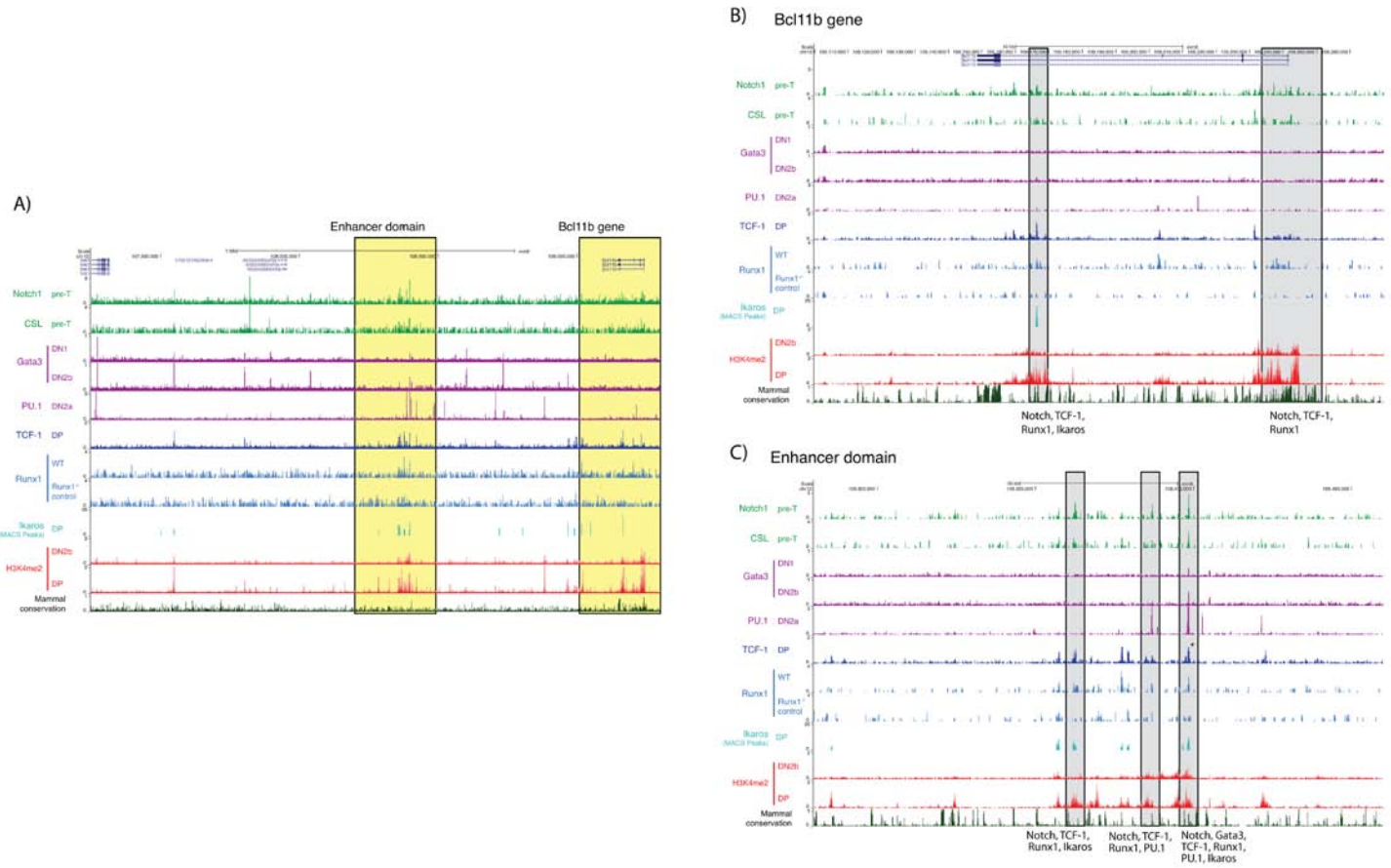
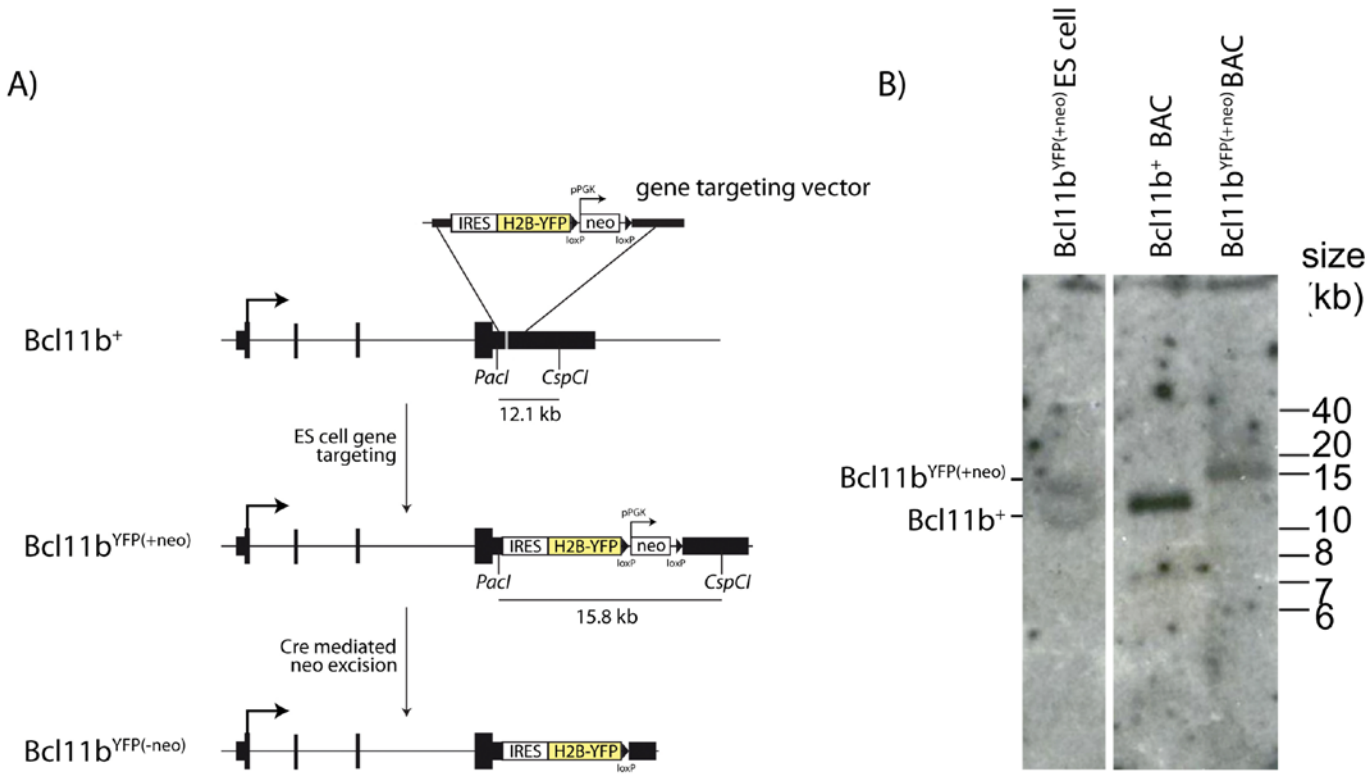


Supplementary Figure 1

Binding profiles of key transcription factors to the *Bcl11b* gene and enhancer.

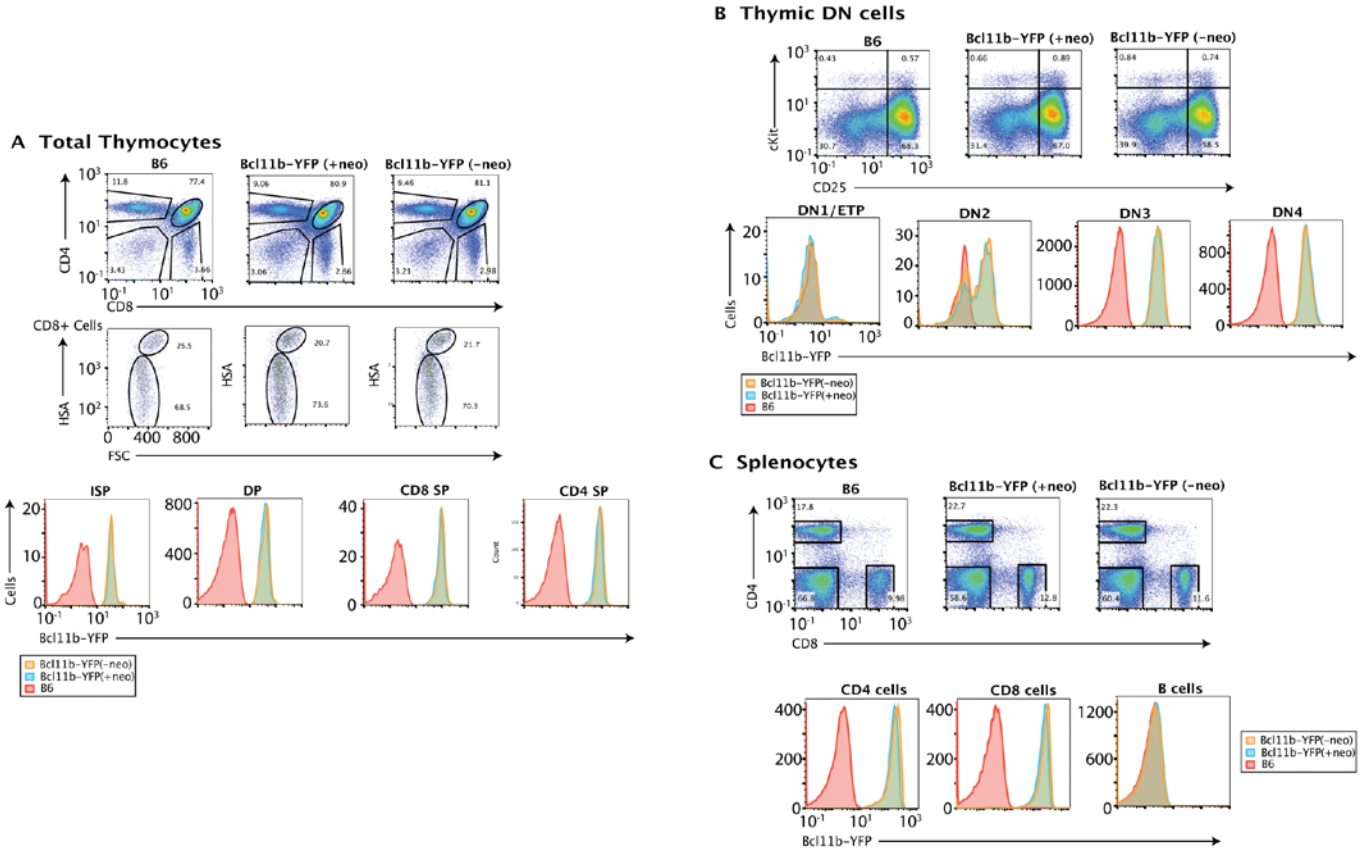
A)-C) UCSC genome browser tracks (mm9 coordinates) showing published ChIP-Seq binding data of the following transcription factors to the *Bcl11b* gene: Notch1[1], CSL[1], Gata3[2], PU.1[2], TCF-1[3], Runx1[4] (both WT and Runx1-/- control) and Ikaros[5]. The H3K4me2 histone mark is also included[2]. The primary cell types on which ChIP-Seq was performed are also indicated. A) Global view of Bcl11b locus, showing gene body in relation to distal enhancer and downstream gene desert. Shaded yellow bars indicate gene and enhancer locations. B) Bcl11b gene body, and C) *Bcl11b* enhancer domain. Gray shaded bars indicate regions containing clusters of transcription factor binding sites and H3K4me marks. Asterisk indicates binding sites tested in enhancer transfection assays[6]. Note that a high resolution version of this figure is also provided separately.



Supplementary Figure 2

Generation and validation of Bcl11b-YFP knock-in reporter.

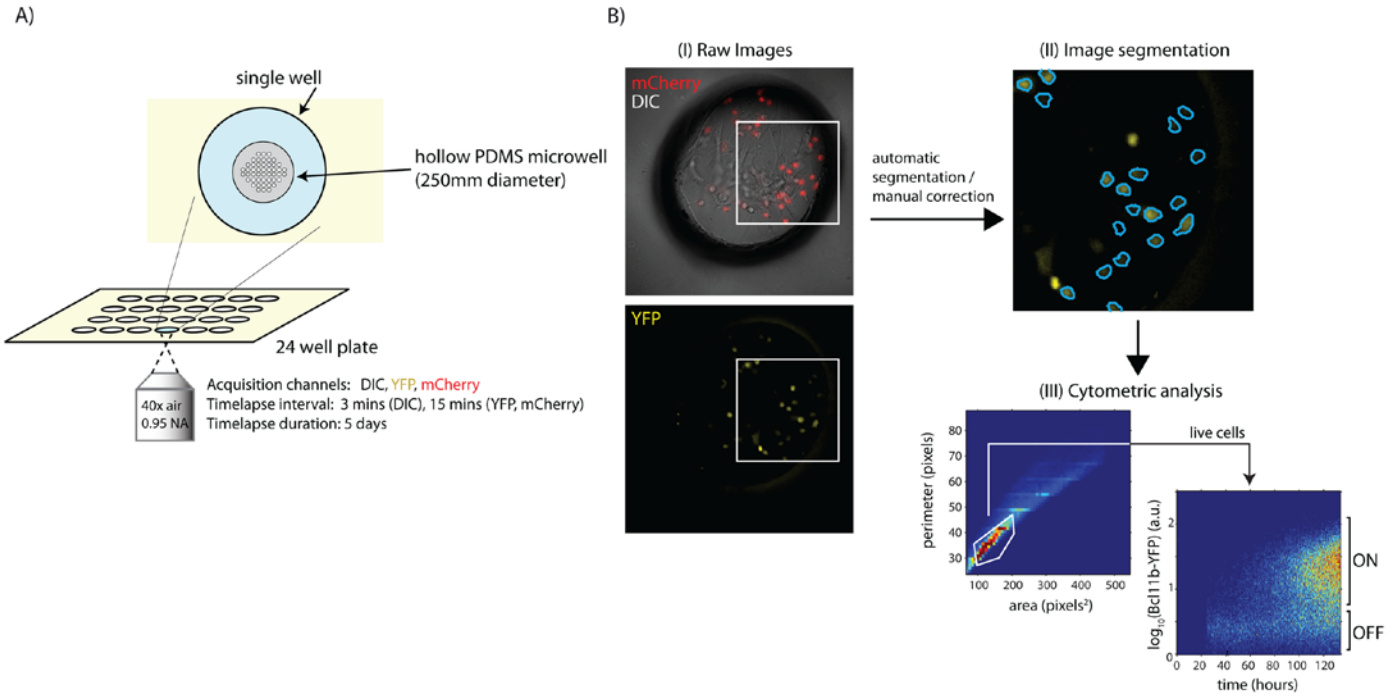
A) Schematic showing targeted insertion of fluorescent protein reporter into 3'-untranslated region (UTR) of Bcl11b locus (top), along with excision of the loxP-flanked PGK-neomycin cassette using Cre recombinase. Cut sites for restriction enzymes *PacI*, *CspCI*, used for Southern Blot validation of targeted insertion in B) are shown. Diagram is not drawn to scale. B) Correct insertion of fluorescent protein cassette was validated by Southern Blot analysis on the targeted ES cell clone (left), which was later used for knock-in mouse generation. Bacterial Artificial Chromosomes (BAC) containing either the wildtype *Bcl11b* locus (center), or a *Bcl11b*^{YFP} (+neo) locus (right) were used as controls.



Supplementary Figure 3

Bcl11b-YFP levels are not affected by presence of the neomycin drug resistance cassette.

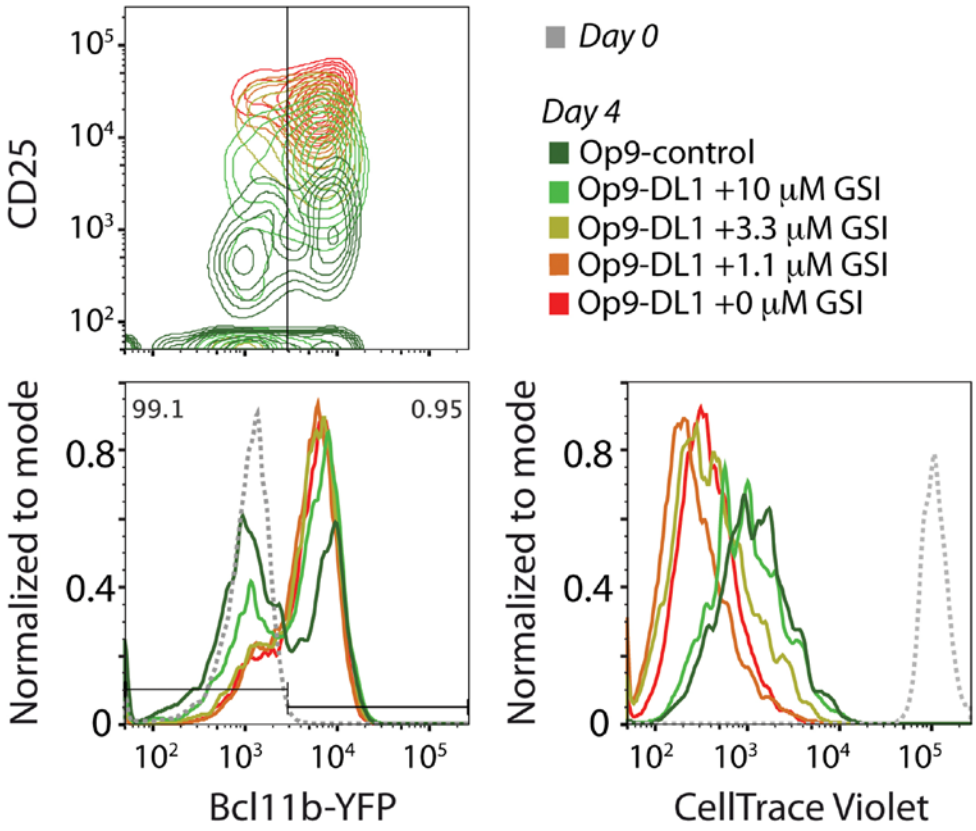
Flow cytometry analysis of *Bcl11b*$^{YFP/+}$ mice either with or without the neomycin drug selection cassette [Bcl11b-YFP(+neo), Bcl11b-YFP(-neo)], together with non-fluorescent controls (B6), using cells from the whole thymus (A), DN thymus (B), or the spleen (C). Bcl11b-YFP reporter expression is identical with or without the drug resistance cassette. ISP – immature single positive, DP – double positive.



Supplementary Figure 4

Experimental and analysis workflow for time-lapse imaging of Bcl11b-YFP levels in progenitor cells.

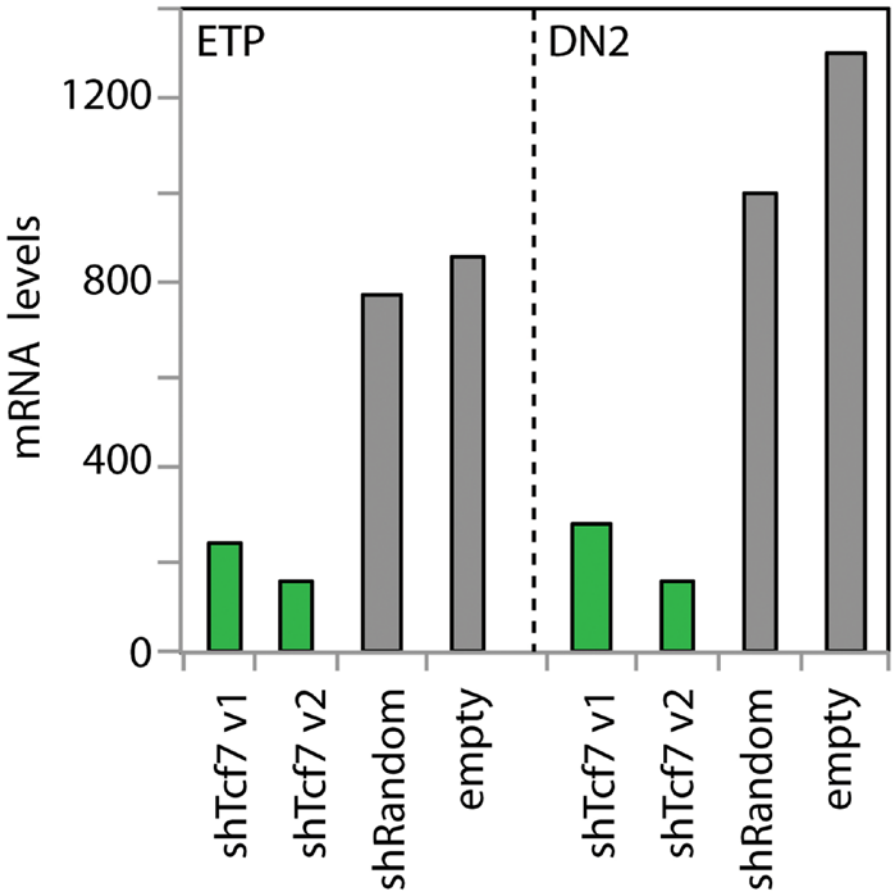
A) Experimental setup, microscope configuration and illumination settings used for timelapse image acquisition. To confine cells to a single imaging field-of-view, progenitors were seeded into hollow PDMS microwells (Microsurfaces; Flemington, Australia) adhered to a glass-bottomed 24 well dish (Mattek; Ashland, MA). B) Workflow for image analysis showing (I) raw differential interference contrast (DIC), mCherry and YFP images; (II) representative images overlaid with automatically segmented cell boundaries; and (III) heat maps showing cytometric analysis of segmented cells, showing OFF and ON populations.



Supplementary Figure 5

Notch signaling promotes all-or-none *Bcl11b* activation in a dose-dependent manner.

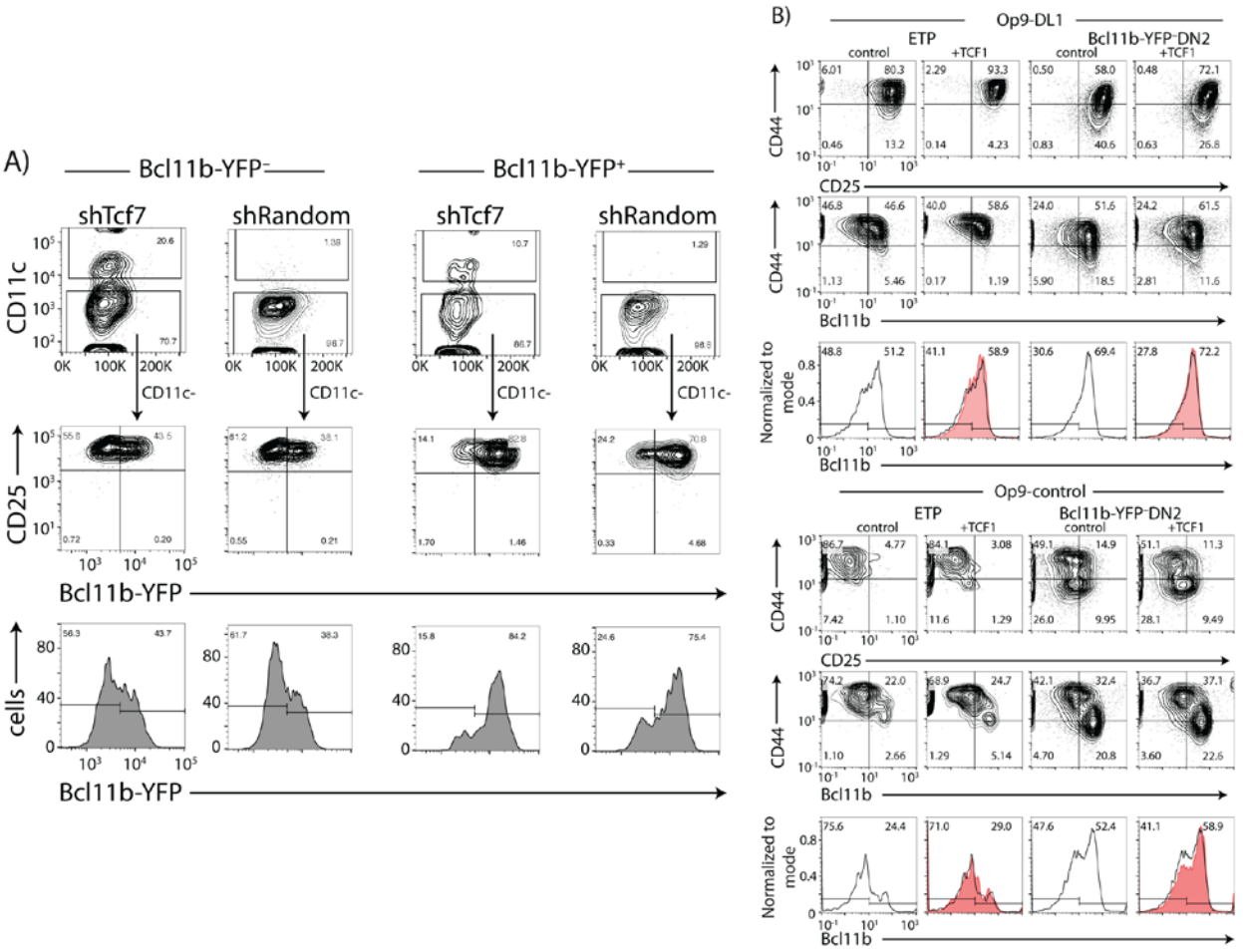
Flow cytometry analysis of bone marrow derived Bcl11b-YFP- DN2 cells cultured under the indicated conditions with 5 ng/mL IL-7 and Flt3L for four days. CD25 versus Bcl11b-YFP levels (top), and histograms of Bcl11b-YFP and CellTrace Violet levels (bottom) are shown. Initial levels (Day 0) are shown as gray dotted lines. GSI: γ-secretase inhibitor (Calbiochem/EMD Millipore, Billerica, MA), a small molecule inhibitor of Notch signaling.



Supplementary Figure 6

shTcf7 constructs reduce *Tcf7* transcript levels in ETP and DN2 cells.

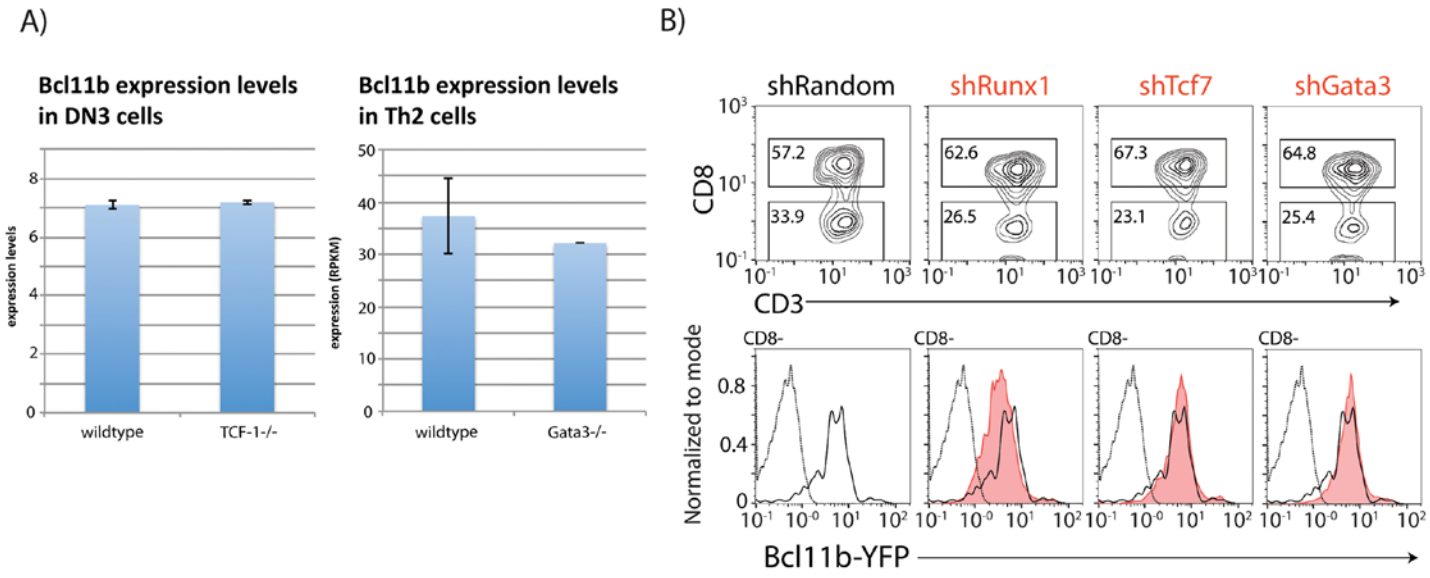
Quantitative real time (RT)-PCR analysis of *Tcf7* transcript levels in DN progenitors from E14.5 fetal livers infected with the indicated shRNA constructs for 2 days and sorted. RNA was extracted and processed as previously described[7]. Forward and reverse primers used for *Tcf7* detection are CAAGGCAGAGAAGGAGGCTAAG and GGCAGCGCTCTCCTTGAG respectively, as previously described[8].



Supplementary Figure 7

Effects of TCF-1 perturbation on *Bcl11b* activation in pro-T cells.

A) Flow cytometry analysis of bone marrow DN progenitors transduced with either shRNA to *Tcf7* (shTcf7) or a non-targeting control (shRandom), then cultured on plates coated with 6 µg/mL DL1 with 5 ng/mL SCF, IL-7 and Flt3L for four days. Levels of the dendritic cell marker CD11c against FSC (top), as well as CD25 levels versus Bcl11b-YFP levels (middle) or Bcl11b-YFP level distributions (bottom) for CD11c$^-$ populations are shown. Knockdown of *Tcf7* promotes DC trans-differentiation in DN2 cells, but does not impede Bcl11b activation. B) Flow cytometry analysis of Bcl11b-YFP expression levels in bone marrow DN progenitors transduced with a control or VEX-GFP retroviral construct for over-expression of TCF-1 (encoded by *Tcf7*), and cultured on OP9- or OP9-DL1 monolayers in 5 ng/mL IL-7 and Flt3L for three days. TCF-1 over-expression does not affect Bcl11b activation from either ETP or DN2 progenitors.



Supplementary Figure 8

Runx1, but not TCF-1 or GATA-3, controls *Bcl11b* expression amplitude in mature T cells.

A) Published transcriptomic data on *Bcl11b* expression in wildtype or *Tcf7*$^{-/-}$ DN3 thymocytes[9] (left), or in wildtype or *Gata3*$^{-/-}$ Th2 cells[10] (right). Levels of *Bcl11b* expression are similar in the absence or presence of either factor. B) Flow cytometry analysis of CD4 and CD8 splenic T-cells activated with anti-TCRβ and anti-CD28 for 1 day, transduced with shRNA constructs, and cultured for 3 days. Plots show CD8 versus CD3 for cells transduced with retrovirus (top), along with Bcl11b level distributions for gated non-CD8 T-cell populations (bottom). Corresponding data for CD8 T-cells are shown in Fig. 8. Solid black lines indicate cells transduced with random shRNA, and dotted lines indicate background levels from non-fluorescent T-cells. Data from (A) represent mean and S.D. of two independent experiments.

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SUPPLEMENTARY FIGURE LEGENDS, SUPPLEMENTARY TABLE LEGENDS, SUPPLEMENTARY TABLES 3 and 4, AND ALL REFERENCES FOR SUPPLEMENTARY FIGURES AND TABLES

Kueh, H. Y., Yui, M. A., Ng, K. K. H., Pease, S. S., Zhang, J. A., Damle, S. D., Freedman, G., Siu, S., Bernstein, I. D., Elowitz, M. B., and Rothenberg, E. V.

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Nature Immunology: doi:10.1038/ni.3514

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SUPPLEMENTARY TABLES:

Supplementary Table 1: Transcript levels for genes differentially expressed in Bcl11b-YFP$^-$ versus Bcl11b-YFP$^+$ cells. To compile this list, differential gene expression between two independent Bcl11b-YFP- and Bcl11b-YFP$^+$ samples was evaluated, and a cutoff value of $p < 0.0005$ was used. FPKM values for different populations are shown, and *p*-values for differential expression across the indicated pairs of conditions are shown. *p*-values were generated using the statistical model in the Cufflinks analysis software[1]. Genes with a mean read count < 5 were not included in this analysis. (separate Excel file)

Supplementary Table 2: Transcript levels for transcriptional regulators expressed in developing T-cells. FPKM values for different populations are shown, and *p*-values for differential expression across the indicated pairs of conditions are shown. *p*-values were generated using the statistical model in the Cufflinks analysis software[1]. Genes with a mean read count < 5 were not included in this analysis. A) Transcript levels for selected T-cell regulatory genes from RNA-Seq analysis. B) Transcript levels for all expressed genes classified as transcription regulators from gene ontology (GO:0006355) from RNA-Seq analysis. Interestingly, although the key transcription factor gene *Lef1* is turned on shortly after *Bcl11b*, it is not significantly upregulated yet in the stages analyzed here. (separate Excel file)

Supplementary Table 3: List of vectors used for this study. Underlined sequences indicate shRNA loop sequences. References to supplementary figures are designated as Fig. S(number).

Supplementary Table 4: Antibodies used for this study. All reagents were obtained from eBioscience (San Diego, USA), unless otherwise indicated. References to supplementary figures are designated as Fig. S(number).

Supplementary Tables 1 and 2: See separate Excel files.

Supplementary Tables 3 and 4 are below

SUPPLEMENTARY REFERENCES FOLLOW SUPPLEMENTARY TABLE 4

SUPPLEMENTARY TABLE 3: List of vectors used for this study

Vector name	Notes / sequence	Usage
Bcl11b-3'UTR-YFP-BAC	Modified BAC containing YFP reporter inserted into Bcl11b 3'UTR	Generation of Bcl11b 3'UTR targeting vector
Bcl11b-YFP 3'UTR targeting vector	Gene targeting vector	ES cell gene targeting
Banshee shRandom-mCherry	CCTAGGGTTAGGTCGCCCTC GTTCAAGAGACGAGGGCGAC TTAACCTTAGG	Control for shRNA knockdown (Fig. 7, 8)
Banshee shTcf7-v1 mCherry	GCCACGAGTCTGAACAATAAT TTCAAGAGAATTATTGTTTAG ACTTGTGGC	*Tcf7* knockdown in T-cell progenitors (Fig. 7)
Banshee shTcf7-v2 mCherry	CAATGTGTTCATGCTTTGCAT TTCAAGAGAATGTAAAGCATG AACGCATTG	*Tcf7* knockdown in T-cell progenitors (Fig. 7)
Banshee shGata3 mCherry	GAAGTTCAGTGTCCGCTGACGTTCGT AGCCGTCAGCGGATACTGAGCTTC, from[2]	*Gata3* knockdown in T-cell progenitors (Fig. 7)
Banshee shRunx1 mCherry	GTCTTTGCAAATTCACCACAATTCAA GAGATTGTGGCGGATTTGTAAAGAC	*Runx1* knockdown in T-cell progenitors (Fig. 8)
MSCV Runx1-IRES-mCherry		*Runx1* over-expression in T-cell progenitors (Fig. 8)
MSCV RunxDN-IRES-mCherry		*Runx* protein inhibition in T-cell progenitors (Fig. 8)
MSCV IRES-mCherry (empty vector)	From[3]	Control for protein over-expression experiments (Fig. 8)
MSCV H2B-mCherry-IRES-ngfr		Nuclear-localized fluorescence signal for automated cell tracking (Fig. 5)
MSCV VEX	From[4]	Control for TCF-1 over-expression experiments (Fig. S7)
MSCV TCF-1 VEX	From[4]	TCF-1 over-expression experiments (Fig. S7)

Supplementary Table 3: Underlined sequences indicate shRNA loop sequences.

Nature Immunology: doi:10.1038/ni.3514

SUPPLEMENTARY TABLE 4: Antibodies used for this study

Antibody epitope/conjugate	Clone	Dilution/ Concentration	Usage
CD44 eFluor-450	1M7	1:300	DN thymocyte analysis (Figs. 1, S3) Cultured DN progenitors (Figs. 1, 7, S7) Sorting of DN progenitors (Figs. 2-7)
CD117 (cKit) PE	2B8	1:150	DN thymocyte analysis (Figs. 1, S3) Sorting of DN progenitors (Figs. 2-7) Cultured DN progenitors (Fig. 3)
CD25 APC-eFluor780	PC61.5	1:300	DN thymocyte analysis (Figs. 1, S3) CD4+CD25+ T-reg analysis (Fig. 1) Cultured DN progenitors (Fig. 1, 3, 4, 8, S5, S7) Sorting of DN progenitors (Figs. 2-7)
CD4 PE	GK1.5	1:300	CD4 SP thymocyte analysis (Figs. 1, S3)
CD8α APC	53-6.7	1:300	CD8 SP thymocyte analysis (Figs. 1, S3)
CD24 (HSA) eFluor-450	M1/69	1:400	Analysis of ISP thymocytes (Figs. 1, S3)
CD19 eFluor-450	1D3	1:300	Analysis of B-cells (Figs. 1, S3)
TCR $\gamma\delta$ APC	GL3	1:300	$\gamma\delta$T-cell analysis (Fig. 1)
NK1.1 PE	PK136	1:300	NK, NKT cell analysis (Fig. 1)
TCRβ eFluor-450	H57-197	1:300	NKT cell analysis (Fig. 1)
CD45 APC	30-F11	1:300	Cultured DN progenitors (Fig. 1, 3) Sorting of DN progenitors (Figs. 2-7)
CD11c APC-e780	N418	1:300	Alternate lineage potential assays (Fig. 2, limiting dilution assays)
CD11c e450	N418	1:300	Alternate lineage potential assays (Fig 2, 300 cell assay; Fig. S7)
DX5 (CD49B) APC	DX5	1:300	Alternate lineage potential assays (Fig. 2, 300 cell assay)
NK1.1 APC	PK136	1:300	Alternate lineage potential assays (Fig. 2)
CD45 eFluor-450	30-F11	1:300	Alternate lineage potential assays (Fig. 2)
CD11c eFluor-450	N418	1:300	Cultured DN progenitors (Fig. 6)
CD25 Brilliant Violet-510 (Biolegend)	PC61	1:300	Cultured DN progenitors (Fig. 7)
CellTrace Violet (Invitrogen)		5 μM	Cultured DN progenitors (Fig. 4, Fig. S5)
CD8a biotin	53-6.7	1:150	Thymocyte depletion (Fig. 1, S3)

			Splenic T-cell enrichment (Fig. 8, S8)
TCRγδ biotin	GL3	1:100	Thymocyte depletion (Fig. 1, S3)
TCRβ biotin	H57-597	1:150	Thymocyte depletion (Fig. 1, S3)
Gr-1 Ly6D biotin	R86.8C5	1:100	Thymocyte depletion (Fig. 1, S3)
TER-119 biotin	Ter119	1:100 1:100	Thymocyte depletion (Fig. 1, S3) Bone marrow depletion (Figs. 2-7, S5, S7)
CD122 biotin	5H4	1:100	Thymocyte depletion (Fig. 1, S3)
NK1.1 biotin	PK136	1:100	Thymocyte depletion (Fig. 1, S3) Bone marrow depletion (Figs. 2-7, S5, S7)
CD11c biotin	N418	1:100	Thymocyte depletion (Fig. 1, S2) Bone marrow depletion (Figs. 2-7, S5, S7)
CD11b biotin	M1/70	1:100	Bone Marrow depletion (Figs. 2-7, S5, S7)
CD19 biotin (Biolegend)	6D5	1:100	Bone Marrow depletion (Figs. 2-7, S5, S7)
CD3 biotin (Biolegend)	145-2C11	1:100	Bone Marrow depletion (Figs. 2-7, S5, S7)
Streptavidin PerCP-Cy5.5		1:100	Sorting of DN progenitors (Figs. 2-7)
CD4 biotin	GK1.5	1:100	Splenic T-cell enrichment (Fig. 8, S8)
TCRβ (Biolegend)	H57-597	1 μg/mL coating	CD8 T-cell activation (Figs. 8, S8)
CD28 (Biolegend)	37.51	1 μg/mL	CD8 T-cell activation (Figs. 8, S8)
CD8a e450	53-6.7	1:300	CD8 T-cell activation (Figs. 8, S8)
CD3 APC-e780	17A2	1:300	CD8 T-cell activation (Figs. 8, S8)

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14. Weber, B.N. *et al.* A critical role for TCF-1 in T-lineage specification and differentiation. *Nature* **476**, 63-8 (2011).