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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)<br>Mutation analysis (SSCP and cDNA sequencing) | Allelic loss: 54% (99/182)<br>Homozygous deletions: 8/108[1]<br>Missense point mutations in DBD: 5/108[1]<br>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<br>RNA expression (RT-PCR)<br>Protein expression (Western blotting)<br>Point mutations | LOH for Ikzf1 in 20/37 tumors<br>No or dn transcripts in 9/37 tumors (correlated with absence of Ikaros proteins or presence of dn Ikaros proteins)<br>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)<br>Deletions (Southern)<br>Allelic loss (microsatellite) | 8 DBD point mutations (8/104)<br>3 frame-shift mutations (3/104)<br>27% allelic loss (12/40)<br>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)<br>Point mutations (SSCP and sequencing) | 42% (32/75) of LOH (1 homozygous deletion)<br>1 missense mutation in DBD |
| Kakinuma *et al*[45], 2005 | Mutagen-induced thymic lymphoma | LOH<br>Point mutations | LOH: 2/27<br>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<br>Western blotting | Frame-shift point mutations: 85% (17/20)<br>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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