Protein expression

- Western blotting, is a widely used and accepted technique to detect levels of protein expression in a cell or tissue extract. This technique measures protein levels in a biological sample through antibody binding to a specific protein of interest. The precise binding that occurs between an antibody and its target protein epitope allows detection of highly specific amino acid sequences within a protein.

Post-translational modification

- Antibodies can also detect specific post-translational modifications (PTM) of a protein. Phospho-specific antibodies have been used to identify components of specific signaling pathways and to study changes in phosphorylation events in various biological contexts. Antibodies specific to other PTMs have been developed recently, allowing researchers to monitor changes in acetylation, methylation, and ubiquitination status of a protein.

Primary antibody validation

- A poor primary antibody can result in dirty, uninterpretable or misleading results.
- To be confident that an antibody is specific to the target of interest, the antibody specificity should be validated.
- It takes more than just seeing a band at the expected molecular weight to validate antibody specificity.
- Antibodies should undergo a stringent validation procedure using a number of different approaches to ensure that the antibody detects the target accurately.

Be in Control of Your Western Blot

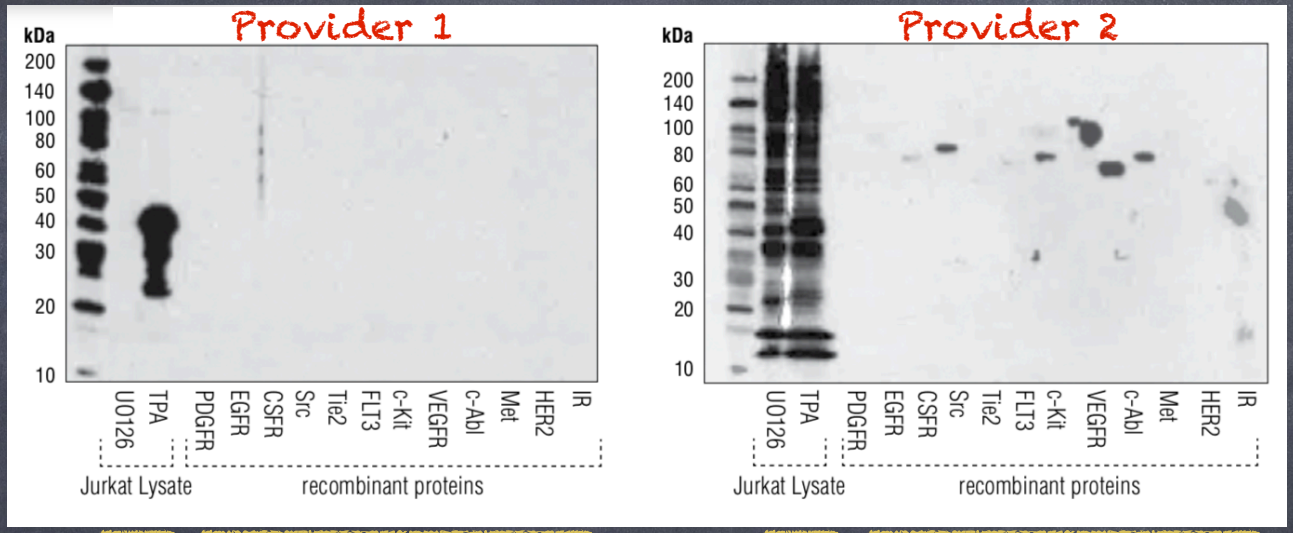
An important consideration in any experiment is the inclusion of appropriate controls.

Primary antibody specificity validation includes:

- Specificity testing on cell and tissue extracts with documented protein expression levels – not just on recombinant protein;
- Specificity confirmation through the use of siRNA transfection or knockout cell lines;
- Specificity testing of antibodies directed against a post-translational modification by treatment of cell lines with growth factors, chemical activators, or inhibitors, which induce or inhibit target expression.
- Phospho-specificity testing of phospho-antibodies by phosphatase treatment;

Positive and negative controls ensure confidence that your antibody is detecting a specific signal.

mAb anti-P-Erk1/2

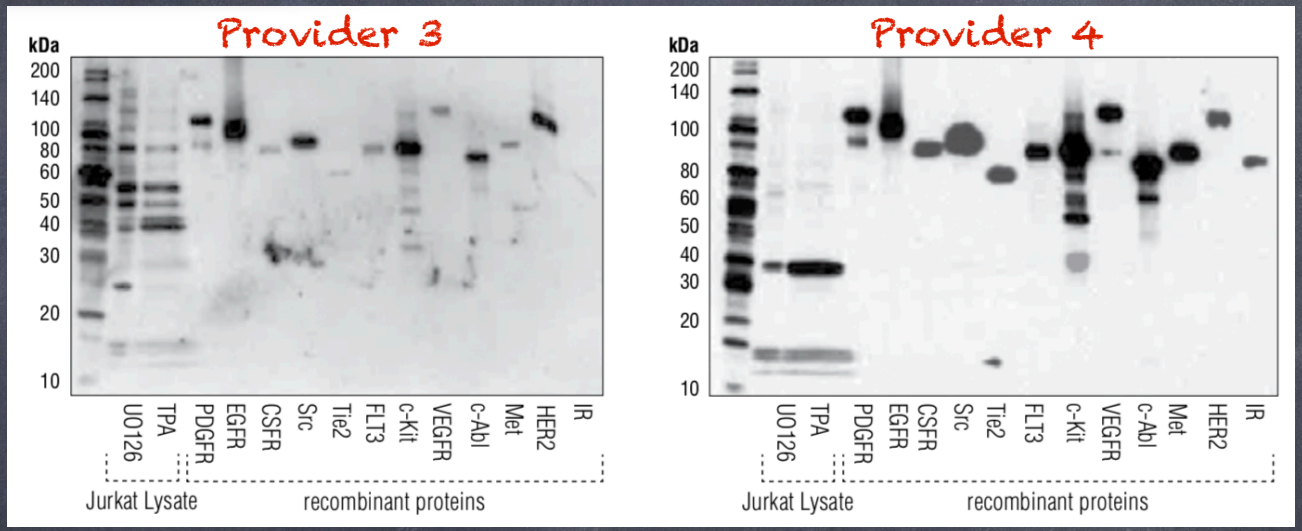


Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibodies are evaluated using negative (U0126-treated) and positive (TPA-treated) Jurkat lysate, as well as purified recombinant tyrosine-phosphorylated proteins.

TPA: small molecule drug that activates the signal transduction enzyme protein kinase C (PKC) and ERK phosphorylation

Positive and negative controls ensure confidence that your antibody is detecting a specific signal.

mAb anti-P-tyrosine



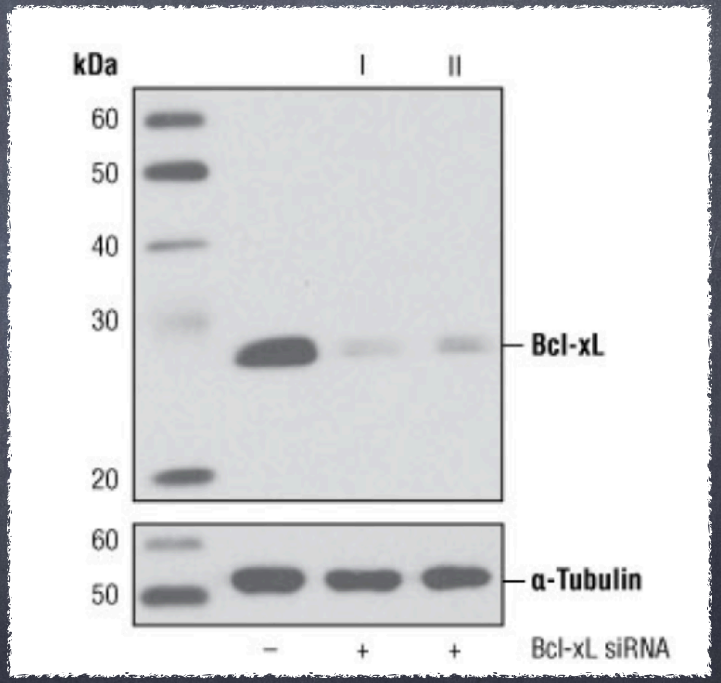
Phospho-tyrosine antibodies are evaluated using negative (U0126-treated) and positive (TPA-treated) Jurkat lysate, as well as purified recombinant tyrosine-phosphorylated proteins.

Loading controls are used to:

- Ensure equal loading of a gel
- Ensure Integrity of the sample
- Quantitatively compare samples

Ensure equal loading of a gel

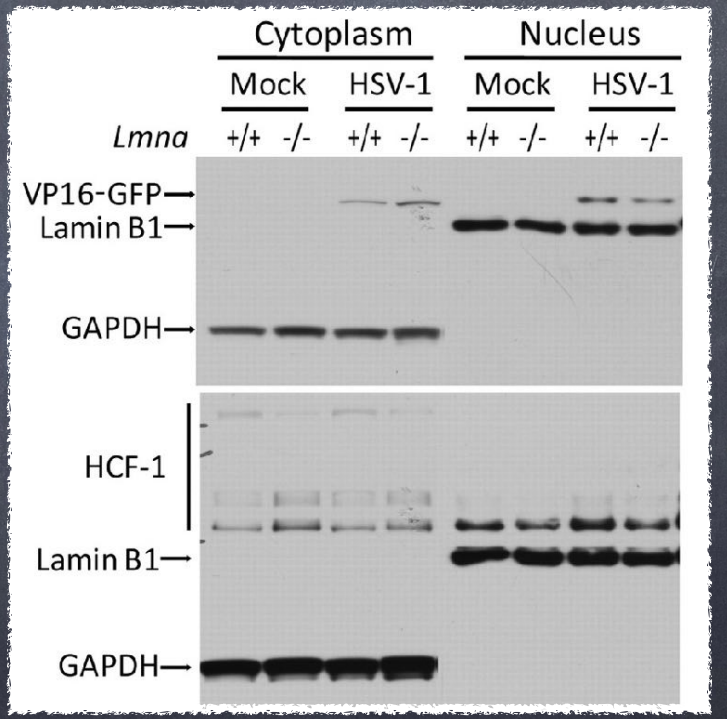
- Proteins that express well across many cell lines and tissues, such as β-actin, α-tubulin, and GAPDH* are often used as loading controls in experiments designed to compare total protein levels across multiple samples.



(*) Glyceraldehyde 3-phosphate dehydrogenase

Ensure equal loading of a gel

- **Cellular fractionation markers** should be used when you are preparing nuclear and cytoplasmic extracts to confirm that the lysate was prepared appropriately. Histone H3 is a nuclear protein and Lamin A is a nuclear membrane protein; both of these offer strong nuclear fraction markers. Cytoplasmic proteins like MEK1/2, GAPDH and cytoskeletal proteins like β-actin and α/β-tubulin are common cytoplasm markers.

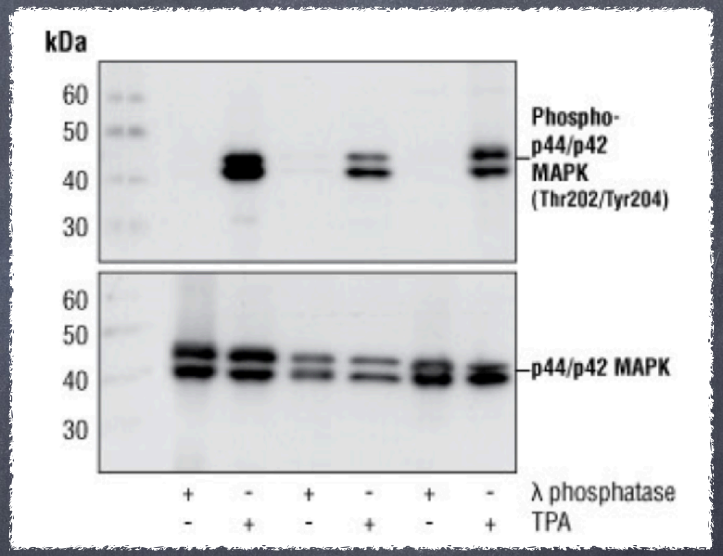


Decreased nuclear association of VP16-GFP and HCF-1 in the absence of lamin A/C. (Left) Lmna+/+and Lmna−/− MEFs were infected with the HSV-1 DG1 virus at an MOI of 50 in the presence of cycloheximide (100 μg/ml), harvested at 3 hpi, and fractionated into cytoplasmic and nuclear fractions. Cytoplasmic and nuclear fractions were loaded at a 1:2 ratio onto an SDS-polyacrylamide gel, and the proteins were resolved in the gel. VP16-GFP and HCF-1 were detected by Western blotting with GFP- and HCF-1-specific antibodies, respectively. GAPDH and lamin B1 were detected as fractionation and loading controls

DOI: 10.1128/mBio.00300-11

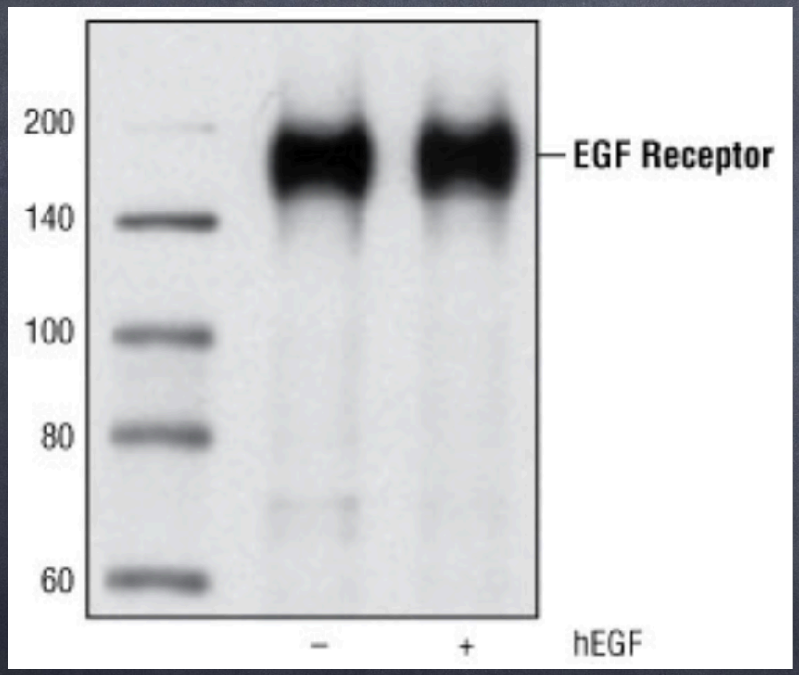
Ensure equal loading of a gel

- Blot with antibodies against the protein of interest is used as control for the loading control of enzymatic modification of the protein, such as phosphorylation.



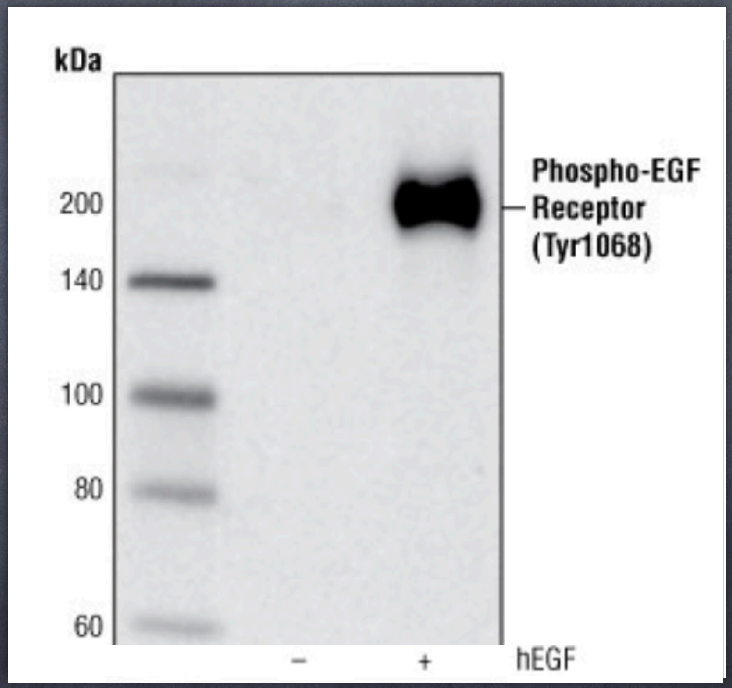
Western blot analysis of EGF Receptor

Blot 1



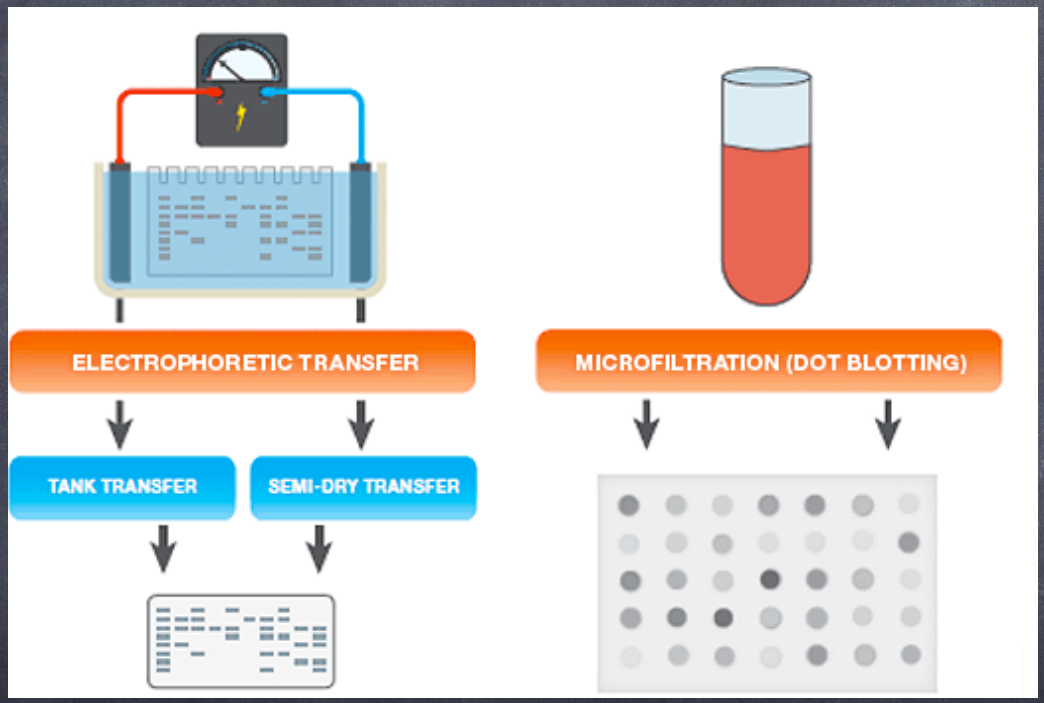
Cell extracts using EGF receptor mAb

Blot 2



Cell extracts using Phospho-EGF receptor (Tyr1068) mAb

Western blotting Dot blotting



IP and co-IP

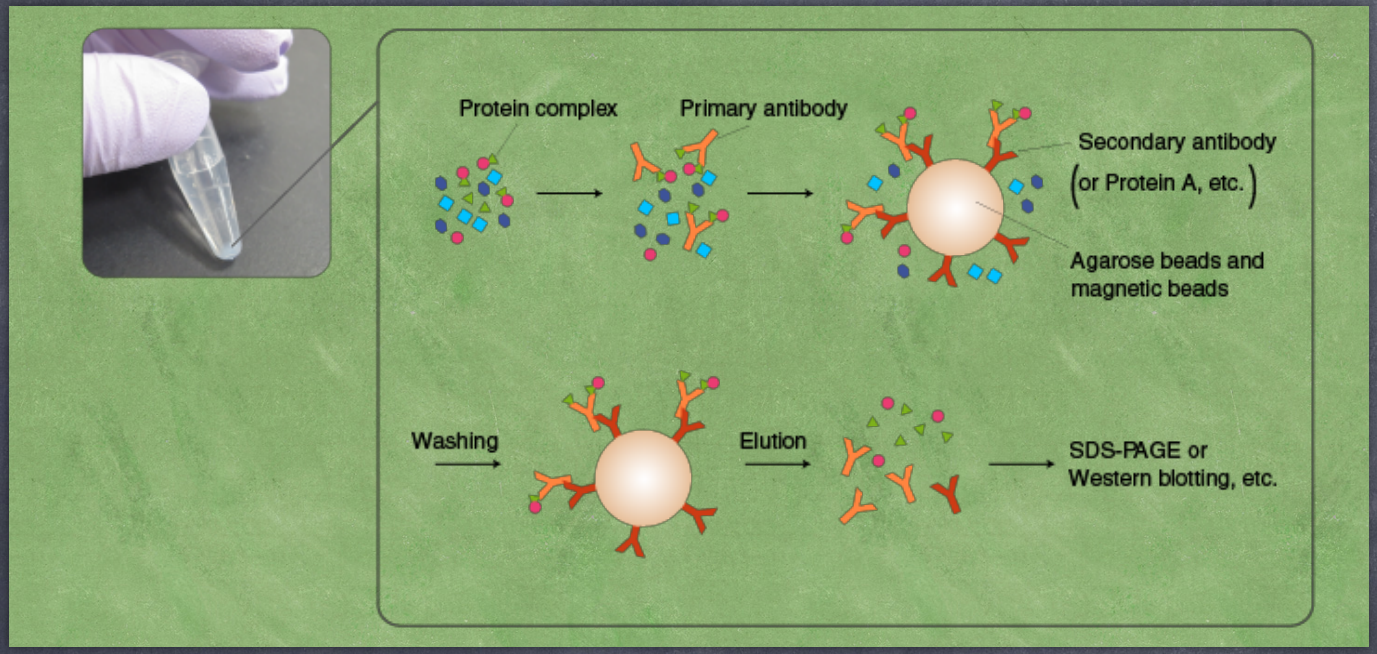
Immunoprecipitation and co-immunoprecipitation

Immunoprecipitation

IP is an affinity purification technique widely used to enrich for a protein of interest for further experiments such as WB and Co-IP techniques but also mass spectrometry or structure-function studies.

IP flowchart

Immunoprecipitation: affinity purification technique



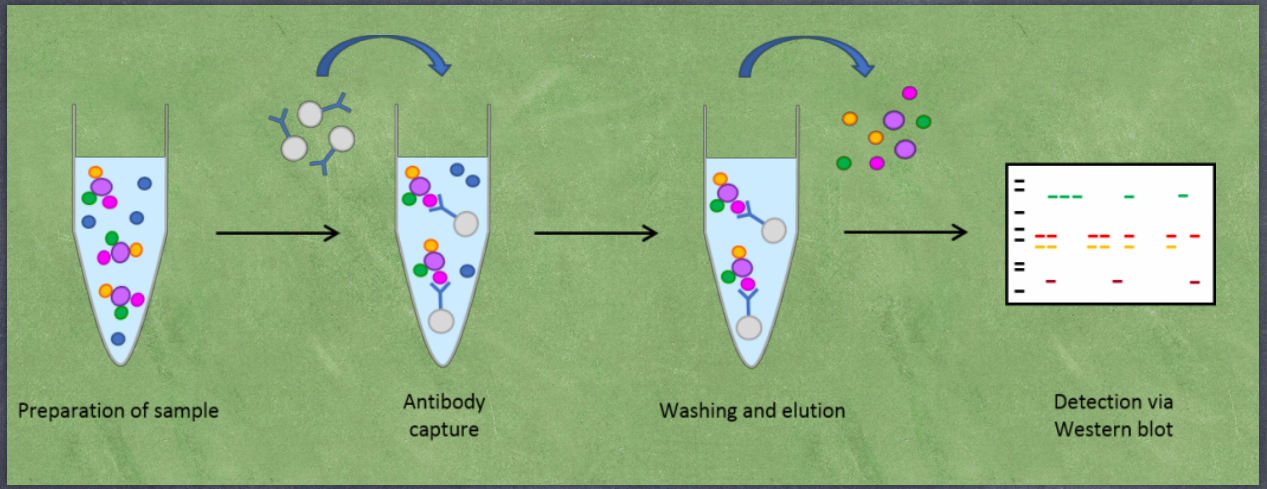
Used to isolate a single protein (the target antigen of the antibody) to investigate its identity, structure, expression, or activation or modification state

Co-IP versus IP

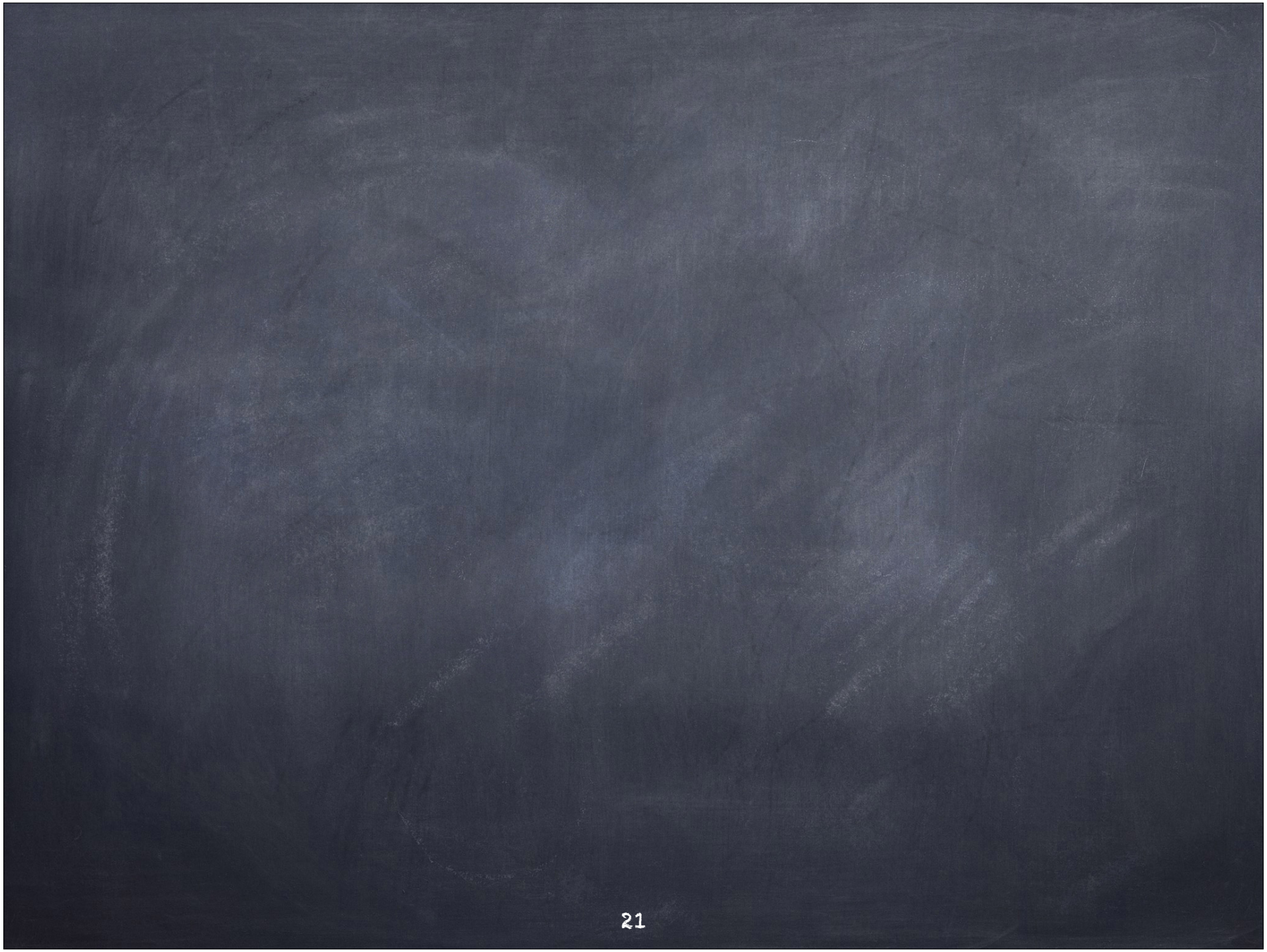
Co-IP is conducted in essentially the same manner as IP, except that antigen precipitated by the antibody is used to co-IP its binding partner(s) or associated protein complex from the lysate. The assumption that is usually made when associated proteins are co-IP is that these proteins are related to the function of the target antigen at the cellular level. This is only an assumption, however, that is subject to further verification.

Co-IP flowchart

Co-IP



Co-IP: used to study the interactions of the primary antigen protein with other proteins or nuclei acids (chromatin IP also called ChIP). In these methods, the goal is to study the interactors or associated cellular components that are bound to the primary antigen.

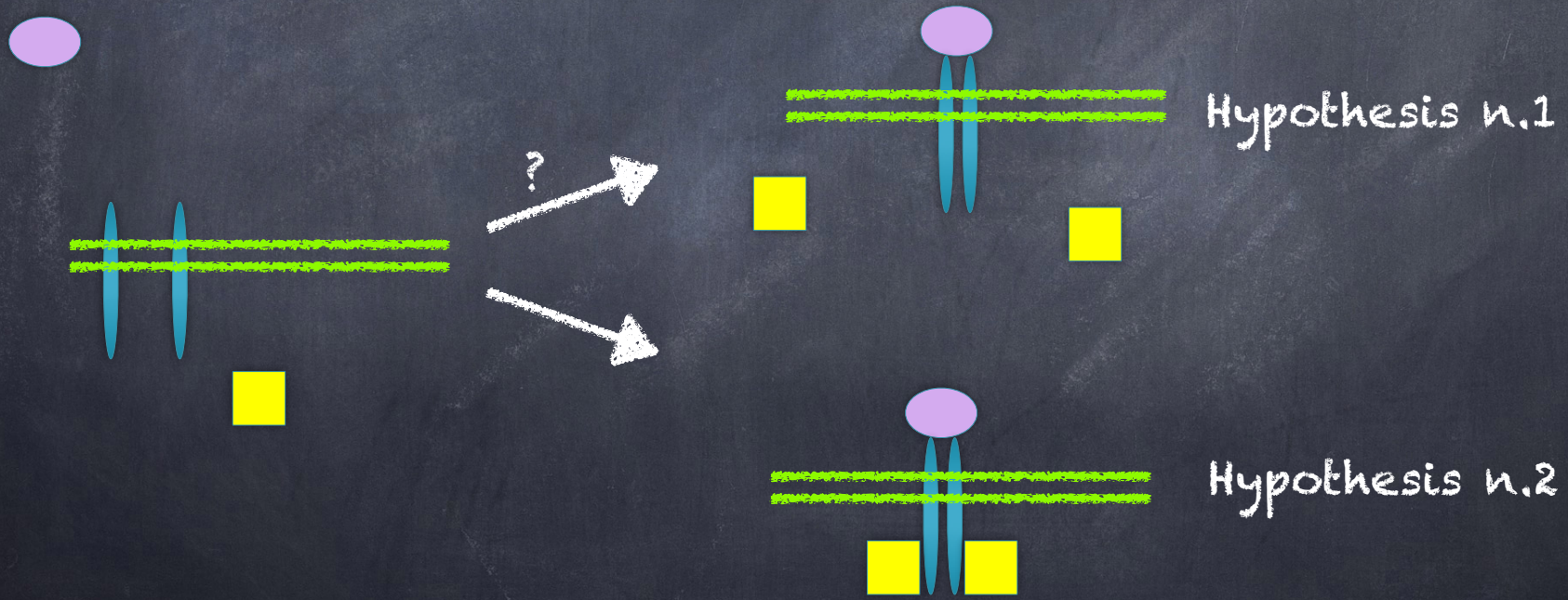


Example of scientific questions:

Is there a protein-protein association between receptor and signal molecule after treatment?

T=0 ?

T=1, T=5, T=10, T=30 min ?



Hypothesis n.1

Hypothesis n.2

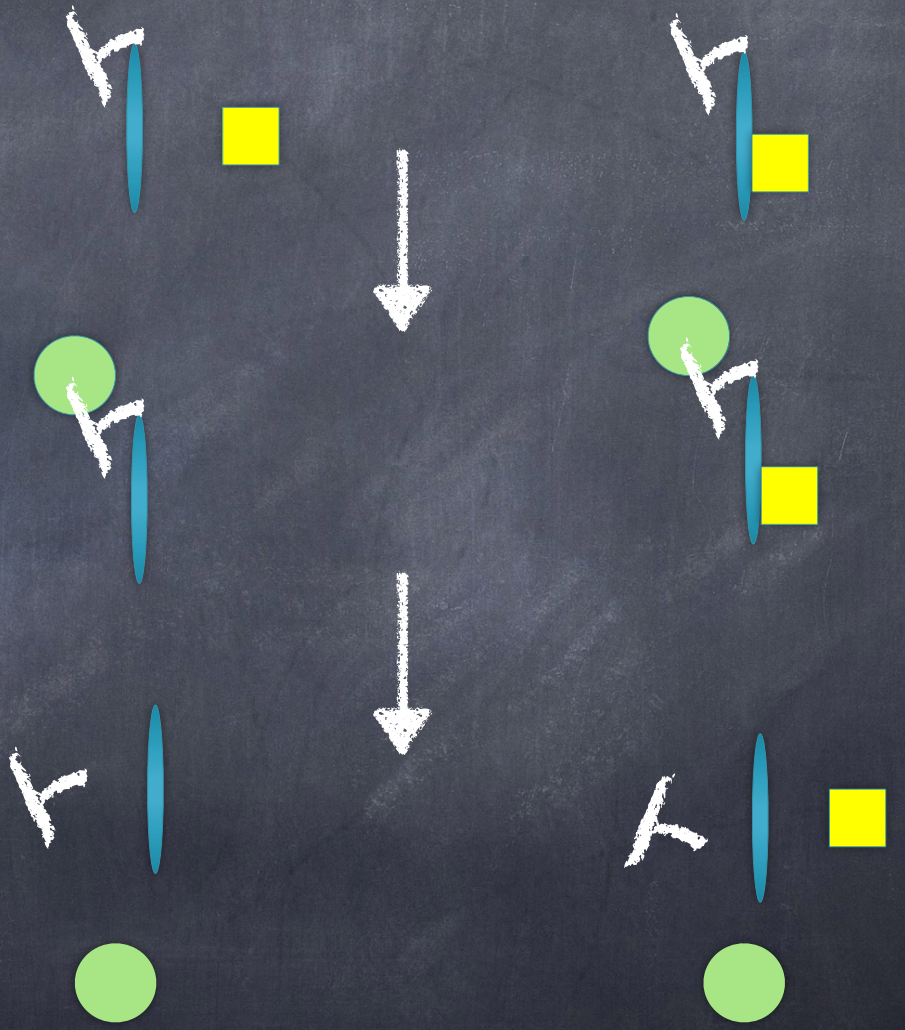
1) Treatment with Ab anti-receptor

2) Immunoprecipitation

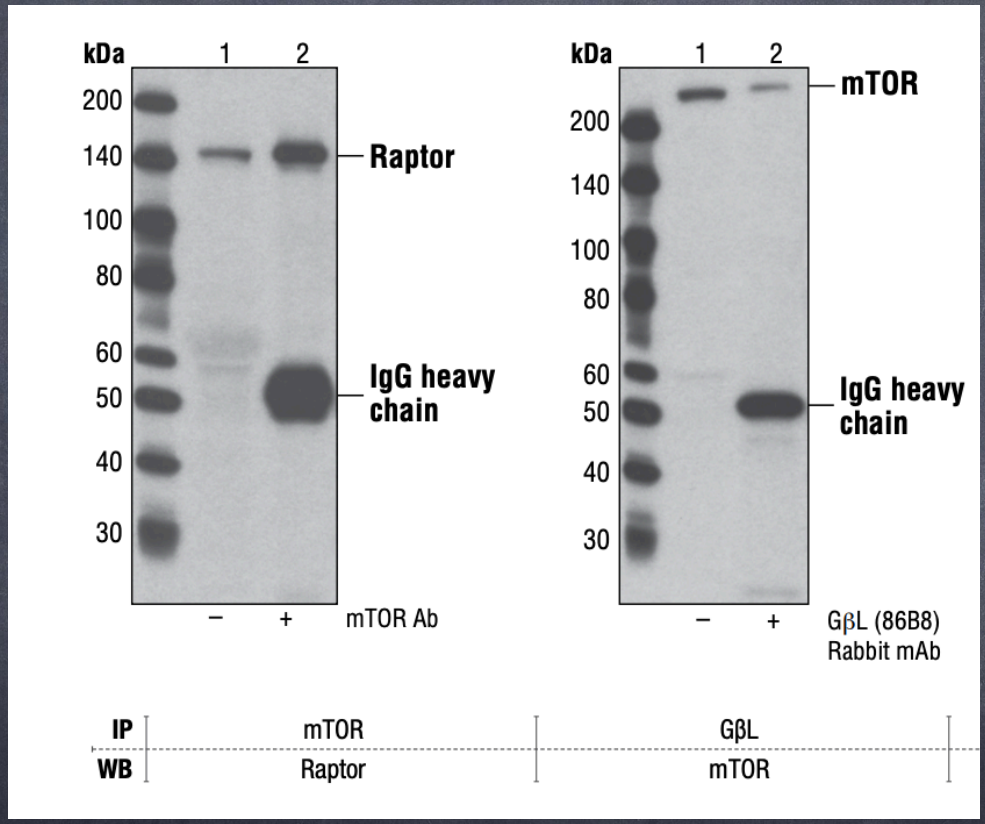
3)Dissociation with SDS-PAGE loading buffer

Surnatant

Pellet



IP versus WB


kDa
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Raptor
IgG heavy chain
– + mTOR Ab
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200
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100
80
60
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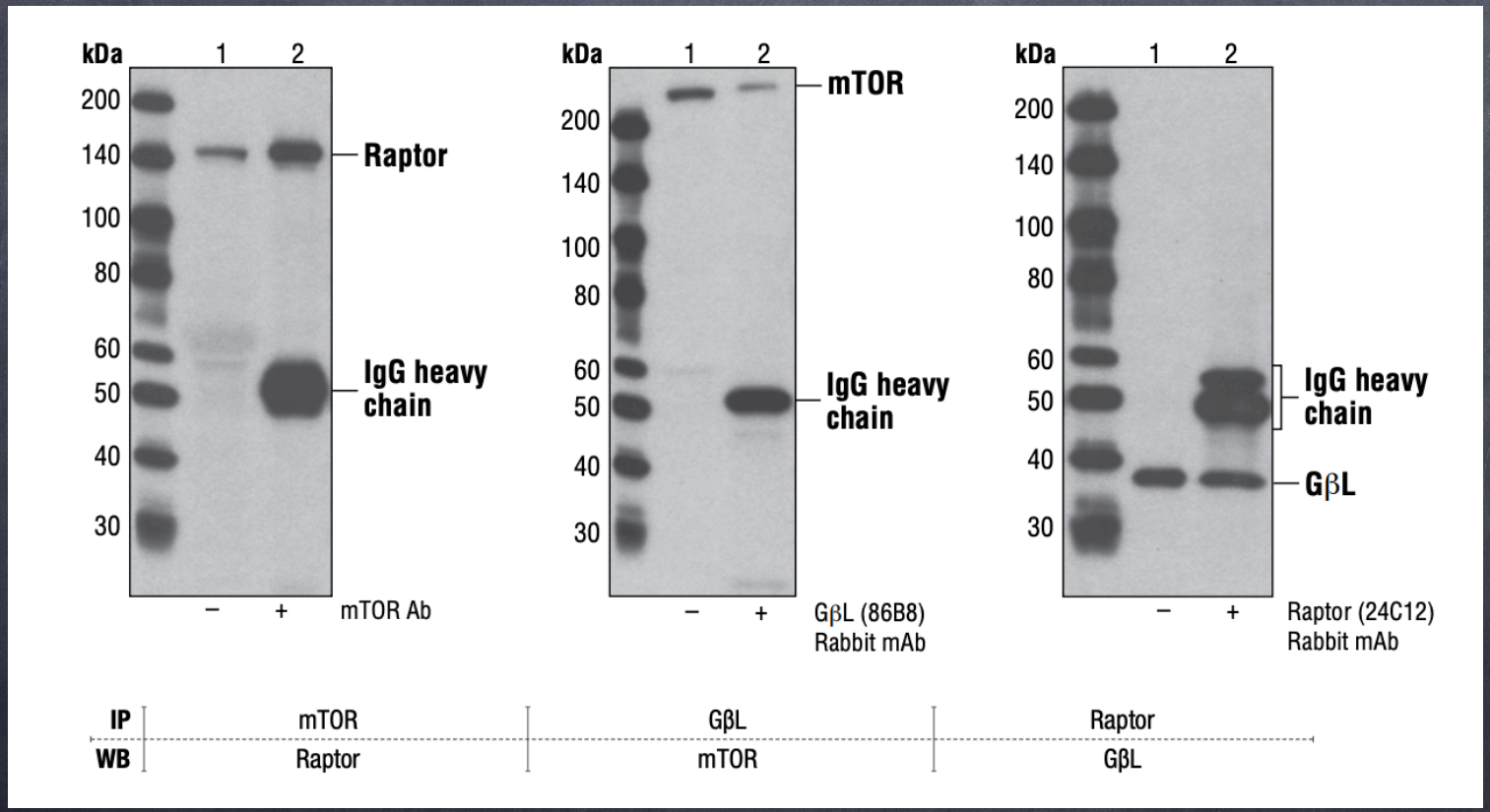
30
IgG heavy chain
– + GβL (86B8) Rabbit mAb
IP
WB
mTOR
Raptor
GβL
mTOR

IP versus WB


kDa
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Raptor
IgG heavy chain
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mTOR Ab
mTOR
GβL (86B8) Rabbit mAb
GβL
Raptor (24C12) Rabbit mAb
IP
WB
mTOR
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GβL
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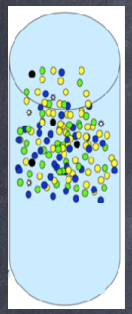
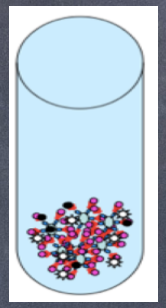
Raptor
GβL

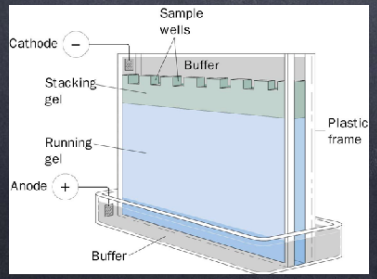
Surnatant

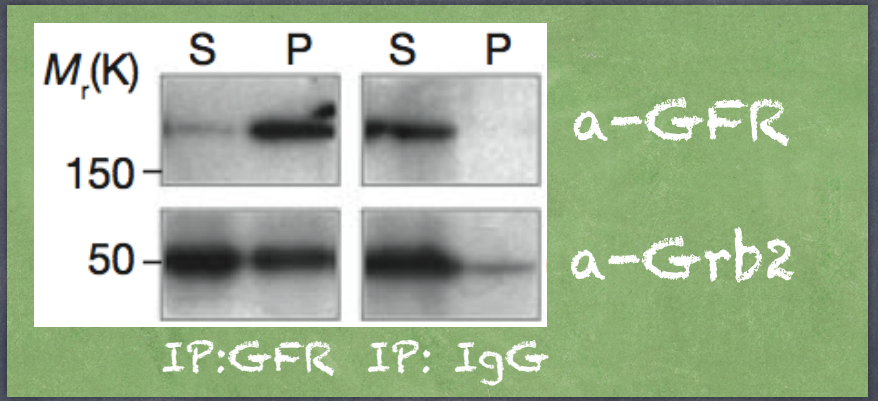
Pellet





S P





Pioneer experiment

MEK1-ERK1 binding was observed. These results are consistent with a model where MP1 can form a ternary complex with MEK1 and ERK1, facilitating the interaction of the two enzymes; however, at high concentrations of MP1, binary MP1-ERK1 and MP1-MEK1 complexes would be favored.

In yeast, scaffolding proteins such as STE5 can enhance signaling by locally sequestering a subset of components. In the mammalian MAP kinase pathway, analogous functions may be distributed among several proteins, acting at different steps in the pathway, with MP1 facilitating the functional interactions involving MEK. The existence of multiple small proteins that serve adapter functions would provide enhanced flexibility in regulating the efficiency and specificity of the MAP kinase cascade.

References and Notes

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2. K. Y. Choi, B. Satterberg, D. M. Lyons, E. A. Elion, *Cell* **78**, 499 (1994); I. Herskowitz, *ibid.* **80**, 187 (1995); H. D. Madhani and G. R. Fink, *Trends Genet.* **14**, 151 (1998).
3. A. D. Catling, H. J. Schaeffer, C. W. Reuter, G. R. Reddy, M. J. Weber, *Mol. Cell. Biol.* **15**, 5214 (1995).
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5. A. D. Catling, unpublished data; A. Dang, J. A. Frost, M. H. Cobb, *J. Biol. Chem.* **273**, 19609 (1998).
6. C. T. Chien, P. L. Bartel, R. Sternglanz, S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9578 (1991); J. W. Harper, G. R. Adami, N. Wei, K. Keyomarsi, S. J. Elledge, *Cell* **75**, 805 (1993). The two-hybrid screen was done using yeast strain CG1945 and a pACT mouse lymphoma cDNA library (Clontech MATCHMAKER GAL4 two-hybrid user manual). Full-length MEK1 was subcloned into pAS1 and expressed as a Gal4 DNA binding domain fusion protein. Approximately 2×10^6 clones of a mouse lymphoma library were screened, and seven independent clones coding for MP1 were isolated. MP1 clones with additional 5′ sequence were isolated from the same library by plaque hybridization. MP1 failed to interact with various control proteins including Lamin, p53, SNF1, Ras, c-Raf1, and the Gal4 DNA-binding domain alone. The cDNA of the longest clone isolated had an open reading frame of 372 nucleotides and coded for a protein with a predicted molecular size of 13.5 kD. The putative initiation codon is flanked by a consensus Kozak sequence. An in-frame stop codon 5′ of the

A Mammalian Scaffold Complex That Selectively Mediates MAP Kinase Activation

Alan J. Whitmarsh, Julie Cavanagh, Cathy Tournier, Jun Yasuda, Roger J. Davis*

The c-Jun NH_2-terminal kinase (JNK) group of mitogen-activated protein (MAP) kinases is activated by the exposure of cells to multiple forms of stress. A putative scaffold protein was identified that interacts with multiple components of the JNK signaling pathway, including the mixed-lineage group of MAP kinase kinase kinases (MLK), the MAP kinase kinase MKK7, and the MAP kinase JNK. This scaffold protein selectively enhanced JNK activation by the MLK signaling pathway. These data establish that a mammalian scaffold protein can mediate activation of a MAP kinase signaling pathway.

The c-Jun NH_2-terminal kinase (JNK) group of MAP kinases represents one of three groups of MAP kinases that have been identified in mammalian cells (*1*). JNK is activated in cells exposed to environmental stress or in cells treated with proinflammatory cytokines. Targets of the JNK signaling pathway include the transcription factors ATF-2, Elk-1, c-Jun, and NFAT4. JNK is required for a number of cellular processes in both *Drosophila* and mammalian cells. These include early embryonic development, apoptosis, oncogenic transformation, and the immune response (*1*).

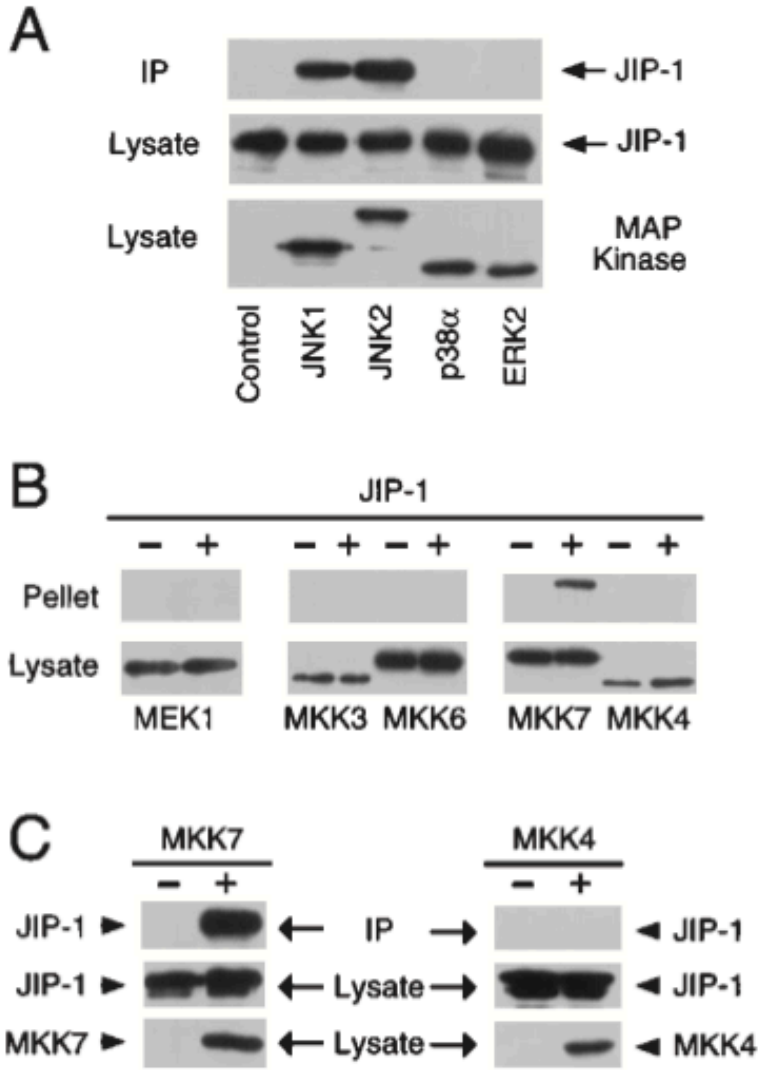
Similar to other MAP kinases, JNK is activated by dual phosphorylation on Thr and Tyr within protein kinase subdomain VIII by a MAP kinase kinase (MAPKK). Each MAPKK is phosphorylated and activated by a MAP kinase kinase kinase (MAPKKK). Distinct signaling modules activate the different MAP kinase groups. Components of the JNK signaling module include the MAPKKs MKK4 and MKK7, together with members of the MEK kinase (MEKK) and mixed-lineage kinase (MLK) groups of MAPKKKs. JNK activity can therefore be regulated by many protein kinases, some of which also regulate other MAP kinase signaling pathways. Because JNK also displays some overlap in substrate specificity with other MAP kinases, mechanisms must exist to achieve signaling specificity and to ensure the correct biological response to extracellular stimulation.

An emerging property of signal transduction pathways that might account for specificity is the formation of signaling complexes (*2*). These complexes may result from the physical interaction between components of particular signaling pathways or by the assembly of signaling molecules on anchor or scaffold proteins that localize their binding partners to specific subcellular compartments or to specific substrates. Multienzyme com-

Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School and Howard Hughes Medical Institute, Worcester, MA 01605, USA.

*To whom correspondence should be addressed.

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.



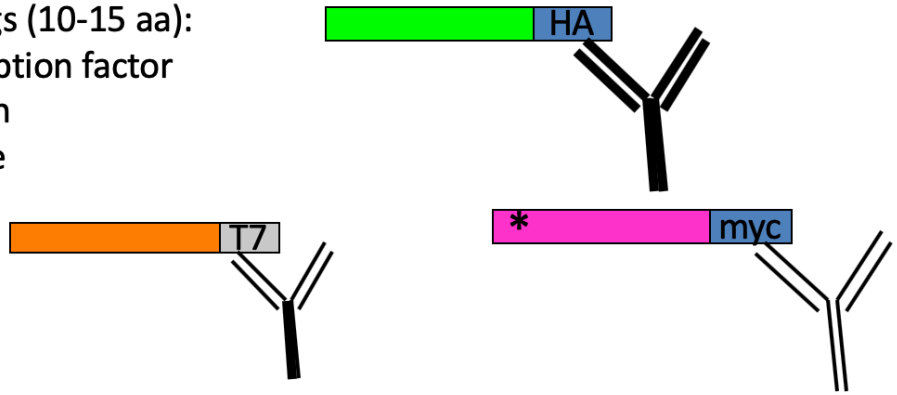
Tagged-proteins

Tagged-proteins

Definition:

Fusion of the target protein to either terminus (the C- or N- terminal) of a short peptide (epitope tag) which is recognized by an antibody (immunoprecipitation and Western blot analysis)

Exemple of epitope-Tags (10-15 aa):
myc: from myc transcription factor
HA: from hemagglutinin
T7: from T7 polymerase



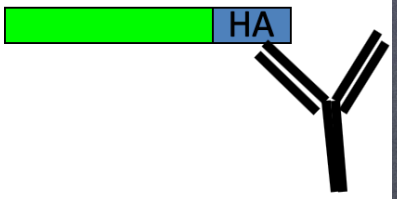
Notice: The fusion protein must be continuous with the target protein - the same open reading frame must be maintained. Stop codons between the target protein and the fusion partner must be omitted.

When are Tag-proteins used?

Ab against endogenous protein not available

If the Ab againt the protein of interest is not available

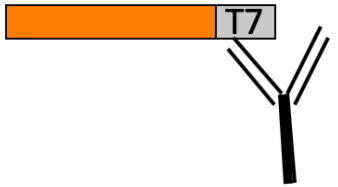
endogenous 

HA-wt 

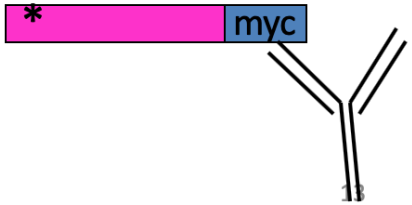
to distinguish recombinant constructs from endogenous proteins

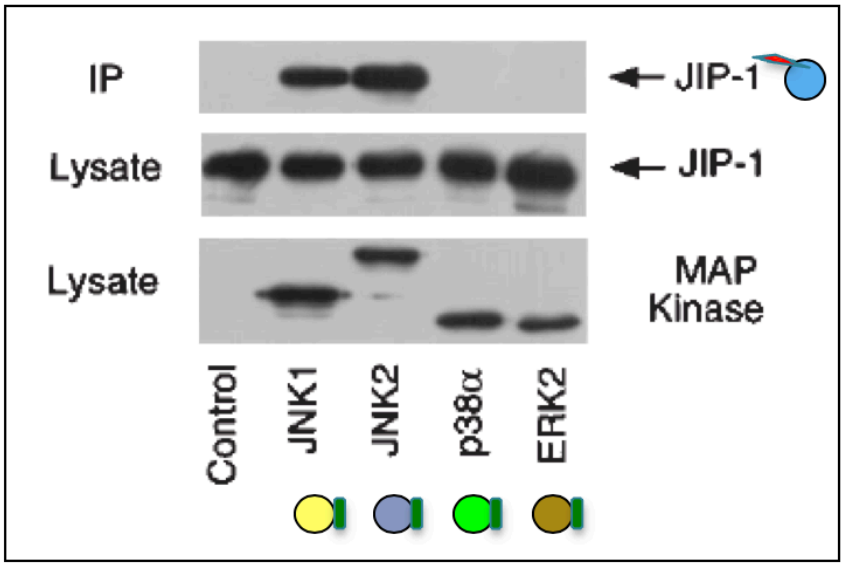
To study recombinant construct of the protein of interest (deletions, site-directed mutations) even in the presence of expression of the endogenous WT protein.

endogenous 

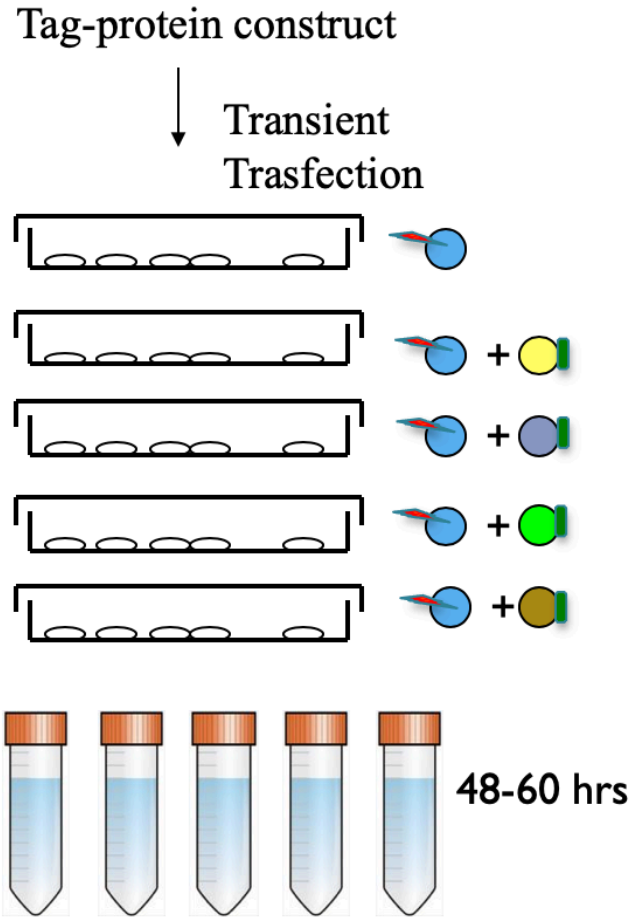
T7-deleted 

endogenous 

myc-mutated 

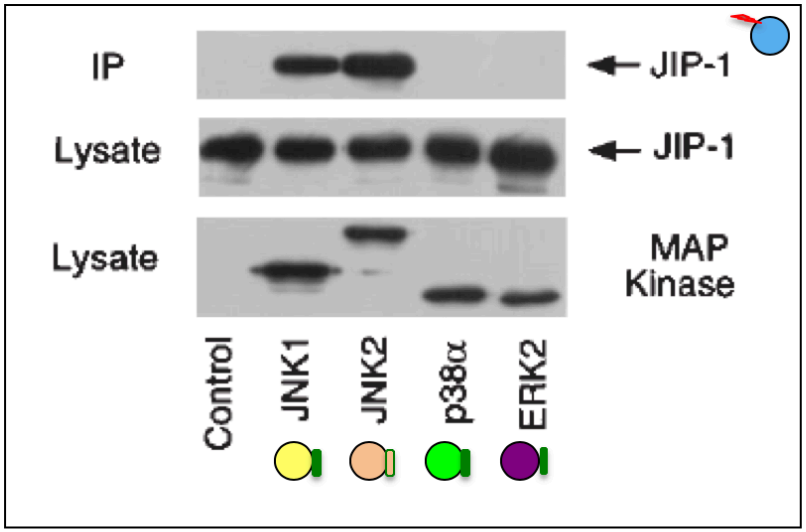


Epitope-tagged JIP-1 (T7-Tag) was ex-pressed in cells with the HA-tagged MAP kinases ERK2, p38α JNK1, and JNK2. The MAP kinases were immunoprecipitated with an antibody to HA. The presence of JIP-1 in the immunoprecipitates (IP) was detected on immunoblots probed with an antibody to T7-Tag. The amount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



IP: Antibody against the Tag

Selective binding of JIP-1 to the MAP kinase JNK



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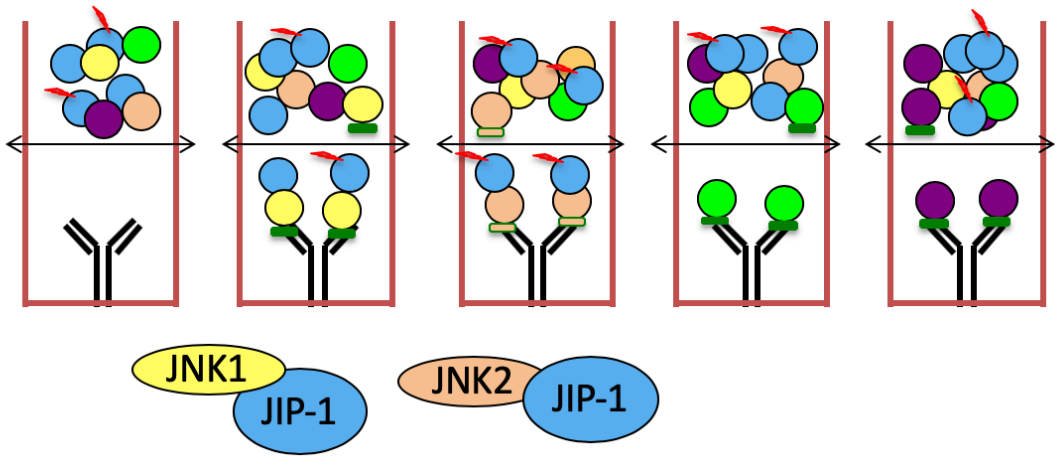
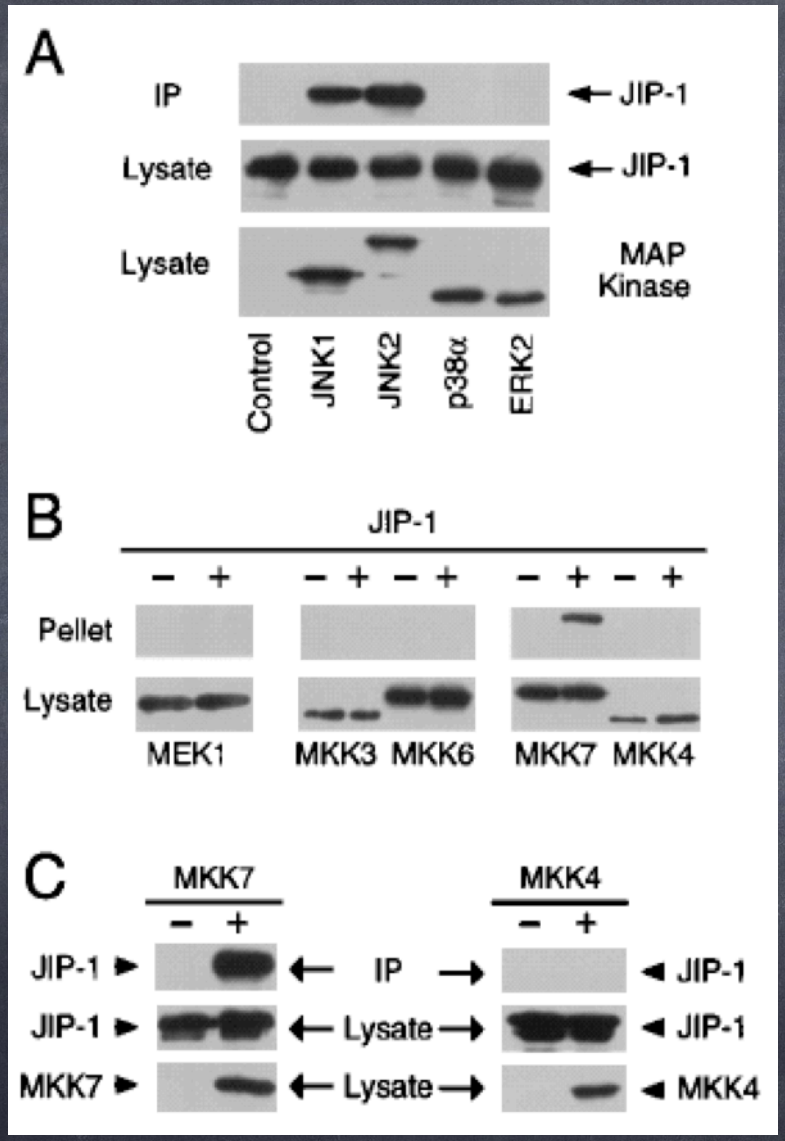


IP


A
IP
JIP-1
Lysate
JIP-1
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MAP Kinase
Control
JNK1
JNK2
p38α
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MKK7
MKK4
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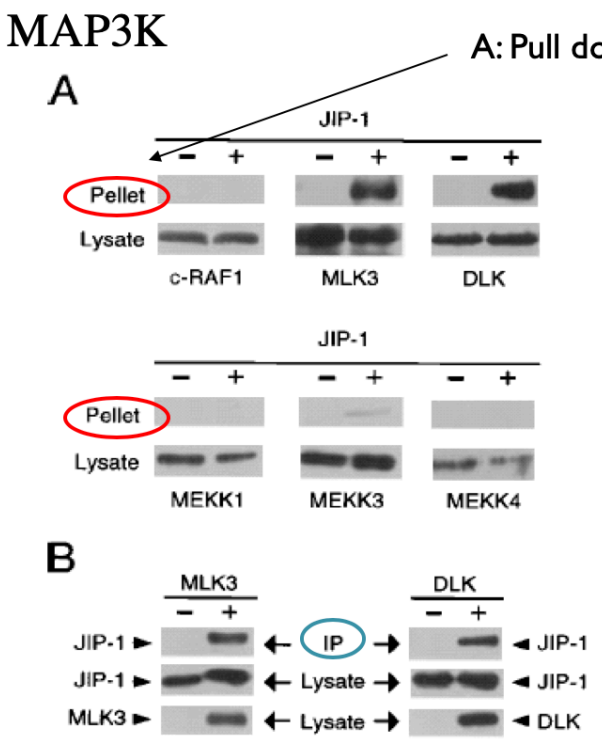
JIP-1
MKK7
Lysate
MKK4



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