Supplementary Information for:

Mechanosensing Controlled Directly by Tyrosine Kinases

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Supplementary Figure Legend:

Supplementary Figure 1: (A) Typical single reference pillar displacement as a function of time. (B) Distributions of D_{max} of reference pillars with different heights. (C) Distributions of T_{50} of reference pillars of different heights. (Black for 750 nm high pillars; Red for 1500 nm high pillars; Blue for 2000 nm high pillars; Error bars represent SD of the fractions from analyses of 10 movies in 3 independent experiments) (D) Bar graph showing the changes in cell aspect ratio of control, AXL and ROR2 knockdown HFF cells on rigid (2MPa) and soft (5kPa) PDMS surfaces after 6 hrs plating. (n=20 in each case; *** $p<0.001$)

Supplementary Figure 2: (A and B) Box-and-whisker plots of median values of T_{50} distributions for similarly treated cells on rigid pillars (A) and soft pillars (B). (n=10 in each case)

Supplementary Figure 3: (A and B) Box-and-whisker plots of median values of D_{max} distributions for similarly treated cells on rigid pillars (A) and soft pillars (B). (n=10 in each case)

Supplementary Figure 4: (A) AXL level but not ROR2 level is knocked down in Western blots of control siRNA vs AXL siRNA transfected cells.

Supplementary Figure 5: (A) Typical pause example of a single pillar under a control cell spreading on soft pillars. (B) Typical pause example of a single pillar under a AXL knockdown cell spreading on soft pillars. (C) Typical pause example of a single pillar under a ROR2 knockdown cell spreading on soft pillars.

Supplementary Figure 6: (A) and (B) Immunostainings of the whole cell that showed in Figure 5 (A) and (C), respectively. Yellow square highlighted the zoomed region. (C) and (D) more super-resolution examples of phospho-AXL (green) overlap with p-MLC (red) in contractile regions at cell periphery.

Supplementary Figure 7: (A) GFP-myosin IIA transfected HFF cells lysates were immunoprecipitated with GFP nanotrp beads, followed by immunoblotting with anti-AXL antibody. (B and E) GFP-AXL transfected HFF cell lysates were immunoprecipitated with GFP nanotrap beads, followed by immunoblotting with anti-filamin A (A) or anti-α-actinin 1&4 (E) antibodies. (C and F) GFP-ROR2 transfected HFF cell lysate were immunoprecipitated with GFP nanotrap beads, followed by immunoblotting with anti-Myosin IIA (C) or anti-α-actinin (F) antibodies. (D) YFP-Tpm 2.1 transfected HFF cells lysate were immunoprecipitated with GFP nanotrap beads, followed by immunoblotting with anti-ROR2 antibody.

Supplementary Figure 8: (A) EGFP-myosin IIA transfected HFF cells lysates were immunoprecipitated with GFP nanotrap beads, followed immunoblotting with anti-phophotyrosine antibody under control (Black) or R428 (Orange) treated conditions. (B) Relative intensity analysis of myosin IIA tyrosine phosphorylation level change (n=3 in each case; * $p<0.05$).

Supplementary Figure 9: (A) Bar graph of total number of contractile pairs that tracked in control and Tpm 2.1 transfected MDA-MB-231 cells during imaging time. (Mean ± SEM) (n=4 in each case; ** $p<0.01$) (B) Histogram plots of D_{max} of Tpm 2.1 transfected MDA-MB-231 cells with or without R428 treatment on 750 nm high pillars.

Supplementary Figure 10: Tpm 2.1 tyrosine mutant affects adhesion formation at later stage in MDA-MB-231 cells. (A) Immunostaining images of MDA-MB-231 cells transfected GFP empty vector, YFP-Tpm 2.1 or different Tpm 2.1 tyrosine mutants (B) Bar graph summary of total cell spreading area across different groups. (Mean ± SEM) (>200 focal adhesions from > 30 cells in each group; ***$p<0.001$; **$p<0.01$) (C) Bar graph summary single focal adhesion area across different groups. (Mean ± SEM) (> 200 focal adhesions from > 30 cells in each group; ***$p<0.001$; **$p<0.01$) (D) Immunostaining images of MDA-MB-231 cells transfected with YFP-Tpm 2.1, YFP-Tpm 2.1-Y214F and YFP-Tpm 2.1-Y214E, respectively. (Scale bar is 10 μm)

Materials and Methods:

Cell culture:

Human Foreskin Fibroblasts (HFFs) and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FBS), 2mM glutamine and 100IU/ml Penicillin-Streptomycin at 5% CO_2 in 37 °C. DMEM, FBS and antibiotic were all from Life Technologies.

Pillar Fabrication:

Pillars fabrication was performed as previously described with some modifications [1-3]. PDMS (mixed at 10:1 base with curing agent, Sylgard 184, Dow Corning) was poured over different silicon molds (750 nm, 1500 nm and 2000 nm), spun coat at 1000 rpm for 1 min to uniformly coat the mold's surface, degassed in vacuum for 15 mins, and then cured at 80 °C for 3 hrs to reach a Young modulus of 2 MPa. Pillars arrays were cured and peeled off from the silicon mold after curing. Individual pillars were 500 nm in diameter with a center-to center distance of 1 μ m. Pillar bending stiffness, k, was calculated by Euler-Bernoulli beam theory:

$$k = \frac{3}{64}\pi E \frac{D^4}{L^3}$$

Where D and L are the diameter and length of the pillar, respectively, E is the Young's modulus of PDMS (2MPa).

Video Microscopy and Force Traction Measurements:

Time-lapse imaging of pillars and high speed image was performed as explained before [2, 3].

Plasmids, siRNA and transfection:

GFP tagged mROR2 plasmid was a generous gift from Dr. Yasuhiro Minami and Dr. Michiru Nishita (Kobe University, Kobe, Japan); GFP tagged mAXL plasmid was a generous gift from Dr. Qingxian Lu (University of Louisville, KY, USA); YFP tagged human Tpm 2.1 plasmid was a generous gift from Dr. Peter Gunning (The University of New South Wales, Sydney, Australia).

Plasmid transfections for HFF cells were carried out 24 hrs prior to the experiment using Neon electroporator system (Life Technologies) according to the manufacturer's instructions.

As described by [4], knockdown of AXL or ROR2 was performed using SMART pool siRNAs (Dharmacon RNAi Technologies).

R428 (Selleckchem) and Gas 6 (R&D) were diluted in DMSO or PBS according to the manufacturer's instructions, respectively. HFF cells were serum starved for 16 hrs and pre-treated with R428 (100 nM) or Gas 6 (200 ng/ml) for 2hrs before pillars imaging. Cells were kept in the same concentration of inhibitor or ligand during whole imaging time. As described before[5], AXL is important in breast cancer tumor growth and the expression level of AXL in MDA-MB-231 cells is higher than normal tissue. To achieve an efficient AXL inhibition, a higher concentration of R428 (2 μM) was used in the experiments using MDA-MB-231 cells.

The YFP-Tpm 2.1-Y162F, YFP-Tpm 2.1-Y214F, YFP-Tpm 2.1-Y261F mutants were generated using the primers 5'- CGACCGCAAATTCGAAGAGGTGG-3' (Y162F), 5'-GCATCTTCAGCAATGTGC-3' (Y162F),5'-AGAGGATAAGTTCTCGCAGA AGG-3' (Y214F), 5'-GCAGCCATTAATGCTTTC-3' (Y214F), 5'-AGACGA GCTGTTCGCTCAGAAAC-3' (Y261F), 5'-TCTAAGTCATCAATGCTTT TCTCC-3' (Y261F) 5'-AGAGGATAAAGGAGTCGCAGAAG-3' (Y214E) and 5'-GCAGCCATTAATGCTTTC-3' (Y214E), following the protocol provided in Q5 Site-Directed Mutagenesis Kit (NEB).

MDA-MB-231 cells were plated in 6 well-plate at day 0 and transfected with GFP vector, YFP-Tpm 2.1 or different Tpm 2.1 mutants using lipofactamine 2000 on day 1 following the manufacturer's instructions (Life Technologies). At day 2, cells trypsinized and re-plated on fibronectin-coated stiff surface for tyrosine phosphorylation pull-down at initial spreading stage or focal adhesion formation analysis after overnight plating.

Fluorescence Microscopy:

After 15mins of spreading on fibronectin-coated pillars, HFF cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 37 °C for 15 mins, and permeabilized

with 0.2% Triton X-100 for 10 mins at room temperature. Samples were blocked with 1% bovine serum albumin (BSA) in PBS for 1h, incubated with primary antibodies for specific proteins: anti-α-actinin 1&4 (Sigma); anti-phospho-AXL (R&D) or anti-ROR2 (CST) at 4 °C overnight and then incubated with secondary antibodies (Molecular Probes) for 1h at room temperature. Fluorescence images were acquired using a spinning-disc confocal microscope (PerkinElmer Ultraview VoX) attached to an Olympus IX81 inverted microscope body.

Imaging for 3B analysis:

HFF cells were fixed and stained with anti-phospho-AXL (R&D) and p-MLC (CST) antibodies after 15mins spreading on pillar substrates. 100 mM mercaptoethanol was added to the sample as a reducing agent to induce blinking events [6]. The imaging and 3B analyses were performed as previously illustrated [2, 7].

Co-immunoprecipitation and western blot

Transfected cells were lysed in RIPA buffer (Sigma) and proteins extracted were separated by 4-20% SDS polyacrylamide gel electrophoresis (Bio-rad) and transferred to PVDF membranes (Bio-rad) at 75V for 2h. Membranes were incubated with primary antibodies to specific proteins: anti-AXL (CST, dilution 1:1000), anti-ROR2 (Abcam, dilution 1:1000), anti-α-tubulin (Sigma, dilution 1:3000) or anti-phosphotyrosine (Millipore, dilution 1:1000) at 4 °C overnight and then followed by an incubation of HRP-conjugated secondary antibodies (anti-mouse or rabbit, Bio-rad, 1:5000/1:2000) for 1h at room temperature.

Lysates for GFP immunoprecipitation and pull down were prepared from cells that were transfected with GFP, GFP-ROR2, GFP-AXL, EGFP-myosin IIA, YFP-Tpm 2.1 or YFP-Tpm 2.1 mutants. Cells were lysed in a RIPA buffer (Sigma) with added protease and phosphatase inhibitors (Roche). Clarified cell lysate was incubated with 20 µl GFP-nanotrap-A beads (ChromoTek) under constant mixing at 4°C for 2 hrs according to the manufacturer instructions. The GFP-Trap complexes were washed three times with iced old washing buffer and then re-suspended in 2X SDS-sample buffer. Re-suspended beads were boiled at 95 °C for 5 mins and separated by 4-20% SDS-gel (Bio-rad).

Statistical analysis:

Prism (GraphPad Software) and Matlab (Math Works) were used for data analysis and graph plotting. Analyses of significant difference levels were carried out using ANOVA test (for more than 2 experimental groups) or Student's t-test.

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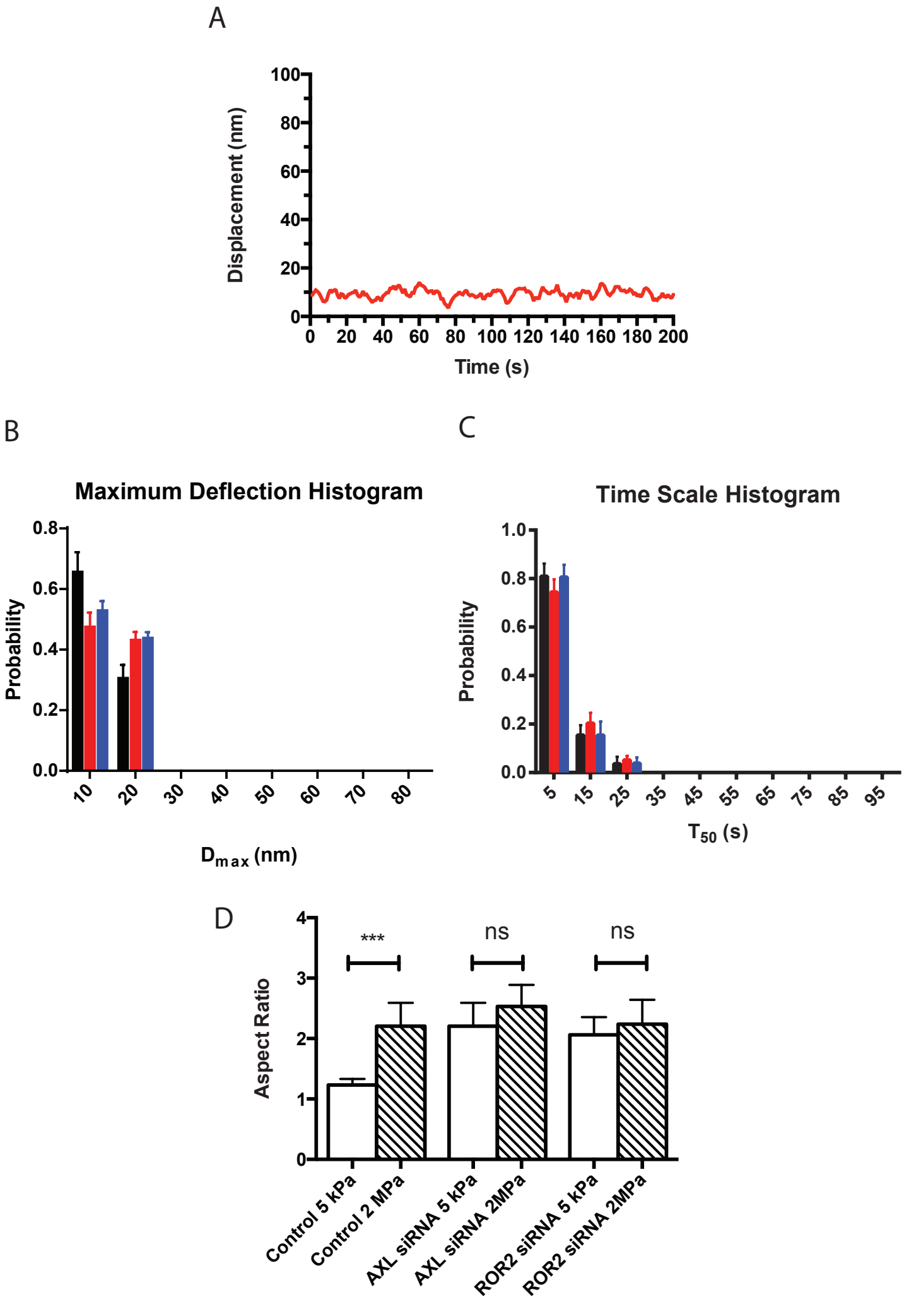

Supplementary Figure 1

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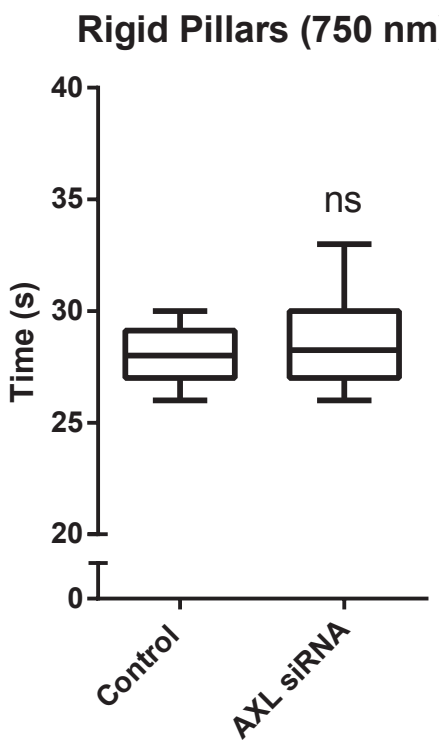

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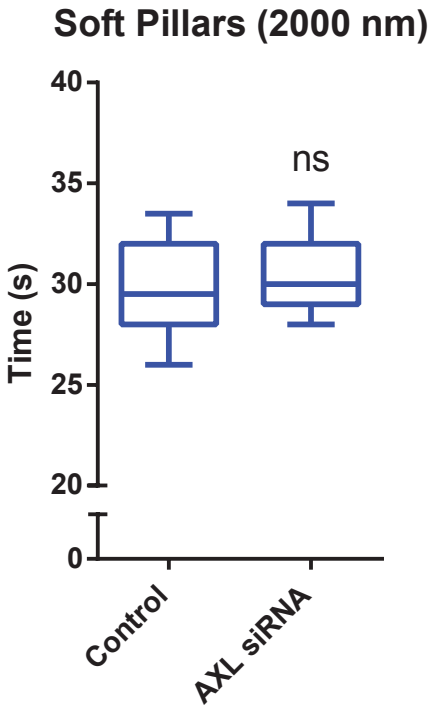

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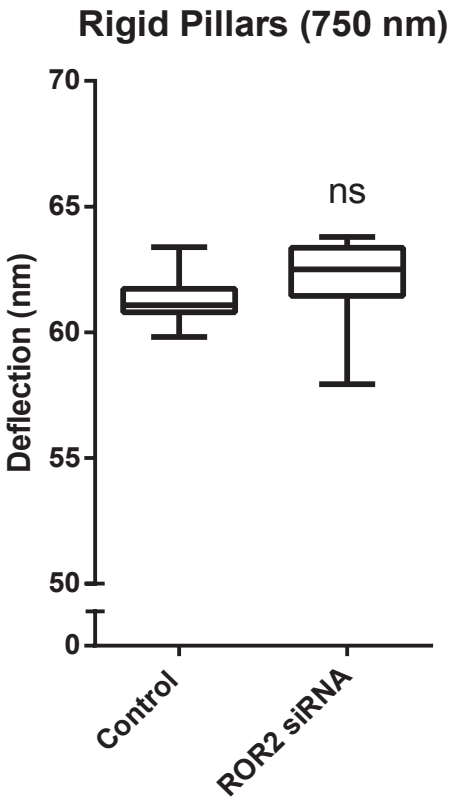

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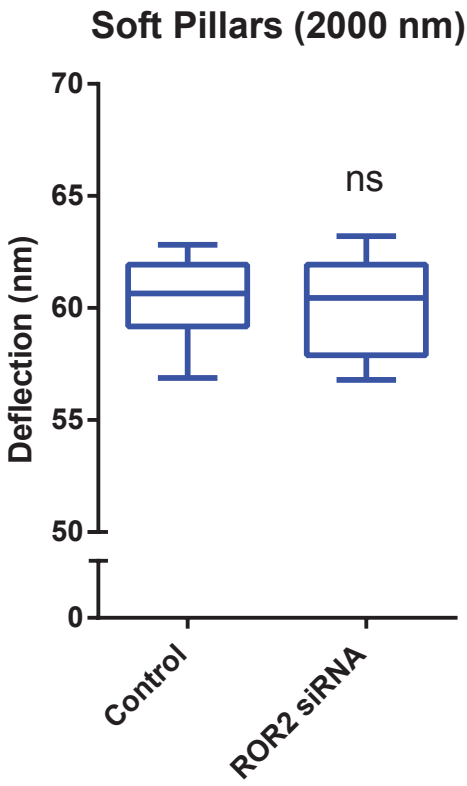

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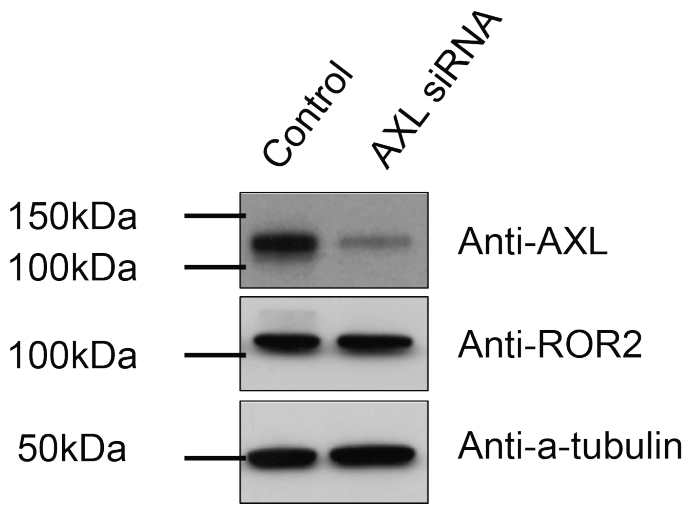

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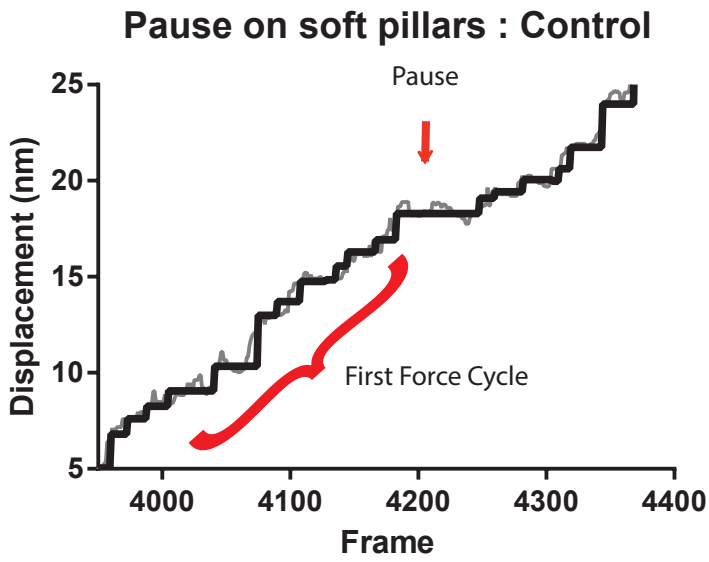

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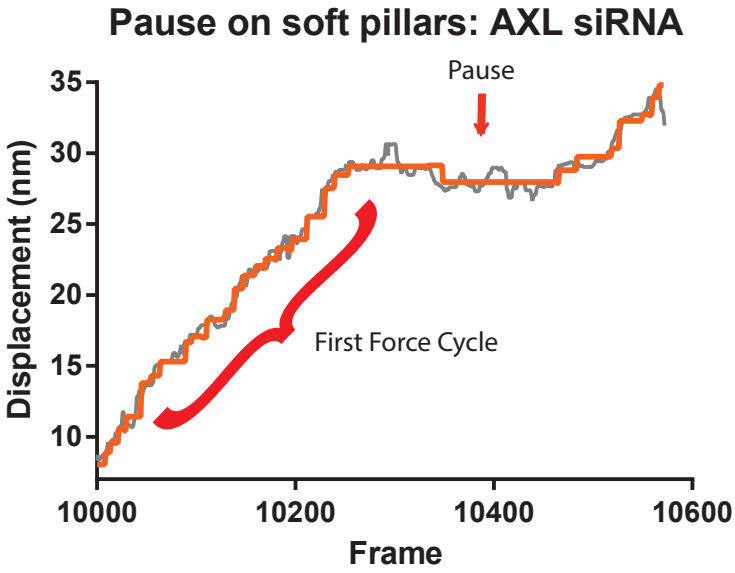

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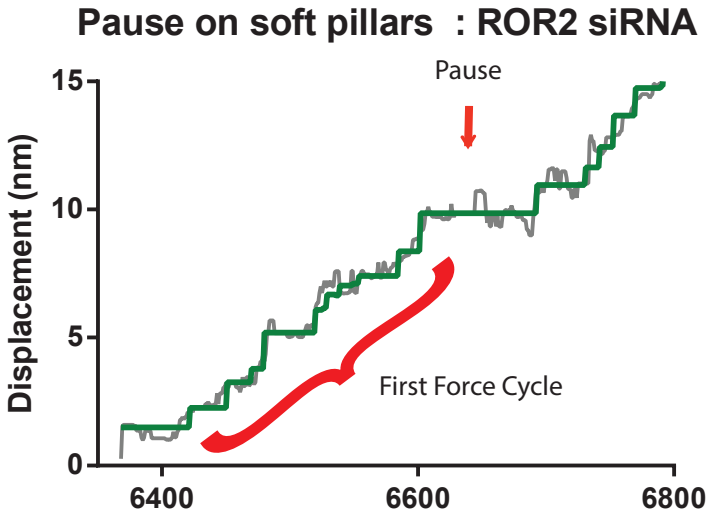

Supplementary Figure 5

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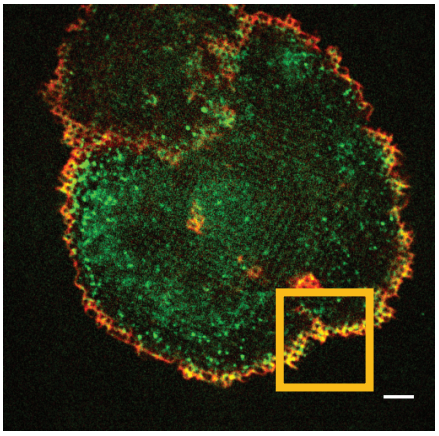

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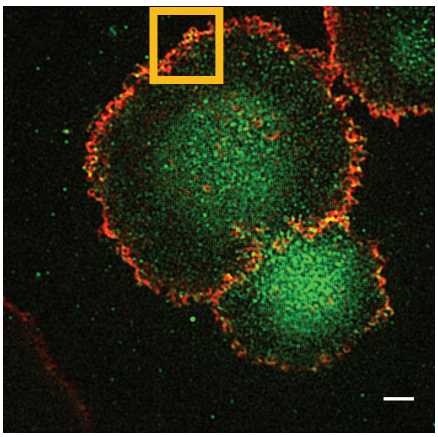

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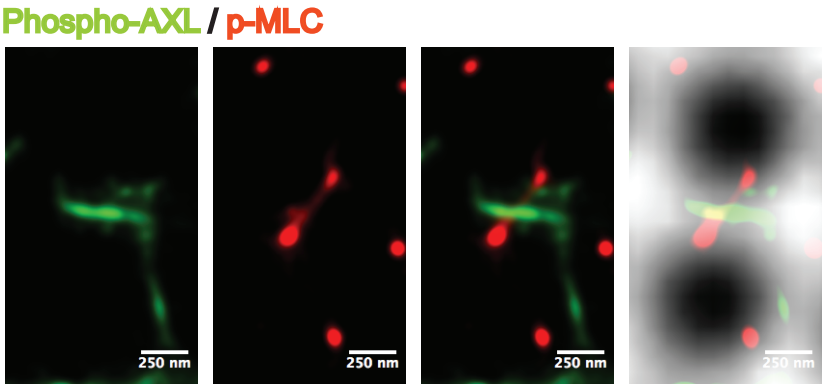

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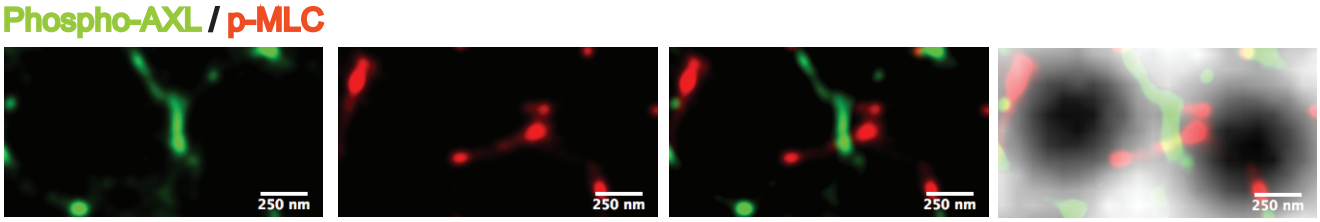
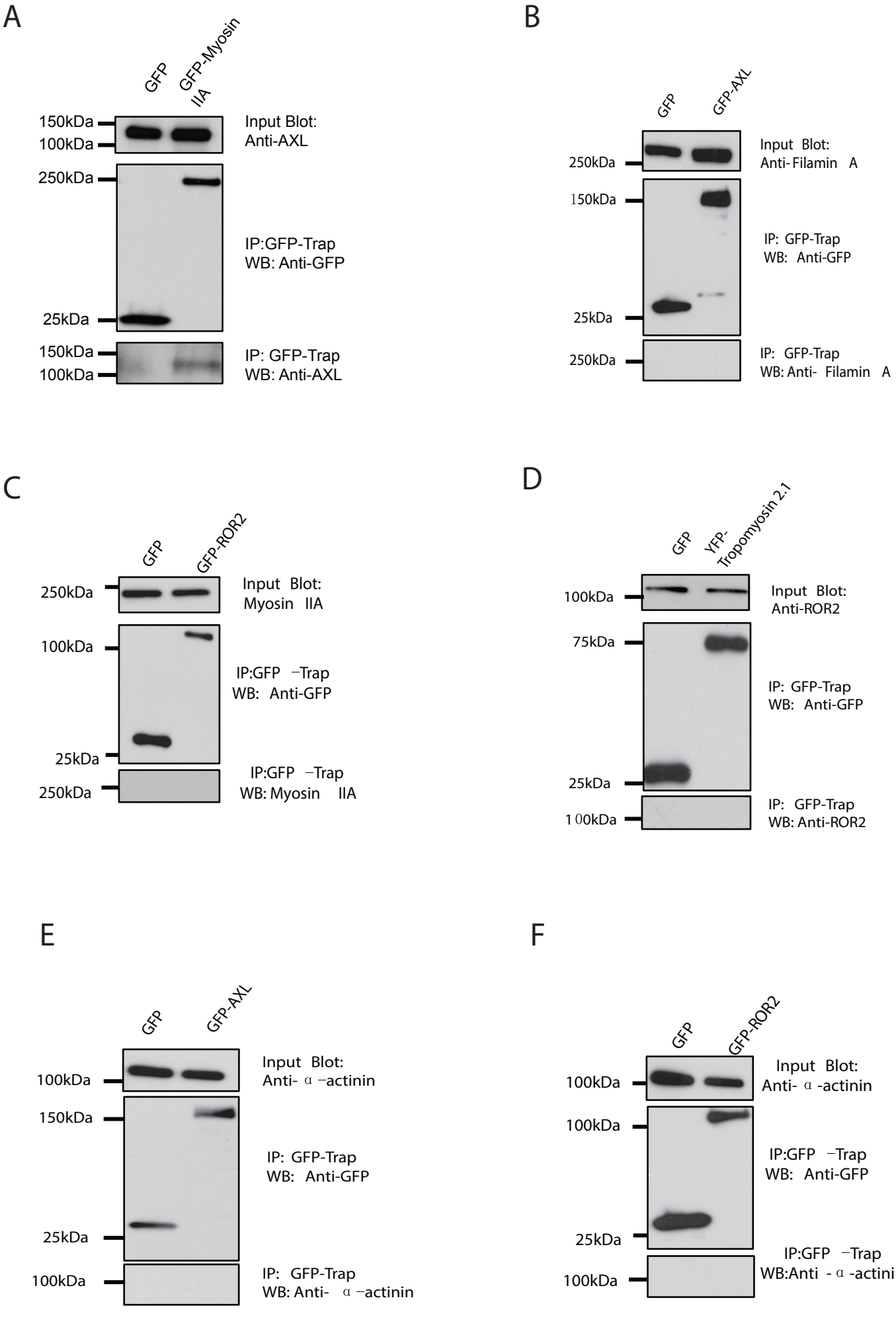


Supplementary Figure 6



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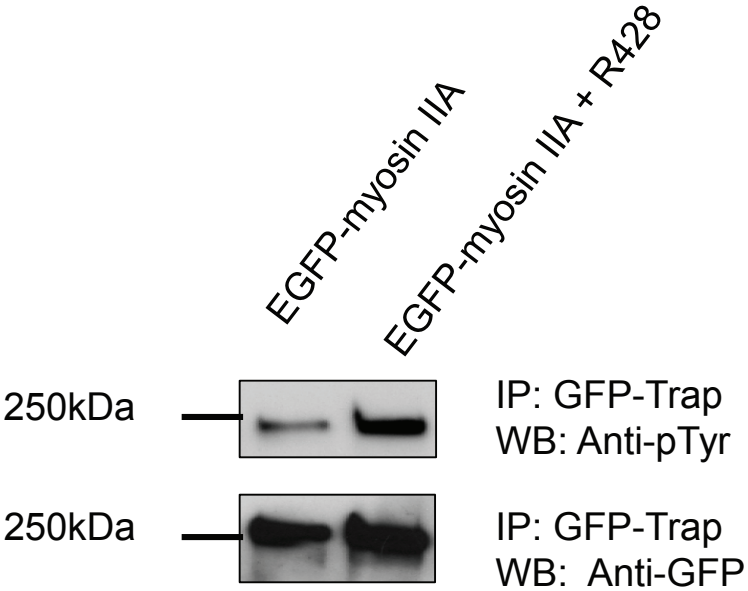

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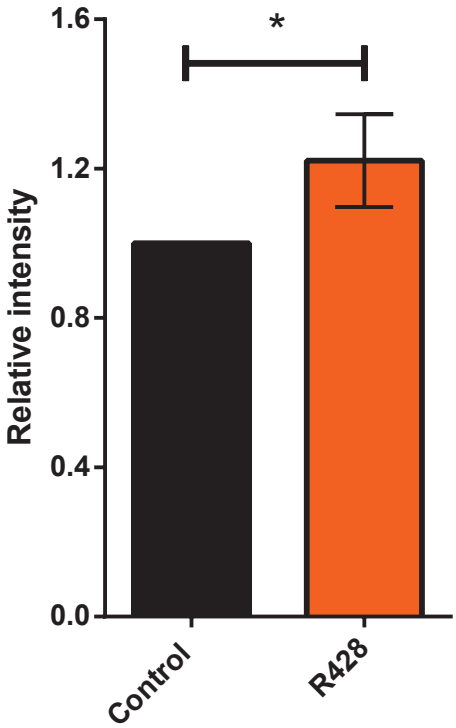

Supplementary Figure 8

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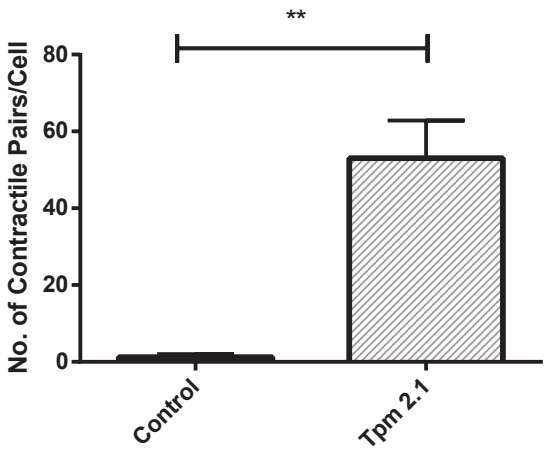

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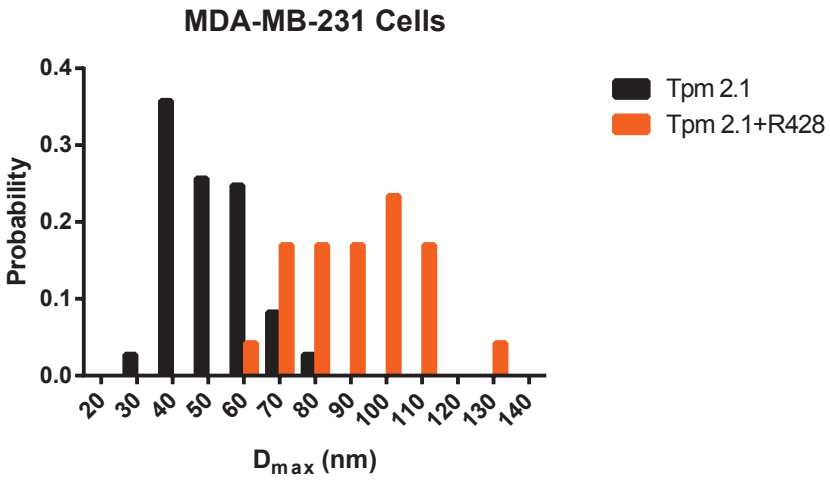

Supplementary Figure 9

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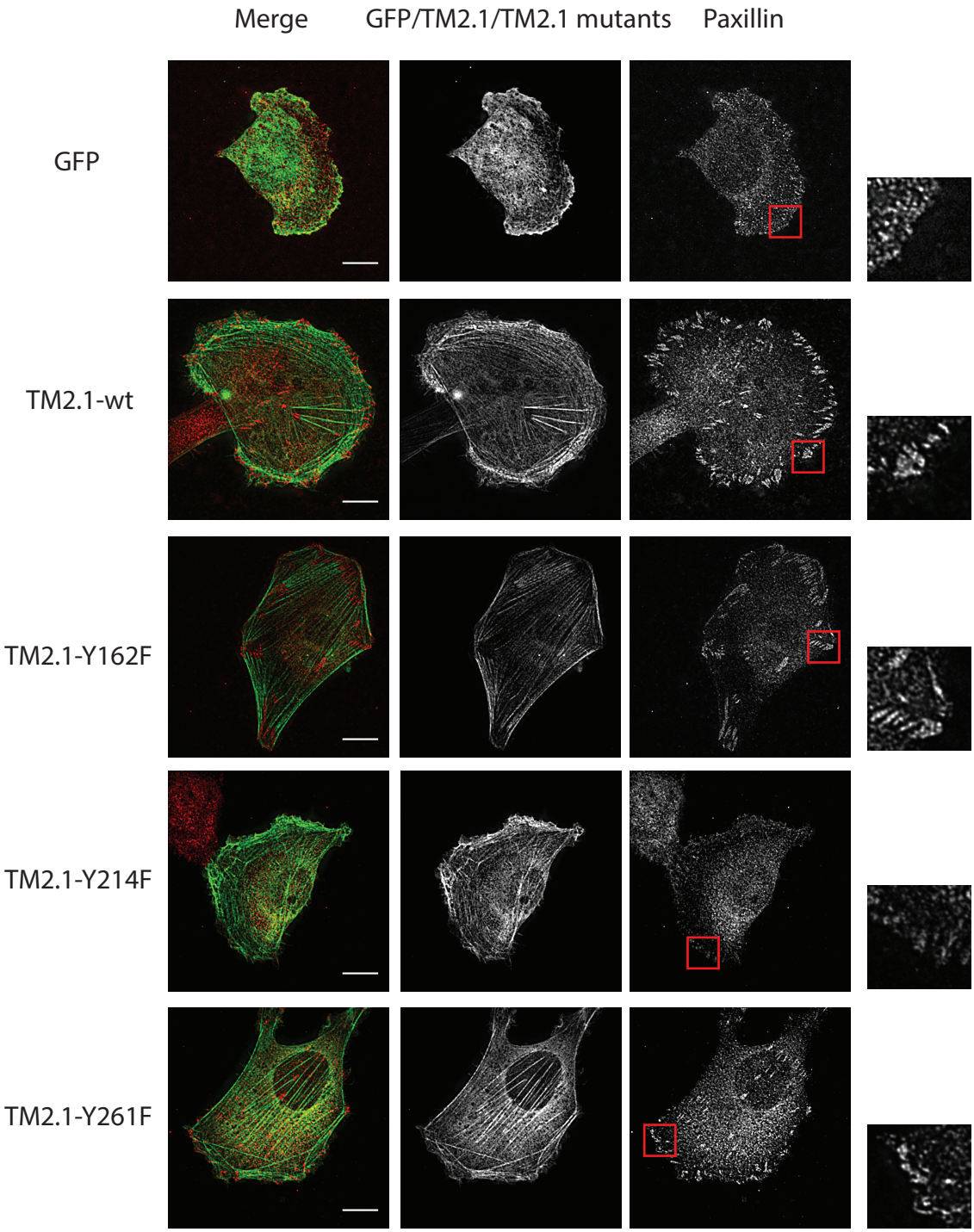

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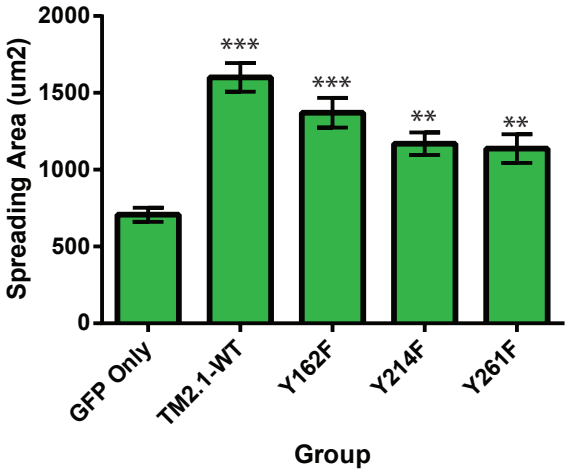

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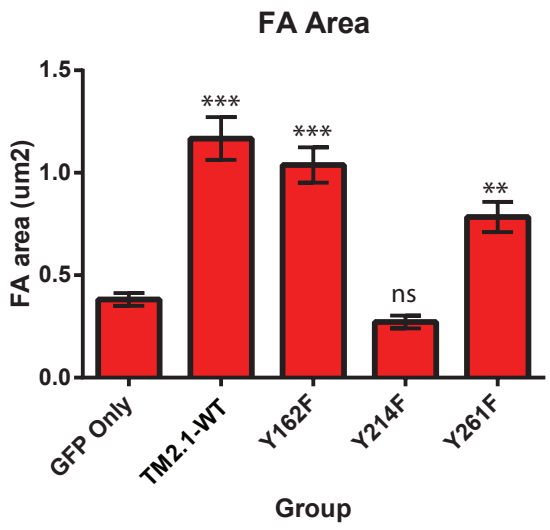

Supplementary Figure 10

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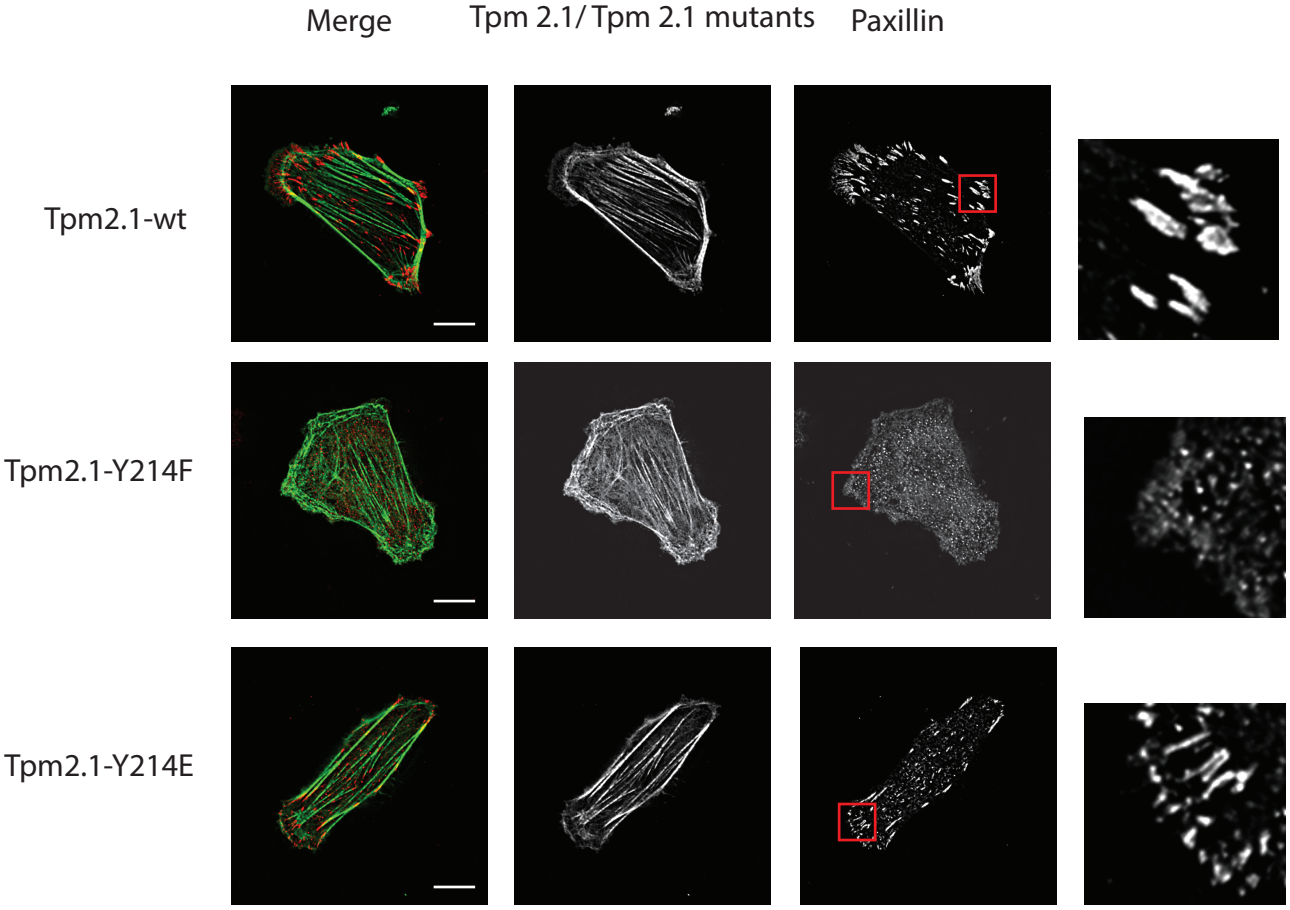

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