SUPPLEMENTAL MATERIAL

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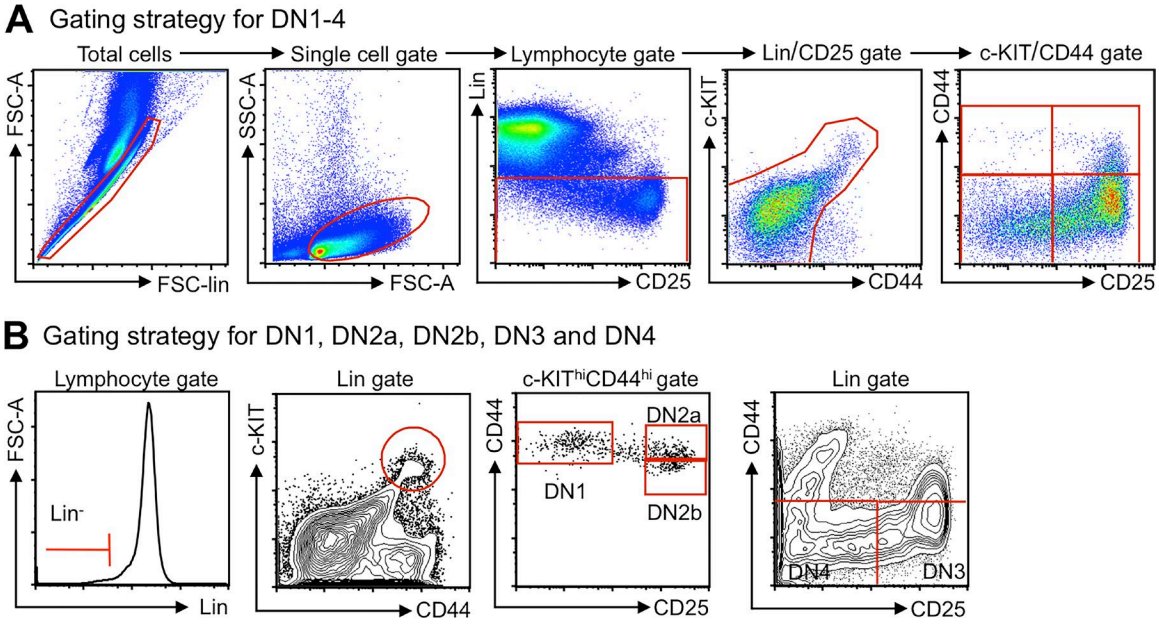

Figure S1. **Gating strategy for DN1–4 thymocytes.** (A and B) Lin$^-$ thymocytes were stained with fluorochrome-conjugated antibodies for CD25, CD44, and c-KIT. (A) Lin$^-$ CD25$^{+/-}$ thymocytes were gated for c-KIT versus CD44 expression to exclude CD44$^+$c-KIT$^-$ cells. DN1–4 thymocytes were defined as DN1 (CD25$^-$CD44$^+$c-KIT^{hi}), DN2 (CD25$^+$CD44$^+$c-KIT^{hi}), DN3 (CD25$^+$CD44$^-$c-KIT$^-$), and DN4 (CD25$^-$CD44$^-$c-KIT$^-$). (B) Lin$^-$ thymocytes were gated for c-KIT$^+$CD44$^+$ cells to define DN1 (CD25$^-$CD44^{hi}c-KIT^{hi}), DN2a (CD25$^+$CD44^{hi}c-KIT^{hi}), and DN2b (CD25$^+$CD44^{lo}c-KIT^{lo}). Lin$^-$ DN3 was defined as CD25$^+$CD44$^-$c-KIT$^-$ and Lin$^-$ DN4 as CD25$^-$CD44$^-$c-KIT$^-$.

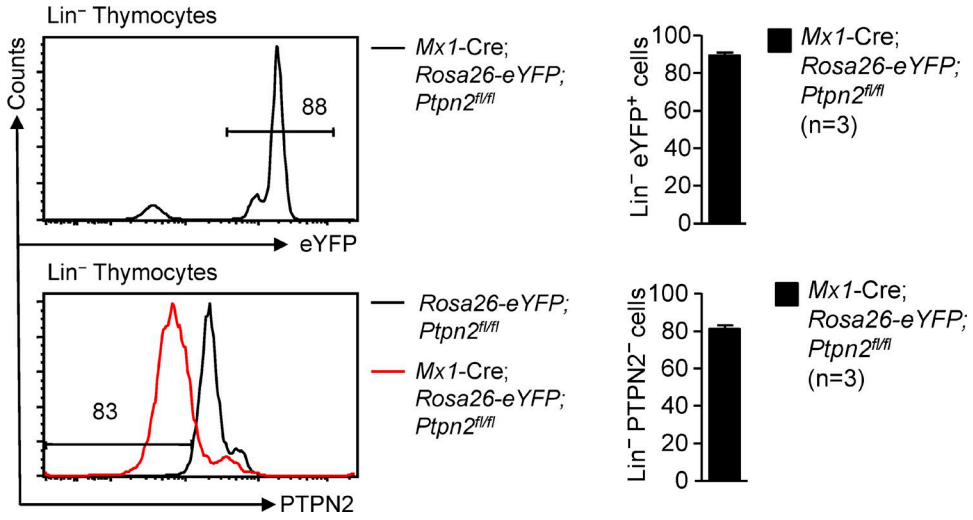

Figure S2. **PTPN2 deletion in *Mx1*-Cre;*Rosa26-eYFP;Ptpn2*$^{fl/fl}$ DN thymocytes.** Lin$^-$ thymocytes from poly (I:C)–treated *Rosa26-eYFP;Ptpn2*$^{fl/fl}$ (C57BL/6) and *Mx1*-Cre;*Rosa26-eYFP;Ptpn2*$^{fl/fl}$ (C57BL/6) mice were stained intracellularly with fluorochrome-conjugated α-PTPN2. eYFP fluorescence in Lin$^-$ *Mx1*-Cre;*Rosa26-eYFP;Ptpn2*$^{fl/fl}$ (C57BL/6) thymocytes and PTPN2 levels in eYFP$^+$ (*Mx1*-Cre;*Rosa26-eYFP;Ptpn2*$^{fl/fl}$) (C57BL/6) versus eYFP$^-$ (*Rosa26-eYFP;Ptpn2*$^{fl/fl}$) (C57BL/6) Lin$^-$ thymocytes were quantified by flow cytometry. Representative results (means ± SEM; *Mx1*-Cre;*Rosa26-eYFP;Ptpn2*$^{fl/fl}$, $n = 3$) from at least three independent experiments are shown.

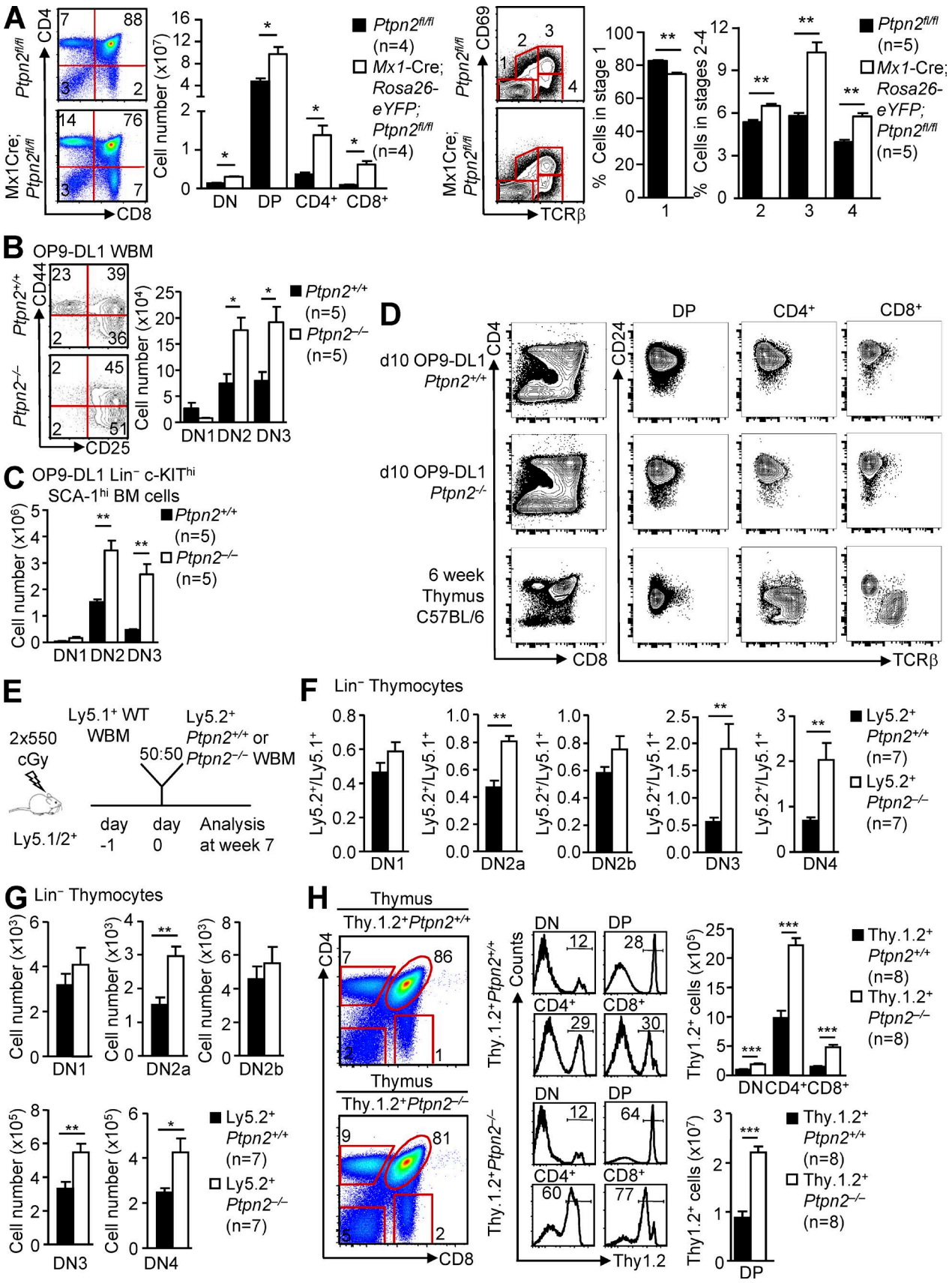
A
Ptpn2fl/fl
Mx1Cre; Ptpn2fl/fl
CD4
CD8
7
88
3
2
14
76
3
7
Cell number (x10⁷)
DN
DP
CD4⁺
CD8⁺
Ptpn2fl/fl (n=4)
Mx1-Cre; Rosa26-eYFP; Ptpn2fl/fl (n=4)
CD69
TCRβ
% Cells in stage 1
% Cells in stages 2-4
Ptpn2fl/fl (n=5)
Mx1-Cre; Rosa26-eYFP; Ptpn2fl/fl (n=5)
B
OP9-DL1 WBM
Ptpn2+/+
Ptpn2−/−
CD44
CD25
23
39
2
36
2
45
2
51
Cell number (x10⁴)
DN1 DN2 DN3
Ptpn2+/+ (n=5)
Ptpn2−/− (n=5)
C
OP9-DL1 Lin⁻ c-KIThi SCA-1hi BM cells
Cell number (x10⁶)
DN1 DN2 DN3
Ptpn2+/+ (n=5)
Ptpn2−/− (n=5)
D
DP
CD4⁺
CD8⁺
d10 OP9-DL1 Ptpn2+/+
d10 OP9-DL1 Ptpn2−/−
6 week Thymus C57BL/6
CD4
CD8
CD24
TCRβ
E
Ly5.1⁺ WT WBM
Ly5.2⁺ Ptpn2+/+ or Ptpn2−/− WBM
2x550 cGy
50:50
Ly5.1/2⁺
day -1
day 0
Analysis at week 7
F
Lin⁻ Thymocytes
Ly5.2⁺/Ly5.1⁺
DN1
DN2a
DN2b
DN3
DN4
Ly5.2⁺ Ptpn2+/+ (n=7)
Ly5.2⁺ Ptpn2−/− (n=7)
G
Lin⁻ Thymocytes
Cell number (x10³)
DN1
DN2a
DN2b
Cell number (x10⁵)
DN3
DN4
Ly5.2⁺ Ptpn2+/+ (n=7)
Ly5.2⁺ Ptpn2−/− (n=7)
H
Thymus
Thy.1.2⁺Ptpn2+/+
Thy.1.2⁺Ptpn2−/−
CD4
CD8
Thy.1.2⁺Ptpn2+/+ Counts
Thy.1.2⁺Ptpn2−/−
Thy1.2
DN
DP
CD4⁺
CD8⁺
12
28
29
30
12
64
60
77
Thy1.2⁺ cells (x10⁵)
DN CD4⁺ CD8⁺
Thy1.2⁺ cells (x10⁷)
DP
Thy.1.2⁺ Ptpn2+/+ (n=8)
Thy.1.2⁺ Ptpn2−/− (n=8)

Figure S3. **PTPN2 deficiency results in enhanced positive selection.** (A) Thymocytes from poly (I:C)–treated *Rosa26-YFP*;*Ptpn2*$^{fl/fl}$ (C57BL/6) and *Mx1*-Cre;*Rosa26-eYFP*;*Ptpn2*$^{fl/fl}$ (C57BL/6) mice were stained for CD4, CD8, TCR-β, and CD69 and then analyzed by flow cytometry. Cells were gated for the DN (CD4$^-$CD8$^-$), DP CD4$^+$CD8$^+$, CD4$^+$ SP, and CD8$^+$ SP subsets and thymocyte positive selection stages 1–4 according to CD69 and TCR-β expression. (B and C) WBM cells (B) or FACS-purified Lin$^-$ c-KIT^{hi} and SCA-1^{hi} BM cells (C) from *Ptpn2*$^{+/+}$ (C57BL/6) and *Ptpn2*$^{-/-}$ (C57BL/6) mice were cultured on OP9-DL1 stromal cells for 9 d. Cells were harvested and stained with fluorochrome-conjugated antibodies against CD25 and CD44. DN1–3 cell numbers were determined by flow cytometry. (D) FACS-purified DN1 thymocytes from *Ptpn2*$^{+/+}$ (C57BL/6) and *Ptpn2*$^{-/-}$ (C57BL/6) mice were cultured for 10 d on OP9-DL1 stromal cells and stained for TCR-β, CD24, CD4, and CD8. CD24 versus TCR-β subsets were determined by flow cytometry. (E) Equal numbers of donor WBM cells (2×10^6) from Ly5.2$^+$*Ptpn2*$^{+/+}$ (C57BL/6) or Ly5.2$^+$*Ptpn2*$^{-/-}$ (C57BL/6) mice and congenic Ly5.1$^+$ (C57BL/6) competitor cells were transferred into lethally irradiated (2×550 cGy) Ly5.1/Ly5.2$^+$ (C57BL/6) recipient animals. Donor cell contribution in the thymus was assessed at 7 wk after transplantation. (F) Donor Ly5.2$^+$*Ptpn2*$^{+/+}$ (C57BL/6) or Ly5.2$^+$*Ptpn2*$^{-/-}$ (C57BL/6) and Ly5.1$^+$ competitor (C57BL/6) DN subset ratios. (G) Absolute numbers of donor Ly5.2$^+$*Ptpn2*$^{+/+}$ (C57BL/6) versus Ly5.2$^+$*Ptpn2*$^{-/-}$ (C57BL/6) DN subsets. (H) Representative profiles, percentages, and absolute numbers of donor-derived Thy1.2$^+$*Ptpn2*$^{+/+}$ (BALB/c) or Thy1.2$^+$*Ptpn2*$^{-/-}$ (BALB/c) DN, DP, CD4$^+$, and CD8$^+$ SP thymocyte subsets in lethally irradiated (2×550 cGy) BALB/c recipient animals that had received equal numbers of donor WBM cells (2×10^6) from Thy1.2$^+$*Ptpn2*$^{+/+}$ (BALB/c) or Thy1.2$^+$*Ptpn2*$^{-/-}$ (BALB/c) mice and congenic Thy1.1$^+$ competitor cells. Representative results (means ± SEM; *Ptpn2*$^{+/+}$, $n = 5$; *Ptpn2*$^{-/-}$, $n = 5$; *Rosa26-eYFP*;*Ptpn2*$^{fl/fl}$, $n = 4$–5; *Mx1*-Cre;*Rosa26-eYFP*;*Ptpn2*$^{fl/fl}$, $n = 4$–5; Ly5.2$^+$*Ptpn2*$^{+/+}$, $n = 7$; Ly5.2$^+$*Ptpn2*$^{-/-}$, $n = 7$; Thy1.2$^+$*Ptpn2*$^{+/+}$, $n = 8$; and Thy1.2$^+$*Ptpn2*$^{-/-}$, $n = 8$) from at least two (B–G) or three (A and H) independent experiments are shown. Significance was determined using a two-tailed Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$.

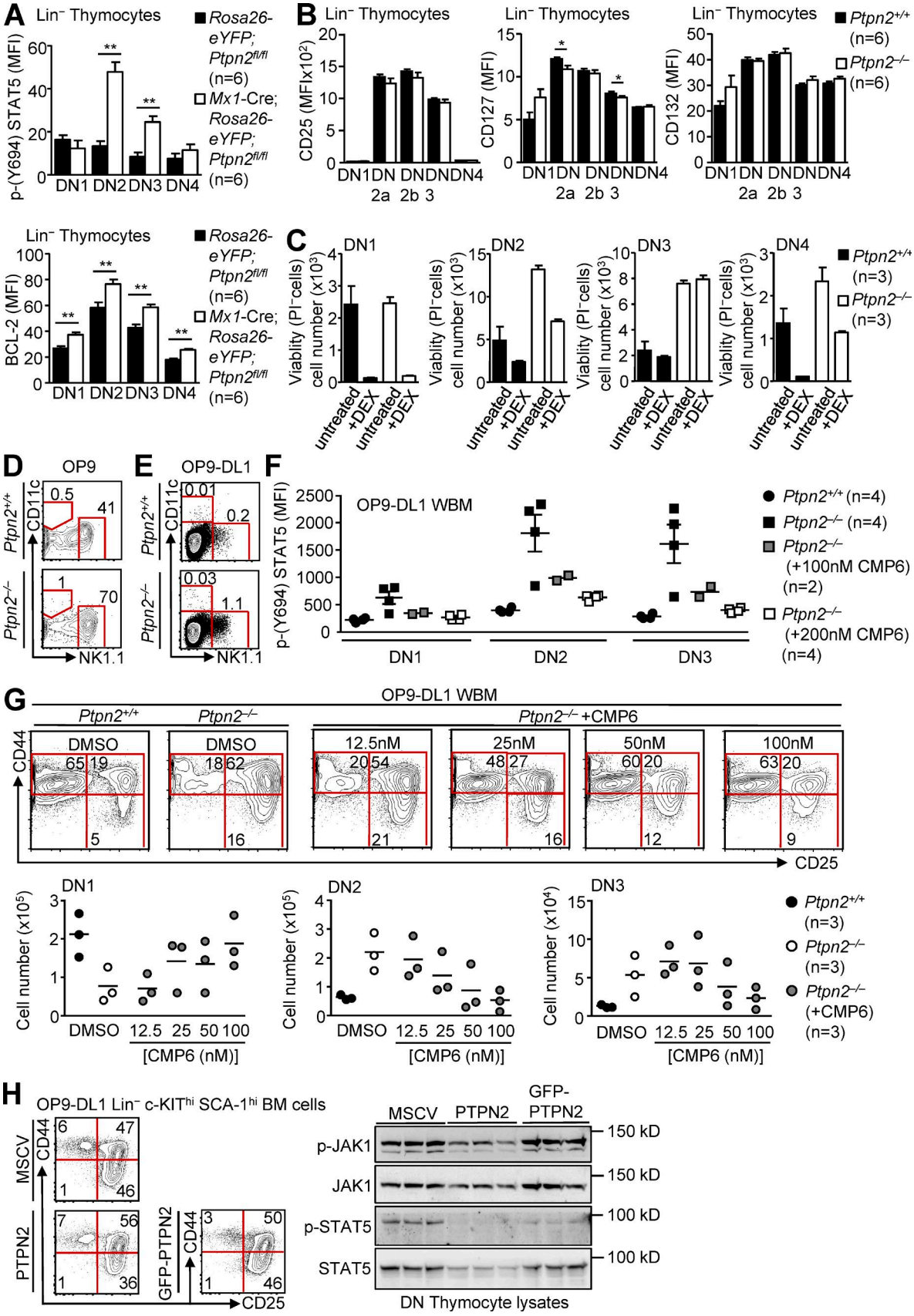
A
Lin⁻ Thymocytes
p-(Y694) STAT5 (MFI)
Rosa26-eYFP; Ptpn2fl/fl (n=6)
Mx1-Cre; Rosa26-eYFP; Ptpn2fl/fl (n=6)
DN1 DN2 DN3 DN4
B
Lin⁻ Thymocytes
CD25 (MFIx10²)
CD127 (MFI)
CD132 (MFI)
Ptpn2+/+ (n=6)
Ptpn2–/– (n=6)
DN1 DN 2a DN 2b DN 3 DN4
C
BCL-2 (MFI)
DN1
DN2
DN3
DN4
Viability (PI⁻cells) cell number (x10³)
Ptpn2+/+ (n=3)
Ptpn2–/– (n=3)
untreated
+DEX
D
OP9
Ptpn2+/+
Ptpn2–/–
CD11c
NK1.1
0.5
41
1
70
E
OP9-DL1
0.01
0.2
0.03
1.1
F
OP9-DL1 WBM
p-(Y694) STAT5 (MFI)
Ptpn2+/+ (n=4)
Ptpn2–/– (n=4)
Ptpn2–/– (+100nM CMP6) (n=2)
Ptpn2–/– (+200nM CMP6) (n=4)
G
OP9-DL1 WBM
Ptpn2+/+
Ptpn2–/–
Ptpn2–/– +CMP6
DMSO
12.5nM
25nM
50nM
100nM
CD44
CD25
65 19 5
18 62 16
20 54 21
48 27 16
60 20 12
63 20 9
Cell number (x10⁵)
Cell number (x10⁴)
[CMP6 (nM)]
Ptpn2+/+ (n=3)
Ptpn2–/– (n=3)
Ptpn2–/– (+CMP6) (n=3)
H
OP9-DL1 Lin⁻ c-KIThi SCA-1hi BM cells
MSCV
PTPN2
GFP-PTPN2
6 47 1 46
7 56 1 36
3 50 1 46
MSCV
PTPN2
GFP-PTPN2
p-JAK1
JAK1
p-STAT5
STAT5
150 kD
100 kD
DN Thymocyte lysates

Figure S4. **STAT5 signaling in DN thymocytes.** (A) Lin^- thymocytes from poly (I:C)–treated *Rosa26-eYFP;Ptpn2*$^{fl/fl}$ (C57BL/6) and *Mx1*-Cre; *Rosa26-eYFP;Ptpn2*$^{fl/fl}$ (C57BL/6) mice were stained with fluorochrome-conjugated antibodies against CD25, CD44, and c-KIT. The levels of intracellular p-(Y694) STAT5 (p-STAT5; i.e., activated STAT5) or BCL-2 were determined by flow cytometry. DN subset p-STAT5 and BCL-2 MFIs were determined. (B) Lin^- *Ptpn2*$^{+/+}$ (C57BL/6) and *Ptpn2*$^{-/-}$ (C57BL/6) thymocytes were stained with fluorochrome-conjugated antibodies against CD25, CD44, c-KIT, CD127, and CD132. CD25 (IL-2R-α chain), CD127 (IL-7R-α chain), and CD132 (common γ chain) MFIs in DN1–4 were determined by flow cytometry. (C) Lin^- thymocytes (2×10^5) from *Ptpn2*$^{+/+}$ (C57BL/6) and *Ptpn2*$^{-/-}$ (C57BL/6) mice were cultured overnight in the presence of dexamethasone or vehicle control (ethanol; untreated). Thymocytes were harvested and stained with fluorochrome-conjugated antibodies against CD25 and CD44 plus propidium iodide $(PI)^-$ (i.e., live). DN1–4 cell numbers were quantified by flow cytometry. (D and E) 10^2 DN2a thymocytes from *Ptpn2*$^{+/+}$ (C57BL/6) and *Ptpn2*$^{-/-}$ (C57BL/6) mice were FACS purified, cultured on OP9 (D) or OP9-DL1 (E) stromal cells for 5 d, and then stained with fluorochrome-conjugated antibodies against the Lin markers CD11c and NK1.1. Cell subsets were quantified by flow cytometry. (F and G) WBM cells from *Ptpn2*$^{+/+}$ (C57BL/6) and *Ptpn2*$^{-/-}$ (C57BL/6) mice were cultured on OP9-DL1 stromal cells in the presence of the JAK PTK inhibitor CMP6 or vehicle control (DMSO). After 7 d, Lin^- thymocytes were harvested and stained with fluorochrome-conjugated antibodies to CD25 and CD44. (F) MFIs for the intracellular staining of p-STAT5 for DN1–3 subsets were determined by flow cytometry. (G) DN cell subset numbers were quantified by flow cytometry. (H) Lin^- $SCA\text{-}1^+$ $c\text{-}KIT^+$ BM cells from C57BL/6 mice were transduced with control retrovirus (MSCV) or retroviruses expressing WT PTPN2 or nuclear-restricted GFP-PTPN2. FACS-purified $mCherry^+$ (MSCV, WT-PTPN2) or $mCherry^+/GFP^+$ (GFP-PTPN2) were cultured on OP9-DL1 stromal cells for 9 d in triplicates. DN2/3 thymocytes were harvested and stained with fluorochrome-conjugated antibodies against CD25 and CD44, and DN cell subsets were determined by flow cytometry. DN2/3 thymocyte lysates were resolved by SDS-PAGE and immunoblotted for p-STAT5, STAT5, p-(Y1022/Y1023) JAK-1 (p-JAK1), and JAK-1. Representative flow cytometry profiles (D, E, G, and H) and immunoblots (H) are shown. Representative results (means ± SEM; *Ptpn2*$^{+/+}$, $n = 3–6$; *Ptpn2*$^{-/-}$, $n = 2–6$; *Rosa26-eYFP;Ptpn2*$^{fl/fl}$, $n = 6$; and *Mx1*-Cre;*Rosa26-eYFP;Ptpn2*$^{fl/fl}$, $n = 6$) from at least two independent experiments are shown. Significance was determined using a two-tailed Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$.

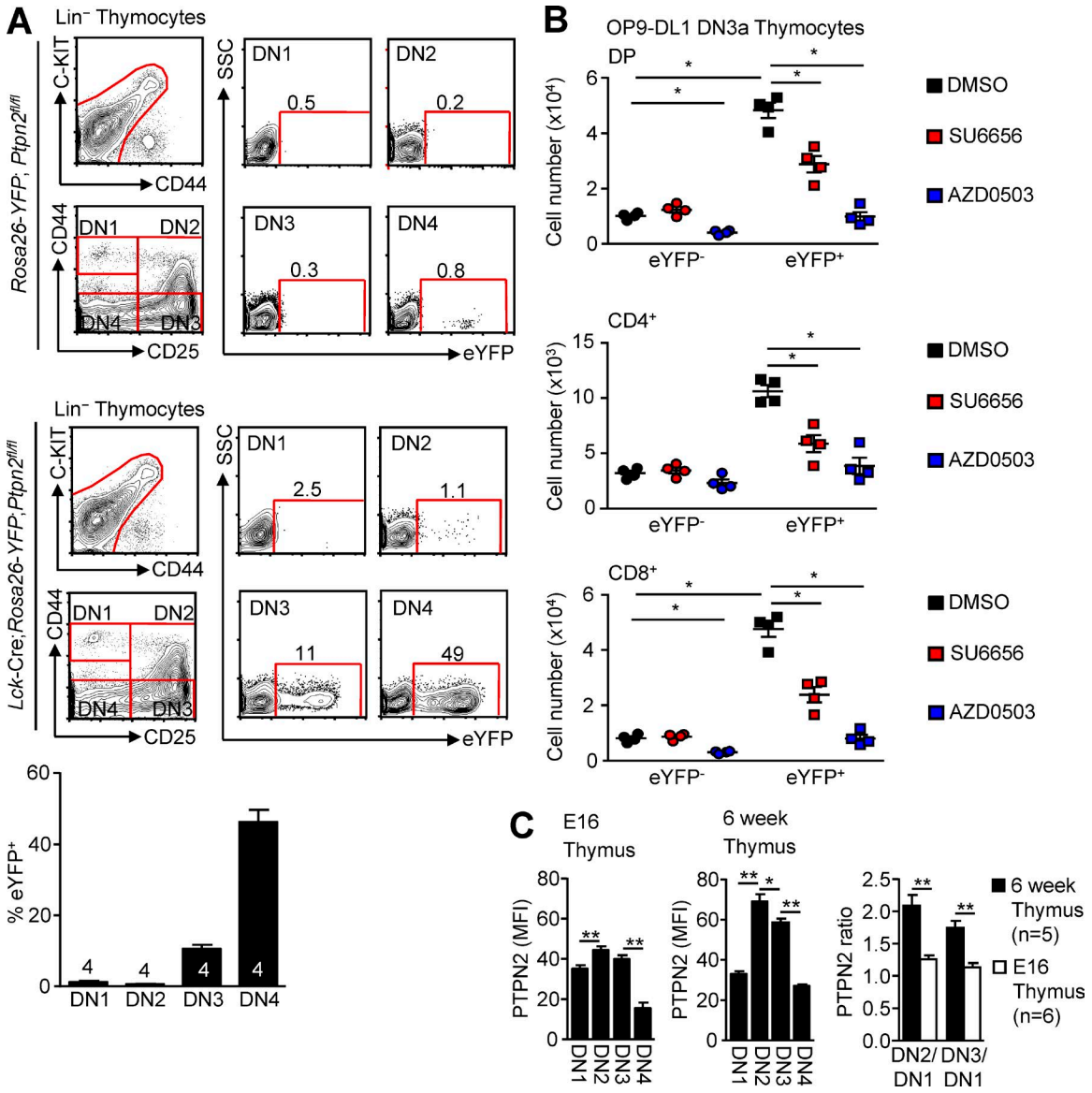

Figure S5. **SFK signaling in DN thymocytes from *Lck*-Cre;*Rosa26-YFP;Ptpn2*$^{fl/fl}$ mice and PTPN2 levels in DN thymocytes.** (A) Lin$^-$ thymocytes from *Rosa26-YFP;Ptpn2*$^{fl/fl}$ (YFP$^-$) (C57BL/6) and *Lck*-Cre;*Rosa26-YFP;Ptpn2*$^{fl/fl}$ (YFP$^+$) (C57BL/6) mice were stained with fluorochrome-conjugated antibodies against CD25, CD44, and c-KIT. Lin$^-$ DN1 (c-KIT$^+$CD44$^+$CD25$^-$), Lin$^-$ DN2 (c-KIT$^+$CD44$^+$CD25$^+$), Lin$^-$DN3 (CD25$^+$CD44$^-$), and (CD25$^-$CD44$^-$) Lin$^-$DN4 thymocytes were gated for YFP$^-$ (i.e., PTPN2 expressing) and eYFP$^+$ (i.e., PTPN2 deleted) cells, and the relative YFP$^+$ cells in DN subsets were determined by flow cytometry. (B) DN3a *Rosa26-YFP;Ptpn2*$^{fl/fl}$ (C57BL/6) eYFP$^-$ (PTPN2 expressing) and DN3a *Lck*-Cre;*Rosa26-YFP;Ptpn2*$^{fl/fl}$ (C57BL/6) eYFP$^+$ (PTPN2 deleted) thymocytes were cultured on OP9-DL1 cells for 3 d in the presence of DMSO vehicle control or the SFK PTK inhibitors SU6656 or AZD0503. Thymocytes were harvested and stained with fluorochrome-conjugated antibodies against CD4 and CD8. DP, CD4$^+$ SP, and CD8$^+$ SP cell numbers were determined by flow cytometry. (C) Lin$^-$ thymocytes from E16 and 6-wk-old C57BL/6 mice were stained for CD25, CD44, and intracellular PTPN2. Relative PTPN2 levels (MFI) adjusted to cell size (PTPN2 MFI/FSC) and PTPN2 DN2/DN1 and DN3/DN1 ratios in E16 versus 6-wk-old C57BL/6 mice were determined by flow cytometry. Representative results (means ± SEM; *Rosa26-YFP;Ptpn2*$^{fl/fl}$, $n = 4$; *Lck*-Cre;*Rosa26-YFP;Ptpn2*$^{fl/fl}$, $n = 4$; and C57BL/6, $n = 5$–6) from at least two independent experiments are shown. Significance in B and C was determined using a two-tailed Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$.