Babich et al., http://www.jcb.org/cgi/content/full/jcb.201201018/DC1

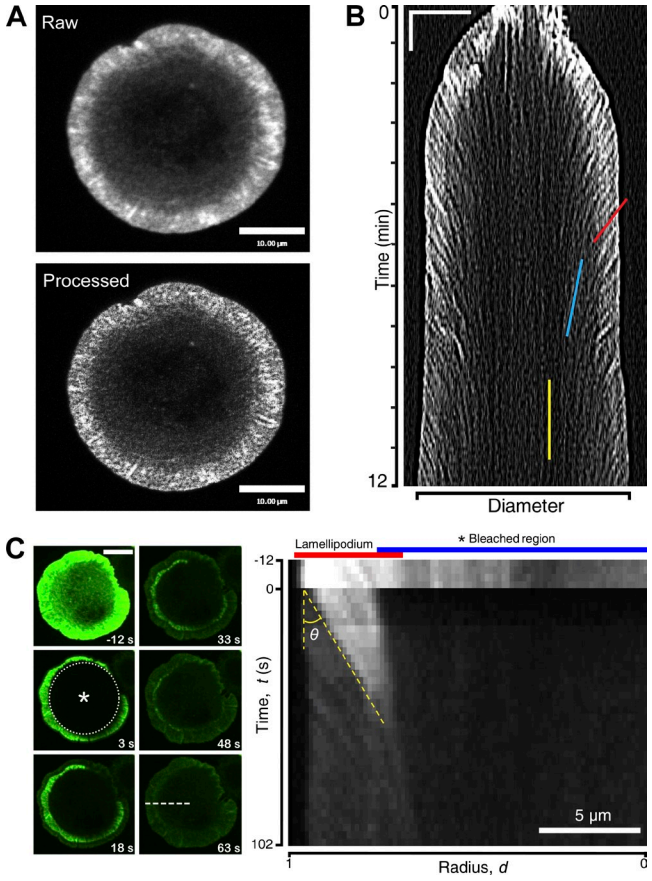



Figure S1. **Measurement of F-actin dynamics at the IS.** (A, top) Raw image of a fully spread IS of a Jurkat T cell expressing GFP-actin. (bottom) Same image digitally processed in Photoshop using a filter with 300% intensity increase for local maxima within a 3-pixel radius. Bars, 10 µm. (B) A kymograph generated from the video sequence and along the diameter of the cell depicted and processed as described in A. Brackets represent 10 µm × 1 min. (C, left) Image series of a spreading GFP-actin Jurkat T cell subjected to photobleaching in the region marked with an asterisk. Bar, 10 µm. (right) A kymograph generated along the dashed line from the sequence on the left. θ represents the angle of deflection from the vertical position. This figure is related to Fig. 1.

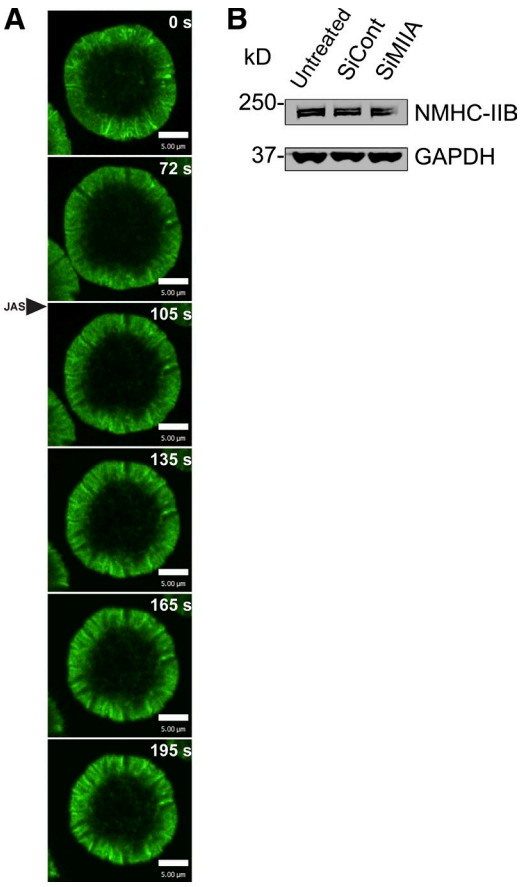



Figure S2. **Assessment of F-actin dynamics in response to JAS treatment of cells suppressed for myosin IIA.** (A) Time-lapse series of a Jurkat T cell spreading on an OKT3-coated coverglass. At the indicated time, 1 µM JAS was added to the imaging well (arrowhead). Bars, 5 µm. (B) Western blot analysis of myosin IIB expression in Jurkat T cells suppressed for myosin IIA (same lysates as in Fig. 3 D [bottom]). This figure is related to Fig. 3.

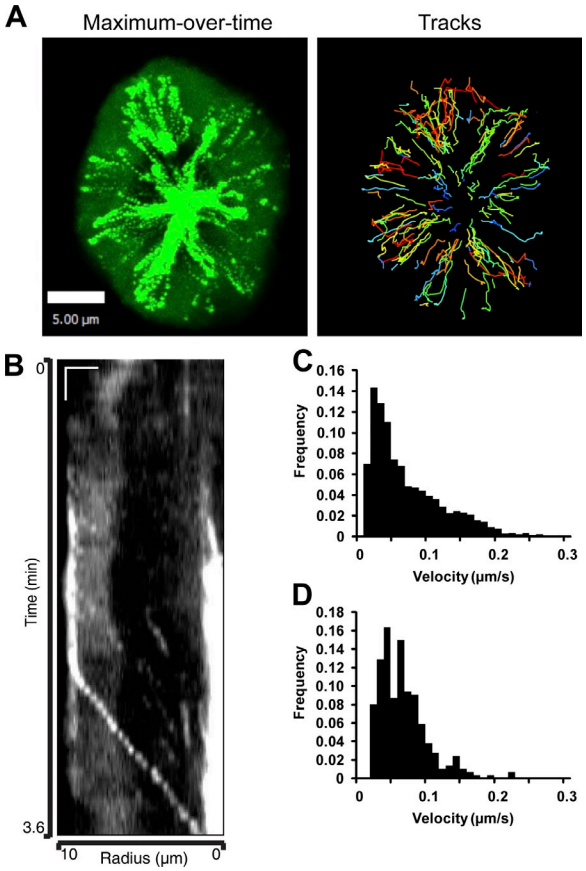



Figure S3. **Measurement of SLP-76 MC velocities at the IS.** (A, left) Maximum-over-time projection of a video of SLP-76 MCs centralizing at the IS. (right) Tracks calculated from the same cell as on the left using the Shortest Path algorithm in Volocity. Bar, 5 µm. (B) A kymograph generated along the path of a mobile SLP-76 MC. Brackets represent 2 µm × 15 s. (C) Histogram of apparent instantaneous velocity distribution from the cell in A ($n$ = 6,407). (D) Histogram of mean velocity distribution from a cell population ($n$ = 287) found using kymographic analysis. This figure is related to Fig. 5.

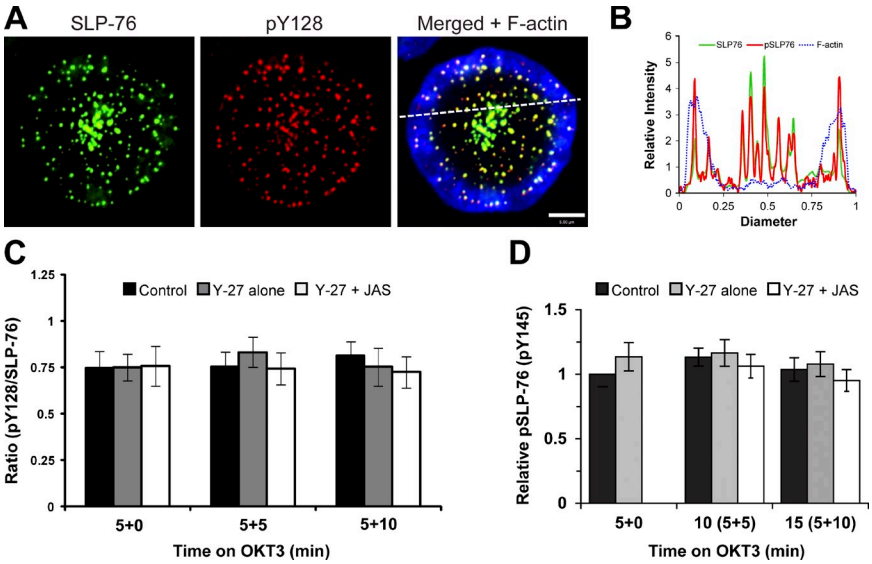
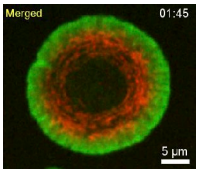
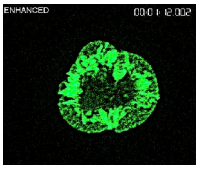


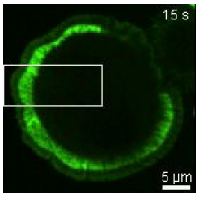

Figure S4. **Assessment of SLP-76 phosphorylation in response to F-actin immobilization.** (A) Jurkat T cells expressing GFP–SLP-76 were allowed to interact with the stimulatory surface. Cells were fixed after 5 min and stained for F-actin and phospho-Y128 (pY128) of SLP-76; a sample cell is shown. Bar, 5 µm. (B) Relative fluorescence intensities along the dashed line in A. (C) Mean ratios of fluorescence intensities of phospho-Y128 SLP-76 and total GFP–SLP-76. Cells were pretreated with Y-27632 (Y-27) or left untreated and then allowed to spread for 5 min. JAS was added where indicated, and cells were allowed to spread further for the indicated times. Mean ± SEM is shown (mean $n$ = 3,410 MCs from a mean of 23 cells per condition). (D) Relative total fluorescence intensities of phospho–SLP-76 (phospho-Y145 [pY145]) obtained from synapses of GFP-actin–expressing Jurkat T cells fixed and stained as in A. Mean ± SEM is shown (mean of 30 cells per condition). This figure is related to Fig. 7.



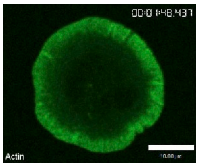

Video 1. **Myosin IIA localizes to the IS and accumulates behind the LP.** Jurkat T cells stably expressing GFP-actin were transiently transfected with mKate2–NMHC II-A (myosin IIA heavy chain). After 16 h, cells were allowed to spread on an OKT3-coated coverglass. Confocal image sequences were acquired every 3 s for 5 min. This video is related to Fig. 1.



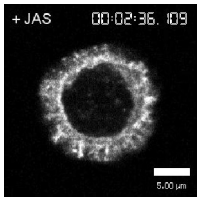

Video 2. **F-actin features at the IS slow down toward the center of the IS.** Jurkat E6.1 cells stably expressing GFP-actin were allowed to interact with an OKT3-coated coverglass. Confocal 1-µm-thick z stacks were acquired every 6 s for 12.5 min. The video of a spreading cell is shown as a raw extended projection (left) or as an enhanced extended projection (right) and corresponds to still images and the kymograph in Fig. S1 (A and B).



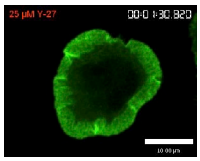

Video 3. **Bleaching of GFP-actin prominently reveals F-actin velocity at the IS.** GFP-actin Jurkat T cells were allowed to spread on OKT3 for 5 min before image acquisition was initiated. Confocal images were acquired every 3 s for 1–2 min. (left) A spread T cell subjected to a high-intensity laser to bleach GFP-actin in the cytosol. The corresponding time series is in Fig. S1 C. (right) An enlarged view of the boxed region on the left, corresponding to the kymograph shown in Fig. S1 C.



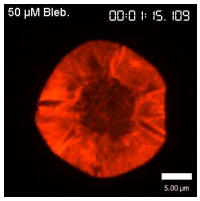

Video 4. **The actomyosin network collapses to the center of the IS of Jurkat T cells upon stabilization of actin filaments.** Jurkat T cells stably expressing GFP-actin (left) or transiently transfected with GFP–NMHC II-A (right) were allowed to contact OKT3-coated coverglasses and imaged after full spreading was reached. JAS was added to the wells when indicated. Confocal 1-µm-thick z stacks were acquired every 3 s for 7 min and are shown as extended projections. This video is related to Fig. 2.



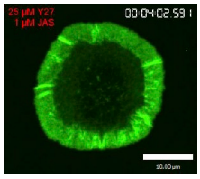

Video 5. **The actomyosin network collapses to the center of the IS in human primary T cells upon stabilization of actin filaments.** Human primary CD4$^+$ T cell blasts expressing GFP-actin were dropped onto an OKT3-coated coverglass and allowed to spread for 3 min. After full spreading was reached, 0.5-µm-thick z stack projections were collected every 3 s. JAS was added to the imaging chamber when indicated. Similar results were obtained in two independent experiments. This video is related to Fig. 2.



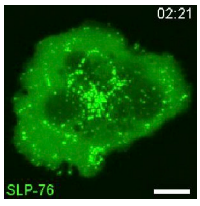

Video 6. **F-actin retrograde flow is unperturbed in cells pretreated with Y-27632.** Jurkat T cells stably expressing GFP-actin were pretreated with Y-27632 for 15 min to inhibit myosin II activity. Cells were then allowed to spread on an OKT3-coated coverglass and imaged to assess the dynamics of the F-actin network. During imaging, cells were left untreated for comparison with JAS-treated cells in Video 9. Confocal 1-µm-thick z stacks were acquired every 3 s for 7 min and are shown as extended projections. This video is related to Fig. 3.



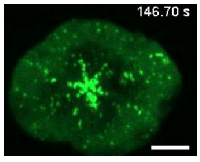

Video 7. **Neither myosin II inhibition with blebbistatin nor myosin IIA suppression with siRNA affects actin retrograde flow.** (Part A) Jurkat T cells transiently transfected with F-tractin tdTomato were pretreated with blebbistatin (Bleb.) for 30 min to inhibit myosin II activity. Cells were then allowed to spread on an OKT3-coated coverglass and imaged to assess the dynamics of the F-actin network. Confocal 1-µm-thick z stacks were acquired every 3 s for 1–5 min and are shown as extended projections. (part B) Jurkat T cells expressing GFP-actin were transfected with siRNA against the heavy chain of myosin IIA (siMyH9) and used 48 h later. Cells were allowed to spread on an OKT3-coated coverglass, and 1-µm-thick z-stack projections were collected every 3 s. When indicated, 1 µM JAS was added to the imaging well. This video is related to Figs. 4 and S2.




Video 8. **Pretreatment with Y-27632 and acute treatment with JAS arrest F-actin retrograde flow in Jurkat and human primary T cells.** (Part A) Jurkat T cells stably expressing GFP-actin were pretreated with Y-27632 for 15 min to inhibit myosin IIA activity. Cells were then allowed to spread on an OKT3-coated coverglass and imaged to assess the dynamics of the F-actin network. During imaging, cells were treated with JAS when indicated. Confocal 1-µm-thick z stacks were acquired every 3 s for 7 min and are shown as extended projections. (part B) Human CD4$^+$ T cell blasts expressing GFP-actin were pretreated with Y-27632 for 15 min. Cells were then allowed to spread on an OKT3-coated coverglass for 3 min before imaging. 0.5-µm-thick z-stack projections were acquired every 3 s. When indicated, 1 µM JAS was added to the imaging well. Similar results were obtained in two independent experiments. This video is related to Fig. 4.




Video 9. **Actin dynamics govern SLP-76 MC movement.** Jurkat T cells stably expressing GFP-actin (left) or GFP–SLP-76 (right) were mixed in culture and pretreated with Y-27632 for 15 min. The cell mixture was then dropped onto an OKT3-coated coverglass to initiate spreading. Fields of view were scanned for cells that had dynamic GFP-actin and SLP-76. Confocal 1-µm-thick z stacks were acquired every 3 s for 2.5 min and are shown as extended projections. JAS was added to the well when indicated. After acquisition, videos of individual cells were cropped and tiled to provide side-by-side comparison. Bar, 5 µm. This video is related to Fig. 5.




Video 10. **SLP-76 MCs move to the center of the IS at constant velocity.** Jurkat T cells stably expressing GFP–SLP-76 were dropped onto an OKT3-coated coverglass and imaged after full spreading was reached. Confocal 1-µm-thick z stacks were acquired every 3.2 s for 4 min and are shown as extended projections. Bar, 5 µm. This video is related to Fig. 5.