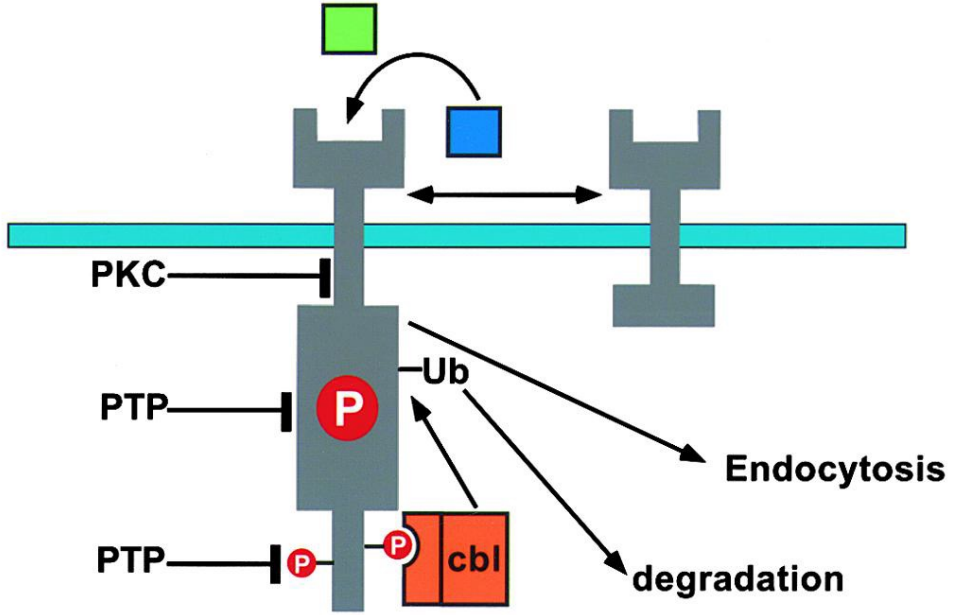
Negative Signalling

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

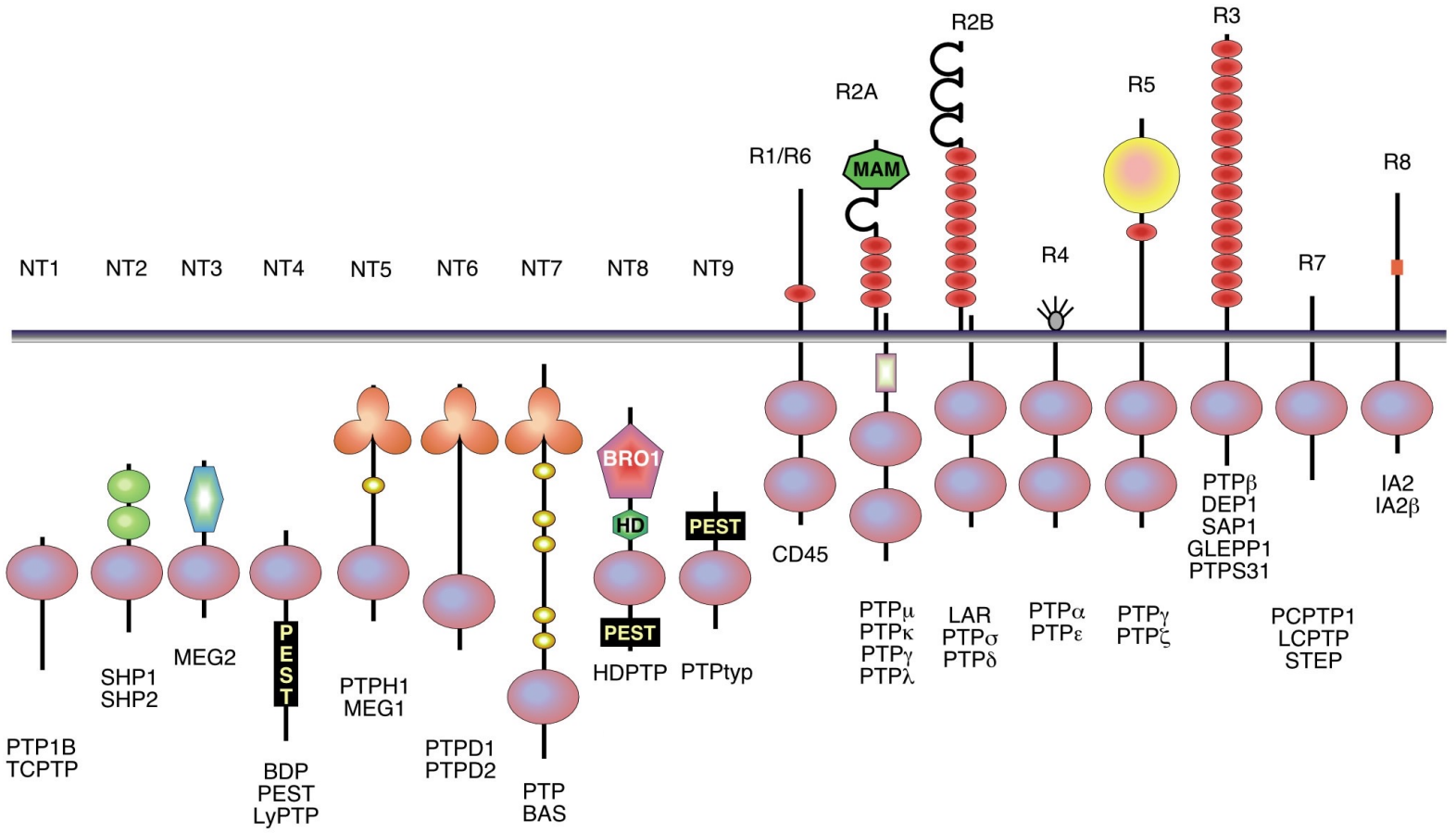
Mechanisms for Attenuation & Termination of RTK Activation



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



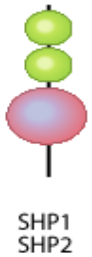
Andersen et al., Mol Cell Biol, 21, 7117, 2001

Functional Diversity Through Targeting and Regulatory Domains

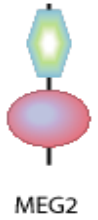
C-terminal
- ER targeting
- Proteolytic cleavage

Proline rich segment
- SH3 binding sites

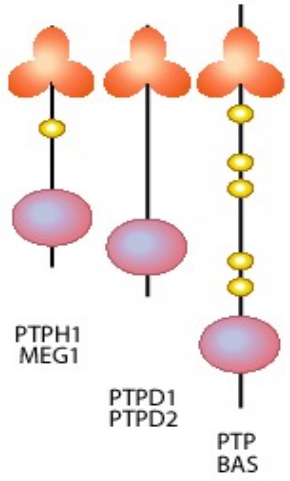
Alternative splicing
- Nucleus vs Cytoplasmic



SH2 domains
- Plasma membrane signaling complexes
- Auto-inhibition

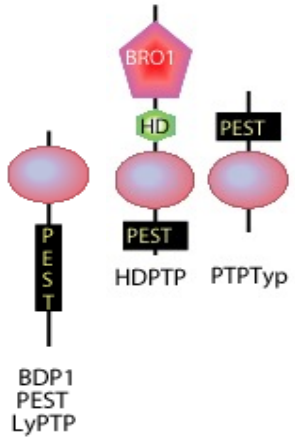


Cellular retinaldehyde binding protein-like
- Golgi targeting
- Secretory vesicles
- Putative lipid-binding domain



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

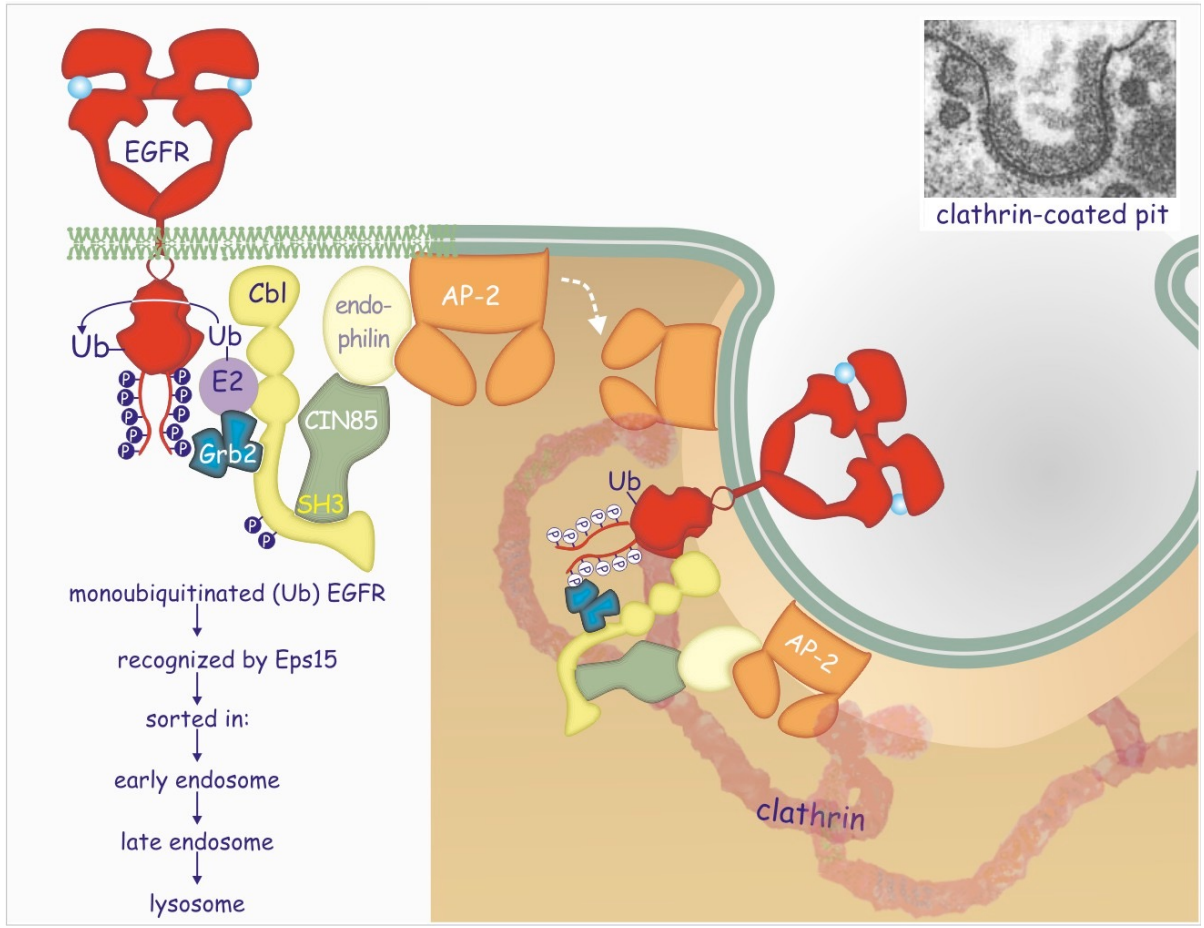
PTEN	Tumor Suppressor	Mutated in various human cancers. Cowden disease
DEP1	Tumor suppressor	Colon cancer susceptibility locus Scc1 (QTL in mice)
PTPκ	Tumor Suppressor	Primary CNS lymphomas
SHP2	Noonan Syndrome Stomach Ulcers	Developmental disorder affecting 1:2500 newborn Target of *Helicobacter pylori*
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.

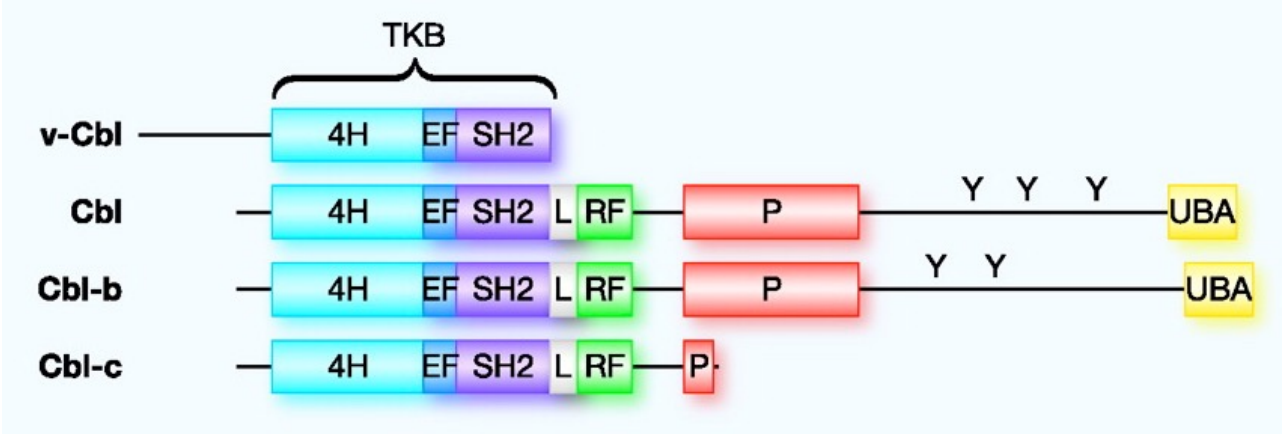
The complex then recruits clathrin monomers. As a result, active EGFRs accumulate in clathrin-coated membrane pits which then pinch off from the plasma membrane as endocytic vesicles. Within the intracellular network of vesicular transport pathways, the receptors are sorted into a pathway that takes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

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

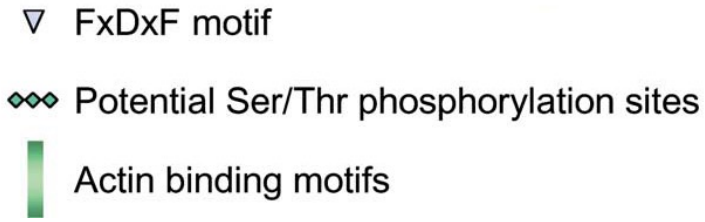
CIN85 (Cbl-interacting protein of 85 kDa) is an adaptor protein.

Minireview

CIN85/CMS family of adaptor molecules

Ivan Dikic*

CIN85
(Ruk, SETA, SH3KBP1)



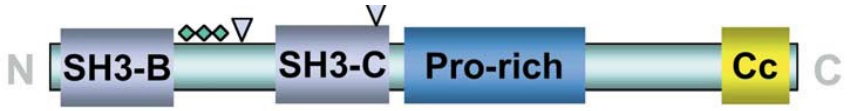
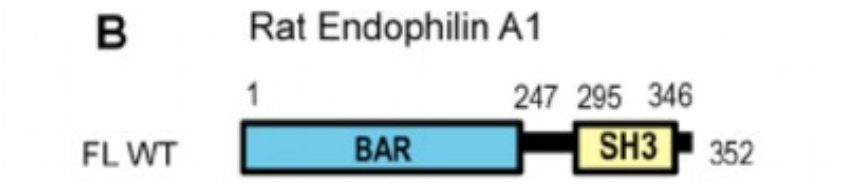
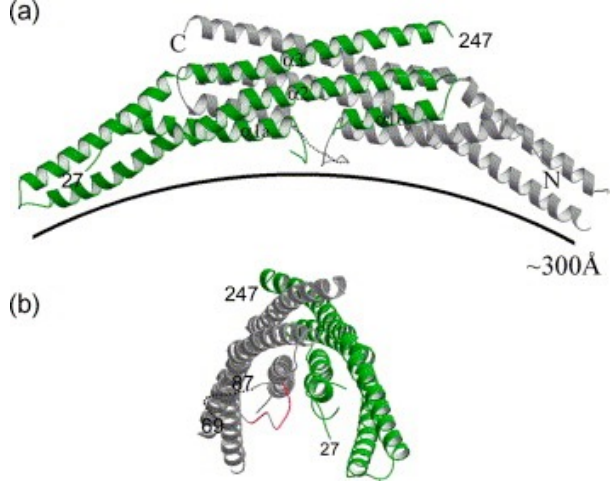


Table 1
CIN85/CMS binding partners

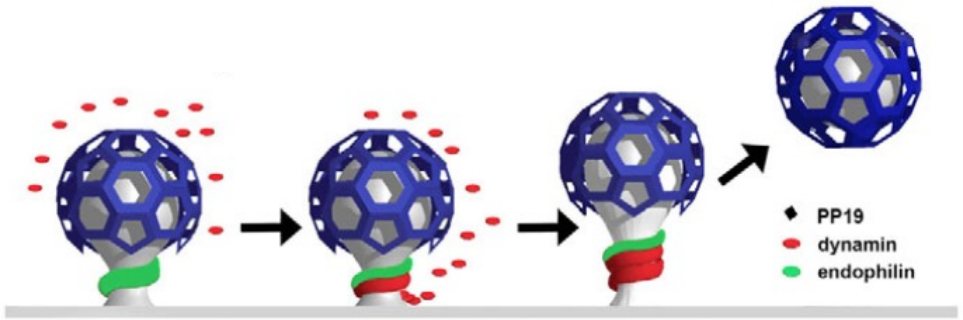
Protein	Interacts with	Function
c-Cbl	SH3-ABC of CIN85/CMS	Downregulation of RTKs
Cbl-b	SH3-ABC of CIN85	Downregulation of RTKs
BLNK: B-cell linker protein	SH3-ABC of CIN85	B-cell receptor signaling
SB1 (similar to NY-REN-45)	SH3-ABC of CIN85	Not yet defined
CD2	SH3-B of CD2AP and CIN85	T-cell receptor 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

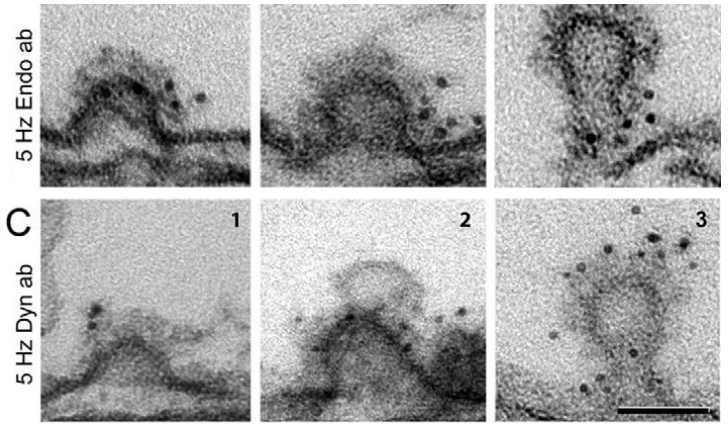
Endophilins



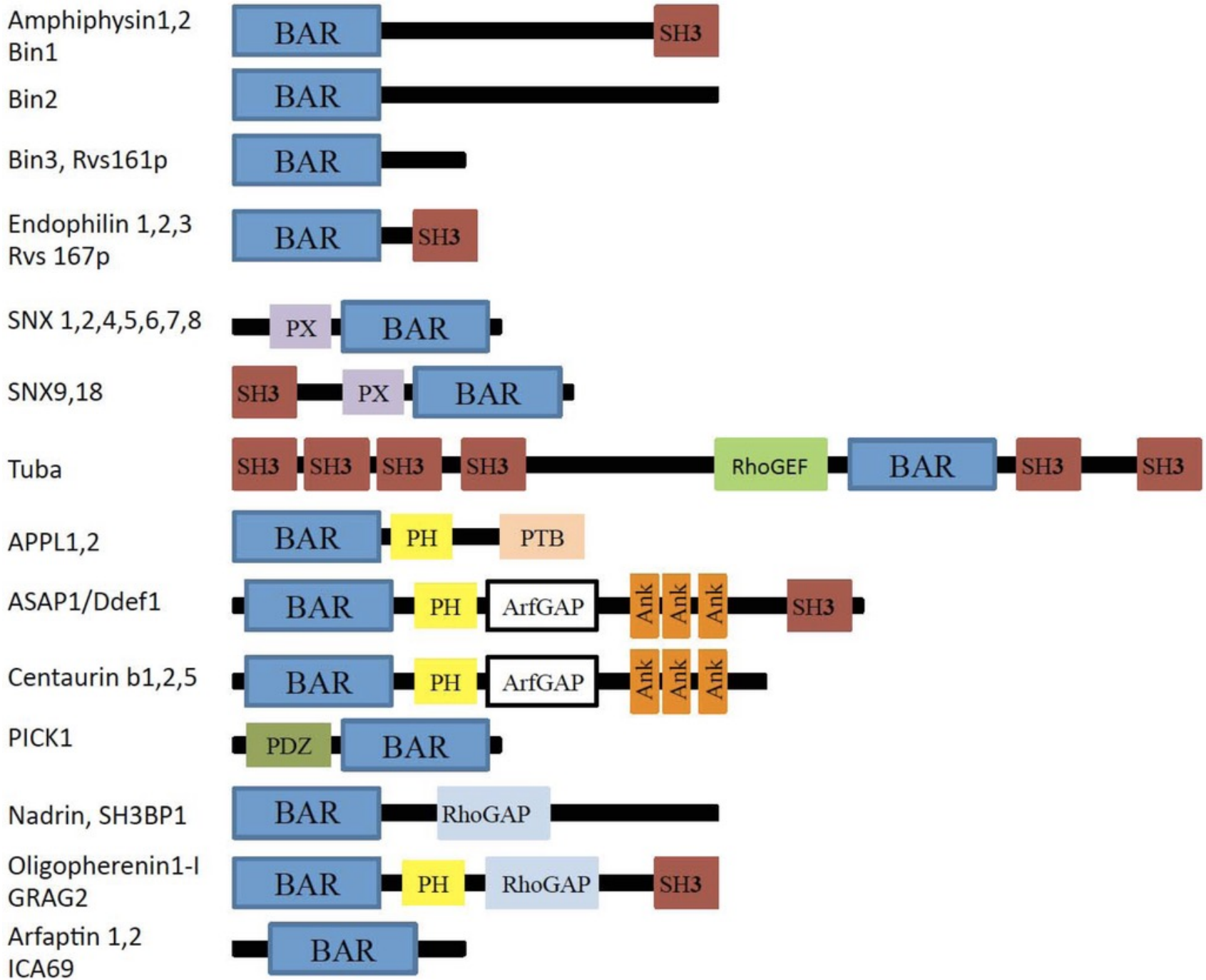


- Endophilin localizes in the vesicle pool at rest and in spirals at the necks of clathrin-coated pits (CCPs). Endophilin and dynamin colocalize at the base of the clathrin coat.
- Tubulation efficiency and the amount of dynamin recruited to lipid tubes are dramatically increased in the presence of endophilin.
- 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 GTP□S.
- 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,*]
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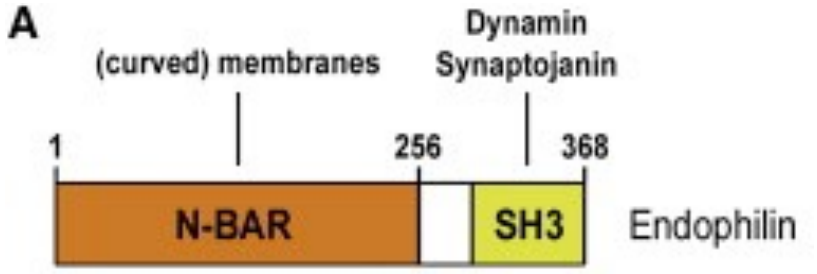
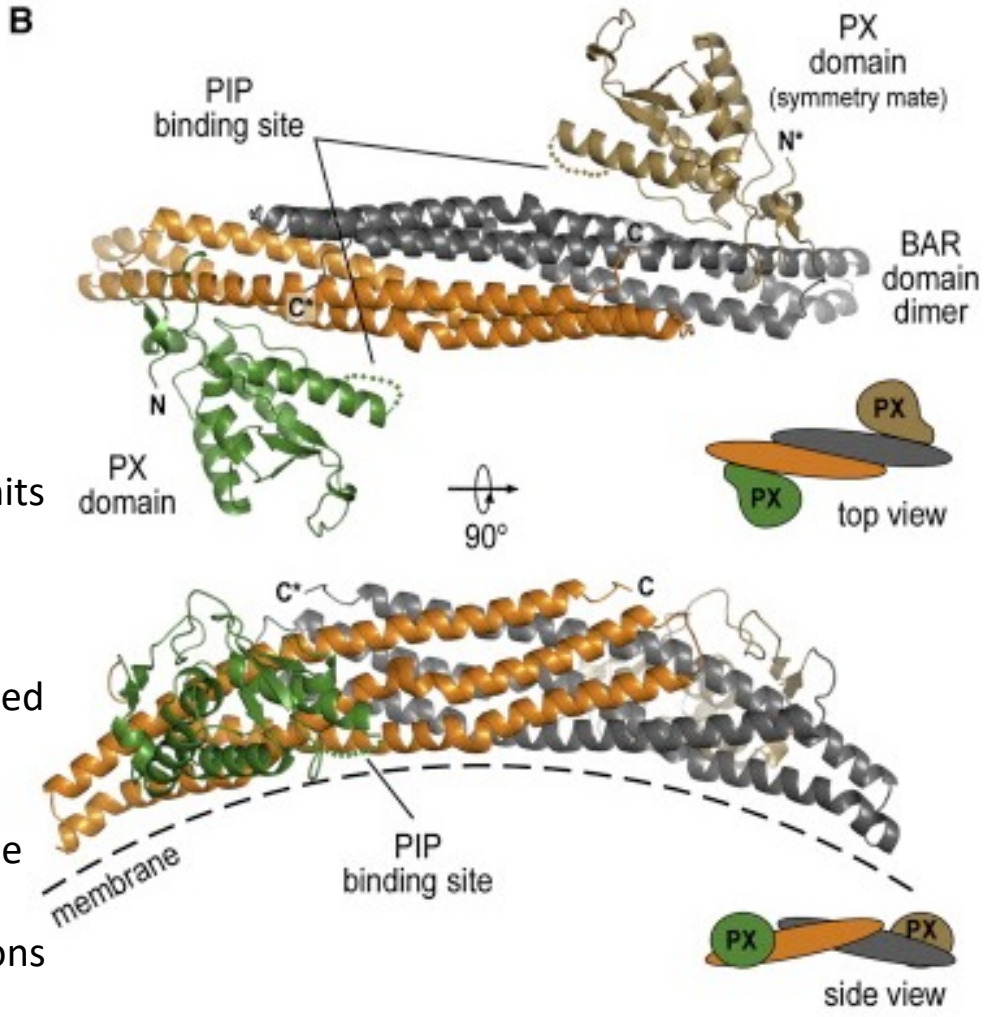
DOI 10.1016/j.str.2008.07.016





- 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

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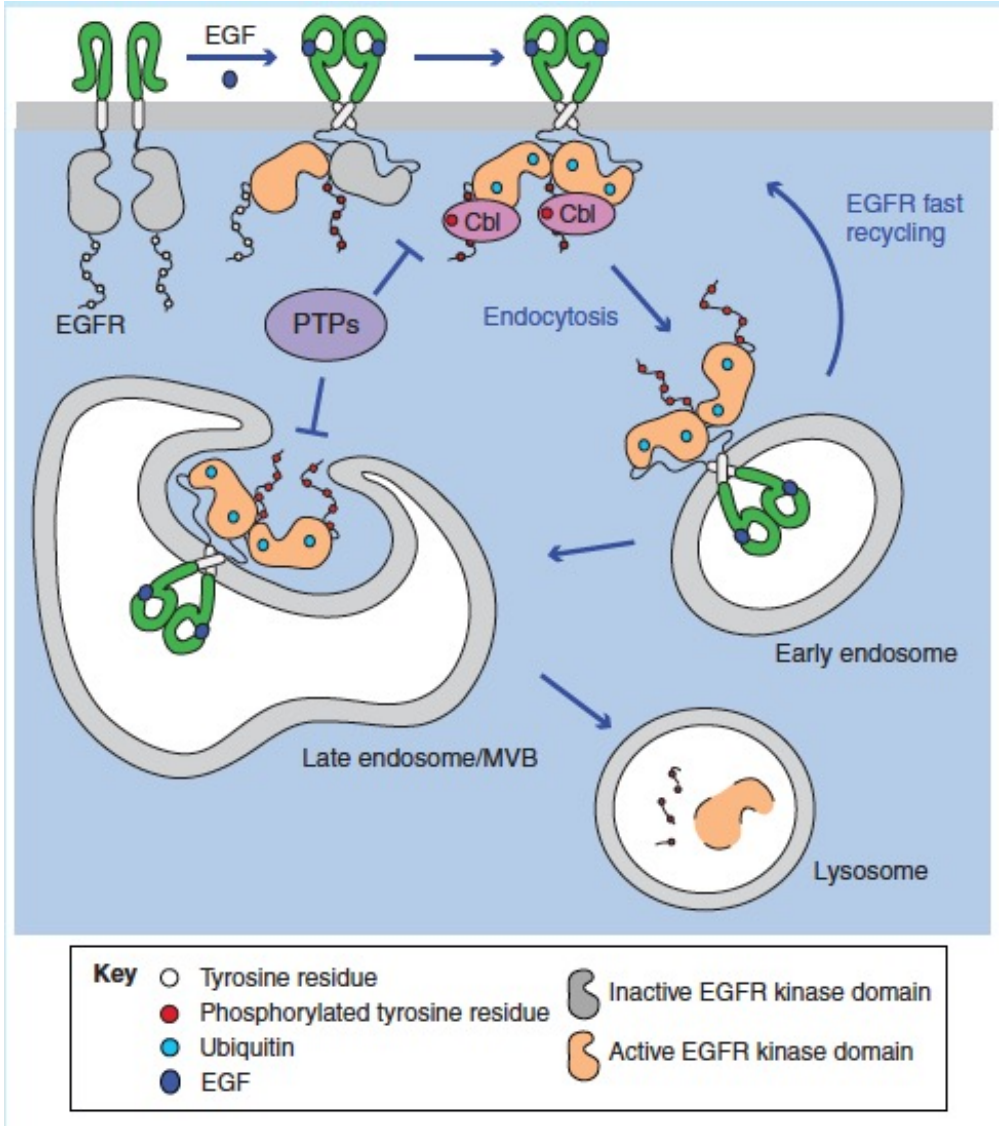
Journal of Cell Science 124, 1785-1793

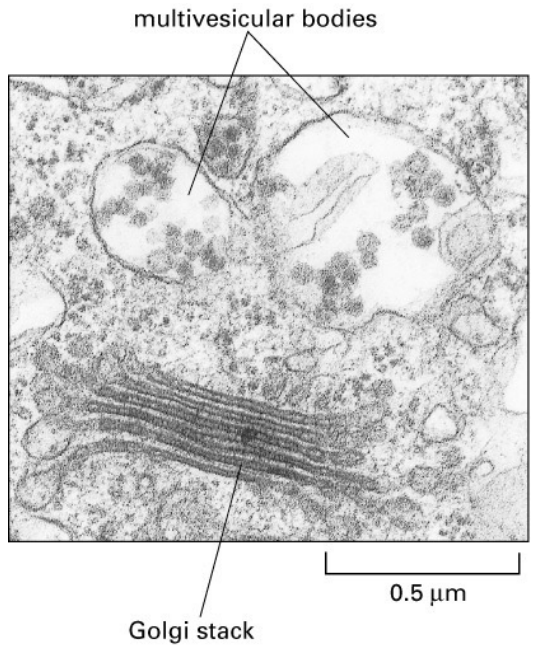
doi:10.1242/jcs.083303



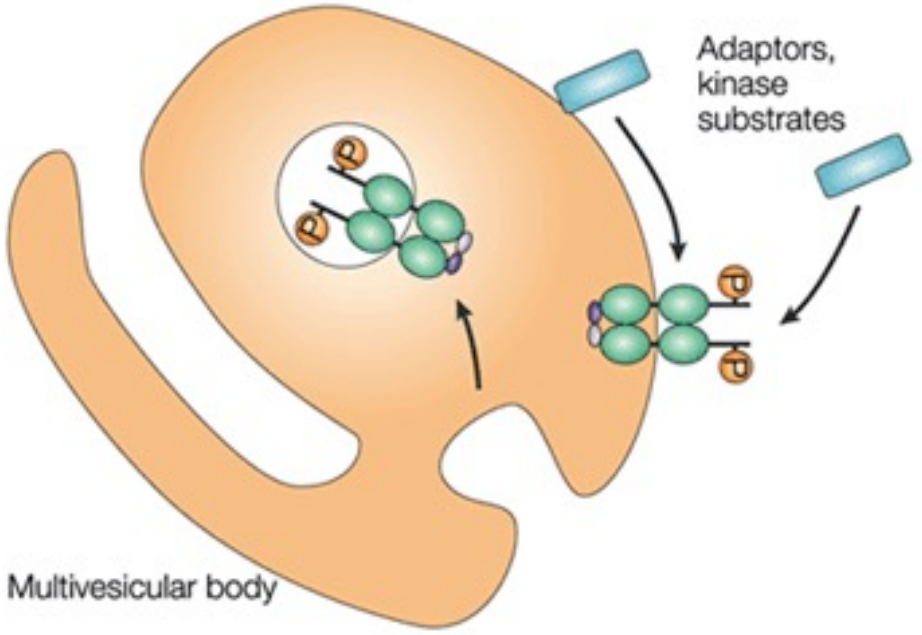
- Tyrosine phosphatases (PTPs) reverse EGFR tyrosine phosphorylation at the cell membrane, as well as during endocytic trafficking.
- Activated EGFRs undergo rapid endocytosis.
- Internalised EGFRs reach early endosomes, from where receptors are rapidly recycled to the cell surface unless tagged robustly 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.

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.

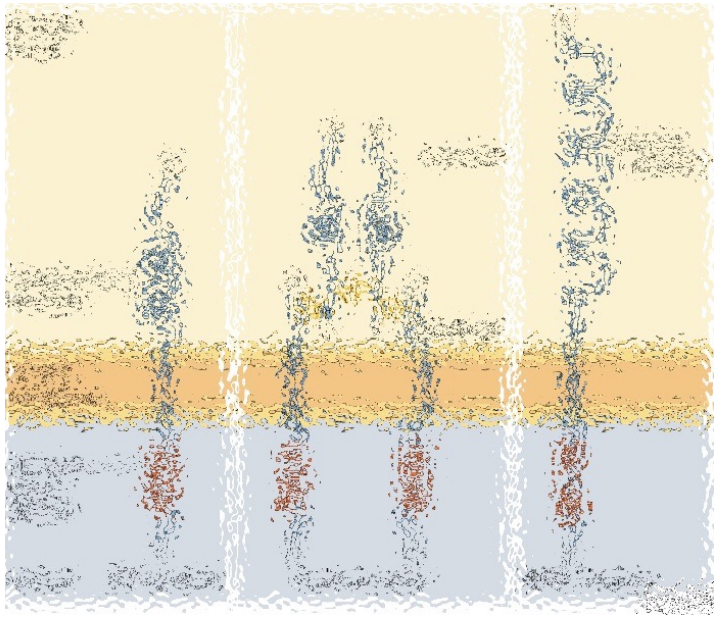


Receptors incorporated into vesicles are not accessible for interactions with adaptor proteins and cannot phosphorylate cytoplasmic signalling proteins.
So, sequestration of active receptors inside MVBs might terminate RTK signalling before their degradation in lysosomes.

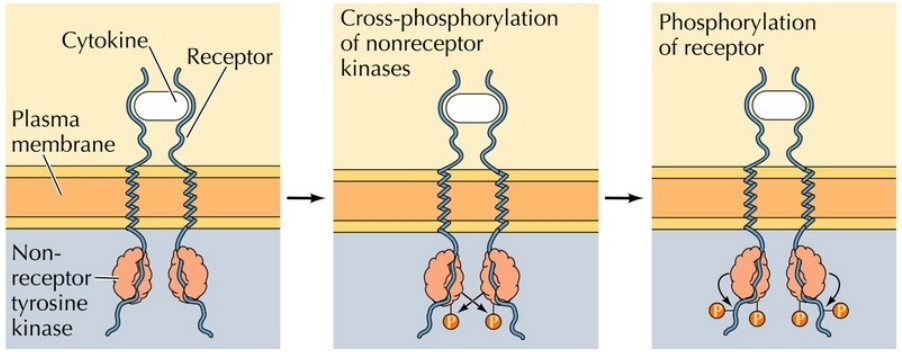


Enzyme-linked receptors fall into 3 categories:

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



- **Cytokine superfamily receptors**
  - No catalytic domain
  - Interact with <u>non</u> 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*.

Cytokines: main functions

- **Hematopoiesis** (ex. CSFs, colony stimulating factors).
- **Inflammatory reaction** (ex. IL1, TNF).
- **Chemotaxis** (ex. IL8, MIP1- macrophage inflammatory protein 1, BLC – B-lymphocyte chemoatractant).
- **Immunostimulation** (ex. IL12, IFNγ).
- **Suppression** (ex. IL10).
- **Angiogenesis** (ex. VEGFs - vacsular endothelial growth factor).
- **Embryogenesis** (ex. TGF-β, LT - lymphotoxin).

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	

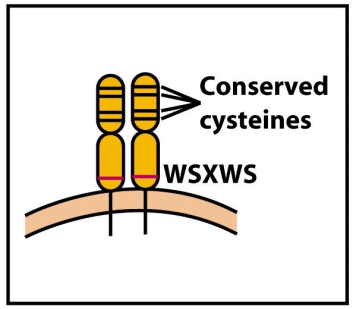


Figure 12-6b
Kuby IMMUNOLOGY, Sixth Edition

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

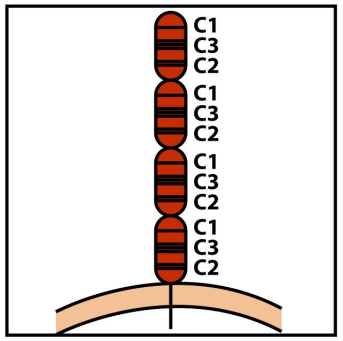


Figure 12-6d
Kuby IMMUNOLOGY, Sixth Edition

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

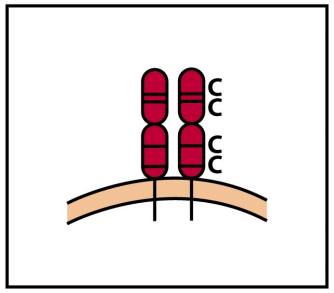


Figure 12-6c
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RECEPTOR FAMILY	LIGANDS
Immunoglobulin superfamily receptors	IL-1
	M-CSF
	C-Kit
	IL-18

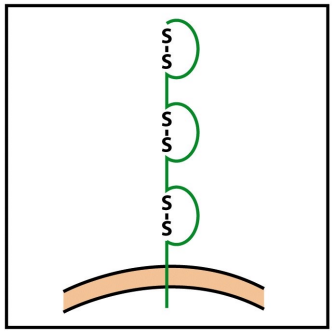

Figure 12-6a
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RECEPTOR FAMILY	LIGANDS
Chemokine receptors	IL-8
	RANTES
	MIP-1
	PF4
	MCAF
	NAP-2

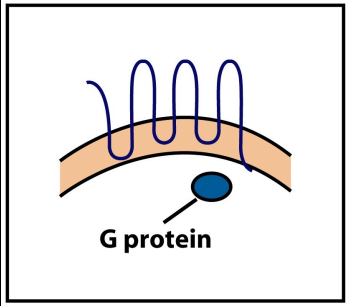

Figure 12-6e
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Cytokine receptors belong to families of receptor proteins, each with a distinctive structure.

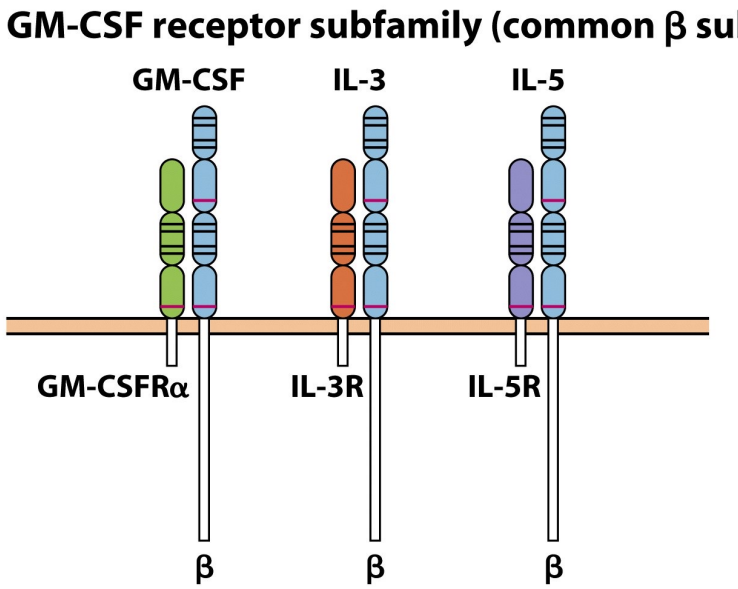

Figure 12-7a
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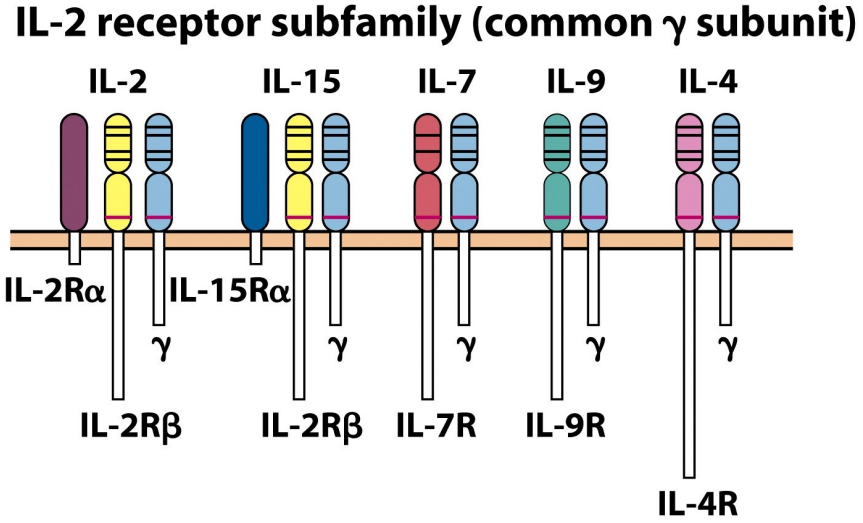

Figure 12-7c
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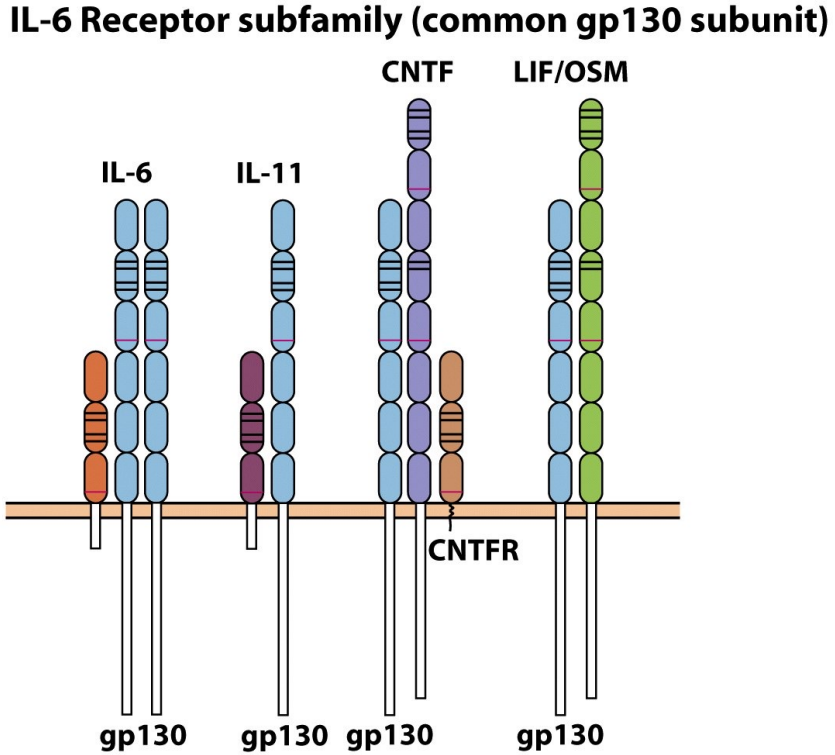

Figure 12-7b
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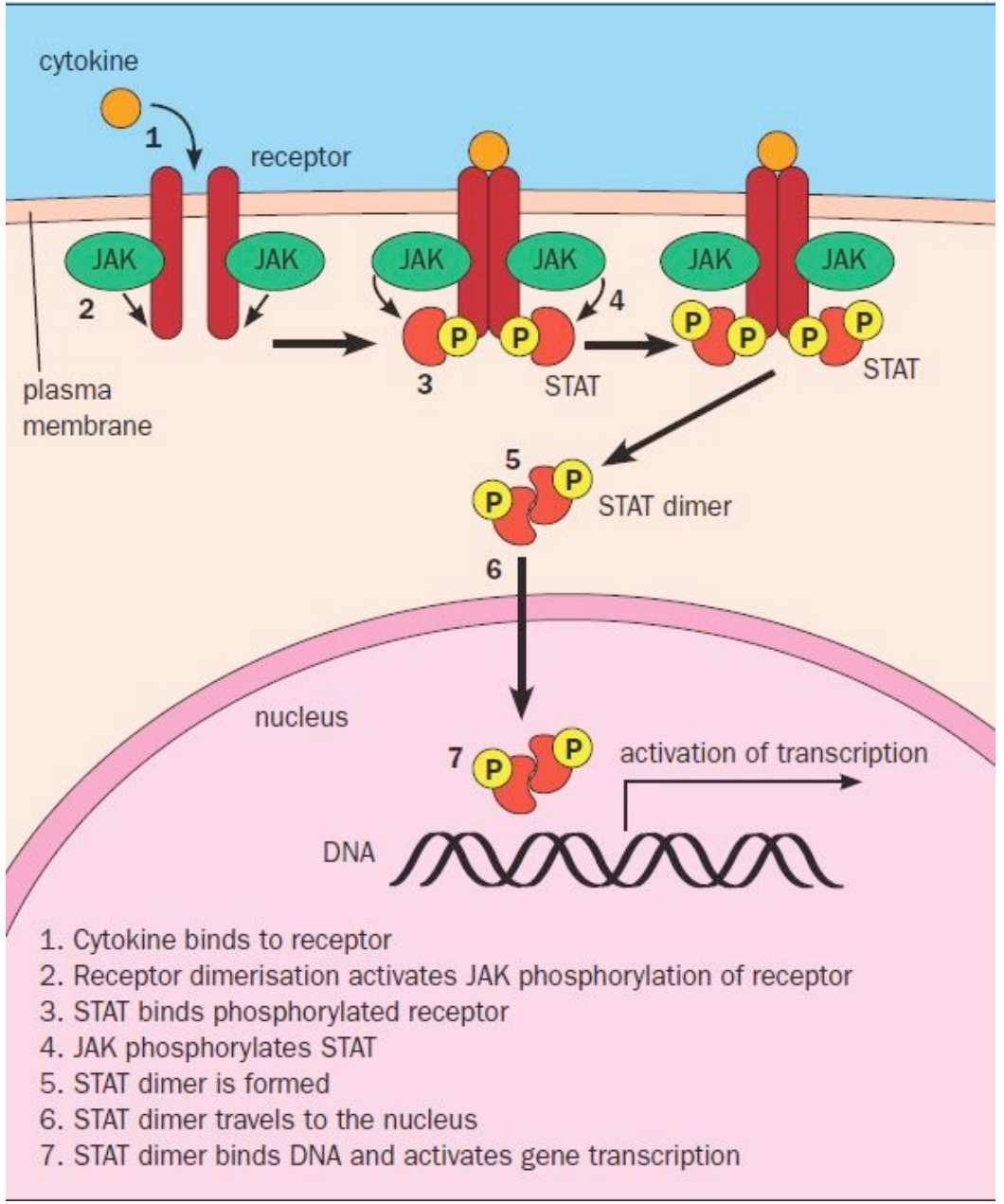
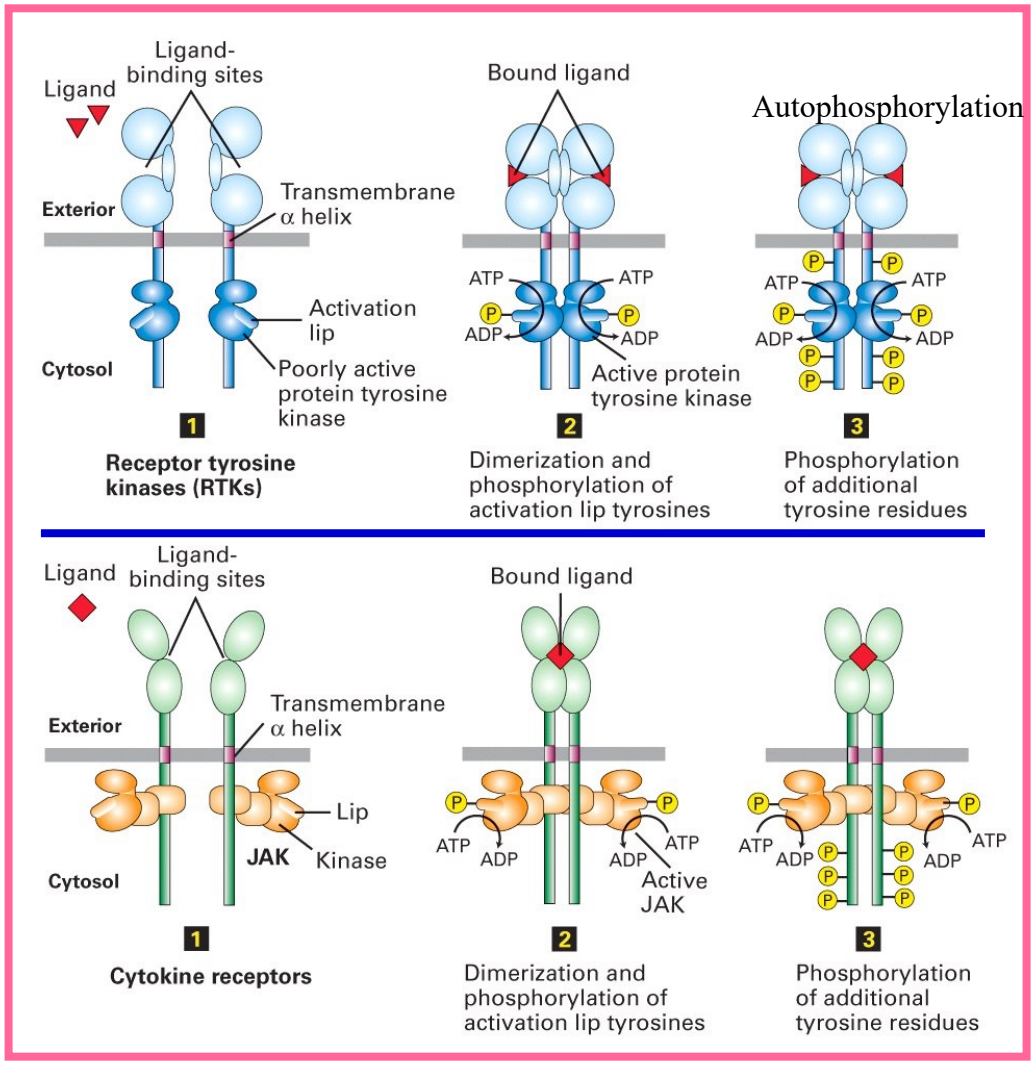
Cytokine receptors subfamilies have shared signaling subunits

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



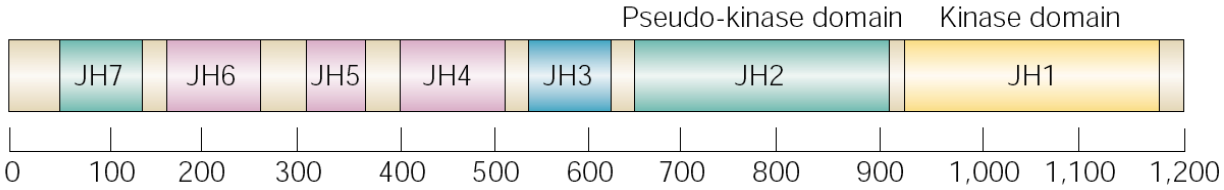
Cytokine Receptors and Receptor Tyrosine Kinases Share Many Signaling Features



- Ligand binding to both cytokine receptors and receptor tyrosine kinases triggers formation of functional oligomers
- In some cases, the ligand induces association of two monomeric receptor subunits diffusing in the plan of the plasma membrane; in other cases, the receptor is a dimer in the absence of ligand and ligand binding alters the conformation of the extracellular domains of the two subunits
- In either cases, formation of the functional dimeric receptor causes the cytosolic kinases to phosphorylate the second kinase

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

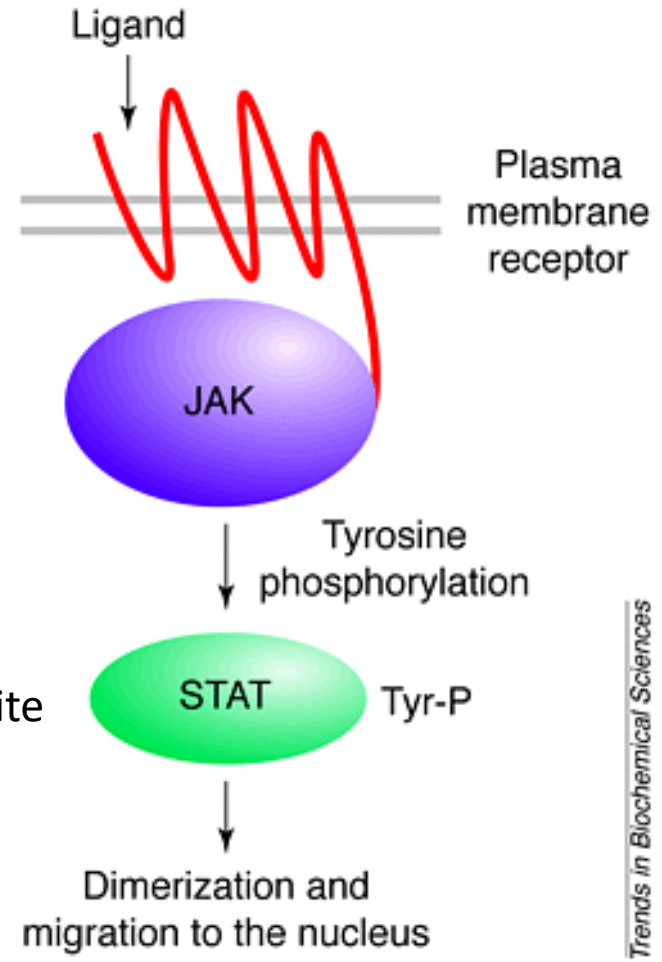
STAT proteins

STATs: Signal Transducers and Activators of Transcription

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

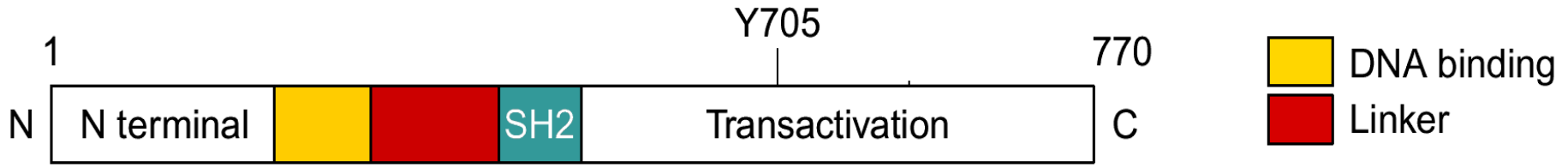
2. Transcriptional activators
   activation at the cell membrane, response in the nucleus

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

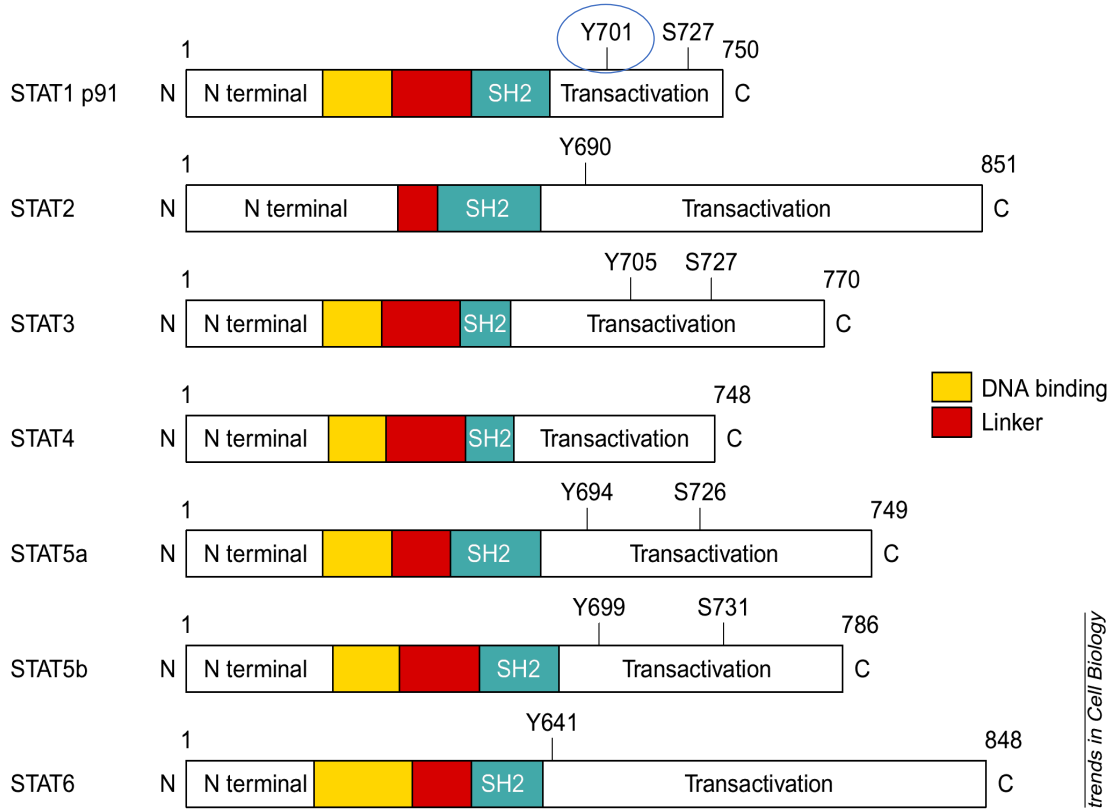


STAT proteins

- 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



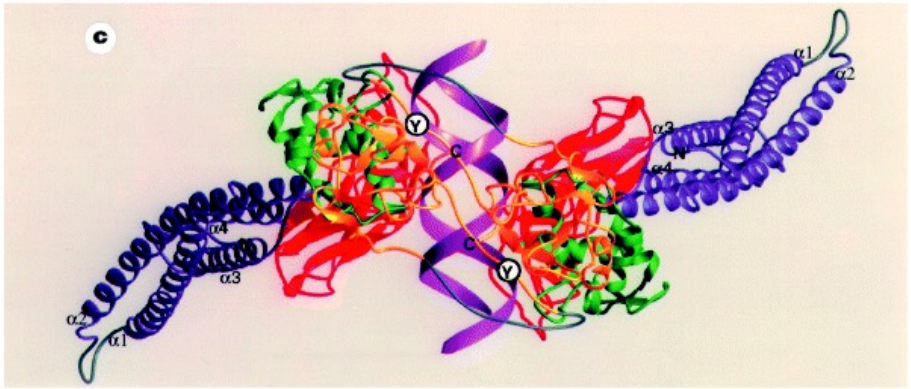
STAT-family members



- 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 in 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

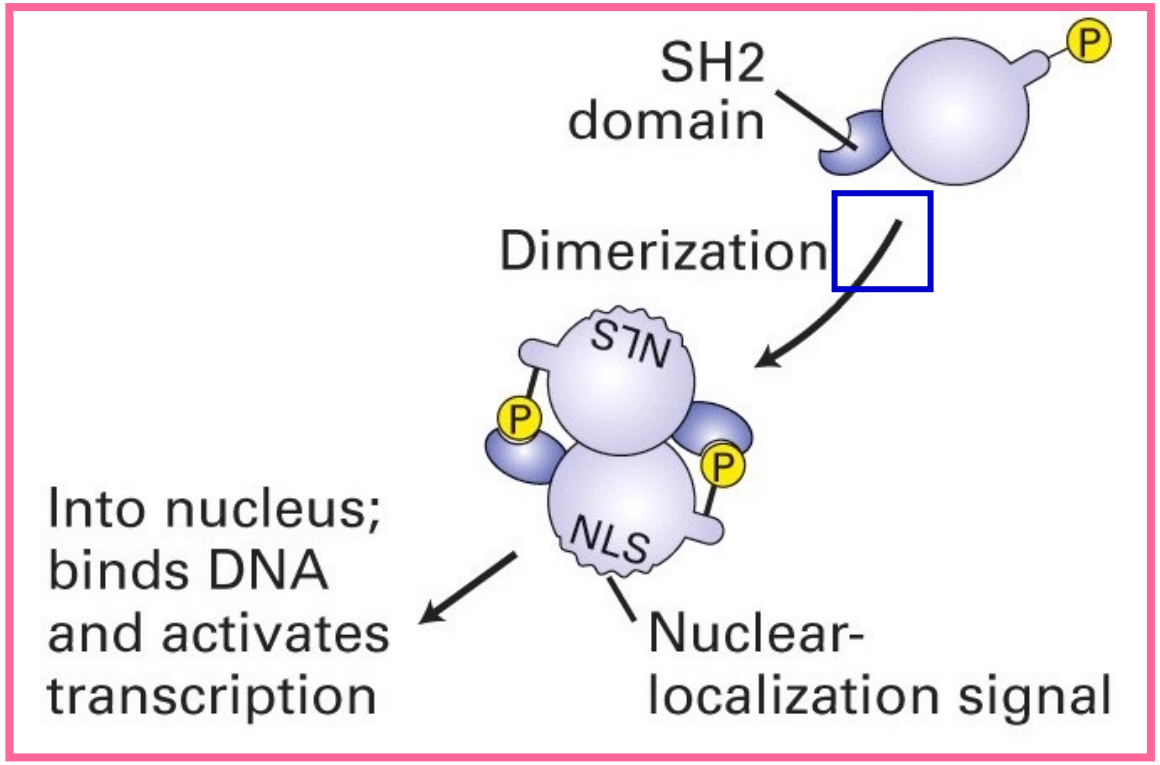


- **DNA-binding domain**

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



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

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

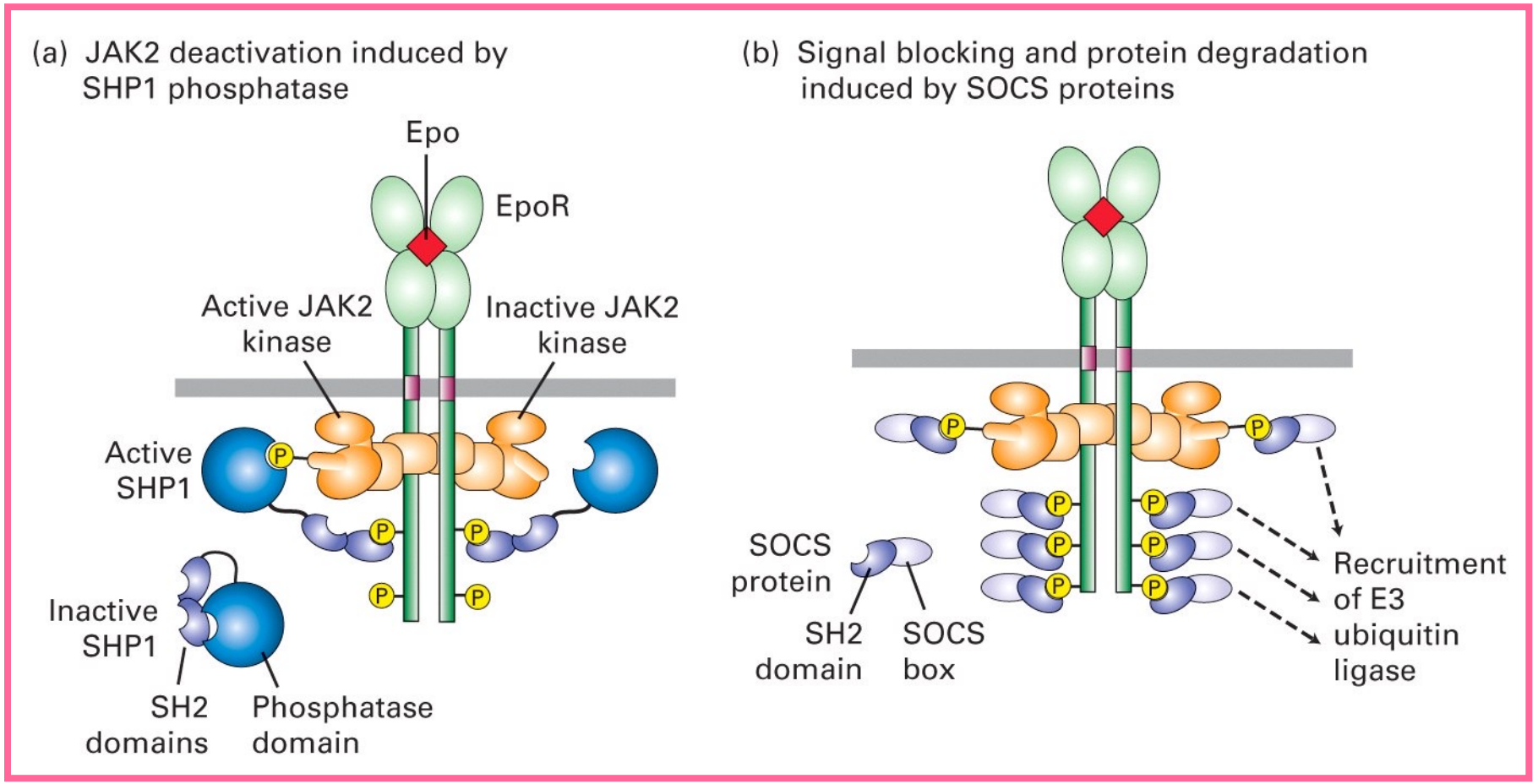
STAT1 knock-out mice illustrate biological specificity: $STAT1^{-/-}$ phenotype: total lack of IFN-response $\rightarrow$ highly sensitive to virus-infection

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

Negative Regulation of the JAK-STAT pathway

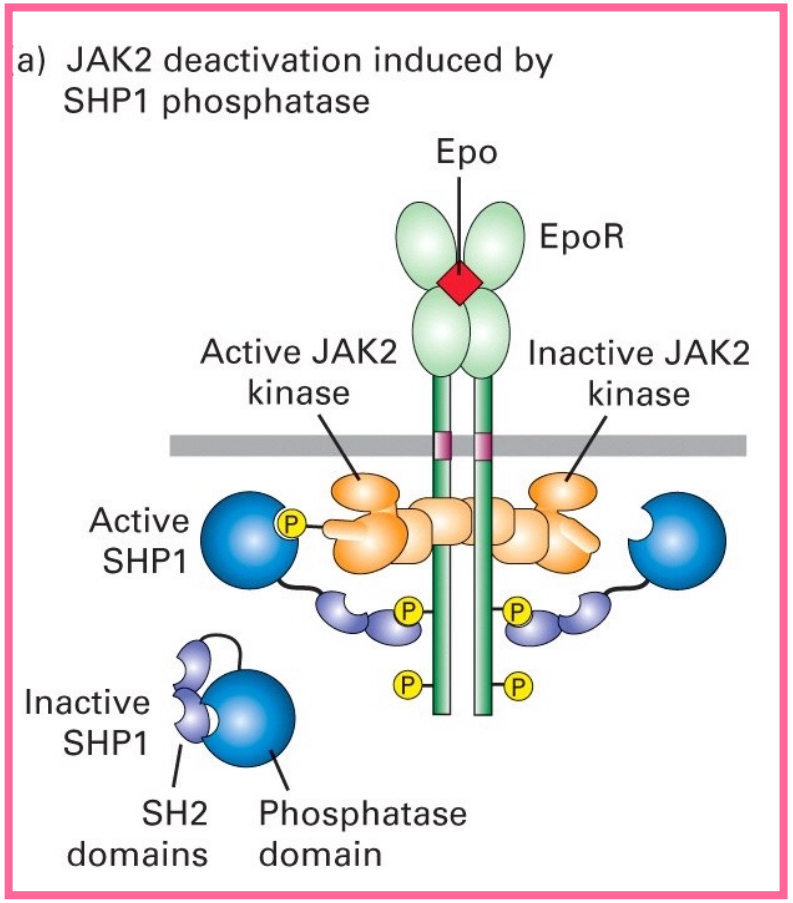


- Signal-induced transcription of target genes can not last for too long and needs de-sensitized
- Signaling from cytokine receptor is usually dampened by two classes of proteins: short term regulation by SHP1 phosphatase and long term regulation by SOCS proteins

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

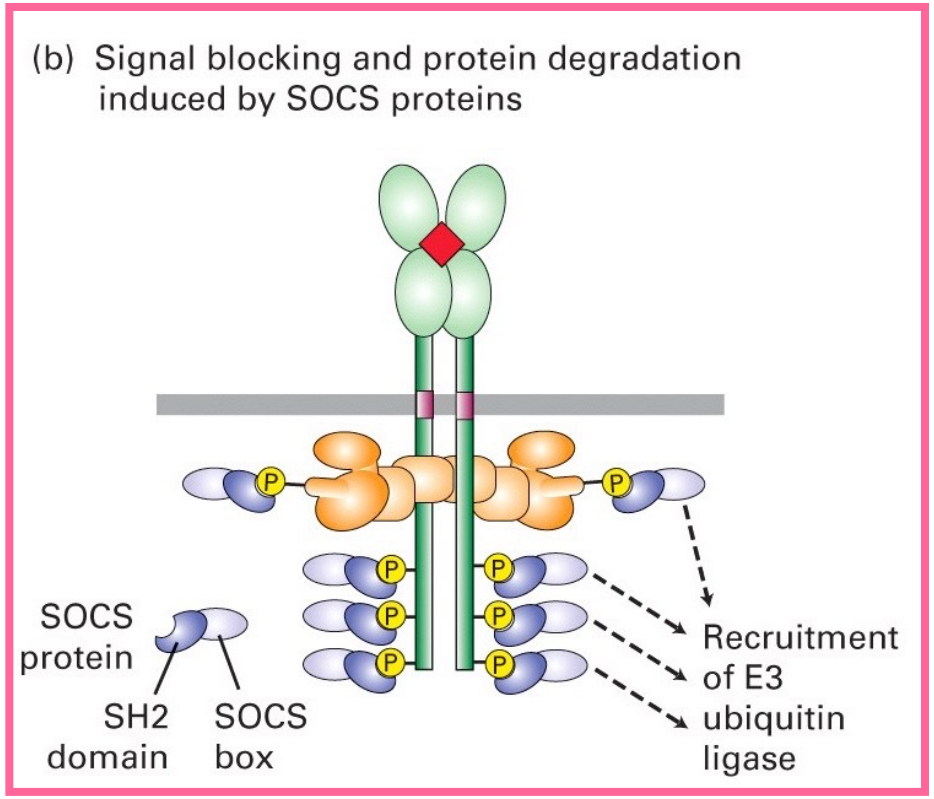


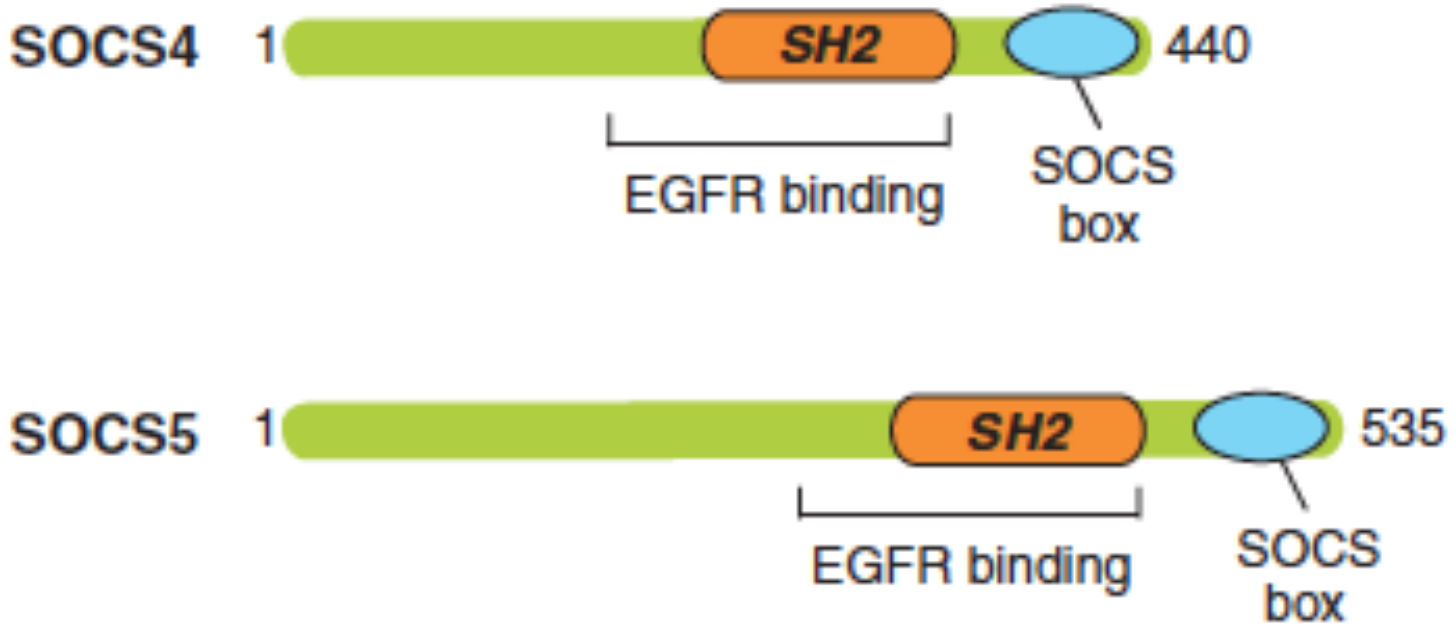
Signaling from Cytokine Receptors Is Modulated by Negative Signals

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

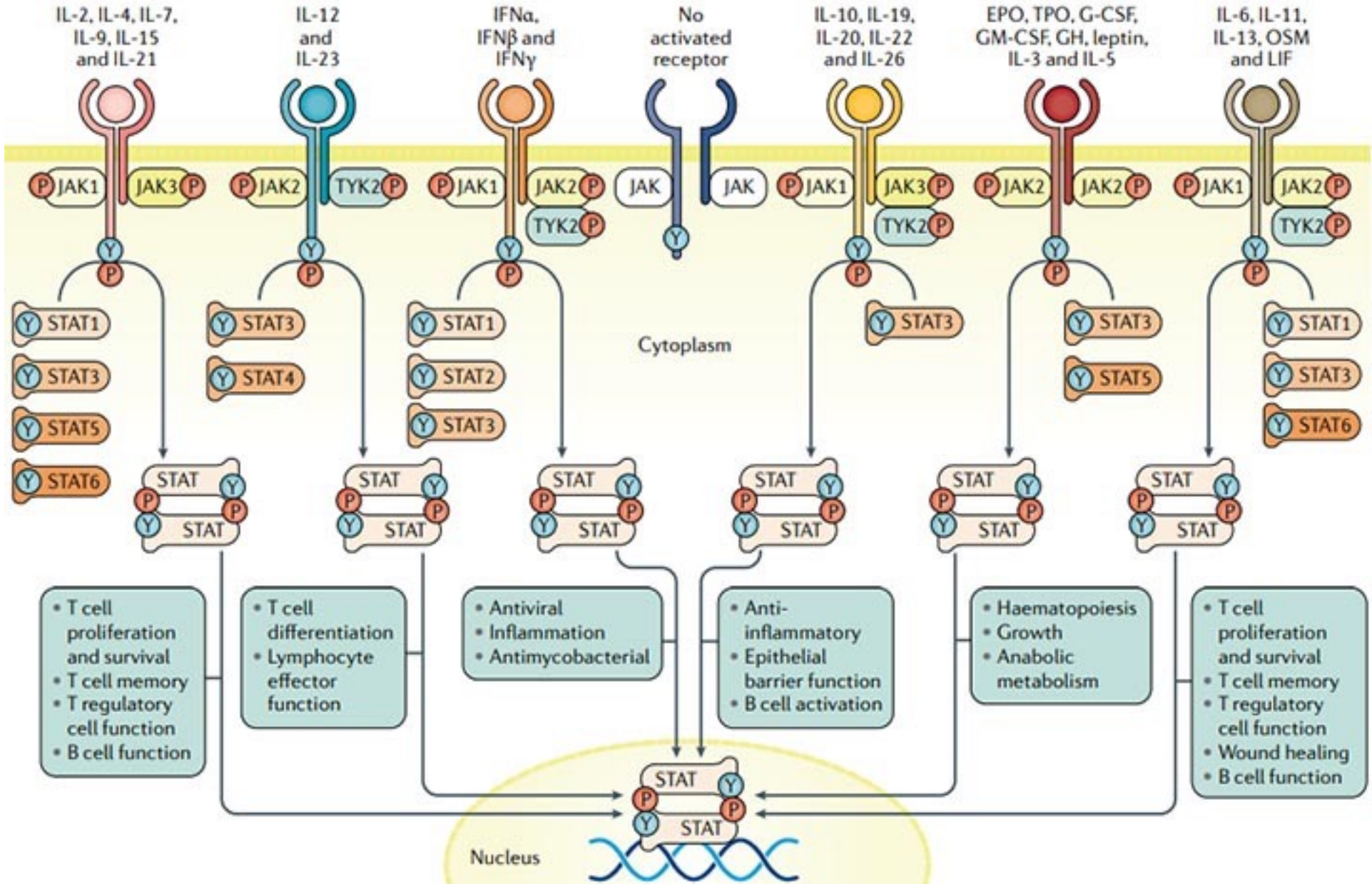
CIS proteins act in two ways to negatively regulate cytokine receptor stimulated signaling:

- 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

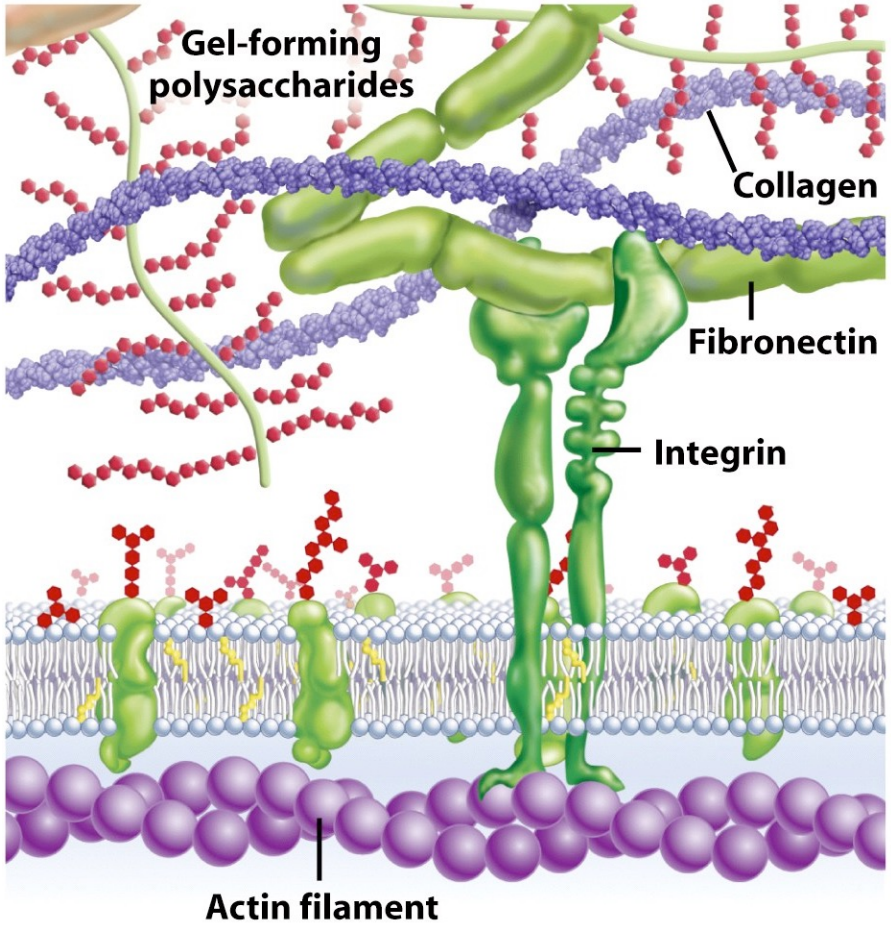




SOCS4 and SOCS5 bind to the EGFR through their respective SH2 domains, which share 87% sequence homology and a poorly defined Nterminal region.
The SOCS box recruits an E3 ligase and thereby leads to EGFR ubiquitylation.


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
STAT
Cytoplasm
• 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

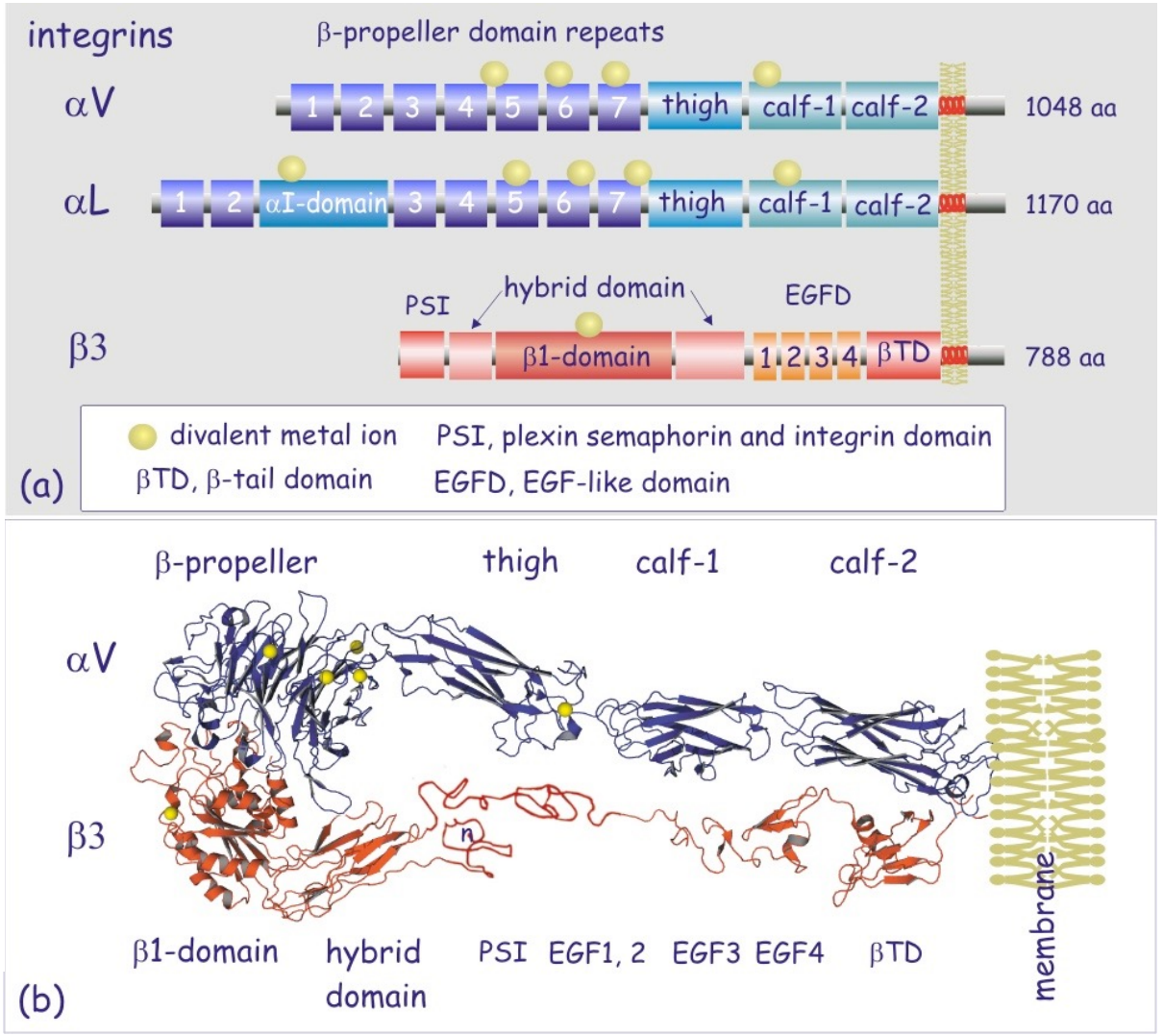
Integrins



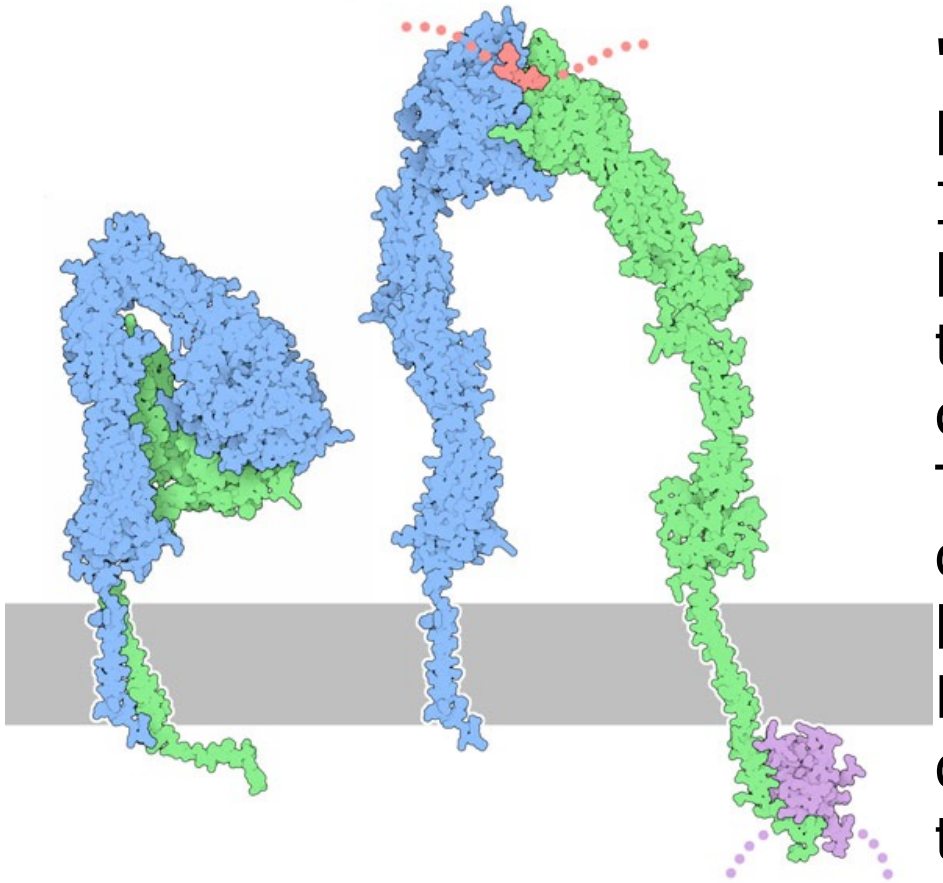
Integrins are transmembrane receptors that mediate the attachment between a cell and other cells or the extracellular matrix (ECM) components such as fibronectin, vitronectin, collagen, and laminin. In addition to transmitting mechanical forces across otherwise vulnerable membranes, they are involved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

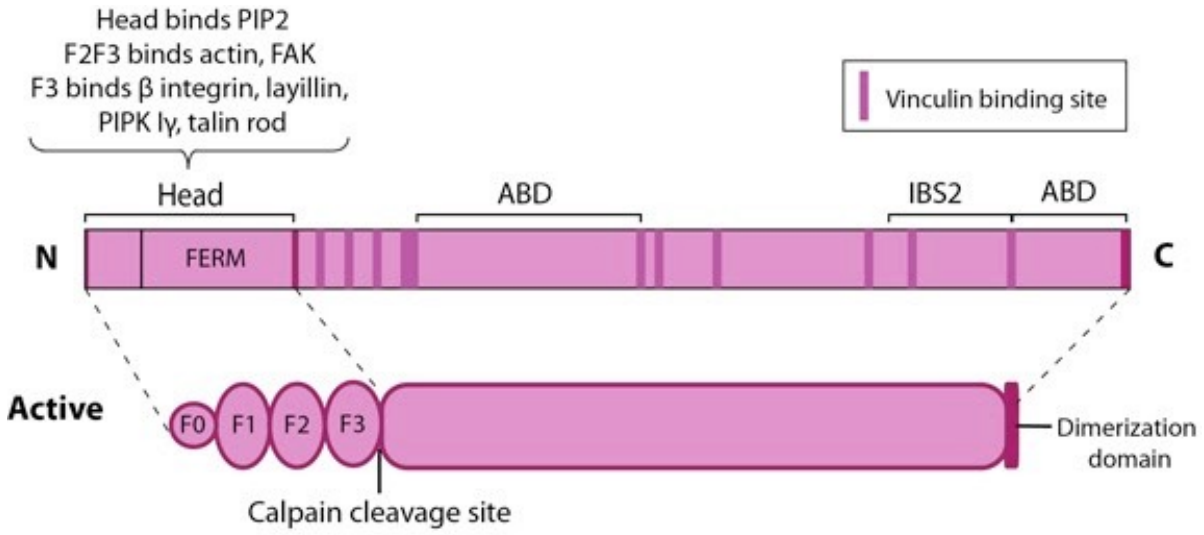
In cells, the priming is accomplished by **talin**, which binds to the β tail of the integrin dimer and changes its conformation.

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

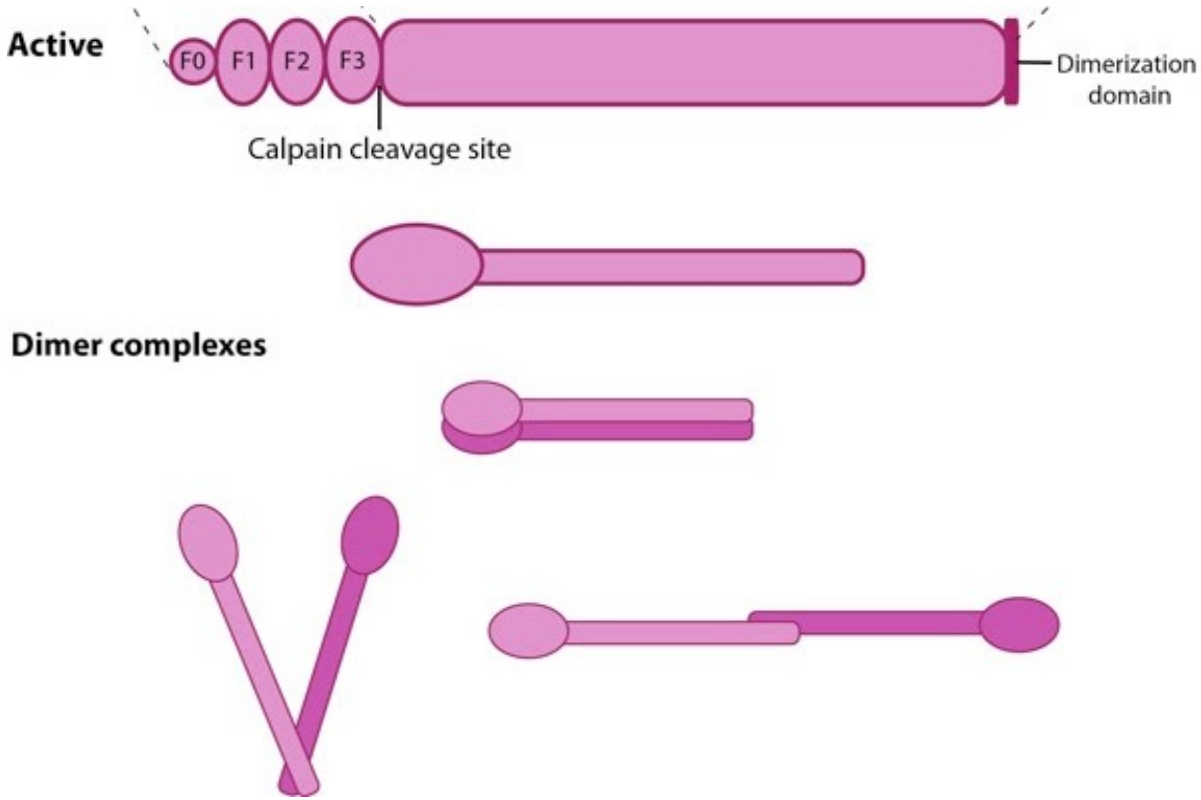
Talin is a 270kDa cytoskeletal protein concentrated at regions of cell–substratum contact and, in lymphocytes, at cell–cell contacts. It is a structural platform that is required for the initial linkage between the contractile cytoskeleton and sites of integrin/fibronectin adhesion



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.