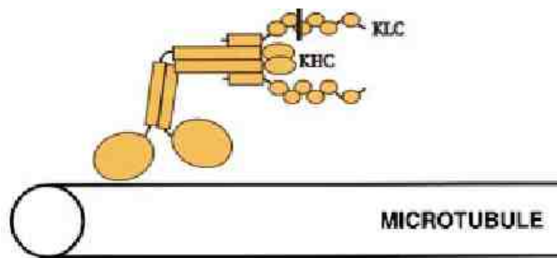
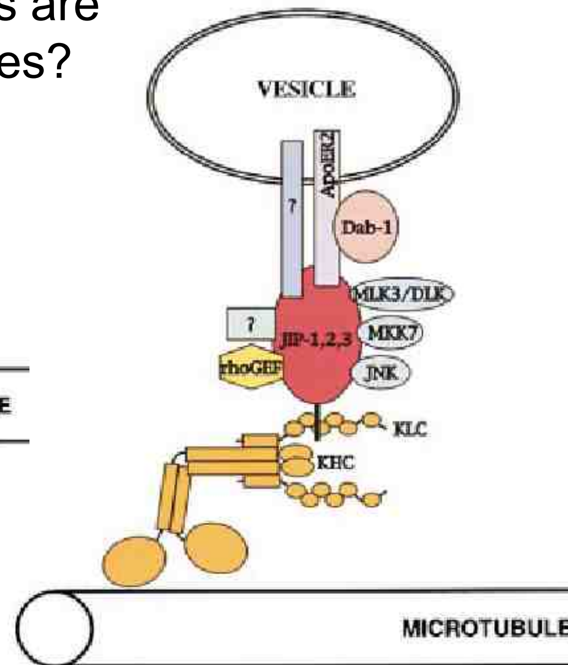
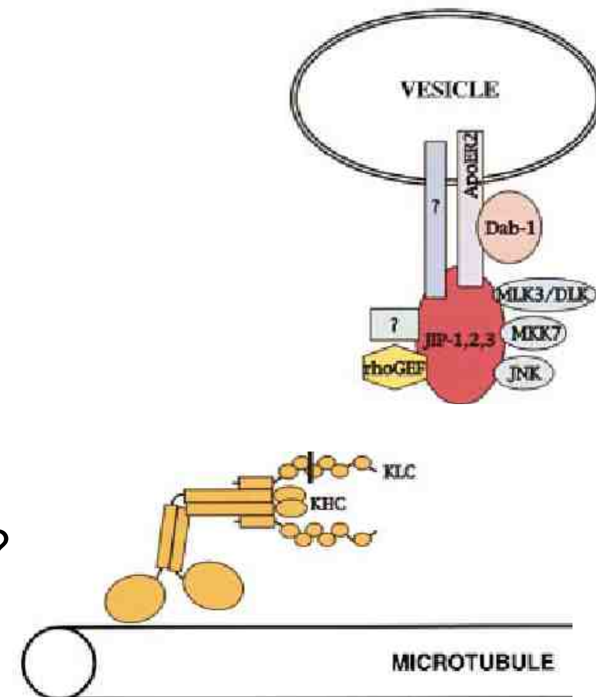
Three more questions:

Without cargoes, kinesins are running along microtubules?



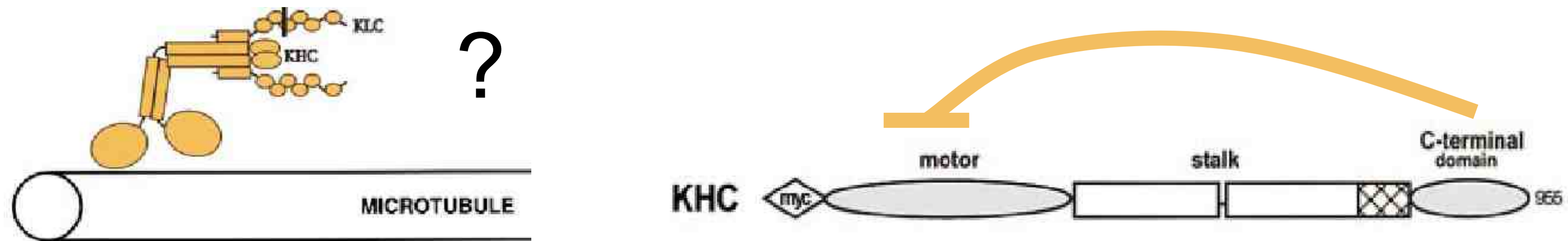


Co-operative or competitive transport of different cargoes?



Signal for cargoes to detach?

1st question: Without cargoes, kinesins are running along microtubules?



Two binding partners cooperate to activate the molecular motor Kinesin-1

T. Lynne Blasius,[1] Dawen Cai,[1,2] Gloria T. Jih,[1] Christopher P. Toret,[3] and Kristen J. Verhey[1]

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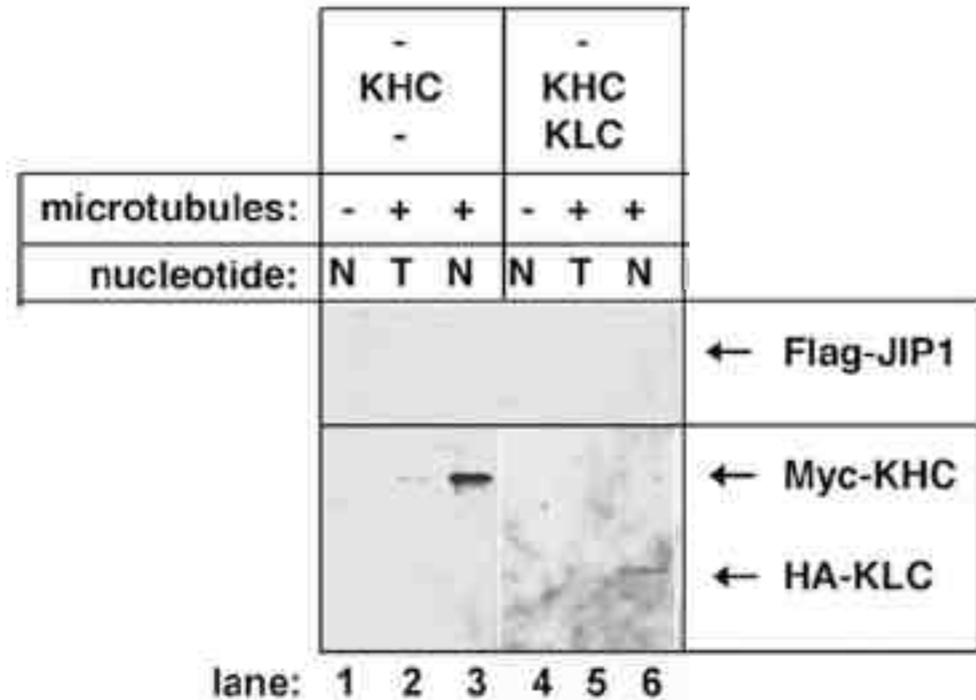
[3]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720

The Journal of Cell Biology, Vol. 176, No. 1, January 1, 2007 11–17

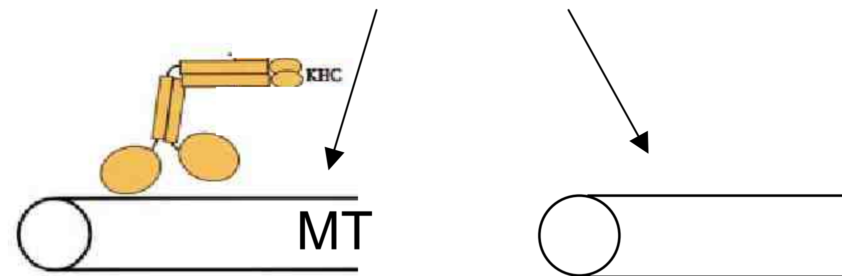
Myc-KHC expressed alone is not autoinhibited Coexpression of myc-KHC + HA-KLC recreates the autoinhibited Kinesin-1 holoenzyme

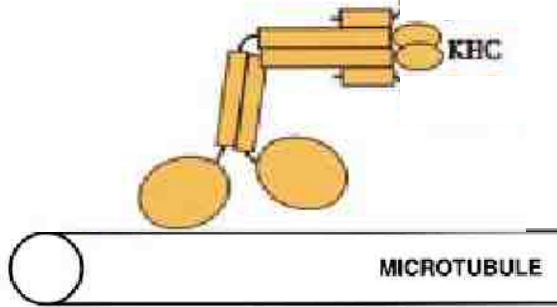
Microbubule binding

- Prepolymerized taxol-stabilized MTs were added (+) or not added (–) to the indicated lysates with either ATP (T) or AMPPNP (N), a nonhydrolyzable analogue of ATP
- After sedimentation, the MT pellets were immunoblotted with antibodies to the Flag (top) or myc and HA tags (bottom).

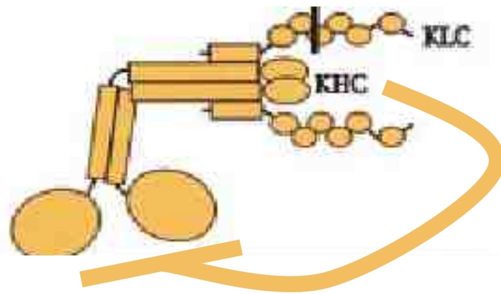


Myc-KHC expressed alone is not autoinhibited and can be cosedimented with MTs





Myc-KHC expressed alone is not autoinhibited and can be cosedimented with MTs



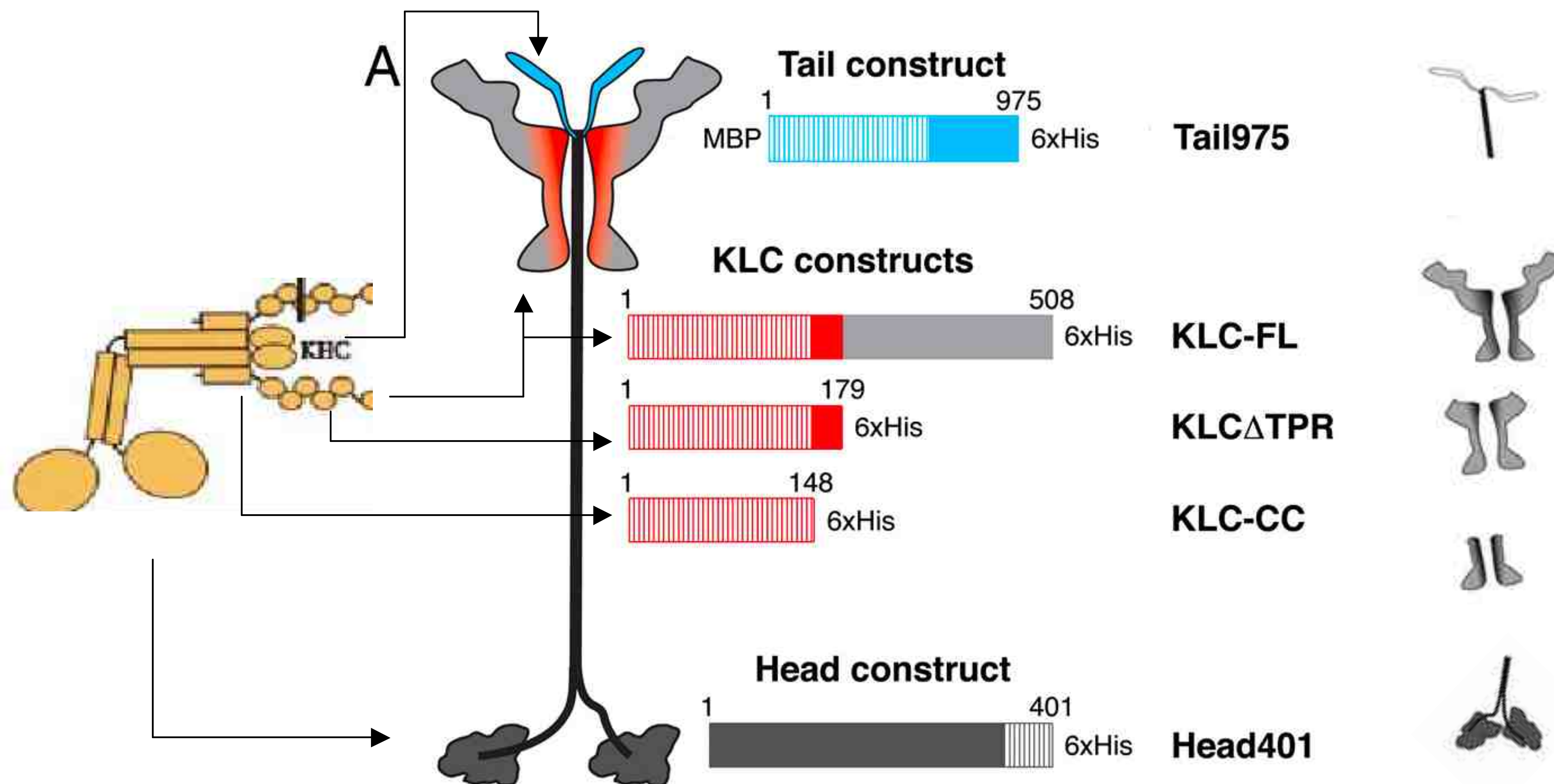
In the absence of cargo, Kinesin-1 is inactive as a result of a folded conformation that enables autoinhibition of the N-terminal motor domain by C-terminal tail domains.

PNAS Early Edition

Kinesin's light chains inhibit the head- and microtubule-binding activity of its tail

Yao Liang Wong and Sarah E. Rice[1]

www.pnas.org/cgi/doi/10.1073/pnas.1005854107



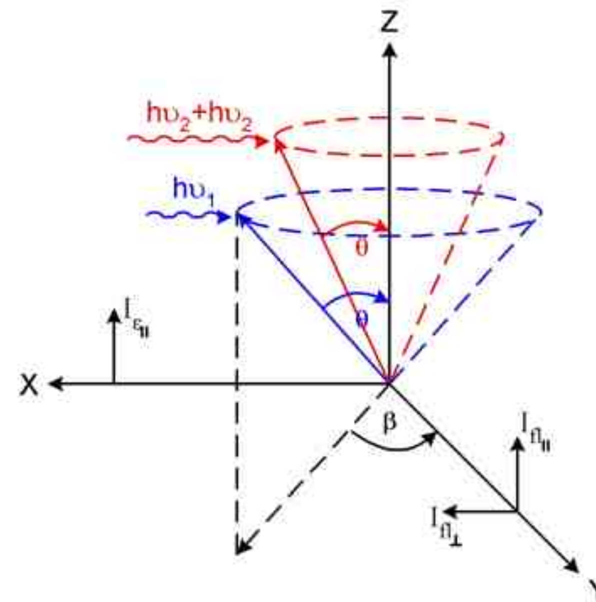
All constructs were C-terminally 6xHis-tagged

……KLCs have been previously identified as regulators of kinesin-1 activity (17, 18). These authors found that MT binding by KHC/KLC complexes in cell extracts and in live cells was significantly weaker than by KHCs alone. They proposed that KLCs might enhance inhibition of the kinesin-1 head by the tail.

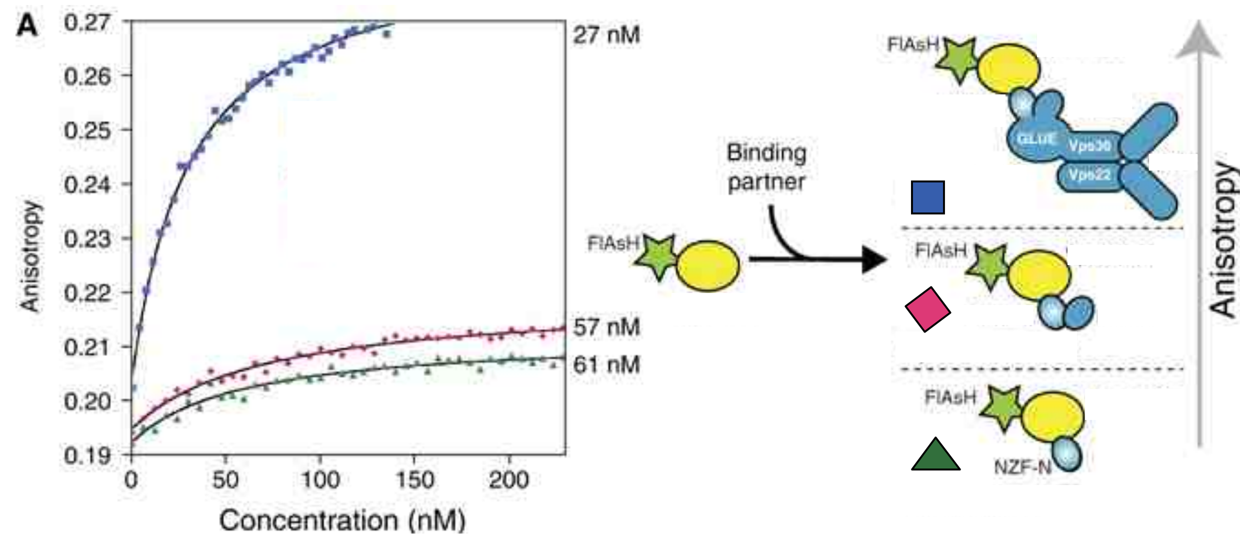
17. Verhey KJ, et al. (1998) Light chain-dependent regulation of Kinesin's interaction with microtubules. *J Cell Biol* 143:1053–1066.
18. Cai D, Hoppe AD, Swanson JA, Verhey KJ (2007) Kinesin-1 structural organization and conformational changes revealed by FRET stoichiometry in live cells. *J Cell Biol* 176:51–63.

We examined this possibility with purified components in vitro, by using fluorescence anisotropy to directly measure the effect of the KLCs on head-tail binding affinity.

Fluorescence anisotropy can be used for measuring the binding interaction between two molecules, to determine the binding constant (or the inverse, the disassociation constant) for the interaction.
The basic idea is that a fluorophore excited by polarized light will also emit polarized light. However, if a molecule is moving, it will tend to "scramble" the polarization of the light by radiating at a different direction from the incident light. The "scrambling" effect is greatest with fluorophores freely tumbling in solution and decreases with decreased rates of tumbling.



Protein interactions can be detected when one of the interacting partners is fused to a fluorophore: upon binding of the partner molecule a larger, more stable complex is formed which will tumble more slowly (thus, increasing the polarization of the emitted light and reducing the "scrambling" effect). This technique works best if a small molecule is fused to a fluorophore and binds to a larger partner (this maximizes the difference in signal between bound and unbound states).



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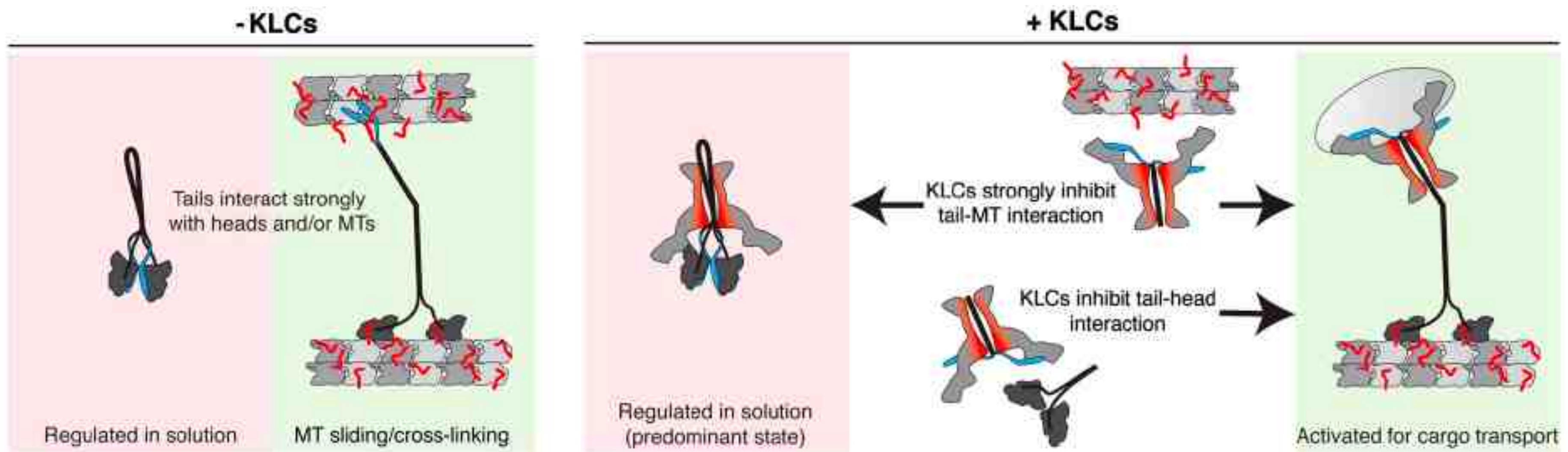
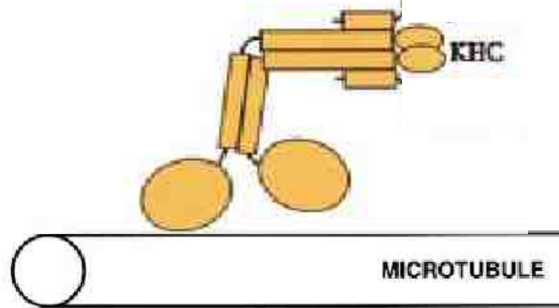
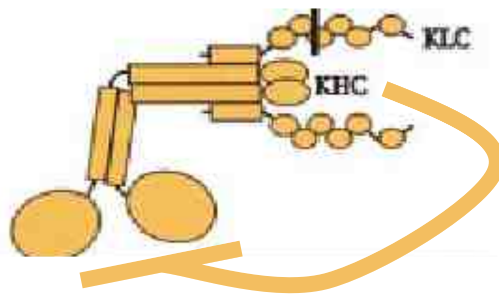


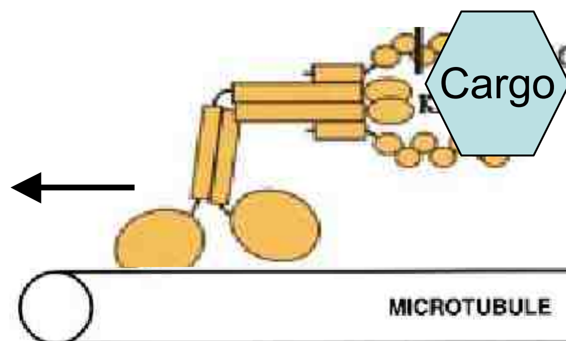
A model for KLC-mediated regulation of the kinesin-1 tail. Kinesin-1 is colored as in Fig. 1. Without KLCs (Left), kinesin-1 would be either regulated in solution or bound to MTs with the tail tethered. Due to the high affinity of tails for heads/MTs, the motor cannot access its cargo transport-competent state. In the presence of KLCs (Right), tail-head and tail-MT interactions are inhibited. Strong inhibition of tail-MT binding means that the regulated conformation of kinesin-1 becomes the predominant form, but tail-head affinity is also reduced such that the motor is in a poised state that can be easily activated for cargo transport.



Myc-KHC expressed alone is not autoinhibited and can be cosedimented with MTs



In the absence of cargo, Kinesin-1 is inactive as a result of a folded conformation that enables autoinhibition of the N-terminal motor domain by C-terminal tail domains.



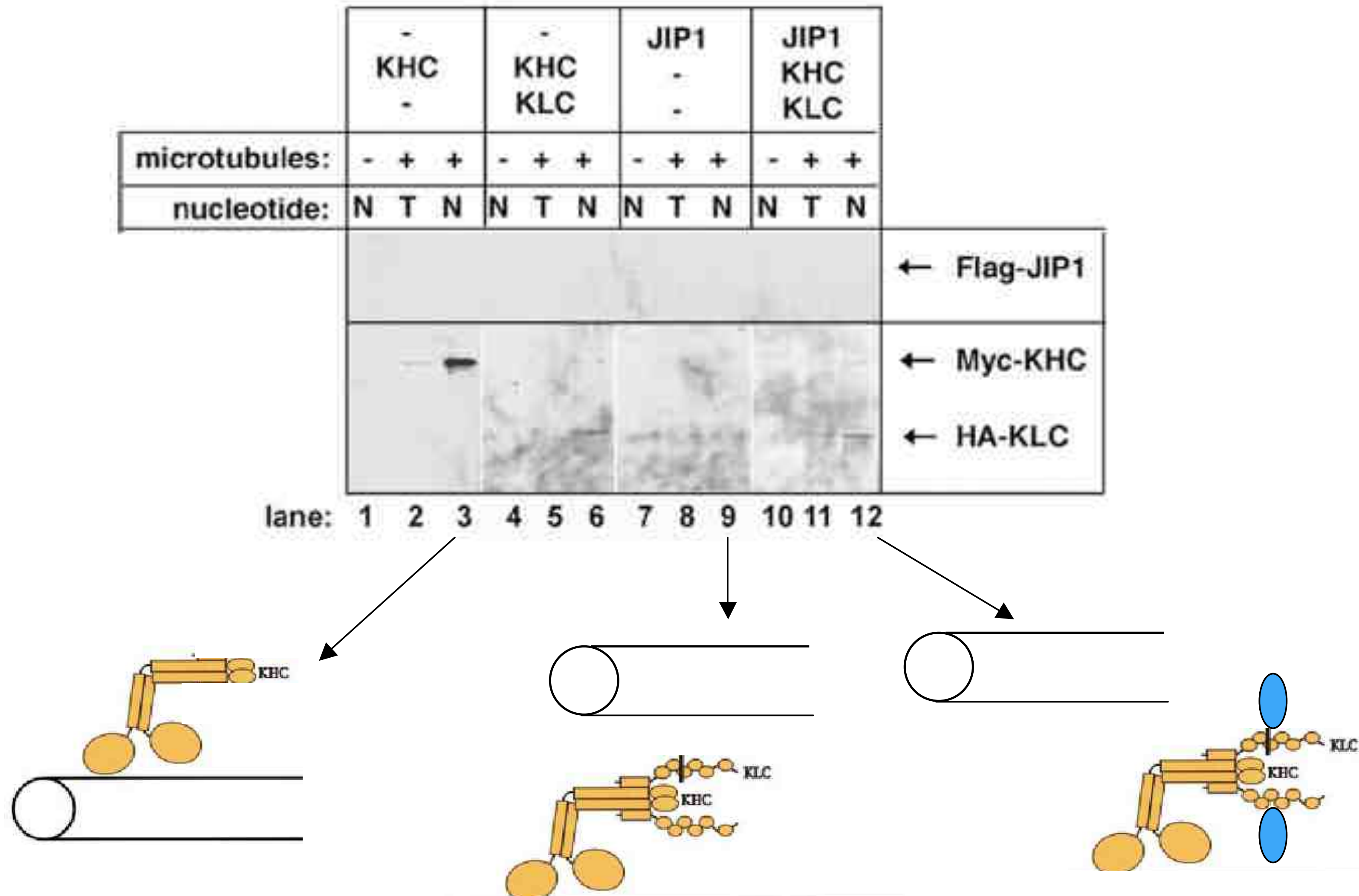
?

Hypothesis:

Cargo binding to the Kinesin-1 tail frees the motor domains for ATP-driven motility?

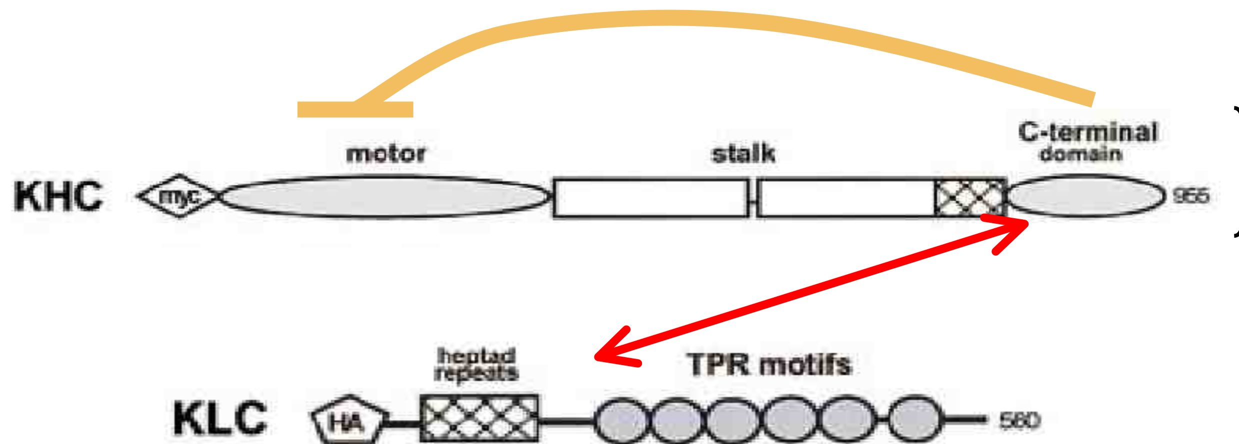
Cargo binding is not be sufficient to activate Kinesin-1, and subsequent events are required?

Binding of the JIP1 cargo protein is not sufficient to activate Kinesin-1



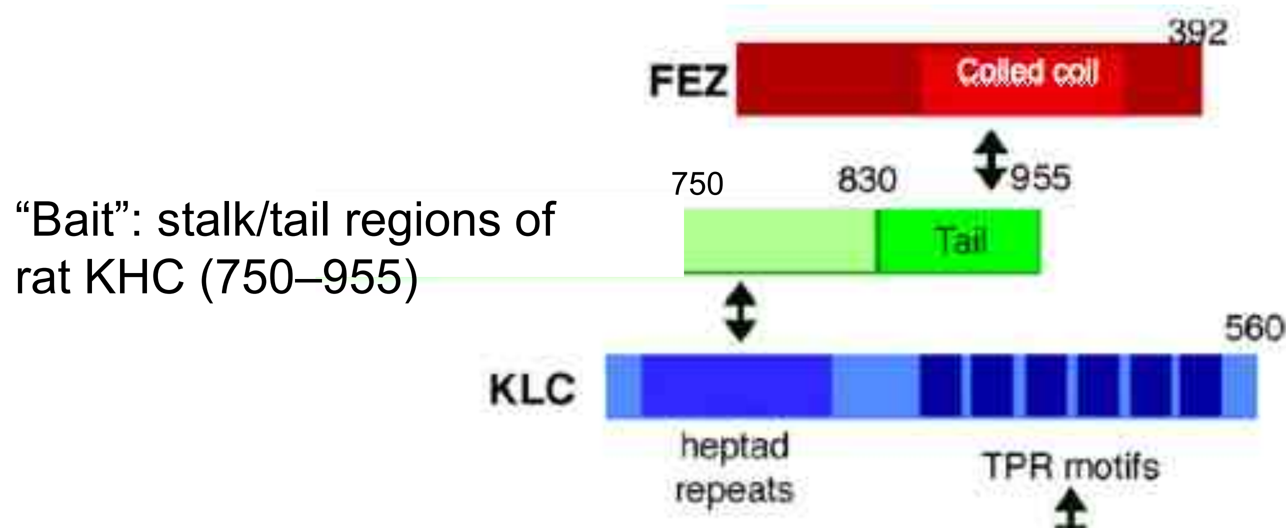
These results suggest that an additional event is required to activate Kinesin-1.

As the complete autoinhibition of Kinesin-1 requires both the KHC inhibitory tail and the KLC subunit, the hypothesis is that the autoinhibitory effects of both of these regions must be relieved for activation.



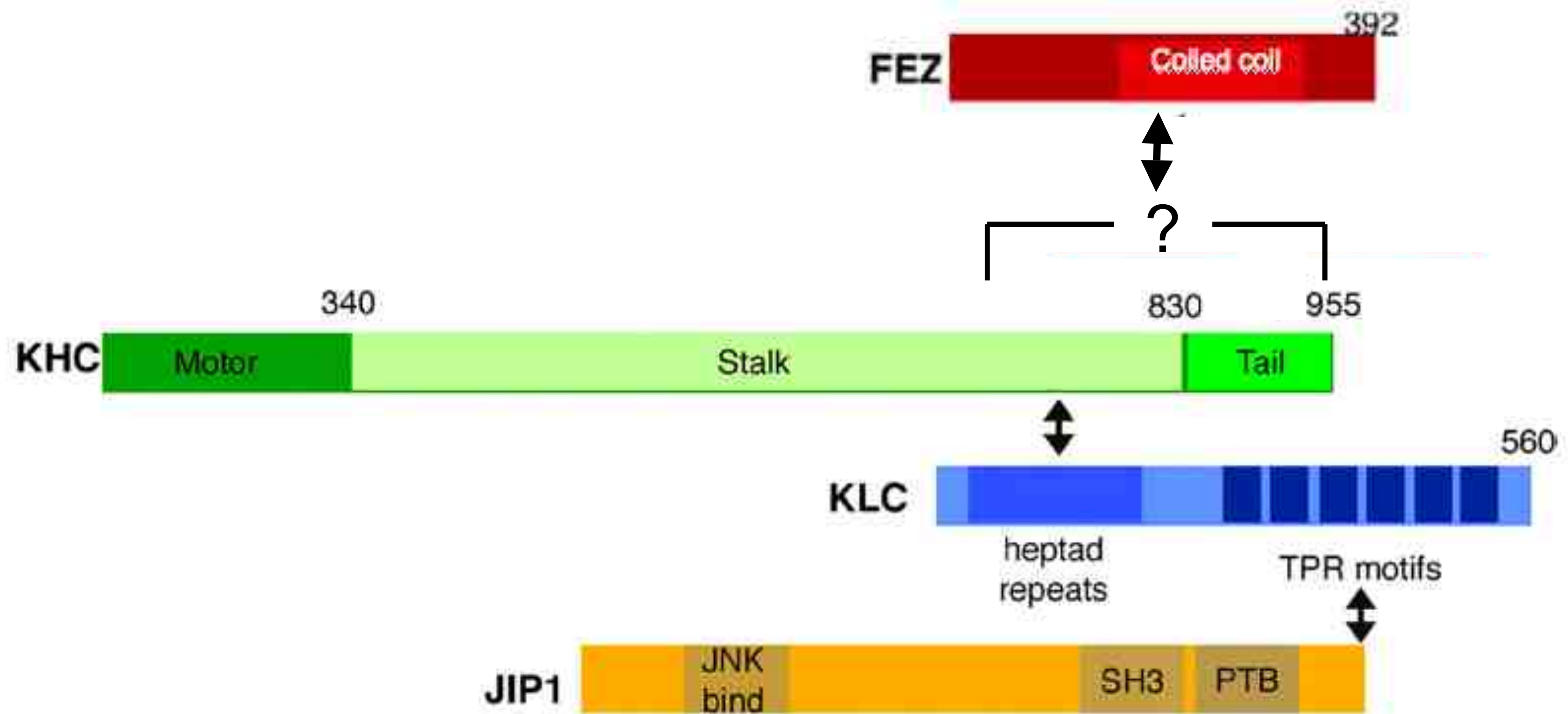
To identify potential cargoes and/or regulators of the KHC tail, **a two-hybrid screen** of a human brain library using the stalk/tail regions of rat KHC (750–955) as a bait, has been performed.

Two-hybrid screen
Plasmid pGBKT7-KHC(750–955) was expressed in yeast strain AH109. A Matchmaker pretransformed human fetal brain library (CLONTECH Laboratories, Inc.) in strain Y187 was screened by yeast mating. 46 of the positive clones contained sequences encoding KLC as expected. 22 clones containing fragments of FEZ1 and five clones containing fragments of FEZ2 were isolated from 7.5×10^6



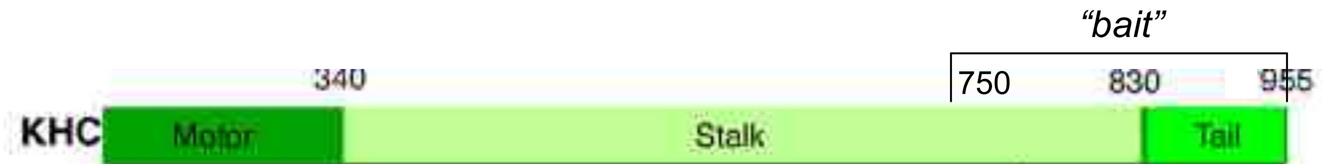
FEZ: Fasciculation and elongation protein

Which part of KHC (stalk-tail) is involved in FEZ binding?



Sequence analysis of KHC tail domains: sequence alignment

“bait”



Hs: Homo sapiens
Dm: *Drosophila*
Ce: Caenorhabditis elegans
N.c: Gardenia plant

***
conserved coiled tail;

```
Hs Tail  799  HNLRKLFVQDLTTRVKKSV.ELDND............DGGGSAAQKQKISFLENNLEQLTKVHKQLVRDN
Dm Tail  818  HNLRKLFVQDLQQRIRKNVVNEESE............EDGGSLAQKQKISFLENNLDQLTKVHKQLVRDN
Ce Tail  666  KNLKKEFMRVLVARCQANQ.DTEGE............DSLSGPAQKQRIQFLENNLDKLTKVHKQLVRDN
Nc Tail  739  DVMKKSLMRDLQNRCERVV.ELEISLDETRDEYNNVLRSSNNRAQQKKMAFLERNLEQLTQVQRQLVEQN
```

implicated in cargo binding

globular tail

```
Hs Tail  855  ADLRCELPKLEKRLRATAERVKALESALKEAKENAMRDRKRYQQEVDRIKEAVRAKNMARR.........
Dm Tail  876  ADLRCELPKLEKRLRCTMERVKALETALKEAKEGAMRDRKRYQYEVDRIKEAVRQKHLGRR.........
Ce Tail  722  ADLRVELPKMEARLRGREDRIKILETALRDSKQRSQAERKKYQQEVERIKEAVRQRNM.RR.........
Nc Tail  808  SALKKEVAIAERKLMARNERIQSLESLLQESQEKMAQANHKFQVQLAAVKDRLEAAKAGSTRGLGTDAGL
```

folding site

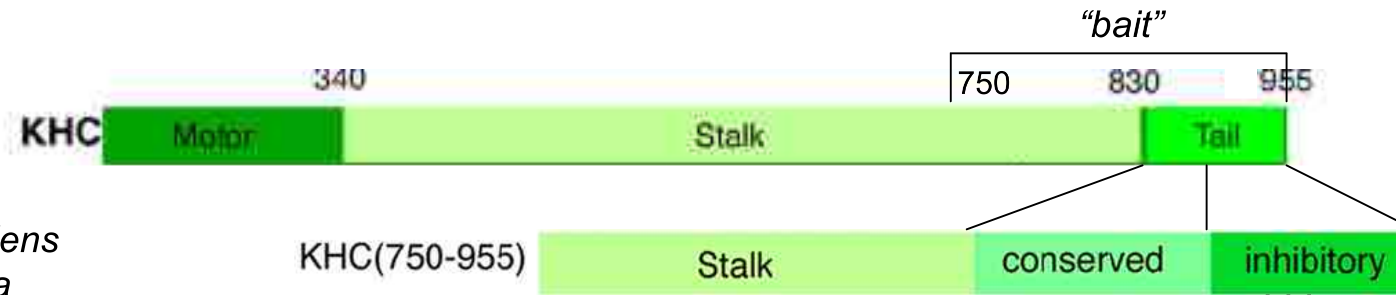
implicated in autoinhibition

```
Hs Tail  917  ...AHSAQIAKPIR....PGHYPASSPTAVHAIRGGGGSSSNSTHYQK.....
Dm Tail  937  ...GPQAQIAKPIR....SGQ.......GAIAIRGGGAVGGPSPLAQVNPVNS
Ce Tail  783  ...MNAPQIVKPIR....PGQV.YTSPSAGM...SQGAPNGSNA.........
Nc Tail  878  GGFSIGSRIAKPLRWRDAVAGATATNPTIATLQQNPPENKRSSWFFQKS....
```

****

IAK: inhibition of its ATPase activity and weak net affinity for microtubules in the presence of ATP

Sequence analysis of KHC tail domains: sequence alignment



Hs: Homo sapiens
Dm: *Drosophila*
Ce: Caenorhabditis elegans
N.c: Neurospora crassa (gardenia plant)

conserved coiled tail;

```
Hs Tail   799   HNLRKLFVQDLTTRVKKSV.ELDND............DGGGSAAQKQKISFLENNLEQLTKVHKQLVRDN
Dm Tail   818   HNLRKLFVQDLQQRIRKNVVNEESE............EDGGSLAQKQKISFLENNLDQLTKVHKQLVRDN
Ce Tail   666   KNLKKEFMRVLVARCQANQ.DTEGE............DSLSGPAQKQRIQFLENNLDKLTKVHKQLVRDN
Nc Tail   739   DVMKKSLMRDLQNRCERVV.ELEISLDETRDEYNNVLRSSNNRAQQKKMAFLERNLEQLTQVQRQLVEQN
```

implicated in cargo binding

globular tail

```
Hs Tail   855   ADLRCELPKLEKRLRATAERVKALESALKEAKENAMRDRKRYQQEVDRIKEAVRAKNMARR.........
Dm Tail   876   ADLRCELPKLEKRLRCTMERVKALETALKEAKEGAMRDRKRYQYEVDRIKEAVRQKHLGRR.........
Ce Tail   722   ADLRVELPKMEARLRGREDRIKILETALRDSKQRSQAERKKYQQEVERIKEAVRQRNM.RR.........
Nc Tail   808   SALKKEVAIAERKLMARNERIQSLESLLQESQEKMAQANHKFQVQLAAVKDRLEAAKAGSTRGLGTDAGL
```

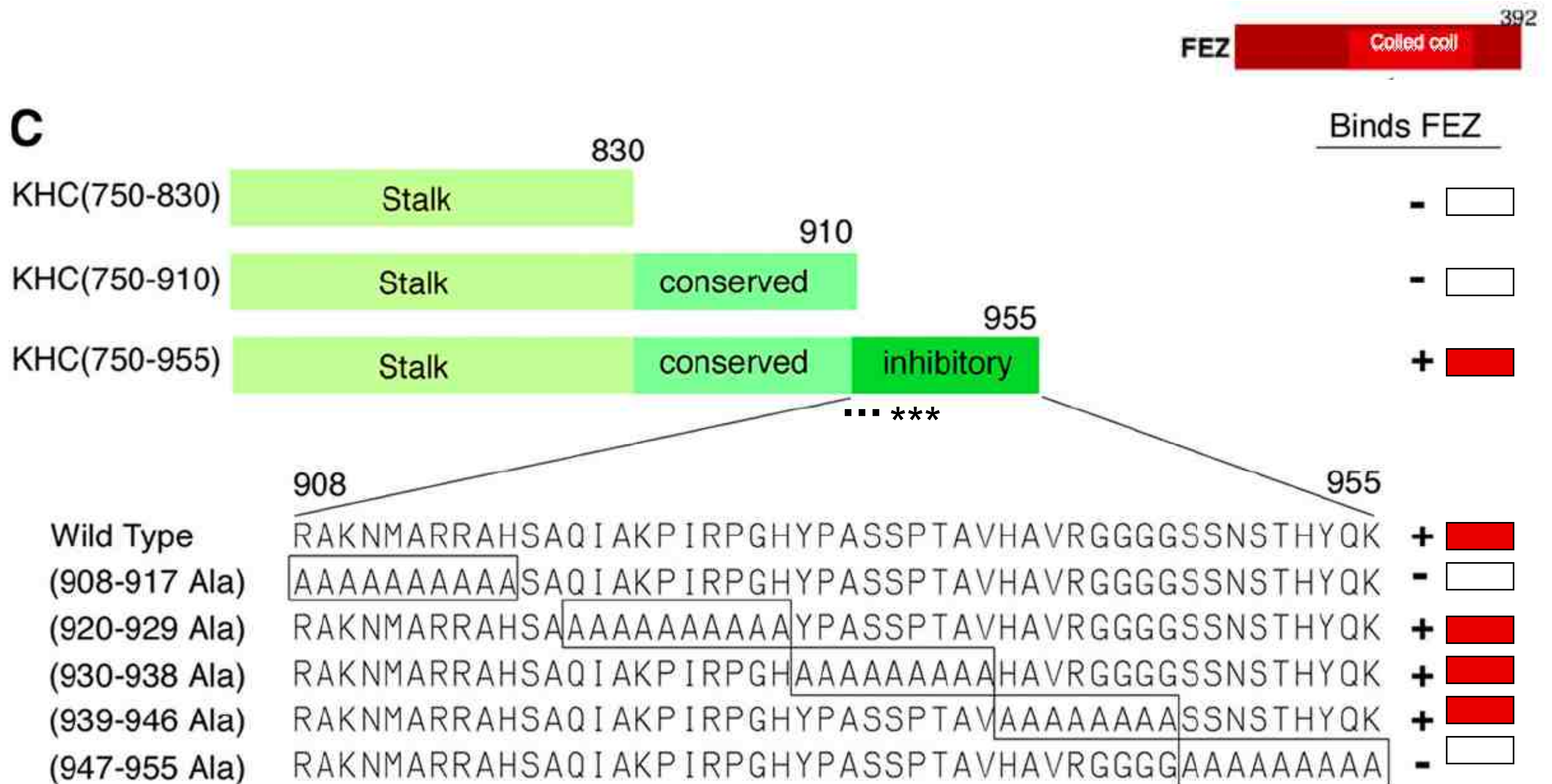
folding site

implicated in autoinhibition

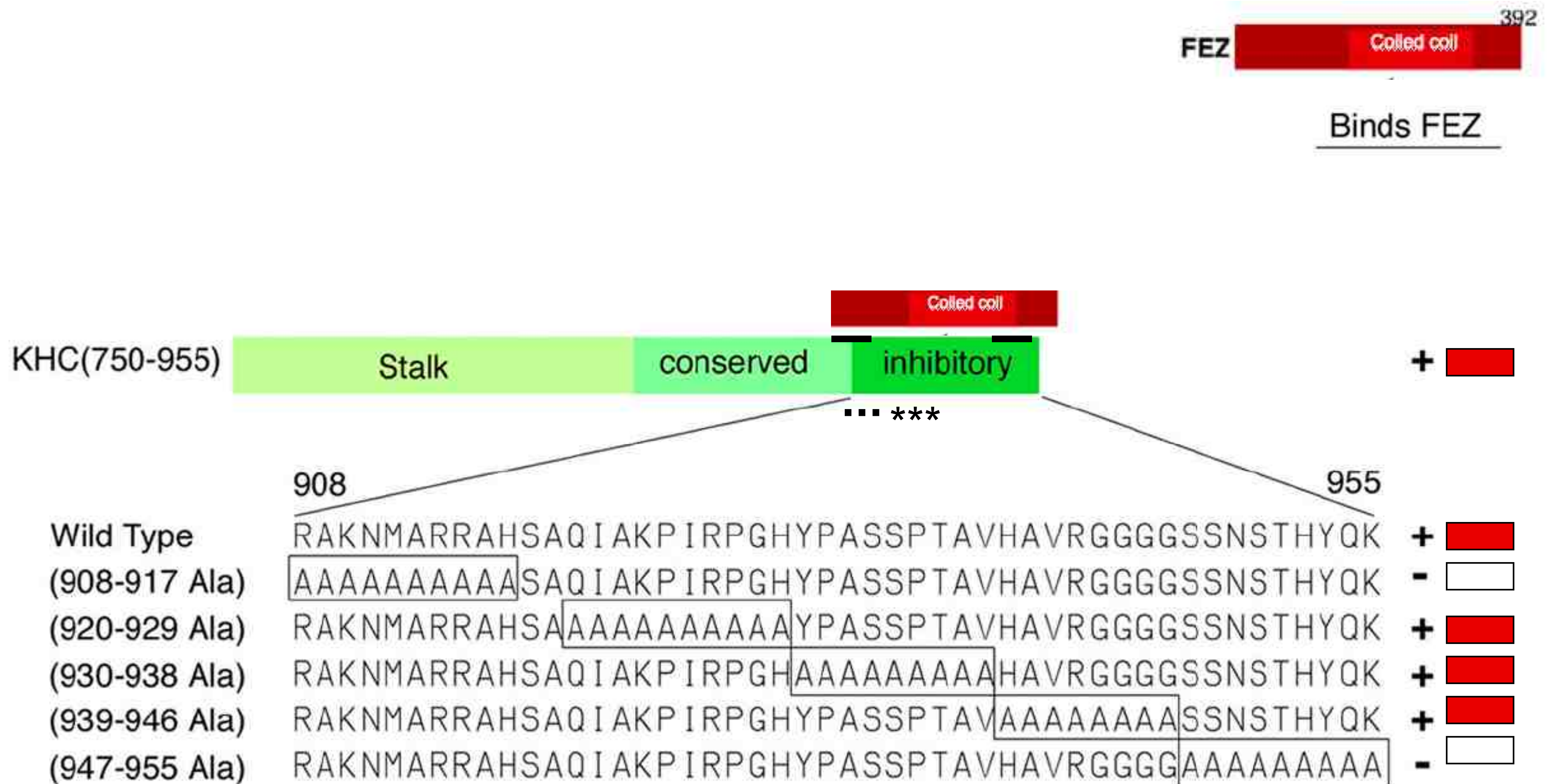
```
Hs Tail   917   ...AHSAQIAKPIR....PGHYPASSPTAVHAIRGGGGSSSNSTHYQK.....
Dm Tail   937   ...GPQAQIAKPIR....SGQ........GAIAIRGGAVGGPSPLAQVNPVNS
Ce Tail   783   ...MNAPQIVKPIR....PGQV.YTSPSAGM...SQGAPNGSNA.........
Nc Tail   878   GGFSIGSRIAKPLRWRDAVAGATATNPTIATLQQNPPENKRSSWFFQKS....
```

IAK: inhibition of its ATPase activity and weak net affinity for microtubules in the presence of ATP

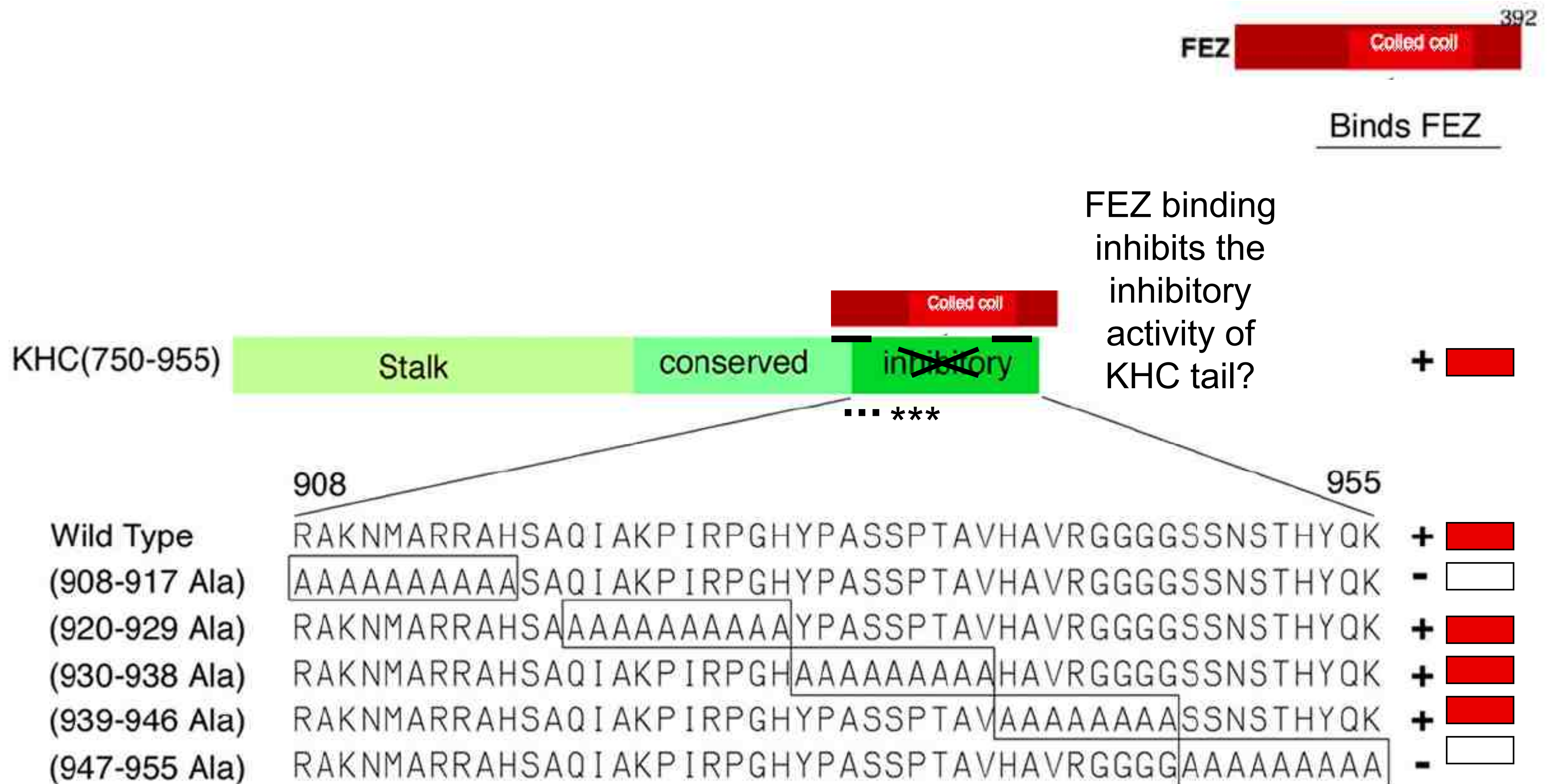
Directed two- hybrid assay: Truncation of the KHC inhibitory tail or mutation of the folding site in the inhibitory tail abolished the interaction with FEZ1



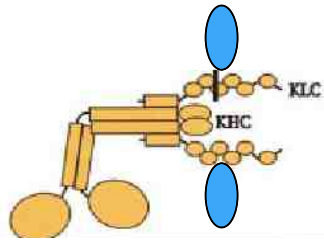
FEZ binds to the inhibitory region of KHC ---> FEZ1 is not likely to be strictly a cargo of Kinesin-1.



FEZ1 binding to the KHC folding site could play a critical role in Kinesin-1 activation, perhaps by relieving the folded conformation?



FEZ1 and JIP1 cooperate to activate Kinesin-1 in vitro: binding to microtubules



(From fig.1)

C microtubule binding

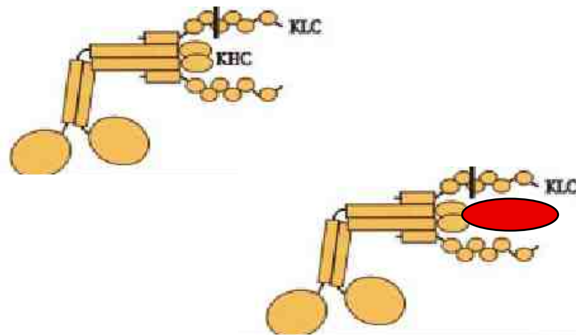
lysates added:									
Flag-JIP1	-			-			+		
FEZ1-Hsv	-			+			+		
Myc-KHC + HA-KLC	+			+			+		
microtubules:	-	+	+	-	+	+	-	+	+
nucleotide:	N	T	N	N	T	N	N	T	N

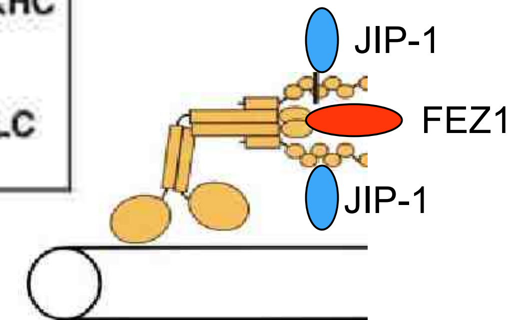
← Flag-JIP1

← FEZ1-Hsv

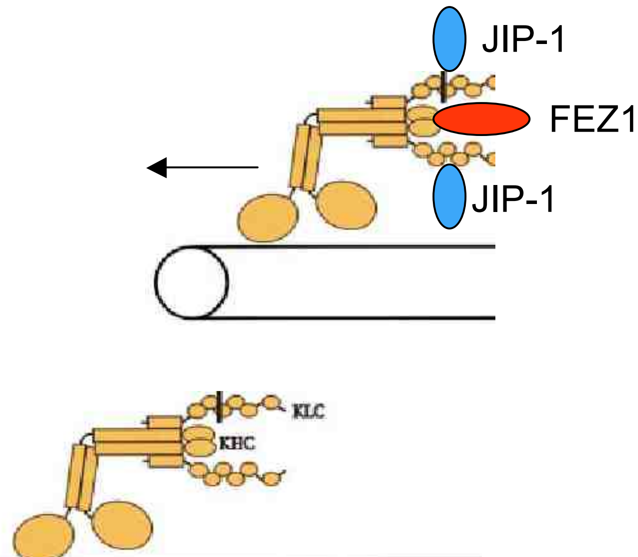
← Myc-KHC

← HA-KLC





FEZ1 and JIP1 cooperate to activate Kinesin-1 in vivo: mobility on MT.



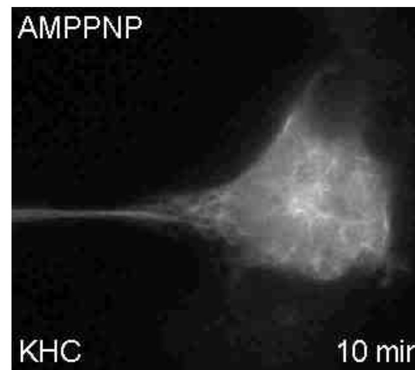
(D) Myc-KHC + 3xmCit-KLC lysates were mixed with lysates of mock-transfected cells (left) or cells expressing Flag-JIP1 and FEZ1-hsv (right). Representative motile events along Cy5-labeled MTs are shown in the kymographs (13 frames; 100-ms intervals). Bar, 1.0 µm.

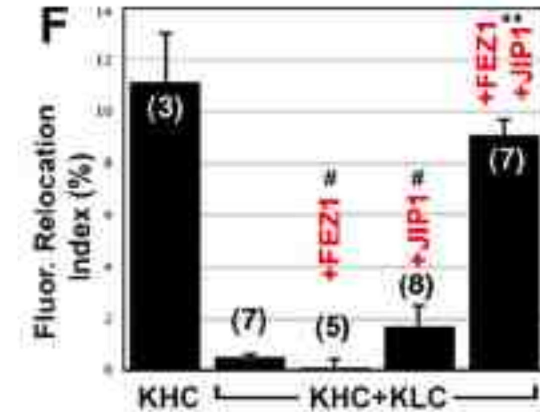
D single mol. motility

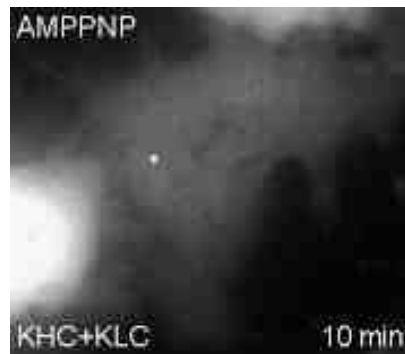
lysates added:	Myc-KHC + 3xmCit-KLC and mock transfected	Myc-KHC + 3xmCit-KLC and Flag-JIP1 + FEZ1-Hsv
time		
# of motility events observved:	2	47

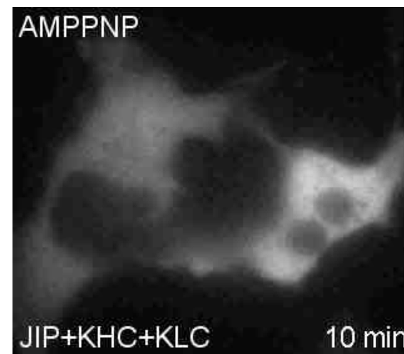
FEZ1 and JIP1 cooperate to activate Kinesin-1 in live cells.

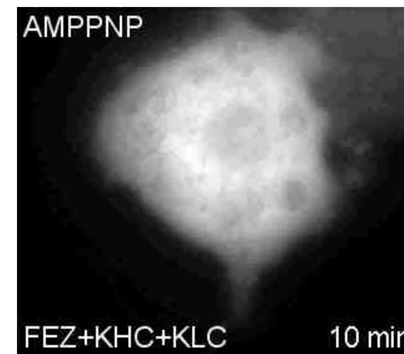
COS cells expressing KHC-mCit were transiently permeabilized with streptolysin O and washed, and then AMPPNP was added. When expressed alone, KHC-mCit is active and becomes locked on the MTs.

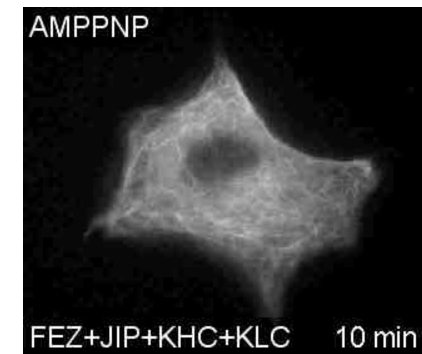












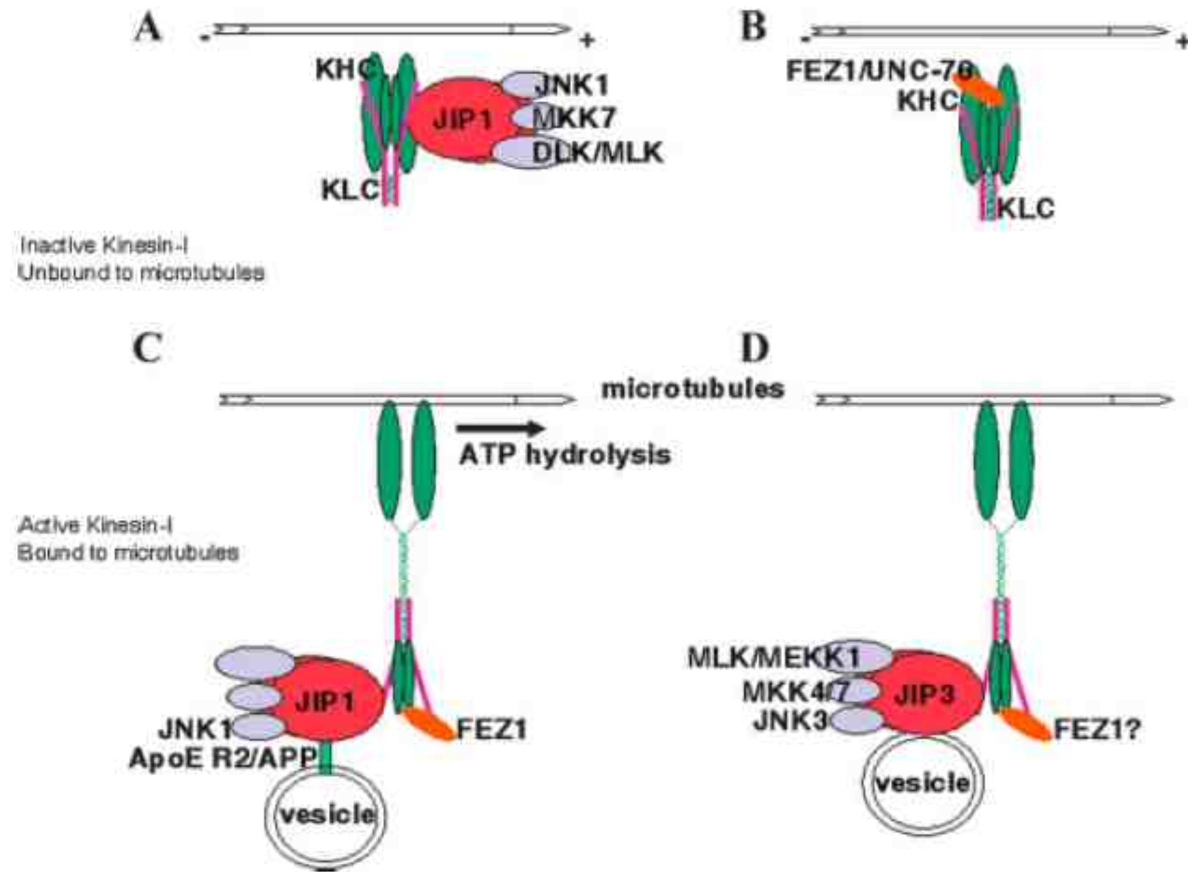
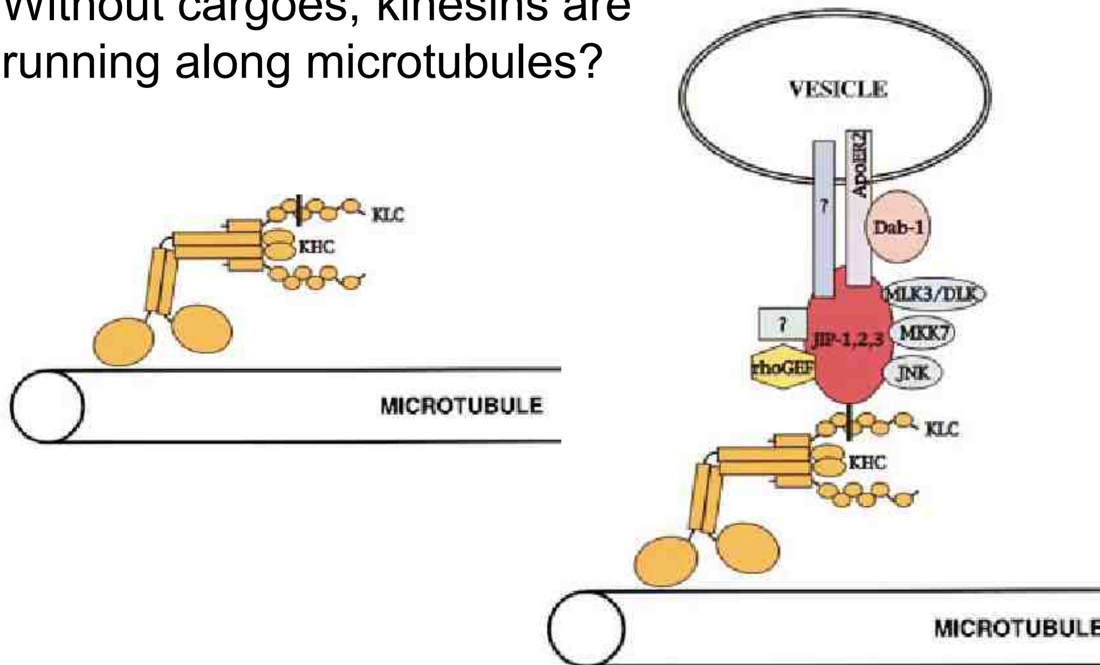


Figure 1. Schematic representation of the activation of Kinesin-I by JIP1 and FEZ1. The tail domain of KHC auto inhibits the motor domain. This inhibition is aided by KLC binding. **A,B:** Binding to JIP1 or FEZ1 independently does not allow KHC–microtubule interactions. Binding both JIP1 and FEZ1 enables KHC to bind microtubules. This ensures that only the Kinesin-I motor loaded with cargo or multiple cargoes (UNC-76, JNK signaling kinases and possibly APP containing vesicles) begins movement on microtubules. **C:** JIP1 binds to vesicular cargoes through proteins such as APP or ApoE R2. **D:** JIP3 may also act in concert with FEZ1/UNC-76 to allow interaction of KHC with microtubules, thereby activating Kinesin-I. JIP3 can directly interact with membranous cargoes. JIP1 and JIP3 are known to homodimerize and have been represented as such.

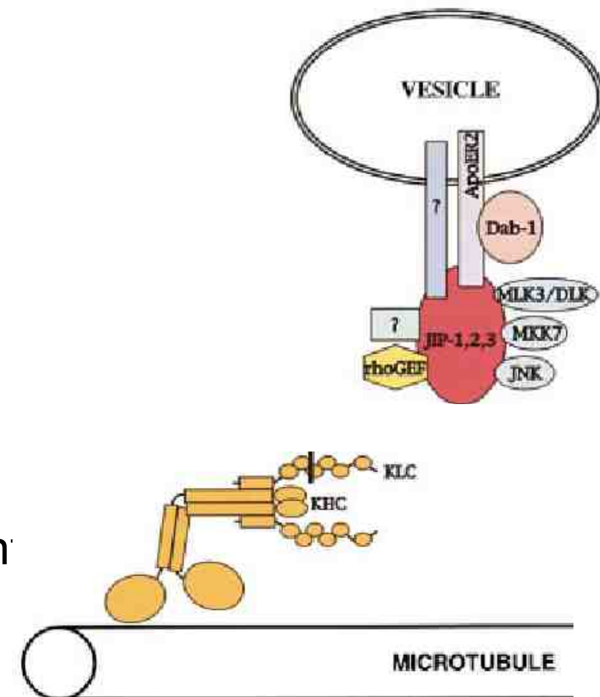
BioEssays 30:10–14, 2007

2° question: Kinesin-1 cargoes compete, cooperate or are transported independently of each other?

Without cargoes, kinesins are running along microtubules?



Cooperative, competitive or indifferent transport of different cargoes?



Signal for cargoes to detach?

Co-operative Versus Independent Transport of Different Cargoes by Kinesin-1

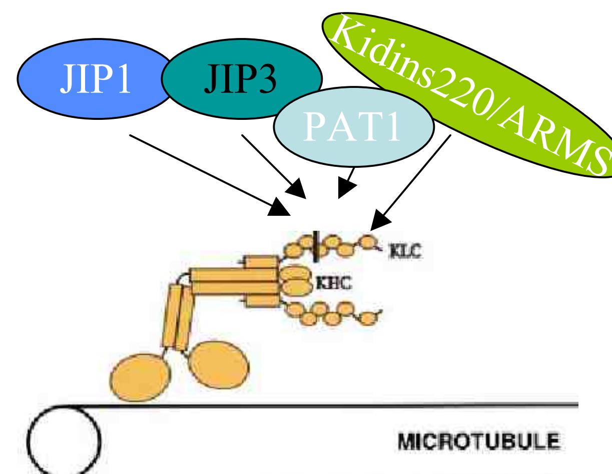
Traffic 2008; 9: 725-741

Jennetta W. Hammond[1], Kelly Griffin[1], Gloria T. Jih[1], Jeanne Stuckey[2] and Kristen J. Verhey[1,*]

How kinesins discriminate among their many potential cargoes is unknown.

Kinesin-1 activity is required for the transport of JIP1, JIP3 and Kidins220/ARMS to neurite tips in neuronal cells

Kinesin-1 cargoes that bind directly to the kinesin light chain (KLC) subunit:



Kidins220/ARMS *is a transmembrane protein.* ***PAT1*** *was identified as a binding partner of KLC in a yeast two-hybrid screen using the TPR motifs of KLC as the bait.*

To test whether distinct Kinesin-1 cargo proteins are transported competitively, co-operatively or independent of each other, **competition experiments in neuronal cells** have been used.

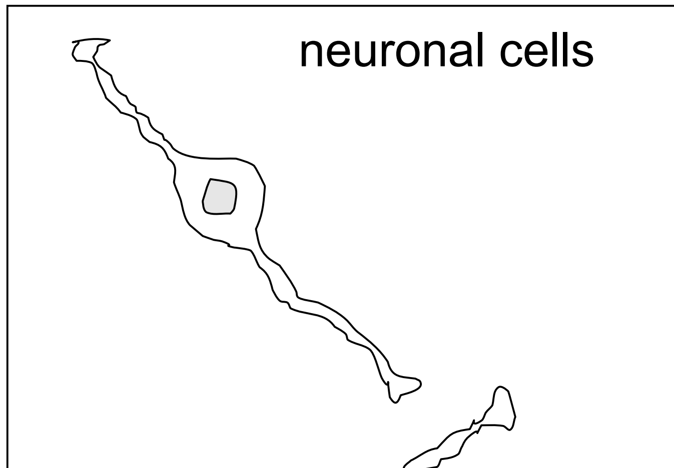
- **Competition:** overexpression of one cargo should result in reduced transport and mislocalization of other cargoes

- **Co-operation:** overexpression of one cargo should result in enhanced transport of other cargoes

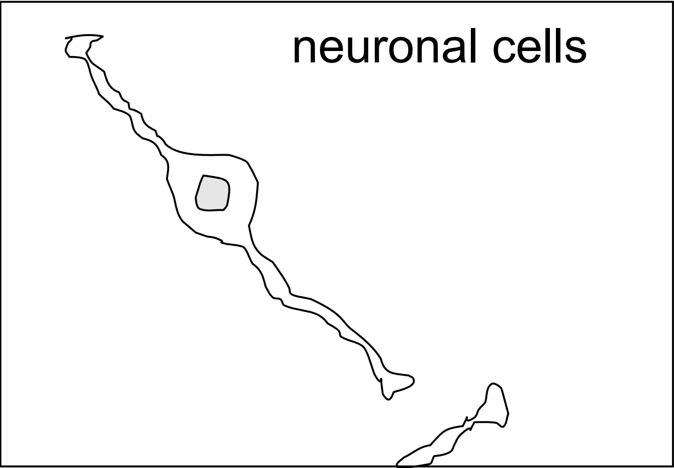
- **Independent:** overexpression of one cargo should not affect transportation of other cargoes

Immunolocalization of endogenous JIP-1

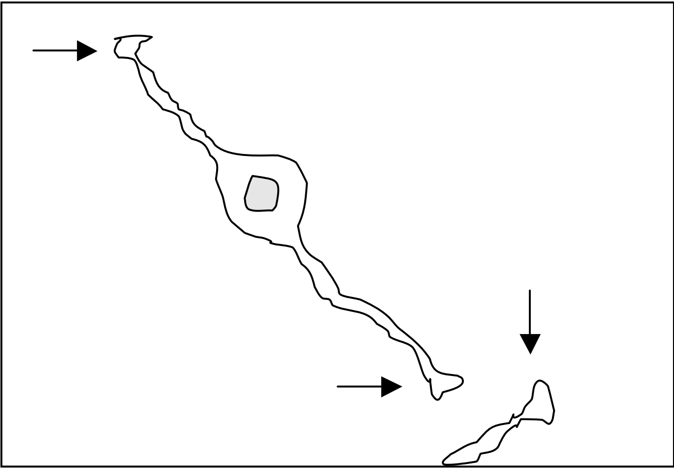
Immunohistochemistry for Endogenous JIP-1



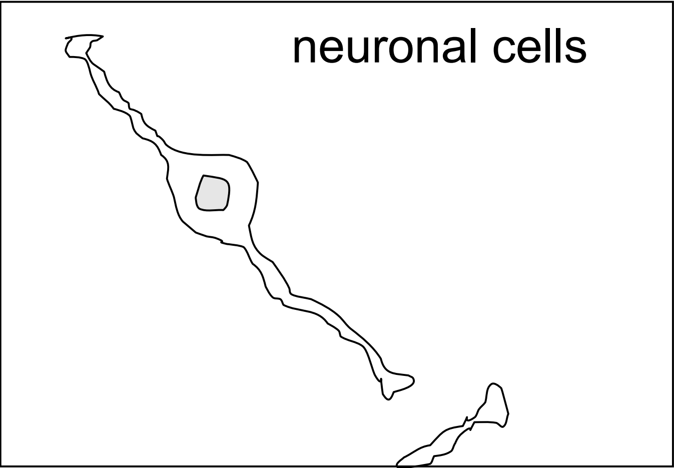
Immunohistochemistry for Endogenous JIP-1



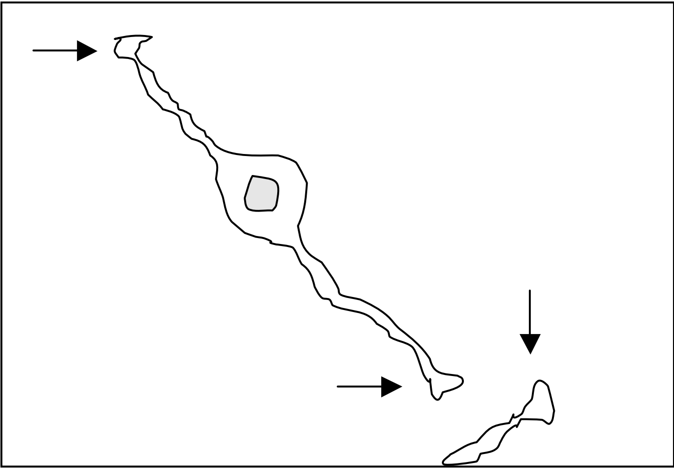
IHC Anti-JIP1



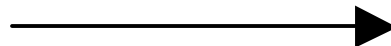
Immunohistochemistry for Endogenous JIP-1

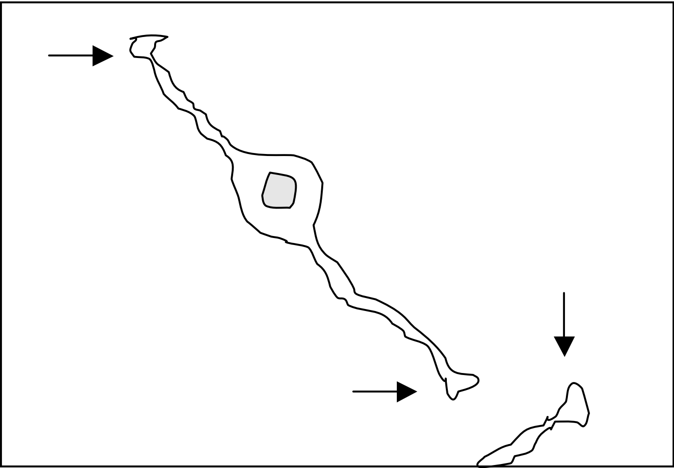


IHC Anti-JIP1

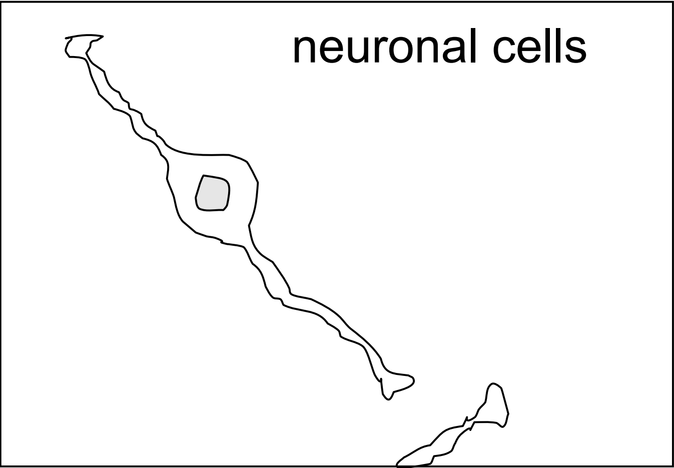


2°ab-FITC

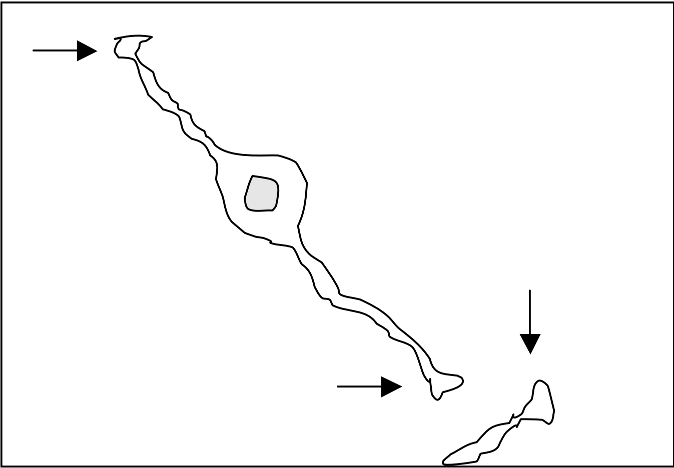




Immunohistochemistry for Endogenous JIP-1



IHC
Anti-JIP1



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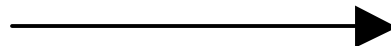
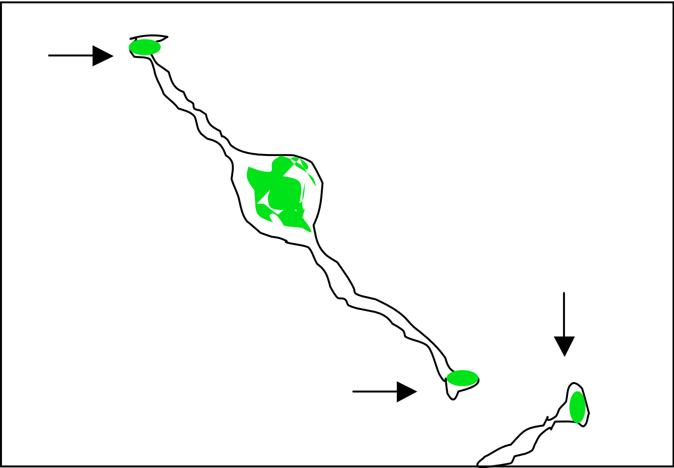
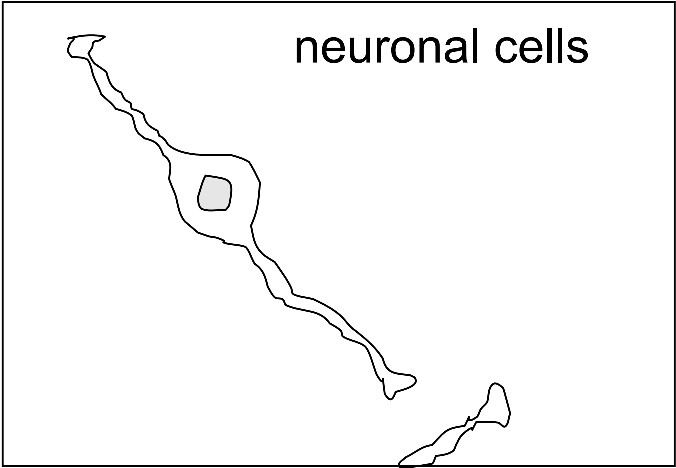


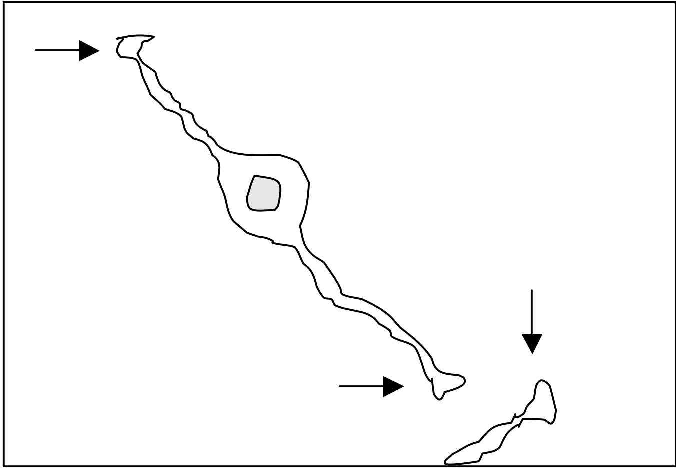


Immunohistochemistry for Endogenous JIP-1



IHC

Anti-JIP1



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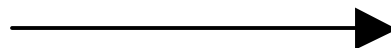
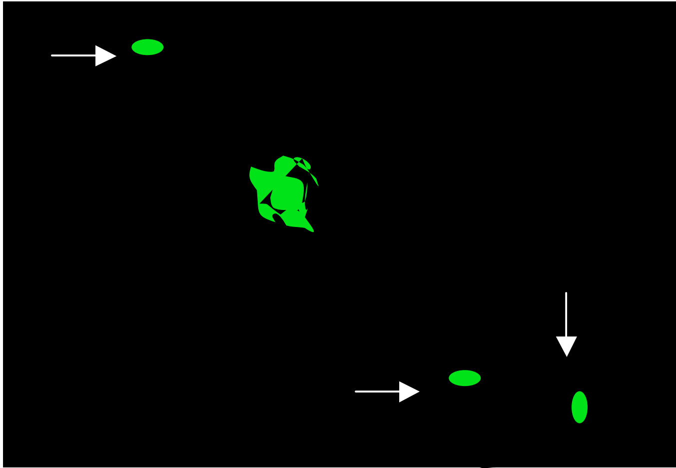
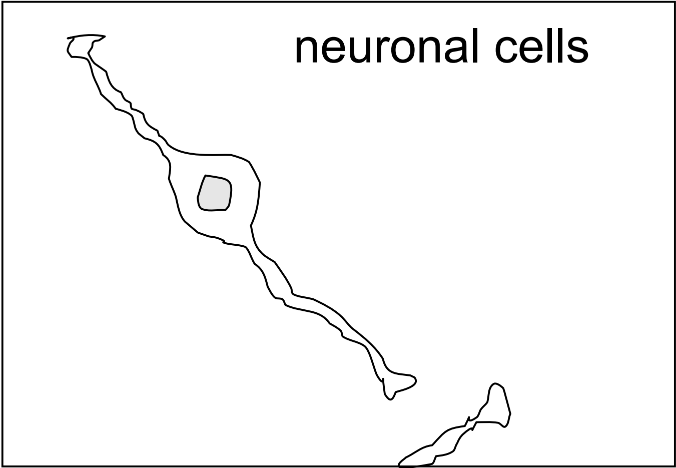
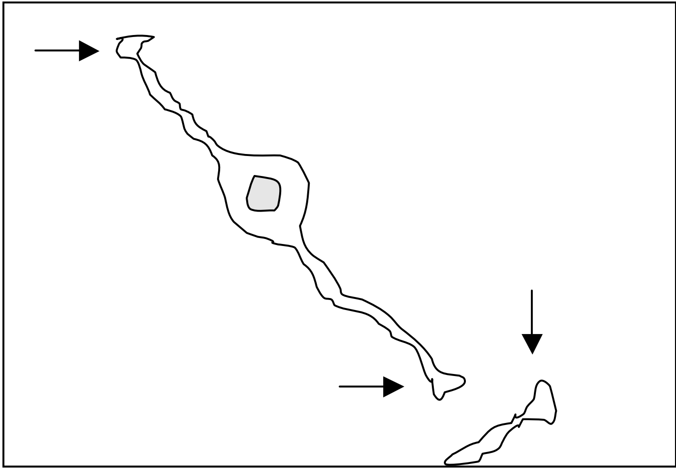




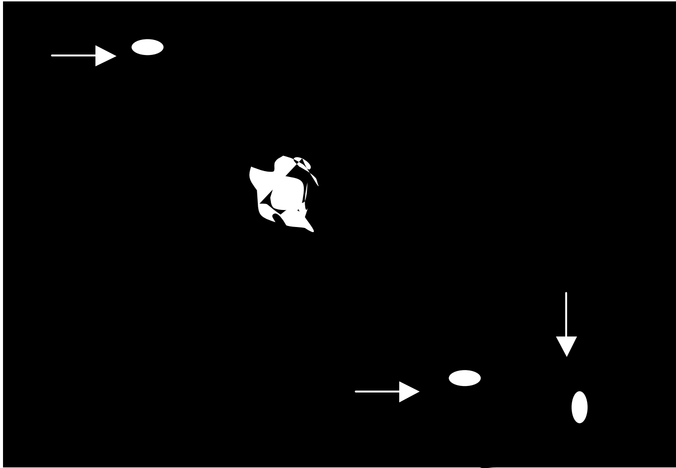
Immunohistochemistry for Endogenous JIP-1



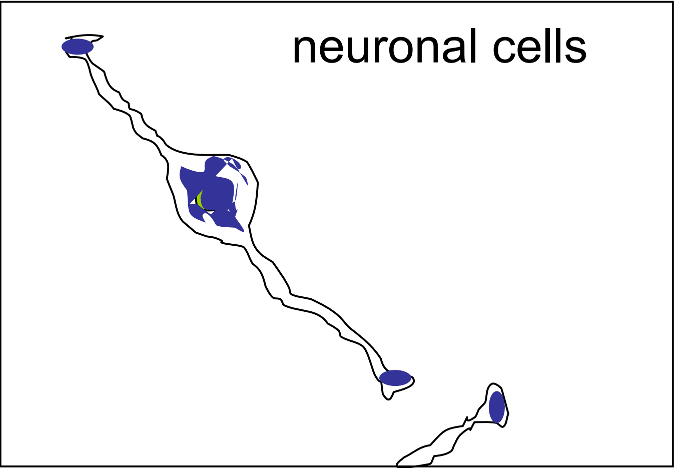
IHC Anti-JIP1



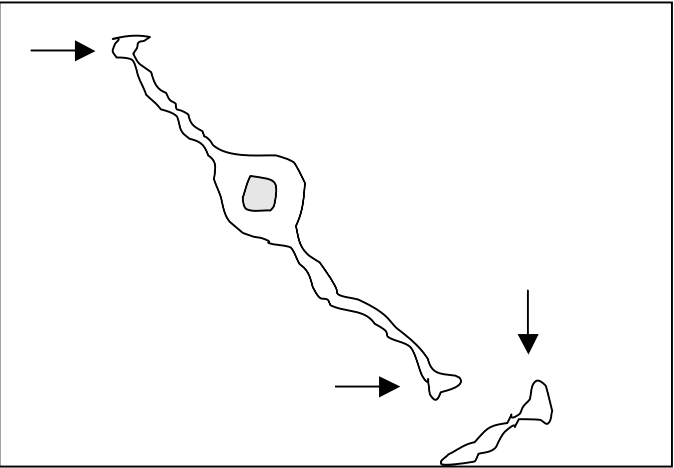
2°ab-FITC



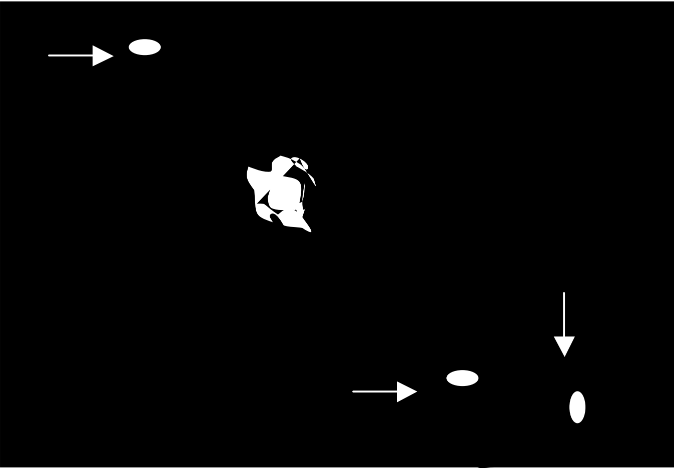
Immunohistochemistry for Endogenous JIP-1



IHC Anti-JIP1



2°ab-FITC



JIP1 is transported to neurite tips

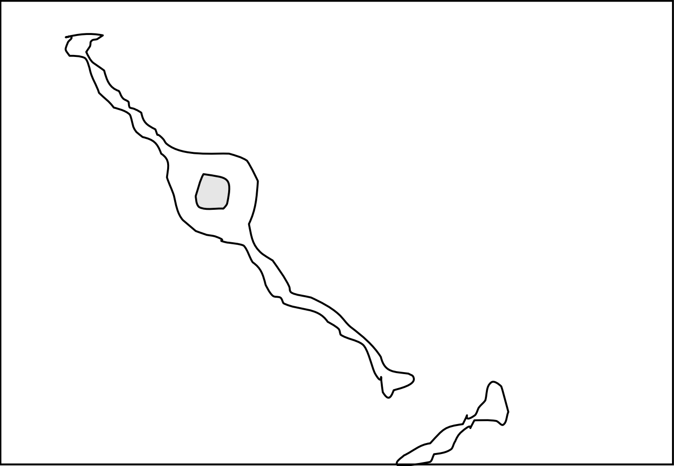
Exemple of Competition:

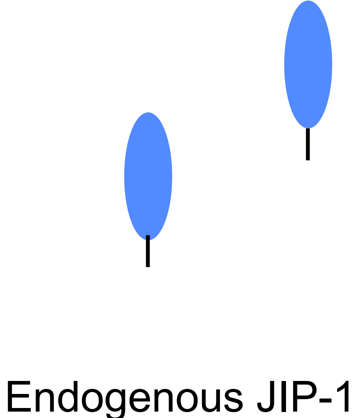
Effect of overexpression of a cargo protein on the localization of its endogenous protein.

Transfection of truncated KLC-binding JIP-1 constructs
&
IHC for Endogenous JIP-1

Exemple with two cells that are transfected

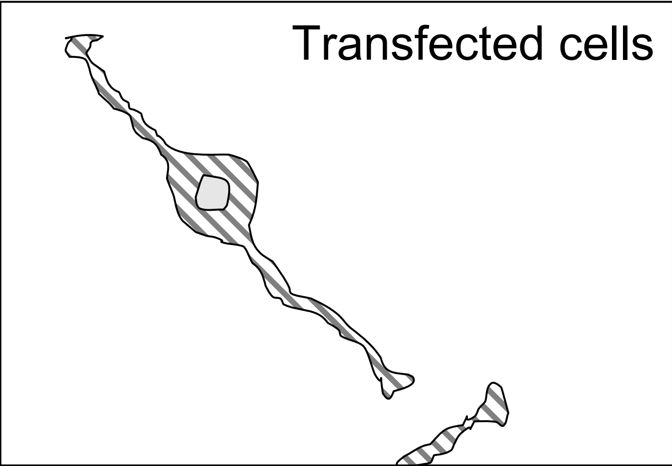
Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1

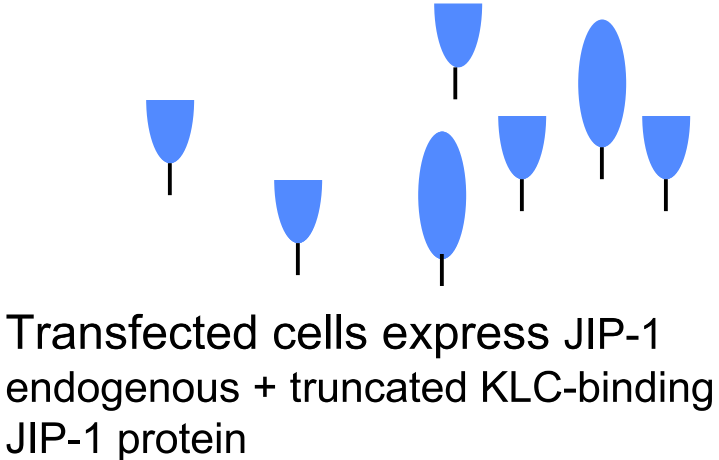




Endogenous JIP-1

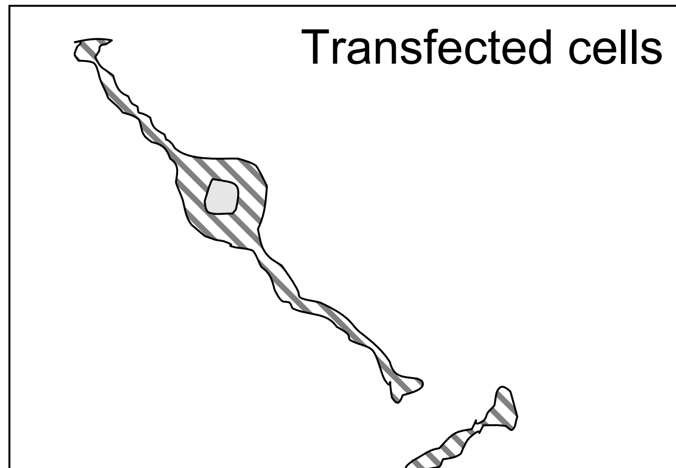
Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1



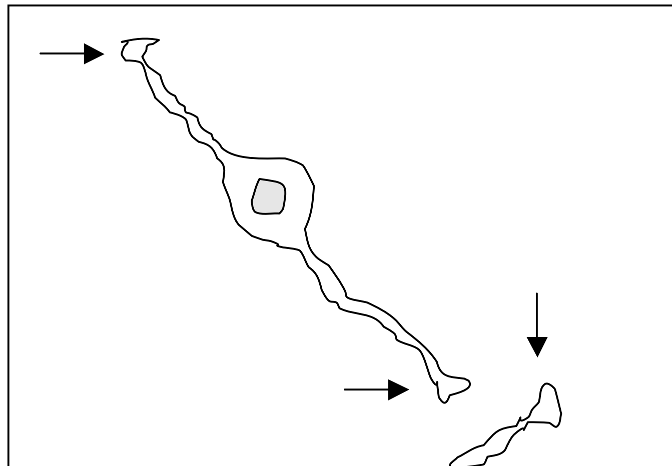


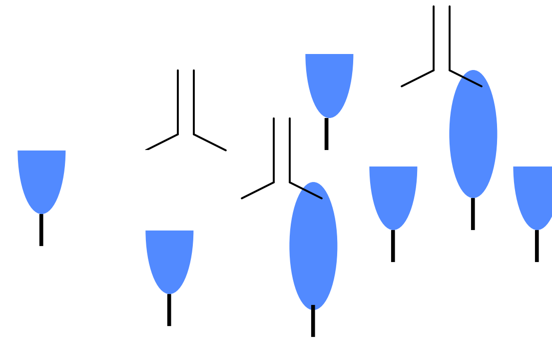
Transfected cells express JIP-1 endogenous + truncated KLC-binding JIP-1 protein

Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1



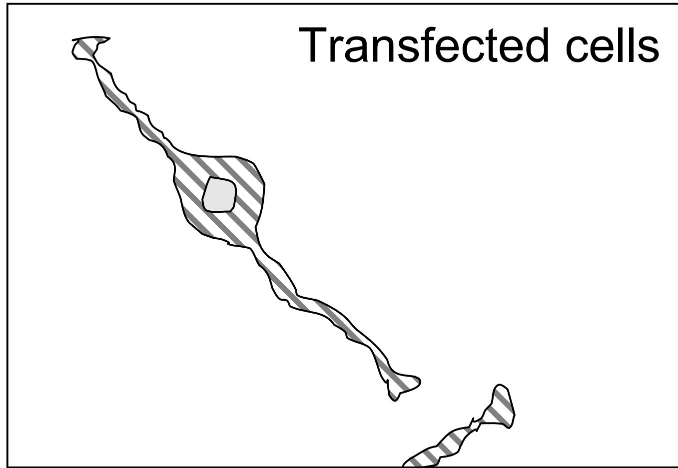
IHC Anti-JIP1

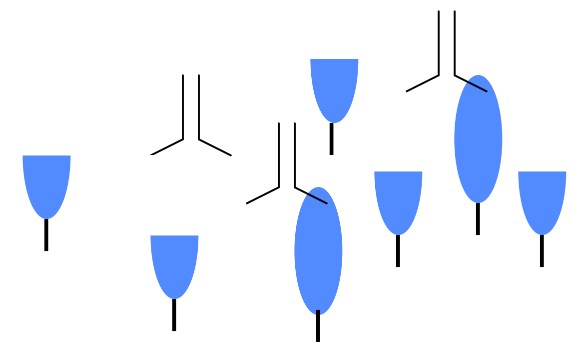




It is necessary to use an antibody anti-JIP-1 that recognizes the endogenous JIP-1 but not the truncated costruct

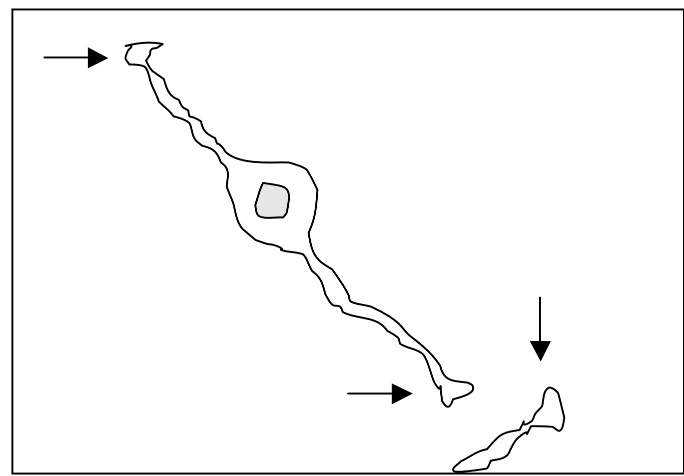
Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1



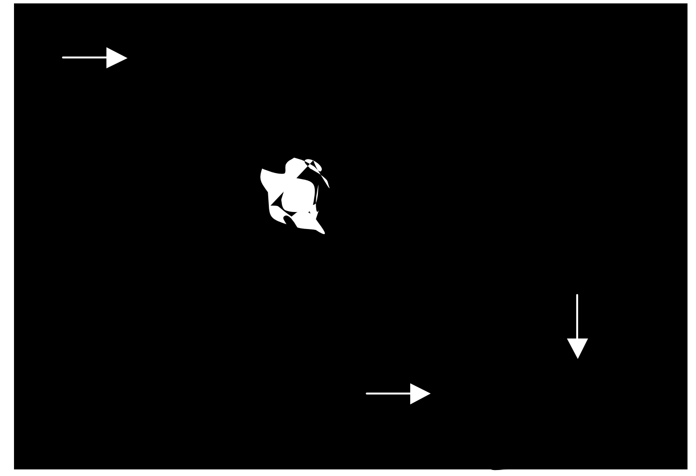


It is necessary to use an antibody anti-JIP-1 that recognizes the endogenous JIP-1 but not the truncated costruct

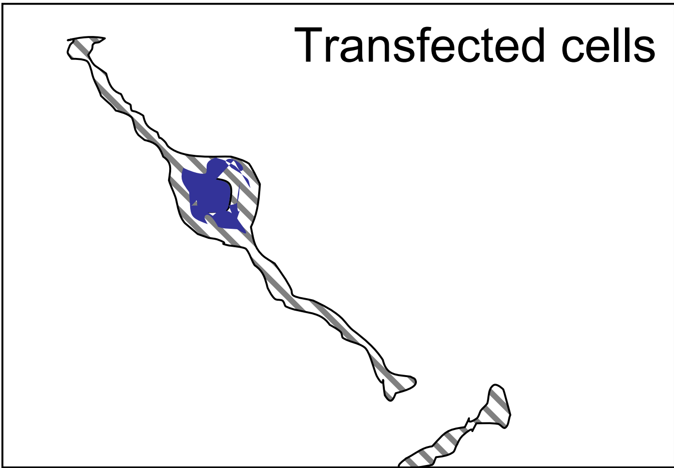
IHC Anti-JIP1



2°ab-FITC

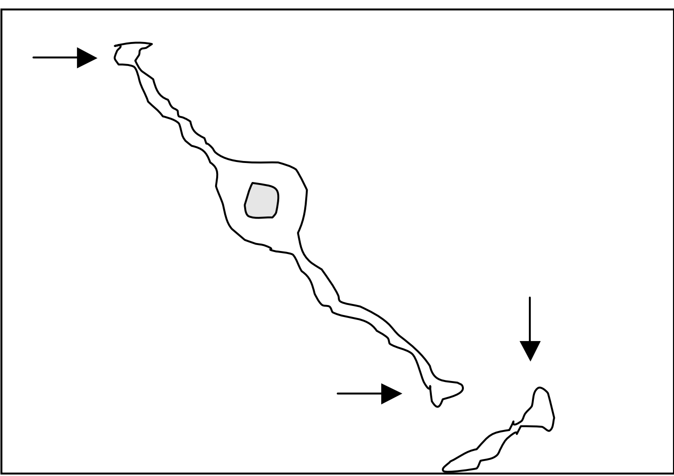


Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1

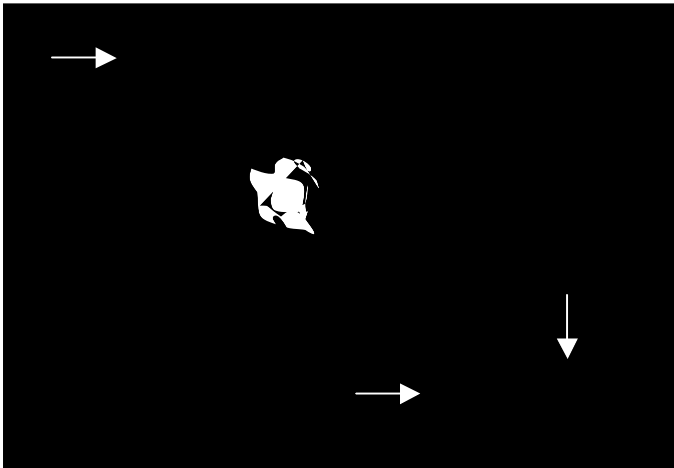


Endogenous JIP1 is NOT anymore transported to neurite tips

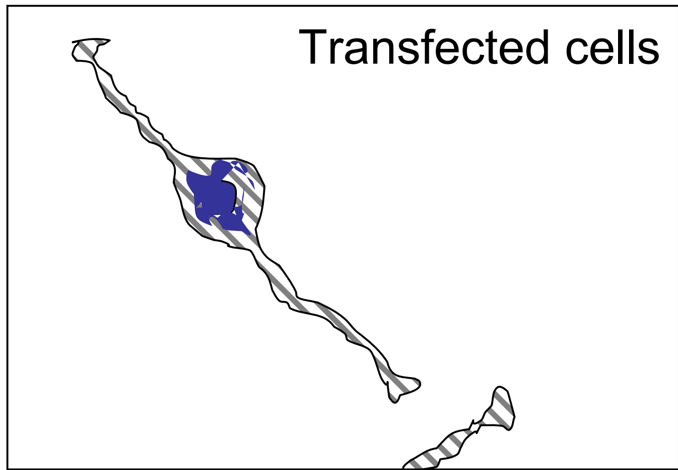
IHC Anti-JIP1

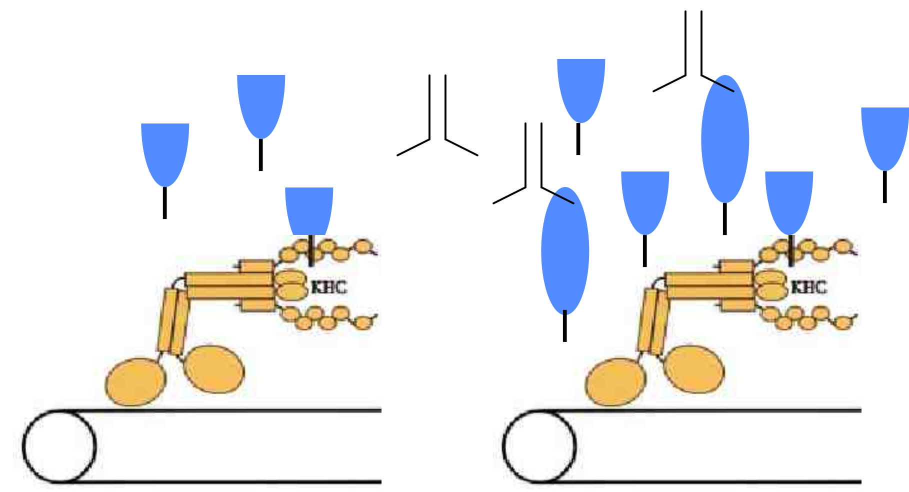


2°ab-FITC

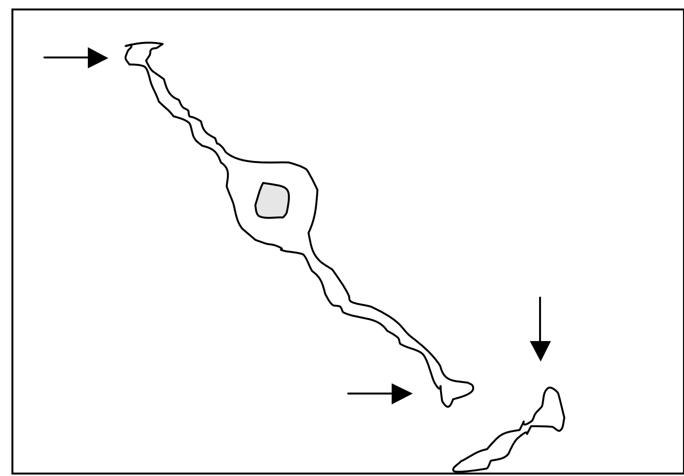


Truncated KLC-binding JIP-1 constructs compete with endogenous JIP-1 for transportation

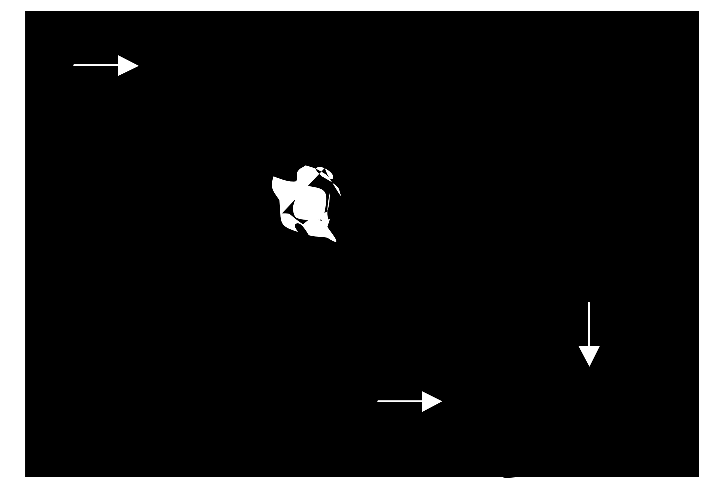




IHC ↓ Anti-JIP1



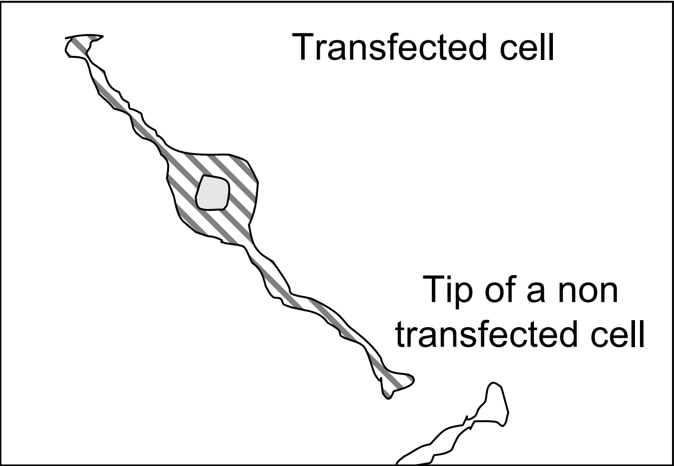
2°ab-FITC →



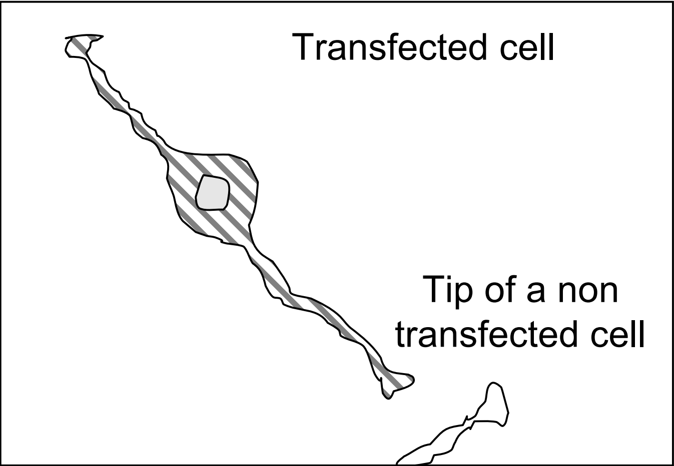
Transfection of truncated KLC-binding JIP-1 constructs
&
IHC for Endogenous JIP-1

Exemple with one cell transfected
and one not transfected

Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1

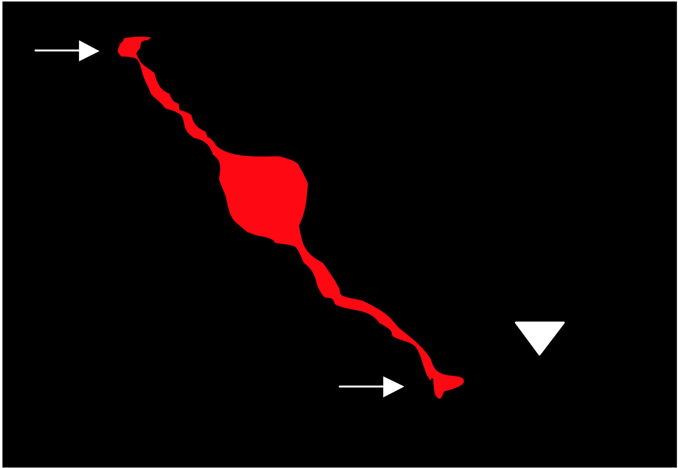


Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1

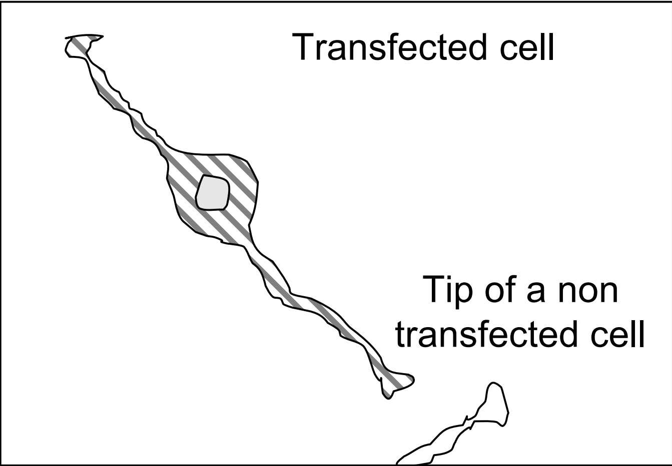


IHC

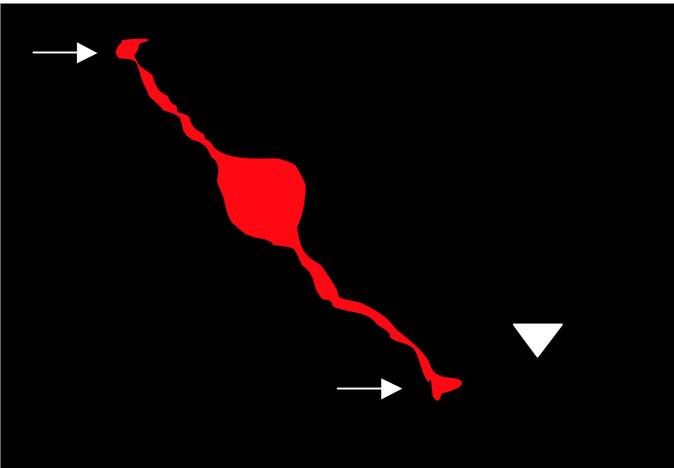
Anti-HA
2° TRITC



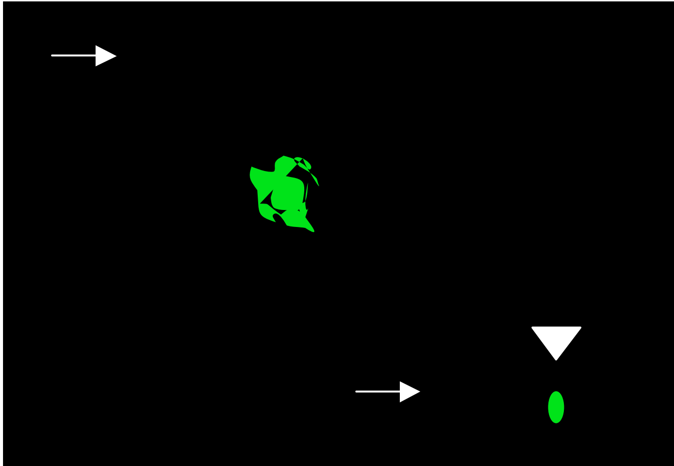
Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1



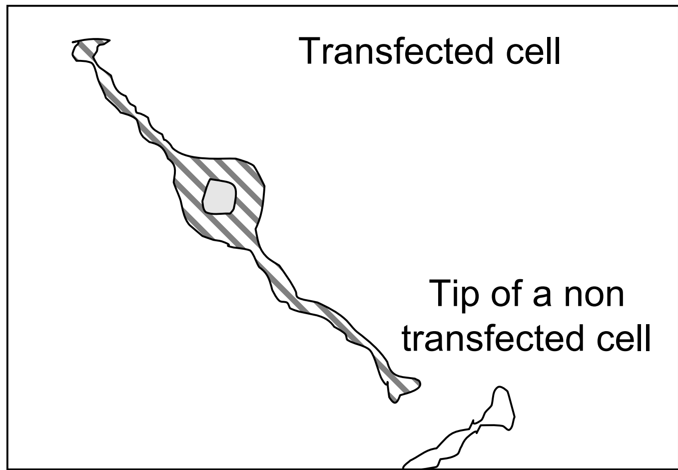
IHC — Anti-HA, 2°TRITC



Anti-JIP-1, 2° FITC

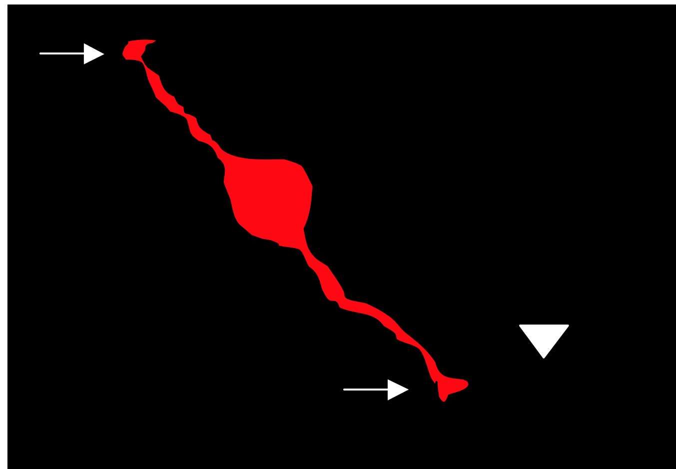


Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1

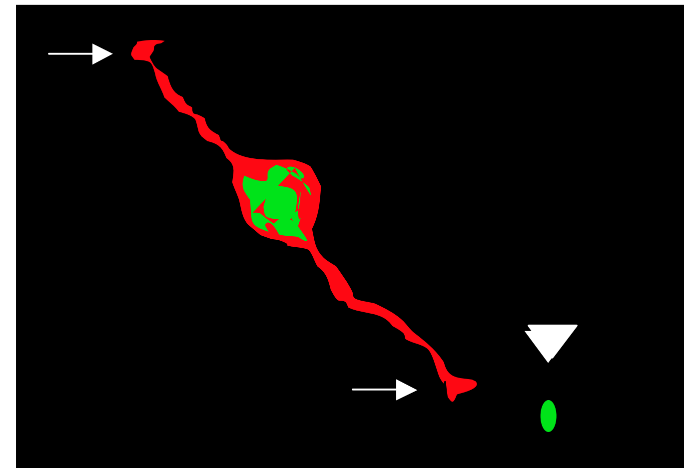


IHC

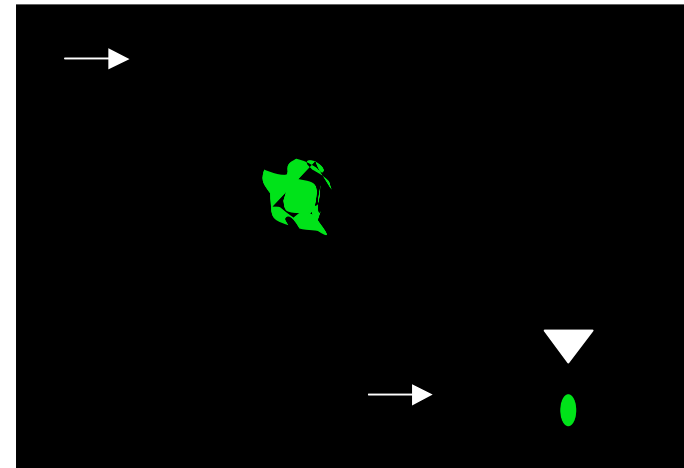
Anti-HA
TRITC



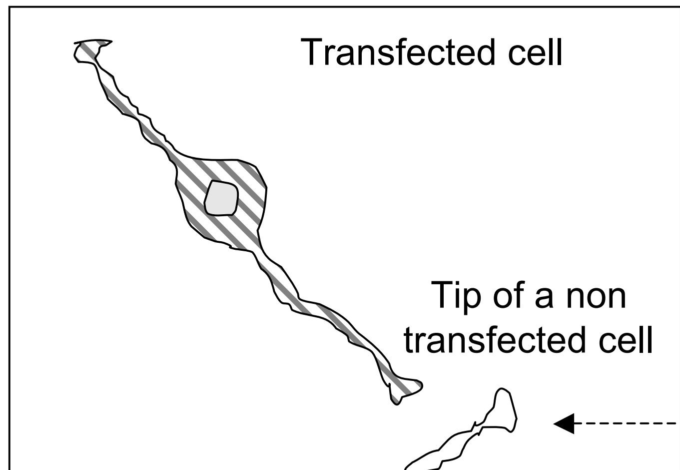
Anti-JIP-1
2° FITC



merge

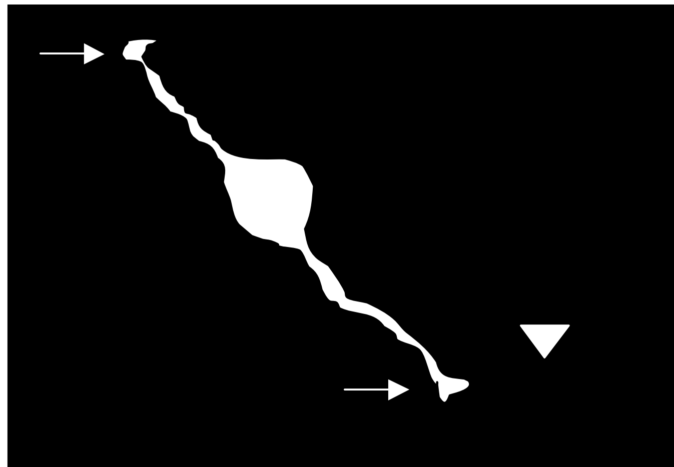


Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1

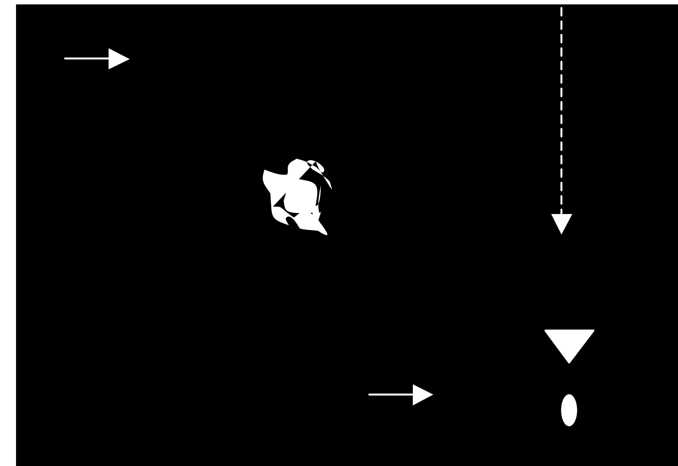


Endogenous JIP1 is transported to neurite tips of non transfected cells (internal control)

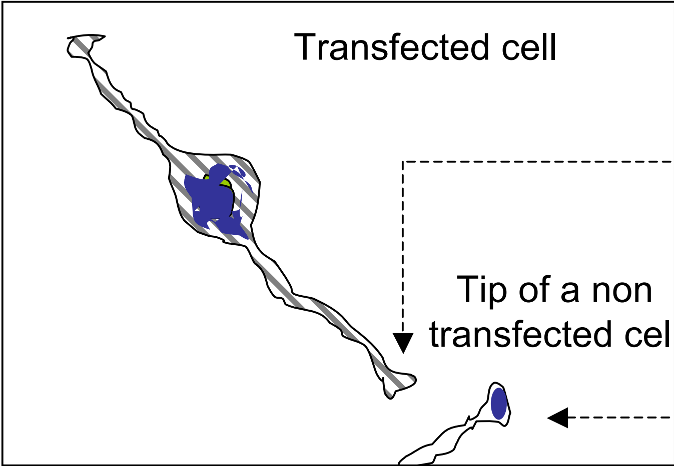
truncated KLC-binding



JIP1



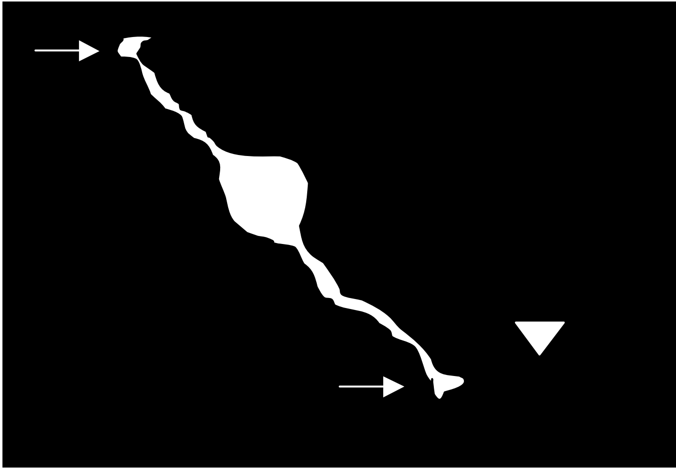
Transfection of truncated KLC-binding JIP-1 constructs & IHC for Endogenous JIP-1



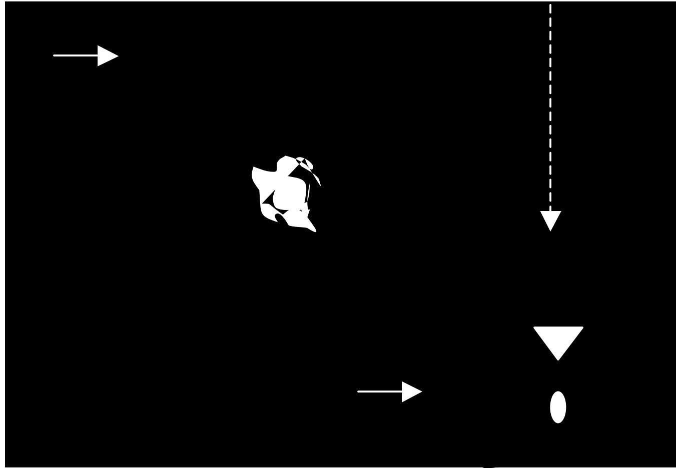
Endogenous JIP1 is NOT transported to neurite tips of transfected cells

Endogenous JIP1 is transported to neurite tips of non transfected cells (internal control)

truncated KLC-binding

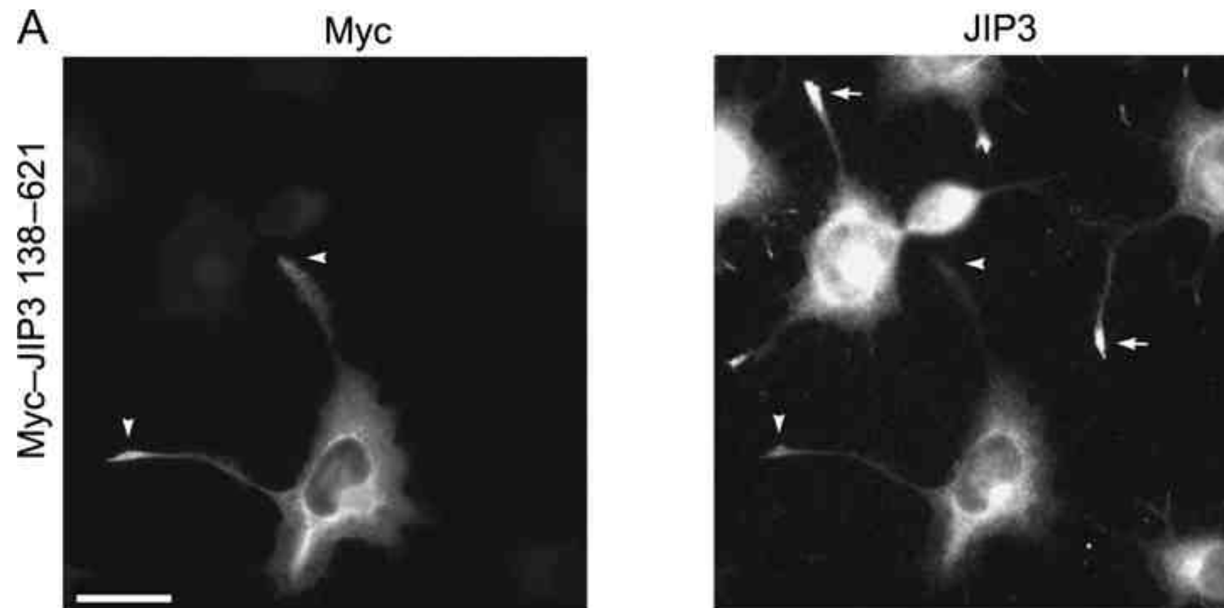


JIP1



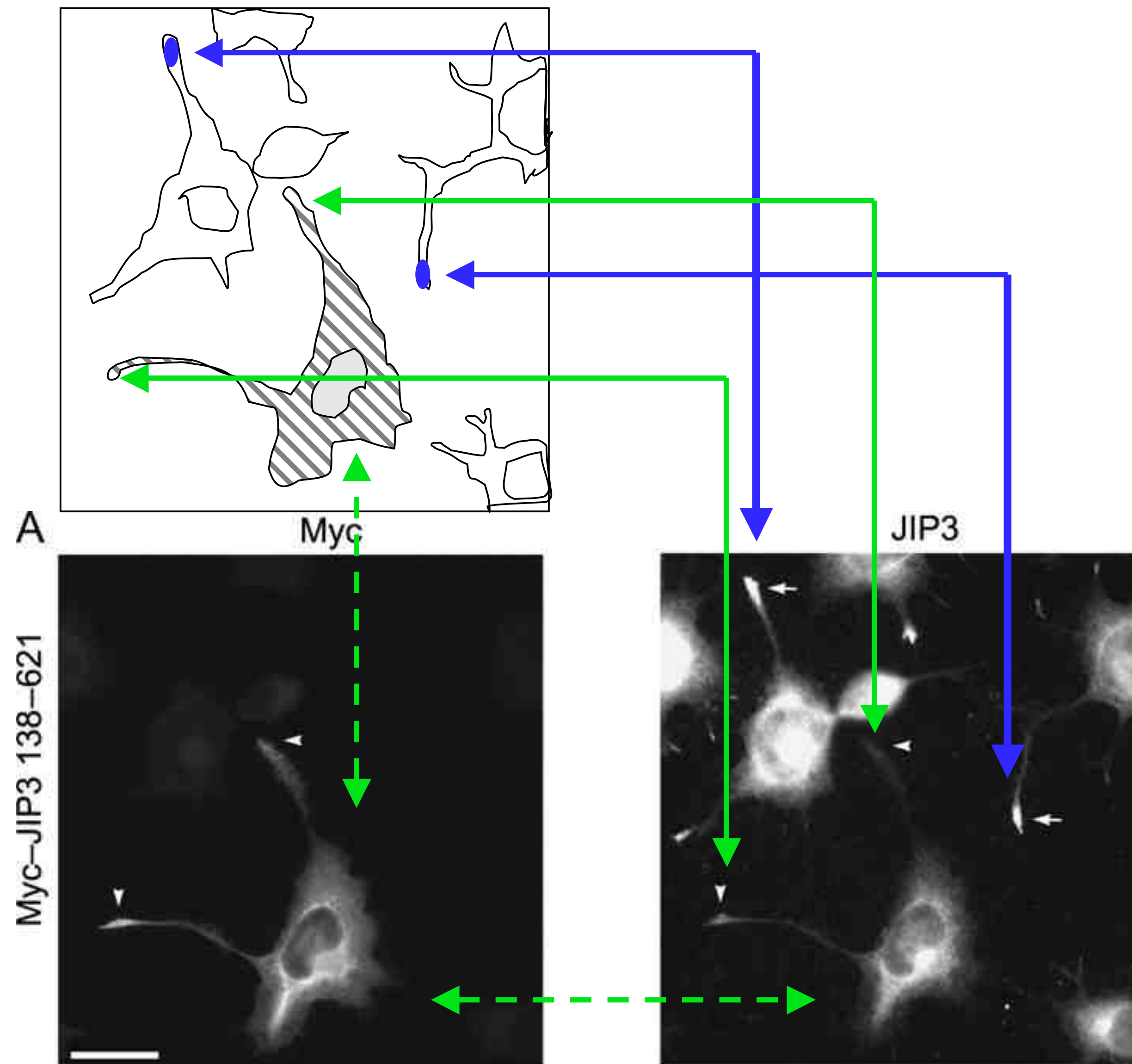
Transfection of truncated KLC-binding JIP-3 constructs
&
IHC for endogenous JIP-3

Truncated KLC-binding JIP-3 constructs compete with endogenous JIP-3



(A) Differentiated CAD cells overexpressing the KLC-binding region of JIP3 [Myc–JIP3 (138–621)] were fixed and stained for the Myc tag and the endogenous JIP3 protein. Arrowheads, neurite tips of transfected cells; arrows, neurite tips of non-transfected (NT) cells. (B) Quantification of endogenous JIP3.

Truncated KLC-binding JIP-3 constructs compete with endogenous JIP-3



(A) Differentiated CAD cells overexpressing the KLC-binding region of JIP3 [Myc–JIP3 (138–621)] were fixed and stained for the Myc tag and the endogenous JIP3 protein. Arrowheads, neurite tips of transfected cells; arrows, neurite tips of non-transfected (NT) cells. (B) Quantification of endogenous JIP3.

Truncated KLC-binding JIP-3 constructs compete with endogenous JIP-3

(A) Differentiated CAD cells overexpressing the KLC-binding region of JIP3 [Myc–JIP3 (138–621)] were fixed and stained for the Myc tag and the endogenous JIP3 protein. Arrowheads, neurite tips of transfected cells; arrows, neurite tips of non-transfected (NT) cells. (B) Quantification of endogenous JIP3.