Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors

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Background: Normal hematopoietic development depends on the activity of the Ikaros transcription factor, which contains distinct zinc-finger domains that mediate DNA binding and protein dimerization. Mice homozygous for a transgene encoding a dominant-negative version of Ikaros that lacks the DNA-binding domain but not the dimerization domain have a more severe phenotype than *Ikaros* null mice. This observation suggests the presence of factor(s) that can dimerize with Ikaros and partially complement its function. One previously identified factor, Aiolos, probably serves this role in the lymphoid system; a related factor involved in hematopoietic progenitors remains unknown, however.

Results: Here, we describe the cloning of an *Ikaros*-related gene, *Helios*. Analysis of the primary sequences of Helios, Ikaros and Aiolos revealed that the DNA-binding, transcriptional activation and dimerization domains are functionally conserved. Helios activated transcription from Ikaros DNA-binding sites and could dimerize with itself, Ikaros or Aiolos. Expression of *Helios* was detected in the earliest hematopoietic sites of the embryo, in hematopoietic stem cells in the adult and was subsequently restricted to a subset of cells in the T cell lineage. Helios co-localized with Ikaros and Aiolos proteins in macromolecular nuclear structures and formed stable complexes *in vivo* with the dominant-negative version of Ikaros.

Conclusions: Distinct but overlapping expression patterns of members of the *Ikaros* gene family during hematopoiesis might result in the formation of different multimeric complexes that have specific roles in lineage progression. The preferential expression of *Helios* in the earliest stages of hematopoiesis suggests that this gene functions predominantly in early progenitors.

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Background

Hematopoiesis involves the generation of pluripotent hematopoietic stem cells (HSCs) and their subsequent restriction and differentiation along one of at least seven distinct lineages. Hematopoiesis begins in two sites of the early embryo: the blood islands of the yolk sac and the splanchnopleural mesoderm that becomes the aorta–gonad–mesonephros (AGM) region [1,2]. By late gestation, the fetal liver becomes the major site of hematopoiesis. After birth, HSCs are found in the bone marrow where they undergo self-renewal and replenish the various lineages throughout adult life. At this site, the fate of the HSC is controlled by environmental cues that ultimately signal to transcription factors that are pivotal for lineage decisions [3,4].

Among the lineage-determining transcription factors, Ikaros is required for the normal development and homeostasis of the lymphoid system [5–9]. *Ikaros* mRNA is detected in the earliest defined HSC progenitor and continues to be expressed throughout the development of the lymphoid lineages [5,10,11]. The *Ikaros* gene is alternatively spliced and the resulting mRNA species produce at least six distinct isoforms containing between one and four amino-terminal Krüppel-type zinc fingers which dictate the sequence specificity and DNA-binding affinity of the isoforms [12,13]. All of the Ikaros isoforms have two Krüppel-like zinc fingers at their carboxyl termini which mediate protein dimerization [14]. Formation of homodimers and heterodimers between the DNA-binding Ikaros isoforms — Ik-1, Ik-2 and Ik-3 — dramatically increases both their affinity for DNA and their ability to activate transcription from their cognate DNA-binding sites. An Ikaros heterodimer in which one of the monomers lacks a DNA-binding domain cannot bind DNA, however, and is therefore transcriptionally inert.

The importance of Ikaros in the ontogeny and homeostasis of the immune system has been demonstrated through the analysis of two different targeted mutations in the *Ikaros* gene [6,9]. Mice homozygous for a functionally null mutation in the *Ikaros* gene, which results in the deletion of the dimerization domain and destabilization of the

protein, lack natural killer (NK) cells and B cells [9]. T cells are absent in the fetus, but small numbers of T cell progenitors appear in the thymus a few days after birth. Further abnormalities in the differentiation of the postnatal T cell compartment include a skewing of thymocytes toward the $CD4^+CD8^-$ lineage and a hyperproliferative response to T cell receptor stimulation [9].

A second *Ikaros* mutation, involving a deletion of the amino-terminal DNA-binding domain of the Ikaros protein, results in a more severe phenotype when expressed in mice. This amino-terminal deletion mutant acts in a dominant-negative fashion, as these proteins are able to dimerize with wild-type isoforms and thereby inhibit Ikaros activity. Mice homozygous for the *Ikaros* dominant-negative (DN) mutation (*Ikaros* $DN^{-/-}$) have a complete block in B, T and NK cell differentiation in both the fetus and the adult and have additional defects in the HSC compartment ([6]; A. Nichogiannopoulou and K.G., unpublished observations). The more severe phenotypes manifested by the *Ikaros* DN homozygotes (*Ikaros* $DN^{-/-}$) compared with the *Ikaros* null mice suggest that Ikaros DN dimerizes and interferes with the activity of other Ikaros-like factors in the hematopoietic system.

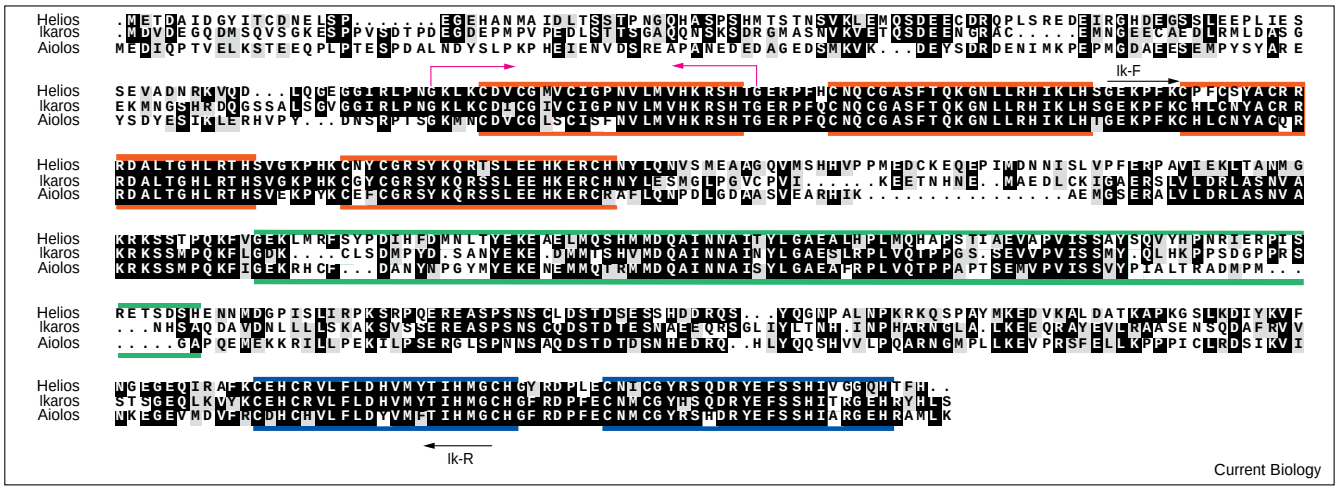
This hypothesis led to the identification of the Ikaros-related factor Aiolos, which is highly conserved in both the DNA-binding and protein dimerization zinc-finger domains [10]. *Aiolos* expression is restricted to the lymphoid lineages, and the protein can functionally interact with Ikaros. Aiolos is critical during B cell differentiation and its function in lymphocyte differentiation can be disrupted by dimerization with Ikaros DN (J.H. Wang, N.A., C. Friedrich, T.I., A. Renold, K. Andrikopoulos, L. Liang, B.A.M., K.G., unpublished observations). In addition to the more severe lymphoid defects, however, the *Ikaros* $DN^{-/-}$ mice also show defects in HSCs that are not observed in *Ikaros* null mutants (A. Nichogiannopoulou and K.G., unpublished observations). Whereas Ikaros is expressed in the bone marrow population highly enriched for HSC activity ($c\text{-}Kit^+Sca\text{-}1^+lineage^-$; [15,16]), Aiolos is not [10]. This suggests that the HSC abnormalities in the *Ikaros* $DN^{-/-}$ mice result from the interference of Ikaros DN with another factor(s). We therefore sought to identify new Ikaros-related-interacting factors in the HSC population. This led to the identification of *Helios*, a novel member of the *Ikaros* gene family.

Results

Identification of *Helios*, a novel *Ikaros*-related gene

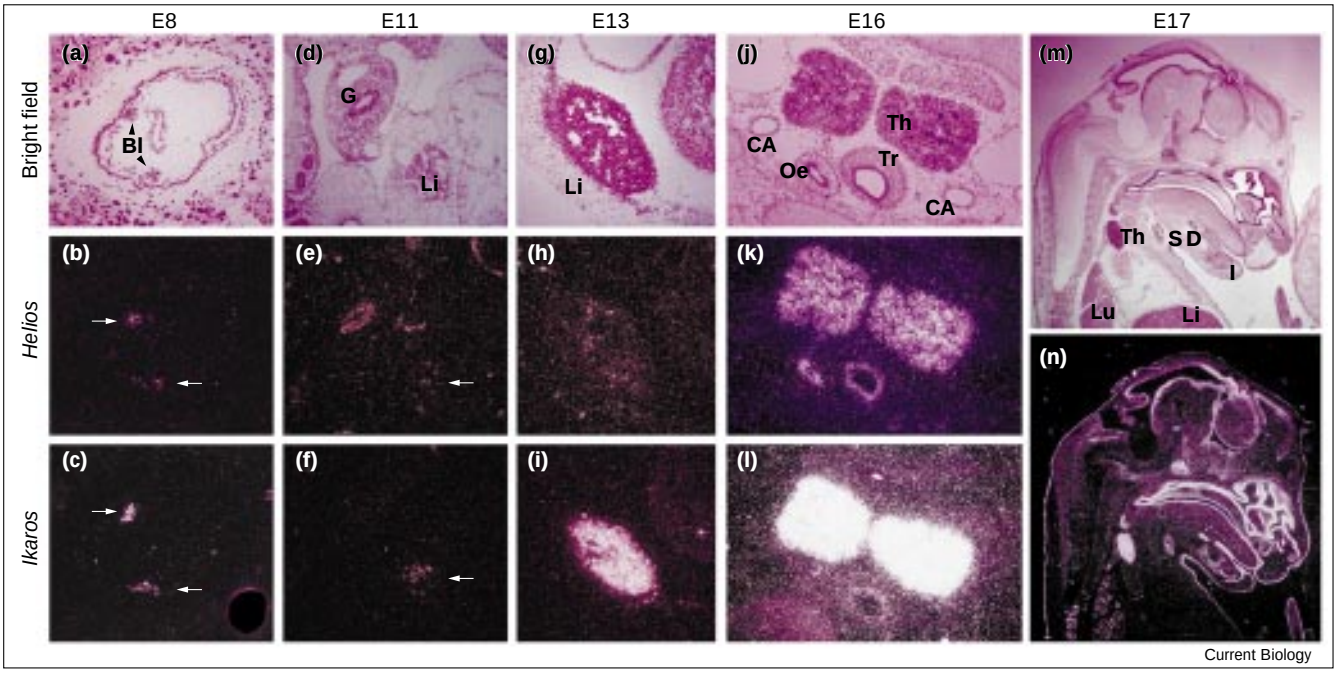
Degenerate primers encoding conserved sequences in the Ikaros amino-terminal (Ik-F) and carboxy-terminal zinc-finger (Ik-R) domains [17] were used to amplify cDNAs generated from the spleen of *Aiolos* null mutant mice (Figure 1; J.H. Wang, *et al.*, unpublished observations). A PCR product of the expected size (980 bp) was cloned and shown to have a unique DNA sequence that was homologous to the *Ikaros* gene. Full-length coding sequence was obtained by rapid amplification of cDNA ends (RACE)–PCR using nested specific internal primers. The encoded protein, designated Helios, shows a high degree of conservation to the Ikaros and Aiolos proteins (73% and

Figure 1



Structure of the *Helios* gene product. Alignment of the predicted amino-acid sequence of Helios with the sequences of Ikaros and Aiolos. The four amino-terminal zinc fingers comprising the DNA-binding domain are outlined in red, and the carboxy-terminal zinc fingers that mediate protein dimerization are outlined in blue. The conserved transcriptional activation domain is outlined in green. Arrows indicate the conserved sequences to which the degenerate oligos Ik-F (encoding GEKPFK) and Ik-R (encoding YTIHMG) were designed [17] to clone the *Helios* gene. The sequence between the purple arrows is present in the Hel-1 isoform, but absent from the Hel-2 isoform. Amino acids outlined in black represent identical residues and those outlined in gray represent conservative substitutions.

Figure 2



Developmental expression of *Helios* detected by *in situ* hybridization. (a,d,g,j,m) Bright-field views of embryonic sections at various stages with (b,e,h,k,n) dark-field views of *Helios* expression in the same section. (c,f,i,l) Dark-field views of *Ikaros* expression in an adjacent section for each stage. *Helios* is first detected in the yolk sac blood islands (Bl) of the day 8 embryo (b). Arrows point to the blood islands in the dark-field views showing *Helios* and *Ikaros* expression. (e,f) At day 11, both *Helios* and *Ikaros* are detected in the fetal liver (Li, arrow). *Helios* is also expressed in the epithelial lining of the gut (G). (h,i) At day 13, *Helios* is detected in bright spots within the liver, whereas *Ikaros* is expressed at higher levels throughout the liver. *Helios* is expressed in bright spots within the thymus (Th) at E16 (k) and appears highest in spots near the center of the thymus, while *Ikaros* is expressed at high levels throughout the thymus (l). *Helios* is also detected in the epithelial linings of the oesophagus (Oe) and trachea (Tr); CA represents the carotid artery. In addition to expression in the thymus, *Helios* can be detected in a number of epithelial tissues of the day 17 embryo (n), but is no longer detected in the liver at this stage (lower right). These include epithelial tissue in the lung (Lu), mouth, salivary glands (S) and ducts (D), tooth bud epithelium (I represents incisor), olfactory epithelium and skin. Some expression of *Helios* in the brain can also be seen.

67% overall similarity, respectively). The three proteins are almost identical throughout the amino-terminal zinc-finger DNA-binding domain. The protein dimerization domain, comprising the carboxy-terminal zinc fingers, is 86% identical between Helios and Ikaros and 75% identical between Helios and Aiolos. In a third region that contains the transcriptional activation domain, Helios has 68% similarity with Ikaros and 70% identity to Aiolos. Two alternatively spliced forms of Helios were identified. The full-length isoform (*Hel-1*) is analogous to the *Ikaros* isoform *Ik-1* in that it encodes a protein that contains all four DNA-binding zinc fingers. The second isoform (*Hel-2*) is similar to *Ik-2* in that its protein product is missing the first zinc finger (Figure 1). The strong conservation of the amino-terminal zinc-finger motifs encoded by *Hel-1* and *Hel-2* with those of the *Ikaros* isoforms *Ik-1* and *Ik-2* predicts that they will display similar DNA-binding properties [12].

Expression of *Helios* during embryogenesis

The expression of *Helios* during mouse embryogenesis was compared with *Ikaros* by *in situ* hybridization to adjacent sections. *Helios* is expressed in all hematopoietic centers of the developing embryo. The blood islands of the yolk sac constitute the first site of embryonic hematopoiesis. *Helios* and *Ikaros* are expressed in this extraembryonic site at day 8 of gestation (Figure 2a–c). By day 11, however, *Helios* expression has significantly decreased, whereas Ikaros expression is maintained until embryonic day 13 in this region (data not shown). Both *Helios* and *Ikaros* are expressed in the liver at day 11, although *Helios* mRNA is only present in a subset of cells in this tissue (Figure 2d–f). Throughout hematopoietic development, *Helios* expression in the liver is detected in a small number of scattered cells. In contrast, *Ikaros* is expressed at high levels in most of the cells present in this tissue during mid- to late-gestation (Figure 2g,i). In the thymus, *Helios* is first detected at low levels at embryonic day 13, whereas *Ikaros* expression is readily detected in this site 2 days earlier (data not shown). By day 16, *Helios* is expressed at high levels towards the center of the thymus, a region where early progenitors enter from the vasculature: in contrast, *Ikaros* is detected in most thymocytes (Figure 2j–l). This pattern of *Helios*

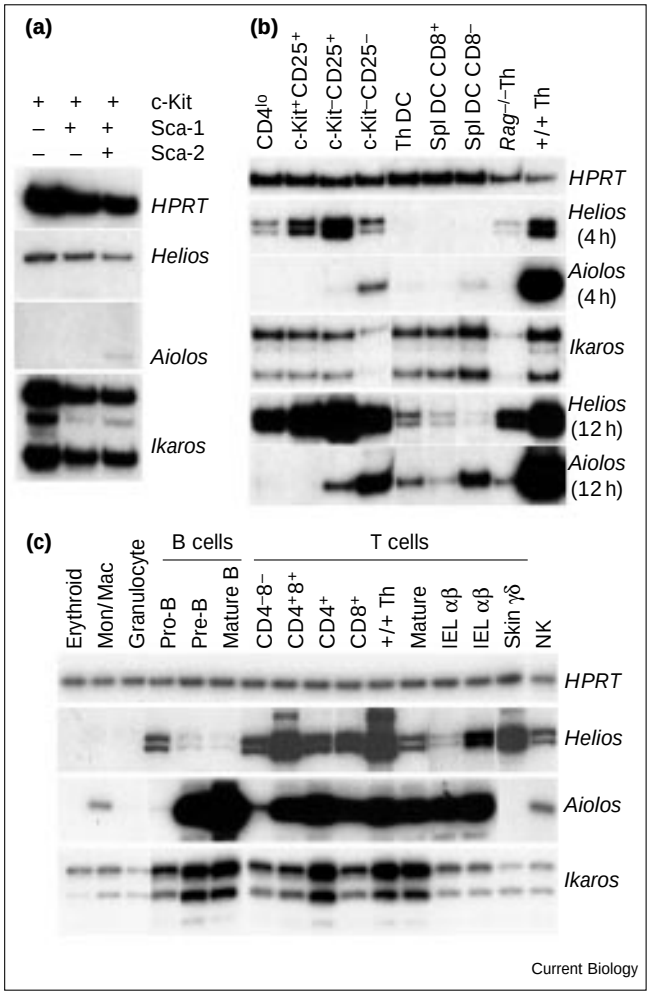
expression is maintained in the postnatal thymus. *Helios* is also detected in a very small subset of cells within the spleen of the adult (data not shown).

Outside of the hematopoietic system, *Helios* expression is high in a number of epithelial tissues. These include the endoderm lining the gut, the tubules of the kidney, the lining of the respiratory tract and the olfactory epithelium. During late-gestation, high levels of *Helios* expression can be detected in the salivary glands and ducts (Figure 2m,n; T.I. and B.A.M., unpublished observations). In adult tissues, *Helios* was detected in the thymus by northern blot analysis. Although *Helios* is expressed in the lung, liver, kidney and brain at various times during embryogenesis, it is not detected by northern analysis in these tissues in the adult (data not shown).

Expression of *Helios* in hematopoietic subpopulations

The expression of *Ikaros* gene family members in sorted hematopoietic and lymphoid progenitors of the adult was examined by reverse transcription (RT)–PCR. *Helios* mRNA is detected in the bone-marrow progenitor population that is highly enriched for stem cell activity (c-Kit$^+$Sca-1$^+$lineage$^-$) and is also present in hematopoietic progenitors that have a more restricted lymphoid or erythromyeloid potential, c-Kit$^+$Sca-1$^+$Sca-2$^+$ and c-Kit$^+$Sca-1$^-$Sca-2$^-$ respectively [15,16,18,19] (Figure 3a). *Ikaros* displays a similar pattern of expression in these hematopoietic progenitor populations whereas *Aiolos* is detected only in the progenitors that are more committed to lymphoid development, c-Kit$^+$Sca-1$^+$Sca-2$^+$ ([10]; Figure 3a). *Helios* is not expressed in definitive erythroid precursors (Ter119$^+$) and very low levels of *Helios* mRNA are present within the monocyte (Mac1$^+$GR$^-$) and granulocyte (Mac1$^+$GR$^+$) populations of the adult bone marrow (Figure 3c). *Ikaros* is detected at low levels in all three of these cell types. *Helios* is present at low levels in pro-B cells (CD45R$^+$CD43$^+$ from *Rag*$^{-/-}$ bone marrow) and decreases as they progress to pre-B cells (CD45R$^+$IgM$^+$). In contrast, *Aiolos* expression is low in pro-B cells and dramatically increases in pre-B and mature B cells.

Figure 3



RT–PCR analysis of Helios expression in hematopoietic cell populations. *Helios*, *Ikaros* and *Aiolos* expression is shown in sorted hematopoietic populations using *HPRT* expression as a control. Th, thymus; Spl, spleen; DC, dendritic cells; Mon/Mac, monocyte/macrophage; IEL, intestinal intra-epithelial lymphocytes; NK, natural killer cells; +/+ Th, wild-type thymocytes. See text for a full description of the sorted populations.

As HSCs differentiate along the myeloerythroid and B lymphoid lineages, *Helios* expression is diminished. *Helios* is present at varying levels in all T cell subsets analyzed, however (Figure 3b,c). The earliest lymphoid progenitors entering the thymus are CD4^{lo} (and c-Kit$^+$) and are not necessarily committed to the T cell lineage [20]. *Helios* and *Ikaros* are both detected in these earliest lymphoid progenitors. Among the progenitors that are CD4$^-$CD8$^-$, an increase in *Helios* expression is apparent during the progressive transition to the c-Kit$^+$CD25$^+$ and then to the c-Kit$^-$CD25$^+$ population [21], in which *Aiolos* is first detected (Figure 3b). A marked increase in *Aiolos* levels is observed at the next stage of thymocte development (c-Kit$^-$CD25$^-$), whereas *Helios* expression decreases. *Ikaros* levels remain constant during these early stages of T cell differentiation. In *Rag*$^{-/-}$ mice, all thymocytes are CD4$^-$CD8$^-$ and the majority of these are c-Kit$^+$CD25$^+$ [21,22] due to a block to thymocyte development at this stage, whereas in a wild-type thymus the majority of cells are at the double-positive CD4$^+$CD8$^+$ stage of development [23] (Figure 3b). *Helios* mRNA increases as thymocytes progress from the CD4$^-$CD8$^-$ double-negative to the CD4$^+$CD8$^+$ double-positive stage and declines as these become single-positive (CD4$^+$ or CD8$^+$) thymocytes (Figure 3c). Peripheral T cells have a lower expression of *Helios* than immature thymocytes, with the highest levels detected in γδ T cells of the skin (Vγ3) and the gut (IEL; Figure 3c) [25,26]. *Ikaros* and *Aiolos* are present in these T

cell populations but *Aiolos* is not detected in the fetally derived skin γδ T cells. All three genes are expressed in NK cells. The lymphoid-derived thymic dendritic cells (DCs) as well as the splenic $CD8^+$ and $CD8^-$ dendritic subsets express very low levels of *Helios*. *Ikaros* is present in all three populations, but is highest in the splenic $CD8^-$ DC subset. Among the DC subpopulations, *Aiolos* is also highest in the splenic $CD8^-$ DCs.

Both Helios isoforms (*Hel-1* and *Hel-2*) are expressed at approximately equivalent levels in all cell types tested. During hematopoietic development, *Helios*, *Ikaros* and *Aiolos* have overlapping but distinct patterns of expression. The differential patterns of expression of these three factors within the hematopoietic system might underscore their specific regulatory roles during differentiation.

Helios forms homodimers and heterodimerizes with Ikaros and Aiolos

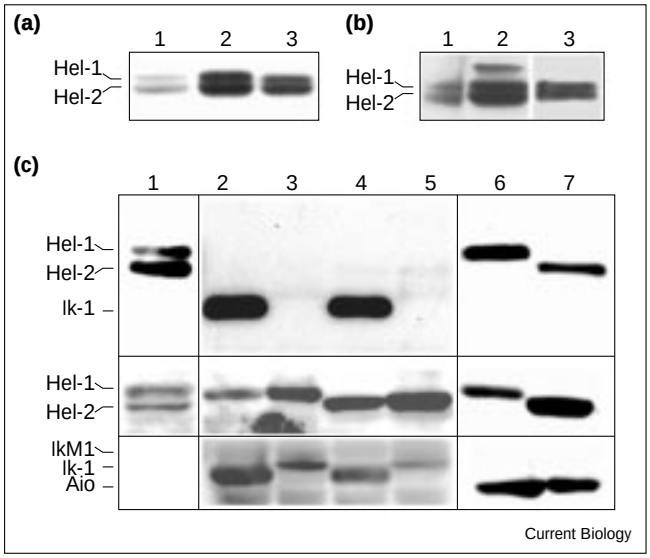
The carboxy-terminal zinc fingers of Ikaros and Aiolos, which mediate their homodimerization and heterodimerization, are highly conserved in Helios [10,14]. Helios-specific polyclonal antibodies recognize two Helios isoforms in thymocyte nuclear extracts from wild-type, *Ikaros* null and *Ikaros* $DN^{+/-}$ mice (Figure 4a). The Helios isoforms detected in thymocytes are approximately 64 kDa and 68 kDa, and co-migrate with the proteins produced by the *Hel-1* and *Hel-2* cDNAs co-expressed after transfection into the epithelial cell line 293T (Figure 4b, lanes 1,2).

In cell lysates from the thymuses of mice expressing an epitope-tagged Ikaros DN isoform, FLAG–Ik-7, both Helios isoforms were found in complexes immunoprecipitated with a mouse monoclonal antibody to the FLAG epitope (Figure 4b, lane 3). Thus, Ikaros DN forms a stable complex with Helios protein isoforms and might interfere with their normal activity *in vivo*. Helios also forms complexes with itself, Aiolos or Ikaros when these proteins are co-transfected into 293T cells in paired combinations (Figure 4c). Either FLAG–Hel-1 or FLAG–Hel-2 co-precipitate with Ik-1 (Figure 4c, lanes 2,4) and both Helios isoforms also dimerize with Aiolos (Figure 4c, lanes 6,7). IkM1 contains two point mutations in the carboxy-terminal zinc fingers of Ikaros that disrupt its ability to dimerize [14]. In contrast to Ik-1, this dimerization-deficient form of Ikaros was unable to interact with either Helios isoform (Figure 4c, lanes 3,5). Thus, the carboxy-terminal zinc fingers in Helios, Ikaros and Aiolos are functionally conserved and mediate the stable interactions between these proteins which might be critical for hematopoiesis as well as lymphocyte differentiation and function.

Helios is part of a higher order nuclear structure that contains Ikaros and Aiolos

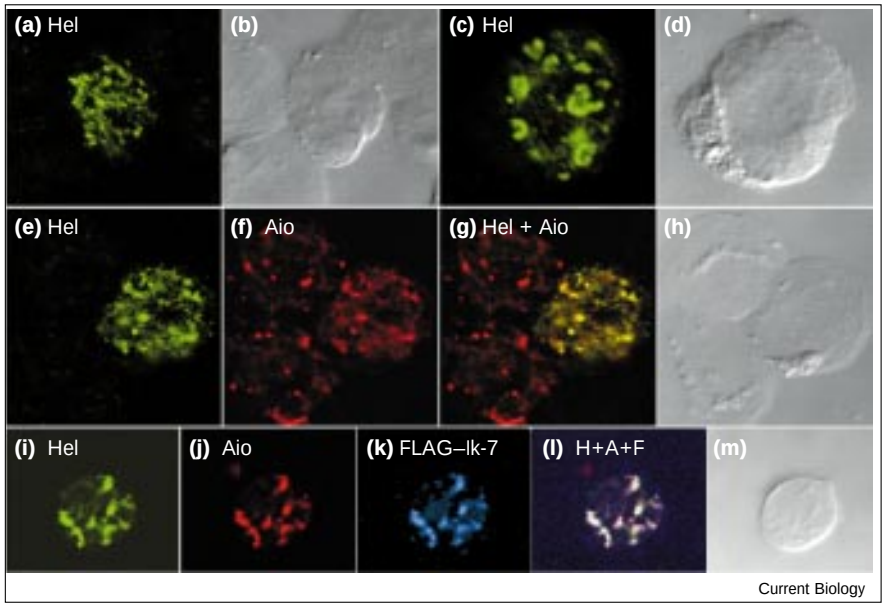
Ikaros and Aiolos are part of a higher order structure in resting lymphocytes that undergoes dramatic changes upon activation (N.A. and K.G., unpublished observations and J.H. Wang, *et al.*, unpublished observations). Helios also participates in these nuclear macromolecular structures in primary lymphoid cells. In contrast to Ikaros and Aiolos, bright staining for Helios is detected only in a small number of either resting or activated thymocytes (approximately 1 in 25 cells). In resting cells, Helios is detected in a punctate pattern within the nucleus, similar to that previously described for Ikaros and Aiolos [10,12,14] (Figure 5a). Upon thymocyte activation, Helios is redistributed into ring-like structures in the nucleus (Figure 5c), as are Ikaros and Aiolos. Helios is also detected in a very small number of splenocytes. These cells are probably T or NK cells, as RT–PCR analysis indicated that *Helios* is not expressed at significant levels in myeloid, erythroid or mature B cells.

Figure 4



Helios homodimerization and heterodimerization with Ikaros and Aiolos. **(a)** A polyclonal antibody generated against Helios detects two bands of approximately 64 kDa and 68 kDa by western blot analysis of thymic nuclear extracts from wild-type mice (lane 1), *Ikaros* null mice (lane 2) and *Ikaros* $DN^{+/-}$ mice (lane 3). **(b)** The Hel-1 and Hel-2 isoforms co-expressed in 293T cells (lane 1), co-migrate with the endogenous Helios isoforms detected in thymic nuclear extracts (lane 2). Both Helios isoforms are co-precipitated with Ikaros DN protein from thymuses of FLAG–Ik-7 transgenic mice (lane 3). **(c)** Constructs containing genes encoding Helios, Ikaros or Aiolos were expressed in pairs in 293T cells. Homodimerization of Helios proteins was demonstrated by precipitation of the Hel-2–FLAG–Hel-1 complex with the anti-FLAG antibody (lane 1). Antibodies to Ikaros were used to detect Ik-1 in complexes with either FLAG–Hel-1 (lane 2) or FLAG–Hel-2 (lane 4) immunoprecipitated using the FLAG antibody. The dimerization mutant IkM1 was not detected in immunoprecipitates with either FLAG–Hel-1 (lane 3) or FLAG–Hel-2 (lane 5). FLAG–Aio was expressed with either Hel-1 (lane 6) or Hel-2 (lane 7). Both Helios isoforms were detected in complexes precipitated with the FLAG antibody. The middle panel confirms the expression of Hel-1 (lanes 1,2,3,6), or Hel-2 (lanes 1,4,5,7) in the cell lysates. The lower panel confirms the expression of Ik-1 (lanes 2,4), IkM1 (lanes 3,5), and Aiolos (lanes 6,7) in the lysates.

Figure 5



Confocal immunofluorescence demonstrates co-localization of Helios within nuclear structures with Ikaros and Aiolos. (a) Bright staining for Helios (Hel) is detected in nuclear speckles in some primary resting thymocytes and (c) in ring structures of activated thymocytes. (e) Helios is detected only in a small number of resting splenocytes, whereas Aiolos (Aio) is expressed in most splenocytes (f). (g) Double staining shows co-localization (yellow) of Helios (green) and Aiolos (red) in a subset of resting splenocytes. (i–k) Staining for Helios, Aiolos and FLAG–Ik-7, respectively, in primary splenocytes from mice expressing the FLAG–Ik-7 minigene from the T-cell-specific CD2 promoter. (l) Triple staining shows co-localization of all three proteins within a subset of cells. (b,d,h,m) Corresponding bright-field images for the immunofluorescence studies.

Most cells in the spleen express Aiolos, but only a small subset of splenocytes also express Helios (Figure 5e–g). In most cases, there is complete overlap in the expression of these two proteins in a punctate pattern within the nucleus. There are a few small spots where either Helios or Aiolos is detected alone, however. In addition, a few cells were observed that showed bright staining for Helios, but only faint staining for Aiolos (data not shown). Cells stained for Ikaros and Helios showed a similar co-localization of the proteins (data not shown). In T cells from the spleen of mice expressing the FLAG–Ik-7 transgene, the endogenous Helios and Aiolos proteins co-localize with Ik-7 in nuclear structures (Figure 5i–l).

These studies establish the presence of all three family members in the same structures within the nucleus and demonstrate that Ikaros DN has the potential to interfere with the activity of the endogenous Helios and Aiolos proteins by co-localization within the same nuclear complexes. As inferred from the expression profiles of sorted cells, this immunofluorescence data also confirms the co-expression of different Ikaros family proteins in varying combinations within cells of distinct sub-populations in the thymus and spleen.

Helios can function as transcriptional activator

Helios protein contains a domain that is similar to the transcriptional activation domain of Ikaros and Aiolos. Given the near identity in the DNA-binding domain between Helios and Ikaros, we tested the ability of Helios to activate transcription from Ikaros-binding sites. The expression of a reporter gene under the control of four high-affinity Ikaros-binding sites (IkBS2) was tested in the presence of Helios or Ikaros in NIH3T3 cells [12]. A fivefold increase in reporter gene expression was detected in the presence of Helios, and a ninefold increase was detected in the presence of Ikaros (data not shown). These results confirm the functional conservation of both the DNA-binding and transcriptional activation domains in Ikaros family members.

Discussion

In the present study, we describe the identification and characterization of *Helios*, a new member of the *Ikaros* gene family. The proteins encoded by all three genes in this family have similar properties mediated by conserved functional domains. All three members bind to the consensus DNA-binding sites characterized for Ikaros and activate transcription from an adjacent promoter in co-transfection assays. Like Aiolos and Ikaros, Helios can dimerize with itself as well as with other family members including a dominant-negative version of Ikaros. Although the conservation of these domains emphasizes the similarity of these proteins, other regions differ between the proteins encoded by these genes and might confer functional specificity among them. The functional significance of these genes is supported by the fact that the regions that diverge between family members are conserved in the orthologs of these genes in other species. *Ikaros* orthologs have been reported in the chicken, frog and fish and their identity is confirmed by our analysis of *Aiolos* and *Helios* orthologs in these species as well ([26,27]; T.I. and B.A.M., unpublished observations). This analysis has also revealed that the previously reported partial '*Ikaros*-related gene' [17] is the *Xenopus* homolog of *Helios*. The divergent regions include domains that interact with other

proteins which might therefore mediate this functional specificity (J.K. and K.G., unpublished observations).

The importance of Ikaros and Aiolos in hematopoietic development has been established by mutational analysis ([6,8,9,28]; J.H. Wang, *et al.*, unpublished observations). Ikaros and Aiolos are co-expressed at varying levels in most branches of this lineage and co-localize in macromolecular nuclear complexes ([10]; J.H. Wang, *et al.*, unpublished observations). Recent co-localization studies suggest that these macromolecular complexes sequester lineage-specific genes in a repressed state that might be reversed during lineage progression [29]. Although the activation of these genes may then require dissolution of these macromolecular complexes, it is likely that Ikaros and Aiolos contribute directly to the transcriptional activation of lineage-specific genes as well, albeit in a different macromolecular context ([5,30]; A. Nichogiannopoulou and K.G., unpublished observations). These observations have led to the model proposing that varying levels of *Ikaros* and *Aiolos* expression in the cell lead to distinct combinations of heteromeric and homomeric complexes between these proteins; the functional differences between these complexes contribute to changes in gene expression as the lymphoid lineages progress. Consistent with this model, *Aiolos* is expressed at higher levels than *Ikaros* only in the B cell lineage and defects in *Aiolos* null mutant mice are predominantly found in this lineage ([10]; J.H. Wang, *et al.*, unpublished observations). *Ikaros* null and DN mutant mice have defects in all lymphoid lineages that can be attributed to the predominance of *Ikaros* expression in lymphoid progenitors and the T cell lineage. Genetic interactions between different mutant alleles of *Ikaros* and *Aiolos* confirm the data from molecular analyses showing that these genes co-operate to regulate lymphoid development (J.H. Wang *et al.*, unpublished observations).

One prediction resulting from analysis of *Ikaros* DN mice was that another member of the *Ikaros* gene family would co-operate with *Ikaros* in the earliest stages of the hematopoietic lineage when *Aiolos* is not expressed. The expression of *Helios* at sites where HSCs arise suggests that this gene is an important regulator of the earlier stages of hematopoietic development. Hematopoietic progenitors accumulate in the yolk sac at embryonic day 8 and the fetal liver at embryonic day 11. Both *Ikaros* and *Helios* are expressed in moderate numbers of cells in these regions at early stages, although *Ikaros* expression is consistently greater than that of *Helios*. As gestation continues, these sites are increasingly populated by more committed erythroid progenitors. Whereas *Ikaros* expression increases dramatically in both sites, *Helios* remains expressed in a limited number of cells. This may reflect its preferential expression in the less-committed hematopoietic progenitors.

The expression of *Helios* in sorted hematopoietic cells in the adult supports this interpretation. *Helios* is expressed in adult HSCs but its expression decreases in the maturing erythroid, macrophage and B cell lineages. *Helios* expression peaks in the early stages of T cell development and decreases as T cells mature in the thymus and are exported to the periphery. Significant levels of *Helios* are maintained in only a small subset of mature T cells. Upon immunization, *Helios* is detected in a very small number of cells in germinal centers of the spleen that might be T helper cells (data not shown). When compared with that of *Ikaros* and *Aiolos*, this profile of *Helios* expression suggests that transcriptional complexes including *Ikaros* and *Helios* will predominate in the earlier stages of hematopoiesis. This combination may be important for the self-renewing capacity of early progenitors that is compromised in the *Ikaros* $DN^{-/-}$ mice (A. Nichogiannopoulou and K.G., unpublished observations). The increasing expression of *Aiolos* and *Ikaros* as development proceeds may lead to complexes that promote lineage progression and differentiation.

Whereas *Ikaros* and *Aiolos* are predominantly expressed in hematopoietic sites, *Helios* is also expressed elsewhere in the embryo. Although a more detailed analysis of this expression pattern is required, it is tempting to speculate that the *Ikaros* gene family regulates lineage progression in other tissues as well. The dynamic expression of *Helios* in the embryo is consistent with such a role. Mutational analysis of the *Helios* gene will help to dissect its role in regulating progenitor development in the hematopoietic system and elsewhere in the embryo.

Materials and methods

Cloning of the Helios *gene*

A 980 bp *Helios* fragment was obtained from cDNA from the spleen of an *Aiolos*$^{-/-}$ mouse (J.H. Wang, *et al.*, unpublished observations) by PCR using degenerate oligos encoding the GEKPFK and YTIHMG motifs (single-letter amino-acid code) as previously described [17]. The RACE–PCR technique (Marathon cDNA Amplification Kit; Clontech) was used to obtain a full-length clone. PCR analysis of *Helios* expression in hematopoietic cells using various combinations of specific 5′ and 3′ primer pairs routinely yielded two bands (corresponding to *Hel-1* and *Hel-2*). These two bands were cloned and sequenced to show that the two alternatively spliced transcripts differ in the presence of sequence encoding the first amino-terminal zinc finger.

Expression analysis

See supplementary materials for full details of PCR conditions, primers, and isolation of hematopoietic cell populations. *In situ* analysis was carried out essentially as described [31] with single-stranded [^{33}P]UTP-labeled antisense RNA probes. Slides were exposed for 5 weeks.

Preparation of a Helios-specific polyclonal antibody and western blot analysis

A Helios peptide from amino acids 223–459 (Figure 1) was generated using the Xpress system (Invitrogen). Rabbit polyclonal antibodies raised to this protein were affinity-purified by pH elution [32]. Specificity of this antibody for Helios and not other Ikaros homologs was confirmed by western blot analysis of protein extracts from transfected 293T cells (see below) and by immunofluorescence of transfected cells (data not shown).

Thymic nuclear extracts were prepared as previously described [14]. Protein lysates were resolved on a 10% SDS–PAGE gel, transferred to an Immobilon-P membrane (Millipore) and probed with the affinity-purified polyclonal Helios antibodies (1:500 dilution in PBS/0.05% TWEEN-20). To detect Helios in primary cells, the signal was amplified by incubation of the filter with a 1:5 000 dilution of biotinylated goat anti-rabbit antibody followed by the same dilution of peroxidase-coupled streptavidin (Jackson Labs). The ECL kit (Amersham) was used for detection.

Expression constructs and transfection of 293T cells

The full-length *Hel-1* or *Hel-2* isoforms were cloned into CDM8, creating CDM8-Hel-1 and CDM8-Hel-2. Additional constructs were generated containing the FLAG or hemagglutinin (HA) tags (FLAG–Hel-1, FLAG–Hel-2, HA–Hel-1, HA–Hel-2). Transfections of 293T cells and immunoprecipitations were performed as described [14]. Western blot analysis was as described above except that, for 293T extracts, incubation with affinity-purified polyclonal antibodies specific for Ikaros [12], Aiolos [10] or Helios was followed by incubation with peroxidase-coupled goat anti-rabbit secondary antibody. For immunoprecipitation from primary cells, thymocytes or splenocytes were obtained from transgenic mice expressing the FLAG-tagged dominant-negative version of Ikaros, Ik-7, from the CD2 minigene [33]. Cells were harvested, washed in PBS/2% FCS, and lysed in 100 µl lysis buffer as described [14].

Confocal immunofluorescence

Primary thymocytes or splenocytes were obtained as previously described [6]. Thymocytes were activated for 40 h as described [8]. Cells were harvested, cytospun onto slides and fixed in 4% paraformaldehyde.

Supplementary material

A detailed methodological section is published with this paper on the internet.

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Supplementary material

Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors

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Materials and methods

Cloning of the Helios *gene*

PCR using degenerate oligos encoding the sequences GEKPFK and YTIHMG and cDNA from the spleen of an *Aiolos* null mouse (J.H. Wang, N.A., C. Friedrich, T.I., A. Renold, K. Andrikopoulos, L. Liang, B.A.M., K.G., unpublished observations) was used to obtain a 980 bp fragment of the *Helios* gene as described [17]. The RACE–PCR technique (Marathon cDNA Amplification Kit; Clontech) was used to obtain a full-length clone using the primers : 5′-RACE R51: GGGTGAAGGCCTCAGGT and R52: CCATCATATGAGACTGCATCAGCTCAGCCTCC; 3′-RACE R31: GGAGGCTGAGCTGATGCAGTCTCATATGATGG and R32: CACCTACCTTGGAGCTGAGGCCCTTCACCC (1.5 min, 95°C, 1 cycle; 20 sec, 98°C, 2.5 min, 72°C, 5 cycles; 20 sec, 98°C, 2.5 min, 70°C for 5 cycles; 30 sec, 98°C, 2.5 min, 68°C for 32 cycles; 10 min, 72°C, 1 cycle). A second amplification was 1.5 min, 95°C, 1 cycle; 20 sec, 98°C, 2.5 min, 68°C, 20 cycles; 10 min, 72°C, 1 cycle). PCR analysis of *Helios* expression in hematopoietic cells using various combinations of specific 5′ and 3′ primer pairs routinely yielded two bands. These two bands were cloned and sequenced.

RT–PCR analysis of gene expression

RT-PCR conditions and *Ikaros* and *Aiolos* primers were as previously described [10]. *HPRT* primers (For: TGGCCCTCTGTGTGCTCAAG; Rev: CACAGGACTAGAACACCTGC) were used as a control for RNA recovery. *Helios* primers were: Figure 3b Forward (2F): GGAACACGCCAATATGGCC (nt 60–78 of cDNA) and Reverse (8R): GGCCTTGGTAGCATCCAAAGC (nt 1327–47). For PCR in Figure 3c, forward primers 1F: AACAGACGCTATTGATGGC (nt 6–24) and 8R were used. The annealing temperature was 60°C and amplification was determined to be in the linear range. One fifth of the PCR reaction was loaded on the gel. For bone-marrow-derived progenitor populations where cells were limiting in number, cDNA from 50 cell equivalents was amplified for 32 cycles. For thymocyte precursors shown in Figure 3c, amplification was done for approximately 1000 cell equivalents for 26 cycles for each primer pair. All analyses were performed on at least two (and as many as five) independently sorted populations with consistent results. The exceptions to this were the IELs in Figure 3c and the fractionated T cell progenitors in the first four lanes of Figure 3b where cells from a single sort were analyzed by independent PCR reactions.

Isolation of hematopoietic cell populations

Stem cell populations (c-Kit$^+$Sca-1$^+$lineage$^-$), early progenitors (c-Kit$^+$Sca-1$^-$lineage$^-$ and c-Kit$^+$Sca-1$^+$Sca-2$^+$lineage$^-$) were purified from the bone marrow of wild-type mice as previously described [10]. Lineage-committed erythroid (ter119$^+$), pre B (B220$^+$), granulocyte (Mac1$^+$GR$^+$), monocyte/macrophage (Mac1$^+$GR$^-$) populations were purified from bone marrow of wild-type mice using antibodies to cell surface markers, magnetic secondary antibodies and separated using a MACS magnetic separation column (Miltenyi Biotec) as previously described [10]. B220$^+$ pro-B cells were (CD45R$^+$CD43$^+$) sorted from the bone marrow of *Rag*$^{-/-}$ mice, B220$^+$ mature B cells were from the spleens of wild-type mice. Splenocytes from *Rag*$^{-/-}$ mice were depleted of red cells and used as an enriched source of NK cells. IEL $\alpha\beta$ and $\gamma\delta$ cells were purified according to Lefrancois *et al*. [S1]. Thymic and splenic dendritic cells were purified as described [28]. Double-positive (CD4$^+$CD8$^+$) and single-positive (CD4$^+$ or CD8$^+$) thymocytes were sorted from wild-type thymus and double-negative (CD4$^-$CD8$^-$) were obtained from thymocytes of *Rag*$^{-/-}$ mice that are arrested at this stage of differentiation. Developmental stages of double-negative thymocytes (CD4^{lo}, c-Kit$^+$CD25$^+$, c-Kit$^-$CD25$^+$, c-Kit$^-$, CD25$^-$) were sorted to 98–99% purity as described [20,S2].

Preparation of mammalian expression constructs and transfection of 293T cells

The full-length *Hel-1* or the *Hel-2* isoforms were amplified by PCR from thymocyte cDNA using primers generated to the 5′ or 3′ ends (5′F: AATTGAATTCATGCACTGCACTTTGACTATGG and 3′R: TTTTCCTTTTGCGGCCGCATGTCGCCATCCGAGGGAAGG) and cloned into CDM8 creating CDM8-Hel-1 and CDM8-Hel-2. Additional constructs were generated containing the FLAG or hemagglutinin (HA) tags (FLAG–Hel-1, FLAG–Hel-2, HA–Hel-1, HA–Hel-2). 293T cells were transfected and immunoprecipitations were performed as described [14]. Western blot analysis was as described above except that, for 293T extracts, incubation with affinity-purified polyclonal antibodies specific for Ikaros [12], Aiolos [10] or Helios was followed by incubation with peroxidase-coupled goat anti-rabbit secondary antibody. For immunoprecipitation from primary cells, thymocytes or splenocytes were obtained from transgenic mice expressing the FLAG-tagged dominant-negative Ikaros isoform Ik-7 from the CD2 minigene [33]. Cells were harvested and washed in PBS/2% FCS, and 1×10^7 cells were lysed in 100 µl lysis buffer as described [14].

Confocal Immunofluorescence

Primary thymocytes or splenocytes were obtained as previously described [6]. Thymocytes were activated for 40 h on plates precoated with 20 µg/ml CD3 [8]. Cells were harvested and washed in PBS. Onto each slide, 1×10^5 cells were cytospun and fixed in 4% paraformaldehyde, 0.5% TWEEN in PBS at 4°C and then washed in PBS. Prior to antibody incubation, cells were blocked for 1 h in 3% BSA, 1% goat serum, 1% donkey serum in PBS. Slides were then incubated with a 1:50 dilution of primary affinity-purified anti-Helios antibody in blocking buffer overnight at 4°C, followed by a 60 min incubation at room temperature with a 5 ng/µl biotinylated goat anti-rabbit IgG (Jackson Labs). Each antibody incubation step was followed by three washes in PBS. A 45 min incubation with 5 ng/µl avidin–FITC (Southern Biotechnology Associates) in 1% dialyzed FCS/3% BSA in PBS was carried out for detection. For double staining, an overnight 4°C incubation with affinity-purified polyclonal anti-Aiolos antibody directly coupled to the Alexa 568 fluorophore (Molecular Probes) was performed as the final step. For triple staining of Helios, Aiolos and the FLAG-tagged Ik-7 in cells from transgenic mice, slides were additionally incubated for 60 min at room temperature with 5 ng/µl anti-FLAG M5 monoclonal antibody (Kodak), washed and then incubated for 60 min with a 5 ng/µl Cy5-coupled goat anti-mouse antibody (Zymed). Specific staining was visualized by confocal immunofluorescence microscopy (Leica).

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