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Ikaros in T-Cell Leukemia

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1. Introduction

Although extensive clinical data have established that the loss of Ikaros tumor suppressor activity *via* genetic inactivation is a major contributor to leukemogenesis leading to B-cell ALL, less is known about the role of Ikaros in T-cell leukemia. The T-cell malignancies observed in Ikaros-deficient mice suggest that Ikaros is likely to function as a tumor suppressor in T-cells. In human, multiple studies have identified genetic inactivation of Ikaros (deletion or mutation) to be associated with ~5% of T-ALL cases. Additional studies provide evidence that the functional inactivation of Ikaros due to defects in signal pathways that normally regulate Ikaros activity is likely to be a critical factor in the development of T-cell malignancies. These studies provide a rationale for new chemotherapeutic strategies in the treatment of T-cell leukemia. In this chapter, we summarize the newest advances that shed light on the role of Ikaros in T-cell leukemia.

2. Structure and function of Ikaros

The *Ikaros* gene, discovered independently by K. Georgopoulos and S. Smale, encodes multiple Ikaros protein isoforms *via* alternate splicing (Lo et al. 1991; Georgopoulos et al. 1992; Hahm et al. 1994). The structure of Ikaros protein reveals several known structural motifs corresponding to distinct functional domains as described below.

2.1 Molecular structure of Ikaros

2.1.1 DNA-binding domain

The N-terminal region of the Ikaros protein contains four zinc finger motifs (Fig. 1). Three of these exhibit a typical C2H2 structure with two cysteines and two histidines covalently bound to a zinc atom, while the fourth zinc finger has a CCHC structure. The four N terminal zinc fingers in the Ikaros protein function in DNA binding. Point mutational analysis revealed that zinc fingers #2 and #3 are essential for the DNA-binding function of Ikaros, as well as its localization to pericentromeric heterochromatin (see 2.2.1). Based on DNA-se footprint analysis, it has been suggested that the first zinc finger contributes to DNA binding specificity. The role of the fourth zinc finger in DNA-binding remains unknown, although it has been noted that Ikaros isoforms that lack the fourth zinc finger exhibit a unique expression pattern during hematopoiesis (Payne et al. 2003). Recently, a point mutation in a single allele of the fourth zinc finger has been associated with primary immunodeficiency and pancytopenia in humans (Goldman, et al. In press.).

2.1.2 Dimerization domain

The C-terminal region of the Ikaros protein contains two zinc finger motifs (Fig. 1). These zinc fingers do not bind DNA, but mediate protein-protein interactions with other Ikaros isoforms, and/or with Ikaros family proteins (Helios, Aiolos, etc.) that share the same motif (Sun et al. 1996). Every described Ikaros isoform contains the two C terminal zinc fingers, thus allowing for the formation of diverse Ikaros dimers with the potential for unique and specific functions. Ikaros binds DNA as a dimer, which underscores the importance of this domain for Ikaros function.

2.1.3 Bipartite activation-repression domain

All of the Ikaros proteins share a bipartite activation domain, that is adjacent to the two C-terminal zinc fingers (Fig. 1). This domain is responsible for stimulating basal levels of transcription of Ikaros target genes (Sun et al. 1996; Georgopoulos et al. 1997). Within the bipartite activation domain, there are two distinct stretches of amino acids – acidic and hydrophobic. It has been established that acidic amino acids are responsible for transcriptional activation, while the hydrophobic amino acids do not exhibit such an effect (Sun et al. 1996). However, the presence of the hydrophobic amino acids next to the acidic stretch increases their transcriptional activation activity, suggesting that the hydrophobic residues have a functional role in transcriptional regulation (Sun et al. 1996; Georgopoulos et al. 1997).

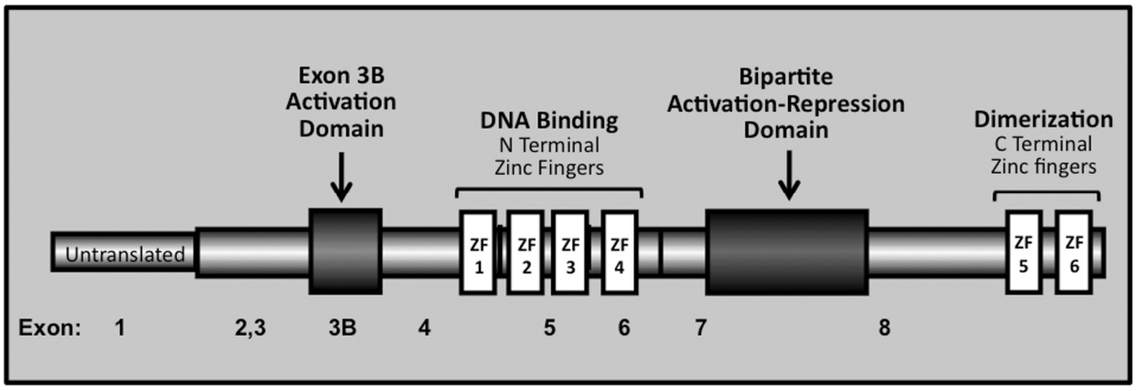


Fig. 1. Major structural features of the Ikaros protein. The *Ikaros* gene (*Ikzf1)* includes eight coding exons. Exon 1 is untranslated and was not identified in initial reports. Current Genbank sequences do not identify Exon 3B which encodes the 20 amino acid activation domain.

2.1.4 Exon 3B activation domain

Exon 3B of the *Ikaros* gene encodes a short, 20-amino acid stretch in the N region of the Ikaros protein (Fig. 1). This domain has been described (Hahm et al. 1994; Sun et al. 1999; Payne et al. 2001) and functionally analyzed in human Ikaros (Ronni et al. 2007). This domain has been shown to take part in determining the DNA-binding specificity of the Ikaros protein complex and in regulating the expression of Ikaros target genes (Ronni et al. 2007).

We would like to emphasize that there are functionally significant domains of the Ikaros protein that are as yet unidentified. It is known that Ikaros can act as a transcriptional repressor and that it has a role in chromatin remodeling (see 2.2.2). However, the structures

and/or particular amino acids within the Ikaros protein that are responsible for these functions remain unknown. Further studies are needed to provide more detailed maps of the functional domains within the Ikaros protein.

2.2 Ikaros function in regulating gene expression

2.2.1 Ikaros regulates gene expression *via* chromatin remodeling

Ikaros has been shown to regulate transcription by binding DNA at the upstream regulatory elements of its target genes (Georgopoulos et al. 1992; Ernst et al. 1993; Hahm et al. 1994; Molnar and Georgopoulos 1994). Thus, DNA binding is essential for Ikaros function. While Ikaros can directly activate or repress its target genes, subsequent experiments established that Ikaros function as a regulator of gene expression occurs *via* its role in chromatin remodeling. Studies by A. Fisher's group demonstrated that Ikaros is abundantly localized in pericentromeric heterochromatin in cells (Brown et al. 1997). In pericentromeric regions, transcriptionally inactive genes have been shown to selectively associate with Ikaros foci, while transcriptionally active genes do not (Brown et al. 1997). The expression status of several genes that are differentially expressed during T and B cell maturation correlate with their association with Ikaros. Mutations in Ikaros binding sites interferes with the developmentally-regulated shut-down of the λ5 gene (Sabbattini et al. 2001), as well as the gene that encodes TdT *(dntt)* (Trinh et al. 2001) during lymphoid differentiation. These data further support a role for Ikaros in gene silencing *via* chromatin remodeling. Experiments by Georgopoulos' group also demonstrated that Ikaros regulates expression of its target genes by recruiting them to pericentromeric heterochromatin, but that recruitment to pericentromeric heterochromatin can lead to activation of the target genes (Koipally et al. 2002). Studies in human T-cells revealed that Ikaros binding to human pericentromeric heterochromatin is regulated in a complex way by the association of different Ikaros isoforms. A model has been proposed whereby Ikaros regulates expression of its target genes by recruiting them to pericentromeric heterochromatin leading to either their activation or repression, and that switching from repression to activation depends on the presence of particular Ikaros isoforms in the Ikaros DNA-binding complex (Ronni et al. 2007; Kim et al. 2009). **Thus, the current hypothesis is that Ikaros binds the upstream region of target genes and aids in their recruitment to pericentromeric heterochromatin, resulting in repression or activation of the target gene (Brown et al. 1997; Liberg et al. 2003).**

2.2.2 Molecular mechanisms of chromatin remodeling and gene regulation by Ikaros

Ikaros associates with histone deacetylase (HDAC)-containing complexes (NuRD and Sin3A and Sin3B) (Koipally et al. 1999). Ikaros directly interacts with the NuRD complex ATPase, Mi-2β, and with Sin3A and Sin3B through both its N-terminal and C-terminal regions (Kim et al. 1999; Koipally et al. 1999). The histone deacetylase complex acts as a transcriptional repressor *via* chromatin remodeling. It has been hypothesized that Ikaros recruits histone deacetylase complex to the upstream regulatory elements of its target genes resulting in **chromatin remodeling and repression** of the target genes (Brown et al. 1997; Liberg et al. 2003).

Ikaros has been shown to interact with the CtBP corepressor *in vivo*. This interaction with CtBP is achieved through amino acids at the N-terminal region of Ikaros (Koipally and Georgopoulos 2000). The Ikaros-CtBP complex acts as a transcriptional repressor. This

repression is HDAC-independent, and represents **an additional means by which Ikaros represses transcription** of its target genes (Koipally and Georgopoulos 2000).
Ikaros also associates with Brg-1, a catalytic subunit of the SWI/SNF nucleosome remodeling complex that acts as an activator of gene expression (Kim et al. 1999; O'Neill et al. 2000). It has been hypothesized that Ikaros recruits the SWI/SNF nucleosome remodeling complex to the upstream regions of its target genes (in a similar fashion to the NuRD complex) resulting in **chromatin remodeling and activation** of its target genes. **Thus, Ikaros can act as either an activator or a repressor of its target genes, depending on whether it associates with the NuRD, the CtBP or the SWI/SNF complex.**

3. Ikaros in T-cell development

The role of Ikaros in normal T-cell development is demonstrated by evidence that Ikaros regulates the expression of key genes in T-cell differentiation and by the impaired T-cell differentiation observed in Ikaros-defficient mice.

3.1 Ikaros regulates genes critical for the development of T-cells (*TdT*)

The role of Ikaros in regulating expression of the gene encoding terminal deoxynucleotide transferase (*TdT*) during thymocyte differentiation has been extensively studied (Ernst et al. 1993; Ernst et al. 1996; Trinh et al. 2001; Su et al. 2005). Ikaros has been shown to bind *in vivo* to the D′ upstream regulatory element of the *TdT* (*dntt*) gene. This region contains a perfect Ikaros consensus DNA-binding site, and mutational analysis has demonstrated that the presence of this sequence is essential for Ikaros binding to the upstream regulatory element of the *TdT* gene. This same region contains a consensus binding site that is bound *in vivo* by Elf-1, a member of the Ets family of transcription factors. The binding of Elf-1 and Ikaros have been shown to be mutually exclusive. Ikaros binding to the *TdT* upstream regulatory element leads to repositioning of the *TdT* gene to pericentromeric heterochromatin and to repression of *TdT* transcription. Elf-1, in contrast, acts as positive regulator of *TdT* expression. Thus, Ikaros and Elf-1 compete for occupancy of the same upstream control region during thymocyte development and have the opposite effect on *TdT* expression (Trinh et al. 2001). In CD4+CD8+ thymocytes, the upstream region of *TdT* is occupied by Elf-1 leading to expression of the *TdT* gene. During the induction of T-cell differentiation, Ikaros displaces Elf-1 from binding the upstream regulatory element of *TdT*, resulting in the loss of *TdT* expression. The absence of Ikaros results in failure to repress *TdT* expression during thymocyte differentiation leading to impaired T-cell development. The exact molecular mechanisms by which Ikaros displaces Elf-1 during thymocyte differentiation remains to be determined, although there is compelling evidence that the reversible phosphorylation of Ikaros (see 6.1 and 6.2 below) is responsible for this regulation.

3.2 Ikaros regulation of CD4, CD8, and IL-2 expression

During T-cell development, Ikaros has been demonstrated to bind to the regulatory element of the CD8α gene *in vivo* by chromatin immunoprecipitation (ChIP) assay. Ikaros has been hypothesized to positively regulate expression of the CD8α gene during thymocyte differentiation (Harker et al. 2002). This has been supported by evidence that Ikaros-defficient mice have decreased numbers of CD8+ T-cells (see 3.3 below).

Studies by Georgopoulos' group demonstrated that Ikaros binds *in vivo* to a silencer that is located in the first intron of the *Cd4* gene resulting in the suppression of *Cd4* transcription. Further analysis revealed that Ikaros can bind concommitantly with the Mi-2β chromatin remodeler leading to suppression of the Cd4 silencer and upregulation of *Cd4* gene expression. The Ikaros-Mi2β complex aids in recruitment of histone acetyl transferases. Thus Ikaros appeaars to be able to both activate or repress expression of *Cd4 via* chromatin remodeling (Naito et al. 2007).

In mature CD4+ cells, Ikaros was shown to bind *in vivo* to the upstream regulatory element of the *IL-2* gene. Further analysis demonstrated that Ikaros represses expression of the *IL-2* gene in mature CD4+ cells *via* chromatin remodeling. This established Ikaros as a regulator of anergy induction in CD4+ T-cells (Thomas et al. 2007).

3.3 T-cell development in Ikaros-defficient mice

Studies of Ikaros-deficient mice established Ikaros as a master regulator of T-cell development and identified several biological functions of Ikaros:

1. Thymocytes that lack Ikaros expression progress from the double negative (DN) CD4– CD8– to the double positive (DP) CD4+CD8+ stage in the absence of β selection (Wang et al. 1996). The proliferative response of thymocytes following TCR β ligation is ~9 fold higher in thymocytes from Ikaros null mice, compared to the wild-type. Thus, Ikaros has been identified as a key regulator of the β selection checkpoint in T-cell differentiation and a regulator of thymocyte expansion following β selection (Winandy et al. 1999).
2. Thymocytes from Ikaros-deficient mice downregulate CD8 to become CD4 single positive (SP) thymocytes without positive selection signals that are normally required for differentiation from the DP to the SP stage (Winandy et al. 1999; Urban and Winandy 2004). Ikaros has also been shown to regulate negative selection that occurs during the DP stage (Urban and Winandy 2004). T-cell production in these mice is skewed toward the production of CD4 T-cells. Thus, Ikaros functions as a regulator that controls the TCR selection checkpoint in T-cell development, as well as a regulator of CD4 versus CD8 T-cell fate decisions (Wang et al. 1996; Winandy et al. 1999; Urban and Winandy 2004).
3. Ikaros-deficient T-cells from all three Ikaros knock-out mice strains possess a lower activation threshold than normal T-cells and enter the cell cycle at an accelerated pace (Avitahl et al. 1999; Winandy et al. 1999). Thus, Ikaros sets the activation threshold in mature T-cells and regulates cell cycle progression.

4. Ikaros and T-cell leukemia

4.1 Ikaros-deficient mice develop T-cell leukemia

The Ikaros-defficient mouse described above clearly established that Ikaros has an essential role in T-cell development. Subsequent analysis of heterozygous Ikaros-defficient mice revealed that Ikaros has tumor suppressor activity. Between the second and third month of age, these mice display an aberration in thymic differentiation with an accumulation of triple-positive thymocytes that have intermediate expression levels of CD4, CD8, and the TCR complex (Winandy et al. 1995). At the same time, a polyclonal expansion of mature T lymphocytes occurs in the spleen of these mice. Proliferation

assays established that thymocytes from heterozygous Ikaros-defficient mice have hyperproliferative potential, and that they can proliferate in the absence of TCR stimulation. It is worth noting that these changes precede the malignant transformation of thymocytes described below (Winandy et al. 1995).

After 3 month of age, all of the Ikaros-defficient heterozygous mice develop T-cell leukemia and lymphoma (Winandy et al. 1995). This is manifested by severe generalized lymphadenopathy and splenomegaly, along with an increased number of malignant lymphoblasts in the peripheral blood. Flow cytometry analysis of malignant lymphoblasts established that they are monoclonal in origin, and that malignancy arises in the thymus. The expression analysis of maliganT-cells revealed that the wild type Ikaros copy was lost in these cells, thus they have a loss of Ikaros heterozygosity (Winandy et al. 1995).

An additional Ikaros-defficient biological model provided evidence for the loss of Ikaros tumor suppressor activity in T-cell leukemia. This *Ikaros*-targeted mouse ($IK^{L/L}$) had the β-galactosidase (βgal) reporter gene inserted in-frame into exon 2 that is present in all known Ikaros isoforms. These mice produce very low levels of Ikaros proteins (Kirstetter et al. 2002; Dumortier et al. 2003; Dumortier et al. 2006). The $IK^{L/L}$ mice exhibit T lineage defects that are identical to those reported in Ikaros null mice including a lowered threshold to activation stimuli and the invariable development of thymic tumors.

These findings strongly suggested that Ikaros acts as a tumor suppressor for T-cell leukemia. They also provide support for the hypothesis that Ikaros regulates normal thymocyte differentiation, and controls the proliferation of thymocytes and mature T-cells in response to TCR signaling.

Further studies directly addressed the question of whether normal Ikaros function is essential and sufficient to induce tumor suppression of T-cell leukemia. The re-introduction of Ikaros *via* retroviral insertion into T leukemia cells that were derived from Ikaros-defficient mice resulted in their cessation of growth (Kathrein et al. 2005). Expression of Ikaros in T-cell leukemia induced T-cell differentiation that was characterized by increased expression of CD4, CD69, CD5, and TCR. These data suggested that Ikaros tumor suppressor activity involves positive regulation of normal thymocyte differentiation, and that a potential mechanism for malignant transformation of Ikaros-defficient thymocytes involves failure of T-cell differentiation.

The induction of T-cell differentiatiom following re-introduction of Ikaros was accompanied by induction of cell cycle arrest at the G0/G1 stage of the cell cycle (Kathrein et al. 2005). The exact mechanism by which Ikaros induces cell cycle arrest remains unknown, although it has been observed that the induction of Ikaros expression in leukemia cells correlates with the increased expression of the cell cycle-dependent kinase inhibitor $p27^{kip1}$ (Kathrein et al. 2005). One possibility is that Ikaros affects global chromatin remodeling, since restoration of Ikaros activity in T-cell leukemia correlates with a global increase in histone H3 acetylation (Kathrein et al. 2005).

These complementary studies established Ikaros as a *bona fide* tumor suppressor in T-cell leukemia and demonstrate that the lack of Ikaros is the major causative factor of T-cell malignancy in Ikaros-deficient mice.

4.2 Ikaros deficiency in human T-cell leukemia

Since the discovery of the *Ikaros* gene and the identification of its function as a master regulator of lymphocyte differentiation and a tumor suppressor in mice, a number of studies

have examined human leukemia samples to determine if an alteration of Ikaros' function is associated with the development of hematopoietic malignancies in humans. Increased expression of dominant-negative Ikaros isoforms has been associated with a variety of hematopoietic malignancies in humans. These include childhood ALL (Kuiper et al. 2007; Mullighan et al. 2007; Mullighan et al. 2008; Dovat and Payne 2010; Marcais et al.), adult B cell ALL (Nakase et al. 2000), myelodysplastic syndrome (Crescenzi et al. 2004), AML (Yagi et al. 2002), and adult and juvenile CML (Nakayama et al. 1999). Deletion of an Ikaros allele was detected in over 80% of BCR-ABL1 ALL and the deletion or mutation of Ikaros has been identified as a poor prognostic marker for childhood ALL (Mullighan et al. 2007; Mullighan et al. 2008; Martinelli et al. 2009; Martinelli et al. 2009; Martinelli et al. 2009; Mullighan et al. 2009). These data established Ikaros as a major tumor suppressor in human leukemia. The most compelling data supporting the tumor suppressor activity of Ikaros in human hematopoietic malignancies was established for B-cell ALL.

In T-cell ALL, the initial studies produced somewhat conflicting data. The first report described expression of dominant-negative Ikaros isoforms (using Western blot and RT-PCR) in all 18 pediatric T-cell ALL patients that were studied (Sun et al. 1999), suggesting a strong correlation of the loss of Ikaros function with the development of T-cell ALL. However, subsequent studies on a total of 14 patients with T-cell ALL (both adult and pediatric) did not detect the presence of dominant-negative Ikaros isoforms (using Western blot and RT-PCR) in T-cell ALL (Nakase et al. 2000; Ruiz et al. 2004), although one study detected an association of the expression of a dominant-negative isoform of the Ikaros-family member – Helios with T-cell ALL (Nakase et al. 2002).

More comprehensive studies that utilized high-resolution CGH-arrays in a total of 81 patients, detected deletion of one copy of Ikaros in 5% of T-cell ALL patients (Kuiper et al. 2007; Maser et al. 2007; Mullighan et al. 2008). The most recent study of 25 cases of human T-cell ALL, that combined Western blot, CGH-array analysis, and sequencing of Ikaros cDNA following RT-PCR, provided a more complete view of the relation of Ikaros and T-cell ALL. This study detected one patient (4%) in which one Ikaros allele had been deleted, while the Ikaros protein produced by the other intact allele exhibited a loss of nuclear localization with an abnormal localization in the cytoplasmic structure (Marcais et al. 2010). This study provided the first definitive functional evidence of the complete loss of Ikaros function and T-cell ALL in humans.

In summary, studies in human T-cell ALL have demonstrated that genetic inactivation of the *Ikaros* gene by deletion or mutation does occur in human T-cell ALL in at least 5% of cases. Although Ikaros deletion is not as frequent an event in T-cell ALL when compared to B-cell ALL (30%) or BCR-ABL1 ALL (80%), it is a notable cause of T-cell ALL, and needs to be tested in newly diagnosed patients with this disease. The prognostic significance of Ikaros deletion in T-cell ALL remains to be determined. Studies also suggest that functional inactivation of Ikaros plays a significant role in T-cell ALL, although the mechanisms by which Ikaros function is impaired in T-cell ALL is still unknown. Recent findings discussed below provide insights into signal transduction pathways that potentially affect Ikaros tumor suppressor function in T-cell ALL.

5. Mechanisms of Ikaros tumor suppressor activity

The mechanisms of Ikaros tumor suppressor activity are not well understood. One major obstacle is the paucity of known Ikaros target genes. The best evidence for the mechanisms

of tumor suppression by Ikaros in T-cell ALL comes from studies that identified the role of Ikaros in the Notch pathway.

5.1 Ikaros-mediated repression of the downstream effectors of the Notch pathway (Deltex and Hes1)

The Notch pathway is essential for normal T-cell differentiation. However, activation of the Notch 1 gene has been found in over 50% of T-ALLs (Weng et al. 2004) and T-ALL leukemia cells often express the Notch target genes Hes-1 and pTα (Chiaramonte et al. 2005). The intracelular domain of $Notch^{IC}$ forms a complex with the Notch transcription factor RBP-Jk/CSL and the cofactor Mastermind. This complex activates expression of Notch pathway target genes. Studies by S. Chen's group demonstrated that the Notch pathway is activated in the T-cell leukemia that develops in Ikaros-defficient mice (Dumortier et al. 2006). Additional analysis showed that Ikaros directly downregulates a Notch target gene, Hes-1. Ikaros directly competes with CSL for binding to the upstream regulator element of Hes-1 (Kleinmann et al. 2008). Since CSL acts as a stimulator of transcription, and Ikaros represses transcription of Hes-1, Ikaros counteracts the pro-oncogenic Notch signaling in T-cell ALL. Repression of Hes-1 by Ikaros likely involves chromatin remodeling since Ikaros binding to the upstream regulatory region of Hes-1 leads to a decrease in histone H3 acetylation (Kathrein et al. 2008), and Ikaros-defficient mice have reduced trimethylation of histone H3 at the K27 residue (Kleinmann et al. 2008).

A link between Ikaros deficiency and Notch activation in T-cell ALL had been suggested by Beverly and Capobianco (Beverly and Capobianco 2003). They found synergism between Notch activation and the inactivation of Ikaros in T-cell leukemogenesis. Sequence analysis of the consensus binding sequence for CSL and Ikaros revealed remarkable similarities and the authors hypothesized that Ikaros may interfere with CSL binding and Notch signaling (Beverly and Capobianco 2003).

Additional experiments showed that Ikaros represses another target gene of the Notch signaling pathway – Deltex1 (Kathrein et al. 2008). Similarly to the regulation of Hes-1, Ikaros competes with CSL for binding to the upstream regulatory region of Deltex1 and represses expression of Deltex1 by chromatin remodeling. Ikaros binding to the upstream region of Deltex1 also results in decreased histone H3 acetylation (Kathrein et al. 2008).

These data strongly suggest that one of the mechanisms by which Ikaros suppresses leukemogenesis in T-cells involves inhibition of the Notch signal transduction pathway.

5.2 Additional mechanisms of Ikaros tumor suppressor activity in T-cell ALL

One possible explanation for why the lack of Ikaros function leads to leukemia is the fact that Ikaros regulates expression of several genes that are essential for normal T-cell development (section 3 above). Often, malignant transformation is characterized by a failure (or arrest) of normal differentiation. Thus, the absence of Ikaros activity and its subsequent impact on T-cell differentiation is likely to be an important step toward the development of leukemia.

It has been demonstrated that Ikaros can negatively regulate cell cycle progression at the G1/S transition (Gomez-del Arco et al. 2004), thus the absence of Ikaros would impair the G1/S check point in the regulation of the cell cycle.

Several reports suggested that Ikaros downregulates $Bcl\text{-}x_L$ expression (Yagi et al. 2002; Ezzat et al. 2006; Kano et al. 2008). Thus, Ikaros might regulate apoptosis, and the lack of

Ikaros activity would have an oncogenic effect similar to the overexpression of Bcl2 in chronic lymphocytic leukemia (CLL). This would also lead to the development of leukemia that is more highly resistance to chemotherapy.
In summary, multiple mechanisms including the loss of Notch pathway inhibition, blocked T-cell differentiation and impaired cell cycle control are likely to play a role in the T-cell leukemogenesis that occurs with the loss of Ikaros activity. However, the specific mechanisms by which Ikaros exerts its tumor suppressor activity remain largely unknown. Identification of additional genes that are regulated by Ikaros will provide more insight on this important process.

6. Regulation of Ikaros' function in T-cell leukemia

6.1 Phosphorylation of Ikaros by CK2 kinase inactivates the Ikaros protein

Despite the fact that Ikaros plays a critical role in regulating T-cell proliferation and differentiation, the level of Ikaros expression remains high throughout the cell cycle and during lymphocyte differentiation. This suggests that Ikaros function is regulated by posttranslational modifications. The role of phosphorylation in regulating Ikaros function has been studied the most extensively. Studies that identified mitosis-specific hyperphopshorylation of Ikaros at an evolutionarily conserved linker sequence, provided evidence that the cell cycle-specific phosphorylation of Ikaros regulates its DNA-binding ability and nuclear localization during mitosis (Dovat et al. 2002). This provided the first evidence that phosphorylation can regulate Ikaros function in cells.
Subsequent studies demonstrated that Ikaros is a direct substrate of Casein Kinase II (CK2) and that CK2-mediated phosphorylation regulates multiple functions of Ikaros in normal and leukemia cells:
Experiments performed by Georgopoulos' group identified several amino acids located in the C-terminal region of the Ikaros protein that are directly phoshorylated by CK2. Mutational analysis of phosphoacceptor sites revealed that CK2-mediated phosphorylation regulates Ikaros' ability to control cell cycle progression during the G1/S transition.
Studies by Dovat's group identified additional phosphorylation sites in the N-terminal region of the Ikaros protein that are phosphorylated by CK2 (Gurel et al. 2008). Experiments with Ikaros phosphomimetic mutants (that mimic constitutive phosphorylation) and phosphoresistant mutants (that mimic constitutive dephosphorylation) revealed that CK2-mediated phosphorylation of two amino acids located in the N-terminal region of Ikaros controls two essential functions of Ikaros: 1) Ikaros' DNA-binding activity and 2) Ikaros' subcellular localization to pericentromeric heterochromatin (Gurel et al. 2008). Increased phosphorylation of Ikaros by CK2 results in severely decreased DNA-binding affinity and the loss of pericentromeric localization, thus **CK2-mediated phosphorylation leads to inactivation of Ikaros function** (Gurel et al. 2008).

6.2 CK2-mediated phosphorylation controls Ikaros function in T-cell differentiation

The significance of CK2-mediated phosphorylation for normal T-cell development was underscored by the discovery that Ikaraos in CD4+CD8+ thymocytes is phosphorylated at multiple sites by CK2 (Gurel et al. 2008). As described above, in thymocytes, Ikaros binds to the upstream regulatory region of its target gene, *TdT,* leading to repression of *TdT* transcription. Phosphorylation of Ikaros in CD4+CD8+ thymocytes by CK2 decreases its

DNA-binding affinity toward the upstream regulatory element of *TdT*, which results in occupancy of this region by the transcription factor Elf-1 and expression of *TdT*. During induction of thymocyte differentiation, Ikaros undergoes dephosphorylation at amino acids #13 and #294 (Gurel et al. 2008). Differentiation-specific dephosphorylation of Ikaros results in its increased DNA-binding affinity toward the upstream regulatory element of *TdT*, displacement of Elf-1, and repression of *TdT* transcription. These data demonstrate that the regulation of Ikaros phosphorylation by CK2 plays an important role in T-cell differentiation and suggest that increased activity of CK2 kinase in thymocytes would lead to impaired T-cell development due to interference with normal Ikaros function.

6.3 CK2-mediated phosphorylation leads to ubiquitination and degradation of the Ikaros protein

Further functional analysis of Ikaros phosphorylation revealed that CK2-mediated phosphorylation occurs at PEST sequences in the Ikaros protein. PEST sequences are comprised of a region that is rich in Proline (P), Gluamate (E), Serine (S) and Threonine (T). It has been demonstrated that phosophorylation of the serines or threonines located within the PEST sequence typically results in increased degradation of the respective protein. Ikaros contains two PEST sequences that are phosphorylated *in vivo* by CK2 (Popescu et al. 2009). Phosphorylation at PEST sequences leads to decreased half-life and increased degradation of Ikaros, resulting in a low level of Ikaros protein in cells. Hyperphoshorylated Ikaros undergoes polyubiquitination, which leads to its degradation *via* the ubiquitin/ proteasome pathway (Popescu et al. 2009).

6.4 Dephosphorylation of Ikaros by PP1 opposes CK2-mediated phosphorylation

The discovery that Ikaros is a direct substrate for Protein Phosphatase 1 (PP1) (Popescu et al. 2009), a well-known tumor suppressor protein, provided additional evidence that phosphorylation plays an extremely important role in the regulation of Ikaros activity in normal hematopoiesis and in leukemia. Ikaros interacts with PP1 directly by binding *via* a PP1 consensus recognition motif that is located near the C-terminal zinc fingers. Dephosphorylation of Ikaros by PP1 is essential for preservation of Ikaros DNA-binding activity and subcelular localization to pericentromeric heterochromatin (Popescu et al. 2009). Mutated Ikaros protein that is unable to interact with PP1 undergoes accelerated degradation *via* the ubiquitin pathway. This results in a 5- to 10-fold decrease in the level of Ikaros protein in cells, when compared to wild type Ikaros (Popescu et al. 2009). Thus, PP1-mediated dephosphorylation of Ikaros counteracts the effect of CK2-mediated phosphorylation of Ikaros. This provides evidence that two major signal transduction pathways – one involving CK2 and the other PP1 – converge on the Ikaros protein (Fig. 2).
These pathways exert opposite effects on Ikaros function and thus regulate T-cell differentiation and proliferation.
Both CK2 and PP1 proteins are known to play a critical role in malignant transformation. Increased expression of CK2 during T-cell differentiation results in the development of T-cell leukemia in mice (Seldin and Leder 1995). Furthermore, CK2 activity is elevated in many other types of human malignancies (Phan-Dinh-Tuy et al. 1985; Pinna 1997; Roig et al. 1999; Kim et al. 2007). The activity of CK2 and PP1 is closely tied to the ability of cells to proliferate, and thus the balance of their function is essential to prevent malignant transformation. Both CK2 and PP1 exerts strong effects on multiple functions of Ikaros – its

role in T-cell differentiation, ability to regulate cell cycle progression, DNA-binding affinity, chromatin remodeling capability (by controlling its subcellular localization to pericentromeric heterochromatin), regulation of gene expression, and protein stability. This suggests that the pro-oncogenic activity of CK2 involves inactivation of the *Ikaros* gene, while the tumor suppressor activity of PP1 is mediated by preserving the tumor suppressor function of Ikaros. These data also strongly suggest that in leukemia cells that have increased activity of CK2, but no deletion/mutation in the *Ikaros* gene, the Ikaros protein that is present is unlikely to be a functionally active Ikaros protein with tumor suppressor activity. Instead, recent discoveries suggest that the functional inactivation of Ikaros by CK2-mediated hyperphopshorylation might be one important mechanism leading to T-cell leukemia. This provides new insight into the role of Ikaros, CK2, and PP1 in T-cell leukemia, and identifies CK2 kinase as a potential target for novel chemotherapy for T-cell ALL.

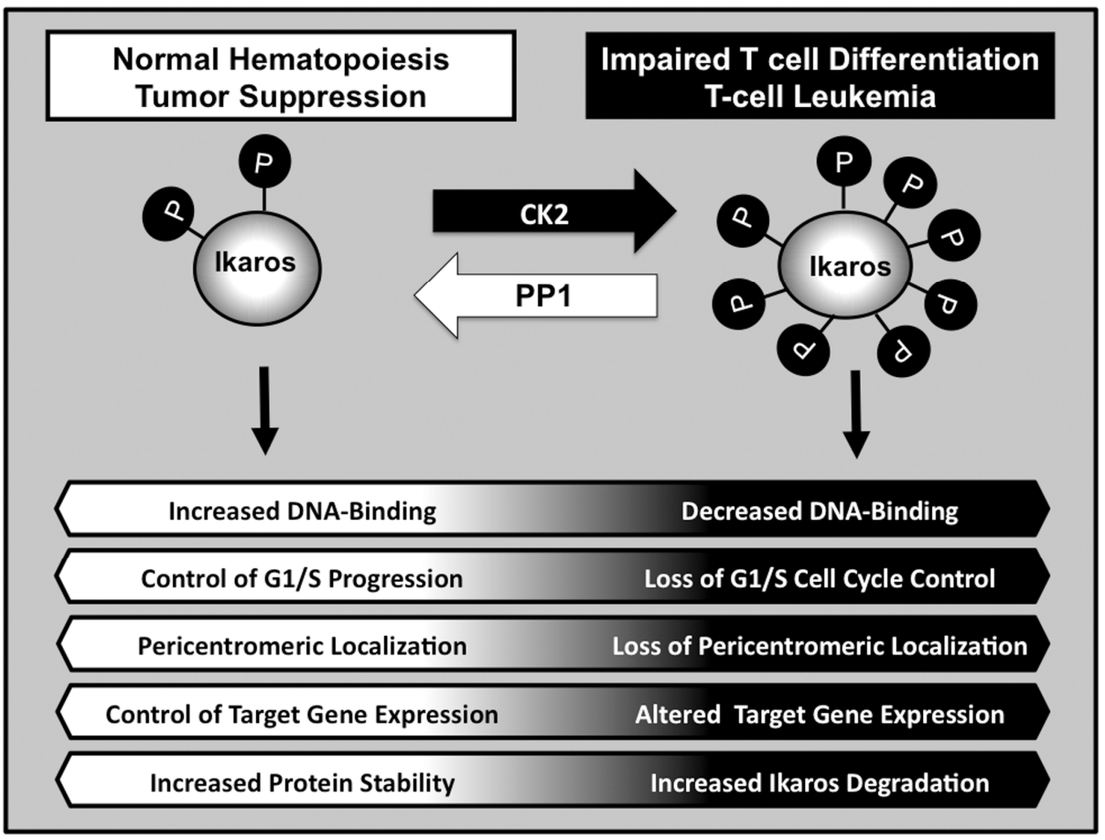


Fig. 2. CK2 kinase-mediated hyperphosphorylation results in functional inactivation of Ikaros and T-cell ALL. Hyperphosphorylation of Ikaros by CK2 kinase leads to the loss of Ikaros' ability to bind DNA, control cell cycle progression, regulate chromatin remodelling, regulate gene expression and to increased degradation of the Ikaros protein. The loss of Ikaros tumor suppressor function leads to the development of T-cell ALL.

7. Conclusion: The role of Ikaros in T-cell leukemia – overall hypothesis

Numerous clinical and experimental studies established the absence of Ikaros activity as a causative and/or contributory factor to the development of T-cell ALL. While genetic inactivation of Ikaros is evident in about 5% of T-cell ALL, recent evidence suggests that the functional inactivation of Ikaros by CK2 kinase-mediated phosphorylation is an important factor for the development of T-cell ALL. **With regard to Ikaros activity, we propose that there are three types of T-cell leukemia (Fig. 3):**

4. T-cell leukemia with the presence of genetic inactivation (deletion or mutation) of at least one Ikaros allele – responsible for ~5% of T-cell leukemia.
5. T-cell leukemia with functional inactivation of the Ikaros protein in leukemia cells due to overexpression of CK2 kinase.
6. T-cell leukemia with intact Ikaros function, but with defects in other genes and proteins that regulate T-cell proliferation and differentiation.

The prognostic and therapeutic significance of these three types of T-cell leukemia will be the subject of intense investigation in the future.

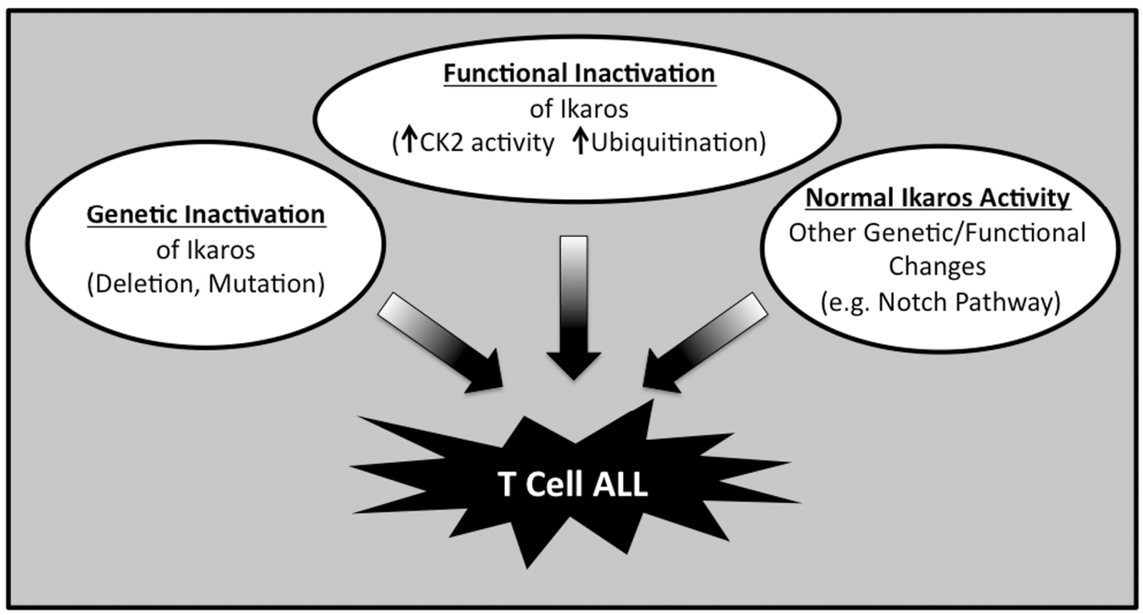


Fig. 3. Three types of T-cell leukemia with regards to Ikaros activity. Genetic and functional inactivation of Ikaros, in addition to changes in previously identified pathways, lead to T-cell leukemogenesis.

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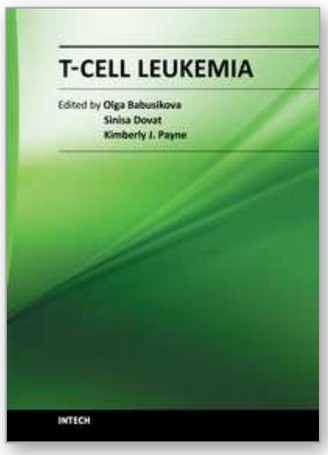
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The purpose of this book is to provide a comprehensive review of the scientific advances in T-cell malignancies and to highlight the most relevant findings that will help the reader understand both basic mechanisms of the disease and future directions that are likely to lead to novel therapies. In order to assure a thorough approach to these problems, contributors include basic scientists, translational researchers and clinicians who are experts in this field. Thus, the target audience for this book includes both basic scientists who will use this book as a review of the advances in our fundamental knowledge of the molecular mechanisms of T-cell malignancies, as well as clinicians who will use this book as a tool to understand rationales for the development of novel treatments for these diseases.

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