Sun et al., https://doi.org/10.1083/jcb.201602002

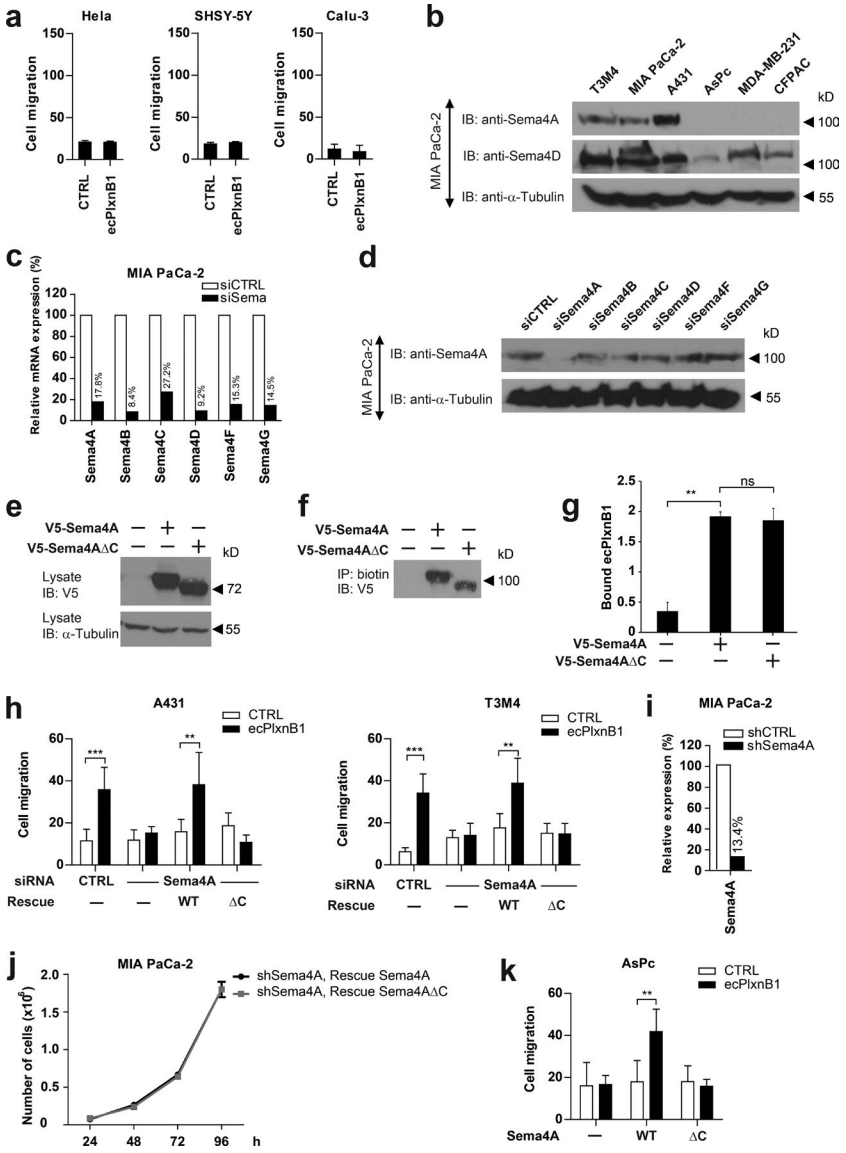


Figure S1. **The extracellular portion of Plexin-B1 increases cancer cell migration through Sema4A.** (a) HeLa, SHSY-5Y, and Calu-3 cancer cell lines were starved, and cell migration toward 150 nM ecPlxnB1 was tested using a transwell system. Shown are mean values ± SD from duplicates of three experiments (total $n = 6$ per condition). (b) Expression analysis of Sema4A and Sema4D in different cancer cell lines by Western blotting. (c and d) Knockdown efficiency of siRNAs directed against class 4 semaphorins analyzed by real-time quantitative RT-PCR (c) and by Western blotting (d). (e) MIA PaCa-2 cells were transfected with cDNA encoding either the full-length human Sema4A (V5-Sema4A) or Sema4A without its intracellular portion (V5-Sema4AΔC). Cells were lysed and proteins were detected using the indicated antibodies. (f) MIA PaCa-2 cells transfected as in panel e were washed, and cell surface proteins were biotinylated as described in Materials and methods. Cells were then washed and lysed, and the biotinylated proteins were precipitated using streptavidin agarose. Bound proteins were then separated and detected using an anti-V5 antibody. IB, immunoblotting; IP, immunoprecipitation. (g) MIA PaCa-2 cells transfected with the indicated Sema4A constructs were incubated with 150 nM ecPlxnB1, and bound ecPlxnB1 was detected as described in Materials and methods (total $n = 6$ per condition). ns, not significant. (h) Cell lines were transfected as indicated, and cell migration toward ecPlxnB1 was measured (total $n = 6$ per condition). (i) Knockdown efficiency of shRNA against Sema4A in MIA PaCa-2 cells was analyzed using real-time quantitative RT-PCR as described in Materials and methods. (j) Stable MIA PaCa-2 cell lines expressing shRNA against Sema4A together with shRNA-resistant full-length human Sema4A (shS4A_S4A) or Sema4A without the intracellular portion (shS4A_S4AΔC) were seeded onto 6-well plates (40,000 cells/well), and the number of cells was counted over four consecutive days (total $n = 3$ per condition). (k) AsPc cells were transfected with wild-type Sema4A (WT) or Sema4A without its intracellular portion (ΔC). Cell migration was measured using a transwell assay. Shown are mean values ± SD from three independent experiments (total $n = 7$ per condition). CTRL, control. Error bars represent means ± SD. **, $P < 0.01$; ***, $P < 0.001$.

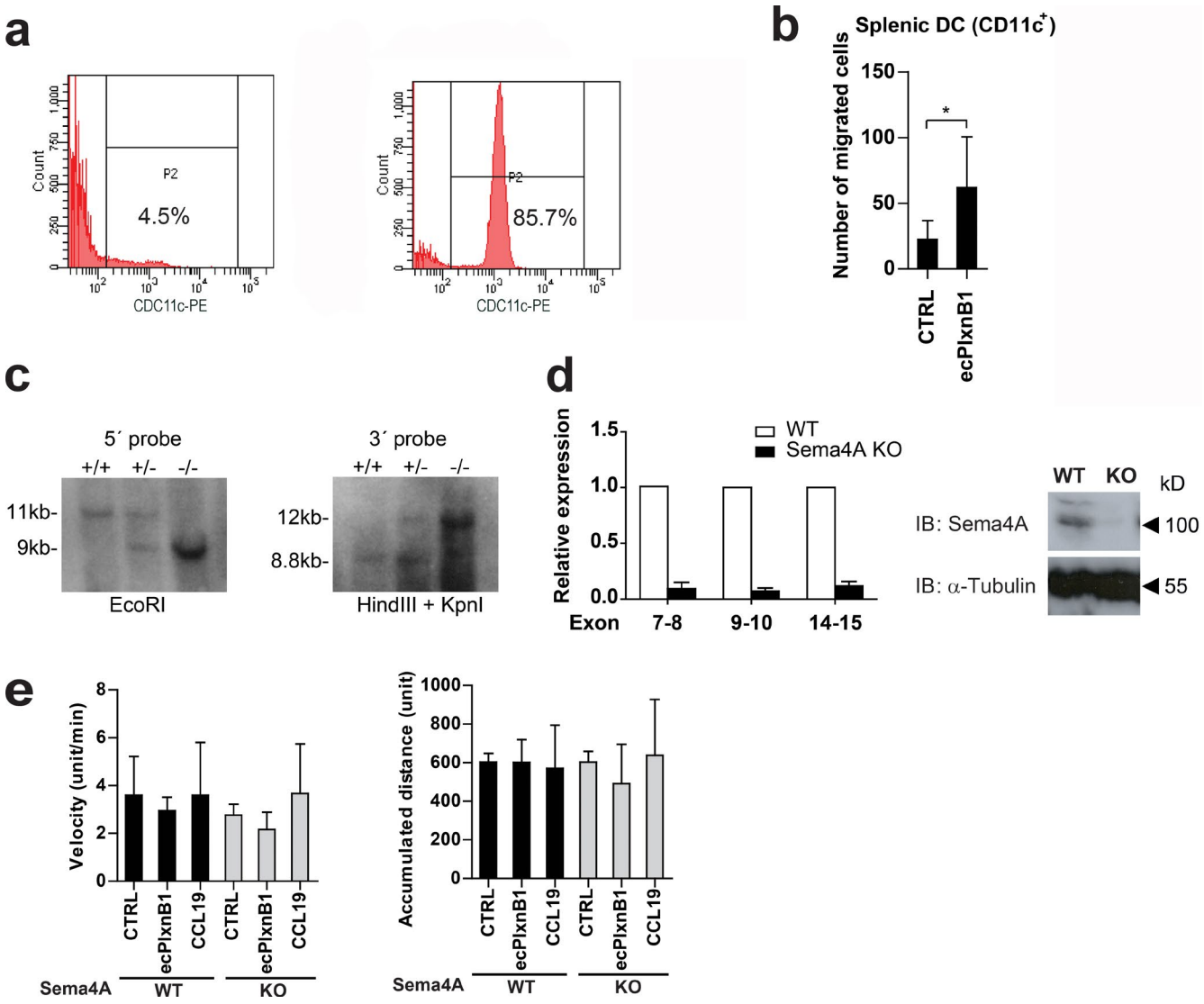


Figure S2. **The extracellular portion of Plexin-B1 stimulates DC migration through Sema4A.** (a) Splenic DCs were labeled and sorted with CD11c-PE. Cell purity before (left) and after (right) sorting was checked using FACS Canto II, as indicated. (b) CD11c-positive DCs (CD11c$^+$ DCs) were sorted from mouse spleen. 10^4 CD11c$^+$ DCs were transferred into the top chamber of a 96 transwell plate (5-µm pore size). DCs were stimulated with 150 nM ecPlxnB1. Migrated cells were stained and counted (total n = 8 per condition). (c) Southern blot analysis of genomic DNA from Sema4A wild-type (*sema4a*$^{+/+}$) or knockout (*sema4a*$^{-/-}$) littermates. To verify homologous recombination, genomic DNA was digested with EcoRI, blotted, and incubated with a probe upstream of the 5′ homology arm (left) or digested with HindIII and KpnI and incubated with a probe downstream of the 3′ homology arm (right). (d) RNA and protein were isolated from the spleen of wild-type (WT) and Sema4A knockout (Sema4A KO) mice. RT-PCRs with three different pairs of intron-spanning primers as indicated were performed to check the mRNA level of Sema4A (left). Sema4A protein expression in wild-type and Sema4A knockout mice was analyzed by Western blotting (right). Shown are mean values ± SD. IB, immunoblotting. (e) Cell velocity and accumulated distance from Fig. 2 c was quantified using the Chemotaxis Tool in ImageJ as described in Materials and methods. Total numbers of analyzed cells are WT/CTRL, n = 47; WT/ecPlxnB1, n = 38; WT/CCL19, n = 43; KO/CTRL, n = 36; KO/ecPlxnB1, n = 30; and KO/CCL19, n = 40. CTRL, control. Error bars represent means ± SD. *, $P < 0.05$.

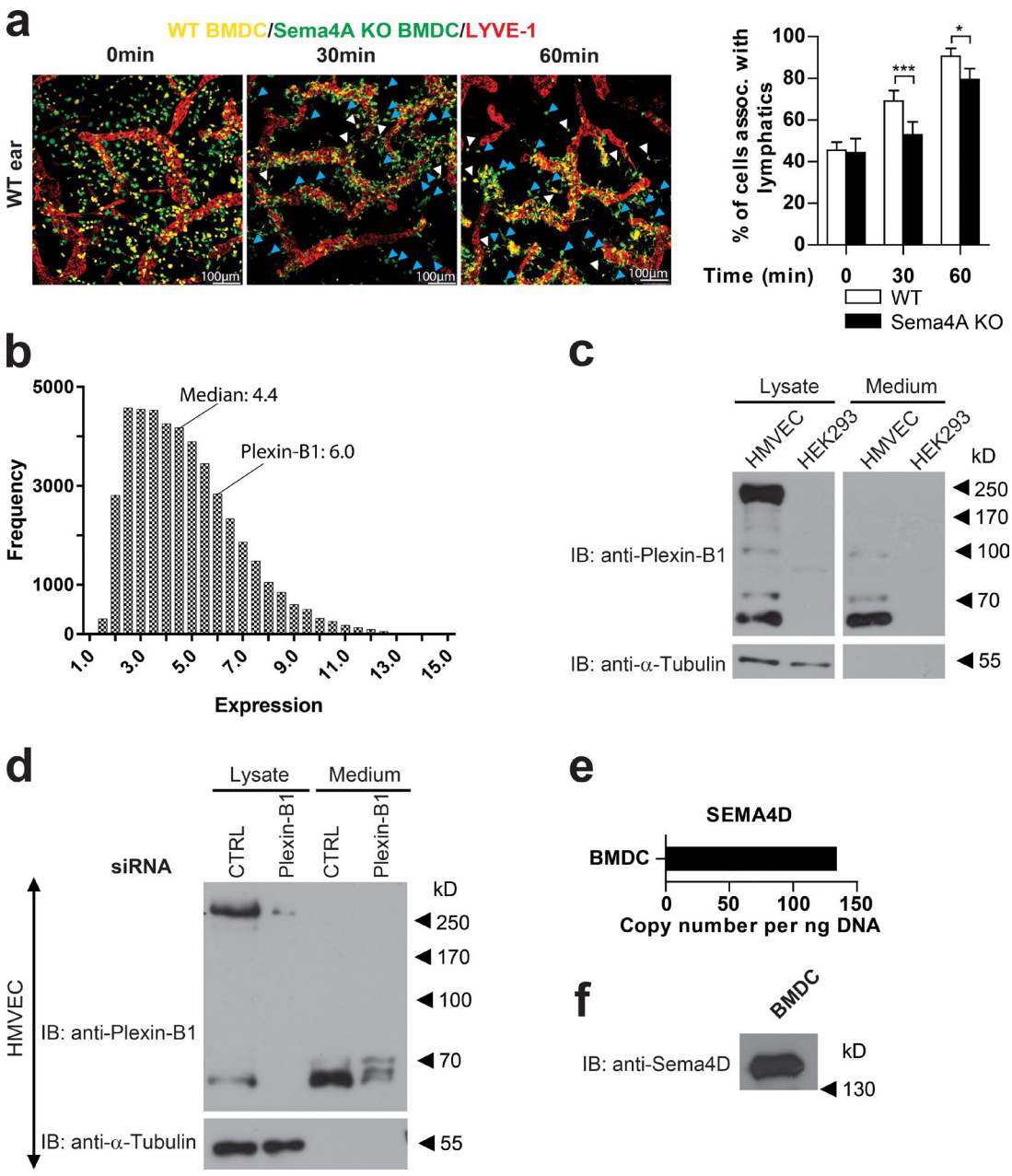


Figure S3. **Analysis of BMDC migration toward ear lymphatic vessels and expression analysis of lymphatic endothelial cells and BMDCs.** (a) Mature BMDCs generated from wild-type (WT) and Sema4A knockout mice (Sema4A KO) were labeled (wild type, yellow; Sema4A knockout, green), mixed 1:1, and used for the ex vivo migration assay. Cells associated with lymphatic vessels were counted at 0, 30, and 60 min. Examples of wild-type (white arrowheads) and Sema4A knockout (blue arrowheads) BMDCs not associated with lymphatic vessels are marked. Shown are mean values ± SD from triplicates of three independent experiments. Total numbers of analyzed cells are WT/0 min: $n = 588$; Sema4A KO/0 min: $n = 630$; WT/30 min: $n = 1{,}566$; Sema4A KO/30 min: $n = 1{,}203$; WT/60 min: $n = 1{,}673$; Sema4A KO/60 min: $n = 1{,}396$. (b) The histogram of a lymph endothelial cell array displays the global gene expression level distribution. Expression levels are represented on the x axis, and the absolute frequency of genes are represented with a particular expression level on the y axis. The median gene expression level and the expression level of Plexin-B1 are marked. (c and d) Human lymphatic endothelial cells (HMVEC-dLyAd) and HEK293 cells were cultured for 48 h. Where indicated, cells were transfected with control siRNA or siRNA directed against Plexin-B1. Conditioned media (medium) and cell lysates (lysate) were analyzed using anti–α-tubulin and anti–Plexin-B1 antibodies. (e and f) Analysis of Sema4D mRNA (e) and protein (f) expression in mature BMDCs. Error bars represent means ± SD. *, $P < 0.05$; ***, $P < 0.001$.

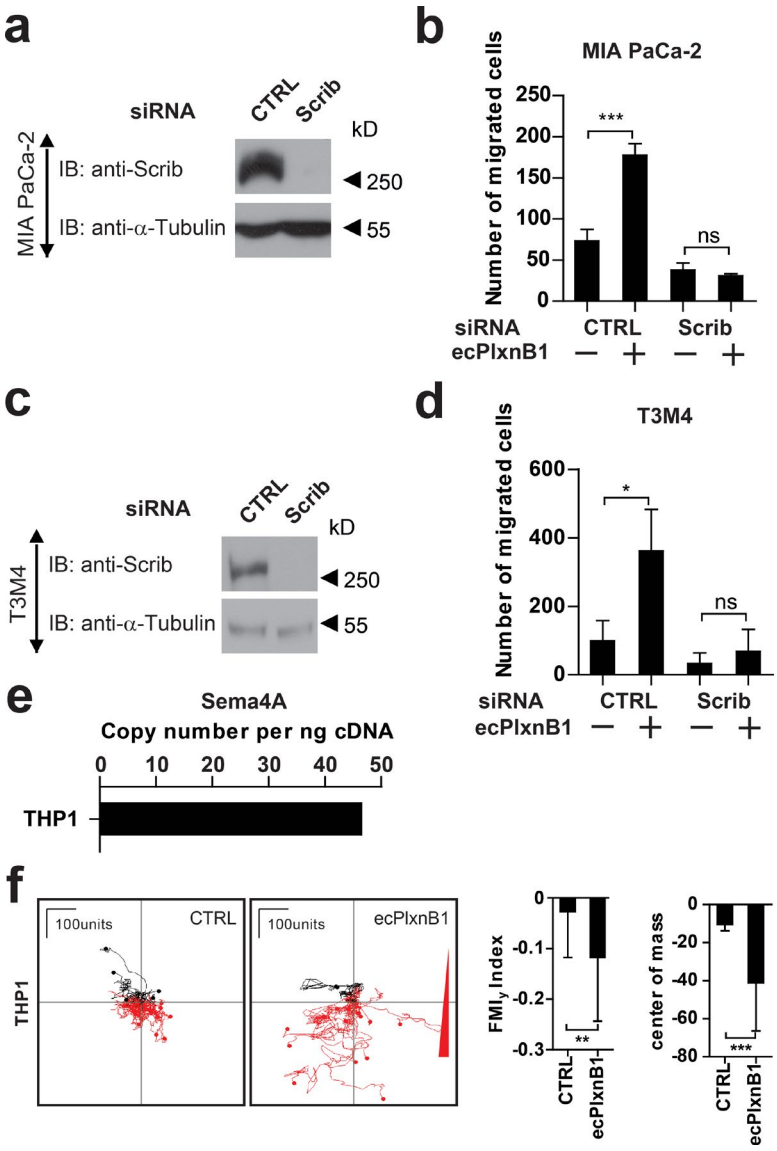


Figure S4. **The promigratory effect of the extracellular portion of Plexin-B1 depends on Scrib.** (a and b) MIA PaCa-2 cells were transfected with a control (CTRL) or Scrib siRNA, which is different from the siRNA used in Figs. 6 and 7 and in panels c and d of this figure. (a) Scrib expression levels were analyzed by Western blotting. (b) Migration of MIA PaCa-2 cells in the absence or presence of 150 nM ecPlxnB1 was analyzed in transwell migration assays (total $n = 8$ per condition). (c) T3M4 cells were transfected with control or Scrib siRNA, and Scrib expression levels were analyzed by Western blotting. (d) Migration of T3M4 cells in the absence or presence of 150 nM ecPlxnB1 was analyzed in transwell migration assays (total $n = 3$ per condition). IB, immunoblotting; ns, not significant. (e) Expression of Sema4A in DCs differentiated and matured from THP1 cells was analyzed by quantitative RT-PCR. Shown are copy numbers of Sema4A per nanogram cDNA. (f) DCs differentiated and matured from THP1 cells were mixed with collagen I gel and seeded into µ-Slide Chemotaxis^{3D} migration chambers. Cells were allowed to migrate in the presence of gradients of ecPlxnB1. Migrating cells were tracked and analyzed as described in Materials and methods. Total numbers of analyzed cells are control, $n = 24$, and ecPlxnB1, $n = 18$. Error bars represent means ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

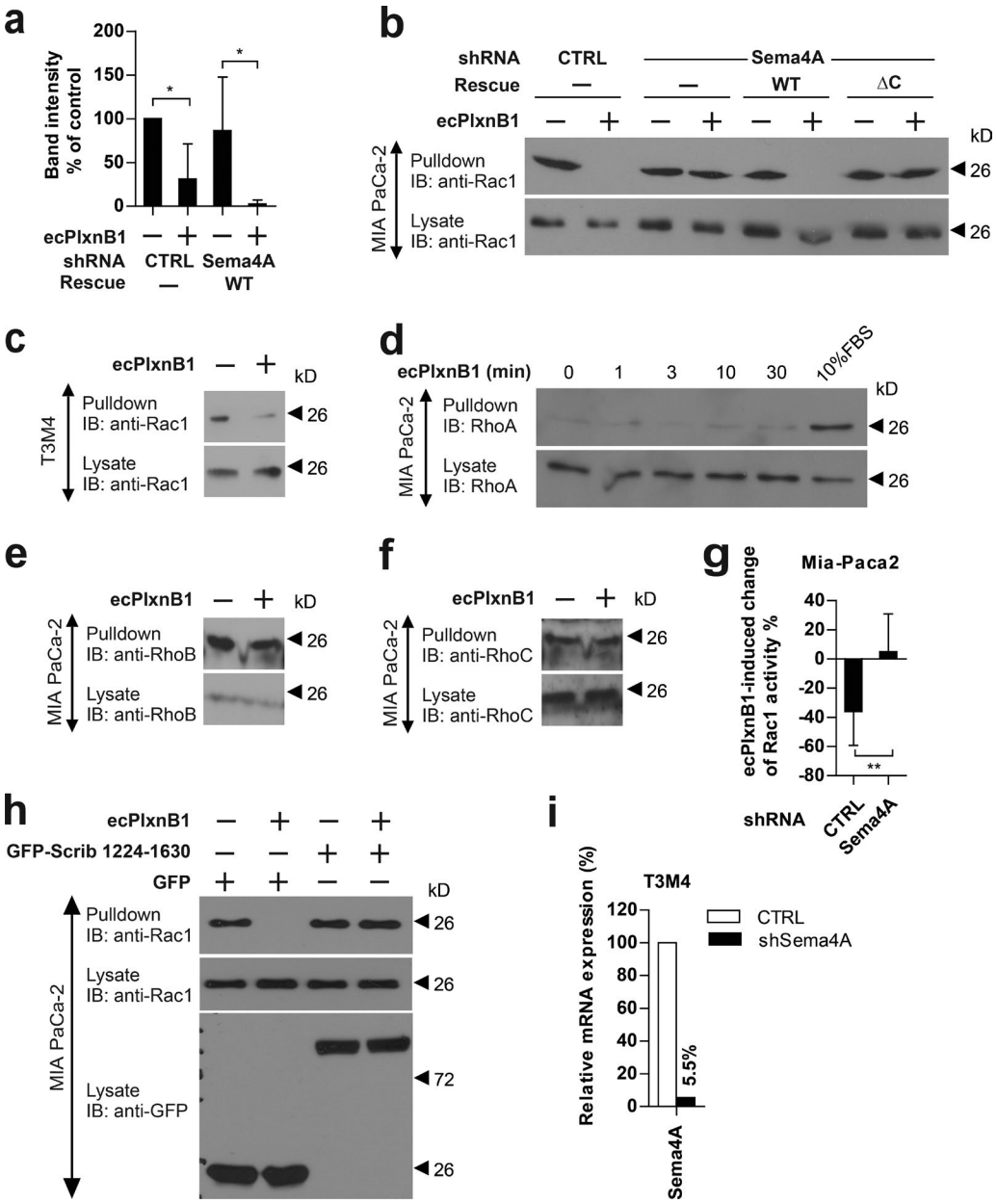
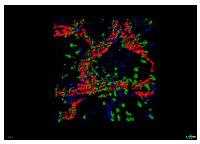


Figure S5. **The extracellular portion of Plexin-B1 induces modulation of Cdc42 and Rac1 activities through Sema4A-Scrib.** (a) MIA PaCa-2 cells stably expressing control shRNA or Sema4A shRNA together with shRNA-resistant wild-type (WT) Sema4A were starved and stimulated with or without 150 nM ecPlxnB1, and active Cdc42 was precipitated. Band intensities were measured using ImageJ as described in Materials and methods. Shown are mean values of three independent experiments ± SD. (b) MIA PaCa-2 cell lines stably expressing Sema4A shRNA alone or together with shRNA-resistant wild-type or mutated (ΔC) Sema4A (as indicated) were starved and stimulated with or without 150 nM ecPlxnB1, and active Rac1 was precipitated as described in Materials and methods. (c) Rac1 activity in T3M4 cells in the absence or presence of 150 nM ecPlxnB1 was measured. (d) MIA PaCa-2 cells were stimulated with 150 nM ecPlxnB1. Active RhoA was precipitated (pulldown) at the indicated time points. Application of 10% FBS served as control. (e and f) MIA PaCa-2 cells were incubated with or without 150 nM ecPlxnB1, and the levels of active RhoB (e) and RhoC (f) were analyzed in pulldown assays. (g) The change of Rac1 activity induced by 150 nM ecPlxnB1 was calculated as percentages of the difference between the FRET ratio at baseline (before treatments) and the FRET ratio after treatment with 100 μM Rac inhibitor EHT1864. The total number of cells analyzed are control (CTRL), $n = 12$ and Sema4A, $n = 6$. (h) MIA PaCa-2 cells were transfected with GFP or the GFP-tagged C-terminal portion of Scrib (GFP-Scrib, amino acids 1224–1630), and Rac1 activity was measured (pulldown). (i) T3M4 cells were stably transfected with control shRNA or shRNA directed against Sema4A. mRNA expression levels were determined by real-time quantitative PCR. Error bars represent means ± SD. *, $P < 0.05$; **, $P < 0.01$.



Video 1. **Migration of BMDCs into ear lymphatic vessels.** Mature BMDCs generated from wild-type and Sema4A knockout mice were fluorescently labeled (wild type, green; Sema4A knockout, blue), mixed 1:1, and used for live-cell imaging in an ex vivo ear crawl migration assay. The video was recorded over 45 min. This video was shot at seven frames per second.