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From stem cells to functional lymphocytes: cell differentiation and gene therapy implementation for RAG-SCID

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Chapter 2

Functional definition of a transcription factor hierarchy regulating T cell lineage commitment

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ABSTRACT

T cell factor 1 (Tcf1) is the first T cell–specific protein induced in multipotent progenitors following Notch signaling in the thymus, leading to the activation of two major target genes, Gata3 and Bcl11b. Tcf1 deficiency results in partial arrests in T cell development, high apoptosis, and increased development of B cells and myeloid cells. Phenotypically, seemingly fully T cell–committed thymocytes with Tcf1 deficiency have promiscuous gene expression, an altered epigenetic profile and can dedifferentiate into more immature thymocytes and non-T cells. Restoring Bcl11b expression in Tcf1-deficient cells rescues T cell development but does not strongly suppress the development of non-T cells; in contrast, expressing Gata3 suppresses the development of non-T cells, but does not rescue T cell development. Thus, T cell development is controlled by a minimal transcription factor network involving Notch signaling, Tcf1, and the subsequent division of labor between Bcl11b and Gata3, thereby ensuring a properly regulated T-cell gene expression program.

INTRODUCTION

T cells are disease-fighting leukocytes that, similar to all blood cells, originate from hematopoietic stem cells (HSCs). However, whereas all other blood cell lineages develop in the bone marrow in specific niches, T cells develop in the thymus, a specialized organ located in the chest where progenitor cells migrate from the bone marrow and definitively commit to the T cell lineage, ultimately forming mature T cells [1]. The development of T cells within the thymus is a highly complex process involving successive stages in which the expression of CD4 and CD8 co-receptors occurs in distinct microenvironments [2]. Via a series of progressive developmental stages, T cell precursors (i.e. thymocytes) differentiate from double-negative (DN; $CD4^-CD8^-$) cells into intermediate immature single-positive (ISP; $CD8^+CD3^-CD4^-$) cells, then into double-positive (DP; $CD4^+CD8^+$) cells, and finally into single-positive (SP; $CD8^+CD4^-CD3^+$ or $CD4^+CD8^-CD3^+$) cells. In the DN stage, developing thymocytes can be further subdivided into four stages of differentiation based on their expression levels of CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). Early stages are not committed to the T cell lineage (i.e., fate restricted), allowing other lineages to develop [3]. Indeed, B cells, dendritic cells, myeloid cells, and natural killer (NK) cells can all be generated from $CD44^+CD25^-c\text{-}kit^{hi}$ early thymic progenitors (ETPs) [4, 5], DN1 cells, and—albeit to a lesser extent—DN2 cells [6]. These multipotent cells, which can enter a number of differentiation programs, are directed towards the T cell lineage via a process called specification. The irreversible capacity to develop solely into T cells occurs somewhat later and is referred to as T lineage commitment; this process also involves the active repression of non-T cell lineages [7-9].

The microenvironment of the thymus provides a cellular context that drives T cell development. This process is initially driven by the expression of Notch ligands, particularly delta-like protein 4 (DLL4) [10], and later in the DP stage by providing the signals required to control positive selection (for self-MHC) and negative selection (against autoreactive T cell clones). The various stages in T cell development have been investigated in great detail using flow cytometry and genomic analyses; thus, T cell development serves as a paradigm for the molecular regulation of cell fate [11, 12]. The fact that T cell development occurs in an anatomically separate niche has allowed researchers to study the detailed successive steps that underlie lineage specification and commitment. All of the events that establish the identity of T cell precursors are driven by Notch signaling [13], involving binding of the transcription factor RBP-J (also known as CBF1) to intracellular Notch ligands, thereby forming an active transcription factor complex in ETPs.

The subsequent stages of T cell development are governed by several key transcription factors that form an intricate gene regulatory network [14]. The core set of transcription factors in the early phases of T cell development are Tcf1 (encoded by the gene confusingly termed *Tcf7*), Gata3, Bcl11b, and two members of the E2A family (E2A and HEB), Ikaros and Runx1 [14-17]. Importantly, the *Tcf7* gene is a direct Notch signaling target and the first T cell–specific transcription factor induced by Notch signaling [18]; in contrast, Bcl11b drives T cell commitment by limiting the NK cell fate and activating the T cell developmental gene program at the DN2-DN3 stage [19], leading to expression of the fully rearranged TCR-beta gene at the DN3 stage. Rothenberg and colleagues showed that

four transcription factors — Tcf1, Gata3, Notch/RBP-J, and to a lesser extent Runx1 — are required for the timed expression of Bcl11b [14]. Of these four transcription factors, Tcf1 is the most complex, as it can act as both a transcriptional repressor (e.g., when bound by a co-repressor such as Groucho) or a transcriptional activator by binding β-catenin in order to respond to canonical Wnt signals [20]. Interestingly, Tcf1 also acts as a tumor-suppressor gene [21, 22], and it can be functionally replaced — at least partially — by Lef1, a related transcriptional regulator expressed at approximately 50-fold lower levels than Tcf1 [23]. Additional complexity arises from many alterative splice forms and alternative promoter usage, leading to at least 6 different Tcf1 isoforms that are differentially expressed throughout the T cell lineage.

The precise role that Tcf1 plays in regulating T cell specification and commitment, and its interaction with other core regulatory factors in T cell development, is not fully understood. Therefore, we examined the role of Tcf1 at the earliest stages of T cell development, focusing initially on fully committed DN3 cells. We found that Tcf1 is necessary for driving thymocytes down the T cell developmental path even after the T cell commitment stage, as Tcf1-deficient DN3 thymocytes can dedifferentiate into DN1/2-like cells that can then develop into the myeloid and B cell lineages. In addition, we found that Tcf1 supports this "lineage fidelity" via two direct — and functionally complementary — target genes, *Gata3* and *Bcl11b*. An epistasis analysis using retroviral gene complementation in Tcf1-deficient stem cells revealed that the role of Gata3 in immature T cells is to repress B cell and myeloid fate, whereas Bcl11b establishes the T cell lineage program, and its expression can overcome the defect in T cell development in Tcf1 deficient thymocytes.

MATERIALS AND METHODS

Mice

C57Bl/6 TCF-1 -/- ΔVII/ΔVII mice were originally described by Verbeek et al (1995) and C57Bl/6-Ly5.1 mice were purchased form Charles Rivers Laboratories. Mice were bred and maintained in the animal facility of Leiden University Medical Center. All animal experiments were performed in accordance with legal regulations in The Netherlands and with approved protocols of the Dutch animal ethical committee.

Mice used for transplantation assay were kept in specified pathogen-free section and were fed with special food and antibiotic water. Genotyping assay of newborn Tcf1 mice was performed with DNA samples from earpieces using GoTaq Flexi DNA polymerase kit (Promega) according to manufacturer's instructions.

Flow cytometry and cell sorting

Single cell suspensions from thymus, spleen, BM and blood were stained with monoclonal antibodies against CD3e, CD4, Cd8a/Ly-2, CD11b/Mac-1, CD19, CD25, CD27, CD44/Ly-24, CD45.1/Ly-5.1, CD45.2/Ly-5.2, B220/CD45R, CD90.2/Thy1.2, CD117/c-kit, CD135/Flt3, Gr1/Ly-6G-6C, NK1.1, Sca1/Ly-6A, TCRβ, TCRvβ5.1/5.2, TCRβ6, TCRβ8 and Ter-119/Ly-76 (See **Table S3**). All antibodies used were directly conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll-a Protein (PerCP), PE-Cy7, allophycocyanin (APC), APC-Cy7 or efluor450. Biotinylated antibodies

were revealed with streptavidin conjugated antibodies (PE, efluor450, APC-Cy7, APC or Pe-Cy7) (all antibodies were purchased from BD, Biolegend or eBioscience).

Cells were blocked with normal mouse serum (NMS, Invitrogen) for 10min at room temperature and subsequently cell surface staining was performed in two steps. Firstly, cells were incubated for 30min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30min incubation step at 4°C was performed with the streptavidin conjugated antibodies mix.

Cell apoptosis was assessed by AnnexinV and 7AAD staining, which was performed following the PE AnnexinV Apoptosis detection Kit protocol (BD Pharmingen) after the cell surface staining. Proliferation assay was done by intracellular Ki67 staining (mIgG as control) with PE Mouse anti-human Set protocol (BD Pharmingen). For that purpose, cells were initially stained for cell surface markers as described previously and subsequently fixated and permeabilized by using fixation/permeabilization buffer (eBiosience) for an hour at 4°C. Cells were then washed with permeabilization (eBiosience) buffer with 2% NMS and stained with Ki67 or IgG1 solution for 30min at 4°C in the dark. The same procedure was used to assess icTCRβ expression.

Double positive CD4&CD8 cells before DN cell sorting and lineage positive cells before LSK/LK sorting were depleted using magnetic-activated cell sorting, autoMACS (Miltenyi Biotec). For DNs sorting, thymocytes were first stained with anti-CD4 and CD8-biotin, following by Streptavidin microbeads staining according to manufacturer instruction (Miltenyi Biotec). For LSK/LK cell sorting, lineage depletion kit (Miltenyi Biotec) was used according to manufacturer instruction. Subsequently, depleted cells were stained again for DNs or LSKs as described before. Cell sorting was performed on FACSAria II (BD Biosciences) or stained cells were measured with FACS-CantoII and LSR Fortessa x-20 (BD Bioscience). Data was analysed using FlowJO (Tree Star). All different hematopoietic populations were defined as described in **Table S4** and **Fig S7**.

Cell culture

Bone-marrow-derived stromal cell line OP9 and OP9-DL1 cells which ectopically express the Notch ligand Delta-Like 1 (DL1) were used as described by J.C. Zuñiga-Pflucker. Sorted DN cells were cultured on OP9 or OP9 WT/OP9-DL1 (10:1) confluent monolayers in αMEM (Lonza)-10%FCS, 1% P/S (Life Technologies) and GlutaMAX (Life Technologies) medium complemented with 50 ng/ml rmFlt3L, 50 ng/ml rmSCF, 10 to 1 ng/ml rmIL-7, and 50μM β-mercaptoethanol (β-ME; Sigma-Aldrich). (all cytokines purchased from R&D). Cells were harvested after 7 to 14 days of coculture and were analysed by flow cytometry.

Transduced LSK and LK with LZRS-ires-eGFP (control), LZRS-Gata3-eGFP or LZRS-Bcl11b-GFP vector were cultured on OP9-DL1 monolayer for 6 to 14 days in αMEM-10%FCS complemented with rmIL7 (10 ng/ml), rm Flt3L (50 ng/ml), rmSCF (10 ng/ml) and β-ME (50μM). Harvested cells were analysed by flow cytometry or sorted.

Retroviral production

LZRS-Gata3 and Bcl11b plasmids were obtained from Addgene and cloned into LZRS-ires-eGFP vector (Addgene, control vector). Control, Gata3 and Bcl11b retroviruses were generated using Phoenix ecotropic and amphotropic packaging cell line (ATTC). Cells were cultured in IMDM (Lonza)-10%FCS-1% Penicillin/Streptomycin -1%Glutamine and transfected with plasmids using X-treme Gene9 DNA transfection reagent (Roche) protocols. Selection of transfected cells was performed with 1mg/mL puromycin (Sigma-Aldrich) for a week and viral supernatant was harvested at 24h and 48h.

Retroviral transduction

LSK and LK sorted cells were stimulated overnight in StemSpan serum-free expansion medium (StemCell Technologies) supplemented with 10ng/ml rmTPO (R&D), 50ng/ml rmFlt3L (R&D) and 100ng/ml rmSCF (R&D). Hematopoietic progenitors were transduced using RetroNectin (Takara Bio Inc) coated wells according to the manufacturer's instructions. Non-tissue culture plates were coated with RetroNectin overnight at 4ºC and then blocked with 2% bovine serum albumin (BSA) in PBS for 30min. Retroviral supernatant (24h or 48h) was centrifuged at 1500xg for 1h at 32ºC and incubated an extra hour at 37ºC. After coating, viral supernatant was removed and stimulated cells were immediately added on the virus-coated plates. Cells were cultured in StemSpan medium supplemented with rmTPO (10 ng/ml), rmFlt3L (50 ng/ml) and rmSCF (100 ng/ml) and transduced overnight at 37ºC. LZRS-ires-eGFP, LZRS-Gata3-ires-eGFP and LZRS-Bcl11b-ires-eGFP transduced cells were used for *in vitro* and *in vivo* approaches.

Quantitative real time q-PCR

RNA from sorted cells was purified using Micro RNeasy kit (Qiagen) and reverse transcribed into cDNA using Superscript III kit (Invitrogen). RT-PCR was performed using TaqMan Universal Master Mix II in combination with specific probes for indicated genes from Universal Probe Library (Roche). Specific primers for ABL-2, Bcl11a, Bcl11b, Gata3, Pax5, PU.1/Spfi1, IL-7Ra, CD117/c-kit, ID2, Axin-2, Hes1, CD3e, CD3d, pTa and ZAP70 were designed and purchased from Sigma-Aldrich (See specific gene sequences on **Table S5**). Samples were analyzed by StepOnePlus RT-PCR system (Life Technologies). Relative transcript abundance was determined by ΔCt and expression levels were normalized for the endogenous reference gene ABL-1. All samples were run in at least in duplicates.

RNA-Seq

RNA from sorted DN3b cells ($Lin^-CD25^+CD44^-CD27^+$) from Tcf1-/- and wild-type littermates thymi was isolated using the Mini RNeasy Kit (Qiagen) The integrity (scores > 9.0) of the RNA was determined on the Agilent 2100 Bioanalyzer (Agilent). Total RNA enrichment for sequencing poly(A) RNAs was performed with the TruSeq mRNA sample preparation kit (Illumina). 1µg of total RNA for each sample was used for poly(A) RNA selection using magnetic beads coated with poly-dT, followed by thermal fragmentation. The fragmented poly(A) RNA enriched samples were subjected to cDNA synthesis using Illumina TruSeq preparation kit. cDNA was synthesized by reverse transcriptase (Super-Script II) using poly-dT and random hexamer primers. The cDNA fragments were then blunt-ended through an end-repair reaction, followed by dA-tailing. Subsequently, specific

double-stranded bar-coded adapters were ligated and library amplification for 15 cycles was performed. The pooled cDNA library consisted of equal concentration bar-coded samples. The pooled library was sequenced in one lane, 36 bp single read on the HiSeq2500 (Illumina). Raw RNA-seq reads are accessible on SRA by accession number SRP158670.

RNA-seq data processing

FASTQ files were aligned to the mm10 genome using STAR 2.5.1b (Dobin et al, 2013). Transcript counts were quantified and annotated using HTSeq-0.6.1. WT sample 3 was removed due to a low number of aligned reads.

Differential expression and statistical analysis

Differential expression of DN3b wild-type vs TCF1-/- was identified by using DESeq2 (Love et al. 2014), after filtering for genes with a low read count (> 5 reads per sample) resulting in 205 differential expressed genes (97 upregulated in KO and 108 downregulated in KO) at p-value < 0.05 (FDR adjusted) and a Log2 Fold Change of > 1.5.

Geneset Enrichment

RNA-seq results from mouse T-cell precursors in different developmental stages including DN1, DN2a, DN2b, DN3 and DP (GEO accession: GSE89198, Rothenberg et al.) were used to create DN1 and DN2 genesets. Of this RNA-seq dataset log2 transformed FPKM values of 25 DN2 and 8 DN3 wildtype mice were used for differential expression analysis with Limma. Genes that were differentially upregulated (p-value < 0.05 and LogFC > 2) between DN1 vs DN3 (365 genes), DN2a vs DN3 (342 genes), DN2b vs DN3 (120 genes) and DN2a/b combined vs DN3(141 genes) were used as genesets for Geneset Enrichement Analysis. GSEAPreranked (GSEA 4.0.3, Broad) was run on all expressed Wild-type vs TCF -/- RNA-seq genes which were ranked by the p-value and LogFC generated by DESeq2. The DN3b TCF -/- was negatively associated with the DN1 geneset (Normalized Enrichement Score of -1.04) and positively associated with all of the DN2 genesets (DN2a NES 1.23, DN2b NES 1.53, DN2a/b combined NES 1.36).

ATAC-Seq

15,000 sorted DN3a ($Lin^-CD25^+CD44^-CD27^-$) and DN3b ($Lin^-CD25^+CD44^-CD27^+$) cells were washed 1 time with cold PBS. Pellets were spin down at 500 g for 5 min at 4°C, and the supernatant was removed carefully. 20 µl of transposase mix (10µl 2xTD buffer, 1 µl TDE (Nextera DNA Library Prep Kit; Illumina), 0.2 µ digitonin (G9441, Promega), 8.8 µl nuclease-free water) was added to the cells. Reactions were incubated at 37°C for 30 min. Transposed DNA was purified using the MinElute Reaction Cleanup Kit (28204, Qiagen), amplified, and again purified according to published protocols (Buenrostro et al 2015, CurrProtocMolBiol). Size selection was done using Low Range Ultra Agarose (161-3107, Bio-Rad). Fragments between 150-600bp in size were used for further analysis. Quality and quantity of the libraries was assessed by Bioanalyzer High Sensitivity DNA Analysis Kit (Agilent) before sequencing. Libraries were sequenced 50 bp, paired-end, on a HiSeq4000.

The reads were filtered by quality using Trim-galore tool (Krueger, 2015) (default values) and the quality control was driven by FastQC (Andrews, 2010) and MultiQC (Ewels, 2016).

The remained reads were mapped to mm10 using bowtie2 (Langmead et al, 2012) with –very-sensitive parameter. After all, before the peak calling, the read duplicates and multiple mapping reads were removed using Picard tools (http://broadinstitute.github.io/picard). The peaks for 2 wild-type and 2 Tcf1 -/- samples were called using MACS2 (Zhang et al, 2008) with the following parameters: -g mm -B –shift -100 -ext 200 –nomodel –q 0.05 and BigWig-tracks with FPKM were generated by deeptools (Ramirez et al, 2014). Coverage plots and heatmaps were generated with deeptools using the BigWig tracks previously generated with the following parameters: --binSize 100 -m 3000 -b 1000 -a 1000. To find differential open chromatin regions, the differential peaks between wild-type and Tcf1 -/- conditions were calculated by DiffBind R Bioconductor package (Start et al, 2011), only the statistically significant peaks (FDR<0.05) were taken in account for downstream analysis. Motif analysis on the differentially accessible regions was performed using Homer (http://homer.ucsd.edu/homer/) using the parameters: size given. MEME-FIMO (Grant et al. 2011) and Tcf1 position probability matrix (MA07769.1) from JASPAR (http://jaspar.genereg.net/) were used to analyze the distribution of the Tcf1 motif on the differentially accessible regions.

Chromatin immunoprecipitation

DN thymocytes ($CD8^-CD4^-$) from Tcf1-/- and wild-type littermates were sorted and subsequently crosslinked with formaldehyde (Sigma). Crosslinking was quenched with Glycine and after cell lysis chromatin was sonicated into fragments. Sonicated chromatin was precleared and incubated with antibodies. TCF-1 (C46C7; #2206 Cell Signalling Technologies). Immuno precipated chromatin complexes were purified and quantified by real-time PCR using Faststart Universal Sybr Green Master mix (Roche). (See specific gene sequences on **Table S5**).

Stem cell transplantation

Competitive transplantation assay is used to determine HSC development and functionality in vivo by measuring multi-lineage reconstitution of hematopoiesis in irradiated transplanted mice. Competitive transplantation Ly5.2/Ly2.1 was used to assess if in vivo re-expression of Gata3 could rescue T cell development in the thymus. Total 52.500 Ly5.2 Tcf1 (wild-type or -/-) transduced cells (mixed LSK and LK progenitors cells) were transplanted into lethally irradiated (8.07Gy) Ly5.1 recipient mice (8-12 weeks), together with 300.000 splenocytes (Ly5.1) as support cells. Chimerism and peripheral T cell were analysed at week 6 after transplantation in peripheral blood. Mice were sacrificed for analysis 7 weeks after transplantation to evaluate hematopoietic system repopulation. Mice were considered repopulated when ≥1% multi-lineage Ly5.2 Tcf1 cells could be detected. Single cell suspension from the thymus, spleen and bone marrow (BM), as well as lysate blood were analyzed by flow cytometry as described previously.

Statistical methods

All statistics were calculated and all graphs were generated using GraphPad Prism6 (GraphPad Software). Statistical significance was determined by Mann-Whitney U test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$), Multiple t-test or Two-Way ANOVA depending on the experimental setting.

RESULTS

Tcf1 deficiency leads to several arrests in T cell development with increased non-T cells

Tcf1 deficiency results in multiple incomplete blocks in T cell development that vary from mouse to mouse. Besides the well documented block at the ISP stage [24-26], T cell development can be arrested at DN1, DN2 and DN3 stages (**Fig S1A**). In contrast to these partial arrests in developing mice, transplanting Tcf1-deficient stem cells into adult recipient mice led to a complete block in T cell development at the DN1-DN2 transition (**Fig.S1B**), presumably the result of an insufficient compensatory expression of Lef1 in these cells [27]. We also observed increased percentages of non-T cell lineages, most notably B cells and myeloid cells (**Fig.S1C&1D**), consistent with previous reports of *ex vivo* cultured Tcf1 deficient cells.

Phenotypically, fully committed DN3 Tcf1-deficient thymocytes have promiscuous gene expression and altered chromatin

Given the effects of Tcf1 deficiency on sequential stages of T cell development, we initially focused on those stages where thymocytes should be fully T cell committed. Therefore, we compared gene expression profiles between Tcf1-deficient thymocytes and wild-type thymocytes. The T cell commitment process starts at the DN2 stage and continues to the DN3a (CD25+CD44-CD27-) stage, in which a rearranged Tcrb gene is expressed in combination with pTA to form the pre-TCR complex in a process known as β-selection. After β-selection, the cells rapidly proliferate, express CD27, and are fully T cell committed based on expression of a functional, rearranged Tcrb gene [28]. We consider thymocytes αβ T cell committed when they express a fully rearranged TCRβ. We realize that there are definitions where T cell commitment occurs at earlier stages but phenotypically defined DN3(b) cells are here considered as the candidate population for committed T cells. We performed whole-transcriptome RNA-Seq on DN3b cells obtained from Tcf1-deficient and wild-type littermates (**Fig.1A**), reasoning that at DN3b thymocytes should be fully T cell lineage committed (see **Fig.S2B**; CD27 and Ptcra). We found 108 genes with downregulated expression (> 1.5 fold, FDR<0.05) in the Tcf1-/- DN3b thymocytes, but also 97 upregulated genes (**Table S1**). For visualization, the top 100 differentially expressed genes are shown and the absence of Tcf7 expression was confirmed in Tcf1 deficient DN3b cells. Furthermore, the RNA-seq analysis shows fewer rearranged Tcrb genes than in wild-type control DN3b thymocytes, as shown for the Trbj expressed gene segments (data not shown). We used the genes differentially expressed between Tcf1 deficient and wild-type DN3b cells in a Gene Set Enrichment analysis (GSEA) and used published gene sets of T cell developmental stages to establish a DN1 and DN2 signatures[29]. The genes highly expressed in Tcf1-/- DN3b clustered strongly with the DN2-specific gene set (DN2a and DN2b but not DN1), indicating that they share many characteristics of earlier developmental stages that are less T cell committed (**Fig.1A** and **Fig.S2A**). The RNA-seq data also indicated that many of the T cell commitment genes were low or not expressed while genes involved in non-T cell lineages (Pax5, Pu.1, Blc11a) were highly expressed in the Tcf1 deficient cells compared to the control DN3b cells. Based on these data we validated the expression of a number of important T cell developmental genes by q-PCR

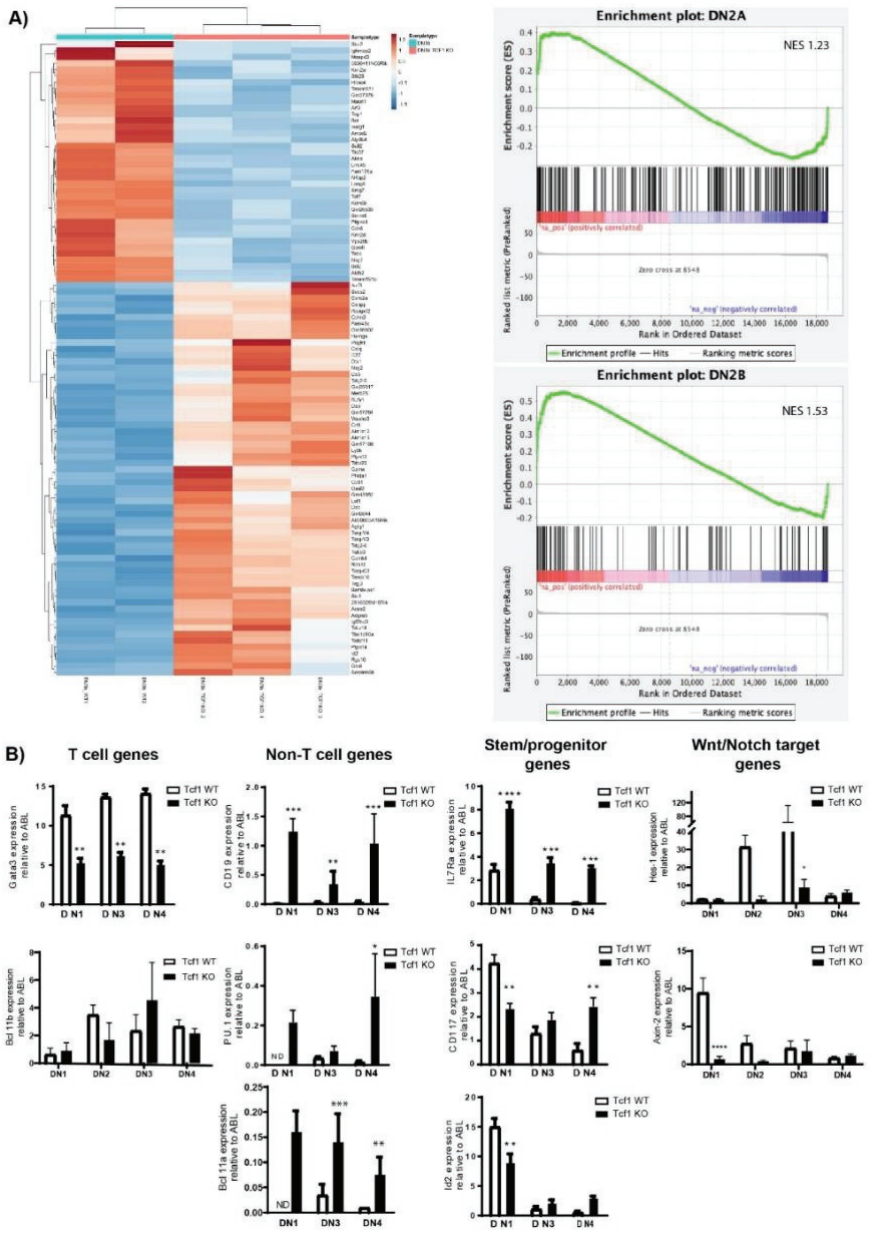

Figure 1: Tcf1-deficient DN3b cells show promiscuous gene expression compared to WT littermate controls. *A) Heat map of the top 100 differentially expressed gene as determined by RNA-seq of sorted DN3b cells from WT and Tcf1-deficient thymi. GSEA of the differentially expressed genes (Tcf1−/− KO over Tcf1 WT for DN3b) is enriched for DN2 genes (DN2a and DN2b with NES +1.23 and + 1.53, respectively). B) qPCR validation of RNA-seq data for selected T cell–specific genes, genes expressed in non-T cells, and legacy genes whose expression is inherited from stem cells/multipotent progenitors. The levels of expression are normalized by ABL-2 expression as*

*housekeeping gene. (Mann-Whitney U test; *P < 0.05, **P < 0.01, and ***P < 0.001. Error bars represent the SD of three pooled mice and from two independent experiments.)*

on sorted DN1, DN2, DN3 and DN4 thymocytes. These results validated the RNA-Seq data and showed lower expression (2-fold change) of the T cell specific transcription factors Gata3 (DN1 to DN4) and Bcl11b (DN2 stage) (with higher expression of its functional counterpart Bcl11a) while the B cell commitment marker CD19 and the myeloid associated factor Pu.1 were significantly higher expressed in the Tcf1 deficient thymocytes (**Fig.1B** and **Fig. S2B**). In addition, genes known to be associated with stem/progenitor cells (sometimes referred to as legacy genes[1]) such as c-kit were also significantly higher expressed (**Fig.1B**), while both Wnt and Notch target genes (HES-1 and Axin2) were decreased. Collectively, these data showed that while in some regards Tcf1-/- DN3b thymocytes were T cell committed (phenotypic markers, expression of some Tcrb genes), they also showed lineage infidelity, with expression of master regulatory genes from non-T cells.

The strongly reduced number of thymocytes due to the lack of Tcf1 is not only explained by the developmental arrests and differentiation into non-T cells, but also by high levels of apoptosis. Compared to wild-type cells, we found increased levels of apoptosis in Tcf1-deficient cells at nearly every stage (**Fig.S3A**), as well as decreased cell proliferation in the DN2 and DN4 stages (**Fig.S3B**).

Gata3 and Bcl11b are direct targets of Tcf1 and downregulated in Tcf1 deficient thymocytes

The downregulated mRNA expression levels of the transcription factors *Gata3* and *Bcl11b* in various DN thymocyte stages in Tcf1 deficient mice, suggested that these factors may be direct target genes of Tcf1. In accordance, the Bcl11b and Gata3 promoter/enhancer sequences contain conserved Tcf/Lef binding sites [30 31]. To check whether in *ex vivo* DN thymocytes these promoters are regulated in a Tcf-dependent manner, we performed chromatin immune precipitation (ChIP) using a monoclonal antibody specific for Tcf1 (**Fig.2A**) followed by q-PCR. This revealed binding of Tcf1 to the Gata3 and Bcl11b promoter sequences in wild-type DN thymocytes but not in Tcf1 deficient thymocytes, consistent with both genes being direct target genes of Tcf1. This supports previous reports on OP9-DL1 cultures [18] and reporter gene assays.

This finding was further substantiated by ATAC-Seq (Assay for Transposase-Accessible Chromatin) data which indicates chromatin accessibility. In general, we found fewer ATAC-Seq peaks in DN3b thymocytes lacking Tcf1 compared to wild-type DN3b cells, 55217 and 50175 peaks were found in wild-type samples and 21142 and 7520 peaks in Tcf1 -/- samples; but in DN3a thymocytes, there is no a clear difference between Tcf1 -/- and wild-type. In total, 68883 and 30357 peaks were found in wild-type samples and for Tcf1 -/- samples, 40716 and 68605 peaks (**Fig.S2C**).

To find regions with differentially chromatin accessibility between Tcf1 -/- and wild-type for DN3a and DN3b thymocytes, we looked for peaks statistically different between the conditions. For this analysis only differential peaks with FDR less than 0.05 were taken in account. In DN3a, 564 accessible sites were lost in Tcf1-/- cells, from which 141 were Tcf1

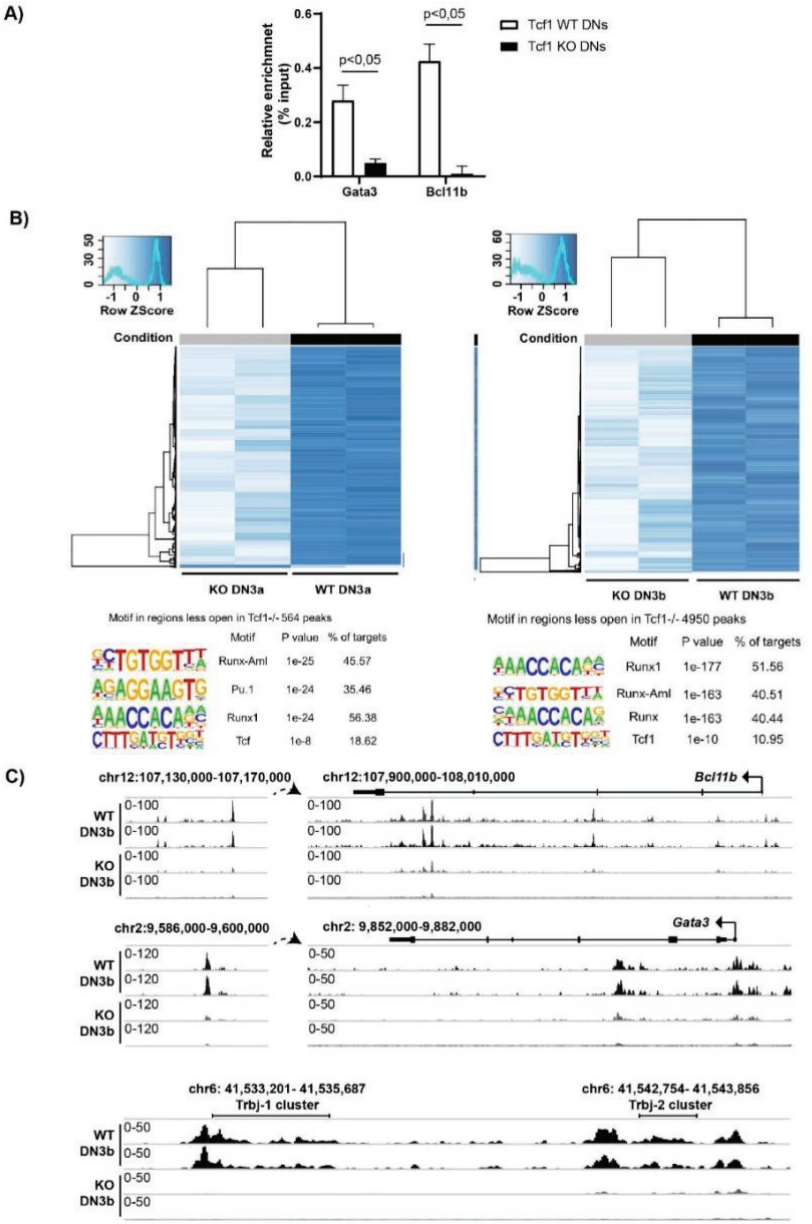

Figure 2: Chromatin accessibility analysis in Tcf1-deficient versus WT DN thymocytes*. A) Chromatin immunoprecipitation with an antibody specific for Tcf1 revealed that the Gata3 promoter and the Bcl11b enhancer are occupied by Tcf1 in vivo, whereas in Tcf1 KO DN thymocytes, no binding can be detected. Negative controls with IgG instead of anti-Tcf1 showed no enrichment. (Multiple t test. Error bars represent the SD of at least three pooled mice and from two independent experiments.) B) Heat map of DESeq2 normalized read counts of ATAC-seq shows differentially accessible regions between WT and Tcf1−/− in DN3a and DN3b. Motif analysis was performed in the differentially accessible regions using HOMER showing the three highest scores and Tcf1 score. C)*

ATAC-seq data mined for the Bcl11b, Gata3, and Trbj (T cell Receptor Beta) genomic regions. Per locus, the relative abundance of transposase accessible regions is indicated. The individual ATAC-seq profile from each genotype is shown. Data are shown as normalized read density.

binding sites. Only 8 sites were statistically significant higher in Tcf1-/- containing 3 Tcf1 binding sites. In the case of DN3b, extra sites were lost in Tcf1-/- compared to Tcf1 wild-type (4950 in total), including 756 Tcf1 binding sites. 21 sites were more accessible, but no Tcf1 binding sites were found. These results indicate that global chromatin accessibility was higher in wild-type thymocytes than in Tcf1 deficient thymocytes (**Fig.2B**). Interestingly, both DN3a and DN3b share the fact that Runx motifs seem to be abundantly lost upon Tcf1 deficiency (**Fig.2B**), in accordance with the diminished *Runx1* expression shown in the RNA-seq data (**Fig.S2B**).

Focusing on the *Bcl11b* and *Gata3* promoter/enhancer sequences, the chromatin in these promoters was less accessible compared to wild-type littermate control DN3b cells (**Fig.2C**). Similarly, the TCRB loci were much less accessible in accordance with the RNA-Seq data. The full genome-wide data analysis is provided in **Table S2**. Interestingly, no major differences in chromatin accessibility were found at genes involved in alternative lineages (not shown), indicating that expression of these genes was not regulated at the level of chromatin opening. Collectively, these data show profound differences due to the lack of Tcf1 in chromatin accessibility and expression of genes and promoters associated with T cell commitment.

Phenotypically, fully committed DN3 Tcf1-deficient thymocytes dedifferentiate into DN1 thymocytes, B cells, and myeloid cells.

Based on the hypothesis derived from these results, that Tcf1 deficient DN3 thymocytes may not be fully T cell committed, we sought to better investigate the differentiation capacity of Tcf1-/- DN3 thymocytes. Therefore, DN3 cells were sorted and cultured under conditions with strong T cell inducing capacity (OP9-DL1 system). Indeed, the majority of wild-type DN3 thymocytes differentiated further into DN4 cells, with a smaller part remaining DN3 (**Fig.3A&B**). Unexpectedly, most Tcf1-/- DN3 thymocytes dedifferentiated into DN1 and DN2 cells, with extensive B and myeloid development while only a minority of cells remained DN3 without any further development along the T cell lineage (**Fig.3A&B**). Especially development into B cells was extensive, with up to 60% of DN3 thymocytes developing into B cells (**Fig.3A&B**). These dedifferentiated DN1 and DN2 cells were not a contaminating fraction in the sorted DN3 cells that expanded, as intracellular staining for Tcrb revealed high Tcrb expression in these DN1/2 cells at similar levels as cells remaining in DN3 stage and wild-type DN3 and DN4 cells (**Fig.3C**). Therefore, these non-T cells (B and myeloid cells) developing in the assay expressed icTCR indicating that they also derived from the seeded DN3 Tcf1 -/- promiscuous cells (data not shown). We conclude that Tcf1 KO cells dedifferentiate to less committed cells and exhibit lineage DN1 and DN2-like cells were derived from the sorted “fully” committed DN3 cells. Similarly, infidelity with significant development into alternative (non-T) lineages. When ETP cells rather than DN3 cells were seeded on OP9-DL1, as expected, Tcf1 deficient cells were arrested in development at DN1 (**Fig.S4A**), with abundant B and myeloid development,

whereas wild-type stem cells differentiated along the T cell lineage with many fewer non-T cells (**Fig.S4B**).

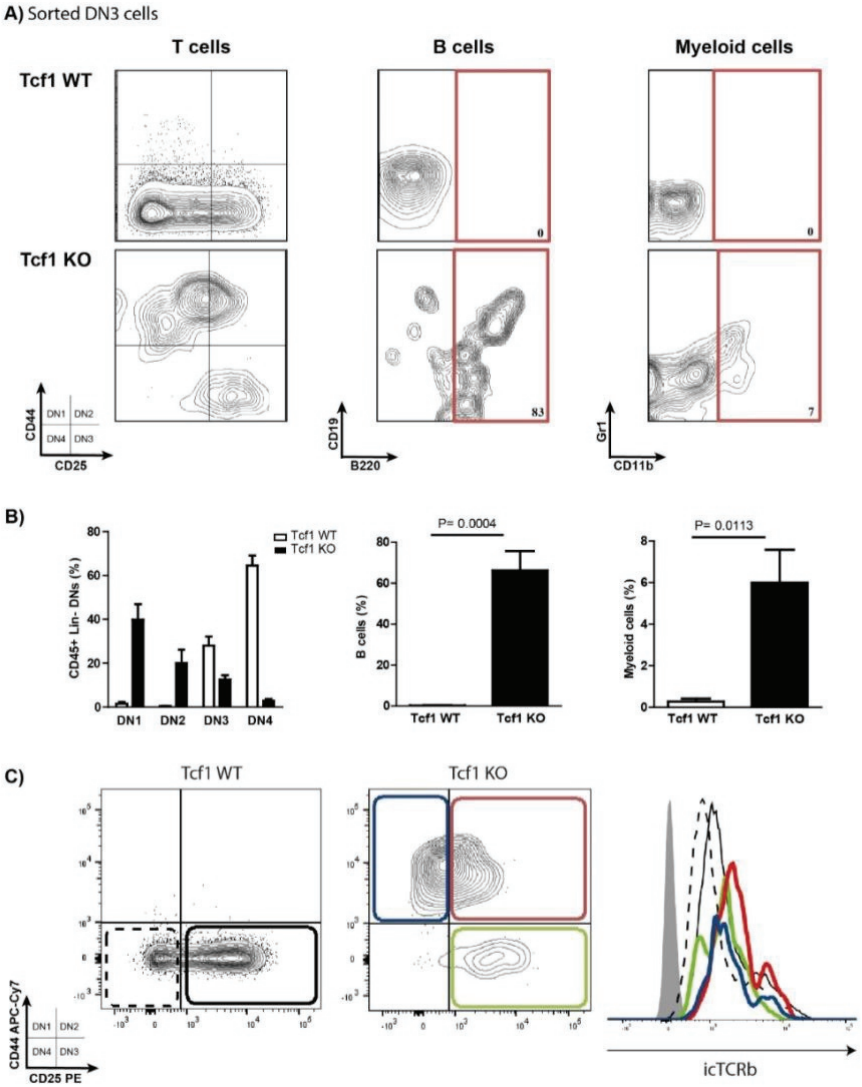

Figure 3: Tcf1-deficient DN3 cells dedifferentiate into DN1/2-like cells with multipotent lineage capacity. *A) WT DN3 cells sorted and seeded on OP9 WT/OP9-DL1 (10:1) cells develop largely further into DN4 or remain DN3 after 7 days in culture, while Tcf1-deficient cells develop into DN1 and DN2 cells (pre-gated Thy1+ Lin− cells) with prominent B cell (B220+ CD19+) and myeloid cell (CD11b+ Gr1+) development. B) Quantification of the developmental plasticity and dedifferentiation effects of DN3 Tcf1-deficient thymocytes into DN1, DN2, myeloid, and B cells. C) Intracellular TCRß staining reveals the dedifferentiated DN1 and DN2 cells to be derived from DN3 cells. (Mann-Whitney U test. Error bars represent the SD of three samples from three independent experiments.)*

Dedifferentiation into alternate lineages can be prevented by expressing Gata3 in Tcf1 deficient thymocytes

Epistasis analysis is a powerful genetic tool, often used in model organisms such as Drosophila to investigate hierarchical relationships between genes [32]. It can be more complex to perform in mammals such as mice, where not only expression per se but also gene dosage is important. For instance, while complete loss of Gata3 blocks T cell development at the earliest stages, transgenic overexpression of Gata3 can lead to development of mast cells in the thymus [33-36]. We therefore expressed Gata3 and Bcl11b using recombinant retroviruses as they have a broad range of expression that would allow different phenotypes to be selected under the strong developmental pressure of the thymic microenvironment. We used retroviruses encoding GFP only, Gata3 together with GFP or Bcl11b together with GFP to investigate complementation of the Tcf1 phenotype by either Gata3 or Bcl11b (**Fig.4A, 5A**). We used retroviruses solely encoding GFP as negative controls. Re-expression of Gata3 could partially rescue the development of Tcf1-/- thymocytes from a DN1 arrest to an apparent CD25+ DN2 stage but not further (**Fig.4B**). However as, Thy1 expression was not increased on the apparent DN2 cells, they cannot be considered real DN2 cells. Similarly key T cell lineage specific (*CD3, PtA*,) gene expression was not induced upon forced Gata3 expression (**Fig.S6A&B**). Strikingly, high Gata3 expression strongly suppressed the enhanced development of B and myeloid cells (granulocytes as well as monocytes) from Tcf1-/- thymocytes. This also occurred to some extend when starting with wild-type cells (**Fig.4C**). Competitive stem cell transplantation (Ly5.2 Tcf1 stem cells / Ly5.1 recipient mice) was used to assess if re-expression of Gata3 could rescue T cell development in the thymus *in vivo.* The suppression of B cell development (Ly5.2 B cells) in the thymus was also observed *in vivo* when Gata3 complemented Tcf1-deficient stem cells were transplanted in irradiated recipient mice (**Fig.4D_right panel**). However, thymic T cell development (Ly5.2 T cells) again was arrested at a DN1/2 transition, barely different than GFP control transduced cells (**Fig.4D_left and middle panel**). Thus, the major role of Gata3 in earliest DN development is the suppression of non-T cell development with only a minor feed forward role into the T cell program.

The T cell lineage-specific defects caused by Tcf1 deficiency can be rescued by expressing Bcl11b

Enforced expression of Bcl11b (**Fig.5A**), in contrast, rescued the T cell developmental defect of Tcf1 deficient cells virtually completely. Bcl11b transduced Tcf1 deficient stem cells developed readily into Thy1 positive (**Fig.5B** and **Fig.S5B**) cells and could develop into DN2 and DN3 thymocytes to a similar degree as wild-type thymocytes (**Fig.5C** and **Fig.S5C**) (while non transduced Tcf1 deficient cells are arrested at the DN1/DN2 stage as the control cells (**Fig.5D**). In addition, expression of TCRβ by intracellular flow cytometry also was restored to wild-type levels in DN3 and DN4 by expressing Bcl11b in the Tcf1 KO background (**Fig.5D**). Accordingly, T cell receptor gene expression was rescued upon Bcl11b overexpression in Tcf1 deficient cells (**Fig.S6B**). In contrast, expression of Bcl11b did not markedly influence B and myeloid development from Tcf1 deficient cells (**Fig.5E** and **Fig.S5C**). Overexpression of Blc11b did suppress the development of NK cells

(**Fig.S5E**), consistent with its described role in promoting T cell fate over NK cell fate at the DN2 stage [19].

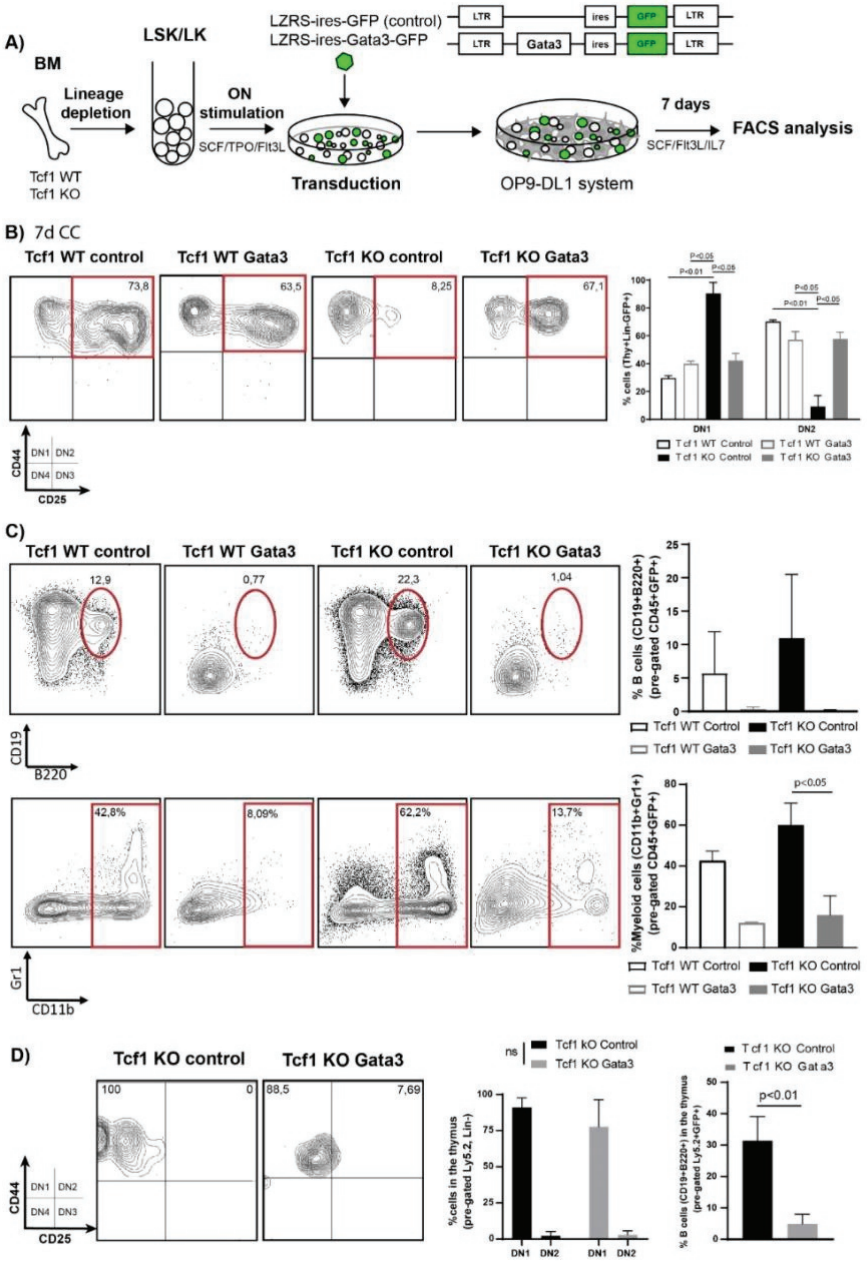

Figure 4: Re-expression of Gata3 suppresses B and myeloid development in Tcf1 deficiency. (A) Layout of retroviral complementation experiments with GFP control and/or Gata3. B) Gata3 expression partially overcomes the DN1 thymocyte block and C) suppressed the enhanced non-T cell

lineages (B and myeloid cell development) after 7 days in the OP9-DL1 culture system. FACS shows representative plots and graphs quantitative data from replicate measurements. (Multiple t test analysis. Error bars represent the SD from two independent experiments.) D) In vivo complementation [Ly5.2 Tcf1 (WT or KO)–transduced stem cells transplanted into Ly5.1 recipient mice] reveals suppression of B cell development also in the thymus (right) 8 weeks after transplantation but minimal and partial rescue of T cell development in the thymus (left and middle). (Middle: Multiple t test analysis. Right: Paired t test. Error bars represent the SD from three individual mice per group.)

DISCUSSION

T cell development has been used as a classic example of a relatively ordered pathway to study cell fate determination [16], thereby giving the impression that transcriptional regulation during T cell development is a well-understood process. Despite this general belief, however, and compared to other developmental processes (for example, B cell development, which has similar requirements in terms of proliferation, lineage restriction, immune receptor rearrangement, and checkpoints for premature and mature immune receptors), the roles of the major transcription factors in T cell development are rather poorly understood. In B cell development, a clearly defined linear hierarchical relationship exists between E2A, EBF1, and Pax5 [37-44]. However, with respect to early T cell development, whether the Notch (RBP-J), Gata3, Bcl11b, Runx1, E2A, Tcf1/Lef1, Ikaros, and/or Hox genes play unique, redundant, or synergistic roles remains unclear and is the subject of intense research that focuses largely on either individual factors or the collective activity of these factors using computational biology. Considering that Notch signaling is required for T cell development, and given that the first T cell–specific target gene is Tcf7 [18], which encodes Tcf1, we investigated the process of T cell lineage commitment in Tcf1-deficient mice.

The study of Tcf1-deficient mice is generally complicated by three factors. First, in the absence of Tcf1, the HMG box transcription factor Lef1 — which is expressed in the thymus, albeit at much lower levels than Tcf1 — plays a compensatory role [23, 27, 45] (**Fig.S2B**). This low-level expression of Lef1 causes incomplete penetrance of the Tcf1-deficient phenotype. However, if adult Tcf1-deficient stem cells are either transplanted into recipient mice or cultured on OP9-DL1 cells to induce T cell differentiation, a complete block occurs at the DN1 stage (see **Fig.1D**), as Lef1 expression is believed to result from reaming fetal expression in the thymus [21,22, 27]. Therefore, in our experiments we used bone marrow-derived cells obtained from Tcf1-deficient mice. Second, Tcf1-deficient mice are prone to developing T cell lymphomas in the thymus [22], which is similar to T-cell acute lymphoblastic leukemia (T-ALL) in patients. As discussed above, this issue can be overcome by using Tcf1-deficient stem cells instead of thymocytes. The third issue associated with studying Tcf1-deficient mice is that Tcf1 functions as both a transcriptional repressor and a transcriptional activator (for example, when bound to the Wnt mediator β-catenin). Indeed, when Tcf1-dependent promoters were tested using in vitro reporter systems, transcription occurred only when β-catenin was also expressed [46, 47]. Consistent with this notion, Tcf1 binds to the promoter/enhancer regions of the target genes Gata3 and Blcl11b, and it seems likely that Tcf1 binds to β-catenin at these promoter regions. Co-chromatin immune precipitation experiments provide initial evidence for this notion, as

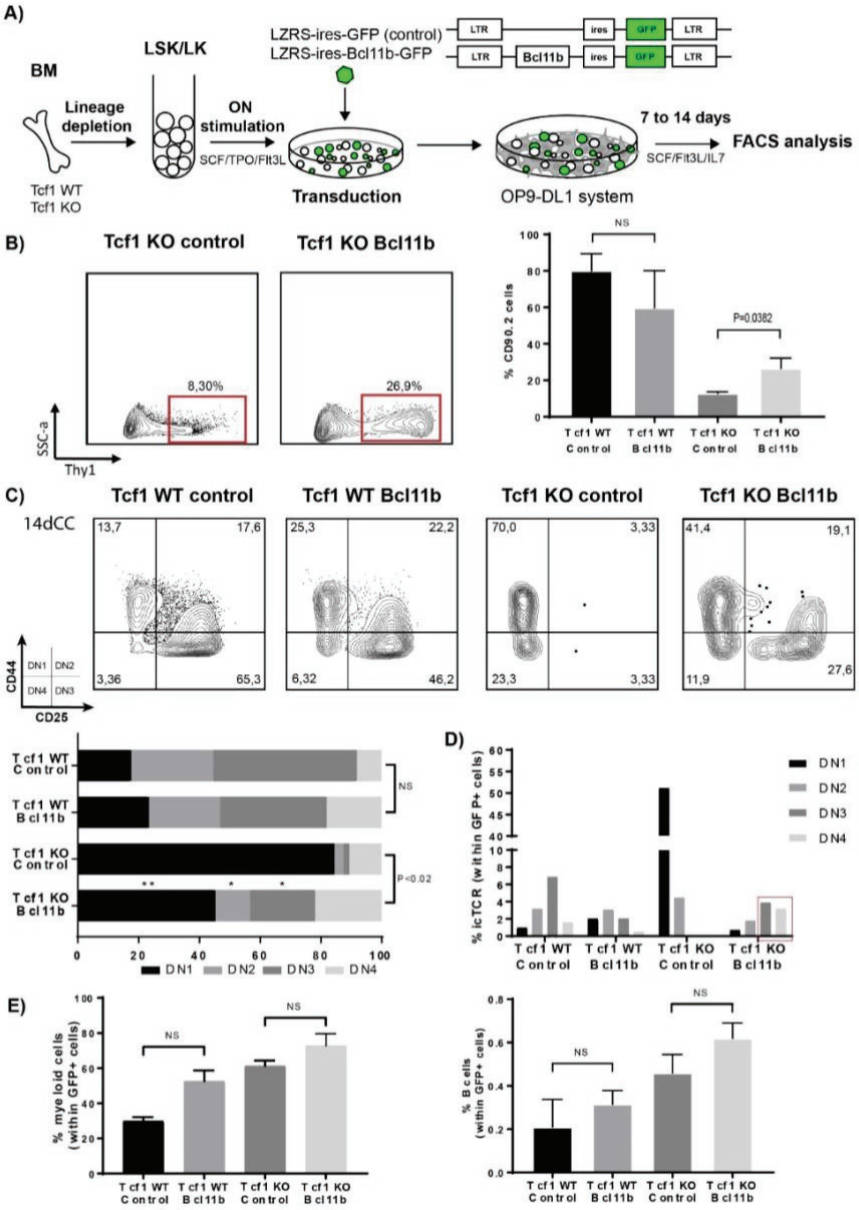

Figure 5: Reexpression of Bcl11b rescues T cell development in Tcf1-deficient stem cells. *A) Layout of retroviral complementation experiments with Bcl11b. B) Thy1 expression is rescued by Tcf1 deficiency by expression of Bcl11b after 14 days in OP9-DL1 culture (pre-gated Lin-GFP+ cells). (C) Bcl11b fully rescues T cell development from Tcf1-/- stem cells that otherwise are arrested in DN1 (pre-gated Thy1+Lin-GFP+). D) Intracellular TCRβ expression can be restored in Tcf1-deficient cells by expression of Bcl11b (pre-gated Thy1+Lin-GFP+ DN subset). E) Bcl11b overexpression does not affect myeloid and B cell development. (Two-way ANOVA. Error bars represent the SD from three independent experiments.)*

β-catenin can also be found at active promoters where Tcf1 binds (data not shown). In addition, DN stages of T cell development show high canonical Wnt signaling, which is driven by β-catenin and Tcf/Lef [48]. Of note, expression of the Wnt target gene Axin2 was markedly reduced in thymocytes lacking Tcf1 (**Fig.1B**). On the other hand, some of Tcf1's functions in the earliest stages of T cell development are independent of β-catenin [18], possibly due to the redundant role of Lef1.

A seminal study by Busslinger and colleagues revealed that Pax5 is a major lineage commitment factor in the development of B lymphocytes [42, 43, 49]. Thus, B cells that lack Pax5 can dedifferentiate into multipotent progenitor cells that can replenish all hematopoietic lineages, even in vivo. In this respect, our findings are somewhat analogous, as Tcf1-deficient DN3 cells —which seemingly are fully committed — have promiscuous gene expression and can dedifferentiate into immature cells that can give rise to non-T cell lineages, including B cells and myeloid cells. In the T cell lineage such de-differentiation has also been shown to occur in E2A or HEB deficient thymocytes [50 51]. Indeed, key transcription factors that drive alternate lineages (e.g., the transcription factors Bcl11a, Pax5, and Pu.1) are robustly expressed in Tcf1-deficient DN3 and DN4 cells, but not in wild-type cells. In contrast with Pax5-deficient cells, however, only a small number of Tcf1-deficient cells survive the dedifferentiation process, which is likely due to the highlevel of apoptosis in Tcf1-deficient thymocytes (**Fig.S3**). Additionally, the assessment of chromatin status by ATAC-seq revealed that in Tcf1 deficient thymocytes, the chromatin is more condensed and several key T cell specific loci (for instance the Tcrb locus) are less accessible and therefore likely not as readily transcribed and expressed (**Fig.2B&C**). Therefore, the mechanisms underlying dedifferentiation in Pax5 deficiency and as reported here in Tcf1 deficiency appear to be mechanistically different. It should also be noted that formal proof of dedifferentiation in Tcf1 deficiency would require use of a conditional knockout model using a Floxed allele with a Cre enzyme under control of a late acting promoter during thymocyte differentiation. As commitment implies loss of plasticity and the capacity to give rise to only one cell type but not to others, Tcf1 deficiency in contrast is associated with lineage infidelity and lack of commitment.

Recent work has investigated the epigenetic status of DP thymocytes in Tcf1 deficiency, similar to our experiments using DN3 thymocytes [52]. In agreement, Tcf1-/- DN thymocytes also display more condensed chromatin (**Fig.2B**). Yet Tcf1 in the context of T cell commitment and immature thymocyte development seems to act mostly as a transcription factor regulating expression of other key T cell specific genes than acting as a chromatin modifying factor per se. Indeed, an intrinsic HDAC activity has been shown for Tcf1 in CD8+ cells [53]. Our analysis in DN3 T cell populations revealed that only a very small number of sites containing a Tcf1 motif (n= 3 in DN3a, n= 0 in DN3b) gained accessibility in Tcf1-/- cells. This supports an activator rather than a suppressor function for Tcf1 in early T cells. Similar observations, i.e. the majority of sites (80%) lost accessibility in Tcf1-/- DP cells, were reported by others in total DP thymocytes [52], again consistent with a function of Tcf1 as a transcriptional activator. One explanation could be that the HDAC activity of Tcf1 is differentially required (e.g. cell type specific, context dependent manner) and would be different in developing T cells in the thymus versus effector cell maturation

in CD8+ peripheral cells. This is consistent with the observation that HDAC-deficient Tcf1 could largely restore differentiation into the CD4+ lineage [53]. Nevertheless, further analyses will be required to fully understand the activator/repressor functions of Tcf1 in immune cell development.

Given that both *Bcl11b* and *Gata3* are key target genes for Tcf1, we expressed these transcription factors in Tcf1-deficient cells in an attempt to rescue the thymic phenotype. Similar analyses of epistasis have been used previously in model organisms (e.g., *Drosophila*) to delineate both hierarchical and functional relationships. The expression of exogenous *Gata3* has been shown to suppress B cell development in the wild-type thymus [35 54 55]; furthermore, we found that Gata3 also suppresses myeloid fate in DN thymocytes. Interestingly, Gata3 does not suppress myeloid fate in the bone marrow, whereas the effect on B cell development also occurs outside of the thymus.

Our finding that the constitutive expression of Bcl11b in Tcf1-deficient cells fully rescued T cell development suggests a division of labor between Bcl11b and Gata3, with Gata3 suppressing non-T cell lineages and Bcl11b inducing the expression of T cell–specific genes. This is schematically illustrated in figure 6 (**Fig.6**). Taken together, the data from our group and others indicate a gene network in which Notch signaling via RBP-Jκ drives the expression of Tcf1, which in turn activates Gata3 and Bcl11b, most likely in collaboration with Notch signals that can also act directly on these genes' promoters. Importantly, in addition to its requirement for initiating the T cell commitment process, Tcf1 expression is also required to maintain lineage fidelity. In skin stem cells, lineage infidelity increases the likelihood of malignancy [56]. Thus, given that loss of Tcf1 leads to the rapid development of T cell lymphomas [22,23], lineage infidelity may also serve as a previously unrecognized factor in leukemogenesis.

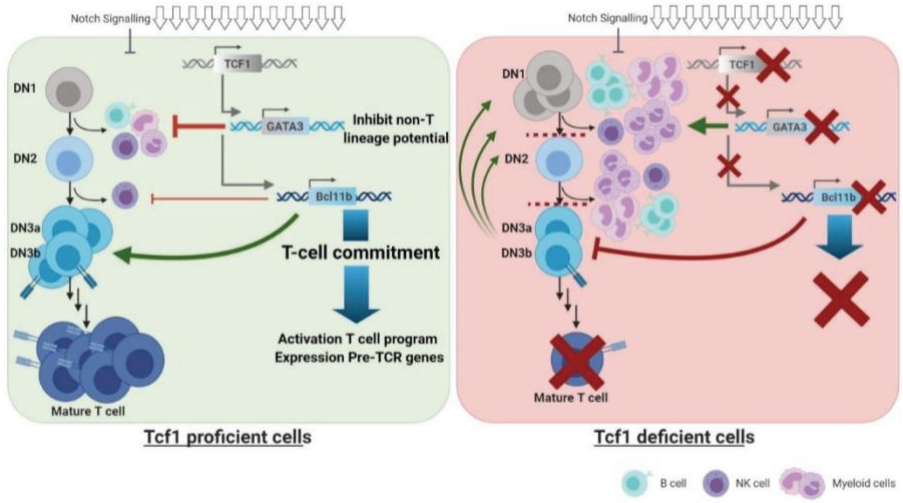

Figure 6: Hierarchy of the core transcription factors in immature T cell development. *On the basis of the proven functional interactions shown in Figs. 4 and 5. Notch signaling (indicated by the open arrow symbols) induces Tcf1 expression that subsequently has two target genes: Gata3 and Bcl11b. Gata3 has a minor role in supporting development along the T cell linage but mainly acts to*

suppress the myeloid and B cell fates. In contrast, Bcl11b induces a T cell–specific program but has minor roles in suppressing alternative lineages with exception of NK cell development that is suppressed by Bcl11b. Collectively, there is a clear functional hierarchy of transcription factors. Potential additional roles for Runx1 and E2A are not shown here.

Supplementary Material

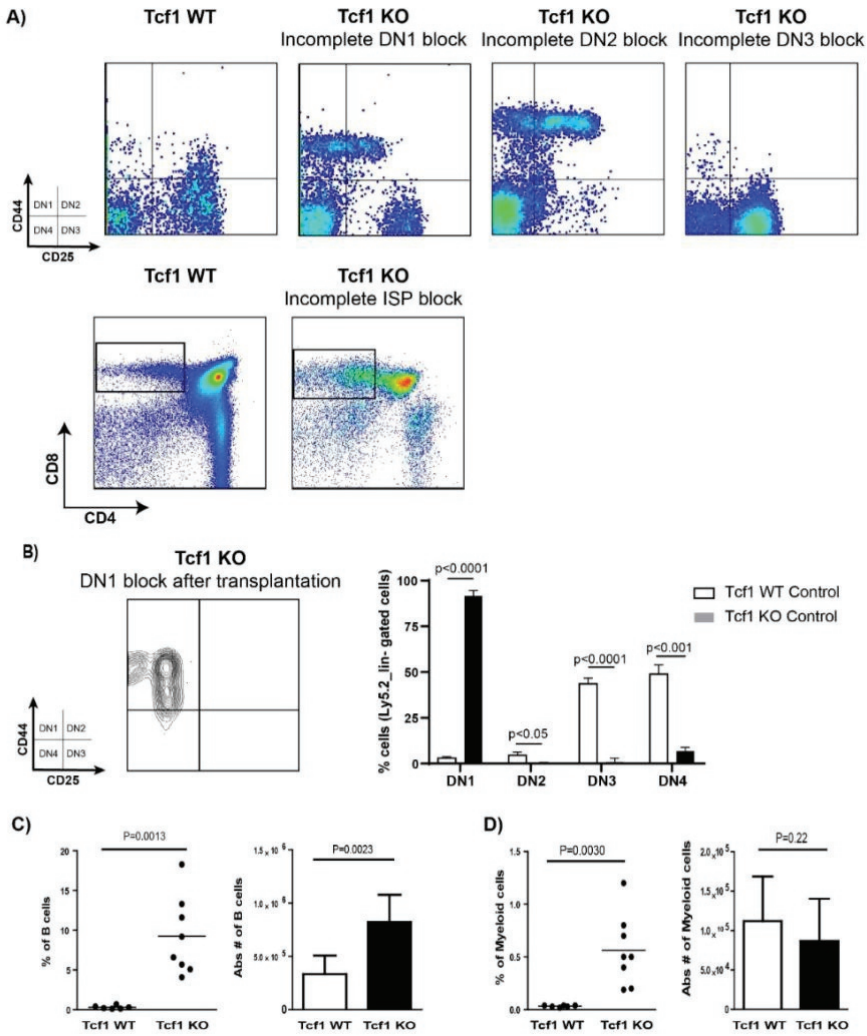

***Figure S1: Tcf1 deficiency leads to several arrests in T cell development with increased non-T cells**. A) Multiple incomplete blocks in T cell development (DN1, DN2, DN3, and ISP) of Tcf1 deficient thymocytes compared to wild-type (pre-gated Thy1+, Lin- cells). B) Transplanted Tcf1 deficient stem cells led to a complete block at the DN1 to DN2 transition in T cell development (pre-gated Thy1+, Lin- cells). C) Increased percentage and absolute number of B cells (B220+CD19+) in Tcf1 deficient thymi compared to wild-type littermates. D) Increased percentage and total number of*

myeloid cells (CD11b+Gr1+) in Tcf1 deficient thymus compared to wild-type littermates. Number of dots indicate number of mice. (Mann-Whitney U test, P<0.05 is statistically significant)

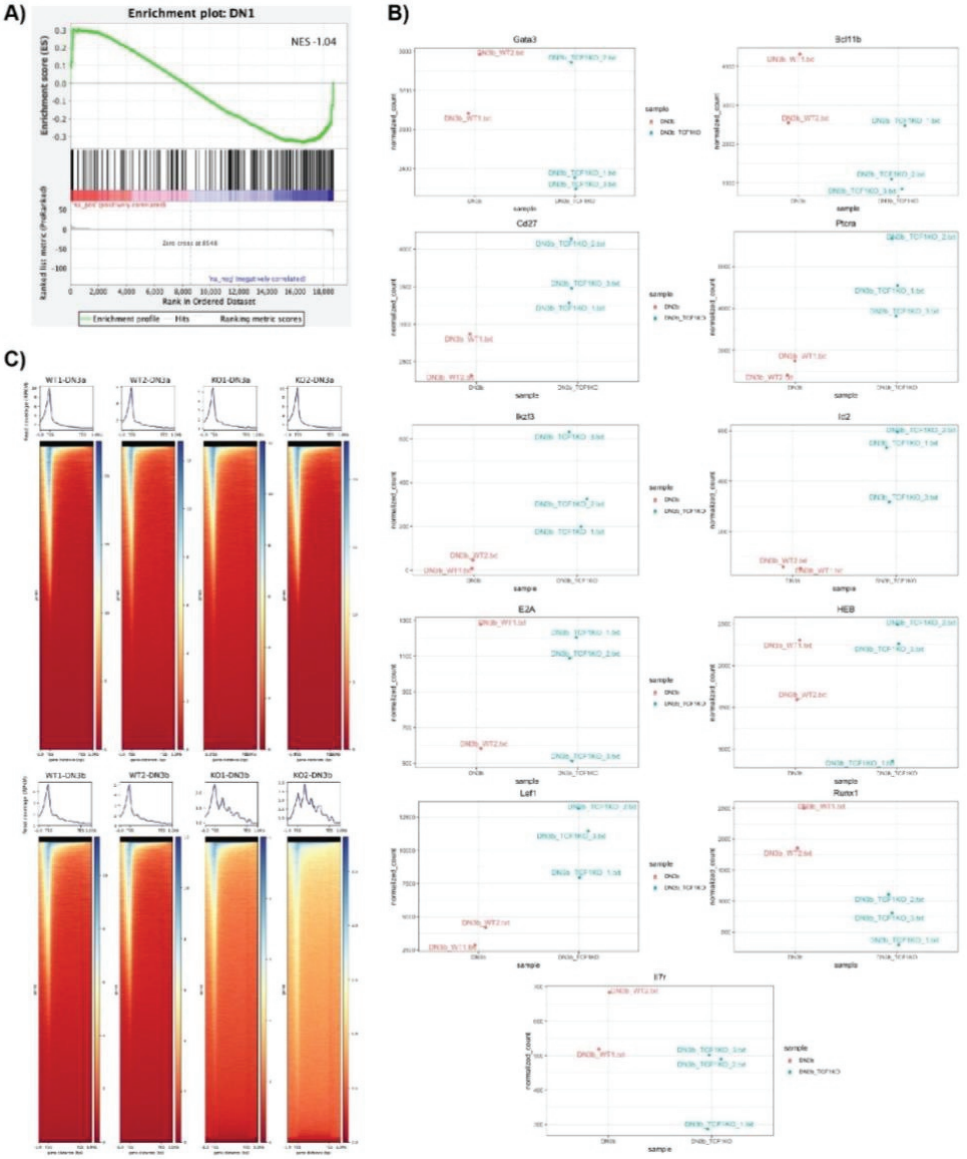

Figure S2: Selected gene expression profile of Tcf1 wt and ko DN3b cells by RNA-seq. *A) GSEA of the differentially expressed genes (Tcf1-/- KO over Tcf1 WT for DN3b) are negatively enriched for DN1 genes (NES -1,04). B) Selected gene expression (normalized count) determined by RNA-seq in DN3b cells from TCF1 wt and deficient cells. C) ATAC-seq read coverage in DN3a and DN3b cells over genes including 1kb downstream and upstream the gene body.*

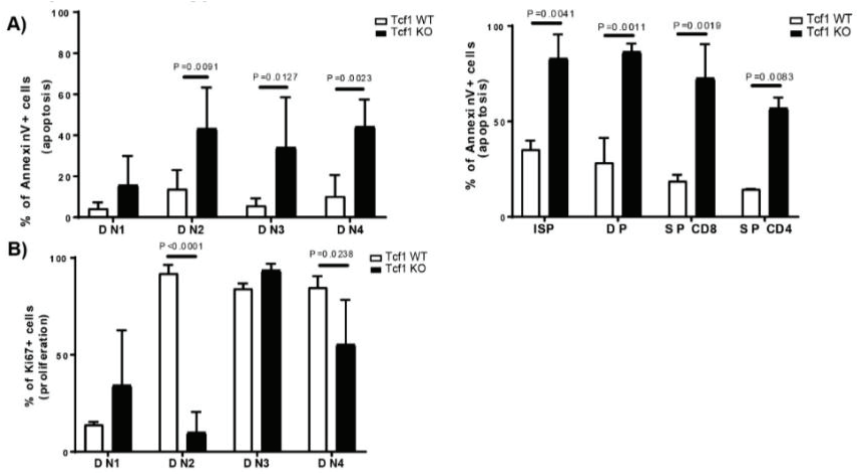

Figure S3: Increased apoptosis and reduced proliferation of Tcf1 deficient thymocytes compared to wild-type cells*. A) Ex vivo wild-type and Tcf1 deficient thymocytes were analysed by flow cytometry for various developmental stages of T cell development in combination with AnnexinV/7AAD. B) Quantification of proliferating cells (Ki67 cells) within the early developmental stage of T cell development of Tcf1 wild-type and Tcf1 deficient thymocytes. Percentage of Annexin V and Ki67 are shown after pre-gating of various subsets *p < 0.05, **p < 0.01 and ***p < 0.001 (Mann-Whitney U test). Error bars represent the SD of three samples from individual mice in two independent experiments.*

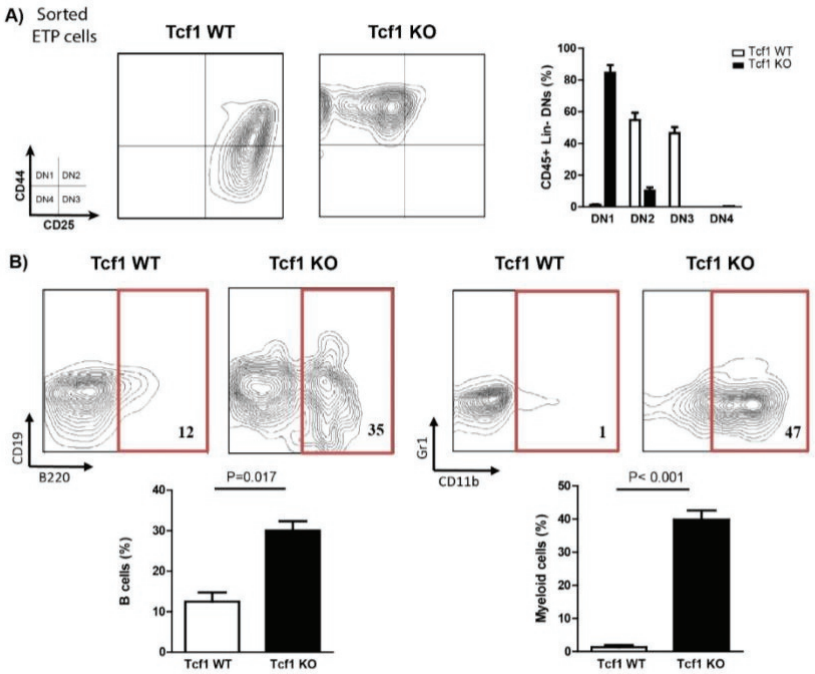

Figure S4: Tcf1 deficient sorted ETP cells are arrested in development at DN1 stage in culture, with prominent B and myeloid development compared to wild-type sorted ETPs*. A) Sorted wild-type ETP cells seeded on OP9-DL1 cells differentiate along the T cell lineage while sorted Tcf1 deficient cells are blocked in development at the DN1 stage. B) Sorted ETPs cells from Tcf1 deficient thymi show abundant B and myeloid development on OP9-DL1 compared to wild-type sorted ETPs. (Mann-Whitney U test. Error bars represent the SD of three samples from three independent experiments.)*

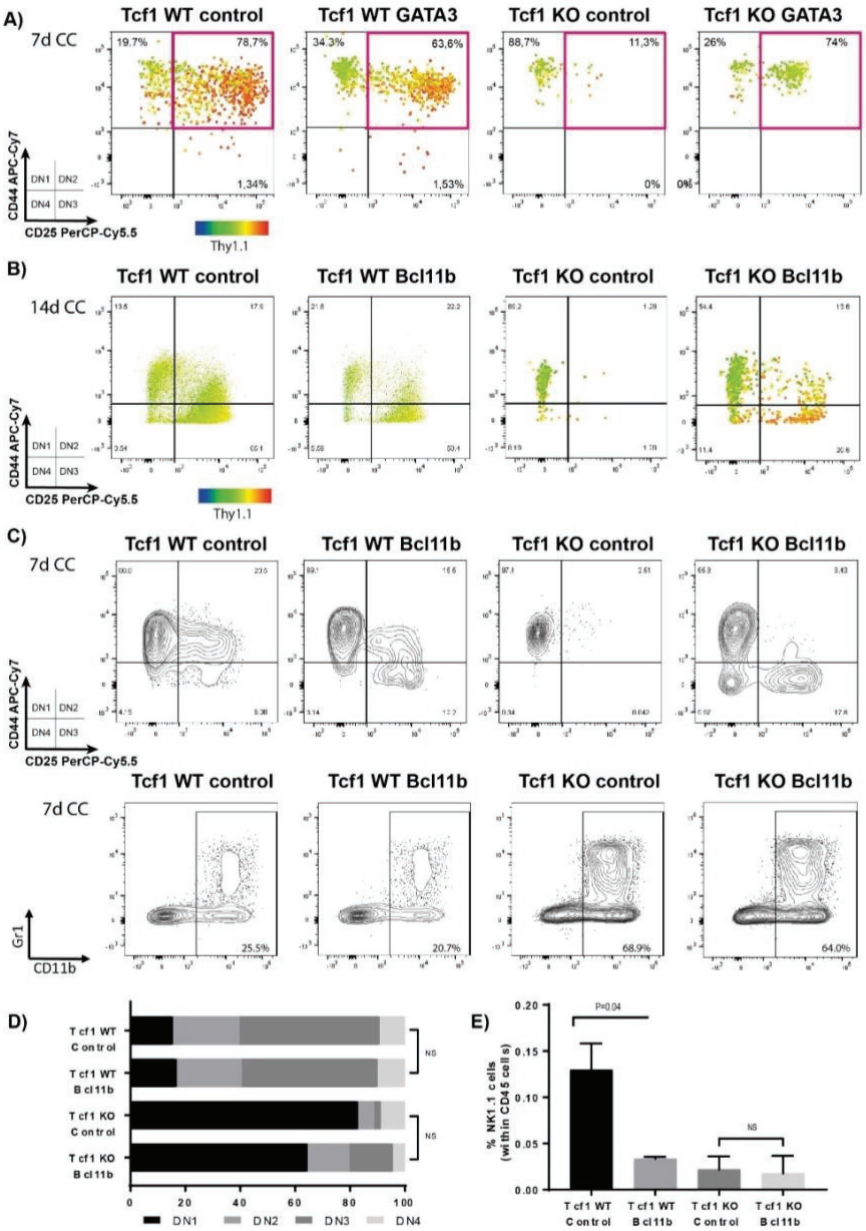

***Figure S5: OP9-DL1 coculture cell development**. A) Gata3 expression partially overcomes the DN1 thymocyte block, but do not induce Thy1 increasing expression through development after 7 days in the OP9-Dl1 culture system. B) Bcl11b fully rescues T cell development from Tcf1 -/- stem cells, with increased in Thy1 expression, that otherwise are arrested in DN1 after 14 days in OP9-Dl1 culture system. (Thy1 is displayed using median color mapping). C) Bcl11b transduced cells are developing through the DN2 stage after 7d in the OP9-Dl1 system. Tcf1-/- cells shows a higher percentage of myeloid cells in culture compared to Tcf1 wt cells, but Bcl11b overexpression does not*

affect that populations. D) Untransduced Tcf1 deficient and wild-type cells (GFP- cells) with Bcl11b preserve control phenotype. Untransduced Tcf1 deficient cells are arrested at DN1/DN2 transition after 14d on OP9-DL1 as the control cells. (pre-gated Thy1+Lin-GFP- cells) E) Bcl11b overexpression does suppress the development of NK cells in wild-type cells after 14d on OP9-DL1. (Two-way ANOVA. Error bars represent the SD from three independent experiments.)

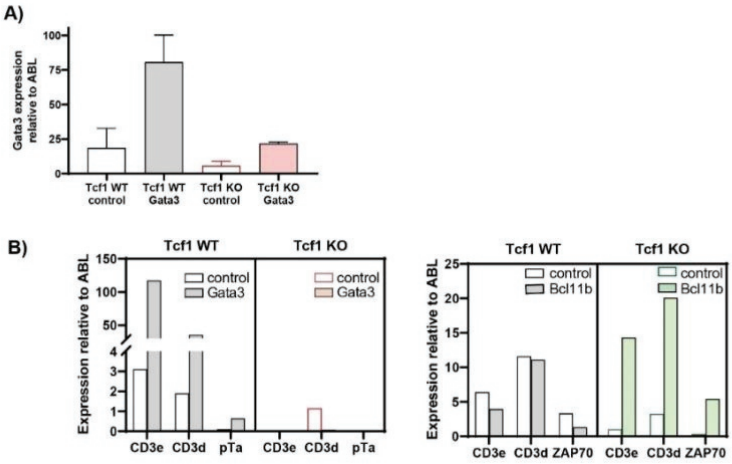

***Figure S6: T cell receptor genes expression in co-culture experiments**. A) Gata3 expression levels relative to ABL determined by qPCR from cells harvested after 7 days in culture. Tcf1-/- reach normal wt levels of Gata3 after transduction. (Error bars represent the SD from two independent experiments.) B) Expression of T cell receptor genes (CD3e, CD3d, pTa, ZAP70) relative to ABL determined by qPCR after 7 days (Gata3) and 14 days (Bcl11b) OP9 DL1 culture. Only Bcl11b overexpression in Tcf1-/- cells (not Gata3) is able to rescue expression of T cell receptor genes.*

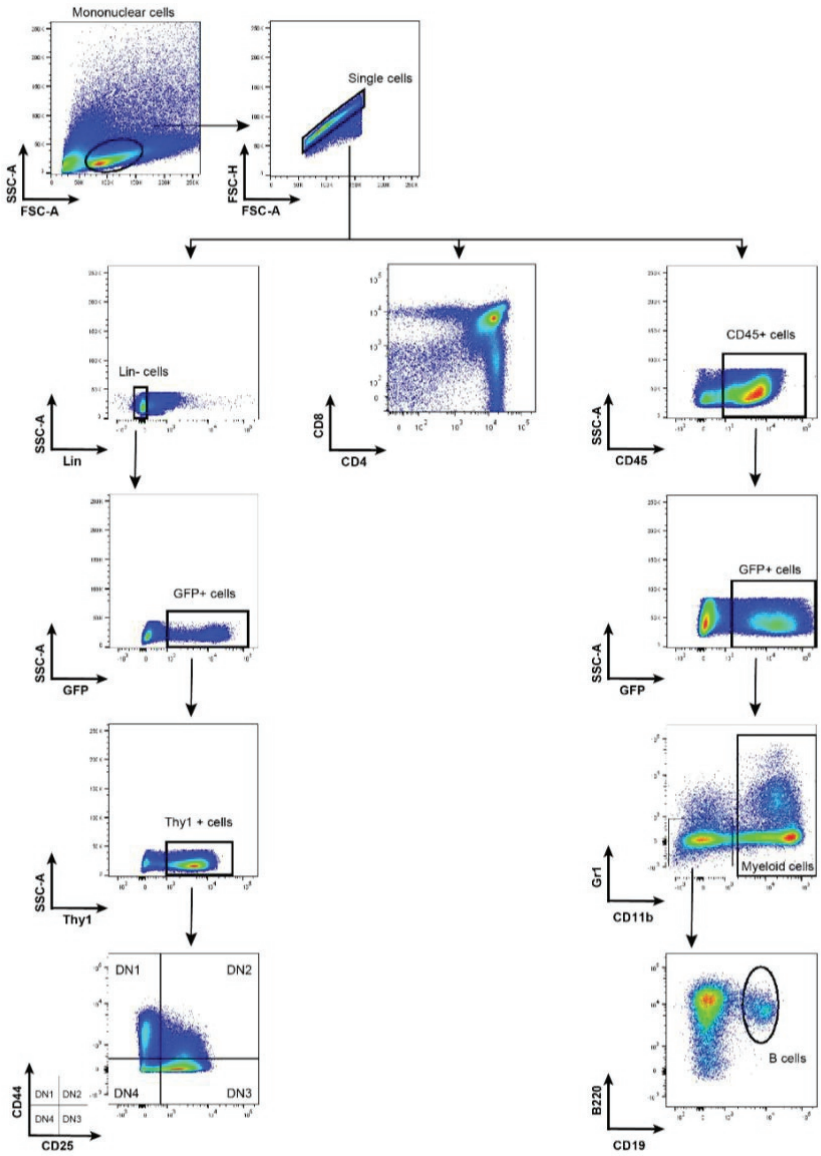

Figure S7: Flow cytometric gating strategy

Table S1. Deseq Differential expression DN3b v.3 (Excel file)

Table S2. The full genome wide ATACseq data analysis of sorted DN3 Tcf1 wt vs ko (Excel file)

Table S 3: List of antibodies used in the study (including manufacturer, clone, catalog number)

Antibodies	SOURCE	IDENTIFIER
Anti-CD3e antibody (clone 145-2C11)	BD Biosciences	Cat# 553060 RRID:AB_394593
Anti-CD3e antibody (clone 145-2C11)	BD Biosciences	Cat# 553062 RRID:AB_394595
Anti-CD4 antibody (clone H129.19)	BD Biosciences	Cat# 553648, RRID:AB_394968
Anti-CD4 antibody (clone RM4-5)	eBioscience	Cat# 25-0042-82, RRID:AB_469578
Anti-CD8a antibody (clone 53-6.7)	BD Biosciences	Cat# 553029, RRID:AB_394567
Anti-CD8a Monoclonal Antibody (Clone 53-6.7)	BD Biosciences	Cat# 553035, RRID:AB_398527
Anti CD11b/MAC1 antibody (clone M1/70)	Biolegend	Cat# 101204, RRID:AB_312787
Anti-CD11b Monoclonal Antibody (Clone M1/70)	BD Biosciences	Cat# 553311, RRID:AB_394775
Anti-CD19 Monoclonal Antibody (Clone 1D3)	BD Biosciences	Cat# 550992, RRID:AB_398483
Anti-CD25 (IL2Ra/p55) antibody (Clone PC61)	BD Biosciences	Cat# 553866, RRID:AB_395101
Anti CD27 Monoclonal Antibody (Clone LG.7F9)	eBioscience	Cat# 11-0271-82, RRID:AB_465001
Anti-CD44 antibody (Clone IM7)	BD Biosciences	Cat# 560568, RRID:AB_1727481
Anti-CD45.1 Monoclonal Antibody (Clone A20)	eBioscience	Cat# 25-0453-82, RRID:AB_469629
Anti-CD45.2 Monoclonal Antibody (Clone 104)	BD Biosciences	Cat# 552950, RRID:AB_394528
Anti-CD45R/B220 antibody (Clone RA3-6B2)	BD Biosciences	Cat# 553085, RRID:AB_394615
Anti-CD45R (B220) antibody (Clone RA3-6B2)	eBioscience	Cat# 25-0452, RRID:AB_2341160
Anti-CD90.2 (Thy-1.2) antibody(Clone 53-2.1)	eBioscience	Cat# 17-0902-81, RRID:AB_469421
Anti-CD117 antibody (Clone 2B8)	BD Biosciences	Cat# 558163, RRID:AB_647250
Anti-CD117 Monoclonal Antibody (Clone 2B8)	BD Biosciences	Cat# 553356, RRID:AB_398536
Anti-Ly-6G, Ly-6C antibody (Clone RB6-8C5)	BD Biosciences	Cat# 553124, RRID:AB_394640
Anti-Ly-6G (Gr-1) Monoclonal Antibody (Clone RB6-8C5)	eBioscience	Cat# 48-5931-80, RRID:AB_1548797
Anti-NK-1.1 antibody (Clone PK136)	BD Biosciences	Cat# 553163, RRID:AB_394675
Anti-TER-119 antibody (Clone TER-119)	BD Biosciences	Cat# 553672, RRID:AB_394985
Anti-TCR beta Monoclonal Antibody (Clone H57-597)	BD Biosciences	Cat# 553174, RRID:AB_398534
Anti-mouse TCR Vb5.1, 5.2 antibody (Clone MR9-4)	Biolegend	Cat# 139504, RRID:AB_10613279
Anti-mouse TCR Vb6 antibody (Clone RR4-7)	Biolegend	Cat# 140003, RRID:AB_10640727
Anti-mouse Vb8 antibody (Clone F23.1)	BD Biosciences	Cat# 555604, RRID:AB_395975
Anti-Ly-6A/E (Sca-1) Monoclonal Antibody (Clone D7)	eBioscience	Cat# 25-5981-82, RRID:AB_469669
TCF1 (C46C7) Rabbit mAb antibody	Cell Signalling Technologies	Cat# 2206S, RRID:AB_2199300

Table S 4: List of markers used to define all different hematopoietic populations in the study.

Subset	Markers
LSK	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $c\text{-}Kit^+$ $Sca1^+$
LK	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $c\text{-}Kit^+$ $Sca1^-$
ETP	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^-$ $CD44^+$ $c\text{-}Kit^+$
DN1	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^-$ $CD44^+$ $c\text{-}Kit^-$
DN2	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^+$ $CD44^+$
DN3a	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^+$ $CD44^-CD27^-$
DN3b	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^+$ $CD44^-CD27^+$
DN4	Lin^- ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $CD11b^-$ $NK1.1^-$ $GR1^-$ $Ter\text{-}119^-$) $CD25^-$ $CD44^-$
ISP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ $Ter\text{-}119^-$) $CD3^-$ $CD4^-$ $CD8^+$
DP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ Ter^-119^-) $CD4^+$ $CD8^+$
CD4 SP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ Ter^-119^-) $CD3^+$ $CD4^+$ $CD8^-$
CD8 SP	Lin^- ($B220^-$ $CD11b^-$ $NK1.1^-$ Ter^-119^-) $CD3^+$ $CD4^-$ $CD8^+$
B cell (Mature)	$B220^+$ $CD19^+$
Granulocytes	$CD11b^+$ $Gr1^+$
Monocytes	$CD11b^+$ $Gr1^-$

Table S 5: Name ad sequences of used primers

	Name	Sequences
Genomic qPCR primers	mGata3	F: CTTATCAAGCCCAAGCGAAG
		R: CCCATTAGCGTTCCTCCTC
	mBcl11a	F: CCCCGCAGGGTATTTGTA
		R: TGAATGGCTGTTTGCAAGTT
	mBcl11b	F: TGTCCCAGAGGGAACTCATC
		R: GGCTGCTTGCATGTTGTG
	mPax5	F: ACGCTGACAGGGATGGTG
		R: GGGGAACCTCCAAGAATCAT
	mPU.1/Spi1	F: GGGATCTGACCAACCTGGA
		R: AACCAAGTCATCCGATGGAG
	mIL-7Ra	F: GATCCATTCCCCATAACGATT
		R: CAGGATCCCATCCTCCTTG
	mCD117/c-kit	F: GGAGCCACAATAGATTGGTAT
		R: CACTGGTGAGACAGGAGTGGT
	mID2	F: GACAGAACCAGGCGTCCA
		R: AGCTCAGAAGGGAATTCAGATG
	mAxin-2	F: AGTCCATCTTCATTCCGCCTAGC
		R: AAGCTGCGTCGGATACTTGAGA
	mHes-1	F: 5'-AAACACTGATTTTGGAGCACT-3'

		R: 5'-TGCTTCACAGTCATTTCCAGA-3'
	Abl-2	F: CAACGTCTTCACCCAGCAC
		R: TCCAGTATTGTCTCCCTCAAA
	CD3e	F: CTTGTACCTGAAAGCTCGAGTG
		R: TGTGATTATGGCTACTGCTGTC
	CD3d	F: TGCTTTGCAGGACATGAGAC
		R: CGATCTCGAAGAGGCTGTAC
	pTa	F: CTGTCAGGGGAATCTTCGAC
		R: GTACCTGCCGCTGTGTCC
	ZAP70	F: AGAAGCACTCATGCTGGTCA
		R: GTTCAGCCACATTGCTCACA
ChIP primers	Gata3-1b promotor:	F: 5' GTACACGGTACTTCGGGGAC 3'
		R: 5' AGGACCTGGGCTTTGATTCG 3'
	Enhancer Bcl11b:	F: 5' CCAACAGCACTGGGGATTCT 3'
		R: 5' ACTTGGGCTGAACTTGCTGA 3'

F: forward primer; R: reverse primer

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