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Transcriptional Drivers of the T-cell Lineage Program

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Abstract

The T-cell development program is specifically triggered by Notch-Delta signaling, but most transcription factors needed to establish T-cell lineage identity also have crossover roles in other hematopoietic lineages. This factor sharing complicates full definition of the core gene regulatory circuits required for T-cell specification. But new advances illuminate the roles of three of the most T-cell specific transcription factors. Commitment to the T-cell lineage is now shown to depend on Bcl11b, while initiation of the T-cell differentiation program begins earlier with the induction of TCF-1 (*Tcf7* gene product) and GATA-3. Several reports now reveal how TCF-1 and GATA-3 are mobilized in early T cells and the pathways for their T-lineage specific effects.

Introduction

Multipotent or lymphoid-biased precursors enter the T-cell developmental pathway in response to thymic microenvironmental signals [1]. The most important trigger is Notch pathway signaling, activated in the precursors by contact with Delta-family Notch ligands expressed by thymic epithelial cells. Pro-T cells then proliferate under continued influence of Notch signaling and remain Notch-dependent through T-lineage commitment, until after successful gene rearrangement enables them to express TCRβ or TCRγδ. However, something more durable and portable than a direct response to Notch pathway signaling must sustain the T-cell gene expression program later, during cell migration through multiple environments and more or less proliferation. The cells establish expression combinations of transcription factors that not only drive T-cell "identity" genes – those encoding TCR/CD3 components, signaling kinases, phosphatases, and adaptors – but also cross-regulate each other to stabilize the T-cell regulatory state.

T cell specification has multiple regulatory requirements, but most of the factors needed by T cells are also needed in other hematopoietic differentiation programs (e.g. Ikaros, Gfi1, Myb, Runx1/CBFβ, E2A and its relatives)[2]. Presumably these regulate T-cell specific genes mainly as components of lineage-specific combinations. Three transcription factors are much more T-cell specific in their expression: Bcl11b, GATA-3, and TCF-1 (encoded by *Tcf7*). These three factors are steeply upregulated in precursors in response to Notch signaling and sustained in T-cell development at varying levels thereafter (Fig. 1). Their sharp profiles of induction during T-cell development offer clear opportunities to reveal how their activities change multipotent progenitors into true pro-T cells.

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The T-cell specification process is actually a succession of three distinct regulatory states (Fig. 1A,B,C). Activation of GATA-3 and TCF-1 marks the transition from the first to the second, and activation of Bcl11b marks the transition from the second to the third. By considering how the advent of each factor affects the appropriate transition, the nature of the process is emerging in sharper focus.

Punctuating the T-lineage commitment process: role of Bcl11b

Bcl11b, a bifunctional C_2H_2 zinc finger factor, was discovered as a potential suppressor of radiation-induced T-cell lymphomas, and independently as a corepressor collaborating with the nuclear receptor COUP-TF (rev. in [3]). Its relative Bcl11a is a proto-oncogene in B lineage cells, essential for B-cell development, and a repressor of fetal-type hemoglobins; but Bcl11b is restricted to T-lineage cells (and at a lower level, NK cells) within hematopoiesis. Bcl11b is crucial for αβ T cell development but dispensable for some γδ T cells, and for some time its main role appeared to be exerted during β-selection [4-6]. It regulates αβ-lineage cell survival, developmental fidelity, and mature T-cell function from the $CD4^+$ $CD8^+$ stage onward [*6,7], restraining the latent NK-like differentiation potential in mature CD8 cells [**8], and supporting both CD8-cell expansion and regulatory T cell differentiation [9,*10]. However, its expression is initially induced in a dramatic upsurge much earlier, within the DN2 stage of T cell development [11]. This event closely coincides with the time frame when most T-lineage precursors become committed to a T cell fate [**12,13].

The linkage is more than a coincidence, as three recent reports have established that unless Bcl11b can be turned on, most T-cell precursors cannot become committed at all [**8,**14,**15]. These studies tracked precursors developing in response to continuous Notch-Delta signaling *in vitro,* where the lineage commitment impact of Bcl11b was readily observed. Bcl11b was perfectly dispensable for pro-T cell survival and proliferation under these conditions. However, deletion of Bcl11b could extensively prolong the time window in which T-cell precursors retain myeloid potential [**14,**15], and it enabled the developing cells to build up a formidable potential as natural killer (NK) cell precursors [**8,**15].

The role and mode of action of Bcl11b are now under investigation by many groups. It may be a timing factor for T-lineage commitment, since in fetal thymocytes, where differentiation is accelerated as compared to adult thymocytes, *Bcl11b* RNA begins to be detectable even at the DN1 stage (D.D. Scripture-Adams, M.M. Del Real, K.J. Elihu, and E.V.R., unpublished results). One aspect of the Bcl11b knockout phenotype is interesting from another perspective, however: namely, how much of the T-cell program can be activated without Bcl11b. Multiple gene expression changes normally occur in the DN2 to DN3 transition, since most genes involved in T-cell identity are upregulated immediately after Bcl11b [2], some but not all as direct Notch target genes [16-18]. Concomitantly, multiple “stem/progenitor-associated” genes are profoundly downregulated [19]. The DN2-like Bcl11b deletion phenotype dissects the T-cell specification process into separable modules. Stem/progenitor-associated genes fail to be shut off in the mutant cells, but T-cell transcription factors including GATA-3, and TCF-1 are fully induced to DN3-like levels, GATA-3 perhaps even higher than in normal DN2 cells [**15]. The T-lineage identity genes show split responses, with initial phases of *Cd3g* and *Cd3e* upregulation and *Rag1* induction occurring, but *Zap70* and *Ptcra* expression remaining weak [**15]. These responses dissect commitment, which depends on Bcl11b, from activation of the T-cell program, which GATA-3, TCF-1, and Notch can initiate even in cells that cannot become committed.

The difficulty with T-cell factors: asymmetric gain and loss of function phenotypes

TCF-1 and GATA-3 have long been recognized as essential for T-cell development [20,21]. Pioneering work using antisense oligonucleotides in fetal thymic organ culture systems showed that these factors were rate-limiting for early T-cell development, and implied additive roles [22]. However, dissecting what they do for T-cell specification has been held back by the peculiarities of their effects when ectopically activated.

GATA-3 plays at least three roles in T cell development: during initial specification, during TCRαβ-dependent positive selection, and in mature T cells where it establishes the Th2 effector program. In the Th2 context the addition of GATA-3 clearly promotes Th2 fate just as loss of GATA-3 inhibits it [23,24]. In TCRαβ-mediated positive selection of $CD4^+$ lineage thymocytes its effects can be more complicated, but again the gain of function of GATA-3 promotes the $CD4^+$ fate relative to other options [25-27]. However, its role has been murkier in the earliest stages. Loss of GATA-3 is profoundly inhibitory to T-cell precursors from the earliest ETP stage (see Fig. 1)[28,29]. GATA-3 binds to genes encoding TCR complex components [30,31], and in acute loss of function at certain stages some of these targets, e.g. the CD3 genes, are downregulated [**32](S. Damle, E.V.R., unpublished). But ectopic expression of GATA-3 in prethymic cells does not turn these genes on without Notch [33]. In early pro-T cells, added GATA-3 does not accelerate T-cell development, but can actually block it, whether or not Notch signaling is provided [34]. Forced GATA-3 expression can activate various inappropriate, non-T programs, depending on the cells into which it is introduced: a nonlymphoid, mast cell program in bone marrow cells or thymocyte progenitors [34], a nonlymphoid, megakaryocyte program in fetal liver precursors [35], and a myeloid program in Pax5-knockout pro-B cells [36]. GATA-3 dose-dependence may account for its problematic behavior in early T cells.

TCF-1 is best known as a signal-dependent transducer of environmental signals from the Wnt pathway via β-catenin [37-39]. In this pathway, without β-catenin, TCF family members act as repressors via recruitment of Groucho/TLE factors, but when Wnt signaling mobilizes β-catenin, β-catenin binding to TCF factors makes them work as activators. By this model the balance of positive and negative regulation of gene targets by TCF-1 should depend on the level of β-catenin. Indeed, ectopic expression of the Wnt antagonist, Dickkopf, could block T-cell development from a very early stage [40]. However, when hematopoietic progenitors have been forced to activate β-catenin, which should optimally activate TCF-1, the cells did not appear to upregulate lineage-specific T-cell genes. Instead, if they were already in the T-cell pathway they upregulated general survival genes [41], and if not, they reverted to a deregulated, multilineage gene expression state [42-44]. Furthermore, in loss of function experiments, mutation of the β-catenin cofactor alone or together with its relatives has had shockingly little effect on T-cell development [45,46], much less than the effect of mutating *Tcf7* alone or together with its relative *Lef1*. Then is TCF-1 mostly important as a repressor? In general, evidence for direct positive regulation of T-cell specification genes by GATA-3 or TCF-1 has been lacking, leaving a major roadblock to understanding the early events in T-cell development.

Three levels of regulation for GATA-3 activity

The essential but complex roles of GATA-3 in T cell development can be explained in part because it does not always regulate the same target genes. Genome-wide maps of GATA-3 binding at different stages now confirm that this factor occupies different genomic sites depending on its developmental context [**32](J.A. Zhang, A. Mortazavi, B. Williams, B.J. Wold, and E.V.R., submitted). Site affinity and factor concentration probably contribute:

e.g., the particularly high levels of GATA-3 in Th2 cells enable binding to many more sites than in other T-lineage cells [**32]. However, even in cells with similar levels of GATA-3, occupancy patterns are distinct according to developmental stage. When it is first upregulated in DN1 cells, we find that GATA-3 occupies sites in many stem/progenitor genes, then vacates them during commitment, shifting to bind more “T-cell identity” gene sites in the postcommitment $CD4^+$ $CD8^+$ stage (J.A. Zhang et al., op. cit.). It follows that GATA-3 normally collaborates with other developmentally-restricted factors to help define its targets. Because even the earlier-stage specific sites are genuine GATA-3 sites, however, it is easy to see how experimental overexpression could cause some GATA-3 binding to be inappropriately deployed. The control of GATA-3 levels itself must be extremely precise.

For years, the mechanisms responsible for inducing *Gata3* expression at the beginning of T cell development were inaccessible. Two promoters were defined: a proximal one driving most T-cell expression, and a distal Th2-specific one where direct input from Notch was demonstrated in Th2 cells [47,48]. An intronic enhancer and an upstream lineage-specific silencer were also reported [49,50]. However, even a 650 kb *Gata3* YAC transgene containing all these elements could not work *in vivo* to promote expression in T lineage cells [51]. Furthermore, in mature peripheral T cells and DN thymocytes alike, the proposed site of Notch input is buried under repressive H3K27me3 marking in all except Th2 cells, and is probably inaccessible in early stages [52,53](J.A. Zhang et al., op. cit.).

In a tour de force, Engel and coworkers have now found a T-lineage specific enhancer for *Gata3* which can mediate activation from the earliest T-cell stages [**54]. The new enhancer is in a gene desert 280 kb downstream (3′) of the *Gata3* gene, and it is necessary and sufficient to enable *Gata3* transgene expression in T lineage cells. Activity of this enhancer may still need to be modulated by interaction with other cis-regulatory elements. However, this is now the region where to seek the mechanism that Notch signaling first uses to turn on *Gata3* expression.

Importantly, GATA-3 is not only regulated through transcriptional control. In thymocyte development there are some mismatches between protein and RNA levels, e.g. during β-selection after successful TCRβ gene rearrangement, when RNA decreases but protein increases [25,55]. The key factor increasing GATA-3 protein seems to be activation via TCR. One reported mechanism is MAP kinase-controlled protein stabilization [56], but this could be more effective to preserve a pool in nondividing cells than to supply new protein during rapid cell division. Cook & Miller have now found another mechanism that enables signaled T cells to produce more GATA-3 protein *de novo* even from a declining pool of RNA [*57]. Through a PI3K-Akt-mTOR dependent pathway, translational efficiency of GATA-3 is specifically enhanced, possibly by unwinding secondary structure from the *Gata3* translational start site. Interestingly, the PI3K-Akt-mTOR pathway can also be activated by IL-7R in DN2 cells and Notch signaling in DN3 cells [58,59]. Thus, GATA-3 protein levels could possibly “measure” integrated transcriptional and survival signals in the cells.

TCF-1: essential driver of T-cell specification downstream of Notch

As a widely used developmental signaling trigger, Notch activation does not embody enough specificity to be the origin of the whole T-cell program. One set of factors that may help to select T-cell specific target genes comprises basic helix-loop-helix E protein genes, including E2A [17]. However only a subset of T-cell genes is turned on by Notch and E2A together, conspicuously omitting Bcl11b, and none of these are sustained by E2A when Notch signals are withdrawn. Something else downstream of Notch must help activate and

then sustain the T cell program, something that can act epistatically to Notch signaling in a gain of function assay. Neither GATA-3 nor Bcl11b can do this.

In the past year several groups have returned to TCF-1 as a candidate for initiating T-cell development downstream of Notch. These studies show that *Tcf7* is directly activated by Notch through an enhancer 31.5 kb upstream of the promoter [*60,**61], and that TCF-1 becomes essential in adult T-lineage progenitors as soon as it is turned on at the ETP stage. An important role for TCF-1 seems to be antiapoptotic even in ETPs [*60]. However, Weber et al. have also demonstrated that forced expression of TCF-1 can activate most of the T-cell identity program in prethymic precursors, in the absence of Notch signaling [**61]. It is the first T-cell transcription factor shown to do so. TCF-1 need not do everything alone: among the first genes it turns on are *Gata3* and *Bcl11b*, but its effect is impervious to high levels of γ-secretase inhibitors that block Notch signaling [**61]. It fails to turn on the sensitive Notch target gene *Ptcra*, yet activates many other “DN3-stage” genes that would normally accompany *Ptcra* expression [16]. Thus, many parts of the T-cell program are dependent on Notch only indirectly, through Notch-activated TCF-1 and its own targets (Fig. 2).

How TCF-1 works here is a crucial question. Results shown by Weber et al. notably imply that β-catenin and relatives are dispensable for forced expression of TCF-1 to turn on T-cell genes [**61], in harmony with the negative evidence *in vivo* [45,46]. However, they also show TCF-1 binding directly to genes like *Gata3* and *Bcl11b* that it positively regulates, so it is not acting as a repressor [**61]. This could imply a novel coactivator to replace β-catenin, or even a new mode of TCF-1 action that might have broader developmental significance. Yet there remain questions about the nature of the β-catenin mutation that has been tested in this context [38]. Clarifying the relationship of this new, early positive regulatory role with classic β-catenin mediated signaling pathways will be important.

Initiating the T-cell program: an emerging circuit

These results excitingly open new opportunities and new questions. TCF-1 only appears to be able to bypass Notch when it is introduced at high levels, typical of DN3 rather than DN1 stage cells. Thus at earlier stages, a classic feed-forward network circuit probably operates, in which lower levels of TCF-1 initially induced synergize with Notch signals on first-tier targets (Fig. 2)[*62]. Then with continued Notch signals, TCF-1 autoregulation, and/or cross-regulation by other factors, TCF-1 levels could rise to the point of being able to sustain the T-cell program without Notch. Most interestingly, the interaction between TCF-1 and GATA-3 may be mutual: not only Notch/RBP-Jκ (CSL) but also GATA-3 binds to the *Tcf7* upstream enhancer (J.A. Zhang et al., op. cit.). There is much yet to be learned about how dosage regulation is effected in this network circuit, and how these factors intersect with E proteins, Bcl11b, and other regulators. But the outlines of the T-cell specification process are coming into clearer focus.

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**[54]. Hosoya-Ohmura S, Lin YH, Herrmann M, Kuroha T, Rao A, Moriguchi T, Lim KC, Hosoya T, Engel JD. An NK and T cell enhancer lies 280 kilobase pairs 3′ to the gata3 structural gene. Mol Cell Biol. 2011; 31:1894–904. [PubMed: 21383068] Engel and colleagues use a BAC-transgenic strategy in mice to explore hundreds of kilobases beyond the *Gata3* gene for the presence of a long-sought enhancer that would allow expression in T lineage cells. The region they find that provides this function can confer T-lineage expression based on in vivo assays with gain and loss of function, yet it maps far downstream of the locus in a gene desert. The pattern of expression driven by this enhancer using a GFP reporter does not completely match the endogenous pattern of expression, but provides the first permissive cis-regulatory function for pan-T lineage activation and NK lineage expression that has been found for the murine *Gata3* locus. As it is activated in earliest DN stages, it must contain the direct or indirect inputs that mobilize *Gata3* in response to early intrathymic Notch signaling.

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*[57]. Cook KD, Miller J. TCR-dependent translational control of GATA-3 enhances Th2 differentiation. J Immunol. 2010; 185:3209–16. [PubMed: 20696860] The authors carefully document the disproportionate increase in GATA-3 protein relative to *Gata3* RNA that occurs in TCR-signaled T cells. In the case analyzed, protein stability is not detectably increased but translational efficiency of Gata3 RNA is enhanced. Using inhibitor assays they show that the increased protein is the result of an mTOR and PI 3-kinase-dependent pathway. The Discussion considers the possible mechanisms in light of the extensive secondary structure of 5′-untranslated region of *Gata3* and the ways access to the initiator codon could be actively controlled.

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*[60]. Germar K, Dose M, Konstantinou T, Zhang J, Wang H, Lobry C, Arnett KL, Blacklow SC, Aifantis I, Aster JC, et al. T-cell factor 1 is a gatekeeper for T-cell specification in response to Notch signaling. Proc Natl Acad Sci U S A. 2011; 108:20060–5. [PubMed: 22109558] The authors carry out a state-of-the-art analysis of the effects of TCF-1 loss of function in adult bone-marrow-derived T cell precursors. Detailed knockout and cell transfer experiments are shown to pinpoint the stage when TCF-1 loss affects the developmental fitness of T-cell precursors. The authors demonstrate that this does not occur prethymically but affects the population by the ETP (Kit^{high} DN1) stage and all subsequent stages. The mechanism is probed via RNA analysis and shown to affect DNA damage repair, CD3 expression, IL-7R, Kit, and Id2 and E2A at the ETP stage. *Tcf7* is shown to be a Notch-dependent target gene, and ChIP-seq analysis is used to demonstrate strong Notch occupancy in the same putative regulatory region of *Tcf7* where TCF-1 itself binds, about 31.5 kb upstream of the promoter, in agreement with ref. **61.

**[61]. Weber BN, Chi AW, Chavez A, Yashiro-Ohtani Y, Yang Q, Shestova O, Bhandoola A. A critical role for TCF-1 in T-lineage specification and differentiation. Nature. 2011; 476:63–8. [PubMed: 21814277] These authors use a gain of function approach to show that forced expression of TCF-1 can bypass most of the requirement for Notch signaling for entry into the T-cell program, provided that the level of TCF-1 is high enough. Levels of *Gata3*, *Bcl11b*, *Cd3*, and *Rag1/2* expression are strongly upregulated by TCF-1 gain of function, to levels similar to those in DN2 or even DN3 cells. TCF-1 can do this even under conditions where essentially no Notch pathway input can be invoked. Surprisingly, this powerful effect is intact in β-catenin mutant cells, and insensitive to the effect of adding the catenin inhibitor ICAT to wildtype cells, implying that TCF-1 is not working through the canonical pathway of interaction with β-catenin or one of its relatives. ChIP-seq and direct transfection experiments are used to show that *Tcf7* itself is a Notch target, with Notch binding at −31.5 kb (as in *61); thus, part of the requirement for Notch signaling is to induce adequate levels of TCF-1. TCF-1 itself exerts its inductive effects, at least in part, by direct binding to potential cis-regulatory elements in *Gata3* and *Bcl11b*.

*[62]. Kueh HY, Rothenberg EV. Regulatory gene network circuits underlying T cell development from multipotent progenitors. Wiley Interdiscip Rev Syst Biol Med. 2011 This review includes an updated compilation of data from the literature for effects of transcription factor perturbation and signaling perturbation on T-cell precursor gene expression, and the results are organized in terms of a new T-cell specification gene regulatory network model. Evidence for potential subcircuit architectures that could mediate particular developmental functions like feed-forward amplification or IL-7 dosage sensing are discussed.

HIGHLIGHTS

- Notch, TCF-1, GATA-3, and Bcl11b are linked in a T-cell gene network
- Bcl11b is needed for commitment but not to initiate the T-cell program
- GATA-3 action is regulated via transcription, translation, and binding site selection
- TCF-1 (*Tcf7*) is a direct Notch target required from the Early T-cell Precursor stage
- TCF-1 can bypass the requirement for Notch itself to activate *Gata3* and *Bcl11b*

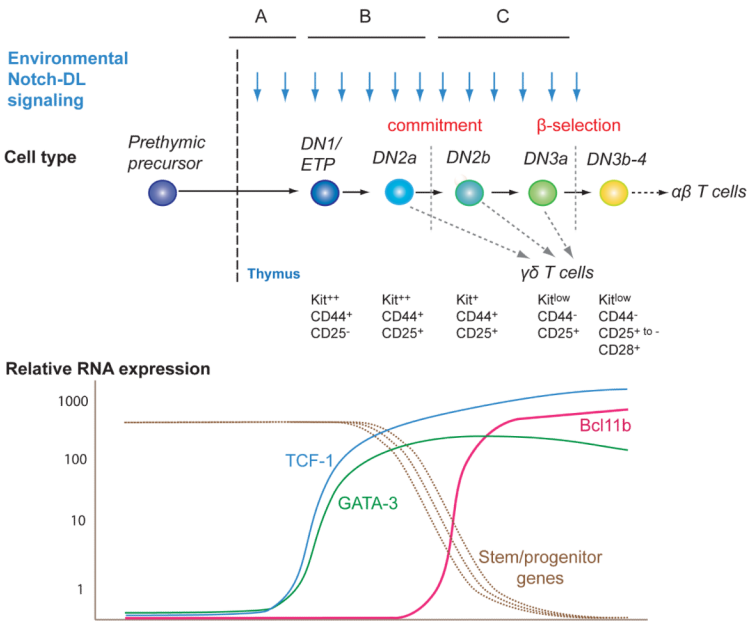

Figure 1.

Stages in progression through T-lineage commitment and β–selection. Phenotypes and key developmental events are indicated. γδ cells can emerge at several branchpoints. Notch interactions are sustained as shown. RNA expression patterns are approximate.

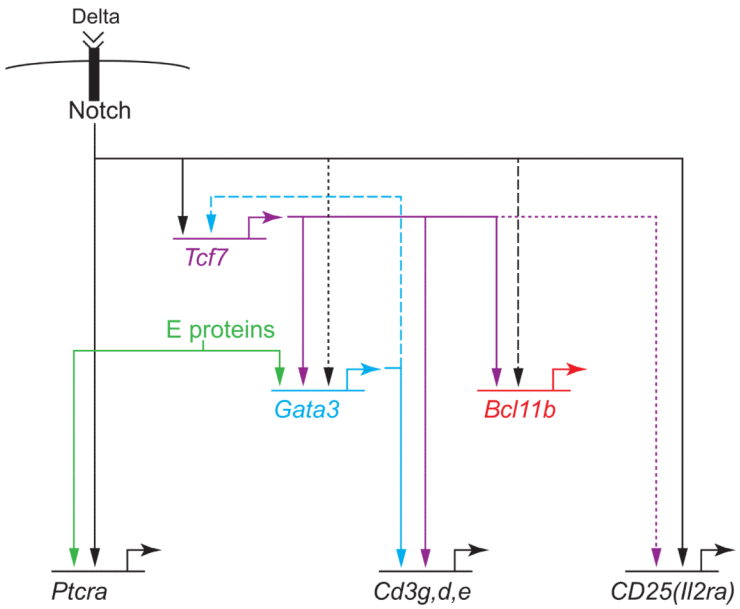

Figure 2.
Gene network relationship among Notch, TCF-1 (*Tcf7*), GATA-3, Bcl11b, and selected target genes. Solid arrows: support from perturbation and binding evidence. Dashed: binding evidence. Dotted: perturbation evidence. Data from [60-*62]. Bcl11b targets are still being defined.