blood

2001 98: 1792-1801
doi:10.1182/blood.V98.6.1792

The zebrafish *klf* gene family

Andrew C. Oates, Stephen J. Pratt, Brenda Vail, Yi-lin Yan, Robert K. Ho, Stephen L. Johnson, John H. Postlethwait and Leonard I. Zon

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

The zebrafish *klf* gene family

Andrew C. Oates, Stephen J. Pratt, Brenda Vail, Yi-lin Yan, Robert K. Ho, Stephen L. Johnson, John H. Postlethwait, and Leonard I. Zon

The *Krüppel-like factor* (*KLF*) family of genes encodes transcriptional regulatory proteins that play roles in differentiation of a diverse set of cells in mammals. For instance, the founding member *KLF1* (also known as *EKLF*) is required for normal globin production in mammals. Five new *KLF* genes have been isolated from the zebrafish, *Danio rerio*, and the structure of their products, their genetic map positions, and their expression during development of the zebrafish have been characterized. Three genes closely related to mammalian *KLF2* and *KLF4* were found, as was an ortholog of mammalian *KLF12*. A fifth gene, apparently missing from the genome of mammals and closely related to *KLF1* and *KLF2,* was also identified. Analysis demonstrated the existence of novel conserved domains in the N-termini of these proteins. Developmental expression patterns suggest potential roles for these zebrafish genes in diverse processes, including hematopoiesis, blood vessel function, and fin and epidermal development. The studies imply a high degree of functional conservation of the zebrafish genes with their mammalian homologs. These findings further the understanding of the *KLF* genes in vertebrate development and indicate an ancient role in hematopoiesis for the *Krüppel-like factor* gene family. (Blood. 2001;98:1792-1801)

Introduction

The *Krüppel* gene of *Drosophila* is required for the correct formation of the embryonic body plan through its activity as a transcriptional repressor.[1-3] Members of the *Krüppel-like factor* (*KLF*) gene family have been discovered in mammals; they are expressed in a wide variety of tissues and encode both transcriptional activator and repressor proteins.[4] Recent targeted-deletion experiments in mice have revealed roles for members of the *KLF* family in erythroid cell maturation,[5,6] T-cell activation,[7] and blood vessel stability,[8] as well as skin permeability.[9] These results indicate that the mammalian *KLF* gene family plays an important role in the control of hematopoietic and other cell type differentiation.

Although the DNA-binding activity of the highly conserved, C-terminal zinc finger domain of KLF proteins is clear, the function of diverged, N-terminal structures remains under investigation. Membership in the KLF family is defined by the presence of 3 tandem zinc finger motifs at the C-terminus of the protein with a F/Y-X-C-X_{2-4}-C-X_3-F-X_5-L-X_2-H-X-R/K-X-H consensus that are connected by a characteristic linker: T/S-G-E-R/K-P.[10] This domain confers sequence-specific DNA binding to GC-rich elements of the general structure CCN CNC CCN,[11] such as the CACCC-box,[12-16] and in vitro site selection has suggested the consensus sequence NNR CRC CYY.[17] The N-termini of KLF proteins are highly variable, and several are characterized by the presence of domains with transcriptional activator and/or repressor functions in vitro.[14-16,18-23] In addition, the N-terminus of some KLF proteins contains sites of phosphorylation and acetylation that may contribute to regulation of KLF activity.[24,25] Despite these studies, it remains an important task to identify functionally significant sites in KLF protein N-termini. Zebrafish is sufficiently diverged from mammals that amino acid sites unimportant for protein function will have diverged, leaving functionally important sites as islands of sequence conservation. Thus, the isolation of zebrafish homologs of the mammalian *KLF* genes and comparison of the N-terminal regions would allow the demarcation and functional testing of evolutionarily conserved domains.

Loss of *Klf1 (erythroid Krüppel-like factor, EKLF)* gene function in mice results in a lethal β-thallassemia.[6,26] To determine whether any of the hypochromic, thalassemialike phenotypes seen in zebrafish[27,28] might be the result of a lesion in a homolog of the *Klf1* gene, we have isolated related members of the *KLF* gene family from the zebrafish and determined their structure, chromosomal position, and expression during embryogenesis. The isolation of novel zebrafish *klf* genes may point to the existence of previously unsuspected mammalian orthologs, and their expression patterns in the developing fish may help to understand more rapidly the estimated 300 zinc finger–containing genes in the human genome.[29] We found that 3 of the 5 zebrafish *klf* genes are expressed in the developing hematopoietic system and another is expressed in developing vasculature, suggesting an ancient evolutionary role for the *KLF* gene family in generation of the blood.

From the Howard Hughes Medical Institute, Children's Hospital, Boston, MA; Ludwig Institute for Cancer Research, Melbourne Tumour Biology Branch, Royal Melbourne Hospital, Victoria, Australia; Department of Molecular Biology, Princeton University, Princeton, NJ; Department of Genetics, Medical School, Washington University, St. Louis, MO; and Institute of Neurosciences, University of Oregon, Eugene.

Submitted March 19, 2001; accepted May 18, 2001.

Supported by a Postdoctoral Fellowship from the Ludwig Institute for Cancer Research (A.C.O.), National Institutes of Health (NIH) grants RO1RR10715 and 5-PO1-HD22486 (J.H.P.), the W. M. Keck Foundation 961582, M. J. Murdoch Charitable Trust 96127, National Science Foundation ST19602828 and NIH 1-G20-RR11724 (renovation of the University of Oregon zebrafish facility), and Associate Scholar of the Howard Hughes Medical Institute and NIH (L.I.Z.).

Reprints: Leonard I. Zon, Howard Hughes Medical Institute, Children's Hospital, Enders Building, Room 761, 320 Longwood Avenue, Boston, MA 02115; e-mail: zon@smtp.ne.mediaone.net.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

Materials and methods

Isolation of zebrafish *klf* cDNA

To isolate zebrafish *klf* cDNAs, a 1120-bp *Bam*HI/*Nco*I fragment from the mouse *Klf1* cDNA clone[12] was hybridized to 5.0×10^5 plaques of an adult zebrafish kidney cDNA library in pBK-CMV using standard techniques. More than 80 positive clones were originally isolated, and 32 were examined more extensively by sequencing. Recently, the Human Gene Nomenclature Committee has standardized the naming of this gene family. We will use the new gene names in this article, but supply the old name of a gene where it appears first in the text. Gene accession numbers of the *klf* cDNA sequences are as follows: *klf2a*, AF392992; *klf2b*, AF392995; *klf4*, AF392994; *klfd*, AF392996; *klf12*, AF392993.

Phylogenetic analysis

Alignment of sequences was performed using the ClustalX near neighbor joining algorithm,[30,31] with some adjustment by eye. Sequence accession numbers of the *KLF* genes used in these analyses are as follows: *Hs KLF1*, U37106; *Mm Klf1*, AF033102; *Hs KLF2*, NM_006075; *Mm Klf2*, U25096; *Hs KLF3*, NM_016531; *Mm Klf3*, U36340; *Hs KLF4*, O43474; *Mm Klf4*, U70662; *Hs KLF5*, Q13887; *Hs KLF6*, U44975; *Hs KLF7*, AF001461; *Hs KLF8*, NP_009181; *Hs KLF12*, NP_009180; *Mm Klf12*, Y14295; *Hs SP1*, P08047; *Ce F56F11*, AF099922; *Ce F54H5*, U80952; and *Dm Krüppel*, AAF47321; where *Hs*, *Mm*, *Ce*, and *Dm* stand for *Homo sapiens*, *Mus musculus*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, respectively.

Phylograms were built using the output.dnd and .phb files from ClustalX in Treeview PPC.[70]

Genetic mapping

Gene mapping on the mother of pearl (MOP) cross-meiotic panel was done as described,[32-34] as was typing on the Goodfellow hybridoma panel.[35]

In situ hybridization

Riboprobes were prepared and hybridization was performed as described previously,[36] with modifications.[37] For each *klf* gene, full-length cDNA was used to generate riboprobe by cutting with *Eco*R1 and transcribing with T7 RNA polymerase.

Results

To isolate the *Klf1* homolog in zebrafish, we hybridized a fragment of the cDNA of mouse *Klf1*[12] to a zebrafish adult kidney cDNA library because the kidney is the site of definitive hematopoiesis in the zebrafish. Sequence analysis of 32 positive clones demonstrated that these cDNAs represented 5 independent genes, each containing Krüppel-like zinc finger motifs. In 4 cases, the assignment of probable orthologies required information from both protein phylogeny and chromosomal localization (see below, *klf2a*, *klf2b*, *klf4*, and *klf12*). For the fifth gene, these data suggested that no ortholog exists in modern mammalian genomes, and this gene is designated *klfd*, consistent with zebrafish nomenclature procedures.

Phylogenetic and structural analysis of zebrafish *klf* genes

In an amino acid comparison with other *KLF* family members, the C-terminal regions of the 5 zebrafish genes were highly conserved (Figure 1A), consisting of 3 tandem zinc fingers. However, the regions outside the finger domain strongly diverged and cannot be reliably aligned across the entire *KLF* family. Of 83 amino acid positions in the conserved zinc finger domain, only 19 were phylogenetically informative, which limits the resolution of phylogenetic reconstruction. A probable phylogeny of this gene family based on the relations between the zinc finger domains is presented in Figure 1B.

Zebrafish Klf2a, Klf2b, and Klf4 proteins were most similar to mammalian Klf2 (lung Krüppel-like factor, Lklf[38]) and Klf4 (gut Krüppel-like factor, Gklf[39]) (Figure 1B,C). They were more closely related to each other than to either Klf2 or Klf4, however, although low bootstrap values precluded the assignment of direct orthologies to either of these mammalian genes on the basis of sequence alone. Examination of an amino acid alignment of the N-termini of this group of proteins revealed 5 highly conserved blocks of residues separated by divergent regions (numbered brackets in Figure 1C). These are potentially functional domains and include (1) the N-terminal–most 16 amino acids, (2) an acidic domain (D20 to T37 [single-letter amino acid code] of Klf2a), (3) a proline-rich domain (P75 to R106), and (4) a conserved block (F218 to D246) immediately N-terminal to (5) a basic domain (K254 to R266).

The Klf12 protein was more similar to mouse Klf12 (AP2-rep) and human KLF12 than to any other protein, including the closely related KLF3/Klf3 (BKLF/Bklf) and KLF8, which together form a clade (Figure 1B). Thus, it appears that zebrafish *klf12* is the ortholog of the mammalian *KLF12* genes (Figure 1B,D). Comparison of the amino acid sequences of the N-termini of these proteins revealed that they are less conserved than those of the KLF2/4 subfamily, with 2 regions of high similarity (indicated with brackets and numbers in Figure 1D): (1) from Q65 to K75 of Klf12, and (2) another cluster immediately N-terminal to the zinc finger domain (E221 to I242).

Although *klfd* was placed at the base of the *KLF2/4* subfamily by virtue of its zinc finger domain, it lies close to the stem of the *Klf1-Klf2/4* divergence in Figure 1B. We note that the bootstrap replication values for the nodes in the tree at this point were sufficiently low to regard this placement as unresolved with respect to membership in the *Klf1* or *Klf2/4* family. To place these putatively novel zebrafish *klf* genes more precisely in the evolutionary history of the *KLF* family, we determined their chromosomal positions and potential syntenic relations to mammalian chromosomes.

Genetic mapping of *klf* family members

Each of the *klf* genes was genetically mapped to the zebrafish genome (Table 1). *klfd*, *klf12*, *klf2b*, and *klf4* were analyzed by meiotic mapping on the MOP-cross DNA panel.[32-34] Both *klf2b* and *klf4* are found on linkage group 2 (LG2), whereas *klf12* is found on LG1 and *klfd* on LG6. *klf2a* was mapped using a radiation hybrid panel,[35] placing it on LG22. None of these positions correspond to the 6 published hematopoietic mutant positions: *sauternes*,[40] *weissherbst*,[41] *dracula*,[42] *yquem*,[43] *riesling*,[44] or *spadetail*.[45]

The orthology assignments of the zebrafish *klf* genes based on amino acid sequences were reassessed and refined using information about syntenic chromosomal regions between fish and mammals[34,46-48] and are described in Table 1. This analysis will be presented in depth elsewhere (manuscript in preparation), but is beyond the scope of the current report. The syntenic relations between the chromosomes containing the zebrafish *klf2a*, *klf2b*, and *klf4* genes and their closest known mammalian homologs provide support for the notion that *klf2a* and *klf2b* are collectively orthologous to *KLF2* and that *klf4* is orthologous to the mammalian *KLF4* gene. The syntenic relations between the chromosomes containing *klf12* and human *KLF12* support the phylogenetic analysis showing that these genes are likely orthologous. Finally, *klfd* is found in a chromosomal region that

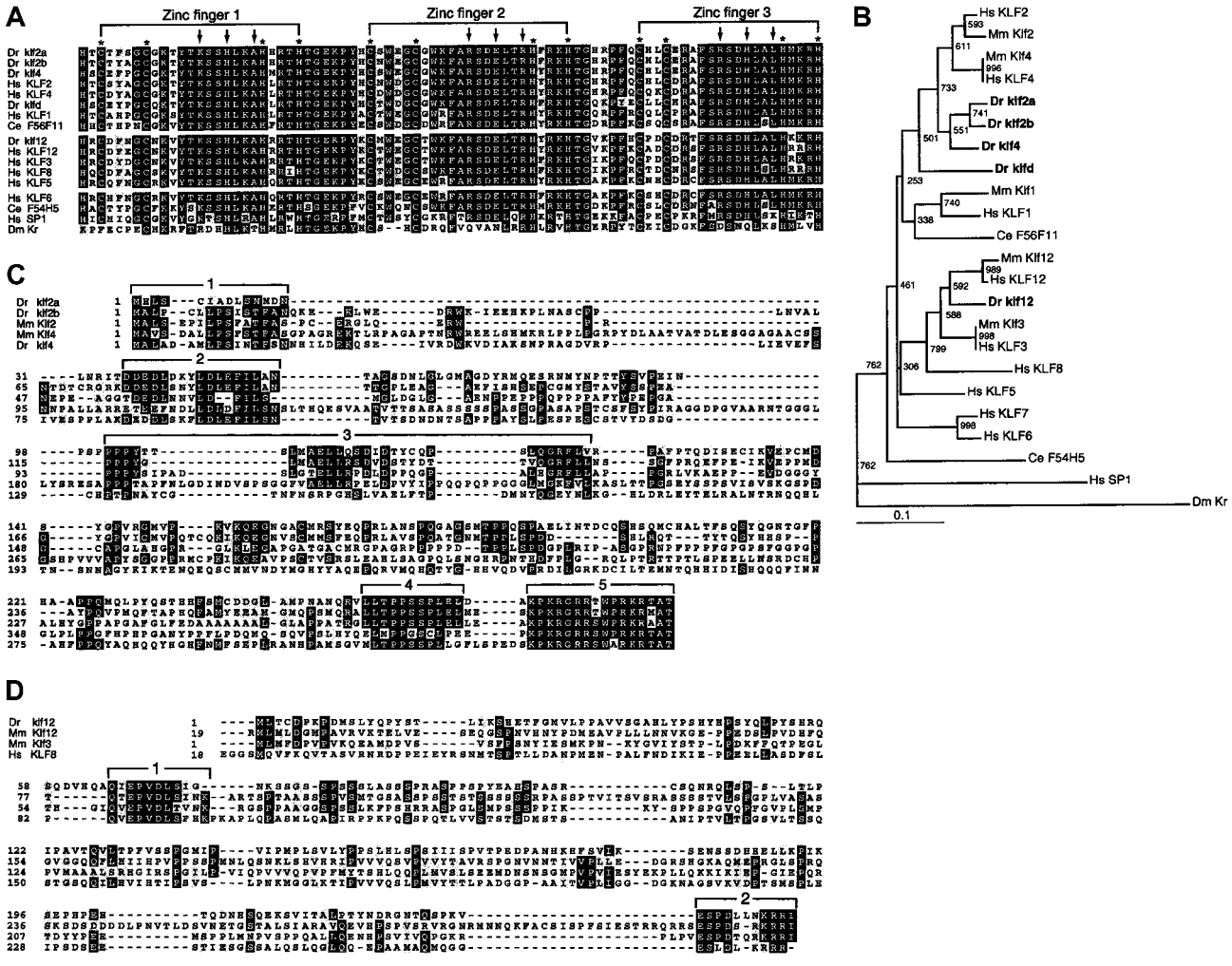

Figure 1. Evolutionary relations between the zebrafish *Krüppel-like factor* (*KLF*) genes and those from other species. (A) Amino acid alignment of the C-terminal zinc finger domains of zebrafish KLF proteins with selected vertebrate KLF proteins and *Drosophila* KRUPPEL. Amino acid identity is marked with black and similarity with gray highlighting. Asterisks mark the invariant zinc-chelating residues, and the arrows indicate those residues that contact the DNA. (B) Phylogenetic tree of the evolutionary relation of zebrafish *KLF* genes to their close vertebrate relatives and *Drosophila Krüppel*, based on the sequences of the zinc finger domains of the respective proteins. Numbers give the bootstrap support for given nodes in the phylogram. (C) Amino acid alignment, excluding the zinc finger domain, of the N-termini of zebrafish Klf2a, Klf2b, and Klf4 proteins with their closest vertebrate relatives Klf2 (Lklf) and Klf4 (Gklf). Regions of high similarity are numbered and indicated with brackets; see text for details. (D) Amino acid alignment, excluding the zinc finger domain, of the N-termini of zebrafish Klf12 protein with its close vertebrate relatives Klf3 (Bklf) and Klf12 (AP-2rep). Regions of high similarity are numbered and indicated with brackets; see text for details.

shares conserved synteny with both *Klf1* and *Klf2* genes in mammals. Although sequence analysis indicates that *klfd* does not have a known ortholog in mammalian genomes, it is most closely related to *Klf1* and *Klf2* (Figure 1B). Combined, these data suggest that *klfd* may be related to an ancestor of these genes.

Expression of the *klf* genes during development

While the structural comparisons, phylogenetic analyses and conserved syntenies presented above argue gene histories, expression analysis can reveal potential developmental properties of the zebrafish *klf* genes. The expression of each of the *klf* genes reported here was determined during embryonic and larval stages by whole-mount in situ hybridization.

klf12, klf4, and *klfd* expression patterns

klf12, klf4, and *klfd* were characterized by high-level expression in the hematopoietic system, as illustrated in Figure 2. Transcripts from each of these genes were detected in a line of cells immediately below the notochord along the ventral aspect of the trunk at 24 hours past fertilization (hpf) (Figure 2A,E,H). This region is known as the intermediate cell mass (ICM) and is the site of primitive erythropoiesis.[49] The pattern of expression along the anterior/posterior axis was similar to that seen with erythrocyte markers *gata1*,[50,51] *jak2*,[37] and embryonic *globins*[40,52] and did not extend into the posterior tail as seen with the stem cell marker *scl*,[53] consistent with expression in primitive erythrocytes. To confirm that the signal was produced in hematopoietic cells, we assayed the expression of each of these genes in the *cloche* mutant background, which exhibits a profound defect in the hematopoietic and vascular lineages.[54-56] Staining was absent from the ICM in one quarter of embryos in clutches from *cloche*$^{+/-}$ parents (Figure 2A,E,H; Figure 3D), verifying a hematopoietic site of expression for *klf12, klf4,* and *klfd*. Expression of *klf4* and *klfd* in circulating primitive erythrocytes was detected at 48 hpf (Figure 2F,I), whereas expression of *klf12* in these cells was absent by this time (Figure 2B). Only *klfd* expression was detected in definitive hematopoietic cells. By 8 days past fertilization (dpf), transcripts were detected in the site of definitive hematopoiesis, the pronephros, and in circulating

Table 1. Genetic mapping and conserved syntenies in the evolution of *KLF* family genes

Dre gene	Dre locus	Dre GB	Dre EST	Hsa gene	Hsa cytogenetics	Hsa GB4 (cR3000)	Reference
klfd							
klfd	HS6_20.5	?		*KLF1*	19p13.12	19_73.90	Current study
				KLF2	19p13.13-p13.11	19_86.89	
ctl2	HS6_23.1	AI722360	fc26c03	*CTL2*	19p13.1	19_68.54	48
klf12							
fgb	HS1_19.7	AA658651	fa55c11	*FGB*	4q28	4_635.80	48
mellar	HS1_27.8	U31822		*MTNR1A*	4q35.1		47
slc34a2	HS1_29.5	AF121796		*SLC34A2*	4p15.3-p15.1		Y.Y. and J.H.P.*
tolloid	HS1_41.3	AF027596		*TLL1*	4q32-q33		48
zef2	HS1_49.6	U84616		*NERF2*		4_615.56	34
klf12	HS1_52.1			*LOC51274*		4_174.08	Current study
ednra1	HS1_58.4	AI545753	fb75g12	*EDNRA*		4_630.42	34
hmx1	HS1_62.4	AI658291	fc21b03	*HMX1*	4p16.1		48
msxb	HS1_72.1	U16311		*MSX1*	4p16.1	4_34.92	34
lef1	HS1_77.8	AF136454		*LEF1*	4q23-q25		48
gpsn2	HS1_93.8	AI106232	db03b10	*GPSN2*		4_457.36	48
klf2a							
dkfzp572c163.1	HS22_08.6	AI721563	fc29c08	*DKFZp572C163.1*	19p13.1	19_110.54	48
tim44	HS22_17.0	AI617238	zehn1275	*TIM44*	19p13.3-19p13.2		48
ela2	HS22_25.0	AI522803	fb62f03	*ELA2*	19p13.3		48
klf2a	HS22_25.2			*KLF2*	19p13.13-p13.11	19_86.89	Current study
kiaa0223	HS22_30.3	AI477812	fb55d04	*KIAA0223*	19p13.3		48
dkfzp586O0120	HS22_30.3	AI722693	fc30d06	*DKFZP586O0120*	19		48
oaz	HS22_30.3	AB017117		*OAZ1*	19p13.3		48
mbd3	HS22_30.3	AI667514	fc41c05	*MBD3*	19p13.3		Y.Y. and J.H.P.*
tle1	HS22_33.2	AI588095	fb96a10	*TLE1*	19p13.3		48
uba52	HS22_60.2	AI330380	fa91f08	*UBA52*	19p13.1-p12		Y.Y. and J.H.P.*
calr	HS22_16.3	AF195882		*CALR*	19p13.3-p13.2	19_71.27	68
klf2b							
Z9615	HS2_48.0	G41653		*PTPRS*	19p13.3	19_34.68	69
ifi30	HS2_59.3	AI384734	fb12b04	*IFI30*	19p13.1	19_104.14	48
myo9b	HS2_83.6	AI497441	fb53f01	*MYO9B*	19p13.1		48
elavl1	HS2_83.6	U17600		*ELAVL1*	19p13.2	19_52.59	34,48
eef2	HS2_131.9	AI332171	fa93h02	*EEF2*	19pter-q12	19_31.19	Y.Y. and J.H.P.*
caps	HS2_134.9	AW174520	fj05b10	*CAPS*	19p13.3		48
klf2b	HS2_136.5			*KLF2*	19p13.13-p13.11	19_86.89	Current study
klf4							
rxraa	HS2_53.9	U29940		*RXRA*	9q34.3	9_414.54	34
phapi2b	HS2_62.0	AI444255	fb40e12	*PHAPI2B*	9pq	9_314.98	Y.Y. and J.H.P.*
klf4	HS2_73.1			*KLF4*	9q31		Current study
ptc1	HS2_78.1	X98883		*PTCH*	9q22.3	9_310.90	34
tnw	HS2_81.1	AJ001423		*TNC*	9q32-q34	9_357.98	47

This table displays the mapping data for genes from both human and zebrafish genomes that demonstrate the existence of chromosomal regions surrounding the zebrafish *klf* genes with conserved synteny to the human genome and the *KLF* genes therein.

Dre indicates *Danio rerio;* Hsa, *Homo sapiens.*

*Y.Y. and J.H.P., unpublished data, 2000.

definitive erythrocytes in the heart lumen and vessels of the tail (Figure 2J).

The cells of the hatching gland expressed both *klf12* and *klf4* at 24 to 36 hpf (Figure 2A,B,E), an organ derived from the prechordal plate mesoderm, or polster, which also showed high levels of *klf4* at 12 hpf (Figure 2D). By 36 hpf, *klf12* was also detected in cells of the pronephric duct, being at highest levels in 2 bilaterally symmetrical clusters of cells of unknown identity at an axial position level with somite 14 (Figure 2B,C). At 24 hpf, *klf4* was present in 2 paired cell clusters lateral to the hindbrain (Figure 2E), consistent with the location of the cranial ganglia of the anterior and posterior lateral line.[57] This was confirmed by observations at 36 hpf of *klf4* expression in the migrating lateral line primordia and in the clusters of neuromast cells deposited in its wake (Figure 2F). By 4 dpf, the full array of lateral line organs was marked by *klf4* expression (Figure 2G).

klf2a and *klf2b* expression patterns

The closely related *klf2a* and *klf2b* genes were expressed during gastrula stages in the early epidermis in a distinctive, partially overlapping pattern (Figure 3). Beginning at approximately 70% epiboly, *klf2a* transcripts were detected in the ventral, animal portion of the epiblast (Figure 3A,B), and as epiboly progressed, this expression domain extended vegetally (Figure 3C,D). At 70% epiboly, *klf2b* was also expressed in the ventral, animal portion of the epiblast, but in a more extensive domain than *klf2a* (Figure 3E,F), extending further across the gastrula both dorsally and vegetally. In addition, a thin line of cells along the dorsal margin also expressed this gene. By the end of epiboly, *klf2b* expression was lost in the animal-most one third of the epiblast and was expressed at higher levels in a lateral band extending around all but the dorsal midline of the embryo (Figure 3G,H). Thus, the domain

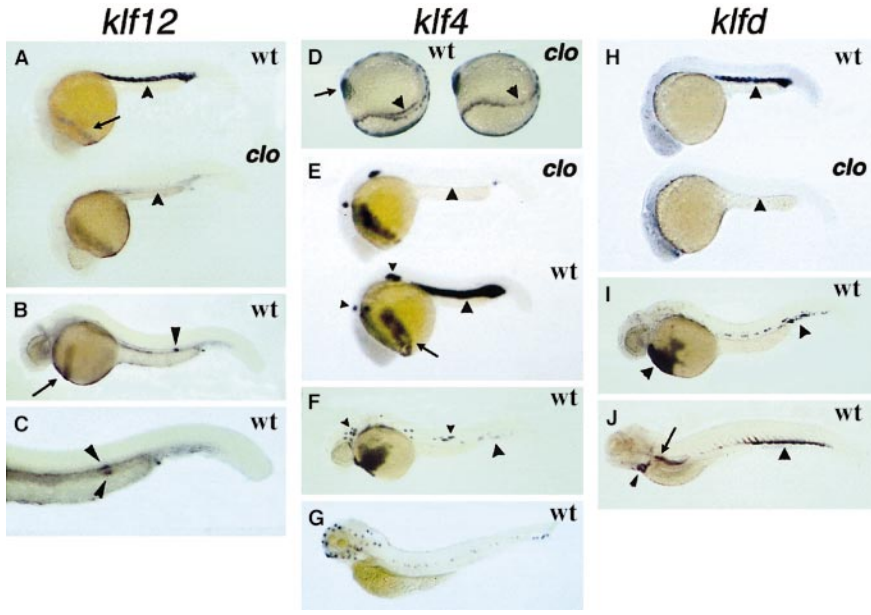

Figure 2. Expression of the *klf12, klf4,* and *klfd* genes in the zebrafish embryo. Whole-mount in situ hybridization of zebrafish embryos with riboprobes for the zebrafish *klf12* gene (A-C), *klf4* (D-G), and *klfd* (H-J). Embryos are shown in lateral view, oriented with anterior to the left and dorsal up, except in panel D, where embryos are viewed obliquely from the posterior, with anterior to the top and dorsal to the right. (A-C) Expression of *klf12.* (A) Comparison of wild-type (wt, upper) and *cloche* mutant animals (*clo*, lower) at 24 hours past fertilization (hpf), showing the absence of *klf12* expression in the intermediate cell mass (ICM, arrowheads) of *clo* embryos and *klf12* expression in hatching gland cells across the yolk (arrow). (B) Expression at 36 hpf showing hatching gland (arrow) and pronephric duct cell clusters (arrowhead). (C) Higher magnification and oblique view of animal in panel B, showing bilateral nature of pronephric duct cell clusters (arrowheads). (D-G) Expression of *klf4.* (D) Comparison of wt (left) and *clo* mutant (right) at 12 hpf in an oblique view, showing the absence of a row of *klf4*-expressing blood cells in the posterior lateral plate mesoderm (arrowheads) and the position of the polster (arrow). (E) Comparison of wt (lower) and *clo* mutant animals (upper) at 24 hpf, showing the absence of *klf4* expression in the ICM (arrowheads) of *clo* embryos and *klf4* expression in hatching gland cells (arrow). Small arrowheads mark the primordia of the anterior and posterior lateral line ganglia. (F) At 48 hpf, circulating erythrocytes expressing *klf4* are visible across the anterior of the yolk and in the vessels of the tail (arrowhead). The anterior and posterior extents of the migrating lateral line primordia are marked with small arrowheads. (G) Larva at 4 days past fertilization (dpf) showing *klf4* expression in complete lateral line system. (H-J) Expression of *klfd.* (H) Comparison of wt (upper) and *clo* mutant animals (lower) at 24 hpf, showing the absence of *klfd* expression in the ICM (arrowheads) of *clo* embryos. (I) Embryo at 48 hpf showing *klfd* expression within circulating erythrocytes on anterior yolk and in trunk and tail vessels (arrowheads). (J) Larva at 8 dpf showing *klfd* expression in circulating definitive erythrocytes in tail vessels (arrowhead), heart lumen (small arrowhead), and pronephros (arrow). Original magnification 50 ×; stained with nitroblue tetrazolium chloride (NBT) and S-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) precipitate.

of *klf2a* expression in the epiblast was nested concentrically within the domain of *klf2b* expression. *klf2b* expression was maintained in the dorsal marginal cells.

klf2a expression was detected at 24 hpf in the anus and in small clusters of superficial cells lateral to the most posterior notochord (Figure 4A), as well as in scattered cells closely associated with the axial and head vessels and in the heart (Figure 4A,B). These expression patterns persisted until 48 hpf, and at this time a faint signal was detected from the distal margin of the caudal fin and in the pectoral fins (Figure 4C; also see below).

klf2b was expressed in the superficial layer of the epidermis in large squamous cells more populous dorsally (Figure 5A). At 36 hpf, this expression was dramatically decreased; *klf2b* transcripts at high level were instead present in 2 cords of cells in the body wall immediately anterior and ventral to the pectoral fin buds (Figure 5B). By 48 hpf, the mesenchymal interior of each fin bud expressed *klf2b* strongly (Figure 5C).

Both *klf2a* and *klf2b* were expressed in a dynamic manner in the developing pectoral fins; the *klf2b* riboprobe gave a clearer and more intense signal, but we could not otherwise distinguish between the 2 patterns in the limb. The expression of *klf2b* is illustrated in Figure 6 from 2 until 5 dpf. At 2 dpf, *klf2b* is expressed in the mesenchymal interior of the fin bud (Figure 6A). By 3 dpf, expression had decreased in the proximal portion of the fin concomitant with the emergence of striated muscle (Figure 6B), but was maintained in the distal portion of the mesenchymal part of the fin. Expression was confined to a thin row of cells at the distal tip of the striated muscles at 4 dpf (Figure 6C), and by 5 dpf, *klf2b* was found only in scattered epidermal cells over the fin (Figure 6D).

Discussion

In this report, we characterize *KLF* gene family members from a nonmammalian vertebrate. The sequences, genetic map positions, and developmental expression patterns of 5 new *KLF* genes represent an opportunity to analyze the evolution of the roles played by this gene family in vertebrate development.

Potential structural domains in the N-termini of the KLF family proteins

In contrast to the highly conserved C-termini of the *KLF* family, which contain 3 tandem DNA-binding zinc fingers, the N-termini are significantly diverged.[4] Determination of activator and repressor functions in the N-termini of KLF proteins has required the generation of extensive deletion series through these regions.[14-16,18-23] Our finding of domains of high amino acid sequence conservation between zebrafish and mammalian KLF proteins offers the possibility of guiding these searches and potentially extending them to detect previously unsuspected activities. We have delineated 5 regions of high sequence conservation in the N-terminus of Klf2/4 subfamily proteins. Three of these correspond to domains previously defined to possess activities essential for transcription factor function in mouse and human KLF4.

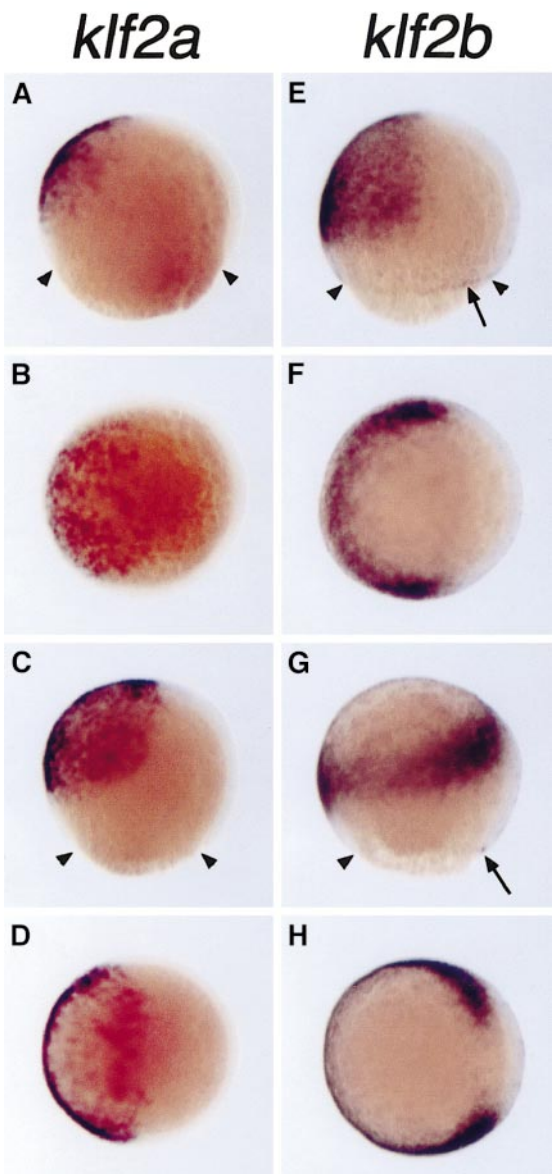

Figure 3. Expression of *klf2a* and *klf2b* in the zebrafish gastrula. Whole-mount in situ hybridization of zebrafish embryos with riboprobes for the zebrafish *klf2a* (A-D) and *klf2b* genes (E-H). The embryos are shown in lateral view (A,E,C,G) with animal pole to the top and dorsal to the right, and in animal pole view (B,F,D,H) with dorsal to the right. Embryos in panels A, B, E, and F are at 70% epiboly and those in C, D, G, and H are at 90% epiboly. The gastrula margin is indicated with small arrowheads (A,E,C,G), and the *klf2b*-expressing cells on this margin are indicated with an arrow (E,G). Original magnification 50 ×; stained with NBT/BCIP precipitate.

Domain 2 is an acidic domain conferring transcriptional activation via the recruitment of p300/CBP[15,23]; domain 3 is a proline-rich domain containing sequences required for transcriptional repression by KLF4[15]; and domain 5 is a basic nuclear localization signal.[58] The function of the 2 novel conservation domains, including the N-terminal–most 16 amino acids and the conserved block immediately N-terminal to the potential nuclear localization signal, is currently unknown.

Similarly, the alignment between zebrafish Klf12 and its closest mammalian relatives revealed 2 domains of high sequence conservation N-terminal to the zinc fingers: (1) a region that includes the mCtBP2 interaction domain as defined for Klf3,[22] and (2) a short block neighboring the DNA-binding domain. This subfamily does not appear to possess the extended basic sequence immediately N-terminal to the zinc finger domain seen in the KLF2/4 group, and we speculate that this short C-terminal homology domain may confer nuclear localization instead.

The N-terminus of Klfd was sufficiently diverged from the members of the KLF2/4 and KLF1 families to preclude a meaningful assessment of blocks of conserved amino acid similarity.

Thus, we have identified 7 conserved domains in the N-termini of KLF2/4 and KLF3/12/8 family proteins. The close correspondence between those domains previously defined by functional testing in cell culture and those identified by evolutionary conservation suggests that the 3 novel conserved domains we have defined by comparison between zebrafish and mammalian KLF proteins may also possess important conserved functions. The differential conservation of residues within these blocks likely reflects requirements for specific amino acids in their functions. These domains would make suitable target sequences for deletion analysis or use in a yeast 2-hybrid experiment,[59] for example; and because members of both protein groups are expressed in hematopoietic cells, these domains may therefore allow the characterization of previously unrecognized activities important in hematopoiesis.

Comparison of developmental expression patterns of zebrafish *klf* genes with mammalian *KLF* genes

The mRNA expression patterns of the zebrafish *klf* genes not only suggest possible functions for these genes in the development of zebrafish, but, by comparison with the expression patterns and loss-of-function phenotypes of their murine homologs, also reveal the likely expression patterns of ancestral vertebrate *KLF* genes. To date, loss-of-function phenotypes are known only for the *Klf1*, *Klf2*, and *Klf4* genes of mice.

Removing *Klf1* function in the mouse results in a lethal β-thalassemia because of inability to complete the switch from embryonic γ-globin to adult β-globin, indicating that *Klf1* is required for adult erythropoietic differentiation.[6,26] We did not find a unique zebrafish ortholog of the mammalian *KLF1* gene, despite our success in isolating several closely related sequences from an adult kidney library. This may be because the ortholog of *KLF1* has been lost from the zebrafish genome or because the regulatory regions of a surviving *KLF1* gene are mutated such that it is no longer expressed in developing definitive erythrocytes, and hence would not be found in the adult zebrafish kidney, the source of our cDNA library. Nevertheless, a role in the development of the primitive wave of erythropoiesis in zebrafish would be predicted for *klf4*, *klf12*, and *klfd* on the basis of their expression patterns, although these genes are not closely related. In addition, the expression of *klfd* in the definitive wave of hematopoiesis marks this gene as only the fourth so far identified in this process in the zebrafish, along with *scl*,[53] *gata1*,[60] and *jak2a*.[37] The characterization of the definitive wave of hematopoiesis in the zebrafish, although currently lagging behind that of the primitive wave, will be of great interest for genetic studies of stem cells and their behavior. The conservation of hematopoietic expression patterns we have identified suggests that a vertebrate *KLF* gene ancestral to the clades containing *klf4*, *klf12*, and *klfd* was expressed in the primitive erythropoietic lineage. The existence of other mammalian *KLF* genes with erythrocytic expression from these clades, such as *Klf3* and *Klf8*,[13,16] and from other clades[61,62] is consistent with this expression pattern as an ancestral character for vertebrate *KLF* genes.

Additional roles can be postulated for *klf4* and *klf12* in the development of the hatching gland, but the identity of a homologous structure in mammals is unknown. *klf4* is also expressed in the

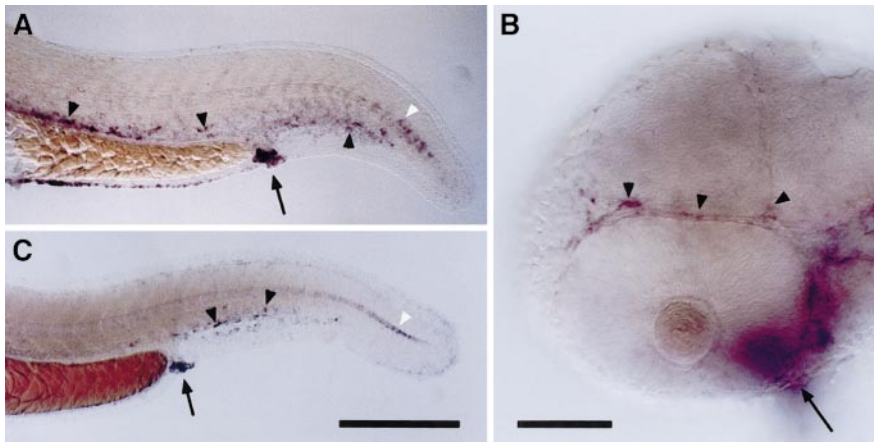

Figure 4. Expression of *klf2a* in the pharyngula-stage zebrafish embryo. Whole-mount in situ hybridization of zebrafish embryos with riboprobe for the zebrafish *klf2a* gene. Embryos are shown in lateral view with anterior to the left and dorsal up. Scale bars are 100 μm (A,C) and 50 μm (B). (A) Trunk and tail of a 24-hour post-fertilization (hpf) embryo showing *klf2a* expression in cells closely associated with the axial vessels (black arrowheads), the anus (arrow), and superficial cell clusters (white arrowhead). (B) Head of a 24-hpf embryo showing *klf2a* expression in head vessels (arrowheads) and heart (arrow, out of plane of focus). (C) Trunk and tail of a 36-hpf embryo showing *klf2a* expression denoted as in panel A. Original magnification in panels A and C 100 ×, panel B 200 ×; stained with NBT/BCIP precipitate.

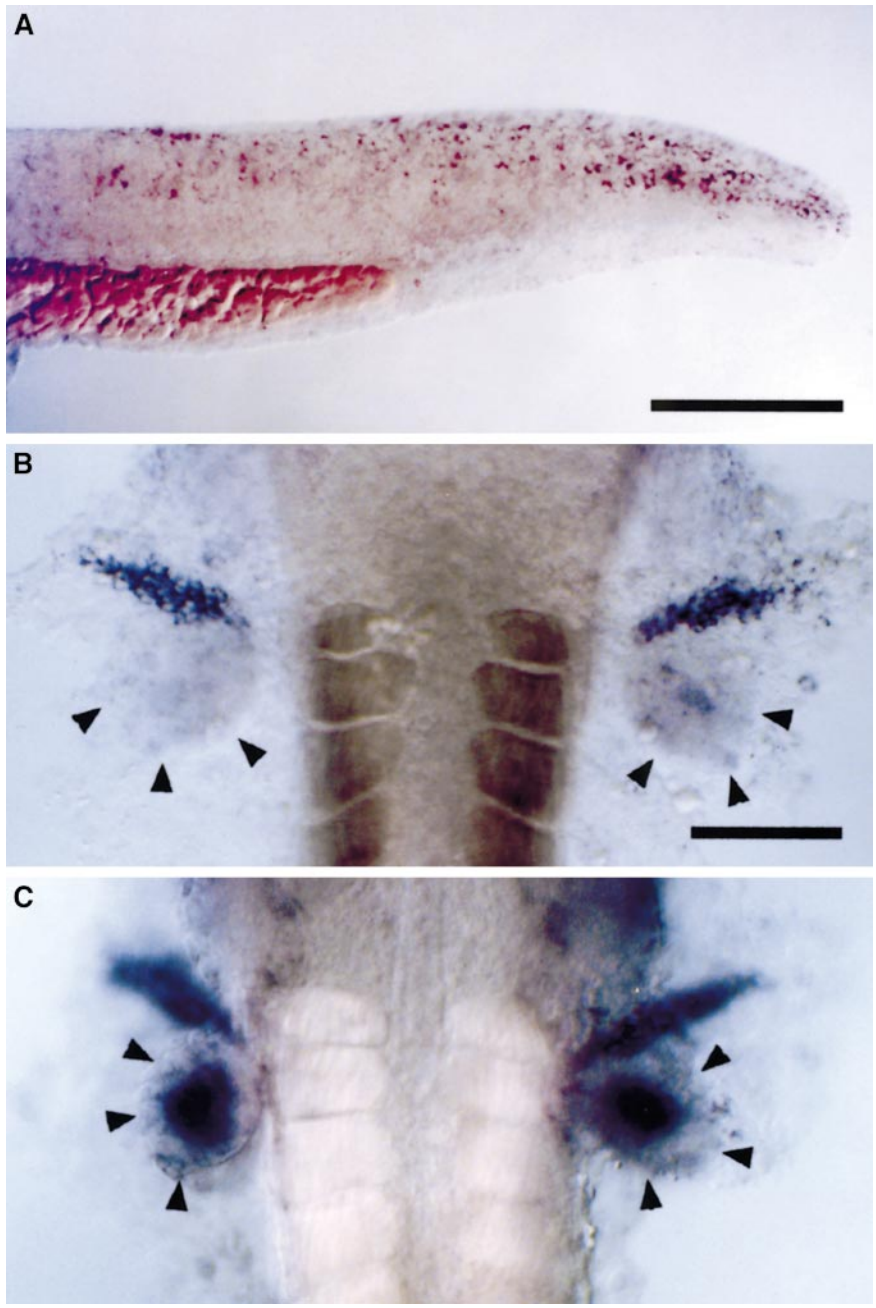

Figure 5. Expression of *klf2b* in the pharyngula and hatching stages of the zebrafish embryo. Whole-mount in situ hybridization of zebrafish embryos with riboprobe for the zebrafish *klf2b* gene. Embryo in panel A is viewed laterally with anterior to the left and dorsal up; embryos in panels B-C are viewed from the dorsal aspect with anterior up. Scale bars are 100 μm (A) and 50 μm (B-C). (A) Embryo at 24 hours past fertilization (hpf) showing *klf2b* expression in squamous cells of the epidermis. (B) Embryo at 36 hpf at the axial level of the anterior trunk (somites are visible as dark blocks) showing bilateral *klf2b*-expressing cords of cells extending laterally from anterior margin of the pectoral fin bud (arrowheads). (C) Embryo at 48 hpf viewed as in panel B, showing expression of *klf2b* in center of pectoral fin bud. Original magnification in panel A 100 ×, panels B and C 200 ×; stained with NBT/BCIP precipitate.

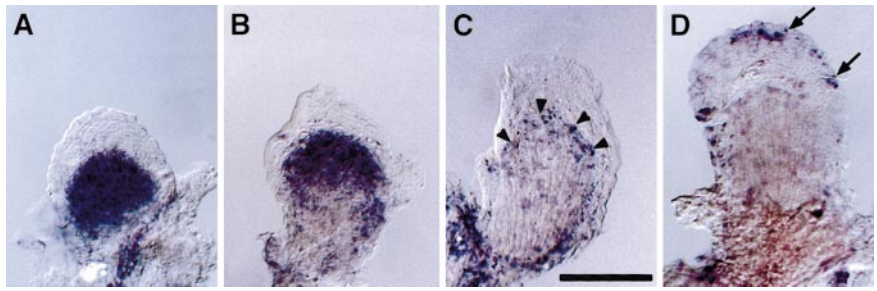

Figure 6. Expression of *klf2b* in the developing pectoral fins of the zebrafish. Whole-mount in situ hybridization of dissected zebrafish pectoral fins with riboprobe for the zebrafish *klf2b* gene. Fins are shown with distal up and anterior margin to the left; scale bar is 50 μm. (A) *klf2b* is expressed in the central mesenchymal region of the fin bud at 2 days past fertilization (dpf). (B) Expression is restricted to distal positions of the fin bud at 3 dpf. (C) At 4 dpf, a narrow row of *klf2b*-expressing cells lines the distal end of the muscular compartment of the fin (arrowheads). (D) By 5 dpf, only scattered cells in the overlying epidermis express *klf2b* (arrows). Original magnification 200 ×; stained with NBT/BCIP precipitate.

cranial ganglia of the zebrafish and in the migrating and differentiating neuromasts of the lateral line that are derived from these ganglia. The expression of murine *Klf4* in cranial sensory ganglia has not been reported, suggesting that this pattern of expression may be a novel acquisition in the zebrafish lineage or has been lost in mouse. The expression of *Klf4* in the mouse is notable for the high levels seen in the differentiated epithelial cells of the gastrointestinal tract[39,63] and epidermis.[9,14] Loss-of-function mutation in this gene results in death of mice shortly after birth from dehydration because of an impaired epidermal barrier function of the skin.[9] There is no evidence of zebrafish *klf4* expression in the epidermis, or any other epithelium, suggesting that the functions of these genes during embryogenesis have diverged significantly. Although no additional *KLF4*-related genes were detected in BLAST searches of the zebrafish EST database, we cannot rule out the existence of another zebrafish *KLF4* ortholog, and we note that any gene expressed only during embryogenesis would not have been recovered in our screen of an adult kidney library.

The spatial expression patterns of *klf2a* and *klf2b* in the zebrafish gastrula cannot yet be compared with those of murine *Klf2* during gastrulation because the latter has not been determined, although *Klf2* mRNA is detectable in the midgastrula by Northern blotting at 7 dpc.[38] At later stages in mouse development, *Klf2* is expressed in the vertebra and the endothelium of developing blood vessels of all sizes.[8] A loss-of-function mutation in *Klf2* in mouse results in the failure to form stable tunica media in the embryonic blood vessels, and the affected mice die in utero from massive hemorrhage.[8] Zebrafish *klf2a* is expressed in cells associated with blood vessels in the head, trunk, and tail, although not with the intersegmental vessels, indicating that a function in blood vessel formation is likely to be ancestral for *KLF2* genes in vertebrates. The expression of the paralogous *klf2b* gene is interesting because it appears to be expressed in the differentiating epidermis of the zebrafish, like *Klf4* in mouse. It is possible, therefore, that epidermal expression was a feature of the ancestral vertebrate *KLF2/4* gene, but was lost from *klf4* and retained in *klf2* in the zebrafish lineage, whereas in the mammalian lineage, epidermal expression was retained by *Klf4* and lost by *Klf2*. This change in epidermal expression patterns may also be related to the aquatic habitat of the zebrafish, where dehydration is not such a problem and the requirements for the barrier function of the skin are likely to be different.[64]

The expression of *Klf2* is also observed in the spleen of mice and rats,[38] and mice without *Klf2* function suffer additionally from hyperactivation of their single-positive T cells, which appear to die in the spleen and lymph nodes.[7] We do not observe the expression of either zebrafish *klf2* gene in the developing thymus; this function may be a mammalian innovation or an ancestral character retained in mammals and lost in zebrafish. Finally, we observe a striking expression pattern of *klf2a* and *klf2b* in the mesenchyme of developing pectoral fin buds, and in underlying mesenchyme of the body wall, suggesting that the *klf2* orthologs may play some role in the differentiation of limb muscle.[65] Expression of mouse *Klf2* has not been reported in the developing limbs; therefore, the ancestry of this and other divergent expression patterns mentioned above cannot be resolved until the expression patterns of *KLF2* genes in additional vertebrates are known.

In summary, we have isolated the first vertebrate *KLF* genes known outside the mammals and have characterized the structure of their protein products, their genetic map positions, and expression patterns in the developing zebrafish. Our results identify novel conserved structural domains in the vertebrate *KLF2/4* and *KLF3/12* subfamilies and describe the probable evolutionary histories of these subfamilies. Furthermore, we present evidence suggesting that developmental function in epidermis formation is ancestral to the vertebrate *KLF2/4* subfamily and that a function for the *KLF2* gene family in blood vessel formation has been conserved in fish and mammals. Expression during erythrocyte development is seen in fish and mammalian genes throughout the *KLF* family, suggesting that a function in hematopoiesis is likely ancestral to all vertebrate *KLF* genes.

The developmental roles of these genes can now be tested in the zebrafish using overexpression and antisense loss-of-function approaches.[66] It will be of particular interest to determine whether any of the zebrafish *klf* genes expressed during erythropoiesis, in particular *klfd*, perform *Klf1*-like functions in the zebrafish.

Note added in proof. During the preparation of this manuscript, Kawahara and Dawid[67] reported the independent cloning of *klf4* as *biklf*.

Acknowledgments

We thank Tracy Roskoph for fish care, Ilya Ruvinsky for discussion and advice on phylogenetic methods, and Ashley Bruce for critical comments on the manuscript.

References

1. Licht JD, Grossel MJ, Figge J, Hansen UM. Drosophila Kruppel protein is a transcriptional repressor. Nature. 1990;346:76-79.
2. Schuh R, Aicher W, Gaul U, et al. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene. Cell. 1986; 47:1025-1032.
3. Stanojevic D, Hoey T, Levine M. Sequence-specific DNA-binding activities of the gap proteins encoded by hunchback and Kruppel in Drosophila. Nature. 1989;341:331-335.
4. Turner J, Crossley M. Mammalian Kruppel-like transcription factors: more than just a pretty finger. Trends Biochem Sci. 1999;24:236-240.
5. Perkins AC, Gaensler KM, Orkin SH. Silencing of human fetal globin expression is impaired in the absence of the adult beta-globin gene activator protein EKLF. Proc Natl Acad Sci U S A. 1996;93: 12267-12271.
6. Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature. 1995;375:316-318.

7. Kuo CT, Veselits ML, Leiden JM. LKLF: a transcriptional regulator of single-positive T cell quiescence and survival. Science. 1997;277:1986-1990.
8. Kuo CT, Veselits ML, Barton KP, Lu MM, Clendenin C, Leiden JM. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. Genes Dev. 1997;11:2996-3006.
9. Segre JA, Bauer C, Fuchs E. Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet. 1999;22:356-360.
10. Ruppert JM, Kinzler KW, Wong AJ, et al. The GLI-Kruppel family of human genes. Mol Cell Biol. 1988;8:3104-3113.
11. Klevit RE. Recognition of DNA by Cys2, His2 zinc fingers. Science. 1991;253:1367, 1393.
12. Miller IJ, Bieker JJ. A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. Mol Cell Biol. 1993;13:2776-2786.
13. Crossley M, Whitelaw E, Perkins A, Williams G, Fujiwara Y, Orkin SH. Isolation and characterization of the cDNA encoding BKLF/TEF-2, a major CACCC-box-binding protein in erythroid cells and selected other cells. Mol Cell Biol. 1996;16:1695-1705.
14. Garrett-Sinha LA, Eberspaecher H, Seldin MF, de Crombrugghe B. A gene for a novel zinc-finger protein expressed in differentiated epithelial cells and transiently in certain mesenchymal cells. J Biol Chem. 1996;271:31384-31390.
15. Yet SF, McA'Nulty MM, Folta SC, et al. Human EZF, a Kruppel-like zinc finger protein, is expressed in vascular endothelial cells and contains transcriptional activation and repression domains. J Biol Chem. 1998;273:1026-1031.
16. van Vliet J, Turner J, Crossley M. Human Kruppel-like factor 8: a CACCC-box binding protein that associates with CtBP and represses transcription. Nucleic Acids Res. 2000;28:1955-1962.
17. Shields JM, Yang VW. Identification of the DNA sequence that interacts with the gut-enriched Kruppel-like factor. Nucleic Acids Res. 1998;26:796-802.
18. Bieker JJ, Southwood CM. The erythroid Kruppel-like factor transactivation domain is a critical component for cell-specific inducibility of a beta-globin promoter. Mol Cell Biol. 1995;15:852-860.
19. Chen X, Bieker JJ. Erythroid Kruppel-like factor (EKLF) contains a multifunctional transcriptional activation domain important for inter- and intramolecular interactions. EMBO J. 1996;15:5888-5896.
20. Matsumoto N, Laub F, Aldabe R, et al. Cloning the cDNA for a new human zinc finger protein defines a group of closely related Kruppel-like transcription factors. J Biol Chem. 1998;273:28229-28237.
21. Ratziu V, Lalazar A, Wong L, et al. Zf9, a Kruppel-like transcription factor up-regulated in vivo during early hepatic fibrosis. Proc Natl Acad Sci U S A. 1998;95:9500-9505.
22. Turner J, Crossley M. Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. EMBO J. 1998;17:5129-5140.
23. Geiman DE, Ton-That H, Johnson JM, Yang VW. Transactivation and growth suppression by the gut-enriched Kruppel-like factor (Kruppel-like factor 4) are dependent on acidic amino acid residues and protein-protein interaction. Nucleic Acids Res. 2000;28:1106-1113.
24. Zhang W, Bieker JJ. Acetylation and modulation of erythroid Kruppel-like factor (EKLF) activity by interaction with histone acetyltransferases. Proc Natl Acad Sci U S A. 1998;95:9855-9860.
25. Ouyang L, Chen X, Bieker JJ. Regulation of erythroid Kruppel-like factor (EKLF) transcriptional activity by phosphorylation of a protein kinase casein kinase II site within its interaction domain. J Biol Chem. 1998;273:23019-23025.
26. Perkins AC, Sharpe AH, Orkin SH. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature. 1995;375:318-322.
27. Weinstein BM, Schier AF, Abdelilah S, et al. Hematopoietic mutations in the zebrafish. Development. 1996;123:303-309.
28. Ransom DG, Haffter P, Odenthal J, et al. Characterization of zebrafish mutants with defects in embryonic hematopoiesis. Development. 1996;123:311-319.
29. Bellefroid EJ, Lecocq PJ, Benhida A, Poncelet DA, Belayew A, Martial JA. The human genome contains hundreds of genes coding for finger proteins of the Kruppel type. DNA. 1989;8:377-387.
30. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876-4882.
31. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673-4680.
32. Johnson SL, Gates MA, Johnson M, et al. Centromere-linkage analysis and consolidation of the zebrafish genetic map. Genetics. 1996;142:1277-1288.
33. Johnson SL, Africa D, Horne S, Postlethwait JH. Half-tetrad analysis in zebrafish: mapping the ros mutation and the centromere of linkage group I. Genetics. 1995;139:1727-1735.
34. Postlethwait JH, Yan YL, Gates MA, et al. Vertebrate genome evolution and the zebrafish gene map [published erratum appears in Nat Genet. 1998;19:303]. Nat Genet. 1998;18:345-349.
35. Kwok C, Korn RM, Davis ME, et al. Characterization of whole genome radiation hybrid mapping resources for non-mammalian vertebrates. Nucleic Acids Res. 1998;26:3562-3566.
36. Schulte-Merker S, Ho RK, Herrmann BG, Nusslein-Volhard C. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development. 1992;116:1021-1032.
37. Oates AC, Brownlie A, Pratt SJ, et al. Gene duplication of zebrafish JAK2 homologs is accompanied by divergent embryonic expression patterns: only jak2a is expressed during erythropoiesis. Blood. 1999;94:2622-2636.
38. Anderson KP, Kern CB, Crable SC, Lingrel JB. Isolation of a gene encoding a functional zinc finger protein homologous to erythroid Kruppel-like factor: identification of a new multigene family. Mol Cell Biol. 1995;15:5957-5965.
39. Shields JM, Christy RJ, Yang VW. Identification and characterization of a gene encoding a gut-enriched Kruppel-like factor expressed during growth arrest. J Biol Chem. 1996;271:20009-20017.
40. Brownlie A, Donovan A, Pratt SJ, et al. Positional cloning of the zebrafish sauternes gene: a model for congenital sideroblastic anaemia. Nat Genet. 1998;20:244-250.
41. Donovan A, Brownlie A, Zhou Y, et al. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature. 2000;403:776-781.
42. Childs S, Weinstein BM, Mohideen MA, Donohue S, Bonkovsky H, Fishman MC. Zebrafish dracula encodes ferrochelatase and its mutation provides a model for erythropoietic protoporphyria. Curr Biol. 2000;10:1001-1004.
43. Wang H, Long Q, Marty SD, Sassa S, Lin S. A zebrafish model for hepatoerythropoietic porphyria. Nat Genet. 1998;20:239-243.
44. Liao EC, Paw BH, Peters LL, et al. Hereditary spherocytosis in zebrafish riesling illustrates evolution of erythroid beta-spectrin structure, and function in red cell morphogenesis and membrane stability. Development. 2000;127:5123-5132.
45. Griffin KJ, Amacher SL, Kimmel CB, Kimelman D. Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. Development. 1998;125:3379-3388.
46. Amores A, Force A, Yan Y-L, et al. Zebrafish *hox* clusters and vertebrate genome evolution. Science. 1998;282:1711-1714.
47. Gates MA, Kim L, Egan ES, et al. A genetic linkage map for zebrafish: comparative analysis and localization of genes and expressed sequences. Genome Res. 1999;9:334-347.
48. Woods IG, Kelly PD, Chu F, et al. A comparative map of the Zebrafish genome. Genome Res. 2000;10:1903-1914.
49. Al-Adhami MA, Kunz YW. Ontogenesis of hematopoietic sites in Brachydanio rerio (Hamilton-Buchanan) (Teleostei). Cell Growth Differ. 1977;19:171-179.
50. Detrich HW 3rd, Kieran MW, Chan FY, et al. Intraembryonic hematopoietic cell migration during vertebrate development. Proc Natl Acad Sci U S A. 1995;92:10713-10717.
51. Thompson MA, Ransom DG, Pratt SJ, et al. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev Biol. 1998;197:248-269.
52. Quinkertz A, Campos-Ortega JA. A new beta-globin gene from the zebrafish, betaE1, and its pattern of transcription during embryogenesis. Dev Genes Evol. 1999;209:126-131.
53. Liao EC, Paw BH, Oates AC, Pratt SJ, Postlethwait JH, Zon LI. SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. Genes Dev. 1998;12:621-626.
54. Liao W, Bisgrove BW, Sawyer H, et al. The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. Development. 1997;124:381-389.
55. Stainier DY, Weinstein BM, Detrich HW 3rd, Zon LI, Fishman MC. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. Development. 1995;121:3141-3150.
56. Parker L, Stainier DY. Cell-autonomous and non-autonomous requirements for the zebrafish gene cloche in hematopoiesis. Development. 1999;126:2643-2651.
57. Holder N, Hill J. Retinoic acid modifies development of the midbrain-hindbrain border and affects cranial ganglion formation in zebrafish embryos. Development. 1991;113:1159-1170.
58. Shields JM, Yang VW. Two potent nuclear localization signals in the gut-enriched Kruppel-like factor define a subfamily of closely related Kruppel proteins. J Biol Chem. 1997;272:18504-18507.
59. Brent R, Finley RL Jr. Understanding gene and allele function with two-hybrid methods. Annu Rev Genet. 1997;31:663-704.
60. Long Q, Meng A, Wang H, Jessen JR, Farrell MJ, Lin S. GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. Development. 1997;124:4105-4111.
61. Asano H, Li XS, Stamatoyannopoulos G. FKLF, a novel Kruppel-like factor that activates human

embryonic and fetal beta-like globin genes. Mol Cell Biol. 1999;19:3571-3579.

62. Asano H, Li XS, Stamatoyannopoulos G. FKLF-2: a novel Kruppel-like transcriptional factor that activates globin and other erythroid lineage genes. Blood. 2000;95:3578-3584.
63. Ton-That H, Kaestner KH, Shields JM, Mahatanankoon CS, Yang VW. Expression of the gut-enriched Kruppel-like factor gene during development and intestinal tumorigenesis. FEBS Lett. 1997;419:239-243.
64. Nemes Z, Steinert PM. Bricks and mortar of the epidermal barrier. Exp Mol Med. 1999;31:5-19.
65. Grandel H, Schulte-Merker S. The development of the paired fins in the zebrafish (Danio rerio). Mech Dev. 1998;79:99-120.
66. Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish [in process citation]. Nat Genet. 2000;26:216-220.
67. Kawahara A, Dawid IB. Expression of the Kruppel-like zinc finger gene biklf during zebrafish development. Mech Dev. 2000;97:173-176.
68. Rubinstein AL, Lee D, Luo R, Henion PD, Halpern ME. Genes dependent on zebrafish cyclops function identified by AFLP differential gene expression screen. Genesis. 2000;26:86-97.
69. Shimoda N, Knapik EW, Ziniti J, et al. Zebrafish genetic map with 2000 microsatellite markers. Genomics. 1999;58:219-232.
70. Page RDM. University of Glasgow. Treeview. Available at: http://taxonomy.zoology.gla. ac.uk/rod/treeview/treeview.html. Accessed November 1998.