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# SIGNAL TRANSDUCTION AND REGULATION OF GENE EXPRESSION IN LEUKEMIA*

# TRANSDUKCIJA SIGNALA I REGULACIJA EKSPRESIJE GENA U LEUKEMIJI *

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*Abstract*

The cause and pathogenesis of leukemia is still unknown. Discovery of a novel tumor suppressor, Ikaros, provided new insights into the process of malignant transformation that leads to the development of leukemia as well as the fundamental mechanisms that regulate cell cycle progression, gene expression and chromatin remodeling. In this review, we provide an overview of the function of Ikaros as a regulator of transcription, the role of Ikaros in hematopoiesis and as a tumor suppressor in childhood leukemia, and Ikaros function as a part of the chromatin remodeling machinery. Special emphasis is placed on the role of signal transduction pathways in regulating Ikaros tumor suppressor activity and how the discovery of these signaling pathways are contributing to the development of novel, targeted therapy for leukemia.

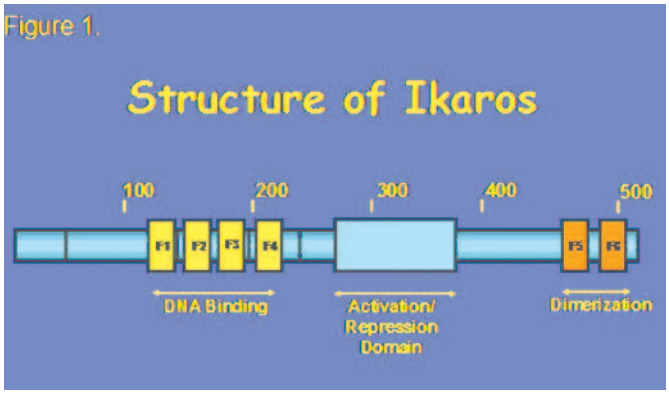
Leukemia is a deadly disease that affects people of all ages. Over the last 60 years, tremendous advances in understanding the mechanisms of malignant transformation and cellular proliferation have led to the development of effective medications and improved survival. Major advances have been achieved in the treatment of childhood acute lymphoblastic leukemia (ALL), the most common form of leukemia in children. However, subsets of high-risk leukemia are still resistant to chemotherapy and associated with high mortality.

The use of next-generation sequencing has identified many genes whose deregulation is associated with the development of leukemia. One of the most important genes whose genetic inactivation is associated with leukemic transformation is Ikaros (ikzf1). Over the last 20 years, extensive studies of the structure and function of this gene have provided insights into the mechanisms by which Ikaros regulates gene expression and exerts its tumor suppressor activity in leukemia. During this period several signal transduction pathways that regulate Ikaros activity were also identified and their effects on Ikaros function have been elucidated. This has led to the design of the drugs that target specific signal transduction pathways in order to enhance Ikaros function as a tumor suppressor. This review will briefly summarize the current knowledge of Ikaros function, with an emphasize on signal transduction regulatory pathways that control its activity in leukemia.

***Structure of Ikaros.*** Ikaros was originally identified through screening an expression library for proteins that interact with an enhancer of the TCR CD3d gene, an early and definitive marker of T cell differentiation[(1)]. The same gene was later found to encode the LyF-1 protein that binds a critical control element in the promoter of the lymphocyte-specific terminal deoxynucleotide transferase (TdT) gene[(2)].

Ikaros homologs have been cloned from various species including trout and *Xenopus*[3], chicken[4] and human[5, 6]. The structure of Ikaros is shown in Fig. 1.

Figure 1.




The Ikaros gene contains two separate zinc finger regions. Four zinc fingers in the amino half of the protein take part in sequence-specific DNA binding[6]. Ikaros binds to purine-rich sequences, and site selection experiments have shown the core recognition sequence to be TGGGAA/T[6, 7]. At the C-terminus of the protein, there are two additional zinc fingers, which do not bind DNA but which are responsible for protein-protein interactions[8]. This region shares homology over ~70 amino acids with the *Drosophila* hunchback gene[1, 2], which is a factor responsible for establishing patterns of gene expression in *Drosophila melanogaster*[9]. Ikaros proteins can form dimers or multimers using the zinc finger domains at the C-terminus to interact with different Ikaros isoforms or Ikaros family members (see below). Between amino acid residues 283 to 362, which are flanked by the two zinc finger domains, is a domain that acts as a transcriptional activator when fused to the DNA binding domain of Gal4[8]. It is not known whether this domain serves as a transcriptional activator in the Ikaros protein.

The Ikaros gene encodes many different isoforms generated by alternative splicing from a single transcript[2, 6, 8, 10]. All isoforms contain the first two coding exons and the last exon, which includes the two C-terminal zinc fingers that take part in protein-protein interactions, but they vary in the number of central coding exons and in the number of DNA binding zinc fingers. DNA binding requires the presence of zinc fingers #2 and #3, but high affinity DNA binding requires in addition either zinc finger #1 or #4[2, 6, 8]. The most common isoforms are: Isoform VI (Ik-1), the largest one, which contains the full length protein, and Isoform V (Ik-2), which lacks exon 3, which contains zinc finger #1. Both isoforms are capable of binding DNA with high affinity, however, their DNase I footprinting patterns vary. In different mouse cell lines and in different stages of mouse lymphocyte development, smaller isoforms, lacking some or all DNA binding zinc fingers have been detected. The level of expression of the small Ikaros isoform varies from small to 5-6 times the amount of the two largest isoforms. The smaller isoforms lack zinc fingers #2 and #3 and do not bind DNA, but they are capable of forming heterodimers with large isoforms via zinc fingers at their C - terminal end. A large number of dimeric or multimeric complexes can be created and each complex may have a different function. Heterodimers between large and small isoforms, bind DNA poorly, which implies that small isoforms can act as dominant negative inhibitors of Ikaros[8]. Homodimers and heterodimers of the large Ikaros isoforms, bind DNA with much higher affinity than Ikaros monomers[8].

***Biological functions of Ikaros.*** Gene disruption experiments have revealed several biological functions of Ikaros:

*Ikaros null knock-out mouse.* Deletion of exon #7 in Ikaros was used to create an Ikaros C-/- knock-out mouse[11]. Exon #7 encodes the C-terminal zinc fingers, as well as a bipartite domain possibly involved in activation. The resulting proteins are functionally inactivated, unstable, and not detectable at the cellular level. This experiment resulted in a true null phenotype for Ikaros protein. In Ikaros C-/- knock-out mice, B cell development is completely impaired. Pro-B cells, pre-B cells, and mature B cells are absent[11]. Development of the thymus in Ikaros C-/- mice is severely delayed with no detectable lymphoid precursors and no cortical or medullary structures until 1 week after birth. During the following 5 weeks, T cell development is evident, but the differentiation pattern is abnormal. The thymus of these mice contains an increased percentage of CD4+CD8– cells, along with a decreased percentage of CD4+CD8+ cells, compared to other cell populations. The overall number of T cells is 50 to 100-fold lower than in wild type mice. There are increased numbers of clonally expanded populations in the thymus of Ikaros C-/- mice. Numbers of T cells in the spleen of C-/- mice is initially low, but increases with age, although the total number of T cells remains below the number in the wild type spleen. Upon stimulation of their TCR complex, T cells from Ikaros C-/- mice show increased proliferation when compared to the wild type. Dendritic epidermal T cells are absent in the spleen and epidermis, but are present in thymus and in vaginal epidermis in Ikaros C-/- mice. Natural killer (NK) cells and thymic dendritic antigen presenting cells (APC) are absent or significantly reduced[12]. The myeloid and erythroid lineages are not affected, although the percentage of granulocytes is lower in Ikaros C-/- mice than in the wild type. These data show that Ikaros is essential for B cell development and that it has an important role in the development of T cells.

*Ikaros dominant negative knock-out mouse.* The dominant negative Ikaros knock-out mouse (DN-/-) was created by deletion of the N-terminal region of the Ikaros gene which encodes the DNA-binding zinc finger domain[13]. These mice are deficient in full length Ikaros protein, but are able to produce Ikaros isoforms containing the protein binding domain, which could act as a dominant negative mutants, affecting the function of other Ikaros-associated proteins, which under physiological conditions, bind Ikaros through the C-terminal zinc finger domain. The phenotype of dominant negative Ikaros knock-out mice (DN-/-) is

severe. Ikaros DN-/- mice are born with the expected frequency, but they failed to thrive. The majority of them die between the first and third weeks of their lives. They have a rudimentary thymus with no T cell precursors. Peripheral lymphoid centers, dendritic epidermal T cells and NK cells are absent. The bone marrow of Ikaros DN-/- mice contain a 10-fold lower number of cells when compared to the wild type. No B cells are detected in the bone marrow and the CD45R+ population is completely absent. In mutant mice older than 3 weeks an increased proportion of myeloid vs. erythroid cells is observed when compared to the wild type. Mature polymorphonuclear cells are absent in the bone marrow of the mutant mice, although peripheral blood contains numerous mature granulocytes. The spleen of mutant mice is enlarged. Myeloid cells predominate, consisting of up to 60% of all cells in spleen as compared to 5% of wild type. In soft agar clonogenic assays, mixed granulocyte and macrophage colonies are established 10-fold more frequently when spleen cells of mutant mice are grown on soft agar in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) than when wild type spleen cells are used. This suggests an increased capacity for proliferative response to GM-CSF stimulation by Ikaros DN-/- myeloid cells. These studies show that the impaired protein-protein interactions between Ikaros and Ikaros associated proteins due to the presence of dominant negative mutants in the absence of Ikaros results in a more severe phenotype, than the simple absence of Ikaros. They demonstrate that Ikaros is necessary for normal T and B cell development. The role of Ikaros during myeloid development in mouse remains unknown.

*Ikaros heterozygous dominant negative knock-out mouse.* Thymocytes and splenocytes of 1-month-old mice heterozygous for the Ikaros DNA-binding mutation (ND +/-) show increased proliferative response to T cell receptor (TCR) stimulation when compared to wild type[14]. After 3 months of age all of the Ikaros DN +/- mice develop lymphadenopathy with enlarged spleen and thymus and the presence of circulating lymphoblasts in the peripheral blood. Analysis of the malignant lymphoblasts shows that they are all T cells expressing TCR, they are clonal in nature, and that the site of malignant transformation is the thymus. The malignant cells show the loss of Ikaros heterozygosity with only a mutant, truncated allele being expressed in malignant cells. These data suggest that the proper expression of Ikaros protein is necessary for normal T cell development. It also raised the possibility that Ikaros might act as a tumor suppressor gene, since the malignant transformation was preceded by the loss of the normal Ikaros allele. It also implies that other Ikaros-associated proteins might act as tumor suppressor genes, since malignant transformation occurred only in DN+/- mice, but not in Ikaros C-/- mice which do not express dominant negative mutant isoforms.

*Additional murine Ikaros knock-out models.* Another *Ikaros*-targeted mouse line had the β−galactosidase (βgal) reporter gene inserted in-frame into exon 2 that is present in all known Ikaros isoforms. Bone marrow cells of these mice produced very low levels of Ikaros proteins[15]. The mice displayed a partial block at the pro-B stage of B cell differentiation. Mature B cells in these mice showed a lower activation threshold and a hyperproliferative response to antigen stimulation. These mice also had extensive deficiencies in myeloid differentiation.

**These experiments established Ikaros as a primary regulator of lymphoid development and suggested that it is involved in the regulation of lymphocyte activation and tumor suppression.**

In humans, altered expression of Ikaros isoforms has been associated with the development of different malignancies including childhood ALL [16-18] infant T-cell ALL [19], adult B cell ALL [20], myelodysplastic syndrome (MDS) [21], AML [22], and adult and juvenile CML [23]. These data show an association between the loss of Ikaros function and the development of human leukemia. Aberrant expression of small dominant negative Ikaros isoforms has been associated with development of human pituitary adenomas [24]. The current hypothesis is that small Ikaros isoforms act as dominant negative mutants in human cells and their overexpression promotes malignant transformation. The full-length Ikaros most likely acts as a tumor suppressor gene in human cells, although little is known of the molecular mechanisms by which Ikaros controls cellular proliferation and the cell cycle.

## *Biochemical and cellular functions of Ikaros*

***Ikaros DNA binding is sequence-specific.*** The DNA binding activity of Ikaros has been studied using purified Ikaros protein, as well as in vivo in murine and in human cells. The use of electromobility shift assay (EMSA), as well as global chromatin immunoprecipitation followed with next-generation sequencing (ChIP-SEQ) have established that Ikaros binds DNA at specific DNA sequences. Further experiments have identified the "core" Ikaros binding sequence as either 5'-GGAA-3' or 5'-GGGA-3'. DNA-binding studies using different DNA probes demonstrated that Ikaros binds with high affinity to two types of sequences: 1) a single consensus sequence 5'-TGGGAA/T-3' or 2) sequence that contains two 5'GGGA-3' motifs that are separated by 2-40 base pairs (5'-GGGA-3'-2-40 bp-5'-GGGA-3'). These sequences thus constitute the "high-affinity Ikaros binding consensus sites." [25]

Ikaros binds DNA poorly as a monomer. Deletion of the C-terminal, protein-interaction domain results in a 50-fold reduction in Ikaros' DNA binding affinity. These data strongly suggest that Ikaros binds DNA as a dimer. This explains the ability of Ikaros to form heterodimers with its own isoforms as well as with other family members. Although several experiments suggest that Ikaros can also bind DNA as a multimer, more data are necessary to determine how common this event is in vivo.

**Ikaros exhibits a specific subcellular localization with predominance at pericentromeric heterochromatin (PC-HC)** The distribution of Ikaros proteins in the nucleus of B lymphocytes in mouse has been studied by immunofluores-

cence confocal microscopy(26). Antibodies against the N-terminus and C-terminus of Ikaros proteins showed the presence of 8 to 20 foci of intense staining within the nuclei. In order to study Ikaros co-localization within chromatin, a combined immuno-FISH approach, which allows the simultaneous detection of protein and DNA in nucleus, was used. In all cells, Ikaros protein co-localized with pericentromeric regions of the chromosome. The Immuno-FISH approach was used to identify the position of different developmentally regulated genes in relation to the Ikaros-centromere clusters at different stages of B cell development. Transcriptionally inactive genes were shown to be selectively associated with Ikaros foci in pericentromeric regions, while transcriptionally active genes were not associated with Ikaros complexes. Genes that are differentially regulated during B cell maturations showed a correlation between expression status and Ikaros association. In cells where these genes were not expressed, the genes were associated with Ikaros complexes. In contrast, in cells where these genes were expressed, they did not associate with Ikaros in pericentromeric regions. These data suggest that Ikaros can regulate its target genes by recruitment to pericentromeric heterochromatin resulting in their transcriptional repression. These studies revealed that Ikaros localization to pericentromeric heterochromatin is an essential part of its function.

### *Ikaros associated with chromatin remodeling complexes*

The above data strongly suggested that Ikaros represses transcription of its target genes via chromatin remodeling. Additional studies further supported the role of Ikaros in gene silencing. Ikaros associates with histone deacetylase (HDAC)-containing complexes (NuRD and Sin3) [27], although HDAC-independent gene repression via binding to the transcriptional corepressors, CtBP and CtIP, has been reported as well [28, 29]. Ikaros directly interacts with the NuRD complex, ATPase Mi-2b, and with Sin3 through both its N-terminal and C-terminal regions [27, 30], while interaction with the CtBP corepressor is achieved through amino acids at the N-terminal region [28].

Despite extensive data demonstrating the role of Ikaros in gene silencing, it has been observed that Ikaros can activate transcription of its target genes. A mechanistic explanation for Ikaros role in gene activation via chromatin remodeling was revealed by the demonstration of a physical association between Ikaros and Brg-1, a catalytic subunit of the SWI/SNF nucleosome remodeling complex that acts as an activator of gene expression [30, 31]. This provided a rationale for Ikaros role as transcriptional activator. The current hypothesis is that Ikaros binds the upstream region of target genes and aids in their recruitment to PC-HC, resulting in repression or activation of the gene [26, 32]. Thus, Ikaros can act both as an activator and a repressor, depending on whether it associates with the NuRD, the CtBP or the SWI/SNF complex. The discovery that human pericentromeric heterochromatin contains an area that confers transcriptional activation provided further support for this hypothesis.

### *Regulation of Ikaros activity by phosphorylation*

Ikaros is abundantly expressed during the various stages of the cell cycle, as well as during different stages of hematopoiesis. Given that Ikaros subcellular localization and function varies during the cell cycle, and that Ikaros functions differently in lymphopoiesis than in erythropoiesis or myelopoiesis, it became obvious that differential Ikaros function is not regulated by the amount of Ikaros protein in the cell, but rather by posttranslational modifications of Ikaros protein.

The phosphorylation of Ikaros is the posttranslational modification that has been most extensively studies. The first report provided evidence for the cell cycle-specific phosphorylation of Ikaros during mitosis[33]. The phosphorylation sites were located at the linker that connects DNA-binding zinc finger units of Ikaros. Functional analyses of the effect of phosphorylation using phosphoresistant mutants (where phosphorylation sites are mutated to mimic constitutive dephosphorylation) or phosphomimetic mutants (where phosphorylation sites are mutated to mimic constitutive phosphorylation) have demonstrated that the cell-cycle specific phosphorylation of Ikaros regulates its DNA binding activity and pericentromeric localization. Further analysis showed that mitosis-specific phosphorylation at the linker region that connects zinc finger motifs is not restricted to Ikaros, but rather represents a global mitotic control mechanism that regulates cell cycle-specific DNA binding of C2H2 zinc finger proteins[33].

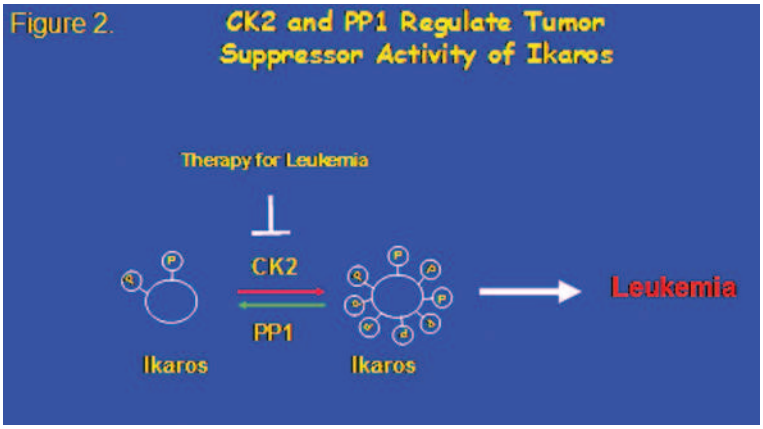
The above data suggest that phosphorylation can play an important role in the regulation of Ikaros function. Further studies showed that multiple phosphorylation sites on the Ikaros protein are directly phosphorylated by Casein Kinase II (CK2), and that the CK2 pathway regulates Ikaros function in normal and malignant cells. A total of 11 amino acids on Ikaros protein were demonstrated to be directly phosphorylated by CK2. An initial report showed that CK2-mediated phosphorylation of Ikaros inhibits its ability to regulate G1/S progression of the cell cycle[34]. A more specific role for the phosphorylation of individual amino acids was provided in subsequent reports, which demonstrated that phosphorylation of amino acids #13 and #294 regulate Ikaros binding to the upstream regions of its target genes, as well as to probes that are derived from pericentromeric heterochromatin[35]. Functional analyses using phosphomimetic and phosphoresistant Ikaros mutants showed that phosphorylation of amino acids #13 or #294 regulate subcellular localization of Ikaros to pericentromeric heterochromatin[35]. Additional data provided evidence that CK2-mediated phosphorylation regulates Ikaros DNA-binding affinity toward its target genes during the S phase of the cell cycle in human hematopoietic cells[36].

New insights into the role of phosphorylation in the regulation of Ikaros function was provided with the discovery that Ikaros physically interacts with and is directly dephosphorylated by Protein Phosphatase 1 (PP1)[37]. PP1 is considered to be a tumor suppressor, and can be localized both to the cytoplasm and the nucleus. The C-terminal end of the

Ikaros protein contains a consensus PP1 interaction site. Point mutation of this PP1 interaction site abolishes in vitro and in vivo interaction between Ikaros and PP1 and results in hyperphosphorylated Ikaros protein. Functional analyses of Ikaros mutants that have point mutations in the PP1 interaction domain provided evidence that: 1) A large majority of Ikaros protein undergoes phosphorylation by CK2 kinase, followed by dephosphorylation by PP1. Thus, there is a constant balance between CK2 and PP1 activity that determines the phosphorylation status of Ikaros and consequently its activity, and 2) Dephosphorylation by PP1 is essential for Ikaros DNA-binding ability, as well as for its pericentromeric localization. The unexpected discovery was that a lack of Ikaros-PP1 interaction results in a reduced half-life of the Ikaros protein due to increased degradation via the ubiquitin pathway. The introduction of phosphoresistant mutations at CK2 phosphorylation sites on the Ikaros protein restored the stability of the Ikaros protein, and prolonged its half-life(37). These results provided evidence that the function, as well as the stability of the Ikaros protein, is regulated by a balance in the activity of CK2 and PP1[38].

CK2 is a pro-oncogenic kinase, and the increased activity and/or overexpression of CK2 has been associated with various types of malignancies in humans, as well as in animal models. The discovery that CK2 inhibits the function of Ikaros led to the hypothesis that CK2 exerts its pro-oncogenic activity in leukemia by inhibiting the tumor suppressor function of Ikaros. Several experiments provided support for this hypothesis – inhibition of CK2 activity with CK2-specific inhibitors restored Ikaros binding ability toward its target genes and this was demonstrated using multiple assays[37]. Thus, the current working hypothesis is that increased activity of CK2 results in hyperphosphorylation of Ikaros, the consequent loss of its tumor suppressor function, and the development of leukemia[39, 40] (Figure 2.).

Figure 2.




According to this hypothesis, inhibitors of CK2 would be potent anti-leukemia medications, and they would act by restoring the tumor suppressor function of Ikaros in leukemia. Additional experiments are needed to confirm this hypothesis and are clearly warranted.

In conclusion, the discovery of the Ikaros tumor suppressor and its role in the regulation of gene expression and chromatin remodeling in hematopoiesis and leukemia have provided novel insights into the mechanisms that regulate proliferation and differentiation of hematopoietic cells. The elucidation of the role of CK2 and PP1 in the regulation of Ikaros activity established the role of these signaling pathways in hematopoiesis and leukemia, and also provide a rationale for the development of new anti-leukemia medications – inhibitors of CK2. In vitro and in vivo testing to fully assess the therapeutic efficacy of CK2 inhibitors as a new treatment for leukemia is expected in the near future.

*Apstrakt*

Uzrok i patogeneza leukemija su još uvek nepoznati. Otkriće novog tumor supresora, Ikaros omogućilo je nova saznanja o procesu maligne transformacije nastanka leukemije, kao i fundamentalnih mehanizama koji regulišu ćelijski ciklus, ekspresiju gena i funkciju hromatina. Ovaj pregled obuhvata naučna saznanja o fukciji Ikarosa kao regulatora transkripcije, hematopoeze, tumor supresora u pedijatrijskoj leukemiji i kao regulatora hromatina. Posebni osvrt je na ulozi signalnih puteva koji regulišu funkciju Ikarosa u supresiji tumora i kako su otkrića signalnih puteva dovela do razvoja nove, eksperimentalne terapije za leukemiju.

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