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# Divergent evolution of the vertebrate polysialyltransferase *Stx* and *Pst* genes revealed by fish-to-mammal comparison

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**Abstract**

Polysialic acid (PSA) is a developmentally regulated carbohydrate attached to the neural cell adhesion molecule (NCAM). PSA is involved in dynamic processes like cell migration, neurite outgrowth and neuronal plasticity. In mammals, polysialylation of NCAM is catalyzed independently by two polysialyltransferases, STX (ST8Sia II) and PST (ST8Sia IV), with STX mainly acting during early development and PST at later stages and into adulthood. Here, we functionally characterize zebrafish *Stx* and *Pst* homolog genes during fish development and evaluate their catalytic affinity for NCAM *in vitro*. Both genes have the typical gene architecture and share conserved synteny with their mammalian homologues. Expression analysis, gene-targeted knockdown experiments and *in vitro* catalytic assays indicate that zebrafish Stx is the principal–if not unique–polysialyltransferase performing NCAM-PSA modifications in both developing and adult fish. The knockdown of Stx exclusively affects PSA synthesis, producing defects in axonal growth and guidance. Zebrafish Pst is in principle capable of synthesizing PSA, however, our data argue against a fundamental function of the enzyme during development. Our findings reveal an important divergence of *Stx* and *Pst* enzymes in vertebrates, which is also characterized by a differential gene loss and rapid evolution of *Pst* genes within the bony-fish class.


*Keywords:* ST8SiaII; ST8SiaIV; Polysialyltransferase; NCAM; PolySia; Morpholino; Zebrafish; Oligonucleotides; Axon guidance; Development; Evolution

## Introduction

Polysialic acid (PSA) is a homopolymer of α-2,8-linked sialic acid residues mainly attached to the neural cell adhesion molecule (NCAM). The addition of PSA to NCAM is developmentally regulated and peaks in the perinatal nervous system (reviewed in Durbec and Cremer, 2001; Muller et al., 2000). After birth, PSA levels decrease progressively during development. In the adult nervous system, PSA is restricted to areas of the brain showing ongoing morphological and/or functional plasticity e.g. the hippocampus and olfactory system (Cremer et al., 2000; Kiss and Rougon, 1997; Rutishauser, 1998). In general, PSA-NCAM was thought to interfere with adhesion events mediated by NCAM due to steric hindrance (Edelman and Crossin, 1991; Rutishauser, 1998). More recent data, however, suggest additional roles for PSA during cellular interactions: PSA can bind and present growth factors e.g. BDNF (Muller et al., 1996), as it is involved in long term potentiation (Becker et al., 1996; Muller et al., 1996), and we have shown that PSA expressed on floor plate cells in the zebrafish hindbrain is essential to guide PSA-negative commissural axons (Marx et al., 2001). Molecular characterization of PSA biosynthesis in mice indicates that in mammals at least two polysialyltransferase genes, known as *ST8SiaII* (*Stx*) and *ST8SiaIV* (*Pst*), are involved in this process (reviewed in Angata and Fukuda, 2003; Tsuji, 1996). Both genes encode for highly conserved proteins in vertebrates (Harduin-Lepers et al., 2005), but differ in their temporal expression profiles on CNS structures. Whereas *Stx* is highly expressed during early

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developmental stages, *Pst* is mainly expressed at later stages and adulthood (Becker et al., 1996; Hildebrandt et al., 1998; Ong et al., 1998).

Transgenic knockout of the polysialyltransferase genes in mice (Angata et al., 2004a; Eckhardt et al., 2000) has provided the first information on the individual contribution of each enzyme in PSA biosynthesis. *Stx* and *Pst* knockout mice exhibit specific and subtle defects in the central nervous system (Angata et al., 2004a; Eckhardt et al., 2000). In addition, both strains do express PSA, demonstrating that both enzymes are independently capable of directing PSA synthesis. Whereas in *Stx* depleted mice PSA levels are remarkably reduced at postnatal day one (P1), PSA levels are close to normal at P1 in *Pst*-knockouts, but then decrease rapidly during development and PSA is virtually absent in adults (Eckhardt et al., 2000). These findings indicate that PST is the major enzyme in the adult, while STX seems to be crucial in the developing organism. However, the genetic mechanisms regulating PSA expression during development and in the adult nervous system of mammals remain unclear.

As an extension of our previous work on PSA functions during development (Marx et al., 2001), in this study we used the zebrafish (*Danio rerio*) model to get insights on the roles of polysialyltransferases in vertebrates. By combining phylogenetic, genomic, developmental, molecular and biochemical approaches we show that zebrafish *Stx* and *Pst* genes differ in their transcriptional pattern during development and in adulthood, as well as in their catalytic activity on NCAM. In contrast to the situation in mammals, our results reveal that zebrafish *Stx* is the principal enzyme performing PSA modifications on NCAM throughout life. Regarding its function, zebrafish *Pst* departs from the known mammalian counterparts, as it is generally expressed at low levels in all ages. Moreover, our genomic analyses show that chromosomal regions containing polysialyltransferases share conserved synteny relationships among vertebrates, indicating a common origin for zebrafish and human *Stx* and *Pst* genes. However, the higher frequency of chromosomal rearrangements detected in the region containing *Pst* genes (rather than in their *Stx* counterparts) between fish and mammals, could explain the divergent transcriptional dynamics observed for zebrafish *Pst*. Our data highlight the suitability of zebrafish as a non-mammalian model to study the functional divergence of the polysialyltransferase ST8SiaII (STX) and ST8SiaIV (PST) in chordates.

## Materials and methods

### *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 accordingly to Kimmel et al. (1995).

### *Cloning of zebrafish polysialyltransferases*

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 *Stx* and *Pst* sequences were obtained via conventional PCR protocols, using as a template a pool of cDNA from 3–72 hpf stage embryos. For this purpose two sets of degenerated primers (see Supplementary Table 1) for vertebrate *Stx* and *Pst* were designed using publicly available polysialyltransferases sequences. Based on these partial sequences, a new set of gene-specific primers (see Supplementary Table 1) were designed to extend the full mRNA information via RACE-PCR (5′-RACE and 3′-RACE kit; Invitrogen). Full cDNAs 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). Antisense and sense (control) riboprobes were synthesized from linearized plasmids containing partial or full cDNA regions of zebrafish *Stx* and *Pst* using the DIG RNA labeling kit (Roche). 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. Embryos were cleared and mounted following standard protocols (www.zfin.org). ISH on cryosections was performed (as described before (Weth et al., 1996) on fresh-frozen brains sectioned sagitally into 20 μm thick slices. Cryostat sections were stained to immuno-detect localization of nuclei (DAPI; Hoechst) and PSA (mAB 12E3), as well as for ISH. Pictures were taken using a Coolpix 4500 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), using the murine anti-PSA antibodies IgG2a mAb 735 (Frosch et al., 1985) and IgM mAb 12E3 (Seki and Arai, 1991). Axons were labeled with zebrafish pAb Tag-1 (Lang et al., 2001). Embryos were analyzed using a confocal microscope (LSM 510, Zeiss) equipped with a high aperture lens 40x/1.2W (C-Apochromat, Zeiss) and the appropriate lasers. Serial optical sections were flattened into projections and processed with Photoshop 6.0 software (Adobe).

### *RT-PCR screening of Stx and Pst expression in zebrafish*

Alternatively, we examined the expression of zebrafish polysialyltransferases via RT-PCR on a palette of cDNAs generated from stage-specific and tissue-specific total RNAs (Invitrogen), by using exon-specific primers (see Fig. 2). The amount and quality of cDNA loaded by PCR reactions was standardized regarding the housekeeping gene *actin* (670 bp; for: 5′-GAAGATCAAGATCATTGCTCCTCC-3′, rev: 5′-CTGGTCTCAAGTCAGTGTACAGG-3′).

### *Morpholino injections*

To knock-down the expression of zebrafish Pst and Stx proteins during development we used a morpholino (MO) antisense approach (Nasevicius and Ekker, 2000). The following MOs were purchased from Gene Tools (Oregon): MO-STX1: 5′-CGCATCGGTCAAGTCATGTCTTTT-3′; MO-STX2: 5′-CGTGAAAGAGGAGAACACATTTCT-3′; MO-PST: 5′-GCGTTTATCTTGGATTAATTGTTTA-3′ MO were dissolved in distillated water and injected into one- to four-cell stage embryos in 1× Danieau solution (Nasevicius and Ekker, 2000) and 0.004% phenol red (Sigma) at different concentrations (2–18 ng/embryo) in a final volume of 5 nl per embryo.

### *Determination of the catalytic activity of recombinant polysialyltransferases in vitro*

We assessed the relative activity of recombinant Stx and Pst enzymes from zebrafish and mammals, by using two complementary approaches: (i) *in vivo*, by co-expressing N-Flag-HA tagged constructs and murine NCAM in mouse LM-TK cell lines, and (ii) *in vitro*, by using recombinant A-polysialyltransferase chimeric proteins. Zebrafish *Stx* and *Pst* genes were subcloned in frame into pFlagHA vector (XhoI/XbaI sites; Windfuhr et al., 2000)) using the following primers for *Pst* gene: KS117for 5′-GCCTCGAGTCTTTTGAATTCCGAATACTGATG-3′; KS118rev 5′-GCTCTAGATCATGTAGGAGGTTTGCATGG-3′; and for *Stx* gene: KS115for 5′-GCCTCGAGCGGCTTTCACGCAAACACTG-3′; KS116rev 5′-GCTCTAGATTAAGTTGATGTGCATTTAGATGT-3′. For *in vitro*

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assays soluble recombinant Stx and Pst protein was generated with the eukaryotic expression system pPROTA (generously provided by Dr. R. Breathnach, INSERM, Nantes, France) as described before (Sanchez-Lopez et al., 1988) on CHO cells of the complementation group 2A10 that lacks an active polysialyltransferase (Eckhardt et al., 1995). LM-TK cells were transfected and analyzed by using a Leica DM IRBE microscope as described before (Muhlenhoff et al., 2001; Windfuhr et al., 2000). PSA was detected by immunofluorescence staining with mAB 735 (38) at a concentration of 5 g/ml. PSA-synthesis was corroborated by endoN treatment (20 min at 37 °C; Gerardy-Schahn et al., 1995). We analyzed all samples by standard SDS-page electrophoresis and autoradiography, and micro-beads (Pierce) were used as a loading control.

### *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 polysialyltransferases homologues in chordate organisms, using as a reference the human sequences NM_006011 (*Stx*) and NM_005668 (*Pst*). 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 (Felsenstein, 1985), after 1000 iterations. 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, and substitution ratios were compared by Chi-square test ($\chi^2$). To establish synteny, the zebrafish, *Takifugu rubripes*, *Xenopus tropicalis*, chick, opossum, human, rat and mouse genomic regions containing *Stx* and *Pst* genes were analyzed for gene content and order at the Ensambl server (www.ensambl.org). When necessary, reciprocal BLAST searches where performed to identify ambiguous genome annotations. All genomic data used here were extracted from public databases.

## Results

### *Two α-2,8-polysialyltransferase genes are transcribed in zebrafish*

By combining *in silico* prediction and direct PCR amplification using degenerated and gene-specific primers, we successfully cloned two full-length mRNAs corresponding to the zebrafish *Stx* and *Pst* homologues (Genebank Accession numbers AY055462 and DQ779599 respectively). During preparation of this article, the zebrafish *Pst* sequence was independently reported by another group (Harduin-Lepers et al., 2005). At the protein level, the zebrafish *Stx* (382 aa) and *Pst* (359 aa) clones are 49.3% identical, sharing 63.4% and 70.2% of sequence identity with their human homologues (see Fig. 1C for more details). Notably, zebrafish Stx has an insertion of six residues (DLNAAP) in the so-called “stem region”, which in mammals may be involved in the interaction with NCAM (Angata et al., 2004b).

As expected for sialyltransferases, the zebrafish genes encode for type II membrane proteins containing all the landmark motifs TM, L, S, VS, III and *ls* (Eckhardt and Gerardy-Schahn, 1998; Harduin-Lepers et al., 2005; Patel and Balaji, 2006; Yoshida et al., 1996), as shown in Fig. 1A. The comparison of our mRNA information with available genomic data from zebrafish revealed that their gene structures (i.e. exon–intron organization and intron phase; Fig. 1A) resemble those described previously in other organisms e.g. human and frog (Eckhardt and Gerardy-Schahn, 1998; Harduin-Lepers et al., 2005; Patel and Balaji, 2006; Yoshida et al., 1996). Even though the reported sequence of the *Amphioxus* polysialyltransferase is the best candidate for the pre-vertebrate ancestor, it shows equally low sequence identity scores with human *STX* (18.6%) and *PST* (18.7%) genes.

We next analyzed the molecular variation among chordate *Stx* and *Pst* genes. The robust phylogenetic relationships obtained for chordate α-2,8-polysialyltransferases shown in Fig. 1C recovers the expected monophyletic nature of vertebrate *Stx* and *Pst* loci with an internal clustering that is consistent with the known taxonomy. The overall ratios of standardized non-synonymous/synonymous substitutions (dN/dS) are rather comparable (0.714 and 0.729 respectively, $\chi^2 = 13.82, p < 0.001$), indicating negative selection against amino acid replacements resulting in a higher occurrence of synonymous changes (Fig. 1B). This explains the high levels of sequence conservation observed. Remarkably, fish *Pst* and *Stx* differ in their evolutionary history since we failed to detect clear *Pst* homologues in either *Takifugu* or *Tetraodon* pufferfish genome databases. Moreover, we obtained a stepped phylogenetic distribution of zebrafish and trout *Pst* genes basal to tetrapods that is likely to be due to the their particularly large amount of codon substitutions (larger branch length in Fig. 1C), which is especially evident in the zebrafish (0.916 in zebrafish and 0.737 in tetrapods, $\chi^2 = 15.14$, $p < 0.0001$). Our results highlight the evolutionary conservation of vertebrate α-2,8-polysialyltransferases at gene organization and protein structure levels. However, by examining their variation at codon level, we were able to detect that fish *Pst* genes seem to have undergone a particularly faster molecular evolution that departs from the trend observed for other tetrapod polysialyltransferases.

### *Zebrafish polysialyltransferases genes are differentially transcribed during early embryogenesis and adulthood*

To examine the transcription of *Stx* and *Pst* during early development whole-mount in situ hybridizations (ISH) were performed. Positive signals for *Stx*, but not *Pst*, were detectable from 13 hpf onwards in the prospective brain primordium, which is approximately 3 h before PSA immunoreactivity becomes detectable (Marx et al., 2001). At the pharyngula stage (22 hpf) and later stages *Stx* is ubiquitously expressed in the anterior nervous system (Fig. 2A) and the floorplate (not shown). From 40 hpf onwards the *Stx* signal is slightly reduced but remains strong in the cerebellum and nuclei of cranial nerves (Fig. 2C). In contrast to *Stx*, *Pst* transcription was weakly detectable via ISH throughout early zebrafish development (Fig. 2B). Upon reaching the larvae stage, a slight increase of the signal becomes visible in brain regions close to the ventricles (Fig. 2D). To exclude methodological artifacts, we used different *Pst* riboprobes, all of them producing similar results. To confirm the transcription of *Pst* gene, we performed additional RT-PCR reactions on our developmental cDNA palette using *Pst* and *Stx* exon-specific primers (Fig. 2E). As

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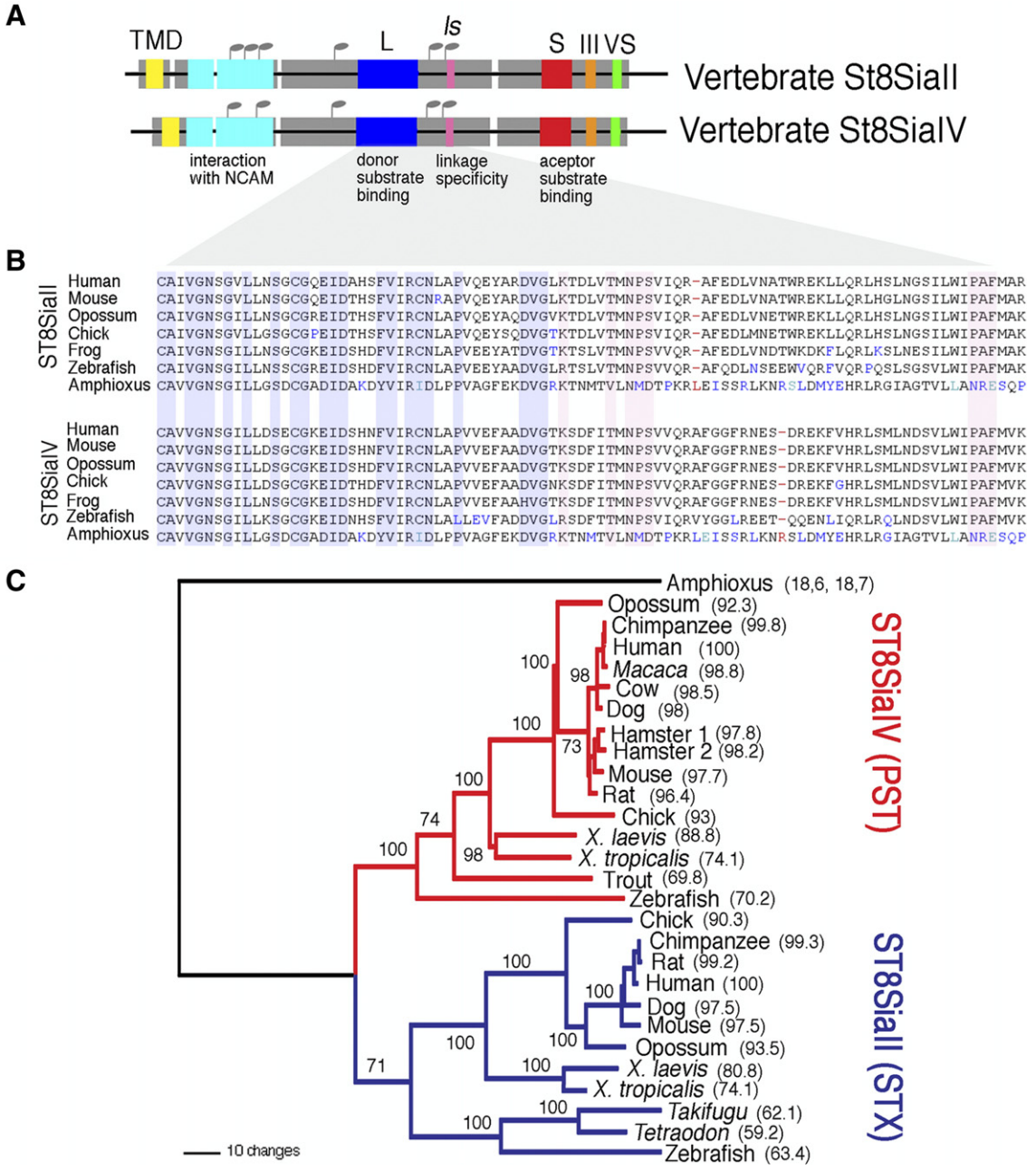


Fig. 1. Structural landmarks of vertebrate polysialyltransferases and their evolutionary relationships. (A) Schematic representation of the relative location of conserved structural and functional domains of vertebrate Stx and Pst proteins. Gray boxes represent protein backbones encoded by the corresponding gene exons; colored boxes on each polypeptide represent different motifs as follows: yellow = transmembrane domain (TMD); turquoise = stem region; blue, red and green = sialyl-motif large (L), small (S) and very small (VS) respectively; pink = St8Sia linkage specific motif; and orange = motif III. Gray icons depict distributions of the expected *N*-glycosylation sites over the polypeptide backbone. (B) Conservation of amino acid composition between representative vertebrate organisms regarding their putative closest chordate relative sequence (*Amphioxus*, Accession number AF391289). Residue substitutions that do not share similar physicochemical properties are highlighted in color. Indels are denoted by –. (C) Evolutionary relationships between vertebrate polysialyltransferases as recovered from ME and NJ analysis. Stx and Pst proteins are recovered as monophyletic groups with comparable evolutionary dynamics, as observed in their branching pattern. Note that the clustering of fish *Pst* differs from the one observed for fish *Stx*. As fish *Pst* genes differ in their overall dN/dS ratio, they do not cluster as a sister clade basal to tetrapods. The consensus tree was based on amino acid sequence alignment. Bootstrap support is shown for relevant nodes. The sequence identity scores (regarding their human homologue) are given in brackets beside the taxa label.

expected, our RT-PCR analysis showed the existence of *Pst* and *Stx* mRNA in all developmental stages. These experiments support the weaker expression pattern of *Pst* in our ISH during zebrafish development. Taken together, our data show that both polysialyltransferases are expressed exclusively in the central nervous system during zebrafish development. Whereas zebrafish *Stx* is comparatively highly transcribed about 3 h before PSA becomes detectable, *Pst* shows only weak expression.

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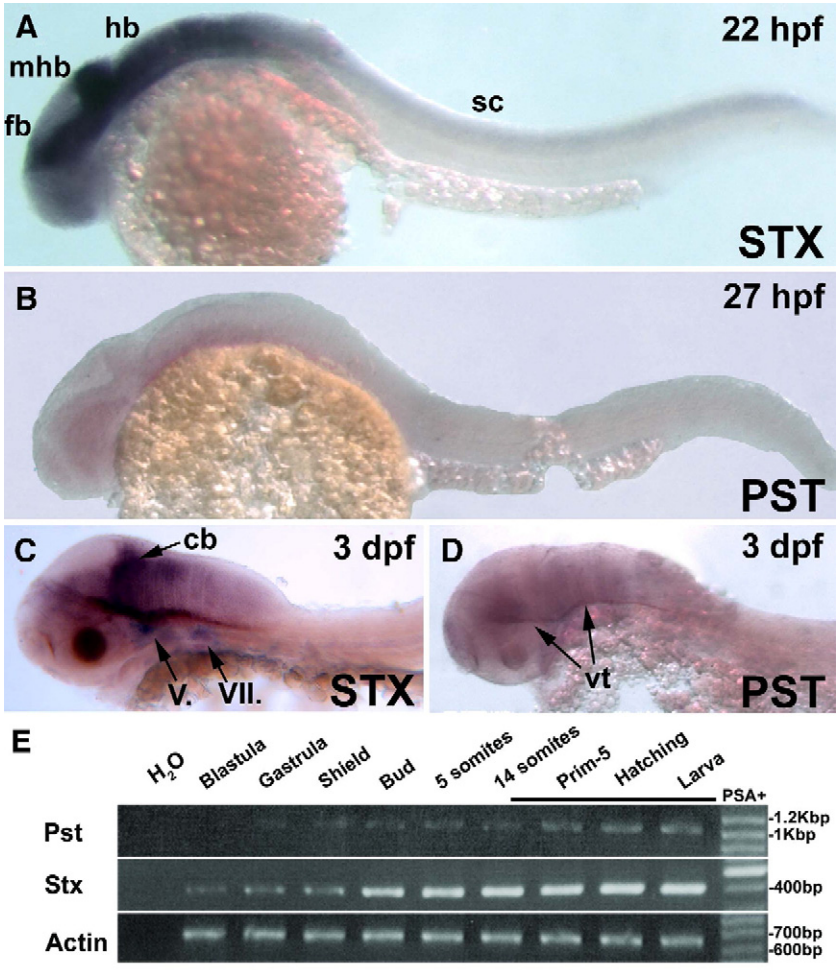


Fig. 2. Expression of zebrafish polysialyltransferases Stx and Pst during the first 3 days of development. At 22 hpf, *Stx* is transcribed in all structures of the central nervous system (A) whereas *Pst* not detectable at 27 hpf (B). At 3 dpf, the expression of *Stx* remains strong in the cerebellum and motor nuclei (C), whereas a weak *Pst* signal becomes visible in brain regions close to the ventricles (arrows in panel D). RT-PCR analysis on developing zebrafish cDNAs reveals a weaker and later transcription of *Pst* compared to the expression of *Stx* mRNA (E). Target amplifications were based on exon-specific primers. *Actin* reactions were conducted using a 1:10 template dilution. The detection of PSA in developing embryos starts around 17 hpf as indicated by a PSA+bar. A–D lateral views, anterior to the left. cb, cerebellum; hb, hindbrain; fb, forebrain; mhb, midbrain–hindbrain boundary; sc, spinal cord; V., trigeminal nucleus; VII., facial nucleus.

To understand the interplay between PSA biosynthesis and polysialyltransferase expression in later stages, we examined their expression profiles in brain sections from adult zebrafish. In general, PSA distribution in the adult brain correlates with those areas known to undergo frequent cell proliferation and/or neurogenesis (Zupanc et al., 2005), PSA was found in neuronal cell bodies and axonal tracts. The most prominent axon tracts positive for PSA were located in the valvula cerebelli and formatio reticularis of the hindbrain (Fig. 3A, arrowheads) (Zupanc et al., 2005). Consecutive sections processed for ISH reveal that PSA expression always correlates with a strong *Stx* signal, whereas *Pst* signals as detected with all used riboprobes are either absent or weak. An example depicting the hypothalamus is shown in Figs. 3B–E. Additionally, we performed RT-PCR on different tissues of adult zebrafish to confirm the transcription of both genes. We observed that *Stx* is comparatively highly expressed in brain, eye, ovary and liver, weakly expressed in skeletal muscle and not detectable in the heart (Fig. 3F). *Pst* transcription, however, differs considerably from *Stx* showing modest expression in skeletal muscle and brain (Fig. 3F). Therefore, we hypothesize that in zebrafish PSA synthesis in the nervous system mainly depends on the regulation of *Stx* expression and activity.

### *The synthesis of PSA during zebrafish development depends exclusively on Stx expression*

We tested this hypothesis by performing morpholino (MO) knockdown of *Stx* and *Pst* genes in developing zebrafish. In general, the MO injected embryos do not show any evident morphological defects or increases in the rate of mortality (Figs. 4A–F). Noteworthy, only in STX-MO treated embryos we fail to detect PSA-immunoreactivity (Fig. 4E). In these morphants the resulting phenotypes were associated with abnormal growth and pathfinding of commissural axon tracts in the hindbrain and the posterior commissure (pc), which resemble our previous results by removing PSA in early larvae via treatment with

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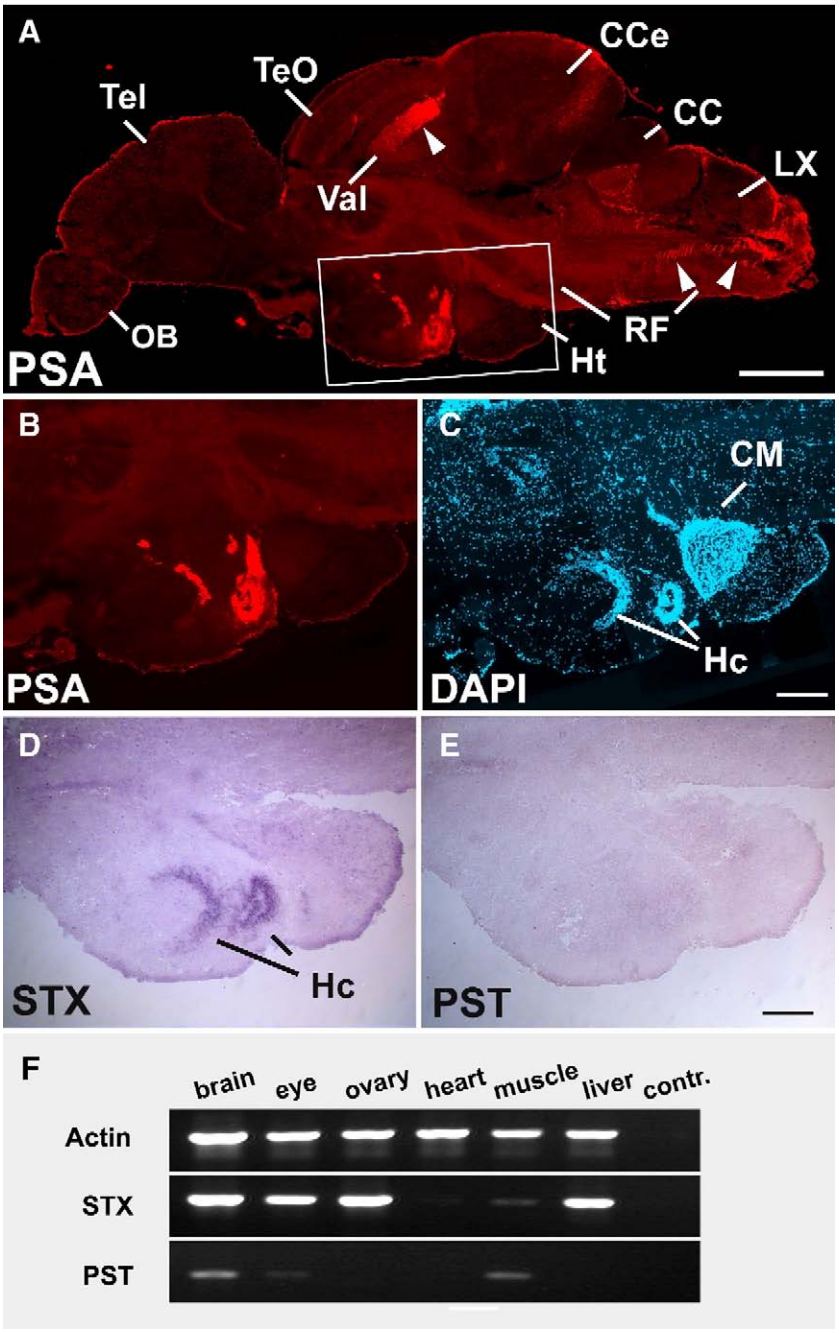


Fig. 3. Expression of PSA and zebrafish polysialyltransferase mRNAs in the adult brain. (A) Sagittal section of an adult brain, stained for PSA. Boxed region in A is enlarged in B–E. (B–E) PSA is expressed in periventricular regions of the hypothalamus (B) on cell bodies (C, nuclear stain). Only *Stx* is found in the Hc (D) whereas *Pst* is not detectable (E). (F) RT-PCR analysis on adult tissue cDNAs reveals expression of *Stx* in all tissues except for heart and skeletal muscle. *Pst* expression is evident in brain and skeletal muscle. Target amplifications were performed as described in Fig. 2. cc, crista cerebellaris; Cce, corpus cerebelli; CM, corpus mamillare; Hc, caudal zone of periventricular hypothalamus; Ht, hypothalamus; LX, vagal lobe; OB, bulbus olfactorius; RF, reticular formation; Tel, telencephalon; TeO, tectum opticum; Val, valvula cerebelli. Scale bars 500 μm (A); 200 μm (B–E).

endoN (Marx et al., 2001). However, the extent of STX-MO defects was more severe than defects obtained with endoN treatments. In control and PST-MO embryos, the posterior commissure (pc) traverses the brain hemispheres in one fasciculated, PSA-positive bundle (Figs. 4G–J). This typical growth pattern is severely affected in 83% ($N=35$) of STX-MO embryos. In these embryos, the pc was either reduced in diameter (17%), split into several bundles (41%), unable to cross the midline (11%), or not developed at all (14%). As the co-injection of STX-MO and PST-MO produced similar phenotypes as obtained with STX-MO alone (data not shown), we conclude that during the development of zebrafish embryos the synthesis of PSA is exclusively controlled by Stx. Under this scenario, the important question arises whether zebrafish Pst protein can perform polysialyltransferase activity at all.

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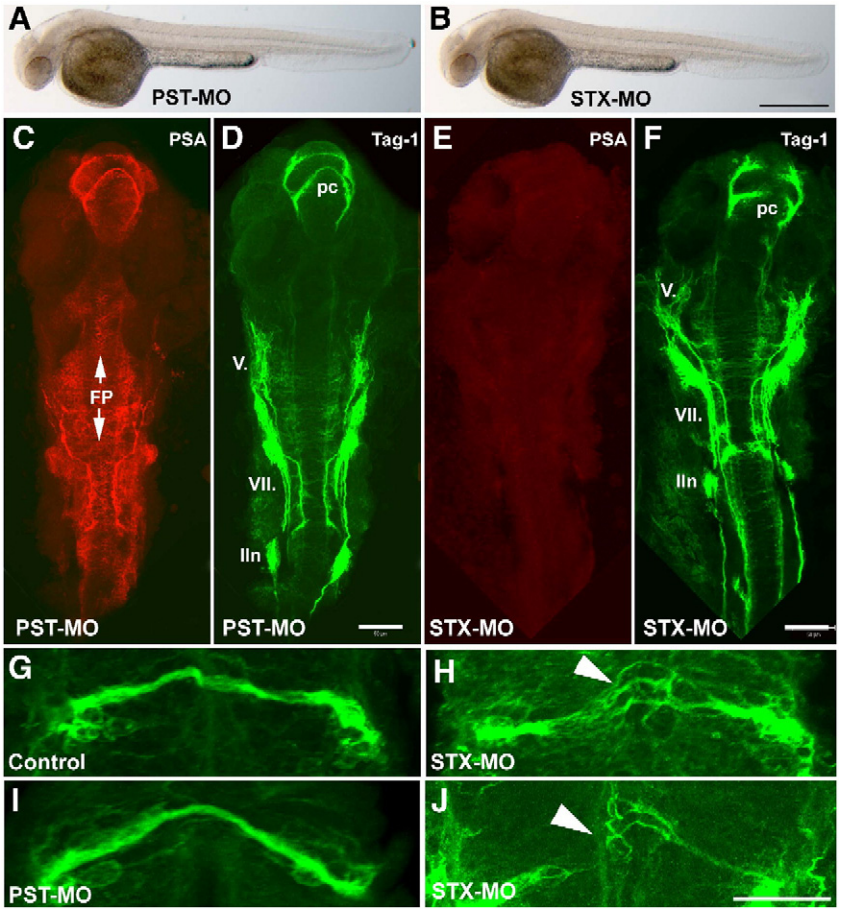


Fig. 4. Knockdown of Stx produces abnormal axonal growth and guidance in developing zebrafish. (A, B) Embryos at 32 hpf injected with PST- (A) or STX-specific (B) morpholinos do not show any evident phenotype defects or increases in the rate of mortality. (C–F) Embryos at 32 hpf after morpholino knockdown of polysialyltransferases Stx and Pst immunolabeled for PSA (mab 735, red) and the axonal marker Tag-1 (in green). (C, D) Knockdown of Pst neither alters PSA immunoreactivity nor the growth pattern of axons. PSA is expressed by cells in the CNS, the floorplate, on axons and nuclei of cranial nerves V. and VII., and on axons of the posterior commissure (pc). (E,F) Knockdown of Stx leads to a complete loss of PSA-immunoreactivity (E), whereas the overall development is not severely disturbed (F). (G–J) Growth pattern of pc in control (G) and in PST-MO embryos (I), axons of the pc grow in a fasciculated bundle. After Stx knockdown, the PC splits into several bundles (H, arrowhead), or axons do not cross the midline (J, arrowhead). FP, floorplate; lln, lateral line nerve; pc, posterior commissure; V., trigeminal nuclei and axons; VII., facial nuclei and axons. Images in C–J are projections of confocal images, rostral to the top. Scale bars: 500 μm (A–B), 50 μm (C–F), 20 μm (G–J).

*Zebrafish Pst shows a reduced catalytic activity and a low affinity for NCAM than their mammalian homologue*

To test, whether the zebrafish Pst protein is able to catalyze the synthesis of PSA on NCAM, we compared its relative catalytic efficiency by using two complementary *in vitro* assays to estimate polysialyltransferase activity (Angata and Fukuda, 2003; Muhlenhoff et al., 2001). We first used a radioactive assay system, where Ig-purified recombinant polysialyltransferases and NCAM proteins were incubated overnight in a media containing the radioactive-labeled donor substrate CMP-[$^{14}$C]Neu5Ac. Whereas under these conditions zebrafish Stx presents catalytic profiles on NCAM and in autopolysialylation, which are comparable to our mammalian control (Fig. 5A, lanes 4, 5), the zebrafish Pst does not give any detectable signal (Fig. 5A, lanes 6, 7).

We next used a cell-based system that is highly sensitive for PSA detection (Muhlenhoff et al., 2001). Here, PSA-negative LM-TK cells were transiently transfected with our *Stx* and *Pst* fusion constructs as well as with or without co-transfection of NCAM constructs. PSA production was then monitored by immunohistochemistry. All tested constructs produced detectable levels of PSA in single LM-TK cells (Fig. 5C), indicating that the heterologous expression of polysialyltransferases in mammalian cell lines preserves the catalytic activity of the enzymes. Surprisingly, high amounts of PSA in LM-TK cells transfected only with zebrafish *Pst* constructs were detected in the plasma membrane suggesting that either autopolysialylated Pst enzymes are transported to the cell membrane, or alternatively–and more interestingly–other (yet unidentified) protein(s) are being used as acceptor for PSA modification by zebrafish Pst.

Our data indicate that both zebrafish polysialyltransferases are catalytically active and capable of producing PSA *in vitro*. However, we only detect NCAM polysialysation with recombinant zebrafish Stx proteins.

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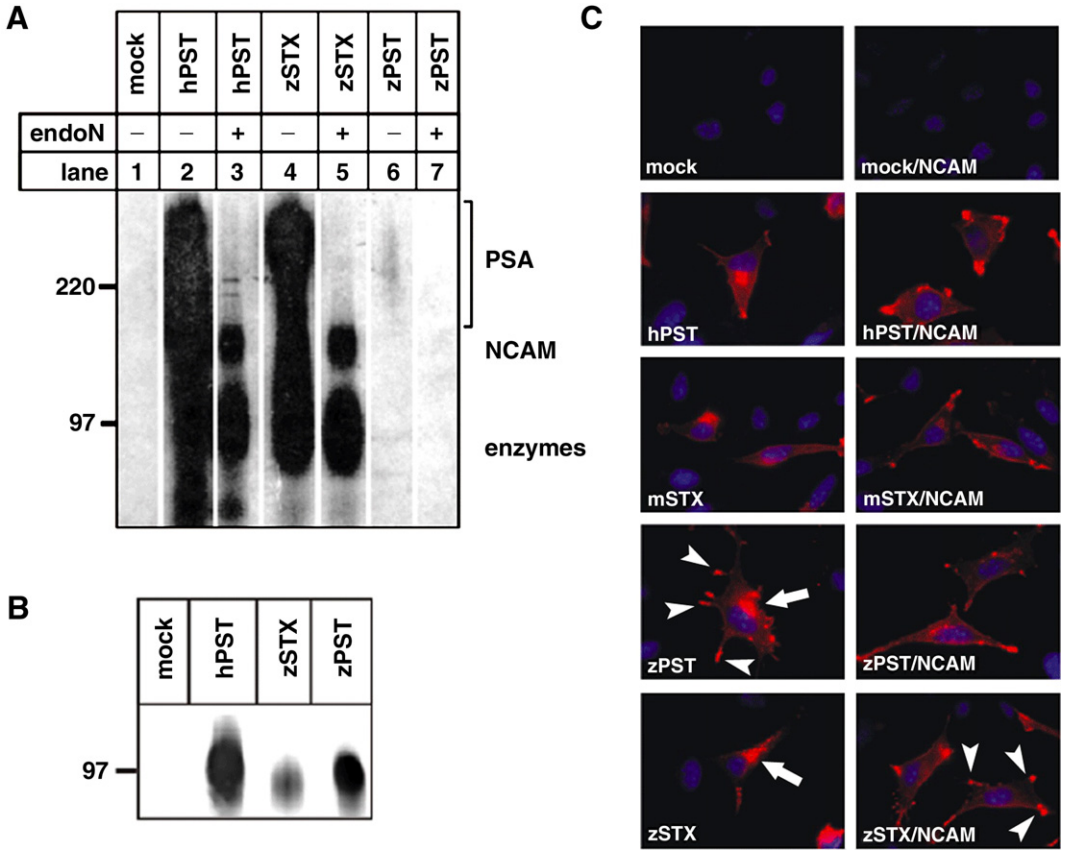


Fig. 5. The divergent zebrafish Pst polysialyltranferase shows a low affinity for NCAM and autopolysialisation *in vitro*. (A) Sepharose fixed Protein A-fusion proteins of zebrafish Pst and Stx were analyzed *in vitro* for NCAM polysialylation. The radiolabelled reaction products were analyzed by SDS-PAGE and autoradiography before (−) and after (+) treatment with endoN. Depolysialylated Protein A fusion proteins of enzymes and NCAM are indicated. Under these conditions only zebrafish Stx is able to polysialylate NCAM to the same amount as the control, hamster Pst. (B) The western blot immunostained with anti-Protein A mouse IgG demonstrates that loading of beads with recombinant protein A-polysialyltransferases was comparable in all reaction mixtures. (C) *In vivo* activity of zebrafish polysialyltransferases using NCAM- and polysialyltransferase-negative LM-TK cells. Cells were analyzed for PSA expression by immunoflourescence using anti-PSA mAb 735 (red). Nuclei were visualized by Hoechst staining (blue). Cells transfected either with an empty vector or solely with NCAM do not express PSA (mock, mock-NCAM). The expression of one polysialyltransferase (zPST, zSTX), either alone or in co-transfection with NCAM, leads to a strong PSA signal comparable to the positive controls (hPST, mSTX). In cells expressing solely one polysialyltransferase, the golgi apparatus (arrows) is labelled for PSA, whereas co-expression with NCAM leads to an additional labelling of the cell membrane (arrowheads). An exception is cells transfected with zebrafish Pst alone, that reveal expression of PSA in both structures.

*Chromosomal regions containing polysialyltransferase genes share conserved synteny relationships among vertebrates*

To get insights into the origin and functional diversification of vertebrate polysialyltransferases, we examined the genomic context of *Stx* and *Pst* in representative chordate taxa. To decide whether *Stx* and *Pst* originated from a common ancestor gene by gene or genome duplication, we compared the gene content and order in genomic regions containing those genes in representative species of major vertebrate classes (e.g. mammals, marsupials, amphibians). We found that in all analyzed species *Stx* and *Pst* reside in different chromosome regions and share conserved syntenic relationships from fish to mammals (Fig. 6). For instance, the gene cluster containing the human *SLCO3A1–STX–CHD2–RGMA* genes was also found in opossum, chick, frog (except for *Slco3a1*), zebrafish and *Takifugu*, indicating that this chromosome region has been conserved for at least ~476 My, which is the expected divergence time for bony-fish and tetrapod lineages (Blair and Hedges, 2005). In a similar way, in humans the gene cluster containing *NUDT2–HISPDI–SLCO4C1–PAM–PST–LIX1–CAST–PCSK1–ELL2–GRLY–RhoBTB3* is highly conserved in other tetrapods (i.e. opossum, chick and frog; Fig. 6), but reduced to the pair *PAM–PST* in zebrafish. This indicates that fish and mammalian polysialyltransferases (and vertebrate in general) are orthologues, which might likely have arisen from a common pre-vertebrate ancestor. Notably, genomic regions containing the *Pam* gene in the *Takifugu* and *Tetraodon* pufferfish do not show direct evidences of having a *Pst*-like gene in their direct neighborhood (data not shown), a fact that might indicate a secondary loss of this gene in the order Tetraodontiformes, e.g. via non-functionalization (Force et al., 1999).

Our data suggest the occurrence of independent dynamics following the duplication of chromosome regions containing the *Stx* and *Pst* loci in fish and mammals, as the *Stx* region seems to have undergone less chromosomal rearrangements than their *Pst* counterparts. These findings might explain the divergent transcriptional pattern of the zebrafish *Pst* gene and its mammalian counterparts, as higher frequencies of chromosomal

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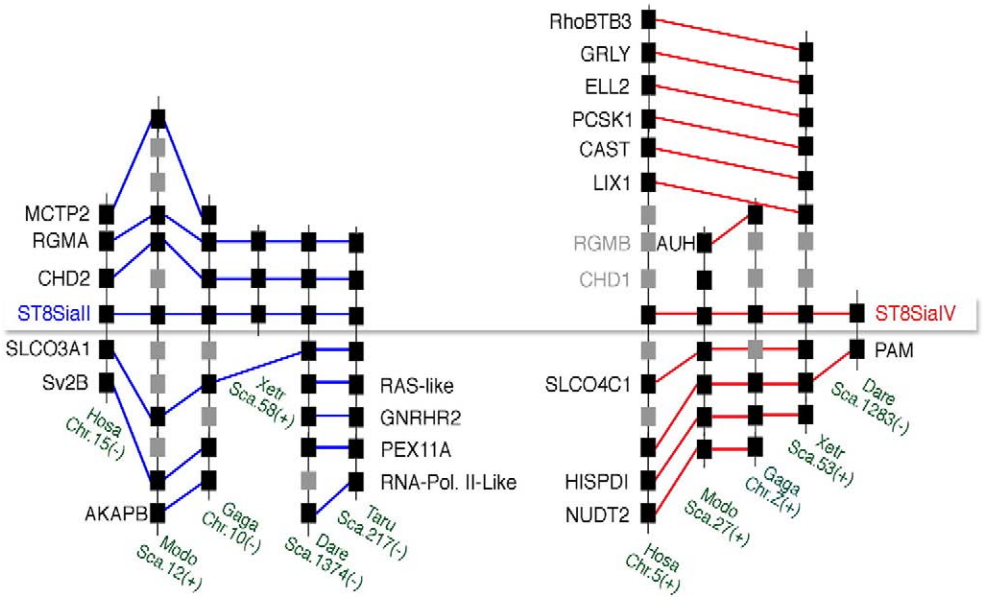


Fig. 6. Conserved syntenic relationships between zebrafish and human chromosome regions containing *Stx* and *Pst* loci. Schematic diagram showing conserved synteny between zebrafish and tetrapod chromosome regions containing *Stx* and *Pst* loci. Each vertical line represents the chromosomal region of one species containing syntenic genes (black boxes) and non-syntenic genes (gray boxes) connected by non-coding intergenic segments. Orthologue genes are connected by colored lines (blue for *Stx* and red for *Pst*) across species. The species identity, genome region and their relative orientation are given below each chromosome as follows: Hosa = human, Modo = opossum, Gaga = chick, 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 and drawings do not reflect the original scale.

rearrangement can promote the gain–or loss–of genomic information (e.g. promoters, enhancers or repressors) affecting the regulatory control of zebrafish *Pst*.

## Discussion

By analyzing and comparing the functional expression of the two zebrafish α-2,8-polysialyltransferases *Stx* and *Pst* at different organization levels, we show that: 1) the nervous system expression pattern of the zebrafish genes throughout development and into adulthood differs from that in mammals; 2) in zebrafish, only *Stx* performs an efficient biosynthesis of PSA under *in vivo* and *in vitro* conditions; 3) the rate of molecular changes, transcriptional dynamics and catalytic properties of the zebrafish *Pst* gene departs from the trend observed for other vertebrate *Pst* genes; 4) chromosomal regions containing polysialyltransferase genes share conserved syntenic relationships between different vertebrate classes; 5) in the fish lineage, the polysialyltransferase subfamily underwent a secondary loss of *Pst* genes that follows a taxonomic-specific fashion. We discuss the importance of our findings in the context of the developmental and physiological functions of α-2,8-polysialyltransferases on the biosynthesis of PSA.

### *Divergent properties of the vertebrate polysialyltransferase genes revealed by loss-of-function and biochemical assays in zebrafish*

We show that zebrafish *Stx* transcription strongly correlates with the development of brain and nervous system structures as well as the floorplate, at the onset of neurogenesis. This pattern precedes the expression of PSA by about 3 h (this study and Marx et al., 2001). It is noteworthy that during development of mammals, PSA and both enzymes are also present in non-neuronal tissues like muscle, lung and kidney (Angata et al., 1997), whereas during zebrafish development PSA expression seems to be nervous system specific. In the adult zebrafish brain, we detected considerable amounts of *Stx* mRNA in PSA-positive regions accompanied by only weak expression of *Pst* mRNA. Therefore, PSA synthesis in the adult zebrafish brain is likely to depend on Stx activity. Early *Stx* expression in the zebrafish nervous system is much more ubiquitous than in mammals, which express *Stx* in very distinct areas of the developing brain, e.g. in the cerebellum, the thalamus or certain layers of the cortex (Hildebrandt et al., 1998; Kurosawa et al., 1997). Moreover, in mammals the majority of adult PSA synthesis is driven by strongly expressed PST (Angata et al., 2004b; Eckhardt et al., 2000; Ong et al., 1998), whereas in adult zebrafish brain Stx prevails, indicating the major role of Stx in PSA synthesis in the adult nervous system. Further developmental studies on *Stx* and *Pst* gene expression and activity in linking vertebrate model organisms–as e.g. frog and chick–would help us to clarify the succession of molecular and functional changes affecting the activity of polysialyltransferases in vertebrates.

In contrast to the alternate expression but conserved activity described for mammalian polysialyltransferases, our loss-of-function and catalytic assays on their zebrafish counterparts showed convincingly that Stx rather then Pst is the principal (if not the unique) catalytic agent undertaking the posttranslational modification of NCAM throughout zebrafish ontogeny. Our data show that knockdown of Stx leads to a complete abolishment of PSA-immunoreactivity during the first 2 days

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of development. In contrast, mice, in which the polysialyltransferases have been knocked out individually (Angata et al., 2004b; Eckhardt et al., 2000) or in combination (Weinhold et al., 2005) confirmed that combined activity of the two enzymes is essential to fully polysialylate NCAM. Another significant difference between zebrafish and mammals is the finding that in zebrafish the STX-morpholino knockdown induced loss of PSA severely affects the growth pattern of certain commissural axon populations. While some of the induced defects resemble those observed after enzymatic removal of PSA (Marx et al., 2001), the posterior commissure is affected more severely in the morphants. The enhancement of this defect is likely caused by the continuous depletion of PSA throughout development. Thus, while morphants lack PSA from the onset of development, endoN injected embryos had already expressed PSA to some extent. Nevertheless, the similarity of defects induced by the two treatments confirms the crucial function of PSA and Stx, respectively, in these steps of morphogenesis. Our finding that the morpholino knockdown of zebrafish Pst did not interfere with the PSA expression, supports the idea that this enzyme does not perform PSA synthesis on NCAM during development. However, the function of zebrafish *Pst* remains to be elucidated.

Taken together, the different pattern of PSA distribution during fish and mammalian development is a hint for an independent evolution of gene regulatory control mechanisms of polysialytransferases between vertebrate classes. Remarkably, the exclusive detection of PSA in non-neuronal structures during mammalian development (and not in fish) may suggest additional differences in the affinity for PSA-acceptor substrates between different vertebrate classes.

### *Conserved synteny relationships among vertebrate Stx and Pst loci revealed a disparate evolution of the fish polysialiltransferases homologues*

Our genomic analysis on available genome databases confirms the existence of two α-2,8-polysialyltransferase genes in bony-fish and tetrapods (Harduin-Lepers et al., 2005), a fact that hints for the origin of these genes to come from a common Actinopterygii–Sarcopterygii ancestor about 480 My. Noteworthy, the prompt availability of further genome databases for more basal vertebrates as e.g. hagfish (Myxini), lamprey (Agnatha), shark (Chondricthyes) and sturgeon (Chondrostei) as well as for alternative pre-vertebrate organisms (e.g. *Amphioxus*, Cephalochordata and *Oikopleura*, Urochordata) will help us to better understand the origin and diversification of the α-2,8-polysialyltransferase subfamily. We detected remarkable differences between zebrafish and mammalian *Stx* and *Pst* genes at both cellular (i.e. gene expression and catalytic activities) and genome-wide level (i.e. codon substitutions and synteny). Among the analyzed *Pst* genes the trout and zebrafish homologues present faster molecular evolutionary rates as noticed in their relative larger branching pattern (Fig. 1C) and dN/dS ratios, a fact that highlights an asymmetric molecular evolution of *Stx* and *Pst* genes between vertebrate classes that is also evident at chromosome level. The asymmetric dynamics detected at different organizational levels could explain the changes observed at the gene expression level (e.g. by mutation at gene promoter elements), the catalytic activity (e.g. by non-synonymous substitutions in key residues for catalytic activity) and the substrate affinity (e.g. by non-synonymous substitutions in key residues for acceptor substrate binding), which could promote opposite selective constrains on *Pst* and *Stx* genes leading to the functional loss of *Pst* in certain fish lineages. Similar asymmetric evolution has also been detected in other protein families as the SPFH (Rivera-Milla et al., 2006b), Rtn4-Nogo (Diekmann et al., 2005), the prion-protein PrP (Rivera-Milla et al., 2006a) and Sox9 (Cresko et al., 2003). Although it is likely that these expected duplicated polysialyltransferase paralogues may have been lost readily after the 3R genome duplication event (Postlethwait et al., 2004), we cannot discard that these paralogues remain undetectable to sequence analysis by losing their phylogenetic landmark information due to fast evolution. More important, our genomic analysis brings a novel explanation to the pattern of distribution of polysialyltransferase genes in the bony-fish group. Notably the fact that in humans the *PST* locus exhibits an alternative splice variant (NM_175052.1) that is not present in *STX* highlights the trend of *Pst* genes to show higher molecular variation than *Stx* genes. The existence of a single *ST8Sia*-like locus in the cephalochordate *Branchiostoma floridae* genome (genome.jgi-psf.org/Brafl1/Brafl1.home.html; our unpublished analysis) supports the idea that the ST8Sia gene family may have quickly expanded during the radiation of vertebrates.

Taken together, it is conceivable that, after gene duplication, *Stx* and *Pst* genes underwent a rapid phase of molecular variation that shaped their enzymatic properties as well as their regulatory control. In this hypothetical scenario, it is possible to predict that: (i) mammalian *Stx* and *Pst* genes solely segregated the control of their temporal expression but kept their shared catalytic activity; (ii) fish *Stx* genes kept most of the ancestral catalytic activity like their mammalian Pst and Stx counterparts; (iii) fish *Pst* genes accumulated considerable molecular changes that influenced their expression control, catalytic activity, and substrate affinity in a way that might differ in different fish orders, some of which might even have lost *Pst* completely.

Our data reveal significant differences regarding the molecular control of the expression and activity of polysialyltransferases in vertebrates and pose important questions, for instance, what are the genetic bases allowing the recruitment of polysialyltransferases to posttranslationally modify NCAM in vertebrates and how is the concerted co-expression of *Stx*, *Pst* and *NCAM* in the nervous system controlled? Further comparative studies analysing variations at non-coding genomic sequences between model vertebrate organisms (e.g. zebrafish and mice) will help to understand the interplay between polysialyltransferases and NCAM functions during development and in adulthood.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.03.032.

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