The balance of STAT5 and NFκB or IKAROS at enhancer networks dictates progenitor B cell survival, proliferation, and differentiation

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Dedication

This thesis is dedicated to anyone that is curious about how the universe works. Follow your curiosity in quest of discovering therapies and technologies that better the lives of sentient beings.

Abstract

B cell Acute Lymphoblastic Leukemia (B-ALL) arises from transformation of progenitor B cells[1]. The transcription factor STAT5 plays a critical role in B-ALL, as high STAT5 activation is correlated with poor patient survival[2]. How STAT5 mediates this effect is unclear. Previous studies suggested that STAT5 simply promotes the survival of progenitor B cells[3,4]. However, other roles for STAT5 in B-ALL have not been explored. This study demonstrates that STAT5 activation drives leukemia in cooperation with defects in a linear signaling pathway emanating from the pre-BCR, including *Blnk*, *Btk*, *Prkcb*, *Nfkb1*, and *Ikaros*. Using microarray analysis and chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq), we demonstrate that STAT5 antagonizes NFκB and IKAROS by opposing regulation of shared target genes. High levels of STAT5 binding was enriched at super-enhancers that are typically associated with an opposing network of B cell transcription factors including PAX5, EBF1, PU.1, IRF4, or IKAROS. The antagonism between STAT5 and NFκB or IKAROS has direct clinical relevance as the balance between these transcription factors affects patient outcome. Patients with high ratios of active STAT5 to NFκB or IKAROS have more aggressive disease characterized by decreased survival. Our studies illustrate how modest perturbations in two opposing transcriptional programs have dramatic consequences for B cell transformation, and that the degree of antagonism between these transcriptional programs correlates with patient survival.

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Introduction

Acute Lymphoblastic Leukemia (ALL) arises from the transformation of B or T cell progenitors. An estimated 6,000 new cases of ALL are diagnosed each year in the United States[5]. Roughly 60% of those cases occur in adolescents[5], making ALL the most common cancer in children[6]. With the advent of new therapies, the 5-year event free survival of children with ALL has improved from less than 10% in the 1960s[7] to 80-90% today[5,8]. While this is a great improvement, ALL remains the leading cause of cancer-related death in children and young adults[5,9].

Current treatments for ALL typically involve a cocktail of four drugs that non-specifically target leukemic cells by inhibiting mitosis, DNA replication, inflammation, and the availability of L-aspargine[10,11]. Consequently, more than two-thirds of the children that survive longer than 5 years from diagnosis suffer from treatment-related side effects such as: impaired intellectual and psychomotor functioning, neuroendocrine abnormalities, impaired reproductive capacity, cardiotoxicity, and second malignant neoplasms[12,13]. Thus, current therapies are non-specific, leading to many off-target effects that can reduce the quality of life for survivors[14]. Therefore, there is a need for new therapies that specifically target ALL and cause minimal side effects.

The outcome for adults with ALL tends to be much worse, as only 30% of adults achieve long-term disease free survival[15]. The poorer prognosis has been attributed to an increased frequency of high-risk leukemia subtypes, poorer tolerance of treatment,

and less effective treatment regimens compared to childhood ALL[12]. Risk factors for ALL include: age, sex, white blood cell count at diagnosis, cytogenetics, and the level of minimal residual disease[6,9]. However, these factors are unable to accurately predict all patients who go on to relapse[6]. Moreover, some patient subgroups that currently receive intensive therapy are likely being over-treated[16]. These patients could potentially be cured using less intensive regimens, resulting in reduced toxicity and fewer long-term side effects[16]. Therefore, new stratification methods need to be developed to more accurately identify patients that are most likely to relapse. Such stratification methods could be used to develop more personalized therapies. For example, those high-risk patients could then be treated more aggressively than low-risk patients. This should reduce the number of patients that are being over treated by therapeutic regimens. To reiterate, current therapies are insufficient and a next generation of ALL therapies needs to be developed to more specifically and effectively treat ALL. In order to develop such therapies, we need to better understand the underling mechanisms that drive the development of ALL, particularly those within high-risk patient groups that are less likely to respond to current therapies.

The vast majority of ALL cases (85%) result from the transformation of one or more B cell progenitors (B-ALL)[17]. B-ALL is characterized as a block in B cell differentiation, leading to an accumulation of progenitor B cells in the bone marrow, blood, and occasionally the central nervous system[17]. The stage at which this block occurs varies; however, most B-ALL cases exhibit a pre-B cell phenotype[18]. Mutations that only cause a block in B cell development usually are not sufficient to cause disease[19–25]. Consequently, transformed B cell progenitors accumulate other critical genetic lesions that drive leukemogenesis[26]. Genes that are frequently mutated in B-

ALL cases typically control the survival, proliferation, and differentiation of B cell progenitors during normal B cell development[26–29].

B lymphopoiesis

The stages of B cell development

In adults, B cell development begins solely in the bone marrow. The earliest B cell progenitors in the bone marrow are hematopoietic stem cells (HSCs) (Figure 1.1). HSCs are pluripotent, as they can differentiate into any cell of the blood lineage: erythrocytes, megakaryocytes, basophils, eosinophils, neutrophils, macrophages, dendritic cells (DCs), NK cells, T cells, and B cells. HSCs also have the capacity to self-renew by dividing at a low rate, thus maintaining this population of cells. The next stage of B cell differentiation is multipotent progenitors (MPPs). MPPs can still mature into all hematopoietic linages, but have lost the ability to self-renew[30]. MPPs can then give rise to lymphoid-primed multipotent progenitors (LMPPs), which have lost megakaryocyte and erythroid differentiation potential[30]. LMPPs are heterogeneous and include cells that are biased toward one of several lineages, including B and T cells[31]. The next B cell differentiation stage is the common lymphoid progenitor (CLP). CLPs are more biased to the lymphoid compartment than LMPPs, as they can only mature into B cells, T cells, NK cells, and some DC subsets[30]. CLPs that encounter the proper signals, such as the cytokine IL7, can differentiate into pre-pro-B cells[30]. B cell progenitors then develop in a linear progression through several stages of differentiation: pre-pro-B to early pro-B to late pro-B to large pre-B to small pre-B to immature B and finally to mature B. B cell progenitors are not fully committed to the B lineage until the pro-B cell stage[32], at which

point no alternative lineage potential exists. All stages take place in the bone marrow until the immature B cell stage. Once generated, immature B cells migrate via the blood to the spleen, where they pass through the transitional 1 (T1) stage and then the T2 stage of differentiation before maturing into mature B cells[33].

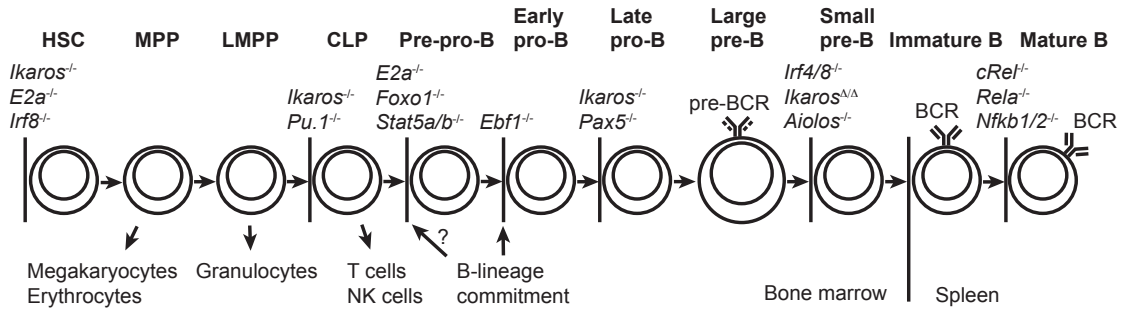


Figure 1.1. Disruption of the B cell transcriptional network blocks B cell development.

B cell development begins in the bone marrow, where progenitor B cells pass through several stages of differentiation before migrating to the spleen to complete maturation. Cells are committed to the B-lineage at the early pro-B cell stage and potentially at the pre-pro-B cell stage. The pre-BCR is expressed exclusively on large pre-B cells, where as the BCR is expressed on immature and mature B cells. Loss of function mutations in key B cell transcription factors lead to blocks in B cell differentiation at various developmental stages[19,34–53].

Immunoglobulin recombination

In order to be able to combat a wide variety of pathogens, B cells possess a very diverse antibody repertoire. This diversity is generated by a process called immunoglobulin (Ig) recombination, which generates more potential antibodies (10^{11}), than there are protein-coding genes (~$2x10^5$) in the human genome[33,54]. To achieve this

feat, recombination shuffles the immunoglobulin gene segments to create unique combinations.

Antibodies are composed of two heavy and two light chains, or immunoglobulins (Igs)[33]. Each heavy chain (IgH) is composed of one variable (V) segment, one diversity (D) segment, one joining (J) segment, and one constant (C) segment[33]. Each light chain also contains one V segment, one J segment, and one C segment, but no D segment[33]. The human heavy chain locus contains ~40 V (V_H) segments, 23 D (D_H) segments, 6 J (J_H) segments, and 9 constant segments[33]. The light chain can be encoded by the κ or λ loci[33]. The κ locus is composed of 38 V (Vκ) segments, 5 J (Jκ) segments, and one C (Cκ) segment[33]. Similarly, the λ locus is contains of 30 V (Vλ) segments, and 4 J (Jλ) segments[33]. Each Jλ segment is linked to a single C (Cλ) segment, therefore there are 4 Cλ segments in total[33]. The organization of the immunoglobulin loci helps facilitate the recombination process[33].

Extensive mouse studies have revealed that recombination is executed by the recombinase complex[33]. This complex is composed of the recombination-activating genes, RAG1 and RAG2, DNA-dependent protein kinase (DNA-PK), and the nuclease ARTEMIS[33]. Each V, D, and J segment is flanked by DNA elements called recombination signal sequences (RSSs). Recombination is initiated by the recognition of the RSSs by a complex of RAG1 and RAG2 (RAG1/2)[33]. RAG1/2, in combination with DNA-PK and ARTEMIS, cut the DNA at two compatible RSSs, which are adjacent to each of the two Ig segments that are to be ligated together (one V_H and one D_H, for example)[33]. Then, the enzyme terminal deoxynucleotidyl transferase (TdT or *Dntt*) adds random nucleotides to the ends of the cut, single-stranded DNA[33]. These additional

nucleotides add further diversity to the antibody repertoire[33]. DNA ligase IV and the recombinase complex then link these two DNA segments together[33]. The RSSs are organized such that only one V_H segment can recombine with one D_H segment, which can then be linked to one J_H segment[33]. Moreover, V_H cannot recombine to J_H directly because of the orientation of the RSSs. This is similarly orchestrated in the light chain locus[33].

During B cell development, the heavy chain is rearranged first, followed by the light chain[33]. The first heavy chain recombination events involve the joining of the D_H and J_H segments[33]. This can occur in LMPPs, CLPs, pre-pro-B cells, or early pro-B cells[33,55]. Then the V_H to DJ_H segments are rearranged in late pro-B cells[33]. This recombined μ heavy chain (μH) then pairs with an invariant, surrogate light chain on the cell surface[33]. The surrogate light chain is composed of VPREB1 and λ5, which exhibit homology to a V segment and Cλ segment, respectively[33]. The μ heavy chain and surrogate light chain pair with Igα and Igβ to form a pre-B cell receptor (pre-BCR)[33]. Cells that express the pre-BCR are termed large pre-B cells[33]. Signaling through the pre-BCR acts as a checkpoint to test whether the cell has successfully recombined a heavy chain that can pair with a surrogate light chain[33]. If recombination of the first heavy chain allele is unsuccessful, then the cell attempts to rearrange the second heavy chain allele[33]. If progenitor B cells fail to produce a functional heavy chain a second time, then they die by apoptosis – at least 45% of pro-B cells are lost at this stage[33].

Functional pre-BCR signaling then stimulates the rearrangement of the light chain[33]. Pre-B cells first attempt to rearrange the κ locus, then the λ locus[33]. If successful, a κ or λ chain pairs with the μ heavy chain, generating a surface IgM

antibody[33]. This IgM forms a complex with Igα and Igβ to form a B cell receptor (BCR)[33]. The first cells to express a BCR are immature B cells[33]. Recombination is limited to one immunoglobulin allele at a time; thus, limiting the number of B cells that express multiple antibodies with different specificities[33].

In order to develop into mature B cells, B cell progenitors must rearrange a heavy chain that pairs with the surrogate light chain to form a pre-BCR[33]. Pre-BCR signaling then initiates light chain recombination to generate a BCR[33]. Therefore, evolution has generated a system that tests for the successful rearrangement of the heavy and light chains separately. Consequently, B cell progenitors can sense whether an unsuccessful rearrangement is due to defects in the heavy or light chain and attempt to rearrange another allele of the same locus. Ultimately, recombination generates a vast repertoire of immature B cells capable of recognizing many different antigens[33].

Negative and positive selection

Once an immature B cell expresses a BCR, it is tested for reactivity to self-antigens in the bone marrow[33]. If an immature B cell is self-reactive (~75% are self-reactive)[56], then it has four potential fates: production of a new BCR (receptor editing), clonal deletion, anergy, or ignorance[33]. If a BCR multivalently binds to a self-antigen, then the cell attempts to rearrange the other κ allele[33]. If that is unsuccessful, then the cell tries to recombine the λ alleles[33]. This process of receptor editing, gives the immature B cells another chance to express a BCR that is not self-reactive[33]. If receptor editing is unsuccessful, then the cell dies by apoptosis in a process called clonal deletion[33]. Multivalent binding tends to occur when the self-antigen is expressed on the

surface of a cell in the bone marrow[33]. Immature B cells that encounter more weakly cross-linking self-antigens of low valence, such as small soluble proteins, are rendered anergic[33]. Anergic B cells are unresponsive, as they cannot be activated by their antigen, even with CD4 T cell help[33]. Finally, if an immature B cell has affinity for a self-antigen, but does not respond to it, then it is considered ignorant[33]. Ignorance can occur when the self-antigen is inaccessible to the immature B cell, is in low concentration, or binds weakly enough that it does not activate the B cell[33]. These processes that eliminate or inactivate potentially autoreactive B cells are mechanisms of central tolerance[33]. Central tolerance is critical in limiting autoimmunity caused by autoreactive lymphocytes[33]. Immature B cells that survive central tolerance in the bone marrow migrate to the spleen via the blood to complete the developmental process[33].

Immature B cells that migrate to the spleen are called transitional B cells[33]. The processes governing transitional B cell development are not well defined[33]. What is known is that transitional B cells must interact with a self-antigen with moderate affinity, otherwise they die by apoptosis[57]. This process is called positive selection[33]. Conversely, if these cells bind to self-antigen too strongly, then they are eliminated from the repertoire by negative selection[57]. The molecular mechanisms that underlie positive and negative selection are not well understood, but recent studies have begun to shed light on these processes.

The pre-B cell checkpoint is regulated in part by the balance of the transcription factors BCL6 and BACH2[58]. Pre-BCR signaling positively regulates *Bcl6* expression[58]. BCL6 in turn promotes the survival of pre-B cells by repressing the expression of DNA damage response and checkpoint genes (CHEK1, ARF, CDKN1A, and TP53) as well as the DNA damage sensor ATR[58]. In contrast, BACH2 opposes BCL6 function and

executes negative selection of progenitor B cells with failed V_H–DJ_H rearrangements[58]. BACH2 mediates these effects by downregulating antioxidant response genes and triggering a program of oxidative-stress-induced cell death[58]. BACH2 also increases the probability of a pre-B cell to undergo successful heavy chain rearrangement by increasing the expression and activity of the RAG recombinases[58].

Immature B cells in the bone marrow that express self-reactive BCRs activate classical NFκB via a NEMO/IKK-dependent pathway[59]. One of the consequences is up regulation of BAFF-receptor on these cells, making them more responsive to BAFF-dependent survival signals[59]. Extended survival of these cells permits continual receptor editing that is required to generate κ+ immature B cells[59].

In the spleen, T1 transitional cells are extremely susceptible to apoptosis upon BCR stimulation, making them the target of negative selection against self-reactivity[59]. BCR-induced cell death is mediated by the mitochondrial pathway utilizing the pro-apoptotic BH3-only domain proteins BAK, BAX, and BIM[59]. NFκB is important at this stage as transfer of *Rel*$^{-/-}$ x *Rela*$^{-/-}$ fetal liver cells into irradiated hosts leads to a block at the T1 to T2 transition of development[59]. The extreme sensitivity to apoptosis of the residual IgM+ cells in this genotype is partially rescued by a *Bcl2* transgene; however, differentiation remains incomplete indicating that c-REL and RELA are required for differentiation as well as cell survival[59]. However, BCR crosslinking in T1 transitional cells does not drive the activation of NFκB, suggesting that another receptor besides the BCR (such as the BAFFR) is driving NFκB activity in these cells[59]. Conversely, BCR signaling in T2 transitional cells induces more long-term c-REL activation compared to T1 transitional cells, leading to increased expression of anti-apoptotic genes as well as

the prosurvival receptor BAFFR and its downstream substrate p100 (NFκB2)[60]. Moreover, lack of *Nfkb1* and *Nfkb2* leads to a block at the T2 stage[59]. Together these studies suggest that NFκB is required for positive selection in T2 cells by promoting cell survival.

A network of B cell transcription factors regulates B cell development

Progression through B cell development is regulated by several transcription factors. The B cell differentiation program is initiated at the earliest stages of B cell development through concerted regulation by the transcription factors PU.1, IKAROS (*Ikzf1*), and E2A (*Tcf3*). All three of these transcription factors are expressed in HSCs[30]. Deletion of any one of these genes leads to a very early block in B cell development[34,35,37–42,45,46,61] (Figure 1.1).

The onset of B-lineage specification coincides with E2A-mediated activation of the transcription factor *Foxo1*[43,62] (Figure 1.2). E2A and FOXO1 cooperatively upregulate the recombinase components *Dntt (TdT)*, *Rag1*, and *Rag2* in LMPPs[30]. Expression of these enzymes leads to D_H to J_H recombination in these early progenitors and may be the underlying mechanism that biases LMPPs to the B and T cell lineages[30,63]. PU.1, IKAROS, E2A, and FOXO1 collaborate to drive the expression of the interleukin-7 receptor (IL7R) α chain on CLPs[30]. IL7R signaling leads to the activation of signal transducer and activator of transcription 5 (STAT5). STAT5 cooperates with IKAROS, E2A and FOXO1 to upregulate the transcription factor early B cell factor 1 (*Ebf1*)[30,43,64–69]. EBF1, in turn, positively regulates *E2a* and *Foxo1*, forming a positive-feedback loop that stabilizes *Ebf1, E2a, and Foxo1* expression[43,69,70].

EBF1 is critical in the specification of the B cell lineage. Its expression is restricted to the B lineage within the hematopoietic system[71,72]. Deficiency in *Ebf1* leads to an early block in B cell development[19] (Figure 1.1). Moreover, the B cell progenitors that do arise in *Ebf1*$^{-/-}$ mice fail to upregulate many B cell specific genes[19,67,70,73]. EBF1 acts as a "pioneer" transcription factor that increases the accessibility of target genes to other transcription factors and histone modifiers[43,74]. EBF1 cooperates with E2A and FOXO1 to activate many B-lineage–restricted genes, thereby promoting B-lineage specification[69,73,75]. This is supported by the observation that ectopic expression of EBF1 in HSCs skews cells to enter the B lineage[76]. Therefore, EBF is necessary in the early events of B cell development, including B lineage specification.

EBF1 is also important in the commitment and maintenance of the B lineage. EBF1 promotes commitment by directly repressing genes that are critical for specification to alternative lineages, including *Tcf7*, *Gata3*, *Cebpa*, and *Id2*[77–79]. *Ebf1*$^{-/-}$ lymphoid progenitors retain T, NK, myeloid, and dendritic cell potential[67,70,79], but ectopic expression of *Ebf1* limits specification to these alternative lineages and promotes B cell differentiation[76,79]. EBF1 is also required for the maintenance of B cell identity. Deletion of *Ebf1* in pro-B cells induces dedifferentiation into progenitors capable of giving rise to T cells and innate lymphoid cells[77]. Therefore, EBF1 is necessary for the specification, commitment, and maintenance of the B lineage.

EBF1 also promotes B cell development by guiding and redistributing other transcription factors such as E2A and PU.1 to genes involved in B cell specification[69,80]. This redistribution likely affects the differentiation program that E2A and PU.1 instruct. PU.1, for example, promotes both B cell and myeloid development[80,81]. However, the distribution of PU.1 is dramatically different between these lineages[80]. Moreover, the

PU.1 distribution in the B lineage is dependent on EBF1[80]. Therefore, EBF1 redistributes the binding of PU.1 and E2A to genes that promote B cell development.

EBF1 cooperates with PU.1, IKAROS, E2A, and FOXO1 to drive the expression of PAX5 [30,32,75,82]. PAX5, in turn, feeds back to further drive the expression of *Ebf1*; thus creating a self-stabilized network of B cell specific transcription factors[30] (Figure 1.2).

PAX5 has a similar although not identical role as EBF1 in B cell development. Like EBF1, PAX5 is exclusively expressed within the B cell lineage of the hematopoietic system[32]. Mice deficient for *Pax5* have a block at the early pro-B to late pro-B transition of B lymphopoiesis, which is slightly later than that seen in *Ebf1*$^{-/-}$ mice[83] (Figure 1.1). Moreover, Pax5$^{-/-}$ pro-B cells fail to recombine middle and distal V_H segments to DJ_H segments[83]. This is because PAX5 is required for μH chain contraction[84], which juxtaposes distal V_H segments with D_H segments to increase distal V_H to DJ_H recombination. PAX5 also promotes μH chain recombination by cooperating with E2A, IKAROS, and EBF1 to increase the accessibility of the μH chain locus[85]. However, ectopic expression of a rearranged μH chain in Pax5$^{-/-}$ lymphoid progenitors does not rescue B cell development[86]. This is probably in part because PAX5 is required for the expression of many pre-BCR signaling components including *Vpreb1*, *Igll1*, *Cd79a*, *Blk*, and *Blnk* [87–90]. This is supported by the observation that ectopic expression of the pre-BCR adaptor BLNK in *Pax5*$^{-/-}$ progenitors partially rescues pre-BCR signaling[90].

In addition to inducing genes involved in pre-BCR signaling, PAX5 also upregulates an additional 165 genes involved in transcriptional regulation, adhesion, migration, antigen presentation, and B cell differentiation[91,92]. This transcriptional regulation by PAX5 promotes B cell specification[91,92]. One third of PAX5 targets are

occupied by EBF1, suggesting that EBF1 and PAX5 cooperate to promote B cell specification[75]. The cooperative regulation by EBF1 and PAX5 is most extensive for genes involved in pre-BCR signaling[75], which are synergistically upregulated by EBF1 and PAX5[78].

PAX5 also cooperates with pre-BCR signaling to upregulate the transcription factors *Irf4* and *Irf8*[87,92–95], which then drive *Ikaros* expression[96]. IKAROS feeds back to positively regulate *Ebf1*, *Pax5*, and *Foxo1*[68]. IRF4, IRF8, IKAROS, FOXOs, and E2A then cooperate to promote Igκ recombination and pre-B cell differentiation[50,68,97–102].

In addition, PAX5 promotes B cell commitment and maintenance. Unlike their WT counterparts, *Pax5*$^{-/-}$ pro-B cells can differentiate into macrophages, granulocytes, dendritic cells, natural killer cells, and T cells[103–106]. This suggests that *Pax5* is required for B lineage commitment. However, forced *Ebf1* expression can restore lineage restriction in *Pax5*$^{-/-}$ progenitors[78,79]. This suggests that EBF1 can commit cells to the B lineage independently of PAX5 and that PAX5 potentially commits cells to the B lineage by upregulating *Ebf1*. In addition, deletion of *Pax5* in committed pro-B or mature B cells results in dedifferentiation and regained T lineage potential[107,108]. Therefore, PAX5 is necessary for the maintenance of B cell identity and for B cell commitment, potentially by upregulating *Ebf1*.

EBF1 and PAX5 continue to be expressed through the remainder of B cell development[30]. It is not until B cells terminally differentiate into plasma cells that EBF1 and PAX5 expression is lost[30]. At that point, B cells turns off most B cell specific genes and terminally differentiate into plasma cells[30]. The transcriptional network in plasma cells is instead dominated by the transcription factor BLIMP[49,109].

The *Cd79a* (Igα) locus has been studied extensively and provides a detailed example as to how a B cell specific gene is regulated in B cell development[110]. Igα is a critical signaling component of the pre-BCR and the BCR. The rate-limiting step in its transcriptional activation is the binding of EBF1 to the *Cd79a* locus[30]. EBF1 then recruits E2A and RUNX1[30]. These transcription factors then collaborate to recruit histone demethylases, which in turn begin to demethylate and open the *Cd79a* promoter[30]. As demethylation propagates, it unmasks a key PAX5/ETS composite binding site[30]. This site then cooperatively binds PAX5 and the pan-lymphoid transcription factor, ETS1[30]. PAX5 and ETS1 then recruit SWI/SNF chromatin remodeling complexes[111], which then further increase accessibility and expression[30].

B cell development is controlled by a network of transcription factors. B cell specification begins with the expression of PU.1, IKAROS, and E2A, which initiate a cascade of events that result in the expression of EBF1 and PAX5. EBF1 and PAX5 form a self-reinforcing network along with PU.1, IKAROS, E2A, and FOXO1 to specify and commit cells to the B lineage. Ultimately this network of transcription factors promotes and facilitates the differentiation of lymphoid progenitors into mature B cells.

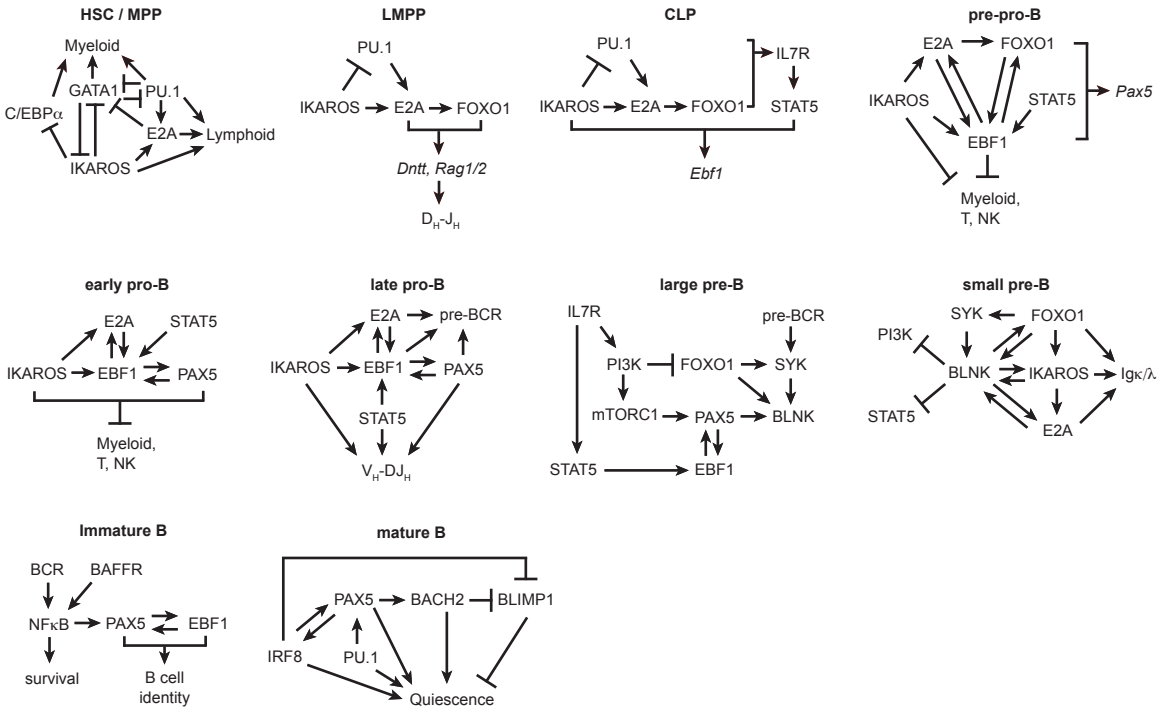


Figure 1.2. The induction of a self-reinforcing B cell transcriptional network promotes B cell development.

Hematopoietic stem cells (HSCs) and multipotent progenitor cells (MPPs) express a variety of transcription factors that can promote myeloid and/or lymphocyte development. The induction of *Foxo1* and *Ebf1* form a self-reinforcing network that specifies cells to the B lineage and limits alternative lineage potential. This network induces *Pax5*, which further reinforces the B cell differentiation program to promote V_H to DJ_H recombination and pre-BCR expression. Pre-BCR signaling positively regulates *Foxo1*, *Ikaros*, and *E2a*, which then induce light chain recombination. The activation of NFκB by the B cell receptor (BCR) and B cell activating factor receptor (BAFFR) is necessary for the survival of immature B cells. The expression of EBF and PAX5 in immature and mature B cells is critical to maintain B cell identity.

Signal transducer and activator of transcription 5 (STAT5)

Structure and function of STAT5

STAT5 exists in two isoforms, STAT5a and STAT5b, which exhibit 96% sequence identity. They are ubiquitously expressed and functionally interchangeable[112]. STAT5a and STAT5b contain an N-terminal domain that promotes dimerization, a DNA binding domain, an SH2 domain, and a C-terminal transactivation domain[112] (Figure 1.3). These proteins are encoded by *STAT5a* and *STAT5b*, which are separated by less than 14,000 base-pairs (bp) in the genome[48,112–114]. Mice lacking either *Stat5a* or *Stat5b* have mild defects in mammary development or growth hormone regulation and liver enzyme production, respectively[115,116]. It remains unclear whether this is due to differences in function or expression of STAT5a versus STAT5b[112]. STAT5a and STAT5b compensate for one another as mice lacking both *Stat5a* and *Stat5b* exhibit a perinatal lethal phenotype and have defects in erythropoiesis and B and T cell development[47,48]. Conditional deletion of *Stat5a* and *Stat5b* in B cells arrests B cell development at the CLP to pre-pro-B cell transition[47] (Figure 1.1). Therefore STAT5a and STAT5b have redundant roles in promoting early B cell development.

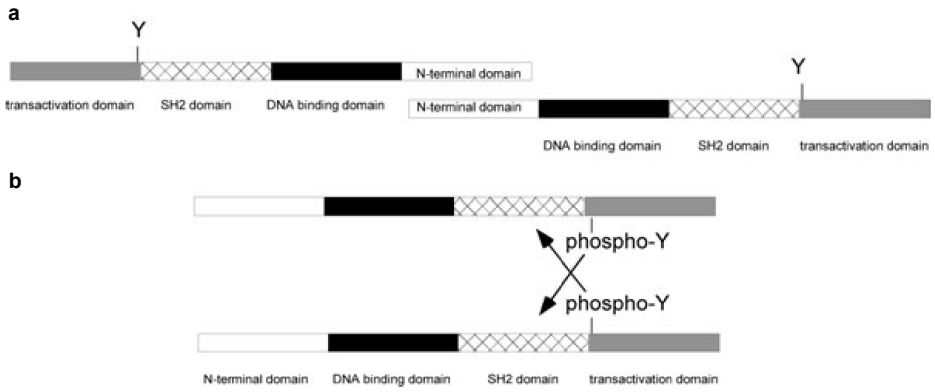


Figure 1.3. Schematic of the STAT5 proteins.

a, Prior to activation, STAT5 exists as a homodimer organized in an anti-parallel head to head fashion that is mediated via interactions between the N-terminal domains. **b,** Phosphorylation of the regulatory tyrosine (Tyr-694 and Tyr-699 in the case of STAT5a and STAT5b, respectively) results in binding of this phosphorylated tyrosine residue to the SH2domain of a neighboring STAT5a or STAT5b molecule and the generation of a novel parallel homodimeric configuration that can translocate to the nucleus, bind DNA, and modulate gene transcription (LHH 2011 review).

STAT5 regulates early B cell development

STAT5 activation is regulated by various cell surface receptors. In progenitor B cells, IL7R signaling activates STAT5. The IL7R is composed of two subunits, the IL7Rα and common γ (γc) chains, which associate with the tyrosine kinases JAK1 and JAK3, respectively. Upon binding to its ligand, IL7, the IL7R brings JAK1 and JAK3 in close proximity to each other. This proximity allows JAK1 and JAK3 to transphosphorylate one another, thus activating them. The active JAKs then phosphorylate a tyrosine on the IL7Rα chain, which then recruits STAT5. Prior to activation, STAT5 exists as a homodimer organized in an anti-parallel head to head fashion that is mediated via interactions between the N-terminal domains of STAT5[112] (Figure 1.3). The JAKs phosphorylate recruited STAT5 (pSTAT5) at Tyr-694 (STAT5a) or Tyr-699 (STAT5b)[112].

This phosphorylation causes these tyrosine residues to bind to the SH2 domain of a neighboring STAT5a or STAT5b molecule and generates parallel homodimers that can translocate to the nucleus, bind DNA, and modulate gene transcription[112,117–121].

Once in the nucleus, STAT5 can function as an activator or repressor of transcription. STAT5 can activate target gene transcription by recruiting the coactivator p300 or histone deacetylases (HDACs)[122–124]. Conversely, STAT5 can also repress transcription by instead recruiting the corepressors SMRT or EZH2[120,125]. The consensus DNA binding sequence for STAT5 is TTCxxxGAA, in which "x" is any base pair[126]. However, STAT5 can also bind to imperfect consensus sites, particularly if a second STAT5 site is within 6-7 bp[120]. Such neighboring STAT5 sites recruit STAT5 as a tetramer[117,118]. There is evidence to suggest that tetrameric sites preferentially lead to target gene repression rather than activation[120]. However, this is not true for all loci[127]. Therefore, STAT5 can recruit multiple co-activators and co-repressors to promote and inhibit target gene expression.

The IL7R is first expressed at the CLP stage and peaks at the pro-B cell stage. Then IL7R levels begin to decrease in pre-B cells and are largely absent in immature and mature B cells. Expression of constitutively active STAT5b mostly restores B-lymphopoiesis in the absence of *Il7r*[128], suggesting that IL7R promotes early B cell development primarily by activating STAT5. One way that STAT5 promotes early B cell development is by positively regulating *Ebf1*[64–67]. In addition, STAT5 drives proliferation in progenitor B cells by upregulating *Myc, Ccnd2,* and *Ccnd3*[101,128,129]*.* STAT5 also promotes the survival of progenitor B cells by positively regulating of *Bcl2l1* (BCL-X_L), *Bcl2, Mcl1,* and *Pim1*[3,128,130]*.* Therefore, STAT5 drives the development and expansion

of early B cell progenitors by positively regulating *Ebf1* and genes involved in cell cycle progression and survival.

STAT5 also regulates immunoglobulin recombination. For example, STAT5 promotes the recombination of distal V_H segments in B cell progenitors[3]. However, STAT5 also binds to the Igκ intronic enhancer (Eκi) and represses Igκ accessibility and recombination[3,97,120]. This potentially limits progenitor B cells from recombining the light chain before heavy chain. Thus, STAT5 promotes μH chain recombination but inhibits light chain recombination. In summary, STAT5 promotes B cell development in early B cell progenitors, but potentially inhibits later stages of B cell differentiation by repressing light chain recombination.

Pre-B cells escape IL7R signaling

In order to promote efficient light chain recombination and differentiation, small pre-B cells escape IL7R signaling by several mechanisms. As large pre-B cells exit the cell cycle and differentiate, they no longer respond to IL7[131]. Yet these cells express a similar level of IL7R compared to proliferating large pre-B cells[131]. One difference is that large pre-B cells that have exited the cell cycle express higher levels of the JAK-STAT inhibitor, *Socs1*, than proliferating large pre-B cells[131]. Another molecule that could be responsible for IL7 insensitivity is the adaptor BLNK. BLNK expression increases during pre-B cell differentiation[102] and has been shown to bind to JAK3 and inhibit the activation of STAT5[132]. Additionally, pre-B cells may escape IL7 signaling by moving away from IL7-producing stromal cells within the bone marrow[133]. Pre-BCR signaling positively regulates the transcription factor IRF4, which upregulates the chemokine receptor *Cxcr4*[133]. Cell may migrate in response to the corresponding chemokine CXCL12 to

areas of low IL7 concentration[133]. Therefore, there are several potential mechanisms by which pre-B cells can lose IL7 sensitivity.

Pre-BCR Signaling

Structure and proximal signaling of the Pre-BCR

Each pre-BCR is composed of two μ heavy chains, two surrogate light chains (each with one λ5 and one VPREB1), and a heterodimer of Igα and Igβ. Disulfide bonds hold the two heavy chains together and each heavy chain with a surrogate light chain. Igα and Igβ each contain an extracellular Ig-like domain that associates with the μH chain through hydrophilic interactions. Importantly, each Igα and Igβ also contains an immunoreceptor tyrosine-based activation motif (ITAM) located in the cytoplasm. The ITAMs associate with the Src family kinase BLK, FYN, and LYN via low-affinity interactions in the absence of ITAM phosphorylation[33].

Pre-BCR signaling can be triggered by ligand dependent and independent mechanisms[134]. Several self-antigens that induce pre-BCR signaling have been identified[133]. However, it is unknown whether self-antigens are required for normal pre-BCR signaling[85,134]. Pre-BCR signaling can also be induced independently of antigens by inter-pre-BCR interactions involving the binding of λ5 (*Igll1*) on one pre-BCR to the μH chain on another pre-BCR[85]. Therefore, the exact mechanisms that initiate pre-BCR signaling *in vivo* are not well understood and may or may not involve the binding of the pre-BCR to self-antigens.

Pre-BCR signaling induces BLK, FYN, and LYN to phosphorylate tyrosine residues within the ITAMs. Phosphorylated ITAMs then recruit the tyrosine kinase SYK to the plasma membrane, thereby activating SYK. Active SYK then phosphorylates the adaptor BLNK, rendering it active. BLNK then facilitates many of the effector signaling pathways downstream of the pre-BCR such as FOXO proteins, ERK, and IKAROS/AIOLOS.[33].

Pre-B cell proliferation

Upon rearrangement of the μH chain, large pre-B cells undergo 2-5 cell divisions, followed by cell cycle arrest, light chain recombination, and differentiation[135]. This proliferative burst requires PI3K signaling[136–138], which is probably driven by the IL7R and potentially the pre-BCR[133,134]. The complete mechanism underlying pre-B cell proliferation is unclear[133]. It is possible that the pre-BCR has two signaling states: one that drives proliferation and another that promotes differentiation[133]. Such a pro-proliferative signaling pathway downstream of the pre-BCR has yet to be identified[133]. However, recent studies have begun to elucidate how progenitor B cells shift from a proliferative state to a quiescent state.

Switch to pre-B cell differentiation

The switch from a proliferative state to a quiescent state that promotes pre-B cell differentiation involves several signaling molecules. IL7R signaling actives STAT5 and PI3K, which positively regulate the transcription factor *Myc*[85,129]. MYC, in turn, promotes proliferation by upregulating *Ccnd3*[85]. However, MYC also initiates a negative feedback loop by activating p19Arf[85]. p19Arf suppresses the E3 ubiquitin ligase MDM2, which

itself inhibits p53 stability. Thus, MYC activation of p19Arf stabilizes p53 and promotes cell cycle arrest[85]. This pathway may be an important mechanism by which pre-B cells promote quiescence.

In addition, PI3K-AKT signaling activates mTORC1, which then upregulates *Pax5*[85]. Similarly, active STAT5 positively regulates *Ebf1*. PAX5 and EBF1 then upregulate many of the genes involved in pre-BCR signaling, including *Vpreb1*, *Igll1*, *Cd79a*, *Cd79b*, *Blk*, and *Blnk*[75,87]. Once expressed, the pre-BCR promotes cell cycle arrest and light chain rearrangement by activating FOXOs, ERK, and IKAROS/AIOLOS.

Pre-BCR signaling activates FOXO proteins

Initially, BLNK is expressed at low levels in pre-B cells[134]. However, BLNK levels increase due to positive regulation by EBF1 and PAX5[87,139]. Once BLNK activity passes a certain threshold, it negatively regulates PI3K signaling[99] (Figure 1.4). This then alleviates the negative regulation of FOXO proteins by PI3K[85]. BLNK can also positively regulate FOXOs by activating p38[98,99,102,140]. Moreover, IL7 activation of STAT5 drives *Ebf1* expression, which also positively regulates *Foxo1*[70]. This forms a positive feedback loop whereby FOXO proteins bind and upregulate *Syk* and *Blnk*[102], which in turn positively regulate FOXOs. FOXOs also promote cell cycle arrest by positively regulating the cell cycle inhibitors *Cdkn1b* (INK4B / $p27^{Kip1}$) and *Cdkn2a* (ARF and INK4A)[85]. In addition, FOXO proteins bind to and upregulate *Rag1* and *Rag2* and promote Igκ recombination[98,99]. To summarize, FOXO proteins are important in forming a positive feedback loop with BLNK that promotes cell quiescence and Igκ rearrangement.

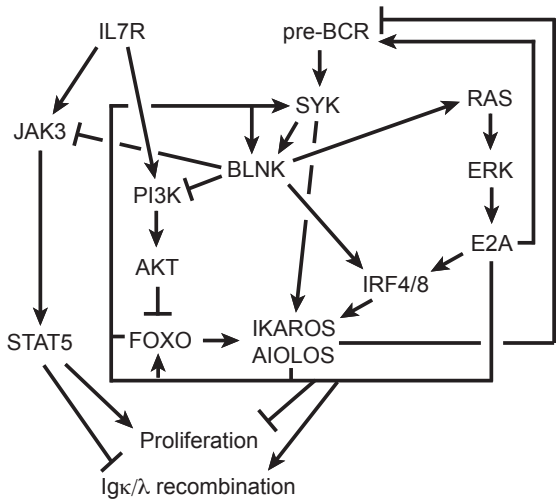


Figure 1.4. **IL7R and pre-BCR signaling pathways have opposing roles in pre-B cells.**

Signaling via the IL7R activates the transcription factor STAT5 and the kinases PI3K and AKT, which promote pre-B cell proliferation and inhibit light chain recombination. PI3K-AKT does this in part by suppressing FOXO proteins. Expression of the pre-BCR leads to the activation of the kinase SYK and the adaptor BLNK. SYK and BLNK in turn positively regulate FOXOs, IKAROS, AIOLOS, and E2A, which promote cell cycle arrest and light chain recombination. BLNK also negatively regulates JAK3-STAT5 and PI3K signaling. This alleviates FOXOs of negative regulation by PI3K-AKT. FOXOs, IKAROS, and E2A form a positive feedback loop by further inducing SYK and BLNK activity. Paradoxically, IKAROS and AIOLOS also form a negative feedback loop by suppressing the expression of the surrogate light chain components *Vpreb1* and *Igll1* (λ5).

Pre-BCR signaling activates the RAS-ERK pathway

BLNK also activates the RAS-ERK pathway by recruiting the adaptor GRB2 and the enzyme PLCγ1/2[141,142]. The RAS-ERK pathway is necessary for pre-B cells to exit the cell cycle and recombine the Igκ locus[101,143]. ERK seems to promote cell cycle arrest by inhibiting *Ccnd3* expression in pre-B cells[101]. However, ERK induces Igκ recombination by a separate mechanism. ERK promotes E2A activity by inducing *E2a* expression and by repressing the E2A inhibitor *Id3*[101]. E2A then upregulates

Foxo1[102]. E2A cooperates with FOXO1 to promote Igκ recombination by upregulating the recombinase components *Rag1*, *Rag2*, and *Dntt* (TdT) and by opening the Igκ locus[85,98,99,101,144]. E2A also increases accessibility to the Igκ locus by binding to the Igκ intronic enhancer (Eκi) and the Igκ 3' enhancer (Eκ3)[101,145,146]. Pre-BCR activation of RAS-ERK is necessary for pre-B quiescence and light chain recombination.

Pre-BCR signaling positively regulates the IRF4/8-IKAROS/AIOLOS pathway

The pre-BCR also promotes pre-B cell differentiation by upregulating *Irf4*, *Irf8*, *Ikaros*, and the Ikaros family member *Aiolos (Ikzf3)*. More specifically, BLNK activity induces *Irf4* and *Irf8*[94,147,148], which upregulate *Ikaros* and *Aiolos*[94,96,149]. AIOLOS has 70% overall homology with IKAROS and this homology is particularly extensive in the DNA binding and C-terminal domains[150]. Unsurprisingly, the binding of IKAROS and AIOLOS overlaps considerably at loci throughout the genome[151]. Moreover, IKAROS cooperates with AIOLOS to inhibit the expression of *Ccnd2*, *Ccnd3*, and *Myc* and promote cell cycle arrest in pre-B cells[94,96,135,152,153]. In addition, IRF4, IRF8, and IKAROS promote Igκ recombination[50,68,97]. Finally, IKAROS and AIOLOS cooperate in a negative feedback loop by inhibiting the expression of the pre-BCR components *Igll1* (λ5) and *Vpreb1*[50,94,96,154]. Pre-BCR induction of *Irf4*, *Irf8*, *Ikaros*, and *Aiolos* is necessary for pre-B cells to exit the cell cycle, induce light chain recombination, and differentiate into immature B cells.

Deregulation of B cell development promotes ALL

A network of B cell transcription factors, STAT5 and pre-BCR signaling coordinately promote the generation of B cells in the bone marrow. However, mutations in these same processes can lead to the transformation of B cell progenitors and cause B-ALL. There are several ways that B cell progenitors can become transformed.

Deregulation of B cell transcriptional network in B-ALL

Recurring chromosomal translocations and mutations are a hallmark of B-ALL and contribute significantly to the diagnosis and prognosis of this disease[9,112,155]. The most commonly altered pathway in B-ALL is the transcriptional regulation of B cell development[9]. B-ALL is also characterized by a block in B cell differentiation[9]. Unsurprisingly, the genes that regulate the early stages of B cell development, *IKAROS*, *PAX5*, and *EBF1*, are frequently mutated in B-ALL[9]. These mutations result in loss of function deletions, dominant negative deletions, or gene fusions that lead to an arrest in B cell development[134].

The role of PAX5 in B-ALL

The most common loss of function mutations in B-ALL are found in the *PAX5* locus (32% of patients)[9,26]. These mutations consist of predominantly mono-allelic deletions that sometimes involve a larger segment of chromosome 9p and point mutations[15,17]. Less frequently, *PAX5* mutations are found in the form of chromosomal translocations, which drive the expression of various fusion proteins[15]. The most common PAX5 fusion proteins in B-ALL include PAX5-ETV6, PAX5-C20orf112, PAX5-FOXP1 and PAX5-PML[17,108]. *PAX5* mutations are detected in virtually all B-ALL

subtypes, but occur more frequently in BCR-ABL+ cases (54%)[26]. BCR-ABL is a fusion protein consisting of break point cluster region (BCR) and the kinase ABL1 that results from a translocation of chromosomes 9 and 22. The *BCR-ABL1* translocation drives the expression of a highly active kinase that phosphorylates many downstream targets to drive leukemia. PAX5 mutations are not associated with adverse outcomes, however, they are important in leukemogenesis[9].

The mechanism by which *PAX5* mutations promote B-ALL varies and depends on the type of mutation. The PAX5 fusion proteins function as dominant-negatives by multimerizing and binding with high avidly to target genes[156]. This prevents the WT PAX5 from binding and modulating gene expression[156,157]. The more common deletions and point mutations typically occur in only one *PAX5* allele, suggesting that these genetic alterations result in haploinsufficiency or hypomorphic alleles[108,158]. However, B cell development is normal in *Pax5*$^{+/-}$ mice[2,159,160]. This may be because monoallelic mutations in *PAX5* require secondary mutations to drive ALL. This is supported in mice, as *Pax5* heterozygosity cooperates with STAT5 activating mutations to initiate B-ALL[2,161,162]. Similarly, *PAX5* deletions are frequently found in human B-ALL samples that also harbor BCR-ABL or TCF3-PBX1 translocations or *CDKN2A* deletions[17,26,158,163], suggesting that *PAX5* deletions cooperate with these mutations to drive B-ALL. *Pax5* is also known to induce the expression of the tumor suppressor *Bach2*[164]. Ectopic expression of *Bach2* suppresses B-ALL induced by MYC or BCR-ABL by activating p53 and thereby causing apoptosis[134,164]. Therefore, PAX5 may suppress ALL, in part, by upregulating *Bach2*. This also might explain why *PAX5* mutations are common in BCR-ABL+ leukemias - potenitally as a means to inactivate *BACH2*. In summary, PAX5

mutations can function as dominant negatives or cooperate with other secondary mutations to promote B-ALL.

The role of IKAROS in B-ALL

The second most common genetic deletions in B-ALL occur in the *IKAROS* locus. Deletions of *IKAROS* are found in 28% of patients with B-ALL[26]. Moreover, *IKAROS* deletions have clinical significance, as they are associated with poor clinical outcome[165–167]. *IKAROS* deletions result in loss of function or dominant negative isoforms of IKAROS[9]. The dominant negative form of IKAROS results from a deletion of exons 4-7[168], which is likely mediated by off target effects of RAG1/2[169]. This causes in-frame splicing of exon 3 to exon 8 and expression of an isoform called Ik6[168]. This Ik6 isoform localizes to the cytoplasm due to the loss of the DNA binding domain and the nuclear localization sequence[170–172]. The Ik6 isoform acts as a dominant negative by sequestering normal IKAROS proteins in the cytoplasm, as well as other IKAROS family members including AIOLOS[150,169,173]. Approximately 33% of all *IKAROS* deletions found in B-ALL result in the expression of Ik6[169].

IKAROS deletions can also result in no IKAROS expression. This is caused by biallelic deletions that result in 2 null alleles[169]. Biallelic deletions comprise 12% of all *IKAROS* deletions in B-ALL[169]. The majority of *IKAROS* deletions (55%), however, result in haploinsufficiency[169]. In mouse models, deficiency in one *IKAROS* allele accelerates BCR-ABL-driven leukemia[174]. These leukemic cells show a 50% reduction in the expression of full-length *IKAROS*, as expected[174]. These studies illustrate that monoallelic deletions in *IKAROS* can result in haploinsufficiency and accelerate leukemia[174].

The various types of *IKAROS* deletions are disproportionally found in different subtypes of ALL. For instance, up to 70% of *IKAROS* mutations found in BCR-ABL+ B-ALL exhibit severe *IKAROS* deletions (null or dominant negative)[170,175,176]. Haploinsufficiency, however, is more common in BCR-ABL negative B-ALL (57-72% of *IKAROS* mutations)[166,170]. These observations suggest that the selective pressure to acquire severe *IKAROS* mutations is higher in BCR-ABL+ B-ALL than other subtypes[169]. IKAROS deletions are highly enriched for B-ALL cases that are BCR-ABL+ or BCR-ABL-like, as 84% of BCR-ABL+ and 39% of BCR-ABL-like B-ALL cases have IKAROS deletions[165,175]. BCR-ABL-like cases express a similar gene signature as BCR-ABL+ B-ALL samples, but are BCR-ABL negative[177]. Some of the genes that are commonly deregulated in BCR-ABL-like cases include deletions of B cell transcription factors and mutations that activate STAT5 such as IGH-CRLF2 and TEL-JAK2 translocations[9,177,178]. Patients with BCR-ABL+ or BCR-ABL-like B-ALL tend to respond more poorly to therapy and have poorer outcomes than patients with other forms of B-ALL[9]. These observations suggest that *IKAROS* deletions cooperate with genes that control a BCR-ABL gene signature that is associated with high-risk cases of B-ALL.

In mice, null mutations and dominant negative forms of *Ikaros* tend to drive T-ALL rather than B-ALL[36,179,180]. In humans, however, *IKAROS* deletions are rarely observed in T-ALL (5% of cases)[181–183]. Therefore, *IKAROS* may play a more important role in suppressing T-ALL in mice compared to in humans.

How *IKAROS* deletions promote B-ALL is not completely clear. Gene expression signatures associated with loss of *IKAROS* in B-ALL have revealed an overexpression of genes indicative of a stem cell phenotype[166,184]. Moreover, IKAROS is known to silence stem cell genes in lymphoid progenitors[40]. Thus, *IKAROS* deficiency might promote self-

renewal by inducing a stem cell gene expression pattern[169]. *IKAROS* mutations may also promote B-ALL by blocking B cell development, as mice with loss-of-function mutations in Ikaros exhibit blocks at various stages of B cell development[34,35,37–40,61] (Figure 1.1). IKAROS is required for HSC activity[38], the rearrangement of the heavy and light chains[68,169,185], and the expression of genes involved in B cell specification and commitment[61,186]. In addition, loss of *IKAROS* may promote transformation by preventing cell cycle arrest[169]. This is supported in mice as IKAROS induces and is required for pre-BCR-mediated exit from the cell cycle in BCR-ABL+ pre-B cells[35,36,94,153,174]. IKAROS promotes cell cycle arrest in pre-B cells by binding to and inhibiting *Myc* and *Ccnd2* expression[152,187]. IKAROS also promotes exit from the cell cycle by redirecting SYK phosphorylation of BLNK to tyrosine 96 (Y96)[153,188]. Phosphorylation of BLNK at Y96 preferentially activates a BLNK-dependent tumor suppressor pathway that induces *Cdkn1b* ($p27^{Kip1}$) and cell cycle arrest[132]. Finally, conditional deletion of *Ikaros* starting in CLPs has been shown to promote B-ALL by enhancing integrin-mediated survival, proliferation, and self-renewal[35]. Therefore, IKAROS suppresses ALL potentially by increasing self-renewal capabilities, causing a block in B cell development, inhibiting proliferation, and/or inhibiting cell survival.

The role of EBF1 in ALL

Deletions of *EBF1* are found in 4-7% of B-ALL cases and can occur on one or both alleles[158,189,190]. These deletions are variable in size, but frequently affect a large portion of the *EBF1* open-reading frame, suggesting a loss-of-function phenotype[17]. Complete loss of *Ebf1* in mice causes an arrest in B-cell development at the pro-B-cell stage[19], suggesting that bi-allelic deletions of *EBF1* may promote B-ALL by blocking B

cell differentiation. *Ebf1*$^{+/-}$ mice, on the other hand, display a 50% reduction in pro-B cells[19], but do not develop leukemia[2]. However, *Ebf1* heterozygosity cooperates with constitutive active STAT5b to drive B-ALL[2]. Similarly, *EBF1* deletions are more prevalent in BCR-ABL+ cases (14%) and BCR-ABL-like B-ALL cases (38%)[190]. Additionally, Monoallelic deletions of *EBF1* occur more frequently in combination with *BTG1* gene deletions (26%)[191], suggesting that these tumor-suppressors may cooperate to promote B-ALL[17]. Deletions of *EBF1* may contribute to B-ALL by blocking B cell development or by cooperating with secondary mutations.

The role of STAT5 in ALL

The two most common oncogenic translocations in B-ALL are ETV6-RUNX1 (TEL-AML1) and BCR-ABL[14]. Both of these translocations activate STAT5[1,192,193]. ETV6-RUNX1 is found in up to 25% of pediatric B-ALL cases and 2% of adult B-ALL cases[6,14]. Where as BCR-ABL is found in 25% of adult B-ALL cases and 2% of pediatric B-ALL patients[14]. Moreover, high levels of active STAT5 are associated with poor prognosis in BCL-ABL+ leukemias[2]. In addition, several other mutations found in B-ALL activate STAT5, including rearrangements of the kinases *ABL1, ABL2 (ARG), CSF1R, EPOR, JAK2, PDGFRB* and mutations in the cytokine receptors *IL7R, CRLF2*, and *FLT3*[9]. As previously mentioned, many of these STAT5-activating mutations are common in BCR-ABL-like B-ALL[177]. In addition, activating mutations in *STAT5* itself have also been identified in leukemia[194–196]. Therefore, STAT5 is commonly hyperactivated in B-ALL and activation of STAT5 is correlated with patients that have high-risk forms of B-ALL and shorter overall survival.

Several of these mutations that activate STAT5 have been shown to require JAK-STAT signaling to promote cell proliferation, including ETV6-RUNX1, CRLF2, IL7R, EPOR, EBF1-PDGRRB, and FLT3-ITD[9,192,197–202]. Moreover, BCR-ABL, TEL-JAK2, and TEL-PDGFRB have been shown to require *Stat5* to cause transformation in mice[203–205]. One of these studies showed that inducible deletion of *Stat5a* and *Stat5b* in BCR-ABL+ B-ALL cell lines induced cell cycle arrest and apoptosis[203]. Therefore, STAT5 may initiate BCR-ABL-driven B-ALL by promoting proliferation and survival. However, the molecular mechanisms underlying the role of STAT5 in B-ALL remain unclear.

Pre-BCR and ALL

Strong evidence suggests that pre-BCR signaling functions to suppress ALL. Moreover, many of the genes that encode pre-BCR signaling components are frequently disrupted in ALL.

Deletions of one such pre-BCR signaling component, the adaptor *BLNK*, have been found ALL. However, the exact frequency of *BLNK* mutations is not clear[153,158,206–208]. In one small study, 16 of 34 B-ALL samples had complete loss or dramatic reductions in *BLNK* expression[207]. The decreased *BLNK* expression is probably due to deletions that cause alternative splicing of *BLNK* and expression of a dominant negative isoform[207,208]. Moreover, BCR-ABL has been shown to induce similar alternative splicing of *BLNK*[209]. In mice, *Blnk* has been shown to function as a tumor suppressor in pre-B cells[132,210]. One way that BLNK has been proposed to suppress leukemia is by binding to JAK3 and inhibiting JAK-STAT5 signaling[132]. BLNK inhibition of STAT5 leads to p27 expression and cell cycle arrest[132]. BLNK might also suppress B-ALL by activating the kinase BTK. This is supported by the observation that constitutively active BTK prevents

leukemia caused by *Blnk* deficiency[211]. BLNK may also suppress B-ALL by activating the tumor suppressor pathway IRF4/8-IKAROS/AIOLOS[50,94,96,149,174,212,213], which promote cell cycle arrest and differentiation[68,152]. In addition, BLNK positively regulates other potential tumor suppressor pathways such as FOXO and RAS-ERK[85,99,101,214], which also promote cell cycle arrest and differentiation[210]. Therefore BLNK suppresses B-ALL potentially by inhibiting JAK-STAT5 signaling and activating downstream pathways that promote cell cycle arrest and differentiation.

FOXO proteins also appear to have a role in suppressing ALL. FOXO3, for example, is decreased in B-ALL and T-ALL compared to healthy controls[189,215]. Furthermore, FOXO proteins function as tumor suppressors in mice[216]. However, all three FOXO homologues, *Foxo1*, *Foxo3*, and *Foxo4*, must be deleted to cause transformation. The resulting ALL originates from the T-lineage. Therefore, FOXO proteins likely have a role in suppressing B-ALL but it has not been demonstrated that FOXOs function as tumor suppressors in B cell progenitors. However, FOXOs are known to promote quiescence and Igκ recombination in progenitor B cells[98,99,102,217], potentially by positively regulating the expression of a full-length isoform of IKAROS[149]. Therefore, FOXOs may suppress B-ALL by promoting cell cycle arrest and Igκ recombination.

Another pre-BCR component that is disrupted in B-ALL is the kinase *BTK*[218]. However, the role of BTK in B-ALL is not clear. In mice, BTK can suppress transformation independent of it kinase activity[211,219], suggesting that it can suppress leukemia by functioning as an adaptor.

BTK positively regulates PLCγ2, which has also been shown to suppress B-ALL. *Plcg2* deficiency in mice causes enhanced IL7 signaling and proliferation[220]. In addition, *Plcg2*$^{-/-}$ pre-B cells have elevated levels of RAG1 and RAG2[220], which may increase the chances of secondary mutations[175,191,221,222]. Moreover, *Plcg2* deficiency cooperates with the pro-proliferative factor MYC to initiate leukemia[220]. Therefore, *Plcg2* can suppress B-ALL potentially by inhibiting proliferation.

PLCγ2 positively regulates NFκB, which promotes cell proliferation and survival in immature and mature B cells[60,90,142,147,223]. NFκB is also known to promote the survival of many other cell types[224]. Consequently, NFκB drives transformation in many cell types, including B cell lymphomas[224]. However, NFκB can also suppress tumorigenesis in some contexts and cell types including pre-B cells[224]. Therefore, the role of NFκB in cancer is context-dependent. There are several factors that can influence the activity of NFκB. Post-translational modifications of NFκB, for example, regulate NFκB subunit dimerization, proteolytic degradation, and localization[224]. Post-translational modifications can also determine whether NFκB induces or represses the expression of target genes[224]. Others factors that modulate the function of NFκB include tumor suppressors, kinases, transcriptional regulators, or transcription factors[224,225]. Tumor suppressors such as p53 and ARF can inhibit the tumor-promoting activities of NFκB subunits while simultaneously facilitate their ability to suppress cancer progression[224]. Therefore, the role of NFκB in cancer is influenced by many factors and varies between cell types.

In pre-B cells, NFκB1 has been shown to suppress v-ABL1-induced transformation, potentially by inhibiting *Ccnd1* expression[226]. Additionally, NFκB binds to the Igκ enhancer and promotes light chain rearrangement and differentiation[227,228].

Thus, NFκB can suppress the transformation of pre-B cells, possibly by inhibiting proliferation and promoting light chain rearrangement.

NFκB induces IRF4 and IRF8[148,229–231], which have been shown to suppress B-ALL in mice. IRF4 deficiency cooperates with MYC to drive B-ALL[213]. IRF4 also suppresses BCR-ABL-induced leukemia in mice, primarily by negatively regulating cell cycle progression[50,232]. IRF4 and IRF8 seem to exhibit redundant roles in pre-B cells, as a deficiency in both factors invariably leads to B-ALL-like disease[212]. Deficiency in just one of the other factors can lead to B-ALL but the penetrance is lower. The mechanism is not clear but deficiencies in *IRF4* and *IRF8* may promote B-ALL by blocking B cell development, as is seen in mice[44,50]. IRF4 and IRF8 may also suppress B-ALL by inducing IKAROS and AIOLOS[96], which induce cell cycle arrest and Igκ recombination[68,152]. Therefore, IRF4 and IRF8 can potentially suppress B-ALL by inhibiting proliferation and by promoting light chain recombination and B cell differentiation.

Deletions in other pre-BCR signaling components such as *SYK* and *VPREB1* have also been reported in B-ALL[165,218]. The mechanism by which these deletions drive disease may involve BCR-ABL, as BCR-ABL+ B-ALL samples consistently have a decrease in the expression of many pre-BCR signaling components: *VPREB1, IGLL1, IGHM, CD79A*, *CD79B, LYN*, *BLK*, *FYN*, *SYK, BLNK*, *PLCG2*, *IKK*, *IRF4,* and *IKAROS*[153,158,175,207,209,233]. Moreover, BCR-ABL activates many of the signaling components downstream of the pre-BCR, including SYK, BTK, and PLCγ1/2[233]. This may cause pre-B cell activation above a signal strength threshold that causes negative selection or apoptosis[233]. Therefore, BCR-ABL+ clones that express a functional pre-

BCR are selected against[153]. Pharmacological agonism of SYK induces cell death in BCR-ABL+ cells, but not when BCR-ABL is concomitantly inhibited with Imatinib[233]. These data suggest that pre-BCR signaling is important in the suppression of BCR-ABL+ B-ALL and that agonism of the pre-BCR signaling pathway might be effective in treating B-ALL. To summarize, the deregulation of B cell transcription factors, STAT5 and pre-BCR signaling can lead to the transformation of B cell progenitors.

Super-enhancers

General features of enhancers and super-enhancers

Transcription factors typically regulate gene expression by binding cis-acting regulatory elements known as enhancers and by recruiting coactivators and RNA polymerase II (RNA Pol II) to target genes[234]. Enhancers are segments of DNA that are generally a few hundred base pairs in length and are typically occupied by multiple transcription factors[234]. The number of enhancers that are active in any one cell type has been estimated to be in the tens of thousands, and enhancer activity is largely cell-type specific[234].

Recent studies have identified a new class of enhancers, termed super-enhancers, which tend to regulate the most highly expressed genes in each cell type in a cell-type-specific manner[234]. These super-enhancers appear to play a key role in cell identity and in diseases such as cancer and autoimmunity[234–237].

Role of super-enhancers in embryonic stem cells

Super-enhancers have been most well characterized in embryonic stem cells (ESCs). In ESCs, only a small number of master transcription factors control the gene expression program that establishes and maintains ESC state[234,238–240]. These transcription factors, which include OCT4, SOX2, and NANOG, bind to enhancers together with the Mediator coactivator complex[234,241]. The Mediator complex facilitates the ability of enhancer-bound transcription factors to recruit RNA Pol II to the promoters of target genes and is essential for maintenance of ESC state and embryonic development[234,241–247].

Whyte and colleagues found that ESC enhancers can be divided into two classes based on Mediator levels—one class comprised the vast majority (8,563) of enhancers, and the other encompassed 231 large enhancer domains, termed super-enhancers[234]. Approximately 40% of Mediator signal associated with the 231 super-enhancers by ChIP-Seq[234]. The key features of the super-enhancers are that (1) they span DNA regions whose median length is an order of magnitude larger than the typical enhancer and that (2) they have levels of Mediator that are at least an order of magnitude greater than those at the typical enhancer[234].

Super-enhancers can also be distinguished from typical enhancers in ESCs by the presence of the transcription factors KLF4 and ESRRB, which play important roles in the ESC gene expression program and in reprogramming of somatic cells to induced pluripotent stem cells[234]. Super-enhancers were also enriched for sequence motifs bound by transcription factors important in ESC function, including OCT4, SOX2, NANOG, KLF4, and ESRRB[234]. This indicates that super-enhancers are formed as a

consequence of binding of specific master transcription factors to dense clusters of their binding site sequences[234].

The set of super-enhancer-associated genes contained nearly all genes that have been implicated in control of ESC identity, including genes encoding the master ESC transcription factors OCT4, SOX2, and NANOG and DNA-modifying enzymes and MicroRNAs that control the ESC gene expression program[234]. OCT4, SOX2, and NANOG form an interconnected autoregulatory loop in which all three factors bind as a group to the promoters of each of their own genes and form the core regulatory circuitry of ESCs[234,248,249]. Therefore, the genes encoding the master transcription factors OCT4, SOX2, and NANOG are themselves driven by super-enhancers, forming feedback loops in which the key transcription factors regulate their own expression[234].

Super-enhancer-associated genes are generally expressed at higher levels than genes associated with typical enhancers[234]. Luciferase assays have confirmed that super-enhancers tend to drive higher levels of transcription than typical enhancers[234]. The presence of KLF4 and ESRRB also correlates with high levels of luciferase activity, suggesting that high levels of the transcription factors KLF4 and ESRRB at super-enhancers drive high-level expression of their target genes in ESCs[234].

Enhancers typically function through cooperative and synergistic interactions between multiple transcription factors and coactivators[234,250–254]. The transcriptional output of enhancers with large numbers of transcription factor binding sites can be more sensitive to changes in transcription factor concentration than those with smaller numbers of binding sites[234,255,256]. Similarly, super-enhancer-associated genes are more sensitive to loss of transcriptional networks than other genes, as reducing the levels of

OCT4 or Mediator lead to more profound effects on expression of super-enhancer-associated genes than on other active genes with typical enhancers[234].

Super-enhancers in progenitor B cells

Similar to those studies in ESCs, high levels of Mediator binding has been used to define super-enhancers in pro-B cells[234]. The occupancy of Mediator highly correlates with the B cell transcription factor PU.1, which is predictive of enhancer activity[234,257,258]. Therefore this suggests that Mediator can also be used to predict enhancer activity in pro-B cells[234]. Those 395 Mediator-defined super-enhancers in pro-B cells shared similar characteristics to those found in ESCs—they spanned DNA domains whose median length is an order of magnitude larger than the typical enhancer, and they had levels of Mediator that are at least an order of magnitude greater than those at typical enhancers[234]. These pro-B cell super-enhancers were enriched for closely spaced clusters of the sequence motifs of several B cell transcription factors, including EBF, E2A and FOXO1[234]. Those genes associated with pro-B cell super-enhancers featured those that are regulators of B cell identity including the B cell transcription factors *Ikaros, Ebf1,* and *Foxo1,* the IL7R kinase *Jak1,* and the pre-BCR signaling components *Vpreb1, Igll1 (λ5), Cd79a (Igα), Cd79b (Igβ), Syk, Blnk,* and *Inpp5d*[234]*.* Therefore pro-B cell super-enhancers seem to be formed by a clustering of B cell transcription factors and associated with genes that are key to B cell development.

Role of super-enhancers in transformation

In transformed B cells (diffuse large B cell lymphoma or DLBCL), super-enhancers have been shown to be enriched for BRD4, which is a member of the

bromodomain and extraterminal (BET) subfamily of human bromodomain proteins[235,236]. BRD4 binds to acetylated chromatin and recruits Mediator and the positive transcription elongation factor P-TEFb to such sites[235,259–262]. This in turn leads to the recruitment of RNA Pol II and transcriptional activation[235,259,263–265].

Chromatin regulators such as BRD4 are attractive as therapeutic targets for cancer because they are deregulated in numerous cancers[235,266–270] and are amenable to small-molecule inhibition[235,261,271,272]. Most chromatin regulators, however, are expressed in a broad range of healthy cells and contribute generally to gene expression, so inhibition of these important genome-associated proteins might be expected to adversely affect global gene expression in healthy cells and thus produce highly toxic effects[235]. Nonetheless, inhibitors of some chromatin regulators, such as BRD4, have been shown to selectively inhibit transcription of key oncogenic drivers such as MYC in multiple tumor types including multiple myeloma, Burkitt's lymphoma, acute myeloid leukemia, and ALL[235,236,261,273–276].

The inhibition of MYC apparently occurs as a consequence of BRD4 depletion at the super-enhancers that drive MYC expression[235,236,273,277]. Treatment of multiple myeloma tumor cells with the BRD4 inhibitor JQ1, for example, causes a preferential loss of BRD4, Mediator, and P-TEFb at super-enhancers and preferential loss of transcription at super-enhancer-associated genes[235]. These included genes that are important in cancer pathogenesis such as *Myc, Ccnd2, Pim1, Mcl1,* and *Bcl2l1*[235,278–285]. Similarly, JQ1 causes the preferential loss of expression of super-enhancer associated genes in DLBCL cell lines, including the DLBCL oncogenes *Myc* and *Cd79b*[236,286].

BRD4 inhibitors have been shown to induce cell cycle arrest and/or apoptosis in several tumor cell types, including neuroblastomas, glioblastomas, medulloblastomas, breast cancer cells, erythroleukemias, acute myeloid leukemias, T cell acute lymphoblastic leukemias, and B-cell non-Hodgkin lymphomas[277,287–295] . BRD4 inhibitors have also been shown to inhibit tumor progression in mouse models of DLBCL, multiple myeloma, and mantle cell lymphoma[235,277,296,297]. Importantly, BRD4 inhibition has been shown to induce cell cycle arrest and inhibit proliferation in human B-ALL samples and decrease leukemic burden and improve survival in B-ALL primary human xenograft models[275]. Although BRD4 is widely expressed in mouse tissues, mice are reasonably tolerant of the levels of BET bromodomain inhibition that are used mouse models to suppress tumor growth[235,261,273,274,276,298]. Therefore, BRD4 inhibitors may be safe and effective in treating a wide variety oncogene-addicted malignancies, including B-ALL[275].

Conclusions

ALL is a common form of cancer in children, but is also found in adults. It is caused by the transformation of lymphocyte progenitors, typically derived from the B-lineage. Normally, the development of B cells in the bone marrow generates a vast repertoire of mature B cells, capable of specifically recognizing and combating many different foreign pathogens. B-ALL is frequently caused by genetic mutations in genes that control B cell development. Common pathways that are deregulated in B-ALL include the B cell network of transcription factors, STAT5, and pre-BCR signaling. Mutations in some of the genes that make up these pathways are associated with high-risk subgroups of B-ALL that currently have the worst prognosis. How these pathways contribute to disease and how they interact to cause B-ALL is not well understood. For

example, why are IKAROS deletions highly enriched in BCR-ABL+ and BCR-ABL-like leukemias?

Super-enhancers are an emerging class of enhancers that have an important role in controlling cell identity, function, and transformation. While super-enhancers have been identified in progenitor B cells, more extensive studies about how these super-enhancers interact with STAT5 or other B cell transcription factors have yet to be reported. A better understanding of these transcriptional networks may elucidate the mechanisms by which oncogenes and tumor suppressors are regulated in progenitor B cells.

My thesis is focused on understanding some of the underlying mechanisms by which the deregulation of B cell transcription factors, STAT5, and pre-BCR signaling cause ALL. Moreover, I aim to elucidate how these pathways interact and cooperate to initiate disease. Such knowledge may lead to the development of more specific and effective therapies, particularly for patients that are currently at the highest risk for treatment failure. Moreover, understanding how these pathways interact may lead to better stratification methods for B-ALL. Such methods could then be used to minimize the overtreatment of low-risk patients.

Chapter 2: STAT5 drives B-ALL by opposing gene regulation by the pre-BCR-NFκB-IKAROS tumor suppressor pathway

Introduction

Previous studies have suggested that the role of STAT5 in B cell progenitors is only to provide a survival signal[3,4]. Therefore, STAT5 could be promoting B-ALL by

upregulating genes that promote cell survival. However, this has yet to be tested. Moreover, other groups have shown that STAT5 is involved in other processes in progenitor B cells such as cell proliferation, the upregulation of *Ebf1*, the rearrangement of distal V_H-DJ_H gene segments, and the suppression of light chain recombination. Therefore STAT5 has roles in B cell development other than promoting cell survival that could underlie STAT5-driven leukemia.

Stat5 is required for the initiation of B-ALL that is driven by BCR-ABL, TEL-JAK2, and TEL-PDGFRB translocations[203–205]. Moreover, high levels of active STAT5 are correlated with poor patient survival in BCR-ABL+ B-ALL[2]. Together these studies indicate that STAT5 is important in the initiation and outcome of B-ALL. However, these studies failed to elucidate the mechanism by which STAT5 drives B-ALL.

BCR-ABL translocations are associated with defects in pre-BCR signaling. One study suggested that defects in the pre-BCR promote B-ALL by limiting pre-BCR signaling below a threshold that induces negative selection in BCR-ABL+ B-ALL[233]. However, pre-BCR signaling is also known to promote cell cycle arrest and differentiation. Therefore, pre-BCR signaling can suppress transformation by multiple mechanisms and potentially in ways that are not currently understood. BCR-ABL is known to activate many downstream targets of the pre-BCR. However, these two pathways may interact in other ways as well to promote ALL. For example, how downstream targets of BCR-ABL such as STAT5 might interact with pre-BCR signaling effectors to regulate target genes in B-ALL is unclear.

Therefore the mechanisms by which STAT5 activation and defects in pre-BCR signaling components contribute to B-ALL are not entirely understood. In addition, how these pathways might interact is also poorly defined. Therefore, we aimed to determine

the mechanisms by which the deregulation of these two pathways promotes the development of ALL.

Methods

Mice

Stat5b-CA, *Blnk*$^{+/-}$, *Prkcb*$^{-/-}$, *Xid, Nfkb1*$^{-/-}$, and *Bcl2l1* transgenic mice have been described previously[299–303]. All mice were backcrossed to the C57Bl/6 background with the exception of *Prkcb*$^{-/-}$. Mice were housed in specific pathogen-free facilities at the University of Minnesota; all animal experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee. All *Stat5b-CAxBlnk*$^{+/-}$, *Stat5b-CAxXid* and *Stat5b-CAxPrkcb*$^{-/-}$ leukemias were confirmed by flow cytometry to be B-ALL. 50% of the *Stat5b-CAxNfkb1*$^{+/-}$ and *Stat5b-CAxNfkb1*$^{-/-}$ leukemias were B-ALL and the other 50% were T-ALL. Spleen, lymph nodes, and bone marrow were isolated from leukemic mice and used for further experiments. B220^{+}CD19^{+} lymph node cells were isolated using magnetic bead separation (Miltenyi Biotech, Bergisch Gladbach, Germany) and used to isolate RNA and DNA. Purity of B lymphocytes was > 95% as assessed by flow cytometry. Kaplan-Meier survival curves were created using Prism software (GraphPad Software, La Jolla, CA).

Flow cytometry

Single cell suspensions were stained with fluorescent antibodies: α-IgM (Jackson ImmunoResearch; West Grove, PA), α-CD19 (1D3), α-CD45R (RA3-6B2), α-CD127 (A7R34), α-pre-BCR (SL165, BD Pharmingen, San Diego, CA), α-Igk (187.1,

SouthenBiotech, Birmingham, AL), α-Igl (JC5-1, SouthenBiotech, Birmingham, AL). α-CD43 (S7, BD Pharmingen), α-Gr-1 (RB6-8C5), and α-Ter119 (TER-119). All antibodies were obtained from eBioscience (San Diego, CA) unless otherwise indicated. SA-PerCP-Cy5.5 was used to detect biotinylated antibodies. For BrdU analysis, mice were injected intraperitoneally with 200 µl of 10 mg/ml BrdU (BD Pharmingen) and analyzed after 16 hours. Cells were assayed on a LSRII or Fortessa flow cytometer (BD Biosciences, San Diego, CA) and data was analyzed using FlowJo software (Treestar; San Carlos, CA).

Microarray

Microarray analysis was performed on total RNA extracted from either sorted $B220^{+}CD19^{+}IgM^{-}CD43^{lo}$ pre-B cells (C57Bl/6, *Stat5b-CA*, or *Xid*) or $B220^{+}CD19^{+}$ leukemic cells from lymph nodes of tumor-bearing mice using an RNeasy kit (Qiagen, Valencia, CA). cRNA probes were synthesized and hybridized to Mouse 430 2.0 arrays following Affymetrix protocols and statistical analyses were performed using GeneSpringGX 11.0 (Agilent, Santa Clara CA). Samples were normalized using RMA, filtered on expression (20.0-100.0th) percentile, or Affymetrix Microarray Suite 5.0 (MAS5.0) software. Significant gene lists were generated using one-way ANOVA with a corrected p-value < 0.05 with a 1.5 fold change in gene expression. Clustering was performed using hierarchical clustering on both entities and conditions, using Euclidean distance metric and Centroid Linkage rule. Synergy scores were calculated as described previously[304]. Briefly, expression values for each probe were averaged across sample groups. Synergy between *Stat5b-CA* and *Xid* was calculated as follows: for any probe, let *a* represent the average *Stat5b-CA* pre-B value, let *b* represent the average

Xid pre-B value and let *c* represent the average leukemia value. Values for each probe were entered into the formula [(*c*/*a*)+(*c*/*b*)] for downregulated genes or [(*a*+*b*)/*c*] for upregulated genes. Synergy scores < 0.9 identified cooperation response genes. A list of NFκB target genes was obtained from www.nf-kb.org. We then restricted our analysis to those NFκB target gene expressed in WT pre-B cells, which were defined as those genes with an expression ≥ 200 in WT pre-B cells by microarray analysis.

Quantitative real-time PCR

$CD19^+Ig\lambda^-Ig\kappa^-Gr\text{-}1^-Ter119^-$ cells were isolated from murine bone marrow by magnetic column separation (Miltenyi Biotech) and plated at a concentration of 1-2 x 10^6 cells/ml in Opti-MEM + 5% FBS + 1% penicillin/streptomycin. Cells were cultured for 16 hours at 37°C and 5% CO_2 with or without 10 ng/ml IL7 (Peprotech, Rocky Hill, NJ). After culturing, RNA was isolated from the cells using an RNeasy kit (Qiagen, Venlo, Netherlands). cDNA was synthesized from 2 ng of RNA using a qScript cDNA synthesis kit (Quanta Bisciences, Gaithersburg, MD). Real-time PCR was performed using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland). Primer sequences were as follows: *Nfkb1* (forward: 5'-GGGAGCCTCTAGTGAGAAGAA-3'; reverse: 5'-TGTGACCAACTGAACGATAACC-3'), *Nfkb2* (forward: 5'-CTTTCCTTCGAGCTAGCGATG-3'; reverse: 5'-CATTCGGGAGATCTTCAGGTTC-3'), *Ikaros* (forward: 5'-AAGAGCGATGCCACAACTAC-3'; reverse: 5'-GTCTTCTGCCATCTCGTTGT-3'), *Irf4* (forward: 5'-GGAAGCTCATCACAGCTCAT-3'; reverse: 5'- AACGTGTTCAGGTAACTCGTAG-3' *Hprt* primers have been previously described[128]. Reactions were run on a 7000 Sequence Detection PCR System (Applied Biosystems, Foster City, CA). Amplification conditions were: 50°C for 2 minutes; 95°C

for 10 minutes; 40 cycles of 95°C for 15 seconds and 58°C for 60 seconds. Normalized values were calculated as previously described[305].

Chromatin Immunoprecipitation (ChIP) assays

ChIP-Seq was performed based on previously published protocols[120]. Briefly, *STAT5b-CAxBlnk*$^{+/-}$ tumor lymph node single cell suspensions were stimulated with 10 ng/mL at 37°C for 30 minutes. Cells were immediately fixed in 1% formaldehyde for 10 minutes at room temperature. Formaldehyde was quenched with 50 mM Glycine. Cells were washed twice with PBS and resuspended in 0.5% SDS, 5.6 mM EDTA, 33.4 mM Tris, 84 mM NaCl. DNA was sonicated to 200 bp fragments. 1-8x10^6 (ChIP-qPCR) or 50 x10^6 cell (ChIP-Seq) samples were immunoprecipitated overnight with 10 mg (ChIP-qPCR) or 50 mg (ChIP-Seq) of a-STAT5 (sc-835 x, Santa Cruz Biotechnology, Santa Cruz, CA) or isotype control antibody (sc-2027 x, Santa Cruz Biotechnology) and salmon sperm DNA blocked protein A agarose bead slurry (EMD Millipore, Billerica, MA). Beads were washed twice with each of low salt, high salt, and $LiCl_2$ buffers then three times with TE buffer. DNA was eluted with 1% SDS, 0.1 M $NaHCO_3$. NaCl was added to 0.6 M final and crosslinks were reversed at 65°C overnight. Tris, pH 8, was added to 40 mM final and EDTA, pH 8, to 8.8 mM final. Samples were digested with 0.1 mg/ml Proteinase K for 1 hour. DNA was purified with QIAquick PCR purification kit (Qiagen). Replicate IKAROS ChIP-seq datasets were produced using cultures of ~5x10^7 GM12878 cells cultured on separate days, essentially as described[36]. The following primers were used for ChIP-qPCR: *Ikaros* promoter B (probe: 5'-AGACGAGGGGGAAGACATTTG-3', forward: 5'-GCTTCCCTCCTTCTTTGCATACTTGG-3', reverse: 5'-CGCAGATTCCTCTTCCTCTTCCTCTT-3'), *Ikaros* intron 5-6 (probe: 5'-

CAAAGGCATTATTAGTCCTGTCTGCCTCC-3', forward: 5'-AAAGAATGCCCACCTTCCCTTCCT-3', reverse: 5'-TTCGAGCTAGTGTTCTGGCCATTG-3'), *Nfkb1* intron 14-15 (probe: 5'-CTCCAGAAGACAGGTTTCCTTCCTGT-3', forward: 5'-GGGCAATTCTAGGTACAGGAAG-3', reverse: 5'- ACTCAGAGGAGATGCTGACT-3'), *Irf4* promoter (probe: 5'-ACCAGGGCTCTGACAATGGAAA-3', forward: 5'-CTTTGAACGAAACTCTGTTGTTTAC-3', reverse: 5'-GGATGCCATGTCGTTCTTTC-3'), *Irf8* promoter (probe: 5'-TTCAGAGAAGGCGGATTTGGCAGG-3', forward: 5'-AGTGATTTCTCGGAAAGAGAGC-3', reverse: 5'-GCGCGAGCTAATTGAGGA-3'), *Aiolos* intron 6-7 (probe: 5'-TGGCCTTCAACCTGATTCTTCAGTGT-3', forward: 5'-TGGTCATCCTTCCTCCTATCC-3', reverse: 5'-GACTGCTTCCCGCTATACAAG-3').

Library Preparation

ChIP-seq libraries were prepared using the Illumina TruSeq ChIP sample kit according to the manufacturer's instructions with the following deviations. Adaptors were ligated to the sample DNA and libraries were PCR amplified prior to size-selection.

Retroviral transduction

pMIGR retroviral plasmids containing either nothing, *Ikaros*, or *Stat5b-CA* cDNAs were transfected into 293 T cells along with helper viruses using Effectene (Qiagen). Supernatants were harvested 24-48 hours later and used to transduce Ba/F3 pro-B cells (ATCC, Manassas, VA) in the presence of 20% WeHi 3B cell supernatant as previously described[306].

Luciferase Constructs

Forward (5'-ATTAGAGCTCTGGTGACCGGGATAGTT-3') and reverse (5'-ATTACTCGAGAAAGAGGAACTTTATAGAGCCG-3') primers were used to amplify a 1372 bp amplicon of the *Irf4* promoter. The resulting product was then digested with SacI and XhoI (New England Biolabs, Ipswich, MA) and ligated to pGL4.10 (Promega, Madison, WI). Forward (5'-ATTAGCTAGCCTGTCTGGGAGCTGACTATCT-3') and reverse (5'-ATTAAAGCTTGGCTCGAGAGTCGGAGTT-3') primers were used to amplify a 930 bp amplicon of the *Cish* promoter. The resulting product was then digested with NheI and HindIII (New England Biolabs, Ipswich, MA) and ligated to pGL4.10. Two consecutive rounds of site-directed mutagenesis (round 1: 5'-CTCCCGCCCAGTTTTCTTTGAAAGTTCTTTGAAATCTGTCAAAGG-3' and 5'-CCTTTGACAGATTTCAAAGAACTTTCAAAGAAAACTGGGCGGGAG-3'; round 2: 5'-CGCGGTTCTATGAAGATGAGGCTTCTTTGAAGGGCTGGGACGCAG-3' and 5'-CTGCGTCCCAGCCCTTCAAAGAAGCCTCATCTTCATAGAACCGCG-3') on the *Cish* pGL4.10 construct were used to generate the *Cish* pGL4.10 with the IKAROS sites mutated.

Luciferase Assay

For *Irf4* luciferase assays, empty pMIGR or *Stat5b-CA* Ba/F3 cells were transfected with Irf4 promoter pGL4.10 or basic (empty) pGL4.10 and pRL-TK (Promega) by electroporation (240 volts, 25 milliseconds). For Cish lucifierase assays, Ba/F3 cells were transfected with a WT or mutated Cish promoter pGL4.10, pRL-TK (Promega), and empty, *Stat5b-CA*, or *STAT5b-CA* + *Ikaros* pMIGR constructs by electroporation.

Relative light units were measured 48 hours post-transfection using Dual-Luciferase Reporter Assay System (Promega).

Reverse phase proteomics

This study used samples collected from the blood and/or bone marrow of ALL patients. Samples were collected for the Leukemia Sample Bank at the University of Texas M.D. Anderson Cancer Center between 1992 and 2007. These samples were collected on institutional review board (IRB)–approved protocol Lab01-473, and consent was obtained in accordance with the Declaration of Helsinki. Samples were analyzed under an IRB-approved laboratory protocol (Lab05-0654). These samples were analyzed using reverse phase protein arrays, as previously described[307].

IKAROS deletion detection

IKAROS deletions in human leukemia samples were detected using the SALSA P335 ALL-IKAROS Multiplex Ligation-dependent Probe Amplification (MLPA) assay (MRC-Holland) according to manufacturer's instructions.

Mouse STAT5 ChIP-seq analysis

Mouse STAT5 and input control ChIP-Seq data were mapped to human genome version hg19 using BWA v0.7.4[308]. Duplicate reads were removed using Picard MarkDuplicates v1.68 (http://picard.sourceforge.net), and reads with mapping quality less than 1 were discarded. MACS v2 was employed for ChIP-seq peak determination[309]. For ChIP-seq distribution plots, identified peaks for each sample are centered by peak summit and logarithm normalized average coverage in units of number of reads per million

sequenced reads was counted within 3kbp relative to the peak center. The heatmap related to Fig. 3f for peak enrichment of different transcription factors with input was generated using python-based script on raw reads and visualized +/- 3kbp centered on each STAT5 peak summit.

Mouse IKAROS ChIP-seq analysis

IKAROS pro-B cell gene expression data and B cell ChIP-seq data (HA-tagged B3 cell line and Primary B cell) were retrieved from Ferreirós-Vidal, I., et al.[187] HA-tagged B3 cell line ChIP-seq data were reanalyzed for consistency with the STAT5 ChIP-seq data presented here. Reads were mapped using BWA (v. 0.7.4)[308]. Reads with a mapQ >1 were used to call peaks with MACS2 using default settings (q=0.05). For the IKAROS data, for each treatment, (HA-tag, IKAROS IP of Primary B cells), MACS2 was run with a p-value cutoff of 0.01, and then the Irreducible Discovery Rate (IDR) procedure[310] was used to compare the two replicates within each treatment (IDR threshold of 0.01). This resulted in two final lists of genes, one for the HA-tagged treatment, one for the IKAROS IP of primary B cells treatment. The high confidence peaks selected from the IDR analysis for the two treatments were compared, and overlapping peaks were merged to create a final set 5,085 of high confidence, non-overlapping peaks.

Human IKAROS, NFkB and STAT5A GM12878 ChIP-seq analysis

Peaks of these ChIP-seq datasets were obtained from the ENCODE consortium (encodeproject.org) and were generated by the Transcription Factor ChIP-seq uniform processing pipeline developed for the ENCODE Integrative Analysis[311].

PAX5, EBF, PU.1, IRF4 ChIP-seq analysis

Published ChIP-seq data for PAX5, EBF, PU.1, and IRF4 in progenitor B cells were retrieved from the GEO database (PU.1, EBF, IRF4: GSE53595; PAX5: GSE38046)[37,312]. Reads were mapped with BWA v.0.7.4[308], and reads with a mapQ<1 were discarded. Peaks were called with a p-value cut-off of 10^{-10} and without a matched control.

Gene annotation of ChIP-seq data

ChIP-seq peaks for all datasets were annotated by selecting all RefSeq genes within +/- 10kb of each peak using bedtools annotate v 2.17.0[313].

Known motif search

We identified 5,908 STAT5 binding sites (peaks) and scanned over-represented transcription factor-binding site motifs from the TRANSFAC database using Pscan-ChIP[314] from those peak regions. Identified motifs were ranked by local enrichment P-value.

Motif scanning analysis

For the motif scanning analysis, STAT5 motifs were centered and 100 bp of DNA sequence 5' and 3' were analyzed for the c-REL motif. Matched motif hits were counted at each nucleotide position and then plotted using a histogram, with breaks set at 200.

Super-enhancer analysis

Enhancers identified in pro-B cells by Whyte *et al.*[234] were re-scored using STAT5 signal

from the present study using ROSE: Rank Order of super-enhancers[234,235]. Tag enrichment for STAT5, PAX5, EBF, PU.1, IRF4, and IKAROS were generated from mapped reads (bams) using ngs.plot.r[315]. Annotated super-enhancers in Figure 3 and Extended Data Figures 3, 4, and 5 were defined by Whyte *et al.*[234] based on binding of Mediator in pro-B cells. Enhancers in the GM12878 cell line were identified by H3K27-acetylation and then scored using STAT5 signal using ROSE as described above.

Venn diagrams

Peak overlaps for Venn diagrams were generated using bedtools[313] to merge the set of all peaks for all datasets being compared; peaks from each dataset were then compared back to the merged dataset using bedtools intersect. We then counted how many merged peaks overlapped with peaks in all of the original datasets, as well as each combination of datasets, in order to generate the counts used to create the Venn diagrams. Venn diagrams were generated in python using the python package matplotlib[316] for 2- and 3-way Venn diagrams or using the Vennerable R package [https://r-forge.r-project.org/projects/vennerable/] for 4-way Venn diagrams.

Statistics

p-values in Kaplan-Meier curves for mouse studies were determined by log-rank Mantle-Cox test. Similar results were obtained with a Gehan-Breslow-Wilcoxon test (p-values ≤ 0.0078 in all cases). A log-rank test for trend of medians was also done for the *STAT5b-CA x Nfkb1*$^{+/-,-/-}$ Kaplan-Meier curves; this test demonstrated a significant decrease in median survival as *Nfkb1* gene dosage decreased (p = 0.0001). The p-values for qRT-PCR, ChIP-qPCR, luciferase assays, or the quantitation of microarray data were

determined by one-way ANOVA with Bonferroni's multiple comparison post-test; *=p≤0.05, **=p≤0.01, ***=p≤0.001, n.s.=not significant; Bars represent mean ± SEM.

For the human studies evaluating the role of pSTAT5 and IKAROS status, human patient samples were first split into two groups based on IKAROS deletion status (either WT or IKAROS deleted). Each of these subgroups was then divided in half again based on pSTAT5 levels. We then merged the two groups with one bad indicator (either deleted IKAROS with low pSTAT5 or high pSTAT5 with WT IKAROS). p-value shown in the figure represents log rank test for trend of medians and indicates that median survival or remission duration decreases as number of bad indicators increases. p-values from log-rank Mantle-Cox test and Gehan-Breslow-Wilcoxon test for survival were also calculated and were 0.0016 and 0.0036, respectively; for remission duration these p-values were 0.0022 and 0.0027, respectively. A similar analysis was carried out in the human studies evaluating the role of pSTAT5 and RELA, except samples were first split into two groups based on the pSTAT5/RELA ratio (low versus high). These two groups were then split into two groups again based on total pSTAT5 levels within each group. We then merged the two groups with one bad indicator (high pSTAT5/RELA with low pSTAT5 or low pSTAT5/RELA with high pSTAT5). P-value shown represents log rank test for trend of medians. P-values for log-rank Mantle-Cox test and Gehan-Breslow-Wilcoxon test were also calculated and were 0.0792 and 0.0636, respectively. All calculations were carried out using Prism software.

Results

Stat5b-CA mice develop spontaneous B-ALL with low penetrance

In order study the role of STAT5 in B-ALL, we utilized transgenic mice that express a constitutively active form of STAT5b throughout B cell development (*Stat5b-CA* mice)[299]. In this model, STAT5b has been rendered constitutively active by replacing histidine 299 and serine 711 with arginine and phenylalanine, respectively[299,317]. Mutation of STAT5b in this manner results in the constitutive phosphorylation of tyrosine 699, thereby mimicking the process by which wild-type (WT) STAT5 is activated[299,317]. The expression of this transgene is restricted to B and T cells by a compound promoter and enhancer cassette consisting of the *Lck* proximal promoter and the Eμ enhancer[299].

Approximately 1-2% of *Stat5b-CA* mice spontaneously develop leukemia[132,299]. To better characterize these leukemias, we preformed flow cytometry on the leukemic cells from *Stat5b-CA* mice. This analysis revealed that *Stat5b-CA* leukemias phenotypically appear to be transformed pre-B cells based on the expression pattern of CD19, IL7Rα, pre-BCR, CD43, and BP-1 (Figure 2.1a). Therefore, the leukemia observed in *Stat5b-CA* mice resembles B-ALL. The leukemic pre-B cells can be found in the blood, spleen, lymph nodes, and bone marrow (Figure 2.1b).

To determine whether these leukemias are clonal, we assessed immunoglobulin heavy chain gene rearrangements by PCR amplification with primers specific for various distal and proximal V_H gene loci (Figure 2.1c). $B220^+CD19^+$ leukemic lymph node cells from one *Stat5b-CA* mouse indicated usage of primarily the V_HQ52 and J_H2 gene segments, while leukemic cells from another mouse were limited to V_HJ558-DJ_H1 rearrangements. Based on these patterns of gene rearrangement, *Stat5b-CA* leukemias

appear to be clonal, suggesting that they arise from a single transformed B cell progenitor.

The low penetrance and clonality of *Stat5b-CA* leukemias suggests that active STAT5 requires secondary mutations to cause disease. Therefore, we preformed microarray analysis to identify potential genes and pathways that are deregulated in these leukemias. As compared to WT pre-B cells, *Stat5b-CA* leukemias exhibit decreased expression of genes involved in pre-BCR signaling, including *Vpreb1, Igll1* (λ5), *Blk, Sykb* (SYK), and *Prkcb* (PKCβ) (Figure 2.1d). This suggested that loss of pre-BCR signaling was an important step in the initiation of STAT5-dependent leukemia, and lead us to hypothesize that STAT5 activation cooperates with defects in pre-BCR signaling components to initiate B-ALL.

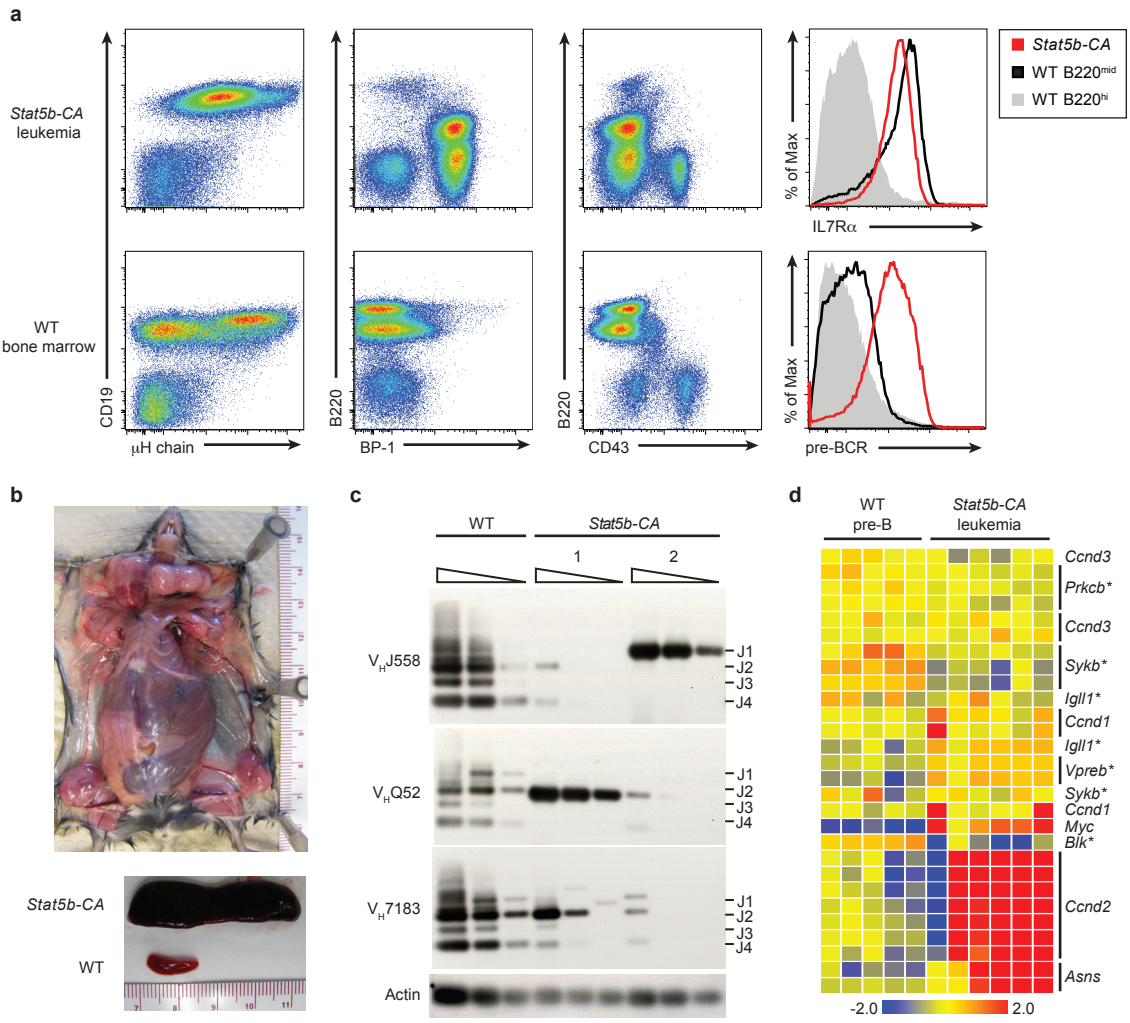


Figure 2.1. Spontaneous leukemia in *Stat5b-CA* mice.

a, *(Left 3 panels)* Flow cytometric analysis of total cells from leukemic *Stat5b-CA* lymph nodes or total lymphocytes from WT C57BL/6 bone marrow (dot-plot). *(Right panels)* Flow cytometric analysis of $B220^+$ lymphocytes from leukemic *Stat5b-CA* lymph nodes or $B220^+$ lymphocytes from WT C57BL/6 bone marrow. **b**, *(Upper panel)* Photo of a *Stat5b-CA* mouse that developed ALL-like leukemia. *(Lower panel)* Spleen from a leukemic *Stat5b-CA* mouse compared to an age-matched WT C57Bl/6 control. **c**, Clonality as determined by *Igh* gene usage. *Stat5b-CA* refers to $B220^+CD19^+$ leukemic lymph node cells from *Stat5b-CA* mice; WT refers to $CD19^+$ splenic B cells from C57Bl/6 mice. Wedges above lanes represent serial dilutions of 100, 10, and 1 ng of genomic DNA. DNA was amplified using primers specific for V_HJ558-DJ_H, V_HQ52-DJ_H, and V_H7183-DJ_H rearrangements and detected by Southern blotting. Numbers on right indicate rearrangements involving different J_H segments. PCR with *Actin*-specific primers was used as a loading control. Results are representative of 4 experiments. **d**,

mRNA expression in *Stat5b-CA* leukemias as determined by microarray analysis. RNA was isolated from $CD19^{+}B220^{+}$ leukemic lymph node cells (n = 6) or sorted pre-B cells from WT C57BL/6 bone marrow ($B220^{+}CD19^{+}IgM^{-}CD43^{-}$; n = 5). Asterisks indicate genes involved in pre-BCR signaling.

Active STAT5 cooperates with defects in pre-BCR signaling components to initiate B-ALL

To test our hypothesis, we bred the *Stat5b-CA* mice to mice that harbor loss-of-function mutations in the pre-BCR adaptor *Blnk,* or the downstream kinases *Btk* (*Xid* mutant) or *Prkcb*. The resulting *Stat5b-CA* x $Blnk^{+/-}$, *Stat5b-CA* x *Xid* and *Stat5b-CA* x $Prkcb^{-/-}$ mice rapidly developed leukemia with high frequency (Figure 2.2a). In contrast, $Blnk^{+/-}$, *Xid* and $Prkcb^{-/-}$ control mice never developed leukemia. Similar to *Stat5b-CA* leukemias, *Stat5b-CA*x$Blnk^{+/-}$, *Stat5b-CA*x*Xid* and *Stat5b-CA*x$Prkcb^{-/-}$ leukemias resemble pre-B cell leukemia based on the expression of B220, IL7Rα and pre-BCR (Figure 2.2a). These leukemias express intermediate levels of μH chain, but this is likely pairing with the surrogate late chain as *Stat5b-CA x* $Blnk^{-/-}$ leukemias do not express Igκ or Igλ (Figure 2.2b,c). Therefore the *Stat5b-CA* x $Blnk^{+/-}$, *Stat5b-CA* x *Xid* and *Stat5b-CA* x $Prkcb^{-/-}$ mice develop highly penetrant forms of leukemia that resemble B-ALL. This confirmed our initial hypothesis that STAT5 activation cooperates with defects in pre-BCR signaling components to initiate leukemia.

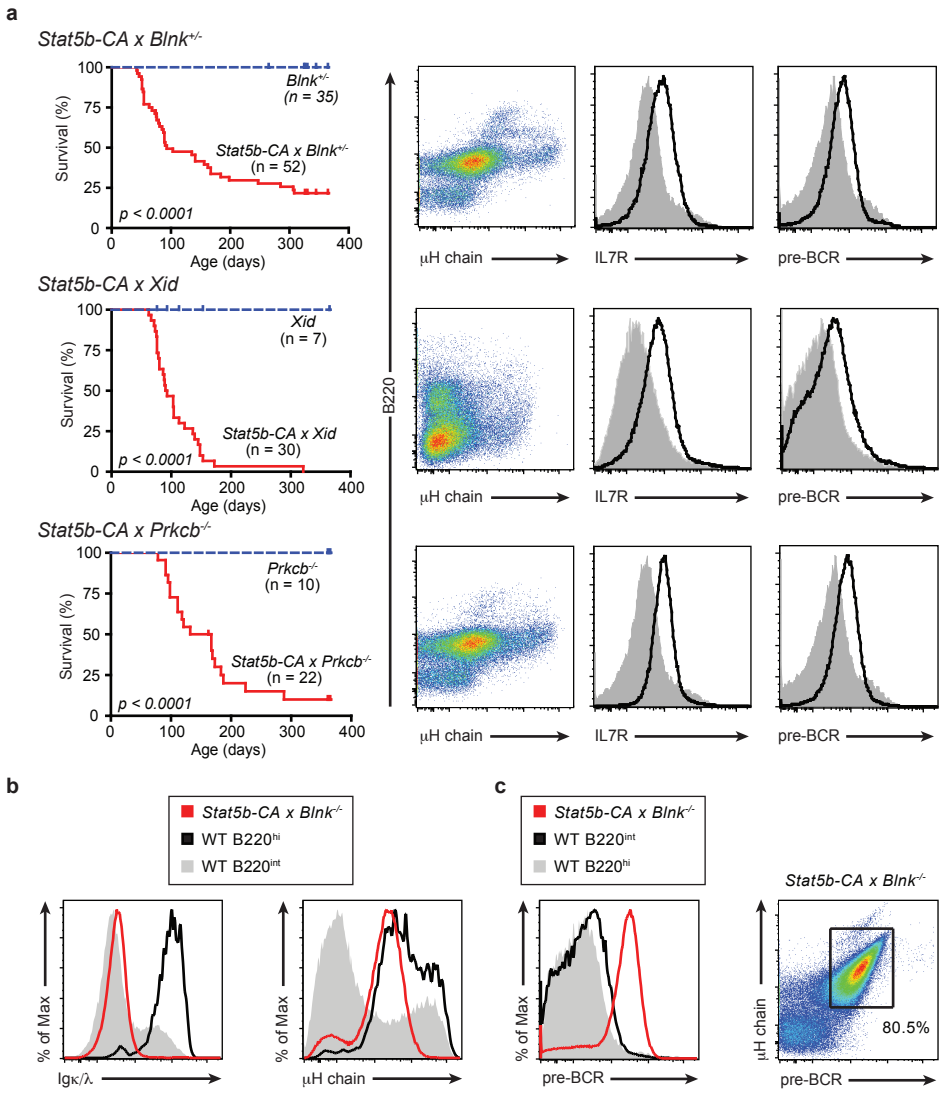


Figure 2.2. Loss of pre-BCR signaling components cooperates with STAT5b-CA to induce pre-B ALL.

a, (*Left panels*) Survival of Stat5b-*CA x Blnk*$^{+/-}$*, Stat5b-CA x Xid,* and *Stat5b-CA x Prkcb*$^{-/-}$ mice compared to littermate controls. Deaths are indicative of leukemia development. (*Right panels*) Flow cytometric analysis of B220, IgM, IL7R, and pre-BCR expression on lymph node cells from *Stat5b-CA x Blnk*$^{+/-}$*, Stat5b-CA x Xid,* and *Stat5b-CA x Prkcb*$^{-/-}$ leukemic mice. Grey histograms represent staining for these markers on mature B220^{+} B cells from lymph nodes of C57Bl/6 mice. **b,** *(Left 3 panels)* Flow cytometric analysis of total cells from leukemic *Stat5b-CA x Blnk*$^{-/-}$ lymph nodes or total lymphocytes from WT C57BL/6 bone marrow. *(Right panel)* Flow cytometric analysis of total cells from leukemic *Stat5b-CA x Blnk*$^{-/-}$ lymph nodes (dot-plot).

STAT5 does not drive B-ALL by solely promoting cell survival or proliferation

We next tested whether STAT5 drives transformation solely by providing survival signals. To do this, we bred *Blnk*$^{+/-}$ mice with transgenic *Bcl2l1* (i.e., *Bcl-X*$_L$) mice[300]. *Bcl2l1* transgenic mice exhibit a comparable expansion of progenitor B cells as seen in *Stat5b-CA* mice[299,300]. Unlike STAT5b-CA, however, BCL-X$_L$ overexpression did not cooperate with *Blnk*$^{+/-}$ to initiate leukemia (Figure 2.3a). We then used BrdU labeling to test if STAT5 could be driving transformation by enhancing cell division. However, these experiments revealed that *Stat5b-CA* x *Xid* leukemias do not proliferate significantly more than WT or *Xid* pre-B cells (Figure 2.3b). Therefore, STAT5 does not induce leukemia by solely promoting survival or enhancing the proliferation of progenitor B cells.

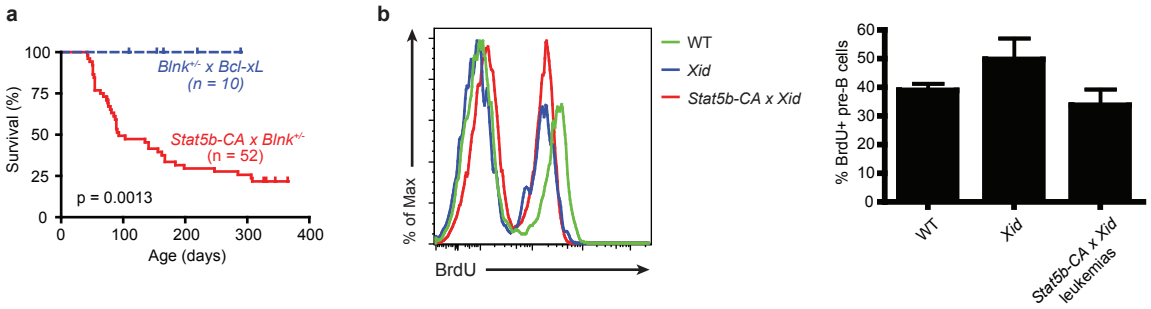


Figure 2.3. STAT5 does not drive leukemia by solely promoting cell survival or -proliferation.

a, Survival of *Blnk*$^{+/-}$ *x Bcl-xL* compared to *Stat5b-CA x Blnk*$^{+/-}$ mice. Deaths are indicative of leukemia development. **b,** BrdU analysis of pre-B cell proliferation. (*Left panel*) Representative histogram of BrdU levels in B220^{mid}CD19^{+}CD25^{+} pre-B cells from *Stat5b-CA x Xid* leukemic mice compared to WT C57Bl/6 and *Xid* non-leukemic controls. (*Right panel*) Percentage of BrdU^{+} pre-B cells in each group of mice. Differences between groups were not significant as determined by one-way ANOVA (p = 0.19).

STAT5 antagonizes NFκB regulation of NFκB target genes

We next sought to find other signaling effectors downstream of the BLNK-BTK-PKCβ pathway that are important in suppressing STAT5-driven leukemia. One protein that is activated by this pathway is the transcription factor NFκB[60,90,142,147,223]. Microarray analysis revealed that the expression of 67 NFκB target genes was altered in *Stat5b-CA* x *Blnk*$^{+/-}$, *Stat5b-CA* x *Xid* and *Stat5b-CA* x *Prkcb*$^{-/-}$ leukemias compared to WT pre-B cells (Figure 2.4a). In addition, the expression of more than 50 of these NFκB target genes was changed in *Stat5b-CA* pre-B cells compared to WT pre-B cells, suggesting that STAT5 regulates dozens of NFκB targets (Figure 2.4b,c). We then used an algorithm developed by Land and colleagues[304] to show that STAT5 activation synergized with decreased pre-BCR signaling to modulate the expression of 25 NFκB target genes, including the oncogenes *Bcl2*, *Ccnd2* and *Myc* (Figure 2.4d). Therefore, STAT5 antagonizes the regulation of several NFκB target genes by a pre-BCR signaling pathway involving BLNK, BTK, and PKCβ.

We next tested whether STAT5 can regulate the expression of NFκB targets directly. To do this, we performed a chromatin immunoprecipitation followed by next generation sequencing (ChIP-Seq) on STAT5 in *Stat5b-CA* x *Blnk*$^{+/-}$ leukemias. This analysis showed that STAT5 bound to ~50% of the NFκB target genes that are deregulated in the *Stat5b-CA* x *Blnk*$^{+/-}$, *Stat5b-CA* x *Xid* and *Stat5b-CA* x *Prkcb*$^{-/-}$ leukemias compared to WT pre-B cells (Figure 2.5a,b). Moreover, the STAT5 binding sites identified by ChIP-Seq showed an enrichment for nearby NFκB binding motifs, suggesting that STAT5 and NFκB share binding sites at numerous loci (Figure 2.5c,d, Table 2.1). We then confirmed that STAT5 binds to the NFκB targets *Irf4* and *Irf8* by

ChIP-qPCR (Figure 2.5e). In addition, qRT-PCR of WT pre-B cell transcripts suggests that IL7 stimulation decreases the expression of *Irf4* (Figure 2.5f). Furthermore, a trend from luciferase assays suggests that STAT5 can repress transcription by binding to the *Irf4* promoter, although this is not statistically significant (Figure 2.5g,h). Together, these data suggest that STAT5 directly regulates several NFκB target genes including the tumor suppressors *Irf4* and *Irf8*[318].

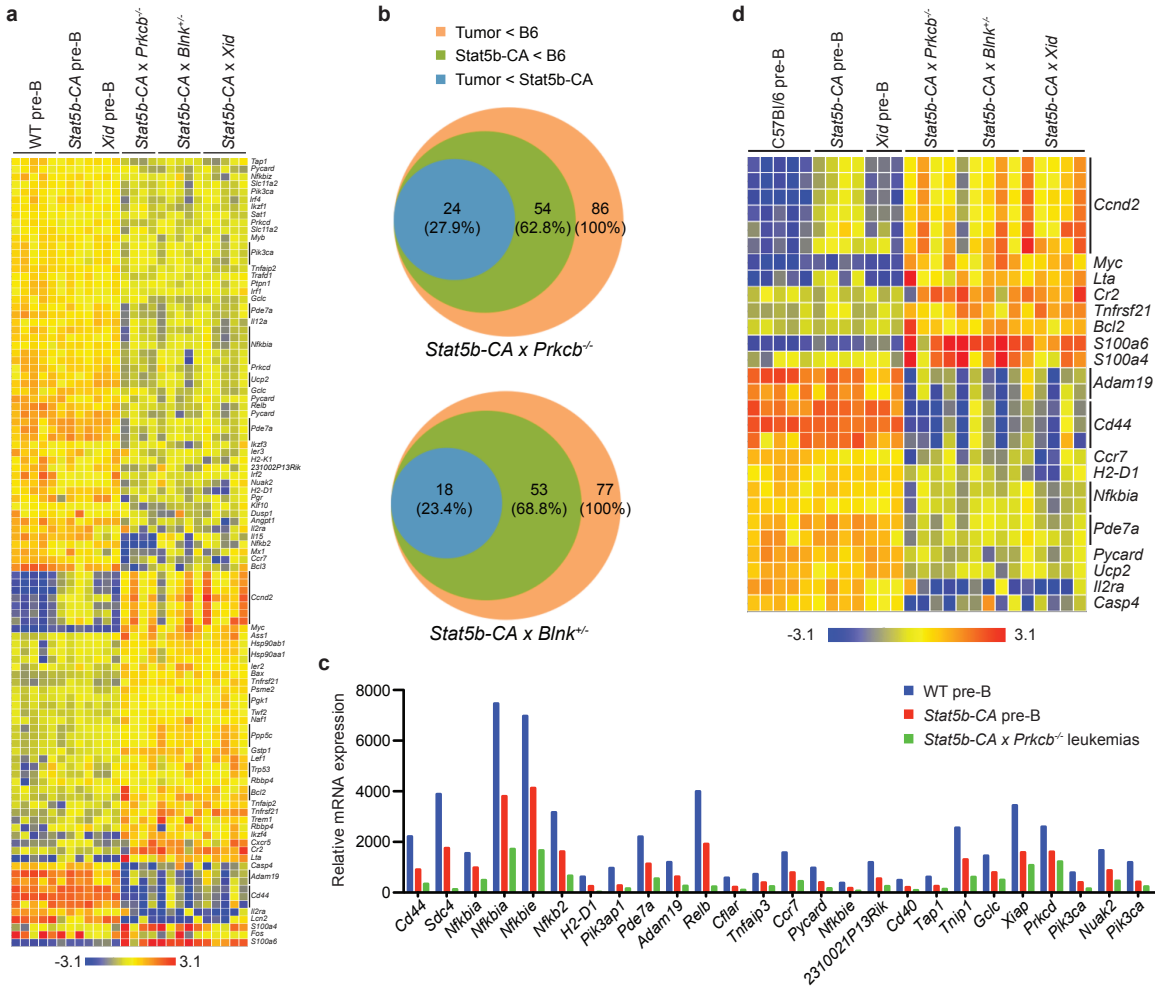


Figure 2.4. STAT5 opposes pre-BCR regulation of NFkB target genes.

a, Microarray analysis of mRNA expression of NFkB targets in pre-B cells or B-ALL-like leukemias. Heatmap represents genes with significantly altered expression compared to WT C57Bl/6 pre-B cells in both RMA- and MAS5.0-transformed sets. **b,** Venn diagrams of NFκB target genes that have reduced expression in *Stat5b-CA x Prkcb*$^{-/-}$ leukemias (top panel) or *Stat5b-CA x Blnk*$^{+/-}$ leukemias (bottom panel) compared to WT C57BL/6 pre-B cells. NFκB target genes that also exhibit reduced expression in *Stat5b-CA* pre-B cells compared to WT pre-B cells or B-ALL-like leukemias compared to *Stat5b-CA* pre-B cells are shown as well. Percentages indicate the fraction each gene subset comprises of all NFκB target genes that are reduced in the B-ALL-like leukemias compared to WT pre-B cells. **c,** mRNA expression by microarray analysis of NFκB target genes with reduced expression in *Stat5b-CA* pre-B cells compared to WT pre-B cells and further reduced expression in *Stat5b-CA x Prkcb*$^{-/-}$ leukemias compared to *Stat5b-CA* pre-B cells. **d,** Synergistic effects of STAT5b-CA and loss of pre-BCR signaling on NFκB target gene expression. Genes identified in panel a were used to calculate synergy scores (see Methods). Heatmap represents mRNA expression of genes with synergy scores < 0.9.

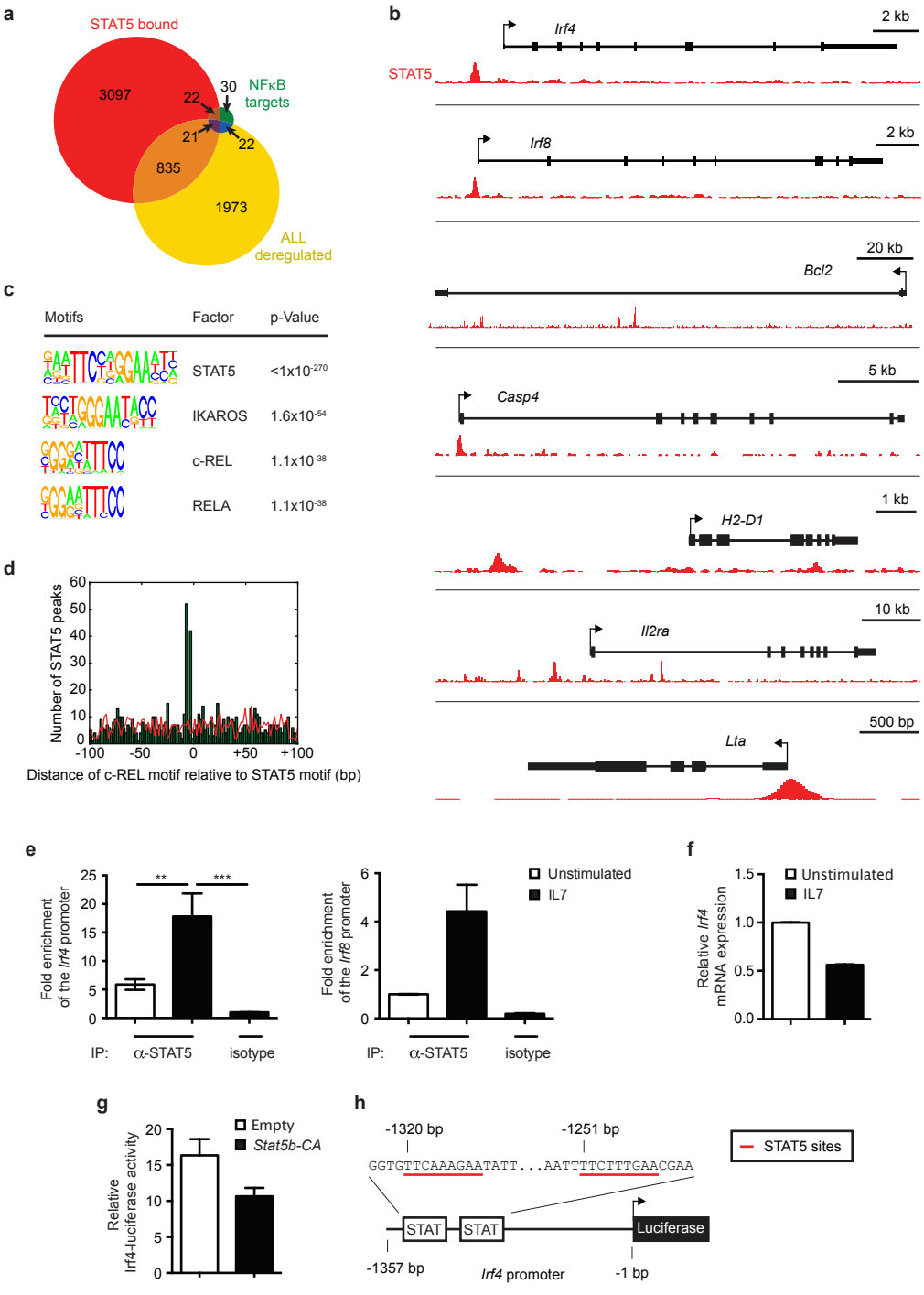


Figure 2.5. STAT5 opposes pre-BCR regulation of NFkB target genes.

a, Venn diagram illustrating overlap of STAT5-bound genes in *Stat5b-CAxBlnk*$^{+/-}$ leukemia with NFκB gene targets and genes deregulated in *Stat5b-CAxBlnk*$^{+/-}$, *Stat5b-*

CAxXid, and *Stat5b-CAxPrkcb*$^{-/-}$ leukemias. **b**, STAT5 occupancy in *Stat5b-CAxBlnk*$^{+/-}$ leukemias at select NFκB target genes by ChIP-seq. **c**, Transcription factor binding motifs enriched by STAT5 ChIP-Seq in *Stat5b-CAxBlnk*$^{+/-}$ leukemias. **d**, Distribution of c-REL binding motifs relative to STAT5 binding sites found by STAT5 ChIP-Seq. **e,** STAT5 ChIP followed by quantitative PCR (ChIP-qPCR) at *Irf4* promoter (left panel) or *Irf8* promoter (right panel) in *Stat5b-CAxBlnk*$^{+/-}$ leukemias. **f**, Quantitative real-time PCR for *Irf4* expression in WT pre-B cells. **g,** Luciferase activity of *Irf4* promoter in Ba/F3 pro-B cells transfected with *empty* or *Stat5b-CA* pMIGR retroviruses. Relative luciferase activity is calculated as the relative firefly/renilla activity of the Irf4 luciferase construct divided by the relative firefly/renilla activity of an empty luciferase construct with no promoter. Paired t-test p-value = 0.23. **h,** Illustration of the WT *Irf4* luciferase construct. STAT5 binding sites are underlined in red. Base pair (bp) positions indicate distances relative to the *Irf4* transcriptional start codon. Results (e left panel, g) represent three independent experiments; results (e right panel) represent two independent experiments; results (f) represent duplicates from one experiment.

STAT5 represses the expression of Nfkb

In addition to regulating NFκB target genes, we investigated whether STAT5 can directly regulate *Nfkb*. ChIP-seq analysis revealed that STAT5 bound to *Nfkb1* and *Nfkb2* (Figure 2.6a,b). Moreover, WT pre-B cells express less *Nfkb1* following IL7 stimulation, and *Stat5b-CA* pre-B cells express less *Nfkb2* compared to WT pre-B cells following stimulation with IL7 (Figure 2.6c). Therefore STAT5 binds to and represses the expression of *Nfkb2*. STAT5 also binds to and likely negatively regulates *Nfkb1* expression in a similar fashion.

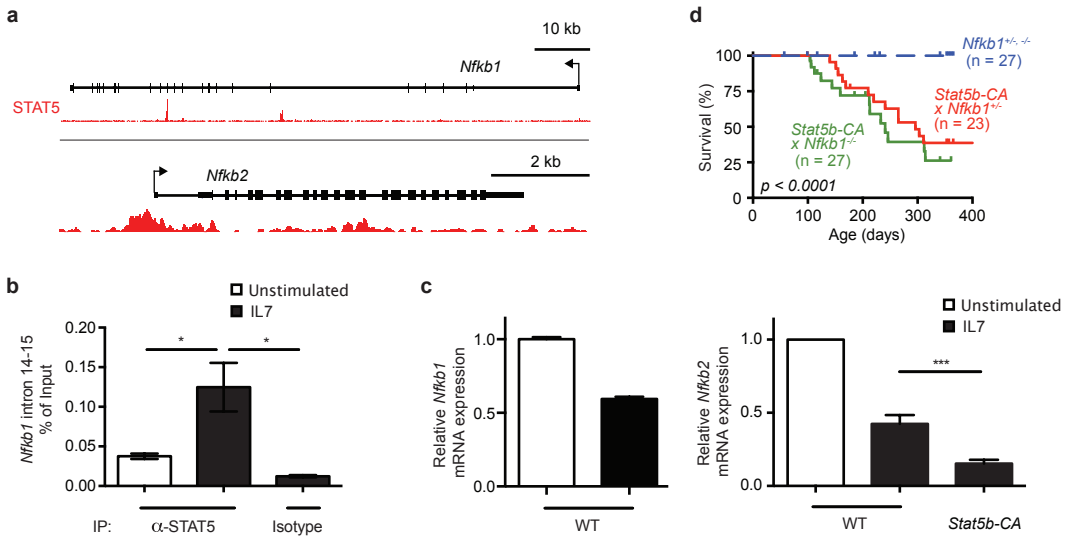


Figure 2.6. STAT5 binds to and represses the expression of the tumor suppressor *Nfkb*.

a, STAT5 occupancy in *Stat5b-CAxBlnk*$^{+/-}$ leukemias at *Nfkb1 and Nfkb2* by ChIP-seq. **b,** STAT5 ChIP followed by quantitative PCR (ChIP-qPCR) at *Nfkb1* intron 14-15 in *Stat5b-CAxBlnk*$^{+/-}$ leukemias. **c**, Quantitative real-time PCR for *Nfkb1* and *Nfkb2* expression in WT or *Stat5b-CA* pre-B cells. **d**, Survival of *Stat5b-CAxNfkb1*$^{+/-}$ and *Stat5b-CAxNfkb1*$^{-/-}$ mice. Results (b and c right panel) represent three independent experiments; results (c left panel) represent duplicates from one experiment.

Active STAT5 cooperates with loss of Nfkb1 to initiate B-ALL

We next tested if the interaction between STAT5 and NFκB is important in leukemogenesis by breeding *Stat5b-CA* mice with *Nfkb1*$^{-/-}$ mice. The resulting crosses, *Stat5b-CA* x *Nfkb1*$^{+/-}$ and *Stat5b-CA* x *Nfkb1*$^{-/-}$ mice, developed leukemia at a high frequency (Figure 2.6d). However, *Nfkb1*$^{+/-}$ and *Nfkb1*$^{-/-}$ control mice did not develop ALL. Thus, NFκB1 functions as a tumor suppressor in pre-B cells to oppose STAT5-dependent pre-B cell leukemia. Conversely, STAT5 antagonizes the transcription of *Nfkb2* and multiple NFκB target genes.

STAT5 and IKAROS opposingly regulate hundreds of shared target genes

NFκB promotes the expression of the transcription factor *Ikaros* by upregulating IRF4 and IRF8[96,147,148]. Our ChIP-seq analysis revealed that IKAROS binding motifs were enriched near STAT5 binding sites in the *Stat5b-CA x Blnk*$^{+/-}$ leukemias (Figure 2.5c). Furthermore, comparison of our STAT5 ChIP-Seq dataset with an IKAROS ChIP-Seq dataset in pre-B cells[187] showed that 37% of genes bound by IKAROS were also bound by STAT5. Moreover, 53% of these genes (896 in total) show direct binding overlap between IKAROS and STAT5 (Figures 2.7a,b, 2.8). This included 249 genes that are bound and regulated by IKAROS in pre-B cells and deregulated in the *Stat5b-CA* x *Blnk*$^{+/-}$, *Stat5b-CA* x *Xid* and *Stat5b-CA* x *Prkcb*$^{-/-}$ leukemias (Figure 2.7b). Of those 249 genes, 180 (72%) show reciprocal regulation by STAT5 and IKAROS. This suggests that STAT5 and IKAROS share hundreds of direct target genes, and that these transcription factors tend to oppose the regulation of one another. Moreover, because STAT5 and IKAROS binding appears to directly overlap at numerous loci by ChIP-seq, this therefore suggests that these transcription factors can potentially compete for binding to target genes.

In order to test this possibility, we cloned the promoter of a shared target gene, *Cish*, into a luciferase reporter construct. Importantly, the STAT5 and IKAROS binding sites overlap at a sequence level in this promoter[123,127] (Figure 2.9a,c). The ectopic expression of STAT5b-CA induced luciferase activity in a pro-B cell line, while co-expression of STAT5b-CA and IKAROS reduced this effect. Conversely, transfection with a *Cish* lucifierase reporter with mutated IKAROS binding sites alleviated the repression by IKAROS (Figure 2.9b,c). Therefore, this suggests that STAT5 and IKAROS can antagonize each other by competing for the binding to target genes.

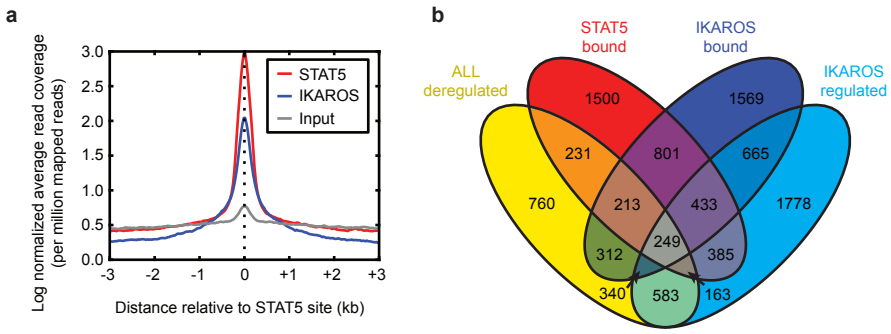


Figure 2.7. STAT5 and IKAROS bind and regulate hundreds of shared target genes.

a, Distribution of STAT5 and IKAROS peak summits relative to STAT5 binding sites by ChIP-Seq. **b**, Venn diagram of genes deregulated in *Stat5b-CAxBlnk*$^{+/-}$, *Stat5b-CAxXid*, and *Stat5b-CAxPrkcb*$^{-/-}$ leukemias compared to WT pre-B cells; genes bound by STAT5 in *Stat5b-CAxBlnk*$^{+/-}$ leukemias; genes bound by IKAROS in pre-B cells; genes regulated by IKAROS.

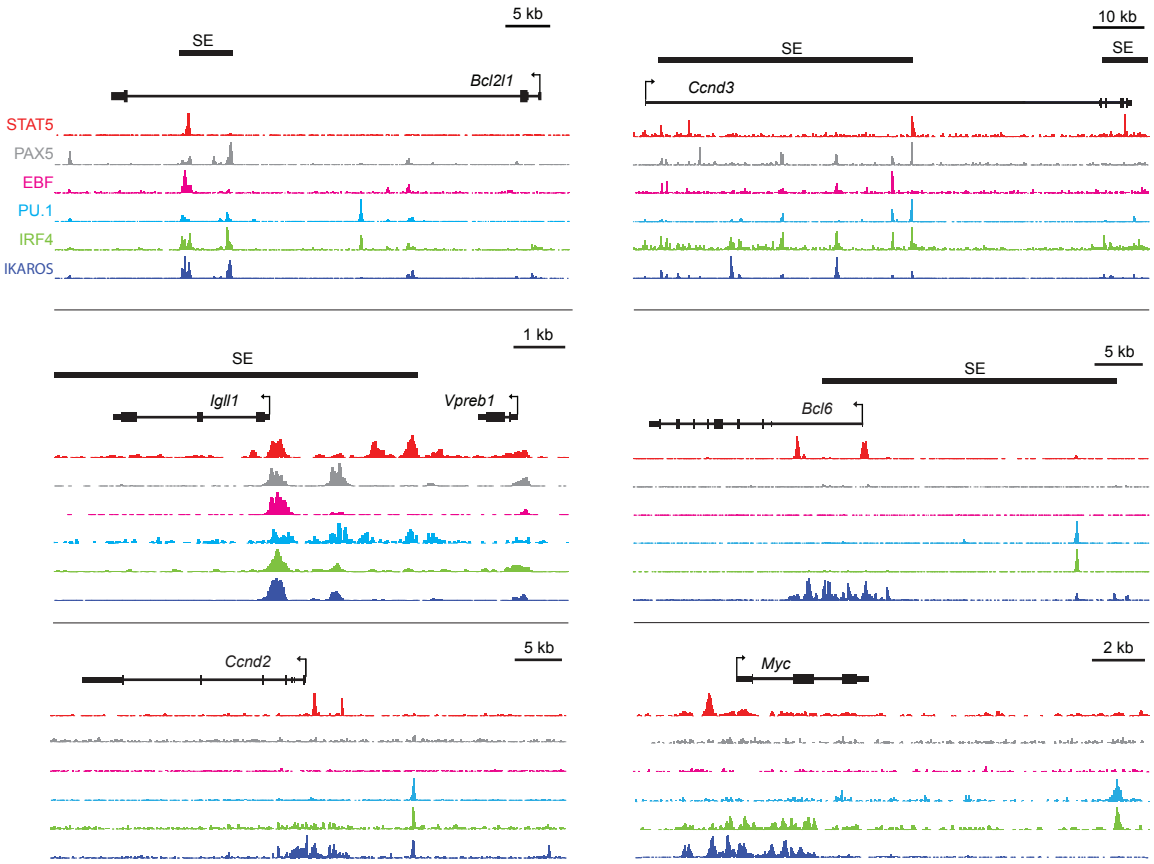


Figure 2.8. STAT5 binding overlaps with the binding PAX5, EBF1, PU.1, IRF4, and IKAROS at genes that promote pre-B cell survival, proliferation, and differentiation.

Occupancy of STAT5, PAX5, EBF, PU.1, IRF4 and IKAROS by ChIP-Seq at *Bcl2l1* (BCL-X_L)*, Igll1* (λ5), *Vpreb1, Ccnd2, Ccnd3, Bcl6, and Myc* loci; SE = super-enhancer.

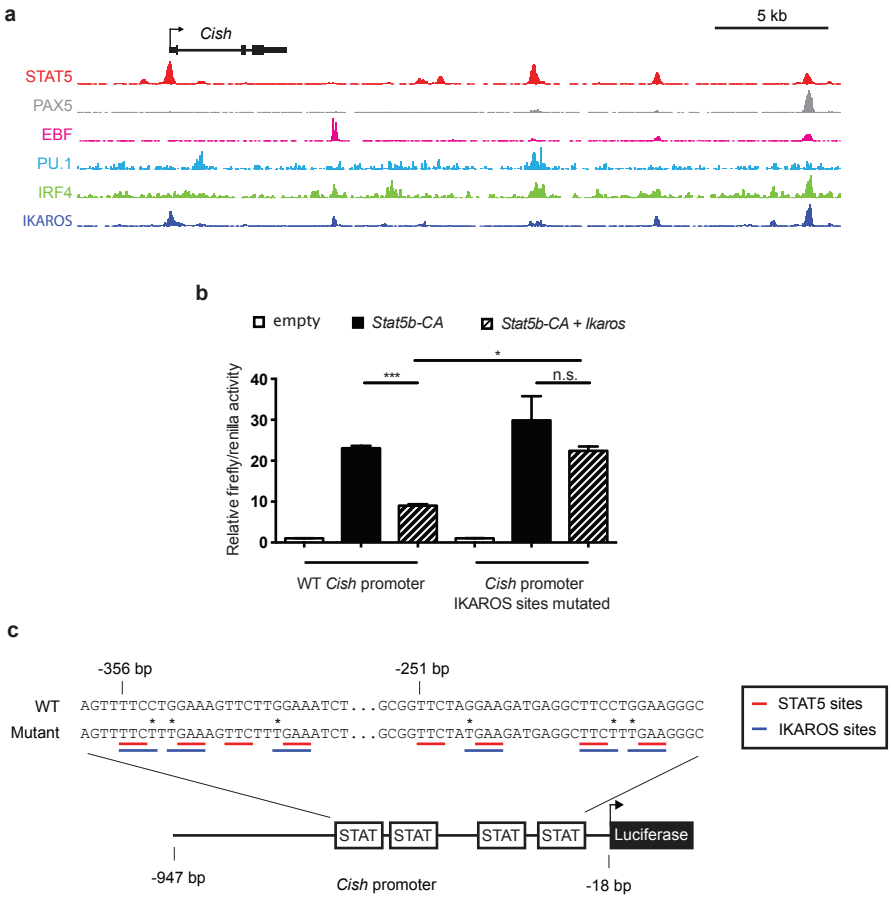


Figure 2.9. STAT5 and IKAROS reciprocally regulate *Cish* by binding to overlapping binding sites in the *Cish* promoter.

a, Occupancy of STAT5, PAX5, EBF, PU.1, IRF4 and IKAROS by ChIP-Seq at the *Cish* locus. **b**, Luciferase activity of WT or mutant *Cish* promoter in Ba/F3 pro-B cells transfected with *empty*, *Stat5b-CA,* or *Stat5b-CA* and *Ikzf1* pMIGR retroviruses. **c,** Illustration of the WT and mutant *Cish* luciferase constructs. STAT5 and IKAROS binding sites are underlined. Mutated base pairs are indicated with asterisks. Base pair (bp) positions indicate distances relative to the *Cish* translational start codon. Results (b) represent three independent experiments.

STAT5 represses Ikaros and Aiolos expression

We next investigated whether STAT5 can repress the expression of *Ikaros* directly. We found that STAT5 binds to a promoter of *Ikaros* (promoter B), which is known to drive the expression of *Ikaros* in the B lineage specifically[319,320] (Figure 2.10a,b). Moreover, qRT-PCR analysis revealed that *Ikaros* transcripts are reduced in *Stat5b-CA* pre-B cells compared to WT pre-B cells (Figure 2.10c). These data therefore suggest that STAT5 can directly bind to and repress *Ikaros* expression. We found that STAT5 similarly binds to and represses the expression of the related *Ikaros* family member, *Aiolos* (Figure 2.10a,d). Therefore, STAT5 opposes IKAROS function by multiple mechanisms. STAT5 opposes target gene regulation by IKAROS, potentially by competing for binding to shared target gene loci and by non-competitive mechanisms involving the binding to non-overlapping binding sites within shared target genes. In addition, STAT5 can also oppose IKAROS function by binding to and repressing the expression of the Ikaros family members *Ikaros* and *Aiolos*.

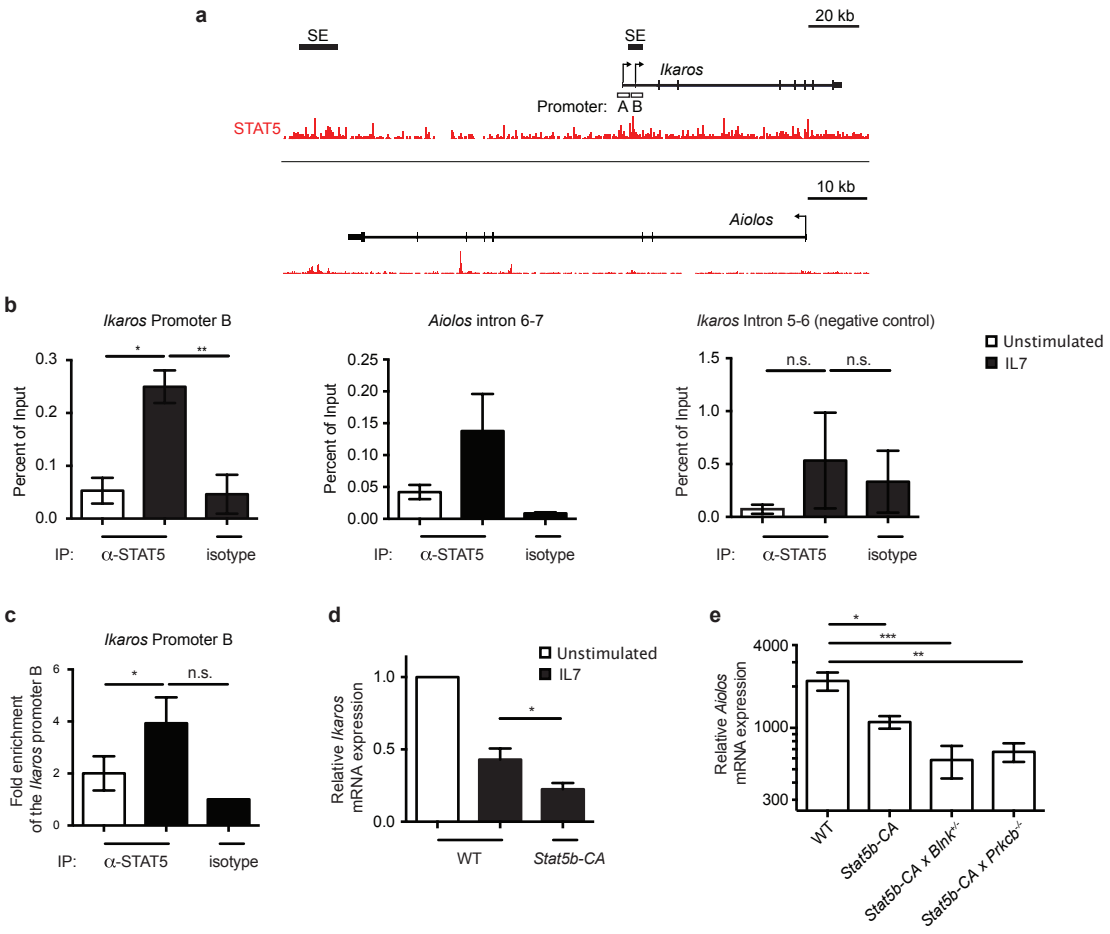


Figure 2.10. STAT5 binds to and represses the expression of *Ikaros* and *Aiolos*.

a, STAT5 occupancy (red) in *Stat5b-CA x Blnk*$^{+/-}$ leukemias at *Ikaros* or *Aiolos* loci by ChIP-seq. The two arrows in *Ikaros* indicate two alternative transcriptional start sites near promoters A and B; SE = super-enhancer. **b,** STAT5 ChIP followed by quantitative PCR (ChIP-qPCR) at *Ikaros* promoter B (left panel), *Aiolos* intron 6-7 (middle panel), or *Ikaros* intron 5-6 (middle panel) in *Stat5b-CAxBlnk*$^{+/-}$ leukemias. **c,** STAT5 ChIP-qPCR at *Ikaros* promoter B in WT C57BL/6 pre-B cells. **d**, Quantitative real-time PCR for *Ikaros* expression in WT or *Stat5b-CA* pre-B cells. **e,** The expression of *Aiolos* transcripts by microarray analysis in WT pre-B cells, *Stat5b-CA* pre-B cells, *Stat5b-CA x Blnk*$^{+/-}$ leukemias, or *Stat5b-CA x Prkcb*$^{-/-}$ leukemias. Results (left and right panels in b, c, d, e) represent at least three independent experiments. Results (middle panel in b) represent two independent experiments.

Active STAT5 cooperates with loss of Ikaros to promote ALL

We next tested whether constitutively active STAT5 can cooperate with loss of function mutations in *Ikaros* to drive B-ALL. However, the resulting *Stat5b-CA x Ikaros*$^{+/-}$ mice developed T cell leukemia rather than pre-B cell leukemia (data not shown). To circumvent this issue, we used a sleeping beauty (SB) transposon mutagenesis screen to identify genes that cooperate with *Stat5b-CA* to initiate B-ALL. The transposon contains two splice acceptors and a bi-directional poly(A) and can terminate transcription when integrated in either orientation in a gene[321]. It also contains a murine stem cell virus (MSCV) promoter that can promote gene expression when integrated upstream or within a gene[321]. The position and orientation of the MSCV promoter relative to the direction of normal gene transcription can be used to predict whether the transposon is likely to drive or disrupt gene transcription[322]. We restricted the expression of the transposon to cells of the B-lineage by using *Mb1-cre*. The resulting leukemias almost entirely originated from the B lineage. *Ikaros* was identified as a common insertion site in this screen, and the orientation and location of the transposon insertions within the *Ikaros* locus were predicted to cause loss of function mutations (L.M.H.H. and M.A.F., manuscript in-preparation). Moreover, *Ikaros* expression was significantly reduced in these *T2/onc x Stop*$^{fl/fl}$*-Rosa26 x SB1/15 x Mb1-cre x Stat5b-CA* leukemias compared to WT controls (L.M.H.H. and M.A.F., manuscript in-preparation). These data suggest that STAT5 activation and loss of function mutations in *Ikaros* cooperate to initiate B-ALL.

STAT5 binding overlaps with the binding of PAX5, EBF1, PU.1, IRF4, and IKAROS at super-enhancers

IKAROS forms a transcriptional network with PAX5, EBF1, PU.1, and IRF4 (referred to as PEPII factors) that regulates B cell differentiation. Therefore, we evaluated whether STAT5 binding also overlapped with the binding of other members of this network. We found enrichment of the binding motifs of all PEPII factors in our STAT5 ChIP-Seq dataset (Table 2.1). Furthermore, comparison of PEPII[37,187,312,323] and STAT5 ChIP-Seq datasets demonstrated a substantial enrichment of PEPII factors at STAT5-bound loci (Figure 2.11a). In contrast, the transcription factor FOXO1, which also supports B cell development, did not overlap significantly with STAT5 binding (Figure 2.11a). Thus, STAT5 binding directly overlaps with the binding of many but not all transcription factors that promote B cell differentiation.

We next evaluated whether STAT5 and the PEPII factors interact with progenitor B cell super-enhancers. We found that 67% of pro-B cell super-enhancers are bound by 4 or more members of the PEPII network (Figure 2.11b). Furthermore, 70% of PEPII-bound super-enhancers are also bound by STAT5 in B-ALL-like leukemia (Figure 2.11b). We next identified enhancers that have the highest levels of STAT5 binding by ranking pro-B cell enhancers based on the amount of STAT5 bound at each enhancer. We found that high levels of STAT5 were enriched at super-enhancers over typical enhancers (Figure 2.11c), suggesting that STAT5 binding levels correlate with super-enhancer activity in pro-B cells. Moreover, PEPII factors and high levels of STAT5 binding were enriched at super-enhancers linked to genes regulating survival, cell cycle, and B cell differentiation (Figures 2.8, 2.11c). Consistent with other super-enhancer networks, STAT5 and PEPII factors cross-regulated each other (Figure 2.12). These

data demonstrate that STAT5 participates in a regulatory B cell transcriptional network at B cell super-enhancers, and suggest that STAT5 regulation is a defining feature of B-ALL.

Transfac database			Jaspar database		
Motif	Motif ID	p-Value	Motif	Motif ID	p-Value
STAT5A	M00457	$< 5.6 \times 10^{-270}$	STAT1	MA0137.2	$< 5.9 \times 10^{-269}$
STAT5B	M00459	$< 5.6 \times 10^{-270}$	STAT3	MA0144.1	5.9×10^{-269}
STAT	M00223	$< 5.6 \times 10^{-270}$	PU.1	MA0080.2	3.3×10^{-57}
STAT1	M00224	5.6×10^{-270}	FEV	MA0156.1	4.1×10^{-56}
STAT3	M00225	6.2×10^{-267}	GABPA	MA0062.2	7.2×10^{-54}
STAT5A	M00460	2.0×10^{-255}	ETS1	MA0098.1	6.9×10^{-46}
IKAROS	M00088	1.6×10^{-54}	ELF5	MA0136.1	1.2×10^{-42}
STAT6	M00500	3.0×10^{-47}	C-REL	MA0101.1	7.5×10^{-41}
cETS p54	M00032	2.6×10^{-46}	p65 / RELA	MA0107.1	1.5×10^{-35}
STAT3	M00497	8.2×10^{-44}	ELK4	MA0076.1	1.3×10^{-30}
ELK1	M00025	3.7×10^{-42}	EBF	MA0154.1	3.0×10^{-22}
C-REL	M00053	1.1×10^{-38}	NFKB	MA0061.1	1.5×10^{-21}
NRF2	M00108	1.6×10^{-36}	NFATC2	MA0152.1	4.9×10^{-21}
cETS p54	M00074	9.6×10^{-36}	SPIB	MA0081.1	6.9×10^{-21}
STAT1	M00496	6.4×10^{-35}	NFKB1	MA0105.1	5.8×10^{-18}
p65 / RELA	M00052	1.1×10^{-33}	ELK1	MA0028.1	2.9×10^{-17}
STAT4	M00498	1.2×10^{-33}	ZNF143	MA0088.1	1.3×10^{-12}
STAT5A	M00499	7.4×10^{-31}	IRF1	MA0050.1	6.4×10^{-10}
EBF	M00261	3.8×10^{-29}	NR3C1	MA0113.1	8.7×10^{-10}
IKAROS	M00141	1.8×10^{-25}	p53	MA0106.1	5.5×10^{-9}
NFAT	M00302	6.9×10^{-25}	RUNX1	MA0002.2	7.6×10^{-9}
ELK1	M00007	2.0×10^{-21}	NHLH1	MA0048.1	2.1×10^{-8}
NFKB	M00054	4.5×10^{-21}	IRF2	MA0051.1	1.5×10^{-7}
HSF1	M00146	9.2×10^{-18}	AR	MA0007.1	1.3×10^{-6}

Table 2.1. Transcription factor binding motifs enriched by STAT5 ChIP-seq.

A list of transcription factor binding motifs enriched following a STAT5 ChIP-seq in *Stat5b-CA x Blnk*$^{+/-}$ leukemias. Motifs were identified using the *Transfac* (left column) or *Jaspar* (right column) databases.

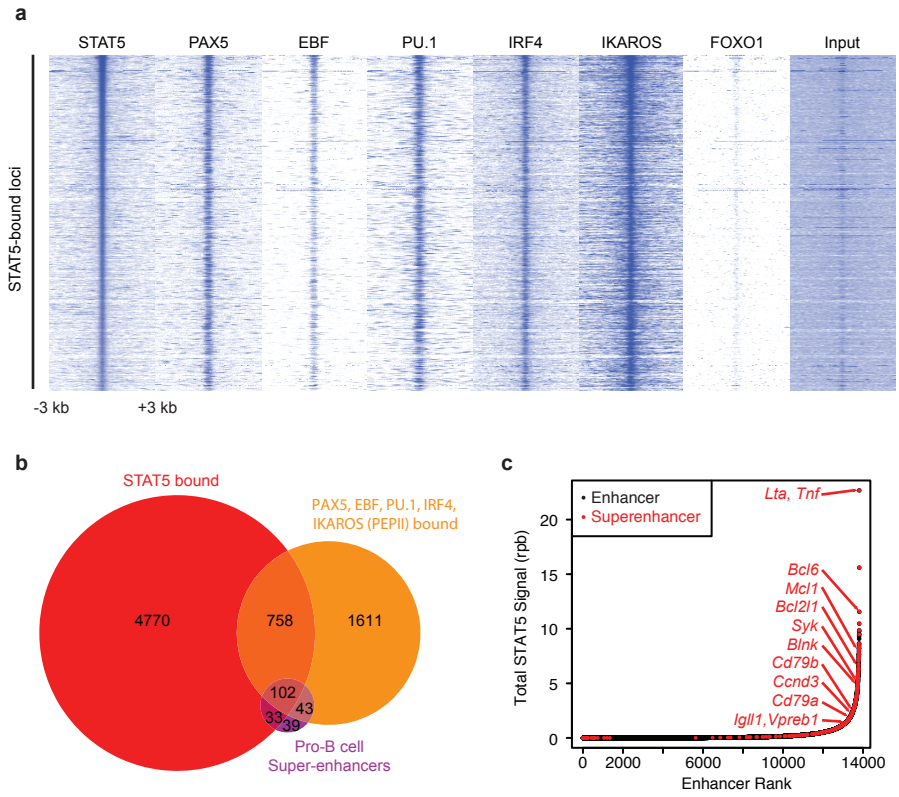


Figure 2.11. STAT5 binding overlaps with the binding of PAX5, EBF1, PU.1, IRF4, and IKAROS at pro-B cell super-enhancers.

a, Heat map of STAT5, PAX5, EBF, PU.1, IRF4, IKAROS and FOXO1 occupancy centered on STAT5 binding sites at STAT5-bound loci by ChIP-seq. Input is a negative control for the STAT5 ChIP-seq. **b**, Venn-diagram illustrating the peak-level overlap of STAT5, 4 or more of PAX5, EBF, PU.1, IRF4, and IKAROS (PEPII), and pro-B cell super-enhancers. **c**, STAT5 ChIP-seq signal in reads-per-billion at pro-B cell enhancers and super-enhancers.

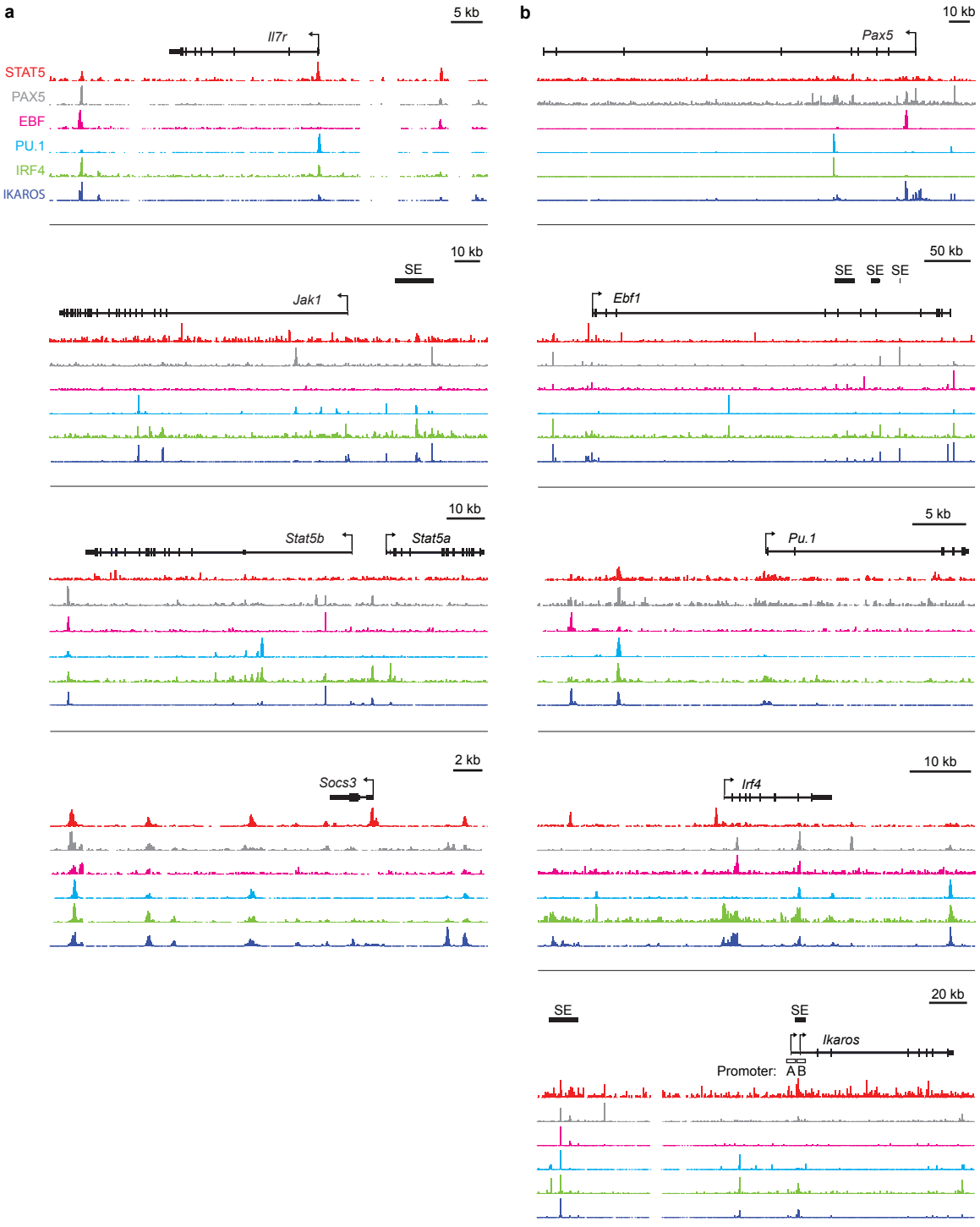


Figure 2.12. Coordinated binding of STAT5, PAX5, EBF, PU.1, IRF4, and IKAROS at genes that govern pre-B cell transcriptional networks.

a, Occupancy of STAT5, PAX5, EBF, PU.1, IRF4 and IKAROS by ChIP-Seq at *Il7r, Jak1, Stat5b, Stat5a* and *Socs3* loci. **b**, Occupancy of STAT5, PAX5, EBF, PU.1, IRF4 and IKAROS by ChIP-Seq at *Pax5, Ebf1, Sfpi, Irf4* and *Ikzf1* loci. SE = super-enhancers.

STAT5, IKAROS, and NFκB binding overlaps in human B cell leukemia

We then determined if the results we observed in murine leukemias are recapitulated in human leukemia. To test this, we evaluated the binding of STAT5, IKAROS, and the NFκB subunit RELA (p65) in a B lymphoblastoid cell line. This analysis revealed that 41% and 33% of STAT5 binding sites overlap with IKAROS or RELA binding sites, respectively (Figure 2.13a,b). In addition, we found that high levels of STAT5 binding tend to occur in B cell super-enhancers rather than typical enhancers. Moreover, we found that super-enhancers with high levels of STAT5 were enriched for associated genes that control survival, proliferation, and B cell differentiation (Figure 2.13c). We further confirmed our results in human B-ALL, as we found an enrichment of STAT5 binding motifs near IKAROS binding sites that were identified by ChIP-Seq in two primary BCR-ABL+ B-ALL samples (Figure 2.13d). Thus, STAT5 binding overlaps with NFκB and IKAROS in transformed human B cells. Moreover, STAT5 appears to regulate a similar network of super-enhancers in mouse and human B cell malignancies.

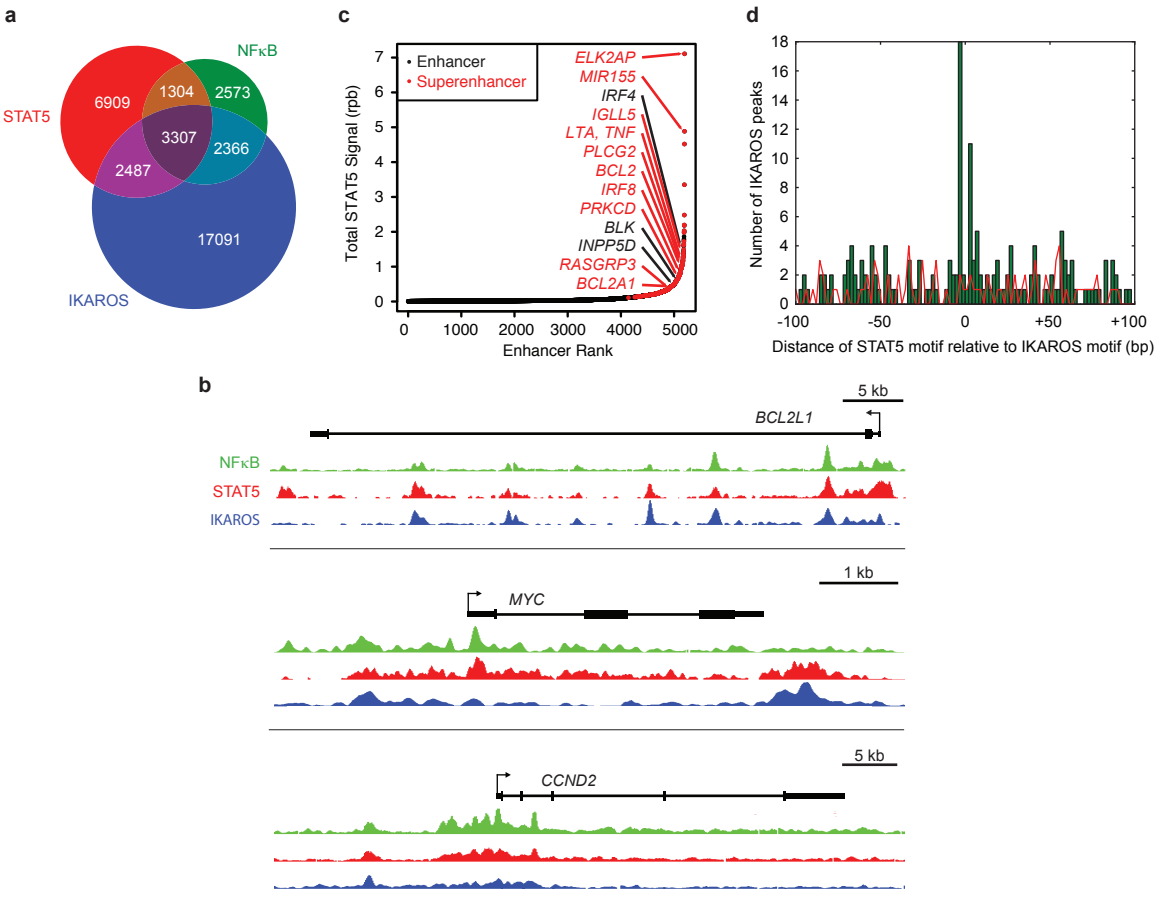


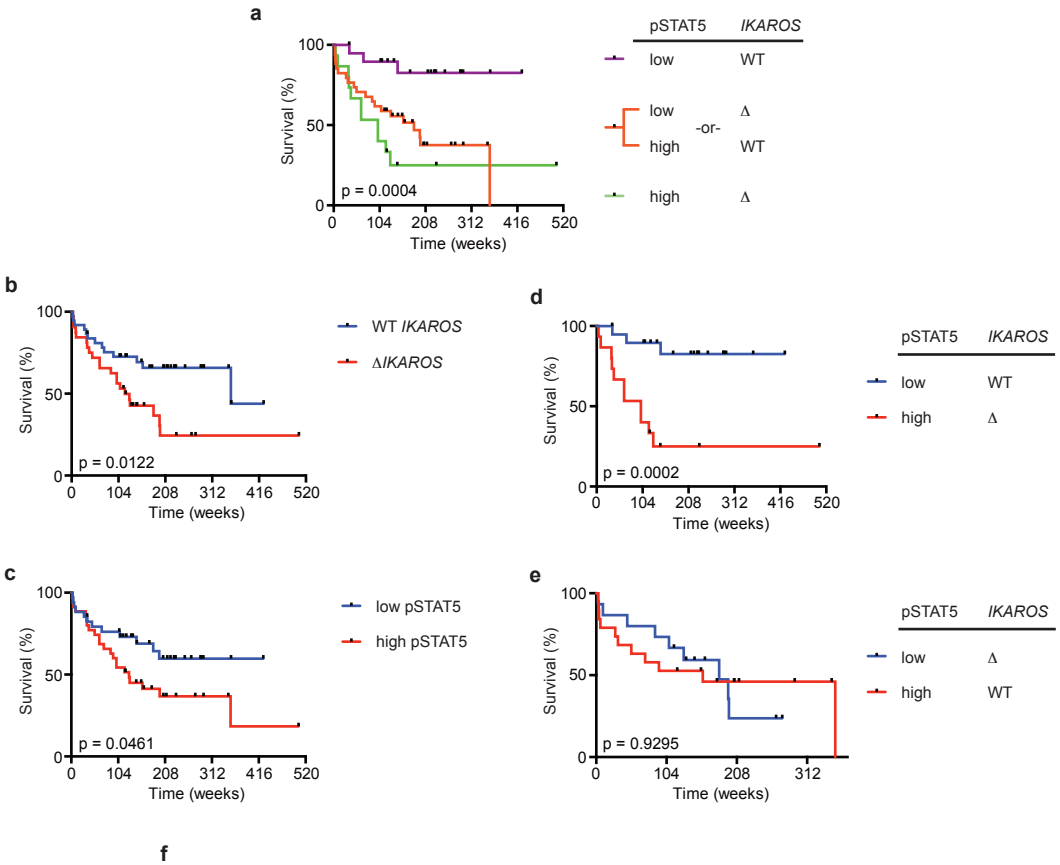
Figure 2.13. STAT5 binding overlaps with NFκB and IKAROS binding and with super-enhancers in human B cells.

a, Venn diagram showing overlap between STAT5, IKAROS and NFκB binding sites by ChIP-seq in the GM12878 human B lymphoblastoid cell line. **b**, Occupancy of STAT5, IKAROS and NFκB at *BCL2L1, CCND2* and *MYC* in GM12878 cells. **c,** STAT5 ChIP-Seq signal in reads-per-billion at pro-B cell enhancers and super-enhancers in GM12878 cells. **d,** Distribution of STAT5 binding motifs relative to IKAROS binding sites found by IKAROS ChIP-Seq in ICN1 and LAX2 primary B-ALL samples.

The ratio of STAT5 and IKAROS or NFκB correlates with B-ALL patient outcomes

We next examined whether the interaction between STAT5 and IKAROS is clinically significant in B-ALL. Based on our current findings, we hypothesized that the ratio of active STAT5 (pSTAT5) to *IKAROS* will inversely correlate with remission duration and patient survival. To test this, we subdivided B-ALL samples from a cohort of 69 patients into three groups: (1) low pSTAT5 and WT *IKAROS* (two good indicators), (2) low pSTAT5 and deleted *IKAROS* (Δ*IKAROS)* or high pSTAT5 and WT *IKAROS* (one bad indicator) and (3) high pSTAT5 and deleted *IKAROS* (2 bad indicators). As predicted, we observed a significant decrease in remission duration and patient survival as the number of bad prognostic indicators increased (Figures 2.14a, 2.15a). In contrast, *IKAROS* or pSTAT5 status alone was less effective at predicting survival or remission duration (Figures 2.14b-f, 2.15b-f). Similarly, we examined if the relationship of pSTAT5 and RELA correlated with survival in 161 B-ALL patients. Patient samples were split into three groups based on total pSTAT5 levels and the ratio between pSTAT5 and RELA: (1) low total pSTAT5 and low pSTAT5/RELA (two good indicators), (2) low total pSTAT5 and high pSTAT5/RELA or high overall pSTAT5 and low pSTAT5/RELA (one bad indicator) and (3) high overall pSTAT5 and high pSTAT5/RELA (two bad indicators). The combination of high pSTAT5 with a high ratio of pSTAT5 to RELA correlated most strongly with decreased survival (Figure 2.16a). In contrast, pSTAT5 levels or the ratio of pSTAT5 to RELA alone did not effectively correlate with overall patient survival (Figure 2.16b-f). Thus, the combination of pSTAT5 levels and *IKAROS* deletion status or pSTAT5 levels and the ratio of pSTAT5 to RELA strongly correlated with patient outcomes.

Our patient cohorts in the aforementioned studies included BCR-ABL+ ALL cases. Moreover, the BCR-ABL+ cases were disproportionally enriched in the groups with two bad prognostic indicators (high pSTAT5 and Δ*IKAROS* or high pSTAT5/RELA). Therefore, the worse outcomes associated with these groups could be due to an increased prevalence of BCR-ABL, which has already been shown to be associated with poor outcomes. However, we obtained similar stratifications after removing the BCR-ABL cases from our analyses (Figures 2.17-2.19). Therefore, pSTAT5 levels and *IKAROS* deletion status or pSTAT5 levels and the ratio of pSTAT5 to RELA correlate with patient survival and remission duration regardless of BCR-ABL status.



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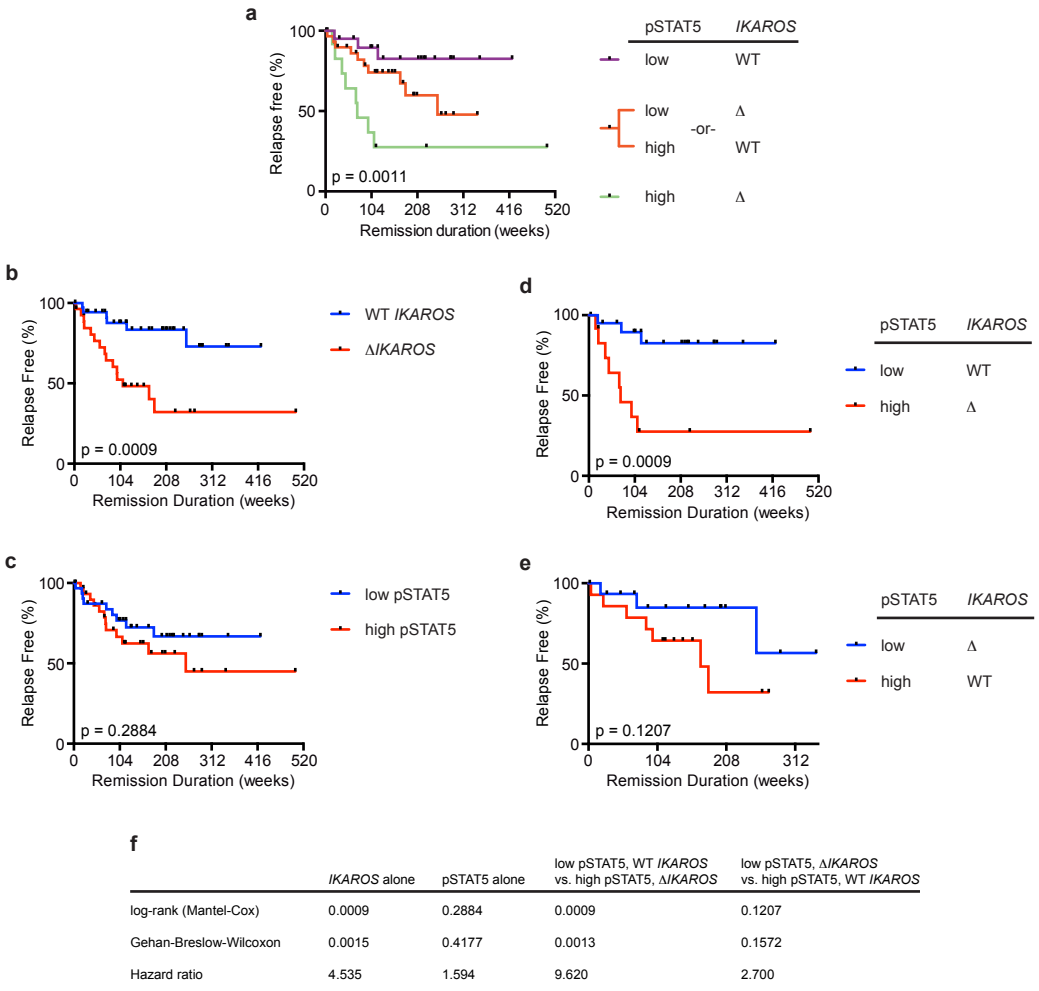
	IKAROS alone	pSTAT5 alone	low pSTAT5, WT *IKAROS* vs. high pSTAT5, Δ*IKAROS*	low pSTAT5, Δ*IKAROS* vs. high pSTAT5, WT *IKAROS*
log-rank (Mantel-Cox)	0.0122	0.0461	0.0002	0.9295
Gehan-Breslow-Wilcoxon	0.0378	0.0951	0.0003	0.5894
Hazard ratio	2.419	1.988	8.369	N.D.

Figure 2.14. STAT5 activation paired with deletion of *IKAROS* negatively correlates with the survival of B-ALL patients.

a, Survival of B-ALL patients stratified by pSTAT5 and *IKAROS* status: WT or deleted (Δ). p-values represent log-rank test for trend of medians. **b**, Survival of B-ALL patients that were stratified based on *IKAROS* status alone: WT or deleted. **c**, Survival of B-ALL patients that were separated into two equal-sized groups based on low or high pSTAT5 levels. **d**, **e**, Survival of B-ALL patients that were stratified by separating them based on *IKAROS* status (WT or deleted) and then further subdividing those groups based pSTAT5 levels (low or high). p-values in panels b-e represent log-rank (Mantel-Cox) test. **f**, Statistical summary of the results shown in panels b-e. ND = not done.



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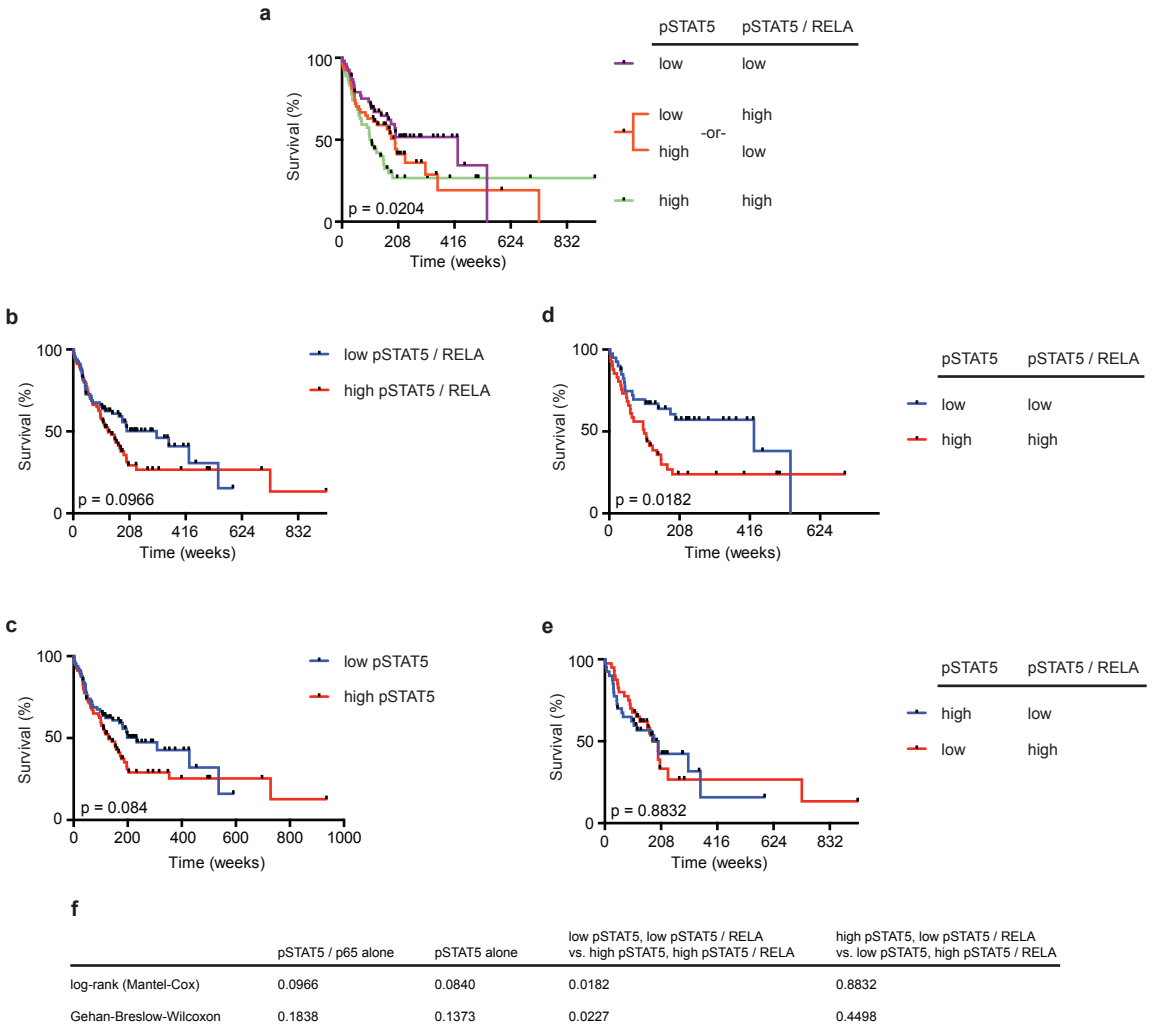
	IKAROS alone	pSTAT5 alone	low pSTAT5, WT *IKAROS* vs. high pSTAT5, Δ*IKAROS*	low pSTAT5, Δ*IKAROS* vs. high pSTAT5, WT *IKAROS*
log-rank (Mantel-Cox)	0.0009	0.2884	0.0009	0.1207
Gehan-Breslow-Wilcoxon	0.0015	0.4177	0.0013	0.1572
Hazard ratio	4.535	1.594	9.620	2.700

Figure 2.15. STAT5 activation paired with deletion of *IKAROS* negatively correlates with remission duration in B-ALL patients.

a, Remission duration in B-ALL patients stratified by pSTAT5 and *IKAROS* status: WT or deleted (Δ). p-values represent log-rank test for trend of medians. **b**, Remission duration in B-ALL patients that were stratified based on *IKAROS* status alone: WT or deleted. **c**, Remission duration in B-ALL patients that were separated into two equal-sized groups based on low or high pSTAT5 levels. **d**, **e**, Remission duration in B-ALL patients that were stratified by separating them based on *IKAROS* status (WT or deleted) and then further subdividing those groups based pSTAT5 levels (low or high). p-values in panels b-e represent log-rank (Mantel-Cox) test. **f**, Statistical summary of the results shown in panels b-e.



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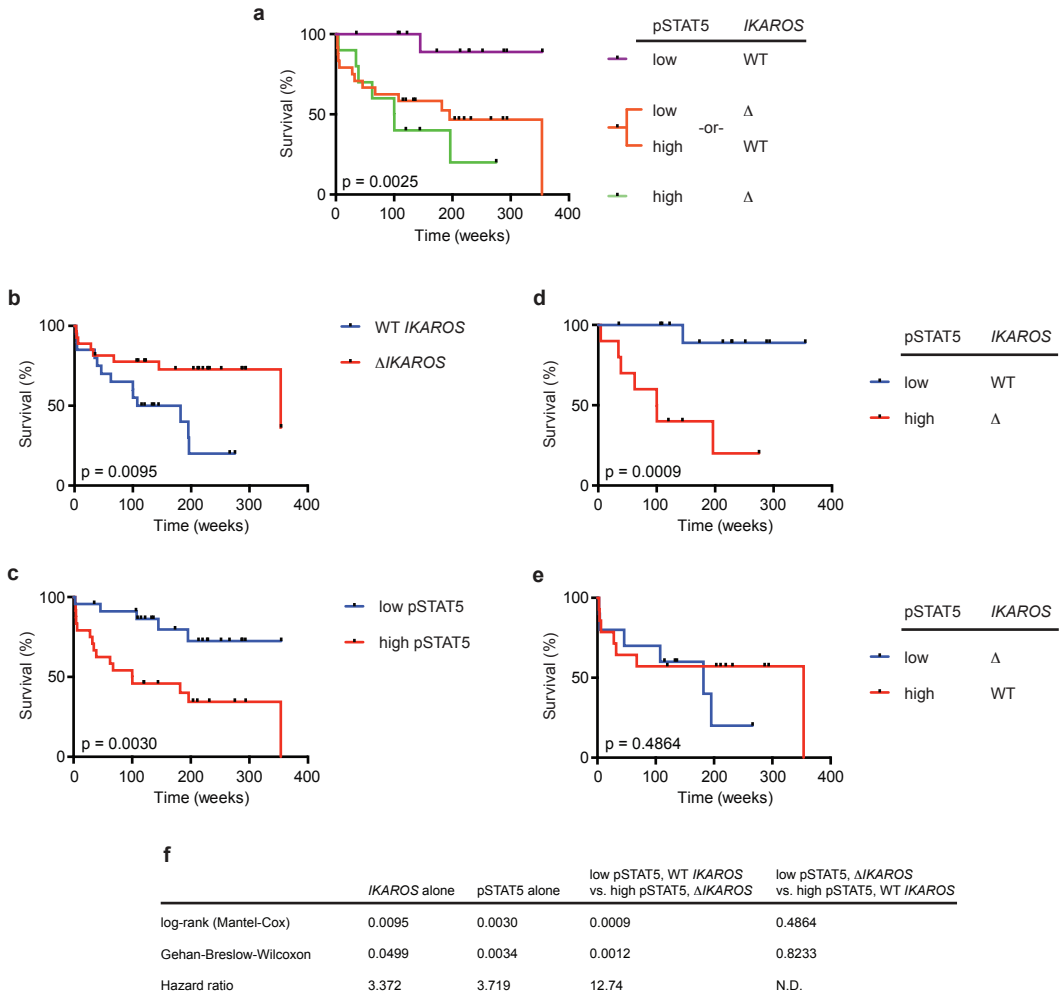
	pSTAT5 / p65 alone	pSTAT5 alone	low pSTAT5, low pSTAT5 / RELA vs. high pSTAT5, high pSTAT5 / RELA	high pSTAT5, low pSTAT5 / RELA vs. low pSTAT5, high pSTAT5 / RELA
log-rank (Mantel-Cox)	0.0966	0.0840	0.0182	0.8832
Gehan-Breslow-Wilcoxon	0.1838	0.1373	0.0227	0.4498

Figure 2.16. Combined pSTAT5 / RELA ratio and total pSTAT5 levels correlate best with survival in patients with progenitor B-ALL.

a, Survival of B-ALL patients stratified by pSTAT5 and pSTAT5 / RELA ratio. p-values represent log-rank test for trend of medians. **b,** Survival of B-ALL patients that were stratified based on pSTAT5 / RELA ratio alone: low or high. **c**, Survival of B-ALL patients that were separated into two equal-sized groups based on low or high pSTAT5 levels. **d**, **e**, Survival of B-ALL patients that were stratified by separating them based on pSTAT5 / RELA ratio (low or high) and then further subdividing those groups based pSTAT5 levels (low or high). p-values in panels b-e represent log-rank (Mantel-Cox) test. **i**, Statistical summary of the results shown in panels b-e.



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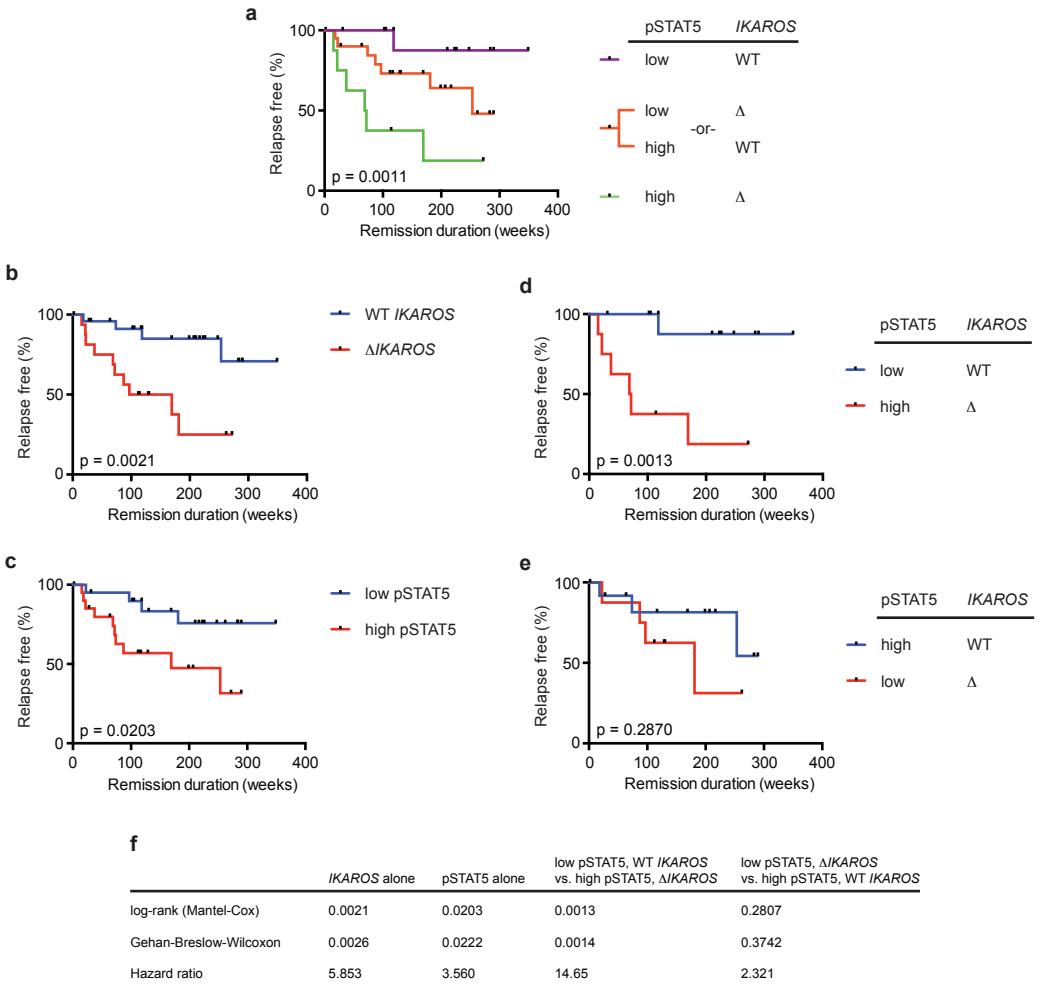
	IKAROS alone	pSTAT5 alone	low pSTAT5, WT *IKAROS* vs. high pSTAT5, Δ*IKAROS*	low pSTAT5, Δ*IKAROS* vs. high pSTAT5, WT *IKAROS*
log-rank (Mantel-Cox)	0.0095	0.0030	0.0009	0.4864
Gehan-Breslow-Wilcoxon	0.0499	0.0034	0.0012	0.8233
Hazard ratio	3.372	3.719	12.74	N.D.

Figure 2.17. STAT5 activation paired with deletion of *IKAROS* negatively correlates with the survival of BCR-ABL negative B-ALL patients.

a, Survival of BCR-ABL negative B-ALL patients stratified by pSTAT5 and *IKAROS* status: WT or deleted (Δ). p-values represent log-rank test for trend of medians. **b**, Survival of BCR-ABL negative B-ALL patients that were stratified based on *IKAROS* status alone: WT or deleted. **c**, Survival of BCR-ABL negative B-ALL patients that were separated into two equal-sized groups based on low or high pSTAT5 levels. **d**, **e**, Survival of BCR-ABL negative B-ALL patients that were stratified by separating them based on *IKAROS* status (WT or deleted) and then further subdividing those groups based pSTAT5 levels (low or high). p-values in panels b-e represent log-rank (Mantel-Cox) test. **f**, Statistical summary of the results shown in panels b-e. ND = not done.



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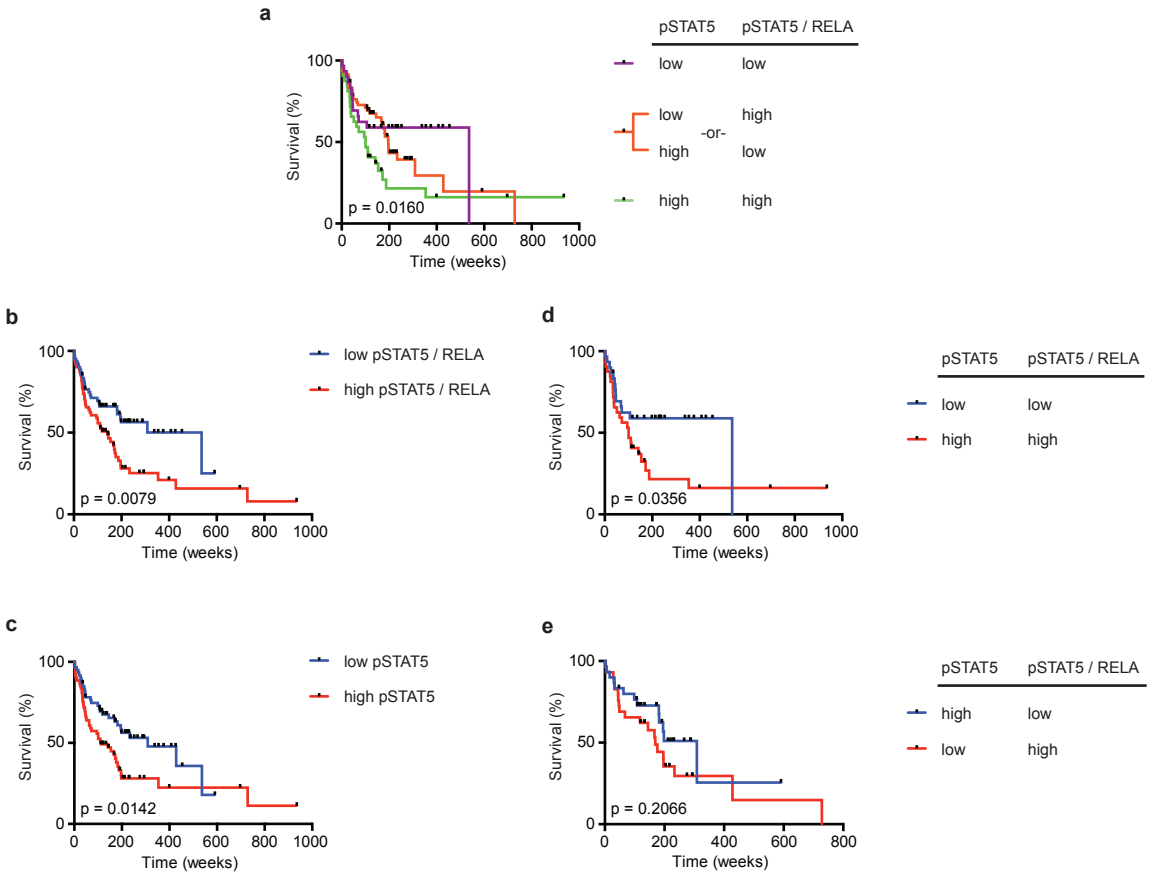
	IKAROS alone	pSTAT5 alone	low pSTAT5, WT *IKAROS* vs. high pSTAT5, Δ*IKAROS*	low pSTAT5, Δ*IKAROS* vs. high pSTAT5, WT *IKAROS*
log-rank (Mantel-Cox)	0.0021	0.0203	0.0013	0.2807
Gehan-Breslow-Wilcoxon	0.0026	0.0222	0.0014	0.3742
Hazard ratio	5.853	3.560	14.65	2.321

Figure 2.18. STAT5 activation paired with deletion of *IKAROS* negatively correlates with remission duration in BCR-ABL negative B-ALL patients.

a, Remission duration in BCR-ABL negative B-ALL patients stratified by pSTAT5 and *IKAROS* status: WT or deleted (Δ). p-values represent log-rank test for trend of medians. **b**, Remission duration in BCR-ABL negative B-ALL patients that were stratified based on *IKAROS* status alone: WT or deleted. **c**, Remission duration in BCR-ABL negative B-ALL patients that were separated into two equal-sized groups based on low or high pSTAT5 levels. **d**, **e**, Remission duration in BCR-ABL negative B-ALL patients that were stratified by separating them based on *IKAROS* status (WT or deleted) and then further subdividing those groups based pSTAT5 levels (low or high). p-values in panels b-e represent log-rank (Mantel-Cox) test. **f**, Statistical summary of the results shown in panels b-e.



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	pSTAT5 / p65 alone	pSTAT5 alone	low pSTAT5, low pSTAT5 / RELA vs. high pSTAT5, high pSTAT5 / RELA	high pSTAT5, low pSTAT5 / RELA vs. low pSTAT5, high pSTAT5 / RELA
log-rank (Mantel-Cox)	0.0079	0.0142	0.0356	0.2066
Gehan-Breslow-Wilcoxon	0.0337	0.0146	0.0877	0.2516
Hazard ratio	1.906	1.818	2.009	N.D.

Figure 2.19. Combined pSTAT5 / RELA ratio and total pSTAT5 levels correlate best with survival in patients with BCR-ABL negative progenitor B-ALL.

a, Survival of BCR-ABL negative B-ALL patients stratified by pSTAT5 and pSTAT5 / RELA ratio. p-values represent log-rank test for trend of medians. **b,** Survival of BCR-ABL negative B-ALL patients that were stratified based on pSTAT5 / RELA ratio alone: low or high. **c**, Survival of BCR-ABL negative B-ALL patients that were separated into two equal-sized groups based on low or high pSTAT5 levels. **d**, **e**, Survival of BCR-ABL negative B-ALL patients that were stratified by separating them based on pSTAT5 / RELA ratio (low or high) and then further subdividing those groups based pSTAT5 levels (low or high). p-values in panels b-e represent log-rank (Mantel-Cox) test. **i**, Statistical summary of the results shown in panels b-e.

Discussion

This research demonstrates that STAT5 activation cooperates with defects in a BLNK-BTK-PKCβ-NFκB pathway to initiate leukemia in progenitor B cells. Contrary to previous reports, STAT5 did not promote leukemia by merely providing a survival or proliferation signal. Instead, we found that STAT5 synergized with the loss of pre-BCR signaling components to modulate the expression of several NFκB target genes, including known oncogenes. Therefore these data suggest that STAT5 antagonizes gene regulation by NFκB. This interaction appears to be direct in many cases, as STAT5 was bound to roughly half of the NFκB target genes that were deregulated in the *Stat5b-CA* x *Blnk*$^{+/-}$, *Stat5b-CA* x *Xid* and *Stat5b-CA* x *Prkcb*$^{-/-}$ leukemias. In addition, we found that STAT5 binds to and represses the expression of *Nfkb2*. Therefore, STAT5 antagonizes the function of NFκB by multiple mechanisms. Similarly, we found that STAT5 opposed gene regulation by IKAROS, which is positively regulated by the pre-BCR and NFκB. Moreover, STAT5 and IKAROS binding directly overlapped at hundreds of loci and luciferase studies suggested that these factors can compete for binding to target gene sequences. STAT5 may also oppose the function of IKAROS by directly binding to and repressing the expression of *Ikaros*. We also found that STAT5 binding overlapped with the binding of the B cell transcription factors PAX5, EBF1, PU.1, IRF4, and IKAROS, particularly at B cell super-enhancers. Moreover, those super-enhancers with the highest STAT5 binding were associated with genes that regulate pre-B cell survival, proliferation, and differentiation. These data were recapitulated in humans, as STAT5 binding directly overlapped with NFκB and IKAROS at thousands of loci in a human B lymphoblastoid cell line. Additionally, high levels of STAT5 binding in

the human B cell line tended to occur at super-enhancers that were associated with a similar regulatory gene network identified in mouse pre-B cells. Finally, the ratio of active STAT5 to functional *IKAROS* alleles negatively correlated with the remission duration and survival of B-ALL patients. Similarly, high levels of active STAT5 and a high ratio of active STAT5 to NFκB correlated with shorter overall survival of B-ALL patients.

Our studies suggest a model in which the balance between STAT5 and a network of B cell transcription factors at super-enhancers acts as a molecular switch to govern appropriate progenitor B cell survival, proliferation and differentiation (Figures 2.20, 2.21). Altering the balance between these two antagonistic pathways drives B cell transformation, while the degree of imbalance underlies how B-ALL patients will respond to therapy (Figure 2.21). Our findings suggest that strategies aimed at altering this balance, involving STAT5 inhibition paired with NFκB agonism, could potentially inhibit B-ALL in patients who are at high-risk of relapse.

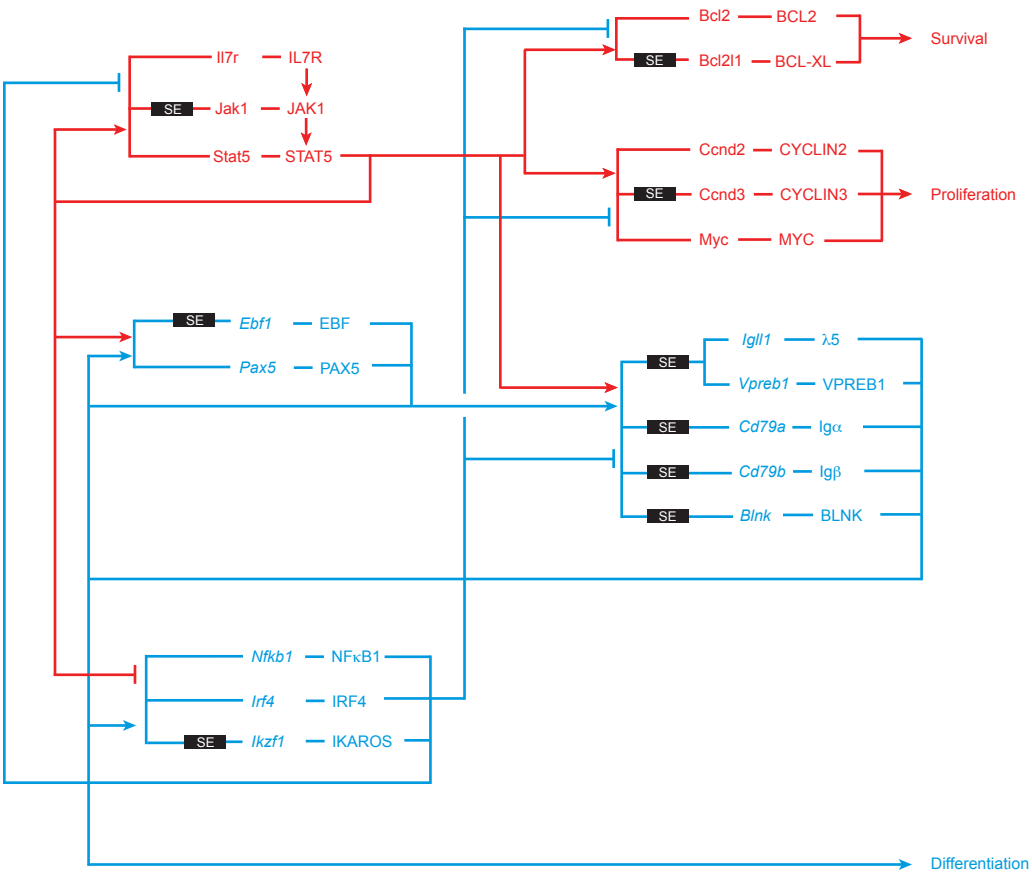

Figure 2.20. STAT5 and a B cell transcriptional network regulate progenitor B cell survival, proliferation and differentiation.

A model illustrating how the IL7R/STAT5 pathway and a B cell transcriptional network regulate progenitor B cell super-enhancer networks. SE = super-enhancer.

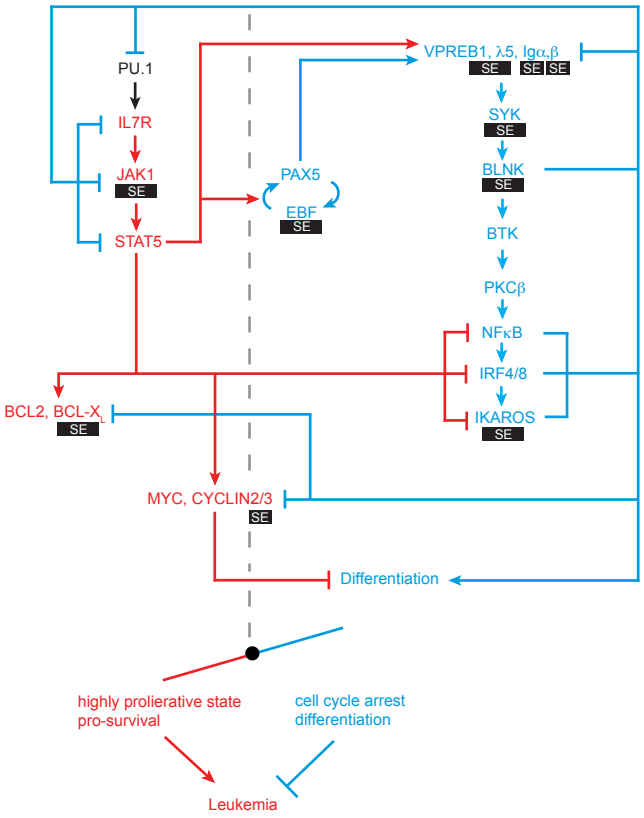

Figure 2.21. The balance between STAT5 signals and a B cell transcriptional network govern appropriate progenitor B cell survival, proliferation and differentiation.

A model illustrating how the balance between the IL7R/STAT5 pathway and the pre-BCR and PAX5, EBF, PU.1, IRF4, and IKAROS (PEPII) transcriptional network govern progenitor B cell survival, proliferation, and differentiation. SE = super-enhancers.

Chapter 3: Implications of the ratio of STAT5 to IKAROS or NFκB dictating patient outcomes

Conclusions

This work demostrates that STAT5 cooperates with defects in numerous pre-BCR signaling components to initiate B-ALL. Previous studies have suggested that the pre-BCR adaptor BLNK suppresses leukemia by binding to JAK3 and inhibiting JAK-STAT5 signaling[132]. However this is probably not the only way that BLNK suppress B-ALL, as *Stat5b-CA* cooperates with deficiencies in signaling effectors downstream of *Blnk* such as *Btk*, *Prkcb*, and *Nfkb1* to initiate B-ALL. Instead, our results suggest that BLNK suppresses leukemia by activating a linear tumor suppressor pathway involving BLNK-BTK-PKCβ-NFκB-IRF4/8-IKAROS/AIOLOS, which inhibits cell cycle progression and promotes differentiation[50,60,90,94,96,142,147–149,174,211–213,223,324–326].

Our results also demonstrate that *Nfkb1* acts as a tumor suppressor in pre-B cells. This is a relatively unique role for NFκB in B cells, as NFκB activity usually promotes transformation in mature B cells[142]. Recent studies support our findings, as *Nfkb1* expression is decreased in B-ALL samples compared to healthy controls[327]. NFκB is likely acting as a tumor suppressor in pre-B cells because of contextual factors that influence the activity of NFκB[224]. However, what these factors are in pre-B cells is unknown. Our data suggests that one way that NFκB suppresses pre-B cell leukemia is by inducing the IRF4/8-IKAROS/AIOLOS pathway. In addition, NFκB binding overlaps with STAT5 in human B lymphoblastoid cells, including at the oncogenes *Bcl2l1*, *Myc*, and *Ccnd2*. Moreover, our STAT5 ChIP-seq dataset revealed that NFκB binding motifs

were enriched within 5 bp of STAT5 binding sites. Furthermore, our microarray data indicates that STAT5 and NFκB antagonize target gene regulation by one another. Together these data suggest that NFκB could be competing with STAT5 for binding to shared target genes and preventing STAT5 from promoting a pro-leukemic gene program. Conversely, NFκB may antagonize STAT5 gene regulation by non-competitive mechanisms as well. Therefore, our data suggest a multifaceted role for NFκB in suppressing STAT5-driven B-ALL.

In mature B cells, on the other hand, NFκB activity promotes transformation. In addition, a previous study suggested that STAT5 and NFκB cooperate to drive transformation in mature B cells[328]. Moreover, we found that STAT5 and NFκB bind near one another in B lymphoblastoid cells. Therefore, these data suggest that STAT5 and NFκB may drive transformation in mature B cells by synergistically regulating oncogenes such as *MYC*, *CCND2*, and *BCL2L1*.

Our microarray and ChIP-Seq data indicate that STAT5 and IKAROS are reciprocally regulating the expression of hundreds of shared target genes. This could be by direct or indirect mechanisms. Approximately half of the shared target genes showed direct binding overlap. Moreover, our luciferase assays suggest that STAT5 and IKAROS binding overlaps at a sequence level and that these factors likely compete for binding. However, STAT5 and IKAROS are likely antagonizing the function of one another by non-competitive mechanisms at other shared targets for which their binding does not overlap. Instead, these transcription factors may opposingly open or close the chromatin by binding to different sites within shared target gene loci. STAT5 can also oppose the function of IKAROS and NFκB by binding to and repressing the transcription

of *Ikaros*, *Aiolos*, *Nfkb2*, and potentially *Nfkb1*. Therefore STAT5 can potentially antagonize IKAROS and NFκB activity by directly competing for binding to target genes, by opposing regulation of shared target genes via non-competitive binding, and by binding to and repressing the expression of *Ikaros* and *Nfkb* transcription factors.

In addition, deletions of *IKAROS* may promote transformation by increasing STAT5 accessibility to target genes and enhancing responsiveness to STAT5 in leukemic cells that already have increased STAT5 activity. Indeed, *IKAROS* deletions do not drive leukemia alone[68,174] but do drive leukemia when paired with STAT5-activating mutations, such as BCR-ABL[174] or *Stat5b-CA*. IKAROS also appears to inhibit the activation of STAT5, as dominant negative isoforms of IKAROS lead to an increase in STAT5 activity[329]. This model of reciprocal antagonism between STAT5 and IKAROS is supported by multiple pieces of evidence. IKAROS cooperates with IL7 withdrawal to promote cell cycle exit, pre-BCR down-regulation, and light chain expression[68]. Additionally, *IKAROS* deletions are strongly associated with mutations that promote STAT5 activation[175,178].

Similarly, abrogations in pre-BCR signlaing may increase sensitivity to IL7-STAT5 signaling by decreasing NFκB and IKAROS activity. This is supported by the observation that *Blnk*$^{-/-}$ and *Plcg2*$^{-/-}$ pre-B cells proliferate more than WT pre-B cells in response to IL7[210,220]. Our model also suggests that one reason BCR-ABL+ leukemias select for pre-BCR signaling defects[153,233] is to enhance responsiveness to STAT5. Moreover, our data predicts that defects in pre-BCR signaling components or *IKAROS* may cooperate with other STAT5 activating mutations involving JAK2, CRLF2, or IL7R to drive leukemogenesis. This is supported by observations that IKAROS deletions are enriched in leukemias that also have translocations in JAK2 and/or CRLF2[9,177,178].

Therefore, deficiencies in IKAROS and/or pre-BCR signaling may promote B-ALL by increasing STAT5 accessibility to target genes and may cooperate with several STAT5-activating mutations to initiate B-ALL.

IKAROS is part of a large network of transcription factors that promote B cell development. We found that STAT5 binding overlapped with many of these factors including PAX5, EBF1, PU.1, IRF4, and IKAROS. However, it is currently unclear how these transcription factors are influencing the binding of one another or how these factors interact to affect gene expression. IKAROS is known to compete with EBF1 for binding to the promoter of the pre-BCR component *Igll1* (λ5)[94,154]. At this locus, EBF1 promotes expression whereas IKAROS inhibits expression. *Igll1* is upregulated in the *Stat5b-CA* x *Blnk*$^{+/-}$, *Stat5b-CA* x *Xid* and *Stat5b-CA* x *Prkcb*$^{-/-}$ leukemias and STAT5 directly binds to *Igll1*, suggesting that STAT5 directly upregulates *Igll1*. We found similar evidence that STAT5 binds to and positively regulates another pre-BCR component, *Vpreb1*, which is also positively regulated by EBF1 and PAX5 but repressed by IKAROS[75,87,96,187]. However, our data also indicates that STAT5 opposes regulation by EBF1 and PAX5 at other loci such at *Irf4*, *Aiolos*, and *Bcl6*[69,87]. This suggests a more complex model whereby STAT5 synergizes with EBF1 and PAX5 in the early stages of B cell development to antagonize gene regulation by IKAROS. However, at later stages of B cell development STAT5 opposes gene regulation by EBF1, PAX5, and IKAROS.

Even though STAT5 can oppose and cooperate with EBF1 and PAX5 to modulate target gene expression, it seems that the antagonism between the pathways dictates the outcome of B-ALL. This is supported by the observation that *Pax5*$^{-/-}$ progenitor B cells proliferate more than WT controls in response to IL7[86]. This suggests that PAX5 suppresses IL7-driven proliferation, which is mediated in part by STAT5[128,330].

Moreover, PAX5 and EBF1 suppress STAT5-driven leukemia, as *Pax5* or *Ebf1* heterozygousity cooperates with STAT5 activation to drive B-ALL-like leukemia in mice[2]. Therefore, deficiencies in pre-BCR signaling or in the B cell transcriptional network increase sensitivity to IL7-STAT5 signaling, potentially by preventing IKAROS, NFκB, PAX5, and/or EBF1 from antagonizing gene regulation by STAT5.

Another unanswered question worth investigating is how do STAT5 and PEPII factors interact at target loci temporally. For example, IKAROS, EBF1 and STAT5 are expressed earlier in B cell development than PAX5. Moreover, STAT5 activation peaks in pro-B cells, whereas IKAROS, EBF1 and PAX5 levels continue to rise as cells differentiate into mature B cells. In addition, NFκB, IRF4, IRF8, IKAROS and AIOLOS are all induced by pre-BCR signaling. STAT5 may occupy shared target genes in pro-B cells, but then active STAT5 levels decrease in pre-B cells, thereby allowing PEPII factors to occupy such sites. Additionally, PAX5 and EBF1 binding redistributes to different loci as progenitors progress throughout B cell development[30,312]. The factors that guide or prevent this redistribution have not been identified. However, it seems likely that NFκB, IRF4, IRF8, IKAROS, and AIOLOS may guide the binding of EBF1 and PAX5 to new sites following pre-BCR signaling. It is also tempting to speculate that STAT5 binding to shared target genes could physically prevent EBF1 and PAX5 from redistributing to new sites in the genome. The redistribution of EBF1 and PAX5 is likely important in the differentiation of B cells and potentially in B-ALL. However, this has yet to be studied.

We observed extensive overlap between STAT5 and PEPII factors particularly at super-enhancers. In addition, we found significant overlap of STAT5 and 4 or more

PEPII factors at hundreds of loci outside of super-enhancers. These sites could be important in the regulation of genes that control B cell differentiation and leukemia. However, what these sites are doing in normal B cell development or transformation remains unclear. Super-enhancers are defined by high levels of the coactivators Mediator or the open chromatin mark H3K27Ac and are usually associated with those genes that are expressed at the highest levels. Therefore, the overlap of PEPII factors outside of super-enhancers may correspond to loci that are not expressed at high levels in normal B cell progenitors, but are still important in transformation. Studying these sites in further detail may provide valuable insights into the mechanisms underlying normal B cell development as well as the transformation of B cell progenitors.

One caveat to our STAT5 ChIP-seq data is that it was done in *Stat5b-CA x* *Blnk*$^{+/-}$ leukemias, which have hyperactive levels of STAT5. Therefore, is this the reason that we observed such a large overlap in binding between STAT5 and PEPII factors? Potentially STAT5 binding would not normally be bound to such sites in WT pre-B cells. However, we know that in WT pre-B cells, STAT5 is bound to *Ikaros*, which was one of the weakest STAT5 binding sites that we observed in the leukemias (Figure 2.10b). Therefore, this suggests that STAT5 may occupy many of the same sites in WT pre-B cells, however, the amount of STAT5 bound at those sites may be lower in WT cells. Therefore, a comparison of STAT5 binding in WT pre-B cells and transformed pre-B cells should be done to test this possibility. Moreover, it may also be insightful to test how STAT5 binding changes in the absence of other transcription factors such as IKAROS, NFκB, PAX5, EBF1, or IRF4. Also, how does the binding of these factors change in response to more or less active STAT5. This would shed light on whether these factors cooperate or compete for binding to sites of overlap. Moreover,

determining how the binding of these transcription factors changes in B-ALL may help to explain why these factors are commonly deregulated in B-ALL.

Several other questions remain in regards to the interaction between STAT5 and PEPII transcription factors. What is happening at sites of STAT5 and PEPII factor binding overlap? Can STAT5 and PEPII factors compete for binding to shared target genes and/or synergistically bind to other loci? Do STAT5 and PEPII factors interact directly by protein-protein interactions or via co-repressors or co-activators functioning as adaptors? Such interactions are possible since both STAT5 and IKAROS have been shown to bind to the co-repressor SMRT[125,331]. In addition, how does the interaction of STAT5 and PEPII factors at shared sites affect gene transcription? What determines whether the binding of these factors turns on or off target gene expression? For example, the mechanism by which STAT5 modulates gene expression is uncertain. STAT5 can interact with other co-regulators besides SMRT such as the co-activator p300[332,333]. However, the mechanisms that determine whether STAT5 interacts with a co-activator or a co-repressor to promote or repress transcription are poorly understood. One mechanism may involve the interaction of STAT5 with PEPII factors. Finally, which co-regulators STAT5 is interacting with to drive B-ALL is also unknown.

In this study, we identified groups of B-ALL patients that are at high-risk of relapse and death. Moreover, our data suggests that the combination of active STAT5 levels and deleted IKAROS or active STAT5 and the ratio of active STAT5 to RELA could be used as prognostic indicators to better stratify B-ALL patients. These markers could potentially be used with current risk stratification methods[6,9] such as age and white blood cell counts to further improve stratification. This method could potentially be used

to aggressively treat those patients that we have identified as high-risk, and minimize the over-treatment of patients that will not benefit from harsh treatment regimens.

Moreover, our data could be used to develop more tailored therapies that treat patients based on the specific underlying causes of disease. For example, those patients with IKAROS deletions could be treated with NFκB agonists to restore IKAROS expression. Additionally, induction of NFκB, IRF4, IRF8, and/or AIOLOS by NFκB agonists may compensate in cases where both alleles of IKAROS are deleted. Moreover, NFκB agonists may synergize with SYK agonists to further increase treatment efficacy. IKAROS and SYK positively regulate one another[68,94,152,153,188,334], which potentially forms a positive feedback loop. Evidence for the synergy between SYK and IKAROS lies in the observation that patients with forms of B-ALL that do not express μH chain or IKAROS have poorer survival than those with leukemia that only express one of these proteins[169,170]. Increasing SYK activity has already proven to induce cell death in BCR-ABL+ B-ALL[233]. Therefore, combining the use of NFκB agonists with SYK agonists may induce the activity of IKAROS more than using either agonist alone.

Other factors that are also likely involved in a positive feedback loop with SYK and IKAROS include BLNK, FOXO1, NFκB, IRF4, PAX5, and EBF1 (Figure 3.1). Therefore, loss of one or two members of this network may reduce the expression of all members in this network. Additionally, because super-enhancers are known to be exquisitely sensitive to disturbances in transcriptional networks[234,235], loss of one or two members in the B cell transcriptional network may disrupt the binding of several members of the network at super-enhancers. This is supported by evidence that

deletion of EBF or E2A significantly affects PU.1 binding and distribution. Likewise, EBF1 binding is severely impaired in E2A deficient B cell progenitors[69,80]. Therefore B cell transcription factors affect the binding and distribution of other B cell transcription factors. Therefore, disturbances in the B cell transcriptional network may disproportionately affect the expression of genes associated with B cell super-enhancers, which are known to be key in the development of B-ALL. Therefore, treatments aimed at restoring this super-enhancer network may be very effective in treating patients that have deletions in the transcription factors that make up this network. Therefore, SYK and NFκB agonists may be effective in treating patients with PAX5 or EBF1 deletions, for example, in addition to those with IKAROS deletions or decreased expression of pre-BCR signaling components.

For those B-ALL cases with high STAT5 levels, then our data suggests that STAT5 inhibitors should be employed as treatment options. Our data also suggests that the ratio of STAT5 to IKAROS or NFκB is important in B-ALL. Therefore, combining the treatment of STAT5 inhibitors along with NFκB and SYK agonists may be effective in B-ALL cases with high levels of active STAT5 or deleted IKAROS in addition to those that have both pathways deregulated. Moreover, the levels of STAT5, NFκB, and SYK may only need to be moderately modulated in order to effectively treat B-ALL. This would minimize off target effects to other STAT5, NFκB and SYK dependent processes. Therefore, moderate STAT5 inhibition combined with moderate agonsim of NFκB and SYK may effectively treat high-risk cases of B-ALL such as BCR-ABL+ and BCR-ABL-like leukemia.

In conclusion, these studies have provided many insights and potential mechanisms by which STAT5 activation cooperates with defects in pre-BCR signaling components and B cell transcription factors to initiate B-ALL. Moreover, this work can potentially be used to develop novel risk stratification methods and treatment options to more specifically and effectively treat B-ALL. Finally, this research suggests new directions for scientific inquiry to further elucidate the underlying causes of B-ALL and ultimately cure B-ALL.

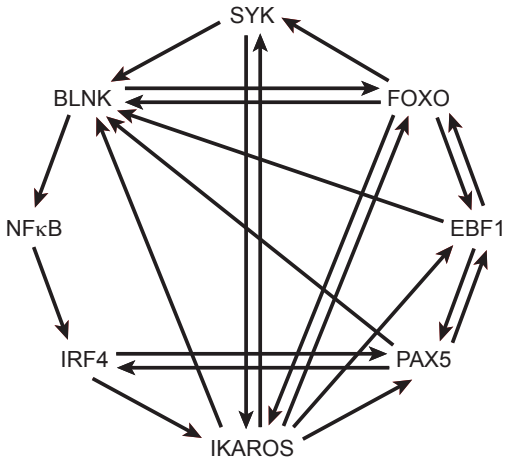

Figure 3.1. SYK and IKAROS form a positive feedback loop with other factors that regulate B cell development.

IKAROS, FOXOs, EBF1, PAX5, and IRF4 form a self-reinforcing transcriptional network in B cell progenitors. This network of transcription factors positively regulates pre-BCR signaling components such a SYK and BLNK, which feedback to positively regulate the factors in the B cell transcriptional network.

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