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Highlights

FRONTLINE:

Ikaros has a crucial role in regulation of B cell receptor signaling

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The transcription factor Ikaros, a key regulator of hematopoiesis, has an essential role in lymphocyte development. In mice, fetal lymphoid differentiation is blocked in the absence of Ikaros, and whereas T cells develop postnatally, B cells are totally absent. The significance of Ikaros in the B cell development is evident, but how Ikaros regulates B cell function has neither been established nor previously been studied with B cells that lack Ikaros expression. Here we show that disruption of *Ikaros* in the chicken B cell line DT40 induces a B cell receptor (BCR) signaling defect with reduced phospholipase Cγ2 phosphorylation and impaired intracellular calcium mobilization, which is restored by Ikaros reintroduction. Furthermore, we show that lack of Ikaros induces hyperphosphorylation of Casitas B lymphoma protein subsequent to BCR activation. These results indicate that the absolute need of Ikaros for development, cell fate decisions and maintenance of B cells is due to the enhancement of BCR signaling.

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Introduction

A central issue is to understand the functional importance of regulatory genes for the hematolymphoid system. The transcription factor Ikaros (Lyf-1) [1, 2] is expressed in all hematopoietic lineages controlling the early development of hematolymphoid progenitors. Several gene targeting experiments in mice have established that the nuclear factors encoded by the *Ikaros* gene are essential for the development and function of the lymphoid system [3–6], as the development of the lymphoid lineage is severely compromised by Ikaros deficiency [3–5], while myeloid and erythroid lineage differentiation is only mildly affected [7]. In *Ikaros*-null mutant mice [3], the long-term reconstitution activity of hematopoietic stem cells is reduced [7], and fetal lymphoid cells are absent [3]. Despite the postnatal appearance of T cell precursors, B cells are not generated in the absence of Ikaros [3].

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Abbreviations: **BLNK:** B cell linker protein · **Cbl:** Casitas B lymphoma protein · **IP_3:** inositol 1,4,5-triphosphate · **PLCγ2:** phospholipase Cγ2 · **sIgM:** surface IgM

The *Ikaros* gene is comprised of seven coding exons, four of which (exons 3–6) can be alternatively spliced to generate several functionally distinct isoforms [2, 8, 9], all containing two C-terminal zinc fingers which mediate functionally indispensable protein-protein interactions between Ikaros family members [10]. Four N-terminal zinc finger modules encoded by exons 3–5 mediate sequence-specific DNA binding, which is altered in differentially spliced isoforms. Only the isoforms with at least three out of four of the N-terminal zinc fingers are capable of binding DNA, others are considered dominant-negative. The dominant-negative isoforms can dimerize with DNA-binding isoforms of Ikaros or with other members of the Ikaros family such as Aiolos [11] and Helios [12, 13], preventing the DNA binding of the formed dimer [4, 10]. Complexes of DNA-binding isoforms recognize high-affinity Ikaros binding sites [8], which have been described within the regulatory elements of several lymphoid-associated genes [14].

The molecular function of Ikaros appears to be dualistic depending on whether it acts as a transcriptional activator or potentiator that enhances the activity of the promoter, or as a suppressor. For the latter, Ikaros associates with the nucleosome remodeling and deacetylation complex and has been considered as a factor to convert genes from active to inactive state [14]. In support of this, Ikaros binds to its consensus sites in the regulatory elements of *TdT* and *λ5* genes *in vitro*, and is important for the down-regulation of their activity in lymphocytes [15, 16]. However, the full activation of the *CD8* locus during the T cell development is known to require Ikaros and its family member Aiolos [17], demonstrating an example of positive gene regulation by Ikaros.

Despite the extensive studies defining mechanistic details of the transcriptional regulation by Ikaros, the functional importance of Ikaros, especially in B cells, is not established. Although several mutant mouse strains carrying a disruption of the *Ikaros* gene lack B cells, the fact that the development of B cell lineage was blocked at an early stage in these mice [3–5] has made it difficult to assess the importance of Ikaros for B cell function. The fact that B cells are absent during fetal development in mice homozygous for a hypomorphic $Ikaros^{lacZ}$ allele, but abnormal hyperproliferative B cells develop postnatally [6], suggests, however, an important role for Ikaros in the B cell development and function.

To investigate the functional significance of Ikaros in B cells, we established an Ikaros-deficient DT40 B cell line. Surface IgM (sIgM) expression was increased in $Ikaros^{-/-/-}$ cells. However, disruption of *Ikaros* strongly reduced the BCR-induced phosphorylation of phospholipase Cγ2 (PLCγ2), a crucial cytoplasmic signaling molecule generating the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol, which promote the release of calcium from intracellular stores and protein kinase C activation, respectively. Accordingly, the intracellular calcium mobilization induced by cross-linking of the sIgM was clearly attenuated in the absence of Ikaros, providing evidence that Ikaros is needed to enhance the strength of the BCR signal. Since signaling through the BCR regulates early B cell ontogeny [18], survival of immature B cells [19], the maintenance [20] and cell fate decisions of mature B cells [21], our results provide an explanation for the absolute requirement for Ikaros in B cells.

Results

Disruption of the *Ikaros* gene in DT40 B cells

To study the role of Ikaros in B cell function, we established an Ikaros-deficient DT40 B cell line showing total loss of functional Ikaros transcripts and proteins. To create this novel *Ikaros*-null mutant B cell line, we disrupted the coding sequence of the last *Ikaros* exon (exon 7), which has been shown to be indispensable for Ikaros function [10]. All three *Ikaros* alleles present in wild-type DT40 cells were sequentially targeted by the constructs *Ik-bsr*, *Ik-neo* and *Ik-pur* (Fig. 1A). Successful integration of the constructs into the target locus was verified by genomic PCR and subsequent Southern blotting, using the *Ikaros*-specific primer 6f with primers (Br, Nr and Pr) specific for each of the three selection markers and the *Ikaros*-specific probe 6p (Fig. 1A, B). Analysis by RT-PCR confirmed that $Ikaros^{-/-/-}$ cells did not express full-length Ikaros transcripts (Fig. 1C). Four of the Ikaros isoforms (Ik-1, Ik-1A, Ik-2 and Ik-6) [22] are expressed at protein level in DT40 cells. Immunoblot analysis of the C-terminal region verified that none of these isoforms were expressed in Ikaros-deficient cells (Fig. 1D), confirming the cell line as a functionally *Ikaros*-null mutant.

Increased surface IgM expression in Ikaros-deficient B cells

The $Ikaros^{-/-/-}$ cells were viable, although they exhibited slower growth than wild-type cells (Fig. 2A) due to a prolonged cell cycle time (data not shown). To gain insight whether or not the cells have retained features of B cells, we characterized the cell surface expression of ChB6 (Bu-1), CD45 and sIgM by flow cytometry. The alloantigen ChB6 is expressed in all avian B cells [23]. Ikaros-deficient cells expressed ChB6 and CD45 at levels comparable to wild-type cells (Fig. 2B). In contrast, sIgM expression was up-regulated, as indicated by the increased mean fluorescence intensity ratios of the

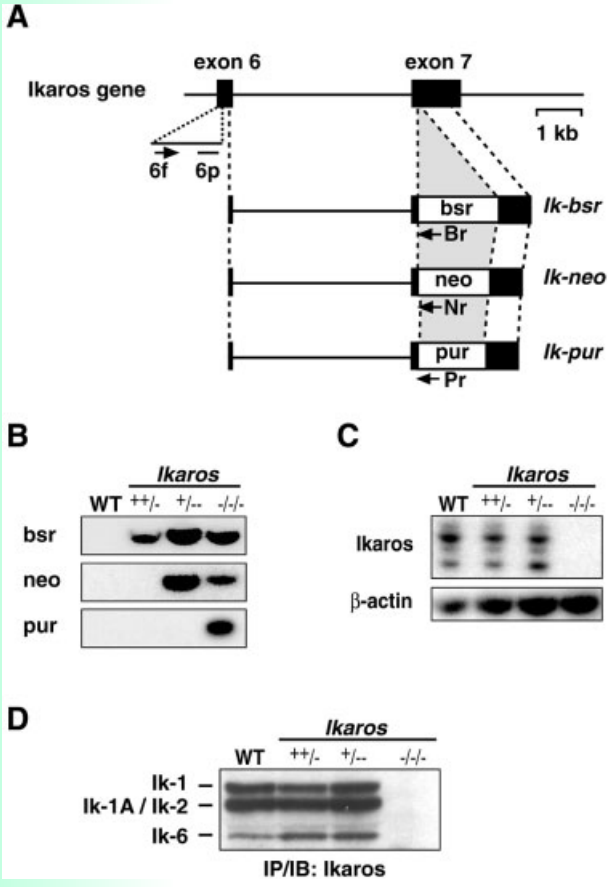

Figure 1. Gene targeting of the Ikaros locus in DT40 B cells. (A) Schematic presentation of the targeting constructs *Ik-bsr*, *Ik-neo* and *Ik-pur* containing blasticidin, neomycin and puromycin resistance markers, respectively. (B) Integration of targeting constructs into the three alleles of *Ikaros* locus monitored by genomic PCR reactions in which the *Ikaros*-specific primer 6f was paired with the selection marker-specific primers Br, Nr or Pr. The obtained genomic PCR products were analyzed by Southern blot analysis using the *Ikaros*-specific probe 6p. (C) Southern blot of RT-PCR analyzing the Ikaros expression within cDNA of wild-type (WT), $Ikaros^{++/-}$, $Ikaros^{+/-/-}$ and $Ikaros^{-/-/-}$ cells. (D) Immunoprecipitation (IP) of Ikaros followed by immunoblot (IB) using an Ikaros-specific Ab.

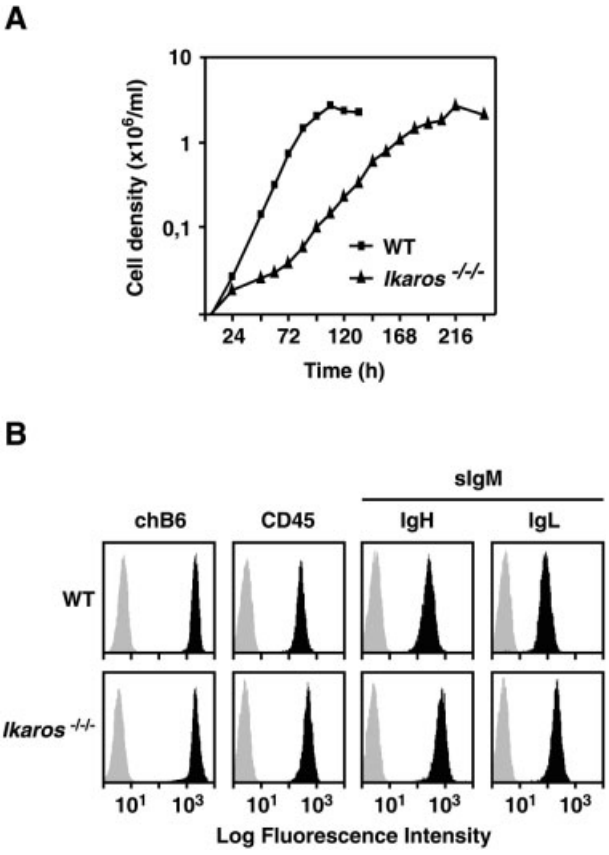

Figure 2. Comparison of cellular growth and surface marker expression of wild-type and $Ikaros^{-/-/-}$ cells. (A) Time *vs.* cell density growth curves of wild-type and Ikaros-deficient cells. (B) Flow cytometric analysis of surface expression of ChB6, CD45 and immunoglobulin (sIgM) μ heavy (IgH) and λ light (IgL) chains as indicated by logarithmic fluorescence intensity *vs.* relative cell number.

sIgM components, *i.e.* the μ heavy chain and the λ light chain (3.1- and 2.7-fold increase in expression, respectively; Fig. 2B). Thus, $Ikaros^{-/-/-}$ cells seemed to retain many characteristics of their B cell phenotype, although sIgM expression was enhanced.

Aberrant phosphorylation of cytoplasmic signaling molecules following sIgM cross-linking of $Ikaros^{-/-/-}$ cells

As part of an effective humoral immune response a diverse repertoire of antigen-specific effector cells with extremely high proliferative potential is generated. To achieve this, B cells are instructed continuously by BCR signals to make cell fate decisions at several checkpoints during their development. Considering the elevated sIgM expression in the absence of Ikaros (Fig. 2B) and the importance of BCR for B cell function, we investigated the BCR signaling of Ikaros-deficient cells.

One of the initial events in BCR signaling is the sequential tyrosine phosphorylation of cytoplasmic signaling molecules. Therefore, we compared the BCR-induced cytoplasmic phosphorylation patterns of Ikaros-deficient and wild-type cells. Phosphotyrosine immunoblots of wild-type and $Ikaros^{-/-/-}$ whole-cell lysates revealed two differentially phosphorylated proteins (Fig. 3A), which were identified by immunoprecipitations with specific Ab as B cell linker protein (BLNK; also known as SLP-65, BASH and BCA) and Casitas B lymphoma protein (Cbl) (Fig. 3B, C). BLNK and Cbl are both involved in the BCR signaling pathway, being rapidly phosphorylated after BCR activation. However, the lack of Ikaros had no effect on protein levels of BLNK or Cbl (Fig. 3B, C). BLNK functions as an adaptor protein, which serves as a scaffold to assemble several downstream targets of the antigen-induced

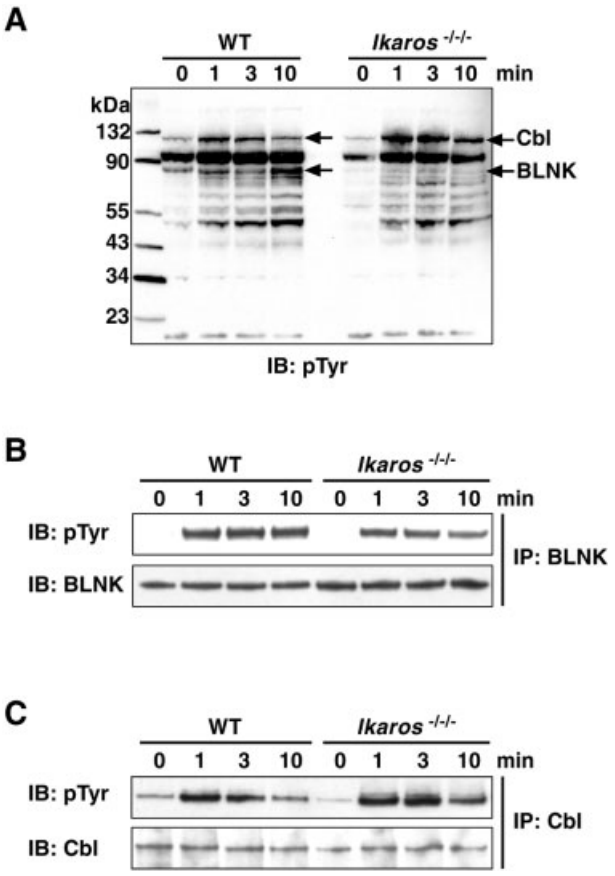

Figure 3. Phosphorylation of cytoplasmic signaling molecules following BCR cross-linking. (A) Phosphotyrosine immunoblots of whole-cell lysates before BCR engagement and 1, 3 or 10 min after BCR activation. Differentially phosphorylated proteins of 120 and 88 kDa molecular mass are indicated by arrows, and were later identified as Cbl and BLNK, respectively. (B) BLNK immunoprecipitation at indicated time points after BCR cross-linking followed by immunoblots with anti-pTyr or anti-BLNK Ab. (C) Immunoprecipitation with anti-Cbl Ab after BCR activation followed by pTyr or Cbl immunoblots.

activation. In BCR-induced activation of PLCγ2, tyrosine phosphorylation of BLNK is crucial, as phosphotyrosine-binding SH2 domains of PLCγ2 are required for its interaction with BLNK [24], which is an essential requirement for PLCγ2 activation [25]. In Ikaros-deficient cells, BLNK showed decreased levels of phosphorylation following the BCR cross-linking compared to that of wild-type cells (Fig. 3A, B). In contrast to BLNK, Cbl was hyperphosphorylated in Ikaros-deficient cells following the BCR activation (Fig. 3A, C). In antigen receptor signaling, Cbl is a prominent substrate for tyrosine kinases, which is thought to facilitate the ubiquitinylation of activated signaling proteins. In DT40 cells, Cbl has previously been shown to interact with BLNK after BCR ligation and consequently inhibit the PLCγ2 activation [26].

To find an explanation for the aberrant BLNK and Cbl phosphorylation, we investigated the activity of Lyn and Syk in Ikaros-deficient cells, as BLNK is a substrate for Syk [27] and Cbl is phosphorylated by Lyn in the DT40 cells [28]. Phosphorylation of Syk, which correlates with its activity [29], was decreased in Ikaros-deficient cells after BCR induction. However, following the BCR signal also the amount of Syk protein diminished in Ikaros$^{-/-/-}$ cells (Fig. 4A), which may be linked to enhanced Cbl phosphorylation, as Cbl is shown to negatively regulate Syk by mediating its ubiquitinylation after the BCR signal [30]. In contrast to Syk, the activity of Lyn was increased in the absence of Ikaros (Fig. 4B). Hence, the aberrant phosphorylation of BLNK and Cbl in Ikaros$^{-/-/-}$ cells is likely a consequence of inefficiently activated Syk and hyperactive Lyn.

Impaired BCR signaling and release of calcium from the intracellular stores

Given that the BCR-mediated phosphorylation and subsequent activation of PLCγ2 requires its association with BLNK and can be negatively regulated by Cbl [25–26], we next focused on the PLCγ2 pathway. Immunoprecipitation of PLCγ2 followed by phosphotyrosine immunoblot revealed that disruption of Ikaros strongly reduced the phosphorylation of PLCγ2 following BCR cross-linking (Fig. 5A). This suggests that PLCγ2 is less active in the absence of Ikaros, as phosphorylation of PLCγ2 correlates with its activity [31, 32]. ((Fig. 5))

To gain more information about the activity of PLCγ2, we examined the inositol phosphates produced after the BCR cross-linking, as PLCγ2 cleaves phospha-

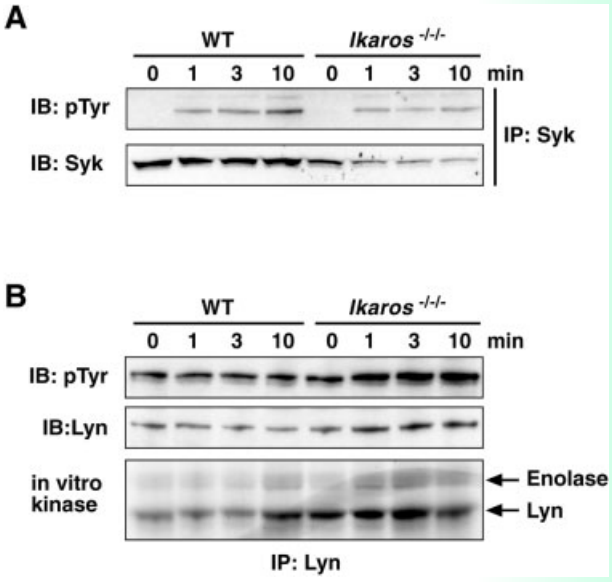

Figure 4. Phosphorylation and activity of Syk and Lyn after BCR engagement. (A) Immunoprecipitation with anti-Syk Ab after BCR activation followed by pTyr or Syk immunoblots. (B) Immunoprecipitation with anti-Lyn Ab followed by pTyr and Lyn immunoblots as well as by *in vitro* kinase assay.

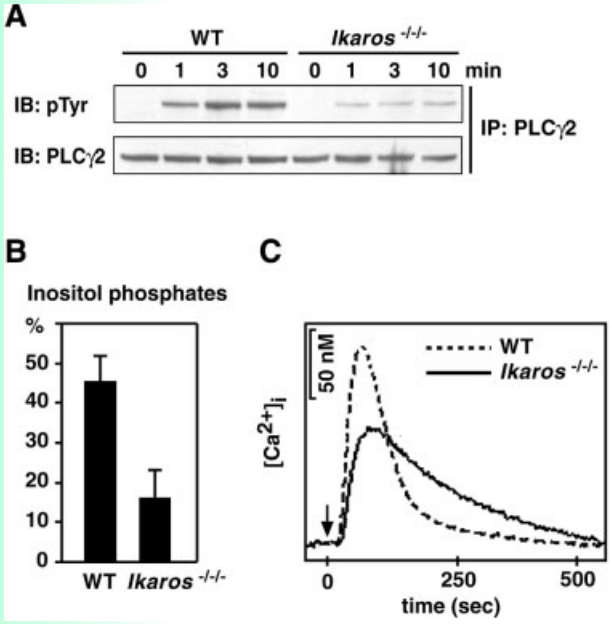

Figure 5. Comparison of the PLCγ2 pathway activation in wild-type and Ikaros$^{-/-/-}$ cells following BCR cross-linking. (A) Phosphotyrosine and PLCγ2 immunoblots of PLCγ2 immunoprecipitation products at indicated time points after BCR activation. (B) Increase in the intracellular inositol phosphate content at 45 s after the BCR cross-linking. A significantly ($p<0.05$) smaller increase of 16±7% ($n=3$) was observed in Ikaros-deficient cells compared to 45±7% ($n=5$) increase observed in wild-type cells. (C) Spectrophotometric measurements of intracellular calcium concentration following BCR cross-linking as indicated by the arrow. About 40% less free calcium was detected from the cytoplasm of *Ikaros*$^{-/-/-}$ cells compared to wild-type cells following the BCR cross-linking.

tidylinositol 4,5-biphosphate to produce IP_3 following the BCR stimulus. In accordance with reduced PLCγ2 phosphorylation, the increase in the amount of inositol phosphates after BCR activation was significantly ($p<0.05$) lower in the Ikaros-deficient cells (16±7%) than in the wild-type cells (45±7%; Fig. 5B), indicating inefficient PLCγ2 activation in the absence of Ikaros.

Since PLCγ2-mediated IP_3 generation is essential for the BCR-mediated mobilization of calcium through binding of IP_3 to its receptors, we measured the BCR-induced release of calcium from intracellular stores. The intracellular calcium mobilization induced by cross-linking of sIgM was clearly diminished in the absence of Ikaros (Fig. 5C), as 40% less free calcium was detected from the cytoplasm of Ikaros-deficient cells compared to wild-type cells following the BCR engagement. Thus, in the absence of Ikaros BCR signal strength was greatly reduced due to inefficient PLCγ2 activation.

Reintroduction of Ikaros expression restores the BCR signaling defect of Ikaros-deficient cells

To verify the observed BCR signaling defect as a consequence of Ikaros deficiency, we transfected the full-length Ikaros isoform Ik-1 into *Ikaros*$^{-/-/-}$ cells (Fig. 6A). The altered sIgM expression of Ikaros-deficient cells was normalized by restoration of Ikaros expression (Fig. 6B). Moreover, the re-expression of Ik-1 fully normalized the attenuated BCR-induced calcium mobilization found in *Ikaros*$^{-/-/-}$ cells (Fig. 6C), in-

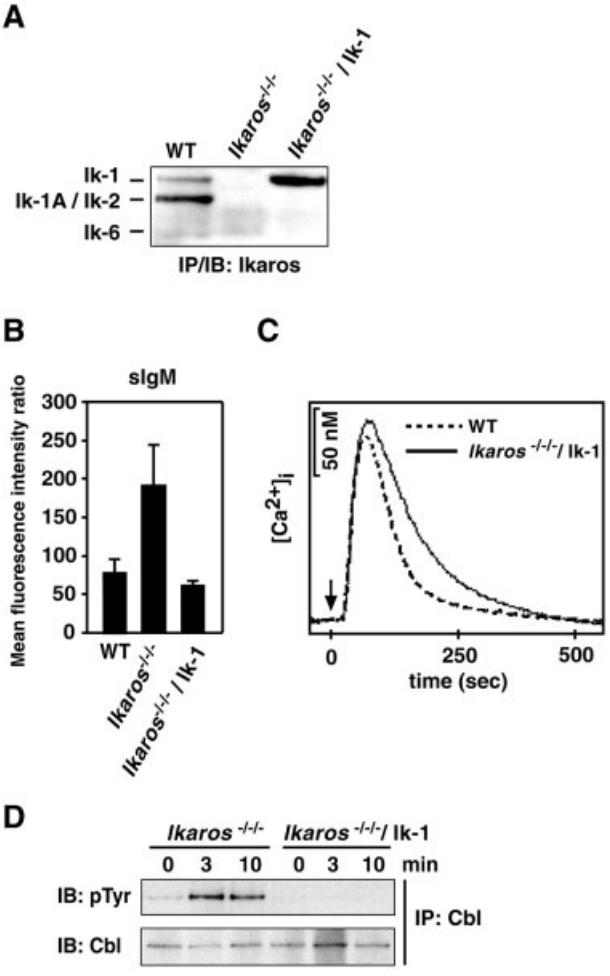

Figure 6. Re-expression of the full-length Ikaros isoform Ik-1 normalizes sIgM expression, restores BCR signaling, and lowers the Cbl phosphorylation observed in Ikaros$^{-/-/-}$ cells. (A) Ikaros immunoblots of the Ikaros immunoprecipitates from wild-type, *Ikaros*$^{-/-/-}$ and *Ikaros*$^{-/-/-}$/Ik-1 cells. (B) Flow cytometric analysis of sIgM expression using Ab M1 in wild-type, *Ikaros*$^{-/-/-}$ and *Ikaros*$^{-/-/-}$/Ik-1 cells. Error bars indicate mean SD of independent experiments ($n=3$ for each of the experiments). (C) Comparison of intracellular calcium mobilization in wild-type and *Ikaros*$^{-/-/-}$/Ik-1 cells following BCR cross-linking as indicated by the arrow. (D) Cbl and pTyr immunoblots of Cbl immunoprecipitates from *Ikaros*$^{-/-/-}$ and *Ikaros*$^{-/-/-}$/Ik-1 cells overexpressing Ik-1.

dicating that the regulation of BCR signaling is dependent on Ik-1.

To investigate whether the hyperphosphorylation of Cbl after BCR engagement would be due to the attenuated calcium signaling, we studied the phosphorylation of immunoprecipitated Cbl from Ik-1-transfected *Ikaros*$^{-/-/-}$ cells. Cbl phosphotyrosine immunoblots revealed that despite restored calcium signaling, Cbl phosphorylation was diminished as a consequence of Ik-1 overexpression in *Ikaros*$^{-/-/-}$ cells after the BCR induction (Fig. 6D). These findings suggest that Ikaros regulates Cbl phosphorylation independently from the BCR-induced calcium signaling. Furthermore, as Cbl is hyperphosphorylated in the absence of Ikaros (Fig. 3A, B) and unphosphorylated as a result of Ik-1 overexpression (Fig. 6D), our results suggest that the expression level of Ik-1 is an important factor in the regulation of Cbl phosphorylation.

Discussion

Here we show that Ikaros is required to regulate the BCR signaling machinery, providing first molecular evidence how Ikaros functions in B cells. Our results demonstrate that in the absence of Ikaros, reduced levels of calcium are released from intracellular stores due to insufficient PLCγ2 phosphorylation after BCR cross-linking. Restored Ikaros expression in Ikaros-deficient cells normalizes the BCR signaling defect, demonstrating unequivocally that Ikaros regulates B cell function by enhancing the BCR signals. In early hematopoietic differentiation the expression of Flk2/Flt3 depends on Ikaros [7], and it has been proposed that the early specification of multipotential progenitors to the B cell lineage is regulated by concerted action of Ikaros and PU.1 [33]. As the defect of Flk2/Flt3 signaling would explain the early requirement for Ikaros in B cell development, the defect in BCR signaling shown by our results provides an explanation for the need of Ikaros at later stages throughout the B cell lineage. This is because signal strength seems to be the decisive factor in B cell fate determination [21], and all resting mature B cells need basal BCR signal for survival [20].

Hypomorphic Ikaros mice expressing low levels of Ikaros have less mature B cells in the spleen than wild-type mice [6]. As a clear reduction in the transition of immature B cells from bone marrow to spleen has been observed in mice having an impaired BCR signaling caused by deletion of Syk or by truncation of Igα [34, 35], immature B cells are thought to migrate from bone marrow to spleen through a mechanism involving BCR signaling. Hence, the compromised BCR signaling due to insufficient Ikaros expression could cause inefficient transition of immature B cells to the spleen, and subsequently lead to decreased population of splenic mature B cells, as observed in mice expressing low levels of Ikaros [6]. Furthermore, since the signaling machineries of BCR and pre-BCR share multiple features, one plausible prediction from our results would be that also pre-BCR signaling is attenuated during Ikaros deficiency. We therefore suggest that (1) the partial developmental block at the pro-B to pre-B transition and (2) the impaired IL-7-dependent differentiation of B cell precursors to IgM$^+$ cells in hypomorphic Ikaros mice [6] are both possible consequences of aberrant pre-BCR signaling.

Thus, our results implicate that several possible checkpoints during B cell development are regulated by Ikaros since (1) B cell progenitors undergo positive selection at the pro-B to pre-B transition due to pre-BCR expression and signaling, and (2) pre-BCR signaling is thought to lower the threshold of IL-7 response permitting the pre-BCR$^+$ cells to thrive in the limited IL-7 supply environment [36], leading to subsequent differentiation into IgM$^+$ immature B cells.

In T cells, Ikaros is thought to set a threshold for T cell receptor (TCR) signaling [37] and our results with *Ikaros*$^{-/-/-}$ DT40 B cells show that Ikaros has a critical role in the regulation of BCR signaling. If Ikaros is involved in the regulation of both BCR and TCR signaling, and since these two signaling machineries have common molecules, it is likely that at least some regulatory targets and mechanisms are similar. One such target molecule is Cbl, which is rapidly phophorylated following both the BCR and TCR activation [28, 38]. Our results indicate that Ikaros is needed to suppress the BCR-induced Cbl phosphorylation. The three consensus tyrosines of Cbl phosphorylated by Src kinases and Syk upon antigen receptor activation are Y700, Y731 and Y774 [38], which are reported to interact with the SH2 domains of Vav, p85 of phosphatidylinositol 3-kinase and Crk, respectively [39, 40]. It is possible that Ikaros deficiency increases the phosphorylation of these tyrosines, thus promoting Cbl interactions, which would contribute to the ubiquitinylation of bound signaling molecules.

Ikaros could also participate in the regulation of self tolerance, as enhanced Cbl phosphorylation has been described in anergic T cells following TCR activation [40]. In fact, another member of the Ikaros family, Aiolos, which binds to the same consensus DNA sequence as Ikaros, is known to be important for the regulation of the BCR signaling threshold [41]. Aiolos-deficient mice lose B cell self tolerance, leading to development of SLE-type autoimmune disease [42]. Moreover, Ikaros$^{+/-}$ and Aiolos$^{-/-}$ mice develop T and B cell lymphomas, respectively [42–44]. All these findings are in line with our proposal that Ikaros affects the immune system by regulating the signaling machin-

ery of BCR (and possibly TCR), whose dysfunction leads to the development of autoimmune diseases and lymphomas.

However, in contrast to our results showing that BCR signal strength is diminished in the absence of Ikaros (Fig. 5C), Aiolos-deficient mouse B cells exhibit enhanced BCR signal strength [41], suggesting that Aiolos decreases the strength of BCR signals. These opposite phenotypes are not due to the differences in model systems, as we have found similar hypersignaling phenotype in Aiolos-deficient DT40 cells (unpublished observations). Hence, Ikaros and Aiolos seem to have opposite function in the regulation of BCR signaling.

At the molecular level, Ikaros appears to be needed in the regulation of primary events in the proximity of cell membrane and BCR complex, as indicated by altered activity of both Lyn and Syk (Fig. 4), which are the first cytoplasmic protein tyrosine kinases that become activated upon BCR engagement in DT40 cells. Given that Cbl negatively regulates Syk after BCR stimulation [30], the BCR-induced decrease in the amount of Syk (Fig. 4A) is possibly related to the BCR-induced hyperphosphorylation of Cbl (Fig. 3A, C) in the absence of Ikaros. However, the fact that tyrosine kinase binding domain, which binds to tyrosine-phosphorylated Syk [45, 46], is essential for Cbl-mediated Syk ubiquitinylation [30, 46], suggests that Cbl hyperphosphorylation would not promote interactions contributing to negative regulation of Syk. Cbl is phosphorylated by Lyn in the DT40 cells [28], and according to our results Lyn is potentially hyperactive in the absence of Ikaros (Fig. 4B). However, the Cbl hyperphosphorylation alone does not explain the observed BCR signaling defect of *Ikaros*$^{-/-/-}$ cells. Furthermore, since phosphatase activity and lipid raft environment critically contributes to the activity of Lyn [47], our results suggest that Ikaros may also regulate these primary events of BCR signaling.

To conclude, our results provide first molecular insight into the functional importance of Ikaros in B cells, showing that it influences BCR-induced PLCγ2-dependent calcium mobilization and Cbl phosphorylation. Our *Ikaros*-null mutant cell line constitutes a new tool to study the function of Ikaros in B cells. This study on the loss of Ikaros, a transcriptional regulator associated with nuclear remodeling complexes, provides new avenues for research concerning the connections of nuclear regulators to proximal signaling events on the cell membrane. Further research will be aimed to understand how gene expression is perturbed in Ikaros-deficient B cells and how Ikaros regulates BCR signaling at the molecular level.

Materials and methods

Cells and antibodies

Wild-type and Ikaros-mutant chicken DT40 B cells were maintained in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, penicillin and streptomycin. The cells were cultured at 40°C with 5% CO_2. Anti-PLCγ2 Ab [25], anti-Syk Ab [31], anti-Lyn Ab [31], anti-BLNK Ab [25] and anti-ChB6 mAb (L22) [23] were described previously. Anti-Ikaros Ab (E-20), anti-Cbl Ab (C-15) and anti-pTyr mAb (PY99) were purchased from Santa Cruz Biotechnology. Anti-chicken IgM mAb (M4 and M1), anti-chicken λ light chain mAb (L1) and anti-CD45 mAb (LT40) were purchased from Southern Biotechnology Associates Inc.

Targeting vector construction and generation of Ikaros-deficient DT40 B cell line

In gene targeting vectors *Ik-bsr*, *Ik-neo* and *Ik-pur*, selection cassettes were flanked by 4.0 and 0.7 kb of the chicken *Ikaros* sequence in the 5′ and 3′ sides, respectively (Fig. 1A). The 5′ arm of the targeting vector was obtained by genomic PCR from DT40 cells using primers 6Lf and 7Lr, which were designed based on the coding sequence of chicken *Ikaros* in the exons 6 and 7, respectively. 6Lf 5′-AAAGGTACCGACTAG-CAAGTAACGTCGCCTAACGTAAG-3′ added an *Acc65I* site and 7Lr 5′-AAAGGATCCATCTTATGGAAGATCAGATAGT-CACTTCTCAC-3′ added a *BamHI* site to the genomic PCR-sequence, which were used to clone the fragment into the pUC18 vector. The 3′ arm of the targeting vector was obtained by RT-PCR from DT40 cDNA, using primers 7Rf and 7Rr designed for the *Ikaros* exon 7 sequence. 7Rf 5′-AAAGGATC-CAACTAAGAGAAGGAGAACTAGATAATGCAG-3′ added a *Bam-HI* site and 7Rr 5′-AAAGTCGACTTAACTCATGTGGAAACGGT-GCTCCCCTCGAGT-3′ a *SalI* site, which were used to clone the fragment into the pUC18 vector. Finally, the selection markers were cloned between the two flanking Ikaros sequences as *BamHI* cassettes [48].

The targeting vectors were linearized by *Acc65I* digestion and introduced into DT40 cells by electroporation at 710 V, 25 μF. *Ik-bsr* was first transfected to wild-type DT40 cells followed by transfections of *Ik-neo* and *Ik-pur*, which were sequentially introduced to the heterozygous cells. Transfectant clones were selected in the presence of 50 μg/mL blasticidin S (*Ik-bsr* transfectants), 2 mg/mL G418 (*Ik-neo* transfectants) or 0.5 μg/mL puromycin (*Ik-pur* transfectants), depending of the transfected construct.

After each transfection event the *Ikaros*-targeted clones were selected from the transfectant clones based on three genomic PCR reactions (Fig. 1B), in which the primer 6f 5′-AACTAACCAGAGTGAAATGGCTGAAGACCTG-3′ specific for the chicken *Ikaros* was used in combination with primers specific for a selection marker Br (for *bsr*) 5′-CGATTGAA-GAACTCATTCCACTCAAATATACCC-3′, Nr (for *neo*) 5′-GCGCATCGCCTTCTATCGCCTTCTTGACGAG-3′ and Pr (for *pur*) 5′-CAGCGCCCGACCGAAAGGAGCGCACGACC-3′ (Fig. 1A). The obtained genomic PCR-products were probed in Southern hybridization with the *Ikaros*-specific probe 6p 5′-CCTGTGCAAGATAGGGTCAGAAAGATCCCTCG-3′.

RT-PCR analysis

Ikaros expression in wild-type DT40, heterozygous *Ikaros*$^{+/+/-}$ and *Ikaros* $^{+/-/-}$, and homozygous *Ikaros*$^{-/-/-}$ clones was analyzed by RT-PCR. Total RNA was first isolated from 5×10^6 cells with Trizol (Invitrogen) according to the manufacturer's protocol. Total RNA from 1×10^6 cells was treated with DNase and then used for first-strand cDNA synthesis with oligo $p(dT)_{15}$ primer (Roche). The cDNA from 1×10^5 cell equivalent was amplified with primers Ik-f 5′-ATGGAAACA-GATGAGGCTCAA-3′ and Ik-r 5′-TTAACTCATGTG-GAAACGGTG-3′ specific for chicken *Ikaros*. A PCR reaction with primers b1-f 5′-GTGCTGTGTTCCCATCTATCGT-3′ and b1-r 5′-TGGACAATGGAGGGTCCGGATT-3′ specific for chicken *β-actin* was used as a positive control. Southern hybridization was performed for the PCR products with the *Ikaros*- or *β-actin*-specific oligonucleotides Ik-p (for *Ikaros*) 5′-TAAATTAAAC-CACTGCGTTCCTCATTATTTGCT-3′ and b1-p (for *β-actin*) 5′-AAGCCAACAGAGAGAAGATGACACA-3′.

Analysis of cellular growth

The wild-type and *Ikaros*$^{-/-/-}$ cell cultures were diluted to 10^4 cells /mL and samples were harvested at 24-h intervals. The concentration of cells was analyzed with flow cytometer using TruCOUNT^{TM} tubes (Becton Dickinson) according to the manufacturer's instructions.

Immunoprecipitation and Western blot analysis

For immunoprecipitations, unstimulated and stimulated cells were solubilized in lysis buffer (1× PBS, 1% Nonidet-P40, 0.5% sodium deoxycholate, 1% SDS, 1 mM EDTA) containing protease and phosphatase inhibitors [2 mM phenylmethylsulfonylfluoride, 1 mM Na_3VO_4 and protease inhibitor 'cocktail' (Roche)], and subsequently lysed for 1 h at 4°C. Before lysis the stimulated cells were incubated with mAb M4 (4 μg/mL) for indicated times (1, 3 or 10 min). In immunoprecipitations of Ikaros, 20×10^6 cells were lysed for each sample, whereas in the immunoprecipitations of PLCγ2 each sample was prepared from 10×10^6 cells, in Lyn and Cbl immunoprecipitations from 5×10^6 cells, and in immunoprecipitations of Syk and BLNK from 3×10^6 cells. After centrifugation, the lysates were precleared and sequentially incubated with appropriate Ab and protein A-agarose (Santa Cruz Biotechnology) at 4°C overnight. The immunoprecipitates were washed four times with lysis buffer, and the samples were denatured at 75°C for 10 min in the SDS sample buffer.

For Western blot analysis, the whole-cell lysates or immunoprecipitates were separated by 4–12% SDS-PAGE, transferred onto nitrocellulose membranes, and detected by appropriate Ab and the enhanced chemiluminescence system (Amersham Pharmacia Biotech). For phosphotyrosine immunoblots of whole-cell lysates, 1×10^6 cells were used for the preparation of each sample.

In vitro kinase assay

For Lyn *in vitro* kinase assay, 5×10^6 cells were lysed and immunoprecipitated in NP40 buffer (1% Nonidet-P40, 50 mM Tris-HCl pH 8, 150 mM NaCl) with freshly added inhibitors [protease inhibitor cocktail (Roche) and 1 mM Na_3VO_4]. The precipitates were washed four times in NP40 buffer and three times in kinase buffer (20 mM Hepes pH 7.4, 5 mM magnesium acetate, 5 mM $MnCl_2$, 1 mM dithiothreitol). To make the reaction mixture, 2.5 μg acid-denatured rabbit muscle enolase (Sigma) was added as a substrate to 25 μL of kinase buffer together with 10 μM cold ATP and 10 μCi [γ-^{33}P]ATP (>3000 Ci/mmol; NEN). The reactions were allowed to proceed at 30°C for 10 min and terminated by addition of sample buffer and boiling for 3 min. Samples were separated on 4–12% SDS-PAGE. The gel was stained, dried and developed by autoradiography.

Calcium measurements

Cells (10^7) were suspended in Hepes-buffered salt solution (HBSS) containing 20 mM Hepes (pH 7.4), 118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$ and 10 mM glucose, and were loaded with 1.5 μM Fura-2 AM (Molecular Probes) for 45 min at room temperature. Following the loading period cells were further incubated for 20 min to ensure the complete cleavage of acetoxymethyl group from Fura-2. Cells were washed twice and suspended to HBSS lacking $CaCl_2$ and containing 0.05 mM EDTA. Continuous monitoring of fluorescence from cell suspension (5×10^6/mL) was performed at 37°C using a Hitachi F2000 fluorescence spectrophotometer at the excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as described previously [49]. Cells were stimulated with 4 μg/mL of mAb M4.

Measurement of inositol phosphates

The cells were pre-incubated with myo-[^{3}H]inositol (10 mCi/100 mm dish) for 36 h, harvested and suspended in HBSS. Following the incubation for 10 min at 37°C, the cells were suspended to HBSS containing 10 mM LiCl and further incubated for 10 min at 37°C. After the incubations, the cells were stimulated with mAb M4 (2 μg/mL) for 45 s. Inositol phosphates were extracted using 10% v/v $HClO_4$ and separated using Amprep (SAX) mini-colums [50]. The radioactivity of the samples was measured by liquid scintillation counting.

Expression vectors and transfections

The sequence coding for the full-length isoform Ik-1 of Ikaros was amplified from DT40 cDNA using primers Ik-fN 5′-AAAGCTAGCATGGAAACAGATGAGGCTCAAGA-3′ and Ik-rN 5′-ATTGCTAGCTTAACTCATGTGGAAACGGTGCT-3′, which created *NheI* sites to both ends of the PCR product. These *NheI* sites were used to clone the PCR product into the *NheI* site of pExpress vector [48]. The expression cassette containing the cloned PCR product between the chicken *β-actin* promoter and SV40 poly-A sequence was excised from pExpress as a *SpeI* cassette and subsequently cloned into the pLoxHisD vector [48]. The resulting plasmid was linearized with *NotI* and transfected into *Ikaros*$^{-/-/-}$ cells at 710 V, 25 μF. Transfectant clones were selected in the presence of 1.5 mg/mL histidinol, and the Ikaros expression level within clones was verified by Ikaros immunoblots.

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