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DETERMINING THE CELL EXTRINSIC MECHANISMS DRIVING E2A DEFICIENT T CELL LEUKEMOGENESIS

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Abstract

T cell Acute Lymphoblastic Leukemia (T-ALL) is a highly heterogeneous hematological malignancy resulting from the transformation of immature thymocytes during their development. Despite this heterogeneity, there are several recurring mutations fond in the majority of T-ALL patient. 60% of all pediatric T-ALL patients have mutations in the *TAL1* gene, and 10% of have mutations in the related *LYL1* gene.[1] TAL1 and LYL1 are transcription factors typically expressed in hematopoietic progenitors that form complexes with the transcription factor E2A, which then recruits other factors including the LMO proteins, LDB1, and GATA proteins.[2] This complex regulates numerous functions in hematopoietic progenitors, including quiescence, self-renewal, and antagonizes lymphoid development.[2–5] In the thymus, *TAL1* and *LYL1* expression is extinguished as thymic progenitors receive T cell lineage specifying signals and commit to the T cell lineage.[6] As TAL1 and LYL1 expression is extinguished, E2A dimerizes with another transcription factor, HEB, to enforce the T cell lineage and promote healthy T cell development.[7,8] One major oncogenic function of ectopic TAL1/LYL1 expression is the inhibition of these E2A:HEB dimers and the repression of the T cell lineage specifying function of E2A. Indeed, mouse models that ectopically express the inhibitors of E2A DNA binding, the ID proteins, or delete E2A in the germline faithfully recapitulate many features of human TAL1^{+} T-ALL.[8–11] I use multiple mouse models that delete E2A at different stages of T cell development to investigate the different functions of E2A and how these functions contribute to the suppression of T-ALL. Deleting *E2a* at the hematopoietic stem cell stage was sufficient to induce robust leukemogenesis, while deleting *E2a* at the DN2/DN3 stage was only weakly oncogenic. Additionally, only early deletion resulted in the upregulation of the T-ALL-associated pathways Notch, Wnt/β-catenin,

NFκB, and Myc. Further, the early deletion induced developmental defects in the thymocytes, which resulted in a 3-5 fold decrease in the number of thymocytes at 3-5 weeks of age. This reduction in the number of thymocytes in the early deletion correlates with an inability of thymocytes to provide a competitive environment. Further, the later deletion showed no signs of impaired competition, and restoring competition in the early deletion was sufficient to significantly inhibit leukemogenesis. Restoring competition induced several transcriptional changes in the E2A deficient thymocytes, most notable of which were an inhibition of the Myc pathway and an induction of apoptotic pathway genes. Overall, my analysis provide insight into how the stage at which the tumor suppressor E2A differentially predisposes thymocytes to transformation and how early deletion promotes leukemogenesis through induction of oncogene activation and rendering thymocytes unable to out compete pre-leukemic cells.

CHAPTER 1:

INTRODUCTION

INTRODUCTION

T CELL ACUTE LYMPHOBLASTIC LEUKEMIA

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy that accounts for 15% of pediatric and 25% of adult acute lymphoblastic leukemia cases. These leukemias are highly heterogeneous, however there are several recurring mutations that are found across most T-ALL cases.[1,12] To date, mutations or dysregulated expression of multiple transcription factors (*cMYC*, *IKZF1*, *GATA3*, *TCF7*, *LEF1),* epigenetic regulators (*EZH2*, *PHF6*, *TET2*), cytokine receptors (*IL7Rα* and *FLT3*), cell cycle regulators (*CDKN2A* and *CDKN2B*), and signaling proteins (*PTEN*) have been recurrently identified in subsets of human T-ALL.[12–15] In addition to these mutations there are 2 pathways mutated in almost all T-ALL patients. The first is the Notch pathway, of which one or more components are mutated in ~80% of all T-ALLs.[16] The second is the basic Helix-loop-helix family. ~60% of pediatric T-ALL patients have mutations in genes that impact E protein DNA binding, including the *TAL1* or *LYL1* genes, which require dimerization with the E-proteins to bind DNA,[17–19] 10-15% of patients overexpress *LMO2*,[12] which is a core component of the TAL1:E2A complex,[20] and many patients overexpress the transcription factor *ID2*,[21] which inhibits E-protein DNA binding. The high frequency of all these mutations indicates that the Notch pathways and E2A DNA binding impact key processes underlying T cell homeostasis and development, that if dysregulated, drive transformation (Figure 1.1). Indeed, the transcription factors commonly dysregulated in T-ALL are highly stage specific and known to impact healthy T cell development. Consistent with this, the subtype of T-ALL frequently mirrors the stage of immature T cell development at which the dysregulated transcription factors are required.[1,21,22]

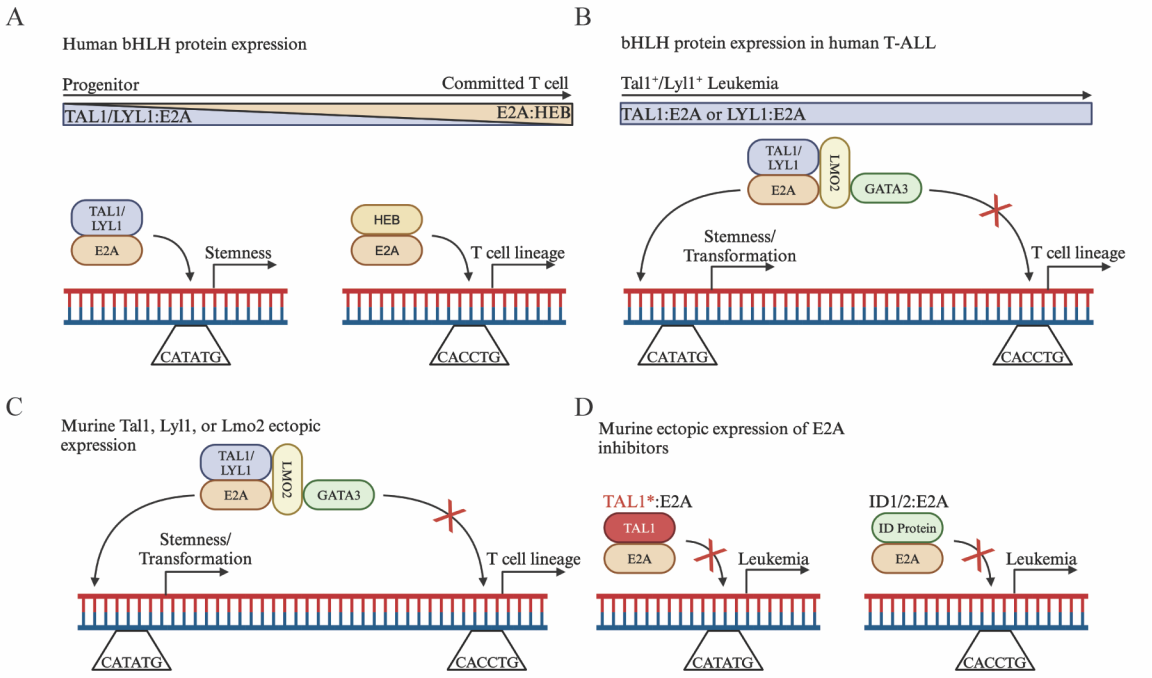

Figure 1.1: Mechanisms of E protein repression in human and murine T-ALLs
A) Schematic of E protein dynamics in normal human hematopoeisis and thymopoeisis. B) Schematic of E2A repression induced by TAL1 or LYL1 overexpression in human TAL1/LYL1^{+} T-ALLs. C) Mechansism of E2A repression induced by TAL1 or LYL1 overexpression in murine TAL1, LYL1, or LMO2 transgenic models. D) Mechanism of E2A repression in DNA-bindiing-domain mutant TAL1-tg (Left) and ID1/2-tg mouse models of T-ALL

TRANSCRIPTIONAL REGULATION OF T CELL DEVELOPMENT

T cells are a major component of the immune system tasked with integrating signals from numerous sources and directing the appropriate response. T cell development is an intricate process requiring tightly regulated, stepwise progression from uncommitted hematopoietic progenitors to lymphoid-lineage primed progenitors in the bone marrow. Once adequately primed for the lymphoid lineage, T cell progenitors egress from the bone marrow and settle in the thymus, a specialized organ that nurtures their development into mature, functional T cells.[23] Interestingly, the development of T-ALL, and its subsequent subtype, is highly associated with the misregulation or mutation of transcription factors that normally drive healthy T cell development.[18]

Starting in the bone marrow, the most immature unit of hematopoiesis is the hematopoietic stem cell (HSC)[24] defined by lack of lineage marker expression (CD3, CD19, CD11b, CD11c, Gr-1, and Ter119 negative) and expression of Sca-1 and Kit ($lin^{-}Sca\text{-}1^{+}Kit^{+}$; LSK).[25] TAL1, and by analogy its E protein partners, plays a critical role in specification of hematopoietic stem cells (HSCs) from hemangiogenic endothelium but TAL1 is not essential for HSC survival after HSC development.[26,27] While not essential, TAL1 can function to help main HSC quiescence through repression of the cell cycle gene *Cdkn1a*.[2] TAL1 also plays important roles in megakaryocyte differentiation and erythropoiesis.[28,29] A transcription factor closely related to TAL1, LYL1, similarly forms complexes with E2A and maintains an HSC's capacity to self-renew.[3,4] LYL1, unlike TAL1, is not essential for HSC specification, although it becomes essential when TAL1 is limiting, indicating that these proteins have some redundant functions.[5] ChIP-seq experiments with TAL1 and LYL1 in HSC-like cell lines revealed extensive overlap in their binding sites indicating that these proteins regulate overlapping set of genes.[30]

The next step towards development of T cells is differentiation of HSCs into lymphoid primed multipotent progenitors (LMPP).[31] LMPPs are found within the LSK pool but are identified by high expression of Flt3 ($LSK^{+}Flt3^{+}$).[25] LMPPs have lost erythroid and megakaryocyte potential but retain the ability to develop myeloid and lymphoid lineages, with a significant bias towards the lymphoid lineage.[31] In LMPPs, TAL1 functions to antagonize lymphoid gene expression, as evidenced by *Tal1*$^{-/-}$ LMPPs showing upregulation of lymphoid lineage genes such as *Rag1 & 2*, *Il7ra*, and *Notch1*.[32] LMPPs are the first population that attain the ability to egress from the bone marrow and traffic to the thymus. Thymic homing is dependent on the expression of chemokine receptors CCR7/CCR9,[33,34,35] which interact with CCL19,[36] CCL21,[36,37] and CCL25[37,38] to drive thymic immigration. CCR7 and CCR9 expression are partially redundant, as deficiency in one reduces, but does not fully ablate, thymus seeding.[33] Regulation of CCR9 on LMPPs is multifaceted, being positively regulated by E-protein signaling and negatively regulated by NOTCH1.[39] While LMPPs are able to colonize the thymus, only a small fraction of the LMPP pool express CCR7 and CCR9. If thymus seeding is a rare event, then the small fraction of LMPPs that coexpress CCR7 and CCR9 may be sufficient to seed the thymus. However, LMPPs give rise to another, more differentiated population of cells, called the Common Lymphoid Progenitors (Lin^{-} $Sca\text{-}1^{+}Kit^{lo}IL\text{-}7r\alpha^{+}$; CLP). CLPs also express these chemokine receptors and are implicated in thymus seeding.[40]

Thymus seeding cells enter the thymus at the cortico-medullary junction where the T cell lineage developmental program is initiated.[41,42] The earliest stage of thymocyte development is the early T cell precursor ($Lin^{-}Kit^{+}CD25^{-}CD44^{+}$; ETP). ETPs retain the ability to differentiate into alternate lineages including: Dendritic cells,[43,44,45] Natural Killer cells,[46] and B cells[46,45] under certain conditions. The core signal that initiates T cell lineage development in ETPs is NOTCH1.[47]

NOTCH1 functions as both a surface receptor and transcription factor and is essential for T cell development.[16,48] The ligands for NOTCH1 are members of the Jagged and Delta-like family, with DELTA-LIKE 4 (DLL4) being the most important in the thymus.[49] NOTCH1 is translated as a single protein that is cleaved in the Golgi to create extracellular and intracellular components that are held together in the membrane by heterodimerization domains (HD).[16] Upon ligand binding the extracellular portion undergoes a conformational change that allows cleavage by a disintegrin and metalloprotease, which exposes a cleavage site for g-secretase, which then cleaves and liberates the intracellular domain of NOTCH1 (called ICN). ICN translocates to the nucleus, where it complexes with the DNA bound transcriptional repressor CBF1/RBP-Jk and recruits Mastermind (MAML) proteins to initiate the transcription of multiple genes that promote T cell specification.[16] Activation of NOTCH1 is transient owing to the presence of a PEST sequence at the 3' end that is recognized by the FBW7 ubiquitin ligase and targets ICN for proteasomal degradation.[50] Targets of the ICN/CBF1/MAML complex in murine T cells include *Hes1* and *Tcf7* (protein: TCF1), both of all of which play critical roles in T cell development.[51–53] Interestingly, Notch signaling also shuts down transcription of *Ccr9*.[33,39]

The Notch family genes in ETPs are required for the development of ETPs, as shown by experiments in mice that have been transduced with a dominant negative Notch inhibitor. These mice are lacking all ETPs and downstream T cells.[54] Mice with mutations in the *Ikzf1* (IKAROS) gene have similar phenotypes, notable due to IKAROS' known interactions with the *Notch1* gene.[55–57] NOTCH1 signaling induces expression of *Tcf7*,[58] which in turn promotes expression of *Gata3*.[58,59] However, these transcription factors alone are insufficient to force T cell lineage commitment at the ETP stage, but the coordinated effort of Notch1 signaling combined with *Tcf7* and *Gata3* expression drives transition to the next stage of T cell development, the CD4/CD8

Double Negative 2 ($CD4^-CD8^-Kit^+CD25^+$; DN2) stage.[59,60,61,62] At this stage, *Tcf7*,[63] *Gata3*,[63] and *Bcl11b*[64] coordinate to fully commit developing thymocytes to the T cell lineage through significant reorganization of the chromatin landscape and inhibition of alternate lineage potential.[65,66,6] Commitment to the T cell lineage is concomitant with the transition from the DN2 stage to the Double Negative 3 ($CD4^-CD8^-Kit^-CD25^+$; DN3) stage. At this stage, the majority of DN3s undergo RAG-mediated V(D)J recombination of the β chain of the TCR, while some recombine their γ and δ chains at this stage[67]. After recombination, the TCRβ chain associates with the pre-TCRα[68] to allow surface expression and check if the TCRβ chain is functional and able to interact with cognate MHC molecules.[69,70] Failure to generate functional TCRβ chains results in apoptosis[71] and a failure to pass into later stages,[72] while successful rearrangement induces expression of the transcription factor ID3,[73] the antiapoptotic molecule BCL2,[71] hyper-profileration,[74] and progression to the next stages of development. Passage through β-selection is concomitant with repression of Notch1 target genes such as *Il2rα* (CD25), which may be due to actions of IKAROS. ChIP-seq experiments show significant overlap in IKAROS bound and NOTCH1 bound genes, albeit at different locations within the genes. Loss of IKAROS function also correlates with increased expression of the co-bound genes, showing that IKAROS functions to restrain Notch1 target genes at this stage.[56] This is notable because IKAROS and TCF1 have also been shown to be vital for promoting expression of an alternate isoform of NOTCH1 that lacks the extracellular domain, and thus requires no ligand interactions to activate signaling. Expression of this isoform is dispensable during the ETP-DN2 stages, but vital for development of DN3s and passage through β-selection.[57] Indeed, IKAROS deficient DN3s can pass the β-selection checkpoint in the absence of V(D)J recombination,[55] but whether IKAROS allows

passage through β-selection via its' role in *Notch1* regulation or through other mechanisms is undefined.

Upregulation of the CD8 coreceptor after successful β-selection defines the highly proliferative Immature Single Positive stage ($CD4^-CD8^+TCR\beta^-$; ISP). Notably, ISPs represent the first stage of TCD that are no longer dependent on Notch for their survival.[75] Further, IL-7 signaling is repressed at this stage as evidenced by high IL-7 signaling preventing passage into the next stage.[76] Thus, ISPs appear to act as an intermediate stage where thymocytes transition to a new phase that is not reliant on the external signals needed previously. The final stage of early thymocyte development is the Double Positive ($CD4^+CD8^+$; DP) stage. It is at this stage where the second TCR segment, the α chain, is rearranged and the fully developed TCR is selected in a similar process as β-selection in the DN3 stage.[70] NOTCH1 signaling is actively repressed in DPs, and IKAROS, TCF1, and other transcription factors repress the alternate *Notch1* isoform used in DN3 cells.[57] Further, TCF1 interactions with IKAROS, ETS, RUNX, and the E-protein HEB (in a complex with E2A[77,78]) establish the epigenetic identity of DP thymocytes through nucleosome remodelling.[79] TCF1 has other functions in DPs, promoting survival through regulation of the anti-apoptotic protein BCL-XL.[80] This allows DPs to survive long enough to fully rearrange the α chain of the TCR, facilitating positive selection and transition to the next stage of T cell development.[81] Upon successful positive selection DPs traffic to the thymus medulla,[70] where they are checked for self-reactivity before finally exiting into the periphery.[70]

Successful T cell development is highly dependent of tight regulation of several transcription factor networks. Dysregulation or mutations in any one of these transcription factors is sufficient to initiate a cascades that can eventually lead to T-ALL development. However, one family of transcription factors, the E-protein family, is vital for T cell development by regulating

the previously discussed transcription factor networks, and the main factor in this family, *E2a*, acts as potent tumor suppressors.

E-PROTEINS IN T CELL AND T-ALL DEVELOPMENT

The *Tcf3* (*E2a*; protein E2A) gene encodes 2 bHLH proteins (E12 and E47) through alternative splicing of exons encoding the bHLH domain.[82] The HLH domain is involved in dimerization with other HLH proteins, and the basic region is largely responsible for DNA binding, although some DNA contacts are made with the HLH domain.[17] The bHLH domains of E12 and E47 share approximately 80% identity and they bind the same DNA motif, although with differing affinity, and interact with the same proteins.[83,84] There are two additional genes encoding for E proteins in humans and mice, *Tcf12* (protein: TCF12 or HEB) and *Tcf4* (protein: TCF4 or E2-2), that each code for two E box binding proteins through alternative transcription start sites, resulting in proteins with differing activation domains but identical bHLH domains.[78] Other proteins that dimerize with E proteins include the Class IV HLH proteins (ID1-4), which lack a DNA binding domain and therefore prevent E proteins from stably binding DNA, and Class II bHLH proteins, which are largely cell type specific (i.e. MYOD in muscle cells and TAL1 in hematopoietic stem cells).[17] E proteins are broadly and constitutively expressed and are generally found in complexes with tissue- restricted Class II proteins. However, E proteins function as homodimers in lymphocytes; E2A homodimers predominating in B lymphocytes and dimers of E2A and HEB being prevalent in T lymphocytes.[78,82] Consistent with this, $E2a^{-/-}$ mice have severe defects in lymphopoiesis with a complete lack of B lymphocytes and a 3-5X decrease in thymocyte numbers prior to the onset of leukemogenesis.[8,10,85,86] *Tcf12*-deficiency or *Tcf4*-deficiency also impacts T

cell development but to date, neither of these deficiencies is sufficient to promote T-ALL like disease.[7,78,87,88]

In *E2a*$^{-/-}$ mice, HSC specification is intact but lymphopoiesis is impacted at the stage when HSCs become specified to the lymphoid fate with fewer LMPPs and a failure to initiate expression of multiple lymphoid genes.[89,90] In LMPPs E2A likely functions in cooperation with LYL1 since *Lyl1*$^{-/-}$ mice have a strikingly similar phenotype to *E2a*$^{-/-}$ mice at this stage.[91] In contrast, TAL1 antagonizes T lymphocyte specification within the HSC and LMPP populations.[32] Therefore, despite the similarity in TAL1 and LYL1 structure and their overlapping function in HSC specification, these proteins function in an opposing manner to regulate lymphoid specification.

E2A is required for proper expression of *Notch1* at the inception of T cell development.[89,92] Consistent with this, when *E2a*$^{-/-}$ multipotent progenitors are cultured under T cell differentiation conditions in vitro they fail to generate T cells unless they are transduced with a NOTCH1 producing retroviral vector.[93] *E2a*$^{-/-}$ DN2 thymocytes struggle to enter the T cell lineage and fail to regulate the expression of GATA3, which is substantially elevated in *E2a*$^{-/-}$ DN2 and DN3 thymocytes.[94] This elevated expression of GATA3 contributes to diversion of these cells toward the innate lymphoid lineages, which is particularly evident when *E2a* and *Tcf4* are both deleted or when ID1 is over expressed in T cell progenitors.[94–97] Indeed, heterozygous deletion of *Gata3* restores differentiation of *E2a*$^{-/-}$ DN2 cells into the T cell lineage.[94] Ectopic expression of GATA3 under the control of the CD2 promoter promotes T cell transformation suggesting that failure to repress *Gata3* could be a key event in the generation of *E2a*$^{-/-}$ leukemias.[98] In established *E2a*$^{-/-}$ leukemia lines, re-expression of E2A proteins alters the transcription of numerous genes including *Gata3*, which is indirectly regulated by E2A-mediated induction of GFI1B.[99] Whether GFI1B, alone or in combination with the related transcription factor GFI1, functions to dampen *Gata3*

expression at the inception of T cell development remains to be fully explored but it is notable that both *Gfi1b*$^{-/-}$ and *Gfi1*$^{-/-}$ mice have defects in T cell development that overlap with those of *E2a*$^{-/-}$ mice.[100,101] During B cell development *Gata3* is repressed by EBF1 suggesting that GFI1/GFI1B and EBF1 might play similar roles in progenitors prior to their entry into the T and B cell developmental pathway with EBF1 leading to more severe, or sustained, repression of *Gata3*.[102]

The few T cell progenitors that develop from *E2a*$^{-/-}$ DN2 thymocytes highly express LEF1, an effector of the Wnt signaling pathway, and it is required for the survival of *E2a*$^{-/-}$ leukemias.[103,104] LEF1 is not essential for T cell development owing to the high expression of the related transcription factor TCF1 in T cell progenitors.[105] *Tcf7* is regulated by NOTCH1 and plays a major role in T cell lineage specification.[51,58] TCF1 is expressed normally in *E2a*$^{-/-}$ thymocytes despite the increased expression of LEF1; nonetheless, LEF1 impacts *E2a*$^{-/-}$ T cell development. Indeed, deletion of *Lef1* from *E2a*$^{-/-}$ T cell progenitors results in a profound loss of DN3 thymocytes while, surprisingly, not affecting overall T cell numbers.[104] These findings suggest that LEF1 plays a role in controlling the maturation of *E2a*$^{-/-}$ T cells. *Lef1* mRNA is elevated in multiple mouse models that develop T-ALL, and LEF1 can play both oncogenic and tumor suppressor roles in these models depending on the timing of its expression.[104,106–109]

TRANSCRIPTION FACTORS ASSOCIATED WITH *E2A*$^{-/-}$ T-ALLs

Tal1/Lyl1

TAL1 was first identified as a gene involved in the t(1:14) and t(1;7) chromosomal translocations in T-ALL, which place TAL1 under the control of the *TCRA/TCRD* or *TCRB* locus, respectively.[110–112] LYL1 was also identified through a chromosomal translocation in T-ALL in which LYL1 on chromosome 19 is juxtaposed to the *TCRB* constant regions on Chromosome 7 (Table 1.1).[113]

These translocations are found in approximately 3-7% of TAL1/ LYL1^{+} T-ALL cases, however, there are frequent alterations at the *TAL1* locus in T-ALL including deletions such as *TAL1^{d}*, which arises from a site-specific DNA recombination event causing a 90kb deletion upstream of *TAL1*.[111] These deletions place the coding region of the *TAL1* gene downstream of regulatory elements in the *SCL interrupting locus* (*STIL*). The *STIL* regulatory elements are constitutively active in thymocytes, resulting in ectopic TAL1 expression. These *TAL1* upstream deletions are specific for T-ALL cells and are most likely caused by erroneous V(D)J recombinase activity.[114,115] Alterations in the *TAL1* gene that result in ectopic T lymphocyte expression of TAL1 are now recognized to be present in as many as 60% of human T- ALLs.[116] While these genomic alterations account for a majority of T-ALL associated TAL1 expression, a subset of patients have ectopic TAL1 expression without these alterations. Studies on the mechanisms of *TAL1* deregulation in these patients revealed small insertions (<20bp) in a region 8kb upstream of *TAL1* that create a de novo MYB binding site that results in strong enhancer activity in these leukemias (Table 1.1).[117] ChIP-sequencing experiments showed that MYB binds to this novel site along with chromatin remodelers and other components of the DNA transcriptional machinery. Deletion of the novel MYB binding site abrogated MYB binding and significantly reduced *TAL1* expression. MYB is highly expressed in thymocytes and MYB is often dysregulated in cancer and thus this mutation can lead to robust *TAL1* transcription in leukemic cells.[118] Taken together, these findings outline multiple mechanisms leading to the errant expression of *TAL1* in T-ALL.

TAL1 positive leukemias frequently express CD4 and CD8 and have a cortical phenotype similar to what is observed in *E2a$^{-/-}$* mice.[1,8,10,119] In mice, expression of *TAL1* under the control of the *Lck* promoter, which drives gene expression starting at the DN2/DN3 stages of T cell development, is sufficient to predispose mice in T-ALL-like disease (Table 1.1).[120] This

observation led to the hypothesis that TAL1 drives leukemogenesis through sequestration of E proteins. This idea is supported by studies that showed that TAL1-driven leukemia is not dependent on the DNA binding ability of TAL1.[121] Moreover, transgenic expression of ID1 or ID2, which prevent E proteins from binding to DNA, also predisposes mice to develop a T-ALL-like disease (Table 1.1).[9,11] It is also notable that the *Lck-TAL1* transgene promotes leukemia in a dose-dependent manner that is enhanced by deletion of *Tcf4* indicating that E protein dose is a major determinant of leukemogenesis in this model.[121] Analogous to what is observed in *Lck-TAL1* transgenic mice, ectopic expression of *LYL1* in T cell progenitors blocks the formation of E-protein homodimers, suppresses the expression of E2A-dependent genes, and leads to T-ALL-like disease.[119,122] These findings suggest that at least a part of the mechanism through which TAL1 and LYL1 promote leukemogenesis is through inhibition of E protein homodimer function. However, $LYL1^+$ and $TAL1^+$ leukemias have unique gene expression profiles and $LYL1^+$ leukemias tend to be related to immature $CD4^-CD8^-$ T cell progenitors.[1,123] These observations suggest that LYL1 and TAL1 have unique functions or that they are expressed in different cellular contexts, either distinct stages of development or stages of transformation. Interestingly, nearly 30% of pediatric $TAL1^+$ T-ALL patients have heterozygous loss-of-function mutations in USP7, a deubiquitinating enzyme that interacts with E proteins and other leukemia-associated proteins, and is associated with decreased E protein target gene expression and increased cell growth.[124] Therefore, there appears to be multiple mechanisms contributing to reduced E protein function in T-ALL.

While inhibition of E protein function is sufficient to predispose T cell progenitors to transformation, TAL1 and LYL1 may contribute to transformation through their participation in transcriptional complexes that activate or inhibit gene expression. In hematopoietic progenitors

Gene	Mutation consequence	Interactions with E-proteins	Found in:	Citations
Tal1	Overexpression	Changes DNA binding sequence to prevent binding at T cell lineage specifying loci	*Lck-Tal1* Transgenic mice *Tal*^{d} expressing patients Patient-derived T-ALL lines *Tal1-TCR* translocations	(120,121) (111) (117) (110–112)
Lyl1	Overexpression	Changes DNA binding sequence to prevent binding at T cell specifying loci	*Lyl1-TCR* translocations Patient derived samples	(113) (1,12)
Lmo2	Overexpression	Stabilizes the Tal1:E2A or Lyl1:E2A complexes	*Lmo2* transgenic mice Patient derived samples	(123) (1,12,116,123,125,126)
Id1/2	Overexpression	Dimerizes with E2A to inhibit DNA binding	*Id1/2* transgenic mice *E2a*$^{-/-}$ mice *Tcf7*$^{-/-}$ mice ETP-ALL patients	(9–11) (94,99) (106) (21)
E2a	Deletion	Prevents expression of functional E2A	*E2a*$^{-/-}$ and E47$^{-/-}$ mice	(8,127)

Table 1.1: Common T-ALL mutations impacting E2A function
Table of common oncogenic mutations found in human and/or murine T-ALLs that interact with E proteins

and T-ALL, both TAL1 and LYL1 bind DNA in large complexes that include the LMO (LIM only), LIM domain binding (LDB1), and GATA protein families.[20,128–131] Indeed, LMO proteins are critical members of the TAL1 complex, acting as bridging factors that connect TAL1 to other DNA binding proteins like GATA1/2/3.[132] This is of particular interest because LMO proteins are overexpressed in approximately 10% of T-ALLs and these leukemias frequently have TAL1 overexpression (Table 1.1).[116] In a subset of pediatric (3.7%) and adult (5.5%) T-ALL the *LMO2* gene contains intronic indels that result in *de novo* binding sites for the leukemia-associated transcription factors MYB, ETS1 or RUNX1 and thus dysregulated LMO2 expression.[126] Interestingly, both TAL1 and LMO1 or LMO2 are required to induce luciferase reporter activity in T-ALL cell lines.[133] Experiments in human T-ALL cell lines have been vital to elucidating the core components of the TAL1 complex in leukemia. ChIP-seq in these lines has identified E2A, GATA3, LMO1/LMO2, RUNX1, and MYB as co-bound to TAL1 bound regions suggesting multi-transcription factor complex formation.[134] siRNA knock down of *TAL1* mRNA or other members of the TAL1 complex identified genes regulated by this complex in leukemias.[135] Interestingly, siRNA-mediated knockdown of *TAL1* results in decreased expression of the genes that encode other components of the TAL1 complex. This finding suggests a positive feed-forward mechanism where the oncogenic TAL1 complex promotes expression of its own components, in addition to promoting expression of other known oncogenes. TAL1-dependent genes include *Myb*, which positively regulates cell cycle and the anti-apoptotic genes *Trib2*, which supports the survival of T-ALL cell lines, and *Arid5*, a gene associated with a variety of leukemias.[135–137]

There is evidence indicating that LYL1 forms oncogenic complexes similar to TAL1. Indeed, Jurkat cells forced to express LYL1, LMO2 and LDB1 induced robust target gene expression that was dependent on LMO2 and LDB1.[130] Further, LMO2 is frequently overexpressed

in TAL1 expressing leukemias but in LMO2 transgenic mice TAL1 is dispensable for leukemogenesis.[123] In contrast, deletion of *Lyl1* significantly increases leukemia latency in LMO2 transgenic mice suggesting that LYL1 supports transformation. Microarray analysis revealed that LMO2 expressing thymocytes have higher *Lyl1* expression compared to wild-type thymocytes indicating a potential feed-forward mechanism reminiscent of the mechanism seen in TAL1 expressing leukemias. Consistent with this idea, the *Lyl1* promoter contains ETS and GATA binding sites, which promote the expression of LYL1 in HSCs and both ETS1 and GATA3 are implicated in T-ALL.[3,138,139] Taken together, these data suggest that LYL1 may function in a manner analogous to TAL1 during T cell progenitor transformation.

Notch1

NOTCH1 is constitutively activated in a majority of T-ALL, including those that overexpress TAL1, and in leukemias from *E2a*$^{-/-}$ mice.[127,140] NOTCH1 was identified as an oncogene in T-ALL by its involvement in a t(7;9)(q34;q34.3) translocation that placed the 3' end of the NOTCH1 gene under control of the *TCRB* locus, resulting in constitutive activation of NOTCH1 in T cell progenitors.[141] This translocation is present in approximately 2% of leukemias, however, it is now appreciated that >60% of all human T-ALLs have mutations in *NOTCH1*.[16] These mutations cluster in the HD and PEST domains. Mutations affecting the HD domains prevent the association of the extracellular and intracellular portions of NOTCH1 thus allowing for spontaneous γ-secretase mediated cleavage to produce active ICN. The PEST domain mutations promote stabilization of ICN by removing the phosphorylation sites that lead to docking of FBW7 (gene: *FBXW7*), a ubiquitination enzyme that induces ICN proteasomal degradation.[142] These mutations are not mutually exclusive, with ~20% of human leukemias having mutations in

both domains.[143] Additional mutations have been identified that inactivate *Fbw7* resulting in the constitutive stabilization of ICN.[16,142,144] The TAL1 complex also represses *Fbxw7* through miRNA-223 suggesting that there may be numerous mechanisms contributing to stabilization of NOTCH1 in T-ALL.[145]

Leukemias arising in *E2a*$^{-/-}$ mice have mutations in the PEST domain of NOTCH1 but no mutations have been identified in the HD domain.[127] Thus, Notch activation in *E2a*$^{-/-}$ leukemias does not occur through spontaneous cleavage of membrane bound Notch and must occur through DLL4 binding or alternate mechanisms. One such alternate mechanism is the use of alternative transcription initiation sites at the *Notch1* locus in *Ikzf1*$^{-/-}$ and *E2a*$^{-/-}$ leukemias.[146,147] Multiple *Notch1* transcripts were identified that initiate from a cryptic promoter upstream of exon 26 leading to a protein that lacks the extracellular domain of NOTCH1. This cryptic promoter can be activated by deletion of the promoter upstream of exon 1, which occurs through a RAG1-dependent mechanism.[148] Surprisingly, in *Ikzf1*$^{-/-}$ mice, deletion of the first exon of *Notch1* did not impact T cell development, unlike what is seen in *Ikzf1*$^{+/+}$ mice, due to use of this alternative mechanism for transcribing *Notch1* in the absence of IKAROS. *Ikzf1*$^{-/-}$ thymocytes have increased histone acetylation near IKAROS binding sites located near the alternative *Notch1* promoter raising the possibility that IKAROS represses the use of this alternative mechanism through epigenetic modification.[146,147] E2A binding sites are also present within the alternate promoter and these alternative NOTCH1 isoforms are expressed in E2a$^{-/-}$ leukemias indicating that E proteins may cooperate with IKAROS to repress alternative promoter use.[146] In addition to IKAROS and E2A binding sites, ChIP-seq experiments identified TCF1 and RUNX binding sites as enriched in the alternate promoter. Notably, this alternate promoter regulates development of wild-type DN thymocytes and is critical for passage through β-selection and into the DP stage. This suggest that

ligand independent Notch1 expression plays a vital role in T cell development but is coopted in T-ALL development to drive leukemogenesis.[57]

While E2A may repress the alternative promoter, E2A promotes *Notch1* expression in thymic seeding progenitors.[93] This deficiency in NOTCH1 could provide a strong selective pressure for NOTCH1 mutations or altered transcription initiation site used to support T cell development. Genomic deletions have also been found in mouse leukemic cells that result in splicing of Exon 1 to downstream exons and again result in proteins that lack the extracellular domain and are constitutively active but dependent on γ-secretase.[16,148]

The mechanisms by which NOTCH1 promotes leukemogenesis have been studied intensively. Interestingly, Notch signaling can impact expression of E2A, at least in mice, where it has been shown that mitogen activated protein kinase phosphorylation of E2A leads to NOTCH1-dependent ubiquitination and proteasomal degradation of E2A.[149,150] Whether this mechanism contributes to ICN induced leukemogenesis in mice or humans requires further investigation. In human T-ALL, a major target of Notch signaling is c-MYC, which itself is oncogenic in T lymphocyte progenitors.[16,151,152] ICN binds to an enhancer 140 Mb downstream of c-*MYC*, whose activity correlates with responsiveness to NOTCH1 inhibitors.[153] Moreover, mutation of this enhancer prevents leukemogenesis by ectopic expression of ICN demonstrating that it is an essential target. This enhancer is also regulated by NOTCH3 in NOTCH3-dependent leukemias.[154] It is likely that there are many essential targets of NOTCH1 in T-ALL. Indeed, in *E2a*$^{-/-}$ leukemias c-MYC expression is stably amplified through trisomy at chromosome 15 and therefore does not require ICN for expression, yet these leukemias are still dependent on Notch1 signaling.[8,103]

ChIP-seq analysis for ICN has revealed multiple novel targets of NOTCH1 in leukemia.[155] ICN bound regions are in close proximity to RUNX, ETS, and ZNF143 binding motifs and these

regions have extensive histone acetylation and H3K4me3 chromatin modifications, indicative of open chromatin and active gene transcription.[109,155] Thus, it is possible that NOTCH1 promotes accessibility to target gene regulatory regions, which allows other T cell specific transcription factors or DNA transcriptional machinery to bind and promote gene expression. Consistent with this idea, NOTCH1 was required for recruitment of RUNX1 and MYB to enhancers located within the *TCRG* and *TCRB* locus.[156]

Many ICN target genes, including *DTX1*, *IGF1R*, *IL7Ra*, and *GIMAP*, have been identified by evaluating changes in gene expression after treatment of leukemias with γ-secretase inhibitors.[109,151] Importantly, many of these genes are co- regulated by T cell specific factors like RUNX and ETS1. Deletion of RUNX1 in DN2/3 thymocytes impairs IL7R expression,[157] and expression of dominant-negative RUNX1 and NOTCH1 inhibitors (RUNT and DN-MAML, respectively) suppressed IL7R mRNA expression,[109] Further, ETS1 binds to multiple NOTCH1 occupied sites in T-ALL.[138] Indeed, mice overexpressing NOTCH1 fail to develop leukemia when lacking functional ETS1 suggesting that both of these factors are required for leukemia initiation. ETS1 is frequently over expressed in human T-ALL samples and cell lines indicating that ETS1 may act in concert with NOTCH1 in the human disease as well. Indeed, shRNA–mediated knockdown of ETS1 in human T-ALL lines promoted cell death and significantly down-regulated expression of the oncogenes *c-MYC* and *IGFR1*, as well as other NOTCH1 target genes like *HES1* and *DELTEX1*.[138]

Gata3

GATA3 is essential for T cell specification and in its absence multipotent progenitors fail to generate committed T lymphocytes.[60–62] Ectopic expression of GATA3 can also derail T cell

development and force T cell progenitors down alternative lineages, such as the mast cell lineage.[158] However, transgenic expression of *Gata3* under the control of the CD2 promoter, which drives expression in all lymphocytes, predisposes mice to develop T-ALL-like disease with trisomy of chromosome 15 and activation of NOTCH1, similar to what is seen in *E2a*$^{-/-}$ leukemias, although with longer latency.[98,159] *Gata3* is elevated in *E2a*$^{-/-}$ T cell progenitors and has a negative impact on the ability of DN2 cells to generate T lineage-restricted cells.[94] These findings support a role for GATA3 in T cell leukemogenesis and implicate it as a potential contributing factor to T cell transformation in *E2a*$^{-/-}$ mice even though this has not been formally demonstrated. Indeed, in non-ETP-ALL GATA3 expression is elevated compared to T cells from healthy donors and defines a stem-like progenitor.[160] The mechanism by which GATA3 promotes thymocyte transformation and leukemia survival is not well understood. One potential mechanism involves GATA3's association with TAL1 as a member of the oncogenic TAL1 complex. Indeed, siRNA knockdown of GATA3 in T-ALL cells represses transcription of TAL1 target genes suggesting that GATA3 is required for proper TAL1 complex function.[135] GATA3 and other members of the TAL1 oncogenic complex also bind to the NOTCH1-regulated enhancer downstream of *c-Myc*.[139,153] Mutating the GATA3 binding sites in this enhancer impacted nucleosome eviction and chromatin accessibility, resulting in decreased c-MYC expression and abrogated leukemia development in mice.[139] These observations indicate that GATA3 cooperates with TAL1 and NOTCH1 to promote transformation through regulation of c-MYC.

In contrast to these cases of increased GATA3 expression, 5% of T-ALL patients have silencing mutations in the *GATA3* gene.[116] Consistent with this, another study found that 33% of patients in their cohort with the ETP-ALL subtype had reduced GATA3 expression associated with increased methylation throughout the *GATA3* gene.[160] Thus, GATA3 may play multiple roles in T-

ALL development, suppressing ETP-ALL or promoting T-ALL at later stages. Decreased GATA3 expression in ETP-ALL is consistent with GATA3's function in promoting T cell lineage differentiation as GATA3 silencing could contribute to a developmental block at the ETP stage that supports transformation. It also seems likely that GATA3 is not a driver mutation and its function may be dependent on the spectrum of additional mutations that occur during transformation.

Lef1/Tcf1

The NOTCH1 target gene *Tcf7* is also implicated as a suppressor of T cell transformation.[106,161] TCF1 is a member of the HMG box family of proteins along with the closely related protein LEF1. Both TCF1 and LEF1 can promote transcription in response to canonical WNT signaling activation or repress transcription through recruitment of the Groucho related co-repressors such as TLE3.[162,163] In the absence of TCF1, thymocytes have a developmental block at the ETP, DN2, and ISP stages whereas mice lacking LEF1 have no obvious defects in DN thymocytes.[51,58,105,164] Combined deletion of *Tcf7* and *Lef1* exacerbates the phenotype seen in *Tcf7*-deficient mice, leading to a nearly complete block in T cell development.[105] This observation indicates that TCF1 and LEF1 have overlapping functions and that LEF1 partially compensates for the loss of TCF1. In addition to the defects seen in T lymphopoiesis, approximately 50% of *Tcf7*$^{-/-}$ mice develop T-ALL.[106,161] *Tcf7*$^{-/-}$ leukemias are heterogeneous; phenotypically resembling DN3, DN4, or DP thymocytes. Despite this cell surface phenotype, RNA profiling revealed that the transcriptome of *Tcf7*$^{-/-}$ T-ALLs is related to that of human ETP-ALLs, which is consistent with the early requirement for TCF1 in T cell development.[106] *Tcf7*$^{-/-}$ leukemias have activated NOTCH signaling and inhibiting this pathway with GSI at least partially impacts their viability.[161] Further, *Tcf7*$^{-/-}$ leukemias highly express ID2 and LEF1, particularly in a subset of T cell progenitors with

a gene signature predictive of high leukemic potential, suggesting that that suppression of E protein activity may be a feature of transformation in this model.[106,107] Indeed, *Tcf7*$^{-/-}$*Id2*$^{-/-}$ mice showed an increased latency of leukemogenesis consistent with this hypothesis.

Like *Tcf7*$^{-/-}$ leukemias, *E2a*$^{-/-}$ leukemias have high expression of *Lef1* and LEF1 is required for the survival and proliferation of these leukemias.[103] LEF1 is an oncogene in acute myeloid leukemia and in multiple forms of B lymphocyte leukemia and is suppressed by TCF1.[165–168] Ectopic expression of LEF1 in HSCs induced acute myeloid leukemia-like or B cell ALL-like disease in mice, demonstrating LEF1's oncogenic potential.[167] In an adult cohort of T-ALL patients, high LEF1 expression was associated with increased expression of the oncogenes encoding c-MYC and CYCLIN D1 suggesting that LEF1 is positively associated with T cell leukemia.[169] Moreover, 4 unique mutations that augment LEF1 function were found in these patients. In contrast, approximately 11% of pediatric T-ALL patients were found to have inactivating mutations in the *LEF1* gene.[14,170] These mutations consist of deletions or truncation mutations, both resulting in lower LEF1 function. These conflicting findings suggest that LEF1 can play multiple roles in T cell leukemia. Indeed, while *E2a*$^{-/-}$ leukemias are dependent on LEF1, inactivation of *Lef1* in *E2a*$^{-/-}$ mice prior to transformation did not prevent transformation; rather, it reduced leukemia latency and resulted in leukemias with a unique gene expression program compared to *E2a*$^{-/-}$ leukemias.[104] Taken together, these experiments reveal that the timing of genetic alterations in the evolution of T-ALL can determine latency, phenotype, and genetic susceptibilities within these cells.

IKAROS Family Transcription Factors

Ikzf1, encoding the transcription factor IKAROS, is required for the development of thymocytes, however it is also a potent tumor suppressor.[171,172] *Ikzf1* deletions in human T-ALLs are relatively rare, being detected only in approximately 5% of T-ALL patients.[173] It is notable that many of these deletions are heterozygous, which mirrors commonly used mouse models. Indeed, a heterozygous mutation of *Ikzf1* that deletes exons 3 and 4 (encoding the DNA binding domain) in mice is sufficient to induce hyperproliferation in thymocytes, with T-ALL developing within 3 months with 100% penetrance.[171] The mutant protein maintains its' ability to dimerize with full length IKAROS, but the complex fails to bind DNA, resulting in a dominant-negative inhibition of IKAROS function.[174] Similar phenotypes have been identified with IKAROS hypomorphic mutations ($Ik^{L/L}$ mice), which may mimic a heterozygous loss of protein and IKAROS null mutants, albeit with longer disease latency.[146,175,176] This indicates that even slight loss of IKAROS function is weakly oncogenic, with full inhibition a more potent oncogenic event. Similar to *E2a*$^{-/-}$ leukemias, tumors from $Ik^{L/L}$ have mutations in the *Notch1* PEST domain, but to date no mutations in the HD domain have been identified.[147] This is presumably due to *Ikzf1* deficient thymocytes activating a ligand independent isoform of *Notch1* through alternate promoter usage.[57,108,146] The alternate NOTCH1 isoform is functionally similar to HD mutations that allow continuous cleavage of NOTCH1 from the membrane via γ-secretase. In support of this, γ-Secretase inhibitor treatment significantly inhibits the growth of these leukemias *in vitro*.[146,147,176] Notably, *Notch3* is dispensable for this process, suggesting that the combination of ligand independent NOTCH1 activation and PEST mutations are sufficient to promote the development and survival of *Ikzf1*$^{-/-}$ T-ALLs.[147] It is unknown if *Notch3* would be required in situations of *Notch1* deletion in mice, but when RBPJ was deleted in $Ik^{L/L}$ mice leukemia was significantly

delayed but not fully abrogated.[147] However, the relationship between *Notch3* and *Ikzf1* is more complicated. Human T-ALLs frequently express *NOTCH3* but activating mutations in *NOTCH3* have not yet been described. Regardless, *NOTCH3* expression promotes the survival and proliferation of human T-ALL lines.[177] Using *Notch3* transgenic mice, it has been found that *Notch3* upregulates the RNA-binding protein HuD that alternatively splices *Ikzf1* into an isoform that lacks its DNA binding domain, thus acting as a dominant negative inhibitor.[178] It is unknown if *Notch1* regulates *Ikzf1* in a similar mechanism, but the *Notch3* mediated alternate splicing of IKAROS could act initiate a feed forward loop to amplify Notch pathway signaling in the absence of distinct *Ikzf1* mutations. Together, these data suggest that the predominant tumor suppressive function of IKAROS is to adequately restrain Notch signaling.

Ikzf2 (HELIOS) is a highly homologous molecule to Ikaros, sharing a similar structure and expression pattern in lymphocytes.[179] Mostly studied in the context of regulatory T cells, *Ikzf2* deletion has been shown to have little to no effect on T cell development.[180] Despite this, there have been reports of *IKZF2* mRNA and protein being significantly upregulated in human T-ALL patients and patient-derived T-ALL cell lines.[181] The upregulated HELIOS isoforms are short isoforms that lack the DNA binding domain, reminiscent of leukemia associated IKAROS mutations. Retroviral expression of similar short isoforms of *Ikzf2* in HSCs followed by transplantation into wild-type mice resulted in the development of T-ALL. Notably, this was not seen in mice transplanted with HSCs transduced with wild-type *Ikzf2*, which had mild defects in T cell development but did not develop T-ALL. These observations suggests that HELIOS can act as an oncoprotein by a similar mechanism to dominant negative isoforms of IKAROS.[182] The exact mechanism used by mutant HELIOS to promote leukemogenesis has not been studied, but Jurkat cells transduced with either a wild-type isoform or short isoform of HELIOS had minimal gene

expression changes, suggesting that Helios target genes do not drive leukemogenesis.[183] However, HELIOS is known to dimerize with IKAROS, therefore, expression of the HELIOS short isoform may act as a dominant negative inhibitor of IKAROS to promote T cell transformation.

THYMUS AUTONOMY IN T CELL AND T-ALL DEVELOPMENT

There has been significant work into understanding the regulation of transcriptional networks that contribute to T cell development and how mutations in, or dysregulation of, these networks drive T-ALL development. However, one underappreciated aspect to maintaining healthy T cell development and suppressing leukemogenesis is maintaining competition between thymocytes. As previously discussed, T cell development relies on continuous import of thymus seeding progenitors, either from LMPPs or CLPs that traffic to the thymus.[33–35,40] Using models of thymus transplants into immunodeficient hosts that cannot import new bone marrow progenitors into the transplanted thymi, two groups studied how thymocytes develop in the absence of progenitor input, a phenomenon termed "Thymus Autonomy" (Table 1.2).[184,185] The transplanted thymi continued to produce thymocytes and peripheral T cells, despite being unable to generate *de novo* thymus seeding progenitors. Initial characterization of thymus transplant mice showed thymi enriched for cells with a $CD4^{-}CD8^{-}CD44^{+/int}CD25^{int}$ phenotype, suggesting that the cell population that maintains the pool of T cells is an immature precursor after the ETP stage. Further analysis identified $CD4^{-}CD8^{-}Kit^{int}CD25^{int}$ "early" DN3s as the population maintained long term in thymus autonomy, which is consistent with earlier reports.[184,185,186] DN3s being the self-renewing population is notable given that DN3s undergo β-Selection, which suggests that T cells derived from autonomous development may have altered TCR repertoires. However, different groups report conflicting results when investigating this hypothesis. One group reported normal V(D)J

Donor	Host Mouse	Treatment	Outcome	Latency (Days)
Wild type thymus	Wild-type	N/A	No leukemia	---
Wild type thymus	*Rag2*$^{-/-}\gamma c^{-/-}$ *kit*$^{w/w}$	N/A	Leukemia	200
Wild type thymus	*Rag2*$^{-/-}\gamma c^{-/-}$ *kit*$^{w/w}$	Wild type bone marrow transplant 1 week post thymus transplant	No Leukemia	---
Wild type thymus	*Rag2*$^{-/-}\gamma c^{-/-}$ *kit*$^{w/w}$	Wild type bone marrow transplant 6 weeks post thymus transplant	Reduced Leukemia penetrance	200
NA	*CD2-Lmo2*	N/A	Leukemia	200
LMO2-CD2 bone marrow	Wild type	N/A	Leukemia	300
3:1 wild type:CD2-LMO bone marrow	Wild type	N/A	Reduced leukemia	450

Table 1.2: Models of Thymus Autonomy
Table depicting common T-ALL models shown to rely upon thymus autonomy for leukemia development.

rearrangement[185] and the other reported expansion of specific clones.[184] Notably, one group sequenced peripheral T cells[185] and the other DPs.[184] Therefore, the differences in repertoire may be affected by cell location, with over representation of certain V(D)J sequences in the thymus that may be purged through positive or negative selection prior to T cells exiting to the periphery. Despite the differences in reported V(D)J usage, intrathymic competition clearly enforces the β-selection checkpoint.[187] DN3 thymocytes under thymus autonomy are significantly more proliferative while increasing the time needed to differentiate into later stages, suggesting self-renewal.[187] This is supported by RNA-sequencing of DN3s after one month in thymus autonomy, which were reported to have a stem-like transcriptome. Further, autonomous DN3s have significantly more V(D)J recombinations than in the wild-type context, and single cell TCR sequencing identified a skewing towards more distal Vβ and Jβ gene usage.[187] This argues that the self-renewing DN3s maintaining thymus autonomy are continuously recombining their TCRβ chain and indicates a longer dwell time.[187] There have not been reports of dysfunctional TCRα chain usage, suggesting that the defects in selection only occur at the DN3-β-selection checkpoint, and do not affect the α-selection in DPs.[184,185] Thus, the break in β-selection may not be a result of intrinsic differences in the DN3s caused by autonomy but may be a result of the continued proliferation and self-renewal of the DN3s. How the break in β-selection gets resolved by the time cells egress into the periphery has yet to be investigated.

Regardless of potential differences in the TCR repertoire, T cells derived from progenitors in autonomy retain the ability to protect from *Listeria monocytogenes* (LM) infection, suggesting that these T cells are healthy and functional.[185] One caveat, however, is that the mice were infected only one month post thymus transplant, and the T cells that acted to clear infection may have developed immediately post-transplant before progenitors spent prolonged time in autonomy.

Indeed, donor derived T cells can be seen in the periphery in as few as two weeks post thymus transplant,[185] and progenitors in autonomy progressively acquire gene expression changes.[188] Thus, it is currently unknown if T cells derived from progenitors in autonomy long-term can still confer the same antibacterial protection.

Increased dwell time in the thymus induces significant transcriptional changes in wild-type thymic progenitors. However, the gene expression changes seen are exacerbated in the absence of competition.[187,188] Thus, preventing the accumulation of these transcriptome changes, or purging cells that do acquire them prior to transformation, is a crucial aspect of T cell development regulated by competition. How competition prevents these gene expression changes and T-ALL development has yet to be fully elucidated.

One proposed mechanism by which competition regulates T cell development is through regulation of thymocyte proliferation. Old thymocytes (those with longer dwell times) have undergone many divisions, which increases the probability of stochastic mutations, which may explain gene expression changes seen in old thymocytes and thymocytes in autonomy. In a competitive, non-autonomous thymus, rapid proliferation occurs when cells transition to the next stage of development, with periods of quiescence and relatively few divisions during critical checkpoints (in particular VDJ recombination).[186,189] DN3 thymocytes rapidly proliferate after passing β-selection, which is regulated in part by the repression of E-protein function.[74,190] Thus, the tight regulation of thymocyte division is a crucial for healthy T cell development. Notably, the regulation of pre-β-selection DN2/DN3 thymocyte proliferation is regulated in part by DP thymocytes. In models of neonatal thymus transplant into wild-type or immunodeficient mice, increasing the dwell time of donor DPs using a BCL2-transgenic thymus donor decreased the

number of DN2/DN3s and EdU incorporation, suggesting that DPs negatively regulate DN thymocytes' ability to proliferate.[186]

Another potential mechanism though which competition regulated T cell development is through regulation of IL-7 signaling. Comparison of old thymocytes and young thymocytes identifies *Bcl2* as a gene downregulated with age.[188] BCL2 is downstream of IL-7 receptor signaling, is vital for T cell development,[191] and inhibits apoptosis.[192] Thus, reduced BCL2 expression old thymocytes suggest they are purged from the thymus due to a failure to inhibit apoptosis. The downregulation of BCL2 in the absence of reported IL-7R downregulation implies that older thymocytes lose sensitivity to IL-7 signaling and would require more IL-7 to prevent apoptosis. Experiments generating mixed bone marrow chimeras with marrow from mice with naturally high IL-7R expression (AKR/J mice) or low IL-7R expression (B10.BR mice) show that AKR/J derived thymocytes are better at reconstituting the thymus of the recipient mice. This was true even in mixed chimeras with as little as 10% AKR/J marrow to 90% B10.BR. This establishes that high sensitivity to IL-7 signaling confers a competitive advantage. Notably this difference in thymic reconstitution was not due to differences in proliferative capacity of AKR/J derived and B10.BR derived thymocytes, suggesting a difference in survival may be driving this phenotype.[193] Together, these data suggest that older thymocytes lose their sensitivity to IL-7, leading to a downregulation of BCL2. This renders them less competitively fit than young thymocytes which promotes their apoptosis and they are purged from the thymus. How old thymocytes lose their sensitivity has not yet been fully elucidated as there have been no reports to date investigating IL-7 receptor expression on young vs. old thymocytes. It has been reported, however, that DN3s in prolonged thymus autonomy upregulate *Socs2*, a molecule that represses cytokine signaling.[187]

Therefore, it is possible that the reduced sensitivity to IL-7 may be a result of active repression in older thymocytes.

All together, these data suggest that intra-thymocyte competition safeguards healthy T cell development by dynamically regulating the proliferation of DN thymocytes, and by removing old, unfit thymocytes from the thymus. Broadly, these regulatory steps coordinate to limit the number of thymocytes that acquire mutations, either by limiting opportunity by inhibiting proliferation, or by deleting thymocytes that have begun to acquire them. This is notable because failure to enforce competition in the thymus results in T-ALL (Table 1.2).[188]

Bulk RNA-sequencing of DN3 thymocytes in prolonged autonomy show significant gene expression changes, including upregulation of a stem-like phenotype, increased expression of *Notch1* target genes *Dtx1* and *Ptcra*, increased expression of E-protein inhibitors *Id1* and *Id2*, and down regulation of *Bcl2*, *Bcl11b*, and *Tet2*.[187] This suggests that prolonged autonomy is a strong selective pressure for canonical T-ALL associated transcriptomic changes. Indeed, leukemias arising from thymus autonomy share many features with $E2a^{-/-}$ leukemias, including *Notch1* mutations, trisomy of chromosome 15[127,188], and E2A repression, which may be the result of *Tal1, Lmo2, Id1*, and *Id2* upregulation.[188]

Leukemia develops in models of thymus autonomy with a latency of ~2.5-3 months after thymus graft. However, transplantation of bone marrow into immunocompromised hosts to restore the input of fit, young thymocyte progenitors was sufficient to fully prevent leukemogenesis (Table 1.2).[188] This outcome was highly time dependent, as transplanting bone marrow 1 week after thymus graft fully prevented T-ALL, but transplanting bone marrow 6 weeks after thymus graft failed to prevent leukemia development.[188] These data imply there is an inflection point that thymus progenitors reach where sufficient time in autonomy has resulted in mutations that make

them resistant to restored competition, presumably by being more competitive than the incoming donor thymocytes. This is consistent with the defined kinetics of thymus reconstitution in the context of wild-type thymi transplanted into wild-type hosts (Table 1.2). In these experiments DN3s reached 50:50 chimerism by day 13 and were fully donor derived by day 14. Thus, after 2 weeks, DN3s are old enough to be outcompeted by incoming progenitors.[186] In these models of thymus autonomy, the pre-leukemic stem cell identified is the DN3.[184,184,187] Thus, it is presumably after 2 weeks the DN3s fully acquire the ability to self-renew to maintain T cell development. Considering the highly oncogenic transcriptome described in DN3s after 1 month in autonomy, the switch between pre-leukemic self-renewing DN3 to leukemic stem cell probably occurs between 2-4 weeks in autonomy. Thus, there is a very limited window (< 1 month post graft) in these models to restore competition to prevent leukemogenesis.

These insights into how intrathymic competition acts to regulate T cell development and prevent T-ALL development came from highly artificially models of thymus transplants into severely immunocompromised host mice. These insights are strongly reminiscent of 2 cohorts of SCID-X1 patients treated with retroviral therapy to restore expression of their missing common γ-chain.[125,194] T cell development was restored in all patients, but 20% (5/20) of patients in these studies developed T-ALL. Initially it was assumed that T-ALL was a result of the retrovirus incorporating into oncogenes, promoting their expression. Indeed, 3/5 patients had insertions upstream of the *LMO2* gene, while the other 2 had insertions near other oncogenes. However, none of these patients were preconditioned for bone marrow transplant prior to transplant, and after chemotherapy treatment for T-ALL, functional T cell development persisted.[195] Preconditioning consists of treatments with chemotherapy to induce HSC death to allow better engraftment of donor HSCs.[196] Thus, in the patients that developed T-ALL, it is possible that the donor LMPPs

and CLPs transduced with the common γ-chain settled in the thymus and initiated T cell development, without significant bone marrow engraftment, creating a similar system to the murine thymus graft experiments. In addition to thymus autonomy potentially induced by poor bone marrow engraftment, Overexpression of LMO2 in murine thymocytes also leads to a failure of competition and leukemia development, in this case, of ETP-ALL.[123,197] Transplant experiments placing wild-type donor thymi in the kidney capsule of CD2-*Lmo2* transgenic mice recipients show that thymocytes with LMO2 overexpression fail to successfully reconstitute the donor thymus. This induces thymus autonomy for the donor thymocytes, resulting in a pre-leukemic stem cell with a DN2 phenotype.[197] Finally, transplantation of CD2-*Lmo2* transgenic bone marrow induces leukemia in recipient mice with a mean latency of 10-12 months. This was abrogated when CD2-*Lmo2* bone marrow was transplanted alongside wild-type "competitor" bone marrow despite successful bone marrow engraftment. Together, this suggests that a failure to provide competition contributes to leukemogenesis in the CD2-*Lmo2* transgenic model. Further, it provides further evidence that the LMO2 overactivation seen in the 3/5 SCID-X1 patients that develop T-ALL drove leukemogenesis through a failure to provide competition, even if the donor bone marrow successfully engrafted.[125,194,196]

It is notable that almost all models of T-ALL using dysregulations of T cell lineage specifying transcription factors have significant defects in one or more thymocyte populations. Broadly, these mutations result in a loss in the total number of thymocytes, primarily the result of reduced numbers of DP thymocytes. DPs are the most populous thymocyte population, typically up to 90% of a murine thymus, and the reduced numbers are either a result of defects specifically within DPs or defects in earlier populations resulting in failure to develop downstream populations. Regardless, the recurring finding that reduced thymic cellularity, regardless of origin, is coincident

with T cell transformation implicates a failure to provide competition may drive transformation in these models. To date, no such experiments have shown a failure to provide competition is a driver of transformation using a physiologically relevant model of T-ALL.

CHAPTER 2:

MATERIAL AND METHODS

MATERIALS AND METHODS

Mice

Vav-Cre (Jax #035670), *Lck-Cre* (Jax, #003802), E2a$^{f/f}$,[198] and wild-type CD45.2^{+} mice were backcrossed at least 10 generations onto an FVB/NJ background. *Ikzf2*$^{f/f}$ [199] mice were backcrossed 5 generations for all experiments. Mice were housed at The University of Chicago Animal Resource Center and all experiments were performed in accordance with the guidelines of The University of Chicago Institutional Animal Care and Use Committee. No randomization or blinding was used. Male and female mice were used in all assays. Control mice were age and sex matched litter mates when possible.

Leukemogenesis Studies

VcKO, LcKO, and wild-type mice were monitored for up to 52 weeks for leukemia development. For all leukemogenesis studies mice were euthanized when hunched, presenting with respiratory distress, or presenting with enlarged lymph nodes. Leukemia was confirmed by autopsy with identification of enlarged spleen, lymph nodes, or thymus.

Cell Lines

1.F9, 0531, and 115-2 cell lines were previously stablished from primary leukemias from E47$^{-/-}$ mice. Lines were thawed and maintained in Opti-mem media supplemented with 10% fetal bovine serum, 100units/ml penicillin, 100ug/ml streptomycin, 29.2 mg/ml glutamine and 80uM of 2-mercaptoethanol (Complete medium). Plat-E cells were maintained in DMEM complete. All cells

were maintained in a humidified incubator at 37°C with 5% CO_2 and passages when approaching 80% confluence.

Flow Cytometry, Cell Sorting, and Antibodies

Flow Cytometry and Antibodies

Single cell suspensions were treated with FCR block (2.4G2) and fluorescence- conjugated antibodies for 25 min in the dark on ice. Propidium Iodide or Zombie NIR(Biolegend, Cat: 423106) was used to exclude dead cells when appropriate. Lineage cocktail contained the following biotinylated antibodies: Ter119 (Invitrogen/13–5921–85), CD11b (Invitrogen/13–0122–85), CD11c (Invitrogen/13-0114- 85), DX5 (Invitrogen/13-5971-85), GR1 (Invitrogen/13-5931-82), B220 (Invitrogen/13-0452-82), and CD19 (Invitrogen/13-0193-85). Antibodies specific for the following antigens were purchased from BD Biosciences, eBiosciences, Biolegend and Cell Signaling Technology: CD4 (GK1.5), CD8(53-6.7), CD25(PC61.5), CD117/c-Kit(ACK2), TCRβ(H57-597), Sca-1(D7), Flt3(A2F10), CD45.1(A20), CD45.2(104), Annexin V (Thermofisher, Cat: A13199), The FoxP3/Transcription factor staining kit was used for intracellular staining using the following antibodies: Ki67(SolA15), and Helios(22F6). ICN(D3B8) and E2A (G127-32) primary antibodies were used with αMouse IgG Fab2-AlexaFluor 488 (Cell Signaling Technology/44085) secondary antibody. BrDU analysis was performed by injecting 100μL of 10mg/mL of BrDU 24 and 12 hours prior to harvest. BrDU staining was performed using the APC BrDU Flow Kit (BD Pharmigen/51-9000019AK) following manufacturers protocol. Active caspase staining was performed with FLICA-660 following manufacturers protocol (Immunochemistry Technologies). Data were acquired on an LSRFortessa

4-15, LSRFortessa X-20 (BD Biosciences), or NovoCyte Penteon (Agilent) flow cytometer and analyzed using FlowJo v10.8.1.

Cell Sorting

CD45.1$^+$ DP thymocytes were FACS sorting using a FACSAriaIII or FACSAria Fusion cell sorter (BD Biosciences) as propidium iodide$^-$CD4$^+$CD8$^+$ or propidium iodide$^-$CD4$^+$CD8$^+$CD45.1$^+$ in bone marrow transplant mice.

Irradiation and Bone Marrow Transplantation

Total bone marrow cells were isolated from the left femurs and tibias of CD45.2$^+$ or CD45.1/2$^+$ congenic wild-type FVB mice. For TPT^{KO} transplants CD45.2$^+$E47$^{-/-}$ or CD45.1/2$^+$ *Vav-Cre*$^+$*E2a*$^{+/f}$*E47*$^{+/-}$ were used. Single cell suspensions were depleted of T cells using biotinylated antibodies to TCRβ, TCRγδ, CD3ε, CD4, and CD8 (1:400 dilution), followed by the addition of streptavidin microbeads and passage over LD magnetic columns (Miltenyi). T cell- depleted bone marrow cells were resuspended (10^6 cells/100 μL) in ice- cold 1X PBS + 0.5% FBS and injected through the retro-orbital vein into sublethally irradiated (750 rads or 550 rads) mice. Mice were monitored daily for leukemia for up to 32 weeks post-transplant. At 4 weeks and 20 weeks post-transplant approximately 10μL of whole blood was isolated from the tail vein and treated with 400μL ACK lysis buffer for 90 seconds. ACK reaction was neutralized through addition of at least 3mL of FACS+EDTA and cells were prepared for flow cytometry.

RNA isolation, DNA isolation, and PCR

RNA isolation and cDNA synthesis

Cells were centrifuged at 400g for 5 minutes and resuspended in RLT lysis buffer from the RNeasy mini kit (QIAGEN) before passage through QIAshredder columns (Cat. 79656). For cell populations < $5x10^5$ RNA was isolated using the RNeasy micro kit (QIAGEN, Cat. 74004). For cell populations >$5x10^6$ RNA was isolated using RNeasy mini kit (QIAGEN, Cat. 74104) following manufacturers protocol.

RT-qPCR

RNA concentration was measured using a NanoDrop system (ThermoFisher). RNA concentration was normalized across samples and cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, Cat. 18080-051) following manufacturers protocols. QPCR was performed with gene-specific primers in an iCycler (BioRad), using the iQ SYBR Green Supermix (BioRad, Cat. 1708880). Reactions were performed in triplicate under the following conditions: 95°C for 3 min, 37 cycles at 95°C for 10 sec and 60°C for 30 sec. *β-actin* was used to normalize gene expression across samples. Results were analyzed using the ΔΔCT method. qPCR primers are listed below:

Table 2.1: qPCR primer sequences

Gene	Primer Sequence
β-Actin Fwd	ACGGCCAGGTCATCACTATTG
β-Actin Rev	AATGTAGTTTCATGGATGCCAC
Ikzf2 Fwd	ACACCTCAGGACCCATTCTG
Ikzf2 Rev	TCCATGCTGACATTCTGGAG
Notch1 Fwd	CTGTTGTGCTCCTGAAGAACG
Notch1 Rev	AGTCTCATAGCTGCCCTCAC

DNA isolation and PCR

Sorted cells were centrifuged at 400g for 5 minutes. Cells were then resuspended in 0.5mL sterile H_2O and boiled in a 100°C heat block for 120 seconds. After boiling cells were placed on ice and 2μL of 10mg/mL proteinase K was added. Cells were incubated for 30 minutes on 55°C heat block. After incubation cells were placed on ice and DNA was isolated using the Zymo Research Quick-DNA Miniprep kit (Cat. 11-317AC) following manufacturers protocols. PCR to assay E2a genomic recombination used the following primers:

Table 2.2: *E2a* deletion PCR primer sequences

Primer	Sequence
E2a Floxed	5′–TCGTCCTCGTCCTCGTCT–3′
E2a Recombined	5′–CTCACAGAGACCTCCCGACT–3′
Universal Reverse	5′–CGGATCCATCCTCGTCTTTGGTACTG–3′

Retroviral transduction

Generation of retrovirus

Retroviral supernatants for MigR1-GFP and MigR1-E47 were produced in Plat-E cells through $CaPO_4$ transfection protocol. Briefly, Plat-E cells were plated at a concentration of $6x10^6$ cells/10cm petri dish approximately 16-24 hours before transfection. For transfection, 1.5mL of HBSS (pH 7.05) was added dropwise while bubbling to a 1.5mL solution containing 30μg DNA vector, 186μL $CaCl_2$, and sterile H_2O. DNA+HBSS solution was then added to Plat-E cells dropwise. Cells were rested overnight and old media was aspirated and refreshed with 10mL of fresh media. After 48 hours viral supernatant were collected and stored at -80°C.

Retroviral transduction of Leukemia Lines

Leukemia lines were counted on a hemocytometer and $1x10^6$ cells were isolated and transferred to 1.5mL Eppendorf microfuge tubes. Cells were then centrifuged at 400g for 5 minutes and were then resuspended inn 1mL of MigR1-GFP or MigR1-E47 retroviral supernatant supplemented with 5µg sterile polybrene. Cells were plated in 6 well plates and spinoculated at 33°C at 2,500RPM for 90 minutes. After centrifugation supernatant was aspirated and 1mL fresh media was added. Cells were cultured for 48 hours after transduction before analysis.

RNA sequencing

RNA-seq libraries were constructed using Nugen's Ovation Ultralow Library systems and sequenced on a NovaSeq 6000 and and were subsequently subjected to 76 cycles of NextSeq500 sequencing. Raw sequence reads were trimmed using Trimmomatic v0.33[200] and STAR,[201] and to mouse genome assembly mm10 using HTSeq v0.6.1. Reads were assigned to genes using the high throughput sequencing count tool from HTSeq v0.6.1 and gene annotations from Ensembl release 78.[202] The R package DESeq2[203] each gene for each pairwise comparison of sample groups within an nf-Core Differential Abundance workflow.[204] Metascape was used for KEGG analysis.[205] Gene set enrichment analysis (GSEA) was described previously.[206] Genes were considered differentially expressed if the Log_2FC was > 0.5 had a p-value of <0.05. Heatmaps were generated using the R package PHeatmaps and represent all differentially expressed genes between any samples shown. Browser tracks for RNA-sequencing and HEB ChIP-sequencing data were analyzed using Integrative genome browser v2.15.2.

Statistics

EdgeR or GraphPad Prism software was used to calculate statistics. A Student's t-test or ANOVA with multiple comparisons was used to establish the level of significance. Kaplan-Meier curves

were analyzed using Log- Rank (Mantel-Cox) test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

CHAPTER 3:

REGULATION OF T CELL TRANSFORMATION BY E2A

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INTRODUCTION

E2a has been appreciated as a tumor suppressor in murine thymocytes since it was discovered that germline deletion of *E2a* (or germline deletion of the dominant E2A isoform, E47) resulted in rapid, aggressive T cell leukemias.[8] E2a is expressed ubiquitously throughout T cell development where its stage-specific function is governed by the proteins E2A interacts with.[6] In multipotent hematopoietic progenitors, E2A interacts with either Tal1 or Lyl1, where it broadly functions to antagonize or promote lymphoid specification, respectively.[89,90,207] In the thymus, E2A forms a complex with other E protein family members, HEB and E2-2.[7,88] Inhibition of E2A signaling, either through E2a deletion or generation of an HEB dominant negative allele (HEB-DN), results in stark defects in T cell development, including a 3-5X reduction in the number of T cells in the thymus.[7,8,198] This phenotype is more mild in HEB or E2-2 deficient mice, indicating that E2A:E2-2 and E2A:HEB heterodimers can partially compensate for the loss of each other.[7,88,208] The HEB-DN mutation was generated through a point mutation of the basic domain. This prevents dimers containing the HEB-DN from binding DNA.[7] Unsurprisingly, the HEB-DN phenotypes were similar to those seen when ectopically expressing canonical inhibitors of E2A DNA binding, ID1 or ID2.[9,11] Indeed, ID protein transgenic mice had significant reductions in the total number of thymocytes. More notably, however, ID transgenic mouse models develop T-ALL, consistent with what has been reported in *E2a*$^{-/-}$ mice.[8,9,11] Together, these observations suggest that partial loss of E protein function is sufficient to induce mild defects in T cell development, but full inhibition of E2A induces severe defects that cannot be adequately compensated for by other

E proteins. Loss of HEB or E2-2 have not been reported to cause T cell leukemias, only inhibition of E2A through deletion or ectopic expression of E2A inhibitors have resulted in T-ALL.

Repression of E2A function is one of the most common features of human T-ALLs, with nearly 60% of pediatric T-ALL patients ectopically expressing TAL1, and many leukemias that do not express Tal1 overexpress ID2.[21,22,116] TAL1:E2A dimers have their own target genes that may influence leukemogenesis, however, in mice the DNA binding activity of TAL1:E2A dimers is dispensable for leukemia development.[120,121,134,135] Notably, prior to leukemia development, TAL1 expressing mice have similar developmental defects as *E2a*$^{-/-}$, ID1/2 transgenic, and HEB-DN mice.[7–9,11,121] Altogether, these data suggest that E2A is required to safeguard thymocyte development and prevent thymocyte transformation.

One of the most significant differences in the HEB-DN and ID transgenic models is the timing at which E2A function is repressed. The *E2a*$^{-/-}$ model is a germline deletion, which effects all cells, but most importantly the deletion is in the HSC compartment.[7,8] The ID1/2 and Tal1 transgenic models use an *Lck* promoter, which initiates expression in the DN2 stage of T cell development.[9,11,121] These models suggest that inhibition of E2A before the DN3 stage is sufficient to induce leukemogenesis, however, there are conflicting reports. Deleting *E2a* in mice using the *Lck-Cre* did not induce leukemogenesis within the 16 months of the study.[198] *In vitro* analysis of sorted DN3s 2 days after inducing *E2a* deletion showed significant transcriptional changes, including upregulation of the oncogene *Gata3*, confirming that E2A still plays a role in repressing oncogene activation at this stage.[209] Similar phenotypes have been seen using *Tcf7*$^{f/f}$ mice combined with different Cre drivers. Notably, germline *Tcf7*$^{-/-}$ and *Vav-Cre*$^{+}$*Tcf7*$^{f/f}$ mice, which deletes *Tcf7* in HSCs, develop leukemia but *Lck-Cre* and *CD4-Cre*- mediated deletion did not.[210] Together, these observations suggest that the stage and timing at which a tumor suppressor is

deleted plays a significant role in its ability to repress transformation. How germline *E2a* deletion predisposes mice to leukemia but deletion in the DN3 stage does not has yet to be resolved.

Previous work in the Kee lab identified that *E2a*$^{-/-}$ leukemias recapitulate several aspects of human TAL1^{+} T-ALL. Indeed, *E2a*$^{-/-}$ leukemia cell lines are dependent on Notch signaling for their survival and have mutations in the Notch1 PEST domain, similar to human T-ALLs.[127,143] In T-ALL lines, Notch promotes expression of cell cycle machinery, tRNA synthesis, cell growth, and metabolism genes.[152] However, when during transformation these *Notch1* mutations occur has not yet been described. Notably, the *Notch1* mutations commonly found in human T-ALLs are weakly oncogenic by themselves. Mice transplanted with bone marrow transduced with mutant *Notch1* alleles that mirror patients' mutations rarely developed T-ALL.[211] These *Notch1* mutations have not been described in pre-leukemic thymocytes, thus they appear to occur later in T cell transformation and may provide further amplification of Notch1 signaling. Given that Notch1 is such a strong oncogenic signal, it is plausible that other mechanisms promote increased Notch1 signaling in pre-leukemic thymocytes prior to selection for these mutations.

In addition to *Notch1* mutations, the Kee lab identified *Lef1* as a *Notch1* target gene that promotes the survival of *E2a*$^{-/-}$ leukemias.[103] This was shown to be an active process, as withdrawal of Notch signaling *in vitro* and *in vivo* resulted in a downregulation of *Lef1* mRNA and protein. Inhibition of *Lef1* expression in *E2a*$^{-/-}$ leukemia lines using siRNA vectors resulted in significant cell death, demonstrating the pro-survival function of *Lef1*. Consistent with this, over-expression of LEF1 promoted leukemia survival upon *Notch1* inhibition.[103] Together, these findings suggest that a major function of *Notch1* in leukemia lines is to maintain *Lef1* expression to promote survival. These findings prompted an investigation into how *Lef1* contributes to the transformation of T cells. We have shown that *Lef1* is highly upregulated in *E2a*$^{-/-}$ DN3 thymocytes by 5 weeks of

age and enforces their development *in vitro*.[104] Surprisingly, however, deletion of *Lef1* in *E2a*$^{-/-}$ mice did not delay leukemia latency, but rather increased it, suggesting that *Lef1* was acting as a tumor suppressor prior to transformation. This contrasts with deleting *Lef1* in fully transformed leukemia lines, where *Lef1* was acting as an oncogene. *Lef1's* ability to act as an oncogene or a tumor suppressor depending on when during transformation it is deleted indicates that the timing or specific order of mutations is critical to developing leukemias.

As primary oncogenic mutations, the stage at which *E2a* or *Tcf7* deletion occurs dramatically impacts its ability to drive thymocyte transformation, suggesting that different stages of thymocyte development are more susceptible to transformation than others. Alternatively, transcription factors in the thymus are highly stage specific, regulating unique networks of genes depending on the specific stage.[6] Thus, deletion earlier in the developmental program may induce transcriptional changes that pre-dispose later stages to transformation. How secondary mutations affect the transformation of thymocytes is dependent on when during the course of transformation they occur. In the case of *Lef1*, its role as a tumor suppressor or oncogene is dependent on such timing.[104] This is reminiscent of the idea that cancers become "addicted" to specific oncogenes. Specifically, this phenomenon is the idea that cancers depend on specific oncogenic signals despite acquisition of other mutations that promote transformation.[212] In the case of *Lef1*, E2a$^{-/-}$ T-ALLs developing in the presence of *Lef1* become addicted to it resulting in death up its' withdrawal, while deleting it sufficiently early in leukemogenesis allows the tumors to develop independent of *Lef1*.[104] This is not the case for all oncogenes in T-ALL, however. Studies using a model of LMO2 induced T-ALL, in which constitutive LMO2 expression can be ablated through continuous addition of doxycycline show three "groups" of leukemias arise upon secondary transfer. The first is LMO2 dependent, where ablating LMO2 expression prevented secondary hosts from developing

leukemia. The second is an evolving phenotype, where there is still development of T-ALL, but with delayed latency upon LMO2 withdrawal. The final is LMO2 independent, where leukemia latency was unaffected by the presence of absence of LMO2.[213] This study also found that the human derived T-ALL lines CCRF-CEM and RPMI-8402, which are TAL1^{+} T-ALLs, can survive CRISPR-Cas9 mediated deletion of *Tal1* with no loss in growth potential. This was not true in all lines, as the P12-Ichikawa line (LMO2 overexpressing) and the TAL1^{+} HSB2 T-ALLs lost growth potential upon deletion of *Lmo2* and *Tal1*, respectively. It is notable that *Lef1* deletion was universally not tolerated in E47$^{-/-}$ leukemia lines,[104] while loss of LMO2 and TAL1 were tolerated in some murine and human cell T-ALL lines. This may be due to the LMO2 and TAL1 mutations being the primary mutations in their respective leukemias, while Lef1 deletion was a secondary event. Given that a major oncogenic function of Tal1 and Lyl1 is repression of E2A,[121,214] another hypothesis is that the LMO2 and TAL1^{+} leukemias may have acquired other mutations to repress E2A, such as ID2 or ID3 upregulation. This would allow them to tolerate loss of LMO2 and TAL1 without a restoration of E2A function. Regardless, understanding when during leukemogenesis secondary events occur could provide key insight into genes the leukemic cells are addicted to.

Together, this suggests that the kinetics of T-ALL-associated genetic dysregulations and mutations are understudied, and understanding these kinetics can provide novel insights into how and why T cells transform. Here I show the kinetics of T-ALL development in 2 different models of *E2a* deletion and explore the kinetics of activation of numerous oncogenic pathways in model that rapidly develops T-ALL. Increased cycling and oncogene activation is observed as early as 4 weeks of age only when *E2a* is deleted in hematopoietic stem cells. Oncogenes from these pathways were further dysregulated with time, however by 8 weeks of age, novel proliferation

pathways were enriched, and apoptosis and senescence pathways were depleted, indicating that the cells are closer to full transformation.

RESULTS

Generation of E2a$^{-/-}$ Mouse Models and Confirmation of Deletion

Germline deletion of *E2a* results in severe deficiencies in T cell development and an aggressive and rapid T-ALL with a DP/Cortical phenotype.[8,104] However, the kinetics of T cell development dysfunction in relation to the kinetics of leukemogenesis have not been elucidated. To investigate this, I developed mouse models that conditionally delete *E2a* at different stages of T cell development using two different Cre drivers (Fig. 3.1.A). *Vav-Cre^{+}E2a$^{f/f}$* mice (VcKO) delete *E2a* at the HSC stage, while *LckCre^{+}E2a$^{f/f}$* mice initiate *E2a* deletion at the DN2-DN3 transition. (Figure 3.1.B). To confirm at what stage of T cell development *E2a* is fully deleted in LcKO mice, I FACS sorted LcKO DN3s, DPs, thymic CD4s, wild-type DPs, and VcKO DPs and performed PCR to assess genomic deletion of exons encoding the *E2a* DNA binding domain. By the DN3 stage, approximately 75% of DNA isolated had deleted this genomic region (Figure 3.2.A; bottom band). By the DP stage, 100% of the DNA had undergone deletion, which continued into the CD4 SP stage. Control wild-type DPs show no deletion, while VcKO DPs also show 100% deletion. (Figure 1.2.A). Together, these data suggest that approximately 75% of LcKO thymocytes delete *E2a* by the DN3 stage, and 100% fully delete *E2a* by the DP stage. To evaluate protein deletion in LcKO DN3s, I analyzed *E2a* protein expression using flow cytometry. Consistent with the ~75% deletion seen in the PCR assay, 75% of LcKO DN3s had lost expression of E2A compared to wild-type controls.

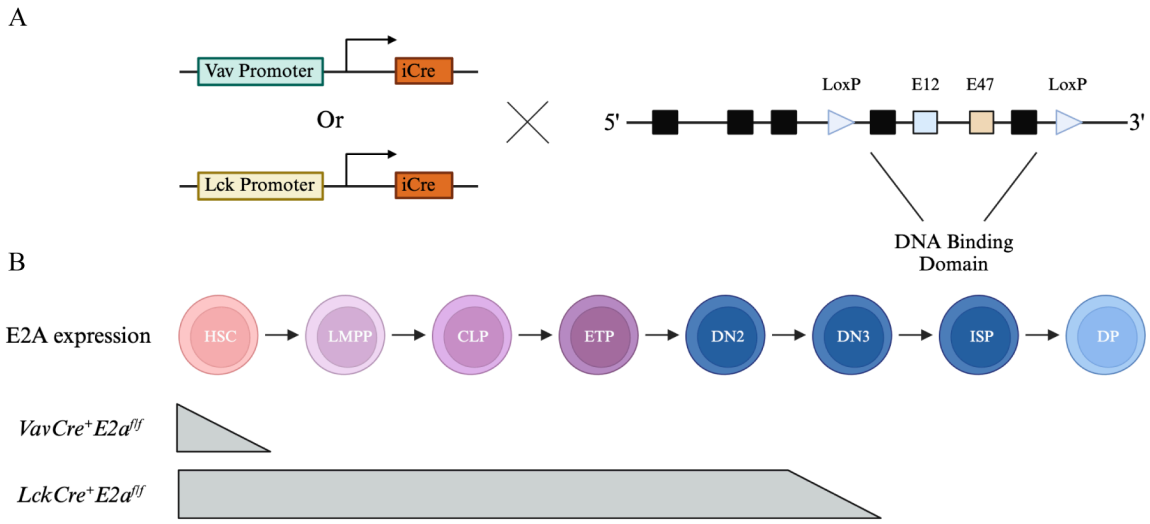

Figure 3.1: Models of E2A deletion
A) Schematic representation of Cre-mediated deletions and *E2a* gene. B) Timing of *E2a* deletion for Cre drivers with respect to stage of T cell development.

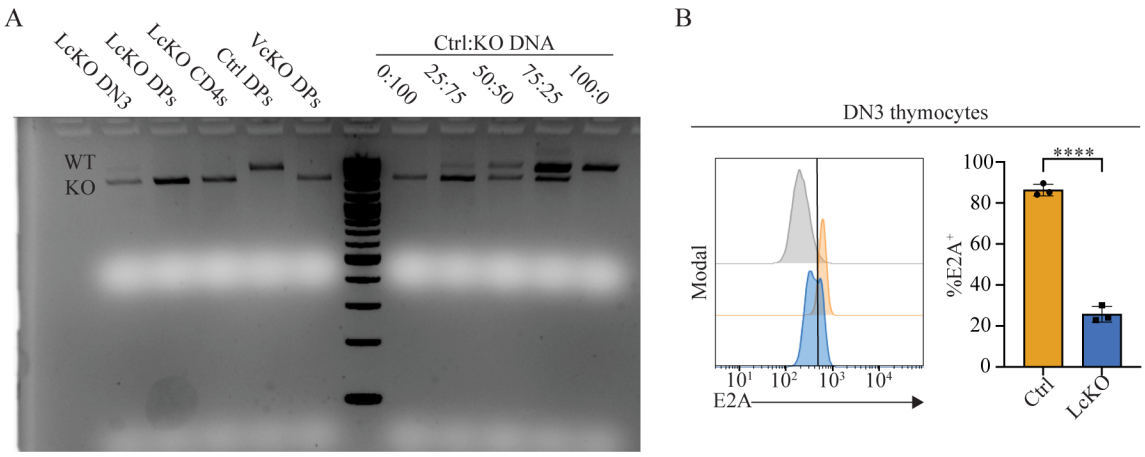

Figure 3.2: Analysis of *E2a* deletion in LcKO thymocytes
A) Representitive gel image of PCR analysis of genomic rearrangements at the *E2a* DNA binding domain. Genomic DNA was isolated from the indicated populations. Standard was generated by mixing VcKO and WT DP DNA at the indicated ratios. B) Flow cytometric analysis of E2A expression in control and LcKO DN3s.

Early Deletion of E2a is Required for Leukemia Development

After confirming that both mouse models efficiently delete *E2a* prior to the DP stage, I investigated if the VcKO conditional deletion mirrored the germline deletion in regard to leukemia latency. Previous reports with germline *E2a* deletions report that mice succumb to T-ALL with an average latency of 18.5 weeks,[104] and consistent with this report, VcKO mice had a similar latency (18.5 vs. 17.3 weeks) (Figures 3.3.A). This observation suggests that the leukemia that develops in $E2a^{-/-}$ mice is the result of a loss of *E2a* in the hematopoietic compartment. A comparison of leukemogenesis in VcKO and LcKO mice revealed a striking difference in disease penetrance and latency. In contrast to previous reports that $Lck\text{-}Cre^{+}E2a^{f/f}$ mice did not develop leukemia, 4/15 LcKO mice (26%) succumbed to T-ALL within the 52 weeks of the study (Figure 3.3.B and 3.3.C). Surprisingly, the LcKO mice that succumbed to T-ALL did so with a significantly increased latency (mean 37.3 weeks vs. 17.3 weeks). Therefore, initiating deletion of *E2a* at the DN2/3 stages (Figure 3.1.A) is insufficient to induce robust leukemogenesis but remains weakly oncogenic in a subset of mice.

VcKO, but not LcKO mice have significant developmental defects

Several models of T-ALL first present with significant defects in T cell development prior to leukemogenesis, including reductions in the total number of thymocytes and specific defects early in T cell development.[8,106,121,171] Indeed, VcKO mice have reduced thymocyte numbers at several timepoints post-birth, including a neonatal time point (< 1week), peak thymocyte development (3-5 weeks) and an adult timepoint (8-9 weeks) (Figure 3.4.A). By 10 weeks of age, however, VcKO thymocyte numbers have begun to expand, which could indicate full initiation of leukemia. Analysis of VcKO thymi at a preleukemic timepoint (3-5 weeks of age) showed a reduction in the frequency and number of most immature thymocyte populations. Further, there is

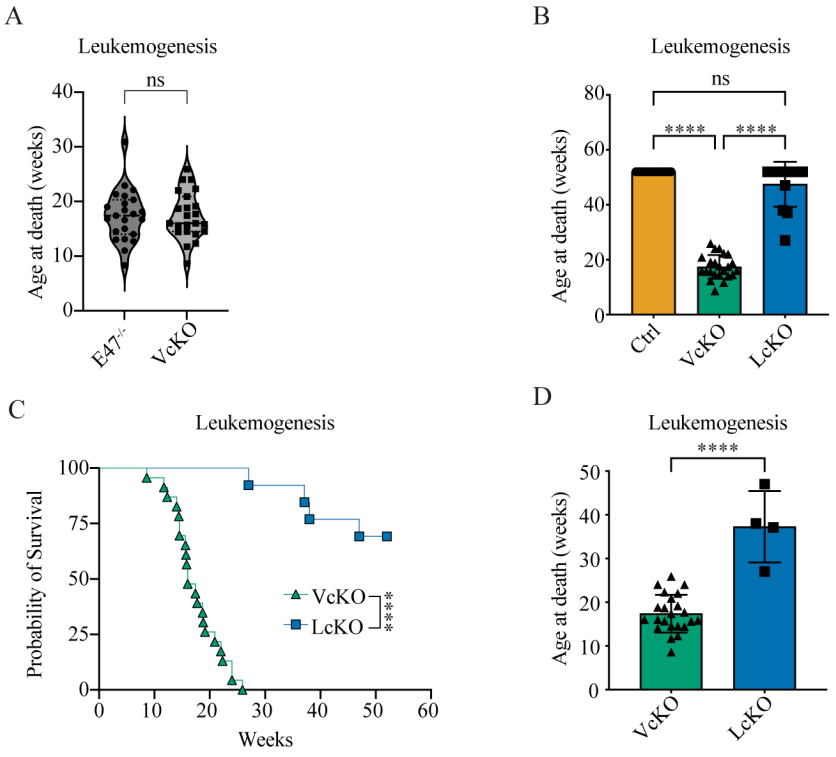

Figure 3.3: Comparison of leukemogenesis in multiple models of *E2a* deficiency
A) Comparison of leukemia latency between germline *E2a* deletion and VcKO mice. B) Comparison of leukemia latency betweem VcKO, LcKO, and Control mice. C) Kaplan-Meyer curve analysis of leukemogenesis in VcKO and LcKO. D) Age at death of VcKO mice and the 4/17 mice that succumbed to T-ALL in LcKO leukemogenesis study.

a near absence of DN3s and loss of DP thymocytes (Figure 3.4.B and Figure 3.4.C). This defect in T cell development is consistent with what has been reported in the germline *E2a* deletion and when taken together with the leukemogenesis data (Figure 3.3.A), confirms that the VcKO model faithfully recapitulates the *E2a*$^{-/-}$ model. The developmental defects noted in the VcKO model are absent in the LcKO model, which phenotypically resembles wild-type mice (Figure 3.4.A, Figure 3.4.B, and Figure 3.4.C). The only notable difference between the LcKO and wild-type mice is an increase in the DN3 population of LcKO mice, which is consistent with loss of *E2a* at the DN3 stage inducing hyper-proliferation.[215] This slight increase in numbers is resolved and no longer present by the ISP stage, however (Figure 3.4.C). Taken together, these results support the idea that the early deletion of *E2a* is driving developmental deficits and suggests that *E2a* is mostly dispensable for leukemogenesis after the DN3 stage. Finally, it supports the hypothesis that tumor suppressors need to be deleted at specific stages of T cell development to induce transformation.

VcKO DPs are more proliferative and have increased Notch1 signaling than LcKO DPs

To investigate how the early loss of *E2a* in the VcKO model predisposes DPs to transformation I analyzed several markers of leukemic transformation in VcKO, LcKO, and Ctrl thymocytes in 3-5 week old mice. Consistent with there being no significant T cell developmental defect in LcKO mice, there was no difference in Ki67 expression between Ctrl and LcKO DPs, while the VcKO DPs had higher Ki67 expression (Figure 3.5.A). DPs recombine their DNA to generate the TCRα chain, which requires the generation of double strand DNA breaks.[216] It has been well established that errant V(D)J recombinase activity can cause T-ALL associate mutations, raising the possibility that the increase in the frequency of cycling DPs could impact the frequency of mutations that drive transformation.[214] Further, LcKO DN3s and DPs showed no significant

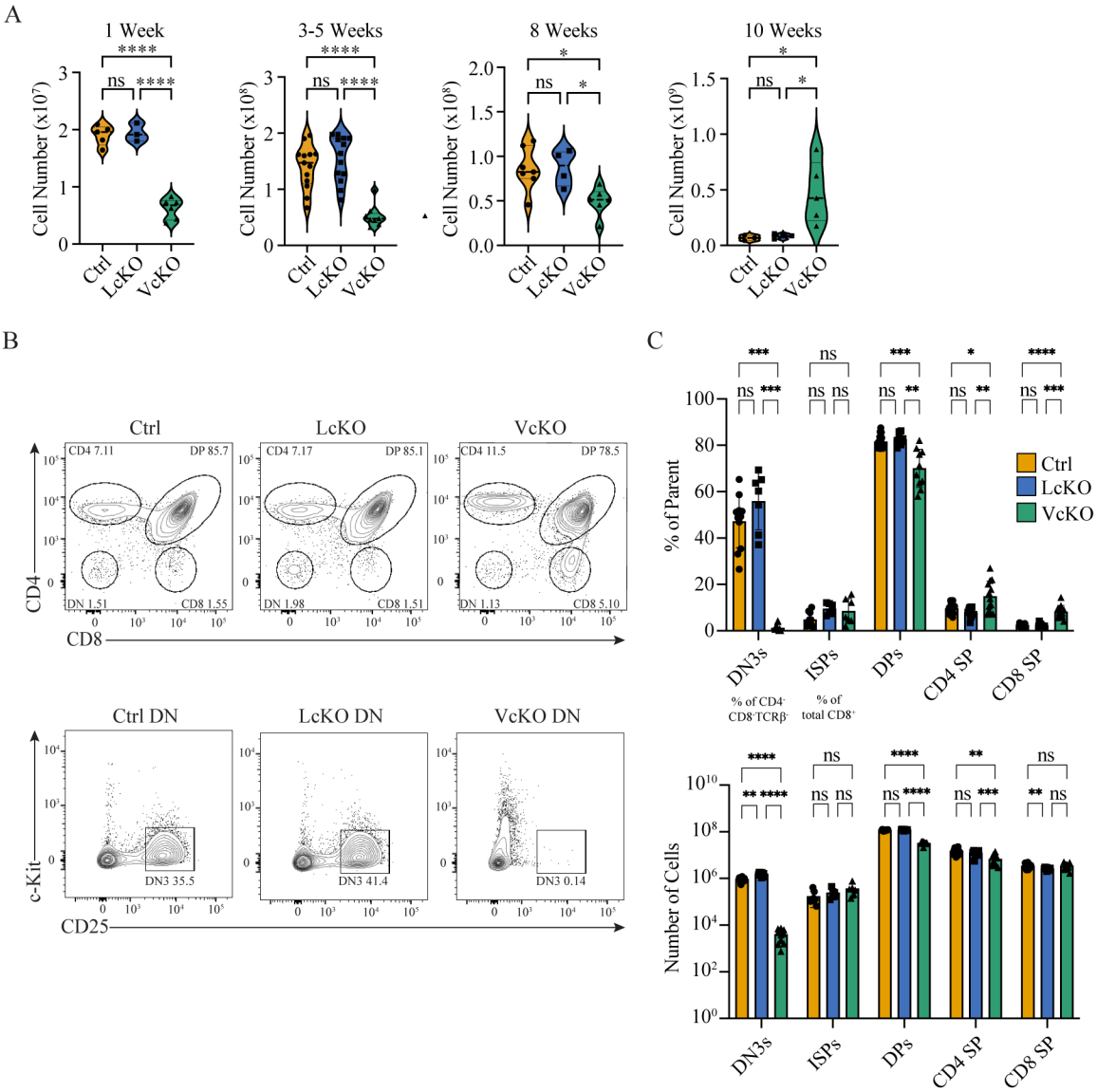

Figure 3.4: Phenotypic analysis of control, LcKO, and VcKO thymocytes

A) The number of total thymocytes from mice of the indicated genotype at 1 week, 3-5 weeks, 8 weeks, or 10 weeks of age. B) Representitive FACS plots of thymi from 3-5 week old mice of the indicated genotype pregated on Lineage$^-$ (Top) and pregated on Lineage$^-$CD4$^-$CD8$^-$TCRβ$^-$ (Bottom). C) Quantification of frequencies (Top) and total numbers (Bottom) of the indicated thymocyte population from the indicated genotype.

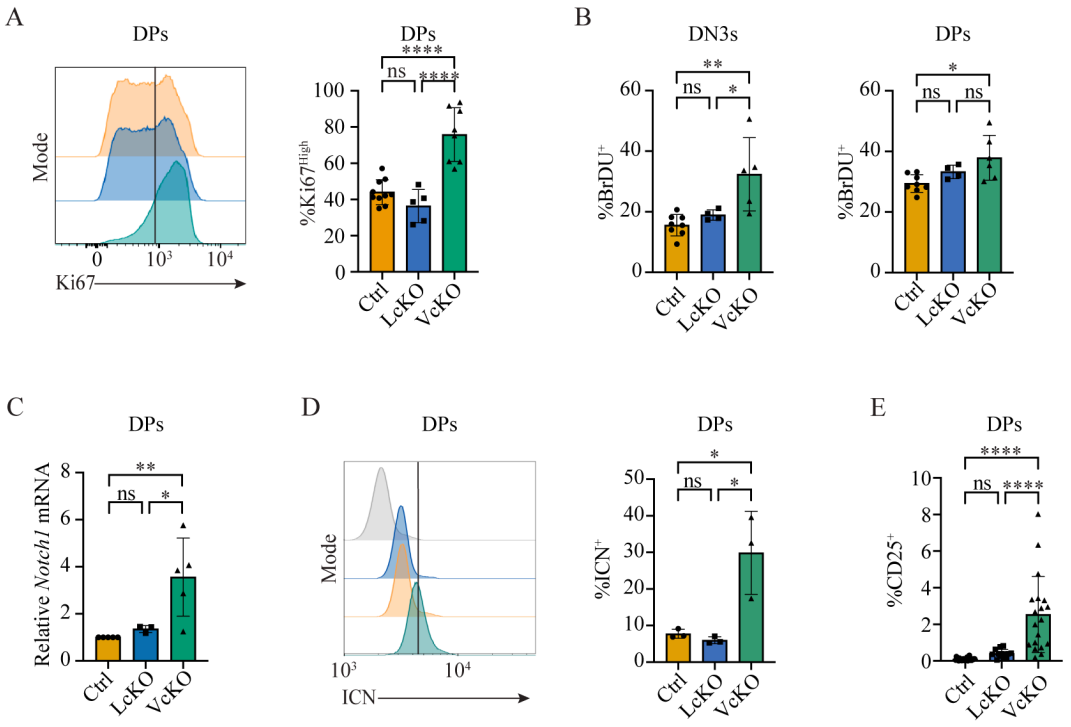

Figure 3.5: Phenotypic Analysis of DP thymocytes

A) Expression of Ki67 in total DP thymocytes from 3-5 week old mice. B) Percentage of DN3s (left) or DPs (right) that incorporate BrDU after injections of 1mg BrDU 24 and 12 hours before harvest. C) RT-qPCR for *Notch1* expression in sorted DPs from 3-5 week old Ctrl, LcKO, and VcKO mice. Data analyzed using ΔΔ CT method D) Expression of ICN in DP thymocytes. E) Expression of the NOTCH1 target gene CD25 in DP thymocytes.

increase in BrDU incorporation, suggesting no difference in the rate of proliferation (Figure 3.5.B, Left). Notably, VcKO DN3s showed a significant increase in BrDU incorporation consistent with previous reports,[94] while VcKO DPs only had a mild increase over Ctrl DPs, but not LcKO DPs (Figure 3.5.B). Together, this suggests that at 3-5 weeks of age, more VcKO DPs are in cycle than Ctrl or LcKO DPs, but there is little difference in the rate of proliferation. Given that Notch signaling is tightly associated with proliferation and leukemic transformation, I analyzed the expression of *Notch1*, ICN, and the Notch1 target gene CD25 in 3-5 week old DP thymocytes. VcKO DPs have a significant increase in the amount of *Notch1* mRNA, and a corresponding increase in ICN expression, consistent with early transformation events, while LcKO mice did not (Figure 3.5.C and Figure 3.5.D). This correlated with an increase in CD25 expression, which was significantly enriched in VcKO, but not LcKO or Ctrl DPs (Figure 3.5.E). Thus, the early deletion of *E2a*, but not the later deletion, predisposes DPs to increased Notch expression that may drive entry into the cell cycle and promote initial transformation events.

Early deletion of E2a results in significant transcriptomic changes

To get a better understanding of the genetic differences caused by the different timing of the *E2a* deletion prior to the onset of leukemia, I performed bulk RNA sequencing on 4-5 week old VcKO, LcKO, and Ctrl DPs. Transcriptomic analysis revealed that LcKO DPs more closely resemble Ctrl DPs than VcKO DPs (Figure 3.6.A). There were 421 differentially expressed genes (DEGs) between LcKO and Ctrl DPs, while VcKO DPs had 1659 DEGs (Figure 3.6.B and Figure 3.6.C). 764 DEGs upregulated in VcKO DPs showed no change in LcKO DPs, and 643 downregulated DEGs in VcKO DPs showed no change in LcKO DPs (Figure 3.6.B and Figure 3.6.C). There were 61 and 108 genes upregulated and downregulated, respectively, in LcKO but not VcKO DP thymocytes (Figure 3.6.B). Thus, early *E2a* deletion induces greater transcriptomic

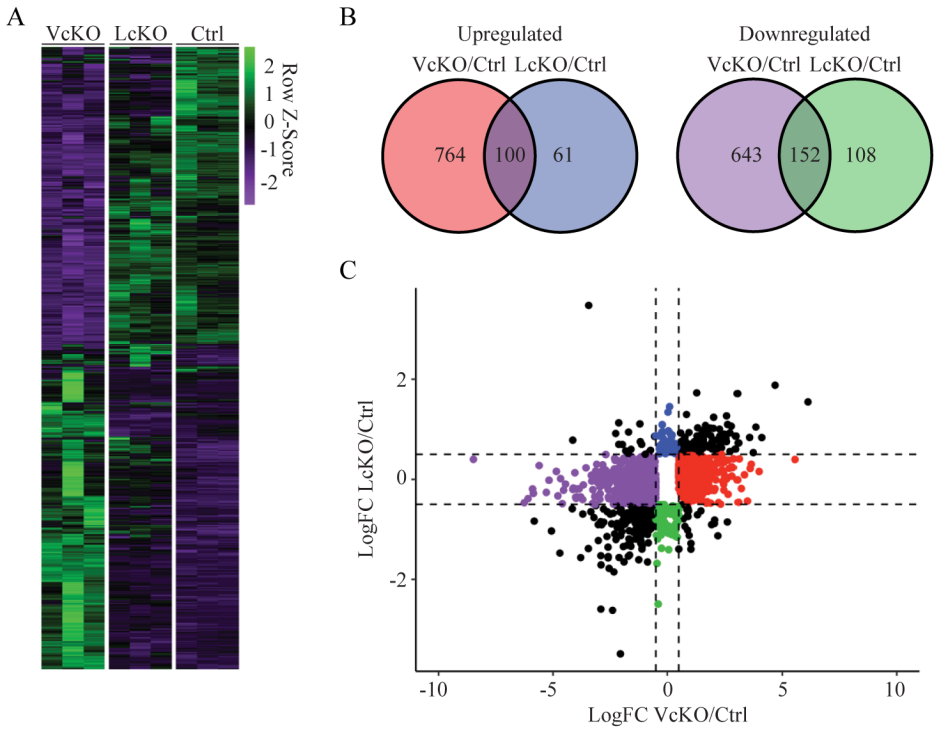

Figure 3.6: Early *E2a* deletion induces significant transcriptomic changes in DPs
A) Heatmap of all genes differentially expressed between VcKO and LcKO DPs relative to their respective controls. B) Comparison of the number of up- and down-regulated genes between VcKO and LcKO DPs compared to controls. C) Plot of the log fold change of all DEGs in VcKO and LcKO DPs.

changes than DN3 deletion, which may contribute to the decreased latency and increased rate and penetrance of leukemogenesis. To investigate if the DEGs in VcKO DPs are associated with biological pathways known to contribute to T cell transformation, I performed KEGG pathways analysis and Gene Set Enrichment Analysis (GSEA) on DEGs in VcKO DPs vs. Ctrl DPs, excluding those that were also differentially expressed in LcKO DPs. KEGG pathway analysis identified enrichment of pathways associate with biosynthesis of unsaturated fatty acids and fatty acid elongation (Figure 3.7.A). This could be indicative of metabolic reprogramming to allow for further transformation, which is metabolically demanding.[217] Other highly enriched pathways included Notch signaling, consistent with the increased *Notch1* mRNA, ICN protein, and CD25 in 3-5 week old DPs (Figure 3.7.A, Figure 3.5.C Figure 3.5.D, and Figure 3.5.E). VcKO DPs were also enriched for the JAK-STAT, Chemokine signaling, and Th1 and Th2 differentiation pathways, and many of the genes contained within the Th1 and Th2 differentiation pathway were cytokine receptors (Figure 3.7.A). GSEA indicated enrichment of Wnt_β_catenin, Myc, Notch and TNFA_via_NFκB pathway genes in VcKO DPs (Figure 3.7.B). Together, the transcriptomic data suggests that the early deletion is required for the activation of canonical T-ALL pathways, while the later deletion has a smaller effect on the transcriptome of DPs.

Phenotypic changes in VcKO DPs with age

The oncogene activation seen in young, pre-leukemic thymocytes suggests that it is either a direct result of early *E2a* deletion or that selection for these pathways is an early event that occurs during transformation. To better understand oncogenic events that occur later as mice age, I analyzed the thymic phenotype, Ki67, and CD25 expression in 8-week-old VcKO mice. Notably, the frequency of DPs in VcKO mice decreased vs. wild-type mice which correlates with an increase the frequency of CD25 expressing DPs (Figure 3.8.A and Figure 3.8.B, Left). In addition

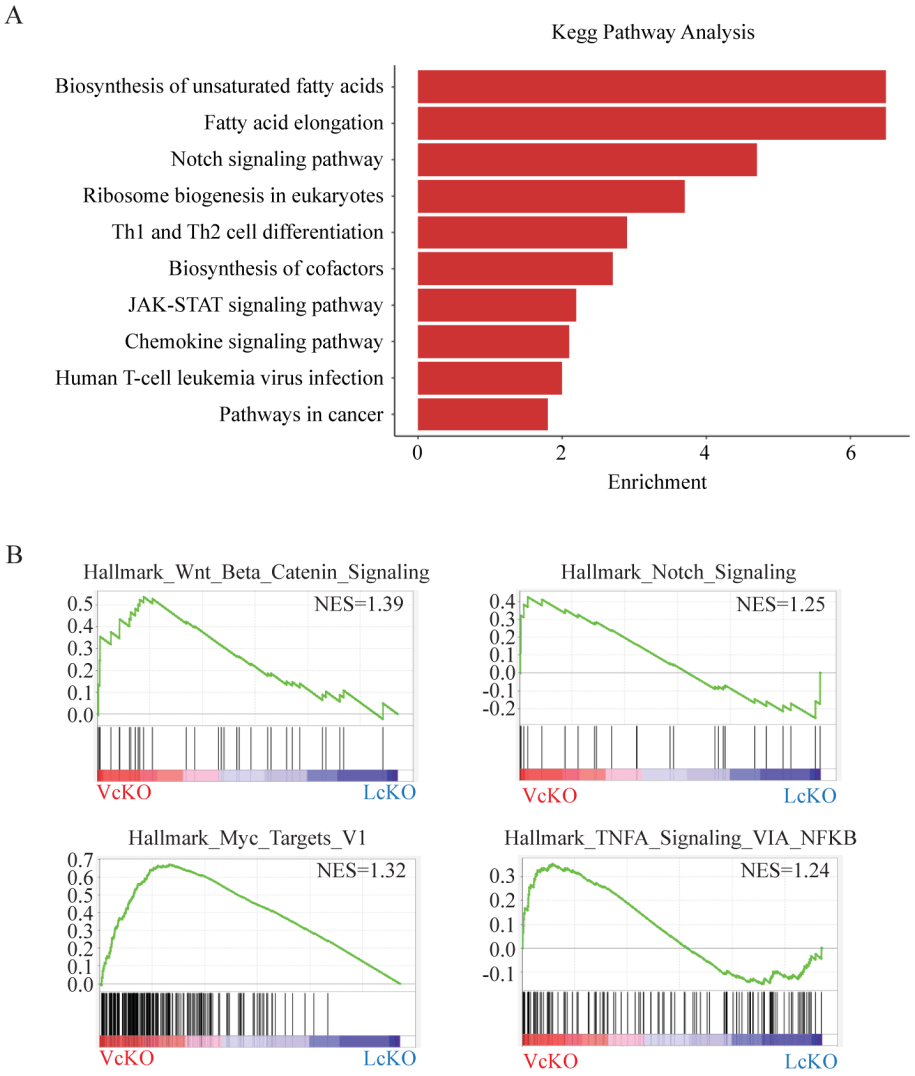

Figure 3.7: Early deletion induces oncogenic pathway activation and cytokine signaling pathways in *E2a*$^{-/-}$ DPs

A) KEGG pathway analysis of genes uniquely upregulated in VcKO DPs vs ctrl compared to LcKO DPs vs ctrl. B) Select enriched gene sets identified by GSEA comparing the transcriptomes of VcKO and LcKO DPs.

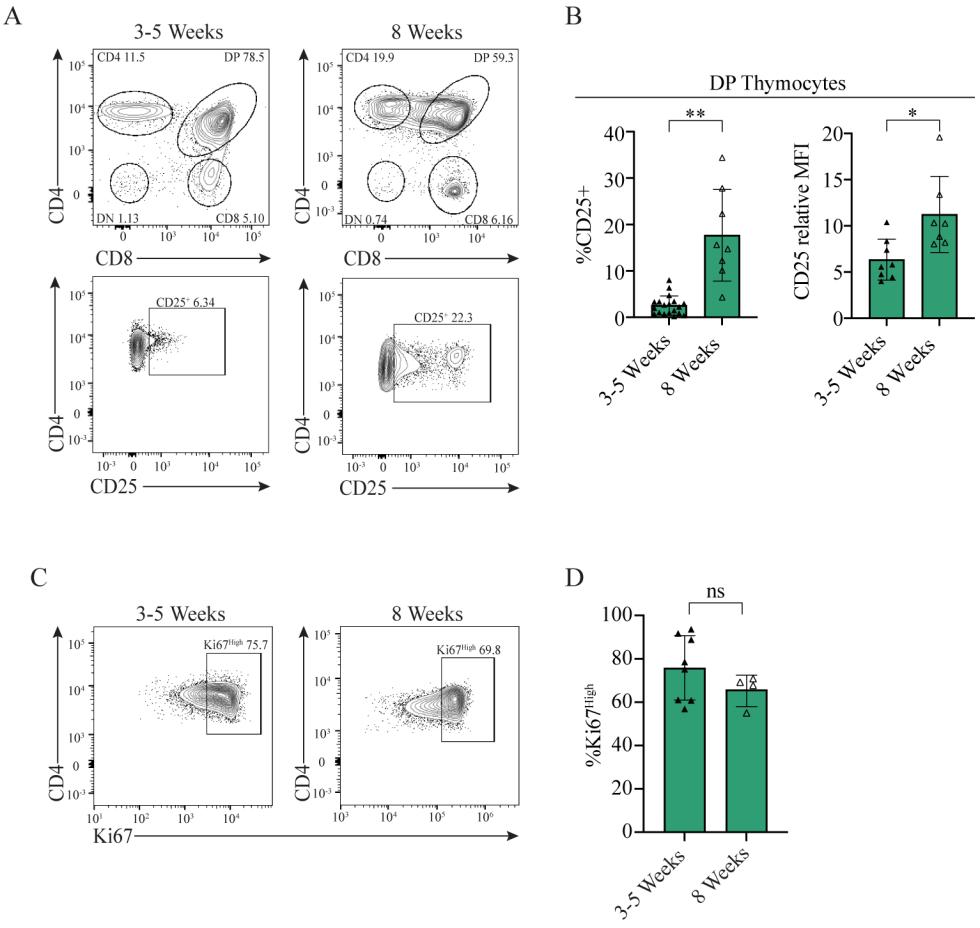

Figure 3.8: Comparison of 3-5 week and 8 week old DP phenotypes
A) Representitive FACS plots of Lineage$^-$ thymocytes from VcKO mice of the indicated age (Top). CD25 expression was assessed on Lineage$^-$ DP thymocytes (Bottom). B) Quantification of the percentage of CD25 expressing cells and CD25 relative MFI on DPs. MFI is relative to CD25$^-$ DPs from the same mouse. C) Representitive FACS plots and D) quantification of Ki67 expression on DP thymocytes from VcKO mice of the indicated age.

to a higher frequency of CD25 expressing DPs, the relative MFI of CD25 among $CD25^{+}$ DPs was increased in 8-week-old VcKO DPs compared to 3-5 week old DPs (Figure 3.8.B, Right). Surprisingly, there was no change in the frequency of $Ki67^{High}$ DPs, suggesting no change in the frequency of cycling cells (Figure 3.8.C). This finding is consistent with there being no change in the number of thymocytes from 3-5 weeks of age to 8 weeks (Figure 3.5.A). Together, these data suggest that by 8 weeks of age, VcKO DPs have selected for further increase in Notch signaling, without increasing the frequency of cells in cycle.

VcKO DP thymocytes show increased gene dysregulation in 8 week old DPs compared to 4 week old DPs

The increase in CD25 expression in 8 week old DPs compared to 4 week old DPs suggests an increase in Notch signaling, which implies further oncogene dysregulation (Figure 3.8.A and Figure 3.8.B). To investigate the gene expression changes that occur as VcKO thymocytes age, I performed bulk RNA-sequencing on 8 week old VcKO and Ctrl DPs. There were 3,973 DEGs between 8-week VcKO and Ctrl DPs, a substantial increase compared to 4-week-old mice (Figure 3.9.A). Of the 8-week DEGs, 1392 were dysregulated in both 4- and 8-week-old VcKO DPs (Figure 3.9.A). Many DEGs that increased in 4 week VcKO DPs were further upregulated in 8 week VcKO DPs, and the same trend was observed in DEGs that decreased in 4-week VcKO DPs (Figure 3.9.B and Figure 3.9.C). *Notch1* mRNA was more enriched in 8-week VcKO DPs, but notably *Fbxw7* mRNA was decreased in 8 week, but not 4 week, VcKO DPs (Figure 3.9.C). *Fbxw7* encodes a ubiquitin ligase that targets ICN for degradation and is frequently repressed or mutated in T-ALL.[142,218,219] Other notable oncogenes that are upregulated at both ages but are more strongly enriched in older VcKO DPs are *NFκB1*, *c-Myc*, and *IL7r*, all of which are associated with T-ALL and leukemic transformation.[152,220,221] In addition to enriched expression of classic oncogenic

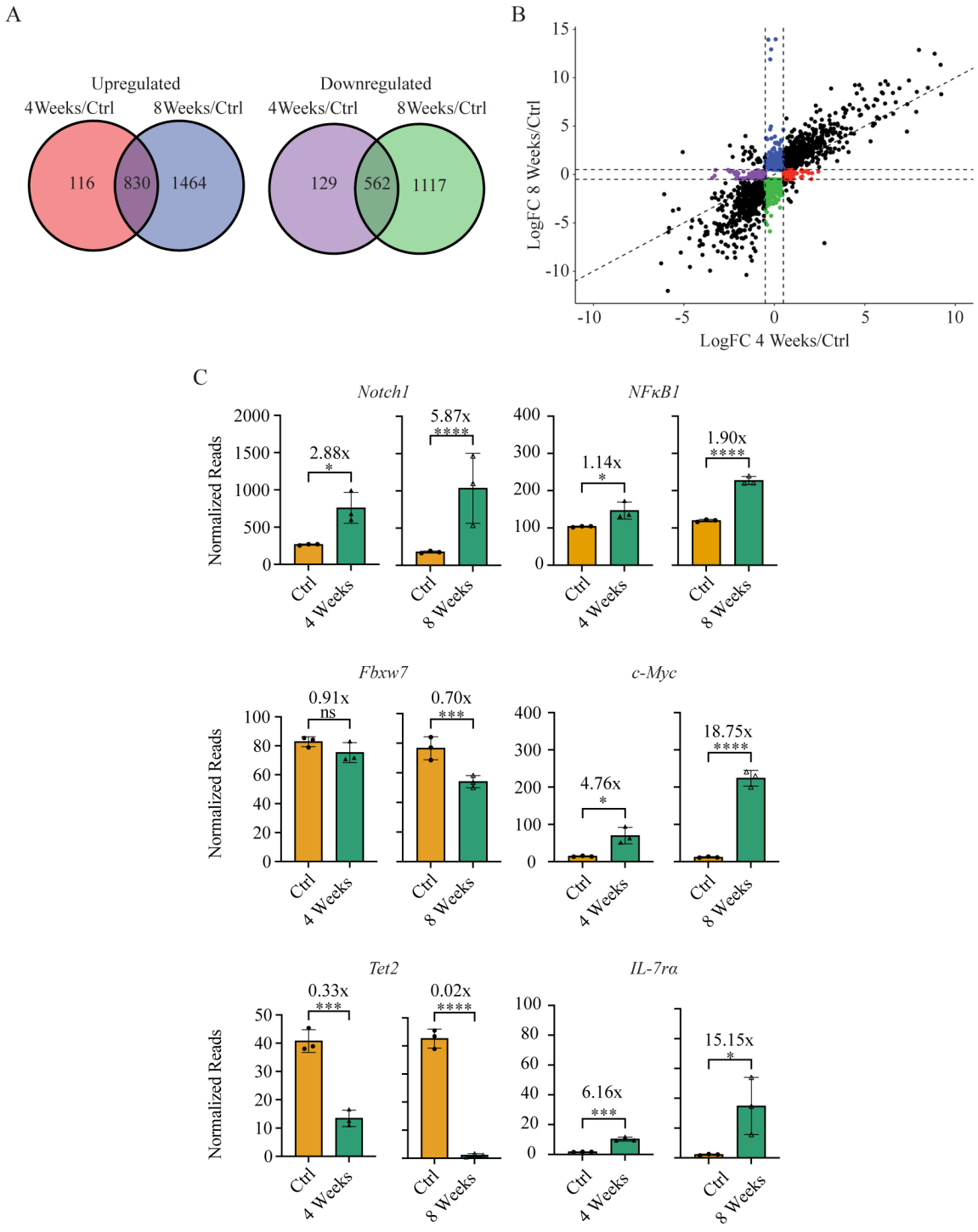

Figure 3.9: Oncogenic pathway dysregulation is exacerbated in 8 week old VcKO DPs vs 4 week old DPs

A) Venn Diagrams depicting the number of differentially expressed genes between VcKO DPs of the indicated age relative to their respective controls. B) Plot depicting the logFC of genes dysregulated in both ages relative to controls. C) Normilized read counts of select genes from bulk RNA sequencing fold change relative to controls.

pathways, tumor suppressors such as *Tet2* were also progressively repressed in VcKO DPs as mice age (Figure 3.9.C).

The increase in *Notch1* and decrease in *Fbxw7* mRNA may explain the increased CD25 expression seen (Figure 3.8.A, Figure 3.8.B, Figure 3.9.C) and suggests that *Notch1* upregulation is selected for prior to transformation. However, fully transformed T-ALL cells present with clonal *Notch1* mutations in both humans and *E2a*$^{-/-}$ mice.[127,143] Notably, there were no *Notch1* PEST domain indels identified in 4 or 8 week old VcKO DPs (Figure 3.10.A), suggesting that these mutations occur later in transformation and that by 8 weeks there is still no significant expansion of *Notch1* mutated clones.

To identify alterations that occur later in transformation, I analyzed the genes that were uniquely dysregulated in 8 week VcKO DPs vs. Control DPs. KEGG pathway analysis identified the enrichment of DNA replication pathways, metabolic pathways (TCA cycle and Carbon metabolism), and pathways involved in resolving genetic instability (Mismatch repair, Nucleotide excision repair, and p53 signaling pathway) (Figure 3.11.A). These pathways being enriched is consistent with the expansion in the number of thymocytes from 8 to 10 weeks (Figure 3.4.A). Notably, the most repressed pathways, Autophagy, Apoptosis, and Cellular senescence are associated with response to cell stress and are important checks on transformation (Figure 3.11.A).

A

Deleterious indels in Notch1 exon 34

Sample	Age (weeks)	#sequences with indel	#unique indels
4 week			
VCtrl-1	4	2	2
VCtrl-2	4	3	3
VCtrl-3	4	4	1
VcKO-1	4	3	3
VcKO-2	4	12	8
VcKO-3	4	6	5
8 week			
VCtrl-1	8	1	1
VCtrl-2	8	0	0
VCtrl-3	8	0	0
VcKO-1	8	13	10
VcKO-2	8	39	3
VcKO-3	8	8	3

Figure 3.10 *Notch1* mutations are rare in VcKO DPs by 8 weeks of age
A) Table of indels in the *Notch1* exon 34 (encoding the PEST domain) in pre-leukemic DPs from mice of the indicated age and genotype.

A

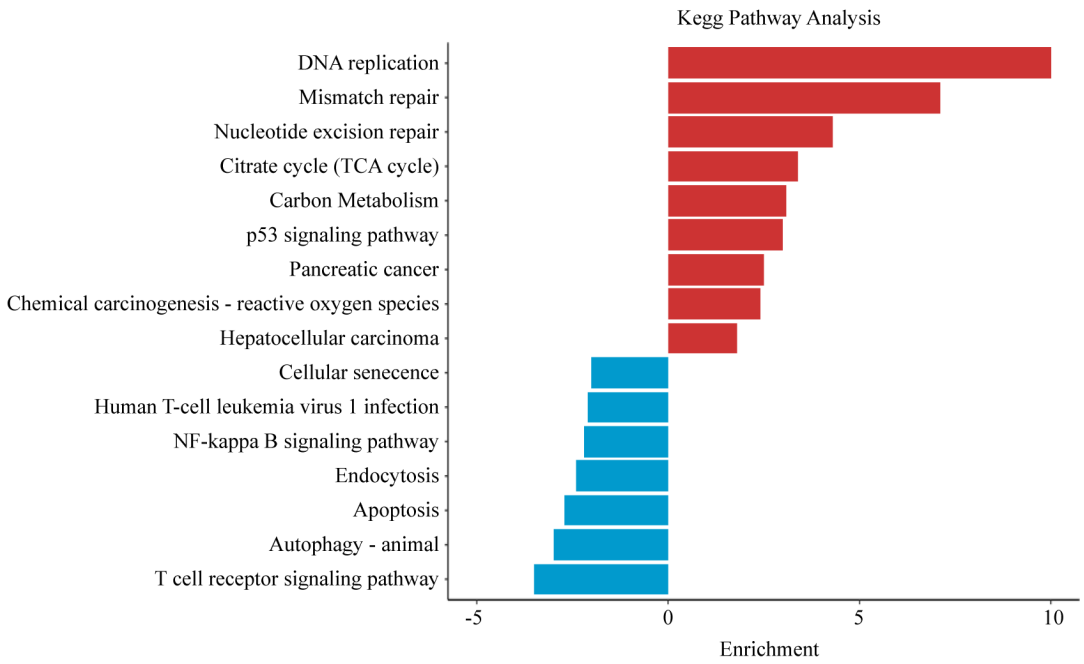

Figure 3.11: 8 Week old VcKO DPs are enriched for cell cycle genes and repress stress pathways
A) GSEA of genes uniquely differentially expressed in 8 week old VcKO DPs vs controls compared to 4 week old VcKO DPs.

DISCUSSION

I have demonstrated that an early deletion of *E2a*, using a *Vav-Cre* driver, is sufficient to induce the rapid, aggressive T-ALL development seen in the germline deletion, but the *Lck-Cre*, which deletes starting at the DN2/DN3 stage, is not. This finding is reminiscent of what is seen using *Tcf7*$^{f/f}$ mice, where *Lck-Cre* and *CD4-Cre* were unable to induce transformation, but germline and *Vav-Cre* deletions were.[210] These data suggest that despite the leukemias in both models presenting with a DP phenotype, the primary oncogenic mutation in these models needs to occur prior to the DN3 stage. It's unknown, however, how early in T cell development *E2a* or *Tcf7* needs to be deleted to drive oncogenesis. It seems unlikely that the *E2a* deletion needs to occur in the bone marrow progenitors, as many oncogenic mutations, such as *Gata3* or *Lmo2* overexpression, are oncogenic when expressed under a CD2 promoter that initiates in the CLP/ETP stage.[123,222] Thus, is possible that *Gata3* activation or E2A repression simply needs to occur in the early thymocytes, potentially before full lineage commitment or β-selection, which occurs at the DN2 and DN3 stages, respectively.

Many human TAL1$^+$ and LYL1$^+$ leukemias are the result of translocations that place them under TCR gene promoters.[111,214] Its notable then, that these translocations would promote expression of E2A inhibitors at the same stages of T cell development that *Lck-Cre* initiates deletion at. This is consistent with several models of T-ALL that ectopically express E2A inhibitors, including ID1, ID2, or Tal1 transgenic mice. All of these models are driven by the *Lck* promoter, which activates at the same stage that *E2a* is deleted in the LcKO mice, however, these models drive robust leukemogenesis. Therefore, it is surprising that the *Lck-Cre* failed to induce robust T-ALL. One possible explanation is the incomplete deletion at the DN3 stage in LcKO mice (Figure 3.2.B). Indeed, the transgenic models that drive E2A-inhibitor expression rapidly block

E2A DNA binding, while the deletion depends on endogenous protein degradation to eliminate E-protein activity. Indeed, it is possible that in the LcKO DN3s, there is sufficient E2A protein to exert a tumor suppressor function despite DNA deletion. If true, this argues that E2A signaling needs to be fully ablated by the DN3 stage at the latest to drive transformation. Some evidence supporting this idea can be found when combining *Lck-Tal1* ectopic expression with constitutive Casein-kinase II (*CKIIα*) expression.[120] CKIIα is a serine/threonine kinase that phosphorylates E-proteins to inhibit DNA binding. Notably, *Tal1-tg/CKIIα* mice developed T-ALL with decreased latency vs. *Tal1-tg* mice, indicating that disease latency correlates with reduced E-protein function at the DN3 stage.[120] Thus, the LcKO mice potentially have too much E2A protein at the DN3 stage despite initiating DNA deletion. This would the reduced penetrance of the T-ALL, but it would not explain, the minor DP phenotype seen in all LcKO mice (Figure 3.4 and Figure 3.5) given that E2A:HEB dimers are still required to help establish the identity of DPs.[79] There are still 421 DEGs in LcKO DPs vs Ctrl DPs (Figure 3.6.B), which suggests that E2A is not entirely dispensable for DPs, which is consistent with previous data. The transcriptional changes seen in LcKO DPs are not entirely benign, given that 4 of the mice studied did develop T-ALL with reduced latency (Figure 3.3.C and Figure 3.3.D). It is possible that the 4 LcKO mice that did develop T-ALL during the study stochastically deleted E2A early and had lower initial protein expression which predisposed them to a slower transformation.

The sweeping transcriptional changes seen in the VcKO DPs are surprising given the early (4 week) timepoint analyzed. There was already significant oncogenic pathway activation compared to LcKO DPs, with the predominant enriched pathways being Notch and cytokine signaling (Figure 3.7). Increased cytokine signaling in the thymus, specifically IL-7, is associated with T-ALL transformation[220] and suggests that the increased cytokine signaling may be a critical

early event in transformation and explain the difference in latency between VcKO and LcKO mice. It's surprising, however, that cytokine signaling pathways are no longer enriched in 8 week old VcKO DPs (Data not shown). This could be due to a reduction in the available signals in the thymus or due to a genetic drift away from needing the external signals to survive. Indeed, during the initial oncogenic events, the pre-leukemic thymocytes may be less stable and more prone to apoptosis, and the cytokine signals may compensate and allow survival despite this stress. After sufficient transformation, upregulation of Myc and Notch signals, seen amplified in 8 week vs. 4 week DPs (Figure 3.9.C), may provide sufficient survival signals that the cytokines are no longer needed. Consistent with this, 8 week old DPs repress apoptosis pathways (Figure 3.12.A), which is a main function of IL-7 signaling in the thymus.[191] At both 4 and 8 weeks, the IL-7 receptor is upregulated (Figure 3.9.C), and while the signaling pathways are no longer enriched in 8 week VcKO DPs, there still may be a basal level of signaling that may not show up in bulk RNA-sequencing.

The comparison of 4 week and 8 week VcKO DPs provides key insight into the initial vs. secondary changes that occur during the course of transformation. It is striking the NFκB, Myc, and Wnt/β-catenin pathways are enriched in 4 week old DPs, despite being far from full transformation. It is not surprising that these pathways are all active, however, given that E2A has been shown to regulate them all independently.[94,99,103,159,223] Thus, E2A may be such a strong tumor suppressor given its role in regulating several oncogenic pathways. The exacerbation of the pathways by 8 weeks is not surprising, given their oncogenic potential, however in the case of *Notch1*, it is surprising that the increased signaling is not due to canonical exon 34 mutations (Figure 3.10.A). These mutations are found in almost all T-ALLs,[143] and therefore are thought to be the most potent drivers of transformation. There has been some debate in this, as experiments

transducing HSCs with constructs encoding mutated Notch proteins require secondary *Kras* mutations to induce T cell transformation.[211] My data agree with the idea that mutations in Notch are not early events, but occur very late during leukemogenesis, which agrees that they are not selected for initially and are weak tumor initiators. The exon 34 mutations consistently prevent ICN degradation[224] to allow for increased signaling. This and my data argue that it is more critical to increase Notch1 expression early than it is to increase the lifespan of ICN signaling. Notch1 ligands are ubiquitous in the thymus, thus increasing *Notch1* expression should lead to an abundance of activation. Amplifying ICN signaling comes later, as by 8 weeks VcKO DPs have downregulated *Fbxw7*, a ubiquitin ligase that marks ICN for degradation to limit signaling (Figure 3.9.C).[142,218,219] Despite this, it is not known when exactly Notch1 PEST domain mutations are selected for other than it occurs after 8 weeks of age. This correlates with the timepoint when the leukemic cells start to rapidly proliferate and expand in numbers (Figures 3.4.A and Figure 3.11.A). The expansion of cells could result in a relative depletion in the available ligands, which would provide a selective pressure for these mutations. Further, the rapid proliferation leads to strains on the mismatch repair machinery, which was one of the most upregulated pathways in 8 week old DPs (Figure 3.11.A). This may lead to mutations in the Notch gene that are then selected for. This creates a paradigm where Notch signaling is upregulated in three stages: 1) Initial increased Notch1 mRNA, 2) Further increase transcription combined with inhibiting degradation, and finally 3) Mutations promoting signaling complex stability. Why Notch signaling is increased in this progressive manner in unknown, however, it has been shown that excessively strong Notch signals causes stress in T-ALL cells and induces apoptosis.[225] This suggests that a threshold of Notch signaling can be reached that may be counter-productive and kill cells before transformation. Therefore, a graded approach to increasing Notch signaling may be optimal, as it would allow T-

ALL cells to further mutate to survive the *Notch1* induced stress. Indeed, T-ALL cells can protect themselves from *Notch1* induced stress through the action of DNA and chromatin modifiers such as KDM6B.[225] Further, it has previously been shown that deletion of DNA and chromatin modifiers protects cancer cells from environmental stresses by preventing the reorganization of the chromatin to a pro-apoptotic state.[226] VcKO DPs show significant dysregulation of chromatin and DNA modifiers, such as *Tet2* and *Kdm6b* that is exacerbated with age (Figure 3.9.C and data not shown). Thus, the threshold of Notch signaling a pre-leukemic cell can tolerate may progressively increase as the cells become less able to reorganize their genome to a pro-apoptotic state.

CHAPTER 4:

IKZF2 AND ITS CONTRIBUTIONS TO VCKO LEUKEMOGENESIS

CHAPTER 4: *IKZF2* AND ITS CONTRIBUTIONS TO VCKO LEUKEMOGENESIS

INTRODUCTION

E2a$^{-/-}$ thymocytes have significant upregulation of T-ALL associated oncogenes including *Notch1*,[127] *Lef1*,[103,104] and *Gata3*.[94,99] However, there are still numerous differentially expressed genes that may contribute to transformation that require exploration. Indeed, the IKAROS family of transcription factors, in particular IKAROS and HELIOS, have been associated with leukemia development. The *Ikzf* family of transcription factors contains 5 proteins, Ikaros, Helios, Aiolos, Eos, and Pegasus (Gene: *Ikzf1-5, respectively*). They broadly fall into 2 categories, with Ikaros, Helios, and Aiolos being primarily expressed in hematopoietic lineage cells while Eos and Pegasus are predominantly restricted to peripheral tissues.[179] All members contain 6 zinc finger domains, except for Eos, which only has 5. The 2 c-terminal domains facilitate hetero- or homodimerization with other members of the IKAROS family while the N-terminal domains facilitate DNA binding.[227] Multiple models of IKAROS loss of function, including a dominant negative mutant,[171] hypomorph mutations,[176] and full deletion[175] show its requirement to suppress transformation. This hypothesis is supported by data from human T-ALL patients, in which IKAROS loss is rare but associated with immunodeficiency that eventually progresses to T-ALL.[172,173] In contrast, HELIOS is highly expressed in human-derived T-ALL cell lines, in primary human T-ALL samples, and in human Adult T cell Leukemia samples.[181,228] Therefore, HELIOS may play an oncogenic role in several T cell leukemias while IKAROS plays a tumor suppressive one. IKAROS' role as a tumor suppressor is well characterized, acting predominantly to enforce T cell development and to precisely regulate NOTCH1 signaling.[56,57,146,176] However, HELIOS's role as an oncogene is less

well defined. Analysis of patient samples and human derived T-ALL cell lines revealed that in addition to overexpression, some samples had mutations in *Ikzf2* that encode for short isoforms that lack some of the zinc fingers in the DNA-binding domain.[181,183,228] Forced expression of similar isoforms in HSCs using retroviral vectors promoted T-ALL in mice within 5 months, showing the oncogenic potential of the mutant isoforms.[181] Notably, expression of full length HELIOS did not induce T-ALL, but did result in developmental blocks at the ETP and DN4 stages of T cell development.[181] This data shows that overexpression of HELIOS is insufficient to drive transformation by itself, however it is unknown if overexpression of HELIOS can synergize with other mutations to affect transformation. Regardless, if and how wild-type or mutant forms of HELIOS affect transformation has yet to be fully investigated.

HELIOS is associated with T cell activation, typically being upregulated by TCR stimulation.[229] In DP thymocytes, HELIOS is specifically associated with strong TCR signaling during selection, restricting HELIOS expression to a small subset of DPs.[230] This suggests that signals downstream of the TCR regulate HELIOS in DPs. One such pathway downstream of TCR signaling is NFκB.[231] In regulatory T cells (T_{reg}), loss of NFκB signaling results in a loss of HELIOS expression.[179,232] However, it is unknown if HELIOS is similarly regulated by NFκB in DPs. Regardless, NFκB signaling is upregulated in the absence of E2A and is vital for the survival of human T-ALL cells.[223,233] Therefore, it is possible that increased NFκB signaling in pre-leukemic *E2a*$^{-/-}$ thymocytes may drive HELIOS expression to promote the survival of the leukemia cells. However, it is unknown if the high NFκB signaling in *E2a*$^{-/-}$ leukemias is exerting its pro-survival effects through HELIOS upregulation. Here I show that *Ikzf2* is upregulated in VcKO DPs and in *E2a*$^{-/-}$ leukemia lines due to the loss of E-proteins and the constitutive activation of the NFκB pathway. Despite developmental defects resulting from HELIOS ectopic expression,

HELIOS deletion did not reverse the developmental defects seen in VcKO mice. Moreover, HELIOS deletion did not impact of the incidence or latency of leukemogenesis of VcKO mice.

RESULTS

Ikzf2 is upregulated in VcKO DPs

HELIOS has been reported to contribute to T-leukemogenesis when it is mutated through action as a dominant negative IKAROS repressor.[181–183] To investigate if HELIOS contributes to leukemogenesis in VcKO mice, I first analyzed expression of *Ikzf2* mRNA in 4 and 8 week old VcKO DPs using bulk RNA sequencing. *Ikzf2* was upregulated at both ages, however it had a higher fold change at 8 weeks vs 4 weeks (Figure 4.1.A). At 3-5 weeks, HELIOS protein expression was increased in the DP population (Figure 4.1.B, Figure 4.1.C, and Figure 4.1.D). This was not consistent across all thymocytes, however, as VcKO DN3 and CD8 SP thymocytes had reduced expression of HELIOS, indicating that E2A-deficiency differentially impacts HELIOS expression depending on the stage of T cell development. In addition to pre-leukemic thymocytes, HELIOS was highly expressed in *E2a*$^{-/-}$ leukemia lines (Figure 4.1.E).

No evidence of clonal Helios short isoform mutations

Current evidence suggests that HELIOS predominantly acts as an oncogene if it is mutated.[181–183] These mutations invariably delete the zinc finger domains that interact with DNA, preventing HELIOS from binding DNA. This would then cause it to act as a dominant negative inhibitor when dimerized with other IKAROS family transcription factors. Notably, all leukemias derived from short-isoform HELIOS transgenic HSCs expressed IKAROS.[182] This finding suggests that these short isoforms/mutants act as oncogenes by inhibiting Ikaros DNA binding. To test if HELIOS is mutated in VcKO DPs, I analyzed the browser tracks for the *Ikzf2* locus from

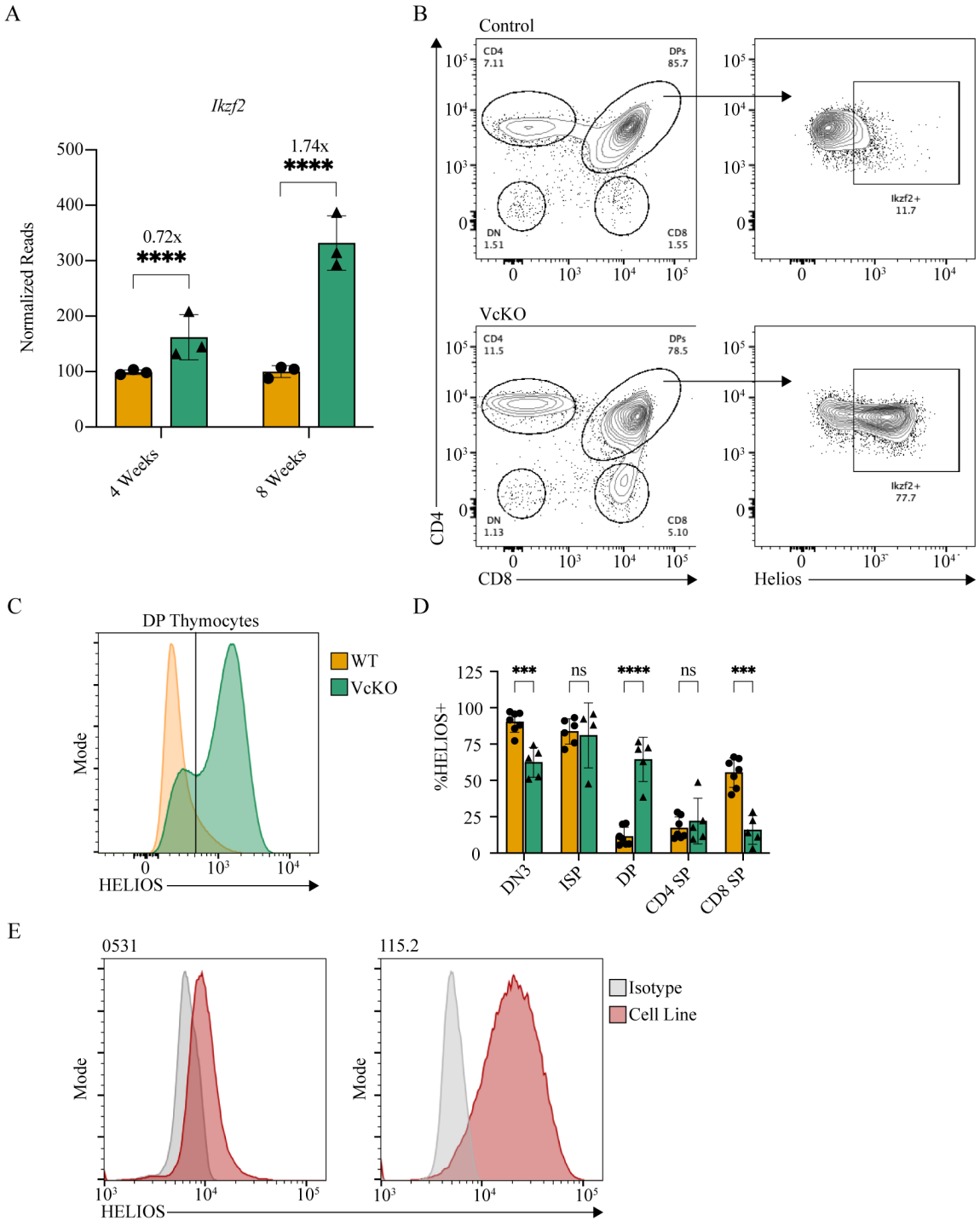

Figure 4.1: *Ikzf2* is upregulated in VcKO DPs and *E2a*$^{-/-}$ cell lines
A) Normalized RNA-seq reads for *Ikzf2* from DPs from mice of the indicate genotype and age. B) Representitive FACS plots showing HELIOS expression in Ctrl (top) and VcKO (bottom) DPs at 3-5 weeks. C) Representitive histogram of HELIOS expression in B. C) Quantification of the percentage of HELIOS expressing thymocytes within the indicated populations. E) Representitive FACS plots of HELIOS expression in select *E2a*$^{-/-}$ cell lines.

A

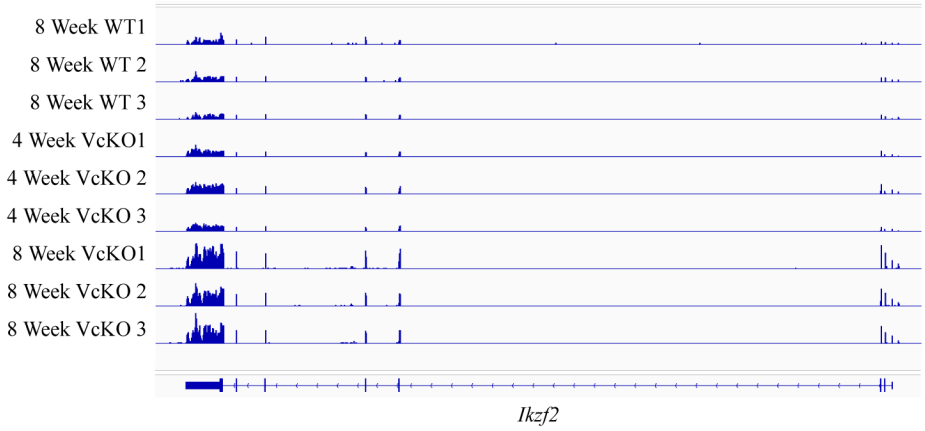

Figure 4.2: No evidence of clonal HELIOS short isoforms
A) Browser tracks of the *Ikzf2* locus from RNA-seq performed on 8 week WT, 4 week VcKO, and 8 week VcKO DPs.

RNA-seq of 4- and 8-week-old VcKO DPs and Ctrl DPs. The browser tracks confirmed *Ikzf2* upregulation but showed no evidence of clonal mutations that prevent transcription of the DNA-binding domain (Figure 4.2.A). This does not exclude the possibility of single nucleotide polymorphisms or heterozygous mutations that may be hidden in the browser track analysis. Regardless, at these pre-leukemic time points it is likely HELIOS is still able to bind DNA and would not act as an IKAROS inhibitor.

HELIOS is downstream of E proteins

To investigate if HELIOS is directly or indirectly regulated by E proteins, I cultured *E2a*$^{-/-}$ leukemia cell lines *in vitro* and transduced them with a retroviral vector encoding for E47 or an empty vector control. FACS analysis of leukemia lines 48 hours after transduction showed significant downregulation of HELIOS in E47 expressing cells vs. empty vector controls (Figure 4.3.A and Figure 4.3.B). This correlation suggests that HELIOS is a target of E proteins and argues the upregulation of HELIOS is a result of E2A inhibition and not resultant from secondary mutations occurring during transformation. To determine if *Ikzf2* is a direct or indirect target of E proteins, I analyzed publicly available ChIP-Sequencing data of HEB, E2A's preferred dimerization partner in thymocytes. In wild-type DP thymocytes, there are 2 peaks near the *Ikzf2* gene that may indicate HEB binding (Figure 4.3.C), suggesting that E proteins could directly impact *Ikzf2* transcription. However, E2A is typically a transcriptional activator in thymocytes, leading us to hypothesize that *E2a* may repress *Ikzf2* through indirect mechanisms.[82]

The NFκB Pathway is overactive in VcKO DPs and regulates Ikzf2 expression

In immune cells, HELIOS expression and function has predominantly been studied in regulatory T cells (T_{reg}), and RNA sequencing of T_{reg} cells deficient in NFκB signaling showed *Ikzf2* is significantly repressed in the absence of NFκB signaling.[232] Further, repression of E protein

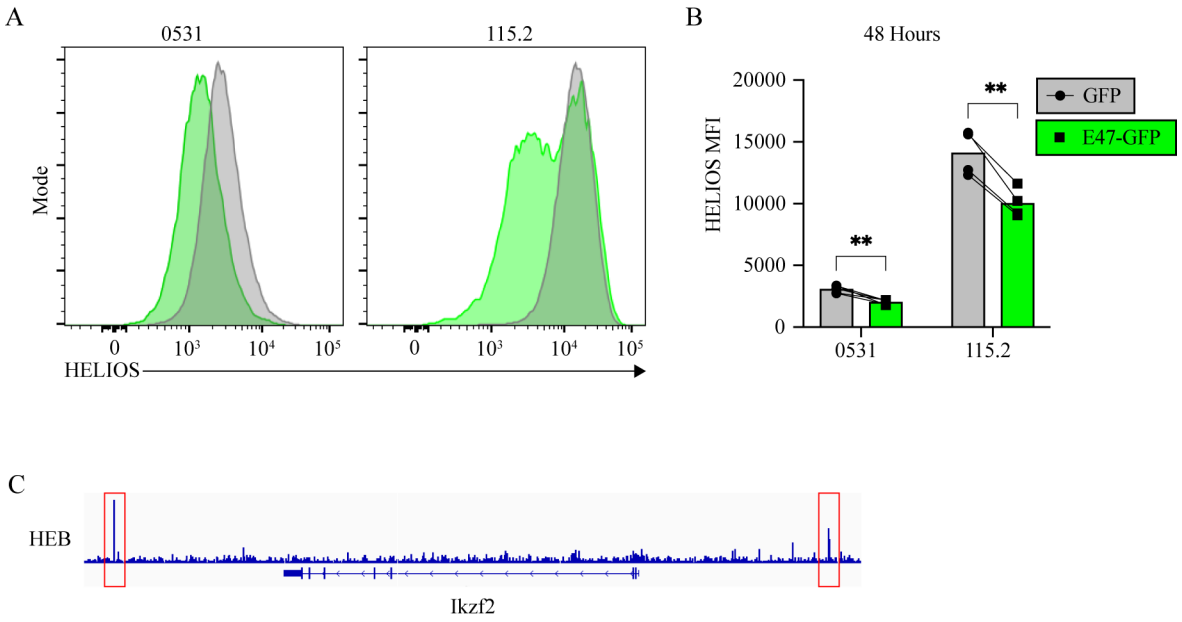

Figure 4.3: HELIOS expression is regulated by E2A
A) Representitive Histograms of *E2a*$^{-/-}$ leukemia lines 48 hours post transduction with E47-GFP or GFP only expressing retrovirus. B) Quantification of the change in HELIOS MFI 48 hours post transduction with E47-GFP or GFP only expressing retrovirus. C) Gene tracks from ChIP-sequencing data showing HEB bound regions near the *Ikzf2* locus in wild-type DP thymocytes.

function through transgenic ID1 or TAL1 expression induced NFκB expression in DN3 thymocytes[223]. Therefore, I investigated if NFκB was responsible for promoting HELIOS expression in VcKO DPs. Consistent with previous reports, mRNA expression of potentiators of NFκB signaling *Nfκb1* and *Ikbke* were increased in 8 week VcKO DPs, while *RelA* expression was unaffected by the loss of E2A (Figure 4.4.A). Further, several inhibitors of NFκB signaling were repressed, including *Nfκbia, Nfκbie*, and *Nfκbiz* (Figure 4.4.B).

The NFκB pathway has been shown previously to promote the survival of human T-ALL lines.[233] Therefore, to test if NFκB is required for *E2a*$^{-/-}$ leukemia survival I cultured 3 *E2a*$^{-/-}$ leukemia lines with the NFκB inhibitor BMS-345541 or DMSO vehicle control for 24 hours and measured apoptosis via Annexin V staining. In all 3 lines tested, there were significant increases in the frequency of Annexin V^{+} cells, indicating an increase in apoptosis (Figure 4.4.C and Figure 4.4.D). Together, these data indicate that the NFκB pathway is upregulated in the absence of E2A, which provides a survival advantage for leukemic cells.

To investigate if HELIOS is regulated by NFκB in wild-type DPs I cultured thymocytes in the presence of the NFκB activator PMA, BMS-345541, or a combination of both for 5 hours. Compared to untreated controls, the frequency of HELIOS expressing DPs was significantly increased upon PMA treatment and significantly decreased by BMS-345541 treatment (Figure 4.5.A and Figure 4.5.B). Notably, combination treatment blocked HELIOS upregulation, indicating that NFκB signaling promotes HELIOS expression in wild-type DPs (Figure 4.5.A and Figure 4.5.B). To determine if HELIOS is similarly regulated in VcKO DPs, I sorted 10^5 8 week VcKO DPs and cultured them in the presence of BMS-345541 or the DMSO vehicle control and performed RT-qPCR for *Ikzf2* mRNA. BMS-345541 treated VcKO DPs showed a significant reduction in *Ikzf2* mRNA, confirming NFκB regulation in VcKO DPs (Figure 4.5.C).

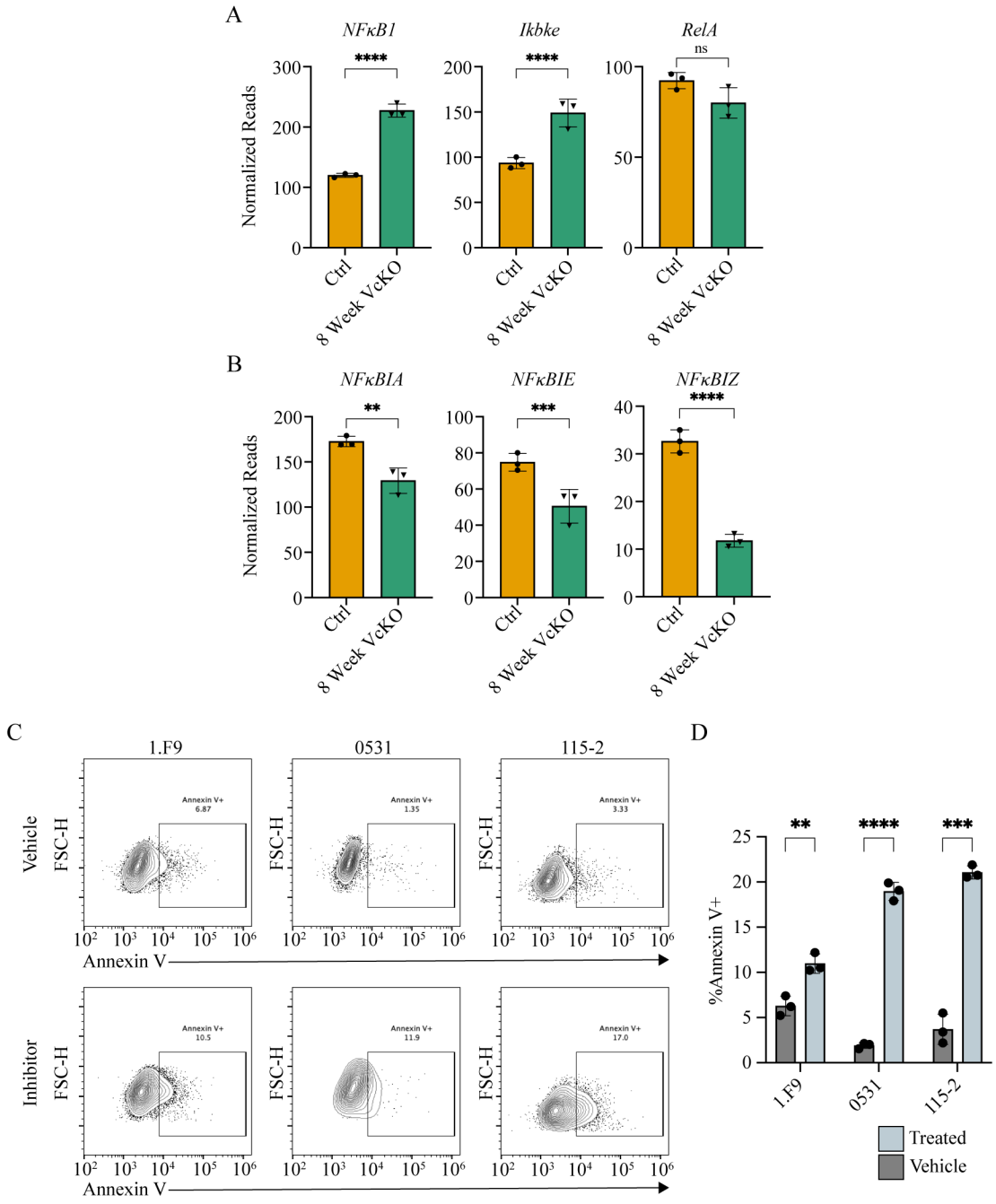

Figure 4.4: NFκB is upregulated in VcKO DPs and promotes the survival of *E2a*$^{-/-}$ leukemias
Normalized read counts from bullk RNA-seq for A) Activators or B) Inhibitors of NFκB signaling pathway in 8 week old VcKO DPs. C) Representitive FACS plots of Annexin V staining in *E2a*$^{-/-}$ cell lines treated for 24 hours with Vehicle (DMSO, Top) or 5μM NFκB inhibitor (BMS-345541, Bottom). D) Quantification of the frequency of Annexin V+ cells from C.

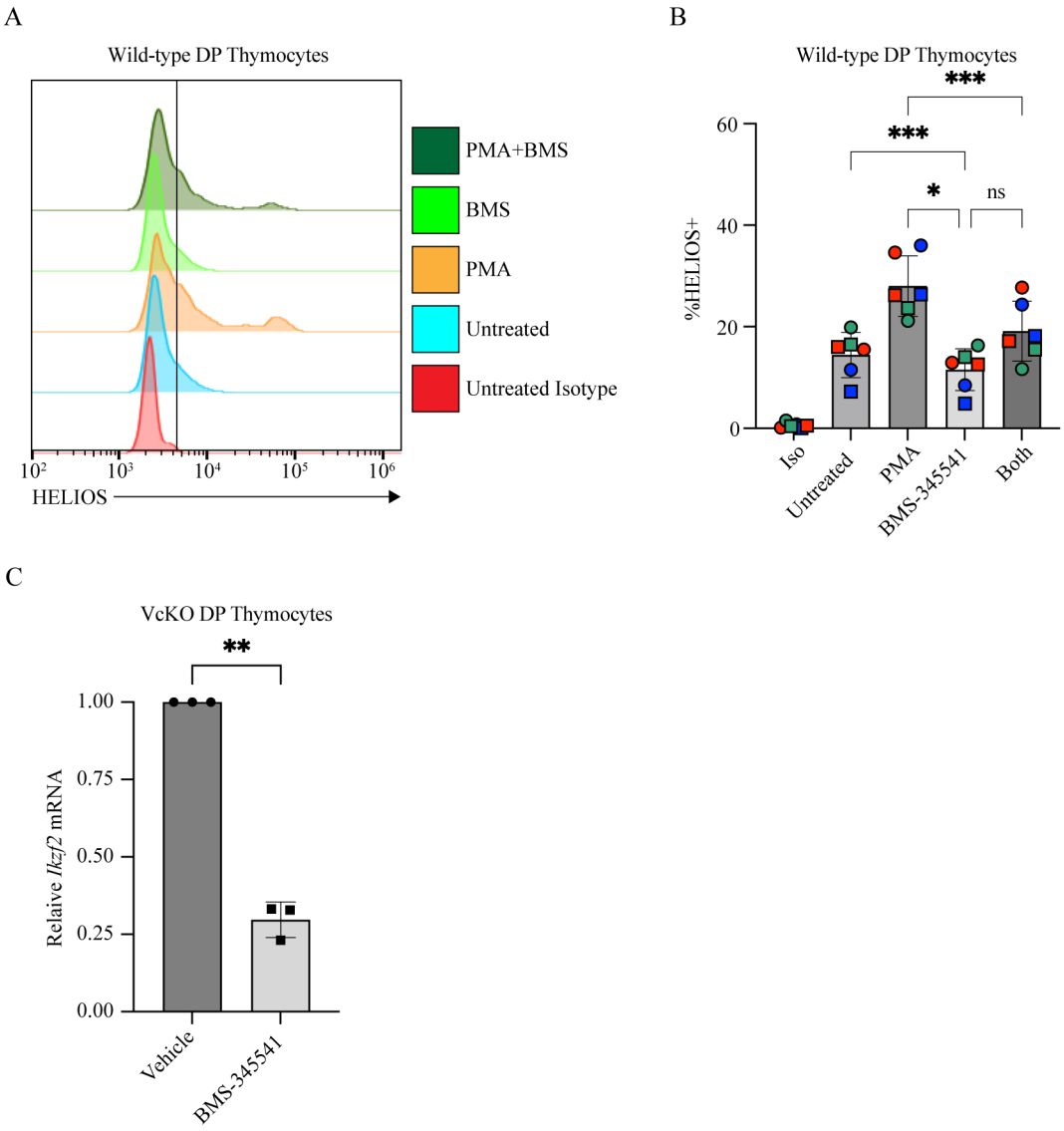

Figure 4.5: HELIOS is regulated by NFκB signaling in wild-type and VcKO DPs
A) Representitive histogram of HELIOS expression in wild-type DPs after 5 hour in vitro culture with 20ng/mL PMA, 5μM BMS-344541, or both. B) Quantification of the frequency of HELIOS expression in wild-type DPs as treated in (A). Colors indicate technical replicates and shapes indicate biological replicates. C) RT-qPCR for *Ikzf2* expression was performed on 10^5 sorted 8 week old VcKO DPs cultured for 3 hours in the presence of 5μM BMS-345541 or DMSO vehicle control. Data was analyzed using the ΔΔCT method.

HELIOS overexpression does not contribute to the developmental defects in VcKO thymocytes or leukemogenesis

Human T-ALL lines are highly susceptible to NFκB inhibition, demonstrating the strong oncogenic signal NFκB provides.[233] Indeed, NFκB synergizes with increased Notch signaling to promote the development of Notch-driven T-ALLs.[233] To investigate if NFκB promotes VcKO DP transformation through induction of HELIOS, I crossed the *Ikzf2* floxed allele onto the VcKO background to generate *Vav-Cre*$^{+}$*E2a*$^{f/f}$*Ikzf2*$^{f/f}$ (DKO) mice. Phenotypic analysis of the thymocyte populations of DKO mice show no rescue of the developmental defects seen in VcKO mice (Figure 4.6.A and Figure 4.6.B) Therefore, the increased HELIOS expression seen in VcKO DPs does not contribute to their reduced frequency. Further, CD4 SP and CD8 SPs continued to be increased, suggesting that HELIOS does not affect maturation to SP stages. This finding is consistent with reports that the deletion of HELIOS does not affect wild-type T cell development.[180] However, analysis of 3-5 week old mice revealed increased expression of CD25 on DKO DPs as compared to Control or VcKO, with DKO DPs expressing CD25 at levels similar to 8 week old VcKO DPs (Figure 4.6.C and Figure 4.6.D). Therefore, DKO DPs may be receiving stronger Notch signals at a younger age, which could contribute to faster T-ALL development than VcKO mice.

To investigate the impact HELIOS has on the development of T-ALL in VcKO mice, I monitored DKO and VcKO *Ikzf2*^{Het} mice for leukemia development. VcKO *Ikzf2*^{Het} mice had no significant difference in leukemia latency compared with VcKO mice (Figure 4.7.A and Figure 4.7.B). DKO mice also showed no change in leukemia latency when analyzing age at death or probability of survival (Figure 4.7.A and Figure 4.7.B). This suggests that HELIOS does not play a role in *E2a*$^{-/-}$ leukemogenesis.

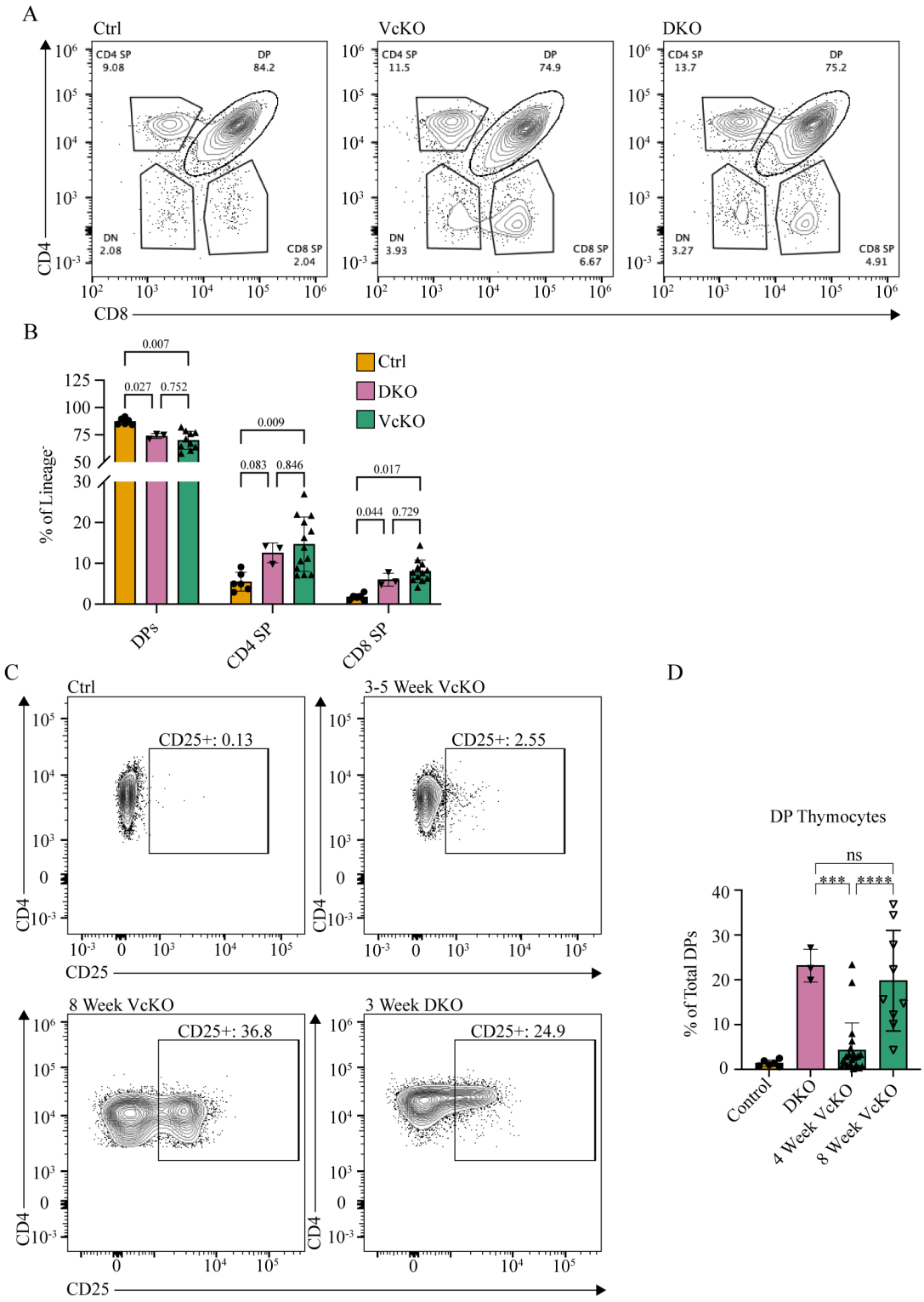

Figure 4.6: Deletion of *Ikzf2* does not resuce the developmental defects seen in VcKO mice
A) Represetitive FACS plots of Lineage- thymocytes from 3-5 week old mice of the indicated genotype. B) Quantification of the frequency of the indicated population amongst all lineage- thymocytes. C) Representitive FACS plots of CD25 expression in DPs of mice of the indicated age and genotype. D) Quantification of CD25 expression on 3-5 week old WT, DKO, and VcKO DPs and 8 week old VcKO DPs.

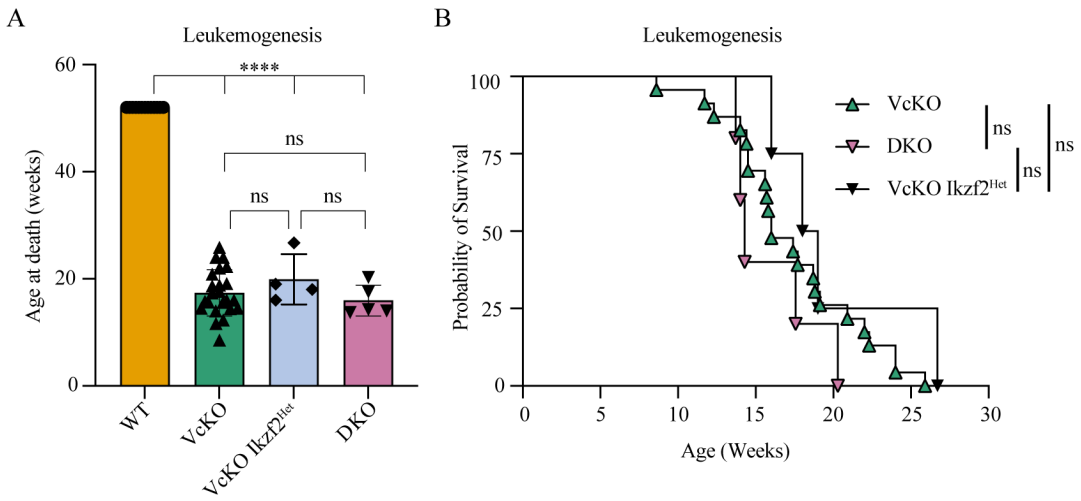

Figure 4.7: *Ikzf2* deletion has no effect on T-ALL development
A) Age at death and B) Kaplan-Meyer curve analysis of leukemogenesis in WT, VcKO, VcKO Ikzf2^{het}, and DKO mice. Significance determined by A) One-way Anova or B) Log-rank Mantel-Cox test.

DISCUSSION

I have shown here that HELIOS is progressively upregulated in VcKO DPs as they get closer to transformation consistent with the expression patterns of other oncogenes in the VcKO model (Figure 3.9.C and Figure 4.1.A). This implicated HELIOS as a potential oncogene, similar to what has been seen in other models of HELIOS-mediated oncogenesis.[181,182] Notably, those models of T-ALL rely on mutant HELIOS isoforms that fail to bind DNA. Presumably, these models act as dominant negative inhibitors of other *Ikzf* family transcription factors, most likely IKAROS. This is due to all the mutant HELIOS-Tg leukemias also overexpressing IKAROS,[182] which is contrary to IKAROS' tumor suppressive function. Notably, one major function of IKAROS is to limit *Notch1* expression,[176] so overexpression of functional IKAROS in these models would counteract a major oncogenic signal. This, taken together with HELIOS' known ability to dimerize with IKAROS,[234] strongly support the idea that HELIOS short isoforms/mutations acts as a dominant negative inhibitor. Analysis of 8 week VcKO DP RNA-sequencing browser tracks shows no evidence of similar mutations, suggesting that HELIOS is not acting as an inhibitor of IKAROS in the VcKO model, at least by the 8 weeks of age. Whether or not HELIOS is mutated later in transformation remains undiscovered.

It is important to note that the idea of HELIOS as a dominant negative inhibitor has only been proposed in mutant HELIOS-driven leukemia models. Notably, little work has been done on investigating HELIOS as a secondary or later mutation. Generation of *E2a Ikzf2* DKO mice was the first attempt to study the role of *Ikzf2* in an autochthonous model of T-ALL. DKO mice show no change in T-ALL latency, suggesting HELIOS is not contributing to transformation or acting as a tumor suppressor in this model (Figure 4.8.B), consistent with mice transplanted with wild-type HELIOS-Tg bone marrow not developing T-ALL.

The understanding that HELIOS is dispensable for transformation raises the question of why it is overexpressed in both transforming cells and fully transformed leukemia lines (Figure 4.1.C and Figure 4.1.D). Loss of E proteins induces significant transcriptional changes, and based on my data HELIOS is expressed due to NFκB activation and not due to selective mutations in the *Ikzf2* gene. I do not exclude the possibility that E2A directly regulates HELIOS, however, in wild-type DPs, HELIOS expression is restricted to the $CD69^{+}TCR\beta^{+}$ post-selection DPs, which upregulate ID3 to repress E2A DNA binding.[235] Similar results are seen in DN3s after β-selection.[235] Thus, there is a correlation between loss of E protein activity and HELIOS expression, which is consistent with my data. Notably, both of these developmental checkpoints induce NFκB through activation of TCR signaling.[231] This, together with my data showing activation of the NFκB pathway (Figure 4.4.A and Figure 4.4.B) in the absence of E2A delineate the regulatory pathways regulating HELIOS expression. At two major checkpoints in wild-type T cell development, TCR stimulation inhibits E2A DNA binding through ID3 upregulation. The TCR activation induces NFκB, while the inhibition of E2A further allows upregulation of *Nfκb* genes, amplifying NFκB activation. Finally, this induces HELIOS expression.

What HELIOS is doing in T cell development remains yet to be discovered. Previous reports suggest HELIOS is dispensable for T cell development.[180] Notably, DKO mice have no differences in thymocyte populations compared to VcKO mice (Figure 4.7.A and Figure 4.7.B), showing that HELIOS overexpression is not contributing to the defect in T cell development in the context of E2A deletion either. Taken all together, my data show that HELIOS has minimal impact on T cell development and leukemogenesis.

CHAPTER 5:

E2A PROMOTES THE COMPETITIVE FITNESS OF THYMOCYTES TO PREVENT LEUKEMOGENESIS

CHAPTER 5: E2A PROMOTES THE COMPETITIVE FITNESS OF THYMOCYTES TO PREVENT LEUKEMOGENESIS

INTRODUCTION

In addition to intrinsic oncogene dysregulation caused by the primary oncogenic mutations there is a growing appreciation that the thymic microenvironment can contribute to T-ALL. Recent studies have shown that a competitive environment, i.e. where there are sufficient numbers progenitors entering the thymus, can act as a tumor suppressor.[236] This phenomenon was discovered using models involving transplantation of neonatal thymic lobes into the kidney capsule of immunocompromised (*Rag2*$^{-/-}\gamma c^{-/-}$) mice, creating a situation where the recipient mouse cannot develop thymus-settling progenitors. In these experimental settings, aging donor-derived wild-type thymocytes maintain T cell production, called "thymus autonomy",[184,185] acquire somatic mutations, and eventually progress to T-ALL.[188] The resultant leukemias highly resemble human TAL1^{+} and murine *E2a*$^{-/-}$ leukemias, including a DP/cortical phenotype, trisomy chromosome 15, and a selection for *Notch1* mutations.[104,116,127,188] This similarity is likely due to the leukemias arising from cells that had increased expression of *Tal1* and *Lmo2*.[20,121,188] This suggests either that thymus autonomy selects for E-protein repression, or that there is a synergistic effect between thymus autonomy and the repression of E-proteins that is highly oncogenic.

In mouse models of thymus autonomy, leukemogenesis can be suppressed by restoring competition by transplanting wild-type bone marrow into the host mouse.[187,188] DN2 thymocytes derived from the donor marrow can be found in the thymus lobe after approximately one week, and DPs can be identified after approximately 2 weeks.[186] However, restoring competition in this method is ineffective at suppressing transformation after 6 weeks of thymus autonomy.[188] This

timing suggests that critical transforming events are established by 6 weeks of thymus autonomy but may occur as late as 7-8 weeks given that it takes approximately 1-2 weeks for donor-derived progenitors to appear in the thymus. This demonstrates the rapid oncogenic potential of autonomy.

Recent studies in *Lmo2* transgenic mice indicate that compromised T cell differentiation also leads to a failure of competition and leukemic out-growth, in this case, of ETP-ALL.[123,197] In these mice, the recipient of the thymus lobe transplant was not immunocompromised but instead had the highly oncogenic CD2-LMO2 transgene. This transgene causes overexpression of LMO2 in ETPs, which drives transformation into ETP-ALL by a *Lyl1* dependent mechanism.[123] Notably, TAL1 is dispensable for this process, but given the high expression of *Lyl1* and *Lmo2*, repression of E-protein signaling is likely a strong oncogenic signal in this model. Transplantation of CD2-LMO2 transgenic bone marrow into lethally irradiated hosts was sufficient to induce transformation in 12/12 (100%) of mice, but when transplanted concurrently with wild-type competitor cells leukemia developed in only 3/11 (27%).[197] This indicates that the presence of the wild-type competitor cells were sufficient to prevent transformation in the majority of recipient mice. It is unknown, however, how the competitor cells protect from transformation.

Insight into how competitor cells provide competition come from studies transplanting wild-type donor thymi into genetically wild-type mice and monitoring the fate of the cells originating from the donor thymus lobe. Within 2 weeks the DN compartment is entirely replaced with cells originating from the recipient mouse, and within 1 month there are no donor derived thymocytes remaining.[186] Thus, it is likely that in the context of sufficient competition, thymocytes develop into the next stage and exit into the periphery after maturation. In the absence of competition, wild-type DN3 thymocytes take on a stem-like phenotype marked by increased self-renewal and oncogene expression,[187] suggesting that competition inhibits this dedifferentiation and

prevents leukemia through this mechanism. The DN3 thymocytes being the population to maintain autonomy is of note given that wild-type DN3s are programed to die if they fail to generate a functional TCRβ chain.[71] Indeed, DN3s in autonomy have fewer productive TCRβ rearrangements, and there are more DPs derived from autonomous DN3s that fail to express TCRβ compared to control DPs, suggesting compromised β-selection. Compromised β-selection may predispose autonomous DN3s to apoptosis, which would be a strong selective signal for oncogenic mutations that promote survival and transformation, such as *Notch1* or IL-7R mutations.[187] Consistent with this idea, overexpression of the anti-apoptotic factor BCL2 delays CD2-LMO2 driven leukemogenesis.[197]

It is unknown if leukemogenesis in *E2a*$^{-/-}$ mice is the result of compromised inter-thymocyte competition. Indeed, *E2a*$^{-/-}$ thymocytes have significant developmental defects (Figure 3.4.C),[7,8] and E2A regulates the expression of CCR9 in LMPPs.[39] Thus, *E2a*$^{-/-}$ thymi have defects in progenitor import and reduced thymocyte numbers, both of which have been shown to cause impaired inter-thymocyte competition.[188,197] Here, I show that restoring competition significantly inhibits the development of T-ALL in VcKO mice and show that is does that through regulation of the Myc pathway, cellular metabolism, and promotion of the deletion of pre-leukemic progenitors. These studies have implications not only for T-ALL arising from genomic alterations affecting E2A and other regulators of T cell development, but also for T-ALL arising in the context of lymphocyte progenitor deficiency.

RESULTS

Restoration of competition in VcKO thymi inhibits leukemogenesis

It has been shown previously that intrathymocyte competition can act to suppress T-leukemogenesis.[188] A lack of competition occurs is situations where there are full blocks in progenitor import into the thymus or partial blocks in progenitor import combined with defects in T cell development. This causes early thymocytes to take on a stem-like phenotype to maintain thymopoiesis.[187,188] To investigate if a lack of competition is contributing to the transformation of VcKO DPs, I transplanted wild-type congenically labeled bone marrow into sublethally irradiated VcKO recipients and monitored them for leukemogenesis. The frequencies of DPs, CD4 SPs, and CD8 SPs were broadly restored in the VcKO mice receiving wild-type bone marrow transplants (TPT^{WT}) (Figure 5.1.A) However, TPT^{WT} mice did not show a rescue of the total number of thymocytes (Figure 5.1.B). Despite this, there was a partial rescue of DP, CD4 SP, and CD8 SP numbers. This was primarily due to the presence of wild-type cells in thymi (Figure 5.1.B, cyan), although 4 weeks post-transplant there were still robust populations of VcKO host cells (Figure 5.1.B, pink). Thus, wild-type cells can effectively colonize the thymus and develop normally in the presence of pre-leukemic VcKO host cells. Consistent with my hypothesis, this significantly delayed leukemogenesis in TPT^{WT} mice within the 35 weeks of the study (Figure 5.1.C and Figure 5.1.D). Notably, TPT^{WT} mice that survived until 35 weeks showed no signs of leukemia upon autopsy (data not shown). Inhibition of leukemogenesis was dependent on the introduction of wild-type bone marrow, as transplantation of $E2a^{-/-}$ or $Vav\text{-}Cre^{+}E2a^{+/f}E2a^{+/-}$ bone marrow into VcKO mice (TPT^{KO}) failed to delay leukemogenesis (Figure 5.1.C and Figure 5.1.D). Thus, the prevention of leukemogenesis was specifically due to the presence of competitively fit wild-type donor cells.

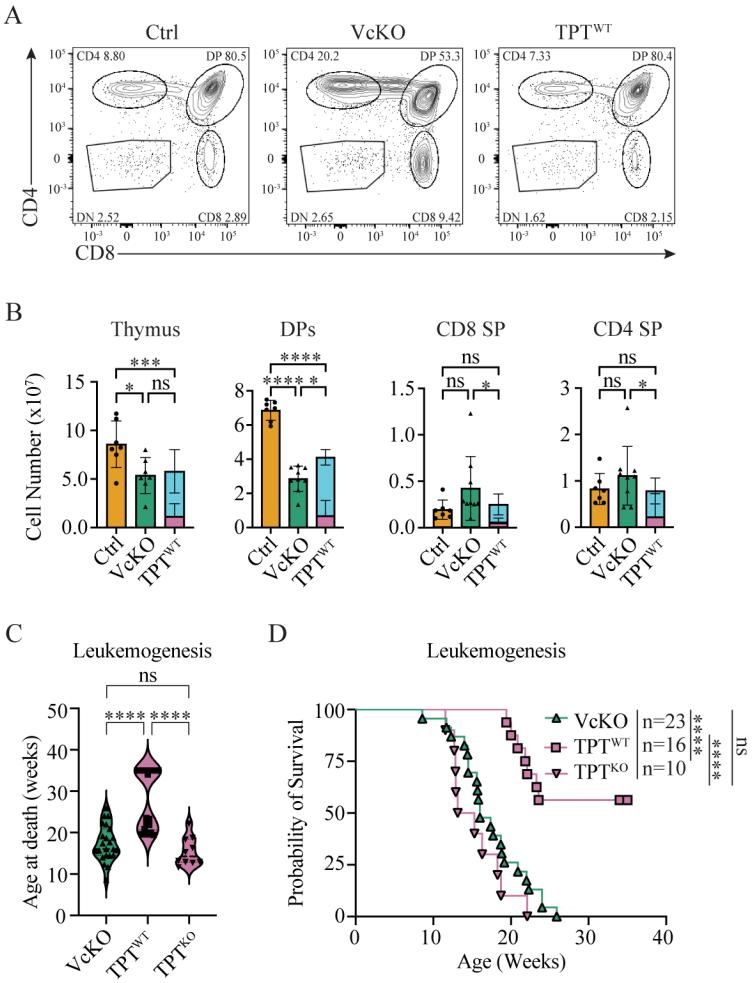

Figure 5.1: Restoring competition inhibits leukemogenesis
A) Representitive FACS plots of lineage$^-$ thymi from 8 week old control, VcKO, and TPT mice 4 weeks post transplant B) Quantification of the indicated populations. Cyan indicates the contribution of donor thymocytes and pink represents the contribution of host thymocytes to total number. C) Age of death of mice from the indicated genotype. D) Kaplan-Meyer curve of mice of the indicated genotype.

Competition does not regulate canonical T-ALL associated pathways

Thymocytes in prolonged thymus autonomy undergo significant transcriptional changes that predispose them to transformation.[187,188] Therefore, competition may regulate the expression of oncogenic pathways. Indeed, VcKO mice have significantly fewer thymocytes than LcKO mice and VcKO DPs have significantly more DEGs vs ctrl DPs than LcKO DPs, suggesting that the lack of competition in VcKO thymus may contribute to the oncogenic program and transformation of these cells (Figure 3.4.B, Figure 3.4.C, and Figure 3.7.B). To investigate if competition regulates canonical T-ALL associated oncogenic pathways, I performed bulk RNA-sequencing on host DPs from TPT^{WT} mice 4 weeks post-transplant and age matched VcKO and Ctrl DPs. Restoring competition induce highly heterogeneous transcriptomic changes, but notably did not restore the Notch, NFκB, or Wnt/β catenin pathways back to Ctrl levels (Figure 5.2.A and Figure 5.2.B). This finding suggests that competition does not regulate these pathways in VcKO mice, and their differential expression is a result of the loss of *E2a*. However, GSEA comparing the transcriptomes of VcKO and TPT^{WT} DPs identified several metabolic pathways that were no longer enriched in TPT^{WT} DPs (Figure 5.2.C). The top pathways enriched in VcKO DPs compared to TPT^{WT} DPs were Fatty_Acid_Metabolism, Glycolysis, and Oxidative_Phosphorylation, and are crucial sources of energy for cells. Thus, an absence of competition in VcKO thymi may provide an excess of energy that supports the rapid proliferation and growth of transforming cells.

Competition regulates the Myc pathway and induces apoptosis in pre-leukemic DPs

The repression of metabolic pathways in TPT^{WT} DPs relative to VcKO DPs and the inhibition of leukemogenesis in TPT^{WT} mice implicates intrathymocyte competition as a key regulator core growth pathways (Figure 5.1.D and Figure 5.2.C). Indeed, GSEA identified the Myc

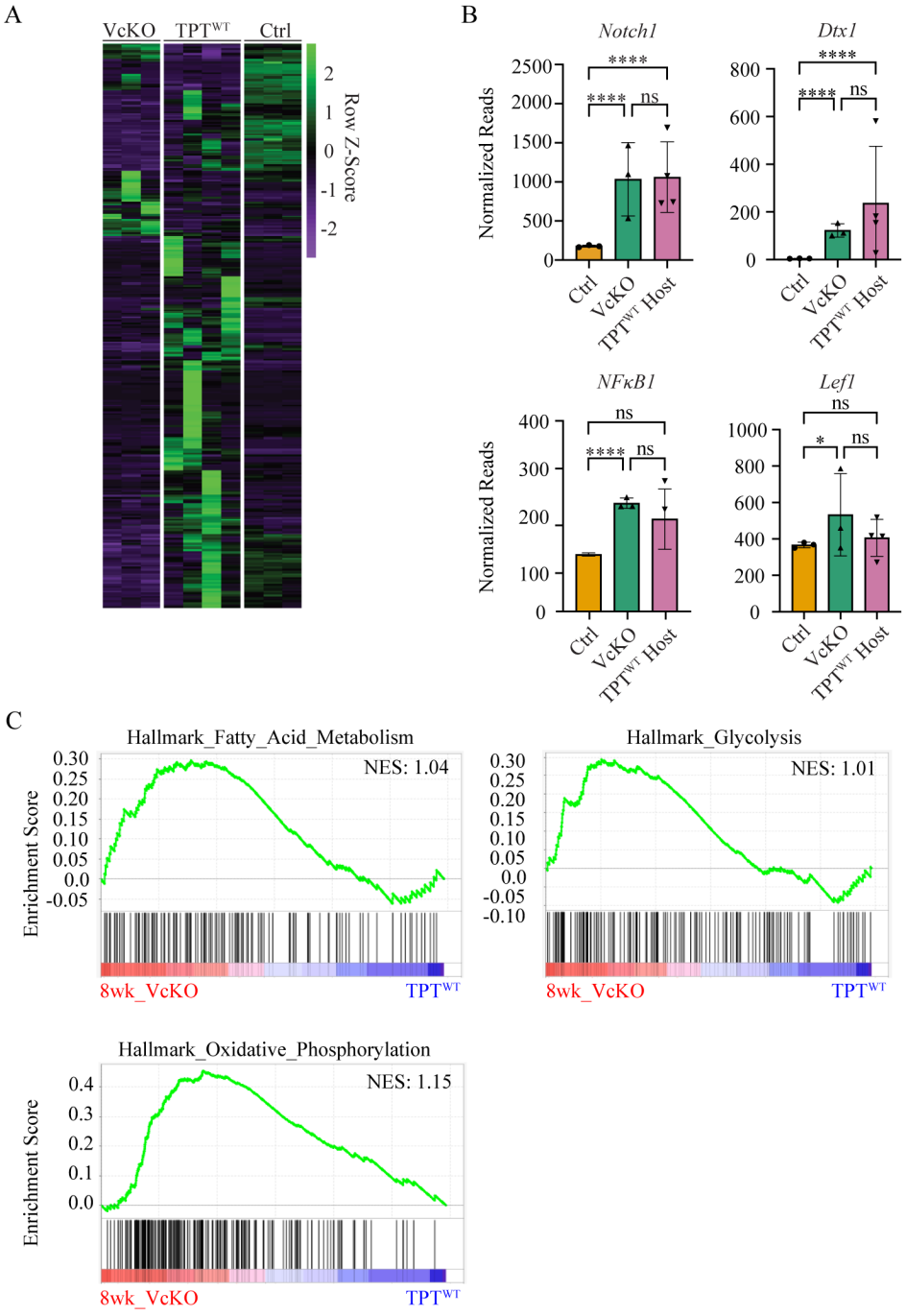

Figure 5.2: Resorting competition represses metabolic pathways in VcKO DPs
A) Heatmap of all genes differentially expressed between VcKO and TPT^{WT} DPs vs controls. B) Normalized reads for key genes in the canonical T-ALL associated pathways shown in Figure 3.7.B. C) GSEA comparing the transcrpitomes of VcKO DPs and TPT^{WT} DPs.

pathway as enriched in 8 week VcKO DPs vs. TPT^{WT} DPs (Figure 5.3.A). Myc has been shown to regulate cellular metabolism and cell cycle.[152,217,237] Consistent with this, Ki67 expression in TPT^{WT} Host DPs was rescued back to wild-type levels, suggesting that competition inhibits DPs from entering the cell cycle (Figure 5.3.B). Myc is also a strong survival signal for T-ALL cells, suggesting that a repression of Myc in TPT^{WT} DPs may be associated with an increase in apoptosis.[237] Indeed, GSEA identified the Apoptosis pathway as enriched in TPT^{WT} DPs compared to VcKO DPs (Figure 5.3.C). To confirm that TPT^{WT} DPs are undergoing apoptosis, I performed FACS for activate caspase activity using Flica. TPT^{WT} DPs showed higher Flica staining, indicating they are undergoing more apoptosis (Figure 5.3.D). Notably, host DPs from TPT^{KO} mice did not show increased Flica staining, indicating that the increase in apoptosis is specifically due to the presence of competitive wild-type thymocytes. Together, these data suggest that restoring competition negatively regulates pre-leukemic cell cycling and induces apoptosis, potentially explaining the inhibition of leukemogenesis.

Inhibition of leukemogenesis is dependent on sufficient intrathymic competition

The identification of the increased apoptosis in VcKO DPs when competition is restored suggests that competition purges pre-leukemic thymocytes from the thymus before they can transform. This is consistent with TPT^{WT} mice surviving until 35 weeks of age and showing no signs of leukemic upon autopsy (Figure 5.1.C). However, approximately 50% of TPT^{WT} mice still succumbed to T-ALL at approximated 25 weeks of age (21 weeks post-transplant) (Figure 5.4.A). This observation suggests that these mice developed leukemia due to a failure of the donor thymocytes to outcompete the host DPs. Notably, grouping the mice by their age of death (Long-lived >30 weeks, Short-lived < 30 weeks) identified trends strongly in support of this hypothesis. Analysis of the chimerism within the peripheral CD4 compartment shows a stark difference in the

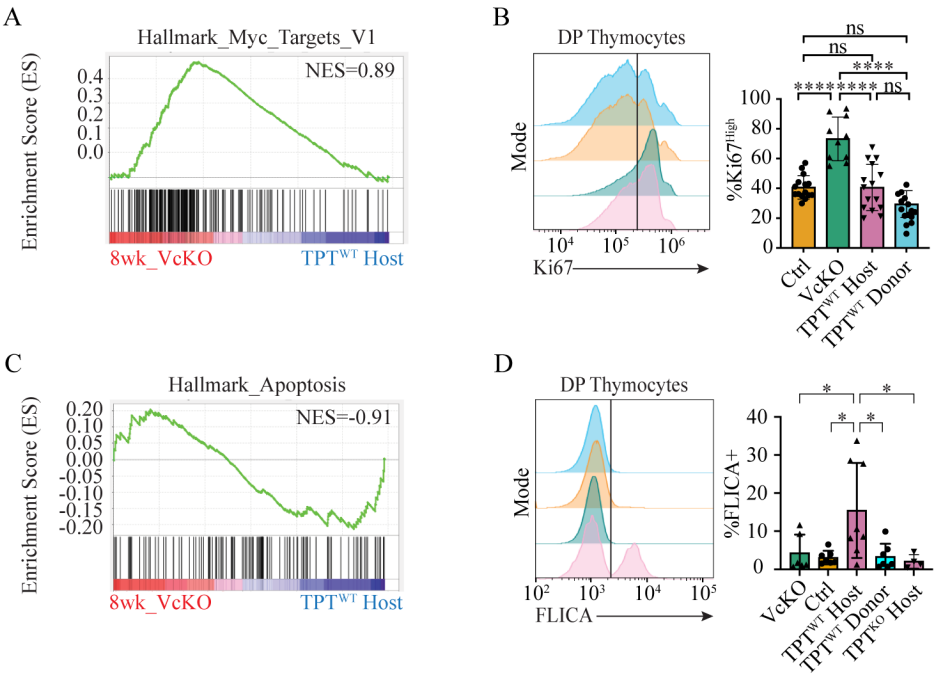

Figure 5.3: Restoring competition inhibits the Myc pathway and induces apoptosis
A) and C) GSEA comparing 8 week old VcKO DPs and TPT^{WT} Host DPs. B) Representitive histograms of Ki67 expression in DPs from mice with the indicated genotype. D) Representitive histograms of Flica staining in DPs from mice with the indicated genotype.

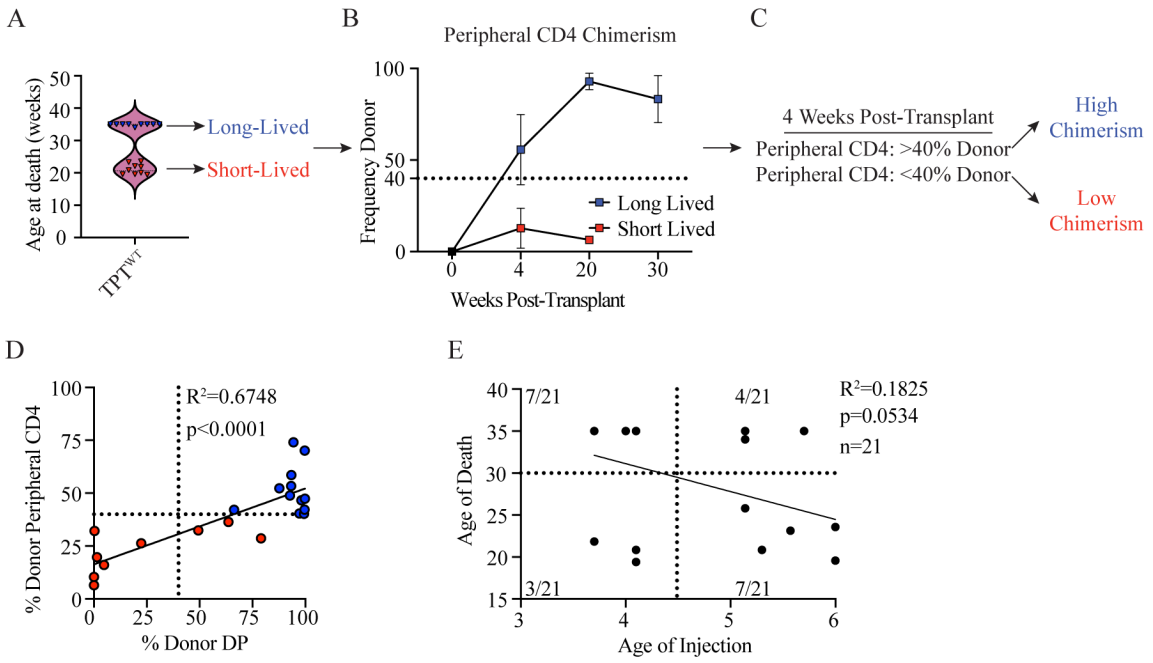

Figure 5.4: Age at death can be predicted by peripheral CD4 chimerism and correlates with age of injection

A) TPT^{WT} mice age at death colored by Long-lived (Blue) or Short-lived (Red). B) Plot showing frequency of donor cells in peripheral CD4 T cell pool seperated by long lived and short lived. C) Definition of chimerism groups based on peripheral CD4 chimerism at 4 weeks post transplant. D) Correlation between frequency donor of peripheral CD4 and thymic DPs at 4 weeks post transplant. Red indicates low chimerism mice and blue indicates high chimerism mice. E) Correlation between age of injection and age of death for TPT^{WT} mice.

frequency of donor CD4s by 4 weeks of age (Figure 5.4.B). This trend is exacerbated as the mice age, with Long-lived mice showing almost 100% donor $CD4^+$ T cells in the periphery at 20 weeks post-transplant, while the opposite is seen in Short-lived mice. This trend allows stratification of mice into separate groups based on their peripheral CD4 chimerism, with greater than 40% donor CD4s in the periphery (High Chimerism; HC) indicating that a mouse would be in the Long-lived group, while less that 40% donor CD4s in the periphery (Low Chimerism; LC) indicating that a mouse would fall into the Short-lived group (Figure 5.4.C). Analysis of TPT^{WT} mice 4 weeks post-transplant shows a significant correlation between peripheral CD4 and thymic DP chimerism, confirming the use of peripheral CD4s for grouping mice (Figure 5.4.D). Strikingly, in addition to peripheral CD4 chimerism, the likelihood of a mouse falling into either the Long-lived or Short-lived groups correlated with the age at which it was transplanted (Figure 5.4.E).

Analysis of HC and LC mice at 4 weeks post-transplant showed remarkable differences in the CD4xCD8 phenotype and in thymic DP chimerism. HC mice had much more normal-looking CD4xCD8 phenotypes, indicating that in HC mice wild-type donor thymocytes are effectively colonizing the thymus (Figure 5.5.A, Top). This is consistent with the high frequency of donor cells amongst all thymocyte populations (Figure 5.5.B). Indeed, LC mice show more transformed thymi consistent with a low frequency of wild-type donor thymocytes (Figure 5.5.A, Bottom and Figure 5.5.B). The significant differences in chimerism between HC and LC mice were exclusive to the thymus and periphery. Analysis of LSK populations from TPT^{WT} mice show no correlation between LSK chimerism at 4 weeks post-transplant and thymic or peripheral chimerism (Figure 5.5.B). This rules out the possibility that chimerism status and response to bone marrow transplant are due to differences in donor bone marrow engraftment. Notably, segregation into HC or LC was identified as early as the ISP stage, indicating that competition happens within the thymus, in the

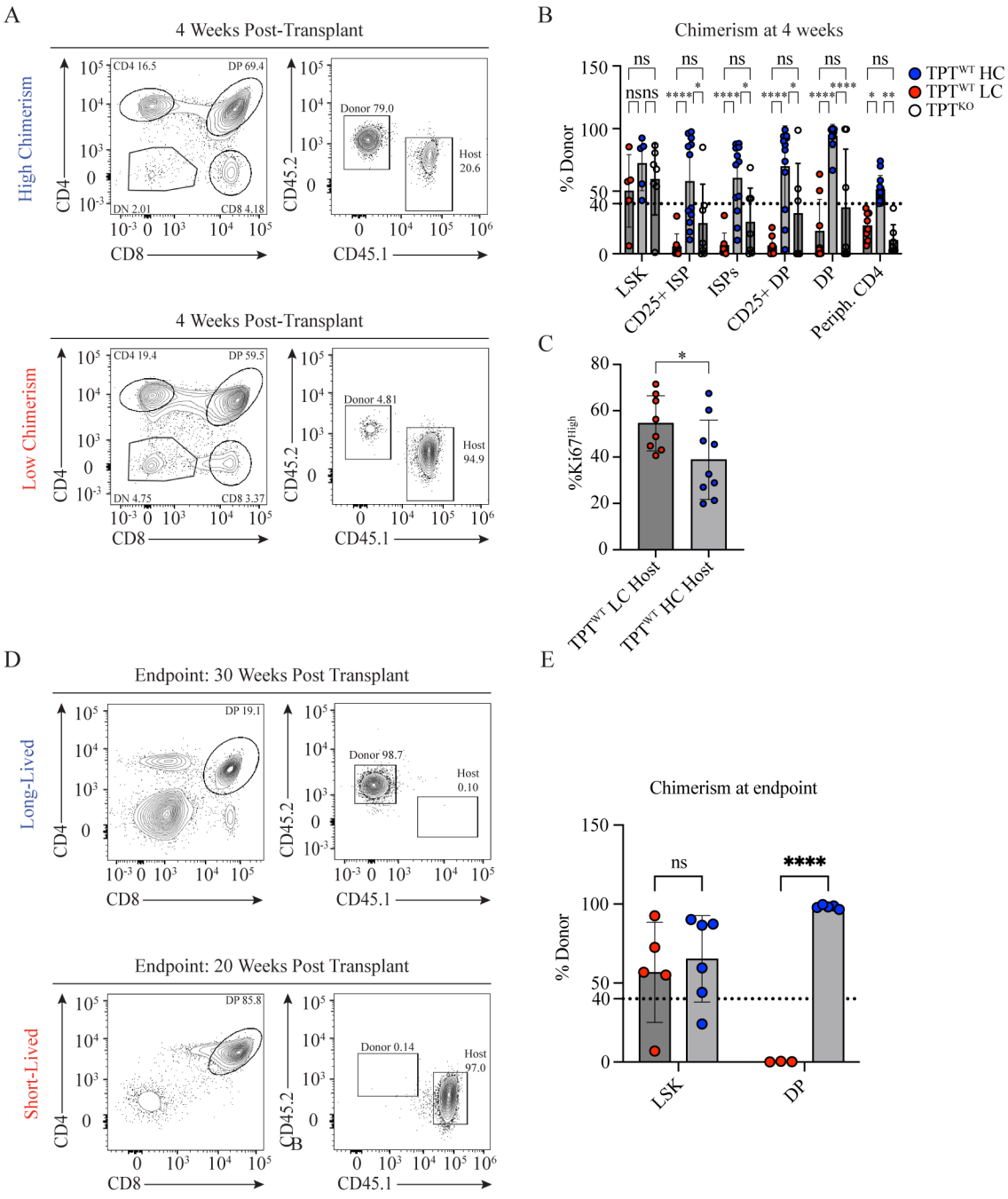

Figure 5.5: Competition occurs in the thymus prior to the ISP stage

A) Representitve FACS plots of thymi from high chimerism (Top; Blue) and low chimerism (Bottom; Red) mice defined by peripheral CD4 chimerism 4 weeks post transplant. B) Quantification of the chimerism of the indicated populations at 4 weeks post transplant seperated by chimerism group and genotype. Red represents low chimerism group mice and blue represents high chimerism group C) Quantification of Ki67 expression from TPT^{WT} Host DPs seperated by chimerism group. D) Representitive FACS plots of thymi from Long-lived (Top) and Short-lived (bottom) mice defined age of death (30 weeks post transplant or ~20 weeks post transplant, respectively). E) Measure of chimerism in the LSK and DP compartments at time of death grouped by age of death.

CD4/CD8 double negative compartment (Figure 5.5.B). Notably, TPT^{KO} thymocyte populations showed chimerism similar to TPT^{WT} LC mice (Figure 5.5.B). Analysis of Ki67 expression in TPT^{WT} HC and LC mice showed HC mice had a smaller percent of DPs in cycle than LC chimerism mice, showing that response to bone marrow transplant correlates with the ability of donor thymocytes to inhibit pre-leukemic host thymocyte's ability to enter the cell cycle (Figure 5.5.C). FACS analysis of mice at 20 weeks post-transplant (Short-lived) or 30 weeks post-transplant (Long-lived) showed thymic chimerism that mirrored the peripheral CD4 chimerism (Figure 5.4.B and Figure 5.5.D). Long-lived mice had no pre-leukemic host-derived DPs remaining and Short-lived mice showed no wild-type donor DPs (Figure 5.5.D and Figure 5.5.E). Consistent with the chimerism at 4 weeks post-transplant, there was no difference in the LSK chimerism between Long-lived and Short-lived mice (Figure 5.5.E). Together, these data suggest that the differing responses to bone marrow transplant is due to differences in the donor thymocytes ability to outcompete the pre-leukemic host thymocytes in the thymus. Given that the donor thymocytes are derived from wild-type mice, it is likely that there are differences in the host thymocytes at time of transplant that contribute to the difference in response.

To confirm if the Short-lived mice develop T-ALL due to an inability of the wild-type thymocytes to colonize the thymus and outcompete the host thymocytes, I performed similar bone marrow transplants with reduced amounts of radiation. None of the mice receiving transplants with low dose radiation (550 Rads) had thymic DP or peripheral CD4 chimerism over 40% at 4 weeks post-transplant (Figure 5.6.A). Further, comparison of 550 Rad TPT^{WT}, TPT^{WT} LC, and TPT^{WT} HC mice showed that 550 Rads TPT^{WT} and TPT^{WT} LC thymi both had significantly fewer donor-derived DPs compared to TPT^{WT} HC. This suggests that a sufficient number of competitively fit donor-derived DPs are required in the thymus to provide sufficient competition. Consistent with

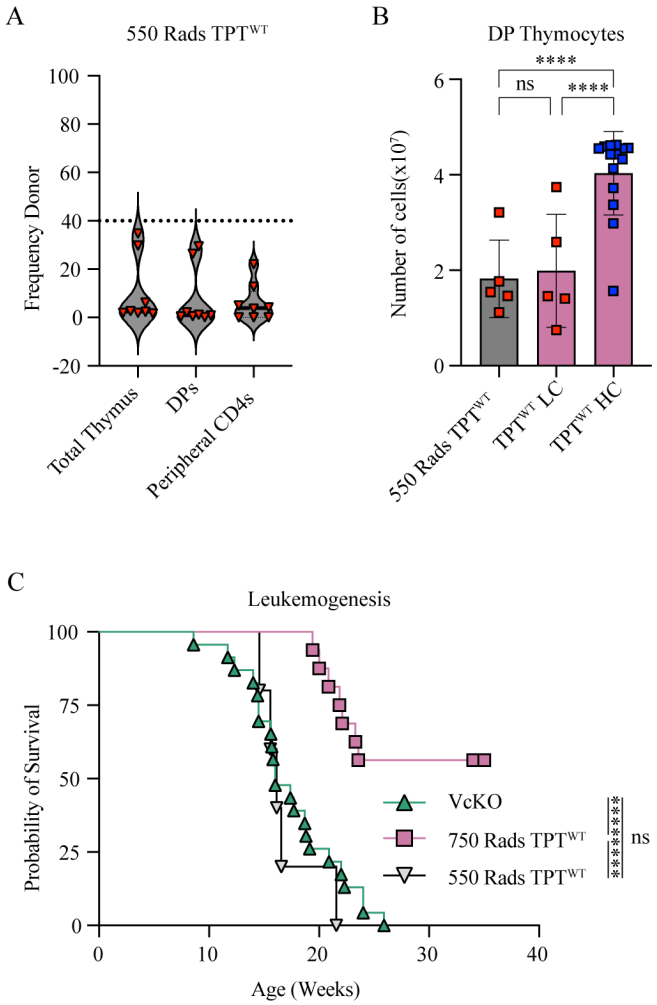

Figure 5.6: Low dose radiation fails to restore competition and prevent leukemogenesis
A) Chimerism whithin the indicated populations from TPT^{WT} mice irradiated with 550 rads. B) Comparison of the number of donor-derived thymocytes from 550 Rads TPT^{WT}, TPT^{WT} LC, and TPT^{WT} HC mice 4 weeks post transplant. C) Kaplan-Meyer curve of untreated VcKO, 750 Rads TPT^{WT}, and 550 Rads TPT^{WT} mice.

this, there is no significant delay in leukemogenesis in 550 Rads TPT^{WT} mice. Together, these data suggest that low dose radiation fails to allow sufficient engraftment of donor thymocytes, thus preventing sufficient competition from occurring and allowing T-ALL development.

Final comparison of the age of death for all mice indicates that both TPT^{WT} LL and TPT^{WT} short-lived mice have significant delays in leukemogenesis, however TPT^{WT} LL mice show full inhibition of T-ALL (Figure 5.7.A). This suggests any amount of competition is sufficient to delay leukemogenesis, but a threshold is needed to fully prevent it. Further, TPT^{KO} and 550 Rads TPT^{WT} have no significant delays, indicating that in these models' insufficient competition was provided, either due to non-competitive donor cells, or a failure to sufficiently engraft in the host thymus.

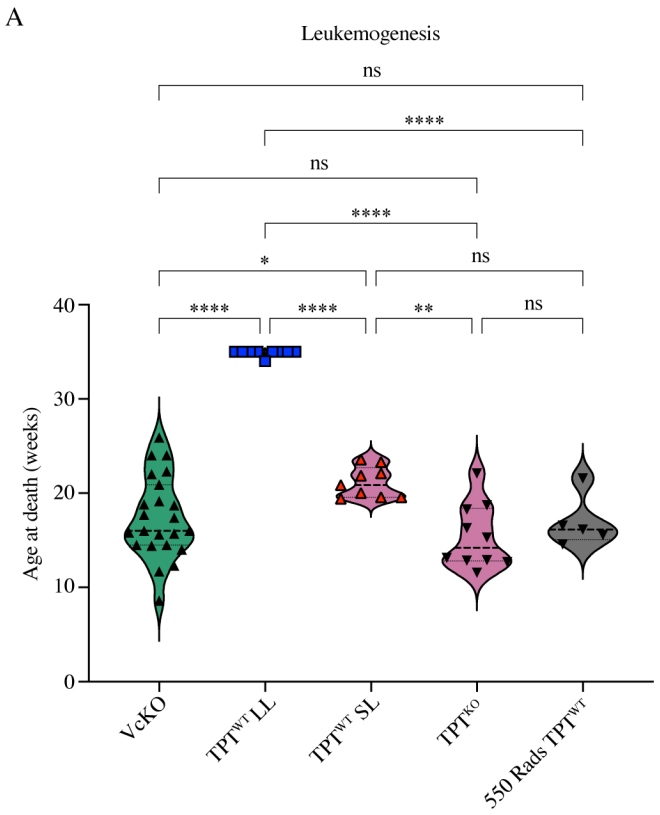

Figure 5.7: Summary of leukemogenesis in transplant models
A) Summary of age at death for untreated VcKO, TPT^{WT} mice seperated by Long or Short lived, TPT^{KO}, and 550 Rads TPT^{WT} mice

DISCUSSION

Thymocyte competition is emerging as an important mechanism to prevent leukemogenesis. Here, I demonstrate that leukemogenesis in *E2a*$^{-/-}$ mice is exacerbated by the lost competition caused by *E2a* deficiency. I show that competition can be restored by transplanting wild-type bone marrow into VcKO mice (Figure 5.1). Restoring competition delayed competition in a subset of VcKO mice, while it fully prevented T-ALL in another (Figure 5.1.C and Figure 5.4.A). In addition, my data show that the difference in leukemia prevention in these mice was not due to differences in bone marrow engraftment, but differences in the frequency and number of donor cells in the thymus (Figure 5.5.B, Figure 5.5.E, and Figure 5.6.B). This confirms previous reports that competition in this manner is occurring in the thymus,[186,197] although it does not confirm at which stage of thymocyte development. Previous work by others indicates that the stage is the DN2 or DN3 populations, depending on the specific model.[186,197] My data are consistent with this, as I see strong segregation between HC and LC groups by the ISP stage at 4 weeks post-transplant. The specific loss of DNs in the VcKO mice make analysis of the DN populations difficult, but competition is likely occurring at the DN2 and DN3 stages. Indeed, one aspect of the competition is the DP population acting upon DN2 to limit proliferation.[186] *E2a*$^{-/-}$ and VcKO mice have significantly reduced DP populations[8] (Figure 3.4.C) which correlates with *E2a*$^{-/-}$ DN2s and DN3s being hyper proliferative (Figure 3.5.B).[94] Thus, one aspect of competition lost in the VcKO model is the repression of DN2 and DN3 proliferation by DPs.

Notably, the addition of *E2a*$^{-/-}$ bone marrow failed to prevent leukemogenesis despite LSK engraftment similar to both TPT^{WT} HC and LC mice (Figure 5.5.B). This suggests that *E2a*$^{-/-}$ thymocytes are inherently unable to provide competition, regardless of thymic reconstitution. This may be due to a deficit in progenitor import, as *E2a*$^{-/-}$ LMPPs have reduced CCR9 expression[39]

and thus host and donor *E2a*$^{-/-}$ LMPPs may not migrate to the thymus efficiently. While this remains to be investigated, the other possibility is that *E2a* regulates the genes that allow thymocytes to maintain competitiveness. Indeed, in TPT^{WT} host DPs, there was an increase in Flica staining, indicating increased apoptosis. This was exclusive to TPT^{WT} host DPs and was notably absent in TPT^{KO} host DPs (Figure 5.3.C and Figure 5.3.D). This suggests that wild-type DPs providing competition cause less fit VcKO DPs to undergo apoptosis, while *E2a*$^{-/-}$ DPs, being equally unfit, are unable to induce this. What exactly makes *E2a*$^{-/-}$ thymocytes unfit is unknown, but, notably, VcKO DPs have upregulation of the IL-7rα, which has been shown to increase fitness in competitive transplant models (Figure 3.9.C).[193] Therefore, there must be other mechanisms regulated by E2A that promote thymocyte fitness that have yet to be discovered. Regardless, I have identified several mechanisms by which competition promotes tumor suppression. Competition does not regulate the canonical oncogenic pathways: Notch, NFκB, or Wnt/β-catenin (Figure 5.2.B and Figure 5.2.C). Surprisingly, the *Notch1* pathway was not affected, given that Notch signaling is dependent on external ligand binding. Wild-type donor-derived DN thymocytes express Notch1 and would presumably act as "sinks" that sequester some of the available NOTCH1 ligands, Thus I would anticipate that NOTCH1 target genes would have reduced expression, although this is not the case (Figure 5.2.B). This is not due to Notch1 PEST domain mutations, which were not found in 8 week VcKO DPs (Figure 3.10.A). It is plausible that the wild-type DNs that could act as Notch ligand sinks simply were not abundant enough to fully sequester the ligands, as they are a small fraction of the thymus in relation to the VcKO DPs. It is also plausible that the reduced number of host-derived DPs in TPT^{WT} mice allowed the few cells that remained to receive sufficient Notch1 ligands to promote the high signaling (Figure 5.1.B). Regardless, competition does regulate Myc and several metabolic pathways (Figure 5.2.B, Figure 5.2.C, and Figure 5.3.A). Oncogenesis is a

highly energy dependent process[217] and these data suggest that restoring competition limits energy uptake or metabolism by pre-leukemic cells and potentially explains VcKO host DPs are purged from the thymus.

The distinction between short-lived and long-lived mice is evident even by 4 weeks post-transplant. Given that it takes up to 3 weeks for DPs to reconstitute in models of thymus autonomy,[186,187] the events that dictate commitment to either fate likely occur within that time period. Competition in this model occurs before the ISP stage (Figure 5.5.B), therefore these events may occur even earlier, within the 2-3 week periods given the kinetics of thymus reconstitution.[186] At these time points, the VcKO host mouse is approximately 6-7 weeks of age and the thymocytes in these mice have presumably been in autonomy for the same amount of time. This timing I identified lines up strikingly well with the timepoints at which restoring competition fails to delay leukemogenesis described by other groups in the thymus transplant models of autonomy.[188] Taken together, this suggests that the commitment to either the short-lived or long-lived fate is correlated with the age of the mouse. This is consistent with trends seen when analyzing the age of injection and age of death of TPT^{WT} mice (Figure 5.4.E). Presumably then, the thymocytes from mice short-lived have sufficient mutations to be competitive with the donor wild-type thymocytes, while the thymocytes from long-lived mice do not have these mutations, which broadly correlates with the age of the mouse. It is also possible that the apoptosis induced by the restoration of competition acts as a selective pressure that promotes mutation. Thus, while the thymocytes in the Short-lived mice have no significant *Notch1* mutations at the time competition is restored, they could have other mutations or develop them which ultimately allows the pre-leukemic cells to overcome the competition. The exact nature of these mutations is unknown, although my data suggest that it is

not the Notch exon 34 mutations (Figure 3.10.A). Further, the exact mutations may vary from mouse to mouse making it difficult to identify.

The idea of sufficient competition being needed is supported by both the TPT^{KO} transplant model and the 550 Rads TPT^{WT} model. Notably, while the TPT^{KO} model relies on the transplant of non-competitive cells, the 550 Rads TPT^{WT} model specifically prevents engraftment of sufficient numbers of competitive wild-type thymocytes (Figure 5.6.B). However, it is unknown if this is due to insufficient bone marrow engraftment or other factors. Regardless, few donor-derived thymocytes are found in host thymi and the mice develop leukemia with the same latency as untreated VcKO mice (Figure 5.6.A, Figure 5.6.C, and 5.7.A). This is in contrast with the short-lived TPT^{WT} mice, which still have a delayed onset of T-ALL compared to VcKO and TPT^{KO} controls (Figure 5.7.A). Altogether, my data suggest that intrinsic loss of the transcription factor *E2a* causes thymocytes to be non-competitive in several ways. They are intrinsically unable to provide competition, as shown by TPT^{KO} experiments, and they are unable to sufficiently develop and provide competition in this manner. Further, my data show there is a threshold of competitive cells in the thymus that needs to be reached to fully eliminate pre-leukemic stem cells. Finally, I have elucidated how the presence of competitive cells effects the transcriptome of less fit competitive cells. Competition does not regulate canonical T-ALL pathways other than Myc, and competition does regulate cellular metabolism eventually inducing apoptosis in pre-leukemic cells.

CHAPTER 6:

DISCUSSION

DISCUSSION

E2A's tumor suppressive functions have been well appreciated and studied for decades.[8] This is evidenced by the fact that most current models used to study T-ALL either directly delete *E2a*,[214,238] overexpress oncogenes that directly impact E2A function,[19,123,135,197] or have secondary mutations that impact E2A function.[57,106,146] Significant research has gone into understanding the molecular events that drive transformation using these models, with a focus on identifying genes directly regulated by *E2a* that contribute to transformation or dysregulations in canonical T-ALL associated oncogenes. E2A has been previously shown to regulate genes such as *Gata3*[99] and *Lef1*,[103] which coordinate with *Notch1* to regulate c-*Myc*.[139,152,153,159,237] These genes then promote transformation through regulation of proliferation, survival, and metabolism.[152–154,159,237,239] In addition to these oncogenes, E2A deficient leukemias develop mutations that mirror human leukemias, including *Notch1* PEST domain mutations[127] and trisomy of chromosome 15.[104] Further, E2A coordinates with IKAROS to regulate usage of a non-canonical *Notch1* exon that leads to loss of the extracellular domain of NOTCH1 and ligand independent activation, which mirrors *Notch1* HD mutations found in human T-ALLs.[57,143,146] While all of these features promote transformation, little work has been done to understand when during transformation these events occur. This is vital to understand as it has previously been shown in $E2a^{-/-}$ leukemias that some genes can be oncogenes or tumor suppressors based on the timing of their mutation.[104] By sequencing the transcriptomes of $E2a^{-/-}$ DPs using 2 mouse models and at multiple pre-leukemic time points, I have identified key insights into how the timing of *E2a* deletion contributes to the dysregulation of oncogenic and tumor suppressive pathways (Figure 6.1). First, using VcKO and LcKO mice, I have shown that loss of E2A function must occur prior to the DN3 stage to induce

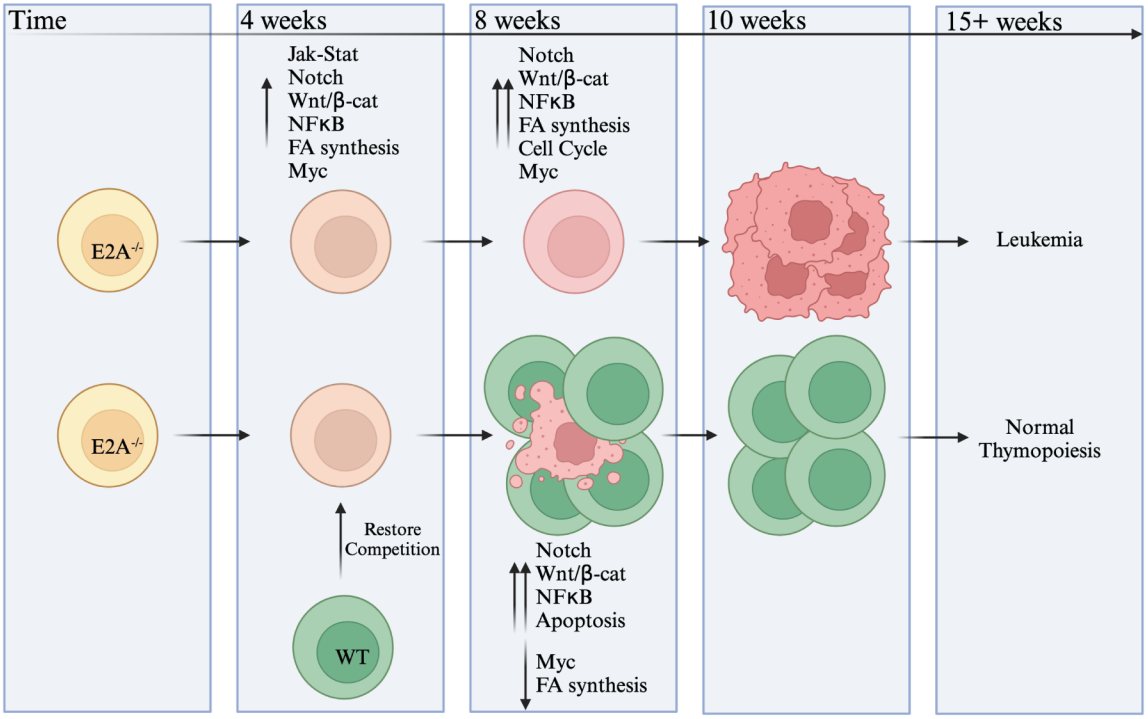

Figure 6.1: Final Model of E2A$^{-/-}$ thymopoiesis with and without restored competition
Top) Model depicting thymopoiesis over time in *E2a*$^{-/-}$ mice without competition. E2A$^{-/-}$ thymocytes gradually increase expression of oncogenic pathways over time to eventually transform. Bottom) Restoring competition at 4 weeks of age does not effect the expression of Notch, Wnt/β-catenin, or NFκB pathway genes but inhibits Myc pathway expression 1 month post transplant. Pre-leukemic cells undergo apoptosis, purging them from the thymus before they transform.

robust leukemogenesis. This is analogous to other models of T-ALL, including the *Tcf7* deficient model.[210] In both models, DN3 deletion or later failed to induce robust T-ALL, surprising given that *Tcf7* expression peaks at the DP stage.[210,240] One key difference between the *LcKO* and *Lck-Cre Tcf7$^{f/f}$* models is that a small subset of LcKO mice did eventually develop T-ALL, while no *Lck-Cre Tcf7$^{f/f}$* mice were reported to. This may be a consequence of genetic background, as all the *Tcf7* models are on the C57BL/6 background,[210] while the LcKO mice are on the FVB background. Regardless, the presence of low-frequency leukemogenesis in LcKO mice suggests that even a later *E2a* deletion is weakly oncogenic while the robust leukemogenesis in VcKO mice shows that early deletion is a much stronger leukemia initiator. Indeed, the comparisons between VcKO and LcKO mice are strikingly reminiscent of phenotypes seen when comparing *Vav-Cre Tcf7$^{f/f}$* and *Lck-Cre Tcf7$^{f/f}$* mice. Single-cell RNA-seq of wild-type, *Vav-Cre Tcf7$^{f/f}$*, and *Lck-Cre Tcf7$^{f/f}$* DN cells identified separate *Vav-Cre* and *Lck-Cre* DN3 clusters. Pseudo-bulk analysis of the transcriptomes of the *Vav-Cre* populated cluster and *Lck-Cre* populated cluster identified the *Vav-Cre* cluster as having high oncogenic potential, defined by high expression of *Lef1*, *NfκB1*, *Dtx1*, and *Id2*.[210] These transcriptional difference overlap significantly with my bulk analysis of VcKO and LcKO DPs, suggesting that breaking T cell development at a sufficiently early timepoint (pre-DN3) predisposes later thymocytes to transformation through induction of a common oncogenic program typically repressed by E2A and TCF1.

I have shown that the predisposition for leukemogenesis in *E2a* deficient mice is associated with a difference in thymic competition. Specifically, VcKO thymocytes are unable to provide a competitive environment, while LcKO thymocytes can. The *E2a$^{-/-}$* model of T-ALL was a promising candidate for investigating if inter-thymocyte competition regulated leukemogenesis because a competitive thymus relies on 2 factors: 1) sufficient progenitor import and 2) sufficient

expansion of thymocytes. *E2a*$^{-/-}$ LMPPS are known to have defects in CCR9 expression[39] and therefore have difficulties trafficking into the thymus. Further, thymocytes with CD2-driven *Lmo2* overexpression have no reported defects in thymic trafficking but fail to properly expand and provide a competitive environment,[197] indicating that the developmental defects caused by E2A loss impair thymocytes' ability to compete. Both of these deficiencies, found in VcKO mice, create a scenario where there are relatively few developing thymocytes. This causes the few progenitors that do colonize the thymus to hyperproliferate. Indeed, *E2a*$^{-/-}$ DN2s[94] and VcKO DN3s are hyper-proliferative, consistent with this idea. Similar phenomena have also been seen in mouse models of neonatal thymus transplants into the kidney capsules of wild-type mice (Table 6.1). Artificially increasing the number of DPs in the donor thymus by transplanting it into a BCL2-tg host, which prevents DPs from dying if they fail positive selection, inhibits DN2 proliferation,[186] suggesting that a lack of DPs induces DN2 proliferation. Given this fact and the decrease in DPs in VcKO mice, it is likely that the reduced number of DPs in VcKO thymi also induce hyper proliferation in VcKO DN2s that may amplify transformation through generation of spontaneous mutations or genomic instability that may select for oncogene activation.

The idea that competition is a key suppressor of T cell transformation is gaining more acceptance throughout the field, but until now, has only been identified in the context of the highly artificial thymus transplant experiments, either through transplantation of wild-type thymi into immunocompromised mice or CD2-*Lmo2* transgenic mice.[188,197] My work is the first to show competition suppresses transformation in an autochthonous model of T-ALL (Table 6.2). How competition acts as a tumor suppressor was unknown, but it has been shown that thymocytes developing in the absence of competition undergo significant transcriptional changes,[187,197] with the eventual development of pre-leukemic stem cells with a DN2 or DN3 phenotype.[184,187,197] This

Donor	Host Mouse	Treatment	Outcome	Mean Latency (Days)
Wild-type thymus	Wild-type	N/A	No Leukemia	---
Wild-type thymus	BCL2-tg	N/A	Increased DPs in donor thymus Reduced DN2 proliferation No Leukemia	---
Wild-type thymus	*Rag2*$^{-/-}$*γc*$^{-/-}$ *kit*$^{w/w}$	N/A	Leukemia	200
Wild-type thymus	*Rag2*$^{-/-}$*γc*$^{-/-}$ *kit*$^{w/w}$	Wild type bone marrow transplant 1 week post thymus transplant	No Leukemia	---
Wild-type thymus	*Rag2*$^{-/-}$*γc*$^{-/-}$ *kit*$^{w/w}$	Wild type bone marrow transplant 6 weeks post thymus transplant	Reduced Leukemia penetrance	200
NA	*CD2-Lmo2*	N/A	Leukemia	200
CD2-*Lmo2* bone marrow	Wild-type	Lethally irradiated prior to transplant	Leukemia	300
3:1 wild type:CD2-L*mo2* bone marrow	Wild-type	Lethally irradiated prior to transplant	Reduced leukemia	450
N/A	VcKO	N/A	Leukemia	120
Wild-type bone marrow	VcKO	Sublethally irradiated prior to transplant	Reduced Leukemia penetrance	150
E2a$^{-/-}$	VcKO	Sublethally irradiated prior to transplant	Leukemia	100

Table 6.1: Expanded list of models of thymus autonomy and competition
Table depicting models used to study thymus autonomy and common models of T-ALL that rely upon compromised thymic competition for their development

implies that competition acts to inhibit these transcriptional changes, suggesting that cellular competition directly augments the transcriptional landscape of thymocytes to limit oncogene expression. This makes it likely that the lack of competition in VcKO thymi contributes to the significant transcriptional changes seen in VcKO DPs at 4 weeks old. If the thymocytes in the VcKO thymi are derived from the earliest T cell progenitors, which can be seen in embryonic thymi by day E12,[241] then by the time of my sequencing they may have been in autonomy for up to 4-5 weeks. Notably, this is the timepoint at which other models of thymus autonomy found the induction of oncogenic pathways in wild-type DN3s and DPs.[187] The LcKO mouse has no such failure of competition and LcKO DPs have very few DEGS relative to VcKO DPs. Thus, the transcriptomic changes seen in LcKO DPs may be the "core" genes regulated by E2A at the DP stage, while the VcKO DP transcriptome may contain those "core" genes as well as hundreds of other genes dysregulated by prolonged autonomy. Indeed, there were 252 genes dysregulated in both VcKO and LcKO DPs, and 1,407 genes uniquely dysregulated in VcKO DPs. Why restoring competition failed to rescue expression of the DEGs found in VcKO DPs remains unknown. It is possible that there are mutations in 4 week old VcKO DPs driving differential expression of these genes, but the likelihood that all of the DEGs are mutated is low. It is more probable that after sufficient time in autonomy, thymocytes irreversibly change their transcriptomes to compensate. This may occur through the reorganization of the chromatin landscape in cells in prolonged autonomy. To date, analysis of the chromatin landscape of pre-leukemic *E2a*$^{-/-}$ thymocytes over the course of transformation has not been performed, so the extent of any chromatin changes are unknown. However, this idea is consistent with there being an inflection point when competition fails to prevent leukemogenesis, as seen in models of thymus autonomy[188] and is an attractive explanation for why some VcKO mice fail to respond to restored competition. In this scenario,

DPs from short-lived TPT^{WT} mice would have repressed enough chromatin modifying enzymes to inhibit the reorganization of the chromatin landscape back to wild-type. The short-lived TPT^{WT} DP chromatin landscape would be much more permissive of transformation, i.e. higher accessibility at oncogene loci, while DPs from TPT^{WT}, which would not have repressed the chromatin modifiers, may be able to repress accessibility at those oncogene loci upon restored competition.

While the broad lack of "competition regulated" genes may not be surprising if the hypothesis that VcKO cells are defective in their ability to reorganize their chromatin is true, the maintenance of increased Notch signaling in the context of restored competition is a surprise, given that the pathway specifically relies upon interactions with a potentially limiting source of extracellular ligands. Restoring competition significantly increased the number of cells in the cortex (both DN and DP thymocytes), which could either inhibit the ability of the pre-leukemic thymocytes to physically interact with NOTCH1 ligands or the increased number of NOTCH1 expressing DN cells could have sequestered NOTCH1 ligands and prevented Notch activation in pre-leukemic TPT^{WT} host DPs. At 8 weeks of age, no *Notch1* mutations are present, meaning that the TPT^{WT} DPs must actively rely on interaction with NOTCH1 ligands for Notch activation. Together, these data suggest that in the VcKO + BM transplant model cells are either not competing for NOTCH1 ligands, or there is an abundance of NOTCH1 ligands that can overcome the restored competition.

While the Notch pathway was relatively unaffected by restored competition, Myc and metabolic pathways were directly repressed by the addition of wild-type thymocytes. The exact signal regulating Myc pathway is yet to be defined, however, IL-7 signaling is likely playing a significant role. IL-7 is vital for thymocyte development, and has been shown to promote *Myc*

expression in murine thymocytes.[242,243] Further, IL-7 has been previously implicated as the limiting resource for which thymocytes compete,[186] and overexpression of IL-7 is associated with increased competitive fitness[193] and leukemia development.[220,242] Together, these data suggest that the restoration of competition in TPT^{WT} mice may alter IL-7 signaling in the host VcKO DPs, most likely through modulating its availability, in order to repress the Myc pathway. This would consequently make VcKO thymocytes less stable, and more prone to apoptosis, which is consistent with the transcriptional analysis and Flica staining. It should be noted that VcKO DPs have increased expression of the IL-7rα chain, which would suggest that they are actually more fit, or at least more able to act as a sink for IL-7, especially in the context of the TPT^{KO} mice. It is unknown if *Myc* is dysregulated in the TPT^{KO} host DPs, but the transplantation of $E47^{-/-}$ bone marrow into VcKO mice clearly shows that $E47^{-/-}$ derived thymocytes are unable to provide a competitive environment. This may be due to a slight, but not statistically significant, decrease in expression of the common-γ chain, the second subunit of the IL-7R, rendering the cells unable to interact with extracellular IL-7, or may be a result of other defects in $E47^{-/-}$ thymocytes that make them unfit to compete, such as an inability to fully expand despite increased IL-7 sensitivity. Therefore, $E47^{-/-}$ donor thymocytes in the TPT^{KO} model may be better poised to act an IL-7 sink, but simply cannot expand to the numbers needed to sequester all available IL-7.

As stated previously, the phenotype of the pre-leukemic stem cells in artificial models of thymus autonomy are of DN origin. In my mice, I do not see an appearance of a DN2 or DN3-like stem cell in VcKO mice, however, the pattern of expression for many of the differentially expressed oncogenes (*Il7ra, Myc, NfκB1, Notch1*) and tumor suppressors (*Fbxw7, Tet2*) in VcKO DPs closely resembles that of wild-type DN3s when compared to wild-type DPs.[244–246] This pattern of gene expression is highly consistent with cells expressing an extracellular DP phenotype, while

transcriptionally they are less mature thymocytes. In agreement with this hypotheses, I and others have shown that there is a near absence of the DN3 population in *E2a*$^{-/-}$ mice[8,94,238] despite the presence of later thymocyte populations in VcKO mice, albeit at a lower frequency than in wild-type mice.[238,247] Therefore, it is plausible that absence of DN3s is due to *E2a*$^{-/-}$ DN3s rapidly developing into later thymocyte stages. Consistent with this idea, I show that VcKO DN3s are hyper-proliferative, and other's experiments in RAG1$^{-/-}$ mice show that the developmental block at the DN3 stage is absent when crossed with ID1 transgenic mice. Notably, *Id1*^{tg}*Rag1*$^{-/-}$ mice have no DN3s, but have the DP compartment, similar to VcKO mice.[223] Together, these data suggest that E-protein repression causes rapid, autonomous passage to the DP stage. However, no analysis was performed to determine if the *ID1*^{tg}*Rag1*$^{-/-}$ DPs were *bona fide* DPs, or DN3s that upregulate CD4 and CD8. Further, VcKO mice have a significantly higher proportion of CD25 expressing DPs. CD25 is a defining marker for Notch signaling but is also a marker used to distinguish DN3s from other DN populations, thus it is possible that the CD25^{+} population that I identify as expanded in the VcKO thymi are *de facto* DN3s that express CD4 and CD8, and not a sign of "true" DPs that have increased Notch signaling. The fact that this population expands in frequency and number as VcKO mice age and all of the *E2a*$^{-/-}$ leukemia cells are CD25^{+} suggests that these are the cells transforming, regardless of if they are CD4^{+}CD8^{+} DN3s or DPs. If they are *bona fide* DN3s, this would explain the difference in leukemia latency between VcKO and LcKO mice. LcKO DN3s only have ~86 DEGs compared to wild-type DN3s, none of which are notable T-ALL associated oncogenes (Data not shown), suggesting that DN3s are not significantly broken by the loss of *E2a* driven by *Lck-Cre*, presumably due to the E2A protein not being fully ablated by the DN3 stage in LcKO mice. Thus, LcKO deletion is insufficient to fully delete E2A at the DN3 stage, induce rapid CD4 and CD8 expression, and promote transformation. In the few LcKO mice that did eventually

develop T-ALL, it is possible that they stochastically deleted *E2a* earlier than other mice, which could create a small population of $CD4^+CD8^+$ DN3 pre-leukemic stem cells that are able to eventually transform. This would explain the low penetrance of LcKO T-ALLs; however, it fails to explain the increased latency.

The hypothesis that the transforming cells in the VcKO mouse are actually $CD4^+CD8^+$ DN3s makes sense when considering the dynamics of the chromatin landscape between the DN3 and DP stage. Early T cell development is comprised of 3 distinct phases, each with a unique chromatin landscape. The first phase contains the ETP and DN2a stages, which are more multipotent and stem-like, the second phase contains DN2b and DN3a stages, which is where commitment to the T cell lineage occurs, and the third phase is the post-β-selection DN3b to DP.[6] The transition from phase 1 to phase 2 occurs as progenitors commit to the T cell lineage, repress progenitor-associated transcription factors (PU.1, MEF2C, BCL11A), repress accessibility at multipotency-associated loci, and increased accessibility at loci associated with E-protein and BCL11B binding.[6] This is concomitant with increased expression of *Lef1*, *Bcl11b*, and *Ets1*.[6,63,94] The transition from phase 2 to phase 3 occurs after β-selection and coincides with the greatest change in the chromatin accessibility in early thymocytes.[6,65] These changes are orchestrated through the collaboration of TCF1, BCL11B, and E2A activity.[6,65,79] The requirement for E2A for these phase 2 to phase 3 chromatin topology transitions suggests that there may be defects in the chromatin architecture in $E2a^{-/-}$ DPs. Indeed, E2A has previously been shown to associate with CBP/p300 to promote its histone acetyltransferase activity,[248,249] which can promote chromatin reorganization, and numerous DNA and chromatin modifying enzymes (including *Tet2*) are dysregulated in VcKO DPs. Thus, when the β-selection checkpoint is compromised by loss of

E2A, then the VcKO DN3 cells may fail to undergo the chromatin accessibility changes needed to fully develop into DPs.

An inability to fully transition to the DP stage is implied by my sequencing and phenotypic data and may be a direct cause of the difference in the leukemia latency between VcKO and LcKO mice. Indeed, there are no differentially expressed DNA/chromatin modifying enzymes seen in LcKO DN3s or DPs vs wild-type controls. Thus, VcKO DN3s may be unable to fully reorganize the chromatin to transition to a DP state while the LcKO DN3s can. This may cause genomic instability in VcKO DPs, which would be consistent with the enriched p53, mismatch repair, and nucleotide excision repair pathways seen in 8 week VcKO DP transcriptomes. If true, an inability to reorganize chromatin may leave the chromatin at oncogenic loci accessible in VcKO DPs when it should be inaccessible. DN3s have high accessibility at oncogene loci such as *Notch1* and *Lef1* that is repressed during the transition to the DP stage.[65,246] An inability to repress accessibility at these loci may explain their increased expression, and eventually drive transformation. Its notable that VcKO thymocytes dysregulate several DNA/chromatin modifying enzymes, including *Tet2*, which acts to demethylate DNA to promote gene expression.[15] During the course of normal T cell development, *Tet2* expression increases as DN3s transition to the DN4 and DP stages,[250] suggesting that it is a key regulator of this transition. This further supports the hypothesis that the DPs in the VcKO mouse are DN3s unable to reorganize their DNA to fully transition to the DP state.

Surprisingly, *Tet2* is one of the few genes whose expression is partially restored by the restoration of competition. It suggests that in the wild-type context, intrathymic competition regulates the expression of DNA-modifying enzymes to facilitate the DN3-DP transition. When broken due to E2A loss, the thymocytes are “frozen” as DN3s which predisposes them to eventual

transformation through maintenance of high accessibility at putative oncogene loci, as discussed previously. However, *Tet2* and chromatin modifying enzyme downregulation may promote transformation through another mechanism. It has been reported that inhibition of DNA/chromatin modifiers promotes cancer cell fitness through a phenomenon deemed "phenotypic inertia".[226] When cultured in stress-inducing conditions, such as low pH or nutrient deprivation, cancer cell lines with chromatin modifiers repressed had a significant advantage over wild-type cancer cell lines. This was eventually mapped to the cells inability to reorganize their chromatin to allow expression of stress associated genes, which typically induce apoptosis in response to prolonged stress.[226] My data suggest a similar phenomenon is occurring in the thymus. The repression of *Tet2* in the absence of competition suggests that VcKO thymocytes may be unable to activate stress genes due to this phenotypic inertia. This hypothesis is supported by the repression apoptosis and senescence pathways in 8 week old VcKO DPs compared to wild-type DPs, and perfectly explains the differences in leukemogenesis seen in the TPT^{WT} and the TPT^{KO} mice. In the TPT^{WT}, loss of *E2a* stresses the cell. In the VcKO mouse prior to transplant, which cannot provide competition, *Tet2* is repressed, the cell cannot reorganize its DNA, and apoptosis is not induced. When competition is restored through transplantation, *Tet2* is expressed which allows the DNA to be demethylated to allow expression of stress associated genes. These genes would induce apoptosis, which prevents pre-leukemic cells from expanding and inhibits leukemogenesis. In the context of the TPT^{KO} mice where competition is not restored, *Tet2* is presumably not expressed and therefore apoptosis is not induced, promoting the development of T-ALL.

CONCLUSIONS

The transcription factor E2A is a tumor suppressor whose function is altered in most murine models of T-ALL[8,106,121] and the majority of human T-ALL patients.[12,18,251] It has been well established that E2A regulates specific oncogenes, including *Notch1*,[89,92] *Gata3*,[94] and *Lef1*[103,104] and more broadly promotes T cell development. However, previous attempts to study E2A's tumor suppressor role in conditional deletions found that *Lck-Cre* mediated deletions did not phenocopy the germline deletion.[198] Further, deletion of *E2a* at the DN3 stage previously showed no defects in T cell development.[198] This was surprising, given the strong phenotype and oncogenic potential of the germline deletion. I have found that the high oncogenic potential of $E2a^{-/-}$ DPs is encoded transcriptionally and requires the early deletion, as the later deletion fails to induce significant oncogene activation. Most notably, the LcKO DPs had no Notch, Gata3, Wnt/β-catenin, NFκB, or Myc pathway activation, while the VcKO DPs were enriched for all of them. These pathways were further enriched as VcKO DPs grew closer to fully transforming, while I also saw an enrichment of cell cycle and proliferation associated genes at this timepoint as well. Finally, the final checks on transformation, inducing apoptosis and senescence, were repressed at this later timepoint. This temporal analysis of VcKO and LcKO DPs provided key insights into the cell intrinsic mechanisms leading to transformation in early deletions. I also found that a major promoter of transformation in the VcKO mouse is the absence of competition. $E2a^{-/-}$ bone marrow derived thymocytes fail to provide competition in bone marrow transplant experiments, while wild-type bone marrow derived thymocytes can. This competition represses cellular metabolism and represses the *Myc* pathway, and eventually induces apoptosis in the pre-leukemic thymocytes. In the absence of this competition, no apoptosis is observed and leukemia develops unchecked. Finally, I identify a

crucial turning point in the kinetics of leukemogenesis, where at approximately 4-6 weeks of age, murine *E2a*$^{-/-}$ thymocytes begin to acquire mutations that make them more competitively fit than donor thymocytes. This change in fitness effects the response to competition restoration and dictates the eventual fate of the pre-leukemic mouse. Altogether, I have provided key insights into the cell intrinsic and cell extrinsic mechanisms regulated by E2A that drive leukemogenesis.

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