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EDITORIAL

Ikaros in hematopoiesis and leukemia

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Abstract

Ikaros is a gene whose activity is essential for normal hematopoiesis. Ikaros acts as a master regulator of lymphoid and myeloid development as well as a tumor suppressor. In cells, Ikaros regulates gene expression *via* chromatin remodeling. During the past 15 years tremendous advances have been made in understanding the role of Ikaros in hematopoiesis and leukemogenesis. In this Topic Highlights series of reviews, several groups of international experts in this field summarize the experimental data that is shaping the emerging picture of Ikaros function at the biochemical and cellular levels. The articles provide detailed analyses of recent scientific advancements and present models that will serve as a basis for future studies aimed at developing a better understanding of normal hematopoiesis and hematological malignancies and at accelerating the application of this knowledge in clinical practice.

Key words: Ikaros; Hematopoiesis leukemia; Chromatin remodeling

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Since the discovery of the *Ikaros* gene, less than 20 years ago, there has been tremendous advance in understanding the function of this gene and its protein products at the molecular, cellular, and biological levels, and in elucidating the clinical significance of these findings. The first published papers identified Ikaros as a master regulator of hematopoiesis, and in particular a gene essential for lymphocyte development[1]. Advanced biochemical studies revealed that Ikaros binds DNA and regulates its target genes *via* chromatin remodeling[2]. These discoveries helped in understanding Ikaros function at both the biochemical and cellular levels and advanced our general knowledge of the mechanisms by which transcription factors control gene expression. Extensive association studies that examined the consequences of the loss of Ikaros function due to deletions, to mutations, or to overexpression of dominant negative isoforms, established that Ikaros acts as a tumor suppressor in acute lymphoblastic leukemia and possibly in other types of hematological malignancies[3-5]. The discovery of multiple signal transduction pathways that regulate Ikaros function identified signaling networks that are involved in normal and malignant hematopoiesis. These findings provided a mechanistic rationale for inhibition of these pathways as a component of treatment for hematological malignancies[6,7]. In this Topic Highlight series, we have assembled a group of international experts to provide an update on the progress in understanding the role of Ikaros in normal hematopoiesis, immune regulation, and leukemia, and to provide insights into the cellular and biochemical function of Ikaros.

The first two papers in this series provide in-depth reports on the role of Ikaros in different types of leukemia. The first review concentrates on the role of Ikaros

in T-cell leukemia[8]. The association of the loss of Ikaros activity and the development of T cell leukemia in mice and in humans is discussed. Authors present evidence that deletion of Ikaros is less frequent in T-cell acute lymphocytic leukemia (ALL) than B-cell ALL in humans. They discuss the possible downstream pathways that are controlled by Ikaros and whose deregulation contributes to leukemogenesis. The review by Greif *et al*[9] focuses on the role of Ikaros as a regulator of cell proliferation in myeloid leukemia. The authors discuss the alteration of Ikaros function in CALM/AF10 positive acute myeloid leukemia (AML), as well as in chronic myeloid leukemia (CML). The models presented describe how the alteration of Ikaros function by CALM/AF10 accounts for impaired thymocyte differentiation and AML with lymphoid characteristics, in addition to providing insights into the role of Ikaros in regulating pre-B-cell receptor signaling.

The next three reports provide an overview of the role of Ikaros in B cell development, myeloid differentiation, and immune function. It has been hypothesized that Ikaros regulates B cell development at multiple levels. Sellars *et al*[10] systematically summarize the current knowledge of Ikaros function in this process. They outline three roles for Ikaros in B cell development: (1) control of B lineage commitment; (2) regulation of the pro-B to pre-B cell transition through regulation of immunoglobulin (Ig) gene recombination *via* activation of the *Rag1* and *Rag2* genes; and (3) regulation of pre-BCR signaling by repressing transcription of the λ5 gene. The second part of this detailed review describes Ikaros' role in the regulation of B cell activation and isotype selection during immunoglobulin class switch recombination. In the next review, Francis *et al*[11] focus on the role of Ikaros as a regulator of normal myeloid differentiation and function. Authors emphasize Ikaros function in myeloid lineage commitment in the classic and lymphoid-myeloid progenitor hematopoietic pathways. The potential of Ikaros involvement in regulating expression of the Gr-1 gene, as well as inducible nitric oxide synthase in macrophages is reviewed. The role of the myeloid-specific Ikaros isoform, Ik-x in myeloid differentiation is discussed. The interesting role of Ikaros in regulating the activity of vasoactive intestinal peptide in human CD4+ lymphocytes is nicely presented by Dorsam *et al*[12]. The authors review the current evidence that Ikaros directly regulates the expression of vasoactive intestinal peptide receptor-1 (VPAC1) in T lymphocytes. The significance of Ikaros-controlled regulation of VPAC1 expression as a potential pathway by which the nervous system regulates immune response is discussed.

The last three reviews provide insights into the molecular mechanisms that regulate Ikaros activity and structural determinants of Ikaros function. Li *et al*[13] provide an in-depth review of the functional significance of the two largest human Ikaros isoforms - IK-1 and IK-H. The authors describe data demonstrating that coordinated expression of IK-1 and IK-H regulates Ikaros DNA binding, pericentromeric localization, and chromatin remodeling. The evidence that forms the basis of the current hypothesis - that the presence of the IK-H isoform determines whether Ikaros complexes function as activators or repressors of gene transcription - is discussed. The role of two signal transduction pathways in the regulation of Ikaros function is described by Song *et al*[14]. The authors summarize the evidence that phosphorylation by casein kinase 2 and dephosphorylation by protein phosphatase 1 directly regulates the activity of Ikaros protein. The control of Ikaros' DNA-binding affinity and subcellular localization, as well as its degradation *via* the ubiquitin pathway by two opposing signal transduction pathways is described. The role of Ikaros phosphorylation in T cell differentiation and regulation of target gene expression has been emphasized. A model by which the phosphorylation of Ikaros regulates its tumor suppressor activity and controls malignant transformation is outlined. Finally, Payne[15] presents an excellent review of the structural aspects of zinc finger motifs present in the Ikaros protein and uses knowledge of zinc finger structure to explain their significance in Ikaros function. In this review, the structure of DNA-binding N-terminal zinc fingers, as well as protein-interacting C-terminal zinc fingers is outlined. Based on detailed analysis, the author proposes an interesting hypothesis that the fourth N-terminal zinc finger serves the dual function of promoting DNA binding and participating in the formation of ternary complexes of Ikaros dimers with DNA.

The present Topic Highlight series "The role of Ikaros in hematopoiesis and leukemia" does not contain a complete reference of all experimental data regarding the activity of Ikaros in these processes. It represents a summary of current knowledge of the function of Ikaros in normal hematopoiesis and in hematopoietic malignancy. These reviews provide a foundation for future studies that will be aimed at validating some of the proposed models and advancing our understanding of hematopoiesis and leukemogenesis.

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TOPIC HIGHLIGHT

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Role of Ikaros in T-cell acute lymphoblastic leukemia

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Abstract

Ikaros is a zinc finger transcriptional regulator encoded by the *Ikzf1* gene. Ikaros displays crucial functions in the hematopoietic system and its loss of function has been linked to the development of lymphoid leukemia. In particular, Ikaros has been found in recent years to be a major tumor suppressor involved in human B-cell acute lymphoblastic leukemia. Its role in T-cell leukemia, however, has been more controversial. While Ikaros deficiency appears to be very frequent in murine T-cell leukemias, loss of Ikaros appears to be rare in human T-cell acute lymphoblastic leukemia (T-ALL). We review here the evidence linking Ikaros to T-ALL in mouse and human systems.

Key words: Ikaros; Notch; T-cell leukemia

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INTRODUCTION

Ikaros is a zinc finger transcriptional regulator that binds to specific DNA target sequences harboring the TGGGAA consensus motif[1,2]. How Ikaros regulates its target genes once bound to DNA is not entirely understood, and Ikaros has been found to activate or repress transcription, depending on the target gene. Part of its function may depend on the interaction of Ikaros with chromatin remodeling complexes, which may be recruited by Ikaros to target sites[3,4]. A large number of studies have shown that Ikaros is a key regulator within the hematopoietic system. Ikaros is crucial for controlling the development or the function of almost all hematopoietic cell types (see other reviews in this issue). Mutations or polymorphisms that lead to reduced Ikaros function or expression have also been found to be a major genetic feature in human B-cell acute lymphoblastic leukemia (B-ALL)[5-8]. Loss of Ikaros also promotes the development of T-cell lymphoma/leukemia in mice, which suggests that Ikaros acts as a tumor suppressor in T-cell acute lymphoblastic leukemia (T-ALL) and B-ALL. However, evidence linking Ikaros to human T-ALL has been elusive and sometimes contradictory, and only recently has the involvement of Ikaros in a small proportion of human cases of T-ALL become clear. We review below the role of Ikaros in murine and human T-ALL.

ROLE OF IKAROS IN MOUSE T-ALL

Targeted Ikaros mutations lead to T-ALL

Ikaros function in mice has been addressed through several mutant lines. The first mutant allele (Ikdn$^-$) generated was a deletion of exons 3 and 4, which encode the DNA binding domain (DBD)[9]. This mutation is dominant-negative, because it allows the synthesis of mutant proteins that retain the C-terminal dimerization domain that can dimerize with, and inhibit, other Ikaros proteins or related proteins (such as Aiolos or Helios). Strikingly, heterozygous Ikdn$^{+/-}$ mice rapidly develop T-cell leukemia, which provided the first genetic demonstration that Ikaros is a tumor suppressor in T cells[10]. A second dominant-negative Ikaros allele, named Ik^{Plastic}, was isolated in an ENU mutagenesis screen. This allele harbors a point mutation in the third zinc finger of the DBD. These mutant Ikaros proteins could therefore dimerize with their wild-type (WT) counterparts, but dimers fail to bind DNA[11]. Like Ikdn$^{+/-}$ mice, Ik$^{Plastic/+}$ mice develop T-ALL with a rapid onset. The third mutant allele (Ik$^-$) was later generated by deleting the sequences that encode the C-terminal zinc fingers that are required for dimerization; this is considered a null mutation[12]. Clonal expansion of thymocyte populations occurs in these mice as early as 5 d after birth, which also suggests rapid onset of leukemia. Finally, a hypomorphic allele, Ik^L, has been generated by Kirstetter *et al*[13], in which, the LacZ gene was targeted into the second exon. In these mice, in-frame transcripts between exons 1 and 3 are generated, which produce low amounts of truncated Ikaros proteins (lacking the protein segment encoded by exon 2). All homozygote Ik$^{L/L}$ mice develop T-cell lymphoma/leukemia in the thymus, with a median survival of 20 wk[14]. Thus, all mice that carry loss-of-function mutations in Ikaros develop T-cell leukemia with high incidence, which highlights a strong tumor suppressor function for Ikaros in the T-cell lineage.

Spontaneous Ikaros mutations are frequent in mouse T-ALL

The prevalent role for Ikaros as a tumor suppressor in mouse T-cell leukemia has been underscored by studies that have identified frequent loss-of-function Ikaros mutations in murine thymic lymphoma induced by irradiation, mutagens or deficiency in DNA repair pathways (Table 1). Ikaros mutations have been detected in 20-85% of the tumors, depending on the particular model and study. Two types of defects have been observed: focal genomic deletions (mostly heterozygous) in the proximal part of chromosome 11 where Ikaros is located; and point mutations in the coding regions that are missense mutations leading to amino acid changes in the DBD (and therefore are functionally similar to the Ik^{plastic} mutation), or mutations leading to premature stop codons that probably behave as null alleles. Ikaros has also been identified in several studies as a recurrent locus mutated by retroviral insertion, which cooperates with other primary oncogenic events such as activated Notch1 or K-ras proteins, or p19^{ARF} deficiency (Table 1). These insertions appear to promote abnormal splicing and the synthesis of aberrant, dominant-negative isoforms[15]. Together, these studies indicate that Ikaros is an important tumor suppressor gene whose inactivation can be triggered by several mechanisms in a variety of murine T-cell leukemias.

Function of Ikaros in murine T-cell leukemogenesis

The studies of murine T-ALL models have implicated certain molecular pathways associated with the tumor suppressive activity of Ikaros. Several reports have found a strong link between Ikaros deficiency and the activation of the Notch pathway; the latter of which plays a crucial role in human and murine T-ALL development. (1) High levels of Notch target gene expression, as well as frequent selection of activating mutations in the Notch1 gene, have been documented in T-cell lymphoma from the mouse models with germline Ikaros deficiencies[14,16,17]; (2) The selection of secondary Ikaros mutations appears to be strongly associated with Notch1 mutations in mice[15,18-20]; and (3) Loss of Ikaros function directly cooperates with Notch activation to promote leukemia[15]. At the molecular level, Ikaros appears to bind similar DNA sequences as RBP-J, the transcriptional mediator of Notch signaling[14,15], and Ikaros expression inhibits the proliferation of leukemic cells and represses the expression of Notch target genes such as Hes1[14,17]. Furthermore, Ikaros has been shown to silence some Notch target gene transcription during T-cell differentiation, which suggests that increased sensitivity to Notch signals is crucial for promoting the outgrowth of Notch-dependent leukemic cells[14,21,22]. Ikaros-mediated silencing of Notch target gene expression appears to be particularly important at the DN4 stage of T-cell differentiation; a stage at which Notch target genes are downregulated and Ikaros expression is strongly upregulated[21]. In this respect, Kleinmann and coworkers have shown at the single cell level that WT DN4 cells can no longer transcribe Hes1 in response to Notch signaling, and that this desensitization to Notch is crucially dependent on Ikaros function[21]. Indeed, the DN4 compartment is expanded in the thymus of Ikaros-deficient mice, which suggests that Ikaros-regulated cell proliferation in this compartment might be particularly relevant to tumor suppression[21,23].

Recently, three groups have addressed the *in vivo* role of Notch signaling in Ikaros-deficient T-ALL, by deleting floxed RBP-J alleles in the T cells of mice carrying various Ikaros mutations. Chari *et al*[24] have studied the impact of RBP-J deletion on the expansion of clonal populations in Ikaros null mice. Germline disruption of RBP-J is lethal *in utero*, therefore, RBP-J was deleted specifically in DN4/DP (CD4$^+$CD8$^+$) thymocytes *via* CD4-Cre-mediated deletion of floxed RBP-J alleles. Surprisingly, clonal populations still emerge in RBP-J-deleted thymuses within the same time-frame as in RBP-J-proficient thymuses. However, these cells do not expand as efficiently as those from mice with undeleted RBP-J alleles, which

Table 1 Mutations of Ikaros in murine T-cell lymphomas

Study	Model	Alteration studied	Type and frequency of Ikaros anomalies
Matsumoto *et al*[40], 1998	γ-irradiation (Balb/c-MSM F1 hybrids)	Genome-wide LOH mapping (microsatellites)	Allelic loss in proximal chromosome 11 region: 40% (8/20)
Okano *et al*[41], 1999	γ-irradiation (Balb/c-MSM F1 hybrids)	LOH (polymorphic restriction site) Mutation analysis (SSCP and cDNA sequencing)	Allelic loss: 54% (99/182) Homozygous deletions: 8/108[1] Missense point mutations in DBD: 5/108[1] Frame-shift or stop codon point mutations: 6/108[1]
Shimada *et al*[42], 2000	X-ray-induced thymic lymphomas	LOH (microsatellite mapping)	Distal region of chromosome 11 containing the *Ikzf1* gene identified as a common deleted region in 50% of cases
Kakinuma *et al*[43], 2002	X-ray-induced thymic lymphomas	LOH RNA expression (RT-PCR) Protein expression (Western blotting) Point mutations	LOH for Ikzf1 in 20/37 tumors No or dn transcripts in 9/37 tumors (correlated with absence of Ikaros proteins or presence of dn Ikaros proteins) Point mutations in 9/37 tumors (mostly zinc finger point mutations)[2]
Karlsson *et al*[44], 2002	Mutagen-induced thymic lymphoma	Point mutation analysis (SSCA and sequencing) Deletions (Southern) Allelic loss (microsatellite)	8 DBD point mutations (8/104) 3 frame-shift mutations (3/104) 27% allelic loss (12/40) 3 homozygous deletions (3/68)
Beverly *et al*[15], 2003	Notch1-IC transgenic mice	cooperating retroviral insertions	40% (synthesis of dn proteins)
López-Nieva *et al*[18], 2004	γ-irradiation (C57Bl6-Balb/c hybrids)	LOH (polymorphic restriction site) Point mutations (SSCP and sequencing)	42% (32/75) of LOH (1 homozygous deletion) 1 missense mutation in DBD
Kakinuma *et al*[45], 2005	Mutagen-induced thymic lymphoma	LOH Point mutations	LOH: 2/27 Point mutations: 5/27 (all in zn finger regions)
Kang *et al*[46], 2006	γ-irradiation (C57BL/6)	CGH-array (BAC)	Focal loss of chromosome 11: 20% (2/10)
Kakinuma *et al*[47], 2007	Mlh1-deficient mice (20 spontaneous or radiation-induced lymphomas)	Point mutation analysis Western blotting	Frame-shift point mutations: 85% (17/20) Lack of Ikaros protein: 75% (15/20)
Ohi *et al*[48], 2007	γ-irradiation (Balb/c-MSM F1 hybrids)	LOH (polymorphic restriction site)	43% (15/35)
Yoshida *et al*[49], 2007	X-irradiation (C57Bl6-C3H F1 hybrids)	Karyotype	Interstitial deletion of the proximal chromosome 11: 27% (7/15)
Uren *et al*[19], 2008	p19ARF- and p53- deficient mice	Common retroviral insertions (CIS)	33/510[3] (mostly in p19ARF-deficient mice; strong association with Notch1 activation
Dail *et al*[20], 2010	$Kras^{G12D}$-induced thymic lymphomas	Retroviral insertional mutagenesis	30% (9/30) insertions into Ikaros gene leading to synthesis of dn proteins

[1]The 108 tested samples include the 99 with loss of heterozygosity (LOH); [2]The tumors with point mutations are distinct from those exhibiting abnormal transcripts; [3]11 out of the 33 tumors had more than one hit in the Ikaros gene. CGH: Comparative genomic hybridization; SSCP: Single strand conformation polymorphism; SSCA: Single strand conformation analysis; dn: Dominant-negative; RT-PCR: Reverse transcription polymerase chain reaction.

suggests that RBP-J (and thus Notch signaling) is required for the expansion but not the initiation of Ikaros-deficient T-ALL. These results, however, must be interpreted with caution, as RBP-J levels were not measured in the clonal populations, and transformation may occur in cells with partially deleted RBP-J alleles. Similarly, we deleted RBP-J in $Ik^{L/L}$ mice with the CD4-Cre transgene[25]. In this case, RBP-J deletion significantly delays leukemia onset, and the leukemias that develop in these mice still carry the undeleted RBP-J alleles, which suggests a selection of cells that express RBP-J. Finally, Gómez-del Arco *et al*[26] have deleted RBP-J in $Ikdn^{+/-}$ and $Ik^{-/-}$ mice using the CD2-Cre transgene, which deletes at the early thymic progenitor stage, and have also observed a significant decrease in leukemia incidence. Altogether, these results suggest an essential role for RBP-J and Notch signaling in the development of Ikaros-deficient T-ALL.

We and Gómez-del Arco *et al*[26] have further addressed the role of Notch1 in Ikaros-deficient thymocytes by deleting floxed Notch1 alleles that correspond to 3.5 kb of the Notch1 promoter and exon 1[25]. Surprisingly, this deletion greatly accelerates, rather than delays, leukemia development in both studies. Although the Notch1 alleles are efficiently deleted in T cells, truncated Notch1 proteins are generated from *de novo* transcripts that arise from cryptic intragenic promoters located between exons 25 and 27, or upstream of the deleted canonical promoter. Activation of the cryptic 3' promoters is a direct consequence of the deletion of the canonical promoter, and an unexpected pro-oncogenic event[25]. Importantly, spontaneous Rag-mediated deletions of the Notch1 promoter region are common in murine T-ALL, including 75% of $Ik^{L/L}$ leukemias[25,27,28]. Ikaros binds to both the 5' and the intragenic cryptic promoters in WT thymocytes[25,26], which suggests that it plays a role in repressing their activity. If so, Ikaros deficiency may cooperate with Notch1 promoter deletions in promoting the activation of these alternative promoters. This hypothesis however awaits experimental demonstration.

Mouse studies have also implicated other pathways as-

Table 2 Studies of Ikaros status in human T-cell acute lymphoblastic leukemia

Study	No. of patients	Methods used	Cases with Ikaros abnormalities
Sun *et al*[30]	18 (pediatric)	WB, EMSA, IF, RT-PCR	18/18
Nakase *et al*[50]	5 (adult)	RT-PCR	0/5
Ruiz *et al*[51]	9 (pediatric)	RT-PCR, WB	0/9
Maser *et al*[52]	24 (16 cell lines and 8 primary leukemias)	CGH-array	2/24
Kuiper *et al*[53]	7 (unknown origin)	CGH-array	0/7
Meleshko *et al*[54]	14 (pediatric)	RT-PCR	1/14 (expression of dn Ik6 isoform)
Mullighan *et al*[6]	50 (unknown origin)	CGH-array	2/50
Marçais *et al*[31]	25 (adult, pediatric)	CGH-array, RT-PCR, WB, IF, cDNA sequencing	1/25

WB: Western blotting; EMSA: Electrophoretic mobility shift assay; IF: Immunofluorescence; RT-PCR: Reverse transcription polymerase chain reaction.

sociated with Ikaros deficiency. Uren *et al*[19] have compared cooperating retroviral insertion sites between $p19^{ARF}$- and p53-deficient tumor models, and have found Ikaros inactivation almost exclusively in $p19^{ARF}$-deficient tumors, which suggests that loss of Ikaros cooperates selectively with $p19^{ARF}$ inactivation but not with that of p53. Interestingly, Dumortier *et al*[14] also have detected a big decrease in $p19^{ARF}$ RNA expression in leukemic T cells from $Ik^{L/L}$ mice. Furthermore, concomitant loss of Ikaros and of $p19^{ARF}$ (encoded by the Cdkn2a gene) are hallmarks of human Bcr-Abl-positive B-ALL, which suggests a conserved cooperating pathway in lymphoid malignancies[29].

Finally, Winandy *et al*[23] have addressed the role of T cell receptor (TCR) signaling in the development of T-cell leukemia in $Ikdn^{+/-}$ mice. Leukemia fails to develop when the $Ikdn^{+/-}$ mutation is introduced onto a $Rag1^{-/-}$ background, which indicates an important role for the pre-TCR/TCR in leukemia development. Intriguingly, deletion of the α chain of the TCR dramatically accelerates leukemia progression, while ablation of the TCR β chain promotes the clonal expansion of cells expressing a γδTCR. These data indicate that signals from the pre-TCR or the γδTCR may synergize with Ikaros deficiency to promote the outgrowth of leukemic cells.

IKAROS IN HUMAN T-ALL

Evidence for Ikaros loss-of-function mutations in human T-ALL

The data demonstrating a clear role for Ikaros deficiency in the development of murine T-cell leukemia have prompted investigation into the possibility of a similar role in human T-ALL. A first study by Sun *et al*[30] has documented a strikingly high prevalence of aberrant, dominant-negative Ikaros isoforms and their cognate transcripts in all 18 cases of pediatric T-ALL studied. Furthermore, aberrant Ikaros proteins were localized in the cytoplasm of the leukemic cells. This study therefore seemed to indicate that Ikaros plays an important role in human T-ALL.

Additional studies by other groups, however, have not confirmed these results. Ikaros status in human T-ALL has now been analyzed by seven other groups over the years, in a total of 134 samples. All together, these studies have revealed six cases with Ikaros anomalies, most often with genomic deletions (Table 2). Several of these studies, however, have employed a limited set of assays, and could thus have missed some defects. Recently, Marçais *et al*[31] have performed a multi-parameter analysis of 25 T-ALL cases from diverse genetic subtypes, investigating DNA (by CGH-array), RNA (by reverse transcription polymerase chain reaction and cDNA sequencing) and protein (by Western blotting and intracellular localization by immunofluorescence), and found only one case with defective Ikaros. It appears therefore that Ikaros mutations that directly modify the gene and/or proteins are relatively rare in human T-ALL, and occur in approximately 5% of cases. It is unclear why Sun *et al*[32] have found so many T-ALL cases with aberrant Ikaros. Given the results of the other studies, it appears that the original study may have contained a systematic bias that led to the artefactual detection of aberrant proteins and RNAs. It should be noted that, in another study by the same authors, similarly high frequencies of Ikaros defects were found in B-ALL, which is also at odds with the current estimated frequency of approximatively 30% for these leukemias.

In addition to direct genetic inactivation, Ikaros can also be inactivated at the functional level. This possibility was suggested by Marçais *et al*[31] in an intriguing case of Ikaros protein delocalization in leukemic cells (TL92) that had lost one of its Ikaros alleles from a genomic deletion. When analyzed by immunofluorescence, the Ikaros proteins synthesized from the remaining allele appeared to localize to a discrete cytoplasmic location; possibly the centrosome. Unfortunately, the limited material from this patient prevented further characterization of the structure and mechanism involved in this delocalization. This observation suggests that delocalization of Ikaros proteins might contribute to Ikaros inactivation in some T-ALL patients. It will thus be interesting to conduct a systematic investigation of Ikaros localization in primary T-ALL cases.

It has also been suggested that Ikaros can be functionally inactivated following sequestration by the CALM-AF10 fusion protein in the cytoplasm, although this scenario is mostly based on overexpression experiments in cell lines[33]. CALM-AF10 translocation occurs in a subset of T-ALL, therefore, Ikaros localization in three primary T-ALL samples with the CALM-AF10 translocation was studied; no abnormality in Ikaros localization was ob-

served[31]. However, the expression and localization of CALM-AF10 was not analyzed, and further studies are thus required to assess the link between CALM-AF10 and Ikaros in T-ALL.

Finally, Ikaros has been shown to be the target of secondary modifications that could alter its activity, such as phosphorylation and sumoylation[34,35], and the possible relevance of altered post-translational modifications in suppressing Ikaros function in leukemic cells has been proposed[36]. The role of indirect mechanisms that inactivate Ikaros will be important to investigate in the future.

Is loss of Ikaros important in human T-ALL?

Despite the low prevalence, Ikaros loss of function is clearly a recurrent anomaly in human T-ALL. Thus, Ikaros inactivation is unlikely to be a bystander defect and could play a causal role in disease progression in a subset of T-ALL cases. T-ALL is a particularly heterogeneous disease with multiple subtypes, therefore, it will be important to determine if loss of Ikaros is associated with a specific class of T-ALL, or if Ikaros defects spread across molecular subtypes.

Given the strong link between Ikaros inactivation and Notch activation in murine T-ALL, a possible role for Ikaros loss in promoting Notch activation in human T-ALL is obviously of interest. However, Notch target gene activation is not particularly elevated in the TL92 case when compared with others[31]. Conversely, cases with high Notch target gene expression appear to express normal Ikaros[31]. Thus, Ikaros deficiency in human T-ALL may not be as strongly associated with Notch activation as it is in mice, and other pathways might be critical in human T-ALL. It will therefore be essential to achieve a better understanding of how Ikaros suppresses leukemogenesis independent of Notch. In this respect, insights gained from the role of Ikaros mutations in B-ALL will be relevant, as Notch has so far not been implicated in these leukemias.

CONCLUSION

The importance of Ikaros as a tumor suppressor in murine T-cell leukemia has been known for about 15 years, but it is only becoming clear that Ikaros inactivation is a recurrent event in human T-ALL. Given that Ikaros is also a major tumor suppressor in human B-ALL[6,37,38], and that reduced Ikaros function dramatically accelerates B-cell leukemia development in mice[39], we hypothesize that Ikaros functions *via* common mechanisms to suppress B-ALL and T-ALL. Defining these mechanisms will be the central aim of future research on the role of Ikaros in lymphoid malignancies.

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TOPIC HIGHLIGHT

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Up a lymphoid blind alley: Does CALM/AF10 disturb Ikaros during leukemogenesis?

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Abstract

The *Ikaros* gene is required for normal development of lymphocytes and frequent intragenic deletions of *Ikaros* have been identified in acute lymphoblastic leukemia. However, little is known about the role of *Ikaros* in myeloid malignancies. Here we discuss the role of *Ikaros* as a lineage master regulator during the onset and progression of myeloid leukemias, namely CALM-AF10 positive acute myeloid leukemia and chronic myeloid leukemia. Alterations of *Ikaros* at the gene or protein level may act as a bi-directional lineage switch subverting developmental plasticity for malignant transformation. Finally, we propose that promiscuous signaling involving Ikaros and FOXO transcription factors might be a critical link between early lineage fate and uncontrolled proliferation.

Key words: Acute lymphoblastic leukemia; Acute myeloid leukemia; CALM/AF10; Chronic myeloid leukemia; Ikaros

IKAROS IN CALM/AF10 RELATED LEUKEMIAS

Impaired differentiation is a hallmark in cancer. Hematopoietic tumors often involve deregulation of lineage specific transcription factors such as *Ikaros* in the lymphoid or *CEBPA* in the myeloid compartment[1,2]. Mutations in these key regulators of differentiation are frequently found in leukemias which morphologically resemble immature cells of the affected lineage. For example, genomic alterations of *Ikaros* are found in acute lymphoblastic leukemia (ALL) and *CEBPA* mutations are associated with acute myeloid leukemia (AML)[3,4]. Unsurprisingly, an impaired differentiation machinery may cause accumulation of non-functional early cells. However, disturbed *Ikaros* function may not only lead to blocked lymphoid differentiation, but also to myeloid transdifferentiation as a result of the blocked lymphoid differentiation pathway.

In particular, this mechanism could be relevant in CALM/AF10 positive AML, since expression of the

CALM/AF10 fusion protein alters the subcellular localization of Ikaros[5]. The fusion of the CALM and AF10 genes results from the t(10;11)(p13;q14) translocation and can be found as the sole cytogenetic abnormality in ALL, AML and in malignant lymphomas and correlates with poor prognosis[6,7]. In a murine bone marrow transplantation model, CALM/AF10 expression results in the development of an aggressive bi-phenotypic leukemia. While the leukemic bulk is myeloid, the leukemia propagating cells show lymphoid traits including B220 surface markers and immunoglobulin heavy chain rearrangements[8] (Figure 1). This bi-phenotypic leukemia might be explained in part by the interaction between CALM/AF10 and Ikaros that results in increased cytoplasmic localization of Ikaros[5]. While the putative transcription factor AF10 shows a nuclear localization[9], CALM (Clathrin Assembly Lymphoid Myeloid Leukemia Gene) plays a role in endocytosis and localizes mainly to the cytoplasm[10]. Similar to CALM, the CALM/AF10 fusion protein shows a predominately cytoplasmic localization, however, both CALM and CALM/AF10 were shown to shuttle between the nucleus and the cytoplasm[11]. Thus, CALM/AF10 is able to interact with nuclear proteins such as Ikaros. The interaction of CALM/AF10 and Ikaros may tether Ikaros to the cytoplasm and thereby disturb Ikaros function as a transcription factor, and further, as a tumor suppressor. Similar to dominant negative Ikaros isoforms, CALM/AF10 may interfere with the formation of Ikaros homodimers that are essential for the recruitment of Ikaros target genes to pericentromeric heterochromatin[12,13]. If this occurs in early progenitor cells, lymphoid differentiation could be partially or completely blocked. Depending on the extent of the block, progenitors may either seek a detour towards the myeloid route or escape into the T-cell compartment. Interestingly, in Ikaros *knock-out* mice, B lymphocytes are absent, whereas T-lymphocytes are present, but severely defective[12,14]. In a similar manner, clinically healthy transgenic CALM/AF10 mice show impaired thymocyte differentiation[15] (Figure 1). CALM/AF10 is a common fusion transcript in T-ALL with a particularly high frequency in the TCRγδ lineage[16]. Recently, aberrant cytoplasmic Ikaros localization was reported to be common in T-ALL, while genomic deletions of *Ikaros* are rare in T-ALL[17]. It is tempting to speculate that CALM/AF10 may modulate lineage fate through interaction with Ikaros in a dose-dependent manner, depending on the expression levels of the two proteins, the exact developmental stage of the target cell and possible additional genetic alterations. A complete block of lymphoid differentiation would then result in AML, and a partial block would facilitate the onset of T-ALL. Considering the numerous known Ikaros isoforms including the myeloid specific isoform Ikaros X[18], it will be challenging to elucidate the role of the interaction between CALM/AF10 and Ikaros in malignancy.

Altered Ikaros function is likely necessary but not sufficient to cause CALM/AF10 related leukemia. Leukemic fusion proteins are successful in causing leukemia, because they often affect several different regulatory circuits in their target cells. For example, CALM/AF10 was recently shown to cause chromosomal instability through its interaction with the histone methyltransferase DOT1L[19].

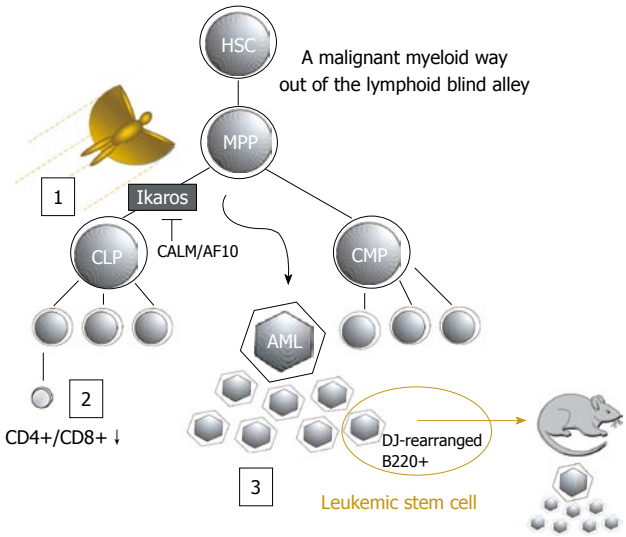

Figure 1 Model of the CALM/AF10 Ikaros interaction during leukemogenesis (adapted from Greif *et al*[5], 2008). Hematopoietic stem cells (HSC) give rise to multipotent progenitors (MPP) that divide into common lymphoid (CLP) and myeloid progenitors (CMP). Ikaros is required for the maturation of lymphoid progenitors. We propose that CALM/AF10 alters its subcellular localization[5] and thereby disturbs Ikaros function at this stage leading to impaired thymocyte differentiation[15] and phenotypically acute myeloid leukemia (AML) with lymphoid characteristics[8].

LINEAGE PROMISCUITY AND ABERRANT SIGNALING IN HEMATOPOIETIC MALIGNANCY

Another Ikaros-mediated lineage switch can be observed in chronic myeloid leukemia (CML). Lymphoid blast crisis during the progression of CML is often associated with acquisition of *Ikaros* deletions[1,20]. Apparently, in CML *Ikaros* mutations facilitate the development of a lymphoid blast crisis. This is in contrast to the myeloid phenotype observed in CALM/AF10-driven AML which might be a consequence of altered Ikaros function. How can these multiple consequences of disturbed Ikaros function be explained? We propose that impaired function of Ikaros might have two main consequences: (1) mutations or altered protein interactions of Ikaros prevent the cell from entering the lymphoid compartment; and (2) impaired lymphoid differentiation interrupts feedback loops involving upstream regulators of Ikaros such as the pre-B-cell-receptor (pre-BCR). Loss of the physiological inhibition of pre-B-BCR-signaling by Ikaros and its protein family member Aiolos[21,22] (Figure 2) may result in continued or even increased signaling. Continuous pre-BCR-signaling may not only persistently push the cells towards lymphoid development, but might also cause the cell to differentiate along alternative pathways

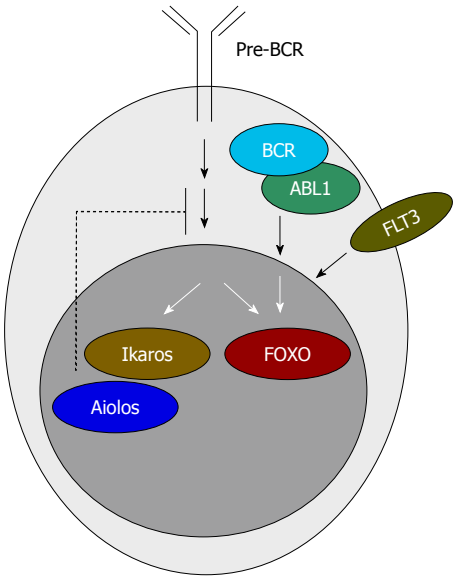

Figure 2 Pre-B-cell-receptor signaling is regulated by a feed-back loop *via* Ikaros and Aiolos. The pre-B-cell-receptor (pre-BCR)-signaling cascade also involves FOXO transcription factors which may act as an interface between early B-cell development and FLT3-signaling that is frequently altered in acute myeloid leukemia (AML)[25]. FOXO-proteins are also regulated by downstream signaling of BCR-ABL1[27]. Disruption of Ikaros function by either deletions or protein interactions may interrupt the inhibitory feed-back loop to the pre-BCR pathway and thereby enhance pre-BCR-signaling.

(e.g. the myeloid lineage). The pre-BCR-signaling cascade includes promiscuous players like the forkhead box O (FOXO) transcription factors[23,24] (Figure 2). In AML for example, the frequently mutated FLT3 receptor tyrosine kinase was reported to promote proliferation by signaling through FOXO proteins[25]. Increased phosphorylation of FOXO3A correlates with adverse prognosis in AML[26]. Hence, FOXO proteins might act at the interface between early lymphoid and malignant myeloid cell fate. In CML, persistent BCR/ABL1-mediated signaling towards lymphoid development might eventually break through the differentiation block after Ikaros becomes mutated resulting in lymphoid blast crisis. In particular, phosphorylation and thereby inactivation of FOXO3A through the PI3-K/Akt pathway is a potential link between BCR-ABL1 signaling and pre-BCR-signaling[27]. Gilliland and co-workers[28] postulated that at least two classes of mutations are required for leukemia development: class I mutations which increase proliferation (e.g. activating mutations in tyrosine kinases) and class II mutations which lead to a differentiation block (e.g. fusion genes like AML1/ETO)[28]. We suggest that the distinction between these two classes of mutations is often not as clear and that a single mutation might have effects on both proliferation and differentiation. One example might be mutations or functional alterations of the Ikaros protein. *Ikaros* mutations might not only result in a block in differentiation but might, at the same time, redirect up-stream signaling to drive the cell to differentiate along alternative pathways and thereby contribute to increased proliferation.

In summary, the above discussed examples illustrate that the hematopoietic hierarchy is highly dynamic and that its plasticity does not only allow the diversity of specialized blood cells, but also lineage promiscuity in leukemia. The example of Ikaros demonstrates how a regulator of healthy differentiation can be converted into a driver of malignancy. There is a growing list of key hematopoietic regulators which are altered in leukemia including PAX5, GATA-1, C/EBPa and PU.1[29,30].

Ikaros is a lineage switch controlling differentiation towards the lymphoid or myeloid lineage. However, lineage fate is not always binary, rather, an intermediate position of the switch may cause the cell to be confused about its fate and become malignant. For example, the complete absence of the *ets* transcription factor PU.1 does not lead to the development of leukemia in knock-out mice, while lowering the levels of PU.1 to just 20% of normal levels results in leukemia[31].

If an early hematopoietic cell gets trapped in the lymphoid blind alley, it may escape through the myeloid back door and emerge as a leukemia cell.

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Regulator of myeloid differentiation and function: The secret life of Ikaros

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Abstract

Ikaros (also known as Lyf-1) was initially described as a lymphoid-specific transcription factor. Although Ikaros has been shown to regulate hematopoietic stem cell renewal, as well as the development and function of cells from multiple hematopoietic lineages, including the myeloid lineage, Ikaros has primarily been studied in context of lymphoid development and malignancy. This review focuses on the role of Ikaros in myeloid cells. We address the importance of post-transcriptional regulation of Ikaros function; the emerging role of Ikaros in myeloid malignancy; Ikaros as a regulator of myeloid differentiation and function; and the selective expression of Ikaros isoform-x in cells with myeloid potential. We highlight the challenges of dissecting Ikaros function in lineage commitment decisions among lymphoid-myeloid progenitors that have emerged as a major myeloid differentiation pathway in recent studies, which leads to reconstruction of the traditional map of murine and human hematopoiesis.

Key words: Ikaros; Myeloid differentiation; Lineage commitment; Hematopoiesis; Post-transcriptional regulation; Post-translational regulation

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OVERVIEW OF IKAROS FUNCTION

The *Ikaros* gene product is alternately spliced to produce multiple zinc finger proteins (Figure 1) that bind to promoter regions and regulate target gene expression[1,2]. Ikaros is essential for normal hematopoiesis[3] and has been implicated as a tumor suppressor[4,5]. Ikaros has been shown to both activate[6-9] and repress[10-14] gene expression and to participate in chromatin remodeling where it targets genes for epigenetic modifications and recruitment to pericentromeric heterochromatin[15]. Little is known about the mechanisms that regulate the expression of the *Ikaros* gene at the transcriptional level[16], al-

though a variety of mechanisms for post-transcriptional regulation of Ikaros activity and/or protein levels have been identified.

Ikaros activity is dependent on its ability to bind DNA. Ikaros binds DNA *via* four N-terminal zinc fingers[1]. The loss of zinc fingers through alternate splicing or chromosomal deletion can result in Ikaros isoforms with reduced DNA binding affinity[1,2]. DNA-nonbinding Ikaros isoforms exert a dominant negative (DN) effect, inhibiting the ability of other Ikaros isoforms and Ikaros family members (e.g. Aiolos and Helios) to bind DNA[4]. Phosphorylation of Ikaros by casein kinase 2 (CK2)[17] has been shown to inhibit the ability of Ikaros to interact with DNA, including pericentromeric hetorochromatin[15], and to regulate Ikaros susceptibility to ubiquitin-mediated degradation[18]. Thus, in addition to transcriptional regulation of the *Ikaros* gene product, the level of Ikaros DNA binding activity can be regulated by at least four other mechanisms: (1) alternative splicing that results in altered Ikaros DNA binding; (2) DN Ikaros isoforms that inhibit the DNA-binding activity of other Ikaros isoforms; (3) phosphorylation of Ikaros that results in decreased DNA binding affinity; and (4) phosphorylation-mediated changes in the stability of Ikaros proteins.

Ikaros activity can be regulated by post-transcriptional events that are independent of its DNA binding affinity. In addition to changes in DNA binding affinity, alternative splicing gives rise to Ikaros isoforms with altered DNA binding specificity[1,2,19]. This provides a potential mechanism for fine tuning Ikaros targets in different cell types. Repression of target genes by Ikaros can be mediated by recruitment of histone deacetylase (HDAC)-containing complexes[20], as well as through the HDAC-independent mediator, CtBP[21]. Sumoylation of Ikaros has been shown to inhibit the ability of Ikaros to interact with both HDAC-dependent and HDAC-independent repressors of transcription[22]. The multiple mechanisms by which Ikaros activity can be regulated post-transcriptionally suggest that the complete picture of Ikaros function is likely to be complex and underscores the importance of Ikaros studies at the protein level.

IKAROS AS A REGULATOR OF MYELOID CELL DIFFERENTIATION AND FUNCTION

Ikaros was identified using strategies designed to detect transcription factors that regulate lymphoid genes and was initially described as a lymphoid-specific transcription factor[2,23,24]. Ikaros mutant mice are characterized by profound lymphoid defects and as a consequence Ikaros has primarily been studied in context of lymphoid development and function and lymphoid malignancy. The central role of Ikaros in the lymphoid lineages has largely overshadowed the more subtle, yet crucial roles that Ikaros plays in the myeloid lineage.

Defects in myelopoiesis are present in all of the Ikaros mutant mouse models that have been described to date. Homozygous Ikaros^{DN} mutants show a reduction in the number of myeloid lineage cells in the bone marrow. Terminal granulocyte differentiation is absent in homozygous Ikaros^{DN}[25] and Ikaros^{null}[3] mutants, in IK$^{L/L}$ mice that express very low levels of Ikaros[26], and in the *plastic* mutant mouse strain[27] that harbors a point mutation that prevents Ikaros from binding DNA.

A comprehensive analysis of neutrophil differentiation in the IK$^{L/L}$ mice showed defects in neutrophil survival and migration as well as a failure of immature granulocytes to upregulate Gr-1 (also a characteristic of other Ikaros mutants); a differentiation event that is preceded by high levels of Ikaros protein expression in wild-type mice[26]. Ikaros has also been shown to regulate the expression of inducible nitric oxide synthase downstream of lipopolysaccharide/interferon-γ stimulation in a macrophage cell line[28]. These data provide evidence that Ikaros regulates differentiation and immune function in the myeloid lineages as it does in the lymphoid lineages.

IKAROS AS A TUMOR SUPPRESSOR

Studies of Ikaros mutant mice together with clinical data provide compelling evidence that Ikaros acts as a tumor suppressor. The rapid development of T-cell lymphoma in mice that are heterozygous for a defect that produces DN Ikaros isoforms (IK^{DN}) provided the first data to support a role for Ikaros in tumor suppression[4]. IK$^{L/L}$ mice that express low levels of Ikaros also develop T-cell lymphoma[11]. Similarly, multiple clinical studies have linked Ikaros mutations and deletions to human B-cell acute lymphoblastic leukemia and to a lesser extent T--cell acute lymphoblastic leukemia[29-39]. Ikaros has been shown to regulate expression of molecules that control cell cycle progression and cell survival[40,41], as well as hematopoietic differentiation; all of which are likely to contribute to the tumor suppressor activity of Ikaros. More recently, Ikaros defects have been linked to myeloproliferative neoplasms[42,43] and childhood acute myelogenous leukemia[40], providing evidence that Ikaros tumor suppressor activity extends to the myeloid lineage. In these cases, Ikaros activity is lost due to deletion of the *Ikaros* gene[42,43] or expression of DN Ikaros isoforms[40]. The mechanisms that regulate Ikaros tumor suppressor activity have not been defined[16]. However, the roles of CK2 in post-transcriptional regulation of Ikaros protein levels[18] and in functionally inactivating Ikaros[44] suggest that CK2 may be involved in regulating Ikaros tumor suppressor function. Overexpression of CK2 has been associated with myeloid malignancies[45,46]. Thus, overexpression of CK2 is a potential mechanism for the functional inactivation of Ikaros that leads to the loss of Ikaros tumor suppressor activity in myeloid leukemia.

EXPRESSION OF IKAROS ISOFORM-X IS ASSOCIATED WITH MYELOID POTENTIAL

It is important to note that Ikaros proteins are often

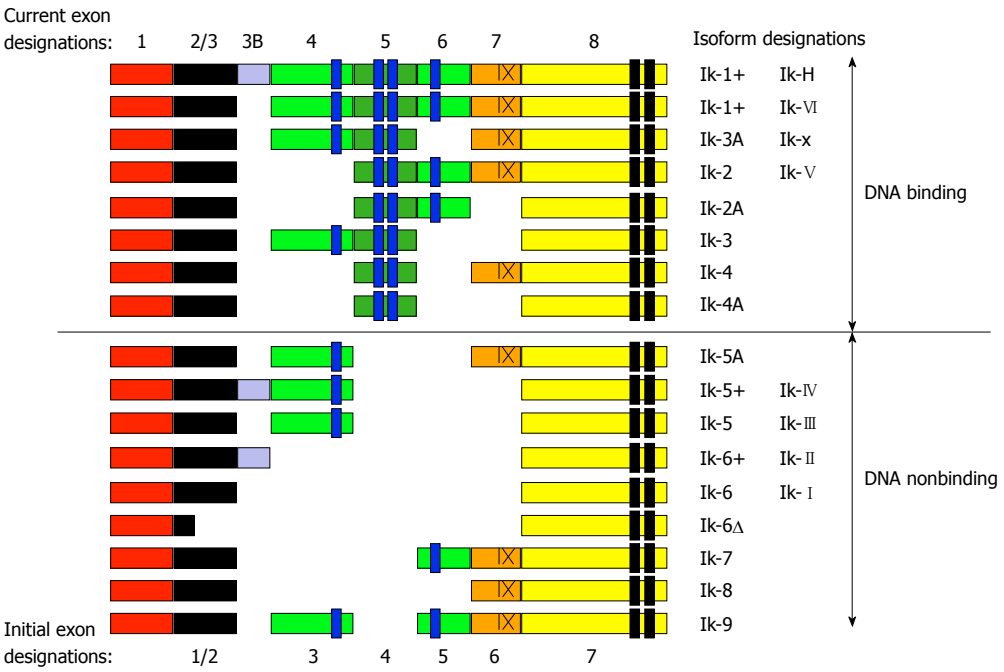

Figure 1 Ikaros isoforms. The *Ikaros* gene in mice (*Ikzf1)* and humans (*IKZF1*) includes eight coding exons (exons 2-8 and 3B) and one upstream exon that is not translated (Figure 1). The untranslated exon (shown in red) has not been identified in initial reports, and the alternate exon designations that have appeared in early reports are shown at the bottom of the figure. Exon 3B is currently not identified as an exon in Genbank. Splice forms that include exon 3B have been designated as "plus" forms and many such splice forms, in addition to the ones shown, have been identified at the protein and/or mRNA level (i.e. Ik-x$^+$, Ik-2$^+$, Ik-4$^+$, Ik-7$^+$, Ik-8$^+$) in humans and mice. An alternate splice site gives rise to splice forms that lack the last 30 bases of exon 7 (indicated with an X). Such splice variants have been designated minus forms (e.g. Ik-1$^-$ and Ik-x$^-$). The four N-terminal zinc fingers (shown in blue) contribute to DNA binding and the two C-terminal zinc fingers (shown in black) are responsible for dimerization[1,2,19,47-53].

divided into two broad categories, DNA-binding and DNA-nonbinding, and indeed this is a key distinction in studies of malignant hematopoiesis. However, the DNA-binding Ikaros is often treated as if it were one protein, although this is not the case. The *Ikaros* gene in mice (*ikzf1*) and humans (*IKZF1*) includes eight coding exons and one upstream exon that is not translated (Figure 1). Ikaros coding exons are alternately spliced to produce more than 10 DNA binding and nonbinding Ikaros isoforms[1] detectable by reverse transcription polymerase chain reaction (RT-PCR)[1,2,19,47-53]. The detection of Ikaros transcripts by RT-PCR correlates poorly with protein expression as measured by immunoblotting[54].

At the protein level, Ikaros expression in normal human bone marrow is dominated by the expression of Ikaros-x (Ik-x), followed by Ik-1, Ik-H, and Ik-2/3 (Ik-2 and Ik-3 are indistinguishable by molecular weight) with very little expression of DNA-nonbinding isoforms[54]. The expression pattern of Ikaros proteins is similar in murine hematopoietic cells, with the exception that expression of the Ik-H protein is largely absent in mice[19,54]. Although all of these isoforms bind DNA, they differentially incorporate exon 3B and/or the exons that encode the four N-terminal zinc fingers that contribute to DNA binding. The alternate use of these exons has been reported to fine tune the DNA binding specificity and/or affinity of Ikaros proteins[1,2,19]. Thus, differential expression of Ikaros isoforms is a potential mechanism for regulating the expression of Ikaros target genes.

The differential expression of Ikaros proteins observed in different hematopoietic lineages provides further evidence that alternative splicing is a mechanism for regulating Ikaros activity (Figure 2). B, natural killer (NK), activated T cells, and nucleated erythroid lineage cells express all of the major isoforms[54]. Surprisingly, Ikaros is largely absent in resting human T cells but is upregulated upon activation[19,54]. In contrast, Ik-x proteins in the hematopoietic system are detected exclusively in myeloid lineage cells or progenitors with myeloid differentiation potential. Human Lin$^-$ CD34$^+$ hematopoietic stem cells (HSCs) express both Ik-x and Ik-1. When placed in cultures that selectively support lymphoid or myeloid differentiation, there is a loss of Ik-x under lymphoid conditions and an upregulation of Ik-x under myeloid conditions[54]. CD14$^+$ monocytes express both Ik-1 and Ik-x while terminally differentiated granulocytes express only Ik-x[19,54]. These data identify the Ik-x isoform as a potential candidate for mediating myeloid lineage commitment decisions.

Studies that examine Ikaros DNA-binding activity have largely focused on one isoform-Ik-1. Several factors have likely contributed to this. Ikaros studies have targeted the lymphoid lineages where Ik-x is not expressed and have been performed in mice, in which Ik-H is largely absent. If we are to obtain a clearer picture of Ikaros function in myelopoiesis, it will be important to widen our scope to include Ik-x; the most abundantly expressed Ikaros isoform. It will also be important to consider how

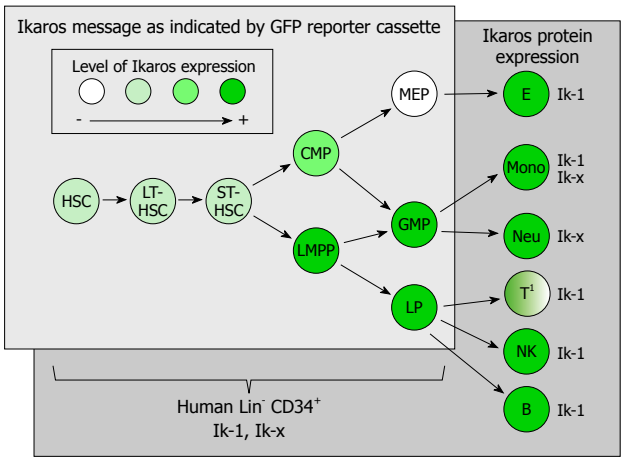

Figure 2 Overview of Ikaros expression in hematopoiesis. The relative expression levels of Ikaros mRNA in hematopoietic progenitors (light gray box in foreground) are indicated by levels of green shading. Progenitors from both the classic and the LMPP pathways are included. Relative Ikaros expression levels are from studies by Yoshida *et al*[63] that have used mice with an Ikaros reporter cassette [green fluorescent protein (GFP) down stream of an Ikaros promoter] as an indicator of Ikaros mRNA expression in early stages of murine hematopoiesis. Shown in the dark gray box in the background is the protein level expression of human Ikaros isoforms in differentiated hematopoietic cells and in Lin^-CD34^+ hematopoietic progenitors as described by Ronni*et al*[19] and Payne *et al*[54]. Human cells that express Ik-x and/or Ik-1 also express the plus form of these isoforms[19,54] (i.e. $Ik\text{-}1^+$/Ik-H and $Ik\text{-}x^+$, see Figure 1). [1]Mature T cells express little Ikaros protein unless they are activated[19,54]. HSC: Hematopoietic stem cell; LT-HSC: Long-term-HSC; ST-HSC: Short-term-HSC; CMP: Common myeloid progenitor; GMP: Granulocyte/macrophage progenitor; LMPP: Progenitor with lymphoid and myeloid potential; MEP: Megakaryocyte/erythroid progenitor; LP: Lymphoid progenitor; NK: Natural killer.

the interplay between Ik-x and Ik-1 and/or other less abundant Ikaros isoforms might contribute to myeloid lineage commitment and granulocyte *vs* macrophage specification in human myeloid differentiation.

IKAROS AND MYELOID COMMITMENT IN THE CLASSIC AND LMPP HEMATOPOIETIC PATHWAYS

Protein studies are essential for elucidating the complete picture of Ikaros function as a mediator of hematopoietic lineage commitment. Nevertheless, transcriptional studies of normal and Ikaros null mice that express an Ikaros reporter cassette, coupled with bioinformatics approaches, are contributing to the outline of an important new pathway for myelopoiesis. Primitive multipotent HSCs give rise successively to long-term-HSCs and short-term (ST)-HSCs, which repopulate HSCs with multilineage potential, but increasingly limited self-renewal capacity[55] (Figure 2). According to the classic model of hematopoietic differentiation, the segregation of lymphoid and myelo-erythroid differentiation is an early event that occurs as ST-HSCs become either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). The CMPs then give rise to megakaryocyte/erythroid progenitors and granulocyte/macrophage progenitors (GMPs) and their lineage restricted progeny, while the CLPs generate all of the lymphoid lineages (T, B, NK and lymphoid dendritic cells). Evidence for the classic differentiation pathway has been reported in both mice and humans[56-59]. However, accumulating evidence suggests the existence of differentiation pathways that are distinct from the classic model of hematopoietic differentiation. Early branching of the megakaryocyte/erythroid lineages from progenitors with lymphoid and myeloid potential (LMPPs) have been demonstrated downstream of ST-HSCs in mice[60,61] and just recently in humans[62] (Figure 2). The extent to which the classic hematopoietic pathway or the more recently identified LMPP pathway contributes to normal myelopoiesis remains controversial. Whether a particular pathway predominates at a given point in ontogeny and/or whether one pathway overlays another throughout life is not yet clear.

Mice with an Ikaros reporter cassette produced by expressing the green fluorescent protein (GFP) under the control of an Ikaros promoter have been used to evaluate the relationship between Ikaros expression and hematopoietic lineage potential[63], thus shedding light on the role of Ikaros in lineage commitment decisions (Figure 2). Based on GFP expression and corresponding levels of Ikaros, early hematopoietic progenitors from these mice have been isolated, into $Ikaros^-$, $Ikaros^{INT}$, and $Ikaros^+$ populations. $Ikaros^-$ progenitors show expression of early erythroid-lineage genes (e.g. *Gata1* or *Gata2*) but not myeloid promoting genes. Progenitors with intermediate levels of Ikaros have been shown to co-express early erythroid and myeloid genes, and consistent with the classic model of hematopoiesis, to contain functional CMPs with the capacity to generate myeloid and erythroid lineage cells[63].

Surprisingly, progenitors with high levels of Ikaros express early genes that promote early myeloid differentiation events (e.g. *Gfi1b, Csf3r* or *Cebpa*) as well as lymphoid promoting genes (e.g. *Flt3, Rag1* or *Il7r*), but not erythroid-associated genes. Consistent with gene expression, functional assays have demonstrated that the progenitors that express the highest levels of Ikaros have both lymphoid and myeloid but not erythroid potential[63]. These data are consistent with the emerging picture of hematopoiesis in which the classic hematopoietic model co-exists with a hematopoietic program in which lymphoid and myeloid potential is segregated from erythroid differentiation early in hematopoiesis (Figure 2).

The role of Ikaros in the classic and LMPP hematopoietic pathways has been investigated using Ikaros null mice that are engineered to express the GFP Ikaros reporter cassette[63]. These mice, while lacking Ikaros, have expressed the GFP Ikaros reporter in a manner similar to that observed in wild-type mice. Analysis of (GFP^{INT}) progenitors from wild-type mice and their Ikaros null counterparts show that the ability of the classic CMPs to produce myeloid-committed GMPs is substantially reduced in the absence of Ikaros. In contrast, LMPPs

which express high levels of Ikaros in wild-type mice (GFP+) show increased ability to generate myeloid-committed cells in the Ikaros null mice[63].

These studies provide a glimpse of the complex role of Ikaros in myeloid differentiation, in which its impact has previously been obscured due to reciprocal effects on the CMPs and LMPPs. The above data suggest that low level Ikaros expression can promote myeloid instead of erythroid differentiation among CMPs with myelo-erythroid potential in the classic hematopoietic pathway. The role of Ikaros in lymphoid *vs* myeloid lineage commitment in LMPPs is less clear. Although Ikaros is required for the production of lymphoid lineage cells from LMPPs, this is not the case for myeloid cells. In fact, their numbers are increased with the loss of Ikaros. Thus Ikaros is clearly not a requirement for myeloid commitment among LMPPs.

The story of Ikaros in lineage commitment entered a new era when bioinformatics approaches made it possible to perform highly sensitive whole genome analysis of gene expression in small numbers of hematopoietic progenitors. Studies of the newly defined LMPP pathway[64] have shown that normal HSCs express not only genes that are associated with HSC function, such as self-renewal, but also low level expression of genes associated with early erythroid, myeloid and lymphoid differentiation. This expression is thought to prime progenitors for subsequent differentiation events that proceed in a stochastic manner. A comparison of normal progenitors in the LMPP pathway and their Ikaros null counterparts (identified using the GFP Ikaros cassette[63] as described above) gives important clues to Ikaros function in hematopoietic differentiation. Ikaros null mice fail to appropriately shut down expression of HSC-associated genes in downstream progenitors. These mice also fail to downregulate early erythroid-associated genes and to upregulate early lymphoid-associated genes. Dysregulated myeloid gene expression is observed in these mice, that is, genes such as *Cfs1r, Cebpd* and *Id2* that are usually associated with late myeloid differentiation are upregulated in HSCs as well as in the LMPPs[64].

The failure of Ikaros null mice to suppress myeloid genes is consistent with the commonly accepted idea that Ikaros promotes lymphoid differentiation by inhibiting the expression of myeloid genes, and thereby, myeloid differentiation. However, models for lymphoid *vs* myeloid cell fate specification that account for the maintenance of myeloid potential in LMPPs and downstream GMPs that express high levels of the *Ikaros* gene product[63] have not been described. The lineage commitment studies discussed above were limited in that they examined Ikaros transcripts. The extent to which Ikaros transcripts correspond to Ikaros protein expression, and the particular isoforms expressed in normal hematopoietic progenitors, are hard to determine because of the technical limitations of obtaining adequate cell numbers. The status of Ikaros protein activity due to post-translational modifications is an important question to address because of the potential for phosphorylation and SUMOylation to affect Ikaros function. Given the high levels of Ikaros transcripts reported in the largely myeloid-committed GMPs[63], it seems likely that differential isoform expression or post-translational regulatory mechanisms will at least be a part of the Ikaros story in lymphoid *vs* myeloid lineage commitment.

CONCLUSION

Ikaros is a key regulator of normal and malignant hematopoiesis in multiple lineages, including the myeloid lineage. Currently, very little is known of the mechanisms that regulate *Ikaros* gene expression[16]. Information on the factors that control Ikaros expression is likely to provide important new insights. However, the fact that Ikaros transcripts are detected at similar levels in a wide range of progenitors and lineages, where Ikaros can have opposing effects, suggests that much of the Ikaros story may be found at the protein level. This could pose challenges for studies that involve hematopoietic progenitors or primary human samples in which cell numbers are often limiting. To get a complete picture of the roles that Ikaros plays in normal and malignant hematopoiesis, including myelopoiesis, it will be important to distinguish the parts played by the various Ikaros isoforms and to identify other cast members such as CK2, chromatin remodeling complexes, and other Ikaros family members that regulate and cooperate in Ikaros activities.

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TOPIC HIGHLIGHT

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Regulation of Ikaros function by casein kinase 2 and protein phosphatase 1

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Abstract

The *Ikaros* gene encodes a zinc finger, DNA-binding protein that regulates gene transcription and chromatin remodeling. Ikaros is a master regulator of hematopoiesis and an established tumor suppressor. Moderate alteration of Ikaros activity (e.g. haploinsufficiency) appears to be sufficient to promote malignant transformation in human hematopoietic cells. This raises questions about the mechanisms that normally regulate Ikaros function and the potential of these mechanisms to contribute to the development of leukemia. The focus of this review is the regulation of Ikaros function by phosphorylation/dephosphorylation. Site-specific phosphorylation of Ikaros by casein kinase 2 (CK2) controls Ikaros DNA-binding ability and subcellular localization. As a consequence, the ability of Ikaros to regulate cell cycle progression, chromatin remodeling, target gene expression, and thymocyte differentiation are controlled by CK2. In addition, hyperphosphorylation of Ikaros by CK2 leads to decreased Ikaros levels due to ubiquitin-mediated degradation. Dephosphorylation of Ikaros by protein phosphatase 1 (PP1) acts in opposition to CK2 to increase Ikaros stability and restore Ikaros DNA binding ability and pericentromeric localization. Thus, the CK2 and PP1 pathways act in concert to regulate Ikaros activity in hematopoiesis and as a tumor suppressor. This highlights the importance of these signal transduction pathways as potential mediators of leukemogenesis *via* their role in regulating the activities of Ikaros.

Key words: Ikaros; Leukemia; Zinc finger; Transcription factor; Casein kinase 2; Protein phosphatase 1; Phosphorylation

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IKAROS AS A TUMOR SUPPRESSOR AND REGULATOR OF HEMATOPOIESIS

The *Ikaros* gene encodes a zinc finger, DNA-binding protein that acts as regulator of gene transcription and chromatin remodeling[1]. Studies of Ikaros mutant mice have established Ikaros as a master regulator of hematopoiesis[1]. Partial arrest or defects in normal hematopoiesis often lead to aberrant cellular proliferation and leukemia/lymphoma, therefore, it is not surprising that Ikaros knockout mice that lack one copy of Ikaros develop T cell leukemia[2]. The remarkable observation is that these mice developed T cell leukemia with 100% penetrance, and in each case, the leukemic clones arose from cells that had lost the single wild-type *Ikaros* allele[2]. This suggests an essential role for Ikaros as a tumor suppressor in T cell differentiation. In humans, defects in the *Ikaros* gene (90% of observed defects involved deletions of one allele, while the remainder involved nonsense or functionally inactivating mutations of a single allele) can result in the production of dominant negative (DN) Ikaros isoforms that act to suppress the function of full-length Ikaros. *Ikaros* defects have been associated with the development of a variety of hematopoietic malignancies. These include childhood acute lymphoblastic leukemia (ALL)[3,4] infant T-cell ALL[5], adult B cell ALL[6], myelodysplastic syndrome[7], acute myeloid leukemia[8], and adult and juvenile chronic myeloid leukemia[9]. *Ikaros* defects leading to a loss of Ikaros activity have been detected in 30% of pediatric B-cell ALL, in > 80% of BCR-ABL1 ALL, and approximately 5% of T-cell ALL[10,11]. In addition, defective *Ikaros* has been identified as a poor prognostic marker for childhood ALL[4,12-14]. A noteworthy observation is that, in almost all primary human leukemia cells in which an *Ikaros* defect is observed, one wild-type Ikaros copy is retained. These data not only show a strong association between the loss of Ikaros function and the development of human leukemia, but also suggest that even a moderate alteration of Ikaros function (e.g. haploinsufficiency) is sufficient to promote malignant transformation. The aberrant expression of small DN Ikaros isoforms has also been associated with the development of human pituitary adenoma[15]. The current hypothesis is that small Ikaros isoforms act as DN mutants in human cells and their overexpression promotes malignant transformation, while the full-length Ikaros acts as a tumor suppressor.

Several crucial questions remain unanswered. (1) Is the loss of Ikaros activity an essential step in the malignant transformation of hematopoietic cells? (2) How is the function of Ikaros regulated in normal and leukemia cells? (3) Can alterations in the regulation of Ikaros function contribute to the development of leukemia?

A partial answer to the first question came when the T leukemia cells derived from Ikaros-deficient mice were transduced with retrovirus to express wild-type Ikaros. The introduction of wild-type Ikaros at physiological levels led to cessation of growth, induction of T-cell differentiation, and cell cycle arrest in Ikaros-deficient T-leukemia cells[16]. These results suggest that the presence of functional wild-type Ikaros, at physiological levels, is sufficient to arrest the aberrant proliferation of malignant cells. This experiment involved a single leukemia cell line that completely lacked Ikaros expression, therefore, this does not fully answer the question of whether the loss of Ikaros function is an essential step in leukemogenesis, although it does underscore the importance of functional Ikaros in tumor suppression. To address these issues regarding the importance of the regulation of Ikaros activity in the development of leukemia, the first step will be to identify the mechanisms that regulate Ikaros activity in normal and malignant hematopoiesis, and to dissect their role in regulating the function of Ikaros.

IKAROS IS PHOSPHORYLATED AT MULTIPLE SITES

The function of many proteins is regulated by their phosphorylation status. Protein phosphorylation is a reversible, dynamic process. The balance between phosphorylation states of a protein regulates its overall function. The *in vivo* phosphopeptide mapping of Ikaros provided the first evidence that Ikaros is phosphorylated at multiple sites[17]. The observation that phosphorylated amino acids within Ikaros are evolutionarily conserved suggests that phosphorylation is an important mechanism regulating Ikaros function. Further phosphopeptide mapping demonstrated that Ikaros phosphorylation sites are very similar in primary thymocytes, in leukemia cells, and in the HEK 293T embryonic kidney carcinoma cell line following transduction or transfection to express Ikaros[17,18]. This suggests that phosphorylation of Ikaros occurs by kinases that are present in multiple tissues and that phosphorylation is an integral feature of Ikaros regulation (Figure 1).

CELL-CYCLE-SPECIFIC PHOSPHORYLATION OF IKAROS

The first study to examine the role of phosphorylation in regulating Ikaros function focused on the cell-cycle-specific phosphorylation of Ikaros. *In vivo* phosphopeptide mapping of Ikaros at different stages of the cell cycle has revealed that during mitosis, Ikaros undergoes hyperphosphorylation[17]. Point mutation analysis has demonstrated that the cell-cycle-specific phosphorylation of Ikaros occurs at an evolutionarily conserved linker sequence that connects DNA-binding zinc finger motifs. Mutational analysis of phosphomimetic and phosphoresistant Ikaros mutants has shown that the cell-cycle-specific phosphorylation of Ikaros regulates its DNA-binding ability and nuclear localization during mitosis[17]. The linker sequence that connects the zinc finger motifs is preserved in all Kruppel-like zinc finger proteins[19], therefore, this mitosis-specific phosphorylation is not unique to Ikaros, but rather it appears to serve as a global

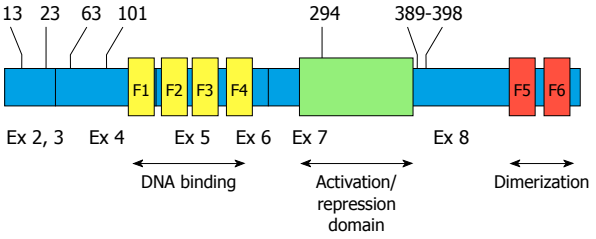

Figure 1 Location of casein kinase 2 phosphorylation sites on Ikaros. The phosphorylated amino acids are indicated by numbers at the top. The location of zinc fingers is indicated by yellow (F1-F4) and red bands (F5-F6). Exons (Ex) are indicated at the bottom. Exon 1 (untranslated) is not shown.

control mechanism of cell cycle progression during mitosis. The kinase that is responsible for the mitosis-specific phosphorylation of Ikaros has not been identified and is considered to be a different kinase from the one responsible for Ikaros phosphorylation during G1 and S phases.

PHOSPHORYLATION OF IKAROS BY CASEIN KINASE 2 AND CELL CYCLE PROGRESSION

The studies described above have established that phosphorylation can control the function of Ikaros in a cell-cycle-specific manner, but have not identified the signal transduction pathway that regulates the function of Ikaros during G1 and S phases of the cell cycle. Studies by Georgopoulos and colleagues have identified several amino acids in Ikaros that are phosphorylated by casein kinase 2 (CK2) (Figure 1). Substitution analysis has revealed that phosphorylation of Ikaros by CK2 at its C-terminal region regulates its ability to control G1/S cell cycle progression[20]. These results have identified CK2 as the enzyme that directly controls Ikaros function, and have demonstrated that CK2-mediated phosphorylation can regulate cell cycle progression.

CK2-MEDIATED PHOSPHORYLATION REGULATES IKAROS FUNCTION IN TRANSCRIPTIONAL REGULATION AND DIFFERENTIATION

A subsequent study has identified four novel evolutionarily conserved CK2 phosphorylation sites located at the N-terminal end of Ikaros (Figure 1)[18]. Functional analysis of Ikaros phosphomimetic mutants (where phospho-sites are mutated to aspartate to mimic phosphorylation) and phosphoresistant mutants (where phospho-sites are mutated to alanine to mimic the dephosphorylated state) has revealed that phosphorylation by CK2 affects Ikaros function at many different levels. Phosphorylation of amino acids 13 and 294 results in decreased Ikaros DNA binding affinity for probes that are derived from pericentromeric heterochromatin (PC-HC). *In vivo,* phosphorylation of the same amino acids causes Ikaros to lose its ability to localize into PC-HC, resulting in diffuse nuclear distribution of Ikaros. These results have provided the first evidence that CK2-mediated phosphorylation regulates not only the ability of Ikaros to bind DNA, but also its subcellular localization and function in chromatin remodeling[18]. We want to emphasize that the cell-cycle-specific phosphorylation of Ikaros that occurs during mitosis is not due to the activity of CK2 because: (1) CK2 is active during G1 and S phases of the cell cycle while this phosphorylation is mitosis-specific; and (2) the CK2 consensus site is well established, and the mitosis-specific phosphorylation of Ikaros occurs at a consensus linker sequence that shows no resemblance to the consensus recognition motif of CK2.

This study also examined the role of CK2-mediated phosphorylation in regulating the ability of Ikaros to bind the upstream regulatory element of the Ikaros target gene, TdT (*dntt*). Results have shown that phosphoresistant Ikaros mutants have much higher DNA-binding affinity toward the TdT regulatory elements when compared to wild-type Ikaros in thymocytes or in HEK 293T cells. Further analysis has revealed that phosphorylation of Ikaros changes during T-cell differentiation. Following the induction of thymocyte differentiation with phorbol myristate acetate, Ikaros undergoes dephosphorylation at amino acids 13 and 294. This results in increased Ikaros binding to the TdT regulatory element and repression of TdT transcription[18]. These data demonstrate that CK2-mediated phosphorylation of Ikaros regulates expression of a key gene in T-cell development - TdT. Thus, CK2-mediated phosphorylation of Ikaros is one of the regulatory mechanisms that govern Ikaros function in normal hematopoiesis.

PROTEIN PHOSPHATASE 1 DEPHOSPHORYLATES IKAROS AND REGULATES ITS ACTIVITY

The studies described above were limited in that they examined only the phosphorylation of Ikaros amino acids that are detected *in vivo*. Phosphopeptide mapping suggests the presence of more phosphorylated amino acids on Ikaros and thus the potential for additional phosphorylation-regulated functions of Ikaros. The discovery that Ikaros is dephosphorylated *in vivo* by protein phosphatase 1 (PP1), and identification of the PP1-Ikaros interaction site, has provided an opportunity for further studies of the role of phosphorylation in regulating Ikaros function[21]. Ikaros with a mutated PP1 interaction site cannot be dephosphorylated by this enzyme. When this mutant is transfected into HEK 293T cells, its protein binds DNA very poorly compared to wild-type Ikaros. This indicates that a very large percentage of Ikaros undergoes phosphorylation *in vivo* and that dephosphorylation of Ikaros is essential for its activity. In addition, this mutant is unable to localize to PC-HC, which confirms

that phosphorylation controls the subcellular localization of Ikaros. When phosphoresistant mutations of the CK2 phosphorylation sites are introduced into the PP1-nonbinding Ikaros mutant, DNA-binding ability and PC-HC localization is restored to a level similar to that observed for wild-type Ikaros. These data have established that CK2-mediated phosphorylation is the major regulator of Ikaros function as a DNA-binding protein and in chromatin remodeling/PC-HC localization[21]. They also suggest that PP1 has the opposite effect on Ikaros function, and that these two signaling pathways are the major regulators of Ikaros activity.

One striking feature of the PP1-nonbinding Ikaros mutant is its low level of expression, as compared to wild-type Ikaros, when transfected into HEK 293T cells. That difference is prominent at the protein level, whereas the mRNA level of both constructs is similar. A possible explanation is that Ikaros that is unable to interact with PP1 undergoes increased degradation compared to wild-type Ikaros. This hypothesis is supported by the observation that Ikaros contains two very strong PEST sequences. The presence of PEST sequences is associated with increased degradation of protein following phosphorylation at amino acids [proline (P), glutamic acid (E), serine (S), and threonine (T)] that are located within the PEST sequence. Analysis of the known CK2 phosphorylation sites that have been identified by us and by the Georgopoulos group has revealed that PEST sequences contain multiple phospho-sites that are phosphorylated *in vivo* by CK2. This suggests that CK2-mediated phosphorylation might promote degradation of Ikaros. *In vivo* degradation assays have demonstrated that the Ikaros mutant that does not interact with PP1 has a severely decreased half-life compared to wild-type Ikaros. The introduction of phosphoresistant mutations at the known CK2-mediated phosphorylation sites prolongs the half-life of the PP1-nonbinding mutant[21]. These results have identified another important role of CK2: to regulate the protein stability and turnover of Ikaros. These results have demonstrated that the hyperphosphorylation of Ikaros leads not only to the loss of its function, but also to a reduction in Ikaros levels due to increased degradation. Subsequent experiments have demonstrated that Ikaros is polyubiquitinated, which has provided evidence that Ikaros degradation occurs *via* the ubiquitin pathway. Overall, the studies of CK2-mediated phosphorylation and PP1-mediated dephosphorylation of Ikaros illustrate the importance of these signal transduction pathways and the role of phosphorylation in regulating Ikaros activity in cells.

ADDITIONAL IKAROS PHOSPHORYLATION SITES

An additional study to identify and analyze the phosphorylation of Ikaros has been performed by the Smale group[22]. This has involved a comprehensive approach using LC-MS/MS analysis of Ikaros phosphorylation in the murine VL3-3M2 T leukemia cell line. This analysis has identified several additional phosphorylation sites in Ikaros, including several threonine and serine residues, as well as two tyrosines. The functional significance of one of these novel phosphorylation sites (amino acid 441) has been studied using phosphomimetic and phosphoresistant mutants, but no alteration of Ikaros function has been observed in a transient transfection assay. The functional significance of the additional phospho-sites identified in this study has not been elucidated. These data illustrate the potential for additional mechanisms of phosphorylation-mediated regulation of Ikaros function.

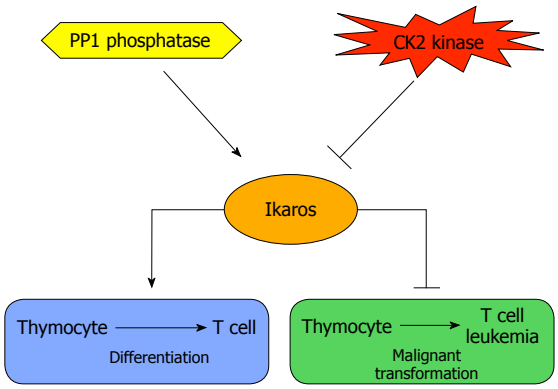

Figure 2 A model for the role of casein kinase 2 and protein phosphatase 1 in regulating Ikaros activity in T-cell differentiation and tumor suppression. CK2: Casein kinase 2.

REGULATION OF CK2 AND PP1 ACTIVITY

CK2 and PP1 both have numerous substrates and their activity involves a complex network of different metabolites. The focus of this review is the regulation of Ikaros function by phosphorylation, therefore, we briefly mention the major regulators of CK2 and PP1 activity that are known to affect cellular proliferation. Phosphorylation of PP1 by cdc2 kinase results in PP1 inactivation in a cycle-dependent manner[23]. Three known tumor suppressors directly bind and inhibit CK2 activity: (1) it has been demonstrated that the tumor suppressor p53 inhibits CK2 by binding to its regulatory β subunit[24]; (2) similarly, another tumor suppressor p21WAF1 binds to the β regulatory subunit of CK2 and inhibits its activity[25]; (3) adenomatous polyposis coli protein also inhibits CK2 by interacting with its CK2 α-subunit[26]. Activators of CK2 include stimulators of cellular proliferation such as: polyamines[27] and fibroblast growth factor-2[28]. These findings suggest that Ikaros acts as a part of multiple signal transduction networks that regulate cellular proliferation and malignant transformation.

CONCLUSION

Studies of Ikaros phosphorylation have provided a partial answer to the question concerning the regulation of Ikaros function in normal and leukemia cells, as well as

how alteration in the regulation of Ikaros might contribute to leukemia. The studies described above have established phosphorylation/dephosphorylation as one of the major mechanisms that controls Ikaros activity. These data strongly suggest that CK2 is the principal regulator of Ikaros function. CK2 is an extensively studied regulator of cellular proliferation and a tumor-promoter protein[29]. Overexpression of CK2 has been associated with the development of various types of tumors, including mammary gland, prostate, lung, and kidney cancers[30]. Forced expression of the catalytic subunit of CK2 in transgenic mice leads to the development of T-cell leukemia and lymphoma[31-34], similar to that observed in mice with impaired Ikaros function[2,16,35,36]. Thus, we hypothesize that Ikaros function is controlled by its phosphorylation status and that overexpression of CK2 leads to hyperphosphorylation of Ikaros, which results in its loss of tumor suppressor activity and the subsequent development of leukemia (Figure 2). Additional studies utilizing Ikaros phosphomimetic and phosphoresistant mutants in mouse models and in primary human and murine cells are necessary to confirm and/or refine this hypothesis. The abundance of Ikaros phosphorylation sites, as well as the lack of a complete understanding of Ikaros function in chromatin remodeling and tumor suppression, suggests that many additional signal transduction pathways that regulate Ikaros function remain to be discovered.

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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-

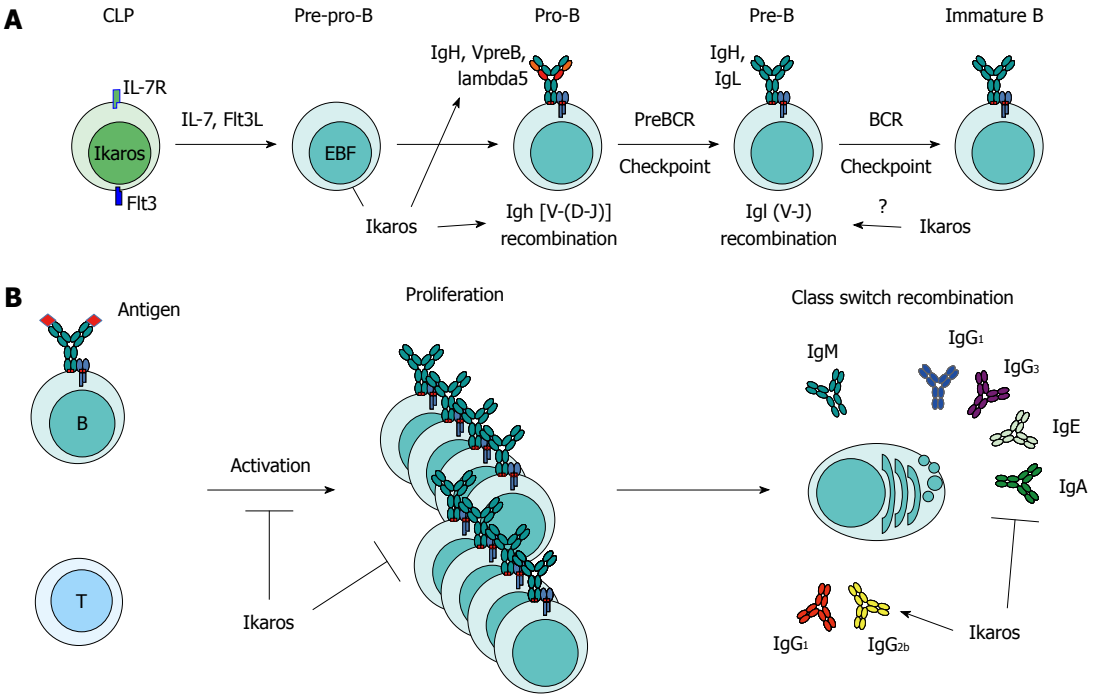

Figure 1 Ikaros controls multiple levels of B cell lymphopoiesis and function. A: Ikaros plays a crucial role in the specification of the B cell lineage by promoting the expression of the IL-7R and Flt3 receptors in common lymphoid progenitors (CLPs) and of the EBF transcription factor in pre-pro-B cells. 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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TOPIC HIGHLIGHT

Sinisa Dovat, MD, DSc, *Series Editor*

Ikaros isoforms: The saga continues

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Abstract

Through alternate splicing, the *Ikaros* gene produces multiple proteins. Ikaros is essential for normal hematopoiesis and possesses tumor suppressor activity. Ikaros isoforms interact to form dimers and potentially multimeric complexes. Diverse Ikaros complexes produced by the presence of different Ikaros isoforms are hypothesized to confer distinct functions. Small dominant-negative Ikaros isoforms have been shown to inhibit the tumor suppressor activity of full-length Ikaros. Here, we describe how Ikaros activity is regulated by the coordinated expression of the largest Ikaros isoforms IK-1 and IK-H. Although IK-1 is described as full-length Ikaros, IK-H is the longest Ikaros isoform. IK-H, which includes residues coded by exon 3B (60 bp that lie between exons 3 and 4), is abundant in human but not murine hematopoietic cells. Specific residues that lie within the 20 amino acids encoded by exon 3B give IK-H DNA-binding characteristics that are distinct from those of IK-1. Moreover, IK-H can potentiate or inhibit the ability of IK-1 to bind DNA. IK-H binds to the regulatory regions of genes that are upregulated by Ikaros, but not genes that are repressed by Ikaros. Although IK-1 localizes to pericentromeric heterochromatin, IK-H can be found in both pericentromeric and non-pericentromeric locations. Anti-silencing activity of gamma satellite DNA has been shown to depend on the binding of IK-H, but not other Ikaros isoforms. The unique features of IK-H, its influence on Ikaros activity, and the lack of IK-H expression in mice suggest that Ikaros function in humans may be more complex and possibly distinct from that in mice.

Key words: Ikaros; Chromatin, Pericentromeric; Transcription; IK-H; Leukemia; γ satellite

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IKAROS ISOFORM EXPRESSION AND IKAROS ACTIVITY

An important factor in the functional diversity of indi-

vidual genes is their ability to encode different proteins. Many genes produce multiple proteins that can have dissimilar functions through alternate splicing. This process is responsible for the generation of complex structural and regulatory networks that control normal cellular function. Determination of the functional similarities and differences of individual isoforms is essential for understanding the role of a gene in a particular process.

The *Ikaros* gene encodes a zinc finger protein that is essential for normal hematopoiesis and that acts as a tumor suppressor[1-3]. The Ikaros protein binds DNA at the upstream regulatory elements of its target genes and regulates their transcription *via* chromatin remodeling. Soon after discovery of the *Ikaros* gene, it was determined that it encodes multiple isoforms *via* alternate splicing[4-6]. In mice, the full-length Ikaros isoform comprises three domains: (1) a DNA-binding domain (at the N-terminal end of the protein) that consists of four C2H2 Kruppel-like zinc finger motifs; (2) a protein-interaction domain (at the C-terminal end of the protein) that consists of two zinc finger motifs with an unusual structure that is most similar to those described in the hunchback gene in *Drosophila*; and (3) an activation domain that is a loosely defined domain that is located between the DNA-binding and protein-interaction domains[4]. Every Ikaros isoform described thus far contains the protein-interaction domain, but they differ in the presence of the DNA-binding domain, or other regions of the gene. Ikaros associates *in vivo* with its various isoforms, as well as with other Ikaros family members that contain a protein-interaction domain similar to that of Ikaros[7]. Ikaros binds DNA as a dimer and possibly as a multimer, therefore, it has been hypothesized that diverse complexes that comprise full-length Ikaros together with different types of isoforms could have distinct functions[8,9]. The ability of the different Ikaros isoforms to interact with each other and with other Ikaros family members[7,10,11] creates many possible combinations of proteins with the potential for diverse functions: four major possibilities are summarized in Figure 1.

Studies of mice with mutations in the *Ikaros* gene, together with biochemical experiments, have revealed that small Ikaros isoforms that contain a protein-interaction domain and that lack a DNA-binding domain exert a dominant-negative (DN) effect by inhibiting the activity of the full-length Ikaros protein[6]. This is demonstrated in *Ikaros* knockout mice that express small DN isoforms. These mice have a more severe phenotype when compared to "true null" *Ikaros* knockout mice[1,12,13]. Overexpression of DN isoforms disrupts normal hematopoiesis and has been shown to inhibit normal Ikaros function in numerous *in vitro* and *in vivo* systems[14,15]. In biochemical experiments, overexpression of DN isoforms inhibits DNA-binding of full-length Ikaros. High expression of DN Ikaros isoforms has been associated with hematopoietic malignancies in humans and mice, as well as with pituitary gland tumors[3,15-20]. It has been hypothesized that this is most likely due to inhibition of the tumor suppressor function of Ikaros and possibly other family members. Thus, the first established functional relationship between Ikaros isoforms was relatively clear cut - full-length Ikaros is functional and a tumor suppressor, whereas small DN isoforms are inhibitory and pro-oncogenic. A mechanistic explanation for this involved inhibition of the DNA-binding ability of the full-length Ikaros isoform by small DN ones. This involved the possibility depicted in Figure 1C.

Deciphering the functional significance of other large Ikaros isoforms has proved to be more complicated. One particular problem is the abundance of endogenous full-length Ikaros isoforms in most hematopoietic cells, which makes it difficult to study the function of individual large Ikaros isoforms *in vivo*. However, over time, data have emerged to reveal the potential functional significance of other Ikaros isoforms. Ikaros-2 (Ikaros-V in the nomenclature of the Smale group), which lacks the first N-terminal DNA-binding zinc finger, has been shown to produce a footprint distinct from that of IK-1 in DNase protection assays[6], although the functional significance of this has not been studied. A report by Payne *et al*[21] has revealed that the Ikaros-X isoform has a unique expression pattern compared to other large Ikaros isoforms, and has suggested that this isoform plays a role in myeloid differentiation. This study was limited to expression analysis and did not provide detailed functional analysis of the Ikaros-X isoform.

Most functional studies of Ikaros isoforms have been performed using murine transcripts and proteins. An early murine study has detected the presence of Ikaros protein and cDNA that includes coding sequence that lies between currently designated *Ikaros* exons 3 and 4[6]. Human *Ikaros* gene transcripts that incorporate the human homolog of this additional exon (which we designate exon 3B) have been subsequently detected in leukemia samples[19]. Studies of Ikaros protein expression in normal human hematopoietic cells have identified several Ikaros splice forms that include exon 3B, including one larger in size than any of the previously described murine Ikaros isoforms[21,22]. Comparisons of Ikaros expression in mice and humans have shown that strong protein level expression of this isoform occurs only in human cells[21,23]. For this reason, the largest Ikaros splice variant is termed Ikaros-H (H for human). Due to the high sequence homology between the murine and human Ikaros genes, the activity of individual isoforms has been assumed to be very similar. The high level protein expression of Ikaros-H in human, but not murine cells is an intriguing observation that suggests that the regulation of Ikaros activity in human cells might be more complex than in mouse cells. Other than Ikaros-1 (IK-1, and in the nomenclature of the Smale group, Ikaros-VI), Ikaros-H has been the subject of the most extensive functional studies to date. The following summarizes the functional characteristics of the Ikaros-H isoform.

EXPRESSION PATTERN AND DNA BINDING AFFINITY OF IK-H

The Ikaros-H isoform (IK-H, also designated IK-1+ in

alternate nomenclature[22]) contains the sequence of the "full-length" Ikaros isoform (IK-1) plus an additional 20 amino acids N-terminal to the DNA-binding zinc fingers (Exon 3B in Figure 2A). In mice, the most abundant Ikaros isoforms in primary T and B lineage cells and in lymphoid leukemia cells are IK-1 and IK-2, while IK-H is essentially absent[21,23]. In contrast, in humans, IK-1, IK-2 and IK-H are expressed at comparable levels in primary leukemia cells and in lymphoid cell lines. IK-H is also abundant in primary human T and B cells[21]. Thus, IK-H exhibits a species-specific expression pattern.

Ikaros contains four C2H2 Kruppel-like zinc finger motifs at its N-terminal end (exons 4-6) that directly interact with DNA. Zinc fingers 2 and 3 are essential for DNA binding of Ikaros protein, although other zinc fingers probably contribute to DNA-binding specificity of Ikaros[24]. Ikaros isoforms bind to the Ikaros consensus sequence TGGGAA/T with the core sequence comprising GGGA[24]. DNA binding analysis has revealed that the two largest human isoforms, IK-1 and IK-H, have different DNA-binding affinities[23]. Both isoforms can bind to the DNA sequences that contain the TGGGAA/T consensus site, as well as to high-affinity sites where two core consensus GGGA sequences are present within 40 bp of each other. However, only IK-1 is able to bind efficiently to DNA sequences that contain a single core sequence, GGGA; the absence of the second GGGA consensus sequence abolishes DNA binding by IK-H. This result was surprising and suggests that the presence of the 20 amino acids that are located upstream from the DNA-binding zinc fingers in IK-H have an inhibitory effect on DNA binding of Ikaros protein. Substitution mutational analysis[23]. Has identified three specific amino acids within the N region that are responsible for the differences in DNA binding of IK-H and IK-1. This suggests that it is not the presence of the additional 20 amino acids N-terminal of the DNA-binding zinc fingers that affects the ability of the Ikaros protein to bind DNA, but rather interaction of specific residues within the 20-amino-acid region with unknown proteins.

IK-H REGULATES DNA BINDING OF OTHER IKAROS ISOFORMS

Evidence that the two largest human Ikaros isoforms have different DNA-binding affinities has led to us to speculate that the DNA-binding affinity of Ikaros proteins for a specific DNA sequence could depend on the relative expression levels of IK-1 and IK-H isoforms. We hypothesize that IK-H could either synergize with IK-1 or act to inhibit its DNA-binding, depending on its unique affinity for a particular DNA sequence. This hypothesis has been tested *in vitro* by mixing recombinant IK-1 and IK-H isoforms and comparing the DNA-binding affinity of the IK-1/IK-H mixture with the same quantity of IK-1 protein. IK-1 and IK-H act synergistically to bind DNA that contains two Ikaros binding sites. However, the IK-H isoform inhibits the binding of IK-1 to DNA that contains a single Ikaros binding site[23]. Thus, IK-H can either potentiate or inhibit the DNA binding of the IK-1 isoform *in vitro*.

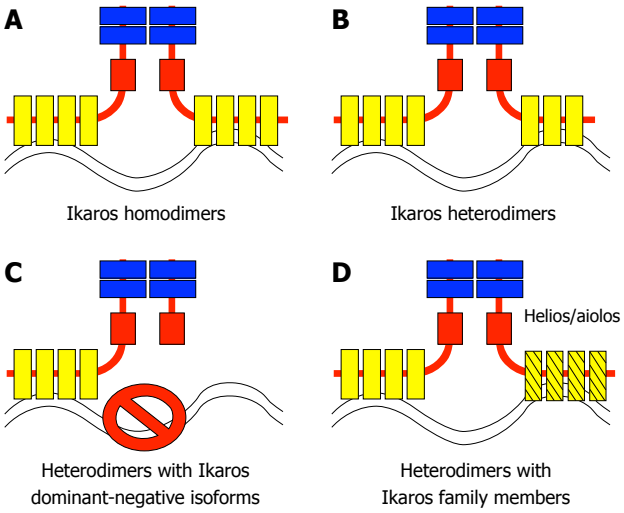

Figure 1 The association of Ikaros proteins with Ikaros isoforms and Ikaros family members controls the activity of the Ikaros protein complex. The four main types of Ikaros family-containing protein complexes are shown. A: Homodimers of the most abundant Ikaros isoform (IK-1); B: Heterodimers of IK-1 with DNA-binding Ikaros isoforms; C: Heterodimers of IK-1 and DNA-nonbinding (DN) Ikaros isoforms - this complex does not bind DNA; D: Heterodimers of Ikaros with its other family members (e.g. Helios or Aiolos).

It has been demonstrated that Ikaros binds DNA *in vivo* as a dimer and possibly as a multimeric complex[25]. To test whether the interaction of IK-1 and IK-H regulates the DNA-binding affinity of Ikaros *in vivo,* the ability of Ikaros to bind DNA has been compared using electrophoretic mobility shift assay and chromatin immunoprecipitation (ChIP) to assess cells that expressed only IK-1, only IK-H, or both isoforms. Expression of IK-H selectively affects the DNA-binding of Ikaros proteins - DNA-binding to the DNA sequences that contain a single Ikaros binding site is reduced, but binding to DNA with two Ikaros consensus binding sites is strong. These experiments were performed in activated T cells, thus confirming the physiological relevance of IK-H interactions with IK-1. These results demonstrate that IK-H does not function as a typical DN isoform (as described for the small Ikaros isoforms that lack DNA binding zinc fingers), but rather as a unique control mechanism that determines DNA-binding specificity of Ikaros proteins *in vivo*. This provides confirmation that Ikaros binds DNA *in vivo* as a dimer or multimer, and for the first time has demonstrated the functional significance of the coexpression of different large Ikaros isoforms: to provide precise control of the DNA-binding specificity of Ikaros in cells.

SUBCELLULAR LOCALIZATION OF THE LARGEST IKAROS ISOFORMS

The condensed chromatin that flanks centromeres is known as pericentromeric heterochromatin (PC-HC). Genes localized to PC-HC are generally transcriptionally

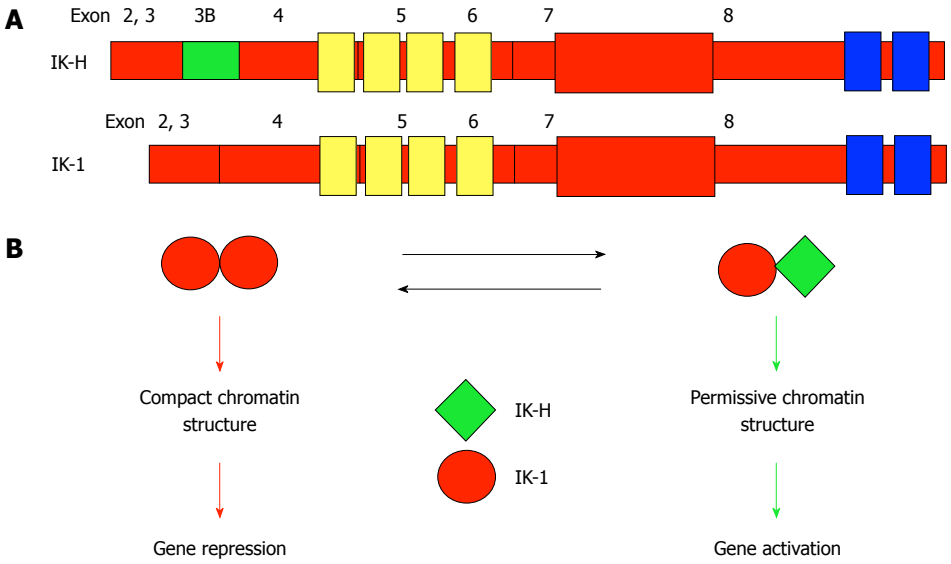

Figure 2 A model for IK-H regulation of human gene expression. A: Exon structure of IK-1 and IK-H. The 20 amino acids encoded by Exon 3B distinguish IK-H from IK-1. Zinc fingers responsible for DNA binding are shown in yellow. Zinc fingers responsible for dimerization are shown in blue; B: A protein complex that contains IK-1 homodimers binds DNA tightly, leading to a compact chromatin structure and gene repression. Binding of Ikaros protein complexes that contain IK-H changes chromatin into a permissive structure leading to activation of gene expression.

inactive. The subcellular localization of Ikaros to PC-HC has been shown to correlate directly with its DNA-binding affinity towards the repetitive sequences that are part of PC-HC[24]. Confocal microscopy has revealed that IK-1 and IK-H exhibit distinct subcellular localization. The IK-1 isoform displays the typical punctate distribution pattern consistent with PC-HC localization. In contrast, IK-H exhibits dual - non-centromeric and centromeric - localization. The non-centromeric localization of IK-H is not in the form of the diffuse nuclear staining that is typically observed in other transcriptional factors, but rather localization to other specific nuclear structures[23]. More intriguing is that the non-centromeric localization of IK-H is observed in hematopoietic cells, but not when this isoform is transduced into fibroblasts. When transduced in murine hematopoietic cells, IK-H retains the same localization pattern as in human cells. This suggests that the distinct localization pattern of IK-H is tissue-specific and that it is a result of the unique properties of the IK-H protein, and not due to the differences in heterochromatin between different species. This also provides evidence that IK-H exists in complexes that do not contain the IK-1 isoform and thus, it may have its own unique cellular function. This provides the first evidence to suggest that different Ikaros isoforms may function in distinct subcellular regions.

ROLE OF IK-H IN GENE ACTIVATION AND CHROMATIN REMODELING

It has been demonstrated that Ikaros can act both as a positive and a negative regulator of gene expression. This is likely related to the ability of Ikaros to associate with the SWI/SNF activator complex or with the histone deacetylase repressor complex, NuRD[26-28]. The evidence that Ikaros can both activate and repress expression of its target genes by recruiting them to PC-HC suggests a role for chromatin remodeling in this process[29]. The molecular mechanisms that determine whether Ikaros functions as an activator or repressor of transcription are unknown.

Experiments that have tested the DNA-binding specificities of IK-1 and IK-H isoforms to regulatory sequences of known Ikaros target genes have revealed differential binding of these isoforms to regulatory elements of repressed *vs* activated Ikaros targets. Binding of both IK-H and IK-1 to the upstream regulatory elements of *IKCa1*, *Granzyme B, STAT4,* and *FAAH* has been demonstrated by ChIP and electromobility supershift assay. In contrast, IK-1 and other Ikaros isoforms, but not IK-H, bind the upstream regulatory element of the VPAC-1 receptor gene. *In vivo*, Ikaros proteins downregulate transcription of the VPAC1 receptor gene[30], while positively regulating expression of *IKCa1*[31], *Granzyme B*[32], *STAT4*[33] and *FAAH*. The observation that the IK-H isoform binds to the regulatory region of genes upregulated by Ikaros, but not to the regulatory region of genes repressed by Ikaros has led to the intriguing hypothesis that the IK-H isoform acts as an activator, while other Ikaros isoforms act as transcriptional repressors of Ikaros target genes[23]. According to this hypothesis, IK-H would have the opposite function of other Ikaros isoforms in regulating transcription. The mechanism by which Ikaros would activate its target genes upon their recruitment to PC-HC has not been resolved.

A detailed analysis of the function of human gamma-satellite DNA repeats reveals that gamma-satellite DNA has anti-silencing activity[34]. ChIP analysis has documented binding of both IK-H and IK-1 to the gamma-satellite DNA. Mutational analysis reveals that the anti-silencing

activity of gamma-satellite DNA is directly dependent on the presence of two consensus Ikaros binding sites that are in close proximity (bipartite motif), and on the binding of IK-H. The mutation of one Ikaros consensus binding site that abolishes the binding of IK-H, but not binding of other Ikaros isoforms, leads to the loss of the anti-silencing activity of gamma-satellite DNA[34]. These results clearly showed that: (1) human gamma-satellite DNA allows a transcriptionally permissive chromatin conformation, thus it can have an anti-silencing function; and (2) binding of the IK-H Ikaros isoform (but not other Ikaros isoforms) is essential for the anti-silencing activity of gamma-satellite DNA. This sheds light on the molecular mechanisms by which Ikaros can both activate or repress its target genes *via* recruitment to PC-HC and establishes distinct (and possibly opposing) roles for IK-H and IK-1 isoforms in the regulation of gene expression and chromatin remodeling. The current (simplified) model of transcriptional regulation by IK-H and IK-1 is outlined in Figure 2B.

CONCLUSION

In summary, the Ikaros gene encodes a large number of different proteins *via* alternate splicing. *Ikaros*-encoded proteins form dimers and multimers that regulate gene expression and chromatin remodeling. The presented analysis emphasizes that the function of Ikaros complexes depends on the presence of particular Ikaros isoforms, and that individual isoforms likely have unique functions. High expression of the IK-H isoform in human lymphoid cells, but not their murine counterparts, suggests that the regulatory function of Ikaros in humans is more complex and possibly distinct from that in mice.

The discovery of functional differences between IK-1 and IK-H isoforms raises many questions that need to be resolved. Future research will be directed to achieve the following goals: (1) dissection of the DNA-binding specificity of IK-1 and IK-H using global functional genomic approaches; and (2) identification of additional genes that are regulated by Ikaros and a correlation of the binding of IK-1 and IK-H to upstream regulatory regions with changes in target gene expression. Further analysis of the function of Ikaros proteins is essential for understanding their role in normal human hematopoiesis and their tumor suppressor function in human leukemia.

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TOPIC HIGHLIGHT

Sinisa Dovat, MD, DSc, *Series Editor*

Vasoactive intestinal peptide signaling axis in human leukemia

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Abstract

The vasoactive intestinal peptide (VIP) signaling axis constitutes a master "communication coordinator" between cells of the nervous and immune systems. To date, VIP and its two main receptors expressed in T lymphocytes, vasoactive intestinal peptide receptor (VPAC)1 and VPAC2, mediate critical cellular functions regulating adaptive immunity, including arresting CD4 T cells in G_1 of the cell cycle, protection from apoptosis and a potent chemotactic recruiter of T cells to the mucosa associated lymphoid compartment of the gastrointestinal tissues. Since the discovery of VIP in 1970, followed by the cloning of VPAC1 and VPAC2 in the early 1990s, this signaling axis has been associated with common human cancers, including leukemia. This review highlights the present day knowledge of the VIP ligand and its receptor expression profile in T cell leukemia and cell lines. Also, there will be a discussion describing how the anti-leukemic DNA binding transcription factor, Ikaros, regulates VIP receptor expression in primary human CD4 T lymphocytes and T cell lymphoblastic cell lines (e.g. Hut-78). Lastly, future goals will be mentioned that are expected to uncover the role of how the VIP signaling axis contributes to human leukemogenesis, and to establish whether the VIP receptor signature expressed by leukemic blasts can provide therapeutic and/or diagnostic information.

Key words: Neuropeptides; Ikaros; Cancer; Hut-78; Epigenetics

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THE VASOACTIVE INTESTINAL PEPTIDE NEUROIMMUNE-NETWORK

Vasoactive intestinal peptide (VIP) is a 3.3 kDa protein originally discovered in swine intestines by Said *et al*[1]. Upon characterization, this small peptide consisting of 28 amino acids, had vasoactive properties when added to arteries, which prompted its name, VIP. Within 10 years after its discovery in 1970, VIP detection was measured in a number of human and rodent blood cells, including

mast cells[2], neutrophils[3], eosinophils[4], thymocytes[5] and T and B lymphocytes[6]. Additional discoveries revealed that biological fluids derived from immune-privileged organs (e.g. eye and spine) were rich in VIP[7], and importantly inhibited the proliferation of immune cells in mixed lymphocyte cultures[8]. Intriguingly, in addition to immune-privileged compartments that actively repeal immune cells, the immunosuppressive VIP peptide was also detected in secondary immune organs that actively recruit high numbers of immune cells. Two immune compartment examples are the mucosa associated lymphoid tissue (MALT) of the pulmonary and gastrointestinal tissues[9]. The immunoreactive (IR) VIP nerves detected within these compartments co-stained with markers for noradrenergic, non-cholinergic nerves that innervated these organs, thus identifying an additional neuronal source for the immunosuppressive VIP peptide, in addition to certain immune cells, including developing thymocytes, activated T cells and mast calls[5,10,11].

These studies detecting IR VIP$^+$ nerves within the eye and MALT represented the first major discovery that firmly established an anatomical basis for a neuroendocrine-immune network. Additional observations confirmed IR-VIP$^+$ nerves innervating additional immune organs, including the thymus, spleen, bone marrow, skin and Peyer's Patches within the gastrointestinal mucosa associated tissue. A second major contribution was the discovery that both immune and non-immune cells, in proximity to VIP$^+$ nerve endings, expressed receptors for the VIP neuropeptide[12]. A third important observation was that VIP possessed chemotactic properties for resting T lymphocytes and actively recruited them to Peyer's Patches located in the gut[13,14]. Lastly, VIP suppressed T lymphocyte activation by blocking interleukin (IL)-2, IL-4 and interferon (IFN)-γ production, inhibited apoptosis thereby enhancing T_{h2} memory cells and promoted the inducible FoxP3$^+$ regulatory T cell (iTreg) lineage (Figure 1). This collective body of research is the fundamental core for the field called neuroimmunomodulation, of which VIP has been firmly established as a master mediator in this regulatory axis (for review see[15-17]).

This review will focus on the VIP signaling axis and its relevance to human T cell leukemia. We will begin with a review of the VIP signaling axis in healthy T lymphocytes followed by the current understanding of VIP ligand and receptor expression profiles in T cell leukemia patients and cell lines. Penultimately, there will be a discussion on the contemporary dogma of the transcriptional regulation of VPAC1 by the anti-leukemic chromatin remodeling factor, Ikaros. Lastly, concluding comments will place into perspective a current working model that we expect will yield important insight into the potential role of the VIP signaling system in the diagnostic, treatment and clinical outcome of T cell acute lymphoblastic leukemia (ALL).

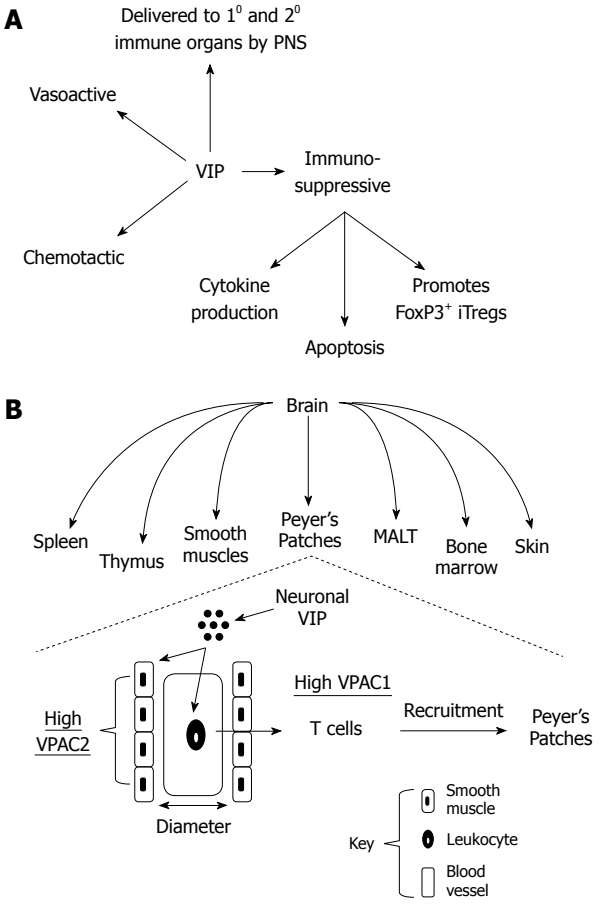

Figure 1 Neuroimmunomodulation by vasoactive intestinal peptide. A: Vasoactive intestinal peptide (VIP) is delivered to primary (1^0) and secondary (2^0) immune organs by the peripheral nervous system (PNS), which affects the metabolism of cells in close proximity through its vasoactive properties (vascular smooth muscle cells) and its chemotactic activities on resting T lymphocytes. During TCR signaling, VIP is immunosuppressive directly on T lymphocytes by: inhibiting proinflammatory cytokine secretion/production, inhibiting apoptosis and promoting FoxP3$^+$ inducible T regulatory cells; B: Delivery of VIP ligand to immune cells in indicated anatomical compartments (immune and non-immune) that represents a division of labor for VIP/vasoactive intestinal peptide receptor (VPAC)2 signaling (vasoactive; smooth muscle cells) and VIP/VPAC1 signaling (chemotactic and immunosuppressive; directly on lymphocytes) in an effort to effectively target trafficking naïve T cells to appropriate immune compartments such as Peyer's Patches within the gut. MALT: Mucosa associated lymphoid tissue.

VIP SIGNALING AXIS

VIP

The ligand, VIP, is classified as a neuropeptide member of the secretin superfamily that performs crucial biological activities, including regulation of the immune system[18]. The secretin superfamily is made up of nine diverse small peptides that share similar, as well as, distinct biological activities. Cloning of the human VIP gene occurred in 1995 and is located on chromosome 6q25 (Entrez Gene ID 7432)[19]. Rat and mouse VIP genes had been previously cloned in 1991, and are positioned on syntenic regions of the rat chromosome 1p11 (Entrez Gene ID 117064) and on the mouse chromosome 10A1 (Entrez Gene ID 22353), respectively[20]. The structure of the human VIP gene consists of 7 exons interrupted by 6 introns and spans 9 kb. The VIP gene is translated into a 170 amino acid preproprotein and proteolytically tailored to generate at least two biologically active peptides, called VIP and peptide histidine methionine

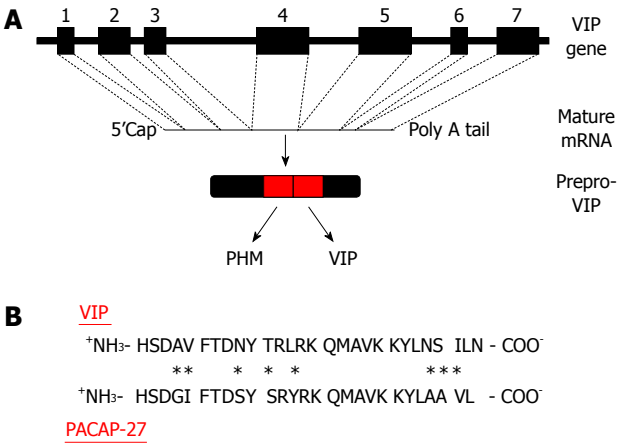

Figure 2 Molecular biology of vasoactive intestinal peptide. A: Vasoactive intestinal peptide (VIP) is transcribed from a gene consisting of 7 exons and translated into a 170 amino acid prepropeptide that produces at least two biologically active peptides as shown; B: The amino acid comparison between pituitary adenylate cyclase activating polypeptide (PACAP)27 and VIP with bold letters representing identical amino acids between peptides, and asterisks indicating amino acid differences.

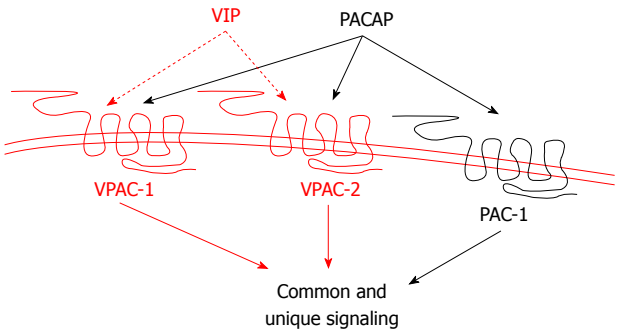

Figure 3 Binding selectivity of the vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptors. Pituitary adenylate cyclase activating polypeptide receptor 1 (PAC1) selectively binds pituitary adenylate cyclase activating polypeptide (PACAP) with 1000-fold greater affinity than vasoactive intestinal peptide (VIP), whereas vasoactive intestinal peptide receptor (VPAC)1 and VPAC2 bind VIP and PACAP with equal affinity.

(PHM). Thus, the VIP gene appears to be organized into exon modules in which exon 5 exclusively encodes the VIP peptide, and exon 4 the PHM peptide (Figure 2). VIP shares nearly 70% amino acid sequence identity with another secretin family peptide called pituitary adenylate cyclase activating polypeptide (PACAP). The rodent (Adcyap1) (Entrez Gene ID mouse - 11516; rat - 24166), and human (ADCYAP1) (Entrez Gene ID 116) PACAP genes were cloned in the early 1990s[21-23], and have a similar gene structure and translational processing to VIP, generating at least three biologically active peptides called PACAP-38 (38 amino acids in length), PACAP-27 and PACAP related peptide. PACAP has remained nearly unchanged (96% identical) for over 700 million years of evolution and is considered the progenitor of the secretin superfamily of peptides[24]. PACAP-27 and VIP possess 68% amino acid sequence identity and VIP is thought to have evolved by exon duplication from PACAP concomitant with the evolution of the adaptive immune system as invertebrates evolved into vertebrates around 500 million years ago[24]. A co-evolution of VIP and its receptors with the establishment of the adaptive immune system may explain why VIP/PACAP modulates numerous immune functions such as proliferation[25], cytokine expression[26], inhibition of apoptosis[27] adhesion[14] and chemotaxis[13]. VIP is delivered by peripheral neurons to immune organs (and non-immune organs), in addition to being secreted by resting and activated leukocytes[28]. VIP is one of the most abundant peptides in immune organs such as the spleen, thymus and MALT[29]. This chemotactic and immunosuppressive neuropeptide ameliorates several autoimmune and inflammatory disease models in mice, including rheumatoid arthritis[30,31], atopic dermatitis[32], Crohn's disease[33], multiple sclerosis (MS)[34] host *vs* graft disease, and antagonists to VIP receptors inhibit the proliferation of many common, solid-tissue, human cancers, including 51 of 56 human lung cancer cell lines[35].

Recently, VIP and PACAP were discovered to increase the generation of inducible $CD4^+/CD25^+$ regulatory T cells (iTregs) that are positive for FoxP3 expression[36].

VIP receptors

The receptors that bind VIP and PACAP receive their name based on their affinity for these two biologically active peptides, respectively. For example, pituitary adenylate cyclase activating polypeptide receptor 1 (PAC1) binds PACAP with a 1000-fold greater affinity than VIP, and is therefore categorized as the selective VIP/PACAP receptor[24]. In addition to PAC1, there are two non-selective receptors that bind VIP and PACAP with equal affinity, called VIP/pituitary adenylate cyclase activating polypeptide receptor (VPAC)1 and VPAC2 (Figure 3). All three receptors share a presumed similar 7-transmembrane structure based on hydropathy plots, with three external (EC1-3) and three internal loops (IC1-3), an extended N-terminal extracellular ectodomain and a relatively short intracellular C-terminal domain[37,38]. The VPAC1 (Vpr1) and VPAC2 (Vpr2) genes were cloned in rodents (Vpr1 - Entrez Gene ID mouse - 22354; rat - 24875; Vpr2 - Entrez Gene ID mouse 22355; rat 29555) and humans (VPR1 - Entrez Gene ID - 7433; VPR2 - 7434) in the early 1990s, and have very similar genetic structures with the human VPAC1 gene consisting of 13 exons and 12 introns[39-41]. Human VPAC1 has been mapped to chromosome 3q22, and to a syntenic region on chromosome 9 in mouse. Upon ligand binding, VPAC1 and VPAC2 couple with at least three G proteins, including $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha q}$ that regulate signaling molecules as diverse as adenylate cyclase, PKA, PKC, PLC, PLD and EPAC, and elevate the intracellular secondary messengers, cAMP, IP_3, DAG and Ca^{2+}, that appear to be largely cell-context dependent[17,42,43]. There is also solid evidence for nuclear factor κB dependent and independent signaling effects by VIP[44].

VIP receptor expression profile and its transcriptome in T lymphocytes

In naïve, mouse and human CD4 and CD8 T lympho-

cytes, the constitutively expressed VPAC1 receptor is 300-500-fold higher than VPAC2[12,45-47] (unpublished data) at the mRNA and protein levels that appears to be inversely related to the expression level of IL-2. Our laboratory has recently identified the VIP/VPAC1 transcriptome in naïve and activated mouse splenic CD4 T cells. In naïve T cells, VIP/VPAC1 signaling appears to induce directed cell movement through EGFR signaling. In early activated T cells (5 h), the cAMP dependent CREM/ICER transcription factor is upregulated, which has been shown to "short-circuit" helix-loop-helix transcription factors that are critical for pro-inflammatory cytokine expression[48]. These microarray observations, we propose, can explain at a molecular level how the VIP signaling axis can act in an anti-inflammatory manner, as well as, induce the differentiation of activated T cells toward different effector phenotypes, including T regulatory cells and T_{h17} cells[36,49]. Since developing and mature T lymphocytes are heterogeneous cell populations, it will be important to generate the next generation of anti-VIP receptor antibodies capable of detecting protein within these hematopoietic subpopulations to confirm which receptor is evoking VIP-initiated signal transduction and altering metabolic cellular changes. To this end, our laboratory has generated by gene-gun technology a highly specific mouse anti-VPAC1 polyclonal antibody capable of detecting cell-surface VPAC1 protein on primary CD4 and CD8 T lymphocytes (manuscript submitted).

During *in vitro* T cell activation (e.g. anti-CD3/anti-CD28) T lymphocytes engage the cell cycle to begin a proliferative program that results in a precipitous drop of VPAC1 mRNA levels $\geqslant$ 80% as assessed by qPCR[46,47]. Likewise, *in vivo* activation of ovalbumin-specific CD8 T cells (OT-I) also showed undetectable levels ($\geqslant$ 99%) of VPAC1 mRNA and protein using an adoptively transferred T_{h1} pathogen mouse model[50]. The mechanism for VPAC1 downregulation in mouse CD4 T cells is through a Src/ZAP70/JNK signaling pathway based on a pharmacological inhibitor study conducted by our laboratory[51]. Importantly, Anderson *et al*[52] recently demonstrated that VIP/VPAC1 signaling potently inhibited G_1/S transition in human CD4 T cells. Thus, VPAC1 signaling appears to block the very signal (TCR activation) that causes its downregulation at the mRNA and protein levels, and might be the reason why many human and rodent T cell leukemia cell lines and human T cell blasts from patients with T cell leukemias have significantly reduced levels of VPAC1 mRNA (see below). After the expansion phase of antigen-specific CD8 T cells, we have collected data in mice that VPAC1 levels are restored in primary, but not secondary memory pools. These data may suggest an interesting possibility that the *in vivo* timing of VIP/VPAC1 signaling during T cell activation or in memory cells can have significant consequences regarding proliferative expansion or recruitment/retention in certain immune compartments based on the number of times exposed to antigen. In sharp contrast to VPAC1, VPAC2 has been termed the inducible VIP receptor, and was shown to become upregulated on T_{h2} cells, but not T_{h1} cells. A cause and effect for VPAC2 upregulation has been confirmed in its ability to protect T_{h2} cells from apoptosis and therefore is a survival factor and promoter of T_{h2} memory cells[53]. We have confirmed that mouse VPAC2 upregulation indeed does not take place in activated antigen-specific CD8 T cells during an *in vivo* T_{h1} pathogen, *Listeria monocytogenes,* infection[50]. This observation supports the idea that VPAC2 is induced against T_{h2}, but not T_{h1}, pathogens. Clinical relevance for VIP to skew towards a T_{h2} lymphocyte lineage is shown by amelioration of T_{h1}-driven autoimmune disorders, including MS[34]. In summary, the timing and location of T cell activation is paramount to whether the VIP/VPAC1 signaling axis modulates the metabolism of the T cell population, as well as, whether VIP/VPAC2 signaling in T_{h2} cells can promote T cell survival.

VPAC receptor expression profile in hematopoiesis

Human hematopoietic stem cells that are enriched for $CD34^+$ cells derived from either bone marrow or cord blood (CB) have been shown to predominately express VPAC1 verses VPAC2 as assessed by semi-quantitative PCR, subtractive hybridization and western analysis[54,55]. Also, the immature, non-dividing $CD34^+CD38^-$ hematopoietic precursors express 4 times greater VPAC1 expression compared to the more mature $CD4^+CD38^+$ population, which contain elevated numbers of colony-forming cells (CFCs). The signaling induced by VPAC1 due to added VIP ligand (10^{-9} mol/L) to these hematopoietic stem precursor cells showed a synergic effect on myeloid and mixed colony growth of $CD34^+$ CB cells with little to no detectable effect on BM cells in the presence, but not absence of three early cytokines, FLT3 ligand, stem cell factor (STF) and thrombopoietin (TPO)[55]. Another study confirmed high levels of VPAC1 mRNA in BM cells, but exogenously added VIP (10^{-13} to 10^{-7} mol/L) instead suppressed erythroid and myeloid colony growth, with a concomitant increase in transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α from an unidentified stromal cell type (possibly macrophages)[54]. These authors concluded that the suppressive activities by VIP/VPAC1 signaling was partly due to the increase in TGF-β and TNF-α as neutralizing antibodies to these cytokines suppressed the effect by VIP. These two studies did in fact validate functional VPAC1 expression in early hematopoietic populations. Their disagreement regarding a positive or negative influence on colony formation of $CD34^+$ cells might be due to deriving these cells from different hematopoietic groups; bone marrow *vs* cord blood. Regardless, it was suggested that microenvironments immediately surrounding nerve endings that supply VIP in bone marrow would best allow for this neuropeptide to alter hematopoietic cellular growth as VIP is readily degraded in serum (10^{-11} mol/L), and the early cytokine signaling is inhibited by serum. Further research is needed to better understand the coordination power of VIP/VPAC1 signaling in the context of different hematopoietic microenvironments.

VPAC receptor expression profile during thymocyte development

It is agreed that peripheral, mature T cells from rodents and humans express higher levels of VPAC1 compared to VPAC2, however, there is some disagreement between their expression profile in developing mouse thymocytes, as well as, a potential species difference between rodents and humans[5,6,12,56-58]. Several labs have measured rat, mouse and human VPAC receptor mRNA by qPCR and RNase protection assays in thymocytes, and all agree on the expression of functionally active VIP receptors in total thymocytes. The discrepancy comes from distinguishing which VIP receptor, VPAC1 or VPAC2 (PAC1 is not expressed), was predominately expressed. Total thymocytes from rat and mouse revealed constitutive VPAC1 levels with increases in VPAC2 only upon TCR activation[5,57]. In contrast, human thymocytes showed greater VPAC2 verses VPAC1 mRNA expression, and TCR activation decreased VPAC1 but not VPAC2 mRNA message[58]. The latter study measured VPAC receptors by qPCR, which may account for the greater sensitivity for VPAC2 in the absence of TCR signaling, but does not explain the higher VPAC2 levels upon T cell activation. Additional studies further fractionated thymocytes into specific groups based on CD4 and CD8 expression. With the exception of rat double negative cells ($CD4^-CD8^-$, DN), double positive ($CD4^+CD8^+$, DP), single positive ($CD4^+CD8^-$, SP4) and SP8 subsets showed readily detectable VPAC1 mRNA message by PCR followed by southern hybridization confirmation[5]. This consistently high VPAC1:VPAC2 ratio in developing rat T cells was opposite for human thymocytes, which showed approximately 4-6-fold more VPAC2 mRNA compared to VPAC1 in DP, SP4 and SP8 subsets as assessed by qPCR[58]. DN cells contained low but equivalent levels of both receptors. These results suggest a species difference during T cell development. Furthermore, definite VIP receptor ratio discrepancies are seen within mouse thymocytes. For example, two studies using Balb/c mouse thymocyte subsets disagreed on the VPAC1:VPAC2 ratios in DN and SP8 thymocyte subsets. In contrast, their data did agree with respect to DP and SP4 subsets that showed higher VPAC2 *vs* VPAC1 mRNA levels[57,59]. These discrepancies can most likely be attributed to PCR primers used and/or experimental conditions such as media culture conditions. Our laboratory has collected data suggesting further discrepancies of VIP receptor expression in mouse thymocytes. Using the C57Bl/6 mouse strain instead of Balb/c mice, we detected more VPAC1 than VPAC2 in total thymocytes and greater VPAC1 than VPAC2 in all four major thymocyte populations: DN, DP, SP4 and SP8, respectively by qPCR (Manuscript submitted). In addition, we further subdivided this population based on CD44, CD25 and CD117 expression called DN1-4 subsets[60] that revealed a fascinating VIP receptor reversal with high VPAC1 mRNA expression found in the earliest T cell progenitor ($CD44^+/CD25^-/CD117^+$, DN1) subset that became transiently silenced in DN2 ($CD44^+/CD25^+$) and DN3 ($CD44^-/CD25^+$) subsets with the concomitant induction of VPAC2 mRNA. DN4 cells showed the restoration back to high VPAC1 and low VPAC2 expression as observed for the later thymocyte populations. It is enticing to speculate that the VIP receptor ratio during T cell development may contribute to a T_{h1} skewing in C57BL/6 mice (high VPAC1:VPAC2) *vs* a T_{h2} skewing in Balb/c mice (low VPAC1:VPAC2). This idea is supported by the VPAC2 transgenic mouse model where forcing the expression of VPAC2 in a C57BL/6 T_{h1} skewed this mouse strain towards a T_{h2} phenotype[61] (see below). Functionally, two reports have shown evidence that VPAC2 mediates IL-2 suppression upon TCR activation in DP cells, and that VPAC2 signaling enhances DP →SP4 differentiation without altering apoptosis, viability, proliferation or cell numbers[57,59]. A third study revealed that VPAC1 signaling was contributing to the protection of spontaneous and glucocorticoid-induced apoptosis[62]. In summary, there appears to be functional VIP receptors expressed on developing thymocytes, but their expression ratio may be species specific, and VIP signaling influences IL-2 expression, differentiation and protection from apoptosis.

Genetically altered VPAC2 mice

While VPAC1 knockout and transgenic mice have not yet been reported, we and others have created VPAC2 knockout and transgenic C57BL/6 mouse strains. Opposite phenotypes were observed for the VPAC2 knockout and transgenic mouse models that provided further evidence for VIP/VPAC2 signaling playing an important role in immune responses[61,63]. Mice that developed in the absence of the immune-inducible VPAC2 receptor demonstrated enhanced delayed type hypersensitivity (DTH), which is mediated primarily by activated T cells and macrophages[63]. In contrast, there was a significant decrease in immediate type hypersensitivity (IH). These mice also demonstrated a polarization toward a T_{h1} response as evidenced by an increase in the T_{h1} cytokine, IFN-γ, and a decrease in T_{h2} cytokines, IL-4 and IL-5 as determined by *ex vivo* experiments of TCR stimulated $VPAC2^{-/-}$ CD4 T cells[63]. In VPAC2 transgenic mice under the control of the LCK promoter, VPAC2 protein was predominately expressed in the helper T cell compartment (25 fold higher in CD4 *vs* CD8 T cells). VPAC2 transgenic mice exhibited a shift in CD4 T cell polarization towards a T_{h2} phenotype as evidenced by (1) a depressed DTH response and an enhanced IH response; (2) an increased number of eosinophils and serum IgE and IgG_1 levels; and (3) higher T_{h2} cytokines, IL-4 and IL-5, and lower T_{h1} cytokine, IFN-γ production by activated CD4 T cells[61]. Thus, VPAC2 significantly modulates CD4 T cell responses. VPAC1 expression levels were consistent with wild type levels in both genetically mutated mouse models. In review, VPAC2 knockout and transgenic mice show a reciprocal differentiation influence towards a

T_{h2} polarization with respect to cytokine expression and delayed-type hypersensitivity through an unknown mechanism. In addition to T_{h2} differentiation, VIP/VPAC2 signaling protects T_{h2}, but not T_{h1}, cells from apoptosis and appears to contribute to this memory cell pool.

VIP$^{-/-}$ *mice and immunity*

The targeted removal of the VIP gene has been engineered[64-68]. A cadre of studies marshaled by James Waschek and other colleagues has focused on pulmonary disorders and asthma. These studies have uncovered an inflammatory component to the VIP$^{-/-}$ knockout mouse. Homozygous VIP$^{-/-}$ knockout mouse have enhanced lymphocyte and eosinophil infiltration into the lung. Moreover, microarray analyses have revealed that lung tissue in the absence of VIP show elevated inflammatory genes representing a chemokine (Ccr6), protease (Mcpt8) and two TNF superfamily members. These data suggest that VIP normally suppresses inflammation in tissues such as lung[68]. Evidence for similar inflammatory exacerbations in VIP$^{-/-}$ mice was observed in gastrointestinal disorders (Crohn's disease) as well[69].

VIP signaling axis and cellular proliferation

The effect on cellular proliferation and cell cycle entry by the VIP signaling axis is complex. In rat neurons, it is well-established that VIP induces proliferation[70,71], whereas it is a potent inhibitor of proliferation in human vascular smooth muscle and CD4 T cells[52,68]. These apparent nonsensical influences toward cellular mitotic control is contributed to by the differential expression of at least three different receptors capable of binding VIP (VPAC1, VPAC2 and PAC1), as well as a fourth called formyl peptide receptor-like 1 (FPRL-1)[43]. Once bound, the ability for these receptors to engage signal transduction cascades is cell-specific as they differentially couple multiple G proteins[17]. VPAC1 expressed on a lymphoblastic T cell line (H9) can transmit alternate internal signals by differentially coupling to $G_{\alpha s}$ or $G_{\alpha i}$ based on whether PHM or VIP binds. However, irrespective of the particular G protein pathway activated, both ligands increased proliferation as assessed by BrdU incorporation[42]. The fact that VIP and PHM evoked different pathways suggests that VPAC1 (and possible other family receptors) can distinguish subtle residue differences in natural ligands thereby tailoring the signaling cascade elicited. In addition to VIP and PHM, activated mast cells and rat basophilic leukemia cell lines secrete a truncated VIP_{10-28} that acts as a potent VPAC1 antagonist, with low VPAC2 binding[11]. Couple this complexity to at least one splice variant of VPAC1, two for VPAC2 and 11 for PAC1, and the ability for the VIP ligand released by neurons innervating an immune organ can have a multitude of functional consequences[72,73]. Unpublished data from our laboratory has identified up to four additional VPAC1 splice variants present in lymphoid and brain cells. The VIP field, therefore, is in its infancy with respect to understanding the biochemical and cellular effects of the VIP signaling axis.

VIP signaling axis and T cell leukemia

VIP signaling is evident in most common types of human cancer, including breast, prostate, lung, and colon[74,75]. These cancer etiologies have been shown to predominately express functional VPAC1, with only the rare human leiomyomas expressing functional VPAC2 receptor. PAC1 receptors are typically expressed in paragangliomas, pheochromocytomas and endometrial carcinomas. Antagonists that inhibit all three VIP receptors have been shown to be effective at suppressing the proliferation of these common cancers, as well as, CNS, melanoma, ovarian and renal tumors and leukemia[35,76]. These reports indicate that VIP and their related peptides enhance the survival and/or promote cellular proliferation in most cancers. In contrast, the VIP receptor(s) responsible for promoting cellular proliferation has not been strenuously studied T cell leukemia. In 1992, Sue O' Dorisio's group showed functional VIP binding sites that evoked increases in i[cAMP] levels in 22 out of a 32 patient cohort diagnosed with ALL of T or B cell origin[77]. The receptor identity, however, was unknown as the VIP receptors had yet to be cloned at the time of this study. Once the VIP receptors had been cloned later in the 1990's, the identity of the VIP receptor(s) expressed in human T cell leukemia blasts could be determined. This research has been conducted primarily utilizing a handful of human leukemic T cell lines, including Stanford University Pediatric (SUP) T1, Molt-4b, Jurkat, Hut-78 and H9 lines (Table 1). Based on these four parent cell lines (H9 is a derivative of Hut-78 cells) there appears to be either high levels of VPAC2 mRNA expression (Sup T1 and Molt 4b with an immature phenotype), or low levels of both VPAC1 and VPAC2 mRNA (Jurkat, Hut-78 and H9 cells with a mature phenotype)[42,78-81]. Our laboratory has verified that CD4^{+}/CD19^{-} cells recovered from biopsied lymph tissue from 2 human T cell leukemia patients also expressed high levels of functional VPAC2 receptor with exceeding low levels of VPAC1 mRNA as assessed by qPCR and cAMP ELISA (manuscript in preparation). In rodent leukemia T cell lines of various etiologies, all cell lines studied exclusively expressed VPAC2, some of which were validated to be functional[57,82-84]. A VPAC2 predominant expression profile in cancer is unusual as VPAC2 expressing tumors are rare[74], and that the expression profile of healthy peripheral lymphocytes express extremely high VPAC1 at both the mRNA and protein levels[45]. Malignant T cells from ALL patients occur due to a blockade in thymocyte development (thymic in origin), or from a blockade in HSC within the bone marrow (prethymic)[85]. These hyperproliferating, low VPAC1:VPAC2 ratio expressing leukemic blasts egress from the thymus and enter the vasculature and bone marrow, where they co-mingle with healthy HSC (CD34^{+}/CD38^{-}) and peripheral mature T

Table 1 Vasoactive intestinal peptide receptor expression in T cell lines

Name	Procedure	Receptor	Ref.
Human T cell lines			
Sup T1	RT-PCR and Northern	VPAC2	Xia *et al*[78] 1996
Molt-4b	q-PCR	VPAC2	Summers *et al*[129] 2003
Jurkat	RT-PCR and q-PCR[1]	Low levels of both	Finch *et al*[80] 1989
Hut-78	RT-PCR and q-PCR[1]	Low levels VPAC1 > VPAC2	Xia *et al*[81] 1996
H9	RT-PCR	VPAC1	Goursaud *et al*[42] 2005
Mouse T cell lines			
EL-4.IL-2	Northern/ RT-PCR	VPAC2	Waschek *et al*[82] 1995, Xin *et al*[57] 1997
MBI-1.15	Northern	VPAC2	Waschek *et al*[82] 1995
BW5147	Northern	VPAC2	Waschek *et al*[82] 1995
CTLL-2	Northern	ND	Waschek *et al*[82] 1995
CTLL-M	Northern	ND	Waschek *et al*[82] 1995
DBA/2	Northern	VPAC2	Waschek *et al*[82] 1995
YAC-1	Northern	ND	Waschek *et al*[82] 1995
F10	Northern	VPAC2	Waschek *et al*[82] 1995
BL/VL3	cAMP	VPAC2	Abello *et al*[83] 1989
NS8	cAMP	VPAC2	Robberecht *et al*[130] 1989
TL-2	cAMP	VPAC2	Robberecht *et al*[130] 1989
D10.TCR31	RT-PCR	VPAC2	Xin *et al*[57] 1997
D10.G4.1	RT-PCR	VPAC2	Xin *et al*[57] 1997
Rat T cell lines			
GK 1.5	RT-PCR	VPAC2	Xin *et al*[57] 1997
3.155	RT-PCR	VPAC2	Xin *et al*[57] 1997

[1]Quantitative polymerase chain reaction (qPCR) is unpublished data from our laboratory. RT-PCR: Reverse transcription-polymerase chain reaction; ND: Not detected. Receptor refers to which vasoactive intestinal peptide receptor is predominantly expressed.

cells that all express high VPAC1:VPAC2 ratios (Figure 4). One possible explanation for a low VPAC1:VPAC2 ratio in human T cell leukemia blasts compared to healthy peripheral T cells, might be due to normally low VPAC1:VPAC2 expression during T cell development as human thymocytes were found to express much higher levels of VPAC2 compared to VPAC1[58]. This altered expression profile in leukemia blasts for the VIP receptor signaling axis could contribute to a growth advantage as VPAC1 is a potent G_1/S transition arrestor by blocking the upregulation of several cyclins, while VPAC2 acts as a survival factor of mouse T_{h2} cells[36,52]. High levels of VIP ligand are also detected in human thymus. It is for these reasons the authors hypothesize that the increased expression of the VPAC2 receptor is a possible diagnostic marker for human (and rodent) T cell ALL. A similar supposition has previously been suggested by Waschek *et al*[82] where they rationalized that a potential molecular switch could take place during healthy T cell development, activation and/or homeostasis to explain the apparent VIP receptor reversal between healthy and leukemic T cells.

There is, however, some discrepancy with our proposal for high VPAC2 and low VPAC1 levels in ALL based on data showing human non-Hodgkin lymphoma patients exclusively expressing VPAC1 in 100% of the

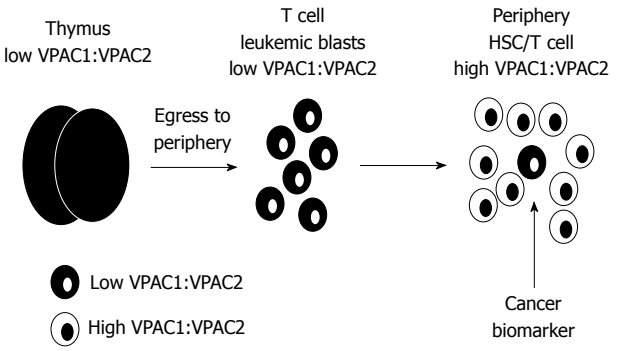

Figure 4 Working hypothetical model for differential vasoactive intestinal peptide receptor expression in T cell acute lymphoblastic leukemia blasts. The radical difference between low vasoactive intestinal peptide receptor (VPAC)1:VPAC2 ratio in developing thymocytes may act as a biomarker and prognostic indicator, readily distinguishable from peripheral hematopoietic stem cells (HSC) and mature T cells that express high VPAC1:VPAC2 ratios.

patient samples tested (6 out of 6)[75]. The authors did not distinguish between T *vs* B cell patients, however. Moreover, this study utilized ligand binding specificity for VIP receptor identification, which may contribute to discordant results compared to reverse transcription-polymerase chain reaction (RT-PCR) gene expression analysis, due to receptor internalization, dimerization and changes in ligand affinity[37]. Another study reported that Hut-78 T cells expressed 75 000 VPAC1 binding sites per cell based on RT-PCR, western analysis and ^{125}I-VIP binding measurements[81]. This high VPAC1 expression level is not consistent with our qPCR data showing < 1% that of healthy CD4 T cells (manuscript in preparation), which have been estimated to have only approximately 15 000 binding sites[14]. Also, H9 cells, a derivative of Hut-78s, were also estimated to have fewer binding sites of approximately 10 000 sites per cell[42]. Therefore, the levels of VPAC1 in Hut-78 T cells in all likelihood are lower than healthy primary T cells, and that the report suggesting they contain high levels of VPAC1 expression (75 000 sites/cell) is perhaps an overestimation.

IKAROS REGULATION OF VPAC1

IK and its role in T cell leukemia

IK is a kruppel-like, zinc-finger transcription factor that functions as a master regulator for the development and maintenance of the hemo-lymphoid compartment[86-88]. The IK gene generates at least 11 isoforms through alternative splicing[89]. All IK isoforms have a common C-terminus containing an activation domain and two zinc fingers that facilitate dimerization with other IK isoforms. All Ikaros protein products (at least 11) differ in their N-terminal domain consisting of 4 zinc-fingers, three of which are necessary to bind DNA[90]. Of the three genetically modified IK mouse models that have been generated[91-93], the more severe model resulted in the complete arrest of fetal and adult lymphocyte development. Importantly, heterozygous mice developed an aggressive lymphoblastic leukemia (100% penetrance) 3 to 6 mo

after birth[94]. In human leukemia patients, several reports have shown mutations in the IK gene[95-98]. More recently, it has been revealed that alternative splicing dysregulation alters the ratio between IK DNA binding to non-DNA binding isoforms[99]. Also, a 2009 study confirms that deletions/mutations in the IK gene is associated with a poor prognosis for B cell ALL patients[100], but interestingly IK mutations occurs a very small percentage of T cell ALL patients (≤ 4%)[101]. These mouse and human data have established IK as a master regulator for lymphopoiesis and an authentic tumor suppressor that sets the threshold for T cell activation, but indicates a species specific difference in Ikaros biology[102].

Mechanisms for transcriptional regulation by IK

Previous investigations in naïve mouse CD4 T cells have shown that IK recognizes at least five different chromatin remodeling and histone-modifying enzyme complexes. Regarding transcriptional permissive complexes, IK has been shown to bind the stimulatory chromatin remodeler, termed switch/sucrose nonfermentable[103]. Regarding transcriptional repressive complexes, IK has been shown to interact with the repressive nucleosome remodeling and deacetylase complex, c-terminal binding protein, c-terminal interacting protein, and mSin3a/b complexes[104-106]. Immunofluorescence staining shows IK protein present in a diffuse reticular nuclear pattern in naïve, non-cycling CD4 T cells. During T cell activation and entry into the cell cycle, IK is redistributed into a donut shaped nuclear pattern that co-localizes with pericentrimeric heterochromatin[86,107]. IK is differentially phosphorylated in a cell cycle dependent manner. The phosphorylation pattern of IK changes as T cells cycle from G_1 to G_2/M phase, and modulates DNA-binding affinity (36). IK is thought to act as an activator or repressor of gene expression based on its subnuclear distribution and binding partner(s) in naïve CD4 T cells[86,108]. There are also differences in basal Ikaros isoform expression levels between resting and activated mouse and human T cells. For example, mouse primary T cells express equivalent protein levels of IK-1 and IK-2 (IK-VI and IK-V based on Sinisa Dovat's nomenclature) irrespective of the activation status of T cells. In contrast, human primary T cells clearly show low levels of IK-1 and the largest known Ikaros isoform, Ikaros-H, that are upregulated upon TCR signaling[109]. Moreover, Sinisa Dovat's group has very nicely demonstrated how phosphorylation by casein kinase Ⅱ and dephosphorylation by PP1 serves to regulate IK's ability to bind DNA, regulate gene expression as well as dictate its subnuclear distribution[110,111].

Identification of Ikaros binding elements in the VIP receptor gene loci

VPAC1 and VPAC2 promoters possess a high frequency of putative Ikaros (IK) binding sites (5'-TGGGAT/A-3'). An inspection of a 5 kb nucleotide sequence of the human VPAC1 and VPAC2 promoters reveal 12 putative IK consensus sequences (5'-TGGGAA/T-3') spanning the transcriptional start site (Figure 5). In comparison, the rhodopsin gene that encodes a group I GPCR has only 1 putative IK binding site over a similar DNA length. Moreover, the entire gene loci of VPAC1 (55 kbp) and VPAC2 (48 kbp), including 10 kb immediately flanking these genes both upstream and downstream, possess 138 and 262 putative IK binding motifs, respectively. The random frequency of any 6 nucleotide DNA sequence being found on both strands of DNA in a 60 kb region is approximately 30 times. Using this random frequency as a comparison, there are 5-fold and 9-fold more IK consensus sequences present at these receptor gene loci. In addition, the IK binding elements are equally distributed throughout both VIP receptor loci with a nearly equal probability of finding an IK sequence on the template (60%) or non-template (40%) strand. Curiously, the 5 kb region mentioned above that spans the transcriptional start site for VPAC1 and VPAC2 has 12/12 (100%) and 8/12 (66%) IK binding motifs that are oriented on the non-template strand, respectively. We propose that this frequency of IK binding motifs preferentially on the non-template DNA strand is not a random event, but rather demarcates a powerful regulatory domain for Ikaros regulation of the VPAC1 and VPAC2 genes.

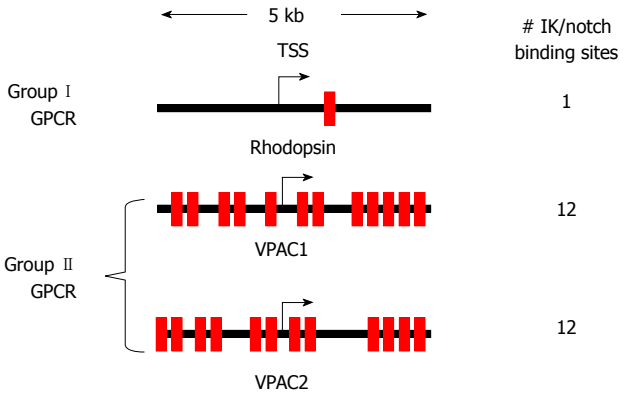

Figure 5 High Frequency IK binding sites at the vasoactive intestinal peptide receptor promoters. Schematic diagram of a 5 kb region for the vasoactive intestinal peptide receptor (VPAC)1 and VPAC2 promoters spanning the transcriptional start site (TSS). Red boxes are IK consensus sequences, with the number of IK binding sites per gene promoter indicated.

IK protein binds to the promoter regions of VPAC1

Evidence for IK protein binding to high-affinity IK-consensus elements in the promoter of VPAC1 is based primarily on electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation assays (ChIP). In 2002, we showed that nuclear protein from human Jurkat T cells, but not WI-38 fibroblasts, produced a retardation signal using a positive IK DNA probe (IKBS4[112]) or the most distal IK site within the VPAC1 promoter[113]. Jurkat protein was supershifted by anti-IK IgG but not a non-specific IgG confirming that IK protein was indeed part of the complex engaging the VPAC1 DNA probe. Moreover, recombinant GST-IK1 and IK2 bound to both probes and were competed away by unlabeled

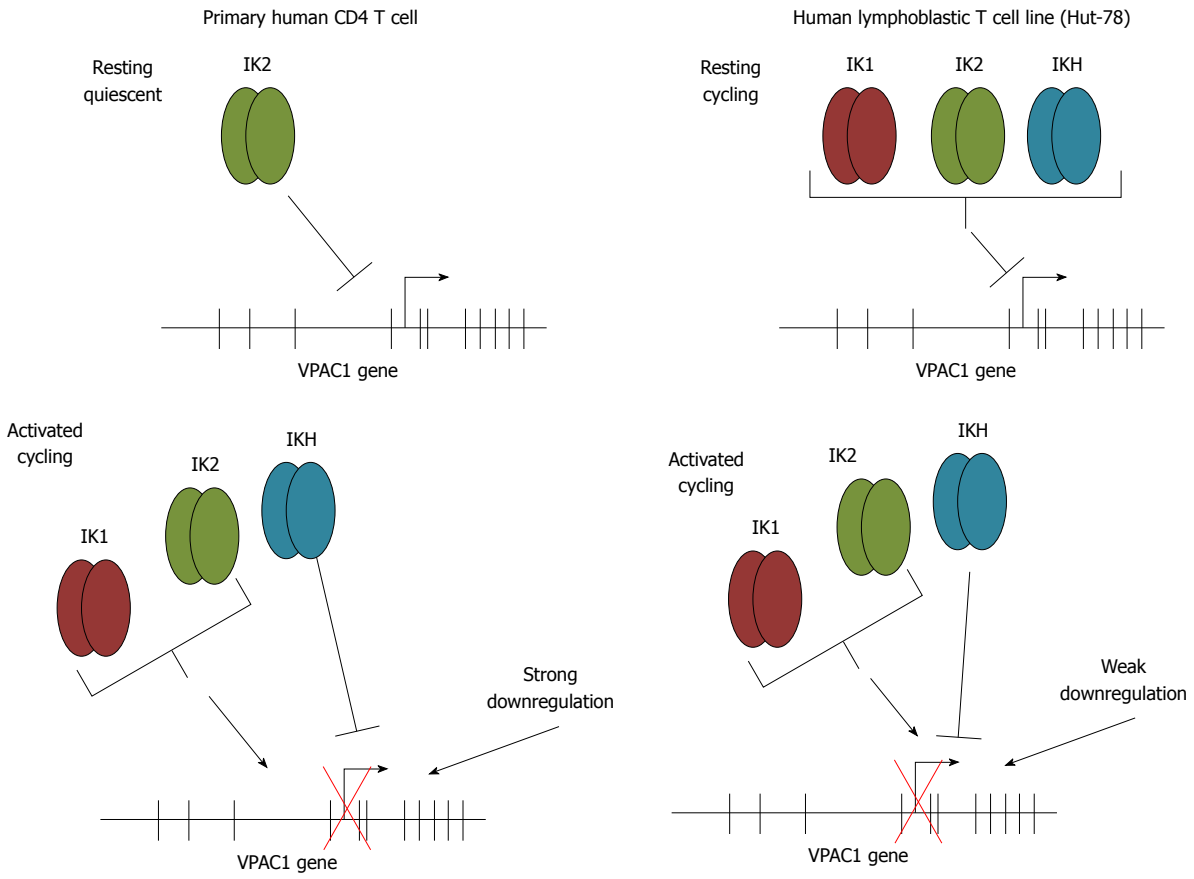

Figure 6 Ikaros engagement of the vasoactive intestinal peptide receptor 1 promoter in primary and T cell lines. Schematic representation of electrophoretic mobility shift assays and chromatin immunoprecipitation assays data comparing Ikaros engagement to the vasoactive intestinal peptide receptor (VPAC)1 promoter in primary CD4 T cells compared to the human lymphoblastic T cell line, Hut-78 cells. Top panels represent the expression profile for Ikaros in resting cells, and the bottom panels represent activated T cells.

probe, further validating that IK protein could positively recognize and bind to the VPAC1 promoter. Subsequent EMSA studies further validated these observations using nuclear protein from human primary T cells. Interestingly, only nuclear extracts from activated CD4 T cells, but not resting cells, showed a retardation signal with the VPAC1 DNA probe. Anti-IK antibody supershifted this signal whereas IgG did not. Collectively, these data reveal that human recombinant and endogenous IK protein from either activated CD4 T cells or from malignant T cell lines can bind to IK-consensus sequences within the VPAC1 promoter in a sequence dependent manner. This observation was confirmed by *in vivo* ChIP assays that allows for a "snap shot" to be taken within a cell to determine protein/DNA interaction by forming reversible cross links with formaldehyde. This study supported the EMSA data revealing VPAC1 DNA amplification of immunoprecipitated chromatin using the anti-IK-CTS pAB, but not anti-IK-H or IgG negative controls[109]. That IK-1 is upregulated during T cell activation supports the notion that IK-1 engages the VPAC1 promoter resulting in its repression. Possible mechanisms to explain the differential binding affinity for IK-2 verses IK-1 dimers might be differences in consensus sequence recognition, subnuclear distribution or post-translational modification pattern changes that renders it not conducive to engage the VPAC1 promoter position in euchromatin (Figure 6)[51].

Ikaros binding causes a functional change in VPAC1 expression

Using a negative IK cellular background of mouse NIH-3T3 cells, overexpression of DNA binding isoforms (IK-1 and IK-2), but not a DNA binding isoform that fails to enter the nucleus (IK-3), or the non-DNA binding isoform (IK-5), significantly downregulated VPAC1 expression by greater than 90% as assessed by qPCR[113]. These decreases in steady-state mRNA were paralleled at the protein level as well (50% decrease). Follow-up studies by our laboratory using a Hut-78 T lymphoblastic cell line background overexpressing the dominant negative IK-5 isoform introduced by nucleoporation resulted in a dramatic 15-fold induction of VPAC1 steady-state mRNA levels (manuscript in preparation, Figure 7). Surprisingly, the IK-2 DNA-binding isoform also increased VPAC1 levels by 2-fold, thus mimicking the positive upregulation that the DN IK-5 isoform displayed, albeit to a lower magnitude. These overexpression studies support a working model where decreasing the net DNA binding potential of the IK pool (IK-5), or altering the homo/heterodimer combination (IK-2, IK-H binds with

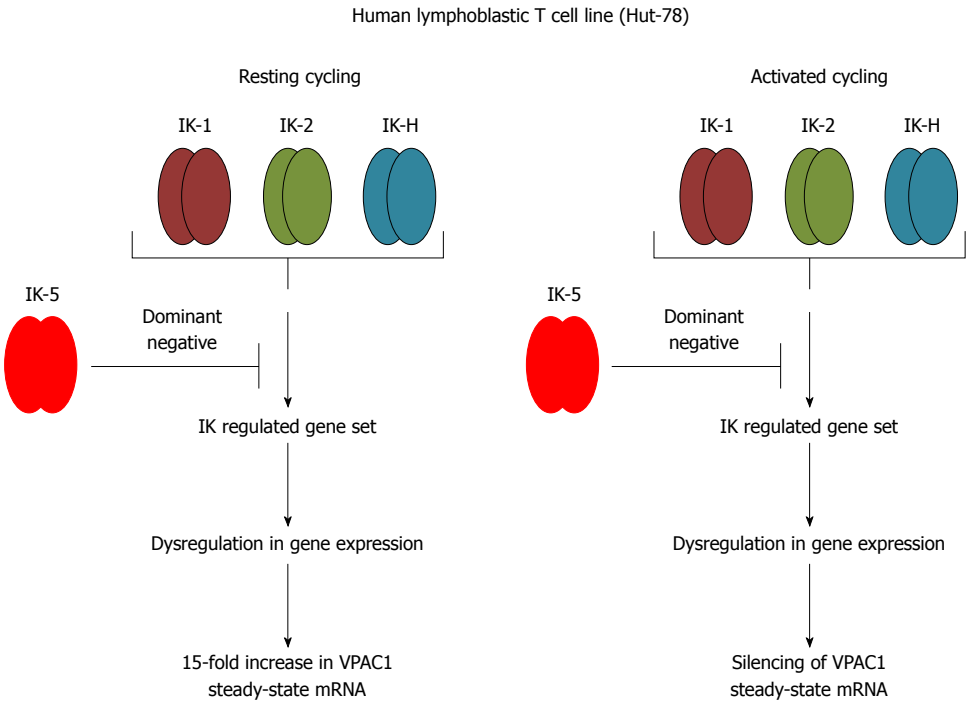

Figure 7 Working model for Ikaros mediated regulation of vasoactive intestinal peptide receptor 1 expression. IK-5 overexpression results in the upregulation of vasoactive intestinal peptide receptor (VPAC)1 steady-state levels in resting Hut-78 cells. The DNA binding activity of Ikaros therefore can alter the expression of growth modulating genes like VPAC1 in a direct (binding to the VPAC1 gene) or indirect mechanism (regulating a repressor/activator that binds the VPAC1 gene). We predict VPAC1 silencing will occur in activated IK-5 overexpressing cells.

greater affinity to IK-2 then IK-1[109]) results in greater IK-1 homodimers and causes elevated VPAC1 steady-state mRNA expression. We have unpublished data demonstrating IK enrichment to the VPAC1 promoter by ChIP assays in activated (PMA/ionomycin), but not resting, human Hut-78 T lymphoma cells could imply that dysregulating IK DNA binding by overexpressing IK isoforms result in increases in VPAC1 expression by an indirect manner. Future studies will investigate how IK dysregulation affects VPAC1 expression during T cell activation. We predict that VPAC1 expression will be silenced without functional IK DNA binding protein, which would support the idea that IK regulates transient gene expression changes and its plasticity upon resolution of T cell activation (memory cells).

FUTURE DIRECTIONS

Elevated VPAC2 expression in the diagnosis and therapeutic intervention of human T cell ALL

There still remains a critical gap in the fundamental knowledge base regarding VIP ligand and receptor expression levels in human T cell ALL. It is becoming readily apparent that functional VPAC2 expression is elevated in rodent and human T cell blasts with an immature phenotype. Unfortunately, there are only a few reports documenting VIP receptor expression in human leukemia, and none to our knowledge have conducted molecular measurements of mRNA and protein expression levels. It will be imperative to collect qPCR and flow cytometry data from a large human T cell ALL cohort to confirm high VPAC2 expression to support its use as a diagnostic tool and/or drug target for this particular leukemia etiology. Moreover, parallel studies showing a concomitant reduction of VPAC1 levels in T cell ALL patient samples would further imply that a low VPAC1:VPAC2 receptor ratio could be utilized as a leukemic indicator for routine diagnosis. Also, the specific VPAC1:VPAC2 receptor signature could be used to follow a cohort of human T cell ALL patients in an attempt to determine the extent to which this receptor ratio can predict patient outcome. Absence of such research will continue to put these human leukemia patients at risk.

Ikaros and Notch regulation of the VIP receptors

The Ikaros transcription factor binds a 6 nucleotide DNA sequence that is identical to the Notch trimer complex[114]. An antagonistic competition between Ikaros (differentiation) and Notch signaling (proliferation) to gain access to DNA binding sites in gene targets is thought to control the T cell developmental plan in the thymus. Gain of function in Notch signaling is observed in 60% of human T cell ALL, which may shift the delicate equilibrium of differentiation/proliferation toward cellular division. Future research to identify whether the Notch DNA binding trimer is actively displacing Ikaros protein from the VPAC receptor loci (and other gene targets) is an important question to answer.

A growth advantage for T cell leukemia blasts with a low VPAC1:VPAC2 ratio

Investigations focusing on how low VPAC1:VPAC2

expression levels alter VIP signaling and whether this chemical information is interpreted by leukemic blasts to initiate a survival/proliferative cellular program is paramount to uncovering future therapeutic drug targets downstream of VIP receptors. Additionally, antagonists and agonists to VIP receptors can be used in combination with other known chemotherapy drugs in an attempt to obtain greater apoptosis induction in leukemia blasts.

Functional significance of VPAC receptor expression in human B cell ALL

Lastly, VIP receptor expression data needs to be collected from B cell ALL patients as Ikaros mutations/deletions have been deleted in 30% of these patients[100]. A decrease in Ikaros protein would increase the relative Notch trimer complex binding to gene targets, including the VIP receptor loci, and again potentially causing a hyperproliferative phenotype. These expression changes in VIP receptors may allow for prognostic prediction and future drug targets downstream of VIP receptors. That VIP and its receptors are also expressed in myeloid and erythroid blood cell lineages, future research focused on these leukemic etiologies is expected to result in important insight in combating these types of human leukemias as well.

CONCLUSION

Numerous studies have demonstrated that a number of human cancers overexpress VIP, or pituitary adenylate cyclase-activating peptide (PACAP) receptors[74,75,80,115]. Interestingly, VIP and PACAP analogs have been shown to affect tumor growth in *in vitro* and *in vivo* animal tumor models, suggesting that these receptors could be used as novel therapeutic targets or for localization of tumors[116-119]. The effect of VIP varies with the type of tumor, by either directly promoting tumor growth[76,120-122], suppressing growth[123], or promoting its differentiation through VPAC1 receptor signaling[124,125]. More recently, VIP has been shown to modulate tumor cell migration[125]. However, the role of the VIP signaling pathway in human leukemia is unknown, and only a few *in vitro* studies have examined the role of this signaling pathway in the survival of leukemic blasts[126]. VIP has been shown to modulate EGFR/HER2[120], VEGF[127,128], FOS expression[48] in breast cancer cell lines. These findings further underscore the importance of this signaling pathway in human cancer and warrants further investigation.

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TOPIC HIGHLIGHT

Sinisa Dovat, MD, DSc, *Series Editor*

Zinc finger structure-function in Ikaros

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Abstract

The zinc finger motif was used as a vehicle for the initial discovery of Ikaros in the context of T-cell differentiation and has been central to all subsequent analyses of Ikaros function. The Ikaros gene is alternately spliced to produce several isoforms that confer diversity of function and consequently have complicated analysis of the function of Ikaros *in vivo*. Key features of Ikaros *in vivo* function are associated with six C2H2 zinc fingers; four of which are alternately incorporated in the production of the various Ikaros isoforms. Although no complete structures are available for the Ikaros protein or any of its family members, considerable evidence has accumulated about the structure of zinc fingers and the role that this structure plays in the functions of the Ikaros family of proteins. This review summarizes the structural aspects of Ikaros zinc fingers, individually, and in tandem to provide a structural context for Ikaros function and to provide a structural basis to inform the design of future experiments with Ikaros and its family members.

Key words: Ikaros; Zinc finger; DNA binding protein; Transcription factor ⅢA; C2H2; Tandem

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INTRODUCTION

The zinc finger motif was used as a vehicle for the initial discovery of Ikaros in the context of T-cell differentiation[1] and has been central to all subsequent analyses of Ikaros function. The C2H2 zinc finger motif was first described in 1985[2] with the discovery of a total of nine tandem zinc fingers in transcription factor ⅢA (TFⅢA) in Xenopus oocytes. A retrospective review of the original discovery, subsequent structural studies, and the application this information in the design of zinc fingers with novel DNA binding functions has recently been provided[3]. Although no complete structures are available for the Ikaros protein or any of its family members, considerable evidence has accumulated about the structure of zinc fingers and the role that this structure plays in the functions of the Ikaros family of proteins.

The Ikaros gene is alternately spliced to produce several isoforms that confer diversity of function and consequently have complicated analysis of the function of Ikaros *in vivo*. The basic architecture of the longest Ikaros isoform [519 amino acids, Gene: IKZF1 (UniProt: gi|3913926), for the full-length human Ikaros] consists of an N-terminal DNA binding domain containing four centrally located C2H2 zinc fingers and a C-terminal protein interaction (dimerization) domain with two additional zinc fingers near the C-terminus of the protein (Figure 1). For purposes of analysis and discussion the zinc fingers have been numbered from N to C: 1-6. The two presumed domains of

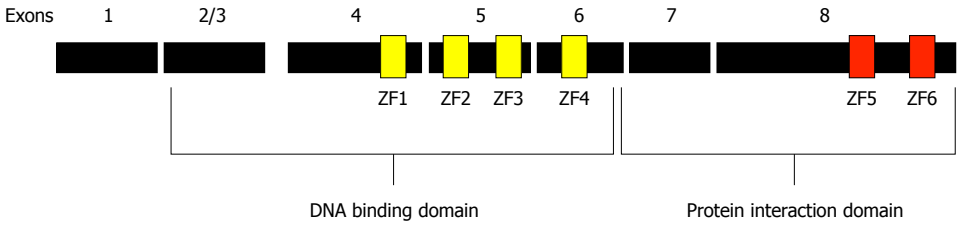

Figure 1 Architecture of the Ikaros protein. The full-length Ikaros is shown in context of the exons present in the longest form of Ikaros. The positions of the six zinc fingers are shown in their approximate locations. Fingers 1-4 are contiguous as well as fingers 5 and 6.

```
HZF1    LKCDICGIICIGPNVLMVHKR--SH   TGERP
HZF2    FQCNQCGASFTQKGNLLRHIK--LH   SGEKP
HZF3    FKCHLCNYACRRRDALTGHLR--TH   SVGKP
HZF4    HKCGYCGRSYKQRSSLEEHKER-CH

HZF5    YKCEHCRVLFLDHVMYTIHMG--CH   GFRDP
HZF6    FECNMCGYHSQDRYEFSSHITRGEH
```

Figure 2 Primary sequence of the six zinc fingers of Ikaros. The sequences of the six zinc fingers of human Ikaros are shown along with their respective linkers (UniProt: gi|3913926). The letters in red are the consensus Cys and His residues that chelate zinc in the fingers. The blue highlighted letters represent the -1, 2, 3, and 6 positions of the finger helices read from left to right.

Ikaros have distinctly different functions and are treated as such here.

ARCHITECTURE OF THE N-TERMINAL DNA BINDING DOMAIN

To understand the role of the C2H2 zinc finger in Ikaros-DNA interactions, we examine the structure of a C2H2 zinc finger peptide, which was engineered based on C2H2 consensus sequences, during interaction with DNA (Figures 2 and 3)[4]. This structure has three tandem zinc fingers bound in the major groove of the DNA. Individual fingers have two antiparallel β sheets folded in on an α helix. In the interior of the fingers two cysteines in the β sheets and two histidines in the helix are coordinated with a zinc that confers considerable rigidity to the structure. Deeper in the interior of the fold are three hydrophobic side chains that are also important in maintaining the structure of the finger. This gives a total of seven characteristic amino acids that are essential for the basic C2H2 zinc finger structure. The residues responsible for the sequence-specific DNA interactions generally occur at locations toward the N-side of the helix (positions -1, 2, 3 and 6, which make the start of the helix). The side chains of amino acids at positions -1, 3 and 6 interact with a triplet of nucleotide bases on one strand of the DNA and the position 2 side chain interacts with a base adjacent to the triplet on the opposite strand of the DNA (see[5] and[3] for detailed descriptions of these interactions). The interactions with the nucleotide bases in this binding arrangement are in the major groove. These patterns have allowed construction of a loose code for the sequence-specific protein-DNA interactions to be developed for C2H2 zinc fingers, although considerable

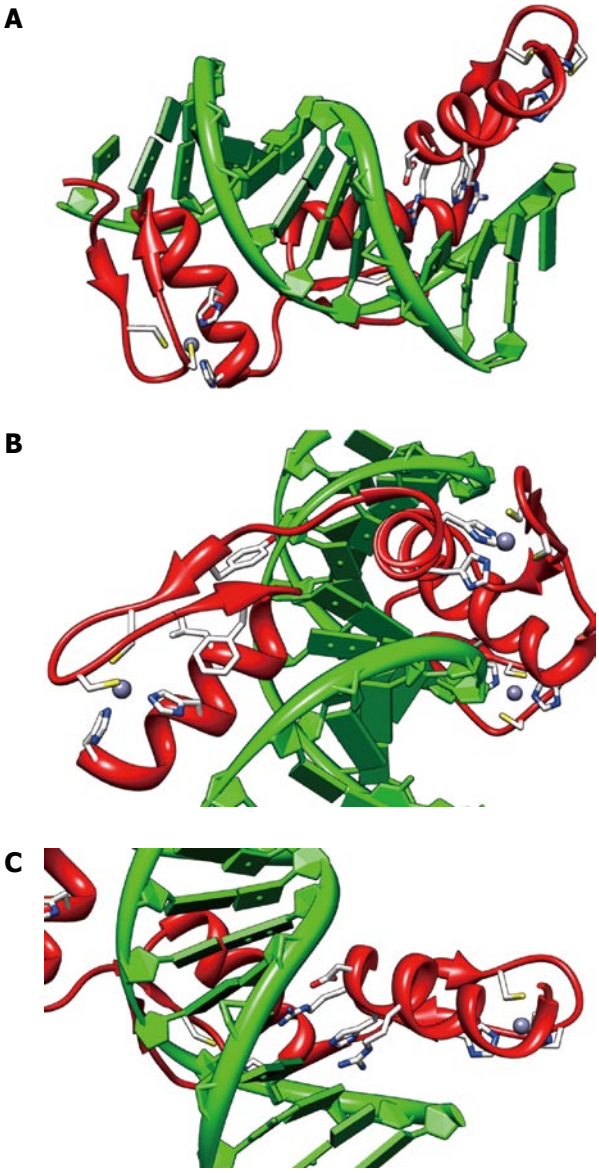

Figure 3 Structure of an engineered peptide with three tandem zinc fingers similar to Ikaros ZF2-3. The views shown are from 1MEY (pdb), an engineered three tandem zinc finger peptide (shown in red) in complex with cognate DNA[4] shown in green. All three fingers show the zinc (grey sphere) complexed to two sulfurs of cysteine (yellow) and two imidazole nitrogen (blue). A: An overview of the zinc fingers nestled in the major groove of the DNA. The N terminus is on the left. The C-terminal finger has the DNA-interacting side chains shown; B: A view of the N-terminal finger showing the seven essential residues for zinc finger structural integrity; C: A view of the DNA-interacting residues on the C-terminal finger, -1:arg, 2:asp, 3:his, 6:arg. The views were produced using CHIMERA[35].

variation has been observed in both natural and engineered zinc finger specificity determinants[6]. This makes the zinc finger code a good starting point for analysis but it clearly has limited explanatory power in individual cases (as with ZF1 and ZF4 in Ikaros).

Numerous studies of both naturally occurring and engineered zinc fingers have shown that modular zinc fingers often appear in tandem separated by short flexible linkers. For a series of two or three tandem fingers, the mode of binding in the major groove described above can easily be maintained. However for four or more tandem fingers in a protein such as Ikaros, the topological constraints imposed by the twist of the B-DNA helix dictates more complex DNA interactions. For short peptides composed of tandem zinc fingers, up to three fingers can follow the major groove of the DNA around the helical axis without introducing undue strain in the DNA other than some underwinding. Several studies have shown that with C2H2 zinc fingers the DNA is slightly under wound to accommodate the zinc finger. This also makes the major groove slightly more open than in B-DNA[6,7]. For four fingers, at least one of the fingers generally binds outside of the canonical binding determinants described above[8,9]. Detailed studies of the zinc-finger domain of the Wilms tumor suppressor protein (WT1, which contains four tandem C2H2 zinc fingers) showed that ZF1 was in the major groove but does not use any of the normal amino acid-base interactions for binding[9]. The conclusion is that ZF1 contributes only to non-specific affinity of WT1 for DNA with the other three zinc fingers that confer sequence-specificity[9]. For larger tandem arrays of six zinc fingers it has been shown that all six can reside in the major groove. However such an arrangement is accompanied by some strain on the DNA and several non-standard DNA-zinc finger interactions[10]. Alternatively, the first and last of a series of zinc fingers can bind to the major groove with intervening fingers placed out of the major groove and interacting primarily with the minor groove. Placing Ikaros in the context of the data from other proteins and synthetic peptides, it is highly likely that at least one of the fingers does not bind in the major groove of the DNA in the classical arrangement. There is strong evidence that the second and third fingers of Ikaros do conform closely to the classical binding mode in the major groove[11] as discussed below. The function and possible DNA binding modes for fingers 1 and 4 are less clear.

The role of the N-terminal tandem zinc fingers (ZF1-ZF4) of Ikaros in targeting pericentromeric heterochromatin has been studied in detail using mutational analysis. Cell lysates from 3T3 cells transfected with mutant Ikaros have been used in gel shift assays with probes derived from multiple Ikaros binding sites to provide a functional assessment[11]. The full-length Ikaros has been shown to tolerate substantial deletions while maintaining DNA targeting behavior. Perhaps due to the natural architecture of Ikaros that supports multiple isoforms, ZF1 and ZF4 are not required for targeting. However, deletions of ZF2 and/or ZF3 and ZF5/ZF6 are not tolerated. To identify the parts of ZF2 and ZF3 that are essential for targeting, several substitution mutants have been generated to replace F2 and/or ZF3 with all or a part of the ZF5 finger (the most divergent in sequence from ZF2 and ZF3). The results showed that the residues most important for DNA binding are located in the region between the second β sheet and the N-terminal half of the α helix in both ZF2 and ZF3. This is the region of the finger that interacts directly with the DNA bases in classic C2H2 fingers. Alanine-scanning mutagenesis has been used to probe further the critical sequences in ZF2 and ZF3. Alanine scanning mutagenesis is coupled with confocal microscopy to assess DNA targeting, and with gel-shift assays to evaluate binding affinity for target DNA sequences. In the substitution and point mutations, gel-shift binding assays have shown high correlation with confocal data, which give confidence to the conclusion that the DNA targeting is dependent on the direct DNA binding of ZF2 and ZF3. To develop a rationale for the role of individual residues of ZF2 and ZF3 in DNA binding, structural models of ZF2 and ZF3 have been produced *via* homology modeling[11]. For ZF2, the essential residues include those located at positions -1, 2, 3 and 6 of the α helix, along with residues that are a part of the hydrophobic interior of the finger as mentioned earlier for the classical C2H2 zinc finger. ZF3 does not conform as closely to the classic C2H2 zinc finger binding pattern. Only position -1 is essential for binding to all DNA probes tested. Positions 2 and 3 give variable binding results, which depends on which probe is used. Position 6 shows no correlation with DNA binding (presumably because it is a glycine with no side chain). Available data and the models of ZF2 and ZF3 suggest that F2 has higher affinity for DNA and also perhaps greater sequence specificity than ZF3, but no direct quantitative DNA-binding affinity data are available to test this hypothesis at present. The roles of ZF1 and ZF4 are still not well understood, although some data suggest that ZF1 contributes to DNA binding affinity for select DNA probes[11].

FUNCTION OF THE LINKERS BETWEEN ZINC FINGERS

A complete picture of the binding of tandem zinc fingers to DNA (as in the case with Ikaros) must include the role of the linkers[12]. The classic linker consensus sequence is TGEKP as observed in TFⅢA and many other proteins with tandem zinc fingers. In fact, the above linker consensus is highly conserved, occurring in several thousand proteins. In the absence of DNA, the linkers are fairly flexible but become much more ordered when flanking zinc fingers are bound to DNA[6]. The linker is important in increasing DNA-binding affinity of tandem zinc fingers but the exact sequence of the linker can vary widely and still serve that function.

The linkers between Ikaros ZF1-ZF2, ZF2-ZF3 and

ZF3-ZF4, have the sequences TGERP, SGEKP and SVGKP respectively. A striking feature of these linkers is that all three have been shown to be phosphorylated in cells arrested at the G2/M boundary of the cell cycle as they enter mitosis[13]. Studies of several mutants, including phosphomimetic mutants with charged residues that mimic phosphorylation at the threonine and serine in position 1 of the linker, indicate that phosphorylation causes Ikaros to dissociate from the pericentromeric heterochromatin[13]. Gel-shift assays of the phosphomimetic mutants have shown that this behavior is correlated with decreased affinity for DNA. Furthermore, the low affinity of Ikaros for DNA that is observed in cells arrested in the G2 stage of the cell cycle is increased dramatically upon treatment with phosphatase[13]. This phosphorylation phenomena has also been observed with sp1, a transcription factor that is also inactivated in G2 arrested cells[14]. This has led Dovat *et al*[13] to propose that phosphorylation of linkers is a fundamental mechanism by which the affinity of tandem zinc fingers for DNA is modulated during mitosis and a potentially important mechanism for the regulation of transcription in general.

The above conclusion is supported by studies of YY1, a widely distributed transcription factor with a C-terminal DNA binding domain containing four tandem zinc fingers[15]. The DNA binding domain of YY1 is phosphorylated in the first position of the linkers between fingers 2 and 3 (both threonine) in addition to a third site at a serine outside of the DNA binding domain. Phosphorylation (or phosphomimetic mutation) at either threonine in the linker dramatically decreases DNA binding and prevents nuclear localization of YY1. The phosphorylated serine outside the DNA binding domain lacks the above effects. Thus YY1 function has also been postulated to be regulated by linker phosphorylation[15].

Insight into the mechanism by which linker phosphorylation causes dissociation from DNA has been provided by a study of the effects of linker phosphorylation on DNA binding affinity using direct measurement of fluorescence anisotropy of fluorescent probes attached to synthetic DNA[16,17]. This method has been used to measure the affinity of all possible phosphorylation states of a synthetic three-zinc-finger protein for a DNA probe[16]. Direct measurements of dissociation constants have confirmed that phosphorylation at the first position of either of the two linkers decreases DNA binding affinity by 30-49-fold while phosphorylation of both linkers produces a 130-fold decrease in affinity, thus supporting the hypothesis that linker phosphorylation can modulate DNA binding affinity *in vivo*. Investigations of the crystal structures of tandem zinc fingers complexed with cognate DNA have revealed that the addition of the phosphate should not actually pose any serious steric problems[16]. Other studies have indicated that the hydroxyl of the threonine or serine at position 1 of the linker forms an H-bond with the last residue of the α-helix of the zinc finger providing a "cap" to the helix[18]. This may also be important in establishing the orientation of the two flanking zinc fingers with respect to each other. Indeed, simply substituting an alanine for the threonine or serine at position 1 of the linker is sufficient to disrupt DNA binding of Ikaros[13], which supports the idea of an important structural role for the hydroxyl of threonine or serine at position 1 of the linker.

Several other phosphorylation sites on Ikaros have been demonstrated to exist outside of the zinc finger regions. These appear to have indirect effects on the DNA-binding affinity of Ikaros[19-21]. A detailed analysis of the interplay between phosphorylation and Ikaros function awaits a complete 3D structure of Ikaros or one of its family members.

FUNCTION OF THE C-TERMINAL ZINC FINGERS

The C-terminal protein interaction (dimerization) domain of Ikaros supports both homodimerization and heterodimerization with close family members[22-24], including Aiolos[25], Helios[24,26-28], Eos[29,30], and Pegasus[29]. Although a single molecule of Ikaros binds DNA as a module, the full biological function of Ikaros requires the C terminus of Ikaros that encompasses zinc fingers 5 and 6. This segment of the protein is both necessary and sufficient for Ikaros dimerization[22]. McCarty *et al*[22] have produced a construct that contains a short N-terminal sequence fused to a 64-amino-acid peptide that contains only fingers 5 and 6 which they have termed the dimerization zinc finger (DZF) domain. This DZF domain can duplicate all the normal dimerization behavior of Ikaros as measured by gel filtration, co-immunoprecipitation and cross-linking. Several other constructs have been made by exchanging zinc fingers 2 and/or 3 for fingers 5 and/or 6. Only constructs with intact zinc fingers 5 and 6 support dimerization. Experiments have been performed with homologs, Hunchback (from Drosophila)[31] and TRPS-1 (from human)[32], both of which homodimerize but do not dimerize with Ikaros[22]. A series of mutants have been produced that identified amino acids critical for binding. Important positions reside largely in the N-terminal portion of the α helices of both F5 and F6 with the selectivity region of Ikaros extending from the C portion of the helix in F5 to the middle of the helix in F6. McCarty *et al*[22] have also been able to produce a chimeric DZF that selectively homodimerizes, which suggests that the DZF comes in direct contact with the corresponding DZF in the binding partner. Homology modeling of the DZF has shown that the critical amino acids cluster on one face of the model. Precisely how the two DZF domains on separate subunits interact remains unknown.

INTERPLAY BETWEEN DNA BINDING AND PROTEIN INTERACTION DOMAINS

With a protein such as Ikaros that has multiple binding partners and phosphorylation sites there is certain to

be linkage between the DNA binding domain and the protein interaction domains with each modulating the respective affinities of the other. A picture of this interplay can be developed from examining a synthetic construct between a zinc finger DNA-binding domain and a leucine zipper protein interaction domain (designated Zif268-GCN4)[33]. This protein selected by phage display techniques is capable of dimerizing with high affinity and binding a bipartite DNA probe that is cognate to the zinc finger domains[33]. This protein has two tandem zinc fingers that bind to DNA as a dimer, which recognizes overlapping DNA sequences on opposite strands of the DNA. The second zinc finger serves as a transition from the DNA binding domain to the protein interaction domain by interacting with both DNA and the other subunit of Zif268-GCN4. A high-affinity probe for Ikaros (BS-4)[34] has wider spacing between the cognate DNA sequences (three base pairs) than Zif268-GCN4. However, binding ZF2 and ZF3 of Ikaros to the BS-4 DNA is likely to place ZF4 in close proximity with the corresponding ZF4 from the interacting Ikaros partner with probably the same orientation as Zif268-GCN4. Thus ZF4 of Ikaros could serve a dual function of promoting DNA binding and the formation of the terniary Ikaros$_2$-DNA complex, providing a crucial linkage between the Ikaros domains.

CONCLUSION

Ikaros and its family members are an interesting study in the application of both the reductionist and the holistic approaches to studying important cellular functions. The ultimate answers to the biological questions are clearly rooted in the fine-tuned molecular interactions such as those discussed here. The zinc finger domains form a functional core around which multiple cellular effects are manifest through the interactions/activities mediated by the remainder of the protein.

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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