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Expression analysis of the family of 14-3-3 proteins in zebrafish development

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Abstract

14-3-3 proteins comprise a family of dimeric multi-functional proteins present in all eukaryotes, that are important in a whelm of ubiquitous biological processes. We have analyzed the genomic structure of all 14-3-3s from zebrafish comprising 11 genes and have analyzed their phylogeny. The gene family was cloned and its expression pattern in zebrafish embryogenesis was analyzed by whole mount *in situ* hybridization and microarray analysis with gene specific probes. We demonstrate that maternal mRNA of 14-3-3s is expressed evenly at the first cell division. At later stage all genes are expressed in a patterned way with, in most cases, intricate patterns in the developing brain. Our result shows distinct expression patterns of various genes. Microarray results show that differences in expression levels of highly similar 14-3-3 genes also occur in the adult stage.
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Keywords: 14-3-3 gene family; *Danio rerio* zebrafish embryogenesis expression patterns; Genomic location; Phylogenetic tree; Whole mount *in situ* hybridization; Microarray analysis; Ywha genes

1. Results and discussion

The term 14-3-3 denotes a large family of ±30 kDa acidic proteins that exists primarily as homo- and heterodimers within all eukaryotic cells (reviewed in Aitken et al., 1992). In mammals seven isoforms have been identified (beta, gamma, epsilon, zeta, eta, sigma, and tau), while yeast and plants contain between 2 and 15 genes. Despite this genetic diversity, there is a surprisingly large amount of sequence identity and conservation between all the 14-3-3 isotypes. Stringently conserved regions either form a dimer interface, or line the central ligand binding channel of the dimeric 14-3-3 molecule. High homology suggests functional redundancy, and indeed, many ligands appear to bind to several different isoforms with similar affinity (Finlin and Andres, 1999; Garcia-Guzman et al., 1999; Rittinger et al., 1999; Li et al., 1995). However, there has also been evidence for isotype specific ligand binding and function for some family members (Toyo-oka et al., 2003; Yuan et al., 2003; O'Kelly et al., 2002; Chan et al., 1999).

14-3-3s have been assigned a multitude of functions. Generally speaking, 14-3-3 proteins bind to ligands containing phosphoserine/phosphothreonine – motifs; this binding often regulates cellular processes by influencing localization and activity of ligands. 14-3-3 proteins have been shown to be involved in a multitude of cellular pathways including metabolism, cell cycle, differentiation, cellular signaling, apoptosis, and neoplasic transformation (reviewed in Darling et al., 2005; Mhawech, 2005; Dougherty and Morrison, 2004). 14-3-3 family members appear to be predominantly expressed in adult brain structures of higher animals but are found in many other tissues as well. Expression analysis in the embryonic stage of animals has been performed in rainbow trout (Koskinen et al., 2004) and *Xenopus laevis* (Kousteni et al., 1997).

Some evidence exist that 14-3-3's are involved in early axis formation in vertebrates. Research in *X. laevis* has indicated a role for 14-3-3E in left–right axis formation in early development (Bunney et al., 2003). At the two-cell stage, 14-

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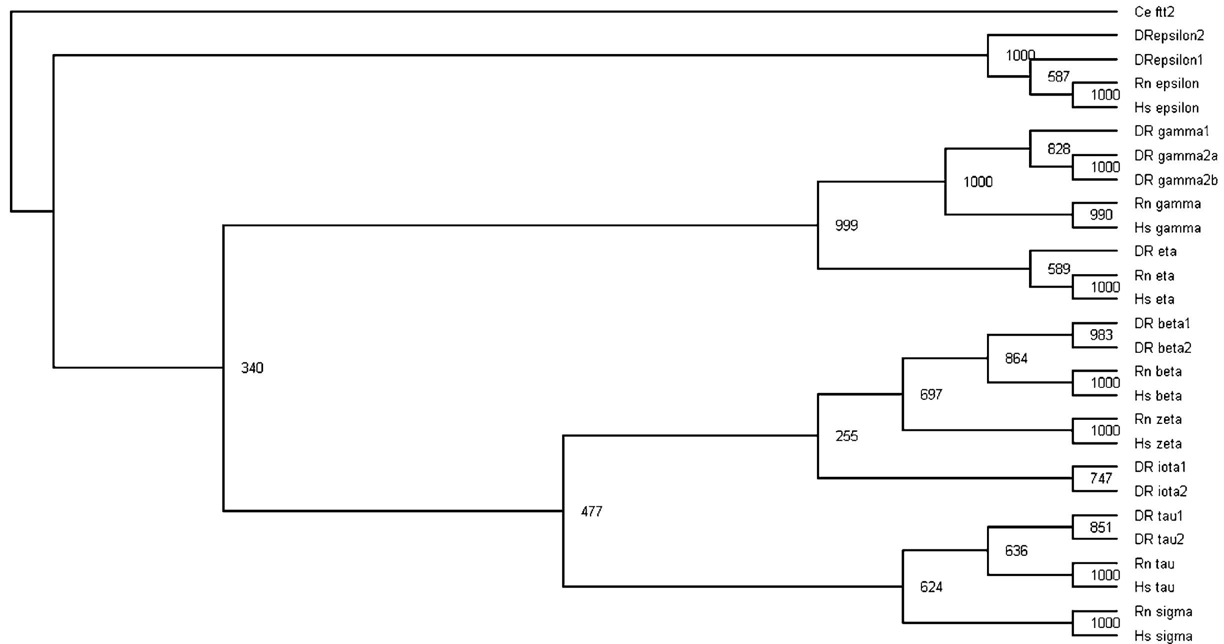

Fig. 1. Phylogenetic tree. The analysis is based on the protein sequences for human, rat, and zebrafish 14-3-3 family members.

3-3E mRNA and protein was found to be localized exclusively in one of the two blastomeres. Forced overexpression of 14-3-3E mRNA or addition of a phosphorylated blocking peptide abolished the exclusive expression and caused heterotaxia in the adult animal (Bunney et al., 2003). We were particularly interested to determine, if 14-3-3 expression in *Danio rerio* is similarly asymmetrical after the first cell division. We found, however, that all isotypes were expressed either in both blastomeres or not at all.

In this study, we analyzed the genomic structure of the zebrafish 14-3-3 gene family and investigated the expression pattern of all family members in developing zebrafish by *in situ* hybridization and microarray analysis.

1.1. Genomic location of zebrafish 14-3-3 genes and phylogenetic analysis

We identified the genomic location of the 14-3-3 family in zebrafish in the last two draft assemblies of the zebrafish genome (Zv5 and Zv6) (Supplemental Table S1). Based on the sequences provided in the zebrafish genome project, we devised specific primers to clone the complete coding sequences. We were able to clone all 14-3-3 family members except gamma 2.

A phylogenetic analysis of the 14-3-3 family was performed using the new sequence information we obtained for the zebrafish 14-3-3 family genes (Fig. 1). The analysis is based on the protein sequences for human, rat and zebrafish 14-3-3 family members. The accession numbers used for the human and rat genes are given in Table S1 and the zebrafish protein sequences were based on our sequenced clones. In the resulting rooted phylogenetic tree, all zebrafish 14-3-3 family members clustered with their respective human and mouse orthologues. The nematode 14-3-3 family member ftt-2 was used as an outgroup (*C.elegans* ftt2: Accession No. NM_077537) for the analysis.

For two of the zebrafish genes, 14-3-3 gamma 2 and epsilon 1, two genomic locations with identical sequences were found probably representing genome assembly errors. As indicated in Table S1, the duplicated zebrafish genes were termed gamma 2a and 2b, and epsilon 1a and 1b, respectively. In the case of epsilon 1, the two loci are present within an extended duplicated area of the genome. The recent acquirement of several gene duplications is more extensive than other duplications in the zebrafish genome, such as duplication of the hox clusters. It therefore resembles more closely the situation found with the TLR genes were also abundant zebrafish-specific genes duplications are found (Meijer et al., 2004).

Subsequently, we performed a detailed genomic analysis of the intron/exon boundaries of all 14-3-3 genes. A schematic overview of this gene architecture is shown in Fig. 2. Most genes, namely the betas, the iotas, and the taus, appear to be arranged in an identical or nearly identical fashion of five exons of different but conserved sizes. The gammas and the eta family member, on the other hand, only have two exons each at similar locations and of similar sizes. The two epsilon isoforms have again a different arrangement, namely six (epsilon 1) and seven (epsilon 2) exons that are, despite having a different number of exons, arranged in an almost identical fashion. Intron/exon boundaries are given in Supplemental Tables 2 and 4 (Tables S2 and S4) and for comparison the intron/exon boundaries of the human 14-3-3 genes in Supplemental Table 3 (Table S3). The results show that the clustering of the phylogenetic tree is consistent with the conservation of the intron–exon boundaries in the zebrafish and human orthologues. It is clear that compared to the mammalian

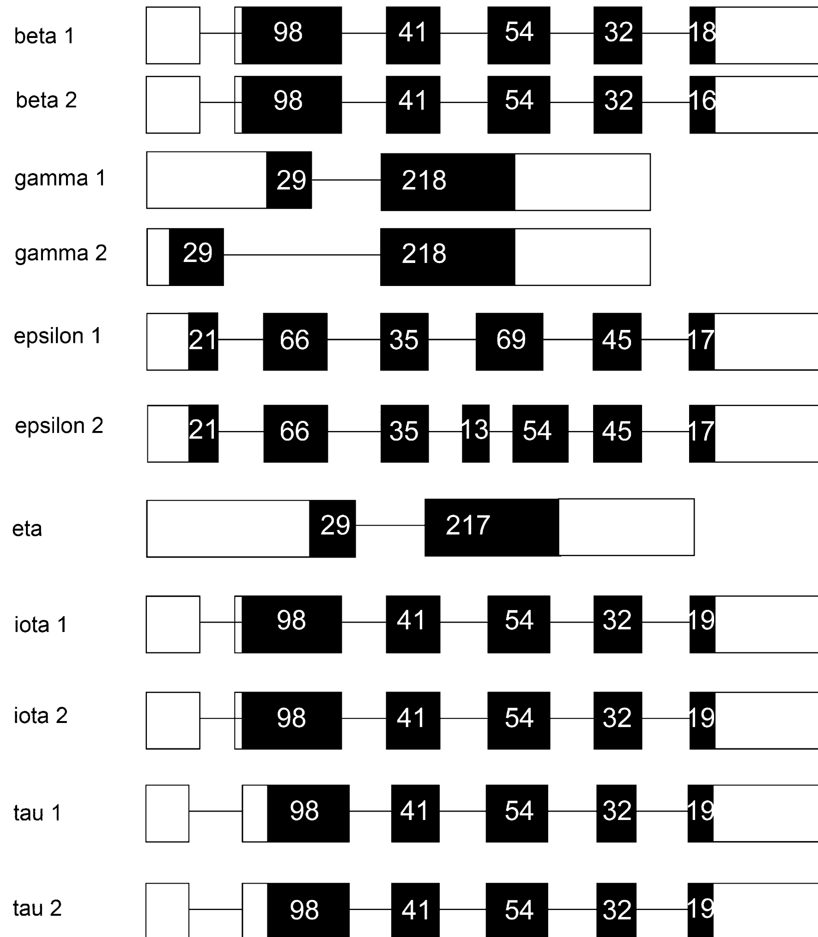

Fig. 2. Schematic overview of gene architecture of all 11 14-3-3 genes. Indicated are the numbers of amino acids per exon. Introns are not drawn to scale.

14-3-3 group the zebrafish has no counterpart of the 14-3-3 sigma isoform that is encoded by a single exon.

1.2. Microarray analysis

In order to quantify relative total expression levels of all the 14-3-3 genes we performed microarray expression analysis using custom made oligonucleotides that are specific for each of the genes. The results (Fig. 3) show that expression of all 14-3-3 genes was detectable during each of the tested stages. The microarray read outs for each of the genes are not expected to represent absolute measures for expression levels but are used to compare expression variation of the 14-3-3 genes during different developmental stages. Nevertheless, for representation purposes, we have divided the 14-3-3 genes in two classes that showed either a high or low expression relative to beta actin (Fig. 3A and B, respectively). In general, the expression levels show high standard deviations between the biological replicates and therefore only in a limited number of cases solid conclusions can be drawn on the statistical significance of expression differences. Most noteworthy is the difference between the expression patterns of the 14-3-3 gammas 1 and 2 genes: whereas gamma 1 is not expressed in 26 h or adult stage the gamma 2 gene has it highest expression level at these stages. This indicated that although the gamma 2 gene is highly related to the gamma 1 it has a specialized function in adult physiology. A similar observation can be made with the epsilon 1 and 2 genes, where epsilon 1 is apparently specialized for the embryonic stages whereas epsilon 2 is equally expressed in embryonic and adult stages. The difference between the tau 1 and 2 genes is less clear although the relative expression of tau 2 is significantly higher in adult stage than is the case for tau 1. In contrast, the two iota and two beta isoforms do not seem significantly different in their expression patterns. In summary, the expression data indicate that several 14-3-3 genes are specialized for embryonal development namely, the gamma 1, epsilon 1, and (less clearly) the eta genes whereas all the other 14-3-3 genes also have high relative expression levels in the adult stage.

1.3. 14-3-3 expression patterns in zebrafish development

We performed detailed *in situ* hybridization studies with 10 14-3-3 family members at zebrafish embryonal stages two-cell, shield (6 hpf), 19-somites (18 hpf), 24 h (Prim 5 stage), and 48 h. An overview of all expression results is given in Table 1.

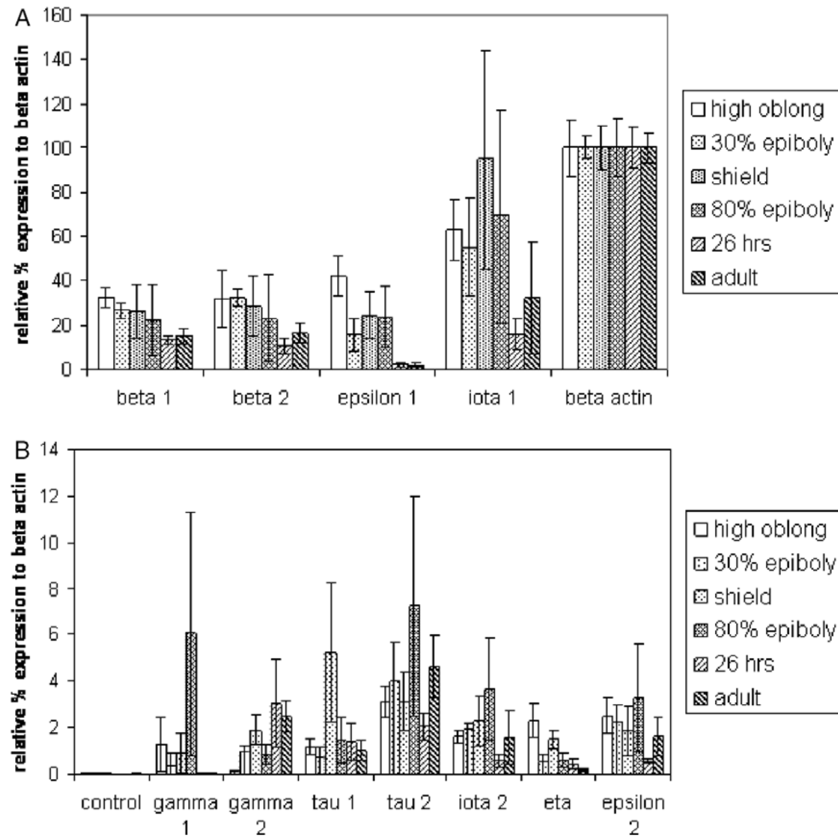

Fig. 3. Microarray expression analysis. (A) Betas 1 and 2, epsilon 1, and iota 1 are highly expressed isoforms. (B) Gammas 1 and 2, taus 1 and 2, iota 2, eta, and epsilon 2 are much less expressed throughout development. Data of embryonic and adult 14-3-3s relative to actin.

We were interested in the expression pattern of 14-3-3 mRNA isoforms at the two-cell stage of *D. rerio* embryonal development to specifically determine if any one or more isoforms are expressed in only one of the two cells, indicating involvement of 14-3-3 in early axis formation. However, all 14-3-3mRNA isoforms are either present in both cells (beta 1 and 2, epsilon 1, iotas 1 and 2, and tau 2) or not at all (gamma 1, eta, epsilon 2, and tau 1) (Fig. 4).

14-3-3 is speculated to act in concert with the H^+/K^+ ATPase to establish an ion gradient across gap junctions in *Xenopus* (Bunney et al., 2003; Levin et al., 2002). The H^+/K^+ ATPase itself is also localized to one blastomere at the two-cell stage of *Xenopus* (Levin et al., 2002). However, in zebrafish, mRNA levels of this pump are evenly expressed in all cells up to the 1k-cell stage, yet a role of this ATPase in early axis formation was proven (Kawakami et al., 2005). Translational or posttranslational regulation possibly ensures asymmetric activity of this protein. The same cannot be excluded for the 14-3-3 family members: while the mRNAs are expressed evenly, mechanisms might exist that ensure 14-3-3 translation or activity only in a subset of cells in the early embryo.

At the shield stage at 6 h of development betas 1 and 2 mRNA are expressed strongly, whereas epsilon 1 and iota 1 mRNA are expressed at lower levels (Fig. 5). Subtypes gamma 1, eta, epsilon 2, iota 2, and tau 1 and 2 are not expressed at this stage.

At 18 h (19-somite stage), all 14-3-3 isoforms are expressed in the developing neural structures in the embryo (Fig. S1). Strongly expressed are the beta, the epsilon, and the iota isoforms in the mid- and hindbrain as well as in the floor plate extending into the tail. A more restricted expression is seen in the gamma, eta, and tau subclasses. Gamma and eta are expressed in the ventral diencephalon and the spinal chord neurons (Fig. S1C). Eta alone is furthermore, expressed in the telencephalon, the tegmentum, the cranial ganglia, and the spinal chord neurons (Fig S1D). The tau isoforms, along with the betas and the epsilons, have a prominent expression in the optic cup.

At 24 hpf (Prim 5 stage), an interesting pattern emerges (Fig. 6). All isoforms that were found in the optic cup (betas, epsilons, and taus) now show expression in the eye or in substructures of the eye (Fig. 5A, C, J, K, L, P, Q, and R). Additionally, all of these isoforms (but only these) are also expressed in the tectum, but never in the diencephalon or telencephalon. The other isoforms, namely gamma 1, eta, iota 1 and 2, are all expressed in the diencephalon but not in the tectum. Some, like gamma 1 and eta, can furthermore be seen in the telencephalon and the tegmentum. In some neural structures, such as the hindbrain and the spinal chord neurons, most 14-3-3 isoforms (compare Table 1 with Fig. 6) are expressed. Other tissues, such as the developing liver bud, expresses only one isoform, namely beta 2 (Fig. 6D).

Table 1
Overview of all 14-3-3 *in situ* data

	Two-cell	Shield	19-Somite	24 h (Prim 5 stage)	48 h
Beta 1	Yes	Yes	In head In tail Optic cup Mid/hindbrain Floor plate	In head In tail Lens Outer retina boundary Tectum Hindbrain Otic vesicle Ventricular zone Spinal chord neurons	In head Not in tail 5th Ganglion Otic vesicle Fin buds Gut
Beta 2	Yes	Yes	In head In tail Optic cup Mid/hindbrain Floor plate	In head In tail Eye Tectum Hindbrain Spinal chord neurons Liver bud	In head Not in tail Otic vesicle Fin buds Liver Gut
Gamma 1	No	No	In head Diencephalon Spinal chord Neurons	In head Telencephalon Diencephalon Tegmentum Cranial ganglia Hindbrain Otic vesicle Spinal chord neurons	In head Diencephalon Hypothalamus Tegmentum 5th Ganglion Hindbrain Otic vesicle
Eta	No	No	In head Telencephalon Diencephalon Tegmentum Spinal chord Neurons	In head Telencephalon Diencephalon Tegmentum Hindbrain Otic vesicle Spinal chord neurons	In head Diencephalon Hypothalamus Tegmentum Tectum Midbrain/hindbrain Boundary Hindbrain
Epsilon 1	Yes	Yes	In head In tail Optic cup Mid/hindbrain Floor plate	In head In tail Eye Tectum Hindbrain Somites Spinal chord neurons Ventricular zone	In head Not in tail Tegmentum 5th Ganglion Otic vesicle Fin buds
Epsilon 2	No	No	In head In tail Optic cup Mid/hindbrain Floor plate	In head Not in tail Eye Tectum (?) Ventricular zone	In head Not in tail Otic vesicle Ventricular zone
Iota 1	Yes	Yes	In head In tail Mid/hindbrain Floor plate	In head In tail Diencephalon Hindbrain	In head Not in tail 5th Ganglion Otic vesicle
Iota 2	Yes	No	In head In tail Mid/hindbrain Floor plate	In head In tail Diencephalon Hindbrain	In head Not in tail 5th Ganglion Otic vesicle
Tau 1	Yes	Yes	In head In tail Optic cup Mid/hindbrain	In head Not in tail Telencephalon Lens Outer retina boundary Tectum	In head Not in tail Diencephalon Retina 5th Ganglion Otic vesicle

(*continued on next page*)

Table 1 (*continued*)

	Two-cell	Shield	19-Somite	24 h (Prim 5 stage)	48 h
				Hindbrain Spinal chord neurons Ventricular zone	Hindbrain Fin buds Liver
Tau 2	Yes	No	In head In tail Optic cup	In head Not in tail Eye Tectum Hindbrain Spinal chord neurons Ventricular zone	In head Not in tail Tegmentum Otic vesicle Fin buds Liver

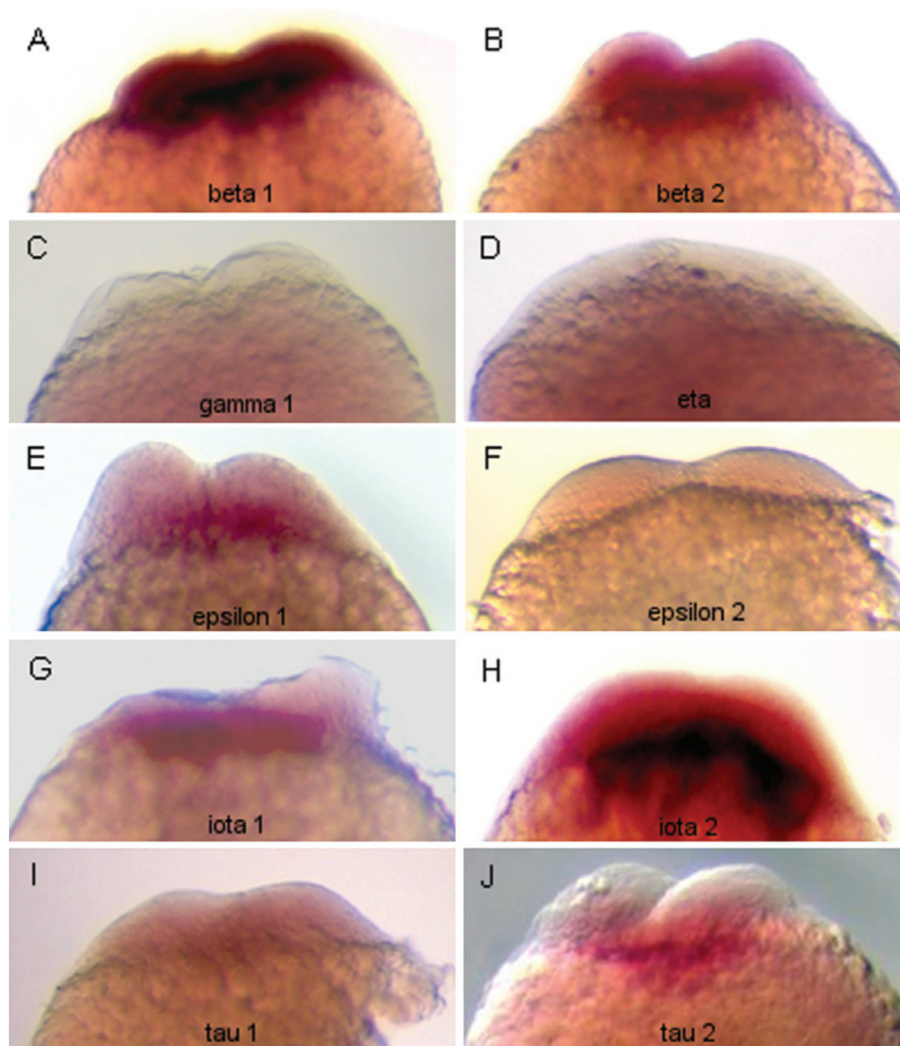

Fig. 4. Expression of 14-3-3 family members in two-cell stage embryos. (A) Beta 1, (B) beta 2, (C) gamma 1, (D) eta, (E) epsilon 1, (F) epsilon 2, (G) iota 1, (H) iota 2, (I) tau 1, and (J) tau 2.

While some isoforms, such as gamma 1 and eta, are expressed in almost exactly the same tissues, they do so at different levels. The eta probe stains the embryos more intensely in all structures, and is therefore likely to be more highly expressed than the gamma 1 subtype (compare Fig. 6E–I). Since the gamma 1 gene is highly similar to the gamma 2 gene and its gene duplicate and the eta gene we cannot exclude that cross-hybridization of the probes within this group has occurred. This is suggested by the microarray results that show hardly any expression of the gamma 1 gene at the 26 h stage. Epsilon 2 appears different from the other isoforms as it is barely expressed anymore at 24 hpf and shows only faint expression in the eye and the tectum and the ventricular zone (Fig. 6L and M).

At 48 h, all family members except eta are now expressed in the otic vesicle. The beta subtypes are additionally

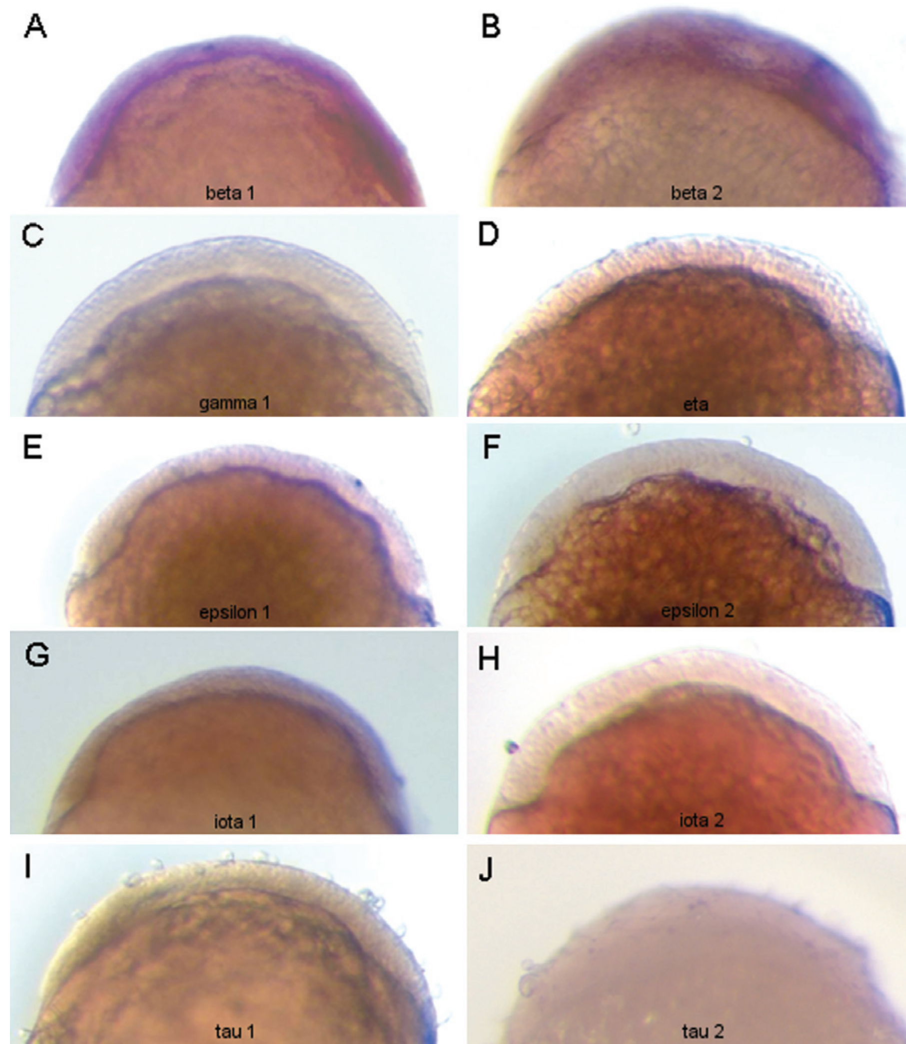

Fig. 5. Expression of 14-3-3 family members in shield stage (6 h) embryos. (A) Beta 1, (B) beta 2, (C) gamma 1, (D) eta, (E) epsilon 1, (F) epsilon 2, (G) iota 1, (H) iota 2, (I) tau 1, and (J) tau 2.

expressed in the fin buds and in some isolated neural structures in the zebrafish head which includes the 5th ganglion, a neural structure located posterior to the eye (Fig. 7A, B, and D). Furthermore, they are found in the gut (beta 1) or in both gut and liver (beta 2) (Fig. 6C and E, respectively). Gamma and eta 14-3-3 are again both prominently expressed in the diencephalon, the midbrain tegmentum, the hypothalamus, and the hindbrain (Fig. 6F–I). Gamma shows a very distinct expression in a structure that is most likely only the dorsal portion of the otic vesicle (Figs. 6 and 7F and G) and in the 5th ganglion (Fig. 7F). Eta, on the other hand, is expressed additionally in the tectum and uniquely in the midbrain/hindbrain boundary region (Fig. 7H and I). Epsilon 1 is localized in the fin buds and in neural structures around the eye, probably again the 5th ganglion and the midbrain tegmentum (Fig. 7J). The epsilon 2 probe, on the other hand, appears to be trapped non-specifically in ventricles in the developing brain structures and may, in addition to the mentioned otic vesicle, only stain the ventricular zone (Fig. 7K and L). Iotas 1 and 2 expression is limited to the otic vesicle and the 5th ganglion (Fig. 7M, N, and O). Tau 1 displays a general expression in the head, including the diencephalon, the retina of the eyes, the 5th ganglion, the hindbrain, and, interestingly, the liver (Fig. 7P, Q, and R). Tau 2 has a similar but distinct expression pattern. It is also found in the otic vesicle, the fin buds and the liver. Unlike tau 1, however, it is also found in the midbrain tegmentum but not in the diencephalon, the retina, or the 5th ganglion. (Fig. 7S, T, and U). Table 1 summarizes this expression data, listing all of the structures in which expression. As reported before for rainbow trout (Koskinen et al., 2004) and *X. laevis* (Kousteni et al., 1997), these data confirm the expression of 14-3-3 family members in neural tissue but also clearly demonstrates distinct expression patterns of individual isoforms.

2. Experimental procedures

2.1. Animals

Zebrafish (*D. rerio*) embryos were raised according to standard procedures and handled in compliance with local animal care regulations and staged in hours post fertilization (hpf) (Kimmel et al., 1995).

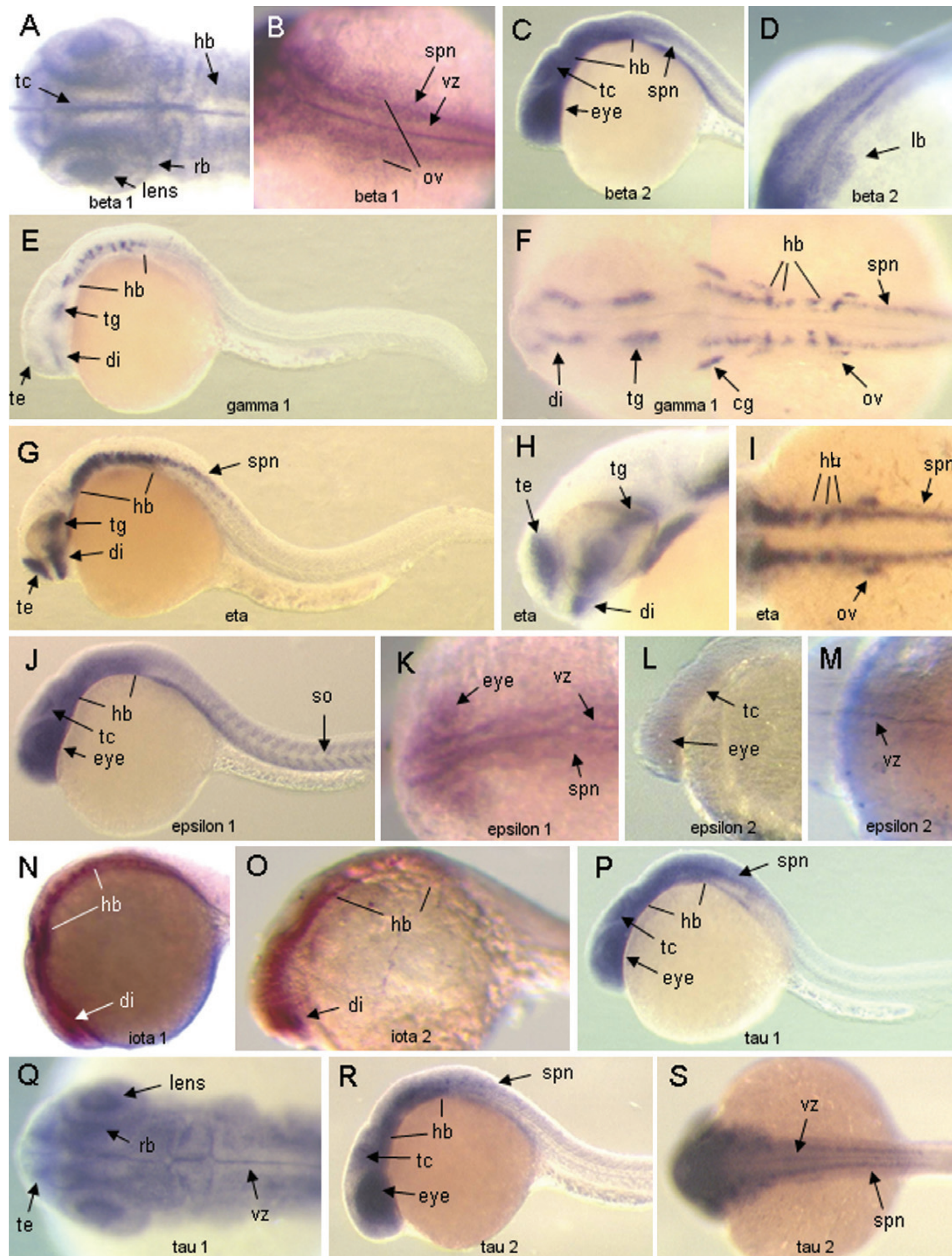

Fig. 6. Expression of 14-3-3 family members in 24 h stage embryos. All views are lateral, except when noted. (A) Beta 1, dorsal view; (B) beta 1, dorsal view; (C) beta 2; (D) enlargement of beta 2; (E) gamma 1; (F) gamma 1, dorsal view; (G) eta; (H) enlargement of eta; (I) dorsal view of eta; (J) epsilon 1; (K) epsilon 1, dorsal view; (L) epsilon 2, (M) epsilon 2, dorsal view; (N) iota 1; (O) iota 2; (P) tau 1; (Q) tau 1, dorsal view; (R) tau 2; and (S) tau 2, dorsal view. di, diencephalon; cg, cranial ganglia; he, heart; hb, hindbrain; lb, liver bud; ov, otic vesicle; rb, outer retina boundary; spn, spinal chord neurons; so, somites; tc, tectum; te, telencephalon; tg, tegmentum in the midbrain; vz, ventricular zone.

2.2. Genomic location and phylogenetic analysis

BLAST searches of the zebrafish genomic sequence were carried out with the tblastn program at the Sanger Institute Ensembl BLAST server (http://www.ensembl.org/Danio_rerio/blastview). The current release of the zebrafish genome project (Zv6) was used for tblastn searches with the full-length sequences of the zebrafish clones. To predict intron/exon boundaries of the zebrafish genes we used GENSCAN predictions of the Sanger Ensembl. The predicted sequences were further adjusted manually.

The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using Phylip distance (http://hyper-nig.nig.ac.jp) at the web server of the DNA Data Bank of Japan (DDBJ). Analysis was done with Kimura's correction. Bootstrap sampling was reiterated 1000 times. For the matrix table 'blosum' was used. The gap

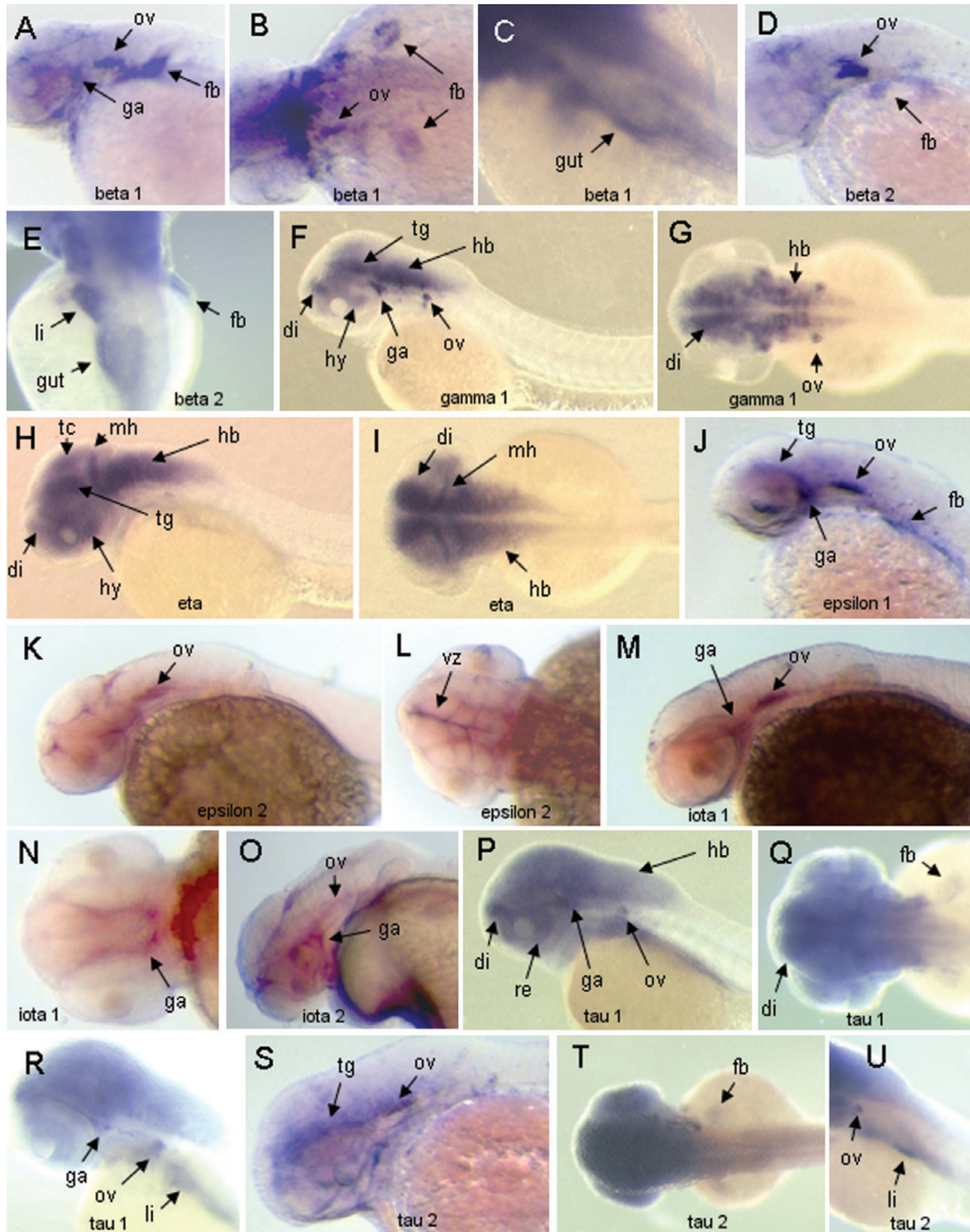

Fig. 7. Expression of 14-3-3 family members in 48 h stage embryos. All views are lateral except where noted. (A) Beta 1; (B) beta 1, dorsal view; (C) beta 1, detail; (D) beta 2; (E) beta 2, detail; (F) gamma 1; (G) gamma 1, dorsal view; (H) eta; (I) eta, dorsal view; (J) epsilon 1; (K) epsilon 2; (L) epsilon 2, dorsal view; (M) iota 1; (N) iota 1, dorsal view; (O) iota 2; (P) tau 1; (Q) tau 1, dorsal view; (R) tau 1, detail; (S) tau 2; (T) tau 2, dorsal view; and (U) tau 2, detail. di, diencephalon; fb, fin bud; ga, 5th ganglion; hb, hindbrain; hy, hypothalamus; li, liver; mh, midbrain/hindbrain boundary; ov, otic vesicle; re, retina; tg, tegmentum; vz, ventricular zone.

extension penalty was set at 0.2 and the gap distance was set at 8. Trees were exported to Treeview 1.66.

2.3. Cloning of the 14-3-3 gene family in zebrafish

Total RNA was isolated from adult zebrafish, using TRIZOL® Reagent protocol (GIBCOBRL, Life technologies™). 14-3-3 coding sequences were amplified by RT-PCR using specific primers shown in Supplemental Table S5 and cloned into pCRII-TOPO® (Invitrogen). Cloned ORF's were checked for orientation and correct nucleotide sequences (sequencing service from BaseClear, Leiden, The Netherlands).

2.4. Whole-mount in situ hybridizations

14-3-3 – TOPO constructs were linearized using restriction enzyme *Hin*dIII (for all clones except iota 1) or *Spe*I (for iota 1) for the generation of anti-sense probes and restriction enzyme *Xho* for the generation of the sense probes (for control). Full coding sequence anti-sense and sense digoxigenin labeled RNA probes were synthesized from the linearized templates (DIG RNA Labeling Mix, Roche, Mannheim, Germany) with T3 and T7 RNA polymerase. *In situ* hybridization was performed as described previously (Thisse et al., 1993).

2.5. Microarray analysis

Microarray expression analyses were performed using spotted oligonucleotides representing each of the 14-3-3 genes. For this purpose custom made oligonucleotides (synthesized by Illumina) of 50 nucleotide length were spotted on glass plates (Codelink, Amersham) using an Omnigrid 100 Micro-Arrayer (genemachines) as described previously (Carvalho et al., 2004). The sequence of the oligonucleotides was designed using the software program Osprey (http://osprey.ucalgary.ca/)(Gordon and Sensen, 2004). The sequences of the oligonucleotides are shown in Supplementary data table (Table S1). Data were analyzed using the software program Genepix and Rosetta Resolver as described previously (Carvalho et al., 2004). For each embryonic stage at least four independent biological replicates were analyzed. Expression levels for each experiment were set relative to expression of the beta actin gene represented by two different oligonucleotides on the custom made microarrays. The microarray data are part of a larger developmental study which will be submitted to the GEO database.

2.6. Immuno analysis

In order to explore the possibilities for immuno localization of the zebrafish 14-3-3 proteins three different antibody preparations against human 14-3-3 proteins were tested in Western blot assay. For this purpose, we have expressed the cloned 14-3-3 proteins under control of the T7 promoter in *Escherichia coli* strain BL21 codon+ (Stratagene). Using a Pan antibody described by van Heusden et al. (2003) we tested the cloned genes beta 1, iota 1, gamma 1, epsilon 1, and eta. Of these clones, only the iota 1 and eta 14-3-3 genes could be detected on Western blot (Supplementary Fig. S2A). Since this antibody was unsuitable for whole mount immuno localization due to aspecific background we also tested commercially available antibodies against the human zeta and beta isoforms (C-16 and K-19, respectively, obtained from Santa Cruz Biotechnology). Unfortunately these antibodies did not show binding to any of the *E. coli* expressed zebrafish 14-3-3 proteins (results shown for C-16 in Fig. S2B).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.modgep.2006.10.007.

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