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Structural characteristics of zebrafish orthologs of adaptor molecules that associate with transmembrane immune receptors

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Abstract

Transmembrane bound receptors comprised of extracellular immunoglobulin (Ig) or lectin domains play integral roles in a large number of immune functions including inhibitory and activating responses. The function of many of the activating receptors requires a physical interaction with an adaptor protein possessing a cytoplasmic regulatory motif. The partnering of an activating receptor with an adaptor protein relies on complementary charged residues in the two transmembrane domains. The mammalian natural killer (NK) and Fc receptors (FcR) represent two of many receptor families, which possess activating receptors that partner with adaptor proteins for signaling. Zebrafish represent a powerful experimental model for understanding developmental regulation at early stages of embryogenesis and for efficiently generating transgenic animals. In an effort to understand developmental aspects of immune receptor function, we have accessed the partially annotated zebrafish genome to identify six different adaptor molecules: Dap10, Dap12, Cd3 ζ , Cd3 ζ -like, FcR γ and FcR γ -like that are homologous to those effecting immune function in mammals. Their genomic organizations have been characterized, cDNA transcripts have been recovered, phylogenetic relationships have been defined and their cell lineage-specific expression patterns have been established.

Keywords

DAP10; DAP12; FcR γ ; CD3 ζ

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1. Introduction

The cells of the mammalian immune system rely on an intricate network of signaling pathways in order to differentiate between “self” and “non-self”. The activation or inhibition of these signaling pathways relies on specific membrane receptors on immune cells engaging specific ligands. In general, these receptors can be classified as inhibitory or activating based on the functional outcome of ligand recognition. For example, when an activating natural killer (NK) cell receptor binds its ligand, the NK cell is activated to kill the target cell; in contrast, when an inhibitory NK receptor binds its ligand, NK cell-mediated killing is repressed. Similarly, engagement of T-cell antigen receptor (TCR) or Fc receptor (FcεRI) with the appropriate ligand (e.g. peptide-MHC complex or IgE, respectively) leads to a direct cell-mediated immune response.

Despite differences in receptor structures, the cytoplasmic signaling utilized by NK receptors, TCR and Fc receptors, are well conserved (Billadeau and Leibson 2002; Cerwenka and Lanier 2000; Yokoyama and Kim 2006). Inhibitory NK and Fc receptors typically possess one or more cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs). In contrast, activating NK, Fc and other receptors including TCR partner with adaptor proteins, which ultimately transduce signals to the cell nucleus. These receptors physically associate with an adaptor protein via oppositely charged residues within their transmembrane domains, e.g. a positive charge in the transmembrane domain of the activating receptor and a negative charge in the transmembrane domain of the adaptor protein. The majority of activating NK receptors, including most KIRs and Ly49, utilize the adaptor protein, DAP12; the activating NK receptor NKG2D, another lectin-type receptor, utilizes the adaptor DAP10 (Hyka-Nouspikel and Phillips 2006; Takaki et al 2006). TCR and FcR along with other immune related activating receptors, including: CD16, NKp30, NKp46 and NKR-P1C and KIR2DL4, utilize FcRγ or CD3ζ (Cerwenka and Lanier 2000; Tassi et al 2006). The DAP12, FcRγ and CD3ζ adaptors utilize cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs: YxxLX₆₋₁₂YxxL/I) for signaling (Pitcher and van Oers 2003), whereas DAP10 uses a YxxM motif similar to CD28 (Wu et al 1999).

The zebrafish is becoming a more broadly recognized model for infection and immunity and is particularly well suited for examining gene function during embryogenesis (Deiters and Yoder 2006; Lieschke and Currie 2007; Phelps and Neely 2005; Traver et al 2003a; Trede et al 2004; Van Der Sar et al 2004; Yoder et al 2002). As part of an ongoing effort to characterize immune receptors in zebrafish and to understand the signaling pathways mediated by their immune cells, we have identified and characterized the adaptor molecules: Dap12, Dap10, CD3ζ, CD3ζ-like, FcRγ, and FcRγ-like.

2. Materials and methods

2.1 Cloning zebrafish *dap10* (*hcst*) cDNA

A catfish (*Ictalurus punctatus*) *DAP10* cDNA sequence (GenBank: **AAZ16504**) was used as the query for a tBLASTn search of the NCBI zebrafish sequence database (Wheeler et al 2007). Zebrafish bacterial artificial chromosome (BAC) clone DKEY-29H14 (GenBank: **BX571853**) from chromosome 16 encodes a sequence similar to catfish DAP10 (E value = 1e-10). A sequential RACE strategy was used to clone the full-length open reading frame (ORF) of *dap10* (*hcst*) from zebrafish kidney/spleen RNA (GeneRacer, Invitrogen). Initially, 3' RACE was completed with nested forward primers (designed from the DKEY-29H14 sequence) ZFDAP10-F1 and ZFDAP10-F2 which identified the 3' untranslated region of the *dap10* mRNA. Subsequently, nested, reverse primers ZFDAP10-3'UTR-R1 and ZFDAP10-3'UTR-R2 were designed within the 3' UTR of *dap10* and 5' RACE was completed to generate

a cDNA encoding the ORF of *dap10*. Primer sequences are listed in Table 1. PCR products were cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen) and sequenced.

2.2 Cloning zebrafish *dap12* (*tyrobp*) cDNA

tBLASTn analyses of the zebrafish nucleotide (nr), genome and EST databases, using human DAP12 as a query, failed to identify a similar sequence (no sequence identified with an E value <0.05). As *DAP10* and *DAP12* are adjacent genes in mammals and pufferfish (Gusel'nikov et al 2003b), the zebrafish BAC DKEY-29H14, which encodes *dap10*, was examined for *dap12* sequence. A BLASTx analysis using the nucleotide sequence of DKEY-29H14 as a query identified a short sequence (108 nucleotides) within this BAC with extremely low similarity (E value = 1.5) to the transmembrane domain of mouse TCR δ (GenBank: [AAH62807](#)) which, like the transmembrane domain of DAP12, includes a negatively charged residue. We predicted that this novel transmembrane domain encoded by BAC DKEY-29H14 would represent an exon of zebrafish *dap12* (*tyrobp*) based on the following observations: 1) the transmembrane domains encoded by this novel zebrafish sequence and mammalian DAP12 include a negatively charged residue; 2) this candidate zebrafish transmembrane domain is adjacent to *dap10*; and 3) no other sequences within DKEY-29H14 shares similarity with DAP12. Subsequently, a full-length open reading frame cDNA of this sequence was cloned (as follows) and confirmed to encode *dap12*. Initially, 3' RACE was completed from zebrafish kidney/spleen RNA with nested forward primers (designed from the DKEY-29H14 novel transmembrane sequence) ZFDAP12-F1 and ZFDAP12-F2 which identified the 3' untranslated region of the *dap12* mRNA. Subsequently, nested, reverse primers ZFDAP12-R3 and ZFDAP12-R4 were designed within the ORF of *dap12* and 5' RACE was completed to generate a cDNA encoding the 5' UTR of *dap12*. Finally, nested, forward primers ZFDAP12-5' UTR-F3 and ZFDAP12-5'UTR-F4 were designed within the 5' UTR of *dap12* and a cDNA encoding the ORF of *dap12* was derived by 3' RACE. Primer sequences are listed in Table 1. PCR products were cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen) and sequenced.

2.3 Cloning zebrafish FcR γ (*fcer1g*) cDNA

A zebrafish kidney EST (GenBank [BG799671](#)), which encodes FcR γ (*fcer1g*), was identified from a tBLASTn search using a catfish FcR γ sequence (GenBank [AF538721](#)) as a query. In order to confirm this sequence, a 5' RACE strategy was performed as described above using spleen RNA and the nested reverse primers, ZFFcRg-3'UTR-R1 and ZFFcRg-3'UTR-R2, which are complementary to the 3' UTR of *FcR γ* . Primer sequences are listed in Table 1. PCR products were cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen) and sequenced.

2.4 Cloning zebrafish FcR γ -like (*fcer1gl*) cDNA

A zebrafish FcR γ -like (*fcer1gl*) EST sequence was identified (GenBank [BC124700](#)) from a tBLASTn search using catfish FcR γ -like sequence (GenBank [AAN38001](#); Shen et al 2003) as a query. As this zebrafish EST encoded the entire open reading frame (ORF) of *FcR γ -like*, forward and reverse primers, ZFFcRg-B-F, and ZFFcRg-B-R, were designed to amplify the entire ORF from zebrafish spleen cDNA. Primer sequences are listed in Table 1. The *FcR γ -like* ORF was amplified from zebrafish spleen cDNA using 40 cycles and cloned into pCRII-TOPO (Invitrogen) and sequenced.

2.5 Cloning zebrafish *cd3 ζ* (*cd247*) cDNA

A zebrafish *cd3 ζ* (*cd247*) EST sequence was identified (GenBank [EE715400](#)) from a tBLASTn search using a trout CD3 ζ sequence (GenBank [CA344798](#); Rexroad, III et al 2003) as a query. As this zebrafish EST encoded the entire open reading frame (ORF) of *cd3 ζ* , forward and

reverse primers, ZFNEWCd3z-F, and ZFNEWCd3z-R, were designed to amplify the entire ORF from zebrafish spleen cDNA. Primer sequences are listed in Table 1. The cd3 ζ ORF was amplified from zebrafish lymphocyte cDNA using 40 cycles and annealing at 70° C and subsequently cloned into pCRII-TOPO (Invitrogen) and sequenced.

2.6 Cloning zebrafish cd3 ζ -like (cd247l) cDNA

A predicted zebrafish *cd3 ζ -like* (*cd247l*) sequence was identified (GenBank [XM_688285](#)) from a tBLASTn search using the catfish CD3 ζ -like sequence (GenBank [AAZ16505](#)) as a query. The same RACE strategy as described above for *dap10* and *dap12* was used to clone the full-length open reading frame of *cd3 ζ -like* from zebrafish spleen RNA. Initially, 3' RACE was completed with nested forward primers (complementary to a conserved cytoplasmic immunoreceptor tyrosine-based activation motif [ITAM]) ZFCD3z-F1 and ZFCD3z-F2 which confirmed the 3' untranslated region of the *cd3 ζ -like* mRNA. Subsequently, nested, reverse primers ZFCD3z-3'UTR-R1 and ZFCD3z-3'UTR-R2 were designed within the 3' UTR of *cd3 ζ -like* and 5' RACE was completed to generate a cDNA encoding the ORF of *cd3 ζ -like*. Primer sequences are listed in Table 1. PCR products were cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen) and sequenced.

2.7 Phylogenetic analyses

Predicted leader and transmembrane domains of protein sequences were identified with SMART software (<http://smart.embl-heidelberg.de/>; Letunic et al 2004) and proteins were aligned by ClustalW (<http://www.ebi.ac.uk/clustalw/#>; Chenna et al 2003). Neighbor-joining trees (Saitou and Nei 1987) were constructed from pairwise Poisson correction distances with 2000 bootstrap replications by MEGA2.1 software (Kumar et al 2001). Sequences used for this alignment and analyses are listed in Table 2.

2.8 Reverse transcriptase-polymerase chain reaction

Zebrafish (AB strain) embryos were collected by natural mating and maintained at 28° C as described (Westerfield 2000). Tissues from adult zebrafish were dissected and myeloid and lymphoid cell lineages were purified from anterior kidney by cell sorting using forward and side scatter as described (Traver et al 2003b) and cDNAs were generated as described (Panagos et al 2006). One μ l of cDNA was subjected to thermal cycling with gene-specific primers (see RT-PCR primers in Table 1) and Titanium Taq DNA polymerase (BD Biosciences) in a 20 μ l PCR reaction and 10 μ l were analyzed by agarose gel electrophoresis. The number of PCR cycles used for detecting *dap10*, *dap12*, *FcR γ* , *FcR γ -like*, *Cd3 ζ* , *Cd3 ζ -like*, and *actin* transcripts were 30 (annealing at 65° C), 40 (annealing at 65° C), 40 (annealing at 65° C), 30 (annealing at 70° C), 40 (annealing at 70° C), 40 (annealing at 65° C), and 25 (annealing at 65° C), respectively. Myeloperoxidase (*mpx*) transcripts were detected using 25 PCR cycles (annealing at 70° C) and TCR α transcripts were detected using 40 PCR cycles (annealing at 60° C). Forward and reverse primers span at least 1 intron and are listed in Table 1.

3. Results and Discussion

3.1 Zebrafish encode six adaptor proteins: Dap10, Dap12, FcR γ , FcR γ -like, Cd3 ζ and Cd3 ζ -like

As a first step in characterizing immune signaling patterns in zebrafish, we data-mined the zebrafish genome and EST databases to identify sequences corresponding to six candidate adaptor proteins. Full-length cDNAs encoding these proteins termed, Dap12 (*tyrobp*), Dap10 (*hcst*), FcR γ (*fcrlg*), FcR γ -like (*fcrlgl*), Cd3 ζ (*cd247*) and Cd3 ζ -like (*cd247l*), were cloned by RACE or RT-PCR and sequenced. The predicted protein sequences for these zebrafish adaptors are shown aligned to orthologous proteins in Figure 1. All six zebrafish adaptors

encode a peptide leader sequence, a transmembrane domain with a negatively charged amino acid (Asp) and a cytoplasmic tail with at least one activating motif.

Zebrafish Dap12 is 33% identical (46% similar) to human DAP12 and possesses a single cytoplasmic ITAM (Figure 1A). The zebrafish Dap10 sequence identified here differs from a previously described Dap10 EST (GenBank [BQ261988](#)) by a single residue, is 26% identical to human DAP10 (40% similar) and possesses the cytoplasmic tyrosine motif, YMNV, which is similar to the YINM motif encoded by mammalian DAP10 (Figure 1B and Guselnikov et al 2003b). Although this is not an exact match to the mammalian DAP10 signaling motif, this same peptide sequence is encoded by a catfish DAP10 sequence (GenBank [DQ114899](#)) and a related sequence, YMNT, is encoded by DAP10 in *Takifugu* (Guselnikov et al 2003b). It is likely relevant that the YMNV motif encoded by zebrafish and catfish DAP10 is highly similar to the CD28 YMNM motif that, (like DAP10's YINM motif), when tyrosine phosphorylated, binds phosphatidylinositol 3-kinase (PI3K; Cai et al 1995; Prasad et al 1994). The original experiments that led to the definition of the YxxM motif as necessary for PI3K (p85) binding is based on studies using mutated CD28 motifs. When the fourth residue of this motif was mutated from an apolar methionine to a polar cysteine CD28 lost the ability to bind PI3K (Cai et al 1995; Prasad et al 1994). It might be that if this motif is mutated from YxxM to YxxV, (which is a more conserved substitution and matches the zebrafish and catfish DAP10 motif), PI3K binding would be maintained. These observations suggest that the zebrafish DAP10 sequence reported here may signal via PI3K.

Zebrafish FcR γ and FcR γ -like are 44% and 40% identical (72% and 61% similar) to human FcR γ , respectively, and each possesses a single cytoplasmic ITAM (Figure 1C). Zebrafish Cd3 ζ and Cd3 ζ -like are 34% and 29% identical (57% and 52% similar) to human CD3 ζ , respectively. Zebrafish Cd3 ζ and Cd3 ζ -like possess two cytoplasmic ITAMs and Cd3 ζ possesses an additional ITAM-like sequence (Figures 1D and 1E; Underhill and Goodridge 2007).

A phylogenetic analysis was performed in order to further characterize the relationships between all six zebrafish adaptor proteins and their respective orthologs (Figure 2A). The DAP10 and DAP12 proteins form one large family and the CD3 ζ and FcR γ proteins form a second family, supporting the hypotheses that DAP10 and DAP12 are evolutionarily derived from the same progenitor gene and that, likewise, CD3 ζ and FcR γ are likely derived from a single gene (Guselnikov et al 2003a). The observation that DAP10 from fish and *Xenopus* group with mammalian DAP12, rather than DAP10 was unexpected (Figure 2A). A second phylogenetic analysis focused only on DAP10 and DAP12 shows the expected grouping for DAP10/DAP12 across all species (Figure 2B), suggesting that the fine-scale organization of Figure 2A is not reliable: this is likely a result of the gaps introduced into the DAP10/DAP12 sequences required to align them with the longer CD3 ζ /FcR γ sequences. Of these six adaptors, Dap12, Dap10, FcR γ and Cd3 ζ are likely orthologs of their mammalian counterparts, whereas, FcR γ L and Cd3 ζ L are likely paralogs of FcR γ and Cd3 ζ , having arisen via a gene duplication event. This interpretation is strongly supported by evidence for a whole-genome duplication event in the lineage leading to the teleosts (Amores et al 1998; Woods et al 2005). In contrast, no evidence is seen for a second copy of either *DAP10* or *DAP12* in zebrafish (see Section 3.2 for additional discussion).

Zebrafish Dap12 is most closely related to the mammalian DAP12 sequences and groups with these orthologs with ~53% confidence (Figure 2A). To our knowledge, the full-length DAP12 cDNA sequence reported here represents the first from any non-mammalian species although a similar (partial) sequence was predicted from the *Takifugu* genome that was shown to partner with human KIR2DS2 (Feng et al 2006; Guselnikov et al 2003b) in an *in vitro* assay. Although

the DAP10 family is less conserved, all of the DAP10 proteins possess similar signaling motifs (Figure 1), supporting both this classification and related functions.

Zebrafish FcR γ and FcR γ L group with all other FcR γ orthologs with 54% confidence (Figure 2A). A sequence comparison of fish and mammalian FcR γ and FcR γ L sequences suggests that the carp cDNA, originally defined as FcR γ (Fujiki et al 2000), is less similar to FcR γ than other fish genes. A second carp FcR γ gene may exist (Figure 2A). Furthermore, this analysis suggests that of the two proposed catfish sequences designated FcR γ , an unpublished FcR γ sequence (GenBank [AF538721](#)), here identified as “FcR γ ”, is more closely related to the mammalian sequences than to the published FcR γ sequence, here identified as “FcR γ L” (Figures 1 and 2A; Shen et al 2003). The CD3 ζ family of proteins from mammals, *Xenopus*, chicken, and multiple fish species cluster with 73% confidence, however, CD3 ζ L sequences are more divergent from the CD3 ζ sequences and from each other (Figure 2A). The three CD3 ζ L sequences identified here from zebrafish, catfish and trout are not well conserved (Figures 1E and 2A) suggesting that less selective pressure has been encountered during the evolution of this gene. The FcR γ L and CD3 ζ L genes may be restricted to bony fish.

Further support for the nomenclature for these genes is provided by BLASTp analyses of mammalian proteins using the peptide sequences of the zebrafish adaptors as queries. Although the similarity is not strong, the mammalian protein with the strongest similarity to zebrafish Dap10 is a primate DAP10 (Table 3) and much of this similarity is focused on the transmembrane domain and cytoplasmic signaling motif (Figure 1B). In contrast to DAP10, DAP12 is much more conserved between fish and mammals (Figures 1A and 2), suggesting that there has been more selective pressure on DAP12. In addition, this analysis confirms that zebrafish Cd3 ζ and Cd3 ζ L are most similar to mammalian CD3 ζ and that zebrafish FcR γ and FcR γ L are most similar to mammalian FcR γ . Zebrafish CD3 ζ and FcR γ are more closely related to their mammalian orthologs than are CD3 ζ L and FcR γ L (Table 3)

3.2 Genomic organization of zebrafish adaptor proteins

Zebrafish *dap10* (*hcst*) and *dap12* (*tyrobp*) are encoded by BAC DKEY-29H14 (GenBank [BX571853](#)) which maps to chromosome 16. Zebrafish *FcR γ* (*fcer1g*) is encoded by BAC CH211-286P15 (GenBank [BX324220](#)), which maps to chromosome 7 and *FcR γ L* (*fcer1gl*) is encoded by a chromosome 2 contig (GenBank [NW_001513019](#)). Only a single exon of zebrafish *cd3 ζ* (*cd247*) has been placed on a chromosome 1 contig (GenBank [NW_001511947](#)) whereas *cd3 ζ L* (*cd247l*) is encoded by BAC CH211-218D20 (GenBank [BX294189](#)), which maps to chromosome 9. The genomic organization of these genes is depicted in Figure 3. Zebrafish *dap10* and *dap12* are immediately adjacent genes as they are in mammals and *Takifugu* (Guselnikov et al 2003b).

The presence of two paralogs for a single mammalian gene (e.g. CD3 ζ and FcR γ) is not uncommon in zebrafish as much evidence indicates a whole genome duplication event occurred in the lineage leading to the teleosts (Amores et al 1998; Woods et al 2005). The zebrafish paralogs of FcR γ are present on chromosomes 7 and 2 which have been shown to encode other duplicated genes (e.g. *ap1g1* and *ap1g2*). Similarly, the zebrafish paralogs of Cd3 ζ are present on chromosomes 1 and 9 which encode other duplicated genes (e.g. *dlx2a* and *dlx2b*) and are predicted to be the result of an ancient chromosomal duplication (Woods et al 2005). In contrast the characterization of zebrafish *dap10* and *dap12* as single copy genes is supported by the estimate that only ~25% of the duplicated genes are currently identifiable within the zebrafish genome (Woods et al 2005). The completion of its genome will conclusively determine if zebrafish possess paralogs for *dap10* and *dap12*.

DAP10 and *DAP12* are tightly associated genes in mammals and zebrafish. They are immediately adjacent genes and separated by ~200 and ~ 8,700 bp in human and zebrafish,

respectively. In contrast, *FcR γ* and *Cd3 ζ* , which are encoded by the same chromosome in human and mice, are present on two chromosomes in zebrafish. This difference in physical linkage is likely due to the larger intergenic distance between these genes in mammals (~6,200,000 bp in human) and the extensive number of interchromosomal rearrangements since the ancestral genome of bony vertebrates (Woods et al 2005).

Further evidence supporting the identity of these zebrafish adaptors is provided by the syntenic relationships between the human and zebrafish loci for these genes. Four zebrafish genes which flank each adaptor gene (two genes on each side) were identified and the orthologous genes from human were defined by BLASTp analyses (Table 4). For example, zebrafish *FcR γ* (*fcrlg*) is adjacent to *zgc:112036* which shares high identity with human *NDUFS2*: both human *FcR γ* (*FCER1G*) and *NDUFS2* map to human chromosome 1q23. There is evidence for conserved synteny in all six adaptor gene loci in zebrafish: one or more of the genes which flank each zebrafish adaptor gene maps to the same chromosomal region as the human adaptor gene (Table 4).

3.3 Zebrafish adaptor proteins are differentially expressed in hematopoietic lineages

The expression of *dap10*, *dap12*, *FcR γ* , *FcR γ L*, *cd3 ζ* , and *cd3 ζ L* during zebrafish embryogenesis and in adult tissues (ovary, liver, kidney, spleen and intestine) (Figure 4A) was examined using an RT-PCR strategy. Maternal transcripts of all six adaptors can be detected in the ovary and *dap10* transcripts continue to be detected in the 1-cell stage embryo (0 hours post fertilization, hpf). Zebrafish *FcR γ* is the only adaptor that is detected at later stages of embryogenesis (72 hpf) whereas *FcR γ* , *cd3 ζ* , and *cd3 ζ L* transcripts are detected at the larval stage of 6 days post fertilization (dpf). This may reflect either very low levels of expression, expression in small sets of cells or simply a lack of expression at the early stages of development. Similar expression patterns of *dap10*, *dap12*, *cd3 ζ L*, *FcR γ* and *FcR γ L* transcripts can be detected in adult liver, kidney, spleen and intestine, whereas *Cd3 ζ* is primarily detected in ovary and spleen, with lower levels of expression in intestine. Analysis of adaptor expression in zebrafish lymphoid and myeloid cells was completed using flow cytometrically separated cell populations (Figure 4B) and reveals that *dap10*, *dap12* and *cd3 ζ* are restricted to lymphocytes whereas *cd3 ζ L*, *FcR γ* and *FcR γ L* are expressed in both lymphoid and myeloid lineages (Figure 4C). Based on these distributions, it is likely that the adaptor homologs may function in immune signaling systems in zebrafish.

3.4 Concluding remarks

As *DAP12*, *DAP10*, *CD3 ζ* and *FcR γ* are all expressed by mammalian NK cells (Hamerman and Lanier 2006; Wu et al 2000), these data raise the possibility that zebrafish lymphocytes (purified by forward and side scatter) include NK cells. Although clonal NK-like cell lines have been described from catfish (Shen et al 2004), there are currently no reagents for identifying or purifying pure NK cell populations from bony fish. Finally, the observation that zebrafish *dap10* and *dap12* transcripts are not detected in myeloid cells was unexpected as the mammalian orthologs of these genes are expressed in both lymphoid and myeloid cells (Takaki et al 2006; Wu et al 1999): it is possible that *dap10* and *dap12* are expressed at very low levels or in a small population of cells within the zebrafish myeloid lineage.

The observation that *Takifugu* *DAP12* can associate with a human KIR in a cell culture system suggests a conserved function for *Dap12* (Feng et al 2006); however, KIRs have not been identified in bony fish. Other candidate activating NK receptors, that possess positively charged residues within their transmembrane domains, have been identified from zebrafish including an immunoglobulin-type receptor, *Nitr9*, and a lectin-type receptor, *Illr3* (Panagos et al 2006; Yoder et al 2004): it will be of interest to determine if these receptors physically partner with *Dap12*. The presence of paralogs for *CD3 ζ* and *FcR γ* in zebrafish raises additional

questions of functional specificity. Do zebrafish TCR and Fc ϵ RI utilize a single adaptor for signaling? If so, which one? And what role does the second adaptor paralog play in immune response? Knowledge of the structures of the zebrafish adaptors and the relationships of these structures to each other will be useful for the design of specific reagents and engineering of constructs for examining the specific interactions and functions of these adaptor molecules in the immunological and developmental context of the zebrafish model.

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Abbreviations

FcR, Fc receptor; Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; NK, natural killer; TCR, T-cell antigen receptor.

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Figure 1. Comparison of zebrafish and mammalian adaptor protein sequences

The predicted sequences of zebrafish adaptor proteins are aligned with orthologous adaptor proteins. Protein symbols are defined in Table 2. Comparisons include members of the (A) DAP12, (B) DAP10, (C) FcR γ , FcR γ L, (D) CD3 ζ and (E) CD3 ζ L families. Black shading indicates identical amino acids and gray shading indicates functionally similar amino acids. Peptide leader sequences (L) and transmembrane (TM) domains are indicated above the alignment. A negatively charged residue (D) is present within the transmembrane domain of all adaptor proteins and indicated with asterisks (below) and pink highlighting. Cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs; Y_{xx}LX₆₋₁₂Y_{xx}L/I) and ITAM-like sequences (Y_{xx}LX₆₋₁₂Y_{xx}A) which have been defined in mammalian signaling proteins (Pitcher and van Oers 2003; Underhill and Goodridge 2007) and are conserved in other species are indicated with down black arrowheads and yellow highlighting. Conserved cysteines that are utilized for dimerization are indicated by red arrows (Feng et al 2005; Feng et al 2006; Rutledge et al 1992). The tyrosine present in the DAP10 signaling motif (Y_{xx}M) is indicated with a black circle (above) and blue highlighting: note that this motif is modified in catfish, zebrafish (YMN_V) and *Takifugu* (YMNT). Amino acid numbering is on the left. Introduced gaps in the sequence alignment are indicated with a dash (-).

Figure 2. Phylogenetic analyses of adaptor proteins

(A) The six zebrafish adaptor proteins (black text boxed in gray) are compared to the orthologous proteins defined in Table 2 and Figure 1. Note that FcR γ L and CD3 ζ L may be restricted to fish species. (B) Zebrafish Dap10 and Dap12 are compared to orthologous proteins defined in Table 2 and Figure 1. Protein groups are identified on the right (white text boxed in gray). Bootstrap values less than 50 are not shown. Branch lengths are measured in terms of amino acid substitutions, with the scale indicated below the tree.

Figure 3. Genomic organization of zebrafish adaptor genes

The cDNA sequences for zebrafish (A) Dap10 (*hcst*) and Dap12 (*tyrobp*), (B) FcR γ (*fcrl1g*), (C) FcR γ -like (*fcrl1gl*) and (D) CD3 ζ -like (*cd247l*) were mapped onto their corresponding genomic sequence in order to define their exon-intron organization. The transcriptional orientation of all genes is left to right. All of these genes encode a translational start site within the first exon and a stop codon in the last exon. The identity of the BAC clone or contig used to deduce the exon-intron organization and the chromosome to which each gene maps is listed below each gene. (E) The relative genomic locations of the genes for all six adaptors are shown on each chromosome (chromosome numbers are listed below each figure). Note that only a single exon of CD3 ζ (*cd247*) can be identified in the current release of the zebrafish genome database and is localized to chromosome 1.

Figure 4. Detection of adaptor protein expression

(A) RT-PCR was used to detect transcripts of the six zebrafish adaptor proteins, *dap10*, *dap12*, *Fcrg*, *Fcrg-like*, *CD3 ζ* and *CD3 ζ -like* from embryonic and larval zebrafish including 0 hours post fertilization (hpf) through 6 days post fertilization (dpf) as well as from adult tissues (ovary, liver, kidney, spleen and intestine). (B) Myelomonocytic (gray gate) or lymphocytic (black gate) cells were purified from whole kidney marrow based on light scatter characteristics. The scatter profile of zebrafish whole kidney marrow (left) is shown in comparison to post-myeloid (middle) and post-lymphoid (right) sorts using forward (FSC) and side (SSC) scatter. Myeloid and lymphoid cells, which represent ~21.5% and ~13.5% of the whole kidney marrow were purified to ~95.6% and ~75.6% purity, respectively, and used for RT-PCR analyses. (C) RT-

PCR was used to assess which adaptor proteins are expressed in the zebrafish lymphoid and myeloid lineages. RT-PCR of *mpx* provides a positive control for myeloid cells and RT-PCR of TCR α provides a positive control for lymphocytes. β -*actin* is shown as a positive control for all RNA samples.

Table 1**Oligonucleotide Primer Sequence**

Purpose	Primer Name	Primer Sequence
DAP10 3' RACE	ZFDAP10-F1	CGGTCCTCATTGTTACCACCACC
	ZFDAP10-F2	GCGCAGACAGAAGAAGGAAAAACG
DAP10 5' RACE	ZFDAP10-3'UTR-R1	GTCTTATTACACAAGGCCACTATG
	ZFDAP10-3'UTR-R2	CCATTGTTGACGTCAGTCTGCC
DAP12 3' RACE	ZFDAP12-F1	CCGTCTACTGTTTTGTTTCTCGCC
	ZFDAP12-F2	GATGCTGTAGCTCAGGTAAGCCTG
	ZFDAP12-5'UTR-F3	CTTGATTTCGAGTCAGGGCTGTG
	ZFDAP12-5'UTR-F4	TCTTTCCTAAACCTGTCTGCACTC
DAP12 5' RACE	ZFDAP12-3'UTR-R3	ACTGTATATGTCTGACTGCACTCC
	ZFDAP12-3'UTR-R4	TGCCCTCATAGATGTCTGCTGAAG
CD3 ζ 3' RACE	ZFCD3z-F1	GACTTAGATCTACCTCAGGTGGGC
	ZFCD3z-F2	CAGGTGGGCTCCGATTATCAACAAC
CD3 ζ 5' RACE	ZFCD3z-3'UTR-R1	AGAAATCAAGACACAAGTGCATCC
	ZFCD3z-3'UTR-R2	AGTTGCTCCTCCGCTCTTTGCTGTG
FcR γ 5' RACE	ZFFcRg-3'UTR-R1	GATGGTGGCTTTTTCTTCTGGTGGCG
	ZFFcRg-3'UTR-R2	GGCTTTTTCTTCTGGTGGCGAGTCTC
RT-PCR DAP10	ZFDAP10-F3	AGGGCTTCTAGTGTTCCTCTCTC
	ZFDAP10-R3	GCCAAAGATCTTCATGTTTTGCAG
RT-PCR DAP12	ZFDAP12-F3	GGATACTGTTTATAATGTTCCCCG
	ZFDAP12-R3	TTCCGATACTGCTGAAGATCACTG
RT-PCR CD3 ζ L	ZFCD3z-F3	AGCAGGACAAGCACTCTATTGTTG
	ZFCD3z-R3	AAACGTTTCCATCTCCACAGCCTC
RT-PCR CD3 ζ	ZFNEWCD3z-F	GAGACTTGATTGATGGCGAGAGC
	ZFNEWCD3z-R	ATCAGTTTAGCGCGGTGGCAAC
RT-PCR FcR γ	ZFFcRg-F3	ATGGTGGTGTGCTGATGAAGATCTTC
	ZFFcRg-R3	GGCGGATTTTCATCTTGATGGTCTC
RT-PCR FcR γ L	ZFFcRg-B-F	GAGAGTAAAGATGAAGCTGCTGG
	ZFFcRg-B-R	GTTAATGTGGGACATGTTACAGC
RT-PCR β -actin	Actin For	GGTATGGAATCTTGCGGTATCCAC
	Actin Rev	ATGGGCCAGACTCATCGTACTCCT
RT-PCR TCR α	TCRa For	GGAACACAAGTTCATGTTGAAAC
	TCRa Rev	GCTCATCCACGCTTTGAAAGTCA
RT-PCR <i>mpx</i>	<i>mpx</i> -For	CCAGAACCAGTGAGCCTGAGACACG
	<i>mpx</i> -Rev	CAGTCTAACCATGGGCAGCGCTGCAC

Table 2

Protein sequences used for phylogenetic comparisons

Symbol in Manuscript	Species	Gene Symbol	mRNA, GenBank	EST, GenBank	Reference
Hs Dap10	<i>Homo sapiens</i> (human)	<i>HCST</i>	NP 055081		(Chang et al 1999; Wu et al 1999)
Mm Dap10	<i>Mus musculus</i> (mouse)	<i>Hcst</i>	NP 035957		(Chang et al 1999; Wu et al 1999)
Xl DAP10	<i>Xenopus laevis</i> (frog)	<i>dap10-A</i>	AAP33502		(Gusel'nikov et al 2003b)
Tr DAP10	<i>Takifugu rubripes</i> (pufferfish)			AL835849	(Gusel'nikov et al 2003b)
Ip Dap10	<i>Ictalurus punctatus</i> (catfish)			DOI14899	Unpublished
Dr Dap10	<i>Danio rerio</i> (zebrafish)	<i>hcst</i>	EF158445		This manuscript
Hs DAP12	<i>Homo sapiens</i> (human)	<i>TYROBP</i>	NP 003323		(Lanier et al 1998)
Mm DAP12	<i>Mus musculus</i> (mouse)	<i>Tyrobp</i>	NP 035792		(Lanier et al 1998)
Dr Dap12	<i>Danio rerio</i> (zebrafish)	<i>tyrobp</i>	EF158446		This manuscript
Hs FeRγ	<i>Homo sapiens</i> (human)	<i>FCER1G</i>	NP 004097		(Kuster et al 1990)
Mm FeRγ	<i>Mus musculus</i> (mouse)	<i>Fcer1g</i>	NP 034315		(Ra et al 1989)
Xl FeRγ	<i>Xenopus laevis</i> (frog)	<i>FeRgamma</i>	AAO14673		(Gusel'nikov et al 2003a)
Tr FeRγ	<i>Takifugu rubripes</i> (pufferfish)			CA 330711	(Gusel'nikov et al 2003b)
Ip FeRγ	<i>Ictalurus punctatus</i> (catfish)		AF538721		Unpublished
Dr FeRγ	<i>Danio rerio</i> (zebrafish)	<i>fcer1g</i>	EF158447		This manuscript
Cc FeRγL	<i>Cyprinus carpio</i> (carp)		BAA92270		(Fuiki et al 2000)
Ip FeRγL	<i>Ictalurus punctatus</i> (catfish)		AF543420		(Shen et al 2003)
Dr FeRγL	<i>Danio rerio</i> (zebrafish)	<i>fcer1gl</i>	EF601085		This manuscript
Hs CD3ε	<i>Homo sapiens</i> (human)	<i>CD247</i>	NP 932170		(Weissman et al 1988b)
Mm Cd3ε	<i>Mus musculus</i> (mouse)	<i>Cd247</i>	NP 112439		(Weissman et al 1988a)
Xl CD3ε	<i>Xenopus laevis</i> (frog)	<i>cd3ε-A</i>	AAO14672		(Gusel'nikov et al 2003a)
Gg CD3ε	<i>Gallus gallus</i> (chicken)	<i>CD247</i>	NP 996762		(Gobel and Bolliger 1998)
Ss CD3ε	<i>Salmo salar</i> (salmon)		EF421412		Unpublished
Om CD3ε	<i>Oncorhynchus mykiss</i> (trout)			CA344798	(Gusel'nikov et al 2003b)
Dr CD3ε	<i>Danio rerio</i> (zebrafish)	<i>cd247</i>	EF601086		This manuscript
Ip CD3εL	<i>Ictalurus punctatus</i> (catfish)		DQ114900		Unpublished
Om CD3εL	<i>Oncorhynchus mykiss</i> (trout)			CA 381679	Unpublished
Dr CD3εL	<i>Danio rerio</i> (zebrafish)	<i>cd247l</i>	EF158448		This manuscript

Table 3

Mammalian proteins most similar to zebrafish adaptor proteins

Proteins most similar to zebrafish Dap10	GenBank Accession	Species	BLASTP E value
DAP10	NP_001028007	rhesus monkey	0.010
DAP10	NP_055081	human	0.047
DAP10	NP_999307	pig	0.053
Proteins most similar to zebrafish Dap12			
DAP12	NP_777052	cattle	2e-10
DAP12	NP_001028039	rhesus monkey	3e-10
DAP12	XP_512605	chimpanzee	4e-10
Proteins most similar to zebrafish FcRγ			
FcR γ	P20411	rat	1e-13
FcR γ	NP_004097	human	4e-13
FcR γ	XP_001115585	rhesus monkey	3e-12
Proteins most similar to zebrafish FcRγL			
FcR γ	P20411	rat	9e-11
FcR γ	P20491	mouse	3e-10
FcR γ	Q9BDR7	cow	1e-09
Proteins most similar to zebrafish Cd3ζ			
CD3 ζ	BAD93281	pig	5e-11
CD3 ζ	NP_740770	rat	7e-11
CD3 ζ	P24161	mouse	2e-10
Proteins most similar to zebrafish Cd3ζL			
CD3 ζ	AAV59894	water buffalo	1e-04
CD3 ζ	P24161	mouse	2e-04
CD3 ζ	Q9TUF8	rabbit	2e-04

Table 4Evidence for conserved synteny at adaptor gene loci.¹

Zebrafish chromosome	Zebrafish gene ²	Human gene ³	Human chromosome
1	<i>zgc:63856</i>	<i>LRR1Q2</i>	3q12.3
	<i>LOC100001781</i>	<i>NFKBIZ</i>	3p12-q12
	<i>cd3c (cd247)</i>	<i>CD247</i>	1q22-q23
	<i>LOC100007643</i>	<i>CREG1</i>	1q24
	<i>LOC573838</i>	<i>HIST1H2AA</i>	6p22.2
2	<i>LOC794920</i>	<i>ZNF429</i>	19p13.1
	<i>LOC794993</i>	<i>PVRL4</i>	1q22-q23.2
	<i>FcRg-like (fcer1g)</i>	<i>FCER1G</i>	1q23
	<i>LOC566106</i>	<i>DIS3L2</i>	2q37.1
	<i>LOC566167</i>	<i>MIER1</i>	1p31.3
7	<i>zgc:64208</i>	<i>KIRREL</i>	1q21-q25
	<i>zgc:112036</i>	<i>NDUFS2</i>	1q23
	<i>FcRg (fcer1g)</i>	<i>FCER1G</i>	1q23
	<i>LOC799932</i>	<i>LY75</i>	2q24
	<i>LOC100000010</i>	<i>MRC2</i>	17q23.2
9	<i>LOC564820</i>	<i>GPA33</i>	1q24.1
	<i>LOC564893</i>	<i>POU2F1</i>	1q22-q23
	<i>cd3z-like (cd247l)</i>	<i>CD247</i>	1q22-q23
	<i>LOC563328</i>	<i>LAMP1</i>	13q34
	<i>LOC791767</i>	<i>GRTP1</i>	13q34
16	<i>LOC567390</i>	<i>ZNF525</i>	19q13.41
	<i>LOC100007321</i>	<i>HKR1</i>	19q13.12
	<i>dap10 (hcst)</i>	<i>HSCT</i>	19q13.1
	<i>dap12 (tyrobp)</i>	<i>TYROBP</i>	19q13.1
	<i>zgc:110182</i>	<i>FAM84B</i>	8q24.13
	<i>LOC563341</i>	<i>LOC340359</i>	8q24.13

¹ Bold text indicates a conserved syntenic relationship between adaptor gene loci in zebrafish and human.² Gene order was determined with version Zv6 of the zebrafish genome and NCBI Map Viewer.³ Human genes shown have the highest identity to the zebrafish genes as determined by BLASTp analyses.