

# Multiple layers of transcriptional regulation by PLZF in NKT-cell development

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**The transcription factor PLZF [promyelocytic leukemia zinc finger, encoded by zinc finger BTB domain containing 16 (*Zbtb16*)] is induced during the development of innate and innate-like lymphocytes to direct their acquisition of a T-helper effector program, but the molecular mechanisms involved are poorly understood. Using biotinylation-based ChIP-seq and microarray analysis of both natural killer T (NKT) cells and PLZF-transgenic thymocytes, we identified several layers of regulation of the innate-like NKT effector program. First, PLZF bound and regulated genes encoding cytokine receptors as well as homing and adhesion receptors; second, PLZF bound and activated T-helper-specific transcription factor genes that in turn control T-helper-specific programs; finally, PLZF bound and suppressed the transcription of *Bach2*, a potent general repressor of effector differentiation in naive T cells. These findings reveal the multilayered architecture of the transcriptional program recruited by PLZF and elucidate how a single transcription factor can drive the developmental acquisition of a broad effector program.**

PLZF | NKT | lymphocyte | development

Natural killer (NK) T (NKT) cells are a conserved population of innate-like T cells that express CD1d-restricted semi-invariant  $\alpha\beta$  T-cell receptors (TCRs), using mostly the V $\alpha$ 14-J $\alpha$ 18 chain in mouse (V $\alpha$ 24-J $\alpha$ 18 in human) combined with variable V $\beta$ 8, V $\beta$ 7, and V $\beta$ 2 (V $\beta$ 11 in human) chains, which confer recognition of conserved self and foreign lipids (1–3). NKT cell precursors arise during thymic development at the CD4<sup>+</sup>CD8<sup>+</sup> double-positive stage and undergo massive expansion on interaction with CD1d ligands expressed on cortical thymocytes. They also characteristically acquire an effector CD44<sup>hi</sup>CD62L<sup>lo</sup> phenotype along with receptors of the NK cell lineage.

The innate-like effector functions of NKT cells are illustrated by the cells' ability to promptly secrete large quantities of both IL-4 and IFN- $\gamma$  either after TCR activation or on exposure to tissue- and antigen-presenting cell-derived cytokines, such as IL-25+IL-33 and IL-12+IL-18, respectively. Whereas C57BL/6 mice produce predominantly NKT cells with a T-bet-dependent type 1 helper phenotype (NKT1), other strains, including BALB/c, also express substantial populations of so-called NKT2 and NKT17 cells with polarized type 2 and type 17 helper programs controlled by GATA3 and ROR $\gamma$ t, respectively.

The BTB-zinc finger transcription factor PLZF (promyelocytic leukemia zinc finger, encoded by *Zbtb16*), is specifically expressed in NKT cells, but not in conventional T cells or NK cells, and directs the acquisition of several key components of the NKT cell effector program during development, including cytokine and migration properties (4–7). Mutations or deletion of *Zbtb16* abrogate both the expansion and the effector-memory differentiation of NKT cells, resulting in reversal to a naive phenotype and redistribution to the lymph nodes and circulating blood. PLZF expression is also essential for the development of other innate-like T cells, including MR1-specific semi-invariant  $\alpha\beta$  T cells and  $\gamma\delta$  T cells expressing the V $\gamma$ 1.1-V $\delta$ 6.3 TCR (4, 8). Importantly, ectopic expression of PLZF in the CD4 lineage during thymic development converts conventional naive CD4<sup>+</sup> thymocytes into CD44<sup>hi</sup>CD62L<sup>lo</sup>

effector cells capable of producing both type 1 and type 2 cytokines (9, 10), suggesting that PLZF is not only required, but also sufficient, for acquisition of the innate T-cell effector program. Notably, PLZF was recently found to be transiently expressed during the development of innate lymphoid cells (ILCs), defining a common dedicated precursor to ILCs, the ILCP, and to significantly impact the development and function of ILC lineages (11, 12). Taken together, these findings indicate a broad defining role of PLZF in the differentiation of several innate and innate-like lymphocytes.

The molecular mechanisms underlying these remarkable properties of PLZF are only partially understood. In a  $\gamma\delta$ TCR transgenic mouse model, the genetic inactivation of PLZF was found to compromise expression of the transcription factors *Id2* and *c-Maf*, which are important for the survival of NKT cells in the liver and the production of IL-4, respectively (13). PLZF is essential for the expression of genes encoding cell surface receptors that shape responses to external stimuli, including costimulatory receptor *Icos* and cytokine receptors *Il12rb1* and *Il18r1*, as well as *Cd44*. In addition, PLZF down-regulates *Sell*, which encodes the homing receptor CD62L. The precise molecular mechanisms underlying these regulatory properties, and in particular the question of whether PLZF directly or indirectly controls the expression of effector genes, have not yet been elucidated, however.

Here, using biotinylation-based ChIP-seq and microarray analysis, we identified the PLZF target genes in NKT cells. We found that many of the genes involved in the acquisition of an innate effector program, including T-helper-specific transcription factors,

## Significance

Natural killer T (NKT) cells are a specialized population of innate-like T cells that acquire their effector program during development under the control of the transcription factor PLZF (promyelocytic leukemia zinc finger, encoded by *Zbtb16*). To elucidate the mechanisms underlying this unique property of PLZF, we performed ChIP-seq and microarray analysis of NKT cells and PLZF-transgenic T cells, which revealed direct regulation of effector genes and of T-helper-specific transcription factors. Notably, PLZF also bound and repressed *Bach2*, a global repressor of effector differentiation. Thus, multiple layers of positive and negative regulation coordinate the induction of the innate effector program by PLZF.

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The authors declare no conflict of interest.

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Data deposition: The ChIP-seq and microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE81772).

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cytokine receptors, and other key surface receptors, are directly bound by PLZF. Notably, we also found that PLZF binds and suppresses *Bach2*, a broad and potent regulator of effector programs. These results elucidate how a single transcription factor can act at multiple levels to regulate the developmental acquisition of the innate effector gene program.

## Results

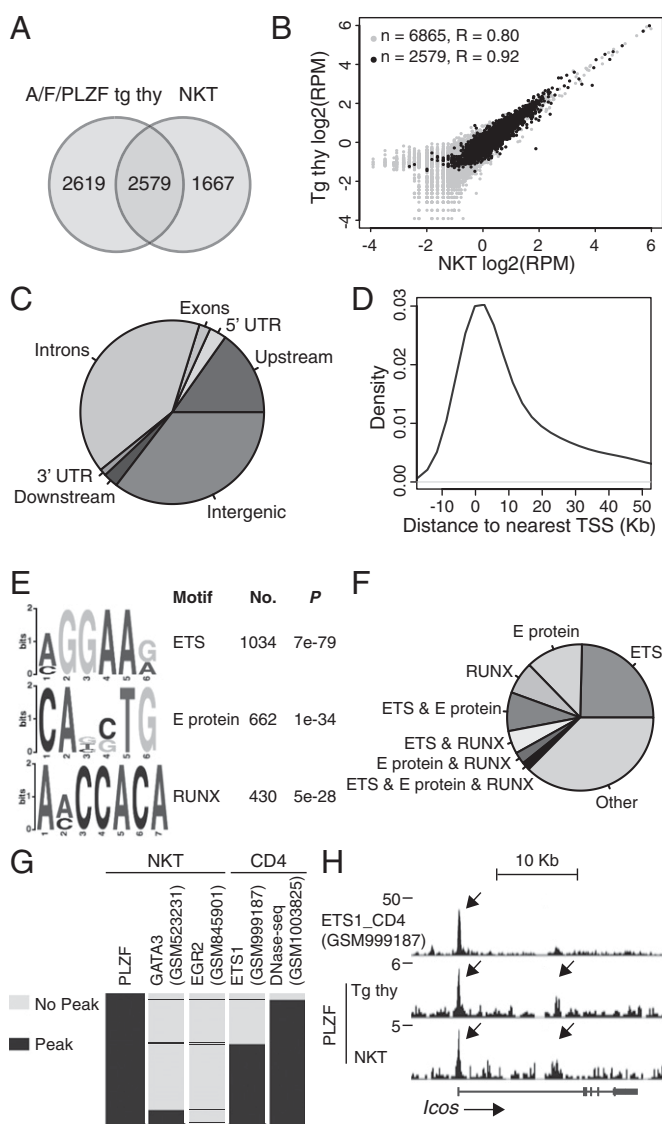
**PLZF Predominantly Binds DNA at Regulatory Sites Carrying Consensus Sequences for ETS, RUNX, and E Proteins.** We identified the direct targets of PLZF in NKT cells through ChIP-seq analysis. Because ChIP-seq with various anti-PLZF antibodies was not successful in NKT cells, we generated a transgenic strain expressing a biotin acceptor peptide and a 3×Flag at the N terminus of PLZF (A/F/PLZF tg) under control of the *Cd4* promoter, and crossed it to mice expressing a bacterial biotin ligase BirA transgene. In these mice, PLZF could be specifically biotinylated *in vivo* without altering its interaction with major binding partners, such as CUL3 and HDAC1, or its effector-promoting function (Fig. S1). We used magnetic streptavidin bead pull-down of chromatin from A/F/PLZF tg; BirA tg thymocytes (referred to as Tg thy) or from purified Vα14-Jα18 tg; A/F/PLZF tg; BirA tg NKT thymocytes (referred to as NKT cells) before DNA sequencing. Using the model-based analysis of ChIP-seq (MACS) peak calling software with a *P* value threshold of  $1e-5$ , we identified 5,198 peaks in the Tg thy and 4,246 peaks in the NKT cells, of which 2,579 peaks were shared (Fig. 1A and B).

Visual comparison of peaks in the Tg thy and NKT cells confirmed that the binding landscape of PLZF was largely overlapping in both mouse models. Even when a peak was called in only one model, close inspection often revealed a subthreshold accumulation of reads in the other model. In fact, the analysis of enrichment for consensus sequences produced similar results in both sets of peaks and in the shared peaks. PLZF binding sites were over-represented in the upstream and intronic regions of genes (Fig. 1C). Density plot analysis suggested that most gene-related peaks were within 10 kb of transcription start sites (TSS) (Fig. 1D).

To explore whether PLZF binding sites contain consensus binding motifs, we examined peaks for the presence of known and novel motifs. The top three motifs, which together account for more than 60% of PLZF-occupied sites, contained consensus sequences for ETS, E proteins, and RUNX factors (Fig. 1E and F). In support of these findings, comparison with published ETS1 ChIP-seq analysis of CD4 T cells (14) revealed that nearly 60% of PLZF binding sites identified in NKT thymocytes overlapped with ETS1 peaks in CD4 T cells (Fig. 1G and H). In contrast, only minor fractions of PLZF binding sites overlapped with published GATA3 (15) and EGR2 (16) ChIP-seq peaks in NKT cells. Similar findings were obtained by comparing the ChIP-seq profiles of Tg thy and NKT cells with a large database containing thousands of ChIP-seq data from a variety of cell types. In addition, >95% of shared PLZF binding sites were found to be DNase-accessible in CD4 T cells (Fig. 1G). These results suggest that rather than binding its own specific DNA motif, PLZF predominantly binds close to canonical transcription factors prominently expressed in lymphoid cells.

We also performed a ChIP-seq analysis of the human myeloid KG1a cell line (17), which naturally expresses PLZF and in which anti-PLZF antibodies efficiently pull down chromatin, to compare peaks across different cell types. Interestingly, the most strongly enriched consensus motif in the PLZF KG1a ChIP-seq peaks was for nuclear respiratory factor 1 (NRF1). This motif was not significantly enriched in NKT cells, indicating that PLZF likely binds different landscapes of regulatory elements in the two cell types (Fig. S2). A canonical ETS motif similar to the top consensus sequence in NKT cells was also found in KG1a, however, indicating overlap between the different cell types.

**PLZF Binds a Broad Set of Immune Effector Genes Expressed by NKT Cells.** Although microarrays of Vα14-Jα18 NKT cells have been published and their functional program has been well described compared with that of CD4 T cells (13, 18, 19), the specific



**Fig. 1.** Distribution of PLZF ChIP-seq peaks. (A) Overlap of PLZF-bound peaks in A/F/PLZF Tg thy and NKT cells. Peaks were called with the MACS program with a *P* value cut-off of  $1e-5$ . The minimum overlap required was 1 bp. (B) Scatterplot showing the correlation of peak reads per million (RPM) of all (gray) and overlapping (black) PLZF-bound peaks in Tg thy and NKT cells. (C and D) Distribution of PLZF peaks (C) and distances to the TSSs (D). PLZF was considered associated with a gene if a MACS peak was located anywhere from 5 kb upstream of TSS to 2 kb downstream of a TTS. (E) The top three motifs identified by MEME-ChIP. (F) Proportion of peaks containing the indicated motifs or their combinations. (G) Heatmap showing the overlap of PLZF peaks with GATA3, EGR2, and ETS1 and the DNase-seq peaks in indicated cell types. The ChIP-seq data for GATA3, EGR2, and ETS1 and the DNase-seq data for CD4 T cells were downloaded from the GEO database with accession numbers as shown. Peaks were called with the MACS program with a *P* value cut-off of  $1e-6$ . A total of 3,946, 1,262 and 14,061, and 135,874 peaks were called for GATA3, EGR2, and ETS1 ChIP-seq and DNase-seq, respectively. (H) ETS1 and PLZF ChIP-seq reads aligned at the *Icos* gene locus. The peaks are indicated by arrows.

contribution of PLZF to the NKT cell transcriptional program has not yet been elucidated by gain-of-function and loss-of-function studies. Using microarray analysis, we compared Vα14-Jα18 stage 1 NKT thymocytes in WT and PLZF-deficient backgrounds (loss of function), as well as CD4 SP thymocytes in WT and PLZF-transgenic backgrounds (gain of function). At a twofold change cut-off, PLZF up-regulated or down-regulated a total of 336 genes distributed in regions C1–C6, as shown in the scatterplot in Fig.

24. Thus, PLZF was required for 168 genes (in regions C1, C3, C4, and C6), sufficient for 223 genes (in C1, C2, C5, and C6), and necessary and sufficient for 55 genes (in C1 and C6). There was no example of discordant gene regulation by PLZF in NKT vs. PLZF Tg cells. Notably, similar frequencies of genes were up-regulated or down-regulated in the presence of PLZF. Of the 336 PLZF-regulated genes identified in C1–C6 and displayed in a heat map across the four T-cell populations (Fig. 2B), on average 43% were directly bound by PLZF, as determined by ChIP-seq analysis.

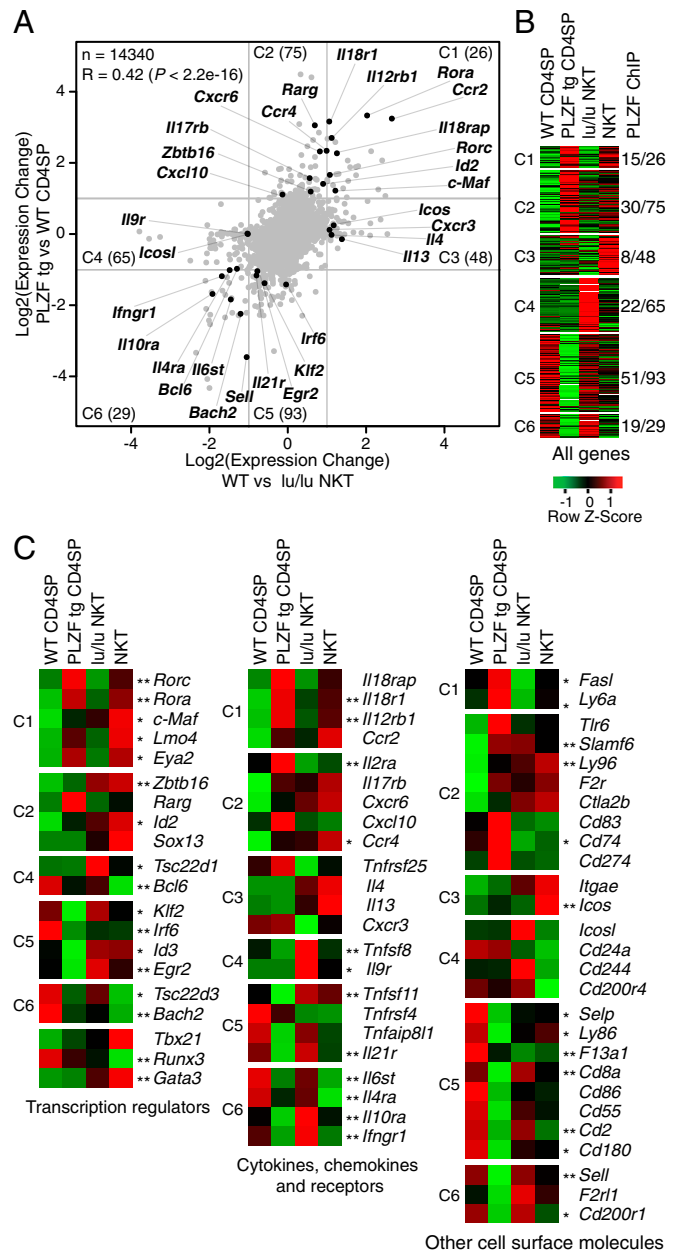
We isolated a group of 70 PLZF-regulated “immune” genes and broke them down by such categories as transcription regulators, cytokines/chemokines and their receptors, and other cell surface molecules (Fig. 2C). These immune genes were bound by PLZF, as denoted by asterisks in the figure, at frequencies ranging from 52% for surface receptors to 80% for transcription factors. Thus, PLZF directly binds a majority of PLZF-regulated immune effector genes, many of which have critical functions in the NKT cell effector program, as detailed below.

**PLZF Binds and Controls Key Homing and Adhesion Receptor Genes.** Among the key genes whose down-regulation characterizes the NKT cell effector program is *Sell*, which encodes CD62L and is required for recirculation to lymph nodes. PLZF directly bound this gene in both NKT cells and Tg thy (Fig. 3A). Furthermore, PLZF also bound and down-regulated the transcription factor *Klf2*, a major positive regulator of *Sell* (20, 21) (Fig. 3B). Another key direct target of PLZF, *Cd44*, encodes an adhesion receptor (Fig. 3C). Thus, PLZF directly regulates the CD44<sup>hi</sup>CD62L<sup>lo</sup> nonrecirculating effector phenotype of NKT cells.

**PLZF Binds and Controls Critical Cytokine Receptors and Chemokine Receptors.** A major function of NKT cells is to respond to cytokines, such as IL-12 and IL-18, produced by dendritic cells (DCs) by secreting abundant IFN- $\gamma$ , even in the absence of exogenous antigen (22–25). PLZF directly bound and regulated *Il12rb1* and *Il18r1*, as well as *Ifngr1*, in both NKT cells and Tg thy (Fig. 3D–F). PLZF also bound and repressed *Il21r* (Fig. 3G). In addition, PLZF bound and activated *Ccr4*, which encodes a chemokine receptor that enables cell attraction toward CCL17-producing DCs and enhances the cross-presentation of antigen to CD8 T cells (Fig. 3H) (26). PLZF also bound and induced *Icos*, which can engage the ICOS ligand to activate DCs (Fig. 1H) (27).

**PLZF Indirectly Controls Cytokine Secretion and T-Helper Function by Binding T-Helper–Specific Transcription Factors.** Although PLZF did not bind cytokine loci encoding IL-4, IL-13, IFN- $\gamma$ , or IL-17 (Fig. S3), it did directly bind and/or activate multiple T-helper–specific transcription factors (Fig. 4). These factors included *Gata3*, *c-Maf*, *Runx3*, *Rorc*, and *Rora*, as was also confirmed by ChIP-quantitative PCR (qPCR) for *Gata3* and *c-Maf*. Notably, there was no detectable binding to *Tbx21*, suggesting that its up-regulation in NKT cells may be mediated by another pathway, perhaps through *Runx3*, which was bound by PLZF. PLZF also directly bound and repressed *Bcl6* (Fig. 4G), which controls T follicular helper (Tfh) cell function, perhaps explaining the relatively weak Tfh potential of NKT cells (28).

**PLZF Binds and Represses *Bach2*, a Broad Suppressor of T-Helper Effector Genes.** One of the most profoundly repressed genes in both NKT cells and PLZF-transgenic T cells was *Bach2* (Fig. 2), a transcription factor that is normally mostly expressed in naïve T cells and down-regulated in effector T cells. *Bach2* has recently emerged as a broad, direct repressor of T-helper effector genes and is one of the genes most conspicuously associated with autoimmune diseases in multiple genome-wide association studies (29–31). We confirmed that *Bach2* was down-regulated at the protein level in both NKT thymocytes and PLZF Tg CD4 SP thymocytes compared with WT CD4 SP thymocytes (Fig. 5A). Furthermore, PLZF showed extensive binding to multiple enhancer regions of *Bach2* in the ChIP-seq analysis of both NKT thymocytes and PLZF-transgenic thymocytes, which was confirmed



**Fig. 2.** PLZF-regulated transcriptional program. (A) Scatterplot showing differentially expressed genes in the indicated cell type comparisons. Gray lines delineate the twofold change thresholds. Select immune genes are highlighted. Data are shown as mean values of two to three independent experiments. (B) Heatmap of all differentially expressed genes grouped according to indicated regions C1–C6 in the scatterplot with the corresponding number of genes bound by PLZF. Binding of a gene was defined by a peak ( $P = 1e-4$ ) between 50-kb upstream of the TSS and 2 kb downstream of the TTS. Data are shown as mean values of two to three independent experiments. (C) Heatmap of select immune genes. Single and double asterisks denote genes bound by PLZF in either Tg thy or NKT thymocytes or in both, respectively. Data are shown as mean values of two to three independent experiments.

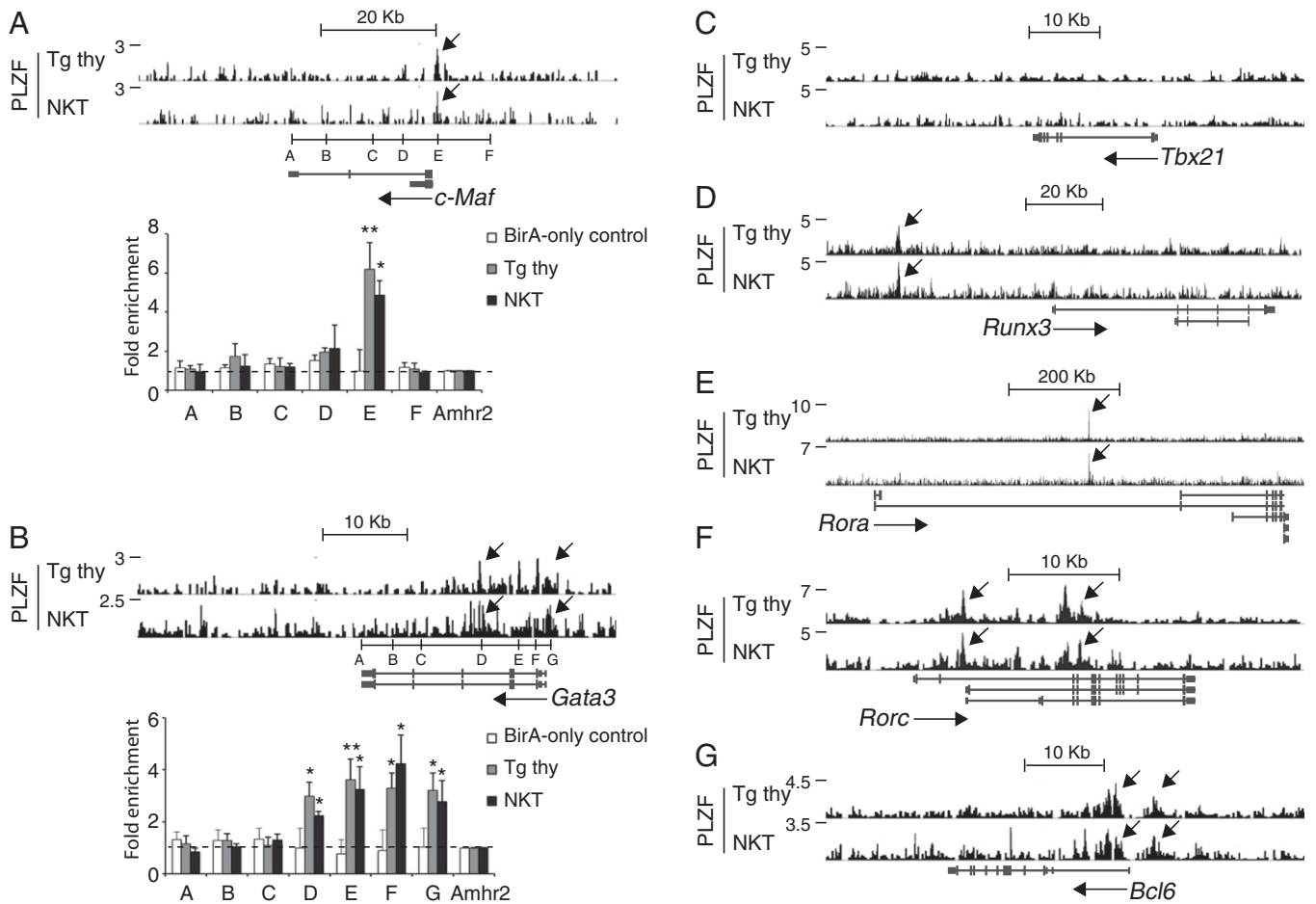
by ChIP-qPCR (Fig. 5B), supporting direct repression of *Bach2* transcription by PLZF in NKT cells.

**Discussion**

By combining ChIP-seq analysis with PLZF gain-of-function and loss-of-function studies, we have elucidated several molecular mechanisms that together explain how PLZF alone can direct



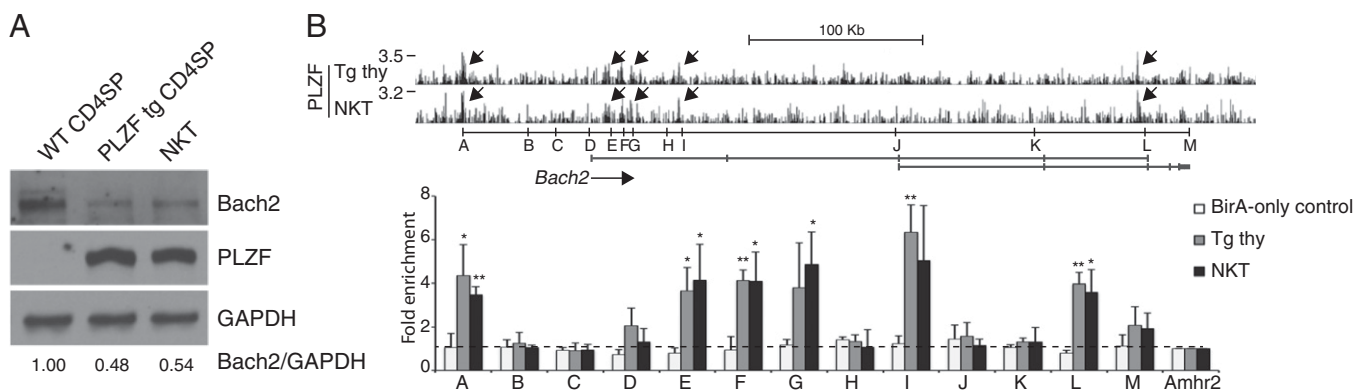




**Fig. 4.** PLZF directly binds genes encoding T-helper-specific transcription factors. Shown are PLZF ChIP-seq reads aligned at the *c-Maf* (A), *Gata3* (B), *Tbx21* (C), *Runx3* (D), *Rora* (E), *Rorc* (F), and *Bcl6* (G) loci. Peaks are indicated by arrows. ChIP-qPCR analysis of PLZF binding is also shown at indicated sites in *c-Maf* (A) and *Gata3* (B) loci, with BirA-only control referring to thymocytes expressing BirA but not the biotin acceptor sequence. The fold enrichment is normalized relative to enrichment at the promoter region of reference gene *Amhr2*. Data are shown as mean  $\pm$  SD of two to four independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

**Microarray Analysis.** RNA was extracted from FACS-sorted thymic CD4SP cells ( $CD4^+CD8^-TCR\gamma\delta^-CD25^-CD1d-\alpha GalCer^-$ ) obtained from PLZF transgenic mice and littermate controls and stage 1 NKT cells ( $CD1d-\alpha GalCer^+CD24^{low}CD44^-NK1.1^+$ ) obtained from WT and *lul1u*  $V\alpha 14-J\alpha 18$  tg mice using TRIzol (Thermo Fisher

Scientific), followed by column purification using the RNeasy Mini Kit (Qiagen). RNA integrity was assessed using an Agilent 2100 Bioanalyzer; all samples used for hybridization had an RNA integrity number  $>9.3$ . Total RNA was used to synthesize cDNA, which was then fragmented and hybridized according to



**Fig. 5.** PLZF directly binds *Bach2*. (A) Immunoblot of lysates from indicated cell types with anti-Bach2, anti-PLZF, and anti-GAPDH. Ratios of Bach2 over GAPDH band density are indicated. Data are representative of two independent experiments. (B, Upper) PLZF ChIP-seq reads aligned at the *Bach2* gene locus. Peaks are indicated by arrows. (Lower) For ChIP-qPCR analysis, the fold enrichment is normalized relative to enrichment at the promoter region of reference gene *Amhr2*. Data are shown as mean  $\pm$  SD of two to four independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

Affymetrix instructions at the University of Chicago's Functional Genomics Facility. CD45P samples were hybridized to GeneChip Mouse Exon 1.0 ST arrays (Affymetrix), and NKT samples were hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix).

Arrays were scanned using a GeneChip Scanner 3000 (Affymetrix), and intensity values were generated by MicroArray Suite 5.0 software (Affymetrix). Microarray datasets were normalized using RMA, and differential expression was estimated using the Bioconductor package limma. When multiple probes were present for a given gene, their fold changes were averaged. Two or three biological replicates were analyzed for each cell type, and the average fold changes were used for comparison.

**ChIP-seq and ChIP-qPCR.** For PLZF ChIP-seq in thymocytes, approximately 500 million thymocytes from A/F/PLZF tg/BirA tg mice were used. For PLZF ChIP-seq in NKT cells, approximately 100 million autoMACS-enriched NKT cells from A/F/PLZF tg/BirA tg/ $\alpha$ 14-J $\alpha$ 18 tg mice were used. For PLZF ChIP-seq in KG1a cells, approximately 20 million cells were used. Cells were fixed for 5 min at room temperature with 10% (vol/vol) formaldehyde. Glycine was then added to a final concentration of 0.125M to quench the reaction. Cells were washed twice with ice-cold PBS, lysed, and then sonicated to fragment DNA to 200–1,000 bp. The chromatin was then incubated overnight with magnetic streptavidin beads (14204; Invitrogen) or PLZF antibody (AF2944; R&D Systems). Precipitated ChIP and input DNA were washed, reverse cross-linked, and digested with proteinase K and RNase A. The DNA was then purified with phenol/chloroform extraction or a Qiagen reaction cleanup kit. ChIP was validated by qPCR for known targets before library preparation. For PLZF ChIP-seq, ChIP DNA was pooled from multiple batches of mice before deep sequencing of more than 100 million raw reads. Sequencing libraries were prepared with an

Illumina ChIP-seq DNA Prep Kit and sequenced using Illumina Genome Analyzer Ix or HiSeq. The primers used for ChIP-qPCR are listed in Table S1.

**Analysis of ChIP-seq Reads.** ChIP-seq raw reads for transcription factors EGR2, GATA3, and ETS1 were downloaded from the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) database. For all mouse ChIP-seq experiments, the raw reads were aligned to the mm10 mouse genome using bowtie, allowing for two mismatches. For PLZF ChIP-seq in KG1a cells, the raw reads were aligned to the hg19 human genome. Only uniquely aligned reads were analyzed. Redundant reads due to PCR amplification were compressed to single reads. The total numbers of uniquely aligned PLZF ChIP-seq reads were 59 million in Tg thy cells, 38 million in NKT cells, and 9 million in KG1a cells. The ChIP-seq bedgraphs were generated using HOMER, with the total number of aligned reads normalized to 10 million. Input controls were used for peak calling to exclude peaks with a high input control signal.

**Motif Analysis.** We extracted 200 bp centered on PLZF ChIP-seq peak summits and used it as input for MEME-CHIP (43), set to use the Vertebrate (in vivo and in silico) motifs. FIMO (44) was used to scan for occurrences of these motifs among all PLZF ChIP-seq peaks.

**Statistical Analysis.** Two-tailed Student's *t* tests were performed with R.

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