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Author manuscript

Nat Rev Immunol. Author manuscript; available in PMC 2015 August 01.

Published in final edited form as:

Nat Rev Immunol. 2014 August ; 14(8): 529–545. doi:10.1038/nri3702.

Developmental gene networks: a triathlon on the course to T cell identity

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Summary

Cells acquire their ultimate identities by activating combinations of transcription factors that initiate and sustain expression of the appropriate cell-type specific genes. T-cell development depends on the progression of progenitor cells through three major phases associated with distinct transcription factor ensembles that control their recruitment to and proliferation in the thymus, their lineage commitment, and their responsiveness to T-cell receptor (TCR) signals, before the allocation of cells to particular effector programs. All three phases are essential for proper T cell development, as are the mechanisms that determine the boundaries between each phase: cells failing to shut off one set of regulators before the next gene network phase is activated are predisposed to leukemic transformation.

Introduction

The T-cell developmental program, initiated and sustained by the unique thymic environment, guides small numbers of pluripotent cells through multiple rounds of proliferation and differentiation, leading to T-lineage commitment, T-cell receptor (TCR) rearrangements, and generation of $\alpha\beta$ TCR- or $\gamma\delta$ TCR-expressing T-cells that function as killers, regulatory cells, or producers of specific cytokines¹⁻⁶. In the past five years, the transcriptional and epigenetic mechanisms that forge T-cell identity and suppress other developmental pathways have come into focus. It is not enough for cells to simply activate the set of transcription factors that maintain T-cell gene expression in mature T-cells; instead, the developmental program depends on the sequential operation of several distinct developmental gene networks.

From the time a lymphoid precursor arrives in the mouse thymus to the first expression of an $\alpha\beta$ TCR, it traverses at least 8 phenotypically distinct stages defined by expression of CD4, CD8 and other markers¹⁻⁶ — Flt3⁺ early thymic progenitor (ETP), ETP, double negative 2a (DN2a), DN2b, DN3a, DN3b, transitional DN4 and immature single-positive (ISP), and double positive (DP) (DN: CD4⁻ CD8⁻, DP: CD4⁺ CD8⁺) (Fig. 1a). Most of these stages undergo proliferation, but the degree of proliferation and the time required to reach the DP $\alpha\beta$ TCR⁺ stage vary between lymphoid precursor cohorts. It takes a little over a day for the first wave of lymphoid precursors that populate the fetal mouse thymus to generate DN2 cells (E12.5-E14) and only a total of four days for the first DP cells to appear (E16). In

The authors declare no competing interests.

contrast, the lymphoid precursors that continuously trickle into the thymus throughout young adult life can take ten days to reach DN2 stages and two weeks to develop into DP cells, with the extra time providing the opportunity for much more extensive proliferation^{7, 8}.

Despite these kinetic differences, the gene expression patterns at given developmental stages of fetal and adult thymocytes are similar⁹. This similarity extends to the transcription factor genes that are characteristically expressed at each stage (Fig. 1b) as well as to the differentiation genes that these factors regulate. Thus, the transcriptional control of proliferation and of developmental progression is to some extent modular and may depend upon checkpoints to ensure orderly differentiation. This implies that distinct phases of T-cell development are governed not only by key transcription factors, but also by the coordination among such transcription factors, synchronized by gene regulatory network connections.

All the events that establish the T-cell identity of precursors are driven by Notch signaling¹⁰⁻¹³. Notch1 molecules on lymphoid precursors interact with Notch ligands in the thymic microenvironment, leading to activation of the T-cell-specific developmental program. During the first developmental stages, Notch signaling interacts with a “legacy” stem and progenitor-cell gene network inherited from multipotent precursors. Both legacy genes that will play ongoing roles in T cells and progenitor-specific legacy genes with roles confined to the earliest stages participate in this network that we term “phase 1” (Fig. 1b). Although still incompletely understood, the phase 1 network may support the extensive proliferative expansion of the ETP and DN2a cells, as well as impact upon the order, timing, and level of T-cell gene activation. Notch signaling also activates the first T-lineage specific transcription factors by its interaction with the phase 1 network, although the newly induced factors only express full T cell specification activity under the continuing influence of Notch signals in a second phase network.

T-cell specific transcription factors in the phase 2 network mediate commitment-linked functions that drive T-cell specific gene expression and open the TCR gene loci for rearrangement, as well as repress the expression of the progenitor-cell-specific phase 1 genes. The phase 2 network thus creates the distinctive T-cell identity. However, this phase 2 network also must be profoundly modified, once TCR gene rearrangement occurs and enables some cells to express either a pre-TCR (TCR β with invariant pre-TCR α) or a $\gamma\delta$ TCR. The resulting TCR signal transduction, again under the influence of Notch signaling, triggers another regulatory factor network shift, transitioning to phase 3. The phase 3 network includes newly activated genes that support the T cell phenotype but leads to the extinction of the expression of some factors that had played crucial roles in phase 2, reduces Notch target gene expression, and enables the cells to become Notch-independent.

The switching between the three phases must be correctly regulated to ensure the fidelity of T-cell development and to avoid leukemogenesis. Activation of new regulatory genes and repression of those from earlier phases must be accurately coordinated to insure proper passage through the developmental checkpoints. An important question is how homogeneous the precise trajectory of gene expression changes is between the surface receptor-defined stages, and this awaits single-cell transcriptional analysis¹⁴. However, a

strong relationship has recently emerged between the generation of T-cell leukemias and thymocyte precursor competition and self-renewal as well as inappropriate persistence of gene expression from the phase 1 restricted genes of normal T-cell development¹⁵⁻¹⁹. This relationship provides insights into the origins of certain forms of human T-cell acute lymphoblastic leukemias (T-ALL) as well as suggesting the roles of specific stem and progenitor cell genes in normal T-cell developmental regulatory networks.

Thus, a three-phase succession of gene networks processes multipotent precursors into T-lineage committed cells, and sets them on the paths to distinct functional roles. Expression of certain individual transcription factors can extend across the boundaries of these phases, but their functions in each phase are modulated according to their level and the overall gene network state. In this review we explore what these functions are and how they are interlinked.

Major features of early T-cell development: a framework

Progenitor interaction with the thymic environment

T-cell progenitors from bone marrow or fetal liver continuously seed the thymus via the blood. The major types of prethymic cells that appear competent to do this are multipotent precursors: primarily common lymphoid precursors, which express higher levels of the interleukin-7 receptor (IL-7R) and lower levels of the receptor tyrosine kinase Kit (CD117), and lymphoid-primed multipotent precursors, with lower IL-7R expression but expressing high levels of Kit²⁰⁻²³. Whereas the respective contributions of these precursors in vivo is an important question, once in the thymus the ETPs derived from them share a Kit-high, IL-7R-low phenotype and develop into Kit-high, IL-7R-high DN2a cells before commitment. In fact, both of the defining growth factor receptors are crucial for early pro-T cell expansion within the thymus. Both types of thymic immigrants also express the functionally important growth factor receptor Flt3 (FMS-related tyrosine kinase 3; Flk2), as well as Notch1²⁴. The unique thymic environment not only provides supportive cytokines, Kit ligand and IL-7, which maintain and expand the precursors, but it critically provides an environment rich in the Notch ligand, Delta-like 4 (DLL4)⁶. Engagement of Notch1 by environmental Notch ligands triggers proteolytic release of intracellular Notch1, which binds to DNA-bound RBPJ (or CSL) and recruits a transcriptional co-activator complex to activate Notch target genes²⁵.

Stages of early T-cell development

The process of differentiation from thymic entry to expression of a full heterodimeric TCR can be divided coarsely into Notch-dependent and TCR-dependent parts (Fig. 1a). The early cells do not express a TCR, and they proliferate and differentiate in a TCR-independent, Notch-dependent fashion. As soon as TCR gene rearrangement yields its first successful products, cell survival and differentiation become TCR-dependent, and Notch influence is downregulated. For $\alpha\beta$ T-cells, TCR β rearrangement normally occurs in the DN3a stage, with TCR α rearrangement delayed until the DP stage. DP $\alpha\beta$ TCR⁺ cells then await positive selection based on TCR recognition specificity, which is a prerequisite for further differentiation into effector T-cell subsets (such as CD4⁺ T cells, CD8⁺ T cells, natural

killer T cells and regulatory T cells). Rearrangements of both TCR γ and TCR δ , leading to $\gamma\delta$ TCR-expressing cells, take place more synchronously in the DN2 or DN3a stages (Box 1).

A major landmark in the Notch-dependent developmental process is T-lineage commitment, which occurs at the DN2a to DN2b transition (Fig. 1a). Commitment marks the final loss of factors and gene regulatory networks that allow alternative lineage fate choices under permissive conditions^{4, 26} (Box 2). Under normal conditions in the thymus Notch ligands push and retain progenitor cells in the T cell pathway and block alternative fate choices, but during commitment the accumulation of Notch-dependent gene regulatory changes makes these fates inaccessible regardless of the environment. The commitment transition was originally detected with an *Lck*-GFP transgene²⁷ and is marked in non-transgenic animals by a deceptively subtle decrease in Kit expression^{1, 28}. In fact, however, this transition is a major gene regulation upheaval which causes both substantial upregulation of many T-cell genes and the loss of progenitor-specific gene expression. Dendritic-cell, myeloid, NK (ILC1) and probably ILC2 and mast-cell potentials, indicative of sustained diverse and permissive regulatory states, are all terminated here^{4, 26, 29, 30} (Fig. 2). Thus, the Notch-dependent period of T-cell development is divided into two phases (phase 1: pre-commitment and phase 2: T-identity), which are profoundly distinct in their transcription factor gene network states and developmental properties (Fig. 1b).

By the time commitment has occurred (DN2b–DN3a stages), most T-cell identity genes are fully turned on^{31, 32}. Such genes include all of the invariant TCR components and mediators of TCR-dependent signaling, the expression of which prepares the cells for TCR-dependent survival. Proliferation slows in progression to the DN3a stage, and many cells enter G₁ arrest. The cells of this stage then persist until they successfully rearrange TCR genes, or die. Growth and differentiation beyond the β -selection checkpoint at DN3a normally requires expression of a functional TCR, which activates the phase 3 gene network. This occurs when newly made TCR β protein assembles at the cell membrane with TCR complex components that are already expressed, including pre-TCR α (*Ptcr* α gene product), CD3 γ , CD3 δ , CD3 ϵ and TCR ζ . This pre-TCR complex assembly triggers cell enlargement, progression into rapid cell-cycling, termination of CD25 (IL-2RA) expression, and increased surface expression of CD27 (TNFRSF7) and the co-stimulatory molecule CD28, as the cells enter DN3b and then DN4/ISP stages³³⁻³⁷. Although Notch signals are required for passage through the β -selection checkpoint³⁸⁻⁴⁰, cells responding to this “pre-TCR” then free themselves from their Notch-dependence and turn off expression of Notch target genes. A somewhat different pathway is followed by cells that productively rearrange the TCR γ and TCR δ genes in the DN2-DN3 stages; these become one of a variety of TCR $\gamma\delta$ cell types (Box1). They too transition from Notch-dependent to Notch-independent differentiation at this point.

Critical transcription factors in the induction and establishment of T cell regulatory networks

Expression of T-cell differentiation genes and many aspects of mature T-cell function eventually depends on a toolkit of transcription factors that includes TCF-1 (*Tcf7* gene product) and its relative LEF-1, GATA-3, Bcl11b, E2A (*Tcf3* gene product) and HEB (*Tcf12*

gene product), Ikaros (*Ikzf1*, or family members), Myb, Gfi1, and complexes of Runx1 (or family members) with CBF β (Table 1) (see reviews ^{3, 4, 41-47}). The process of T-cell programming, through phase 2 and beyond, must establish gene networks that stably maintain appropriate levels of these factors. However, these are only part of a much larger set of transcription factors that is differentially regulated during the T-cell specification process^{31, 48-50}(Fig. 1b)(Table 1).

The induction of critical T cell specification genes such as *Tcf7*, *Gata3* and *Bcl11b* by thymic Notch ligands occurs in the context of previously established legacy stem/progenitor cell transcriptional networks. These legacy networks are not immediately dismantled and many of these progenitor-specific factors persist through multiple cell cycles and stages within the thymus (Figure 1), even though continued expression of these regulatory genes beyond the ETP and DN2a stages may be oncogenic. Furthermore, some of these progenitor genes are now known to play important roles during the precommitment phase, as described below. Understanding what these “phase 1-restricted factors” contribute to normal T-cell development is an important frontier in T-cell developmental homeostasis.

Phase 1-restricted transcription factors

Because development of each T-cell precursor cohort is coupled with proliferation, the steady-state percentage of thymocytes that are still in precommitment stages is tiny. However, in these early stages TCR-independent proliferation from any given immigrant can be extensive, spanning more than 10 cell cycles before commitment^{7, 51}, and this proliferative expansion could be a major function supported by the legacy progenitor network. Many of the transcription factors that are expressed only in phase 1 ETP and/or DN2a cells have roles in the proliferation, survival, and self-renewal of other hematopoietic cells and leukemias, where the genes that encode them are functionally implicated as proto-oncogenes (see below; Table 2).

From thymic entry to commitment the cells progress through several variants of the phase 1 regulatory state, due to the staggered losses of legacy phase 1 restricted genes and gain of Notch target genes (Fig. 1b; Fig. 3a-c). The most restricted subset of phase 1 regulators, including *Gata2*, *Meis1*, and *Hoxa9*, is expressed solely or predominantly in ETPs. Some of these genes may be co-expressed with Flt3 in a pattern restricted to the most primitive ETPs, as these genes are known to be involved in regulatory networks in Flt3⁺ prethymic progenitors^{52, 53}. The possibilities for their functions in prethymic progenitors are intriguing, but most likely they are important for supporting engraftment in the thymus. *Mef2c* and *Lmo2* are turned off a little later, persisting into the DN2a stage, especially in adult thymocytes, with their protracted period of expansion^{31, 32}. The phase 1 factors *Lmo2*, *Meis1* and N-myc (see below) have a direct connection to “stem-ness”: they have been identified recently as potent components of a transcription factor “cocktail” that can reprogram differentiated blood cells back to hematopoietic stem cells⁵⁴.

The most characteristic group of phase 1 transcription factors is expressed throughout the precommitment stages. These include *Spi1* (PU.1, also called *Sfp1*), *Hhex*, *Bcl11a*, *Gfi1b*, *Erg*, *Mycn* (N-myc) and *Lyl1* (and its relative *Tal1*, expressed at much lower levels)(Figure 1b; Table 2). Most of these genes are repressed partially or completely during the transition

to phase 2 (between DN2a and DN2b), although *Lyl1* is only silenced in the DN3a stage²⁸, and *Erg* only during β -selection^{31, 32}.

Bcl11a is crucial for normal lymphoid progenitor proliferative expansion in both fetal liver and bone marrow⁵⁵. Similarly, although the basic helix–loop–helix (bHLH) factor *Lyl1* plays its best-known role in hematopoietic progenitors and B-cells^{56, 57}, deletion of *Lyl1* severely impairs development of lymphoid precursors in bone marrow and specifically reduces ETP expansion, progression to DN2, and early T-cell survival^{56, 58, 59}. Lyl1, working in dimers with E2A or HEB, probably helps to maintain expression of the crucial ETP–DN2 growth factor receptor Kit, similar to the function of the related protein SCL (*Tal1*), and promotes expression of *Gfi1*, a regulatory factor required for normal generation of ETPs and for proper responses to Notch signaling^{58, 60, 61}. *Erg* and *Hhex* are implicated in early T-cell self-renewal in both normal and malignant contexts^{62, 63}. Thus, despite their transient expression, these factors should be regarded as integral parts of the T-lineage program.

PU.1: linking phase 1 and developmental options

PU.1 is normally associated with differentiation of non-T cells, i.e. dendritic, myeloid, and B-cells⁶⁴, and its expression correlates very well with the ability of uncommitted T-cell precursors to divert to dendritic cell and myeloid fates when Notch signaling is withdrawn (Fig. 2). However, loss of PU.1 from prethymic or DN1 stages also causes an arrest in T-cell development⁶⁵. PU.1 is important for expression of *Flt3* in immigrants, and it probably sustains the pro-survival actions of *Bcl11a* in developing thymocytes until they reach commitment (phase 2), as well as regulating other phase 1 regulatory genes⁶⁶. PU.1 is a highly stable protein and can act as a pioneer factor in some contexts⁶⁷⁻⁷⁰. It binds to most enhancers and promoters active in ETP and DN2a cells *in vivo*³¹. Thus, it may in part shape the epigenetic landscape in these hematopoietic progenitors in ways that affect the activities of many other factors. It may also play a more intimate role in controlling the T-cell program, via network interactions with Notch and T-cell commitment genes including *Tcf7*, *Myb* and *Gata3*⁶⁶. PU.1 can dampen the intensity of Notch signaling; this contributes to its ability to restrain the induction of many T-cell identity genes as long as it is expressed⁶⁶ (A. Champhekar & E.V.R., unpublished results). Because this Notch antagonism is graded and not absolute, PU.1 may itself contribute to the proliferative expansion of T-cell precursor clones by delaying the onset of commitment, as described below.

Notch target genes in early T cell precursors

Notch signaling activates distinct target genes in disparate developmental contexts, and even in DN thymocytes it activates a succession of different target genes at different stages. DN thymocytes are primed for responsiveness to intrathymic Delta ligands by expression of Fringe proteins encoded by *Lfng*, *Mfng* and *Rfng*⁷¹. Notch signaling tightly controls the expression of genes encoding the canonical DN2 marker CD25 (*Il2ra*), the ubiquitination adaptor Deltex1 (*Dtx1*), pre-TCR α (*Ptcra*), the ankyrin repeat protein Nrarp, Notch3, and to some extent Notch1 itself, as well as the transcription factors *Myc* and *Hes1*, all of which have different patterns of expression in the DN stages^{39, 72, 73}. Notch signaling also strictly controls activation of a distinct promoter for *Tcf12* (HEB) that drives the expression of a

truncated protein, HEBalt, which appears to be more supportive of cell proliferation than canonical HEB or E2A^{46, 74}.

Hes1 and *Nrarp* are among the earliest Notch-activated target genes induced in ETPs. *Hes1* is a bHLH transcriptional repressor that is needed for early T-cell population expansion, and may also antagonize myeloid cell development⁷⁵⁻⁷⁷. *Nrarp* is an adaptor protein that can act as a substantial negative feedback regulator of Notch signaling itself^{78, 79}. Whereas *Hes1* expression continues from ETP through the DN3a stage, *Nrarp* is shut off at commitment. Thus in phase 1, Notch signaling is self-limiting and exacerbates the Notch-damping effect of PU.1. Both restraints are relieved, however, when the cells undergo commitment. The later-activated Notch target genes may thus be awaiting an increase in Notch signal strength for their induction as well as other inputs.

Building infrastructure for the Phase 2 network

The regulatory bridge to the phase 2 gene network is built from within the phase 1 network. Notch signaling in ETP cells initiates expression of the crucial regulatory genes *Gata3* and *Tcf7* (encoding TCF-1), which then transform the developmental status of the cells (Fig. 3a, b). Even in the context of the phase 1 developmental network, these two genes begin to exert T-lineage promoting functions as soon as they are expressed, as knockouts of either gene severely reduce the survival and differentiation of ETP cells as well as their descendants^{80, 81}. They not only begin to antagonize the progenitor-specific, phase 1-restricted factors (Fig. 3b), but also collaborate with the regulatory genes needed for T cell development that were also part of the legacy set, expressed from a prethymic stage (*Myb*, *Runx1/CBF β* , *Ikaros*, *Gfi1*, *E2A*). A particularly important member of this group of inherited factors is the basic helix-loop-helix factor *E2A*, which is expressed at relatively constant levels throughout T-cell specification, but has its activity substantially modulated by availability of different heterodimerization partners^{45, 46, 58, 82-86}. Despite their stable expression, all these progenitor-inherited factors can also act in developmentally modulated ways, and they become incorporated with TCF-1 and GATA-3 into the network for T-cell lineage commitment.

Activation and function of TCF-1

Tcf7 is activated by Notch directly^{81, 87}, but once activated, its expression is no longer severely affected by Notch signal inhibition^{29, 66}. This suggests that it may become self-sustaining by direct or indirect positive feedback (Fig. 3c). In ETPs, TCF-1 drives expression of a suite of genes needed for survival and expansion⁸¹. Some fetal T-cells can develop without it^{88, 89}, but TCF-1 becomes increasingly important in postnatal waves of T-cell precursors⁹⁰. TCF-1 primarily acts as a crucial positive regulator of T-cell specification, collaborating with Notch to activate T-cell genes through a feed-forward network circuit. Although TCF-1 cannot duplicate all effects of Notch signaling, high amounts of a full-length isoform of TCF-1 can activate many T-cell genes including *Gata3*, *Bcl11b*, *Il2ra* (CD25), *Cd3g*, *Lat*, *Lck* and endogenous *Tcf7*, even without Notch signals⁸⁷.

It is still controversial whether TCF-1 acts together with β -catenin in these positive regulatory roles^{87, 91}. Although it can mediate canonical Wnt signaling via the formation of

a complex with β -catenin, TCF-1 may operate in a different mode in ETP and DN2a cells. At a later stage TCF-1 can directly or indirectly help to repress Id2, a potent E2A antagonist that can otherwise be induced readily in T-cell precursors⁸⁹. Certain natural isoforms of TCF-1 also repress or delay activation of the related TCF-1 gene, *Lef1*, which can otherwise become oncogenic^{88, 89}. Finally, as a highly abundant transcription factor in developing T-cells, TCF-1 may also play an architectural role at cis-regulatory elements across the genome⁹²; its high-mobility group DNA binding domain can bend the DNA to facilitate contacts among other transcription factors⁹³.

In prethymic cells the gene encoding TCF-1 (*Tcf7*) begins with substantial CpG methylation on some of its putative regulatory elements⁹⁴, but its promoter and major enhancer become fully active during ETP stage³¹, and CpG methylation is removed from *Tcf7* by the time cells reach DN2 and DN3 stages⁹⁴. This may reflect the action of Notch/RBPJ(CSL) complexes at the enhancer, in addition to direct and indirect positive feedback signals from TCF-1 and GATA-3^{81, 87}. Initially, Notch signals remain important to protect *Tcf7* expression against a variety of experimental regulatory perturbations ranging from excessive PU.1 activity⁶⁶ to excessive GATA-3 activity²⁹. However, in later stages of T-cell development (phase 3 and beyond), *Tcf7* can be expressed strongly in a Notch-independent way, in a pattern often correlated with levels of GATA-3.

GATA-3 functions and regulation

GATA-3 has vital dose-dependent roles in early T-cell survival, growth, specification, and commitment, as well as continuing roles in T-cell subset diversification after TCR expression and antigen stimulation^{95, 96}. It plays a key non-redundant role in excluding the B-cell fate⁹⁷, and probably explains why B-cell potential is lost within the ETP stage^{24, 98, 99}. It is required for full activation of *Bcl11b* in DN2a-DN2b stage and to prime DN3 cells for β -selection, and may also upregulate *Ets1* and repress *Spi1* in DN2b stage^{29, 97, 100}(D. D. Scripture-Adams, S. Damle, and E.V.R., unpublished)(Fig. 3c-e). *Gata3* expression is activated by a mechanism involving a far downstream enhancer¹⁰¹ and by removal of H3K27me3 repression marks from its promoter^{102, 103}, although the sequence(s) through which it is activated by Notch in ETP cells have not been identified. Part of the regulation of *Gata3* could be mediated indirectly, e.g. by TCF-1. Although GATA-3 levels can be maintained when Notch signals are removed, Notch signaling is needed to enable *Gata3* to be expressed in the phase 1 context of high PU.1 activity⁶⁶.

GATA-3 must be tightly regulated at several levels. First, the negative effect of GATA3 overexpression on T-cell development is as severe as loss of this transcription factor^{29, 104}. In precommitment stages, *Gata3* expression can be restrained by phase 1 transcription factors PU.1 and Gfi1b^{66, 105} (Fig. 3a, b). During and after commitment, surprisingly, it is restrained by other T-cell specification regulators including E2A and Bcl11b¹⁰⁴(Zhang, J.A., Li, L., and E.V.R., unpublished data)(Fig. 3d, e). Second, GATA-3 is subject to considerable post-transcriptional regulation by signaling pathways that are downstream of many growth factor and activation signals, including Akt pathway regulation of its translation and p38 MAP kinase regulation of its nuclear localization and activity¹⁰⁶⁻¹⁰⁸. Thus, GATA-3 activity levels in a cell integrate responses to environmental signals with intrinsic transcriptional

programming. Third, the sites that GATA-3 binds across the genome are distinct at different stages of T cell development, even at matched levels of GATA3 expression. Major redistributions of GATA-3 binding are seen between successive DN and DP thymocyte stages, as well as between thymocytes and mature T cells^{31, 109}. This is not because GATA-3 is particularly excluded by repressed (H3K27me3-marked) chromatin sites, but because of shifting preferences among potential open sites. The implication is that GATA-3 “assignments” depend on interactions with changing stage-dependent collaborating factors. Thus, as a member of a regulatory ensemble, this factor may contribute in different ways in each of the three phases from ETP to DP stage.

Phase 2 - commitment

Transition to phase 2

Several cell cycles after TCF-1 and GATA-3 are activated, cells transition to the DN2b stage, when proliferation rates slow, survival becomes strictly Notch signal-dependent, and Kit expression declines²⁸. *Bcl11b* expression is activated and most phase 1 genes are silenced (Fig. 3c-d). As the cells progress through the DN2b stage there are major shifts in the expression of Ets family transcription factor genes: *Ets1* and *Ets2* are sharply turned on as *Spi1* is turned off⁴⁸. *Lef1* is induced in a highly Notch-dependent way^{29, 66}, adding a partially redundant transcriptional regulation activity to that of its relative *Tcf7*^{88, 89, 110}.

During *in vitro* differentiation, DN2b cells can continue to proliferate, but *in vivo* these transformations presage a faster shift to the DN3a stage. The cells become desensitized to IL-7R signaling, in a process dependent on E proteins¹¹¹. E2A/HEB activity is probably boosted by the silencing of the competitor dimer partner *Lyl1*^{28, 112}. E protein and Notch-dependent gene expression soars (e.g., *Rag1*, *Rag2*, *Ptcra*, *Dntt*, *Cd3e*)^{72, 83, 104, 113-115}, and as proliferation slows, RAG-mediated TCR gene recombination intensifies (Fig. 3e).

Bcl11b expression is necessary for many if not all of these changes, and the timing of *Bcl11b* activation is crucial for lineage commitment¹¹⁶⁻¹¹⁸. *Bcl11b*, a six zinc-finger transcription factor with functions including corepressor activity¹¹⁹, is turned on in late DN2a. It may repress *Kit* directly, thus creating the DN2b phenotype; virtually all DN2b cells express high levels of *Bcl11b* (H. Y. Kueh and E.V.R., unpublished results). Both the promoter and a far-distant enhancer appear to participate in *Bcl11b* activation and to contain binding sites for Notch, TCF-1, Runx/CBF β , and possibly GATA-3¹²⁰, all of which are positive regulators for *Bcl11b* expression^{87, 97, 118, 121}. These known positive regulators are expressed before *Bcl11b* is turned on, but their combined levels may need to exceed a threshold in order to remove initial DNA methylation and H3K27me3 marks from both the promoter and the enhancer of the *Bcl11b* gene^{94, 120}.

Commitment: end of progenitor status and other options

The commitment transition from DN2a to DN2b marks the end of a process of exclusion of access to different alternative fates (Figure 2). The completion of commitment in DN2b stage terminates access to “innate lymphocyte” (NK or ILC1 and ILC2) development^{28, 30}, while perhaps surprisingly, potential for non-lymphoid pathways to dendritic cell, granulocyte, macrophage and possibly mast-cell development is only extinguished at this

point as well (rev. in ²⁶). At least three kinds of molecular mechanisms implement different aspects of alternative lineage exclusion, including the repression of PU.1 (Box 2).

The silencing of PU.1 has an obvious link to commitment, but commitment also silences many other phase 1 factors such as Gfi1b, Hhex, Bcl11a, Mycn, Lmo2, and Mef2c, which have less clear roles in promoting alternative cell fates. Even in some leukemias where these factors are aberrantly expressed (see below), they immortalize pro-T cells without obscuring their T-lineage identity. This implies that T-cell commitment excludes a progenitor-like form of self-renewal as well as specific alternative differentiated fates. Commitment mechanisms that silence phase 1 genes are thus most likely to underlie the cell growth control alteration that intensifies Notch dependence, slows proliferation, and finally brings proliferation under strict TCR dependence²⁸.

The phase 1-specific regulatory genes that are silenced after DN2a show diverse repression patterns. The levels of positive enhancer and promoter marks [H3K4me2, H3K(9,14)Ac] on these genes are reduced as transcription decreases, but H3K27me3 repression marks generally only begin to appear later (if at all), often not until the DP stage^{31, 103}. Bcl11b is required for silencing other phase 1 genes during commitment, including *Lyl1*, *Hhex*, *Bcl11a*, and possibly *Spi1* (PU.1), but it is not yet clear whether Bcl11b directly acts on them as a repressor (J. A. Zhang, L. Li and E.V.R., unpublished data)^{116-118, 122}. Thus, a variety of negative regulatory mechanisms ensure that phase 1 proto-oncogenes are silent by the time cells reach the β -selection checkpoint.

DN3a stage: phase 2 beyond commitment

DN3a stage cells are committed, $\alpha\beta$ lineage-biased, and primed for TCR gene rearrangement based on a climax of phase 2 regulatory activity. To attain and survive in this stage, cells need Runx1/CBF β , Notch signals, GATA-3, Gfi1, Bcl11b, and E2A; Myb and Ets1 also play roles in maintaining viability by this stage if not earlier (Fig. 3e). CHIP-seq analysis shows that GATA-3 binding is now redeployed from its phase 1 network targets to a new set of target genes³¹. Some transcription factors, including Ahr and SpiB, are transiently expressed only during this DN3a stage (Fig. 1b), but their functions at this point are unknown. The maximal expression of the *Cd3* gene cluster is regulated by E proteins, GATA-3, and TCF-1; the *Rag* genes are induced by E proteins and GATA-3; and *Hes1*, *Notch3*, and *Ptcra* are jointly regulated by E proteins and Notch^{31, 83, 104, 123, 124}.

Involvement of E proteins is a hallmark of gene expression in DN3a cells in particular, and E proteins, Notch signaling, and Bcl11b may have a particularly tight collaboration. E proteins are needed to promote and sustain Notch1 expression in DN3a cells, and Notch signaling can be antagonized by E protein antagonist Id2^{66, 125}. Bcl11b may thus support the DN3a state by keeping *Id2* expression silent^{117, 118, 122} (Fig. 3d, e). Change in $\alpha\beta$ versus $\gamma\delta$ T-cell potential between the DN2 and DN3a stages could also be related to their differential needs for Bcl11b and Notch, as well as the strong activation of Id3 or Id2 factors linked to selection of most $\gamma\delta$ T-lineages¹²⁶. The Notch/E protein target gene *Ptcra* is very poorly activated in Bcl11b-knockout cells. $\alpha\beta$ T-cells are accordingly disproportionately affected by Bcl11b deletion^{127, 128}, whereas several $\gamma\delta$ T cell lineages, especially fetal lineages and lineages that emerge early from the DN2 stage, are much less affected^{117, 122, 129, 130}. Thus,

special features of the DN3a stage may set up the conditions for selection processes specific for the $\alpha\beta$ T cell lineage.

Phase 3: Crossing the β -selection checkpoint

Construction of the checkpoint

The proliferation and differentiation checkpoint imposed at DN3a is enforced by E2A and Ikaros^{84, 131, 132}. There are several clues from target gene analyses that suggest why proliferation stops at this point. E2A may slow cell cycling by inducing Cdkn1a¹²³ (coexpressed with Cdkn2d). Other features of the cells suggest that they are progressively starved for growth signals. As they lose support from IL-7R signaling, the cells upregulate a PI3-kinase inhibitor, Pik3ip1, and a growth-suppressive kinase, Dapk1, in response to E2A¹⁰⁴, and these may make the cells dependent on ever-stronger viability support signals from Notch and eventually from pre-TCR¹³³. Ikaros, although stably expressed from the earliest stages, also has a vital, dose-dependent role in enforcing the β -selection checkpoint^{132, 134}. The complexes Ikaros forms are pivotal for patterns of nucleosome remodeling activity deployment across the genome¹³⁵. How exactly Ikaros contributes to cell cycle arrest and TCR gene rearrangement is still unclear, but it acts strongly to suppress Notch response genes rapidly once pre-TCR signals are received¹³⁶⁻¹³⁸, and this is crucial to block leukemogenesis.

β -selection

Signaling through the newly expressed pre-TCR and passage through the β -selection checkpoint disrupt the quiescence of DN3a-stage $\alpha\beta$ T-cell precursors. Expression of both Notch target genes and *Il7r* is shut off as they move to DN3b and beyond (Fig. 1; Fig. 3f)³⁴. Despite the downregulation of these growth-supporting systems, the cells shift into very rapid proliferation, as the cells become highly responsive to chemokine signaling through the receptor Cxcr4^{139, 140}. Proliferation not only expands the potential TCR repertoire pool, it also helps $\alpha\beta$ T-cells confirm their lineage commitment by allowing the cells to dilute out the last vestiges of earlier regulatory molecules and to reset epigenetic marks³⁷.

Creating the new DP state, the expression of *Ets1*, *Tcf7* (TCF-1), and *Lef1* further increases, whereas that of *Ets2* and *Tcf12* (canonical HEB) reaches new peaks. Several new transcription factors are turned on during β -selection, including *Ror γ t*, *Nfatc3*, *Pou6f1*, and *Aiolos* (*Ikzf3*), while some like *Egr2* and *Id3* are transiently activated in response to the TCR signal during the rapidly proliferating DN4 stages (Fig. 1). Although E protein activity is transiently antagonized by this temporal increase in *Id3* expression, E protein dominance is subsequently restored¹⁴¹. ROR γ t expression in thymocytes is highly specific for DP cells, where it works in a different gene network context than it does in mature Th17 cells^{142, 143}. In DP cells, it works with Myb to promote viability through *BclxL* induction while suppressing conventional effector responses like proliferation or cytokine production¹⁴⁴⁻¹⁴⁶. The roles of TCF-1 and LEF-1 may be qualitatively altered at this point by a stage-specific interaction with the canonical Wnt pathway mediator β -catenin, which is more strongly implicated in effects at β -selection than in earlier stages^{88, 147-150}. This altered regulatory factor ensemble turns on the expression of CD4, CD8, CD2 (in mice), and CD28.

However, arguably the most dramatic feature of the change in the developmental gene network at β -selection is repression. Any residual expression of phase 1 genes vanishes along with DN3-specific regulatory genes like *SpiB* and *Ahr* and the Notch-dependent genes. The whole Notch-dependent regulatory framework is dismantled like a redundant assembly scaffold (Fig. 3f). The broad downregulation of multiple Notch targets may result from repression of *Lfn3*, which disables Notch on DP cells from access to its intrathymic ligands⁷¹. As cells become DP, H3K27me3 repression marks pile up at the promoters of phase 1 genes and Notch targets such as *Hes1*^{31, 103}. The resultant new regulatory state prepares DP thymocytes for the complex positive and negative selection events that they must undergo.

Although relatively few “new” factors enter the regulatory mix, the β -selection cascade is dangerous because of the way new growth signals impinge on a rapidly altering regulatory state. Cells with inadequate E protein or Ikaros activity cannot control access to this transition or re-establish quiescence correctly, and they become malignantly transformed. Notch target genes must be silenced: inappropriately prolonged Notch activity, in the context of TCR complex activation during β -selection, tilts the cells toward leukemia. Furthermore, as described below, any persistence of phase 1 gene expression through this transition can itself lead to leukemic transformation.

T-cell leukemia

Checkpoint failure and T-cell leukemias

The earliest stages of early T-cell development are replete with potent proto-oncogenes (Fig. 1b; Table 2) inherited from pre-thymic progenitors. Many of these genes encode factors that regulate proliferation, survival and differentiation in the earliest stages of T-cell development in conjunction with the Notch-mediated T-cell specification program, although termination of this progenitor program is imperative for both completing T-cell specification and avoiding leukemogenesis. Overexpression of individual phase 1 transcription factors in mouse hematopoietic stem and progenitor cells commonly lead to self-renewal and transformation, with the specific leukemia type dependent upon the cell in which it is first overexpressed (Table 2). In addition, recent data show that if early thymic cells are not displaced from their intrathymic niches by new immigrants, they retain self-renewal potential coupled with a threat of leukemic transformation¹⁵⁻¹⁷.

Repression of legacy proto-oncogenes occurs at several developmental points (Figure 1). While a subset of these genes terminates expression within the ETP stage, others are expressed through to the commitment checkpoint, after which time most of these phase 1-specific genes are repressed. By the time cells transit through the β -selection checkpoint, remnants of the stem/progenitor program are fully and permanently extinguished. Epigenetic mechanisms likely play important roles in establishing and maintaining repression of the progenitor gene programs, although the process needs clarification and likely varies between stages and specific genes. For example, progenitor genes that continue to be expressed until commitment are generally not strongly marked by H3K27me3 until after β -selection, even though most are repressed in the DN2 stage³¹. These results hint that proliferation may be needed for establishment of repressive chromatin.

T-ALLs in humans are a heterogeneous group of aggressive T-cell malignancies that are thought to arise from oncogenic transformation of immature thymocytes¹⁵¹. These leukemias can result from mutation and/or uncontrolled activation of a T-cell proto-oncogene, such as Notch, or from translocation of an exogenous oncogene to the control of T-cell specific promoters or enhancers like those of a TCR locus or *BCL11B*. Sub-types of human T-ALL, with differing treatment responses and relapse rates, have been identified based on surface phenotypes and genetic signatures, including overexpression of specific transcription factors and surface receptors characteristic of different stages of the T-cell differentiation program¹⁵²⁻¹⁵⁶. While these genetic signatures provide hints as to the primary and cooperating genetic lesions in the multi-step process that drives malignant transformation, manipulatable mouse models are invaluable for investigating the sequence of genetic lesions that can initiate and sustain T-ALLs. Although some markers of T-cell development differ between humans and mice^{157, 158}, the broader requirements for Notch and cytokines, as well as underlying transcriptional networks are very similar¹⁵⁹, making results with mouse models relevant to human T-ALL.

Failure to transit correctly through the β -selection checkpoint due to sustained Notch signaling or loss of E proteins or Ikaros has long been known to result in malignant transformation of early T-cells^{134, 160-162}. Constitutively activated Notch signaling is particularly potent at triggering T-cell leukemia in both humans and mice, with over 50% of human T-ALL cases exhibiting activating mutations in *NOTCH1* or its pathway^{151, 160, 163}. However, in both mice and humans distinct “early” subtypes of T-ALL exist, with characteristics of very immature T-cells, and these may arise from a failure to permanently repress legacy phase 1 stem and progenitor genes, which have only recently been recognized to be a major part of early T-cell differentiation¹⁵⁶.

High expression of phase 1 genes *LYL1* and *LMO2* was originally found to distinguish subtypes of human T-ALLs that are associated with higher rates of treatment failure and relapse¹⁵³. Subsequently, a T-ALL subtype was designated as ETP T-ALL (ETP-ALL), based on overexpression of genes expressed by murine thymic ETP cells, including *KIT*, *GATA2*, *CEBPA*, *SPI1 (PU.1)*, *LYL1*, and *ERG*^{1, 18}. ETP-ALLs also express myeloid surface markers and have lower levels of typical T-cell genes. Genome-wide sequencing of human ETP-ALLs showed that they commonly have activating mutations in cytokine receptor genes (*FLT3*, *IL7R*) and genes encoding members of RAS signaling pathways, as well as inactivating mutations in some important T-cell genes and specific histone modifying genes, and that they resemble myeloid stem cells and acute myeloid leukemia more than other forms of T-ALL¹⁹. Thus, the genetic characteristics of ETP-ALL, including the genes shared with myeloid progenitors, indicate a failure to properly terminate the normal phase 1 regulatory state, despite the onset of the T-cell specification program. As in murine early T cells, the human T-cell development program could be built on a proto-myeloid foundation¹⁶⁴.

Specific genes involved in epigenetic silencing are mutated in many ETP-ALL, especially genes *EZH2*, *SUZ12*, and *EED*¹⁹, which are part of the Polycomb Repressive Complex 2 (PRC2) that mediates H3K27 trimethylation. This further supports a role for these pathways in extinguishing expression of stem/progenitor phase 1 genes during normal commitment, as

implied by repression marking patterns in mice³¹. Loss-of-function mutations in components of PRC2 are found in other kinds of T-ALLs as well, however, suggesting more diverse stage-specific roles in normal T-cell development and tumor suppression¹⁶⁵. Other genes involved in establishing gene repression, such as the DNA methyltransferase *DNMT3A*, are also mutated in some subsets of T-ALL¹⁶⁶, although the role of this gene and other epigenetic factors in early T-cell development and leukemia initiation in mice and humans have yet to be determined.

Three mouse models provide additional insights into the mechanisms of leukemogenesis due to loss of phase 1-specific transcription factor gene repression. First, when overexpressed in mouse DN thymocytes, the ETP-specific gene *Lmo2* generates a self-renewing population of Kit^{hi} DN thymocytes, and the mice develop T-cell leukemias after a long latency period^{63, 167}. While the frank leukemia probably depends on accumulating additional mutations, the preleukemic self-renewing cells already express other phase 1 genes including *Hhex* and *Lyl1*, which play cooperative roles with *Lmo2* in self-renewal and leukemogenesis^{59, 63}. Although most T-ALLs also carry activating Notch pathway mutations, it is noteworthy that ETP-ALLs need not do so. T-lineage expression of an *Lmo2* transgene induces T-ALL in mice by two distinct pathways: one requires Notch activation but the other, ETP-ALL type, requires only the sustained expression of classic phase 1 genes *Lmo2*, *Lyl1*, *Hhex*, and *Mycn*¹⁵⁵.

Second, early T cells from *Cdkn2a*^{-/-} mice could be transformed by retroviral transduction with *Il7r* carrying constitutively activating mutations identified from human ETP-ALLs. The cells generated transplantable leukemias resembling human ETP-ALL¹⁶⁸, in which myeloid potential was combined with an early T-cell phenotype. These mutations blocked cell differentiation at an uncommitted DN2a-like stage, and the leukemic cells themselves showed high levels of *Lmo2*, low to undetectable levels of *Bcl11b*, and most did not have activating Notch mutations. This mouse model shows that abnormal cytokine receptor signals can prevent or reverse *Lmo2* silencing, *Bcl11b* upregulation and T lineage commitment and that this can efficiently lead to transformation.

Third, Rag-deficient NOD mice also exhibit spontaneous emergence of an aberrant self-renewing thymocyte population from the DN2 stage. These mice also develop T-cell leukemias with high penetrance, which share some gene expression features with human ETP-ALL^{169, 170}. These abnormal thymocytes emerge from the DN2 stage as Kit^{hi} cells that fail to repress many of the phase 1-specific genes including *Lmo2*, *Lyl1*, *Hhex*, and *Mef2c*. These emerging cells also continue some aspects of the T-cell program by activating genes characteristic of committed DN3 cells, like *Ptcra*, and *Spib*. However, they bypass the β -selection checkpoint even without TCR gene rearrangements and aberrantly turn on *Cd4*, *Cd8*, and *Cd5*. Thus, this model provides additional evidence that failure to repress the stem/progenitor program at the commitment checkpoint can initiate T-cell transformation. Furthermore, transformation at this checkpoint does not necessarily preclude aberrant activation of some aspects of the T-cell differentiation program.

Taken together, these studies of mouse and human T-ALL suggest that a breakdown of repressive mechanisms normally enforced at the commitment and β -selection checkpoints

can lead to specific forms of T-ALL. A more complete understanding of epigenetic and checkpoint control mechanisms in normal T-cell development may shed light on leukemia initiation and subsequent transformational events leading to generation of leukemia stem cells.

Conclusions

The T cell commitment program is not a simple cumulative progression toward endowment of T-cell character, but rather a series of distinct regulatory phases with different, sometimes mutually antagonistic features (Figure 3). An initial phase of proliferative expansion in which differentiation is actively deferred is important to establish an adequate pool of cells, and it is this phase 1 developmental program that is “tuned” to promote varying numbers of cell divisions according to the needs of fetal and postnatal T-cell development. At least some of the stem- and progenitor-cell factors needed to sustain this phase are incompatible with T-lineage commitment and must be repressed. The lasting features of the commitment process include the irreversibility of silencing of phase 1 factors. The commitment process establishes a growth-limited state dominated by different transcription factors, at least one of which, Bcl11b, is only turned on at commitment. Other features (like intensified Notch signaling) are normally temporary, until cells acquire a pre-TCR or $\gamma\delta$ TCR, allowing them to exit the commitment process and pass into a third phase. The effective termination of each phase as the cells transition to the next can be viewed as the cells’ vital bulwark against malignant transformation. Failure to terminate the phase 2 program as the cells pass through β -selection, i.e. by continuing Notch activity, has long been recognized to contribute to T-ALL. It is now clear that particularly severe forms of T-ALL are those in which the phase 1–2 transition is porous or too easily re-crossed. Thus, the early lineage commitment events, occurring in only a tiny fraction of cells in the steady-state thymus at any given time, not only offer an illuminating paradigm for developmental fate determination but also take on extreme significance for the fate of the organism as a whole.

Acknowledgments

We apologize to colleagues whose work helped to inspire this review but could not be cited due to space constraints. We thank present and former members of our group whose published and unpublished data, as well as helpful discussion, shaped the ideas presented here. The authors gratefully acknowledge support from NIH grants AI064590 to M.A.Y., AI083514, AI095943, and HD076915 to E.V.R., and the Albert Billings Ruddock Professorship.

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Key Terms

Commitment	Stage when cells give up any intrinsic capability to give rise to more than one kind of descendant. This concept depends on recognizing that precursor cells often begin with intrinsic potential to give rise to a variety of different types of descendants. In embryology and hematopoiesis, the actual choices of fates such precursor cells adopt will be different, depending on signals they receive from the environment. Commitment is the developmental transition within a given pathway during which the cells' choice of fate becomes intrinsically irreversible, independent of the environment
Notch signalling	A signalling system comprising highly conserved transmembrane receptors that regulate cell-fate choice in the development of many cell lineages, and so are vital in the regulation of embryonic differentiation and development. Unusually among signaling systems, the cytoplasmic domain of each Notch transmembrane protein can itself become a transcriptional coactivator in the nucleus, because it is proteolytically cleaved from the transmembrane region when Notch interacts with its ligands of the Delta or Jagged family
T-cell acute lymphoblastic leukemias	Leukemias with an immature T cell phenotype
Common Lymphoid Progenitor	(CLP). A progenitor cell type that appears to be committed to lymphoid fates as measured by <i>in vivo</i> transfer, and that can give rise to all lymphocyte cell types, including T cells, B cells and natural killer cells
Lymphoid-primed Multipotent Precursor	(LMPP) A multilineage precursor cell type that can generate myeloid as well as lymphoid descendants <i>in vivo</i> and <i>in vitro</i> , but not erythroid or megakaryocytic cells
Positive selection	A step in the process of T-cell differentiation in the thymus that selects CD4 ⁺ CD8 ⁺ double-positive T cells for survival and maturation, based on the appropriate degree of interaction between their T-cell receptor and peptide–MHC complexes on thymic epithelial cells. Depending on the class of MHC molecule recognized, thymocytes are positively selected either to a CD4 ⁺ or to a CD8 ⁺ single-positive cell fate
Gene regulatory network	A system of relationships among a set of regulatory factor coding genes and the transcription factors that are their products, defined and understood such that the interactions between them explain the stability or change in the developmental properties of a cell type that expresses these genes

Pioneer Factor	A transcription factor that has the ability to bind to its target site even when the site is initially engaged in nucleosome-packed chromatin, serving as a focal point for recruitment of other transcription factors; crucial for the multi-step process needed to activate some positive regulatory elements in the genome during development
WNT	A signalling mediator named both for its mutant phenotype in <i>Drosophila melanogaster</i> (Wingless) and for its role as a preferential retrovirus integration site in murine leukaemia virus-induced leukaemias (Int-1). WNT signalling activates the TCF1 and LEF1 family transcription factors through stabilizing their co-activator, β -catenin, and mobilizing it from the cytoplasm to the nucleus
ChIP-seq analysis	A genome-wide method of mapping the sites where a transcription factor is binding to the DNA in a cell, based on cross-linking proteins to chromatin, immune-precipitating the chromatin with antibodies to the factor of interest, comprehensively sequencing the DNA that is recovered in the immune precipitates, and aligning it to the genome to identify the enriched regions
Non-obese diabetic (NOD) mice	NOD mice spontaneously develop type 1 diabetes mellitus as a result of autoreactive T-cell-mediated destruction of pancreatic beta-islet cells

Box 1 **$\gamma\delta$ T-Cell Development Overview**

Two classes of T cells expressing distinct TCR dimers, $\alpha\beta$ and $\gamma\delta$, have been maintained in all jawed vertebrate taxa since TCR-expressing cells first evolved¹⁷¹. $\gamma\delta$ T-cells share common intrathymic precursors with $\alpha\beta$ T-cells, but undergo different differentiation programs following TCR expression¹⁷². Successful assembly of a $\gamma\delta$ TCR triggers signals that lead to down-regulation of CD25; however, $\gamma\delta$ TCR⁺ cells proliferate much less than pre-TCR⁺ cells and do not turn on CD4 or CD8. Specific characteristics of individual precursor cells at different stages may bias them towards the $\gamma\delta$ - or $\alpha\beta$ -lineage. DN2 cells in general, particularly those with high levels of IL7R and/or Sox13, can frequently adopt a $\gamma\delta$ fate, but individual DN3a cells are more likely to produce $\alpha\beta$ T-cells^{38, 173-175} (Fig. 2). Interplay of Notch signals with signals from the newly expressed pre-TCR or $\gamma\delta$ TCR is important for $\alpha\beta$ vs. $\gamma\delta$ divergence, although the outcomes of this interaction differ between mouse and human¹⁷⁶. A $\gamma\delta$ TCR can produce a stronger signal than a pre-TCR, leading to more intense upregulation of *Id3* and *Erg*, and initiation of a $\gamma\delta$ rather than an $\alpha\beta$ T-cell program^{177, 178}.

Interestingly, $\gamma\delta$ TCR signaling triggers one of several different genetic programs available for $\gamma\delta$ TCR-expressing cells, differentially depending upon *Id3*, *Sox13*, *Zbtb16* (encoding PLZF), and/or *Bcl11b* for further development and effector functions^{127, 130, 174, 179-181}. The range of accessible $\gamma\delta$ -subtype fates narrows with differentiation from DN2a to DN3a¹³⁰. These $\gamma\delta$ -lineage-specific programs mirror the range of Th1, Th2, NKT and Th17 effector programs that $\alpha\beta$ T-cells acquire at later stages, either after positive selection or in the periphery. However, $\gamma\delta$ T-cells can implement specific effector programs directly upon first TCR expression and signaling within the thymus. Unlike $\alpha\beta$ T-cells, these functions correlate with particular TCR γ and δ segment usages¹⁷⁹(see reviews^{172, 182, 183}).

Box 2**Mechanisms of Alternative Lineage Exclusion**

CLPs and LMPPs retain the potential to generate diverse lymphocyte types, as well as natural killer, dendritic, and myeloid cells under somewhat different ranges of permissive conditions^{21-23, 52}. Contact with the thymic environment efficiently blocks the cells' access to these non-T cell lineage potentials through a variety of mechanisms, however, and under normal conditions the overwhelming majority of precursors only generate T cells in situ^{184, 185}. Nevertheless, a gene network state providing latent competence to switch to other fates persists throughout the first few stages of T-cell development, which can be detected by experimentally removing the cells from the normal thymus.

Alternative lineage potentials are lost at different times (Fig. 2) and by at least three different mechanisms. At the earliest thymic stage, as ETP cells turn off *Flt3* expression, they unconditionally lose their B-cell potential^{100, 125, 126}. The mechanism depends on Notch signals and *GATA-3*^{97, 186}, but this remains incompletely defined^{21, 99, 187}.

Exclusion of myeloid and dendritic cell potentials is caused by removal of a positive regulator. Access to both options correlates with the expression profile of PU.1, and at early stages *C/EBP* factors, present at low levels, may also contribute^{188, 189}. But while PU.1 is present Notch signaling keeps myeloid options under check. First, Notch-induced *Hes1* represses *Cebpa*⁷⁵. Notch signaling also modulates the spectrum of PU.1 transcriptional activities irrespective of *C/EBP* α ^{66, 188, 190}. PU.1 is silenced during DN2b, under the influence of Runx and possibly TCF-1 or *GATA-3*^{29, 191-193} as well, closing the myeloid options.

The loss of access to NK and ILC2 fates may instead be due to activation of a repressor, i.e. activation of *Bcl11b*. Both innate lymphocyte programs require *Id2* in order to neutralize E protein activity, and *Bcl11b* is implicated directly in keeping *Id2* silent^{117, 118, 122}.

Ironically, essential T-cell lineage regulatory inputs may themselves represent a threat for alternative lineage diversion during the uncommitted stages of T-cell development if their levels are dysregulated. Overexpression of *GATA-3* can drive the cells toward a mast-cell fate²⁹, antagonizing Notch-dependent survival systems. *GATA-3* and TCF-1 can also work in a low-E protein regulatory state to promote development to ILC2s^{30, 194, 195} instead of T cells, and even excessive Notch signals may favor ILC fates in human precursors¹⁹⁶. These alternatives too are removed during the commitment transition, through mechanisms that need to be clarified.

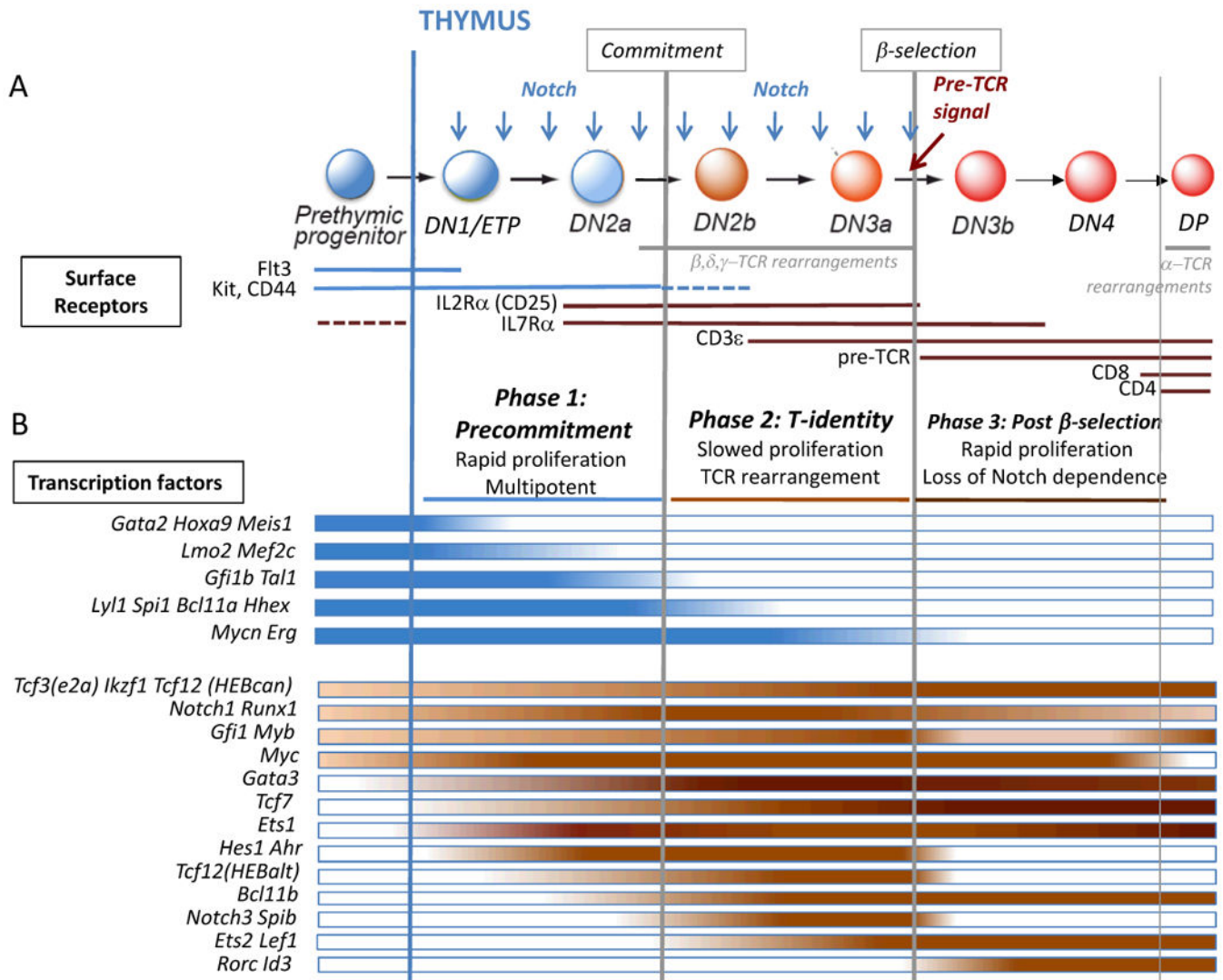


Figure 1. αβT-cell development: stages, surface markers, and transcription factor expression
 a. Adult mouse T-cell development begins in the bone marrow from lymphoid-primed prethymic progenitors that migrate to the thymus and begin differentiation in the thymic environment, which provides Notch ligands (blue arrows). Cells transit sequentially through DN1/ETP, DN2a, DN2b, DN3a, DN3b, DN4, and DP stages on the way to becoming αβT-cells (DN: CD4⁻ CD8⁻; DP: CD4⁺ CD8⁺; ISP transitional-stage cells not shown). DN1 (CD44⁺ CD25⁻) cells include a subset with high Kit expression that contains the Early T-cell Precursors (ETP; CD44⁺Kit⁺⁺CD25⁻), which contain essentially all the T-cell progenitor activity and are the only kind of DN1 cells that will be considered further here. ETPs lack or have downregulated IL7R, but as they differentiate to DN2, they turn on IL7R. Key cell surface receptors used to identify these stages are shown indicating the stages during which each receptor is expressed. Dotted lines indicate stages with lower expression levels. The stages during which TCR rearrangements occur are also marked. Development is divided by the commitment and β-selection checkpoints into three major regulatory phases (Phases 1, 2, and 3: post-β-selection), each with unique gene networks and cellular characteristics. Cells

in phase 1 proliferate extensively and retain multipotentiality, while phase 2 cells are committed, slow their proliferation, and undergo TCR rearrangements. Only cells with a rearranged TCR β that can combine with pre-T α and transduce a signal can continue through the β -selection checkpoint into phase 3, a second highly proliferative but increasingly Notch-independent phase leading to CD4 and CD8 upregulation, then proliferative arrest, and TCR α rearrangement.

b. Stage-specific patterns of expression of important transcription factor genes are shown below the developmental stages. The color intensity variations provide an approximation of the dynamic changes in expression of the genes, grouped together based on similar expression patterns although not necessarily similar levels; for more accurate quantitation of gene expression, see sources of this figure in Zhang et al.³¹ and ImmGen^{32, 197} (www.immgen.org). The transcription factor genes are divided into legacy stem and progenitor genes that are mostly turned off in Phase 1 and are all off by β -selection (blue bars), and genes that are critical to different aspects of the T-cell specification and commitment programs (red bars). Additional information about each of these genes is provided in Tables 1 and 2.

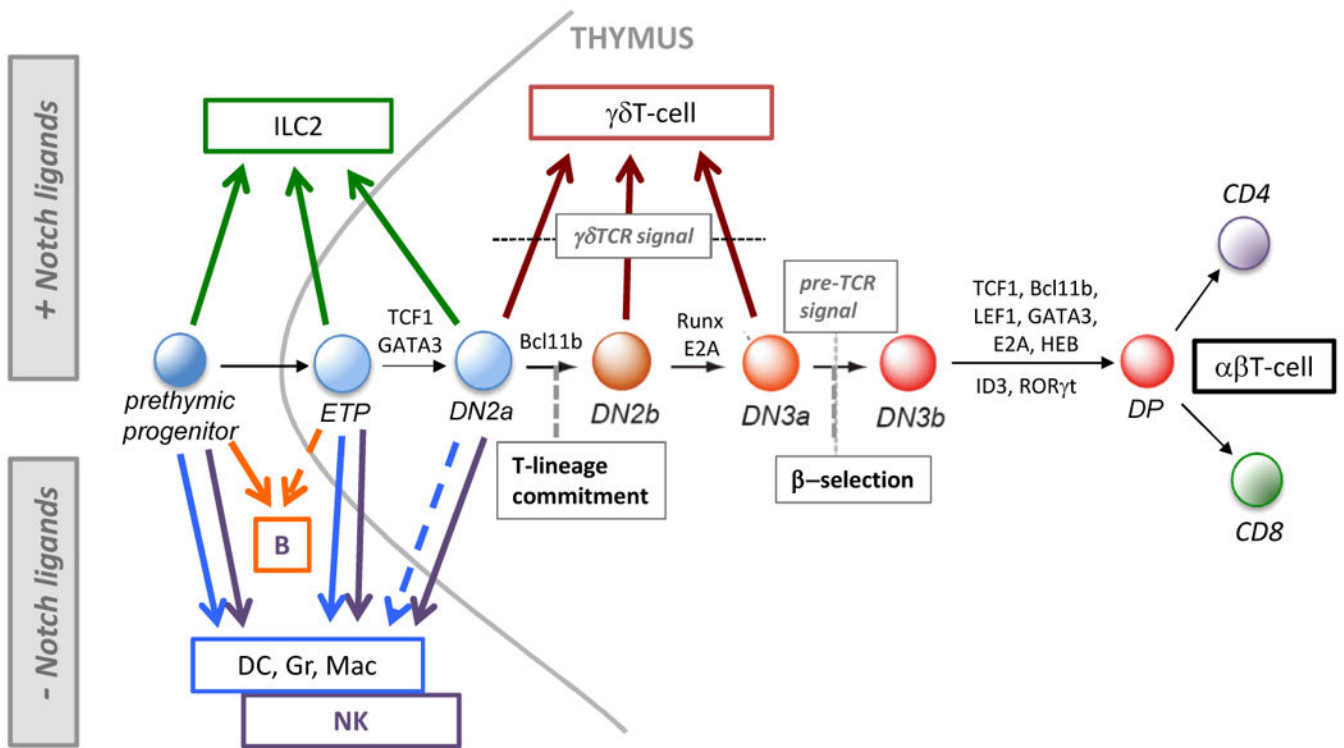


Figure 2. T-lineage commitment and alternative lineage potentials

Diagram of early T-cell development for $\alpha\beta$ T-cells from bone marrow prethymic progenitors to entry into and development in the thymus, showing the stages from which alternative lineages can develop. The figure shows which fates can be accessed by cells in the presence of Notch signaling (upward pointing arrows), including $\gamma\delta$ T-lineage cells that can develop from DN2a/b and DN3 cells (brown arrows), as well as those fates that can only be accessed by experimental withdrawal from Notch signaling (downward pointing arrows). Arrows shown emerging from the thymus show conditional outcomes usually only observed if the cells are removed from the thymic environment. Alternative non-T-lineage innate lymphocytes (ILC2) can be generated from bone marrow precursors, as well as ETP and DN2 cells, but only in the presence of Notch ligands and specific cytokines. In the absence of Notch ligands, B-cells can be generated from bone marrow precursors and a subset of the earliest (Flt3+) ETP cells, while dendritic cells (DC) and granulocytes (Gr) or macrophages (Mac), can be derived from prethymic progenitors as well as from thymic ETP cells and to a lesser extent from DN2a cells, but not from later stage cells. Natural killer (NK) cells also develop well from prethymic, ETP and DN2a cells, but not from later stage cells. Broken-line arrows denote potentials seen in low-frequency precursors within a population. Shown also are some key transcription factors that are critical for specific transitions (see text and Fig. 3).

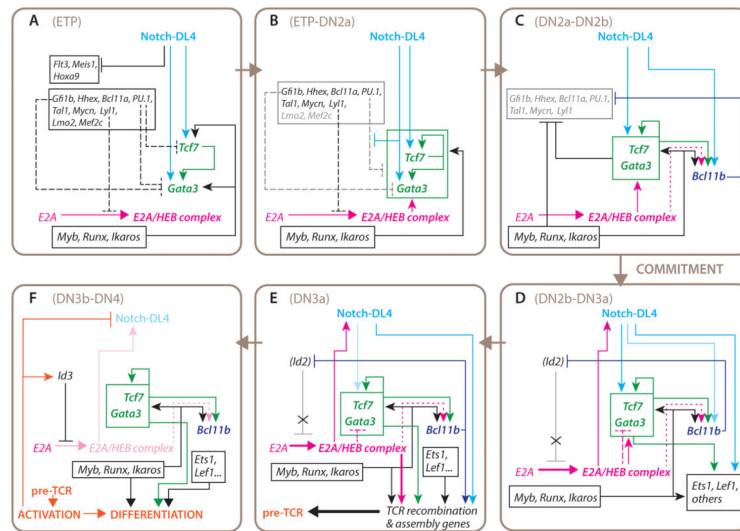


Figure 3. Progression of gene regulatory network states in T-cell commitment: current model Panels a-f (clockwise progression) show a model of the sequential changes in regulatory relationships between the genes, based on evidence discussed in the text, from the early Flt3⁺ ETP stage (a) through commitment (c-d) to β -selection (f). Passage through the indicated changes in gene network states (a-f) occurs during periods that correspond approximately to developmental stages, as defined by cell-surface markers, which are indicated at the top of each panel, but the regulatory states may not be homogeneously equivalent to these stages. Phase 1: panels a, b. Transition to phase 2: panel c. Phase 2: panels d, e, in which panel d emphasizes the regulatory gene expression changes and panel E emphasizes the consequences of those changes. Phase 3, post β -selection (for $\alpha\beta$ T lineage): panel f. Boxes enclose genes that operate coordinately at a given stage even if the details of regulation that link them are not known. Arrows indicate activation. Lines ending in bars indicate repression. Dashed lines indicate “soft repression” which limits the maximal activity or deployment of a transcription factor but does not silence its expression. Dotted lines: likely but undemonstrated linkages. Faded colors: decreasing expression or functional impact of a given gene or gene group as a result of regulatory effects shown elsewhere in the panel. (Id2), potential for activation of Id2 in response to cytokine signaling or other stimuli, which is held in check by Bcl11b. Note that E2A/HEB heterodimers can play a positive role in *Gata3* and *Tcf7* induction as well as exerting restraint on maximum levels of *Gata3* expression. TCR recombination and assembly genes steeply increase in expression from DN2a to DN3a, but are most functionally significant in DN3a. As revealed by T-ALL phenotypes, cells that take a “short cut” from the states represented in panels a-b to the activation events in panel f, bypassing the commitment process, are susceptible to oncogenic transformation. For details, see text.

Table 1
Critical T-cell specification transcription factor genes in murine early T-cells and their progenitors

Gene	Gene Family	Knockout phenotype	Overexpression phenotype	Selected References
<i>Notch1</i>	NOTCH, ankyrin repeat	Block in T development	T-ALL	1-3 Reviewed in 4, 5
<i>Runx1</i> (AML1)	RUNT	Early stem cell defects; Block before DN3; Myeloproliferation	AML	6 7
<i>Ets1</i>	ETS	Impaired DN3 to DP development and allelic exclusion; Defective NK and CD8 cell development		8, 9 10
<i>Ikaros</i> (IKAROS)	IKAROS C2H2 ZnF	Loss of lymphoid cells and progenitors; T- and B-ALL		11, 12
<i>Tcf3</i> (E2A)	E protein basic helix-loop-helix (bHLH)	Defects in hematopoietic stem cell pools; DN2 defects; T-ALL		13, 14 Reviewed in 15
<i>Myb</i>	MYB SANT (helix-turn-helix, HTH)	Multiple blocks from HSC to DP		16-19
<i>Myc</i> (<i>c-myc</i>)	MYC bHLH	Defects in proliferation and survival of HSC, lymphoid progenitors, after β -selection	T-ALL	20-22
<i>Tcf7</i> (TCF1)	TCF HMG	Almost complete loss of T cell development; T-ALL	Limited T development in the absence of Notch signals	23-26 27
<i>Gata3</i>	GATA ZnF	No DN cells; if deleted later, β -selection block and loss of CD4 cells	Poor viability, diversion to mast cells	28, 29 Reviewed in 30 31
<i>Hes1</i>	HAIRY bHLH	Defective T-cell development		32, 33 34
<i>Bcl11b</i> (CTIP2)	ZnF, C2H2-like	No $\alpha\beta$ T cells; Accumulation of DN2a and NK cells		35, 36 37, 38
<i>Tcf12</i> (HEBcan/alt)	E protein bHLH	Partial developmental blocks at DN1 and between DN3 and DP	Enhanced T-cell differentiation, reduced proliferation (esp. HEBcan)	39 Reviewed in 40
<i>Lef1</i>	TCF HMG	No phenotype alone; ISP block with hypomorphic <i>Tcf7</i>	T-ALL	26, 41
<i>Gfi1</i>	SNAG, C2H2-like ZnF	Partial block between ETP and DN2	Potentiates T-ALL	42, 43

bHLH, basic helix-loop-helix; C2H2, Cys2 His2, a form of Zinc finger; ETS, E26 transformation-specific; HMG, high mobility group; HTH, helix turn helix; SNAG, Snail/Gfi1 domain, a repression domain; Znf, zinc finger

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Table 2
Critical Phase 1-specific transcription factor genes in murine early T-cells and their progenitors

Gene	Gene Family	Knockout Phenotype	Overexpression Phenotype	Selected References
<i>Lmo2</i> (Rbtn2)	LIM	Severe stem cell defects; No T cell effects if deleted after DN2	T-ALL	1-4
<i>Gata2</i>	GATA	Early stem cell defects		5, 6
<i>Mef2c</i>	MADS-box	T, B and NK defects	AML, T-ALL association	7 8
<i>Meis1</i>	MEIS Homeodomain	Early stem cell defects	AML	9, 10
<i>Hoxa9</i>	HOX Homeobox	Defect in HSC proliferation; Block in DN, partial DN2 block	AML	11, 12
<i>Tal1</i> (SCL)	TAL bHLH	Early stem cell defects	T-ALL	13, 14
<i>Gfi1b</i>	SNAG, C2H2-like ZnF	HSC expansion		15
<i>Lyl1</i>	TAL bHLH	Defects in LMPP, ETP, DN2a cells	ALL (B and T)	16 17, 18
<i>Spi1</i> (<i>Sfp1</i> , PU.1)	ETS	Absence of T-cell and NK development; AML	Diversion to DC or myeloid lineages	19-22 Reviewed in ²³
<i>Bcl11a</i> (Evi9, Ct1p1)	ZnF, C2H2-like	Required for B, T, NK development	Myeloid, B, T oncogene	6, 24 25
<i>Hhex</i> (Prhx)	Homeobox	Impaired monocyte development	T-ALL	2 26
<i>Mycn</i> (N-myc)	MYC bHLH	Defects in HSC survival, proliferation (with c-myc)	AML	27, 28
<i>Erg</i>	ETS	Early stem cell defects	T-ALL and other leukemias	29 30

bHLH, basic helix-loop-helix; C2H2, Cys₂ His₂, a form of Zinc finger; ETS, E26 transformation-specific; SNAG, Snail/Gfi1 domain, a repression domain; Znf, zinc finger

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