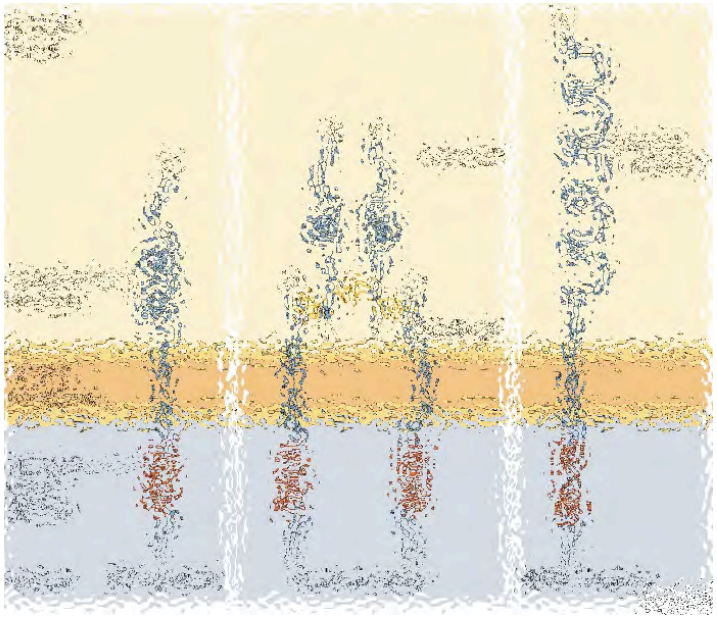
ERBB2/3 receptors are recruited into these complexes leading to phosphorylation of ERBB2 by Src bound to FAK.

Src also phosphorylates STAT3 and this signal is enforced by a second phosphorylation on serine through ERK2. Both phosphorylations enhance its transcriptional activity.

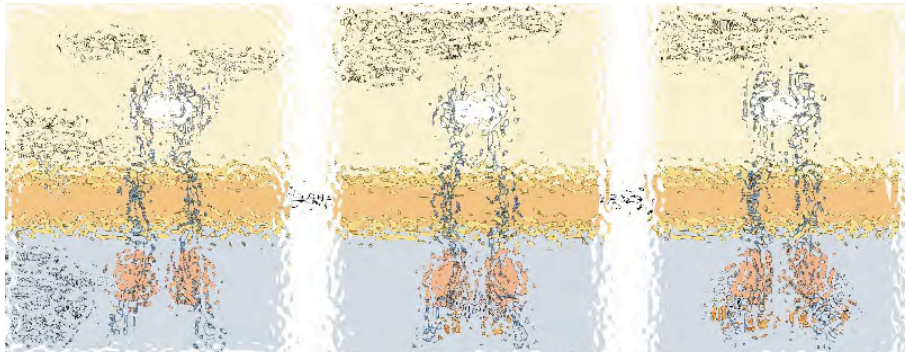
In the case of breast tumor cells, this pathway promotes cellular invasion.

Enzyme-linked receptors fall into 3 categories:

- **Tyrosine Kinase Receptors**
  - Not only a receptor
  - Also an enzyme: Tyrosine kinase



- **Cytokine superfamily receptors**
  - No catalytic domain
  - Interact with non receptor protein-tyrosine kinases
    - Src family
    - JAK family



- **TGF-β receptors**

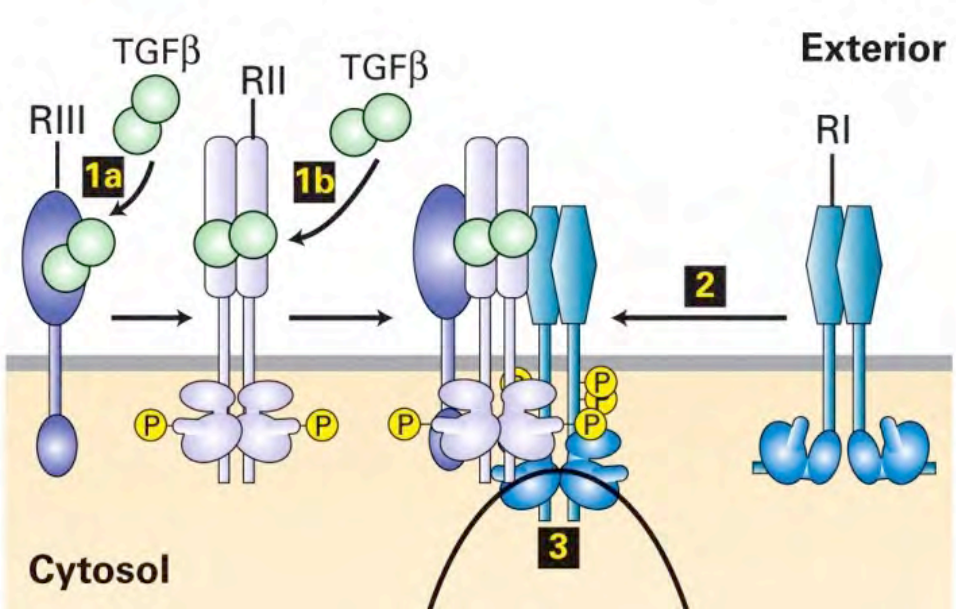
TGFβ

- TGFβ (Transforming Growth Factor β) superfamily proteins play important roles in regulating development of vertebrates and invertebrates
  - Bone Morphogenic Protein (BMP) is one of the TGFβ superfamily important in regulating formation of mesoderm and the earliest blood forming cells
  - GDF11
  - TGFβ-1 is another member of the TGFβ superfamily proteins which can induce a transformed phenotype of certain cells in culture
- There are three human TGFβ isoforms known to have potent anti-proliferative effects on many types of mammalian cells. Mutation of TGFβ will result in releasing cells from growth inhibition (frequently occurs in human tumors)
- TGFβ also promotes expression of cell-adhesion molecules and extracellular matrix molecules

TGFβ Signaling Receptors Have Serine/Threonine Kinase Activity

- Three different polypeptides with apparent molecular weights of 55, 85 and 280 kDa were purified, referred to as types RI, RII and RIII TGFβ receptors
- Type RIII TGFβ receptor is a **cell-surface proteoglycan**, also called **β-glycan** which bind and concentrate TGFβ near the cell surface
- Type RI and type RII receptors are dimeric transmembrane proteins with serine/threonine kinases as part of their cytosolic domains

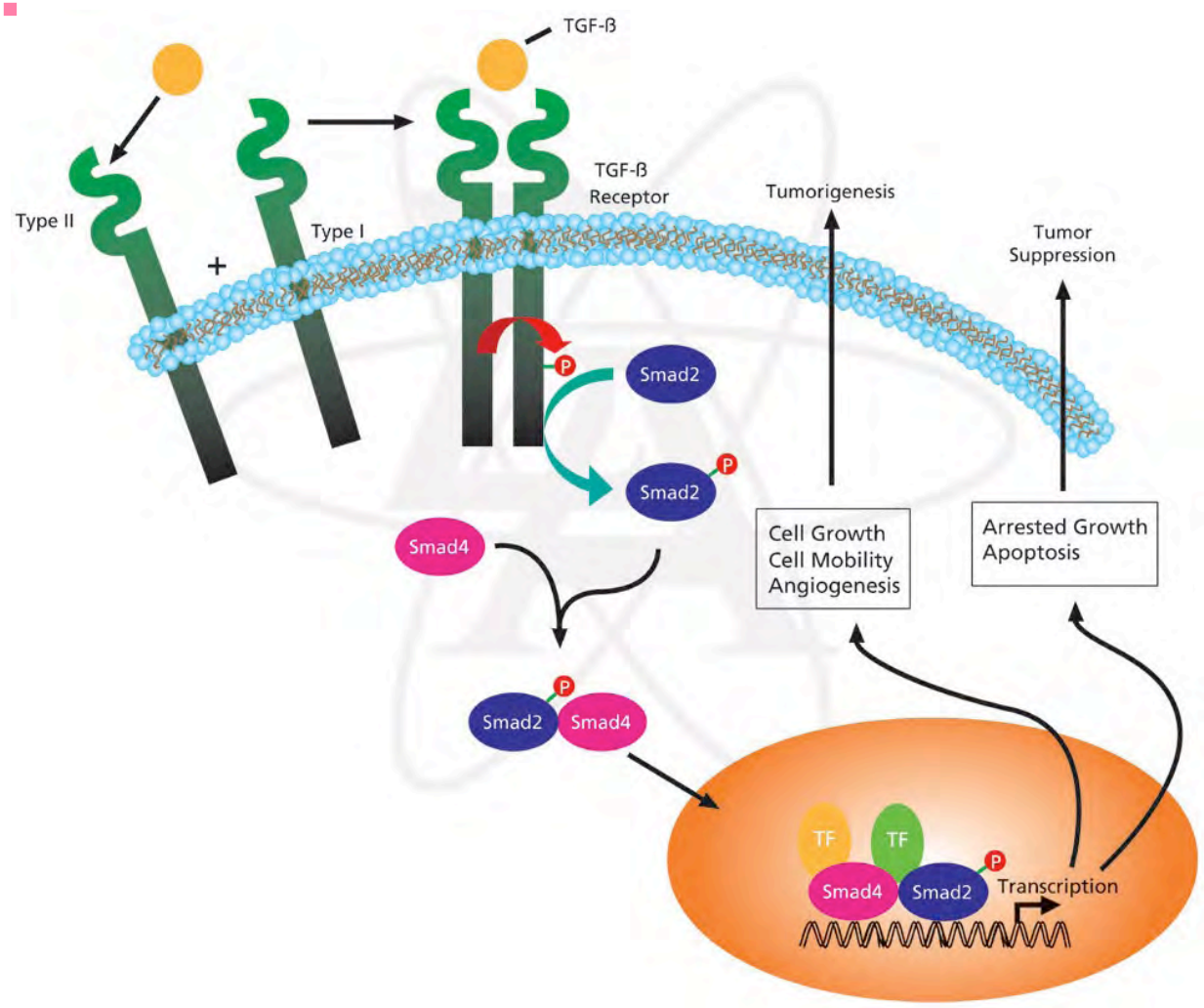
- RII is a constitutively active kinase that phosphrylates itself in the absence of TGFb
- Binding of TGFβ induces the formation of two copies each of RI and RII. A RII then phophorylates serine/threonine of RI adjacent to the cytoplasm and thus activate the RI kinase activity



TGFβ Receptor Signaling:
a logic resembling the STAT-family

STAT-related logic

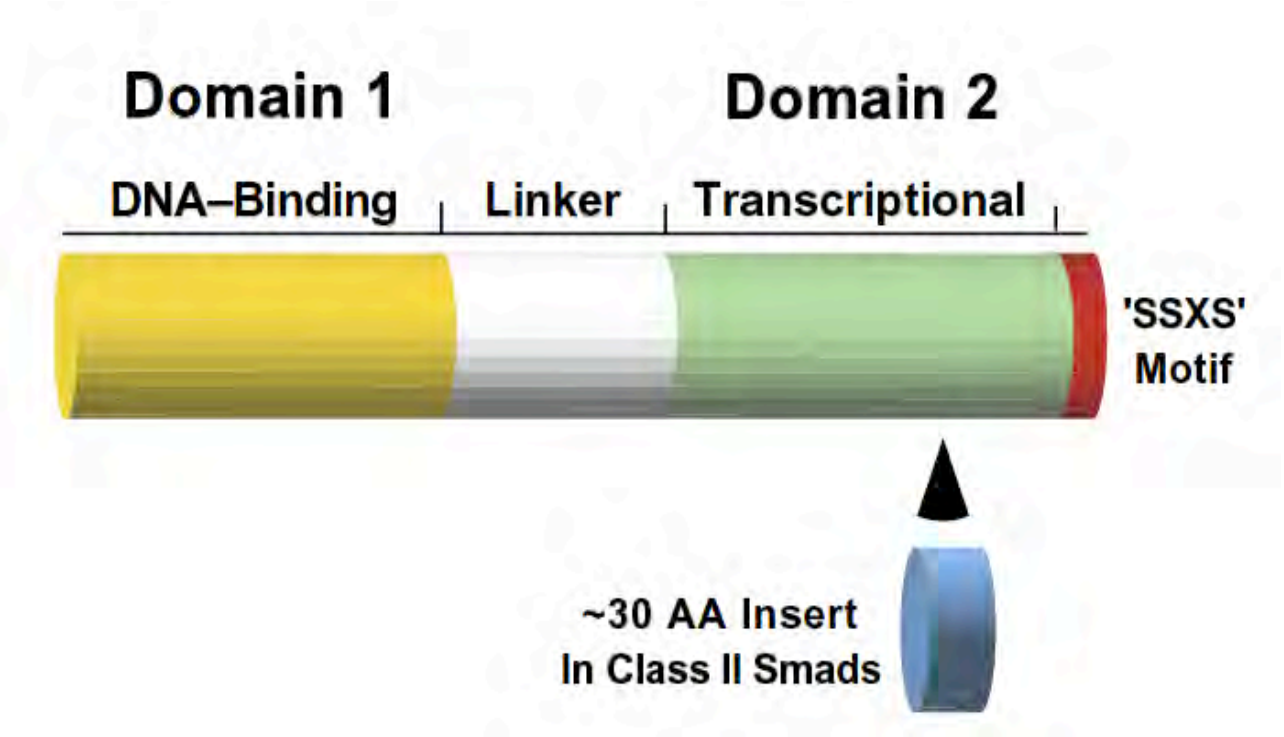
- TGFß-receptors are activated by binding of ligand (TGFb).
- Activated receptor kinases phosphorylate specific Smad-factors
- Phosphorylated Smad-factors associate with a common Smad-factor (Smad4)
- The generated heteromeric complexes migrate to the nucleus as transcription factors



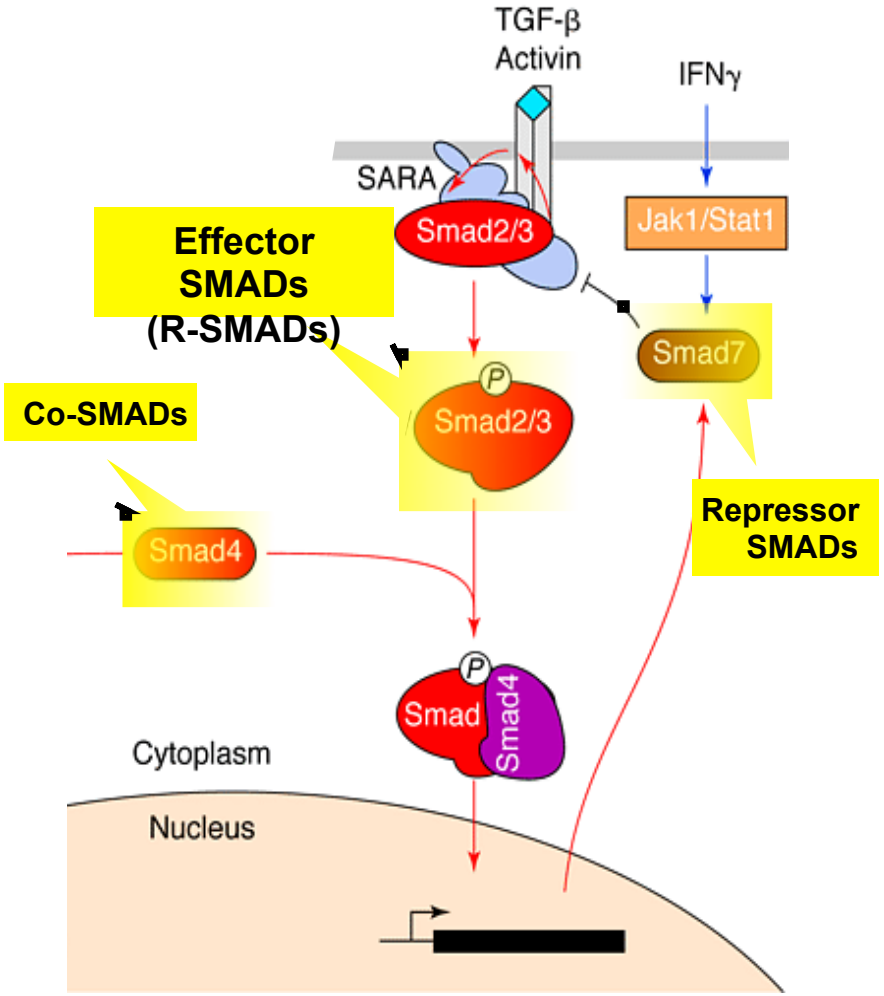
Classification

- Smad-factors: design and classification

  - Nine different Smad-factors identified in vertebrates
  - common conserved domains: N-terminal MH1-domain (DBD) + C-terminal MH2-domain



Three groups of SMADs

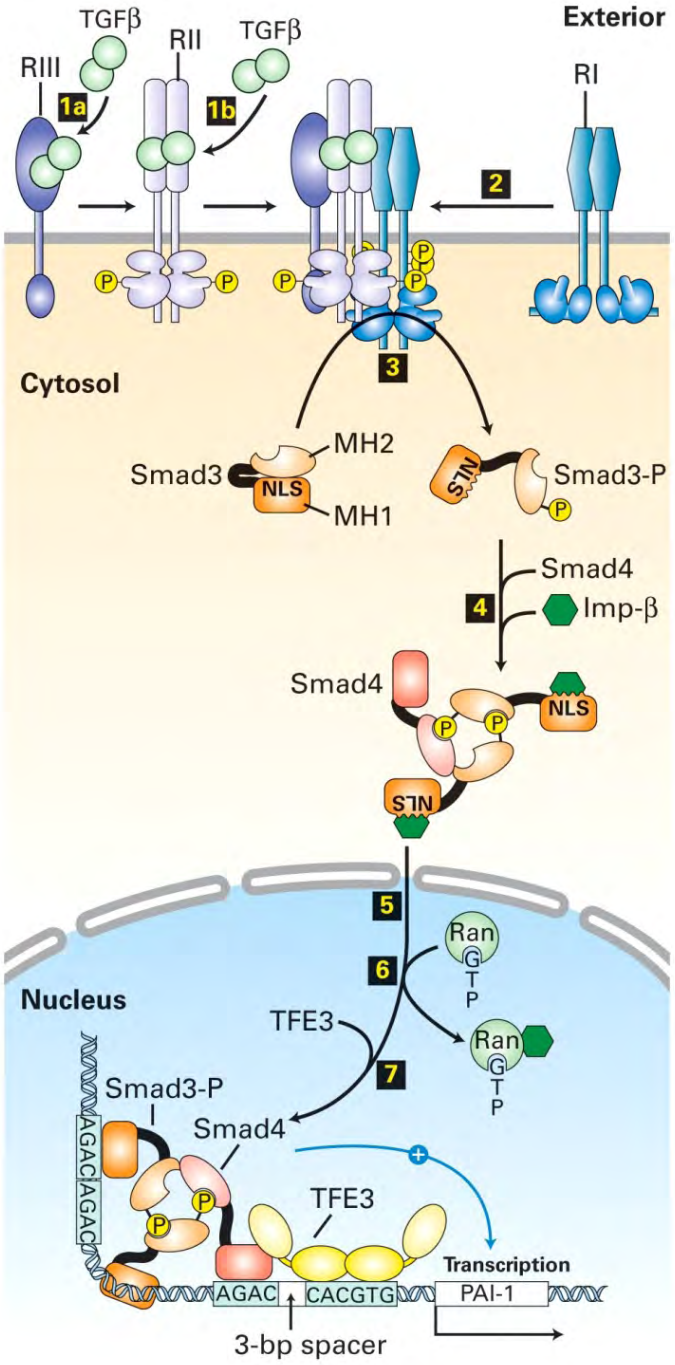


1: **Effector SMADs** (also called the Receptor-SMADs) are ser-phosphorylated by the activated receptor. Smad1, 5, 8, 9 are phosphorylated in response to bone morphogenetic morphogenetic protein (BMP) and growth and differentiation factor (GDF); Smad2 and 3 are phosphorylated in response to the activin/nodal branch of the TGF-β pathway.

2: **Regulatory or co-SMADs** (common SMADs). They are Smad4 and Smad4β.

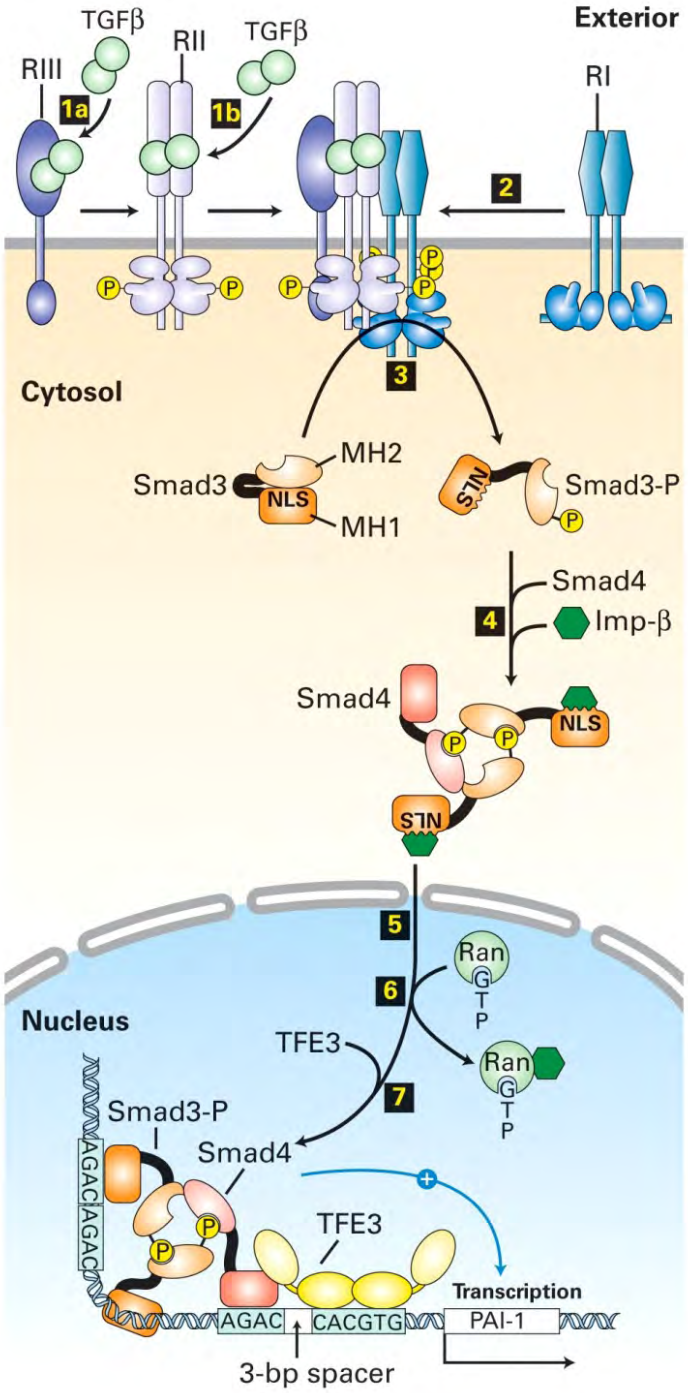
The regulatory Smad4 binds to all effector SMADs in the formation of transcriptional complexes, but it does not appear to be required for nuclear translocation of the effector molecules.

3: Two **inhibitory SMADs**, Smad6 and Smad7, provide negative regulation of the pathway by blocking Smad4 binding.



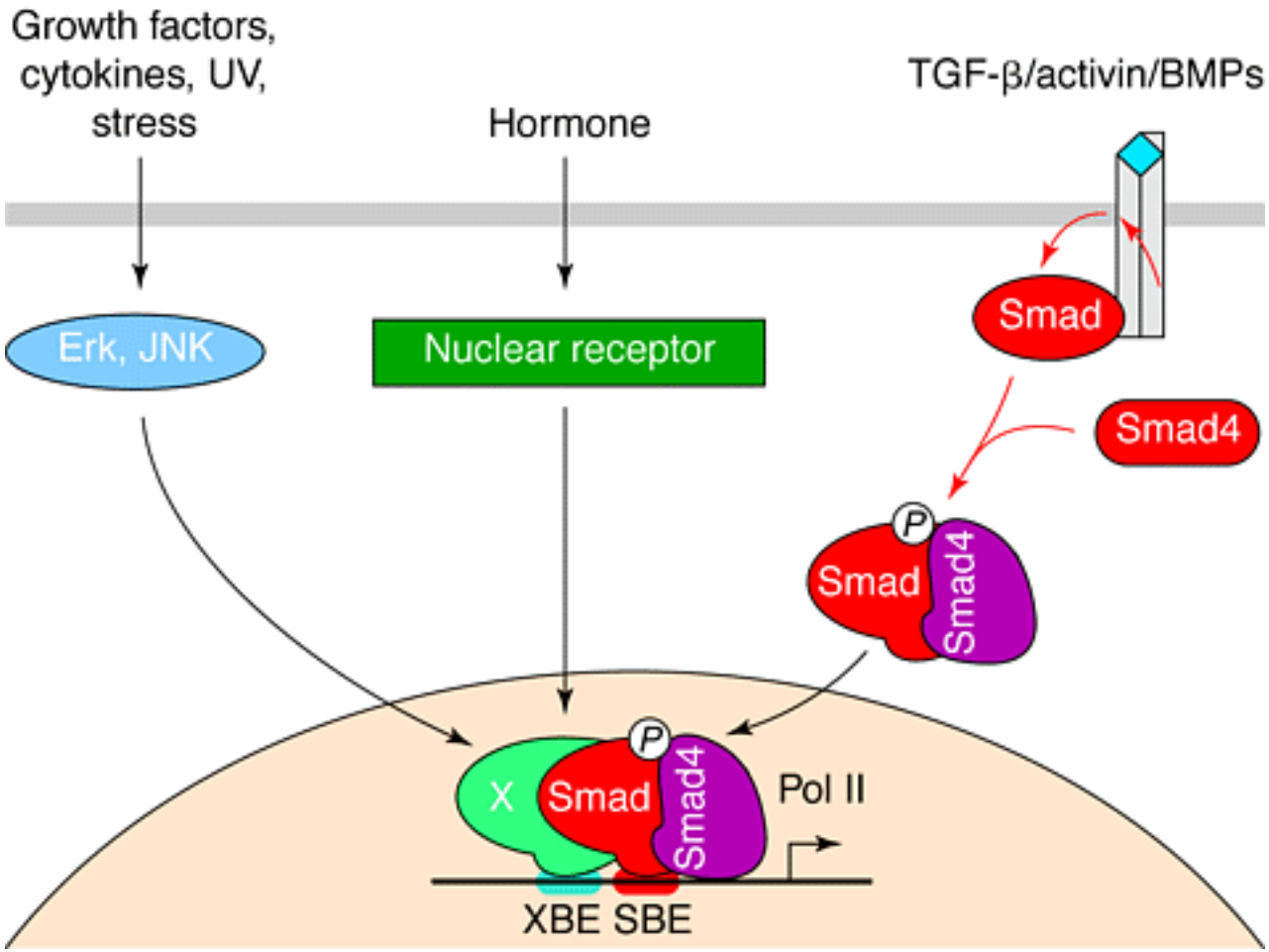
Activated Type I TGFβ Receptors Phosphorylate Smad Transcription Factors

- R-Smads contain two domains, MH1 and MH2, separated by a flexible linker region. The N-terminus of the MH1 contains a specific DNA binding segment and a NLS sequence
- When R-Smads are in inactive state, the NLS is masked and the MH1 and MH2 domains associate in a way that they can not bind to DNA or to a co-Smad
- Phosphorylation of three serine residues near the C-terminus of a R-Smad (Smad2 or Smad3) by activated type I TGFβ receptors separates the domains, allowing binding of importin β to the NLS

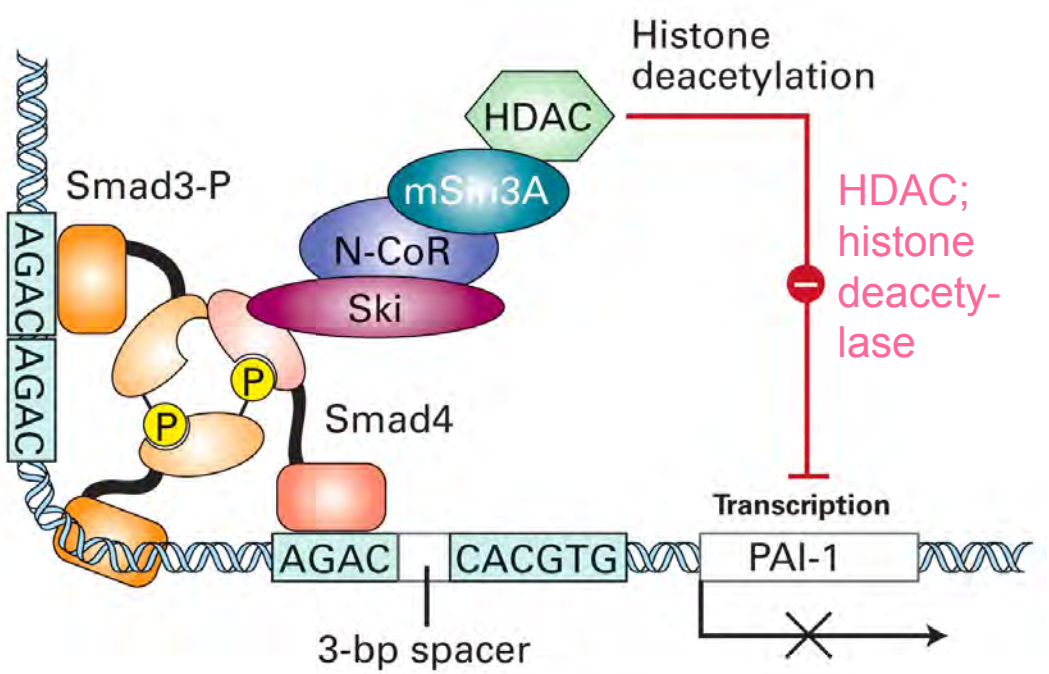


- A complex containing two molecules of Smad3 (or Smad2) and one molecule of a co-Smad (Smad4) forms in the cytosol
- The complex is stabilized by binding two phosphorylated serines in both the Smad3 and the Smad4 MH2 domains
- The importin β–bound heteromeric R-Smad3/ Smad4 complex translocates into nucleus
- After importin β dissociates from the complex in the nucleus, the Smad2 (or 3)/Smad4 will cooperate with other transcription factors to turn on specific target genes
- In the nucleus, R-Smads are continuously dephosphorylated, resulting in the dissociation of the R-Smad /co-Smad complex and export of these Smads from the nucleus. Therefore, the concentration of the active Smads in the nucleus closely reflects the levels of the activated TGFβ receptors on the cell surface

The Smad-factors activate their target genes in combination with other TFs



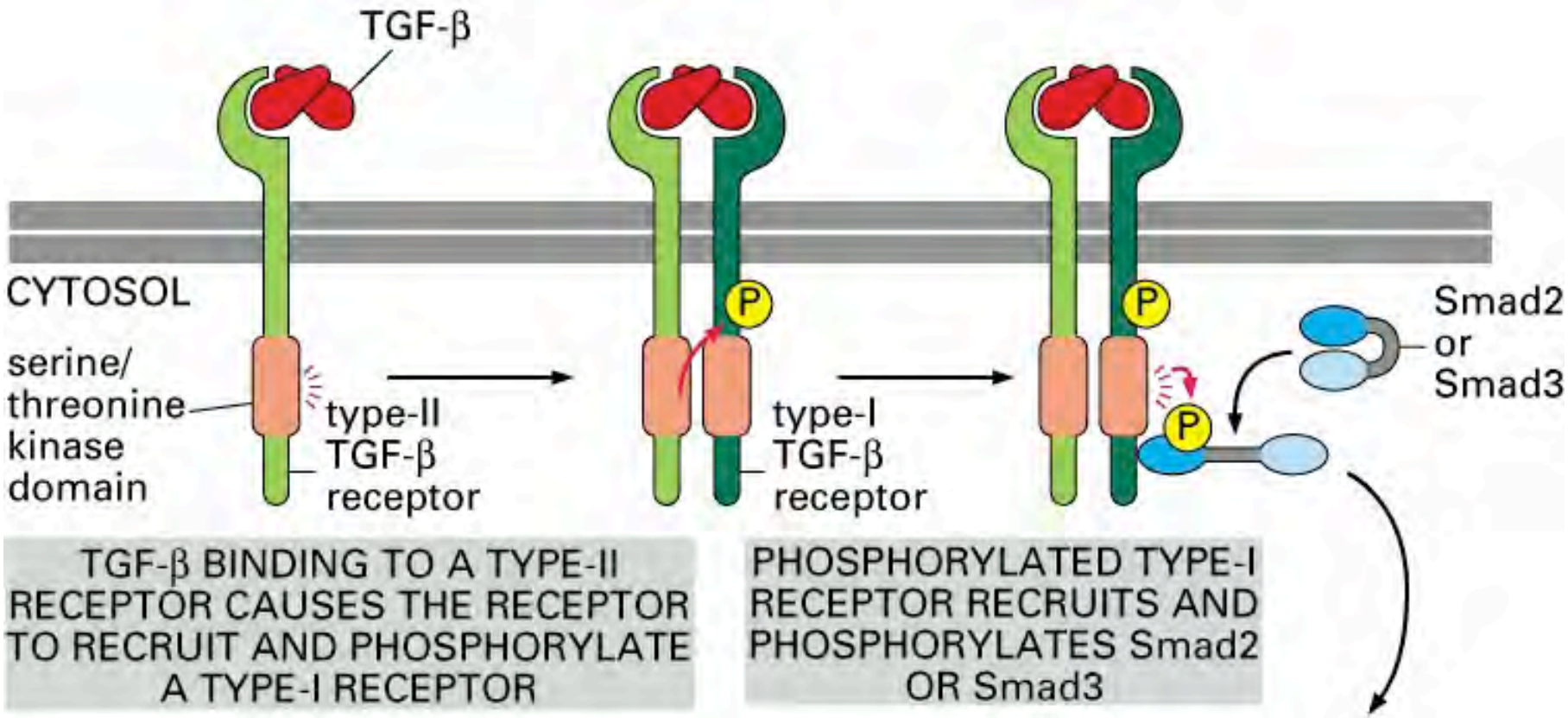
Oncoproteins and I-Smads Regulate Smad Signaling via Negative Feedback Loop



- Smad signaling is regulated by additional intracellular proteins including *SnoN and Ski (Ski stands for "Sloan-Kettering Cancer Institute")*
- These proteins are oncoproteins since they cause abnormal cell proliferation when over expressed in cultured fibroblasts

- SnoN and Ski can bind to Smad2/Smad4 or Smad3/Smad4 complex after TGFβ stimulation
- Binding of SnoN and Ski to Smad2/Smad4 or Smad3/Smad4 will block transcriptional activation of target genes, making cells resistant to growth inhibition induced by TGFβ
- PAI-1 gene: encodes plasminogen activator inhibitor-1

Summary


TGF-β
CYTOSOL
serine/
threonine
kinase
domain
type-II
TGF-β
receptor
P
type-I
TGF-β
receptor
P
P
Smad2
or
Smad3
TGF-β BINDING TO A TYPE-II RECEPTOR CAUSES THE RECEPTOR TO RECRUIT AND PHOSPHORYLATE A TYPE-I RECEPTOR
PHOSPHORYLATED TYPE-I RECEPTOR RECRUITS AND PHOSPHORYLATES Smad2 OR Smad3

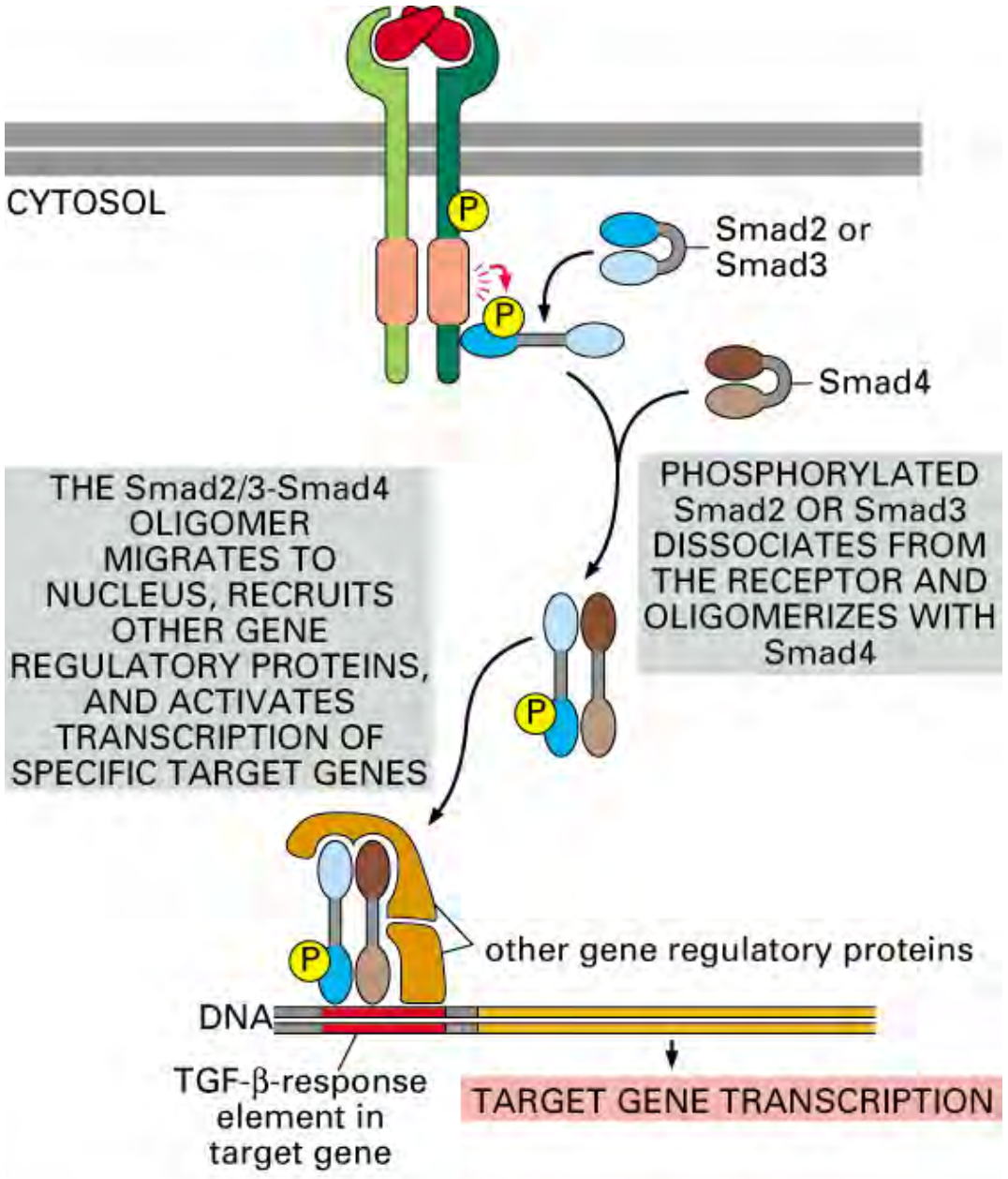
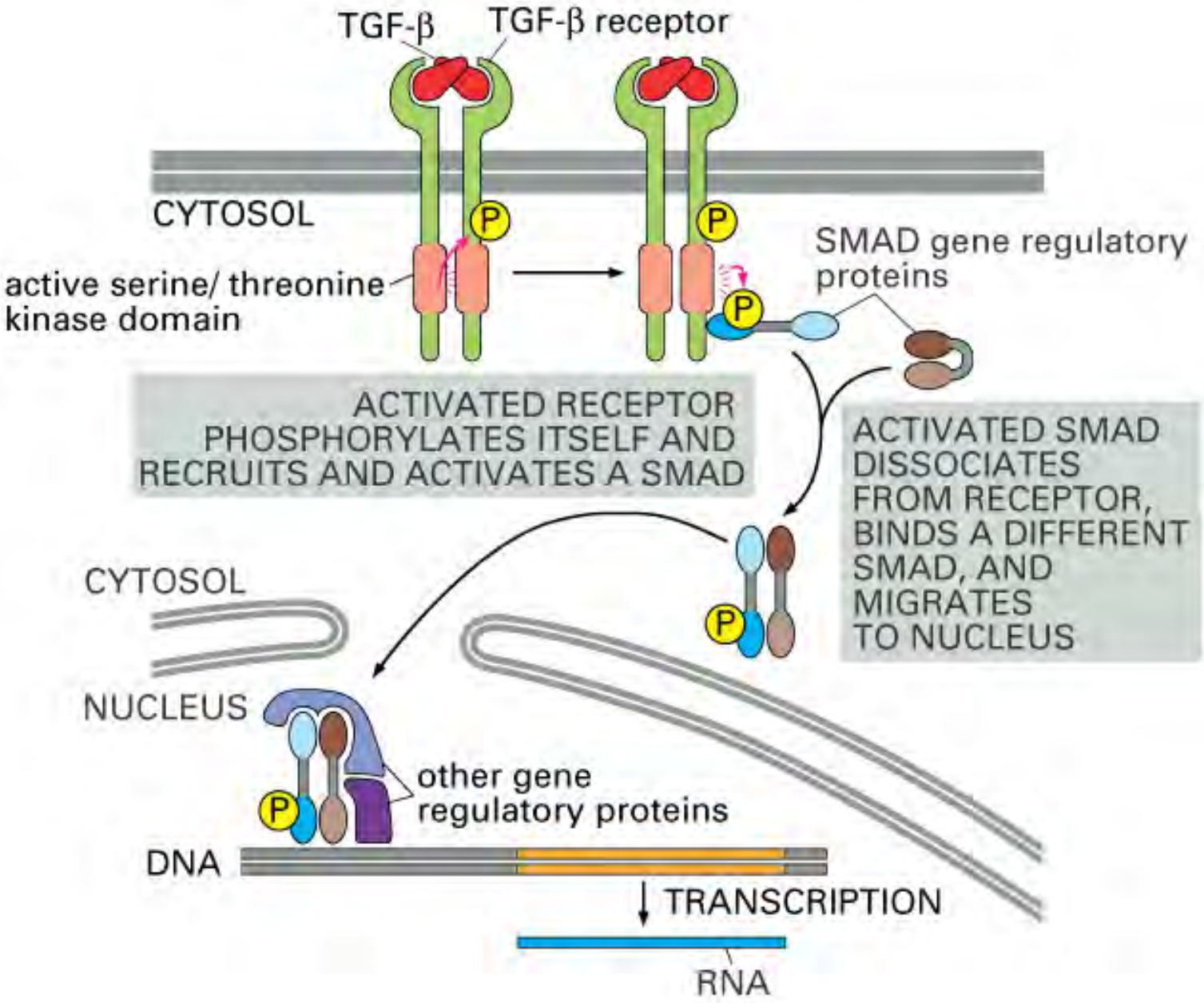


Figure 15–65 part 2 of 2. Molecular Biology of the Cell, 4th Edition.


TGF-β
TGF-β receptor
CYTOSOL
active serine/ threonine kinase domain
P
SMAD gene regulatory proteins
ACTIVATED RECEPTOR PHOSPHORYLATES ITSELF AND RECRUITS AND ACTIVATES A SMAD
ACTIVATED SMAD DISSOCIATES FROM RECEPTOR, BINDS A DIFFERENT SMAD, AND MIGRATES TO NUCLEUS
CYTOSOL
NUCLEUS
other gene regulatory proteins
DNA
TRANSCRIPTION
RNA