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Dynamic control of the T-cell specification gene regulatory network

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Abstract

Specification of multipotent blood precursor cells in postnatal mice to become committed T-cell precursors involves a gene regulatory network of several interacting but functionally distinct modules. Many links of this network have been defined by perturbation tests and by functional genomics. However, using the network model to predict real-life kinetics of the commitment process is still difficult, partly due to the tenacity of repressive chromatin states, and to the ability of transcription factors to affect each other's binding site choices through competitive recruitment to alternative sites ("coregulator theft"). To predict kinetics, future models will need to incorporate mechanistic information about chromatin state change dynamics and more sophisticated understanding of the proteomics and cooperative DNA site choices of transcription factor complexes.

Keywords

Gene regulatory network; Transcription factors; Notch signaling; Functional genomics; Epigenetic constraints; System dynamics; Topological model; T cell development

Introduction

T cell development is a particularly accessible system for studying the sequence of regulatory changes through which stem and progenitor cells resolve their multipotency to select a particular differentiation pathway. Multipotent blood (hematopoietic) precursor cells are driven to a T-cell fate by sustained Notch-pathway signaling, after they enter the thymus [1](see reviews [2–5]). In the process, the cells downregulate a large battery of stem- and progenitor-associated regulatory factors, upregulate T-lineage associated regulatory factors, undergo commitment to the T-cell fate, and then upregulate recombinase-activating gene (RAG) 1 and 2 expression to execute recombination at the T-cell receptor (TCR) loci, which

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will enable them to work as T cells. T-lineage-affiliated transcription factors GATA-3 and TCF1 (encoded by *Tcf7*) are induced early in this process, but several waves of other T-cell transcription factors are activated at distinct stages during and after commitment. Stages of this process in mouse development (ETP, DN2a, DN2b, DN3a, DN3b, DP) are defined in Fig. 1A and have similar counterparts in human development. The T cell precursors enter the thymus as multipotent cells that can develop into other cell types if their Notch signaling is blocked or interrupted. Between DN2a and DN2b stage, however, they lose this alternative developmental capacity, and they become intrinsically committed to generate T cells or die [6–8]. Commitment is an indicator of a major transition in gene regulatory network state as well as genome-wide epigenetic state.

Regulatory gene expression changes provide robust landmarks for the T-cell specification process [4](Fig. 1B). Recent single-cell transcriptome analysis confirms that the same ETP cells that initiate the T-cell program begin by expressing multiple progenitor-specific factors [9]. Progenitor-factor silencing can then be directly linked with the narrowing of developmental potential, since after commitment, addback of a progenitor factor like PU.1 can restore access to alternative developmental fates [10–12](rev. in [13]). Perturbation tests can be done relatively easily in early T-cell development because of excellent stromal coculture-based systems that support early differentiation in vitro [14], and efficient transduction with retroviral vectors for gain of function, shRNA knockdown, and Cre or CRISPR-mediated deletion experiments.

Transcription factors with different expression patterns each contribute independently to the changing gene expression patterns in the cells. For example, PU.1 is downregulated during commitment, Bcl11b is upregulated during commitment, and E proteins are expressed consistently throughout (Fig. 1C). Yet when the genes that encode them are disrupted by stage-specific Cre- or Cas9-dependent knockouts [15–17], whole-transcriptome analyses show that these factors interact combinatorially in different ways on different target genes (Fig. 1D), for promotion of the T-cell fate or for suppression of some alternative fate(s).

Topological T-cell gene regulatory network model

Perturbation evidence has been used to construct a “bottom-up” gene regulatory network model for T-cell specification from multipotent precursors [18]. The network model captures available functional data, giving causal polarity, not simply correlations, with the goal to clarify how well available evidence explains T-cell developmental control. A newer version of the model (Fig. 2) has been improved by mapping of the genomic binding of key transcription factors at increasingly relevant developmental stages, new tests for the regulatory functionality of these sites, new genome-wide analyses of genes affected by gain and loss of factor function in relevant stages, and improved methods of causing potent, synchronous perturbations within relevant developmental intervals (references in Table 1) [15–17,19–22]. There remain issues: not all edges may be direct; functional linkages measured in one developmental state may not be the ones operating at a different state; direct transcription factor binding to a gene may not indicate function; and gain- and loss-of-function phenotypes may not agree due to regulator dosage sensitivities (detailed in [23]). Some crucial limitations are discussed below. However, the network model illustrates the

dynamic logical relationships between several distinct groups of regulators that guide T-lineage specification.

Regulator Groups

Group A (Fig. 2, upper left; see inset) is Notch signaling, working on direct targets as a transcriptional coactivator. Signaling is triggered when the transmembrane Notch1 protein comes into contact with Notch ligands DLL4 or DLL1 provided in the thymic microenvironment (thymic epithelium). The response depends on the expression of *Notch1* and transcription factor-coding gene *Rbpj* (CSL, Suppressor of Hairless) in lymphoid precursors. Notch-activated target genes include *Hes1*, encoding a repressor, and *Nrarp*, encoding a feedback inhibitor.

Group B (Fig. 2, upper right and *Spi1*; see inset) is a group of stem/progenitor regulatory genes, expressed in the earliest pro-T cells [4,9]. Many are implicated in self-renewal and also participate in certain non-T cell fates. Among their products, PU.1 (encoded by *Spi1*) has the strongest genomic signature, as its binding occupancy is most prevalent and its canonical target motif is most enriched across the genomic sites that are specifically active during pre-commitment stages [16,19,21].

Group C (Fig. 2, middle right; see inset) encodes the core group of T-lineage-affiliated transcription factors activated directly or indirectly by Notch signaling, including TCF1 (*Tcf7*), GATA-3, and Bcl11b. Regulatory genes *Tcf7* and *Gata3* are activated in the earliest responses to Notch signaling, and are essential from the earliest stages [24–27], while *Bcl11b*, *Ets1* and others are activated later [4].

Group D (Fig. 2, lower left, see inset) centers on the genes encoding the E proteins, E2A (*Tcf3*) and HEB (*Tcf12*), which are expressed continuously but participate in different transcription factor dimers. As E2A/HEB dimers, they regulate many T-cell genes post-commitment [15,28]. They can be neutralized by dimerization with Id2 and Id3, which totally block their DNA binding. E proteins regulate not only some of the same post-commitment genes as Bcl11b, but also some of the same pre-commitment genes as PU.1 (Fig. 1C), possibly reflecting their ability to participate in distinct complexes with additional dimerization partners, e.g. group B gene Lyl1 [29–31].

Group E gene-encoded factors (Fig. 2, middle; see inset) are also expressed at similar levels from prethymic multipotent progenitor stages throughout early T-cell development. These include Runx family factors, Myb, Gfi1, and Ikaros family factors, which can play indispensable roles without being lineage-instructive. Runx1 is particularly important for the gene expression impacts of group C regulators. It collaborates with group C factors (GATA-3 and probably TCF1)[32–34] to repress group B gene *Spi1* (encoding PU.1) during commitment [20,35,36]. However, before commitment, it co-localizes with PU.1 and contributes to its activity [20]. Some of the latest insights into mechanisms making commitment so transformative show how factors in group E can have highly regulated and dynamic functions in T-cell development despite their modestly changing levels.

Cells in the T-cell pathway also require stage-specific viability support from three cytokine receptors until successful TCR expression. Initially, Kit and Flt3 are both required. Flt3 is downregulated midway through the ETP stage, Kit declines during commitment, while upregulation of IL7R (encoded by *Il7r* and constitutively expressed *Il2rg*) sustains the cells from DN2a onward.

Essential Intergroup Network Circuits

Circuit 1: Notch signaling (group A) activates the T-cell affiliated regulatory genes (group C) in a positive feed-forward circuit. For a key subset of the group C genes, *Tcf7* and *Gata3*, this begins while the multipotent progenitor-associated gene set is still actively expressed [26,27]. Another important node in this network, represented by *Bcl11b*, is activated later, as described below.

Circuit 2: Within the initial stages of T-cell development, Notch signaling also modulates the activity of PU.1 ("Notch-sens PU.1"), whereas high-level PU.1 may inhibit Notch signaling ("PU.1-sens Notch"). PU.1 has direct positive effects on a subset of other group B regulatory genes, including *Bcl11a*, *Lmo2* and *Mef2c* [12,16]. Notch signaling focuses PU.1 activity on target genes associated with multipotent progenitor status (group B), rather than on target genes associated with an alternative myelomonocytic differentiation pathway [12], enabling PU.1 to support proliferation in early T-cell stages [37]. Notch may accomplish this by repressing C/EBP family members [12,16] needed to collaborate with PU.1 for myeloid differentiation [38,39], possibly via the Notch-induced repressor Hes1 [40]. The mechanism through which PU.1 damps down Notch signaling is not clear yet. The balance between PU.1 and Notch signals, sustained over multiple cell cycles, is one of the key distinctions of the early T-lineage regulatory state (Fig. 2 legend).

Circuit 3: Basic helix-loop-helix (bHLH) E proteins, E2A and HEB (encoded by *Tcf3* and *Tcf12*), group D in the network, are crucial for B as well as T cell development, but they play key roles in activating T lineage-specific genes as well as B-T shared genes. Their own expression changes relatively little during T-cell specification, but their connectivities in the network are stage- and context-dependent. While contributing to group B gene expression, likely via complexes with Lyl1 and Lmo2 ("L/L/E"), they are also needed especially to sustain Notch1 expression itself (group A)[15,41], and they become dominant regulators of T-cell-specific genes like the invariant TCR components activated specifically during and after commitment. E protein activity particularly strongly distinguishes the T-cell pathway from the Innate Lymphoid Cell (ILC) differentiation pathways, and needs to be sustained in order to prevent diversion of the pro-T cells to an ILC pathway [42–44](see Box 1).

Although levels of expression of the E proteins themselves are not strongly affected by perturbations of the other network components, their activities are highly modulated by other, dynamically regulated network members. The most prominent E protein inhibitors are helix-loop-helix antagonists Id2 or Id3. Although not normally expressed, Id2 is potentially activatable in DN T-cell precursors under various growth-stimulatory conditions. Id3 is activated specifically by signals like those delivered by newly assembled TCR, which normally terminate the T-lineage determination process at the end of the DN3 stage.

Importantly for the T-cell developmental process, regulators of three other network groups can work to block Id protein expression: PU.1 (group B)[16,37], Bcl11b (late-activated member of group C)[17], and possibly Notch signals (group A)[12,44], themselves. Thus, E protein activity may be seen to be protected through a stage-to-stage relay mechanism for inhibiting its inhibitors.

Latent circuits, suppressed in normal development

Three powerful circuits for diversion to alternative lineages are maintained in latent form in pro-T cells (details in Box 1). All of these are normally kept in check during normal early T-cell development but become fully active in case of regulatory perturbations. (1) Elevated PU.1, when combined with loss of Notch signal effectiveness at any stage through DN3, causes coordinated, fast multi-node inhibition of the T-cell program coupled with induction of a monocytic or dendritic-cell-like program. (2) Elevated GATA-3 at any stage through DN2 causes the T-cell program to abort, with cells favoring a transition to the mast-cell program. (3) Removal of Bcl11b at any time up through DN3 stage causes cells to redirect to an abnormal T-cell program with strong innate lymphoid cell-like features. Because of the large numbers of regulatory genes affected in these latent circuits, there may be many indirect and cooperative effects involved (Box 1).

Commitment

Commitment is the transition when developing pro-T cells lose the intrinsic ability to shift to an alternative (non-T) fate if Notch stimuli are withdrawn. Fluorescent reporter assays show that at the single-cell level commitment occurs when cells upregulate *Bcl11b* [6]. It is also accompanied by the decline in expression of PU.1 and multiple other group B regulators, and closely followed or accompanied by upregulation of other late-tier group C factor coding genes, *Ets1*, *Ets2*, and *Lef1* (Fig. 1B,C and [4]). Thus, the metastable network state dominated by Group B factors, together with Notch (group A) and Group E contributions, gives way to the alternative, committed network state dominated by Group C factors, together with Notch, Group E, and full-fledged Group D contributions. Recent genome-wide evidence shows that this is when chromatin sites enriched for PU.1 binding motifs close and chromatin sites enriched for E protein and TCF1 binding motifs open [16,19,21]. This transformation also entails a global transformation of 3D chromatin organization [19]. Thousands of genomic sites gain accessibility while thousands lose accessibility, while looping frequencies within topologically-associated domains undergo considerable changes. Whole topologically associated domains undergo compartment flips, from decondensed, “active” configurations to condensed, “silenced” configurations [19].

Network dynamics: beyond network topology

Despite the intricacy of the documented network connections, the topological network model is not sufficient to explain the dynamics of the T-cell development process. The main problems are that in actual thymocytes of young adult mice, the state changes are slow, occupying days and multiple cell divisions, and that individual regulatory nodes exhibit unpredictably delayed responses to changes in known positive or negative inputs. Most obviously, the commitment transition occurs later than it would be predicted to occur based

on simple assumptions about gene regulation biochemistry. For example, *Bcl11b* is turned on by Notch signaling, Runx, GATA-3, and TCF-1, but all of these positive inputs are already strongly active, days before *Bcl11b* is turned on [6]. Similarly, GATA-3, TCF-1 and Runx1 can repress *Spi1* [32–36], even though *Spi1* expression persists in the presence of these factors for many cell cycles before commitment. In these respects, the T-cell regulatory network starkly differs from other classic models, like the Boolean model of sea urchin endomesoderm specification [45] or ODE models of Drosophila syncytial blastoderm patterning by gap genes [46].

Several possible reasons could contribute. The T-cell network model is certainly incomplete, and the known regulators could be opposed by trans-acting antagonists at crucial target nodes, as yet uncharacterized. T-cell development also shows a narrow optimum dosage range in its responses to several of its key regulators, shown conspicuously for effects of GATA-3 [33,34,47,48] but also for E proteins [49] and Runx1 [50]. This could reflect a need to keep a regulator's dosage low enough to avoid activation of confounding circuits while keeping it high enough to out-compete possible sources of antagonism. More profoundly, however, there may be a problem with the basic assumption of topological gene network models: namely, the assumption that when a transcription factor is expressed, it will "find" its target sites on the genome in a predictable way. There are at least two issues with this assumption in the T-cell case, discussed in the next sections. One is that T-cell development starts from an epigenetic state that imposes real barriers to transcription factor activity on the network genes. The other is that even when sites are accessible, the deployment of transcription factors across possible sites is not autonomously determined, but influenced strongly by other factors. This creates novel forms of indirect antagonism as well as cooperativity, but with rules yet to be characterized. Together, these are formidable sources of nonlinearity in network state dynamics.

Accessibility

Several factors with important roles in the T-cell specification network model are known to be able to open chromatin. PU.1 (group B)[16,51,52] and TCF-1 (early-initiating group C) [21] can displace nucleosomes, create nuclease accessibility, and initiate activating histone modifications around their binding sites. Chromatin opening is probably an important aspect of their mechanisms of action as positive transcriptional regulators. However, not all their genomic sites are equally accessible to them in any given cell state. For example, PU.1 has been compared by ChIP-seq in various cellular lineages and across different levels of expression within the same cell lineage, where collaborating factors are more likely to be similar [16,52,53]. In pro-T cells as in other cell types [54,55], PU.1 is excluded from chromatin with H3K27me3 histone modifications, even from sites that are highly bound by PU.1 in B or myeloid cells [53], but otherwise it binds to approximately equal numbers of "open" and "closed" sites in pro-T cells, as classified by ATAC accessibility [16]. This ability to bind closed sites is consistent with its "pioneer factor" role in other cells, albeit showing that binding is not always sufficient to cause opening. But quantitative analysis shows that PU.1 only occupies closed sites if they have substantially better matches to the ideal PU.1 target motif than is needed for open sites [16]. Thus, even for a "pioneer factor" like PU.1, the impact of prior site opening can be considerable.

Chromatin state not only affects transcription factor binding, but can also affect the timing of changes in target gene expression once transcription factor binding occurs. A striking, although possibly extreme, example is in the regulation of *Bcl11b*. The four known positive inputs for *Bcl11b*, namely GATA-3, TCF1, Runx1, and Notch signaling [6], are all active within ETP stage [53,56]. However, *Bcl11b* itself is only turned on in late DN2a stage, measurably multiple days and cell cycles after the ETP to DN2a transition [6]. At least part of the delay has now been shown to be based on a cis-acting epigenetic constraint. Despite some local DNase accessibility, permissive histone marks, and PU.1 binding at the *Bcl11b* superenhancer region as early as ETP stage [19,53,57], even accompanied by lincRNA transcripts [58], the whole chromatin subdomain from *Bcl11b* to *Vrk1* has a closed configuration in ETP and most DN2a cells [19]. *Bcl11b* may also require escape from a nuclear matrix-associated compartment to be activated [58]. Its initial state is evidently a substantial kinetic barrier to the activation of the locus. This has been shown by tagging the two alleles of *Bcl11b* in the same mouse with two different fluorescent protein reporters [59]. A trans-acting constraint, whatever it was, would affect activation of both alleles equally, whereas if the two alleles were unsynchronized in their activation in the same cell, this would need to be explained by a cis-acting constraint. In fact, longitudinal imaging of individual clones shows that multiple cell cycles can intervene between expression of the first and second-activated alleles within the same cells [59]. As allele choice in a clone is random, the same epigenetic resistance that is measured by the ***difference*** in allele activation times would likely have slowed the response of the first allele as well. Thus, the real biological system is both slower and more stochastically noisy than the topological model, or an ODE implementation of the topological model, might predict.

Nonautonomous transcription factor “choreography”

Durable epigenetic states can establish barriers to interconversion between cell types as just described. However, ChIP-seq analyses show that many factors also redistribute their binding patterns over the genome during natural development, relatively quickly and within a cell lineage. GATA-3 occupies markedly different site patterns at different pro-T cell [53] and later T-cell stages [60]. Runx1, like GATA-3, shifts its binding sites markedly during normal pro-T cell development as cells progress through the commitment transition [20], with many sites vacated despite overall increases in total binding. Are the losses of GATA-3 or Runx1 binding due to loss of co-occupying factors, which might be represented in a gene network model as AND logic partners? Are the losses due to displacement by competing antagonists, which might be represented as AND NOT inputs? Or could something else be occurring?

Collaboration with other factors in normal T-cell development both positively and negatively influences the site choices of specific transcription factors. In fact, signs of this phenomenon have been seen widely (e.g. [61–64]) and may be a general principle of developmental state shifts. In pre-commitment stages, PU.1 strongly recruits other factors to bind with it at its target sites, including Runx1, Satb1, and GATA-3 [20]. In later T-cell development (DP stage), TCF1 is a strong influence on other factors, including E proteins [21,22]. Gene network impacts of TCF1 in DP cells also cause it to stabilize E proteins, complicating this effect [22]. However, without changing TCF1 levels, the E protein HEB also influences the

sites where TCF1 binds to the DNA [22]. Importantly, both for the effect of PU.1 and for the effect of HEB, partner recruitment to co-binding sites can also draw partners competitively away from alternative sites ("coregulator theft")[20]. When a cell line similar to newly committed pro-T cells (PU.1-negative) is transduced to (re-)express PU.1, mimicking retrograde differentiation, Runx1, Satb1, and GATA-3 move to different sites, vacating many while joining PU.1 at others. This effect is not "eviction", for it does not appear to involve any PU.1 binding at the emptied sites [20]. The response is relevant to normal development, for the shifts of Runx1 binding that PU.1 causes in the artificial retrograde differentiation response are largely the reverse of the natural shifts of Runx1 that occur during normal forward differentiation, as endogenous PU.1 is turned off. Motif analysis shows that re-introduced PU.1 in the cell line model draws Runx1 to combined sites that are "poorer-quality" Runx sites, based on Runx motif scores, than those that Runx1 occupies in the absence of PU.1 [20]. This too mirrors the natural deployment changes of Runx1 from pre-commitment to post-commitment stages.

Transcription factor pool effects like these emphasize that the physiologically regulated pool sizes of these factors are limited, so that there is competition for these factors among distinct sets of potentially accessible sites. Thus, the ability of genomic sequence at a site to predict the likelihood of occupancy by a given factor depends not only on the site's match to an optimal motif and its accessibility in chromatin, but also on the presence of specific other "theft-capable" factors in the cell that may nucleate more stable ternary complexes, in the context of the pool size of the factor of interest. Although probably important for predicting gene network dynamics, at present the key parameters are hard to measure. We do not yet know the rules determining which factors are "theft-capable" or "theft-vulnerable" in different contexts. Proteomics of transcription factor complex assembly must become much more sensitive, to capture and measure these interactions in a genomic site-specific way.

Concluding remarks

Gene network logic in early embryos is extremely powerful not only to describe but also to predict developmental dynamics, partly because of the orderly increase in complexity from the unique state of the fertilized egg, and partly because of the epigenetic openness of the system. Not only does the genome act as though it is functionally unconstrained during zygotic gene activation, but also pre-existing transcriptional regulators are sparse. In contrast, T cells in young adult mice develop from non-naïve stem and progenitor cells that have fully established tissue-specific epigenetic constraints already, and harbor a rich complement of stem and progenitor-associated transcription factors already investing the genome. From this point of view, natural T cell development is more like induced pluripotent cell reprogramming than like emergence of an original embryonic cell lineage.

Here, the current understanding of gene network modules that guide progenitors to acquire T-cell identity is reviewed. Activities of these modules, containing nodes encoding transcription factors, are seen to cooperate and compete in shifting circuitries. However, the powerful experimental systems available for early T-cell development reveal that the trans-factors encoded by nodes in the network currently provide only a qualitative understanding of the dynamics of T-cell developmental progression. The relationships between factor

action and chromatin opening still need to be factored in, not omitting their intrinsic stochasticity. Furthermore, highly nonlinear system behavior emerges because of the unexpectedly strong system-level effects of transcription factor collaboration given limited pool sizes. Technologies that measure these interactions accurately will make a whole new generation of gene network models possible.

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Identity of T Cells. Immunity 2018; 48:243–257 e10. [PubMed: 29466756] TCF-1 motifs are shown to predominate in regulatory elements that are opened definitively during T-cell commitment and sustained afterwards, and detailed analysis of DP cells shows the ability of TCF-1 to alter chromatin configuration. Even when introduced into fibroblasts, TCF-1 is shown to open many T-lineage specific chromatin sites and to induce detectable transcription of T-cell specific genes.

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Box 1:

Latent gene network circuits.

Latent circuit 1: myeloid cell potential.

Latent circuit 1 is triggered when high levels of PU.1 cause an inhibition of Notch signaling (mechanism still to be determined). The combination of high PU.1 and low Notch signaling reveals novel responses. T-cell program genes *Tcf7*, *Bcl11b*, and *Ets1* (group C), *Tcf3* and *Tcf12* (encoding E2A and HEB, group D), and *Gfi1*, *Runx1*, and *Myb* (group E) are all substantially repressed. At the same time, innate immunity-associated *Cebp* family and *Irf* family regulatory genes are sharply up-regulated. Most of the repressive effects may be indirect, as shown with a dominant repressor form of PU.1 [37]; a proximate inhibitor of the T-cell program could be C/EBPα [11]. Note that normal levels of Notch signaling in the thymus completely block these responses to PU.1 via Circuit 2, even up to at least 3× physiological *Spi1* expression levels.

Latent circuit 2: mast-cell potential.

Although latent circuit 2 is not documented at genome-wide scope, elevated GATA-3 blocks T-cell development [47] and can redirect cells toward a mast cell program [33]. Elevated GATA-3 blocks expression of group B regulatory factors PU.1 and Bcl11a, which is also needed for early T-cell viability [66], and severely downregulates the crucial growth factor receptor IL-7R, while it upregulates *Kit* and mast-cell genes [33]. If Notch signaling is sustained, the cells die in these conditions. Withdrawal of Notch signaling causes them to lose *Tcf7* expression as well, but they can then activate a full mast-cell program if supported by Kit ligand.

Latent circuit 3: innate lymphoid/ innate-like T cell program.

Bcl11b activation is linked to commitment, but the response to deletion of *Bcl11b* in late DN2b/DN3 cells does not restore the stem/progenitor cell state. Instead, it causes upregulation both of genes expressed in non-T programs and of genes expressed in the effector stages of later T and ILC or natural killer cell development, including *Id2*, *Nfil3*, *Zbtb16*, and others [17,18,67]. *Tcf7*, *Gata3*, and *Ets1* expression is preserved intact [17]. In general, ILC and effector T cell programs share use of the E protein antagonist, Id2 [3,15,42,68–71]. In pro-T cells, specific parts of the Bcl11b regulome in fact reflect its ability to help sustain maximal E protein activity by keeping *Id2* repressed: for example, both the increase in *Zbtb16* expression and the decrease in T-cell receptor signaling component coding genes *Cd3g*, *Cd3d*, *and Cd3e* when *Bcl11b* is deleted are much attenuated if both *Id2 and Bcl11b* are deleted together [17].

Highlights:

- A gene regulatory network model for T-cell specification is based on functional data
- Five modules of regulatory genes are linked by defined, stage-dependent subcircuits
- Pro-T cells also prime three latent network circuits that are normally suppressed
- Actual system kinetics are also constrained by slow epigenetic state changes
- Regulator genomic binding site choices are competitive, affected by other factors

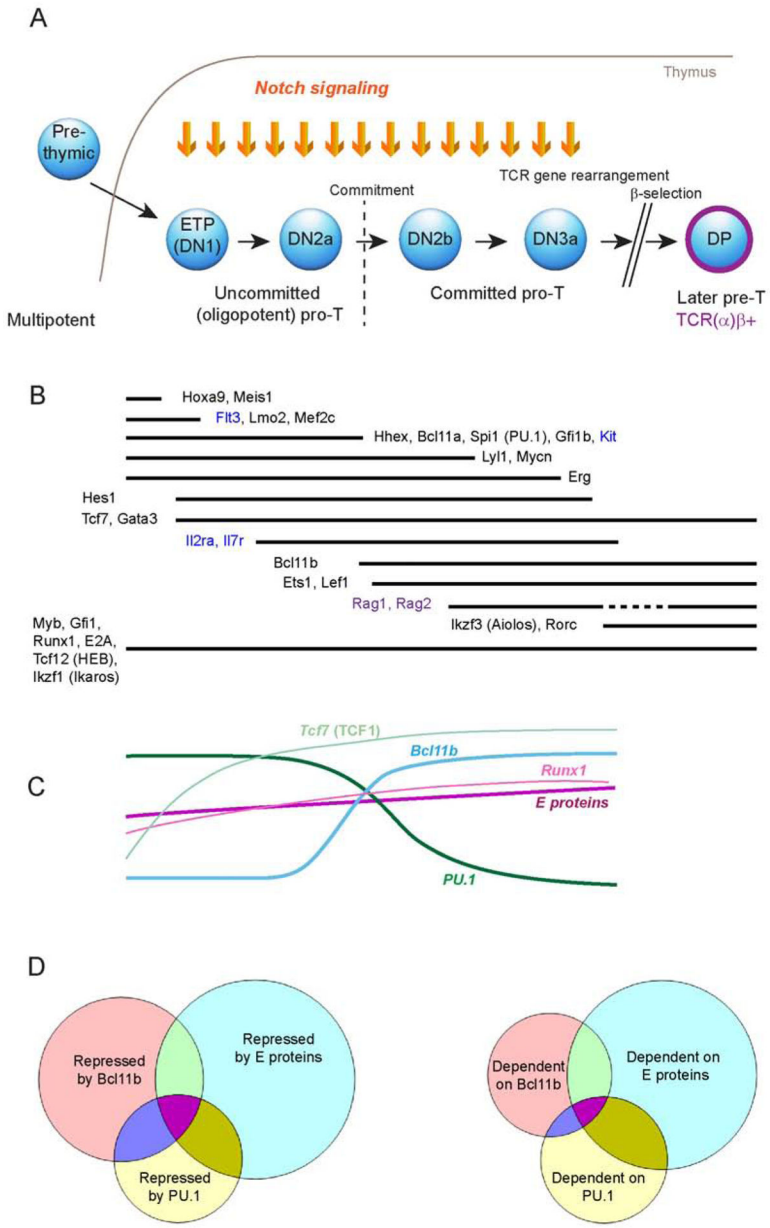

Fig. 1. Outline of T-cell development and T-cell gene regulatory network

A. Diagram of mouse T-cell developmental stages in the mouse. Cells in early T-cell developmental stages are called “DN” for CD4, CD8 “double negative” (in later stages, the cells express either or both of these markers). The most primitive cells in any cohort of DN cells are Early T-cell Precursors (ETPs: Kit^{+} $CD44^{+}$ $CD25^{-}$), then DN2a (Kit^{++} $CD44^{+}$ $CD25^{+}$), DN2b (Kit^{int} $CD44^{+}$ $CD25^{+}$), and DN3a (Kit^{low} $CD44^{low}$ $CD25^{+}$ $CD28^{-}$). If and only if TCR gene recombination happens successfully in DN3a cells so that a TCR protein can be expressed, the cells can differentiate further in the T cell pathway. Cells successfully expressing a TCRβ chain through a process dependent on Rag1, Rag2, Dntt, and auxiliary signaling molecules (CD3γ, δ, ε and others) proceed to $CD4^{+}$ $CD8^{+}$ (DP) stage, when both

chains of the αβ TCR are first expressed, and then to other stages beyond the scope of this review.

B. Simplified summary of expression patterns of regulatory genes and cytokine receptors through early T-cell developmental stages until full TCR expression. Approximate “on” and “off” states are depicted; more quantitative modulation occurs for some factors.

C. Schematic contrasting the expression patterns of PU.1, Bcl11b, and E proteins relative to T-cell lineage commitment. Vertical axis approximates log scale values (~100 fold range). “E proteins”: combination of E2A (stably expressed) with HEB (moderately increasing during T-cell specification). Expression patterns of TCF1 (*Tcf7*) and Runx1 are also shown.

C. Venn diagrams showing the independent but intersecting effects of PU.1, Bcl11b, and E proteins (E2A and/or HEB) relative to each other as determined by genome-wide transcriptome analysis in acute stage-specific gene knockout experiments. Left panel, dark purple intersection: genes repressed by all three factors are enriched for innate lymphoid program genes. Data for PU.1, from [16] Table S5, represent effects of acute PU.1 deletion in adult bone marrow-derived DN2a-DN2b cells. Data for Bcl11b, from [17] Table S3, represent genes that are significantly differentially expressed in at least two of three *Bcl11b* gene knockout protocols used in adult intrathymic or adult bone marrow-derived DN2b-DN3 cells. Data for E proteins represent the effects of a double knockout of E2A and HEB (*Tcf3* and *Tcf12*) using *Il7r*-Cre in fetal ETP cells from [15](GSE95337). Note that fetal ETPs precociously express many genes that are only expressed in more advanced adult pro-T cells, as well as genes expressed in adult ETPs [15,34,65].

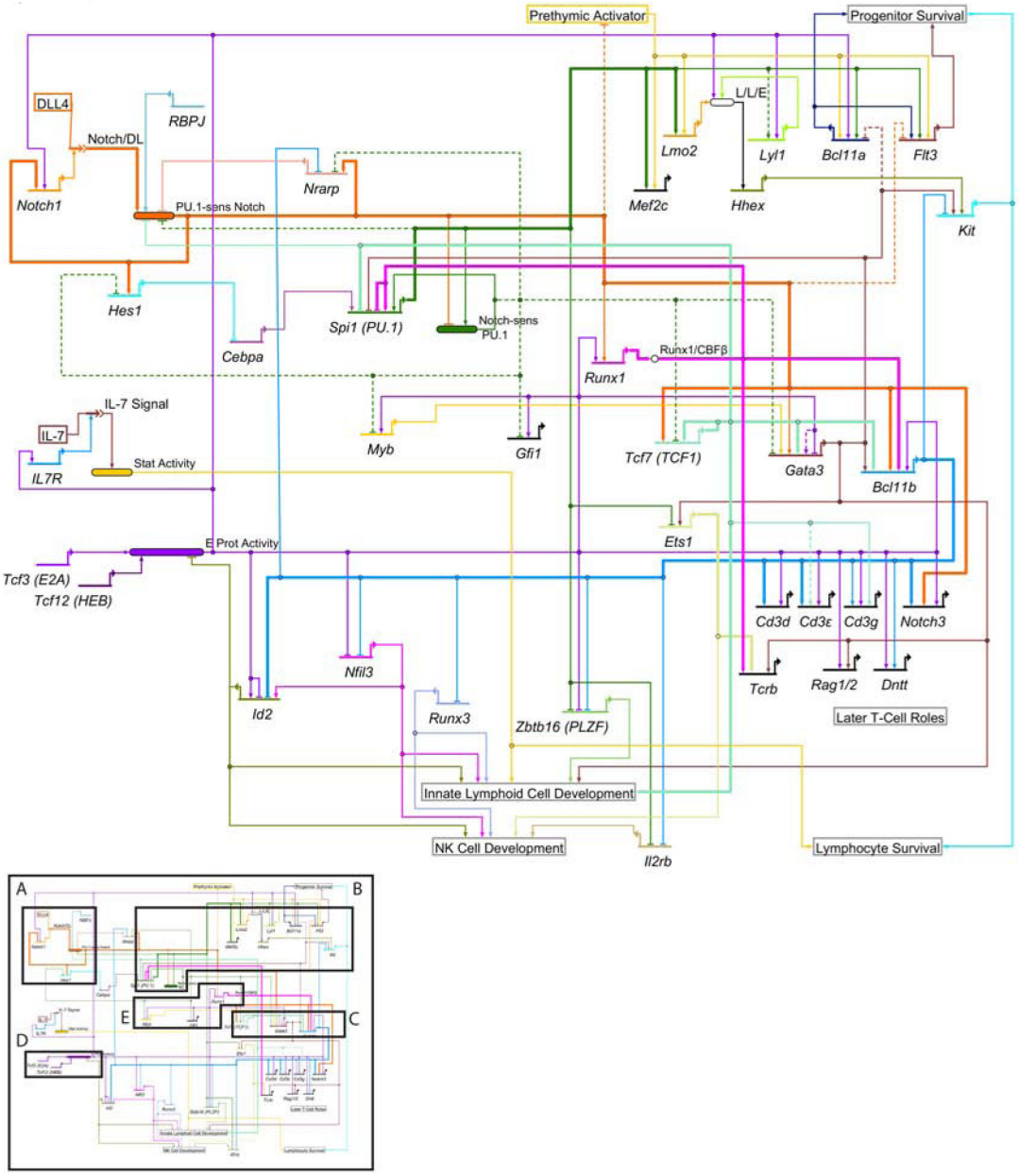

Fig. 2. Gene network model for early T-cell specification

Shown is an updated, streamlined version of the network developed in ref. [18], focusing on important nodes and consistently supported linkages (sources cited in Table 1). Inset at bottom shows boundaries of groups described in the text. Group A regulators centered on Notch are in upper left. Group B regulators are in upper right and include PU.1 (encoded by *Spi1*). Group C regulators *Tcf7*, *Gata3*, *Bcl11b*, and *Ets1* are in middle right. Group D regulators, centered around E proteins E2A (encoded by *Tcf3*) and HEB (encoded by *Tcf12*), are in lower left. Group E regulators, represented by *Myb*, *Gfi1*, and *Runx1*, are in center (Ikaros family regulators, not shown). Lower right: genes associated with assembly of a signaling-competent TCR complex, essential for later T cell development. Lower center: genes associated with alternative innate lymphoid fates. *Id2* (adjacent to group D) is an

antagonist of Group D which is also critical for innate lymphoid cell and natural killer cell maturation and expansion (lower center).

Intrinsic PU.1 and ligand-dependent Notch signaling can modulate each other (upper left and center), but the relationship is asymmetric. Notch signaling narrows the range of PU.1 action but does not reduce its intensity, whereas high-level PU.1 can globally inhibit Notch signaling effects. The effect of PU.1 gain of function on target genes has been dissected out from the Notch inhibitory effect in [12], and built into the experimental design in [16]. However, in normal pro-T cells, Notch signaling is predominant over natural levels of PU.1 activity.

TABLE 1:

Table of sources for Gene Regulatory Network Model

SOURCE	TARGET	Direction	Notes	References
Notch1	Intercellular Notch/DL	activate	definition	
DLL4	Intercellular Notch/DL	activate	definition	
NotchDL	PU.1-sens-Notch	activate	definition	
RBPJ	PU.1-sens-Notch	activate	definition	
Nrarp	PU.1-sens-Notch	repress		[72]
Prethymic Activator	Bcl11a	activate	assumed by expression	
Prethymic Activator	Mef2c	activate	assumed by expression	
Prethymic Activator	Lmo2	activate	assumed by expression	
Prethymic Activator	Flt3	activate	assumed by expression	
Mef2c	(none noted)			
Lmo2	L/L/E Activity	activate	definition	
L/L/E activity	Hhex	activate		[73]
Lyl1	L/L/E Activity	activate	definition	
Bcl11a	Progenitor survival	activate		[66,74]
Bcl11a	Flt3	activate		[75]
Hhex	Kit	activate		[76]
Kit	Progenitor survival	activate		
Kit	Lymphocyte survival	activate		
Flt3	Progenitor survival	activate		
Hes1	Cebpa	repress		[40]
PU.1-sens Notch	Notch1	activate		[12,77,78]
PU.1-sens Notch	Notch3	activate		[12,77]
PU.1-sens Notch	Tcf7	activate		[26,27]
PU.1-sens Notch	Hes1	activate		[12,77,78]
PU.1-sens Notch	Gata3	activate	functionally direct?	[79–82]
PU.1-sens Notch	Runx1	activate		[12]
PU.1-sens Notch	Nrarp	activate		[12,78]
PU.1-sens Notch	Bcl11b	activate		[6,59]
PU.1-sens Notch	Prethymic activator	repress	conjectural	
PU.1-sens Notch	Notch-sens PU.1	repress	definition	[12,16,83]
PU.1-sens Notch	Flt3	repress		[84]
Cebpa	Spi1	activate		[11,85,86]
Spi1	Flt3	activate	in prethymic cells	[87]
Spi1	Bcl11a	activate		[12,16]
Spi1	Lyl1	activate	only w/ Notch signal	[12]
Spi1	Mef2c	activate		[16,37,88]
Spi1	Notch-sens PU.1	activate	definition	
Spi1	Lmo2	activate		[12,16,37,89]
Spi1	Zbtb16	repress		[16,37]

SOURCE	TARGET	Direction	Notes	References
Spi1	Il2rb	repress		[16,37]
Spi1	Ets1	repress		[12,16,37]
Spi1	PU.1-sens Notch	repress		[11,12,83]
Notch-sens PU.1	Spi1(PU.1)	activate	autoregulation limited in early T	[90,91]
Notch-sens PU.1	Gfi1	repress	may not be direct	[12]
Notch-sens PU.1	Hes1	repress	direct and indirect	[12,83]
Notch-sens PU.1	Gata3	repress	may not be direct	[12]
Notch-sens PU.1	Nrarp	repress	may not be direct	[12]
Notch-sens PU.1	Tcf7	repress	may not be direct	[12,37,83]
Notch-sens PU.1	Myb	repress	may not be direct	[12,37,83]
Runx1	Runx1/CBFb	activate	definition	
Runx/CBFb	Tcrb	activate		[92]
Runx/CBFb	Spi1	activate	bivalent	[35,93]
Runx/CBFb	Bcl11b	activate		[6]
Runx/CBFb	Spi1	repress	bivalent	[35,36]
IL7r	IL7signal	activate	definition	
Il7	IL7signal	activate	definition	
IL7signal	Stat activity	activate	definition	
Stat activity	Lymphocyte survival	activate		
Stat activity	Innate lymphoid cell devel	activate		
Myb	Gata3	activate		[12,94,95]
Gfi1	(none noted)			
Tcf7	Tcf7	activate		[27]
Tcf7	Cd3g	activate		[27]
Tcf7	Cd3e	activate		[26]
Tcf7	Innate Lymphoid cell devel	activate		[96–98]
Tcf7	Gata3	activate		[27]
Tcf7	Bcl11b	activate		[6,27,99]
Tcf7	Spi1	repress		[32]; support in [6]
Tcf7	PU.1-sens Notch	repress		[22,100]
Gata3	Innate lymphoid cell devel	activate		[101–104]
Gata3	Tcrb	activate		[105]
Gata3	Kit	activate		[33,34]
Gata3	Bcl11b	activate		[6,34,48]
Gata3	Rag1/2	activate		[48]
Gata3	Ets1	activate		[34,48]
Gata3	Spi1	repress		[33,34]
Gata3	Bcl11a	repress		[34,48]
Bcl11b	Cd3e	activate		[17,18]
Bcl11b	Cd3d	activate		[17,18]
Bcl11b	Cd3g	activate		[17,18]
Bcl11b	Dntt	activate		[17,18]

SOURCE	TARGET	Direction	Notes	References
Bcl11b	Notch3	activate		[17,18]
Bcl11b	Runx3	repress		[17,18]
Bcl11b	Id2	repress		[17,18,67,106]
Bcl11b	Kit	repress		[17,18]
Bcl11b	Zbtb16	repress		[17,18,106]
Bcl11b	Nfil3	repress		[17,18,67]
Bcl11b	Nrarp	repress		[17,18]
Bcl11b	Il2rb	repress		[17,18,67]
Tcf3	E prot activity	activate	definition	
Tcf12	E prot activity	activate	definition	
E prot activity	Cd3g	activate		[47]
E prot activity	Lyl1	activate		[15,47]
E prot activity	Notch3	activate		[47]
E prot activity	Il7r	activate		[107]
E prot activity	Id2	activate	potential in Il7r- pro-B cells	[107,108]
E prot activity	Cd3d	activate		[15]
E prot activity	Gfi1	activate		[47,109]
E prot activity	Notch1	activate		[12,15,41]
E prot activity	Gata3	activate	bivalent	[110]
E prot activity	Bcl11a	activate		[15,17,61,111]
E prot activity	Cd3e	activate		[15,28,47]
E prot activity	Dntt	activate		[47,107]
E prot activity	Rag1/2	activate		[109,111]
E prot activity	Myb	activate		[15,28]
E prot activity	Runx1	activate		[12,15,47]
E prot activity	Bcl11b	activate		[28,47]
E prot activity	L/L/E activity	activate	definition	
E prot activity	Zbtb16	repress		[15,47]
E prot activity	Nfil3	repress		[15,47]
E prot activity	Id2	repress	bivalent	[15,47]
E prot activity	Gata3	repress	bivalent	[15,47]
Ets1	Tcrb	activate		[92]
Ets1	NK cell development	activate		[112,113]
Id2	NK cell development	activate		[42,112,114–116]
Id2	Innate lymphoid cell devel	activate		[15,69,71]
Id2	E prot activity	repress	definition	
Nfil3	Id2	activate		[117]
Nfil3	NK cell development	activate		[117,118]
Nfil3	Innate lymphoid cell devel	activate		[119–122]
Runx3	NK cell development	activate	limited roles	[123]
Runx3	Innate lymphoid cell devel	activate	limited roles	[124]
Zbtb16	Innate lymphoid cell devel	activate		[125]

SOURCE	TARGET	Direction	Notes	References
Il2rb	NK cell development	activate		[112]
Notch3	(none noted)			
Cd3d	(none noted)		to TCR	
Cd3e	(none noted)		to TCR	
Cd3g	(none noted)		to TCR	
Tcrb	(none noted)		to TCR	
Rag1/2	(none noted)		to TCR	
Dntt	(none noted)		to TCR	

Sources are given for relationships shown in Fig. 2. Repression targets are shown in red. “Definition”: well-established property of a molecule’s function, such as the ligand-receptor interactions for IL-7 receptor or Notch. Transcriptional activation of any part of the *Tcrb* locus is indicated as “Tcrb”, with the understanding that this extremely large and complex locus has many additional sites of regulation as well. “Limited roles”: demonstrated roles are in particular subsets, not necessarily all subsets of this cell type.