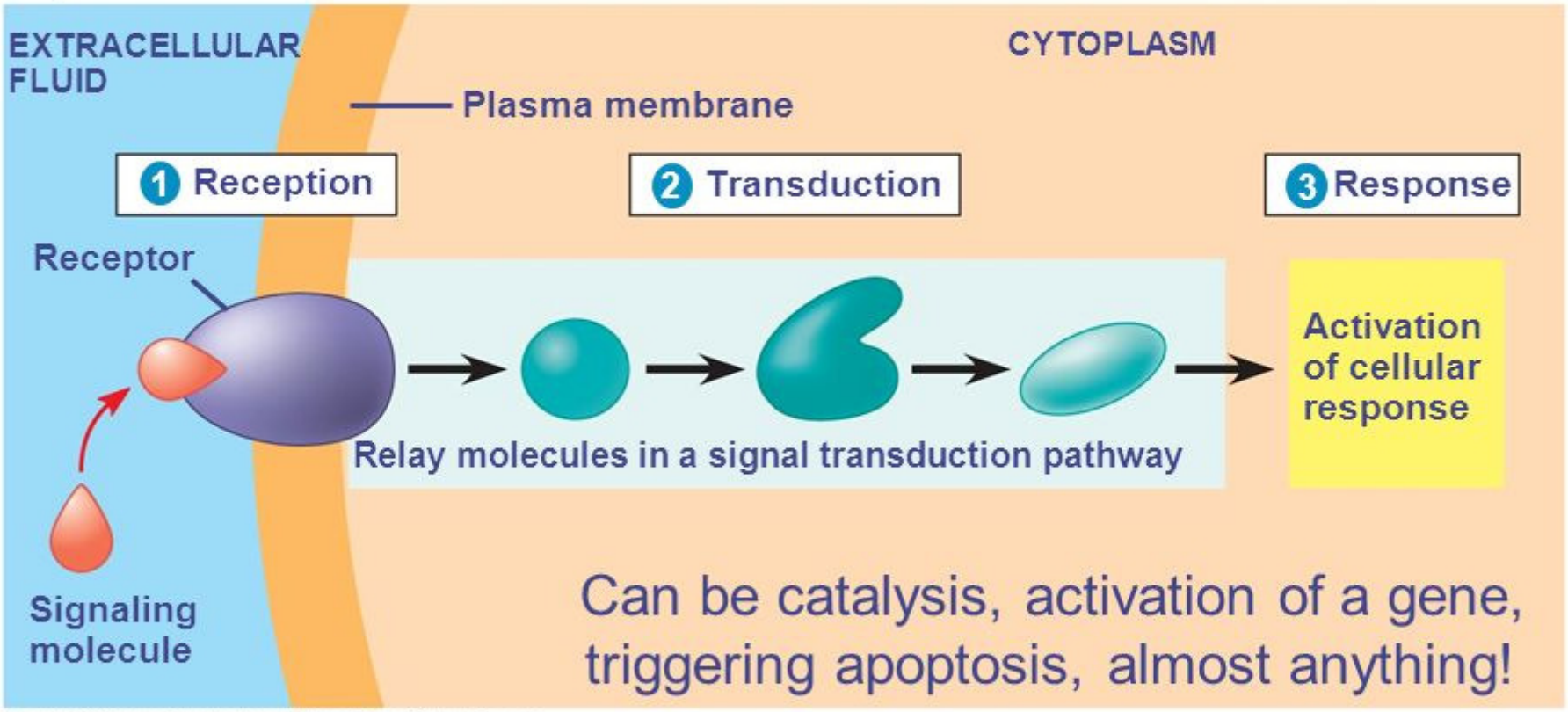
METODI DI STUDIO

DELLA TRASDUZIONE DEL SEGNALE

Three Stages of Cell Signaling



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- **The transduced signal triggers a cellular response**

STUDIO DELLA TRASDUZIONE DEL SEGNALE: localizzazione subcellulare

1 RECEPTION

MEMBRANA CELLULARE

- Metodiche di studio dei recettori
- Studi di binding

2 TRANSDUCTION

CITOPLASMA

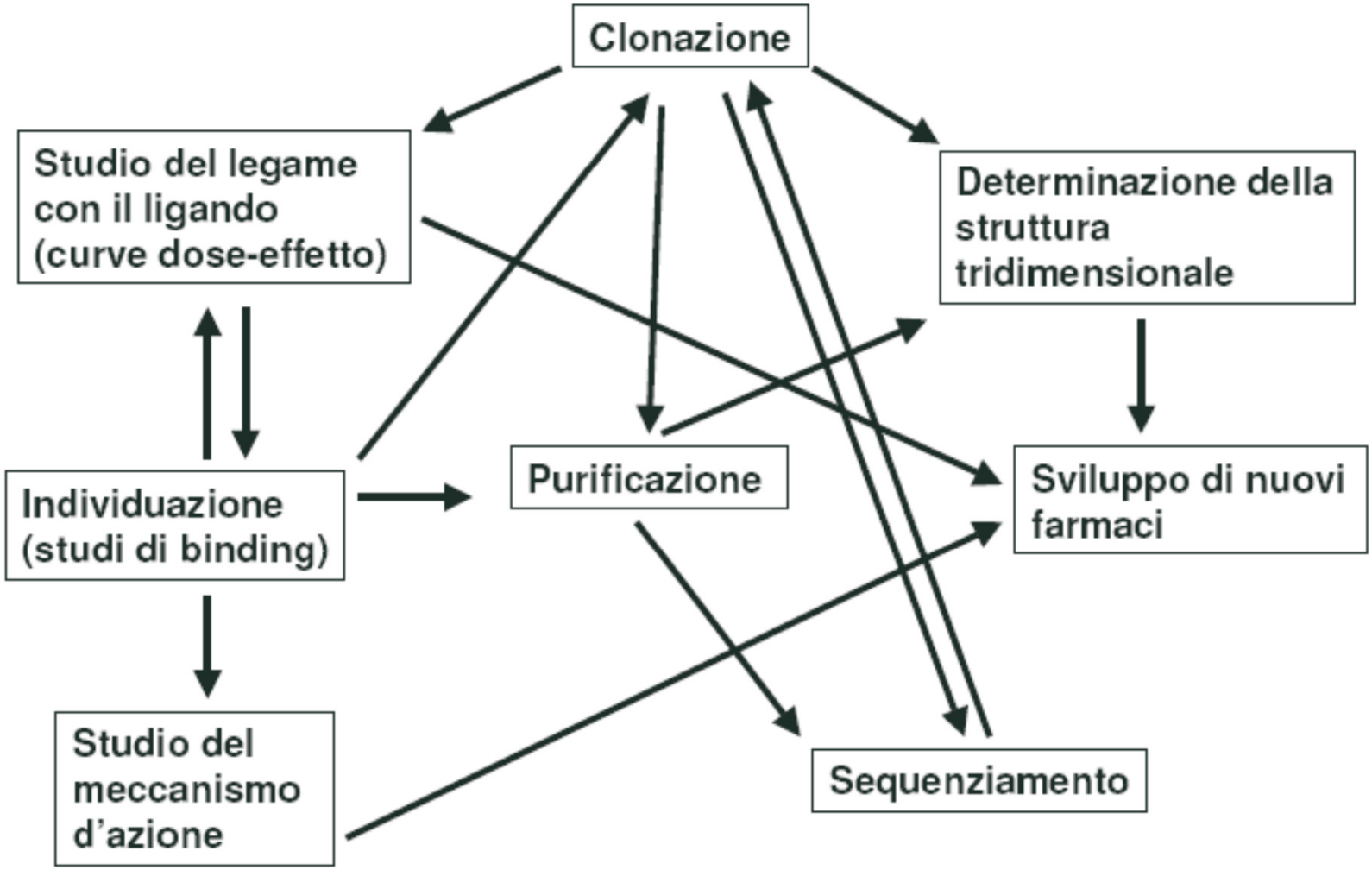
- Analisi dei fattori citoplasmatici che trasducono all'interno della cellula lo stimolo extracellulare

3 CELL RESPONSE

NUCLEO

- Analisi dei fattori di trascrizione TRASLOCATI nel nucleo in seguito allo stimolo extracellulare

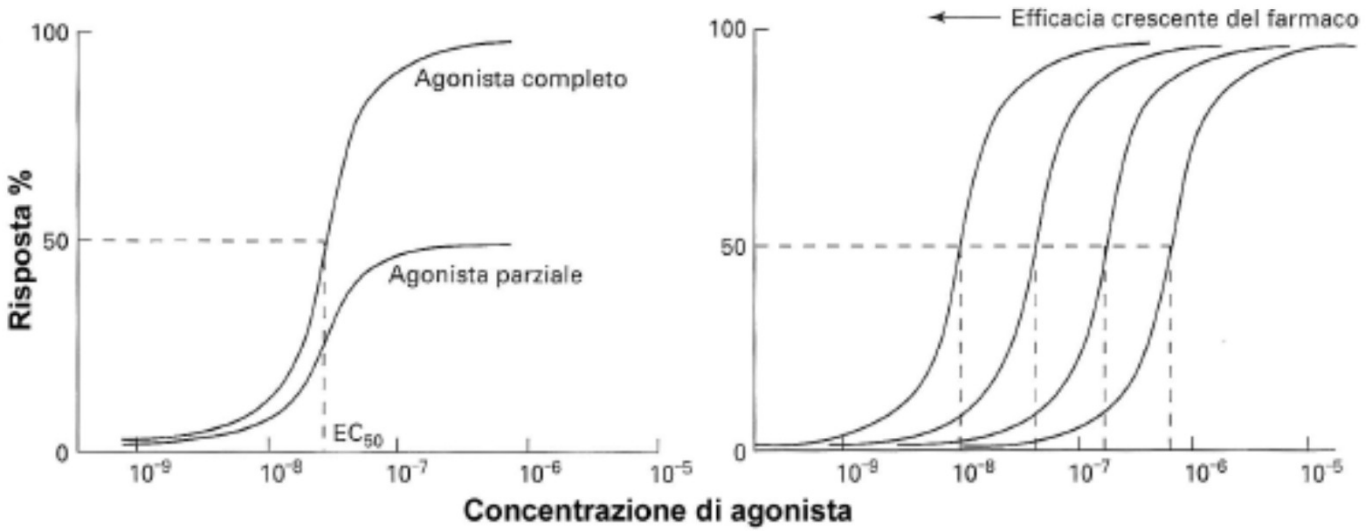
Approcci allo studio dei recettori



Studio del legame recettore-ligando

Obiettivi:

- Studio dell'attività intrinseca
- Determinazione EC_{50} (potenza)
- Determinazione K_d (efficacia)



Tecniche per lo studio del legame recettore-ligando

CAMPIONI:

- Sezioni di tessuto
- Cellule isolate
- Colture cellulari
- Membrane isolate
- Recettori isolati

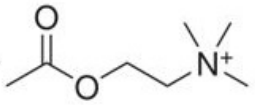
TECNICHE:

- FLUORESCENZA
- RADIOATTIVITA’

ISOLAMENTO E PURIFICAZIONE DEI RECETTORI – Approccio classico

1. Isolamento dell'organo
2. Omogenizzazione tramite detergenti
3. Purificazione del recettore tramite cromatografia di affinità

Approccio utilizzato per la prima volta con successo per il recettore dell'acetilcolina.



Torpedo sp.

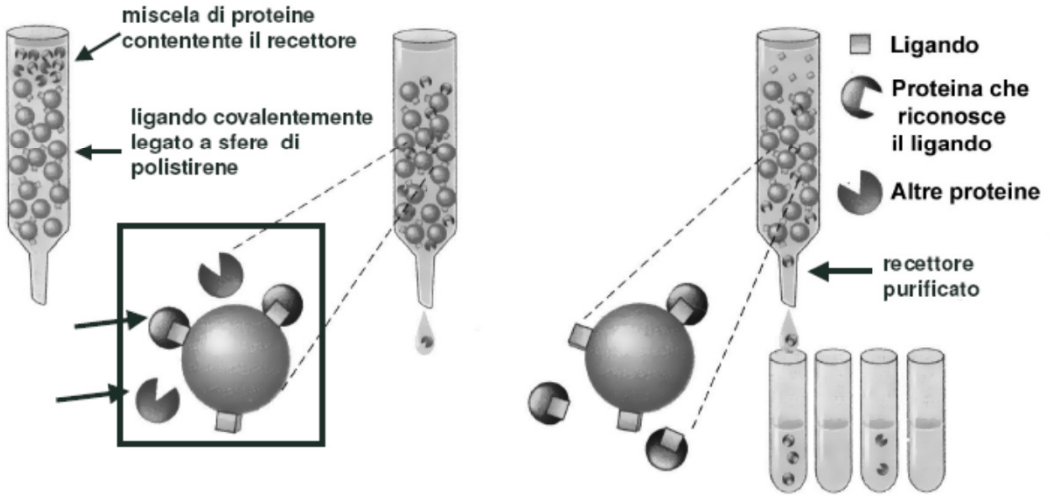
Electrophorus sp.

Organi elettrici particolarmente ricchi di recettori per l'acetilcolina.

Bungarus multicintus

Scoperta ligandi con alta specificità per il recettore in questione = α-tossine (α-bungarotossine) = hanno reso possibile la purificazione (cromatografia di affinità)

Tecniche di purificazione: la cromatografia di affinità

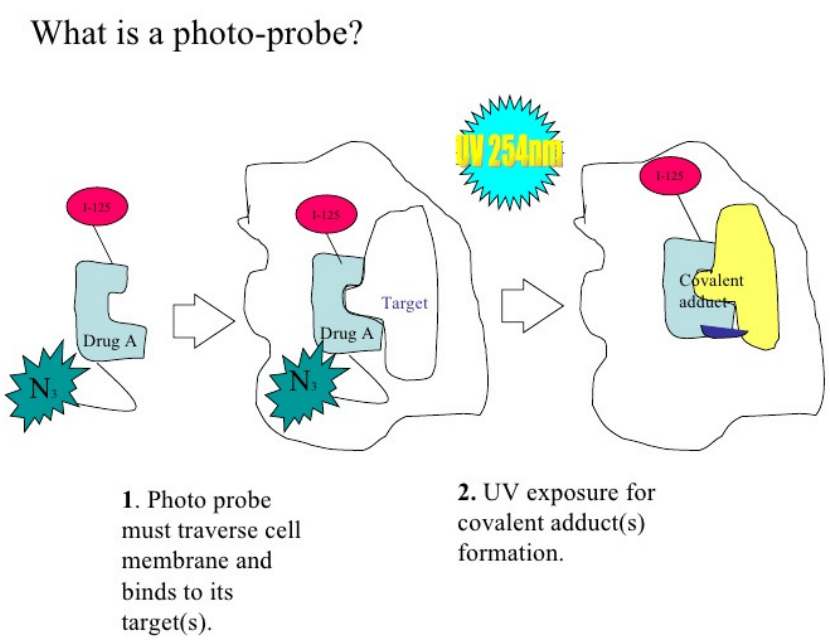


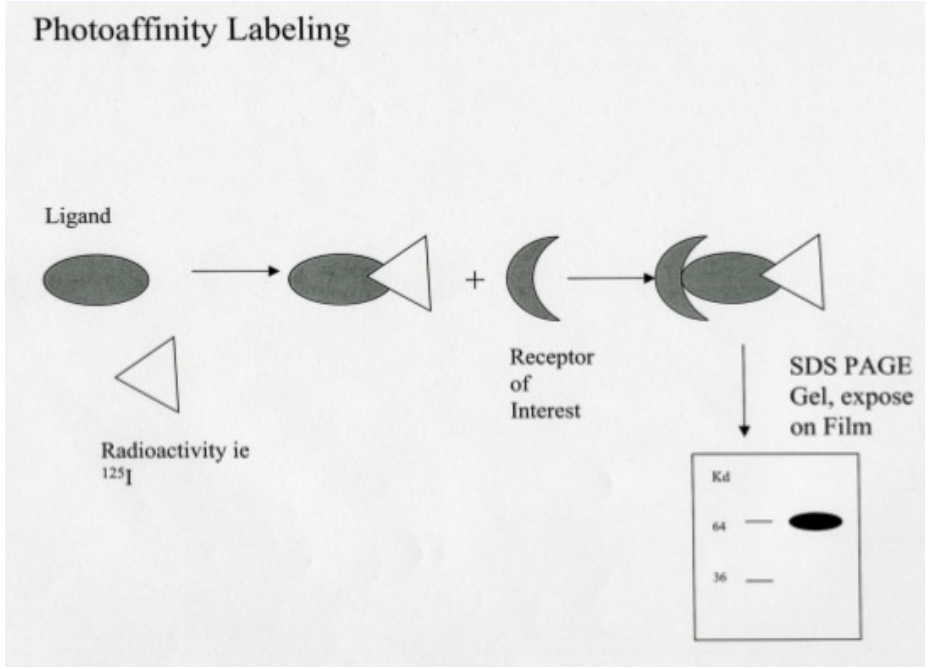
Affinity Chromatography

Separates proteins on the basis of reversible interaction between a protein and a specific ligand that is coupled to a chromatography matrix

- Used to purify receptor of interest with high selectivity
- Requires a selective ligand covalently linked to chromatography matrix
- Ligand-Receptor binding must be reversible
- Receptor is eluted via changes in pH or ionic strength, or via competition

Binding techniques: Photoaffinity Labeling





Direct probing of a target protein through a covalent bond which is photochemically introduced between a ligand and its specific receptor

- The ligand is linked to a photoreactive group sensitive to UV light
- After binding to the receptor, UV light induces covalent linkage
- The Ligand-Receptor complex can be identified by SDS-PAGE

Advantageous for Target Identification via quantification of the complex formed; more complex will form if a ligand has high affinity vs low affinity

Binding techniques: Receptor Autoradiography

Distribuzione e localizzazione dei recettori

Studi sulla schizofrenia:

autoradiografia di encefalo umano marcato con [3H]MDL: distribuzione e localizzazione dei recettori 5HT2A.

Juan F. López-Giménez et al.
Naunyn-Schmiedeberg's Archives of Pharmacology. September 1997, Volume 356, Issue 4, pp 446-454

Direct binding of a selective radioligand to its receptor in native tissue

- Shows the distribution of the receptors in native tissue; ie brain slices.
- Allows for quantitative analysis of the receptors
- Ligand of choice determines selectivity

In theory similar to Immunohistochemistry, with the use of selective ligands instead of antibodies

Tecniche di studio impiegate per la localizzazione dei recettori

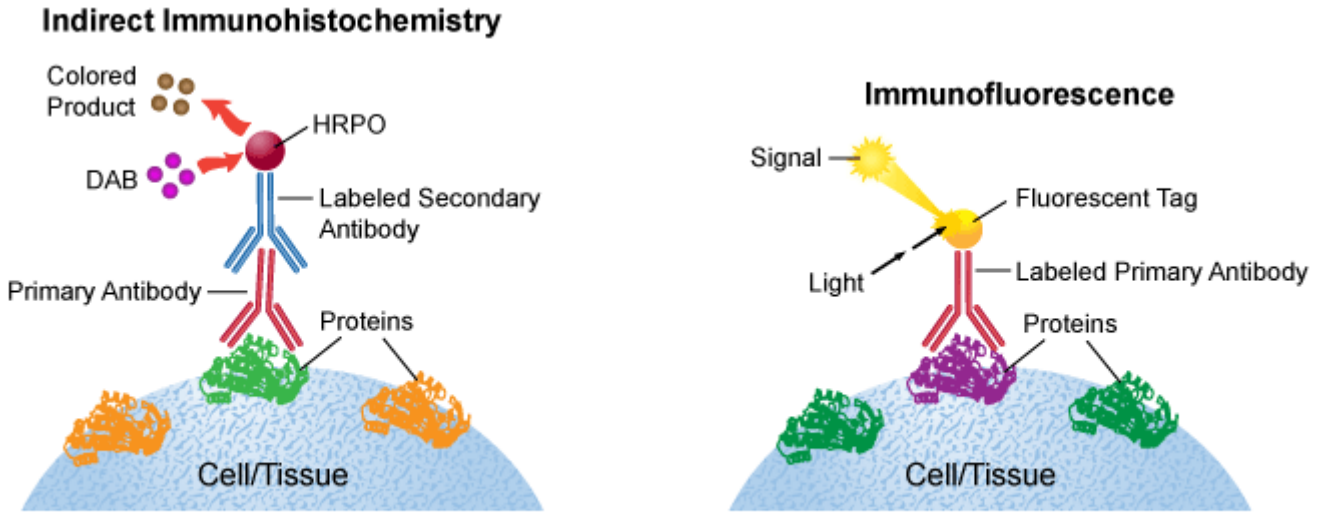
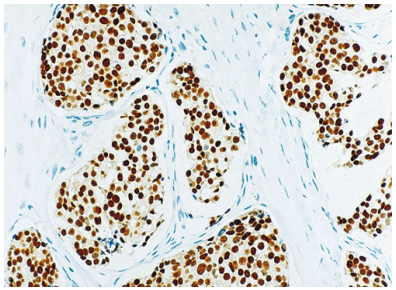
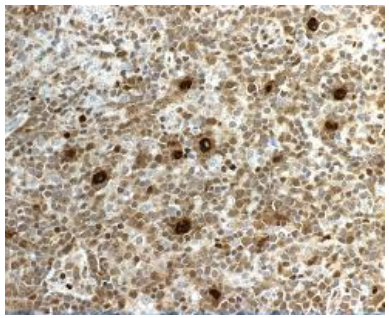
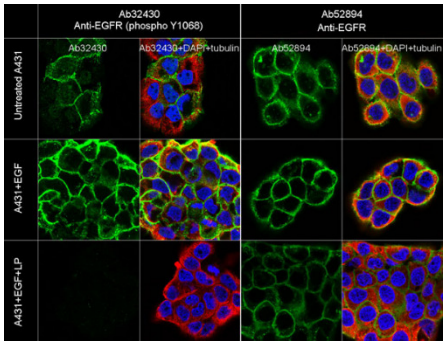
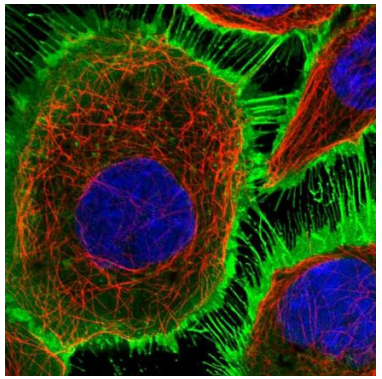


Diagram 1: *Illustration of Indirect Immunohistochemistry and Immunofluorescence methods.*







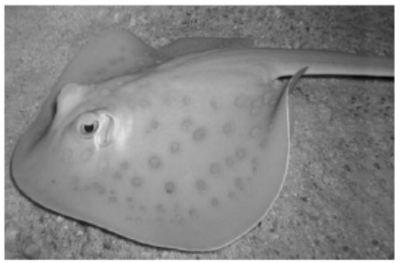


Anti-hEGFR staining (green)

RECETTORI RICOMBINANTI– Approccio moderno

Svantaggi della purificazione dei recettori

Le tecniche classiche di isolamento dei recettori sono vincolate all'esistenza di casi eccezionali.



L'organo elettrico della torpedine è ricco di recettori dell'acetilcolina

Il veleno dei cobra è ricco di a-bungarotossina, antagonista del recettore dell'acetilcolina

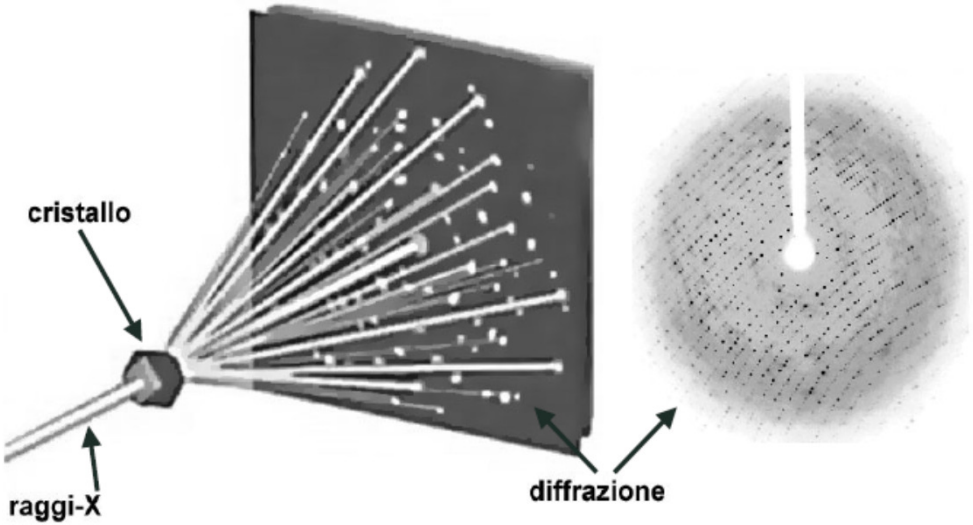
Fasi sperimentali per la produzione dei recettori ricombinanti:

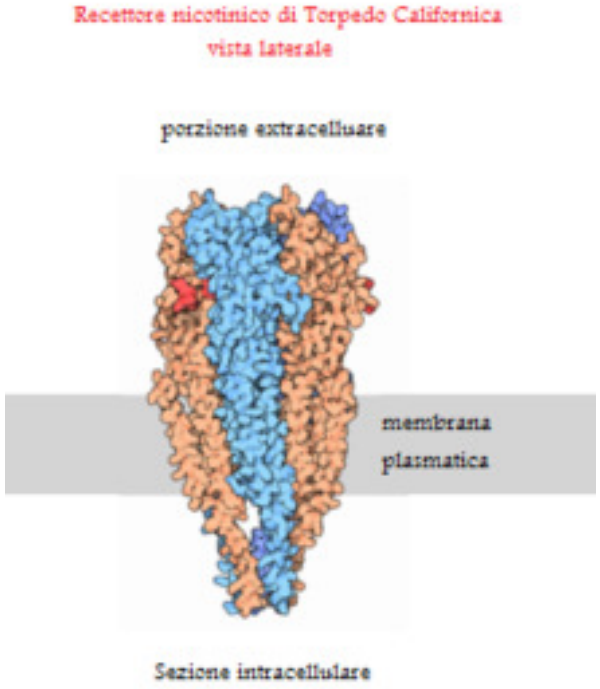
1. Estrazione dell' mRNA da cellule che esprimono il recettore di interesse
2. L' mRNA è enzimaticamente retrotrascritto in cDNA ed inserito in plasmidi di espressione
3. I plasmidi di espressione sono trasfettati:

   - in cellule eucariotiche per l'espressione proteica del recettore ed eventuale esposizione sulla membrana
   - in cellule batteriche come produttrici del recettore ricombinante

DETERMINAZIONE DELLA STRUTTURA TRIDIMENSIONALE DEL RECETTORE

Sequenza aminoacidica + dati di diffrazione → **Modello atomico del recettore**





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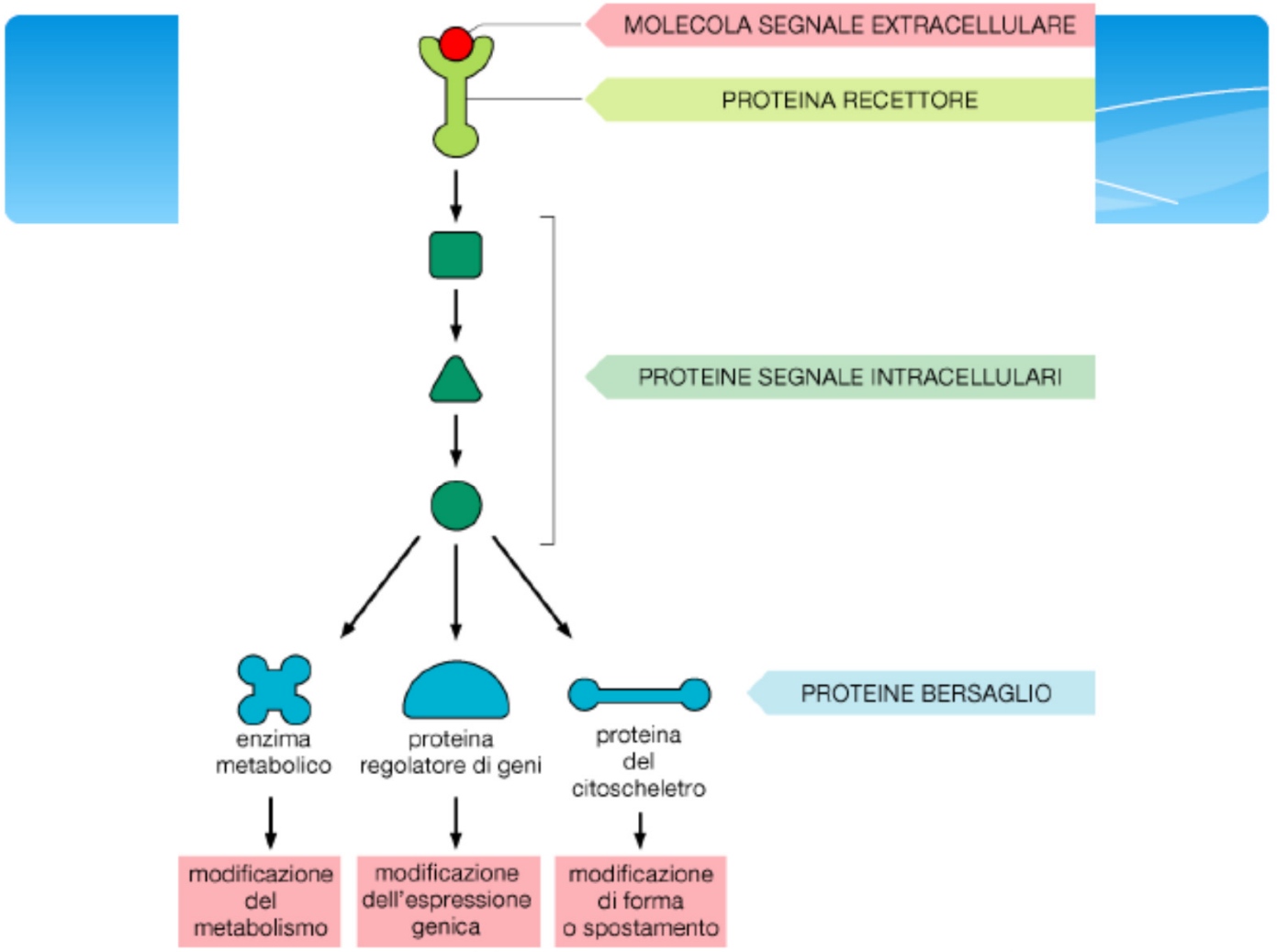
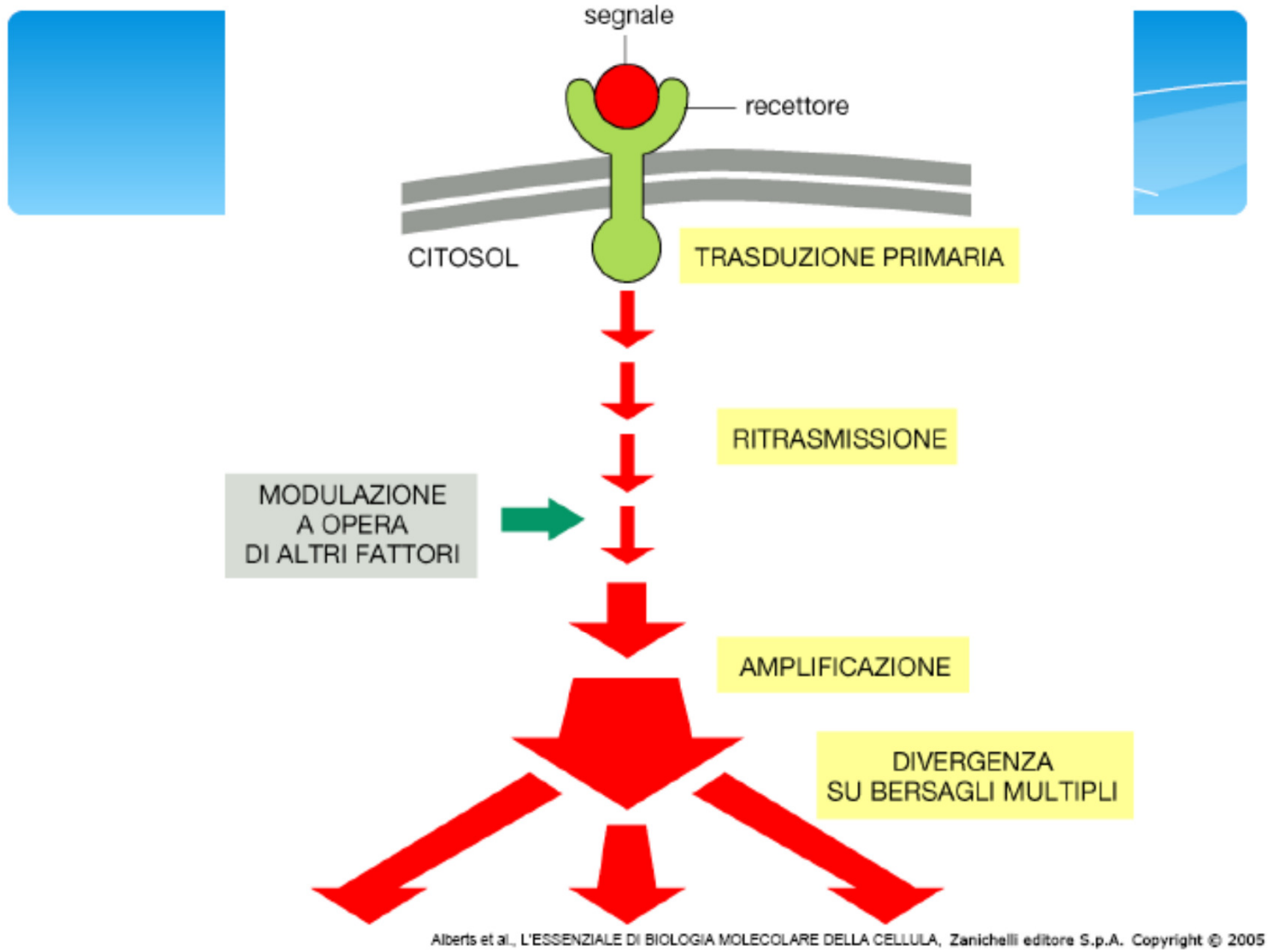


Alberts et al., L'ESSENZIALE DI BIOLOGIA MOLECOLARE DELLA CELLULA, Zanichelli editore S.p.A. Copyright © 2005

Analisi dei fattori citoplasmatici che trasducono all'interno della cellula lo stimolo extracellulare



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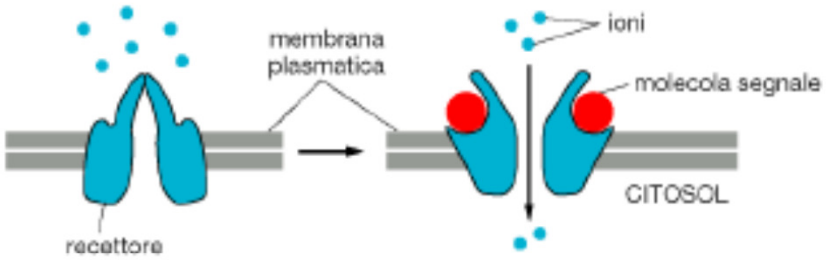
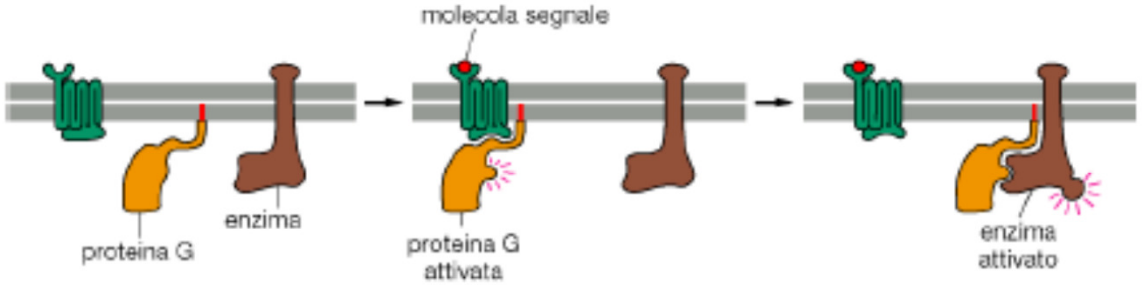
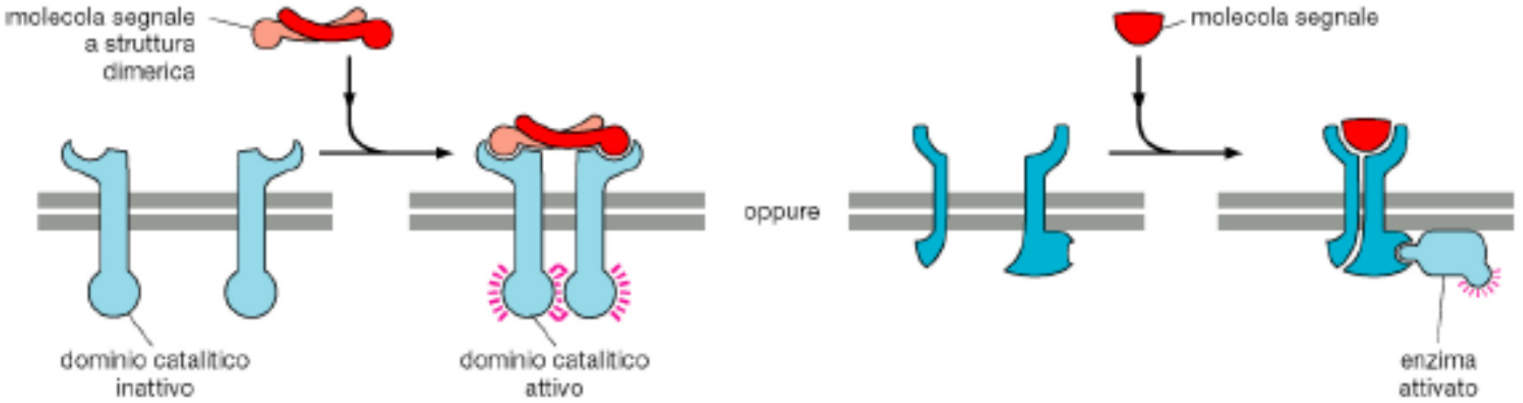
(A) RECETTORE ANNESSO A CANALI IONICI



(B) RECETTORE ACCOPPIATO A UNA PROTEINA G



(C) RECETTORI LEGATI A ENZIMI



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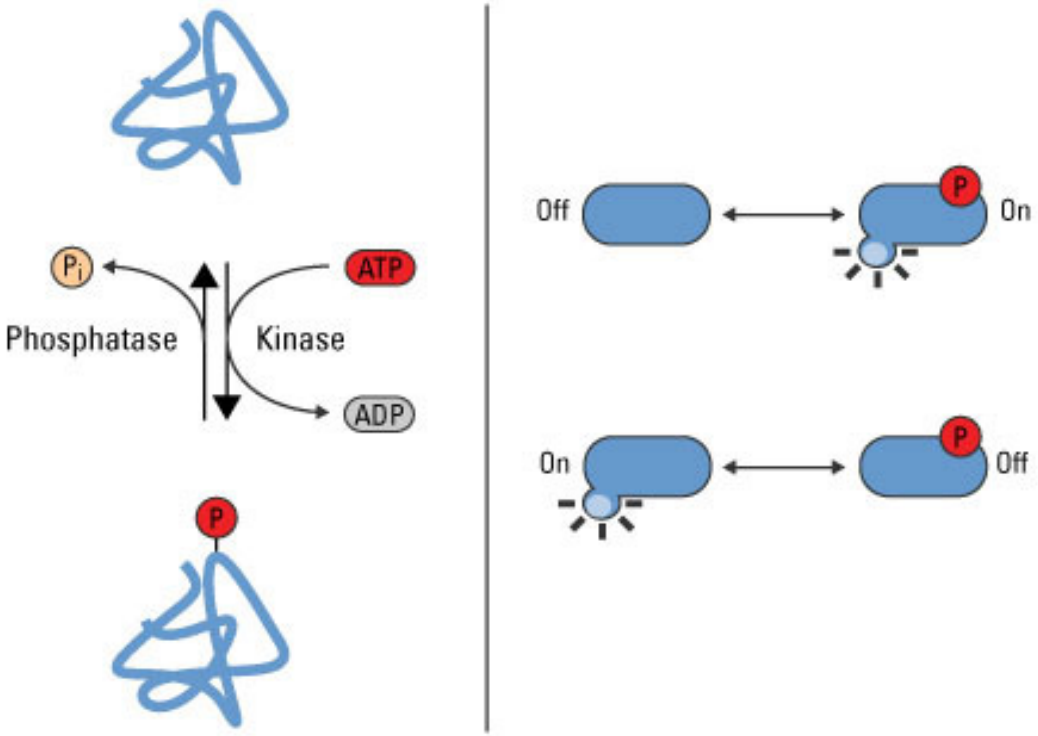
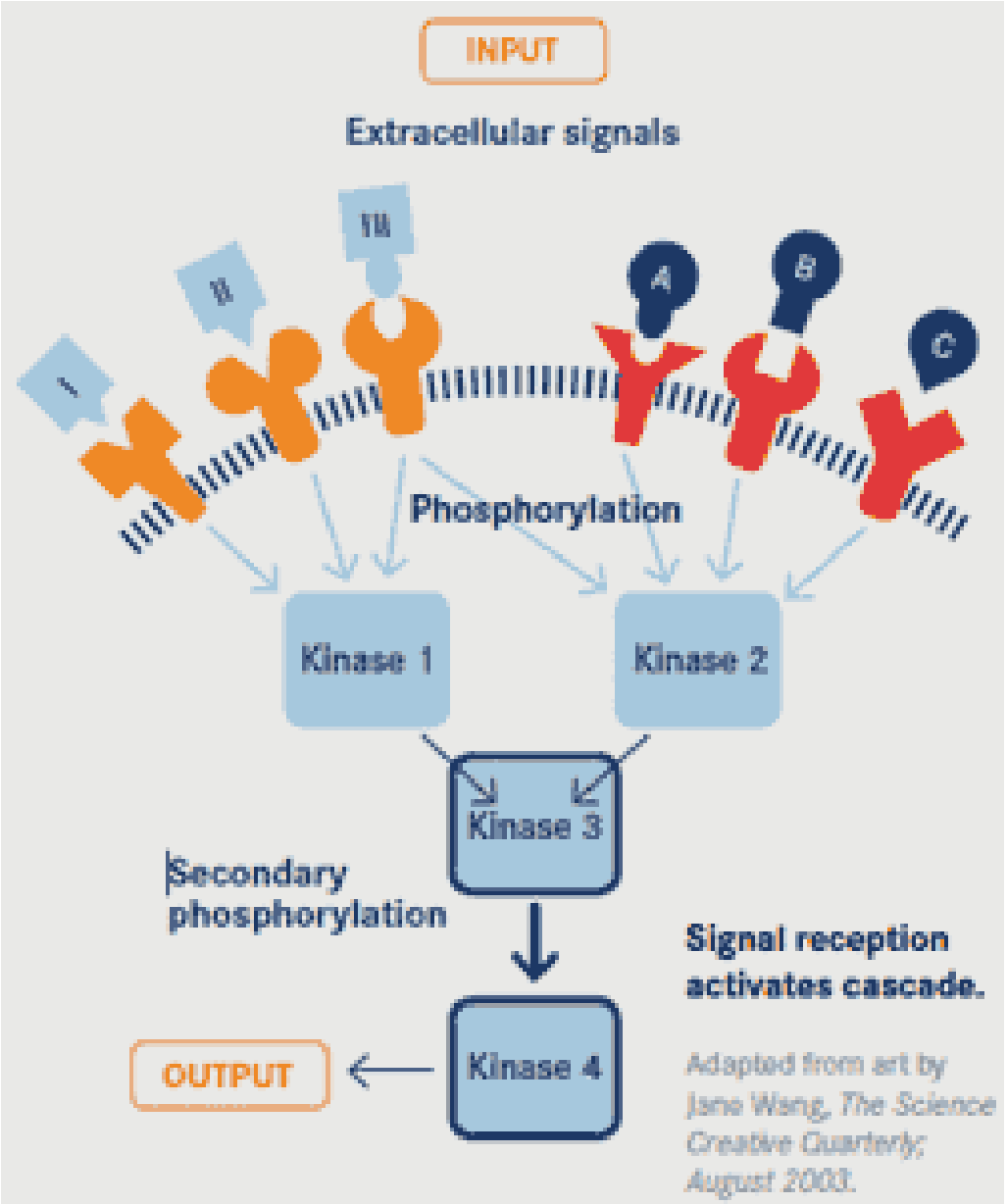
Importanza della fosforilazione nella trasduzione del segnale



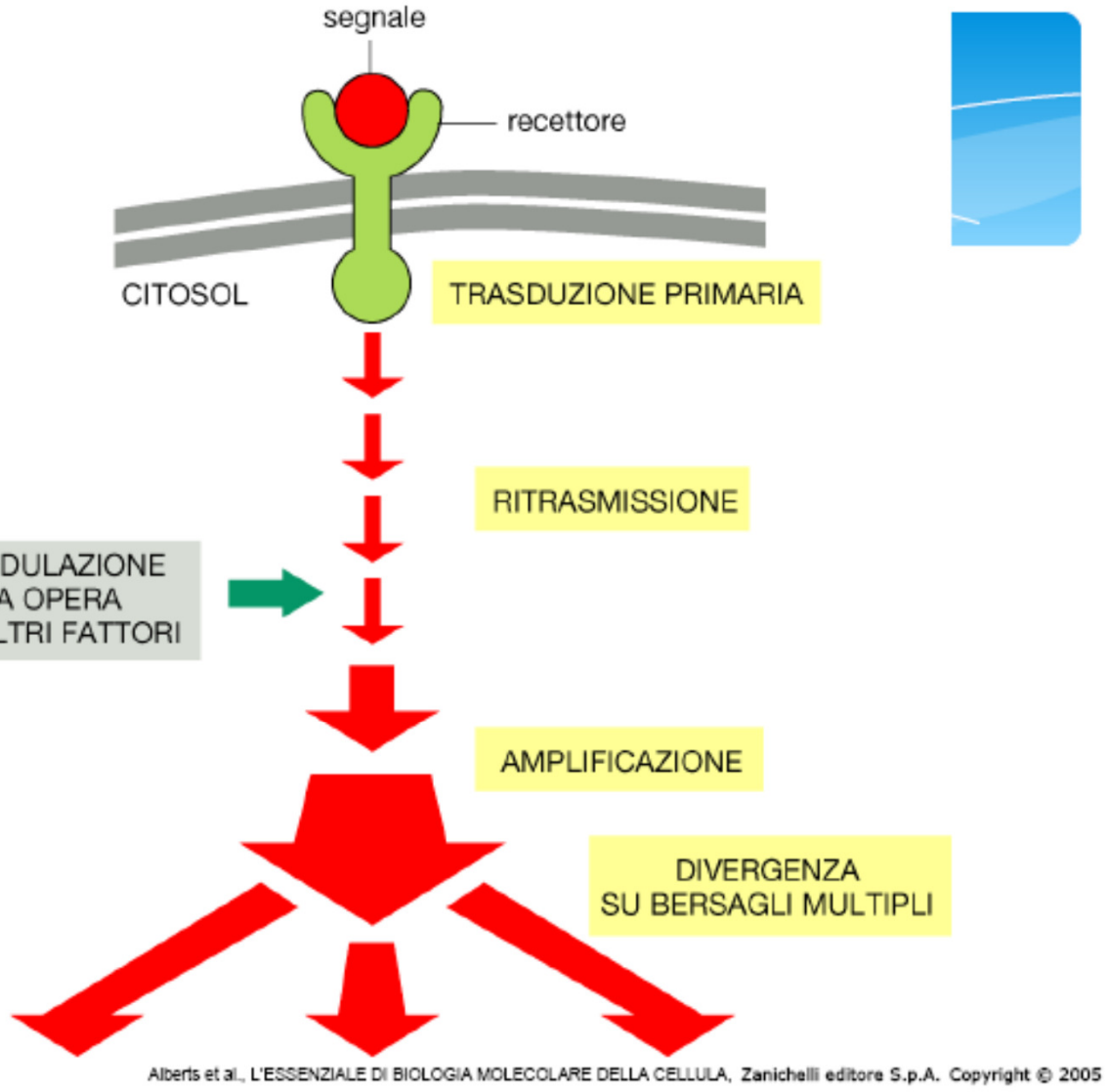
Protein phosphorylation is the most commonly studied area of post-translational modification since it plays a vital role in intracellular signal transduction and is involved in regulating cell cycle progression, differentiation, transformation, development, peptide hormone response, and adaptation. It has been estimated that **one third of mammalian proteins may be phosphorylated** and this modification often plays a key role in modulating protein function. **Reversible protein phosphorylation, principally on serine, threonine or tyrosine residues**, is one of the most important and well-studied post-translational modifications.



Phosphorylation regulates protein function and cell signaling by causing conformational changes in the phosphorylated protein. These changes can affect the protein in two ways. **First,** conformational changes regulate the catalytic activity of the protein. Thus, a protein can be either activated or inactivated by phosphorylation. **Second,** phosphorylated proteins recruit neighboring proteins that have structurally conserved domains that recognize and bind to phosphomotifs. These domains show specificity for distinct amino acids

Tecnica di studio delle proteine citosoliche coinvolte nella transduction

WESTERN BLOT



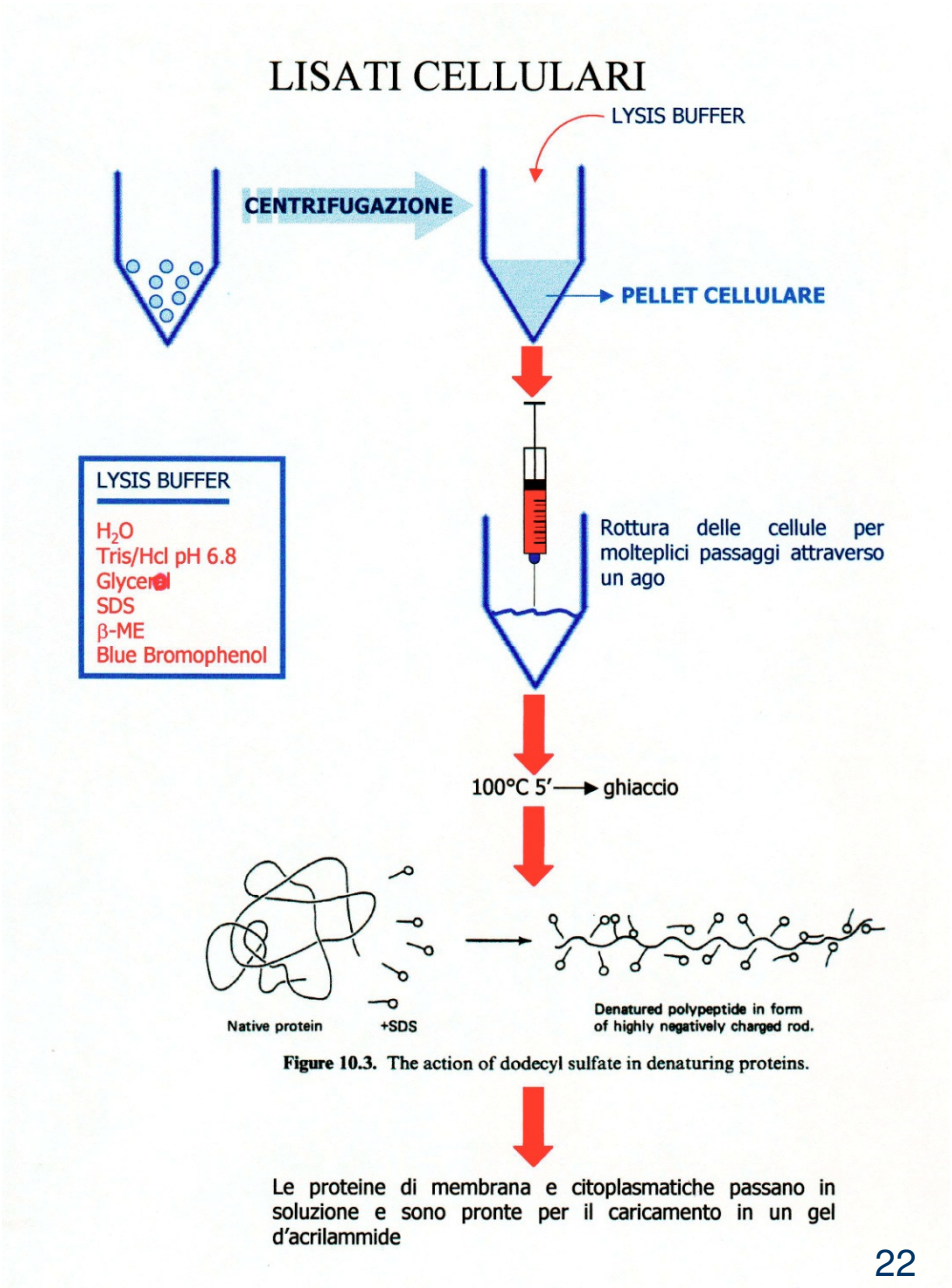
WESTERN BLOT

- E' una tecnica di immunodetection che permette di evidenziare in una cellula l'attivazione di proteine coinvolte nel *signaling* (ad es.: fosforilazione di recettori, chinasi, fattori di trascrizione, etc.), conseguentemente alla recezione di uno stimolo extracellulare.

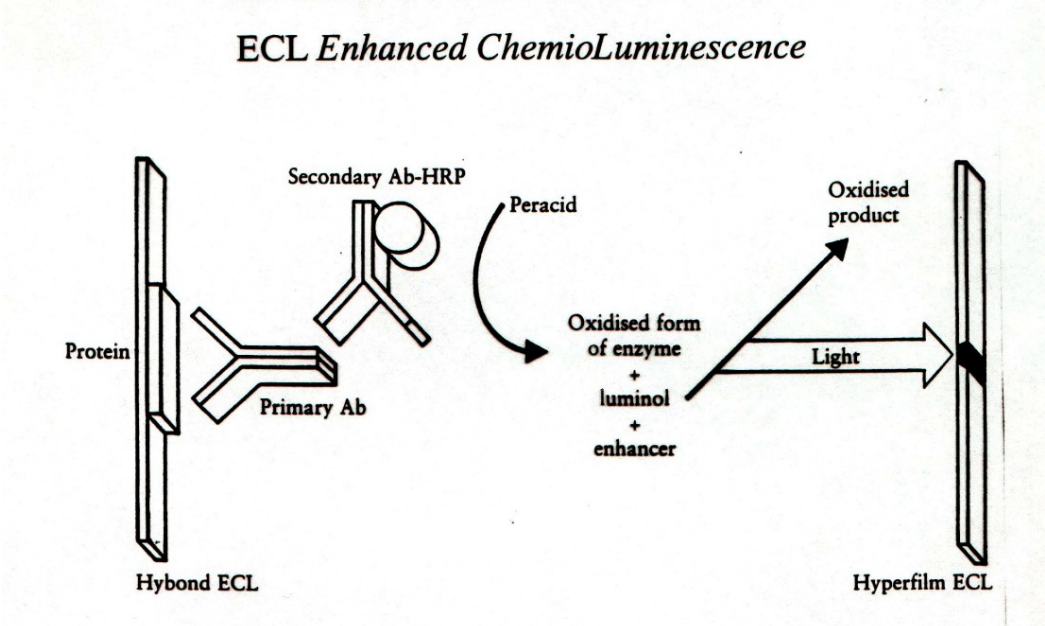
- Per mezzo di anticorpi capaci di riconoscerne la forma attivata (ad es.: fosforilata), è possibile rivelare le proteine che si attivano nel *signaling* mediante reagenti chemioluminescenti.

- Le principali fasi sperimentali del Western Blot sono:
    1. stimolazione delle cellule (attivazione del *signaling*);
    2. lisi cellulare con un opportuno reagente, per liberare le proteine intracellulari
    3. Corsa elettroforetica su gel del lisato proteico, per separare le proteine;
    4. trasferimento su membrana delle proteine migrate;
    5. rivelazione delle proteine coinvolte nel *signaling* con specifici anticorpi (ad es. capaci di riconoscerne la forma fosforilata) e con reagenti chemioluminescenti.

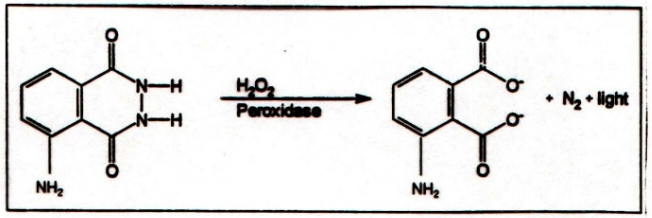
Western Blot: preparazione dei campioni



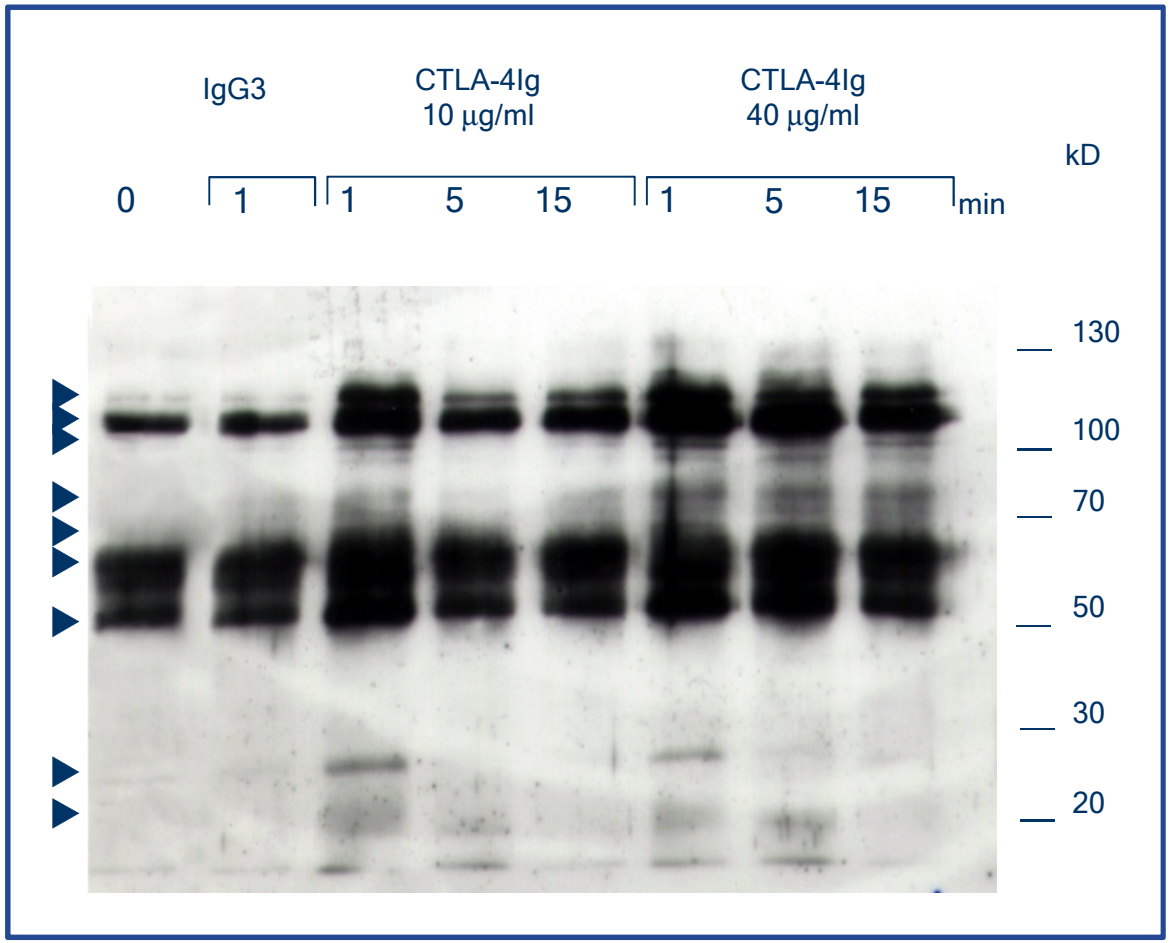
Western Blot:
rivelazione della proteina per chemioluminescenza



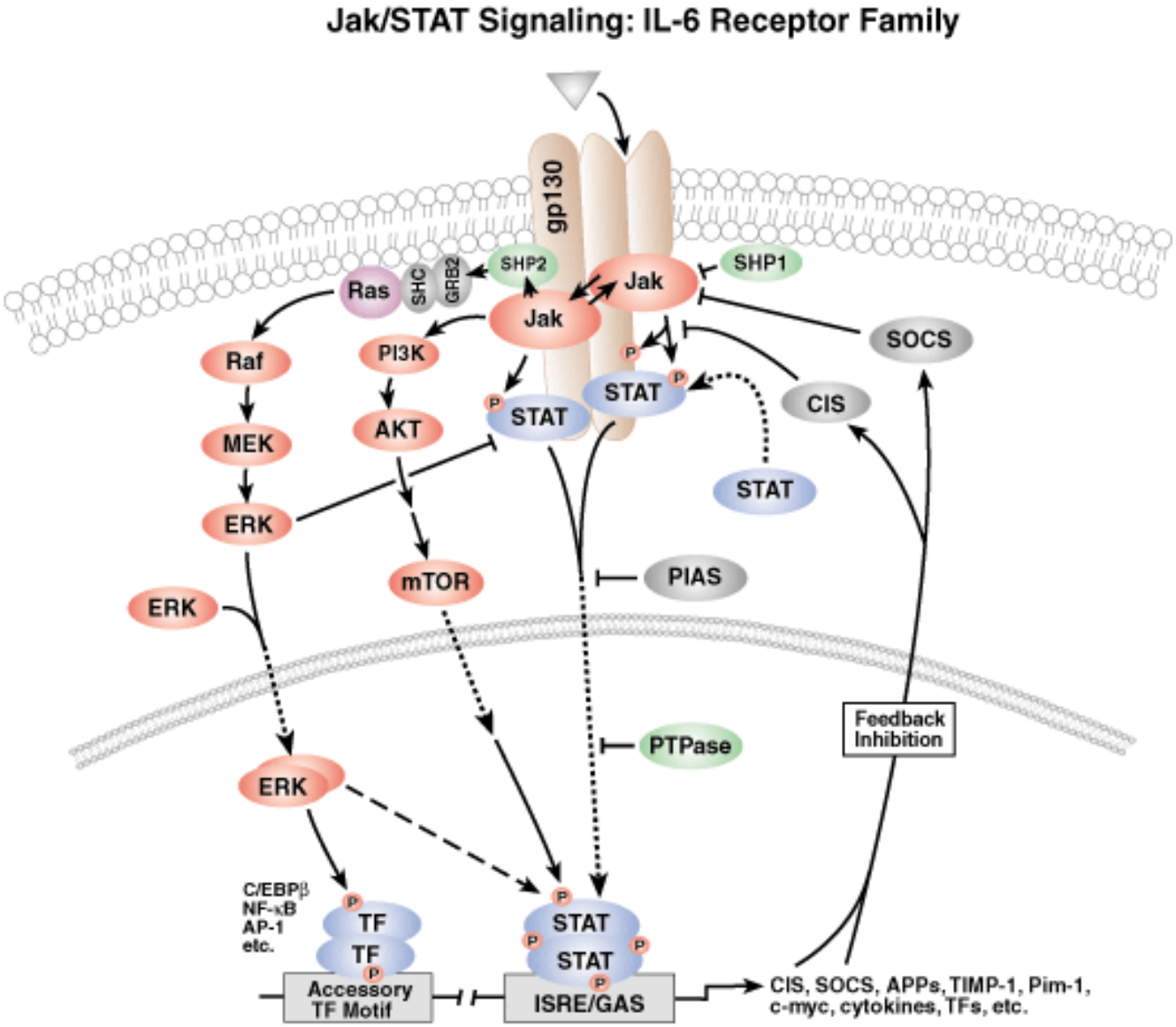
Reazione chimica



Western Blot: esempio di rivelazione con anti-fosfotirosina

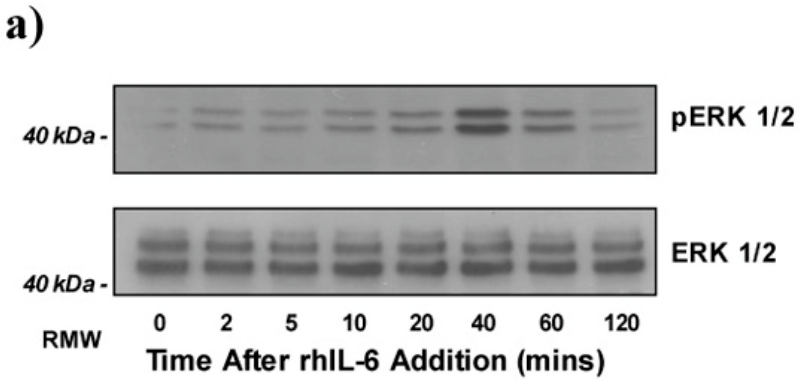


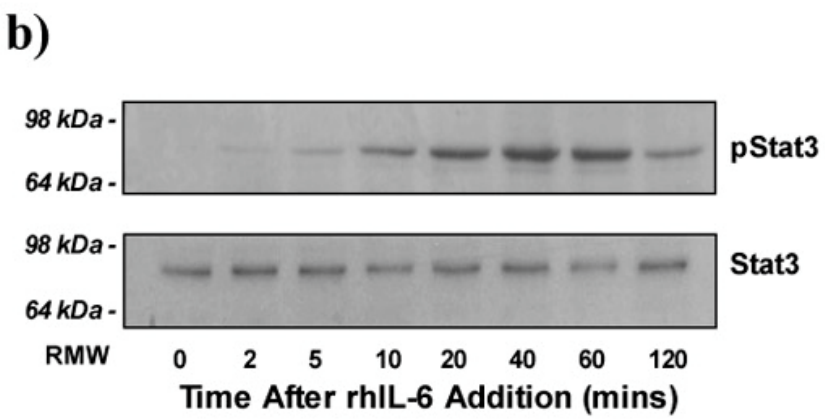
L'Ab primario riconosce tutte le proteine fosforilate in tirosina presenti nel campione, ma non identifica la proteina.


Jak/STAT Signaling: IL-6 Receptor Family
gp130
SHP2
SHP1
Ras
SHC
GRB2
Jak
Jak
SOCS
Raf
PI3K
STAT
STAT
CIS
MEK
AKT
STAT
ERK
mTOR
PIAS
ERK
Feedback Inhibition
PTPase
ERK
C/EBPβ
NF-κB
AP-1
etc.
TF
TF
STAT
STAT
Accessory TF Motif
ISRE/GAS
CIS, SOCS, APPs, TIMP-1, Pim-1, c-myc, cytokines, TFs, etc.

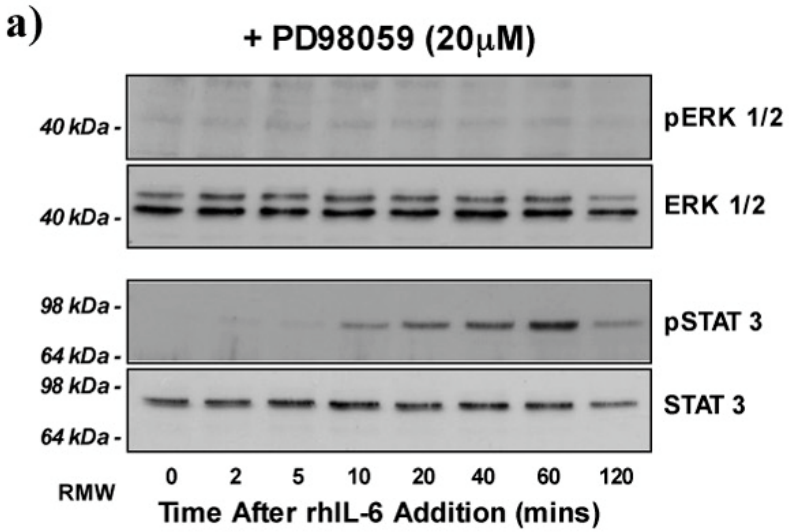
INTERLEUKIN-6 (IL-6) ACTIVATES STAT 3- AND ERK DEPENDENT PATHWAY

Stimulation of hepatocellular carcinoma cells with IL-6

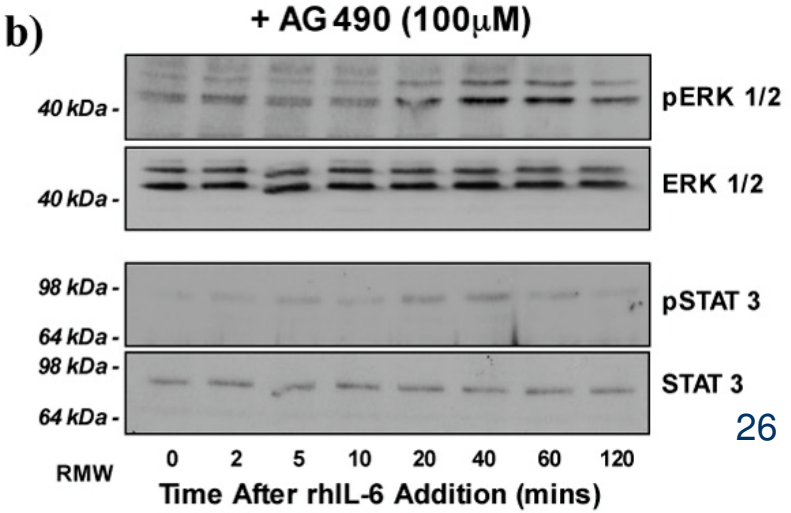


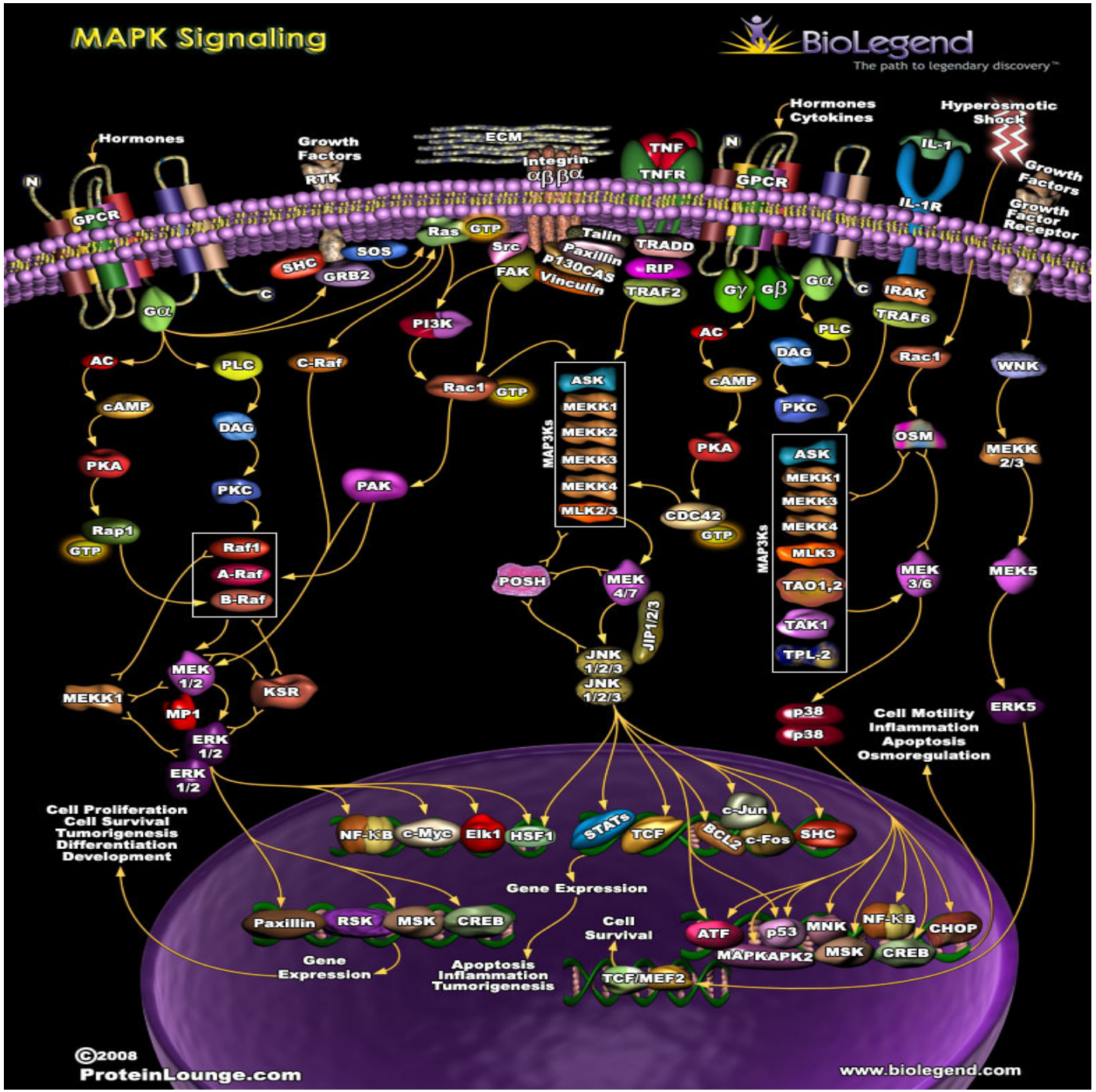


MEK specific inhibitor PD98059



Jak-STAT3 specific inhibitor AG490




MAPK Signaling
BioLegend
The path to legendary discovery™
Hormones
Growth Factors
RTK
ECM
Integrin-αββα
TNF
TNFR
Hormones Cytokines
GPCR
IL-1
IL-1R
Hyperosmotic Shock
Growth Factors
Growth Factor Receptor
N
C
GPCR
Gα
Ras
GTP
SOS
SHC
GRB2
Src
FAK
Talin
Paxillin
P130CAS
Vinculin
TRADD
RIP
TRAF2
Gγ
Gβ
Gα
IRAK
TRAF6
AC
PLC
DAG
cAMP
PKA
PKC
C-Raf
PI3K
PAK
Rac1
GTP
ASK
MEKK1
MEKK2
MEKK3
MEKK4
MLK2/3
MAP3Ks
CDC42
GTP
OSM
MEKK 2/3
MLK3
TAO1,2
TAK1
TPL-2
MEK 3/6
MEK5
ERK5
Rap1
GTP
Raf1
A-Raf
B-Raf
POSH
MEK 4/7
JIP1/2/3
JNK 1/2/3
MEK 1/2
MEKK1
MP1
KSR
ERK 1/2
p38
Cell Motility
Inflammation
Apoptosis
Osmoregulation
Cell Proliferation
Cell Survival
Tumorigenesis
Differentiation
Development
NF-κB
c-Myc
Elk1
HSF1
STATs
TCF
c-Jun
BCL2
c-Fos
SHC
Gene Expression
Paxillin
RSK
MSK
CREB
Cell Survival
Apoptosis
Inflammation
Tumorigenesis
TCF/MEF2
ATF
p53
MAPKAPK2
MNK
NF-κB
MSK
CREB
CHOP
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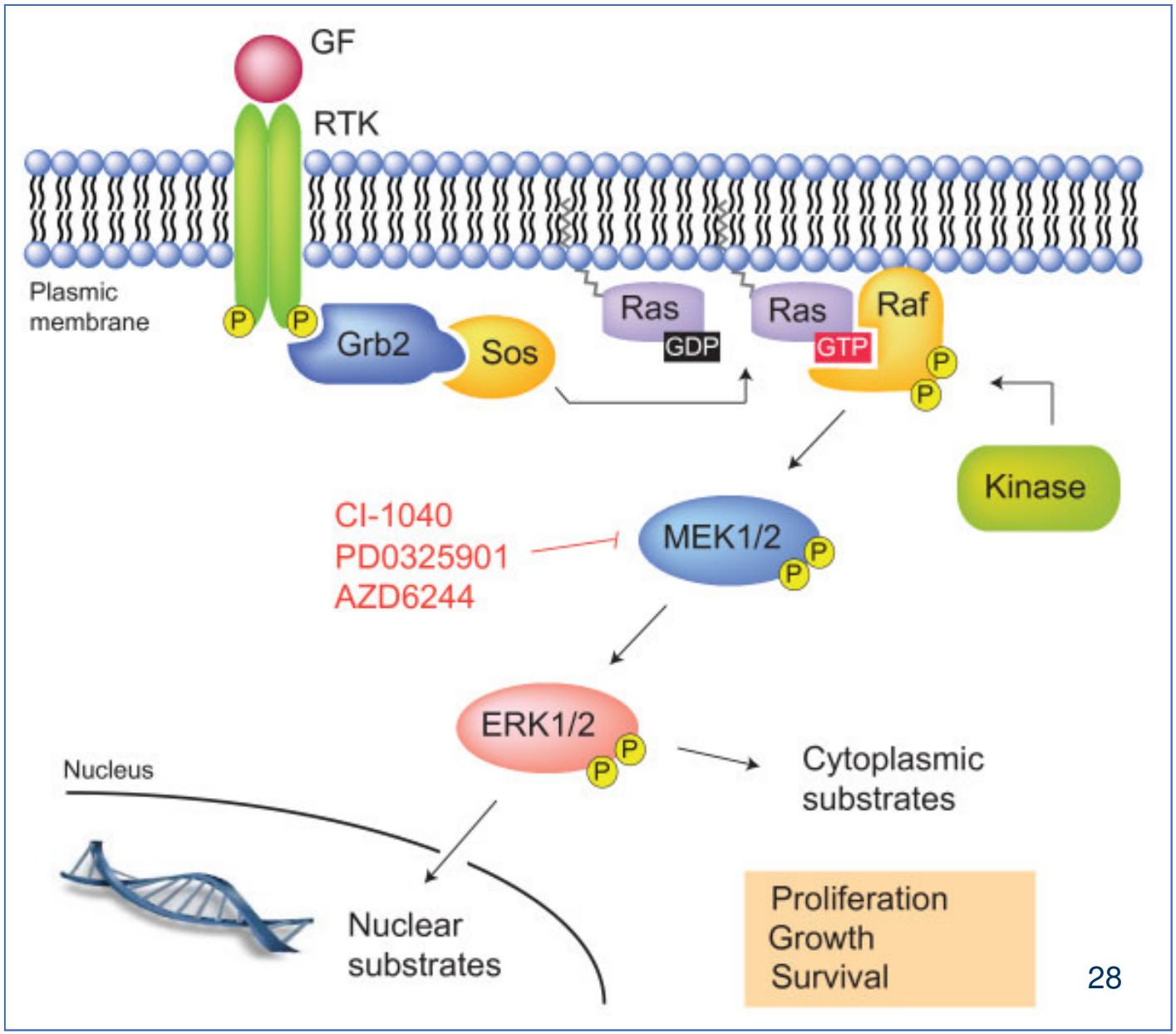
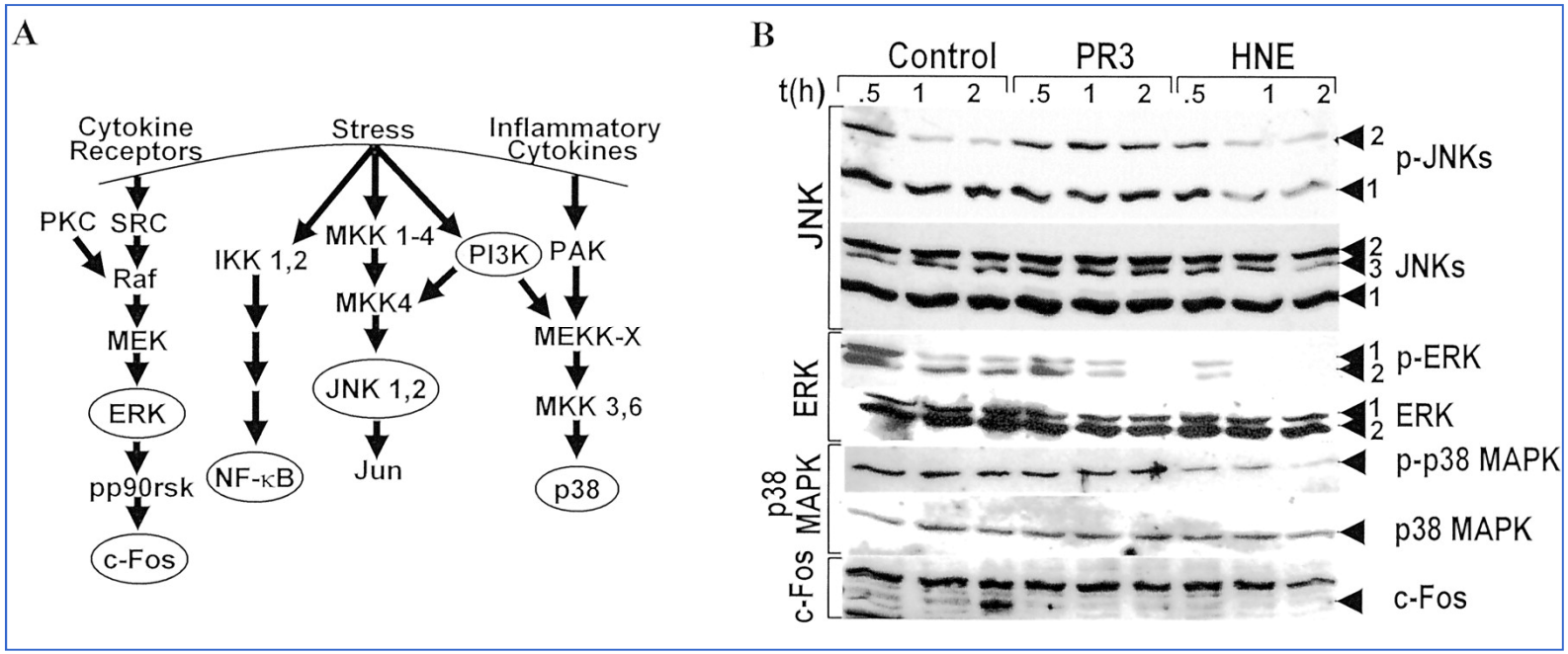
MAPK pathway



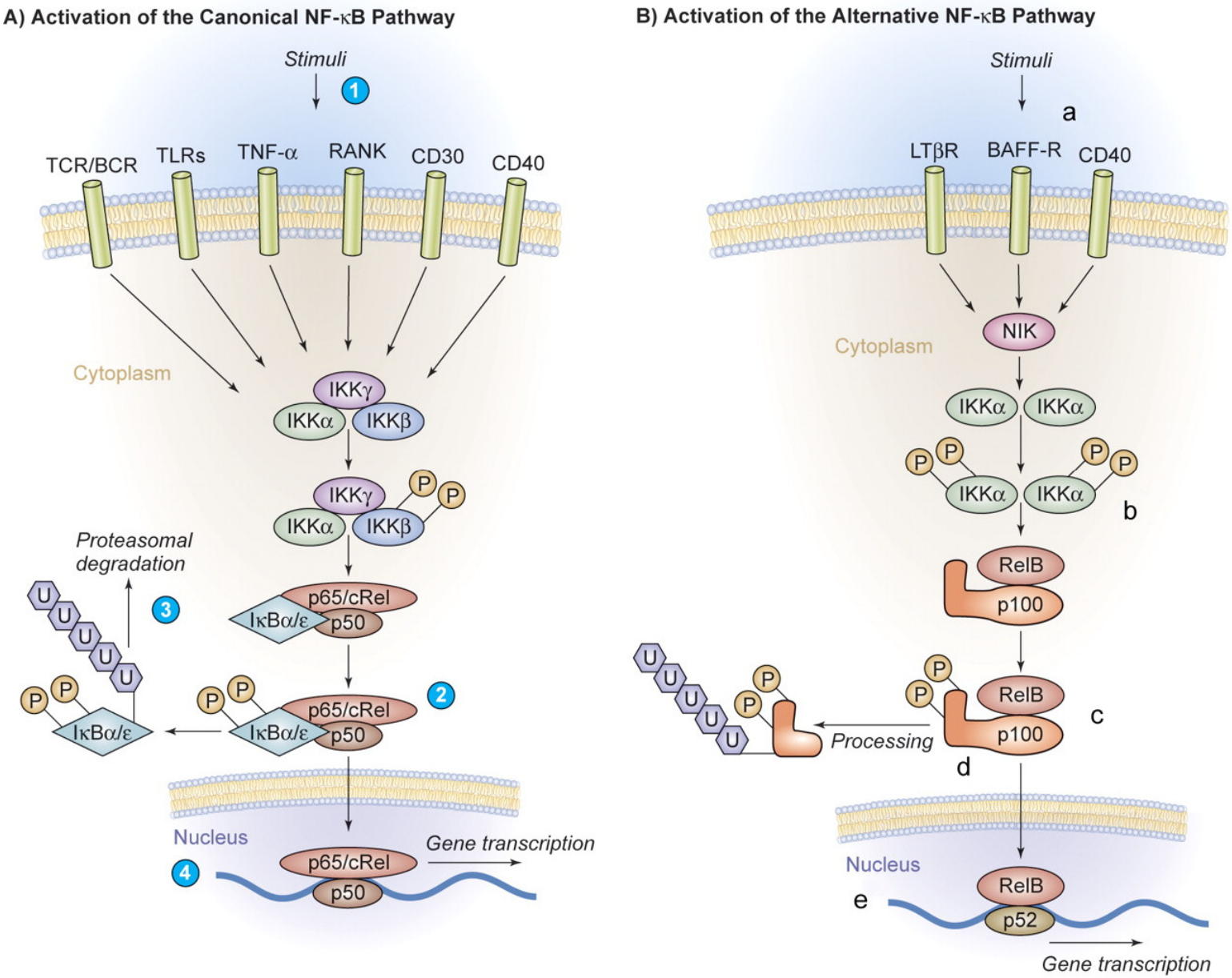
Novel Effects of Neutrophil-Derived Proteinase 3 (PR3) and Elastase (HNE) on the Vascular Endothelium

J Am Soc Nephrol 13: 2840–2849, 2002

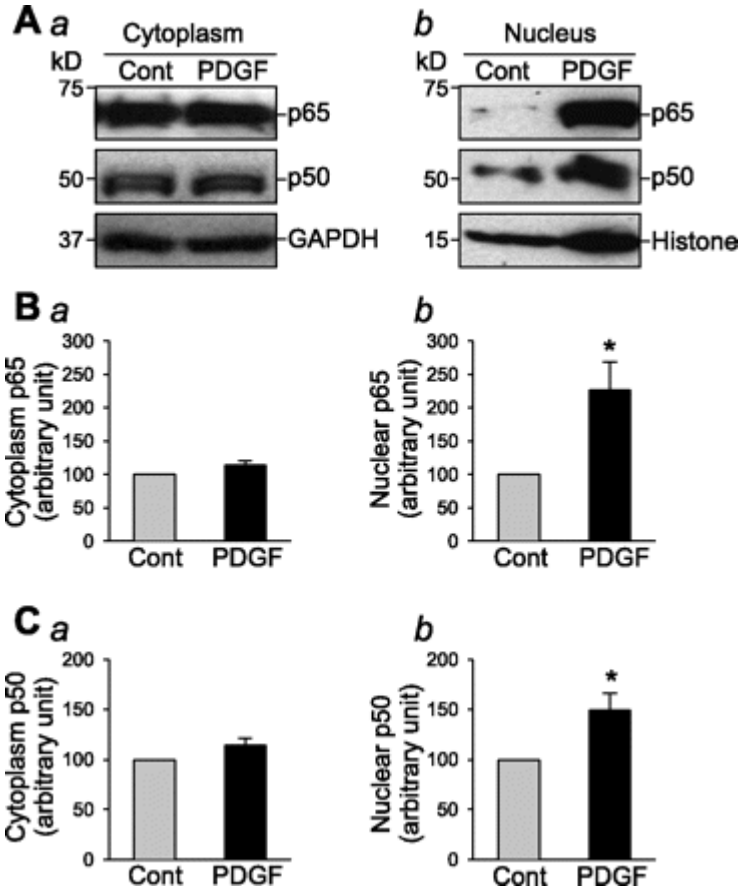


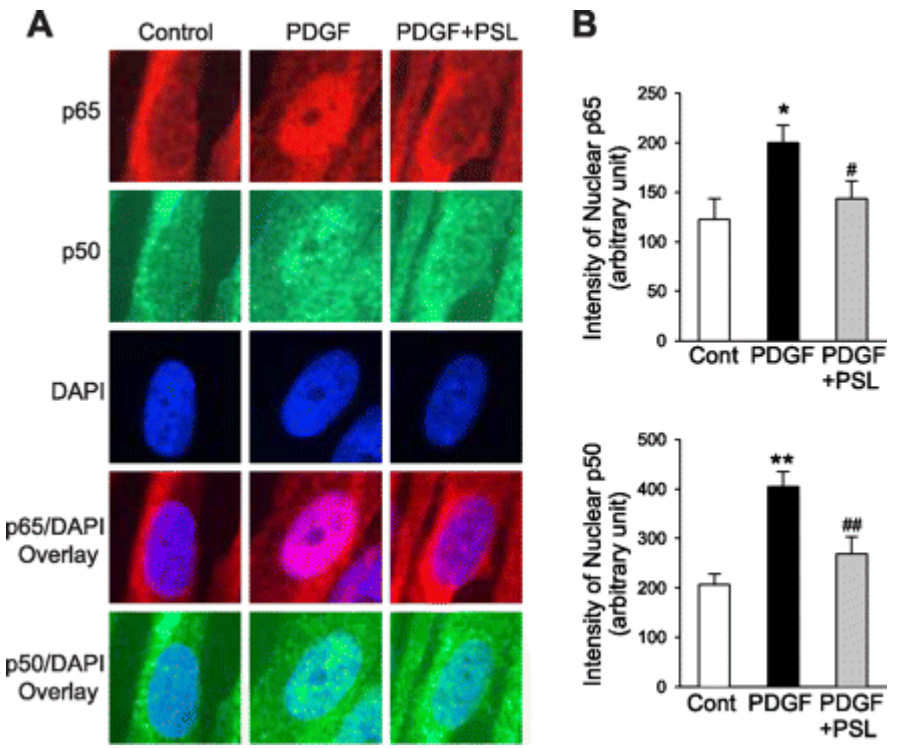
Stress-related signal transduction pathways are required for PR3- and HNE-induced apoptosis. (A) Schematic of kinases analyzed for activity (circles) and the major pathways associated with these kinases (B). Phosphorylated/active p-JNK, p-ERK, and p-p38 MAPK were analyzed using phospho-specific Abs. Immunoblots were reprobed with Abs to detect both active and inactive forms..

NF-kB pathway



Prednisolone inhibits PDGF-induced nuclear translocation of NF-κB in human pulmonary artery smooth muscle cells





PDGF induces nuclear translocation of NF-κB in pulmonary arterial smooth muscle cells. Western blot analysis (**A**) shows the expression level of p65 and p50 in the cytoplasm (Aa) and in the nuclei (Ab) in PASMC cultured in 0.1% FBS-DMEM with (PDGF) or without (Cont) PDGF (10 ng/ml) for 24 h. Summarized data (n = 3 experiments) show the average protein level of p65 (**B**) and p50 (**C**) (normalized to the protein level of GAPDH or histone) corresponding to the bands shown in the Western blots.

Immunofluorescent imaging shows a prednisolone-mediated suppression of PDGF-mediated nuclear translocation of p65 and p50. A: representative immunofluorescence staining of p65 and p50 in PASMC. DAPI staining was performed to indicate cell nuclei. Overlay of the images (p65 or p50 with DAPI) identifies nuclear translocation of p65 and p50 in PASMC treated with (PDGF), without (Control) PDGF (10 ng/ml), and with both PDGF and prednisolone (PDGF+PSL). **B**: summarized immunofluorescent staining of p65 and p50

STUDIO DELLA TRASDUZIONE DEL SEGNALE: localizzazione subcellulare

1 RECEPTION

MEMBRANA CELLULARE

- Metodiche di studio dei recettori
- Studi di binding

2 TRANSDUCTION

CITOPLASMA

- Analisi dei fattori citoplasmatici che trasducono all'interno della cellula lo stimolo extracellulare

3 CELL RESPONSE

NUCLEO

- Analisi dei fattori di trascrizione TRASLOCATI nel nucleo in seguito allo stimolo extracellulare

TECNICHE DI STUDIO DEI FATTORI DI TRASCRIZIONE TRASLOCATI NEL NUCLEO

1. **Luciferase Assay**
   Si basa sulla possibilità di evidenziare per luminescenza la trascrizione di un gene *reporter* regolato dal promoter cui si legano i fattori di trascrizione attivati da un certo stimolo in esame.

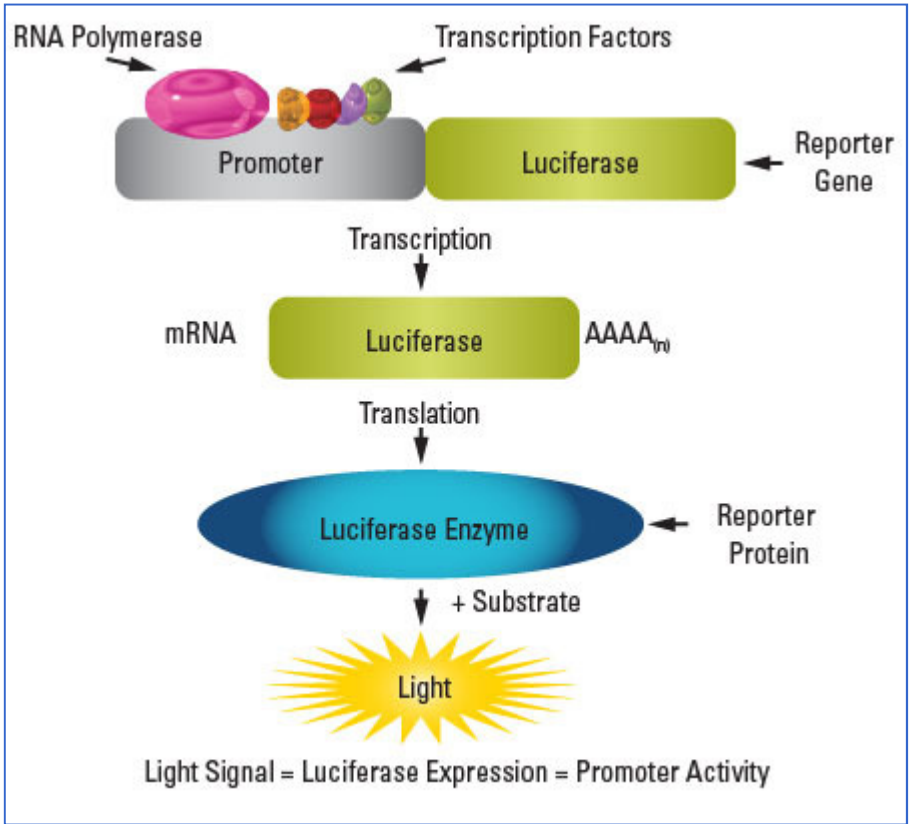
2. **EMSA (Electrophoretic Mobility Shift Assay)**
   Si basa sulla possibilità di evidenziare per corsa elettroforetica i fattori di trascrizione attivati da un certo stimolo in esame.

3. **CHIP (Chromatin Immunoprecipitation)**
   Si basa sulla possibilità di amplificare per PCR sequenze di DNA precipitate con specifiche binding proteins.

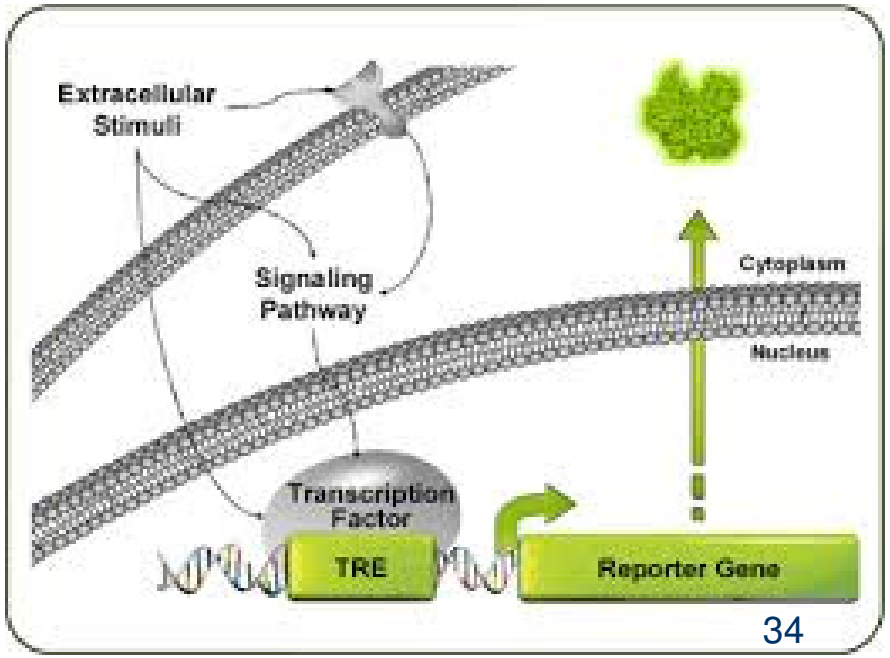
LUCIFERASE ASSAY

- E' un sistema che rivela l'attività trascrizionale di un **gene reporter** ed è ampiamente utilizzato in studi di espressione genica e di fisiologia cellulare.

- Ampliamente utilizzato nell'analisi di fattori di trascrizione nel *signaling* intracellulare.



Applicazione in signal transduction



DUAL-LUCIFERASE REPORTER ASSAY

Il termine "*Dual Reporter*" si riferisce alla simultanea espressione e misura di due differenti enzimi *reporter* (sperimentale e controllo) in un unico sistema.

Renilla reniformis (controllo)
La "sea pansy" (un antozoo) è una colonia di individui del diametro di alcuni centimetri.
Vive in ambiente sabbioso marino.
Diventa intensamente bioluminescente se disturbata.

Photinus pyralis (sperimentale)
La "firefly" (lucciola) è un coleottero della lunghezza di due centimetri.
La porzione terminale dell'addome emette una luminescenza giallo-verde allo scopo di attrarre altre lucciole.

ESEMPIO DI COPPIA DI VETTORI IMPIEGATI IN UN DUAL-LUCIFERASE REPORTER ASSAY

Gene reporter controllo
Renilla Luciferase

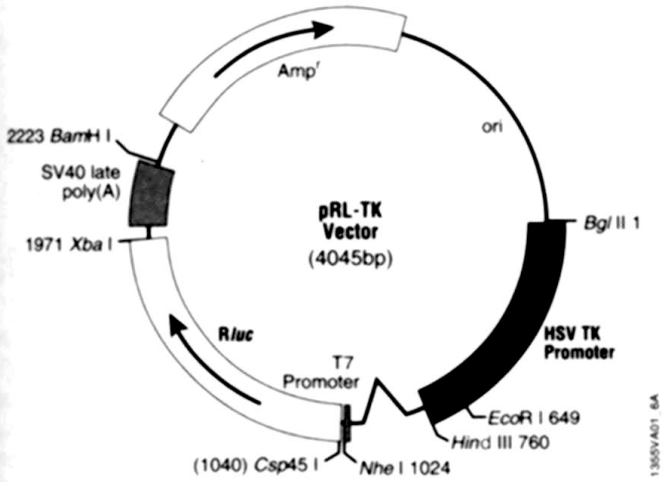
pRL-TK Vector

Description: The pRL-TK Vector[a] is intended for use as an internal control reporter and may be used in combination with any experimental reporter vector to cotransfect mammalian cells. All of Promega's pRL Reporter Vectors contain a cDNA (R*luc*) encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis* (sea pansy; 1).

The pRL-TK Vector contains the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide low to moderate levels of *Renilla* luciferase expression in co-transfected mammalian cells. *Renilla* luciferase is a 36kDa monomeric protein that does not require post-translational modification for activity (2). Therefore, like firefly luciferase, the enzyme may function as a genetic reporter immediately following translation. For information about the use of this plasmid in conjunction with a reporter vector containing the firefly luciferase gene, refer to the Dual-Luciferase® Reporter Assay System Technical Manual (#TM040).

GenBank®/EMBL Accession Number: AF025846.

[a] Licensed under U.S. Pat. Nos. 5,292,658, 5,418,155 and other patents.

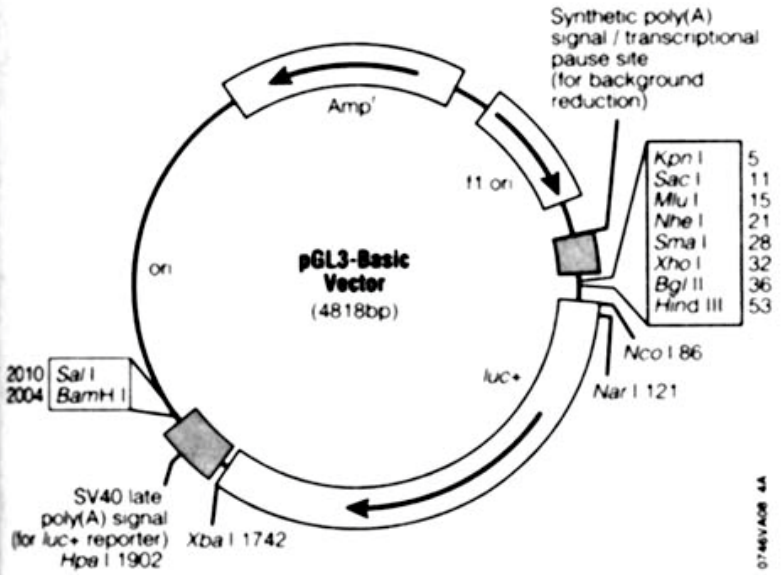


Gene reporter sperimentale
Firefly Luciferase

pGL3-Basic Vector

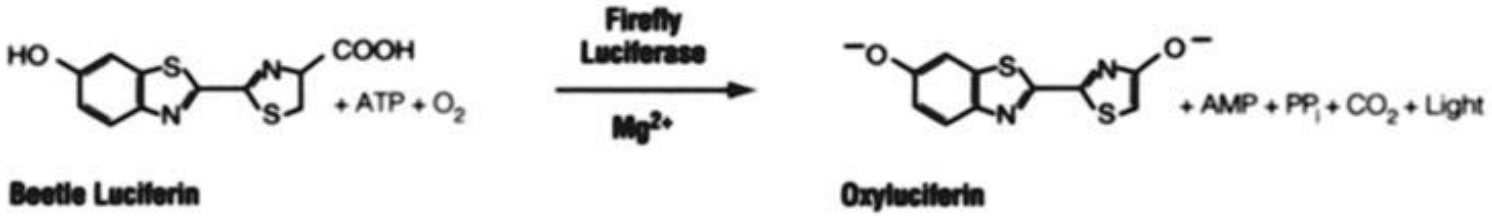
Description: The pGL3-Basic Vector[a,b] lacks eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter upstream from *luc+*. Potential enhancer elements can also be inserted upstream of the promoter or in the *Bam*H I or *Sal* I sites downstream of the *luc+* gene.

GenBank®/EMBL Accession Number: U47295.



- Di questi, il *reporter* "sperimentale" è correlato con l'effetto di specifiche condizioni sperimentali (es. stimoli extracellulari), mentre l'attività del *reporter* "controllo" cotrasfettato fornisce un controllo interno che serve per la stima della risposta basale del sistema.
- Normalizzando l'attività del *reporter* "sperimentale" con quella del controllo interno, è possibile minimizzare la variabilità provocata dalla diversa vitalità cellulare o dall'efficienza di trasfezione.

Gene reporter sperimentale



Gene reporter controllo

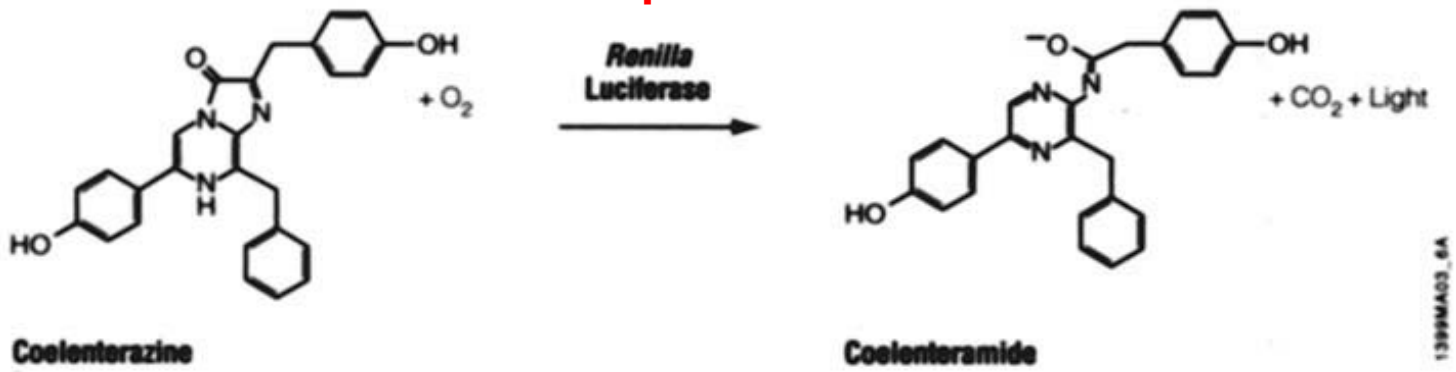


Figure 1. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferases.

FASI SPERIMENTALI DEL DUAL LUCIFERASE ASSAY

1. trasfezione delle cellule in studio con due plasmidi contenenti i geni reporter sperimentale e di controllo

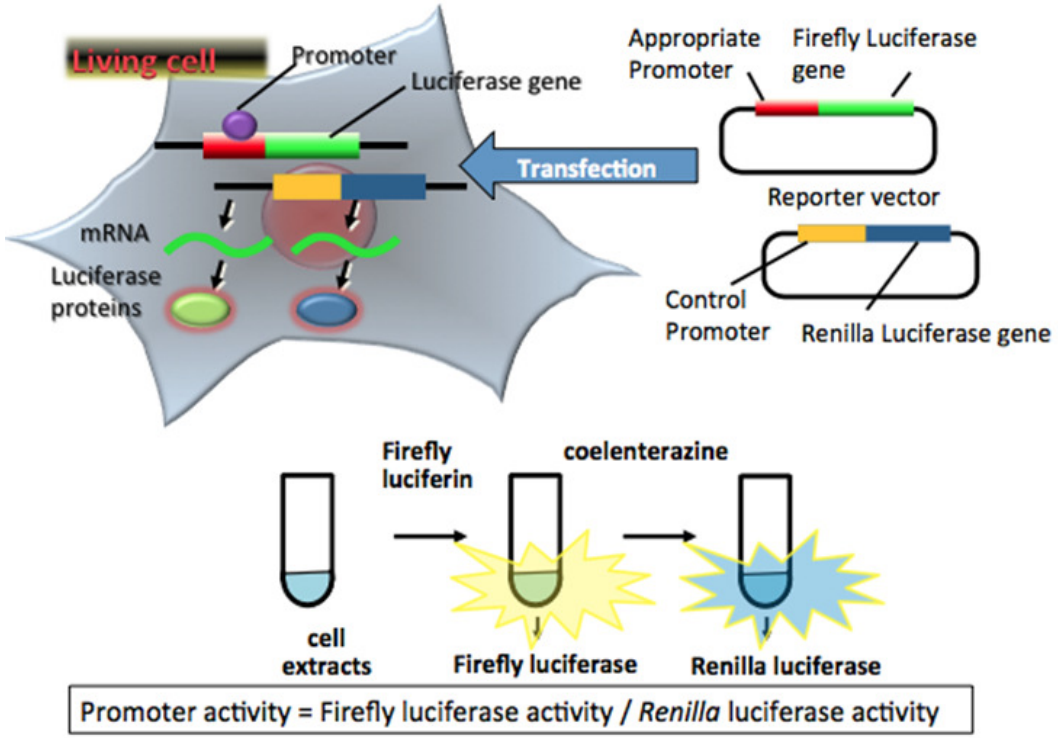
2. stimolazione delle cellule (attivazione del *signaling* e induzione della trascrizione della luciferasi sperimentale)

3. lisi cellulare con un opportuno reagente contenente il substrato per l'attività della luciferasi sperimentale

4. quenching della luciferasi sperimentale e aggiunta del substrato della luciferasi di controllo

5. valutazione dell'emissioni luminose (luciferasi sperimentale/luciferasi di controllo) come misura dell'attivazione del fattore di trascrizione esaminato

RAPPRESENTAZIONE SCHEMATICA DI UN DUAL-LUCIFERASE REPORTER ASSAY



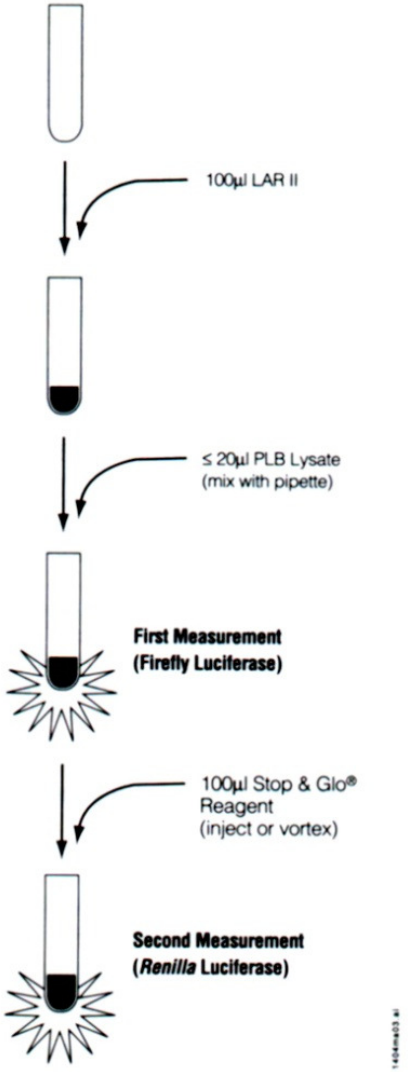


Figure 6. Format of the DLR™ Assay using a manual luminometer or a luminometer equipped with one reagent injector. If the instrument is equipped with two injectors, it may be preferable to predispense the lysate into luminometer tubes, followed by sequential auto-injection of the LAR II and Stop & Glo® Reagents.

MISURA DELL'ATTIVITA' DELLA LUCIFERASI PRIMA E DOPO L'AGGIUNTA DI *Stop & Glo Reagent*

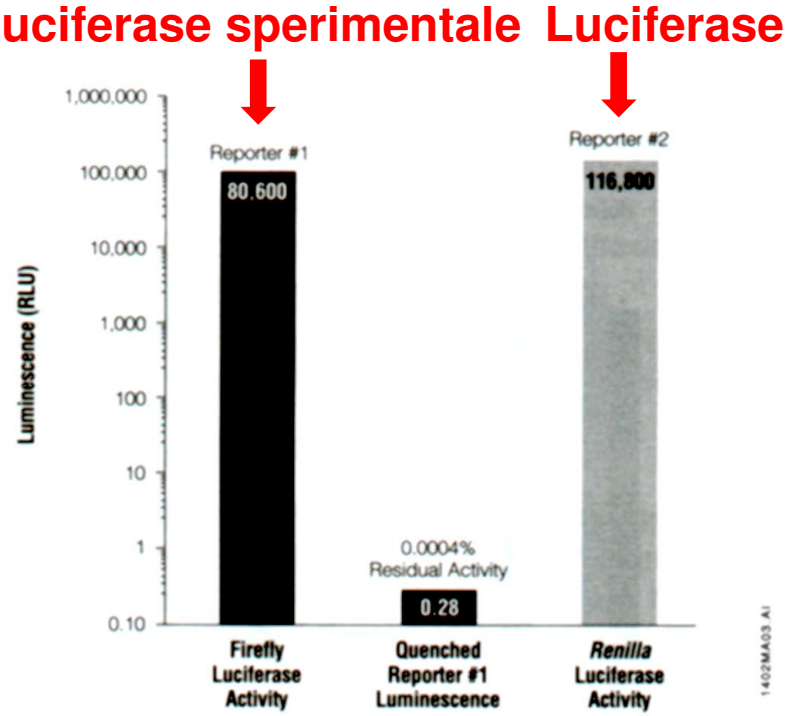
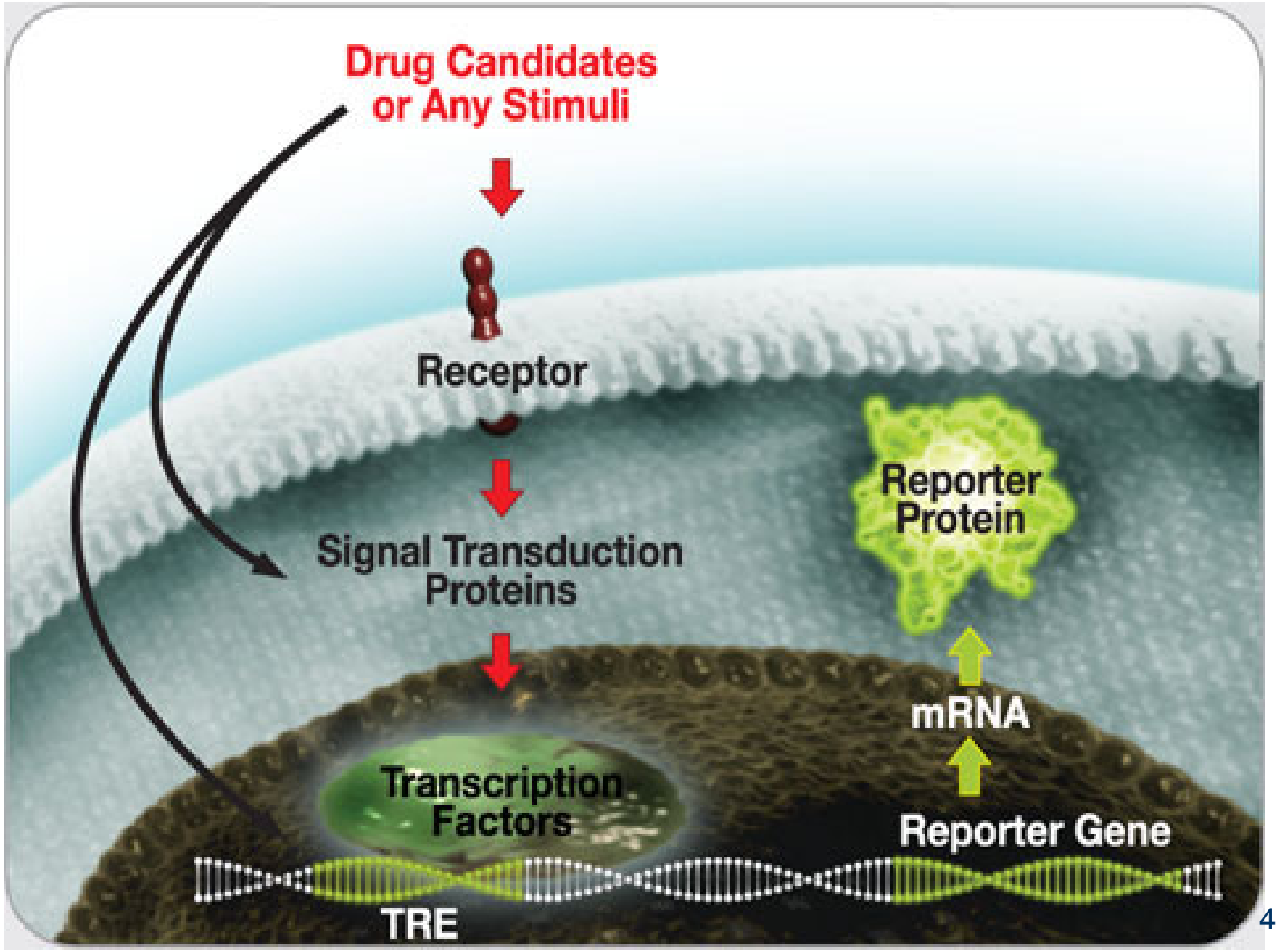
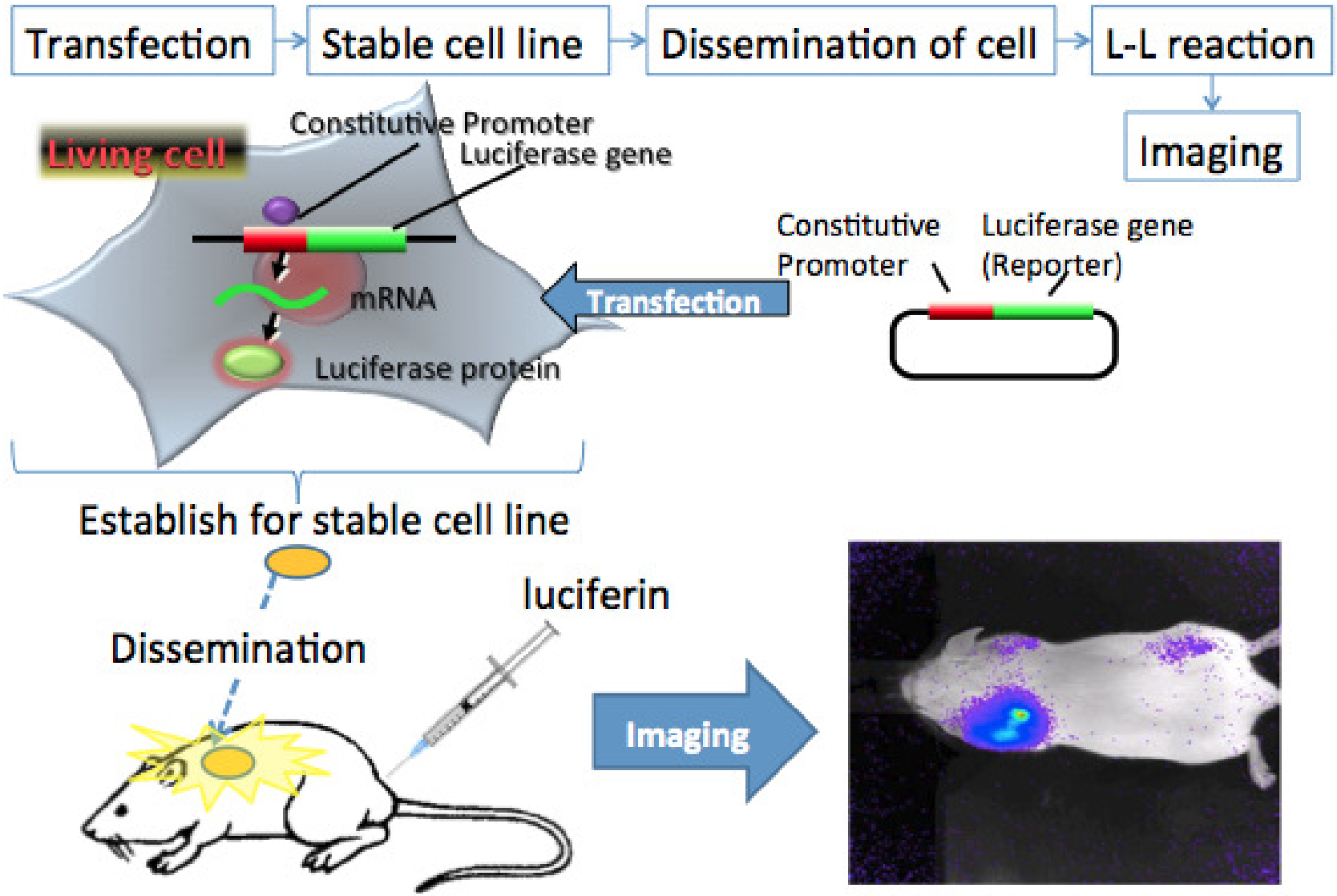


Figure 4. Measurement of luciferase activities before and after the addition of Stop & Glo® Reagent. The DLR™ Assay allows sequential measurement of firefly luciferase (Reporter #1), followed by *Renilla* luciferase activity (Reporter #2) on addition of Stop & Glo® Reagent to the reaction. Both reporter activities were quantitated within the same sample of lysate prepared from CHO cells co-transfected with pGL3 Control Vector[d,e] (Cat.# E1741) and pRL-SV40 Vector[g] (Cat.# E2231). To demonstrate the efficient quenching of Reporter #1 by Stop & Glo® Reagent, an equal volume of Stop & Glo® Buffer (which does not contain the substrate for *Renilla* luciferase) was added. Firefly luciferase luminescence was quenched by greater than 5 orders of magnitude.

APPLICAZIONI DEL LUCIFERASE ASSAY: Drug candidates screening

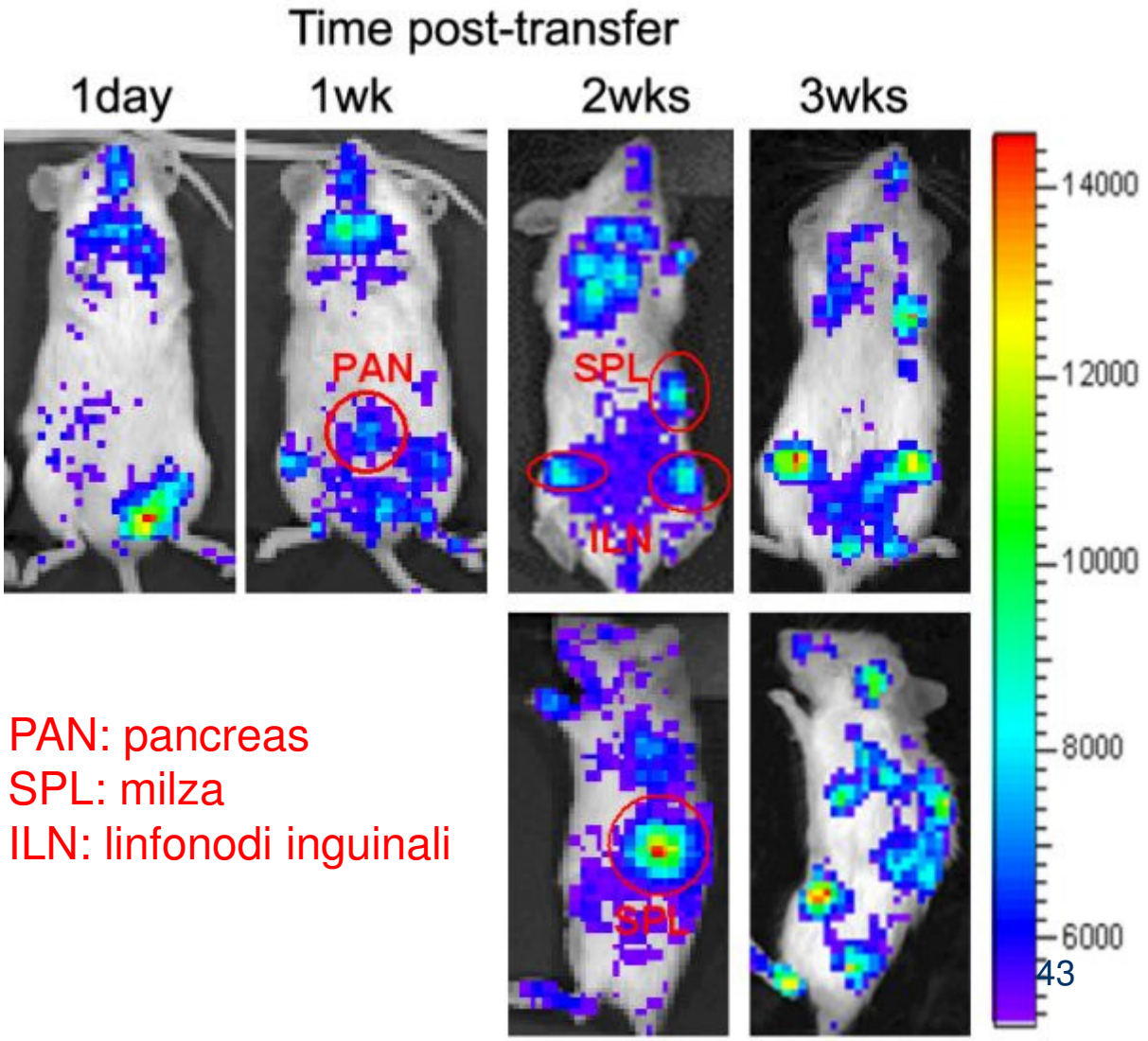


APPLICAZIONE IN VIVO DEL LUCIFERASE ASSAY



APPLICAZIONE *IN VIVO* DEL LUCIFERASE ASSAY

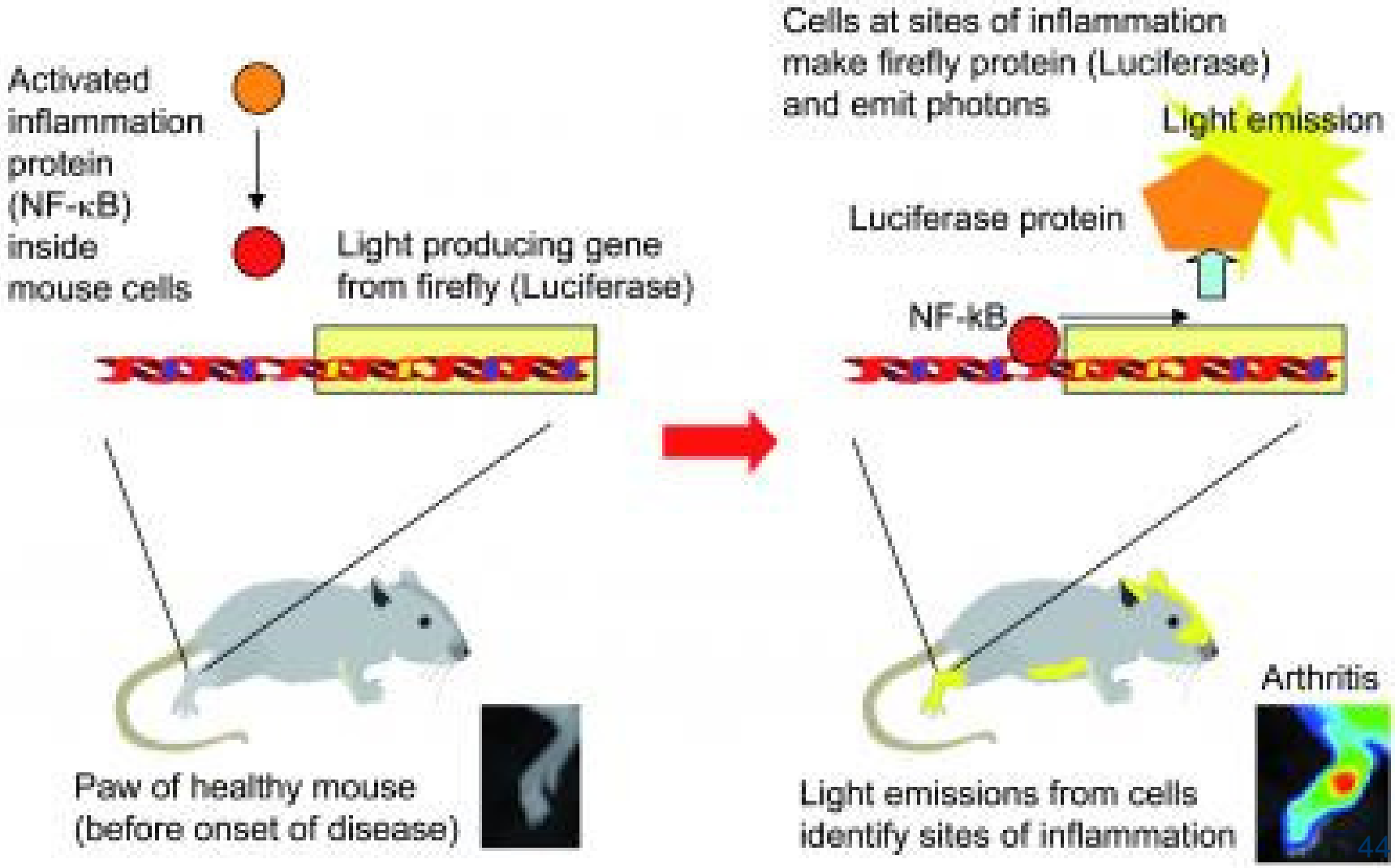
In Vivo Monitoring of Inflammation and Regulation in Type 1 Diabetes



PAN: pancreas
SPL: milza
ILN: linfonodi inguinali

APPLICAZIONE *IN VIVO* DEL LUCIFERASE ASSAY

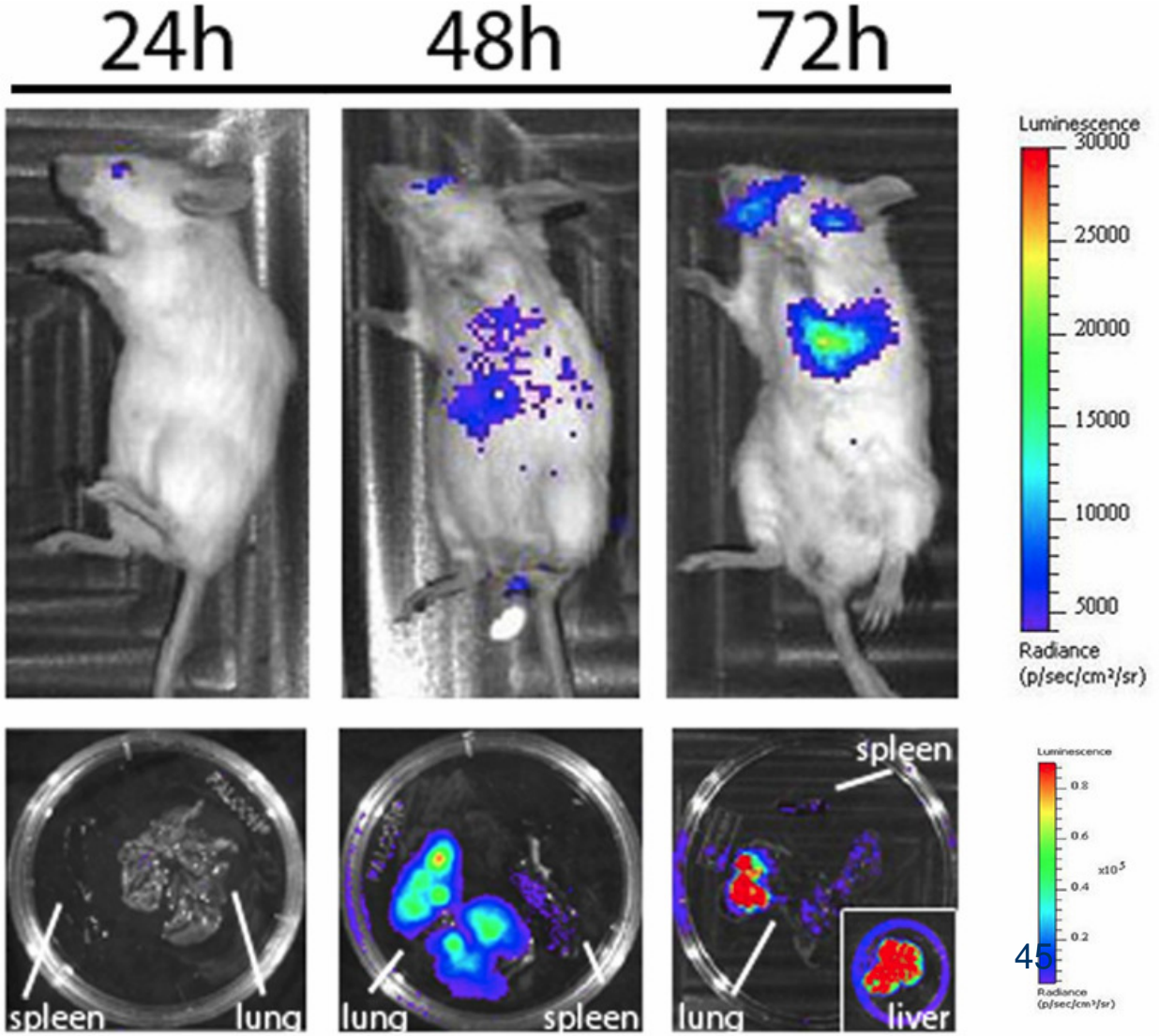
In Vivo Monitoring of Inflammation



In Vivo Monitoring of Infection

In vivo bioluminescence imaging of Burkholderia mallei (G- bacterium) respiratory infection and treatment in the mouse model

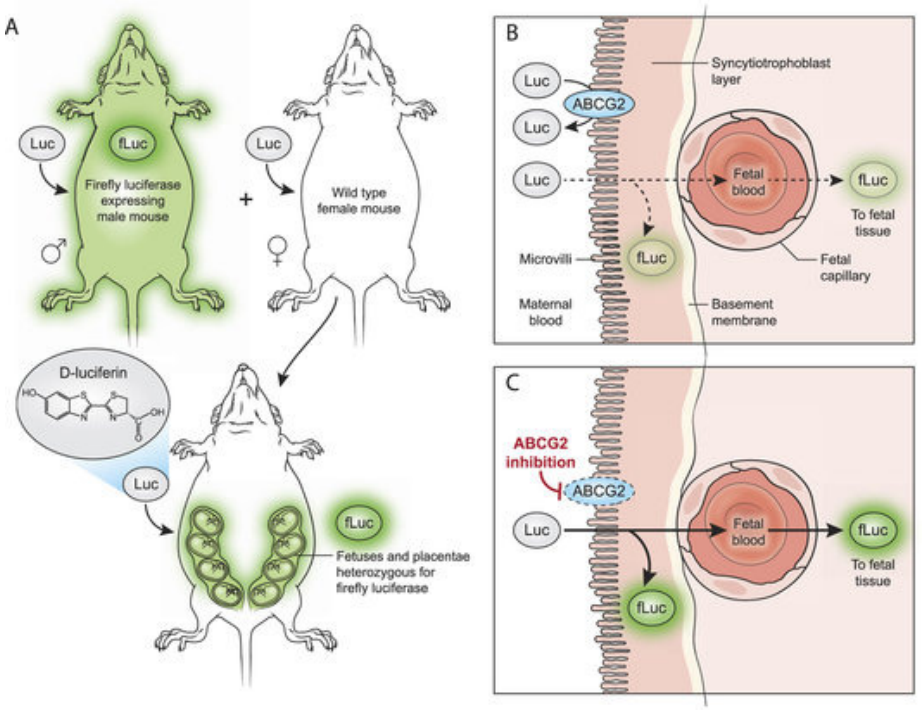
In vivo imaging of CSM001 infection. CSM001 was i.n. inoculated into mice. The in vivo bioluminescence imaging was conducted at 24, 48, and 72 h post-infection.

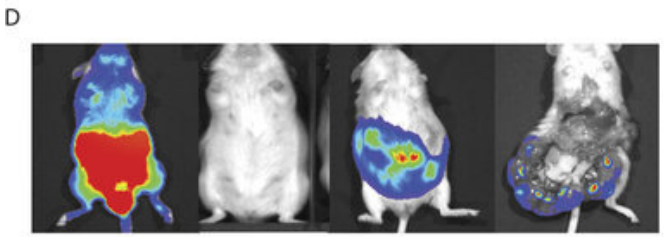


Bioluminescent imaging of ABCG2 efflux activity at the Blood-Placenta Barrier (BPB)

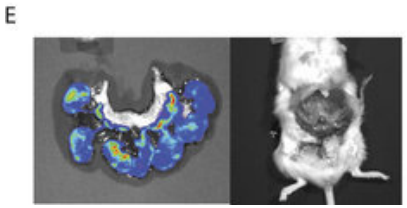
Jeyan S. Kumar et al., Scientific Reports | 6:20418 | DOI: 10.1038/srep20418

- ABC transporters are transmembrane proteins found at the BPB and function to efflux xenobiotics and maintain chemical homeostasis
- D-luciferin was used as a specific substrate of ABCG2, and was used to image the transporter function of ABCG2 at the BPB
- A mouse model was designed by mating a transgenic male expressing fLuc with a wild-type female to generate pregnant mice carrying hemizygous pups and placentae expressing fLuc.





D Bioluminescence images (left to right) of a male CAG-fLuc mouse, non-pregnant female FVB mouse, pregnant FVB mouse, and pregnant FVB mouse imaged immediately post euthanasia with peritoneum opened to visualize uterus, all after being administered 5 mg/kg D-luciferin i.p. for 20 min.



E Bioluminescence images from (left to right) of intact uterus demonstrating BLI signal from fetuses and placentae, and female mouse after uterus was removed with no signal seen, after 5 mg/kg IP d-luciferin for 20 minutes.

3 CELL RESPONSE

NUCLEO

- Analisi dei fattori di trascrizione TRASLOCATI nel nucleo in seguito allo stimolo extracellulare

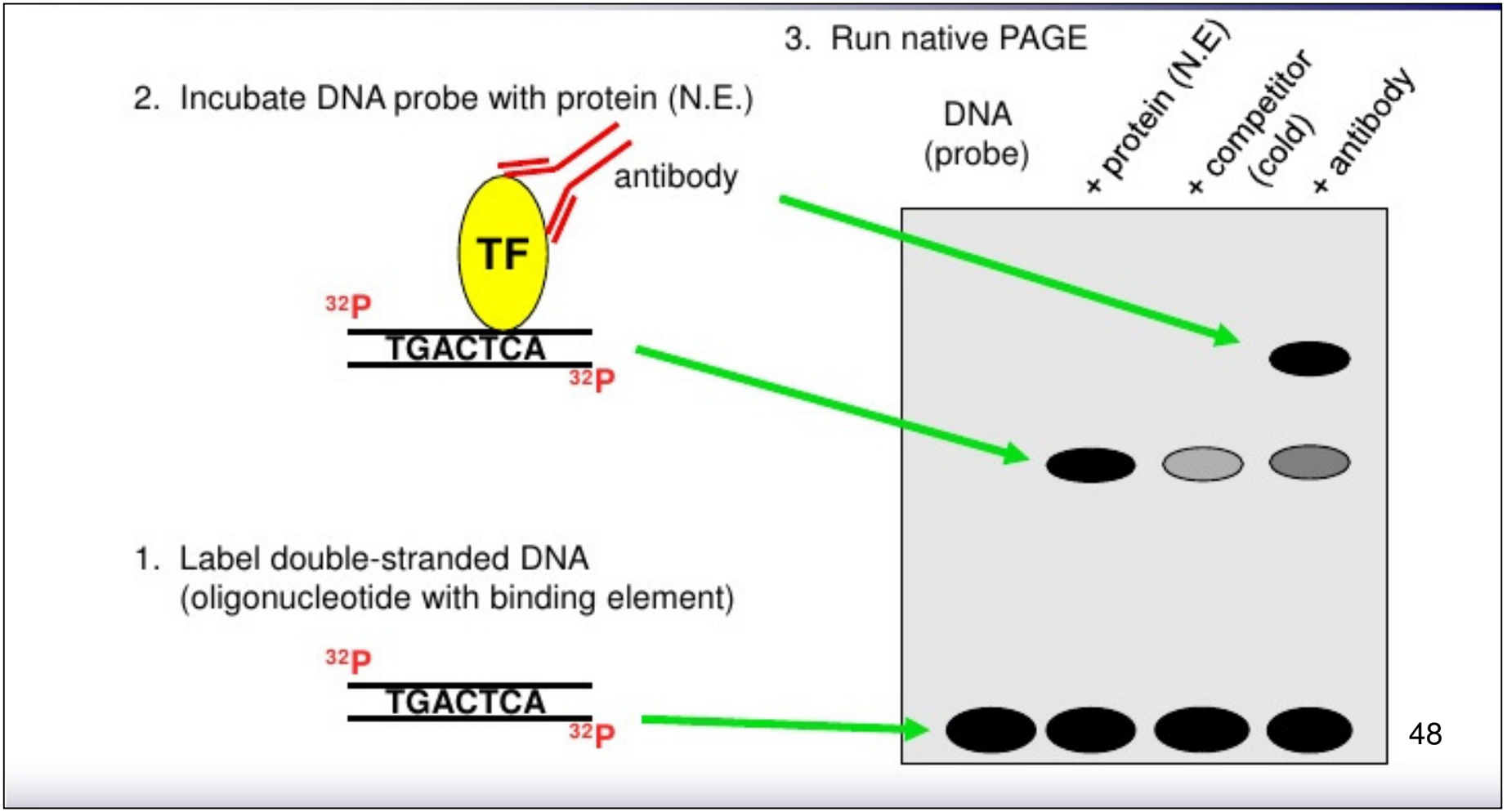
1. **Luciferase Assay**
   Si basa sulla possibilità di evidenziare per luminescenza la trascrizione di un gene *reporter* regolato dal promoter cui si legano i fattori di trascrizione attivati da un certo stimolo in esame.

2. **EMSA (Electrophoretic Mobility Shift Assay)**
   Si basa sulla possibilità di evidenziare per corsa elettroforetica i fattori di trascrizione attivati da un certo stimolo in esame.

3. **CHIP (Chromatin Immunoprecipitation)**
   Si basa sulla possibilità di amplificare per PCR sequenze di DNA precipitate con specifiche binding proteins.

EMSA (Electrophoretic Mobility Shift Assay)

- E' una tecnica che permette di studiare i fattori di trascrizione attivati da un certo segnale, mimando *in vitro* l'interazione che avviene all'interno della cellula tra fattori di trascrizione e sequenze *promoter*.
- Con l'ausilio di una sonda radiomarcata (**1**), è possibile visualizzare su lastra autoradiografica il fattore di trascrizione coinvolto nel *signaling* in esame (**2**), e impiegando opportuni anticorpi, distinguerlo tra i membri di una stessa famiglia ("supershift") (**3**).



EMSA (Electrophoretic Mobility Shift Assay)

FASI SPERIMENTALI:

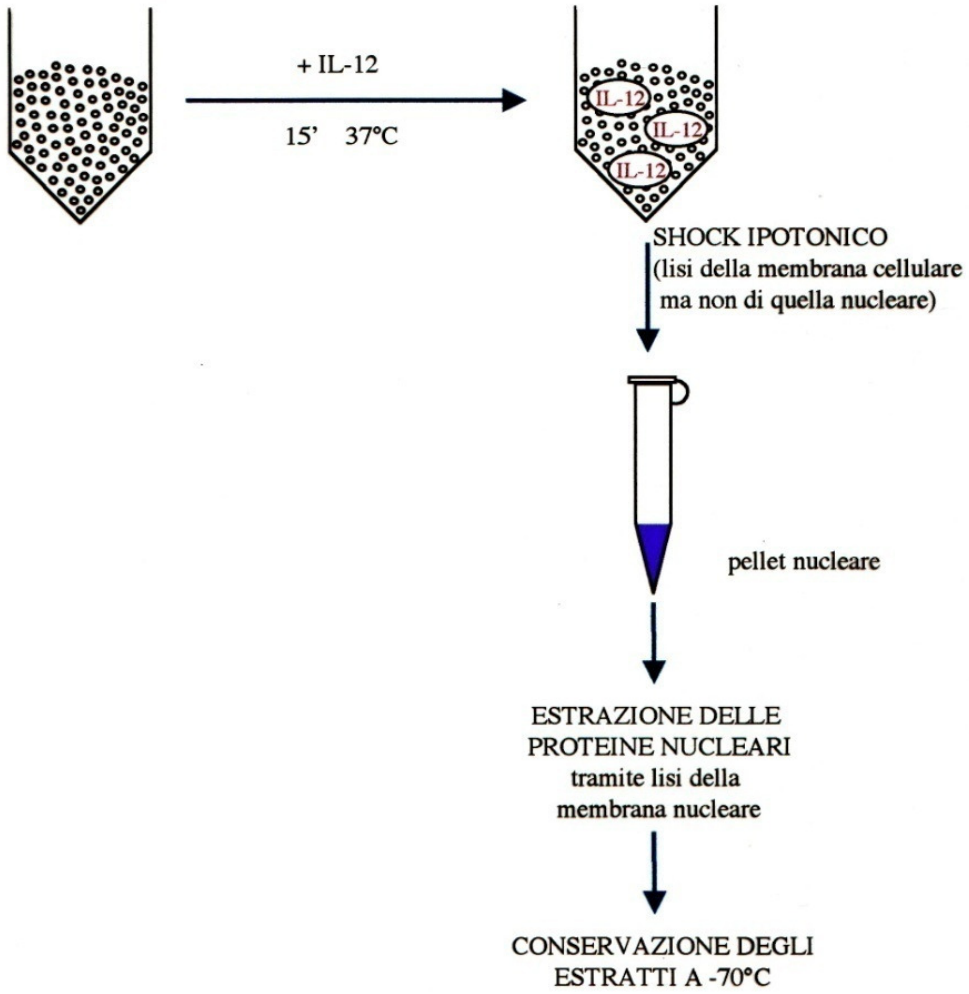
1. stimolazione delle cellule (attivazione del *signaling* e migrazione dei fattori di trascrizione in sede nucleare)

2. lisi delle cellule con opportuni reagenti ed estrazione delle proteine nucleari

3. incubazione con un oligonucleotide a doppia catena di DNA radiomarcato (sequenza *promoter*) ed, eventualmente, con un anticorpo anti-fattore di trascrizione

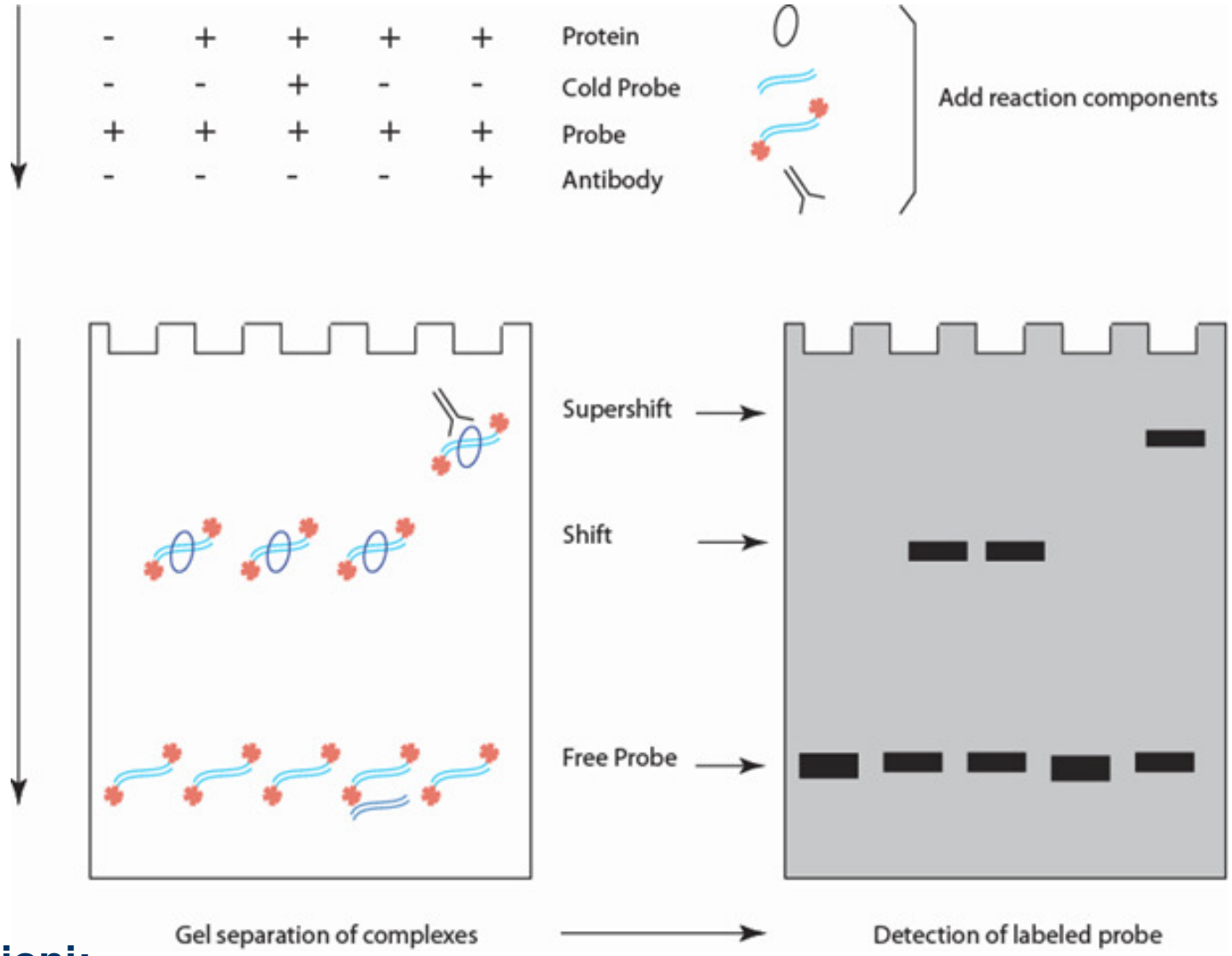
4. caricamento su gel per evidenziare il complesso “sonda-fattore di trascrizione”, oppure “sonda-fattore di trascrizione-anticorpo” (complesso ritardato).

EMSA: PREPARAZIONE DEI CAMPIONI



Rappresentazione schematica del metodo di preparazione degli estratti nucleari.

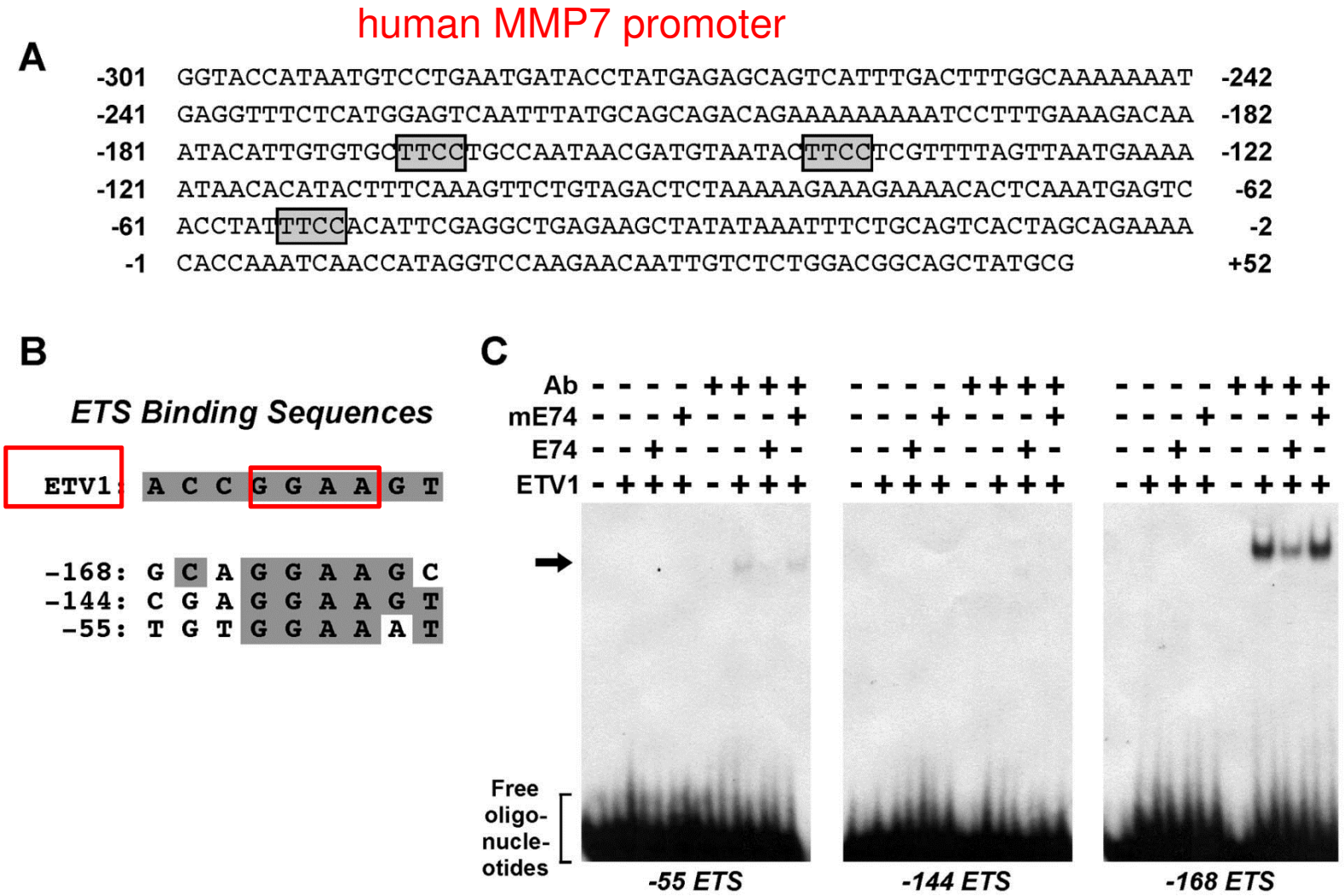
Interpretazione delle bande ottenute in un esperimento EMSA



Applicazioni:

- studio dei fattori di trascrizione che si legano ad una specifica sequenza promotrice
- studio della sequenza promotrice che risponde ad uno specifico fattore di trascrizione

Direct binding of ETV1 to the MMP-7 promoter in LNCaP prostate cancer cells



(A) DNA sequence of the human MMP-7 promoter from –301 to +52. The 5′-GGAA-3′ (5′-TTCC-3′ in reverse) core of ETS binding sites at -55, -144 and -168 is shown in boxes. **(B)** Alignment of the indicated MMP-7 ETS sites (complementary strand) to the consensus ETV1 binding site. (C) Gel electrophoretic mobility shift assays with indicated ETS sites. Recombinant ETV1, unlabeled oligonucleotides (E74 or mE74) and ETV1 antibody (Ab) were incubated with 32P-labeled oligonucleotides and then separated on native gels. Shown are respective autoradiograms. Arrow indicates binding of ETV1 to 32P-labeled DNA.

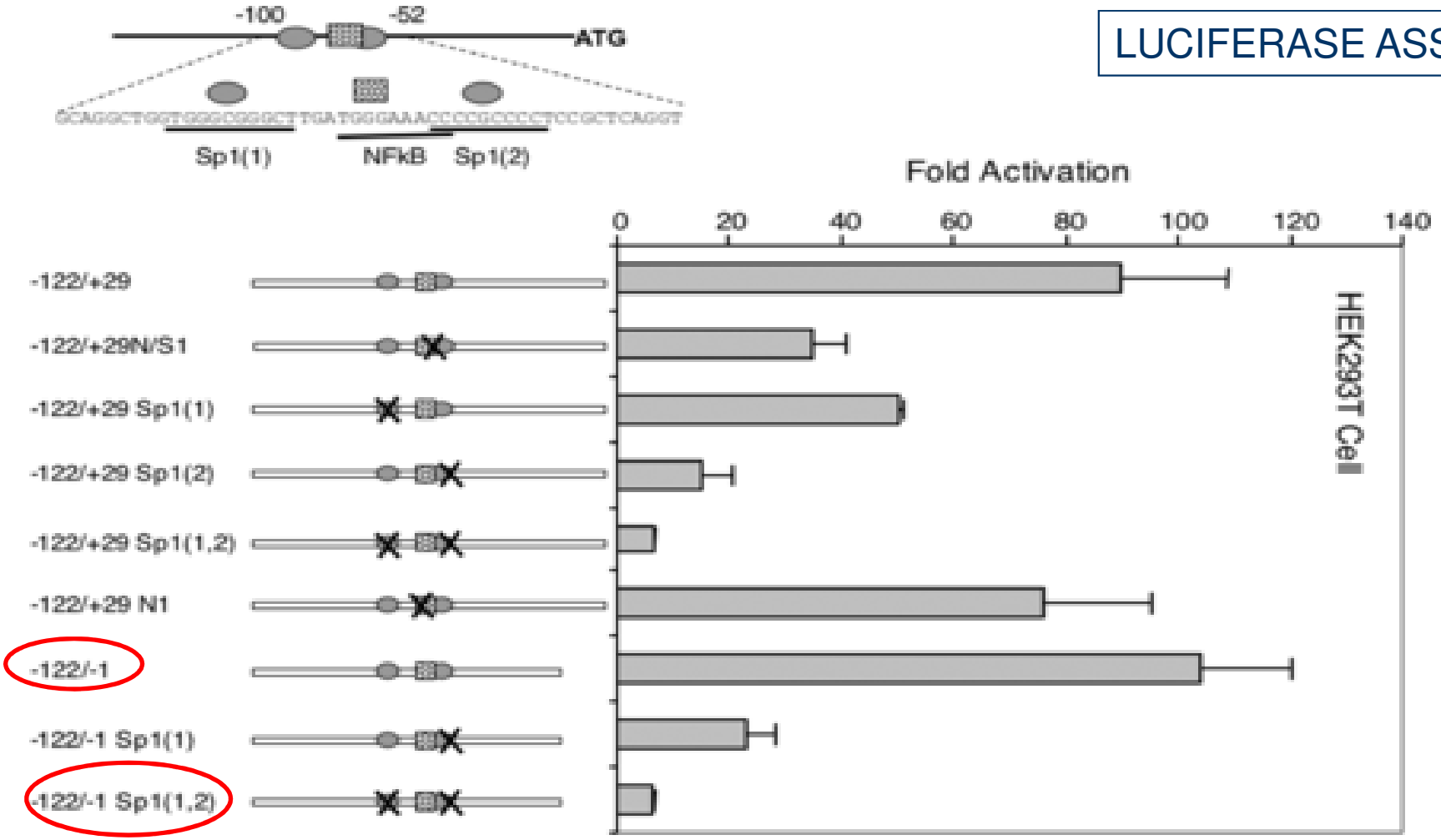
SOOK SHIN et al.; ONCOLOGY REPORTS 29: 306-314, 2013

A simple promoter containing two Sp1 sites controls the expression of sterol-regulatory-element-binding protein 1a (SREBP-1a)

ZHANG et al. Biochem. J. (2005) 386, 161–168

SREBPs (sterol-regulatory-element-binding proteins) are important regulators of mammalian lipid metabolism

LUCIFERASE ASSAY

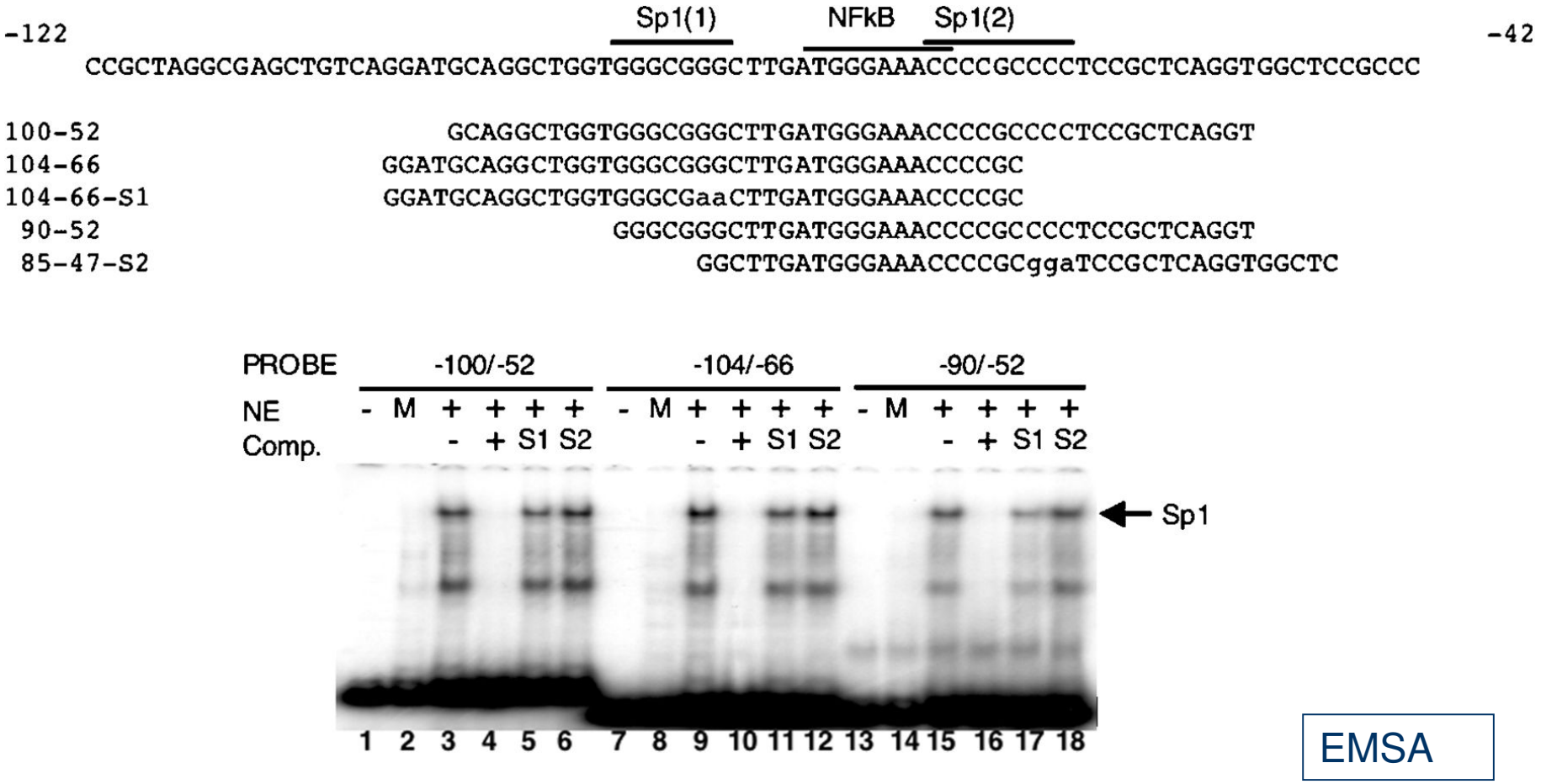


Functional analysis of binding sites for Sp1 and NF-κB in the SREBP-1a promoter

DNA containing the wild-type or indicated mutant versions of the mouse SREBP-1a promoter were fused to luciferase and analysed for promoter activity
N denotes a mutation in the putative NF-κB site.

A simple promoter containing two Sp1 sites controls the expression of sterol-regulatory-element-binding protein 1a (SREBP-1a)

ZHANG et al. Biochem. J. (2005) 386, 161–168



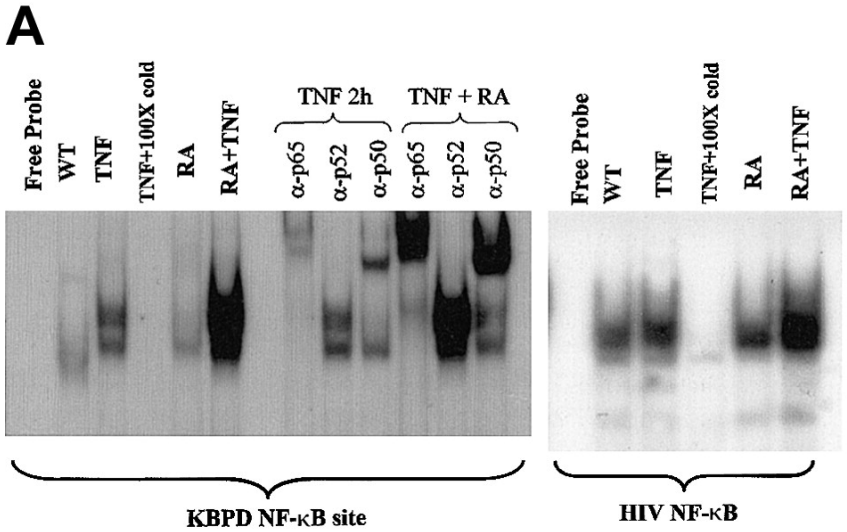
EMSA

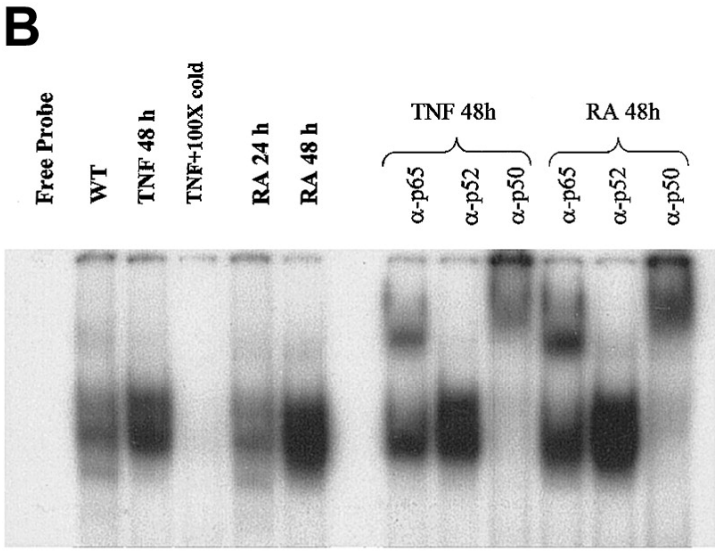
Binding of Sp1 to the SREBP-1a promoter analysed by EMSA

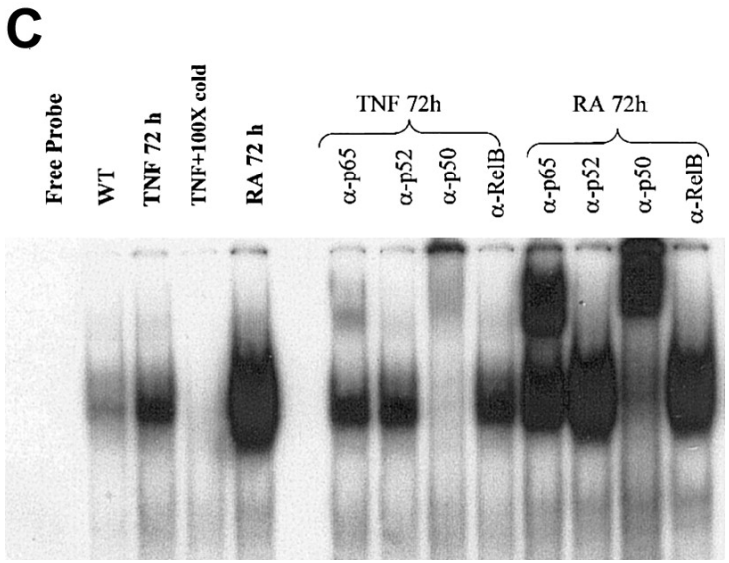
The DNA sequence for the proximal region of the mouse SREBP-1a promoter, along with locations of the putative Sp1 and NF-κB sites, is shown in the upper panel. The sequences for individual probes derived from this sequence and used in the EMSA studies are shown below the wild-type sequence. Mutations generated in probes S1 and S2 are noted in lower-case. An autoradiogram from a typical EMSA is shown in the lower panel. The identity of the three probes that were 32P-labelled and used in the EMSA are shown. 'NE' denotes nuclear extract prepared from mock-transfected SL2 cells (M) or from SL2 cells transfected with the pPACSp1 expression vector. (+) denotes that the cold competitor was the same DNA used as a probe. S1 and S2 denote the use of mutant probes containing the mutations shown in the top of the Figure.

ATRA (*All-Trans Retinoic Acids*) regulation of NF-κB binding

Witcher et al. BLOOD, 1 JULY 2003;VOLUME 102, NUMBER 1







EMSAs and supershift assays showing binding to a palindromic NF-κB response element using nuclear extracts from NB4 cells treated with ATRA and TNF for various times. (**A**) ATRA is seen to heighten TNF-induced NF-κB binding after 2 hours and to increase these complexes alone after 48 and 72 hours (**B-C**). Supershift assays indicated that complexes induced by ATRA were composed primarily of p65 and p50 proteins.

3 CELL RESPONSE

NUCLEO

- Analisi dei fattori di trascrizione TRASLOCATI nel nucleo in seguito allo stimolo extracellulare

1. **Luciferase Assay**
   Si basa sulla possibilità di evidenziare per luminescenza la trascrizione di un gene *reporter* regolato dal promoter cui si legano i fattori di trascrizione attivati da un certo stimolo in esame.

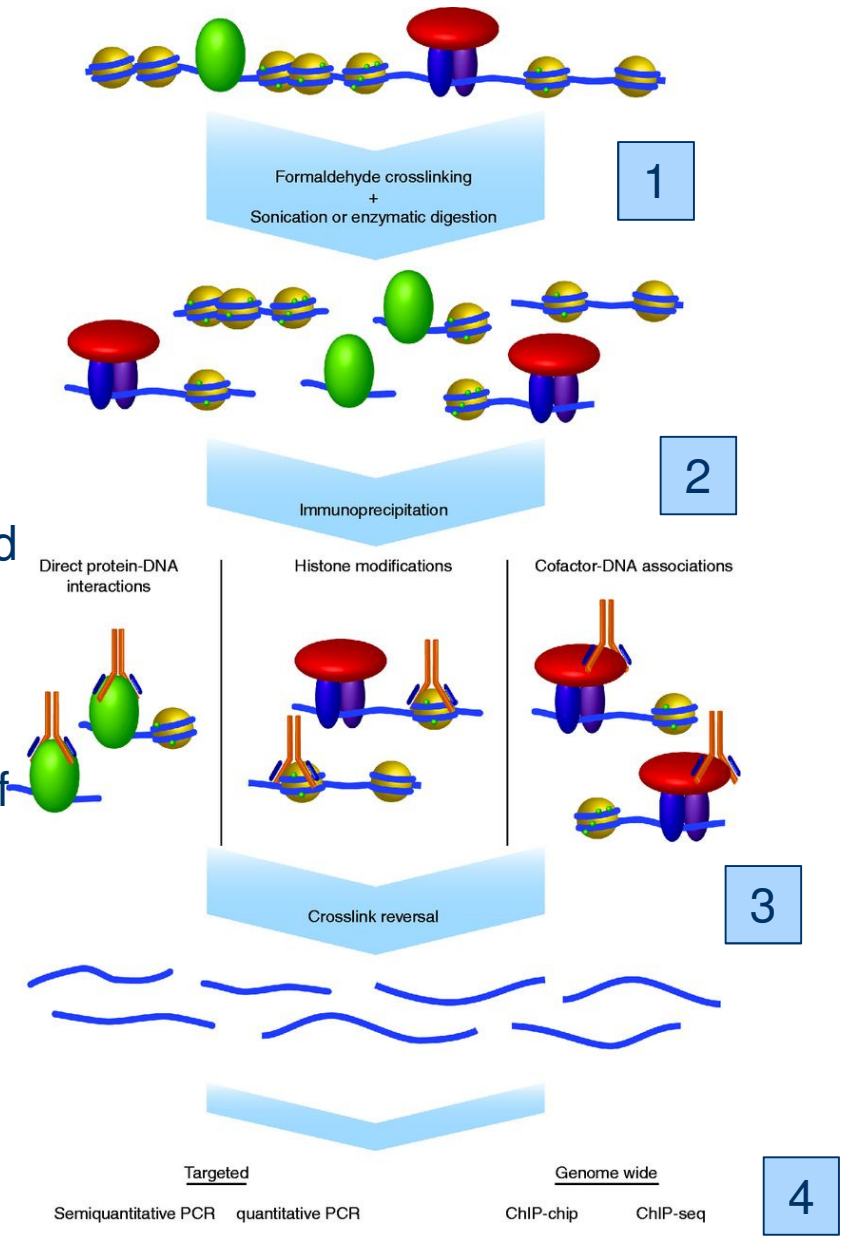
2. **EMSA (Electrophoretic Mobility Shift Assay)**
   Si basa sulla possibilità di evidenziare per corsa elettroforetica i fattori di trascrizione attivati da un certo stimolo in esame.

3. **CHIP (Chromatin Immunoprecipitation)**
   Si basa sulla possibilità di amplificare per PCR sequenze di DNA precipitate con specifiche binding proteins.

CHIP
fasi sperimentali

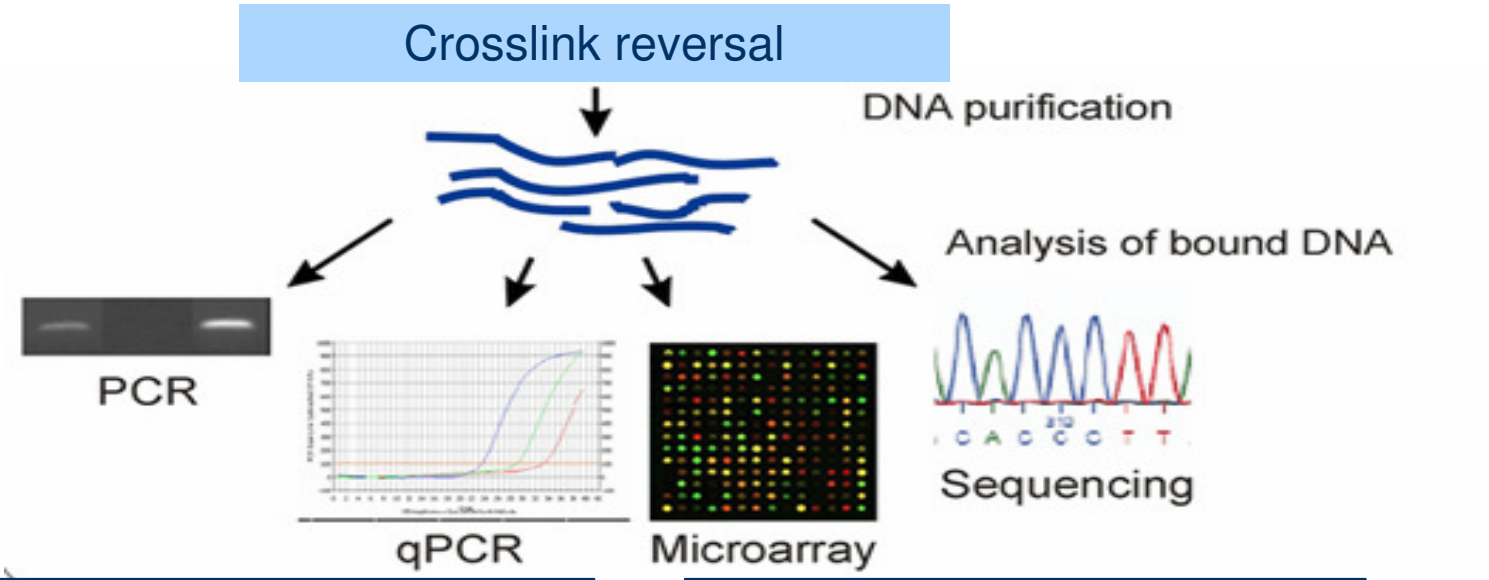
ChIP assay is a powerful method for analyzing epigenetic modifications and genomic DNA sequences bound to specific regulatory proteins.

1. protein-DNA complexes are covalently crosslinked and the chromatin is fragmented
2. Antibodies against various proteins associated with DNA (histones, transcription factors, cofactors, etc) can be used to immunoprecipitate any associated fragments of DNA
3. Crosslink reversal for isolating DNA sequences
4. Targeted or genome-wide analysis of the DNA



Aurimas Vinckevicius, and Debabrata Chakravarti J Mol Endocrinol 2012;49:R113-R123

4. Targeted or genome-wide analysis of the DNA



Targeted analysis:
- End-point PCR
- Real-time PCR

Genome-wide analysis:
- CHIP-chip
- CHIP-seq