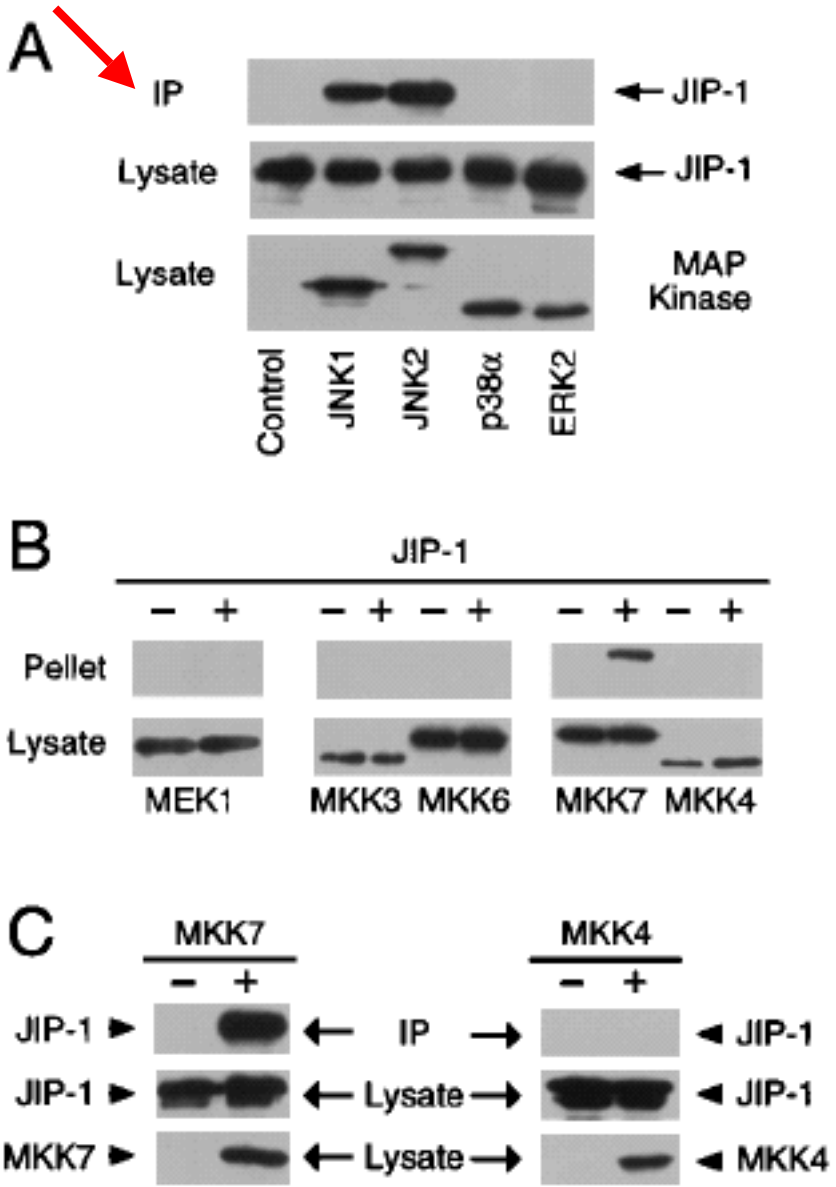
Fig. 1. Selective binding of JIP-1 to the MAP kinase JNK and the MAP kinase kinase MKK7. (**A**) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with the HA-tagged MAP kinases ERK2, p38α, JNK1, and JNK2 (*15*, *16*). The MAP kinases were immunoprecipitated with an antibody to HA. The presence of JIP-1 in the immunoprecipitates (IP) was detected on immunoblots probed with an antibody to T7-Tag. The amount of JIP-1 and MAP kinases in the cell lysates was examined by protein immunoblot analysis. (**B**) JIP-1 was expressed in cells as a GST fusion protein together with epitope-tagged MEK1, MKK3, MKK4, MKK6, or MKK7 (*15*, *16*). JIP-1 was precipitated from cell lysates with glutathione-agarose, and the MAPKKs present in the pellet were detected by protein immunoblot analysis. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis. (**C**) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with Flag-tagged MKK4 or MKK7 (*15*, *16*). The presence of JIP-1 in Flag IP was detected by protein immunoblot analysis with an antibody to T7-Tag. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis.

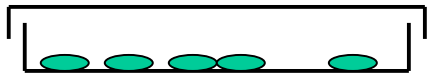


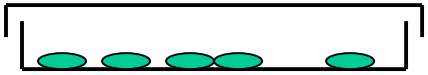
Experimental approach to protein-protein interaction

Immunoprecipitation of tagged-proteins

Costrutto “taggato”

Trasfezione
transiente



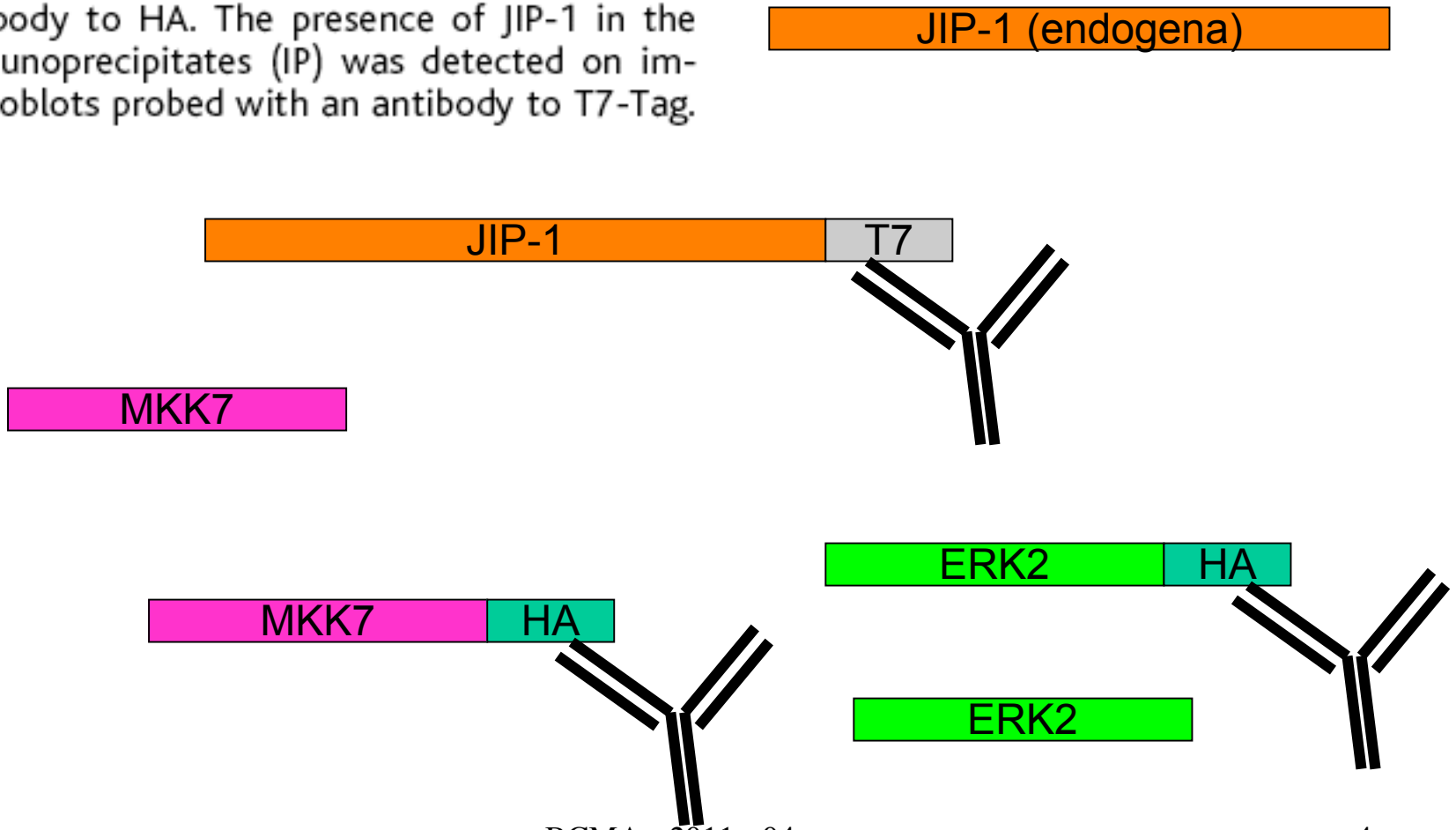


IP:
anticorpo anti-proteina
d’interesse

IP:
anticorpo anti-Tag

Differenza?

Fig. 1. Selective binding of JIP-1 to the MAP kinase JNK and the MAP kinase kinase MKK7. (**A**) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with the HA-tagged MAP kinases ERK2, p38α, JNK1, and JNK2 (*15*, *16*). The MAP kinases were immunoprecipitated with an antibody to HA. The presence of JIP-1 in the immunoprecipitates (IP) was detected on immunoblots probed with an antibody to T7-Tag.



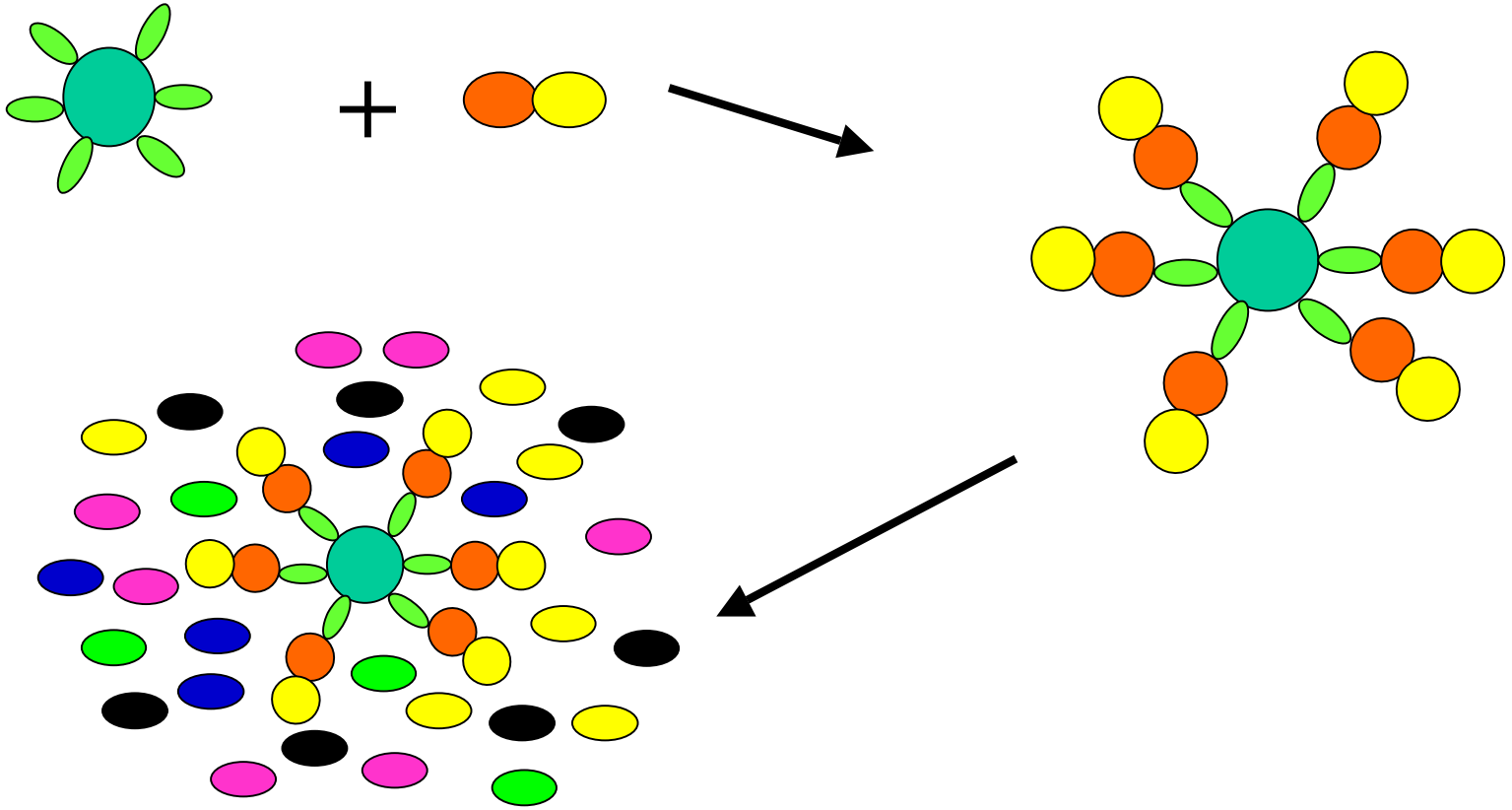
Pull-down

PULL-DOWN

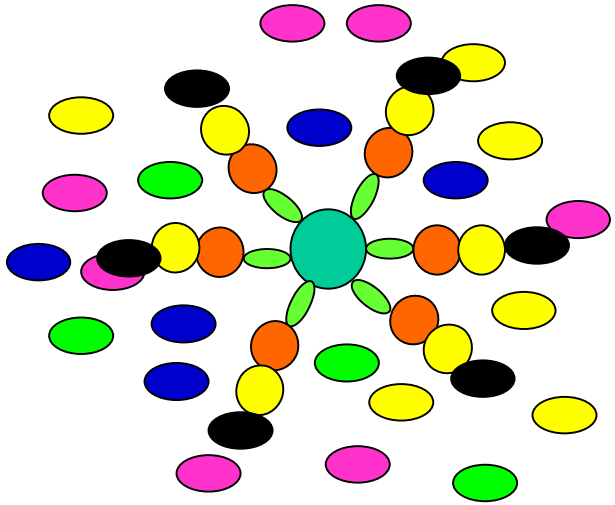
sferette di sepharose- glutatione

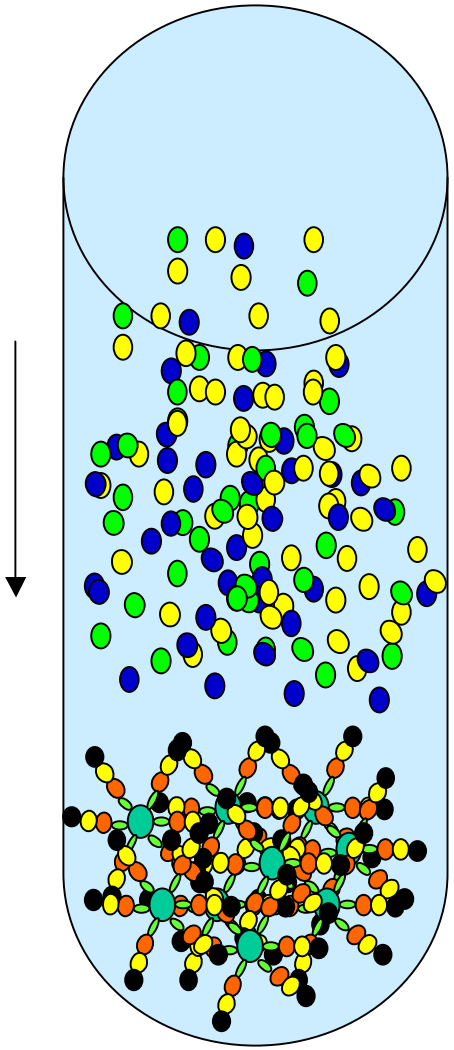
\+

GLUTATIONE TRANSFERASI | PROTEINA ESCA



• metto in agitazione (rotazione) a 4°C 2h o O/N affinché la proteina “esca”, (fusa con la glutatione transferasi) vada in contatto con tutte le proteine presenti nell’estratto

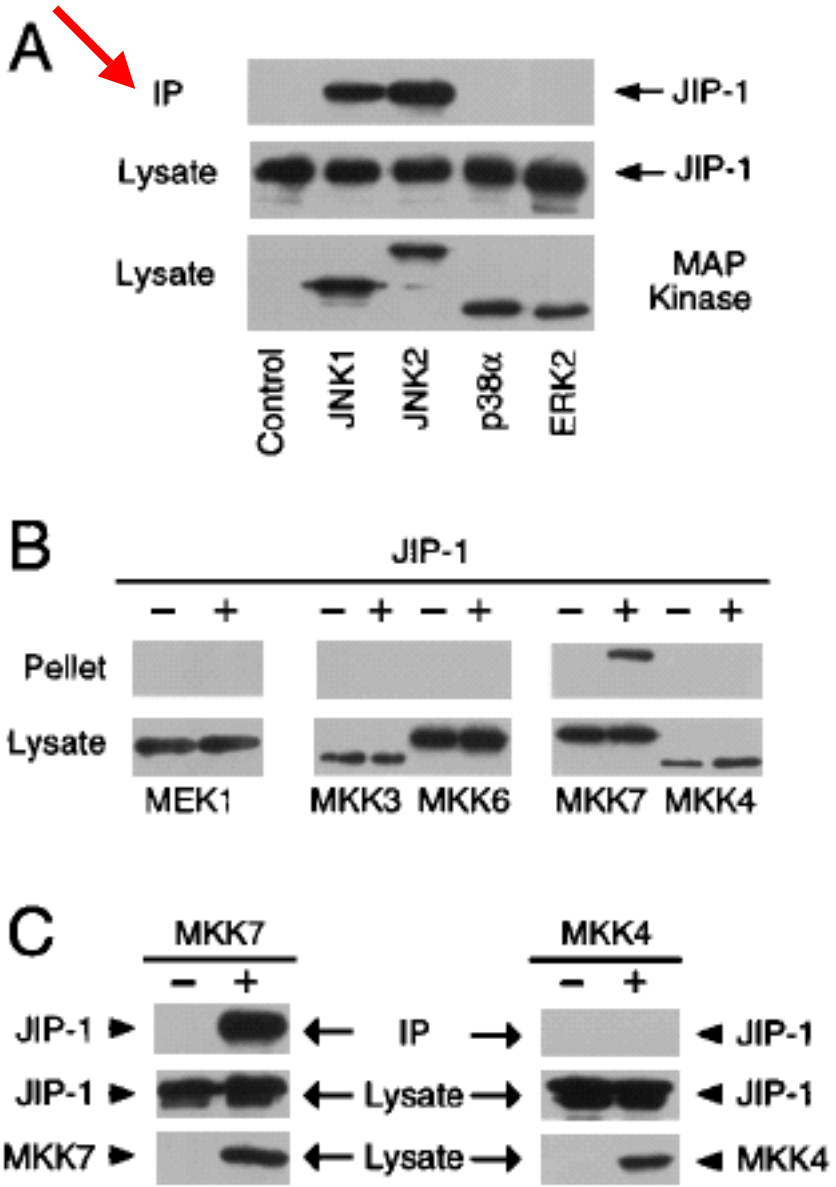


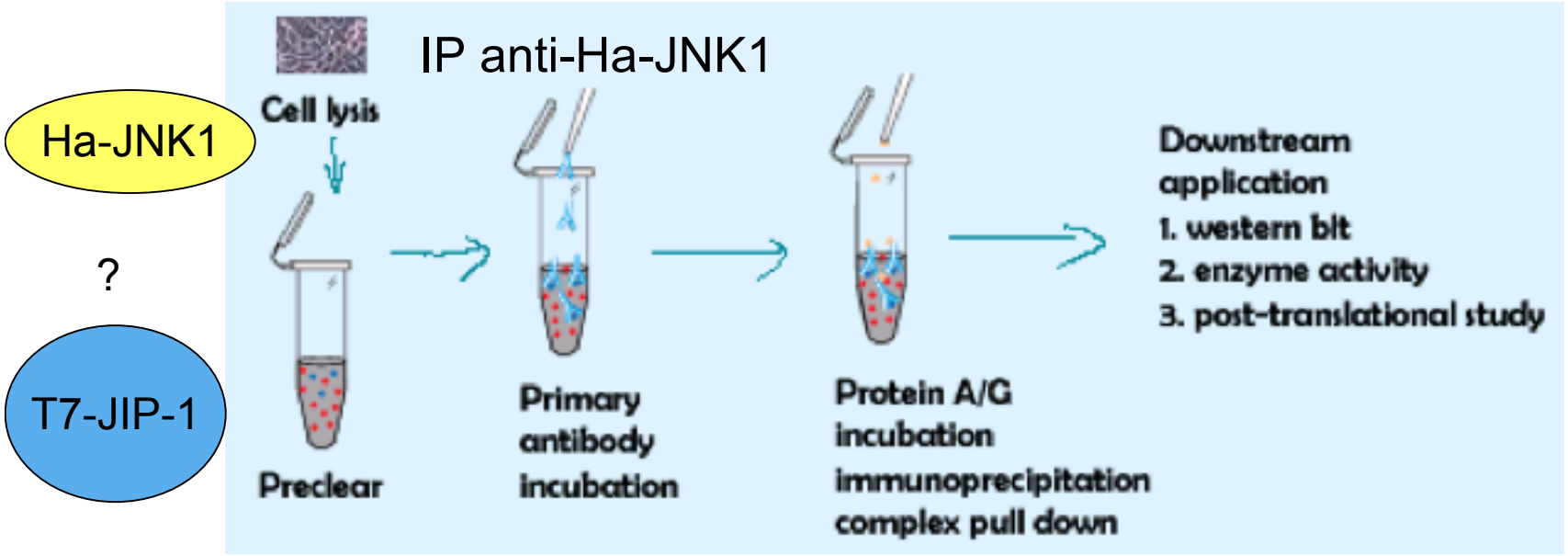


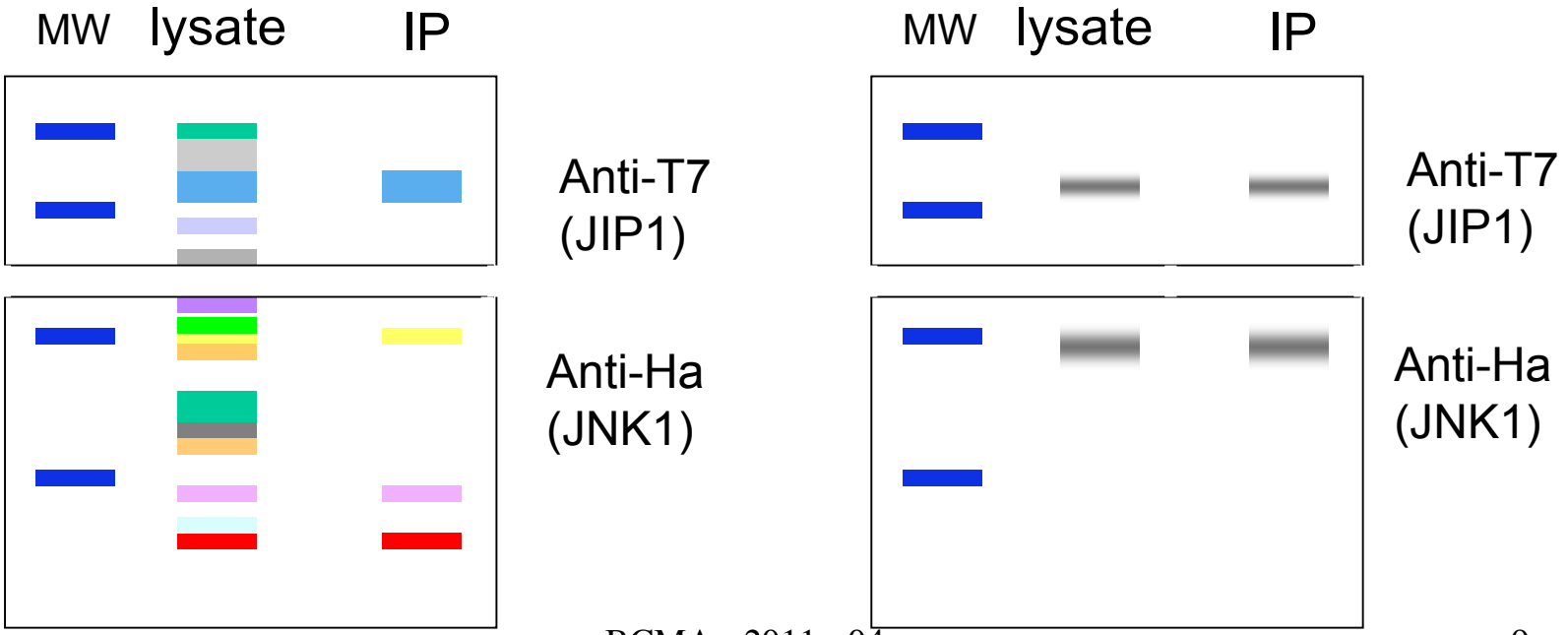
• centrifugo a bassa velocità (3000 rpm, 1 min, 4°C). Sul fondo della provetta si depositano:sferette di sepharose-glutatione--glutationetransferasi-proteina esca; in soluzione restano:proteine non legate alla proteina “esca”

•procedo come per l’immunoprecipitazione

Fig. 1. Selective binding of JIP-1 to the MAP kinase JNK and the MAP kinase kinase MKK7. (**A**) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with the HA-tagged MAP kinases ERK2, p38α, JNK1, and JNK2 (*15*, *16*). The MAP kinases were immunoprecipitated with an antibody to HA. The presence of JIP-1 in the immunoprecipitates (IP) was detected on immunoblots probed with an antibody to T7-Tag. The amount of JIP-1 and MAP kinases in the cell lysates was examined by protein immunoblot analysis. (**B**) JIP-1 was expressed in cells as a GST fusion protein together with epitope-tagged MEK1, MKK3, MKK4, MKK6, or MKK7 (*15*, *16*). JIP-1 was precipitated from cell lysates with glutathione-agarose, and the MAPKKs present in the pellet were detected by protein immunoblot analysis. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis. (**C**) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with Flag-tagged MKK4 or MKK7 (*15*, *16*). The presence of JIP-1 in Flag IP was detected by protein immunoblot analysis with an antibody to T7-Tag. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis.



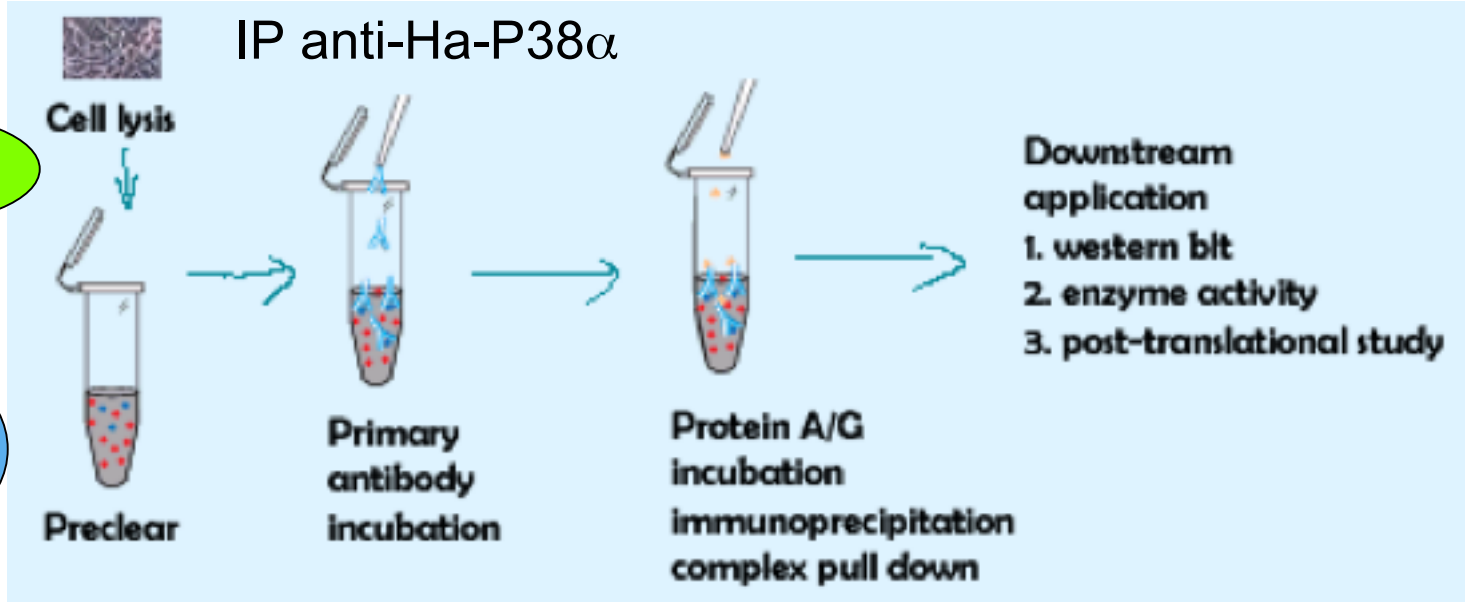

Ha-JNK1
?
T7-JIP-1
IP anti-Ha-JNK1
Cell lysis
Preclear
Primary antibody incubation
Protein A/G incubation immunoprecipitation complex pull down
Downstream application
1. western blt
2. enzyme activity
3. post-translational study

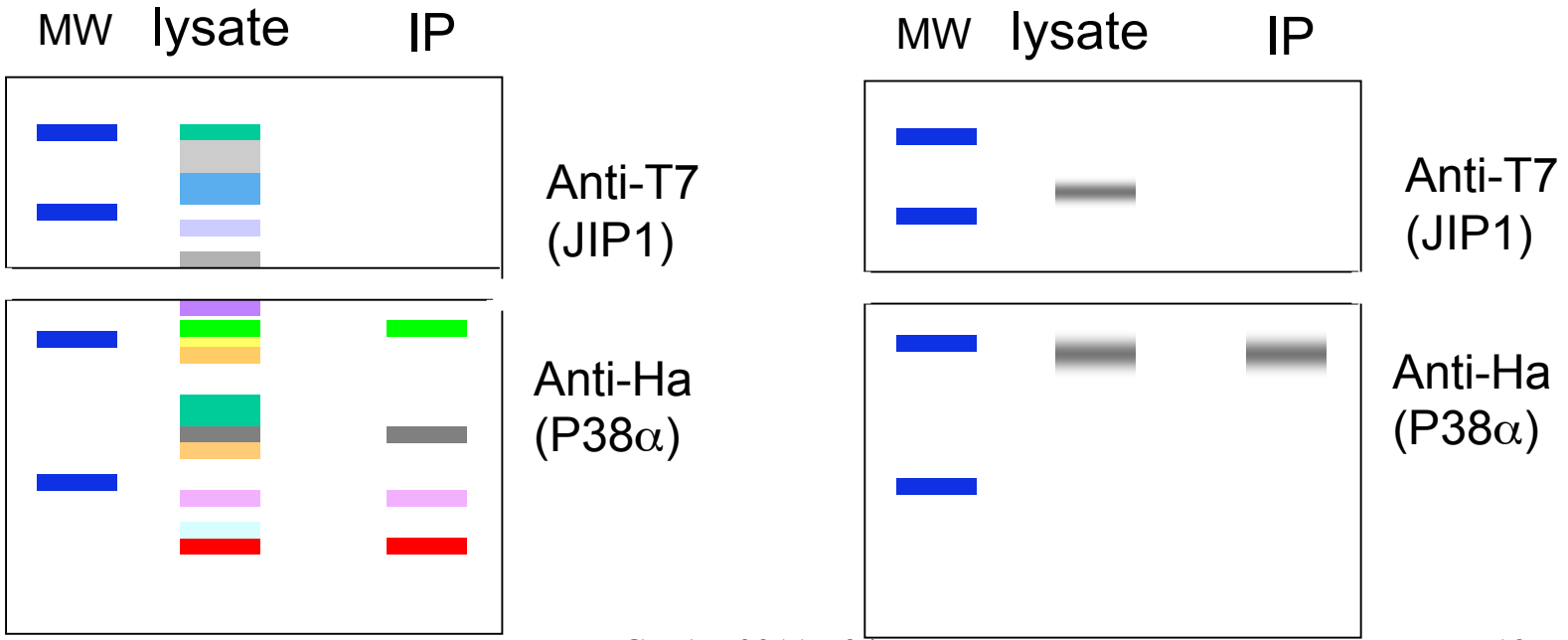

MW
lysate
IP
Anti-T7 (JIP1)
Anti-Ha (JNK1)
MW
lysate
IP
Anti-T7 (JIP1)
Anti-Ha (JNK1)

Ha-P38α

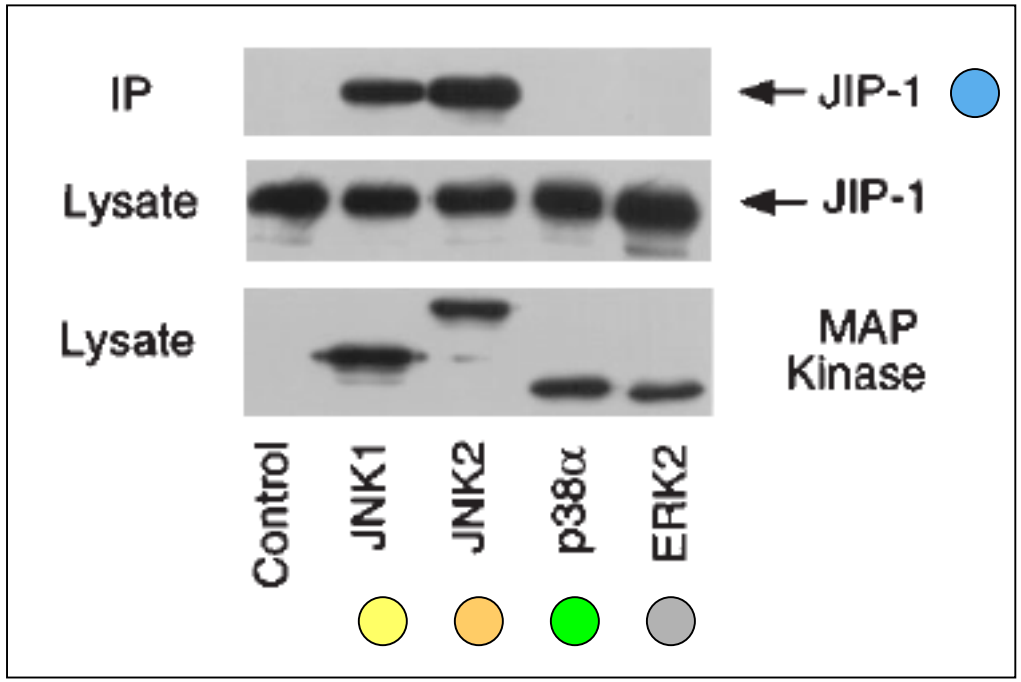
?

T7-JIP-1

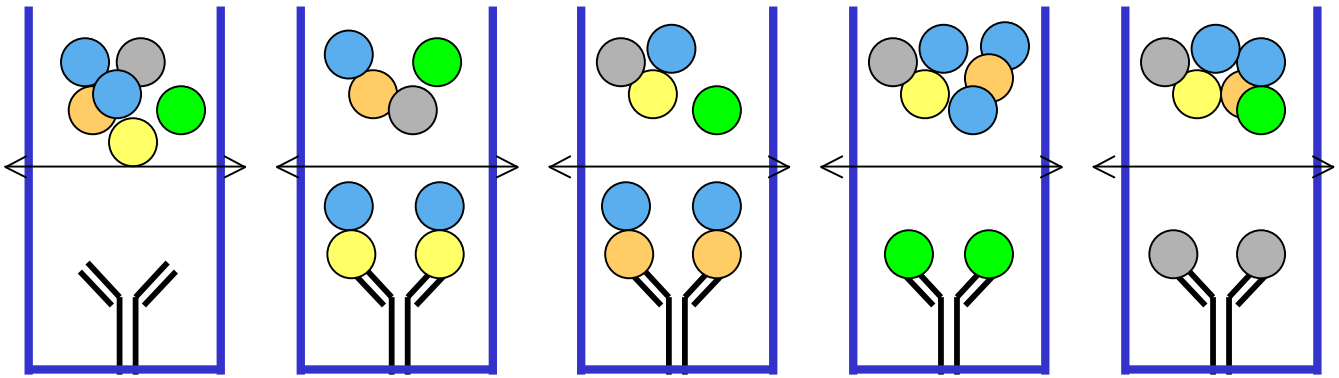




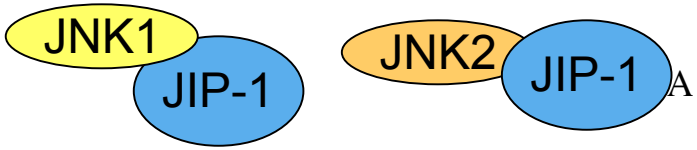
Selective binding of JIP-1 to the MAP kinase JNK

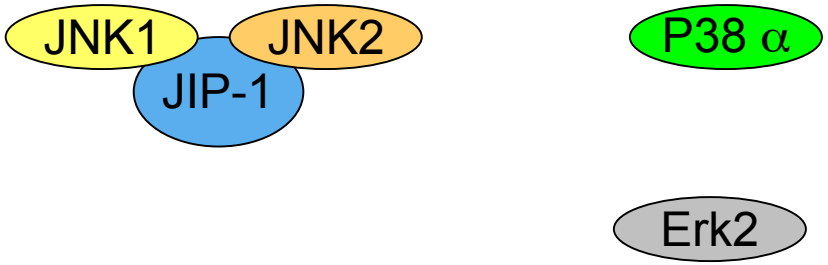


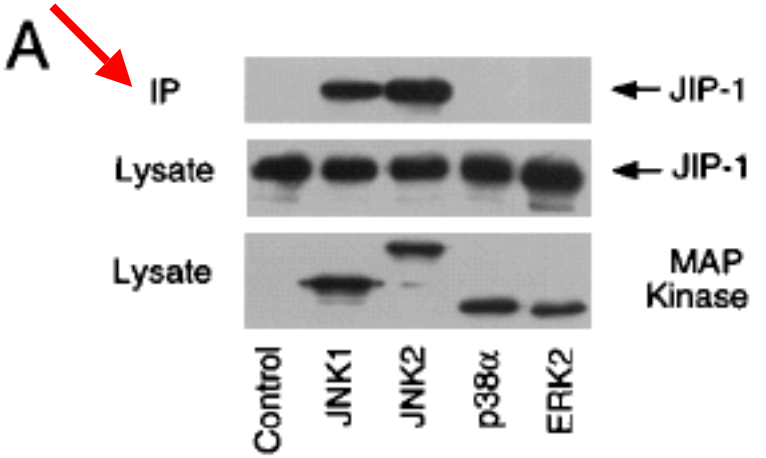
Epitope-tagged JIP-1 (T7-Tag) was ex- pressed in cells with the HA-tagged MAP kinases ERK2, p38α JNK1, and JNK2. The MAP kinases were immunoprecipitated with an antibody to HA. The presence of JIP-1 in the immunoprecipitates (IP) was detected on im- munoblots probed with an antibody to T7-Tag. The amount of JIP-1 and MAP kinases in the cell lysates was examined by protein immunoblot analysis.

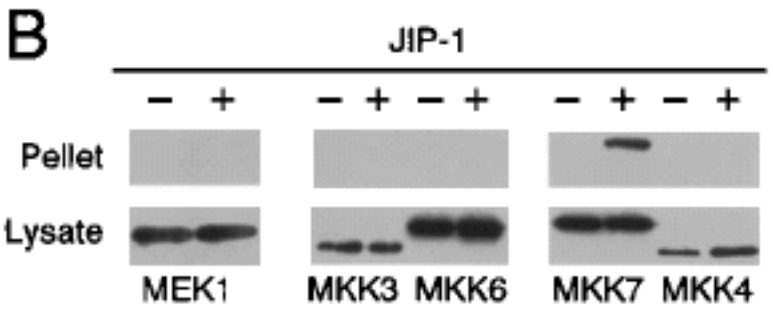


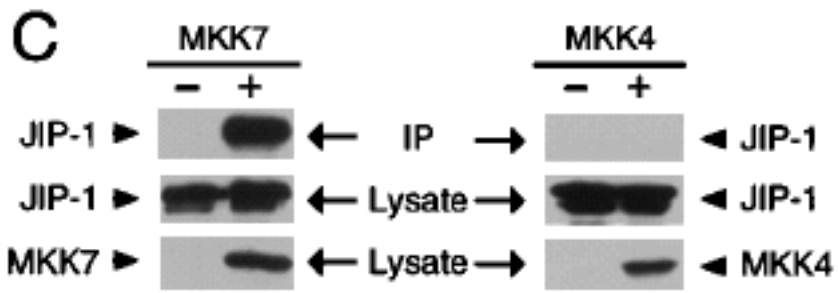
IP



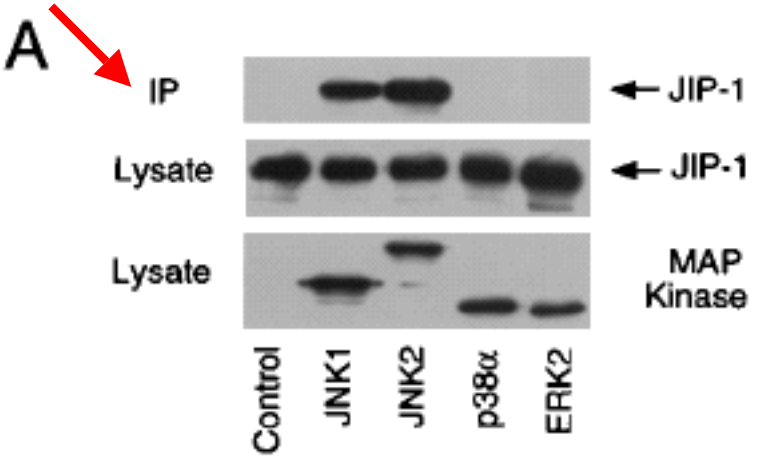


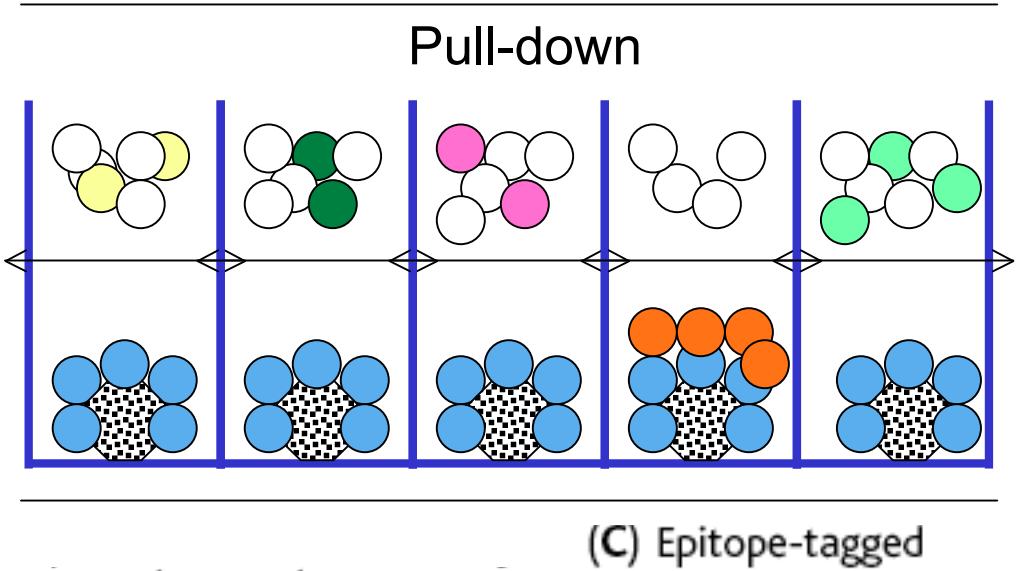


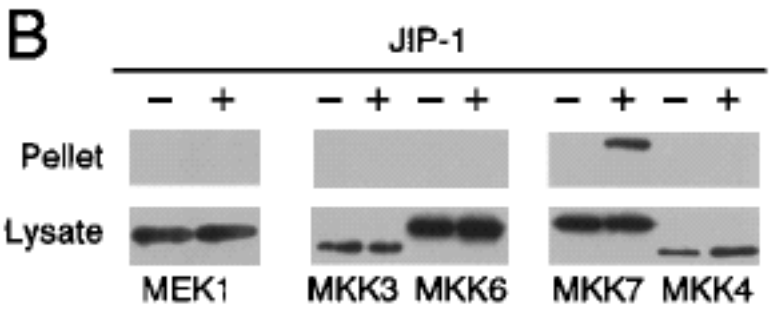


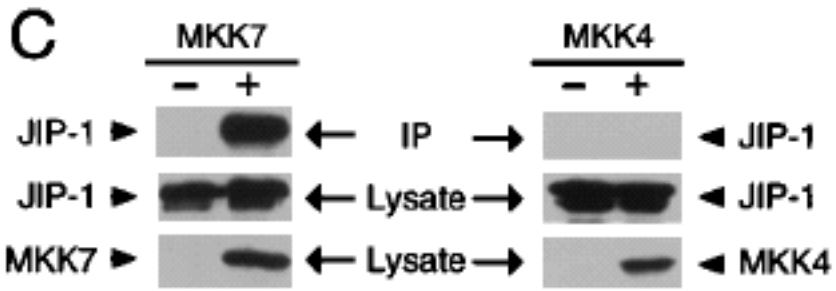


(**B**) JIP-1 was expressed in cells as a GST fusion protein together with epitope-tagged MEK1, MKK3, MKK4, MKK6, or MKK7 (*15*, *16*). JIP-1 was precipitated from cell lysates with glutathione-agarose, and the MAPKKs present in the pellet were detected by protein immunoblot analysis. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis. (**C**) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with Flag-tagged MKK4 or MKK7 (*15*, *16*). The presence of JIP-1 in Flag IP was detected by protein immunoblot analysis with an antibody to T7-Tag. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis.

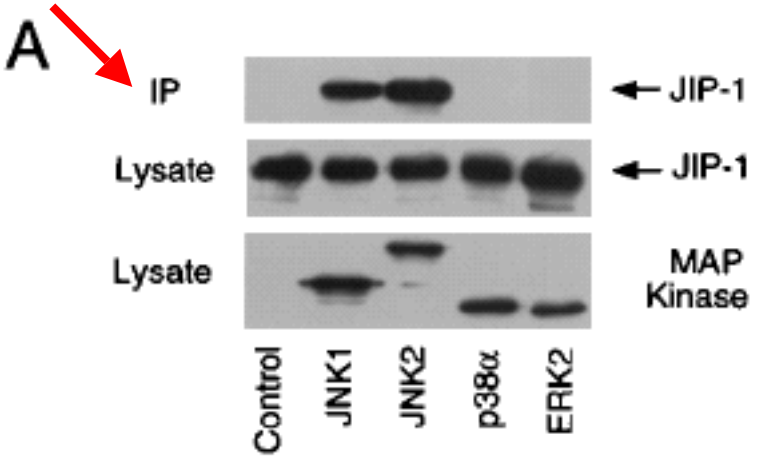




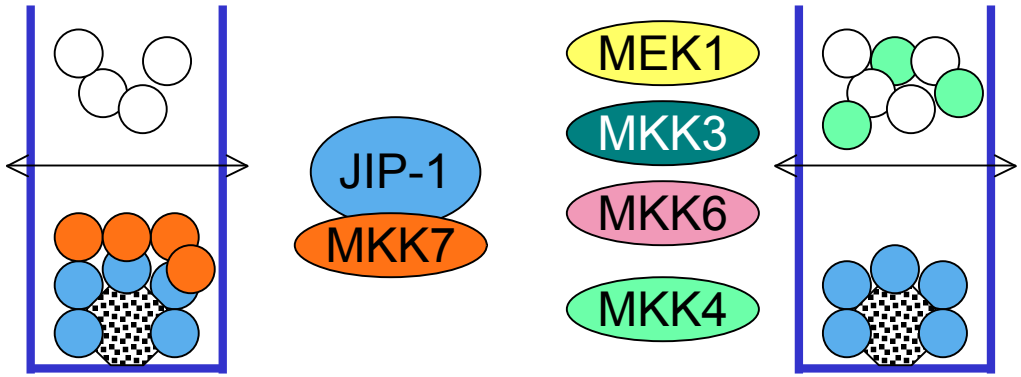


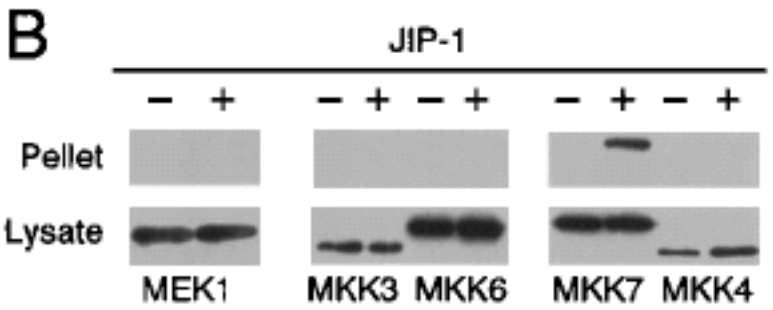


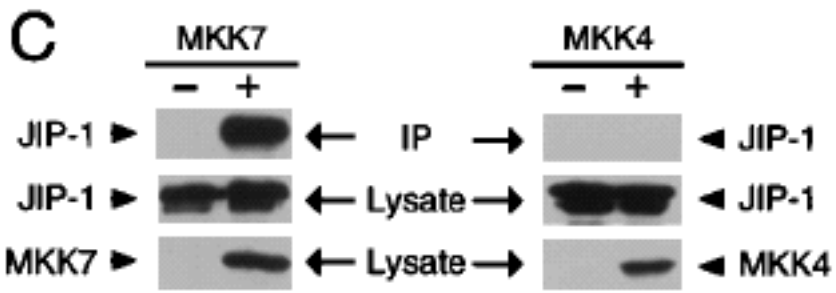
(C) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with Flag-tagged MKK4 or MKK7 (*15*, *16*). The presence of JIP-1 in Flag IP was detected by protein immunoblot analysis with an antibody to T7-Tag. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis.



Pull-down

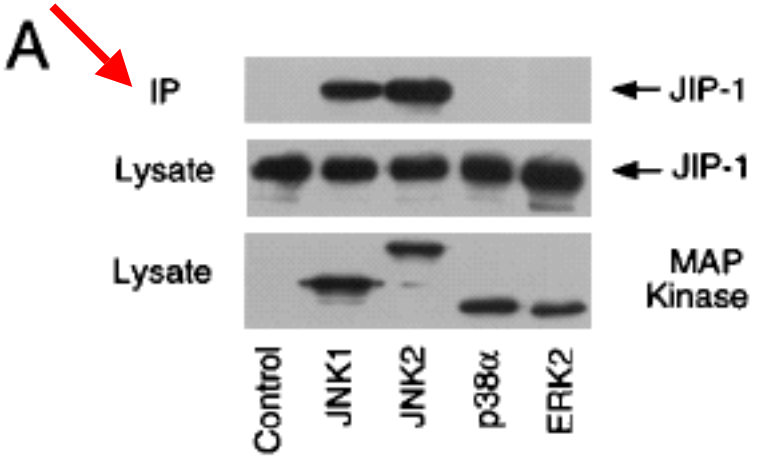






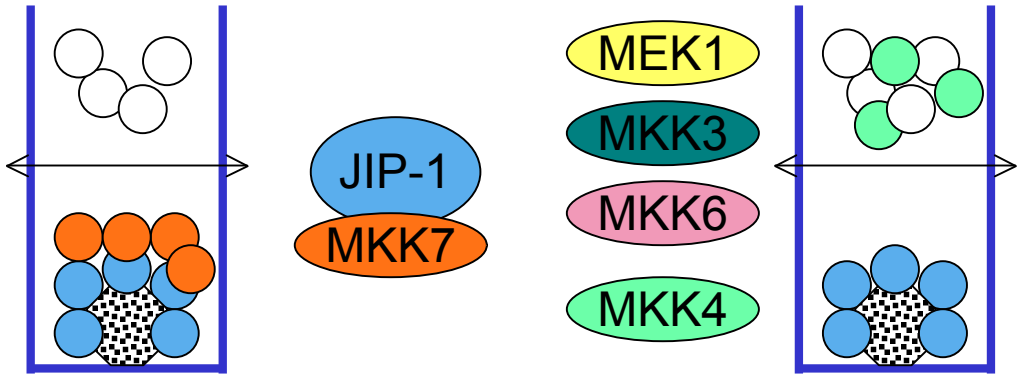
(C) Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with Flag-tagged MKK4 or MKK7 (*15*, *16*). The presence of JIP-1 in Flag IP was detected by protein immunoblot analysis with an antibody to T7-Tag. The amount of the MAPKKs in the cell lysates was examined by protein immunoblot analysis.

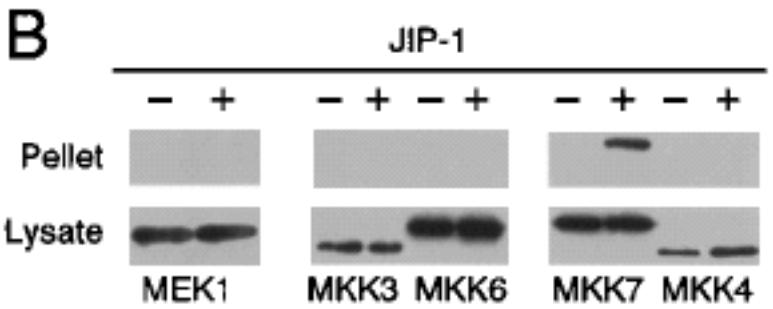
MAPK



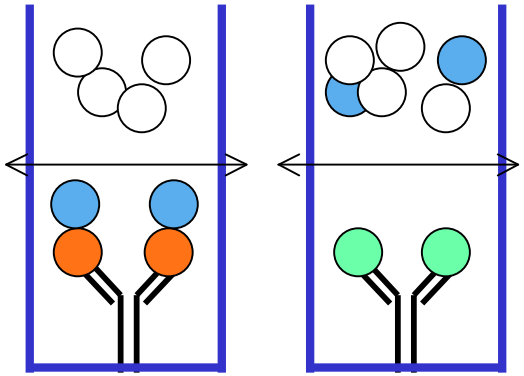
MAP2K

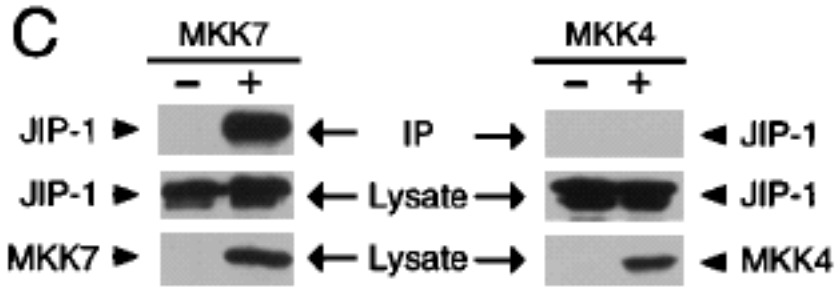
Pull-down





IP





MAP3K

A: Pull down

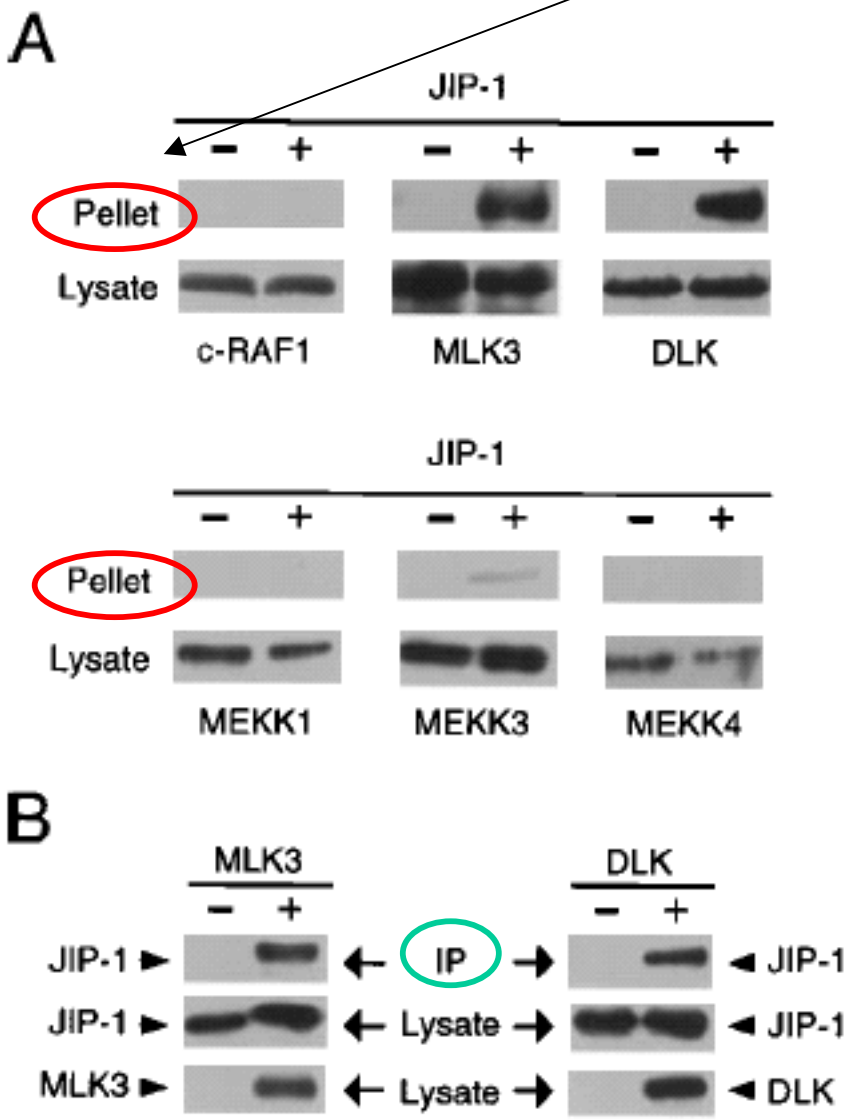
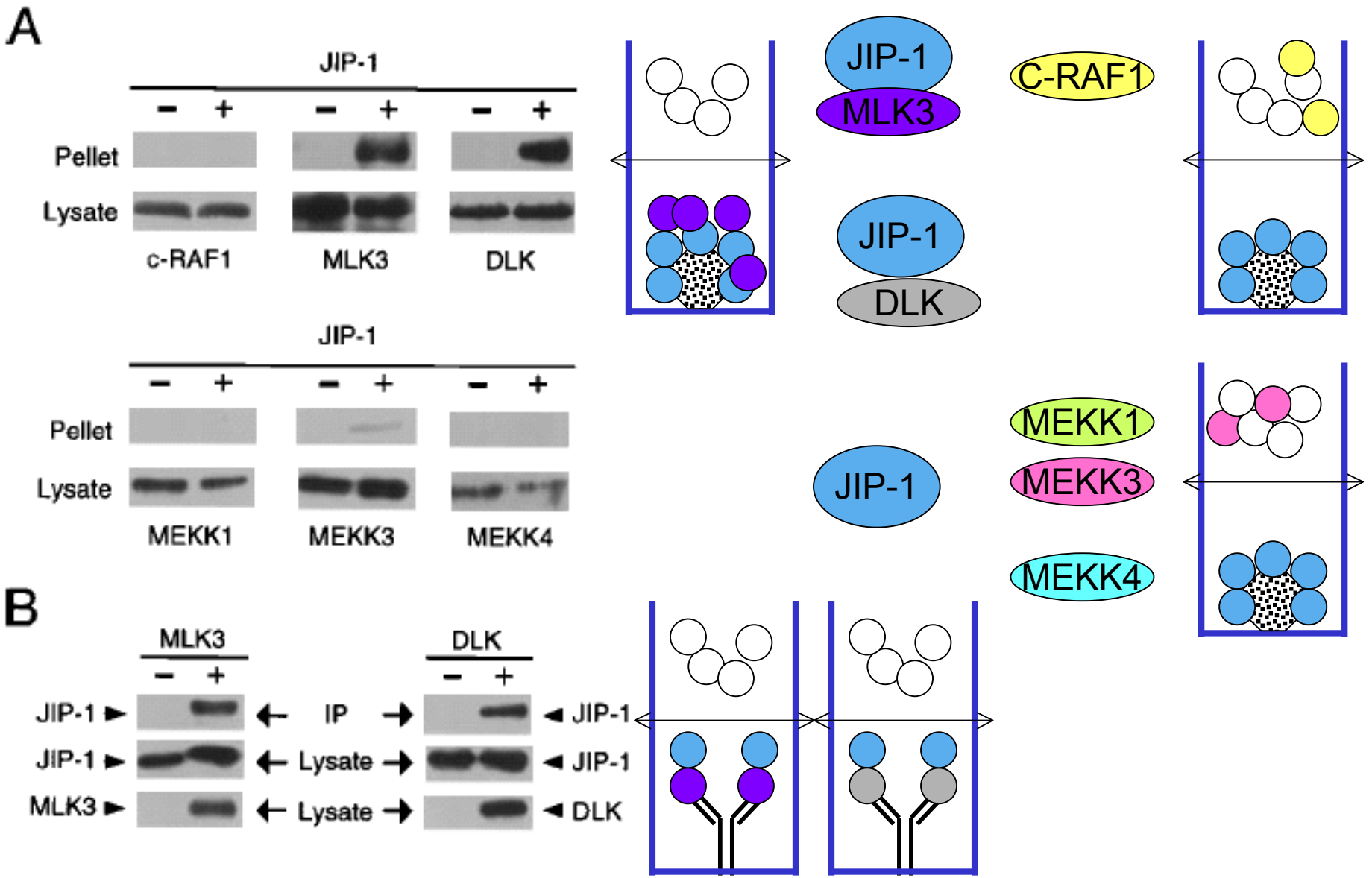
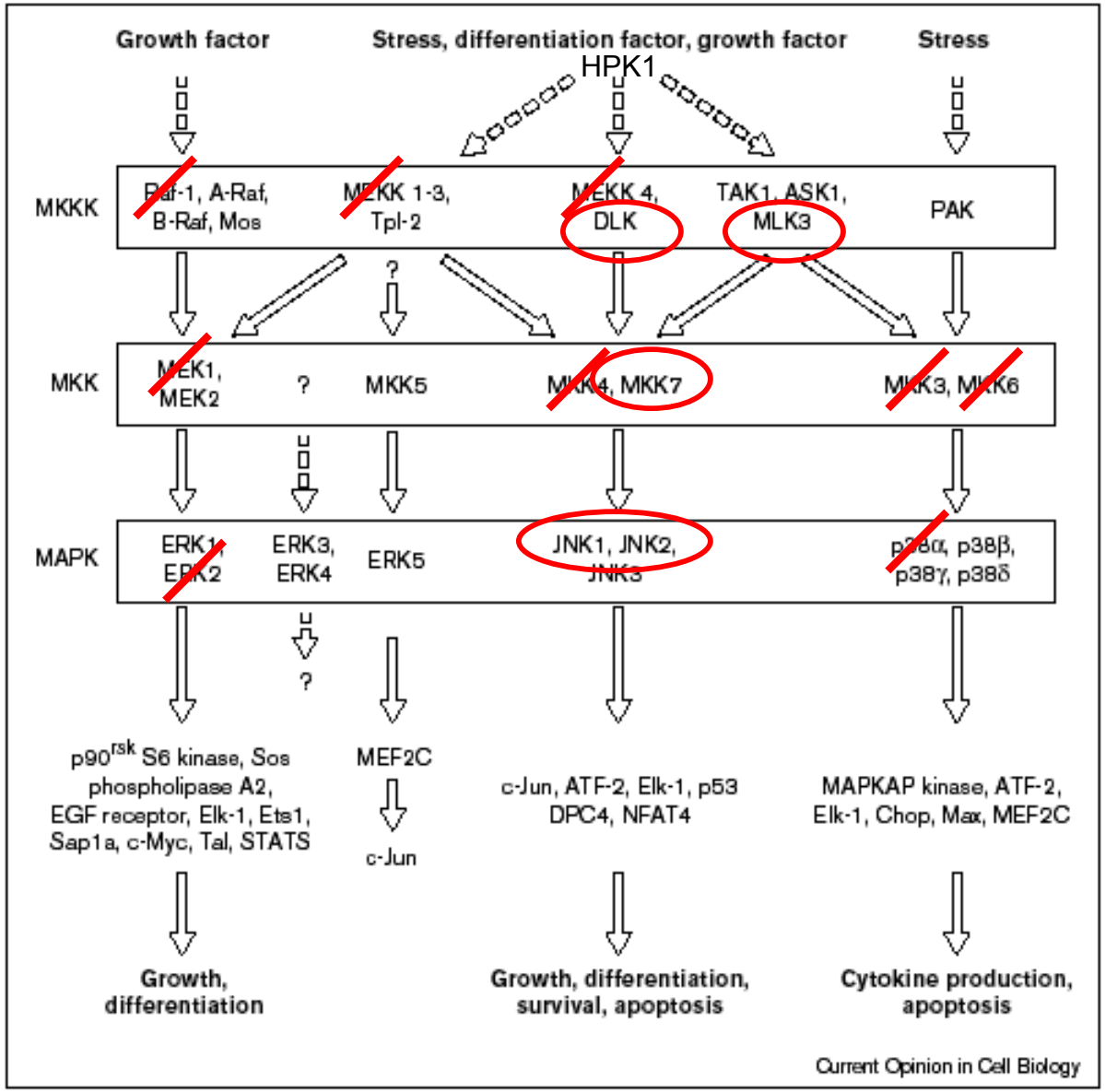


Fig. 2. Selective binding of JIP-1 to the mixed-lineage group of MAPKKKs. (**A**) JIP-1 was expressed in cells as a GST fusion protein together with the epitope-tagged MAPKKKs (*15*, *16*). The presence of MAPKKKs in glutathione-agarose precipitates (pellet) was assayed by protein immunoblot analysis. The amount of the MAPKKKs in the cell lysates was examined by protein immunoblot analysis. (**B**) Epitope-tagged JIP-1 was coexpressed in cells with epitope-tagged MLK3 or DLK (*15*, *16*). The presence of JIP-1 in the MLK3 and DLK immunoprecipitates (IP) was examined by protein immunoblot analysis. The amount of the MAPKKKs in the cell lysates was examined by protein immunoblot analysis.

B: immunoprecipitation

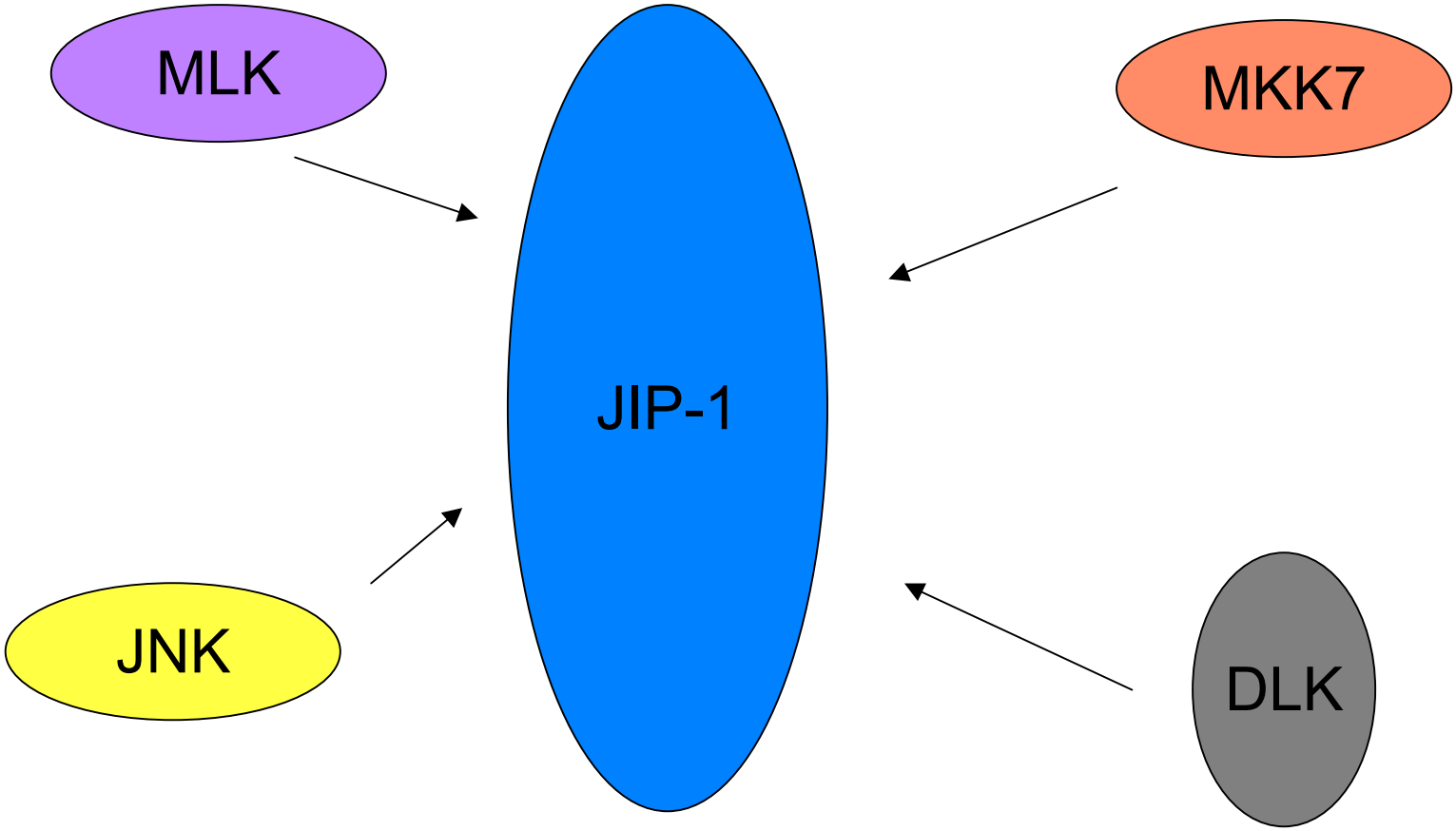
MAP3K


A
JIP-1
- + - + - +
Pellet
Lysate
c-RAF1
MLK3
DLK
JIP-1
- + - + - +
Pellet
Lysate
MEKK1
MEKK3
MEKK4
JIP-1
MLK3
JIP-1
DLK
C-RAF1
JIP-1
MEKK1
MEKK3
MEKK4
B
MLK3
DLK
- + - +
JIP-1
JIP-1
MLK3
IP
Lysate
Lysate
JIP-1
JIP-1
DLK



Mitogen-activated protein kinase modules. The MAPK module consists of an MKKK, an MKK, and a MAPK. MKKKs respond to a variety of extracellular signals, including growth factors differentiation factors and stress. The activated MKKKs can then activate one or several MKKs. In contrast, the MKKs are relatively specific for their target MAPKs. Once activated, MAPKs can then phosphorylate transcription factors (for example ATF-2, Chop, c-Jun, c-Myc, DPC4, Elk-1, Ets1, Max, MEF2C, NFAT4, Sap1a, STATs, Tal, p53), other kinases (MAPKAP kinase, p90^{rsk} S6 kinase), upstream regulators (EGF receptor, son of sevenless [Sos] Ras exchange factor), and other regulatory enzymes such as phospholipase A2. These downstream targets then control cellular responses including growth, differentiation, and apoptosis [1••,2,3].

Each of these MAPK cascade member interacts with JIP-1but is it a competitive or a cooperative interaction?



Binding of JIP-1 to MKK7, MLK and HPK1 independently of JNK

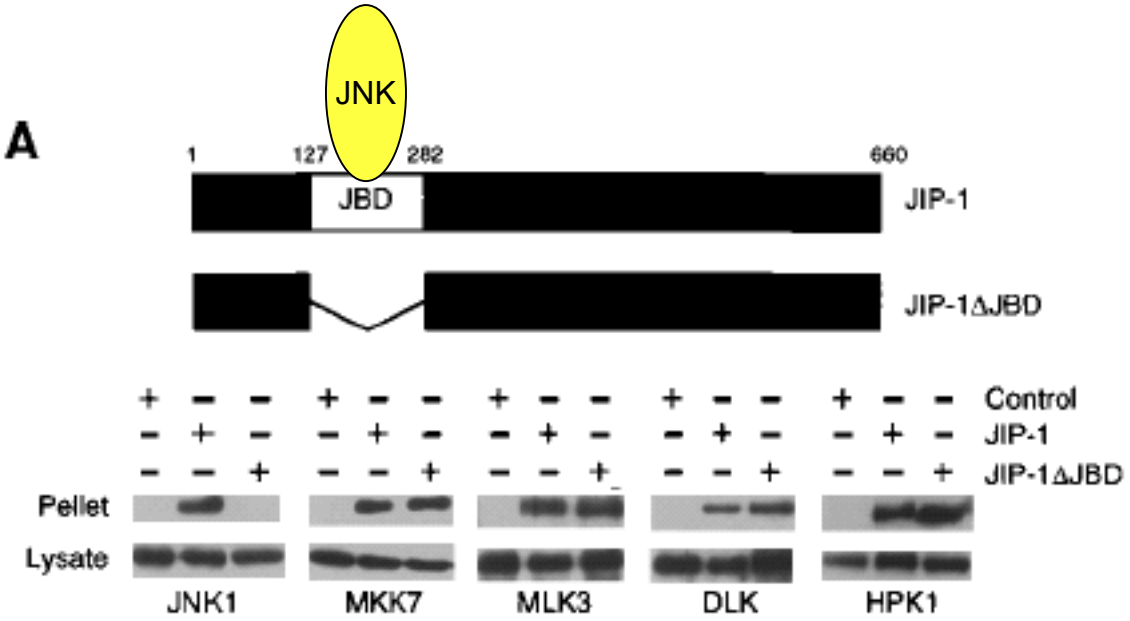
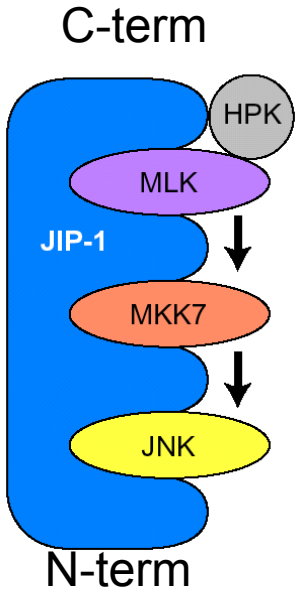
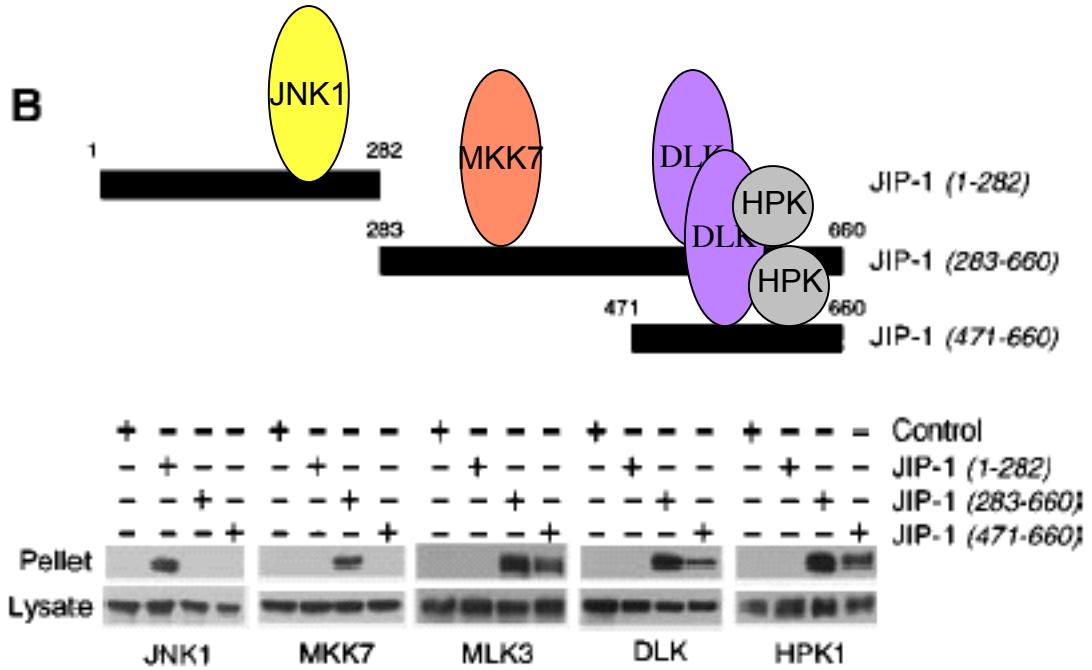


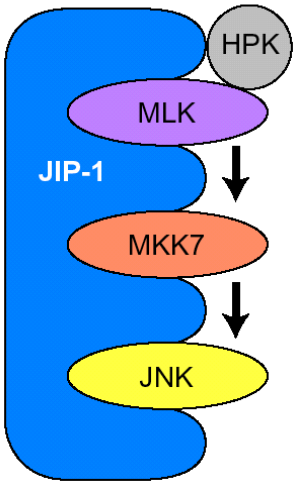
Fig. 3. Binding of JIP-1 to MKK7, MLK, and HPK1 independently of JNK. (**A**) The binding of JIP-1 to HPK1, MLK3, DLK, and MKK7 is independent of JNK. GST (Control), GST-tagged JIP-1, and a GST-tagged JIP-1 mutant with an in-frame deletion of the JNK binding domain (JIP-1ΔJBD) were coexpressed in cells together with HPK1 and epitope-tagged JNK1, MKK7, MLK3, and DLK (*15*, *16*). The presence of kinases in the reduced glutathione-agarose precipitates (pellets) and cell lysates was examined by protein immunoblot analysis.

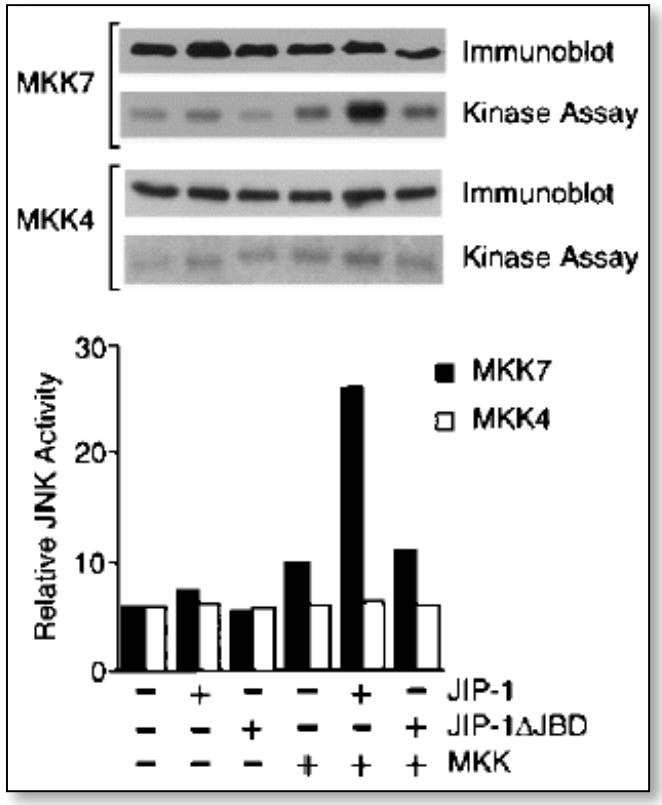




(**B**) Deletion analysis of the binding of JIP-1 to JNK1, MKK7, MLK3, and DLK. JIP-1 was expressed in cells as a GST fusion protein together with HPK1 or epitope-tagged JNK1, MKK7, MLK3, and DLK (*15*, *16*). The presence of these kinases in glutathione-agarose precipitates was examined by protein immunoblot analysis. (**C**)

Kinase assay: JIP1 enhances the activation of JNK by MKK7 and MLK3





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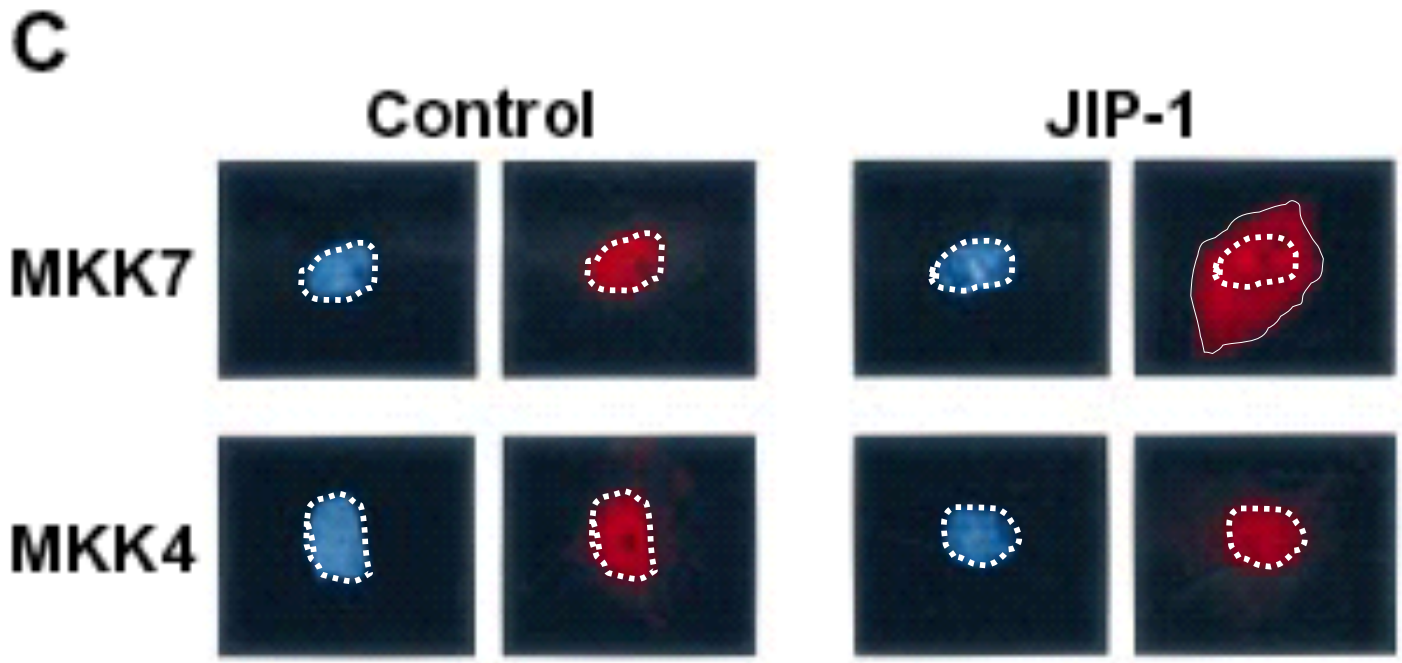
A Cytoplasmic Inhibitor of the JNK Signal Transduction Pathway

Martin Dickens, Jeffrey S. Rogers, Julie Cavanagh, Art Raitano, Zhengui Xia, Jocelyn R. Halpern, Michael E. Greenberg, Charles L. Sawyers, Roger J. Davis*

The c-Jun amino-terminal kinase (JNK) is a member of the stress-activated group of mitogen-activated protein (MAP) kinases that are implicated in the control of cell growth. A murine cytoplasmic protein that binds specifically to JNK [the JNK interacting protein-1 (JIP-1)] was characterized and cloned. JIP-1 caused cytoplasmic retention of JNK and inhibition of JNK-regulated gene expression. In addition, JIP-1 suppressed the effects of the JNK signaling pathway on cellular proliferation, including transformation by the *Bcr-Abl* oncogene. This analysis identifies JIP-1 as a specific inhibitor of the JNK signal transduction pathway and establishes protein targeting as a mechanism that regulates signaling by stress-activated MAP kinases.

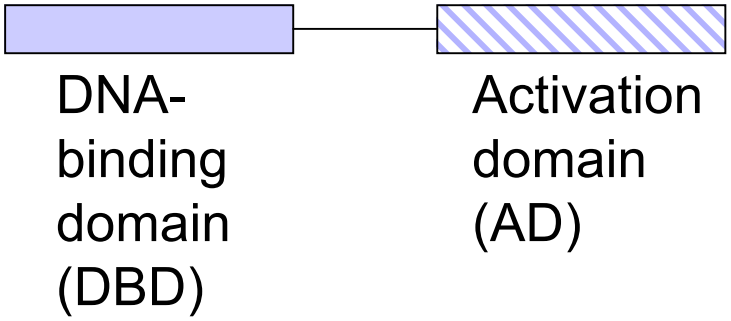
Overexpression of JIP1 inhibits JNK traslocation to the nucleus and in vivo activity

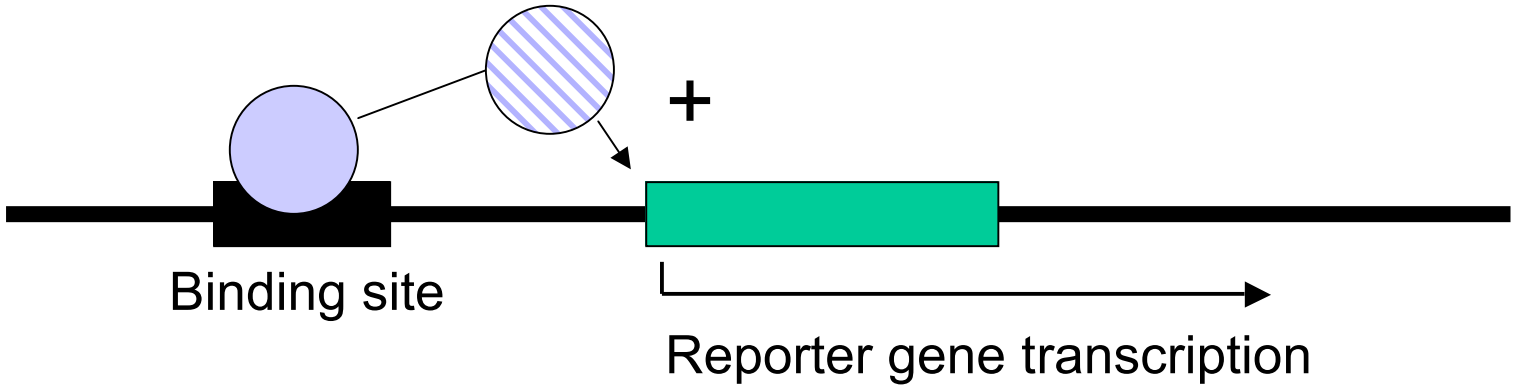


Experimental approach to protein-protein interaction

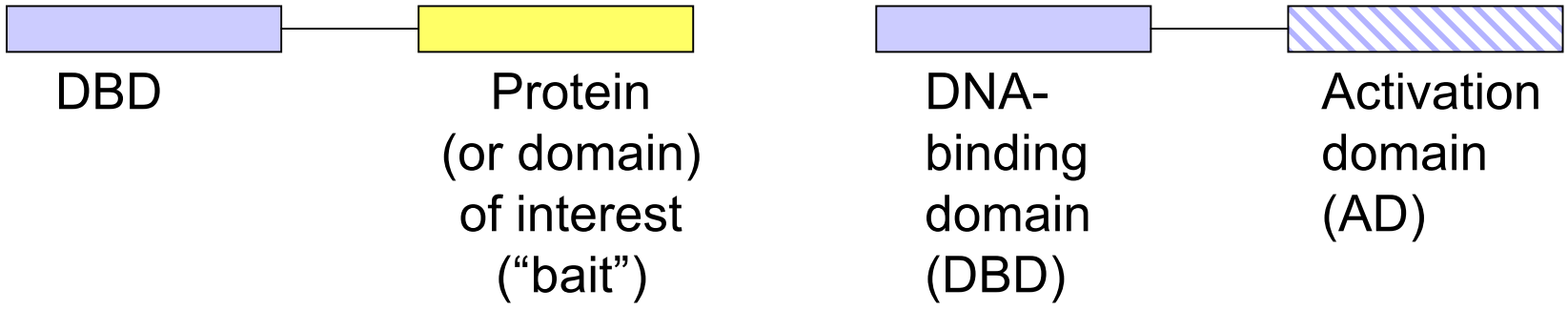
Two-hybrid system

Y2H systems exploit the modular nature of eukaryotic transcription factors, which consist of a sequence-specific DNA-binding domain (DBD) and an RNA Pol II-recruiting, transcription activation domain (AD).

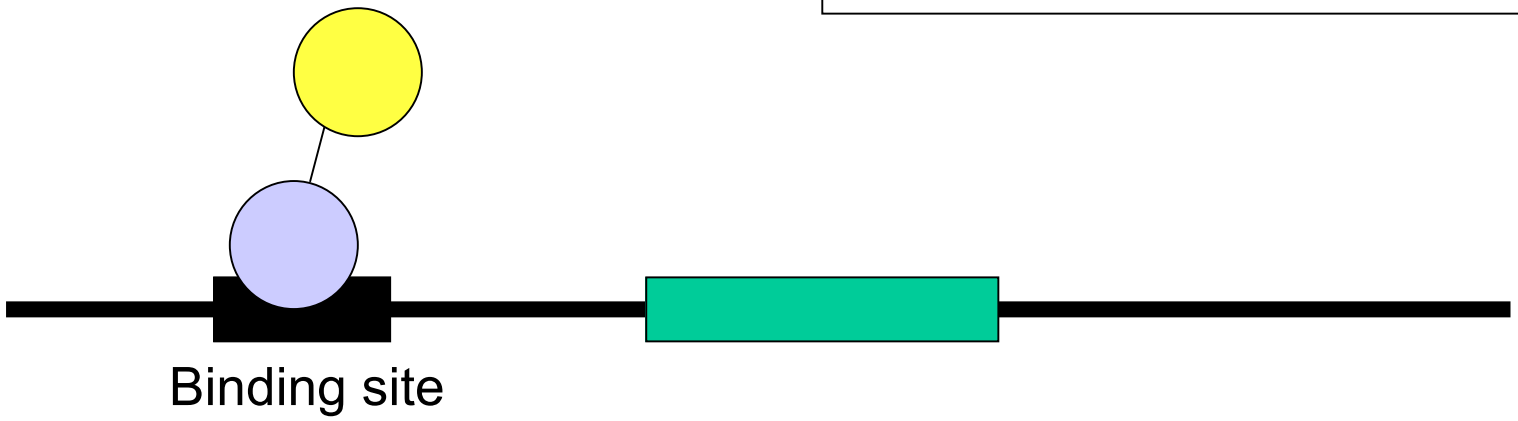




Two-hybrid system: two types of hybrids



By itself, the DBD:bait fusion does not stimulate expression.



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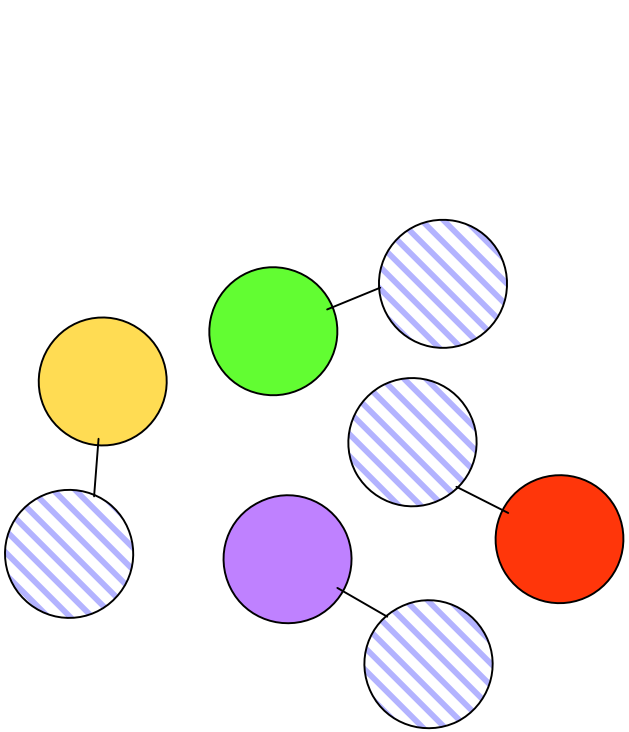
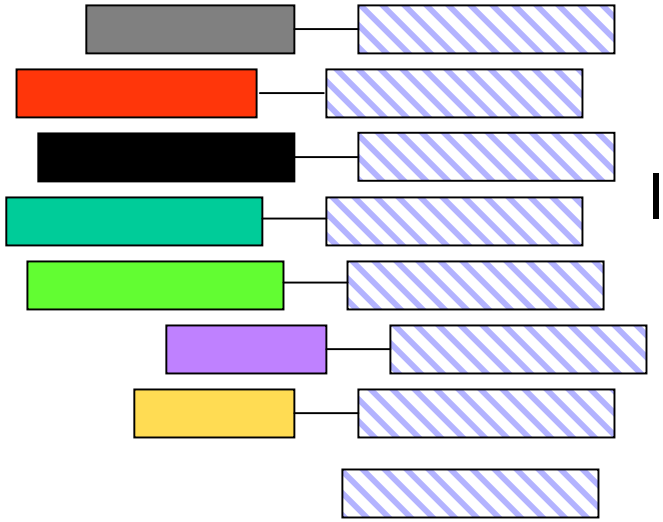




library

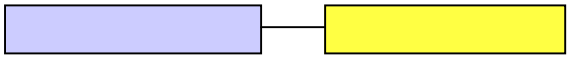
Interacting protein (or domain) (“prey”)

Activation domain (AD)

Binding site

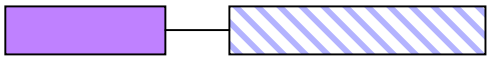
By itself, the AD:prey fusion does not stimulate expression.

Pairs of bait and prey fusion proteins are coexpressed in yeast cells.



DBD

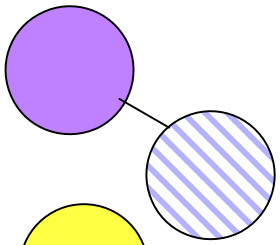
Protein (or domain) of interest ("bait")



Interacting protein (or domain) ("prey")

Activation domain (AD)

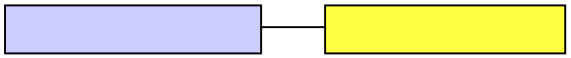
Screening of the library



No transcription of the reporter gene ---> no interaction.

Binding site

Pairs of bait and prey fusion proteins are coexpressed in yeast cells.



DBD

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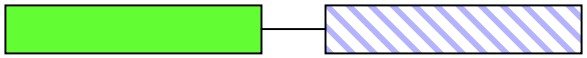
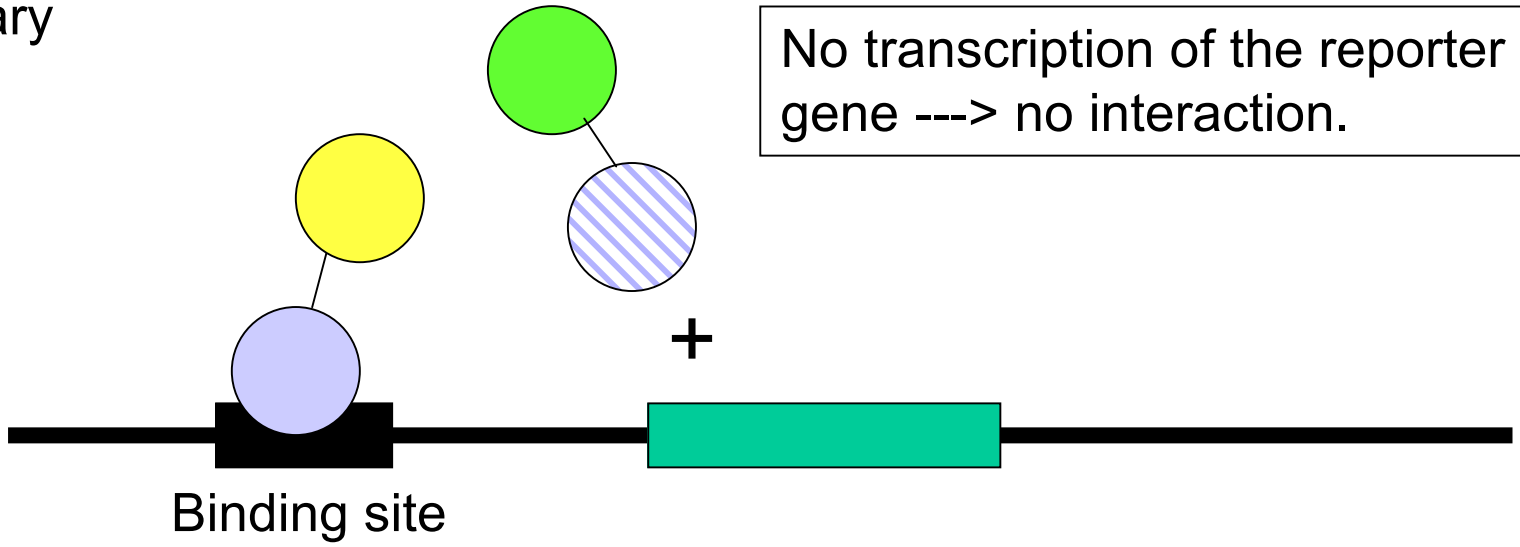


Interacting protein (or domain) ("prey")

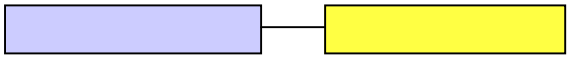
Activation domain (AD)

Screening of the library



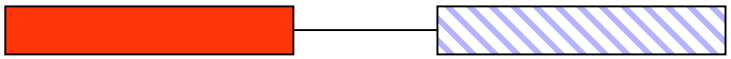
No transcription of the reporter gene ---> no interaction.

When pairs of interacting bait and prey fusion proteins are coexpressed in a yeast cell, the interacting fusion proteins are able to activate transcription of the reporter genes.



DBD

Protein (or domain) of interest ("bait")

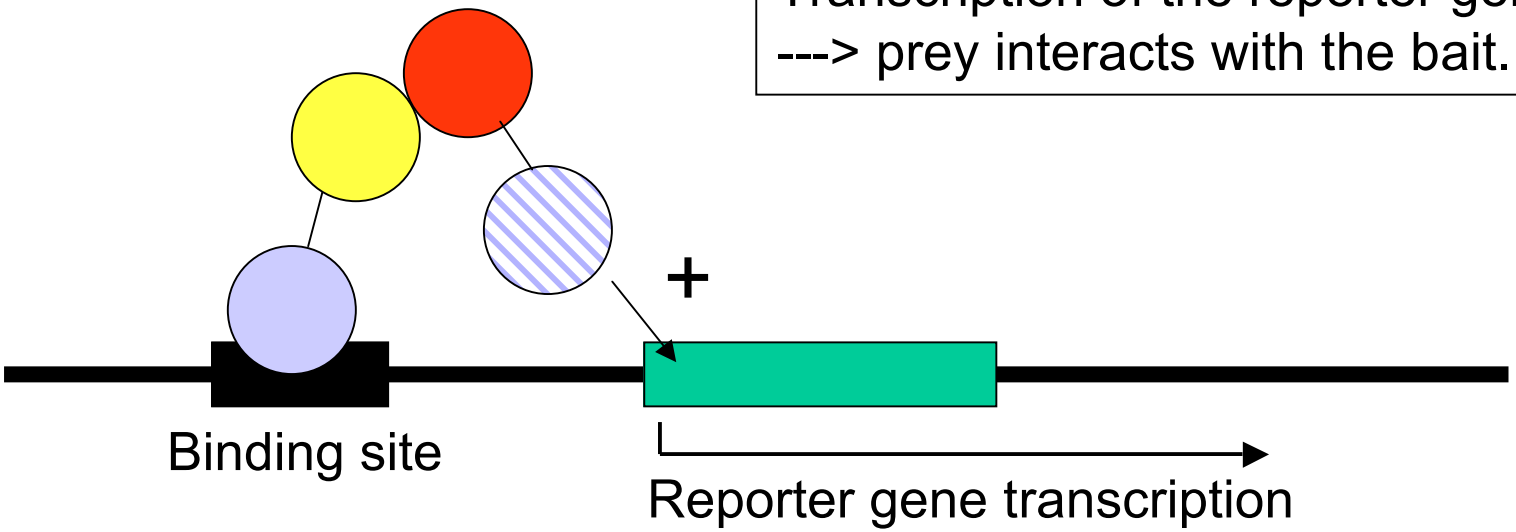


Interacting protein (or domain) ("prey")

Activation domain (AD)

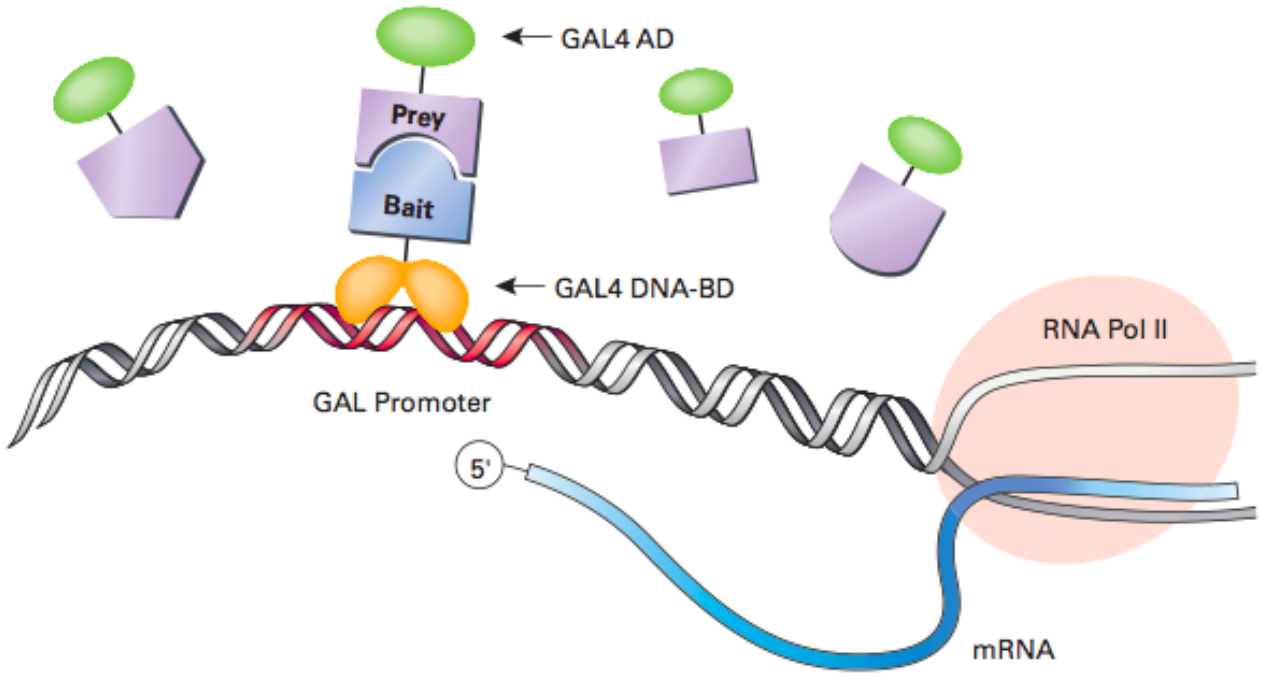
Screening of the library

Transcription of the reporter gene ---> prey interacts with the bait.

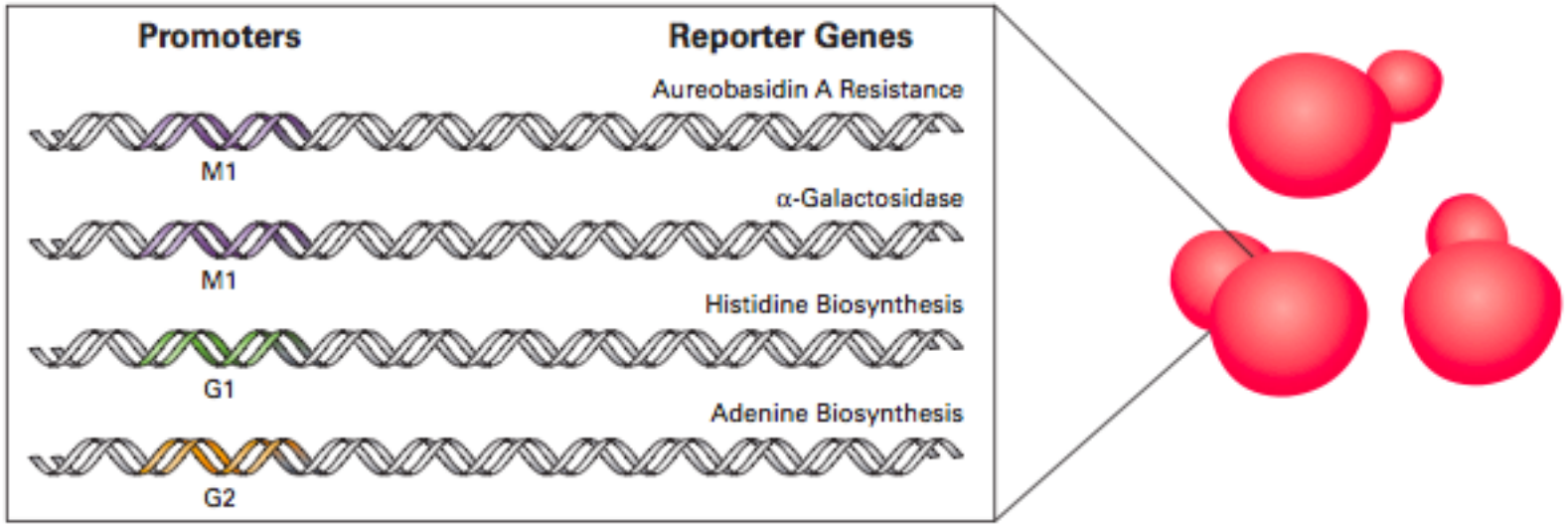


Yeast Two-Hybrid System

Yeast two-hybrid system design. Library-derived, transcription-activating prey fusion proteins that interact with the DNA-binding bait fusion protein activate the expression of reporter genes.



a known protein of interest is fused to the DNA-BD of the yeast GAL4 transcription factor to create a “bait” protein. Interacting partner proteins, often derived from a library, are expressed as fusions to the AD of yeast GAL4, to create “prey” proteins



Four reporters give high stringency. Interacting bait and prey fusion proteins drive the expression of four different reporters from three different GAL4-responsive promoters (M1, G1, and G2), which are stably integrated in the genome of the reporter strain, Y2HGold. Aureobasidin A (AbA) resistance and the two auxotrophic reporters for histidine and adenine biosynthesis confer growth selection in the presence of AbA and on histidine- and adenine-deficient media, while the α-galactosidase reporter produces blue colonies in the presence of X-alpha-Gal.

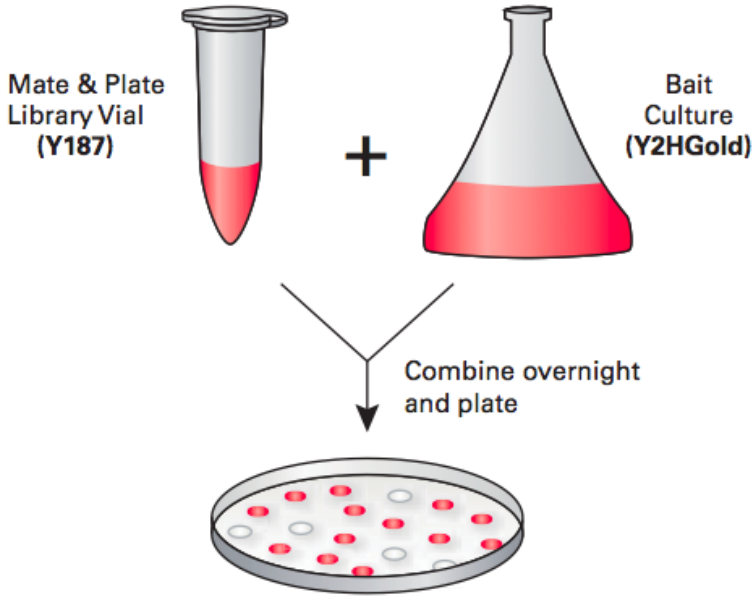
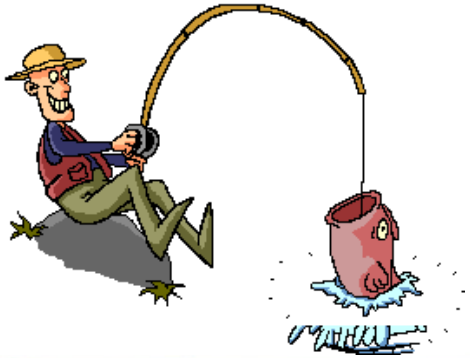
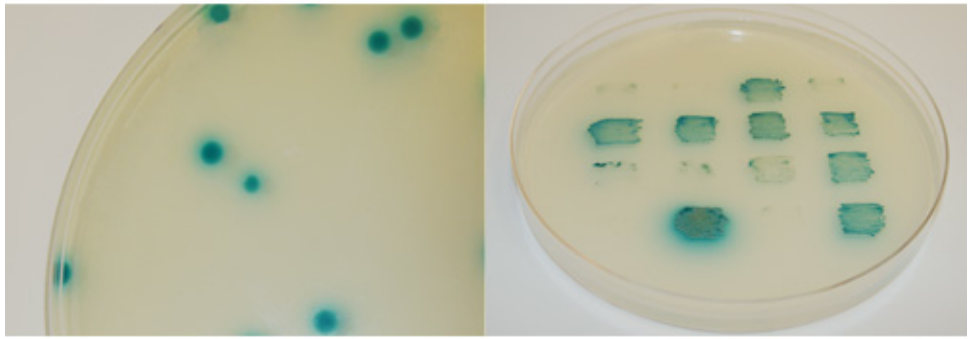


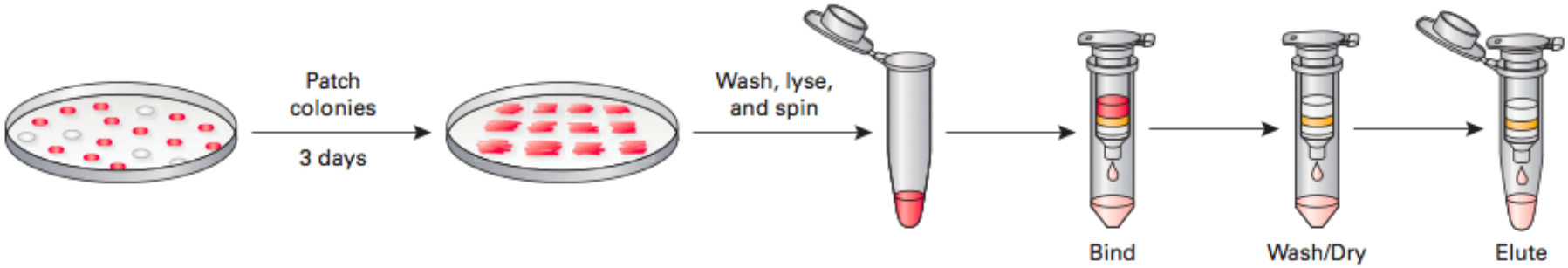
Figure 3. The Mate & Plate Protocol. To screen a Matchmaker Mate & Plate Library, an aliquot of the library in the Y187 strain (*MATα*) is simply mixed with a bait-expressing culture of the Y2HGold strain *(MATa)*. The mated strains are cultured overnight and plated on selective agar medium containing AbA.





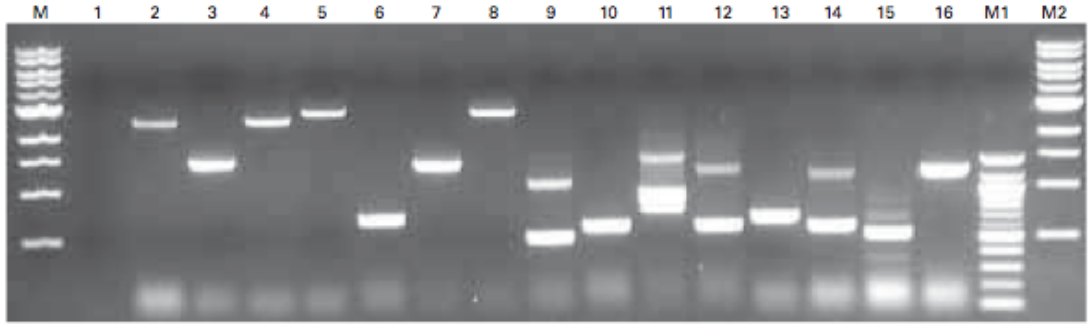
X-α-Gal detects secreted α-galactosidase activity following a GAL4-based two-hybrid interactions in Y2HGold yeast patches and colonies.

Rescuing plasmid DNA from yeast (Saccharomyces cerevisiae).positive clones



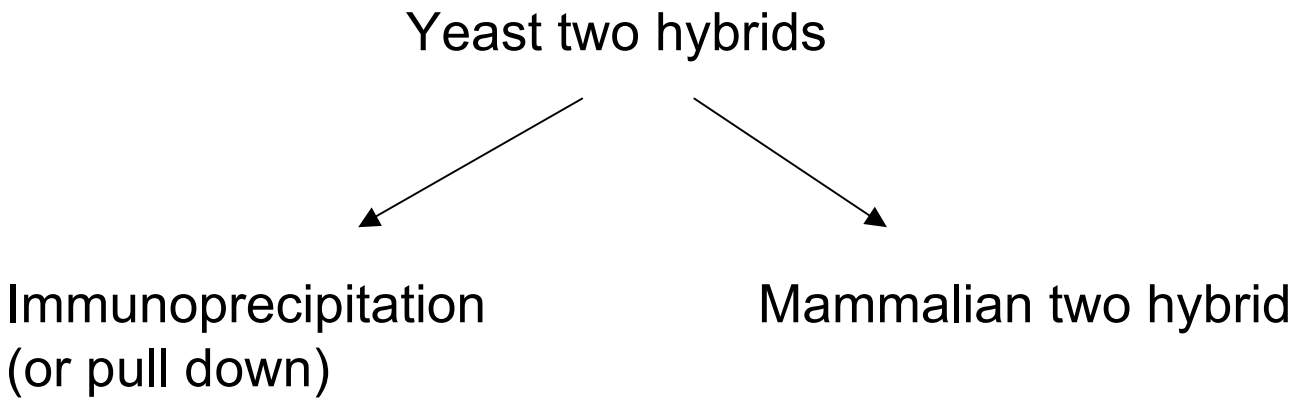
The protocol uses Zymolyase to efficiently digest the cell walls of the yeast and generate spheroplasts, which are then subjected to SDS/alkaline lysis. A spin column purifies the plasmid DNA, which can then be analysed directly or used to transform E. coli for propagation and scaled-up plasmid preparations, or used as a template for PCR.

Analysis of positive candidate clones.

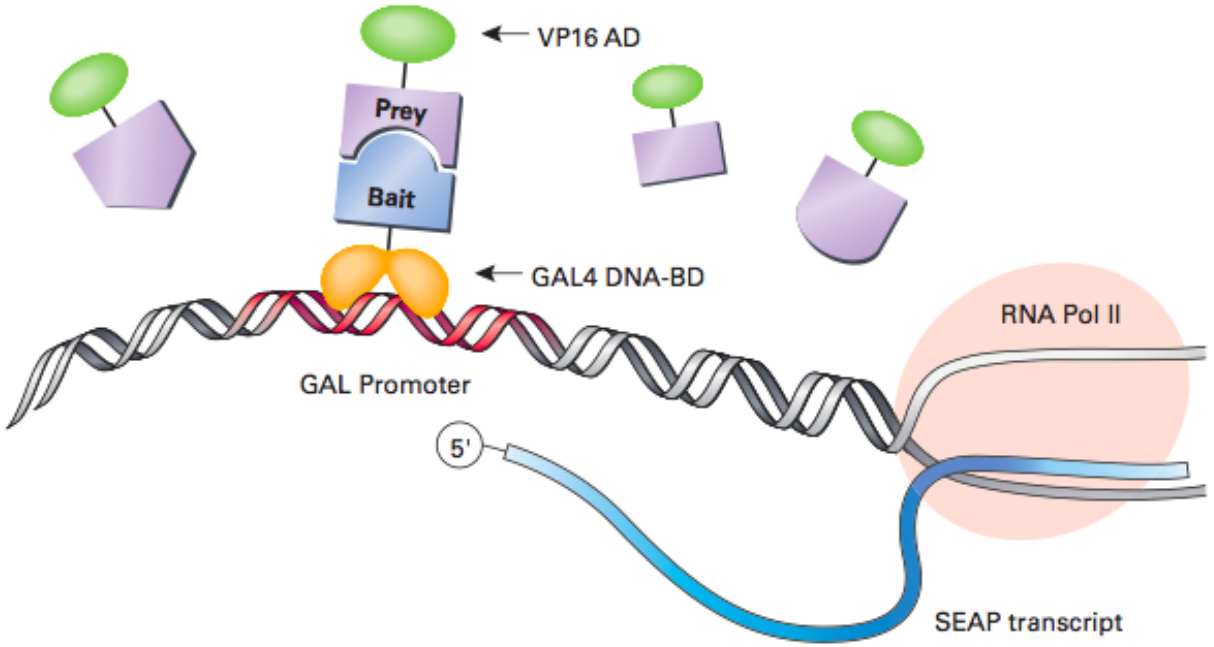


Lane 2: No template control.
Lanes 3-17: randomly selected colonies containing candidate clones
Lanes M1: 1 kb ladder.
Lane M2: 100 bp ladder.

Performing two-hybrid screens in yeast is a powerful method of identifying novel protein binding relationships that involve a particular protein of interest. However, the conformation of a mammalian protein expressed in yeast may be quite different from its normal conformation in a mammalian cells. Thus, it is important to perform a two-hybrid assay in mammalian cells to confirm that the suspected interactions also take place when the proteins are folded and modified as they would be in their native environment. The mammalian assay often reflects interactions between mammalian proteins with greater authenticity than can be achieved in yeast.



The mammalian two-hybrid principle. The bait protein is fused to the DNA binding domain of yeast GAL4 and the prey protein is fused to the transcriptional activation domain of HSV VP16. If the two proteins interact at the PGAL4-E1b promoter (GAL promoter), SEAP is secreted into the growth medium.



secreted alkaline phosphatase (SEAP), which is readily detected in the culture medium (chemiluminescence).