

## **TAP isolation of Ras protein complexes from mammalian cells**

### **Transfection:**

Transfect  $5 \times 10^8$  NIH 3T3 cells with TAP-Ras fusion constructs using Lipofectamine (Invitrogen).

Perform all steps in a laminar flow hood, with gloves pulled over clamped disposable sleeves. Use only unused pipets, pipet tips, dishes and disposable columns and clean all pipettors and equipment thoroughly with 70% ethanol before use.

### **Cell lysis**

Wash cells 3x with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$ ). Remove last traces of PBS. Freeze dishes at  $-80^\circ\text{C}$  overnight. Lyse cells:

Scrape cells into 1 ml lysis buffer\* + 1 mM EGTA. Keep on ice 30 min.

Remove insoluble material by centrifugation at 14000xg for 10 min at  $4^\circ\text{C}$ .

### **Binding to $\text{Ni}^{++}$ beads**

Transfer 400  $\mu\text{l}$  1:1  $\text{Ni}^{++}$ -charged sepharose beads to a 10ml Bio-Rad disposable filtered column, wash with 5 ml lysis buffer + 40 mM imidazole.

Cap bottom of column, add cell lysate, cap the top, and incubate 1 hr at  $4^\circ\text{C}$  in constant rotation.

Remove cap and drain column.

Wash beads with 30 ml lysis buffer + 40 mM imidazole.

### **Elution from $\text{Ni}^{++}$ beads with imidazole**

Elute in 5 ml lysis buffer + 400 mM imidazole + 2 mM  $\text{CaCl}_2$ .

### **Binding to calmodulin resin**

In a fresh column, add 100  $\mu\text{l}$  1:1 calmodulin beads. Wash with 5 ml lysis buffer + 400 mM imidazole + 2 mM  $\text{CaCl}_2$ .

Cap columns. Add  $\text{Ni}^{++}$  eluates to columns and cover. Incubate 1 hr  $4^\circ\text{C}$  in constant rotation.

Wash columns with 5 ml lysis buffer + 400 mM imidazole + 2 mM  $\text{CaCl}_2$ .

Wash with 5 ml lysis buffer + 400 mM imidazole + 2 mM  $\text{CaCl}_2$  with no detergent.

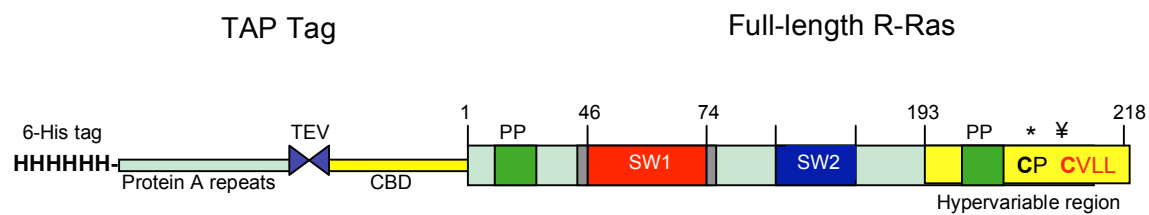
### **Elution from calmodulin columns**

Elute columns in 200  $\mu\text{l}$  lysis buffer + 4 mM EGTA with no detergent.

Send snap-frozen eluates for LC/MS analysis.

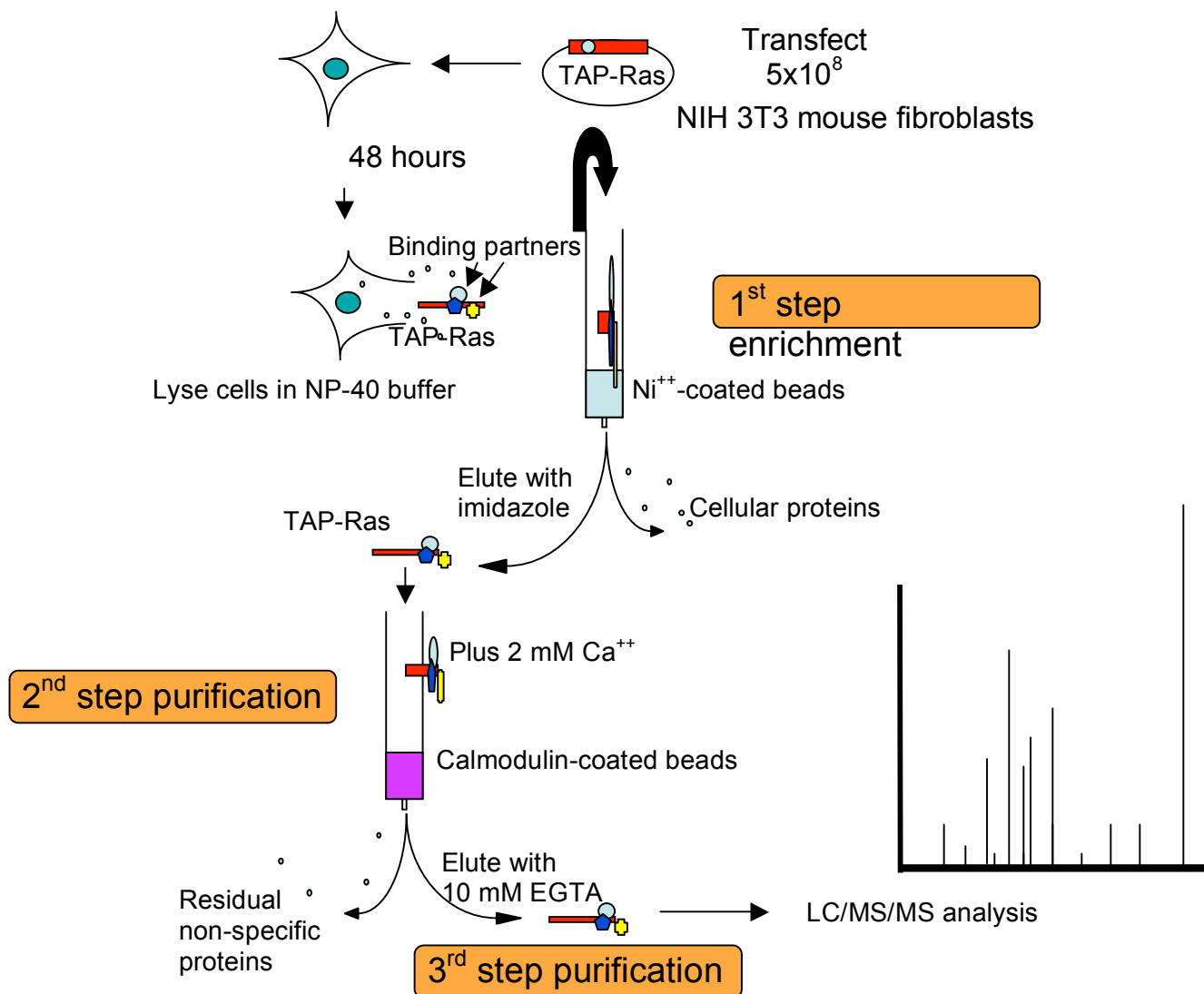
\* Lysis buffer: 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM  $\text{MgCl}_2$ , 0.5% NP-40, plus freshly added protease inhibitor cocktail (Roche) and 10  $\mu\text{M}$  GTP.

## TAP-Ras constructs



CBD = Calmodulin-binding domain  
PP = Proline-rich sequence  
SW1/2 = switch1/2  
TEV = TEV protease cleavage site  
\* = palmitoylation site  
¥ = methylation and geranylgeranylation site

## Ras-TAP Purification Strategy



## N-terminal TAP-tagged GTPase constructs

nTAP: pEF4 expression construct containing the N-terminal TAP tag. Coding sequences were cloned in frame for generation of a fusion construct using BamH1 (5').

nTAP-RRas(G38V)\*\*

nTAP-RRas(T43N)\*\*

nTAP-HRas(G12V)\*\*

nTAP-HRas(T17N)

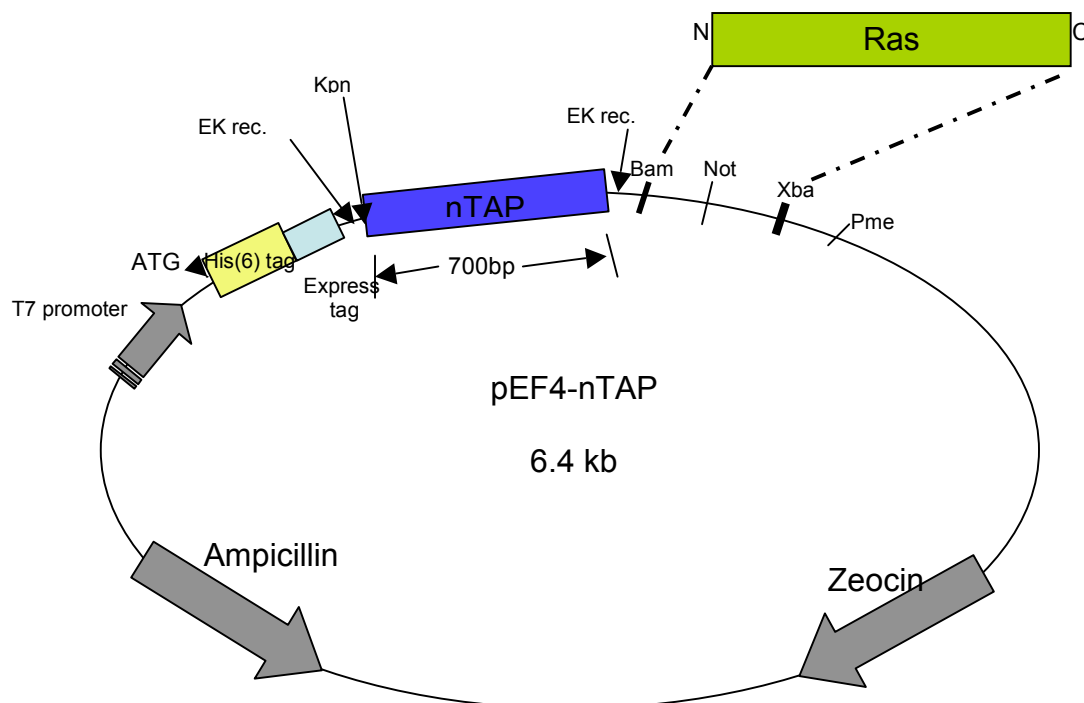
nTAP-Rap1a(G12V)

nTAP-Rap1a(T17N)

nTAP-KRas(G12V)

nTAP-KRas(T17N)

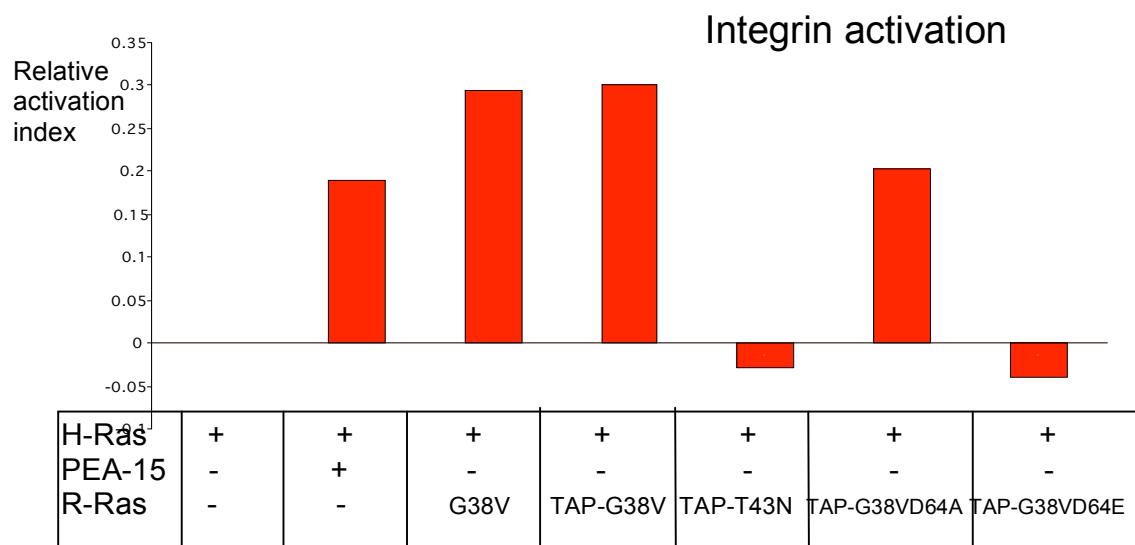
### TAP-Ras restriction map



These constructs can be obtained from Lawrence Goldfinger ([goldfinger@ucsd.edu](mailto:goldfinger@ucsd.edu)) or Mark Ginsberg ([mhginsberg@ucsd.edu](mailto:mhginsberg@ucsd.edu)).

### Confirmation that TAP-Ras fusion constructs are functional *in vivo*.

To confirm that full-length Ras constructs maintain Ras functions *in vivo* in the presence of a TAP fusion, we analyzed the ability of TAP fusions of distinct Ras isoforms to modulate integrin activation, as previously observed (Oertli *et al.*, 2000). Expression of activated H-Ras(G12V) suppresses integrin activation in CHO cells bearing chimeric integrins  $\alpha\text{IIb}\beta\text{3}\beta\text{1}$ , which are constitutively active, as measured by cell labeling with the ligand-mimetic antibody, PAC1, a generous gift of Dr. S. Shattil (Shattil *et al.*, 1985; Hughes *et al.*, 1997). Activated R-Ras reverses integrin suppression, and an additional mutation in the effector loop (G38VD64A) does not disrupt this function of R-Ras. However, an alternate mutation of the same residue (G38VD64E) blocks the ability of R-Ras to reverse suppression of integrin activation by activated H-Ras (Oertli *et al.*, 2000). As shown below, TAP fusion to H-Ras and R-Ras did not alter the abilities of these GTPases to modulate integrin activation in cells.



Integrin activation was measured in CHO cells expressing constitutively active chimeric integrins. nTAP-H-Ras(G12V) suppresses integrin activation, similar to untagged H-Ras(G12V) (Oertli *et al.*, 2000). Activated R-Ras(G38V) with or without a TAP tag fusion or PEA-15 (control) rescues H-Ras-mediated suppression, as does an effector loop double mutant nTAP-R-Ras(G38VD64A). nTAP-R-Ras(T43N) (dominant negative) and nTAP-R-Ras(G38VD64E) do not rescue suppression, consistent with previous observations (Oertli *et al.*, 2000).

\*\* These constructs have been confirmed for functionality by the integrin activation assay.

#### Reference List

Hughes,P.E., Renshaw,M.W., Pfaff,M., Forsyth,J., Keivens,V.M., Schwartz,M.A., and Ginsberg,M.H. (1997). Suppression of integrin activation: A novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell* 88, 521-530.

Oertli,B., Han,J., Marte,B.M., Sethi,T., Downward,J., Ginsberg,M., and Hughes,P.E. (2000). The effector loop and prenylation site of R-Ras are involved in the regulation of integrin function [In Process Citation]. *Oncogene* 19, 4961-4969.

Shattil,S.J., Brass,L.F., Bennett,J.S., and Pandhi,P. (1985). Biochemical and Functional Consequences of Dissociation of the Platelet Membrane Glycoprotein IIb-IIIa Complex. *Blood* 66, 92-98.