Supporting Information

Foster et al. 10.1073/pnas.1003747107

SI Experimental Procedures

To measure T cell proliferation, spleens from wild-type (Eprhin-B2$^{+/+}$) and mutant (*Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre*) adult mice were dissected and mashed through a cell strainer; 10^8 cells were stained with CD19, B220, Ter119, Mac1, NK1.1, CD11c, TCRγδ, and Gr1 biotinylated antibodies (eBiosciences) before incubating with streptavidin-conjugated Dynabeads (Invitrogen). T cells were purified by three rounds of magnetic-separation steps and stained with carboxy fluorescein diacetate succinimidyl ester (CFSE; Invitrogen); 5×10^6 cells were incubated with plate-bound anti-CD3 (10 μg/mL) and CD28 (2 μg/mL) for 72 h before flow-cytometric analyses. Cells were first gated for CD4 and CD8 expression, and the fluorescence intensity of CFSE was plotted on a histogram (FlowJo).

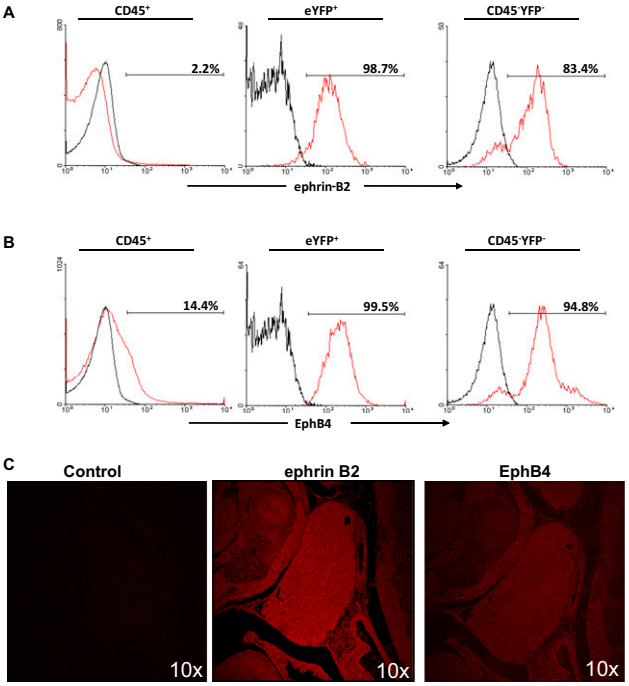


Fig. S1. Expression of ephrin ligands and Eph receptors on neural crest (NC)-derived mesenchyme and thymic epithellial cells (TECs). Thymus lobes from E15.5 *Wnt1-Cre;Rosa26^{eYfp}* embryos were digested with collagenase, stained with antibodies recognizing CD45, ephrin-B2, and EphB4, and analyzed by flow cytometry. Cells were first-gated CD45$^+$ (thymocytes) (*A Left* and *B Left*), enhanced YFP (eYFP$^+$; NC-derived) (*A Center* and *B Center*), or eYFP$^-$CD45$^-$ (thymic epithelium) (*A Right* and *B Right*) cells and were further analyzed for expression of ephrin-B2 (*A*) and EphB4 (*B*). Red line, specific antibody; black line, isotype control. Two mice of each genotype were analyzed, giving similar results. Embyros from E15.5 were stained with antiephrin B2 and EphB4 (*C*) antibodies, and representative confocal images are shown.

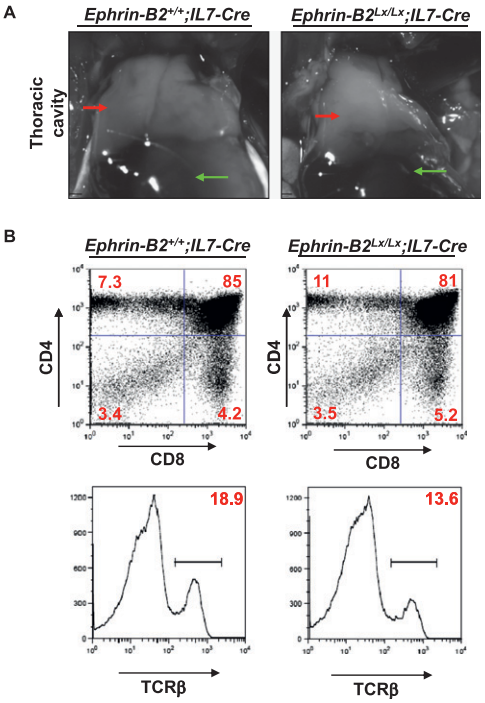


Fig. S2. The thymus seems normal in the absence of ephrin-B2 expression on TECs; (*A*) 3-mo-old adult *Ephrin-B2*$^{+/+}$*;IL7-cre* (*Left*) and *Ephrin-B2*$^{Lx/Lx}$*;IL7-Cre* (*Right*) mice were dissected to reveal the thoracic cavity to visualize the location of the thymus lobes. Red arrows, thymus; green arrows, heart. (*B*) Thymi from 3-mo-old *Ephrin-B2*$^{Lx/+}$ and *Ephrin-B2*$^{Lx/Lx}$*;IL7-Cre* mice were dissected, digested with collagenase, stained with antibodies recognizing CD4, CD8 (*Upper*), and TCRβ (*Lower*), and analyzed by flow cytometry. Similar results were obtained in two independent experiments consisting of six and four mice, respectively.

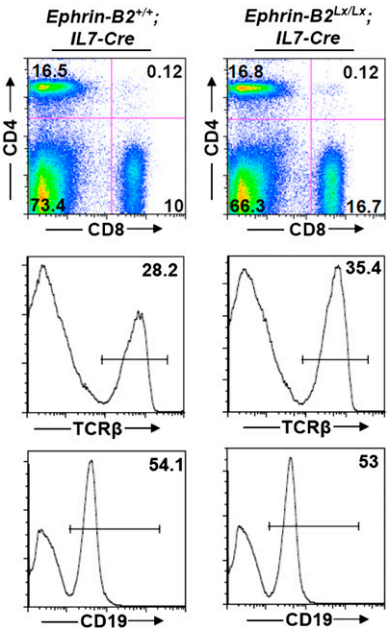


Fig. S3. Normal peripheral T and B cell populations in the absence of ephrin-B2 expression on TECs. Spleens from 3-mo-old adult *Ephrin-B2*$^{+/+}$*;IL7-cre* (*Top*), *Ephrin-B2*$^{Lx/Lx}$*;IL7-Cre* (*Middle*), and C57Bl6 (*Bottom*) mice were dissected, digested with collagenase, and stained with antibodies recognizing CD4 and CD8 (*Top*), TCRβ (*Middle*), and CD19 (*Bottom*). Similar results were obtained from three mice of each genotype.

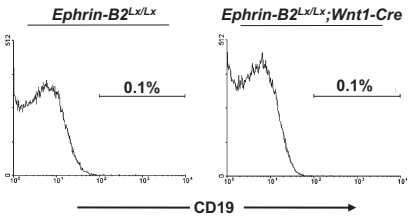


Fig. S4. Ectopic structures are not lymph nodes. Thymi from adult control *Ephrin-B2*$^{Lx/Lx}$ (*Left*) mice and ectopic structures from mutant *Ephrin-B2*$^{Lx/Lx}$*; Wnt1Cre;Rosa26*^{eYfp} (*Right*) mice were digested with collagenase, stained with an antibody detecting CD19, and analyzed by flow cytometry. Similar results were obtained from three control and six mutant mice.

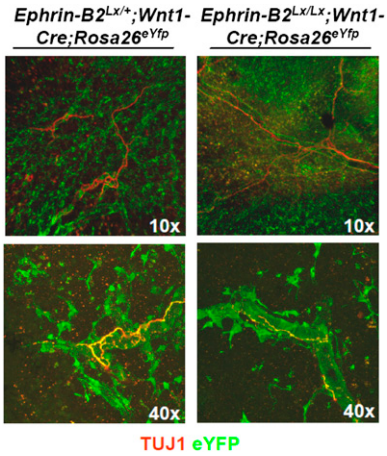


Fig. S5. The ectopic thymus is innervated normally; 3-mo-old adult thymus from control *Ephrin-B2*$^{+/+}$*;Wnt1-Cre;Rosa26*^{eYfp} (*Left*) and mutant *Ephrin-B2*$^{Lx/Lx}$*; Wnt1-Cre;Rosa26*^{eYfp} (*Right*) mice were fixed with 4% paraformaldehyde, cut into 100-μm sections, stained with antibodies recognizing Tuj1 (neurons, red) and eYFP (green), and analyzed by confocal microscopy. (*Upper*) 3D reconstruction of a 100-μm section. (*Lower*) Single confocal sections. Two mice of each genotype were analyzed, giving similar results.

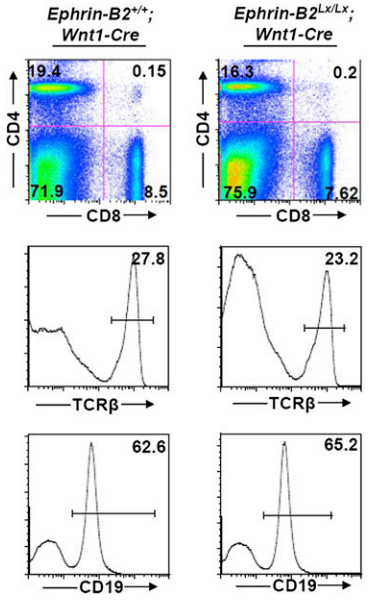


Fig. S6. Normal peripheral T and B cell populations in the absence of ephrin-B2 expression on NC-derived cells. Spleens from 3-mo-old adult *Ephrin-B2*$^{+/+}$*; Wnt1-cre* (*Top*), *Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre* (*Middle*), and C57Bl6 (*Bottom*) mice were dissected, digested with collagenase, and stained with antibodies recognizing CD4 and CD8 (*Top*), TCRβ (*Middle*), and CD19 (*Bottom*). Similar results were obtained from three mice of each genotype.

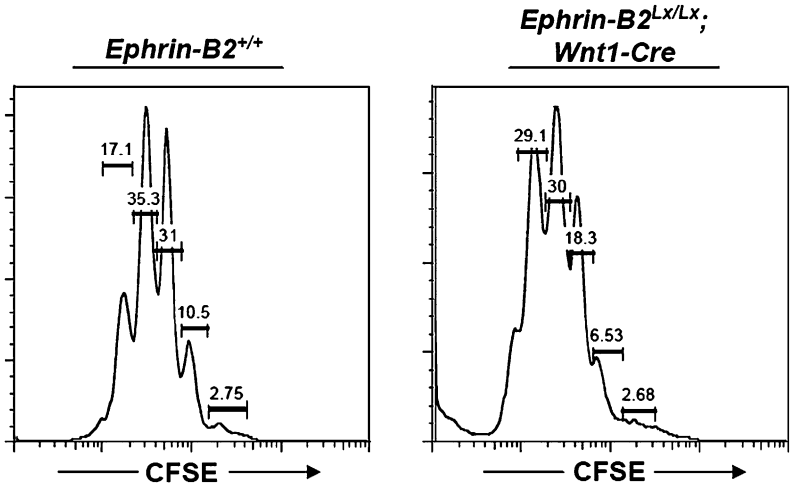


Fig. S7. Normal proliferation of T cells from mice with ectopic thymus. T cells in mice with Ephrin-B2–deficient NC-derived mesenchymal cells (NCCs) proliferate normally. T cells from mutant (*Left*) and wild-type (*Right*) mice were labeled with CFSE and incubated in the presence of plate-bound anti-CD3 and CD28 for 72 h before flow-cytometric analysis. Similar results were obtained from three experiments consisting of three mice per genotype.

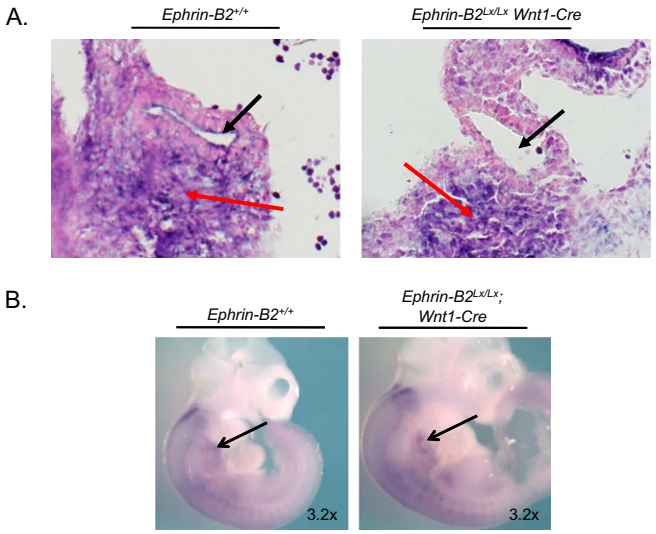


Fig. S8. (*A*) NCCs migrate to the third pharyngeal pouch by E10.5. Sagittal sections through the third pharyngeal pouch showing migrating NCCs, marked by *Crabp1*. (*Left*) Control *Ephrin-B2*$^{+/+}$. (*Right*) Mutant *Ephrin-B2*$^{Lx/Lx}$*;Wnt1Cre*. Black arrows, third pharyngeal pouch; red arrows, migrating NCCs. Two experiments consisting of two mice per genotype were analyzed, giving similar results. (*B*) Hoxa3 expression seems normal in mice with Ephrin-B2–deficient NCCs. (*Right*) E10.5 mutant (*Ephrin-B2*$^{Lx/Lx}$*;Wnt1-Cre*) and (*Left*) control (*Ephrin-B2*$^{+/+}$) embryos were fixed and processed for in situ hybridization with a probe detecting Hoxa3 RNA. Black arrows, third pharyngeal region.

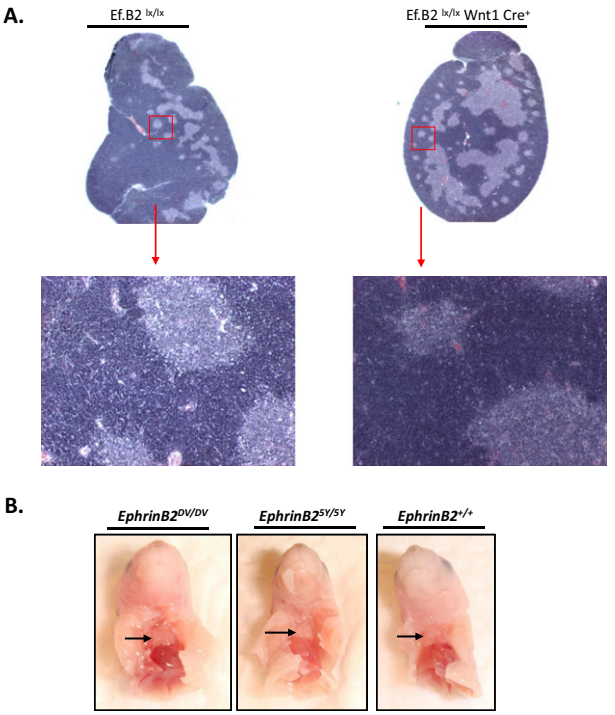
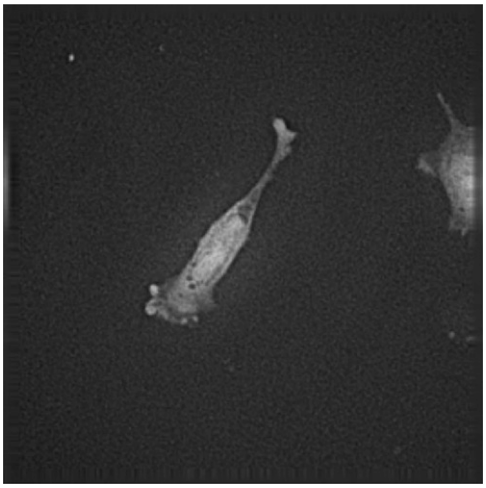
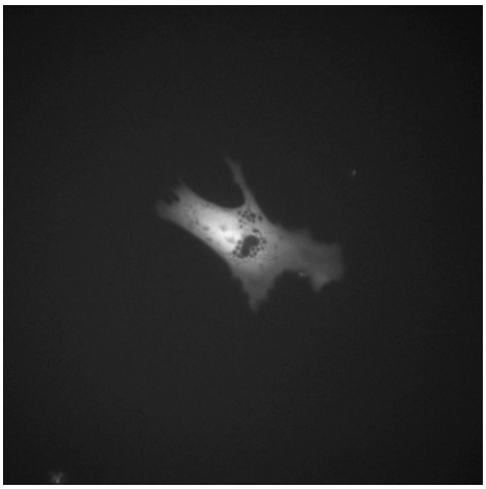


Fig. S9. (*A*) H&E sections from Wnt1cre Ephrin-B2$^{lx/lx}$ mice reveal normal thymus architecture. Thymi from (*Right*) mutant (*Ephrin-B2$^{Lx/Lx}$;Wnt1-Cre*) and (*Left*) control (*Ephrin-B2$^{+/+}$*) mice were fixed, wax-embedded, and sectioned before staining with H&E. The mutant thymus has normal cortical and medullar regions and no evidence of any parathyroid tissue. (*B*) Reverse signaling through ephrin-B2 and the anatomical location of the thymus. E18.5 mutant *ephrin-B2$^{\Delta V/\Delta V}$* (*Left*), mutant *ephrin-B2$^{5Y/5Y}$* (*Center*), and control *ephrin-B2$^{+/+}$* (*Right*) embryos were dissected to reveal the thymus in the thoracic cavity. Black arrows, thymus.



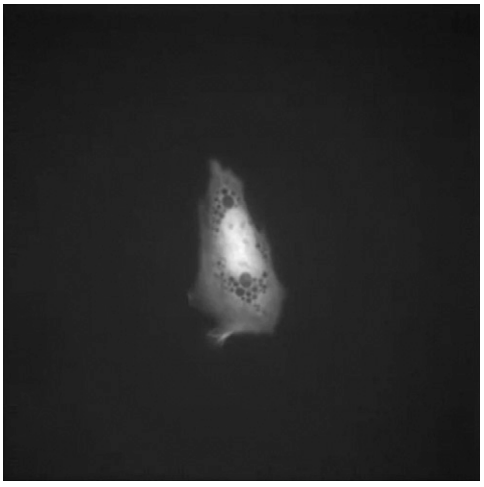
Movie S1. Time-lapse movie of cells depicted in Fig. 4*A*.

Movie S1



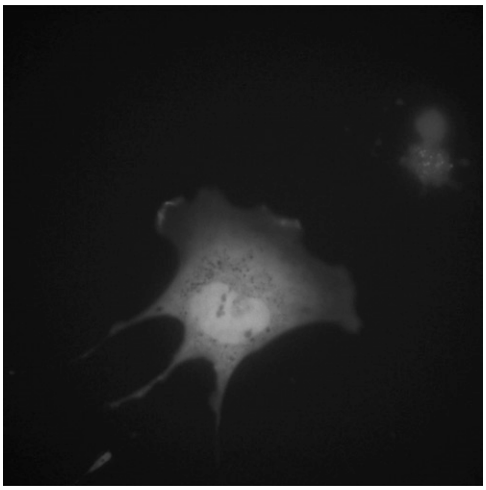
Movie S2. Time-lapse movie of cells depicted in Fig. 4*B*.

Movie S2



Movie S3. Time-lapse movie of cells depicted in Fig. 4*C*.

Movie S3



Movie S4. Time-lapse movie of cells depicted in Fig. 4*D*.

Movie S4