Cargo of Kinesin Identified as JIP Scaffolding Proteins and Associated Signaling Molecules

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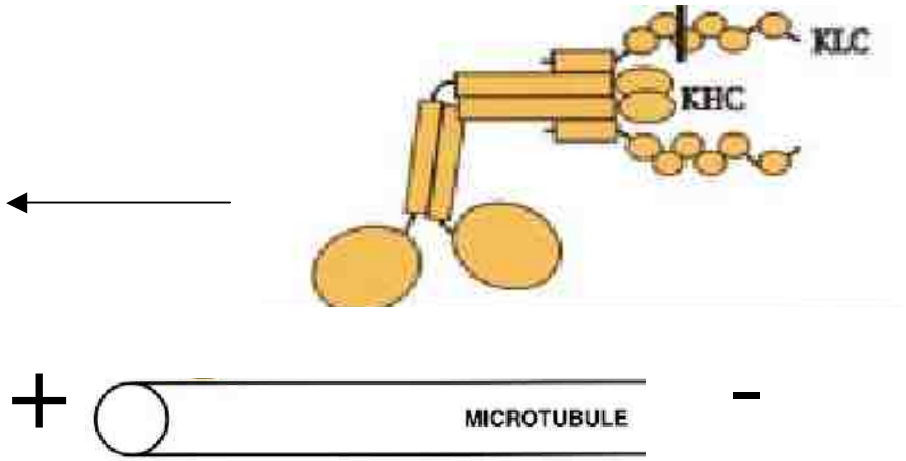
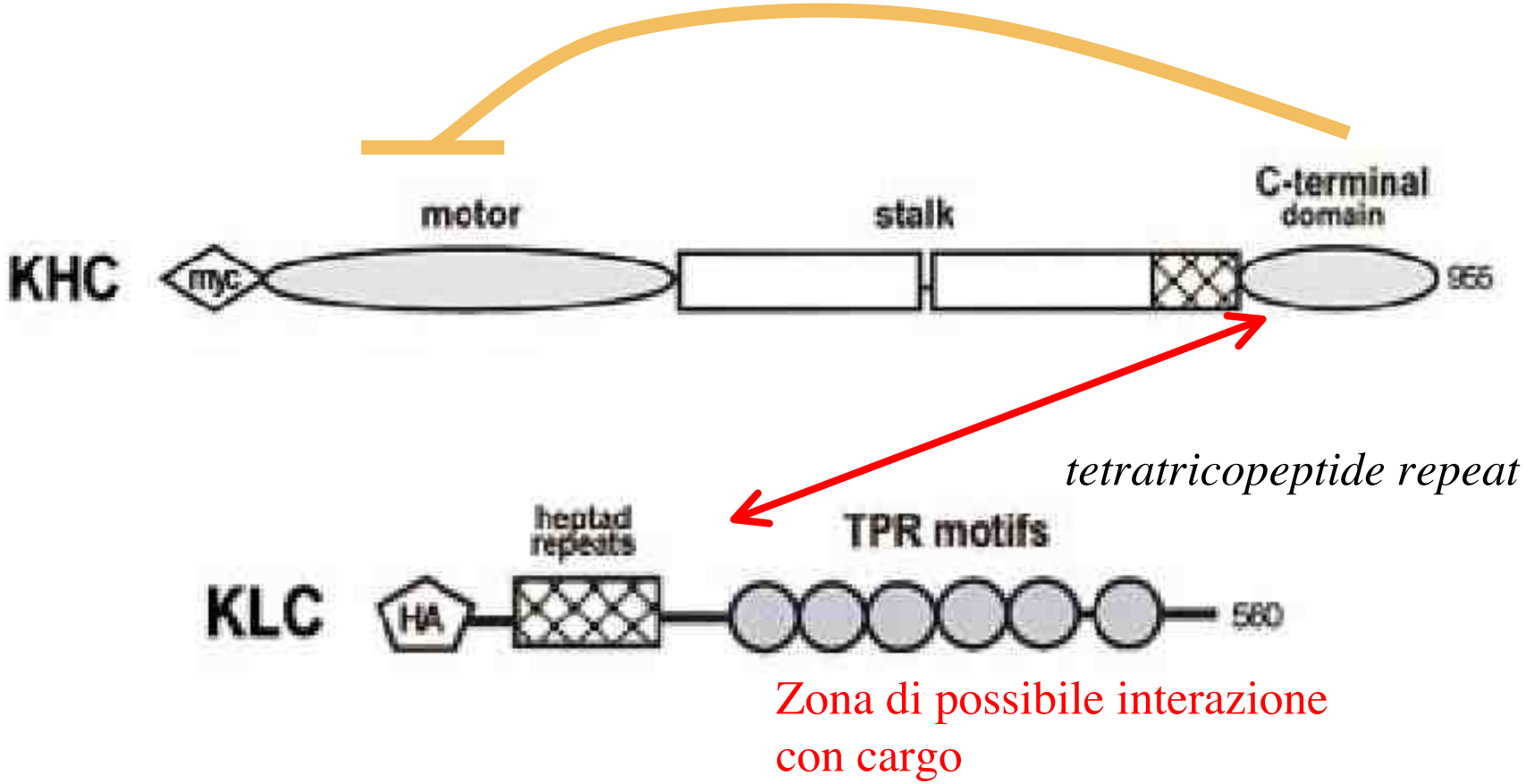


Kinesin light chain

Kinesin heavy chain

Kinesin heavy chain

Kinesin light chain


motor
stalk
C-terminal domain
KHC
myc
955
tetratricopeptide repeat
heptad repeats
TPR motifs
KLC
HA
560
Zona di possibile interazione con cargo

Two-hybrid screen

Direct Interaction of KLC and the JIP Proteins

To identify proteins that interact directly with kinesin, we screened a mouse brain cDNA library using the yeast two-hybrid procedure with the TPR motifs of KLC as a bait. Nine of the clones isolated correspond to overlapping fragments of three different cDNAs encoding JIP-1, JIP-2, and JIP-3 (Fig. 1 B). No interaction of these clones was seen with either of two control bait proteins, the GAL4 DNA binding domain alone, or the TPR motifs of PP5 (data not shown).

Clones of cDNA from JIP-1,2,3

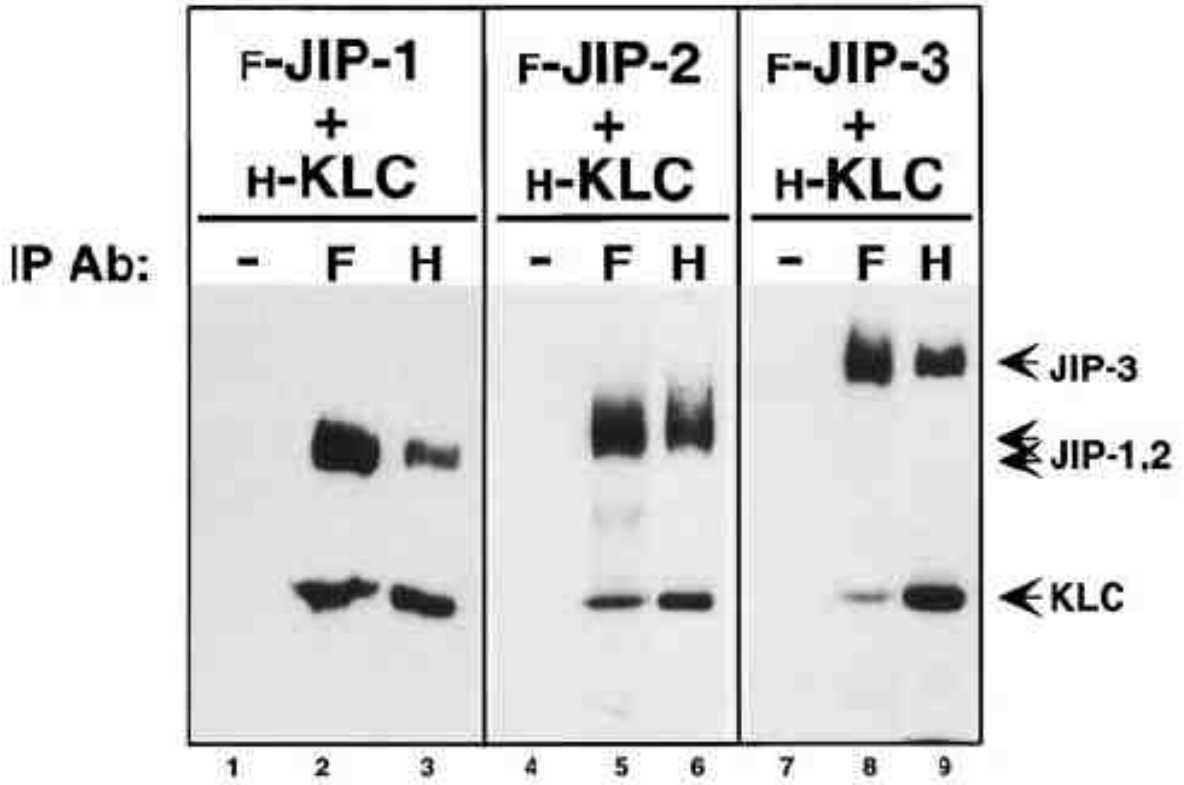
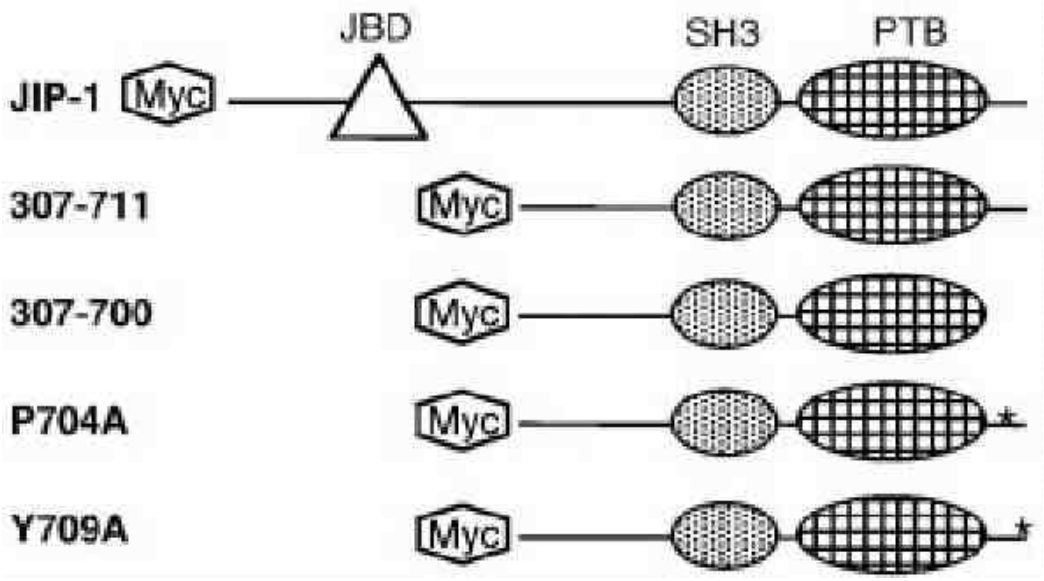


Figure 2. Coimmunoprecipitation of KLC and the JIP proteins. Lysates of COS cells expressing Flag-tagged JIP-1, JIP-2, or JIP-3 together with HA-tagged KLC were immunoprecipitated (IP) with no primary antibody (−), with an anti-Flag mAb (F), or with an anti-HA mAb (H). Precipitates were immunoblotted to detect the expressed proteins using polyclonal antibodies to both epitope tags.

The extreme COOH-term of JIP-1 and JIP-2 are identical and conserved across species

h: human
m: mouse
p: porcine
d: drosophila
ce: C. Elegans

Are JIP-1 COOH-term residues required for binding to KLC TPR?



Site-directed mutagenesis
(*in vitro* mutagenesis)

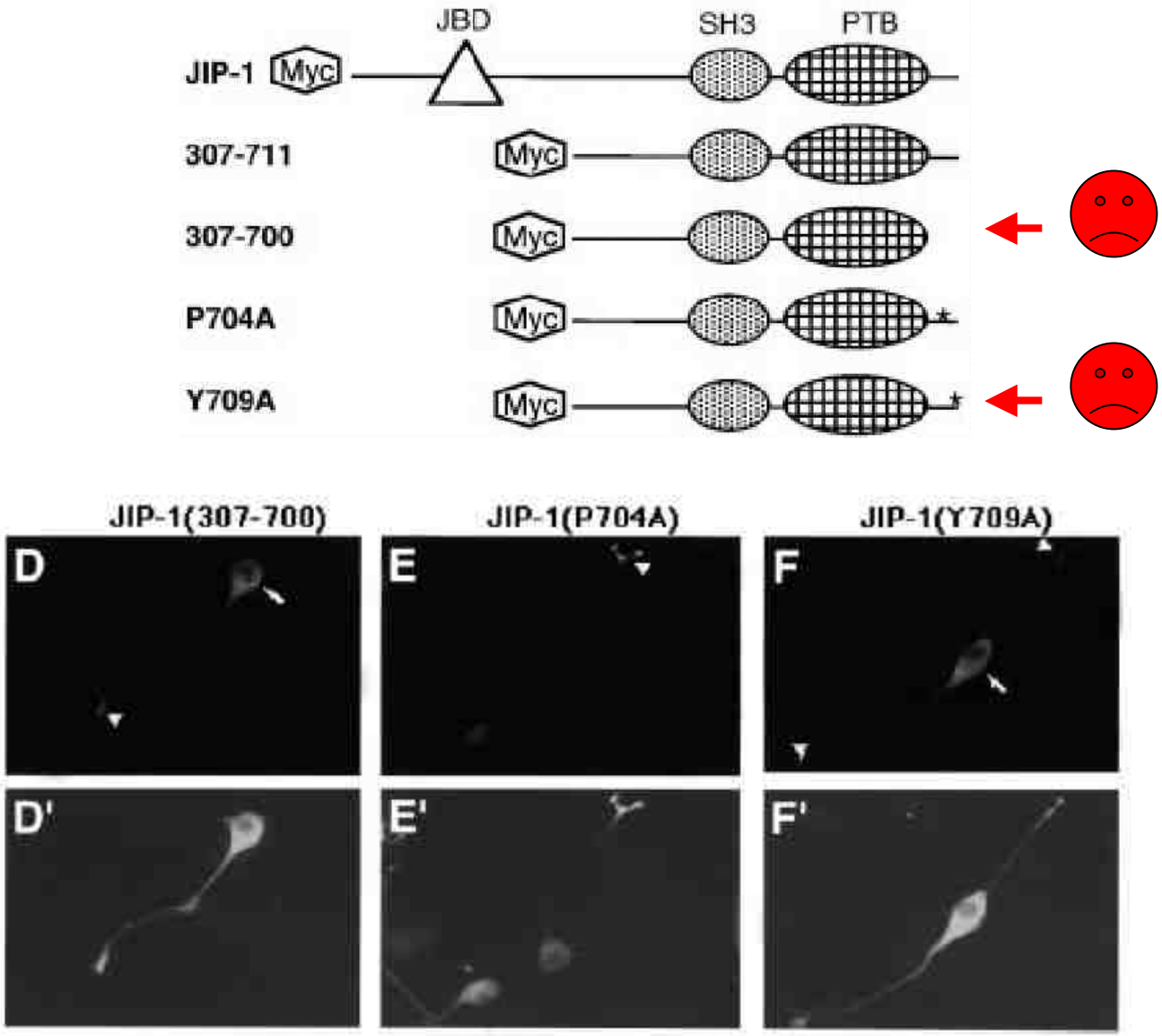
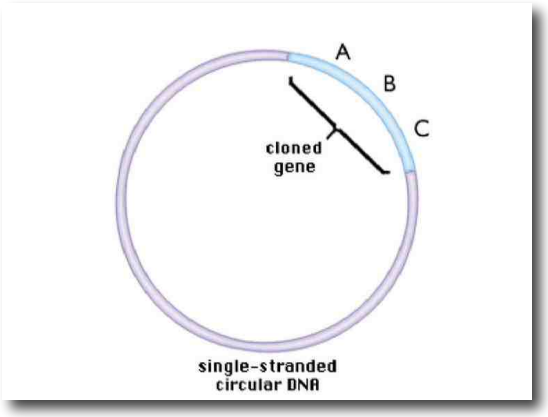
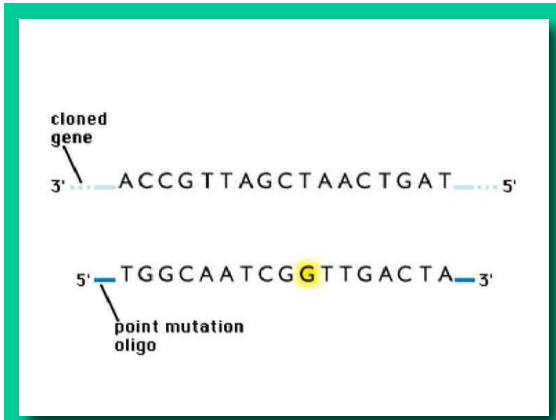


Figure 4. The COOH-terminal residues of JIP-1 are required for proper subcellular localization. NIE 115 cells were transiently transfected with the parental plasmid (control) or with plasmids encoding the indicated JIP-1 variants, differentiated, and the expressed proteins were detected by indirect immunofluorescence microscopy using an anti-Myc mAb. Nonspecific background staining is visible in the control cells and is enhanced in A′–F′ to aid in visualization of the cells. Myc–JIP-1 variants were scored as positive for correct cellular localization (JIP-1, JIP-1 [307–711], and JIP-1 [P704A]) if fluorescence was more pronounced at the neurite tips (arrowheads), whereas transfected proteins were considered negative for localization (JIP-1 [307–700] and JIP-1 [Y709A]) if fluorescence was observed to be more prominent in the cell body (arrows).

Site-Directed Mutagenesis

- Tests role of particular residues in structure, catalytic activity and ligand-binding capacity of protein
- Hundreds of methods described – all based on simple concept
  - Synthetic oligo encoding desired mutation annealed to target region of wt template DNA – serves as primer for initiation of DNA synthesis *in vitro*
  - Extension of oligo by DNA polymerase generates dsDNA that carries desired mutation
  - Mutated DNA is inserted at appropriate location of target gene and mutant protein is expressed
- Requires cloned gene


A
B
C
cloned gene
single-stranded circular DNA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ACCGTTAGCTAACTGAT
5'
5'
TGGCAATCGGTTGACTA
3'
point mutation oligo


cloned gene
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ACCGTTAGCTAACTGAT
5'
5'
TGGCAATCGGTTGACTA
3'
point mutation oligo


cloned gene
3'
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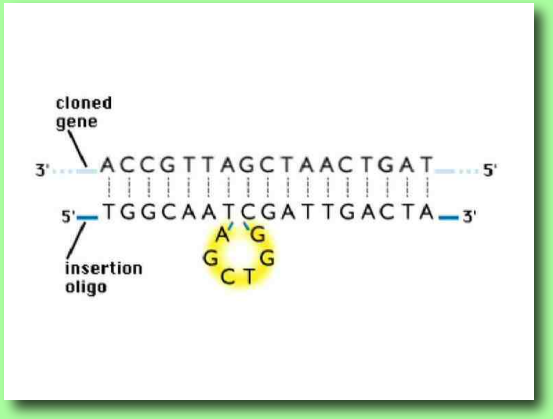
5'
5'
TGGCAATAGCTGGCGATTGACTA
3'
insertion oligo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A
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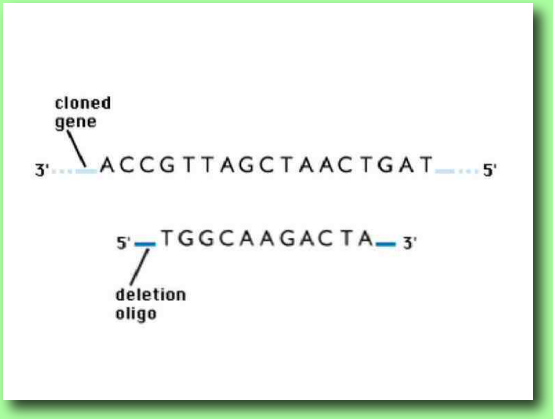
G
C
T
insertion oligo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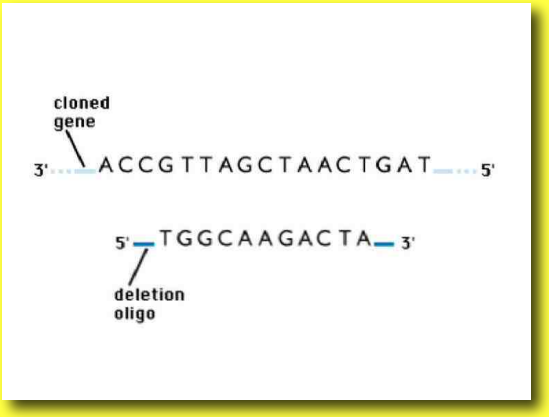
deletion oligo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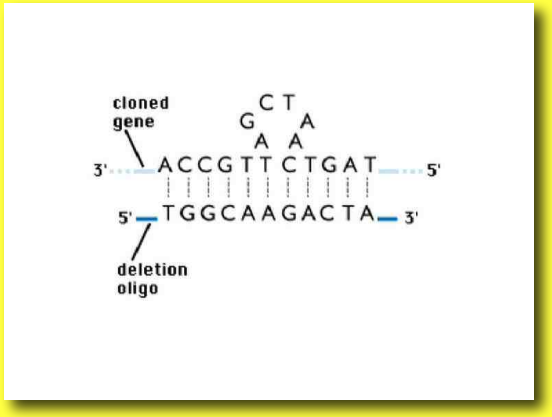
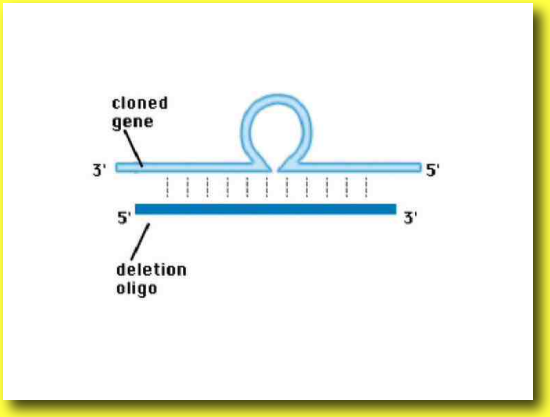
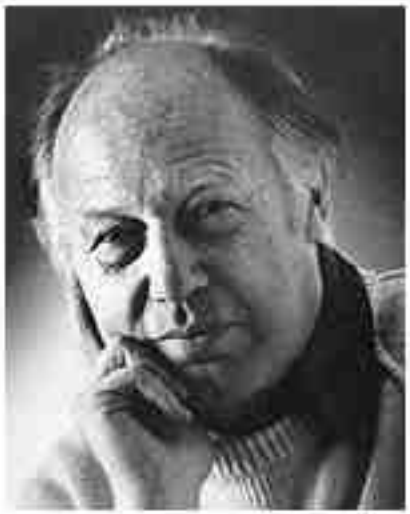

cloned gene
C
T
G
A
A
A
3'
ACCGTTCTGAT
5'
5'
TGGCAAGACTA
3'
deletion oligo


cloned gene
3'
5'
5'
3'
deletion oligo

The Nobel Prize in Chemistry 1993

The Royal Swedish Academy of Sciences awards this year's Nobel Prize in Chemistry to

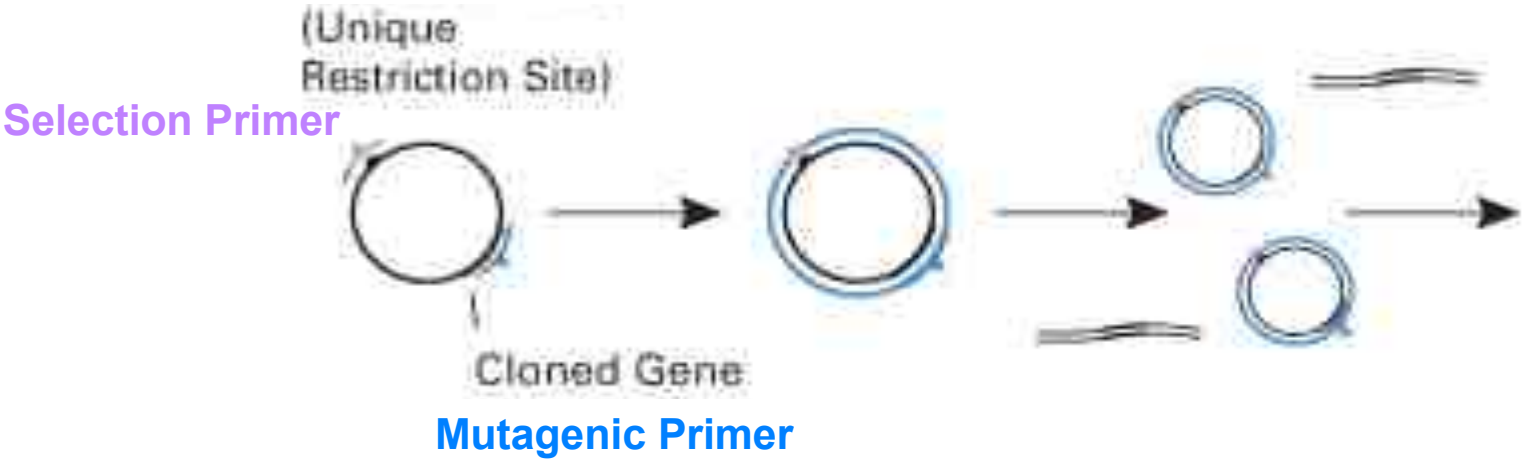


Michael Smith
Canada, for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies

Kary B. Mullis
USA, for his invention of the polymerase chain reaction (PCR) method

The Transformer Method (Clontech)

The Transformer Kit uses two oligonucleotide primers which are simultaneously annealed to one strand of a denatured double-stranded template. One primer introduces the desired mutation and the other mutates the unique restriction site in the plasmid, creating a new restriction site or eliminating the site completely. Elongation by T4 DNA polymerase, which lacks strand displacement activity, results in the incorporation of both mutations in the same newly synthesized strand. The DNA is then digested with a restriction enzyme that cuts at the original restriction site. The uncut, mutated DNA will transform E. coli more efficiently than the linear DNA with no mutations.

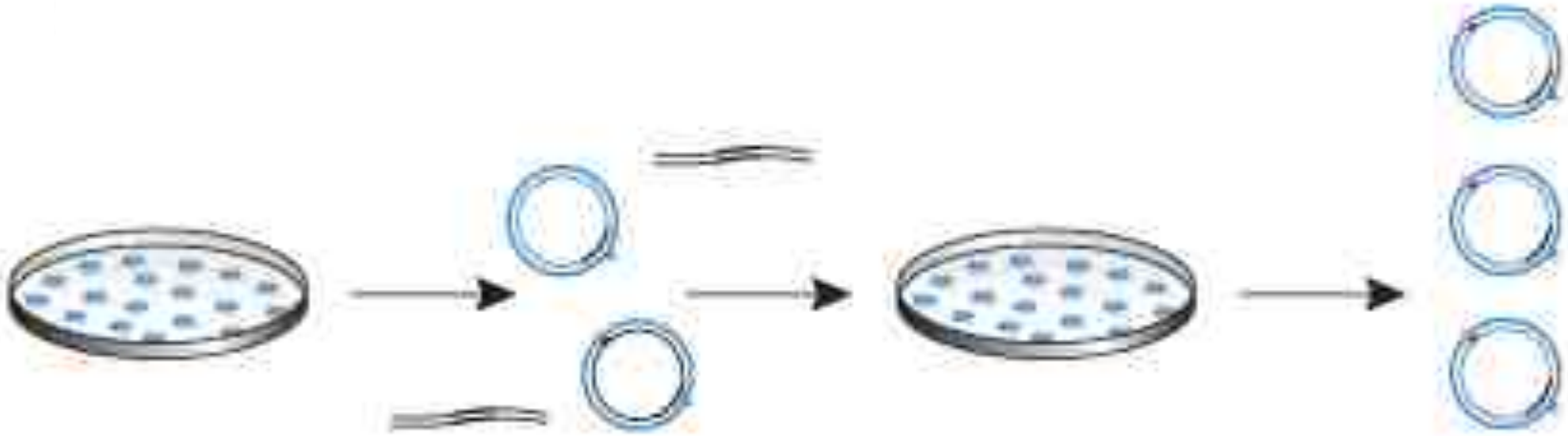


Denature double stranded plasmid anneal primers

Synthesize second strand

Digest DNA primary digestion

arialE. coli BMH 71-18 mutS, which is mismatch repair deficient, is used to propagate the mutated plasmid. Two rounds of DNA digestion and transformation ensure that a very high frequency of transformants carry the mutated plasmid, which nearly always contains both mutations—the desired mutation and the selection mutation.



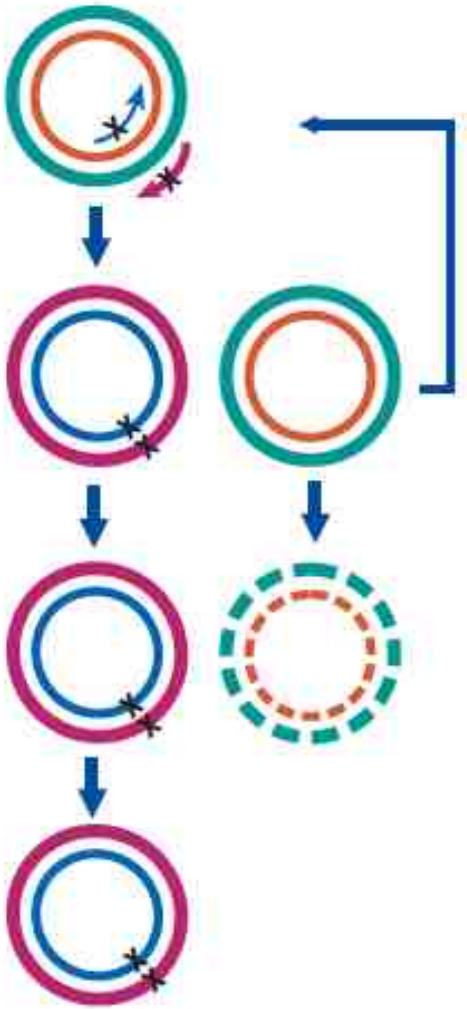
Transform *E. coli* *mutS* to propagate plasmids

Isolate and digest DNA (secondary digestion)

Transform *E. coli*

Isolate DNA

The QuikChange II site-directed mutagenesis method (Stratagene).



1. Mutant strand synthesis
Perform thermal cycling to:
• denature DNA template
• anneal **mutagenic primers** containing desired mutation
• extend and incorporate primers with *PfuUltra* DNA polymerase
(total reaction time: 1 hour)

2. ***Dpn*I digestion** of template
Digest parental methylated and hemimethylated DNA with *Dpn*I
(total reaction time: 5 min)

3. **Transformation**
Transform mutated molecule into competent cells for nick repair
(total reaction time: 1.5 hours)

QuikChange® Lightning Site-Directed Mutagenesis Kit
MATERIALS PROVIDED

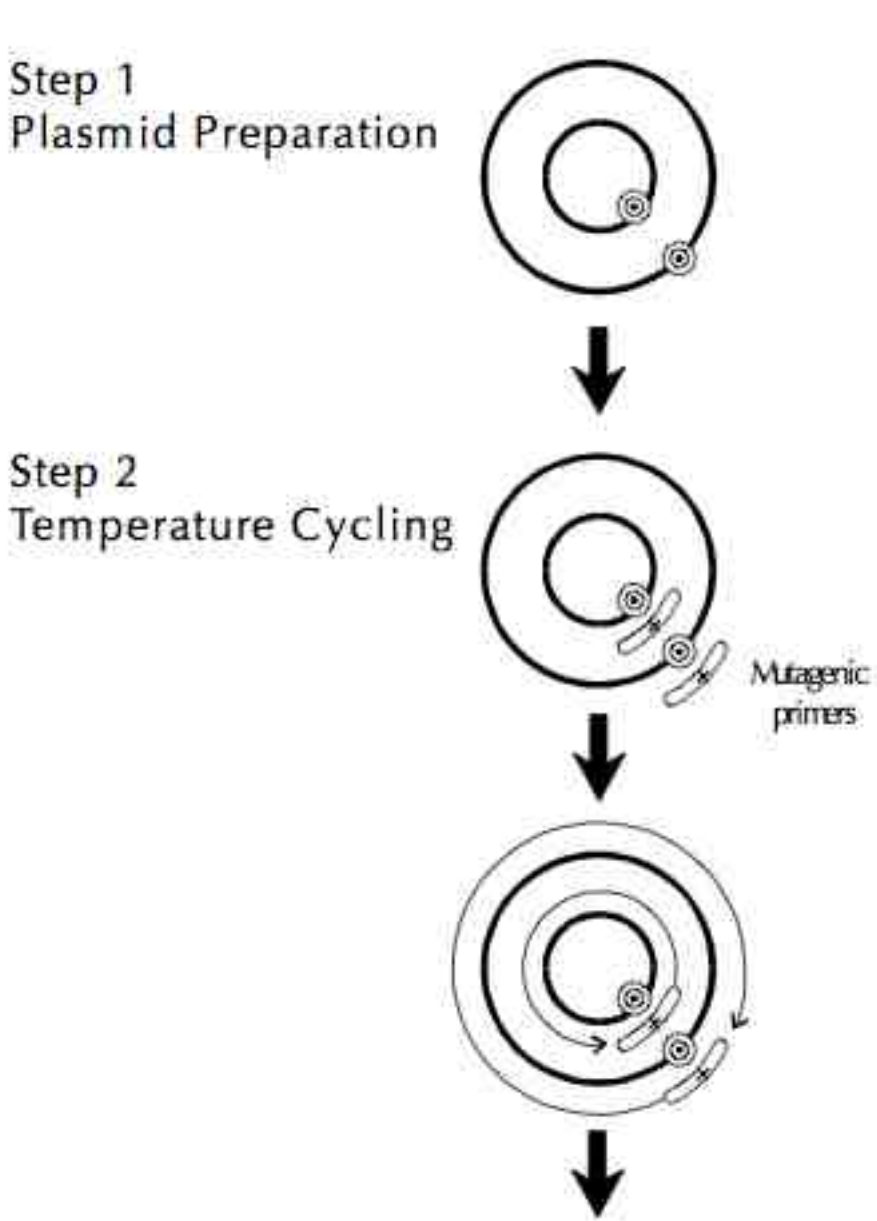
Materials Provided	Catalog #210518[a]
QuikChange® Lightning Enzyme	10 reactions
10× QuikChange® Lightning Buffer	500 µl
dNTP mix[c,d]	10 µl
QuikSolution™ reagent	500 µl
Dpn I restriction enzyme[d]	10 reactions
pWhitescript™ 4.5-kb control plasmid (5 ng/µl)	50 ng
Oligonucleotide control primer #1 [34-mer (100 ng/µl)] 5′ CCA TGA TTA CGC CAA GCG CGC AAT TAA CCC TCA C 3′	750 ng
Oligonucleotide control primer #2 [34-mer (100 ng/µl)] 5′ GTG AGG GTT AAT TGC GCG CTT GGC GTA ATC ATG G 3′	750 ng
XL10-Gold® ultracompetent cells[e] (yellow tubes)	4 × 135 µl
XL10-Gold® β-mercaptoethanol mix (β-ME)	50 µl
pUC18 control plasmid (0.1 ng/µl in TE buffer[f])	10 µl

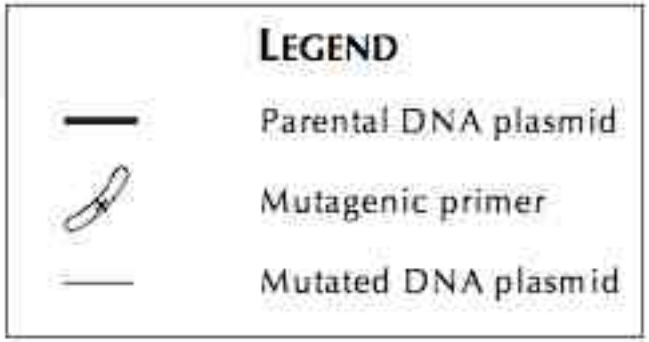
The QuikChange Lightning Site-Directed Mutagenesis Kit (Catalog #210518) contains enough reagents for 10 total reactions, which includes 2 control reactions.

Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at –20°C. Do not subject the dNTP mix to multiple freeze-thaw cycles.

STORAGE CONDITIONS: XL10-Gold® Ultracompetent cells, XL10-Gold® β-ME, and pUC18 Control Plasmid: –80°C. All Other Components: –20°C.

ADDITIONAL MATERIALS REQUIRED: 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) Isopropyl-1-thio-β-D-galactopyranoside (IPTG).

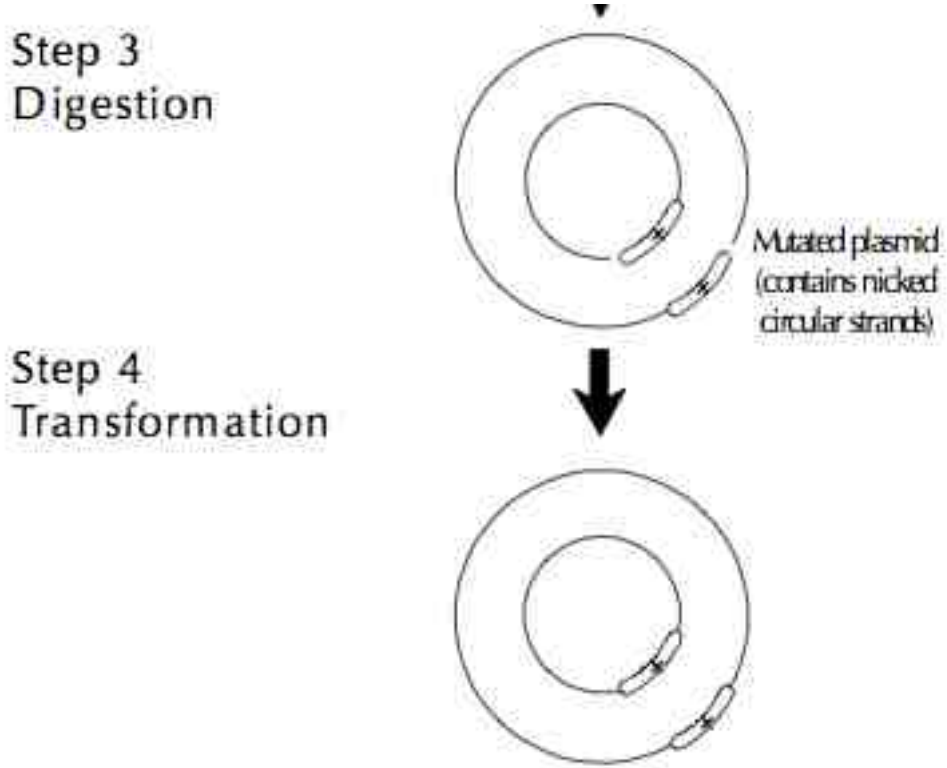




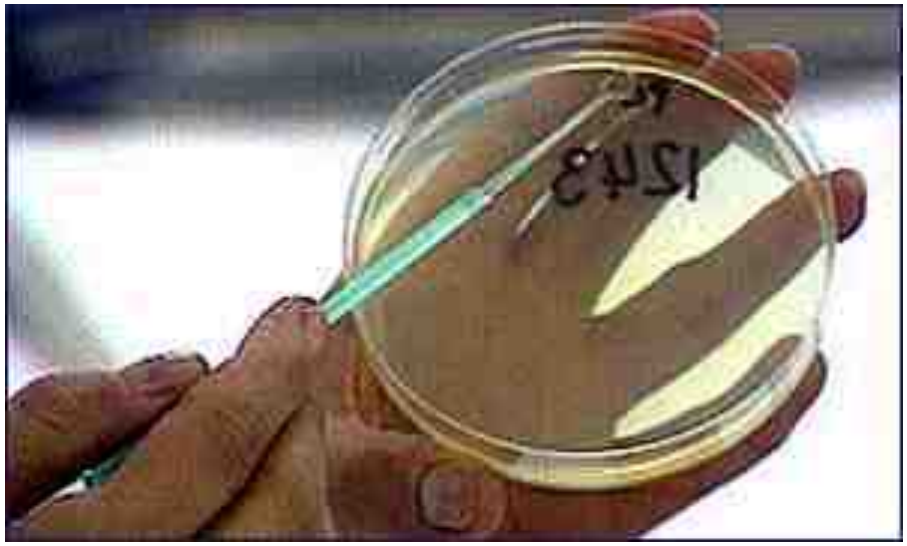
Mutant-strand synthesis is followed by *Dpn*I digestion of the parental DNA template.

transformation of the resulting annealed doublestranded nicked DNA molecules. After transformation, the XL-1 Blue *E. coli* cell repairs nicks in the plasmid.

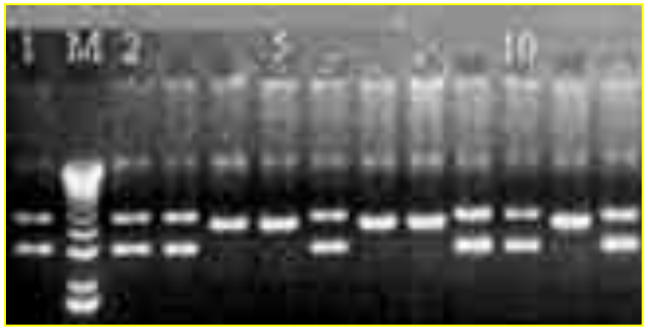
After transformation the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid.



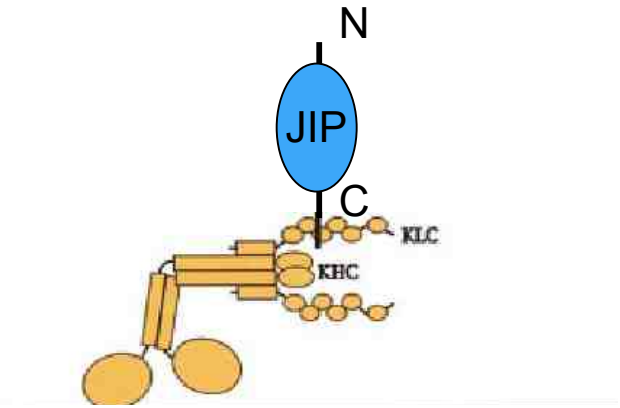
Analysis of Mutants

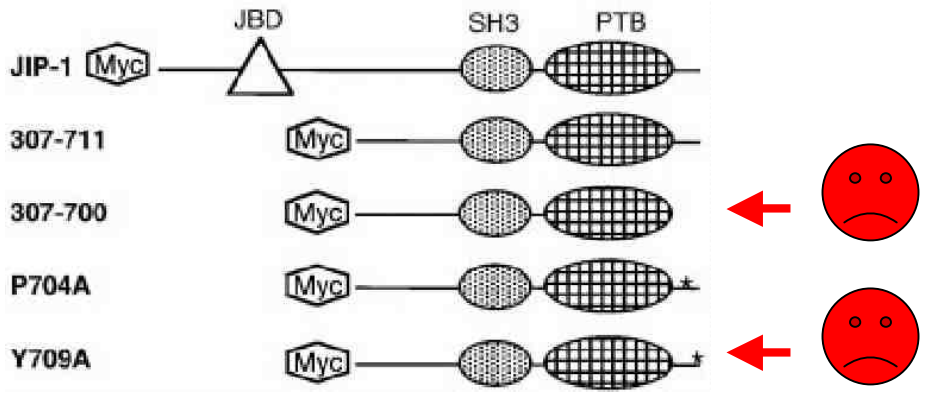


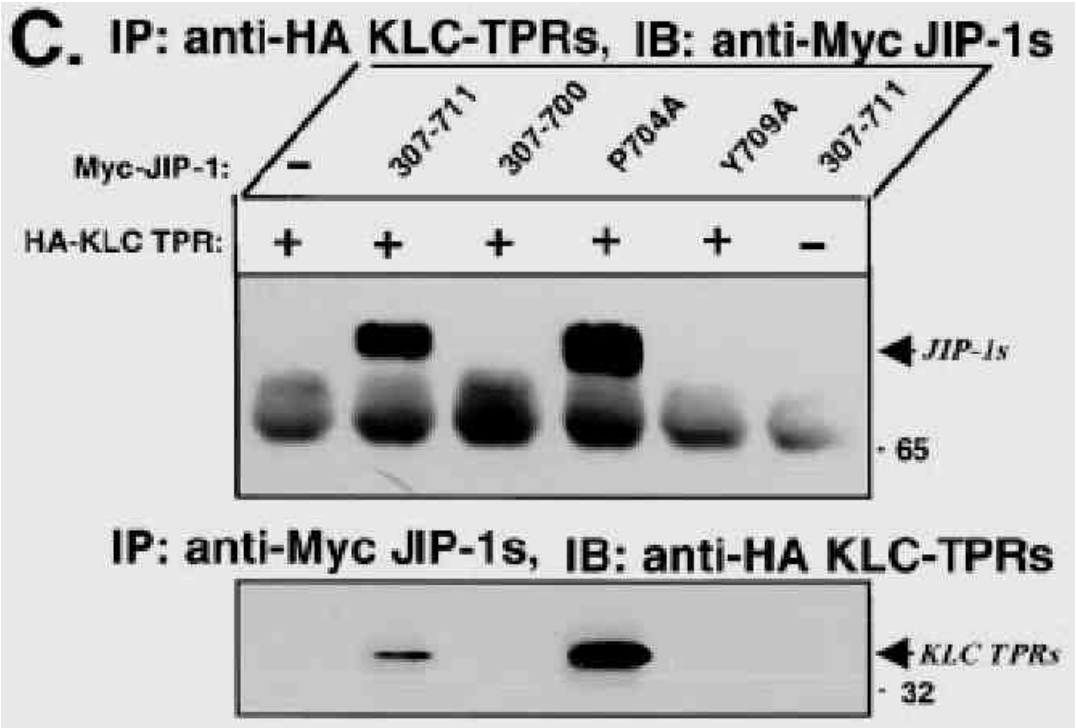
Miniprep and enzymatic digestion



JIP-1 COOH-term residues are required for binding to KLC TPR







Localization of JIP-1 is dependent on kinesin-1 interaction?

Dominant negative approach

Definition of dominant negative :

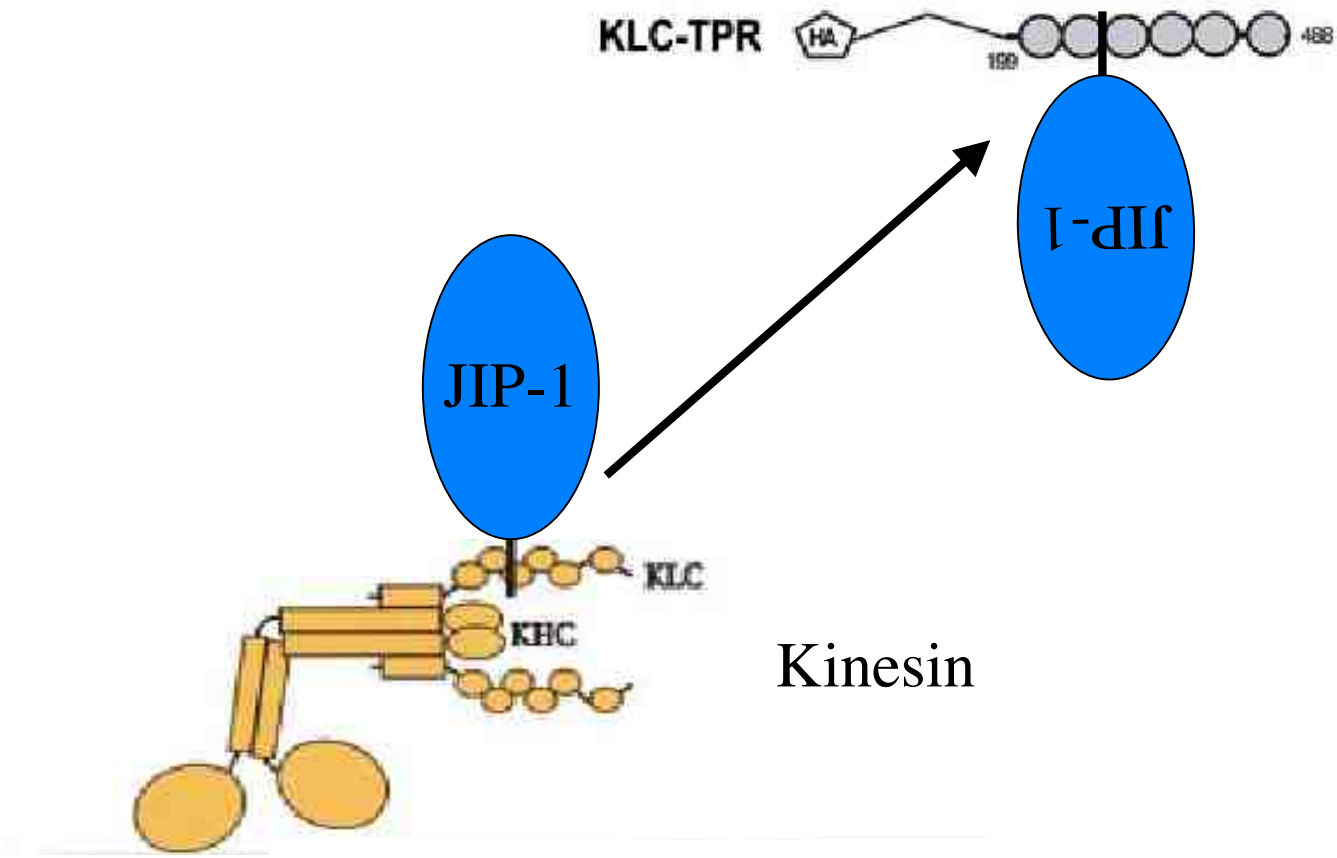
A mutation whose gene product adversely affects the normal, wild-type gene product within the same cell. This usually occurs if the product can still interact with the same elements as the wild-type product, but block some aspect of its function.

Examples:

1. A mutation in a transcription factor that removes the activation domain, but still contains the DNA binding domain. This product can then block the wild-type transcription factor from binding the DNA site leading to reduced levels of gene activation.

2. A protein that is functional as a dimer. A mutation that removes the functional domain, but retains the dimerization domain would cause a dominate negative phenotype, because some fraction of protein dimers would be missing one of the functional domains.

Dominant negative kinesin light chain: no interaction with KHC



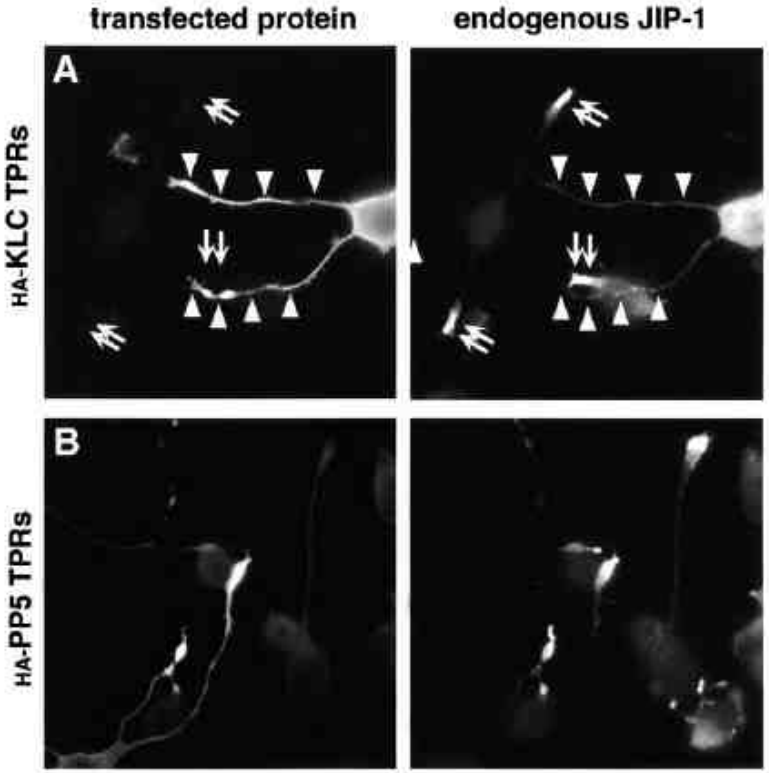


Figure 5. Expression of kinesin dominant negative constructs causes mislocalization of endogenous JIP-1 protein. CAD cells were transiently transfected with plasmids encoding the HA-tagged KLC TPRs (A), HA-tagged PP5 TPRs (B), HA-tagged KLC truncation KLC-176 (C), or Myc-tagged KHC truncation KHC-891 (D). After differentiation, the expressed proteins were detected by indirect immunofluorescence microscopy using mAbs to the epitope tags (left). Endogenous JIP-1 protein was detected with an affinity-purified polyclonal antibody (right). Note that the background fluorescence has been enhanced to show the entire neuronal cell. Arrowheads denote tips of transfected cells; arrows denote tips of untransfected cells.

Mislocalization of endogenous JIP-1

Immunofluorescenza anti-Ha
transfected protein

Immunofluorescenza anti-JIP-1
endogenous JIP-1

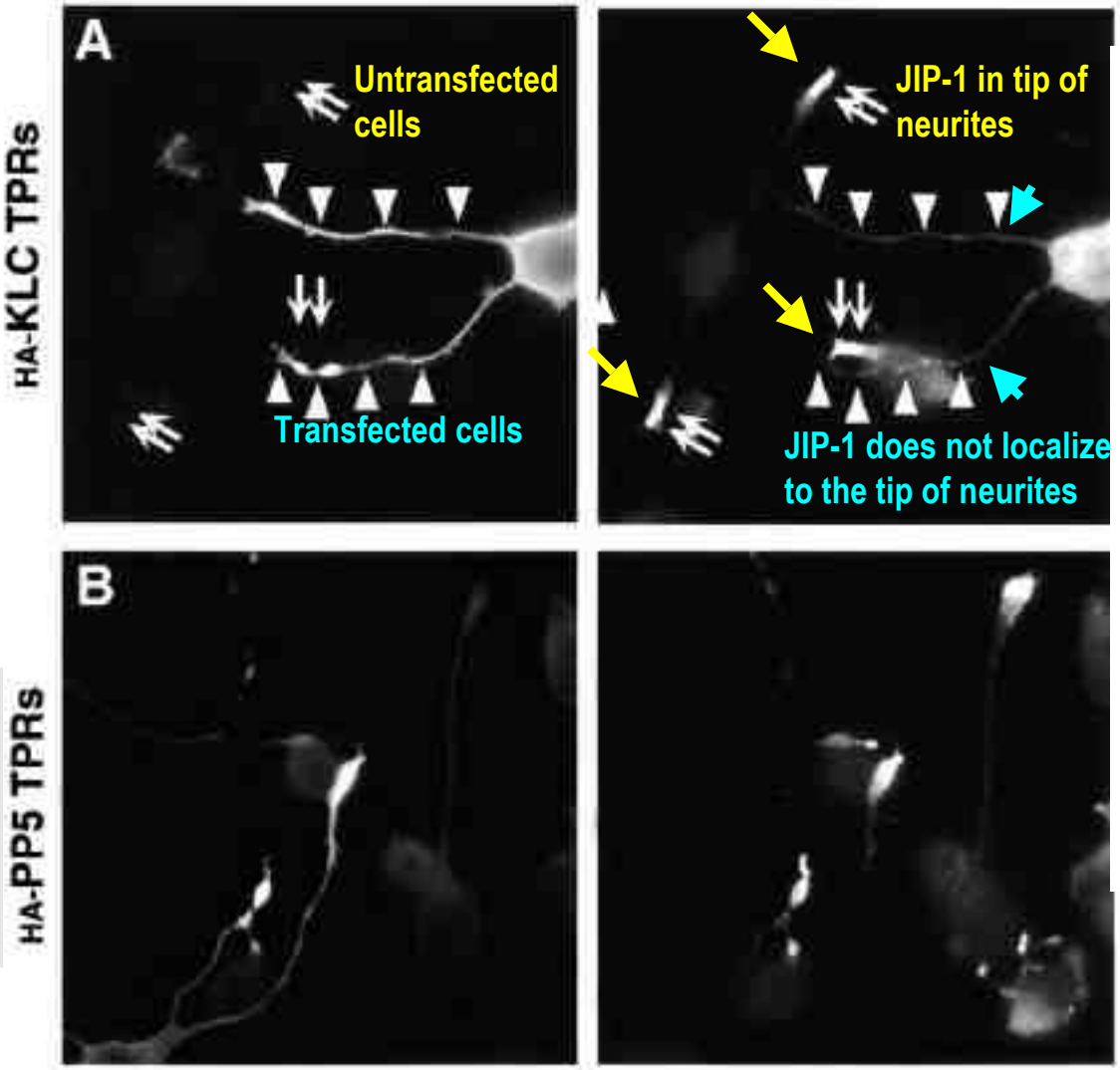
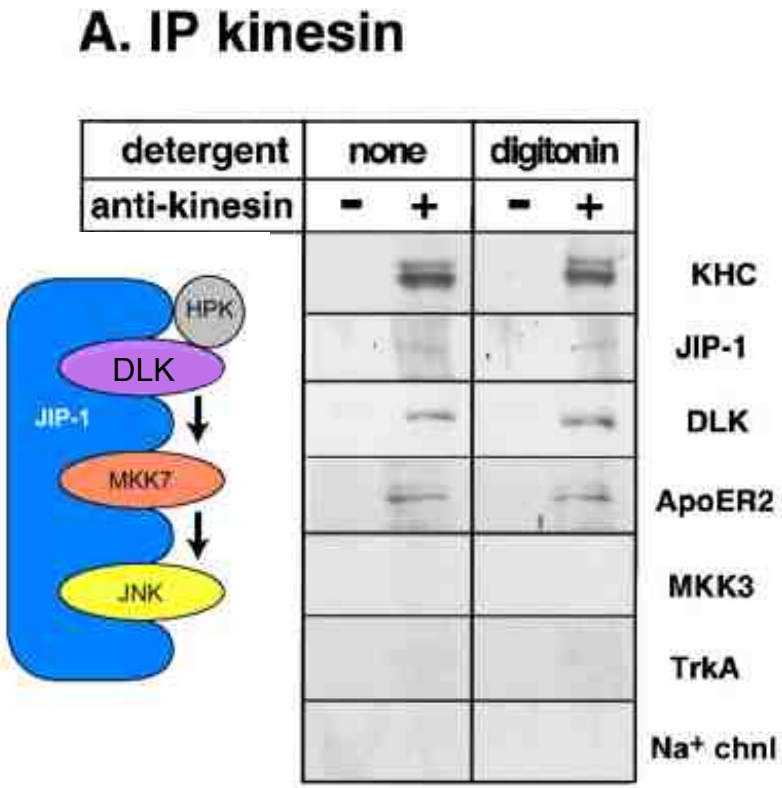


Figure 5. Expression of kinesin dominant negative constructs causes mislocalization of endogenous JIP-1 protein. CAD cells were transiently transfected with plasmids encoding the HA-tagged KLC TPRs (A), HA-tagged PP5 TPRs (B), HA-tagged KLC truncation KLC-176 (C), or Myc-tagged KHC truncation KHC-891 (D). After differentiation, the expressed proteins were detected by indirect immunofluorescence microscopy using mAbs to the epitope tags (left). Endogenous JIP-1 protein was detected with an affinity-purified polyclonal antibody (right). Note that the background fluorescence has been enhanced to show the entire neuronal cell. Arrowheads denote tips of transfected cells; arrows denote tips of untransfected cells.

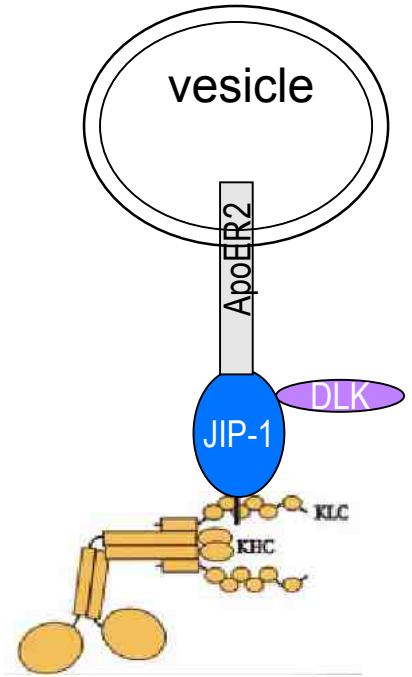
Negative control *TPR of PP5 does not recognize JIP-1*

Kinesin associates with proteins assembled on the JIP scaffold and with ApoER2 (transmembrane receptor for Reelin), thus with vesicles.



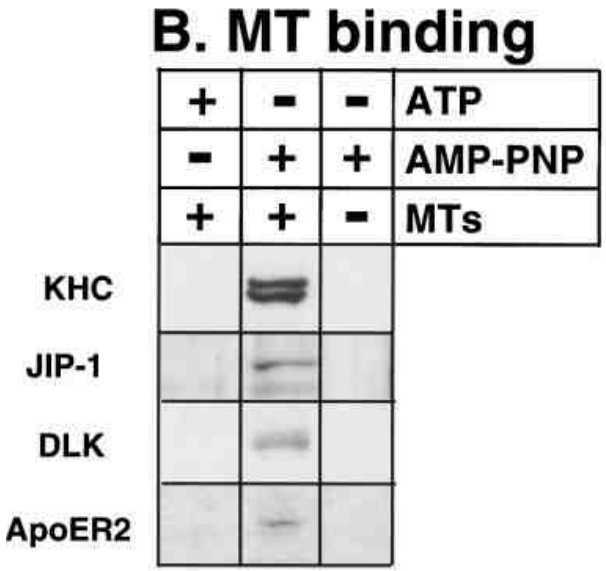
High speed supernatant fraction from rat brain used for IP with mAb to KHC

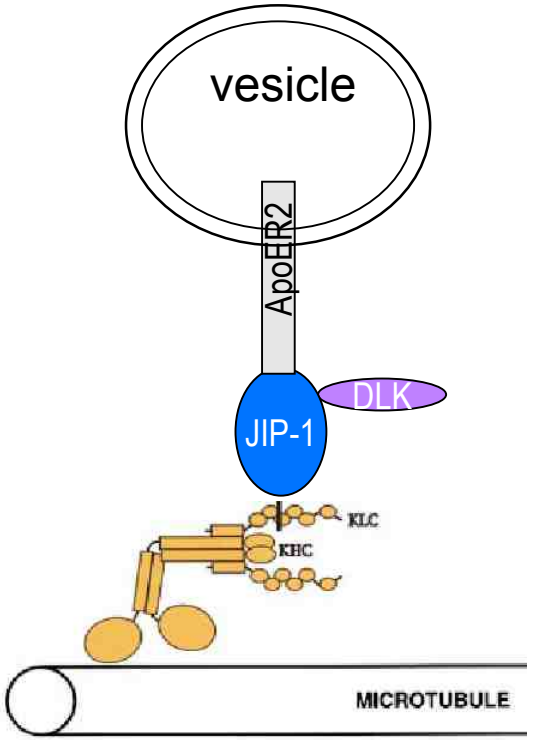
JIP1 is a linker between kinesin-1 and certain vesicle membrane proteins, such as Alzheimer's APP protein and a reelin receptor ApoER2



Kinesin associates with proteins assembled on the JIP scaffold and with microtubules

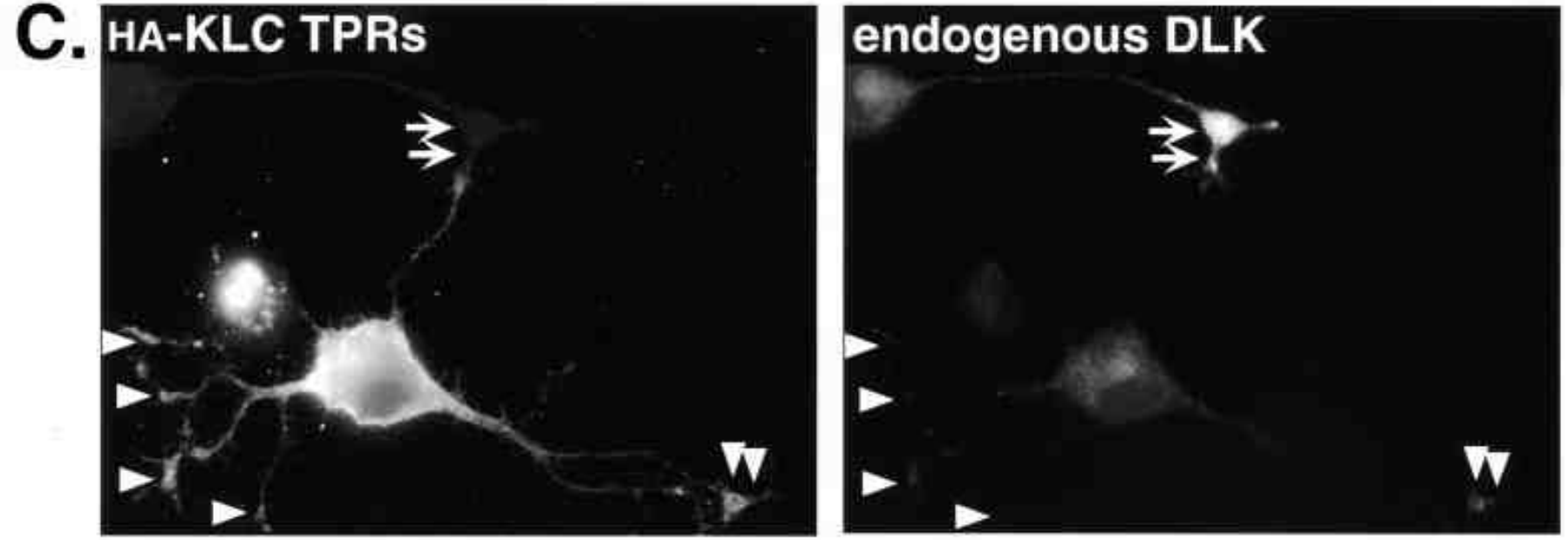
Microtubule binding assay





(B) Rat brain high speed supernatant was subjected to an MT binding assay in the presence of Triton X-100 by adding ATP, AMPPNP (non hydrolysable ATP), and/or MTs as indicated. MTs and bound proteins were sedimented through a sucrose cushion, and the presence of the indicated proteins in the MT pellets was detected by immunoblotting.

…..we looked for an effect of the dominant negative HA-tagged KLC TPRs on the localization of DLK (the antibodies to ApoER2 were not suitable for these experiments)…..



CAD cells were transiently transfected with a plasmid encoding the HA-tagged KLC TPRs. After differentiation, the expressed protein was detected by indirect immunofluorescence microscopy using a mAb to the HA tag (left). Endogenous DLK kinase was detected with a polyclonal antibody (right). Arrowheads denote tips of transfected cells; arrows denote the tip of an untransfected cell.