DEVELOPMENTAL DYNAMICS 237:808–818, 2008

Molecular Evolution and Expression of Zebrafish St8SiaIII, an Alpha-2,8-Sialyltransferase Involved in Myotome Development

Joachim Bentrop,[1*†] Monika Marx,[1†] Sebastian Schattschneider,[1] Eric Rivera-Milla,[2] and Martin Bastmeyer[1]

Enzymes of the St8Sia family, a subgroup of the glycosyltransferases, mediate the transfer of sialic acid to glycoproteins or glycolipids. Here, we describe the cloning of the zebrafish *St8SiaIII* gene and study its developmental activity. A conserved synteny relationship among vertebrate chromosome regions containing *St8SiaIII* loci underscores an ancient duplication of this gene in the teleost fish lineage and a specific secondary loss of one paralog in the zebrafish. The single zebrafish St8SiaIII enzyme, which is expected to function as an oligosialyltransferase, lacks maternal activity, is weakly expressed during nervous system development, and shows a highly dynamic expression pattern in somites and somite-derived structures. Morpholino knock-down of *St8SiaIII* leads to anomalous somite morphologies, including defects in segment boundary formation and myotendious-junction integrity. These phenotypes hint for a basic activity of zebrafish *St8SiaIII* during segmentation and somite formation, providing novel evidence for a non-neuronal function of sialyltransferases during vertebrate development. *Developmental Dynamics 237:808–818, 2008.* © 2008 Wiley-Liss, Inc.

Key words: zebrafish; sialic acid; polysialic acid; sialyltransferase; myotome development; somitogenesis; morpholino; in situ hybridization; muscle development; evolution

Accepted 18 December 2007

INTRODUCTION

Chains of sialic acid, varying in length from two units to over a hundred, are an evolutionarily widespread post-translational modification. Sialic acid chains are found on glycoproteins and glycolipids from bacteria to humans. By structurally and functionally modifying their target molecules, sialic acid chains regulate numerous cell adhesion and cell recognition events. They function by preventing cell–cell contacts as well as by serving as a ligand that mediates selective cell–cell interactions (Rutishauser, 1998; Bruses and Rutishauser, 2001; Bonfanti, 2006).

The addition of sialic acid to glycoproteins or glycolipids is catalyzed by sialyltransferases (STs), a subgroup of the glycosyltransferases (Tsuji et al.,

ABBREVIATIONS: hpf hours post fertilization **NCAM** neural cell adhesion molecule **PolySia** polysialic acid **PSTD** polysialyltransferase domain **SM** sialylmotif **ST** sialyltransferase.

[1]Zoologisches Institut 1, Lehrstuhl für Zell- und Neurobiologie, Universität Karlsruhe (TH), Karlsruhe, Germany
[2]Netherlands Institute for Developmental Biology and Stem Cell Research of the Royal Netherlands Academy of Arts and Sciences, Utrecht, The Netherlands
[†]Joachim Bentrop and Monika Marx contributed equally to this work.
Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: Ba 1034/13-2.
***Correspondence to: Joachim Bentrop, Zoologisches Institut 1, Lehrstuhl für Zell- und Neurobiologie. Universität Karlsruhe (TH), Haid- und Neu-Str. 9, 76131 Karlsruhe, Germany. E-mail: joachim.bentrop@bio.uka.de**

DOI 10.1002/dvdy.21451
Published online 7 February 2008 in Wiley InterScience (www.interscience.wiley.com).

1996; Harduin-Lepers et al., 2001). Animal STs are type II transmembrane proteins of the trans Golgi apparatus with a distinctive protein domain composition, which includes a short cytoplasmic domain, a transmembrane domain, a so-called stem region, and a catalytic domain of about 300 amino acids. Whereas the stem region displays particular variations in length and amino acid composition among different ST subfamilies, the catalytic domain is rather conserved. It is formed by four conserved sialyltransferase motifs (SMs): SM-L (large) is mainly involved in recognition of the sugar donor CMP-Neu5Ac; SM-S (small) is involved in donor and acceptor binding; SM-III (Jeanneau et al., 2004) and SM-VS (very small) play important roles in the catalytic activity, which have not yet been unraveled in detail. A polysialyltransferase domain (PSTD), located immediately upstream of SM-L in polysialyltransferases, is obligatorily required for polysialylation of target molecules (Nakata et al., 2006). The catalytic activity of STs depends on the formation of two disulfide bonds, through which these motifs are arranged in close proximity of each other (Angata et al., 2001; reviewed in Harduin-Lepers et al., 2005; Patel and Balaji, 2006, Nakata et al., 2006).

Among animal STs, enzymes of the St8Sia family mediate the transfer of sialic acid (N-acetylneuraminic acid, Neu5Ac) in α2,8-linkage to other Neu5Ac residues, which are terminally located in antennary carbohydrates of glycoproteins or glycolipids (Harduin-Lepers et al., 2001; Angata and Fukuda, 2003). The St8Sia family splits up into 6 subfamilies, of which ST8SiaIIs, ST8SiaIIIs, and ST8SiaIVs form monophyletic groups residing on a common branch of the phylogenetic tree (Harduin-Lepers, 2005; nomenclature according to Tsuji et al., 1996). Among these, St8SiaII and St8SiaIV are well characterized; they are expressed in the nervous system where they catalyze the synthesis of polysialic acid (polySia) on neural cell adhesion molecule (NCAM) in a developmentally regulated fashion. PolySia on NCAM plays fundamental roles in neural plasticity affecting axonal growth and fasciculation, cell migration, synaptic plasticity, and neurogenesis (recently reviewed by Bruses and Rutishauser, 2001, Bonfanti, 2006). In the zebrafish, polySia is transiently formed on NCAM in the developing nervous system, where it acts in guiding outgrowing axons (Marx et al., 2001). Towards understanding the regulation of sialic acid modifications in the zebrafish as well as the molecular evolution and diversification of the relevant sialyltransferases, we have cloned and functionally analyzed genes that encode putative polysialyltransferases of the St8Sia type (Marx et al., 2007). In this study, we have identified an unexpected pattern of molecular variation between *St8SiaII* and *St8SiaIV* genes along the evolution of vertebrates, which is characterized by rapid molecular evolution and independent events of gain- and loss-of-activity between fish and mammals. In the zebrafish, St8SiaII is the major sialyltransferase expressed in the developing nervous system, and it is the principle enzyme performing polySia modifications on the neuronal cell adhesion molecule NCAM throughout life. St8SiaIV, on the other hand, is generally expressed at low levels in all ages and its function remains elusive (Marx et al., 2007).

Whereas St8SiaII and St8SiaIV are rather well characterized, little is known about a third sialyltransferase, St8SiaIII. The first in vitro studies on human St8SiaIII indicate that this enzyme is capable of synthesizing oligosialic acid, implying a function as an oligosialyltransferase rather than as a polysialyltransferase (Angata et al., 2000; Sato et al., 2000). In mammals, *St8SiaIII* expression has been described in adult mouse brain and testis (Yoshida et al., 1995, 1996), in mouse adipocytes and neuroblastoma cells (Sato et al., 2001, 2002), and in human fetal and adult brain as well as in fetal liver (Lee et al., 1998). In contrast to the well-documented activity of St8SiaII and St8SiaIV during development and adulthood, functional studies on vertebrate St8SiaIII have so far only been carried out in recombinant systems or in cell cultures. Here, Sato et al. (2001) demonstrated an activity of mouse St8SiaIII in transfering sialic acid to an O-glycan of adipoQ to form a disialic acid residue. In an attempt to learn more about the emergence and the function of St8SiaIII enzymes in vertebrates, we have studied the activity of *St8SiaIII* in the zebrafish model and analyzed general patterns of molecular variation of this gene along vertebrate evolution. To that end, we identified and successfully cloned a novel zebrafish *St8SiaIII* homolog and examined its transcriptional pattern and function during zebrafish development. The single zebrafish *St8SiaIII* gene shows an early phase of expression in the developing nervous system and a later phase of prominent expression in somite bodies. Morpholino knock-down experiments reveal an essential requirement of zebrafish *St8SiaIII* activity during somite formation and stability. This is the first report of a non-neuronal function of a vertebrate α-2,8-sialyltransferases at a whole animal level.

RESULTS

The Gene Encoding Sialyltranferase St8SiaIII Is Conserved in Vertebrates and Duplicated in Teleost Fish Except for Zebrafish

In order to continue our previous studies on the diversity and function of sialyltransferases in the zebrafish, we performed an exhaustive bioinformatic search throughout public genome databases and identified novel α-2,8-sialyltransferase homologues. Data mining revealed the existence of a single gene in the zebrafish genome, which encodes for a highly conserved protein that contains all structural landmarks of the human St8SiaIII enzyme (see below, Tsuji et al., 1996). Based on this information, we designed a set of gene-specific primers to amplify this novel zebrafish homolog combining direct PCR and RACE reactions. Using cDNAs of different developmental stages, we cloned a mRNA that includes the complete CDS as well as 235 bp of the 5′-UTR and 447 bp of the 3′-UTR (1,807 bp, GenBank Acc. No. DQ853412). During our experimental work on cloning the zebrafish *St8SiaIII* gene, an in silico prediction of this gene was independently reported by Harduin-Lepers et al. (2005). The sequence reported herein differs from the predicted gene

in one base (C983→T), which induces an amino acid exchange of proline to leucine in a non-functional protein domain.

Remarkably, all *St8SiaIII* loci are highly conserved from fish to mammals. At the genomic level, zebrafish *St8SiaIII* shows the same intron-exon-architecture as its human homolog (Fig. 1A), and at the protein level, the newly isolated sialyltransferase shows a mean identity of 77.7 % with other vertebrate ST8SiaIII homologs. Sequence identity with the zebrafish sialyltransferases cloned earlier by our lab, ST8SiaII and ST8SiaVI, is 31 and 32%, respectively. Like its homologs, zebrafish ST8SiaIII is a type II membrane protein that contains all the landmark motifs of sialyltransferases, namely motifs TM, L, S, III, and VS (Eckhardt and Gerardy-Schahn, 1998; Harduin-Lepers et al., 2005; Patel and Balaji, 2006; Yoshida et al., 1996) (Fig. 1B). Furthermore, all amino acids regarded as essential in the "linkage specific sequence" motif, a motif identified by computational analysis (Patel and Balaji, 2006), are conserved in zebrafish ST8SiaIII. The PSTD domain, which is highly conserved in polysialyltransferases St8SiaII and St8SiaIV, is nearly identical in St8SiaIII proteins of different species (Fig. 1C). All St8SiaIII proteins, however, lack 2 of 4 amino acids required for polysialylation of target proteins as determined by Nakata et al. (2006) (exchanged: R-252 and I-275; conserved: K-276 and R-277; positions refer to human St8SiaIV).

Having found that the zebrafish *St8SiaIII* gene shares high sequence identity and protein domain conservation with its human homolog, we set out to identify the general patterns and dynamics of molecular evolution that shaped and maintained these features along vertebrate evolution. To that end, bioinformatic surveys of public databases revealed a high diversity of *St8SiaIII* genes in the teleost fish genome: most genomes contain as much as two copies of the single human homolog (Fig. 1D), as is expected to have resulted from the hypothesized fish-specific extra genome duplication event (Amores et al., 1998). Phylogenetic analyses show that the two *St8SiaIII* loci in fish are recovered as robust, independent subgroups of genes (named *St8SiaIIIa* and *St8SiaIIIb*; Fig. 1D), indicating the duplicate nature of this gene. Remarkably, the single zebrafish gene we found clusters in the *St8SiaIIIa* subgroup, which is the paralog cluster showing the closer genetic distance to human *St8SiaIII* (Fig. 1D). Even exhaustive searches in zebrafish genomic databases did not yield clear evidence for a second St8SiaIII paralog or pseudogene. When compared with other STs, vertebrate *St8SiaIII* genes form the sister (basal) group of the clade composed by *St8SiaII* and *St8SiaIV* genes, which encode for the enzymes that catalyze the production of NCAM-polySia (Fig. 1D). Notably, the topologies of our phylogenic analysis of vertebrate sialyltransferases are consistent with previous reports (Harduin-Lepers et al., 2005; Marx et al., 2007).

As STs share a remarkable structural conservation of their protein sequences (Marx et al., 2007), we analyzed the distribution of non-synonymous/synonymous substitution (dN/dS) between vertebrate *St8SiaIII* genes. We found that the standardized ratio of dN/dS per site among *St8SiaIII* genes was 0.683 ($\chi^2 = 14.26$; $P < 0.0001$). This result indicates a negative selection against amino acid replacements resulting in a higher predominance of synonymous changes (sense mutations), thereby explaining the high level of sequence conservation observed in *St8SiaIII* (Fig. 1C). Although *St8SiaIII* shows lower dN/dS ratios than *St8SiaII* and *St8SiaIV* (0.714 and 0.729, respectively; Marx et al., 2007), these differences are not statistically significant ($\chi^2 = 3.97$; $P << 0.01$), suggesting that the function of each enzyme is largely sequence-dependent and of high biological importance, which makes these molecules refractory to major amino acid replacements.

Our results highlight the evolutionary conservation of vertebrate St8SiaIII enzymes at gene organization and protein structure levels as well as the exclusive duplication of

Fig. 1. Evolutionary conservation of vertebrate *St8SiaIII* genes and their duplication in the teleost fish lineage. **A:** Schematic representation of the genomic organization of the human and zebrafish *St8SiaIII* loci, according to Ensemble genome projects. Exons (four in each gene) are depicted by squares, and introns (three in each gene) by connecting lines; mRNA-untranslated regions (open squares) flank the coding sequence (black squares). The location of the start codon is highlighted by a red arrow mask on exon-1. **B:** Scale drawing of the conserved structural and functional domains of vertebrate St8SiaIII proteins. Gray boxes represent protein backbones encoded by the corresponding gene exons; colored boxes represent different motifs as follows: yellow = transmembrane domain (TMD); blue, red, and green = sialyl-motif large (L), small (S), and very small (VS), respectively; pink = St8Sia linkage specific motif; light blue = polysialyltransferase domain (PSTD); orange = motif III. **C:** Highly conserved amino acid composition of the vertebrate St8SiaIII proteins highlighted by the molecular variation detected in the PSTD domain of representative vertebrate species. **D:** Evolutionary relationships between vertebrate polysialyltransferases as recovered from phylogenetic distance-based analysis. All four different proteins analyzed are recovered as monophyletic groups with comparable evolutionary dynamics, as observed in their branching pattern (St8SiaI = black, St8SiaII = blue, St8SiaIII = red, and St8SiaIV = green). St8SiaIII is recovered as the sister group of the clade formed by St8SiaI and St8SiaIV. Note that fish St8SiaIII are duplicated forming two clusters, St8SiaIIIa and St8SiaIIIb, with St8SiaIIIa as the closer group to their human homologue. The consensus (NJ) tree was based on amino acid sequence alignment. Bootstrap support (1,000 iterations) is shown for relevant nodes. For accession numbers, see Experimental Procedures section.

Fig. 2. Conserved syntenic relationships among vertebrate genome regions containing *St8SiaIII* loci. Chart diagram showing conserved synteny relationships between representative vertebrate species, based on the analysis of gene content and order of chromosome regions containing *St8SiaIII* loci (highlighted in red). Each vertical line represents the chromosomal region of one species containing syntenic genes (black boxes) and non-syntenic genes (gray boxes) connected by non-coding intergenic segments (black lines). Ortholog genes are connected by colored lines across species (blue for fully syntenic loci; violet and light green for partially syntenic loci). The species identities, genome regions, and their relative orientation are given above each chromosome as follows: Hosa = human;, Xetr = frog; Dare = zebrafish; Taru = *Takifugu rubripes*; Chr. = chromosome number; Sca. = genomic scaffold number; +/− = relative orientation of the raw database information. The graphic complexity of the synteny has been simplified, drawings do not reflect the original scale, and species are arrayed arbitrarily. Only fully and partially syntenic loci are labeled.

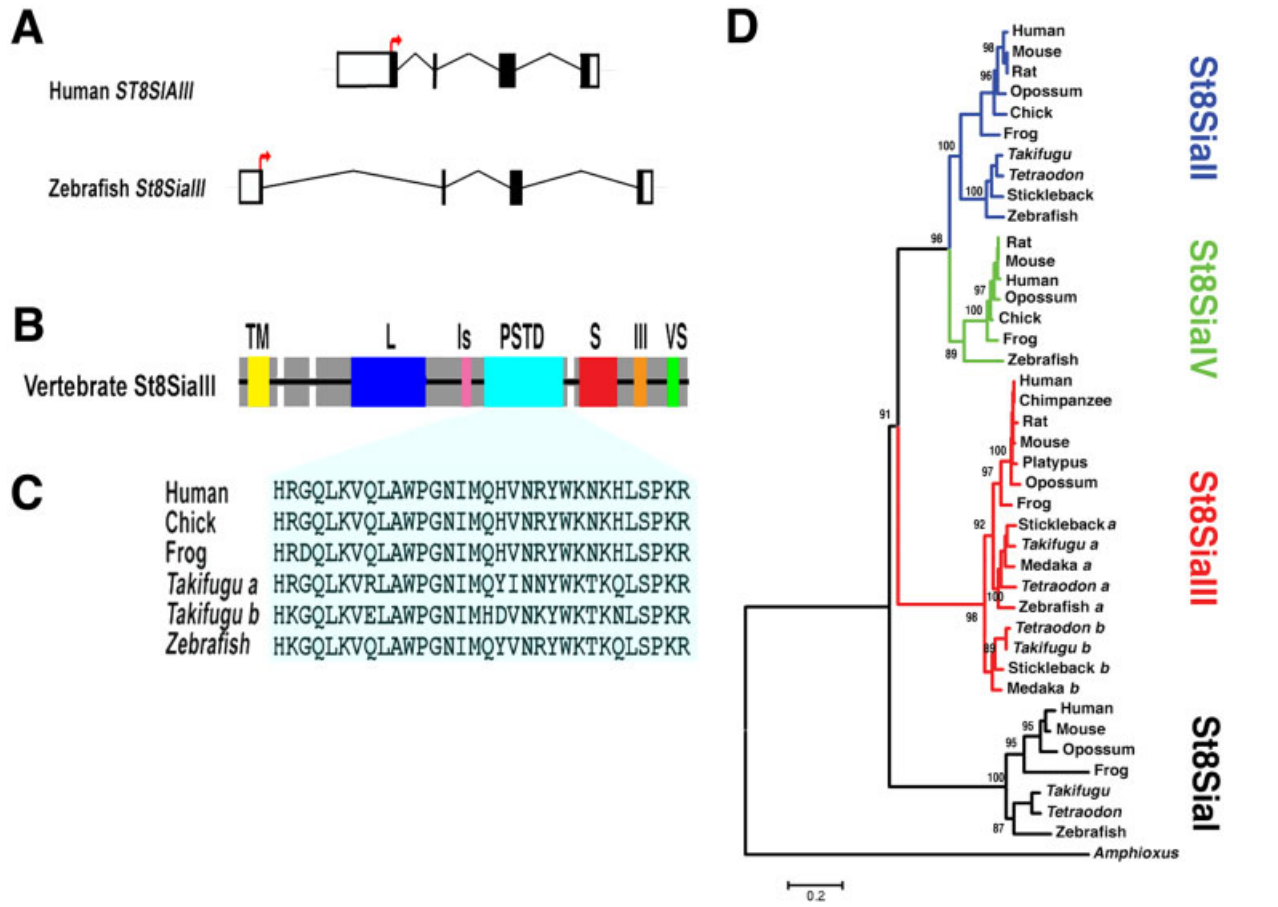

Fig. 1.

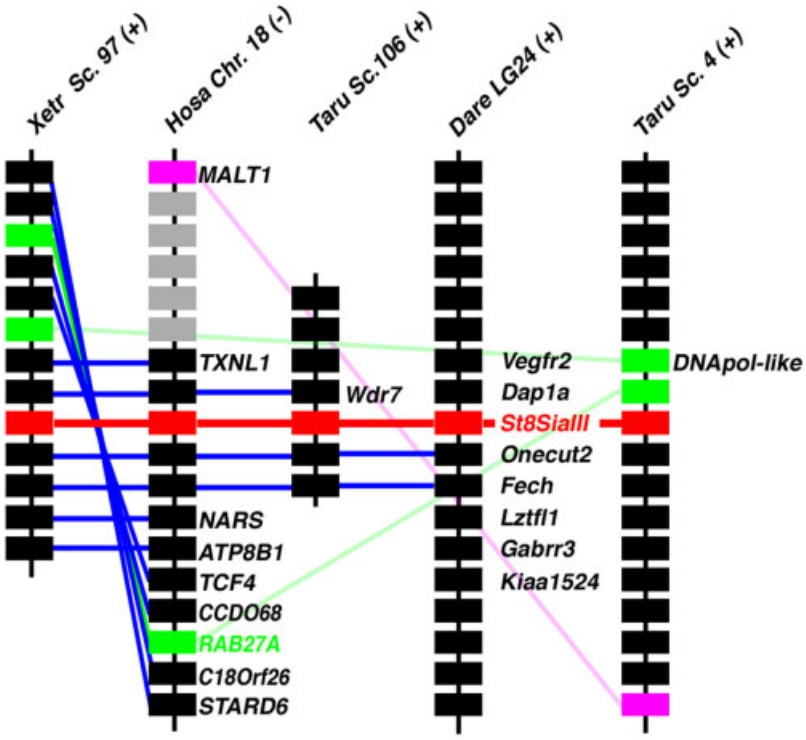

Fig. 2.

this gene in teleost fish. Moreover, by comparing their variation at codon level with other STs, we were able to detect that vertebrate *St8SiaIII* genes seem to have undergone a slower molecular evolution than *St8SiaII* and *St8SiaIV* genes. The existence of a single *St8SiaIII* gene in the zebrafish genome provides a valuable model to study the physiological role of this enzyme in vertebrates.

Conserved Synteny Underscores a Secondary Loss of the Zebrafish *St8SiaIIIb* Locus

To better understand the genetic basis underlying the unique pattern of the molecular diversification of fish *St8SiaIII* genes among vertebrate STs, we compared the gene content and order in chromosome regions containing *St8SiaIII* loci in phylogenetic key vertebrate species. Our genomic analysis revealed conserved synteny relationships between fish and mam-

mals, as is evident by the preserved co-lineal array of the *Wdr7-St8SiaIII-OneCut2-Fech* loci (Fig. 2) between vertebrate genomes as well as by the partial retention of syntenic markers like *Malt1* and *Rab27a* between human and *Takifugu* genomes. Remarkably, our phylogenetic and synteny analyses underscore a secondary loss of the *St8SiaIIIb* paralog from the zebrafish genome. No syntenic relationships were detected when compared with lower vertebrates species (e.g., lamprey), non-vertebrate chordates (e.g., *Ciona intestinalis* and Amphioxus), or invertebrates (e.g., fruitfly; data not shown). Moreover, further analysis of the genomic regions containing *St8SiaIII, St8SiaII*, and *St8SiaIV* loci does not provide clear evidences of meta-syntenic relationships between these three genes. Our findings suggest that several events of gene-genome duplication in early vertebrate radiation might have promoted the rapid expansion of the sub-family of (poly)sialyltransferases originating from a single pre-vertebrate ancestor. These events were followed by independent patterns of secondary gene loss, which are distinctive for individual vertebrate classes. Taken together, our genomic, phylogenetic, and syntenic analyses reveal a unique pattern of molecular variation of *St8SiaIII* genes, which is characterized by (1) a relatively low rate of non-sense codon substitutions; (2) a shared duplication of the *St8SiaIII* locus in the teleost fish lineage; and (3) a secondary loss of the zebrafish *St8SiaIIIb* paralog.

The Zebrafish *St8SiaIII* Gene Displays a Highly Dynamic, Zygotic Transcription Pattern in the Brain and Somites

To elucidate the function of St8SiaIII in zebrafish, we determined the spatiotemporal expression pattern of the *St8SiaIII* gene by whole-mount in situ hybridizations using an antisense-probe that shows no homology to other known zebrafish sialyltransferases. We found that *St8SiaIII* expression is first detectable along the anterior-posterior axis of the developing embryo at the end of gastrulation (12 hours post fertilization, hpf) (Fig. 3A). At the beginning of segmentation (15 hpf), the *St8SiaIII* signal is mostly detectable in anterior structures like the head anlage, whereas labelling in the trunk is weak (Fig. 3B). *St8SiaIII* expression in the brain persists up to 44 hpf, mainly in the midbrain and in the hindbrain, being most evident in the cerebellum anlage and later in the cerebellum (Fig. 3E,F,H,I). In the brain of embryos 50 hpf and older, *St8SiaIII* expression is down-regulated, except for the anterior dorsal hindbrain (not shown), the retina, and the lens of the developing eye (Fig. 3J,K). Whereas *St8SiaIII* expression in the central nervous system coincides with the expression of polysialic acid, a second expression domain is detected in regions of the zebrafish that are devoid of polysialic acid: *St8SiaIII* shows a highly dynamic expression in the trunk. Here, expression starts between 16 and 20 hpf, uniformly throughout the complete somites (Fig. 3D). Around 24 hpf, this pattern gives way to a non-uniform expression with strongly increased intensity. The highest staining intensity is detected in the central parts of somites; staining along the anterior and the posterior somite boundaries is weaker (Fig. 3E, E'). As is obvious from cross-sections, *St8SiaIII* is expressed uniformly throughout the fast muscle fibres of the entire myotome (Fig. 3D). Whether expression extends to the slow muscles remains unclear. Around 32 hpf, *St8SiaIII* expression starts to decrease (Fig. 3F,F'). Proceeding in an anterior to posterior fashion, St8SiaIII is down-regulated in the medial parts of the somites, whereas its expression continues in the dorsal and ventral regions (Fig. 3H,H',I). Remarkably, somites 1–3 deviate from the described pattern, as they show continued expression of *St8SiaIII* beyond 35 hpf (Fig. 3I'). St8SiaIII expression is no longer detectable in the trunk of the embryo at 50 hpf (Fig. 3J). Additional organs of expression in embryos 3 dpf and older are the lens and the retina of the eye, the branchial arches, and the fins (Fig. 3K). To extend our expression analysis of *St8SiaIII* to developmental stages that are not easily accessible to in situ hybridization, we performed additional RT-PCR analyses on cDNA isolated from different developmental stages. These experiments indicate that *St8SiaIII* lacks maternal expression in the embryo. Expression in the embryo starts at 50% epiboly (about 5 hpf) and lasts into adulthood (Fig. 3L).

Knock-Down of the St8SiaIII Gene Activity Disrupts Myotome Architecture in the Developing Zebrafish

The prominent expression of St8SiaIII in the myotomes of the developing zebrafish suggested a function of this novel enzyme during somite and/or myotome formation. To test this hypothesis, we used morpholinos (MO) to knock down St8SiaIII protein expression and subsequently determined the effects on embryonic development. We

Fig. 3. Expression of *St8SiaIII* during zebrafish development. **A–K:** In situ hybridizations using a St8SiaIII antisense probe. From 12 hpf to 15 hpf, *St8SiaIII* is expressed uniformly throughout the head anlage (A,B). Expression in the anterior nervous system is reduced to the midbrain hindbrain boundary around 20 hpf (C–E) and later to the cerebellum (F,H,I). As development proceeds, expression in the nervous system ceases, it is restricted to the retina and lens of the developing eye at 3 dpf (K). *St8SiaIII* expression in the somites starts at 20 hpf (D, arrows indicate first somites), peaks at 24 hpf (E, E') and is down-regulated thereafter (F,F', H,H', I,I'). Down-regulation starts in the central parts of the somites (H') and proceeds in an anterior-to-posterior fashion (H,I). At its peak expression, *St8SiaIII* is expressed uniformly throughout the fast muscle fibres of the entire myotome (G). *St8SiaIII* expression persists in somites 1 and 2 (I') and is later found in the pectoral fins (J,K). Embryos in (A–F, H–K) are lateral views with rostral to the left and dorsal up; G shows a cross-section. Boxes in E, F, H, I indicate the regions shown in higher magnification in E', F', H', I', respectively. cb, cerebellum; dmy, dorsal myotome; fb, forebrain; ha, head anlage; hb, hindbrain; hms, horizontal myoseptum; le, lens; mhb, midbrain-hindbrain boundary; nc, notochord; pf, pectoral fin; re, retinal, s1–s4, somites 1–4; sc, spinal cord; tms, transversal myoseptum; vmy; ventral myotome. (L) reverse transcriptase PCR analysis of St8SiaIII transcripts in embryos, larvae, and adult zebrafish. St8SiaIII products were amplified with primers *SiaIIIGSP3for* and *SiaIIIGSP3rev*; controls show RT-PCR amplification of actin cDNA (Marx et al., 2007). St8SiaIII is expressed from 50% epiboly on throughout all developmental stages into adulthood.

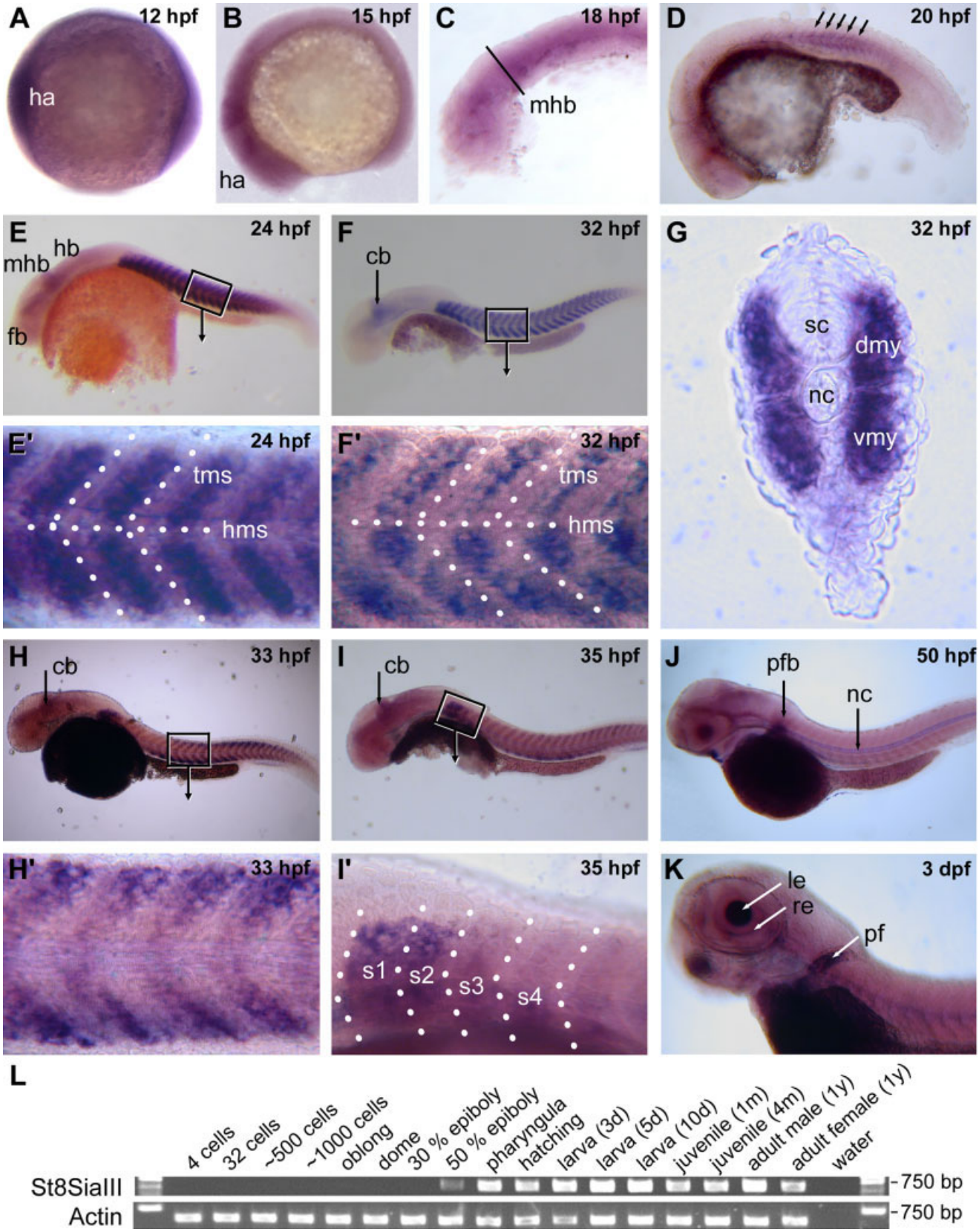
A
12 hpf
ha
B
15 hpf
ha
C
18 hpf
mhb
D
20 hpf
E
24 hpf
mhb
hb
fb
F
32 hpf
cb
G
32 hpf
sc
dmy
nc
vmy
E'
24 hpf
tms
hms
F'
32 hpf
tms
hms
H
33 hpf
cb
I
35 hpf
cb
J
50 hpf
pfb
nc
H'
33 hpf
I'
35 hpf
s1
s2
s3
s4
K
3 dpf
le
re
pf
L
4 cells
32 cells
~500 cells
~1000 cells
oblong
dome
30 % epiboly
50 % epiboly
pharyngula
hatching
larva (3d)
larva (5d)
larva (10d)
juvenile (1m)
juvenile (4m)
adult male (1y)
adult female (1y)
water
St8SiaIII
Actin
750 bp
750 bp

Fig. 3.

found that St8SiaIII knock-down resulted in replicable developmental delays and defects in head and trunk structures ($n > 10$). About 2/3 of morpholino-injected embryos show developmental defects like overall growth retardations, a lack of forebrain, midbrain, and eye priordia, as well as anomalous myotomes coupled with malformations of the tail region (Fig. 4B). The myotomes of morphant are affected to various extents ranging from mild to severe phenotypes. Mild myotome effects in morphants include a reduction of the length of single myotomes. In addition, the v-shape of the myosepta is characterized by a more obtuse angle than is observed in controls (Fig. 4D,F). In about 40% of knock-down embryos, the muscle fibrils have lost their well-ordered bundling and are highly disorganized (Fig. 4F,H). Associated with this phenotype, we find that the motoneurons, which innervate the myotomes, partially defasciculate and grow in a disordered pattern (Fig. 4H). Removal of St8SiaIII during early zebrafish development thus interferes with axial muscle architecture and innervation and provides evidence for a singular activity of St8SiaIII in non-neural structures in the zebrafish. None of these effects was observed in controls using a control morpholino (Fig. 4A,C,E,G).

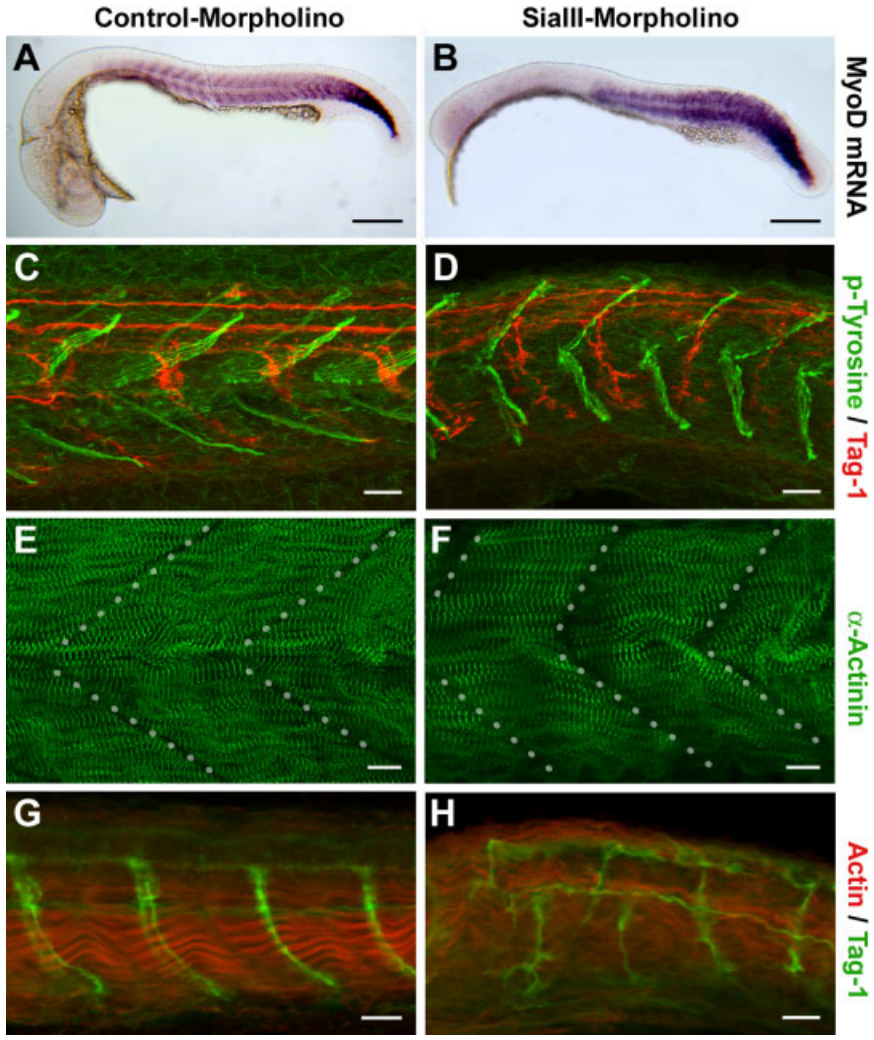

Fig. 4. Knockdown of St8SiaIII induces malformations of myotomes and an abnormal growth pattern of motoneurons in developing zebrafish. **A,C,E,G:** Embryos injected with control morpholinos: (A, C, E) GeneTools control morpholino, (G) *PST*-morpholino (Marx et al., 2007). **B,D,F,H:** Embryos injected with *St8SiaIII*-specific morpholino (2 ng/μl). All embryos are shown rostral to the left. A, B: In situ hybridization using a mytome-specific probe (MyoD) shows a reduction of brain structures in morpholino-injected embryos at 25 hpf. C–F: Embryos at 33 hpf after morpholino knockdown immunolabelled for (C, D) phospho-Tyrosine (green) and the axonal marker Tag-1 (red) or (E, F) α-Actinin (green). Dotted lines in E and F indicate the transversal mysepta. Morphants are characterized by shortened myotomes and myosepta forming a more obtuse angle than in the controls. G,H: Embryos at 35 hpf after morpholino knockdown were immunolabelled for Actin (red) and the axonal marker Tag-1 (green). Muscle fibrils lose their well-ordered bundling, and motor-axons grow in a disordered pattern. Scale bars = 200 μm (A, B), 20 μm (C, D, G, H), 10 μm (E, F).

DISCUSSION

Continuing our previous work on the evolution and activity of animal α-2,8-sialyltransferases, we here provide novel functional data for a devel-
opmental function of vertebrate *St8SiaIII* genes. By combining bioinformatic and phylogenetic analyses, molecular developmental techniques, and loss-of-function experiments, we cloned the zebrafish homolog of the human *St8SiaIII* gene and analyzed its activity in the developing zebrafish. We show: (1) the vertebrate *St8SiaIII* genes are evolutionarily highly conserved in genomic, gene, and protein domain structure; (2) the St8SiaIII gene is duplicated in teleost fish; (3) the zebrafish has secondarily lost one of the *St8SiaIII* paralogs; (4) the zebrafish *St8SiaIII* is zygotically transcribed showing a weak early expression ubiquitously in the brain and a prominent late expression in the somites; and (5) the knock-down of zebrafish *St8SiaIII* induces severe brain and myotome phenotypes. Our results highlight a novel and crucial activity of St8SiaIII enzymes during myotome formation and stability, which is the first non-neuronal activity reported for a vertebrate α-2,8-sialysialyltransferase at a whole-animal level.

Divergent Function of St8SiaIII Genes, Despite Coding for Highly Homologous Proteins, Uncovers the Emergence of Independent Gene Regulatory Pathways During Vertebrate Evolution

Comparative analysis of sialyltransferase sequences reveals that the

Developmental Dynamics

highest homology between zebrafish St8SiaIII and other members of the St8SiaIII family, as well as other sialyltransferases like ST8SiaII and ST8SiaVI, is found in the sialyl motifs. These regions are known to be crucial for substrate recognition and enzymatic activity. In addition, 5 cystein residues are conserved, which form disulfite bonds essential for the catalytic activity (Drickamer, 1993). A high degree of conservation within the linkage specificity domain (amino acid residues 198–207) indicates that St8SiaSiaIII, such as St8SiaII and St8SiaIV, catalyzes the formation of α-2,8-linked sugars and thus is a St8-sialyltransferase (Tsuji et al., 1996; Patel and Balaji, 2006). The fact that the PSTD domain of St8SiaIII in zebrafish and other species lacks 2 of 4 amino acids required for polysialylation of target proteins (Nakata et al., 2006) supports in vitro biochemical data, which point towards a function of vertebrate St8SiaIII enzymes as mono- or oligosialyltransferase rather that as polysialyltransferases (Angata et al., 2000; Sato et al., 2002). This idea is corroborated by the fact that the major expression domain of *St8SiaIII* in zebrafish is devoid of polysialic acid.

The enzymatic activity of St8SiaIII proteins has so far only been studied in recombinant systems, or it was speculated about from indirect evidence. A picture emerges in which human St8SiaIII can form some polySia on itself, not on NCAM, but preferably produces disialyl products (Angata et al., 2000). Other in vitro substrates are N-glycans isolated from different glycoproteins as well as fetuin (alpha2-HS-Glycoprotein), a binding protein that mediates the transport and availability of a wide variety of cargo substances in the bloodstream (Yoshida et al., 1995; Angata et al., 2000). Data from murine cell culture systems indicate that St8SiaIII forms disialic acid residues on O-glycans of adipose tissue–specific protein adipoQ and on CD166, a cell-adhesion molecule of the immunoglobulin superfamily. Murine St8SiaIII is, therefore, implied to function in differentiation processes involved in neuronal and non-neuronal (adipocyte) tissues (Sato et al., 2001, 2002). The substrate acceptor of St8SiaIII catalyzed glycosylation in zebrafish myotomes yet awaits identification, as does the nature of the sugar epitope formed.

Our phylogenetic analyses identified an interesting pattern of low rates of molecular divergence within *St8SiaII, St8SiaIII*, and *St8SiaIV* genes, suggesting a tightly regulated and gene-specific interplay between the sequence composition and catalytic activity of these enzymes in vertebrates. Considering this apparent evolutionary constraint acting on *St8SiaIII* genes, it would be interesting to analyze and compare the expression patterns and developmental roles of the duplicated *St8SiaIII* genes found in other teleost fish species, e.g., medaka. Accordingly to the Duplication-Degeneration-Complementation (DDC) model for gene function evolution (Force et al., 1999), St8SiaIIIa and St8SiaIIIb paralogs are expected to differ by a partial and complementary loss of function after gene duplication, which might allow for the study of the contribution and activity of this gene in neuronal and non-neuronal structures in greater detail. The basal phylogenetic position of *St8SiaIII* in respect to *St8SiaII* and *St8SiaIV* raises the question whether the somite activity of STs correspond to a newly acquired feature or to an ancient one. In this regard, the lack of a prominent polysialyltransferase activity of *ST8SiaIIIs* (Angata et al., 2000) suggests that the polySia activity on NCAM (exerted by *St8SiaII* and *St8SiaIV*) in the developing nervous system might be a derivate property of this gene family, which was gained relatively late in vertebrate diversification. Further molecular developmental analyses on evolutionary key species, lower vertebrates (e.g., lampreys, sturgeon) and other related chordates (e.g., Amphioxus, *Ciona intestinalis, Oikopleura dioica*), would help to clarify this matter.

Zebrafish *St8SiaIII* differs from all other *St8SiaIII* genes described so far as it shows the highest expression levels in the somites and in somite-derived structures of the developing larva. Expression starts at 20 hpf, reaches its peak between 24 and 27 hpf, and is down-regulated thereafter, progressing from anterior to posterior. In addition to the somites, *St8SiaIII* is expressed in the pectoral fins and in the pharyngeal arches. The muscles of the pectoral fins and pharyngeal arches develop from cells that immigrate from the somites (Yelick and Schilling, 2002; Hollway and Currie, 2003). Since *St8SiaIII* expression continues in somites 1 and 2 even after it ceases in all other somites, somites 1 and 2 are likely candidates for the place of departure of those cells. An expression in muscles has not been shown for other *St8SiaIII* genes, which are rather expressed in adult and developing brain (mouse: Yoshida et al., 1995; human: Lee et al., 1998), and to a lesser extent in testis, lung, heart, liver, and kidney (Yoshida et al., 1995; Lee et al., 1998). Adult expression of *St8SiaIII* was not extensively analyzed in the present study. We found, however, evidence for a weak expression in the adult zebrafish skeletal muscle, heart, and brain. Zebrafish St8SiaIII resembles its mammalian counterparts in its, less pronounced, neural expression in the early head anlagen, and later on in the cerebellum. The pronounced differences in expression pattern between zebrafish *St8SiaIII* and other sialyltransferases, including other *St8SiaIII*s, indicate that at least one of the duplicated *St8SiaIII* genes has acquired a distinct neofunctionalization during the development of teleosts.

Novel Roles of α-2,8-Sialyltransferases During Muscle Development Revealed by Loss-of-Function Studies on Zebrafish *St8SiaIII*

Towards an elucidation of the function of St8SiaIII, we show that morpholino knockdown results in disturbances of the stereotyped muscle fibre arrangement in axial muscles of the developing zebrafish. These morphogenetic defects coincide with disturbances in the growth pattern of the motoneurons innervating the myotome. Major steps in somitogenesis, myogenesis, and axogenesis are, however, accomplished before *St8SiaIII* mRNA expression reaches its peak. The first somite is visible at about 10.5 hpf, the following somites emerge one by one in 30-min intervals, and somitogenesis is more or less finished at 24 hpf (Kimmel, 1995; Stickney et al., 2000). Differentiation of the major muscle cell types of the zebrafish larva, fast

muscle, slow muscle, muscle pioneer and medial fast fibre cells, is completed as well at that time (Stickney et al., 2000; Ochi and Westerfeld, 2007). Likewise, the primary motoaxons RoP, MiP, and CaP, which extend out from the spinal cord, have reached their target regions in the myotomes between 18–24 hpf (Myers et al., 1984; Kimmel et al., 1994). We thus conclude that zebrafish St8SiaIII exerts a function in the stability and maintenance of mytotome architecture rather than in segmentation or initial events of myogenesis. Whether St8SiaIII has direct effects on axonal path finding, e.g., by generating guidance cues in form of yet unknown sialylated molecules, or whether disturbed axonal outgrowth in St8SiaIII knock-downs merely results from morphologically disorganized target regions, remains to be elucidated. Our finding that the zebrafish expresses a single St8SiaIII gene, which is required for normal myotome architecture and innervation, highlights the suitability of this model organism to dissect mechanisms underlying the expression and the enzymatic activity of St8SiaIII proteins in vivo.

EXPERIMENTAL PROCEDURES

Animal Care and Maintenance

Wild type and golden zebrafish strains were raised and crossed as described before (Marx et al., 2001). Developmental stages are indicated in hours post-fertilization (hpf), days post-fertilization (dpf), and embryonic stages according to Kimmel et al. (1995).

Cloning of Zebrafish Polysialyltransferases, Reverse Transcriptase PCR

Nucleic acids were extracted from a palette of adult fish tissues and dechorionated embryos with TRIZOL (Invitrogen); total RNA was reverse transcribed with the SuperScriptII™ cDNA synthesis kit (Invitrogen). Partial *St8SiaIII* sequences were obtained by screening publicly available data banks with murine St8SiaIII sequences (Yoshida et al., 1995). Based on the retrieved sequences, a set of gene-specific primers were designed to extend the coding region as well as parts of the 5′-UTR and the 3′-UTR (*SiaIIIGSP1afor*,5′-ATCTGAAAGGATGCGGGTTT-3′; *SiaIIIGSP1for*, 5′-CTGCTGCAGTCGGTTGTTTA-3′; *SiaIIIGSP1rev*, 5′-ATTAACTGACCCACGCGAAC-3′; *SiaIIIGSP2for*, 5′-GTTCGCGTGGGTCAGTTAAT-3′; *SiaIIIGSP2rev*, 5′-AAAGCTGCTTCGTTTTCCAA-3′; *SiaIIIGSP3for*, 5′-TCTTCAAGATGCACGCAGAC-3′; *SiaIIIGSP3rev*, 5′-AGGGCCTTCGAAAATACACC-3′). Overlapping PCR products covering the complete coding region were cloned into the transcription vector pCRII dual promoter vector (Invitrogen) and confirmed by sequencing (MWG-Biotech).

Whole Mounts In Situ Hybridization and Immunostaining

Whole-mount in situ hybridization was carried out following standard protocols (www.zfin.org) as described earlier (Marx et al., 2007). Antisense and sense (control) riboprobes were synthesized from linearized plasmids containing partial or full cDNA regions of zebrafish *St8SiaIII* using the DIG RNA labeling kit (Roche). Embryos shown in Figure 4A,B were hybridized using the myotome-specific probe MyoD (Weinberg et al., 1996). Riboprobes were hybridized overnight at 65°C, and a color reaction was carried out the next day using the NBT/BCIP substrate (Roche) and FAB antibodies (Roche). Embryos were cleared and mounted following standard protocols (www.zfin.org). Pictures were taken using a Coolpix 995 digital camera (Nikon) adapted to a Zeiss Axioplan microscope. Images were edited with Photoshop 6.0 (Adobe). Zebrafish embryos were processed for whole-mount immunohistochemistry as previously described (Marx et al., 2001; Weiland et al., 1997). Embryos were labeled with zebrafish pAb Tag-1 (axons, Lang et al., 2001), anti-α-actinin (Biotrend), anti-phospho-Tyrosin (myotome boundaries, Santa Cruz Biotechnologiy), and phalloidin-Alexa 488 (actin, Invitrogen). Embryos were analyzed using a confocal microscope (LSM 510, Zeiss) equipped with a high aperture lens 40×/1.2W (C-Apochromat, Zeiss) and the appropriate lasers. Serial optical sections were flattened into projections and processed with Photoshop 6.0 software (Adobe).

Morpholino Injections

To knock down the expression of zebrafish *St8SiaIII* during development, we used a morpholino (MO) antisense approach (Nasevicius and Ekker, 2000). MO-ZfSt8SiaIII: 5′-AACCATCTCAGCCCAGAAGAATCAA-3′ (Gene Tools) was injected into one- to four-cell stage embryos in 1 × Danieau solution (Nasevicius and Ekker, 2000) and 0.04% phenol red (Sigma) at a concentration of 2 ng/nl in a final volume of 5 nl per embryo.

Phylogenetic and Genomic Analysis

DNA sequences were edited and analyzed using the DNA-STAR package (Lasergene 5.02). Protein and nucleotide databases (Genbank, www.ncbi.nlm.nih.gov; JGI, genome.jgi-psf.org; Ensemble, www.ensemble.org) were BLAST searched (blastn and tblastn; E-values = e-30 as a threshold) for novel polysialyltransferase homologues in chordate organisms, using as a reference the human *St8SiaIII* sequence AF004668. Nucleotide and amino acid (aa) sequences were unambiguously aligned using ClustalW (www.ebi.ac.uk/clustalw) and the score matrix PAM-45 and PAM-250. Phylogenetic analyses were performed under Neighbor-Joining and Minimum-Evolution algorithms using the MEGA software (Kumar et al., 1994); reliability of tree topologies was assessed by the bootstrap method (1,000 iterations; Felsenstein, 1985). Codon-based selection analysis was conducted through the Z-test of synonymous and non-synonymous differences (Jukes Cantor distance), using the Nei-Gojobori and Li-Wu-Luo methods in MEGA. Standardized substitution ratios were compared by Chi-square test (χ^2) as described (Rivera-Milla et al., 2006). To establish synteny, the zebrafish, *Takifugu rubripes, Xenopus tropicalis*, and human genomic regions containing the *St8SiaIII* gene(s) were analyzed for gene content and order at the Ensembl server (www.ensembl.org).

When necessary, reciprocal BLAST searches where performed to identify ambiguous genome annotations. All genomic data used here were extracted from public databases. Accession number of sequences used in this study: **St8SiaI:** Human = *Homo sapiens* (ENSG00000111728); Mouse = *Mus musculus* (ENSMUSG00000030283); Opossum = *Monodelphis domestica* (ENSMODG00000020449); Frog = *Xenopus tropicalis* (ENSXETG00000020897); *Takifugu* = *Takifugu rubripes* (SINFRUG00000133772); *Tetraodon* = *Tetraodon nivroviridis* (GSTENG00020858001); Zebrafish = *Danio rerio* (XM_684714). **St8SiaII:** Human = *Homo sapiens* (U33551); Rat = *Rattus norvigecus* (L13445); Mouse = *Mus musculus* (X83562); Opossum = *Monodelphis domestica* (ENSMODG00000013182); Chick = *Gallus gallus* (ENSGALG00000006882); Frog = *Xenopus tropicalis* (ENSXETG00000024127); Zebrafish = *Danio rerio* (AY055462). **St8SiaIII:** Human = *Homo sapiens* (AF004668); Chimpanzee = *Pan troglodytes* (ENSPTRG00000010043); Mouse = *Mus musculus* (ENSMUSG00000056812); Rat = *Rattus norvigecus* (ENSRNOG00000018305); Platypus = *Ornithorhynchus anatinus* (ENSOANG00000001713); Opossum = *Monodelphis domestica* (ENSMODG00000020449); Frog = *Xenopus tropicalis* (ENSXETG00000020897); Medaka a = *Oryzias latipes* (AJ871607); Stickleback a = *Gasterosteus aculeatus* (ENSGACG00000013843); *Takifugu a* = *Takifugu rubripes* (SINFRUG00000151173); *Tetraodon a* = *Tetraodon nivroviridis* (AJ715540); Zebrafish a = *Danio rerio* (DQ853412); Medaka b = *Oryzias latipes* (AJ871608); Stickleback b = *Gasterosteus aculeatus* (ENSGACG00000018241); *Takifugu b* = *Takifugu rubripes* (SINFRUG00000152965); *Tetraodon b* = *Tetraodon nivroviridis* (AJ715541). **St8SiaIV:** Human = *Homo sapiens* (NM_005668); Mouse = *Mus musculus* (NM_009183); Rat = *Rattus norvigecus* (AJ699423); Opossum = *Monodelphis domestica* (ENSMODG00000019277); Chick = *Gallus gallus* (AF008194); Frog = *Xenopus tropicalis* (ENSXETG00000023870); Stickleback = *Gasterosteus aculeatus* (ENSGACG00000010746); *Takifugu* = *Takifugu rubripes* (Q6KC10_FUGRU); *Tetraodon* = *Tetraodon nivroviridis* (GSTENG00034465001); Zebrafish = *Danio rerio* (AJ715545). Amphioxus St8SiaI/II/III/IV = *Branchiostoma floridae* (AJ703815).

ACKNOWLEDGMENTS

E.R.-M. is a postdoctoral fellow of the Concordia Foundation.

REFERENCES

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH. 1998. Zebrafish hox clusters and vertebrate genome evolution. Science 282:1711–1714.

Angata K, Suzuki M, McAuliffe J, Ding Y, Hindsgaul O, Fukuda M. 2000. Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct alpha 2,8-sialyltransferases, ST8Sia IV (PST), ST8Sia II (STX), and ST8Sia III. J Biol Chem 275:18594–18601.

Angata K, Fukuda M. 2003. Polysialyltransferases: major players in polysialic acid synthesis on the neural cell adhesion molecule. Biochimie 85:195–206.

Angata K, Yen TY, El Battari A, Macher BA, Fukuda M. 2001. Unique disulfide bond structures found in ST8Sia IV polysialyltransferase are required for its activity. J Biol Chem 276:15369–15377.

Bonfanti L. 2006. PSA-NCAM in mammalian structural plasticity and neurogenesis. Prog Neurobiol 80:129–164.

Bruses JL, Rutishauser U. 2001. Roles, regulation, and mechanism of polysialic acid function during neural development. Biochimie 83:635–643.

Drickamer K. 1993. A conserved disulphide bond in sialyltransferases. Glycobiology 3:2–3.

Eckhardt M, Gerardy-Schahn R. 1998. Genomic organization of the murine polysialyltransferase gene ST8SiaIV (PST-1). Glycobiology 8:1165–1172.

Felsenstein J. 1985. Confidence-limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151:1531–1545.

Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi MA, Samyn-Petit B, Julien S, Delannoy P. 2001. The human sialyltransferase family. Biochimie 83:727–737.

Harduin-Lepers A, Mollicone R, Delannoy P, Oriol R. 2005. The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology 15:805–817.

Hollway GE, Currie PD. 2003. Myotome meanderings. Cellular morphogenesis and the making of muscle. EMBO Rep. 4:855–860.

Jeanneau C, Chazalet V, Auge C, Soumpasis DM, Harduin-Lepers A, Delannoy P, Imberty A, Breton C. 2004. Structure-function analysis of the human sialyltransferase ST3Gal I: role of N-glycosylation and a novel conserved sialylmotif. J Biol Chem 279:13461–13468.

Kimmel CB, Warga RM, Kane DA. 1994. Cell cycles and clonal strings during formation of the zebrafish central nervous system. Development 120:265–276.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310.

Kumar S, Tamura K, Nei M. 1994. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. Comput Appl Biosci 10:189–191.

Lang DM, Warren JT, Jr., Klisa C, Stuermer CA. 2001. Topographic restriction of TAG-1 expression in the developing retinotectal pathway and target dependent reexpression during axon regeneration. Mol Cell Neurosci 17:398–414.

Lee YC, Kim YJ, Lee KY, Kim KS, Kim BU, Kim HN, Kim CH, Do SI. 1998. Cloning and expression of cDNA for a human Sia alpha 2,3Gal beta 1, 4GlcNA: alpha 2,8-sialyltransferase (hST8Sia III). Arch Biochem Biophys 360:41–46.

Marx M, Rutishauser U, Bastmeyer M. 2001. Dual function of polysialic acid during zebrafish central nervous system development. Development 128:4949–4958.

Marx M, Rivera-Milla E, Stummeyer K, Gerardy-Schahn R, Bastmeyer M. 2007. Divergent evolution of the vertebrate polysialyltransferase Stx and Pst genes revealed by fish-to-mammal comparison. Dev Biol 306:560–571.

Myers PZ, Eisen JS, Westerfield M. 1986. Development and axonal outgrowth of identified motoneurons in the zebrafish. J. Neurosci. 6:2278–2289.

Nakata D, Zhang L, Troy FA. 2006. Molecular basis for polysialylation: a novel polybasic polysialyltransferase domain (PSTD) of 32 amino acids unique to the alpha2,8-polysialyltransferases is essential for polysialylation. Glycoconj J 23:423–436.

Nasevicius A, Ekker SC. 2000. Effective targeted gene 'knockdown' in zebrafish. Nat Genet 26:216–220.

Ochi H, Westerfield M. 2007. Signaling networks that regulate muscle development: lessons from zebrafish. Dev Growth Differ 49:1–11.

Patel RY, Balaji PV. 2006. Identification of linkage-specific sequence motifs in sialyltransferases. Glycobiology 16:108–116.

Rivera-Milla E, Oidtmann B, Panagiotidis CH, Baier M, Sklaviadis T, Hoffmann R, Zhou Y, Solis GP, Stuermer CAO, Malaga-Trillo E. 2005. Disparate evolution of prion protein domains and the distinct origin of Doppel- and prion-related loci revealed by fish-to-mammal comparisons. Faseb J 19:317–319.

Rutishauser U. 1998. Polysialic acid at the cell surface: biophysics in service of cell

interactions and tissue plasticity. J Cell Biochem 70:304–312.

Sato C, Fukuoka H, Ohta K, Matsuda T, Koshino R, Kobayashi K, Troy FA, Kitajima K. 2000. Frequent occurrence of pre-existing alpha 2→8-linked disialic and oligosialic acids with chain lengths up to 7 Sia residues in mammalian brain glycoproteins. Prevalence revealed by highly sensitive chemical methods and anti-di-, oligo-, and poly-Sia antibodies specific for defined chain lengths. J Biol Chem 275:15422–15431.

Sato C, Yasukawa Z, Honda N, Matsuda T, Kitajima K. 2001. Identification and adipocyte differentiation-dependent expression of the unique disialic acid residue in an adipose tissue-specific glycoprotein, adipo Q. J Biol Chem 276: 28849–28856.

Sato C, Matsuda T, Kitajima K. 2002. Neuronal differentiation-dependent expression of the disialic acid epitope on CD166 and its involvement in neurite formation in Neuro2A cells. J Biol Chem 277:45299–45305.

Stickney HL, Barresi MJ, Devoto SH. 2000. Somite development in zebrafish. Dev Dyn 219:287–-303.

Tsuji S, Datta AK, Paulson JC. 1996. Systematic nomenclature for sialyltransferases. Glycobiology 6:v–vii.

Weiland UM, Ott H, Bastmeyer M, Schaden H, Giordano S, Stuermer CA. 1997. Expression of an L1-related cell adhesion molecule on developing CNS fiber tracts in zebrafish and its functional contribution to axon fasciculation. Mol Cell Neurosci 9:77–89.

Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, Andermann P, Doerre OG, Grunwald DJ, Riggleman B. 1996. Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetailembryos.Development122:271–280.

Yelick PC, Schilling TF. 2002. Molecular dissection of craniofacial development using zebrafish. Crit Rev. Oral Biol. Med. 13:308–322.

Yoshida Y, Kojima N, Kurosawa N, Hamamoto T, Tsuji S. 1995. Molecular cloning of Sia alpha 2,3Gal beta 1,4GlcNAc alpha 2,8-sialyltransferase from mouse brain. J Biol Chem 270:14628–14633.

Yoshida Y, Kurosawa N, Kanematsu T, Taguchi A, Arita M, Kojima N, Tsuji S. 1996. Unique genomic structure and expression of the mouse alpha 2,8-sialyltransferase (ST8Sia III) gene. Glycobiology 6:573–580.