## Supplemental methods

### Cell preparation

To generate BM-derived DCs, BM cells were cultured for 7 days with granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml; PeproTech) and purified with anti-CD11c microbeads (Miltenyi Biotec). In some experiments, purified DCs were treated with LPS (200 ng/ml; Sigma-Aldrich) for 24 hours before use. BW5147$\alpha^-\beta^-$ cell lines stably expressing DOCK8, DOCK8 ΔDHR-2, DOCK2 and/or βPIX were generated by electroporation of the HA- or Flag-tagged constructs.

### Protein expression and purification

The genes encoding DOCK8 DHR-2 (residues 1633–2071) and DOCK2 DHR-2 (residues 1195–1621) were cloned into the pET-SUMO vector and were expressed in *E. coli* Arctic Express (Agilent Technologies) as fusion proteins with His tag at their N-terminus for purification with nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography (Qiagen). The GST-Cdc42, GST-Rac1, GST-RhoA, and GST were expressed in BL21 (Agilent Technologies) and purified on Glutathione Sepharose™ 4B (GE Healthcare) immediately before use.

For crystallization, the boundaries for the DOCK8 DHR-2 domain were extensively analyzed by expressing various DOCK8 DHR-2 fragments in the small-scale dialysis mode of the *E. coli* cell-free reaction. Consequently, the genes encoding the DOCK8 DHR-2 lacking lobe A (residues 1787–2067) and Cdc42 (residues 1–188) fragments were cloned into the expression vector pCR2.1 (Invitrogen), as fusions with the N-terminal His affinity tag and a tobacco etch virus (TEV) protease cleavage site. The T17N mutation was introduced into Cdc42 by using a QuikChange Site-directed mutagenesis Kit (Agilent Technologies). The DOCK8 DHR-2 and Cdc42 (T17N) proteins were co-synthesized using the large-scale dialysis mode of the *E. coli* cell-free reaction. These proteins were purified together by chromatography on a His-trap column (GE Healthcare) and subjected to TEV protease digestion. The proteins were subsequently

applied onto a HiTrap Q column (GE Healthcare), and DOCK8 DHR-2 and Cdc42 were eluted separately with a linear gradient of sodium chloride. DOCK8 DHR-2 and Cdc42 were then each purified by size-exclusion chromatography on a HiLoad 26/10 Superdex 75 pg column (GE Healthcare), pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl and 2 mM DTT.

**Subcellular fractionation**

BM-derived DCs ($1 \times 10^7$ cells) were resuspended in ice-cold hypotonic buffer (42 mM KCl, 10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 10 μg/ml each aprotinin and leupeptin) and incubated on ice for 15 minutes. Cells were transferred to a 1 ml syringe and sheared by being passed five times through a 27 gauge needle. The lysates were centrifuged at 200 × g for 10 minutes to remove nuclei and cell debris. After being centrifuged at 13,000 × g for 60 minutes at 4°C, the supernatant was then collected as the cytosolic fraction. The pellet was then resuspended in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, and 10 μg/ml each aprotinin and leupeptin), vortexed for 40 minutes at 4°C, and centrifuged again at 13,000 × g for 60 minutes at 4°C. The supernatant representing the membrane fraction was saved. Each fraction was separated by SDS-PAGE, and blots were probed with anti-DOCK8, anti-βPIX, anti-pan-Cadherin and anti-Cdc42 antibodies.

**Immunofluorescence microscopy**

BW5147$\alpha^-\beta^-$ transfectants were stained with Alexa Fluor 488–conjugated succinylated concanavalin A. To assess subcellular localization of DOCK8 and βPIX, cells were then fixed with 4% paraformaldehyde, made permeable for 5 minutes with 0.1% Triton-X-100, and were stained with anti–HA antibody followed by Alexa Fluor 546–conjugated anti–rat IgG antibody. The cells were washed and mounted using fluorescent mounting medium (DAKO) with DAPI (WAKO). All images were taken with a laser scanning confocal microscope (LSM 510 META).

**Retroviral transfection**

The retroviral vector pMXs was used to generate the plasmid encoding Raichu-Cdc42. This plasmid was transfected into Platinum-E packaging cells using FuGENE 6 transfection reagent (Roche). The cell culture supernatants were harvested 48 hours after transfection, supplemented with 5 μg/ml polybrene and 20 ng/ml GM-CSF, and used to infect BM-derived DCs. After centrifugation at 2,000 rpm for 1 hour, plates were incubated at 32˚C for 8 hours and at 37˚C for 16 hours. Before FRET imaging, two additional retroviral infections were performed at daily intervals.

**Legends to supplemental videos**

**Video 1**

Time-lapse video showing PKH-26 labeled *Dock8*$^{+/-}$ DCs (red) entering the lymphatic vessel (green) within dermal tissues of ear explants. Time stamp indicates elapsed time in minute:second.

**Video 2**

Time-lapse video showing PKH-26 labeled *Dock8*$^{-/-}$ DCs (red) entering the lymphatic vessel (green) within dermal tissues of ear explants. Time stamp indicates elapsed time in minute:second.

**Video 3**

Time-lapse video shows that, while *Dock8*$^{+/-}$ DCs (green) squeeze between the LYVE-1$^{+}$ sinus lining cells (blue) by deforming their shape, morphological adaptation process is impaired in the case of *Dock8*$^{-/-}$ DCs (red). Time stamp indicates elapsed time in hour:minute:second. One square line corresponds to 6.41 μm. This image was obtained by two-photon microscopy 6 hours after DC transfer.

**Table S1. X-ray data collection and refinement statistics of the DOCK8 DHR-2•Cdc42 complex**

| | |
|---|---|
| **Data collection** | |
| Space group | $C_2$ |
| Cell dimensions $a, b, c$ (Å) | 104.2, 73.3, 65.0 |
| $\alpha, \beta, \gamma$ (°) | 90, 105, 90 |
| Wavelength (Å) | 1.0000 |
| Resolution (Å) | 30.0 – 2.09 (2.16 – 2.09) |
| Unique reflections | 28,080 |
| Redundancy | 5.5 (5.0) |
| Completeness (%) | 99.5 (98.4) |
| $I/\sigma$ ($I$) | 24.6 (4.96) |
| $R_{sym}$ | 0.060 (0.338) |
| **Refinement** | |
| Resolution (Å) | 29.7 – 2.09 (2.14 – 2.09) |
| No. of reflections | 26,517 |
| No. of atoms | |
| Protein | 3577 |
| Water | 194 |
| Phosphate ion | 10 |
| $R_{work}$ (%) | 17.9 |
| $R_{free}$ (%)[a] | 23.2 |
| Average $B$ factor (Å$^2$) | |
| Protein | 36.9 |
| Water | 39.4 |
| Phosphate ion | 40.7 |
| Rmsd bond length (Å) | 0.010 |
| Rmsd bond angle (°) | 1.275 |
| Ramachandran plot statistics (%) | |
| In most favored regions | 96.6 |
| In additional allowed regions | 2.8 |
| In generously allowed regions | 0.5 |
| In disallowed regions | 0.0 |

All numbers in parentheses represent last outer shell statistics.

[a]Free $R$-factor is calculated for 5 % of randomly selected reflections excluded from refinement.

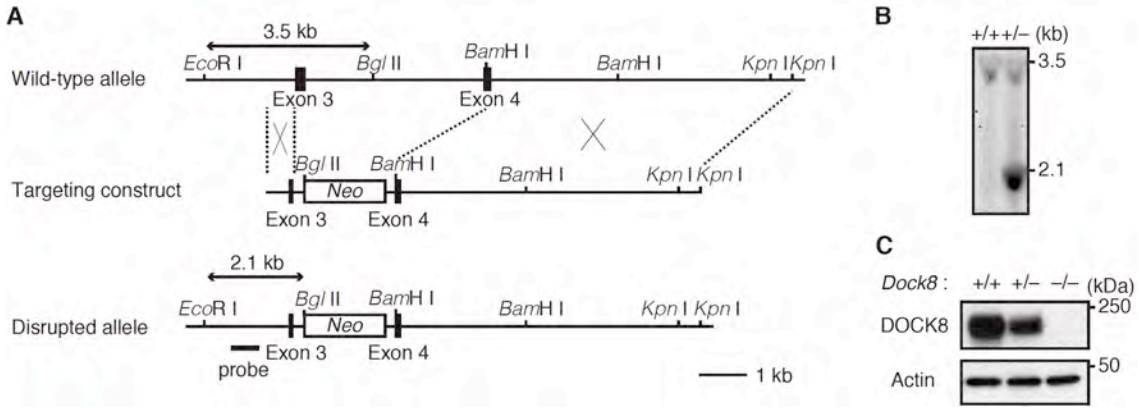


**Figure S1. Strategy of generation of DOCK8-deficient mice**

(**A**) The structure of *Dock8* genomic locus and the targeting vector are illustrated. Homologous recombination causes the replacement of a region spanning from 3'-part of exon 3 through 5'-part of exon 4 with a neomycin resistance gene expressing cassette (*Neo*). The length of diagnostic restriction fragments and location of probe used for Southern blot analysis are shown. (**B**) Southern blot analysis for *Eco*R I/*Bgl* II-digested DNA of control (+/+) and targeted (+/–) ES cell clones. (**C**) Spleen cell lysates from *Dock8*$^{+/+}$, *Dock8*$^{+/-}$, and *Dock8*$^{-/-}$ mice were analyzed by immunoblot. The antibody recognizing the C-terminal fragment of DOCK8 was used in this study.

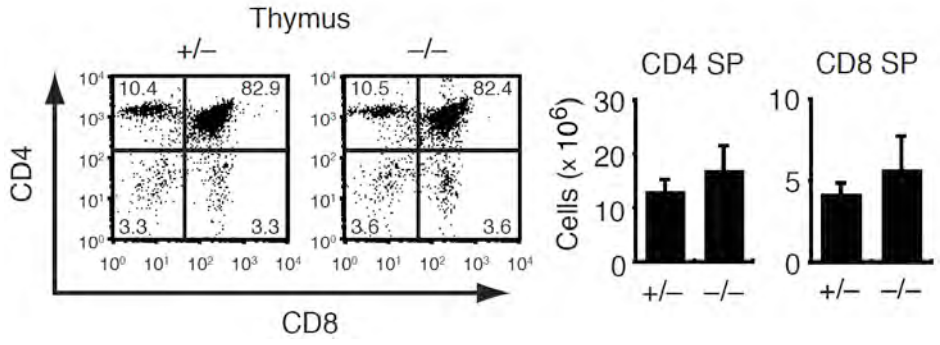


**Figure S2. DOCK8 deficiency does not affect T cell development in the thymus**

Thymocytes from *Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ mice were analyzed for CD4 and CD8 expression. The numerals in quadrants indicate the percentage of cells in each. The numbers of $CD4^+CD8^-$ (CD4 SP) and $CD4^-CD8^+$ (CD8 SP) thymocytes were compared between 6–7 week-old *Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ mice (n = 10 mice per group). Data are from nine separate experiments and are expressed as mean ± SD.

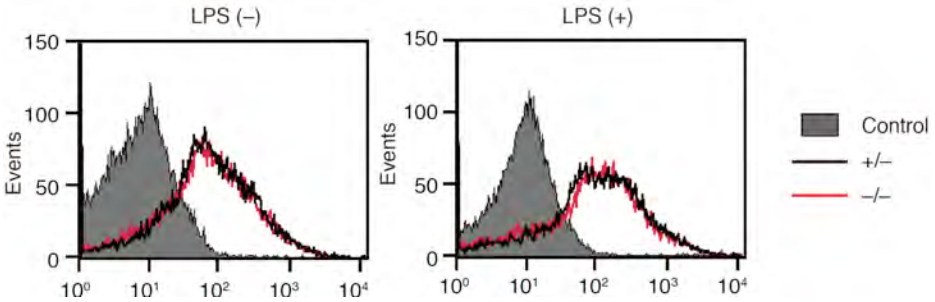


**Figure S3. Normal expression of CCR7 on DOCK8-deficient DCs**

*Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ BM-derived DCs were stimulated with or without LPS (200 ng/ml) for 24 hours, and CCR7 expression was analyzed on a FACSCalibur. Control, isotype-matched control antibody. Data are representative of two independent experiments.

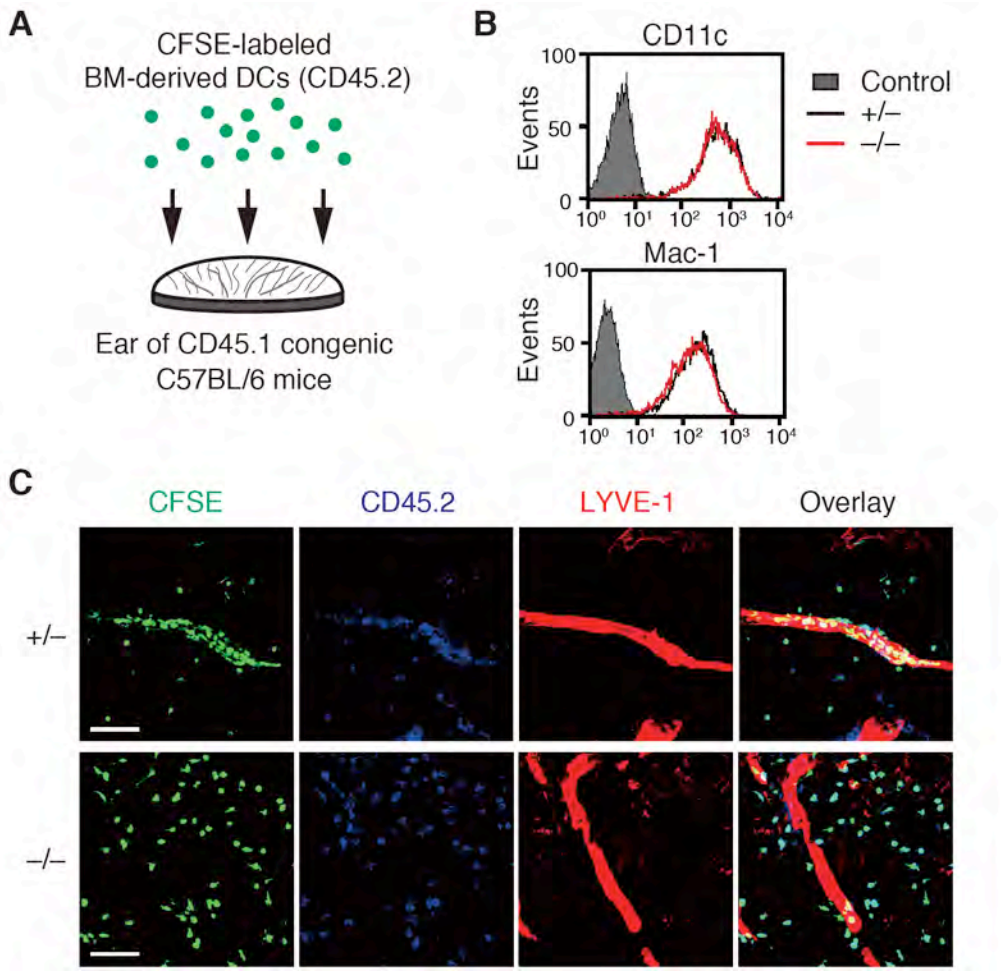


**Figure S4. Localization of DOCK8-deficient DCs within the dermis of ear explants**

(**A**) Experimental protocol for ex vivo DC migration. (**B**) Before assays, *Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ BM-derived DCs were analyzed for the expression of CD11c and Mac-1. Control, isotype-matched control antibody. (**C**) CFSE-labeled DCs (CD45.2) were placed on the dermal ear explants of CD45.1 congenic mice. After a 90–minute incubation, the ear sheets were stained with anti-CD45.2 and anti-LYVE-1 antibodies. Scale bar, 100 μm. Data are representative of three independent experiments.

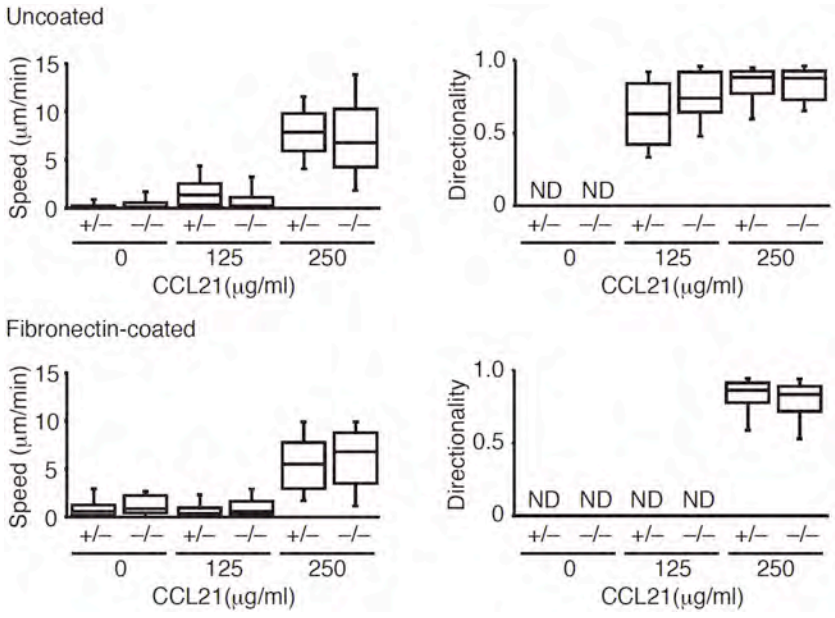


**Figure S5. DOCK8 deficiency does not affect motility and directionality during DC migration on 2D surfaces**

Velocity and directionality were compared between LPS-stimulated *Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ BM-derived DCs (n = 18–53 cells per group) chemotaxing toward 1 μl of indicated concentration of CCL21. The assays were performed in EZ-Taxiscan chamber with or without fibronectin coating. ND, not determined. The lower left panel contains some of the same data used in Figure 4D. Data are representative of two independent experiments. Each box plot exhibits the median (central line within each box), the 25th and 75th percentile values (box ends), and the 10th and 90th percentile values (error bars).

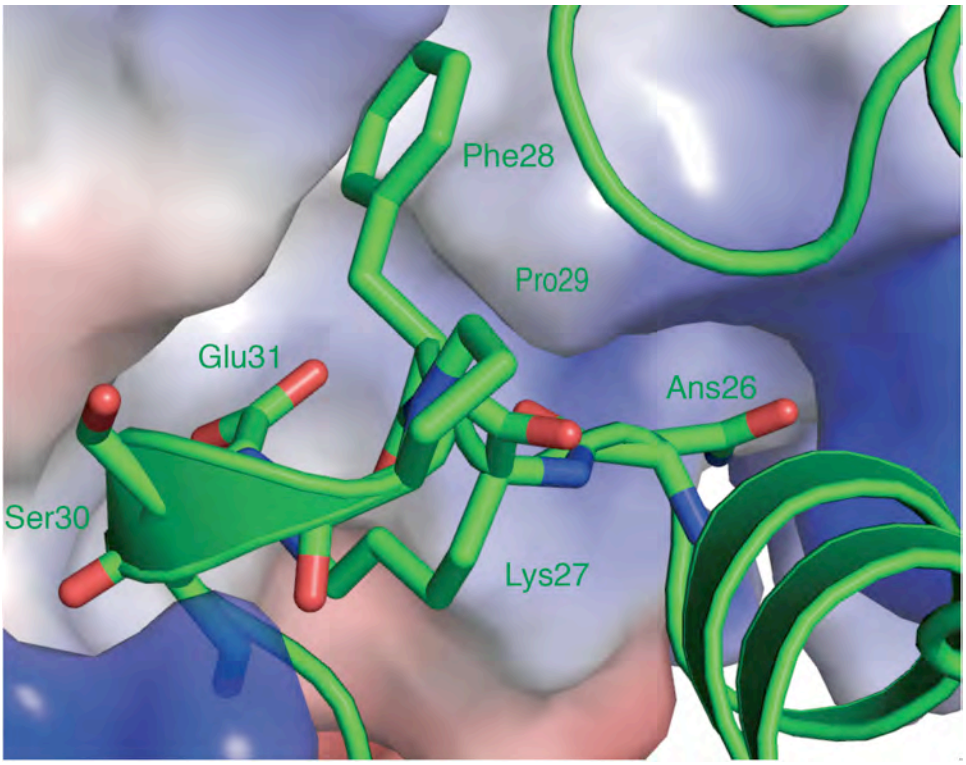


**Figure S6. Interaction of DOCK8 with switch I region of Cdc42**

Molecular surface representation of a part of DOCK8 in interaction with Phe28 and its surrounding region of Cdc42 (green) is shown.