Title

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Journal

Proceedings of the National Academy of Sciences of the United States of America, 114(23)

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Publication Date

2017-06-06

DOI

10.1073/pnas.1610617114

Peer reviewed

Bcl11b and combinatorial resolution of cell fate in the T-cell gene regulatory network

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Edited by Neil H. Shubin, The University of Chicago, Chicago, IL, and approved January 30, 2017 (received for review October 25, 2016)

T-cell development from hematopoietic progenitors depends on multiple transcription factors, mobilized and modulated by intrathymic Notch signaling. Key aspects of T-cell specification network architecture have been illuminated through recent reports defining roles of transcription factors PU.1, GATA-3, and E2A, their interactions with Notch signaling, and roles of Runx1, TCF-1, and Hes1, providing bases for a comprehensively updated model of the T-cell specification gene regulatory network presented herein. However, the role of lineage commitment factor Bcl11b has been unclear. We use self-organizing maps on 63 RNA-seq datasets from normal and perturbed T-cell development to identify functional targets of Bcl11b during commitment and relate them to other regulomes. We show that both activation and repression target genes can be bound by Bcl11b in vivo, and that Bcl11b effects overlap with E2A-dependent effects. The newly clarified role of Bcl11b distinguishes discrete components of commitment, resolving how innate lymphoid, myeloid, and dendritic, and B-cell fate alternatives are excluded by different mechanisms.

Bcl11b | Notch-delta signaling | PU.1 | E2A | commitment

T lymphocyte development from blood stem cells depends on the regulatory inputs from a suite of transcription factors needed by all T-cell types, as well as a set of factors that subdivides mature T cells into different functional groups. Before functional subdivision, a common core gene regulatory network (GRN) guides multipotent precursors to generate committed pro-T cells that will ultimately serve as progenitors for all T-cell lineages. Commitment occurs in the thymus before the cells acquire specific T-cell receptors (TCR) for antigen, and is driven by a combination of extrinsic signals and intrinsic transcription factor activity changes. This early T-cell GRN not only turns on T-cell–specific genes but also coordinates early precursor proliferation with stepwise renunciation of alternative developmental potentials. We and others have found that the commitment process concludes with the onset of expression of a zinc finger transcription factor, Bcl11b (1–4). Here we identify the genes that are immediately sensitive to Bcl11b activity, and integrate the Bcl11b activation process into the context of the broader T-cell specification GRN.

The thymic environment instructively promotes T-cell differentiation of multipotent immigrant cells by presenting Notch ligand Delta-like 4 (DLL4), and providing supportive cytokines (Kit ligand, IL-7). The resulting Notch signaling drives precursors to proliferate through a canonical series of stages [double negative (DN) = $CD4^-$ $CD8^-$; double positive (DP) = $CD4^+$ $CD8^+$]: from Kit^{hi} DN1 (or early T-cell precursor, ETP), to DN2a, DN2b, and DN3a stage (reviewed in ref. 5). If the precursors can begin TCR expression successfully in DN3a, they continue through DN3b and DN4 to DP, when the cells finally acquire complete TCR recognition complexes (5, 6). Importantly, if individual T-cell precursors in the ETP or DN2a stage are removed from thymic Notch ligands, they can still generate non-T cells, but from the DN2b stage on, they can no longer do this unless genetically manipulated (1, 7). This transition defines "commitment."

The robust change in potential from DN2a to DN2b is also accompanied by dynamic transcription factor expression changes (8, 9) At least 20 regulatory genes have expression patterns that can be classed as "phase 1" (expressed in ETP and DN2a, then down-regulated) or "phase 2" (turned on or significantly up-regulated around commitment in DN2b) (5). To date, the most-studied regulators of the phase 1 to phase 2 transition have been Notch signaling, GATA-3, TCF-1, and E2A, and PU.1 as a natural, endogenous "opponent" of developmental progression. Of these, all are present in ETP and DN2a cells, and only Bcl11b is up-regulated de novo during this commitment transition itself.

The activity of Bcl11b in commitment has had two aspects poorly resolved to date. After commitment, Bcl11b seems to repress specific types of effector programs in T cells and innate lymphoid cells (ILC), blocking genes associated with natural killer (NK)-like or effector cytolytic T cells or with differentiation to specific IL-17–producing subsets of invariant NK T cells (reviewed in refs. 6 and 10, 11). This effector-subtype blockade is important to support memory CD8 cell function and regulatory T-cell differentiation, but it does not explain why Bcl11b is important for T-lineage commitment as a whole. Cells losing Bcl11b acutely long after commitment activate an NK-cell like gene-expression profile (2, 12) or a precociously specialized T-cell profile (13), but not an immature gene-expression profile like the one extinguished during commitment.

To resolve how Bcl11b is working, we have focused specifically on the stages at the fulcrum of commitment to carry out a genome-wide RNA-seq analysis of the effects of Bcl11b deletion. We have identified the most strongly influenced target genes in a

This paper results from the Arthur M. Sackler Colloquium of the National Academy of Sciences, "Gene Regulatory Networks and Network Models in Development and Evolution," held April 12–14, 2016, at the Arnold and Mabel Beckman Center of the National Academies of Sciences and Engineering in Irvine, CA. The complete program and video recordings of most presentations are available on the NAS website at www.nasonline.org/Gene_Regulatory_Networks.

Author contributions: A.M. and E.V.R. designed research; W.Z., J.A.Z., H.H., L.L., M.R.-W., and H.Y.K. performed research; W.J.R.L., P.L., and A.M. contributed new reagents/analytic tools; W.J.R.L., W.Z., J.A.Z., H.H., C.S.J., M.R.-W., H.Y.K., A.M., and E.V.R. analyzed data; and W.J.R.L., W.Z., and E.V.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (RNA-seq data accession no. GSE89198; ChIP-seq data accession no. GSE93572).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1610617114/-/DCSupplemental.

de novo analysis of 63 RNA-seq datasets of wild-type as well as perturbed developing T cells. We have resolved distinct subsets of Bcl11b targets as they relate to the regulomes of other developmentally important transcription factors in T-cell development, using self-organizing maps (SOM) (reviewed in ref. 14). These SOM have long been used for analysis of gene-expression (15, 16) and chromatin data (17) because they can resolve relationships in very large datasets with large numbers of clusters, and here they provide an elegant way to identify multiple, related modules of Bcl11b targets with distinct roles in development. In fact, many of the new Bcl11b target genes are unique to the pericommitment developmental context. We place these findings in the context of an updated version of the T-cell specification GRN model (5, 18–20) enhanced with recent insights into connections between PU.1, Bcl11b, Notch signaling, and other crucial factors: that is, GATA-3, TCF-1, Runx1, and the basic helix–loop–helix factor E2A.

Results

T-Cell Specification GRN Transitions at Commitment: A Distinct Context for Bcl11b. A model for the GRN that guides T-cell commitment has been assembled over the last decade (18, 19) but new linkages that control aspects of commitment timing have now been illuminated. Recent reports have identified key mechanisms that make the transition from precommitment to commitment into such a discontinuous, switch-like event. These features are summarized in the revised and updated GRN model shown in Fig. 1. The precommitment phase 1 period is defined by strong expression of the B-cell–, dendritic-cell–, and myeloid-cell–associated transcription factor PU.1 (encoded by *Spi1*, alias *Sfpi1*), and stem-cell–associated regulatory genes that are activated prethymically, including *Erg*, *Mycn*, *Hhex*, *Bcl11a*, *Gfi1b*, *Mef2c*, *Lmo2*, and *Lyl1* (reviewed in ref. 5) (Fig. 1*A*). The most important growth factor receptor sustaining this phase is encoded by *Kit.* These genes all need to be down-regulated during commitment. Meanwhile, Notch signaling turns on expression of T-lineage genes, including *Gata3* and *Tcf7* (encoding TCF-1) starting in the DN1 (ETP) stage, but these are not sufficient to impose commitment. In contrast, *Bcl11b*, a phase 2 regulatory gene, is transcriptionally silent until the late DN2a stage, and then turns on abruptly. It is then expressed throughout the rest of T-cell development and in mature T cells thereafter (Fig. 1*B*). Both silencing of PU.1 and activation of Bcl11b are operationally important for commitment (2–5, 21).

This updated model incorporates recent data on functional impacts of E2A (22), on PU.1 and its interactions with Notch, Myb, and GATA-3 (21, 23, 24), on Notch blockade of the myeloid program via Hes1 against *Cebpa* (25, 26), on GATA-3 and its mutual antagonism with the B-cell program (27–30), and on positive regulators of *Bcl11b* itself (1, 31). Discussed in detail in *SI Appendix*, *Supplementary Text*, these results reveal two major switch circuits. First, a mutual inhibition circuit between PU.1 and Notch, without mutual repression, buffers the intensity of Notch signaling but restricts the lineage-diversion activity of PU.1 if Notch signaling is present, while letting PU.1 support genes linked to multipotentiality (21, 23) (Fig. 1*A*). This balance defines the phase 1 pro–T-cell state, which is surprisingly persistent in pro-T cells through days of proliferation. Second, *Bcl11b* up-regulation depends on a stringently combinatorial action of GATA-3, TCF-1 (encoded by *Tcf7*), Runx1, and Notch signaling. Here, the most collaborative events appear necessary to "prime" the locus during the DN1 phase, before actual transcriptional activation in late DN2a phase (1). New results on E2A targets are considered further below. Detailed review of the operation of the network as a whole and evidence for specific links are provided in *SI Appendix*, *Supplementary Text*.

The commitment event is tightly coupled with Bcl11b up-regulation (Fig. 2*A*) (1), but the exact role of Bcl11b in this process depends on the unique regulatory context, which is not duplicated in any of the later T-cell contexts where Bcl11b works (reviewed in ref. 10) nor in type 2 ILC, where it also has a role (11, 32, 33). Until commitment (Fig. 1*A*), endogenous PU.1, possibly other phase 1 genes, and even GATA-3 still represent regulatory bridges to distinct alternative programs. Notch signaling selectively intervenes in their actions to prevent alternative lineage specification (21, 26, 34, 35), but still allows PU.1 to sustain lymphoid-compatible phase 1 regulatory genes like *Bcl11a*, *Lmo2*, and *Mef2c* (21) in phase 1 pro-T cells. Gene regulation at this stage can also reflect balances between different factors competing to regulate common targets: for example, PU.1 and GATA-3 may compete, respectively, to repress or activate *Zfpm1*, and *Ets1*, and to activate or repress *Bcl11a* (21, 23, 27). This balance is tipped during commitment, with the silencing of the phase 1 regulatory genes and down-regulation of Kit, but only limited aspects of this process are understood. GATA-3, Runx1, and TCF-1 (or its relative LEF-1) eventually play roles in silencing expression of phase 1 regulatory genes encoding PU.1 and Bcl11a during commitment, as demonstrated by gain- and loss-of-function data (27, 36–39) (Fig. 1*B*). However, all of these factors are present at comparable levels for multiple cell cycles before commitment (examples shown in Fig. 2*B*). Thus, although the precommitment and postcommitment states are clearly distinguished from each other, the exact role of rate-limiting components like Bcl11b in triggering the transition between them has not been fully defined.

Determination of Bcl11b Targets During Commitment. Cells failing to turn on Bcl11b during commitment are arrested in vivo (13, 40) but proliferate well in vitro with strong Notch and cytokine signals. In key respects the proliferating cells resemble DN2a cells (3, 4) (DN2a* in Fig. 2*A*). They tend to differentiate to NK-like cells if Notch signal intensity is reduced (2, 3). One prediction is that Bcl11b should itself repress regulatory genes like *Spi1* (PU.1) or *Lyl1* that are sharply down-regulated in commitment (Fig. 2*B*). Another prediction is that, if Bcl11b is important to block access to the NK cell fate, then the NK-promoting genes it represses might be expressed in T-cell precursors until the time that Bcl11b is turned on. However, the actual results differ from these predictions.

Conditional knockout (KO) Bcl11b DN2 cells and control Bcl11b^{+} cells spanning the DN2a–DN3a interval plus two control DP samples were generated for RNA-seq comparisons (Fig. 2*C*) using two protocols (Fig. 2 *D1* and *D2* and *SI Appendix*, *Supplementary Methods*). In most samples, *Bcl11b* was deleted before its normal onset of expression, by introducing Cre into conditional KO hematopoietic progenitor cells or wild-type controls before T-cell development began (protocol I) (Fig. 2*D1*). We used fetal liver-derived hematopoietic precursors and cultured control and conditional KO cells in parallel in a well-characterized in vitro T-cell differentiation system, with cytokines and OP9-DL1 or OP9-DL4 stromal cells (3, 41) (*SI Appendix*, Fig. S1*A*). For protocol II (Fig. 2*D2*), we deleted *Bcl11b* from DN2b or DN3 cells after commitment, usually deleting *Bcl11b* by CreERT2 activation in vitro (2) in freshly isolated DN3 thymocytes. One other protocol II sample pair used Lck-Cre deletion in vivo (*SI Appendix*, *Supplementary Methods*). Both protocols generated characteristically large, DN2a-like Bcl11b KO cells with high expression of Kit (*SI Appendix*, Fig. S1*A*, days 9 and 12 and *B*, day 4). However, fetal-derived cells that had lost *Bcl11b* before commitment could proliferate extensively with high viability in OP9–DL4 coculture (3), whereas adult-derived cells that had lost *Bcl11b* after commitment grew poorly in the same conditions.

For RNA-seq analysis, multiple matched control vs. KO pairs, additional Bcl11b KO samples, and additional controls were sorted from in vitro cultures at corresponding developmental stages, as well as three sets of adult samples with Bcl11b deleted after commitment (Fig. 2*C*). Only cells with a DN2 or DN3 phenotype were sorted (*SI Appendix*, Fig. S1*A*, *Inset*) to exclude any cells already transformed to NK cells. RNA-seq analysis (*SI Appendix*, Fig. S2) showed that the targeted exon of *Bcl11b* was efficiently deleted, and genes including *Tnni1*, *Itga2b*, *Tyrobp*, *Fcer1g*, *Cxcr5*, *Zbtb16*, *Nfil3*, *Id2*, and *Il2rb* were up-regulated in

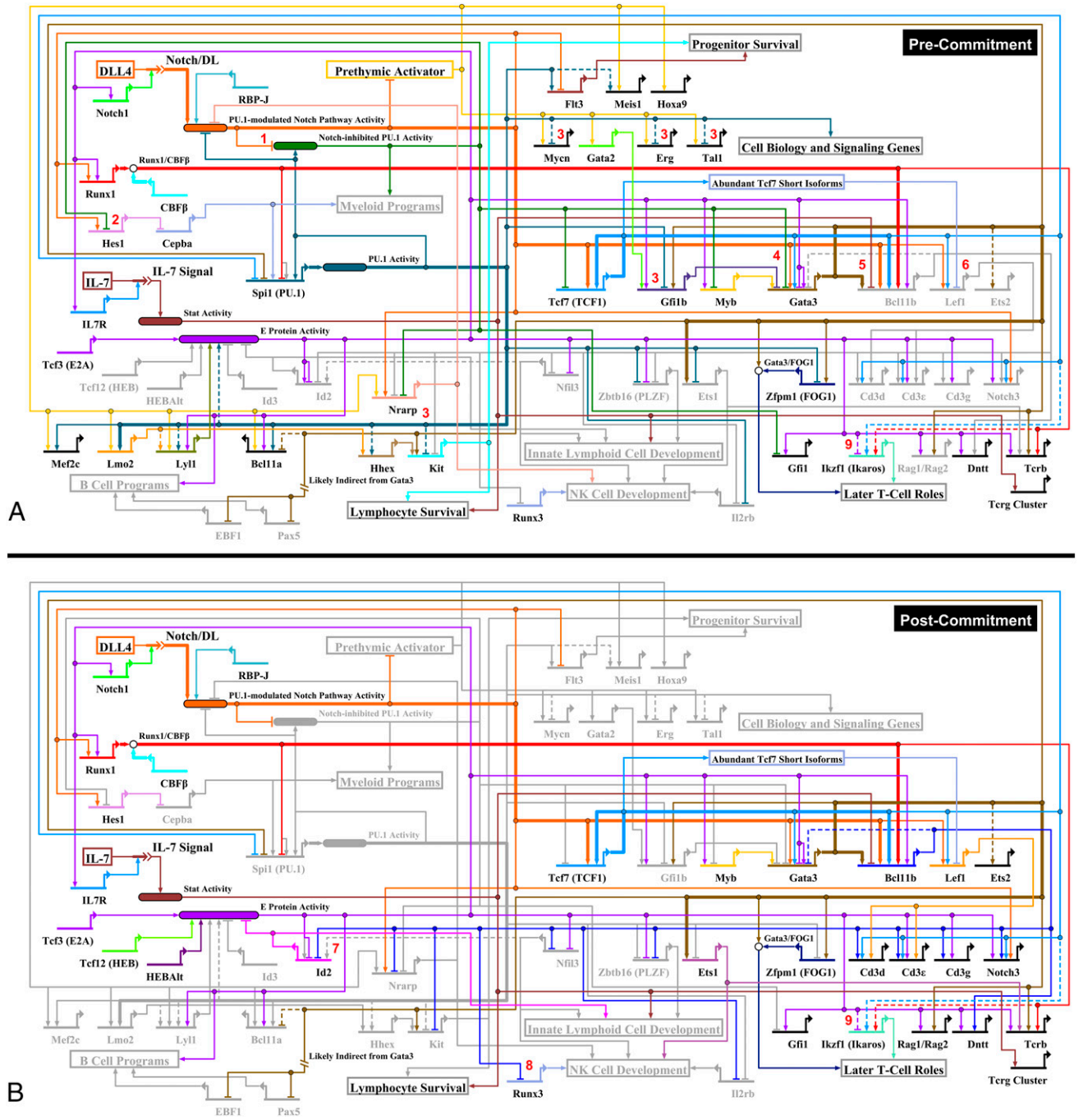

Fig. 1. GRN model of the early T-cell specification network using BioTapestry format (62). (*A*) Relationships among genes active in phase 1 (ETP to DN2a stages). Active genes and connections are in color, inactive ones in gray. Both "AND" logic and "OR" logic relationships between inputs and target genes are included. Thick links: validated direct effects. Regular links: perturbation evidence, at least indirect. Dashed links: weaker or uncertain effects. Circles at intersections: path branch points. The off/on dichotomy in the display does not capture gradations in expression, and note that negative effects at many of the repressive nodes here represent "soft" damping repression rather than silencing (see numbered notes below). A reciprocal antagonism circuit between Notch and PU.1 that maintains T-cell differentiation in phase 1 is in the upper left quadrant. (*B*) Relationships among genes active in phase 2 (DN2b and later), after effects of Bcl11b are manifest and the phase 1 regulatory genes are silenced. Numbers indicate specific connection properties as follows. 1: Notch signals and PU.1 activity modulate each other. Notch signaling inhibits a gene-specific subset of PU.1 activities, here called "Notch-inhibited PU.1 activity". 2: Repression by PU.1 is more severe if Notch signal is absent. 3, 5, 6, 8, and 9: Soft repression, i.e., indicated input limits the maximal activity of target gene but does not silence its expression. Also, 3: Inferred from response to forced PU.1 expression. 4: Complex conditional and soft repressions: see *SI Appendix, Supplementary Text*. 5: Delayed activation in high IL-7. 6: Soft repression by Tcf7 short isoforms. 7: Nfil3 can activate Id2 but is unlikely to explain the transient Id2 in some DN2b cells. 8: Soft repression of distal Runx3 promoter. 9: Soft repression by E proteins. See *SI Appendix, Supplementary Text* for details.

the KO cells, whereas, e.g., *Gbp4* and the *Cd3* gene cluster were down-regulated. Because of the dynamic developmental context (Fig. 2*D*), there were variances in developmental progression overall in different experiments (*SI Appendix*, Fig. S3*A*), as shown by a panel of 90 indicator regulatory genes (*SI Appendix*, Fig. S3*B*). Thus, although some gene-expression changes were

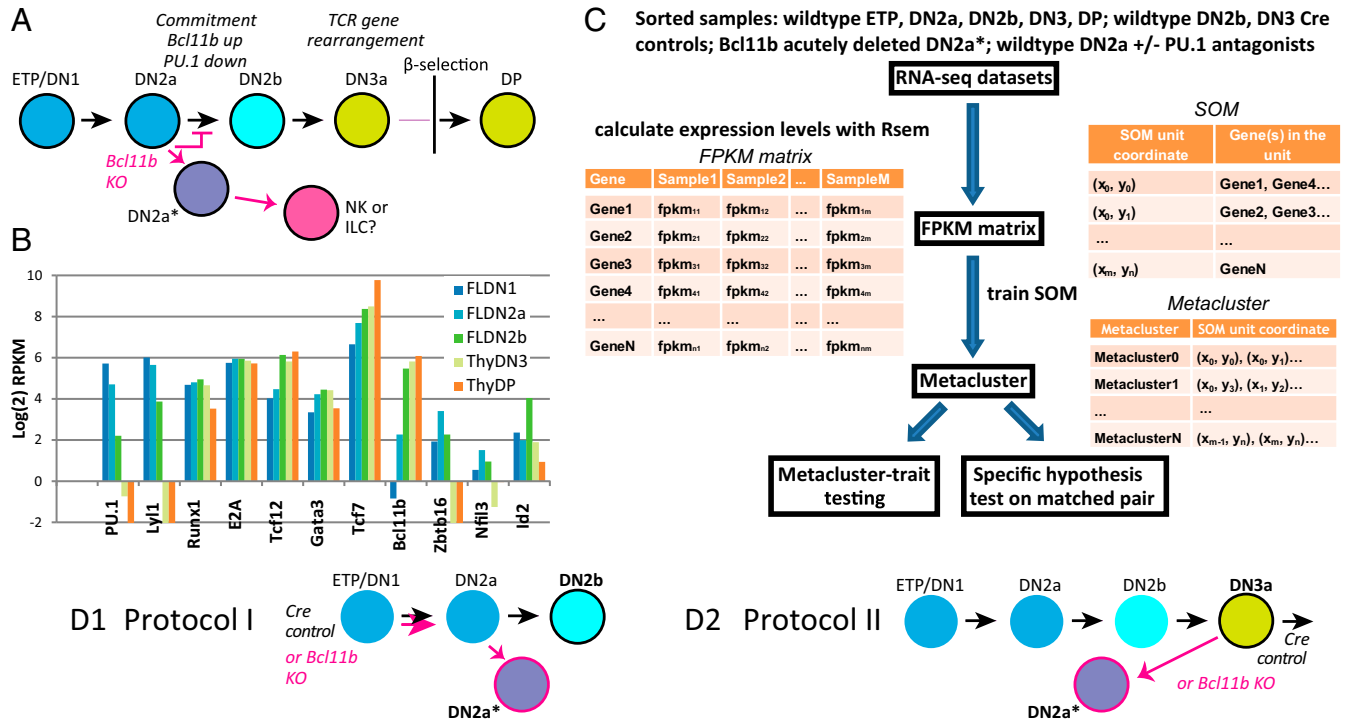

Fig. 2. The T-cell developmental system and experimental design. (*A*) Stages in T-cell development and branch point affected by loss of Bcl11b. For definition of markers, see ref. 5. (*B*) Expression levels of important regulatory factors in early T cells, as measured by RNA-seq. (8). (*C*) Brief workflow of RNA-seq data processing and SOM analysis. (*D*) Comparison of two deletion protocols for Bcl11b$^{f/f}$ samples to test the impact of Bcl11b loss in early T development. (*D1*) In protocol I, Cre is introduced before T-cell development is started, by retroviral transduction in precursors that then develop as indicated on OP9-DLL1 stroma. (*D2*) In protocol II, Cre is activated in adult thymic DN3 cells after commitment, either by Lck-Cre activity in vivo or by tamoxifen induction of Cre-ERT2 in purified DN3 thymocytes in vitro, then culturing cells for 3–4 more days before analysis (*SI Appendix, Supplementary Methods*).

seen in all Bcl11b KO samples, some were significant only in particular types of samples: adult vs. fetal, protocol I vs. protocol II, or where controls were more DN2b-like vs. more DN3a-like (e.g., *SI Appendix*, Fig. S4: *Kit*, *Cd7*, *Bcl11a*). However, overall, the expression changes observed were reproducible among multiple independent KO-control sample pairs (e.g., *Kit*, *Zbtb16*, *Cpa3*, *Tnni1*, *Cd3g*, *Cd3d*, *Cd3e*, all $P < 10^{-2}$, $n = 10$) (*SI Appendix*, Fig. S3*C*). To identify a "gold-standard" list of differentially expressed genes, we grouped the paired-sample comparisons into three pools and selected genes that showed significant up or down-regulation (EdgeR, false-discovery rate < 0.05) in at least two of the three separate pools (Dataset S1*A*; component lists in Dataset S1 *B* and *C*; all genes differentially expressed in ≥one pool in Dataset S1*D*). Of differentially expressed transcripts with known protein-coding potential (omitting TCR loci, Gm and miR transcripts), 77 were up-regulated significantly when Bcl11b was knocked out and were thus presumably repressed by Bcl11b normally, whereas 22 lost expression and thus were Bcl11b-dependent (Dataset S1*A*). Normal expression patterns of all these genes are shown in *SI Appendix*, Fig. S5.

The high-confidence Bcl11b-dependent genes were more likely to increase expression through normal commitment, whereas Bcl11b-repressed genes tended to be down-regulated (*SI Appendix*, Fig. S5*A*), consistent with the expression of Bcl11b itself (Fig. 2*B*). Several genes associated with initial TCR assembly were notably Bcl11b-dependent in this developmental context, although this has not been seen in later stages: that is, genes coding for CD3 signaling components and for Dntt, the terminal deoxynucleotidyl transferase that helps in generating a highly diverse TCR repertoire (*SI Appendix*, Figs. S2 and S5*C*). We excluded *Tcrb* and *Tcrg* loci from the gold-standard gene set because of annotation complexity, but Dataset S1*B* suggests that Bcl11b may support these differentiation-associated transcripts as well. In contrast, genes that were repressed by Bcl11b (i.e., up-regulated in the KOs) fell into several differently regulated groups, only a subset of them phase 1-restricted. Purified DN3 cells from *Bcl11b*$^{fl/fl}$ mice that did not express significant levels of Bcl11b-repressed targets in vivo could be induced to express these genes by acute deletion of *Bcl11b* in vitro, as confirmed by quantitative PCR (qPCR) analyses (*SI Appendix*, Fig. S5*B*).

Context Dependence of Bcl11b Targets. Previous studies have examined Bcl11b deletion in later-stage thymocytes, CD4$^+$ CD8$^+$ DP cells (2, 12, 13), where an NK-like gene signature emerged. Indeed, the Kyoto Encyclopedia of Genes and Genomes pathway most highly enriched in our Bcl11b-repressed gold-standard gene set was NK cell-mediated cytotoxicity (*SI Appendix*, Fig. S6*A*), and multiple genes were shared with gene sets that distinguish ILC from T cells (42, 43). However, the gold-standard set as a whole was distinct from top Bcl11b-regulated genes in DP cells. A subset (24 of 77) of genes up-regulated in our Bcl11b KO pro-T cells were affected similarly in Bcl11b KO DP cells, in at least one of these other studies (2, 13) (*SI Appendix*, Fig. S6*B* and Dataset S1*E*). However, the majority (53 of 77) of Bcl11b-repressed gold-standard genes from DN2/3 cells were not identified in DP cell analyses (e.g., *Cd7*, *Cpa3*, *Kit*, *Tnni1*, and *Zbtb16*) (compare *SI Appendix*, Figs. S2 and S4). Moreover, only 2 of 22 Bcl11b-dependent genes in DN2-3 cells were affected in DP cells (*SI Appendix*, Fig. S6*C* and Dataset S1*F*). Thus, the genes that Bcl11b must keep silent are more consistent across developmental stages than those that it may help to activate. When examining many hematopoietic cell samples from the ImmGen consortium (9, 44), we found that the gold-standard Bcl11b-dependent genes often peaked in expression at DN3a stage (*SI Appendix*, Fig. S7*A*, star), whereas expression patterns of the Bcl11b-repressed genes were notably diverse across multiple hematopoietic lineages (*SI Appendix*, Fig. S7*B*). Many Bcl11b-repressed genes (*Tnni1*, *Il2rb*, *Zbtb16*, *Id2*, *Cxcr5*) did not

simply continue expression from uncommitted phase 1 progenitors, but were either up-regulated from ETP–DN2a levels, or else activated dramatically de novo if Bcl11b were absent.

Bcl11b Binding to Target Genes in Vivo. ChIP and sequencing (ChIP-seq) showed clear Bcl11b binding to sites around positive and negative regulation targets in DN3 cells (Fig. 3*A* and *SI Appendix*, Fig. S8 *A–G*). Bcl11b specifically occupied sites at most if not all gold-standard loci, including *Zbtb16*, *Tnni1*, *Dntt*, and *Cd3d* (ChIP-PCR in Fig. 3*B*). Sites were often in open chromatin at positively regulated loci but also at closed or closing sites in many repressed targets (*SI Appendix*, Fig. S8 *A–C*). Such sites are candidates for mediators of direct regulation.

Global Picture of Bcl11b Effects in the Context of Early T-cell Development. To relate the genes that Bcl11b controls to the global context of T-cell development, we used all of the RNA-seq data in our 63 samples to generate a SOM, clustering gene-expression patterns in all our samples into a large toroidal 40 × 60-unit map. This is a format that is particularly well-suited for clustering high-dimensional datasets first into units of groups of genes that have nearly identical expression profiles among all samples (clusters), then placing similarly regulated units near each other on the map geographically in >one dimension (metaclusters). Importantly, the 2D mapping relates clusters by more than one criterion of similarity. SOM analysis thus enabled us to group the whole transcriptome into 300 metaclusters based on fine-grained similarities of regulation not only in development but also under perturbation (17) (Fig. 3*C* and *SI Appendix*, *Supplementary Methods* and Fig. S9*A*).

The gene-expression patterns defined a complex landscape (*SI Appendix*, Fig. S9*A*) in which changes in expression of different groups of genes were shown by coordinated increases or decreases in signal between stages from particular metacluster regions (Fig. 3 *C–G* and *SI Appendix*, Fig. S9*B* and Dataset S2). Phase 1-specific genes were concentrated in a subset of metacluster regions (blue in Fig. 3*C* and region B in *SI Appendix*, Fig. S9*B*), while later-expressed genes could be mapped to another subset of regions (red in Fig. 3*C* and region A in *SI Appendix*, Fig. S9*B*). Patterns of expression of the eigengenes for specific metaclusters, across all our samples, are shown in *SI Appendix*, Fig. S10. For example, PU.1 (*Sfpi1*, *Spi1*) is in metacluster 112 (region B, in *SI Appendix*, Fig. S9*B*), one whose expression is significantly down-regulated in DN2b and later samples (*SI Appendix*, Fig. S10*A*). Other phase 1-specific regulatory genes, such as *Bcl11a*, map nearby (in metacluster 212) (region B in *SI Appendix*, Fig. S9*B* and expression in *SI Appendix*, Fig. S10*B*). In contrast, the phase 2-specific genes, including *Bcl11b* itself (in metacluster 26) (*SI Appendix*, Fig. S10*D*), are in the upper-right cluster region up-regulated in DN2b T cells (region A in *SI Appendix*, Fig. S9*B*).

The SOM analysis clearly showed that Bcl11b deletion up-regulated expression of genes in two distinct metacluster neighborhoods with different relationships to normal development (Fig. 3*D* and *SI Appendix*, Fig. S9*B*, regions C1 and C2). When the DN2b vs. DN1 fold-change map is compared with the

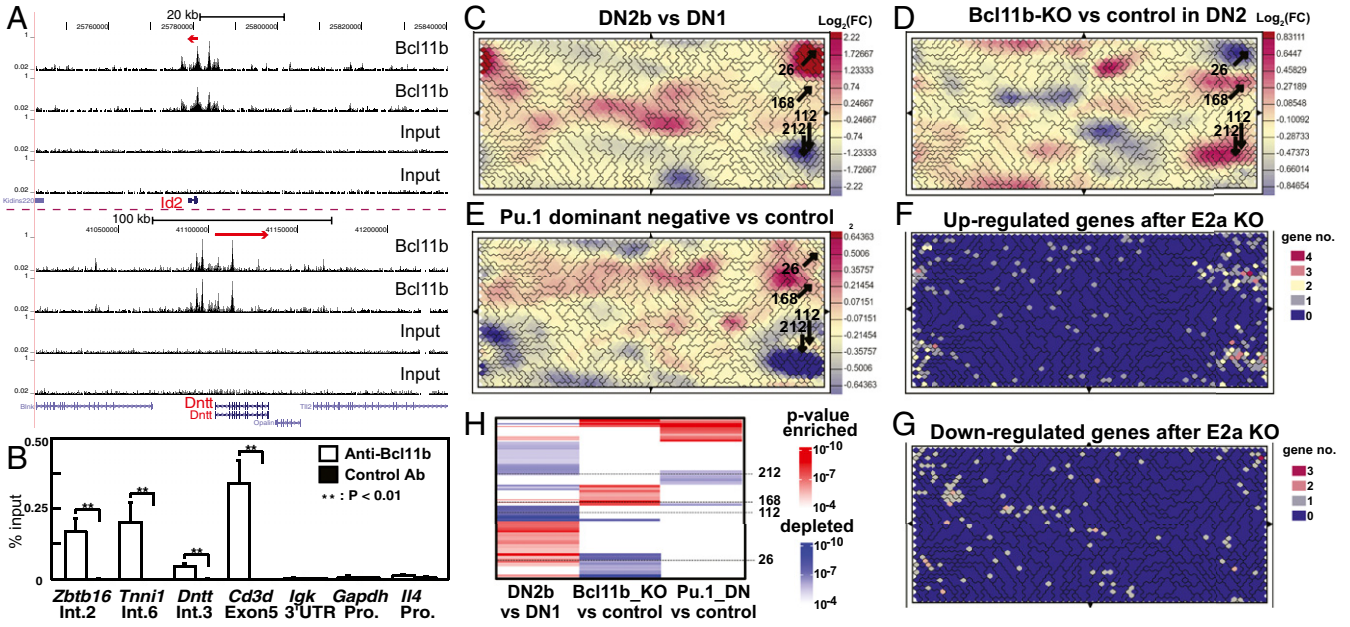

Fig. 3. Bcl11b targets and SOM analysis of their organization in distinct regulomes. (*A*) ChIP-seq shows Bcl11b binding in DN3 cells to a repression target, *Id2* (*Upper*), and a Bcl11b-dependent target, *Dntt* (*Lower*). Two independent samples each of Bcl11b ChIP and control (1% of input) are shown aligned to the mm9 mouse genome. Red arrows: primary transcription units of *Id2* and *Dntt*, respectively. [Scale bars: 20 kb (*Upper*), 100 kb (*Lower*).] Vertical scales: 0.02–1.0 fpm. (*B*) Specific Bcl11b binding in vivo to target gene regulatory sites. ChIP for Bcl11b was analyzed by qPCR for enrichment of candidate regulatory sequences of *Zbtb16*, *Tnni1*, *Dntt*, and *Cd3d*. No binding was seen to *Igk*, *Gapdh*, and *Il4* negative control sites. Averages from three independent experiments are shown (error bars: SD). (*C*) Fold-change SOM, color-coded to show normal gene-expression changes from the DN1 to DN2b stage. Red regions contain genes up-regulated in DN2b pro-T cells. Blue regions: genes down-regulated. For delimitation of SOM metaclusters, see *SI Appendix*, Fig. S9*A*. Four representative metaclusters—112, 168, 212, and 26 (*SI Appendix*, Fig. S10)—are labeled with arrows. The heatmap shows fold-change of average fragments per kilobase of transcript per million mapped reads (FPKM) within each unit, in logarithmic scale. (*D*) Fold-change SOM comparing Bcl11b KO DN2 and control DN2-3 pro-T cells. Red units are up-regulated after Bcl11b KO. (*E*) Fold-change SOM comparing PU.1 antagonist-expressing and control DN2 pro-T cells. Red units are up-regulated in PU.1 antagonist-expressing cells, blue units are repressed. (*F*) Genes up-regulated in E2A$^{-/-}$ DN2 pro-T cells from ref. 22, mapped onto the SOM metaclusters. Note high similarity with pattern of red (enriched) clusters in *D*. (*G*) Genes down-regulated in E2A$^{-/-}$ DN2 cells, mapped onto the SOM. Note overlap with certain cluster regions that are blue (depleted) in *D*. (*H*) Heatmap of hypothesis tests. The metaclusters with significant enrichment of the differentially expressed genes are displayed in red, and those that are significantly depleted are in blue. The color scale corresponds to the *P* value of the enrichment/depletion of differential genes in each metacluster. Dashed lines shown the location of the four representative metaclusters. An expanded version is in *SI Appendix*, Fig. S12*A*.

Bcl11bKO vs. control fold-change map (Fig. 3 *C* and *D*), one region of genes enhanced in the Bcl11b KO overlapped with genes with phase 1-biased expression (Fig. 3 *C* and *D*; region C1 substantially overlapping region B in *SI Appendix*, Fig. S9*B*). These metaclusters include or adjoin the clusters containing most of the signature phase 1 regulatory genes (Dataset S2*A*), identifying the specific immature properties that are perpetuated in Bcl11b KO cells. However, the other salient region (region C2 in *SI Appendix*, Fig. S9*B*) consisted of genes up-regulated de novo in Bcl11b-deficient DN2-like cells. This includes genes associated with NK function and ILC/NK fate, like *Nfil3* and *Zbtb16* themselves (metacluster 168) (*SI Appendix*, Fig. S10*C*). Thus, through distinct sets of genes Bcl11b loss retards aspects of T-cell differentiation and promotes acquisition of a new fate.

Explaining Bcl11b Impact on Development.

No global epistasis with PU.1. Although Bcl11b KO cells perpetuated some aspects of the phase 1 state, including high *Kit* expression (DN2a* in Fig. 2*A*), few phase 1 signature transcription factor genes themselves were specifically up-regulated in Bcl11b KOs (although *Bcl11a* down-regulation tended to be delayed) (*SI Appendix*, Fig. S4). Bcl11b KO cells also seemed normally competent to express T-cell–specifying regulatory genes (Dataset S2*B*). In general, they often up-regulated *Gata3* (metacluster 214), while specifically activating the distal promoter isoform of *Runx3* (metacluster 188) (browser view shown in *SI Appendix*, Fig. S11). Bcl11b KO effects were not mimicked by Notch inhibition, as measured by short-term γ-secretase inhibition (GSI) in DN2a or DN2b cells, shown by the lack of impact on similar metaclusters in this SOM analysis (*SI Appendix*, Fig. S12*A*). Therefore, we tested two other ways that Bcl11b might normally advance the T-cell gene regulatory program: by suppression of phase 1 regulator effectiveness or enhancement of E protein activity.

During commitment, the trajectories of *Bcl11b* and *Spi1* (PU.1) expression cross, one up-regulated as the other is down-regulated (1, 45) (Fig. 2*B*). To test whether these act as mutual antagonists, we directly compared Bcl11b KO effects with those of an acutely acting PU.1 obligate repressor in DN2a cells (PU.1–ENG), which we had used as a dominant negative to identify genes that depend on wild-type PU.1 (23). SOM analysis identified a supercluster of genes that were severely down-regulated by the obligate PU.1 repressor (Fig. 3*E*, lower right; near regions B and C1 in *SI Appendix*, Fig. S9*B*), a SOM placement consistent with expression in ETP–DN2a, as expected if driven by high PU.1 activity (compare with Fig. 3*C*). To see if these PU.1-dependent genes were up-regulated by Bcl11b deletion, we used trait-enrichment heat maps to compare these perturbations on each SOM gene metacluster (Fig. 3*H*; complete version in *SI Appendix*, Fig. S12*A*). In fact, only rare metaclusters showed opposite responses to the PU.1 antagonist and to Bcl11b deletion (Fig. 3*H*) (e.g., metacluster 212). In the gold-standard gene set, the overlaps were limited and extraordinarily symmetrical, showing no bias toward cooperative or antagonistic relationships between Bcl11b and PU.1 (*SI Appendix*, Fig. S12*B* and Dataset S3). Interestingly, some genes under dual control of Bcl11b and PU.1 were repressed by both (e.g., *Il2rb* and *Zbtb16*, and more weakly, *Kit*) (23). Thus, neither Bcl11b nor PU.1 is globally epistatic to the other. Instead, they provide mostly orthogonal inputs to the pro–T-cell program.

Strong network intersection with E2A. In contrast, Bcl11b deletion effects had strong overlap with reported effects of E2A deletion in DN2-stage pro-T cells (22) (*SI Appendix*, Fig. S12*C*). The gold-standard gene responses to Bcl11b loss (Dataset S4*A*) overlapped greatly with the effects of E2A deletion (*SI Appendix*, Table S4*B*), highly biased to respond in the same direction (Fig. 3 *F* and *G*; compare with Fig. 3*D*; numbers given in *SI Appendix*, Fig. S12*C*) (P = 3E-04 by χ^2 test). Most of the overlap was between genes up-regulated in both Bcl11b KOs and E2A KOs (Dataset S4*C*). There was less overlap between Bcl11b-dependent genes and genes affected in any way by E2A KO, but the overlap again was in genes dependent on both (Dataset S4*D*). Interestingly, this linked Bcl11b with the repressive effects of E2A even more than with E2A's activating effects (46–48). Thus, Bcl11b appears to cooperate with E2A in its effects to promote developmental progression and to suppress innate-cell like gene expression, as summarized in the model shown in Fig. 4.

GRN Roles of Bcl11b as a Regulator of Cell Fate. Bcl11b showed surprisingly few specific cross-regulatory effects on other transcription factor-coding genes previously implicated in the progression from phase 1 to phase 2 in T-cell development. Metaclusters up-regulated in Bcl11b KO cells (Dataset S2) contained some phase 1 regulatory genes that we verified to respond consistently to Bcl11b, although not meeting gold-standard criteria, including *Bcl11a*, *Nrarp*, and *Jup* (*SI Appendix*, Figs. S3*C* and S4), and distal promoter transcripts of *Runx3* (*SI Appendix*, Fig. S11). *Bcl11a* is associated with progenitor and B-cell fates, whereas *Nrarp* and distal *Runx3* are expressed in NK cells. Metaclusters up-regulated in Bcl11b KO pro-T cells (Dataset S2) also contained later-acting T-cell regulators *Gata3* and *Lmo4*, also verified by inspection to be frequently but weakly up-regulated (*SI Appendix*, Fig. S3*C*). These regulators are expressed not only in later T cells but also highly in ILC2 cells (*Gata3*) and in invariant NK T cells, ILC2, and ILC3 cells (*Lmo4*), and are also up-regulated in E2A KO samples (22). Thus, Bcl11b during commitment could be damping the non-T lineage roles of these factors.

The most striking effects of Bcl11b on other regulators were to maintain silence of genes seemingly extrinsic to the T-cell program. These include regulatory factor and cytokine receptor genes *Zbtb16*, *Nfil3*, *Pou2af1*, and *Il2rb*. These are genes without obvious drivers within the rest of the known program, and are normally poorly expressed, if at all, before Bcl11b is expressed (compare with *SI Appendix*, Fig. S2, DN1). Whereas most of these genes have at least some baseline expression, some sources of positive regulation are logically required even for targets that

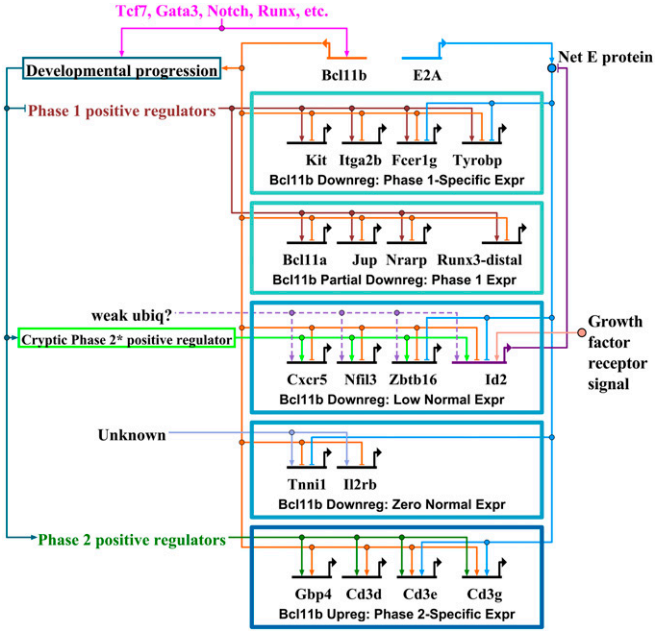

Fig. 4. GRN model of roles of Bcl11b and E2A in the T-cell specification gene network. BioTapestry plots use conventions as in Fig. 1. Targets shown are from the gold-standard list and additional genes validated as shown in *SI Appendix*, Figs. S2, S3*C*, S4, and S11, and represent sets with shared and unshared responses to Bcl11b and E2A. They include genes with likely impact on the rest of the network that are concentrated in Bcl11b-repressed metaclusters in the SOM analysis. Additional genes with similar expression properties identified by SOM cluster membership are noted in the text.

normally have undetectable expression (e.g., *Tnni1*). The Bcl11b KO effects thus logically imply a cryptic positive regulator for them (Fig. 4).

Discussion

Bcl11b is a critical component of the T-lineage commitment machinery, but the target genes it regulates during the commitment process itself have not been globally defined until now. Here we have shown that Bcl11b exerts highly stage-specific positive effects on genes involved in TCR complex assembly and more complex negative effects on a range of target genes, some only during commitment and others at later stages as well, involved in effector response and cellular identity. Bcl11b has many binding sites around both positive and negative regulatory targets, which can be extensively mined in future detailed characterization for insights into its activating and repressive modes of action.

Bcl11b plays a key repressive role during commitment that affects two large groups of genes under distinct baseline patterns of regulation. The first group comprises genes, including the key growth factor receptor gene *Kit*, that are most expressed during phase 1 and turned off after Bcl11b is induced (Fig. 4, phase 1-specific expression). Some, though not all of these genes are only open to Bcl11b regulation during the DN2 to DN3 transition, and later may be permanently silenced. The second group consists of genes that are normally not activated to substantial levels at all during the phase 1 stages. Curiously, these are highly enriched for genes used in NK cells, ILCs, and innate-like T cells, including the powerful regulatory genes *Id2*, *Zbtb16*, and *Nfil3* (6, 42, 49–55). Many of these genes, especially NK-associated genes, require continual Bcl11b action to keep them silent later (Fig. 4, zero or low normal expression). Many other genes are expressed earlier and silenced during commitment. Why are these genes not expressed before Bcl11b is activated? The fact that they are silent before Bcl11b turns on could imply that they need another positive driver that gains in net activity during T-cell specification in parallel with Bcl11b itself. Is this truly a new function? Some of these targets, like *Zbtb16* and *Il2rb*, could have positive regulators that are masked before Bcl11b is turned on, as phase 1 regulator PU.1 also represses them (23) (Fig. 1*A*). It could thus be loss of PU.1-dependent restraint in phase 2 that creates a "need" for Bcl11b to "take over" the repressive role. Further analysis will be required to relate individual Bcl11b occupancy sites at these loci to specific activating or repressive functions, which could ultimately indicate how Bcl11b works to antagonize their activators. However, the fact that Bcl11b is not needed to repress these genes until *Bcl11b* itself is activated suggests that Bcl11b acts on these genes in classic incoherent feed-forward circuit architecture.

The role of Bcl11b is mostly orthogonal to the role of PU.1, and it does not appear to be a direct repressor of most phase 1 transcription factor-coding genes. Many of its phase 1-associated targets encode cell surface markers, signaling, and cell biology components. However, the convergence of Bcl11b actions with E2A actions at the DN2a/b stages is striking. The mode of action does not appear to require coordinated binding to DNA, because the peaks identified here on dual E2A–Bcl11b target genes (Fig. 3*A* and *SI Appendix*, Fig. S8) do not coincide substantially with peaks bound in DN3 cells by E2A (46). Does Bcl11b amplify or sustain E protein action mainly by blocking expression of Id2, the E protein antagonist? Id2 is crucial for the regulatory states of NK cells and ILCs, but it cannot fully account for Bcl11b's role in the phase 1 to phase 2 transition, as there is no decrease in Id2 expression then (Fig. 2*B*). Also, *Id2* is very easily up-regulated with any reduction of Bcl11b level, suggesting that Bcl11b may only weakly repress this gene. Recently, too, the type 2 ILC lineage has been shown to depend on Bcl11b as well as on Id2 (11, 32, 33), proving that the two can be coexpressed. Most genes de-repressed in Bcl11b KOs (Datasets S1 and S2) are more typical of NK, ILC1, and ILC3 cells than of ILC2 cells (54, 56), suggesting that low levels of Bcl11b may block these alternatives while still allowing Id2 expression in ILC2 cells. Thus, additional Bcl11b-repressed genes besides *Id2* may be equally important in excluding innate fates.

Up-regulation of *Zbtb16*, *Nfil3*, *Id2*, and *Il2rb* in Bcl11b-deficient cells provides both growth support and identity functions for NK and ILC fates outside of the conventional αβ T-cell pathway (43, 53, 57, 58). Many Bcl11b KO cells also up-regulate the phase 1-specific Notch-negative feedback regulator, *Nrarp*, which is also highly expressed in NK cells. These genes are not all up-regulated uniformly in all Bcl11b KO cases, however. For example, *Zbtb16* was up-regulated in our DN2–DN3 KO samples made by both protocols I and II (*SI Appendix*, Fig. S2 and Dataset S1 *A–C*), but more strongly in fetally derived cells and not in DP KO cells (2, 13; but see ref. 59). In contrast to *Zbtb16*, *Nfil3* was more strongly up-regulated in protocol II and in DP cells, again suggesting underlying differences in their needs for positive regulators that could promote alternatives to T-cell fate.

The fate alternatives restricted by Bcl11b have further differences from those controlled by PU.1. Pro-T cells in phase 1 overtly express multiple myeloid-associated genes regulated by PU.1 (23), even as Notch signaling restrains others. However, these are silenced during commitment and most will never be expressed by T cells again. In contrast, many of the ILC response genes that Bcl11b represses are normally expressed much later by mature T cells during postthymic immune effector responses, despite the cells' committed status (*SI Appendix*, Fig. S7). Thus, it may be physiological for these Bcl11b-repressed genes to be latently primed for activation during T-cell specification. If so, then another way to see Bcl11b's role at the DN2b stage is that it imposes a temporal delay between the time that these genes could be initially specified for activity (in DN2b pro-T cells) and the time that mature TCR-αβ T cells (their much later descendants) will be allowed to deploy them. As we and others have discussed previously (60, 61), the timing of deployment of the effector response gene subnetwork distinguishes αβ-TCR$^+$ T cells, γδ-TCR$^+$ T cells, and ILCs more profoundly than the actual effector response networks themselves. Some of the targets we see up-regulated in Bcl11b KOs in fact include genes specific for certain γδ lineages (e.g., *Cd163l1*, *Tnni1*, and certain *Tcrg* transcripts). Thus, Bcl11b appears to control the conditionality of access to effector activation gene subnetworks even more than progression within a single canonical T-cell pathway itself.

The role of Bcl11b in commitment thus reflects a different mechanism of innate/adaptive divergence than the control of access to myeloid fates. Unlike PU.1, Zbtb16 and Nfil3 are not normally part of the phase 1 regulatory state. However, considerable overlap between T-cell and ILC programs (6), including use of Runx3 and GATA-3, suggests ways that the T-cell specification process itself can generate mechanisms to prime these genes for expression. Loss of Bcl11b can allow levels of both *Gata3* and *Runx3* to rise. Maintaining repression then appears to depend on E protein, directly or indirectly, as well as Bcl11b. Thus, in the T-cell GRN, the most dynamically regulated T-cell factor collaborates with the most unchanging one to establish and preserve the committed state.

Methods

See *SI Appendix, Supplementary Methods* for details.

Animals and Cell Preparations. Bcl11b conditional KO mice, C57BL/6 (B6) controls, and PLBD [(B6,129).*Bcl11b*$^{fl/fl}$;ROSA26-CreERT2 (2)] mice with or without a ROSA26R-eYFP Cre-reporter gene were bred and used as sources of control and Bcl11b-deficient T-cell precursors as previously described (3, 27) (*SI Appendix, Supplementary Methods* and Dataset S5). B6.*Lck-Cre* mice were obtained from Taconic Laboratories. Both KO and control cells were Cre-treated and usually sorted based on Cre-induced YFP$^+$ phenotype as well as DN2a/b phenotype. RNA was prepared and sequenced as described previously (8). Animals were bred and maintained under specific pathogen-free conditions in our colony at the California Institute of Technology under protocols approved by the Institutional Animal Care and Use Committee.

Data Analysis. RNA-seq and ChIP-seq fastq files were aligned and processed, and the data matrix submitted to SOM analysis (17), as described in detail in *SI Appendix, Supplementary Methods*. The gold-standard gene set was defined by EdgeR comparisons of eight KO and control sample pairs (*SI Appendix, Supplementary Methods* and Datasets S1 *B* and *C* and S5).

Gene Network Construction. BioTapestry v. 7.1.0 (www.biotapestry.org) software (62) was used as described in *SI Appendix, Supplementary Methods*.

ACKNOWLEDGMENTS. We thank Georgi Marinov and Barbara Wold for advice; Igor Antoshechkin of the Millard and Muriel Jacobs Genetics and Genomics Center for sequencing; Henry Amrhein, Diane Trout, and Sagar Damle for data curation; Diana Perez, Josh Verceles, and Rochelle Diamond for flow cytometry and cell sorting; Rochelle Diamond for laboratory management; and Lorena Sandoval, Scott Washburn, and Robert Butler III for supervision of the mice (all at the California Institute of Technology). This work was supported by NIH New Innovator Award DP2GM111100 (to A.M.); NIH Grants R01HD073113 (to W.J.R.L.), K99HL119638A (to H.Y.K.), and RC2CA148278, R01CA90233, R01AI083514, and R01AI95943 (to E.V.R.); the L. A. Garfinkle Memorial Laboratory Fund and the Al Sherman Foundation; the Caltech-City of Hope Biomedical Research Initiative; and the Albert Billings Ruddock Professorship (E.V.R.).

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