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Gfi1b Negatively Regulates the V(D)J Recombinase

By

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Committee in charge:

Professor Mark Schlissel, Chair
Professor Don Rio
Professor Russell Vance
Professor Hei Sook Sul

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Abstract

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Regulating RAG activity in B lineage cells is crucial to prevent deleterious events that can be caused by the presence of DNA double strand breaks. To identify negative regulators of RAG expression, we conducted an unbiased cDNA library screen in Abelson murine leukemia-virus transformed pro-B cells. We found that overexpression of the transcriptional repressor Gfi1b downregulates RAG expression in pro-B cell lines and primary B lineage cells from bone marrow. Gfi1b binds directly to a region of the RAG locus upstream of the B-cell specific Erag enhancer and its activity depends on its association with chromatin modifying cofactors. In addition, Gfi1b's effect on RAG levels appears to be mediated in part by repression of FoxO1, a recently identified positive regulator of RAG expression. Gfi1b-deficient cell lines exhibit increased RAG levels as well as an increase in the overall number of DNA double strand breaks per cell when compared to their wildtype counterparts, suggesting that Gfi1B may be critical to maintain genome integrity. Moreover, microarray experiments revealed that Gfi1b controls the expression of a suite of B lineage-specific genes, including the immunoglobulin kappa locus and the transcription factor SpiB. We identify Gfi1B as a novel regulator of RAG expression that may also be involved in the execution of genetic programs that govern B cell development.

Dedication

For Baby Jack, who will soon grow up to make his own discoveries.

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CHAPTER 1 Introduction to the Adaptive Immune Response

Adaptive vs. Innate Immunity:

Vertebrate immunity consists of two main branches: the adaptive immune system and the innate immune system. While the innate system responds to infection by relying heavily on the recognition of common pathogenic motifs such as Pathogen Associated Molecular Patterns (PAMPs) (1, 2), the adaptive immune system is defined by both specificity and memory (3, 4).

Lymphocytes and their Function in the Immune System

B and T cells (lymphocytes) are central to the specific reactions conferred by the adaptive immune system. The function of both these cell types is entirely dependent on the receptors carried on their cell surface. While T cells produce the T cell receptor, which recognizes peptide antigens that have been processed intracellularly and bound to the major histocompatibility complex (MHC) (5), B cells in turn produce the B cell receptor (BCR), which can recognize antigen in its native form (6). Thus T cells are able to respond to antigens present in the intracellular space, while B cells respond to antigens outside the cell. The antibody molecule comes in two forms: one that includes a transmembrane domain that forms the BCR on the cell surface, and a second, secreted form that lacks the transmembrane domain. In addition, the antibody molecule can be divided into two regions: a variable region that differs from cell to cell, and a constant region that is invariant until a B cell becomes activated (6).

Engagement of the TCR, along with a series of appropriate costimulatory signals, can lead to T cell activation followed by differentiation into a variety of subtypes, including T helper (T_H) cells, cytotoxic T lymphocytes (CTLs), memory (T_M) cells, and regulatory (T_{reg}) (7-11). T helper cells assist in a variety of processes, including but not limited to, B cell maturation, and activation of CTLs and macrophages. CTLs can destroy tumor cells, as well as virally infected cells, through the release of cytotoxins such as perforin, granzymes, and granulysin. Memory cells remain in the body long after an infection has been cleared and can rapidly differentiate if the appropriate antigen reappears (12). Regulatory T cells are important for shutting down the immune response toward the end of an infection, as well as maintaining immunological tolerance (13). Engagement of the B cell receptor, along with appropriate costimulatory signals, results in cell proliferation and differentiation into plasma cells. These cells are capable of producing large quantities of antibodies, as many as 1000/sec, and these antibodies can be transported either to the blood plasma or the lymphatic system. During an antigen-specific immune response, B cells undergo a process called somatic hypermutation (SHM). SHM results in the mutation of the variable regions of the antibody molecule within an individual cell. These mutations can affect the affinity of the BCR for its antigen, and those with the highest affinity are selected for (6, 14, 15). Following activation, B cells also undergo a process

called class switch recombination, wherein the constant region of the antibody molecule is changed, but the variable region remains the same. This allows the antibodies to interact with a variety of effector molecules. Important effector functions involve neutralization, engagement with phagocytes, mast cells, basophils, and eosinophils, which can result in the release of inflammatory mediators, activating complement (6, 16, 17).

Specificity of the Immune Response

One very important aspect of adaptive immunity is that, in theory, a BCR or a TCR only recognizes a specific antigen. It should be noted however, that many examples of cross-reactivity exist, for example in many autoantibodies (18). Only the recognition of a specific antigen can lead to B or T cell activation; this ensures that the antibody molecules that are produced following activation are specific to the relevant pathogen. In addition, the memory cells that are produced by the differentiation of B and T cells following activation are likewise specific for this same particular pathogen (reviewed in (6)).
.

V(D)J Recombination

Because the adaptive immune response relies so heavily on specificity, it is necessary to produce enough BCRs and TCRs to accommodate the enormous number of antigens that an animal will be challenged with during the course of its lifetime. This is accomplished through a process called V(D)J recombination, wherein gene segments that together code for the variable regions of the BCR or TCR are randomly selected and recombined in a site-specific manner to generate a wide variety of distinct variable domain protein sequences (6). This is accomplished by the recognition of Recombination Signal Sequences (RSSs) by the site-specific RAG recombinase, encoded by the genes *RAG1* and *RAG2*. RSS sequences are found immediately adjacent to the gene segments that encode the variable region of the BCR or TCR, and are composed of a conserved heptamer and nonamer region, between which lies a 12 or 23 nucleotide spacer. Once the recombinase binds to this region, it can catalyze a double-strand DNA break (19). Two gene segments that have undergone site-specific cleavage at their respective RSSs are then joined together by the Non-Homologous End Joining (NHEJ) machinery to form a coding joint. The RSS sequences are also joined together and these sequences, along with the intervening DNA, are excised from the genome to form a signal circle. Signal circles are eventually lost as the cells undergo division (20) (Figure 1). Through the use of this combinatoric mechanism the immune system is able to generate a huge number of different receptors with different specificities. Diversity is further increased by N and P nucleotide additions catalyzed by TdT during coding joint formation (21).
.

The Antibody Molecule

Antibody molecules are composed of two identical heavy chains and two identical light chains. The variable region of the heavy chain is composed of three gene segments at the *IgH* locus: a V, a D, and a J segment. The light chain is composed of only two gene segments: a V segment and a J segment. The light chain segments are found at either the *kappa* locus or the *lambda* locus. After heavy chain recombination, B cells generally recombine kappa segments first, and recombine segments at the *lambda* locus only if kappa recombination has failed to produce a productive, non-self specific protein (6).

RAG Expression is Lineage and Stage Specific

Because RAG proteins are capable of making double-strand breaks that can represent a threat to genome integrity, they are regulated in a lineage and stage specific manner. Only B and T cells express the *RAG1* and *RAG2* genes that code for the V(D)J recombinase. In addition, the RAG proteins are expressed only within a narrow developmental window within the life of a B or T cell (22, 23). In this way, the cell is able to minimize potentially deleterious DNA breakage events and confine these programmed developmental breaks to a narrow window of development. Mechanisms of lineage and stage specificity of RAG expression are discussed in detail in chapter two. Another important way in which recombinase-mediated breaks are kept in check is through the regulation of accessibility of the recombinase to the RSSs at recombining gene loci. Mechanisms of accessibility regulation are discussed in detail in chapter 4 and are reviewed in (24). Consequences of aberrantly repaired RAG-Mediated breaks are discussed below.

RAG-Mediated Recombination and Disease

For some time, it has been known that mistakes in V(D)J recombination can result in leukemia or lymphoma (reviewed in (25)). As early as 1985, Tsujimoto and colleagues reported that a t(14,18) translocation that can result in either follicular lymphoma or pre-B cell leukemia is a result of an aberrant V(D)J recombination event that joins a J_H segment to the proto-oncogene *Bcl2* (26). Interestingly, it was later demonstrated that the recombinase is able to bind and cleave to the major breakpoint region of *Bcl2.* This region does not contain an RSS, but does have a non-B structure, indicating that recombinase binding/cleaving activity is not always sequence-specific.

Abberant recombination events can also occur at cryptic RSS sequences (27), and one such event (d(1)p) involving cryptic RSSs at the *TAL1* and *SIL* genes can result in acute T cell leukemia (28).

One way in which recombinase-mediated malignancies can be generated is when deficiency in the DNA damage sensor and tumor suppressor protein, p53,

is combined with a deficiency in a component of the NHEJ DNA repair machinery. In *p53-/-* mice, deletion of NHEJ proteins frequently leads to pro-B cell lymphomas that are initiated by *IgH-myc* translocations and that lead to other anomalies termed complicons (29). Additionally, deletions of *p53* in combination with *H2AX*, the protein product of which is phosphorylated at DNA breaks by the damage sensor ATM, leads to leukemias and lymphomas that are associated with aberrant V(D)J recombination or class switch recombination in mice (30) .

In this study, our goal was to identify factors that negatively regulate the RAG recombinase. Such factors are critical for ensuring that the recombinase is not expressed at the wrong place or at the wrong time. Deregulated expression of the recombinase can lead to deleterious events such as translocations and, in some cases, cancer, as outlined above. Therefore, knowledge of how RAG proteins are regulated is critical for understanding how the cell generates a diverse repertoire of antigen receptors, a process which requires double-strand breaks, while avoiding the generation of cancerous cells that can result from aberrantly repaired breakage events.

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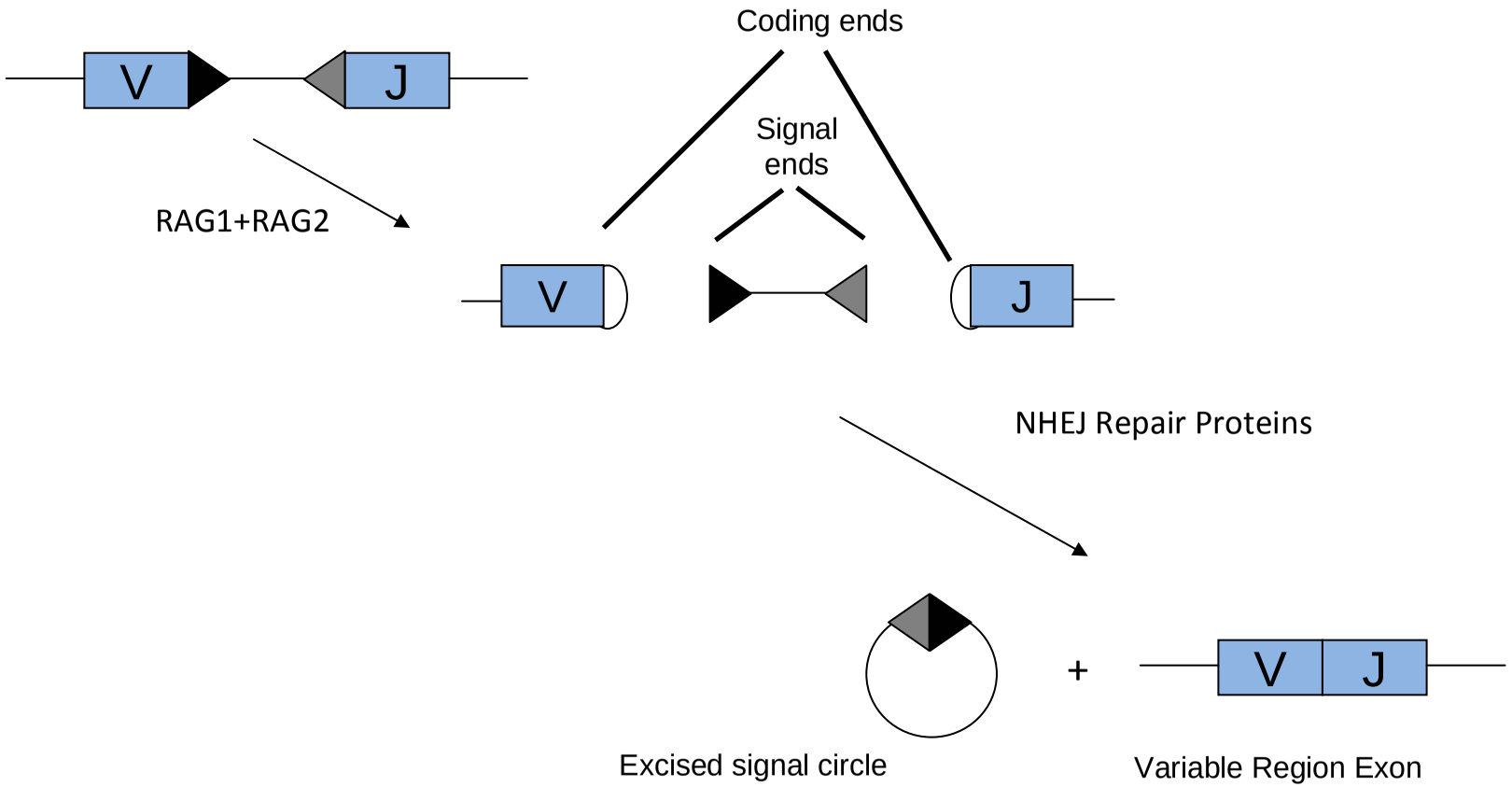

Figure 1: The V(D)J recombination reaction is initiated by RAG1 and RAG2 catalyzing double-strand breaks at RSSs (triangles). This generates two broken signal end two hairpinned coding ends. The coding and signal ends are joined together by the Non-Homologous End Joining (NHEJ) DNA repair pathway. This leads to production of a variable region exon and a signal circle, which is subsequently lost from the cell.

CHAPTER 2 A Screen for Negative Regulators of RAG

BACKGROUND

In 1989 Schatz et al identified *RAG1* and demonstrated that the RAG proteins catalyzed rearrangement of a co-transfected reporter construct in non-lymphoid 293T cells (1). The following year Oettinger et al demonstrated that both RAG1 and RAG2 proteins are required for full recombinase activity (2), and mouse models followed demonstrating that *RAG* knockout mice produced no B or T cells (3, 4). Since then, much scientific effort has gone into understanding the mechanism by which RAG cleavage and subsequent joining takes place (reviewed in (5)), while comparatively little is known about the mechanisms that control RAG expression. Regulation of RAG expression can be divided into four broad categories: the role of cis-acting DNA elements, the role of trans-acting factors, the role of the cell cycle, and finally the signaling events that are initiated at the cell surface. These mechanisms of regulation are discussed in the paragraphs below.

It should be noted that, in addition to the restriction of RAG expression to the appropriate cell lineage and developmental window, recombinase activity is kept in check by controlling the accessibility of rearranging gene loci to the recombinase (reviewed in (6)). Thus, only the IgH, kappa, and lambda loci are accessible the recombinase in B cells, while only the TCR beta, alpha, and gamma/delta loci are accessible to the recombinase in T cells. In addition, only the locus that is programmed to rearrange at a particular developmental window is accessible to the RAG recombinase. For example, at the pro-B cell stage of development where heavy chain rearrangement takes place, the *IgH* locus is accessible to the recombinase, while the kappa and lambda loci are not. Mechanisms for controlling accessibility at rearranging gene loci are discussed in detail in chapter 4.

Overview of RAG expression:

RAG1 and *RAG2* are first expressed in the developing mouse embryo at around E10.5 and first appear in the liver at E11.0 (7). Evidence of RAG expression can be found not only in B and T cells, but also to a lesser extent in natural killer (NK) and dendritic cells (DCs) (8). RAGs are expressed at the common lymphoid progenitor (CLP) stage and are highest in progenitors immediately downstream of CLPs, but not in earlier progenitors that retain myeloid lineage potential. Activity in the CLP stage is dependent on the B cell specific *Erag* enhancer (8). More recently, Mansson et al. found RAG mRNA expression in LSK-, $CD34^{+}$, $FLT3^{Hi,}$ lymphoid-primed, multi-potent progenitor cells (9).

RAGs continue to be expressed as cells transition from the CLP stage to the pro-B cell stage and rearrange their heavy chain loci. They are subsequently shut off during the proliferative burst that marks the transition from the pro-B to the pre-B

cell stages. The RAGs are expressed again at the pre-B cell stage as cells undergo light chain rearrangement (10-14). In the event that a self-specific BCR is generated following light chain rearrangement, the *RAG* locus remains active during receptor editing, a process during which successive kappa, or lambda chain rearrangements are made in an attempt to generate a non-self-specific BCR. Once such a BCR has been generated, the *RAG* genes are shut off, and RAG proteins are not expressed as the cells progress on to the mature stage of B cell development (15, 16)(Figure 1).

A number of pulished reports suggest that RAG proteins could be expressed in activated B cells in both germinal centers and Peyer's Patches (17). Experiments supporting this notion were carried out in anti-MHC antibody transgenic mice, where it was found that secondary rearrangements in splenic B cells were turned off by high affinity receptor engagement, while low affinity antigen could induce receptor revision (18). Subsequently however, 3 different groups found no evidence for RAG expression in activated B cells using a variety of fluorescent RAG reporter constructs (10-13). Despite this, more recent work has suggested that in a systemic lupus erythematosus (SLE) mouse model, RAGs are upregulated in early memory B cells during an autoimmune response (19). In addition, there is evidence that TCR revision occurs in germinal centers and that RAG proteins are expressed during this process (20).

More work is required to understand RAG dynamics in aged mice, but it appears that as mice age, RAG2 expression decreases within the pro-B cell compartment. This is an effect of the age of the BM microenvironment, as evidenced by transplantation experiments (21).

RAG Expression and the Cell Cycle:

RAG2 protein expression is restricted to the G_0/ G_1 phase by phosphorylation-mediated degradation at the G1-S boundary. RAG2 is stabilized by CDK inhibitors, and cyclinA/CDK2 expression prevents the accumulation of RAG2. Ectopic expression of RAG2 throughout the cell cycle results in aberrant recombination products, analogous to those found in NHEJ deficient mice (22). RAG2 is ubiquitinated by the Skp2-SCF ubiquitin ligase and is destroyed at the G_1 /S phase of the cell cycle (23). Further, RAG proteins catalyze double-strand breaks only during the G_0/ G_1 phase of the cell cycle (24). Mouse models have been generated wherein RAG2 is not subject to this type of regulation and, while these mice have defects in lymph node architecture, they do not show marked increases in genomic instability or any defects in ordered gene rearrangement (22, 25). The reasons for this are likely two-fold. First, another layer of RAG regulation depends on cell type specific chromatin structure. This chromatin structure is able to target the RAG proteins to rearrange only the appropriate receptor in the appropriate cell type (26). Therefore, it is possible that RAG proteins are not limiting for recombination. In addition, recent work has

demonstrated that while RAG2 binding is ubiquitous throughout the genome, RAG1 binding is restricted to highly transcribed regions of rearranging loci (27).

RAG Regulation in Cis:

Using a RAG2 blastocyst complementation assay, Monroe et al. were able to define important cis-acting control elements of the *RAG* locus by transfecting various genomic clones along with the *RAG2* and *RAG1* genes in ES cells. They found that a genomic clone containing only the promoter region upstream of *RAG2* could rescue V(D)J recombination in pro-B cell lines, but not *in vivo.* Including a 2kb region upstream of the promoter, as well as the region from 2-7kb upstream of the promoter rescued B cell development, but not T cell development (28). The *RAG1* promoter has limited activity by itself, but supports RAG expression in pre-B, pre-T, and mature B cells when combined with a heterologous enhancer (29).

While binding of PAX-5 to the *RAG2* promoter is necessary for RAG expression in B cells, an overlapping region is required for activity in T cells, along with a more distal element located -107-156bp upstream of the transcription start site. In B cells, deletion of this more distal element has no effect (30).

Many lymphoid specific factors have been shown to have conserved transcription factor binding sites within the *RAG2* and *RAG1* promoters including, PAX5, MYB, SP1, LEF1, NF-Y, C/EBP and GATA3 (29-38).

Using a BAC transgene with a fluorescent *RAG2*-GFP reporter as well as a *RAG1*-YFP reporter, Yu et all found that coordinated expression of RAG1 and RAG2 was dependent on 5' cis elements upstream of *RAG2*, and that RAG expression at the DP stage of T cell development was disrupted when the 5' cis element was deleted (10). Analysis of DNAse hypersensitive sites was subsequently used to identify a lymphoid specific enhancer termed D3, located ~8kb upstream of the *RAG2* first exon, that is bound by C/EBP, and appears to be essential for T cells, but not for B cells (39). Work followed using RAG reporter constructs, and Hsu et al were able to identify a B cell specific transcriptional enhancer, *Erag*, that is bound by E2A and that is necessary to promote RAG activity in early B cell precursors (40).

More recently, Kisielow et al. discovered a gene, *NRW*, within the *RAG2* intron that is expressed in all cells except lymphocytes. It is possible that promoter activity of RAG1 is able to turn off this gene, and it remains to be seen whether it has a fundamental role in RAG regulation (41).

RAG Regulation in Trans:

One of the first insights into how RAG activity was regulated came in 1990 when Menetski et all demonstrated that increased levels of cAMP could increase

recombination, while high levels of PKC decreased RAG activity (42). In T cells PKB controls calcineurin induced NFAT signaling downstream of AKT. Expression of NFAT suppresses RAG expression and ChIP experiments have demonstrated that $NFAT_{c1}$ can bind the *RAG*2 promoter. A constitutively active, myristolated form of PKB decreases NFAT activity and can rescue RAG expression (43). The transcription factor Zfp608 has also been shown to suppress RAG expression in T cells and mutations in Zfp608 that lead to its overexpression result in ablation of RAG expression (44). In mature B and T cell lines, inhibition of protein phosphatases 1 and 2A lead to increased levels of RAG expression (45).

The B cell specific transcription factor PAX-5 is a critical regulator of early B cell development. This transcription factor binds to the *RAG2* promoter and is necessary for *RAG2* promoter activity of a reporter construct in pro-B cell lines (30). While transcribed, acetylated proximal V segments can rearrange at the *IgH* locus without PAX-5 expression, distal V segments require PAX-5 for rearrangement (46). Similarly, in T cells, c-myb occupies the T cell specific *RAG2* promoter element and activates it in a tissue specific manner (38). LEF-1 has been shown to associate with both PAX-5 and c-myb at the *RAG2* promoter (34), and there is some evidence that the myc -associated zinc finger protein, MAZ, may act synergistically with these factors to activate the *RAG2* promoter (47).

The transcription factor RUNX1 may also play a role in regulating the RAG proteins. A CBFβ-SMMHC fusion protein that inhibits the function of RUNX1 results in decreased levels of RAG1 and RAG2, as well as a host of other B cell specific genes, including *CD79A*, *Igll1*, *VpreB1*, and *Blk* (48). In addition, cells deficient in *FoxP1* have a block in the Pro-B to Pre-B cell transition and express lower levels of both RAG1 and RAG2. FoxP1 has also been shown to bind to *Erag* (49).

Ectopic expression of the E2A protein, E12, in a macrophage cell line leads to the induction of *RAG1*, along with other B-cell specific genes such as *EBF*, *IL7-R*α and *lambda 5* (50). Using an embryonic kidney cell line, Romanow et al. demonstrated that transfection of expression vectors encoding the RAG recombinase along with the transcription factor E2A was sufficient for recombination in these cells. This was due to the fact that E2A significantly increased accessibility of Ig loci to the recombinase (mechanisms of accessibility control are discussed in detail in chapter 4). While E2A activates the *kappa* locus for rearrangement, EBF has been shown to be more important for *lambda* locus rearrangements. Both E2A and EBF activate D-J joining at the heavy chain locus, but not V-DJ joining (51). It was later demonstrated that E2A binds to *Erag* (40). While E47 binding at *Erag* is necessary for RAG activity at the CLP stage, it is entirely dispensable within the T lineage (52). Additionally, the E proteins have also been shown to have a role in editing cells. In E2A+/- mice expressing an autoreactive BCR, there are severe defects in RAG1 and RAG2

expression, and consequently in secondary rearrangements and RS deletions (53).

Recent work has shed light on the role of the FoxO family of transcription factors on RAG expression in B cell development. A cDNA overexpression screen in Abelson Murine Leukemia Virus (AMuLV) transformed B cells later revealed that FoxO1 directly upregulates RAG1 and RAG2 and that diminished levels of FoxO1 diminished RAG1 and RAG2 upregulation in editing cells (54). RAG1 and RAG2 activation of FoxO1 can be antagonized by AKT phosphorylation of FoxO1, which sequesters the protein in the cytoplasm (54). This cytoplasmic sequestration can be counteracted by SLP-65, an adaptor that enhances activation of signaling pathways that regulate FoxO1, leading to an accumulation of FoxO1 and FoxO3A in the nucleus and RAG activation (55). FoxO1, but not FoxO3A, has been shown to play a critical role in all stages of B cell development *in vivo*. FoxO1 deletion at the pro-B cell stage results in a failure to express IL-7Rα, and deletion in late pro-B cells results in a block at the pre-B cell stage because the RAG proteins are not produced at a high enough level to support rearrangement and developmental progression (56). Additionally, chIP experiments have demonstrated that FoxO1 binds directly to *Erag* (56).

Using an IκBα superrepressor, Vekoczy et al were able to show that NF-κB/Rel proteins promote RAG transcription, and that in editing cells, p50, p65, and c-rel NF-κB subunits bind to the *RAG* locus. However, in B1 cells, (a type of B cell enriched in marginal zone of the spleen, peritoneal, and pleural cavities (57)), NF-κB deficiency leads to induction of RAG and inappropriate editing (58).

Three hematopoietic-specific factors, Ikaros (a factor shown to be important for controlled accessibility and locus compaction of IgH), PU.1 (a master regulator of cell fate), and SpiB (necessary for lymphopoiesis), have also been found to have a role in RAG regulation. In an Ikaros delete mouse model, CD19+ pro-B cells develop when EBF is expressed, but RAG expression in ablated. These authors also demonstrated that Ikaros binds to the *RAG* locus (59). Mice expressing low amounts of Ikaros have lower levels of RAG1 and RAG2 transcription (60). Pro-B cell lines lacking PU.1 and SpiB have decreased levels of RAG expression when compared to their wildtype counterparts (61).

Signaling at the Cell Surface and its Effect on RAG Expression:

In T cells, RAG proteins are turned off at the single-positive stage of development, and this downregulation is triggered by crosslinking of the TCR during the process of positive selection (62). Similarly, crosslinking of surface IgM in c-myc Eμ cancer cell lines leads to downregulation of *RAG1* and *RAG2* gene expression (63) and immunoglobulin engagement can terminate RAG mRNA expression in human tonsil B cells (64). Basal signaling via ERK (65) and Abl suppress RAG expression, and RAG expression can be induced when this pathway is inhibited by the Abl-specific inhibitor STI-571 (66).

Cytokines have an important role in regulating RAG expression and it has been shown that IL-3, IL-6, and IL-7, but not IL-2, IL-4 or GM-CSF, induce *RAG* gene expression (67). Using cultured pro-B cells from human bone marrow, Billips et al. were able to demonstrate that increased levels of IL-7 increase CD19 expression, but decrease expression of both TdT and RAG proteins. While this seems to contradict the findings of Tagoh et al, it is important to note that the decrease in RAG levels appears to be a *delayed* effect, as it is only seen on the third day following addition of IL-7 to the culture medium. They also found that CD19 crosslinking at the cell surface ablates the ability of IL-7 to decrease RAG levels (68). Expression of CD19 suppresses RAG activity and is required for tonic signaling of the BCR. In its absence, B cells undergo receptor editing without appropriate signals to do so (69).

Using an inducible μ heavy chain transgene, Galler et al were able to demonstrate that IgHC expression expression results in a decrease in TdT, RAG1 and RAG2, even when no surrogate light chain (SLC) is present. However, signaling from Igα is required for this downregulation (70). In the presence of a transgenic BCR, RAG levels and kappa rearrangement remain low, even in the absence of IL-7 (71). In T cells, IL-7Rα deficiency ablates RAG expression at the DN stage of development. However, if a transgene encoding a TCR is present, RAGs are expressed at normal levels at the DP stage of development. Thus, RAG expression depends on the presence IL-7 early in T cell development, but TCR expression is sufficient to turn on the recombinase once T cells have made it past the DN stage of development (72).

Equally important to being able to turn the RAGs on at the appropriate window of development, is being able to subsequently turn them off as cell mature. In order for cells to undergo receptor editing, it is necessary that the recombinase continue to be expressed even after the *kappa* locus has undergone gene rearrangement. In IgM+, IgD- cells, crosslinking of IgM can induce RAG expression, while in IgM+, IgD+ cells, crosslinking generally leads to apoptosis (73). With respect to the BCR, lowering tonic signals of the BCR suppresses RAG expression, while higher levels of signaling from the BCR are able to induce RAG expression (74) in a BLNK dependent manner (75). RAG expression downstream of receptor crosslinking is dependent on expression of the transcription factor IRF-4 (76). Schram et al. later demonstrated that BCR crosslinking in immature cells causes loss of the BCR from the cell surface and thus a decrease in basal BCR signaling. This decrease in signaling was shown to induce RAG expression for light chain editing (77).

PI3K signaling is required to suppress RAG expression. BCR directed PI3K signaling activates BTK, PLCγ2, and AKT, and the two latter factors can both suppress RAG expression. Activation of PI3K signaling in immature B cells carrying an innocuous receptor results in the accumulation of PIP3, which in turn recruits and activates PLCγ2 to repress RAG expression (78). A deficiency in the

PI3K catalytic subunit, p110, results in inappropriate *kappa* locus editing and increased expression of *lambda* light chains (79).

In mature peripheral and tonsillar human B cells, crosslinking of CD40 and the BCR leads to the induction of IL-6, followed by upregulation of IL-6R on the cells surface, which in turn activates RAG expression (80).

In this study, we sought to identify trans acting negative regulators of the *RAG* locus in early B cells using an unbiased screening approach. We reasoned that identification of such factors would not only help us understand the dynamic regulation of RAG expression, but also give us insight into how general mechanisms of complex gene regulation are carried out within a particular developmental program.

MATERIALS AND METHODS:

Cell culture:

The AMuLV-transformed *Rag1*/*GFP*–knock-in cell line was generated by infection of bone marrow from a mouse heterozygous for *Rag1*/*GFP* knock-in with AMuLV (13). Transformed B cells were cloned by limiting dilution and screened for high basal GFP expression and responsiveness to treatment with 2.5 μM STI-571 (Novartis). A single RAG high clone was chosen for all experiments. Cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FCS, L-glutamine (2 mM), penicillin (100 g/ml), streptomycin (100 g/ml) and 2-mercaptoethanol (50 μM) and were grown at 37 °C in 5% CO_2.

Retroviral Production and Infection:

Retrovirus was harvested from the EcoPack2 packaging cell line (Clontech). EcoPack2 cells were transfected with retroviral plasmid resuspended in Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and viral supernatant was collected and filtered 48 h post-transfection.

AMuLV-transformed *Rag1*/*GFP* pro–B cells (RAG high) were infected by resuspension of the cells in viral supernatant containing polybrene (4 g/ml; Sigma) and cultured overnight. Cells were then expanded into normal media.

Retroviral cDNA library screen:

The cDNA library screen was performed as described in (54) with the following modifications: RAG1/GFP pro-B cells were sorted for GFP *low*, Thy1.1 positive cells 1 week post-infection, and the selection and “rescue” procedure was performed 4 times. Additional screening was performed by single cell cloning cDNA infected, RAG1/GFP negative cells by flow cytometry. Retroviral cDNAs in these clones were amplified by PCR as in (54) and the inserts were identified by sequencing and subsequently tested in the RAG high parental line.

Flow Cytometry

Single-cell suspensions were prepared from cultured cells and were labeled with fluorochrome- or biotin-conjugated antibodies by standard techniques. A FC500 or an Elite XL flow cytometer (Beckman Coulter) was used for flow cytometry; a MoFlo high-speed cell sorter (Dako-Cytomation) was used for sorting. Data were analyzed with FlowJo software (Tree Star). All antibodies were from eBiosciences, except anti-CD43 and anti-Thy-1.1 (both from BD Pharmingen).

RESULTS

In order to identify negative trans acting regulators of the locus, we developed a screen to identify cDNAs that, when overexpressed, were able to downregulate RAG transcription. We took advantage of the fact that early B cell progenitors can be transformed by infecting total bone marrow with the Abelson Murine Leukemia Virus (AMuLV). Infection with this virus results in transformation of pro-B cells that are arrested in their development, and are able to cycle continuously in culture without the aid of cytokines (81). Treatment of AMuLV pro-B cell lines with the highly specific abl kinase inhibitor STI-571, results in these cells undergoing a process that, in many respects, mimics developmental progression. STI-571 treated cells stop cycling and upregulate a host of genes necessary for developmental progression such as *RAG1, RAG2, Igα, Igβ,* germline *Igk*, and *BLNK*, among others (82). AMuLV transformed cells were generated from a RAG1/GFP reporter mouse in which the first exon of RAG1 is replaced with GFP (13). Thus, any cell expressing RAG also expresses GFP, and any cell in which RAG1 has been downregulated will show lower levels of GFP expression.

Generation of the RAG high clone

Transformed cells from RAG1/GFP mice were single cell cloned and the clones were screened to identify a clone with high constitutive RAG transcription (Figure 2). A large range of RAG1/GFP transcription was observed in the transformed cells derived from the RAG1/GFP mice. In addition to RAG transcription, clones were screened for STI-571 induced increase in RAG1/GFP expression (Figure 3).

Screening: Infections and Sorting

A cDNA library generated from pro and pre B cells of wildtype mice and cloned into a retroviral backbone (Figure 4) was transfected into a packaging cell line and used to infect the RAG high AMuLV transformed clone (hereafter referred to as “RAG high” cells). This cDNA library has a complexity of 5 X 10^6 and was previously used successfully in a screen to identify positive regulators of the *RAG* locus (54). After a week in culture, infected, GFP low cells were sorted by flow cytometry and placed back in culture. After another week in culture infected,

GFP low cells were sorted again. Multiple rounds of sorting resulted in a population enriched in RAG1/GFP low cells (Figure 5).

Isolation of cDNAs from sorted cells and subsequent rounds of screening

Genomic DNA was isolated from this enriched population and cDNAs were amplified using primers hybridizing to the retroviral backbone (Figure 6A). Enriched cDNAs were recloned in bulk into the retroviral backbone and used to infect the original parental "RAG high" cell line. This procedure was carried out a total of 4 times and the resulting amplicons from each round of screening are shown in Figure 6A. While the complexity of each pool of cDNAs decreased somewhat with each round, it never reached the level of single bands, and at the end of the fourth round of screening, it was necessary to subdivide each pool of enriched cDNAs and test them individually as subpools.

Subdivision of pools and assay for RAG1/GFP downregulation in the parental RAG high line

Because the complexity of the enriched cDNA clones was relatively high, each pool of cDNAs was subdivided into groups of similarly sized clones, recloned (Figure 6B), and tested in the original parental line for RAG downregulating activity (Figure 7). 4 subpools were identified that downregulated RAG1/GFP expression when used to infect the original parental line. Clones from these subpools were then sequenced to identify the relevant cDNAs. Each sequenced cDNA was tested individually within the original "RAG high" parental line for RAG downregulating activity. Table 1 shows the identified clones and the number of times they were individually sequenced. Out of these sequenced clones, only clones identified as having the cDNA encoding Gfi1b (Growth Factor Independent-1B) showed RAG downregulating activity when used to infect the original parental line (Figure 8). It should be noted that all the cDNAs were tested in the original parental line with the exception of the cDNA identified as AKT. AKT is a known negative regulator of the *RAG* locus (54) and was therefore not explored further in this study.

Screening by single cell cloning infected, RAG1/GFP negative cells

In parallel, a separate screening strategy was employed wherein, after the fourth round of infection and sorting, infected, RAG1/GFP negative cells were single-cell cloned. After each RAG1/GFP clone had grown out sufficiently, it was re-analyzed by flow cytometry (representative examples shown in Figure 9). Genomic DNA was then harvested from RAG1/GFP negative single cell clones, and the cDNA was PCR amplified, cloned, and sequenced (Table 2). cDNAs were then individually used to infect the original parental cell line. Of the cDNAs identified in this way, only Gfi1b showed RAG1/GFP downregulating activity when expressed in the original parental line (data not shown).

DISCUSSION

In this study, we performed an unbiased cDNA overexpression screen to identify negative regulators of the *RAG* locus. This approach allowed us to identify factors previously unknown to play a critical role in B cell development, and thus enabled us to explore novel and previously unappreciated pathways of RAG regulation. The fact that we overexpressed only those cDNAs expressed in early B cell development allowed us to identify the most relevant factors for this particular developmental window. However, in taking this approach, it is entirely possible that we may have missed factors responsible for keeping RAG off in other lineages, since those factors may not be expressed in early B cell development, and therefore may not have been included in the library. An interesting future study might involve using the same screening strategy to overexpress an shRNA library in a non-lymphoid lineage, and identify those factors whose knockdown results in inappropriate RAG expression within these lineages. Alternatively, a cDNA library from whole bone marrow or from myeloid cells could be utilized. However, such a screen would require immortalization of a non-B cell type from the RAG1/GFP reporter mouse. Fibroblasts might work well for this purpose. That said, one important limitation for this approach is that, due to redundancy, knockdown of a single factor might not have any effect.

It is worth noting that this screen, in addition to revealing new negative regulators of the *RAG* locus (Gfi family members), also revealed a factor already known to have a role in negatively regulating the *RAG* locus-- AKT. AKT phosphorylates FoxO1, leading to its association with the 14-3-3 proteins and sequestering it in the cytoplasm. Such sequestration prevents FoxO1 from translocating to the nucleus and activating its target genes, among which include the *RAG* genes. Indeed a constitutively active, myristolated version of AKT represses RAG1 and RAG2 expression in primary cells (54). The downregulation in RAG transcription caused by overexpression of the AKT cDNA is consistent with this known pathway, and provides validation for the screening strategy. A further validation for the screen was that Gfi1b was independently pulled out of all 5 cDNA pools tested. This confirmed the consistency of the method, and the validity of the hits.

While this screen successfully revealed novel negative regulators of the *RAG* locus, it also had a number of limitations. The first was that even though the target cell line was generated from a single cell clone, a subset of cells within the "RAG high" population did not express RAG, as evidenced by a lack of GFP expression in a minor subset of the population (Figure 2). That is, RAG1/GFP pro-B cells demonstrate variegated transcriptional regulation of RAG expression. This has been observed with all single cell clones generated from pools of transformed RAG1/GFP pro-B cells (unpublished data). Thus, an inherent background level of RAG downregulation was revealed when we overexpressed an empty vector and sorted out RAG1/GFP negative cells (Figure 5). This background level of GFP negative cells rose as high as 30% after multiple sorts. In general, the percent of GFP negative cells in populations of RAG1/GFP cells

infected with various cDNA library pools was higher then 30% after sorting, and the percentage of RAG1/GFP negative cells remained stably higher than 30% after 1-2 weeks in culture. However, many of the cDNAs sequenced from RAG1/GFP negative clones did not show a RAG1/GFP downregulation phenotype when overexpressed in the original parental line, and this is likely due to the fact that their low level of RAG1/GFP expression had nothing to do with the cDNA being overexpressed, but rather reflected the variegated RAG1/GFP phenotype. Another contributing factor to this background level might be the unlikely event of a retroviral insertion within or near the *RAG1/GFP* locus that inactivated its expression, or insertions elsewhere in the genome had an effect on RAG regulation. Finally, random mutations in the *RAG1/GFP* locus or in regions nearby could have caused silencing of the locus and a downregulation in RAG1/GFP expression without overexpression of any exogenous cDNA.

A further technical problem that arose during screening was that the half-life of GFP is very long, and thus it was necessary to wait at least a week following overexpression of the cDNAs before sorting GFP negative cells. This meant that any cDNA that caused a downregulation of RAG1/GFP, but whose overexpression was toxic to the cells, may easily have been overlooked. We believe that this may have been the case for Gfi1. None of the sequenced clones from the screen were identified as Gfi1, but we tested overexpression of Gfi1 because of its similarity to Gfi1b, which was independently cloned 19 times during the course of the screen. The results of Gfi1 overexpression and the reasons for why it may not have been pulled out of the screen are discussed in chapter 3. Because pools of RAG1/GFP cells infected with cDNAs were sorted twice, the total time between infection and harvesting of genomic DNA from RAG1/GFP cells was about 2.5-3 weeks. Thus, in addition to Gfi1, there may have been other factors whose overexpression caused RAG1/GFP downregulation, but that were eliminated from the RAG1/GFP negative pool due to toxicity/growth issues.

Another limitation of this screen was that it was a “gain of function” screen, meaning that once a cDNA was identified that downregulated RAG1/GFP expression, shRNAs, or in our case, knockout constructs had to be generated to verify a loss of function phenotype. Employing the same screening strategy with shRNA libraries could potentially make the process technically easier. While hits generated during an shRNA screen would have to be verified by overexpression of the corresponding cDNA, obtaining such a cDNA is generally easier on a technical level than generating a knockout mouse or cell line, or screening through large numbers of shRNAs in order to find one with activity. However, the use of an shRNA library would eliminate the tissue specificity of a given library, and thus potential hits may not even be expressed at the appropriate developmental window under study.

CONCLUSIONS

We and others (54) have demonstrated that retroviral expression library screening is an effective method for identifying transcriptional regulators in an unbiased fashion. While rescuing inserts from bulk populations works well for identifying interesting factors, rescuing inserts from single cell clones may not be as effective. Another method that proved problematic in our hands was cyclical packaging rescue, or CPR. In this screening method, retroviral cDNA inserts are mobilized from the target cell by infection with adenoviruses that encode the necessary packaging proteins (83). The viral supernatant containing the mobilized, packaged cDNAs is then used to infect the original parental line. While this method worked well in the cell line in which it was originally reported, cDNAs were not efficiently mobilized in our B cell lines. This could be due to the fact that many cell types have a number of mechanisms to prevent retroviral mobilization after insertion into the genome (84). We conclude that using PCR as a method to rescue the cDNA inserts is a more effective and less labor-intensive method than the adenovirus method.

Based on our studies, we believe that the false positive background involved in screening for repressors is markedly higher than the false positive background generated when screening for activators. However, it is still an effective, if labor-intensive, method for identifying transcriptional repressors. A number of screens are ongoing in our lab using the same method to look at diverse phenotypes, and these screens have likewise been quite successful.

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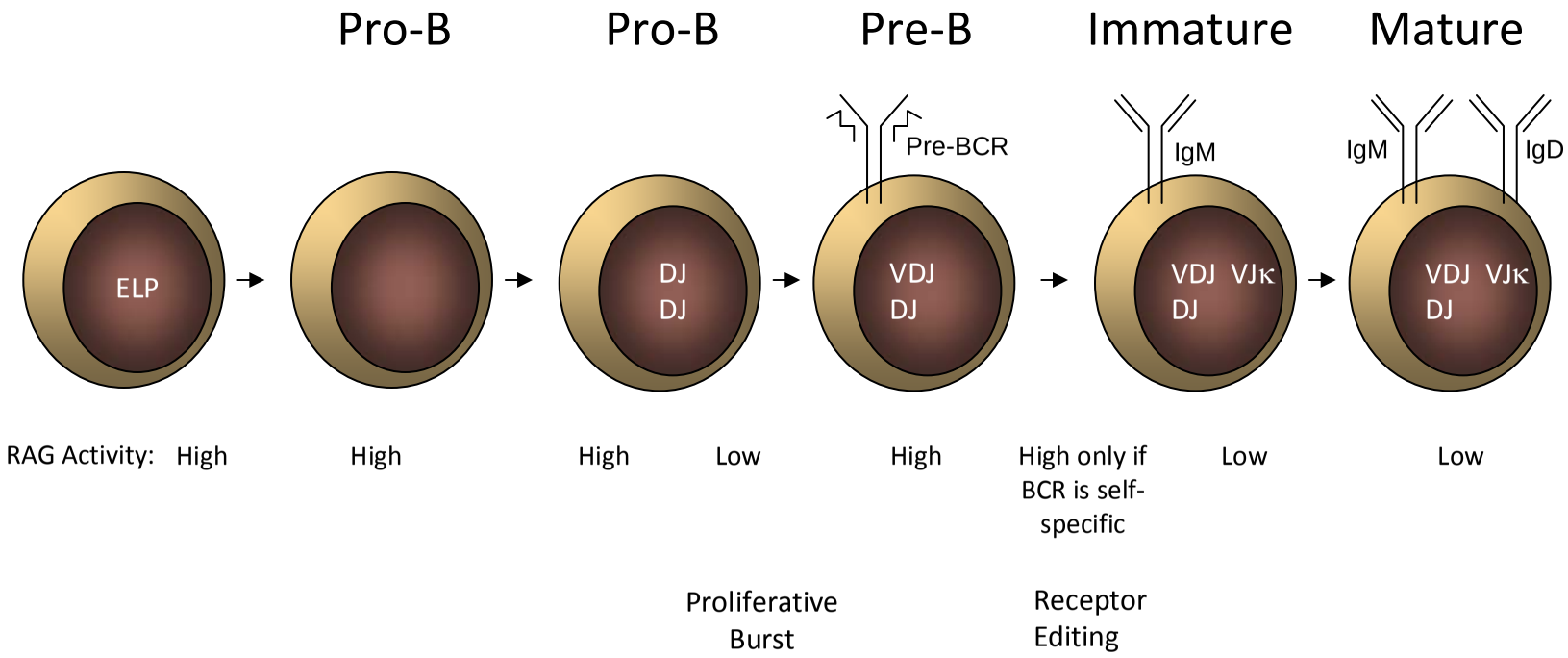


Figure 1: RAG activity during B cell development. RAGs are turned on early in early lymphoid progenitor (ELP) stage. The remain high as cells rearrange their heavy chains but are downregulated during the subsequent proliferative burst. RAGs come on again in the pre-B cell stage for light chain rearrangement, and are then kept on or reinduced should a self-specific BCR be produced so that cells can undergo receptor editing. Figure 1 is adapted from (Schlissel, Nature Immunology, 2003).

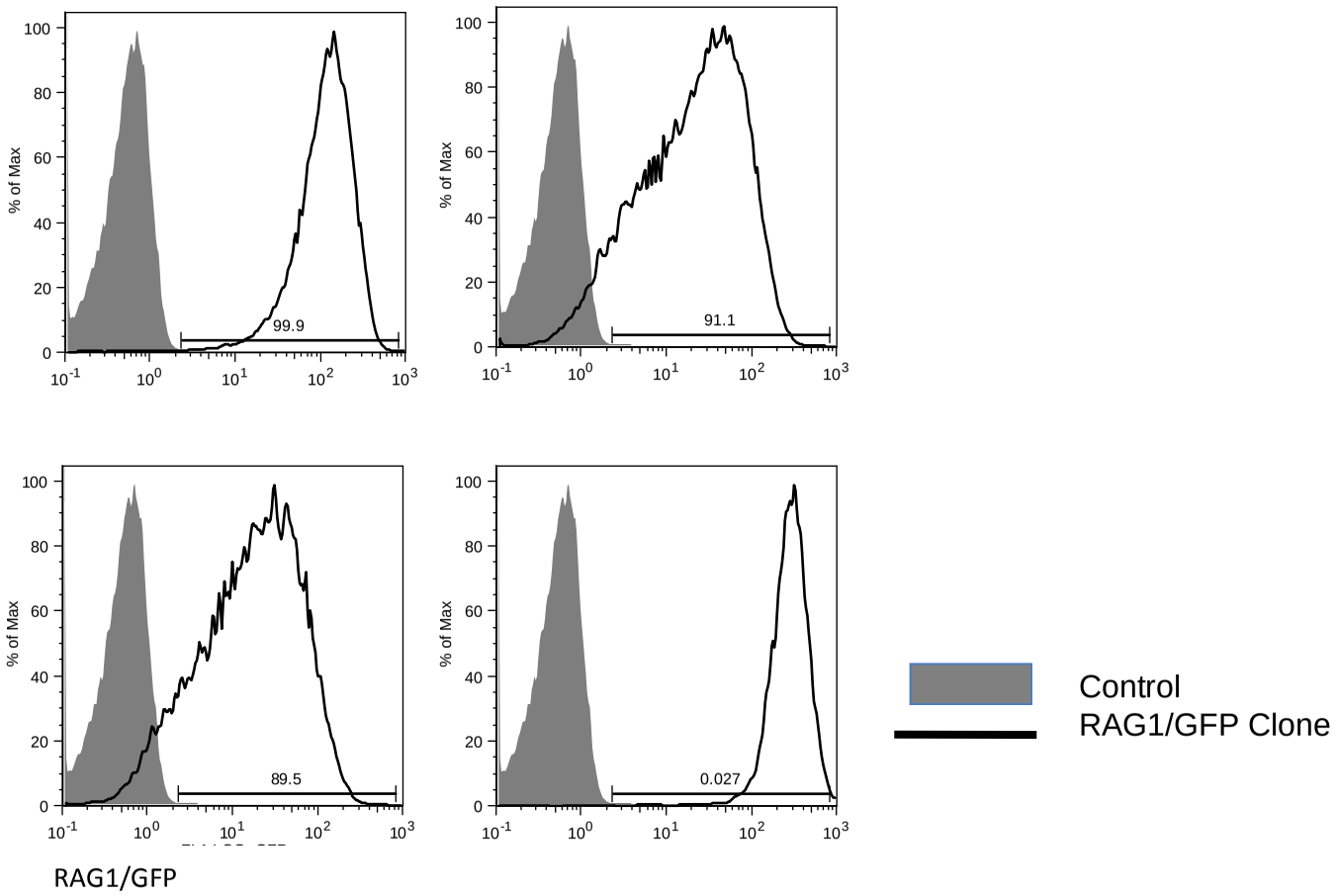


Figure 2: Flow cytometry of 4 representative single cell clones from a pool of AMuLV transformed RAG1/GFP reporter cells.

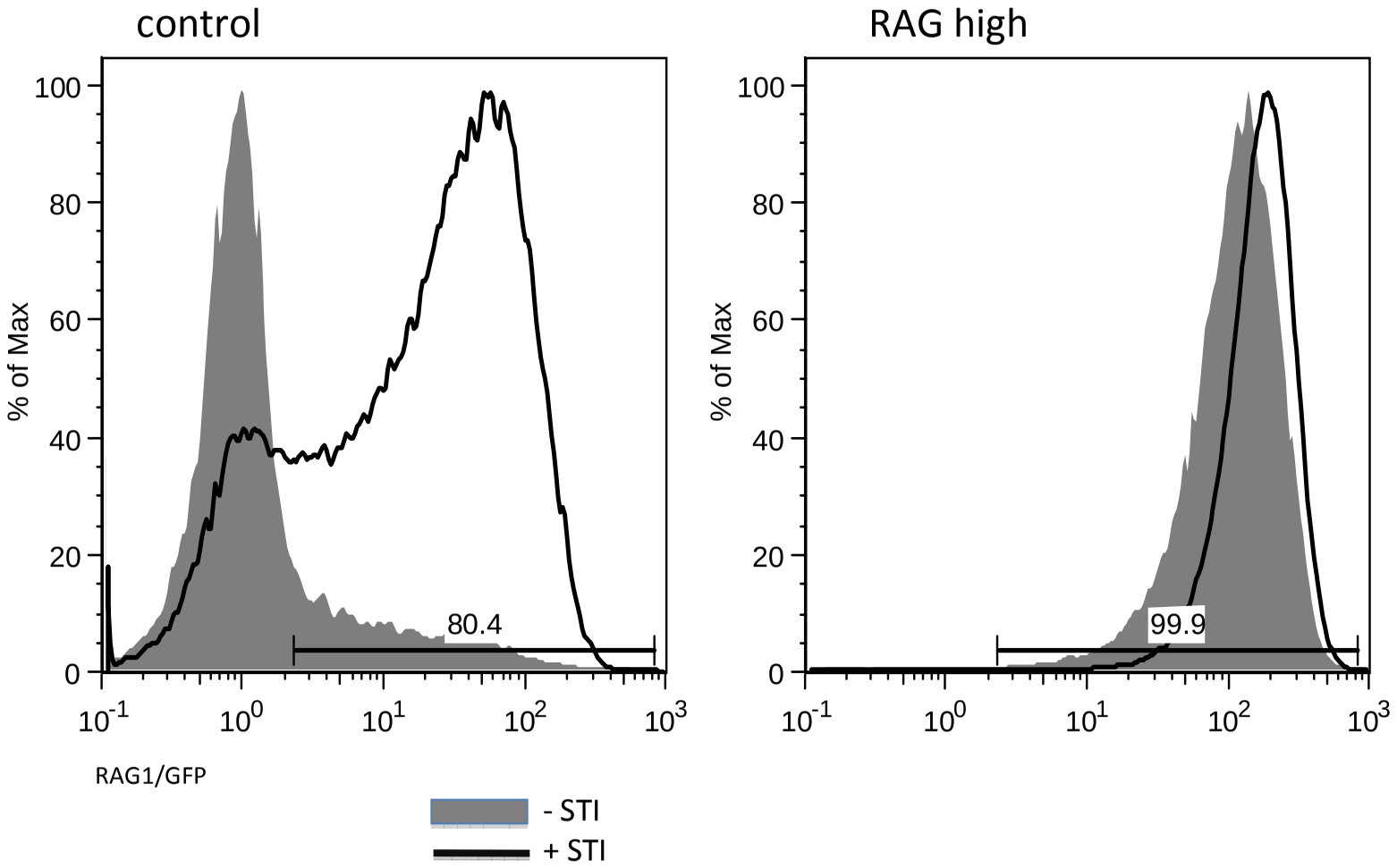


Figure 3 Flow cytometry of two RAG1/GFP single cell clones treated with 2.5 μM STI-571 for 16 hours.

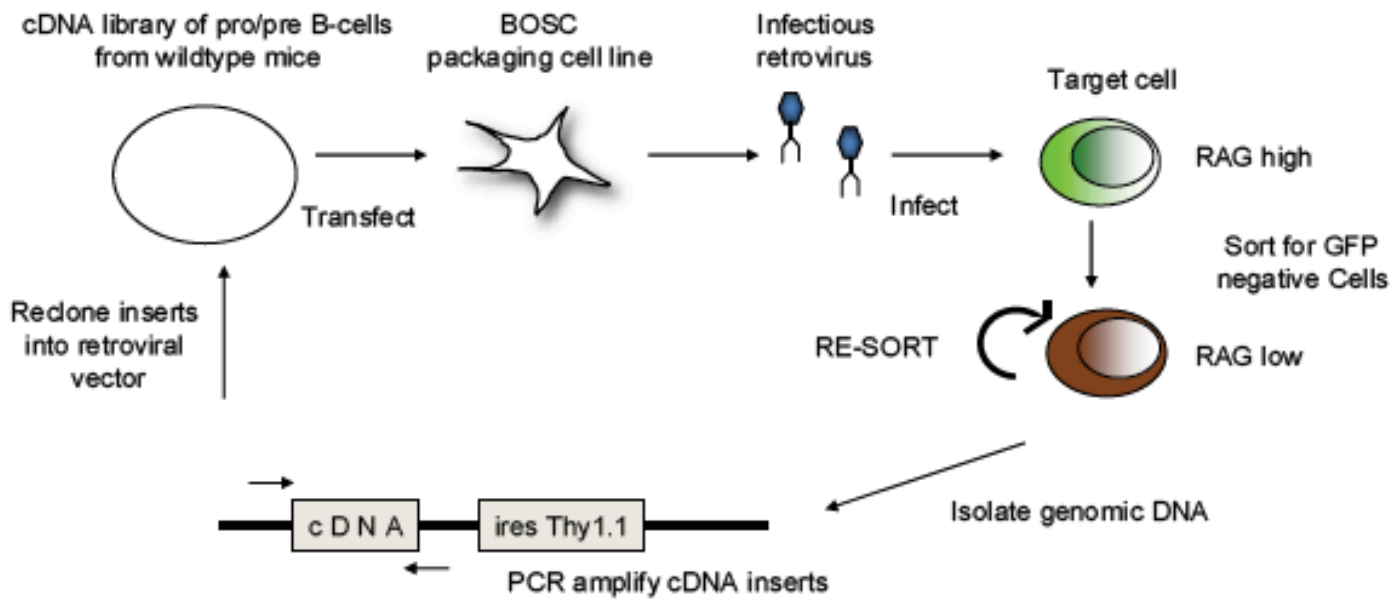


Figure 4. Schematic for the cDNA overexpression screen. Briefly, cDNAs cloned from pro and pre-B cells of wt mice are expressed in a retroviral vector and infectious retrovirus generated from these cDNAs was used to infect a RAG1/GFP reporter cell line. Thy1.1 positive, GFP negative cells were sorted, cDNA inserts were amplifed and cloned into a retroviral backbone for successive rounds of enrichment.

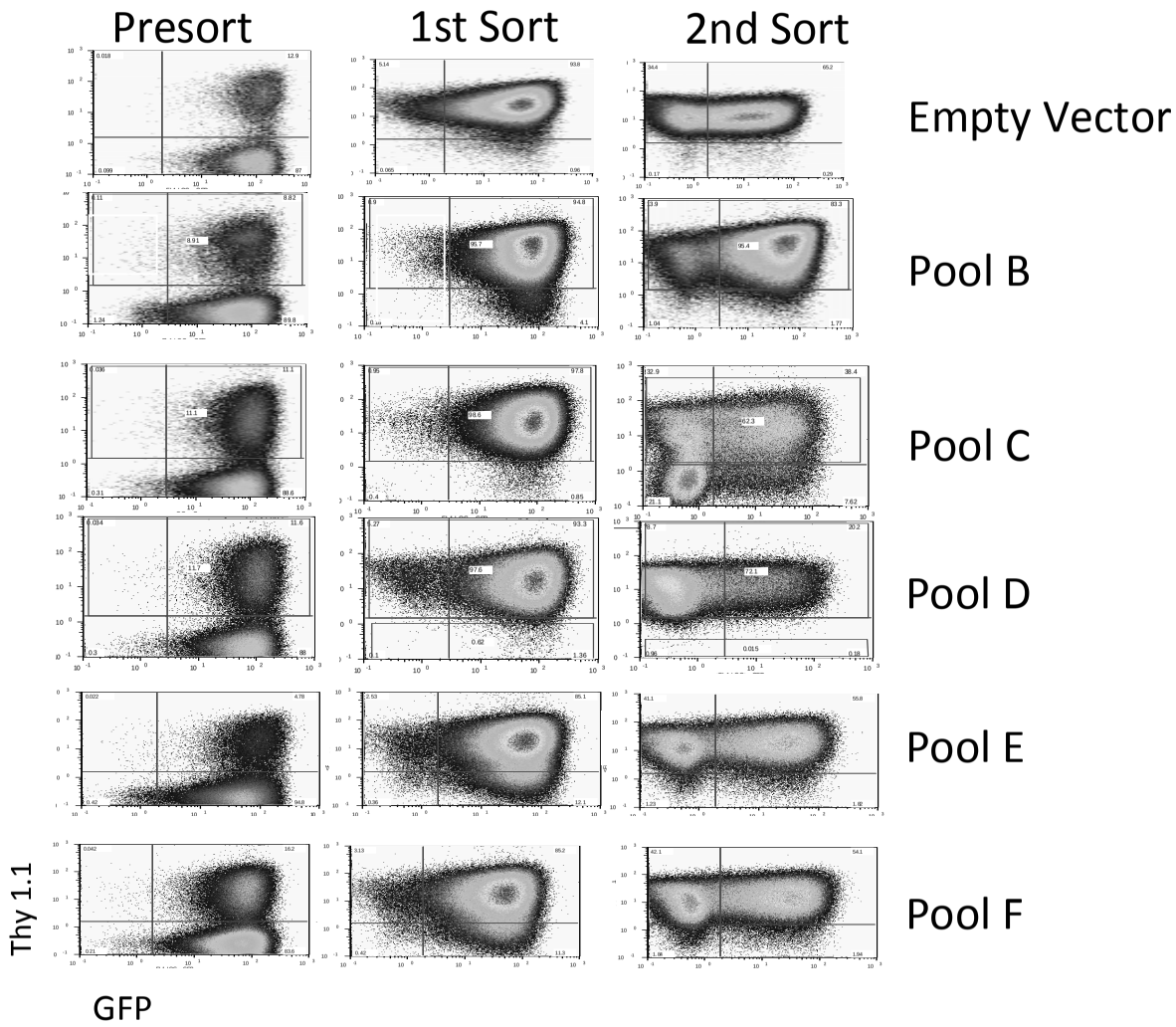
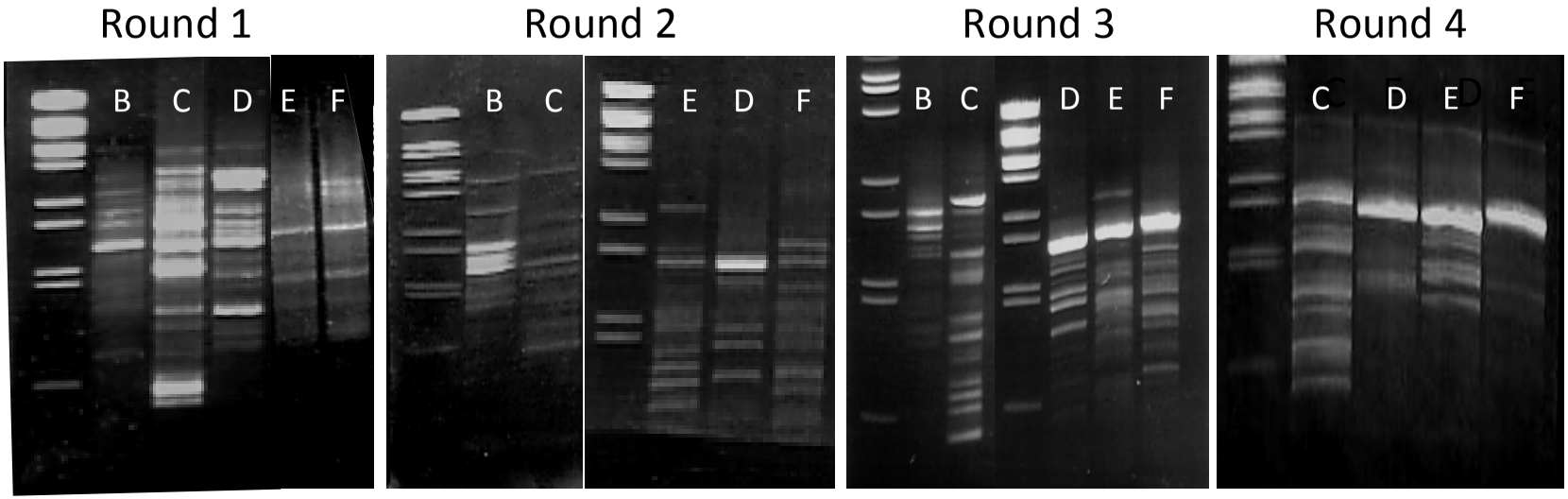


Figure 5 : Successive sorting leads to an enrichment of RAG1/GFP negative cells**.** Flow cytometry of RAG1/GFP high cells infected with 5 separate pools of cDNAs or an empty vector. Infected cells express the Thy1.1 marker. Th1.1+, RAG1/GFP negative cells were sorted twice for each pool.

A.



B.

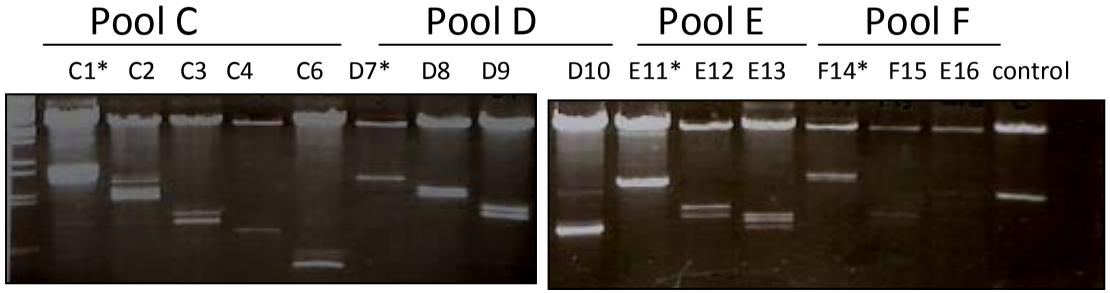


Figure 6 Some cDNAs become enriched over multiple rounds of screening. A. PCR amplicons for cDNAs in sorted RAG1/GFP low cells over multiple rounds of screening. B. Pools of similarly sized cDNAs were individually cloned to test within the parental line. Asterisk indicates those clones later shown to have RAG1/GFP suppressing activity.

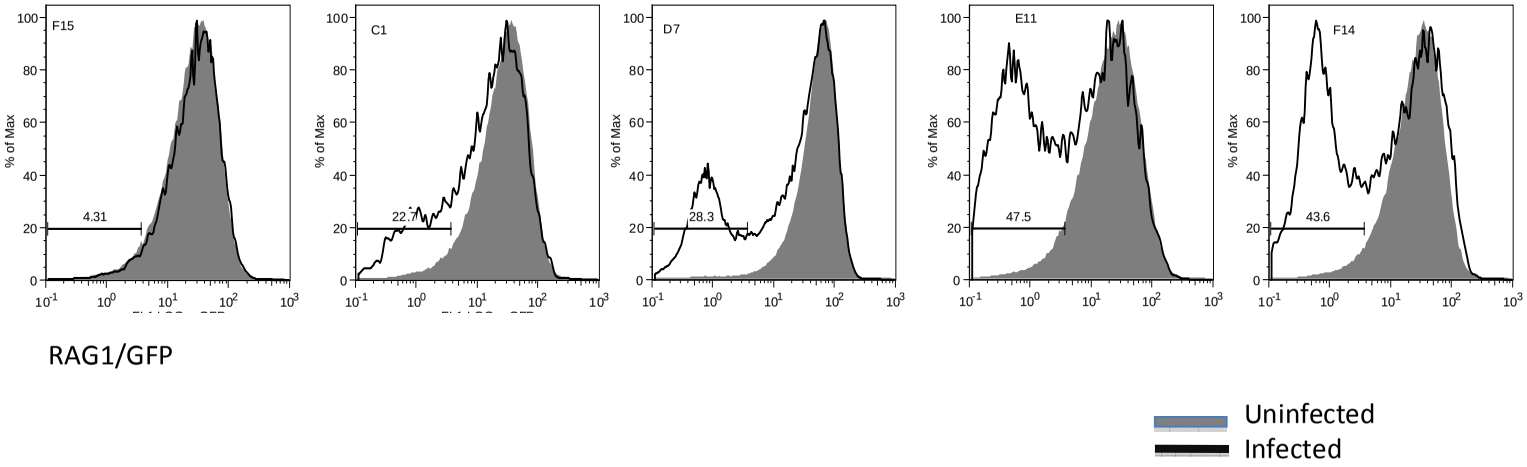


Figure 7. Flow cytometry of RAG high cells infected with subpools of clones amplified from sorted, RAG1/GFP negative cells. The subpool is indicated in the upper left of each plot.

Table 1: Gene identities of cDNA clones enriched in RAG1/GFP negative cells after 4 rounds of screening. Columns 3, 4, and 5 refer to the number of times a given clone was independently sequenced.

Gene Name	Gene ID	1	>1	>15
Gfi1b	14582			X
Abtb1	80283		X	
Aldolase A	11674		X	
AKT	11651		X	
Proteasome subunit beta type 1	19170		X	
Ribosomal protein L12	269261		X	
Zinc finger protein 91	109910		X	
Sin3B	20467	x		
Polymerase delta 1	18971	x		
Msto 1	229524	x		
F box and WD40 domain protein 9	68628	x		
Ring finger protein 123	84585	x		
Similar to Inositine 5-phosphate dehydrogenase 2	382049	x		
Uncoupling protein 2	22228	x		
Ferritin light chain 1	110270	x		
Hematopoietic cell specific-Lyn substrate 1	15163	x		
Sorting assembly machinery component 50 homolog	68653	x		
KCNN3	140493	X		

Table 2: Gene identities of cDNAs amplified from RAG1/GFP negative single cell clones. Columns 3, 4, and 5 refer to the number of times a given clone was independently sequenced.

Gene Name	Gene ID	1	>1
Gfi1b	14582		X
DEAD box polypeptide 56	52513		X
Ribosomal protein L12	269261		X
Ribosomal protein S10	64657		X
Aldolase A	11674		X
Zinc finger protein 91	109910		X
SFRS7	225027	X	
STPK	100047666	X	
Ribosomal protein S15	66407	X	

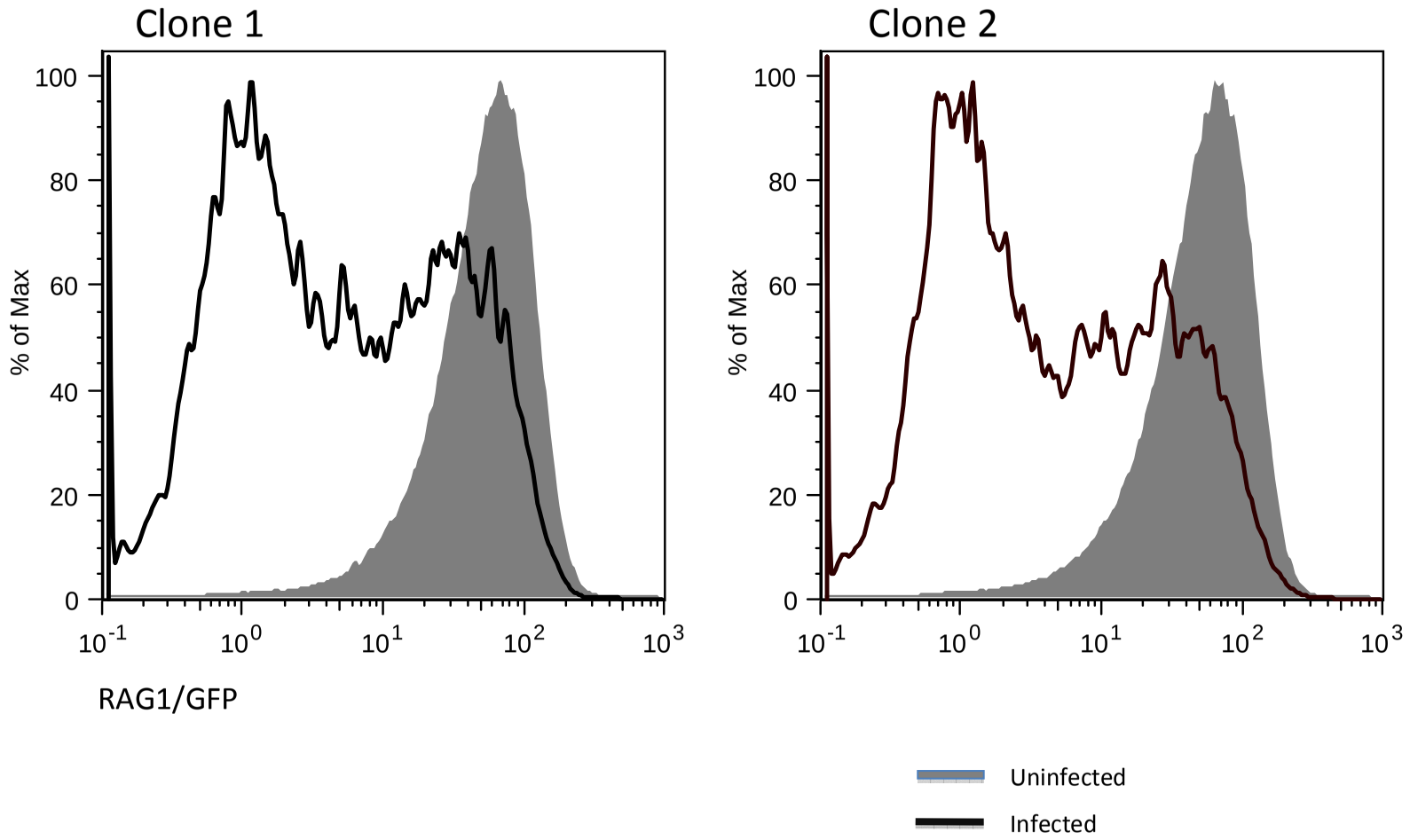


Figure 8: Flow cytometry of RAG high cells infected with two representative rescued cDNAs encoding Gfi1b.

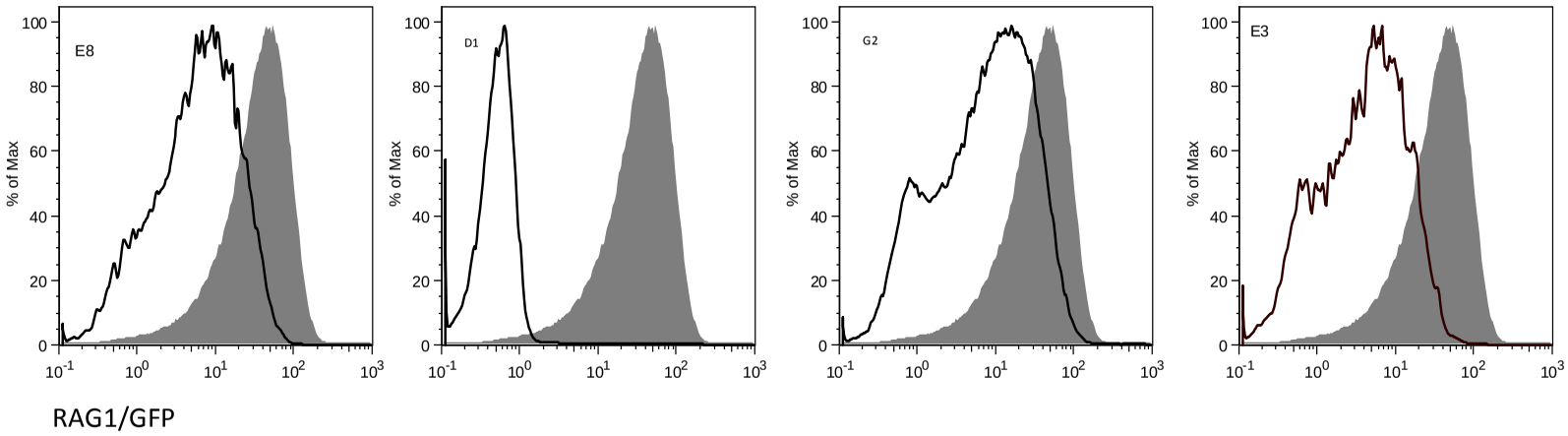


Figure 9: Flow cytometry of Sorted thy1.1 RAG1/GFP negative RAG high cells. Four representative examples are shown.

CHAPTER 3 Gfi1B and its Role in RAG Regulation

BACKGROUND:

Gfi family members and disease

Gfi1b is a member of the Gfi (growth factor independent) family of transcriptional repressors that also includes Gfi1. Gfi1 was first discovered as a pro-oncogenic retroviral insertion site in a screen for retroviral insertions that conferred IL-2 independence in T cell thymomas (1). It was subsequently found to be a common integration site in AKXD T cell lymphomas (2) and to cooperate with c-myc and pim-1 in T cell lymphomagenesis (3). Mutations in *Gfi1* are found in patients with acute myeloid leukemia (AML) (4) and Gfi1 is frequently overexpressed in chronic myelogenous leukemia (CML) (5). Gfi1b and a particular splice variant of the protein has also been found to be highly expressed in patients with acute and chronic leukemias (CML and AML) (6). It is also overexpressed in erythropoietic and megakaryocytic malignancies and appears to increase proliferation of tumor cells in these diseases (7). Gfi1 has also been implicated in severe congenital neutropenia (SCN), as patients with disease are frequently found to have mutations in the gene (8). Finally, Gfi1 deficiency can result in autoimmunity, as Gfi1 deficient B cells in the periphery show a hyperproliferative defect, as well as an abnormal expansion of plasma cells, IgG deposits in the brain and kidney, and an overabundance of nuclear-specific autoantibodies (9).

Gfi protein structure

Gfi1 is a 55 kDa protein, while Gfi1b is 37 kDa. Gfi1 and Gfi1b recognize the same consensus site, TAAATCAC(A/T)GCA, with AATC acting as the core binding site. Both proteins share a conserved C terminal zinc finger domain that binds directly to DNA (10). Crystallography indicates that Gfi proteins bind the major groove of DNA, in a manner similar to the binding of many canonical C(2)H(2) zinc finger domains (11). In addition. both proteins share an identical N-terminal SNAG domain that is responsible for their association with various cofactors (12).

Gfi family members' mechanism of repression

Gfi transcription factors associate with chromatin modifiers such as HDACs (HDAC1 and 2), histone demethylases (LSD1), and histone methyltransferases (G9a and suv39H1), through their N-terminal SNAG domains in order to repress their target loci (13-16). Gfi1 interacts with ETO, a factor which attaches to the nuclear matrix, and both Gfi1 and Gfi1b can be localized to the nuclear matrix, where they interact with HDACs (13). Gfi1b binds to gamma satellite regions of pericentric heterochromatin and Gfi1b deficient cells isolated from fetal liver have lower levels of H3K9 trimethylation and fewer heterochromatic structures (16).

Gfi1b can bind DNA without recruitment of these cofactors in a cell context-dependent manner, and there is evidence that binding without recruitment of cofactors does not result in transcriptional repression (17). In some cases, Gfi1 interacts with the tumor suppressor PRDM5, which is also capable of recruiting G9a and HDACs (18).

Gfi family member expression

Mouse models in which GFP has been knocked into either the *Gfi1* or *Gfi1b* locus have elucidated where these proteins are expressed within the hematopoetic system. Gfi1 is highly expressed in early B cells and early T cells, where its activity peaks at the pre-TCR stage. Gfi1 expression is absent in mature B and T cells, but is re-induced when T cells become activated (19). Gfi1 is also expressed in Hematopoetic Stem Cells (HSCs), Common Lymphoid Progenitors (CLPs), as well as in monocytes, granulocytes, and their progenitors. It is absent in Common Myeloid Progenitors (CMPs) as well as in megakaryocytes, erythroid cells, and their progenitors (20).

Gfi1b is also expressed in early B and T cells, but it is not induced upon T cell activation. Unlike Gfi1, it is not expressed within the macrophage/granulocyte lineages. Instead, it is expressed in erythroid and megakaryocyte cells and their monocyte progenitors (MEPs) (21).

Gfi1 and fate decision

Several groups have demonstrated that Gfi1 is a critical regulator of fate decision within the hematopoietic system. One of the mechanisms for this regulation is the promotion of the B cell fate by Gfi1 through repression of PU.1(22). At high levels, PU.1 represses Gfi1 and promotes macrophage development through the induction of EGR factors, while at lower levels, mixed differentiation into macrophages and neutrophils is observed (23). Promotion of the B cell fate by Gfi1 is enhanced by the expression of Ikaros, which promotes Gfi1 expression and inhibits PU.1 expression (22). Gfi1 has also been shown to suppress the HoxA9-Pbx1-Meis1 progenitor program (24) and antagonize the macrophage/monocyte program (25), while simultaneously inducing the granulopoietic transcription program (24) during myeloid development. Gfi1 deficient mice accumulate immature monocytes and fail to produce granulocytes (26), while overexpression of Gfi1 promotes differentiation of granulocytes and blocks macrophage differentiation (27). miR21 and miR126b, two miRNAs shown to be important for myelopoiesis, are also regulated by Gfi1 (28).

Gfi1 additionally has a well-established role in the development of neutrophils. *Gfi1* knockout mice have severe neutropenia (26) and Gfi1 has been shown to be required for neutrophil development (25). Several mutations in *Gfi1* have been observed in patients with SCN (8). Finally, development of dendritic cells (DCs) depends on the expression of Gfi1, as mice lacking this protein show defective

DC maturation and an overabundance of macrophages (29). Gfi1 deficient mice exhibit decreased thymic cellularity due to reduced proliferation, increased apoptosis and an early block at the DN stage of development. This block is due largely to overexpression of ID1 and ID2 and results in an early recombination defect. Gfi1 deficient mice also have a skewed ratio of CD4+/CD8+ cells, with CD8+ accumulating to higher than normal levels (30). Schwartz et al subsequently demonstrated that both Gfi1 and Gfi1b lie downstream of E47 in T cells (31).

Gfi1 and T cells

A number of mouse models have helped define Gfi1's role within the T cell compartment. Expression of a *Gfi1* transgene results in a block at the double negative stage of development (32), and its overexpression leads to accelerated proliferation and inhibited cell death in activated T cells (33). The inhibited cell death phenotype is probably due to Gfi1 repression of Bcl2 members Bax and Bac (34, 35). Gfi1's role in T cell activation is due, in part, to its interactions with the splicing factor U2AF26, which leads to alternative splicing of the CD45 tyrosine phosphatase (36). *Gfi1* transgenic mice also have a potentiated response to both TCR stimulation and IL-2 (37). Gfi1 appears to be especially important for the differentiation of Th2 cells, and operates downstream of IL-4 induced STAT6 (38). There is also evidence that Gfi1 acts downstream of the MAPK/ERK pathway to stabilize GATA3 during Th2 differentiation (39). In a conditional *Gfi1* knockout model, there is a block in Th2 expansion (but not in Th1) following IL-2 induction in Gfi1 deficient cells that can be rescued by overexpression of STAT5 (40). Quite recently, Gfi1 has been shown to have a role in repressing differentiation of activated T cells to Treg and Th17 cells by binding to the intergenic region of IL-17 and repressing its transcription. Downregulation of Gfi1 expression by TGF-β is necessary for differentiation to Th17 and CD103+ inducible regulatory T cells (41).

Gfi1 and HSCs

An exciting new development in the Gfi field has been the discovery that Gfi proteins are involved in Hematopoietic Stem Cell (HSC) homeostasis. Reports first appeared in 2004 showing that in the absence of Gfi1, HSC frequency is reduced and differentiation to the CLP stage is severely impaired. *gfi1-/-* transplanted cells showed an abnormally high number of cycling HSCs and were functionally compromised in these assays (20, 42). It appears that one important biological role for Gfi1 within hematopoesis is to restrain proliferation of HSCs. Gfi1 was subsequently found to work downstream of p53, a factor shown to be important for stem cell quiescence (43). In addition, Gfi1b was found to be a target of Scl/Tal1 in HSCs`, a factor that controls specification`, and subsequent differentiation of HSCs into the erythroid and megakaryocytic lineages (44). Knocking out the HSC factor cdc42 leads to upregulation of Gfi1 (45). However,

some repression of Gfi1 is necessary to prevent myeloid development from HSCs. PLZF is thought to control Gfi1 levels in these cells (46).

Autoregulation

Both Gfi1 and Gfi1b are able to regulate each other, as well as autoregulate. This phenomenon was first reported in 2003 when both Gfi1 and Gfi1b were shown to repress Gfi1 (47). Subsequently, it was shown that Gfi1b can repress itself as well as Gfi1 (48). In an *Lck-Gfi1* transgenic mouse model, expression of the G*fi1* transgene silenced endogenous *Gfi1* in T cells (19), while a *vav* driven *Gfi1b* transgene silenced endogenous *Gfi1b* in the spleen, but not in the bone marrow (48).

Mouse models of Gfi1b

Studies on Gfi1b have been hampered by the fact that knocking out Gfi1b in the mouse results in embryonic lethality at E15, due to a complete failure in these animals to develop red blood cells. *Gfi1b* knockout mice also fail to develop megakaryocytes, though myelopoiesis is relatively normal. Arrested erythroid and megakaryocyte precursors are found in the fetal liver of these animals (49). *Lck* driven *Gfi1b* transgenic animals show defects in T cell activation, reduced IL-7Rα expression, and a decrease in the number of CD8+ T cells (37).

Gfi1b's Regulation of Various Factors

Overexpression of Gfi1b in human hematopoetic progenitor CD34+ cells leads to a drastic expansion of erythroblasts, through a mechanism that appears to enhance the proliferation of immature erythroblasts through sustained expression of GATA2 (50). Gfi1b is also known to interact with GATA-1 and it can suppress GATA-1 mediated stimulation of its own promoter through a direct protein interaction to restrict levels of Gfi1b in erythroid cells (51, 52). GATA-1 expression in erythroblasts leads to induction of Gfi1b, which in turn, represses BCL-XL. Gfi1b must be downregulated late in differentiation to relieve this repression in late erythroblasts (53). Gfi1b interacts with ETO-2/SCL in megakaryocytes (54) and in red blood cells precursors, and this association stops during differentiation of red blood cells when SCL activity is required (55). Recently, Randrianarison-Huetz et al. found that Gfi1b regulates TGFβ signaling at the erythro/megakaroyctic progenitor stage (56). Finally, knockdown of Gfi1b in human K562 cells delays terminal differentiation, and forced expression of Gfi1b in these cells leads to a proliferation arrest and differentiation (57).

Gfi family members and B cells

Both Gfi1 and Gfi1b are expressed in the early stages of B cell development (19, 21, 58). Deletion of *Gfi1* in the mouse results in a defect in differentiation from lin-, sca+, c-kit+ (LSK) multipotent progenitors (MPPs) to early B220+ B cells

(59). In addition, defects in IL-7 receptor cascades at the pro-B cell stage in *Gfi1* knockout mice results in a slight block in development at this stage. While Gfi1's effect on B cell development has been well characterized (59), determining Gfi1b's role within the B cell compartment has been hampered by the fact that knocking out *Gfi1b* in the mouse results in an embryonic lethal phenotype due to failure of these animals to produce definitive enucleated cells (49). Indeed, Gfi1b has been shown to be critical for development of MPPs into the erythroid and megakaryocytic cell lineages (49, 50).

In this study, we demonstrate that Gfi1b is able to downregulate RAG proteins in primary and transformed B cells. This downregulation depends on an intact SNAG domain and may be mediated partially through the transcriptional activator FoxO1. Deletion of *Gfi1b* leads to high levels of rearrangement and the generation of multiple double strand breaks per cell. Finally, Gfi1b overexpression causes widespread transcriptional changes within the B cell compartment as measured by microarray analysis.

MATERIALS AND METHODS

Cell culture:

Cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FCS, L-glutamine (2 mM), penicillin (100 g/ml), streptomycin (100 g/ml) and 2-mercaptoethanol (50 mM) and were grown at 37 °C in 5% CO_2.

Retroviral Production and Infection:

Retrovirus was harvested from the EcoPack2 packaging cell line (Clontech). EcoPack2 cells were transfected with retroviral plasmid resuspended in Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and viral supernatant was collected and filtered 48 h post-transfection.

AMuLV-transformed *Rag1*/GFP pro–B cells were infected by resuspension of the cells in viral supernatant containing polybrene (4 g/ml; Sigma) and cultured overnight. Cells were then expanded into normal media.

For primary cell infection, bone marrow was harvested from 1- to 2-month-old mice and lymphocytes were enriched by density centrifugation with Histopaque-1083 (Sigma). 10 million bone marrow cells were then cultured for 1-2 days in standard RPMI media as above with 10% FCS and recombinant IL-7 (2ng/ml; R&D systems). Cultured cells were then resuspended in viral supernatant with polybrene and centrifuged at 32^{o}C for 1.5 h at 2300 RPM. Cells were incubated at 37^{o} C overnight and then resuspended in fresh viral supernatant with polybrene for an additional 24h before being diluted into normal RPMI. Cells were labeled with anti-IgM (II/41), anti-B220 (RA2-6B2) and anti-CD43 (S7), plus anti-Thy-1.1 (OX-7). Cells were analyzed 3–4 d after retroviral infection.

Lentiviral constructs were co-transfected with VSVG and pMD2G into 293T cells using Lipofectamine 2000 as described above and virus was harvested and used to infect cell lines as described above.

Generation of AMuLV transformed knockout cell lines:

MxCre tg Gfi1$^{fl/fl}$ or Gfi1b$^{fl/fl}$ (or combined mutants) were injected with pIpC (Sigma) at a dose of 500 µg per injection every other day for a total of 5 injections. Bones from *MxCre tg Gfi1$^{fl/fl}$ or Gfi1b$^{fl/fl}$* (or combined mutants) mice were generously provided by Tarik Moroy . Bone marrow was infected as described above with the AMuLV previously described in (60). Cells were cultured for 4-12 weeks in standard RPMI until transformed cells grew out. The *gfi1-/-, gfi1b-/-* double knockout cell line was generated by treating AMuLV transformed *MxCre tg Gfi1$^{fl/fl}$ Gfi1b$^{fl/fl}$* cells with 3680 units of β-interferon from PBL Interferon source (catalogue 12400-1), single cell cloning, and screening for double knockouts by PCR using primers listed in Table 3.

Gene Expression Analysis. RNA was isolated by lysing cells in TRIzol reagent (Invitrogen) followed by chloroform extraction. Reverse transcription was performed using MoMLV-RT (Invitrogen) according to manufacturers instructions. Quantitative real-time PCR was performed using JumpStart Taq polymerase (Sigma) according to the manufacturer's protocol and fluorescent labeling with EvaGreen (Biotium). PCR cycling conditions were 95 °C for 4 min followed by 45 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1 min. Primer sequences are given in Table 3.

Expression plasmids.
All retroviral plasmids were based on the MSCV retroviral vector and were modified to contain an IRES in-frame with a surface marker protein (Thy-1.1, or human CD4) to 'mark' retrovirus-infected cells. The cDNA was cloned upstream of the IRES sequence.

The Gfi1b-ER fusion construct was created by PCR amplification of the estrogen receptor hormone-binding domain and amplification of the ORF of Gfi1b from the cDNA library. *Pfu* TurboUltra (Stratagene) was used for PCR according to the manufacturer's protocol and fragments were cloned into the MSCV retroviral vector upstream of IRES thy1.1. Constructs were subsequently verified by DNA sequence analysis. The position 2 proline to alanine mutation (P2A)-mutated Gfi1b construct was created by PCR amplification with a primer containing the mutation.

The Gfi1 cDNA was generously provided by Tarik Moroy, PCR amplified with Pfu as described above, and cloned upstream of the IRES Thy1.1 within the MSCV retroviral vector.

The rearrangement reporter construct was created by Pfu mediated PCR of the Eμ heavy chain enhancer and a V_HKI promoter (61) and subsequent insertion

into the pMX-delCJ (62) rearrangement reporter. The ires was inserted upstream of GFP using appropriate restriction sites. The Eμ, V_HKI, ires-GFP, RSS 12, RSS23, and hCD4 fragments were excised in a single unit and inserted into the pLV-UT-tTR-KRAB lentiviral vector obtained from Addgene.

MSCV-FoxO1-ires-hCD4 was generously provided by Rupesh Amin (63).

Drugs:

Tamoxifen (Calbiochem) was used at a concentration of 1μM. STI-571 (Novartis) was used at 2.5μM. Aphidicolin (Sigma) was used at a concentration of 4 μM.

ChIP and ChIP-chip.

Chromatin immunoprecipitation was performed as described in (64). Briefly, 100 million cells were fixed with formaldehyde, sonicated, incubated with either an anti-FLAG or IgG control antibody (Sigma), collected using magnetic, protein G beads (Invitrogen cat no. 100.04D) and 3 times with low salt buffer, once with high salt buffer, and once with LiCl buffer as described in (64). DNA-protein crosslinks were reversed, and DNA was precipitated and subjected to quantitative real-time PCR using primers listed in Table 3. For ChIP-chip, DNA was fragmented and ligated using the Whole Genome Amplification kit from Sigma. Samples were labeled and hybridized to a custom genome tiling array generated by Nimbelgen.

Rearrangement and LM-PCR:

Genomic DNA was isolated and subjected to 30 cycles of PCR using the VκS and Jκ1 primers listed in Table 3. For LM-PCR, broken ends were ligated to the BW linker and then amplified with the BW-H and k05 primers (Table 3) as described in (65). Ligated DNA was subjected to 12 cycles of PCR with the following cycling conditions 94C for 1 min; 66C for 2.5 min. BW-H and ko3 primers (Table 3) were used to amplify 2 μl of DNA from the first reaction for 30 cycles under the same cycling conditions.

Flow Cytometry

Single-cell suspensions depleted of red blood cells were prepared from mice or from cultured cells and were incubated for at least 10 min with Fc receptor–blocking antibody (2.42G; purified from a hybridoma supernatant) and then were labeled with fluorochrome- or biotin-conjugated antibodies by standard techniques. A FC500 or an Elite XL flow cytometer (Beckman Coulter) was used for flow cytometry; a MoFlo high-speed cell sorter (Dako-Cytomation) was used for sorting. Data were analyzed with FlowJo software (Tree Star) and, with the exception of cell cycle analyses, dead cells were gated out using forward and side scatter for all analyses. All antibodies were from eBiosciences, except anti-CD43 and anti-Thy-1.1 (both from BD Pharmingen).

Immunoblot:

AMuLV-transformed pro–B cells were lysed in Rapid ImmunoPrecipitation Assay (RIPA) buffer (66), analyzed by Bradford, centrifuged to clear insoluble material, and boiled for 10 minutes. Lysate was separated by 8% or 10% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked in 5% milk and labeled with primary and secondary antibodies according to the manufacturer's instructions. Membranes were analyzed with the Odyssey Infrared Imaging System (LI-COR Biosciences). Anti-FoxO1 (9462) was obtained from Cell Signaling Technologies, anti-Gfi1b (sc-8559) anti-actin (sc-1615) were obtained from Santa Cruz Biotechnology. Anti-FLAG (F1804) and anti-tubulin (T3526) antibodies was obtained from Sigma. Infrared dye–conjugated secondary antibodies were from Molecular Probes–Invitrogen.

Immunofluorescence:

Cells were affixed to frosted X slides using a cytospin. Cells were then fixed with 4% paraformaldehyde and blocked before staining with anti-H2AX (Abcam ab11174). Cells were washed and stained with DAPI and anti-rabbit infrared dye-conjugated secondary antibody from Molecular Probes-Invitrogen. Cells were visualized with a Nikon Eclipse E800 microscope.

RESULTS

Using the library screening strategy described in chapter one, we found that overexpression of a cDNA encoding the zinc finger transcriptional repressor, Gfi1b, led to a striking downregulation of RAG1/GFP expression in RAG High cells (Figure 1). To ensure that this phenomenon was not cell line specific, we overexpressed Gfi1b in a distinct wild-type AMuLV pro-B cell line (PD31) and compared RAG1 and RAG2 transcript levels in infected and uninfected cells using quantitative RT-PCR. We found that both RAG1 and RAG2 transcript levels were lower in cells overexpressing the Gfi1b cDNA when compared to uninfected cells (Figure 2). It should be noted that the experiment in Figure 2 was performed with a Gfi1b construct containing a portion of the 5'UTR, and thus the downregulation of *RAG1* and *RAG2* transcription was slightly attenuated when compared to that achieved with overexpression of an ORF only version of Gfi1b. Because Gfi1 is closely related to Gfi1b (67), we tested whether Gfi1 overexpression could downregulate RAG1/GFP. We found that while Gfi1 expression does lead to a transient downregulation of RAG1/GFP expression, this effect is not stable, and RAG activity is largely restored after one week (Figure 3). This is likely to do the fact that Gfi1 high expressing cells gradually disappear from the population over time (Figure 4).

The percentage of thy1.1 expressing cells in cultures of RAG high cells infected with a Gfi1b overexpression construct decreases over time, indicating that Gfi1b

overexpression confers some form of growth disadvantage in these cells (Figure 5). In light of this, we decided to further probe the role of Gfi1b by engineering an overexpression vector containing Gfi1b fused to a mutant form of the human estrogen receptor hormone binding domain (Gfi1b-ER) so that Gfi1b overexpression could be induced upon the addition of tamoxifen (OHT) to the culture medium. Addition of tamoxifen to RAG high cells overexpressing the Gfi1b-ER construct caused an approximately 3 fold downregulation of both RAG1 and RAG2 transcripts within 12 hours in 3 biological replicates (Figure 6A).

Since RAG transcript levels are transiently downregulated at the early pre-B cell stage of development, we hypothesized that downregulation of RAG by Gfi1b might be reversible. To test whether this was the case, we treated Gfi1b overexpressing RAG High cells with STI-571 (Figure 6B). We observed that downregulation of RAG1/GFP by Gfi1b was almost completely reversed upon addition of the drug. In addition, washing out tamoxifen from the culture medium of Gfi1b-ER expressing cells completely restored RAG1/GFP expression after cells were put back in culture for 9 days (Figure 6C), indicating that Gfi1b's effect on RAG transcription is completely reversible.

We next asked whether Gfi1b overexpression modulates RAG transcription in primary developing B cells. We infected cultured total bone marrow isolated from RAG1/GFP reporter mice with a Gfi1b overexpressing, ires thy1.1-marked retrovirus and compared levels of RAG1/GFP in these cells to bone marrow cells infected with an empty vector. We found that RAG1/GFP levels were much lower in early B cells that overexpressed Gfi1b when compared to those infected with the empty vector (Figure 7A). In addition we asked whether overexpression of Gfi1 in early B cells would cause RAG downregulation. We found that cultured early B cells from total bone marrow overexpressing Gfi1 had significantly lower levels of RAG1/GFP compared to cultured cells overexpressing an empty vector (Figure 7A).

To test whether lower RAG levels in Gfi1b overexpressing bone marrow cells impairs their ability to differentiate to the IgM positive stage, we compared numbers of IgM positive cells that accumulated four days post infection in bone marrow cultures infected with a Gfi1 overepressing virus, a Gfi1b overexpressing virus, or an empty vector. We found that fewer IgM positive, Gfi1b overexpressing cells accumulate when compared to those infected with the empty vector, and that this is also true for Gfi1 overexpressing cells (Figure 7B). These data indicate that deregulated Gfi1 or Gfi1b levels can lead to impaired accumulation of IgM positive cells in these cultures. Whether this effect is directly related to Gfi1b's effect on RAG levels or due to secondary effects remains to be explored.

Gfi1b binds directly to the *RAG* locus

We first tried to the test the idea that Gfi1b directly downregulates RAG expression by treating Gfi1b-ER overexpressing RAG high cells with tamoxifen in the presence or absence of cycloheximide, a protein synthesis inhibitor (Figure 8). We expected that if the effect was direct, Gfi1b would downregulate RAG expression in the presence of the drug. However, because RAG mRNA is extremely labile when cells are treated with cycloheximide, the results were difficult to interpret because RAG transcription decreased even without the addition of tamoxifen, although levels of RAG2 were lower when both drugs were added (Figure 8).

We therefore decided to test whether Gfi1b directly downregulates RAG transcription using an alternate method, and performed a ChIP-Chip analysis using a FLAG epitope-tagged Gfi1b construct overexpressed in both the RAG High cell line as well as a *Gfi1b* null cell line. We found high levels of Gfi1b binding at a region ~35kb upstream of the *RAG2* first exon in a region neighboring the B cell specific enhancer element *Erag* (68) in both cell lines tested (Figure 9A). To confirm these results, we performed ChIP experiments with these same cell lines using an anti-FLAG or IgG control antibody followed by quantitative real-time PCR using three primer sets specific to the peak of Gfi1b binding noted above and two primer sets lying outside the peak (Figure 9A). We found that Gfi1b binding within the peak is much higher compared to the regions on either side of the peak (Figure 9B).

RAG regulation by Gfi1b requires chromatin modifying complexes and DNA replication.

Previously it had been established that target gene repression by Gfi1b is mediated through its association with cofactors that introduce local chromatin modifications (15). Because Gfi1b associates with chromatin modifying proteins through its N-terminal SNAG domain (67), we tested whether mutating this domain would abrogate Gfi1b 's effect on RAG transcription. Mutation of amino acid 2 in Gfi1b from a proline to an alanine (P2A mutation) does not disrupt its DNA binding activity but does eliminate its association with chromatin modifying cofactors (15, 67). We found that overexpression of the P2A mutant fails to downregulate RAG1/GFP in RAG High cells (Figure 10A), Expression of the mutant version of P2A was confirmed by western blot (Figure 10B). It should be noted that the Gfi1b antibody recognizes the mutated N-terminal end of the protein, and thus may not recognize the large isoform. These data imply that Gfi1b's effect on RAG transcription depends on its association with chromatin modifying cofactors. In some systems, changes in patterns of epigenetic gene regulation require DNA replication (69). To test whether this was the case with respect to Gfi1b's effect on RAG transcription, we asked whether treating RAG High cells with a DNA replication inhibitor would affect Gfi1b's ability to downregulate RAG transcript levels. While tamoxifen induction of Gfi1b activity resulted in a significant decrease in RAG mRNA levels, we found that this same

treatment had little effect on RAG transcription in the presence of the DNA synthesis inhibitor, aphidicolin (Figure 11). We went on to use chromatin immunoprecipitation (ChIP) to test whether Gfi1b overexpression alters the level of acetylated H3 at the *Erag* enhancer. We observed lower levels of H3 acetylation at *Erag* in Gfi1b overexpressing cells (Figure 12). Taken together, these data imply that Gfi1b's effect on RAG transcription is mediated primarily through a chromatin modifying mechanism.

Gfi1b deficiency results in super-physiologic levels of RAG expression

We reasoned that if overexpression of Gfi1b causes downregulation of RAG transcription, then cells lacking Gfi1b should have higher levels of endogenous RAG expression. To test this idea, we infected cultured bone marrow from poly-IC treated, *Gfi1b* floxed, MX-Cre mice with AMuLV to generate a *gfi1b*$^{-/-}$ transformed cell line. A *gfi1*$^{-/-}$ cell line was generated in parallel using bone marrow from a *Gfi1* knockout mouse. To create double knockout cells, AMuLV transformed, *gfi1*$^{-/-}$*, Gfi1b* floxed MX Cre cells were treated with interferon to delete *Gfi1b* and then single cell cloned and screened by PCR, which can discriminate between heterozygous and homozygous allele deletion (data not shown). RAG1 and RAG2 transcripts were expressed at a higher level in *gfi1b*$^{-/-}$ cells when compared to their wt or *gfi1*$^{-/-}$ counterparts (Figure 13A). To ascertain whether higher levels of RAG expression in these cells results in greater Ig gene rearrangement potential, we infected wild-type or *gfi1b*$^{-/-}$ AMuLV cell lines with a recombination reporter construct. Upon rearrangement, an ires-GFP sequence surrounded by two RSSs is deleted. This deletion brings a human *CD4* gene under control of a heavy chain gene promoter element. Cells having undergone a rearrangement event therefore lose GFP and gain hCD4 expression. We observed much higher levels of recombination in cells lacking Gfi1b when compared to wild-type cells (Figure (13B) 11B). Wild-type cells did rearrange the reporter construct when treated with STI-571 (data not shown). In addition, we detected higher levels of V-to-J rearrangements at the endogenous Ig *kappa* locus in these same mutant cells (Figure 13C), as well as higher levels of Jκ signal end breaks as measured by LM-PCR (Figure 13D). We did not observe higher levels of Jκ signal end breaks in *gfi1-/-* cells (Figure 13D) and levels of kappa rearrangement were comparable to wildtype cells (data not shown). We conclude from these experiments that higher levels of RAG transcripts in cells lacking Gfi1b result in a higher rearrangement potential in these cells, indicating that one role for Gfi1b in early B cell development may be to prevent deregulated rearrangement during periods of proliferation, and/or following production of a functional BCR.

Treatment of AMuLV transformed cells with STI-571 mimics key aspects of the transition from the large pro-B to the small pre-B stage in B cell development (70). To understand how loss of Gfi1b during this transition might affect RAG expression, we treated wild-type and *gfi1b*$^{-/-}$ AMuLV cell lines with STI-571 and

monitored RAG transcription over the course of the treatment by quantitative real-time RT-PCR. We found that RAG transcription was induced to strikingly high levels in cells deleted for *Gfi1b* (Figure 14). This leads us to hypothesize that the biological role for Gfi1b might be to limit RAG levels during rearrangement so that promiscuous DNA cleavage does not occur at rearranging loci, at cryptic RSSs, or elsewhere in the genome. To test whether global levels of double strand breaks are higher in cells deleted for *Gfi1b*, we performed immunofluorescence experiments using an antibody to phospho-H2AX, a modified histone found near double strand DNA breaks (71). We found higher numbers of p-H2AX foci per cell in *gfi1b*$^{-/-}$ cells when compared to wild-type cells (Figure 15A,B). Since RAG proteins are capable of generating double-strand breaks that could have a deleterious effect on cells, we interpret these data to indicate that Gfi1b's expression within the B cell compartment may prevent RAG proteins from reaching levels high enough for them to generate multiple off-target double-strand breaks per cell.

We went on to test whether the increased levels of RAG expression result in sufficient DNA damage to induce a G1 cell-cycle arrest in cells treated with STI-571. We performed PI staining on *gfi1b-/-* cells treated with STI-571 for a short (6h) or long (20h) period and compared the cell cycle profile in these cells to that of wildtype cells treated with STI-571. We observed that treatment with STI-571 leads to a much greater number of mutant cells in the G1 phase of the cell cycle compared to their wildtype counterparts (Figure 16). Correspondingly, many fewer cells were observed in S phase when Gfi1b was absent compared to wildtype cells after 20h of STI treatment (Figure 16). In addition, a much greater number of cells in the sub-G1 compartment was observed in cells lacking Gfi1b, indicating that increased numbers of breaks in these cells may lead to apoptosis (Figure 16). We verified that these cell cycle effects were specific to Gfi1b by reconstituting a *gfi1b-/-* cell line with Gfi1b and treating these cells with STI-571. Cell cycle profiles in the reconstituted cell line were nearly identical to those observed in the wildtype cell line (Figure 17).

Multiple modes of RAG inhibition by Gfi1b

We and others have shown that FoxO1 directly activates RAG transcription during B cell development (63, 72). We reasoned that Gfi1b's effect on RAG transcription might be mediated through an interaction with FoxO1 in addition to its direct effects on the *RAG* locus. We addressed this hypothesis by asking whether FoxO1 protein levels are altered when Gfi1b is overexpressed in AMuLV pro-B cells. We observed that total levels of FoxO1 protein and mRNA were decreased in cells overexpressing Gfi1b (Figure 18A and B). Conversely, levels of FoxO1 are higher in cells lacking Gfi1b but not in cells that lack Gfi1 (Figure 18C). To test whether Gfi1b's effect on RAG transcription is solely mediated through FoxO1, we sorted RAG High cells overexpressing Gfi1b and asked whether FoxO1 overexpression could restore RAG transcripts to a high level. We found that overexpression of FoxO1 led to a partial rescue of RAG

transcription (Figure 19), indicating that Gfi1b likely uses multiple pathways to down-regulate RAG transcription in early B cells. We surmise that direct binding of Gfi1b to the *RAG* locus (Figure 9) and indirect effects mediated by FoxO1 are both responsible for maintaining low levels of RAG protein at the appropriate stages in early B cell development.

ChIP-Chip analysis at the *FoxO1* locus revealed a peak of Gfi1b binding at the 5' intergenic region of *FoxO1* (Figure 20A). These results were confirmed using conventional ChIP and comparing enrichment of DNA sequences within the peak and in a region 3' of the peak that was not enriched in our ChIP-Chip analysis (Figure 20B).

We observed that a 20h treatment of cells lacking Gfi1b with STI-571 led to decreased levels of FoxO1 protein (Figure 21A), while this was not the case for wild-type cells. In addition, we detected a steady decrease in FoxO1 protein throughout the course of STI-571 treatment specifically in *gfi1b*$^{-/-}$ cells, but not in wild-type cells (Figure 22). If Gfi1b is required to limit RAG levels during rearrangement, then in its absence the cell may attempt to compensate for dangerously high RAG levels by decreasing the positive regulator, FoxO1 via an alternate mechanism. The fact that RAG levels remain quite high in the absence of Gfi1b (Figure 14A) indicates that decreasing FoxO1 levels is not sufficient to limit RAG levels during V(D)J recombination and that an additional factor is required. If one role for Gfi1b is to limit RAG levels during rearrangement, we would predict that in wild-type cells, Gfi1b would be upregulated following STI-571 treatment. To test this, we monitored Gfi1b protein levels during the course of STI-571 treatment and subsequent washout of the drug. We observed that Gfi1b protein levels increase in response to STI-571 treatment, and are restored to baseline levels once STI-571 is removed from the medium (Figure 21B). We verified that RAG levels were modified by STI-571 treatment using quantitative real-time PCR (Figure 23). We conclude from these data that there exists a biological sensor for RAG levels within the cell, and that when they increase beyond a particular threshold, repressors are induced to limit RAG expression.

Gfi1b controls a broad program of gene expression in AMuLV transformed pro-B cells.

Gfi1b has been shown to be a critical regulator of gene expression within the erythroid lineage. Indeed within this lineage it has been demonstrated using ChIP-Chip analysis that Gfi1b binds to a large number of genes (15). We asked whether Gfi1b has more global effects on gene expression in AMuLV transformed pro-B cells by performing a gene expression micro-array experiment in which Gfi1b-ER- expressing RAG high reporter cells were subjected to 12h of tamoxifen treatment. RNA was isolated from treated and untreated cells and transcript levels were compared via hybridization to an Affymetrix mouse gene chip. We found that approximately 300 genes showed significant differences in transcript levels ($p<.01$) when Gfi1b was overexpressed (Figure 24) as measured

by an Anova statistical test (73). Of these, only 54 showed changes that were 2 fold or higher (Tables 4 and 5). Several genes known to be important for B cell development showed significant changes on the array including *SpiB*, *c-Rel*, *Aiolos*, *Igβ*, *Blk*, *Id2*, and *Zap70*. We went on to validate our microarray results using quantitative real-time RT-PCR. We found that several genes upregulated upon Gfi1b overexpression have lower transcript levels when *Gfi1b* is deleted; similarly, those genes that are downregulated when Gfi1b is overexpressed show higher transcript levels in cells deleted for *Gfi1b* (Figure 25). We were able to verify our microarray results in primary cells by overexpressing Gfi1b in a reporter mouse wherein hCD4 is knocked into the *kappa* locus such that levels of germline kappa transcription can be measured by monitoring levels of hCD4 (74) (Figure 26). We found that levels of germline kappa transcription in cells overexpressing Gfi1b lower than those overexpressing an empty vector (Figure 26). This likely represents an underestimate of the effect of Gfi1b on kappa germline transcripts because the *hCD4* gene is deleted once recombination occurs. Because less rearrangement is taking place when Gfi1b is overexpressed, one would expect that if there were no difference in germline kappa transcription between cells overexpressing Gfi1b and cells overexpressing an empty vector, then the percentage of cells expressing hCD4 would be higher in Gfi1b overexpressing cells, contrary to what is observed. These results gave us confidence that Gfi1b controls transcript levels of a large number of genes in developing B cells, and may have a more global role in B cell development beyond its affect on RAG transcription.

DISCUSSION

We have identified the transcriptional repressors of the Gfi family, Gfi1 and Gfi1b, as potent negative regulators of RAG expression in B-lineage cells (Figures 1,3, and 7). Gfi1 and Gfi1b associate with chromatin modifiers such as HDACs, histone demethylases, and histone methyltransferases through their N-terminal SNAG domains to reversibly repress their target loci (15). Accordingly, we demonstrate that the integrity of the SNAG domain is required for Gfi1b-mediated repression of RAG expression and that Gfi1b interacts with chromatin at the *RAG* locus (Figures 9, 10).

We observed that while Gfi1 overexpression did cause significant downregulation of RAG1/GFP in the “RAG high” cell line, this downregulation was transient (Figures 3). We believe this to be due to the fact that cells expressing high levels of Gfi1, as measured by high expression of the thy1.1 retroviral marker, were rapidly lost from the population over the course of a week (Figure 4), presumably because overexpression of this factor was either toxic, or conferred a significant growth disadvantage. However, we have not formally excluded the possibility that the Gfi1 cDNA is simply not present within the library. As discussed in chapter 2, pools of RAG1/GFP cells infected with cDNAs were sorted twice, and the total time between infection and harvesting of genomic DNA from RAG1/GFP cells was about 2.5-3 weeks. Thus, between the time infection and the time of harvest, all Gfi1 overexpressing cells may have

already been eliminated from the pool. While overexpression of either Gfi1b or Gfi1 can cause RAG repression in cultured primary bone marrow cells, only the deletion of *Gfi1b*, and not *Gfi1*, leads to high RAG levels in Abelson cells, and our studies focused accordingly on Gfi1b.

Both Gfi1 and Gfi1b are expressed during the early stages of B cell development (19, 21). Targeted disruption of *Gfi1* in the mouse results in a defect in differentiation from lin-, sca+, c-kit+ (LSK) multipotent progenitors (MPPs) to early B220+ B cells (59). In addition, defects in IL-7 receptor signaling impairs B cell development at the pro-B cell stage in *Gfi1* knockout mice. While Gfi1's effect on B cell development has been well characterized (59), Gfi1b's role within the B cell compartment has yet to be determined since *Gfi1b* knockout mice die at day 15 of gestation due to failure to produce mature red blood cells (49).

Since Gfi1b's effect on RAG expression is reversible, we imagine that repression of RAG by Gfi1b is compatible with the dynamic changes in its expression that are necessary for B cells to progress through successive stages of development. There are two stages during B cell development when RAG expression must be reactivated following repression. RAG expression is inactivated during the proliferative burst that follows in-frame *IgHC* locus rearrangement but is re-expressed at the pre-B cell stage to allow the *kappa* locus to rearrange. The second scenario involves the activation of receptor editing in the event that the BCR generated during gene rearrangement is self-specific (75). Here, RAG must be expressed so that successive light chain rearrangements can be attempted in order to generate a non self-specific BCR. Whether this involves re-activation of the *RAG* locus, or simply a failure to shut off the locus initially is unclear. In either case, the reversibility of Gfi1b's effects on RAG expression could theoretically allow for modulation of RAG expression during development, although this hypothesis has not been directly tested in this thesis. Once a developing B cell has reached the immature stage of B cell development, Gfi1b expression ceases (21). Since Gfi1b associates with histone methyltransferases that provide modifications leading to recruitment of HP1 proteins, we hypothesize that Gfi1b may initiate the permanent silencing of the *RAG* locus in mature B cells, but may not be required to maintain the silenced state.

In addition to directly binding to the *RAG* locus and modifying local chromatin structure, Gfi1b also indirectly affects RAG levels by repressing FoxO1 (Figure 18), a critical transcriptional activator of RAG expression in developing B cells (63). The interplay between these two proteins may be crucial for coordinating when and where the RAG proteins are expressed. One possible model is that FoxO1 is induced prior to the initiation of gene rearrangement and that this event is followed by induction of Gfi1b, which serves to limit RAG levels during rearrangement by limiting the level of FoxO1 expression. The reduction in RAG is consistent with developmental progression, since RAG is downregulated following both heavy chain and light chain rearrangement.

AMuLV transformed pro-B cells exit the cell cycle, activate RAG expression, and then ultimately undergo apoptosis when cultured with the Abl-kinase inhibitor STI-571. We find that cells lacking Gfi1b accumulate multiple DNA breaks per cell, and that STI-571 treatment of these cells results in a more rapid G1 arrest (Figures 15, 16). These observations lead us to suggest that Gfi1b may be responsible for keeping RAG levels in check during gene rearrangement to prevent the promiscuous generation of DNA double strand breaks. It may also be required to reduce RAG to baseline levels so that early B cells can progress forward in development following gene rearrangement without risking additional DNA damage. This may be especially important for early B cells undergoing the proliferative burst that marks the transition between the pro-B and the pre-B cell stages, since DNA damage is particularly problematic during DNA replication.

RAG-mediated DNA double-strand breaks, if not repaired correctly, can be deleterious to B cells and lead to premature senescence, apoptosis, or chromosomal abnormalities, such as large deletions or translocations. In some cases, translocations activate oncogenes and result in the development of leukemias and lymphomas (76, 77). B and T cells are unique in that DNA damage is part of a programmed developmental pathway that each must undergo in order to reach maturity. It is therefore hardly surprising that the expression of the RAG proteins responsible for these programmed DNA breaks is so tightly controlled during development. This tight control is underscored by the fact that leukemias and lymphomas are rare despite the enormous number of lymphoid cells produced in an individual's lifetime. Understanding the factors responsible for control of the RAG proteins not only provides insight into the transitions that occur during development, but also how millions of B and T cells are produced over the lifetime of an individual while only rarely resulting in malignant disease.

Our microarray analysis revealed a suite of B cell-specific genes whose expression is influenced by overexpression of Gfi1b. However, further experiments are required to elucidate whether changes in transcription levels of these factors have physiological consequences for early B cell development.

It remains to be seen whether Gfi1b has a role in suppressing RAG expression in non-B lineage cells. Certainly Gfi1b is expressed in several other cell types (21) and has been found to have a major role in the development of the erythroid and megakaryocytic lineages (49). Whether the suppression of RAG expression by Gfi1b plays a role in directing hematopoietic stem cells down a particular lineage pathway is an intriguing question that remains unanswered.

CONCLUSIONS AND FUTURE DIRECTIONS

We conclude that Gfi1b can act as a negative regulator of the *RAG* locus in early B cells. Gfi1b binds directly to the *RAG* locus, and is further able to modulate

RAG activity by downregulating the transcription factor FoxO1. This activity may be particularly important for maintaining genomic integrity by limiting RAG levels in rearranging cells and preventing excessive numbers of DNA double strand breaks. One model for how Gfi1b may work *in vivo* is diagrammed in Figure 27. A wave of FoxO1 activity is responsible for activating the *RAG* locus so that rearrangement can occur. This is followed by a wave of Gfi1b activity, which limits RAG levels by acting directly on the locus and decreasing FoxO1 protein levels. By limiting both RAG transcription and kappa germline transcription, Gfi1b promotes termination of kappa locus rearrangement so that cells can progress forward in development (Figure 27).

Microarray analysis reveals that Gfi1b can influence the transcription levels of a host of B cell specific genes. Further work is required to elucidate whether Gfi1b controls a broad- spectrum genetic program during early B cell development. Similarly, our ChIP-chip analysis revealed that Gfi1b can bind to a number of genes shown to have a role in B cell development. These include *TdT*, *Runx1*, *EBF*, and *ID3*. Future studies will be required to elucidate whether Gfi1b has a functional effect on the expression of these genes at various stages of B cell development.

Although we have started to tease out the biological role for Gfi1b within the early B cell compartment, its role outside the B lineage remains unexplored. In order to understand whether Gfi1b plays a role in keeping RAG transcription off in non-B and T lineages, we decided to take advantage of a rearrangement reporter mouse developed in the lab of Dr. Rachel Gerstein (78). Expression of the RAG proteins in this transgenic animal leads to the irreversible inversion and expression of a GFP marker, thus permanently marking any cell with RAG activity. Breeding of this transgenic animal to an inducibly floxed *Gfi1b* knockout mouse will allow us to ask whether, in the absence of Gfi1b repressing activity, RAG proteins are inappropriately expressed in non B and T lineages and/or in various hematopoetic progenitor cells. If Gfi1b is responsible for repressing RAG proteins until lymphoid lineage commitment, we would expect to see an increase in GFP positive cells in the absence of Gfi1b in uncommitted early progenitor cells of various types. These experiments should shed light on Gfi1b's biological role outside the lymphoid lineage.

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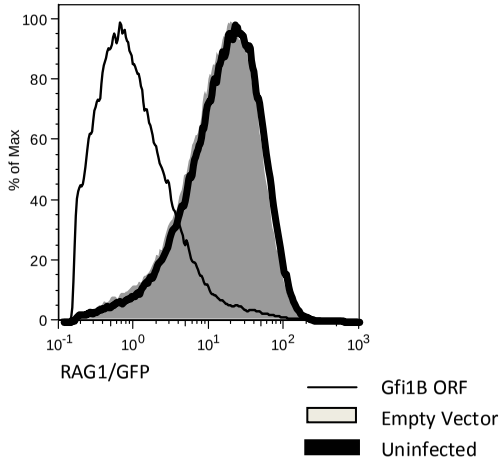

Figure 1. Overexpression of Gfi1B causes downregulation of RAG1/GFP transcripts. Flow cytometry of RAG1/GFP expression in RAG High cells infected with a retroviral construct overexpressing the Gfi1B open reading frame.

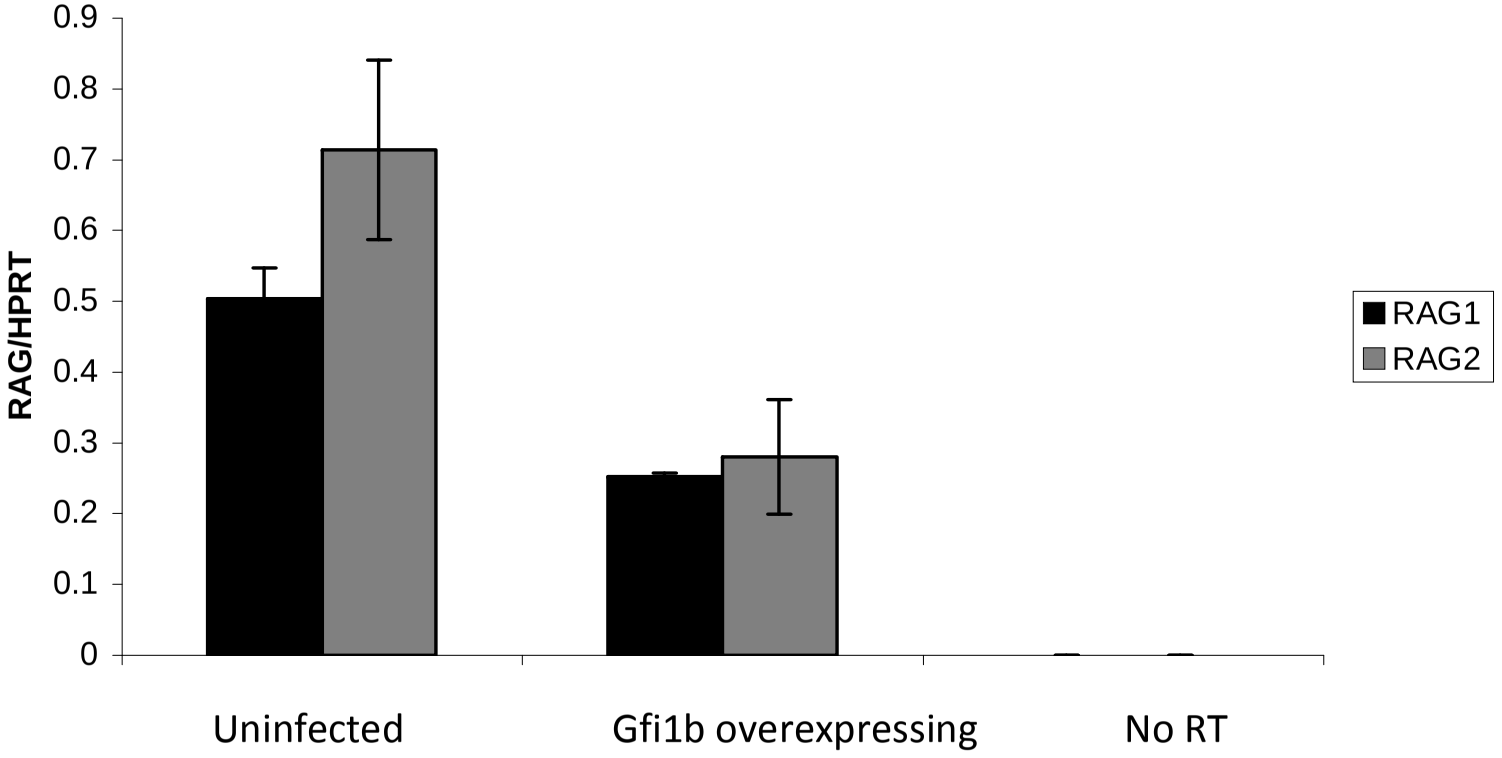

Figure 2 Quantitative Real-Time PCR for RAG1 and RAG2 transcripts in the PD31 AMuLV pro-B cell line infected with a Gfi1b overexpression construct. Infected and uninfected cells were sorted and RNA was isolated from each population.

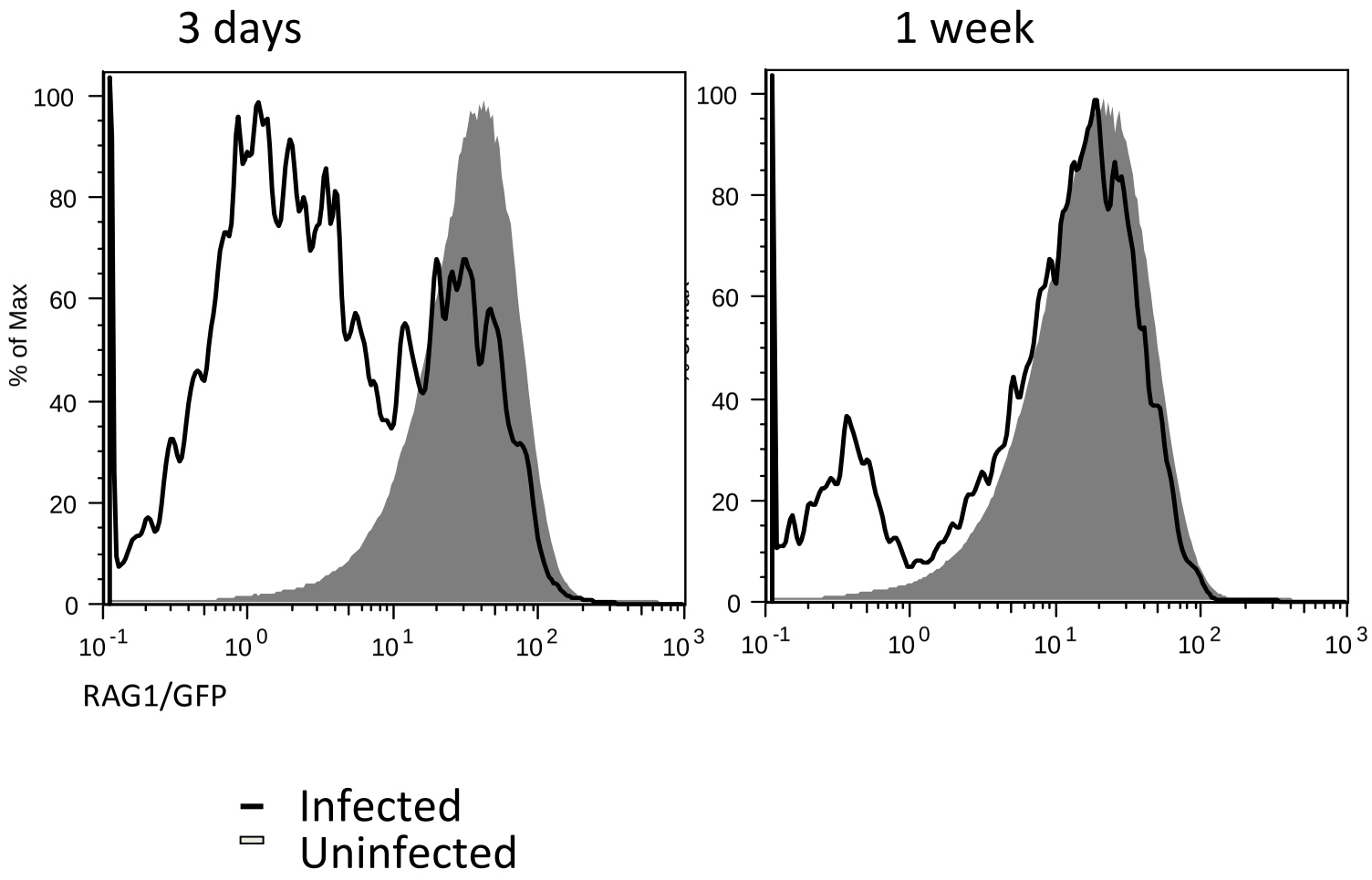

Figure 3 Flow cytometry for RAG1/GFP in RAG High cell lines overexpressing Gfi1. RAG1/GFP levels were measured 3 days and 1 week after infection.

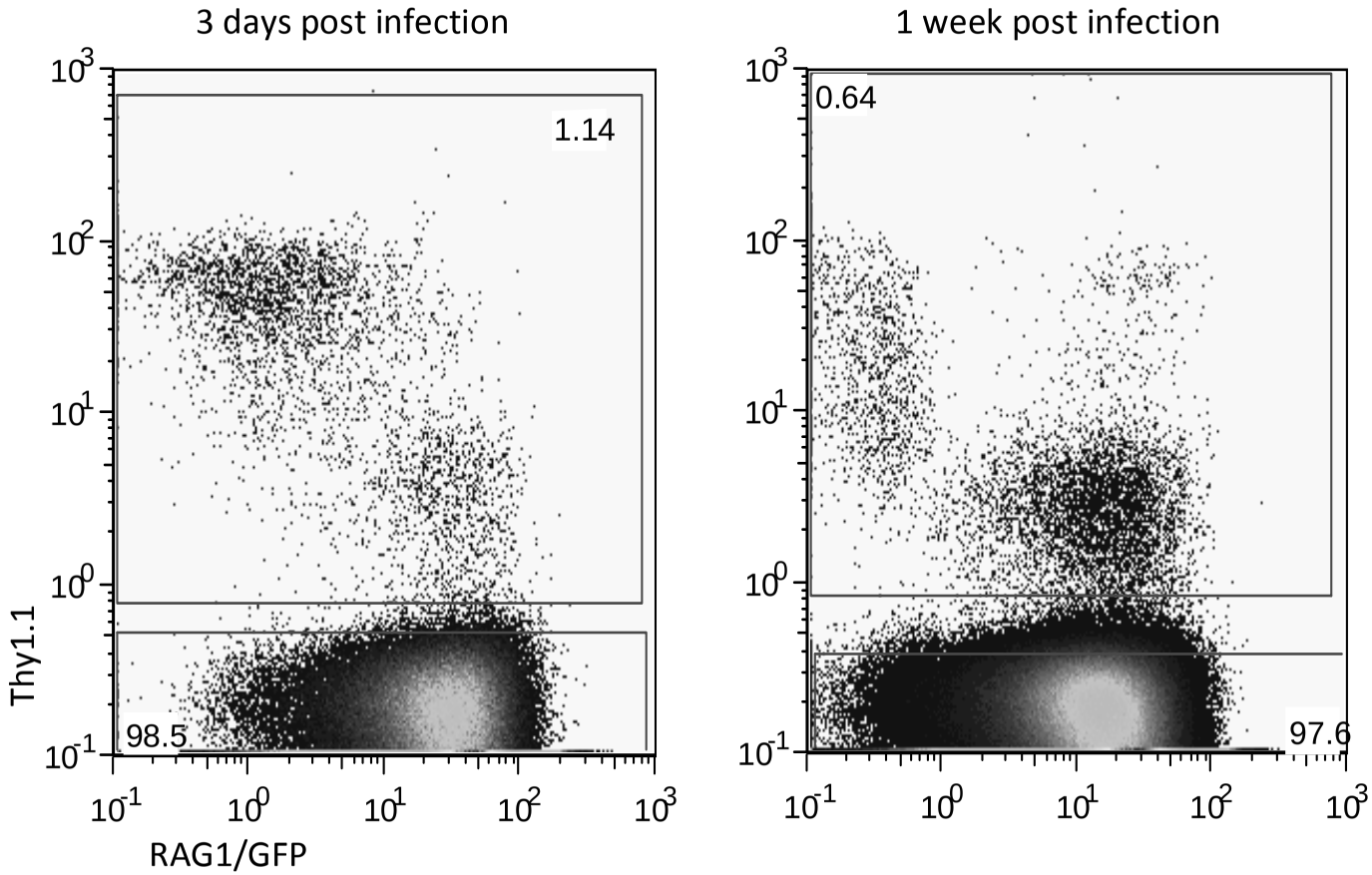

Figure 4: Flow cytometry of RAG high cells infected with a Gfi1 overexpression construct marked with thy1.1. Cells were assayed 2 days and 1 week post infection.

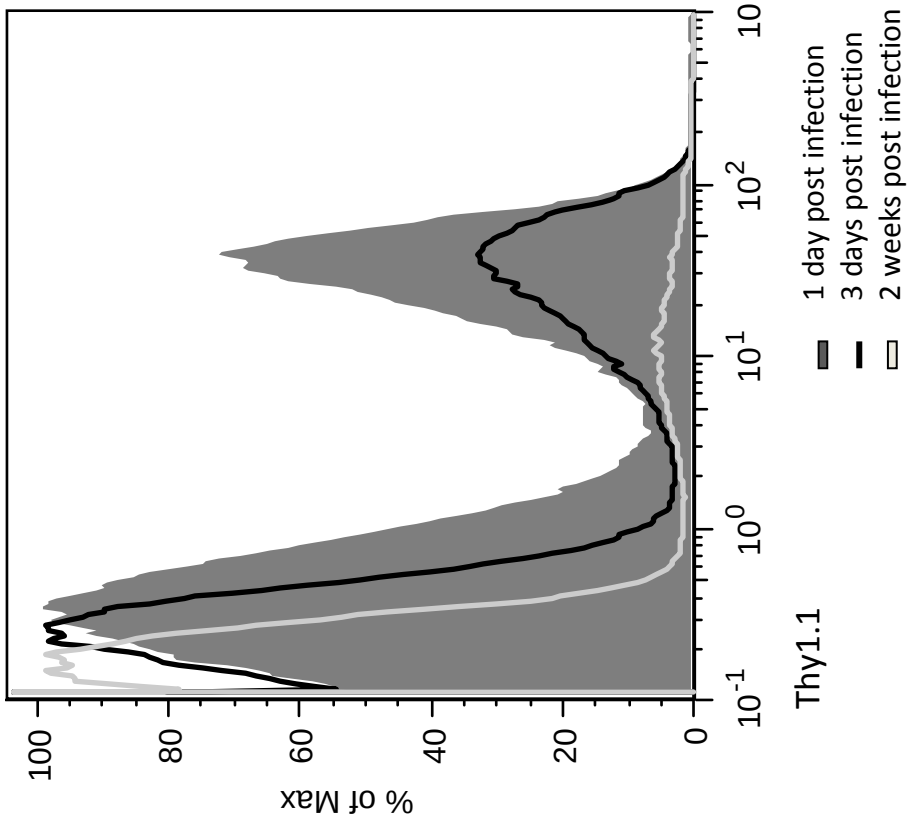

Figure 5 Flow cytometry showing levels of thy1.1 retroviral marker expression in live RAG high cells at various times after infection with Gfi1b cDNA marked with ires thy1.1.

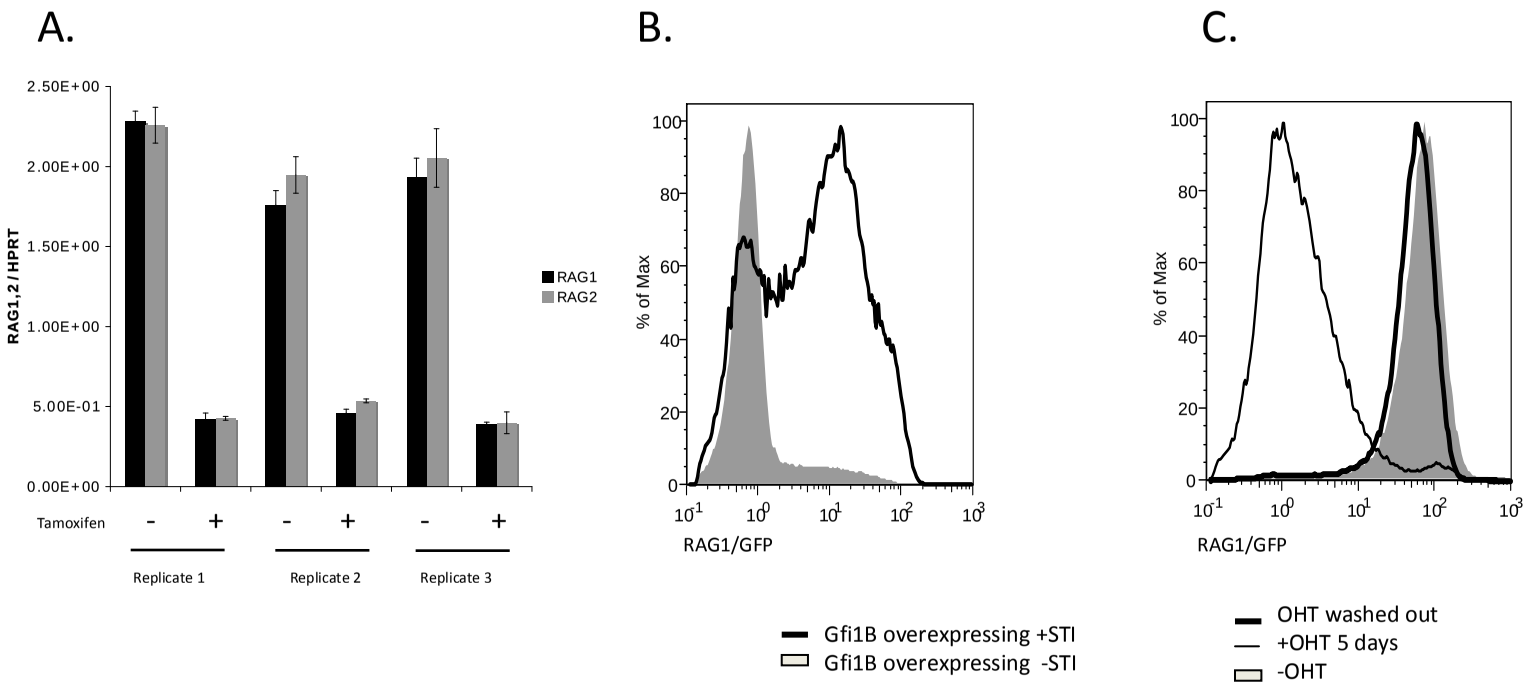

Figure 6 Gfi1b reversibly downregulates RAG expression. A. Quantitative real-time PCR for RAG1 and RAG2 transcript levels of RAG High cells infected with a Gfi1B-ER construct and treated with tamoxifen for 12h. B. Flow cytometry of RAG High cells overexpressing Gfi1B alone, or overexpressing Gfi1B and treated with 2.5 μM STI-571. C. Flow cytometry of GFP levels in RAG High cells infected with a retroviral Gfi1B-ER construct. Cells were treated with tamoxifen for 5 days, washed, and cultured for 9 days thereafter.

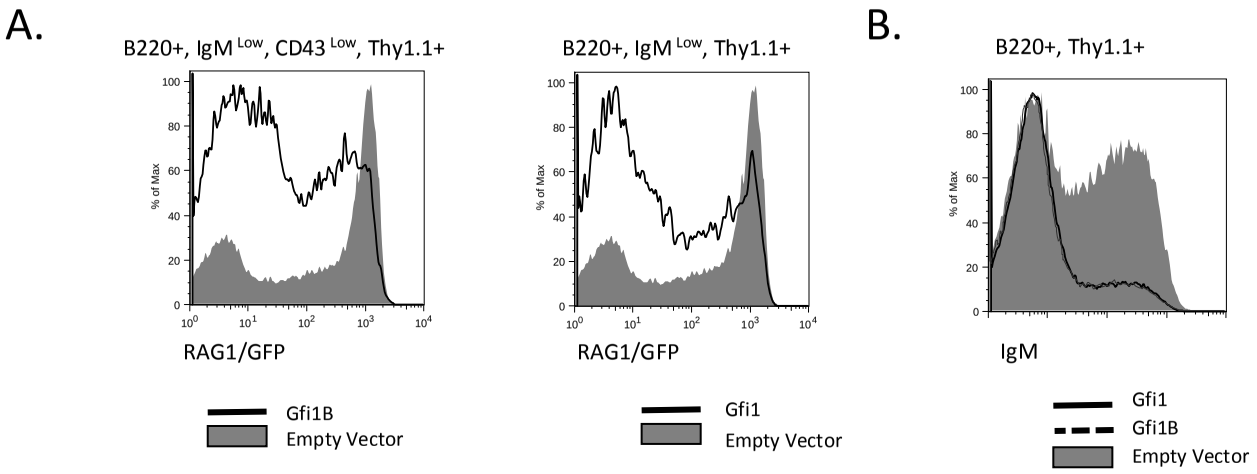

Figure 7 Gfi1b overexpression causes RAG downregulation in primary cells. A. Flow cytometry of GFP levels in cultured bone marrow cells from RAG1/GFP heterozygous mice infected with a thy1.1 marked retrovirus overexpressing either Gfi1b, Gfi1, or an empty vector and gated using B220, IgM, CD43, and Thy1.1 as indicated. B. Flow cytometry of IL-7 cultured bone marrow cells from RAG1/GFP heterozygous mice infected with a retroviral Gfi1B or Gfi1 overexpression construct, or an empty vector.

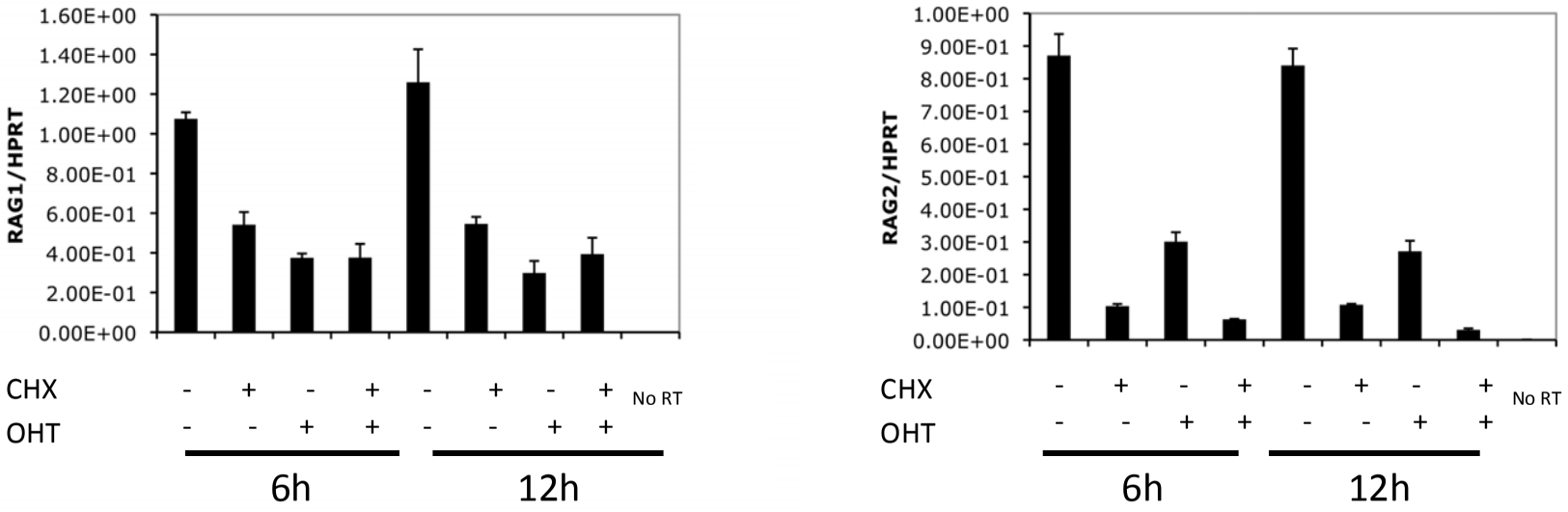

Figure 8: Real-time PCR of RAG1 and RAG2 transcripts in RAG high, Gfi1b-ER overexpressing cells in the presence or absence of cycloheximide.

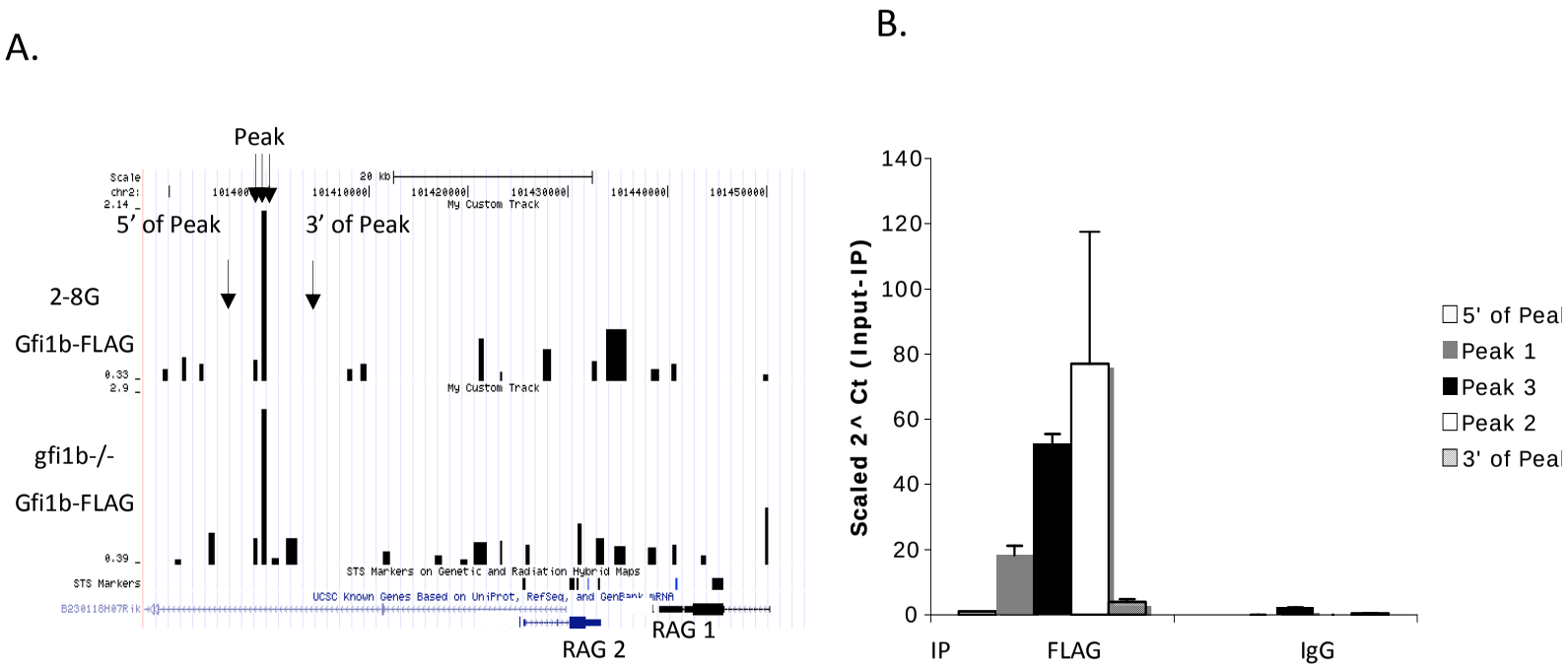

Figure 9 Gfi1B binds directly to the *RAG* locus. A.) α-FLAG ChIP of RAG high and *gfi1b-/-* cells overexpressing FLAG-tagged Gfi1B. Data is shown for the region upstream and including RAG1 and RAG2. Arrows indicate location of primer sets used in B. B.) Quantitative real-time PCR of ChIP conducted on *gfi1b-/-* cells overexpressing FLAG-tagged Gfi1b.

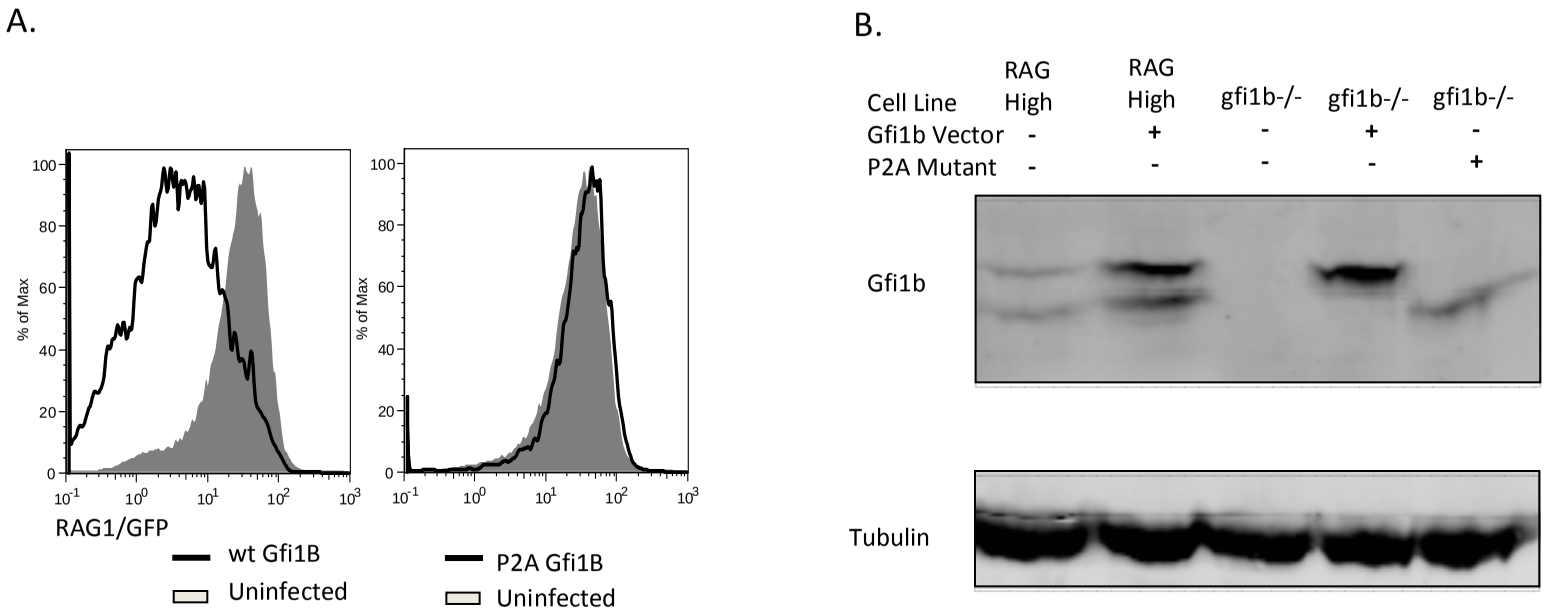

Figure 10 Gfi1b depends on cofactor recruitment to downregulate RAG. A.) Flow cytometry of RAG1/GFP levels in RAG High cells lines infected with a retrovirus expressing a wt or P2A mutant Gfi1B protein. B. Western blot of RAG high or *gfi1b-/-* cells expressing either a wt or a P2A mutant version of Gfi1b.

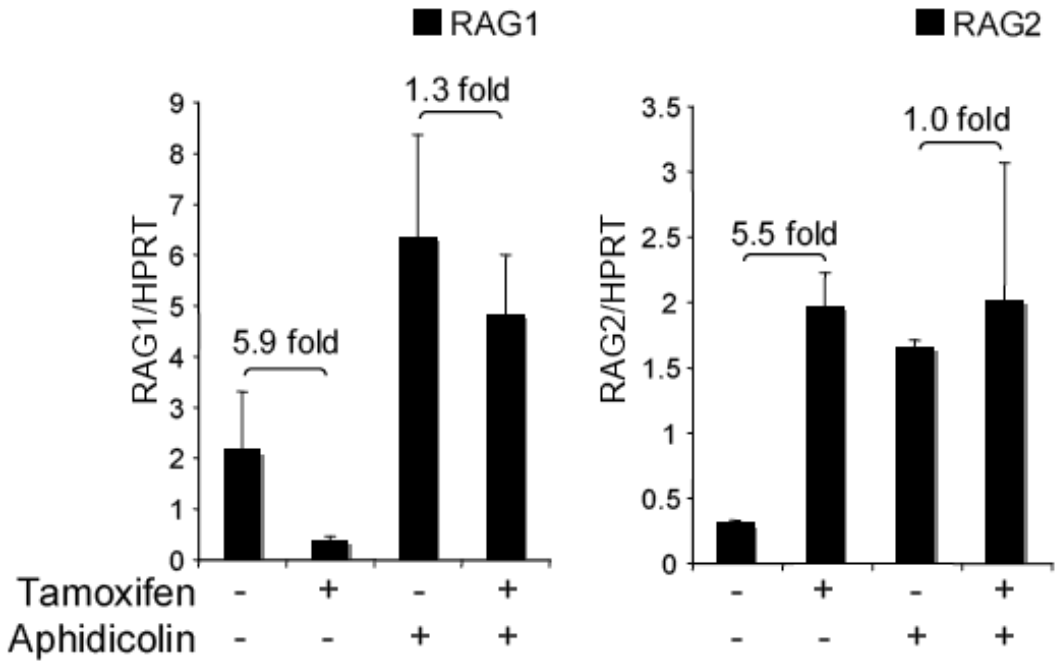

Figure 11. Quantitative real-time PCR of RAG1 and RAG2 transcripts in RAG High cells infected with a retroviral Gfi1B-ER construct and treated with tamoxifen for 12h in the presence or absence of aphidicolin

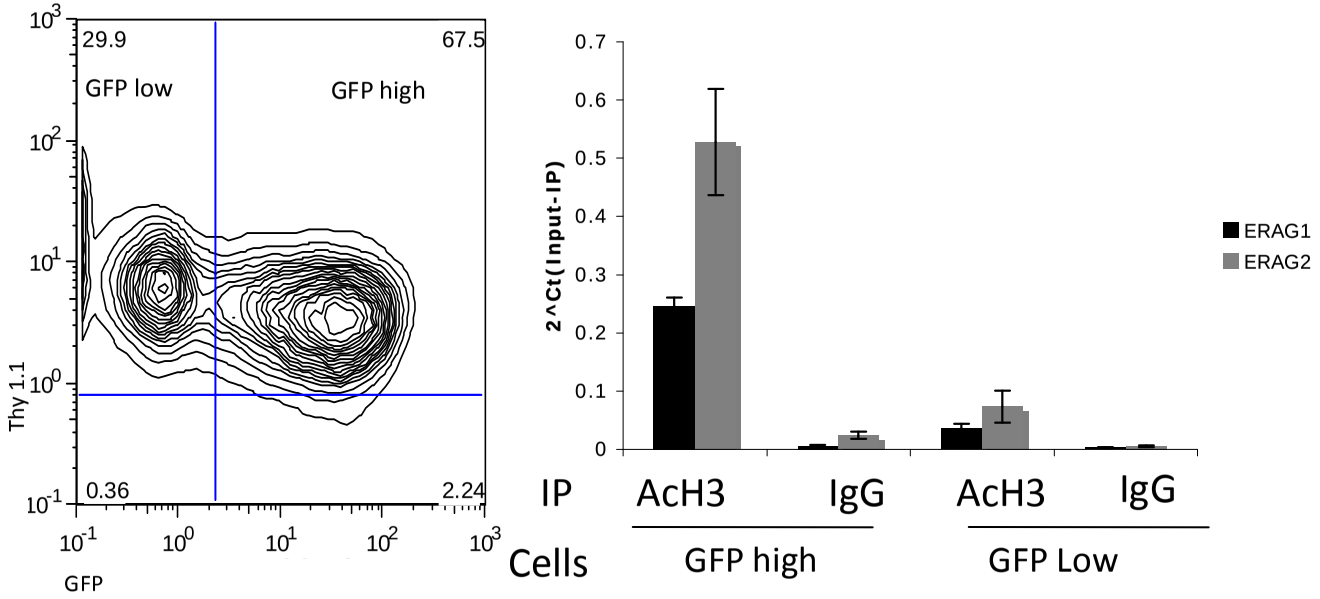

Figure 12: Gfi1b acts on RAG through a chromatin modifying mechanism. Left, Flow Cytometry of GFP in RAG1/GFP AMuLV cells infected with a retrovirus overexpressing Gfi1B. Right, Anti-AcH3 ChIP experiment using primers located with eRAG of RAG1/GFP AMuLV cells infected with a Gfi1B overexpressing retrovirus and sorted into GFP low and GFP high populations.

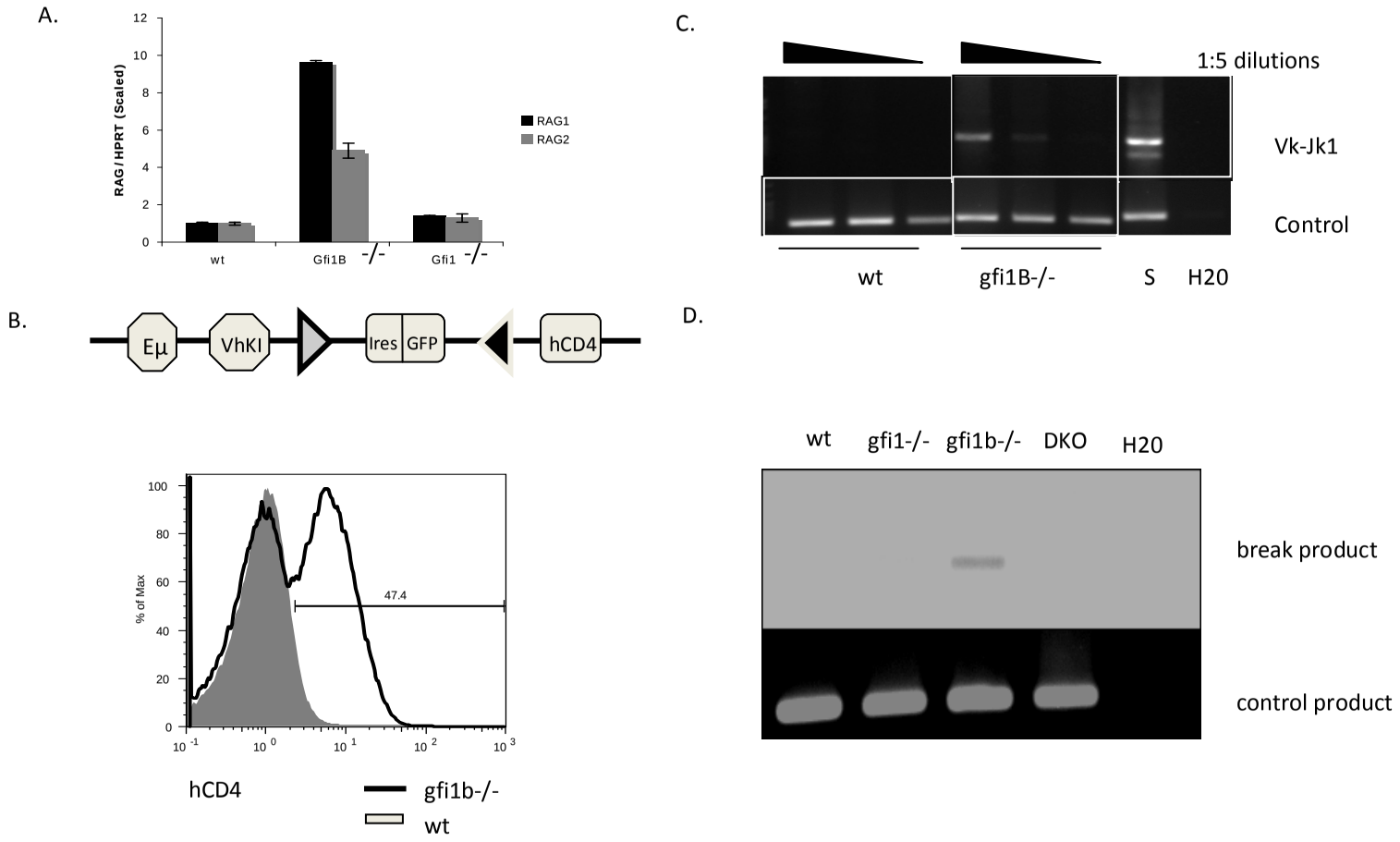

Figure 13 The deletion of Gfi1B results in increased RAG transcription and rearrangement. A. Real-time quantitative RT-PCR of RAG transcripts in AMuLV cell lines generated from *wt*, *gfi1B-/-*, and *gfi1-/-* mice. B. Flow cytometry of hCD4 induction in *wt* and *gfi1B-/-* AMuLV cell lines infected with a rearrangement reporter construct. C. PCR for Vk-Jk1 coding joints in AMuLV cell lines generated from *wt* and *gfi1B-/-* mice. D. LM-PCR to detect breaks at the reporter AMuLV cells generated from *wt, gfi1-/-, gfi1b-/-* and *DKO* mice.

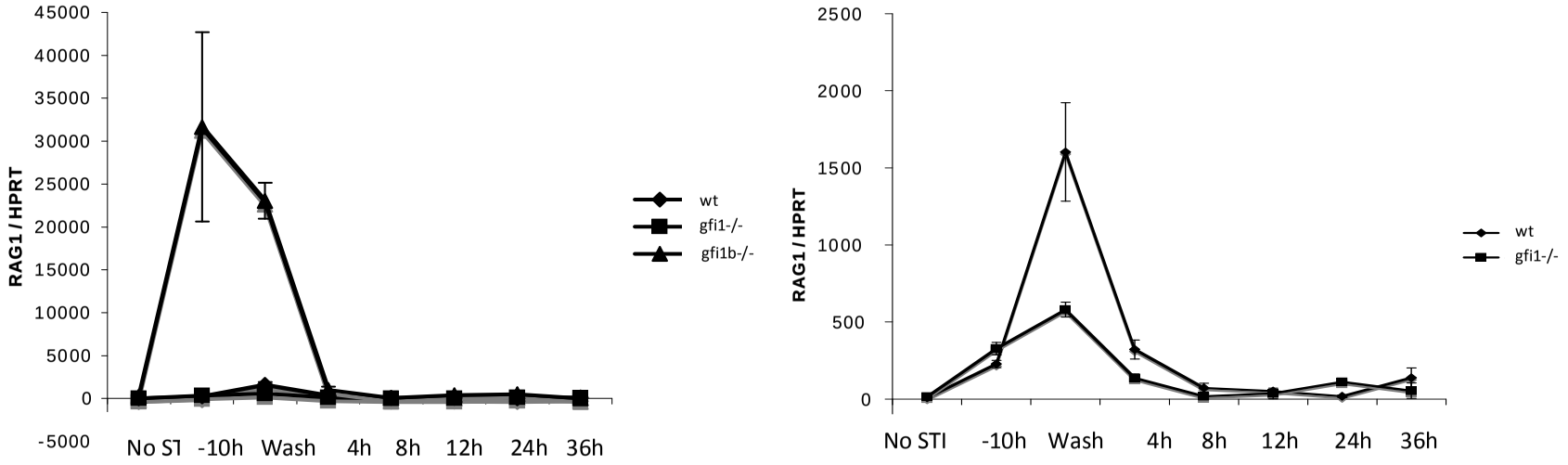

Figure 14. Deletion of *Gfi1B* leads to RAG deregulation. A. Left panel, Real-time quantitative PCR of RAG1 transcription in AMuLV cell lines generated *from wt, gfi1-/-*, and *gfi1b-/-* mice treated with STI-571, washed, and cultured for an additional 36h. Right panel, blow up of data shown on left for the *wt* and *gfi1-/-* cell lines.

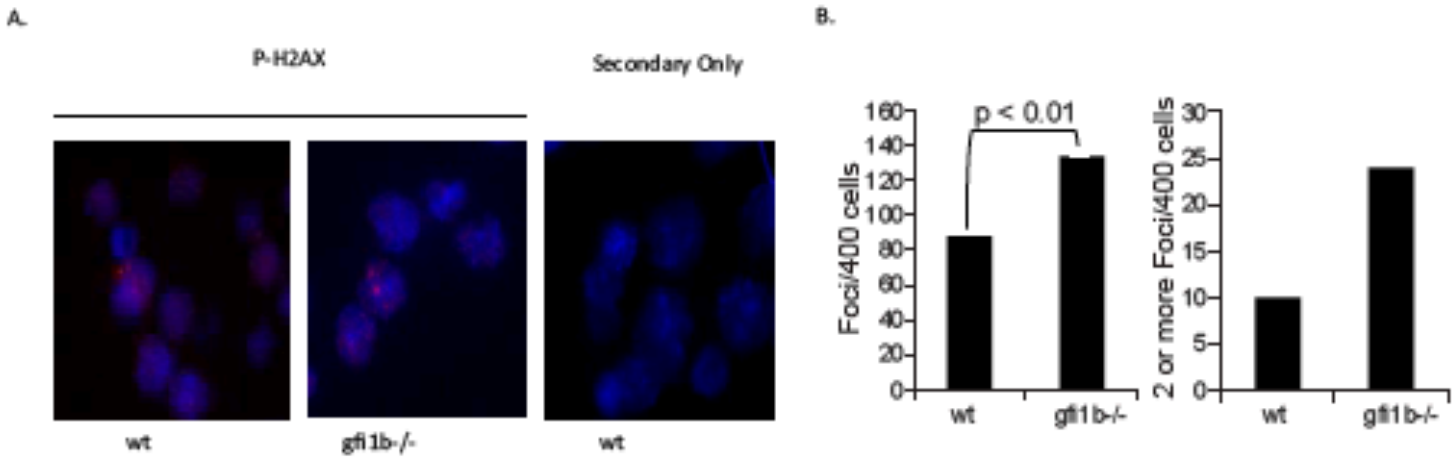

Figure 15: *gfi1b-/-* cells have greater numbers of double-strand breaks. A. Immunofluorescence of AMuLV transformed *wt* and *gfi1b-/-* cells stained with anti-phospho-H2AX antibodies. B. Quantitation of 4B. The total number of H2AX foci/cell was counted (left panel) for 400 cells. Data was analyzed using a student's **t** test. Right panel, number of cells with 2 or more foci/cell, n=400. Data is representative of 3 biological replicates.

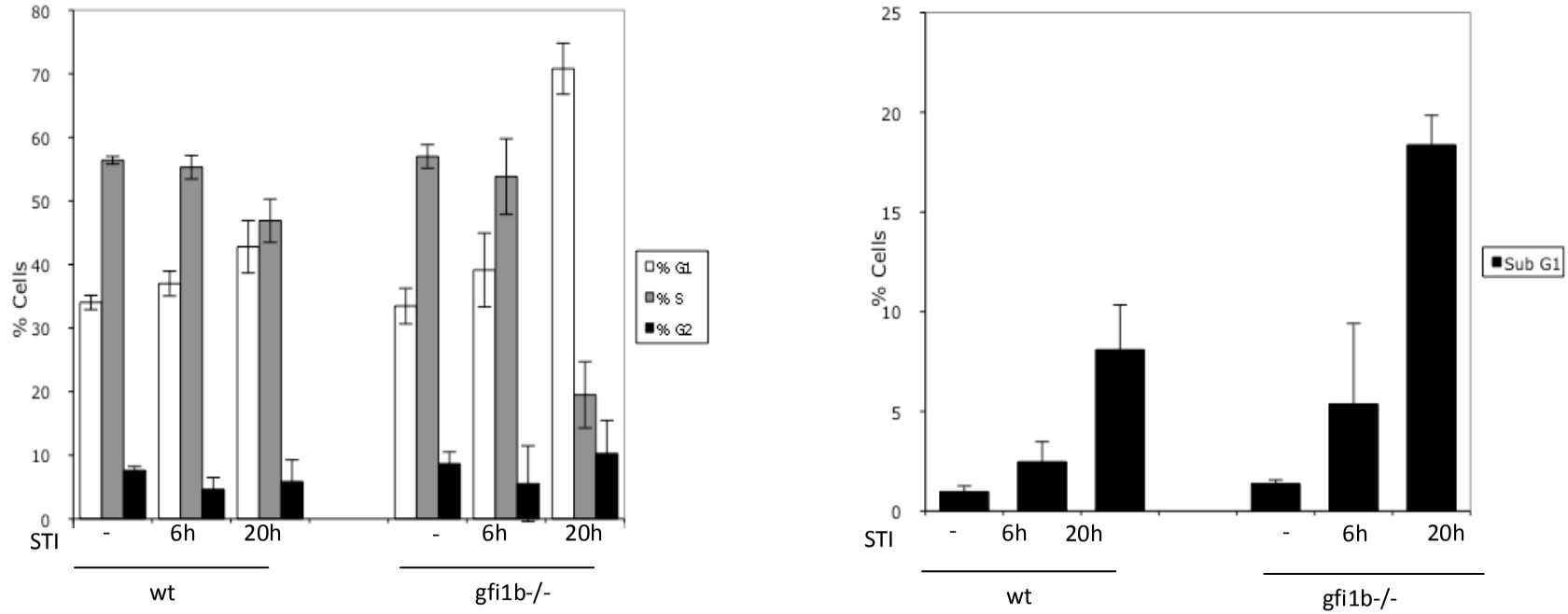

Figure 16: Cell cycle analysis using PI staining in AMuLV transformed *wt* and *gfi1b-/-* cell lines treated with STI-571. 3 Biological replicates are shown.

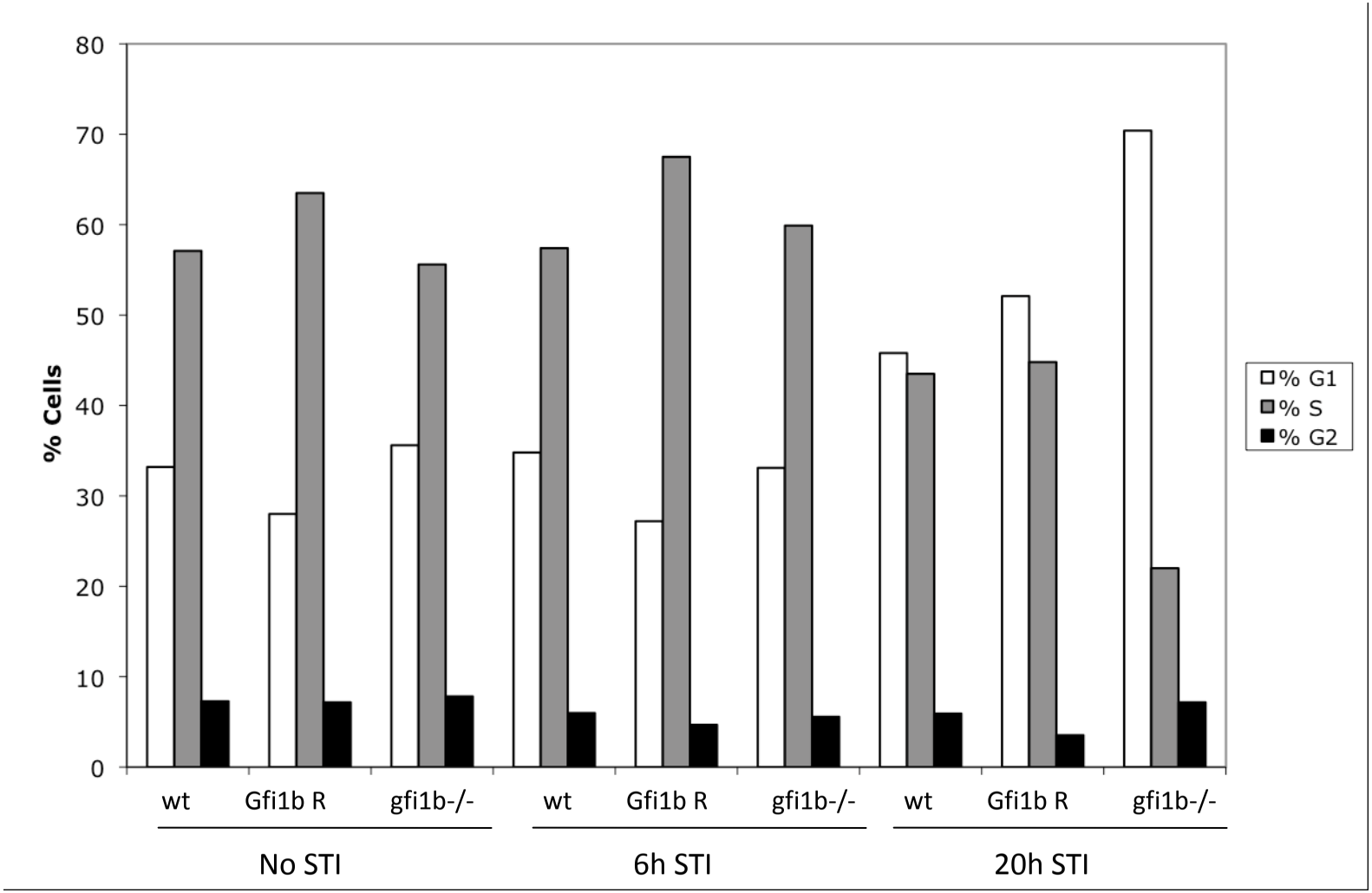

Figure 17 Cell cycle analysis of PI stained AMuLV cells treated with STI-571 and analyzed by flow cytometry. Gfi1b R, *gfi1b-/-* cells reconstituted with a Gfi1b overexpressing vector.

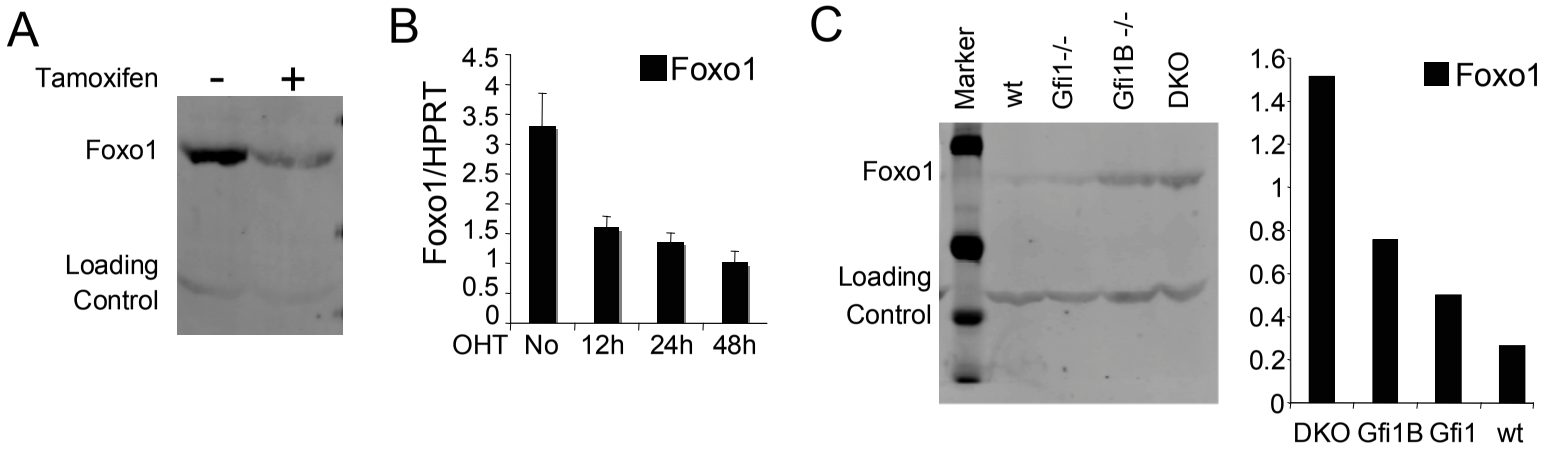

Figure 18. Gfi1b downregulates FoxO1. A. Left, Anti-FoxO1 western blot of RAG1/GFP in RAG High cell lines infected with a Gfi1B-ER retroviral overexpression construct and treated with tamoxifen for 12h. Tubulin was used as a loading control. B. quantitative real-time PCR for FoxO1 on Gfi1b-ER overexpressing RAG High cells treated with tamoxifen for the indicated times. C. Left, Anti-FoxO1 Western blot in indicated AMuLV cell lines, Right quantitation of ani-FoxO1 western blot.

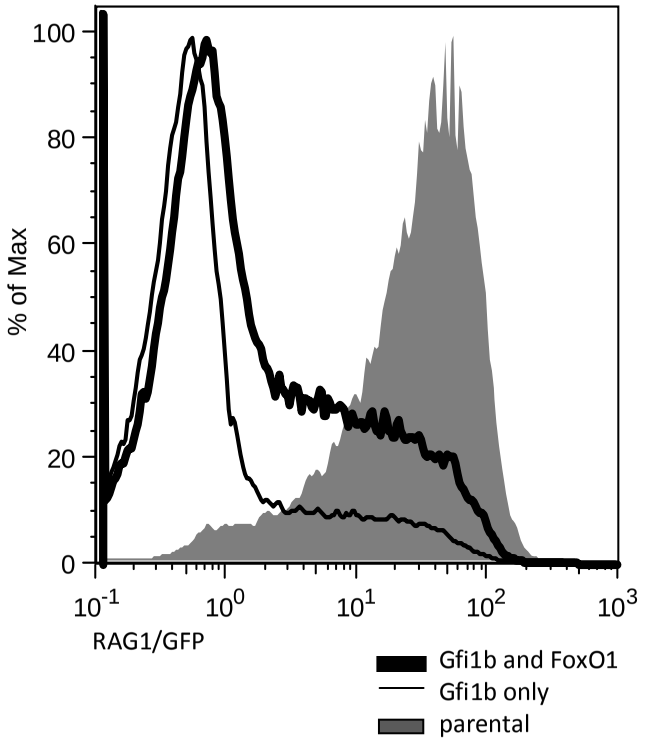

Figure 19 FoxO1 overexpression cannot fully compensate for Gfi1b's downregulation of RAG. Flow cytometry for RAG1/GFP expression in RAG High cells overexpressing Gfi1b alone, or Gfi1b and FoxO1.

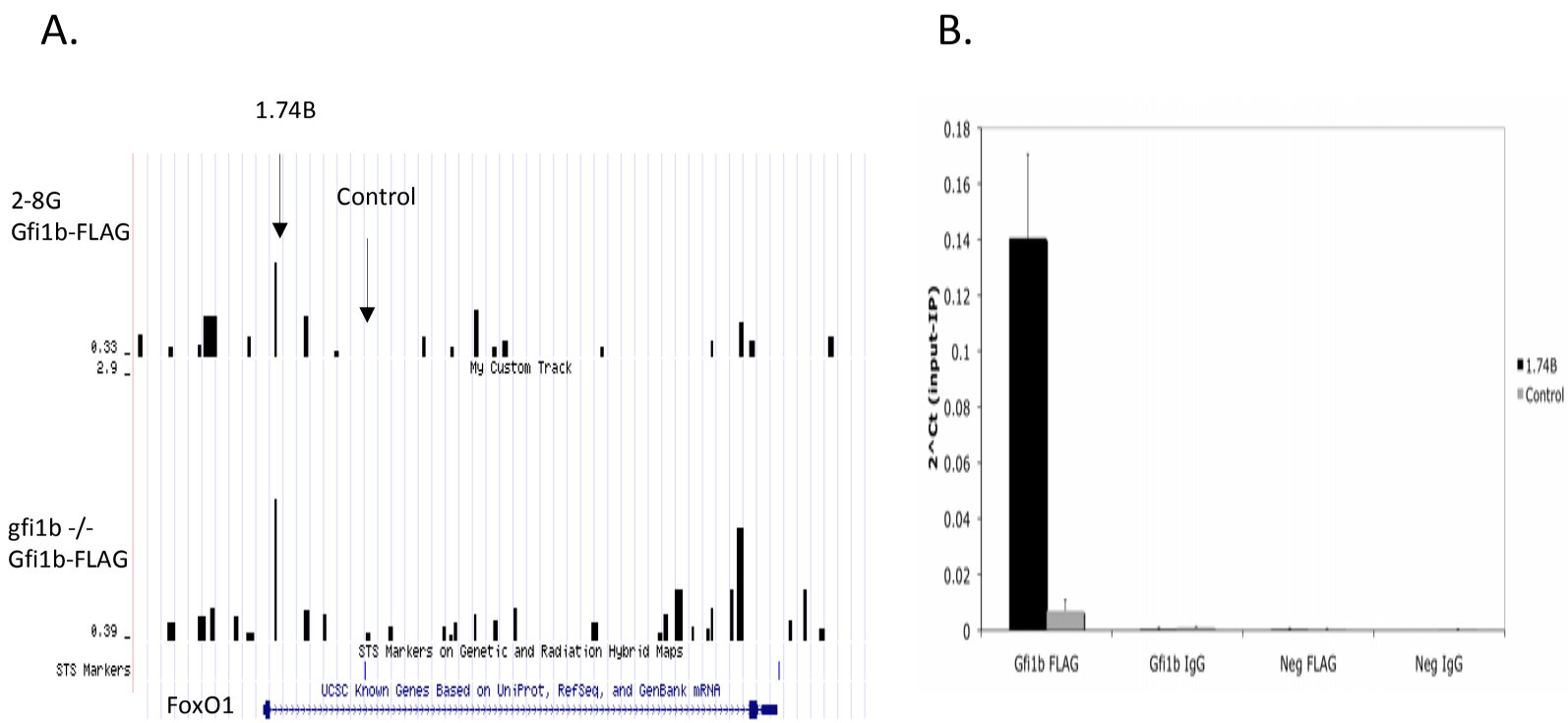

Figure 20 Gfi1b binds to the *FoxO1* locus. A. α-FLAG ChIP of RAG high and *gfi1b-/-* cells overexpressing FLAG-tagged Gfi1B. Data is shown for the *FoxO*1 gene locus. Arrows indicate location of primer sets used. B. Quantitative real-time PCR of ChIP conducted on *gfi1b-/-* cells overexpressing FLAG-tagged Gfi1b.

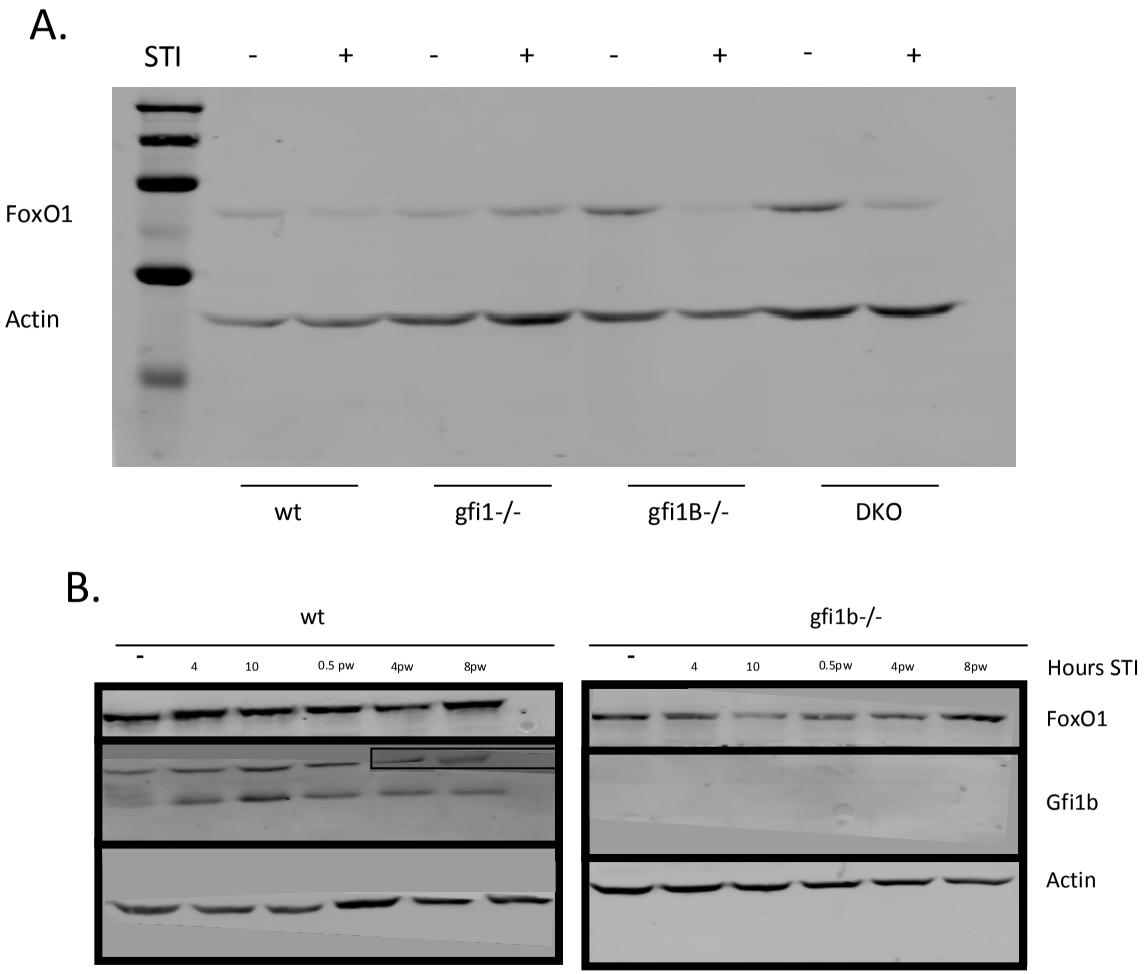

Figure 21. Modulation of FoxO1 and Gfi1b during STI-571 treatment. A. Anti-FoxO1 western blot in indicated AMuLV cell lines treated for 20h with 2.5 μM STI-571. B. Western blot for FoxO1 and Gfi1b protein levels in cells treated with 2.5μM STI for 20 hours washed in PBS, and put back in culture for a total of 8 hours. Pw=postwash.

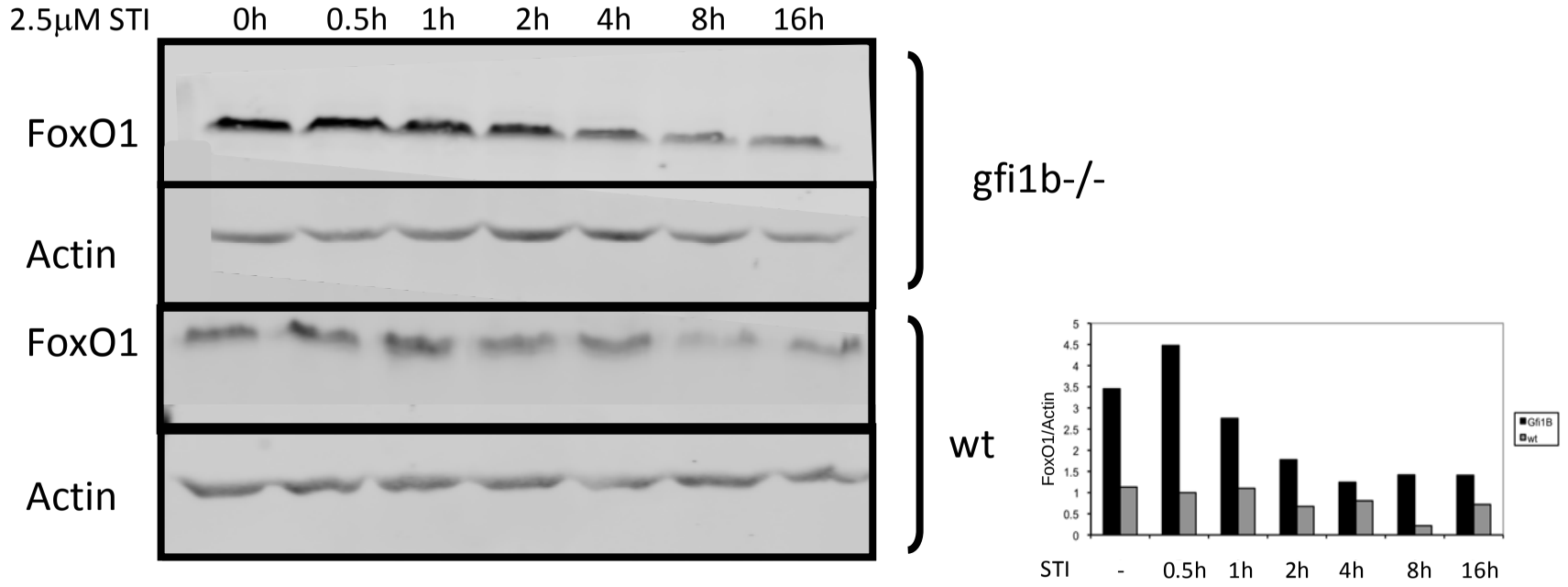

Figure 22: Western Blot for FoxO1 in AMuLV transformed *wt* and *gfi1b-/-* cell lines treated with STI-571. Right panel, quantitation of blot on left.

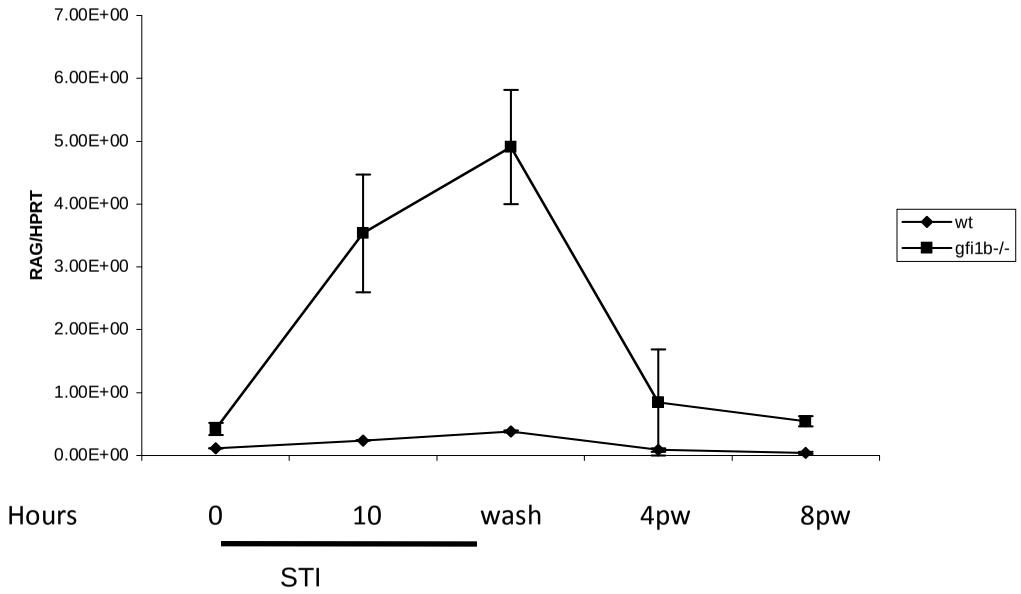

Figure 23. Quantitative real-time PCR for RAG1 transcripts in indicated AMuLV cell lines subjected to 20h of STI-571 treatment followed by subsequent washout. Pw = post wash

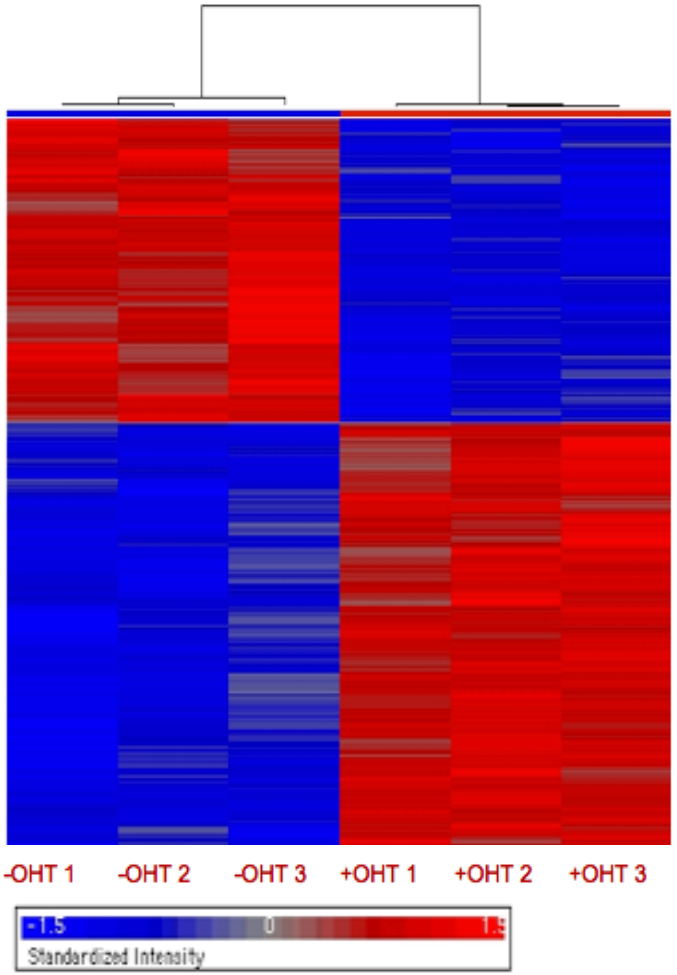

Figure 24 Gfi1b overexpression causes changes in transcription levels for a large number of genes. Heat map of genes whose transcription level is significantly altered ($p<0.01$) upon Gfi1B overexpression.

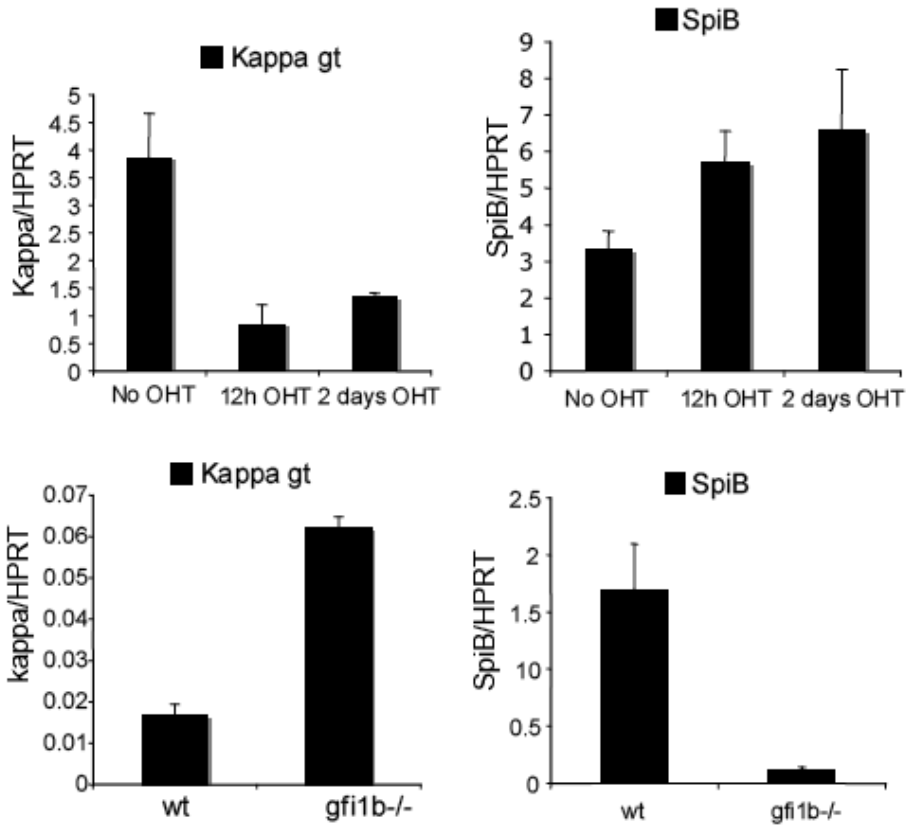

Figure 25: Microarray results verified by real-time PCR for B-cell specific genes. Top panel, quantitative real-time PCR in Gfi1b-ER overexpressing AMuLV cells treated with tamoxifen as indicated. Bottom panel, quantitative real-time PCR in *wt* or *gfi1b-/-* AMuLV cells for two genes whose transcript levels were significantly altered upon Gfi1b overexpression.

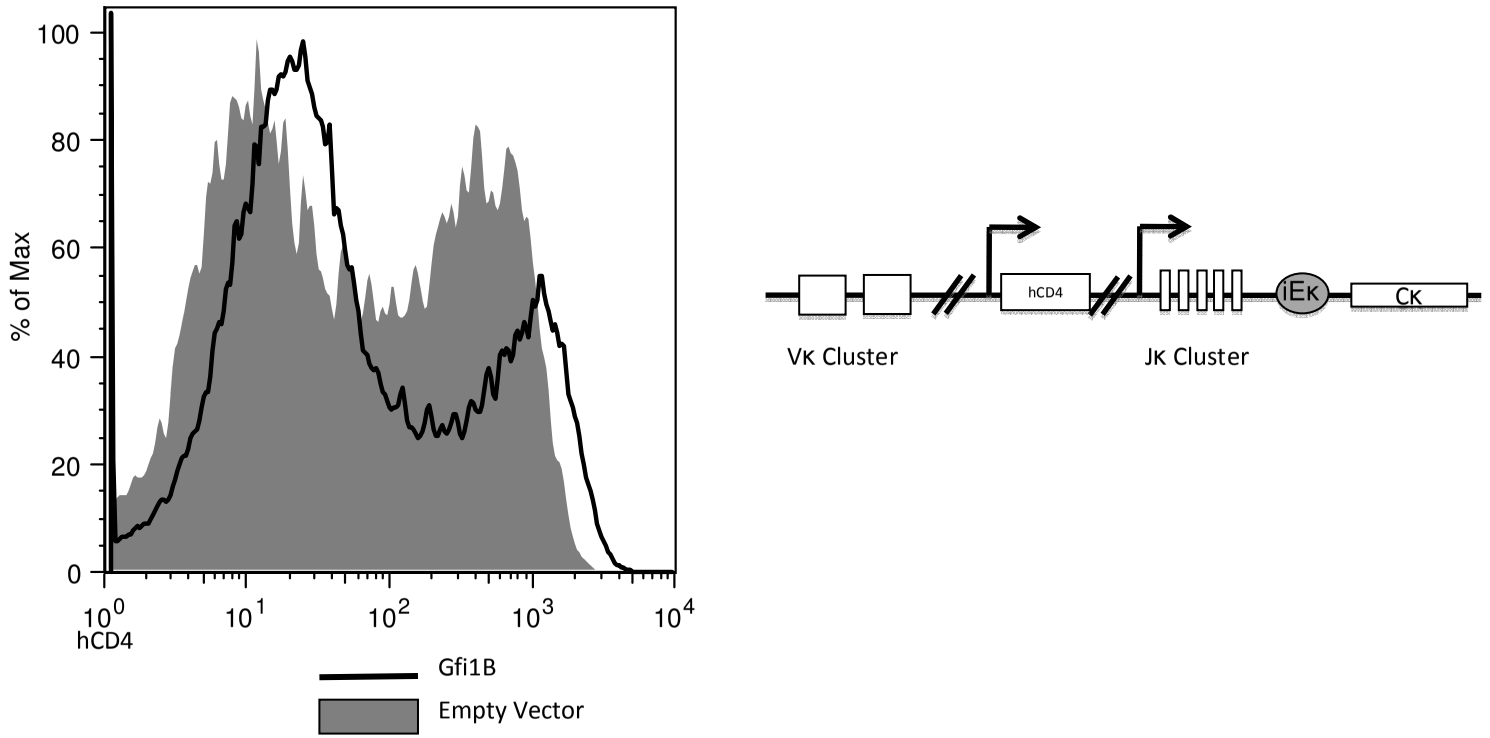

Figure 26: Left, flow cytometry plot of hCD4 expression reporting levels of kappa germline transcripts in cultured primary bone marrow B harvested from dκGT-hCD4 mice cells and overexpressing either Gfi1b or an empty vector. Right, schematic of the *kappa* locus in hCD4 kappa germline transcript knock-in (dκGT-hCD4) mice.

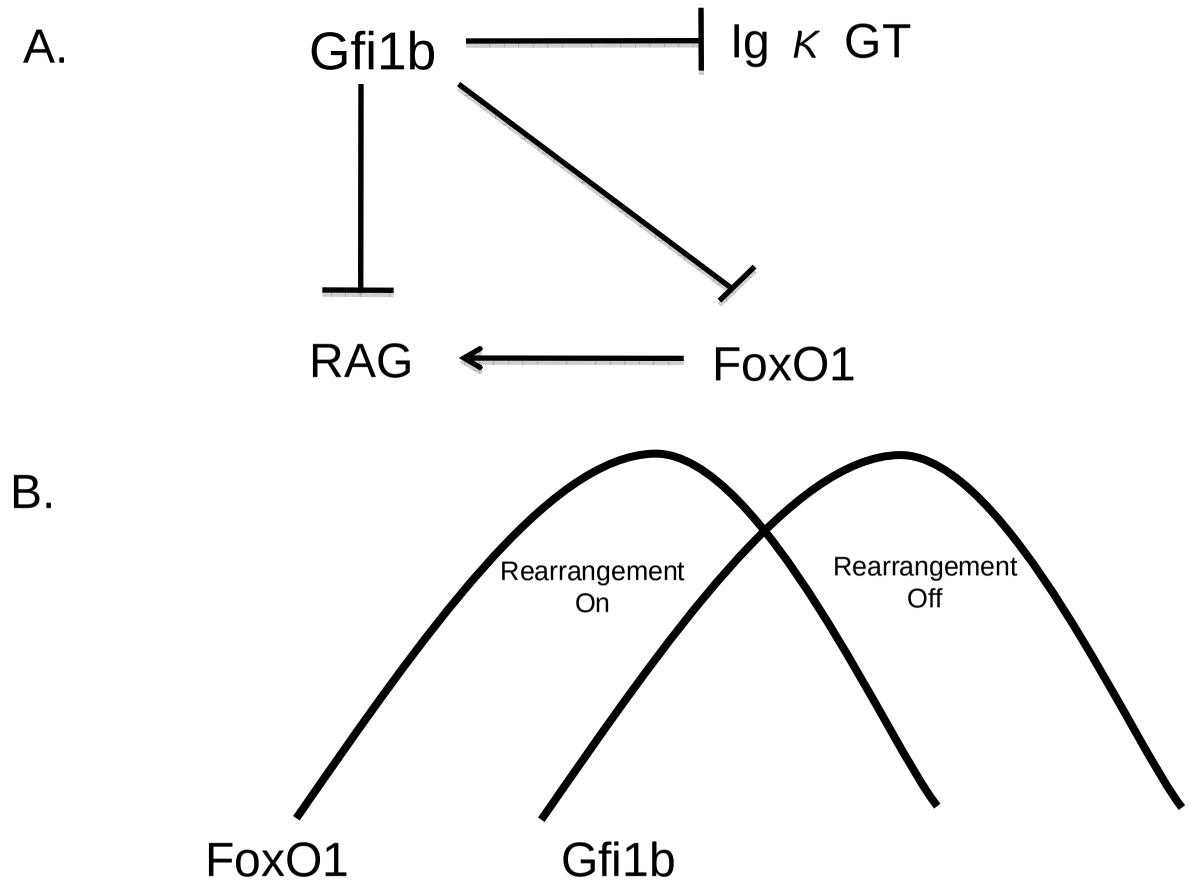

Figure 27: Model for Gfi1b Activity during kappa locus gene rearrangement. A. Gfi1b negatively regulates *RAG, FoxO1,* and germline kappa transcription. B. A wave of FoxO1 activity is responsible for activating the *RAG* locus so that rearrangement can occur. This is followed by a wave of Gfi1b activity, which limits RAG levels by acting directly on the locus and decreasing FoxO1 protein levels. By limiting both RAG transcription and kappa germline transcription, Gfi1b promotes termination of kappa locus rearrangement so that cells can progress forward in development

Table 3: Primers used in ChIP, Genotyping, LM-PCR, Rearrangement, and Transcript Analysis

ChIP	Foxo 3' Peak	ctgtgccaaggaatgctggtgat
		tctcaagtagcccagggcatgtat
	Foxo 1.74 B	tgcactcagagaaaggcttccaca
		taatctggtcacacacagcagcct
	5' RAG peak	tgccttctgaccttaacctccact
		agctgatattggcctgctttaggc
	RAG Peak 1	tagcccatgatgctgaaatgctgc
		tcatctaagtctggcctctgggtt
	RAG Peak 2	caagctggctgccatcacctaaat
		aaatggttggctaagcccaaaggg
	RAG Peak 3	aacgaacaggctaaggattcctcc
		gatttaggtgatggcagccagctt
	3' RAG peak	catagaccaacgtccacagatggat
		tggagttaacacttgccttccacc
	18S	cacggacaggattgacagattg
		catgccagagtctcgttcgtta
Genotyping	MB1 Cre for	ccctgtggatgccacctc
	MB1 Cre rev	gtcctggcatctgtcagag
	RAG1 5' for	aggtagcttagccaacatggctg
	RAG 5' rev	catctgccttcacgtcgatcc
	eGFP	gctcaggtagtggttgtcgg
	LP5-3S	ggtttctaccagtctggccctgaactc
	LP5-3R	ctcacctctctgtggcagtttcctatc
	LP5-4R	tacattcatgcttagaaacttgagtc
	mGfi2	gtaggtttgctgaactgctgtg
	mGfi3	tctgctatctaaccgctaaactg
	PB3	cttccatttgtcacgtcctg
LM-PCR and Rearrangement	Vk deg for	gctgcagsttcagtggcagtggrtcwggrac
	BW-1	gcggtgacccgggagatctgaattc
	BW-2	gaattcagatc
	BW-H	ccgggagatctgaattccac
	ko5	gcccaagcgcttccacgcatgcttggag
	ko3	agtgccactaactgctgagccacc
	JkB2	ctttgccttggagagtgccagaatctgg
Transcripts	CD36	gggaagacaatcaaaagggaagttgtc
		tgctgttctttgccacgtcatctg
	SpiB	agcccttcagttacccagattc
		gatcgaaggcttcatagggag
	RAG1	cattctagcactctggccgg
		tcatcgggtgcagaactgaa
	RAG2	ttaattcctggcttggccg
		ttcctgcttgtggatgtgaaat
	Gfi1b	gtgtggaggttcgtggctctcggg
		ggggctggtggtaagtgtgtgcct
	HPRT	ctggtgaaaaggacctctcg
		tgaagtactcattatagtcaaggca

Table 4: Genes downregulated 2-fold (P<0.01) or more in Gfi1b-ER RAG high cells upon addition of tamoxifen for 12h.

Gene Name	Gene ID	Fold Difference on Addition of OHT
Ctss	NM_021281	-7.08901
Gimap4	NM_174990	-5.41281
Rag1	NM_009019	-4.8565
Cd53	NM_007651	-4.79329
Crisp1	NM_009638	-4.49635
Fcrl1	NM_178165	-4.16806
EG432555	NM_001024230	-4.12304
Cd36	NM_007643	-4.05041
EG432555	NM_001024230	-3.83801
Rgs18	NM_022881	-3.7208
Rag2	NM_009020	-3.56339
Tnfrsf19	NM_013869	-3.43461
P2ry10	NM_172435	-3.33522
Gm885	ENSMUST00000106794	-3.24567
Kynu	NM_027552	-3.06035
EG209380	NR_003967	-2.92093
Il33	NM_133775	-2.90291
Cpm	BC100404	-2.90269
Emp1	NM_010128	-2.6347
Casp1	NM_009807	-2.61513
EG240921	ENSMUST00000037976	-2.61086
P2ry5	NM_175116	-2.58692
Evl	NM_007965	-2.5464
6430550H21Rik	BC062956	-2.52049
Bmpr2	NM_007561	-2.41275
Acsm3	NM_016870	-2.39784
Car5b	NM_181315	-2.34385
Mrc1	NM_008625	-2.29329
Rcbtb1	NM_027764	-2.27865
Gpr174	NM_001033251	-2.27653
Map3k5	NM_008580	-2.23671
Csf1	NM_007778	-2.22449
Ly96	NM_016923	-2.1574
Trpc4	NM_016984	-2.09655
Ighv1-72	ENSMUST00000103541	-2.04089
Il18rap	NM_010553	-2.00103

Table 5: Genes upregulated 2-fold (P<.01) or more in Gfi1b-ER RAG high cells upon addition of tamoxifen for 12h.

Gene Name	Gene ID	Fold Difference on Addition of OHT
Relt	NM_177073	2.00938
Cxcr5	NM_007551	2.04112
Mc1r	NM_008559	2.04851
Fgd2	NM_013710	2.05074
Gna15	NM_010304	2.05478
P2rx1	NM_008771	2.06713
Cd300lf	NM_145634	2.07628
Cish	NM_009895	2.09622
Tmem86a	NM_026436	2.13283
Camkk1	NM_018883	2.22305
Bhlhb8	NM_010800	2.35518
Lyz2	NM_017372	2.39558
Il1r2	NM_010555	2.56765
Mmp13	NM_008607	2.57759
H2-DMb2	NM_010388	2.69428
Ifitm3	NM_025378	2.96388
Pgf	NM_008827	3.31221
Ptgs1	NM_008969	4.14164

CHAPTER 4 The Role of Promoters and Enhancers in V(D)J Recombination

INTRODUCTION:

The Accessibility Hypothesis:

One of the most important insights into the regulation of V(D)J recombination came with the discovery that transcription is correlated with actively rearranging loci, and that this transcription promotes accessibility of RSS sequences to the recombinase (1). Transcription at rearranging loci is dependent on the transcriptional enhancers that are associated with each locus, as well as promoters that exist upstream of V segments and in intergenic regions of rearranging loci (reviewed in (2-4)).

The Role of Enhancers in V(D)J Recombination

A number of studies have demonstrated that removal of enhancers from rearranging gene loci inhibits transcription and causes defects in V(D)J recombination. In mice, deletion of the heavy chain Eμ enhancer abrogates transcription in the D-J_H region, causes an incomplete block in D-J rearrangement (5), and a complete block in V-DJ rearrangements (5-7). In contrast, *kappa* locus accessibility appears to be controlled by three separate enhancers (8). Deletion of the intronic enhancer or the 3' enhancer alone impair V-J joining, while deletion of both these enhancers ablates both transcription and rearrangement (9-12). The 3rd enhancer, called Ed, was identified on the basis of hypersensitivity sites, but its function has not been tested *in vivo*. In pro-B cell lines, it enhances transcription of luciferase constructs downstream of a Vκ promoter in an orientation independent manner (8). The *TCR beta* locus also contains a transcriptional enhancer called *Eβ* (13). Deletion of this enhancer in mice inhibits recombination in both DJ clusters, causing a block in T cell development at the double negative stage. While transcription upstream of Vβ segments in these mice is relatively normal, transcription originating from within the DJ cluster is inhibited (14-18).

The Role of Promoters in V(D)J Recombination:

The role of promoters in rearranging loci has also been extensively studied in cell lines and in mice. Using a TCRβ minilocus, Sikes et al demonstrated that moving a promoter from the beta locus, *pDβ1*, 400 bp upstream of a rearranging gene segment markedly decreased rearrangement of that segment (19). Deleting *pDβ1* in mice inhibits recombination at the Dβ1 cluster but not the Dβ2 cluster (20). While deletion of *pDβ1* causes local effects on chromatin structure around the promoter, chromatin surrounding Jβ1 is unaffected, indicating that the activation of promoters at rearranging loci causes local effects in chromatin structure, and that the effects of enhancer activation are more global (21). These authors postulate a holocomplex between enhancers and promoters in this locus. Promoters upstream of V segments are also important for promoting their ability to rearrange, as deletion of the Vβ13 promoter ablates rearrangement of that segment (22). Studies in the *alpha* locus also support the notion that promoters

are important for promoting accessibility to the recombinase. A number of groups have shown that deletion of the T early alpha (TEA) promoter decreases rearrangement at the *alpha* locus (23, 24). In addition, transcription through a rearranging gene segment appears to be necessary for rearrangement in the *alpha* locus (25).

In the heavy chain locus, *IL-7R* deletion impairs production of germline transcripts and recombination of distal V_H segments (26). While the *pDQ52* promoter that lies 5' of DQ52 has been shown to have both promoter and enhancer activity (27), deletion of this element does not inhibit D-J rearrangement, but does skew usage of particular gene segments (28, 29).

Deletion of both the proximal and the distal promoters at the *kappa* locus leads to ablation of *kappa* gene rearrangement in *cis* in mice and in a pro-B cell line (30, 31). However, neither promoter has been deleted individually, although deletion of the KI/KII elements downstream of the proximal promoter inhibits rearrangement while leaving transcription intact (31). It has recently been demonstrated that it is primarily the distal promoter that has activity in mice (32), so a targeted deletion of this promoter exclusively is required to fully understand its effect on kappa rearrangement.

Transcription and Rearrangement Potential

While the accessibility hypothesis is an attractive model for targeting of the recombinase to the appropriate gene locus at the appropriate developmental window, several groups have carried out studies uncoupling transcription from rearrangement potential. Early on, two groups demonstrated that transcription was not sufficient to activate rearrangement (33, 34). Indeed a pax 5 null mutation in mice results in a complete defect in rearrangement at the heavy chain locus, even though some heavy chain gene segments are still transcribed (35, 36). In addition, placement of the *alpha* locus enhancer, *E*α, next to a Vβ gene segment is insufficient to induce rearrangement of this segment (37).

Conversely, several groups have demonstrated that rearrangement can take place in the apparent absence of transcription. Inversion of a Dβ promoter at a rearranging minilocus does not prevent rearrangement, even though the rearranging genes are no longer transcribed (19). In addition, tethering a chromatin remodeler to a recombination substrate can induce recombination in the absence of transcription (38). A very interesting observation was further made in mice lacking the Eβ enhancer. While these mice have nearly undetectable levels of coding joint formation, their signal joint formation is less severely impaired, and the level of double-strand break generation is also less severely affected (18). These results indicate that there may be an additional function for the presence of promoters and enhancers at rearranging loci beyond that of accessibility.

A Role for Promoters and Enhancers Beyond Accessibility?
A common organizational theme that has emerged from the study of rearranging gene loci is that promoters are generally found upstream of rearranging gene segments and enhancers are found downstream of rearranging gene segments. In light of this observation and the data outlined above, we considered two hypotheses about the position and function of promoter and enhancer elements at rearranging loci that go beyond their role in promoting accessibility. We hypothesize that DNA binding proteins may be recruited to these elements and that association of these DNA binding proteins could be important for the process of recombination. Specifically, association between factors bound at promoters and enhancers may promote the looping out of intervening DNA and aid in the "capture" of an RSS for paired cleavage by the recombinase (39). Association of these factors could also be important for stabilizing the post-cleavage complex so that coding ends do not dissociate prematurely before joining by the NHEJ machinery. Evidence suggests that the RAG proteins remain tightly bound to signal ends following cleavage (40). If RAG proteins are responsible for preventing disassociation of signal ends, then interactions between DNA binding proteins bound to promoters and enhancers could serve an analogous purpose in holding coding ends together. Thus, association between DNA binding factors bound at promoter and enhancer elements could tether the coding ends together, preventing aberrant repair. We sought to test this hypothesis using a set or rearrangement reporter constructs depicted in Figure 1 and described in detail below.

MATERIALS AND METHODS:

Plasmid Construction:
The rearrangement reporter construct was created by Pfu mediated PCR of the Eμ heavy chain enhancer and a V_H gene-segment (V_HKI) promoter (41) and subsequent insertion into the pMX-delCJ (42) rearrangement reporter. The internal ribosome entry site (IRES) was inserted upstream of GFP using appropriate restriction sites. The Eμ, V_HKI, ires-GFP, RSS 12, RSS23, and hCD4 fragments were excised in a single unit and inserted into the self-inactivating pLV-UT-tTR-KRAB lentiviral vector obtained from Addgene.

Viral Infections:
Lentiviral constructs were co-transfected with VSVG and pMD2G into 293T cells using Lipofectamine 2000 as described above and virus was harvested and used to infect cell lines as described in Ch 3.

RESULTS:

In an effort to understand how interactions between promoters and enhancers influence recombination, we engineered two rearrangement reporter constructs depicted in Figure 1. Both constructs contain a 12 and a 23 RSS that flank an ires-GFP sequence. In addition, both constructs contain a V heavy chain promoter, V_HKI (41) upstream of the RSS sequences, and a hCD4 cDNA

downstream of the RSS sequences. Thus, upon recombination, the RSS sequences and the ires-GFP between them are looped out of the chromosome and GFP expression is lost. At the same time, the hCD4 sequence is brought into proximity of the V_HKI promoter and is consequently expressed once the cell has undergone a recombination event. In other words, any cell that has undergone a recombination event loses GFP expression and gains hCD4 expression. Both constructs are cloned into a self-inactivating lentiviral backbone in order to avoid the influence of the viral long terminal repeats on accessibility of the substrates. The difference between the two constructs lies in the placement of the heavy chain Eμ enhancer. In the "opposite side" construct, this enhancer is placed just downstream of the hCD4 element, whereas in the "same side" construct the enhancer is placed just upstream of the V_HKI promoter.

We reasoned that if elements that bind to enhancers and promoters associate with each other to form a stable holocomplex, and if this complex is necessary to prevent disassociation of coding ends following RAG cleavage, then placement of the enhancer upstream of the V_HKI promoter would prevent this stabilization. If this were the case, a possible consequence of moving the enhancer upstream of the promoter would be a normal level of RAG cleavage, but a defect in the ability to form coding joints. With respect to the rearrangement reporters, this phenotype would be reflected in comparable levels of GFP loss between populations of reporter cells, but a decrease in the amount of hCD4 expression in reporter cells infected with the "same side" construct as compared to reporter cells infected with the "opposite side" construct.

Similarly, if elements bound to promoters and enhancers associate with each other to aid in the "synapsis" phase of recombination, reporter cells infected with the "same side" construct may show lower levels of rearrangement than their "opposite side" counterparts.

To compare recombination efficiencies between cells infected with the "opposite side" construct and those infected with the "same side" construct, we first infected the E47+/+ pro-B cell line with each construct and sorted out GFP positive cells by flow cytometry. We then treated sorted, GFP positive cells with STI-571 overnight, washed out the drug, and monitored recombination over 5 days by measuring GFP and hCD4 expression (Figure 2). Treatment with STI-571 resulted in a dramatic increase in the number of GFP negative, hCD4 positive cells in the population, indicating that the rearrangement reporter was adequately accessible to the RAG proteins in cells infected with either construct. However, we did not detect significant differences in rearrangement efficiencies between reporter cells infected with the "same side" construct when compared to cells infected with the "opposite side" construct (Figure 3) as measured by comparing the number of hCD4 positive cells induced by STI-571 treatment. We conclude from these data that placement of the enhancer and promoter on the same side of the rearranging substrate in this particular reporter does not

significantly alter the cell's ability to undergo RAG cleavage events or efficiently join the coding ends generated from those events.

In collaboration with Christian Vettermann, we went on to generate an "enhancerless" version of the rearrangement reporter. This reporter was identical to the "same side" construct, except that the enhancer was excised. We went on to assay recombination efficiencies in cells infected with this "enhancerless" reporter and compared them to those obtained with the "same side" reporter (Figure 4). Interestingly, while reporter cells infected with the "enhancerless" rearrangement construct had lower levels of transcribed GFP (Figure 5), transcription was not abrogated by any means. Despite the fact that transcription was still present in these cells, they showed markedly lower levels of recombination upon treatment with STI-571 when compared to cells infected with the "same side" rearrangement reporter that contained the enhancer upstream of the promoter (Figure 4B). In three independent experiments the enhancerless construct consistently shows an approximately 3-fold reduction in rearrangement when compared to the construct containing the enhancer. These data indicate one of two possibilities. The first is that transcription through the rearranging substrate is not sufficient to generate efficient recombination efficiency in this reporter. If this is the case, we interpret these data to indicate that the presence of the enhancer may play a critical role in governing the ability of a given substrate to undergo recombination, but that this role is independent of its position with respect to the promoter. The second interpretation is that a threshold level of transcription is required for efficient rearrangement, and that this threshold is not reached in the enhancerless construct, thus decreasing its rearrangement potential.

DISCUSSION:

We set out to test the hypothesis that interactions between promoters and enhancers play an important role in V(D)J recombination. More specifically, we wanted to know whether proteins bound to promoters and enhancers could associate with each other to a) aid in bringing two RSS sequences into proximity for RAG cleavage (synapsis) and/or b) stabilize coding ends within the post-cleavage complex to prevent dissociation of these ends prematurely before joining of the coding ends by the NHEJ machinery. While our data do not support our hypothesis, there are a number of limitations to this particular experimental system. Most importantly, recombination substrates in their physiological context are positioned at much greater distances than those represented in our reporter constructs, which were limited by the requirement to be able to efficiently package the virus. It is possible that recombining substrates in their physiological context are more dependent on promoter/enhancer interactions to bring them into close proximity for RAG cleavage, while those in our system are positioned quite close together to start with, and thus may not depend on these interactions. One way to test this idea would be to generate a knock-in mouse wherein the endogenous heavy chain enhancer is deleted, and

replaced with an enhancer just upstream of the promoter in the D-J interval. This would test the same idea in a more physiological context.

Second, while we envisioned a simple looping model, the 3 dimensional architecture of a recombining locus may be substantially more complex, and it may be sufficient to simply have the required accessibility control elements present within a certain vicinity of the recombining substrates to effect the necessary architecture, thus eliminating the need for the promoter and enhancer to be on opposite sides of the recombining substrates. In addition, the physiological architecture found at the endogenous recombining loci may not be recapitulated in our reporter system, since the distances are altered and a large number of the endogenous locus elements are missing in the reporter.
Third, it's possible that our hypothesis is correct and that association between factors bound to promoters and enhancers is necessary to stabilize coding ends within the post-cleavage complex. However, if cells that fail to form coding joints are eliminated from the culture by apoptosis, we would not be able to detect these events, since our flow cytometry analysis is conducted only on live cells. It may be interesting to compare levels of apoptosis in cells infected with the different reporters and treated with STI-571. Alternatively, if large deletions result from destabilized coding ends, our assay system would not allow us to specifically detect these events using the fluorescent markers within the reporters.

Intriguingly, while moving the heavy chain enhancer within the recombination reporter had little effect on rearrangement, eliminating the heavy chain enhancer severely crippled reporter rearrangement (Figure 4), even though transcription of the GFP reporter remained intact (Figure 5). Like others, (18, 19, 33-38).
we have thus uncoupled transcription from rearrangement potential in this reporter system. This observation adds additional credence to the idea that the presence of an enhancer has a role beyond that of accessibility, and may aid in the process of coding joint formation. Two easy ways to test this hypothesis would be to a.) compare the level of double-strand breaks generated in constructs with or without the enhancer, and b.) compare frequencies of signal joint formation between the two constructs. If the presence of the enhancer has a specific role in promoting coding joint formation, we would expect level of double-strand break and signal joints to be comparable regardless of the presence of the enhancer, as was observed in Eβ deficient mice (18). We have so far not been able to determine exactly why the absence of the enhancer has such a strong effect on recombination potential, but a number of possibilities leap to mind.

It has been recently shown that RAG proteins bind to trimethylated H3K4 (43), and that this modification influences RAG cleavage (44, 45). It is possible that the presence of the enhancer is required to recruit the necessary methyltransferase. In the absence of this modification, RAGs may not be efficiently recruited to the recombining substrate. This could be easily tested by

performing ChIP and comparing levels of trimethylated H3K4 within the reporter in the presence or absence of the enhancer.

Another possibility is that the enhancer recruits nucleosome remodelers, and that in the absence of such remodelers, RAG accessibility to the RSS sequences is restricted. This is supported by experiments demonstrating that RAG cutting is prevented by the presence of a nucleosome over an RSS (46).

CONCLUSIONS AND FUTURE DIRECTIONS:

We conclude from this set of experiments that the presence of an enhancer on the same side or the opposite side of a promoter in a rearrangement reporter construct does not have a significant effect on the frequency of recombination. However, removal of the enhancer significantly decreases the recombination potential of this reporter construct.

One set of experiments that could help to elucidate the role for the enhancer in this reporter system would be to identify and characterize protein complexes bound to the promoter sequence in the presence or absence of the enhancer. This could help determine whether factors being recruited by the enhancer are indeed associating in complexes with promoter binding factors.

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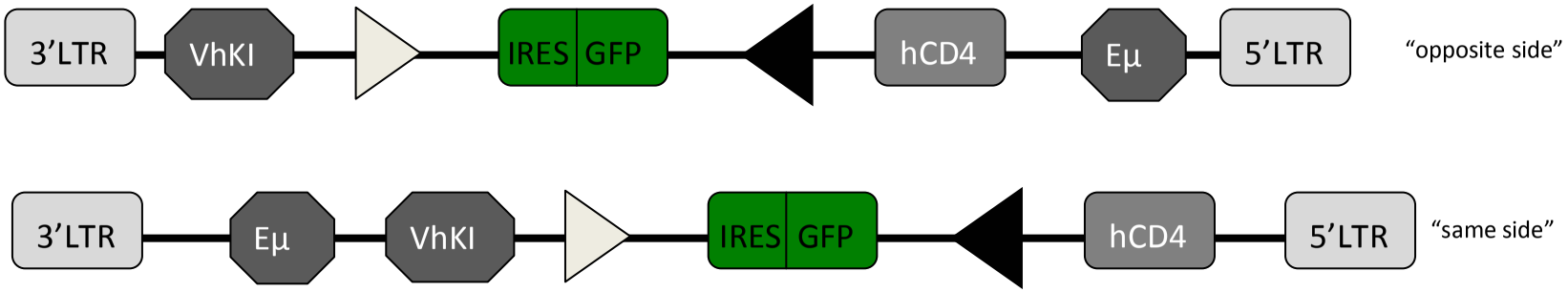

Figure 1: Schematic of opposite side (top) and same side (bottom) rearrangement reporter constructs. Cells having undergone a rearrangement event will lose GFP expression and gain hCD4 expression. White triangle, RSS12, black triangle, RSS23, octagons, accessibility control elements, rounded rectangles, reporters. LTR, long terminal repeat, hCD4=human CD4, ires, internal ribosome entry sequence, GFP, green fluorescent protein.

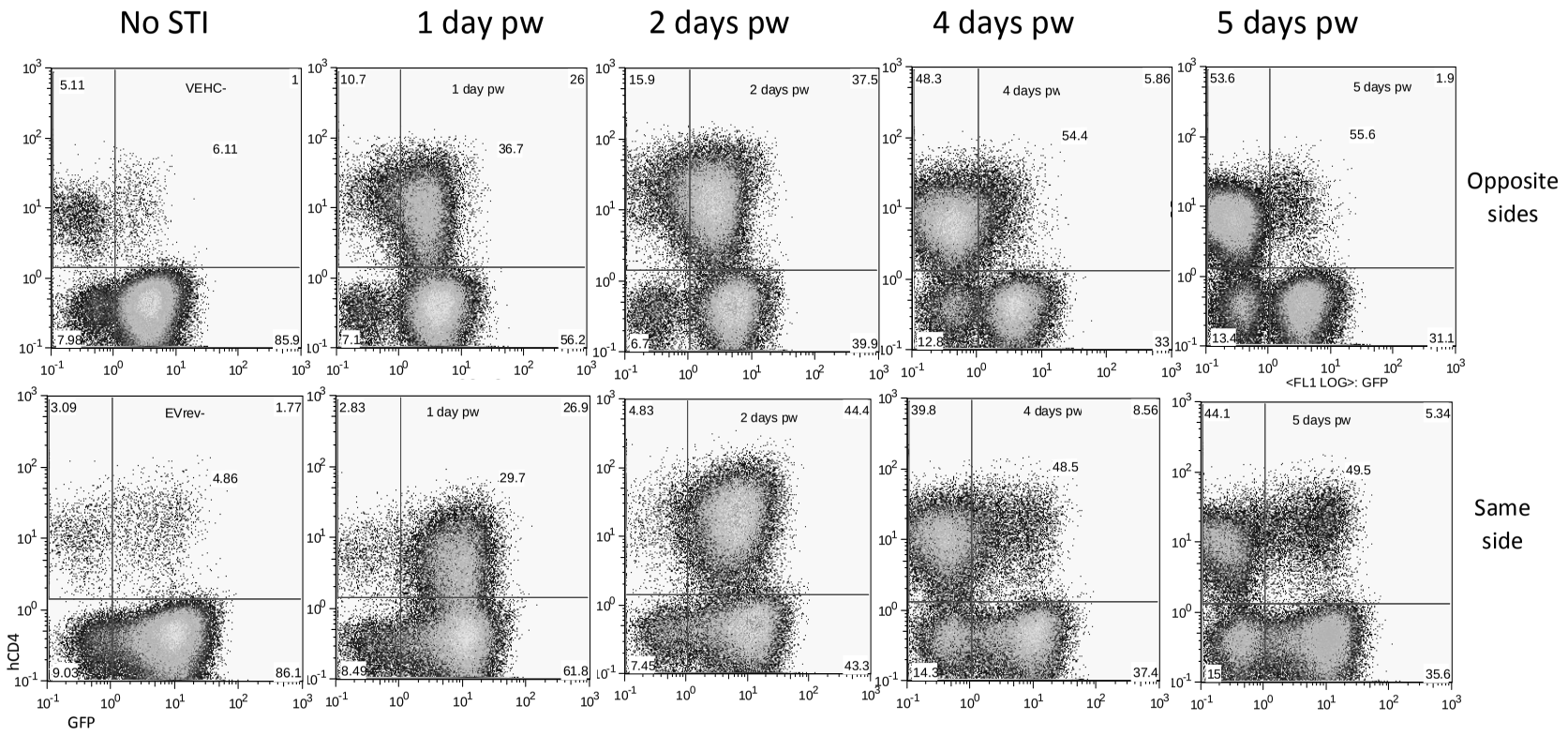

Figure 2: Flow cytometry of GFP and hCD4 expression in E47+/+ cells infected with either the opposite side rearrangement reporter (top panels) or the same side rearrangement reporter (bottom panels). Cells were treated overnight with 2.5 μM STI-571, washed, and cultured for 5 days. Pw, post wash.

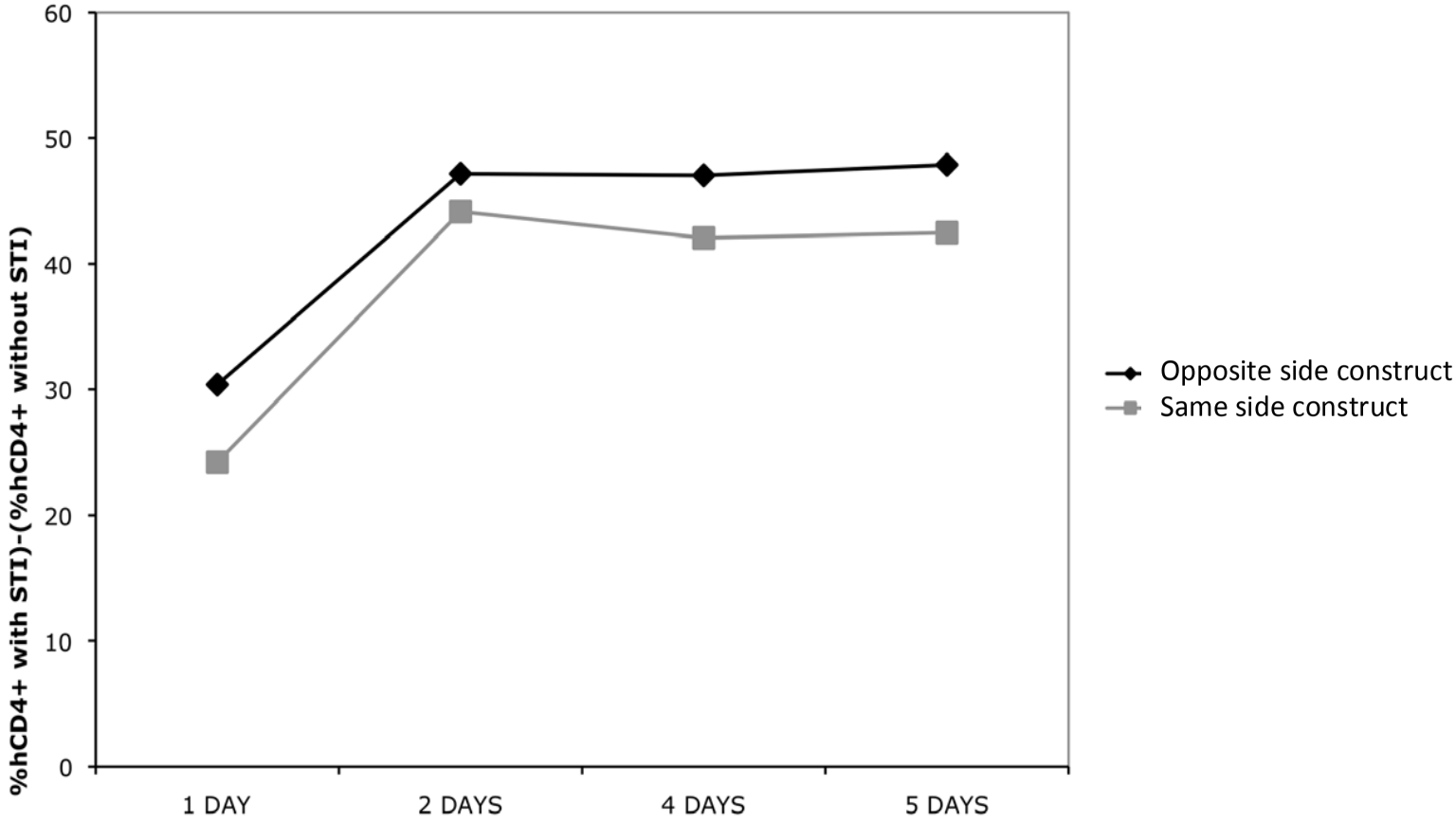

Figure 3 Quantification of data in figure 2. The difference in total number of hCD4 positive cells plus and minus STI for each time point is shown.

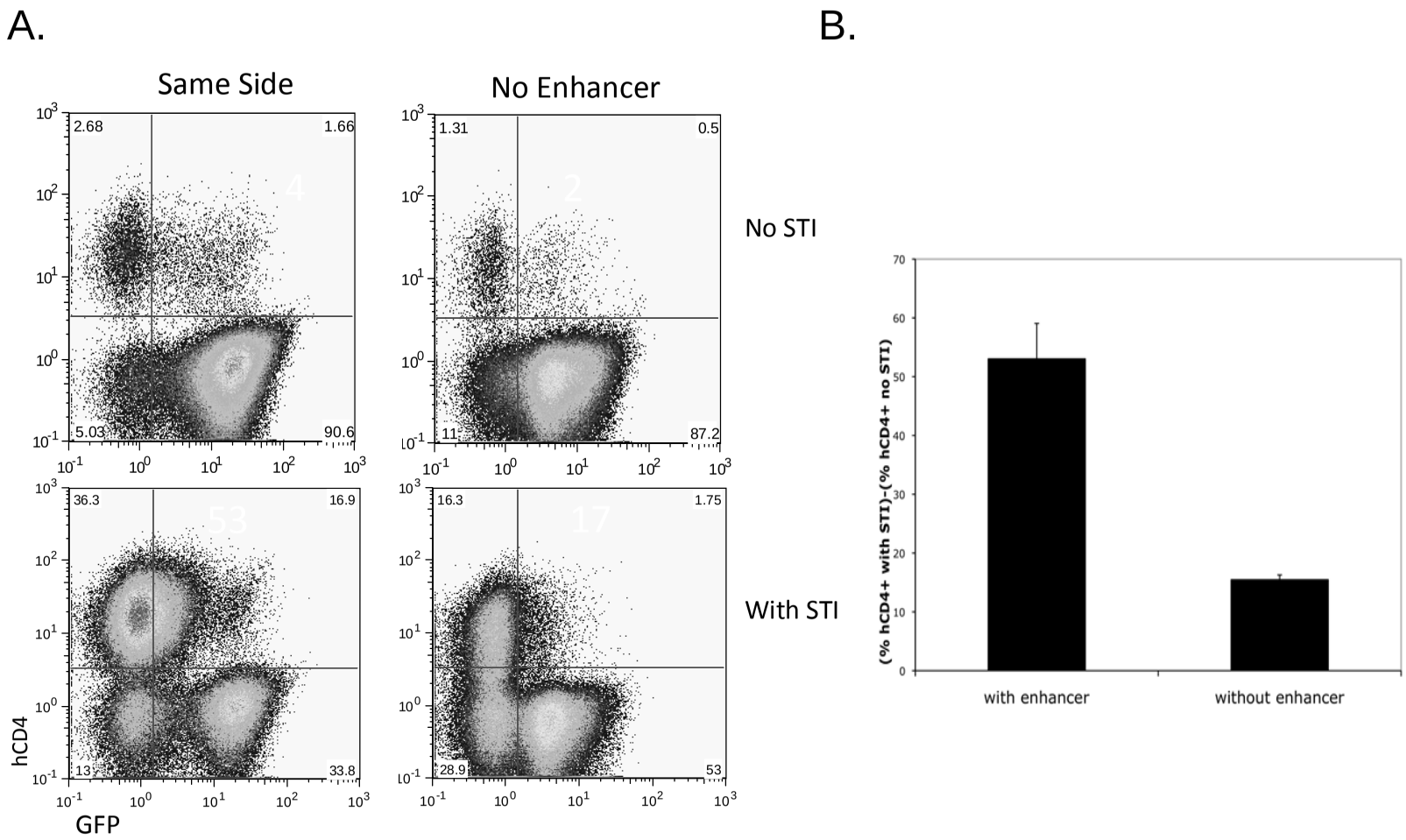

Figure 4. A. Flow cytometry of GFP vs. hCD4 expression in E47+/+ cells infected with either the "Same Side" rearrangement reporter or a rearrangement reporter lacking an enhancer. Cells were treated overnight with 2.5 μM STI-571, washed and cultured for 5 days. Data is representative of 3 independent experiments. B. Quantification of % hCD4+ cells following treatment with STI-571 in in reporter cells with the enhancer compared to those without the enhancer. Average is given for 3 separate experiments.

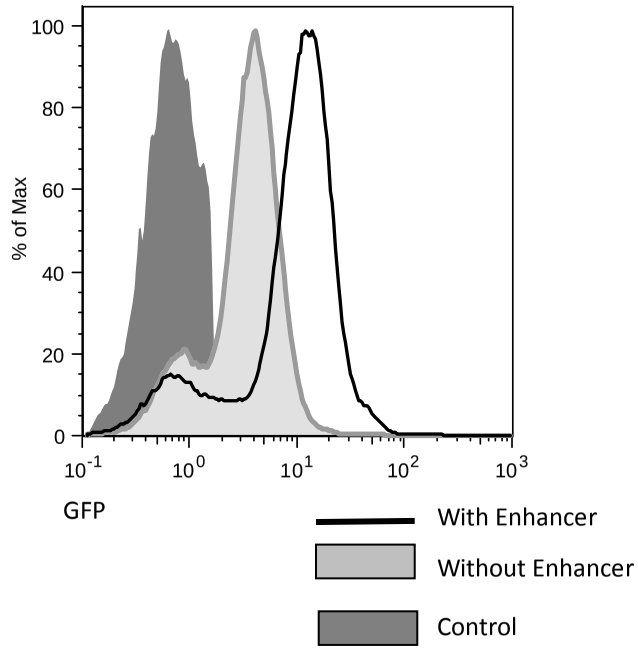

Figure 5: Flow cytometry of AMuLV pro-B cells infected with either the “opposite side” or “same side” construct.