Supplemental material

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**Figure S1. Expression and roles of Rho GTPases in T cell TEM.** (a) TEM of CEM T cells transfected with the indicated siRNAs on Transwells. TEM is shown relative to control siRNA-treated cells (n = 3–6; comparing Rho GTPase siRNA and siControl). (b) Lysates of CEM T cells and T lymphoblasts (T-LB) were Western blotted with antibodies to the indicated Rho GTPases. PC3 cell lysates were used as positive controls for RhoJ and RhoE, and lysates of HEK293T cells transfected with a RhoF expression vector were used as a positive control for RhoF. RT-PCR was used to detect mRNA expression of RhoD, RhoV, Rnd1, Rnd2, and RhoBTB2. (c) Lysates from CEM cells transfected with the indicated siRNAs were Western blotted with antibodies to the indicated Rho GTPases. For RhoBTB2 and Rnd1, mRNA expression was examined by qPCR. Data shown are from two independent experiments. White lines indicate that intervening lanes have been spliced out. (d) CEM cells were infected with lentiviruses encoding two different shRNAs (shRNA-1 and shRNA-2) to knockdown RhoA expression or a control scramble shRNA. After 72 h, TEM was quantified in Transwells, or cells were lysed to determine levels of RhoA expression relative to GAPDH (loading control) by Western blotting. A representative blot is shown (n = 3; comparing Rho GTPase shRNA and control shRNA). (e) Lysates from CEM cells transfected with control or RhoA siRNA were incubated with GST-PBD beads to bind active Cdc42 and Rac1. Total and active Cdc42 and Rac1 levels were quantified after Western blotting. Total cell lysates were also blotted for RhoA and RhoGDI1. A representative set of blots from one experiment is shown (n = 3). Cdc42, Rac1, RhoA, and RhoGDI1 expression is calculated relative to the loading control, GAPDH. Active (GTP bound) Rac1 and Cdc42 expression is calculated relative to total protein levels. Total and percentage of active Cdc42 and Rac1 in RhoA siRNA lysates are shown relative to control siRNA. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Error bars indicate mean ± SEM.
Figure S2. Localization of RhoA activity in T cells migrating on and across ECs. (a) CEM cells expressing the RhoA Raichu probe (wild type [WT]) were fixed 30 min after adding to EC, and images of crawling and transmigrating cells were acquired by FLIM. In some cells, two images were acquired to visualize the apical and basal surfaces better. RhoA activity is higher in three areas: the leading edge (arrowheads), the uropod where it is associated with rear contraction (arrows), and in dynamic puncta in the midregion (dashed arrows). (b and c) CEM cells expressing the RhoA-T19N Raichu probe showed reduced FRET efficiency compared with the wild-type probe in live cell imaging (b) and in fixed cells (c). (d) CEM cells expressing the wild-type RhoA probe (left) or RhoA-T19N probe (right) were added to EC and fixed after 30 min. Images shown are maximum projections of five to eight confocal z-stack images acquired at 0.5-µm intervals. Both RhoA Raichu probes localized to the plasma membrane. (e) Graph showing FRET efficiency plotted against pixel intensity for each acquired pixel from one representative cell expressing the RhoA Raichu probe (wild type) crawling on activated EC and imaged by FLIM. (f) CEM cells expressing the RhoA probe (wild type) were added to EC and fixed after 30-min cells and imaged by FLIM microscopy. The mean FRET efficiency was plotted against mean probe intensity for each cell. (g) CEM cells were imaged crawling on EC by time-lapse microscopy 48 h after transfection with control pEGFPN1 or RhoA Raichu probe (wild type) plasmids and kymographs produced. (h) CEM cells transfected with pEGFPN1 or RhoA Raichu probe (wild type) or untransfected cells were fixed 30 min after adding to EC and stained for F-actin and α-tubulin. Nontransmigrating cells were scored for polarity (n = 3). (i) Quantification of Transwell TEM of CEM cells transfected with control pEGFPN1 or RhoA Raichu probe plasmids. Data are shown as a percentage of pEGFPN1 cell transmigration (n = 4). Error bars indicate mean ± SEM. Bars, 10 µm.
Figure S3. Roles of RhoA, ROCKs, and GEF-H1 in T cells. (a–c) Graphical representation of RhoA activity and protrusion versus time. Data shown from a representative cell (Fig. 3 d, cell A). The local movement and RhoA activation of each angular segment is plotted against the frame number with the frames being taken every 45 s. 0 ± 45° represents the front of the cell, the two sides are 45–135° and −45 to −135°, and the back is 180 ± 45°. (a and b) 2D plots where the color scale denotes the RhoA activation (FRET efficiency; a) and the local movement (b). Regions of large movements at the front of the cell also show high RhoA activity (black arrows). (c) 3D plot of RhoA activity versus size of membrane movement over time. The height of the z axis indicates the movement magnitude (smoothed using a convolution with a 3 × 3 box filter), and the color scale denotes the FRET efficiency (RhoA activity). (d) CEM cells were fixed 30 min after addition to EC and stained for p-MLC and F-actin. Polarized crawling cells were scored for the localization of p-MLC (n = 3). (e) ROCK1 and ROCK2 expression determined by Western blotting of CEM cell lysates 48 h after transfection with control, ROCK1, or ROCK2 siRNAs. (f) Quantification of CEM cell TEM in Transwells (n ≥ 3). *, P < 0.05; ***, P < 0.001; compared with control siRNA. (g) T lymphoblast (T-LB) cells were labeled with cell tracker dye and added to EC. After 15 min, cells were fixed and stained with antibodies to GEF-H1 (arrow). Bar, 10 µm. (h) Quantification of GEF-H1 expression determined by Western blotting of CEM T cell lysates 72 h after transfection with control and GEF-H1 siRNAs (n = 3). GEF-H1 expression is shown relative to the loading control, GAPDH. Error bars indicate mean ± SEM.
Video 1. **RhoA knockdown inhibits T cell TEM.** CEM T cells crawling on and transmigrating across EC 72 h after transfection with control siRNA (left) or RhoA siRNA (right). One frame was taken every minute.

Video 2. **Protrusion dynamics in a control T cell.** CEM T cells crawling on EC 72 h after transfection with control siRNA. One frame was taken every second.

Video 3. **Tail extension in a RhoA-depleted T cell.** CEM T cells crawling on EC 72 h after transfection with RhoA siRNA. Cell has elongated tail at the rear. One frame was taken every second.

Video 4. **Protrusion dynamics in a RhoA-depleted T cell.** CEM T cell on EC 72 h after transfection with RhoA siRNA. Cell shown has rounded cell body and two protrusions, extending from opposite sides of the cell body. One frame was taken every second.

Video 5. **Localization of RhoA activity in a crawling T cell.** CEM T cells expressing the RhoA Raichu probe (wild type) were imaged crawling on ECs. FLIM images were acquired every 45 s.

Video 6. **Localization of RhoA activity in a crawling T cell.** CEM T cells expressing the RhoA Raichu probe (wild type) were imaged crawling on EC. FLIM images were acquired every 45 s.

Video 7. **Localization of RhoA activity in a transmigrating T cell.** CEM T cells expressing the RhoA Raichu probe (wild type) were imaged transmigrating through EC. FLIM images were acquired every 45 s.
Video 8. **Localization of RhoA activity in a transmigrating T cell.** CEM T cells expressing the RhoA Raichu probe (wild type) were imaged transmigrating through EC. FLIM images were acquired every 45 s.

Video 9. **Localization of control dominant-negative RhoA probe in a crawling T cell.** CEM T cells expressing the RhoA Raichu probe (T19N) were imaged crawling on EC. FLIM images were acquired every 45 s.

Video 10. **Effects of Y27632 on T cell protrusions.** CEM T cells on EC. (left) Example of protrusion formation by extension from opposite sides of the cell body in a RhoA siRNA-transfected cell. (middle) Example of protrusions forming by tail retraction defects in cells treated with 10 µM Y27632. The cell migrates with a long tail at the rear and reverses direction, forming a second tail at the opposite side to the first. (right) Example of protrusions forming by a tail retraction defect at the back and a narrow leading edge at the front in cells treated with 10 µM Y27632. One frame was taken every minute.