Murugesan et al., http://dx.doi.org/10.1083/jcb.201603080

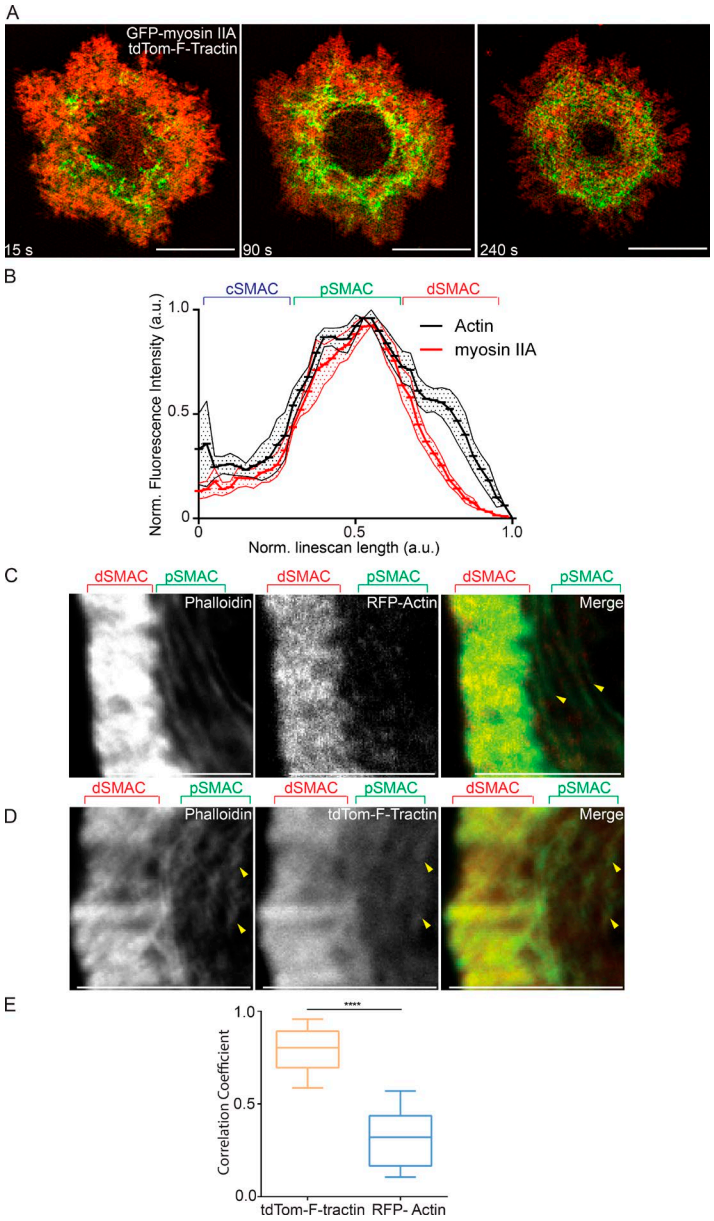


Figure S1. **Actomyosin arc formation in a primary mouse CD8⁺ T cell, and Td Tomato–F-Tractin, but not RFP-actin, reports endogenous, phalloidin-stained actin arcs in the pSMAC.** (A) Still images from a two-color TIRF-SIM video of a primary mouse CD8⁺ T cell expressing GFP–myosin IIA and tdTomato–F-Tractin at the time points indicated after engagement with an activating surface. (B) Radial plot profile showing the distributions of actin and myosin IIA across the IS of a mouse CD8⁺ T cell 5 min after engagement. SMAC zones are bracketed. Data are represented as mean ± SEM (C) Confocal images of F-actin structures at the IS of a Jurkat T cell expressing RFP-actin and stained with phalloidin. (D) Confocal images of F-actin structures at the IS of a Jurkat T cell expressing tdTomato (tdTom)–F-Tractin and stained with phalloidin. (E) Pearson's correlation coefficient for tdTomato–F-Tractin versus phalloidin and RFP-Actin versus phalloidin across the pSMAC. *n* = 15–20 cells/condition. Box and whiskers plot is centered on the mean and display upper and lower quartile ranges and min to max values.The SMAC zones are bracketed in C and D at top. Bars, 5 µm. ****, P < 0.0001. a.u., arbitrary units; Norm., normalized.

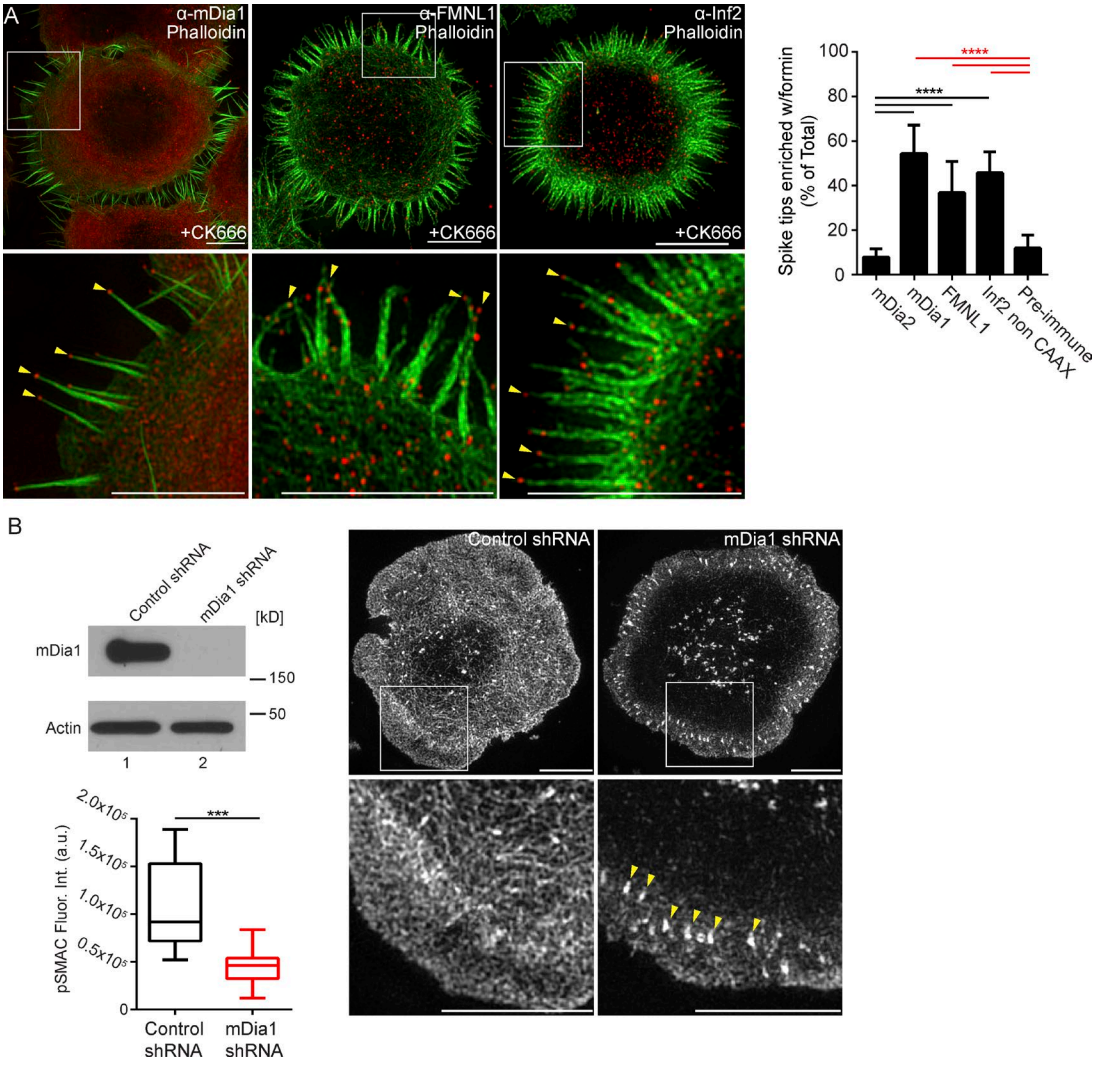


Figure S2. **Three formins can be detected by immunostaining at the tips of actin spikes in CK666-treated Jurkat T cells, and knockdown of mDia1 greatly attenuates arc formation.** (A, first through third panels and magnified insets in fourth through sixth panels) 3D-SIM images of CK666-treated Jurkat T cells immunostained for mDia1, FMNL1, and INF2 non-CAAX and stained with phalloidin. Yellow arrowheads mark formin puncta at spike tips. (A, seventh panel) Percent of spikes enriched with the indicated formin within 1 µm of the tip. *n* = 10 cells/condition. ****, P < 0.0001 when compared with either mDia2 (black) or preimmune sera (red). Data represent means ± SEM. (B, top left) Western blot of whole-cell extracts of Jurkats 48 h after transfection with either a control shRNA plasmid (lane 1) or an shRNA plasmid for mDia1 (lane 2) and probed for mDia1 and actin as an internal control. (B, right panels) Representative 3D-SIM images of activated shRNA control and mDia1 knockdown Jurkat T cells stained with phalloidin. The yellow arrowheads mark actin foci in knockdown cells. (B, bottom left) Mean phalloidin fluorescence in the pSMAC. *n* = 10 cells/condition. Box plots are centered on means and display upper and lower quartile ranges and min to max values. Bars, 5 µm. ***, P < 0.001. a.u., arbitrary units; Fluor. Int., fluorescence intensity.

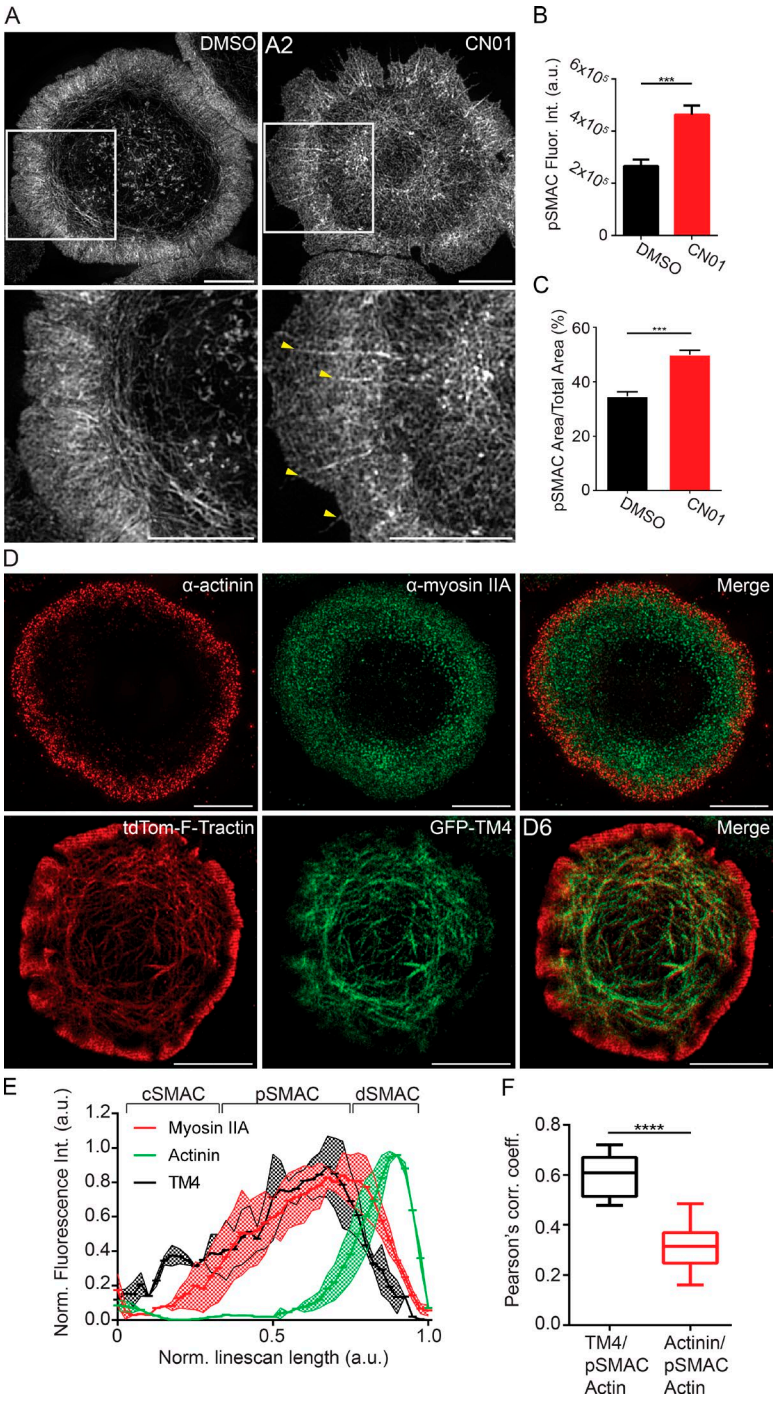


Figure S3. **Linear actin filaments in the dSMAC and actin arcs in the pSMAC are accentuated by a pan activator of Rho GTPases, and arcs are enriched for the tropomyosin TM4 but not for α-actinin.** (A) Representative 3D-SIM image of a Jurkat cell treated with DMSO (left) or 1 unit/ml CN01 (right), a membrane-permeant pan activator of Rho GTPases. Yellow arrowheads point to the accentuated linear actin filaments/bundles in the dSMAC. (B) Mean phalloidin fluorescence in the pSMAC of control, DMSO-treated, and CN01-treated Jurkats. *n* = 14–20 cells/condition. Data are means ± SEM (C) The ratio of pSMAC area to total IS area for control, DMSO-treated, and CN01-treated Jurkats. *n* = 13–24 cells/condition. Data are means ± SEM (D, top) 3D-SIM images of a representative Jurkat T cell immunostained for α-actinin and myosin IIA. (D, bottom) TIRF-SIM images of a representative Jurkat T cell expressing tdTomato (tdTom)–F-Tractin and GFP-TM4. (E) Radial plot profiles of myosin IIA, TM4, and α-actinin in Jurkat T cells. *n* = 7 cells; P < 0.0001, two-way ANOVA. (F) Pearson's correlation coefficient (corr. coeff.). *n* = 10–16 cells/correlation. Box plots are centered on means and displays upper and lower quartile ranges and min to max values. ***, P < 0.001; ****, P < 0.0001. a.u., arbitrary units; Fluor. Int., fluorescence intensity; Norm., normalized. Bars, 5 µm.

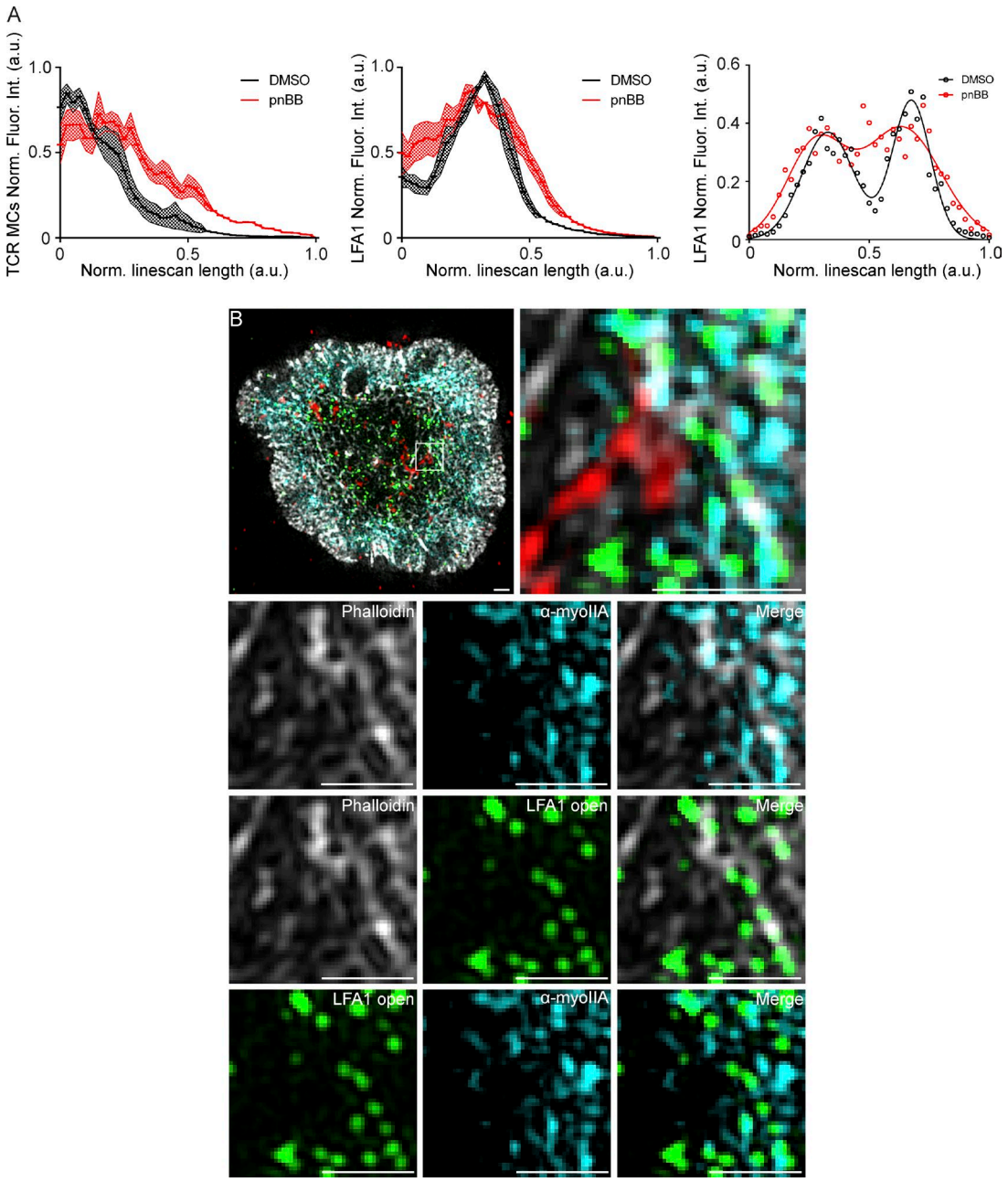


Figure S4. **Myosin II inhibition phenocopies formin inhibition with regard to receptor cluster distribution, and myosin II tends to coincide with actin and open, active LFA-1 in the pSMAC.** (A, first and second panels) Radial plot profiles of TCR MCs and LFA-1 clusters in Jurkat T cells treated with DMSO or 50 µM pnBB. *n* = 13 cells; P < 0.0001, two-way ANOVA. (A, third panel) Line scans across cells in A2 fit to Gaussian distributions. (B, top) Four-color 3D-SIM of F-actin (white), TCR MCs (red), open, active LFA-1 clusters (green), and myosin IIA (myoIIA; blue). (B, bottom nine panels) Individual combinations of the actin, myosin IIA, and open, active LFA-1 channels and their overlays. See also Fig. 8 B. Bars, 1 µm. a.u., arbitrary units; Norm. Fluor. Int., normalized fluorescence intensity.

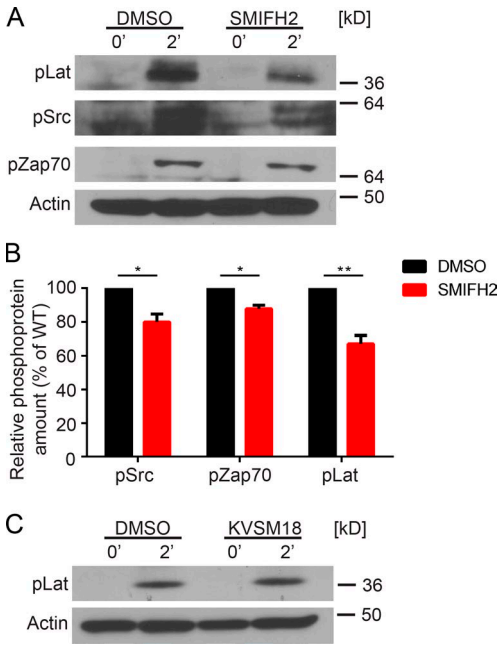
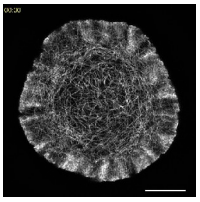
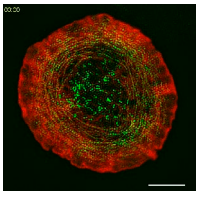


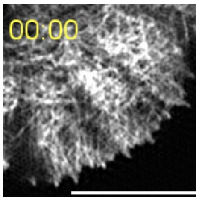
Figure S5. **SMIFH2-treated Jurkats activated with soluble anti-CD3 also exhibit a reduction in TCR proximal signaling, and KVSM18 does not inhibit Lat phosphorylation.** (A) Representative Western blot of whole-cell extracts of DMSO- and SMIFH2-treated Jurkats activated for the indicated times using soluble anti-CD3 and probed for the tyrosine-phosphorylated versions of Src (Y416), Zap70 (Y319), or Lat (Y191). (B) Quantitation of relative phosphoprotein amounts. $n = 3$ independent experiments. Data are means ± SEM. (C) Western blot of whole-cell extracts of DMSO- and KVSM18-treated Jurkats activated and probed with anti-Lat as described in A. *, $P < 0.05$; **, $P < 0.01$. WT, wild-type.



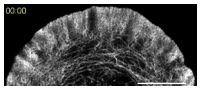
Video 1. **TIRF-SIM reveals the dynamics of F-actin networks at the Jurkat T cell IS.** Jurkat T cell expressing GFP–F-Tractin faithfully reports the two major actin networks at the IS seen with phalloidin staining. Images were collected every 2 s for 1.5 min. See Fig. 1.



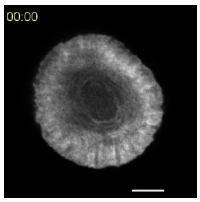
Video 2. **Two-color TIRF-SIM reveals the dynamics of actomyosin arcs in the pSMAC.** Jurkat T cell expressing GFP–myosin IIA and tdTomato–F-Tractin showing enrichment of myosin IIA on concentric actin arcs in the pSMAC. Images were collected every 3 s for 2 min. See Fig. 1.



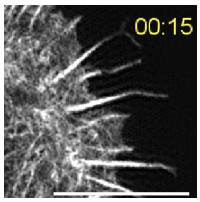
Video 3. **Linear actin filaments/bundles are embedded in the dSMAC.** TIRF-SIM video of a Jurkat T cell expressing GFP–F-Tractin showing the formation of several linear actin filaments/bundles at the distal edge of the IS that span the dSMAC. Images were collected every 1 s for 1 min. See Fig. 3.



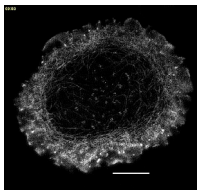
Video 4. **Linear actin filaments/bundles appear to give rise to actin arcs in the pSMAC.** TIRF-SIM video of a Jurkat T cell expressing GFP–F-Tractin showing several linear actin filaments/bundles flowing inward and giving rise to actin arcs in the pSMAC. Images were collected every 3 s for 5 min. See Fig. 3.



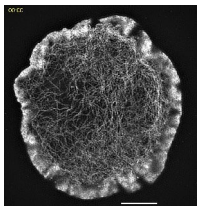
Video 5. **Arp2/3 inhibition reversibly augments linear actin filaments/bundles.** Spinning-disc confocal video of a Jurkat T cell expressing GFP–F-Tractin, before and after treatment with 25 µM CK666, followed by CK666 washout . Images were collected every 2 s for 9 min. See Fig. 4.



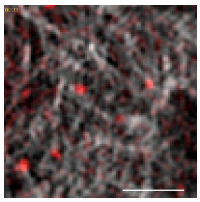
Video 6. **Arp2/3 inhibition augments linear actin filaments/bundles.** TIRF-SIM video of Jurkat T cell expressing GFP–F-Tractin after treatment with 25 µM CK666. Images were collected every 3 s for 2.5 min. See Fig. 4.



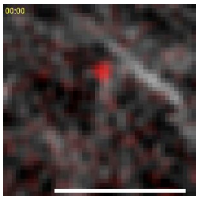
Video 7. **Linear actin filaments/bundles and actin arcs rapidly reappear upon washout from formin inhibition.** TIRF-SIM video of Jurkat T cell expressing GFP–F-Tractin shortly after the washout of 10 µM SMIFH2. Images were collected every 3 s for 5 min. See Fig. 5.



Video 8. **Myosin II inhibition disrupts actin arc organization.** TIRF-SIM video of a Jurkat T cell expressing tdTomato–F-Tractin pretreated with 50 µM pnBB. Images were collected every 3 s for 2 min. See Fig. 6.



Video 9. **TCR MCs are swept inward by actin arcs.** TIRF-SIM videos of Jurkat T cells expressing GFP–F-Tractin stimulated on planar lipid bilayers containing Alexa Fluor 568–labeled anti–CD3 antibody (red) to report TCR MC movement. Video shows a single TCR MC (white open arrowhead) being swept inward from right to left by three successive arcs (white closed, yellow closed, and blue closed arrowheads, respectively, outlined momentarily by yellow lines). The cSMAC of this cell is to the left. Images were collected every 3 s for 1 min 39 s, respectively. See Fig. 9.



Video 10. **TCR MCs are swept inward by actin arcs.** TIRF-SIM videos of Jurkat T cells expressing GFP–F-Tractin stimulated on planar lipid bilayers containing Alexa Fluor 568–labeled anti-CD3 antibody (red) to report TCR MC movement. Movie shows a single TCR MC (white open arrowhead) being swept inward from top to bottom by two successive arcs (white closed and yellow closed arrowheads, respectively, outlined momentarily by yellow lines). The cSMAC of this cell was toward the bottom. Images were collected every 3 s for 1 min 39 s, respectively. See Fig. 9.