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E proteins and the regulation of early lymphocyte development

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Summary

Lymphopoiesis generates mature B, T, and NK lymphocytes from hematopoietic stem cells via a series of increasingly restricted developmental intermediates. The transcriptional networks that regulate these fate choices are composed of both common and lineage-specific components, which combine to create a cellular context that informs the developmental response to external signals. E proteins are an important factor during lymphopoiesis, and E2A in particular is required for normal T and B-cell development. Although the other E proteins, HEB and E2-2, are expressed during lymphopoiesis and can compensate for some of E2A's activity, E2A proteins have non-redundant functions during early T-cell development and at multiple checkpoints throughout B lymphopoiesis. More recently, a role for E2A has been demonstrated in the generation of lymphoid-primed multipotent progenitors and has shown to favor their specification towards lymphoid over myeloid lineages. This review summarizes both our current understanding of the wide-ranging functions of E proteins during the development of adaptive lymphocytes and the novel functions of E2A in orchestrating a lymphoid-biased cellular context in early multipotent progenitors.

Keywords

hematopoietic progenitor cells; lineage commitment; transcription factors; cell differentiation; gene regulation

Introduction

Lymphopoiesis is the process by which B and T lymphocytes as well as natural killer (NK) cells, lymphoid tissue inducing (LTi) cells, and possibly subsets of dendritic cells (DCs) are generated from hematopoietic stem cells (HSCs). The gene expression programs that characterize a given fate are driven by specific combinations of lineage-restricted and ubiquitous transcription factors. Some of these lineage-specifying factors function solely to induce lineage associated genes, whereas others commit cells to a lineage by prevent activation of genes associated with alternative fates. Recent findings also suggest that down-regulation of stem cell-associated transcription factors just prior to lineage commitment may extinguish the ability of ubiquitous or common transcriptional activators to access genes for alternative lineages (1,2). Our understanding of how transcriptional regulatory networks stabilize lineage-associated gene programs and how specific combinations of transcription factors within these networks synergize or antagonize each others' activities is evolving. The E protein transcription factors are key components of the transcriptional networks that promote specification and commitment to the adaptive T and B-lymphoid fates, and they also directly regulate essential features of these lineages such as the rearrangement and expression of the immunoglobulin (Ig) and T-cell receptor (TCR) loci. E proteins are

believed to antagonize innate lymphocyte fates, particularly NK cells and LT α i cells, and the Id inhibitors of E proteins are necessary for the development of these lineages (3,4). The mechanisms by which E proteins antagonize innate lymphocyte development remain controversial but, in analogy with their role in adaptive lymphocytes, may involve the induction of transcriptional networks inappropriate for innate lineages. While E proteins regulate transcriptional networks in adaptive lymphocytes, we propose that at earlier stages of lymphopoiesis, in the common progenitors of both adaptive and innate lymphocytes, E proteins play a different role: collaborating with other factors to establish the context for specification and commitment to lymphoid fates (Fig. 1). In this review, we examine both the evidence supporting this hypothesis and the functions of E proteins in later adaptive lymphocyte development.

E proteins and their interacting partners

E proteins

In mammals, there are 4 class I helix-loop-helix proteins (HLH), or E proteins, encoded by 3 genes: *E2A* (*Tcf3*), *Heb* (*Tcf12*), and *E2-2* (*Tcf4*) (reviewed in 5). The structure of E proteins has been reviewed extensively, and primary references can be found in the review by Massari and Murre (5). E proteins are orthologues of the *Drosophila* protein DAUGHTERLESS, which functions in a wide range of cell fate decisions in flies, including sex determination, selection of sensory organ progenitors, and selection of the R7 photoreceptor. The first mammalian E proteins to be identified, E12 and E47, were cloned on the basis of their ability to bind an 'E box' sequence (κ E2) in the immunoglobulin κ (Ig κ) light chain enhancer. E12 and E47 differ only in their bHLH domain and derive via alternate exon usage from the *E2A* gene. HEB and E2-2 were cloned shortly thereafter based on their ability to bind to similar E box sequences. The E proteins bind DNA as a dimer and protein-protein interactions are mediated by the HLH domain. Immediately upstream of the HLH domain is a basic region that is required for DNA binding and may play a role in nuclear localization. All of the E proteins share a high degree of sequence similarity within their bHLH domains as well as in two upstream transcriptional activation domains (ADs). In the *E2A* proteins, AD1 and AD2 collaborate in activating transcription. However, if both domains are deleted, the remaining N-terminal portion retains some transcriptional activation activity (6). E12 and E47 are primarily transcription activators that function, in part, by recruiting the co-activator protein p300/CBP, which in turn recruits histone acetyltransferases and RNA polymerase II to the promoter or enhancers of target genes (5,7,8). However, there is evidence that *E2A* proteins also repress some genes in pre-B cells, although the mechanism underlying this repression remains to be resolved (9). Interestingly, while E47 readily forms homodimers, an inhibitory domain in E12 may restrict its function to heterodimers (10). Since E47 lacks this inhibitory domain, the proportion of E47 homo- to heterodimers is most likely solely dependent on the quantity of potential binding partners, although the redox state may also alter dimerization and DNA-binding potential under physiological conditions (11). In keeping with this, in B-lymphocyte cell lines, where E47 protein is expressed at higher concentrations than the other E proteins, E47 homodimers are the dominant E box-binding complexes detected by electrophoretic mobility shift assays (EMSA) (5). In contrast, in T lymphocytes where HEB and E47 are both highly expressed, heterodimers bind to the E-box sequence (12,13).

Alternative splice variants have not been reported for *E2-2* and HEB, although northern blot analysis revealed multiple mRNA species reactive with *Heb* cDNA. However, in contrast to the *E2A* gene, there is evidence that alternative promoter usage generates two forms of the HEB protein, which were reported to have different functions (14). The shorter form of HEB has only one activation domain and, by analogy with *E2A*, would be predicted to function in transcriptional activation (6). However, this protein also contains a novel sequence that is

not found in other E proteins, termed an Alt domain (14). While it is tempting to speculate that Alt confers a novel function on HEB, such as transcriptional repression, this remains to be formally investigated. An alternative promoter for the E2-2 protein, resulting in a form of E2-2 that lacks AD1 but has an Alt domain, is also predicted based on DNA sequence comparison but thus far has not been ascribed functional significance (15).

Tissue-specific bHLH proteins

As E proteins are broadly expressed and bind a common E box element, one aspect of their function that is not completely understood is how they mediate so many different developmental outcomes. One means by which specificity can be lent to E proteins is via dimerization with the more tissue-restricted class II bHLH proteins, which, in the context of hematopoiesis, refer to the highly-related stem cell leukemia (SCL)/T-lymphocyte acute lymphoblastic leukemia 1 (TAL1), and lymphoblastic leukemia 1 (LYL1) proteins. Both TAL1 and LYL1 were cloned from translocations associated with T-cell acute lymphoblastic leukemia (T-ALL) and, like the E proteins, possess an HLH domain and adjacent basic region (reviewed in 16). Class II HLH proteins homodimerize poorly and generally require heterodimerization with E proteins to allow DNA binding (5). Binding site selection assays reveal that the E-box sequence preferred by TAL1-E protein heterodimers differs from those preferred by E protein homodimers (17,18). This alteration of E protein DNA binding specificity may explain the ability of TAL1 and LYL1 to interfere with E2A-dependent transcription of target genes and provides one mechanism by which TAL1 or LYL1 over expression might contribute to T-cell leukemogenesis (reviewed in 19).

Id proteins

Class IV HLH proteins are comprised of the mammalian Inhibitor of DNA binding (ID1-4) proteins and *Drosophila* EXTRAMACROCHAETAE (*emc*), which lack the basic domain required for DNA-binding but retain the HLH domain and the ability to dimerize with E proteins (5). Id proteins inhibit E protein activity by directly preventing their DNA-binding but do not interact appreciably with the hematopoietic class II HLH proteins (20-22). Despite some evidence that ID2 can interact with the retinoblastoma (RB) tumor suppressor and that ID2 and ID3 interact with Ets family members, the most well characterized role of ID proteins is in the regulation of E protein activity (23).

E proteins and hematopoietic stem cells

Like all hematopoietic cells, lymphocytes are derived from hematopoietic stem cells (HSCs) that reside in the bone marrow within the lineage marker (Lin)⁻Sca-1⁺CD117 (Kit)⁺ (LSK) population that also contains the CD34⁻ self-renewing long-term HSCs (LT-HSCs) and their short-term reconstituting CD34⁺ progeny (ST-HSCs) (24). For many immunologists, E2A is thought of as a specific factor for B-cell development and mice lacking E2A do have a dramatic defect in B lymphopoiesis (25,26). However, a role for E proteins, particularly E2A, at earlier stages of hematopoiesis has not been thoroughly investigated, even though E protein interacting proteins are essential for HSC formation and function. Specifically, the class II HLH proteins TAL1 and LYL1 have well characterized functions in HSCs, implicating E proteins as their obligate dimerization partners. However, it remains to be determined whether a specific E protein is critical for any TAL1 or LYL1 functions and how this specificity arises.

Tall-deficient embryos die by day 10.5 of gestation, due to a failure of primitive hematopoiesis (27,28). Blastocyst chimeras confirm that *Tall*^{-/-} cells are also severely impaired in definitive hematopoiesis but can contribute to non-hematopoietic tissues. Although critical during the development of HSCs from mesoderm, following hematopoietic

specification *Tal1* is largely dispensable for the maintenance and function of definitive HSCs but remains essential for erythroid and megakaryocyte lineages (reviewed in 29). Interestingly, primitive hematopoiesis can be rescued with a wide range of TAL1 mutants, even those lacking the basic domain and thus DNA-binding capacity, so long as their dimerization function is intact (30). While these findings may be interpreted as evidence that sequestering E proteins is the primary function of TAL1 during primitive hematopoiesis, there is evidence that TAL1-E protein heterodimers can be recruited to DNA through interactions with other transcription factors such as Gata1 (31). In contrast, the basic region of TAL1 is required for definitive hematopoiesis as well as for development of mature erythrocytes, myelocytes, and megakaryocytes, clearly implicating TAL1-mediated transcription in these processes (30).

In contrast to *Tal1*^{-/-} mice, *Lyl1*^{-/-} mice are viable but have reduced numbers of the more primitive LSKCD34⁺ HSCs and a defect in the generation of B cells, while myeloid lineages are largely spared (32). *Lyl1* expression during embryonic development, as determined by a LacZ knock-in model, largely overlaps with that of *Tal1* (33). Despite the high degree of similarity and overlapping expression patterns, endogenous LYL1 does not rescue *Tal1*^{-/-} embryos from lethality (27,28), suggesting that LYL1 protein may not be present during primitive hematopoiesis. It was recently shown that LYL1 and TAL1 do compensate for each other to some extent in adult HSCs, since mice deficient in both *Tal1* and *Lyl1* show a more severe HSC deficiency in that their HSCs fail to repopulate lethally-irradiated recipients and are highly apoptotic (34).

In contrast to the severe consequences to hematopoiesis of deleting *Tal1* or *Lyl1*, deletion of *E2A* results in more subtle alterations of the HSC compartment. In the absence of *E2A*, HSCs and their downstream progeny within the LSK compartment show increased bromodeoxyuridine (BrdU) incorporation, suggesting that there are more cells in cycle than in wildtype (WT) controls (35-37). This increased proliferative capacity was linked to decreased expression of mRNA encoding the cell cycle inhibitor p21 (35,36). Competitive bone marrow transplantation assays revealed that *E2A*^{-/-} HSCs are compromised slightly in their ability to undergo self-renewal (36). In addition, under stress conditions, *E2A*^{-/-} HSCs become exhausted and fail to sustain hematopoiesis (35). However, under steady-state conditions the requirement for *E2A* appears to be minimal, suggesting either that HEB or E2-2 are more important for HSCs, or that all E proteins function redundantly within HSCs.

E proteins and development of lymphoid-primed multipotent progenitors

Lymphoid-primed multipotent progenitors (LMPPs) represent the first lineage restriction point along the route from HSC to committed lymphocyte. LMPPs are a subset of LSKCD34⁺ cells that have lost self-renewal capacity, and most megakaryocyte/erythrocyte (MegE) differentiation potential, but retain the ability to give rise to all lymphocyte subsets as well as granulocyte/macrophage progenitors (GMPs) (38,39). LMPPs can give rise to common lymphoid progenitors (CLPs) (40), which produce B lymphocytes and NK cells in the bone marrow and are likely among the bone marrow precursors that function as thymus seeding progenitors (TSPs) (41,42). A growing list of markers demonstrates the heterogeneity of the LMPP population. In particular, a subset of cells expresses the chemokine receptor CCR9 (43,44), which is required for efficient homing of TSPs to the thymus (45). A partially overlapping subset of LMPPs has low expression of vascular cell adhesion molecule-1 (VCAM-1)/CD106 (44,46), which characterizes more primitive LSKs that retain MegE potential. In addition, a small population of LMPPs express GFP from a *Rag1* reporter gene, indicating that they overlap with early lymphoid progenitors (ELPs), which are more lymphoid-biased than the bulk LMPP population (47). LMPPs with a CD90⁻CD62L⁺ phenotype show heightened T-cell differentiation potential *in vitro* and *in*

vivo (48-50). While this heterogeneity may be a consequence of a developmental hierarchy within the LMPP subset an alternative interpretation is that the LMPP population contains cells with distinct gene expression profiles that may reflect differential biases in their developmental potential.

The two critical attributes that distinguish LMPPs from MPPs are (i) the loss of MegE potential and (ii) the initiation of 'lymphoid priming', a process in which genes associated with multiple distinct lymphoid lineages are co-expressed at low abundance (39,51). E2A is necessary for the development of LMPPs and proper lymphoid priming, but it is not required to extinguish MegE potential (37). Instead, the loss of MegE potential coincides with the loss of thrombopoietic receptor Mpl expression and may also be associated with restricted expression of Gata1, a transcription factor essential for both megakaryocyte and erythroid differentiation (52,53). Similarly, decreased expression of TAL1 in LMPPs may decrease MegE potential and liberate E proteins to function in homodimeric complexes that can contribute to lymphoid gene expression. Consistent with that hypothesis, the expression of E47 protein does not change during differentiation of HSCs into LMPPs, indicating that the essential function of E2A in lymphoid priming does not require increased E2A protein expression (37).

While E2A is not required for the restriction of MegE potential, it does play a role in the myeloid versus lymphoid decision of LMPPs. The few LMPPs present in *E2A*^{-/-} mice show enhanced myeloid cloning efficiency compared to WT LMPPs, and *E2A*^{-/-} MPPs are also skewed toward macrophage differentiation (37). This finding is consistent with a study by Cochrane and colleagues in which inhibition of E protein function via ectopic expression of ID1 favored myelopoiesis at the expense of B cell development (54). However, while ID1 expression is associated with myeloid rather than lymphoid differentiation, *Id1*^{-/-} LMPPs did not show a significant alteration in lineage potential. Therefore, these studies confirm that E proteins promote lymphopoiesis from LMPPs at the expense of myelopoiesis, but indicate that Id1 is unlikely to be the major physiological determinant of E protein function at this stage of development. Interestingly, progenitors from mice transgenic for an inducible version of a hybrid E47, containing TAL1's ID-impervious bHLH domain, preferentially gave rise to B lymphocytes over myeloid cells (54). This finding was interpreted to support the hypothesis that E proteins promote lymphoid differentiation from LMPPs but raises questions about the DNA binding specificity of the E protein complexes involved since this hybrid E47 should bind DNA sequences preferred by TAL1-E protein heterodimers. Nonetheless, most evidence indicates that E2A functions within LMPPs to promote lymphoid rather than myeloid differentiation. Indeed, the fact that MPPs from *E2A*^{-/-} bone marrow are also skewed toward myeloid differentiation raises the possibility that the failure to accumulate *E2A*^{-/-} LMPPs is a consequence of the propensity of these cells to precociously adopt the myeloid fate without forming a stable LMPP intermediate.

In addition to their increased propensity to give rise to myeloid cells, *E2A*^{-/-} LMPPs demonstrated a striking dysregulation of lymphoid-associated genes that includes both known E2A targets such as *Rag1*, *Il7ra*, *Dnnt*, *Igh-6*, and *Notch1*, and genes whose regulation was not previously shown to involve E2A, such as *Ccr9* and *Ets1* (37). Surprisingly however, genes encoding transcription factors known to promote lymphopoiesis early in hematopoiesis, such as *Sfp1* (PU.1), *Ikaros* (IKAROS), and *Gfi1* were not dependent on E2A for their expression. Analysis of the DNA sequences flanking the transcriptional start site for E2A-dependent genes revealed an enrichment of binding sites for IKAROS and PU.1, suggesting that while E2A does not regulate their expression, it collaborates with these factors to initiate lymphoid priming (37). These observations suggest that, unlike commitment to the B-lymphocyte fate, the emergence and function of LMPPs may not depend on E2A to induce an entirely novel transcriptional regulatory network.

Rather, transcription factors already expressed in LSKs and MPPs may cooperate with E2A to drive lymphoid priming and further differentiation to the CLP stage (Fig. 1). Lymphoid priming allows cells within the LMPP and CLP compartment to express a variety of cell surface receptors, including IL7R, NOTCH1, CCR9 (and likely CCR7), and CD69L, which endows them with the ability to respond to their microenvironment, and influences the set of genes regulated by the basal cast of transcriptional regulators inherited from earlier stages. Indeed, individual CLPs or ETPs may have different probabilities of responding to an environmental cue with a given outcome based on small fluctuations in transcription factor expression that may become amplified during their development from LMPPs.

While it is likely that E2A cooperates with Ikaros and PU.1 to promote lymphoid priming, E2A-deficiency has more severe consequences for the development of LMPPs than deficiency of either of the other proteins. As both IKAROS and PU.1 control the expression of *Flt3*, it is difficult to unambiguously identify LMPPs in *Sfp1*^{-/-} or *Ikzf1*^{-/-} mice (55-57), but Yoshida *et al.* (56) cleverly overcame this problem, using an *Ikaros* promoter-GFP reporter mouse that displays increased GFP expression concomitant with the development of LMPPs. When crossed to mice carrying a null allele of *Ikaros*, GFP^{hi} LSKs are clearly present and these cells have appropriate myeloid potential without generating megakaryocytes, indicating that LMPPs are present but lack expression of FLT3. However, these *Ikaros*^{-/-} LMPPs show altered lymphoid gene expression and fail to properly generate B or T lymphocytes *in vitro*. The set of IKAROS dependent genes in LMPPs shows significant but not complete overlap with those regulated by E2A, consistent with these two transcription factors collaborating in gene regulation (58). GFI1 has also been implicated in the generation of LMPPs and appears to function in part by limiting the expression of PU.1, which is both essential for lymphoid differentiation and can block it if expressed at high concentrations (59-61). Therefore, GFI1 may be necessary to allow appropriate cooperative interactions between E2A, IKAROS, and PU.1 to promote lymphoid priming, while limiting PU.1-driven myelopoiesis. Our current understanding of how extracellular signals and the expressed cohort of transcription factors, in collaboration or antagonism with E2A, lead to the transcriptional regulatory networks that dictate specification and commitment to a given lymphocyte fate is the focus of the remaining sections of this review.

E proteins and B-cell development

Specification of the B-cell fate

Specification refers to the process of turning on a lineage-associated gene expression program and, for the B-cell fate, begins in the CLP population. The critical event driving CLPs toward the B-cell fate is the induction of the B-cell-associated transcription factor EBF1, whose mRNA and target gene expression have been detected in a subset of CLPs (62-64). E12 is able to induce *Ebf1* expression in a macrophage cell line (65), and E2A binding sites have been identified in the *Ebf1a* promoter, which is active at the CLP stage (66,67). Therefore, E2A proteins appear to be regulators of the initial expression of the *Ebf1* gene. Moreover, the requirement for E2A in early B-cell development can be bypassed by forced expression of EBF1 in *E2A*^{-/-} progenitors, which rescues the expression of B-cell-associated genes and IgH V(D)J recombination (68,69). However, these data should not be interpreted as evidence that E proteins are superfluous in pro-B cells after the induction of EBF1. As discussed below and reviewed elsewhere, E proteins play many critical roles during B-cell development; however, the E protein activity provided by HEB and E2-2 is sufficient for survival and expression of most E protein target genes in pro-B cells, provided that EBF1 is expressed appropriately.

Ebf1 is not among the lymphoid genes whose expression is primed by E2A in LMPPs, rather, the induction of *Ebf1* occurs in a subset of CLPs that also show increased expression

of E2A protein compared to the bulk of CLPs and LMPPs (37,63,64). This increase in E2A protein levels is dependent on EBF1, suggesting that it is a consequence of and not the cause of EBF1 induction (71). This finding raises the question of why *Ebf1* induction is delayed until the CLP stage if E2A protein levels remain constant from the HSC compartment. The answer likely lies in the requirement for a change in cellular context that occurs between the LMPP and CLP stages, which is, in turn, dependent on signaling through the IL7R.

IL7R surface expression is not observed until the CLP stage even though *Il7ra* is among the lymphoid genes transcribed in LMPPs (51,72). IL7R signaling has been implicated in the expression of *Ebf1* in CLPs, although the mechanism responsible remains controversial (73-76). One possible scenario is that the chromatin context at the *Ebf1* gene is altered as a consequence of IL7R signals, allowing E2A and other regulators of *Ebf1* expression, such as PU.1, to gain access and promote its transcription. Such a change in chromatin context is likely mediated by the STAT5 transcription factors, which may be activated upon expression of IL7R at the CLP stage, even before the initial transcription of the *Ebf1* gene is detected. This hypothesis is supported by the observation that *Ebf1* expression is extremely low in the CLPs of *Il7^{-/-}* and *Il7ra^{-/-}* mice but can be restored by retroviral expression of a constitutively active STAT5 (74,75). However, *Ebf1* is expressed in the pro-B cells of mice in which the *Stat5a* and *Stat5b* genes were deleted using *Rag1*-Cre, suggesting that if STAT5 opens the *Ebf1* gene locus, it must act prior to the function of *Rag1*-Cre and via a hit-and-run mechanism (77). Alternative hypotheses can also explain these data; for example, in mature T lymphocytes, deletion of *Stat5a* and *Stat5b* allows the compensatory use of alternate STAT transcription factors by IL7R, such as STAT6 (78). Thus, the connection between IL7R signaling and histone modifications at the *Ebf1* gene, and the role of Stat5, remain poorly defined. In embryonic stem cells and Lin⁻ bone marrow cells, the *Ebf1* gene is in a poised state containing histone modifications associated with both gene activation (H3K4^{me3}) and repression (H3K27^{me3}) (79). H3K27^{me3} is maintained by the polycomb repressor complex 1 (PRC1), and deletion of *Bmi-1*, encoding a component of PRC1, in hematopoietic cells, leads to the loss of H3K27^{me3} at the *Ebf1* promoters and premature *Ebf1* mRNA expression (79). Therefore, the regulation of PRC1 function may alter the context of the *Ebf1* gene to allow for its E2A-dependent induction at the CLP stage.

Commitment to the B-cell fate

Commitment to a particular lineage requires repressing lineage inappropriate genes to prevent differentiation down alternative pathways. The first cells fully committed to the B-cell lineage express both B220 and CD19 on their surface, along with CD43, HSA and the receptor c-kit (CD117). These cells are absent in *E2A^{-/-}* and *Ebf1^{-/-}* mice and most B-cell associated genes and IgH rearrangements are undetectable in the bone marrow or fetal liver of these mice (26,71,80). Mice heterozygous for the *E2A* and *Ebf1* mutations show a significant reduction of committed pro-B cells (defined as B220⁺CD43⁺) and have reduced expression of many B-cell genes (81). Among the genes regulated by E2A and EBF1 is *Pax5*, which encodes a transcription factor that is essential for development beyond the pro-B-cell stage. Although pro-B cells develop in *Pax-5^{-/-}* mice, there is substantial evidence that they are not committed to the B-cell lineage (82,83). For instance, *Pax-5^{-/-}* pro-B lymphocytes expanded in vitro in IL-7 express many lineage inappropriate genes, which can be repressed by ectopic expression of Pax-5 (84). Remarkably, *Pax5^{-/-}* pro-B cells retain the ability to differentiate into alternative lineages, including myeloid cells, when IL-7 is removed from the culture (82). Moreover, these IL-7-expanded *Pax-5^{-/-}* pro-B cells, and to some degree primary *Pax-5^{-/-}* pro-B cells, can function as HSCs and can reconstitute all hematopoietic lineages when transplanted into lethally irradiated mice. The requirement for Pax-5 to maintain commitment to the B-cell lineage persists throughout B-cell development, since even mature B cells lose their B-cell characteristics and express lineage inappropriate

genes following *Pax-5* deletion (85). These mature cells can regress to a more immature developmental state, and even switch to the T-cell lineage, but most frequently seed the development of B-cell neoplasms. Taken together, these studies clearly demonstrate the essential role of Pax5 in B-cell lineage commitment and E2A as an essential regulator of Pax5 expression.

It has only recently been appreciated that Pax-5 does not act alone in repressing alternative lineage genes in B lymphocytes. Pax-5, like E2A, is involved in regulating the expression of *Ebf1* via a second *Ebf1* promoter, designated *Ebf1 β* . Therefore, in the absence of PAX5, *Ebf1* is only transcribed from the *Ebf1 α* promoter and does not reach the concentrations found in WT pro-B cells (67). EBF1, in turn, has been implicated in the repression of non-B-lineage genes (86,87), although a direct test of *Ebf1*'s role in maintaining B-cell commitment awaits the development of a conditional allele for *Ebf1* that allows its deletion after B-cell commitment. At the present time, studies are limited to an analysis of EBF1 function in cells or cell lines prior to B-cell lineage commitment (57,87). Nonetheless, EBF1 is capable of repressing the *Id2* and *Id3* genes (86). This observation is particularly intriguing, because E2A proteins have been implicated in the induction of *Id2* as well as *Ebf1* (37,88). Therefore, when E2A protein levels increase in CLPs both EBF1 and ID2 should increase, but these proteins have antagonistic effects on E2A function and B-cell development. The ability of EBF1 to repress *Id2* and *Id3* suggests that EBF1 augments E2A function through both positive feedback and repression of E protein inhibitors.

No other targets of EBF1 repression have been identified, and the mechanism of repression remains to be determined. Genome-wide association studies revealed that EBF1 does bind to genes that are repressed during B-cell development, but the relevance of most of these genes remains to be determined (89). In contrast, PAX5 repressed genes include essential regulators of other hematopoietic fates, such as the T-cell-associated genes *Notch1*, *Ptcra*, and the macrophage colony stimulating factor receptor gene *Csfr1* (84). Nonetheless, genome-wide association assays confirmed that EBF1 binding at *cis*-regulatory sequences is frequently associated with E-box binding sites (in 13.5% of cases), consistent with the fact that EBF1 and E2A proteins cooperatively regulate multiple B-cell-associated genes (89). In addition, genome-wide binding data for E2A, comparing *Ebf1*-deficient and sufficient cells, suggest that EBF1 may alter the chromatin context at *cis*-regulatory sites to allow binding by E2A, since the number of E2A bound sequences was nearly 3-fold higher in *Ebf1*-sufficient cells (90). EBF1 was shown to bind to some of its B-cell targets in a T-cell line and promote H3K4 dimethylation, a modification which may facilitate binding of E2A or other regulators of B-cell genes (89).

E proteins in pro-B lymphocytes

E47 homodimers are the major E-box binding proteins in pro-B lymphocytes (5). However, it is the dose of E proteins rather than the specific E protein that appears to be important for B-cell development. This conclusion is based on an analysis of the B-cell phenotype in mice carrying combined mutations in the *E2A*, *Heb*, or *E2-2* genes (91). Deletion of *Heb* or *E2-2* has a minor effect on B-cell development, but when combined with heterozygosity of *E2A*, their deletion results in a more striking effect on B-cell generation. Similarly, the dose of mutant forms of E12 required to rescue B-cell development from *E2A*^{-/-} MPPs is proportional to the transcriptional activation capacity of the mutants (6). The notion that the dose of E proteins is more critical than the identity of the E protein is also supported by experiments in which HEB was able to promote B-cell development when expressed in place of E2A from the *E2A* gene (70). Our data support the notion that high concentrations of E proteins are required for the initial induction of *Ebf1* but that lower levels are sufficient after EBF1 is expressed (68). This finding is likely a reflection of the multiple changes that occur in cells after expression of EBF1 and the fact that EBF1, E2A and PAX5, as well as

other factors such as ETS1 and FOXO1, function cooperatively to regulate a large portion of the B-cell transcriptome (90). Lower concentrations of E protein may be sufficient for these cooperative interactions, whereas higher concentrations of E proteins may be necessary for the initial induction of *Ebf1*. The altered chromatin context and cooperative interactions may explain why deletion of E2A leads to only a modest decline in *Ebf1* in pro-B cells or Abelson transformed B-cell lines (69,92).

In addition to its role in initiating B-cell lineage specification and commitment, E2A coordinates these differentiation events with IL-7-induced proliferation. In the absence of E2A, in the case where EBF1 is expressed ectopically in *E2A*^{-/-} MPPs, HEB, and E2-2 are sufficient to maintain pro-B-cell survival but do not allow expansion in response to IL-7 (68). This deficit is linked to an inability to up-regulate the IL7R signaling dependent gene *N-myc*. E2A is not required in pro-B cells for *Il7ra* expression, surface IL7R, or initial IL7R signalling events, because STAT5 activation is observed in *E2A*^{-/-} EBF1-expressing pro-B cells. Therefore, E2A is likely directly involved in regulating the *N-myc* gene. Inhibition of all E protein activity via ectopic expression of ID3 has much more severe consequences than deleting E2A in pro-B cells, inducing catastrophic growth arrest and caspase-2 dependent apoptosis (69,92,93). There are multiple mRNA changes upon ID3 expression that contribute to growth arrest, including decreased expression of *N-myc* and *c-myc* and decreased expression of genes encoding proteins involved in ribosome function (93). Therefore, E proteins are essential for the viability of pro-B cells and link their survival to the differentiation program.

E2A proteins were identified as regulators of Ig enhancers, and E proteins are essential for IgH expression in pro-B cells, although E2A itself is not absolutely required because *E2A*^{-/-} pro-B cells rescued with EBF1 undergo V_H(D_H)J_H rearrangement and express *IgH* at WT levels (68). In addition, *IgH* is among the E2A-dependent genes primed in LMPPs (37). Nonetheless, EBF1 and PAX5 clearly have important roles in controlling IgH expression and rearrangement. *Rag1* is the gene most affected in pro-B cells expressing ID3, indicating that E protein activity is critical for V(D)J recombination (93). In pre-B cells, E2A is indispensable for Igk light chain expression and rearrangement (94). Removal of the E2A binding sites in the Igk intronic enhancer impairs recombination (68,95) and expression of ID3 or deletion of E2A in pro-B cells or Abelson pre-B-cell lines severely impairs *Igk* gene transcription (93). Expression of either E12 or E47, in combination with the *Rag* genes, is sufficient to induce *Igk* locus recombination in non-lymphoid cells indicating that E proteins are sufficient to promote transcription of *Igk* in this context (96,97). In pro-B cells, IL7R signaling is known to prevent the activation and rearrangement of *Igk* and this has been shown to be mediated by STAT5, through its ability to impede the binding of E2A to the *Igk* intronic enhancer (98). These data demonstrate that E2A proteins are major regulators of *Igk* expression and rearrangement.

E proteins and T-cell development

In contrast to B lymphocytes, T precursors require the thymic environment to develop. In the adult, blood-borne TSPs enter the thymus at the cortical-medullary junction (CMJ), persist for about 10 days, then migrate outwards to the subcapsular zone and back to the medulla over the course of another 5 days (99). The most immature thymocytes lack mature lineage markers and both CD4 and CD8 major histocompatibility complex (MHC) coreceptors and are called 'double negative' (DN) cells (100). DN cells are further subdivided by expression of the adhesion molecule CD44, CD117, and CD25, the α chain of the interleukin-2 (IL-2) receptor. The immediate progeny of TSPs are CD44⁺CD117⁺CD25⁻ early thymic progenitors (ETPs), which also retain myeloid, NK, and DC potential. ETPs are heterogeneous, with a CCR9⁺ and Flt3⁺ subset that resembles the putative TSP and possesses

more B-cell potential and greater proliferative capacity than the CCR9⁻ and Flt3⁻ subset (43,101). DN2 stage cells, characterized by expression of CD25 and CD117, lack B-cell potential but can produce NK and DCs (2). The last non-T potentials are lost in the transition to the CD117⁻CD44⁻CD25⁺ DN3 stage during which thymocytes become further specified to the T-cell lineage as evidenced by expression of *Ptcra*, *Rag1*, and *Rag2* (2). The RAGs, in conjunction with constitutively-expressed DNA repair machinery, rearrange the *Tcrb*, *Tcrg*, and *Tcrd* loci in DN2/3 cells, allowing for further development based on the production of a functional pre-TCR containing preT α and TCR β or a TCR $\gamma\delta$ receptor.

E proteins and T-lymphocyte specification

Multiple transcription factors have been implicated in specification of the T-lymphocyte gene expression program, including NOTCH1, RUNX1, GATA3, IKAROS, GF11, LEF1/TCF1, and E2A. E2A proteins are required for normal thymocyte development. In their absence, the thymus is about 20% smaller than in a WT mouse and the mice develop an aggressive T-cell lymphoma by 3-6 months of age (13,102). E2A was reported to be up-regulated in DN2 cells in parallel with the inception of T-lymphocyte specification and commitment (103,104). However, these early studies examined E2A expression in DN thymocytes without depleting Lin⁺ cells, and therefore the DN1 population was heterogeneous and not highly enriched for ETPs. Using new staining strategies that allow a better resolution of the ETP and DN2 subsets, we found that E2A is expressed at similar levels in ETPs, DN2 and DN3 cells and is up-regulated in a subset of DN4 cells (Fig. 2). These findings are largely consistent with recent mRNA expression analysis for E2A in Lin⁻ thymocytes (2). In addition, we found that *E2A*^{-/-} mice have severely reduced numbers of both ETPs and DN2 cells (Xu and Kee, unpublished data), a finding that is consistent with the reduced number of LMPPs and CLPs in *E2A*^{-/-} mice, and their failure to express the thymus-homing chemokine receptor CCR9 (37). *Flt3l*^{-/-} mice, which also have a reduced number of LMPPs, and *Ccr9*^{-/-} mice, have similar defects in the generation of ETPs and DN2 cells, indicating that LMPP/CLP numbers and homing potential directly determine ETP and DN2 cell numbers (24,42).

In contrast to E2A protein levels, HEB and HEBALT are upregulated at the DN2 and DN3 stages and this induction may be uniquely dependent on signals derived from the thymus microenvironment (2,14). In extracts of total thymocytes, E-box binding complexes are composed predominantly of an E2A:HEB heterodimer (12,13). The incomplete arrest in T-cell development in *E2A*^{-/-} mice has been attributed to partial redundancy with HEB, and this conclusion is consistent with the observation that targeting a DNA-binding defective mutant of HEB, that can dimerize with and inhibit E2A, into the *Heb* locus, causes a severe reduction in the total number of thymocytes (12). However, in contrast to *E2A*^{-/-} thymocytes, *Heb*^{-/-} thymocyte numbers are not reduced until after the DN stage of development, when immature CD8⁺ cells normally undergo proliferation (105), indicating that HEB is not absolutely necessary for differentiation through the DN stages of T-cell development. HEB deficiency also results in thymocyte numbers that are 10%-20% of WT and a loss in the distinction between the thymic cortex and the medulla (105). DN thymocytes and $\gamma\delta$ T-cell numbers are unaffected by the absence of HEB, in keeping with a defect that arises after the DN3 stage (105). There is also evidence that E2-2 contributes to T-cell development, and in mice expressing ID1 as an Lck-driven transgene there is massive apoptosis of thymocytes that is more severe than in the absence of either E2A or HEB (106,107). Taken together, these findings demonstrate that E proteins, primarily E2A homodimers and E2A-HEB heterodimers, play essential roles in T-cell development during lineage specification and commitment. However, the regulatory networks in which E2A functions remain to be fully revealed.

E2A and Notch1

One of the critical specification and commitment factors for early T-cell development is NOTCH1, a transmembrane receptor that also functions as a transcription factor. Mammals have four Notch receptors (12), and five ligands: three Delta-like ligands (Dll1, 3 and 4), and two Serrate-like molecules called JAGGED 1 and JAGGED 2. NOTCH1 and Dll4 are the Notch receptor-ligand pair essential for T-cell development (108). Upon binding of Notch1 to its ligand, the intracellular portion of Notch (ICN) is released by proteolytic cleavage and translocates to the nucleus (reviewed in 109). In the nucleus, ICN binds the constitutively DNA-bound recombination signal binding protein for immunoglobulin kappa J region (RBPJk) or CBF1/suppressor of hairless/Lag-1 (CSL). ICN then forms a complex with CSL, which is stabilized by mastermind-like (MAML) proteins, and recruits coactivators such as p300 and the histone acetyl transferases PCAF and GCN5 to activate target gene transcription. In the context of T-cell development, the critical targets that mediate many of ICN's functions have not been identified, although ICN clearly regulates some essential T-cell-associated genes, including *Ptcra*, *Cd25*, and *Hes1*, an important regulator of DN3 cell proliferation (110).

The importance of Notch signaling in T-cell development has been demonstrated through both gain- and loss-of-function experiments. In the absence of NOTCH1, the ETP population is absent, although the bone marrow LMPP population is intact (111). Progenitors lacking NOTCH1 or CSL, or expressing negative regulators of Notch signals generate an increased number of B lymphocytes in the thymus (112). Extrinsic factors that block progenitors from receiving Notch signals, such as γ -secretase protease inhibitors (113), or other progenitors with a competitive advantage in Notch binding (114), also result in accentuated intrathymic B-cell development. At least a subset of these progenitors has been demonstrated to arise from cells that were destined for the T-cell fate (115). In contrast to Notch deficiency, ectopic NOTCH1 activation enforces the T-cell fate *in vitro* and in the bone marrow, at the expense of B-cell development (116-122), although constitutive Notch signals frequently result in aberrant development that is arrested at the CD4⁺CD8⁺ stage and a high frequency of leukemic transformation involving activation of the proto-oncogene *c-myc*.

There have been many indications that E proteins and the Notch signaling pathway interact during T-cell development. In LMPPs, E2A is required for proper expression of *Notch1*, suggesting that any ETPs that develop in *E2A*^{-/-} mice would be defective in their ability to generate T cells. *In vitro*, *E2A*^{-/-} hematopoietic progenitor cell lines and primary progenitor cells cultured in fetal thymic organ culture (FTOC) do fail to generate DN3 cells, and this deficiency can be overcome by ectopic expression of ICN (123). These findings demonstrate the power of ICN to promote T-cell development and are consistent with the hypothesis that *Notch1* is a critical target of E2A whose decreased expression prevents T-cell development *in vitro*. However, analysis of *Notch1* mRNA expression in *E2A*^{-/-} DN2 and DN3 cells revealed that it is present at near WT levels (Xu and Kee, unpublished data). The molecular basis for this increase in *Notch1* mRNA is not known; however, *Notch1* is a target of the ICN/CSL/MAML complex and therefore the low level of NOTCH1 protein on the surface of *E2A*^{-/-} TSPs may be sufficient to initiate the Notch signaling pathway leading to up-regulation of *Notch1* mRNA. Alternatively, HEB/HEBALT, alone or in combination with other transcription factors, may be sufficient for *Notch1* expression in the thymus. That Notch signaling is functional in *E2A*^{-/-} DN2/DN3 cells seems likely given that they express WT levels of the Notch signaling target gene *Deltex1* and can generate some mature T cells (Xu and Kee, unpublished data). However, another group reported that *Deltex1* is increased in the DN1-DN2 transitional cells in the DN compartment of ID1 transgenic mice and that it may function to inhibit Notch signaling (124). Importantly, these transitional cells are not

apparent in the $E2A^{-/-}$ thymus, indicating that the defects in this population may be unrelated to the functions of E2A in T-cell development.

Notch signaling has been proposed to inhibit E2A function under some conditions. In cells with high levels of activated mitogen-activated protein kinases, such as pro-B cells, Notch signaling promotes the degradation of E2A (125-127). However, during T lymphopoiesis, NOTCH1 and E2A have been shown to collaborate both directly (123,128,129) and indirectly via Deltex1 (130,131). Transduction of $E2A^{-/-}$ progenitors with tamoxifen-inducible E47 (E47ER) directly upregulates targets of the Notch signaling pathway, including *Hes-1*, and *Notch1*, and expression of these genes is reduced in $E2A^{-/-}$ hematopoietic progenitor cell lines (123). Further, Notch and E2A synergize to upregulate *Ptcr* and *CD7*, and to enhance *Tcrb* rearrangement (123,128,132,133). However, ectopic ICN1 restores in vitro CD4⁺CD8⁺ DP development from $E2A^{-/-}$ fetal thymocytes, suggesting that sufficiently strong Notch signals can overcome the requirement for E2A activity, although not excluding that there is still some compensation from HEB and/or E2-2 (123). The accumulating evidence supports a collaborative role for E2A and Notch signaling in T-cell specification.

T-cell commitment

NOTCH1 is the only protein that has been shown to be sufficient for specification and ultimately commitment to the T-lymphocyte fate, at least when activated in hematopoietic progenitors (134). Nonetheless, activation of NOTCH1 upon entry into the thymus does not immediately commit cells to T cell differentiation. Rather, a slow loss of lineage potentials occurs that is likely dependent on progressive changes in transcription factor expression or function, or accessibility to DNA (2). Unlike Pax5, however, Notch1 is not active at all stages of T-cell development and inhibiting Notch signaling after full commitment to the T-cell lineage does not cause lineage instability or expression of non-T-cell gene programs. Indeed, signaling through the pre-TCR results in a down-regulation of *Notch1* mRNA as a consequence of ID3 induction and inhibition of E protein function (135). Therefore, Notch signaling establishes an appropriate context for T-cell lineage commitment but is not required to maintain this program in committed cells. Analysis of gene expression in T-cell progenitors revealed that commitment to the T-cell lineage is associated with a loss of stem cell-associated transcription factor genes such as *Tal1*, *Gata2*, and *Sfp1*, rather than a dramatic induction of T-cell-associated gene expression (1,136). This observation suggests that stem cell transcription factors may be maintaining access to alternative differentiation programs and that extinguishing their expression is necessary to prevent access to these programs by transcription factors that function in T-cell differentiation. Interestingly, one transcription factor that does increase during the commitment process is the transcriptional repressor BCL11b and recent reports demonstrate that deletion of *Bcl11b* in thymocytes leads to activation of NK cell gene expression and lineage conversion (137-139). Therefore, BCL11b plays a role in T-cell lineage commitment akin to that of PAX5 in B lymphocytes. However, the regulatory networks linking BCL11b to NOTCH1 and E proteins are currently not known.

A role for E2A in commitment to the T-cell fate has not been thoroughly investigated. There are more DN3 cells in $E2A^{-/-}$ mice than predicted based on the number of DN2 cells, suggesting that T-cell differentiation is recovering by this stage and that the development of committed cells is not affected by E2A deficiency (Xu and Kee, unpublished data). However, there are multiple observations that suggest a requirement for E2A in the generation of DN3 cells. First, unlike $E2A^{-/-}$ mice, *Flt3L*^{-/-} and *Ccr9*^{-/-} mice, which both have reduced ETP and DN2 cells populations, completely recover WT levels of DN3 cells (42,140). Second, $E2A^{-/-}$ DN3 cells are hyper-proliferative, indicating that the actual number of DN2 cells progressing to the DN3 stage may be lower than evident by the steady

state number of DN3 cells (141). Third, $E2A^{-/-}$ hematopoietic progenitors give rise to more NK cells than T cells when cultured in vitro or in FTOC (123). One possible explanation for reduced ability of $E2A^{-/-}$ DN2 cells to suppress NK cell differentiation could be a subtle difference in NOTCH1 expression levels. However, given the dynamic relationship between ID proteins and commitment factors for the B-cell fate, it seems likely that a similar dynamic exists between T-cell commitment factors and ID proteins in DN2 cells. BCL11b is a clear candidate for this antagonistic factor and Id2 is increased after deletion of *Bcl11b* in T cells (137-139); however, published data do not clearly support a role for E2A in the induction of BCL11b (136). Therefore, the mechanism by which E proteins suppress the NK cell fate remain to be determined.

E2A and Gfi1/1b

To identify E2A target genes in T lymphocytes, two groups have compared mRNA expression profiles in $E2A^{-/-}$ T-cell lymphomas retrovirally transduced with a bicistronic retrovirus encoding GFP alone or E47 and GFP (142,143). Restoring either E47 or E12 expression to $E2A^{-/-}$ T lymphomas results in growth arrest and apoptosis (144,145), leading to the hypothesis that E2A proteins remain capable of inducing their target genes in these lymphomas. In one of these retroviral screens, the transcriptional repressor growth factor independence 1b (GFI1b) was identified as a direct E47 target (142). In the other, Gfi1 was shown to be regulated downstream of E47 (143). In both screens, *Gata3* mRNA decreased after expression of E47. GFI1 and GFI1b are of particular interest as potential E47 targets because they are paralogues of the *Drosophila* gene *sens*, which is a known target of E proteins during fly sensory organ development (146). Transgenic expression of GFI1b under the control of the Ick promoter had already been demonstrated to reduce DP numbers (147), suggesting that it might mediate E47 induced growth arrest and apoptosis in $E2A^{-/-}$ lymphomas. Comparison of $E2A^{-/-}$ lymphoma lines transduced with GFI1b or control vector revealed that GFI1b does inhibit their proliferation and survival (142). GFI1b repressed GATA3 as well as GFI1, which was previously known to be repressed by GFI1b (148), and GATA3 was shown to be essential for the proliferation and survival of the lymphomas (142). Interestingly, in these cells GATA3 promoted the expression of GFI1b, suggesting an autoregulatory loop in which GATA3 might fine tune its own expression to control survival or proliferation. However, if E2A and GATA3 cooperatively regulate *Gfi1b*, *Gfi1/1b* may be less responsive to GATA3 in $E2A^{-/-}$ thymocytes and the GFI1/1b-mediated feedback inhibition of *Gata3* may be defective, causing GATA3 to promote aberrant proliferation and survival of $E2A^{-/-}$ thymocytes. This model is consistent with the tendency of CD2-Gata3 transgenic mice, like $E2A^{-/-}$ mice, to develop an immature T-cell lymphoma.

Gata3 and T-cell lineage commitment

There is abundant evidence that GATA3 plays a role in both T-lymphocyte lineage specification and commitment (149-151). GATA transcription factors are characterized by two highly conserved C2C2 zinc fingers in the C-terminal domain which cooperatively recognize a T/A(GATA)A/G consensus sequence (152). In mice, complete deficiency in GATA3 results in death before e12.0, as a consequence of noradrenalin deficiency (153). RAG-2^{-/-} blastocyst complementation experiments show that *Gata3*^{-/-}, *Gata3*^{+/-} and WT cells contribute similarly to B-cell and myeloid compartments, but *Gata3*^{-/-} cells fail to contribute to the T-cell lineage (151). Recently studies also showed that GATA3 is required at the earliest stage of T-cell development, since ETPs are already decreased in *Gata3*^{-/-} mice (154). *Gata3* is expressed at very low levels in bone marrow LSKFlt3⁺ cells but is increased upon entry of TSPs into the thymus or when MPPs are cultured on stromal cells expressing DL1 (136,155), indicating that Notch signaling controls expression of *Gata3* early in development. *Gata3* is expressed from two distinct promoters during T-cell development and only the *Gata3a* promoter is functional during thymopoiesis (156-158).

Notch signaling has been shown to regulate expression of the *Gata3b* promoter in Th1 cells, but there is currently no evidence for a direct effect of the ICN/CSL/MAML complex on the *Gata3a* promoter (159,160). Therefore, the mechanisms underlying *Gata3* induction during thymopoiesis remain elusive.

While GATA3 is essential for T-cell development, overexpression of GATA3 blocks T-cell differentiation at the DN2 stage, prior to commitment (161,162). Importantly, in the absence of Notch signaling, GATA3^{hi} DN2 cells are reprogrammed to a mast cell fate through the rapid induction of mast cell genes, including *Gata2*, and the transient downregulation of *Sfp11*. However, Notch signaling prevents the reprogramming of DN2 cells by GATA3 and an anti-proliferative effect of ectopic GATA3 on T-cell progenitors is revealed (161). The ability of increases in GATA3 expression to reprogram DN2 cells into mast cells suggests that there must be tight control of *Gata3* expression during T-cell specification and commitment. Our observation that E2A, through induction of GFI1b, can repress *Gata3* in T-cell lymphoma lines, suggests that E2A may play a role in preventing GATA3 from blocking T-cell lineage commitment or diverting DN2 cells away from the T-cell pathway. However, GFI1b is not highly expressed in T-cell progenitors and therefore the importance of this pathway in E2A-dependent T-cell development remains to be demonstrated (136,142). Nonetheless, GFI1 is also regulated by E2A and GATA3 under some conditions and could contribute to limiting *Gata3* at these critical stages of T-cell development. Future studies need to be directed at identifying the *cis* regulatory mechanisms controlling expression of *Gata3* and how the transcription factors expressed at the DN2 stage function to promote differentiation and commitment to the T-lymphocyte lineage.

Concluding remarks

The E protein transcription factors are nearly ubiquitously expressed and yet play unique and essential roles at multiple stages of adaptive lymphopoiesis. For a summary of the functions we have described in this review see Fig. 3. Specificity in the functions of E protein transcription factors arise as a consequence of (i) differences in protein dimerization partners, which can alter the concentration of active E proteins, (ii) differences in expression levels, (iii) differences in chromatin context, which can influence which E protein target genes are accessible, and (iv) differences in the complement of cooperating transcription factors. We have argued here that the earliest functions for E proteins in lymphopoiesis are in the development and maintenance of HSCs as a consequence of their dimerization with TAL1 and LYL1, although no single E protein has thus far been shown to be absolutely essential. As HSC differentiate toward the lymphoid fates, E protein homodimers, composed predominately but not exclusively of E2A proteins, collaborate with other transcription factors in MPPs and LMPPs to promote lymphoid-priming and maintain cells on a trajectory to the lymphoid fates. Indeed, without these E2A proteins, LMPPs rapidly adopt a myeloid fate. Within lymphoid specified cells, E2A proteins are necessary to initiate the transcriptional regulatory network that results in B-lymphocyte lineage specification and commitment, and they likely prevent differentiation toward the NK cell fate, although this remains to be demonstrated. However, after commitment to the B-cell lineage lower concentrations of E protein activity are sufficient for the cooperative interactions that maintain the B cell program. The role of E proteins in specification and commitment to the T-lymphocyte lineage are less well defined, but the decreased number of DN3 cells and propensity to develop T-cell lymphoma indicate that E2A plays a critical role in guiding this process. Future studies should be directed at identifying E2A target genes in T lymphocytes and placing E proteins into the transcriptional networks that result in commitment to the T-cell fate. The mechanisms guiding T-cell lineage commitment are likely to have some parallels with B-cell lineage commitment; however, it is already clear that critical differences exist in the architecture of these programs. For example, elevated expression of

GATA3, a critical regulator of T-cell lineage specification, arrests T-cell development prior to commitment and divert cells away from the T cell pathway in the absence of activated NOTCH1. E2A proteins likely play a role in harnessing GATA3 expression since targets of E2A in T-cell lymphomas are able to modulate the abundance of *Gata3* mRNA. Nonetheless, the myriad of functions of E proteins in the development of adaptive lymphocytes are just beginning to be revealed. Understanding these functions will have important implications for controlling lymphocyte develop and preventing malignant transformation.

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Fig. 1. Mechanism of lymphocyte specification and commitment

E2A collaborates with multiple factors to promote lymphoid priming, which is the low abundance expression of multiple lymphocyte-associated genes. The complement of genes induced in individual LMPPs may differ depending on the concentration of each of the transcriptional regulators leading to a heterogeneous population. Heterogeneous lymphoid priming leads to the expression of multiple receptors and signaling molecules that allows variation in the response to extrinsic signals. These signaling pathways (IL-7R or Notch signaling) can induce transcription factors that collaborate with E proteins to induce novel transcriptional regulatory networks required for B or T lymphopoiesis. Commitment to the adaptive or innate lymphoid pathways involves activation of repression of essential regulators of the alternative fate.

Fig. 2. E47 is expressed at similar levels in all DN thymocyte subsets

(A) Total thymocytes from C57Bl/6 mice were stained with a lineage cocktail including antibodies to CD8, CD3, TCR β , TCR γ , CD11b, CD11c, Gr1, NK1.1, and B220 as well as CD44, CD25 and E47 or control IgG. CD44 and CD25 expression are shown on Lineage negative thymocytes. B) Histogram showing E47 staining in ETPs (Lin⁻CD44⁺CD35⁻), DN2s (Lin⁻CD44⁺CD25⁺), DN3s (Lin⁻CD44⁻CD25⁺), and DN4s (Lin⁻CD44⁻CD25⁻). Isotype control staining is shown in gray.

Fig. 3. Regulatory Networks in Lymphopoiesis involving E2A

The model shows precursor progeny relationships in the lymphopoietic system denoted purple arrows. Transcriptional regulation is depicted using green arrows for positive regulation and red bars for negative regulation.