The neuronal growth and regeneration associated Cntn1 (F3/F11/Contactin) gene is duplicated in fish: expression during development and retinal axon regeneration

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The Cntn1 (Contactin/F3/F11) cell adhesion molecule is involved in axon growth and guidance, fasciculation, synapse formation, and myelination in birds and mammals. We identified Cntn1 genes in goldfish, zebrafish, and fugu, and provide evidence for a fish-specific duplication leading to Cntn1a and Cntn1b. Our analyses suggest a subfunctionalization for the Cntn1 paralogs in zebrafish compared to other vertebrates which have a single Cntn1 gene. Similar to Cntn1a, Cntn1b transcripts are found in subsets of sensory and motor neurons. However, Cntn1b is detected later and more restricted than Cntn1a. This spatio-temporal expression pattern of the two zebrafish Cntn1 paralogs suggests functions related to those of mammalian Cntn1. In adult goldfish, Cntn1b is expressed in oligodendrocytes and is upregulated in retinal ganglion cells after optic nerve transection, which is consistent with an additional role during regeneration.

Introduction

Cell adhesion molecules (CAM) of the immunoglobulin super family (IgSF) are involved in many aspects of nervous system development including axon growth and pathfinding. Many axonal CAMs are built of C2 type immunoglobulin like (Ig) domains associated with fibronectin type III like domains, leading to their definition as Ig/FN molecules. (Brummendorf and Lemmon, 2001; Brummendorf and Rathjen, 1996; Faivre Sarrailh and Rougon, 1997). In particular, the vertebrate Contactin (Cntn) subfamily consists of six Ig domains connected via a hinge region to four FN domains and attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (Brummendorf et al., 1989; Furley et al.,

1990; Gennarini et al., 1989; Ogawa et al., 1996; Yoshihara et al., 1994, 1995). Whereas a single Cntn gene is known in arthropods (Falk et al., 2002 and this study), six different members of this Ig/FN subfamily have been identified in vertebrates: Contactin/F3/F11, TAG 1/Axonin 1/TAX1, BIG 1, BIG 2, NB 2/FAR 2 and NB 3 (hereafter: Cntn1 to Cntn6, respectively, according to the approved nomenclature of human Contactin genes).

Cntn1 (Contactin/F3/F11) is expressed in a spatio temporal regulated pattern in the developing mammalian nervous system. It undergoes *cis* or *trans* heterophilic interactions with other cell surface components including Caspr, NrCAM, RPTP ϵ / β , Cntn2 and Neurofascin 155 on neurons and glial cells (Buttiglione et al., 1998; Faivre Sarrailh et al., 1999; Peles et al., 1995, 1997; Volkmer et al., 1998) and with the extracellular matrix molecules Tenascin C and Tenascin R (Pesheva et al., 1993; Rigato et al., 2002; Xiao et al., 1998). In addition, this glycoprotein has been described to interact with Notch expressed on oligodendrocytes and to activate the associated signaling pathway (Hu et al., 2003). Moreover, Cntn1 associates with microdomains in oligodendrocytes (Kramer et al., 1999) and with the non caveolar raft proteins reggie 1 and reggie 2 in neurons (Lang et al., 1998; Stuermer et al., 2001). In vitro experiments implicated Cntn1 in neurite growth control (Durbec et al., 1992; Gennarini et al., 1991), in repulsion from substrate bound matrix components (Pesheva et al., 1993), and in axon fasciculation (Bizzoca et al., 2003; Buttiglione et al., 1996; Chang et al., 1987; Rathjen et al., 1987). The polarized distribution in some central synapses suggests a potential role in synapse formation (Faivre Sarrailh et al., 1992). Cntn1 knock out mice are only viable until postnatal day 18 and display defects in axon guidance and myelination (Berghlund et al., 1999; Boyle et al., 2001). Developmental misexpression in transgenic mice affects axonal growth of cerebellar granule cells and Purkinje neuron dendritic development (Bizzoca et al., 2003).

The present study focuses on the significance of Cntn1 expression in controlling differentiation/regeneration events using

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different fish neuronal model systems. Retinal ganglion cell (RGC) axons readily regenerate in fish (Gaze, 1970). During this process, many growth associated molecules are re expressed (Stuermer and Leppert, 2000), whereas upregulation upon lesion is the exception rather than the rule in mammals (Jung et al., 1997). To address the significance of Cntn1 in such events, we cloned Cntn1 in goldfish and zebrafish and uncovered two Cntn1 genes in the fugu genome.

While our study was in progress, a second zebrafish Cntn1 gene was identified (Gimnopoulos et al., 2002). Syntenic chromosomal positions and phylogenetic relationships among all available Cntn genes provide convincing evidence that our newly identified fish sequences are true Cntn1 orthologs, indicating that a fish specific duplication of the Cntn1 gene occurred early in actinopterygian evolution leading to fish Cntn1a and Cntn1b. The zebrafish Cntn1

paralogs show both partially overlapping and distinct spatial and temporal expression patterns. Cntn1b expression is delayed with respect to Cntn1a during embryonic development. Cntn1b transcripts are restricted to the nervous system while Cntn1a is additionally expressed in non neuronal tissues. In adult goldfish, Cntn1b is detected in oligodendrocytes and RGCs and its expression is upregulated after optic nerve transection. This indicates a role for this IgSF molecule in RGC axon regeneration and axon oligodendrocyte interaction, resulting in re myelination and recovery of normal vision.

Results

Identification and characterization of Cntn1 in fish

A full length zebrafish Cntn1 sequence was identified by library screening and RACE. According to the zebrafish nomenclature guidelines, this gene was named Cntn1b while the previously identified gene (Gimnopoulos et al., 2002) is Cntn1a. We also screened a cDNA library from adult goldfish (gf) brain using zfCntn1b derived probes and isolated two overlapping Cntn1 clones covering the full length cDNA. Phylogenetic analysis (see below) revealed that the resulting 3.7 kb transcript encodes a zfCntn1b ortholog and was therefore named gfCntn1b. No gfCntn1a transcript was identified in this screen although, guessing from the zebrafish expression profile (see below), expression would be expected in adult goldfish brain. In addition, no gfCntn1a could be amplified by RT PCR neither with degenerate nor with zfCntn1a specific primers from various goldfish libraries and cDNAs of different developmental stages and adult tissues (data not shown). The sequences surrounding the putative start methionines of zfCntn1b and gfCntn1b (atcAagATGG/atcAagATGG) comply with the consensus motif for translation initiation (gccAccATGG; Kozak, 1996). In addition, an upstream stop codon (−42 to −40 bp) ensures that the identified start methionine corresponds to the N terminus of the zfCntn1b protein. Although no upstream stop codon was found for gfCntn1b, the location of the Kozak consensus and comparison to the zfCntn1b sequence suggests that the genuine start methionine has been identified. In the 3' untranslated region (UTR) of the zfCntn1b gene (1443 bp), three alternative polyadenylation signals could be assigned to 3231 to 3236 bp, to 3271 to 3276 bp and to 4498 to 4503 bp (19, 17, and 13 bp upstream of the three alternative poly A tails, respectively). The 3'UTR of the gfCntn1b gene (587 bp) contains two potential,

alternative polyadenylation signals (3412 to 3417 bp and 3760 to 3765 bp), but no poly A tail was detected.

Both sequences were deposited in GenBank (GenBank accession numbers AY301281 for zfCntn1b and AY434496 for gf Cntn1b).

By searching NCBI and Ensembl web pages, two different fugu Cntn1 genomic sequences were retrieved. The respective cDNAs were deduced by comparison with the zfCntn1b transcript. The low sequence homology in the 5' and 3' regions of the Cntn1 molecules did not allow to identify these parts in the fugu genomic sequences (Supplementary Fig. 1). According to the phylogenetic analysis (see below), the two transcripts were called fuguCntn1a and fuguCntn1b, respectively.

The full length zfCntn1b sequence consists of 4771 bp, with an ORF encoding 1031 amino acids (aa). The coding region of the goldfish transcript comprises 1029 aa (Supplementary Fig. 1). Alignment of zfCntn1b, gfCntn1b, fuguCntn1b, and fuguCntn1a protein sequences with zfCntn1a (Gimnopoulos et al., 2002), human, mouse, chicken, and frog Cntn1 showed that these sequences are evolutionary conserved, especially at the domain borders (Supplementary Fig. 1). As predicted for other vertebrate Cntn1 proteins, the fish orthologs consist of an N terminal signal peptide (22 aa in zfCntn1b and 20 aa in gfCntn1b; Nielsen et al., 1997) followed by six C2 type immunoglobulin like (Ig), four fibronectin type III like (FN) domains, and a C terminal GPI anchor attachment site (Eisenhaber et al., 1999) at 1006 aa (zf) and 1007 aa (gf), respectively (Supplementary Fig. 1).

Conservation of the exon intron arrangement of fish and human Cntn1 genes

The Cntn1 gene consists of at least 23 exons in chicken (Plagge and Brummendorf, 1997) and of 24 exons in mouse (Buttiglione et al., 1995; Cangiano et al., 1997) and human (Berglund and Ranscht, 1994). To analyze the gene organization in fish, we performed database searches for genomic sequences and deduced the exons by comparison with the corresponding cDNAs.

The zfCntn1b gene covers 24 exons in total, although additional untranslated exons as described for the mouse Cntn1 gene (De Benedictis et al., 2001) may also exist. According to the exon nomenclature for the human and mouse Cntn1 genes (Berglund and Ranscht, 1994; Buttiglione et al., 1995), the first, untranslated exon is called exon 0 (Fig. 1A and Supplementary Table 3A). Exon 1 contains additional 5'UTR and encodes the signal peptide. The third exon (exon 2) codes for a 14 aa peptide. A splice isoform of zfCntn1b lacking this exon is detectable at low levels (data not

Fig. 1. Exon intron arrangements of fish Cntn1 genes and phylogenetic relationships among Cntn family genes. (A) The exon arrangement and its relationship to the protein domain architecture are schematically shown for the zfCntn1b and dmCntn genes. Exons are represented as numbered boxes with the coding regions shaded in gray. Black lines indicate the extent of individual protein domains (i.e., Ig1-6, FNI-IV). Dotted lines connect the corresponding exons of zfCntn1b and dmCntn. Exon 0 of zfCntn1b contains exclusively 5' UTR, and exon 1 covers 5' UTR and the sequence coding for the signal peptide. Exons 3–22 encode the six Ig and the four FN domains in a 'two exon/one domain' relationship. Exon 23 codes for the GPI attachment signal and includes a large 3' UTR region. Exon 0 of dmCntn contains exclusively 5' UTR, and exon 1 covers 5' UTR, the sequence coding for the signal peptide and for a lectin type 2-like domain absent in vertebrate Cntns. The six Ig and the four FN domains are not encoded in a 'two exon/one domain' relationship. (B) Exon intron arrangements of zfCntn1b, fuguCntn1b and fuguCntn1a are schematically shown. The most 5' and 3' exons (exons 0–2 and exon 23) of the fugu genes were not identified in the genomic sequences (represented by question marks). (C) Evolutionary relationships among vertebrate and invertebrate Cntn genes. The topology represents a consensus tree based upon Neighbor-Joining (NJ) analysis of an amino acid (aa) alignment (910 positions, Supplementary Fig. 2). A similar topology was reconstructed from a nucleotide alignment using NJ analysis. Support for nodes is 100% except where indicated (1000 bootstrap reiterations). Scale represents 5% protein sequence divergence. UTR, untranslated region; SP, signal peptide; Ig, Immunoglobulin type C2-like domain; FN, Fibronectin type III-like domain; GPI, glycosylphosphatidylinositol anchor attachment site; L, lectin type 2-like domain; bp, basepairs; ag, *Anopheles gambiae*; bos, *Bos taurus*; ch, *Gallus gallus*; dm, *Drosophila melanogaster*; fugu, *Fugu rubripes*; gf, *Carassius auratus*; hu, *Homo sapiens*; mu, *Mus musculus*; rat, *Rattus norvegicus*; xl, *Xenopus laevis*; zf, *Danio rerio*.

shown), as has been revealed for the human Cntn1 gene (Berglund and Ranscht, 1994). The consecutive 20 exons encode the six Ig like and the four FN like domains in a 'two exon/one domain' relationship. The last exon of the zfCntn1b gene covers the GPI attachment signal and includes a large 3' UTR region (Fig. 1A). On the basis of the corresponding genomic sequences, exons 3 to 22 were also deduced for fuguCntn1b and fuguCntn1a (Fig. 1B and Supplementary Tables 3B and 3C). Their sizes, intron phasings, and exon domain arrangements are identical to zfCntn1b.

Comparison of fish and human Cntn1 exon intron structures revealed that: (1) exon sizes are nearly identical in zebrafish, fugu, and human genes (Fig. 1B and Supplementary Tables 3A–C); (2) intron sizes vary considerably being smallest in fugu and largest in human; (3) interdomain introns (separating two domains) are exclusively in phase 1 (i.e., after the first nucleotide of a codon), which is in line with the evolution of multi domain proteins via exon shuffling (Roy, 2003). In contrast, introns lying within one domain (intradomain introns) do not show phase preferences.

In comparison, the single *Drosophila melanogaster* Cntn gene consists of eight exons and therefore has a different exon intron arrangement (Fig. 1A). The first exon (exon 0) encodes 5' UTR as in vertebrate Cntn1 genes. The second exon (exon 1) codes for the signal peptide and a lectin type 2 like domain, which is absent in vertebrate Cntn1. Exons 2 to 7 encode the six Ig and four FN domains (Fig. 1A and Supplementary Table 3D) and seem to be the result of exon fusion processes during evolution in the arthropod lineage. The remaining interdomain introns (separating the exons coding for the signal peptide and Ig1, for Ig5 and Ig6, and for FNIII and FNIV, respectively; Fig. 1A) are all in phase 1 (Supplementary Table 3D) and therefore show the same phase preference as in vertebrate Cntn1 genes.

Taken together, the conserved exon intron arrangement between the human and fish Cntn1 genes and the phase preference of the interdomain introns are in compliance with the evolution of this class of IgSF molecules via exon shuffling. Accordingly, a loss of introns has to be postulated for the *Drosophila* Cntn gene.

Phylogenetic and synteny analyses of fish Cntn1 genes

Phylogenetic reconstruction using an unambiguous alignment of all known vertebrate Cntn gene family members and the single Cntn genes of *Drosophila melanogaster* (dmCntn) and *Anopheles gambiae* (agCntn) as an outgroup (Supplementary Fig. 2) resulted in a tree comprised of distinct, well supported clades for the different vertebrate subgroups Cntn 1–6 (Fig. 1C). It appears likely that the vertebrate genes Cntn1 to Cntn6 evolved from one single ancestor gene (Falk et al., 2002 and this study). Our analysis demonstrates that the newly identified fish genes all belong to the Cntn1 subgroup. Consequently, this clade contains one goldfish, two zebrafish and two fugu genes that are all orthologous to the unduplicated Cntn1 genes in other vertebrate species. The relationships among the fish sequences are consistent with the hypothesis that the Cntn1 duplicates (Cntn1a and Cntn1b) were produced after the divergence of the teleost and the tetrapod lineages and before the ancestors of zebrafish and fugu diverged.

To examine whether one of the two zebrafish Cntn1 paralogs evolved faster and is therefore more distantly related to human Cntn1, we calculated nonsynonymous substitutions (aa altering) per nonsynonymous site (d_N) (Nei and Gojobori, 1986). Separate

comparison of each zebrafish Cntn1 gene to the human ortholog revealed comparable sequence distances (Supplementary Table 2), indicating that both fish duplicates evolved equally fast and that neither gene copy is redundant and free to accumulate mutations without constraint.

For synteny analysis, we mapped the zebrafish Cntn1 genes and compared their chromosomal position to the location of the human Cntn1 ortholog (Fig. 2, Supplementary Tables 4A and 4B). ZfCntn1b was mapped to linkage group (LG) 4 and zfCntn1a was identified within LG 25. Both zebrafish genes together with neighboring ESTs each form synteny with the same region of human chromosome 12 (12q11–q12). In addition, zebrafish LG 4 and LG 25 are both syntenic with human chromosome 7 (Fig. 2, Supplementary Tables 4A and 4B).

We further identified fuguCntn1b within the genomic sequences of scaffold 411 and fuguCntn1a within scaffold 856 (Supplementary Table 5). Orthologs of genes from scaffold 411 located downstream of fuguCntn1b map to human chromosome 12, whereas upstream genes, including the IgSF molecule NrCAM, map to human chromosome 7. Within scaffold 856, only one more gene was predicted besides fuguCntn1a. We identified this gene as a second NrCAM and the human ortholog consequently mapped to chromosome 7. Therefore, two paralogous NrCAM genes exist in fugu and both are positioned in the vicinity of the fuguCntn1 paralogs. Accordingly, fugu scaffolds 411 and 856 as well as zebrafish LG 4 and LG 25 are syntenic with human chromosomes 12 and 7, suggesting that a chromosomal rearrangement process (Eichler and Sankoff, 2003; Pevzner and Tesler, 2003) has occurred in tetrapods.

Taken together, these results demonstrate that the newly identified zebrafish gene is a second ortholog of the human Cntn1 gene and that at least the chromosomal region containing Cntn1 underwent a fish specific duplication.

Comparative analyses of zebrafish Cntn1b and Cntn1a mRNA expression

To determine the spatio temporal expression pattern of zfCntn1b and zfCntn1a, we performed RT-PCR analysis on embryos of different developmental stages (3 to 144 hpf) and on various adult tissues. By this means, expression of zfCntn1b mRNA was first detectable at 20 hpf while significant expression levels were observed from 48 hpf onwards (Fig. 3A). In contrast, zfCntn1a mRNA was already present at 3 hpf and its level remained unchanged at least until 144 hpf (Fig. 3A). The constant expression level of zfCntn1a and of the housekeeping gene actin that was used as an internal positive control ensured that the absence of a zfCntn1b PCR product from 3 to 15 hpf was not due to degraded RNA or bad template cDNA.

In adult zebrafish, zfCntn1b expression was found in all neural tissues examined (Fig. 3B) with a high level in brain and eye and a lower level in spinal cord. A weak band indicating rare zfCntn1b gene transcription was also detected in heart. In comparison, zfCntn1a showed a broader expression pattern with the most prominent band in brain, lower but still distinct expression in spinal cord, eye and muscle, and faint expression in heart and gill (Fig. 3B).

These mRNA expression analyses revealed that the zebrafish Cntn1 paralogs show differences in their temporal and spatial expression patterns, indicating a possible subfunctionalization of the duplicated Cntn1 genes.

Fig. 2. Analysis of zebrafish and human synteny relationships. Map locations of ESTs in the radiation hybrid panels (T51 and LN54) were obtained from ZFIN. The relative chromosomal locations of the human orthologs were deduced from LocusLink. Markers that are syntenic to zCntrl1a and zCntrl1b (red), respectively, are shown in green. ESTs defining synteny with human chromosome 7 are blue. Markers present on more than one zebrafish panel are connected by dotted lines. (A) Conserved synteny of zebrafish LG 4 and human Chr 12 defined by zCntrl1b (red). (B) Conserved synteny of zebrafish LG 25 and human Chr 12 defined by zCntrl1a (red). Chr, chromosome; cR, centiRay; K, kilobasepairs; LG, linkage group; # this EST has been published by Woods et al. (2000).

Fig. 3. RT-PCR analyses of *zfCnnt1b* and *zfCnnt1a* mRNA expression. Expression of *zfCnnt1b* and *zfCnnt1a* mRNAs was examined during development (A) and in various adult tissues (B). A reverse transcriptase negative control (without SuperscriptII enzyme, ct) was performed with each primer pair. RT-PCR with actin-specific primers (*zfActin*) served as a positive control and ensured that equal amounts of cDNA template were put into each reaction. (A) Time course of *zfCnnt1b* and *zfCnnt1a* mRNA expression from 3 hpf to 144 hpf. Hours post-fertilization (hpf) are indicated on the top. (B) Expression of *zfCnnt1b* and *zfCnnt1a* transcripts in various adult tissues. br, brain; ey, eye; gi, gill; he, heart; mu, muscle; sc, spinal cord; ct, zero reverse transcriptase negative control; H₂O, no template control.

Spatio temporal divergence of paralogous Cnnt1 expression in the developing zebrafish

The spatial distribution of *Cnnt1b* mRNA during zebrafish development was examined by whole mount in situ hybridization and compared to the expression of *zfCnnt1a*. *Cnnt1a* was detected in the trigeminal ganglion and in Rohon Beard neurons from 16 to

24 hpf and in the anterior lateral line/acoustic ganglion, in six discrete hindbrain clusters and in trunk motor neurons at 24 hpf (Gimnopoulos et al., 2002).

Although *zfCnnt1b* transcripts were detectable at 20 hpf by RT PCR (Fig. 3A), specific labeling of *zfCnnt1b* mRNA was not found before 36 hpf (data not shown). At this stage, neurons in the trigeminal ganglion, in the anterior lateral line ganglion and in the posterior lateral line ganglion showed *zfCnnt1b* expression (Figs. 4A, G and J). In addition, three discrete bilateral clusters were stained in the hindbrain. By their position (compare to older stages; Figs. 4B F), these cells could be reticulospinal neurons (RS) (Mendelson, 1986b). Weak *Cnnt1b* mRNA expression was found in a cluster of cells anterior to the midbrain hindbrain boundary (Fig. 4G). These cells most likely belong to the nucleus of the medial lateral fascicle and are the anterior most localized reticulospinal neurons (Mendelson, 1986b). In comparison, *zfCnnt1a* is still expressed in the trigeminal/anterior lateral line ganglion and in Rohon Beard neurons in the trunk (Figs. 5A, D).

In accordance with higher *zfCnnt1b* RT PCR levels in older embryos (Fig. 3A), the in situ signals increased at 48 hpf (Figs. 4B and K). The Mauthner cells were now clearly distinguishable from other reticulospinal neurons (Fig. 4H) by their specific form and location in rhombomere 4 (Mendelson, 1986a,b; Metcalfe et al., 1986). In addition, labeling of the inner nuclear layer in the eye was detected, but could not be assigned to a specific cell type. At 72 hpf, the number of *zfCnnt1b* expressing reticulospinal neurons and the signal intensity had further increased (Figs. 4C, I, and L). Transcripts were also detected in a structure dorsal to the otic vesicle (Fig. 4L), which probably represents cell bodies of the acoustic ganglion, and in areas lying ventral to the reticulospinal neurons, probably motor nuclei of cranial nerves III, IV, V, and VII (data not shown). The intensity of the *zfCnnt1a* signal, however, rather decreased after 36 hpf (Fig. 5). At 72 hpf, *zfCnnt1a* mRNA is still detectable in the trigeminal/anterior lateral line ganglion, in Rohon Beard neurons and additionally in cells of the posterior lateral line ganglion (Figs. 5B, E). The discrete cell clusters in the hindbrain (inset in Fig. 5B) might be subsets of reticulospinal neurons that might coexpress *zfCnnt1b* (compare Figs. 4C, L with Figs. 5B, E).

While the *zfCnnt1b* mRNA expression pattern remained unchanged from 72 to 144 hpf (Figs. 4C F), *zfCnnt1a* expression further decreased. *zfCnnt1a* mRNA is found in only some Rohon Beard neurons in the posterior part of the trunk (Fig. 5F) and in the hindbrain, it is confined to single cells (inset in Fig. 5C). Control hybridizations with *Cnnt1b* and *Cnnt1a* sense probes yielded no signal at all embryonic stages analyzed (data not shown).

Our in situ hybridization analyses demonstrate differential mRNA expression patterns of *zfCnnt1b* and *zfCnnt1a*. Simultaneous expression of the two paralogs is only found in cells of the trigeminal ganglion and possibly in some reticulospinal neurons.

Fig. 4. In situ hybridization analysis of zebrafish *Cnnt1b* expression. (A, G, J) Expression of *zfCnnt1b* mRNA is first detected in 36 hpf zebrafish embryos in cells of the nucleus of the medial lateral fascicle, trigeminal, anterior and posterior lateral line ganglia and in three discrete clusters of cells in the hindbrain, probably reticulospinal neurons. (B, H, K) At 48 hpf, the intensity of the signal had increased. More reticulospinal neurons are stained and the Mauthner cell can be clearly distinguished. In the eye, cells in the inner nuclear layer are labeled. (C, I, L) In 72 hpf embryos, cells of the nucleus of the medial lateral fascicle and reticulospinal neurons are *zfCnnt1b* positive. At least from this stage onwards, the acoustic ganglion expresses *zfCnnt1b* mRNA. (D, E, F) The staining pattern observed at 72 hpf is maintained at 96, 120, and 144 hpf. (A F) Dorsal views; anterior is up. (G J) Higher magnifications of the area indicated by dotted lines in (A); anterior is to the left. (J L) Lateral views; dorsal is up and anterior is to the left. Scale bars are 100 μ m (A F) and 50 μ m (G L). ac, acoustic ganglion; al, anterior lateral line ganglion; ey, eye; M, Mauthner cell; nucmlf, nucleus of the medial lateral fascicle; pl, posterior lateral line ganglion; RS, reticulospinal neurons; tg, trigeminal ganglion.

Fig. 5. In situ hybridization analysis of zebrafish *Cntn1a* expression. Early expression of *zfCntn1a* (16–24 hpf) has been previously described in the trigeminal ganglion and in Rohon-Beard neurons (Gimnopoulos et al., 2002). (A, D) *ZfCntn1a* mRNA in 36 hpf zebrafish embryos is still expressed in cells of the trigeminal/anterior lateral line ganglion and in Rohon-Beard neurons of the trunk. (B, E) At 72 hpf, labeling is detected in the trigeminal/anterior lateral line ganglion, the posterior lateral line ganglion and in Rohon-Beard neurons. In addition, discrete clusters of cells in the hindbrain are stained (inset is in a different plain of focus), which might be subsets of reticulospinal neurons. (C, F) In 144 hpf embryos, expression has decreased significantly. Less *zfCntn1a* mRNA is found in the trigeminal ganglion and in the hindbrain, it is confined to single cells (inset). Expression in Rohon-Beard neurons declines from anterior to posterior and at this stage is only detectable in cells posterior to the anus. (A–C) Dorsal views, anterior is up. (D–F) Lateral views; dorsal is up and anterior is to the left. Scale bars are 100 µm (A–C) and 50 µm (D–F). al, anterior lateral line ganglion; ey, eye; pl, posterior lateral line ganglion; rb, Rohon-Beard neurons; tg, trigeminal ganglion; [, probably reticulospinal neurons.

Nevertheless, time course and extent of expression differ significantly. Whereas the onset of *zfCntn1a* transcription parallels axonogenesis, expression of our newly identified *zfCntn1b* gene is detected well after birth of these neurons, suggesting that *zfCntn1b* might be involved in later developmental processes such as axon fasciculation, synapse formation, and/or myelination.

Analysis of goldfish Cntn1b expression during optic nerve regeneration

Many IgSF molecules are involved in regeneration of injured axons (Stuermer and Leppert, 2000). Therefore, we analyzed the regulation of *gfCntn1b* mRNA expression at various time points after optic nerve transection by RT-PCR (Fig. 6A) and by in situ hybridization (Fig. 6C).

GfCntn1b transcripts were detected in the optic nerve before transection, but they were consistently downregulated 2 weeks later (Fig. 6A, upper row; spinal cord cDNA was used as an internal positive control template). Then, the mRNA was re-expressed reaching the initial level at 6 weeks after lesion. This time course was specific for *gfCntn1b* and differed from the constant transcription of the housekeeping gene *clathrin* (Fig. 6A, lower row). In comparison, the expression of *Neurolin*, an IgSF

molecule that is re-expressed in RGCs and their axons after optic nerve lesion (Paschke et al., 1992), constantly increases in parallel to RGC axon regeneration (Fig. 6A, middle row). Cultured goldfish oligodendrocytes also contain *Cntn1b* mRNA (Fig. 6B). Therefore, the early downregulation in optic nerve tissue post transection might be caused by oligodendrocyte differentiation known to occur after optic nerve lesion (Ankerhold et al., 1998). Re-expression at later stages may indicate axon-oligodendrocyte interactions that result in re-myelination of regenerating axons. However, expression of *gfCntn1b* in other cell types in the optic nerve (e.g., astrocytes) cannot be excluded.

The *gfCntn1b* mRNA distribution in adult goldfish retinae was also analyzed before and after optic nerve transection by in situ hybridization (Fig. 6C) to determine whether young RGCs that are constantly added to the retinal periphery express *Cntn1b*, and whether *Cntn1b* is upregulated in RGCs upon optic nerve lesion. Unlike many IgSF molecules (Stuermer and Leppert, 2000), *gfCntn1b* expression was not restricted to the youngest RGCs at the margin, but was rather distributed all over the retina (Fig. 6C; middle panel). Control hybridization with a *gfCntn1b* sense probe yielded no specific signal (Fig. 6C, left panel). Therefore, *gfCntn1b* may not be required exclusively for early but also for late processes during RGC development. Two weeks after optic

Fig. 6. Analysis of goldfish *Cntn1b* mRNA expression after optic nerve transection. Regulation of *gfCntn1b* was examined at 0, 2, 4, and 6 weeks after optic nerve transection by RT-PCR (A) and in situ hybridization (C). (A) Expression of *gfCntn1b* after optic nerve transection was compared to goldfish *Neuroilin* and to the housekeeping gene *clathrin* (*gfClathrin*). RT-PCR on cDNA from spinal cord was used as a positive control and a reverse transcriptase negative control was conducted for each specific primer pair (ct). (B) Expression of *gfCntn1b* mRNA was analyzed in goldfish oligodendrocytes and compared to the housekeeping gene *actin* (*gfActin*). (C) In situ hybridization on adult goldfish retinas detects *gfCntn1b* mRNA in goldfish RGCs before (as, 0) and after optic nerve transection (as, 2). se, 0: sense control. Scale bar, 100 μ m. Numbers indicate weeks after optic nerve transection; ct, zero reverse transcriptase negative control; H₂O, no template control; ol, oligodendrocytes; sc, spinal cord positive control.

nerve transection, *gfCntn1b* was significantly upregulated in all RGCs (Fig. 6C; right panel) and its expression level stayed high for at least 6 weeks (data not shown).

The *gfCntn1b* regulation in retinal ganglion and probably optic nerve glial cells during regeneration suggests that *Cntn1b* might be involved in axon glia interactions and perhaps re myelination.

Discussion

Here, we report the identification of *Cntn1b* in goldfish and zebrafish and of *Cntn1a* and *Cntn1b* in the fugu genome. Zebrafish *Cntn1b* and its paralog *Cntn1a* (previously reported by Gimnopoulos et al., 2002) are expressed in distinct neuronal subpopulations during embryonic development. In the goldfish visual system, *Cntn1b* expression is regulated during RGC axon regeneration.

Our phylogenetic analyses show that the identified fish sequences fall into one clade with other vertebrate *Cntn1* genes and therefore are all *Cntn1* orthologs. Since only one *Cntn* gene in arthropods is facing six different *Cntn* genes in vertebrates, one

might assume that vertebrate *Cntn1* to *Cntn6* evolved by duplication events from a single ancestral gene.

The vertebrate *Cntn1* clade contains one goldfish, two fugu, and two zebrafish genes. It has been suggested that fish gene duplicates resulted from a whole genome duplication event that occurred early in teleost evolution (Amores et al., 1998; Gates et al., 1999; Postlethwait et al., 1998; Wittbrodt et al., 1998; Woods et al., 2000). The different chromosomal locations of the zebrafish and fugu *Cntn1* paralogs and our synteny analyses strongly suggest that *Cntn1b* and *Cntn1a* arose in a fish specific duplication event before the divergence of zebrafish and fugu ancestors rather than by tandem duplication. Only *Cntn1b* was identified in goldfish so far, suggesting that the *Cntn1a* paralog either escaped detection or was lost in goldfish.

We showed that the exon-intron arrangement is conserved from fish to human *Cntn1* genes (Berglund and Ranscht, 1994; Buttiglione et al., 1995; Plagge and Brummendorf, 1997). Each one of the six Ig and four FN domains of the modular protein is encoded by two exons (two exon/one domain relationship) in agreement with the hypothesis that immunoglobulin and Ig like molecules have evolved by duplication of an ancestor half domain

(Bourgois, 1975). The introns separating one domain from another (interdomain introns) are all in phase 1, which is considered to be a prerequisite for an exon shuffling mechanism allowing the generation of different multi domain IgSF molecules from one ancestor domain (Barclay et al., 1993; Fedorova and Fedorov, 2003; Patthy, 1991, 1994).

Expression of zebrafish Cntn1b and Cntn1a suggests a partial functional subdivision

Two paralogous genes are more likely maintained during evolution if they have acquired different functions (Ekker et al., 1997; Laforest et al., 1998; Lynch and Force, 2000; Martinez Barbera et al., 1997; Van de Peer et al., 2001). This may be the case for zfCntn1b and zfCntn1a as deduced from their differential expression observed both during development and in specific cell types. Indeed, zfCntn1b is detected relatively late in embryonic development (20 to 144 hpf), well after neuronal differentiation has started. In addition, it is restricted to specific neuronal subsets. In contrast, zfCntn1a is already expressed in early developmental stages (16 and 24 hpf) and coincides with axonogenesis of trigeminal ganglion cells and Rohon-Beard neurons (Gimnopoulos et al., 2002). Although zfCntn1b and zfCntn1a are partially co-expressed by identical neuronal subsets (e.g. trigeminal ganglion; anterior lateral line ganglion), the two Cntn1 genes are activated in different developmental windows, with zfCntn1a preceding zfCntn1b and with zfCntn1b expression being more profound at later developmental stages. As *Xenopus laevis* Cntn1 that is detected at the neurula stage and involved in the development of primary sensory neurons (Fujita et al., 2000), zfCntn1a is an example of early Cntn1 expression, paralleling the onset of axonogenesis. This is consistent with the proposed role for Cntn1 in promoting axonal outgrowth (Brummendorf and Rathjen, 1996; Faivre Sarraillh and Rougon, 1997; Gennarini et al., 1991). However, zfCntn1b expression would rather correlate with later developmental events such as axonal pathfinding and fasciculation, synapse formation, and myelination.

Unlike in zebrafish, one single Cntn1 gene exists in the mammalian nervous system (Berglund and Ranscht, 1994; De Benedictis et al., 2001; Gennarini et al., 1989; Plagge and Brummendorf, 1997). In mouse, its expression peaks within the first postnatal week, both in the whole brain and the cerebellum (Gennarini et al., 1989, 1990; Virgintino et al., 1999). In addition, earlier expression of the Cntn1 gene occurs in peripheral ganglia where a perinatal peak of Cntn1 expression was observed (Durbec et al., 1992). In the mouse nervous system, these differences correlate with alternative promoter usage rather than activation of different genes. Three different promoters associated with the alternative 5' exons A1, C1, and 0 were suggested to drive differential Cntn1 expression in mouse neural tissues (De Benedictis et al., 2001). These different elements are responsible for either early Cntn1 expression (A1) or for its upregulation at the end of the first postnatal week (C1, 0). This complex profile of developmental expression in turn may be of critical importance for mouse Cntn1 functions. Besides axonal growth control and myelination, it might also control earlier developmental events such as the proliferation of neuronal precursors (Bizzoca et al., 2003). In particular, Cntn1 was found to inhibit neuronal precursor proliferation and therefore low Cntn1 levels are necessary for appropriate expansion of the cerebellum during the first postnatal week. On the other hand, its upregulation in the second postnatal week might be rather important

for the differentiation of cerebellar neurons, including the stabilization of granule cell neuron axonal extensions and Purkinje cell terminal differentiation (Bizzoca et al., 2003; Virgintino et al., 1999). A late developmental role for Cntn1 could also be assumed by phenotype analysis of knock out mice that display defects in cerebellar microorganization and in myelin formation (Berglund et al., 1999; Bizzoca et al., 2003; Boyle et al., 2001).

Thus, these different developmental roles may be achieved through differential activation of the unique Cntn1 gene in mice, dependent upon alternative promoter usage. The differences in fish Cntn1b and Cntn1a spatial and temporal expression patterns suggest that a partial functional subdivision occurred with respect to the unduplicated mammalian Cntn1 gene. Different Cntn1 early and late roles might be achieved rather by differential expression of Cntn1a and Cntn1b than by the specifically regulated expression of one Cntn1 gene. In this scenario, Cntn1a could be involved in early developmental events such as initial growth of axonal extensions, whereas Cntn1b might participate in later neurogenetic processes such as fasciculation, synapse formation, and myelination.

Expression of goldfish Cntn1b during RGC axon regeneration suggests a function in axon glia interactions

Axon growth associated proteins that are re-expressed after optic nerve transection have been suggested to play a role in the regeneration of fish RGC axons (Stuermer and Leppert, 2000). Unlike other IgSF CAMs (Deininger et al., 2003; Giordano et al., 1997; Lang et al., 2001; Paschke et al., 1992), gfCntn1b expression is not confined to the youngest, growing RGCs at the margin of normal adult retinae, but is detected at low levels in RGCs all over the retina. Upon optic nerve lesion, it is massively upregulated, suggesting a function for axon regrowth.

The presence of gfCntn1b in cultured goldfish oligodendrocytes and the decrease of its mRNA in optic nerves 2 weeks after lesion indicate that the decrease of gfCntn1b expression during RGC axon regeneration might be due to its downregulation in optic nerve oligodendrocytes. This occurs most likely in parallel with oligodendrocyte differentiation (Ankerhold et al., 1998). Transcription of gfCntn1b is then re-initiated and reaches the initial level at 6 weeks post transection. Therefore, gfCntn1b mRNA might be expressed only by differentiated oligodendrocytes and might play a role in RGC axon re-myelination after lesion. In accordance with this expression profile, a recent report describes Cntn1 as a *trans* interacting ligand for Notch in promoting oligodendrocyte maturation (Hu et al., 2003). It will be interesting to see whether an interaction of Notch and Cntn1b also plays a role in re-myelination of regenerating RGC axons after optic nerve lesion. Following the expression of components of the Notch pathway, the *her1* pair rule gene (Takke and Campos Ortega, 1999) will be of relevance in this respect.

Moreover, Cntn1 is also associated with the lipid raft microdomain proteins reggie 1 and reggie 2 as well as with fyn kinase in growth cones, filopodia and glial cells, indicating that it may participate in signaling events across the plasma membrane (Kramer et al., 1999; Stuermer et al., 2001). Interestingly, the expression of the non caveolar raft marker proteins reggie 1 and reggie 2 is also upregulated upon optic nerve lesion (Schulte et al., 1997). Future experiments are required to determine whether Cntn1 association with lipid rafts plays a role during optic nerve regeneration.

The present identification of Cntn1 genes in zebrafish and goldfish and the analyses of their expression patterns provide an

entry point for functional analysis of the two paralogous Cntn1 genes during development and axon regeneration as well as for the underlying gene regulatory mechanisms and their significance in repair mechanisms in the central nervous system.

Experimental methods

Animals

All animals were kept in the animal research facility of the University of Konstanz in compliance with animal welfare legislation. For optic nerve transection and tissue preparation, adult goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) were anesthetized in 0.03% MS222 (Sigma Aldrich). Zebrafish embryos were staged as described (Kimmel et al., 1995). Embryos were raised in 0.003% 1 phenyl 2 thiourea (PTU, Sigma Aldrich) from 10 h post fertilization (hpf) onwards to inhibit pigmentation.

Cloning of zfCntn1b and gfCntn1b

A rat Cntn2 (TAG 1) cDNA (Furley et al., 1990) was used to screen an oligo dT primed λ gt11 cDNA library from 33 to 36 hpf zebrafish embryos (kind gift of K. Zinn) under low stringency conditions. A partial zfCntn1 clone was obtained covering base pairs (bp) 1528–3326 of the cDNA. The full length sequence was completed by 5' and 3' RACE, respectively (for primer information see Supplementary Table 1A). In brief, we extracted mRNA from a pool of 48 hpf zebrafish embryos (FastTrack™ 2.0 kit; Invitrogen) and used 0.9 μ g/reaction as template for the synthesis of either first strand 5' Ready cDNA using 5' CDS and SMART II oligonucleotides or of 3' Ready cDNA using 3' CDS primer, according to the manufacturer's instructions (SMART RACE cDNA Amplification Kit; BD Clontech). PCR fragments were cloned into the pCRII cloning vector (Invitrogen) and plasmid DNA was prepared using the QIAprep® 8 Miniprep Kit (Qiagen). Both DNA strands were sequenced using the Abi Prism® BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on an AbiPrism 3100 Genetic Analyzer. Single sequences were assembled using SeqMan™ II of the DNASTAR software package (GATC Biotech).

A λ ZAPII cDNA library from adult goldfish brain was screened using zfCntn1b 1528–2287 bp. Two putative overlapping goldfish Cntn1 clones (3.3 and 3.2 kb) were isolated, covering 3.7 kb of gfCntn1b cDNA.

Sequences were deposited in GenBank (GenBank accession numbers AY301281 for zfCntn1b and AY434496 for gfCntn1b).

Nomenclature of the Cntn gene family

Cntn1/F3/F11/Contactin belongs to the Contactin (Cntn) IgSF subgroup. According to the approved nomenclature of human Contactin genes (<http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl>) the six known members are: Cntn1 F3/F11/Contactin; Cntn2 TAG 1; Cntn3 BIG 1; Cntn4 BIG 2; Cntn5 NB 2/FAR 2; Cntn6 NB 3. A prefix is used to distinguish between orthologous sequences in different species (e.g. zfCntn1 for zebrafish (*Danio rerio*) F3). Following the ZFIN zebrafish nomenclature (<http://zfin.org/zfinfo/nomen.html>), duplicated fish genes share the approved symbol for the human ortholog and are distinguished by an additional letter (e.g. zfCntn1a and zfCntn1b).

The described zebrafish F3 gene (Gimnopoulos et al., 2002) is therefore zfCntn1a and our newly identified zebrafish F3 gene is zfCntn1b.

Sequence alignments and phylogenetic analyses

Zebrafish and fugu genomic sequences were obtained using blast algorithms (Altschul et al., 1997) at NCBI (www.ncbi.nlm.nih.gov/BLAST/) and Ensembl (www.ensembl.org/Danio_rerio/blastview; www.ensembl.org/Fugu_rubripes/blastview) web pages. The fugu cDNAs were deduced from the respective genomic sequences by comparison with the zfCntn1b cDNA. Exon-intron structures were examined by comparison of cDNA against genomic sequences, considering the GT-AG rule of splice donor (GT) and acceptor (AG) sites (Stephens and Schneider, 1992). Published *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, *Gallus gallus*, *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster*, and *Anopheles gambiae* Cntn sequences were retrieved from GenBank (Cntn1: gi34191160, gi6680953, gi16923963, gi31342436, gi1708784, gi1843424, gi30725823; Cntn2: gi28373120, gi6981631, gi62852, gi18858448; Cntn3: gi27480743, gi6679206, gi9506948; Cntn4: gi28373121, gi1016011; Cntn5: gi28373127, gi34860279, gi15216159; Cntn6: gi28373130, gi31980845, gi34857645; Cntn: gi28571500, gi31204408).

Nucleotide sequences were translated using BioEdit (Hall, 1999) and aligned as amino acids using ClustalW (Thompson et al., 1994). The alignment was edited by hand and then converted back into nucleotides to produce a codon alignment. Phylogenies of Cntn1 related sequences were reconstructed using Neighbor Joining (NJ) methods with MEGA (version 2.1, Kumar et al., 2001). First, an NJ analysis was performed based upon the amino acid (aa) alignment with substitutions weighted equally. Next, two additional NJ analyses were performed; the first considered characters from the complete nucleotide alignment and the second excluded third codon positions. Support for nodes in the NJ trees was assessed using 1000 bootstrap reiterations (Felsenstein, 1985).

Signal peptides were predicted using SignalP V1.1 at <http://www.cbs.dtu.dk/services/SignalP/> (Nielsen et al., 1997) and GPI anchor sites using big PI Predictor at http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html (Eisenhaber et al., 1999).

Molecular evolutionary analyses were conducted using MEGA (version 2.1, Kumar et al., 2001). In brief, sequences were aligned at the amino acid level using CLUSTALW and gaps were deleted pair wise. Numbers of nonsynonymous substitutions (aa altering) per nonsynonymous site (d_N) were estimated (Nei and Gojobori, 1986) for zfCntn1b, zfCntn1a, and human Cntn1 sequences.

Radiation hybrid mapping and synteny analyses

Zebrafish Cntn1b and Cntn1a were mapped on the LN54 radiation hybrid panel using standard conditions (Hukriede et al., 1999) and the respective web interface (<http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi>).

A conserved synteny is defined by two or more genes located on the same chromosome in fish and their orthologs located on a single chromosome in human (Barbazuk et al., 2000). For synteny analysis (Woods et al., 2000), other zebrafish genes and ESTs already mapped on the LN54 and T51 radiation hybrid maps (<http://zfin.org/cgi-bin/mapper-select.cgi>) were assigned to putative human orthologs by BlastX searches (Altschul et al., 1997) against the NCBI human non-redundant (nr) protein sequence

database (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). For EST clones that have been sequenced at the 5' and 3' ends, both sequences were used for BlastX searches. If the results of these searches had expected scores (*E* values) of $\leq 10^{-5}$, the putative orthologs were further tested with reciprocal searches against the zebrafish nr dbEST sequence database. A human ortholog was confirmed if the original zebrafish gene or EST (or a gene or EST that showed highly significant overlap with the original sequence) was in the top 15 matches of the reciprocal search by tBlastN. Fugu synteny data were retrieved with MartView (http://www.ensembl.org/Fugu_rubripes/martview).

RT PCR

One hundred zebrafish embryos (approximately 100 mg) for each stage or 50 mg of various adult tissues were used for preparation of total RNA with the RNeasy Mini Prep Kit (Qiagen) following the manufacturer's instructions. Muscle tissue was additionally subjected to proteinase K digestion (200 µg/30 mg tissue). First strand cDNA was synthesized under standard conditions with the Superscript First Strand Synthesis System (Invitrogen) using oligo (dT)₁₂₋₁₈ primer. Zero transcriptions (without Superscript II in the reaction) were performed in parallel to control for genomic DNA contaminations in subsequent PCRs. Amount and quality of the different cDNA samples were evaluated comparing the expression level of the housekeeping genes actin and clathrin. Primer information and PCR conditions are listed in Supplementary Tables 1A and 1B.

Whole mount in situ hybridization of zebrafish embryos and goldfish retinae

Retinae were isolated from adult goldfish eyes, attached to nylon filters (Hybond N, Amersham) and fixed as described (Vielmetter and Stuermer, 1989). Zebrafish embryos were dechorionated prior to fixation in 4% PFA at 4°C overnight. Embryos and retinae were dehydrated through a graded methanol series and stored at -20°C in 100% methanol for at least 1 week. A 0.9 kb cDNA fragment of zfcntn1a (corresponding to Ig domains 4-6, 981-1897 bp; AY138255) subcloned into pCRII Topo, a 1 kb cDNA fragment of zfcntn1b (corresponding to FN domains 2-4, bp 2305-3302) subcloned into pBluescript SK (Stratagene) and a 0.6 kb cDNA fragment of gfcntn1b (corresponding to Ig domains 1-2, bp 281-790) subcloned into pCRII Topo (Invitrogen; for primer information see Supplementary Table 1A) were used for the generation of sense and antisense riboprobes. Plasmids were linearized by restriction enzymes (*Xba*I for zfcntn1a sense, *Hind*III for zfcntn1a antisense; *Not*I for zfcntn1b sense, *Eco*RV for zfcntn1b antisense; *Spe*I for gfcntn1b sense; and *Eco*RV for gfcntn1b antisense) and in vitro transcriptions were performed (using the digoxigenin RNA labeling kit, Roche Diagnostics) according to the manufacturer's instructions. Whole mount in situ hybridizations were performed according to standard methods (Schulte Merker, 2002; Westerfield, 1994).

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