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Forging T-Lymphocyte Identity: Intersecting Networks of Transcriptional Control

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Abstract

T lymphocyte development branches off from other lymphoid developmental programs through its requirement for sustained environmental signals through the Notch pathway. In the thymus, Notch signaling induces a succession of T-lineage regulatory factors that collectively create the T-cell identity through distinct steps. This process involves both the staged activation of T-cell identity genes and the staged repression of progenitor-cell-inherited regulatory genes once their roles in self-renewal and population expansion are no longer needed. With the recent characterization of Innate Lymphoid Cells (ILCs) that share transcriptional regulation programs extensively with T cell subsets, T-cell identity can increasingly be seen as defined in modular terms, as the processes selecting and actuating effector function are potentially detachable from the processes generating and selecting clonally unique T-cell receptor structures. The developmental pathways of different classes of T cells and ILCs are distinguished by the numbers of prerequisites of gene rearrangement, selection, and antigen contact before the cells gain access to nearly-common regulatory mechanisms for choosing effector function. Here, the major classes of transcription factors that interact with Notch signals during T-lineage specification are discussed in terms of their roles in these programs, the evidence for their spectra of target genes at different stages, and their cross-regulatory and cooperative actions with each other. Specific topics include Notch modulation of PU.1 and GATA-3, PU.1-Notch competition, the relationship between PU.1 and GATA-3, and the roles of E proteins, Bcl11b, and GATA-3 in guiding acquisition of T-cell identity while avoiding redirection to an ILC fate.

Keywords

T cell development; transcription factor; gene regulation; Notch; GATA-3; PU.1; E2A

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I. Introduction: T-cell identity and processing of T-cell progenitors

A. T-cell development in the context of hematopoiesis

Hematopoietic stem and progenitor cells generate an exceptional diversity of cell types throughout life, and this poses a series of challenges for explanation of developmental dynamics, developmental choice hierarchies, and the mechanisms used to make each of the myriad differentiation programs remain coherent. T cell development is one of the most interesting and complex of the pathways in this system. Hematopoietic cells have traditionally been divided into erythroid/megakaryocytic, myeloid, and lymphoid branches, and T cells have been considered a subspecies of adaptive immune cells that splits off from B cells only once their joint lymphoid fate is confirmed. This classic view has been based on the unique antigen receptor generation strategy based on somatic mutation that T and B cells share, and on the early separation that seemed to occur between precursors that could generate lymphocytes and precursors that could generate all other hematopoietic cell types. However, a more subtle picture of cell type identity and of lineage relationships has emerged in the past decade as increasing knowledge has been gained about the dynamics of lineage-specific developmental processes and the transcriptional regulatory apparatus that drives them. T cell development has turned out to be a particularly revealing branch of hematopoiesis, because the intermediates in the process leading to T-cell commitment are readily isolated, characterized, and then monitored kinetically if their development is allowed to resume. The results from T-cell development have been important to catalyze new understandings of hematopoietic hierarchy in general, and the relationship between T cell fates and other lymphoid and myeloid fates alike.

T-cell development is distinctive within the hematopoietic families both because of its site and because of the nature of the cells produced. The inductive microenvironment needed for T-cell development is not available in the bone marrow or fetal liver. Virtually all known T-cell development occurs in the thymus, where multipotent progenitors immigrate at a low rate. Despite a low absolute cell input per day, the precursors begin to proliferate massively as they begin the T-cell program under the influence of thymic environmental signals, mostly interaction of Notch1 receptors on the surfaces of the hematopoietic cells with Delta-like 4 (DLL4) Notch ligands on the thymic stroma. The thymus then serves both as a development zone and as an immunological filtering zone, where the cells are tested for their newly-expressed antigen-recognition specificities. All are killed but those with the most useful specificities (defined below), and only the cells that have passed through this selection are exported to the periphery to serve in immune responses. Unlike many other types of hematopoietic cells, but like their B-lymphocyte cousins, surviving T cells preserve the potential for extensive cell division when they are triggered as well as the ability to remain quiescent for long periods of time between stimulation. They thus maintain features associated with stem-ness as well as the mature functional properties they acquire through differentiation.

The basic elements of T-cell development and the genes required for T-cell development to proceed have been characterized as summarized in multiple reviews (De Obaldia and Bhandoola, 2015; Miyazaki *et al.*, 2014; Naito *et al.*, 2011; Rothenberg, 2014; Thompson

and Zúñiga-Pflücker, 2011; Yui and Rothenberg, 2014). However, for the most part specific regulatory genes have been studied as isolated influences that are characterized only in terms of "necessity" for the T-cell developmental pathway at one stage or another. In this chapter, we not only review what is known of the multiple transcriptional regulators that regulate this process, but also relate the ways that their roles intersect with each other, especially as subcircuits of specific factors can be viewed as elements of potential modules of functional programming.

B. Origins of T-cell precursors and the initial steps of T-cell development

In mammals, T cell precursors enter the thymus most prominently between the last half of fetal life and puberty. The epithelial structure of the thymus forms in mid-gestation by an outpocketing of branchial cleft endoderm and begins to be populated by hematopoietic progenitors that migrate through disorganized mesenchyme even before the thymus is vascularized. Once blood vessels supply the thymus, there are multiple successive waves of hematopoietic immigration. The cells coming into the thymus in the first wave may be highly biased toward a T-cell fate (Harman *et al.*, 2005; Masuda *et al.*, 2005; Ramond *et al.*, 2014), but by later in gestation and through postnatal life, the thymic immigrants enter as multipotent cells (Ramond *et al.*, 2014). Within the thymus, the strong environmental signals force the cells into the T-cell pathway or pathways, through the mechanisms that are the focus of this chapter. However, before reaching the thymus, the kinds of cells that enter have a constellation of alternative potentials that include lymphoid, myeloid, and dendritic-cell alternatives. There is considerable evidence that both lymphoid-biased precursors (Common Lymphoid Progenitors, CLP) and more balanced lymphomyeloid precursors (Lymphoid-primed MultiPotent Precursors, LMPP) can enter the thymus and give rise to T cells through a common pathway, although the LMPPs take somewhat longer (Saran *et al.*, 2010; Serwold *et al.*, 2009). An important confirmation that the cells enter the thymus as multipotent cells is that even after the initial stage(s) of T cell development, it is still possible to remove early T-cell precursors from the Notch-signaling environment of the thymus and show that they can still give rise to a variety of non-T cells (Bell and Bhandoola, 2008; Benz and Bleul, 2005; Lu *et al.*, 2005; Luc *et al.*, 2012; Sambandam *et al.*, 2005; Tan *et al.*, 2005; Wada *et al.*, 2008). Intrathymically, such early T cell precursors are actively responding to the Notch signaling environment as they begin to turn on T-cell genes, but their status remains reversible at this point: they have not yet internalized mechanisms cutting off their alternative developmental potential. This plasticity is significant because there is a discrete point in T-cell development, a little later, at which they lose their intrinsic potential to access the alternative fates (Masuda *et al.*, 2007; Yui *et al.*, 2010). That transition is what is referred to in the following as "commitment".

Fig. 1A shows the details of the pathway through which cells progress to expression of a T-cell receptor complex. The initial stages are called "DN" to signify that they are "double negative" for expression of either of the two T-cell coreceptors, CD4 and CD8. In mice, these stages are subdivided according to their expression of other surface molecules, the growth factor receptors Flt3 and Kit, the adhesion molecule CD44, and the solitary IL-2R α chain (CD25)(Fig. 1B). In humans, the subdivisions are made based on expression of different molecules: CD34, CD7, and CD1a (Vicente *et al.*, 2010); however, most of this

discussion will focus on the mouse where relationships and mechanisms have been easier to dissect using mouse genetics and in vivo cell transfers. Within the first population [Kit^+ DN1, or “Early T-cell Precursors” (ETP)], the cells that enter the thymus in each wave, initially called thymus-settling progenitors, still express Flt3, but rapidly downregulate this while maintaining or upregulating Kit expression. The resulting cells appear to undergo multiple rounds of cell division under the influence of Notch pathway signaling before progressing to the next recognizable stage. CD25 expression is finally activated by the Notch signals, and this signals the beginning of the DN2 stage. DN2 cells initially proliferate even faster than ETPs, but then go through a transition, at first barely detectable, at which they downregulate Kit expression and start to slow down their proliferation. Although the phenotypic shift is subtle (called DN2a to DN2b), this turns out to signal a major regulatory transition that coincides with lineage commitment. The cells begin to shut off CD44 expression, downregulate Kit almost completely, and slow their proliferation to the point of G1 arrest, at the DN3a stage. At this point the enzymes required to rearrange the TCR genes are strongly upregulated, and the cells await the successful expression of either a TCRβ chain or the combination of TCRγ and TCRδ chains.

Unlike all other essential T cell genes, TCR genes require more than transcriptional regulation. Each chain is encoded in the genome in multiple segments and depends on success of highly error-prone somatic gene rearrangements to generate a full TCR coding sequence, ultimately yielding TCR heterodimers composed either of intact TCRβ and TCRα chains or of TCRγ and TCRδ chains. For the majority of DN3a cells, further survival depends on in-frame TCRβ rearrangements. When this occurs, TCRβ chains assemble with a pre-synthesized set of TCR complex components to trigger a strong burst of proliferation and phenotypic transformation called β-selection. The cells sweep through a succession of states distinguished as DN3b, DN4, and immature single positive as they proliferate on their way to acquire a $CD4^+$ $CD8^+$ Double Positive (DP) phenotype (Fig. 1A). This is a major change in cellular transcriptional and regulatory state, and the resulting cells, the DP cells, finally stop proliferating and accumulate in a G0 state once more to rearrange their TCRα genes.

DP cells are allowed about 3–4 days of survival in which to assemble a TCRαβ complex that successfully forms a low-affinity interaction with major histocompatibility complex (MHC) molecules on the surrounding stroma (positive selection). Most of the cells normally die unsuccessful. For those that survive, the TCR interaction specificity with class II or class I MHC, respectively, determines whether they will be positively selected to become mainstream CD4 cells or CD8 cells, or some form of innate-like T cells (e.g. iNKT cells) with a noncanonical specificity. These choices lead the selected cells to maturation in the thymic medulla, further negative selection against strongly self-reactive cells, and finally export of the surviving cells to the periphery.

If TCRγδ is successfully rearranged first in DN cells, a shorter burst of proliferation is triggered than by TCRβ rearrangement, but this too causes recombinase inhibition and sets in train a series of events that ultimately make the receptor choices final. Interestingly, the TCRγδ lineage opens the door to a variety of effector programs that differ in certain respects from those of TCRαβ subsets (see Section I.C.2). If γδ vs. αβ lineage choice were only

determined stochastically, by the luck of rearrangement, then lineage fate should only be settled at the DN3a stage. However, both genetic and single-cell tests of differentiation in vitro show that by the time cells reach the DN3a stage, ready to carry out the definitive V-DJ rearrangements of their TCRβ genes, their ability to give rise to γδ cells has contracted to a small fraction of what it was earlier (Ciofani *et al.*, 2006; Feng *et al.*, 2011; Shibata *et al.*, 2014). This implies that many cells with γδ potential leave the mainstream after the early DN2 stages, even before the peak of TCR gene rearrangement activity. If so, then transcriptional changes involving differential expression of key regulatory genes (Haks *et al.*, 2005; Melichar *et al.*, 2007; Narayan *et al.*, 2012) may also contribute to bias the outcome of the γδ vs. αβ lineage choice even before completion of rearrangement.

C. Definition of T cell maturity in gene expression terms

1. Pan-T cell genes—The gene regulatory events that operate during T cell differentiation must endow T cells with a combination of pan-T characteristics and subset-specific characteristics. Pan-T characteristics confer the cells' ability to recognize antigen with a T-cell receptor (TCR) complex and trigger a response. Thus, all T cells express either an αβ or γδ TCR, assembly partner proteins CD3γ, δ, and ε and TCRζ (CD247) that enable it to form a signaling-competent complex, and the specialized set of signaling adaptors, kinases and phosphatases that transduce activation signals when TCR ligands are engaged. Finally, all T cells grow and maintain viability throughout both resting and immune response phases by cytokine stimulation through receptors of the γc (*Il2rg*) family. Most T cells must therefore express γc robustly in order to survive, together with a variety of partner chains that assemble with it to complete the IL-7, IL-2, IL-4, IL-15, IL-9, or IL-21 receptors (Yamane and Paul, 2012). While γc expression and its signaling mediators are constitutive in T cells, the roles of the alternative partners for γc are more dynamic and subset-specific, as indicated below for subset-specific effector genes.

Because gene rearrangement is required to enable the cells to express a TCR, all T cells must go through a phase when they also express the lymphoid-specific recombinase enzymes RAG1 and RAG2, and usually terminal deoxynucleotidyl transferase (encoded by *Dntt*) to make the process more mutagenic as well. RAG1-RAG2 complexes are indispensable to catalyze the recombination of TCR V, sometimes D, and J segments to assemble TCR coding sequences. These enzymes collaborate with DNA-dependent protein kinase (encoded by *Prkdc*), Artemis (*Dclre1c*), and DNA ligase 4 (*Lig4*) and other canonical components of the nonhomologous end-joining pathway in order to complete the TCR gene segment joining process. For T cells, the phase of somatic mutation is transient and unique to their intrathymic development; mature T cells can no longer alter their TCR coding sequences. However, their existence depends on the earlier, precisely regulated hit-and-run activity of these mutagenic enzymes.

Signaling molecules required by all mature T cells include the Src-family kinase Lck, the ζ-associated tyrosine protein kinase Zap70, the adaptors LAT and Slp76 (*Lcp2*), Ca^{2+}-activated TEC family kinase Itk, the tyrosine protein phosphatases CD45 and Csk, and additional adaptors including Grap2 (GADS). Most T cells also express the costimulatory receptor CD28 and coreceptors CD4 or CD8α and CD8β. These signaling components

couple to more broadly expressed signaling partners such as 4, 5-phosphatidylinositide 3-kinase (PI3K), Akt, Ras, Raf, multiple MAP kinases, various isoforms of protein kinase C, Orai and CRAC Ca^{2+} channel components, Target of Rapamycin (TOR) complexes 1 and 2, and the transcription factors that these signaling molecules can trigger. Again, some subsets use additional signaling components, such as SLAM Associated Protein (SAP, *Sh2d1*), but these are specialized requirements. Finally, there are additional signaling molecules such as the multiple related GTPases of the IMAP family (Gimap1–9), most of which are strongly expressed in a mature T-cell restricted way and maintain cell viability through pathways that are less well understood (Schwartzberg *et al.*, 2009; Wang *et al.*, 2010; Yano *et al.*, 2014).

To summarize, at a minimum, the core T-cell developmental program must do two things. It must induce the properly timed bursts of RAG1 and RAG2 expression and allow their silencing, and it must set up stable expression of the transcription factors and genomic accessibility states needed to induce and sustain expression of the broad set of pan-T cell genes. These are requirements that apply to essentially all T cells of all subsets.

2. Subset-specific genes—T cell maturity is actually a collection of diverse states, because the T cell developmental pathway is highly branched. T-cell precursors encounter a succession of developmental choice points both during their development in the thymus and after they leave the thymus The effector programs as they are modulated in the peripheral immune response are of extreme clinical interest and have become major fields in themselves. But some points about this effector specialization are important to note, even in simplified form. First, subset specialization choices are made at a variety of developmental stages (Fig. 2). Second, one large fate decision or group of fate decisions has to do with the specific form of the TCR that is to be expressed: γδ or αβ. The γδ vs. αβ decision is made without actually triggering the TCR; however, the relative outputs of cells that have taken these divergent pathways do depend on TCR engagement. Third, all subsequent choices within the T-cell pathway require TCR engagement as one component of the trigger to drive the cells to one fate or another. Fourth, effector subset choice involves a constellation of gene regulation alterations: the cells must express an appropriate γc-containing growth factor receptor; they must set up a poised state in which a specific set of effector genes becomes inducible; and they must do this by establishing stable expression of a network of subset-specific transcription factors.

Subset-specific effector genes often encode cytokines that are used to stimulate or inhibit other cells (IL-4, IFNγ, IL-17, IL-10), chemokine receptors to guide migration preferences, specific costimulatory molecules and auxiliary cytokine receptors, and sometimes cytolytic factors (granzyme B, perforin). Importantly, under normal conditions without antigen challenge, many effector genes remain silent in specific T-cell subsets despite being primed for expression. However, in simplest terms Th1 cells are defined by their ability to express IFNγ on stimulation; Th2 cells, by their ability to express IL-4 (and IL-13); Th17 cells, by their ability to express IL-17a; and cytolytic T cells, by their ability to express granzyme B and perforin.

Effector programs depend on an endogenous transcriptional regulatory state that sets the default gene expression preference of the cells, plus specific real-time interactions with

STAT (signal transducers and activators of transcription) family factors that convey dynamic signals to the nucleus from most different kinds of cytokine receptors. STAT factors collaborate with stably expressed factors in the cells to define the set of enhancers across the genome that are available for activation at any given time (Vahedi *et al.*, 2013; Vahedi *et al.*, 2012). Actual transcription of the effector genes is then triggered by signals through the T-cell receptor, which are mediated by stereotypical signal transduction factors of the NF-κB, NFAT, and AP-1 families. The sustained regulatory states that distinguish Th cells of different classes from each other between bouts of stimulation are defined by specific "lineage-determining" factors (sometimes romantically called "master regulators"). Each effector subclass is characterized by at least one of these stably expressed lineage determining transcription factors and at least one specific type of growth factor receptor capable of mobilizing the right STATs to collaborate with the lineage determining factors (Murphy and Stockinger, 2010; O'Shea and Paul, 2010; Oestreich and Weinmann, 2012; Vahedi *et al.*, 2013). Thus, very broadly, the Th1 state is characterized by T-bet collaborating with STAT1 and STAT4, the Th2 state is characterized by GATA-3 collaborating with STAT6, the Th17 state by RORγt (Rorc isoform) collaborating with STAT3, the Tfh state by Bcl6 collaborating with STAT3, and the Treg state by Foxp3 supported by STAT5. In CD8 cytolytic cells, T-bet or Eomes and Runx3 collaborate with STAT1 and STAT5; and there are also some alternative CD8 activation states which appear to be regulated roughly analogously to the CD4 states.

In depth, the alternative effector types in fact specialize through somewhat more complex regulatory programs, and different effector programs can antagonize each other's operation to create fairly coherent alternative regulatory states (Ciofani *et al.*, 2012; Naito and Taniuchi, 2010; O'Shea and Paul, 2010; Yosef *et al.*, 2013). Importantly, the branching of these alternatives follows a similar pattern whenever cells become specialized for effector function, even though this can happen at a variety of developmental stages (Fig. 2; and section I.D). As described below, there are ways for developing lymphocytes to arm themselves with these effector functions even without their expressing a TCR at all. One key question, then, is what the threshold may be that cells need to cross before they are able to specialize for effector function.

D. Programming for T-cell effector function: intrathymic and postthymic

One notable feature of the transcriptional regulation of effector subset identity is that at least some of the "lineage determining factors" have more than one role in T cell development, working at different stages. RORγt in peripheral T cells is crucial for the Th17 fate, downregulated in the Th1 fate and excluded from the programs for the Th1 and Treg fates; yet it is earlier expressed by all TCRαβ cells during the DP stage. GATA-3 is not only the major regulator of the Th2 fate, but also critical for the choice of all CD4 cell fates during positive selection. It is also indispensable for all DN cell survival in the ETP stage, for differentiation, and for survival from DN2 to DN3 and during β-selection as discussed at length in Section III.C.1. The DN stages and the transition to the DP stage are the periods when pan-T cell features are programmed uniquely. Thus, most if not all regulatory gene activity in these early stages, including roles of factors like GATA-3 and RORγt, is likely to be conferring functions that the cell will use no matter what future effector function it

chooses. The roles of the same factors in selection of effector functions are exerted later, through a series of asynchronous branchpoints.

For most TCRαβ T cells, there is a large divide between the functional programming of CD8 cells, future killers, and CD4 cells, mostly future helpers. This separation occurs in the thymus shortly after successful TCRα rearrangement (Fig. 2), as a result of the interaction of the TCR and CD8 or CD4 coreceptors on DP cells with class I or class II MHC in the thymic microenvironment. The CD4 cell fate is triggered by GATA-3 upregulation and then sealed by the action of a zinc finger-POZ domain factor, Th-POK (Zbtb7b), which antagonizes the CD8 cell fate defined by action of the Runx factor, Runx3. The CD4 (helper) vs. CD8 (killer) decision is generally irreversible after the cells leave the thymus, as a function of the robust gene regulatory network circuitry that enforces mutually exclusive regulatory states (Carpenter and Bosselut, 2010; He *et al.*, 2010; Naito *et al.*, 2011). However, most CD4 cells continue to make effector lineage decisions after CD4/CD8 lineage choice, long after TCR gene rearrangement, both within the thymus and much later, during responses to antigen stimulation. Two additional decisions that are now recognized to occur in the thymus are the specification of some DP cells to become iNKT cells (invariant TCR, natural killer-like T cells) or other innate-like cells (Gangadharan *et al.*, 2006), and others as Tregs (regulatory T cells). DP cells are triggered to differentiate into iNKTs through their TCR's strong interaction with non-classical class I MHC molecules expressed by their fellow DP thymocytes. Other CD4 cells are specified to become tTregs (thymic Tregs, or nTregs, natural Tregs), based on their interaction with self antigens in the thymic medulla, which programs the cells to act as suppressors of other T cells in their peripheral responses. Both of these intrathymic variant decisions are based on fairly strong TCR-ligand interaction ("agonist selection"). In the case of iNKT cells, the agonist interaction activates expression of the transcription factor Promyelocytic Leukemia Zinc Finger (PLZF) or Zbtb16, through a pathway involving Egr2 (Seiler *et al.*, 2012). It is their expression of PLZF that enables iNKT cells to express effector genes with greatly reduced need for priming by TCR signaling (Savage *et al.*, 2011). For the Tregs, the agonist interaction induces expression of the transcription factor Foxp3, through a pathway involving NF-κB and IL-2 (Hsieh *et al.*, 2012; Moran *et al.*, 2011; Nunes-Cabaco *et al.*, 2011; Wirnsberger *et al.*, 2011).

Like the programs induced in the agonist-selected iNKT and Treg cells, the later, post-thymic decisions for these TCRαβ cells all depend on TCR stimulation, which is now joined with stimulation by various polarized combinations of cytokines in the environment. These clinically important events are foundational to the generation of major Th1, Th2, Th17, and Treg subsets of T cells in the periphery, as well as other rarer types of effectors, and to the specific designation of cells as Tfh (follicular helpers) for intense collaboration with B cells in lymph node follicles.

Interestingly, the late lineage decisions made after the cells leave the thymus are not always entirely definitive. Careful analysis of these decisions at a single-cell level have revealed that the initial patterns of activation of the lineage-determining factors (e.g. GATA-3 and T-bet) can be far more overlapping than the effector functions of the cells that emerge, and even after polarization, these decisions have substantial plasticity, especially between Th1

and Th17 and between Th17 and iTreg (peripherally induced Treg)(Antebi *et al.*, 2013; Murphy and Stockinger, 2010; Zhu and Paul, 2010). In some cases, even the CD4/CD8 lineage decision may be reversible (Mucida *et al.*, 2013). In contrast, the decision to be a T cell and to express a core set of pan-T-cell genes appears to be irreversible.

E. Modularity of effector and recognition T cell identity elements

Because most effector subtype choices occur long after programming of multipotent progenitors to become T cells, it would be easy to assume that they are hierarchically subordinate to the prior events that generate a mature TCRαβ$^+$ cell in the first place. Such an impression could seem to be supported by the fact that DP thymocytes are extremely poor at turning on any of the effector genes of mature T cell subsets even when they are stimulated with chemical proxies for TCR signaling that bypass the TCR entirely. However, multiple lines of research over the past 10 years have converged to show that in fact the mechanisms generating TCR and priming the cells for TCR-dependent selection events are functionally quite independent from the mechanisms controlling effector function, and that in certain lineages of cells these mechanisms can be uncoupled.

Early hints for this separability came from the responses to chemical activators of cells much more immature than DP cells. It emerged that although DP cells are unable to express effector cytokines, their precursors are much more competent. IL-2 activation, among other responses, is easy to elicit from DN cells that have not yet undergone β-selection, including cells from RAG-knockout animals that lack the ability to rearrange any kinds of TCR genes (Chen and Rothenberg, 1993; Diamond *et al.*, 1997). By inference, in this respect DP cells are not so much immature as paralyzed. Bevan and coworkers first identified RORγt as one of the transcription factors expressed in DP cells that actively blunts access to Th1 and Th2-like response genes in essentially all TCRαβ lineage precursors throughout the period between β-selection and “positive selection” to a CD4 or CD8 cell fate (He *et al.*, 2000). Positive selection is required to downregulate RORγt and to restore functional responsiveness at the end of the DP stages. If RORγt is ever expressed again, it is only in the context of Th17 differentiation.

The access to effector genes in DN pro-T cells is not necessarily exploited by these immature precursor cells in the thymic microenvironment in vivo. However, it is significant that those DN2 cells that split off to become TCRγδ cells may begin using their effector genes after a much shorter wait than those that become TCRαβ cells (Fig. 2). Recent detailed analyses of γδ cells have revealed that they are at least as diverse functionally as αβ cells, and they begin to be diversified within the thymus, before being exported to the periphery at all (Bonneville *et al.*, 2010; Prinz *et al.*, 2013; Vantourout and Hayday, 2013). Different TCRγδ cell functional subsets have characteristic biases in the gene segments they use for rearrangement to assemble their TCRγδ genes, and so it is easy to separate them and ascertain that their preference for different kinds of effector response are established while they are still in the thymus. The basis for different TCRγδ gene segment choice is determined at least in part by different transcription factor requirements to make these segments accessible: STAT5 activates the TCRγ locus in general, but certain Vγ’s are used more or less depending on the ratio of bHLH E protein activity to Id-family E protein

inhibitors (Bain *et al.*, 1999). However, there are other dramatic regulatory differences between TCRγδ cells of different subsets (Narayan *et al.*, 2012). While many γδ cells express IFNγ, some do so exclusively like Th1 cells, some are IL-4 producers like Th2 cells, while others specialize in IL-17 production like Th17 cells. Thus, for such TCRγδ cells, the decisions that $CD4^+$ TCRαβ cells defer till antigen response in the periphery are precociously shifted to a milieu more like the one in which TCRαβ cells choose CD4 vs. CD8 fates. Notably, this is not because they undergo more extensive programming than TCRαβ cells immediately after TCR expression; the number of cell divisions triggered in TCRγδ cells is significantly less. However, the maturation programs of these cells induce a strongly dynamic transcriptional cascade that yields at least three major types of effectors, apparently based on interaction with “self” microenvironmental molecules alone.

While most TCRαβ cells delay functional specialization much longer, the iNKT cells (and in human, MAIT cells)(Chandra and Kronenberg, 2015) show that rapid intrathymic effector differentiation is possible in principle for TCRαβ cells too. These cells were first noticed to be distinctive because of their rapidly triggered expression of various cytokines that normally would require priming and restimulation before activation, and it has turned out that they represent a discrete branch or set of branches of αβ T-cell development [rev. by (Alonzo and Sant’Angelo, 2011; Chandra and Kronenberg, 2015; D’Cruz *et al.*, 2010b; MacDonald and Mycko, 2007; Yamagata *et al.*, 2006)]. These cells rapidly express IL-4 (a Th2 signature cytokine) as well as IFNγ (a Th1 signature cytokine), and they can also activate the IL-2 gene. Their rapid response capability appears to be correlated with their expression of the zinc finger transcription factor PLZF (Zbtb16). The ability of one population to express all these cytokines initially appeared to be due to a special violation of conventional subset boundaries in the iNKT cell effector program. However, recent data have suggested a more interesting alternative: namely, that diverse types of iNKT cells that were classically thought to be stages in maturation might instead be end states of alternative iNKT cell maturation programs (Lee *et al.*, 2013). All express the hallmark PLZF factor, but at different levels. Whether or not these are absolute end states, the cells in three different intrathymic iNKT subsets all exhibit functional competence, but with significant differences: some better as IFNγ producers, some better as IL-4 producers, and some better as IL-17 producers. Signature transcription factors of peripheral T cell effector subsets as well as PLZF are also differentially expressed in these subsets (Cohen *et al.*, 2013): whereas all express GATA-3, there is more T-bet (and Runx3) expression in those that can express most IFNγ, more PLZF in those that express most IL-4, and more RORγt in those that express most IL-17 (Lee *et al.*, 2013). In general, then, PLZF and GATA-3 can each be implicated as a rate-limiting factor for priming cells to express *Il4* and/or its linked neighboring gene *Il13*. Thus, something very much like the same effector lineage choice involving similar transcription factors can be made either post-thymically, as a late response to repeated antigen stimulation in the periphery; or intrathymically shortly after positive selection, for certain αβ T cells that become iNKT cells; or earlier intrathymically for cells that branch off from the αβ mainstream even before β-selection, without passing through a DP stage at all, to become functionally specialized γδ cells (Fig. 2).

An exciting perspective on T cells that has recently emerged is that a very similar kind of effector lineage choice can be implemented by newly appreciated lymphoid cells that do not enter the T-cell pathway at all. Natural killer cells long offered a prototype of lymphocytes that lack TCR for recognition, yet exercise T cell-like function in the body: they mimic the effector program of activated cytolytic T cells. With the recent description of three other classes of Innate Lymphoid Cells, completely TCR-negative equivalents of Th1, Th2, and Th17 cells have been discovered as well: to simplify slightly, these are ILC1, ILC2, and ILC3, respectively (Di Santo, 2014; Diefenbach *et al.*, 2014; Hazenberg and Spits, 2014; Spits *et al.*, 2013). All of the innate lymphoid classes including NK cells are specified through a pathway that requires the bZIP transcription factor Nfil3 at least transiently (Geiger *et al.*, 2014; Seillet *et al.*, 2014), and among these all the helper-type ILCs are distinguished by origin from precursors with early expression of PLZF, GATA3, and TOX (Constantinides *et al.*, 2015; Constantinides *et al.*, 2014; Seehus *et al.*, 2015). Notably familiar transcription factors are involved in the alternative effector programs of ILCs: T-bet for ILC1 (and NK) cells, GATA-3 and TCF-1 for ILC2 cells, and RORγt for ILC3 cells (Cherrier *et al.*, 2012; Hoyler *et al.*, 2012; Klose *et al.*, 2014; Mjösberg *et al.*, 2012; Serafini *et al.*, 2014; Yagi *et al.*, 2014; Yang *et al.*, 2013). For ILC2 cells especially, there is a close correspondence between the regulatory requirements for generating these cells and for generating committed T-cell precursors, even though they lack TCR expression (Furusawa *et al.*, 2013; Klein Wolterink *et al.*, 2013; Serafini *et al.*, 2014; Walker *et al.*, 2015; Yagi *et al.*, 2014; Yang *et al.*, 2013; Yu *et al.*, 2015). Some of them can even descend from Common Lymphoid Precursors that have had a history of transient RAG1-RAG2 expression (Karo *et al.*, 2014; Yang *et al.*, 2011b), as can some NK cells (Welner *et al.*, 2009). However, these cells are not T cells, and express only scattered members of the pan-T cell gene battery (Robinette *et al.*, 2015). While some ILCs express one or another of the CD3 genes used in the TCR complex, they do so neither as highly nor as coordinately as T cells. Ironically, the ILC2s that have the most extensive transcription factor overlap with T cell precursors express some of the lowest levels of these pan-T cell genes. Thus, it is not necessary to turn on a pan-T cell gene expression program at all to gain access to the choice point between cytolytic program, Th1-like effector function, Th2-like effector function, and Th17-like effector function (Fig. 2). These programs, and the gene network cross-regulation mechanisms enforcing the choices between them, are modular and deployable completely independently of the TCR assembly and selection programs. Making competent T cells thus requires both activation of the regulatory factors needed to drive expression of pan-T cell genes, and also negotiating the developmental conditions and activation thresholds that the cells will need to deploy one of these effector function modules.

II. Transcriptional dynamics of the T-cell program

A. Onset of T cell gene expression

Activation of the pan-T cell genes occurs in the thymus. The precursors of T cells enter the thymus with little if any detectable expression of the pan-T genes or major subset markers. As the cells first arrive, they are expressing chemokine receptors including Ccr9 and supported by the crucial growth factor receptors Flt3 and Kit. Some of the thymus-settling precursors are likely to have had a history of IL-7R and/or RAG gene expression as bone

marrow Common Lymphoid Progenitors before arriving, but as the cells settle in the initial reception compartment and begin to proliferate as Early T cell Precursors (ETP), expression of these genes is silent. The earliest detectable phenotypic transitions within the thymic environment actually involve the repression of Ccr9 and Flt3 in response to Notch signals (Krishnamoorthy *et al*., 2015; Ramond *et al.*, 2014; Sambandam *et al*., 2005), leaving Kit expressed at high levels, before substantial activation of pan-T cell genes is under way. The cells begin to turn on T-cell genes primarily as they make the transition from ETP to DN2a stage, a transition that may occur within just a day in the fetal thymus but may take over a week in the adult thymus. In the adult, based on population expansion kinetics and computational modeling, the cells are likely to undergo ten rounds of cell division on average before they reach this point. CD25 (*Il2ra*) and *Il7ra* are the earliest T-cell genes that reach full expression in murine T-cell precursors. As the cells cross the DN2a to DN2b transition and become committed, the expression of other T-cell genes increases substantially.

The gene expression changes in early T cells from ETP stage through β-selection are complex, with different sets of genes responding to different underlying regulatory state changes, as shown in Figure 3A [data from (Zhang *et al.*, 2012b)]. In this chart, pre-commitment stages (ETP, DN2a) are represented in shades of blue, post commitment DN stages (DN2b, DN3a) in yellow and orange, and DP stage in red (transitional β-selection stages are not shown). To capture the great dynamic range of change for some genes, each gene's expression pattern is shown in terms of differences at these stages from its own geometric mean value across all stages. While there are a few genes in this set that are turned on only in DP cells (top group), most of the T-cell identity and signaling genes undergo their greatest increases from DN2a to DN3a stages (Fig. 3A, light blue to orange). The DN3 and DP stages are the main stages when TCR gene rearrangement occurs, and RAG1 and RAG2, Artemis, and Ligase IV are also strongly upregulated following commitment as if they too were T-cell identity-specific. The CD3 genes and LAT achieve levels at or close to their maxima soon after commitment, by the DN3a stage, with ZAP70 and GADS only somewhat lagging behind. Thus, between the ETP and DN3a stages, the cells are substantially armed with T-cell specific response competence.

This rapid, parallel increase in T-cell gene expression contrasts with the cytokine receptor genes, which behave very individually. As already noted, *Il7ra*, like *Il2ra*, is strongly upregulated during the DN2-DN3 stages, only to decline again in the DP cells. *Flt3* and genes coding for other non-T growth factor receptors such as *Csf1r* (c-fms, M-CSF receptor) are active in the thymus-settling precursors but steeply repressed at the earliest stage transition. *Kit* expression, initially high, continues until after commitment, but is then silenced. Of these receptors, only Kit and IL-7R are functional in early T cells. The *Il2ra* gene product encoding CD25, although it can serve as an α chain for the IL-2 receptor, does not work that way here, for it is not accompanied in these cells by its obligate assembly partner IL-2Rβ. *Il2rb* expression instead serves as a marker for certain γδ cell lineages and cells developing into NK cells. Interestingly, the ETP and DN2a cells initially express a number of kinases that are normally considered specific to non-T cells, but these too are downregulated and silenced during the stages immediately following commitment. The T-

cell differentiation program thus includes precisely timed silencing and transient up- and down-regulation activities as well as the steady increase in T cell identity gene expression. These features hint at the regulatory complexity that underlies the program.

B. Notch signaling: driver and modulator

1. Notch target genes—The indispensable exogenous trigger for T-cell development is the stimulation of the Notch pathway, by interaction of Notch1 transmembrane molecules on the lymphoid precursors with Delta-like 4 molecules on thymic epithelial cells (Fig. 1A). Notch signaling not only induces T-cell development, but also begins blocking access to the B-cell developmental pathway and induces an intrinsic loss of B-lineage potential shortly after precursors enter the thymus. Notch signaling also inhibits NK, myeloid, and dendritic cell alternative developmental pathways for ETP and DN2a cells, and is ultimately required to induce the mechanisms that close off these options by the DN2b stage. Thus, before the cells stop responding to Notch signals during β-selection, Notch-induced inherent regulatory changes render the cells' commitment Notch-independent.

Notch signaling is well known to affect transcription directly. To simplify (Borggrefe and Oswald, 2009; Radtke *et al.*, 2010), engagement of the extracellular domain of a Notch molecule causes a proteolytic cleavage that liberates the cytoplasmic domain from the plasma membrane; its nuclear localization sequence then enables it to enter the nucleus, where it works as a direct transcriptional coactivator. A simple expectation might therefore be that T-cell identity genes could include a large number of direct Notch target genes. Indeed, some T-cell identity-associated genes appear to be strongly dependent on Notch signaling throughout the early developmental stages, including *Il2ra* and the gene encoding the surrogate light chain that is expressed as a transient partner for TCRβ, *Ptcra* (Pre-TCRα). Interrupting the contact with Delta-like molecules or chemically inhibiting the protease-dependent cleavage of Notch causes sharp losses of expression of these genes over a 1–2 day period (Del Real and Rothenberg, 2013; Franco *et al.*, 2006; Liu *et al.*, 2010a; Maillard *et al.*, 2006).

However, the T-cell program as a whole is not simply a Notch signaling response. First, Notch signaling in general is shut off at β-selection, as the cells transition from DN3a through proliferation and intermediate phenotypes to DP. By that time, any permanently expressed T-cell genes must be weaned from Notch-dependence in order to persist. Another complication is that different genes which are clearly Notch-dependent in T-cell development are not co-regulated. Instead, as shown in Fig. 3B, they are expressed in diverse patterns [data from (Mingueneau *et al.*, 2013); note the extended $\log_2$ scale], some like *Hes1* turned on early and sustained throughout the DN stages, some like *Ptcra* turned on only at the last DN stages before β-selection, while others like *Nrarp* are restricted to the earlier stages of T-cell development and paradoxically turned off as other Notch target genes are more strongly activated. Importantly, in the postnatal mouse thymus, ETPs receive Notch signals for an entire week before expressing the definitive T cell genes that define the DN2a stage (Porritt *et al.*, 2003). Thus T cell development as a whole is not an immediate response to Notch signaling.

In fact, Notch plays a variety of distinct roles during the T-cell specification and commitment process, dependent on signal intensity and developmental stage. In mice and humans, in the early ETP stages, Notch signals enhance proliferation but are not essential for viability (Balciunaite *et al.*, 2005; De Smedt *et al.*, 2005; Garcia-Peydro *et al.*, 2006; Schmitt *et al.*, 2004; Taghon *et al.*, 2005), whereas by the time the cells reach DN3a stage, Notch signals become indispensable for survival (Ciofani and Zúñiga-Pflücker, 2005). The intensified Notch dependence correlates with the fact that in mouse, differentiation to undergo β-selection requires a stronger and more prolonged period of Notch signaling than differentiation to undergo γδ selection (Ciofani *et al.*, 2006; Garbe *et al.*, 2006; Taghon *et al.*, 2006); as noted above, in mouse precursors, some γδ cells split off from the DN stage progression before the DN3a stage. Interestingly, this relationship of Notch signaling to TCR choice is reversed in human T cells (Van de Walle *et al.*, 2009; Van de Walle *et al.*, 2013). The evolutionary reversal implies that Notch signaling is being used only as a switch and not as an integrally essential regulator to distinguish these classes of TCR genes themselves. To explain the shifting impacts of Notch signaling on the cells, clearly some additional regulatory influence is directing the impact of the Notch signal to different target genes.

2. Notch mediators—Notch signals are modulated by multiple other gene products, including Numb and its family members as negative regulators, Spen (also called Msx2-interacting protein MINT, SMRT/HDAC1 associated repressor protein SHARP, or RBM15c) as a competitive negative regulator, Mastermind-like (MAML) family proteins as obligate positive regulators, and modulators of Notch protein post-translational processing. Glycosyltransferases of the Fringe family (Lfng, Mfng, Rfng) also strongly modulate the preference of Notch proteins for interaction with Delta-like ligands as opposed to Jagged family ligands. There is indeed an important developmental change in Lfng expression as T-cell precursors differentiate, such that DN cells are generally Lfng high and thus competitively favored for interaction with the DLL4 presented in the thymic microenvironment, whereas DP cells sharply downregulate Lfng and become unable to compete (Visan *et al.*, 2006). However, Lfng is the exception, not the rule: overall the patterns of expression of Notch signaling modulators are remarkably unaffected by progression through T-cell development (Fig. 3C). Thus, the shifting spectra of Notch target genes between ETP and DN3a stage are not readily explained by changes in the Notch signaling machinery itself.

The overall intensity of the Notch pathway signal received by the cells may increase as the cells progress through commitment, due to the downregulation of two antagonists. One of the transcription factors expressed early in the cells is the myeloid and multilineage regulator PU.1, and PU.1 activity levels apparently blunt Notch signaling in a dose-dependent way (see section V.B.1). This Notch-inhibitory effect seems to be general, because it applies similarly to “early” and “late” stage Notch-dependent targets (Champhekar *et al.*, 2015; Del Real and Rothenberg, 2013). The fact that PU.1 is normally turned off during commitment could thus unleash stronger potential for Notch signaling by the DN3a stage. At least two Notch dependent genes also have the potential to act as feedback negative regulators of Notch signaling, i.e. *Nrarp* and *Dtx1*. As differentially

regulated Notch target genes, Dtx1 and Nrarp themselves exemplify the sharp developmental stage-dependence of Notch effects. Dtx1 is particularly labile, and its dynamics are complex, but Nrarp expression is mostly confined to the precommitment period and thus its negative effects on Notch signaling are relieved by the DN3a stage. However, during β-selection most Notch signaling is downregulated for the rest of intrathymic T-cell development. It only re-emerges to be utilized, much later, to modulate mature T-cell functional responses (Osborne and Minter, 2007; Radtke *et al.*, 2013). Thus all Notch induction of pan-T cell genes must be hit-and-run, and the genes that continue to be expressed transition to Notch-independence.

III. Dynamic transcription factor expression

Notch signaling in principle must continue throughout the 12–14 cell cycles that precursors probably undergo between entry into the thymus and arrest at the DN3a stage checkpoint in postnatal mice (Lu *et al.*, 2005; Manesso *et al.*, 2013; Porritt *et al.*, 2003). In contrast, the transcription factors expressed by the cells shift dramatically across this interval. These changes are at least indirectly regulated by Notch signaling, but most of them do not reflect a close dependence of transcription factor gene expression on the immediate status of Notch signaling. They are instead the outcome of cascades of regulatory factor actions. An overview of the dynamics of the major factor groups is shown in Fig. 4A.

A. Stem-progenitor specific factors

The cells enter the thymus continuing expression of a set of transcription factors used in stem and progenitor cells. Some of these factors persist and even increase in expression during T-cell commitment: these include Myb, Gfi1, Runx1, Ikaros (*Ikzf1*) and the ubiquitous E protein E2A (E12/E47, “transcription factor 3”, *Tcf3*). Others play a role only in the earliest stages and are downregulated, and these can be termed stem-progenitor specific factors. We have also referred to these as “phase 1” transcription factors, and their patterns of expression are summarized in Fig. 4B, using data from the Immunological Genome Project (Heng *et al.*, 2008; Mingueneau *et al.*, 2013).

Developing T cells shut off the expression of some stem-progenitor specific factors relatively soon after entering the thymus, but others persist through at least one Notch-dependent transition and multiple rounds of division. The first round of silencing occurs before the cells leave the ETP compartment, probably in parallel with the downregulation of Flt3: this is when Hoxa9, Hlf, Lmo2, Meis1 and Mef2c are turned off (Fig. 4B). A second round of silencing occurs significantly later, during commitment, essentially in parallel with the downregulation of Kit. Here, PU.1 (*Spi1*, *Sfpi1*), Hhex, Gfi1b, N-Myc (*Mycn*), Tal1, and Bcl11a are turned off, with Lyl1 persisting only slightly longer. Clearance of the final progenitor-cell regulatory factors occurs only from DN3a stage to entry into β-selection, when Erg and Etv6 are turned off. Most of these regulatory genes once repressed are never activated in T-cells again (Fig. 4B), except in case of malignant transformation (Yui and Rothenberg, 2014). Any cross-regulatory stimulation that they provide to each other’s expression is lost as they are silenced. This is likely to be one of the features making T-cell specification irreversible.

Despite their temporary expression, the factors in the stem and progenitor set do make a positive contribution to T-cell generation. Elsewhere (Yui and Rothenberg, 2014) we have described substantial evidence that their engagement in self-renewal mechanisms can help to sustain the extensive proliferation that precedes lineage commitment, which is crucial to generate a large pool of T-cell precursors that can be winnowed stringently by TCR-dependent selection. Lyl1 and Bcl11a are both important for sustaining the expansion of normal prethymic and early pro-T cells up to the DN2 stages in vivo (Yu *et al.*, 2012c; Zohren *et al.*, 2012). Recently it has become clear that even PU.1, a factor with explicit ability to promote alternative cell fates (Anderson *et al.*, 2002; Del Real and Rothenberg, 2013; Dionne *et al.*, 2005; Franco *et al.*, 2006; Laiosa *et al.*, 2006; Lefebvre *et al.*, 2005), is needed to contribute to optimal T-cell generation (Champhekar *et al.*, 2015), by retarding the timing of T-cell lineage commitment. Special Notch-dependent mechanisms make it possible for these progenitor factors to work within the confines of the pro-T cell program until they are silenced, as described in Section V.B.2.

B. Factor families with shifting intrafamily dominance: Runx and ETS

T cell development involves roles for some transcription factors that are products of multigene families, such as the ETS family and the RUNX family. ETS and RUNX family transcription factors are likely to play important roles as participants in most lymphoid gene expression, based on the extreme enrichment of their binding motifs in enhancers of lymphoid genes with various patterns of activity. These families are always represented among the transcription factors expressed in the cells, from prethymic stages all the way through to mature T cell effector subsets. With their near-indistinguishable DNA binding affinities, this guarantees that some family member will always be available to occupy an appropriate target site. However, the early T-cell specification stages are times when there is particularly dynamic shifting among members of these two families for dominance in expression.

Runx1, Runx2, and Runx3 family members (depicted collectively as “RUNX” in Fig. 4A) are all expressed in the ETP stage. Later, their expression patterns diverge in different subsets of αβ and γδ T cells, but initially they are coexpressed. During commitment, Runx2 and Runx3 are downregulated while Runx1 rises to its highest level (David-Fung *et al.*, 2009). After the DP stage, Runx3 is specifically re-induced in CD8 T cells, to match the high levels of expression it sustains in most ILCs. Runx activity is implicated in CD4 gene silencing during positive selection of CD8 cells and in the silencing of PU.1 earlier, during the DN2a to DN2b transition (Collins *et al.*, 2009; Egawa *et al.*, 2007; Grueter *et al.*, 2005; Huang *et al.*, 2008; Naito and Taniuchi, 2010; Zarnegar *et al.*, 2010). However, a complete analysis of Runx factor roles is complicated by alternative splicing and promoter use isoforms of all three genes and by uncertainty about whether the family members act redundantly or competitively.

The ETS family is arguably the most complex, as more than a dozen family members are expressed during intrathymic T-cell development (Anderson *et al.*, 1999; David-Fung *et al.*, 2009; Mingueneau *et al.*, 2013; Zhang *et al.*, 2012b). The dominant ETS factor of mature T cells, Ets1, is poorly expressed when the cells enter the thymus, and its closest paralogue

Ets2 is initially silent. However, the cells express multiple other ETS factors that undergo little change in expression during T-cell development (GABPα, Fli1, Elf1, and other Elf, Elk, and Etv subfamily members), and also Erg, Etv6 (TEL), Elk3, and PU.1 which are confined to the early DN stages. During commitment, not only is PU.1 turned off but Ets1 and Ets2 dramatically turn on, Ets2 rising to a summit at DP stage and then falling in more mature cells. A different family member, Etv5 (ERM), is turned on sharply in a γδ-specific way and acts as a consistent feature of most TCRγδ lineage cells thereafter (Narayan *et al.*, 2012); unfortunately nothing is known yet about how this factor could contribute to the special qualities of TCRγδ cell functional maturation. The dominant members of the ETS family thus shift their relative expression levels by orders of magnitude during commitment and progression to the DP stage or to selection of a TCRγδ fate.

It is still uncertain how much these related ETS-family factors compete with each other for the same binding sites. Whereas most ETS factors bind similar DNA sequences containing a GGAA core in vitro, there are characteristic differences between them in their preferences for flanking sequences, so they are unlikely to bind to identical spectra of target sites in living cells (Wei *et al.*, 2010). Nevertheless, for any given target gene it is unlikely that only a single unique ETS factor can provide a regulatory input: in closely studied cases there is clear evidence for redundancy [e.g. (Xue *et al.*, 2004)]. Thus, the impact of ETS family transcription factors on T-cell development is probably vastly larger than has been revealed so far by any single-gene knockout phenotypes.

Of the ETS factors, one which is easiest to interrogate for a specific role is PU.1. It is the founding member of a “Spi” subfamily with a distinctive preference for purines flanking the GGAA core, so it binds to sites that may not be redundantly occupied (Wei *et al.*, 2010), and it is the only member of the Spi subfamily expressed in the early ETP and DN2a/2b stages. PU.1 itself is silenced after commitment and never expressed again except in a rare subset of helper T cells (Th9 cells) (Chang *et al.*, 2010; Chang *et al.*, 2005), but it has a potent and well-studied role in myeloid cells, dendritic cells, and B cells. In fact, PU.1 appears to be directly responsible for the expression of some “myeloid-like” genes in the earliest stages of T-cell development and for the duration of access to dendritic-cell or myeloid fate options that the cells preserve in the stages before commitment. However, as described below, PU.1 is not expressed in early T cell precursors only to be a subverter of the T cell program; its role is illustrative of the interlocking between hematopoietic programs that is characteristic of the lineage commitment process.

C. “T cell” transcription factors

The factors that separate the T-cell program from other hematopoietic programs most clearly are strongly upregulated after the cells enter the thymus. They are members of four different families: GATA-3 (GATA family); TCF-1 (encoded by *Tcf7*, founding member of the TCF/LEF family); HEB (Tcf12) as a partner of E2A (Tcf3), a fellow member of the bHLH factor family; and Bcl11b, a zinc finger transcription factor whose closest relative, Bcl11a, is expressed in a mirror-image pattern. GATA-3, TCF-1, and Bcl11b are virtually T-lineage specific within hematopoiesis, and play roles in most if not all subsets of T cells. TCF-1 is also detectably expressed in NK cells, but the only other blood cells in which they are

expressed substantially are ILCs, especially ILC2 cells. HEB as a partner for E2A is much more common in the T-cell lineage than in any other hematopoietic cell type, and the dimer plays a crucial role throughout specification and commitment, in particular separating the T-cell pathway from all the NK and ILC types. With essential support from progenitor-inherited factors like Myb and Runx1, Gfi1, and members of the Ikaros family, these four factors collaborate to create the T-cell identity.

1. GATA-3 and TCF-1—The GATA-3 and TCF-1 factors are activated first, beginning their expression within the ETP population. TCF-1 is most clearly a Notch target gene (Germar *et al.*, 2011; Weber *et al.*, 2011), and it both positively auto-regulates and positively regulates GATA-3 expression. Whether *Gata3* has direct transcriptional input from Notch is currently uncertain. Later, Notch signaling inhibition can result in higher GATA-3 expression rather than lower, so that *Gata3* at least has some Notch-independent modes of expression. In T-lineage cells, *Gata3* is positively regulated by the progenitor-cell factor Myb as well as by TCF-1 (Del Real and Rothenberg, 2013; Gimferrer *et al.*, 2011; Maurice *et al.*, 2007). Long after lineage commitment, during positive selection and in the periphery, GATA-3 can be activated by other factors, but its association with TCF-1 expression is maintained (Yang *et al.*, 2013; Yu *et al.*, 2009).

GATA-3 is essential for T-cell development from an early stage, and even partial dose reductions can lead to pro-T cell death especially at ETP, DN3, and later stages (García-Ojeda *et al.*, 2013; Hattori *et al.*, 1996; Hosoya *et al.*, 2009; Hozumi *et al.*, 2008; Scripture-Adams *et al.*, 2014). GATA-3 deficient cells cannot progress beyond a stage resembling DN2 stage, although even the apparent DN2 cells are abnormal in gene regulation pattern. Consistent gene expression perturbations have been reported in GATA-3-deficient cells generated both in prolonged OP9-DL1 culture (García-Ojeda *et al.*, 2013; Scripture-Adams *et al.*, 2014) and in short-term acute deletion experiments from ETP-DN2a precursors (Scripture-Adams *et al.*, 2014). For example, GATA-3-deficient “DN2” cells have reduced *Cd3e, Rag1, Ets1, Bcl11b, Itk, Tcf7, Zfpm1*, and *Kit* expression, while they overexpress *SpiB, Bcl11a, Dtx1, Spi1* (PU.1), and possibly also *Lmo2* and *Nfil3*. Some of these abnormalities could simply be indicators of a general developmental retardation, but others clearly indicate derangement of the normal developmental sequence. Later, GATA-3 deficiency causes failure of β-selection as well as failure to generate CD4 cells (Hernández-Hoyos *et al.*, 2003; Pai *et al.*, 2003).

TCF-1 is also crucial for T-cell development, with abnormal and reduced ETP populations, absent DN2 populations, and reduced, developmentally defective DN3 populations (Germar *et al.*, 2011; Jeannet *et al.*, 2008; Verbeek *et al.*, 1995; Yu *et al.*, 2012b). Although some of the residual DN3 cells in *Tcf7*$^{-/-}$ mice appear to express TCRβ chains, they are incompetent to go through β-selection normally (Yu *et al.*, 2012b), and generate small and abnormal later thymocyte populations. The earliest precursors in the *Tcf7*$^{-/-}$ thymic populations are not true ETPs since they lack high Kit expression, and they already express lower levels of *Cd3e* and *Cd3d* than controls while expressing abnormally high *Id2*, *Il7r*, and *Tcf3*. The roles TCF-1 plays in the following stages are complicated by the mixture of activating and repressive isoforms in which it is normally expressed and by the potential for redundancy with its relative, LEF-1, which is turned on sharply starting in DN2b stage (Tiemessen *et al.*,

2012; Yu *et al.*, 2012b). The need for TCF-1 appears to be somewhat dependent on ontogeny', with knockouts grossly defective in post-weaning adult T-cell development but allowing fetal thymopoiesis to occur apparently normally (Germar *et al.*, 2011; Jeannet *et al.*, 2008; Schilham *et al.*, 1998; Weber *et al.*, 2011). This difference could be due mostly to the augmented requirements for proliferation and survival in adult animals, because *Tcf7* mutants generally show their sharpest defects in all proliferative stages of T cell development as well as in survival of DP cells (Germar *et al.*, 2011; Schilham *et al.*, 1998; Staal *et al.*, 2004; Verbeek *et al.*, 1995; Wang *et al.*, 2011).

Although they are activated specifically in early T-cell development, neither factor has a simple relationship of DNA binding to T-cell specific target gene activation. TCF-1 levels become extremely high in early T cell development, with mRNA transcript levels rising to approach those for β-actin (*Actb*). By the DP stage, available TCF-1 binding data shows the protein engaged broadly across nearly all open chromatin throughout the genome, not limited to developmentally specific genes (Dose *et al.*, 2014). TCF-1 family members are renowned for their roles as Wnt signaling pathway effectors, dependent on nuclearized β-catenin as a cofactor [rev. by (Staal and Sen, 2008)], but current evidence strongly disfavors any Wnt/β-catenin dependence of the functions that TCF-1 plays in the earliest stages of T-cell development (Jeannet *et al.*, 2008; Weber *et al.*, 2011). It is possible that TCF-1 is serving instead as a scaffolding or architectural component in the construction of T-lineage specific enhancer/promoter complexes in these cells (Giese *et al.*, 1995). GATA-3 levels remain much lower than TCF-1 levels at all stages, and GATA-3 binding is much more selective, but the sites bound vary substantially from stage to stage (Wei *et al.*, 2011; Zhang *et al.*, 2012b). At a minimum, other factors must be strongly influencing GATA-3 site choice and outcomes of GATA-3 engagement.

2. E proteins—HEB and E2A are the main components of the "E protein" heterodimers in early T cells. Whereas E2A continues to be expressed at unchanging levels from prethymic precursors, HEB is strongly upregulated. HEB-E2A complexes are crucial for the growth arrest, recombinase gene expression, and fidelity of the β-selection checkpoint in DN3a cells, and are vital again for enabling TCRα rearrangement in DP cells (Barndt *et al.*, 2000; D'Cruz *et al.*, 2010a; Jones and Zhuang, 2007). DP cells deprived of these complexes progress prematurely through positive selection-associated changes and shift disproportionately to a CD8-like lineage fate (Jones-Mason *et al.*, 2012). Unlike the situation in B cells, where E proteins are degraded in response to Notch signaling, HEB/E2A complexes are stable in the early T-cell milieu; whether the HEB components that are much more common in T cells play a role in this stability is not known. HEB is expressed in two major isoforms in early T cells: the "canonical" one uses the standard transcriptional start site in exon 1, and its regulation is not well understood. The second "alternative" isoform uses an internal promoter that encodes a truncated product with an alternative N-terminal sequence called "HEBalt". HEBalt is expressed as a classic Notch target gene, paralleling the rise of *Il2ra* in the early T cells from ETP to DN2a and then being abruptly and permanently silenced after DN3a stage during β-selection (Wang *et al.*, 2006a; Yui and Rothenberg, 2004). HEBalt can complex with E2A as well, and the further heterogeneity of E2A isoforms between E12 and E47 alternative splice variants increases the diversity of E

protein heterodimers in the cells. Although mapping of E protein dimer binding sites across the genome is difficult in early T cells, the results available for cells in DN3 stage and β-selection (Miyazaki *et al.*, 2011) and later (Zhang *et al.*, 2012a) show strong binding to many sites of T-cell differentiation genes, and there is good evidence that these genes are in fact functionally regulated by E proteins (Ikawa *et al.*, 2006; Miyazaki *et al.*, 2011; Schwartz *et al.*, 2006; Xu *et al.*, 2013).

Note that in later T-cell development, E protein activity is often dynamically antagonized by induction of the antagonists, Id3 and Id2, which form non-DNA binding heterodimers with E2A and/or HEB (Bain *et al.*, 2001; Kaech and Cui, 2012; Yang *et al.*, 2011a). High-level expression of Id2 especially is characteristic and essential for ILC development, implying that the innate-cell fate depends on neutralizing E proteins [rev. by (De Obaldia and Bhandoola, 2015; Mjösberg *et al.*, 2012; Serafini *et al.*, 2015)]. However, in normal early T cell development from ETPs until DN3a stage, these Id factors are normally held to extremely low levels, thus leaving E2A and HEB free to form active DNA-binding complexes instead.

Complexes of E2A and HEB are crucial for numerous aspects of early T-cell specification, but it is not certain yet which responses are specifically mediated by which isoforms. Nevertheless, the rapid increase in HEB expression during early T-cell specification could have a strong effect on the deployment of the total E2A protein in the cells, because E2A would otherwise be engaged in a different kind of complex entirely. Two of the progenitor-associated factors, Tal1 and Lyl1 (see Fig. 4B), are also bHLH factors that have important roles in the gene regulatory networks in multipotent progenitors. These heterodimerize with E2A and make it possible to form super-complexes with GATA factors (usually GATA-2 in progenitors) and with the bridging components Lmo2 (or Lmo1) and Ldb1, binding as a coordinated unit to a characteristic set of composite GATA/Tal1 sites (Lahlil *et al.*, 2004; Xu *et al.*, 2003). The bridging functions depend on the Tal1 or Lyl1-specific structure of the bHLH heterodimers, and Lyl1 expression is especially high until just after commitment. Thus, by raising levels of HEB to the point that it can compete against Lyl1 for limiting amounts of E2A, the early T cells may be able to break up the progenitor-associated DNA binding complexes and reposition the E2A to different sites. If this should turn out to be the case, then the downregulation of Lmo2 within the ETP stage should collaborate with the initial upregulation of HEB to transform the spectrum of bHLH target genes expressed in the early T cells.

3. Bcl11b—The final member of the T-cell specific set of factors is Bcl11b. This zinc finger transcription factor is not expressed at all until the cells are in the mid-DN2a stage (Rothenberg *et al.*, 2008; Tydell *et al.*, 2007), and then its expression is strongly implicated in the repression of Kit and other changes that define the committed state. Bcl11b is still poorly characterized as a genome-wide regulatory agent, but it has two distinct, stage-dependent roles. When it is first turned on, it is needed to shift the cells from a program of progenitor-cell gene expression which can be sustained by growth in permissive cytokines, to a commitment program of limited growth, obligate progenitor-cell gene silencing, and continued differentiation to a DN3a climax state (Ikawa *et al.*, 2010; Li *et al.*, 2010a). If committed cells are deprived of Bcl11b, they lose viability and shift to a natural killer-like

or ILC1-like gene expression profile (Avram and Califano, 2014; Liu *et al.*, 2010b). Ironically, the cells that do not yet express Bcl11b express its highly related family member Bcl11a, which has roles in B cells, dendritic cells, and in the viability of lymphoid progenitors (Ippolito *et al.*, 2014; Liu *et al.*, 2003; Yu *et al.*, 2012c). The extent to which Bcl11a and Bcl11b play overlapping or competing roles needs to be determined. However, Bcl11b itself has a unique status as the factor the expression of which seems most closely related with T-cell lineage commitment. The connection that may exist between its one-time lineage commitment roles and its roles in regulating access to cytolytic effector programs is discussed below (section IV).

Bcl11b knockouts fail to generate αβ T cells (Wakabayashi *et al.*, 2003), while later deletion has particularly severe effects on DP cells, iNKT cells, and CD4 cells, especially Tregs (Albu *et al.*, 2007; Albu *et al.*, 2011; Avram and Califano, 2014; Kastner *et al.*, 2010; Vanvalkenburgh *et al.*, 2011). One could easily infer that an obligate lineage commitment regulator should be equally essential for all T cells. However, there has been a striking exception to this prediction in Bcl11b knockouts. There is a subset of TCRγδ cells that appears to be able to develop reliably from precursors lacking Bcl11b. Such TCRγδ cells are enriched for use of fetal-specific TCRγδ gene rearrangements that are typical of the first waves of T-cell development in the organism (Li *et al.*, 2010a; Shibata *et al.*, 2014), and they are selectively enriched for the ability to make IFNγ on stimulation. Such fetal-type thymocytes are normally characterized by their unusually high expression of *Id2* and *Il2/Il15rb*, two genes usually associated with NK or ILC1 development. These may only be the progeny of TCRγδ precursors that branch off of the common TCRαβ/γδ pathway at the DN2 stage (Shibata *et al.*, 2014), in harmony with the fact that Bcl11b may not be turned on yet in these precursors even in wildtype mice. Thus, the exception to the requirement of T-cell development for Bcl11b appears to be a very particular lineage of innate-like T cells.

D. Collaborating factors

"T cell factors" cannot complete the job of T-cell specification without collaborating with factors involved in growth factor receptor signaling, the repressor Gfi1, and the multifunctional zinc finger factors of the Ikaros family (Ikzf). Growth factor receptor signaling is not only permissive but also, to some extent, an instructive regulatory input. For example, in postnatal mouse T-cell precursors, signaling through Kit and IL-7R actually needs to be reduced substantially in order to permit arrival at the β-selection checkpoint (Balciunaite *et al.*, 2005; Wang *et al.*, 2006b). STAT5 is a major transducer of signals through the IL-7R, and can act directly to establish epigenetic accessibility for the TCRγδ locus (Schlissel *et al.*, 2000; Ye *et al.*, 2001). Thus, both viability support and STAT5 action as a transcription factor are likely to explain why IL-7/IL-7R signaling is crucial for TCRγδ cells. However, for progression to the αβ-biased, DN3a stage, a more important switch may be the PI 3-kinase cascade, also mobilized by IL-7R signaling, that inhibits activity of FoxO factors (Pallard *et al.*, 1999). FoxO1 is crucial for RAG gene activation in B-cell precursors (Mansson *et al.*, 2012; Welinder *et al.*, 2011), and if the FoxO factors are similarly needed in DN3 pro-T cells, then excessive IL-7/IL-7R signals could retard competence for TCR rearrangement directly. However, once TCRβ is successfully expressed, remaining IL-7R

expression becomes important again to promote the strong burst of proliferation during β-selection (Boudil *et al.*, 2015).

Gfi1 has long been known to play a vital role in lymphocyte development, but its structure suggesting an obligate repressor activity has made it difficult to identify the specific targets that it needs to control in early T cells. There has been evidence that in peripheral T cells it can interact with GATA-3 to stabilize it, and that it can restrain excessive expression of the IL-7 receptor (Park *et al.*, 2004; Shinnakasu *et al.*, 2008). Recently, Gfi1 has been shown to be essential in LMPPs and ETPs to enable cells to be stimulated by Notch signals. Without Gfi1, Notch signaling is toxic, and the cells fail to activate a T-cell gene expression program (Phelan *et al.*, 2013). The mechanism is still being dissected. Collaborative Gfi1-GATA3 interaction could be involved, since GATA-3 deletion or knockdown also prevents successful development under Notch signaling conditions (Hozumi *et al.*, 2008; Scripture-Adams *et al.*, 2014). Gfi1 is needed for granulocyte and B-cell fates as well, where it apparently protects the cells from powerful macrophage-promoting effects of PU.1 (Laslo *et al.*, 2006; Spooner *et al.*, 2009); conceivably a similar mechanism could be involved in early T cells. Finally, Gfi1 is activated by E proteins, and a positive feedback effect on E protein activities is also possible because Gfi1-deficient animals overexpress E protein antagonists Id1 and Id2 in their thymocytes (Hock *et al.*, 2003; Yücel *et al.*, 2003). This suggests that Gfi1 may help to enforce the E protein activity levels that are essential to prepare cells for β-selection. In T cell development as well as in ILC subsets, Gfi1 expression roughly parallels GATA-3 expression until positive selection, when it is turned off; thus, the expression pattern of Gfi1 is consistent with any of these possibilities.

Ikaros family members are crucial for the development of multiple lymphocyte types, and the main reason that any T-lineage cells are made after deletion of the founding member Ikzf1 (Ikaros) is that from the earliest stages they express Ikzf2 (Helios) as well (Hahm *et al.*, 1998; Kelley *et al.*, 1998). The Ikaros family zinc finger factors can multimerize and have complex roles in regulating differentiation (Schjerven *et al.*, 2013), but one function is particularly interesting. They appear to compete for binding to Notch target gene sites, working to damp activation in response to Notch signals (Chari *et al.*, 2010; Geimer Le Lay *et al.*, 2014; Kathrein *et al.*, 2008; Kleinmann *et al.*, 2008). The role of Ikaros is indispensable for maintaining the integrity of the β-selection checkpoint especially. Loss of Ikaros function otherwise converges with gain of Notch signaling activity to drive malignant transformation of early T cells at the β-selection checkpoint. In addition, the implications for normal T-cell development are important. Although Notch signaling drives the T-cell program forward and maintains viability in DN2b and DN3a cells, the T cell program itself may still require Notch-dependent transcription to be restrained.

IV. Commitment: transfer of lineage fidelity from environment to intrinsic factors

Lineage commitment is defined as the point when the developing T cells give up the intrinsic competence to embark on any other developmental pathway. Left in the thymic environment, essentially all of the pro-T cells from ETP through β-selection will normally give rise to T-cell progeny because of the Notch signaling, but there are dramatic differences

from one stage to the next in what they can do if they are removed from the Delta-rich thymic milieu. In fact, lineage commitment is a process rather than a single event, because adult pro-T cells lose access to the B-cell pathway soon after entering the thymus, but only lose access to myeloid, granulocyte, and NK fates significantly later, at DN2b stage [rev. by (Rothenberg, 2011)].

Close analysis of conditional knockouts that affect expression of certain transcription factors in the DN stages shows that the molecular basis of these alterations in response depends on at least three mechanisms. One is “commitment by subtraction” of PU.1: the silencing of PU.1 between DN2a and DN3a cuts a regulatory tie between the T-cell precursors and the granulocyte/monocyte lineage. Runx1, GATA-3, and possibly also TCF-1 are implicated in this repression (Huang *et al.*, 2008; Rosenbauer *et al.*, 2006; Scripture-Adams *et al.*, 2014; Taghon *et al.*, 2007; Zarnegar *et al.*, 2010). A second mechanism is “commitment by addition” of GATA-3: from the earliest stage of GATA-3 expression, even at a low level, it completely blocks access to the B-cell fate through a still-uncharacterized mechanism (García-Ojeda *et al.*, 2013; Scripture-Adams *et al.*, 2014). A third is “commitment by addition” of Bcl11b (Ikawa *et al.*, 2010; Li *et al.*, 2010a; Li *et al.*, 2010b).

A unique role for GATA-3 in the initial exclusion of B-cell potential is implied by the fact that GATA-3-deficient DN2 cells, unlike any normal DN2 cells, can still trans-differentiate into B cells if Notch signaling is withdrawn (García-Ojeda *et al.*, 2013; Scripture-Adams *et al.*, 2014). The direct targets of repression by GATA-3 that are relevant for B-cell development are not known but can be partially inferred from the genes that are upregulated in DN2 cells lacking GATA-3. Potential candidates include *Bcl11a* and possibly also PU.1 and SpiB, all of which play roles in B-cell precursor viability (Liu *et al.*, 2003; Schweitzer and DeKoter, 2004; Yu *et al.*, 2012c).

At the final commitment transition, as Bcl11b is upregulated, the cells become dependent on Notch signals and unable to survive long enough to give rise to anything else if Notch-Delta interaction is withdrawn. The lineage commitment role of Bcl11b also includes lineage-selective repressive effects. There are two effects of Bcl11b: a hit-and-run role in crossing the commitment threshold, and a permanent role in guarding against NK cell gene expression. Pro-T cells that have never expressed Bcl11b at all can become highly proliferative for weeks in culture, as long as strong Notch signals are continuously provided in the environment, and these cells continue to express multiple stem/progenitor genes. Thus, the absence of Bcl11b prevents them from triggering the repressive mechanisms that terminate the progenitor-gene-dominated state (Ikawa *et al.*, 2010; Li *et al.*, 2010a). However, if Bcl11b is deleted after commitment, the stem/progenitor regulatory genes and myeloid-associated genes like PU.1 do not resume expression; only the NK program is activated. This implies that the role of Bcl11b in the silencing of stem and progenitor genes can be a hit-and-run transitional one, unnecessary once the “phase 1” regulatory gene network is dismantled.

In contrast, the mechanism blocking NK and other ILC gene expression is a repressive effect that Bcl11b, once expressed, must exert continuously, especially if Notch signals are not sustained (Li *et al.*, 2010a; Li *et al.*, 2010b). The nature of the stimulus that would activate

this substantial effector gene battery is not clear, but could reflect an ILC-like increased ability to use cytokine signals without TCR signals as priming activators for effector function. Removal of Bcl11b at any stage enables these genes to turn on within 48 hr if cytokine stimulation is present (Li *et al.*, 2010b). Bcl11b-deficient DP cells also spontaneously activate genes that would normally only turn on as a result of positive selection, suggesting a reduced threshold for activation or reduced checkpoint enforcement at the DP stage (Kastner *et al.*, 2010). Even a hypomorphic allele of Bcl11b that allows T cell development in general converts positively selected CD8 cells into “innate-like” cells, with many ILC-like genes upregulated (Hirose *et al.*, 2015). The interactions of Bcl11b with the T-cell gene regulatory network thus link it both to the transitional process of T-cell identity generation and to the subtler differences in activation response that distinguish conventional from innate-like T cells.

V. From transcription factors to developmental process: glimpses of regulatory circuitry

A. Contextual modulation of transcription factor activity

The transcription factors that drive T cell development are all required, but this simple genetic observation leaves the core mechanistic questions about how they work in T-cell development unanswered. First, how are transcription factors deployed to serve different functions at different stages, and in different lineage contexts? In this context, what “placeholders” may keep a memory of lineage defining changes? Second, do transcription factor roles in early T-cell development operate in distinct subcircuits? What are the respective roles of repression and activation in the roles of key factors? Finally, how is modularity of different T-cell identity elements organized in terms of transcription factor action? The kinds of data that need to be marshalled to address these questions are becoming much more accessible, but are all imperfect. It is now possible to map the genome-wide distributions of many transcription factors’ binding in vivo by chromatin immune precipitation analyzed by deep sequencing (ChIP-seq), provided that good antibodies and large, well-characterized cell populations are available. However, sites of binding do not necessarily correspond to sites of specific function. Perturbation experiments are crucial, but because transcription factor effects are stage specific, gain or loss of function experiments must be carried out in a restricted developmental time frame in order to make comparisons with controls meaningful. This is often difficult because the kinetics of Cre deletion, RNA interference, and turnover of pre-existing RNA and protein can be slow compared to developmental progression in some important intervals. Thus current evidence has limitations that will need to be overcome with more sophisticated technology in the future. Nevertheless, it is already possible to glimpse some relationships between the activity spectra of key transcription factors in early T cell precursors that may point toward features of the fundamental modules of T-lineage developmental programming. In this final section, we review available evidence for the ways combinations of transcription factors coordinate and cross-regulate each other’s effects during the pivotal DN2 stages when T-cell identity is being selected.

1. Preferential recruitment by pioneer and placeholder factors—As ChIP-seq has been used increasingly to document transcription factor binding genome-wide, it has become clear that the binding patterns of transcription factors depend on developmental context. Lineage-specific factor combinations and often the presence or absence of particular stimulus-dependent factors cause redeployment of a given transcription factor to different genomic sites. For example, PU.1 is used in B cells, macrophages, progenitors, and early T cells, and occupies largely different sets of genomic sites in these different hematopoietic cells (Ghisletti *et al.*, 2010; Heinz *et al.*, 2010; Heinz *et al.*, 2013; Ostuni *et al.*, 2013; Zhang *et al.*, 2012b). In turn, however, any transcription factors that depend on interaction with PU.1-bound DNA to stabilize their own binding will be recruited preferentially to the appropriate lineage-specific target sites, where PU.1 already resides (Ostuni and Natoli, 2013): thus, genomic sites already rich in transcription factor occupancy can get richer. Stabilization can involve direct factor-factor interactions or simply a displacement of nucleosomes from the neighborhood of DNA sites where transcription factors are engaged (Barozzi *et al.*, 2014). A striking demonstration of the collective binding that can result is the pattern of multiplex transcription factor occupancy seen across the genome in hematopoietic progenitor cells (Beck *et al.*, 2013; Wilson *et al.*, 2010). Active enhancers, whether “super” or compact, are marked in these cells by clusters of 5–10 different transcription factors binding all together. GATA, ETS family, and bHLH family transcription factors with known roles in hematopoiesis are repeatedly seen engaged together, with Runx factors as well. The power of the interaction can be inferred because a significant fraction of the binding of individual factors appears to be occurring with grossly relaxed criteria for recognition of these factors’ normal target motifs in the DNA.

What kinds of mechanisms determine how hematopoietic factors get recruited to lineage-specific sites as T-cell specification begins? In embryonic stem cell differentiation, pioneer factors have been defined that can enter closed chromatin and render it accessible (Zaret and Carroll, 2011). There are several candidates that could provide pioneering-like activity in early T cell precursors. Runx1 is expressed in prethymic cells and throughout early T-cell development, and has target motifs highly enriched in lymphoid gene enhancers. It could at least maintain a hematopoietic identity state. Some data are available for Runx binding in DP cells (Yu *et al.*, 2012a). However, it is not clear yet which sites Runx1 is binding in the early stages of T-cell development. Another potential candidate as an identifier of “starter” hematopoietic cis-regulatory sites is PU.1, even though its role is restricted to the early stages of T-cell specification. PU.1 protein is very stable (Kueh *et al.*, 2013) and can continue to occupy a large fraction of its target sites even as its own RNA levels begin to drop. Initially, PU.1 is found binding to the majority of active enhancers and promoters in the ETP and DN2a cells. Although it selects different sites for engagement in T-lineage cells than it does in B or myeloid cells, this binding pattern is very stable within the first stages of T-cell development (Zhang *et al.*, 2012b). There is a correlation between PU.1 binding sites and gene activity in these early T cells, implying that it is most often contributing to positive regulation, although only a small fraction of the genes near where it binds are PU.1 dependent for their expression (Champhekar *et al.*, 2015). Interestingly, in ETP cells PU.1 binds to some sites that may become active in positive gene regulation only one or two stages later, such as the *Il7r* and *Bcl11b* cis-regulatory elements (Li *et al.*, 2013; Zhang *et*

al., 2012b). In these cases, PU.1 cannot be directing the majority of the transcriptional activity, but it may be helping to give access to the factors that will drive expression later. However, functionally important sites where PU.1 would bind in multipotent progenitors or B cells can be efficiently occluded from PU.1 binding in pro-T cells if they are engaged in repressed chromatin structures, marked by H3K27me3 (Zhang *et al.*, 2012b): examples include the intronic enhancer for the B-cell lineage determining gene *Pax5* (Decker *et al.*, 2009). Thus, conceivably PU.1 could contribute to T-lineage specific gene expression too, both an early placeholder and a sensor to discriminate permissive from nonpermissive chromatin states at regulatory sequences of genes for early T cells.

To open up T-cell genes *de novo*, of course, a T-cell specific input is required. The most abundant candidate among the first wave of factors induced by Notch signaling is TCF-1 (*Tcf7* gene product). In later T cells, TCF-1 is found binding to a very large fraction of all open cis-regulatory elements (Dose *et al.*, 2014), a distribution similar to that of the powerful lineage-determining factor Pax5 in B cells (Cobaleda *et al.*, 2007). However, TCF-1 again is not sufficient to determine all of its own target sites; it also respects context. TCF-1 is normally T-cell specific but is also found expressed in an immortal hematolymphoid progenitor cell line, EML-c1 (Wu *et al.*, 2012). Despite approximately equivalent numbers of binding sites in EML-c1 and in total normal thymocytes (Dose *et al.*, 2014), the occupied sites in the two cell types are largely non-overlapping (Fig. 5A). Thus TCF-1, despite its high abundance, binds only a subset of its possible sites, forming selective complexes at distinctive cell type-specific cis-regulatory elements in the two different cellular contexts. Where it does bind, TCF-1 can be a powerful indicator of sites that are likely to be functionally important for expression. TCF-1 engages its own regulatory elements, and TCF-1 occupancy sites are major elements of the far-distal enhancer recently described for the *Bcl11b* gene (Germar *et al.*, 2011; Li *et al.*, 2013; Weber *et al.*, 2011). Interestingly, TCF-1 binding can also distinguish between different classes of sites engaged by other T-cell specific factors, for example GATA-3.

As noted above, GATA-3 can be a problematic factor to study by ChIP-seq target mapping. In some respects, GATA-3 could play a role as a pioneer, because it does not appear to be excluded by Polycomb-repressed chromatin, and it binds to some future enhancer sites at least one to two stages before they become active at all. For example, its binding to functionally important cis-regulatory sites of *Cd3d* and the *Rag1,2* genes anticipates the activation of these genes by at least one stage (Zhang *et al.*, 2012b). However, as early T-cell development progresses, the sites bound by GATA-3 shift substantially from stage to stage, suggesting that its interactions are highly dependent on partner factors. Fig. 5B shows that only a minority of the sites bound by GATA-3 in the DN1 (ETP) stage overlap with sites bound by GATA-3 in DP cells, and vice versa. However, if one focuses on GATA-3 sites adjacent to sites where TCF-1 binds [sample mostly consisting of DP cells (Dose *et al.*, 2014)], there is a far more consistent pattern of GATA-3 occupancy. In these cases, over 85% of the sites where GATA-3 starts binding in the ETP stage still retain GATA-3 binding by the DP stage (Fig. 5C). Note that these are also predicted to be sites for collaborating factors of the ETS and Runx family (Fig. 5D). Many of these sites are associated with functionally important genes for T-cell development, including *Cd247* (TCRζ), *Itk*, *Jak1*, the

positive selection gene *Tespa1* (Themis, GASP), the TCRα and TCRβ enhancers (near *Dad1* and *Prss2*, respectively), *Il9r*, and transcription factor genes *Zfpm1* (FOG1), *Tox2*, and *Tcf7* itself [data from (Dose *et al.*, 2014; Zhang *et al.*, 2012b)], among many others. Intriguingly, again, although the local GATA-3 binding is already established by ETP stage, some of these genes like *Tespa1* and the elements rearranging to generate TCRα are activated only in later stages of T-cell development, long after the ETP stage: additional cases where GATA-3 occupancy greatly precedes activation. This set of composite sites might thus mediate unusually stable assemblies of GATA-3 and TCF-1 together that guide the construction of a T-cell identity.

2. Notch signaling: modulator as well as independent input—The transcription factors that work in the cell during the earliest phases of T-cell specification have their effects influenced by the ongoing level of Notch signaling. In part this is due to Notch-activated target genes like *Hes1*, which encode transcription factors that themselves add to the regulatory mix. As a repressor, Hes1 can silence the expression of other regulatory genes that could hold back T cell development or promote alternative fates: for example, it can repress the myeloid factor C/EBPα (De Obaldia *et al.*, 2013). There may also be additional mechanisms through which Notch signaling impacts the activities of other factors in the cells so as to change their roles. This can be seen when other transcription factor levels are manipulated in T cell precursors in the presence or absence of Notch signaling. If T cell precursors are forced to express PU.1 at elevated levels, this can shut off the T cell regulatory gene program and rapidly activate a myeloid program if Notch signaling is absent (Del Real and Rothenberg, 2013; Franco *et al.*, 2006; Laiosa *et al.*, 2006). However, if Notch signaling continues, it can provide a buffer against repression of T-cell genes by PU.1, directly or indirectly protecting a wide spectrum of T-cell regulatory genes including *Tcf7*, *Ets1*, *Zfpm1*, *Myb*, and even *Gata3* (Del Real and Rothenberg, 2013). Also, in the presence of Notch signals, overexpressed PU.1 activates a select spectrum of target genes, with less activation of myeloid-specific genes like *Csf1r*, while continuing to activate progenitor-associated target genes like *Bcl11a* (Del Real and Rothenberg, 2013). The selectivity may directly reflect the specific repression of C/EBPα factor expression by Hes1 (De Obaldia *et al.*, 2013). Where PU.1 drives myeloid-specific gene expression, C/EBP family factors very frequently need to be co-recruited (Heinz *et al.*, 2013), but not at PU.1 targets in B cells and progenitors (Heinz *et al.*, 2010). Thus, when Notch induces expression of Hes1, silencing of *Cebpa* may redirect PU.1 to a more progenitor-cell like spectrum of target genes.

Interestingly, Notch signaling can also modulate the responses to transcription factors that are more conventionally T-cell specific, like GATA-3. Despite its crucial function in T-cell development, GATA-3 activity to support the T-cell program is acutely dose-dependent, with inhibitory effects at high doses as well as failures to support survival or developmental progression at low doses (Scripture-Adams *et al.*, 2014; Taghon *et al.*, 2007; Xu *et al.*, 2013). In both respects, GATA-3 effects depend on interaction with Notch signaling status. High-level GATA-3 itself is antagonistic to the T-cell program (Taghon *et al.*, 2007; Xu *et al.*, 2013), and this is a complex function of its tendency to drive alternative lineage fates that are antagonized by Notch as well as its tendency to promote death when Notch

signaling is high. Of particular interest, high-level GATA-3 can become repressive for TCF-1 (*Tcf7*) expression itself, but only when Notch signals are removed (Taghon *et al.*, 2007). This contrasts dramatically with the effects of normal levels of GATA-3 which enhance *Tcf7* in the presence of Notch signals. Conversely, only when Notch signaling is present do hematopoietic precursors become dependent on a basic threshold level of GATA-3 for survival (Scripture-Adams *et al.*, 2014). This is well established in the T-cell system, but interestingly it may also be true of the most Notch-dependent ILC lineage (ILC2), which is GATA-3 dependent (Hoyler *et al.*, 2012; Radtke *et al.*, 2013; Serafini *et al.*, 2014; Yagi *et al.*, 2014), and has recently been shown to require TCF-1 as well (Yang *et al.*, 2013). A well-balanced Notch-GATA-3 collaboration may thus be a fundamental regulatory circuit element deployable in both T and ILC contexts.

B. Competitive circuitries pacing the onset of T-cell gene expression

1. PU.1-Notch mutual antagonism—Whereas Notch signaling modulates the effects of PU.1 on its targets, especially protecting T-cell genes from repression, the relationship between Notch and PU.1 in pro-T cells is actually a bistable, competitive switch. Recent data imply that PU.1 itself works dose-dependently to blunt the efficiency of Notch signal-induced target gene activation (Champhekar *et al.*, 2015; Del Real and Rothenberg, 2013). PU.1 seems to accomplish this by activating a gene or genes that inhibit Notch transcriptional activity indirectly (Champhekar *et al.*, 2015). The identities of these intermediates are still uncertain. However, even in the context of sustained access to Notch ligands, both early and later-stage Notch-activated genes alike increase expression in ETP and DN2 cells when normal endogenous PU.1 is reduced or neutralized (Champhekar *et al.*, 2015). This implies that PU.1 and Notch are competitively restraining each other throughout the early stages of T-cell development, when PU.1 levels are high. The competition is biased against PU.1 as long as Notch signaling persists, because eventually Notch-activated transcription factors including GATA-3 and also Runx1 will ultimately turn *Spi1* off (Huang *et al.*, 2008; Scripture-Adams *et al.*, 2014; Zarnegar *et al.*, 2010); but the mutual antagonism exacerbates the power of PU.1 as a driver of lineage diversion if the Notch signal is interrupted. This could therefore mean that PU.1 has a role as a threshold setter for Notch signal intensity, such that only above a certain level of Delta in the thymic environment can an adequate level of Notch signal be induced to trigger as well as to protect T-cell gene expression (Champhekar *et al.*, 2015; Del Real and Rothenberg, 2013).

2. GATA-3 vs. PU.1—An influential guiding paradigm in the systems biology of hematopoiesis is that GATA family factors are mutually antagonistic with PU.1, and that this antagonism splits erythro-megakaryocytic precursors from granulocyte-macrophage precursors (Huang *et al.*, 2007)(Fig. 6A, left panel). There is abundant evidence that protein-protein interactions between PU.1 and GATA factors in a cell can blunt the transactivation activity of each on its specific target genes, and also evidence that PU.1 and GATA factors can mutually repress each other's expression at the RNA level. Different "common myeloid progenitor" subsets with skewed potential for erythroid vs. macrophage/neutrophil fates can be distinguished accordingly by their divergent expression of GATA-1 vs. PU.1 (Arinobu *et al.*, 2007; Månsson *et al.*, 2007; Nutt *et al.*, 2005). Yet under certain circumstances, GATA factors and PU.1 can also collaborate, such as in certain zebrafish hematopoietic progenitors

(Monteiro *et al.*, 2011) and more stably in mammalian mast cells (Walsh *et al.*, 2002). It is therefore clear that the antagonism depends on several elements, including not only the doses of the two factors but also on the presence of certain exclusionary cofactors, which may or may not be expressed in given contexts.

In early T-cell development, GATA-3 and PU.1 can act as antagonists. Overexpressed GATA-3 sharply downregulates PU.1, and GATA-3 deletion or inhibition with shRNA causes PU.1 mRNA levels to rise, implying that GATA-3 normally exerts at least some restraint on *Spi1* (PU.1) transcription (Scripture-Adams *et al.*, 2014; Taghon *et al.*, 2007). GATA-3 levels, in protein and RNA expression, reach their height as PU.1 begins to be silenced. However, PU.1 has a more complex effect on GATA-3: it can repress *Gata3*, but this effect depends on current Notch signaling status. When Notch signals are withdrawn, PU.1 can repress *Gata3* considerably, yet in the presence of strong Notch signaling, it may even slightly enhance *Gata3* expression (Del Real and Rothenberg, 2013). As a result, in early T cell development under normal conditions the antagonism is not exclusive (Fig. 6A, right panel). Both factors are expressed together through most of the ETP stage and at equally high or higher levels in DN2a stage, before PU.1 expression is turned off. During this period, neither factor is completely inhibiting the other at the level of protein interference either, because both factors have significant normal roles in target gene expression throughout these precise stages (Champhekar *et al.*, 2015; Scripture-Adams *et al.*, 2014).

Genome-wide surveys of the functional targets of both factors imply that they have largely independent functions but some significant competitive interaction at the DN2 stage (Fig. 6B). Provisional relationships can be illustrated by the small number of genes that can be specifically affected both by PU.1 and by GATA-3 perturbations within short timespans in DN2 cells. Of ~240 genes affected by interference with PU.1 activity in DN2 cells, and ~100 genes altered by acute deletion of *Gata3*, only 19 genes were consistently affected by both, showing that these factors are not only global antagonists in DN2 gene expression. Furthermore, the directions of the effects on these genes were not always reciprocal. For three of the genes, i.e. transcription factor *Bcl11a*, tumor necrosis factor receptor family member *Relt*, and immunoglobulin superfamily member *Lair1*, PU.1 appeared to be an activator and GATA-3 a repressor. For seven other genes, *Tcf7*, *Ets1*, *Myb*, *Gfra1* (glial-cell derived neurotrophic factor family receptor α1), *Zfpm1* (FOG-1), *Rag1*, and *Kit*, GATA-3 appeared to be an activator and PU.1 a repressor. However, seven additional genes appeared positively regulated by both factors, and two important genes, *Dtx1* and *Spi1* (PU.1) itself, appeared to receive at least partially negative inputs from both. In general, PU.1 positively regulated genes with their major phases of expression in progenitors and non-T cells, whereas GATA-3 positively regulated T-cell genes and genes used by other GATA-expressing lineages, such as mast cells (*Cpa3*, *Kit*); but the expression patterns were quite varied. Thus, both factors contributed largely independent regulatory activities to the cells, using the full range of possible combinations of interactions.

Importantly, the kind of negative regulation that is seen in such experiments is weak “damping repression” rather than “silencing repression”. For targets like *Myb* and *Zfpm1*, PU.1 has the potential to extinguish expression completely, but it does not do so as long as

Notch signaling is sustained (Del Real and Rothenberg, 2013). Thus, these factors reduce the maximum levels of expression of target genes but allow considerable expression to continue. In marked contrast to the classic bistability models in which GATA factors and PU.1 are each positively autoregulatory (Fig. 6A left) (Chickarmane *et al.*, 2009; Huang *et al.*, 2007), in pro-T cells these factors even exert level-stabilizing damping repression upon their own regulatory elements (Champhekar *et al.*, 2015; Scripture-Adams *et al.*, 2014; Taghon *et al.*, 2007).

In this context, GATA-3 and PU.1 can provide positive and negative inputs in all combinations during a sustained period of cohabitation, and their partial, graduated effects on target genes can work additively over a wide dynamic range. PU.1 is free to work to promote expression of multiple genes that can contribute to the viability, stimulation responsiveness, migration behavior, and cytoskeletal dynamics of the developing thymocytes (Champhekar *et al.*, 2015), even while GATA-3 is being turned on and for multiple cell divisions thereafter. However, the select set of genes on which PU.1 and GATA-3 do exert opposing effects include regulatory genes of great significance for the control of T-cell developmental progression. Because of the effects on these specific genes, the tipping of the PU.1/GATA-3 balance has the potential to act as an important T-cell developmental control point.

C. GATA-3, E protein, and Bcl11b circuitry

Although Notch triggers feed-forward positive circuitry to activate the T-cell program, direct control by Notch-dependent transcription does not account for the levels of expression of most T-cell genes. Thus, the Notch-triggered T-cell factors must be involved in the sequential activation of T-cell genes from ETP to DN3a stage. For example, when prethymic precursor cells are forced to express *Tcf7* in gain of function experiments, they can activate a near-complete spectrum of DN3-like genes, except for particular Notch targets, without apparent input from the Notch pathway (Weber *et al.*, 2011). Although GATA-3 is essential for pro-T cell viability and development, it alone does not account for the activation of the T-cell program. The most target genes regulated sensitively by GATA-3 in DN2 cells include a few with known or likely T-cell functions: positive targets *Kit*, *Zfpm1* (FOG-1, a cofactor of GATA factors), *Tcf7*, *Ikzf2* (Helios, a member of the Ikaros family expressed early in T progenitors and strongly expressed later in Tregs), and transcripts from TCRγ-C1; and negatively regulated targets *Bcl11a*, *Spi1* (PU.1), and a few others. Some of the GATA-3 activated genes indeed are being turned on in DN2 stage for sustained use in the T-cell program or being primed for future activation, whereas others like *Kit*, *Cpa3*, *Fgf3*, *Cd93*, and *Akr1c13* are expressed only transiently in early stages and are actually shut off during commitment. To account for the expression patterns of these genes, and to make the cells turn on additional T-cell genes, additional positive inputs are needed.

One of these inputs is from E proteins. E proteins are needed to activate many genes that distinguish the T-cell gene expression program from progenitors, NK cells, and ILCs alike, mostly during during DN2b and DN3a stages (Fig. 3A). Comparing the transcriptomes of wildtype and E2A-knockout DN2 cells (Xu *et al.*, 2013) shows that *Cd3g*, *Cd3e*, *Lat*, *Rag1*, and genes coding for the transcription factors Bcl11b and Gfi1 are all E2A-dependent. At

least by the DN3a stage, E protein activity is also important to maintain maximal levels of Notch1 itself (Yashiro-Ohtani *et al.*, 2009). E proteins act both positively and negatively, and it is significant that in developing DN2 cells they show repressive effects on regulatory and effector genes *Rora*, *Zbtb16*, *Ahr*, *Fcer1g*, *Id2*, *Gzmb*, *Zfp105* and *Lmo4*, all of which are normally most expressed in innate lymphoid cells. Thus, establishing a strong E protein activity level is a major contributor to the gene expression identity of the developing T cells.

Interestingly, two of the regulatory genes that are subject to damping repression by E2A are those coding for GATA-3 itself and its target and cofactor, Zfpm1 (FOG-1). This result groups the essential T-cell factor GATA-3 and Zfpm1 together with the innate-cell genes which need to be kept repressed, and are only released from repression when E2A is deleted. Kee and colleagues have shown that the 2–3 fold increase in GATA-3 activity that occurs with loss of E2A is a major contributor to the developmental arrest that E2A mutant cells undergo, and the cells can be rescued by using shRNA to knock down the GATA-3 to more normal levels (Xu *et al.*, 2013). In other words, the excess of GATA-3 is more deleterious to T cell development than the reduction of E2A itself.

The effects of E2A have some arresting similarities to those of Bcl11b in the DN2-DN3 stages. Bcl11b also is needed to keep many ILC-specific genes off, and especially to limit activation of the ILC1 and NK cell genes. Although genome-wide analysis of Bcl11b knockout cells has only been published so far for later-stage thymocytes (Hirose *et al.*, 2015; Kastner *et al.*, 2010; Li *et al.*, 2010b), Bcl11b knockout DN2 cells also upregulate *Zbtb16*, *Nfil3*, *Id2*, *Fcer1g*, *Zfp105*, *Gzmb*, and *Prf1* (perforin) as well as turning on expression of numerous NK receptors (Li *et al.*, 2010a) (unpublished results, J. A. Zhang, L. Li, & E.V.R.). Despite the fact that they work together in normal T-cell development to exclude alternative lineage fates, Bcl11b probably also exerts damping repression on GATA-3, very much like E2A. The roles of Bcl11b and E2A are so similar that they can be provisionally considered as part of one pathway. Furthermore, GATA-3 is also linked to Bcl11b, in a classic incoherent feed-forward circuit. GATA-3 is needed to activate Bcl11b itself (García-Ojeda *et al.*, 2013; Scripture-Adams *et al.*, 2014), only to be damped down by Bcl11b activity as it comes on. Thus although GATA-3 and E proteins may collaborate in the activation of crucial pan-T cell genes such as those coding for CD3 proteins, another of the effective collaborators of E proteins is Bcl11b, and its role may be to prevent the effects of high-level GATA-3.

The relationships between direct and indirect action remain to be clarified in this process. One of the key targets of Bcl11b repression is Id2, in the sense that any reduction in Bcl11b dosage rapidly enables cells to upregulate Id2. This enables high-level Bcl11b to collaborate strongly with E2A/HEB to maintain the characteristics of an E protein-dominant state. However, lower levels of Bcl11b can be compatible with Id2 expression, for example in effector CD8 T cells where both are expressed (Kaech and Cui, 2012; Masson *et al.*, 2013; Yang *et al.*, 2011a). It has even been found that Bcl11b at moderate levels is actually required for ILC2 cell development, despite these innate lymphocytes' constitutive requirement for Id2 expression (Califano *et al.*, 2015; Walker *et al.*, 2015; Yu *et al.*, 2015). There, Bcl11b collaborates with GATA-3, TCF-1, and Gfi1 to favor ILC2 as opposed to ILC3, ILC1, or NK development. Thus, Bcl11b itself is probably dose-dependent, playing

an integral role in a network circuit to guide effector subtype specialization at low levels, even while it can support the roles of E proteins in enforcing activation thresholds and disfavoring any innate-like pathway when it is expressed at high levels.

VI. Conclusions: T cell development as an emergent process

The elements identified here reveal ways that Notch-dependent progression through the early stages of T cell development not only turns on key regulators like GATA-3, TCF-1, and increased levels of HEB, but also engages them in a succession of circuit interactions. A feature of most of these circuits is their ability to channel the impacts of the component transcription factors either by level control or by ensuring that they are directed to a particular subset of target genes. This is most obvious with the interaction between Notch signaling and PU.1, but it is also a feature of the circuitry connecting GATA-3 and Notch signaling, and GATA-3 with the commitment climax factors Bcl11b and E proteins. Recurrent features are the dose-dependence of transcription factor effects in this system, policed normally by incoherent feed-forward gene network circuits that constrain factor activities within an appropriate range, and the stage-dependence of factor actions that implies that each successive stage creates new “facts on the ground” as a point of departure for the next stage.

We can speculate that the earliest stages of T-cell development, represented in ETP cells, bring PU.1 activities under the control of Notch modulation. A key component is likely to be the early activation of Hes1, which deprives PU.1 of its pro-myeloid partner C/EBPα and forces it to regulate selective, pro-T-cell compatible genes. The situation can change when Notch signaling can activate GATA-3 and TCF-1, which collaborate with E proteins already present to begin to activate T-cell identity genes. There is still much to learn about the roles of the other “phase 1” factors, but the cells probably cannot experience a maximal intensity of Notch signal as long as PU.1 levels are high. The collaboration of GATA-3, TCF-1, and pre-existing factors like Runx1 can finally tip the balance to a new state, when PU.1 with Lyl1 and other progenitor factors is downregulated. As the restraints on Notch signal strength are lifted during commitment, new regulators like Bcl11b are activated by the convergence of T-lineage regulators, and the cells can become Notch-dependent for viability, thus ending lineage plasticity. Dilution of the competitor Lyl1 as HEB levels rise may now unleash high E protein activity as well. Strong RAG expression can begin, and selection checkpoints are imposed on the cells. Although some of the factors they express could give them access to effector function directly, the cells can no longer take advantage of this. They can now begin a future as T cells in which all the other effector capabilities they may acquire will be linked to a permanent dependence on TCR signals.

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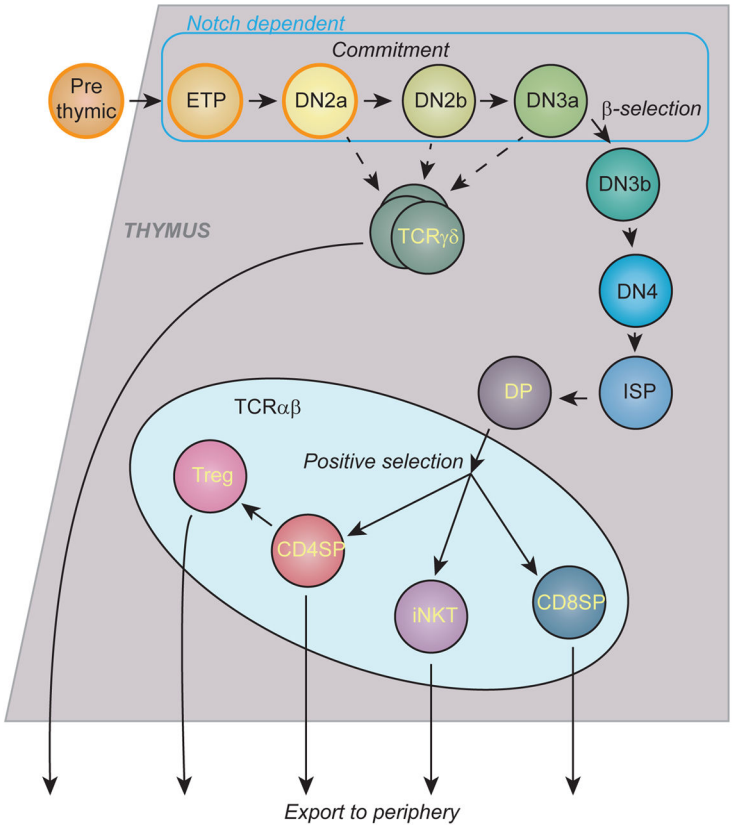
A



B

ETP	Kit++ CD44+ CD25- CD27+ CD4(Lo/-) Flt3+ → - CD24a- → +	
DN2a	Kit++ CD44+ CD25+ CD27+	{Surf TCR- CD24a++ CD4- CD8-}
DN2b	Kit+ CD44+ CD25+ CD27+	
DN3a	Kit^{Lo} CD44- CD25+ CD27^{Lo} CD28-	
DN3b	Kit^{Lo} CD44- CD25+ CD27^{Hi} CD28+	
DN4	Kit- CD44- CD25- CD27^{Hi} CD28+	
ISP	TCR- CD4- CD8+ CD24a++	
DP	TCR^{Lo} CD4+ CD8+ CD24a+	{Surface TCRαβ+}
CD4, CD8 SP	TCR+ CD4+ CD8- *or* CD4- CD8+ CD24+ → -	
iNKT	TCR+ CD4(+/-) CD8- CD44+ CD24a- NK1.1(+/-)	
Treg	TCR+ CD4+ CD8- CD25+	
TCRγδ	Kit$^{(+/Lo)}$ CD44(+/-) CD25- CD4- CD8- CD27+	

Figure 1. Stages of intrathymic T cell development

A) Scheme of stages of mouse T-cell development discussed in the text, showing the stages affected by Notch-DLL4 signaling, intrathymic branchpoints in development, and the timing of three watershed events: commitment, β-selection, and positive selection.

B) Markers used to define the stages shown in A.

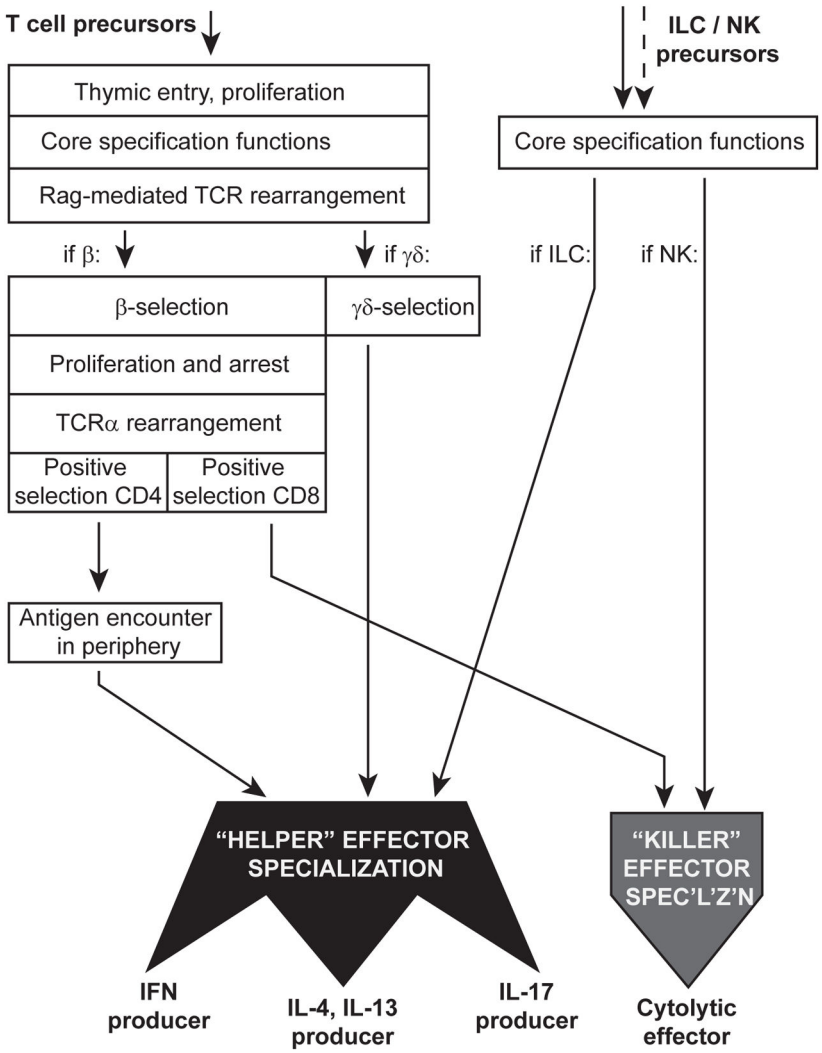


Figure 2. Modularity of the T-cell developmental program: differential access to effector specialization functions depending on choice of T-cell or innate-cell lineage

The figure depicts a surprisingly common set of regulatory programming used to distinguish "killer" and different "helper" subtypes of effector T cells and innate lymphoid cells (ILC and natural killers, NK), and the layers of developmental programming that cells must undergo before gaining access to these common programs. Note that TCRαβ lineage T cells have a highly protracted, multistep pathway requirement before they can access the specialization functions, in contrast to ILC and NK cells. As described in the text, TCRγδ cells appear to be intermediate between the extreme of the longer path taken by $CD4^+$ αβ T cells and the apparently short paths taken by ILCs.

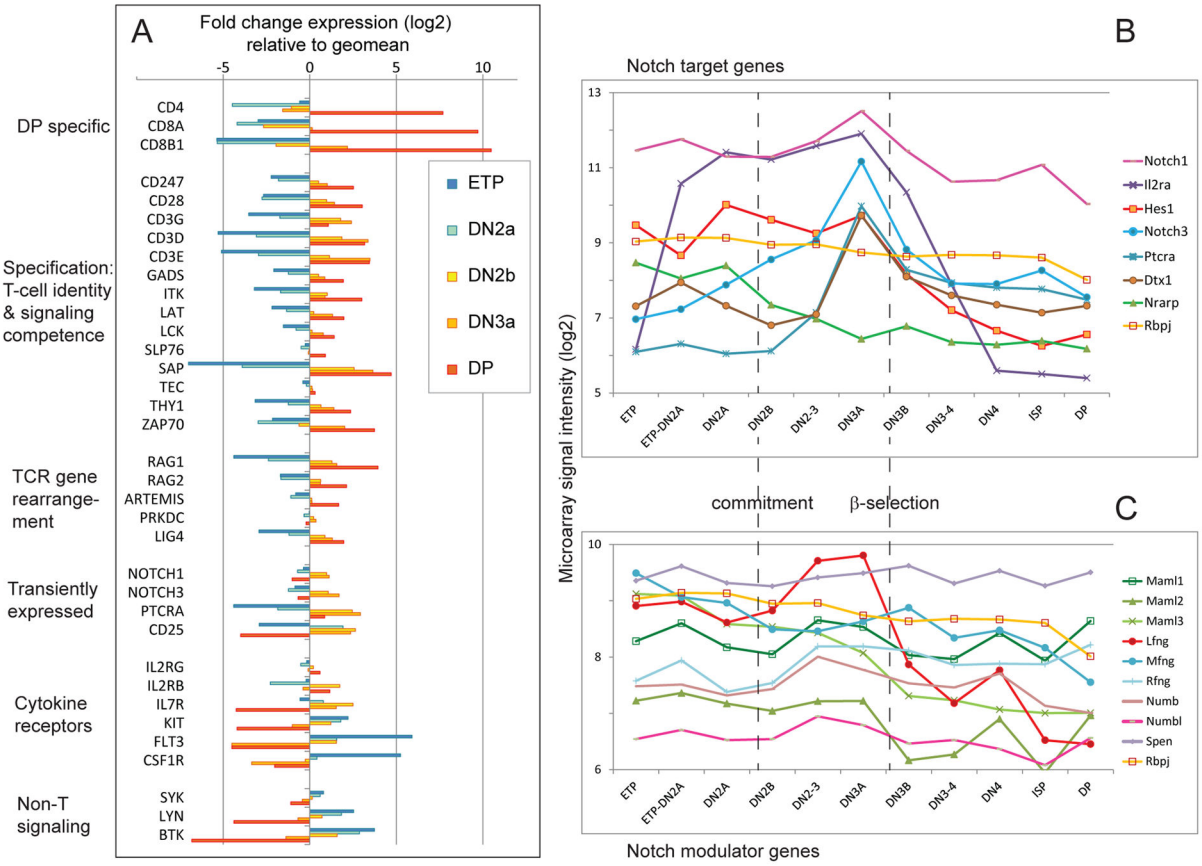


Figure 3. Trajectories of gene regulation in T-cell precursors through commitment and β-selection

Data for (A) are RNA-seq analyses taken from (Zhang *et al.*, 2012b), and data for (B, C) are highly curated microarray analyses taken from www.immgen.org (Mingueneau *et al.*, 2013).

A) Patterns of expression of T-cell identity genes and signaling components through the transition from ETP to DP. Data are presented on a $\log_2$ scale, with increases and decreases in expression plotted as changes relative to the geometric mean of values for each gene. Thus, genes with stable expression at high or low levels have little fold change. Precommitment stages are indicated by blue bars, postcommitment stages by yellow to red bars. Note that T-cell identity and signaling competence genes are drastically upregulated during commitment, while cytokine receptor genes are more diversely regulated.

B) Notch target genes have developmentally distinct patterns of expression. Absolute microarray hybridization intensities at the indicated stages are presented on a $\log_2$ scale. All the genes shown are sharply affected by interruptions or increases in Notch signaling intensity in early T cells except the Notch signal-transducing regulatory gene *Rbpj*, which is shown to indicate the stability of the Notch response machinery.

C) Little developmental change in expression of known Notch signal modulating genes despite dynamic target gene expression. The indicated modifiers are plotted as for the samples in (B), but the scale is expanded for greater sensitivity to change, with *Rbpj* shown again for reference. Note that only *Lfng* is highly regulated across these stages.

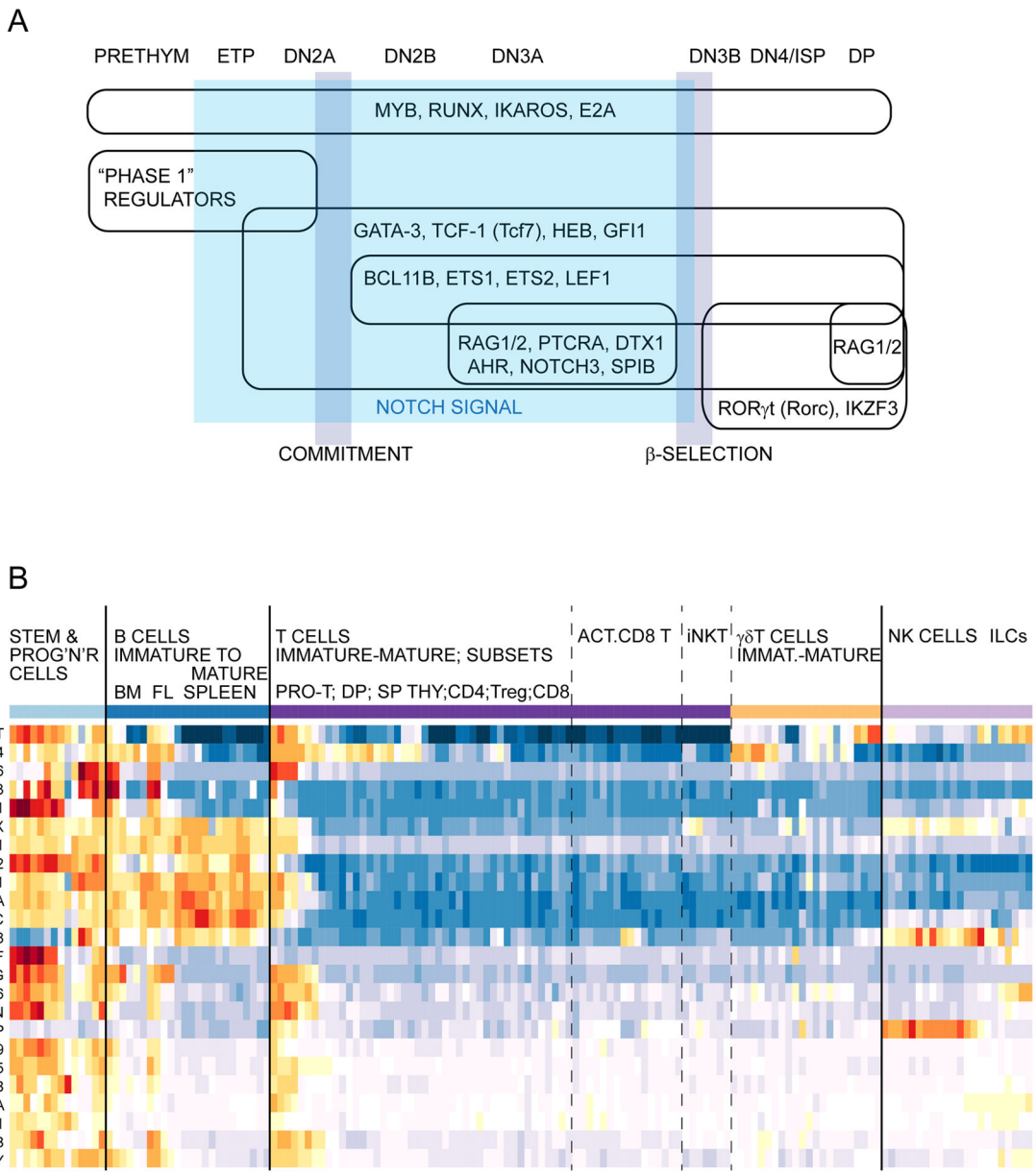


Figure 4. Dynamic regulatory stages in developing T-cell precursors

A) Diagram representing the major groups of regulatory factors at the stages when they are deployed in early T-cell development. The period dominated by Notch signaling is shown by shading. All the factors shown are transcription factors or cofactors except RAG1/2 (recombinases), Ptcra (TCRα surrogate chain used in β-selection), and Dtx1 (Notch pathway target gene). "Phase 1" regulators are the stem/progenitor-associated factors that are highly expressed in thymic immigrants but then silenced during commitment and immediately afterwards. "RUNX" refers to the constant presence of members of the Runx family, although Runx2 and Runx3 are more highly expressed in the ETP stages and Runx1 is more highly expressed at the DN3a stage.

B) Phase 1 regulators have distinct patterns of usage in stem/progenitor cells and in other classes of lymphocytes. Patterns of expression are shown for two cytokine receptor genes (*Kit* and *Flt3*) and 22 phase 1 regulatory factor genes, using the ImmGen database (Heng *et al.*, 2008; Mingueneau *et al.*, 2013; Robinette *et al.*, 2015) and interactive heatmap tool for "MyGeneSet" (http://rstats.immgen.org/MyGeneSet/)(July, 2015). Red and orange cells represent highly expressed genes in the indicated cells relative to their average, blue represents very low levels of expression relative to their average. Note that all these genes have their expression in T cells confined to the left end samples in the "T Cells" series, which correspond to the stages shown in Fig. 3B, C. However, they are all expressed in addition in at least some stem and progenitor cells ("Stem & Prog'n'r Cells") and distinct groups are also shared with early B cells, B cells generally, and/or NK and ILC cells.

Figure 5. GATA-3 and TCF-1 DNA binding sites across the genome in different cellular contexts: higher developmental stability in sites of co-occupancy

The figure summarizes ChIP-seq data from EML-c1 progenitor cells (A), DP T-lineage cells (A–C), and ETP (DN1) pro-T cells (B–C). The Venn diagrams show the extent of overlap between the sequences recovered as binding sites for the two factors, or the same factors in different contexts. Data for GATA-3 in ETP and DP cells were taken from (Zhang *et al.*, 2012b), data for TCF-1 in EML-c1 cells were from (Wu *et al.*, 2012), and data for TCF-1 in DP cells were from (Dose *et al.*, 2014). Peak calling of mm9 aligned sequences was performed with the HOMER package (Heinz *et al.*, 2010) and filtered for a peak score ≥ 15. Peaks were identified using *findPeaks.pl* with the *–style factor* parameter and normalized to sequencing inputs; overlapping peaks were identified and Venn-diagram parameters were retrieved using *mergePeaks.pl* (default parameters with the Venn-diagram option).
A) TCF-1 overlapping and non-overlapping ChIP-seq peaks in EML-C1 progenitor cells (Gene Expression Omnibus accession number GSE31221) and DP thymocytes (GSE46662).
B) GATA-3 overlapping and non-overlapping ChIP-seq peaks in ETP (DN1) T-cell precursors and DP thymocytes (GSE31235). The poor overlap is also noted with a different graphical presentation in (Zhang *et al.*, 2012b).

C) Overlap between Gata3 sites in DP cells shared with TCF-1 bound sites in DP cells and the Gata3 sites in ETP cells shared with TCF-1 bound sites in DP cells. Note that these sites are far more likely to overlap than total GATA-3 sites.
D) Evidence that occupancy involves recurrent partner factors: enrichment of motifs for GATA-3, TCF-1 [TCF (HMG)], and additional factors at GATA-3 and TCF-1 binding sites in DP cells. HOMER was used for *de novo* motif analysis within a 200 bp window of the sites of GATA-3 and/or TCF-1 occupancy in DP cells. Bold type identifies the most common co-enriched motifs, present at >15% of occupancy sites. Note the recurrent co-enrichment of ETS and Runx family motifs.

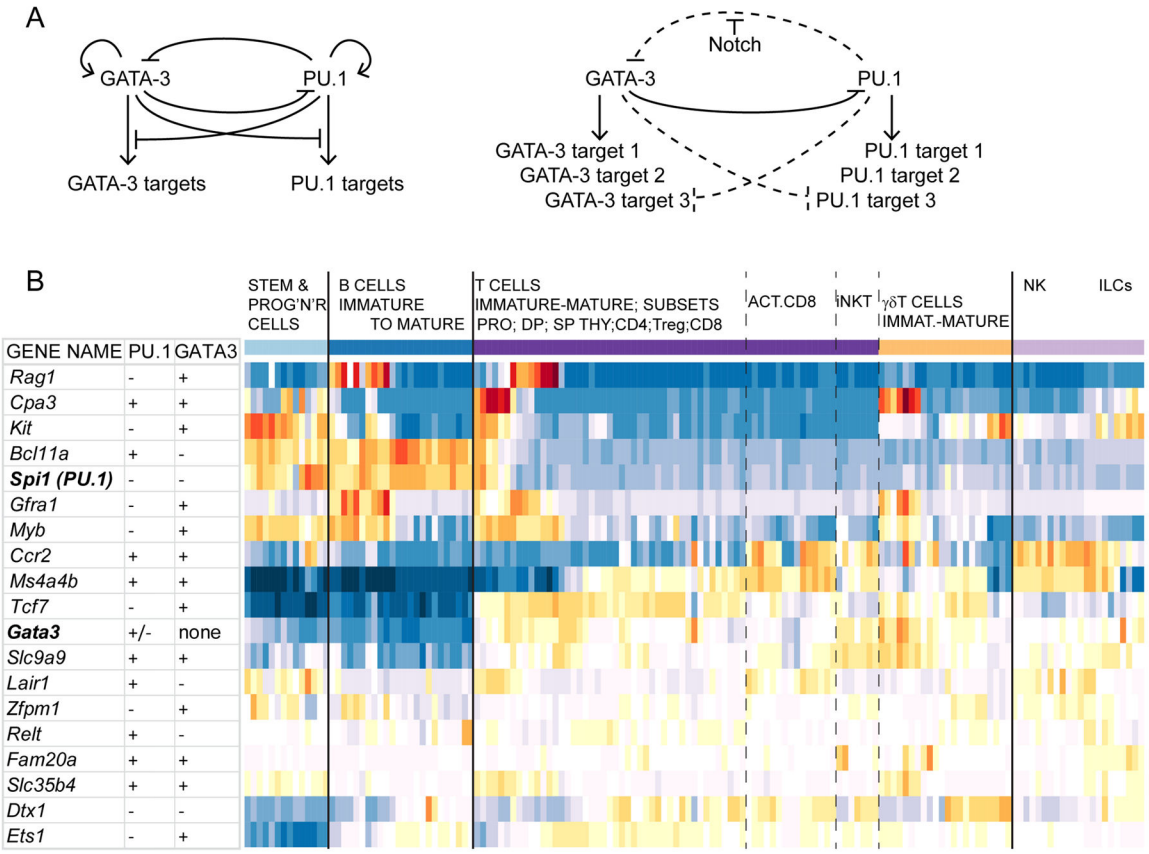


Figure 6. GATA-3 and PU.1 relationship in early T cells: not a simple bistable antagonism

A) Two diagrams contrasting the classic view of GATA/PU.1 interactions in hematopoiesis (left) with the relationship actually seen in early T-cell precursors (right). Note that the actual relationship is missing the positive autoregulation loops for GATA-3 and PU.1 and the categorical antagonism at the protein level. Instead, Notch signaling enables an asymmetric relationship between GATA-3 and PU.1 at the transcriptional level, and effects of PU.1 and GATA-3 on each other's activities are specific for particular target genes, a small subset of their total functional targets.

B) Genes subject to regulation by both PU.1 and GATA-3 in early T-cell precursors, shown with the direction of the effects of PU.1 and GATA-3 inferred from perturbation experiments in DN2 cells developed in vitro from fetal liver precursors (Champhekar *et al.*, 2015; Scripture-Adams *et al.*, 2014). Note that all combinations of effects are seen. The patterns of expression of these genes in stem/progenitor cells, T cells, and other lymphocytes, mostly from adult animals (Heng *et al.*, 2008; Mingueneau *et al.*, 2013; Robinette *et al.*, 2015), are also shown as in Fig. 4B to indicate the diversity of developmental regulation patterns involved, not simply correlated with expression of PU.1 and GATA-3 themselves.