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Supplemental Information

Transcriptional Repression of *Gata3*

Is Essential for Early B Cell Commitment

Anupam Banerjee, Daniel Northrup, Hanane Boukarabila, Sten Erik W. Jacobsen, and David Allman

Inventory of supplemental information

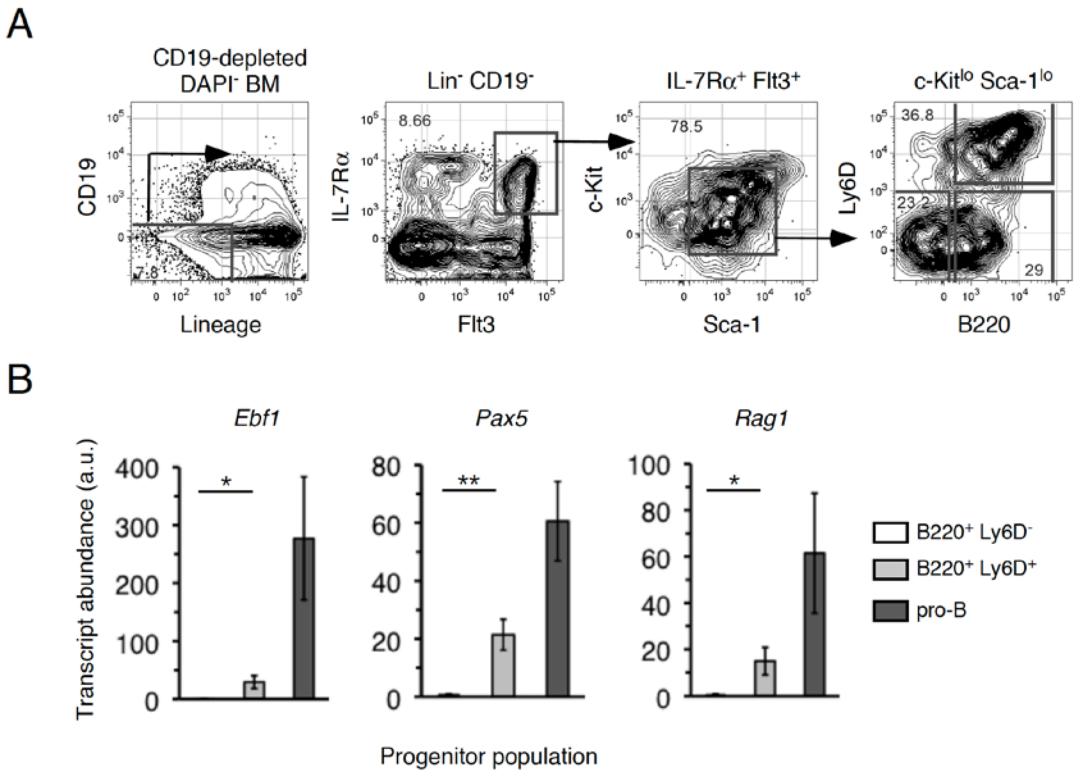

Figure S1. Pre-pro-B cells are subdivided by Ly6D expression. (A) BM cells from a single 12-week-old B6 female were stained with the indicated antibodies then analyzed by collecting 2 x 10^6 events on an LSR2 flow cytometer. The "lineage" cocktail contained antibodies to CD11b, Gr-1, F4/80, Ly6C, CD11c, Ter-119, CD3ε and NK1.1. (**B**) cDNA was prepared from the indicated progenitor populations and subjected to qRT-PCR with the indicated Taqman primer-probe sets. Error bars indicate SEMs, * $p< 0.05$, ** $p< 0.005$. Related to Figure 1.

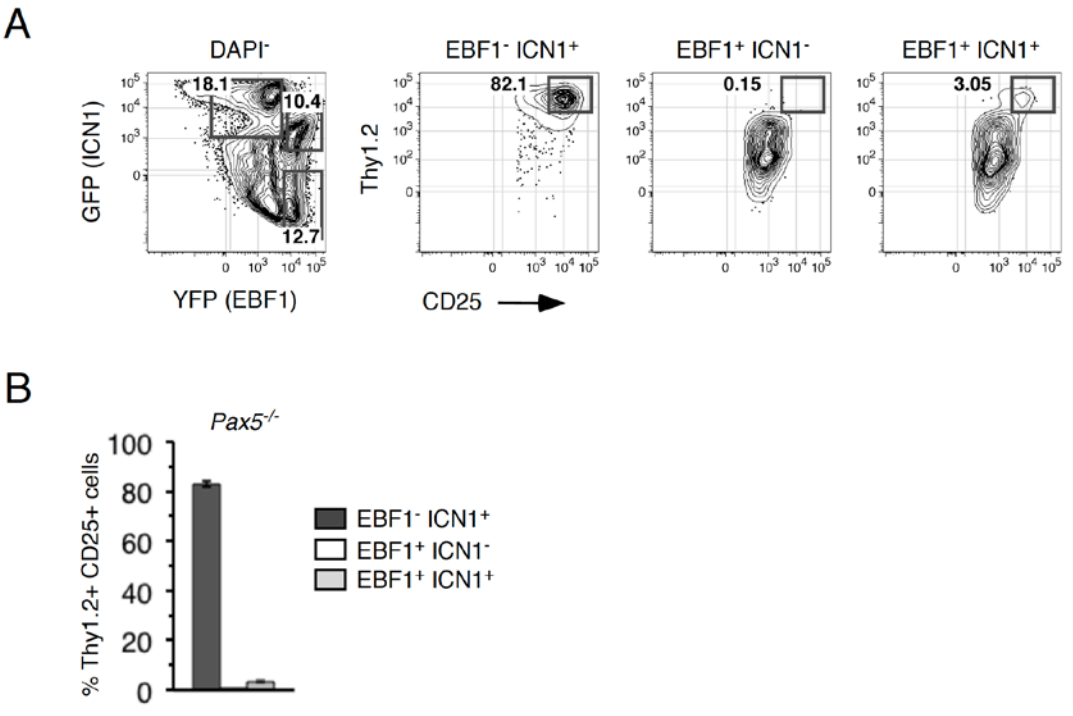

Figure S2. Pax5 is dispensable for the T-inhibitory activity of EBF1. (A) e14.5 *Pax5*$^{-/-}$ fetal liver LSKs were isolated and co-transduced with MigY-EBF1 and MigR-ICN1 viruses. 24 hours later, cells were washed and re-plated on OP9 stromal cells, and then assayed for pro-T cells on day 7 by flow cytometry as shown in (A). (**B**) Summary of data from triplicate wells from each group. Data were gated on DAPI^{-} CD45^{+} cells that were GFP^{+} YFP^{-}, GFP^{-} YFP^{+} or double-transduced (GFP^{+} YFP^{+}). Graph shows means +/- SEMs. Data are representative of 2 separate experiments. Related to Figure 2.

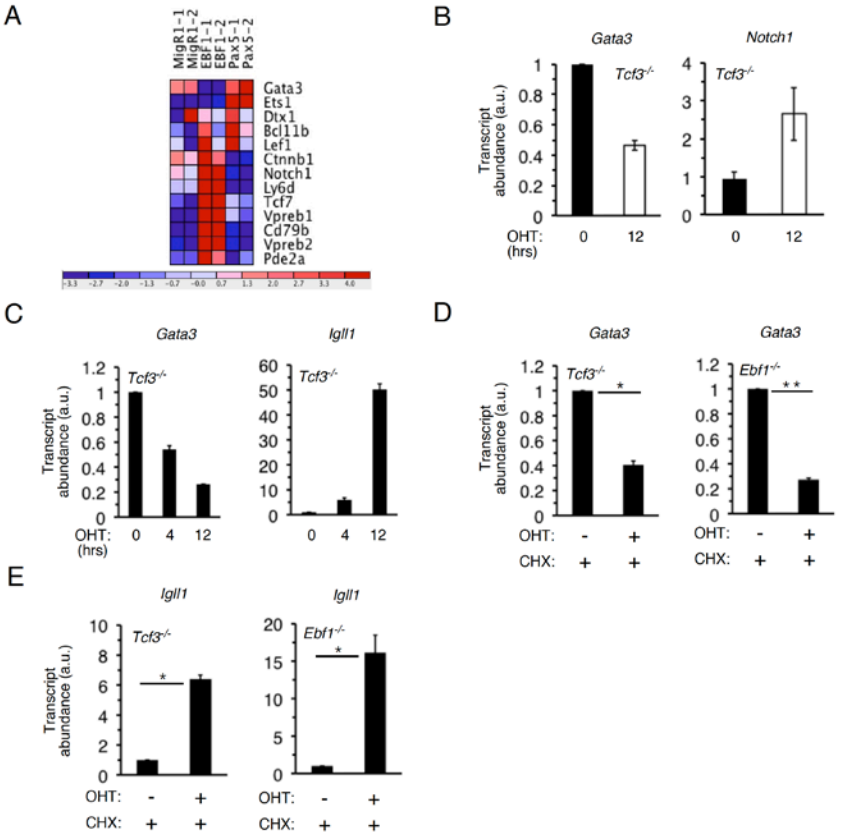

Figure S3. Selective and cycloheximide-insensitive down regulation of *Gata3* by EBF1. (A) *Ebf1*$^{-/-}$ pre-pro-B cells were transduced with either control MigR1, MigY-EBF1 or MigR-Pax5 viruses. 24 hours later, RNA was isolated and processed for Affymetrix microarray analysis. Scale represents $\log_2$ value of normalized signal level. (**B**) *Tcf3*$^{-/-}$ progenitors were transduced with pCS-E47:ER virus and GFP$^+$ cells were sorted after 24 hours on OP9 stromal cells supplemented with IL-7, FL, and SCF and 4-OHT (1μM). 12 hours later GFP$^+$ cells were sorted and cDNA prepared for qRT-PCR for *Gata3* or *Notch1*. (**C**) *Tcf3*$^{-/-}$ pre-pro-B cells were transduced with MigR-EBF1:ER virus and plated on OP9 stromal cells supplemented with IL7, FL and SCF. 50,000 DAPI$^-$ CD45$^+$ GFP$^+$ cells were sorted a day later and re-plated on OP9 cells with cytokines and 4-OHT (1μM). Cells were cultured for 0h, 4h and 12h. At these time points, cells were collected for cDNA synthesis and qRT-PCR analyses for relative *Gata3* and *Igll1* (λ5) expression, with expression levels at time zero arbitrarily set to 1. **(D, E)** *Tcf3*$^{-/-}$ or *Ebf1*$^{-/-}$ pre-pro-B cells were transduced with MigR-EBF1:ER virus and plated on OP9 stromal cells supplemented with IL7, FL and SCF. 24h later, 50,000 DAPI$^-$ CD45$^+$ GFP$^+$ cells were sorted and re-plated on OP9 cells with cytokines and cycloheximide (CHX) (4μM). 8h later, medium with or without 4-OHT (1μM) was added. Cells were harvested 4h later and assayed for *Gata3* or *Igll1* transcripts, with expression level in CHX-treated sample set to 1. Data are means +/- SEMs of triplicate samples, * $p< 0.001$, ** $p< 0.0001$. Data are representative of 2 separate experiments. Related to Figures 3 and 4.

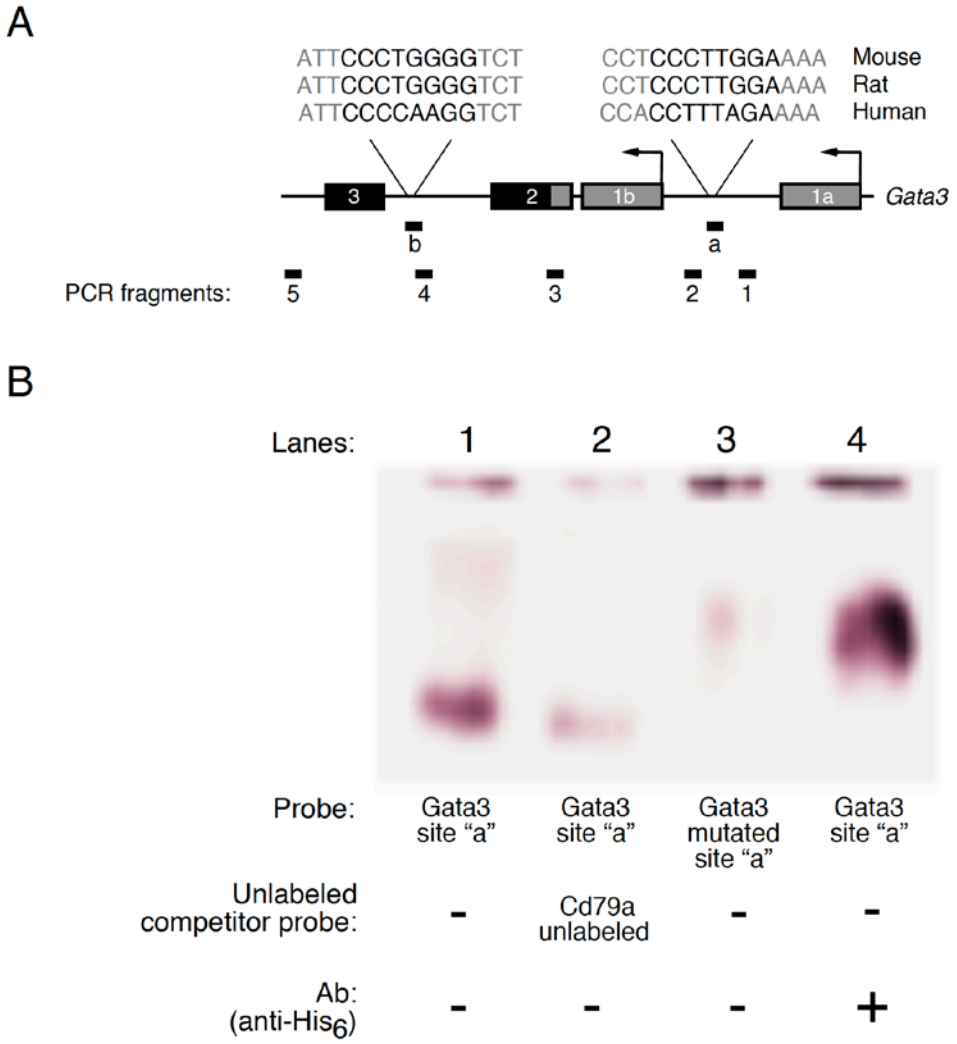

Figure S4. EBF1 binding at the *Gata3* locus. (A) Schematic showing a portion of the *Gata3* locus with conserved putative EBF1 binding sites, as predicted by ECRbrowser (http://ecrbrowser.dcode.org/). Rectangles indicate primer pairs designed to amplify DNA after ChIP with either anti-EBF1 antibodies (designated as "a" and "b") or H3-K27Me^3 antibodies (numbered 1-5). **(B)** 293T cells were transfected with an expression construct encoding a His_6-tagged EBF1 protein. Nuclear extracts were prepared from transfected cells and probed with 24 base-pair 5'-biotinylated ds-oligo encompassing the EBF1 binding "site a" in the *Gata3* locus. EMSA was performed using 4% native-PAGE gels. Relative affinity of EBF1 to "site a" vis-à-vis the canonical EBF1 binding site in *Cd79a* locus was demonstrated when the DNA-protein complex was abrogated with 200-fold molar excess of the latter unlabeled competitor oligo (lanes 1 and 2). When incubated with a mutated "site a" oligo, EBF1 failed to form a stable complex (lane 3). The unique specificity of EBF1 binding at "site a" reaction was further demonstrated upon retardation of this complex with an anti-His_6 antibody. Related to Figure 4.

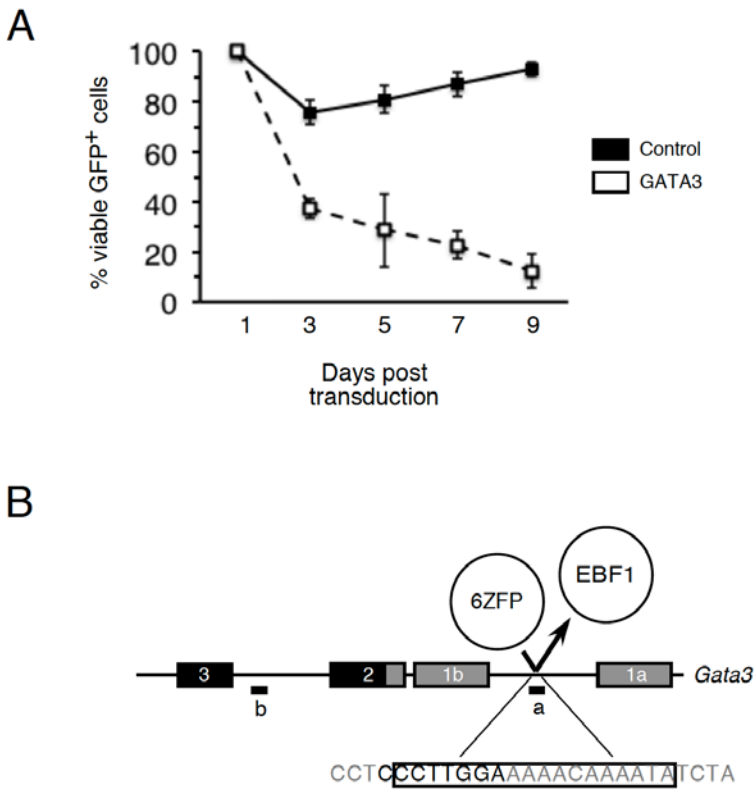

Figure S5. Strategy for increasing GATA3 expression in lymphoid progenitors while avoiding GATA3-induced cell death. (**A**) Ectopic GATA3 expression induces cell death in lymphoid progenitors. Wild type Flt3$^+$ LSKs and CLPs were sorted from adult BM, transduced with control or GATA3 expressing virus, and then plated in triplicate on OP9 stromal cells supplemented with IL-7, FL, and SCF. Viable GFP$^+$ cells were determined by flow cytometry using exclusion of the DNA dye DAPI at the indicated time points. Graph displays means and error bars indicate SEMs. Data are representative of 4 separate experiments. (**B**) Strategy for perturbing EBF1 binding to "site a" in the *Gata3* locus. The schematic shows the proposed activity of 6ZFP, with its 18 base pair target sequence (in box) encompassing the EBF1 binding "site a" (in bold) and the indicated border sequence. Related to Figure 5.

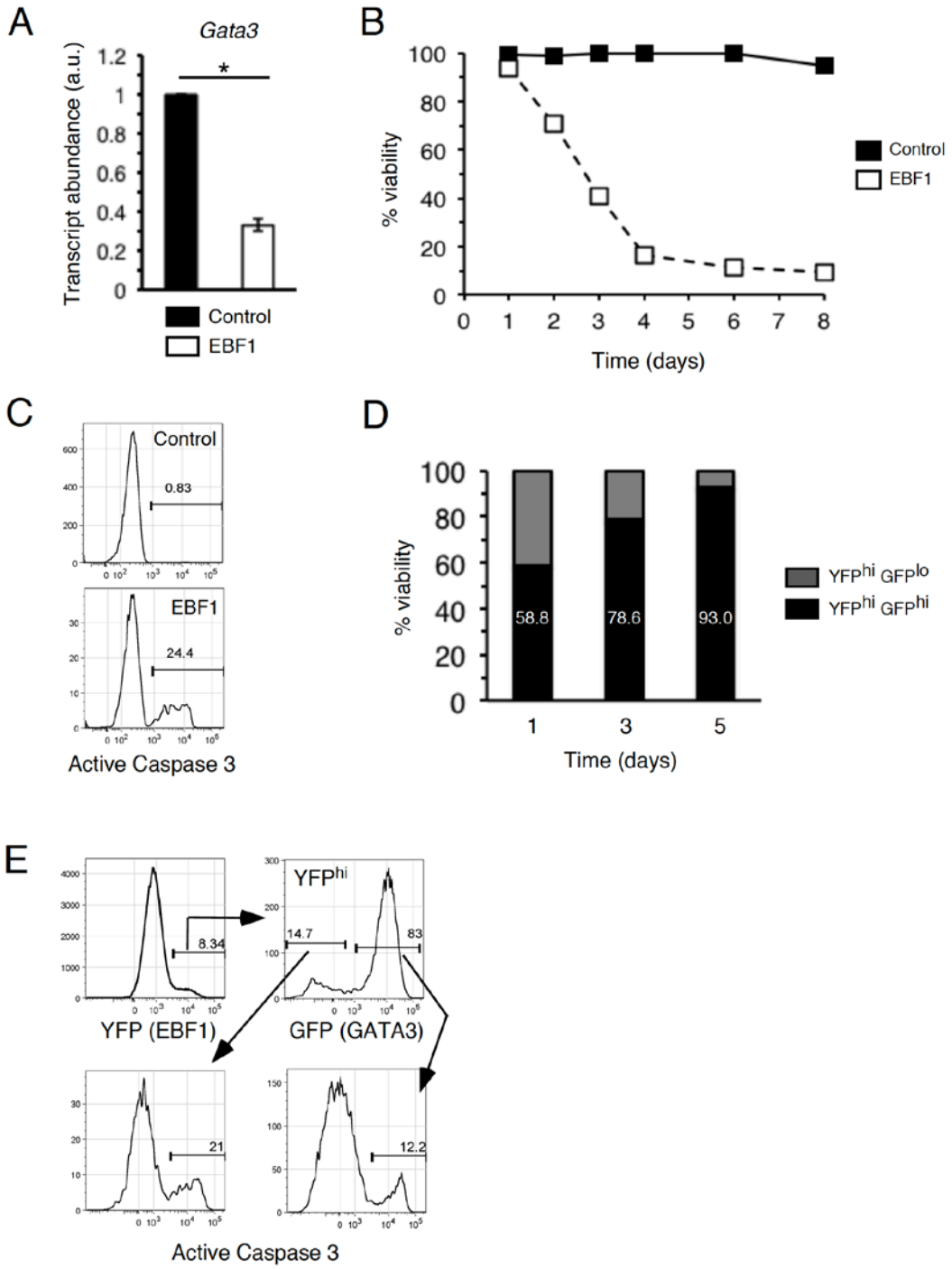

Figure S6. EBF1 perturbs GATA3-dependent thymoma survival. (A) *Tcf3*$^{-/-}$ 1.F9 lymphoma cells were transduced with control MigR1 or MigR-EBF1 cells. 24h later, DAPI^{-} GFP^{+} cells were sorted for RNA and analyzed for *Gata3* transcripts using qRT-PCR. Error bars indicate SEMs, * p< 0.001. **(B)** From each of the above-mentioned transduction groups, 50,000 DAPI^{-} GFP^{+} cells were seeded into 96-well tissue culture plates. Cell survival kinetic curve for each group was revealed by analyzing frequency of DAPI^{-} GFP^{+} cells in each well at days 2, 3, 4, 6 and 8 relative to day 1. Relative percent viable GFP^{+} at each time point were determined by dividing the percent DAPI^{-} GFP^{+} cells on each day by the percent DAPI^{-} GFP^{+} on day 1 (× 100). Data are means +/- SEMs of triplicate samples. **(C)** 48h post-transduction, cells were assayed for apoptosis with an anti-active caspase-3 antibody. **(D)** 1.F9 cells were co-transduced with MiY-EBF1 and MigR-GATA3; and plated into 96-well culture plates 24h

later. Cell survival kinetics for EBF^{hi} $GATA3^{lo}$ versus EBF^{hi} $GATA3^{hi}$ cells were determined by measuring relative frequencies of $DAPI^{-}$ YFP^{hi} GFP^{lo} and $DAPI^{-}$ YFP^{hi} GFP^{hi} cells in each well at days 1, 3 and 5. **(E)** Day 4 cultures were stained for apoptotic cells as in (C), and caspase-3 signal measured by gating on YFP^{hi} GFP^{lo} versus YFP^{hi} GFP^{hi} subpopulations. Data are representative of 2 separate experiments. Related to Figure 5.

Table S1. Oligonucleotides used for ChIP analyses

Primer pair	**Sequence (5' to 3')**
Site a	
Forward primer	CCTCCCTTGGAAAAACAAAATATC
Reverse primer	CCCTCTCTGCCAACCCTAAAC
Site b	
Forward primer	GACGAAAGGCCCAGTCCTAAT
Reverse primer	GTCAAGGGACCTTTCTCCATGT
Site 1	
Forward primer	GCATCCAGTCTAGCTCCTCTGGTG
Reverse primer	GCCAGTCCCTAGTTCACAGCTTCG
Site 2	
Forward primer	GCCTCTCTACTGGGCGTCTTCCAG
Reverse primer	TAGCGAGACCTAGGCTCACTGGTC
Site 3	
Forward primer	AGAGACTGAGAGAGCGAGACATAG
Reverse primer	GCAGGAGCCCAGGACTGACTAAGC
Site 4	
Forward primer	CTCCTTTTCTATACCCTTAACTGC
Reverse primer	AGTACGTCCACCTCTTCCGTCAGC
Site 5	
Forward primer	AGGAGTGGTGTGGTCCTGCTTCAC
Reverse primer	CTGTCCACACTCGCCAACTCTAAG

Shown are the primer sequences used in ChIP reactions to determine EBF1 occupancy and H3-K27Me^3 modifications at the *Gata3* locus. Sites a and b denote the putative EBF1 binding sites in the *Gata3* locus. Sites 1-5 denote the position of primer sets spanning the 5' end of the *Gata3* locus to probe H3-K27Me^3 marks. Related to Figure 4.

Table S2. 6ZFP nucleotide and amino acid sequence

ctggaaccgggcgaaaaaccgtataaatgcccggaatgcggcaaaagctttagccagaaaagcagcctgattgcgcatcagcgcacccataccg gcgaaaaaccgtataaatgcccggaatgcggcaaaagctttagccagcgcgcgaacctgcgcgcgcatcagcgcacccataccggcgaaaaac cgtataaatgcccggaatgcggcaaaagctttagcgatagcggcaacctgcgcgtgcatcagcgcacccataccggcgaaaaaccgtataaatgc ccggaatgcggcaaaagctttagccagcgcgcgaacctgcgcgcgcatcagcgcacccataccggcgaaaaaccgtataaatgcccggaatgcg gcaaaagctttagccgcagcgatcatctgaccacccatcagcgcacccataccggcgaaaaaccgtataaatgcccggaatgcggcaaaagcttta gcaccaaaaacagcctgaccgaacatcagcgcacccataccggcaaaaaaaccagc
LEPGEKPYKCPECGKSFS*QKSSLIA*HQRTHTGEKPYKCPECGKSFS*QRANLRA*HQRTHTGEKPY KCPECGKSFS*DSGNLRV*HQRTHTGEKPYKCPECGKSFS*QRANLRA*HQRTHTGEKPYKCPECGK SFS*RSDHLTT*HQRTHTGEKPYKCPECGKSFS*TKNSLTE*HQRTHTGKKTS

Shown are nucleotide (above) and predicted amino acid (below) sequences for the pMX-ZF insert encoding 6 Zn-finger polypeptide (6ZFP). The amino acid sequence of the individual Zn-finger modules interspersed periodically within the framework regions is depicted in italics. The framework region includes the N- and C-termini backbone, N- and C-termini cloning sequences and the canonical intervening linker sequence as designed with Zinc Finger Tools (http://www.zincfingertools.org). Related to Figure 5.

Supplemental Experimental Procedures:

Cell culture. OP9 and OP9-DL4 stromal cells were a kind gift of J.C. Zuniga-Pflucker (University of Toronto). *Ebf1*$^{-/-}$ pre-pro-B and *Pax5*$^{-/-}$ pro-B fetal liver progenitor lines were obtained from J. Hagman (National Jewish Medical Center) and H. Hu (Wistar Institute), respectively. *Tcf3*$^{-/-}$ progenitors were provided by C. Murre (UCSD). *Ebf1*$^{-/-}$ progenitors were propagated and maintained on OP9 stromal cells in Opti-MEM, 10% FCS, primatone, L-glutamine, and Pen/Strep supplemented with 10 ng/ml each of IL-7, flt3-ligand (FL) and stem cell factor (SCF). For *Tcf3*$^{-/-}$ progenitors, similar cell culture conditions were used, with the exception of IMDM-glutamax used as preferred culture medium. *Pax5*$^{-/-}$ progenitors were also cultured in serum-enriched IMDM-glutamax supplemented with 10 ng/ml IL-7 only. The *E2a*$^{-/-}$ thymoma line 1.F9 was cultured in RPMI, 10% FBS, Pen-strep. All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

T-lymphoid differentiation assays. Bulk and clonal assays measuring T cell differentiation were performed using OP9-DL4 stromal cells as described previously (Weber et al., 2011) and in our supplementnal information file. For bulk assays, OP9-DL4 stromal cells were plated ($20x10^3$ total stromal cells/ml) 2 days prior to initiation of co-culture. 200 sorted progenitors/well were added to pre-established stromal layers and cultures supplemented with 10 ng/ml IL-7, FL and SCF continued for 7 days. For single cell cultures, 96 well plates were first seeded with 3000 OP9-DL4 stromal cells 2 days before the culture began. On the day of the experiment stromal layers were irradiated 3 hours before seeding progenitors. Just before sorting the media was switched to IMDM, 10% FCS, primatone, L-glut, and Pen/Strep supplemented with 10 ng/ml each of the cytokine cocktail mentioned above. After 10 days, non-adherent cells were harvested and analyzed by flow cytometry.

Plasmids. MSCV-IRES-GFP (MigR1), MigR-ICN1 retroviral vectors were obtained from W. Pear (University of Pennsylvania) and MigR-EBF1, MigR-Pax5, MSCV-EBF1-IRES-YFP (MiY-EBF1) were

a kind gift from J. Hagman. pCS-E47:ER and MigR-EBF1:ER vectors were provided by B. Kee (University of Chicago) and M. Kondo (Duke University), respectively. The pcDNA3.1-EBF1-myc-His expression construct was obtained from R. Grosschedl (Max Planck Institute). Retroviral construct expressing full-length isoform (p45) of human TCF1 (TCF1-VEX) was based on a MSCV backbone and provided by A. Bhandoola (University of Pennsylvania).

Microarray analyses. Protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Quality and quantity of extracted RNA was tested on a bioanalyser by UPENN Microarray Core Facility. This was followed by fragmentation and biotinylation with the Affymetrix WT Terminal Labeling kit according to the manufacturers' instructions. Biotinylated targets were heated at 99°C for 5 min and hybridized for 16 h at 45°C. The microarrays were then washed at low (6× SSPE) and high (100 mM MES, 0.1 M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. GeneChips were scanned using the GeneArray Scanner 3000 7G. The data were analysed using Partek Genomics Suite, version 6.5 (Partek). Robust multichip average (RMA) with default settings was used to normalize data. Gene signal values for the arrays were $\log_2$-transformed and heat maps represent the $\log_2$-transformed normalized signals values.

Electrophoretic mobility shift assay (EMSA). Recombinant EBF1 protein (amino acids 26–422) was expressed as a six-histidine-tagged fusion protein in 293T cells and nuclear extracts were made 48 hours later using the NE-PER nuclear extraction reagent (Thermo Scientific, Rockford, IL). 24 base-5'-biotinylated DNA oligonucleotides were purchased from IDT (Coralville, Iowa). Equal molar amounts of complementary strands were mixed together and allowed to anneal for 1 hour at 37°C to form double-stranded probe. EMSA was performed using the Lightshift chemiluminescent

electrophoretic mobility shift assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Briefly, 4 µg of nuclear extract was incubated in binding buffer supplemented with poly(dI:dC), 2.5% glycerol, 0.05% NP-40, 5mM $MgCl_2$, 0.1mM $ZnCl_2$ and 20 fmol wild type or mutated biotinylated ds-oligos for 20 minutes at room temperature. In lane 2, 200-fold molar excess of unlabeled competitor probe was also added. A monoclonal anti-His_6 antibody (GE Healthcare) was also added to the reaction in lane 4 followed by an additional incubation step for 10 minutes at room temperature. Protein-DNA complexes were then separated from the free oligonucleotide by electrophoresis through 4% native polyacrylamide gel containing 0.5× Tris-borate-ethylenediaminetetraacetic acid and transferred to a nylon membrane at 380 mA for 60 minutes. After cross-linking the transferred DNA to the membrane at 120 mJ/cm^2 using a UV-light at 254-nm, the membrane was incubated in blocking buffer for 15 minutes and then conjugated/blocking buffer for another 15 minutes. After four washes, the membrane was incubated in substrate equilibration buffer for 5 minutes, then substrate working solution for 5 minutes, and then exposed to Biomax MR film (Kodak, Rochester, NY). Sequence of EMSA oligonucleotides are as follows: sense *Gata3* "site a" (ACTCCCCTCCCTTGGAAAAACAAA), antisense gata3 "site a" (TTTGTTTTTCCAAGGGAGGGGAGT), mutated sense gata3 "site a" (ACTCCCCTCCCTTCCTAAAACAAA), mutated antisense *Gata3* "site a" (TTTGTTTTAGGAAGGGAGGGGAGT), sense *Cd79a* (AGCCACCTCTCAGGGGAATTGTGG), antisense Cd79a (CCACAATTCCCCTGAGAGGTGGCT).

Flow cytometry. Stained single cell suspensions were analyzed on a 14-color LSR2 flow cytometer (BectonDickinson) equipped with four lasers for excitation of UV, violet, blue, and red-excited dyes. Antibodies included fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-TexasRed, PE-Cy5.5, PE-Cy7, allophycocyanin (APC)/ alexa fluor 647 (AF647), APC-Cy5.5, APC-Cy7/APCeflour780, or Pacific Blue-conjugated versions of purified antibodies to: CD11b (M1/70), Gr-1 (8C5), F4/80 (BM8), Ly6C (AL-21), CD11c (HL3), Ter-119, CD3ε (2C11), NK1.1 (PK136), CD127/IL-7R□ (A7R34),

CD135/Flt3 (A2F10), CD117/c-kit (2B8), B220 (RA3-6B2), Sca-1/Ly6 A/E (E13-161.7), CD19 (1D3), Ly6D (49-H4), $CD45^{B6}$ (104) and $CD45^{SJL}$ (A20). All directly conjugated antibodies were purchased from eBiosciences (San Diego, CA), BioLegend (San Diego, CA), BD Pharmingen (San Diego, CA) or Invitrogen (Carlsbad, CA). Non-viable cells were excluded from all analyses by staining with DAPI. All files were analyzed with FlowJo 8.8.7 (Tree Star, Inc). Cell sorting including single cell sorting was performed on a FACSAria equipped with three lasers and an ACDU. For analyzing or sorting samples positive for both GFP and YFP, the blue laser octagon setup was modified to include a 515/20BP filter with a 505LP dichroic and a 550/30BP filter with a 535LP dichroic mirror.

***In vitro* apoptosis assay**. Apoptotic cells were detected using the Active caspase-3 detection kit (BD Pharmingen). Briefly, transduced cells were fixed in Cytofix-solution and incubated for 20min on ice. They were subsequently washed and stained in Perm/Wash buffer with anti-active caspase-3 antibody for 30min at room temperature and analyzed by flow cytometry.

Supplemental References:

Weber, B.N., Chi, A.W., Chavez, A., Yashiro-Ohtani, Y., Yang, Q., Shestova, O., and Bhandoola, A. (2011). A critical role for TCF-1 in T-lineage specification and differentiation. Nature *476*, 63-68.