Current Biology, Volume *27*

Supplemental Information

Tropomyosin Isoforms Specify Functionally Distinct Actin Filament Populations In Vitro

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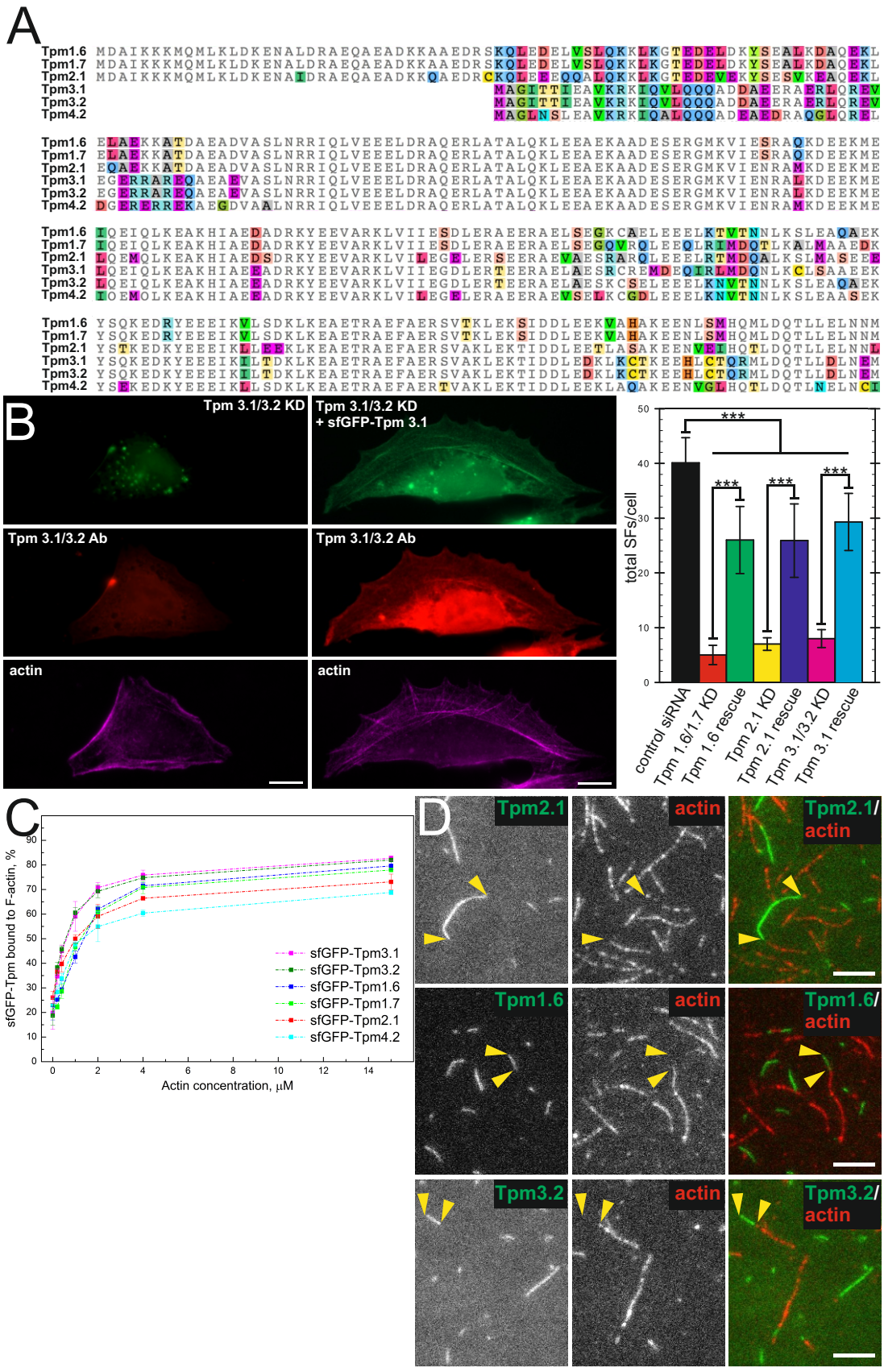

A
Tpm1.6
Tpm1.7
Tpm2.1
Tpm3.1
Tpm3.2
Tpm4.2
B
Tpm 3.1/3.2 KD
Tpm 3.1/3.2 KD
+ sfGFP-Tpm 3.1
Tpm 3.1/3.2 Ab
Tpm 3.1/3.2 Ab
actin
actin
total SFs/cell
***
control siRNA
Tpm 1.6/1.7 KD
Tpm 1.6 rescue
Tpm 2.1 KD
Tpm 2.1 rescue
Tpm 3.1/3.2 KD
Tpm 3.1 rescue
C
sfGFP-Tpm bound to F-actin, %
Actin concentration, μM
sfGFP-Tpm3.1
sfGFP-Tpm3.2
sfGFP-Tpm1.6
sfGFP-Tpm1.7
sfGFP-Tpm2.1
sfGFP-Tpm4.2
D
Tpm2.1
actin
Tpm2.1/
actin
Tpm1.6
actin
Tpm1.6/
actin
Tpm3.2
actin
Tpm3.2/
actin

Figure S1

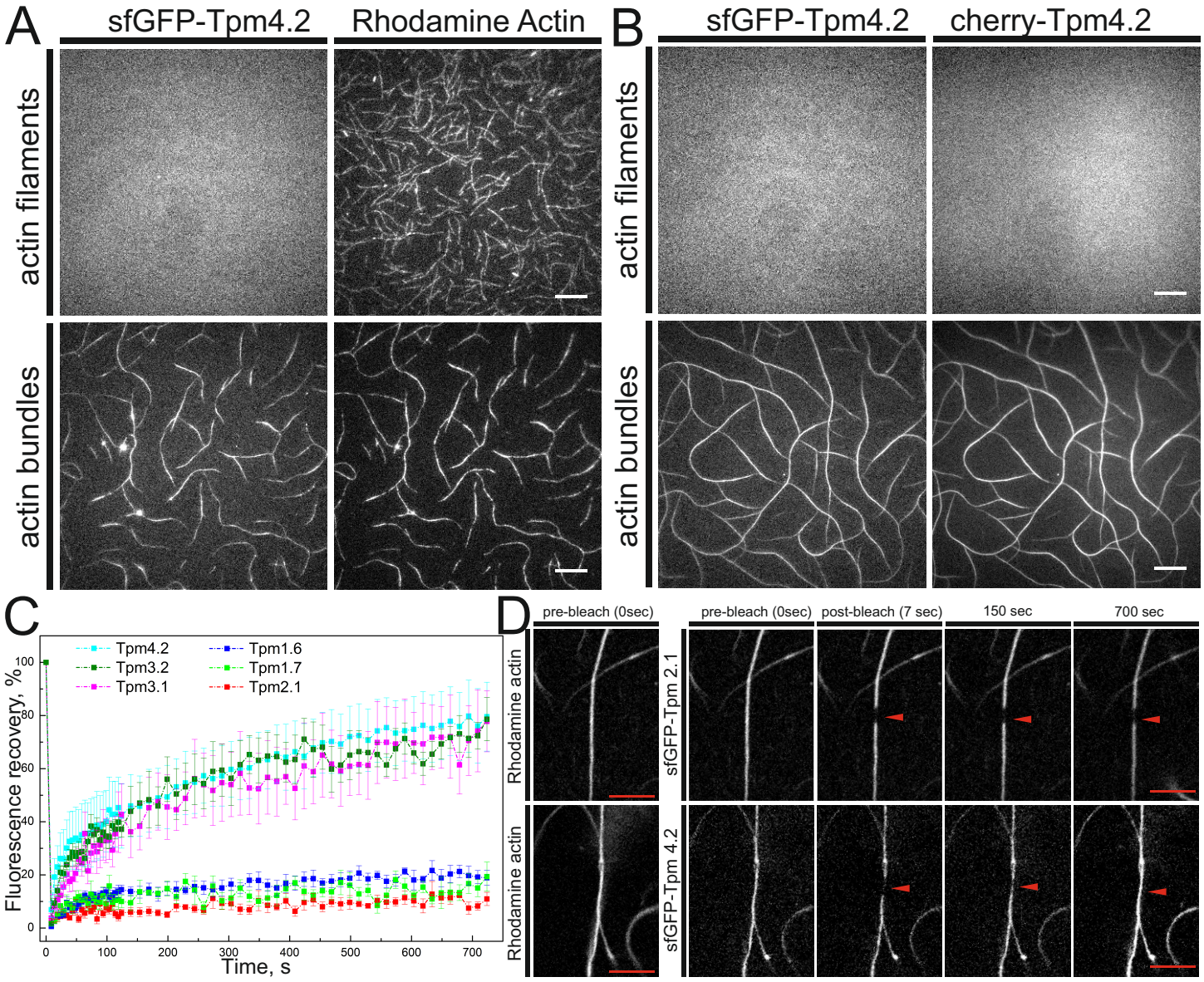
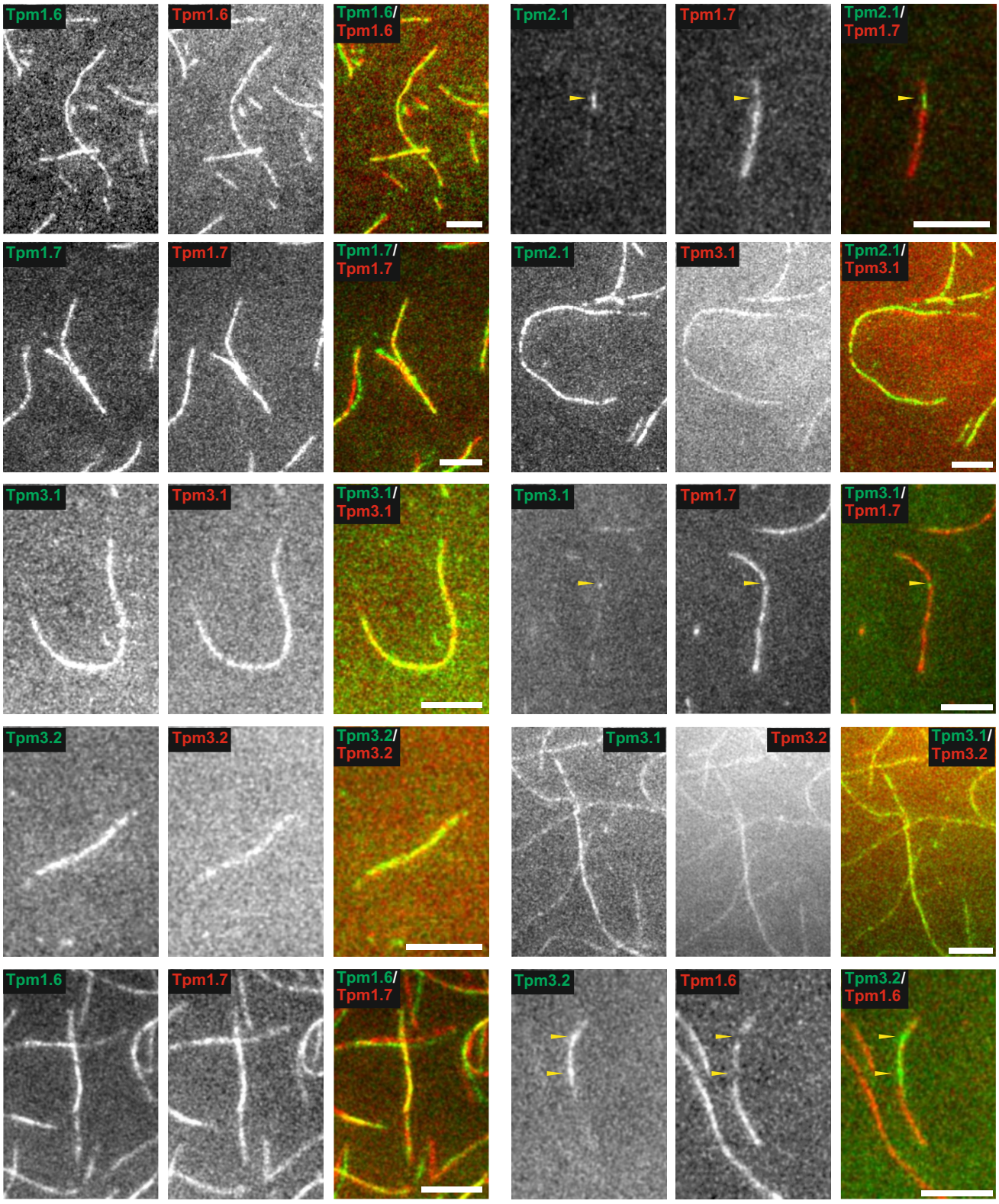

A
sfGFP-Tpm4.2
Rhodamine Actin
actin filaments
actin bundles
B
sfGFP-Tpm4.2
cherry-Tpm4.2
actin filaments
actin bundles
C
Tpm4.2
Tpm3.2
Tpm3.1
Tpm1.6
Tpm1.7
Tpm2.1
Fluorescence recovery, %
Time, s
D
pre-bleach (0sec)
pre-bleach (0sec)
post-bleach (7 sec)
150 sec
700 sec
Rhodamine actin
sfGFP-Tpm 2.1
Rhodamine actin
sfGFP-Tpm 4.2

Figure S2


Tpm1.6
Tpm1.6
Tpm1.6/
Tpm1.6
Tpm2.1
Tpm1.7
Tpm2.1/
Tpm1.7
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Tpm1.7
Tpm1.7/
Tpm1.7
Tpm2.1
Tpm3.1
Tpm2.1/
Tpm3.1
Tpm3.1
Tpm3.1
Tpm3.1/
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Tpm1.7
Tpm3.1/
Tpm1.7
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Tpm3.1
Tpm3.2
Tpm3.1/
Tpm3.2
Tpm1.6
Tpm1.7
Tpm1.6/
Tpm1.7
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Tpm1.6
Tpm3.2/
Tpm1.6

Figure S3

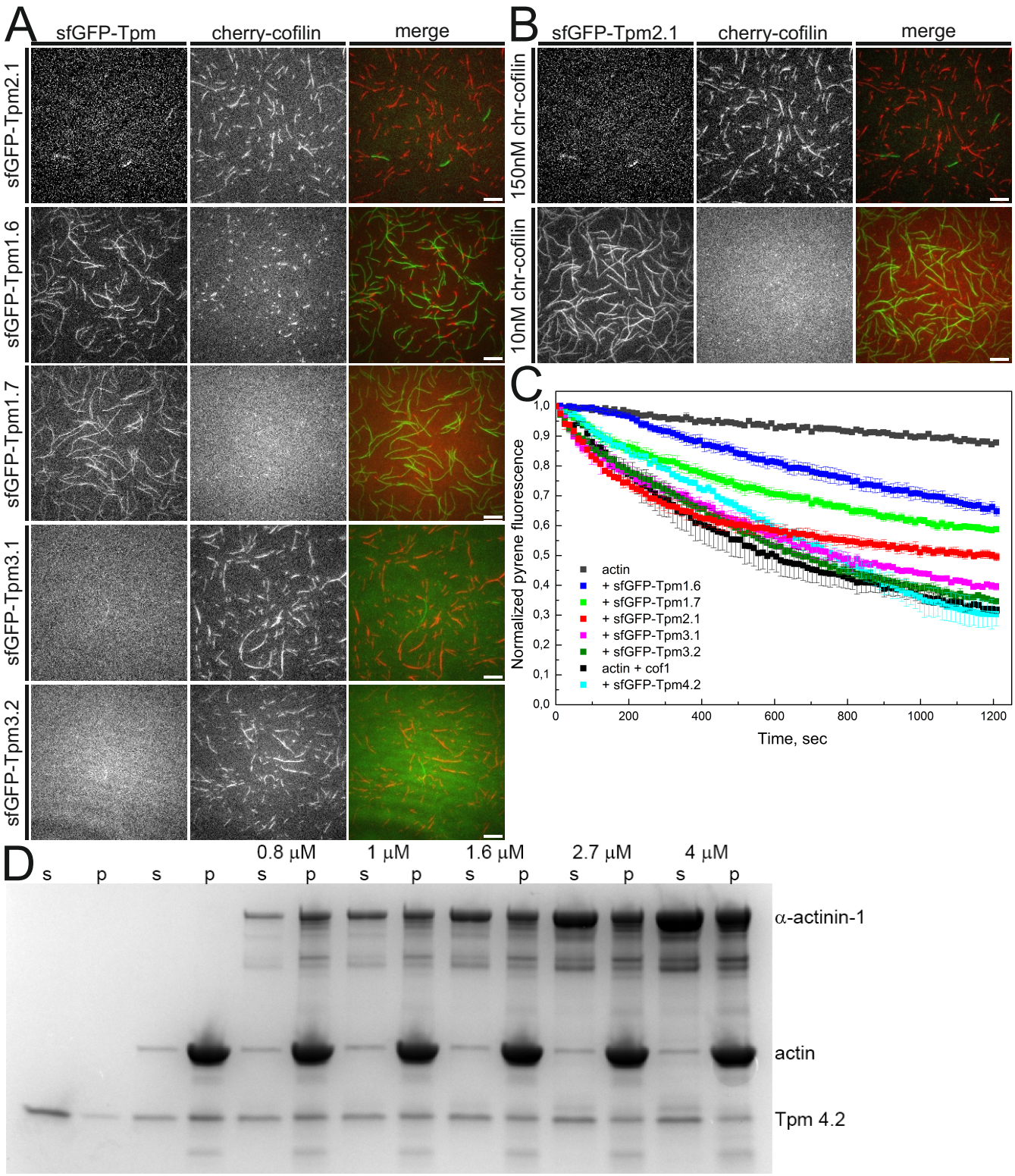
A
sfGFP-Tpm
cherry-cofilin
merge
sfGFP-Tpm2.1
sfGFP-Tpm1.6
sfGFP-Tpm1.7
sfGFP-Tpm3.1
sfGFP-Tpm3.2
B
sfGFP-Tpm2.1
cherry-cofilin
merge
150nM chr-cofilin
10nM chr-cofilin
C
Normalized pyrene fluorescence
Time, sec
actin
+ sfGFP-Tpm1.6
+ sfGFP-Tpm1.7
+ sfGFP-Tpm2.1
+ sfGFP-Tpm3.1
+ sfGFP-Tpm3.2
actin + cof1
+ sfGFP-Tpm4.2
D
0.8 μM
1 μM
1.6 μM
2.7 μM
4 μM
s
p
α-actinin-1
actin
Tpm 4.2

Figure S4

SUPPLEMENTAL FIGURES

Figure S1. Related to Figure 1. Interaction of tropomyosins with labeled actin filaments. (A). Protein sequence alignment of the stress fiber associated tropomyosin isoforms examined in this study. **(B).** sfGFP-fusions of tropomyosins rescue the knockdown stress fiber phenotypes in U2OS cells. Panes on the left show representative examples of phalloidin- and Tpm3.1/2 antibody stained Tpm3.1/3.2 knockdown cells and corresponding knockdown cells expressing sfGFP-Tpm3.1. Please note that the Alexa Fluor-488 labelled siRNA oligonucleotides appear as green dots in the knockdown and the rescue cell. Panel on the right shows quantification of the number of visible stress fibers in control siRNA, knockdown, and knockdown-rescue cells. Data are represented as average ± SD, n=10 cells/condition (Mann-Whitney or t-test= *** $p<0.001$). Bars, 10 mm. **(C).** Co-sedimentation assays demonstrating binding of sfGFP-tropomyosins to b/g-actin filaments**.** The concentration of actin was varied from 0.2 to 15 mM and the concentrations of tropomyosin dimers were 0.5 mM. Data are represented as average ± SEM, n=3 independent experiments. **(D).** Stress fiber associated tropomyosins do not bind rhodamine-labeled actin filaments. Examples of sfGFP-tagged tropomyosins (150 nM) segregating with rhodamine actin on filaments in a TIRF microscopy experiment where 5 % of actin was rhodamine-labeled on lysines. The total actin concentration was 800 nM. Bars, 5 mm.

Figure S2. Related to Figure 1. Tpm4.2 binds only to bundled actin filaments. (A). Comparison of sfGFP-Tpm4.2 (1 mM) binding to individual actin filaments (upper panels) or filament bundles (lower panels) containing 5% rhodamine-actin. Actin filament bundling was induced with 5 % methylcellulose. **(B).** Interactions of sfGFP-Tpm4.2 (1 mM) and mCherry-Tpm4.2 (1 mM) with unlabeled individual actin filaments (upper panels) or unlabeled filament bundles (lower panels). Actin filament bundling was induced with 5 % methylcellulose. The concentration of actin was 800 nM in all experiments. Bars, 10 mm. **(C and D**) Dynamics of tropomyosin binding to b/g -actin bundles was examined by fluorescence-recovery-after-photobleaching (FRAP). For the assay, 3.3 µM sfGFP-fusions of tropomyosin isoforms were mixed with 0.9 µM b/g-actin (containing 5 % of rhodamine labelled actin) in the presence of 5 % methylcellulose to induce bundles, and incubated for 30 minutes. Three frames were recorded prior to bleaching and a single bundle was bleached at maximum laser power for 3 frames at maximum speed. The fluorescence recovery was followed for 12 minutes by obtaining images every 5 seconds for the first 24 frames, and every 15 seconds for the last 40 frames. Representative examples of the FRAP data are shown in panel D, and the averaged recovery curves for the tropomyosin isoforms are shown in panel C. Data are represented as average ± SEM, n=5-6 filament bundles from 3 independent experiments. Bars, 10 mm.

Figure S3. Related to Figure 2. Examples of co-polymerization of tropomyosin isoforms on actin filaments as examined by an *in vitro* TIRF assay. sfGFP- or mCherry-fusions of the tropomyosin isoforms were purified and added in pairs on 800 nM polymerizing b/g-actin filaments, and their segregation on actin filaments were examined by TIRF microscopy. The concentrations of sfGFP- and mCherry-fusions of tropomyosins were 150 - 360 nM and 300 nM - 1.4 mM, respectively. Examples of different tropomyosin pairs that either co-polymerize or segregate on actin filaments are shown. Bars, 5 mm.

Figure S4. Related to Figure 3. Stress fiber associated tropomyosins compete with cofilin-1 and a-actinin-1 for actin filament binding. (A). sfGFP-tagged tropomyosin isoforms (150nM) were mixed with 150 mM cofilin-1 on polymerizing b/g-actin filaments, and the localization of these proteins along filaments was examined by TIRF microscopy. **(B).** Comparison of sfGFP-Tpm2.1 (150 nM) binding to actin filaments in the presence of two different concentrations (10 nM and 150 nM) of mCherry-cofilin-1. Actin concentration was 800 nM. Bars, 10 mm. **(C).** Effects of sfGFP-tropomyosins on cofilin-induced actin filament disassembly. The concentrations of actin and tropomyosins were the same as in Fig. 3C. Data are represented as average ± SEM, n=3-4 independent experiments. **(D).** A representative example of tropomyosin – a-actinin-1 competition assay. The actin and Tpm4.2 concentrations were 8 mM and 1 mM, and the concentration of a-actinin-1 was varied from 0 to 4 mM. S=supernatant and P=pellet.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA constructs

The open reading frames of the human stress fiber associated tropomyosins [S1] were amplified with appropriate primers and cloned into pBAT4 vector, appropriate for expression of non-tagged proteins in bacterial cells. A region encoding an Ala-Ser extension, mimicking the N-terminal acetylation modification of native tropomyosins [S2] was also introduced to all constructs. To generate constructs expressing N-terminally sfGFP- and mCherry-tagged tropomyosins, sfGFP- and mCherry- sequences with an N-terminal 6xHis tag were introduced to the 5'-prime ends of the constructs described above. Special attention was paid to creating the same linker sequence in all constructs, which is identical to the one used in GFP-Tpm 4.2 rescue construct applied in the previous work [S1].

Protein purification

Non-tagged tropomyosins were expressed in *E.coli* BL21-CodonPlus(DE3) cells, grown at 37 ºC in 800 ml LB broth, containing carbenicillin (100 µg/ml) and chloramphenicol (50 µg/ml) until OD 0.6. Expression was induced by the addition of isopropylthio-b-D galactosidase (IPTG, Sigma) to final concentration of 2 mM and the cells were grown for 3h at 37 ºC. Tagged tropomyosins were also expressed in *E.coli* BL21-CodonPlus(DE3) cells, grown at 37 ºC in 800 ml LB broth, containing carbenicillin (100 µg/ml) and chloramphenicol (50 µg/ml) until OD 0.5. Cultures were then cooled and expression was induced by the addition of isopropylthio-b-D galactosidase (IPTG, Sigma) to final concentration of 2 mM, and the cells were incubated with shaking overnight at 16 ºC. The cells were harvested by centrifugation and the pellets were frozen at -80 ºC until purification. The non-tagged tropomyosin pellets were re-suspended in lysis buffer (50 mM Hepes pH 8, 25 % Sucrose, 5 mM EGTA, 5 mM $MgCl_2$, 20 mM DTT, 500 mM NaCl, 1 mM PMSF, 10 % Glycerol, 0.1 % Triton, complete protease inhibitor tablet (Roche)) and the sample was homogenized at *EmulsiFlex* according to the manufacturer's recommendations. The cell suspension was incubated for 15 minutes with 27 mM $MnCl_2$, 1 mg DNaseI (100 µl of 10 mg/ml to 15 ml of cell lysate), followed by 20 minutes incubation with 1:1 volume of lysis buffer containing 2 M NaCl. The cell suspension was subsequently incubated at +85 ºC for 7 minutes, cooled on ice for 15 minutes, and centrifuged at 30.000 g, at 4 ºC for 1 hour. To precipitate the tropomyosins, NaAc pH 4.8 was added to final concentration of 80 mM in the supernant, and the solution was incubated for 30-60 minutes at 4 ºC on a shaker. The precipitated tropomyosins were collected by centrifugation at 30.000 g, at 4 ºC for 1 hour. The pellet was re-suspended and dialysed against buffer A (50 mM Hepes pH 8, 5 mM $MgCl_2$, 10 mM β-mercaptoEtOH, 1 mM DTT, 100 mM NaCl, 10 % Glycerol, 0.1 % Triton) overnight, with two buffer changes. The dialysed protein was centrifuged at 26.700 g, for 20 minutes, at 4 ºC to remove aggregates, and the supernatant was loaded on equilibrated 10 ml HiTrap Q HP anion exchange column. After extensive washing, the tropomyosins were eluted via linear gradient from 0 – 1000 mM NaCl over 10 column volumes, peak fractions were combined, concentrated and loaded on Superdex-75 gel filtration column, equilibrated with 50 mM Hepes pH 8, 300 mM NaCl, 5 mM $MgCl_2$, 10 % glycerol, 10 mM DTT and 1 mM β-mercaptoEtOH. The protein-containing fractions were pooled, concentrated, frozen, and stored at -80 ºC or -20 ºC. The his-tagged sfGFP/mCherry fusions of tropomyosins were purified with Ni-NTA agarose beads (Qiagen) according to manifacturer's instructions. Briefly, pellets were resuspended in lysis buffer, containing 50 mM Hepes, pH 8, 300 mM NaCl, 5 mM Imidazol, 5 mM $MgCl_2$, 1 mM DTT, 15 mM β-mercaptoethanol, 0.1% Triton-X, 2% glycerol, 0.1mM PMSF, complete protease inhibitor tablet (Roche), homogenized at *EmulsiFlex*, the cell debris were separated from the cell lysate via centrifugation and the supernatant was incubated with Ni-NTA beads, equilibrated with lysis buffer. The beads were washed extensively with lysis buffer, containing 20 mM Imidazol, and the bound protein was eluted with lysis buffer, containing 500 mM Imidazol. The eluates were concentrated when needed, aggregates were removed by quick centrifugation, and the samples were loaded on Superdex-200 gel filtration column, equilibrated with gel filtration buffer (50 mM Hepes, pH 8, 300 mM NaCl, 5 mM $MgCl_2$, 10 mM DTT, 20% glycerol). The peak fractions were collected, concentrated, and stored at -80 ºC or -20 ºC. Non-muscle actin (β/γ-actin) and profilin were prepared from bovine spleen as described in [S3] with minor changes. In brief, fresh bovine spleen was homogenized and profilin:actin complex was extracted by using poly-L-proline column. Actin was separated from profilin by polymerization-depolymerization cycles and both proteins were stored at -80 ºC until further use. Human a-actinin-1 construct in pET8C vector was a kind gift from Kristina Djinovic-Carugo lab. Protein was expressed in auto-induction media ZYP5052 and purified according to the previously described protocol [S4]. Mouse cofilin-1 and mCherry-cofilin-1 were expressed and purified as described in [S5].

Co-sedimentation assays

Actin co-sedimentation assay was carried out essentially as described earlier [S5]. Briefly, different amounts of β/γ-actin were polymerized together with 1 µM of each tropomyosin isoform for 30 minutes at RT in the presence of G-buffer (5 mM Tris-HCl pH 7.5 with 0.2 mM DTT and 0.2 mM $CaCl_2$) by addition of 5 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 0.2 mM ATP and 1x Imidazole buffer (10x Imidazole buffer: 100 mM Imidazole pH 7.6, 100 mM DTT, 100 mM β-mercaptoEtOH and 2 mM PMSF). To sediment the polymerized actin filaments and bound proteins, the samples were subjected to ultracentrifugation (30 minutes, 20 ºC, 156425 g). For competition assays with tropomyosin isoforms and α-actinin-1, β/γ-actin (8 µM) was pre-polymerized in G-buffer (5 mM Tris-HCl pH 7.5 with 0.2 mM DTT and 0.2 mM $CaCl_2$) for 30 minutes at room temperature by addition of $MgCl_2$ to the final concentration of 5 mM, in the presence of

100 mM NaCl, 1 mM EGTA, 0.2 mM ATP and 1x Imidazole buffer. One µM of various tropomyosin isoforms and 1.6 µM of α-actinin-1 were sequentially added to the pre-polymerized actin with 30 minutes incubation at room temperature in between. Final samples were further incubated for 30 minutes. Actin filaments were sedimented by centrifugation for 30 minutes at 20 °C in a Beckman Optima MAX Ultracentrifuge at 100000 rpm for Tpm2.1, Tpm4.2, Tpm3.1, Tpm3.2 and at 60000 rpm for Tpm1.6 and Tpm1.7 in a TLA100 rotor. Equal proportions of supernatants and pellets were run on 4-20% gradient SDS-polyacrylamide gels (Mini-PROTEAN TGX Precast Gels, Bio-Rad Laboratories Inc.), which were stained with Coomassie Blue. The intensities of tropomyosin bands were quantified with QuantityOne program (Bio-Rad Laboratories Inc.), analyzed and plotted as tropomyosin bound to actin (µM, tropomyosin in pellet) in the absence and presence of α-actinin-1. Please note that a-actinin-1 addition did not significantly increase the amount of actin in the pellet fraction.

FRAP experiments

The experiments on individual filaments were performed on a confocal spinning disk from Roper Scientific, based on an inversed Nikon Eclipse TI and equipped with a Photometrics EMCCD Evolve camera. For sfGFP imaging, a 491-nm laser line (100mW) and a Plan APO ON 100x/1,4 objective was used. Images are recorded through Metamorph with binning 1. Briefly, non-labeled actin (0.6 mM) was polymerized for 20 minutes and 0.8 µM sfGFP-labeled tropomyosins were added to the pre-polymerized filaments, and the samples were injected into TIRF chamber. Few pre-bleaching frames were imaged before the filament was bleached for 15 seconds and then followed for 10 minutes with images recorded at every 15 seconds. Protein recovery was analyzed with ImageJ software for 5-6 filaments/sample.

Actin filament bundles were induced with increasing the percentage of methylcellulose in TIRF fluorescence buffer as previously described [S6]. Briefly, 3.3 µM sfGFP-fusions of tropomyosin isoforms were mixed with 0.9 µM b/g-actin (containing 5 % of rhodamine lysine-labelled human platelet actin, Cytoskeleton Inc.) in the presence of 5 % methylcellulose to induce bundles and incubated for 30 minutes prior to imaging in a TIRF chamber. Imaging was performed on Leica TCS SP5II HCS A confocal microscope, equipped with PMT and LasAF software. For sfGFP visualization a 488 nm (35mW) laser line and HCX PL APO 63x/1,2 W Corr/0,17 CS (water) Lbd.bl objective were used. Prior to bleaching with full laser power for 3 frames, 3 frames were recorded, and then the fluorescence recovery was followed for 12 minutes after bleaching by obtaining images every 5 seconds for the first 24 frames, and every 15 seconds for 40 frames. Protein recovery was analyzed with ImageJ software for 5-6 filaments/sample.

***In vitro* TIRF microscopy**

In vitro TIRF experiments were performed essentially as previously described [S7]. Briefly, 0.8 mM of β/γ-actin, sfGFP-Tpms and mCherry-Tpms were diluted in fluorescence buffer containing 10 mM imidazole-HCl pH 7.8, 50 mM KCl, 1 mM $MgCl_2$, 100 mM DTT, 3 mg/ml glucose, 20 µg/ml catalase, 100 µg/ml glucose oxidase, and 0.5% methylcellulose. The concentrations of each sfGFP and mCherry isoform was titrated to obtain maximum filament labelling without intense background staining. This resulted in final concentrations between 150 and 360 nM of sfGFP and 300 nM to 1.4 mM of mCherry fusion proteins. After addition of the proteins to G-actin and fluorescent buffer, the solution was injected into TIRF chambers and imaged after 5 minutes incubation. Each video was recorded for 20-30 minutes, and images were acquired with a minimal delay (0.2 mseconds) or every 5-10 seconds. For *in vitro* TIRF experiments with cofilin-1, 150 nM of sfGFP-HMW-Tm isoforms (Tpm2.1, Tpm1.6, Tpm1.7) and 300 nM of sfGFP-LMW-Tm isoforms (Tpm3.1, Tpm3.2) were added to G-actin and polymerization mix, and incubated for 5 minutes at RT, then 150 nM of cherry-cofilin 1 was added to the reaction and imaged immediately. All experiments were imaged at Nikon Eclipse Ti-E TIRF microscope, equipped with 100x Apo TIRF 1.49 Oil Objective, 65mW Argon laser, 150mW 561nm laser, Andor iXon+ 885 EMCCD camera and NIS elements software. Huygens software was used for very light deconvolution (2 cycles) to remove noise from the TIRF data and calculate the percentage of red pixels co-localizing with green pixels.

β/γ-actin disassembly assay

The steady-state rate of β/γ-actin filament disassembly was measured using a modified protocol described for muscle actin in [S5]. Samples of polymerized pyrene actin (4 µM) were mixed and incubated for 5 minutes with 1.25 µM of various tropomyosins before the addition of 1 µM cofilin-1 followed by further 5 minutes incubation. All protein mixtures were assembled in 1.5 ml Eppendorf tubes and after initialization of the reaction by the addition of 6 µM DBP were moved to the fluorometric cuvettes. During the experiments, buffer conditions were constant: 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP and 1x Imidazole buffer (10x Imidazole buffer: 100 mM Imidazole pH 7.6, 100 mM DTT, 100 mM β-mercaptoEtOH and 2 mM PMSF). All measurements were carried out using Perkin Elmer fluorometer or Agilent Cary Eclipse Fluorescence Spectrophotometer with BioMelt Bundle System (Agilent Technologies) with excitation at 365 nm (Ex. Slit = 10 nm) and emission at 407 nm (Em. Slit = 20 nm). Origin 7.5 software (OriginLab Corp.) was used to analyze the data. To calculate the relative actin filament disassembly rates in the presence of different tropomyosins, linear region of each curve during 170 – 400 seconds time interval was fitted with equation $y=a \cdot x+b$, where coefficient "a" represents speed of the process. Please note that the first 170 seconds of the data were excluded from the analysis due to the possible effect of the short mechanically broken actin filaments.

Steady-state ATPase measurements

The regulatory light chain of NM-2A-HMM was phosphorylated for 30 minutes at 30 °C in ATPase Buffer (20 mM MOPS pH 7, 50 mM KCl, 2 mM $MgCl_2$, 0.15 mM EGTA, 2 mM DTT) containing 1 mM $CaCl_2$, 0.2 μM calmodulin (CaM), 2 μM essential light chain (MYL6), 2 μM regulatory light chain (MYL12b), 1mM ATP and 50 nM myosin light chain kinase (MLCK). The steady-state ATPase rates were measured in an NADH-coupled assay using 0.3 μM NM-2a-HMM in ATPase buffer containing 1 mM ATP, 0.8 mM NADH, 0.5 mM phosphoenolpyruvate, 20 μg/ml lactate dehydrogenase and 50 μg/ml pyruvate kinase. All combinations were tested with and without the addition of NM-2A-HMM, and the actin ATPase rate was then subtracted from the NM-2A-HMM ATPase rates. Muscle actin or b-actin was pre-incubated with the different tropomyosins for 30 minutes at RT, before addition to the assay mix. The assays were performed with final concentrations of 20 μM actin and 20 μM tropomyosin. The change in absorption at 340 nm was recorded in a 96-well plate on a microplate reader at 25 °C. Data were analyzed with GraphPad Prism 7 software. The Gaussian distribution of ATPase assay data was confirmed and a one-way ANOVA was performed comparing all conditions to the bare-actin control (* $p<0.05$, *** $p<0.001$).

RNAi – rescue experiments

Tpm1.6/1.7, Tpm 2.1 and Tpm3.1/3.2 knockdown U2OS cells were generated with specific Alexa Fluor-488 labelled siRNA oligonucleotides and the cells were transfected with sfGFP-Tpm constructs essentially as previously described [S1]. Tropomyosins were detected with the following antibodies: TM311 monoclonal antibody (Sigma-Aldrich) for Tpm 1.6/1.7, CGβ6 for Tpm 2.1 and sheep polyclonal γ/9D for Tpm 3.1/3.2, and actin filaments were visualized with Alexa Fluor 647–phalloidin (Invitrogen). The samples were imaged at Zeiss Axio Imager Z2 upright epifluorescence microscope equipped with 100x/1.3 oil EC Plan Neofluar objective, Hamamatsu Orca Flash 4.0 LT camera and Zen software or Zeiss LSM 700 confocal microscope equipped with LCI Plan-Neofluar 63x/1.30 glycerol objective, 639 nm/5mW, 555 nm/10mW, 488 nm / 10mW laser lines and Zen software. To quantify the number of stress fiber per cell, the line profile tool of ImagePro Plus 5.1 was applied to draw lines perpendicular to the dorsal stress fibers, lamella or ventral stress fibers, and the intensity peaks along the lines in the phalloidin channel were counted as previously described [S1].

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