Figures and figure supplements

Calcium influx through CRAC channels controls actin organization and dynamics at the immune synapse

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Figure 1. STIM1 and Orai1 accumulate in puncta in the actin-depleted zone of the immune synapse. (A) TIRF images of Jurkat cells stimulated on anti-CD3 coated coverslips in 0.8 mM Ca\textsuperscript{2+} mCh-STIM1 (green) and Orai1-EGFP (red) puncta accumulate in the center of the synapse over time. Images taken from Video 1. Scale bar, 5 μm. (B) Magnification of the boxed region in A shows a STIM1/Orai1 punctum (arrows) moving toward the center of the synapse. Gamma was adjusted to highlight puncta (mCh-STIM1 gamma = 1.3 and Orai1-EGFP gamma = 1.5). (C) Centripetal trajectories of STIM1 and Orai1 puncta overlaid on a single image of Orai1-EGFP. The frame-to-frame punctum velocity was 47 ± 3 nm/s (n = 24 particles, mean ± SEM). Figure 1 continued on next page.
Dashed line indicates the cell edge. (D) ER tubules containing mCh-STIM1 (green) move centripetally with contraction of the EGFP-actin (red) ring. The dashed line indicates the boundary of the ADZ. (E) Kymograph analysis along the indicated line (left) from the cell in D (see Video 2). STIM1 moves at the same velocity as the edge of the actin ring. In all panels, time after initial image acquisition is indicated in min:sec; scale bar, 5 μm.

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Figure 2. Synaptic ER tubules extend from the ADZ and are moved centripetally by actin. (A) TIRF images of a Jurkat cell coexpressing GFP-actin (red) and ER-mCh (green), after spreading on an anti-CD3-coated coverslip. (B) Magnification of the boxed regions in A depicting an extending ER tubule i, Figure 2 continued on next page.
pink arrows), a tubule extending and retracting along the same trajectory (ii, green arrows), a tubule bending and moving centripetally between actin filaments (iii, cyan arrows) and an immobile tubule in an actin-poor region (iii, yellow arrows). (C) Kymograph analysis of the cell from A along the line shown (left) demonstrating coordinated centripetal movement of the ER and actin (see Video 3). Time after initial image acquisition is indicated in min: sec; scale bar, 5 μm.

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Figure 2—figure supplement 1. The ER extends at the tips of dynamic microtubules that move radially toward the lamella/lamellipod border. (A) TIRF images of a Jurkat cell stimulated on anti-CD3 coated coverslips expressing EB1-EGFP (red) and ER-mCh (green). Magnified view of the boxed region shows an ER tubule moving peripherally (green arrow) with EB1 at the tip (red arrow). Images are from Video 4. (B) A projection of the standard deviation of 40 images of EB1 acquired at 1-s intervals overlaid on a single image of F-tractin-P-tdTom, indicating radial EB1 movement in the ADZ and Figure 2—figure supplement 1 continued on next page.
Figure 2—figure supplement 1 continued

movements perpendicular to cell edge at lamella/lamellipod border. Images are from Video 5. Time after initial image acquisition is indicated in min: sec; scale bar, 5 μm.
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Figure 3. Calcium influx organizes synaptic actin and promotes retrograde flow. (A) TIRF images of a Jurkat cell expressing F-tractin-P-tdTom after spreading on anti-CD3 in 2 mM Ca\textsuperscript{2+} (left), 3.25 min after Ca\textsuperscript{2+} removal (center), and 1 min after readdition of 2 mM Ca\textsuperscript{2+} (right). Ca\textsuperscript{2+} alters F-actin

Figure 3 continued on next page
organization and density. Images taken from Video 6. (B) Spatiotemporal image correlation spectroscopy (STICS) analysis (Hebert et al., 2005) of the cell in A, depicting the direction and relative velocity of actin movement before (left) and after Ca\(^{2+}\) removal (center) and after readdition of 2 mM Ca\(^{2+}\) (right). Color scale represents relative velocities; numerical values were not assigned because small immobile features cause underestimation of velocity by STICS. (C, D) Blocking Ca\(^{2+}\) influx with 2-APB has the same effect on actin as removal of Ca\(^{2+}\). A representative cell is shown before and 2.5 min after treatment with 100 μM 2-APB, and STICS analysis is shown in D. (E) Spinning disk confocal images of a primary human T lymphoblast expressing Lifeact-GFP after spreading on anti-CD3 and ICAM-1 in 0.5 mM Ca\(^{2+}\) (left), 3 min after Ca\(^{2+}\) removal (center), and 1.5 min after readdition of 2 mM Ca\(^{2+}\) (right). The width of the lamellipod (indicated by the green carets) was reduced in 0 Ca\(^{2+}\). Images are maximum intensity projections of 3 successive 0.25 μm sections of the cell footprint taken from Video 7. (F) Kymograph analysis of the cell in E along the indicated yellow line (left) demonstrates centripetal actin flow rate of 426 nm/s in 0.5 mM Ca\(^{2+}\) (left, velocity calculated from the slope of the red dashed lines) that slows to 94 nm/s upon Ca\(^{2+}\) removal (center) and accelerates to 130 nm/s following readdition of 2 mM Ca\(^{2+}\). Time is indicated in min:sec; scale bars, 5 μm.

Figure 4. Calcium influx promotes ER coralling. (A) A cell expressing F-tractin-P-ttdTom (red) and ER-GFP (green) on an anti-CD3 coverslip is shown in 0 Ca\(^{2+}\)o and after readdition of 2 mM Ca\(^{2+}\)o. Peripheral ER tubules in the TIRF images are sparse in 0 Ca\(^{2+}\)o but Ca\(^{2+}\)o readdition causes peripheral...
tubules to appear as they move into the evanescent field. Pink dotted lines outlining the edge of the ADZ in 0 Ca\(^{2+}\_o\) serve as a landmark to highlight centripetal ER movement following Ca\(^{2+}\_o\) readdition. Images taken from Video 9. (B) Peripheral ER tubules were traced in 5 images acquired at 10 s intervals, then color-coded for time and overlaid to indicate movement between frames in 0 Ca\(^{2+}\_o\) (top) and immediately following re-addition of 2 mM Ca\(^{2+}\_o\) (bottom). In 0 Ca\(^{2+}\_o\), peripheral tubules are sparse, extended and move in radial and non-radial directions. Peripheral tubules appearing upon readdition of Ca\(^{2+}\_o\) move centripetally. (C) Kymograph analysis of the cell in A along the indicated line (left) demonstrates centripetal movement of ER tubules between actin structures upon Ca\(^{2+}\_o\) readdition. Black horizontal lines indicate bath exchange. Gamma adjusted to 0.7 to highlight ER tubules. Time after initial image acquisition is indicated in min:sec; scale bar, 5 μm.

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Figure 5. Intracellular calcium reduces the density of F-actin at the synapse. Jurkat T cells expressing F-tractin-P-tdTom and loaded with fura-2 were stimulated on anti-CD3-coated coverslips. (A) Pseudocolor image of F-tractin-P-tdTom intensity in a cell exposed sequentially to 0.5 mM Ca\(^{2+}\) (left), 0 Ca\(^{2+}\) (center) and 5 mM Ca\(^{2+}\) (right), indicating a Ca\(^{2+}\)-dependent decrease in F-actin density. Linear color scale indicates fluorescence intensity (0–1 a.u.); scale bar, 5 μm. (B) Change in F-tractin-P-tdTom fluorescence (green; relative to fluorescence in 0 Ca\(^{2+}\)) and fura-2 ratio (blue) from the cell in A. The data are replotted on the right with an inverted F-tractin-P axis to highlight the delay between changes in [Ca\(^{2+}\)] and F-tractin-P intensity upon Ca\(^{2+}\) removal (top) and readdition (bottom). (C) Change in F-tractin-P-tdTom fluorescence (relative to fluorescence in 0 Ca\(^{2+}\) or 100 μM 2-APB in 0.5 mM Ca\(^{2+}\)) as a function of fura-2 ratio. Each point is an average single-cell value measured at constant fura-2 ratio and F-tractin-P fluorescence in the presence of 0.5–10 mM Ca\(^{2+}\) (green). The red dot indicates the average baseline fura-2 ratio (± s.d.) for all cells in 0 Ca\(^{2+}\) or 2-APB. A linear fit to the data is shown (r\(^2\) = 0.83). DOI: 10.7554/eLife.14850.018
Figure 5—figure supplement 1. Ca\(^{2+}\) influx through CRAC channels reduces F-actin density at the synapse. (A) Jurkat cells expressing F-tractin-P-tdTom were allowed to spread on anti-CD3 coated coverslips in 2 mM Ca\(^{2+}\).
Figure 5—figure supplement 1 continued

followed by perfusion with 100 μM 2-APB to block Ca^{2+} influx through Orai1. 2-APB causes a 20% increase in F-actin at the synapse as indicated by the F-tractin-P-tdTom intensity vs. time normalized to the average fluorescence of the last five images (means ± SEM; n = 8 cells). (B) Spinning disk confocal images of a Jurkat cell expressing F-tractin-P-tdTom stimulated on anti-CD3 coated coverslips in 0.5 mM Ca^{2+}o (left) and 100 s after perfusion of 0 Ca^{2+}o (right). Scale bar, 5 μm; color scale indicates fluorescence intensity (0–1 a.u.). (C) The average F-tractin-P-tdTom intensity vs. time normalized to the average fluorescence of the last five images for cells stimulated and imaged as in B (means ± SEM; n = 10 cells).

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Figure 6. Calcium accelerates actin depolymerization and centripetal velocity at the synapse. Jurkat T cells expressing F-tractin-P-tdTom and PAGFP-actin were stimulated on anti-CD3 coated coverslips and TIRF images of F-tractin-P-tdTom (A, left) were used to identify regions in the lamellipod and lamella.
lamella (red ovals) to photoactivate. The lamella/lamellipod border in 2 mM Ca\(^{2+}\_), and cell edge are indicated by pink dashed lines. (A) Widefield epifluorescence images of PAGFP-actin after photoactivation in 2 mM Ca\(^{2+}\_) (top) and a subsequent photoactivation in 0 Ca\(^{2+}\_) (bottom). Images are from Video 10; color scale indicates fluorescence intensity (0–1 a.u.). Time after photoactivation indicated in min:sec. Scale bar, 5 μm. (B, C) Position of peak PAGFP-actin fluorescence as a function of time after photoactivation in the lamellipod (B) and the lamella (C) (see figure supplement 1A-D). Data are plotted in the presence (blue) and absence (red) of Ca\(^{2+}\_) for the cell pictured in A. Linear fits to the data indicate lamellipod velocities of 72 nm/s (2 Ca) and 22 nm/s (0 Ca) and lamella velocities of 41 nm/s (2 Ca) and 1 nm/s (0 Ca). (D, E) The fluorescence decay of photoactivated PAGFP-actin in the lamellipod (D) and lamella (E) for the cell in A was fitted by a single exponential. In the lamellipod, \( t = 8.3 \) s (2 Ca) and 12.0 s (0 Ca); in the lamella, \( t = 9.2 \) s (2 Ca) and 12.4 s (0 Ca). F/F\(_{\text{max}}\) is the fluorescence intensity after photoactivation relative to the peak. (F) The centripetal velocity of photoactivated actin in the lamellipod (n = 10 cells) and lamella (n = 18 cells) in the presence of absence of Ca\(^{2+}\_), calculated as described in B, C. Error bars indicate SEM; p-values from Student’s two-tailed t-test. (G) Actin filament half-life calculated from the exponential rate of fluorescence decay in photoactivated regions in the lamellipod (n = 10) and lamella (n = 19) with and without Ca\(^{2+}\_). P-values are from paired Student’s two-tailed t-test. DOI: 10.7554/eLife.14850.020
Figure 6—figure supplement 1. Actin filament velocity and half-life at the synapse. Jurkat cells were stimulated on anti-CD3 coated coverslips and PAGFP-actin was photoactivated in small regions within the lamellipod and lamella. (A-D) Fluorescence intensity profiles of photoactivated PAGFP-actin in the lamellipod (A, B) and the lamella (C, D) in the presence of 2 mM or 0 Ca$^{2+}$ as indicated. Each trace is the intensity along a line perpendicular to the direction of actin movement and averaged across its 19-pixel width, displayed every 2 s. Data are from the cell in Figure 6 and Video 10. The 0 position indicates the cell edge, and the dotted line marks the lamellipod/lamella border. Velocity was calculated as in Figure 6 from the peak position versus time. (E) Velocity and (F) actin filament half-life of photoactivated regions were measured in cells before and after perfusion of 2 mM Ca$^{2+}$. P-values from paired Student’s two-tailed t-test.

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**Figure 6—figure supplement 2.** Calcium influx alters actin organization and density independently of myosin activity. (A) TIRF images of Jurkat cells expressing F-tractin-P-tdTom pretreated for 30 min with 50 μM blebbistatin, then stimulated on anti-CD3 coated coverslips in 2 mM Ca\(^{2+}\) and blebbistatin (left) followed by perfusion with 0 Ca\(^{2+}\) and blebbistatin (right). Scale bar, 5 μm. (B) F-tractin-P-tdTom intensity at the synapse versus time for cells as in A, normalized to the average fluorescence of the last five images (means ± SEM; n = 12 cells). DOI: 10.7554/eLife.14850.022
**Figure 7.** Calcium restricts actin polymerization to the distal edge of the synapse. (A, B) Two Jurkat T cells expressing F-tractin-P-tdTom and PAGFP-actin were stimulated on anti-CD3 coated coverslips in 0.5 mM Ca²⁺, and PAGFP-actin was photoactivated in the ADZ regions indicated in the F-tractin-P channels. (C-D) Time versus position plots for (C) 0 Ca²⁺ and (D) 2 Ca²⁺. The ΔF/F values are shown for different time points. (E) Phalloidin and WAV2 staining for 0.5 Ca²⁺ and 0 Ca²⁺ conditions. (F) Bar graph showing the fluorescence intensity ratio of F-tractin-P/Abi1 for 0.5 Ca²⁺ and 0 Ca²⁺ conditions. (G) F-tractin-P and Abi1 staining for 0.5 Ca²⁺, 0 Ca²⁺, and 2 Ca²⁺ conditions. (H) Time versus fluorescence intensity ratio of F-tractin-P/Abi1 for different conditions. (I) Abi1 fluorescence intensity ratio over position for different conditions.

(Figure 7 continued on next page)
tractin-P-tdTom TIRF images (left, yellow circles) 2 min after perfusion of 0 Ca\(^{2+}\_o\) (A) or 2 mM Ca\(^{2+}\_o\) (B). Incorporation of fluorescent PAGFP-actin is shown as a function of time after photoactivation. The lamella/lamellipod border in 2/0.5 mM Ca\(^{2+}\_o\) and cell edge are indicated by pink dashed lines. Images are from Video 11. Time after photoactivation is in min:sec; scale bar, 5 μm; color scale indicates fluorescence intensity (0–1 a.u.). (C, D) Normalized PAGFP-actin fluorescence intensity (see Materials and methods) along the line indicated (top right) as a function of radial position. The fluorescence profile before photoactivation is shown in black; the color scale applies to subsequent profiles acquired every 1.5 s after photoactivation. The cell edge (red arrowhead), the lamellipod/lamella border (blue arrowhead) and the edge of the ADZ (green arrowhead) are indicated. Data are representative of 12–13 cells. (E) Representative TIRF images of Jurkat cells stimulated on anti-CD3 in 0.5 mM Ca\(^{2+}\_o\) then transferred to 0.5 Ca\(^{2+}\_o\) (left) or 0 Ca\(^{2+}\_o\) (right) for 2.5 min, labeled with Alexa-594 phalloidin (top) and anti-WAVE2 (bottom) (see Materials and methods). Ca\(^{2+}\) promotes localization of WAVE2 to the edge of the lamellipod. Images are from Video 12. Scale bar, 5 μm; color scale indicates fluorescence intensity (0–1 a.u.). (F, H) The average fluorescence intensity (a.u.) of EGFP-Abi1 along the line indicated (top right, pink) in 0.5 mM Ca\(^{2+}\_o\) (blue) and 1.5 min after Ca\(^{2+}\_o\) removal (red). DOI: 10.7554/eLife.14850.026
Figure 8. Effects of calcium on actin dynamics and retrograde flow at the synapse. (A) Retrograde actin flow at the immune synapse (yellow arrows) continually removes extended ER tubules (purple) from the periphery, thereby concentrating the ER in the ADZ. An expanded view of the red-boxed region (top) depicts Ca\textsuperscript{2+} effects on actin regulation (bottom). Ca\textsuperscript{2+} drives centripetal actin flow in two ways: (1) by restricting polymerization to the lamellipod edge (green chevrons), it enforces vectorial movement of the actin network; and (2) by increasing the rate of depolymerization, it increases the pool of free actin monomers (grey chevrons), thus enhancing polymerization on free barbed ends at the lamellipod edge (green chevrons). Ca\textsuperscript{2+} restricts polymerization to the lamellipod edge by localizing WAVE2 and Abi1 to this site where they promote ARP2/3-mediated actin nucleation (blue triangles) and possibly by capping free barbed ends elsewhere (pink circles). (B) Experimentally terminating Ca\textsuperscript{2+} influx reduces retrograde actin flow such that extended ER tubules are no longer effectively pushed into the ADZ. In the absence of Ca\textsuperscript{2+}, actin depolymerization is reduced (bottom), nucleation occurs more uniformly throughout the lamellipod/lamella and capping of free barbed ends may be reduced. The overall result is a slowed, non-directional polymerization throughout the lamellipod and lamella resulting in reduced retrograde flow.

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