Supplemental Information

Figure S1. (Left) Lineage marker distributions as a function of ECM stiffness for NSCs differentiated in mixed media for 6 days shows that more undifferentiated, Nestin positive cells remain on stiff substrates, with an additional 10-20% marker negative cells on all stiffnesses. (Right) Immunofluorescence images of cells cultured on 100 Pa and 75000 Pa ECMs.

Figure S2. RhoA-GTP and Cdc42-GTP levels of NSCs expressing DN and CA RhoA and Cdc42 cultured in proliferating conditions on laminin-coated tissue-culture polystyrene. Levels are normalized to control, as determined by GLISA assays of samples loaded with equivalent total protein levels. Error bars are 95% confidence intervals. *$p < 0.05$ (Student's unpaired two-tailed t-test).

Figure S3. 95% confidence bounds, (A, B) solid lines – control, dotted lines – (A) DN RhoA and (B) DN Cdc42, dashed lines – (A) CA RhoA and (B) CA Cdc42, generated from one-way analysis of covariance on log-transformed substrate elastic modulus and cell elastic modulus data shown in Figure 3, reveal lower stiffnesses of NSCs expressing DN RhoA and DN Cdc42 compared to control on substrates above 1000 Pa. Error bars for NSCs on thin gels are 95% confidence intervals for n = 17-50 cells. *$p < 0.05$ (ANOVA-TK) for comparisons to control on each ECM elastic modulus (control data previously shown in Figure 2A).

Figure S4. 95% confidence bounds, (A, B) solid lines – control, dotted lines – (A) DN RhoA and (B) DN Cdc42, dashed lines – (A) CA RhoA and (B) CA Cdc42, generated from one-way analysis of covariance on log-transformed substrate elastic modulus and cell immunostaining data shown in Figure 3, show that (A) DN RhoA and (B) DN Cdc42 increase the proportion of neurons and decrease the proportion of astrocytes on stiffer ECMs compared to control after 6

days. In contrast, (A) CA RhoA and (B) CA Cdc42 decrease the proportion of neurons and increase the proportion of astrocytes on softer ECMs compared to control. (C) Higher power images of insets from Figure 3C-D.

Figure S5. Rho GTPases modulate the effect of ECM elastic modulus on the proportions of neurons and astrocytes in survival conditions. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to control for each substrate elastic modulus (control data previously shown in Figure 1B) (ANOVA-TK). β-tubulin III *(green)*, GFAP *(red)*, DAPI *(blue)*, MBP *(white)*. 95% confidence bounds, (E, F) solid lines – control, dotted lines – (E) DN RhoA and (F) DN Cdc42, dashed lines – (E) CA RhoA and (F) CA Cdc42, generated from one-way analysis of covariance on log-transformed substrate elastic modulus and cell immunostaining data shown in (A) and (B). (G, H) Higher power images of insets from (A, B).

Figure S6. Quantitative RT-PCR measurements of the increases in mRNA levels for neuronal and astrocytic markers in NSCs expressing CA or DN RhoA cultured on laminin-coated tissue culture polystyrene for 6 days in mixed conditions over the same NSCs cultured in self-renewal conditions (20 ng/ml FGF-2). Results show similar trends to immunostaining (Figure 4), with CA RhoA exhibiting a smaller increase in neuronal mRNA levels and a greater increase in astrocytic mRNA levels and DN RhoA exhibiting greater neuronal and smaller astrocytic increases in mRNA levels compared to control. Error bars are 95% confidence intervals, n = 3. *p < 0.05 for comparisons to control NSCs (ANOVA-TK).

Figure S7. Rho GTPases modulate NSC lineage compositions on laminin-coated glass. Immunostaining against β-tubulin III (*green bars*), GFAP (*red bars*), and MBP (*orange bars*) of cells cultured on glass show that DN RhoA and Cdc42 increase the percentage of neurons and

decrease the percentage of astrocytes while CA Cdc42 slightly increases the percentage of oligodendrocytes over control cells after 6 days of differentiation in (A) mixed conditions and (B) survival conditions. DN and CA Rac1 do not affect NSC lineage compositions in either media condition. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to control (ANOVA-TK). Representative immunofluorescence images of NSC lines after 6 days of differentiation in (C) mixed conditions and (D) survival conditions. β-tubulin III *(green)*, GFAP *(red)*, DAPI *(blue)*, MBP *(white)*. (E) Higher power images of insets from (D).

Figure S8. Neither expression of DN and CA Rho GTPases nor variation of ECM stiffness compromises later stages of neuronal maturation and subtype marker expression, with GABAergic (GABA, *red*) and glutamatergic (VGlut1, *green*) neurons detectable on substrates of 700 and 75,000 Pa and all RhoA/Cdc42 genotypes (DAPI, *blue*). Cells were cultured in 1 μM forskolin and 5 μM all-trans retinoic acid for 6 days then switched to 20 ng/ml brain-derived neurotrophic factor with 1 μM forskolin for another 6 days.

Figure S9. Inhibition of proteins that regulate cellular contractility reduces astrocytic differentiation in mixed conditions on soft and stiff ECMs. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to the same NSC population in control media conditions (control data previously shown in Figure 1A) (ANOVA-TK).

Figure S10. (A) Inhibition of proteins that regulate cellular contractility rescues neuronal differentiation in survival conditions on soft and stiff ECMs. Error bars are 95% confidence intervals, n = 6. *p < 0.05 for comparisons to the same NSC population in control media conditions (ANOVA-TK). (B and C) Inhibition of proteins that regulate cellular contractility and adhesion do not appear to modulate the proportion of oligodendrocytes generated from NSCs.

(B) mixed and (C) survival conditions. Error bars are 95% confidence intervals, n = 6. *p < 0.05 for comparisons to the same NSC population in control media conditions (ANOVA-TK).

Supplemental Experimental Procedures

Neural Stem Cell Culture

Standard cultures were grown on tissue culture polystyrene coated with 10 μg/ml poly-ornithine (Sigma-Aldrich, St. Louis, MO) and 5 μg/ml mouse laminin (Invitrogen, Carlsbad, CA), in Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1, Invitrogen) supplemented with N2 supplement (Invitrogen) and 20 ng/ml recombinant human basic fibroblast growth factor (Peprotech, Rocky Hill, NJ). Glass substrates were coated with 20 μg/ml poly-ornithine and 10 μg/ml mouse laminin.

Viral Production, In Vitro Transduction, and In Vivo Delivery

cDNAs were subcloned into the murine retroviral vector plasmid CLGPIT (Peltier et al., 2007) for in vitro transduction and into pCAG-IRES-GFP (modified from Addgene Plasmid 16664 (Zhao et al., 2006)) for in vivo delivery. The resulting vectors were packaged, concentrated, and purified as described (Peltier et al., 2007). In vitro, NSCs were infected at a multiplicity of infection of 1 IU/cell and were selected with 0.6 μg/ml puromycin (Sigma) for 4 days. For in vivo studies, eight-week-old adult female Fisher 344 rats were anesthetized prior to 3 μL bilateral intrahippocampal stereotaxic injections of retrovirus. Injection coordinates were -3.5 mm anteriorposterior and ± 1.8 mm mediolateral relative to bregma and -3.3 mm dorsoventral relative to dura. BrdU (Sigma) was administered intraperitoneally at 50 mg/kg dissolved in saline. All animal protocols were approved by the Animal Care and Use Committee of the University of California Berkeley.

Immunofluorescence and Immunohistochemical Staining

Cells and tissue sections were fixed with 4% paraformaldehyde. BrdU-treated samples were incubated in 2 N HCl, neutralized with 0.1 M borate buffer prior to blocking and permeabilizing in 2% (5% for sections) goat serum (Sigma) and 0.3% Triton X-100 (Calbiochem, San Diego, CA) in pH 7.4 phosphate buffered solution at room temperature. Samples were incubated for 36 hours at 4°C with the following primary antibodies: rabbit anti-β-tubulin III (1:1000 dilution; Covance, Emeryville, CA), mouse anti-glial fibrillary acidic protein (GFAP, 1:1000 dilution; Advanced ImmunoChemical Inc., Long Beach, California), rat anti-myelin basic protein (MBP, 1:100 dilution; Abcam Inc., Cambridge, MA), rabbit anti-cleaved caspase 3 (1:400 dilution; Cell Signaling, Danvers, MA), rat anti-5-Bromo-2'-deoxyuridine (BrdU, 1:250 dilution; Abcam), guinea pig anti-γ-aminobutyric acid (GABA, 1:1000 dilution; Abcam), rabbit anti-vesicular glutamate transporter 1 (VGlut1, 1:2500 dilution; Synaptic Systems, Germany), rabbit anti-green fluorescent protein (GFP, 1:2000, Invitrogen, A11122), mouse anti-neuronal nuclei (NeuN, 1:100, Millipore, Billerica, MA), and guinea pig anti-doublecortin (DCX, 1:1000, Millipore). The primary antibody solution was removed, and cells were rinsed and incubated for 2 hours with the secondary antibodies FITC-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-rat IgG or Cy3-conjugated goat anti-guinea pig, and Cy5-conjugated goat anti-mouse IgG at a dilution of 1:250 (all from Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Nuclei were stained with DAPI (Invitrogen) at 10 μg/ml. Cells were manually scored as positive or negative for lineage markers in regularly spaced and rastered fields of view. Images were collected at 20x magnification on a Nikon Eclipse TE2000-E microscope with a Photometrics Coolsnap HQ2 camera, and exposure settings were chosen to minimize background fluorescence as determined using control samples without primary antibodies. 300-1500 cells were counted per culture until at least 300 cells were obtained. 3-6 experiments were performed in parallel cultures for each study. 40 μm hippocampal sections were stained, and 15-20 confocal images obtained on a LSM710 (Carl Zeiss Inc, Oberkochen, Germany) were z-stacked

and flattened in ImageJ. 200-600 GFP+ cells per rat were counted, which corresponded to 16 total hippocampii sections per animal. 4 rats were sacrificed for each condition.

Quantitative real time Polymerase Chain Reaction

Quantitative real time PCR (QRT-PCR) was used as a complementary technique to immunofluorescence staining to accurately quantify specific mRNA concentrations in cells. Cells were lysed and frozen in TRIZOL (Invitrogen), and mRNA was extracted and reverse transcribed to cDNA using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). Equivalent amounts of total RNA were transcribed into cDNA, which was subsequently used as template for each QRT-PCR reaction. The QRT-PCR assay used GFAP as a marker for astrocytics and β-tubulin III as a marker for neurons (utilizing a Bio-Rad Laboratories iCycler 5, Hercules, CA). To normalize any remaining variations in starting cDNA amounts, each reaction was carried out in duplex format with ribosomal 18S detected using Cal-dye TaqMan probes and the lineage marker was detected using FAM-dye TaqMan probes (Biosearch Technologies, Novato, CA). QRT-PCR reactions were run for each biological sample with n=5-6 for each condition.

The primers and TaqMan probes used are listed as follows: (GFAP, 5'-GACCTGCGACCTTGAGTCCT-3', 5'-TCTCCTCCTTGAGGCTTTGG-3', 5'-FAM490-TCCTTGGAGAGGCAAATGCGC-BHQ-3'), (β-tubulin III, 5'-GCATGGATGAGATGGAGTTCACC-3', 5'-CGACTCCTCGTCGTCATCTTCATAC-3', 5'-FAM490-TGAACGACCTGGTGTCTGAG-BHQ-3'), and (18S, 5'-GTAACCCGTTGAACCCCATTC-3', 5'-CCATCCAATCGGTAGTAGCGA-3', 5'-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3'). Standards for performing QRT-PCR were pPCR4-TOPO plasmids (Invitrogen) containing the amplicon of interest as an insert. The plasmids were linearized by restriction digestion and quantified by absorbance, and tenfold serial dilutions from 1 ng/mL to 10^{-9} ng/mL were prepared to generate a standard curve.

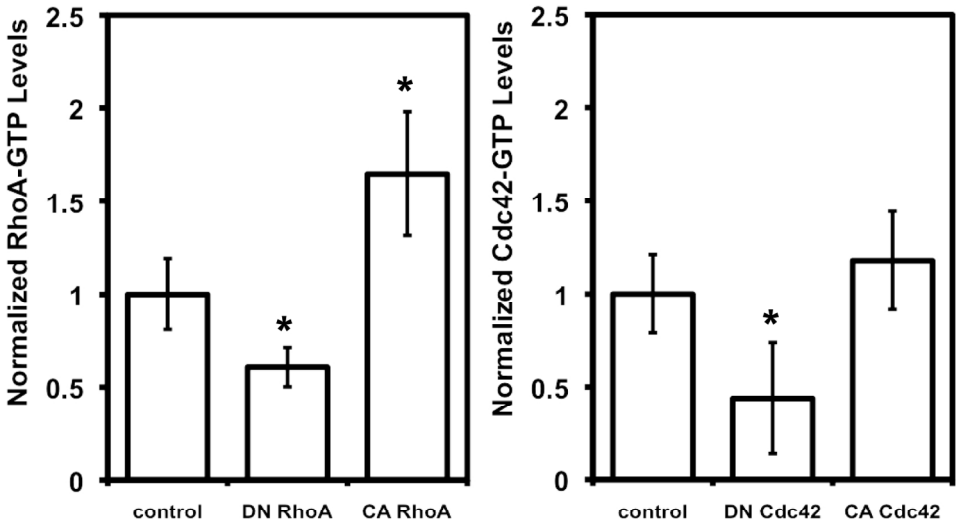

Figure S2

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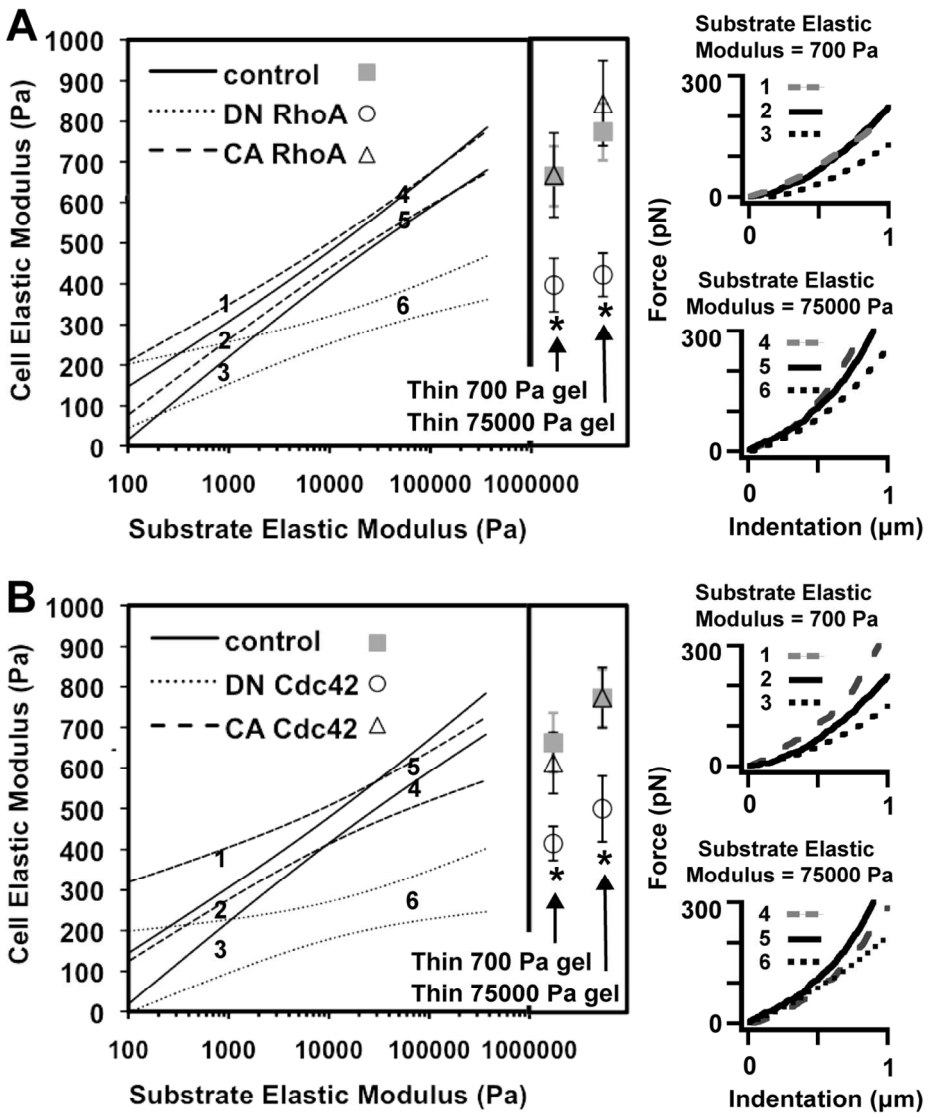

Figure S3

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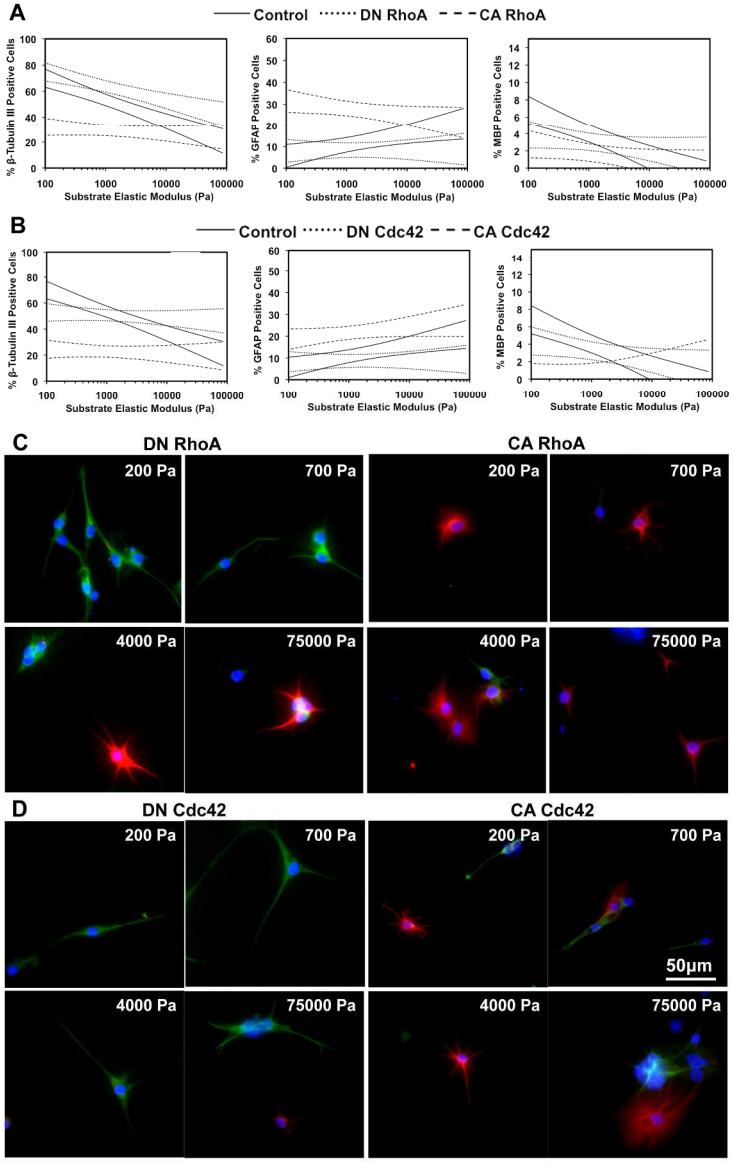

Figure S4

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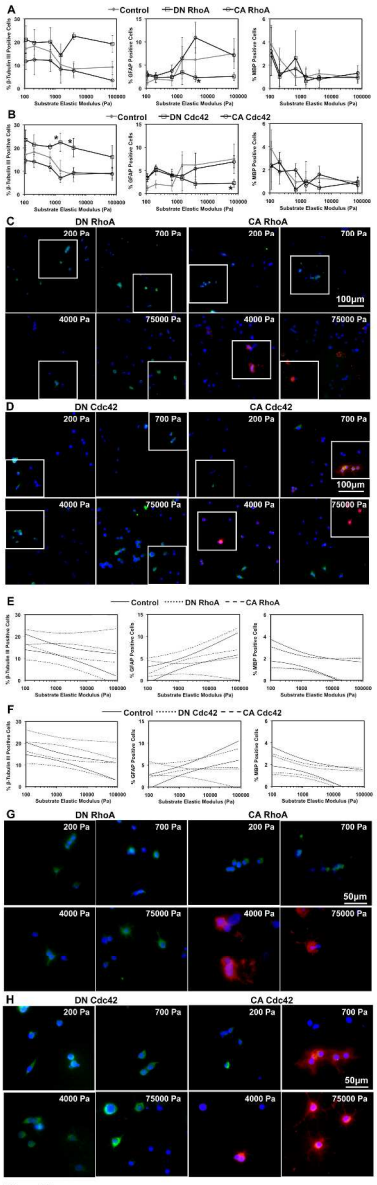

Figure S5

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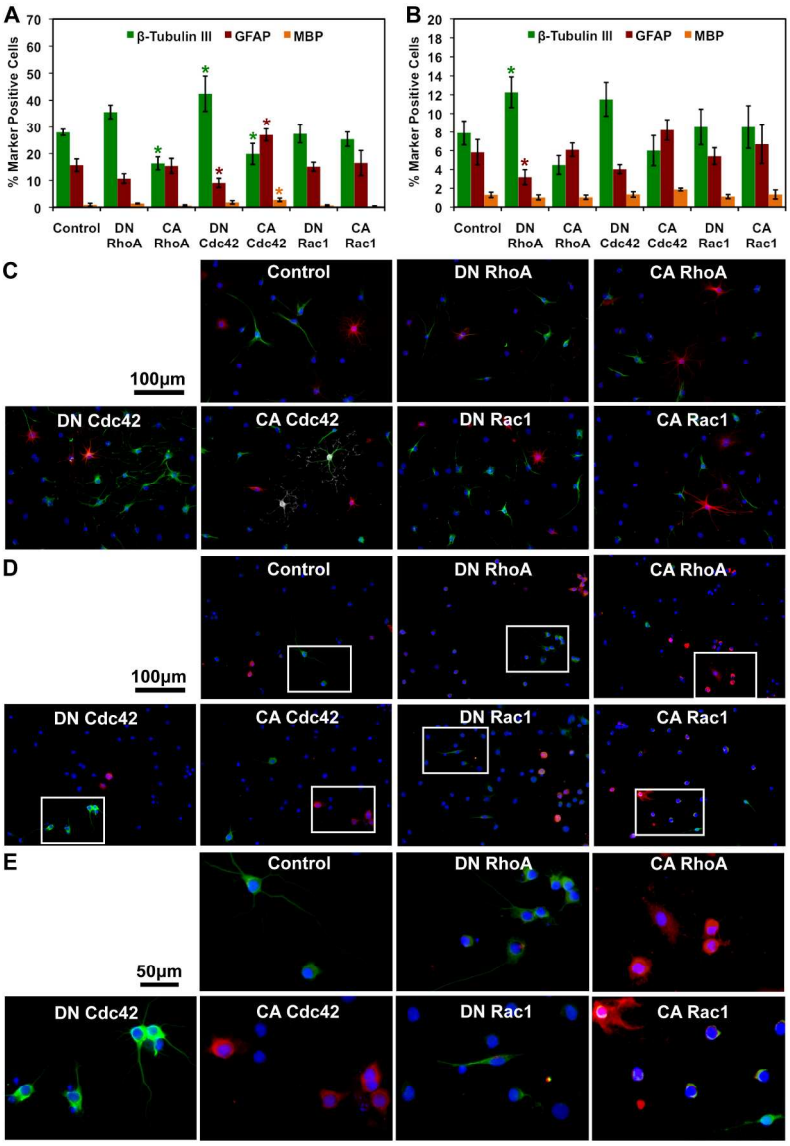

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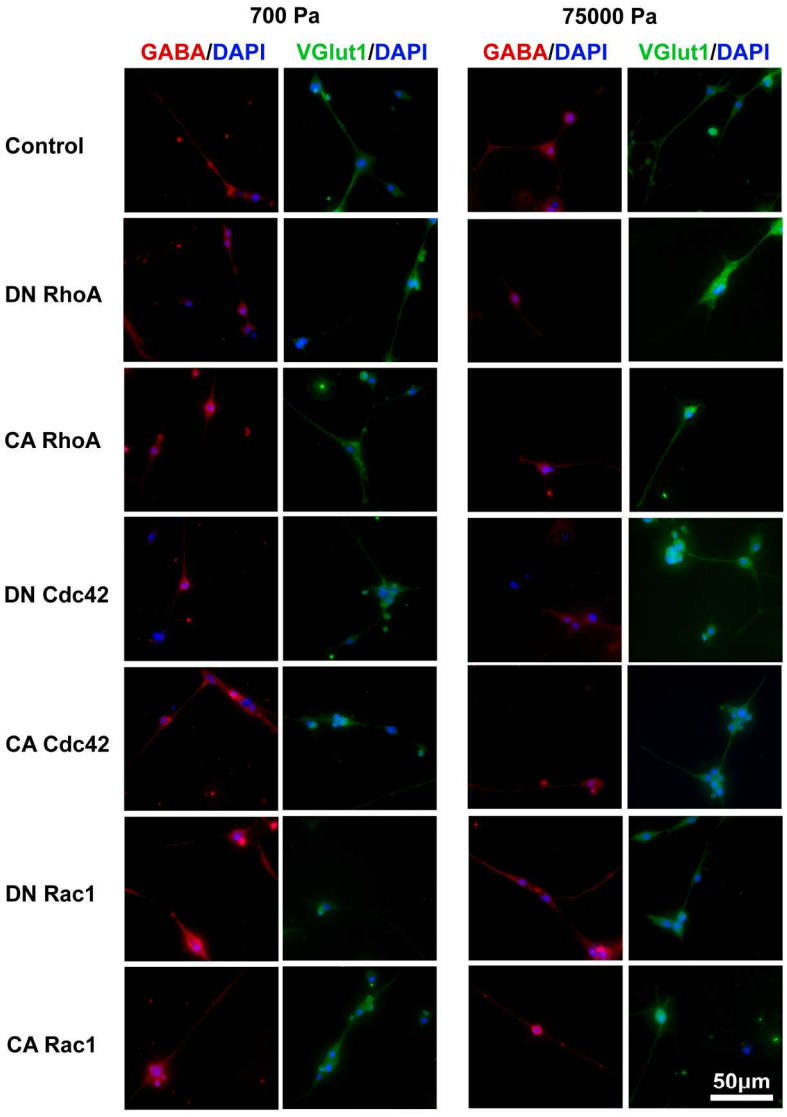

Figure S8

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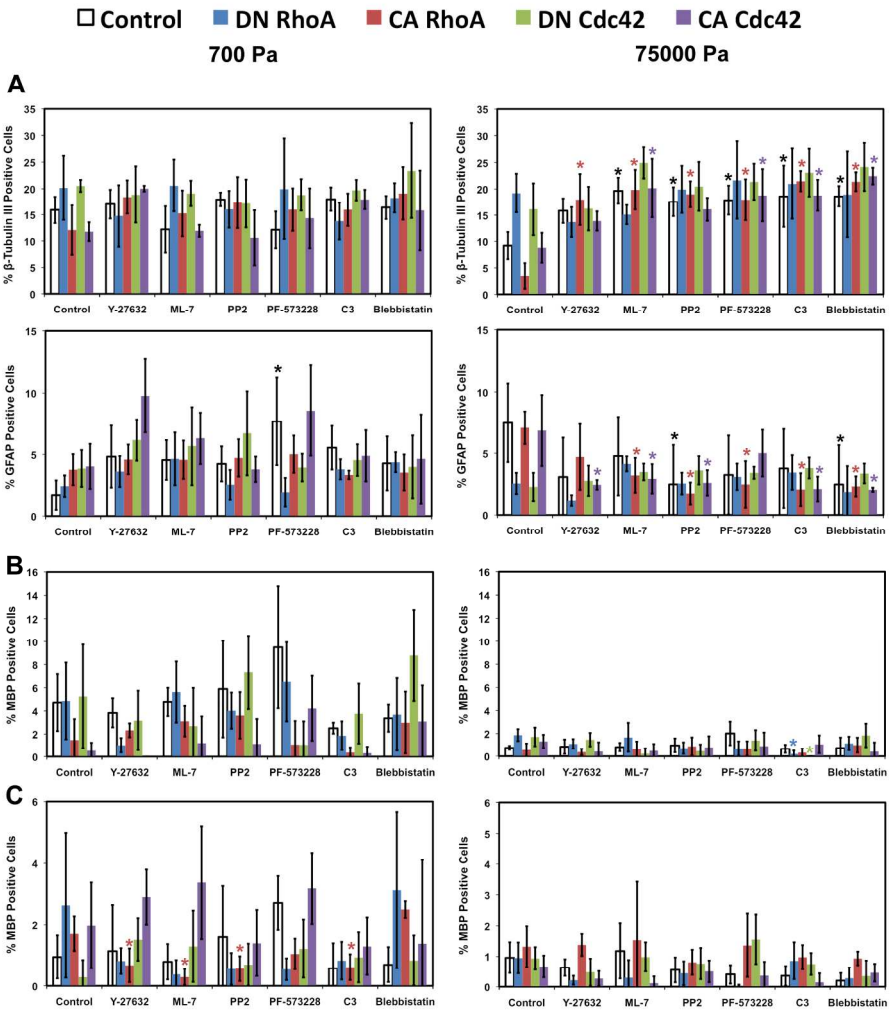

Figure S10

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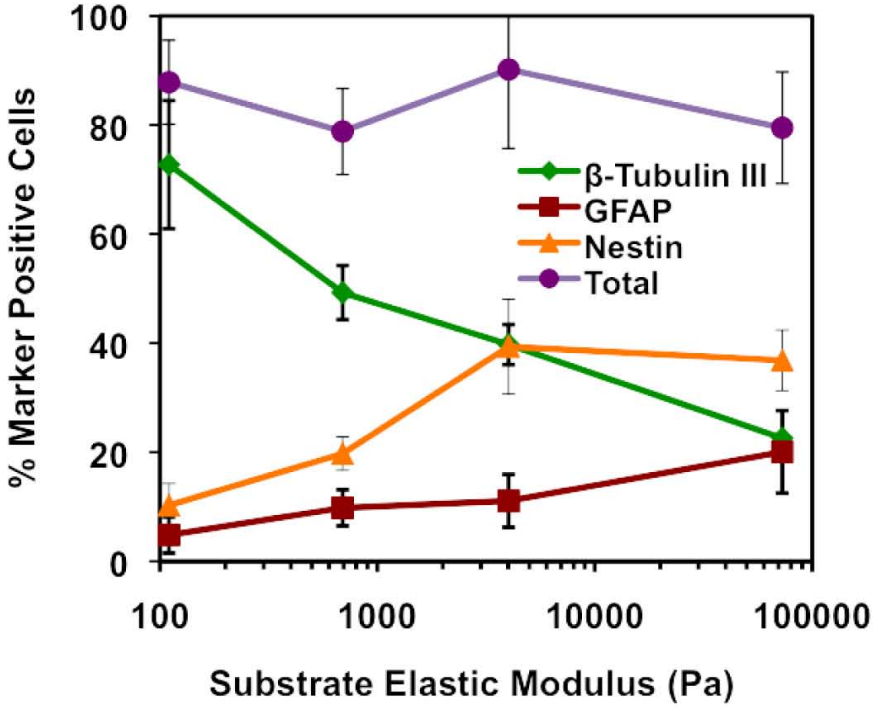
2606x1369mm (72 x 72 DPI)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80
60
40
20
0
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β-Tubulin III
GFAP
Nestin
Total
100
1000
10000
100000
Substrate Elastic Modulus (Pa)

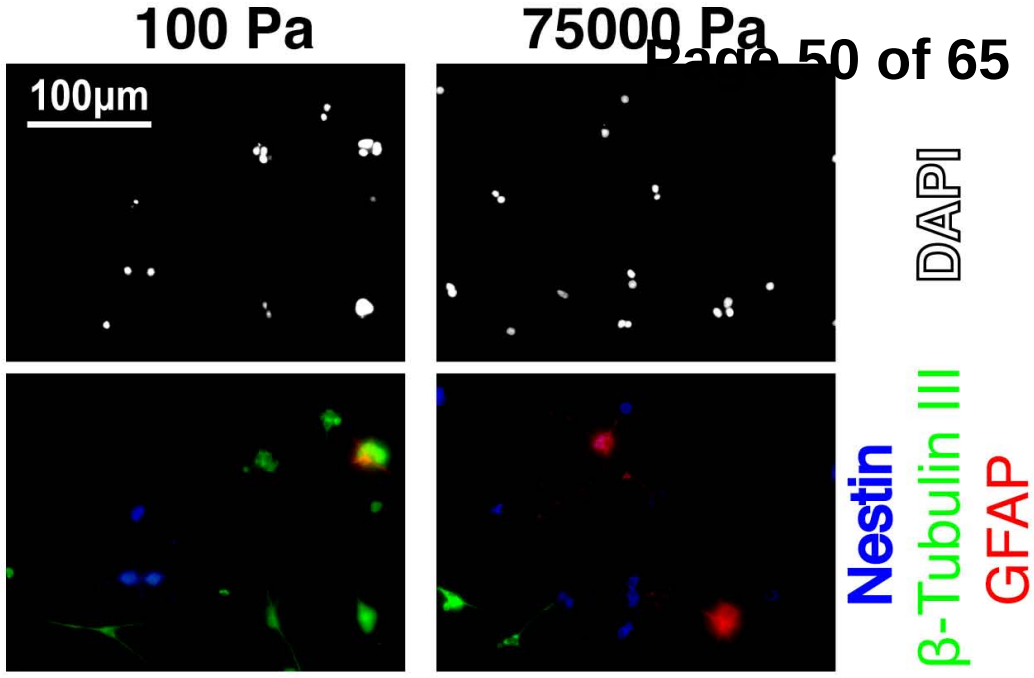
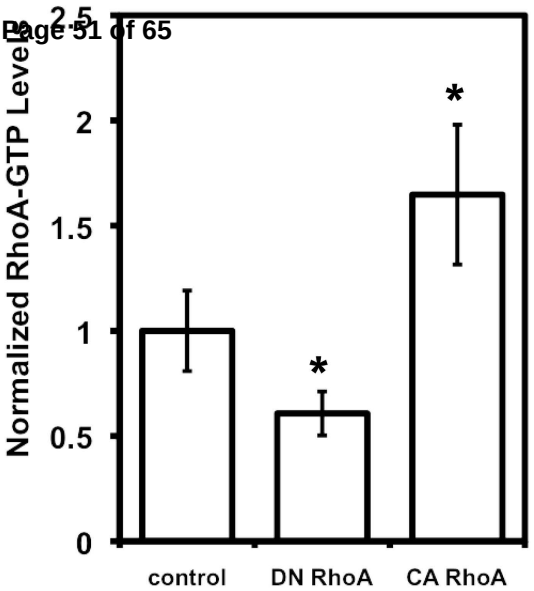

100 Pa
75000 Pa
100μm
DAPI
Nestin
β-Tubulin III
GFAP

Figure S1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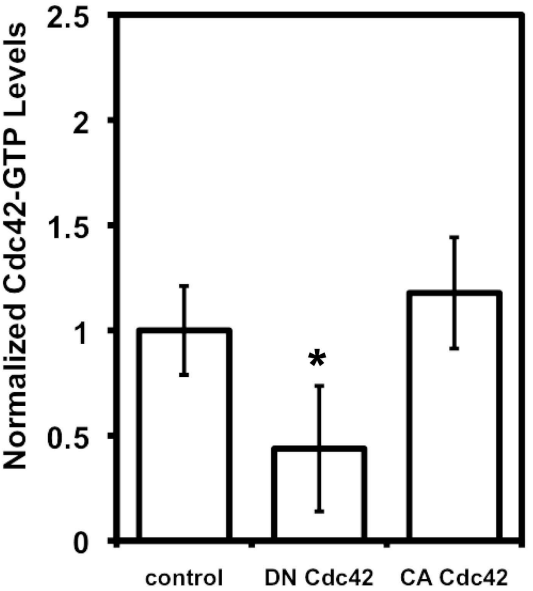
2.5
2
1.5
1
0.5
0
*
*
control
DN RhoA
CA RhoA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1.5
1
0.5
0
*
control
DN Cdc42
CA Cdc42

Figure S2

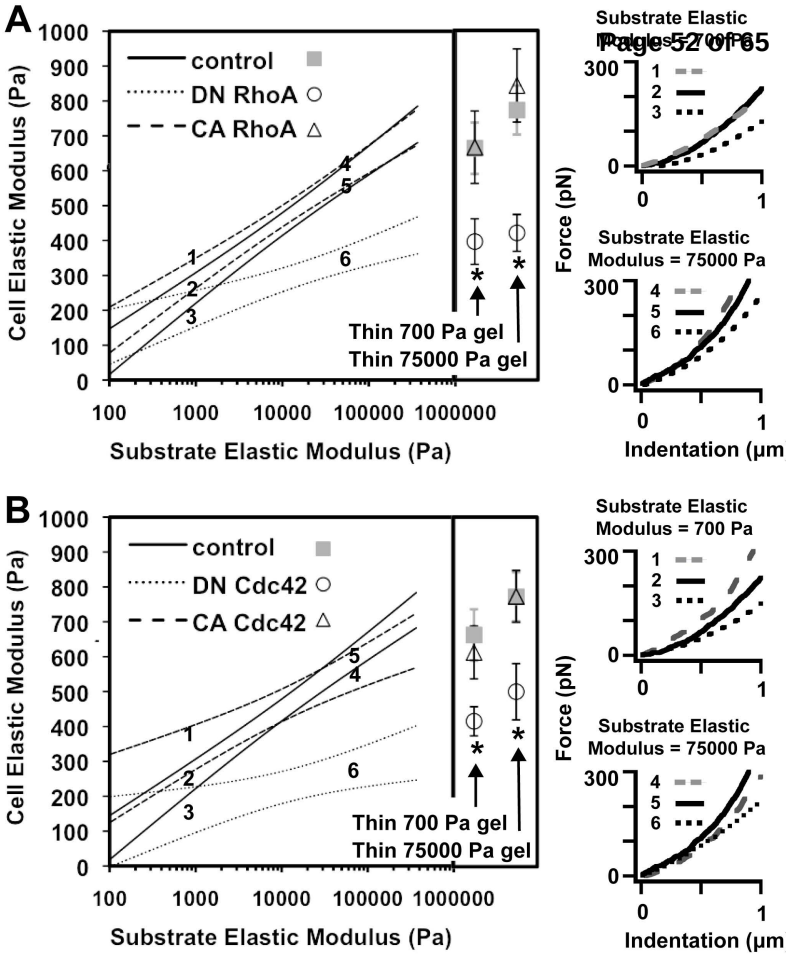
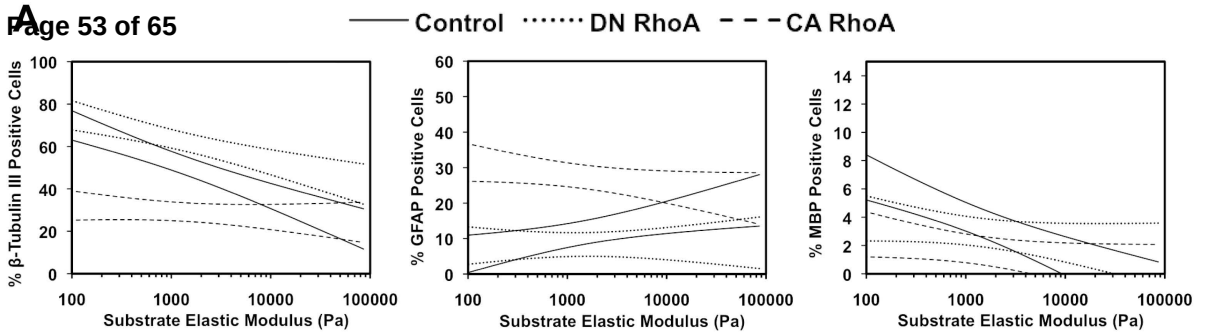
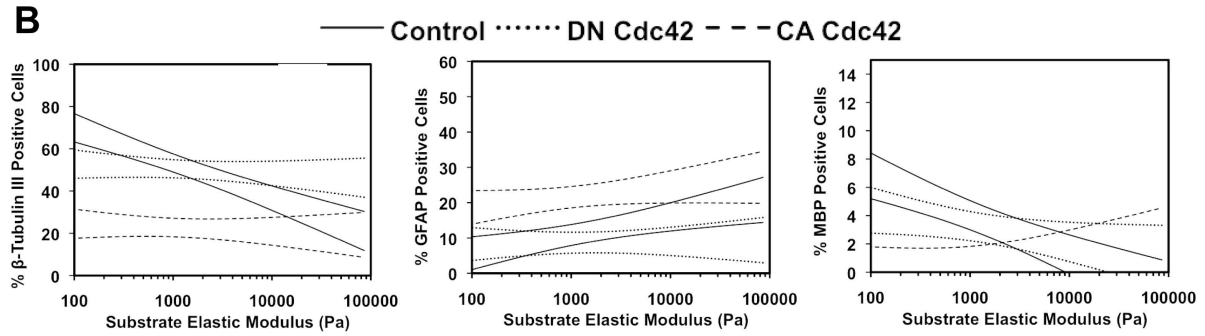
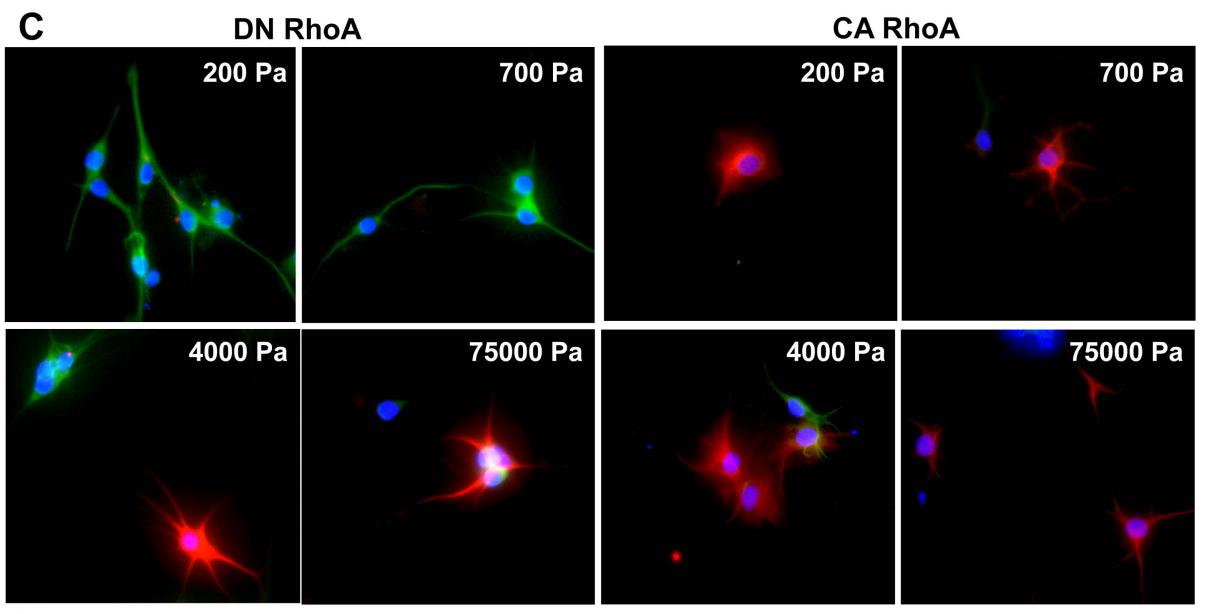

A
Cell Elastic Modulus (Pa)
control
DN RhoA
CA RhoA
1
2
3
4
5
6
Thin 700 Pa gel
Thin 75000 Pa gel
Substrate Elastic Modulus (Pa)
Substrate Elastic Modulus = 700 Pa
Substrate Elastic Modulus = 75000 Pa
Force (pN)
Indentation (μm)
B
Cell Elastic Modulus (Pa)
control
DN Cdc42
CA Cdc42
Thin 700 Pa gel
Thin 75000 Pa gel
Substrate Elastic Modulus (Pa)
Substrate Elastic Modulus = 700 Pa
Substrate Elastic Modulus = 75000 Pa
Force (pN)
Indentation (μm)

Figure S3


A
Control
DN RhoA
CA RhoA
% β-Tubulin III Positive Cells
% GFAP Positive Cells
% MBP Positive Cells
Substrate Elastic Modulus (Pa)
100
1000
10000
100000


B
Control
DN Cdc42
CA Cdc42
% β-Tubulin III Positive Cells
% GFAP Positive Cells
% MBP Positive Cells
Substrate Elastic Modulus (Pa)
100
1000
10000
100000


C
DN RhoA
CA RhoA
200 Pa
700 Pa
4000 Pa
75000 Pa

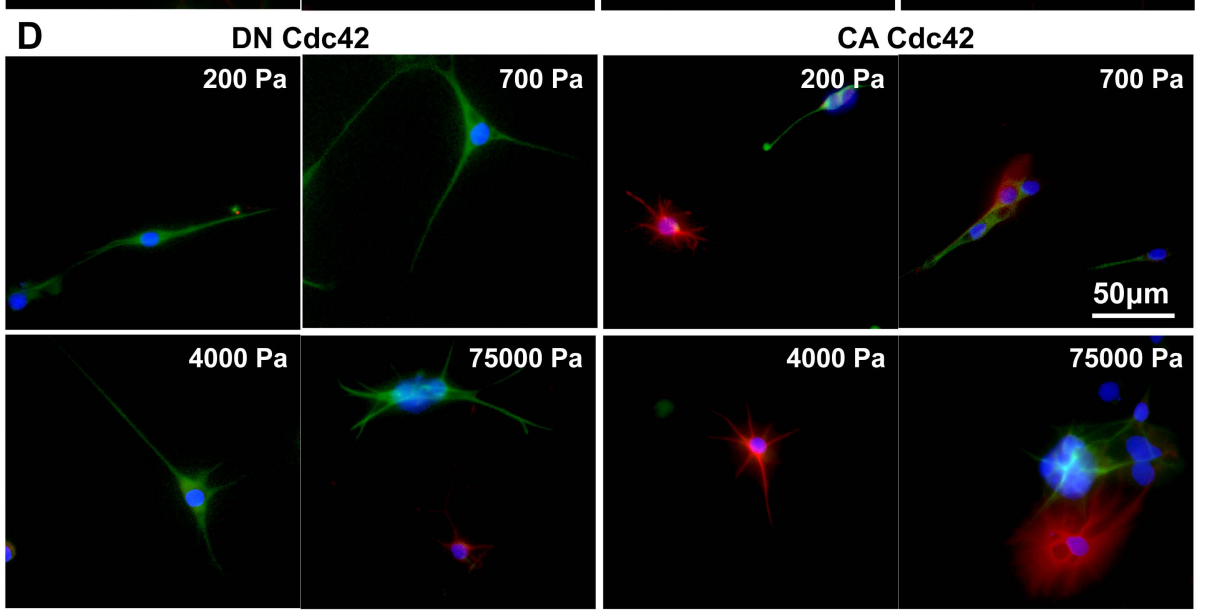
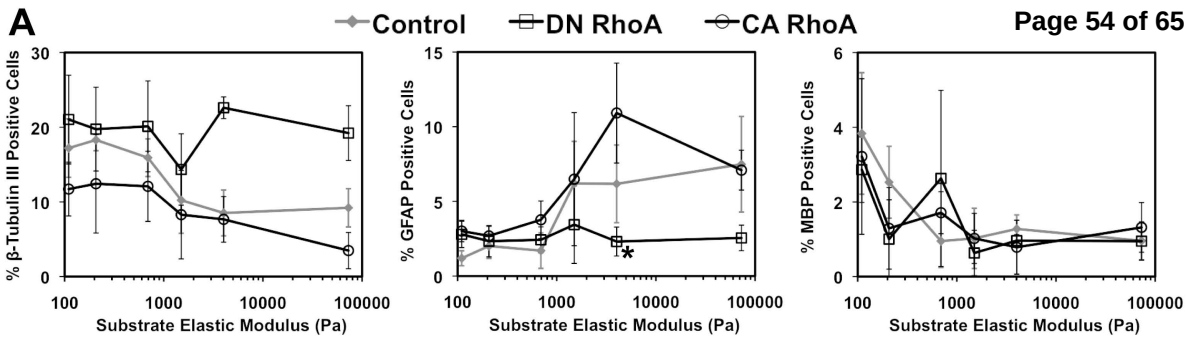

D
DN Cdc42
CA Cdc42
200 Pa
700 Pa
4000 Pa
75000 Pa
50μm

Figure S4

A



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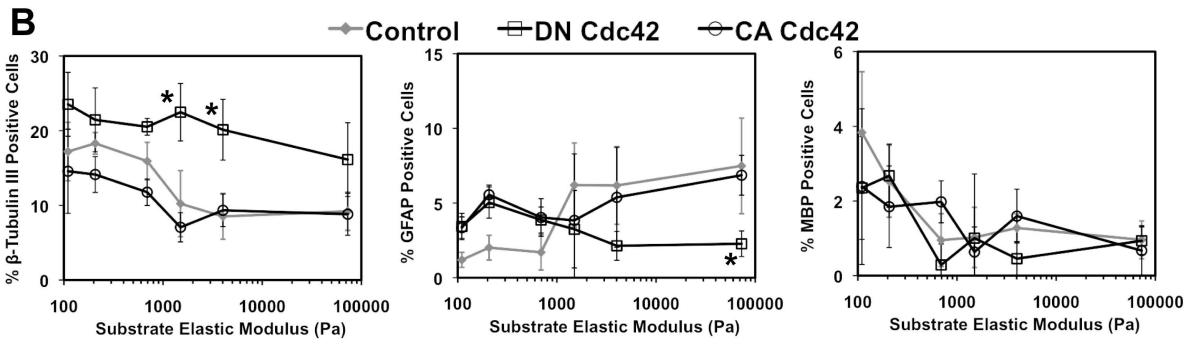

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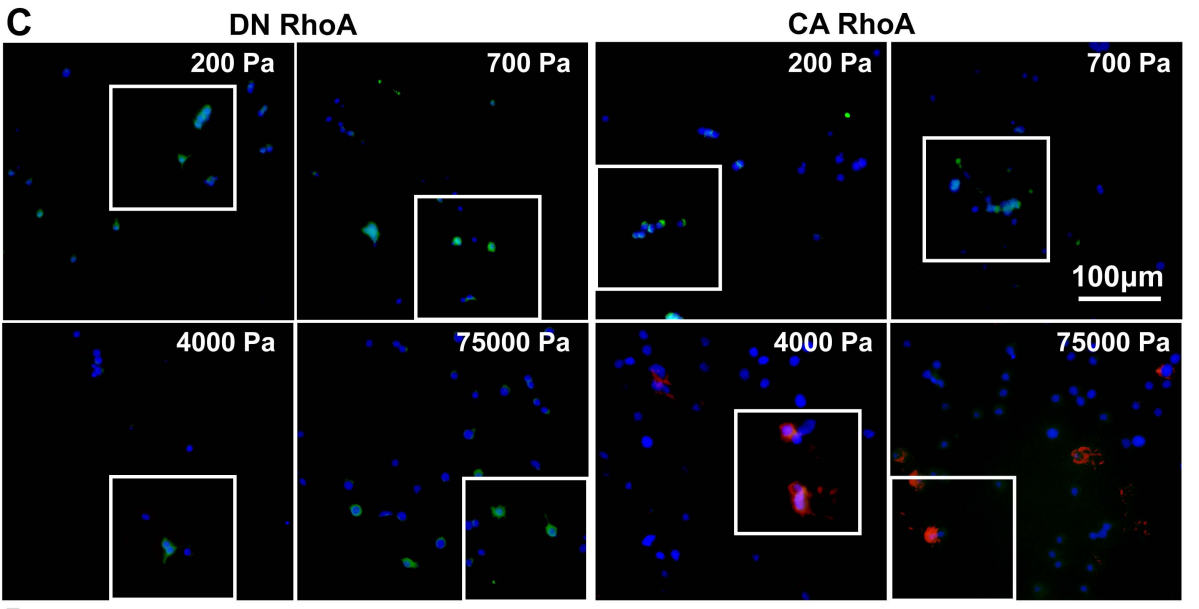
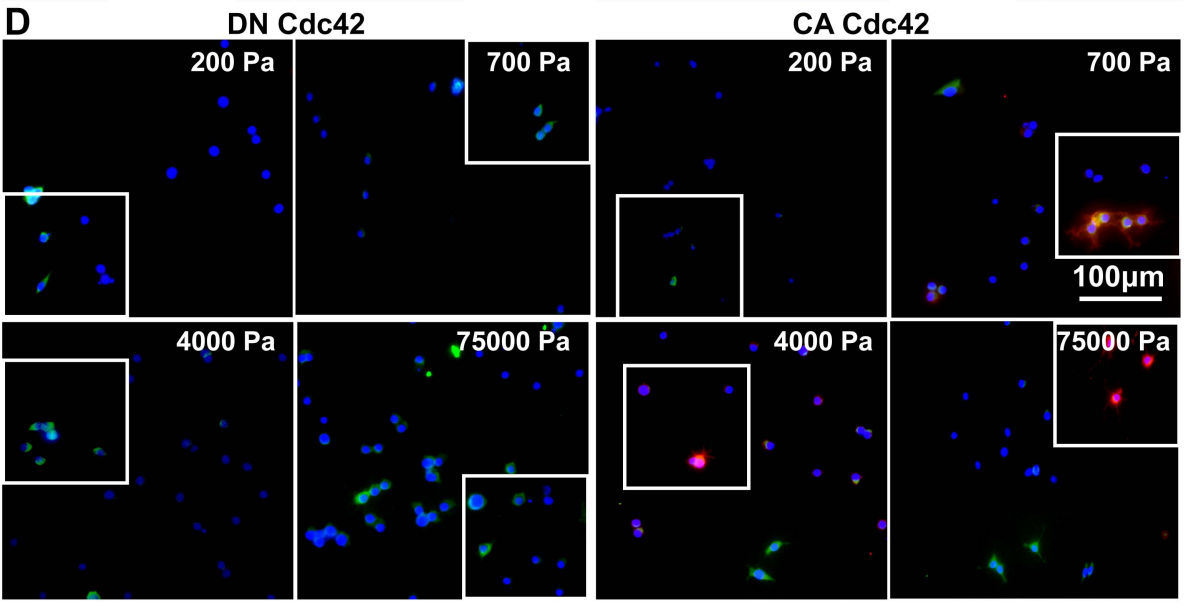
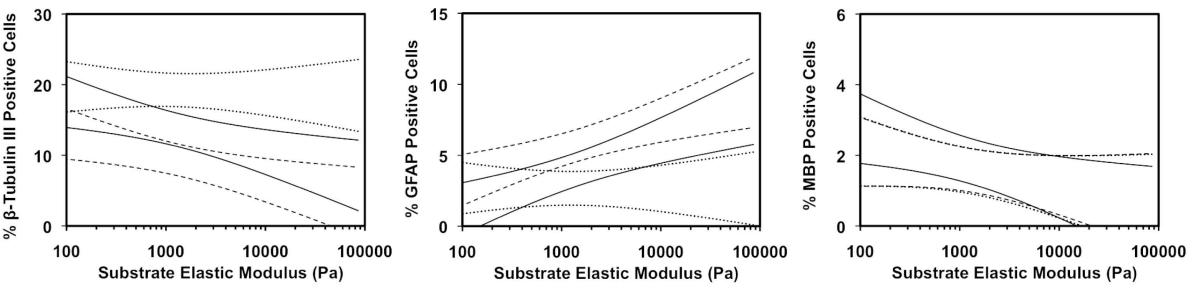


D



E



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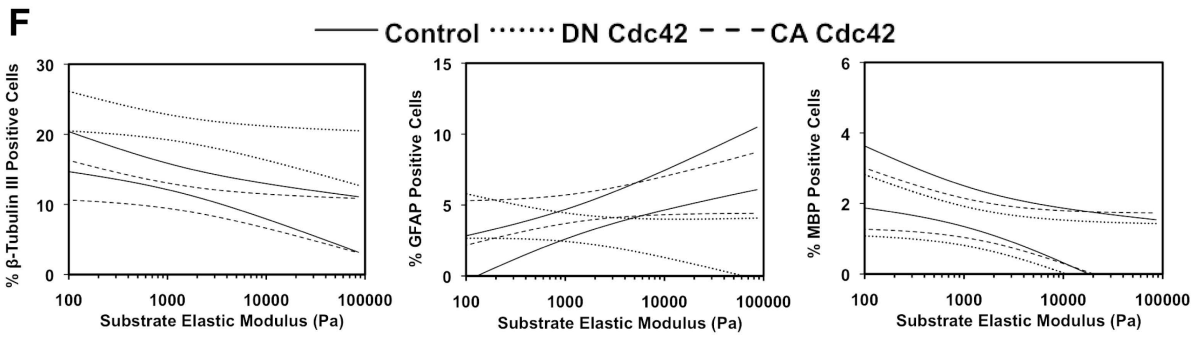

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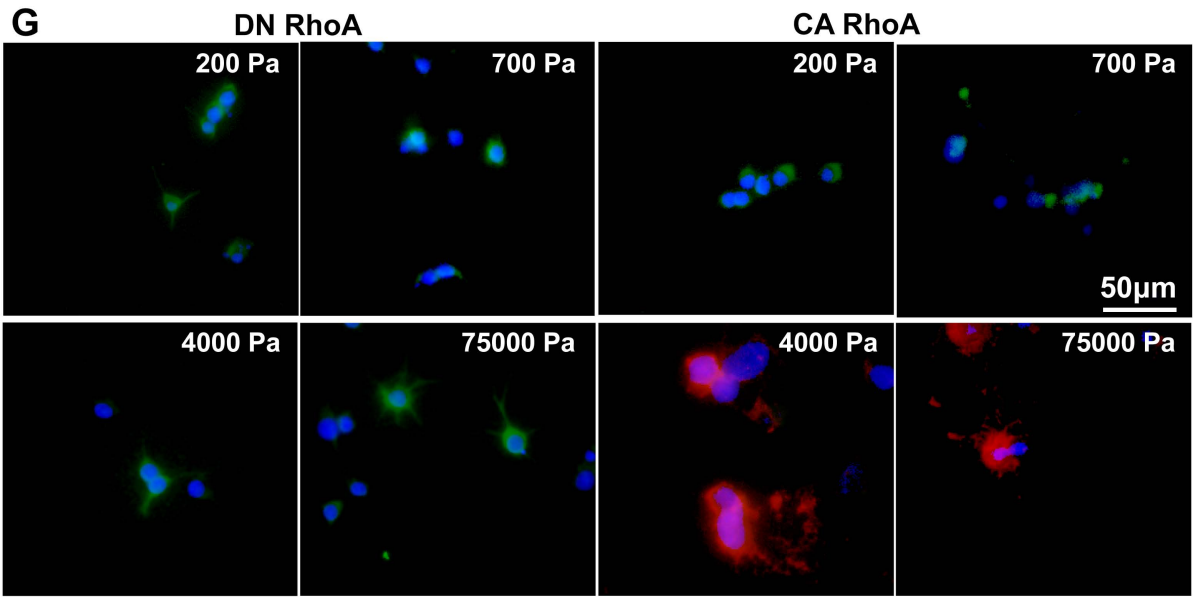


H

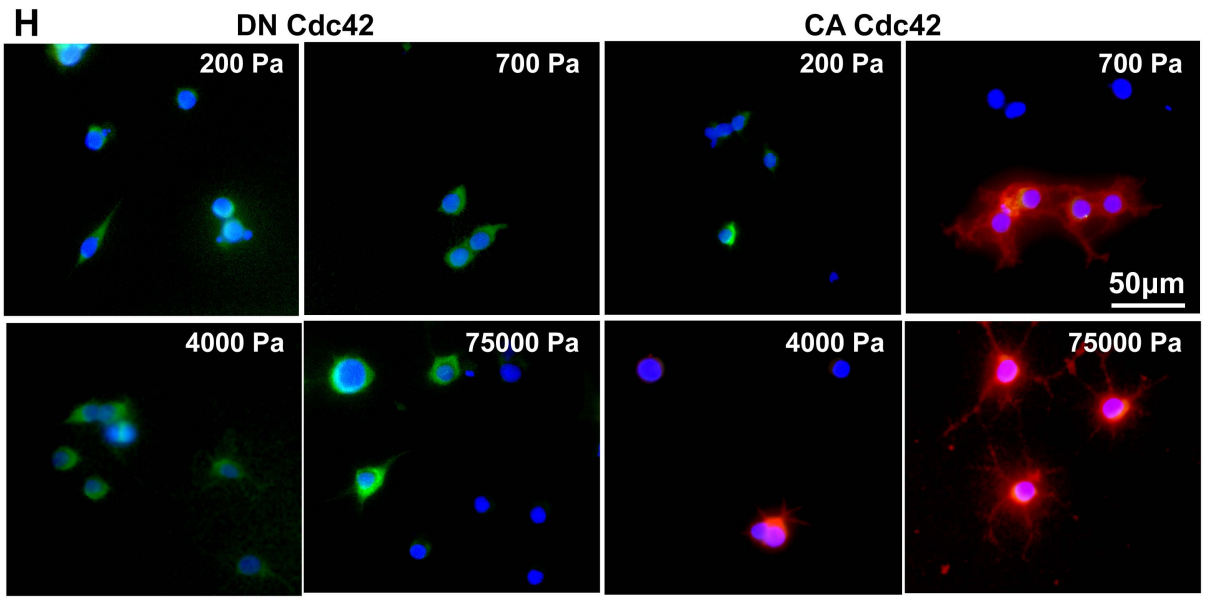
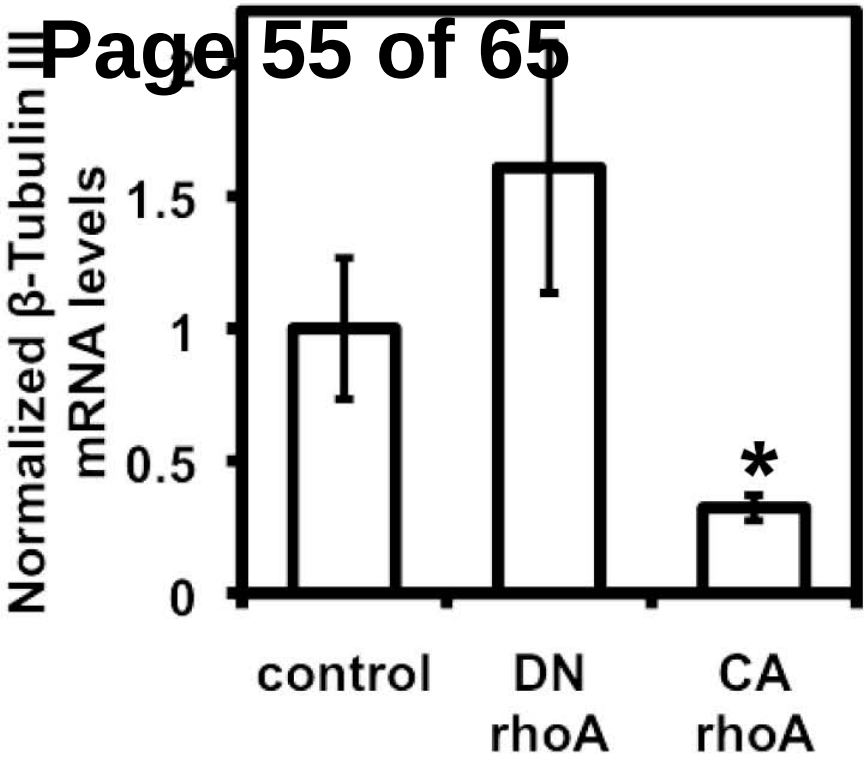
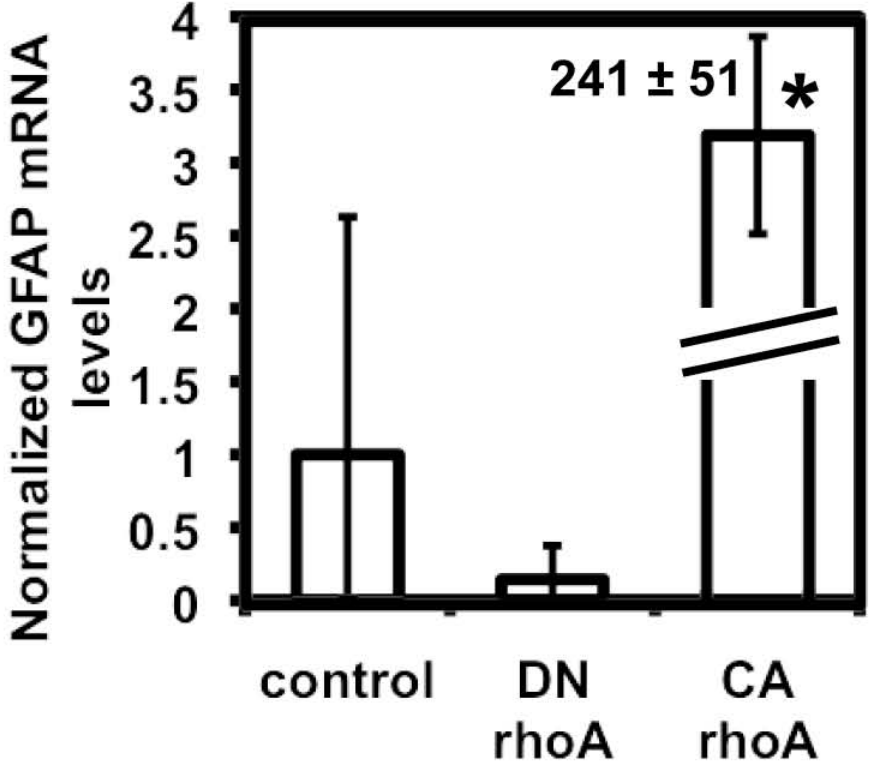


Figure S5


Normalized β-Tubulin III mRNA levels
2
1.5
1
0.5
0
*
control
DN rhoA
CA rhoA


Normalized GFAP mRNA levels
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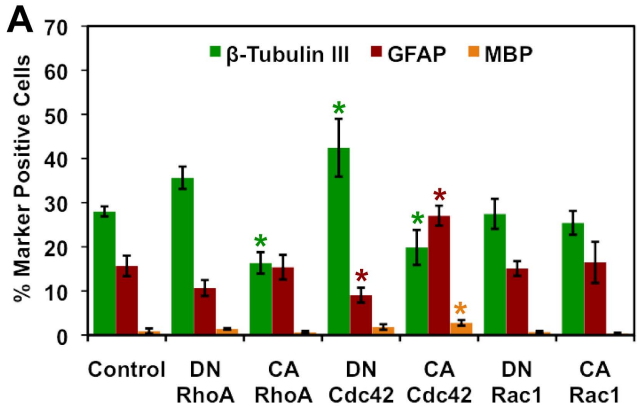
3.5
3
2.5
2
1.5
1
0.5
0
241 ± 51
*
control
DN rhoA
CA rhoA

Figure S6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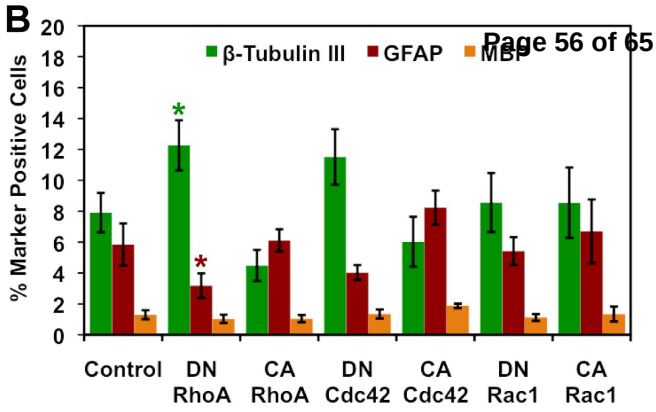
GFAP
MBP
Control
DN RhoA
CA RhoA
DN Cdc42
CA Cdc42
DN Rac1
CA Rac1


B
% Marker Positive Cells
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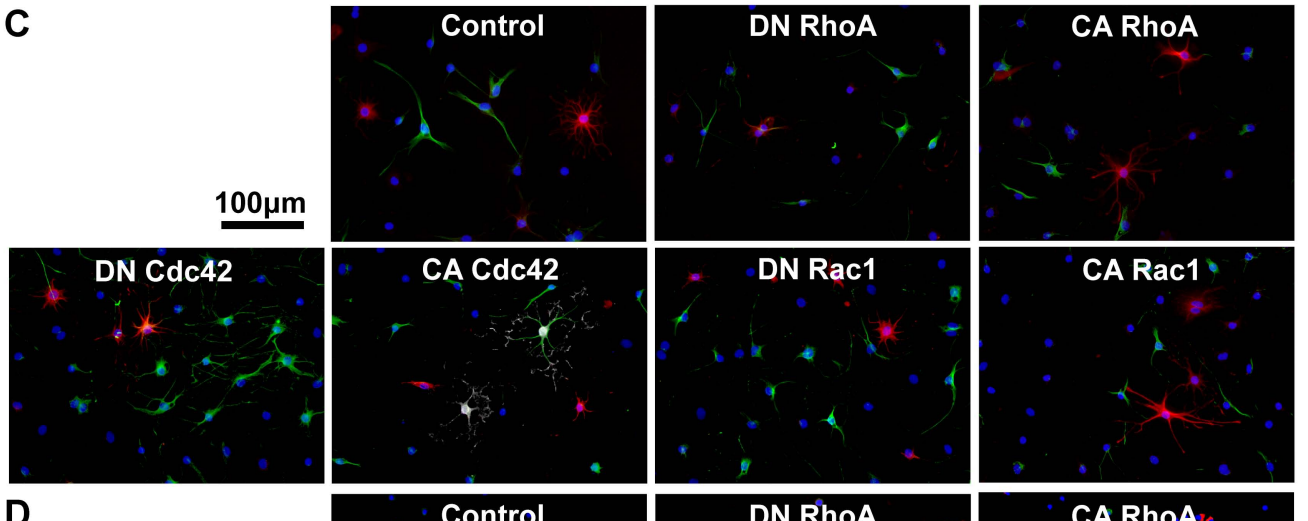
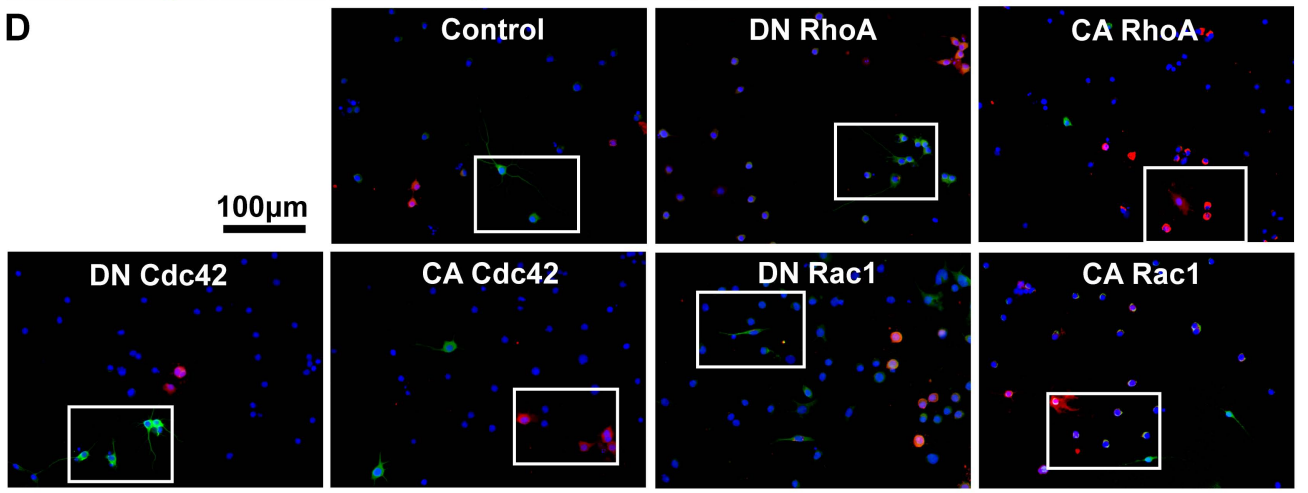
GFAP
MBP
Control
DN RhoA
CA RhoA
DN Cdc42
CA Cdc42
DN Rac1
CA Rac1


C
Control
DN RhoA
CA RhoA
100μm
DN Cdc42
CA Cdc42
DN Rac1
CA Rac1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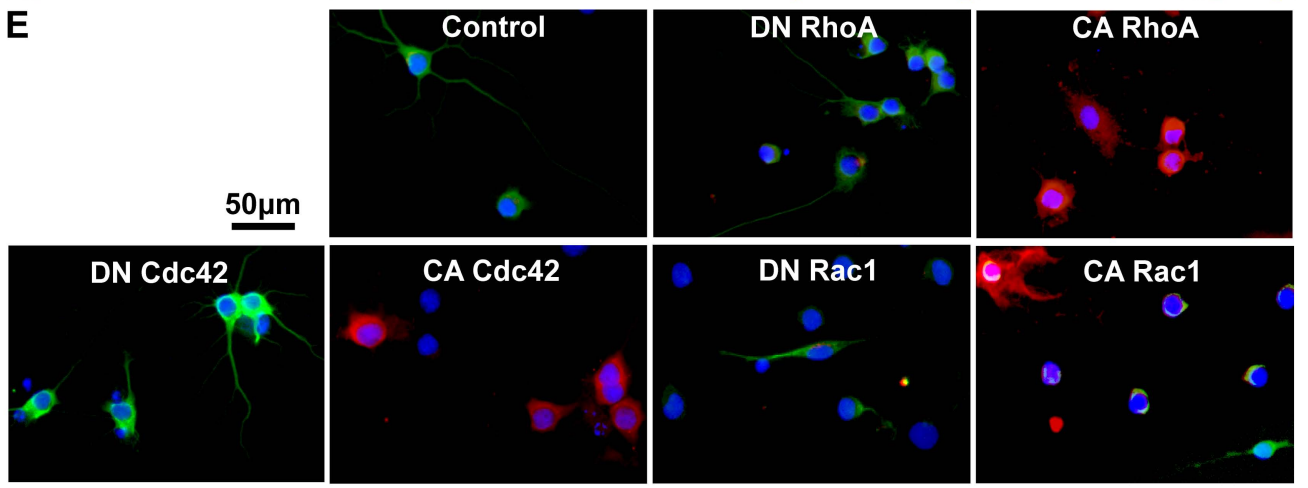
Control
DN RhoA
CA RhoA
100μm
DN Cdc42
CA Cdc42
DN Rac1
CA Rac1


E
Control
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CA RhoA
50μm
DN Cdc42
CA Cdc42
DN Rac1
CA Rac1

Figure S7

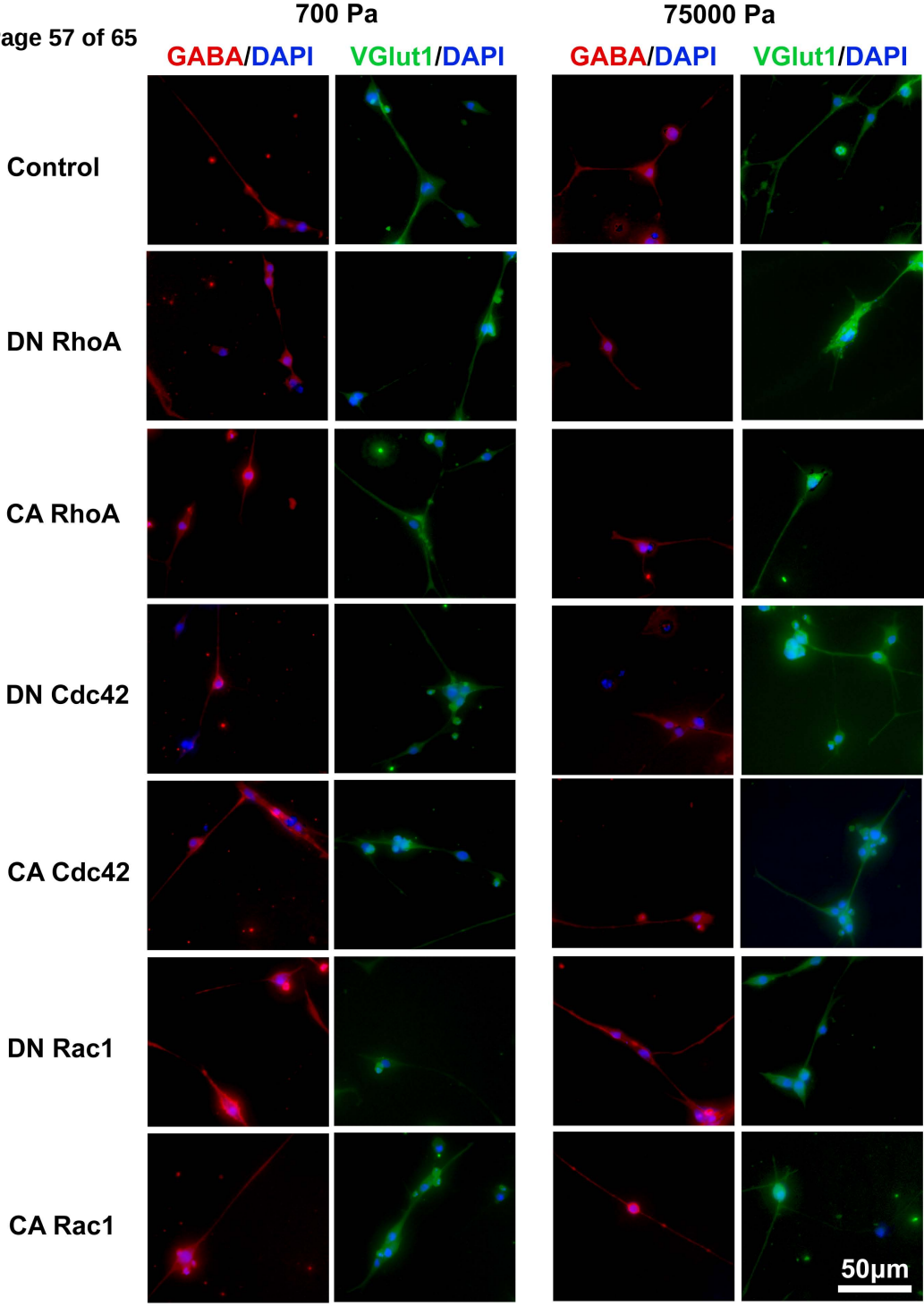

700 Pa
75000 Pa
GABA/DAPI
VGlut1/DAPI
GABA/DAPI
VGlut1/DAPI
Control
DN RhoA
CA RhoA
DN Cdc42
CA Cdc42
DN Rac1
CA Rac1
50μm

Figure S8

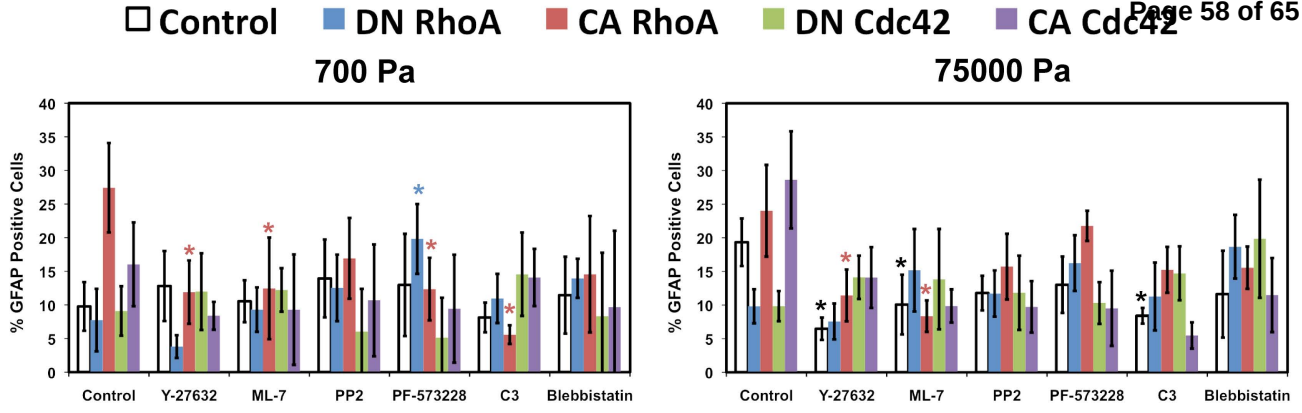

Control
DN RhoA
CA RhoA
DN Cdc42
CA Cdc42
700 Pa
75000 Pa
% GFAP Positive Cells
0
5
10
15
20
25
30
35
40
Control
Y-27632
ML-7
PP2
PF-573228
C3
Blebbistatin

Figure S9

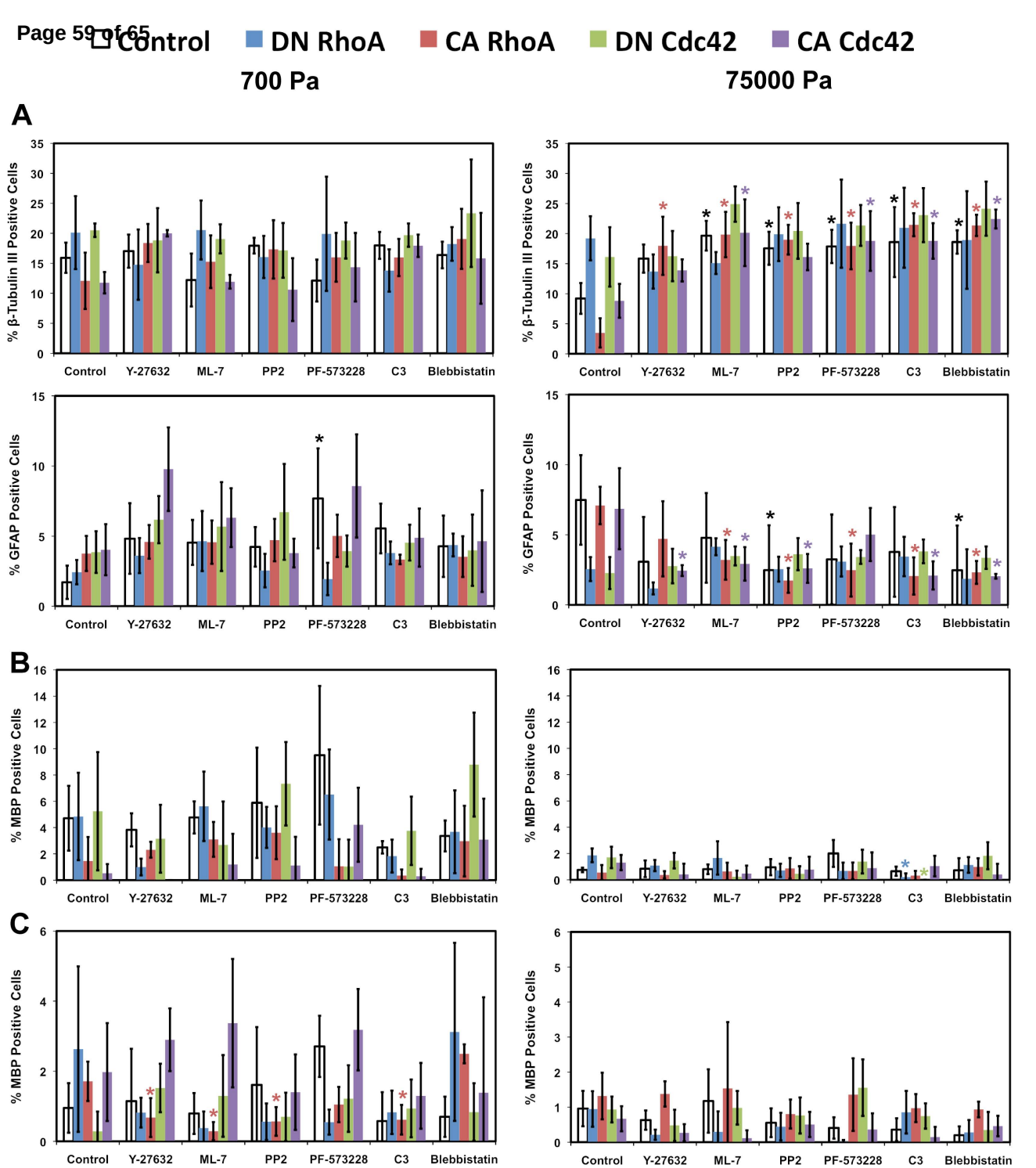
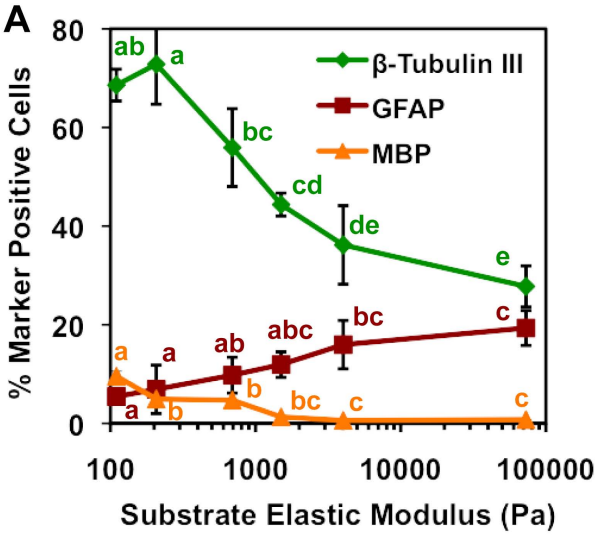
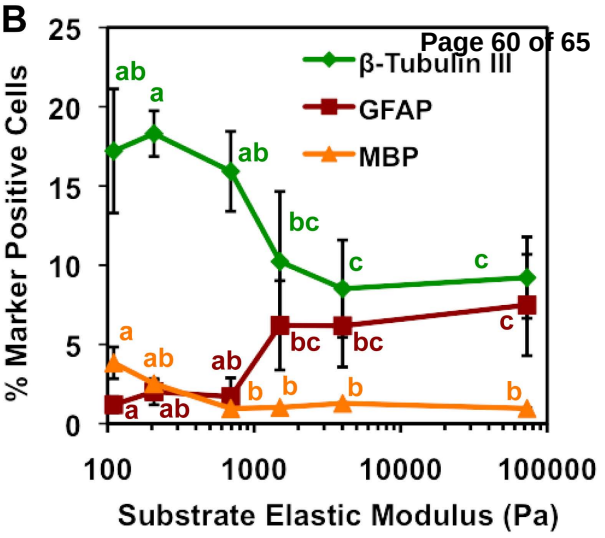

Control
DN RhoA
CA RhoA
DN Cdc42
CA Cdc42
700 Pa
75000 Pa
A
% β-Tubulin III Positive Cells
% GFAP Positive Cells
B
% MBP Positive Cells
C
% MBP Positive Cells
Control
Y-27632
ML-7
PP2
PF-573228
C3
Blebbistatin

Figure S10


A
% Marker Positive Cells
β-Tubulin III
GFAP
MBP
Substrate Elastic Modulus (Pa)


B
% Marker Positive Cells
β-Tubulin III
GFAP
MBP
Substrate Elastic Modulus (Pa)

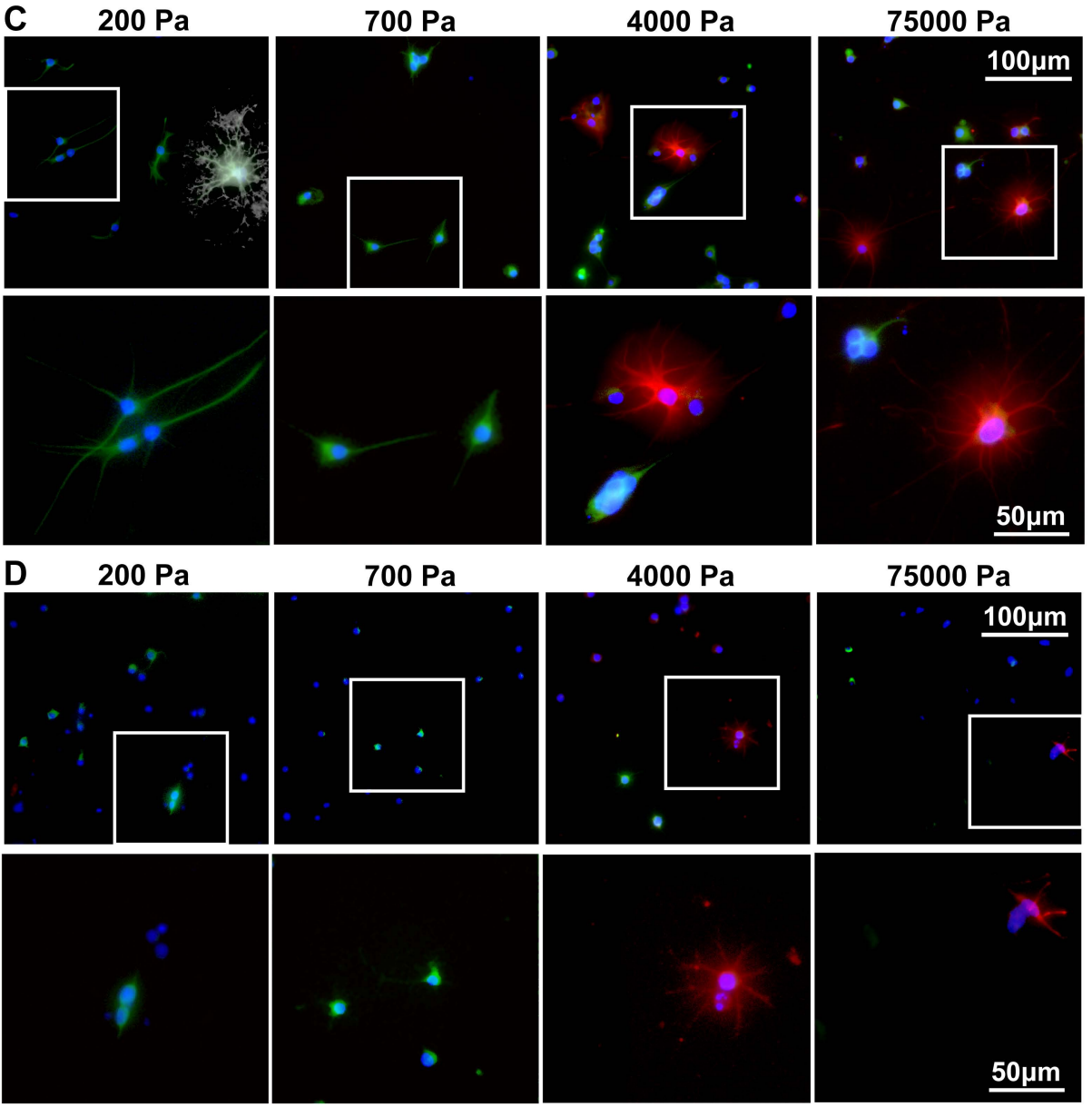

C
200 Pa
700 Pa
4000 Pa
75000 Pa
100μm
50μm
D
200 Pa
700 Pa
4000 Pa
75000 Pa
100μm
50μm

Figure 1

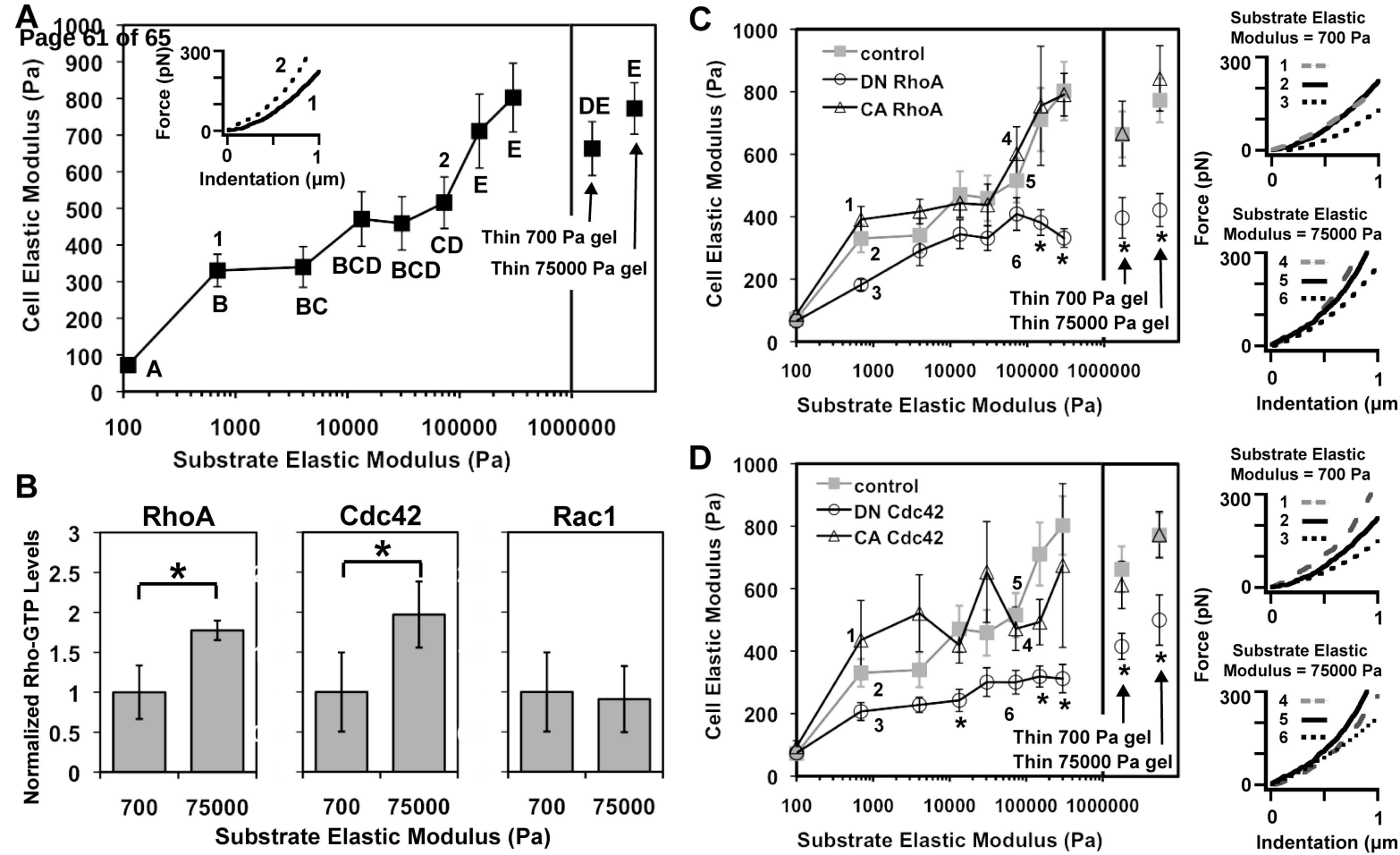
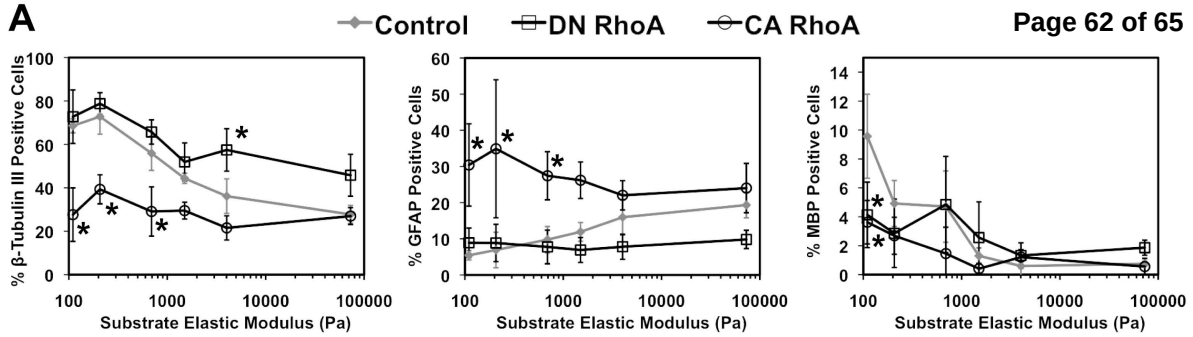
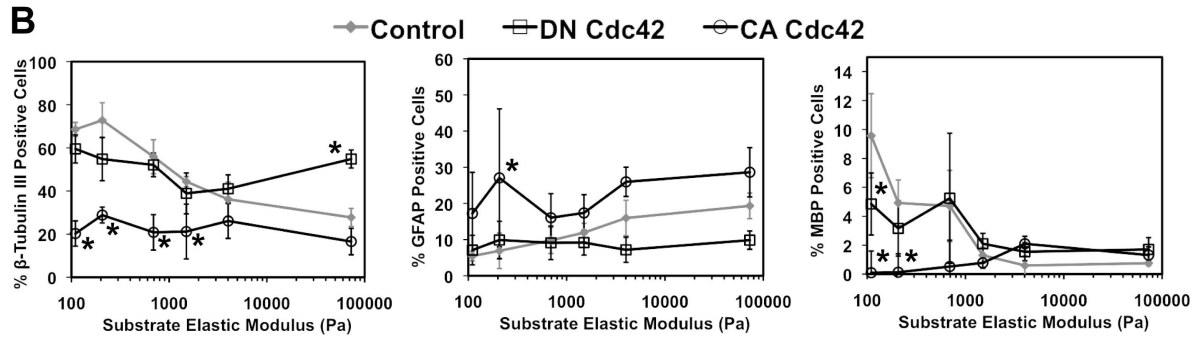

A
Cell Elastic Modulus (Pa)
Substrate Elastic Modulus (Pa)
Force (pN)
Indentation (μm)
A
B
BC
BCD
BCD
CD
E
E
E
DE
Thin 700 Pa gel
Thin 75000 Pa gel
B
RhoA
Cdc42
Rac1
Normalized Rho-GTP Levels
700
75000
Substrate Elastic Modulus (Pa)
C
control
DN RhoA
CA RhoA
Thin 700 Pa gel
Thin 75000 Pa gel
Substrate Elastic Modulus = 700 Pa
Substrate Elastic Modulus = 75000 Pa
Force (pN)
Indentation (μm)
D
control
DN Cdc42
CA Cdc42
Thin 700 Pa gel
Thin 75000 Pa gel
Substrate Elastic Modulus = 700 Pa
Substrate Elastic Modulus = 75000 Pa
Force (pN)
Indentation (μm)

Figure 2


A
Control
DN RhoA
CA RhoA
% β-Tubulin III Positive Cells
% GFAP Positive Cells
% MBP Positive Cells
Substrate Elastic Modulus (Pa)


B
Control
DN Cdc42
CA Cdc42
% β-Tubulin III Positive Cells
% GFAP Positive Cells
% MBP Positive Cells
Substrate Elastic Modulus (Pa)

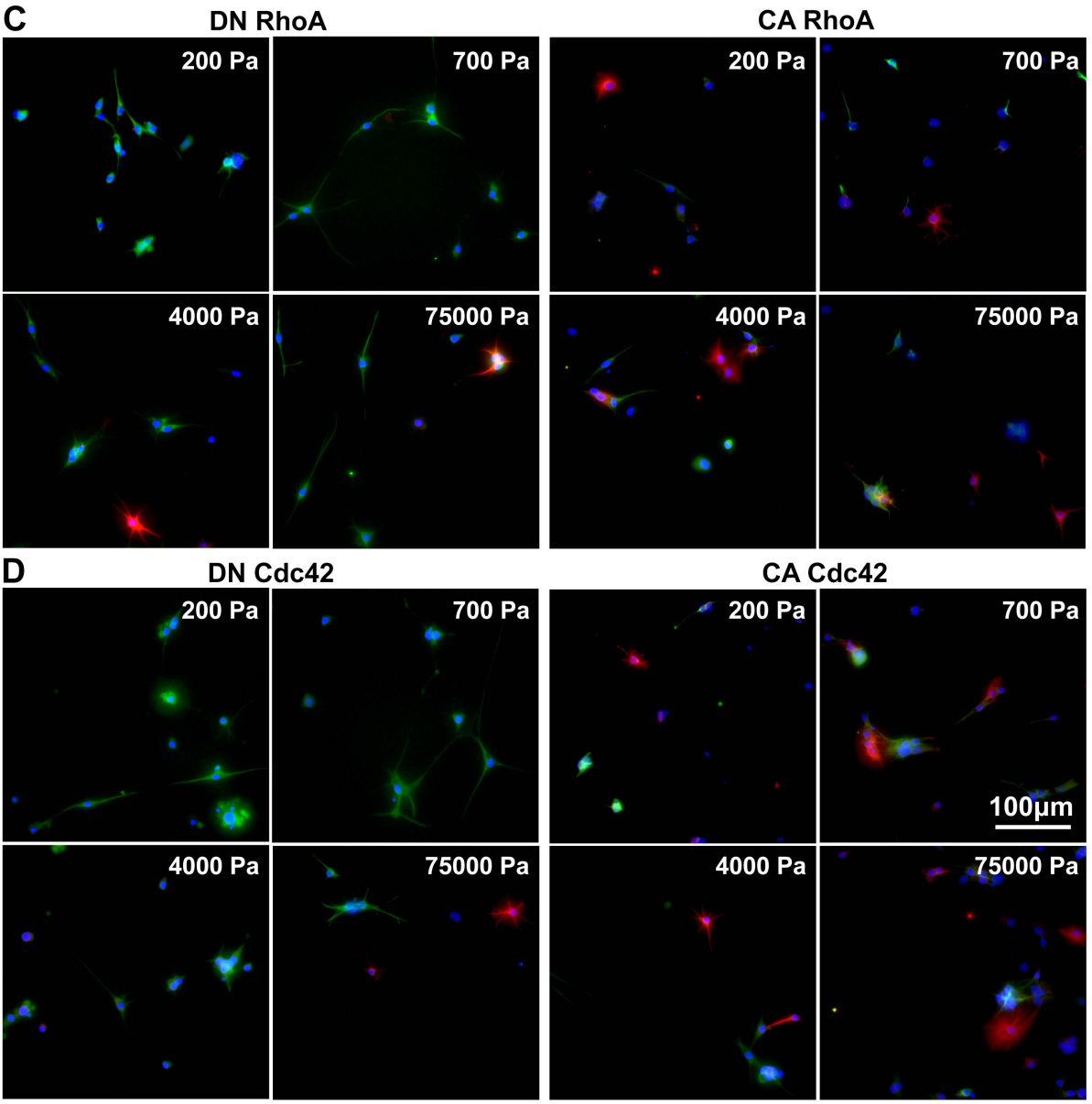

C
DN RhoA
CA RhoA
200 Pa
700 Pa
4000 Pa
75000 Pa
D
DN Cdc42
CA Cdc42
100μm

Figure 3

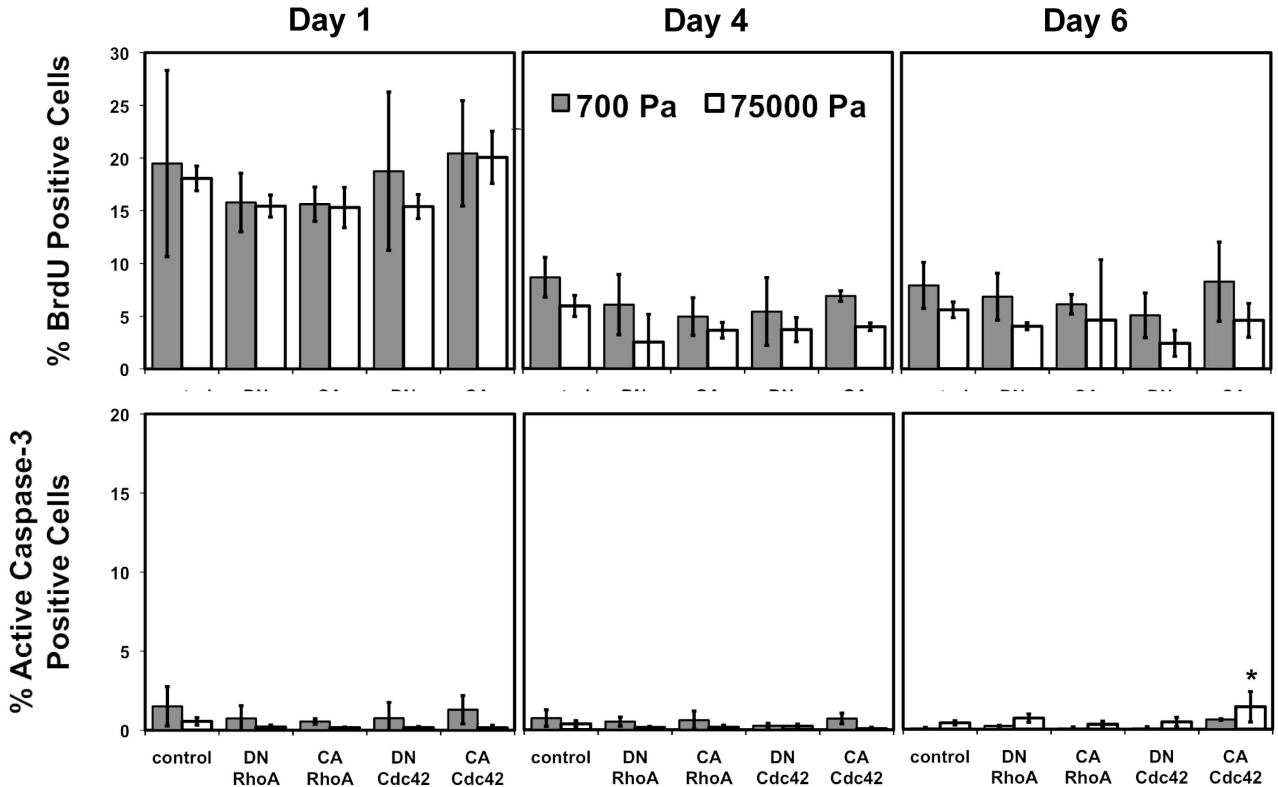

Day 1
Day 4
Day 6
700 Pa
75000 Pa
% BrdU Positive Cells
% Active Caspase-3 Positive Cells
30
25
20
15
10
5
0
20
15
10
5
0
*
control
DN RhoA
CA RhoA
DN Cdc42
CA Cdc42

Figure 4

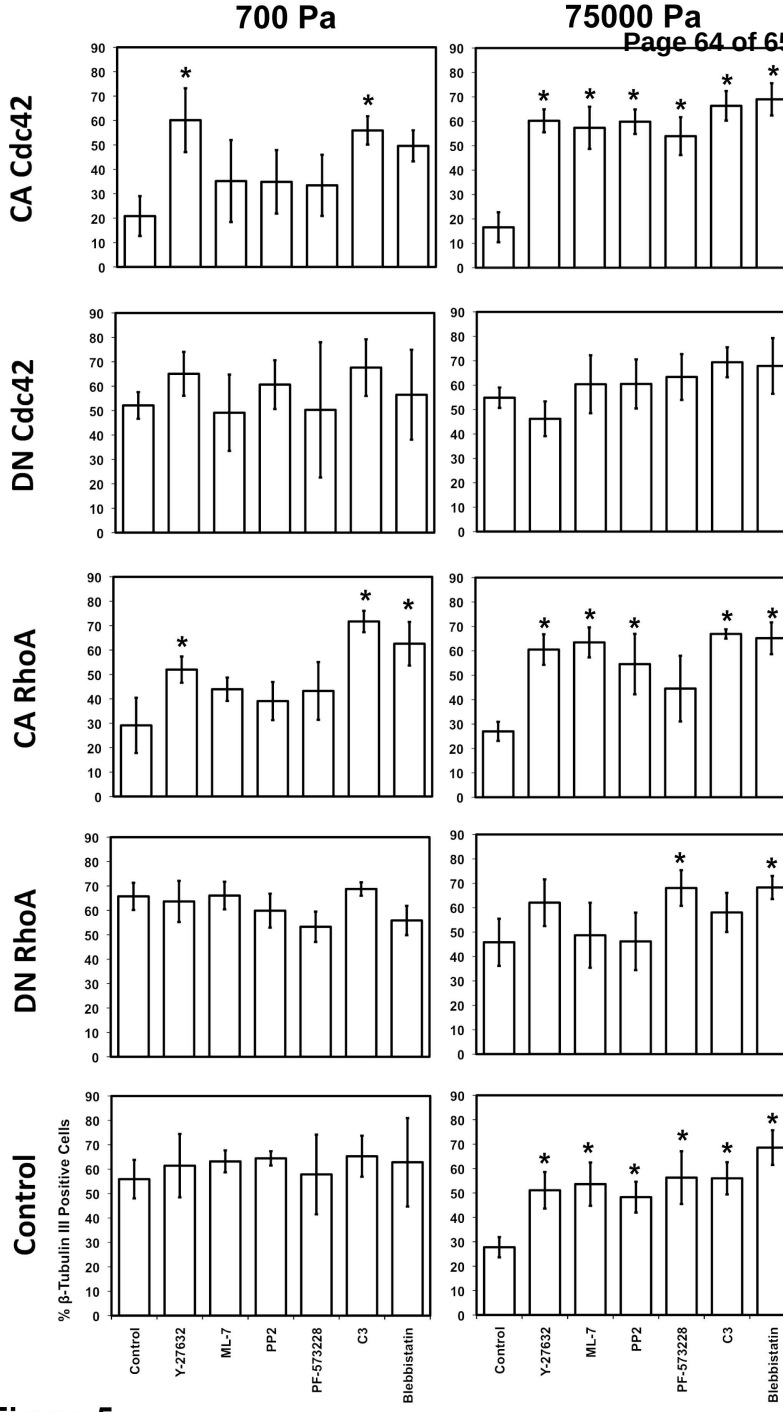
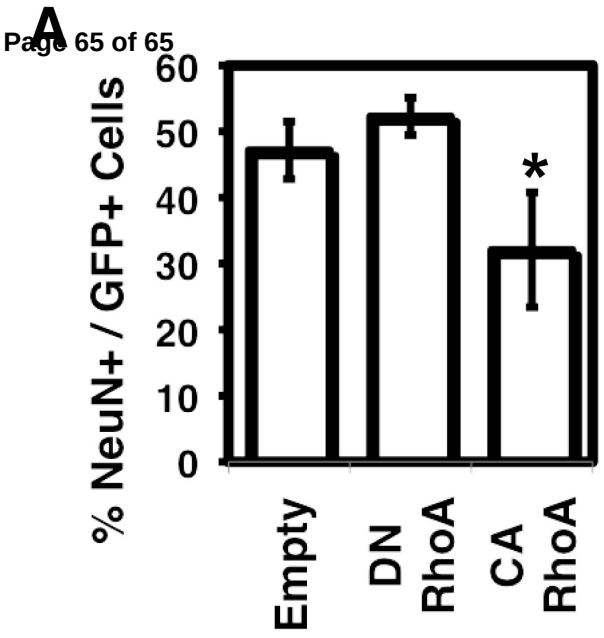
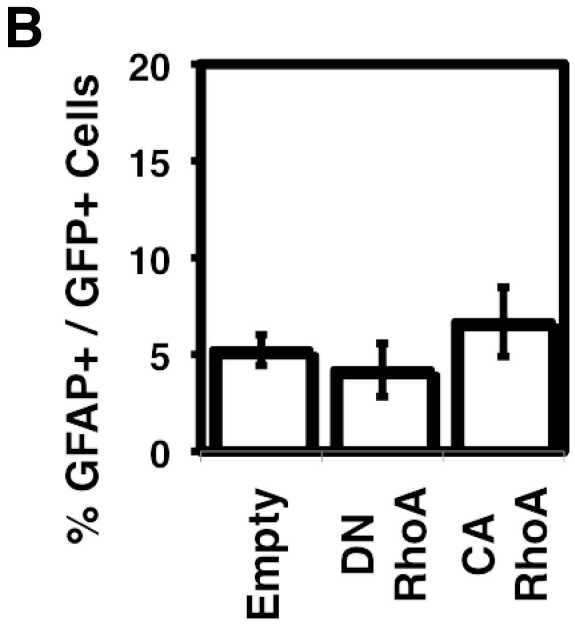
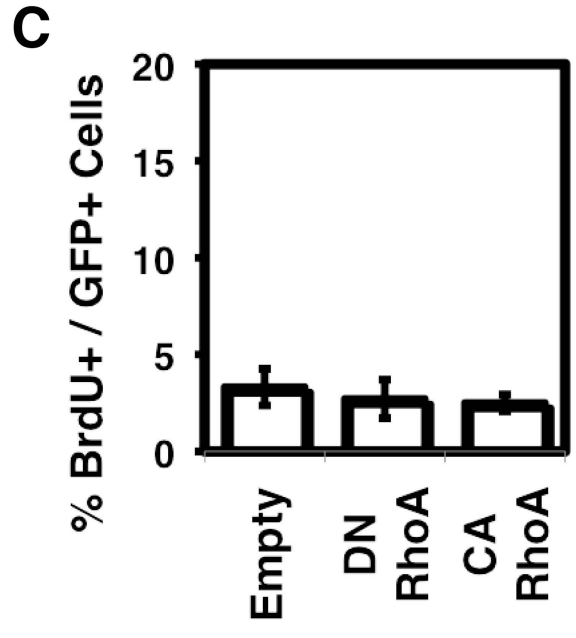
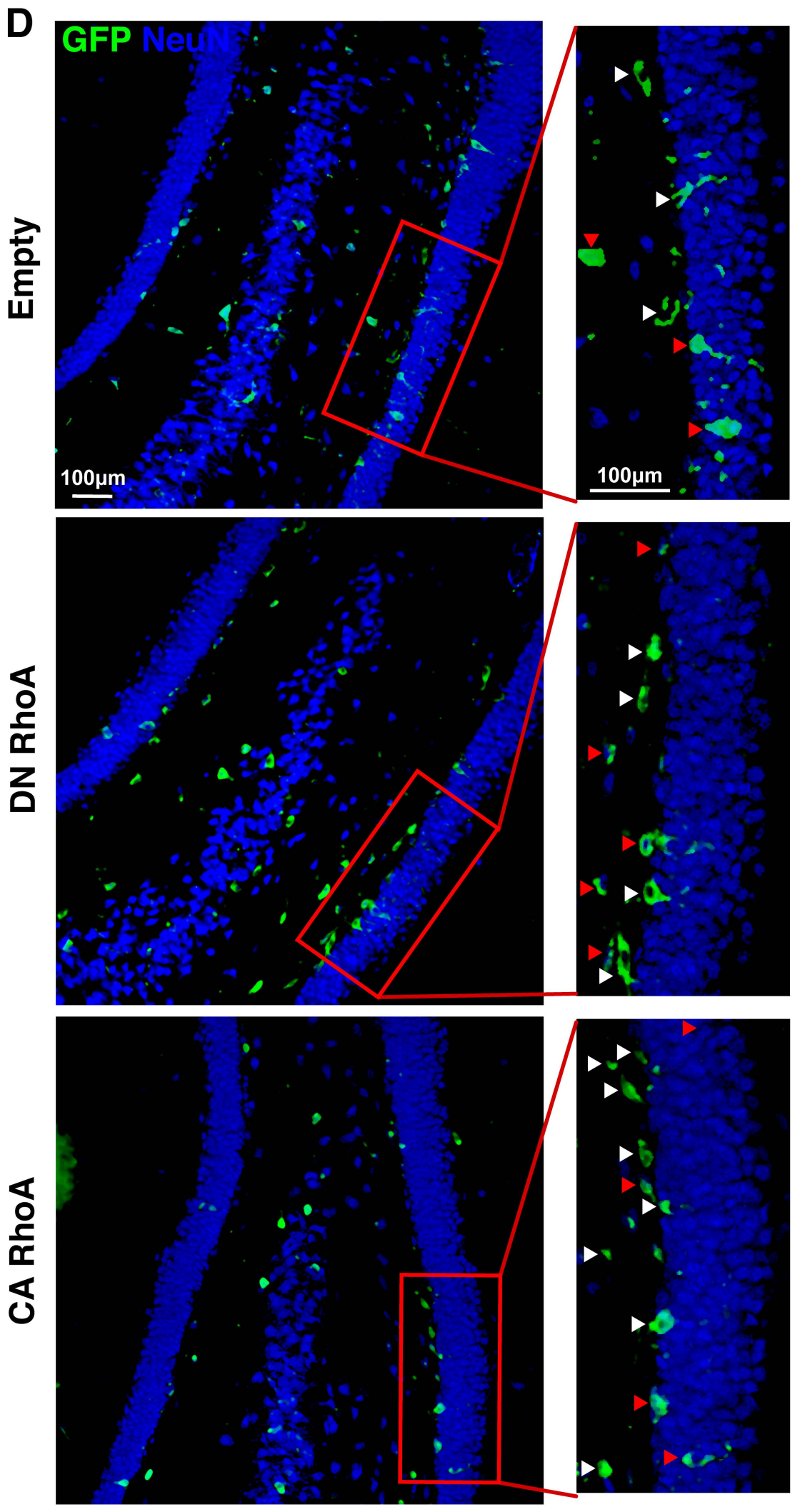

700 Pa
75000 Pa
CA Cdc42
DN Cdc42
CA RhoA
DN RhoA
Control
% β-Tubulin III Positive Cells
Control
Y-27632
ML-7
PP2
PF-573228
C3
Blebbistatin

Figure 5


A
% NeuN+ / GFP+ Cells
60
50
40
30
20
10
0
*
Empty
DN RhoA
CA RhoA


B
% GFAP+ / GFP+ Cells
20
15
10
5
0
Empty
DN RhoA
CA RhoA


C
% BrdU+ / GFP+ Cells
20
15
10
5
0
Empty
DN RhoA
CA RhoA


D
GFP NeuN
Empty
DN RhoA
CA RhoA
100μm
100μm

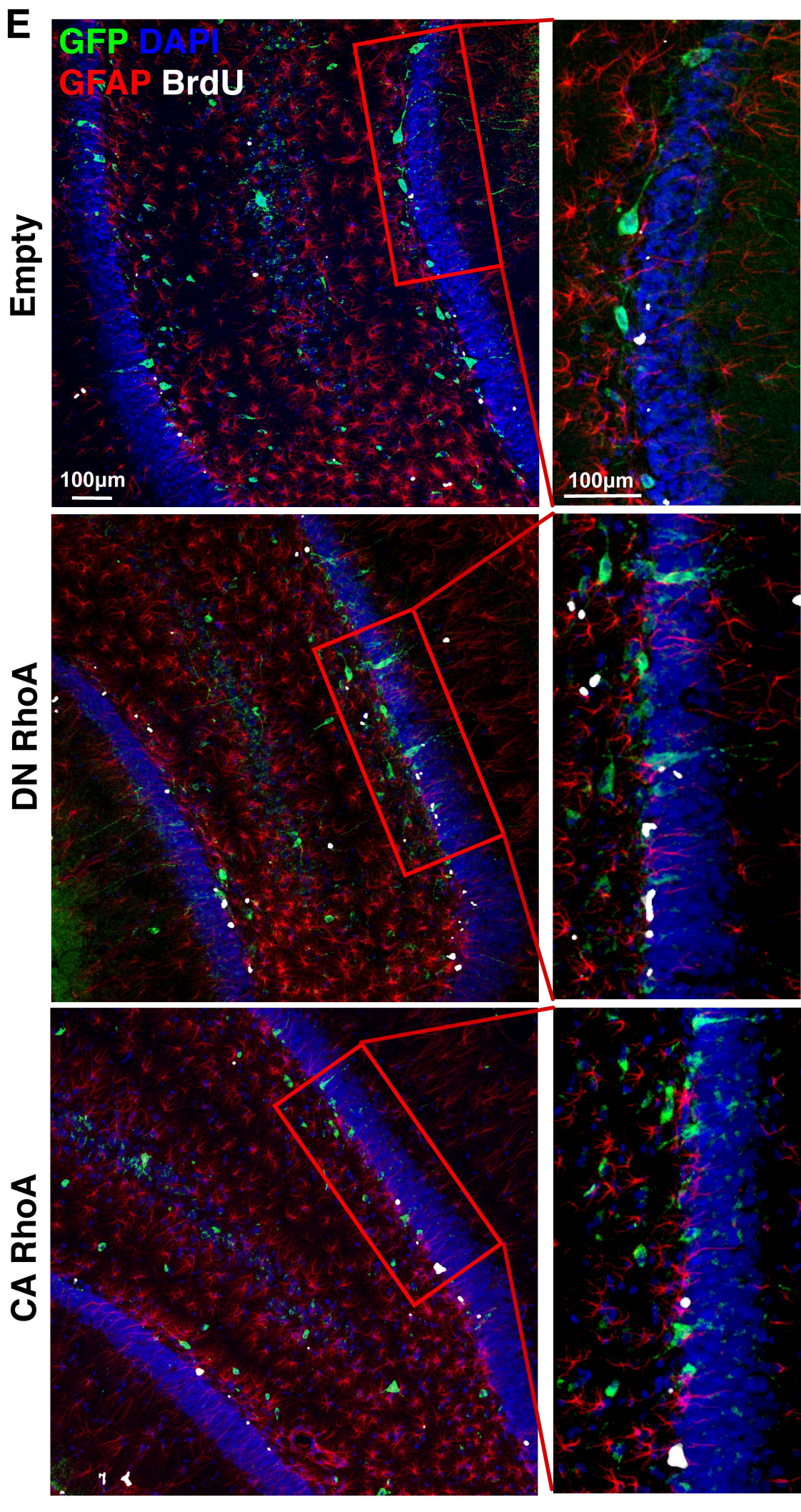

E
GFP DAPI
GFAP BrdU
Empty
DN RhoA
CA RhoA
100μm
100μm

Figure 6