TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB

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Summary

Activation of the basic-helix-loop-helix (bHLH) gene *TAL1* (or *SCL*) is a frequent gain-of-function mutation in T cell acute lymphoblastic leukemia (T-ALL). To provide genetic evidence that tal1/scl induces leukemia by interfering with E47 and HEB, we expressed tal1/scl in an E2A or HEB heterozygous background. These mice exhibit disease acceleration and perturbed thymocyte development due to repression of E47/HEB target genes. In *tal1/scl* thymocytes, we find the corepressor mSin3A bound to the CD4 enhancer, whereas an E47/HEB/p300 complex is detected in wild-type thymocytes. Furthermore, *tal1/scl* tumors are sensitive to pharmacologic inhibition of HDAC and undergo apoptosis. These data demonstrate that tal1/scl induces leukemia by repressing E47/HEB and suggest that HDAC inhibitors may prove efficacious in T-ALL patients who express TAL1/SCL.

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/SCL 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/SCL activation respond poorly to therapy, with only 50% of patients surviving 5 years (Ferrando et al., 2002).

TAL1/SCL heterodimerizes with class I or A bHLH proteins including E12, E47, HEB and E2-2 (Voronova and Lee, 1994; O'Neil et al., 2001; Hsu et al., 1991), and in hematopoietic cells is part of a large transcriptional complex that includes GATA-1 and the LIM-only proteins LMO2 and Ldb-1 (Wadman et al., 1997; Valge-Archer et al., 1994). Mice deficient for tal1/scl have no primitive or definitive hematopoiesis and exhibit angiogenic defects (Shivdasani et al., 1995; Visvader et al., 1998). Surprisingly, conditional inactivation of *tal1/scl* in adult mice does not result in hematopoietic defects, suggesting that tal1/scl is critical for the genesis of the hematopoietic stem cell (HSC), but not required for its maintenance (Mikkola et al., 2003). The function of tal1/scl in the hematopoietic stem cell has suggested that tal1/scl activation in leukemia may stimulate the activation of genes important in stem cell expansion and/or self-renewal.

In mouse *tal1/scl* tumors and in Jurkat cells, a human leukemic cell line that expresses TAL1/SCL, stable Tal1/scl/E47 and Tal1/scl/HEB heterodimers are readily detected (Hsu et al., 1994a; O'Neil et al., 2001), and the related bHLH proteins LYL-1 and BHLH-1 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 to be activated in gene therapy-induced T-ALL (Kohn et al., 2003; O'Neil et al., 2001; Rabbitts, 1998). Yet, how LMO1/2 inhibit E2A function and contribute to leukemia remains unclear. In some leukemic patients, LMO2 and TAL1/SCL are coexpressed (Ferrando et al., 2002), suggesting that leukemic transformation is dependent on the expression of both the TAL1/SCL and LMO2 proteins. Consistent with this idea, a TAL1/SCL/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/scl and LMO2 (Larson et al., 1996). However, not all human T-ALL patients who express TAL1/SCL also express LMO1 or LMO2 (Ferrando et al., 2002). Similarly, only one of six mouse *tal1/scl* tumors examined expresses LMO2 (J.S. and M.K., unpublished data), revealing that LMO2 expression is also not required for *tal1/scl*-induced leukemogenesis in the mouse.

To test whether tal1/scl induces leukemia by interfering with E protein function(s), we expressed the tal1/scl oncogene in an E2A or HEB heterozygous background. We observe thymocyte differentiation arrest and disease acceleration in *tal1/scl/E2A*$^{+/-}$

SIGNIFICANCE

T-ALL patients who activate bHLH proteins like TAL1/SCL respond poorly to current therapies and are at high risk of treatment failure. This study demonstrates that tal1/scl interferes with the transactivation of several E47/HEB target genes, critical for thymocyte differentiation and survival. Repression is mediated by the recruitment of the mSin3A/HDAC1 corepressor complex to E47/HEB target genes, raising the possibility that HDAC inhibitors may be beneficial in treating T-ALL patients with *TAL1/SCL* activation.

and *tal1/scl/HEB*$^{+/-}$ mice, providing genetic evidence that tal1/scl induces leukemia by interfering with E47/HEB. Consistent with the differentiation arrest, gene expression profiling of premalignant *tal1/scl* thymocytes reveals repression of several genes important for thymocyte differentiation. The expression of the E47/HEB target genes, Rag 2 and Pre-Tα, are decreased, on a per cell basis, in premalignant *tal1/scl* cells, and Rag 2 is further decreased in *tal1/scl/E2A*$^{+/-}$ thymocytes. Tal1/scl 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/scl, like the leukemogenic fusion proteins PML-RARα and AML-1/ETO, contributes to leukemia by repressing gene expression and inducing differentiation arrest.

Results

Thymocyte development is perturbed in *tal1/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice

Expression of *tal1/scl* in the thymus results in a 50% decrease in overall thymocyte cellularity (Figure 1A, $p < 0.0000003$) and a 3- to 4-fold decrease in the absolute numbers of double positive thymocytes ($p = 0.002$), as well as decreases in CD4 single positive thymocytes ($p < 0.00003$) (Figure 1B). Increases in the absolute number of immature CD8 single positive thymocytes are also observed when tal1/scl is expressed in the wild-type background (not shown) ($p = 0.03$). Analysis of thymocytes from *tal1/E2A*$^{+/-}$ and *tal1/HEB*$^{+/-}$ mice revealed severe decreases in the percentage of double positive thymocytes (average 43% for *tal1/scl/E2A*$^{+/-}$ and 40% for *tal1/scl/HEB*$^{+/-}$) and increases in the percentage of CD8 single positive thymocytes (Figure 1C), suggesting that tal1/scl 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., 1996), tal1/scl expression in an E2A heterozygous background results in a 3-fold decrease in the absolute number of DP thymocytes (Figure 1B; $p = 0.001$) and 3-fold decrease in CD4 SP thymocytes ($p = 0.02$).

The decrease in DP thymocytes and increase in CD8 SP thymocytes observed in *tal1/scl* mice may reflect an inability to express adequate amounts of the CD4 coreceptor (Figures 1B and 1C). The expression of the CD4 coreceptor, 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- to 5-fold decreases in TCR β chain expression in thymocytes from *tal1/scl/E2A*$^{+/-}$ mice (not shown) as well as decreased CD5 expression (Table 2 and Figure 4), suggesting that tal1/scl interferes with the E47/HEB heterodimer.

Increases in the percentage of DN thymocyte precursors are observed in *tal1/scl* transgenic mice when compared to wild-type littermate controls (average 4% DN in wt versus 12% DN in *tal1/scl*; $p < .00008$). The percentage of DN thymocyte precursors is further increased in *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice (16.6% DN in *tal1/scl/E2A*$^{+/-}$ and 23% DN in *tal1/scl/HEB*$^{+/-}$ mice), suggesting that DN thymocyte development may be more severely affected in these mice than when tal1/scl is expressed on a wild-type background ($p = .027$ for *tal1/scl/E2A*$^{+/-}$; $p = .025$ for *tal1/scl/HEB*$^{+/-}$). *Tal1/scl* transgenic mice exhibit thymocyte arrest at the DN2 stage of thymocyte development with increases in the CD44-positive, CD25-positive DN thymocytes (Figure 1D). We found DN thymocyte development severely affected in *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice, with evidence of arrest at the DN2 and DN3 stages, respectively. Tal1/scl expression in an E2A or HEB heterozygous background perturbs thymocyte development and induces a partial differentiation arrest similar to that observed in E2A- or HEB-deficient mice (Bain et al., 1997; Barndt et al., 1999).

Disease acceleration in *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice

We have previously demonstrated that perturbation of thymocyte development is a central feature associated with the development of *tal1/scl*-induced leukemia in the mouse (O'Neil et al., 2001, 2003). The thymocyte developmental defects observed when tal1/scl is expressed in an E2A or HEB heterozygous background suggested to us that leukemogenesis may be accelerated in these mice.

Compared to *tal1/scl* transgenic mice on the wild-type background, *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice develop disease rapidly within a median survival period of 216 days ($p < 0.0001$) and 143 days ($p < 0.0001$), respectively (Figure 2). In contrast, *tal1/scl* littermates on the mixed background, but wild-type with respect to E2A and HEB, develop disease at a similar frequency as published *tal1/scl* transgenic mice on the FVB/N background (Kelliher et al., 1996). Interestingly, *tal1/scl/HEB*$^{+/-}$ mice develop disease more rapidly than *tal1/scl/E2A*$^{+/-}$ mice ($p < 0.0001$). To provide additional evidence that an E2A or HEB haploinsufficiency collaborates with tal1/scl to induce leukemia and does not reflect effects of the transgene insertion site, we mated an additional tal1/scl transgenic line with *E2A*$^{+/-}$ mice. These *tal1/scl/E2A*$^{+/-}$ mice exhibit similar perturbations in thymocyte development (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/5/6/587/DC1), and six mice developed leukemia with an average latency of 112 days. Thus, disease acceleration reflects effects of tal1/scl on E2A and HEB proteins and does not appear to reflect transgene insertion sites or genetic differences between the strains of mice.

A highly significant increase in disease penetrance was also observed in both the *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice compared to *tal1/scl* transgenic mice. T cell lymphoblastic leukemia is completely penetrant in *tal1/scl/HEB*$^{+/-}$ mice and 80% penetrant in the *tal1/scl/E2A*$^{+/-}$ background. In contrast, only 28% of *tal1/scl* transgenic mice in the wild-type background 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 was indistinguishable from that previously observed in *tal1/scl* transgenic mice (Kelliher et al., 1996). In addition, examination of other tissues revealed evidence of tumor infiltration to organs such as liver and kidney.

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

The disease acceleration observed when tal1/scl is expressed in an E2A or HEB heterozygous background suggested to us

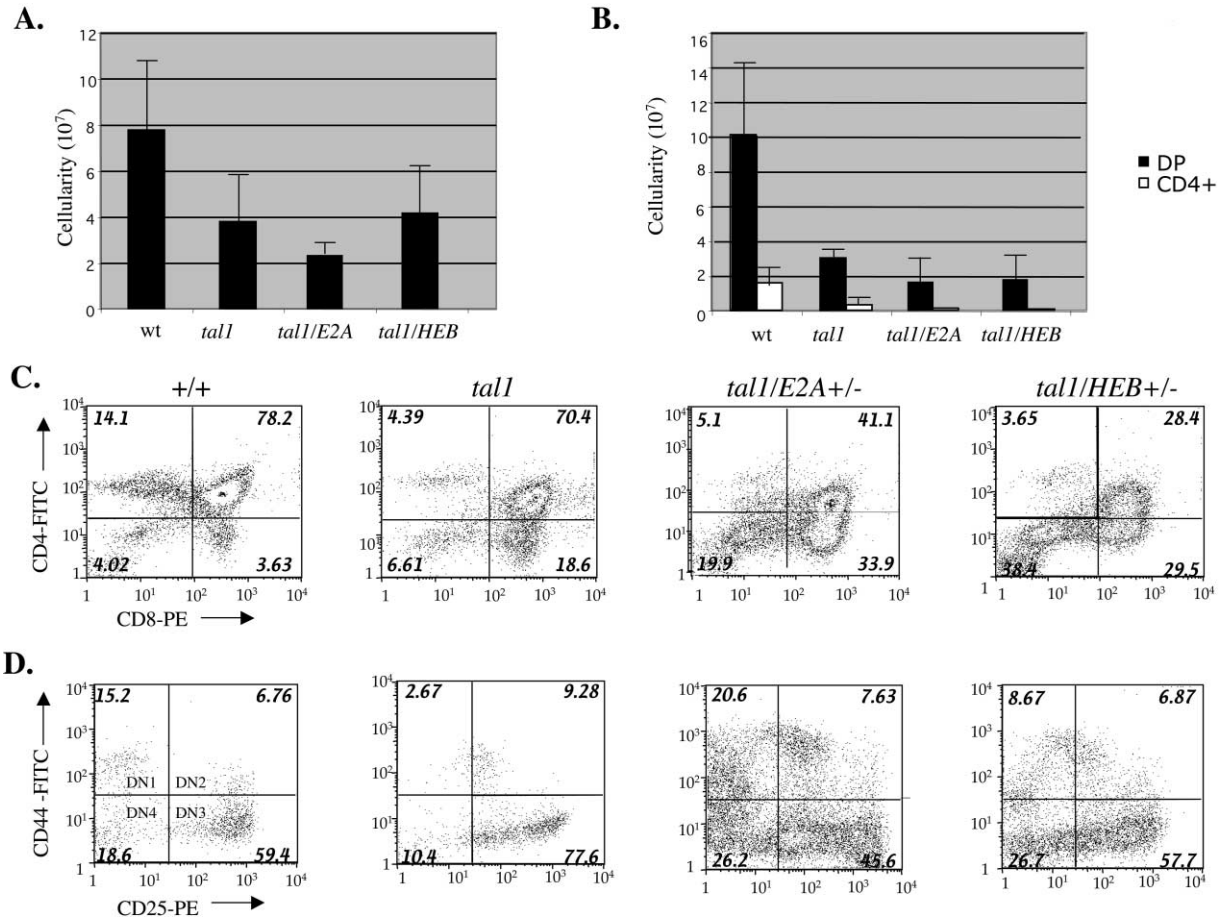

Figure 1. Thymocyte development is severely perturbed when Tal1/scl is expressed on an *E2A*$^{+/-}$ or *HEB*$^{+/-}$ background

A: Decreased thymocyte cellularity in *tal1/scl* transgenic mice. Total thymocyte numbers from four-week-old, wild-type (wt), *tal1/scl, tal1/scl/E2A*$^{+/-}$, and *tal1/scl/HEB*$^{+/-}$ mice. Tal1/scl reduces thymocyte cellularity when expressed on a wt background ($p < .0000003$). Tal1/scl expression on the *E2A*$^{+/-}$ or *HEB*$^{+/-}$ background does not cause further reductions in cellularity that are statistically significant.

B: Tal1/scl expression is associated with reductions in the absolute numbers of DP and CD4 SP thymocytes. The absolute numbers of DP and CD4+ SP thymocytes in wt, *tal1/scl, tal1/scl/E2A*$^{+/-}$, and *tal1/scl/HEB*$^{+/-}$ in preleukemic, four-week-old mice are represented. Compared to littermate controls, Tal1/scl expression results in a reduction in the absolute numbers of DP thymocytes ($p = .002$) and CD4+ SP thymocytes ($p = .00003$). Tal1/scl expression on the *E2A*$^{+/-}$ background results in further decreases in the absolute numbers of DP thymocytes ($p = .001$) and CD4+ SP thymocytes ($p = .02$).

C: DP thymocyte development is further perturbed when Tal1/scl is expressed on an *E2A*$^{+/-}$ or *HEB*$^{+/-}$ background. Thymocytes from four-week-old wt, *tal1/scl, tal1/scl/E2A*$^{+/-}$, and *tal1/scl/HEB*$^{+/-}$ mice were stained with CD4-Cy and CD8-FITC and analyzed by flow cytometry.

D: The percentage of DN thymocyte precursors is increased when Tal1/scl is expressed on an *E2A*$^{+/-}$ or *HEB*$^{+/-}$ background. Thymocytes from 4-week-old wt, *tal1/scl*, *tal1/scl/E2A*$^{+/-}$, and *tal1/scl/HEB*$^{+/-}$ 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. A representative profile of the lineage negative cells stained with CD25-PE and CD44-FITC is shown. Tal1/scl increases the percentage of DN thymocytes compared to littermate controls ($p < .00008$). Tal1/scl expression on an *E2A*$^{+/-}$ or *HEB*$^{+/-}$ background further increases the percentage of DN precursors ($p = 0.03$ for *tal1/scl/E2A*$^{+/-}$ and $p = 0.03$ for *tal1/scl/HEB*$^{+/-}$).

that polyclonal tumors may develop in these mice. To examine this possibility, DNA isolated from *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ tumors was digested with HindIII and analyzed by Southern blot analysis with a TCR Jβ2 probe (Figure 3A). 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/scl/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/scl/E2A*$^{+/-}$ and *tal1/scl/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 1). Diverse tumor phenotypes were also observed in *tal1/scl* transgenic mice (Kelliher et al., 1996). However, about twenty percent of the *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ tumors failed to express CD3, CD4, and CD8, indicating that a more immature cell type may be transformed in some of the *tal1/scl/E2A*$^{+/-}$ or *HEB*$^{+/-}$ mice.

Disease acceleration is not accompanied by loss of heterozygosity in *tal1/scl/E2A*$^{+/-}$ mice

The observation that a percentage of E2A-deficient mice are susceptible to the development of T cell leukemia/lymphoma

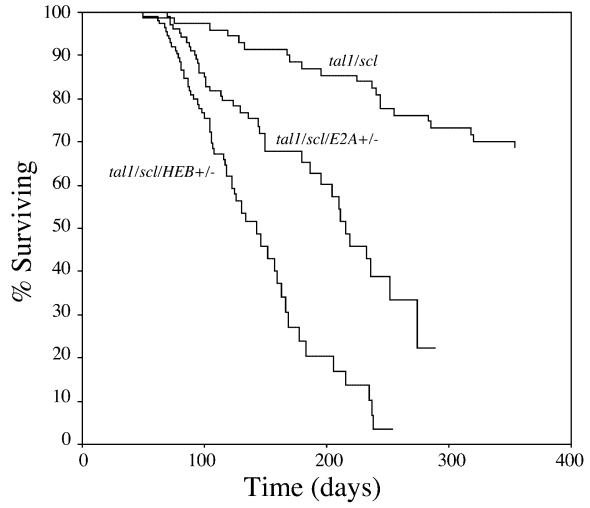

Figure 2. Accelerated leukemogenesis when tal1/scl is expressed in an E2A or HEB heterozygous background

Kaplan-Meier survival plot of *tal1/scl*, *tal1/scl/E2A*$^{+/-}$, and *tal1/scl/HEB*$^{+/-}$ mice. The cohort of *tal1/scl* mice consisted of n = 75 animals, the *tal1/scl/E2A*$^{+/-}$ cohort consisted of n = 102 animals, and the *tal1/scl/HEB*$^{+/-}$ cohort consisted of n = 114 animals. Tarone-Ware statistical analysis confirmed a highly statistically significant difference in survival when *tal1/scl* transgenic mice in the wild-type background are compared to *tal1/scl* in the *E2A*$^{+/-}$ ($p < 0.0001$) or *HEB*$^{+/-}$ ($p < 0.0001$) genetic backgrounds. The difference in survival between *tal1/scl/HEB*$^{+/-}$ mice and *tal1/scl/E2A*$^{+/-}$ mice was also statistically significant ($p < 0.0001$). All animals were monitored daily for signs of disease. Upon onset of disease, the mice were sacrificed and a postmortem examination was performed.

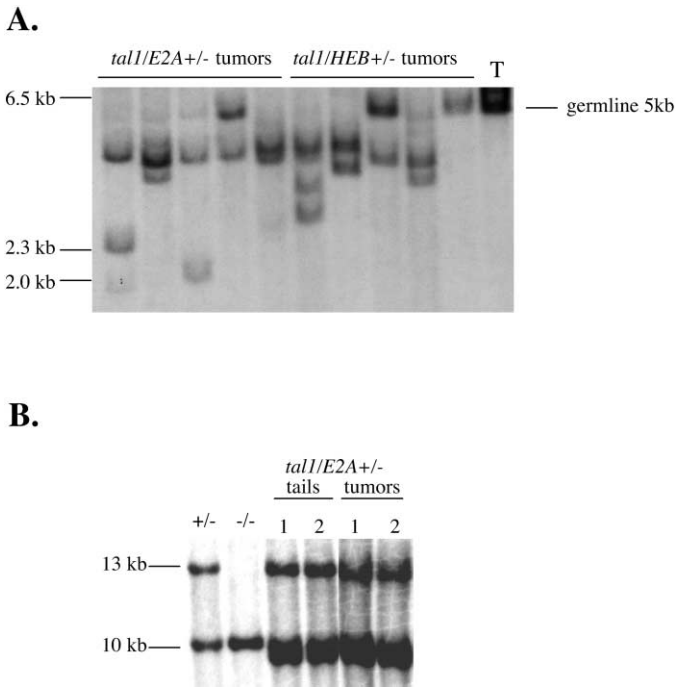

Figure 3. *Tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ tumors are clonal or oligoclonal and do not exhibit loss of heterozygosity

A: Tumors isolated from *tal1/scl/E2A*$^{+/-}$ or *HEB*$^{+/-}$ mice are clonal or oligoclonal. DNA prepared from tumors and wild-type genomic tail DNA was digested with HindIII and analyzed by Southern blot analysis. T cell receptor Jβ chain rearrangements were detected with a probe that identified a 5 kb DNA fragment in the germline position of genomic tail DNA (lane T).
B: Disease acceleration is not accompanied by loss of heterozygosity in *tal1/scl/E2A*$^{+/-}$ mice. *E2A*$^{+/-}$ and *E2A*$^{-/-}$ tail DNA, *tal1/scl/E2A*$^{+/-}$ tail DNA, and DNA prepared from tumors from the same *tal1/scl/E2A*$^{+/-}$ mice was digested with BamHI and analyzed by Southern blot analysis. Using a portion of an E2A genomic clone as a probe, the wild-type E2A allele was identified as a 13 kb fragment and the mutant allele as a 10 kb fragment.

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/scl/E2A*$^{+/-}$ mice may exhibit loss of heterozygosity (LOH). To examine this possibility, we isolated DNA from tumors derived from *tal1/scl/E2A*$^{+/-}$ mice and analyzed the *E2A* locus by Southern blotting (Figure 3B). All *tal1/scl/E2A*$^{+/-}$ tumors tested retained the wild-type allele of *E2A*, indicating that LOH is not a feature associated with disease acceleration in *tal1/scl/E2A*$^{+/-}$ mice. However, loss of E2A expression in the *tal1/scl/E2A*$^{+/-}$ tumors could involve methylation of regulatory sequences. To confirm that the remaining *E2A* allele is expressed, we prepared nuclear lysates from *tal1/scl/E2A*$^{+/-}$ tumors and were able to detect E47 expression by immunoblotting (not shown). Similar results were obtained on tumors from *tal1/scl/HEB*$^{+/-}$ mice, indicating that tal1/scl is not simply cooperating with loss of E2A or HEB proteins to induce leukemia in mice.

Thymic expression of the tal1/scl oncogene is associated with gene repression

To identify potential target genes deregulated by tal1/scl activation, we performed gene expression profiling of premalignant *tal1/scl* 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, wild-type and *tal1/scl* transgenic mice were compared. Consistent with the differentiation arrest observed in the *tal1/scl* transgenic mice (O'Neil et al., 2003), we found the lymphoid-specific cyclin D3 decreased in thymocytes that expressed Tal1/scl (Table 2). Moreover, the expression of several genes important in thymocyte differentiation was also reduced in *tal1/scl* thymocytes, including those encoding CD3, CD6, CD5, RAG1, RAG2, and RORγ (Figure 4 and data not shown). Some of the genes repressed in *tal1/scl* thymocytes have been previously thought to be regulated by E47/HEB heterodimer, notably RAG1/2 and CD5 (Bain et al., 1994; Schlissel et al., 1991; Zhuang et al., 1996). Other genes such as the retinoid-related orphan receptor γ (RORγ) 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, RORγ-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 RORγ expression is decreased in premalignant *tal1/scl* thymocytes, we prepared total cell lysates from thymocytes from *tal1/scl* transgenic and control littermates. We found RORγ levels decreased in cells that express tal1/scl compared to thymocytes from control littermates (Figure 4C). Moreover, repression of RORγ expression was maintained in all seven of the *tal1/scl* tumors examined,

Table 1. Immunophenotypes of *tal1/E2A*$^{+/-}$ and *tal1/HEB*$^{+/-}$ tumors

Animal number	Genotype	CD3	CD4	CD8	CD4, CD8	Phenotype	Survival (days)
737	*tal1/E2A*$^{+/-}$	4	12	4	8	DP	74
6778	*tal1/E2A*$^{+/-}$	2	0	43	0	ISPCD8+	85
6831	*tal1/E2A*$^{+/-}$	32	0	97	2	CD8+	104
8583	*tal1/E2A*$^{+/-}$	32	1	71	0	CD8+	89
388	*tal1 E2A*$^{+/-}$	1	0	0	0	DN	92
9845	*tal1/E2A*$^{+/-}$	31	90	93	78	DP	144
135	*tal1/HEB*$^{+/-}$	45	17	98	18	DP	105
138	*tal1/HEB*$^{+/-}$	69	8	97	8	CD8+	100
8959	*tal1/HEB*$^{+/-}$	4	29	93	30	DP	116
9193	*tal1/HEB*$^{+/-}$	5	19	46	18	DP	81
9205	*tal1/HEB*$^{+/-}$	26	70	92	70	DP	70
9232	*tal1/HEB*$^{+/-}$	8	74	98	75	DP	83
8906	*tal1/HEB*$^{+/-}$	93	1	6	0	DN	67

Tumors were stained with antibodies to CD3, CD4, and CD8 and analyzed by flow cytometry. Tumors were considered positive if >10% of the tumor stained positive for the antibody.

suggesting that RORγ repression may be 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 RORγ genes, suggesting that RORγ may also be regulated by the E47/HEB heterodimer.

The E47/HEB target genes rag 2 and pre-Tα are reduced on a per cell basis in *tal1/scl* and *tal1/scl/E2A*$^{+/-}$ thymocytes

Our microarray experiment suggests that tal1/scl 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 affected by tal1/scl expression, we mated our *tal1/scl* transgenic mice to transgenic mice that carry a BAC in which GFP has been introduced into the mouse *Rag 2* locus by homologous recombination (Yu et al., 1999). Thymocytes from age-matched, premalignant *tal1/scl* mice were stained with antibodies against CD4 and CD8, and the GFP levels in the various thymic subpopulations were examined. The mean fluorescence intensity (MFI) of the Rag 2-driven GFP was consistently reduced on a per cell basis in *tal1/scl* double positive thymocytes compared to control littermates (Figure 5A; MFI = 1087 for wt, compared to MFI = 448 for *tal1/scl*), indicating reduced Rag 2 expression in *tal1/scl* double positive thymocytes. Reduced Rag 2 expression was also observed in the DN thymocyte precursor populations upon tal1/scl expression (not shown). If tal1/scl functions by inhibiting E47/HEB-mediated transcription of Rag 2, then one might predict further decreases in Rag 2 expression in *tal1/scl/E2A*$^{+/-}$ or *tal1/scl/HEB*$^{+/-}$ preleukemic thymocytes. To test this possibility, we mated our *tal1/scl/E2A*$^{+/-}$ mice to the Rag 2-GFP mice. In all mice examined, we found Rag 2-driven expression of GFP further reduced in thymocytes from *tal1/scl/E2A*$^{+/-}$ mice (MFI = 278) compared to *tal1/scl* transgenic mice (MFI = 448), indicating further E47/HEB inhibition in *tal1/scl/E2A*$^{+/-}$ mice. Compared to wild-type mice, tal1/scl expression results in an average 2.73-fold decrease in Rag 2-driven GFP expression, whereas tal1/scl expression on an *E2A*$^{+/-}$ background results in an average 4.38-fold decrease in Rag 2 expression that is statistically significant ($p = 0.0003$).

In addition to the recombinase genes Rag 1/2, the E47/HEB heterodimer has also been implicated in regulation of the pre-Tα chain of the pre T receptor, required for DN thymocyte expansion and survival (Herblot et al., 2000; Petersson et al., 2002; Takeuchi et al., 2001; Tremblay et al., 2003). To determine whether tal1/scl affects the expression of pre-Tα, we used a similar strategy and mated our *tal1/scl* transgenic mice to mice in which the expression of GFP is under the control of the pre-Tα promoter (Reizis and Leder, 2001). We observe a 2- to 3-fold decrease in the pre-Tα-driven GFP expression in *tal1/scl* DN3 thymocytes (Figure 5B; MFI = 747 for wt compared to MFI = 323 for tal1/scl). Pre-Tα expression was also reduced in the *tal1/scl E2A*$^{+/-}$ animals examined (Figure 5B; MFI = 184), but not significantly reduced when compared to tal1/scl in the wild-type background. The reduced transcription of Rag 2 and Pre-Tα in nearly all *tal1/scl* thymocytes (Figure 5) suggests that the thymocyte developmental abnormalities observed upon *tal1/scl* activation may be mediated by reduced Rag 2 and Pre-Tα expression.

Table 2. Genes activated or repressed by Tal1/scl

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 γ (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

Tal1/scl recruits the corepressor mSin3A to the CD4 enhancer

Our previous work demonstrated the presence of stable Tal1/scl/E47 and Tal1/scl/HEB heterodimers in preleukemic thymocytes and tumors isolated from *tal1/scl* transgenic mice (O'Neil

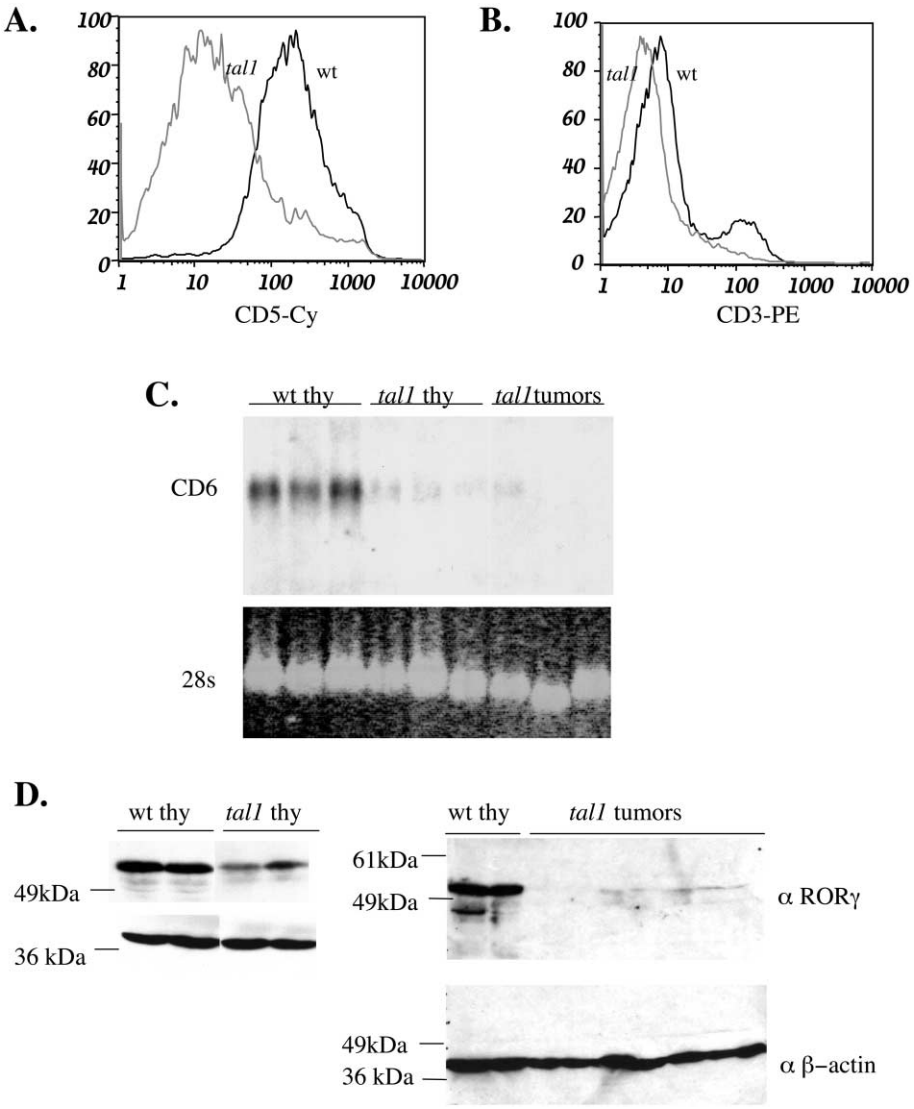

Figure 4. Gene repression in preleukemic *tal1/scl* thymocytes

A: CD5 expression is reduced in *tal1/scl* thymocytes. Thymocytes from four-week-old wild-type and *tal1/scl* transgenic mice were stained with anti-CD5 Cy and analyzed by flow cytometry.

B: CD3 expression is reduced in *tal1/scl* thymocytes. Thymocytes from four-week-old wild-type and *tal1/scl* transgenic mice were stained with anti-CD3 PE antibodies and analyzed by flow cytometry. A representative profile is shown.

C: CD6 expression is decreased in preleukemic tal1/scl thymocytes and in tal1/scl tumors. Total RNA was isolated from wild-type, *tal1/scl* preleukemic thymocytes and from *tal1/scl* tumors. The RNA was separated on a 1% agarose gel and transferred to a membrane that was probed with the mouse CD6 cDNA. To insure equal loading, RNA was stained with ethidium bromide.

D: RORγ expression is decreased in preleukemic *tal1/scl* thymocytes and tumors. Twenty-five micrograms of protein from wild-type, preleukemic *tal1/scl* thymocytes and *tal1/scl* tumors was separated on a 10% SDS-PAGE gel and transferred to a membrane. The membrane was then probed with anti-RORγ. The blot was then stripped and reprobed with an antibody against β actin to control for protein loading.

et al., 2001). Thus, tal1/scl may repress E47/HEB target genes by depleting E47/HEB heterodimers or by modifying E47/HEB transcriptional activity.

To determine whether the Tal1/scl/E47 or HEB heterodimers are localized to regions of gene repression, we used chromatin immunoprecipitation to ask whether E47/HEB or Tal1/scl/E47 heterodimers bound the regulatory regions of the CD4 gene. Thymocytes from age-matched, preleukemic *tal1/scl* and wild-type mice were treated with formaldehyde and extracts were immunoprecipitated using antibodies against Tal1/scl or E2A, or with an irrelevant isotype-matched control antibody (anti-Rip1). 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 wild-type thymocytes. The 200 bp fragment was also detected when *tal1/scl* thymocyte DNA was immunoprecipitated with an anti-Tal1/scl or anti-E2A antibodies, indicating that the Tal1/scl/E47 heterodimer occupies the CD4 enhancer (Figure 6A). These studies reveal that Tal1/scl 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 the Tal1/scl protein associates with mSin3A in undifferentiated MEL cells (Huang and Brandt, 2000). Thus, we hypothesized that Tal1/scl 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 wild-type thymocytes, the 200 bp 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 et al., 1996). Consistent with the CD4 repression observed in the preleukemic *tal1/scl* thymocytes (Figure 1), the coactivator p300 is not detected at the CD4 locus when *tal1/scl* thymocytes are immunoprecipitated. Rather, the corepressor mSin3A is readily detected

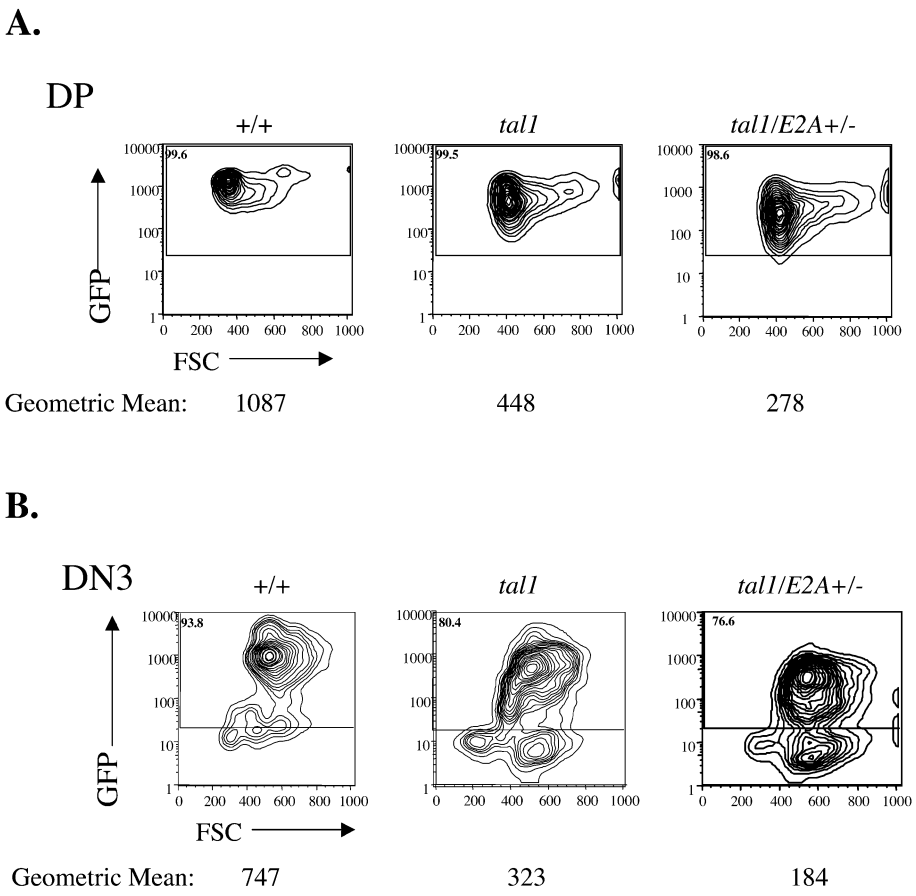

Figure 5. Rag2 and pre-Tα expression is reduced in *tal1/scl* and *tal1/scl/E2A*$^{+/-}$ mice

A: Rag2 expression is decreased on a per cell basis in tal1/scl and tal1/scl/E2A$^{+/-}$ thymocytes. Thymocytes from four-week-old, *rag2-GFP*, *tal1/scl/rag2-GFP*, and *tal1/scl/E2A*$^{+/-}$*/rag2-GFP* mice were stained with anti-CD4 PE and anti-CD8 Cy and analyzed by flow cytometry. The mean fluorescent intensity of the GFP was determined for the double positive (**A**) and double negative thymocyte populations (not shown). In the DP thymocyte population, tal1/scl expression in an *E2A*$^{+/-}$ background results in a statistically significant decrease in rag2-GFP expression when compared to tal1/scl in the wt background ($p = .0003$).

B: Pre-Tα expression is also reduced in tal1/scl DN3 thymocytes. Thymocytes from four-week-old, *preTα-GFP*, *tal1/scl/preTα-GFP*, and *tal1/scl/E2A*$^{+/-}$*/preTα-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 populations.

bound to the CD4 enhancer (Figure 6B). In contrast, the GAPDH locus was amplified from wt and tal1/scl input DNA, but not from the immunoprecipitated extracts (Figure 6B). These results suggest that tal1/scl 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 enhancer when *tal1/scl* preleukemic thymocytes, but not wild-type thymocytes, are immunoprecipitated (not shown).

The recruitment of mSin3A/HDAC1 complex to the CD4 locus and potentially other E47/HEB target genes suggested to us that mouse *tal1/scl* tumors may be sensitive to HDAC inhibitors. Treatment with the HDAC inhibitor TSA induced apoptosis in all six mouse *tal1/scl* tumors tested, resulting in the death of 75% to 95% of the treated tumor cells, whereas thymocytes were only moderately sensitive to TSA treatment (25% death due to TSA treatment) (Figure 6C). To test the specificity of the TSA response, we also treated tumor cell lines that do not express TAL1/SCL for their TSA sensitivity. In contrast to the tal1/scl tumors, the TAL1/SCL–negative tumors, Molt 13 and PEER, appear resistant to TSA treatment (Figure 6C).

TSA treatment of tal1/scl tumors induced apoptosis, as treated tumors stained positive for Annexin V/PI and exhibited caspase 3 activation (Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/5/6/587/DC1). HDAC inhibition may stimulate *tal1/scl* 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/scl contributes to leukemia/lymphoma remains unclear. Studies suggest that Tal1/scl may transactivate the expression of novel target genes in leukemia (Cohen-Kaminsky et al., 1998; Hsu et al., 1994b) and that the LIM-only protein LMO2 is required for *tal1/scl*-induced leukemogenesis (Larson et al., 1996). We demonstrate that tal1/scl transforms mouse T cells in the absence of LMO2 activation by inducing differentiation arrest and by interfering with E47/HEB function(s). Both *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice develop disease rapidly and with increased penetrance. Disease acceleration is accompanied by induction of a more severe thymocyte developmental arrest in *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice compared to transgenic mice expressing Tal1/scl or a DNA binding mutant of Tal1/scl (O'Neil et al., 2001, 2003). Thus, this work also reveals that differentiation arrest is central for disease development and suggests that E2A proteins may directly regulate cell cycle in thymocyte precursors.

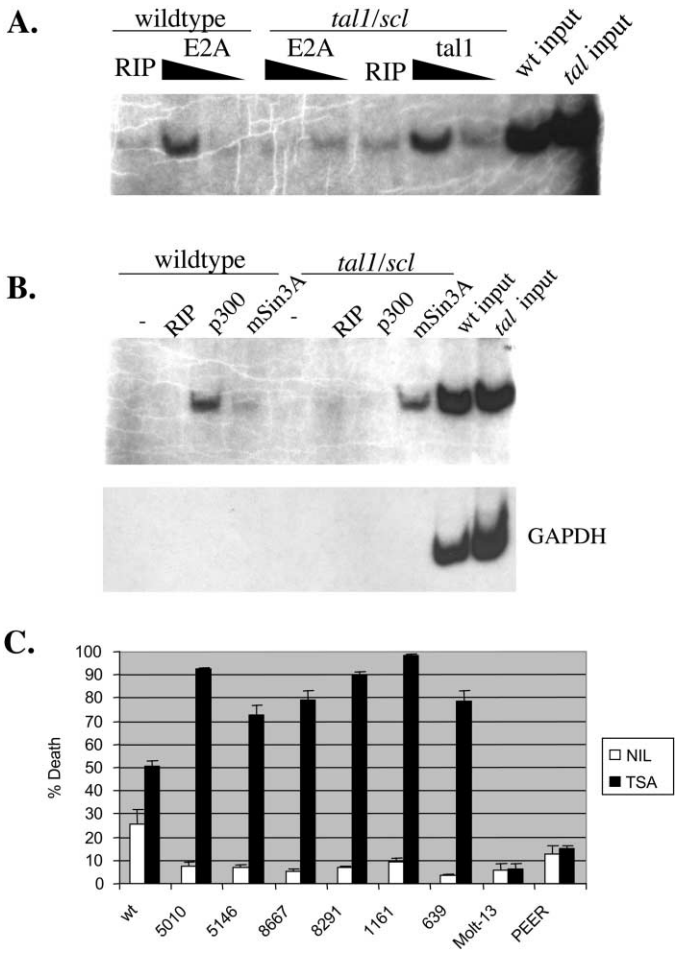

Figure 6. Tal1/scl recruits the corepressor mSin3A to the CD4 locus and tal1/scl tumors are sensitive to the HDAC inhibitor, TSA

A: Tal1/scl/E2A complexes bind the mouse CD4 enhancer. Chromatin immunoprecipitation assays were performed on thymocytes from four-week-old wild-type and preleukemic *tal1/scl* mice using antibodies to E2A and Tal1/scl. No antibody and immunoprecipitations with an irrelevant, anti-Rip1 (receptor interacting protein 1) 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 mouse CD4 enhancer region.

B: The corepressor msin3A is recruited to the CD4 enhancer in *tal1/scl* thymocytes. Chromatin immunoprecipitation was performed with antibodies to the corepressor mSin3A or coactivator p300 or with negative control antibodies. A control PCR reaction was performed on the chromatin immunoprecipitated extracts and inputs using primers to mouse GAPDH.

C: Tal1/scl tumors are sensitive to HDAC inhibition. Six *tal1/scl* tumor cells were left untreated or treated with TSA for 24 hours and tumor cell viability determined by trypan blue exclusion or by the percentage of apoptotic cells determined by staining with Annexin V/PI (Supplemental Data at http://www.cancercell.org/cgi/content/full/5/6/587/DC1). Thymocytes and human tumor cell lines that do not express TAL1/SCL (Molt-13, PEER) were also treated with TSA and cell viability determined.

We found that expression of the E47/HEB target genes CD4, TCRβ, and CD5 decreased in *tal1/scl* DP thymocytes and rag 2 and pre-Tα decreased in *tal1/scl* DN thymic precursors. The expression of rag 2 and pre-Tα are further reduced in *tal1/scl/E2A*$^{+/-}$ or *tal1/scl/HEB*$^{+/-}$ mice, suggesting that decreased expression of these genes may be responsible for the severe differentiation arrest and disease acceleration observed when Tal1/scl is expressed in an E2A or HEB heterozygous background. Consistent with these studies, our gene expression profiling reveals evidence of gene repression during the preleukemic 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 preleukemic *tal1/scl* thymocytes and further reduced in *tal1/scl/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/scl 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/scl contributes to leukemogenesis. Rather than operating like an Id and inhibiting the ability of E proteins to bind DNA, tal1/scl 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, rag 2, and pre-Tα, may also be mSin3A/HDAC1-mediated. The fact that all the mouse *tal1/scl* tumors examined were highly sensitive to HDAC inhibitors raises the possibility that T-ALL patients with TAL1/SCL 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/scl repressive effects may not be limited to E47/HEB target genes that regulate thymocyte differentiation, but may include other genes that regulate proliferation and survival.

A critical remaining question is how thymocyte differentiation arrest induced by tal1/scl 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, 2004). In addition to aberrant cell cycling, survival pathways may also be activated during the differentiation arrest and maintained throughout leukemic progression. The antiapoptotic transcription factor NF-κB is activated in *tal1/scl* thymocytes, and NF-κB activity is maintained in mouse *tal1/scl* tumors, and observed in a majority of human T-ALL samples (O'Neil et al., 2003; Kim et al., 2002; Kordes et al., 2000). Identification of the antiapoptotic NF-κB target genes in *tal1/scl* leukemic cells will be the focus of future work.

Experimental procedures

Mice and tumor cell culture

Proximal *lck-tal1/scl* 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/scl* transgenic mice for disease study. Disease development was monitored in *tal1/scl/E2A*$^{+/-}$ and *tal1/scl/HEB*$^{+/-}$ mice and compared to littermate controls. Rag2-GFP mice were provided by Dr. Michel Nussenzweig (Rockefeller University), and pre-Tα-GFP mice were provided by Drs. Boris Reizis and Philip Leder (Harvard Medical School). *Tal1/scl* tumor cell lines were plated at 1×10^6 cells/ml and left untreated or treated with trichostatin A (Sigma) at a concentration of 90 nM. Cell viability was assessed 24 hr after treatment by

either trypan blue staining or by staining with Annexin V/PI followed by flow cytometry.

Flow cytometry

Thymocytes from four-week-old, wild-type, *tal1/scl,* *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 ^{32}P-labeled 2 kb EcoRI fragment containing the murine TCR $J_\beta 2B$ exon (Malissen et al., 1984). Blots were washed in 1× SSC, 1% SDS, followed by a higher stringency wash containing 0.1× SSC, 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 ^{32}P-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, wild-type and *tal1/scl* 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

Tal1/scl tumor cell lines and thymi from four-week-old, *tal1/scl* 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-RORγ (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 *tal1/scl* transgenic mice and control littermates as well as from *tal1/scl* tumor cell lines. The RNA was electrophoresed on a 1% agarose gel, transferred to a membrane, and then hybridized with a ^{32}P-labeled mouse CD6 cDNA probe (generously provided by Dr. David Fox, University of Michigan Medical Center). To detect caspase activity, extracts were prepared from untreated and TSA-treated tal1/scl tumors and pro-caspase 3 and active caspase 3 detected by immunoblotting with the caspase 3 antibody (Cell Signaling #9662) and the cleaved caspase 3 antibody (Cell Signaling #9661S).

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed using the chromatin immunoprecipitation assay kit from Upstate (Lake Placid, NY). For each immunoprecipitation, 2 × 10^7 wild-type or *tal1/scl* thymocytes were treated with formaldehyde at 37°C for ten minutes. The cells were then lysed and the DNA 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 an antibodies against RIP (Transduction Laboratories), p300 (Santa Cruz N-15), mSin3A (Santa Cruz K-20), tal1/scl, 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, 0.1 M $NaHCO_3$. The protein-DNA crosslinks were reversed by heating at 65°C for 4 hr. 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 (primer 1: TTCTGAGCCCACCCTAAGATG, primer 2: GTCTTTTTTCCAGAGCCCC). A control PCR reaction for GAPDH was performed on the chromatin immunoprecipitated extracts and input sheared DNA (primer 1: ACCACAGTCCATG CCATCAC, primer 2: TCCACCACCCTGTTGCTGTA). To increase sensitivity, 0.25 μl of 10 mCi/ml α^{32}P-CTP was added to each PCR reaction. The reactions were separated by electrophoresis on a 6% acrylamide gel. The gel was then dried and exposed to film.

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