REVIEW

The emerging role of the Ikaros stem cell factor in the neuroendocrine system

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Abstract

In this review, we cover the evidence implicating Ikaros as a key factor whose transcriptional actions and chromatin remodeling properties determine the fate of hypothalamic neuroendocrine and pituitary cell population expansion. We propose that the governing mechanisms involved in the regulation and action of Ikaros are of importance during developmental as well as neoplastic transitions.

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Introduction

The process of anterior pituitary cell development and differentiation follows a highly specific pattern and temporal sequence. Several putative transcription-regulating proteins have been identified in the pituitary and have been implicated as key elements in the definition of cell-specific phenotypes and the regulation of hormone gene expression (Asa & Ezzat 1998, 1999, 2002). Which of the lineage-regulating factors predominates in determining the lineage choice and expansion can be decided not only by the expression and relative concentration of the transcription factor but also by the chromatin accessibility at their cognate sites.

Ikaros was originally described as a transcription factor that binds to regulatory sequences of genes expressed in lymphoid cells (Molnar *et al.* 1996, Georgopoulos *et al.* 1997). The single copy gene contains seven exons that can, by alternative splicing, give rise to eight isoforms (Fig. 1) (Hahm *et al.* 1994, Molnar & Georgopoulos 1994, Sun *et al.* 1996). All Ikaros isoforms share a common C-terminal domain that contains a transcription activation motif and two zinc finger motifs required for hetero- and homodimerization among the Ik isoforms and for interactions with other proteins (Winandy *et al.* 1995, Sun *et al.* 1996, Hahm *et al.* 1998). The N-terminal region includes a domain with zinc finger motifs critical for DNA binding. Ikaros isoforms differ in the number

of N-terminal DNA-binding zinc fingers that differentiate them into members with or without DNA-binding properties (Hahm *et al.* 1994, Molnar & Georgopoulos 1994). Only Ik1–3 contain the requisite three or more amino (N)-terminal zinc fingers that confer high-affinity binding to an Ikaros-specific core DNA sequence motif in the promoters of target genes (Sun *et al.* 1996). Dominant-negative (dn) forms of Ikaros lack the DNA-binding domain.

The various isoforms can act either as transcriptional activators or repressors as part of an integral component of a functionally diverse chromatin remodeling network (Molnar *et al.* 1996). It has become readily evident that the Ikaros system represents an example of a finely tuned system with distinct regulatory functions, particularly during early developmental stages in restricted target tissues.

In the earliest studies, Ikaros expression was identified in the brain and pituitary (Georgopoulos *et al.* 1992), but this was largely left uninvestigated. Our interest in this gene was precipitated by our identification of abundant expression of Ikaros in the anterior pituitary gland and hypothalamic neurons where we have demonstrated important functions for this factor in the regulation of multiple hormones including pro-opiomelanocortin (POMC), growth hormone (GH), prolactin (PRL), and GH-releasing hormone (GHRH). We have also identified a role for Ikaros in modulating neuroendocrine cell growth and survival functions (Ezzat *et al.* 2003, 2006b).

Figure 1 Schematic of the alternatively spliced Ikaros isoforms. E1–7 represent the coding exons. The vertical open bars represent zinc finger domains. As depicted here, Ikaros isoforms lacking the DNA-binding domain have the capacity to act as dominant negative (dn). This occurs through heterodimerization with other Ikaros isoforms. However, interaction with co-repressors and/or components of the nucleosome remodeling complexes (NuRD) has also been suggested as an alternative mode of gene repression.

In this review, we cover the evidence implicating Ikaros as a key factor whose transcriptional actions and chromatin-remodeling properties direct hypothalamic neuroendocrine and pituitary cell population expansion during the development. We propose that the governing mechanisms involved in the regulation and action of Ikaros are of importance during developmental as well as neoplastic transitions.

The importance of Ikaros in hematopoietic stem cell commitment

Gene-targeting experiments have firmly established that nuclear factors encoded by the Ikaros gene are essential for normal lymphoid development (Cortes *et al.* 1999). Mice homozygous for a null mutation in Ikaros display deficient B- and T-cell differentiation (Wang *et al.* 1996, Winandy *et al.* 1999), whereas those homozygous for a dn mutant form of Ikaros lack *all* lymphocytes (Georgopoulos *et al.* 1994). The more severe phenotype of the dn Ik mutant suggested the presence of Ikaros homologs, leading to the identification of the related factors Aiolos (Morgan *et al.* 1997), Helios (Hahm *et al.* 1998, Kelley *et al.* 1998), and Eos (Cortes *et al.* 1999). In addition to its role in

differentiation, Ikaros plays an important role in regulating cell proliferation. Ik^{+/–} mice exhibit hyperactive T-cell receptor-mediated proliferative responses and eventually develop leukemias and lymphomas (Winandy *et al.* 1995, Avitahl *et al.* 1999). Animals heterozygous for the dn Ik-6 that lacks the DNA-binding domain develop T-cell lymphoproliferative disorders similar to human T-lymphoblastic leukemia or lymphoma, presumably as a result of inactivation of the normal allele (Winandy *et al.* 1995). Thus, Ikaros appears to be an essential regulator of early lymphocyte differentiation as well as differentiated cell expansion.

The essential role of Ikaros in anterior pituitary cell growth and survival

Ikaros directs anterior pituitary cell expansion

Our studies focused on detailed characterization of the striking neuroendocrine phenotypes of the Ikaros-deficient mouse model. In particular, the homozygous null mice exhibit a profound dwarf phenotype with nearly 95% mortality by 6 weeks of age (Ezzat *et al.* 2005a). The heterozygous Ik^{+/–} mice survive for 4–5 months and subsequently develop lymphoproliferative disorders with leukemic or atypical lymphoid infiltrates of several tissues.

Although these animals were thought to develop infection with sepsis, complete autopsy examinations of our mice have not identified evidence of infection or sepsis. Rather, they show wasting with features of a profound metabolic/endocrine syndrome. Tissue and blood cultures were found to be negative. These animals have normal myeloid marrow elements, and there is no evidence of a neutrophilic response to infection. Moreover, reconstitution of Ikaros-deficient animals after birth with wild-type marrow demonstrated no measurable impact on size and overall health attributable to successful lymphocyte repopulation, supporting the hypothesis of an endocrine/metabolic basis for the phenotype (Ezzat *et al.* 2005a). These experiments provided compelling evidence for a lymphocyte-independent role for Ikaros in governing neuroendocrine system development.

Ikaros modulates pituitary cell survival

Animals heterozygous for dn forms of Ikaros (isoforms that lack the DNA-binding domain) develop T-cell disorders similar to human T-lymphoblastic leukemia or lymphoma, presumably by inactivation of the normal Ikaros allele (Winandy *et al.* 1995). Over-expression of a dn non-DNA-binding isoform Ik6 has been identified in a third of cases of B-cell acute leukemias (Nakase *et al.* 2000). Ikaros mutant mice display a decrease in expression of flk-2 and c-kit receptors, which may in part contribute to the early lymphopoietic phenotypes manifested in the absence of Ikaros (Nichogiannopoulou *et al.* 1999).

Having identified that Ikaros is expressed in the normal pituitary, we then examined its expression in neoplastic tissues. We detected Ik1 and Ik2/3 protein isoforms in human and mouse pituitary nuclear fractions (Ezzat *et al.* 2003). We also identified Ik6 expression in nearly 50% of human pituitary adenomas (Ezzat *et al.* 2003). Forced expression of this dn form of Ikaros resulted in histone 3 acetylation with the activation of the Bcl-XL promoter (Ezzat *et al.* 2006b). Parallel induction of the endogenous gene resulted in enhanced pituitary cell survival and evasion from apoptotic signals.

Ikaros promotes anterior pituitary cell differentiation through cellular lipids

To further determine the mechanisms of action of Ikaros in pituitary cell growth and differentiation, we used a cDNA microarray to uncover mediators of cholesterol uptake including the low-density lipoprotein receptor (LDL-R) and sterol regulatory element-binding protein 2 as targets of Ik action (Loeper *et al.* 2008). We showed that Ikaros regulates the LDL-R to alter metabolism in pituitary corticotroph cells. The DNA-binding Ikaros

isoform, Ik1, binds and enhances activity of the LDL-R promoter. Ik1 decreases methylation and increases acetylation of histone 3 lysine 9 at the LDL-R promoter (Loeper *et al.* 2008). Confocal microscopy and quantitative fluorometry demonstrated enhanced LDL endocytosis in Ik1-transfected cells that exhibited abundant endoplasmic reticulum, large Golgi complexes, and prominent secretory granule formation, consistent with more robust cholesterol incorporation into functionally relevant membrane-rich organelles. Consistent with these data, LDL-R^{-/-} mice, like Ik^{-/-} mice, have decreased circulating levels of adrenocorticotrophic hormone (Loeper *et al.* 2008). These findings expand the repertoire of Ikaros actions to include regulation of the cholesterol uptake metabolic pathway. This novel link between tumor suppression and differentiation provides a relationship between cellular metabolism and cancer, and has therapeutic implications for lipid-modifying drugs in Ikaros-associated disorders.

Ikaros drives corticomelanotroph population expansion

To quantify the *in vivo* effects of Ikaros on hypothalamic-pituitary development, we examined in detail the effects of disruption of Ikaros on hypothalamic-pituitary architecture, cell number and area by quantitative morphometric analyses, proliferation rates, and apoptotic indices. Morphologic and morphometric examination of these animals reveals pituitary hypoplasia and dysgenesis in Ik-null mice. During fetal development, there is delayed progression of pituitary development with small, but architecturally normal pituitaries compared with wild-type littermates that have normal pituitary development (Shewchuk *et al.* 1999). After birth, the Ik-null mice continue to exhibit a marked reduction in pituitary mass. They have normal cytodifferentiation with the presence of GH-positive somatotrophs, PRL-positive lactotrophs, adrenocorticotrophin (ACTH)-positive corticotrophs, and thyrotrophin (TSH)-reactive thyrotrophs and gonadotrophs containing follicle-stimulating hormone and luteinizing hormone. However, there are marked reductions in the number of adeno-hypophysial cells, most strikingly involving the ACTH- and GH-producing populations. The pituitaries of homozygote mice exhibited a striking reduction of melanocorticotropes, which was most marked in the intermediate lobe. The heterozygote and homozygote animals showed no evidence of the classical expansion of the melanocorticotropes that normally proliferate to occupy the entire intermediate lobe.

To specifically examine the functional consequences of disrupted Ikaros signaling on pituitary corticomelanotroph development, we compared the hormonal and developmental profiles of Ik-null mice with their

heterozygote and wild-type littermates (Ezzat *et al.* 2005a). Homozygote Ik-null mice demonstrated the lowest levels of ACTH in the systemic circulation. Levels of the POMC-derived melanocyte-stimulating hormone were also reduced in heterozygote but more strikingly in Ik-null mice. The functional consequences of a diminished population of POMC-producing corticomelanotroph cells were most evident in homozygote mice. Consistent with the trophic functions of ACTH on adrenocortical development and function, Ik-null mice display reduced circulating corticosterone levels compared with heterozygote and wild-type littermates. These changes were also reflected by the reduction of adrenal gland size with striking loss of adrenocortical mass, especially in homozygous Ik-deficient mice.

To examine the possibility that the diminished POMC production and adrenocortical insufficiency in Ikaros-deficient animals may be mediated through altered cytokine production by a dysregulated lymphocyte population, we examined the impact of wild-type bone marrow reconstitution on the endocrine phenotype. Five weeks after adoptive bone marrow transfer in neonatal mice, the proportion of splenic CD4⁺ and CD8⁺ T-cells in the homozygous Ik-null recipients of Ik wild-type syngenic bone marrow was significantly restored compared with vehicle-treated controls. Despite successful reconstitution with normal lymphocytes, the phenotype of homozygote Ik-null animals was unchanged with persistently small pituitaries and reduction in POMC expression that was indistinguishable from vehicle-treated age-matched homozygote animals. Moreover, neither heterozygote nor homozygote Ik-deficient animals demonstrated a significant change in serum corticosterone levels following reconstitution with wild-type lymphocytes. These data provide evidence for a direct role of Ikaros in pituitary corticomelanotroph development and function independent of its influence on lymphopoiesis.

Recognizing that Ikaros mutant mice display adrenocortical insufficiency, we examined the impact of systemic hormone replacement on growth and survival of the Ik-null mouse. Glucocorticoid hormone treatment compared with vehicle alone resulted in significant weight increase in Ik-null mice; the effect was also present, but less evident, in heterozygote animals. By comparison, the same treatment displayed no significant impact on the growth of wild-type animals.

Overall, homozygote Ik-null mice display diminished life expectancy with nearly 5% mortality at birth, 40% by 3 weeks of age, and 95% by 6 weeks of age. Given the recognized functions of Ikaros on immune cell development and function, we performed complete organ surveys in search of opportunistic infections or malignancy. However, we found no evidence of either of these conditions to explain the observed mortality at these stages of development. Moreover, hormone treatment

with glucocorticoids resulted in improved survival. In particular, Ik-null mice treated with the adrenocortical hormone demonstrated 100% survival beyond 6 weeks of age. For comparison, none of the vehicle-treated homozygote mice survived beyond the same time point.

Ikaros binds and activates the POMC gene

To determine the mechanism of Ikaros action responsible for the corticotroph insufficient phenotype, we analyzed the rat POMC promoter at five potential Ik-binding sites within the −543 bp promoter (Ezzat *et al.* 2005a). The importance of these putative Ikaros-binding sites was examined by electromobility shift assay and luciferase reporter assays. Two of the three sites that formed specific super-shifted complexes (fragment I (−451/−420) and fragment II (−163/−137)) were identified to be functionally significant as determined through mutational analyses and co-transfection studies (Ezzat *et al.* 2005a). These Ikaros-binding sites in the POMC promoter are within close proximity (~70 bp) upstream and downstream of the recently identified Tpit/Pitx1 regulatory element (Lamolet *et al.* 2001). The T-box factor Tpit is restricted to POMC-expressing corticomelanotroph pituitary cells (Lamolet *et al.* 2001). It activates POMC transcription in cooperation with contiguously bound Pitx1 by recruiting the SRC/p160 co-activators (Maira *et al.* 2003). Given the critical role of Ikaros in nuclear dimerization, the potential for physical interaction between Ikaros and Tpit needs to be specifically examined. Nevertheless, forced Ikaros expression, but not (dn) Ik6, results in induction of the endogenous POMC gene at the mRNA and protein levels (Ezzat *et al.* 2005a).

Hypothalamic Ikaros actions

Ikaros directs somatotroph expansion through hypothalamic GHRH regulation

Ikaros-deficient animals display a dwarf phenotype with body weight reaching only ~50% of their wild-type littermates. This dwarfism is associated with diminished GH secretion as evidenced by a reduction of the GH-target growth factor insulin-like growth factor-I (IGF-I) by >50% in homozygote animals. The Ikaros heterozygote-deficient animals display near normal somatic growth with more modest (~12%) reductions in IGF-I levels compared with wild-type mice. Healthy homozygote mice had the same body proportions as those of heterozygote and wild-type mice. At autopsy, internal organs were proportionally equivalent across the genotypes with the exception of the contracted pituitary and adrenal glands of homozygote mice. These features of GH deficiency are not a result of nonspecific

generalized pituitary dysgenesis, since the animals have normal or slightly elevated circulating levels of TSH and PRL. Although these animals exhibit glucocorticoid deficiency that could account for GH insufficiency, glucocorticoid replacement that significantly improved viability resulted in only a minimal increase in body weight (Ezzat *et al.* 2005a).

The changes of proportionate postnatal dwarfism are indicative of GH deficiency in homozygous Ik-null mice. Again, to exclude the possibility of a secondary response to immunodeficiency, we performed bone marrow reconstitution studies and showed that hematopoietic replacement does not alter IGF-I levels and restore normal growth. We carried out administration of exogenous GH with a response of circulating IGF-I levels and body weight reflecting somatic growth (Ezzat *et al.* 2006a).

The pituitary somatotroph population displayed quantitative differences across the Ikaros genotypes during early postnatal development. Somatotrophs were present in approximately the same proportion of cells in all genotypes, but the glands are smaller in heterozygotes than in wild-type mice and are even smaller in homozygotes. The intensity of GH reactivity was also reduced in homozygote mice. GH positivity persists in the intermediate lobe of the lateral wings likely attributable to the lack of melanocorticotrope expansion that normally replaces intermediate lobe somatotrophs during development. Other cell types were not measurably affected; the number and area of immunoreactive thyrotrophs and gonadotrophs were not disproportionately different from those of wild-type littermates (Ezzat *et al.* 2006a).

To examine the regulation of GH by Ikaros, we examined pituitary mammosomatotroph GH4 cells that express abundant Ikaros. We found that Ikaros reciprocally regulates the GH and PRL genes (Ezzat *et al.* 2005b). In contrast to the expected results, we documented that wild-type Ikaros (Ik1) inhibits GH mRNA and protein expression while stimulating PRL mRNA and protein levels. Ikaros does not bind directly to the proximal (−360) GH promoter. Instead, Ikaros significantly abrogates the effect of the histone deacetylation inhibitor trichostatin A on this promoter. Ikaros selectively deacetylates histone 3 residues on the proximal transfected or endogenous GH promoter and limits access of the Pit1 activator. By contrast, Ikaros acetylates histone 3 on the proximal PRL promoter and facilitates Pit1 binding to this region in the same cells (Ezzat *et al.* 2005b). These data provide evidence for Ikaros-mediated histone acetylation and chromatin remodeling in the selective regulation of pituitary GH and PRL hormone gene expression in mammosomatotroph cells.

This paradoxical result did not explain the dwarf phenotype of the Ik-null mice, and therefore another explanation for GH deficiency was sought in the

hypothalamus. Abundant levels of Ikaros expression were identified in the hypothalamus of wild-type littermates. This staining was localized predominantly within the ventral hypothalamus and was colocalized with GHRH in hypothalamic neurons (Ezzat *et al.* 2006a). The number of positive cells reaches a maximum at embryonic day (e)18 and shows a reduction in newborn mice; only scattered cells are positive in the adult brain. Ik-null mice showed a striking lack of hypothalamic GHRH. Over-expression of Ikaros enhanced GHRH promoter activity and induced endogenous GHRH gene expression, proving that there is a critical role of Ikaros in the functional regulation of GHRH (Ezzat *et al.* 2006a). These data unmask a wider role for Ikaros in the neuroendocrine system, highlighting a critical contribution to the development of the hypothalamic–pituitary somatotrophic axis.

Higher central nervous system Ikaros actions

Ikaros transcription factors are also expressed in the brain with predominance in the developing striatum where the putative binding site (5'-GGGA-3') in the promoter of the mouse and rat enkephalin genes is readily identified (Dobi *et al.* 1997). One study focusing on the relevance of Ik to the enkephalinergic system (Agoston *et al.* 2007) concluded that Ik is co-expressed with enkephalin mRNA and may act as a positive regulator of enkephalinergic specification in the developing striatum. While enkephalin is one of several striatal co-transmitters, expressed by a subset of medium spiny neurons (MSNs), there are many others, including gamma-aminobutyric acid (GABA), substance P, and dopamine D1 and D2 receptors (reviewed in (Holt *et al.* 1997)). We found Ik in precursor cells of several neurostriatal compartments, suggesting that it may play a role as a general maturation factor for MSNs.

With these neuro-localization studies in mind, we proceeded to systematically examine the impact of loss of Ikaros on neurostriatal-mediated functions. We performed a battery of standardized neurobehavioral tests including the elevated plus maze (a measure of anxiety-like behavior), the acoustic startle response and pre-pulse inhibition tests (measures of motor and autonomic reaction), the pinch test (a measure of catalepsy), and contextual fear conditioning (measures of learning and emotion). None of these behavioral functions were significantly altered in Ik-null mice, however, it was specifically in the Porsolt's forced swim test (a measure of depression-like behavior), where we found Ik−/− mice spending significantly less time in immobility than their Ik+/+ littermates (Kiehl *et al.* 2008). This phenotype is consistent with

Figure 2 Ikaros is expressed in restricted sites throughout the neuroendocrine and hematopoietic systems. In the brain, the highest expression is in the medium spiny neurons of the striatum where the loss of function results in neurobehavioral changes characterized by an anti-depressant phenotype. In the hypothalamus, the median eminence of GHRH-containing neurons co-localize with Ikaros expression. Loss of Ikaros severely diminishes GHRH production and consequently GH and IGF-I activation. In the anterior pituitary, Ikaros is expressed in POMC-producing corticotrophs that govern the ACTH/adrenocortical axis. Ikaros is also expressed in the somatotrophs where it plays a direct inhibitory role. In the hematopoietic system, Ikaros' peak expression is in stem cells where it directs lymphoid lineage commitment. The multidimensional actions of Ikaros serve to sort and integrate diverse signals to regulate neuroendocrine-immune interactions through direct and indirect mechanisms.

reduced behavioral despair. These findings suggest that Ikaros-mediated neuro striatal cytodifferentiative functions impose significant and selective impact on depressive behavior (Kiehl *et al.* 2008). The neurochemical Ikaros targets involved in mediating these functions will undoubtedly provide novel pharmacologic opportunities for the treatment of psychoaffective disorders.

Conclusions

The identification of Ikaros as a transcriptional regulator of the POMC gene and consequently the ACTH-adrenal axis, its epigenetic control of the GH/PRL axis, and the exciting new finding of Ikaros in selected hypothalamic and striatal neurons, raises new questions about the

pleiotropic neuroendocrine functions of this zinc finger protein (Fig. 2). The established functions of Ikaros as a mediator of early and late hematopoietic cell differentiation and proliferation provide further insights into the role of Ikaros as an integrator of endocrine, immune, and neurobehavioral functions. The studies reviewed here indicate that Ikaros can influence hypothalamic-pituitary cell development, differentiation, proliferation, and/or transformation, and provide evidence that these effects are mediated through overlapping and redundant cellular and nuclear targets. The balance between Ikaros and its dn Ik6 isoform appears to regulate multiple promoters through histone acetylation and methylation-dependent mechanisms. The net influence of these interactions is ultimately expected to favor a chromatin environment that permits recruitment of an activating or repressing complex essential for gene-specific control.

Declaration of interest

The authors have nothing to disclose. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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