

University of Massachusetts Medical School

eScholarship@UMMS

GSBS Dissertations and Theses

Graduate School of Biomedical Sciences

2004-01-22

Mechanisms of TAL1 Induced Leukemia in Mice: A Dissertation

Jennifer Elinor O'Neil

University of Massachusetts Medical School

Let us know how access to this document benefits you.

Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss



Part of the [Amino Acids, Peptides, and Proteins Commons](#), [Animal Experimentation and Research Commons](#), [Genetic Phenomena Commons](#), and the [Neoplasms Commons](#)

Repository Citation

O'Neil JE. (2004). Mechanisms of TAL1 Induced Leukemia in Mice: A Dissertation. GSBS Dissertations and Theses. <https://doi.org/10.13028/c25m-ts25>. Retrieved from https://escholarship.umassmed.edu/gsbs_diss/70

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.

A Dissertation Presented

By

Jennifer Elinor O'Neil

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

January 22, 2004

Copyright

Portions of this thesis appeared in:

O'Neil, J., Billa, M., Oikemus, S., and Kelliher, M. (2001). The DNA binding activity of TAL-1 is not required to induce leukemia/lymphoma in mice. *Oncogene* *20*, 3897-3905.

O'Neil, J., Ventura, J. J., Cusson, N., and Kelliher, M. (2003). NF- κ B activation in premalignant mouse tal-1/scl thymocytes and tumors. *Blood* *102*, 2593-2596.

MECHANISMS OF TAL1 INDUCED LEUKEMIA IN MICE

A Dissertation Presented

By

Jennifer Elinor O'Neil

Approved as to style and content by:

Stephen Jones, Ph.D., Chair of Committee

Timothy Kowalik, Ph.D., Member of Committee

Joonsoo Kang, Ph.D., Member of Committee

Lucio Castilla, Ph.D., Member of Committee

Richard Van Etten, M.D., Ph.D., Member of Committee

Michelle Kelliher, Ph.D., Dissertation Mentor

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program

January 22, 2004

Acknowledgements

I would like to thank my mentor Michelle Kelliher for her guidance and support throughout my graduate career. I also appreciate the advice and criticism of my committee members, Stephen Jones, Timothy Kowalik, Joonsoo Kang, Lucio Castilla and Richard Van Etten. I am grateful to the members of the Kelliher lab for their contributions to this work especially Marilisa Billa and Sarah Oikemus who helped with the generation and analysis of the DNA binding mutant *Tal1* transgenic mice. Sarah contributed Figure 3A and B. Misa is responsible for Figure 3C, Figure 6 and Table 2 and she contributed to Figure 4. Nicole Cusson performed the clonality analysis of the *Tal1/E2A* and *Tal1/HEB* mice (Figure 12A). Nicole Cusson and Leslie Cunningham helped to maintain the mouse colonies used in this work. I would like to thank Lynne Apone for technical advice on chromatin immunoprecipitation assays. I would also like thank Stephen Baker for the statistical analysis of the survival data and the UMass transgenic core for the generation of mice used in this work.

We appreciate the generosity of Dr. Cornelius Murre of the University of California at San Diego who provided us with the *E2A* knockout mice and of Dr. Yuan Zhuang from Duke University who provided us with the *HEB* deficient mice. I also want to thank Rachel Gerstein for help in analyzing some of the FACS data. We thank Dr. Sankar Ghosh of Yale University for providing the plasmid encoding the mutant $\text{IkB}\alpha$ superrepressor and Drs. Boris Reizis and Philip Leder for the *pre-T α -GFP* mice.

I am extremely grateful to my parents for their encouragement and support of my education. I could not have done this without them. I also owe a huge thank you to my husband Eric who has been my biggest cheerleader and also at times my biggest critic. I am grateful for his advice on experiments and critical reading of this thesis.

ABSTRACT

Activation of the basic helix-loop-helix (bHLH) gene *TAL1* is the most common genetic event seen in both childhood and adult T cell acute lymphoblastic leukemia (T-ALL). Despite recent success in treating T-ALL patients, TAL1 patients do not respond well to current therapies. In hopes of leading the way to better therapies for these patients, we have sought to determine the mechanism(s) of Tal1 induced leukemia in mice. By generating a DNA-binding mutant *Tal1* transgenic mouse we have determined that the DNA binding activity of Tal1 is not required to induce leukemia. We have also shown that Tal1 expression in the thymus affects thymocyte development and survival. We demonstrate that Tal1 heterodimerizes with the class I bHLH proteins E47 and HEB in our mouse models of TAL1 induced leukemia. Severe thymocyte differentiation arrest and disease acceleration in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice provides genetic evidence that Tal1 causes leukemia by inhibiting the function of the transcriptional activators E47 and HEB which have been previously shown to be important in T cell development. In pre-leukemic *Tal1* thymocytes, we find the co-repressor mSin3A/HDAC1 bound to the CD4 enhancer, whereas an E47/HEB/p300 complex is detected in wild type thymocytes. Furthermore, mouse *Tal1* tumors are sensitive to pharmacologic inhibition of HDAC and undergo apoptosis. These data demonstrate that Tal1 induces T cell leukemia by repressing the transcriptional activity of E47/HEB and suggests that HDAC inhibitors may prove efficacious in T-ALL patients that express TAL1.

Table of Contents

Acknowledgements		iv
Abstract		vi
List of Tables		ix
List of Figures		x
Chapter I	Introduction	1
Chapter II	The DNA Binding Activity of TAL1 is Not Required to Induce Leukemia/Lymphoma in Mice	37
	Introduction	38
	Results	41
	Discussion	52
	Materials and Methods	54
	Figures and Tables	58
Chapter III	Tal1 Transforms by Repressing E47/HEB Transcriptional Activity	71
	Introduction	72
	Results	75
	Discussion	85
	Materials and Methods	88
	Figures and Tables	91
Chapter IV	NF-κB Activation in Premalignant Mouse Tal1 Thymocytes and Tumors	105
	Introduction	106

	Results	108
	Discussion	113
	Materials and Methods	115
	Figures and Tables	117
Chapter V	Discussion	127
References		138
Appendix	Microarray Results	169

List of Tables

1	Properties of transcription factors discussed in this thesis	33
2	Immunophenotypes of <i>Tal1</i> <i>R188G</i> ; <i>R189G</i> and <i>Tal1</i> <i>R188G</i> ; <i>R189G</i> / <i>CKIIα</i> tumors	63
3	Expression of <i>Tal1</i> and <i>Tal1</i> <i>R188G</i> ; <i>R189G</i> perturbs thymocyte development	64
4	Immunophenotypes of <i>Tal1</i> / <i>E2A</i> ^{+/-} and <i>Tal1</i> / <i>HEB</i> ^{+/-} tumors	95
5	Genes activated or repressed by <i>Tal1</i> expression in the thymus	96
6	<i>Tal1</i> thymocytes undergo apoptosis <i>in vitro</i>	117
7	NF- κ B activation does not contribute to <i>Tal1</i> tumor growth	118

List of Figures

1	Gene targeting experiments in mice have established that <i>Tal1</i> is essential for hematopoiesis and angiogenesis	31
2	Models of transformation by the <i>Tal1</i> oncogene	32
3	Structure and expression of the <i>Tal1 R188G;R189G</i> transgene	53
4	Kaplan-Meier survival plot of <i>Tal1</i> , <i>Tal1 R188G;R189G</i> , <i>Tal1/CKIIα</i> and <i>Tal1 R188G;R189G/CKIIα</i> transgenic mice	55
5	Histology of the lymphoproliferative disease in <i>Tal1 R188G;R189G</i> transgenic mice	56
6	<i>Tal1 R188G;R189G</i> and <i>Tal1 R188G;R189G/CKIIα</i> tumors are clonal or oligoclonal	57
7	<i>Tal1</i> expression perturbs thymocyte development	58
8	Wildtype <i>Tal1</i> and mutant <i>Tal1</i> form stable heterodimers with E2A proteins	60
9	Mutant <i>Tal1/E2A</i> complexes fail to bind DNA	62
10	Thymocyte developmental perturbation is more severe in <i>Tal1/E2A\pm</i> and <i>Tal1/HEB\pm</i> mice than in <i>Tal1</i> transgenic mice	84
11	Accelerated leukemogenesis when <i>Tal1</i> is expressed in an E2A or HEB heterozygous background	86
12	<i>Tal1/E2A\pm</i> and <i>Tal1/HEB\pm</i> tumors are clonal or oligoclonal and do not exhibit loss of heterozygosity	87
13	Gene repression in <i>Tal1</i> transgenic thymocytes	89
14	<i>Rag2</i> and <i>pre-Tα</i> expression is reduced in <i>Tal1/E2A\pm</i> mice	91
15	<i>Tal1</i> recruits the corepressor mSin3A to the CD4 locus and <i>Tal1</i> tumors are sensitive to the HDAC inhibitor, TSA	93
16	<i>Tal1</i> and Mut <i>Tal1</i> perturb thymocyte development	109

17	NF- κ B activation in premalignant <i>Tal1</i> transgenic and <i>E2A</i> null thymocytes	111
18	Constitutive NF- κ B activation and Bcl-2 expression in <i>Tal1</i> and <i>Mut Tal1</i> tumors	113
19	Increased IKK activation in premalignant <i>Tal1</i> thymocytes and tumors	115
20	Mutant I κ B α expression inhibits TNF α -induced NF- κ B activation in <i>Tal1</i> tumors	116
21	Model of <i>Tal1</i> induced leukemogenesis	129

CHAPTER I
INTRODUCTION

100-100-100-100

T cell acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common form of cancer occurring in children accounting for more than 50% of the hematopoietic malignancies observed in the pediatric population. ALL is rare in adults accounting for only 2-3% of hematopoietic malignancies(Downing and Shannon, 2002). T cell acute lymphoblastic leukemia (T-ALL) is a malignant disease of thymocytes that accounts for 10-15% of childhood and 25% of adult ALL cases and is often accompanied by high circulating blast cell counts and central nervous system infiltration (Ferrando et al., 2002). Chromosomal translocations that place transcription factor genes such as *HOX11*, *TALI*, *LYL1*, *LMO1*, or *LMO2* under the control of the T cell receptor loci are observed in some T-ALL patients. In these patients misexpression of the translocated gene is detected in the thymus. These chromosomal abnormalities are quite rare in T-ALL patients, however a recent study using quantitative RT-PCR has shown that one or more of the above mentioned genes is misexpressed in over 90% of both pediatric and adult T-ALL cases(Ferrando and Look, 2003).

TALI can be ectopically expressed in the thymus by the t(1;14) chromosomal translocation, which puts *TALI* under the control of the regulatory elements of the T-cell receptor (TCR) δ locus. Other translocations which juxtapose *TALI* with the TCR β locus have also been described(Begley and Green, 1999). *TALI* can also be activated in T cells by a large interstitial deletion within chromosome 1 that places *TALI* under the control of the promoter of the *SIL* gene which is expressed in T cells(Aplan et al., 1991; Begley and Green, 1999). However, in the majority of T-ALL cases in which *TALI* is misexpressed

in the thymus, no chromosomal aberrations are detected. In these cases it has been hypothesized that mutations in regulatory elements of *TAL1* lead to its aberrant expression (Bash et al., 1995). Recent studies have demonstrated that *TAL1* as well as *HOX11* and *LMO2* are biallelically expressed in some T-ALL cases, suggesting that ectopic expression of these genes in leukemia may result from disruption of the pathways that normally down-regulate expression of *TAL1*, *HOX11* and *LMO2* in mature T cells (Ferrando et al., 2003). In children and adults, *TAL1* misexpression occurs in 60 and 45% of T-ALL cases respectively (Ferrando and Look, 2003).

Great success in the treatment of pediatric T-ALL has been achieved in recent years by intensifying chemotherapy regimens leading to a five-year event free survival rate of 80% (Schrapppe et al., 2000; Silverman et al., 2001). However, a recent study has demonstrated that *TAL1* patients have a less favorable prognosis than T-ALL patients with activation of other oncogenes. Patients with the *MLL-ENL* translocation have a 100% probability of five year survival compared to 92% of patients that have activation of *HOX11* (Ferrando et al., 2002). In contrast, *TAL1* patients have a five-year survival rate of only 43% (Ferrando et al., 2002). Therefore, better treatments for *TAL1* induced leukemia are needed. An understanding of the mechanism(s) by which *TAL1* induces disease will aid in the development of more specific treatments.

T cell development

As thymocytes mature into T cells they go through several stages that are defined by the surface expression of markers and the status of T cell receptor genes. Progenitor

cells that enter the thymus from the bone marrow lack expression of the T cell receptor associated CD3, and the coreceptors CD4 and CD8. These double negative thymocytes can differentiate into $\alpha\beta$ or $\gamma\delta$ thymocytes and can be divided into four stages based on their expression of the adhesion molecule CD44 and the α chain of the interleukin 2 receptor, CD25. The most immature cells express CD44 but not CD25; the next stage, the DN2 stage is defined by the expression of both CD44 and CD25. The DN3 thymocytes exhibit surface expression CD25 but not CD44. During this stage, the T cell receptor β rearranges and interacts with pre-T cell- α . Signaling from the pre-TCR complex leads to cell proliferation and eventually expression of the CD4 and CD8. These CD4+CD8+ cells are referred to as double positive thymocytes. T cell receptor α chain rearrangement occurs in the double positive thymocytes. Positive and negative selection occurs during the double positive stage leading to differentiation of thymocytes that recognize self MHC and apoptosis of thymocytes that recognize self-MHC too well. Positively selected thymocytes down regulate the expression of either CD4 or CD8 to yield CD4 or CD8 single positive thymocytes (Janeway et al., 1999). Thymocytes are induced to proliferate by signals emanating from the pre-TCR and TCR as well as by cytokine signaling. In particular, IL-2 and IL-7 signaling has been shown to be essential for T cell proliferation and development (Janeway et al., 1999; Kim et al., 1998; Peschon et al., 1994).

E proteins

E proteins are widely expressed class I bHLH proteins encoded by the genes *E2A*, *E2-2* and *HEB*. The *E2A* gene encodes for two proteins, E12 and E47, resulting from differential splicing of the exons coding for the bHLH domain(Sun and Baltimore, 1991). *HEB* and *E2-2* are encoded by distinct genes and are 60% identical to E47 and E12 in the bHLH domain(Henthorn et al., 1990; Hu et al., 1992). In addition to the bHLH domain which consists of two amphipathic α -helices separated by a loop structure(Murre et al., 1989b), E proteins contain two highly conserved transcriptional transactivation domains termed AD1 and loop-helix (LH) domains(Aronheim et al., 1993; Quong et al., 1993). Point mutations in either of these two transactivation domains severely affects the transactivation capacity of the proteins(Massari et al., 1996; Quong et al., 1993). The crystal structure of the E47 homodimer revealed the formation of a four-helix bundle which positions the basic region of E47 to contact the major groove of DNA and bind the E box element, CANNTG with each monomer interacting with a three base half-site(Ellenberger et al., 1994).

E proteins can form either homodimers or heterodimers with class II bHLH proteins. In pancreatic cells, E47 interacts with the class II bHLH protein, BETA2 to regulate insulin gene transcription(Dumonteil et al., 1998; Naya et al., 1995), and mutations in BETA2 are associated with type 2 diabetes(Malecki et al., 1999). In muscle cells, E proteins heterodimerize with MyoD and regulate muscle cell differentiation(Lassar et al., 1991). Other bHLH protein containing complexes are

essential for heart and brain development(Ben-Arie et al., 1997; Firulli et al., 1998; Guillemot et al., 1993; Riley et al., 1998; Srivastava et al., 1997).

E proteins are also essential for lymphoid development. When E47 is overexpressed in a pre-T cell line, many of the early events in B cell development occur such as induction of a germ-line heavy-chain gene transcript and immunoglobulin D-to-J rearrangement(Schlissel et al., 1991). *E2A* null mice die within the first few days after birth and lack pre-B cells and mature B cells, and have reduced numbers of B cell progenitors(Bain et al., 1994). In contrast, *HEB* and *E2-2* null mice die within the first two weeks of life, do produce mature B cells but have reduced numbers of pro-B cells(Zhuang et al., 1996a). This difference in phenotype most likely reflects the observation that the major E protein complex in B cells consists of a E47 homodimers(Shen and Kadesch, 1995). *E2A* protein target genes in B cells include RAG1, RAG2, $\lambda 5$, EBF and TdT(Bain et al., 1994; Choi et al., 1996; Greenbaum and Zhuang, 2002; Hsu et al., 2003).

The tight regulation of E47 expression throughout thymocyte development suggests that E proteins play an important role in thymocyte differentiation. DN1 thymocytes exhibit a low level of E47 expression which increases in the DN2 stage. E47 expression levels decrease again in response to pre-TCR signaling at the DN3 stage and decrease further as thymocytes differentiate from the CD4, CD8 double positive to the CD8 or CD4 single positive stage(Engel et al., 2001). Gene targeting studies have demonstrated that E proteins are essential for proper thymocyte maturation and selection. *E2A* null mice have 5-fold fewer total thymocytes and decreased numbers of CD4,CD8

double positive thymocytes compared to wildtype mice (Bain et al., 1997). *E2A* null mice also exhibit increases in both CD8-single positive and CD4-single positive cells, however the increase in CD8-single positive cells is greater leading to a CD4/CD8 thymocyte ratio of 1.8 compared to 4.4 in wildtype mice. Thymocytes from *E2A* deficient mice are partially arrested at the CD44+, CD25-, or DN1 stage demonstrating that loss of *E2A* leads to a partial block at the earliest stage of T cell development (Bain et al., 1997). Studies of *E2A* null mice have also demonstrated a requirement of E proteins for the regulation of V(D)J recombination in $\gamma\delta$ T cells (Bain et al., 1999).

In contrast to B cells in which E47 homodimers are the predominant E protein complexes, in T cells E47/HEB heterodimers predominate (Sawada and Littman, 1993). *HEB* null mice also have 5-fold fewer thymocytes than wildtype mice and decreased numbers of double positive cells (Barndt et al., 1999). In addition, *HEB* deficient mice have increased CD8-single positive cells most of which appear to be immature single positive cells as assessed by HSA staining (Barndt et al., 1999). A partial arrest of double negative thymocytes at the CD44-, CD25+ or DN3 stage is also observed in *HEB* deficient mice (Barndt et al., 1999). E47/HEB heterodimers have been implicated in the regulation of CD4, CD5, Rag1, Rag2 and pre-T α expression (Bain et al., 1994; Herblot et al., 2000; Sawada and Littman, 1993; Schlissel, 1991; Zhuang et al., 1996a). Decreased expression of these genes likely contributes to the perturbation of thymocyte development observed in *E2A* deficient and *HEB* deficient mice.

It has been suggested that E proteins regulate pre-TCR signaling since *E47* deficiency allows the differentiation of double negative thymocytes into double positive

thymocytes in *Rag1*^{-/-} mice(Engel et al., 2001). Further support for this hypothesis comes from a recent study demonstrating that lack of E47 promotes the development of thymocytes in mice lacking the pre-T cell receptor signaling proteins Lck, Fyn and LAT(Engel and Murre, 2003). E47 does not, however, appear to play a role in IL-7 receptor signaling despite similarities in the phenotypes of *E47* deficient and IL-7 receptor deficient mice(Kee et al., 2002).

E proteins and leukemia

E proteins have been shown to inhibit cell proliferation and induce apoptosis. E47 expression reduces the proliferation of NIH3T3 cells(Peverali et al., 1994) and overexpression of E47 in Jurkat cells induces growth inhibition and apoptosis(Park et al., 1999). In addition, a higher percentage of pro-B cells from *E2A*^{+/-} are cycling than in wildtype mice(Herblot et al., 2002). These effects on the cell cycle may be mediated via the cell cycle inhibitors p21 and p16/INK4a since E proteins has been implicated in regulating the expression of these genes(Prabhu et al., 1997) (Alani et al., 2001). More recent data has demonstrated that E47 inhibits cell cycle progression in DN3 thymocytes(Engel and Murre, 2003). Although most *E2A* null mice die shortly after birth, of those that survive about fifty percent develop T cell leukemia within ten months of age demonstrating that *E2A* can act as a tumor suppressor(Bain et al., 1997; Yan et al., 1997). The postnatal lethality of the *HEB* null mice is partially rescued by an *Id3* deficiency, and *HEB* null/*Id3* null mice develop T cell leukemia suggesting that *HEB* can also act as a tumor suppressor(Barndt and Zhuang, 1999).

Disruption of the *E2A* gene has also been observed in human patients. The t(17;19)(q22;p13) chromosomal translocation occurring in a subset of pro-B cell ALL results in the formation of the *E2A-HLF* fusion gene in which the transactivation domains of *E2A* are linked to the DNA binding and protein dimerization domains of hepatic leukemia factor (HLF)(Inaba et al., 1992). Twenty-five percent of pre-B acute lymphoblastic leukemia patients display the t(1;19)(q23;p13) translocation which fuses *E2A* to the homeobox protein *Pbx*. As in the *E2A-HLF* translocation, the activation domains of *E2A* are fused to the DNA binding domain of *Pbx*(Crist et al., 1990; Kamps et al., 1990). Expression of either *E2A-HLF* or *E2A-Pbx* in NIH 3T3 cells induces anchorage independent growth(Kamps et al., 1991; Yoshihara et al., 1995). In addition, expression of either fusion protein in the lymphoid lineage in the mouse results in T cell apoptosis, B cell developmental arrest and the development of T cell leukemia(Dedera et al., 1993; Honda et al., 1999). Retroviral expression of *E2A-Pbx* in the bone marrow of lethally irradiated mice led to the development of acute myeloid leukemia(Kamps and Baltimore, 1993). Taken together, these studies demonstrate the oncogenicity of the *E2A* fusion genes. Similarities between the *E2A-HLF* and *E2A-Pbx* transgenic mice and the *E2A* null mice such as decreased numbers of T cells, B cell maturation arrest and development of T-ALL suggest that loss of one allele of *E2A* may contribute to the oncogenicity of *E2A* fusion genes.

In a rare chromosomal translocation observed in T-ALL patients, *Notch1* is truncated resulting in expression of the activated form of Notch1(Ellisen et al., 1991). Similarly, mice transplanted with bone marrow cells expressing the intracellular domain

of Notch1 develop T cell lymphomas(Pear et al., 1996). Notch1 has been shown to inhibit E2A transcriptional activity and enhance E2A protein degradation suggesting that inhibition of E protein activity may also be involved in intracellular notch induced leukemia(Nie et al., 2003; Ordentlich et al., 1998). Overexpression of Notch3 has been detected in all T-ALL cases examined suggesting that Notch3 may be crucial to leukemic progression(Screpanti et al., 2003). In support of this hypothesis, *Notch3* transgenic mice develop T cell leukemia(Bellavia et al., 2000). Recent data demonstrates that Notch3 inhibits E2A activity by activating pre-TCR signaling which downregulates E2A DNA binding activity(Talora et al., 2003). Therefore, inhibition of E2A activity may be a common mechanism of T-ALL.

Id proteins

The Id family of helix-loop-helix class V proteins contains four members, Id1 to Id4(Zebedee and Hara, 2001). Id1 and Id3 are widely expressed in developing tissues, while Id2 and Id4 display a more restricted pattern of expression (Jen et al., 1997; Riechmann et al., 1994). Id proteins can interact with both class I and class II bHLH proteins but do not contain a basic domain necessary for DNA binding. As a result, Id proteins act as dominant negative inhibitors of bHLH proteins. Id proteins have been shown to inhibit the differentiation of a variety of cell types including B cells(Sun, 1994), muscle cells(Jen et al., 1992), myeloid(Kreider et al., 1992) and erythroid cells(Lister et al., 1995; Shoji et al., 1994). *Id3* deficient mice develop normally but have decreased numbers of CD4 single positive thymocytes and increased CD8 single positive

thymocytes(Rivera et al., 2000). *Id3* deficient mice also display defects in B cell proliferation and humoral immunity(Pan et al., 1999). *Id1* deficient mice are normal, however *Id1/Id3* double knockout mice die at E12.5 due to cranial hemorrhage(Lyden et al., 1999). *Id2* deficient mice also develop normally but lack lymph nodes and Peyer's patches and have reduced numbers of natural killer cells(Yokota et al., 1999).

Id3 most likely modulates T cell development and B cell function through inhibition of E protein activity. The decrease in CD4 single positive cells is likely due to inhibition of E47/HEB activation of the CD4 gene. Thymocyte development is normal in *E2A/Id3* double knockout mice, demonstrating a genetic interaction between the two proteins(Rivera et al., 2000). In addition, the observation that T cell receptor signaling induces the expression of *Id3* and inhibits E47 DNA-binding activity suggests that *Id3* modulates thymocyte differentiation and proliferation through inhibition of E2A (Engel et al., 2001).

Id proteins and cancer

Id proteins also appear to play a role in regulating the cell cycle. Expression levels of *Id* genes are high in proliferating cells and low in terminally differentiated cells(Norton, 2000; Norton et al., 1998). *Id2* has been shown to bind to the tumor suppressor retinoblastoma protein and inhibit its function(Lasorella et al., 1996). In addition, in transfection studies, *Id1* has been shown to inhibit E2A-mediated induction of the cell cycle inhibitors p21 and p16/Ink4a(Alani et al., 2001; Prabhu et al., 1997). *Id2* can induce anchorage-independent growth in NIH3T3 cells(Lasorella et al., 2002).

Furthermore, overexpression of Id1 or Id2 in the thymus of mice causes T cell lymphoma (Kim et al., 1999; Morrow et al., 1999). In addition, transgenic expression of Id1 in B cells or intestinal epithelia of mice results in the development of B cell lymphomas and adenomas respectively (Sun, 1994; Wice and Gordon, 1998). Id protein overexpression has also been observed in a variety of human tumors including breast cancer, melanoma and neuroblastoma (Lasorella et al., 2002; Lin et al., 2000; Polsky et al., 2001).

Id proteins have also been shown to play an essential role in angiogenesis. The blood vessels in the brains of embryonic *Id1*^{-/-} *Id3*^{-/-} are malformed and express lower levels of *Flk1* (VEGF receptor 2) (Lyden et al., 1999). Id proteins also play an important role in tumor angiogenesis. When *Id1*^{+/-} *Id3*^{-/-} mice were injected with B6RV2 lymphoma cells or B-CA breast cancer cells, the cancer cells initially grew but then regressed and the animals remained healthy. In contrast, wildtype mice quickly succumb when injected with the same tumor cells (Lyden et al., 1999). However, Lewis lung cancer cells grew equally well in wildtype and *Id1*^{+/-} *Id3*^{-/-} mice suggesting that not all tumors require Id proteins for invasion (Lyden et al., 1999). Angiogenesis of skin tumors also does not appear to depend on Id1 expression (Sikder et al., 2003). High levels of Id1 are expressed in invasive breast cancer cell lines and overexpression of Id1 in a non-invasive cell line rendered it invasive (Desprez et al., 1998; Lin et al., 2000). Moreover, reducing the expression of *Id1* by infection with *Id1* antisense cDNA decreased the invasiveness of breast cancer cells both *in vitro* and in nude mice (Fong et al., 2003). In contrast, low levels of Id2 are detected in aggressive breast cancer cell lines and

overexpression of Id2 in these cells can reduce their invasiveness (Itahana et al., 2003). Id proteins are expressed at low levels in normal adult tissues (Lyden et al., 1999), therefore they may represent good targets for cancer therapy. However since Id2 appears to inhibit breast cancer cell invasion, drugs for breast cancer may be more effective if they specifically inhibit Id1.

LIM only domain proteins

Lmo2, formerly known as *Ttg2* or *Rbtn2*, is a LIM only domain protein with two cysteine-rich, zinc-coordinating, protein-interaction LIM domains as well as an amino terminal domain that appears to have transcriptional transactivation activity (Visvader et al., 1997). *Lmo2* is expressed in all tissues except mature T cells (Feroni et al., 1992; Neale et al., 1995) although it is highly expressed in DN1 and DN2 thymocytes and expressed at low levels in DN3 and DN4 thymocytes (Ferrando et al., 2003; Herblot et al., 2000). However, recent conditional disruption of *Lmo2* demonstrates that *Lmo2* is not essential for the development of T cells or B cells (McCormack et al., 2003). *Lmo1* has a more restricted pattern of expression with high levels in the brain and low levels in lymphoid tissues (Feroni et al., 1992). *Lmo2* deficient mice have a similar phenotype as *Tal1* knockout mice in that they lack blood cells and are embryonic lethal at embryonic day 10.5 (Warren et al., 1994). By following the fate of *Lmo2*-null ES cells in chimeric mice, it was determined that *Lmo2*, like *Tal1*, is not required for vasculogenesis but is required for angiogenesis (Yamada et al., 2000). *Lmo2* binds to *Tal1*, *Gata1* and *Ldb1* in

erythroid cells and is thought to act as a bridging molecule in the complex(Osada et al., 1997; Wadman et al., 1994a).

Lmo1 and Lmo2 in leukemia

Like *TAL1*, both *LMO1* and *LMO2* can be involved in chromosomal translocations in human T-ALL patients. These translocations place the genes under the control of the T cell receptor regulatory elements and result in misexpression in the thymus. *LMO1* and *LMO2* are involved in the t(11;14)(p15;q11) and t(11;14)(p13;q11) translocations respectively(Rabbitts et al., 1999). Activation of *LMO2* is also implicated in gene therapy induced T-ALL that occurred in two patients in a recent gene therapy clinical trial for X-linked severe combined immunodeficiency(Hacein-Bey-Abina et al., 2003a). In both patients, the retroviral vector inserted near the *LMO2* gene resulting in overexpression of *LMO2*(Hacein-Bey-Abina et al., 2003b).

Transgenic expression of *Lmo2* in the thymus leads to an increase in CD4-, CD8- double negative T cells and development of T cell leukemia with a mean latency of 9-10 months(Fisch et al., 1992; Larson et al., 1994; Larson et al., 1995). Fifty percent of *lck-Lmo1* transgenic mice also develop T cell leukemia with a median latency of 10.5 months(McGuire et al., 1992). *Tal1* can collaborate with both *Lmo1* and *Lmo2* to cause leukemia in mice. Ninety-five percent of *Tal1/Lmo1* bitransgenic mice develop leukemia within 6 months(Aplan et al., 1997) and *Tal1/Lmo2* bitransgenic mice develop leukemia with a mean latency of 7 months(Larson et al., 1996). The thymocyte differentiation arrest observed in the bitransgenic mice is also more severe than in the single *Lmo1* and

Lmo2 transgenic mice, suggesting that differentiation arrest is important in the development of disease (Chervinsky et al., 1999; Larson et al., 1996). In addition, co-expression of *Lmo2* and *Tal1* is often observed in thymic lymphomas from *Lmo2* transgenic mice (Grutz et al., 1998) and from mismatch repair gene null mice (Lowsky et al., 1997). Cooperation of these two oncogenes in human T-ALL is supported by RT-PCR analysis of human T-ALL cell lines and patients samples demonstrating that *TAL1* is usually expressed along with either *LMO1* or *LMO2* (Ferrando and Look, 2003; Ono et al., 1997). Moreover, one of the gene therapy patients with LMO2-induced leukemia had a *SIL-TAL1* chromosomal deletion (Hacein-Bey-Abina et al., 2003b).

Similarities in the phenotypes of *Tal1/Lmo1* bitransgenic mice and *E2A* null mice suggest that *Lmo1* acts by inhibiting E proteins. Like *E2A* null mice, *Tal1/Lmo1* bitransgenic mice display decreases in total thymocyte numbers, decreases in CD4+, CD8+ cells and increased numbers of DN1 thymocyte precursors (Bain et al., 1997; Chervinsky et al., 1999). In transfection studies, expression of both *Tal1* and *Lmo1* inhibited luciferase expression driven by an E box promoter better than expression of *Tal1* alone (Chervinsky et al., 1999). However, CASTing experiments have demonstrated that *Lmo2* expressed in T cells forms part of a complex that binds a two E-box site in DNA (Grutz et al., 1998). This site differs from the E box-GATA site that *Lmo2*-containing complexes have been shown to bind in erythroid cells by similar methods (Wadman et al., 1997), suggesting that *Lmo2* may regulate the expression of novel target genes when expressed in T cells. The DNA-binding, *Lmo2*-containing complex in T cells also contains *E2A*, *Tal1* and *Ldb1* (Grutz et al., 1998). Therefore,

Lmo1/Lmo2 may cause leukemia either by inhibiting E proteins or other proteins to which it binds or by participating in a complex that activates novel gene targets. It is also possible that both mechanisms contribute to Lmo1/Lmo2 induced leukemia.

TAL1

TAL1 (also termed SCL or TCL5) was first discovered due to its involvement in a translocation in a human leukemic stem cell line (Begley et al., 1989). *Tal1* encodes a 42kD basic helix-loop-helix (bHLH) protein of 331 amino acids as well as a smaller 22kD form generated by initiation at an internal methionine (Cheng, 1992). Both *Tal1* gene products contain a basic DNA binding domain as well as a helix-loop-helix dimerization domain. The shorter form of *Tal1* lacks the transcriptional transactivation domain (Wadman et al., 1994b). *Tal1* protein is detected in the embryonic and extraembryonic mesoderm at embryonic day 7.5 (E7.5), in blood islands of the yolk sac at E8.5, and in adult erythroid, myeloid, megakaryocyte and mast cells (Kallianpur et al., 1994). *Tal1* is also expressed in endothelial cells and cells of the developing and adult central nervous system (Drake et al., 1997; Green et al., 1992; Kallianpur et al., 1994).

Tal1 in neuronal development

In situ hybridization experiments on embryonic day 14.5 embryos demonstrated the expression of *Tal1* in the developing brain, most abundantly in the dorsal part of the metencephalon and the roof of the mesencephalon (Green et al., 1992). In addition, northern analysis of adult mouse brain revealed expression of *Tal1* in mature

neurons(Green et al., 1992). In order to further define the expression pattern of *Tal1* in the central nervous system, *Tal1-LacZ* mice were created in which *LacZ* expression is controlled by the *Tal1* promoter(Elefanty et al., 1998). At embryonic day 10.5, *Tal1* was expressed in the presumptive spinal cord and at E12.5 *Tal1* expression was detected in the midbrain. *Tal1* expression was also seen in the ventral surface of the brainstem in both embryonic and adult mice(Elefanty et al., 1999). A more recent and extensive study of the expression pattern of *Tal1* in the brain detected *Tal1* expression in the diencephalic, mesencephalic and metencephalic adult neurons(van Eekelen et al., 2003). *Tal1* is also expressed in post-mitotic neurons but not in the brain regions that give rise to the neural stem cells. Therefore, a role for *Tal1* in late neuronal differentiation and in maintenance of mature neurons has been suggested(van Eekelen et al., 2003). The early embryonic lethality of *Tal1* deficient mice has precluded the analysis of *Tal1* in later stages of development. Conditional disruption of *Tal1* in the brain may be useful in determining the role of *Tal1* in late neuronal development.

The expression patterns of other bHLH proteins such as *Tal2* and *Mash1* are similar to that of *Tal1*. Therefore, these proteins could compensate loss of *Tal1* expression in the brain. Gene targeting studies have demonstrated essential functions for both *Mash1* and *Tal2* in neuronal development. *Mash1* deficient mice lack olfactory receptor neurons and chromaffin cells(Cau et al., 2002). *Tal2* deficient mice develop normally and have no defects in hematopoiesis, but do not survive past 32 days and display defects in midbrain and hydrocephalus development demonstrating that *Tal2*

plays an important role in the development of the mature nervous system(Bucher et al., 2000).

Tal1 in hematopoietic and vascular cell development

The expression pattern of Tal1 suggests that it may be important in blood cell development. In fact, overexpression of *Tal1* in mouse erythroleukemia cells induced erythroid differentiation while expression of antisense *Tal1* inhibited differentiation(Aplan et al., 1992). However, expression of antisense *Tal1* in the human erythroleukemic cell line K562 induced differentiation(Green et al., 1991). Gene targeting studies in mice have demonstrated that Tal1 is essential for embryonic hematopoiesis(Robb et al., 1995a; Shivdasani et al., 1995). *Tal1* deficient mice die between embryonic day 8.5 and 10.5 and are completely bloodless(Robb et al., 1995a; Shivdasani et al., 1995). By studying the capacity of *Tal1* *-/-* embryonic stem cells to differentiate *in vitro* or in chimeric mice, it was established that Tal1 is required for the development of all hematopoietic lineages(Porcher et al., 1996; Robb et al., 1996). However, conditional disruption of *Tal1* in the adult mouse has revealed that *Tal1* expression is essential for the production of hematopoietic stem cells (HSC) and for their differentiation into erythroid and megakaryocyte precursors but not for HSC engraftment, self-renewal or differentiation into myeloid or lymphoid cells(Hall et al., 2003; Mikkola, 2003) (Figure 1).

It has recently been shown that *Tal1* is expressed in the DN1, DN2 and DN3 mouse thymocyte precursors. *Tal1* is not, however, normally expressed in mature T-

cells(Ferrando et al., 2003; Tremblay et al., 2003). The role of *Tal1* in thymocyte precursors is not clear, however when *Tal1* is disrupted in the adult mouse a skewing towards the T cell lineage over the B cell lineage is observed(Mikkola, 2003), suggesting that loss of *Tal1* promotes thymocyte differentiation.

The expression of *Tal1* in both hematopoietic and endothelial cells suggests that *Tal1* may play a role in the hemangioblast, a precursor of both the hematopoietic and endothelial lineages. The existence of the hemangioblast is supported by the phenotypes of the vascular endothelial growth factor (VEGF) receptor-2, *Flk1*, deficient mice and *cloche* mutant zebrafish which have defects in both blood and endothelial cell development(Shalaby et al., 1995; Stainier et al., 1995). In addition, the blast colony forming cells (BL-CFCs) derived from embryonic stem cells can differentiate into both blood and endothelial cells *in vitro*(Choi et al., 1998; Nishikawa et al., 1998). In zebrafish, primitive erythroid cells and endothelial cells originate from the posterior lateral mesoderm (PLM) which gives rise to primitive erythroid cells and endothelial cells of the major trunk vessels, and the anterior lateral mesoderm (ALM) from which endothelial cells and myeloid cells originate(Hsu et al., 2001; Roman and Weinstein, 2000; Zhong et al., 2001). *Tal1* is expressed in both the PLM and ALM supporting the idea that *Tal1* expression is important for the development of the hemangioblast(Gering et al., 1998). Overexpression of *Tal1* in the zebrafish embryo by mRNA microinjection leads to an overproduction of red blood cells, endothelial cells and *Tal1*⁺/*Flk*⁺ cells that appear to represent the hemangioblast cells(Gering et al., 1998). Moreover, expression of *Tal1* in *cloche* mutant zebrafish rescues both the hematopoietic and vascular

developmental defects(Liao et al., 1998). More recent experiments in zebrafish embryos demonstrate that ectopic expression of *Tal1* can convert mesodermal cells into hemangioblasts at the expense of somatic paraxial mesoderm. However, overexpression of both *Tal1* and *Lmo2* is necessary to convert non-axial mesodermal cells into hemangioblasts(Gering et al., 2003).

Despite the effects of *Tal1* overexpression on the development of the hemangioblast in zebrafish, endothelial cells in the yolk sac of *Tal1* deficient mice develop normally(Visvader et al., 1998). The hematopoietic defects in *Tal1* deficient mice were rescued by expressing *Tal1* in hematopoietic cells using the *GATA1* promoter(Visvader et al., 1998). Despite the production of blood cells in *Tal1*^{-/-}/*GATA1*^{-/-}/*Tal1* mice, they do not survive past E9.5 due to defects in angiogenesis(Visvader et al., 1998). In the yolk sac of wildtype embryos, an organized network of vitelline vessels is observed. In contrast, in *Tal1*^{-/-}/*GATA1*^{-/-}/*Tal1* mice, the vessels are smaller and less organized suggesting that *Tal1* is not necessary for the specification of vascular cells but is essential for angiogenesis(Visvader et al., 1998). The presence of endothelial cells in *Tal1*^{-/-}/*GATA1*^{-/-}/*Tal1* mice may reflect compensation by other bHLH proteins such as *Lyl1* that have similar expression patterns.

Tal1 protein interactions

Tal1, *Gata1* and *Lmo2* deficient mice have similar phenotypes suggesting that these proteins work together to specify embryonic hematopoiesis(Fujiwara et al., 1996; Shivdasani et al., 1995; Warren et al., 1994). *Gata1* deficient mice die between E10.5

and E11.5, and the embryos stain weakly with benzidine reagent, exhibit extreme pallor, and contain erythroid cells arrested at the proerythroblast-like stage(Fujiwara et al., 1996). The complete absence of erythroid cells in *Tal1* deficient mice(Shivdasani et al., 1995) demonstrates that *Tal1* plays an earlier role in blood cell development than *Gata1*. *Gata2* may replace *Gata1* in early erythroid cell development. High levels of *Gata2* expression are observed in early progenitors of hematopoietic cells(Leonard et al., 1993). Consistent with this early role in erythroid differentiation, *Gata2* deficient mice die between E10 and E11 of severe anemia(Tsai et al., 1994). *Lmo2* deficient mice die at E10.5 due to a complete absence in erythropoiesis, however, in contrast to *Tal1* deficient yolk sac cells which cannot differentiate into erythroid or myeloid cells *in vitro*, macrophages can develop from *Lmo2* deficient yolk sac cells(Shivdasani et al., 1995; Warren et al., 1994). These results demonstrate that although *Tal1*, *Lmo2* and *Gata1* play essential roles in blood cell development, *Tal1* alone is essential for a myelo-erythroid progenitor.

Tal1 is a class II bHLH protein due to its inability to bind DNA as a homodimer and due to its tissue-restricted expression pattern. Other class II bHLH proteins include *MyoD*, *myogenin*, *NeuroD/BETA2* and *Atonal*. *Tal1* forms heterodimers through their HLH domains with the class I bHLH E proteins, *E47*, *E12*, and *HEB* which are also capable of binding to DNA as homodimers(Hsu et al., 1991; Hsu et al., 1994b; Voronova and Lee, 1994). In erythroid cells, *Tal1* is part of a large transcriptional complex that includes E proteins, *Lmo2*, *Ldb1*, and *Gata1* proteins(Valge-Archer et al., 1994; Wadman et al., 1997). The helix-loop-helix domain of *Tal1* is also required for its interaction with

Lmo2(Wadman et al., 1994a). However since Lmo2 cannot bind DNA, it is thought to act as a bridging molecule in the complex(Wadman et al., 1997). Ldb1/NL1 is a widely expressed protein that interacts with Lmo2 as well as several other LIM domain containing proteins(Visvader et al., 1997). Ldb1 contains a nuclear localization domain, an amino-terminal homodimerization domain and a carboxy-terminal LIM interaction domain(Matthews and Visvader, 2003). The Tal-1/E2A/Gata1/Lmo2/Ldb1 complex binds to an E-box-GATA consensus sequence in erythroid cells, and expression of all five proteins in a reporter assay is necessary for full transcriptional transactivation(Wadman et al., 1997). Tal1 has also been shown to form a complex with E2A, Lmo2, Ldb1 and Rb in erythroblasts. This complex binds to the dual E-box sequence in the c-kit promoter and down-regulates c-kit expression(Vitelli et al., 2000).

A Tal1 mutant that is unable to bind DNA is able to rescue primitive erythropoiesis in *Tal1* null ES cells, however DNA binding is required for the full differentiation of erythroid and megakaryocyte cells(Porcher et al., 1999). These studies suggest that Tal1 may have both DNA binding dependent and DNA binding independent functions in hematopoiesis. The authors propose that Tal1 may function in hematopoiesis without binding DNA either by sequestering proteins or by being part of a large transcriptional complex in which the DNA binding activity of Tal1 is not required(Porcher et al., 1999). In addition, a complex containing Tal1, E47, Lmo2, Ldb1, Gata1/2 and Sp1 regulates c-kit expression in hematopoietic cells even if the DNA binding activity of Tal1 is abolished(Lecuyer et al., 2002). However, the DNA binding

activity of Tal1 is required to activate the expression of the red cell membrane skeleton component, protein 4.2(Xu et al., 2003).

Taken together, these studies suggest that Tal1 regulates genes important in erythroid and megakaryocyte differentiation. Tal1 also plays a critical role in embryonic hematopoietic stem cell development and in vasculogenesis. However, it remains unclear how Tal1 regulates these biological events. Tal1 may regulate the expression of genes required for the proliferation and self-renewal of hematopoietic stem cells; similar targets may contribute to the Tal1 induced transformation of thymocytes. In addition, the observation that the DNA binding activity of Tal1 is dispensable for primitive erythropoiesis raises the possibility that Tal1 does not act as a transcriptional transactivator in leukemia. The interaction partners of Tal1 in erythroid cells, namely E proteins and LIM only domain proteins, have also been implicated in leukemia suggesting that interactions between Tal1 and these proteins in thymocytes may contribute to leukemogenesis.

TAL1 and leukemia

Several groups have attempted to model Tal1 induced leukemia in the mouse with variable success. One group expressed *Tal1* in the mouse thymus using the *CD2* promoter(Robb et al., 1995b). Despite high expression of Tal1 at the both the RNA and protein level, these mice did not develop T cell leukemia. In addition, the *Tal1* transgene did not accelerate Moloney murine leukemia virus induced leukemia(Robb et al., 1995b). Another group expressed the human *TAL1* gene in the thymus of mice using the same

CD2 promoter(Larson et al., 1996). These *TAL1* transgenic mice also did not develop leukemia. However they were able to accelerate leukemia in *CD2-Lmo2* transgenic mice demonstrating that *TAL1* can act as an oncogene(Larson et al., 1996). In contrast, expression of either mouse or human *Tall* in the mouse thymus using the proximal *lck* promoter leads to the development of T cell acute lymphoblastic leukemia(Condorelli et al., 1996; Kelliher et al., 1996).

It is not clear why the *CD2-Tall* transgenic mice do not develop leukemia. Although, *Tall* is expressed at high levels in mature thymocytes in these mice, the expression of *Tall* in thymocyte precursors was not examined. Expression of *Tall* throughout thymocyte development may be necessary to cause leukemia in mice. In addition, one group reported that the shorter, pp22 form of *Tall* was not expressed in their mice(Robb et al., 1995b). It is possible that this form of *Tall* is required to cause leukemia. This hypothesis is supported by a study demonstrating that the pp22 form of *TAL1* collaborates with *LMO1* to cause T cell leukemia in mice(Aplan et al., 1997). In addition, the pp22 form is only species expressed in some human T-ALL patients, but it is not expressed in erythroleukemic cell lines, suggesting that expression of the shorter form is specific to T cells(Bernard et al., 1992; Cheng, 1992).

Twenty-eight percent of *lck-Tall* transgenic mice develop leukemia with a median latency of 350 days(Kelliher et al., 1996). The long latency of *Tall* induced leukemia in the mouse suggests that additional mutations are required for disease progression. Leukemia development is fully penetrant in *Casein kinase II/Tall* bitransgenic mice with a median survival time of 74 days(Kelliher et al., 1996).

Phosphorylation of E47 by casein kinase II (CKII) reduces E47 homodimer DNA binding and transcriptional activation (Johnson et al., 1996). Therefore, casein kinase II may accelerate Tal1 induced leukemia by inhibiting E protein function. Casein kinase II also phosphorylates Myc and regulates its stability (Channavajhala and Seldin, 2002). Moreover, the development of T cell lymphomas is accelerated in *c-Myc/CKII* bitransgenic mice (Seldin and Leder, 1995). So, the disease acceleration in *Tal1/CKII* bitransgenic mice may be a result of increased c-myc protein levels.

In T-ALL patients activation of *TAL1* is often accompanied by *LMO1* or *LMO2* activation, loss of the cell cycle inhibitors *p16/INK4A* and *p14/ARF*, and *Myc* overexpression (Ferrando and Look, 2003). Retroviral insertional mutagenesis studies using the Moloney murine leukemia virus in *Tal1* transgenic mice have been performed in our laboratory. We have identified *Notch1*, *Myc* and dominant negative *Ikaros* as collaborating oncogenes of *Tal1* (Leslie Cunningham, unpublished data). Future work will confirm that these oncogenes can collaborate with *Tal1* to induce leukemia in mice and will determine how they contribute to disease progression. Additional hits may inhibit apoptosis or promote cell cycle progression.

The observations that *E2A* deficient mice and *Id1* and *Id2* transgenic mice develop T cell leukemia, raises the possibility that Tal1 induces leukemia by acting like an Id protein. Further support for this hypothesis comes from the studies demonstrating that the DNA binding activity of Tal1 is not required for some of its functions (Lecuyer et al., 2002; Porcher et al., 1999; Ravet et al., 2004). Therefore, Tal1 and other bHLH proteins such as Tal2, BHLHB1 or Lyl1 that are activated in human T-ALL may act by

inhibiting the function of E proteins. All four proteins interact with E proteins *in vivo* (Hsu et al., 1994b; Miyamoto et al., 1996; Wang et al., 2000; Xia et al., 1994). However, it is also possible that these proteins regulate the expression of novel target genes in the thymus. Both Lyl1/E2A and Tal1/E2A heterodimers preferentially bind to similar DNA sequences that are distinct from the sequences that E2A homodimers bind (Hsu, 1994; Miyamoto et al., 1996), and Tal1/E2A heterodimers do exhibit transcriptional transactivation *in vitro* (Hsu et al., 1994c). In fact, a complex containing Tal1, E47, Lmo2, and Gata3 has been shown to induce the transcription of the retinaldehyde dehydrogenase 2 gene in T-ALL cell lines (Ono et al., 1998). However, Tal1/E2A heterodimers are weaker transcriptional transactivators than E2A homodimers but E2A homodimers are more sensitive to inhibition by Id proteins (Hsu et al., 1994c). Therefore, Tal1 may act to positively or negatively regulate transcription depending on the cellular context.

Tal1 may inhibit E proteins and down regulate the transcription of their target genes by two different mechanisms. Tal1 may act by the sequestration model (Figure 2) in which it interacts with E proteins and sequesters them away from the gene regulatory elements to which they normally bind. Alternatively, Tal1 may actively repress transcription by interacting with E proteins and gene regulatory elements. The transactivation domain of Tal1 may be weaker than that of E proteins or it may be incompatible with the transactivation domain of E proteins. It is also possible that Tal1 displaces a coactivator or recruits a corepressor to gene regulatory elements normally bound by E proteins resulting in gene repression. In fact, E proteins have been shown to

interact with histone acetyltransferases and the transcriptional coactivators p300, CBP, and PCAF in muscle and B cells (Bradney et al., 2002; Eckner, 1996). Tal1 has been shown to interact with the corepressor mSin3A in undifferentiated mouse erythroleukemic cells (Huang and Brandt, 2000). The observation that the C-terminus of TAL1 in addition to the bHLH domain is required for inhibition of E2A-mediated transcriptional transactivation supports the idea that TAL1 must interact with another protein in order to repress transcription (Hofmann and Cole, 1996).

The work presented in this thesis demonstrates that the DNA binding activity of Tal1 is not required to cause leukemia in mice suggesting that Tal1 does not cause leukemia by transactivating the expression of novel target genes. To provide genetic evidence that Tal1 causes disease by interfering with the class I bHLH E proteins, we mated our *Tal1* transgenic mice to *E2A* or *HEB* heterozygous mice. We observe significant disease acceleration in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice. Furthermore, we observe decreased expression of the E protein target genes *Rag1*, *Rag2*, and *pre-T α* in thymocytes from our *Tal1* transgenic mice. Taken together, these studies demonstrate that Tal1 causes leukemia by interfering with the function of the class I bHLH proteins E47 and HEB. In addition, we show that Tal1 can recruit the corepressor mSin3A to the CD4 enhancer suggesting that Tal1 can actively repress the transcription of E47/HEB target genes.

NF- κ B

The NF- κ B family of transcription factors includes p50/p105, p52/p100, c-Rel, RelA(p65) and RelB, all of which contain an N-terminal 300 amino acid Rel homology domain. The p50 and p52 proteins are processed from p105 and p100 respectively. Activation of NF- κ B is controlled in part by the shuttling of NF- κ B dimers from the cytoplasm to the nucleus. In the cytoplasm, NF- κ B dimers associate with the inhibitory proteins I κ B α or I κ B β . In response to a variety of stimuli including TNF- α , IL-1, bacterial infection, or viral infection, the IKK complex consisting of IKK α , IKK β , and the regulatory subunit, IKK γ (NEMO) phosphorylates I κ B leading to its ubiquitination and subsequent degradation (Karin and Ben-Neriah, 2000). The release of I κ B from NF- κ B exposes the nuclear localization signal (NLS) on NF- κ B and leads to the translocation of NF- κ B dimers to the nucleus (Baldwin, 1996). RelA/p50 dimers bind to the consensus sequence GGGRNNYYCC and RelA/c-Rel dimers bind to HGGARNYYCC where H is an A, C, or T; R is a purine and Y is a pyrimidine (Baldwin, 1996). However, *in vitro* experiments have shown that p65 and c-Rel containing complexes can regulate the expression of the same genes, suggesting functional redundancy between the family members (Garoufalidis et al., 1994; Tan et al., 1992). Several NF- κ B target genes involved in immunity and inflammation are known including GM-CSF, IL-6, IL-8 and IL-2 (Baldwin, 1996).

NF- κ B and lymphocyte development

Gene targeting of the NF- κ B proteins in mice have revealed their essential functions in the immune system. *p50/p105* deficient mice develop normally however they exhibit multiple defects in immune system function(Sha et al., 1995). B cells from *p50*^{-/-} mice do not proliferate normally in response to LPS and do not produce normal levels of antibodies. In addition, *p50/p105* null mice are more susceptible to *Listeria monocytogenes* and *Streptococcus pneumoniae* infection(Sha et al., 1995). RelB null mice also survive but have immune system defects. The mice exhibit splenomegaly, infiltration of inflammatory T cells to other organs and decreased antigen presenting dendritic cells in the thymus(Weih et al., 1995). Mature B and T cells from *c-Rel*^{-/-} mice do not proliferate normally in response to a variety of mitogens(Kontgen et al., 1995). The *RelA* deficient mice have a much more severe phenotype in that they die during embryogenesis due to massive apoptosis in the liver(Beg et al., 1995). Therefore, *RelA* appears to play a role in inhibiting apoptosis. Because *RelA* null mice die so early in development, the role of *RelA* in lymphoid development could not be determined.

In order to determine the role of p65 in lymphocyte development, lethally irradiated mice were transplanted with *p65* null fetal liver cells. Modest decreases in fetal liver-derived B and T cells were observed in reconstituted mice(Horwitz et al., 1997). However, when mice were transplanted with cells that lack both *p50* and *p65*, no fetal liver derived B or T cells were observed demonstrating that *p50* and *p65* are essential for lymphopoiesis(Horwitz et al., 1997). Lymphopoiesis was rescued by cotransplant of *p50*^{-/-}*p65*^{-/-} fetal liver cells with wildtype bone marrow cells suggesting

that developing lymphocytes require NF- κ B signaling in stromal cells(Horwitz et al., 1997). To gain more insight into the role of NF- κ B in lymphocyte development, I κ B α was overexpressed in T cells of the mouse from a transgene driven by the *CD2* promoter (Esslinger et al., 1997). Expression of this transgene leads to an inhibition of the activation of all NF- κ B complexes-not only p50/p65. A decrease in overall thymic cellularity was observed. In addition, transgenic mice had decreased numbers of CD4-single positive and CD8-single positive thymocytes(Esslinger et al., 1997) demonstrating that NF- κ B activation is required for proper T cell development.

Another group expressed a N-terminal truncated form of I κ B α that cannot be phosphorylated and degraded in the mouse thymus using a transgene controlled by the proximal *lck* promoter(Boothby et al., 1997). Although thymic cellularity was unaffected in *lck-mI κ B α* transgenic mice, CD8-single positive cells were reduced about 2-fold(Boothby et al., 1997). In the periphery, both CD8-single positive and CD4-single positive cells were reduced however CD8-single positive cells were more severely affected. The authors also observed that the transgenic thymocytes did not proliferate as well as wildtype thymocytes and were more susceptible to apoptosis in response to mitogenic stimuli(Boothby et al., 1997) supporting a role for NF- κ B in protecting thymocytes from apoptosis. Double positive thymocytes from *CD2-mI κ B α* transgenic mice are resistant to α -CD3-induced apoptosis(Hettmann et al., 1999), suggesting that NF- κ B may be pro-apoptotic in double positive thymocytes. NF- κ B dependent thymocyte apoptosis may involve down-regulation of Bcl-X_L(Hettmann et al., 1999) or up regulation of Fas ligand(Ayrolidi et al., 1997).

Recent studies have linked pre-T α signaling to NF- κ B activation. It has been demonstrated that the highest levels of NF- κ B activation are in the DN3 and DN4 populations, the cells that express elevated levels of the pre-T cell receptor (Aifantis et al., 2001; Voll et al., 2000). In addition, expression of the components of the pre-T cell receptor led to NF- κ B activation in a T cell line (Voll and Ghosh, 1999). Inhibition of NF- κ B activity by transgenic expression of the mutant I κ B α that cannot be phosphorylated or degraded led to decreased numbers of DN3 and DN4 cells. In contrast, expression of a constitutively active IKK β resulted in an increase in DN4 cells (Voll et al., 2000). Interestingly, anti-CD3 treatment, overexpression of activated I κ k and γ -irradiation can substitute for pre-TCR signaling in pre-T α null mice (Fehling et al., 1997). All of these signals also activate NF- κ B suggesting that NF- κ B activation may be able to replace pre-TCR signaling (Voll and Ghosh, 1999) and promote differentiation of double negative thymocytes into double positive thymocytes.

NF- κ B and cancer

The first NF- κ B family member discovered was the viral oncogene *v-Rel*. This gene causes lymphoid malignancy in chickens and leads to T cell lymphoma when overexpressed in the mouse (Carrasco et al., 1996; Gilmore, 1999). Moreover, chromosomal rearrangement and amplification of NF- κ B genes has been observed in a variety of human cancers. *c-Rel* amplification has been reported in diffuse large cell lymphoma, primary mediastinal B-cell lymphoma, and follicular lymphoma (Houldsworth et al., 1996; Joos et al., 1996; Rao et al., 1998). *c-Rel* is also involved in chromosomal

rearrangements in some cases of follicular lymphoma and diffuse large cell lymphoma(Lu et al., 1991). Overexpression of c-Rel was found in half of the non-small cell lung carcinomas examined suggesting that c-Rel does not only contribute to lymphoid malignancies(Mukhopadhyay et al., 1995). *RelA* is infrequently involved in chromosomal rearrangements, however it has been shown to be amplified or overexpressed in some solid tumors including squamous head and neck carcinomas, breast adenocarcinomas, and stomach adenocarcinomas(Mathew et al., 1993).

In the past few years there have been many reports of constitutive NF- κ B activation in a wide variety of human cancers making NF- κ B an attractive target for chemotherapy. High levels of nuclear p50/p65 heterodimers are detected in melanoma cells(Yang and Richmond, 2001), prostate cancer cells(Palayoor et al., 1999), pancreatic cancer cells(Wang et al., 1999), and breast cancer cells(Sovak et al., 1997) among many others. In addition, constitutive NF- κ B activation is also observed in chronic myelogenous leukemia(Guzman et al., 2001), Hodgkin's lymphoma(Bargou et al., 1996), and in 39 out of 42 childhood acute lymphoblastic leukemia samples(Kordes et al., 2000). Inhibition of NF- κ B activation by overexpression of a non-degradable form of I κ B α in pancreatic cancer cells, Hodgkin's lymphoma cells and squamous cell carcinoma inhibited tumor growth in mice(Bargou et al., 1997; Duffey et al., 1999; Fujioka et al., 2003). NF- κ B activation is also required for the development of tumors caused by expression of the chimeric oncoprotein Bcr-Abl(Reuther et al., 1998). When mice with prostate tumors were treated with the NF- κ B inhibitor DHMEQ, a significant decrease in tumor size was observed, suggesting that NF- κ B activation contributes to the growth of

these tumor cells(Kikuchi et al., 2003). Inhibition of NF- κ B activation in lung carcinoma cells did not affect their growth but did inhibit metastasis(Andela et al., 2000). In contrast, overexpression of the NF- κ B superinhibitor in the skin of the mouse led to the development of squamous cell carcinoma(van Hogerlinden et al., 1999). Therefore, the effect of NF- κ B inhibition may depend on the cell type.

The prevalence of NF- κ B activation in human cancer including childhood T cell acute lymphoblastic leukemia, prompted us to determine whether NF- κ B was activated in our mouse model of Tal1 induced leukemia. We observed NF- κ B activation in *Tal1* thymocytes and tumors, however inhibition of NF- κ B by expression of the non-degradable form of I κ B α did not affect tumor growth or metastasis *in vivo*. These results suggest that TAL1 leukemia patients may not respond well to the use of NF- κ B inhibitors.

Figure 1. Gene targeting experiments in mice have established that Tal1 is essential for hematopoiesis and angiogenesis. Tal1 is expressed in all cell types shown in red. *Tal1* deficient mice have demonstrated that Tal1 is essential for embryonic blood cell development. Tal1 is also required for embryonic angiogenesis and for the development of hematopoietic stem cells. Conditional disruption of Tal1 in the adult mouse demonstrated that Tal1 is not required for HSC proliferation, engraftment, or the differentiation of HSCs to myeloid or lymphoid lineages. Continued Tal1 expression is required for the differentiation of HSCs to erythroid cells and megakaryocytes. Misexpression of Tal1 in T cells occurs in the majority of childhood T cell acute lymphoblastic leukemia cases. Figure adapted from (Barton et al., 1999).

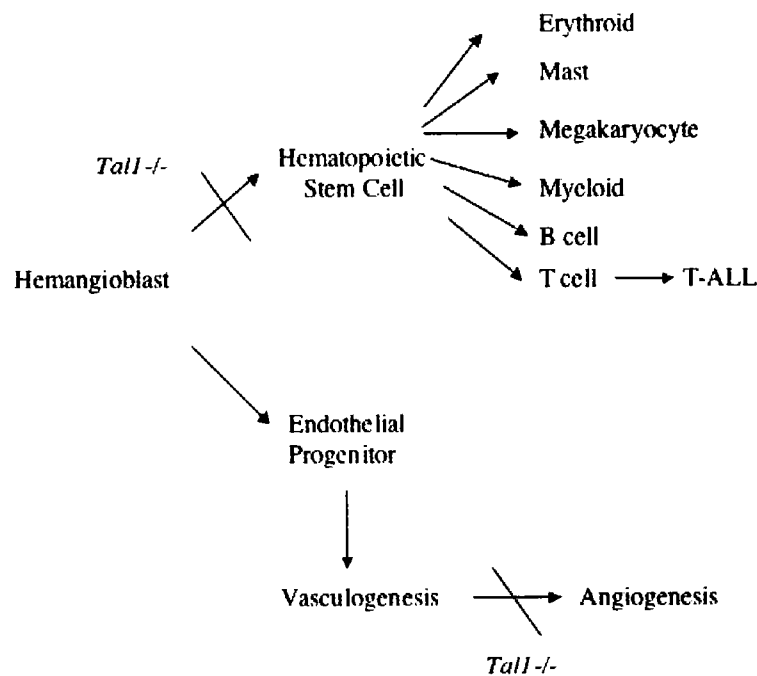


Figure 2. Models of Tal1 leukemogenesis. The sequestration model postulates that ectopic expression of Tal1 in the thymus disrupts E47/HEB heterodimer formation and leads to a down-regulation of genes critical for thymocyte differentiation. In the inhibition model Tal1 represses E47/HEB target genes by recruiting a corepressor. The transactivation model suggests that Tal1/E47 or Tal1/HEB complexes induce novel oncogenes. All three models may contribute to Tal1 leukemogenesis.

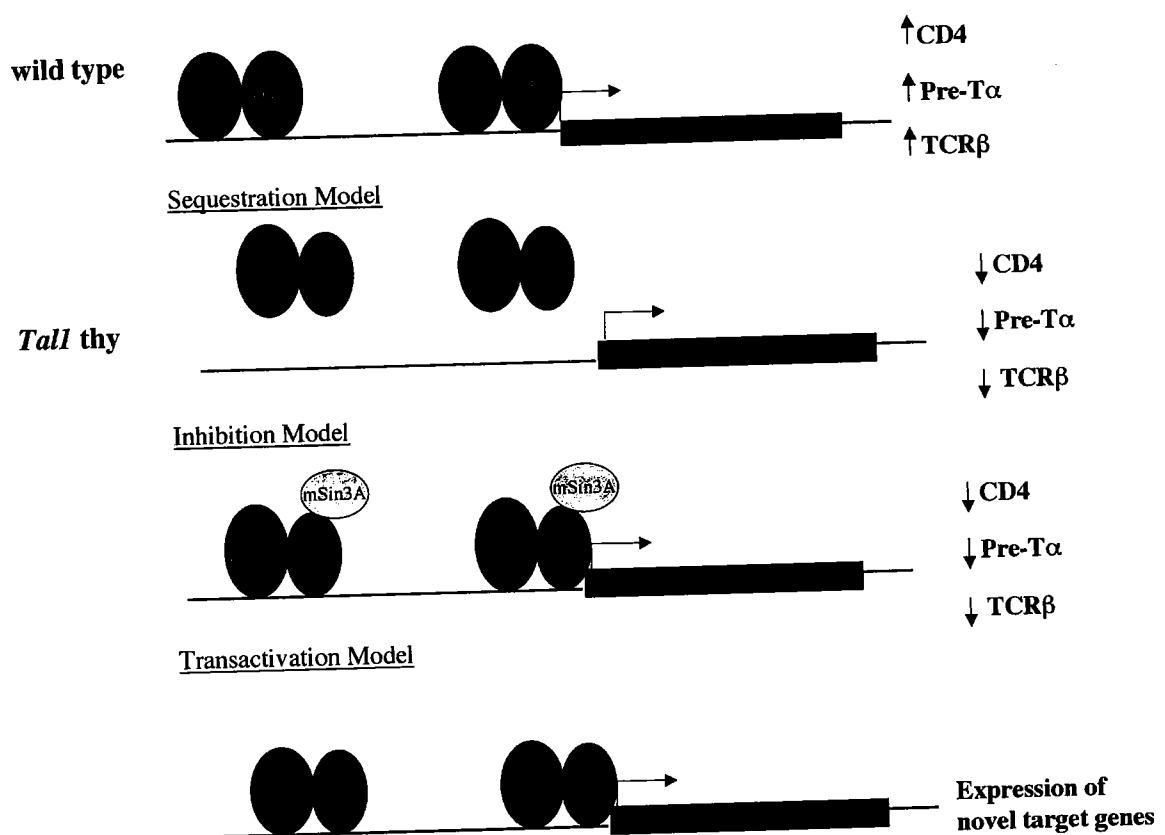


Table 1. Properties of transcription factors discussed in this thesis.

Transcription Factor	Protein Family	Expression Pattern	Knockout phenotype	Role in Leukemia
Tal1	Class II bHLH	Hematopoietic cells, endothelial cells, CNS	Embryonic lethal, bloodless	Activated in human T-ALL patients, transgenic mice develop leukemia
E2A (E47, E12)	Class I bHLH	ubiquitous	Lack B cells, T cell developmental abnormalities, postnatal lethal	50% of null mice that survive develop T cell leukemia
HEB	Class I bHLH	ubiquitous	Reduced pro-B cells, T cell developmental abnormalities, die within first two weeks of life	Lethality rescued in <i>HEB^{-/-} Id3^{-/-}</i> mice; these mice develop T cell leukemia
Lmo1	LIM only domain protein	High levels in brain, low levels in hematopoietic tissues	Not done	Activated in human T-ALL patients, transgenic mice develop T cell leukemia
Lmo2	LIM only domain protein	All tissues except DP and SP thymocytes	Embryonic lethal, bloodless	Activated in human T-ALL patients, transgenic mice develop T cell leukemia
Id1	Class V bHLH	ubiquitous	Normal, <i>Id1/Id3</i> null mice die at E12.5 due to cranial hemorrhage	Transgenic mice develop T cell leukemia
Id2	Class V bHLH	ubiquitous	Lack lymph nodes and Peyer's patches	Transgenic mice develop T cell leukemia

CHAPTER II

THE DNA BINDING ACTIVITY OF TAL1 IS NOT REQUIRED TO INDUCE LEUKEMIA/LYMPHOMA IN MICE

Introduction

The basic helix-loop-helix protein TAL1 is normally expressed in hematopoietic progenitors and erythroid, megakaryocytic and mast cell precursors, as well as endothelial cells and the central nervous system (Begley and Green, 1999). Gene targeting experiments in mice have established *Tall* as an essential regulator of blood cell and vascular development (Porcher et al., 1996; Shivdasani et al., 1995; Visvader et al., 1998). Deregulated expression of *TAL1* in humans by either chromosomal translocation, interstitial deletion or mutation occurs in 60% of patients with T cell acute lymphoblastic leukemia (T-ALL) (Bash et al., 1995). Ectopic expression of *Tall* in the thymus of mice results in the development of clonal T cell leukemia/lymphomas (Condorelli et al., 1996; Kelliher et al., 1996), further demonstrating the oncogenicity of *Tall*. Yet, the mechanism(s) of *Tall*-induced leukemogenesis remains unclear.

In human leukemic Jurkat cells, TAL1 does not homodimerize, but forms stable heterodimers with the ubiquitously expressed bHLH E2A proteins, E12 and E47 (Hsu et al., 1994b). Members of the E2A family include E2-2, HEB and the products of the E2A gene, E47 and E12 (Henthorn et al., 1990; Hu et al., 1992; Murre et al., 1989a). TAL1/E2A heterodimers preferentially recognize the E-box consensus sequence CAGATG (Hsu, 1994) and exhibit transcriptional transactivation activity *in vitro* (Hsu et al., 1994a). Consequently, it was proposed that TAL1 functions as a direct transcriptional activator in leukemia.

In erythroid cells, *Tall* associates with E12 and E47 (Condorelli et al., 1995; Hsu et al., 1991; Hsu et al., 1994b), the cysteine-rich LIM-only protein Lmo2, Ldb1 (Valge-

Archer et al., 1994; Visvader et al., 1997; Wadman et al., 1997) and the erythroid-specific zinc finger protein Gata1(Wadman et al., 1997). The Tal1/E2A/Lmo/Gata1 complex binds a composite E-box GATA site(Wadman et al., 1997) and presumably regulates genes involved in erythroid differentiation. E-box-GATA sites have been identified in several erythroid genes, including enhancers of the erythroid specific EKLF and Gata1 transcription factors(Anderson et al., 1998; Cohen-Kaminsky et al., 1998; Vyas et al., 1999). Hence, Tal1/E2A heterodimers may function as direct transcriptional regulators in both hematopoietic development and leukemia.

However, *in vitro* Tal1/E2A heterodimers are reported to be relatively weak transcriptional activators compared to E2A homodimers(Doyle et al., 1994; Hsu et al., 1994a; Park, 1998). Yet, under physiologic conditions where inhibitory HLH Id proteins are expressed, Tal1/E2A heterodimers exhibit significantly increased transcriptional activity relative to the E2A homodimer, presumably due to the stability of the Tal1/E2A heterodimer(Voronova and Lee, 1994). Thus, two models have been proposed to explain the leukemogenic effects of Tal1 expression in T cells(Begley and Green, 1999). Tal1-induced leukemogenesis may reflect the aberrant transactivation of novel target genes by the Tal1/E2A heterodimer. Alternatively, Tal1 may sequester E2A proteins, resulting in the subsequent alteration of E2A target genes. Support for the sequestration model comes largely from studies of *E2A* deficient mice, where approximately fifty percent of the surviving *E2A*^{-/-} mice develop spontaneous T cell lymphomas/leukemias(Bain et al., 1997; Yan et al., 1997).

To determine whether Tal1 transforms thymocytes by acting as a direct transcriptional activator, we created transgenic mice expressing a known DNA binding mutant of Tal1(Hsu et al., 1994a). Mutagenesis of the myogenic bHLH proteins, myogenin and MyoD1, identified amino acid residues within the basic domain critical for DNA binding(Brennan et al., 1991; Davis et al., 1990). Replacement of two of the conserved, contact arginines with glycines within the basic domain of Tal1, (designated Tal1 R188G;R189G), obliterated binding to the Tal1/E47 consensus sequence and destroyed E-box reporter activity(Hsu et al., 1994a). To elucidate the mechanism(s) of Tal1-induced leukemia, we tested the transforming potential of the *Tal1 R188G;R189G* DNA binding mutant. Three transgenic lines of mice expressing Tal1R188G;R189G in the thymus were generated and characterized. Approximately half of the mice expressing a DNA binding mutant of Tal1 developed disease. This study provides direct evidence that the DNA binding activity of Tal1 is not required to induce leukemia/lymphoma in mice and demonstrates that Tal1 contributes to leukemia by interfering with E2A protein function(s).

Results

***Tal1 R188G;R189G* transgenic mice**

A transgenic construct was generated by placing the human *TAL1* cDNA containing the R188G;R189G mutations under control of the *lck* proximal promoter (Figure 3A). The 3' untranslated region of this construct contains introns, exons and the polyA addition site of the human growth hormone gene (Abraham et al., 1991). The *lck-Tal1 R188G;R189G* construct was microinjected into the pronuclei of fertilized FVB/N oocytes (Taketo et al., 1991). Four transgenic founders were identified initially and three were studied in detail. The three *Tal1 R188G;R189G* lines expanded for study expressed high levels of *Tal1 R188G;R189G* mRNA as shown by ribonuclease protection assay; the fourth line expressed less *Tal1 R188G;R189G* mRNA and was not studied further (Figure 3B, lanes *Mut Tal1/+*). As expected, no *Tal1* message was detected in wild type thymus. Thymocytes from the three *Tal1 R188G;R189G* lines expressed similar levels of *Tal1 R188G;R189G* protein (Figure 3C lanes *Mut Tal1/+*). Furthermore, the protein expression levels of the *Tal1 R188G;R189G* mutant were similar to that achieved in the wild type *Tal1* transgenic lines, in murine erythroleukemic (M) cells, and human Jurkat cells (J) (Figure 3C).

T cell acute lymphoblastic leukemia/lymphoma in *Tal1 R188G;R189G* transgenic mice

The three *Tal1 R188G;R189G* transgenic lines developed leukemia with a median survival of 215 days (Figure 4). Twenty-nine of sixty-two (48%) *Tal1 R188G;R189G*

mice from three lines developed disease compared to twenty one of seventy-five (28%) of wild type *Tal1* transgenic mice (Figure 4). Both the wild type *Tal1* and the *Tal1 R188G;R189G* transgenic animals exhibit respiratory distress, ruffled coat and weight loss. Necropsy revealed the presence of a thymic mass, often accompanied by hepatosplenomegaly. Histological examination of the thymus revealed effacement of the normal thymic architecture by a monomorphic infiltrate of lymphoblastic cells with prominent nucleoli and scant cytoplasm (Figure 5A and D). Similar cells invade the surrounding para-sternal muscle, pericardium and other organs such as spleen, liver and kidney (Figure 5B, C, E and F). Lymphoblasts were detected in the peripheral blood of diseased animals at the time of sacrifice.

The histologic appearance of the thymic tumors as well as the leukemic blood profiles of the *Tal1 R188G;R189G* mice were indistinguishable from that previously observed for wild type *Tal1* transgenic mice (Kelliher et al., 1996). This study demonstrates that the DNA binding properties of *Tal1* are not required to induce leukemia/lymphoma in mice and suggests that *Tal1* transforms via an Id-like mechanism, potentially by sequestering E proteins.

Casein kinase II accelerates leukemia/lymphoma induced by *Tal1 R188G;R189G*

CKII has been shown to modulate the activity of several transcription factors *in vitro* and to synergize dramatically with *Myc* and with wild type *Tal1* in inducing lymphocytic leukemia in bitransgenic mice (Kelliher et al., 1996; Seldin and Leder, 1995). The presence of CKII consensus phosphorylation sites in *Tal1* and E47 and the

fact that CKII phosphorylation has been shown to inactivate E47 DNA binding activity(Johnson et al., 1996) prompted us to test whether CKII might collaborate with a DNA binding mutant of *Tal1* to induce leukemia in mice. To test this, one *Tal1 R188G;R189G* transgenic line (F₀23) was mated with mice which expressed the catalytic subunit of CKII in lymphocytes via the immunoglobulin heavy chain promoter-enhancer(Seldin and Leder, 1995). The CKII transgenic mice develop clonal T cell lymphomas after a long latency (median survival of 400 days)(Seldin and Leder, 1995). Previously, we had shown that wild type *Tal1* and *CKII* cooperate to induce disease in mice(Kelliher et al., 1996). All bitransgenic *Tal1/CKII* animals developed leukemia with a median survival of 72 days (Figure 4). When mated to the *Tal1 R188G;R189G* transgenic mice, a strikingly similar acceleration of disease onset and increase in disease penetrance was observed. All bitransgenic *Tal1 R188G;R189G/CKII* animals developed aggressive disease with a median survival of 69 days (Figure 4). In the *Tal1 R188G;R189G/CKII* animals, the disease was characterized by thymic enlargement, often accompanied by splenomegaly and lymphadenopathy. As observed in wild type *Tal1* transgenic mice, the thymic architecture was obliterated by neoplastic cells and numerous clusters of apoptotic cells were observed.

The nearly identical survival curves observed for *Tal1/CKII* and *Tal1 R188G;R189G/CKII* suggests that CKII does not synergize by potentiating the transcriptional activity of *Tal1*. Furthermore, this experiment supports the idea that wild type *Tal1* and its DNA binding mutant (*Tal1 R188G;R189G*) transform thymocytes by similar mechanisms.

Tal1 R188G;R189G induces clonal or oligoclonal disease

The dramatic acceleration of disease onset in the *Tal1 R188G;R189G/CKII* bitransgenic mice prompted us to determine the clonal nature of the disease. DNA was isolated from cell lines derived from the tumors restricted with Hind III and examined by filter hybridization with the TCR J β 2 probe. Clonal or oligoclonal rearrangements were detected in both the *Tal1 R188G;R189G* and in the bitransgenic *Tal1 R188G;R189G/CKII* tumor cells analyzed and in most cases, both TCR β alleles were rearranged (Figure 6). In animals where disease involved multiple organs such as thymus, spleen, liver and kidney, the specific β TCR bands were evident in all tissues. As expected, the immunoglobulin heavy chain locus was retained in its germline configuration (data not shown).

Tal1 R188G;R189G thymomas do not express CD4

Tumors from the *Tal1 R188G;R189G* and *Tal1 R188G;R189G/CKII* mice were examined by flow cytometry to determine the phenotype(s) of the primary tumor. All of the tumors appeared to be of T cell origin, although at varying stages of thymocyte development (Table 2). None of the *Tal1 R188G;R189G* or *Tal1 R188G;R189G/CKII* tumors expressed the B cell specific antigen B220 or surface immunoglobulin heavy chain (data not shown).

Two predominant immunophenotypes were observed: four of seven tumors consisted of predominantly CD3-positive, CD4-negative and CD8-positive cells, presumably arising from the mature, single positive thymocyte population. The

remaining three *tal-1R188G;R189G* tumors expressed CD3 but failed to express either CD4 or CD8. The bitransgenic *Tal1 R188G;R189G/CKII* tumor cells exhibited similar immunophenotypes. Half (2/4) of the tumors analyzed were CD4-negative and CD8-positive and the remaining two tumors examined were both CD4-negative and CD8-negative.

Interestingly, no CD3-positive, CD4-positive, CD8-negative tumors were observed in either the *Tal1 R188G;R189G* or *Tal1 R188G;R189G/CKII* mice, suggesting that *Tal1 R188G;R189G* expression stimulates CD4-negative, CD8-positive thymocyte differentiation and inhibits development (or survival) of CD4-positive, CD8-negative thymocytes.

Thymic expression of wild type *Tal1* and *Tal1 R188G;R189G* perturbs thymocyte development

The absence of CD4-positive tumor target cells derived from *Tal1 R188G;R189G* mice prompted us to further examine the effects of *Tal1* expression on thymocyte development. Thymus from disease-free, age-matched, *Tal1*, *Tal1 R188G;R189G* transgenics and control littermates was stained with antibodies to CD4 and CD8 and analyzed by flow cytometry. The wild type *Tal1* transgenic mice had 2-3-fold fewer thymocytes compared to *Tal1 R188G;R189G* transgenic or control littermates (not shown). Yet, analysis of the CD4/CD8 thymic profiles revealed the presence of all thymocyte subpopulations. However, mice expressing either wild type *Tal1* or the *Tal1* DNA binding mutant (R188G;R189G) consistently showed significant decreases in the

percentage of the CD4-positive, CD8-negative population (Table 3 and Figure 7A). *Tal1*-expressing thymocytes exhibited concomitant increases in the percentage of CD4-negative, CD8-positive population, resulting in markedly different CD4/CD8 ratios (0.2 for *Tal1*/+; 0.8 for *Tal1* *R188G*;*R189G*/+ compared to 3.45 for wild type littermates (Table 3 and Figure 7A). These data suggest that *Tal1* may interfere with CD4 coreceptor expression, potentially by sequestering other bHLH transcription factors. Consistent with this idea, thymocyte developmental abnormalities have also been observed in both *HEB* deficient mice (Zhuang et al., 1996a) and *E2A* deficient mice (Bain et al., 1997).

Studies in *E2A* null mice have demonstrated that the *E2A* gene products are essential for the proper regulation of V(D)J recombination. Rearrangements in the TCR γ / δ loci that normally occur only in adult thymocytes are rearranged in fetal thymocytes and vice versa (Bain et al., 1999). To test whether V(D)J recombination is also affected in *Tal1* transgenic mice, double negative thymocytes from wildtype and *Tal1*/+ transgenic mice were stained with anti-V γ 2-PE and anti-GL3-FITC (Figure 7B). The percentage of V γ 2 cells is reduced three fold in *Tal1*/+ transgenic mice; rearrangement of this loci is also reduced in the thymocytes of *E2A* null mice (Bain et al., 1999). In addition, PCR analysis of thymus DNA from four-week-old wildtype, *Tal1*/+ and *Mut Tal1*/+ mice revealed an increased rearrangement of the V γ 3 locus (Figure 5C). This locus is normally exclusively rearranged in fetal thymocytes but is also seen rearranged in *E2A* null mice of a similar age (Bain et al., 1999). The aberrant $\gamma\delta$ T cell rearrangements observed in our *Tal1* and *Mut Tal1* transgenic mice suggest that *Tal1*

affects $\gamma\delta$ T cell development as well as $\alpha\beta$ and provides further evidence that Tal1 acts by inhibiting the function of E proteins.

Wild type Tal1 and the DNA-binding mutant Tal1 R188G;R189G form stable heterodimers with E47 and HEB

The tissue-specific bHLH proteins, like Tal1, do not bind DNA because they do not form homodimers (Littlewood, 1998). Thus, heterodimer formation is essential for the DNA-binding activity and functional properties of these proteins. A stable Tal1/E2A complex has been detected in Jurkat cells (Hsu et al., 1994a). However, it remains unclear whether this complex is a consistent feature associated with Tal1 induced leukemia. To test whether a Tal1/E2A complex contributes to leukemia development in the mouse, *Tal1* tumor cell lysates were immunoprecipitated with either a preimmune (PI) or anti-Tal1 polyclonal antiserum (I). Coprecipitating proteins were tested for the presence of E47 by immunoblotting with an anti-E47 monoclonal antibody. E47 coprecipitated with Tal1 in murine erythroleukemia cells (MEL) and in the Tal1 induced thymomas tested (Figure 8A, lanes 2, 4 and 6). Thus, a stable Tal1/E47 heterodimer is present in the leukemic cells of the *Tal1* transgenic mice. By the same method, we were also able to detect the presence of Tal1/HEB heterodimers in *Tal1* tumor cells.

Although cells expressing the *Tal1* R188G;R189G DNA binding mutant have been shown to lack DNA binding activity (Hsu, 1994), it was unclear whether mutation of the Tal1 basic domain might interfere with its ability to interact with E47 and HEB. To test whether the mutant Tal1 R188G;R189G protein is capable of forming stable

heterodimers *in vivo*, lysates were prepared from thymomas derived from the *Tal1 R188G;R189G* mice. *Tal1 R188G;R189G/E47* and *Tal1 R188G;R189G/HEB* complexes were readily detected in the two thymomas tested, demonstrating that these basic domain mutations do not interfere with formation/stability of the *Tal1/E47* or *Tal1/HEB* complex in *Tal1 R188G;R189G* thymomas.

E47/HEB heterodimers are depleted in *Tal1* transgenic mice

We sought to determine whether the interaction of *Tal1* with E47/HEB disrupts the formation of E47/HEB heterodimers, the predominate E2A dimers present in thymocytes (Sawada and Littman, 1993). Thymocytes from wildtype and *Tal1* mice were lysed in a low stringency lysis buffer. Equal amounts of each lysate were immunodepleted with three overnight incubations with anti-*Tal1* to remove all *Tal1* containing complexes. The lysates were then immunoprecipitated with anti-E47 and separated on a SDS-PAGE gel. The blot was then probed with anti-HEB to detect E47/HEB dimers (Figure 8B). Significantly less HEB protein was detected in the *Tal1* lysates indicating that fewer E47/HEB dimers exist in *Tal1* thymocytes compared to wildtype thymocytes. Similar results were also obtained using *Tal1 R188G;R189G* thymocytes. This experiment provides evidence that *Tal1* acts by disrupting the E2A protein complexes that normally form in thymocytes further supporting our hypothesis that *Tal1* acts by inhibiting E proteins. In addition, by gel shift analysis using the sequence from the immunoglobulin heavy chain gene enhancer that contains E boxes, we

were able to demonstrate that fewer E47/HEB dimers bind this sequence in *Tall* transgenic thymocytes compared to wildtype thymocytes (Figure 8C).

Lmo2 expression is not required for Tall induced disease

Stable complexes between TAL1/E47 and the cysteine-rich LIM-only protein LMO2 have been detected in the leukemic cells of some T-ALL patients (Wadman et al., 1994a) and transgenic coexpression of *Tall* and *Lmo2* results in accelerated tumor development (Larson et al., 1996). Moreover, gene targeting experiments support a cooperative relationship between *Tall* and *Lmo2*, as mice deficient for either gene exhibit defects in erythropoiesis (Shivdasani et al., 1995; Warren et al., 1994). Together, these studies suggest that Tall may induce Lmo2 expression in leukemia.

To test whether Lmo2 expression is required for Tall induced leukemogenesis in mice, tumor cell lysates were prepared from *Tall* and *Tall R188G;R189G* thymomas and analyzed for Lmo2 expression by immunoblotting with an anti-Lmo2 antisera. The 22 kD Lmo2 protein was detected in Lmo2-transfected 293T cells and in the MEL cells, however, no Lmo2 expression was detected in any of the *Tall* or *Tall R188G;R189G* tumors examined (Figure 8C). To ensure that samples contained equivalent amounts of nuclear protein, extracts were examined for expression of E47. Similar amounts of E47 were detected in nuclear extracts prepared from MEL cells and *Tall* tumor cells (data not shown). Hence, *Lmo2* activation does not contribute to *Tall* induced leukemogenesis in mice.

Tal1 R188G;R189G /E2A complexes isolated from leukemic cells fail to bind DNA

Although previously shown to obliterate DNA binding *in vitro*, it remained possible that Tal1 R188G;R189G/E2A protein complex retained some ability to bind DNA *in vivo*. To test this possibility, nuclear extracts were prepared from thymomas and subjected to gel mobility shift analysis using the Tal1/E47 consensus E-box motif (CAGATG) as a probe (Hsu, 1994). Incubation of this probe with nuclear extracts from Jurkat cells generated three distinct protein-DNA complexes (Figure 9). All three complexes were eliminated by incubating the extract with an E2A polyclonal antisera. The middle two complexes were also abrogated by incubating the extract with an antiserum raised against human TAL1 but not with the corresponding preimmune serum. Tal1 encodes two phosphoproteins; the full-length pp42 and a truncated polypeptide pp22. The upper Tal1/E2A complex corresponds to a pp42^{TAL}/E2A heterodimer whereas the pp22^{TAL}/E2A forms the lower complex (Hsu, 1994). Nonspecific complexes were also detected (labeled n.s.).

Similar complexes were detected when the Tal1/E47 probe was incubated with nuclear extracts from *Tal1* mouse thymomas (Figure 9, *Tal1* tumor lanes). All three complexes were depleted by incubating the extract with the anti-E2A antisera but not with the corresponding preimmune antisera. The two lower complexes were depleted when the mouse tumor extract was preincubated with an anti-Tal1 antisera, demonstrating the presence of a pp42^{tal}/E2A and pp22^{tal}/E2A complexes in mouse leukemic cells.

To test whether the Tal1 R188G;R189G DNA binding mutant protein exhibited DNA binding activity *in vivo*, nuclear extracts were prepared from thymomas isolated from the *Tal1 R188G;R189G* transgenic mice. As expected, no Tal1 R188G;R189G/E2A heterodimers bound the Tal1/E47 consensus sequence. However, E protein homodimers did bind to the Tal1/E47 consensus sequence in nuclear extracts from DNA binding mutant *Tal1* tumors. Taken together, this study argues that Tal1 contributes to leukemia by interfering with E protein function(s) in thymocytes.

Discussion

We have demonstrated that the DNA binding properties of Tal1 are not required to induce leukemia in mice. Forty eight percent of *Tal1 R188G;R189G* mice from three lines died of clonal T lymphoblastic leukemia. Furthermore, we show that in all the *Tal1* induced thymomas tested, a stable Tal1/E2A complex was detected. This provides direct evidence that transformation by Tal1 does not require DNA binding and demonstrates that Tal1 transforms by an Id-like mechanism, preventing the formation of E protein homodimers.

Tal1 encodes a basic helix-loop-helix protein that is required for embryonic hematopoietic and vascular development(Porcher et al., 1996; Shivdasani et al., 1995; Visvader et al., 1998). A recent structure-function analysis of the regions of Tal1 required for hematopoiesis revealed that DNA binding by Tal1 is not required for primitive erythropoiesis in embryonic stem cells(Porcher et al., 1999). Thus, the DNA binding activity of Tal1 is dispensable in both embryonic hematopoiesis and in leukemia.

When expressed in the thymus, wild type Tal1 and the DNA binding mutant (Tal1 R188G;R189G) perturb thymocyte differentiation, stimulating the development CD8-single positive thymocytes and inhibiting the development of CD4-single positive thymocytes. The E2A proteins participate in lymphocyte development(Bain et al., 1997) and are involved in transcriptional activation of the immunoglobulin and CD4 coreceptor genes(Murre et al., 1989a; Sawada and Littman, 1993). The ratio of CD4 to CD8 single positive thymocytes is also affected in *E2A* deficient mice(Bain et al., 1997), further implicating E2A protein sequestration in Tal1 induced disease.

These studies have major implications for current work focused on identifying *Tal1* target genes. Our work argues that *Tal1* transforms by interfering with genes activated or repressed by the E2A proteins, E12 or E47. One potential E47 target gene is the cyclin-dependent kinase inhibitor p21 (Prabhu et al., 1997). *Tal1* has been shown to inhibit E47-mediated activation of a p21 reporter construct in HeLa cells (Park, 1998), implicating p21^{CIP1/WAF1/Sdi1} as a potential target gene in human T-ALL. However, no differences in p21^{CIP1/WAF1/Sdi1} expression levels were observed in tumors isolated from *Tal1* (or *Tal1* R188G;R189G) transgenic mice (data not shown), indicating that other E47 target genes are likely involved. Future cDNA microarray analyses of genes activated/repressed upon *Tal1* expression or E47 deletion should identify the cooperating oncogene(s).

Materials and Methods

Generation of transgenic mice

The human *TALI* cDNA containing the R188G;R189G mutations (generously provided by Dr. Richard Baer) was isolated as a Bam HI-Bgl II fragment and cloned into the BamHI cloning site of p1017, a plasmid cassette containing the proximal *lck* promoter and the human growth hormone splice and poly A addition sites (Abraham et al., 1991). After being checked for proper orientation and sequenced to confirm presence of the R188G;R189G mutations, the plasmid was linearized by digestion with SpeI and microinjected into the FVB/N pronuclei. Transgenic mice were identified by probing Southern blots of EcoRI-digested tail DNA with a ³²P-labeled random-primed 585 bp EcoRI - EcoRI *TALI* partial cDNA fragment. Southern blots were hybridized and washed as previously described (Kelliher et al., 1996). Transgenic lines were propagated by crossing founder animals with FVB/N animals.

Histology

Upon necropsy, all tissue samples were preserved in Optimal Fix (American Histology Reagent Company, Inc.). Four mm sections were cut and stained with hematoxylin and eosin for histologic evaluation in the Transgenic Core Pathology Laboratory at the University of California at Davis.

Antibodies and Fluorescence-activated flow cytometry analysis

Mouse thymomas were gently teased with frosted glass slides in order to produce single cell suspensions. The cells were washed with PBS and stained with fluorescent-labeled antibodies and subjected to flow cytometry at the FACS facility at the University of

Massachusetts Medical Center. Antibodies used in flow cytometry included FITC-conjugated anti-mouse CD3, FITC-anti-mouse L3T4 (CD4), FITC-anti-mouse Ly-2 (CD8), FITC-anti-mouse IL-2 receptor (CD25), FITC-anti-B220, FITC-polyclonal goat anti-rat immunoglobulin antibody, PE-conjugated anti-mouse L3T4, FITC-anti-GL3 and PE-conjugated V γ 2 (PharMingen, San Diego, CA). Dead cells were eliminated by gating for cells which stained with LDS-751 (Exciton). Data were analyzed using FlowJo software (Treestar, Inc.).

Tumor DNA analysis

Southern blots of *HindIII*-digested DNA (10 μ g) obtained from primary tumors and from tumor cell lines were hybridized with a ³²P-labeled 2 kb *EcoRI* fragment containing the murine TCR J β 2B exon (Malissen et al., 1984). Genomic DNA from tail samples was also digested with *EcoRI*, transferred to GeneScreen Plus (New England Nuclear), and hybridized to a ³²P-labeled 1.5 kb *PstI* μ fragment (Early et al., 1980). Blots were washed in 1X SSC, 1% SDS, followed by a higher stringency wash containing 0.1X SSC, 0.1% SDS.

RNase protection analysis

Total RNA was isolated from thymus from age-matched, disease-free *Tall R188G;R189G* transgenic mice and control littermates. T3 and T7 antisense probes were synthesized and hybridized to RNA samples as previously described (Krieg and Melton, 1987). The probe for human *Tall R188G;R189G* was derived by linearization of a 625 bp partial cDNA clone with *SacI*, resulting in a probe that protects 500 nucleotides.

Rearrangement PCR

500ng of thymocyte DNA was analyzed by PCR with primers specific for the V γ 3 (CCAGCAGCCACTAAAATGTC)(Goldman et al., 1993) and J γ 1 (AGAGGGAATTACTATGAGCT)(Asarnow et al., 1988) loci. Twenty cycles were performed with 1 minute at 94°C, 1 minute at 58°C and 1 minute at 72°C. The entire PCR reactions were separated on a 2.2% Nusieve (Cambrex Bioscience Rockland, ME) gel and blotted. The blot was then hybridized with an oligo specific for V γ 3 (GCGGGAGTGGGACTTGTCCTTGTTT)(Goldman et al., 1993). A control PCR was performed with primers specific for the TNFR1 locus.

Immunoprecipitation and Western Blotting

To analyze transgene expression levels, lysates were prepared from the thymus of 4-week-old *Tall R188G;R189G* transgenic mice and control littermates and Tall1 protein detected by immunoblotting with anti-Tall1 polyclonal antisera (gift of Dr. Richard Baer, Columbia University). Tall1 protein levels were compared to levels expressed in Jurkat and mouse erythroleukemia cells. For the co-immunoprecipitation experiments, *Tall1* tumor cell lines and murine erythroleukemia cells were lysed in a low stringency lysis buffer (10mM Hepes pH 7.6, 250mM NaCl, 0.1% NP-40, 5mM EDTA)(Lassar et al., 1991), pre-cleared with protein A-agarose and immunoprecipitated with either pre-immune or anti-Tall1 polyclonal antiserum. The immune complexes were washed twice in lysis buffer and resolved by SDS-PAGE. The Tall1 R188G;R189G-associated proteins were detected by immunoblotting with either an anti-E47 or anti-E12/HEB monoclonal antibody (PharMingen). To determine whether Lmo2 expression contributed to *Tall1*

induced disease, cell lysates were prepared from murine erythroleukemic cells (M), five *Tal1* tumors and one *Tal1 R188G;R189G* tumor. As a positive control for Lmo2 expression, 293T cells were transfected with the pEFpGKpuro expression vector (Visvader et al., 1997) containing the *Lmo2* cDNA (gift of Dr Stuart Orkin, Harvard Medical School) or with vector alone. Equal amounts of total protein were examined by immunoblotting with an anti-Lmo2 specific antisera (Dr. Stuart Orkin, Harvard Medical School).

Gel Mobility Shift Assay

Nuclear extracts were prepared from Jurkat cells and from tumors isolated from *Tal1* and *Tal1 R188G;R189G* transgenic mice (*Mut Tal1*) as previously described (Grimm et al., 1996). EMSAs were performed with equivalent amounts of nuclear extract (20 μ g), incubated with a 32 P-labeled double-stranded oligonucleotide probe containing the preferred sequence for Tal1/E47 heterodimers (E box underlined, sense strand ACCTGAACAGATGGTCGGCT (Hsu et al., 1994a). Some reactions were supplemented with 1 μ l of a rabbit anti-Tal1 or anti-E2A antisera (gift of Dr. Richard Baer, Columbia University). EMSAs were also performed on thymocytes from age-matched wildtype and *Tal1* transgenic mice. Nuclear extracts from these thymocytes were incubated with the murine immunoglobulin heavy-chain gene enhancer, μ E5 (underlined, sense strand GAACCAGAACACCTGCAGCA).

Figure 3. Structure and expression of the *Tal1* R188G;R189G transgene (A). Diagrammatic representation of the *lck-Tal1* R188G;R189G fusion construct used to create transgenic mice. The human *Tal1* R188G;R189G cDNA subcloned into a vector with the proximal *lck* promoter and human growth hormone (hGH) splice and poly(A) addition sequences was used to establish four lines of transgenic mice designated 2, 5, 6, and 23. **Expression of the *Tal1* R188G;R189G transgene (B).** RNA prepared from thymus of wild-type (+/+) and transgenic (*Mut Tal1* founder lines 2, 5, 6 and 23) was subjected to RNase protection analysis with an antisense riboprobe. The endogenous *Tal1* R188G;R189G mRNA protects 500 bases. RNA levels were compared to the human *Tal1* expressing T-ALL cell line, Jurkat. RNA from the U937 and yeast tRNA served as negative controls. **Expression of the TAL1 protein (C).** The 42 kDa TAL1 polypeptide was detected in the thymocytes of four week old *Tal1* R188G;R189G transgenic mice from lines 2, 5, and 23 (*Mut Tal1*) by immunoblotting with an anti-human TAL1 antibody (gift of Dr. Richard Baer). Similar levels of TAL1 protein expression were detected in the thymocytes of age-matched *Tal1* transgenic mice (*Tal1*), using an anti-mouse TAL1 antibody (gift of Dr. Richard Baer, Columbia University). A murine erythroleukemic cell line (M) and Jurkat cells (J) were used as a positive controls, and wildtype thymus (lane 6) as a negative control for pp42-Tal1 protein expression. Figure contributed by S. Oikemus and M. Billa.

Figure 4. Kaplan-Meier survival plot of *Tal1*, *Tal1 R188G;R189G*, *Tal1/CKII α* and *Tal1 R188G;R189G/CKII α* transgenic mice. Survival plot for the *Tal1*; *Tal1 R188G;R189G* (*Mut Tal1*), *Tal1/CKII α* (*Tal1/CKII*) and *Tal1 R188G;R189G/CKII α* (*Mut Tal1/CKII*) transgenic and bi-transgenic lines. The cohort of *Tal1* mice consisted of n=75 animals, the *Tal1 R188G;R189G* cohort consisted of n=62 animals, the *Tal1/CKII α* bitransgenic cohort consisted of n=14 animals, and the *Tal1 R188G;R189G/CKII* bitransgenic cohort consisted of n=30. All animals were monitored daily for any evidence of disease. Upon onset of disease, the mice were sacrificed and a post-mortem examination was performed.

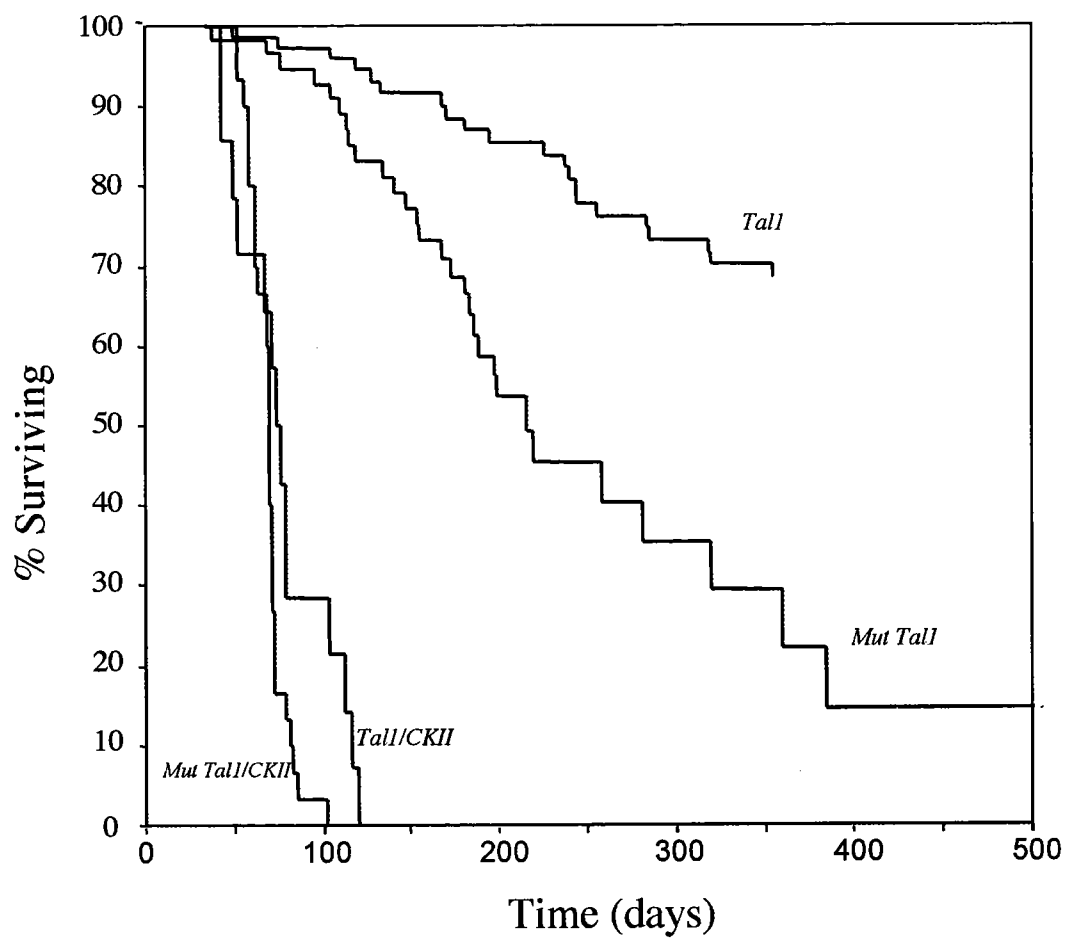


Figure 5. Histology of the lymphoproliferative disease in *Tall R188G;R189G* transgenic mice. A thymus from an adult *Tall R188G;R189G* transgenic mouse that developed a thymoma shows the effacement of the normal thymic architecture (A; 100X) and the proliferation of large lymphoblasts with prominent nucleoli (B; 400X). Similar cells invaded the visceral organs such as the liver (C and D) and the kidney (not shown).

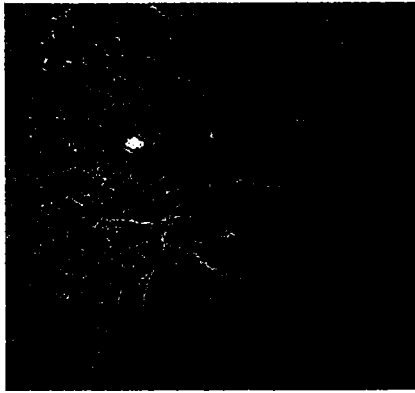
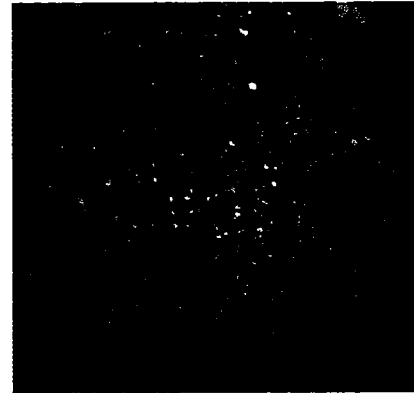
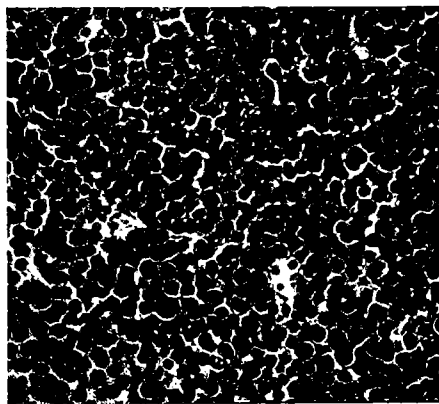
A.**C.****B.****D.**

Figure 6. *Tal1 R188G;R189G* and *Tal1 R188G;R189G/CKII α* tumors are clonal or oligoclonal. DNA prepared from tumor cell lines and wildtype genomic tail DNA was digested with *Hind* III and analyzed by Southern Blot analysis. T cell receptor J β chain rearrangements were detected with a probe that identified a 5 kb DNA fragment in the germline position of genomic tail DNA (lane T). *Mut Tal1* lanes contain DNA isolated from tumor cell lines derived from *Tal1 R188G;R189G* mice, whereas, *Mut Tal1/CKII* lanes contain DNA from *Tal1 R188G;R189G/CKII α* bitransgenic tumor cell lines. Figure contributed by M. Billa.

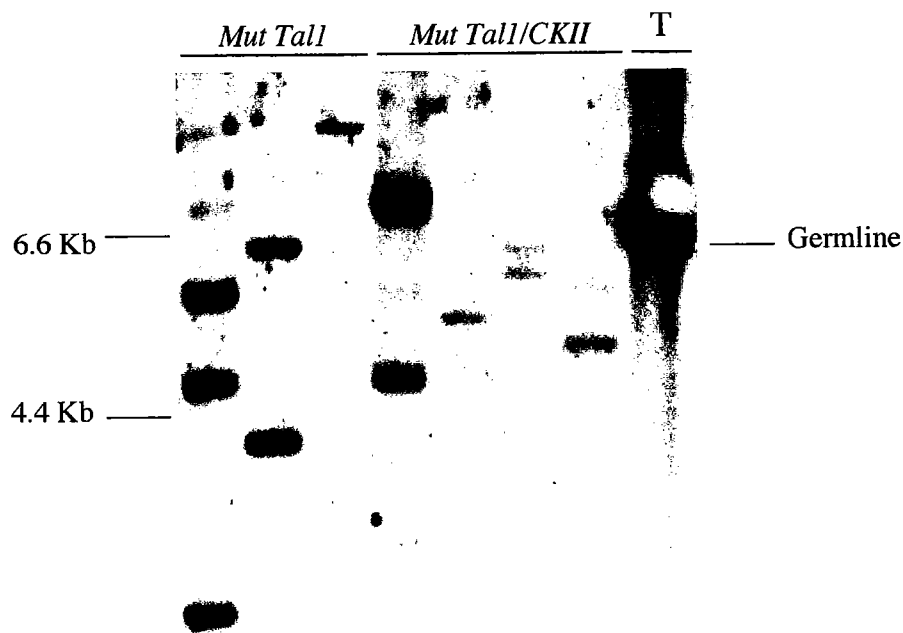
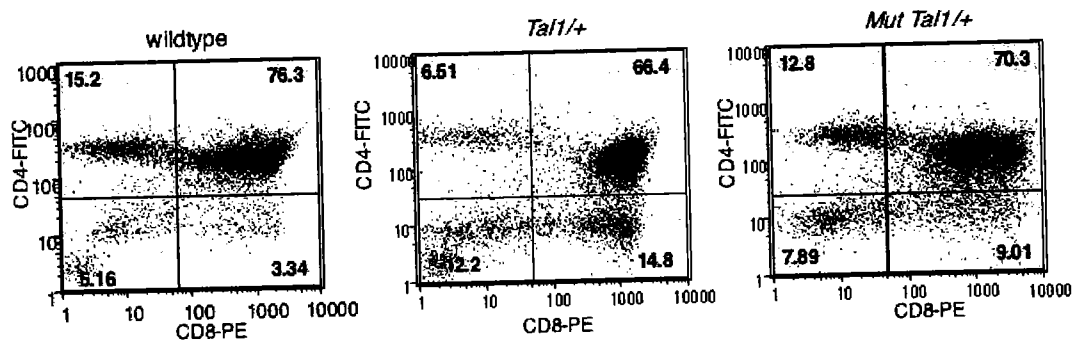


Figure 7. *Tal1* expression perturbs thymocyte development. Thymocytes from four week old wildtype, *Tal1*/+ and *Mut Tal1*/+ were stained with anti-CD4-FITC and anti-CD8-PE and analyzed by flow cytometry (A) The results shown are representative of more than ten experiments. Thymocytes from four-week-old wildtype and *Tal1*/+ mice were stained with anti-CD4 Tri-color, anti-CD8 Tri-color, anti-V γ 2 PE and anti-GL3 FITC and analyzed by flow cytometry. Staining with anti-V γ 2 PE and anti-GL3 FITC is shown on Tri-color-negative cells. DNA prepared from the thymus of four-week-old wildtype, *Tal1*/+, and *Mut Tal1*/+ mice was subjected to PCR with primers specific for the V γ 3 and J γ 1 loci. The PCR reactions were separated on a 2.2% Nusieve gel and blotted. The blot was then hybridized with an internal probe. A control PCR was performed on the TNFR1 locus (B).

A.



B.

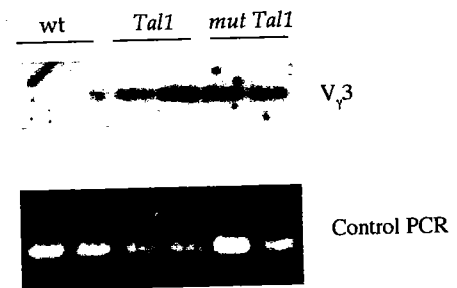
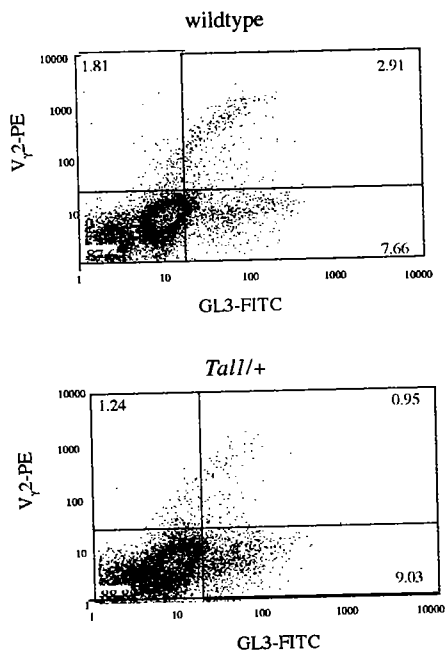
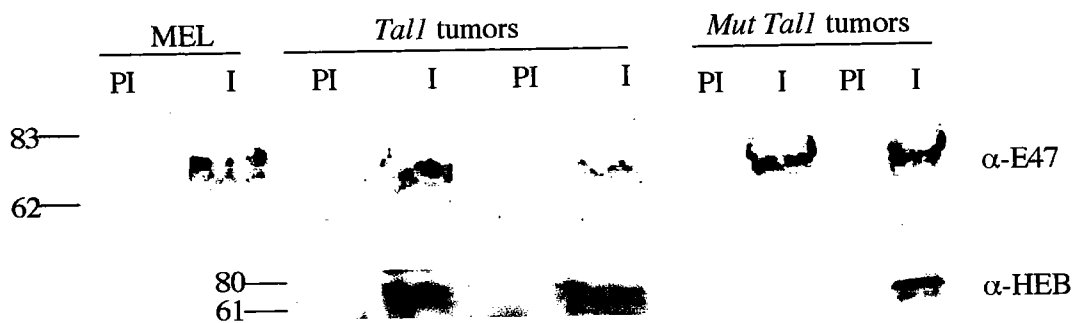
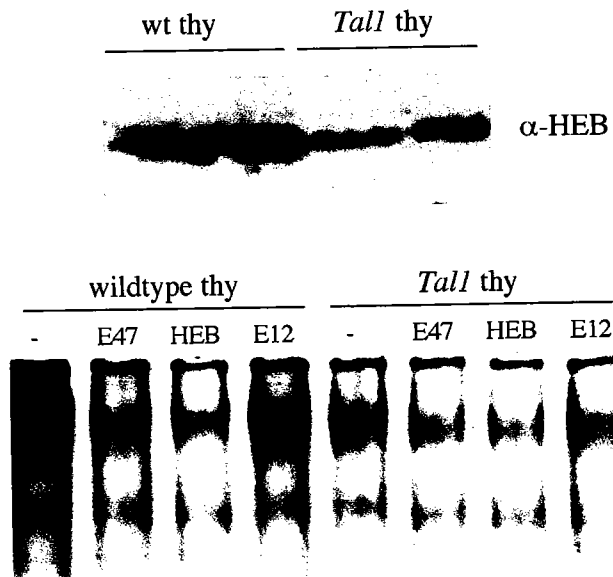


Figure 8. Wildtype Tal1 and mutant Tal1 form stable heterodimers with E2A proteins (A). Wildtype *Tal1* and mutant *Tal1* leukemic cell lines were lysed under low stringency conditions and the lysates were immunoprecipitated with either anti-Tal antiserum or the corresponding pre-immune serum. The samples were fractionated by SDS-PAGE and coprecipitating proteins detected by immunoblotting with an anti-E47 or an anti-E12/HEB monoclonal antibody. A murine erythroleukemic cell line was used as a positive control. **E47/HEB heterodimers are depleted in *Tal1* thymocytes (B).** Equal amounts of total protein lysates from wildtype and premalignant *Tal1* thymocytes were immunoprecipitated three times with anti-Tal1. The Tal1 depleted lysates were then immunoprecipitated with anti-E47 and the E47/HEB complexes were detected by immunoblotting with anti-E12/HEB. Nuclear extracts from wildtype thymocytes and *Tal1* premalignant thymocytes were incubated with a radiolabeled oligonucleotide probe corresponding to the murine immunoglobulin heavy chain gene enhancer (μ E5). In some cases, the reaction was supplemented with the indicated antiserum. The binding reactions were fractionated on a 5% nondenaturing, polyacrylamide gel and the DNA-protein complexes were detected by autoradiography. **Lmo2 expression is not required for *Tal1*-induced leukemia (C).** 293T cells were transfected with the EF1- α puro mammalian expression vector or with the vector expressing LMO2. Nuclear extracts containing an equal amount of protein from the Lmo2-transfected 293T cells, MEL cells, and *Tal1* tumor cells were resolved by SDS-PAGE and Lmo2 protein detected by immunoblotting with an anti-Lmo2 rabbit polyclonal antisera.

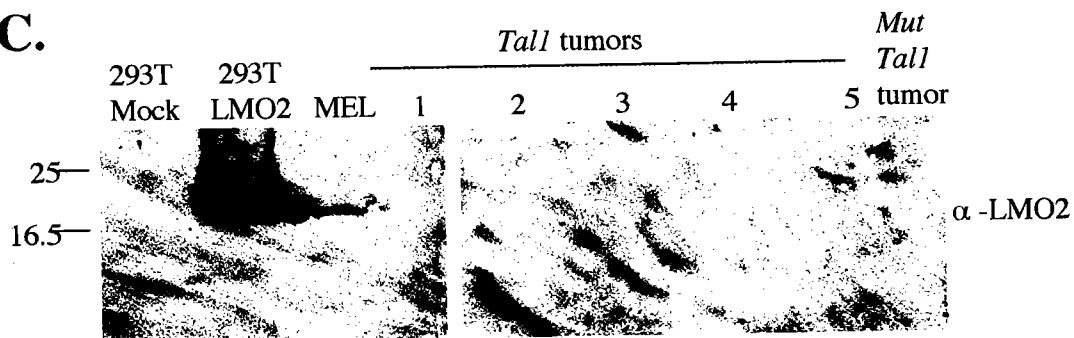
A.



B.



C.



**Table 2. Immunophenotypes of *Tal1* R188G;R189G and *Tal1* R188G;
R189G/CKII α tumors**

Animal	Genotype	% CD3+	% CD4+	% CD8+	Survival (days)
5721	<i>Mut Tal1/+</i>	15	1	98	139
5578	<i>Mut Tal1/+</i>	86	1	96	183
5591	<i>Mut Tal1/+</i>	33	3	86	115
5577	<i>Mut Tal1/+</i>	26	1	77	146
1326	<i>Mut Tal1/+</i>	59	2	3	147
5378	<i>Mut Tal1/+</i>	14	<1	<1	140
1320	<i>Mut Tal1/+</i>	72	4	<1	185
5495	<i>Mut Tal1/CKII</i>	34	3	30	51
5522	<i>Mut Tal1/CKII</i>	99	4	5	85
5535	<i>Mut Tal1/CKII</i>	5	1	20	83
5538	<i>Mut Tal1/CKII</i>	96	7	<1	55

Table contributed by M. Billa.

Table 3. Expression of wildtype *Tall* and *Tall* R188G;R189G (*Mut Tall*) perturbs thymocyte development.

Genotype	%CD4-,CD8-	%CD4+,CD8+	%CD4+	%CD8+	CD4:CD8
+/+	4	83	11	3	3.6
+/+	3	82	11	4	2.8
+/+	3	76	16	4	4.0
<i>Tall</i> /+	9	66	4	21	0.2
<i>Tall</i> /+	4	72	4	19	0.2
<i>Tall</i> /+	8	68	5	19	0.3
<i>Mut Tall</i> /+	7	73	10	11	0.9
<i>Mut Tall</i> /+	6	79	8	8	1.0
<i>Mut Tall</i> /+	6	79	6	10	0.6

CHAPTER III

TAL1 TRANSFORMS BY REPRESSING E47/HEB TRANSCRIPTIONAL ACTIVITY

Introduction

T cell acute lymphoblastic leukemia (T-ALL) accounts for 10-15% of pediatric and 25% of adult ALL cases (Ferrando et al., 2002). Activation of the basic helix-loop-helix TAL1 gene occurs by chromosomal translocation, interstitial deletion or mutation in over 60% of children and adults with T-ALL (Bash et al., 1995). In contrast to T-ALL induced by other oncogenes such as HOX11 or MLL-ENL, patients with TAL1 activation respond poorly to therapy with only 50% of patients surviving 5 years (Ferrando et al., 2002).

TAL1 heterodimerizes with class I or A bHLH proteins including E12, E47, HEB and E2-2 (Hsu et al., 1991; O'Neil et al., 2001; Voronova and Lee, 1994) and in hematopoietic cells, is part of a large transcriptional complex that includes Gata1 and the LIM-only proteins Lmo2 and Ldb1 (Valge-Archer et al., 1994; Wadman et al., 1997). Mice deficient for *Tal1* have no primitive or definitive hematopoiesis and exhibit angiogenic defects (Shivdasani et al., 1995; Visvader et al., 1998). Surprisingly, conditional inactivation of *Tal1* in adult mice does not result in defects in the lymphoid or myeloid lineages, suggesting that Tal1 is critical for the genesis of the hematopoietic stem cell (HSC), but not required for its maintenance (Mikkola, 2003). The function of Tal1 in the hematopoietic stem cell has suggested that *Tal1* activation in leukemia may stimulate the activation of genes important in stem cell expansion and/or self-renewal.

In mouse *Tal1* tumors and in Jurkat cells, a human leukemic cell line that expresses TAL1, stable Tal1/E47 and Tal1/HEB heterodimers are readily detected (Hsu et al., 1994b; O'Neil et al., 2001), and the related bHLH proteins LYL1 and BHLH1 may

contribute to leukemia by interfering with E protein function(s). Consistent with this idea, a percentage of surviving *E2A* deficient mice develop T cell leukemia/lymphoma(Bain et al., 1997; Yan et al., 1997). Disruption of *E2A* function is also believed to be the consequence of chromosomal translocations involving the LIM-only domain proteins, *LMO1* and *LMO2*, recently also found activated in gene therapy-induced T-ALL(Kohn et al., 2003; Rabbitts, 1998). Yet, how *LMO1/2* inhibit *E2A* function and contribute to leukemia remains unclear. In some leukemic patients, *LMO2* and *TAL1* are co-expressed(Ferrando et al., 2002), suggesting that leukemic transformation is dependent on the expression of both the *TAL1* and *LMO2* proteins. Consistent with this idea, a *TAL1/LMO2/E2A* complex is detected in a human T-ALL cell line(Ono et al., 1998) and leukemogenesis is observed in mice that express *Tal1* and *Lmo2*(Larson et al., 1996). However, not all human T-ALL patients that express *TAL1*, also express *LMO1* or *LMO2*(Ferrando et al., 2002). Similarly, only one of six mouse *Tal1* tumors examined express *Lmo2* (Jennifer Shank, unpublished data), revealing that *Lmo2* expression is also not required for *Tal1* induced leukemogenesis in the mouse.

To test whether *Tal1* induces leukemia by interfering with E protein function(s), we expressed the *Tal1* oncogene in an *E2A* or *HEB* heterozygous background. We observe thymocyte differentiation arrest and disease acceleration in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice, providing genetic evidence that *Tal1* induces leukemia by interfering with *E47/HEB*. Consistent with the differentiation arrest, gene expression profiling of premalignant *Tal1* thymocytes reveals repression of several genes important for thymocyte differentiation. The expression of the *E47/HEB* target genes, *Rag2* and pre-

T α are decreased, on a per cell basis, in premalignant *Tal1* cells and further decreased in *Tal1/E2A*^{+/-} thymocytes. Tal1 mediates gene repression by depleting the E47/HEB heterodimer and by recruiting the mSin3A/HDAC1 corepressor complex to target loci. The results of this study demonstrate that Tal1, like the leukemogenic fusion proteins PML-RAR α and AML-1/ETO, contributes to leukemia by repressing gene expression and inducing differentiation arrest.

Results

Thymocyte developmental perturbation in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice

Expression of *Tal1* in the thymus results in a 50% decrease in overall thymocyte cellularity (Figure 10A), a 3-4-fold decrease in double positive thymocytes, as well as decreases in CD4 single positive thymocytes. Increases in the immature CD8 single positive thymocytes are also observed. Analysis of thymocytes from *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice revealed severe decreases in double positive thymocytes (average 43% for *Tal1/E2A*^{+/-} and 40% for *Tal1/HEB*^{+/-}) and increases in CD8 single positive thymocytes, suggesting that *Tal1* expression in an *E2A* or *HEB* heterozygous background results in further decreases in DP and CD4 single positive thymocytes. Although thymocyte development is normal in *E2A*^{+/-} or *HEB*^{+/-} mice (Bain et al., 1997; Zhuang et al., 1996a) *Tal1* expression in an *E2A* or *HEB* heterozygous background results in 8-fold decreases in the absolute number of DP thymocytes (Figure 10B). The decrease in DP thymocytes observed in *Tal1* mice may reflect an inability to express adequate amounts of the CD4 co-receptor (Figure 10B and C). The expression of the CD4 co-receptor, TCR α and β chains and CD5 are decreased in mice deficient for *E2A* or *HEB*, suggesting that these are bona fide *E47/HEB* target genes in thymocytes (Zhuang et al., 1996; Bain et al., 1997). Consistent with the CD4 repression, we also observe 4-5-fold decreases in TCR β chain expression in thymocytes from *Tal1/E2A*^{+/-} mice (not shown) as well as decreased CD5 expression (Table 5 and Figure 13), suggesting that *Tal1* interferes with the *E47/HEB* heterodimer.

Increases in DN thymocyte precursors were also observed in *Tal1/E2A*^{+/-} or *Tal1/HEB*^{+/-} mice, suggesting that thymocyte development may be arrested in these mice. *Tal1* transgenic mice exhibit a partial thymocyte arrest at the DN2 stage of thymocyte development with increases in the CD44-positive, CD25-positive DN thymocytes (Figure 10D). We found DN thymocyte development severely affected in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice, with arrest at the DN2 and DN3 stages, respectively. *Tal1* expression in an *E2A* or *HEB* heterozygous background, induces differentiation arrest similar to that observed in *E2A* or *HEB* deficient mice (Bain et al., 1997; Barndt et al., 1999).

Disease acceleration in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice

We have previously demonstrated that thymocyte differentiation arrest is a central feature associated with the development of *Tal1* induced leukemia in the mouse (O'Neil et al., 2001; O'Neil et al., 2003). The severe thymocyte developmental arrest observed when *Tal1* is expressed in an *E2A* or *HEB* heterozygous background suggested to us that leukemogenesis may be accelerated in these mice.

Tal1/E2A^{+/-} and *Tal1/HEB*^{+/-} mice develop disease rapidly within a median survival period of 216 days ($p < 0.0001$) and 143 days ($p < 0.0001$), respectively (Figure 11). In contrast, *Tal1/E2A*^{+/+} and *Tal1/HEB*^{+/+} littermates develop disease at a similar frequency as *Tal1* transgenic mice (Kelliher et al., 1996). Thus, disease acceleration reflects effects of *Tal1* on *E2A* and *HEB* proteins and does not appear to reflect genetic

differences between the strains of mice. Meanwhile, leukemia is not observed in *E2A* or *HEB* heterozygous mice.

In addition, we observed a highly significant increase in disease penetrance in both the *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice compared to *Tal1* transgenic mice. T cell lymphoblastic leukemia is completely penetrant in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice whereas, only 28% of *Tal1* transgenic mice develop disease in one year (Kelliher et al., 1996).

Upon necropsy, all animals exhibited lymphoblastic cells in the peripheral blood and thymic masses, often accompanied by hepatosplenomegaly and lymphadenopathy. Histopathological examination of the thymus revealed effacement of the normal thymic architecture and the proliferation of lymphoblastic cells with prominent nucleoli and scant cytoplasm. The histological appearance of the thymic tumors were indistinguishable from that previously observed in *Tal1* transgenic mice (Kelliher et al., 1996). In addition, examination of other tissues revealed evidence of tumor infiltration to organs such liver and kidney.

Tumors induced are clonal or oligoclonal and display a variety of immunophenotypes

The disease acceleration observed when *Tal1* is expressed in an *E2A* or *HEB* heterozygous background, suggested to us that polyclonal tumors may develop in these mice. To examine this possibility, DNA isolated from *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} tumors was digested with *Hind*III and analyzed by Southern blot analysis with a TCR β 2 probe (Figure 12A).. All tumors analyzed were clonal or oligoclonal and in most cases

both TCR β alleles were rearranged. Similar to what is observed in *E2A* deficient mice (Bain et al., 1997), clonal or oligoclonal tumors were observed in *Tal1/E2A*^{+/-} or *HEB*^{+/-} mice, revealing that a deficiency of *E2A* and *HEB* proteins is not sufficient to induce leukemia in these mice and that additional genetic changes are required.

Tumors from *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice were also analyzed by flow cytometry to determine the phenotype of the tumor target cell. All tumors were of T cell origin but appear to be at various stages of thymocyte development (Table 4). Diverse tumor phenotypes were also observed in *Tal1* transgenic mice (Kelliher et al., 1996). However, about twenty percent of the *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} tumors failed to express CD3, CD4 and CD8, indicating that a more immature cell type may be transformed in some of the *Tal1/E2A*^{+/-} or *HEB*^{+/-} mice.

Disease acceleration is not accompanied by loss of heterozygosity in *Tal1/E2A*^{+/-} mice

The observation that a percentage of *E2A* deficient mice are susceptible to the development of T cell leukemia/lymphoma led to the idea that the *E2A* locus may act as a lymphoid-specific tumor suppressor (Bain et al., 1997; Yan et al., 1997). Hence, tumors that develop in *Tal1/E2A*^{+/-} mice may exhibit loss of heterozygosity (LOH). To examine this possibility, we isolated DNA from tumors derived from *Tal1/E2A*^{+/-} mice and analyzed the *E2A* locus by Southern blotting (Figure 12B). All *Tal1/E2A*^{+/-} tumors tested retained the wild type allele of *E2A* indicating that LOH is not a feature associated with disease acceleration in *Tal1/E2A*^{+/-} mice. However, loss of *E2A* expression in the

Tal1/E2A^{+/-} tumors could involve methylation of regulatory sequences. To confirm that the remaining *E2A* allele is expressed, we prepared nuclear lysates from *Tal1/E2A*^{+/-} tumors and were able to detect E47 expression by immunoblotting (not shown). Similar results were obtained on tumors from *Tal1/HEB*^{+/-} mice, indicating that *Tal1* is not simply cooperating with loss of *E2A* or *HEB* proteins to induce leukemia in mice.

Thymic expression of the *Tal1* oncogene is associated with gene repression

To identify potential target genes deregulated by *Tal1* activation, we performed gene expression profiling of premalignant *Tal1* thymocytes using high density DNA microarrays. We isolated RNA from sorted CD4- and CD8-positive premalignant thymocytes and used it to interrogate Affymetrix DNA microarrays, representing 6,000 known genes and 6,000 EST clusters. Transcription profiles of sorted double positive thymocytes from age-matched, wildtype and *Tal1* transgenic mice were compared. Consistent with the differentiation arrest observed in the *Tal1* transgenic mice (O'Neil et al., 2003), we found the lymphoid specific-cyclin D3 decreased in thymocytes that expressed *Tal1* (Table 5). Moreover, the expression of several genes important in thymocyte differentiation were also reduced in *Tal1* thymocytes, including those encoding CD3, CD6, CD5, Rag1, Rag2 and ROR γ (Figure 13 and data not shown). Some of the genes repressed in *Tal1* thymocytes have been previously thought to be regulated by E47/HEB heterodimer, notably Rag1/2 and CD5 (Bain, 1994; Schlissel, 1991; Zhuang et al., 1996b). Other genes such as the retinoid-related orphan receptor γ (ROR γ) have been shown to be important in thymocyte development (He, 2002; Littman

et al., 1999; Sun et al., 2000), but have not been linked to E47/HEB regulation. Interestingly, ROR γ deficient mice exhibit decreases in double positive and CD4 single positive thymocytes and develop T cell leukemia/lymphoma at high incidence (Sun et al., 2000; Ueda et al., 2002). To confirm whether ROR γ expression is decreased in premalignant *Tal1* thymocytes, we prepared total cell lysates from thymocytes from *Tal1* transgenic and control littermates. We found ROR γ levels decreased in cells that express *Tal1* compared to thymocytes from control littermates (Figure 13D). Moreover, repression of ROR γ expression was maintained in all seven of the *Tal1* tumors examined, suggesting that ROR γ repression may be an important in leukemogenesis. Although not previously implicated as an E47/HEB target gene, conserved, tandem E box sequences are present in the regulatory region of the mouse and human ROR γ genes, suggesting that ROR γ may also be regulated by the E47/HEB heterodimer.

The E47/HEB target genes Rag2 and pre-T α are reduced on a per cell basis in *Tal1* and *Tal1/E2A*^{+/-} DN thymocytes

Our microarray experiment suggests that *Tal1* expression affects the expression of Rag1/Rag2 recombinases, required for T and B cell development (Shinkai et al., 1992). To validate these findings and to test whether Rag 2 expression is also affected in *Tal1* DN precursors, we mated our *Tal1* transgenic mice to mice in which GFP has been introduced into the mouse *Rag2* locus by homologous recombination (Yu, 1999). Thymocytes from age-matched, premalignant *Tal1* mice were stained with antibodies against CD4 and CD8 and the GFP levels in the various subpopulations were examined.

The mean fluorescence intensity (MFI) of the Rag2 driven GFP was consistently reduced on a per cell basis in *Tal1* double positive thymocytes (Figure 14A). The mean fluorescence intensity was also reduced in double negative thymocytes compared to control littermates (Figure 14B; MFI=659 for wildtype, compared to MFI=438 for *Tal1* thymocytes), indicating reduced Rag2 expression in *Tal1* double negative thymocytes. If *Tal1* functions by inhibiting E47/HEB-mediated transcription of Rag2, then one might predict further decreases in Rag2 expression in *Tal1/E2A*^{+/-} or *Tal1/HEB*^{+/-} premalignant thymocytes. To test this possibility, we mated our *Tal1/E2A*^{+/-} mice to the Rag2-GFP mice. In all mice examined, we found Rag2-driven expression of GFP further reduced in the double negative thymocytes from *Tal1/E2A*^{+/-} mice compared to *Tal1* transgenic mice, indicating further E47/HEB inhibition in *Tal1/E2A*^{+/-} mice.

In addition to the recombinase genes Rag1/2, the E47/HEB heterodimer has also been implicated in regulation of the pre-T α chain of the pre T receptor, required for DN thymocyte expansion and survival (Herblot et al., 2000; Petersson, 2002; Takeuchi et al., 2001; Tremblay et al., 2003). To determine whether *Tal1* affects the expression of pre-T α , we used a similar strategy and mated our *Tal1* transgenic mice to mice in which the expression of GFP is under the control of the pre-T α promoter (Reizis, 2001). We observe a 2-3-fold decrease in the pre-T α -driven GFP expression in virtually all DN3 thymocytes in *Tal1* transgenic mice and a further reduction in *Tal1/E2A*^{+/-} mice (Figure 14C). The reduced transcription of both Rag2 and pre-T α in nearly all *Tal1* thymocytes and the further reduction in *Tal1/E2A*^{+/-} thymocytes suggests that the DN3 arrest is mediated by reduced Rag2 and pre-T α expression.

Tal1 recruits the corepressor mSin3A to the CD4 enhancer

Our previous work demonstrated the presence of stable Tal1/E47 and Tal1/HEB heterodimers in pre-leukemic thymocytes and tumors isolated from *Tal1* transgenic mice (O'Neil et al., 2001). Thus, Tal1 may repress E47/HEB target genes by depleting E47/HEB heterodimers or by modifying E47/HEB activity.

To determine whether the Tal1/E47 or HEB heterodimers are localized to regions of gene repression, we used chromatin immunoprecipitation to ask whether E47/HEB or Tal1/E47 heterodimers bound the regulatory regions of the CD4 gene. Thymocytes from age-matched, pre-leukemic *Tal1* and wildtype mice were treated with formaldehyde and lysates from these cells were immunoprecipitated using antibodies against Tal1, E2A, and with an anti-RIP antibody. PCR was performed on the immunoprecipitated DNA using primers that flank the tandem E-box consensus sequences of the mouse CD4 enhancer (Sawada and Littman, 1993). A 200 bp fragment was amplified from DNA immunoprecipitated with an anti-E2A antibody from wildtype thymocytes. The 200 bp fragment was also detected when *Tal1* thymocyte DNA was immunoprecipitated with an anti-Tal1 or anti-E2A antibodies, indicating that the Tal1/E47 heterodimer occupies the CD4 enhancer (Figure 15A). These studies reveal that Tal1 does not function like an Id protein and inhibit E47/HEB mediated transcription by sequestering E47 into non-DNA binding complexes.

Previous work has demonstrated that Tal1 protein associates with mSin3A in undifferentiated MEL cells (Huang and Brandt, 2000). Thus, we hypothesized that Tal1

may cause gene repression in leukemia by displacing coactivators with corepressor complexes. To test this possibility, we performed additional chromatin immunoprecipitation experiments using antibodies for the coactivator p300 and the corepressor mSin3A. In wildtype thymocytes, the 200bp band is amplified from DNA immunoprecipitated with an anti-p300 antibody, indicating that the coactivator p300 is also bound to the CD4 enhancer sequence. E proteins have been shown to interact with histone acetyltransferases and the transcriptional coactivators p300, CBP, and PCAF (Bradney et al., 2002; Eckner, 1996). Consistent with the CD4 repression observed in the pre-leukemic *Tall* thymocytes (Figure 10), the coactivator p300 is not detected at the CD4 locus when *Tall* thymocytes are immunoprecipitated. Rather, the corepressor, mSin3A is readily detected bound to the CD4 enhancer (Figure 15B). These results suggest that *Tall* represses CD4 transcription by binding to the E box sequences in the CD4 enhancer and bringing in the corepressor mSin3A. Consistent with its association with the mSin3A corepressor complex (Zhang et al., 1997), the histone deacetylase HDAC1 is detected at the CD4 locus when *Tall* preleukemic thymocytes, but not wild type thymocytes, are immunoprecipitated (Figure 15C).

The recruitment of mSin3A/HDAC1 complex to the CD4 locus and potentially other E47/HEB target genes suggested to us that mouse *Tall* tumors may be sensitive to HDAC inhibitors. Treatment with the HDAC inhibitor TSA induced apoptosis in all six mouse *Tall* tumors tested, resulting in the death of 75 to 99% of the treated tumor cells (Figure 15D). HDAC inhibition may stimulate *Tall* tumor cell apoptosis via direct effects on E47/HEB transcriptional activity. Consistent with its function as a lymphoid

specific tumor suppressor, ectopic E47 expression in T cell tumors that arise in *E2A*^{-/-} mice, results in apoptosis (Engel and Murre, 1999), suggesting that in addition to its roles in differentiation, the E47/HEB heterodimer also has proapoptotic activities.

Discussion

Although frequently activated in human T-ALL patients, the mechanism(s) by which *Tal1* contributes to leukemia/lymphoma remains unclear. Studies suggest that *Tal1* may transactivate the expression of novel target genes in leukemia (Cohen-Kaminsky et al., 1998; Hsu et al., 1994c) and that the LIM-only protein *Lmo2* is required for *Tal1* induced leukemogenesis (Larson et al., 1996). We demonstrate that *Tal1* transforms mouse T cells in the absence of *Lmo2* activation by inducing differentiation arrest and by interfering with E47/HEB function(s). Both *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice develop disease rapidly and with complete penetrance. Disease acceleration is accompanied by induction of a more severe thymocyte developmental arrest in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice compared to transgenic mice expressing *Tal1* or a DNA binding mutant of *Tal1* (O'Neil et al., 2001; O'Neil et al., 2003). Thus, this work also reveals that differentiation arrest is central for disease development and suggests E2A proteins may directly regulate the cell cycle in thymocyte precursors.

We found expression of the E47/HEB target genes CD4, TCR β and CD5 decreased in *Tal1* DP thymocytes and Rag2 and pre-T α decreased in *Tal1* DN thymic precursors. The expression of Rag2 and pre-T α are further reduced in *Tal1/E2A*^{+/-} or *Tal1/HEB*^{+/-} mice, suggesting that decreased expression of these genes may be responsible for the severe differentiation arrest and disease acceleration observed when *Tal1* is expressed in an *E2A* or *HEB* heterozygous background. Consistent with these studies, our gene expression profiling reveals evidence of gene repression during the pre-leukemic phase of the disease.

E47/HEB heterodimers appear to regulate CD4 expression in part by recruiting the coactivator p300 to the enhancer. In contrast, CD4 expression is reduced in pre-leukemic *Tal1* thymocytes and further reduced in *Tal1/E2A*^{+/-} or *HEB*^{+/-} thymocytes, supporting the idea that E47/HEB activity is diminished in these mice. In addition to depleting the E47/HEB heterodimer, Tal1 represses E47/HEB target gene expression by recruiting the mSin3A/HDAC1 corepressor to target loci. This observation provides a new mechanism to explain how Tal1 contributes to leukemogenesis. Rather than operating like an Id and inhibiting the ability of E proteins to bind DNA, Tal1 directly represses gene transcription by recruiting corepressor complexes to the E47/HEB target gene, CD4. It seems likely that decreased expression of the other E47/HEB target genes including TCR α and β , CD5, Rag2 and pre-T α may also be mSin3A/HDAC1-mediated. The fact that all the mouse *Tal1* tumors examined were highly sensitive to HDAC inhibitors raises the possibility that T-ALL patients with *TAL1* activation may also be responsive to HDAC inhibitors. This is an important finding as many of these patients fail on modern combination chemotherapy and specific therapies are urgently needed (Ferrando et al., 2002). Finally, Tal1 repressive effects may not be limited to genes regulated by E47/HEB and may include other genes that regulate proliferation and survival.

A critical remaining question is how developmental perturbation induced by Tal1 expression predisposes thymocytes to leukemia. One possibility may be that additional mutations are incurred during the DN arrest, where the thymocyte precursor undergoes extensive cell divisions. Consistent with this idea, increased cell cycling is observed in

E2A-deficient DN3 precursors, suggesting that E2A proteins function as cell cycle inhibitors in thymic precursors(Engel and Murre, 2003). In addition to aberrant cell cycling, survival pathways may also be activated during the differentiation arrest and maintained throughout leukemic progression. The anti-apoptotic transcription factor NF- κ B is found activated in arrested *Tall* thymocytes and NF- κ B activity is maintained in mouse *Tall* tumors, and observed in a majority of human T-ALL samples(Kim et al., 2002; Kordes et al., 2000; O'Neil et al., 2003). Identification of the anti-apoptotic NF- κ B target genes in *Tall* leukemic cells will be the focus of future work.

Materials and Methods

Mice and tumor cell culture

Proximal lck-Tal1 transgenic mice have been described previously (Kelliher et al., 1996). *E2A* heterozygous mice and *HEB* heterozygous mice were generously provided by Dr. Cornelius Murre (UCSD) and Dr. Yuan Zhuang (Duke) respectively. B6 *E2A* and *HEB* heterozygous mice were backcrossed with FVB/N mice for three generations before being mated to *Tal1* transgenic mice for disease study. Disease development was monitored in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice and compared to littermate controls. *Rag2-GFP* mice were provided by Dr. Michel Nussenzweig (Rockefeller University) and *pre-Ta-GFP* mice were provided by Drs. Boris Reizis and Philip Leder (Harvard Medical School). *Tal1* tumor cell lines were plated at 1×10^6 cells/ml and left untreated or treated with trichostatin A (Sigma) at a concentration of 90nM. Cell viability was assessed 24 hours after treatment by trypan blue exclusion.

Flow Cytometry

Thymocytes from four week old, age-matched, wildtype, *Tal1*, *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice were stained with FITC-conjugated anti-mouse L3T4 (CD4) and PE-conjugated Ly-2 (CD8) (Pharmingen). For double negative analysis, cells were stained with antibodies for the lineage markers and the lineage negative cells were stained with CD25-PE and CD44-FITC and analyzed by flow cytometry.

Tumor DNA analysis

For clonality studies, southern blots of *HindIII*-digested DNA obtained from primary tumors were hybridized with a ³²P-labeled 2 kb *EcoRI* fragment containing the murine

TCR J β 2B exon (Malissen et al., 1984). Blots were washed in 1XSSC, 1%SDS, followed by a higher stringency wash containing 0.1XSSC, 0.1%SDS. For loss of heterozygosity analysis, DNA from primary tumors and from tails from the same mice was digested with BamHI and hybridized with a ³²P-labeled *EcoRI/XbaI* fragment of an E2A genomic clone (generously provided by Dr. Yuan Zhuang Duke University).

Microarray Analysis

RNA was prepared from sorted CD4-positive, CD8-positive thymocytes from four week old age-matched wildtype and *Tall* transgenic mice using Trizol reagent (Invitrogen). cDNA was then synthesized from the RNA samples using the Superscript system (Gibco). Biotin labeled cRNA was subsequently made from the cDNA using a RNA transcript labeling kit (Enzo). The labeled cRNA was fragmented and hybridized to the Affymetrix mouse U74Av2 array.

Western and Northern Blotting

Tall tumor cell lines and thymi from four-week-old *Tall*/+ transgenic mice and control littermates were lysed in RIPA buffer. Equivalent amounts of total protein lysates were resolved on a SDS-PAGE gel. Protein levels were detected by immunoblotting with anti-ROR γ (generously provided by Dr. Daniel Littman, New York University School of Medicine). Blots were then stripped and reprobed with anti- β -actin (Sigma) to control for equal protein loading. CD6 expression levels were determined by preparing total RNA from the thymus of four-week-old *Tall* mice and control littermates as well as from *Tall* tumor cells lines. The RNA was electrophoresed on a 1% agarose gel and transferred to a

membrane which was probed with the mouse CD6 cDNA (generously provided by Dr. David Fox, University of Michigan Medical Center).

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed using the chromatin immunoprecipitation assay kit from Upstate (Lake Placid, NY). For each immunoprecipitation, 2×10^7 wildtype or *Tall* thymocytes were treated with formaldehyde at 37°C for ten minutes. The cells were then lysed and the DNA was sheared by sonication. Cellular debris was removed by centrifugation. The samples were precleared with salmon sperm DNA/protein agarose slurry and then incubated overnight with either no antibody or with antibodies against RIP (Transduction Laboratories), p300 (Santa Cruz N-15), mSin3A (Santa Cruz K-20), Tall, or E2A (generously provided by Dr. Richard Baer, Columbia University). The DNA-protein complexes were recovered by incubation with salmon sperm DNA/protein agarose slurry. The beads were washed and the chromatin was eluted by incubation in 1%SDS, 0.1M NaHCO₃. The protein-DNA crosslinks were reversed by heating at 65°C for four hours. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR was performed on the samples using primers specific for the CD4 enhancer region containing tandem E-box consensus sites. To increase sensitivity, 0.25µl of 10mCi/ml $\alpha^{32}\text{P}$ -CTP was added for the last five cycles of each PCR reaction. The reactions were separated by electrophoresis on a 6% acrylamide gel. The gel was then dried and exposed to film.

Figure 10. Thymocyte developmental perturbation is more severe in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} than in *Tal1* transgenic mice. Total thymocyte cell numbers from four-week-old, wildtype and *Tal1*, *Tal/Tal*, *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice (A). *Tal1* expression in an *E2A*^{+/-} or *HEB*^{+/-} background leads to a reduction in DP and CD4-positive cells. The absolute numbers of DP and CD4⁺ SP cells in wt, *Tal1*, *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} in pre-leukemic, four-week-old mice are represented (B). Thymocytes from four-week-old wt, *Tal1*, *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice were stained with CD4-Cy and CD8-FITC and analyzed by flow cytometry (C). Thymocytes from *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} mice are partially arrested at the double negative precursor stage. Cells were stained with antibodies for the lineage markers, IgM, Ter119, Gr1, Mac1, PanNK, CD3, CD4, and CD8 as well as CD25-PE and CD44-FITC. The staining of lineage negative cells with CD25-PE and CD44-FITC is shown (D). The results shown are representative of six experiments. Thymocyte development in *E2A* and *HEB* heterozygous mice is normal.

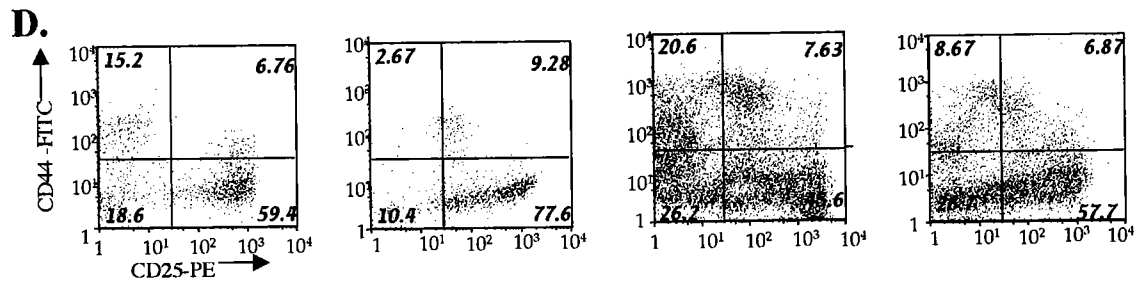
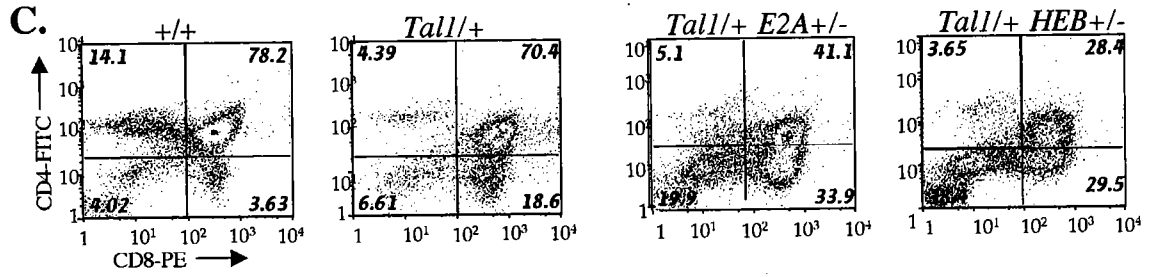
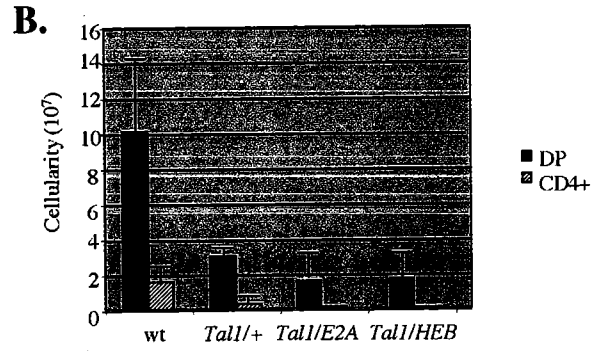
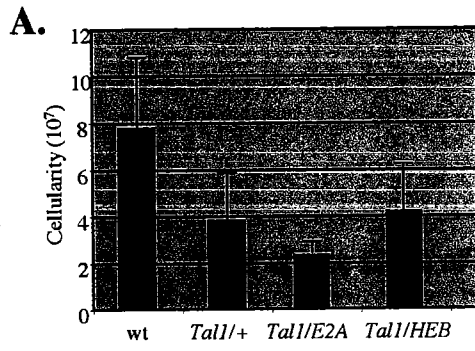


Figure 11. Loss of one allele of *E2A* or *HEB* leads to disease acceleration in *Tal1* transgenic mice. Kaplan-Meier survival plot of *Tal1*, *Tal1/E2A*^{+/-}, and *Tal1/HEB*^{+/-} mice. The cohort of *Tal1* mice consisted of n=75 animals, the *Tal1/E2A*^{+/-} cohort consisted of n=102 animals, and the *Tal1/HEB*^{+/-} cohort consisted of n=114 animals. Tarone-Ware statistical analysis confirmed a highly significant difference in survival between *Tal1/E2A*^{+/-} (p<0.0001) and *Tal1/HEB*^{+/-} (p<0.0001) compared to *Tal1* mice. The *Tal1/E2A* and *Tal1/HEB* survival curves were also significantly different from each other, p=0.0006. All animals were monitored daily for signs of disease. Upon onset of disease the mice were sacrificed and a post-mortem examination was performed.

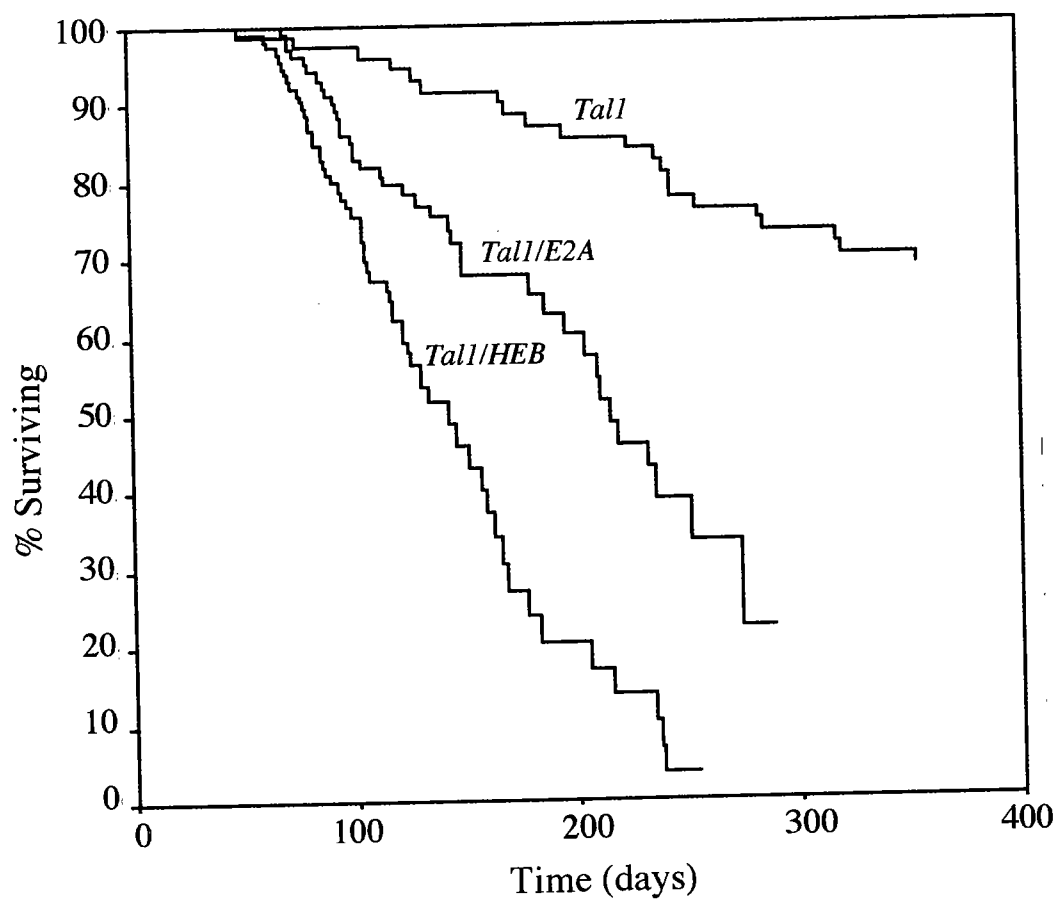


Figure 12. *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} tumors are clonal or oligoclonal and do not exhibit loss of heterozygosity. DNA prepared from tumors and wildtype genomic tail DNA was digested with HindIII and analyzed by Southern blot analysis. T cell receptor J β chain rearrangements were detected with a probe that identified a 5kb DNA fragment in the germline position of genomic tail DNA (lane T)(A). Disease acceleration is not accompanied by loss of heterozygosity in *Tal1/E2A*^{+/-} mice. *E2A*^{+/-}, *E2A*^{-/-} tail DNA, *Tal1/E2A*^{+/-} tail DNA, and DNA prepared from tumors from the same *Tal1/E2A*^{+/-} mice was digested with BamHI and analyzed by Southern blot analysis. Using a portion of an *E2A* genomic clone as a probe, the wildtype *E2A* allele was identified as a 13kb fragment and the mutant allele as a 10kb fragment (B). Panel A was contributed by N. Cusson.

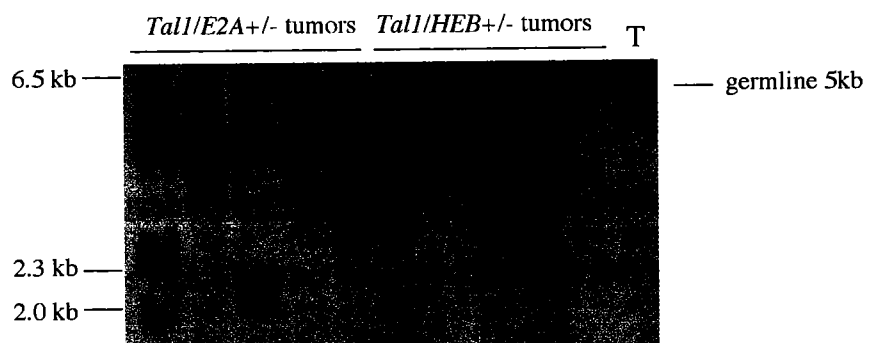
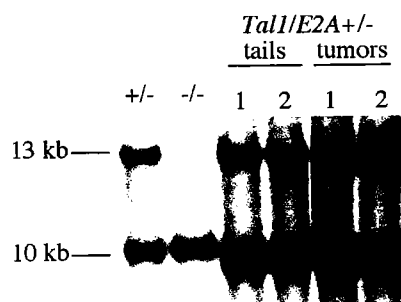
A.**B.**

Figure 13. Gene repression in *Tall* transgenic thymocytes. Thymocytes from four-week-old age-matched wildtype and *Tall* transgenic mice were stained with anti-CD5 Cy or with anti-CD3 PE and analyzed by flow cytometry. The results shown are representative of five experiments (A), (B). Total RNA was isolated from wildtype, *Tall* premalignant thymocytes and from *Tall* tumors. The RNA was electrophoresed on a 1% agarose gel and transferred to a membrane that was probed with the mouse CD6 cDNA (C). ROR γ expression is decreased in premalignant *Tall* thymocytes and tumors. Twenty-five micrograms of protein from wildtype, premalignant *Tall* thymocytes and *Tall* tumors was separated on a 10% SDS-PAGE gel and transferred to a membrane. The membrane was then probed with anti-ROR γ . The blot was then stripped and reprobed with an antibody against β -actin to control for protein loading (D).

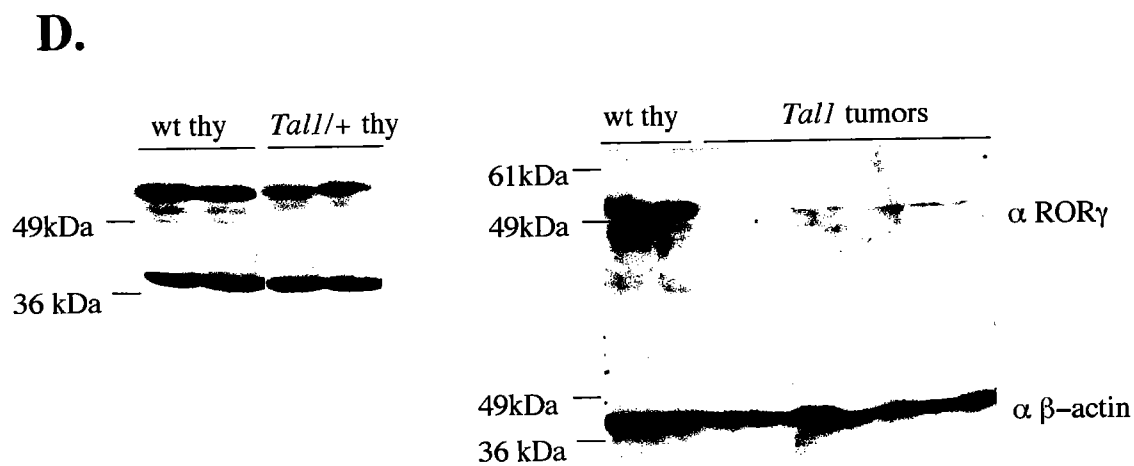
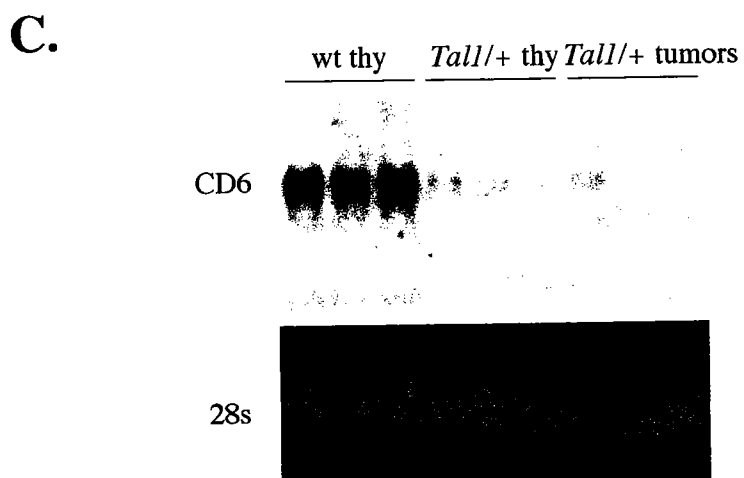
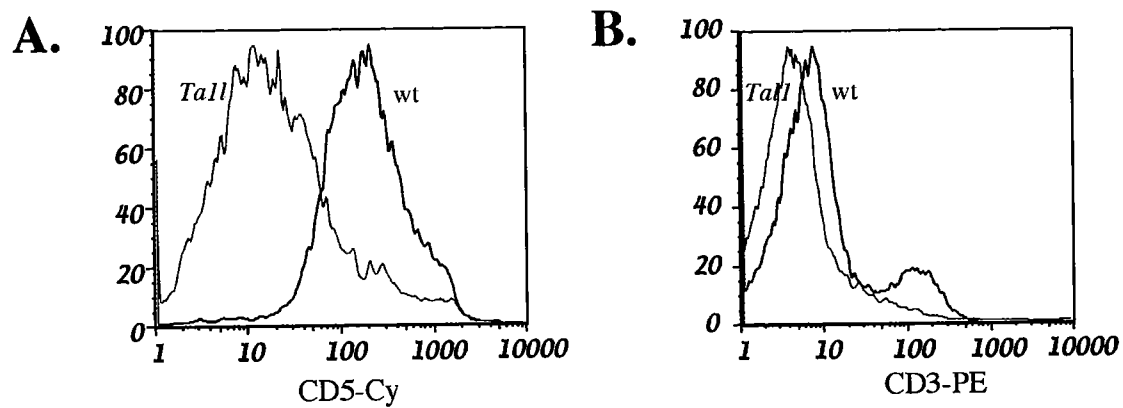
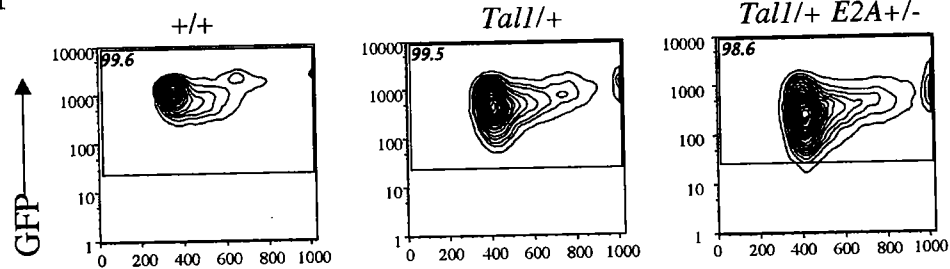


Figure 14. Rag2 and pre-T α expression is reduced in *Tal1/E2A*^{+/-} mice. Thymocytes from four-week-old age-matched *Rag2-GFP*, *Tal1/Rag2-GFP* and *Tal1/E2A*^{+/-}/*Rag2-GFP* mice were stained with anti-CD4 PE and anti-CD8 Cy and analyzed by flow cytometry. The mean fluorescent intensity of GFP was determined for the double positive thymocyte population (A) and double negative thymocyte population(B). Thymocytes from four-week-old age-matched *preT α -GFP*, and *Tal1/preT α -GFP* mice were stained with antibodies for the lineage markers, IgM, Ter119, Gr1, Mac1, PanNK, CD3, CD4, and CD8 as well as CD25-PE and CD44-FITC. The mean fluorescent intensity of GFP was determined for the DN3 population (C). The results shown are representative of three experiments.

A.

DP



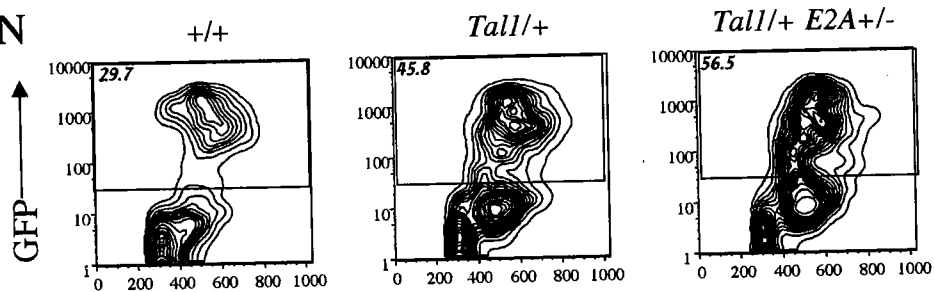
Geometric Mean: 1087

448

278

B.

DN



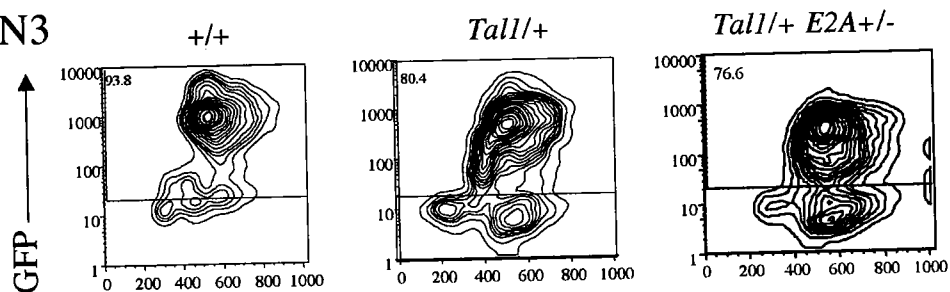
Geometric Mean: 659

438

331

C.

DN3



Geometric Mean: 747

323

184

Figure 15. Tal1 recruits the corepressor mSin3A to the CD4 locus and Tal1 tumors are sensitive to the HDAC inhibitor, TSA. Chromatin immunoprecipitation assays were performed on thymocytes from four-week-old wildtype and pre-leukemic *Tal1* mice using antibodies against E2A and Tal1. No antibody and anti-RIP immunoprecipitations were used as negative controls. Input sheared DNA served as a positive control. Thirty cycles of PCR amplification were performed on the immunoprecipitated DNA with primers specific for the CD4 enhancer region (A). Chromatin immunoprecipitation was performed with antibodies to the corepressor mSin3A or coactivator p300 or with negative control antibodies (B). Chromatin immunoprecipitation demonstrates that HDAC1 also binds to the CD4 enhancer in *Tal1* thymocytes (C). The results shown are representative of five experiments. Six *Tal1* tumor cells were left untreated or treated with TSA for 24 hours and tumor cell viability determined by trypan blue exclusion. The average percentage of cell death is shown with standard deviation for five experiments (D).

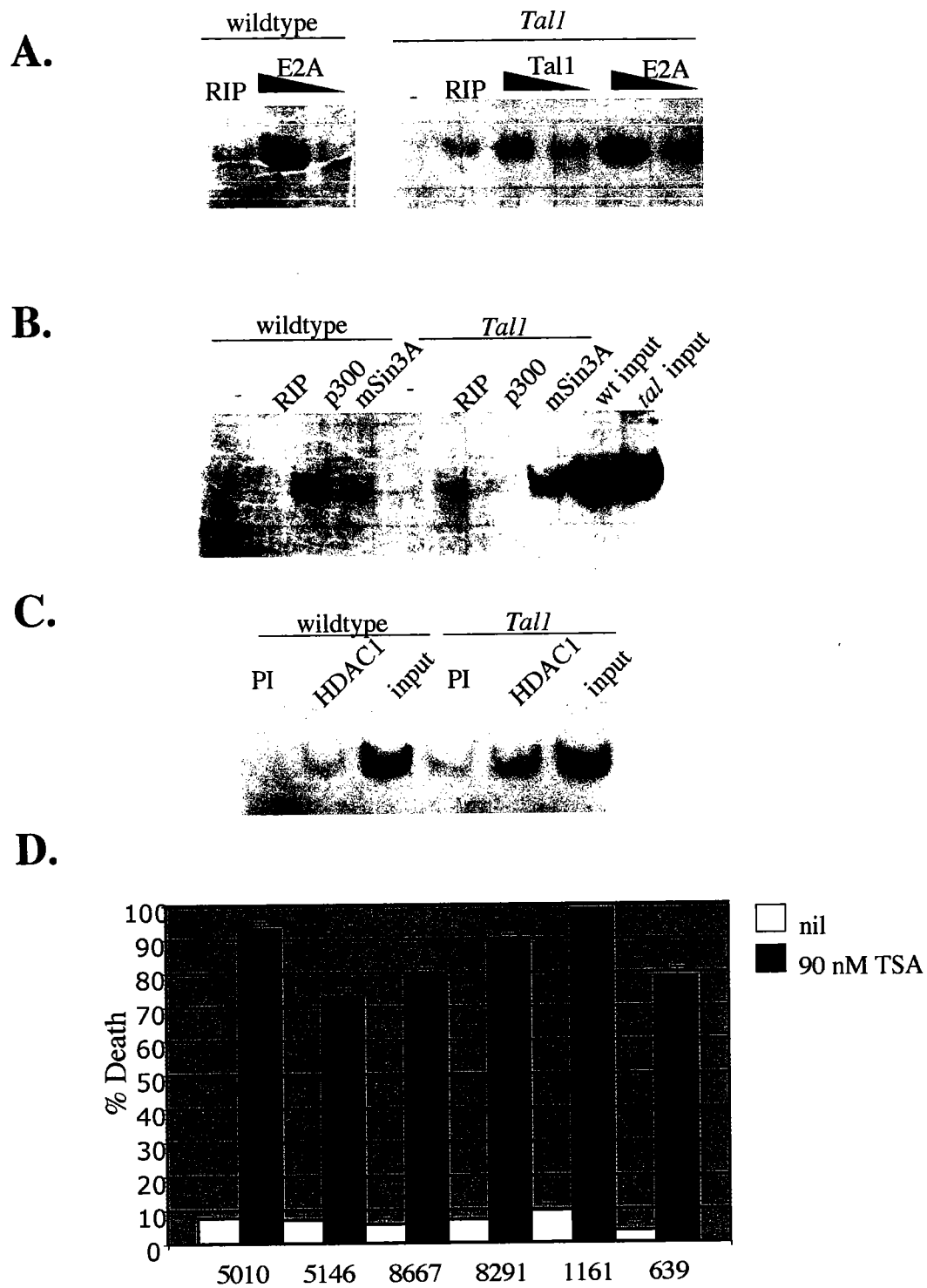


Table 4. Immunophenotypes of *Tal1/+ E2A+/-* and *Tal1/+ HEB+/-* tumors

Animal Number	Genotype	CD3	CD4	CD8	CD4,CD8	Survival (Days)
737	<i>Tal1/+ E2A+/-</i>	4	12	4	8	74
6778	<i>Tal1/+ E2A+/-</i>	2	0	43	0	85
6831	<i>Tal1/+ E2A+/-</i>	32	0	97	2	104
8583	<i>Tal1/+ E2A+/-</i>	32	1	71	0	89
388	<i>Tal1/+ E2A+/-</i>	1	0	0	0	92
9845	<i>Tal1/+ E2A+/-</i>	31	90	93	78	144
135	<i>Tal1/+ HEB+/-</i>	45	17	98	18	105
138	<i>Tal1/+ HEB+/-</i>	69	8	97	8	100
8959	<i>Tal1/+ HEB+/-</i>	4	29	93	30	116
9193	<i>Tal1/+ HEB+/-</i>	5	19	46	18	81
9205	<i>Tal1/+ HEB+/-</i>	26	70	92	70	70
9232	<i>Tal1/+ HEB+/-</i>	8	74	98	75	83
8906	<i>Tal1/+ HEB+/-</i>	93	1	6	0	67

Table 5. Genes Activated or Repressed by Tal1 Expression in Thymus

Description of the Gene (Accession Number)	Fold Change
GM2 activator protein (U09816)	+7.3
Homeobox protein Pknox1 (AF061270)	+4.7
CD3 Antigen (M23376)	-3
Recombination Activating Gene 1 (M29475)	-3.9
Cyclin 3 (M86183)	-4.2
Dishevelled 2, dsh homolog((U24160)	-5.1
RAR-related orphan receptor gamma (AF019660)	-5.6
Cdc2/CDC-28-like protein kinase 3 (AF033565)	-6.8
Recombination Activating Gene 2 (M64796)	-6.9
Interleukin 4 Receptor (M27960)	-8.1
Zinc finger protein (U14556)	-9.2
HOX-4.4 and HOX-4.5 (X62669)	-10.6
G-protein coupled receptor 6-B(Y15798)	-14.7
CD6 antigen (U12434)	-16
CD5 antigen (M15177)	-17.5

CHAPTER IV**NF- κ B ACTIVATION IN PREMALIGNANT MOUSE
TAL1 THYMOCYTES AND TUMORS**

Introduction

The transcription factor NF- κ B is constitutively active in breast, colon and gastric cancers and in lymphoid and myeloid malignancies. Initially discovered as a viral oncogene in an avian retrovirus, NF- κ B genes have been increasingly implicated in the development of malignancy (Rayet and Gelinas, 1999).

The NF- κ B gene family consists of *Rel A* (*p65*), *Rel B* and *c-Rel*. These NF- κ B proteins form homo or hetero dimers and are bound in the cytoplasm by inhibitor of κ B proteins (I κ B). Infection or exposure to proinflammatory cytokines activates the I κ B kinase (IKK) which consists of two catalytic subunits IKK α and IKK β and a regulatory subunit IKK γ or Nemo. The activated IKK complex phosphorylates I κ B at two conserved serines within the I κ B N-terminus. Phosphorylation then targets I κ B for ubiquitination and subsequent degradation. The NF- κ B dimers are then free to translocate to the nucleus where they transactivate the expression of genes involved in inflammation, proliferation and cell survival (Karin et al., 2002).

A recent survey of childhood T-ALL patients revealed that 39/42 patients exhibited NF- κ B activation (Kordes et al., 2000). Yet the cause or effect(s) of elevated NF- κ B activity in the human T-ALL leukemic cells is not known. The basic helix-loop-helix *TALI* gene is activated in as many as 60% of pediatric T-ALL patients by a variety of genetic mechanisms (Begley and Green, 1999). In our mouse models of *Tal1* induced leukemia/lymphoma (Kelliher et al., 1996; O'Neil et al., 2001), we observe dramatic decreases in overall thymocyte cellularity and an arrest of thymocyte development. Similar to what has been reported in human T-ALL (Kordes et al., 2000), mouse *Tal1*

pre-malignant thymocytes and tumors exhibit nuclear p65/p50 and increased IKK activity. Yet, inhibition of NF- κ B in mouse *Tall* tumors had no effect on tumor growth/survival *in vivo*, suggesting that NF- κ B activation may contribute to the pre-malignant phase of Tall induced disease.

Results

Tal1 expression results in decreases in CD4 and CD8 double positive thymocytes and in CD4-single positive thymocytes

Thymocyte expression of the *Tal1* oncogene results in 2-8 fold decreases in overall thymocyte cellularity (Figure 16A). Moreover, perturbations in the relative percentage of CD4-positive and CD8-positive thymocytes as well as CD4-single positive thymocytes are observed (Figure 16B). In mice homozygous for the *Tal1* transgene (*Tal1/Tal1*), there is a 60-fold decrease in the absolute numbers of DP thymocytes and 133-fold decrease in the absolute numbers of CD4-single positive thymocytes. Interestingly, mice expressing a DNA binding mutant of *Tal1* (*Mut Tal1*) also exhibit thymocyte developmental abnormalities (Figure 16), suggesting that induction of novel *Tal1* target genes are not required to arrest thymocyte development.

Tal1 transgenic mice and mice expressing a DNA binding mutant of Tal1(R188G;R189G, designated Mut Tal1) exhibit dramatic arrest at the DN3 stage of thymocyte development with 79-91% of the DN cells expressing CD44 and CD25. The fact that Tal1 and the DNA binding mutant of Tal1 both stimulate thymocyte arrest is consistent with the idea that Tal1 induces arrest by depleting E2A proteins.

Tal1 expression stimulates thymocyte apoptosis

In order to determine whether the decrease in the absolute numbers of DP and CD4 SP thymocytes is due to an increase in thymocyte apoptosis, thymocytes from disease-free, age matched *Tal1* transgenic and littermate controls were cultured *in vitro*

for 24 hours and then the percent apoptotic thymocytes quantified by FITC-AnnexinV and propidium iodide staining followed by flow cytometry. The percentage of apoptotic cells was markedly increased in *Tal1* transgenic thymocytes as compared to thymocytes from control littermates (Table 6).

Thymocytes are highly sensitive to Fas-mediated apoptosis and hence *Tal1* expression in the thymus may induce up regulation of Fas L (Nagata and Golstein, 1995). To determine whether *Tal1* induced apoptosis is Fas-mediated, we generated *Tal1/lpr/lpr* mice which express mutant Fas and are resistant to Fas-mediated apoptosis (Watanabe-Fukunaga et al., 1992). In spite of defects in Fas signaling, expression of *Tal1* oncogene sensitized *lpr/lpr* thymocytes to apoptosis, demonstrating that *Tal1* induced cell death is not Fas-Fas L-dependent (Table 6).

NF- κ B activation in premalignant *Tal1* thymocytes and tumors

Despite its known role in preventing apoptosis, recent observations in NF- κ B superinhibitor transgenic mice have demonstrated that NF- κ B activation is essential for α -CD3 mediated double positive thymocyte apoptosis (Hettmann et al., 1999). To determine if NF- κ B is activated in thymocytes expressing the *Tal1* oncogene, EMSAs were performed on nuclear extracts prepared from premalignant *Tal1* transgenic mice and control littermates. A complex binding to the consensus oligo was detected in the *Tal1* and *Mut Tal1* thymocytes (not shown) but not in thymocytes from control littermates. Supershift analysis demonstrated that the complex contains p65 and p50, but not c-Rel (Figure 17A). Therefore, *Tal1* expression in thymocytes results in an increase in NF- κ B

(p65/p50) DNA binding activity. However, *Tal1* and the DNA binding mutant of *Tal1* induce NF- κ B activation, suggesting that p65 is not a direct target gene of the *Tal1/E47* heterodimer. We also observe NF- κ B activation in thymocytes from *E2A* null mice (Figure 17B) providing evidence that the activation observed in *Tal1* thymocytes is a result of *E2A* depletion.

We then examined three mouse *Tal1* and two *Mut Tal1* tumors for evidence of NF- κ B activation. Nuclear extracts were prepared from primary *Tal1* and *Mut Tal1* tumors and EMSAs performed. We detected the p65/p50 heterodimer in the nucleus of all five *Tal1* and *Mut Tal1* tumors examined (Figure 18A and B), suggesting that NF- κ B activation is associated with *Tal1* induced leukemogenesis in the mouse.

The transcription factor NF- κ B controls the expression of several anti-apoptotic proteins including the inhibitors of apoptosis (cIAPs), Bcl-2 and Bcl-X_L (Chen et al., 2000; Grumont et al., 1999; Zong et al., 1999). To determine whether constitutive NF- κ B activity in *Tal1* tumors correlates with the expression of anti-apoptotic proteins, we examined Bcl-2 and Bcl-X_L expression levels in *Tal1* tumors. In contrast to wild type thymocytes, *Tal1* tumors expressed increased amounts of the Bcl-2 protein (Figure 18C), whereas Bcl-X_L protein was not detected in any of the *Tal1* tumors examined (not shown). Bcl-X_L expression was also reduced in *Tal1* and *Mut Tal1* premalignant thymocytes and in thymocytes from one week old *E2A* null mice compared with expression levels in littermate controls (Figure 18D) suggesting that *Tal1* decreases Bcl-X_L through inhibition of *E2A*. Decreased expression of Bcl-X_L has already been implicated in NF- κ B mediated thymocyte apoptosis (Hettmann et al., 1999) suggesting

that both the NF- κ B activation and the decrease in Bcl-X_L expression may be related to the thymocyte apoptosis observed in our *Tall* mice and *E2A* null mice (Table 6 data not shown).

Elevated IKK activity in premalignant *Tall* thymocytes and *Tall* tumors

To determine the mechanism of NF- κ B activation, we examined I κ B kinase activity in premalignant *Tall* thymocytes and in *Tall* tumors using an *in vitro* kinase assay and GST-I κ B as a substrate. IKK activity was increased in premalignant *Tall* thymocytes compared to thymocytes from control littermates (Figure 19). We also detect elevated IKK activity in *E2A* null thymocytes (data not shown) supporting our hypothesis that *Tall* is contributing to NF- κ B activation by inhibiting E protein function. In addition, IKK activity was increased in the three *Tall* and two *Mut Tall* tumors examined (Figure 19). Taken together, these studies suggest that the nuclear p65 observed in the premalignant *Tall* thymocytes and in the *Tall* tumors may reflect constitutive IKK activation.

Inhibition of NF- κ B activity does not alter *Tall* tumor growth *in vivo*

Although NF- κ B complexes have been detected in human ALL samples (Kordes et al., 2000), the contribution of these complexes to leukemogenesis is unclear. In order to elucidate how NF- κ B activation contributes to disease progression *in vivo*, we introduced a mutated form of I κ B α under the control of the proximal *lck* promoter into three mouse *Tall* tumor cell lines. This I κ B mutant functions as a superinhibitor because

it binds NF- κ B and inhibits DNA binding as well as nuclear translocation (Voll et al., 2000). As expected, expression of the superinhibitor in the *Tall* tumor cells leads to a decrease in nuclear p65/p50 heterodimers. Furthermore, TNF- α -induced NF- κ B activation is repressed in *Tall* tumors expressing the mutant I κ B (Figure 20).

To test whether inhibition of NF- κ B activation affects tumor growth *in vivo*, *Tall* tumors and *Tall* tumors expressing the NF- κ B superrepressor were injected into syngeneic mice. No differences in tumor latency or in tumor size were observed (Table 7). Histopathologic examination of the injected mice did not reveal differences in tumor invasiveness or metastasis. Our failure to observe an effect of the I κ B superinhibitor on *Tall* tumor growth *in vivo* did not reflect the fact that the injected tumors no longer expressed the mutant I κ B superinhibitor. Thus, inhibition of NF- κ B activity in *Tall* tumor cell lines does not appear to alter tumor growth or invasiveness *in vivo*.

Discussion

In this study we demonstrate that *Tal1* expression perturbs thymocyte development, inducing arrest at DN3 (CD44-negative, CD25-positive) and DN2 (CD44-positive, CD25-positive) stages of thymocyte development. Moreover, *Tal1* DP thymocytes are sensitive to apoptosis and exhibit elevated NF- κ B DNA binding activity in the *Tal1* thymus at 3-4 weeks of age, well before tumors appear. This study indicates that constitutive NF- κ B activation is an early event in *Tal1* induced leukemogenesis. Others have observed *c-Rel* activation and apoptosis in thymocytes that express Id1 or the p22 form of *Tal1* and suggest that aberrant pre-TCR signaling stimulates NF- κ B activation (Kim et al., 1999; Kim et al., 2002). We observe partial developmental arrest, apoptosis and NF- κ B activation in *Tal1* transgenic mice. Yet it remains unclear whether NF- κ B activation relates to thymocyte differentiation arrest and/or DP apoptosis. These questions can best be addressed in a conditional *Tal1* transgenic model we are currently establishing. The fact that *Tal1* thymocytes and tumors exhibit NF- κ B activation suggests to us that NF- κ B contributes to leukemogenesis, potentially by protecting thymocytes from apoptosis.

Tumors that develop in *Tal1* transgenic mice exhibit constitutive NF- κ B activation. However, the precise mechanism(s) by which *Tal1* expression results in NF- κ B activation is not clear. In some *Tal1* tumors, increases in IKK activity are observed, whereas in other tumors, TNF α -induced I κ B degradation is not observed (not shown), suggesting that NF- κ B activation can result from mutations in IKK components or in I κ B gene(s). Consistent with the increased NF- κ B activity, expression of the anti-apoptotic

NF- κ B target genes, Bcl-2 and A1 are increased in mouse *Tall* tumor cells, irrespective of tumor cell phenotype. Microarray studies on human T-ALL samples have detected A1 overexpression in *TALI* activated human T-ALL samples (Ferrando et al., 2002). Thus, activation of the anti-apoptotic NF- κ B pathway is also a feature of human ALL. Moreover, resistance to therapy may also be a consequence of NF- κ B activation.

To examine the contribution of NF- κ B to Tall induced leukemogenesis, we inhibited NF- κ B activity in mouse *Tall* tumor cells by overexpressing mutant I κ B α proteins and analyzed their growth *in vivo*. Unlike studies published for NF- κ B in the mouse mammary adenocarcinoma cell line CSMLO (Biswas et al., 2001), we observed no differences in growth, survival or metastasis of *Tall* tumor cells, where NF- κ B activity was inhibited. Although NF- κ B inhibition in established *Tall* tumor cell lines had no effect on tumor growth, NF- κ B inhibition during the premalignant phase may have different effects.

Materials and methods

Flow cytometry

Thymocytes from four-week-old, age-matched, *Tall*, *Mut Tall* transgenic and control littermates mice were stained with Cytochrome-c-conjugated anti-mouse L3T4 (CD4) and PE-conjugated Ly-2 (CD8) (Pharmingen, San Diego, CA). For double negative analysis, cells were stained with antibodies for the lineage markers and the lineage negative cells were stained with CD25-PE and CD44-FITC and analyzed by flow cytometry. Premalignant thymocytes from four week old *Tall*, *Tall/lpr/lpr* transgenic mice and control littermates were plated in 24 well plates at a density of 5×10^6 cells/ml. The cells were then incubated in RPMI medium, containing 10% fetal bovine serum at 37°C for twenty-four hours. Apoptotic cells were quantified by staining with FITC-conjugated AnnexinV and propidium iodide and analyzed by flow cytometry.

Electrophoretic mobility shift assay (EMSA) and Western blotting

Nuclear extracts were prepared from wildtype, premalignant *Tall* or *Mut Tall* thymocytes and from *Tall* or *Mut Tall* tumors were incubated with a ^{32}P -labeled-double-stranded oligonucleotide probe containing the NF- κB consensus sequence (Promega). Antibodies against p65, p50 and c-rel used for supershifting were obtained from Nancy Rice of the National Cancer Institute (Bethesda, MD).

To analyze Bcl-2 and Bcl-X_L expression, wildtype thymocytes and *Tall* tumor cells were lysed and immunoblotted with antibodies against Bcl-2 or Bcl-X_L (Pharmingen). The

blot was then stripped and re-probed with an antibody against β -actin to control for equal loading (Sigma, St Louis, MO).

***In vitro* kinase assay**

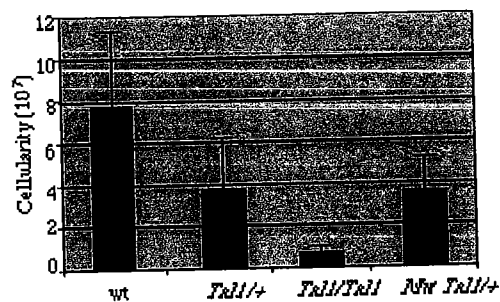
Tall tumors or thymocytes from *Tall* transgenic or control littermates were lysed and immunoprecipitated with anti-IKK γ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and an *in vitro* kinase assay performed as previously described (Mudgett et al., 2000).

Injection of tumor cells into syngeneic mice

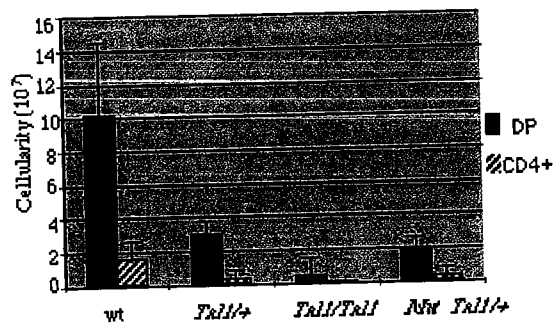
Tall tumor cells or *Tall* tumor cells expressing mutant I κ B α were injected intraperitoneally into two or three syngeneic FVB/N mice. All animals developed palpable tumors within six weeks post-injection. All tissue samples were histologically evaluated at the Transgenic Core Pathology Laboratory at the University of California at Davis.

Figure 16. *Tal1* and *Mut Tal1* perturb thymocyte development. Total thymocytes numbers from four-week-old wildtype and *Tal1/+*, *Tal1/Tal1*, and *Mut Tal1/+* transgenic mice (A). *Tal1* transgenic mice have reduced numbers of DP and CD4+ cells. The absolute numbers of DP and CD4+ cells in wt, *Tal1/+*, *Tal1/Tal1* and *Mut Tal1/+* four-week-old mice are plotted (B). *Tal1* expression perturbs thymocyte development. Thymocytes from four-week-old mice were stained with CD4-Cy and CD8-FITC and analyzed by flow cytometry (C). *Tal1* expression in the thymus blocks the progression of cells through the stages of double negative development. Cells were stained for the lineage markers, IgM, Ter119, Gr1, Mac1, PanNK, CD3, CD4, and CD8 as well as CD25-PE and CD44-FITC. The staining of lineage negative cells with CD25-PE and CD44-FITC is shown. Numbers in quadrants represent percent of cells stained with antibodies (D). The results shown are representative of at least five experiments.

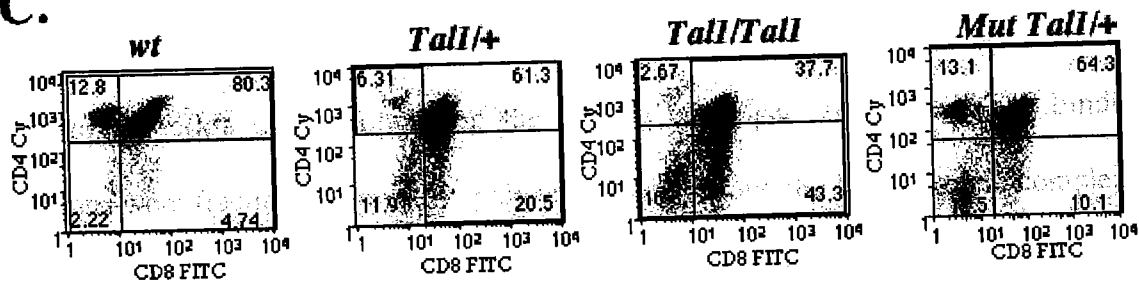
A.



B.



C.



D.

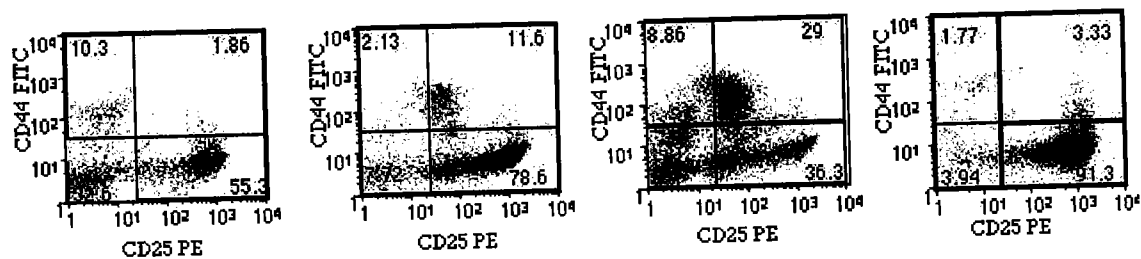


Figure 17. NF- κ B activation in premalignant *Tal1* transgenic and *E2A* null thymocytes. Nuclear extracts were prepared from thymocytes from four-week-old age-matched wildtype, *Tal1*/+ (A), and *E2A* null (B) mice. The extracts were incubated with a oligonucleotide probe containing the NF- κ B consensus sequence. The binding reactions were fractionated on a 5% polyacrylamide gel and the DNA-protein complexes were detected by autoradiography. The presence of the NF- κ B subunits was determined by supershifting with antibodies against p50, p65 and c-Rel.

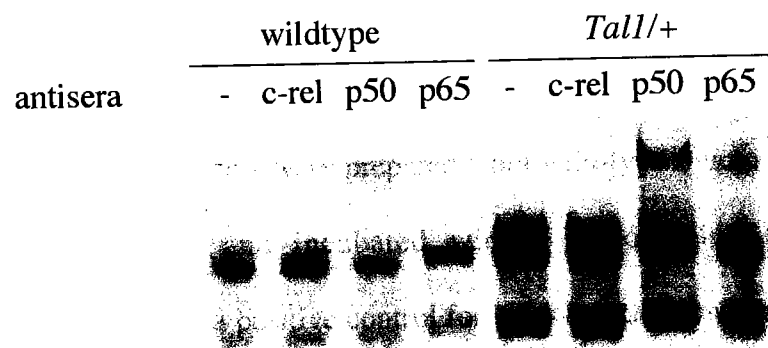
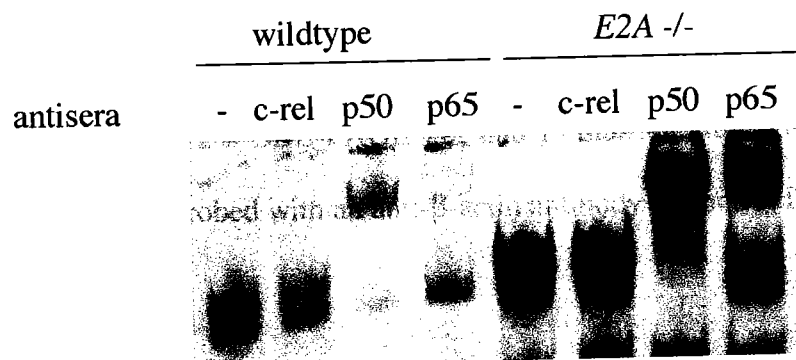
A.**B.**

Figure 18. Constitutive NF- κ B activation and Bcl-2 expression in *Tall* and *Mut Tall* tumors. Nuclear extracts were prepared from wild-type thymocytes, *Tall* tumors, and *Mut Tall* tumors and incubated with a radiolabeled NF- κ B consensus oligonucleotide probe. As a positive control for NF- κ B activation, nuclear extracts were also prepared from murine embryonic fibroblasts (MEF) left untreated or treated with the cytokine TNF α . The binding reactions were fractionated on a 5% polyacrylamide gel and the DNA-protein complexes were detected by autoradiography (A). NF- κ B complex in *Tall* tumors contains p65/p50. Binding reactions were incubated with antibodies against p50, p65 and c-rel prior to running on nondenaturing gel (B). Total cell lysates (50 μ g) were fractionated on a 12% SDS-PAGE gel and immunoblotted with the indicated antibody. Lysates were probed with an anti- β -actin antibody to control for the amount of total protein (C), (D).

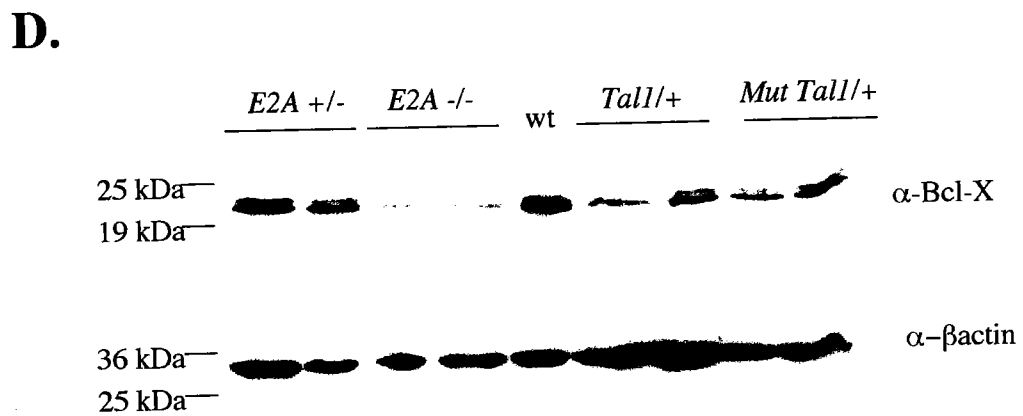
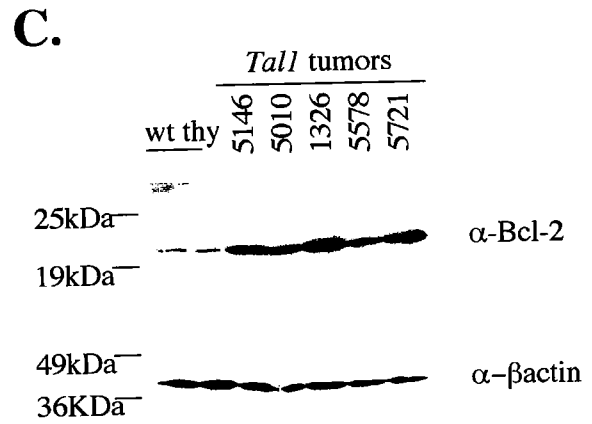
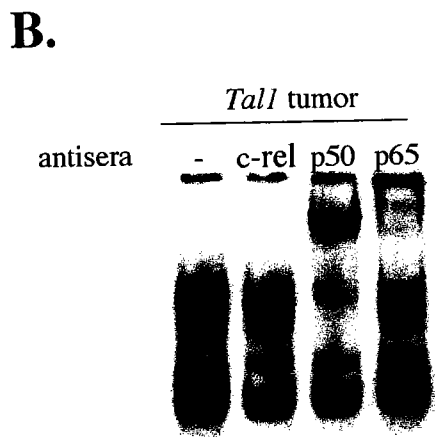
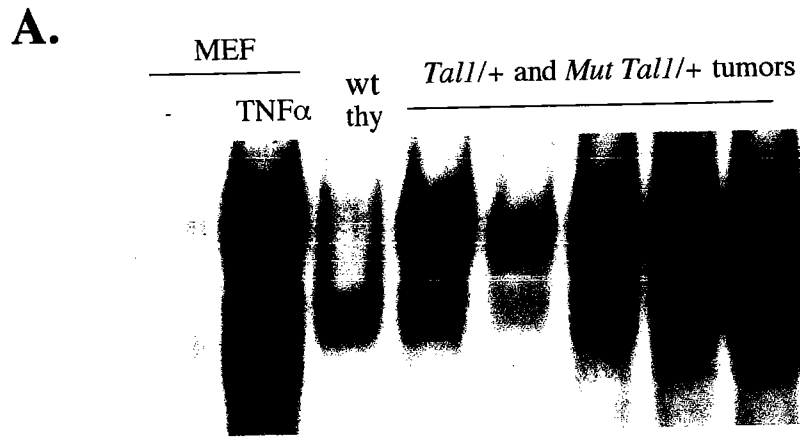


Figure 20. Mutant I κ B α expression inhibits TNF α -induced NF- κ B activation in *Tall* tumors. *Tall* tumor cells were electroporated with *lck-I κ B α RR-AA-F-PEST* and a plasmid containing the neomycin resistance gene. Cells were selected with G418 and treated with TNF α for various time points (times indicated are in minutes). Nuclear extracts prepared from these cells were incubated with a radiolabeled NF- κ B consensus oligonucleotide and fractionated on a 5% non-denaturing polyacrylamide gel.

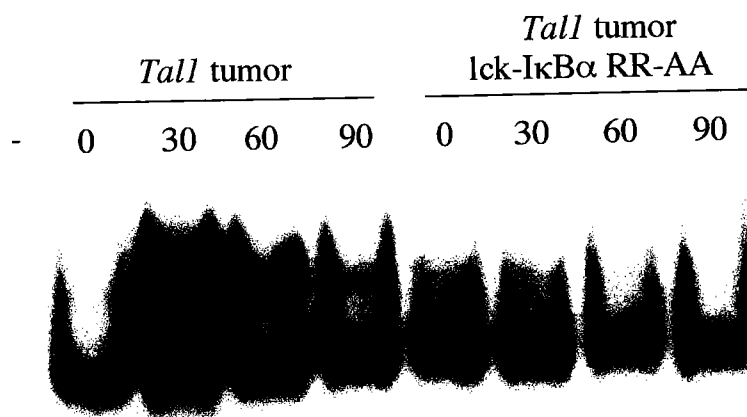


Table 6. *Tal1*/+ thymocytes undergo apoptosis *in vitro*.

	% apoptotic cells
+/+	15
+/+	17
+/+	15
+/+	19
<i>Tal1</i> /+	42
<i>Tal1</i> /+	32
<i>Tal1</i> /+	42
<i>Tal1</i> /+	45
<i>Tal1</i> /+	45
<i>Tal1</i> /+ <i>lpr/lpr</i>	52
<i>Tal1</i> /+ <i>lpr/lpr</i>	35

Thymocytes from disease-free, age-matched *Tal1* transgenic, *Tal1 lpr/lpr* transgenic and wildtype mice were incubated at 37°C for 24 hours and apoptotic cells were quantified by staining with AnnexinV-FITC and PI followed by flow cytometry.

Table 7. NF- κ B activity does not contribute to *Tal1* induced tumor growth.

<i>Tal1</i> tumor line	# mice developing tumors	avg. tumor size (cm)
5721	2/2	2.5
5721 lck-I κ B α RR-AA	2/2	2.75
1326	2/2	2.5
1326 lck-I κ B α RR-AA	1/1	3
5146	2/2	3.75
5146 lck-I κ B α RR-AA	3/3	3.25

Five million tumor cells were IP injected into syngeneic mice. All animals displayed evidence of disease and were sacrificed six weeks after the cells were injected.

CHAPTER V**DISCUSSION**

The work presented in this thesis provides insight into how *Tal1* contributes to leukemia using the mouse as a model system. By expressing a DNA binding mutant form of *Tal1* in the thymus of mice, we have shown that the DNA binding activity of *Tal1* is not required to cause leukemia in mice. In fact, mice expressing the DNA binding mutant *Tal1* exhibit increased disease penetrance (Figure 4). This result was somewhat surprising given the ability of *Tal1* to transactivate genes *in vitro* (Hsu et al., 1994c), however it is in agreement with studies in *Tal1* null ES cells showing that *Tal1* has both DNA binding dependent and independent functions in the specification of hematopoiesis (Porcher et al., 1999). We have also shown genetically that *Tal1* causes leukemia in mice by interfering with the function of the transcription factors E47 and HEB. We find several E47/HEB target genes repressed in thymocytes that express *Tal1*. In particular, the expression of *Rag1*, *Rag2*, pre-T α , and CD5 is decreased in pre-leukemic *Tal1* thymocytes.

The down regulation of genes such as *Rag1/2* and pre-T α that are important for T cell development is likely to be responsible for the perturbation of thymocyte differentiation that we observe in mice expressing *Tal1* or a DNA binding mutant of *Tal1* (O'Neil et al., 2001; O'Neil et al., 2003). The thymocyte differentiation arrest is more severe in *Tal1/E2A*^{+/-} and *Tal1/HEB*^{+/-} transgenic mice indicating that the differentiation arrest is central to the development of leukemia. Interestingly, *E47*^{+/-} *Rag1*^{-/-} mice develop leukemia, but disease has not been reported in *E47* heterozygous mice or in *Rag1* deficient mice suggesting that loss of Rag activity can collaborate with decreased E47 protein levels to induce leukemia in mice (Engel and Murre, 2002). This

data provides evidence that the decreased *Rag* expression levels observed in *Tall* and *Tall/E2A+/-* mice may contribute to the disease phenotype. The development of leukemia is also accelerated when *E47* deficient mice are placed on a *TCR β* deficient background, suggesting that decreased pre-TCR signaling may contribute to leukemia(Engel and Murre, 2002).

Thymocytes in *Tall* and DNA binding mutant *Tall* transgenic mice are partially arrested at the DN2 or DN3 stage (Figure 16). However, the thymocytes do progress through this arrest into double positive and single positive thymocytes. Interestingly, *E47* deficiency has recently been shown to promote the differentiation of thymocytes in *LAT*, *Lck* and *Fyn* deficient mice that normally exhibit a complete developmental block at the double negative stage(Engel and Murre, 2003). These results demonstrate that *E47* is necessary to prevent the differentiation of thymocytes without a functional pre-TCR. Therefore, inhibition of *E47* activity may allow the progression of *Tall* transgenic thymocytes to the double positive stage despite decreased expression of pre-T α . Activation of the Ras, NFAT, and NF- κ B signaling pathways have also been shown to promote the differentiation of double negative thymocytes into double positive thymocytes (Aifantis et al., 2001; Swat et al., 1996). Up regulation of any one of these signaling pathways could promote the differentiation of *Tall* thymocytes. In addition to promoting differentiation, up regulation of any one of these three pathways could contribute to leukemia progression since all have been previously been linked to the development of malignancy(Neal and Clipstone, 2003; Rayet and Gelinas, 1999).

Interestingly, our microarray experiment revealed a 3.7-fold induction of NFAT in double positive *Tall* thymocytes (See Appendix).

We also observe NF- κ B activation in preleukemic *Tall* thymocytes(O'Neil et al., 2003). As mentioned above, NF- κ B activation may be able to substitute for pre-TCR signaling(Voll and Ghosh, 1999). Therefore, it is possible that NF- κ B activation provides the signal that allows double negative *Tall* thymocytes to differentiate into double positive thymocytes despite the differentiation arrest and reduced pre-T α expression. Alternatively, the NF- κ B activation observed may be a consequence of the double negative thymocyte arrest that we observe in *Tall* transgenic mice since high levels of active NF- κ B complexes are detected in DN3 and DN4 thymocytes(Voll et al., 2000).

Finally, NF- κ B activation may be related to the spontaneous thymocyte apoptosis observed in *Tall* thymocytes (Table 6) and unrelated to the double negative thymocyte differentiation arrest since NF- κ B activation is pro-apoptotic in double positive thymocytes(Hettmann et al., 1999). However, since we found no NF- κ B targets genes induced by *Tall* expression in our microarray experiment performed on double positive thymocytes, it seems more likely that the NF- κ B activation is in double negative thymocytes. Future experiments on sorted thymocyte populations will resolve this issue.

Although, it is not clear how *Tall* induces NF- κ B activation, our data showing NF- κ B activation and constitutive IKK activity in *E2A* null thymocytes and tumors (Figure 17 and data not shown) suggests that inhibition of *E2A* somehow triggers IKK activation. Interestingly, *E2A* inhibition and NF- κ B activation are also observed in

Notch3 transgenic thymocytes(Bellavia et al., 2000; Talora et al., 2003). NF- κ B activation may in fact be responsible for the differentiation of *E47* null thymocytes in the absence of TCR signaling(Engel and Murre, 2003).

Interestingly, while this work was being carried out, NF- κ B activation was also observed in *Id1* and *Tall-p22* transgenic mice(Kim et al., 2002). In addition, mice expressing *Id1* or *Tall-p22* and a constitutively active form of IKK β develop a complete thymocyte differentiation arrest at the double negative stage(Kim et al., 2002). Moreover, overexpression of the NF- κ B superinhibitor rescues the developmental arrest in *Id1* transgenic mice(Kim et al., 2002). This data demonstrates that NF- κ B activation is at least in part responsible for the differentiation arrest observed in *Id1* and *Tall-p22* transgenic mice and likely also contributes to the differentiation arrest in our *Tall* transgenic mice.

Yet, NF- κ B activation is observed in tumors, suggesting that it may contribute to tumor survival. However, inhibition of NF- κ B activation in tumors did not appear to affect the growth or metastatic potential of *Tall* tumors, indicating that tumors are no longer dependent on activated NF- κ B. These studies imply that NF- κ B inhibitors such as proteasome inhibitors or IKK inhibitors may not be useful in treating leukemic patients with *TALI* activation.

Our analysis of the contribution of NF- κ B to *Tall* induced leukemia was performed on *Tall* tumor cell lines that had been cultured for several months. These cells may have activated other anti-apoptotic pathways rendering them no longer dependent on NF- κ B activation. Therefore, it is possible that inhibiting NF- κ B activation directly in

developing tumors of *Tall* transgenic mice may yield different results and implicate NF- κ B in *Tal1* pathogenesis.

NF- κ B is activated in response to several chemotherapeutic drugs including ionizing radiation, danorubicin, and the topoisomerase I inhibitor CPT-11 (Wang et al., 1996). Whether inhibition of NF- κ B sensitizes cancer cells to chemotherapeutic agents is controversial (Bentires-Alj et al., 1999; Cusack et al., 1999; Sato et al., 2003; Uetsuka et al., 2003). *Tall* tumor cell lines that express the NF- κ B superinhibitor do not display increased sensitivity to dexamethasone, danorubicin, or gamma irradiation suggesting that NF- κ B inhibition does not promote chemosensitivity in *Tall* tumor cells (our unpublished data). These results suggest that NF- κ B is not antiapoptotic in *Tall* tumor cells and TAL1 patients may not benefit from the use of NF- κ B inhibitors in combination with other more conventional treatments for T-ALL.

When *Tal1* is misexpressed in the thymus, it depletes the E47/HEB heterodimer (Figure 8C). However chromatin immunoprecipitation studies reveal that E47 and HEB can be detected at target loci, suggesting that *Tal1* may repress transcription by recruiting corepressors to gene regulatory elements. Previous work has shown that *Tal1* interacts with the corepressor mSin3A in both Jurkat and undifferentiated MEL cells (Huang and Brandt, 2000). However this interaction had never been demonstrated at a relevant locus or directly associated with disease development. We provide evidence that *Tal1* recruits the corepressor mSin3A and the histone deacetylase HDAC1 to the *CD4* enhancer supporting the inhibition model (Figure 2). Future work will determine whether this is a

general mechanism of Tal1 mediated gene repression. Nonetheless, our data suggests that Tal1 does not cause leukemia by acting like an Id protein and sequestering E proteins into non-DNA binding complexes.

Our results demonstrating Tal1 occupation of the CD4 enhancer may seem in conflict with our observation that the DNA binding activity of Tal1 is not required to cause leukemia in mice. Repression of CD4 expression is more severe in the *Tal1* transgenic mice than in the DNA binding mutant *Tal1* transgenic mice suggesting that the DNA binding activity of Tal1 may contribute to the repression of CD4. We have noted other differences between the *Tal1* and *Mut Tal1* transgenic mice including increased spontaneous apoptosis in *Tal1* transgenic thymocytes and a more severe double negative thymocyte differentiation arrest in *Mut Tal1* transgenic mice, revealing that Tal1 has both DNA binding dependent and independent functions in thymocytes. The DNA binding dependent functions are not required, however to induce leukemia in the mouse.

The demonstration that Tal1 occupies the CD4 enhancer does not necessarily mean that it directly binds DNA. As in erythroid cells, Tal1 may be part of a large transcriptional complex in T cells that includes other DNA binding proteins making the DNA binding activity of Tal1 dispensable. Candidates for the other DNA binding components of the complex include Gata3, the Gata protein expressed in T cells, and Sp1 which is part of the Tal1 containing complex that controls the expression of the c-kit gene (Lecuyer et al., 2002). Chromatin immunoprecipitation experiments with thymocytes from transgenic mice that express the DNA binding mutant of Tal1 will determine whether DNA binding is required for Tal1 to occupy the CD4 enhancer.

It is likely that both sequestration and inhibition contribute to the repression of E47/HEB activity in *Tal1* leukemic cells. In fact, it is still formally possible that transactivation of novel target genes contributes to Tal1 induced leukemia. Although our microarray experiment demonstrated that most genes are repressed by Tal1 expression in the thymus, some genes were up regulated (see Appendix). It is possible that Tal1 regulates the expression of these genes without binding DNA. Future work will determine whether these genes are also up regulated in DNA binding mutant *Tal1* thymocytes and whether they are direct Tal1 target genes.

This work has important implications for the treatment of T-ALL patients with TAL1 activation. Our observation that Tal1 acts mainly by repressing E47/HEB gene transcription and recruits the corepressor mSin3A and HDAC1, suggests that HDAC inhibitors may be effective in treating T-ALL patients. We demonstrate that all mouse *Tal1* tumor cells are sensitive to the HDAC inhibitor TSA, whereas wildtype thymocytes were only mildly sensitive to TSA treatment.

Several other proteins involved in leukemia interact with corepressor complexes. AML1 normally activates and represses gene expression but when it is fused to either ETO or TEL in acute myelogenous leukemia, it represses gene expression through the interaction of the mSin3-HDAC complex with ETO and TEL (Chakrabarti and Nucifora, 1999; Gelmetti et al., 1998; Lutterbach et al., 1998; Wang et al., 1998). In addition, the RAR-PML and RAR-PLZF fusions proteins implicated in acute promyelocytic leukemia recruit the corepressors NCoR and SMRT even in the presence of the retinoic acid, conditions under which the unfused RAR releases the corepressors. As a consequence,

target genes remain repressed in the presence of the fusion genes(He et al., 1998; Lin et al., 1998).

The HDAC inhibitor sodium phenyl butyrate has been used successfully to treat an APL patient and *PLZF-RAR α* transgenic mice that develop acute promyelocytic leukemia(He et al., 2001; Warrell et al., 1998). In addition, treatment of AML-ETO cells with TSA inhibits their growth(Claus and Lubbert, 2003). Several HDAC inhibitors are already in clinical trials for patients with solid tumors and hematological malignancies and preliminary results show that the drugs are well tolerated and have anti-tumor activity(Marks et al., 2001).

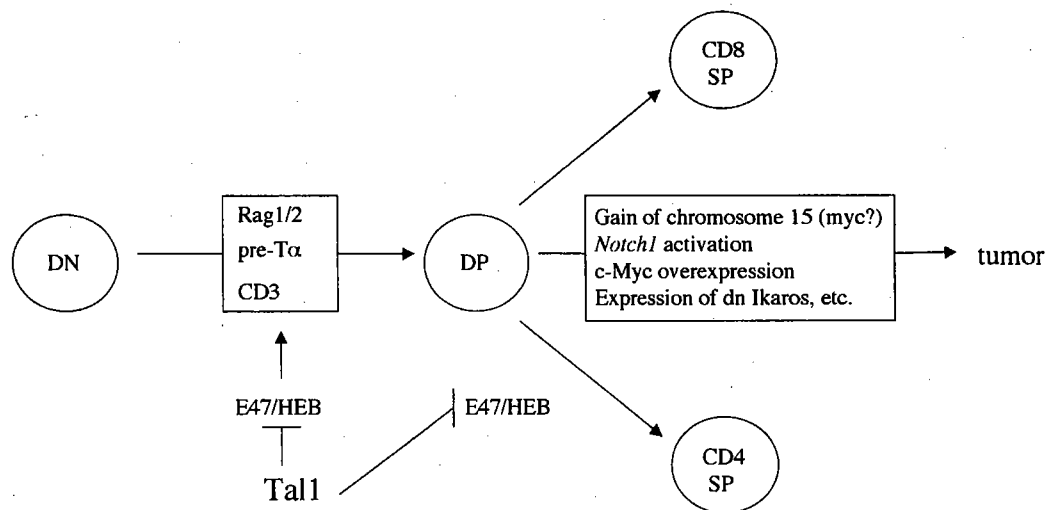
In addition, the Tal1/E47 or Tal1/HEB heterodimer may be amenable to therapeutic intervention. In the past it was believed that protein interactions would be difficult to disrupt with small molecules. However, recent studies have suggested that this may not be the case. Screens for small molecules that inhibit the interaction between the basic helix-loop-helix leucine zipper oncoprotein Myc and its partner Max have yielded promising results(Berg et al., 2002; Yin et al., 2003). Although it is not clear from these studies whether the drugs bound to the helix-loop-helix or the leucine zipper domains, other small molecules that specifically disrupt the Id2-E47 interaction (and not the Id2-HEB, Id2-E2-2 or Id2-E12 interactions) were found in one of the screens demonstrating that HLH interactions can be disrupted by small molecules(Yin et al., 2003). Since Tal1 cannot homodimerize, small molecules that specifically disrupt the Tal1/E47 and/or Tal1/HEB interaction would critically inhibit its function in leukemic cells. Since Tal1 is required for embryonic hematopoietic stem cell development but not

for maintenance or differentiation of the adult hematopoietic stem cell (Mikkola, 2003), inhibition of Tal1 function in patients may not have serious side effects. It may also be possible to inhibit Tal1 function by disrupting the interaction of Tal1 with mSin3A or other corepressors.

The work presented in this thesis provides strong evidence to explain the mechanism of TAL1 induced leukemia by studying the human disease in the mouse. We have demonstrated that the DNA binding activity of Tal1 is not required to induce leukemia in the mouse. When Tal1 is misexpressed in the mouse thymus, it perturbs thymocyte differentiation and represses gene transcription. In addition, we provide genetic evidence that Tal1 inhibits the function of the E47/HEB transcription factor. We also demonstrate that Tal1 recruits corepressor complexes to gene regulatory regions. Consistent with this, mouse *Tal1* tumor cells are extremely sensitive to TSA treatment, suggesting that leukemic patients may benefit from a treatment regimen that includes HDAC inhibitors. Disruption of Tal1/E47 or Tal1/HEB heterodimers may be another means of inhibiting Tal1 induced leukemia. Identification of the other components of the Tal1 transcriptional complex in leukemic cells may provide other interactions that could be amenable to drug therapy. Furthermore, this analysis may result in a better understanding of Tal1 induced leukemia.

Figure 21. Model of *Tal1* induced leukemogenesis. When *Tal1* is expressed in thymocyte precursors it inhibits E47/HEB heterodimer function leading to a down regulation of genes important in T cell development such as *Rag1/2*, *pre-T α* , and *CD3*. Additional mutations such as *Notch1* activation, *c-Myc* overexpression or expression of dominant negative *Ikaros* (identified by L. Cunningham in our laboratory by retroviral insertional mutagenesis) are necessary for the development of leukemia. Using SKY analysis (performed by David Ferguson, University of Michigan) we have seen gain of chromosome 15 in some *Tal1* tumors. Amplification of *Myc* on this chromosome may contribute to *Tal1* induced leukemia.

Tal1 leukemogenesis



References

- Abraham, K. M., Levin, S. D., Marth, J. D., Forbush, K. A., and Perlmutter, R. M. (1991). Thymic tumorigenesis induced by overexpression of p56lck. *Proc Natl Acad Sci U S A* 88, 3977-3981.
- Aifantis, I., Gounari, F., Scorrano, L., Borowski, C., and von Boehmer, H. (2001). Constitutive pre-TCR signaling promotes differentiation through Ca²⁺ mobilization and activation of NF-kappaB and NFAT. *Nat Immunol* 2, 403-409.
- Alani, R. M., Young, A. Z., and Shifflett, C. B. (2001). Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. *Proc Natl Acad Sci U S A* 98, 7812-7816.
- Andela, V. B., Schwarz, E. M., Puzas, J. E., O'Keefe, R. J., and Rosier, R. N. (2000). Tumor metastasis and the reciprocal regulation of prometastatic and antimetastatic factors by nuclear factor kappaB. *Cancer Res* 60, 6557-6562.
- Anderson, K. P., Crable, S. C., and Lingrel, J. B. (1998). Multiple proteins binding to a GATA-E box-GATA motif regulate the erythroid Kruppel-like factor (EKLF) gene. *J Biol Chem* 273, 14347-14354.
- Aplan, P. D., Jones, C. A., Chervinsky, D. S., Zhao, X., Ellsworth, M., Wu, C., McGuire, E. A., and Gross, K. W. (1997). An scl gene product lacking the transactivation domain induces bony abnormalities and cooperates with LMO1 to generate T-cell malignancies in transgenic mice. *Embo J* 16, 2408-2419.
- Aplan, P. D., Lombardi, D. P., and Kirsch, I. R. (1991). Structural characterization of SIL, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. *Mol Cell Biol* 11, 5462-5469.
- Aplan, P. D., Nakahara, K., Orkin, S. H., and Kirsch, I. R. (1992). The SCL gene product: a positive regulator of erythroid differentiation. *Embo J* 11, 4073-4081.
- Aronheim, A., Shiran, R., Rosen, A., and Walker, M. D. (1993). The E2A gene product contains two separable and functionally distinct transcription activation domains. *Proc Natl Acad Sci U S A* 90, 8063-8067.

Asarnow, D. M., Kuziel, W. A., Bonyhadi, M., Tigelaar, R. E., Tucker, P. W., and Allison, J. P. (1988). Limited diversity of gamma delta antigen receptor genes of Thy-1+ dendritic epidermal cells. *Cell* 55, 837-847.

Ayrolidi, E., Migliorati, G., Cannarile, L., Moraca, R., Delfino, D. V., and Riccardi, C. (1997). CD2 rescues T cells from T-cell receptor/CD3 apoptosis: a role for the Fas/Fas-L system. *Blood* 89, 3717-3726.

Bain, G., Engel, I., Robanus Maandag, E. C., te Riele, H. P., Volland, J. R., Sharp, L. L., Chun, J., Huey, B., Pinkel, D., and Murre, C. (1997). E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol* 17, 4782-4791.

Bain, G., Maandag, E. C., Izon, D. J., Amsen, D., Kruisbeek, A. M., Weintraub, B. C., Krop, I., Schlissel, M. S., Feeney, A. J., van Roon, M., and et al. (1994). E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* 79, 885-892.

Bain, G., Maandag, E.C., Izon, D.J., Amsen, D., Kruisbeek, A.M., Weintraub, B.C., Krop, I., Schlissel, M.S., Feeney, A.J., van Roon, M. (1994). E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* 79, 885-892.

Bain, G., Romanow, W. J., Albers, K., Havran, W. L., and Murre, C. (1999). Positive and negative regulation of V(D)J recombination by the E2A proteins. *J Exp Med* 189, 289-300.

Baldwin, A. S., Jr. (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 14, 649-683.

Bargou, R. C., Emmerich, F., Krappmann, D., Bommert, K., Mapara, M. Y., Arnold, W., Royer, H. D., Grinstein, E., Greiner, A., Scheidereit, C., and Dorken, B. (1997). Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 100, 2961-2969.

Bargou, R. C., Leng, C., Krappmann, D., Emmerich, F., Mapara, M. Y., Bommert, K., Royer, H. D., Scheidereit, C., and Dorken, B. (1996). High-level nuclear NF-kappa B and

Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells. *Blood* 87, 4340-4347.

Barndt, R., Dai, M. F., and Zhuang, Y. (1999). A novel role for HEB downstream or parallel to the pre-TCR signaling pathway during alpha beta thymopoiesis. *J Immunol* 163, 3331-3343.

Barndt, R. J., and Zhuang, Y. (1999). Controlling lymphopoiesis with a combinatorial E-protein code. *Cold Spring Harb Symp Quant Biol* 64, 45-50.

Barton, L. M., Gottgens, B., and Green, A. R. (1999). The stem cell leukaemia (SCL) gene: a critical regulator of haemopoietic and vascular development. *Int J Biochem Cell Biol* 31, 1193-1207.

Bash, R. O., Hall, S., Timmons, C. F., Crist, W. M., Amylon, M., Smith, R. G., and Baer, R. (1995). Does activation of the TAL1 gene occur in a majority of patients with T-cell acute lymphoblastic leukemia? A pediatric oncology group study. *Blood* 86, 666-676.

Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376, 167-170.

Begley, C. G., Aplan, P. D., Davey, M. P., Nakahara, K., Tchorz, K., Kurtzberg, J., Hershfield, M. S., Haynes, B. F., Cohen, D. I., Waldmann, T. A., and et al. (1989). Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor delta-chain diversity region and results in a previously unreported fusion transcript. *Proc Natl Acad Sci U S A* 86, 2031-2035.

Begley, C. G., and Green, A. R. (1999). The SCL gene: from case report to critical hematopoietic regulator. *Blood* 93, 2760-2770.

Bellavia, D., Campese, A. F., Alesse, E., Vacca, A., Felli, M. P., Balestri, A., Stoppacciaro, A., Tiveron, C., Tatangelo, L., Giovarelli, M., et al. (2000). Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *Embo J* 19, 3337-3348.

Ben-Arie, N., Bellen, H. J., Armstrong, D. L., McCall, A. E., Gordadze, P. R., Guo, Q., Matzuk, M. M., and Zoghbi, H. Y. (1997). *Math1* is essential for genesis of cerebellar granule neurons. *Nature* 390, 169-172.

Bentires-Alj, M., Hellin, A. C., Ameyar, M., Chouaib, S., Merville, M. P., and Bours, V. (1999). Stable inhibition of nuclear factor kappaB in cancer cells does not increase sensitivity to cytotoxic drugs. *Cancer Res* 59, 811-815.

Berg, T., Cohen, S. B., Desharnais, J., Sonderegger, C., Maslyar, D. J., Goldberg, J., Boger, D. L., and Vogt, P. K. (2002). Small-molecule antagonists of Myc/Max dimerization inhibit Myc-induced transformation of chicken embryo fibroblasts. *Proc Natl Acad Sci U S A* 99, 3830-3835.

Bernard, O., Azogui, O., Lecoite, N., Mugneret, F., Berger, R., Larsen, C. J., and Mathieu-Mahul, D. (1992). A third *tal-1* promoter is specifically used in human T cell leukemias. *J Exp Med* 176, 919-925.

Biswas, D. K., Dai, S. C., Cruz, A., Weiser, B., Graner, E., and Pardee, A. B. (2001). The nuclear factor kappa B (NF-kappa B): a potential therapeutic target for estrogen receptor negative breast cancers. *Proc Natl Acad Sci U S A* 98, 10386-10391.

Boothby, M. R., Mora, A. L., Scherer, D. C., Brockman, J. A., and Ballard, D. W. (1997). Perturbation of the T lymphocyte lineage in transgenic mice expressing a constitutive repressor of nuclear factor (NF)-kappaB. *J Exp Med* 185, 1897-1907.

Bradney, C., Hjelmeland, M., Komatsu, Y., Yoshida, M., Yao, T. P., and Zhuang, Y. (2002). Regulation of E2A activities by histone acetyltransferases in B lymphocyte development. *J Biol Chem*.

Brennan, T. J., Chakraborty, T., and Olson, E. N. (1991). Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. *Proc Natl Acad Sci U S A* 88, 5675-5679.

Bucher, K., Sofroniew, M. V., Pannell, R., Impey, H., Smith, A. J., Torres, E. M., Dunnett, S. B., Jin, Y., Baer, R., and Rabbitts, T. H. (2000). The T cell oncogene *Tal2* is necessary for normal development of the mouse brain. *Dev Biol* 227, 533-544.

- Carrasco, D., Rizzo, C. A., Dorfman, K., and Bravo, R. (1996). The v-rel oncogene promotes malignant T-cell leukemia/lymphoma in transgenic mice. *Embo J* 15, 3640-3650.
- Cau, E., Casarosa, S., and Guillemot, F. (2002). Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development* 129, 1871-1880.
- Chakrabarti, S. R., and Nucifora, G. (1999). The leukemia-associated gene TEL encodes a transcription repressor which associates with SMRT and mSin3A. *Biochem Biophys Res Commun* 264, 871-877.
- Channavajhala, P., and Seldin, D. C. (2002). Functional interaction of protein kinase CK2 and c-Myc in lymphomagenesis. *Oncogene* 21, 5280-5288.
- Chen, C., Edelstein, L. C., and Gelinas, C. (2000). The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol Cell Biol* 20, 2687-2695.
- Cheng, J. T., Hsu, H. L., Hwang, L. Y., Baer, R. (1992). Products of the TAL1 oncogene: basic helix-loop-helix proteins phosphorylated at serine residues. *Oncogene* 8, 677-683.
- Chervinsky, D. S., Zhao, X. F., Lam, D. H., Ellsworth, M., Gross, K. W., and Aplan, P. D. (1999). Disordered T-cell development and T-cell malignancies in SCL LMO1 double-transgenic mice: parallels with E2A-deficient mice. *Mol Cell Biol* 19, 5025-5035.
- Choi, J. K., Shen, C. P., Radomska, H. S., Eckhardt, L. A., and Kadesch, T. (1996). E47 activates the Ig-heavy chain and TdT loci in non-B cells. *Embo J* 15, 5014-5021.
- Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C., and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* 125, 725-732.
- Claus, R., and Lubbert, M. (2003). Epigenetic targets in hematopoietic malignancies. *Oncogene* 22, 6489-6496.

Cohen-Kaminsky, S., Maouche-Chretien, L., Vitelli, L., Vinit, M. A., Blanchard, I., Yamamoto, M., Peschle, C., and Romeo, P. H. (1998). Chromatin immunoselection defines a TAL-1 target gene. *Embo J* 17, 5151-5160.

Condorelli, G., Vitelli, L., Valtieri, M., Marta, I., Montesoro, E., Lulli, V., Baer, R., and Peschle, C. (1995). Coordinate expression and developmental role of Id2 protein and TAL1/E2A heterodimer in erythroid progenitor differentiation. *Blood* 86, 164-175.

Condorelli, G. L., Facchiano, F., Valtieri, M., Proietti, E., Vitelli, L., Lulli, V., Huebner, K., Peschle, C., and Croce, C. M. (1996). T-cell-directed TAL-1 expression induces T-cell malignancies in transgenic mice. *Cancer Res* 56, 5113-5119.

Crist, W. M., Carroll, A. J., Shuster, J. J., Behm, F. G., Whitehead, M., Vietti, T. J., Look, A. T., Mahoney, D., Ragab, A., Pullen, D. J., and et al. (1990). Poor prognosis of children with pre-B acute lymphoblastic leukemia is associated with the t(1;19)(q23;p13): a Pediatric Oncology Group study. *Blood* 76, 117-122.

Cusack, J. C., Liu, R., and Baldwin, A. S. (1999). NF- kappa B and chemoresistance: potentiation of cancer drugs via inhibition of NF- kappa B. *Drug Resist Updat* 2, 271-273.

Davis, R. L., Cheng, P. F., Lassar, A. B., and Weintraub, H. (1990). The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* 60, 733-746.

Dedera, D. A., Waller, E. K., LeBrun, D. P., Sen-Majumdar, A., Stevens, M. E., Barsh, G. S., and Cleary, M. L. (1993). Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. *Cell* 74, 833-843.

Desprez, P. Y., Lin, C. Q., Thomasset, N., Sympton, C. J., Bissell, M. J., and Campisi, J. (1998). A novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1. *Mol Cell Biol* 18, 4577-4588.

Downing, J. R., and Shannon, K. M. (2002). Acute leukemia: a pediatric perspective. *Cancer Cell* 2, 437-445.

Doyle, K., Zhang, Y., Baer, R., and Bina, M. (1994). Distinguishable patterns of protein-DNA interactions involving complexes of basic helix-loop-helix proteins. *J Biol Chem* 269, 12099-12105.

Drake, C. J., Brandt, S. J., Trusk, T. C., and Little, C. D. (1997). TAL1/SCL is expressed in endothelial progenitor cells/angioblasts and defines a dorsal-to-ventral gradient of vasculogenesis. *Dev Biol* 192, 17-30.

Duffey, D. C., Chen, Z., Dong, G., Ondrey, F. G., Wolf, J. S., Brown, K., Siebenlist, U., and Van Waes, C. (1999). Expression of a dominant-negative mutant inhibitor-kappaB α of nuclear factor-kappaB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth in vivo. *Cancer Res* 59, 3468-3474.

Dumonteil, E., Laser, B., Constant, I., and Philippe, J. (1998). Differential regulation of the glucagon and insulin I gene promoters by the basic helix-loop-helix transcription factors E47 and BETA2. *J Biol Chem* 273, 19945-19954.

Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., and Hood, L. (1980). Two mRNAs can be produced from a single immunoglobulin mu gene by alternative RNA processing pathways. *Cell* 20, 313-319.

Eckner, R., Yao, TP, Oldread, E, Livingston, DM (1996). Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. *Genes Dev* 10, 2478-2490.

Elefanty, A. G., Begley, C. G., Hartley, L., Papaevangeliou, B., and Robb, L. (1999). SCL expression in the mouse embryo detected with a targeted lacZ reporter gene demonstrates its localization to hematopoietic, vascular, and neural tissues. *Blood* 94, 3754-3763.

Elefanty, A. G., Begley, C. G., Metcalf, D., Barnett, L., Kontgen, F., and Robb, L. (1998). Characterization of hematopoietic progenitor cells that express the transcription factor SCL, using a lacZ "knock-in" strategy. *Proc Natl Acad Sci U S A* 95, 11897-11902.

Ellenberger, T., Fass, D., Arnaud, M., and Harrison, S. C. (1994). Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev* 8, 970-980.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D., and Sklar, J. (1991). TAN-1, the human homolog of the *Drosophila* notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 66, 649-661.

Engel, I., Johns, C., Bain, G., Rivera, R. R., and Murre, C. (2001). Early thymocyte development is regulated by modulation of E2A protein activity. *J Exp Med* 194, 733-745.

Engel, I., and Murre, C. (1999). Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. *Proc Natl Acad Sci U S A* 96, 996-1001.

Engel, I., and Murre, C. (2002). Disruption of pre-TCR expression accelerates lymphomagenesis in E2A-deficient mice. *Proc Natl Acad Sci U S A* 99, 11322-11327.

Engel, I., and Murre, C. (2003). E2A proteins enforce a proliferation checkpoint in developing thymocytes. *Embo J*.

Esslinger, C. W., Wilson, A., Sordat, B., Beermann, F., and Jongeneel, C. V. (1997). Abnormal T lymphocyte development induced by targeted overexpression of IkappaB alpha. *J Immunol* 158, 5075-5078.

Fehling, H. J., Iritani, B. M., Krotkova, A., Forbush, K. A., Laplace, C., Perlmutter, R. M., and von Boehmer, H. (1997). Restoration of thymopoiesis in pT alpha^{-/-} mice by anti-CD3epsilon antibody treatment or with transgenes encoding activated Lck or tailless pT alpha. *Immunity* 6, 703-714.

Ferrando, A. A., Herblot, S., Palomero, T., Hansen, M., Hoang, T., Fox, E. A., and Look, A. T. (2003). Biallelic transcriptional activation of oncogenic transcription factors in T-cell acute lymphoblastic leukemia. *Blood*.

Ferrando, A. A., and Look, A. T. (2003). Gene expression profiling in T-cell acute lymphoblastic leukemia. *Semin Hematol* 40, 274-280.

Ferrando, A. A., Neuberg, D. S., Staunton, J., Loh, M. L., Huard, C., Raimondi, S. C., Behm, F. G., Pui, C. H., Downing, J. R., Gilliland, D. G., *et al.* (2002). Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell* 1, 75-87.

Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D., and Olson, E. N. (1998). Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat Genet* 18, 266-270.

Fisch, P., Boehm, T., Lavenir, I., Larson, T., Arno, J., Forster, A., and Rabbitts, T. H. (1992). T-cell acute lymphoblastic lymphoma induced in transgenic mice by the RBTN1 and RBTN2 LIM-domain genes. *Oncogene* 7, 2389-2397.

Fong, S., Itahana, Y., Sumida, T., Singh, J., Coppe, J. P., Liu, Y., Richards, P. C., Bennington, J. L., Lee, N. M., Debs, R. J., and Desprez, P. Y. (2003). Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. *Proc Natl Acad Sci U S A* 100, 13543-13548.

Froni, L., Boehm, T., White, L., Forster, A., Sherrington, P., Liao, X. B., Brannan, C. I., Jenkins, N. A., Copeland, N. G., and Rabbitts, T. H. (1992). The rhombotin gene family encode related LIM-domain proteins whose differing expression suggests multiple roles in mouse development. *J Mol Biol* 226, 747-761.

Fujioka, S., Scwab, G. M., Schmidt, C., Niu, J., Frederick, W. A., Dong, Q. G., Abbruzzese, J. L., Evans, D. B., Baker, C., and Chiao, P. J. (2003). Inhibition of constitutive NF-kappa B activity by I kappa B alpha M suppresses tumorigenesis. *Oncogene* 22, 1365-1370.

Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C., and Orkin, S. H. (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci U S A* 93, 12355-12358.

Garoufalidis, E., Kwan, I., Lin, R., Mustafa, A., Pepin, N., Roulston, A., Lacoste, J., and Hiscott, J. (1994). Viral induction of the human beta interferon promoter: modulation of transcription by NF-kappa B/rel proteins and interferon regulatory factors. *J Virol* 68, 4707-4715.

Gelmetti, V., Zhang, J., Fanelli, M., Minucci, S., Pelicci, P. G., and Lazar, M. A. (1998). Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. *Mol Cell Biol* 18, 7185-7191.

Gering, M., Rodaway, A. R., Gottgens, B., Patient, R. K., and Green, A. R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. *Embo J* 17, 4029-4045.

Gering, M., Yamada, Y., Rabbitts, T. H., and Patient, R. K. (2003). Lmo2 and Scf/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. *Development* 130, 6187-6199.

Gilmore, T. D. (1999). Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. *Oncogene* 18, 6925-6937.

Goldman, J. P., Spencer, D. M., and Raulet, D. H. (1993). Ordered rearrangement of variable region genes of the T cell receptor gamma locus correlates with transcription of the unrearranged genes. *J Exp Med* 177, 729-739.

Green, A. R., DeLuca, E., and Begley, C. G. (1991). Antisense SCL suppresses self-renewal and enhances spontaneous erythroid differentiation of the human leukaemic cell line K562. *Embo J* 10, 4153-4158.

Green, A. R., Lints, T., Visvader, J., Harvey, R., and Begley, C. G. (1992). SCL is coexpressed with GATA-1 in hemopoietic cells but is also expressed in developing brain. *Oncogene* 7, 653-660.

Greenbaum, S., and Zhuang, Y. (2002). Identification of E2A target genes in B lymphocyte development by using a gene tagging-based chromatin immunoprecipitation system. *Proc Natl Acad Sci U S A* 99, 15030-15035.

Grimm, S., Bauer, M. K., Baeuerle, P. A., and Schulze-Osthoff, K. (1996). Bcl-2 down-regulates the activity of transcription factor NF-kappaB induced upon apoptosis. *J Cell Biol* 134, 13-23.

Grumont, R. J., Rourke, I. J., and Gerondakis, S. (1999). Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev* 13, 400-411.

Grutz, G. G., Bucher, K., Lavenir, I., Larson, T., Larson, R., and Rabbitts, T. H. (1998). The oncogenic T cell LIM-protein Lmo2 forms part of a DNA-binding complex specifically in immature T cells. *Embo J* 17, 4594-4605.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* 75, 463-476.

Guzman, M. L., Neering, S. J., Upchurch, D., Grimes, B., Howard, D. S., Rizzieri, D. A., Luger, S. M., and Jordan, C. T. (2001). Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* 98, 2301-2307.

Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J. L., Fraser, C. C., Cavazzana-Calvo, M., and Fischer, A. (2003a). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 348, 255-256.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., *et al.* (2003b). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302, 415-419.

Hall, M. A., Curtis, D. J., Metcalf, D., Elefanty, A. G., Sourris, K., Robb, L., Gothert, J. R., Jane, S. M., and Begley, C. G. (2003). The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. *Proc Natl Acad Sci U S A* 100, 992-997.

He, L. Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., and Pandolfi, P. P. (1998). Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat Genet* 18, 126-135.

He, L. Z., Tolentino, T., Grayson, P., Zhong, S., Warrell, R. P., Jr., Rifkind, R. A., Marks, P. A., Richon, V. M., and Pandolfi, P. P. (2001). Histone deacetylase inhibitors induce

remission in transgenic models of therapy-resistant acute promyelocytic leukemia. *J Clin Invest* 108, 1321-1330.

He, Y. W. (2002). Orphan nuclear receptors in T lymphocyte development. *J Leukoc Biol* 72, 440-446.

Henthorn, P., Kiledjian, M., and Kadesch, T. (1990). Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. *Science* 247, 467-470.

Herblot, S., Aplan, P. D., and Hoang, T. (2002). Gradient of E2A activity in B-cell development. *Mol Cell Biol* 22, 886-900.

Herblot, S., Steff, A. M., Hugo, P., Aplan, P. D., and Hoang, T. (2000). SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-T alpha chain expression. *Nat Immunol* 1, 138-144.

Hettmann, T., DiDonato, J., Karin, M., and Leiden, J. M. (1999). An essential role for nuclear factor kappaB in promoting double positive thymocyte apoptosis. *J Exp Med* 189, 145-158.

Hofmann, T. J., and Cole, M. D. (1996). The TAL1/Scl basic helix-loop-helix protein blocks myogenic differentiation and E-box dependent transactivation. *Oncogene* 13, 617-624.

Honda, H., Inaba, T., Suzuki, T., Oda, H., Ebihara, Y., Tsuiji, K., Nakahata, T., Ishikawa, T., Yazaki, Y., and Hirai, H. (1999). Expression of E2A-HLF chimeric protein induced T-cell apoptosis, B-cell maturation arrest, and development of acute lymphoblastic leukemia. *Blood* 93, 2780-2790.

Horwitz, B. H., Scott, M. L., Cherry, S. R., Bronson, R. T., and Baltimore, D. (1997). Failure of lymphopoiesis after adoptive transfer of NF-kappaB-deficient fetal liver cells. *Immunity* 6, 765-772.

Houldsworth, J., Mathew, S., Rao, P. H., Dyomina, K., Louie, D. C., Parsa, N., Offit, K., and Chaganti, R. S. (1996). REL proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. *Blood* 87, 25-29.

- Hsu, H., Huang, L., Tsan, J. T., Funk, W., Wright, W. E., Hu, J. S., Kingston, B., Baer, R. (1994). Preferred Sequences for DNA Recognition by the TAL1 Helix-Loop-Helix Proteins. *Mol Cell Biol* *14*, 1256-1265.
- Hsu, H. L., Cheng, J. T., Chen, Q., and Baer, R. (1991). Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. *Mol Cell Biol* *11*, 3037-3042.
- Hsu, H. L., Wadman, I., and Baer, R. (1994a). Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. *Proc Natl Acad Sci U S A* *91*, 3181-3185.
- Hsu, H. L., Wadman, I., and R., B. (1994b). Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. *Proc Natl Acad Sci U S A* *91*(8), 3181-3185.
- Hsu, H. L., Wadman, I., Tsan, J. T., and Baer, R. (1994c). Positive and negative transcriptional control by the TAL1 helix-loop-helix protein. *Proc Natl Acad Sci U S A* *91*, 5947-5951.
- Hsu, K., Kanki, J. P., and Look, A. T. (2001). Zebrafish myelopoiesis and blood cell development. *Curr Opin Hematol* *8*, 245-251.
- Hsu, L. Y., Luring, J., Liang, H. E., Greenbaum, S., Cado, D., Zhuang, Y., and Schlissel, M. S. (2003). A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity* *19*, 105-117.
- Hu, J. S., Olson, E. N., and Kingston, R. E. (1992). HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. *Mol Cell Biol* *12*, 1031-1042.
- Huang, S., and Brandt, S. J. (2000). mSin3A regulates murine erythroleukemia cell differentiation through association with the TAL1 (or SCL) transcription factor. *Mol Cell Biol* *20*, 2248-2259.

- Inaba, T., Roberts, W. M., Shapiro, L. H., Jolly, K. W., Raimondi, S. C., Smith, S. D., and Look, A. T. (1992). Fusion of the leucine zipper gene HLF to the E2A gene in human acute B-lineage leukemia. *Science* 257, 531-534.
- Itahana, Y., Singh, J., Sumida, T., Coppe, J. P., Parrinello, S., Bennington, J. L., and Desprez, P. Y. (2003). Role of Id-2 in the maintenance of a differentiated and noninvasive phenotype in breast cancer cells. *Cancer Res* 63, 7098-7105.
- Janeway, C. A., Travers, P., Walport, M., and Capra, J. D. (1999). *Immunobiology*, 4th edn (New York, Garland Publishing).
- Jen, Y., Manova, K., and Benezra, R. (1997). Each member of the Id gene family exhibits a unique expression pattern in mouse gastrulation and neurogenesis. *Dev Dyn* 208, 92-106.
- Jen, Y., Weintraub, H., and Benezra, R. (1992). Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. *Genes Dev* 6, 1466-1479.
- Johnson, S. E., Wang, X., Hardy, S., Taparowsky, E. J., and Konieczny, S. F. (1996). Casein kinase II increases the transcriptional activities of MRF4 and MyoD independently of their direct phosphorylation. *Mol Cell Biol* 16, 1604-1613.
- Joos, S., Otano-Joos, M. I., Ziegler, S., Bruderlein, S., du Manoir, S., Bentz, M., Moller, P., and Lichter, P. (1996). Primary mediastinal (thymic) B-cell lymphoma is characterized by gains of chromosomal material including 9p and amplification of the REL gene. *Blood* 87, 1571-1578.
- Kallianpur, A. R., Jordan, J. E., and Brandt, S. J. (1994). The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. *Blood* 83, 1200-1208.
- Kamps, M. P., and Baltimore, D. (1993). E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. *Mol Cell Biol* 13, 351-357.

- Kamps, M. P., Look, A. T., and Baltimore, D. (1991). The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. *Genes Dev* 5, 358-368.
- Kamps, M. P., Murre, C., Sun, X. H., and Baltimore, D. (1990). A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. *Cell* 60, 547-555.
- Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 18, 621-663.
- Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2, 301-310.
- Kee, B. L., Bain, G., and Murre, C. (2002). IL-7Ralpha and E47: independent pathways required for development of multipotent lymphoid progenitors. *Embo J* 21, 103-113.
- Kelliher, M. A., Seldin, D. C., and Leder, P. (1996). Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase IIalpha. *Embo J* 15, 5160-5166.
- Kikuchi, E., Horiguchi, Y., Nakashima, J., Kuroda, K., Oya, M., Ohigashi, T., Takahashi, N., Shima, Y., Umezawa, K., and Murai, M. (2003). Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. *Cancer Res* 63, 107-110.
- Kim, D., Peng, X. C., and Sun, X. H. (1999). Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Mol Cell Biol* 19, 8240-8253.
- Kim, D., Xu, M., Nie, L., Peng, X. C., Jimi, E., Voll, R. E., Nguyen, T., Ghosh, S., and Sun, X. H. (2002). Helix-loop-helix proteins regulate pre-TCR and TCR signaling through modulation of Rel/NF-kappaB activities. *Immunity* 16, 9-21.
- Kim, K., Lee, C. K., Sayers, T. J., Muegge, K., and Durum, S. K. (1998). The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. *J Immunol* 160, 5735-5741.

- Kohn, D. B., Sadelain, M., and Glorioso, J. C. (2003). Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat Rev Cancer* 3, 477-488.
- Kontgen, F., Grumont, R. J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D., and Gerondakis, S. (1995). Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes Dev* 9, 1965-1977.
- Kordes, U., Krappmann, D., Heissmeyer, V., Ludwig, W. D., and Scheidereit, C. (2000). Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. *Leukemia* 14, 399-402.
- Kreider, B. L., Benezra, R., Rovera, G., and Kadesch, T. (1992). Inhibition of myeloid differentiation by the helix-loop-helix protein Id. *Science* 255, 1700-1702.
- Krieg, P. A., and Melton, D. A. (1987). In vitro RNA synthesis with SP6 RNA polymerase. *Methods Enzymol* 155, 397-415.
- Larson, R. C., Fisch, P., Larson, T. A., Lavenir, I., Langford, T., King, G., and Rabbitts, T. H. (1994). T cell tumours of disparate phenotype in mice transgenic for Rbtn-2. *Oncogene* 9, 3675-3681.
- Larson, R. C., Lavenir, I., Larson, T. A., Baer, R., Warren, A. J., Wadman, I., Nottage, K., and Rabbitts, T. H. (1996). Protein dimerization between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. *Embo J* 15, 1021-1027.
- Larson, R. C., Osada, H., Larson, T. A., Lavenir, I., and Rabbitts, T. H. (1995). The oncogenic LIM protein Rbtn2 causes thymic developmental aberrations that precede malignancy in transgenic mice. *Oncogene* 11, 853-862.
- Lasorella, A., Boldrini, R., Dominici, C., Donfrancesco, A., Yokota, Y., Inserra, A., and Iavarone, A. (2002). Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. *Cancer Res* 62, 301-306.
- Lasorella, A., Iavarone, A., and Israel, M. A. (1996). Id2 specifically alters regulation of the cell cycle by tumor suppressor proteins. *Mol Cell Biol* 16, 2570-2578.

Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* 66, 305-315.

Lecuyer, E., Herblot, S., Saint-Denis, M., Martin, R., Begley, C. G., Porcher, C., Orkin, S. H., and Hoang, T. (2002). The SCL complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1. *Blood* 100, 2430-2440.

Leonard, M., Brice, M., Engel, J. D., and Papayannopoulou, T. (1993). Dynamics of GATA transcription factor expression during erythroid differentiation. *Blood* 82, 1071-1079.

Liao, E. C., Paw, B. H., Oates, A. C., Pratt, S. J., Postlethwait, J. H., and Zon, L. I. (1998). SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev* 12, 621-626.

Lin, C. Q., Singh, J., Murata, K., Itahana, Y., Parrinello, S., Liang, S. H., Gillett, C. E., Campisi, J., and Desprez, P. Y. (2000). A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. *Cancer Res* 60, 1332-1340.

Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. H., Jr., and Evans, R. M. (1998). Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature* 391, 811-814.

Lister, J., Forrester, W. C., and Baron, M. H. (1995). Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. *J Biol Chem* 270, 17939-17946.

Littlewood, T., Evan, G. I. (1998). *Helix-Loop-Helix Transcription Factors*. (Oxford University Press).

Littman, D. R., Sun, Z., Unutmaz, D., Sunshine, M. J., Petrie, H. T., and Zou, Y. R. (1999). Role of the nuclear hormone receptor ROR gamma in transcriptional regulation, thymocyte survival, and lymphoid organogenesis. *Cold Spring Harb Symp Quant Biol* 64, 373-381.

Lowsky, R., DeCoteau, J. F., Reitmair, A. H., Ichinohasama, R., Dong, W. F., Xu, Y., Mak, T. W., Kadin, M. E., and Minden, M. D. (1997). Defects of the mismatch repair

gene MSH2 are implicated in the development of murine and human lymphoblastic lymphomas and are associated with the aberrant expression of rhombotin-2 (Lmo-2) and Tal-1 (SCL). *Blood* 89, 2276-2282.

Lu, D., Thompson, J. D., Gorski, G. K., Rice, N. R., Mayer, M. G., and Yunis, J. J. (1991). Alterations at the rel locus in human lymphoma. *Oncogene* 6, 1235-1241.

Lutterbach, B., Westendorf, J. J., Linggi, B., Patten, A., Moniwa, M., Davie, J. R., Huynh, K. D., Bardwell, V. J., Lavinsky, R. M., Rosenfeld, M. G., *et al.* (1998). ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. *Mol Cell Biol* 18, 7176-7184.

Lyden, D., Young, A. Z., Zagzag, D., Yan, W., Gerald, W., O'Reilly, R., Bader, B. L., Hynes, R. O., Zhuang, Y., Manova, K., and Benezra, R. (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* 401, 670-677.

Malecki, M. T., Jhala, U. S., Antonellis, A., Fields, L., Doria, A., Orban, T., Saad, M., Warram, J. H., Montminy, M., and Krolewski, A. S. (1999). Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nat Genet* 23, 323-328.

Malissen, M., Minard, K., Mjolsness, S., Kronenberg, M., Goverman, J., Hunkapiller, T., Prystowsky, M. B., Yoshikai, Y., Fitch, F., Mak, T. W., and *et al.* (1984). Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the beta polypeptide. *Cell* 37, 1101-1110.

Marks, P., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelly, W. K. (2001). Histone deacetylases and cancer: causes and therapies. *Nat Rev Cancer* 1, 194-202.

Massari, M. E., Jennings, P. A., and Murre, C. (1996). The AD1 transactivation domain of E2A contains a highly conserved helix which is required for its activity in both *Saccharomyces cerevisiae* and mammalian cells. *Mol Cell Biol* 16, 121-129.

Mathew, S., Murty, V. V., Dalla-Favera, R., and Chaganti, R. S. (1993). Chromosomal localization of genes encoding the transcription factors, c-rel, NF-kappa Bp50, NF-kappa Bp65, and lyt-10 by fluorescence in situ hybridization. *Oncogene* 8, 191-193.

- Matthews, J. M., and Visvader, J. E. (2003). LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins. *EMBO Rep* 4, 1132-1137.
- McCormack, M. P., Forster, A., Drynan, L., Pannell, R., and Rabbitts, T. H. (2003). The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. *Mol Cell Biol* 23, 9003-9013.
- McGuire, E. A., Rintoul, C. E., Sclar, G. M., and Korsmeyer, S. J. (1992). Thymic overexpression of Ttg-1 in transgenic mice results in T-cell acute lymphoblastic leukemia/lymphoma. *Mol Cell Biol* 12, 4186-4196.
- Mikkola, H., Klintman, J., Yang, H., Hock, H., Schlaeger, TM, Fujiwara, Y, Orkin, SH (2003). Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature* 421, 547-551.
- Miyamoto, A., Cui, X., Naumovski, L., and Cleary, M. L. (1996). Helix-loop-helix proteins LYL1 and E2a form heterodimeric complexes with distinctive DNA-binding properties in hematolymphoid cells. *Mol Cell Biol* 16, 2394-2401.
- Morrow, M. A., Mayer, E. W., Perez, C. A., Adlam, M., and Siu, G. (1999). Overexpression of the Helix-Loop-Helix protein Id2 blocks T cell development at multiple stages. *Mol Immunol* 36, 491-503.
- Mudgett, J. S., Ding, J., Guh-Siesel, L., Chartrain, N. A., Yang, L., Gopal, S., and Shen, M. M. (2000). Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. *Proc Natl Acad Sci U S A* 97, 10454-10459.
- Mukhopadhyay, T., Roth, J. A., and Maxwell, S. A. (1995). Altered expression of the p50 subunit of the NF-kappa B transcription factor complex in non-small cell lung carcinoma. *Oncogene* 11, 999-1003.
- Murre, C., McCaw, P. S., and Baltimore, D. (1989a). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 56, 777-783.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., and et al. (1989b). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* 58, 537-544.

Nagata, S., and Golstein, P. (1995). The Fas death factor. *Science* 267, 1449-1456.

Naya, F. J., Stellrecht, C. M., and Tsai, M. J. (1995). Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev* 9, 1009-1019.

Neal, J. W., and Clipstone, N. A. (2003). A constitutively active NFATc1 mutant induces a transformed phenotype in 3T3-L1 fibroblasts. *J Biol Chem* 278, 17246-17254.

Neale, G. A., Mao, S., Parham, D. M., Murti, K. G., and Goorha, R. M. (1995). Expression of the proto-oncogene rhombotin-2 is identical to the acute phase response protein metallothionein, suggesting multiple functions. *Cell Growth Differ* 6, 587-596.

Nie, L., Xu, M., Vladimirova, A., and Sun, X. H. (2003). Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *Embo J* 22, 5780-5792.

Nishikawa, S. I., Nishikawa, S., Hirashima, M., Matsuyoshi, N., and Kodama, H. (1998). Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* 125, 1747-1757.

Norton, J. D. (2000). ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* 113 (Pt 22), 3897-3905.

Norton, J. D., Deed, R. W., Craggs, G., and Sablitzky, F. (1998). Id helix-loop-helix proteins in cell growth and differentiation. *Trends Cell Biol* 8, 58-65.

O'Neil, J., Billa, M., Oikemus, S., and Kelliher, M. (2001). The DNA binding activity of TAL-1 is not required to induce leukemia/lymphoma in mice. *Oncogene* 20, 3897-3905.

- O'Neil, J., Ventura, J. J., Cusson, N., and Kelliher, M. (2003). NF- κ B activation in premalignant mouse tal-1/scl thymocytes and tumors. *Blood* 102, 2593-2596.
- Ono, Y., Fukuhara, N., and Yoshie, O. (1997). Transcriptional activity of TAL1 in T cell acute lymphoblastic leukemia (T-ALL) requires RBTN1 or -2 and induces TALLA1, a highly specific tumor marker of T-ALL. *J Biol Chem* 272, 4576-4581.
- Ono, Y., Fukuhara, N., and Yoshie, O. (1998). TAL1 and LIM-only proteins synergistically induce retinaldehyde dehydrogenase 2 expression in T-cell acute lymphoblastic leukemia by acting as cofactors for GATA3. *Mol Cell Biol* 18, 6939-6950.
- Ordentlich, P., Lin, A., Shen, C. P., Blaumueller, C., Matsuno, K., Artavanis-Tsakonas, S., and Kadesch, T. (1998). Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol* 18, 2230-2239.
- Osada, H., Grutz, G. G., Axelson, H., Forster, A., and Rabbitts, T. H. (1997). LIM-only protein Lmo2 forms a protein complex with erythroid transcription factor GATA-1. *Leukemia* 11 Suppl 3, 307-312.
- Palayoor, S. T., Youmell, M. Y., Calderwood, S. K., Coleman, C. N., and Price, B. D. (1999). Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. *Oncogene* 18, 7389-7394.
- Pan, L., Sato, S., Frederick, J. P., Sun, X. H., and Zhuang, Y. (1999). Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. *Mol Cell Biol* 19, 5969-5980.
- Park, S. T., Nolan, G. P., and Sun, X. H. (1999). Growth inhibition and apoptosis due to restoration of E2A activity in T cell acute lymphoblastic leukemia cells. *J Exp Med* 189, 501-508.
- Park, S. T., Sun, X. H. (1998). The tal1 oncoprotein inhibits E47-mediated transcription. Mechanism of inhibition. *J Biol Chem* 273, 7030-7037.
- Pear, W. S., Aster, J. C., Scott, M. L., Hasserjian, R. P., Soffer, B., Sklar, J., and Baltimore, D. (1996). Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med* 183, 2283-2291.

Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B., and et al. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 180, 1955-1960.

Petersson, K., Ivars, F., Sigvardsson, M. (2002). The pTalpha promoter and enhancer are direct targets for transactivation by Ebox-binding proteins. *Eur J Immunol* 32, 911-920.

Peverali, F. A., Ramqvist, T., Saffrich, R., Pepperkok, R., Barone, M. V., and Philipson, L. (1994). Regulation of G1 progression by E2A and Id helix-loop-helix proteins. *Embo J* 13, 4291-4301.

Polsky, D., Young, A. Z., Busam, K. J., and Alani, R. M. (2001). The transcriptional repressor of p16/Ink4a, Id1, is up-regulated in early melanomas. *Cancer Res* 61, 6008-6011.

Porcher, C., Liao, E. C., Fujiwara, Y., Zon, L. I., and Orkin, S. H. (1999). Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding. *Development* 126, 4603-4615.

Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W., and Orkin, S. H. (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* 86, 47-57.

Prabhu, S., Ignatova, A., Park, S. T., and Sun, X. H. (1997). Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. *Mol Cell Biol* 17, 5888-5896.

Quong, M. W., Massari, M. E., Zwart, R., and Murre, C. (1993). A new transcriptional-activation motif restricted to a class of helix-loop-helix proteins is functionally conserved in both yeast and mammalian cells. *Mol Cell Biol* 13, 792-800.

Rabbitts, T. H. (1998). LMO T-cell translocation oncogenes typify genes activated by chromosomal translocations that alter transcription and developmental processes. *Genes & Dev* 12, 2651-2657.

- Rabbitts, T. H., Bucher, K., Chung, G., Grutz, G., Warren, A., and Yamada, Y. (1999). The effect of chromosomal translocations in acute leukemias: the LMO2 paradigm in transcription and development. *Cancer Res* 59, 1794s-1798s.
- Rao, P. H., Houldsworth, J., Dyomina, K., Parsa, N. Z., Cigudosa, J. C., Louie, D. C., Popplewell, L., Offit, K., Jhanwar, S. C., and Chaganti, R. S. (1998). Chromosomal and gene amplification in diffuse large B-cell lymphoma. *Blood* 92, 234-240.
- Ravet, E., Reynaud, D., Titeux, M., Izac, B., Fichelson, S., Romeo, P. H., Dubart-Kupperschmitt, A., and Pflumio, F. (2004). Characterization of DNA-binding dependent and independent functions of SCL/TAL1 during human erythropoiesis. *Blood*.
- Rayet, B., and Gelinas, C. (1999). Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 18, 6938-6947.
- Reizis, B., Leder, P. (2001). The upstream enhancer is necessary and sufficient for the expression of the pre-T cell receptor alpha gene in immature T lymphocytes. *J Exp Med* 194, 979-990.
- Reuther, J. Y., Reuther, G. W., Cortez, D., Pendergast, A. M., and Baldwin, A. S., Jr. (1998). A requirement for NF-kappaB activation in Bcr-Abl-mediated transformation. *Genes Dev* 12, 968-981.
- Riechmann, V., van Cruchten, I., and Sablitzky, F. (1994). The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res* 22, 749-755.
- Riley, P., Anson-Cartwright, L., and Cross, J. C. (1998). The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat Genet* 18, 271-275.
- Rivera, R. R., Johns, C. P., Quan, J., Johnson, R. S., and Murre, C. (2000). Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. *Immunity* 12, 17-26.
- Robb, L., Elwood, N. J., Elefanty, A. G., Kontgen, F., Li, R., Barnett, L. D., and Begley, C. G. (1996). The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *Embo J* 15, 4123-4129.

Robb, L., Lyons, I., Li, R., Hartley, L., Kontgen, F., Harvey, R. P., Metcalf, D., and Begley, C. G. (1995a). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the *scl* gene. *Proc Natl Acad Sci U S A* 92, 7075-7079.

Robb, L., Rasko, J. E., Bath, M. L., Strasser, A., and Begley, C. G. (1995b). *scl*, a gene frequently activated in human T cell leukaemia, does not induce lymphomas in transgenic mice. *Oncogene* 10, 205-209.

Roman, B. L., and Weinstein, B. M. (2000). Building the vertebrate vasculature: research is going swimmingly. *Bioessays* 22, 882-893.

Sato, T., Odagiri, H., Ikenaga, S. K., Maruyama, M., and Sasaki, M. (2003). Chemosensitivity of human pancreatic carcinoma cells is enhanced by IkappaBalpha super-repressor. *Cancer Sci* 94, 467-472.

Sawada, S., and Littman, D. R. (1993). A heterodimer of HEB and an E12-related protein interacts with the CD4 enhancer and regulates its activity in T-cell lines. *Mol Cell Biol* 13, 5620-5628.

Schlissel, M., Voronova, A., and Baltimore, D. (1991). Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. *Genes Dev* 5, 1367-1376.

Schlissel, M., Voronova, A., Baltimore, D. (1991). Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. *Genes Dev* 5, 1367-1376.

Schrapppe, M., Reiter, A., Ludwig, W. D., Harbott, J., Zimmermann, M., Hiddemann, W., Niemeyer, C., Henze, G., Feldges, A., Zintl, F., *et al.* (2000). Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group. *Blood* 95, 3310-3322.

Screpanti, I., Bellavia, D., Campese, A. F., Frati, L., and Gulino, A. (2003). Notch, a unifying target in T-cell acute lymphoblastic leukemia? *Trends Mol Med* 9, 30-35.

Seldin, D. C., and Leder, P. (1995). Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle. *Science* 267, 894-897.

Sha, W. C., Liou, H. C., Tuomanen, E. I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell* 80, 321-330.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376, 62-66.

Shen, C. P., and Kadesch, T. (1995). B-cell-specific DNA binding by an E47 homodimer. *Mol Cell Biol* 15, 4518-4524.

Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and et al. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68, 855-867.

Shivdasani, R. A., Mayer, E. L., and Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncprotein tal-1/SCL. *Nature* 373, 432-434.

Shoji, W., Yamamoto, T., and Obinata, M. (1994). The helix-loop-helix protein Id inhibits differentiation of murine erythroleukemia cells. *J Biol Chem* 269, 5078-5084.

Sikder, H., Huso, D. L., Zhang, H., Wang, B., Ryu, B., Hwang, S. T., Powell, J. D., and Alani, R. M. (2003). Disruption of Id1 reveals major differences in angiogenesis between transplanted and autochthonous tumors. *Cancer Cell* 4, 291-299.

Silverman, L. B., Gelber, R. D., Dalton, V. K., Asselin, B. L., Barr, R. D., Clavell, L. A., Hurwitz, C. A., Moghrabi, A., Samson, Y., Schorin, M. A., et al. (2001). Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01. *Blood* 97, 1211-1218.

Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. (1997). Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. *J Clin Invest* 100, 2952-2960.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D., and Olson, E. N. (1997). Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nat Genet* 16, 154-160.

Stainier, D. Y., Weinstein, B. M., Detrich, H. W., 3rd, Zon, L. I., and Fishman, M. C. (1995). Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* 121, 3141-3150.

Sun, X. H. (1994). Constitutive expression of the Id1 gene impairs mouse B cell development. *Cell* 79, 893-900.

Sun, X. H., and Baltimore, D. (1991). An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell* 64, 459-470.

Sun, Z., Unutmaz, D., Zou, Y. R., Sunshine, M. J., Pierani, A., Brenner-Morton, S., Mebius, R. E., and Littman, D. R. (2000). Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science* 288, 2369-2373.

Swat, W., Shinkai, Y., Cheng, H. L., Davidson, L., and Alt, F. W. (1996). Activated Ras signals differentiation and expansion of CD4+8+ thymocytes. *Proc Natl Acad Sci U S A* 93, 4683-4687.

Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., Roderick, T. H., Stewart, C. L., Lilly, F., Hansen, C. T., and et al. (1991). FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci U S A* 88, 2065-2069.

Takeuchi, A., Yamasaki, S., Takase, K., Nakatsu, F., Arase, H., Onodera, M., and Saito, T. (2001). E2A and HEB activate the pre-TCR alpha promoter during immature T cell development. *J Immunol* 167, 2157-2163.

Talora, C., Campese, A. F., Bellavia, D., Pascucci, M., Checquolo, S., Groppioni, M., Frati, L., Von Boehmer, H., Gulino, A., and Screpanti, I. (2003). Pre-TCR-triggered ERK signalling-dependent downregulation of E2A activity in Notch3-induced T-cell lymphoma. *EMBO Rep*.

Tan, T. H., Huang, G. P., Sica, A., Ghosh, P., Young, H. A., Longo, D. L., and Rice, N. R. (1992). Kappa B site-dependent activation of the interleukin-2 receptor alpha-chain gene promoter by human c-Rel. *Mol Cell Biol* 12, 4067-4075.

Tremblay, M., Herblot, S., Lecuyer, E., and Hoang, T. (2003). Regulation of pT alpha gene expression by a dosage of E2A, HEB, and SCL. *J Biol Chem* 278, 12680-12687.

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* 371, 221-226.

Ueda, E., Kurebayashi, S., Sakaue, M., Backlund, M., Koller, B., and Jetten, A. M. (2002). High incidence of T-cell lymphomas in mice deficient in the retinoid-related orphan receptor RORgamma. *Cancer Res* 62, 901-909.

Uetsuka, H., Haisa, M., Kimura, M., Gunduz, M., Kaneda, Y., Ohkawa, T., Takaoka, M., Murata, T., Nobuhisa, T., Yamatsuji, T., *et al.* (2003). Inhibition of inducible NF-kappaB activity reduces chemoresistance to 5-fluorouracil in human stomach cancer cell line. *Exp Cell Res* 289, 27-35.

Valge-Archer, V. E., Osada, H., Warren, A. J., Forster, A., Li, J., Baer, R., and Rabbitts, T. H. (1994). The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells. *Proc Natl Acad Sci U S A* 91, 8617-8621.

van Eekelen, J. A., Bradley, C. K., Gothert, J. R., Robb, L., Elefanty, A. G., Begley, C. G., and Harvey, A. R. (2003). Expression pattern of the stem cell leukaemia gene in the CNS of the embryonic and adult mouse. *Neuroscience* 122, 421-436.

van Hogerlinden, M., Rozell, B. L., Ahrlund-Richter, L., and Toftgard, R. (1999). Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. *Cancer Res* 59, 3299-3303.

Visvader, J. E., Fujiwara, Y., and Orkin, S. H. (1998). Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev* 12, 473-479.

Visvader, J. E., Mao, X., Fujiwara, Y., Hahm, K., and Orkin, S. H. (1997). The LIM-domain binding protein Ldb1 and its partner LMO2 act as negative regulators of erythroid differentiation. *Proc Natl Acad Sci U S A* *94*, 13707-13712.

Vitelli, L., Condorelli, G., Lulli, V., Hoang, T., Luchetti, L., Croce, C. M., and Peschle, C. (2000). A pentamer transcriptional complex including tal-1 and retinoblastoma protein downmodulates c-kit expression in normal erythroblasts. *Mol Cell Biol* *20*, 5330-5342.

Voll, R. E., and Ghosh, S. (1999). Role of NF-kappa B in T-lymphocyte development. *Cold Spring Harb Symp Quant Biol* *64*, 485-490.

Voll, R. E., Jimi, E., Phillips, R. J., Barber, D. F., Rincon, M., Hayday, A. C., Flavell, R. A., and Ghosh, S. (2000). NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. *Immunity* *13*, 677-689.

Voronova, A. F., and Lee, F. (1994). The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. *Proc Natl Acad Sci U S A* *91*, 5952-5956.

Vyas, P., McDevitt, M. A., Cantor, A. B., Katz, S. G., Fujiwara, Y., and Orkin, S. H. (1999). Different sequence requirements for expression in erythroid and megakaryocytic cells within a regulatory element upstream of the GATA-1 gene. *Development* *126*, 2799-2811.

Wadman, I., Li, J., Bash, R. O., Forster, A., Osada, H., Rabbitts, T. H., and Baer, R. (1994a). Specific in vivo association between the bHLH and LIM proteins implicated in human T cell leukemia. *Embo J* *13*, 4831-4839.

Wadman, I. A., Hsu, H. L., Cobb, M. H., and Baer, R. (1994b). The MAP kinase phosphorylation site of TAL1 occurs within a transcriptional activation domain. *Oncogene* *9*, 3713-3716.

Wadman, I. A., Osada, H., Grutz, G. G., Agulnick, A. D., Westphal, H., Forster, A., and Rabbitts, T. H. (1997). The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *Embo J* *16*, 3145-3157.

Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 274, 784-787.

Wang, J., Hoshino, T., Redner, R. L., Kajigaya, S., and Liu, J. M. (1998). ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. *Proc Natl Acad Sci U S A* 95, 10860-10865.

Wang, J., Jani-Sait, S. N., Escalon, E. A., Carroll, A. J., de Jong, P. J., Kirsch, I. R., and Aplan, P. D. (2000). The t(14;21)(q11.2;q22) chromosomal translocation associated with T-cell acute lymphoblastic leukemia activates the BHLHB1 gene. *Proc Natl Acad Sci U S A* 97, 3497-3502.

Wang, W., Abbruzzese, J. L., Evans, D. B., Larry, L., Cleary, K. R., and Chiao, P. J. (1999). The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. *Clin Cancer Res* 5, 119-127.

Warrell, R. P., Jr., He, L. Z., Richon, V., Calleja, E., and Pandolfi, P. P. (1998). Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J Natl Cancer Inst* 90, 1621-1625.

Warren, A. J., Colledge, W. H., Carlton, M. B., Evans, M. J., Smith, A. J., and Rabbitts, T. H. (1994). The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell* 78, 45-57.

Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356, 314-317.

Weih, F., Carrasco, D., Durham, S. K., Barton, D. S., Rizzo, C. A., Ryseck, R. P., Lira, S. A., and Bravo, R. (1995). Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. *Cell* 80, 331-340.

Wice, B. M., and Gordon, J. I. (1998). Forced expression of Id-1 in the adult mouse small intestinal epithelium is associated with development of adenomas. *J Biol Chem* 273, 25310-25319.

Xia, Y., Hwang, L. Y., Cobb, M. H., and Baer, R. (1994). Products of the TAL2 oncogene in leukemic T cells: bHLH phosphoproteins with DNA-binding activity. *Oncogene* 9, 1437-1446.

Xu, Z., Huang, S., Chang, L. S., Agulnick, A. D., and Brandt, S. J. (2003). Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation. *Mol Cell Biol* 23, 7585-7599.

Yamada, Y., Pannell, R., Forster, A., and Rabbitts, T. H. (2000). The oncogenic LIM-only transcription factor Lmo2 regulates angiogenesis but not vasculogenesis in mice. *Proc Natl Acad Sci U S A* 97, 320-324.

Yan, W., Young, A. Z., Soares, V. C., Kelley, R., Benezra, R., and Zhuang, Y. (1997). High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. *Mol Cell Biol* 17, 7317-7327.

Yang, J., and Richmond, A. (2001). Constitutive IkappaB kinase activity correlates with nuclear factor-kappaB activation in human melanoma cells. *Cancer Res* 61, 4901-4909.

Yin, X., Giap, C., Lazo, J. S., and Prochownik, E. V. (2003). Low molecular weight inhibitors of Myc-Max interaction and function. *Oncogene* 22, 6151-6159.

Yokota, Y., Mansouri, A., Mori, S., Sugawara, S., Adachi, S., Nishikawa, S., and Gruss, P. (1999). Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 397, 702-706.

Yoshihara, T., Inaba, T., Shapiro, L. H., Kato, J. Y., and Look, A. T. (1995). E2A-HLF-mediated cell transformation requires both the trans-activation domains of E2A and the leucine zipper dimerization domain of HLF. *Mol Cell Biol* 15, 3247-3255.

Yu, W., Nagaoka, H., Jankovic, M., Misulovin, Z., Suh, H., Rolink, A., Melchers, F., Meffre, E., Nussenzweig, M.C. (1999). Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature* 400(6745), 682-687.

Zebedee, Z., and Hara, E. (2001). Id proteins in cell cycle control and cellular senescence. *Oncogene* 20, 8317-8325.

Zhang, Y., Iratni, R., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (1997). Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. *Cell* 89, 357-364.

Zhong, T. P., Childs, S., Leu, J. P., and Fishman, M. C. (2001). Gridlock signalling pathway fashions the first embryonic artery. *Nature* 414, 216-220.

Zhuang, Y., Cheng, P., and Weintraub, H. (1996a). B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. *Mol Cell Biol* 16, 2898-2905.

Zhuang, Y., Cheng, P., and Weintraub, H. (1996b). B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. *Mol Cell Biol* 16, 2898-2905.

Zong, W. X., Edelstein, L. C., Chen, C., Bash, J., and Gelinas, C. (1999). The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. *Genes Dev* 13, 382-387.

APPENDIX

Microarray Results

Genes Activated or Repressed by Tal1 Expression in the Thymus

Gene	Accession Number	Fold Change
GM2 activator protein	U09816	+7.3
IMAGE clone	AI152867	+6.3
Casitas B-lineage lymphoma	X57111	+5.7
RAB7	Y13361	+5.6
IMAGE clone	AI451701	+5.1
Pknox1	AF061270	+4.7
MAdCAM-1	D50434	+4.7
Interleukin 11 receptor	U14412	+4.3
ASF	X66091	+4.1
New-Rhobin	X95761	+4
MAS1 oncogene	X67735	+4
ZT2 zinc finger protein	AJ005350	+4
LIM and SH3 protein 1	U58882	+4
Otoconin-90	AF091847	+3.9
Caspase 3	U54803	+3.8
IMAGE clone	AW106745	+3.8
Nuclear factor of activated T cells	U36576	+3.7
Thymopoietin	U39074	+3.6
Receptor activator of nuclear factor kappa B	AF019046	+3.6
Ig germline H-chain gene V-region	M16724	+3.6
Aristaless	U96109	+3.6
TCF1	X61385	+3.5
Matrix metalloproteinase 11	Z12604	+3.3
IMAGE clone	AI644072	+3.3
S-AKAP84	U95145	+3.2
IMAGE clone	W49316	+3
Protein tyrosine phosphatase, receptor type, R	D31898	+3
LERK-3	U92885	+2.9
G-protein coupled receptor 12	D21061	+2.8
Zinc transporter 3	U76009	+2.7
IMAGE clone	AI427892	+2.6
T-cell receptor germline beta chain	M21000	+2.6
T-cell receptor delta chain	L36135	+2.5
T-cell receptor beta-chain	M21203	+2.2
T-cell receptor insulin reactive alpha chain	M16118	-2.1
T-cell receptor alpha chain variable region	AF099808	-2.3
Bcl-X _L	L35049	-2.4
CD4 antigen	M17080	-2.5

Cysteine rich protein	D88793	-2.5
Jun proto-oncogene related gene d1	J04509	-2.7
IMAGE clone	AI120844	-2.8
Tip associating protein	AF093140	-2.8
Transaldolase	U67611	-2.9
WW-domain binding protein 2	U40826	-2.9
Isocitrate dehydrogenase 2	U51167	-2.9
CD3 antigen	M23376	-3
Thymus leukemia antigen	X03052	-3.1
Histocompatibility 2, T region locus 17	M35247	-3.1
SIT protein	AJ236881	-3.1
YL-1 protein	D43643	-3.2
Hepatoma-derived growth factor	D63850	-3.2
Replication factor C	X72711	-3.2
Interferon-gamma receptor	M28233	-3.3
Microtubule associated protein 4	M72414	-3.3
I kappa B alpha	U57524	-3.3
LKB1 serine threonine kinase	AB026255	-3.4
COP9 complex subunit 3	AF071313	-3.4
IMAGE clone	AI41025	-3.5
Histocompatibility 2, T region locus 10	M35244	-3.5
Cut alternatively spliced product	U66249	-3.6
E26 avian leukemia oncogene 2	J04103	-3.6
PEST phosphatase interacting protein	U87814	-3.6
Oxidative stress induced protein	U40930	-3.6
IMAGE clone	AI020259	-3.7
Hemin-sensitive initiation factor 2 alpha kinase	AF028808	-3.7
PQBP-1	AJ250406	-3.8
Protective protein for beta-galactosidase	J05261	-3.8
MAD homolog 4	U79748	-3.9
RAG1	M29475	-3.9
Immunoglobulin mu encoding the C-terminus of the membrane bound form	V00821	-3.9
Proapoptotic protein (Siva)	AF033115	-3.9
Mad3	U32394	-3.9
Early growth response 1	M28845	-4.1
TL antigen	D86082	-4.1
NAKAP95	AB028921	-4.2
Cathepsin L	X06086	-4.2
Cyclin 3	M86183	-4.2
A10	L21027	-4.3
Retinal short chain dehydrogenase	X95281	-4.3

RAN GTPase activating protein 1	U08110	-4.3
TGF beta-1	AJ009862	-4.6
Non-receptor protein tyrosine kinase Ack	AF037260	-4.6
IMAGE clone	AI591702	-4.6
ARF5	D87902	-4.6
UBF transcription factor	X60831	-4.7
Protein L	AB009392	-4.8
p116Rip	U73200	-4.9
IMAGE clone	AA940036	-5
Antigen identified by MRC OX-2	AF0292	-5
Dishevelled 2	U24160	-5.1
14-3-3 epsilon	D87663	-5.1
Psme3 gene for PA28 gamma subunit	AB007139	-5.2
Zinc finger protein 36	X14678	-5.2
Heterogeneous nuclear ribonucleoprotein G	AJ237846	-5.2
Serine/threonine kinase 5	D21099	-5.3
Cytoplasmic tyrosine kinase, Dscr28C related	X55663	-5.5
Transcription factor GIF	Af064088	-5.6
RAR-related orphan receptor gamma	AF019660	-5.6
Thymoma viral proto-oncogene 2	U22445	-5.7
Inositol polyphosphate-5-phosphatase	AF040094	-5.7
Schwannoma-associated protein (SAM9)	AF026124	-5.9
Ring finger protein 3	AL009226	-6
Flotillin	U90434	-6.1
Growth factor independent 1	U58972	-6.1
Stromal interaction molecule 1	U47323	-6.2
Kallikrein 8	X03994	-6.3
Casein kinase	AB028241	-6.5
Cdc2/CDC28-like protein kinase 3	AF033565	-6.8
RAG2	M64796	-6.9
Id3	M60523	-7
Prl3	AF035645	-7
Retinoid X receptor beta	D21831	-7.3
IMAGE clone	AA983101	-7.3
Insulin-like growth factor	X71922	-7.4
KIFC1	D49544	-7.4
Coiled-coil like protein 1	U79024	-7.6
Interleukin 4 receptor	M27960	-8.1
3BP-1	X87671	-8.2
Small proline-rich protein 2A	AJ005559	-8.5
AZ1	D88509	-8.5
Mast cell protease 5	M68898	-8.7

RNPS1	X70067	-9
Phosphatidylinositol-4-phosphate 5-kinase I-gamma	AB006916	-9.1
Somatostatin receptor 3	M91000	-9.2
Zinc-finger protein	U14556	-9.2
IMAGE clone	AA204010	-10.7
IMAGE clone	AI646422	-11.3
G-protein couple receptor kinase 6-B	Y15798	-14.7
CD6 antigen	U12434	-16
CD5 antigen	M15177	-17.5
IMAGE clone	AA881438	-23.5

RNA was prepared from sorted CD4-positive, CD8-positive thymocytes from four-week-old, age-matched wildtype and *Tal1* transgenic mice using Trizol reagent (Invitrogen). cDNA was then synthesized from the RNA samples using the Superscript system (Gibco). Biotin labeled cRNA was subsequently made from the cDNA using a RNA transcript labeling kit (Enzo). The labeled cRNA was fragmented and hybridized to the Affymetrix mouse U74Av2 array.