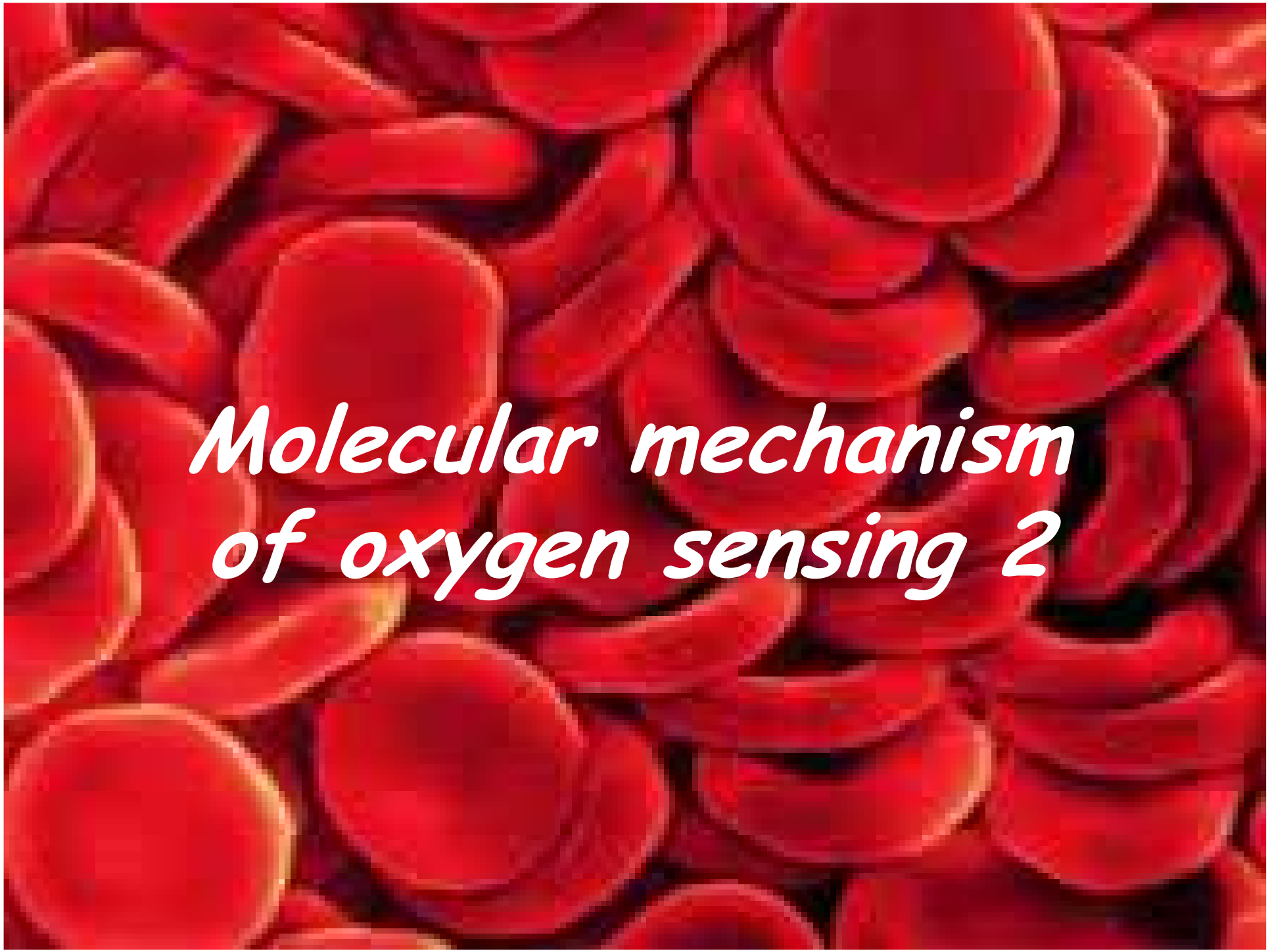

Molecular mechanism
of oxygen sensing 2

Tumor hypoxia plays a crucial role in tumorigenesis.
Under hypoxia, hypoxia-inducible factor 1 alpha (HIF-1 alpha) regulates activation of genes promoting malignant progression.

von Hippel-Lindau disease is characterized by a spectrum of hypervascular tumors, including renal cell carcinoma, hemangioblastoma, and pheochromocytoma,

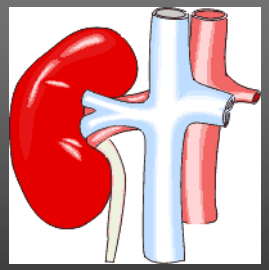
VHL loss causes a failure to regulate the hypoxia inducible factors (HIF-1α and HIF-2α), resulting in accumulation of both factors to high levels.

HIF dysregulation is critical to VHL disease-associated renal tumorigenesis,.

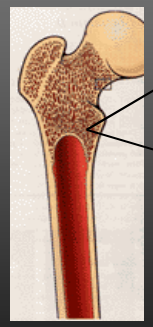
ERITROPOIETINA (Epo)

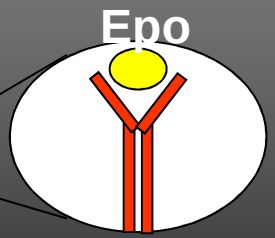
- ✓ Ormone glicoproteico di 34 kDa (165 aa)
- ✓ Struttura a 4 α-eliche (A,B,C,D)
- ✓ Funzione: stimola l'eritropoiesi

SINTESI *LEGAME CON IL RECETTORE*

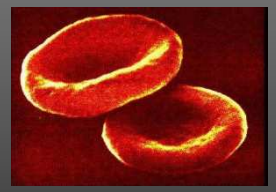


Reni



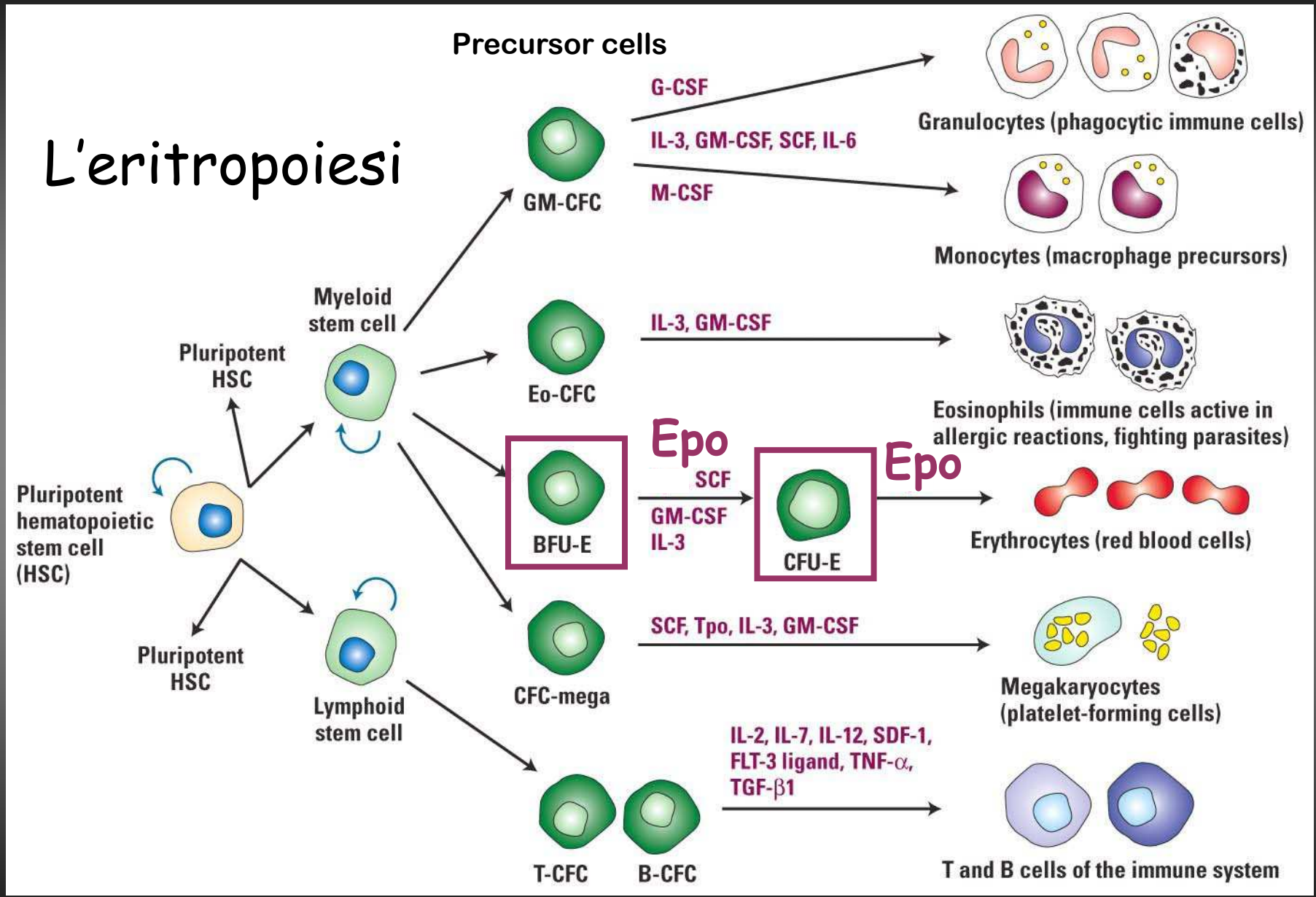


Midollo osseo

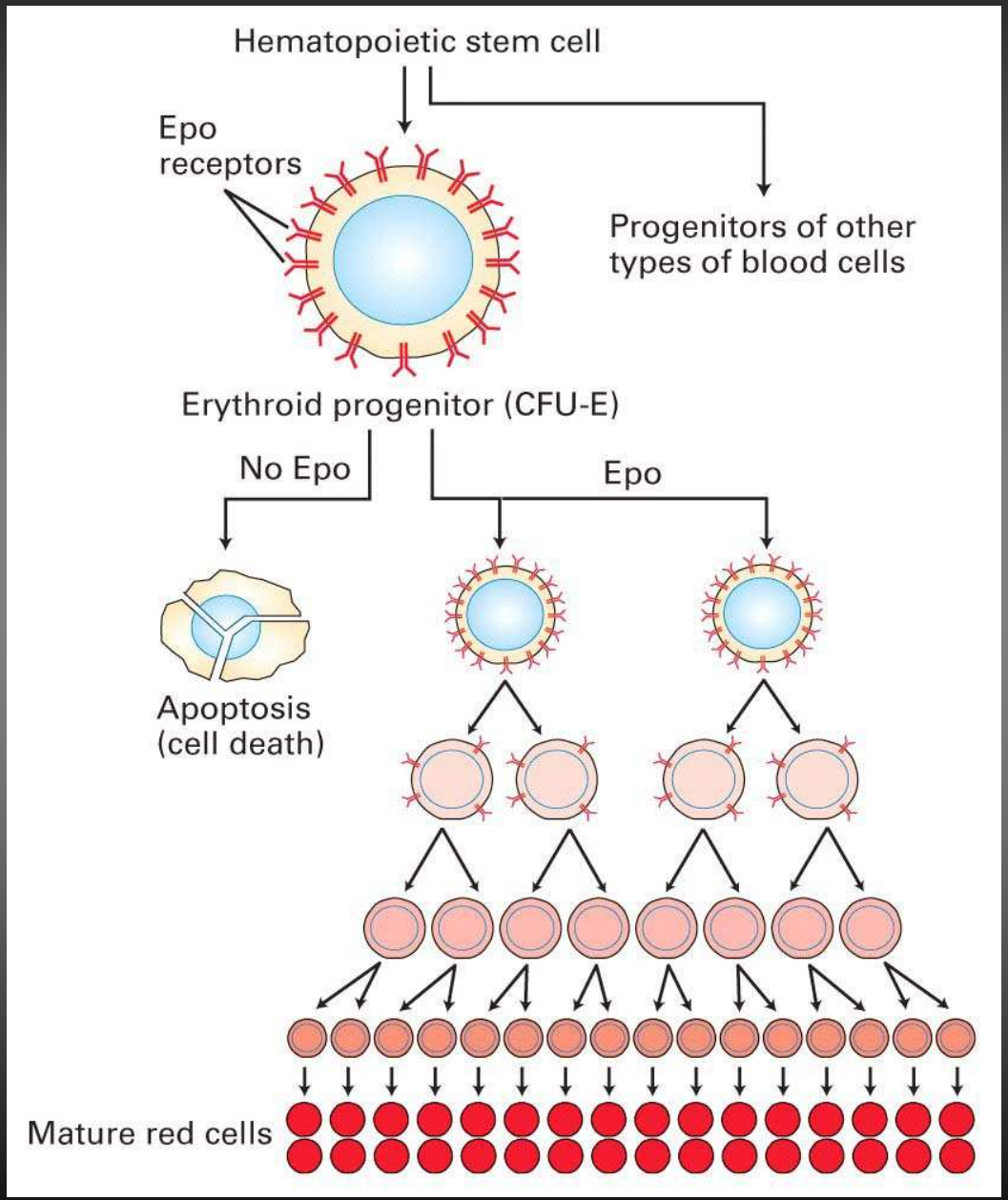


PRODUZIONE DI ERITROCITI

L'eritropoiesi



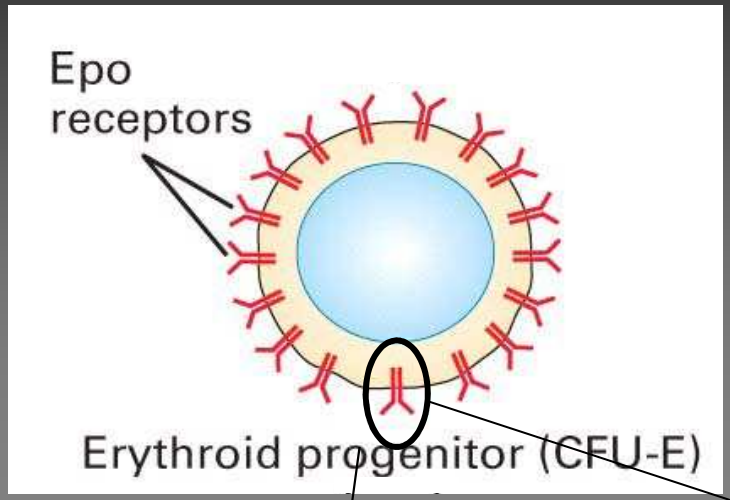
Ruolo dell'Epo nell'eritropoiesi



EpoR è espresso sulla superficie delle cellule eritroidi (massima espressione sulle CFU-E, diminuita sugli stadi più differenziati)

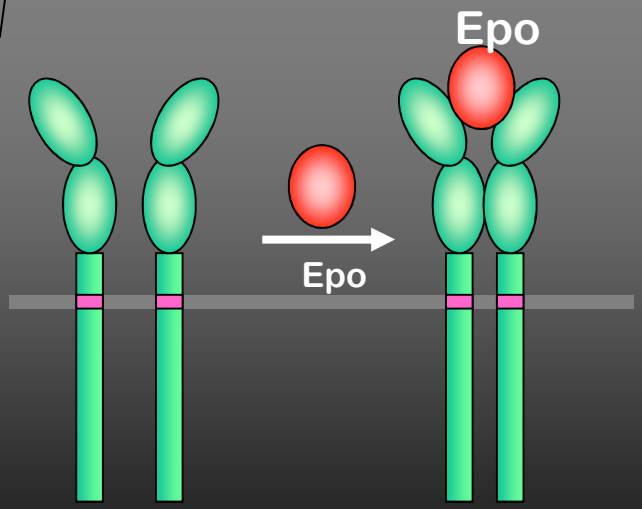
Epo agisce "salvando" dall' apoptosi le cellule progenitrici eritroidi, e stimolandone la maturazione

Il recettore dell'Epo (EpoR)



Glicoproteina transmembrana

Monomero: 66 kDa (507 aa)



Famiglia dei recettori delle citochine:

Legame del ligando

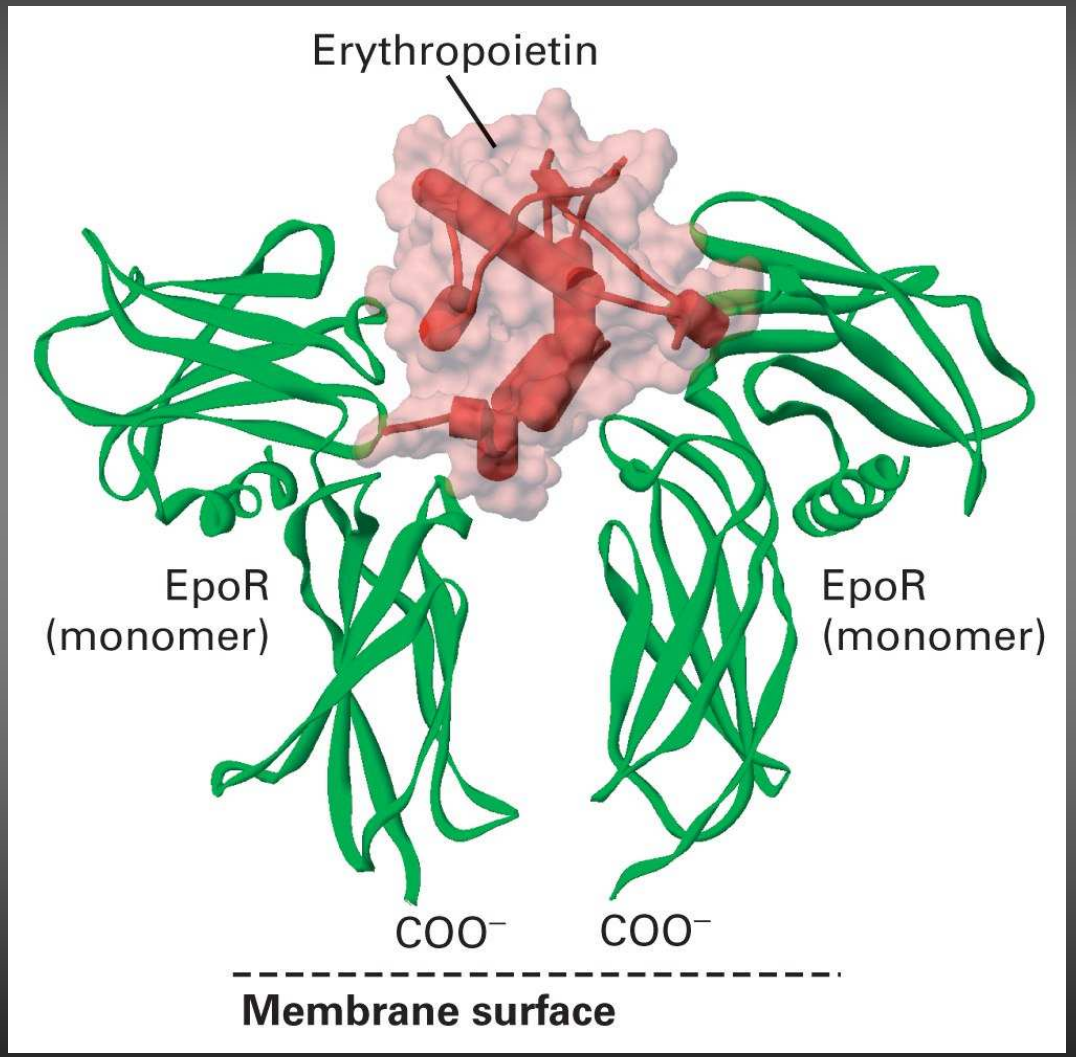
↓

Dimerizzazione

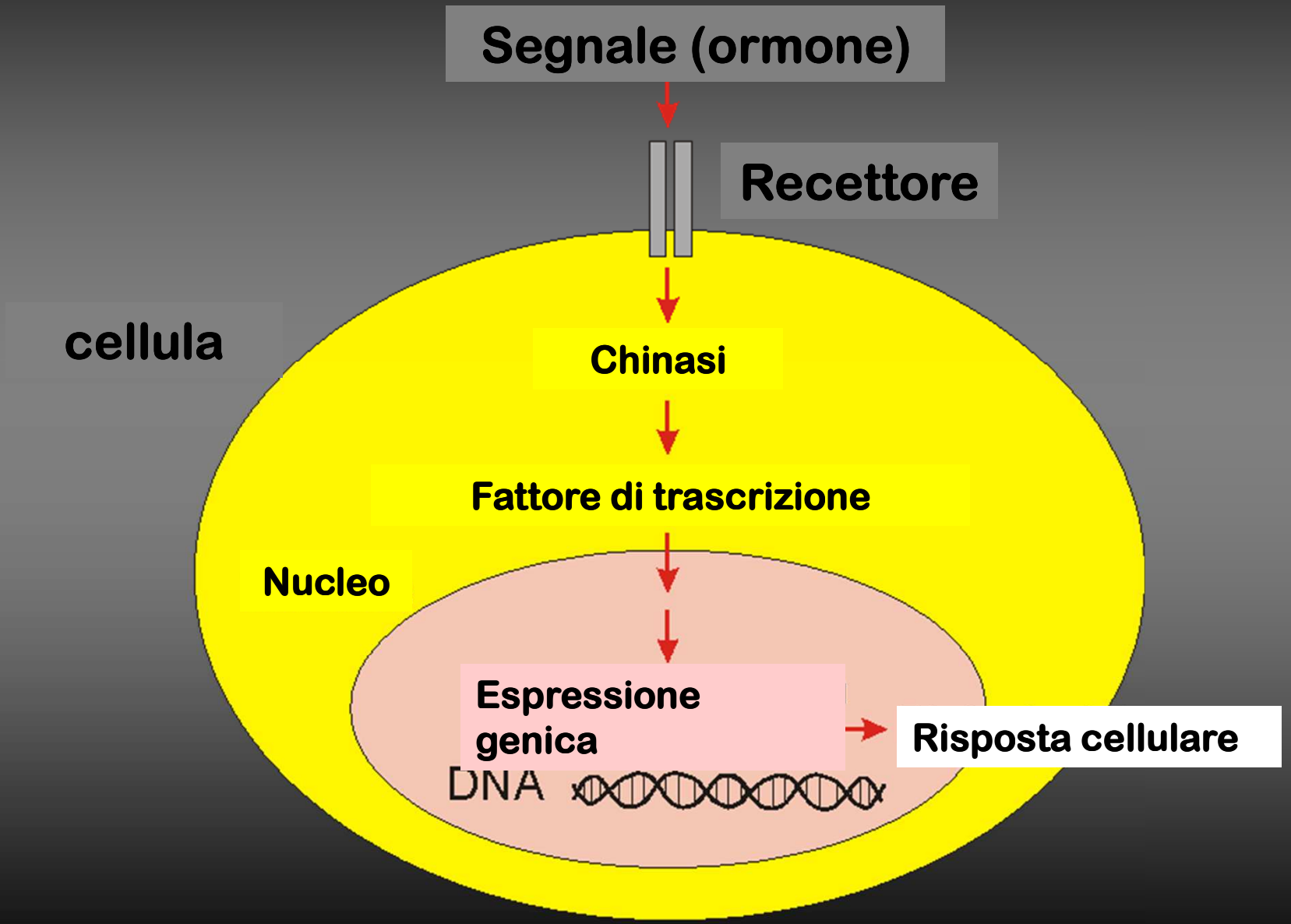
↓

Attivazione del recettore

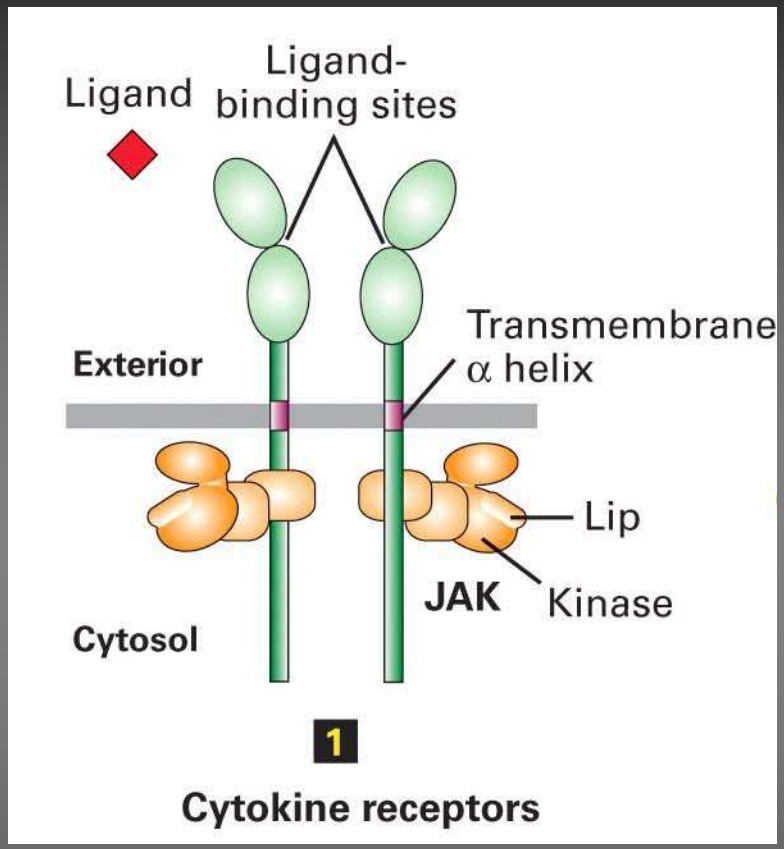
Erythropoietin-Epo Receptor complex



TRASDUZIONE DEL SEGNALE



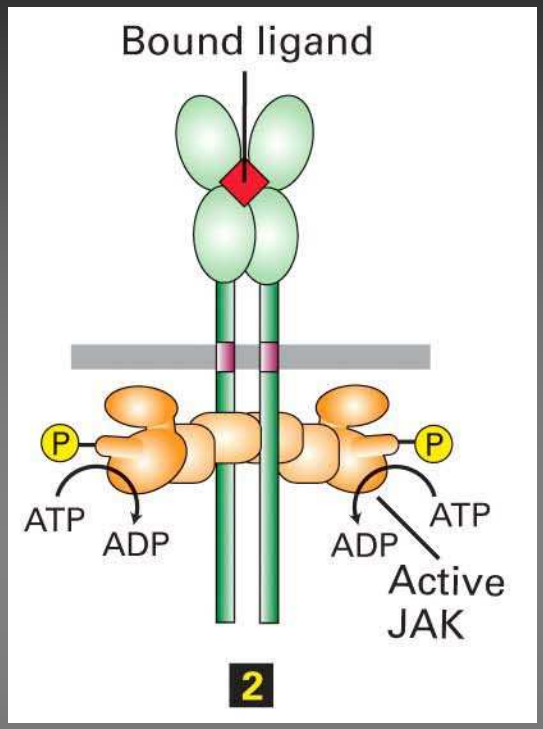
Trasduzione del segnale



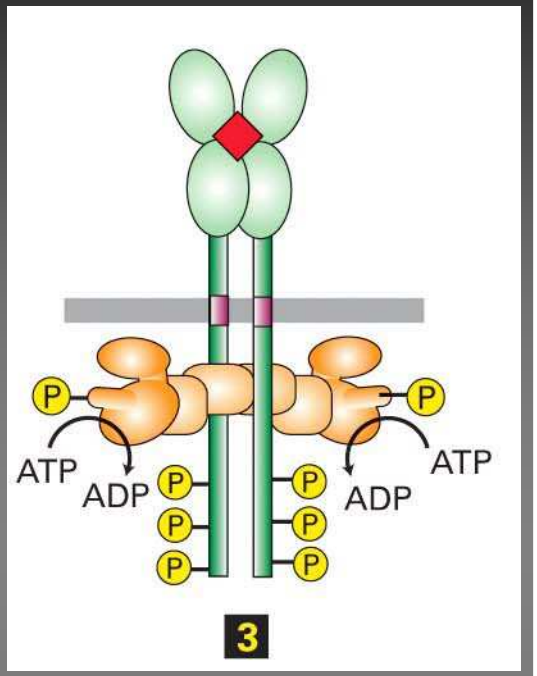
Dominio intracellulare privo di attività catalitica

⇩

Una JAK chinasi è associata al dominio citosolico di EpoR

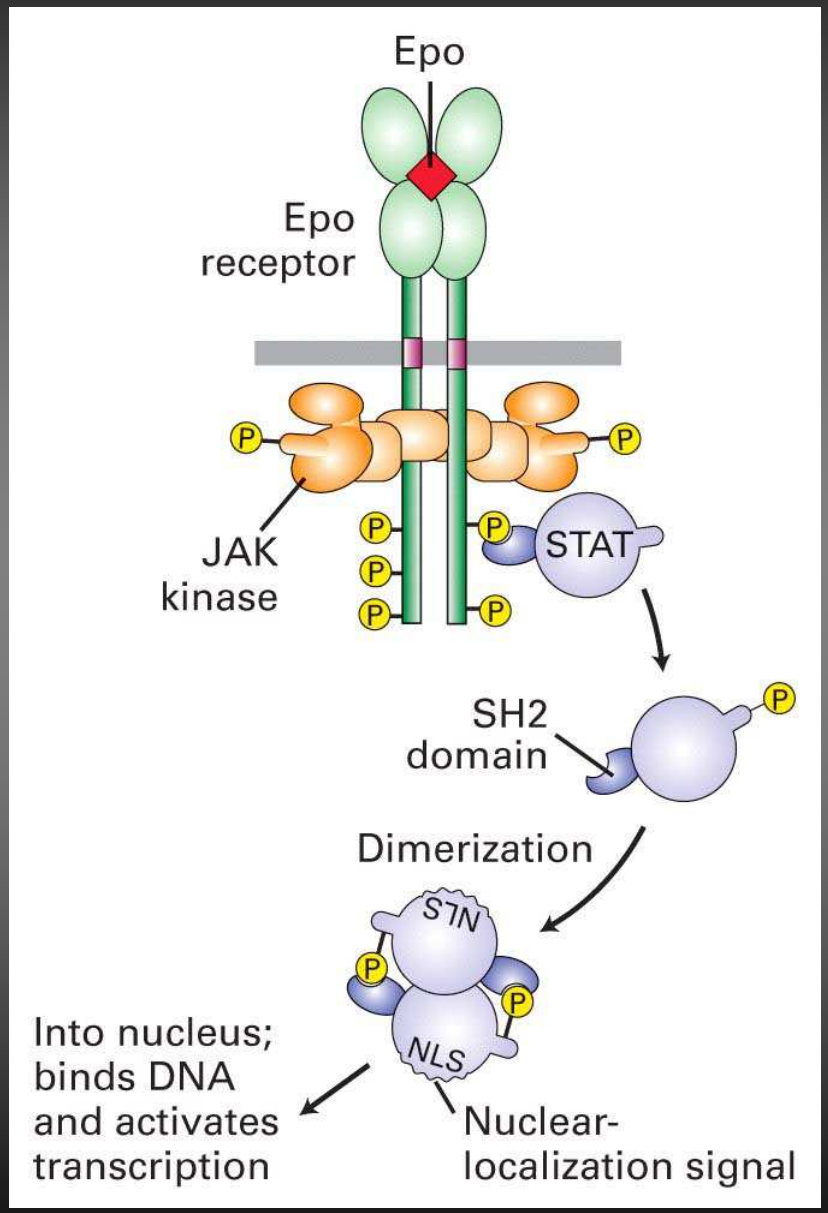


Dimerizzazione di EpoR
Fosforilazione di JAK e attivazione di JAK chinasi



JAK fosforila i residui di Tyr del dominio intracellulare di EpoR

Trasduzione del segnale



4) Legame di STATai residui di fosfo-Tyr di EpoR, mediante il dominio SH2 di STAT

5) Fosforilazione di STAT (fattore di trascrizione)

6) Dissociazione di STAT da EpoR e dimerizzazione di STAT

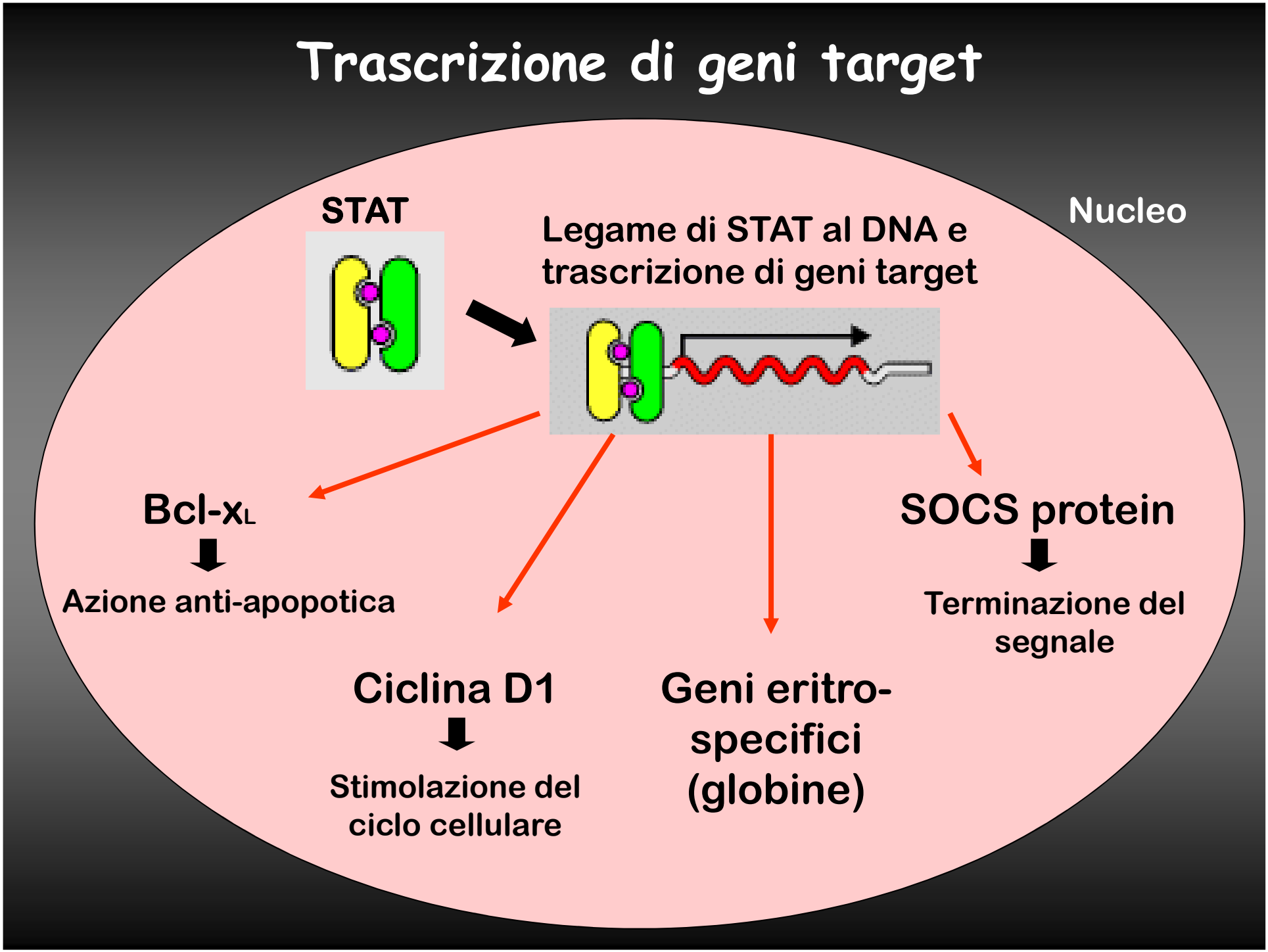
⇩

Esposizione di NLS (nuclear-localization signal)

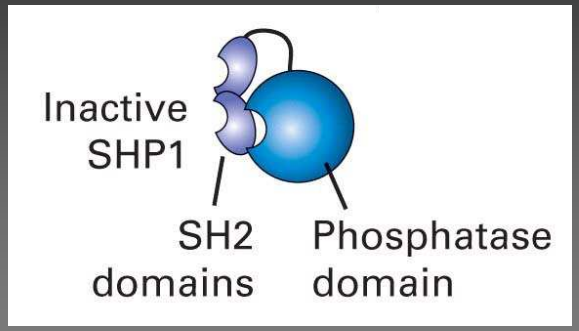
⇩

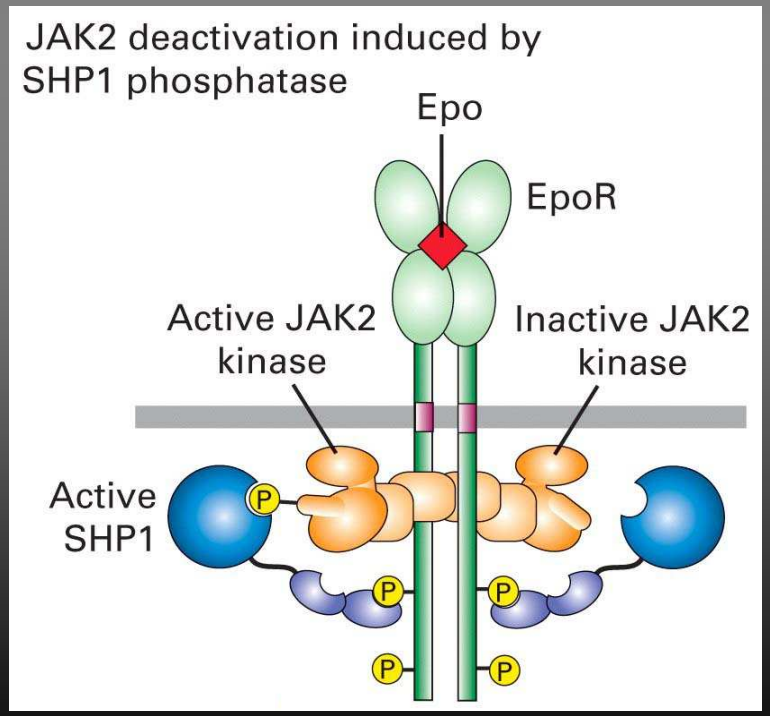
Spostamento di STAT al nucleo e legame a sequenze enhancer specifiche

Trascrizione di geni target



Terminazione del segnale
A breve termine: *SHP1 fosfatasi*





Struttura:

-2 domini SH2

-1 dominio catalitico ad attività fosfatasica

Forma inattiva:

1 dominio SH2 è legato al sito catalitico e lo nasconde

Forma attiva:

il dominio SH2 si lega ad una fosfo-Tyr del recettore

⇩

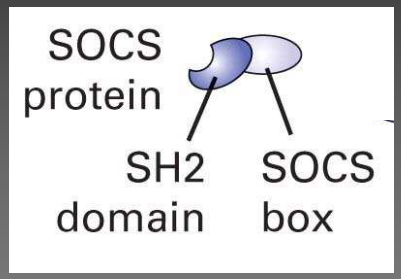
esposizione del sito catalitico, attività fosfatasica nei confronti di JAK

⇩

Inattivazione di JAK e terminazione della trasduzione del segnale

Terminazione del segnale

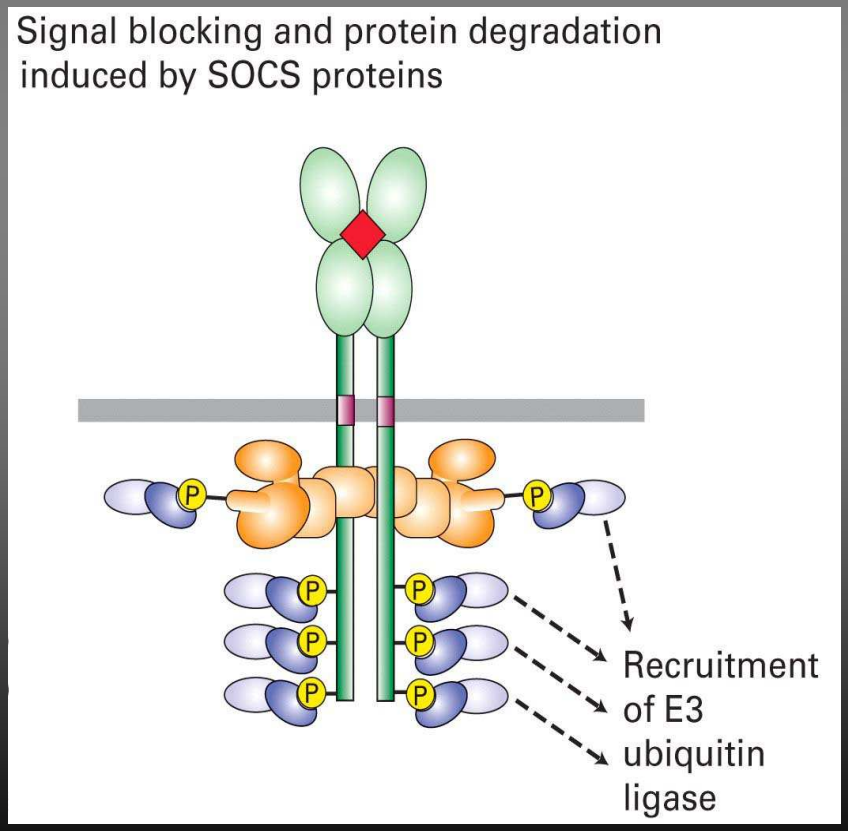
A lungo termine: *SOCS proteins*



Struttura:

-1 dominio SH2

-1 dominio SOCS (SOCS box) → richiama E3 ubiquitina ligasi



Meccanismo d'azione:

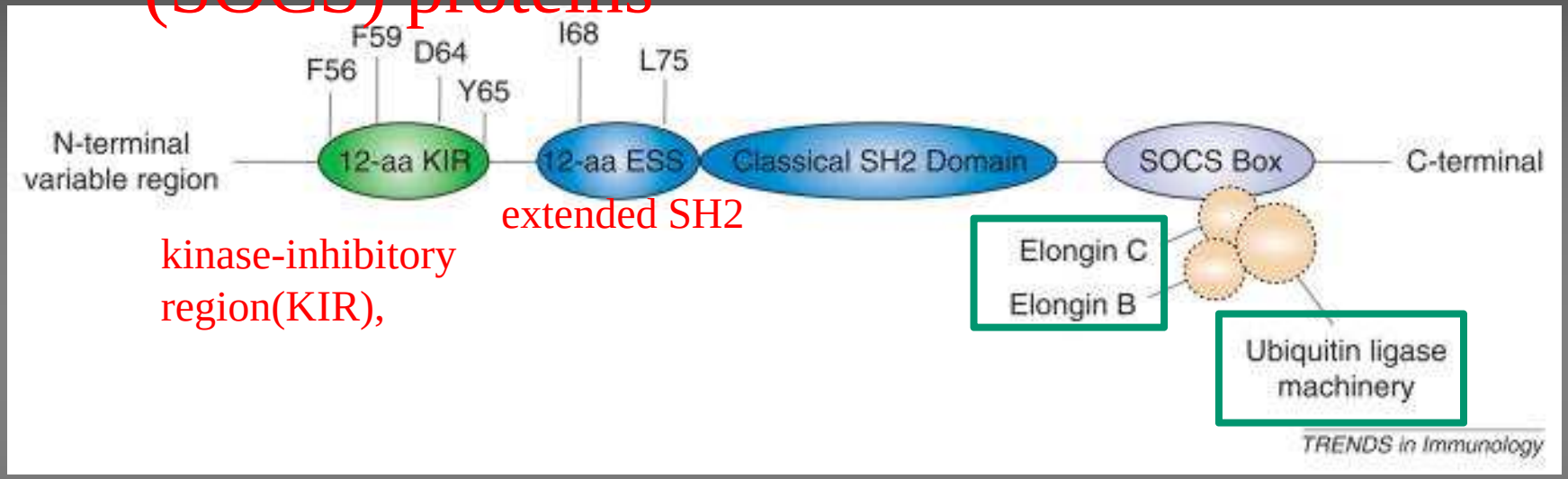
a) Il dominio SH2 si lega alle fosfo-Tyr del recettore: impedisce il legame di STAT

b) Il dominio SOCS richiama E3

⇩

Ubiquinizzazione e degradazione proteosomica di JAK

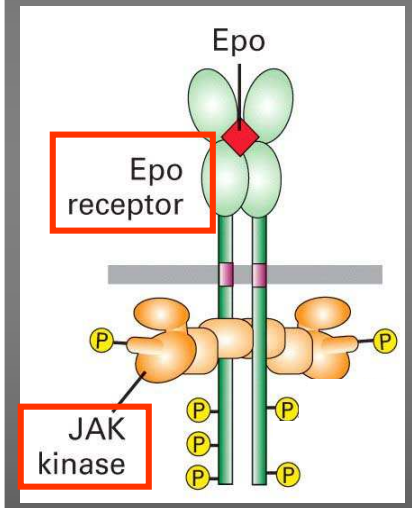
- The suppressor of cytokine signaling (SOCS) proteins



extended SH2

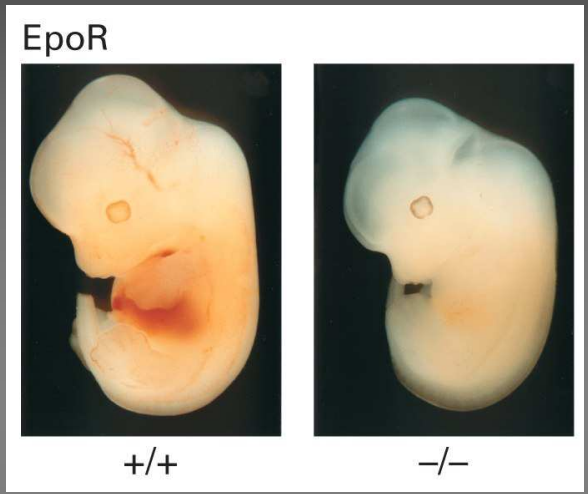
kinase-inhibitory region(KIR),

Trasduzione del segnale Epo-EpoR

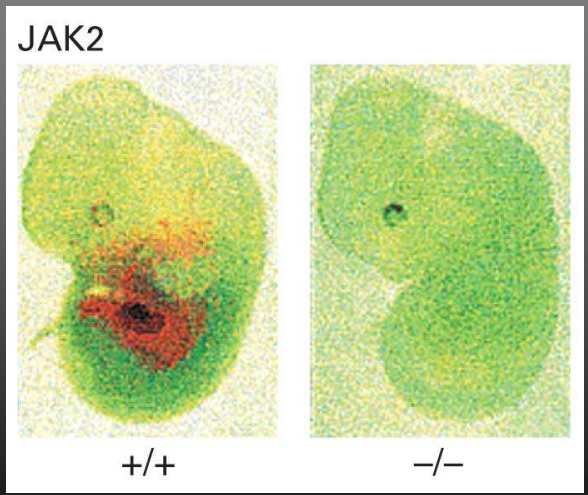


Topi knock-out per EpoR (Wu et al. Cell 1993)

Mancata formazione degli eritrociti → *Morte dell'embrione al 13°giorno per anemia*

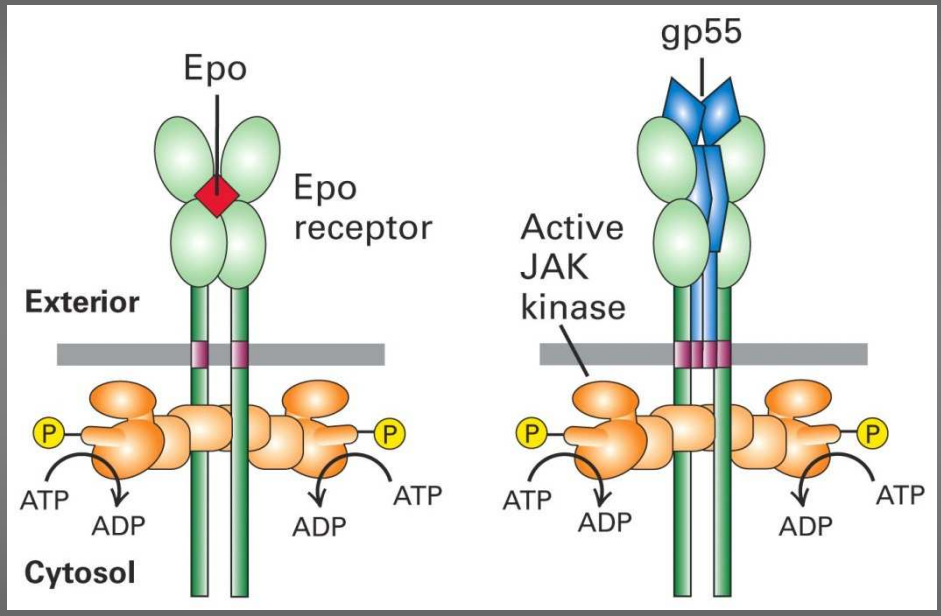


Topi knock-out per JAK (Neubauer et al. Cell 1998)



Trasduzione del segnale Epo-EpoR

Friend spleen focus-forming virus (SFFV) → **Retrovirus murino che causa eritroleucemia**

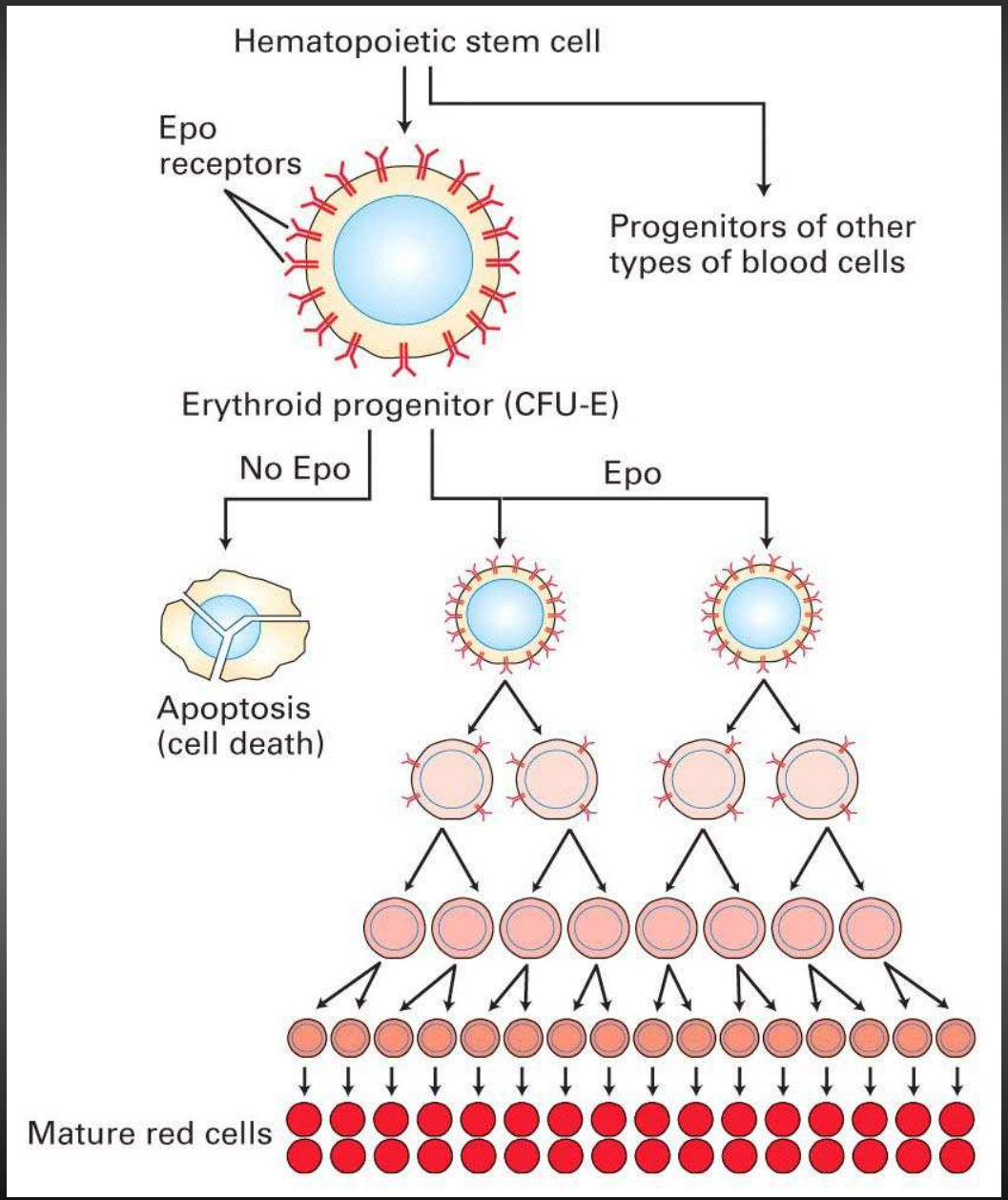


-Legame di gp55 a EpoR

-Attivazione di EpoR

-Proliferazione aberrante degli eritrociti → policitemia

Ruolo dell'Epo nell'eritropoiesi



EpoR è espresso sulla superficie delle cellule eritroidi (massima espressione sulle CFU-E, diminuita sugli stadi più differenziati)

Epo agisce "salvando" dall' apoptosi le cellule progenitrici eritroidi, e stimolandone la maturazione

Epo controls erythrocyte production by preventing apoptosis through activation of Janus kinase 2 (JAK2) and Stat5, which induce expression of the antiapoptotic Bcl2 family member Bcl-xl.

Epo/Bcl-xl-dependent survival is both necessary and sufficient for terminal erythroid differentiation.

Consequently, in mouse models, absence of Epo or its receptor, the Epo effector, Stat5, or the Epo/Stat5 target, Bcl-xl, results in apoptosis of erythrocyte progenitors and anemia.

Epo

down-modulates adhesion factors

Chemokine receptor-4 (*Cxcr4) Integrin* alpha-4 (*Itga4) **mediates binding to vascular cell adhesion molecule 1 (VCAM-1), fibronectin, and paxillin**

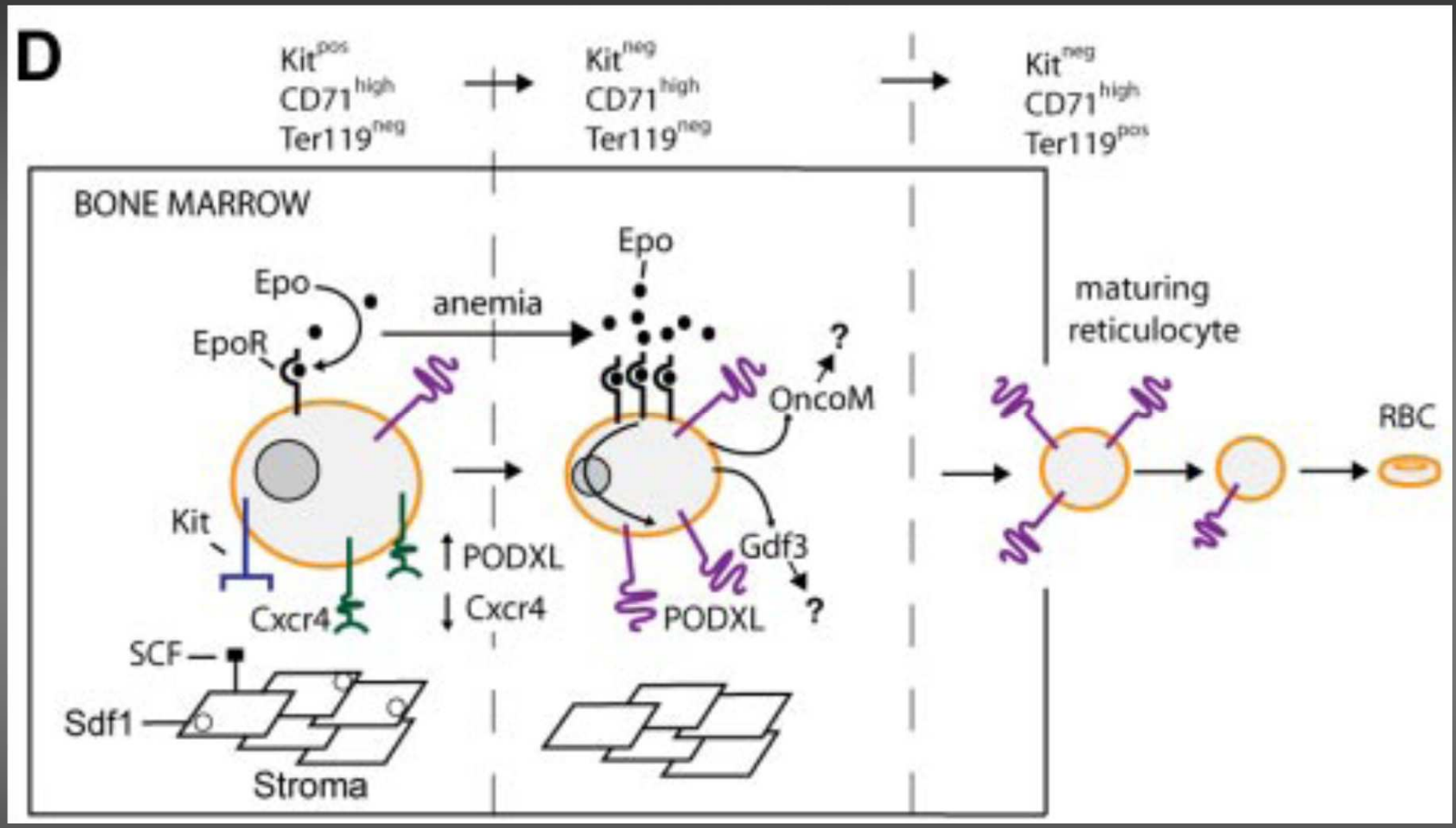
up-modulates
growth differentiation factor-3 (*Gdf3),*
oncostatin-M (*OncoM)* – acts via JAK- Stat- heterodimeric receptor 19 and affects cell growth, differentiation,

Podocalyxin* like-1 (*PODXL)?

Mature mucins are composed of two distinct regions:
The amino- and carboxy-terminal regions are very lightly glycosylated, but rich in cys.
The cys residues participate in establishing disulfide linkages within and among mucin monomers.
A large central region formed of multiple tandem repeats of 10 to 80 residue sequences in which up to half of the aa ser thr.
This area becomes saturated with hundreds of O-linked oligosaccharides. N-linked oligosaccharides are also found

Sialomucin - acid mucopolysaccharide containing sialic acid

Model for Epo regulation of erythroid progenitor cell adhesion and migration within stromal niche



PODXL is a sulphated sialomucin, antiadhesive


USO TERAPEUTICO DI
Epo

Stati Patologici legati all'eritropoietina

Anemia

Inadeguata produzione endogena
(es. patologia renale)

↓

Carenza di globuli rossi

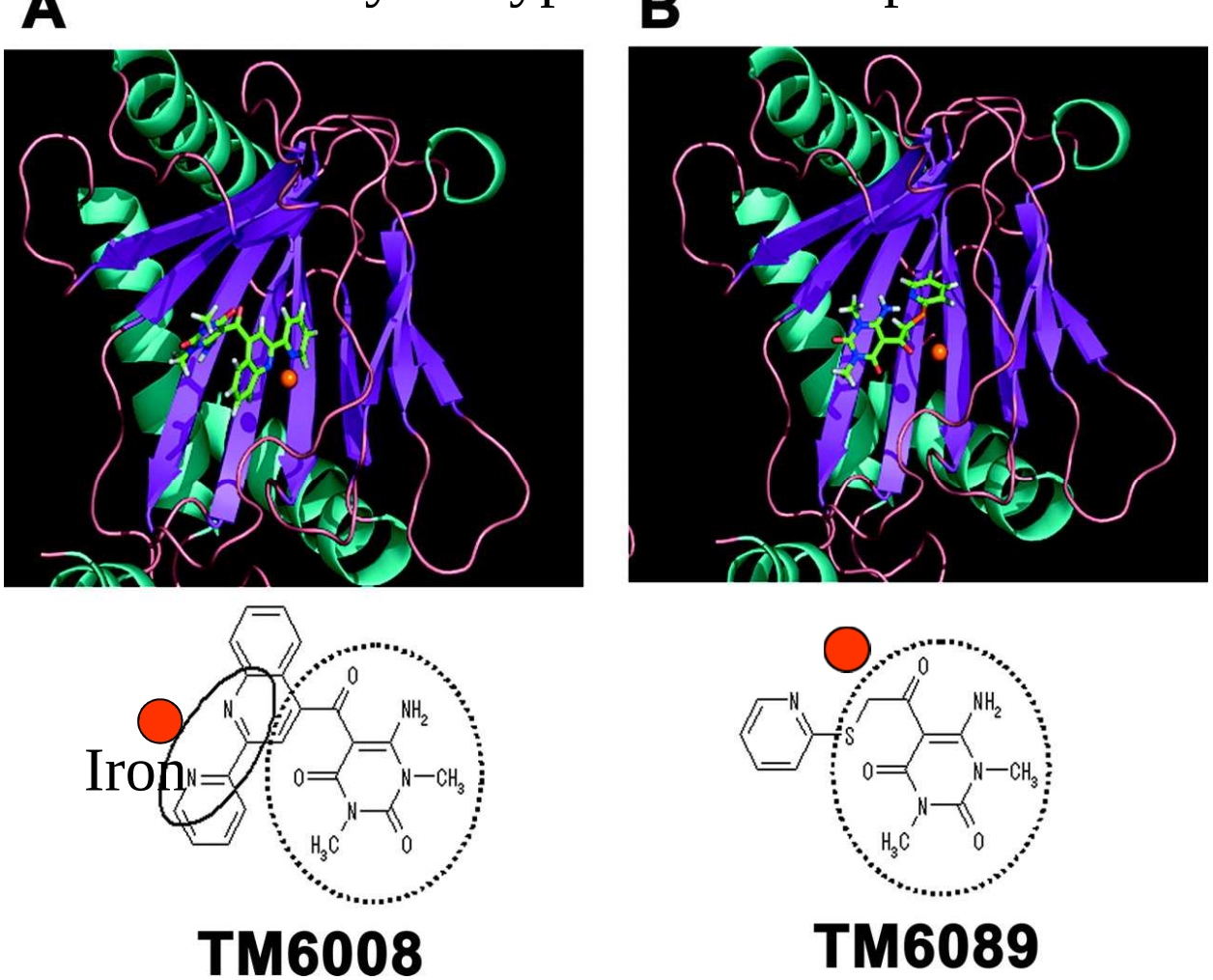
↓

Anemia

HIF prolyl hydroxylase inhibition results in endogenous erythropoietin induction, erythrocytosis

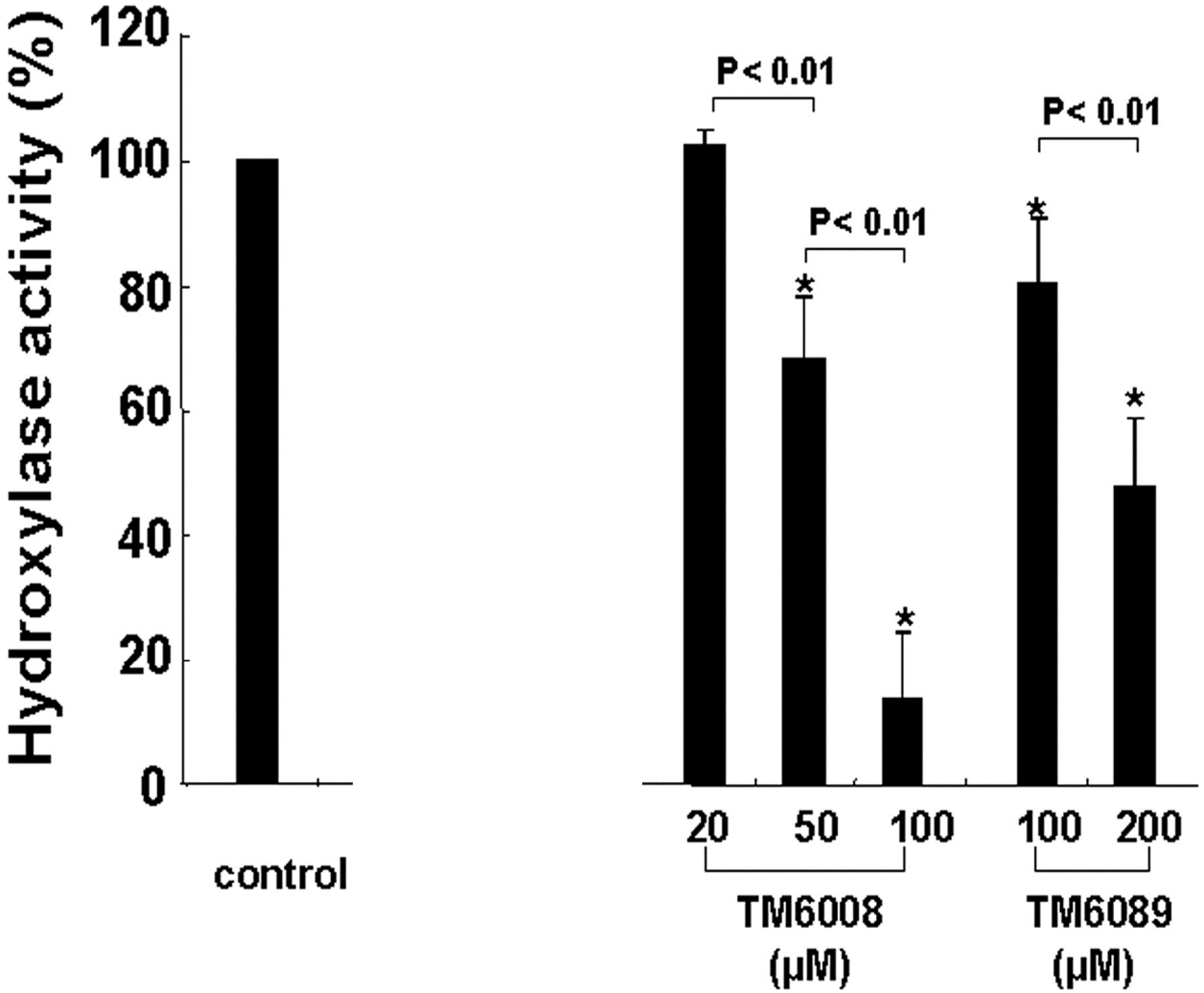
Figure 3. The predicted binding modes of TM6008 (A) and TM6089 (B) in PHD2.

PHD produces trans-4-hydroxyproline in the presence of Fe(II)



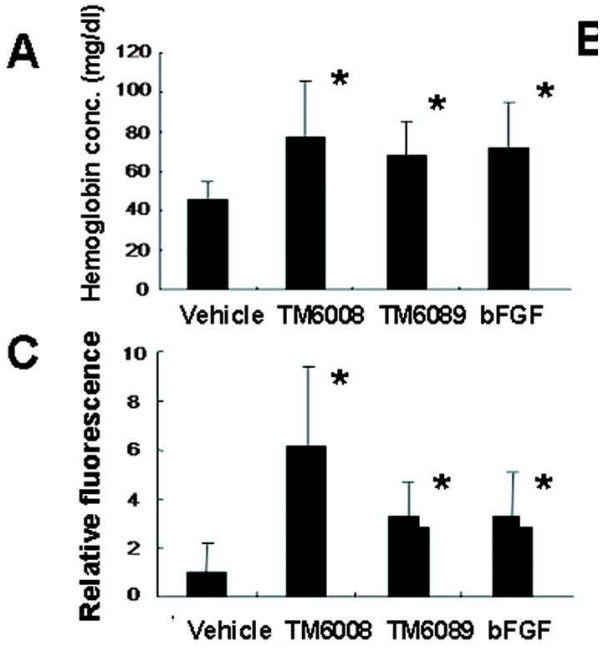
Nangaku M et al. Arterioscler Thromb Vasc Biol 2007;27:2548-2554

Figure 2. Inhibition of PHD activity.



Nangaku M et al. Arterioscler Thromb Vasc Biol 2007;27:2548-2554

Figure 4. Stimulation of angiogenesis in the mouse



Nangaku M et al. Arterioscler Thromb Vasc Biol 2007;27:2548-2554

Trattamento dell'anemia

Epo ricombinante (rHuEPO)

Produzione su larga scala di Epo umana ricombinante

rHuEPO

- ✓34000 Da
- ✓prodotta in cellule mammarie in cui è stato introdotto il gene dell'Epo

Novel Erythropoiesis Stimulating Protein (NESP)

NESP (darbepoetin):

- ✓38500 Da
- ✓Aumentato contenuto di carboidrati, che conferiscono un aumento dell'emivita
- ✓Somministrazione meno frequente

Epo contains one O-linked and three N-linked carbohydrate chains, each having 2–4 branches that often end in a negatively charged sialic acid.

These carbohydrate chains are not required for receptor binding in vitro or stimulation of growth of EpoR-expressing cultured cells but are required for the in vivo bioactivity

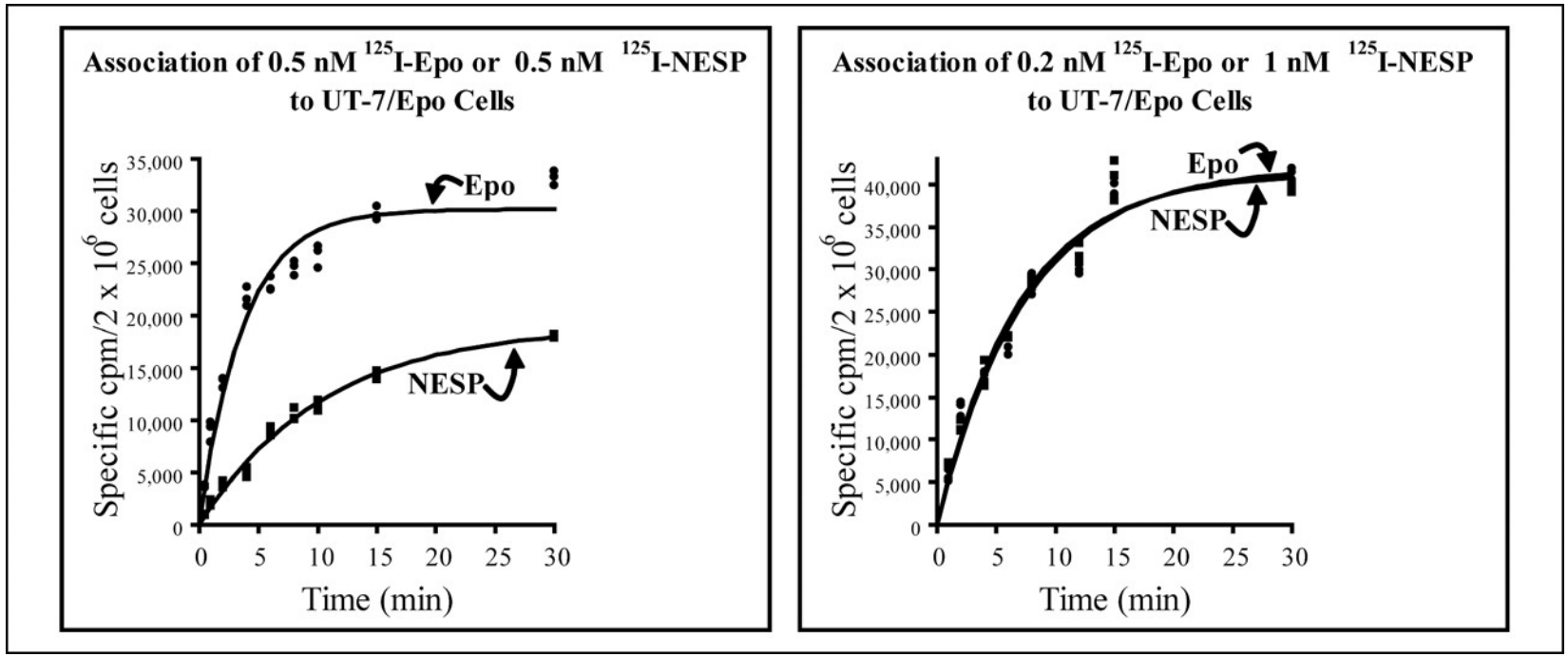
Heterogeneous branching of Epo N-linked carbohydrates results in Epo isoforms with different sialic acid contents up to a maximum of 14.

residues are mutated to provide for 2 additional N-linked glycosylation sites

Epo isoforms with higher sialic acid content have a lower affinity for EpoR but a longer serum half-life and are more effective for stimulating the production of red blood cells in vivo.

How Epo is cleared from the circulation and degraded?

Net binding of 125I-Epo or 125I-NESP with UT-7/Epo cells at 37 ℃.

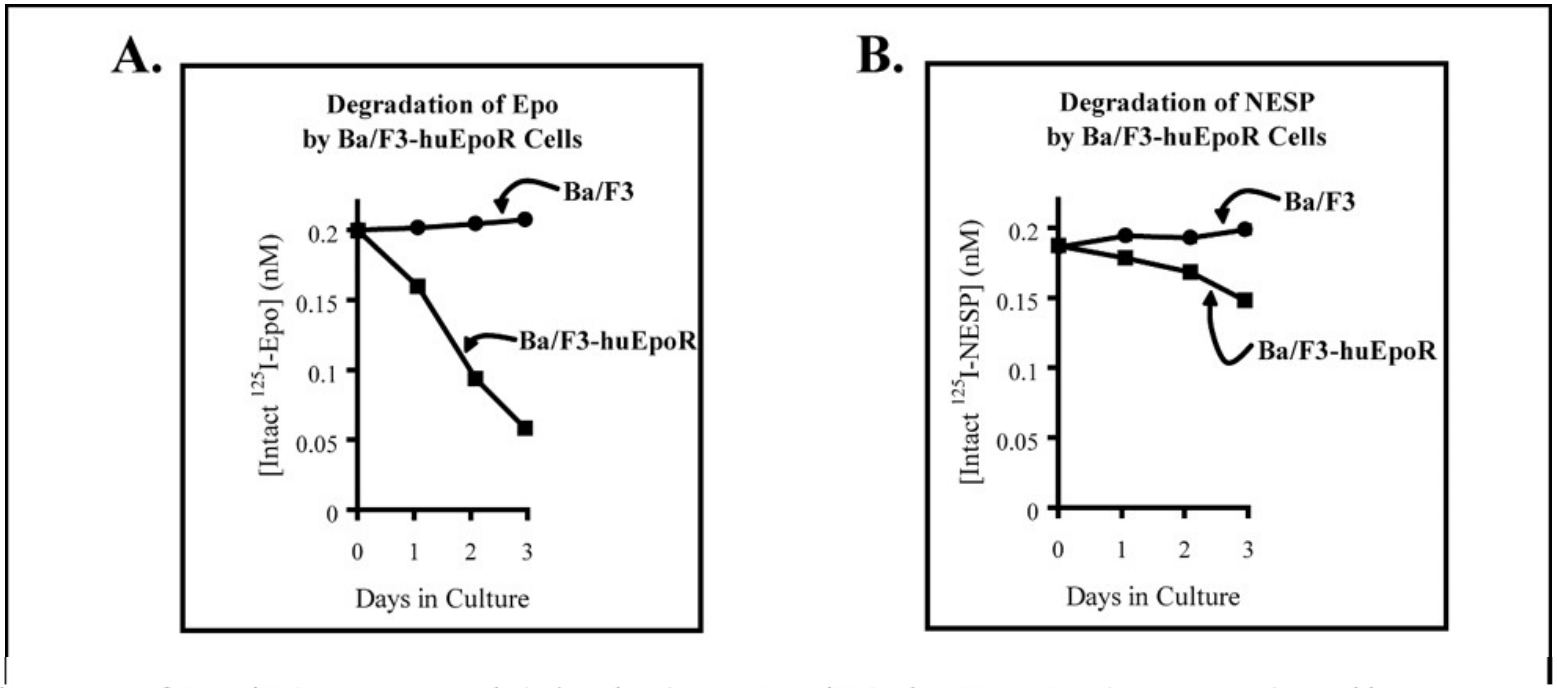


Cells were preincubated at 37 °C for 5 min with endocytosis inhibitors (0.1% sodium azide and 10 μg/ml cytochalasin B) then 125I-labeled ligand was added. Cells were collected and rapidly separated from the medium after the indicated then cell-associated radioactivity was measured. The

Gross A W , Lodish H F J. Biol. Chem. 2006;281:2024-2032

jbc

Degradation and endocytosis of Epo and NESP by Ba/F3-huEpoR cells.

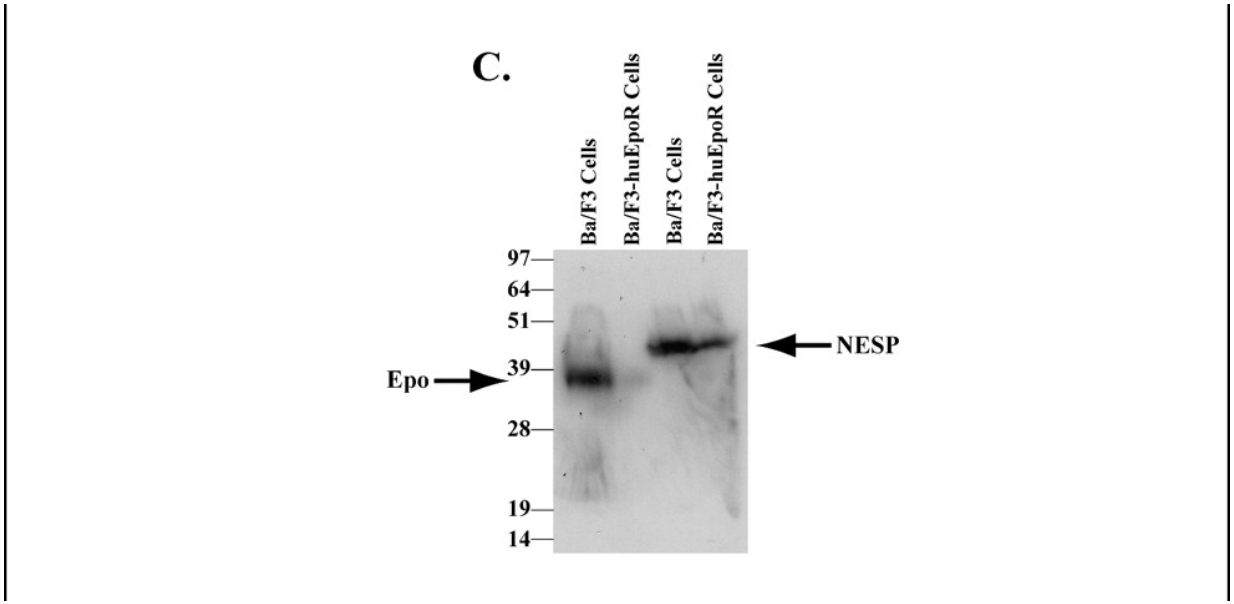


cultures of Ba/F3 parental (circles) or Ba/F3-huEpoR (squares) cells were initiated with excess IL-3 and 0.2 nm 125I-Epo (A) or 0.2 nm 125I-NESP (B)

Gross A W , Lodish H F J. Biol. Chem. 2006;281:2024-2032

jbc

Degradation and endocytosis of Epo and NESP by Ba/F3-huEpoR cells.



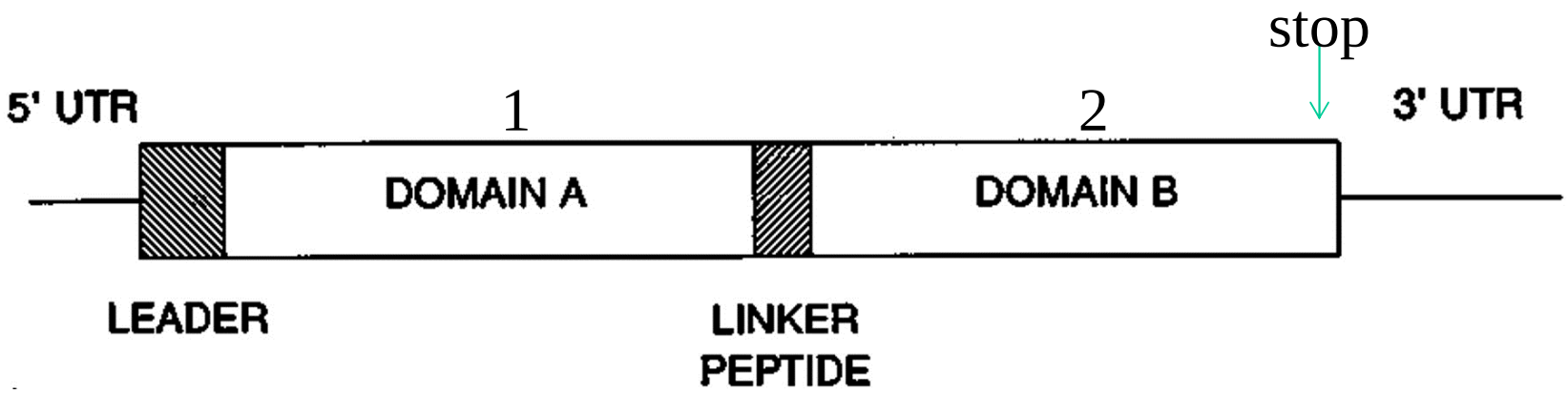
cultures of Ba/F3 parental (circles) or Ba/F3-huEpoR (squares) cells were initiated with excess IL-3 and 0.2 nm 125I-Epo (A) or 0.2 nm 125I-NESP (B)after the third day in culture, proteins precipitated by trichloroacetic acid from the media of the cultures shown in A and B were separated by SDS-PAGE and analyzed by autoradiography. The type of cells cultured with each sample is indicated at the top of each lane. The position of intact Epo and NESP proteins are indicated by arrows. Numbers indicate the size in kDa and position of prestained molecular weight markers.

Gross AW, Lodish HF. J. Biol. Chem. 2006;281:2024-2032

jbc

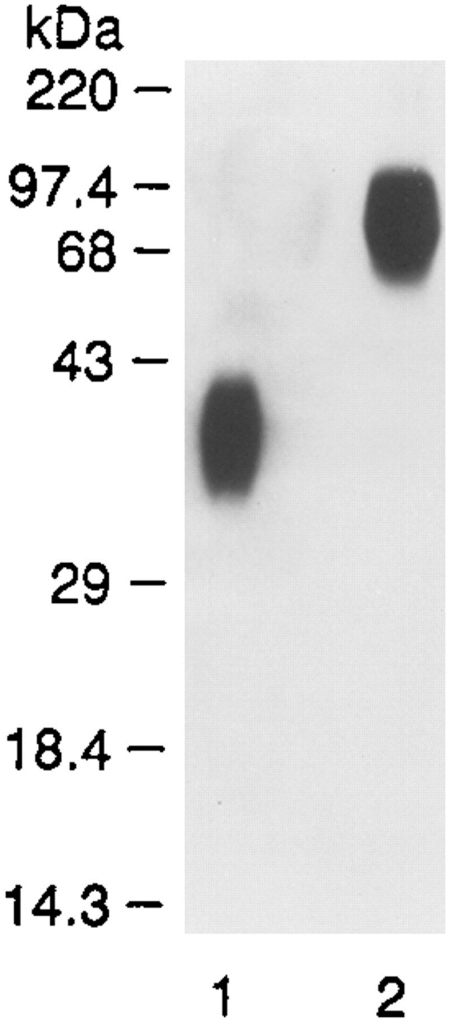
Epo-Epo” -a peptide-linked head-to-tail dimer

Diagram of cDNA encoding the Epo-Epo fusion protein.



Sytkowski A J et al. J. Biol. Chem. 1999;274:24773-24778

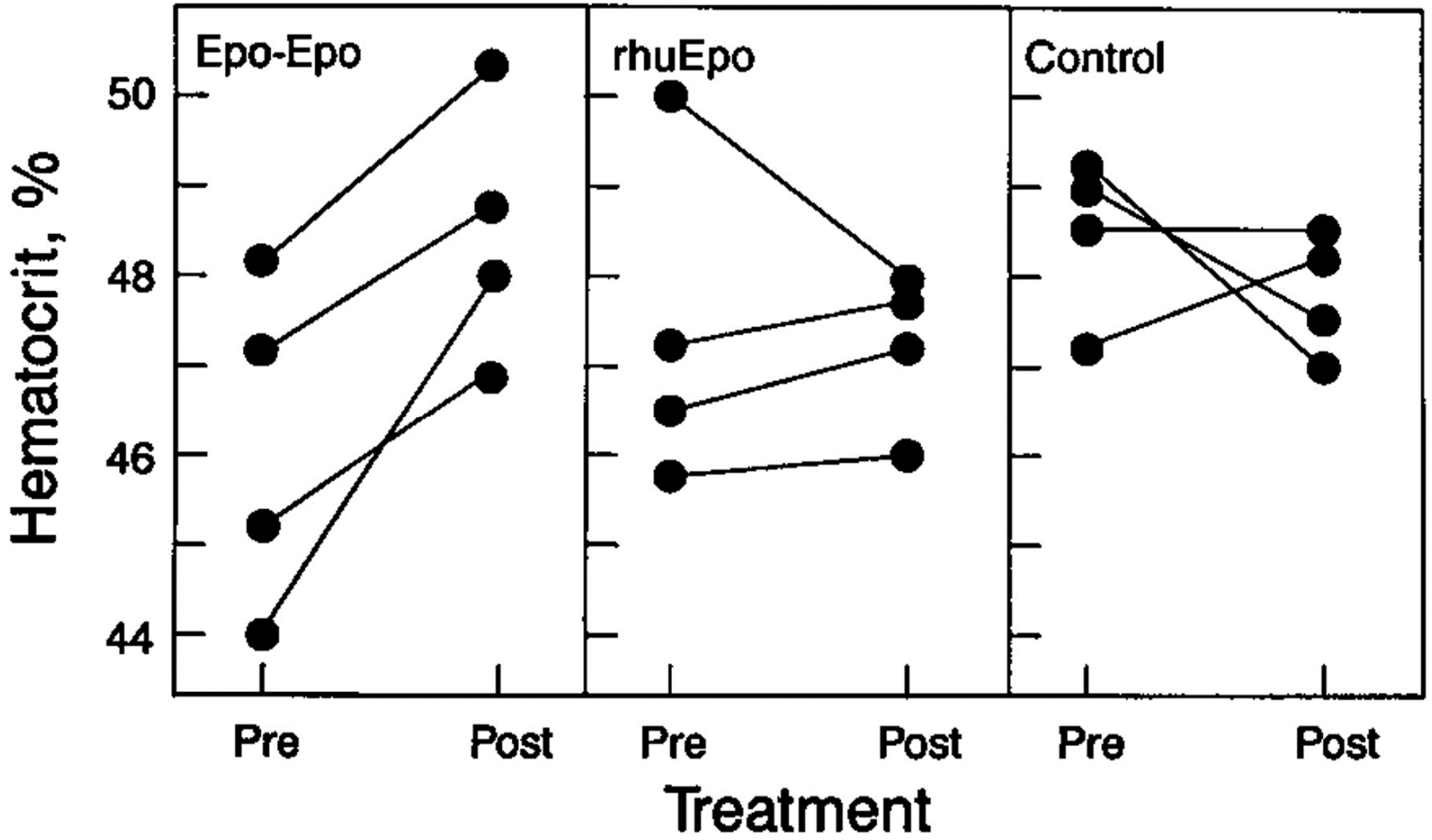
Western blot of purified recombinant Epo (lane 1) and the supernatant of COS1 cells transfected with Epo-Epo cDNA (lane 2).



Sytkowski A J et al. J. Biol. Chem. 1999;274:24773-24778

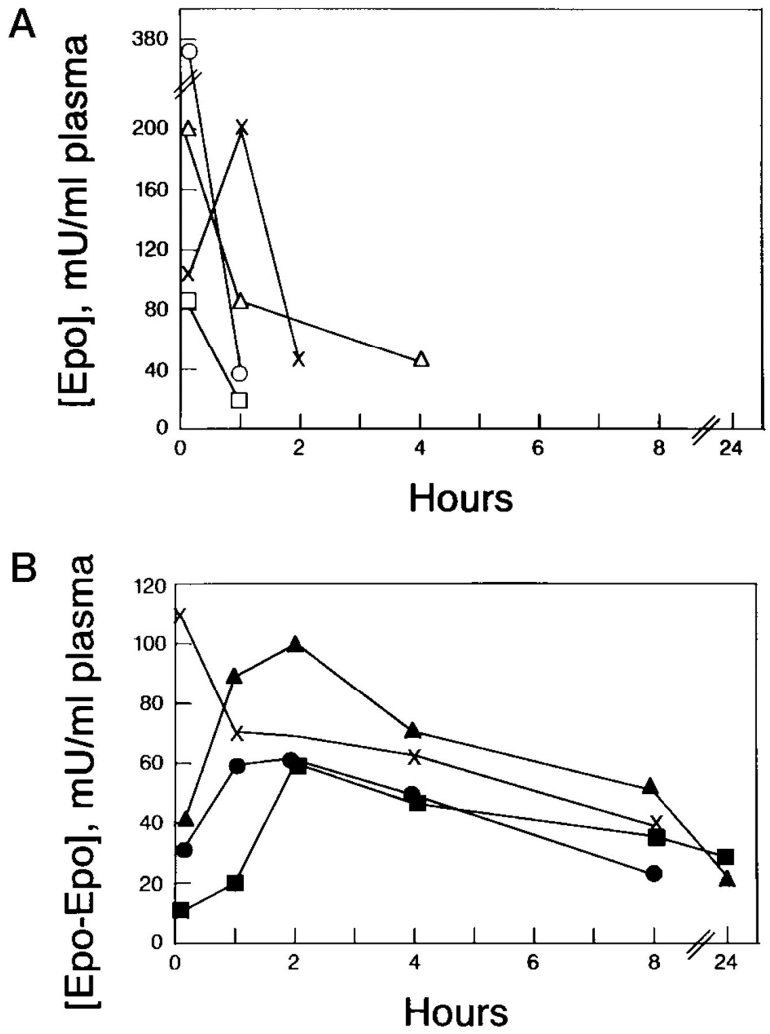
jbc

In vivo efficacy of Epo-Epo compared with that of conventional Epo .



Sytkowski A J et al. J. Biol. Chem. 1999;274:24773-24778

Pharmacokinetics of Epo (A) and Epo-Epo (B) in mice.



Sytkowski A J et al. J. Biol. Chem. 1999;274:24773-24778

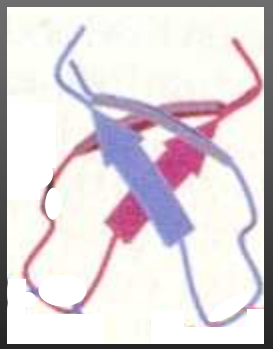
"Hormone mimicry"

Una piccola molecola può "mimare" la funzione di un grande ORMONE POLIPEPTIDICO

Wrighton et al, Science 1996

Sintesi di piccoli peptidi (20 aa) che si legano al recettore dell'Epo e lo attivano → "mimano" l'effetto biologico dell'Epo

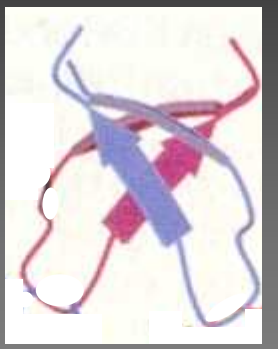
EMP1

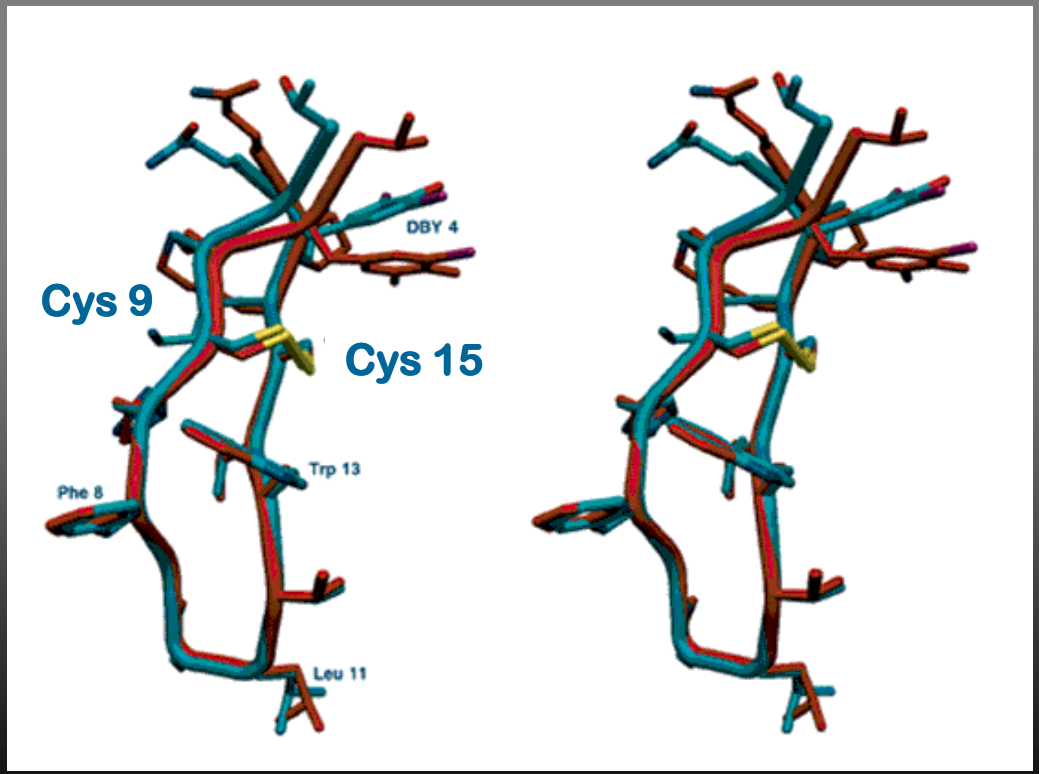


Eritropoietina

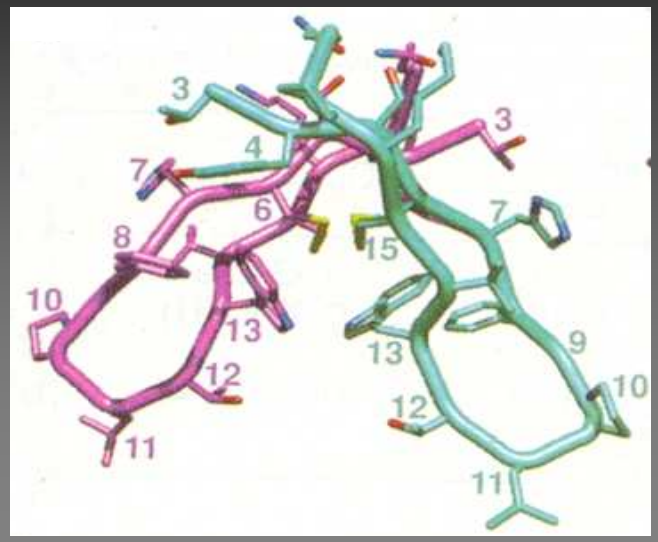
EMP1 (EPO mimetic peptides (EMPs)

- ✓ Peptide di 20 aa (2 kDa): GGTYSCHFGPLTWVCKPQGG
- ✓ Struttura: 2 corti ß-foglietti uniti da un ponte disolfuro
- ✓ Sintesi: ottenuto da una libreria di peptidi random prodotti in sistema fagico (phage display); selezionato mediante saggi di legame alla porzione extracellulare di EpoR



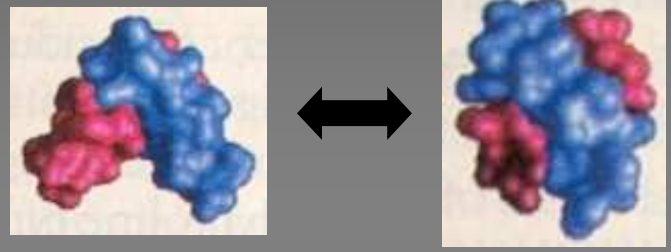


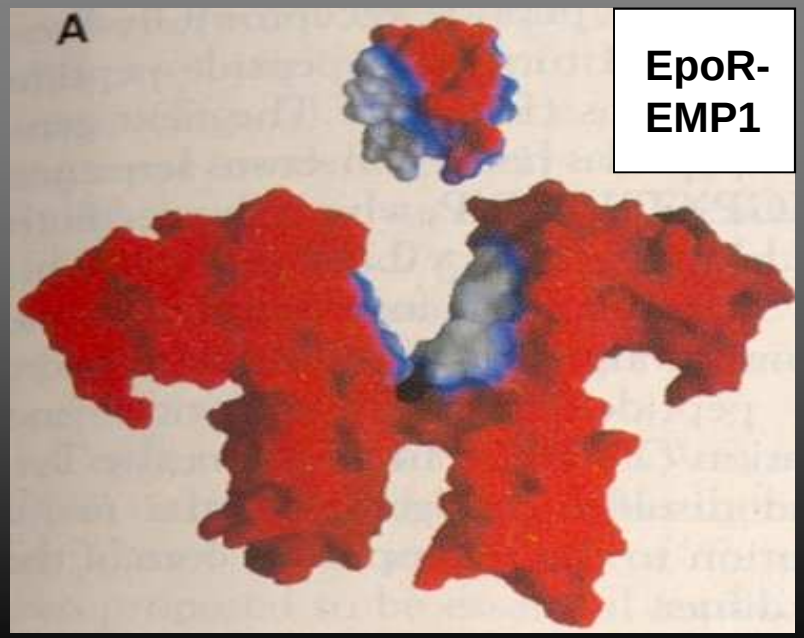
Complesso EpoR-EMP1



EMP1 dimerizza per legarsi a EpoR

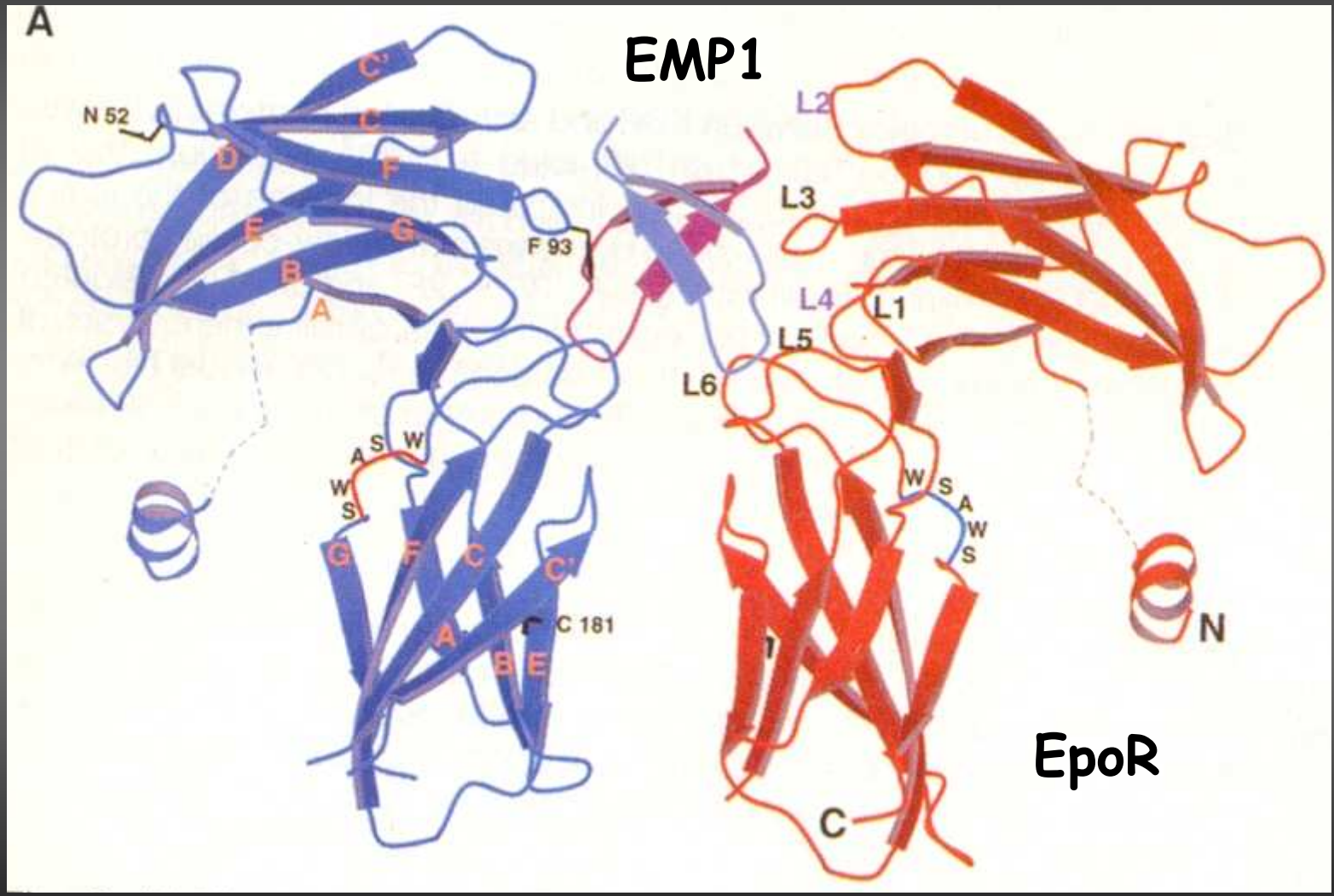
Struttura dimerica molto forte, stabilizzata da 4 legami idrogeno





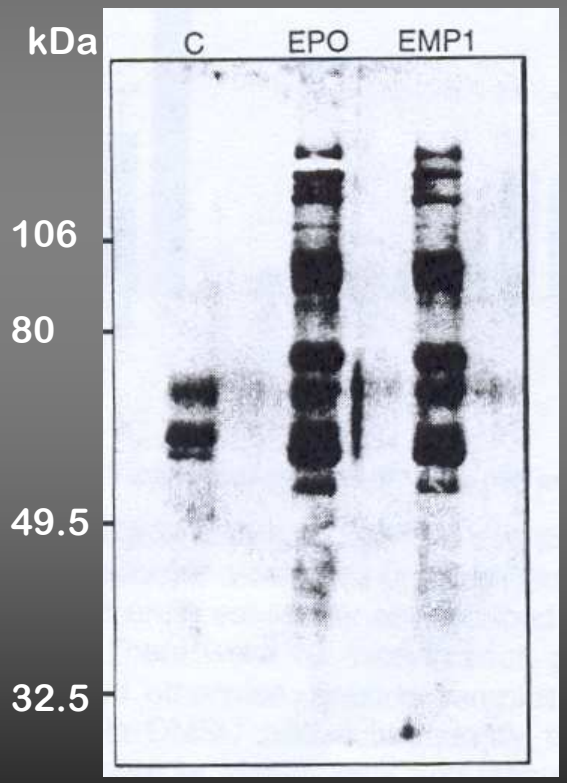
Ogni monomero di EMP1 interagisce sia con l'altro monomero che con EpoR

Complesso EpoR-EMP1



EMP1 stimola l'eritropoiesi attraverso la *stessa via di trasduzione del segnale* indotta da Epo

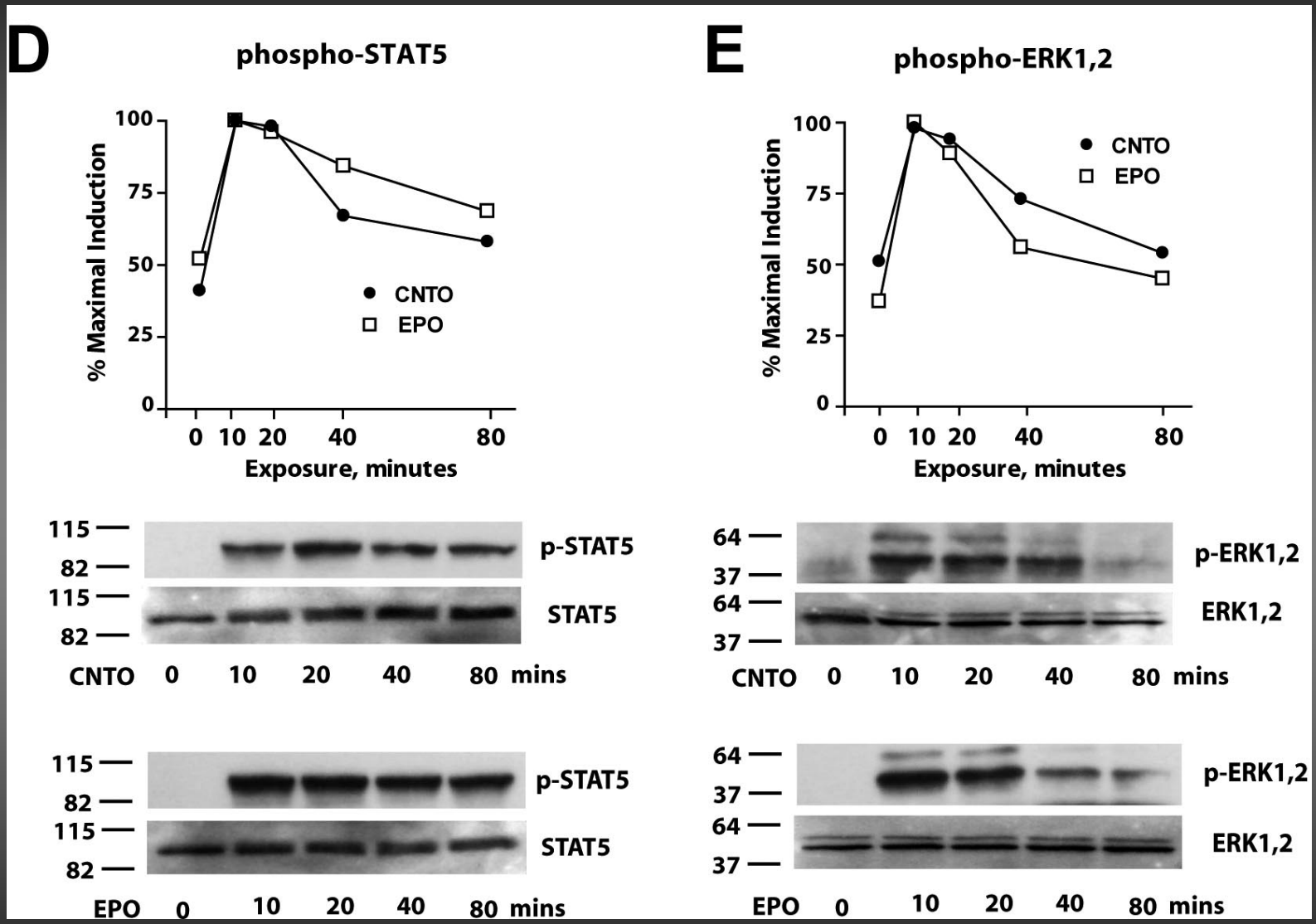
Western blot (anticorpo anti-fosfoTyr)



Cellule stimolate con EMP1 e con Epo presentano lo stesso pattern di fosforilazione

Wrighton et al., Science 1996, 273:458-463

CNTO 530 activates known EPO signal transduction pathways



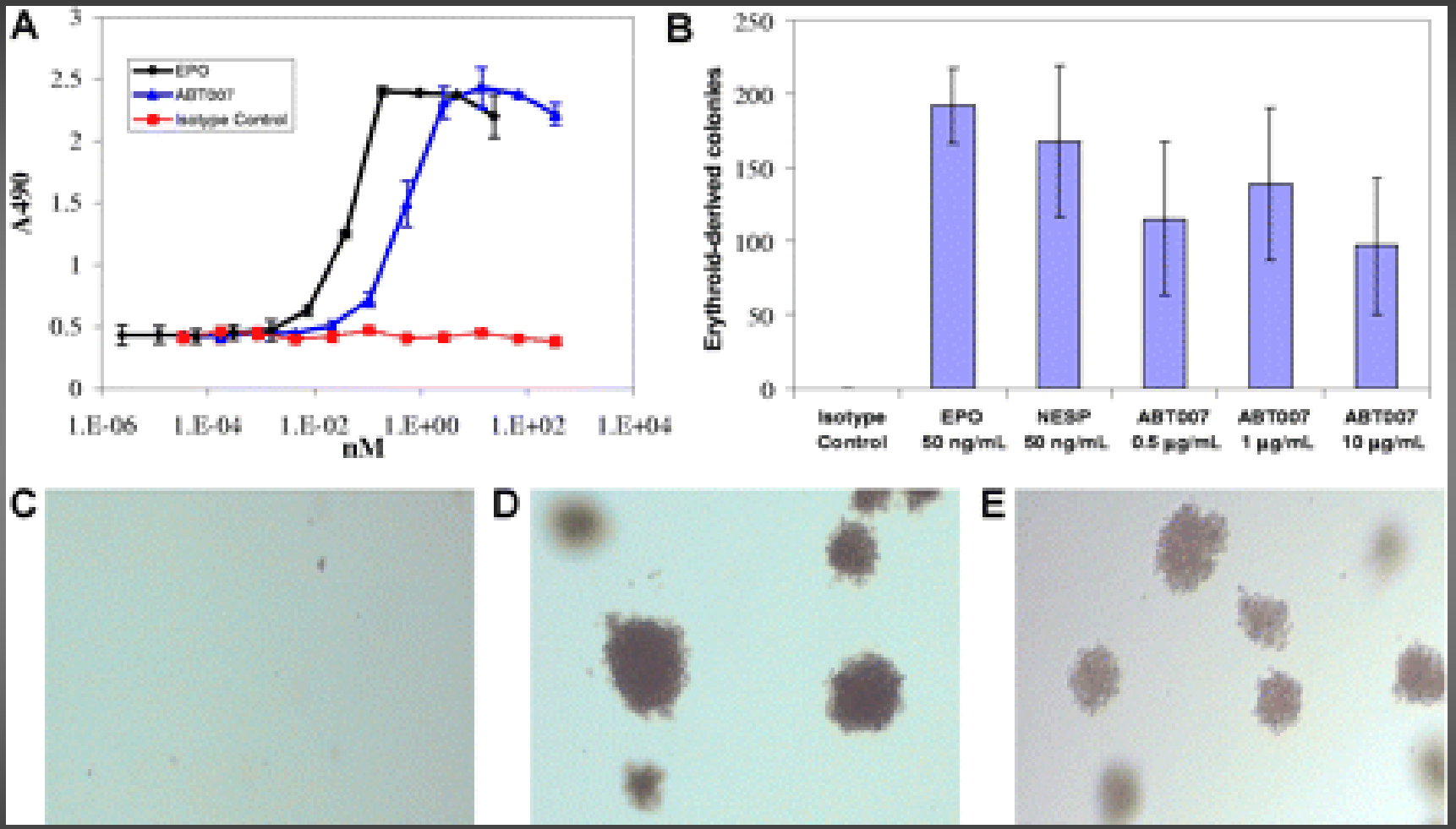
CNTO 530 is a dimeric EMP fused to a human lgG4 Fc

"Hormone mimicry"

EMP1 **è la dimostrazione che una molecola di 20 aa può mimare la funzione di un ormone**

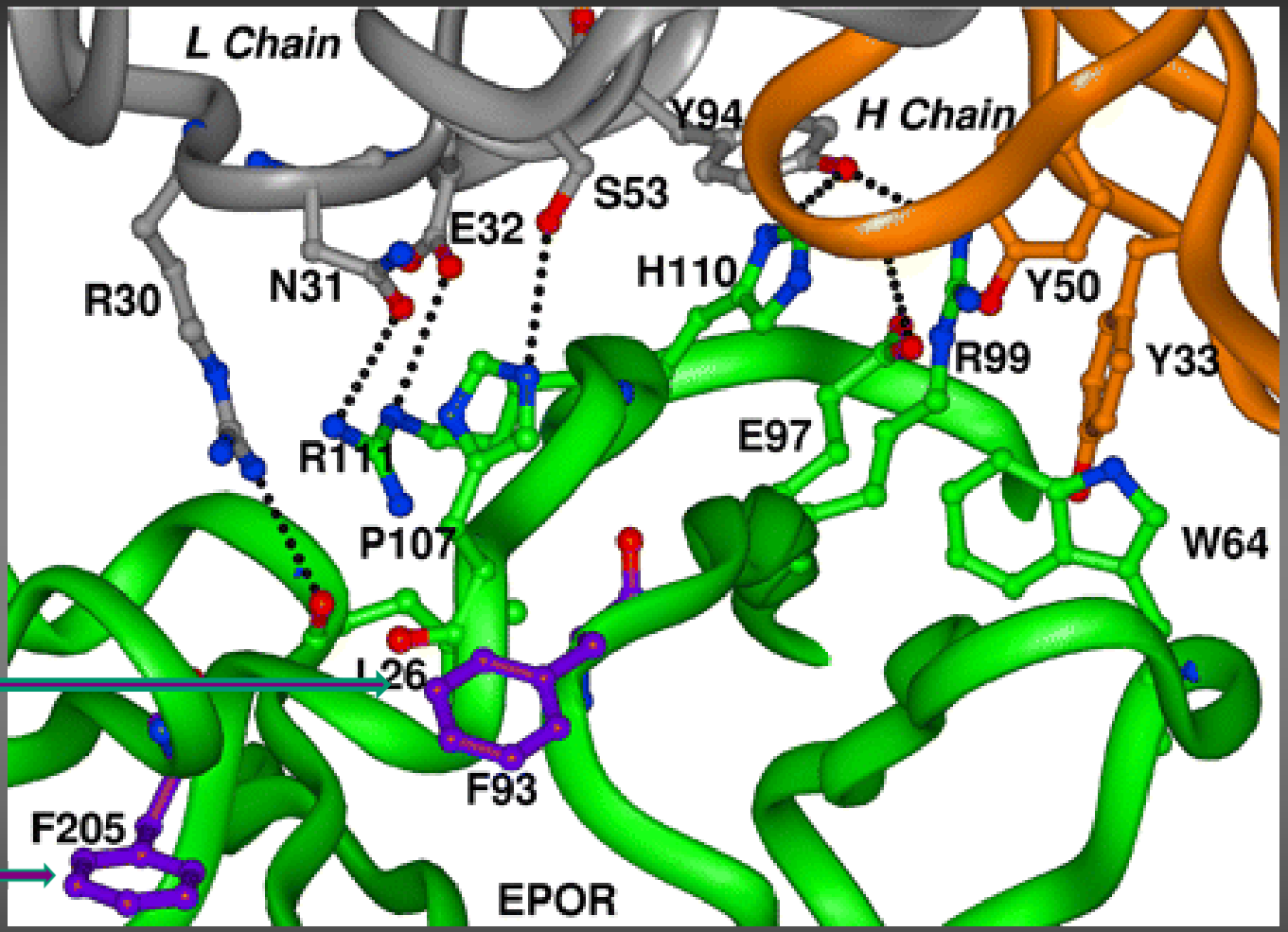
- ✓ **Stimolando la stessa via di trasduzione del segnale (JAK, STAT…)**
- ✓ **Senza avere nessuna omologia di sequenza o struttura con l'ormone**

A potent erythropoietin-mimicking human antibody



ABT007 stimulates in vitro erythropoiesis

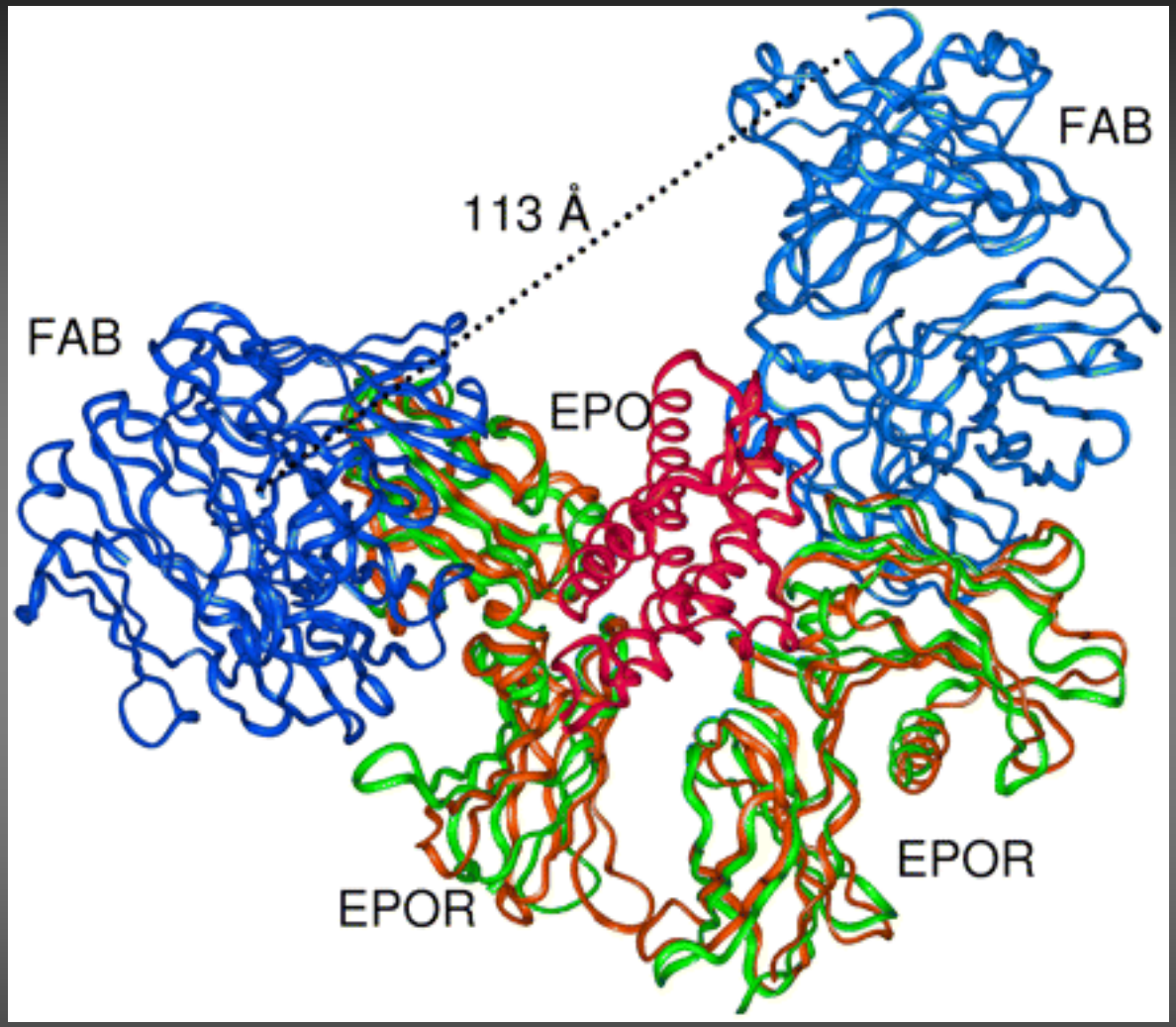
The antibody interacts through a novel binding site



Epo binding

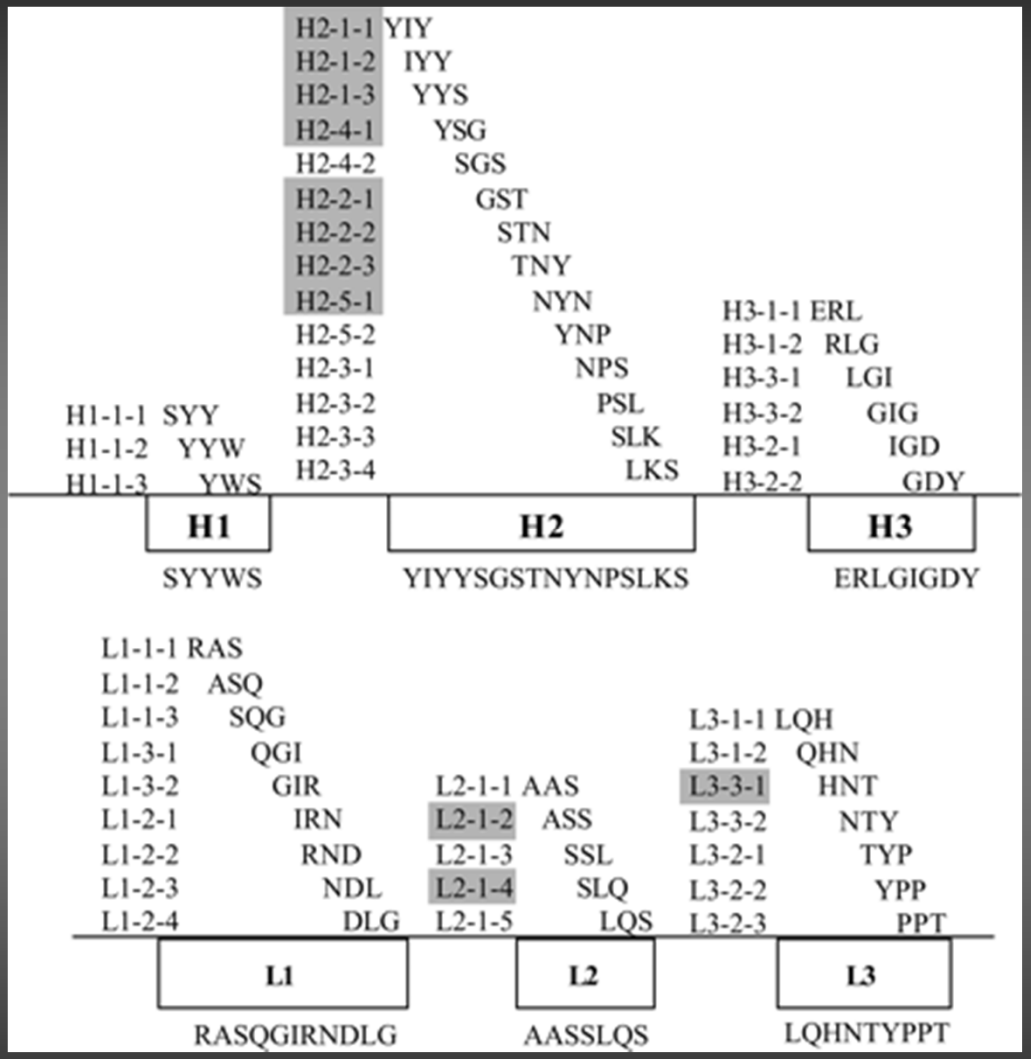
F93 and F205 of EPOR, highlighted in purple, are key residues involved in binding EPO and are not involved in Fab binding.

Comparison of the Fab-EPOR complex with the EPO-activated EPOR

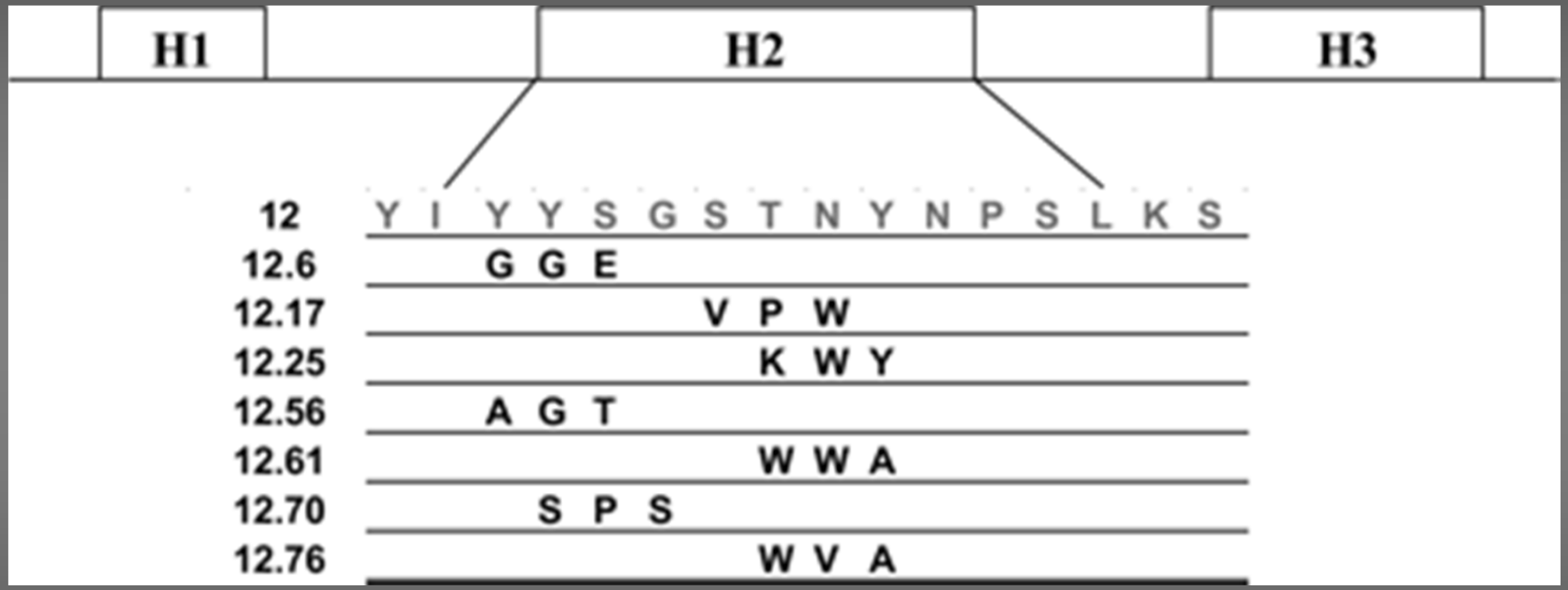


A model of activation based on a conformation induced onto EPOR by ABT007 in a 2:1 ratio that is different from that caused by EPO.

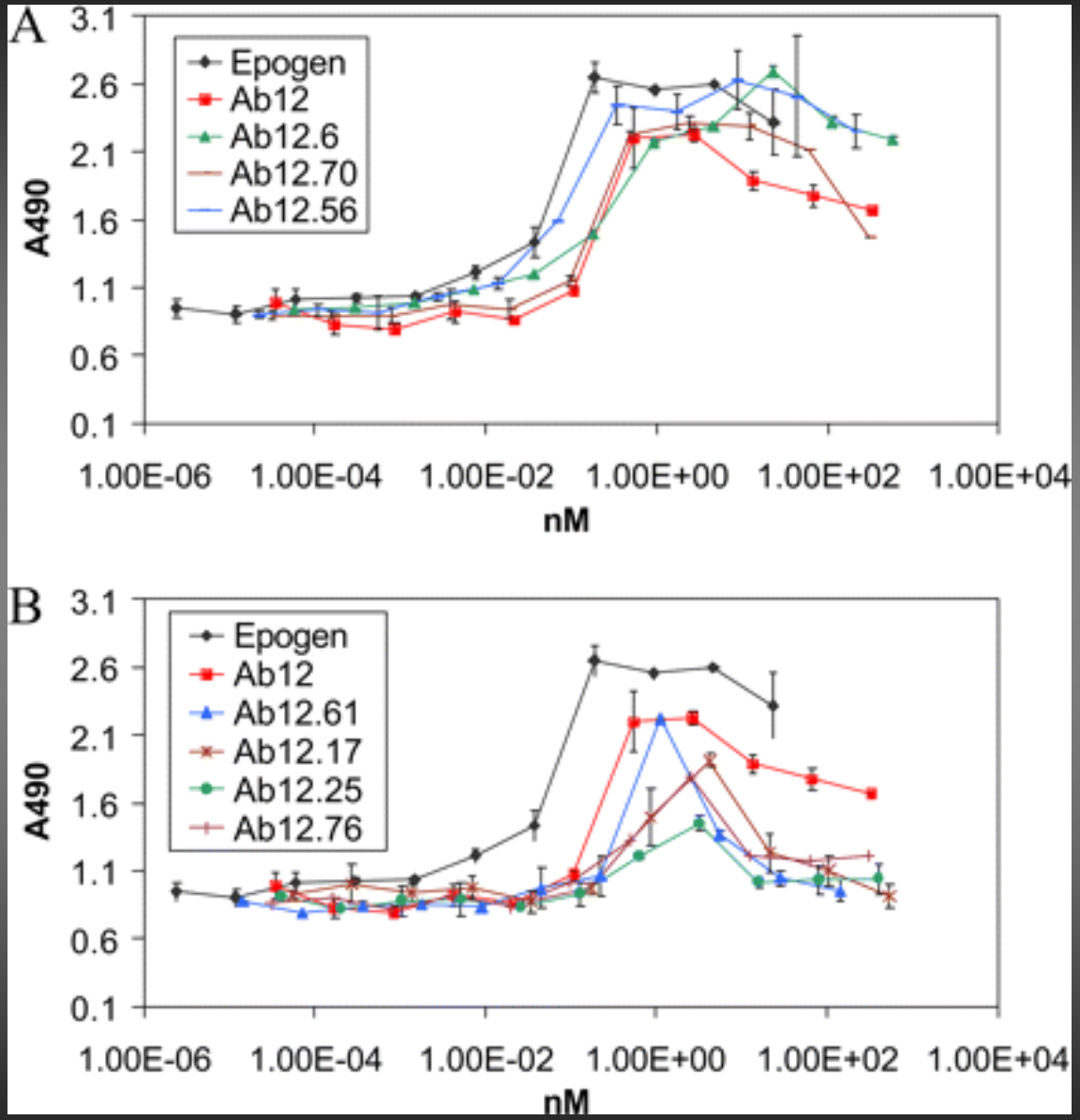
Ab12 scFv CDR VH and VL yeast libraries



Ab12 CDR H2 variants



EPO-dependent cell proliferation activity of Ab12 variants

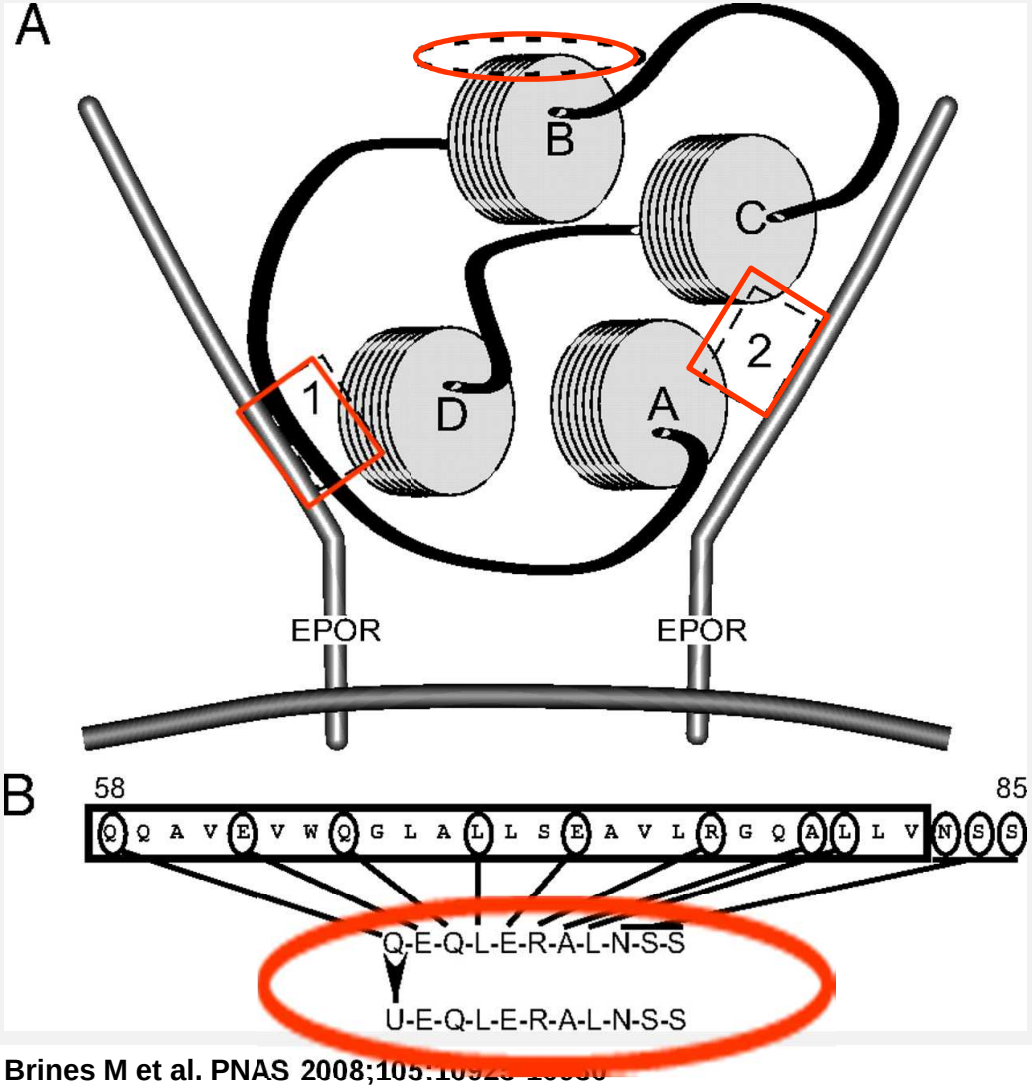


activity of Ab12 variants correlates inversely with Kd

EPO's tissue-protective actions have been shown to be mediated by a tissue-protective receptor complex consisting of the EPO receptor and the β common-receptor (CD131) subunit that is also used by GM-CSF, IL-3, and IL-5.

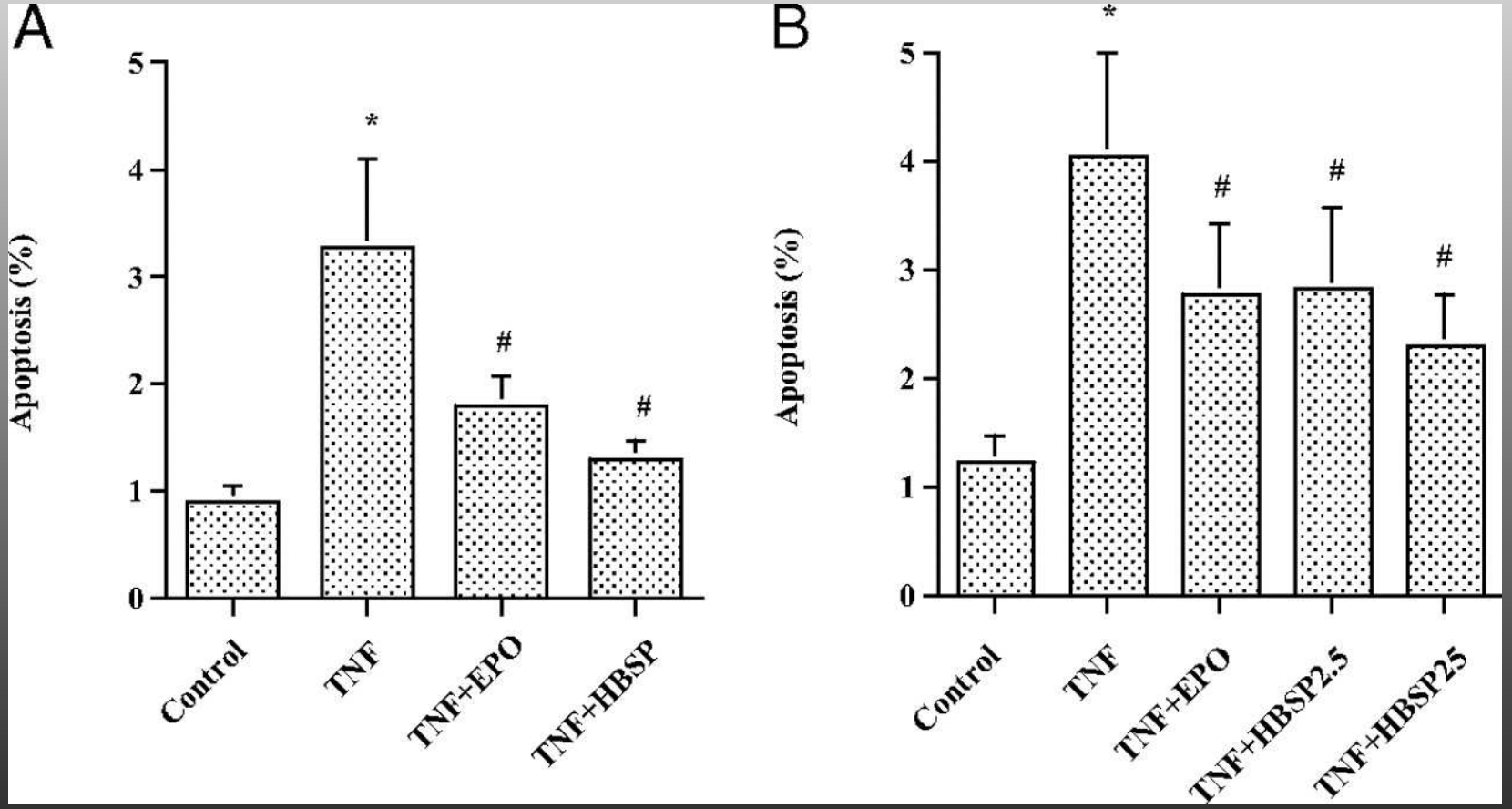
helix B-surface peptide (HBSP). This peptide is composed of 11 amino acids (QEQLERALNSS) derived from the aqueous face of helix B of EPO and exhibits tissue-protective activities

Structure of EPO indicating tissue protective domains and sequences.



Brines M et al. PNAS 2008;105:10925–10930

Effect of HBSP on TNF-α-induced cardiomyocyte apoptosis.



Ueba H et al. PNAS 2010;107:14357-14362