Paper 01

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Force-dependent vinculin binding to talin in live cells: a crucial step in anchoring the actin cytoskeleton to focal adhesions

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Hirata H, Tatsumi H, Lim CT, Sokabe M. Force-dependent vinculin binding to talin in live cells: a crucial step in anchoring the actin cytoskeleton to focal adhesions. *Am J Physiol Cell Physiol* 306: C607–C620, 2014. First published January 22, 2014; doi:10.1152/ajpcell.00122.2013.—Mechanical forces play a pivotal role in the regulation of focal adhesions (FAs) where the actin cytoskeleton is anchored to the extracellular matrix through integrin and a variety of linker proteins including talin and vinculin. The localization of vinculin at FAs depends on mechanical

eton in fibroblasts (17, 34, 65). The talin-mediated link between the actin cytoskeleton and clustered integrin is broken repeatedly by a small force of ~2 pN generated by the retrograde flow of actin filaments (34). On the other hand, the integrin-actin cytoskeleton linkage is strengthened when a mechanical force is loaded to it (7, 61). The strengthened linkage can sustain much larger forces (~20 pN), which

MATERIALS AND METHODS

Cell culture. Human foreskin fibroblasts (HFFs) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (Nipro, Osaka, Japan) at 37°C in 5% CO_2. For immunofluorescence experiments, HFF cells were grown for 15 h on glass coverslips or elastic silicone (polydimethylsiloxane elastomer) chambers (Strex, Osaka, Japan), which were precoated with 100 μg/ml fibronectin (Sigma Chemical). In some cases, cells were treated with 100 μM blebbistatin (Toronto Research Chemicals, North York, Canada) or 40 μM Y-27632 (Calbiochem, San Diego, CA) for 30 min.

Antibodies. Mouse anti-vinculin and -β-actin mAbs were purchased from Sigma Chemical. Mouse anti-talin mAbs were from Sigma Chemical and Chemicon (Temecula, CA). The rabbit anti-α_5-integrin polyclonal antibody was from Chemicon. The mouse anti-$\alpha_5\beta_1$-integrin mAb was from Millipore (Billerica, MA). The mouse anti-green fluorescent protein (GFP) mAb was from Clontech Laboratories (Mountain View, CA). Control mouse IgG1 was from R&D Systems (Minneapolis, MN). Alexa488-chicken anti-rabbit IgG, Alexa546-goat anti-mouse IgG, and Alexa594-chicken anti-rabbit IgG antibodies, and Alexa488- and Alexa647-phalloidin were from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated anti-mouse IgG antibody was from GE Healthcare (Little Chalfont, UK). Horseradish peroxidase-conjugated Mouse TrueBlot ULTRA was from eBioscience (San Diego, CA). The anti-vinculin mAb hVIN-1 recognizes full-length vinculin, but not the vinculin domain D1, in immunoblot (data not shown).

Antibodies

- ❑ Mouse anti-vinculin mAbs
- ❑ Mouse anti-beta-actin mAbs
- ❑ Mouse anti-talin mAbs
- ❑ Rabbit anti-α5-integrin polyclonal antibody
- ❑ Mouse anti-α5β1-integrin mAb
- ❑ Mouse anti-green fluorescent protein (GFP) mAb.
- ❑ Control mouse IgG1
- ❑ Alexa488-chicken anti-rabbit IgG
- ❑ Alexa546-goat anti-mouse IgG
- ❑ Alexa594-chicken anti-rabbit IgG antibodies
- ❑ Alexa488-phalloidin and Alexa647-phalloidin
- ❑ Horseradish peroxidase-conjugated anti-mouse IgG antibody
- ❑ Horseradish peroxidase-conjugated Mouse TrueBlot ULTRA.
- ❑ The anti-vinculin mAb hVIN-1 recognizes full-length vinculin, but not the vinculin domain D1, in immunoblot

Fluorescence microscopy

- Mouse anti-vinculin mAbs
- Mouse anti-beta-actin mAbs
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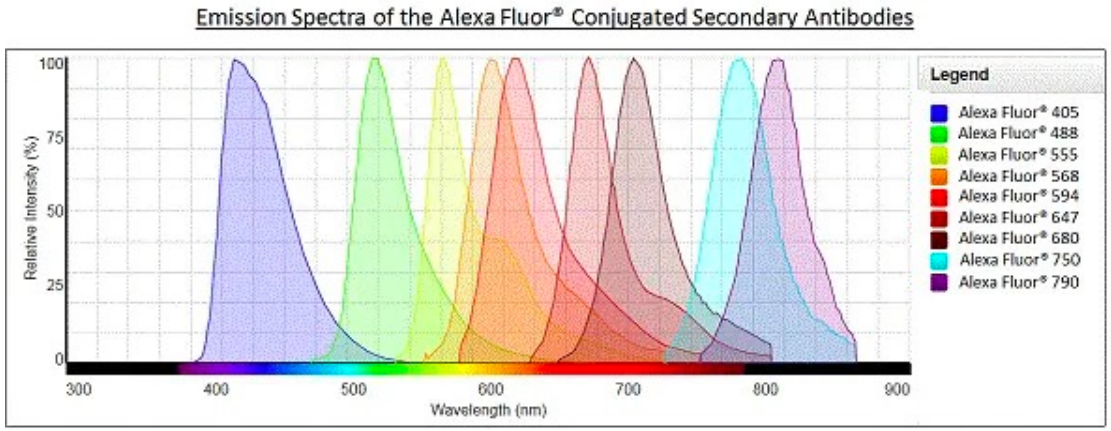
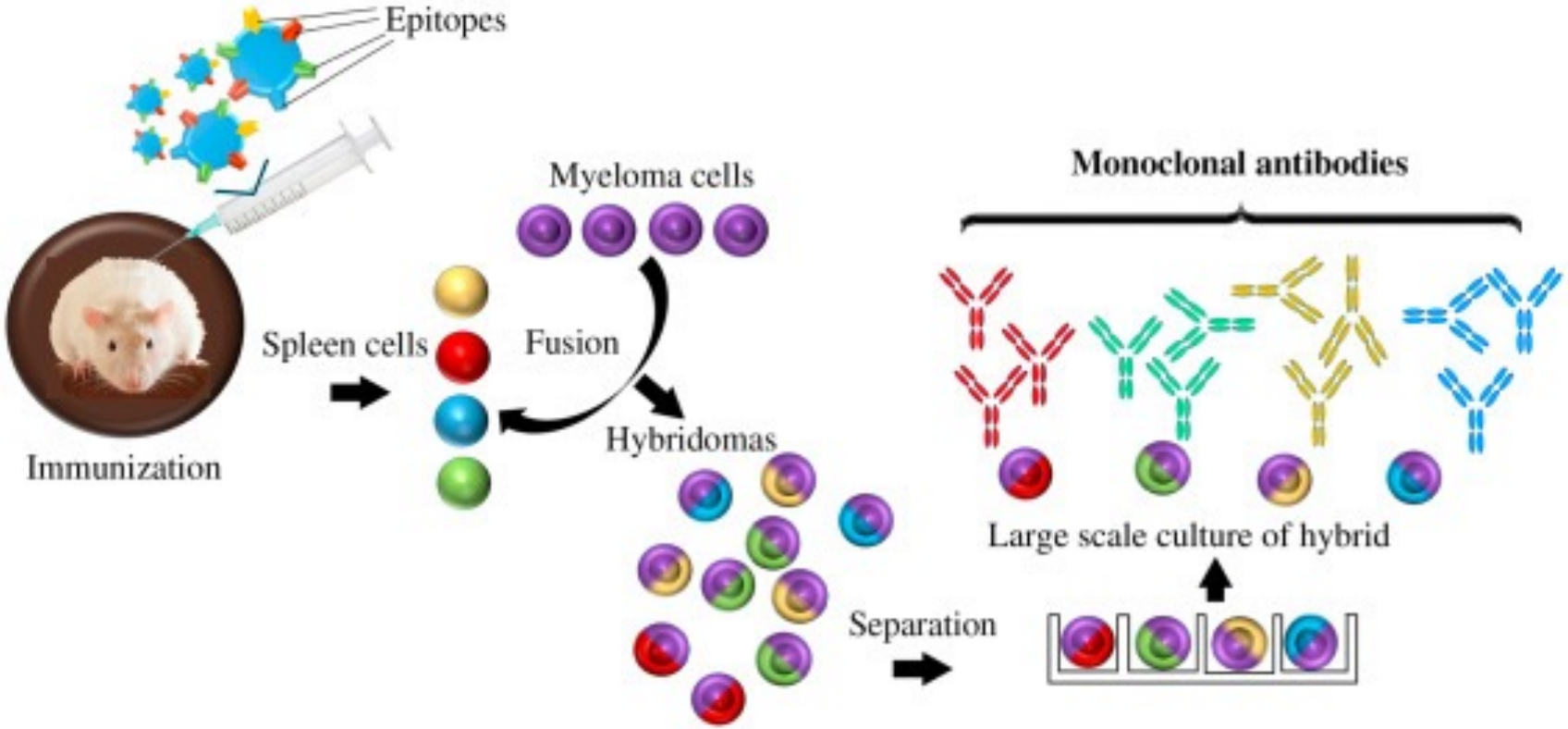


Polyclonal antibody production?

Monoclonal antibody production?

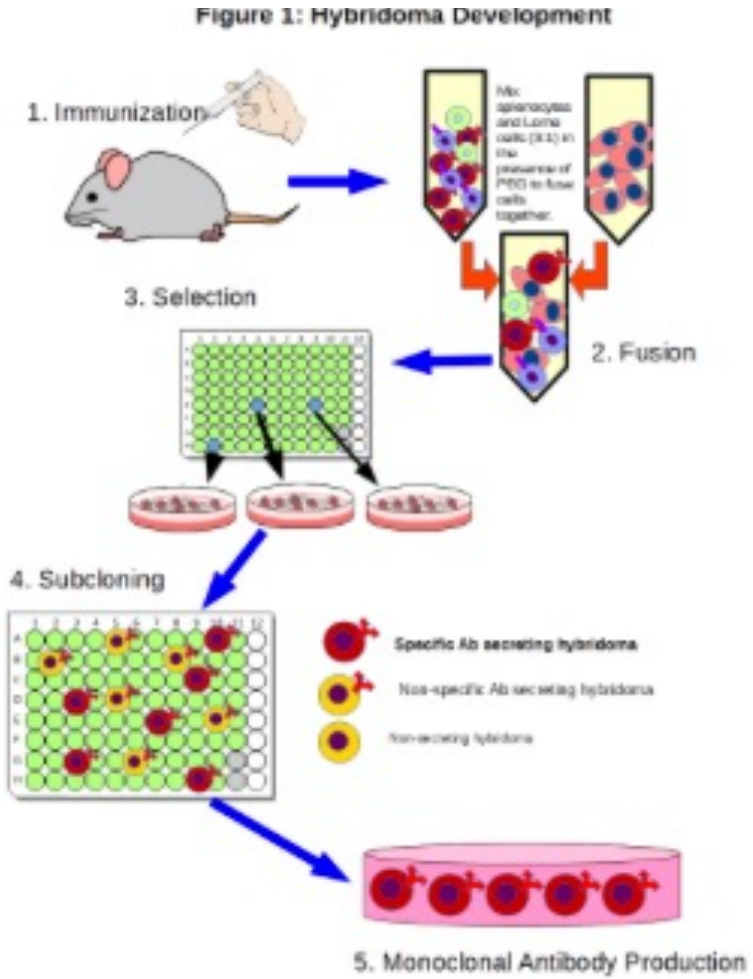
Polyclonal

Monoclonal



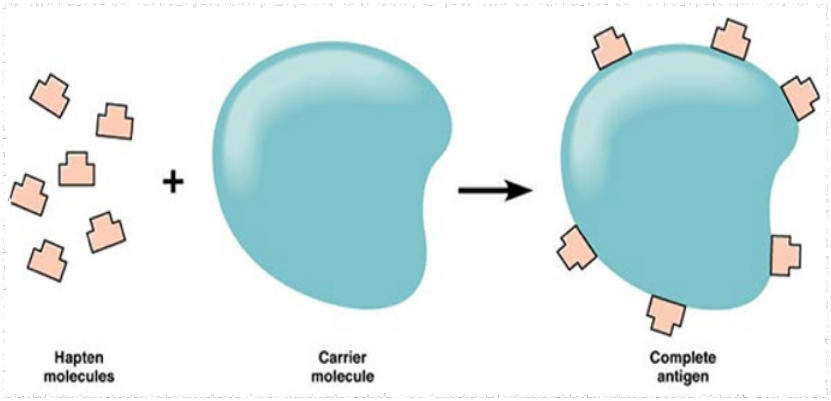
Monoclonal antibody production flowchart

- No needs for antigen purification
- Requires myeloma cells
- HAT selection
- Screen for the desired clones



Immunogen vs antigen

- Immunogen induces immune response
- Antigen reacts with products of the immune response



Immunogenicity

- nature of the immunogenicity
- Ability of immune system to react
- possible to manipulate

Epitope (antigenic determinant)

- recognised by antibody
- recognised by T-cell receptor

Immunofluorescence

For immunofluorescence:

- cells were **fixed** and ***permeabilized*** for 30 min with 4% **formaldehyde** and 0.2% **Triton X-100** in cytoskeleton stabilizing buffer
- **blocking with 1% skim milk** in cytoskeleton stabilizing buffer for 30 min.
- Cells were then **incubated with primary antibodies** for 40 min
- washed,
- and further **incubated with secondary antibodies** for 40 min.

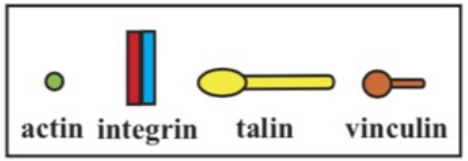
Antibodies were diluted to 1:100 in cytoskeleton stabilizing buffer containing 1% skim milk.

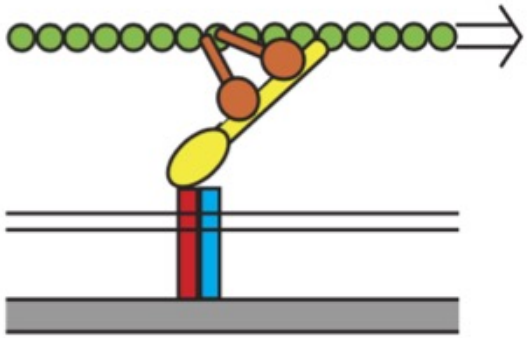
Why is it necessary to *permeabilize cells* ?

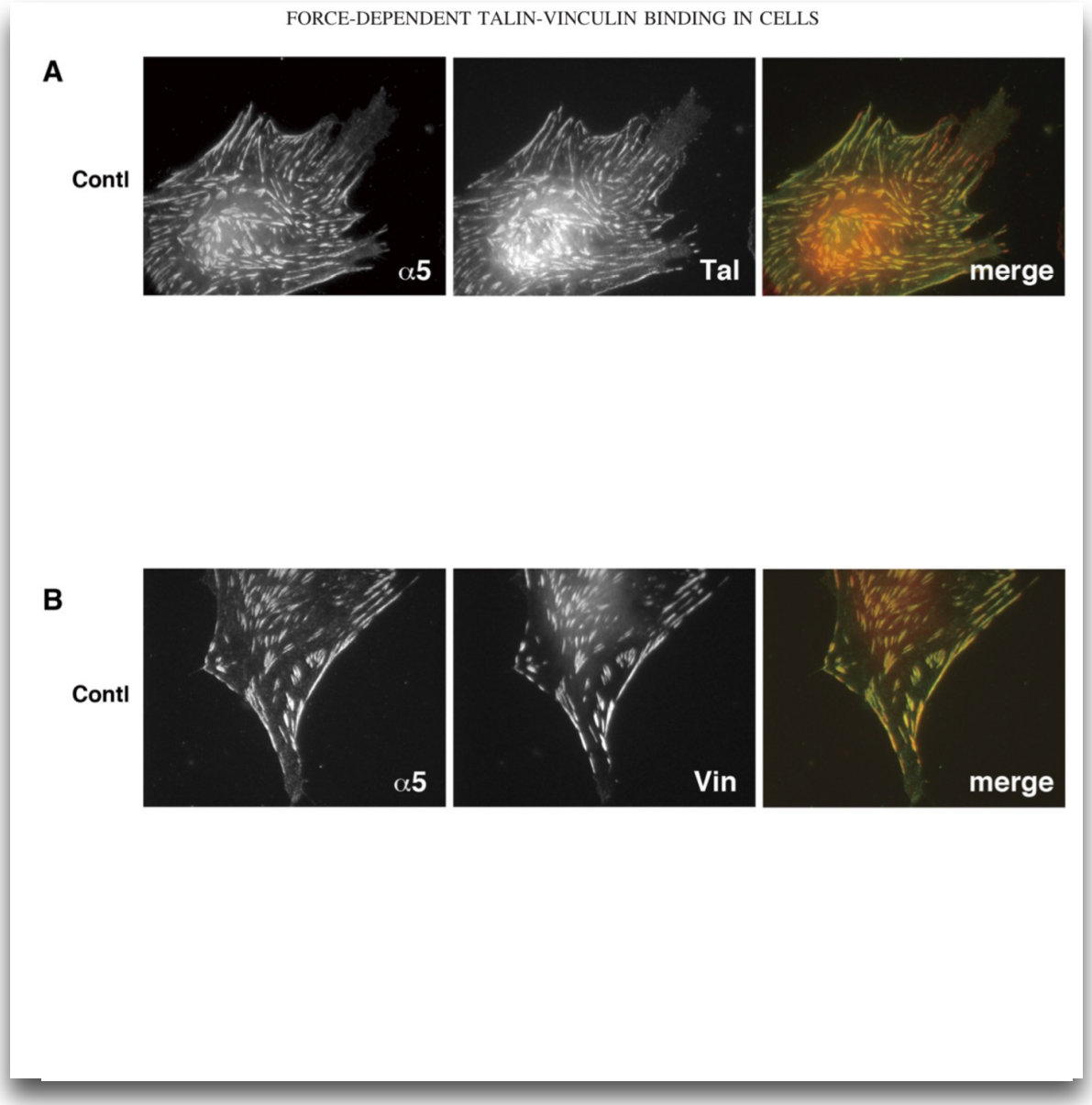
Meaning of *blocking with 1% skim milk?*

Meaning of: ***Antibodies were diluted to 1:100 in cytoskeleton stabilizing buffer containing 1% skim milk ?***

Fig.1



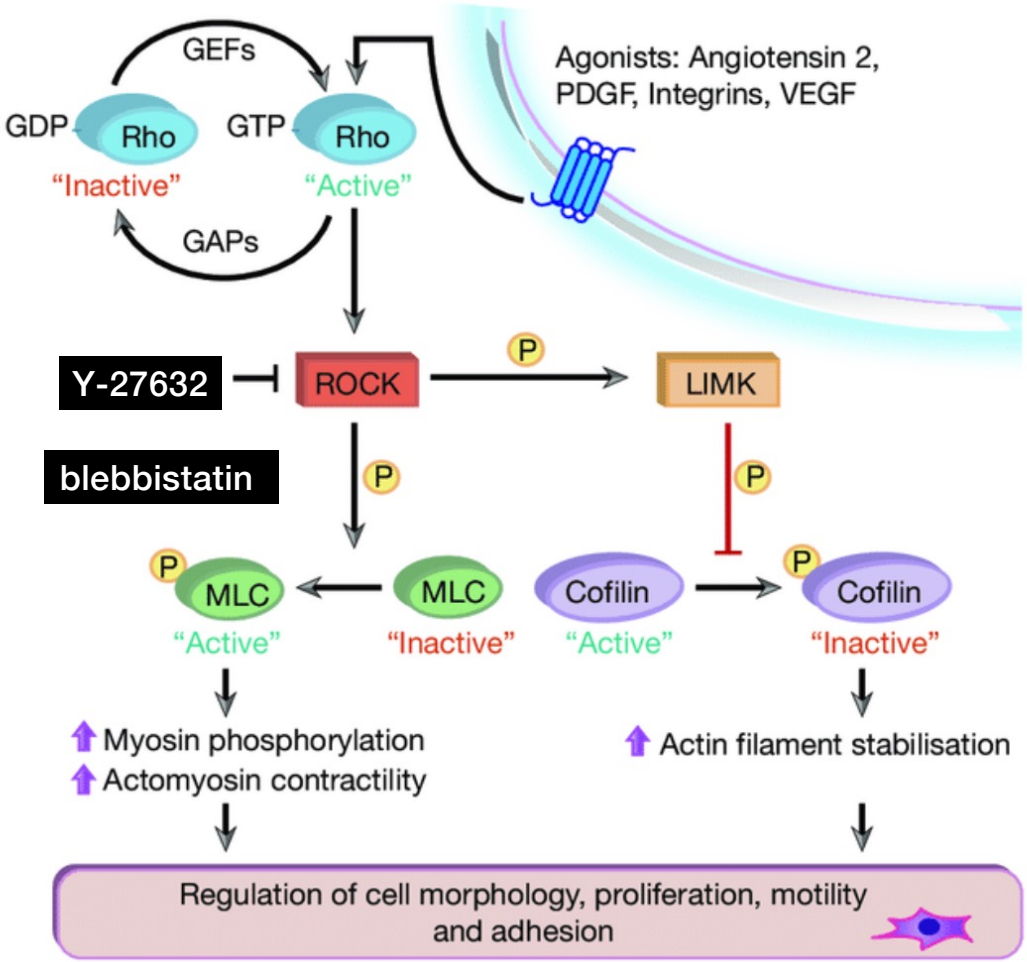




Blebb: Blebbistatin treatment

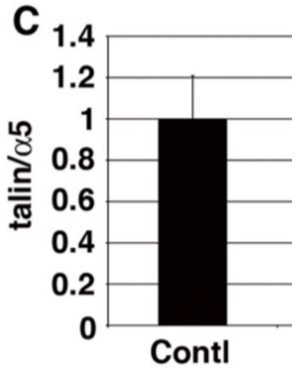
Pharmacological approach

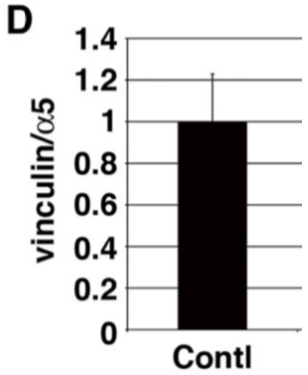
Inhibitors



Key components of the Rho/ROCK signalling pathway

Fig.1





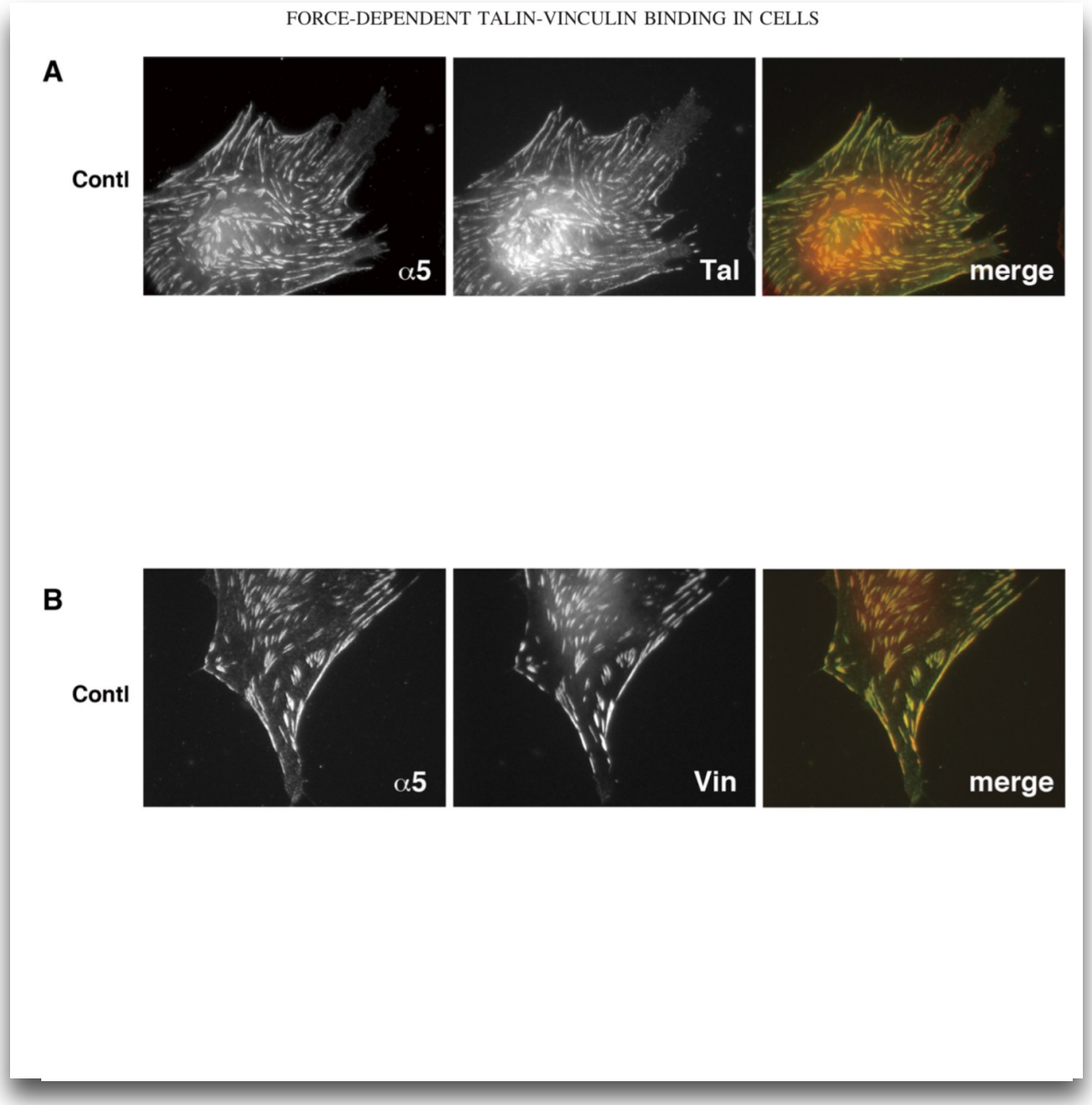


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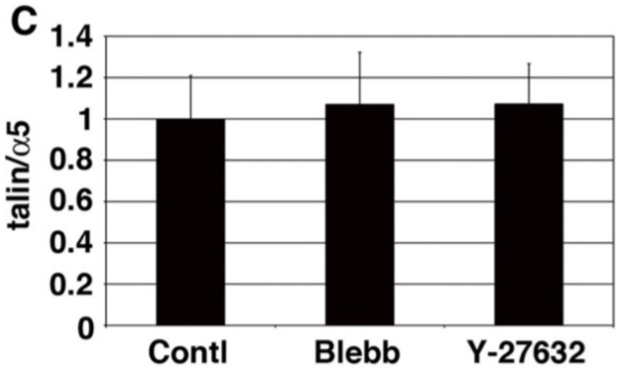
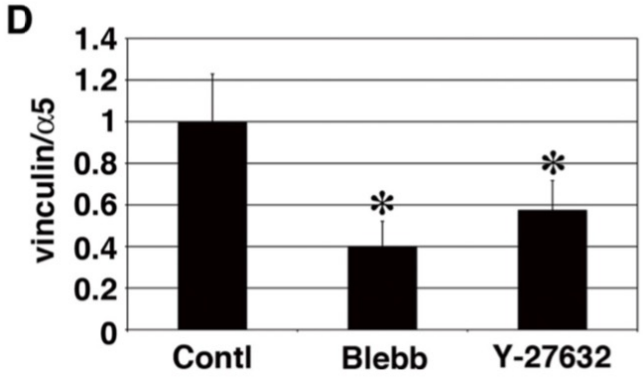
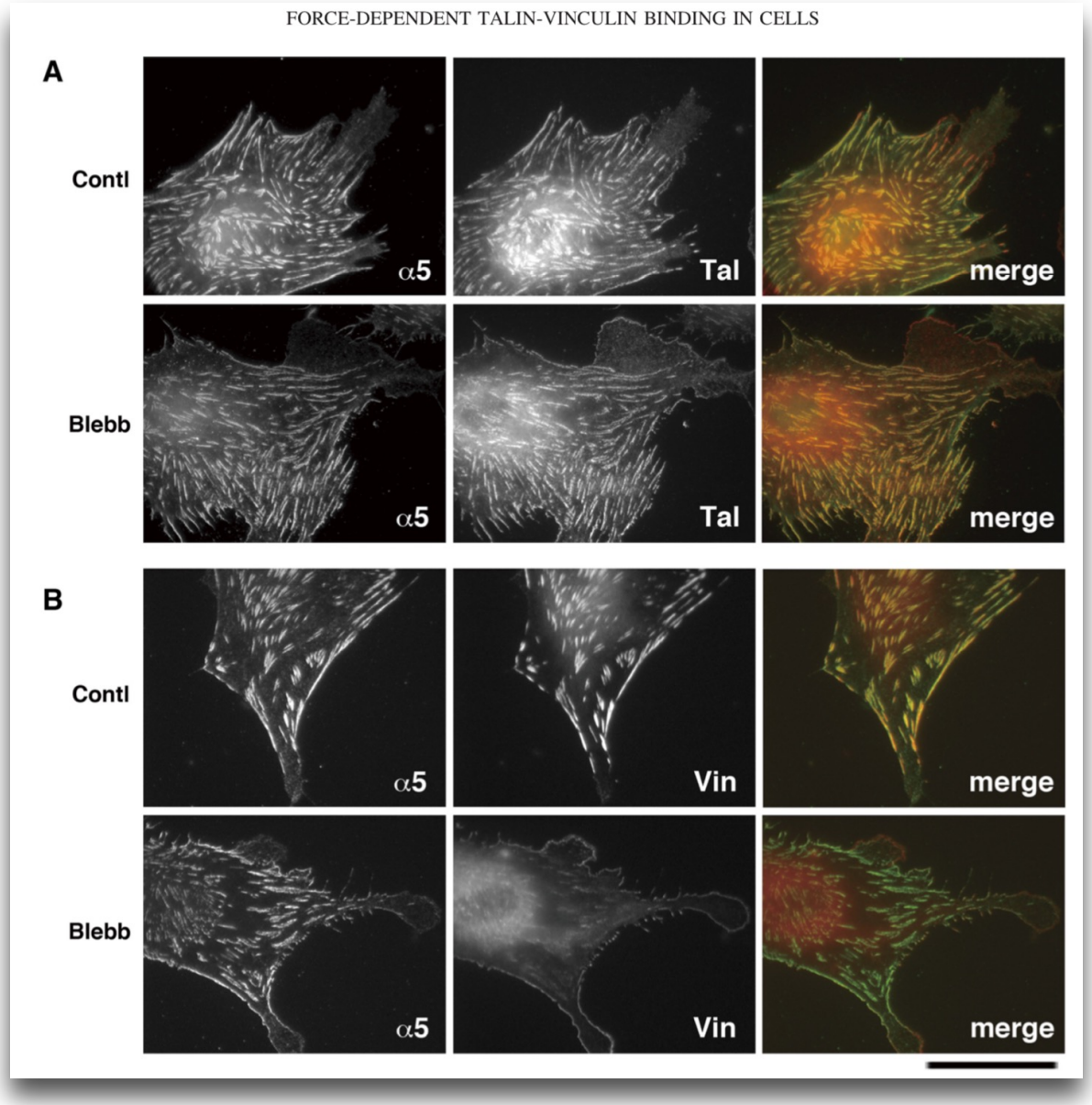
Fig.1



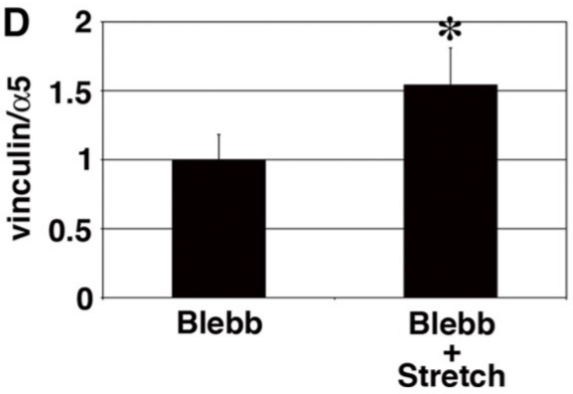


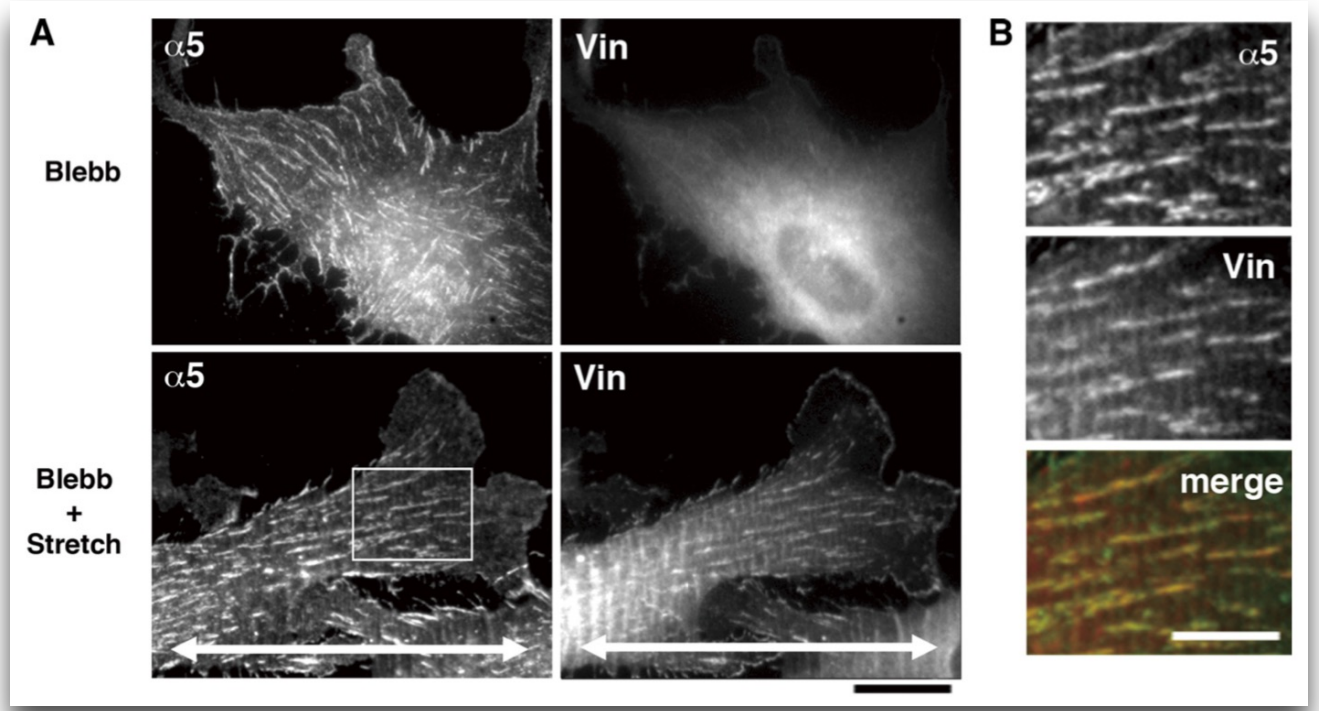


Blebb: Blebbistatin treatment

Fig.2

Stretching substrata induces vinculin accumulation at FAs in myosin II-inhibited cells.





A: HFF cells grown on fibronectin (FN)-coated elastic substrata were treated with 100 μM blebbistatin for 30 min, and then the substrata were uniaxially stretched (50% for 3 min) in the presence of blebbistatin.

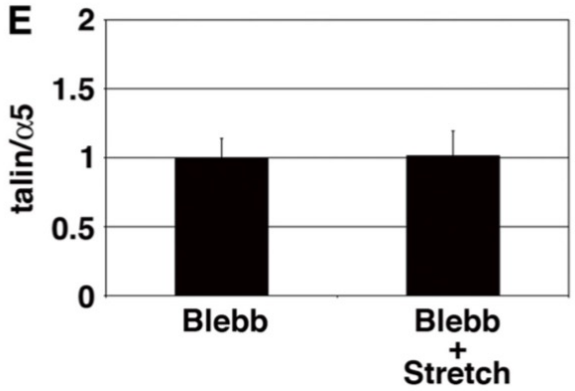
Cells without (Blebb) or with stretching substratum (Blebb + Stretch) were double-stained for α5-integrin (α5) and vinculin (Vin).

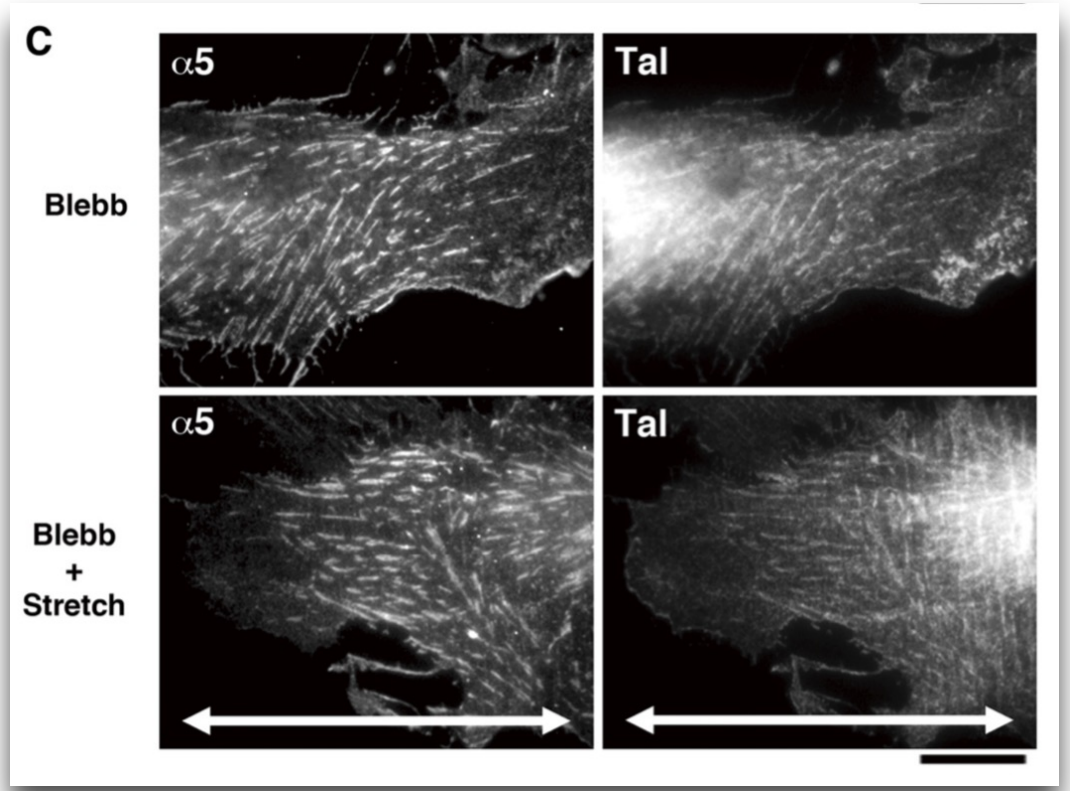
Double-headed arrows indicate the direction of the stretch axis.

B: high magnification of the boxed area in A. Merged image (green for alfa 5-integrin and red for vinculin) is also shown.

Fig.2

Stretching substrata do not affect talin localization at FAs in myosin II-inhibited cells.





C: HFF cells grown on fibronectin (FN)-coated elastic substrata were treated with 100 μM blebbistatin for 30 min, and then the substrata were uniaxially stretched (50% for 3 min) in the presence of blebbistatin.

Cells without (Blebb) or with stretching substratum (Blebb - Stretch) were double-stained for α5-integrin (α5) and talin (Tal).

Double-headed arrows indicate the direction of the stretch axis.

Dominant negative?

Plasmids and transfection. The vinculin domain D1 (amino acid 1–258) (19, 32) was amplified by PCR using mouse vinculin cDNA (a gift from Cheng-Han Yu, National University of Singapore) (64) as a template and subcloned into the pcDNA3-EGFP vector. The A50I mutant form of the domain D1 was generated by the QuickChange mutagenesis method (Agilent Technologies, Santa Clara, CA) using primers 5′-CGCCGTGCAGGCGATCGTCAGCAACCTCGTC-3′ and 5′-GACGAGGTTGCTGACGATCGCCTGCACGGCG-3′. pcDNA3-EGFP and pcDNA3-α-actinin-1-mCherry were provided by Hiroaki Machiyama (National University of Singapore).

For introducing EGFP, EGFP-D1, EGFP-vinculin, and/or α-actinin-mCherry into HFF cells, cells were transiently transfected with their expression plasmids using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

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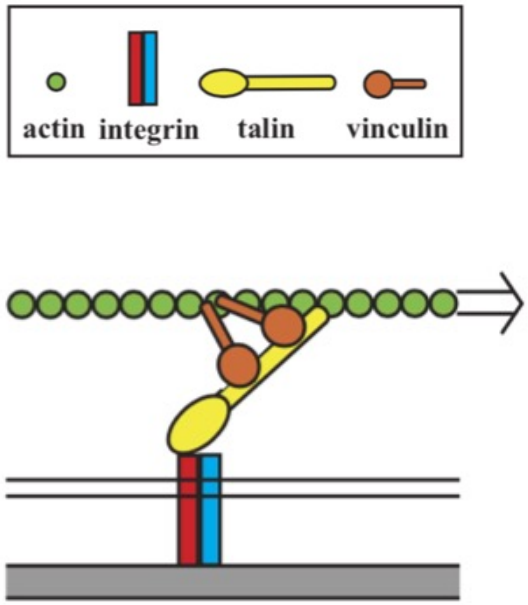

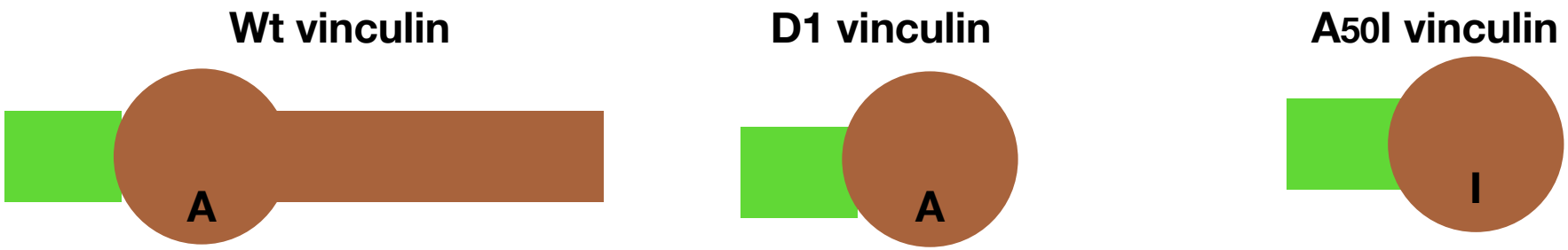

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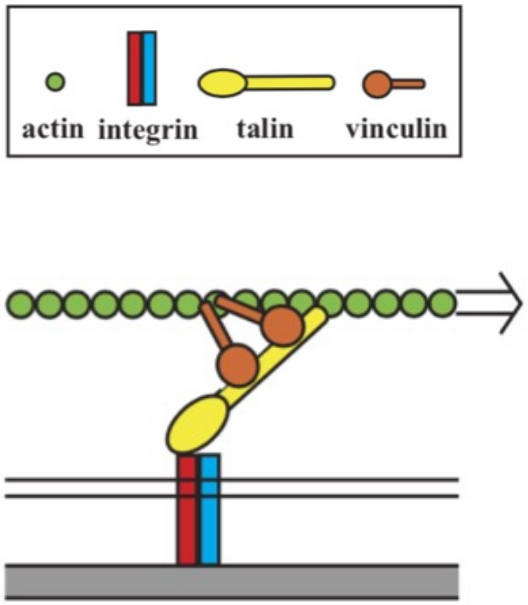
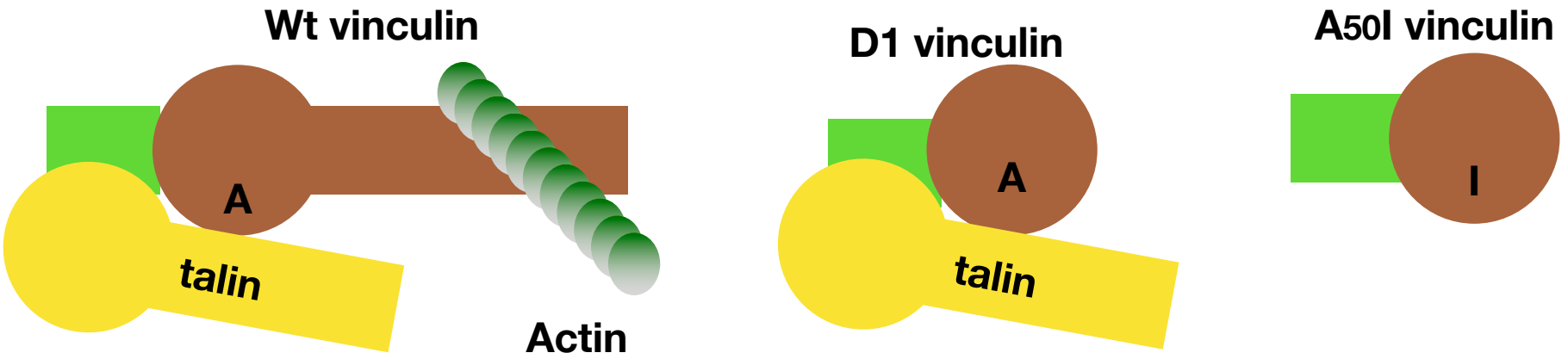
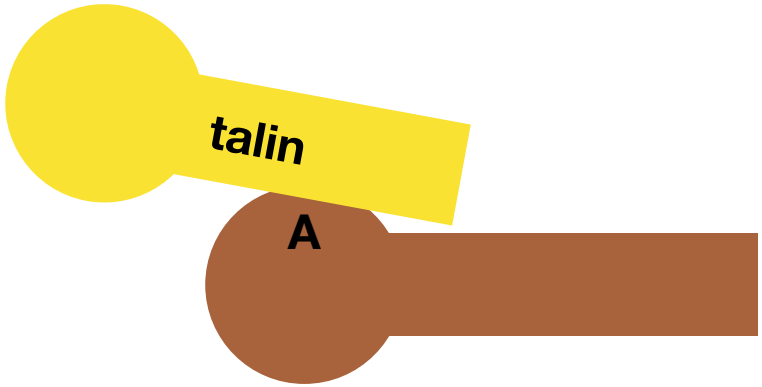


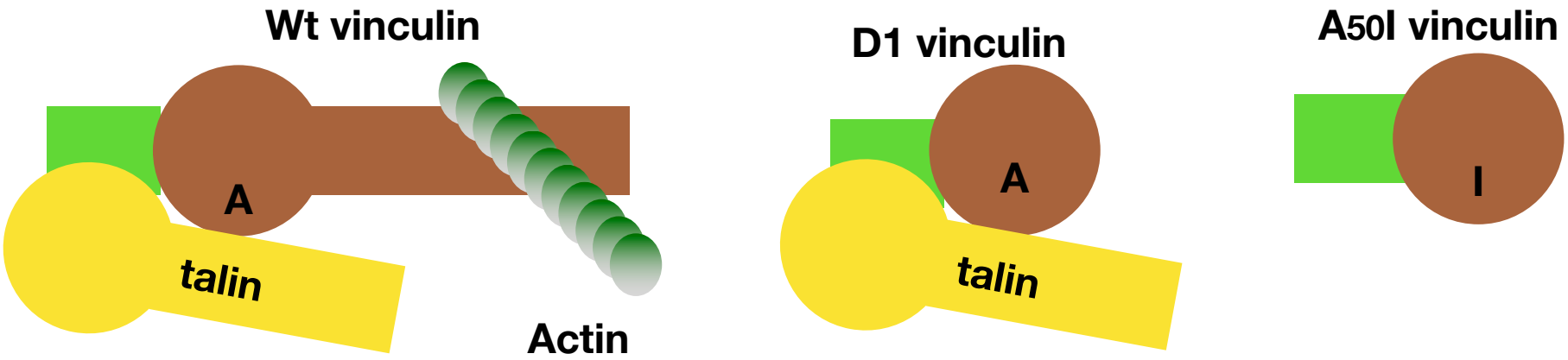


Endogenous expression



Wt vinculin

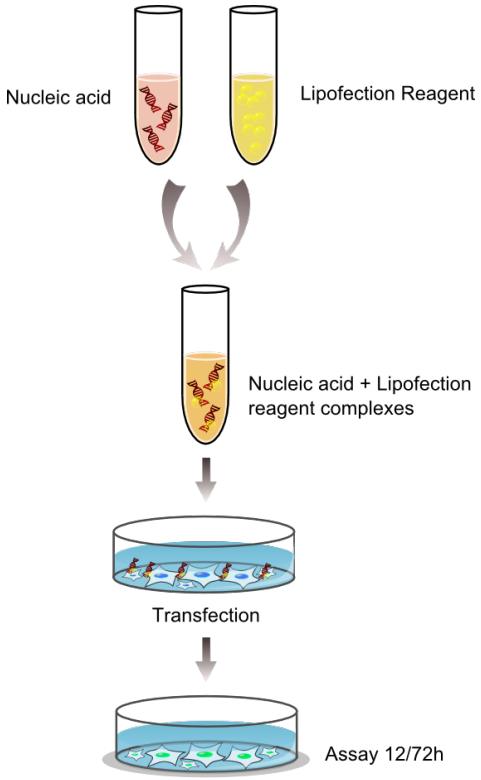
Transfected constructs

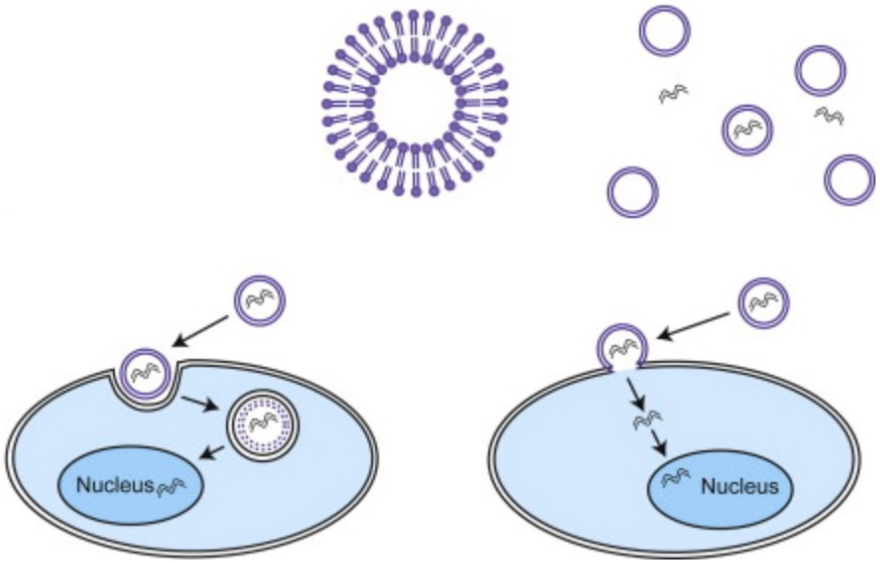


Transfection

For introducing EGFP, EGFP-D1 into HFF cells, cells were transiently transfected with their expression plasmids using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Lipofectamine is a commercial name
The technique is lipofection





Transfection

Example of alternative methods to introduce exogenous DNA in eukaryotic cells

In which cells are suitable for microinjection?
Stable versus transient transfection?