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Evolution of the Ikaros family transcription factors: From a deuterostome ancestor to humans

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ABSTRACT

Ikaros family proteins (Ikaros, Helios, Aiolos, Eos) are zinc finger transcription factors essential for the development and function of the adaptive immune system. They also control developmental events in neurons and other cell types, suggesting that they possess crucial functions across disparate cell types. These functions are likely shared among the organisms in which these factors exist, and it is thus important to obtain a view of their distribution and conservation across organisms. How this family evolved remains poorly understood. Here we mined protein, mRNA and DNA databases to identify proteins with DNA-binding domains homologous to that of Ikaros. We show that Ikaros-related proteins exist in organisms from all four deuterostome phyla (chordates, echinoderms, hemichordates, xenacoelomorpha), but not in more distant groups. While most non-vertebrates have a single family member, this family grew to six members in the acoele worm *Hofstenia miamia*, three in jawless and four in jawed vertebrates. Most residues involved in DNA contact from zinc fingers 2 to 4 were identical across the Ikaros family, suggesting conserved mechanisms for target sequence recognition. Further, we identified a novel KRKxxxPxK/R motif that inhibits DNA binding in vitro which was conserved across the deuterostome phyla. We also identified a EψψxxxψM(D/E)QAIXXAIxYLG(A/D/E)xL motif conserved among human Ikaros, Aiolos, Helios and subsets of chordate proteins, and motifs that are specific to subsets of vertebrate family members. Some of these motifs are targets of mutations in human patients. Finally we show that the atypical family member Pegasus emerged only in vertebrates, which is consistent with its function in bone. Our data provide a novel evolutionary perspective for Ikaros family proteins and suggest that they have conserved regulatory functions across deuterostomes.

1. Introduction

The mammalian Ikaros-related transcription factors are Ikaros (IKZF1), Helios (IKZF2), Aiolos (IKZF3) and Eos (IKZF4). They are C2H2 zinc finger (ZF) proteins that share a 4-ZF DNA-binding domain (DBD) near the N-terminus, and a 2-ZF C-terminal dimerization domain (DD) [1]. The other domains (N-terminal and inter-ZF regions) are less well understood and more variable. While Ikaros family factors have mostly been studied for their critical roles in the development and function of the immune system [2], they have more recently been implicated in the control of key developmental decisions in other cell types, like neurons or the outer hair cells of the cochlea [3–5]. Thus, Ikaros family proteins

may exert essential and conserved functions across disparate cell types. Understanding how their protein sequences evolved may offer insights into these core functions, since the most important features will be conserved as a result of selective pressure. Further, since a given protein will probably exert similar functions in different organisms, the analysis of Ikaros-related proteins within the animal kingdom may offer new clues about their functions.

Very little is known about the evolution of Ikaros family proteins. They share some features with the *Drosophila* Hunchback transcription factor which, like Ikaros, has a C-terminal 2-ZF DD and a central 4-ZF DBD [6]. However, while the DDs of Hunchback and Ikaros share clear homology, their DBDs are unrelated beyond the generic ZF

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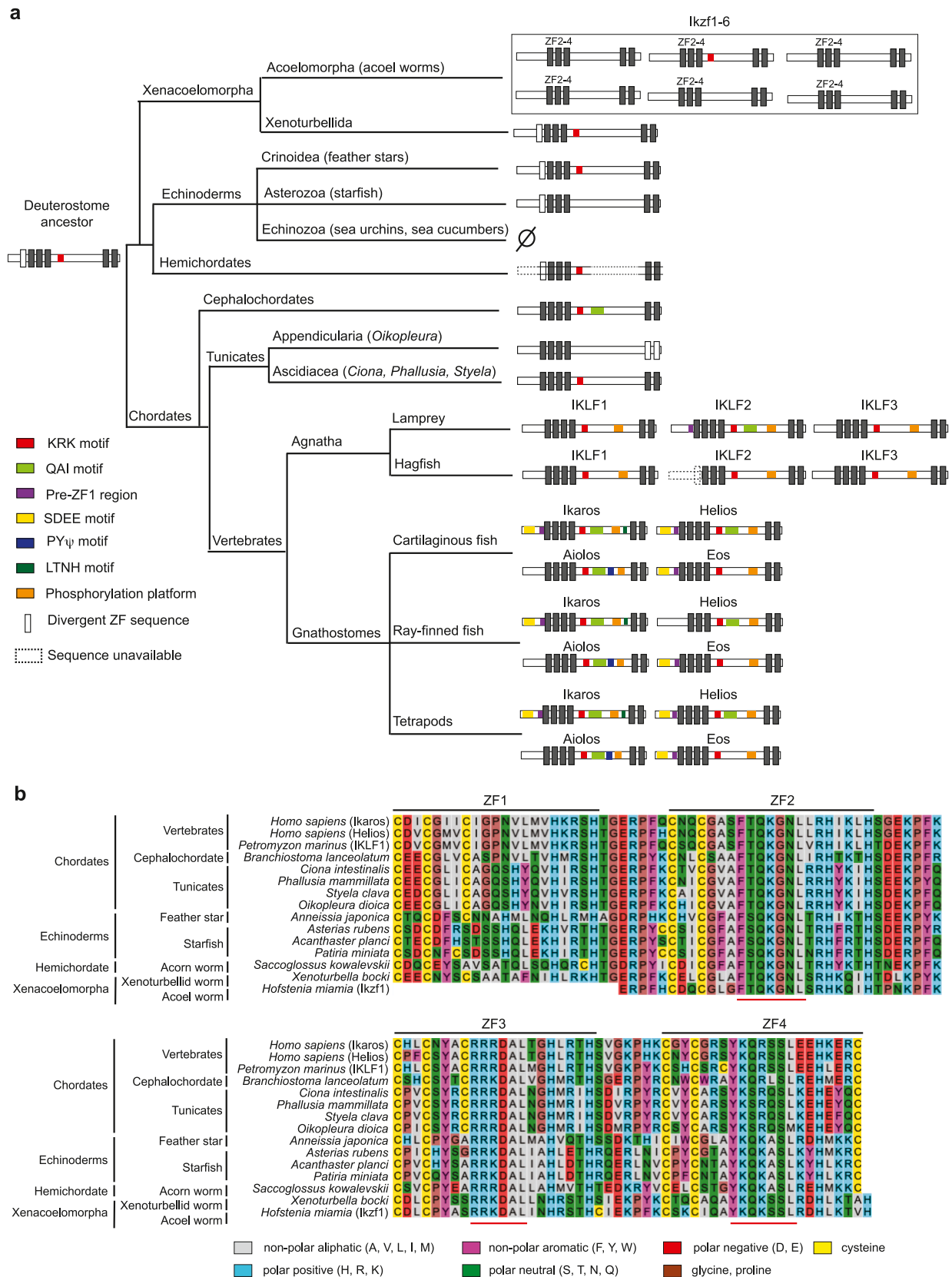


Fig. 1. Existence of Ikaros family members across deuterostomes. (a) Schematic representation of family members in deuterostomes. **(b)** Alignment of family member DBDs. Conserved regions in the alpha-helices involved in DNA binding are underlined. Amino acids are color-coded according to their physico-chemical properties (CINEMA code). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

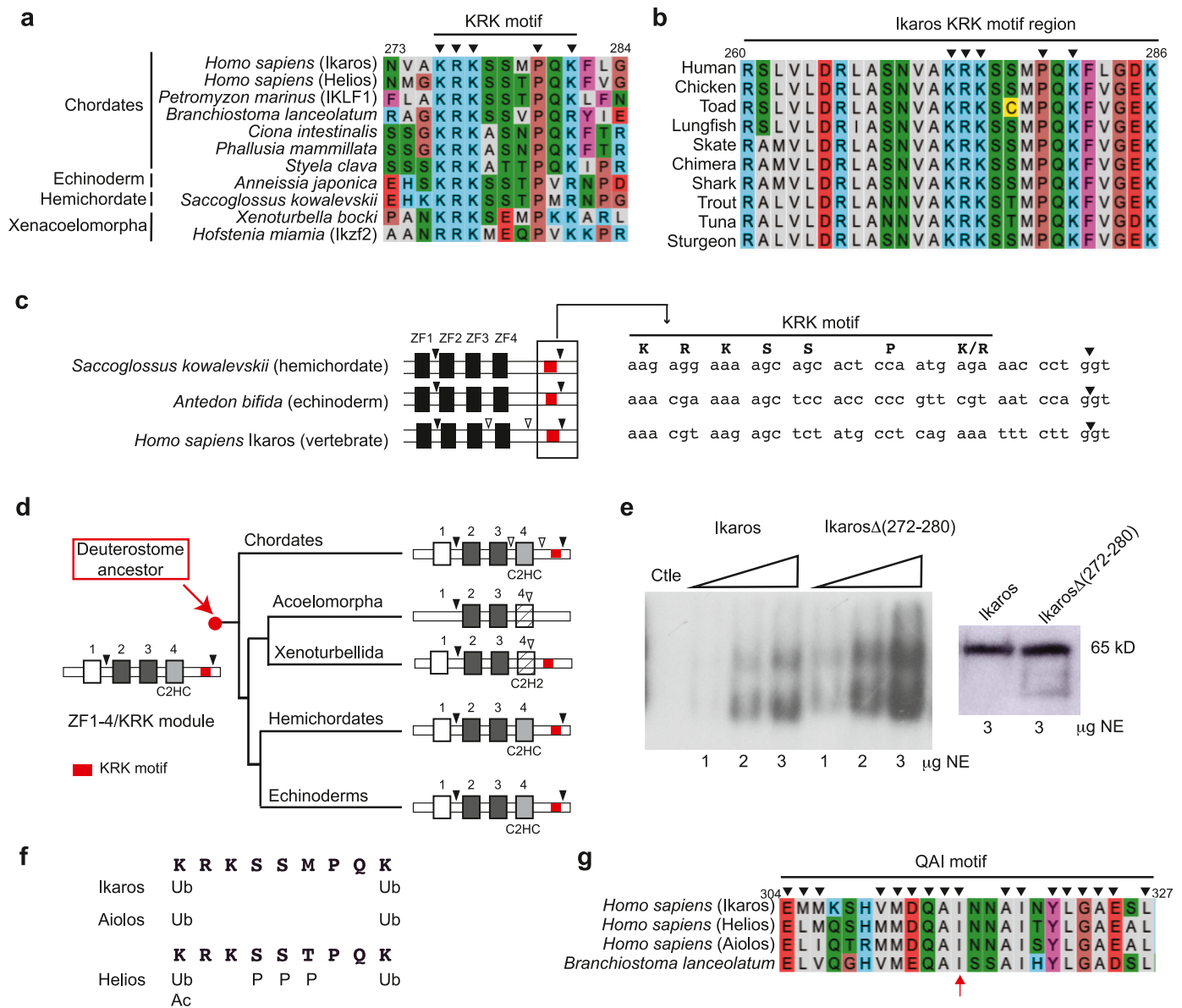


Fig. 2. Novel motifs conserved in deuterostome or chordate family members. (a) Alignment of the regions containing the KRK motif from the indicated family members. (b) Alignment of the KRK motif region of Ikaros proteins. (c) Splicing pattern of the ZF2-4/KRK region in echinoderms, hemichordates and vertebrates. (d) Proposed evolution of the core ZF1-4/KRK region in deuterostomes. Black and white arrowheads respectively indicate conserved and non-conserved splice junctions. The phylogenetic relationship of the four deuterostome phyla is from Ref. [9]. (e) Left: EMSA showing binding of increasing quantities of mouse Ikaros proteins (WT or deleted for the KRK motif) to the BS4 probe. CtIe: nuclear extracts (NE) from Cos cells transfected with the empty vector. Right: Western blot showing that WT and mutant proteins were expressed at similar levels. Representative of 3 experiments. (f) Post-translational modifications in the KRK motif in human Ikaros, Aiolos and Helios (PhosphositePlus). Ub: ubiquitination; Ac: acetylation; P: phosphorylation. (g) Alignment of the regions containing the QAI motif from indicated family members. In (a) and (g), arrowheads point to motif-defining residues.

structure. Proteins with an Ikaros/Hunchback-like C-terminal DD, and varying numbers of other ZFs unrelated to those of the Ikaros DBD, also exist in most animals including vertebrates, in which Pegasus (IKZF5) is a distant family member [7,8].

The animal kingdom is divided into eleven phyla (Fig. S1). The most distant phyla from humans are porifera (sponges) and cnidaria (jellyfish, corals, sea anemones); the others can be further divided into two broad groups based on developmental similarities and molecular taxonomy. One group corresponds to protostomes, which include nematodes, annelids, plathelminths, mollusks and arthropods. The second group corresponds to deuterostomes, which includes *Homo sapiens*. Within deuterostomes, the chordate phylum is the largest and is characterized by the presence of a dorsal neural chord during development; it comprises vertebrates, tunicates (sea squirts) and cephalochordates

(elongated marine invertebrates related to lancelets). Other deuterostome phyla are echinoderms (starfish, sea urchins, feather stars), hemichordates (acorn worms) and xenacoelomorpha (xenoturbellid worms, acel worms) [9].

Here we evaluated the proteins homologous to Ikaros in both their DBD and DD (hereafter referred to as “Ikaros family proteins”). While it is well established that jawed vertebrates (gnathostomes) possess four family members orthologous to Ikaros, Helios, Aiolos and Eos [10], the physiognomy of this family beyond gnathostomes remains poorly explored. Two family members have been documented in jawless vertebrates (agnatha), but it is unclear if they represent the full complement of agnatha family members, and if they are orthologs of the gnathostome Ikaros, Helios, Aiolos or Eos [11,12]. This question is important for understanding how Ikaros family proteins became involved in

controlling adaptive immunity, a biological function that also exists in agnatha. Beyond vertebrates, Ikaros family proteins have also been reported in tunicates, the class of chordates closest to vertebrates [7,13,14]. It remains unknown if they also exist in the more distant cephalochordates, or other deuterostomes.

Here we report that Ikaros family proteins exist in all deuterostome phyla, but not in protostomes, and that they show a remarkable conservation of the DNA-binding domain. This finding indicates that Ikaros family proteins have conserved similar mechanisms of target gene binding over >600 million years of evolution in many different classes of organisms.

2. Methods

2.1. Mining of databases

To identify family members among referenced proteins from a given taxon, we used Blastp, using sequences from the Ikaros DBD as query. To identify nucleotide sequences (mRNA or DNA) encoding Ikaros-related proteins, we used tBlastn, which compares all translated reading frames from the target databank to a query protein sequence. This strategy reliably detected family members across taxa, as the IKZF1-4 genes were the top hits when the human genome was probed with echinoderm sequences. Genomes were accessed through the Refseq Genome Database, or through non-assembled genomic sequences for organisms where fully assembled genomes were lacking (*Hofstenia miamia*, *Saccoglossus kowalevskii*, *Eptatretus burgeri*). Multi-sequence alignments were done with Clustal-omega (online tools from Uniprot or EMBL-EBI). Identity percentages were obtained with EMBOSS Needle (EMBL-EBI). Sequence accession numbers are provided in Table S1 cDNA and protein sequences inferred from genomic sequences are provided in Supplementary file S1. Post-translational modifications were from PhosphositePlus, a repository that compiles data from large scale proteomic studies of mouse and human cells.

2.2. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (3 µg) from Cos cells transfected with the appropriate expression vector were first preincubated for 30' on ice with 0.5 µg poly-dIdC (Merck-Sigma) in a total volume of 20 µl of binding buffer (20 mM Hepes pH 7.9, 0.2 mM EDTA, 20 % glycerol, 100 mM KCl, 1 mM DTT). 100 fmoles (2 µl) of ³²P-labelled BS4 probe (5'-TCAGCTTTTGG-GAATGTATCCCTGTCA) were then added, incubated on ice for 30', before electrophoresis on a 5 % polyacrylamide gel and autoradiography.

3. Results and discussion

3.1. Ikaros family factors exist throughout deuterostomes

To search for Ikaros family proteins in non-vertebrate taxa, we mined NCBI protein or mRNA databanks, using the DBD of human Ikaros as bait. To circumvent potential holes in these databases, we also searched for IKZF-related genes in available genomic DNA sequences. While no Ikaros family proteins were found in porifera, cnideria or protostomes, such proteins were identified in all deuterostome phyla (Fig. 1, Table S2). Ikaros family proteins thus emerged ~670 million years ago in a deuterostome ancestor [15]. Vertebrates had multiple family members, but non-vertebrates had one, in most cases (Fig. 1a, Table S2). The exceptions were echinozoa (sea urchins, sea cucumbers) which lacked Ikaros family members, and acol worms (*Hofstenia miamia*), which had a record number of six (Figs. S2a–b).

Almost all deuterostome organisms displayed Ikaros family proteins with a DBD containing 4 ZFs and a C-terminal DD, except *Hofstenia miamia* whose family members lacked ZF1 (Fig. 1b, Fig. S2a and S3a–b). ZF2-4 were highly conserved, especially the central α -helices involved in

protein-DNA contacts where most residues were identical among family members (underlined in Fig. 1b). This remarkable feature indicated that Ikaros family members had conserved a similar mode of DNA-binding throughout their evolution. In contrast, ZF1 was less conserved among family members from different phyla, or even within chordates. This, together with the loss of ZF1 in family members from *Hofstenia* and in Helios proteins from ray-finned fish (Fig. 1a), and its absence in several xenoturbellid family member isoforms (Fig. S2c), indicates that ZF1 is less central to DNA-binding than the other 3 ZFs. Of note, xenacoelomorpha family members also had an atypical C2H2 ZF4 rather than the typical C2HC (Fig. 1b); this resulted from the appearance of a novel splice donor site, which leads to the loss of the generic cysteine and the appearance of a histidine encoded by a subsequent exon (Fig. S2d).

The existence of Ikaros family members with highly conserved DBDs in all deuterostome phyla suggested that an ancestral function was conserved. We speculate that one function may be related to regulation of cellular plasticity and/or stem cell gene expression programs, as suggested by data from the mouse and human systems. For instance, Ikaros represses stem cell gene expression programs in lymphocytes, while Aiolos confers lymphocyte-like properties to lung cancer cells [16,17]. Since acol worms possess exceptional cellular reprogramming properties [18], it is tempting to speculate that the unusual expansion of the Ikaros family in these organisms might be related to their ability to remodel cell fates.

3.2. Identification of novel “KRK” and “QAI” motifs in deuterostomes

Alignment of family member amino acid (aa) sequences revealed a novel KRKxxxPx(K/R) motif (“KRK” motif hereafter), ~50 residues downstream of the DBD, that was present in organisms from all deuterostome phyla (Fig. 1a and 2a), indicating that it was present in the ancestral protein. It was absent in five of the six *Hofstenia* proteins, and family members from starfish and the tunicate *Oikopleura dioica* (Fig. 1a). This motif was present in all vertebrate proteins and, for any given vertebrate family member, its surrounding aa were among the most conserved sequences within these proteins, highlighting its functional importance (see conservation for Ikaros proteins in Fig. 2b).

Analysis of splicing patterns revealed that the KRK motif and ZF2-4 were encoded by a single exon in the echinoderm *Antedon bifida* and the hemichordate *Saccoglossus kowalevskii* (Fig. 2c; see Figs. S3c and S4 for more complete data on family member splicing patterns). The 5' splice site of this exon was conserved throughout the family while the 3' splice site was conserved only in vertebrates (Fig. 2c and Fig. S3c). Since the chordate phylum (to which vertebrates belong) is the most distant from the echinoderm and hemichordate phyla [9], this observation suggested that the KRK motif and ZF2-4 form part of an ancestral core module of Ikaros family members (Fig. 2d). Despite the presence of several positively charged residues reminiscent of nuclear localization signals, the nuclear localization of Ikaros was unaffected upon deletion of the KRK motif (unpublished results). However, Ikaros proteins lacking this motif showed enhanced DNA binding in vitro (Fig. 2e), indicating that the KRK motif inhibits DNA binding. The first and last lysine residues of the human Ikaros, Aiolos and Helios KRK motifs were also identified as targets of ubiquitination (Fig. 2f). The role of ubiquitination, and how these modifications may influence DNA binding, remains unclear.

We also found conserved motifs that were shared between subsets of taxa. All Ikaros family proteins from echinoderms shared motifs in the N-terminal and inter-ZF regions (Fig. S5). Mammalian Ikaros, Aiolos, Helios and the cephalochordate *Branchiostoma lanceolatum* family member shared a E ψ ψxxxψM(D/E)QAIxxAIxYLGA(D/E)xL motif (ψ = V, L, I, M) in their inter-ZF region (“QAI” motif hereafter; Fig. 2g). This motif was not present in Eos or family members from echinoderms, xenacoelomorpha and tunicates (sequences for this region were not available for hemichordates). Since cephalochordates are more distant than tunicates from vertebrates [14], this motif was likely present in the chordate

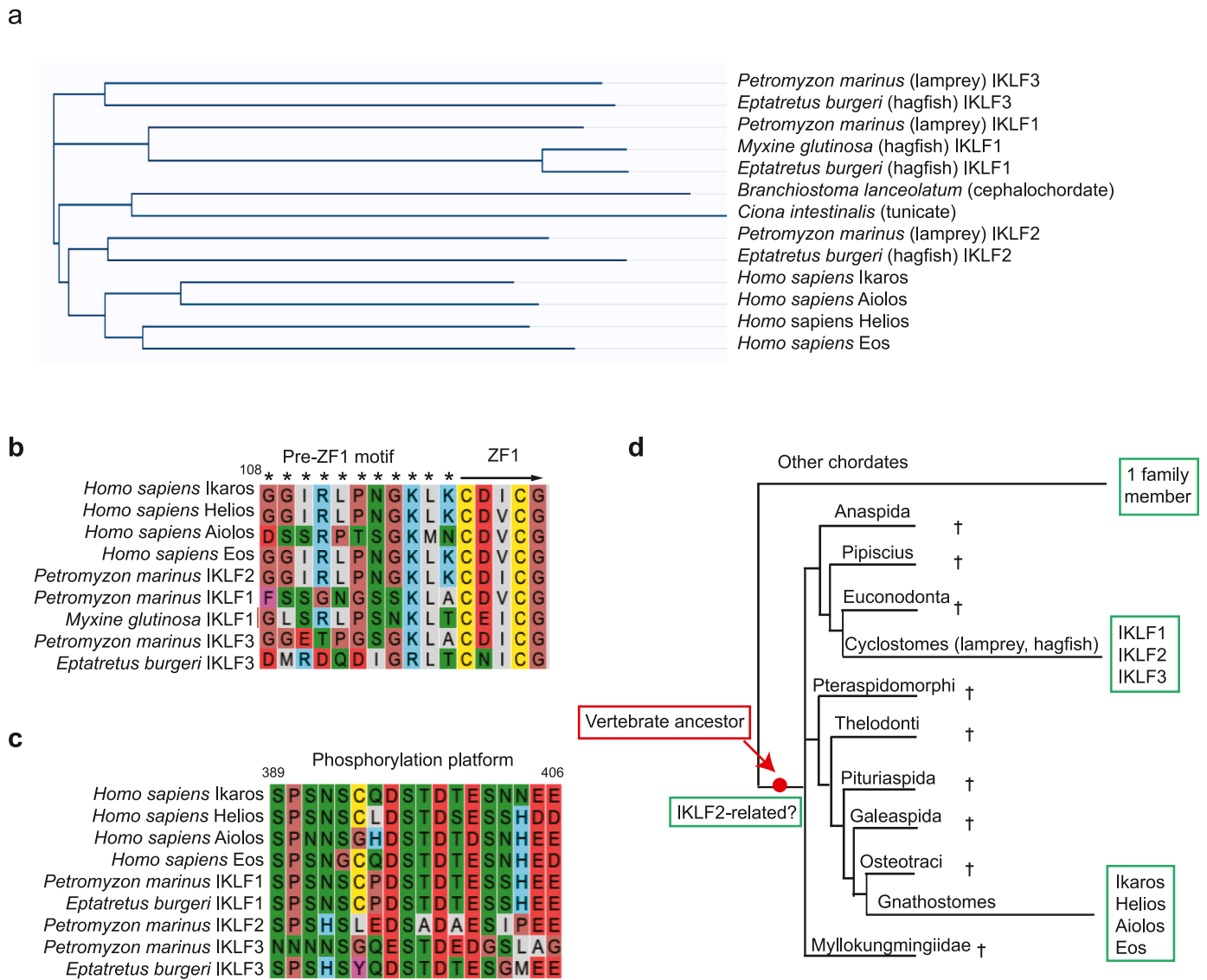


Fig. 3. Relationship between family members from agnatha and gnathostomes. (a) Phylogenetic tree of indicated family proteins. **(b)** Alignment of the region preceding ZF1 for the indicated proteins. Asterisks indicate residues conserved between *Petromyzon* IKLF2 and human Ikaros, Helios and Eos asterisks. **(c)** Alignment of the region homologous to the phosphorylation platform of indicated family members. **(d)** Proposed evolution of vertebrate family members from a single IKLF2-related ancestor. Scheme adapted from Ref. [23] and Wikipedia.

ancestor, and subsequently lost in tunicates. Its integrity is crucial for the function of Helios, as a homozygous mutation of the central isoleucine to valine in humans leads to severe immune dysfunction and altered interactions with partners (Fig. 2g [19]).

3.3. Divergent evolution of the family in jawless and jawed vertebrates

Previous studies have shown that all gnathostomes have four family members orthologous to Ikaros, Aiolos, Helios and Eos [10]. It is currently unclear if orthologs also existed in agnatha. Understanding the physiognomy of the family in agnatha is important, as it may help understand the family make-up in primitive vertebrates, where adaptive immunity is thought to have evolved [20]. We found three orthologous family members, IKLF1-3 (for “Ikaros-like factor”) in lamprey and hagfish, the only modern agnatha (Fig. 3a and Fig. 3S6; see Legend to Fig. S6 for further details). Phylogenetic analyses indicated that the four gnathostome proteins were more related to agnatha IKLF2 than to IKLF1 or -3 (Fig. 3a). The relatedness of IKLF2 and gnathostome Ikaros family proteins was especially apparent in the sequences surrounding the RKR

and QAI motifs (Figs. S6c–d), and in a GGIRLPNGK sequence immediately N-terminal of ZF1 (hereafter “pre-ZF1” sequence), which was identical between lamprey IKLF2 and mammalian Ikaros, Helios and Eos (Fig. 3b). This sequence was also detected in a degenerate form in IKLF1 and -3, but not in non-vertebrate family members. Similarly, we identified a region near the C-terminus known to act as a phosphorylation platform [21,22], that was conserved among all agnatha (notably IKLF1) and gnathostome family members (Fig. 3b), but absent in non-vertebrates. Thus the vertebrate-specific domains probably appeared in a common ancestor for agnatha and gnathostome Ikaros family proteins.

However, the agnatha family members lacked the defining features of gnathostome Ikaros, Helios, Aiolos or Eos (see below), and are therefore not their orthologs. We propose that the agnatha and gnathostome family members evolved independently from a single, IKLF2-related, vertebrate ancestral protein. This is consistent with the facts that non-vertebrate chordates have a single family member, and that the lineages leading to modern agnatha (both cyclostomes) and gnathostomes diverged very early during vertebrate evolution (Fig. 3d [23]).

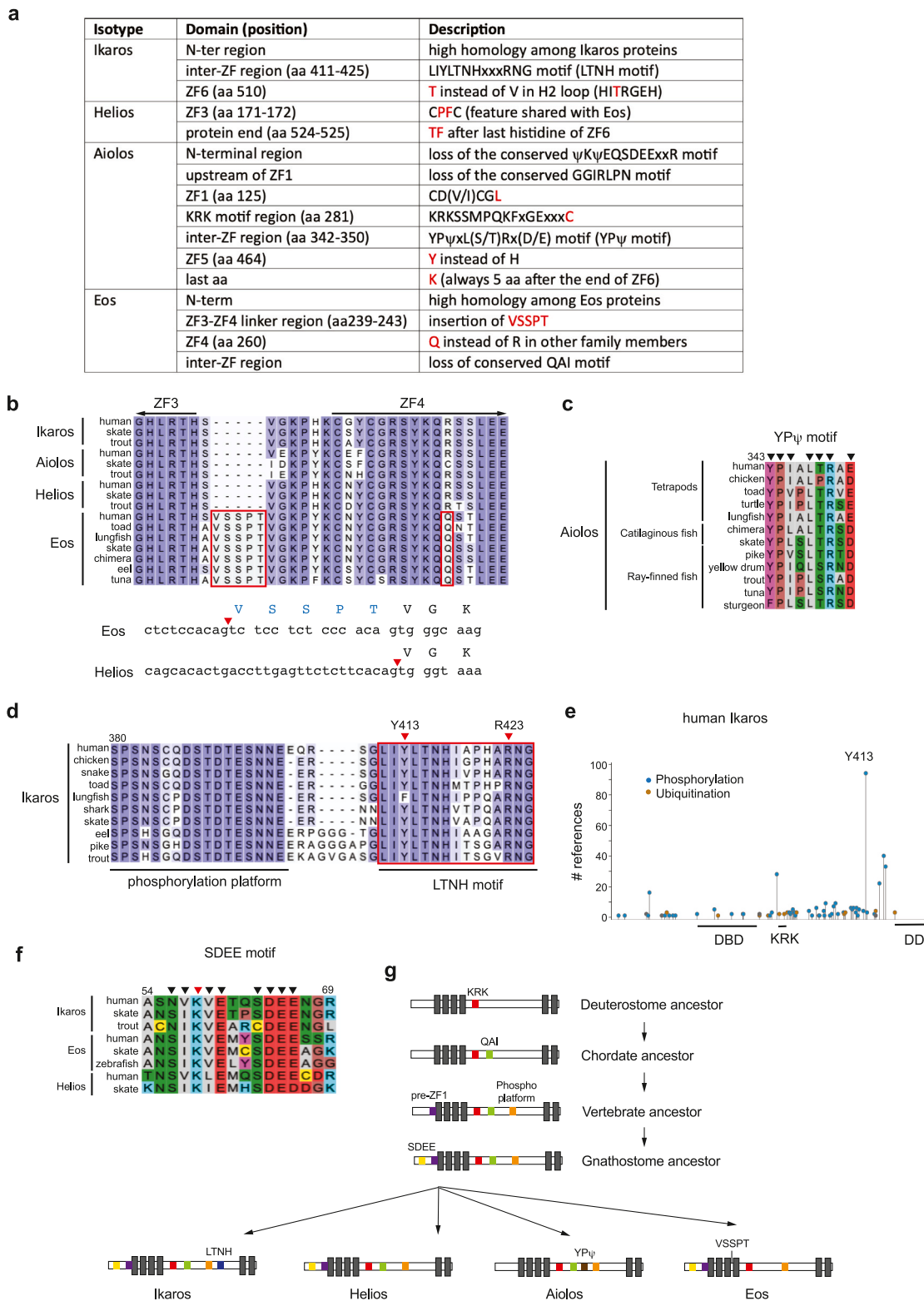


Fig. 4. Specific features of gnathostome family members. (a) Main features defining Ikaros, Helios, Aiolos and Eos. Amino acid numbers correspond to human proteins. (b) Top: alignment of the ZF3/ZF4 region of indicated proteins. Red boxes: Eos-specific features. Bottom: 5' splice sites of the ZF4-encoding exon for human Eos and Helios. (c) Alignment of the YP ψ motif sequences from Aiolos proteins from the indicated species. (d) Conservation of a C-terminal Ikaros-specific LTNH motif. Y413 and R423 correspond to residues mutated in human patients with therapy-resistant multiple myeloma or Diamond-Blackfan anemia. (e) Map of the post-translational modifications detected in human Ikaros (PhosphositePlus). (f) Alignment of the region containing the SDEE motif from the indicated proteins. Red arrowhead: sumoylated lysine in mouse Ikaros. (g) Scheme depicting the gradual appearance and disappearance of specific motifs in the mammalian Ikaros family members. In (c) and (f), arrowheads point to motif-defining residues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Ikaros, Helios, Aiolos and Eos

The independent expansion of the Ikaros family in acoele worms, agnatha and gnathostomes suggested that each family member acquired some functional specificity, as reflected by the unique features of these proteins. We took advantage of the large panel of available gnathostome sequences to identify features that distinguish Ikaros, Helios, Aiolos and Eos, which were conserved in cartilaginous fish, ray-finned fish and tetrapods (Fig. 4a and Fig. 3S7a). Most strikingly, Eos acquired a 5-aa insertion between ZF3 and ZF4, which resulted from a 5' extension of the ZF4-encoding exon (Fig. 4b). Aiolos contains a specific YP ψ xL(S/T) Rx(D/E) motif in the central part of its inter-ZF region ("YP ψ " motif; Fig. 1a and Fig. 4c), while Ikaros proteins have a unique LIYLTNHxxxxxRNG motif ("LTNH" motif) near their C-terminus (Fig. 1a and Fig. 4d). Mutations in this motif have been found in human patients with Diamond-Blackfan anemia (R423 to C, [24]) and therapy-resistant multiple myeloma (Y413 to C [25]). Interestingly, Y413 is also a target of phosphorylation, and by far the most frequently modified aa in Ikaros (Fig. 4d). In addition, family member identity was also defined by the loss of features. Eos lost the QAI motif, while Aiolos lost the pre-ZF1 motif (Fig. S7a). All Aiolos proteins and Helios proteins from ray-finned fish also lost a ψ K ψ EQSDEExxR motif ("SDEE" motif) in the N-terminal region that was shared by Ikaros, Eos and Helios proteins from tetrapods and cartilaginous fish (Fig. 1a and 4f and Fig. S7b). The lysine within this motif is a target for sumoylation in Ikaros [26].

Among the four family members, Aiolos was the least conserved, which was most apparent in the N-terminal sequence (Fig. S7b), and in the loss of the QAI motif and phosphorylation platform in ray-finned fish (Fig. S7c). Since Aiolos is selectively expressed in mature lymphocytes in mammals, these differences may be linked to changes in adaptive immunity in vertebrates. On the other hand, Ikaros, Helios and Eos regulate functions of more cell types (eg. hematopoietic and neuronal cells), which may impose stronger constraints on their sequence. Altogether, these comparisons showed that every family member has a distinct identity that is conserved across gnathostomes. Our data provide a picture of how these identities were constructed, with gains and/or loss of motifs at various junctures during deuterostome evolution (Fig. 4g).

3.5. Pegasus

Pegasus is a distant family member in mammals, with a unique 3-ZF DBD. Human Pegasus mutations are associated with impaired maturation of platelets [27], and unpublished phenotyping data of Ikzf5 KO mice from the KOMP consortium have revealed a role for Pegasus in bone mineralization (data available at <https://www.mousephenotype.org/data/genes/MGI:1914393>). To gain insight into when Pegasus appeared, we searched for its homologs in various taxa, and found Pegasus proteins with highly conserved DBDs and DDs in both gnathostomes and agnatha (Figs. S8a–b). We also found tunicate proteins with DBDs and DDs bearing loose homology with those of Pegasus (Fig. S8c). No other Pegasus-related proteins were found elsewhere. Vertebrate Pegasus also showed strong conservation of sequences in the N-terminal and inter-ZF regions (Fig. S8d), which were not observed in the tunicate homologs. Thus, the origin of Pegasus can be traced to a common ancestor of tunicates and vertebrates, but its identity was clearly established in vertebrates. Pegasus was particularly well conserved in mammals, which have nearly identical Pegasus sequences (Fig. S9a). Strikingly, sequence conservation was markedly higher for mammalian Pegasus than Ikaros (Fig. S9b). These observations point to particularly strong constraints to preserve the Pegasus sequence in mammals. This selective pressure may be related to its function in platelets, which exist only in mammals [28].

The evolutionary history of Pegasus is therefore different from that of Ikaros. In contrast to Ikaros which has ancient roots, Pegasus appeared more recently. We speculate that these differences reflect the different functions of Pegasus vs Ikaros-related proteins. While Ikaros-related

proteins may control fundamental processes such as maintenance of cell identity, Pegasus appears to be involved in more narrow, vertebrate-specific, functions.

4. Conclusion

We found that Ikaros family proteins appeared in a deuterostome ancestor. These ancient roots, together with the remarkable conservation of the DNA-binding domain, point to the existence of core ancestral functions that extend beyond their well studied role in the adaptive immune system.

CRedit authorship contribution statement

Philippe Kastner: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Adina Aukunova:** Investigation. **Susan Chan:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2023.149399>.

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