First publ. in: Mechanisms of Development 118 (2002), 1-2, pp. 219-223

Gene expression pattern

Cloning, expression, and alternative splicing of neogenin1 in zebrafish

Hua Shen, Harald Illges, Alexander Reuter, Claudia A.O. Stuermer*

Department of Biology, University of Konstanz, P.O. Box M662, D-78457 Konstanz, Germany

Received 9 January 2002; received in revised form 12 July 2002; accepted 12 July 2002

Abstract

Caenorhabditis elegans UNC-40, *Drosophila* Frazzled, and vertebrate Neogenin and DCC constitute a subgroup of the immunoglobulin superfamily (IgSF). They possess four immunoglobulin-like domains and six fibronectin-type III repeats at the extracellular region, a single transmembrane region, and a ~300 amino-acid intracellular region. UNC-40, Frazzled and DCC can function in axon guidance as the receptor of Netrin (Cell Mol. Life Sci. 56 (1999) 62; Curr. Opin. Cell Biol. 10 (1998) 609). Neogenin binds to Netrin-1 with the same affinity as DCC in vitro (Cell 87 (1996) 175), and is expressed by neurons as they project axons (J. Cell Biol. 127 (1994) 2009), suggesting that it is also a DCC-like Netrin receptor. A zebrafish homologue of DCC (zDCC) is reported recently (Mech. Dev. 109 (2001) 105), but so far there is no report of zebrafish Neogenin. To elucidate a possible neural function of vertebrate Neogenin, we cloned and characterized a zebrafish homologue of neogenin, zneo1, and identified four alternative splice sites within it. In the adult, despite broad tissue distribution, our reverse transcription polymerase chain reaction and Northern analyses demonstrated the dominant expression of zneo1 mRNA in brain. We detected zneo1 mRNA in the embryos from 10 hpf onward and revealed its spatiotemporally regulated expression pattern in both neuronal and non-neuronal tissues by in situ hybridization. Our data showed that during early brain development, zneo1 mRNA was not only present in the proliferative ventricular zones but also in the domains of several first postmitotic neuron clusters when they extended axons. Alternative splicing generates several isoforms of zneo1. Most of them are developmentally regulated, showing distinct distribution in brain and other tissues.

Keywords: Zebrafish; Neogenin; Alternative splicing; Expression; Brain development

1. Molecular cloning of zneo1

Based on sequences conserved among vertebrates, degenerate primers were designed to amplify partial neogenin cDNA from the first strand cDNA of goldfish brain. The resulting reverse transcription polymerase chain reaction (RT-PCR) product was used to screen a zebrafish brain cDNA library and an embryonic cDNA library (both were kindly provided by the Resource Center of the German Human Genome Project, RZPD, Berlin), giving the full-length cDNA sequence of zneo1. It spans 6300 bp and contains a 4287 bp open reading frame. Four regions of the cDNA seem to be alternatively transcribed (sp1, bp 2778–2834; sp2, bp 3458–3490; sp3, bp 3927–4019; sp4, bp 4020–4178). Analysis of the alternative sequences and the adjacent exon sequences suggests that they are proper exons; however, as we did not determine the genomic structure, we cannot formally rule out that the additional sequences are generated by the usage of different splice donors or acceptor sites. The sp1, sp2, and sp4 were already described earlier in various organisms (Keeling et al., 1997, Vielmetter et al., 1994, Vielmetter et al., 1997), whereas sp3 is new. The GenBank/EMBL accession number of zneo1 is AY029280.

Zneo1 is predicted to encode a 1428-amino-acid protein, and presents identical secondary structure and ~70% amino-acid-sequence identity to other Neogenins. Although DCC and zNeo1 share only 50% overall and 40% cytoplasmic sequence identities, they show high similarity (~90%) at the cytoplasmic P1 and P3 domains critical for axon guidance functions (Hong et al., 1999; Stein et al., 2001; Stein and Tessier-Lavigne, 2001). Phylogenetic analysis further revealed that vertebrate DCCs and Neogenins are highly related; both are the descendants of invertebrate UNC-40 and Frazzled (data not shown).

2. Zneo1 mRNA expression

2.1. Overall expression in adult and embryonic zebrafish

By Northern analysis, two zneo1 transcripts of approximately 6.5 and 7.5 kb were detected only in adult brain and

* Corresponding author. Fax: +49-7531-883894.
E-mail address: claudia.stuermer@uni-konstanz.de (C.A.O. Stuermer).

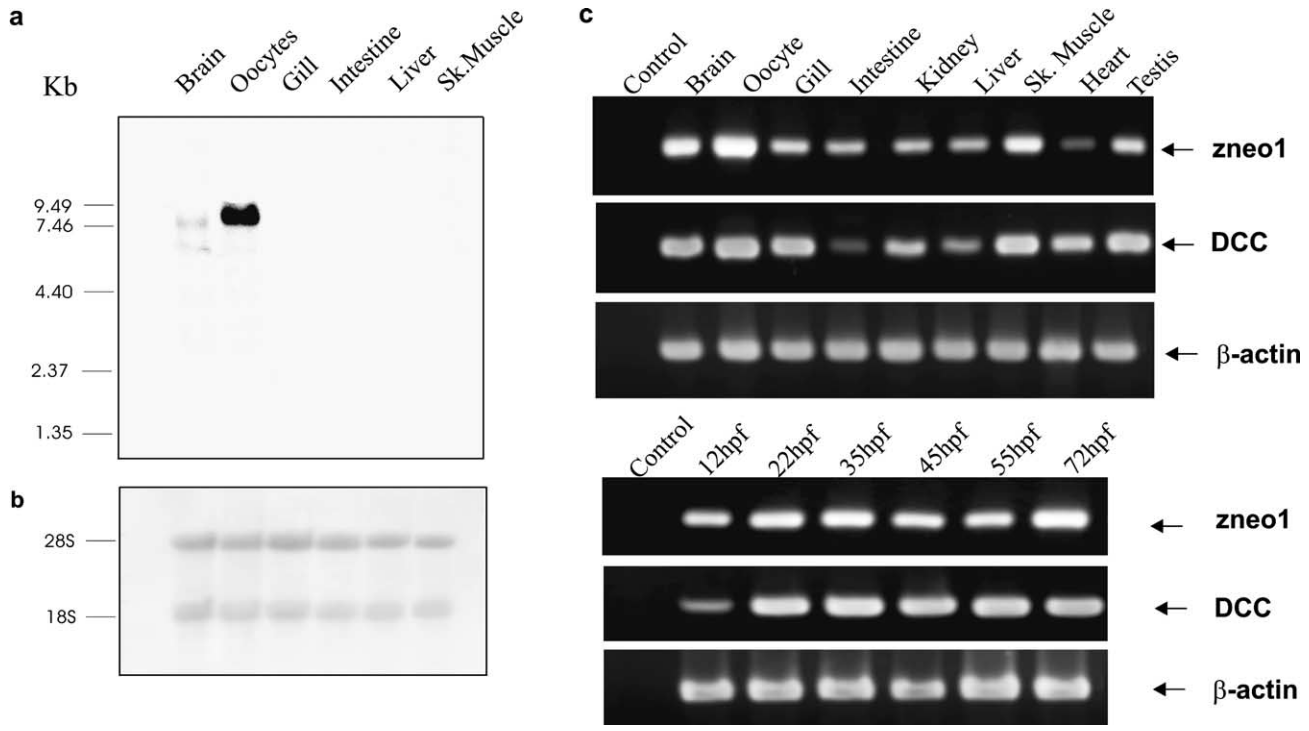

Fig. 1. Expression of neogenin and DCC mRNAs in zebrafish. (a) Northern analysis of zneo1 transcripts in zebrafish tissues. Total RNA, 20 μg, from various tissues were fractionated on 1% denaturing agarose–formaldehyde gel and hybridized with a 1 kb ^{32}P-labeled zneo1 probe according to standard protocols. Two zneo1 transcripts of 6.5 and 7.5 kb were detected only in brain and oocytes. The relative mobility of molecular weight marker is indicated on the left in kb. (b) Methylene blue staining of 18 and 28 s rRNAs revealed the integrity and comparable amount of total RNA used in (a). (c) Semiquantitative RT-PCR analysis for zneo1 and DCC mRNA expression in adult and embryonic zebrafish. RT-PCRs were performed with gene-specific primers. No-RT (data not shown) and no-template controls were involved in all the reactions giving no non-specific product. β-actin RT-PCR was used to standardize the reactions. hpf, hours post-fertilization.

oocytes (Fig. 1a). With more sensitive RT-PCR, zneo1 mRNA was detected in all adult tissues studied (Fig. 1c), with the strongest signal in brain and oocytes. Thus, zneo1 is predominant in adult brain and is also a maternal message. When comparing zneo1 and DCC, both genes were found to be relatively abundant in brain, oocytes and skeletal muscle; DCC is also rich in testis and gill but rare in intestine and liver, whereas unlike DCC, zneo1 expression in heart is very low (Fig. 1c). During development, both DCC and neogenin mRNAs are detectable but of low level at 12 h post-fertilization (hpf), soon after the onset of segmentation. By 22 hpf when the early neuronal clusters appear in the brain and project their axons, both mRNA expressions are dramatically upregulated. Highest zneo1 expression appears at 35 and 72 hpf, while DCC expression is high at 22 and 35 hpf but relatively low at 72 hpf. Thus, both DCC and neogenin mRNAs are temporally regulated and show different expression dynamics during embryogenesis (Fig. 1d).

2.2. Expression pattern during development

To gain the first hints of zneo1 function during development, in situ hybridization analysis was performed with an anti-sense probe of zneo1 in embryos from 10 to 60 hpf. The specificity of the staining was confirmed by using the corresponding sense probe, which gave no staining. Moreover, another anti-sense probe hybridizing to the intracellular region of zneo1 also gave the same staining (data not shown).

In the developing brain, zneo1 transcripts are detectable in the rostral neural tube at 10 hpf (data not shown). By 16 hpf, significant expression appears in the caudal diencephalon, ventral two-thirds midbrain, and midbrain–hindbrain boundary (Fig. 2d). Most of the stained cells are located at the ventricular zone (VZ) (Fig. 2a), probably belonging to the proliferating neural stem cells, whereas a subset of cells in the ventrorostral midbrain (solid arrow in Fig. 2d) within the domain of the first postmitotic neurons of the ventrocaudal cluster (vcc) emerges by that time (Ross et al., 1992).

At 22 hpf, as most of the first neuronal clusters appear and project axon (Ross et al., 1992; Chitnis and Kuwada, 1990), in the forebrain, zneol positive cells occur in four confined regions (2–5 in Fig. 2f). Regions 2 and 3 coincide with the first neuronal clusters, dorsorostral cluster (drc) and ventrorostral cluster (vrc), respectively (Fig. 2g). In the midbrain, in line with the neurogenesis of vcc (Fig. 2g), the ventral positive cells seen at 16 hpf expanded (compare solid arrow in Fig. 2d and 1 in Fig. 2e, f). Intensive expression was also observed in the ventrocaudal

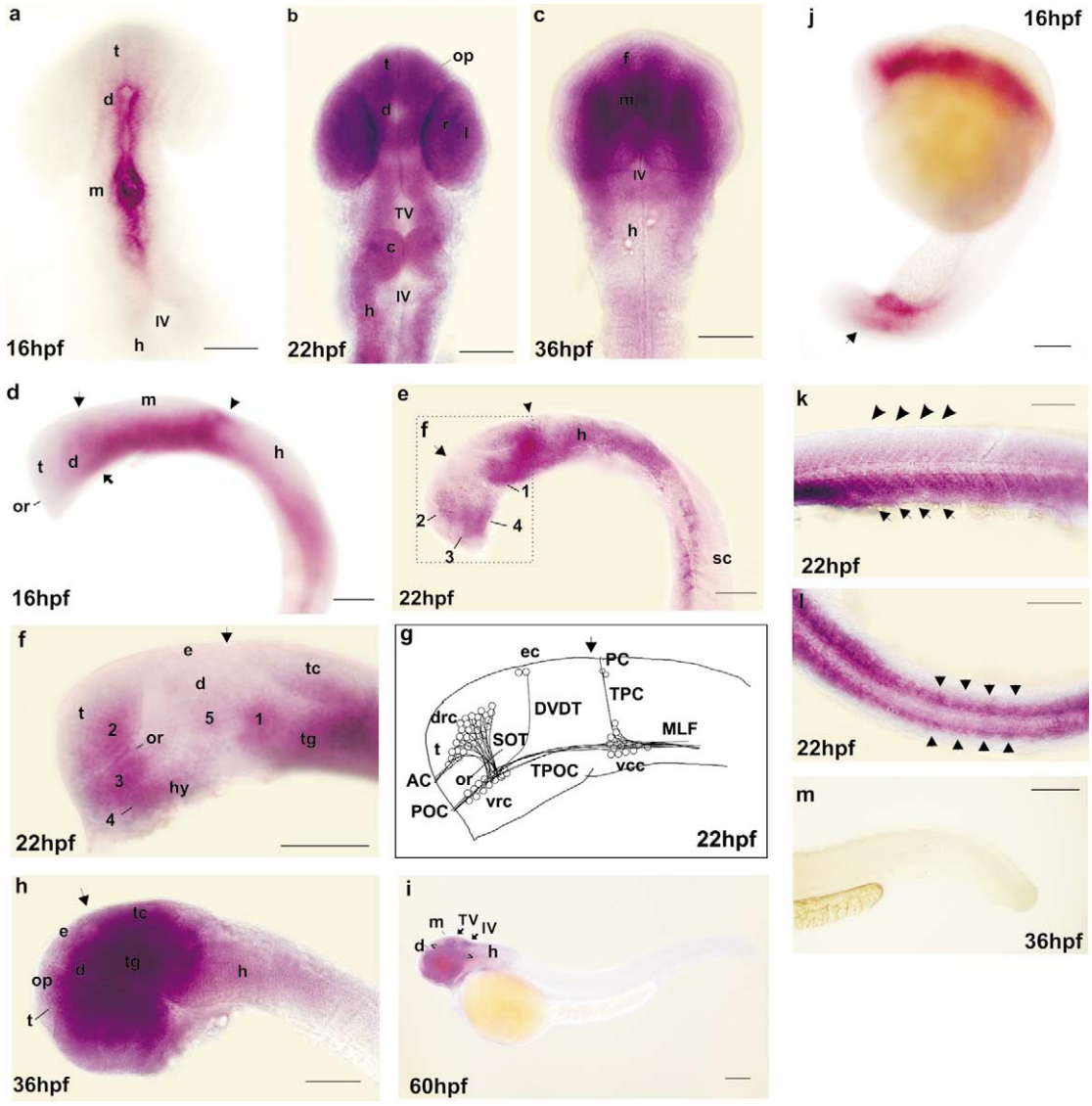

Fig. 2. Expression of zneo1 transcripts in the zebrafish embryo. Whole-mount in-situ hybridization was performed with a 600 bp digoxygenin-labeled cRNA probe for zneo1 as described by Thisse et al., 1993. (a–c), dorsal view, while rostral is up. (d–i, k, m), lateral view, rostral to the left. (l), dorsal view, rostral to the left. (j), lateral view, and dorsal is up. (a), (d), and (j), 16 hpf. Zneo1 expression is evident in the ventricular zone of forebrain and midbrain (a), and tail region (arrow in (j)), weak in hindbrain (d), and not detected in the telencephalon (a and d). Arrow, forebrain–midbrain boundary; arrowhead, midbrain–hindbrain boundary; solid arrow, location of ventrocaudal cluster of the first neurons. (b) 22 hpf. Staining is seen along the rostrocaudal axis in most regions of the brain, olfactory placode (op), lens primordium (l), and the still undifferentiated retina (r). (e) and (f), 22 hpf. In the forebrain and midbrain, zneo1 is expressed by five clusters of cells, that is, 1, in the ventral tegmentum extending caudally toward the hindbrain and dorsally toward the rostral tectum (tc); 2, in the telencephalon dorsal to the optic recess (or); 3, ventral to or, in the rostral diencephalon; 4, ventral to 3, extending caudally into the hypothalamus (hy); 5, central domain of diencephalon. (f) is a higher magnification of the box marked in (e). The arrow denotes an expression gap closer to the forebrain–midbrain boundary. Arrowhead, midbrain–hindbrain boundary. (g) 22 hpf. Schematic illustration of the first neuronal clusters and their axonal processes in approximate 22 hpf embryo, according to Chitnis and Kuwada (1990) for comparison. Four neuronal clusters are present in the forebrain and midbrain: drc, the dorsorostral cluster; vcc, the ventrocaudal cluster; vrc, the ventrorostral cluster; and ec, the epiphyseal cluster. The drc projects axons pioneering the supraoptic tract (SOT) and anterior commissure (AC); vrc grows axons contributing to the tract of the postoptic commissure (TPOC) and postoptic commissure (POC); vcc extends axons that form the medial longitudinal fasciculus (MLF), the tract of the posterior commissure (TPC), and the posterior commissure (PC); and ec grow axons that contribute to the dorsoventral diencephalic tract (DVDT). Arrow indicates the forebrain–midbrain boundary. (c) and (h), 36 hpf. Staining is intensified in the midbrain but is decreased in hindbrain. Arrow, forebrain–midbrain boundary. (i) 60 hpf. Expression is decreased to the tectal and the fourth VZs, (TV and IV), a longitudinal strip ventral to midbrain and hindbrain (bracketed) and diencephalon. (k) and (l), 22 hpf. Transient zneo1 expression is stronger in the ventral part of the somites (arrows) than the dorsal part (arrowheads). (m), 36 hpf. Staining in the somites is no more detectable. c, cerebellum; d, diencephalon; e, epiphysis; f, forebrain; h, hindbrain; hpf, hours post-fertilization; hy, hypothalamus; IV, the fourth ventricle; l, lens; m, midbrain; op, olfactory placode; or, optic recess; r, retina; sc, spinal cord; t, telencephalon; tc, tectum, tg, tegmentum; TV, tectal ventricle. Scale bar, is 50 μm in (a), (d) and (j), 200 μm in (i), and 100 μm in all others.

tectum, the tegmentum (Fig. 2f), the cerebellar anlagen (Fig. 2b), and in cells in the lateral hindbrain (Fig. 2e). As seen in a dorsal view (Fig. 2b), the zneo1-expressing cells occupy a more lateral region (mantle zone) of the neural tube at this time (compare 16 hpf, Fig. 2a), again implying the presence of zneo1 in postmitotic neurons.

By 36 hpf, zneo1 expression increased in diencephalon and midbrain. Stained cells extended to the forebrain/midbrain boundary (compare Fig. 2h, f, arrows), the epiphysis (Fig. 2h), entire tectum and tegmentum (Fig. 2h), and the fourth VZ (Fig. 2c). Its expression in the lateral hindbrain, however, decreased by this time (Fig. 2c, h).

At 60 hpf, zneo1 transcripts persist in the ventral half of the tectal VZ and the fourth VZ (Fig. 2i confirmed by section analysis, data not shown), and a longitudinal strip ventral to midbrain and hindbrain (bracketed, Fig. 2i), and most regions in diencephalon (Fig. 2i).

Outside the brain, zneo1 is transiently expressed in the tail region of 16 hpf embryos, (arrow, Fig. 2j), somites at 22 hpf (Fig. 2k, l), with stronger expression in ventral (arrows, Fig. 2k) than in dorsal somites (arrowheads, Fig. 2k). From 36 hpf onward, these staining patterns are no longer visible (Fig. 2m, i).

3. Alternative splicing in zneo1

Four alternative spliced sequences were identified in zneo1. As alternative splicing was shown to play a role in the regulation of the function of IgSF members, we performed RT-PCR (Fig. 3a) to determine the expression profiles of these splice variants in both adults and embryos.

As shown in Fig. 3a, b, the 57 bp sp1 was located between the fourth and the fifth fibronectin type III domains. Alternative splicing of sp1 generates the Sp1+ and Sp1− isoforms. Most of adult tissues only express the short Sp1− isoform, whereas brain, heart and testis have both Sp1+ and Sp1− isoforms. Only brain contains abundant Sp1+ isoform, which is also developmentally upregulated. This seems to be a unique feature of teleosts. In mouse, it is reported that the Sp1− isoform is predominant in all the adult tissues including brain (Keeling et al., 1997).

The 33 bp sp2 is present between the sixth fibronectin type III domain and the transmembrane region (Fig. 3a). As shown in Fig. 3b, both Sp2+ and Sp2− isoforms are detectable in all adult tissues and embryos studied, whereas the relative abundance of each form is tissue-specific. With high expression of Sp2+ in brain, skeletal muscle and

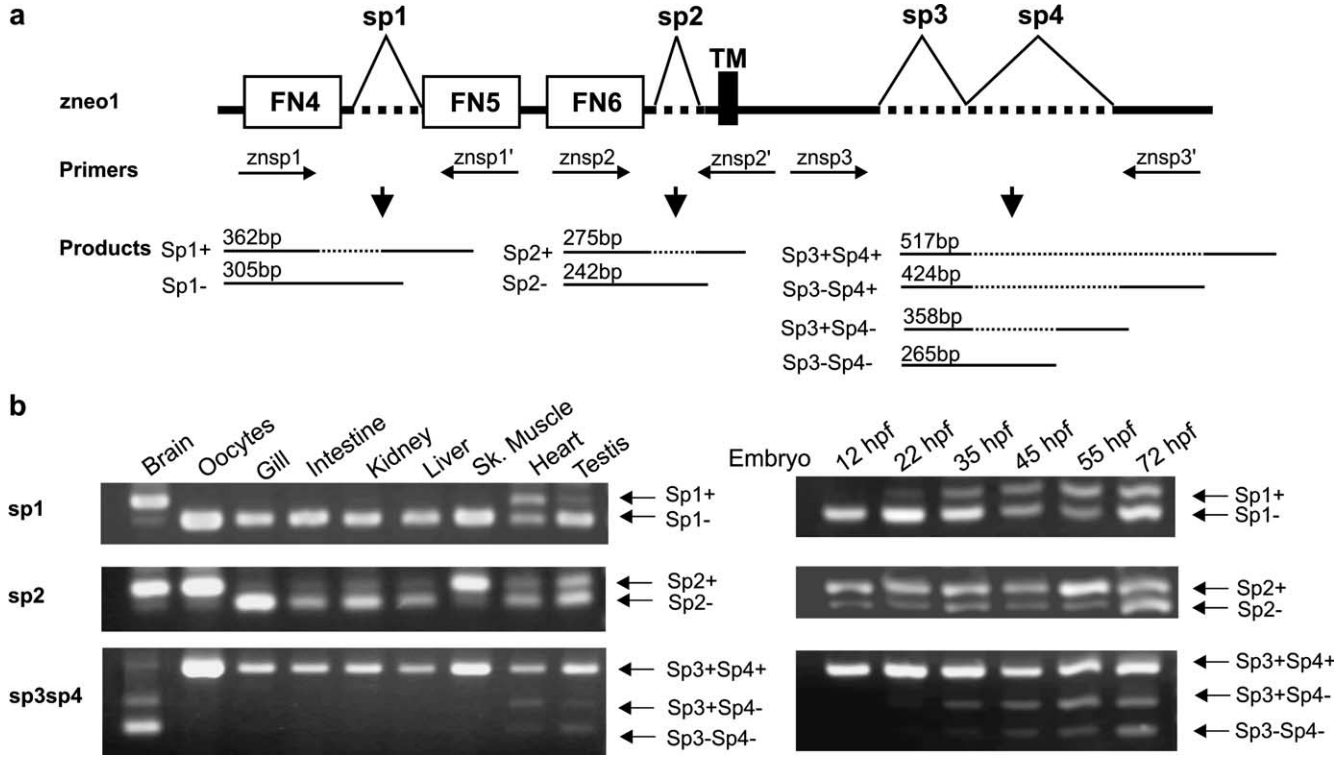

Fig. 3. Expression of zneo1 splice variants in zebrafish. (a) Schematic representation of RT-PCRs used for alternative splicing study. RT-PCR was performed with the indicated primers: znsp1, TCACGCACAGACCATCAAAG; znsp1′, TGGTCTTCCAACGCACTGTA; znsp2, CTGTAGTGAGCAAAGAGG; znsp2′, AGGTCTGGTGGTTTGAGA; znsp3, GGGCAACTCCAAAGATCTCAA; znsp3′, TGAAAGGTCAGTGGTGCAAGA under standard conditions. The resulting products derived from the first strand cDNAs of various adult tissues and staged embryos were subsequently cloned and sequenced. The possible splice isoforms are illustrated in the figure and named after the region amplified and the presence or absence of the alternative splices. FN4–FN6, fibronectin type III domains 4–6; hpf, hours post-fertilization; sp1–sp4, alternative splice 1–4 (indicated by dotted lines); TM, transmembrane domain. (b) Expression of zneo1 splice variants in adult and embryonic zebrafish.

oocytes, in gill, intestine, kidney, heart, and liver, the shorter Sp2− form is the major form, while in testis both forms are expressed nearly equally. In embryos, the Sp2+ form predominated throughout from 12 to 55 hpf, but at 72 hpf, both forms were equivalently detected, suggesting that the expression of sp2 is also developmentally regulated.

The sp3 and sp4 are two adjacent sequences located in the intracellular region, with respective sizes of 93 and 159 bp (Fig. 3a). As shown in Fig. 3b, the longest full-length Sp3+Sp4+ form is predominately expressed in the embryos of all stages as well as oocytes and all adult tissues examined, except brain, in which the shortest Sp3−Sp4− form, lacking both sp3 and sp4 is dominant. Only detectable in adult brain, heart and testis, the sp4 missing forms (Sp3+Sp4− and Sp3−Sp4−) are both developmentally upregulated. PCR products containing only the sp4 (Sp3−Sp4+ form) were not detected. For control, RT-PCRs primed with primers only flanking either sp3 or sp4 were also performed, and consistent results were obtained (data not shown).

In general, we detected zneo1 mRNA in both embryonic and adult brain and demonstrated its presence in the domains of the first neuron clusters coincident with early neuronal differentiation and axon–scaffold formation. Four alternative splices were identified, which generate several developmentally regulated isoforms showing distinct distributions in neuronal and non-neuronal tissues. These data provide useful hints for further functional study on neogenin.

References

Chitnis, A.B., Kuwada, J.Y., 1990. Axonogenesis in the brain of zebrafish embryos. J. Neurosci. 10, 1892–1905.

Hong, K., Hinck, L., Nishiyama, M., Poo, M.-M., Tessier-Lavigne, M., Stein, E., 1999. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 97, 927–941.

Keeling, S.L., Gad, J.M., Cooper, H.M., 1997. Mouse Neogenin, a DCC-like molecule, has four splice variants and is expressed widely in the adult mouse and during embryogenesis. Oncogene 15, 691–700.

Ross, L.S., Parrett, T., Easter Jr, S.S., 1992. Axonogenesis and morphogenesis in the embryonic zebrafish brain. J. Neurosci. 12, 467–482.

Stein, E., Tessier-Lavigne, M., 2001. Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a robo/DCC receptor complex. Science 291, 1928–1938.

Stein, E., Zou, Y., Poo, M.-M., Tessier-Lavigne, M., 2001. Binding of DCC by netrin-1 to mediate axon guidance independent of adenosine A2B receptor activation. Science 291, 1976–1982.

Thisse, C., Thisse, B., Schilling, T.F., Postlethwait, J.H., 1993. Structure of the zebrafish snail1 gene and its expression in wild-type spadetail and no tail mutant embryos. Development 119, 1203–1215.

Vielmetter, J., Kayyem, J.F., Roman, J.M., Dreyer, W.J., 1994. Neogenin, an avian cell surface protein expressed during terminal neuronal differentiation, is closely related to the human tumor suppressor molecule deleted in colorectal cancer. J. Cell Biol. 127, 2009–2020.

Vielmetter, J., Chen, X.-N., Miskevich, F., Lane, R.P., Yamakawa, K., Korenberg, J.R., Dreyer, W.J., 1997. Molecular characterization of human neogenin, a DCC-related protein, and the mapping of its gene (NEO1) to chromosomal position 15q22.3–q23. Genomics 41, 414–421.