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TOPIC HIGHLIGHT

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Ikaros in B cell development and function

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Abstract

The zinc finger transcription factor, Ikaros, is a central regulator of hematopoiesis. It is required for the development of the earliest B cell progenitors and at later stages for VDJ recombination and B cell receptor expression. Mature B cells rely on Ikaros to set the activation threshold for various stimuli, and to choose the correct antibody isotype during class switch recombination. Thus, Ikaros contributes to nearly every level of B cell differentiation and function.

Key words: Ikaros; B cells development; B cell activation; Class switch recombination

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INTRODUCTION

The Ikaros (*Ikzf1*) zinc finger transcription factor is a critical regulator of hematopoiesis. Originally identified as a protein binding regulatory regions of the lymphocyte specific genes *dntt* (terminal deoxynucleotidyl transferase)[1] and *cd3d*[2], Ikaros was soon shown to be required for fetal T cell development[3], as well as important steps in adult thymic development, such as the pre-T cell receptor (TCR) checkpoint and CD4 *vs* CD8 T cell differentiation[4-6]. Far from being T cell specific, Ikaros is expressed in virtually all hematopoietic cells in mice[7,8] and humans[9-13], and even in the murine neuro-endocrine system[14]. In mice, Ikaros has been shown to be crucial for hematopoietic stem cell function and renewal, and promotes the differentiation of conventional and plasmacytoid dendritic cells, natural killer cells, neutrophils and erythrocytes[15-21]. Furthermore, Ikaros is a critical tumor suppressor because mice bearing mutations in the *Ikzf1* gene develop T lymphomas with near complete penetrance[22-24], and *Ikzf1* mutations are found in multiple types of human B and T cell lymphomas and leukemias[25-27]. In addition to these key roles in T cells and non-lymphoid lineages, Ikaros is a critical regulator of B cell lymphopoiesis and function, and this is discussed in detail below. Importantly, unless otherwise stated, experiments referred to in this review were performed in the murine system.

MECHANISMS OF IKAROS-MEDIATED TRANSCRIPTIONAL REGULATION

Ikaros acts as both a transcriptional activator and repressor, depending in large part on the co-factors with which it interacts (Table 1). Mechanisms of Ikaros-mediated repression fit into three broad categories: chromatin modification, co-repressor recruitment, and competition. First, Ikaros colocalizes with pericentromeric heterochromatin in lymphocytes[28], and interacts with components of histone deacetylase (HDAC) complexes, including Sin3A and Sin3B (Sin3 complex), the chromatin remodeling Mi-2b ATPase (NuRD complex), and HDAC-1 and HDAC2 (both Sin3 and NuRD complexes)[29-32]. Thus, Ikaros could contribute to transcriptional repression by recruiting genes to heterochromatin[33], and/or by recruiting chromatin-modifying complexes to specific genes to enforce repressive chromatin. Second, *in vitro* assays have shown that Ikaros can recruit the C terminal binding protein (CtBP) and CtBP interacting protein (CtIP) co-repressors, which may in turn repress transcription by interacting directly with the basal transcriptional machinery (TATA binding protein and transcription factor IIB)[32,34,35]. Finally, Ikaros competes with positive factors to repress transcription of genes such *Igll1* [Lambda5; competes with early B cell factor (EBF)][36], *dntt* (terminal deoxynucleotidyl transferase; competes with Ets)[37], and *Hes1* (competes with the RBP-Jk/Notch complex)[38].

Ikaros may also function as a transcriptional activator. Ikaros can activate transcription of reporter plasmids[39], *cd8a* transcription in developing T cells[40], and adult globin genes in developing erythrocytes[21]. Interestingly, the histone acetyltransferase that contains the SWI/SNF chromatin remodeling complex interacts with Ikaros in both T cells[31] and erythrocyte precursors[41] and is associated with transcriptional activation[42]. Furthermore, Ikaros also interacts with the positive transcriptional elongation factor complex in yolk sac erythroid cells, recruiting it during the induction of globin genes[43]. Thus, Ikaros may activate the transcription of certain target genes, possibly by recruiting SWI/SNF, or promoting transcriptional elongation.

Post-translational modifications of Ikaros itself provide an additional layer of complexity to Ikaros-mediated transcriptional regulation. Ikaros can be phosphorylated and dephosphorylated at multiple residues, by casein kinase 2 and protein phosphatase 1, respectively[30,44-46]. Phosphorylation of Ikaros in turn inhibits its DNA binding, ability to block the cell cycle and repress genes such as *tdt*, and recruitment to peri-centromeric heterochromatin[44-46]. In addition to phosphorylation, SUMOylation at two separate residues (K58 and K240) antagonizes interactions between Ikaros and Sin3A, Sin3B, Mi-2b and CtBP, and relieves Ikaros mediated repression of reporter plasmids[47]. Thus, Ikaros can repress or activate transcription through a variety of mechanisms depending on post-translational modifications, cell type, protein partners and target gene.

IKAROS IN B CELL DEVELOPMENT

B cell development in the bone marrow takes place in sequential steps that are characterized by gene expression programs, and developmental checkpoints centered on antigen receptor rearrangement (Figure 1A, Table 2)[48,49]. To start, hematopoietic stem cells become progressively more restricted to the lymphoid lineage, differentiating into lymphoid primed multi-potent progenitors (LMPPs) and then common lymphoid progenitors (CLPs)[48]. The transcription factor E2A and interleukin-7 receptor (IL-7R) signaling in CLPs induce the expression of the B lineage specifying transcription factor, EBF1[50-52], and together with Flt3 signaling, differentiation into the earliest committed B cell developmental stage, the pre-pro B cell[53]. EBF1 allows further progression to the pro-B stage and induces the expression of B lineage genes. Crucially, one of these genes is Pax5[54,55], which is required for further development[56] and locks in the B lineage by repressing other cell fates[57,58]. At the pro-B stage, cells undergo immunoglobulin heavy chain (*Igh*) rearrangements, and successfully rearranged heavy chains pair with the surrogate light chain proteins Lambda5 and VpreB1/2 to provide a maturation signal[59,60]. This pre-B cell receptor (BCR) signaling, combined with IL-7R signals, induces differentiation into pre-B cells, several rounds of division, and rearrangement of the Ig light chain[61-63]. Those cells that a express functional BCR, consisting of Ig heavy and light chains, suppress further rearrangements (allelic exclusion) and migrate to the spleen to undergo final maturation steps.

Control of B lineage specification and commitment by Ikaros

Ikaros plays crucial roles in B lineage specification and commitment. $Ik^{-/-}$ mice lack pre-pro-B cells and exhibit a complete block in B lymphopoiesis[64]. Similarly, there is a striking reduction in pre-pro-B cells in mice bearing a hypomorphic mutation in *Ikzf1* ($Ik^{L/L}$), which results in Ikaros expression at about 10% of normal levels[65]. Together, these data indicate that Ikaros is crucial for B lineage specification. This was originally thought to be due to a role for Ikaros in promoting Flt3 and/or IL-7R expression on early hematopoietic progenitors because: (1) Flt3 and IL-7R signaling are required for pre-pro-B cell development[53]; (2) $Ik^{-/-}$ LSK ($Lin^{-}Sca1^{+}c\text{-}kit^{+}$) cells lack *Flt3* mRNA expression[16]; and (3) $Ik^{-/-}$ LMPPs express reduced levels of *Il7r* mRNA[66]. Retroviral expression of IL-7R or Flt3 independently in $Ik^{-/-}$ LSK cells however, does not rescue B cell development, indicating that (1) it is the reduced expression of both receptors together (not either one individually) which blocks $Ik^{-/-}$ B lymphopoiesis, and/or (2) that Ikaros has other important functions in B lineage specification[67]. In support of the latter view, retroviral expression of EBF in $Ik^{-/-}$ LSK cells does rescue pro-B cell development, indicating that

Table 1 Ikaros co-factors

Protein/complex	Citation	System
NuRD complex (Mi2b, HDACs)	[31]	Over-expression in murine T cells
	[30]	Over-expression in a murine DP thymocyte cell line
	[41]	Endogenous proteins in a murine erythro-leukemia cell line
	[32]	Over-expression in non-lymphoid cell lines
Sin3 complex (Sin3a/b, HDACs)	[34]	Over-expression in non-lymphoid cell lines and in murine T cells
	[32]	Over-expression in non-lymphoid cell lines
CtBP	[34]	Over-expression in non-lymphoid cell lines and in murine T cells
	[32]	Over-expression in non-lymphoid cell lines
CtIP	[35]	Over-expression in non-lymphoid cell lines and in murine T cells
SWI/SNF complex (BRG1, BAFs)	[31]	Over-expression in murine T cells
	[41]	Endogenous proteins in a murine erythro-leukemia cell line
pTEFb (cdk9)	[43]	Endogenous proteins in murine yolk sac erythroid cells

CtBP: C terminal binding protein; CtIP: CtBP interacting protein; pTEFb: Positive transcriptional elongation factor complex; HDAC: Histone deacetylase.

Table 2 Critical members of the transcription factor network controlling B cell development

Gene	Ref.	Roles
Ikaros (*Ikzf1*)	[64]	Required for earliest B cell progenitors
	[65,67]	Required for efficient pro-B to pre-B transition
	[67]	Required for repression of non-B cell fates
	[65,67]	Promotes Igh rearrangment and Rag expression
E2A (*Tcfe2a*)	[51]	Required for earliest B cell progenitors
	[86]	Required for EBF1 expression
EBF1 (*Ebf1*)	[87]	Required for expression of B lineage genes development past pro-B stage
Pax5 (*Pax5*)	[56]	Required for differentiation past pro-B stage
	[57,58]	Required for commitment to B cell lineage (e.g. repression of alternative fates)

EBF1: Early B cell factor 1.

Ikaros contributes to B lineage specification by promoting the expression of EBF[67]. Ikaros likely contributes to EBF expression in part by activating IL-7R expression, as IL-7 signals are required for EBF transcription[50,52]. However, as retroviral expression of IL-7R could not rescue $Ik^{-/-}$ B cell development, there is likely an IL-7 signaling independent function for Ikaros in EBF upregulation[67]; these functions are not understood at this time. Finally, it should be noted that EBF mediated rescue of $Ik^{-/-}$ pro-B cell development was inefficient, indicating that Ikaros is likely to play further roles in B lineage specification that are independent of EBF regulation.

Beyond the activation of B cell specific gene programs, Ikaros is also critical for B lineage commitment. In comparison to Ikaros-sufficient pro-B cell lines, EBF-induced $Ik^{-/-}$ pro-B cell lines exhibit promiscuous myeloid gene expression (e.g. *csf1r*). More strikingly, like Pax-$5^{-/-}$ pro-B lines, $Ik^{-/-}$ pro-B lines can be differentiated into macrophages when cultured with macrophage colony-stimulating factor[67]. Thus, in addition to contributing to B lineage specification through the activation of IL-7R, EBf1 and Flt3 expression, Ikaros also locks in the B lineage by shutting off alternative cell fates.

Ikaros in Ig gene recombination

Ikaros clearly plays critical roles in B cell development beyond specification and commitment. Ikaros hypomorphic mice ($Ik^{L/L}$) exhibit a partial block in differentiation between the pro-B and pre-B stages, indicating that Ikaros promotes this transition[65]. Similarly, EBF-induced $Ik^{-/-}$ pro-B cell lines do not mature into pre-B cells, further demonstrating that Ikaros contributes to developmental checkpoints in pro-B cells[67]. Interestingly, early experiments has found that compared with wild type (WT), $Ik^{L/L}$ pro-B cells express lower levels of *Rag1* and *Rag2*, which mediate VDJ recombination[65], indicating that Ikaros may contribute to pre-B development by promoting heavy-chain rearrangements. This was confirmed by the observations that EBF-induced $Ik^{-/-}$ pro-B cells lack *Rag1* and *Rag2* expression and Ikaros binds directly to their promoters[67]. Thus, it appears that Ikaros controls pre-B development by activating *Rag* gene expression and *Igh* rearrangments. Indeed, Reynaud and colleagues have found that D_H-J_H and especially V_H-DJ_H recombination is perturbed or absent in EBF-induced $Ik^{-/-}$ pro-B lines. Interestingly, retroviral expression of Rag1 and 2 in these cell lines does not rescue V_H-DJ_H recombination, and V_H and DJ_H gene segments are found further from each other in the nucleus than in Ikaros-sufficient pro-B cell lines[67]. Thus, in addition to promoting *Rag* gene expression, Ikaros contributes to *Igh* locus contraction, a critical step required for V-DJ recombination[68], and for pro-B to pre-B differentiation.

Beyond the pro-B stage and *Igh* rearrangement, Ikaros is likely to play continued roles in B cell development. Ikaros is thought to down-regulate preBCR signaling by repressing *Igll1* (Lambda5) transcription in preB cells[69,70]. Ikaros may also contribute to light-chain rearrangment and allelic exclusion. Deletion of an Ikaros-binding, *cis*-acting regulatory element in the *Igk* locus, abolishes monoallelic silencing of V-J rearrangement, suggesting that Ikaros participates in allelic exclusion[71,72]. Ikaros-

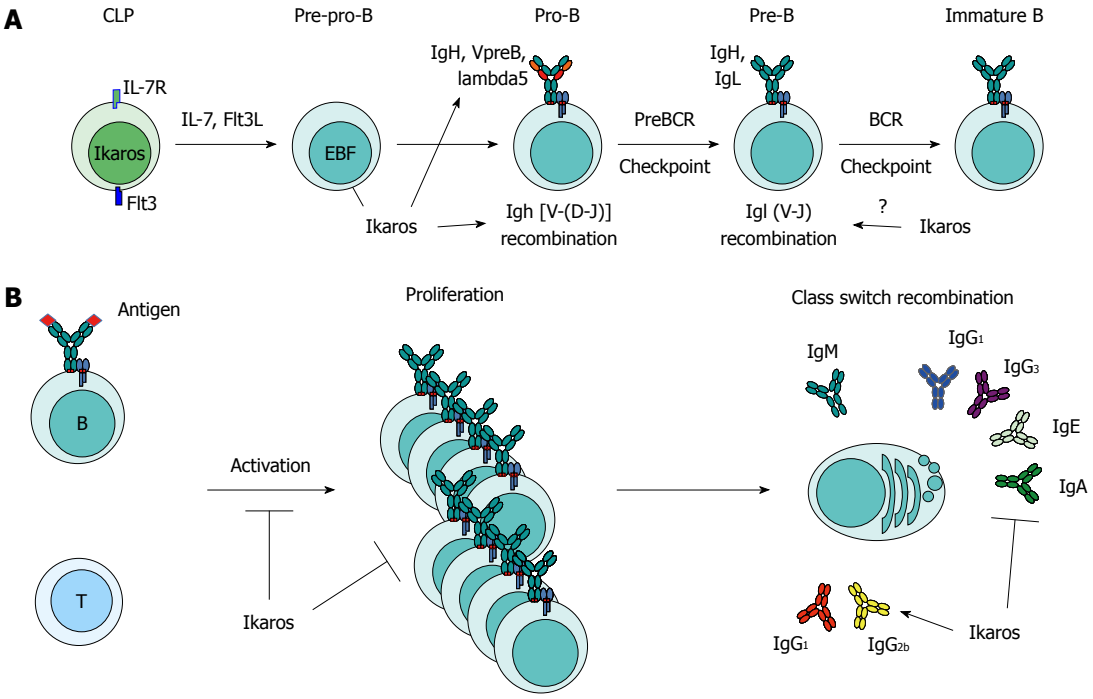

Figure 1 Ikaros controls multiple levels of B cell lymphopoiesis and function. A: Ikaros plays a crucial role in the specification of the B cell lineage by promoting the expression of the IL-7R and Flt3 receptors in common lymphoid progenitors (CLPs) and of the EBF transcription factor in pre-pro-B cells. Later, Ikaros regulates *Igh* recombination by activating *Rag* gene expression and contracting the *Igh* locus. After the preBCR check point, Ikaros also downregulates the expression of the preBCR component, Lambda5. During light-chain rearrangment, it is thought that Ikaros regulates allelic exclusion; B: In the periphery, Ikaros sets the B cell activation threshold to antigen and T cell co-stimulation, and inhibits hyper-proliferation of activated B cells. Finally, during class switch recombination, Ikaros controls isotype choice by inhibiting switching to IgG2b and IgG2a and promoting switching to all other isotypes.

mediated control of this process has not been rigorously tested, however, as the entire silencer (> 4 kb) rather than specific Ikaros binding sites, is deleted, and allelic exclusion has yet to be studied in Ikaros-deficient B cells. Finally, considering that Ikaros activates *Rag* gene expression in pro-B cells during *Igh* recombination, this role may well be reprised at the pre-B stage to allow for light-chain recombination. Taken together, these studies demonstrate that Ikaros is crucial to multiple early steps of B cell development.

IKAROS IN MATURE B CELLS

Mature B cells respond to antigen with co-stimulation from T cells, eventually undergoing multiple divisions, and producing high-affinity antibodies with various constant domains that provide unique effector functions. Ikaros controls both the threshold at which B cells respond, as well as the choice of antibody isotype they will express (Figure 1B).

Ikaros sets the threshold for B cell activation

Ikaros is a crucial regulator of B cell activation. Ikaros-deficient $Ik^{L/L}$ B cells exhibit lower activation thresholds to stimulation than WT cells (e.g. proliferate to lower concentrations of anti-IgM stimulation)[65]. Similarly, B cells from mice bearing a B cell specific transgene encoding the dominant negative (DN) Ikaros 7 isoform, are hyper-responsive to stimulation by mitogens such as lipopolysaccharide[73]. Thus, Ikaros sets B cell activation thresholds for antigen and mitogen stimuli. Interestingly, Ikaros plays a similar role in setting activation thresholds for TCR signals in T cells[5,74].

While it is clear that Ikaros regulates B cell responses to stimulation, the mechanism remains a mystery. Data from T cells have suggested that Ikaros maintains activation thresholds by integrating the inputs of multiple signaling cascades into a transcriptional response to stimulation[5,74]. The rationale for this is two fold: (1) in comparison with WT cells, $Ik^{+/-}$ and $Ik^{+/DN}$ T cells are resistant to inhibitors of signaling pathways that lie downstream of the TCR (MAPK, Ras, PI3K/Akt, LCK/FYN, PKC, calcineurin), indicating that no one pathway is responsible for increased proliferation; and (2) Ikaros, colocalizes with heterochromatin and replication foci, and thus its loss might result in widespread gene deregulation[5,74]. This latter point may be especially relevant in cycling cells, which must synthesize their DNA and re-establish heterochromatin with each cycle. To date however, it is unclear if Ikaros deficiency grossly changes the transcriptional response to BCR, TCR or mitogen stimulation in lymphocytes, and thus, this model lacks strong experimental support.

There are other intriguing possibilities to explain how Ikaros controls B cell activation thresholds. First, retroviral Ikaros expression in $Ik^{-/-}$ T lymphoma cell lines upregulates the cell cycle inhibitor $p27^{kip}$ and blocks cell cycle progression[75]. Thus Ikaros might set activation

thresholds in B cells by maintaining the expression of specific cell cycle inhibitors. Another intriguing possibility focuses on the Notch pathway. Ikaros represses Notch target genes in T cells[23,38] and Notch activity can synergize with BCR and CD40 signaling to enhance B cell activation[76]. Thus, Ikaros could control BCR-induced activation by suppressing Notch pathway activity. Finally, it should be noted that while Ca^{++} mobilization is similar between WT and $Ik^{L/L}$ B cells after stimulation, the activation of other signaling pathways has not been examined[65]. Furthermore, these comparisons have not been rigorously made in T cells. Interestingly, Ikaros appears to regulate BCR signaling positively in chicken DT40 cells by directly suppressing SHIP phosphatase expression[77,78]. While the hypo-responsive phenotype of $Ik^{-/-}$ DT40 cells contrasts with the hyper-responsive phenotype of Ikaros-deficient murine B and T cells, work in $Ik^{-/-}$ DT40 cells clearly demonstrates that Ikaros can control the sensitivity of signaling pathways downstream of antigen receptors. Thus, it is possible that Ikaros deficiency controls lymphocyte activation thresholds by regulating cell cycle inhibitors, Notch signals, or BCR, TCR or Toll-like receptor signaling pathways.

It is important to note that the abnormal activation threshold in Ikaros-deficient B cells may have real consequences for tolerance. Mutations that compromise B cell activation thresholds often lead to autoantibody production[79,80]. In keeping with this, mice with B cells expressing DN Ikaros express higher levels of auto antibodies than do WT mice[73]. Similarly, Ikaros hypomorphic $Ik^{L/L}$ mice express auto antibodies (Sellars M, Kastner P and Chan S unpublished data). While this suggests a possible role for Ikaros in the induction of B cell tolerance, it is unclear from current studies if this would be B cell intrinsic role for Ikaros and not one of its related family members. DN Ikaros isoforms retain the ability to interact with and inhibit other Ikaros family members, including Aiolos, which also regulates B cell activation thresholds[81]. Thus elevated auto-antibodies in mice expressing a B cell restricted DN Ikaros transgene[73], could be due in part to Aiolos inhibition. Furthermore, $Ik^{L/L}$ T cells are hyper-responsive[23], and thus auto-antibodies in these mice could be the result of defective T cell tolerance. Further studies with conditional knockouts of Ikaros will be necessary to understand its fully role in B cell tolerance.

Ikaros regulates isotype selection during immunoglobulin class switch recombination

Class switch recombination (CSR) allows the humoral immune response to clear pathogens effectively by pairing a single antibody variable region gene with different constant region genes (C_H) responsible for unique effector functions[82]. Recombination occurs between induced double stranded breaks in repetitive DNA sequences called switch (S) regions; which are located upstream of each C_H gene (except δ). These breaks are initiated by activation-induced cytidine deaminase (AID) in a transcription-dependent manner, the mechanism of which is not fully understood[82,83]. Importantly, it is transcription across specific S regions in response to antigen, cytokine, and co-stimulatory signals that targets those S regions for CSR[84]. Despite its importance, the factors controlling S region transcription have remained largely unidentified.

Recent work has demonstrated that Ikaros is a central regulator of S region transcription and thereby of isotype selection during CSR. The first clue to this came when $Ik^{L/L}$ mice were found to exhibit abnormal serum antibody titers, characterized by striking > 50% reductions in IgG_3 and IgG_1, and > 50% increases in IgG2b and IgG2a[65]. *In vitro* culture assays then revealed that Ikaros deficiency results in increased and ectopic CSR to IgG2b and IgG2a, and reduced CSR to all other isotypes[85]. Mechanistically, Ikaros binds directly to the *Igh* locus, including the 3' enhancer and S region promoters and suppresses activating epigenetic marks (e.g. histone acetylation), transcription and AID accessibility across Sγ2b and Sγ2a. In fact, this transcriptional repression at a subset of S regions allows other S regions to compete for AID-induced CSR. Thus, Ikaros is a master regulator of isotype specification during CSR, and mediates this function by modulating transcriptional competition between S regions.

CONCLUSION

Ikaros controls major aspects of B cell development and B cell responses to antigen. It contributes to B lineage specification, commitment and maturation (Figure 1A, Table 2). Ikaros activates the expression of IL7R and Flt3, signals through which are critical for the development of the earliest B cell progenitors. Ikaros further promotes B cell differentiation by inducing EBF1, which itself activates a B cell transcriptional program. Beyond specification, Ikaros plays roles similar to Pax5 in repressing alternative (especially myeloid) fates in B cell progenitors. Finally, Ikaros contributes to further maturation by activating *Rag* gene expression and constricting the *Igh* locus to allow for antigen receptor recombination. Clearly Ikaros is a critical regulator of early B cell development.

In the periphery, Ikaros controls activation thresholds and isotype choice during CSR. Ikaros appears to control CSR by directly regulating activating epigenetic marks and transcription at constant region gene promoters (Figure 1B). Notably however, the mechanism by which Ikaros regulates B cell activation remains largely undefined. Does Ikaros shape the transcriptional response to stimulation, repress Notch-mediated proliferation signals, directly control the expression of cell cycle regulators, and/or modulate B cell receptor signaling itself? Or does Ikaros act through some other mechanism to control B cell activation? Future research will be needed to resolve these questions.

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