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Molecular Cloning and Biochemical Characterization of Sialidases from Zebrafish (*Danio rerio*)

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ADRESSES

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SHORT TITLE

Sialidase gene family in zebrafish

KEYWORDS

sialidase, zebrafish, gene family, comparative genomics, evolution, gene and protein expression

FOOTNOTE

The nucleotide and amino acid sequences for zebrafish sialidases have been deposited in the GenBank database under GenBank Accession Numbers: EF107693 (*neu3.1*), EF107692 (*neu3.2*), EF107698 and EF107700 (*neu3.3*), EF107696 (*neu3.4*), EF107694 (*neu3.5*), EF116275 (*neu4*). The sequence of *neu1* gene transcript was already available in GenBank with the Accession Number NM_001044909.

GenBank Accession Numbers for polypeptide sequences included in multiple sequence alignment and phylogenetic reconstruction were NP_000425 (human NEU1), NP_005374 (human NEU2), BAA82611 (human NEU3), CAC81904 (human NEU4), NP_035023 (mouse NEU1), NP_056565 (mouse NEU2), NP_057929 (mouse NEU3), NP_776133 (mouse NEU4), XP_001231585 (*Gallus gallus* Neu2), XP_428099 (*Gallus gallus* Neu3), EF434604 (*Takifugu rubripes* Neu1), EF434605 (*Takifugu rubripes* Neu3) and EF434606 (*Takifugu rubripes* Neu4). *Gallus gallus* Neu4 polypeptide sequence has been predicted by Gnomon (hmm18986 model) on *Gallus gallus* Gallus_gallus-2.1 genomic contig Gga9_WGA169_2.

SUMMARY

Sialidases remove sialic acid residues from various sialo-derivatives. To gain further insights into the biological roles of sialidases in vertebrates, we exploited zebrafish as animal model. A zebrafish transcriptome- and genome-wide search using as a query the sequences of the human NEU polypeptides reveal the presence of 7 different genes related to human sialidases. While *neu1* and *neu4* are the putative orthologs of the mammalian sialidases *NEU1* and *NEU4*, respectively, the remaining genes are organized in a clusters located on chromosome 21 and they are all more closely related to mammalian sialidase *NEU3*. They were thus named *neu3.1*, *neu3.2*, *neu3.3*, *neu3.4* and *neu3.5*. By RT-PCR we detect transcripts for all genes but *neu3.4* and whole mount *in situ* hybridization experiments show a localized expression pattern in gut and lens for *neu3.1* and *neu4*, respectively. Transfection experiments in COS7 cells demonstrate that Neu3.1, Neu3.2, Neu3.3 and Neu4 polypeptides are sialidase enzymes. Neu3.1, Neu3.3, and Neu4 are membrane-associated and show a very acidic pH optimum, below 3.0, whereas Neu3.2 is a soluble sialidase with a pH optimum of 5.6. These data are further confirmed by subcellular localization studies carried out by immunofluorescence. Moreover, expression of these novel zebrafish sialidases but Neu3.2 induces a significant modification of the ganglioside pattern of living COS7 cells, consistent with the results obtained in the case of membrane-associated mammalian enzymes. Overall, the redundancy of sialidases together with their expression profile and their activity exerted on gangliosides of living cells indicate the biological relevance of this class of enzymes in zebrafish.

INTRODUCTION

Sialidases or neuraminidases (EC 3.2.1.18) comprise a family of glycohydrolytic enzymes that remove terminal sialic acid residues from various sialo derivatives, such as glycoproteins, glycolipids (gangliosides) and oligosaccharides. These exoglycosidases are widely distributed in nature, including viruses, protozoa, bacteria, fungi, mycoplasma, other microorganisms and vertebrates [1]. Starting from 1993, eleven mammalian sialidases from different species have been cloned [2]. Despite the significant degree of homology and the presence of highly conserved regions along the primary structure, mammalian sialidases cloned so far differ for their subcellular localization and substrate specificities. The sialidase protein family can be divided into four main groups: the lysosomal sialidase NEU1, the soluble or cytosolic sialidase NEU2, the plasma membrane-associated sialidase NEU3 and the intracellular membrane-associated sialidase NEU4. The human lysosomal sialidase NEU1 is part of a multienzyme complex containing β -galactosidase and protective protein cathepsin A and is implicated in the severe lysosomal storage disorders sialidosis and galactosialidosis [3]. The role of the cytosolic sialidase NEU2 is rather puzzling because the content of natural substrates in this subcellular compartment is expected to be very low. Nevertheless, the enzyme plays a pivotal role in the *in vitro* differentiation of rat L6 myogenic cells into myotubes [4] and its stable expression in A431 human carcinoma cell line leads to a diminished GM3 ganglioside level and enhances EGF receptor tyrosine autophosphorylation [5]. The plasma membrane-associated sialidase NEU3 is the most extensively studied member of the family and is characterized by a high degree of specificity toward ganglioside substrates [2]. The over-expression of NEU3 dramatically modifies the cell ganglioside content and is able to affect also gangliosides exposed on the extracellular leaflet of the plasma membrane of adjacent cells by a cell-to-cell interaction [6]. The enzyme has been recently shown to be associated with caveolin in lipid rafts [7] and plays a pivotal role in different cellular processes including neuronal differentiation [8] and tumorigenic transformation [9]. NEU4 has been originally described as a particulate enzyme associated to the inner cell membranes [10, 11]. More recently, a human isoform with 12 additional amino acid residues at the N-terminus has been identified and shown to be associated to the mitochondrial membranes [12]. Intriguingly, NEU4 has been demonstrated to localize in the lysosomal lumen and its over-expression in fibroblasts derived from sialidosis and

galactosialidosis patients results in clearance of storage materials from lysosomes, suggesting a role of NEU4 in lysosomal function [13].

To gain further insights into the biological roles of the different sialidases in vertebrates, we decided to utilize zebrafish (*Danio rerio*) as animal model. This study for the first time describes the systematic identification and characterization zebrafish *neu* genes and provides interesting clues on the possible function of these novel sialidases in zebrafish.

MATERIALS AND METHODS

In general, standard molecular biology techniques were carried out as described by Sambrook et al [14]. DNA restriction and modifying enzymes were from Roche. Powder and reagents were from Sigma unless otherwise indicated. Cell culture media, antibiotics and animal serum were from Gibco. High performance silica gel-precoated thin-layer plates (HPTLC Kieselgel 60, 20 X 10 cm) were purchased from Merck GmbH. Sphingosine was prepared from cerebroside and [1-³H] sphingosine (radiochemical purity > 98%, specific radioactivity 2.08 Ci/mmol) was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro[³H]hydride as previously described [6]. Radioactive sphingolipids were extracted from cells fed with [1-³H]sphingosine, purified and used as chromatographic standards.

Bioinformatic analysis

Nucleotide sequence assembly and editing was performed using both the AutoAssembler version 2.1 (Perkin Elmer-Applied Biosystem) and DNA Strider 1.4 [15] software programs. Zebrafish genomic sequences were analyzed using the University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu/>) [16] on the Zv6 (March 2006) zebrafish (*Danio rerio*) assembly. The zebafish sialidase gene sequences have been also validated on the Zv7 (July 2007) genome assembly. Fugu (*Takifugu rubripes*) sialidase genes have been identified by BLAST analysis on the v4.0 release of the Japanese pufferfish genome sequence [17].

Multiple sequence alignment was performed using the ClustalW algorithm [18]. Phylogenetic analysis was performed using the Phylogeny.fr web service. Briefly, the multiple sequence alignment was generated using Muscle (version 3.6) [19], alignment refinement was obtained using Gblocks [20] and phylogenetic reconstruction was performed using the maximum

likelihood program PhyML[21]. Nucleotide and amino acid sequences were compared to the non-redundant sequence databases present at the NCBI (National Center for Biotechnology Information) using the BLAST algorithm [22]. The prediction of transmembrane domains was performed using the Kyte-Doolittle algorithm implemented in DNA Strider. Primary structure analysis and post-translational modification prediction was performed using the ExPASy Proteomics tools listed at <http://au.expasy.org/tools/>. For N-Glycosylation sites prediction the NetNGlyc server was used, at <http://www.cbs.dtu.dk/services/NetNGlyc/>; phosphorylation sites were predicted using the NetPhos 2.0 server at <http://www.cbs.dtu.dk/services/NetPhos/>. Finally, potential O- β -GlcNAc attachment sites prediction was performed using the YinOYang 1.2 Prediction Server at <http://www.cbs.dtu.dk/services/YinOYang/>.

Isolation of zebrafish sialidase cDNAs

IMAGE cDNA clones have been obtained from Geneservice Ltd, UK. We have determined the full-insert sequence of clones 7067630, 7036960, 7257445 and 7275043. Automated sequencing (using an Applied Biosystem ABI 3100 fluorescent sequencer) was performed using both vector and gene specific oligonucleotide primers.

Reverse transcription RT-PCR analyses

Total RNA was isolated from zebrafish embryos at different developmental stages using the Trizol protocol (Gibco). cDNA was prepared from 1 μ g of total RNA, using Access RT-PCR System kit (Promega) in the presence of random examers oligonucleotides, following the manufacturer's instructions. PCR was carried out using the following forward and reverse primers: neu3.1-1F (5'-GTTCCTTGCCCTTGCTGAAG-3') and neu3.1-1R (5'-CTGTTCCCTCAGCCATTGGAT-3'); NEU3.2-5'HindIII (5'-CCCAAGCTTGGACCATGGAAAAACAACCTTGGAAGCA-3') and zfB-R3 (5'-AAAGGCGTGCCTGATTCTTA-3'); neu3.3-1F (5'-GGGTGGATGCCAAGACTAAA-3') and neu3.3-1R (5'-GGTGTGAAGCAGCAGAATGA-3'); zfD-F3 (5'-CATCAAGCCATTCAGCTCCTCC-3') and zfD-R5 (5'-CATAGACCGGACATGGATTCATGGAT-3'); neu3.5-1F (5'-CAGCTCCTCCCGAAATATCA-3') and neu3.5-1R (5'-CCAGGTTTCACCAGTGCCT-3'); neu4-2F (5'-TGACGCGTCTTTGTTACGTT-3') and neu4-2R (5'-CGAGGTCTTGACACGTCTTG-3').

For zebrafish β -actin amplification (GenBank Accession Number AF057040) the following specific primers were used: (5'-TGTTTTCCCCTCCATTGTTGG-3' and 5'-TTCTCCTTGATGTCACGGAC-3'). Amplification was performed for 35 cycles (57°C annealing) following the instructions for AmpliTaq Gold (Applied Biosystems).

Fish breeding and embryo collection

Adults zebrafish were bred by natural crosses. Immediately after spawning, the fertilized eggs were harvested, washed, and placed in 100 mm-diameter Petri dishes (corning life sciences) in fish water [23]. The developing embryos were incubated at 28.5° C until use. Zebrafish embryos were fixed in 4 % paraformaldehyde (PFA)/PBS over-night at 4° C, rinsed twice in PBS + 1% Tween-20 (PBT), then dehydrated in methanol and stored at -20° C until processing. Developmental stages of zebrafish embryos were expressed as hours- or days post-fertilization (hpf or dpf, respectively) at 28.5° C.

Whole-Mount in Situ Hybridization

Single hybridizations and detections were carried out on wild-type embryos [24]. Anti-sense and sense RNA probes were prepared by *in vitro* transcribing linearized cDNA clones or PCR products with T7, T3 or SP6 polymerase as indicated, using digoxigenin labeling mix (Roche). In particular, a 1376 bp *neu3.1* antisense riboprobe was synthesized with T7 polymerase by transcribing the EcoRI-linearized IMAGE cDNA clone 7036960. The corresponding sense riboprobe was synthesized with SP6 RNA polymerase using the XhoI-cleaved IMAGE clone 7036960. To prepare *neu4* RNA probes, a 833 bp fragment was amplified by PCR using the IMAGE clone 7275043 as template and the following primers carrying the appropriate T3 and T7 consensus promoter sequences. T3-*neu4/ex3F* (5'-CAGAGATGCAATTAACCCTCACTAAAGGGAGAAGTGGGCCACTTTTGCTGTGG-3') and T7-*neu4/ex3R* (5'-CCAAGCTTCTAATACGACTCACTATAGGGAGAGCAGATAAAGCAGCGCTCCCAG-3'). *neu4* antisense and sense probes were then synthesized by transcribing the PCR products with T7 and T3 polymerases, respectively. Stained embryos were mounted in glycerol, observed on a Leica DMR compound microscope equipped with Nomarski optics, and acquired with a Leica DC500 digital camera.

Expression constructs

The coding regions of *neu3.1*, *neu3.2*, *neu3.3* and *neu4* were amplified by PCR using cloned *Pfu Turbo* polymerase (Stratagene) and the corresponding IMAGE cDNA clone as template (clone 7067630, 7036960, 7257445 and 7275043 respectively). Since no EST clones corresponding to the *neu3.4* and *neu3.5* transcripts were available, the two corresponding genes were amplified from *Danio rerio* genomic DNA as template. The PCR products were then digested with the appropriate restriction enzymes and cloned into the pcDNA3.1/myc-His (Invitrogen) (*neu3.1*, *neu3.2* and *neu3.3*) or pMT21 vector [25] (*neu4*) to generate an open reading frame (ORF) encoding *neu3.1*, *neu3.2*, *neu3.3* and *neu4* with a COOH-terminal Myc-tag.

The following forward and reverse oligonucleotides were used in PCR amplification of the gene ORFs:

neu3.1: NEU3.1-5'BamHI (5'-CGGGATCCACCATGTTTTTTACTTACGTTTATG-3') and NEU3.1-3'XhoI (5'-CCCTCGAGAGAGACAATAAATCATTAAAGT-3'). *neu3.2*: NEU3.2-5'HindIII (5'-CCCAAGCTTGGACCATGGAAAAACAACCTTGGGAAGCA-3') and NEU3.23'-XhoI (5'-CCCTCGAGAATAACCTCTTTGTGTTCAAAC-3'). *neu3.3*: NEU3.3-5'BamHI (5'-CGGGATCCACCATGGGCAACAAGACACCGTCAA-3') and NEU3.33'-XhoI (5'-CCCTCGAGCAGCTTTTTCTCAGGGAGTTTG-3'). *neu3.4*: NEU3.4-5'BamHI (5'-CGGGATCCACCATGATGGCGTATTCATCAAGCCA-3') and NEU3.4-3'XhoI (5'-CCCTCGAGCAGCTTTTTCTCAGGGAGTTTG-3'). *neu3.5*: NEU3.5-5'BamHI (5'-CGGGATCCACCATGATGGGTTGCTGTTGCCTG-3') and NEU3.5-3'XhoI (5'-CCCTCGAGATTCTGTTTAGTAAATTTGG-3'). *neu4*: NEU4 5'-EcoRI (5'-CGGAATTCATGGGGTCTCAATACTACCCA-3') and NEU4 3'-SalI (5'-ACGCGTCGACGGCAGATAAAGCAGCGCTCCCAGAT-3'). Amplifications were carried out according to cycling parameters indicated for *Pfu turbo* DNA polymerase for 35 cycles. Annealing steps were performed at 53°C (*neu3.1*), 52°C (*neu3.2* and *neu3.3*) and 56°C (*neu3.4* and *neu3.5*). Each clone sequence was confirmed by automated fluorescent sequencing.

Cell culture and transient transfections

African green monkey kidney cells COS7 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37° in a 5% CO₂ humidified incubator. For transfection COS7 cells were seeded on 100 mm culture dishes

(Corning Life Sciences) or on sterile glass coverslips (BDH) in six-wells plates (Corning Life Sciences) and grown for 24 h before transfection. Transfections were performed using 2–6 μg of plasmid DNA and FuGENE 6 transfection reagent (Roche), according to the manufacturer's guidelines.

For *neu1* transfection experiments, we directly used the IMAGE cDNA clone 7218457 carrying the complete ORF in the pME18S-FL3 expression vector.

For Neu3.2 colocalization experiments, *HsNEU2*-pIRESneo (Clontech) was co-transfected together with Neu3.2-Myc construct. Transfections with the same amount of pcDNA3.1/myc-His or pMT21 expression vector were used as negative controls (mock). Cells were either collected for protein analysis or enzymatic activity assay or fixed for immunofluorescence at different times after transfection.

Immunofluorescence and Confocal Analysis

Transfected cells grown on coverslips were rinsed with ice-cold PBS, immediately fixed and permeabilized with in pre-cooled 100% methanol at -20°C for 10 min and washed in PBS. Cells were subsequently immunolabeled using an indirect procedure in which all incubations (primary, secondary antibodies and washes) were performed in PBS containing 1% BSA. Primary antibodies used were: rabbit anti-human NEU2 [26], rabbit anti-Myc (Sigma), mouse anti-Myc (Sigma), mouse anti-LAMP1 (BD Biosciences), rabbit anti-calnexin (Stressgen), mouse anti-PDI (Stressgen), mouse anti-cytochrome c (Promega). Staining was obtained after incubation with Alexa Fluor 488 and/or 555-conjugated isotype-specific antibodies (Molecular Probes). Controls included staining with each secondary antibody separately. Coverslips were then washed in PBS, mounted on a glass microscopic slide (BDH) with Fluorescent Mounting Medium (Dako) and examined using a Zeiss Axiovert 200 microscope equipped with Confocal Laser System LSM 510 META. Image processing was performed with Adobe Photoshop software.

Protein extraction

Transfected COS7 cells were harvested by scraping, washed in PBS and resuspended in the same buffer containing 60 $\mu\text{g}/\text{ml}$ chymotrypsin, 8 $\mu\text{g}/\text{ml}$ pepstatin A, 32 $\mu\text{g}/\text{ml}$ aprotinin and 32 $\mu\text{g}/\text{ml}$ leupeptin. Total cell extracts were prepared by sonication. The supernatant obtained after a centrifugation at 800 g for 10 min represented the crude cell extract and was subsequently centrifuged at 100,000 g for 60 min on an Optima T 80 ultracentrifuge

(Beckman). Aliquots of the original crude cell extract, 100,000 g supernatant and pellet, were used for protein assay (BioRad Protein Assay), Western blot analysis and enzymatic activity. All the fractionation passages were carried out at 4°C.

Western blot analysis

Protein samples were subjected to SDS-PAGE (10% (w/v) polyacrylamide gel) and subsequently transferred by electroblotting onto Immobilon-P blotting membrane (Amersham Biosciences). The membranes were incubated for 30 min in PBS containing 0.05% (v/v) Tween 20 (PBST) and 5% (w/v) dried milk (blocking buffer) and subsequently incubated with rabbit anti-Myc antibody (Zymed). After final washing in PBST and incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulins (Amersham Biosciences), proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate detection kit (Pierce).

Sialidase enzymatic assays

The enzymatic activity of the sialidase in total cell lysates and in cellular subfractions was determined using 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4-MU-NeuAc) as substrate. Assays were performed with up to 30 μ g of total proteins for Neu3.1, Neu3.2 and Neu3.3, and up to 5 μ g of total proteins in the case of Neu4. Briefly, reactions were set up in triplicate in a final volume of 100 μ l with 0.2 mM 4-MU-NeuAc, 600 μ g bovine serum albumin in presence of 20 mM Tris/glycine buffer for assays ranging from pH 2.2 to 3.0 and 12.5 mM sodium citrate/phosphate buffer for assays ranging from pH 2.6 to 7. Reaction mixtures were incubated at 37°C for 30 min and stopped by addition of 1.5 ml of 0.2 M Glycine/NaOH pH 10.4. Fluorescence emission was measured on a Jasco FP-770 fluorometer with excitation at 365 nm and emission at 445 nm, using 4-methylumbelliferone to set up a calibration curve [27].

Treatment of cell cultures with [1-³H] sphingosine

A proper amount of [1-³H] sphingosine, dissolved in methanol, was transferred into a sterile glass tube, and dried under a nitrogen stream. The resulting residue was then dissolved in an appropriate volume of pre-warmed (37°C) 10% FBS/DMEM medium to obtain a final concentration of 3×10^{-8} M (corresponding to 0.4 μ Ci/100 mm dish). Transfected COS7 cells were incubated for a 2 h pulse followed by a 40 h chase, except for Neu4 transfected COS7

cells that were followed by 20 h chase. Cells were harvested by scraping, washed in PBS, snap frozen, lyophilized and subjected to lipid extraction and sphingolipid analyses.

Lipid extraction and analyses

Total cell extracts obtained after [^3H]sphingosine labeling were lyophilized and extracted twice with chloroform/methanol 2:1 (v/v). The resulting lipid extracts were dried under nitrogen stream and dissolved in chloroform/methanol 2:1 (v/v). The total lipid extracts were counted for radioactivity and subjected to a two-phase partitioning in chloroform-methanol-water, 2:1 (v/v), and 20% water; the aqueous and organic phases obtained were counted for radioactivity. [^3H]sphingolipids of aqueous and organic phases were analyzed by HPTLC using the solvent systems chloroform/methanol/0.2% aqueous CaCl_2 , 60:40:9 (v/v), and chloroform/methanol/water, 110:40:6 (v/v), respectively. [^3H]sphingolipids were identified referring to radiolabeled standards and quantified by radiochromatography (Beta-Imager 2000, Biospace) [6].

RESULTS

Identification and organization of sialidase genes in zebrafish

To isolate genes encoding for sialidases in zebrafish we carried out a genome-wide search using the sequences of the human NEU1, NEU2, NEU3 and NEU4 polypeptides as a query. Our study was performed using both the TBLASTN and BLAT algorithms vs. the March 2006 *Danio rerio* Zv6 assembly of genomic sequences. This analysis led to the identification of 7 putative sialidase genes whose general features are summarized in Supplementary Table S1. One gene is located on chromosome 19 (Fig. 1A), and several EST sequences have been identified in dbEST corresponding to the gene transcript. We characterized IMAGE clone 7218457 that contains the entire ORF. The putative encoded protein shows a high degree of amino acid sequence identity (58%) to human NEU1 (Supplementary Table S2). Moreover, the gene is organized in six exons as the human counterpart and also the intron positions are conserved in the two species (Fig. 2). Based on these evidences, this novel zebrafish gene has been named *neu1*.

Interestingly, five sialidase-related sequences are clustered within 33 kb on chromosome 21 (Fig. 1B). Only for three of them we identified cDNA clones in GenBank either as ESTs or full-insert cDNA sequences. DNA sequence analysis of IMAGE clones 7067630, 7036960,

7257445 indicates that they contain the entire protein coding region. The predicted polypeptides encoded by the three genes show the highest sequence identity with human NEU3 (Supplementary Table S2). We thus adopted a numerical nomenclature (*neu3.1*, *neu3.2* and *neu3.3*) for these three novel zebrafish genes, based on their mapping location within the cluster (Fig. 1B). In the case of the additional two genes found in the same cluster, since no cDNA clones were available in GenBank, their putative coding sequences have been deduced *in silico* by i) comparing the genomic sequences harboring the two genes with human sialidase polypeptides and by ii) the analysis of gene predictions available within the UCSC Genome Browser. The genomic stretches of the two genes from the putative ATG initiation codon to the stop codon, including the single intron present in both genes, were amplified by PCR from zebrafish genomic DNA and subcloned into pcDNA3.1 Myc-His expression vector. The resulting recombinant vectors were subsequently used to transfect COS7 cells, and RT-PCR carried out on the RNA extracted from these cells confirmed the *in silico* predicted splicing sites. Also in this case the encoded polypeptides show the highest sequence identity to human NEU3 (Supplementary Table S2); the two corresponding genes were thus named *neu3.4* and *neu3.5*. The entire coding region of *neu3.5* has been then successfully amplified by RT-PCR performed on adult zebrafish RNA (data not shown). All NEU3-like genes clustered on chromosomes 21 are organized in two exons like human NEU2 and NEU3 and, in addition, the intron positions are also conserved in the two species (Fig. 2). A seventh sialidase family member was also identified. While in the Zv6 sequence assembly its chromosomal location remains unassigned, the analysis of the Zv7 assembly allowed to locate it on chromosome 15 (Fig. 1C). The nucleotide sequence analysis of the IMAGE cDNA clone 7275043 allowed us to derive its entire ORF. The encoded polypeptide shows the highest degree of amino acid sequence identity (48%) to human NEU4 (Supplementary Table S2). In addition, the putative gene coding region is the only one among zebrafish sialidases organized in three exons like human NEU4 (Fig. 2). The gene was thus named *neu4*. Supplementary Table S3 reports the values of amino acid sequence identity among the seven zebrafish sialidases we identified.

To gain further information about sialidases in ray-finned fish, we have extended the bioinformatic search to *Takifugu rubripes* (fugu), a distantly related teleost to zebrafish, whose genome has been completely sequenced [17]. This analysis led to the identification of only three sialidases that, by reciprocal BLAST comparisons with the mammalian enzymes, resulted to be the putative orthologs of human NEU1, NEU3 and NEU4. These novel fugu genes have been accordingly named *neu1*, *neu3* and *neu4*. Interestingly, only these three

sialidase genes have been found in the genomic sequence of another pufferfish, *Tetraodon nigroviridis* (data not shown).

We analyzed the genomic regions surrounding the sialidase genes both in teleosts and in man. A partial synteny can be observed in the region harboring *NEU1*. In both zebrafish and fugu the gene is flanked by *bat2* on one side and *skiv2l* and *rdp* genes on the other. In the human genome, *BAT2* is located on the distal side some 230 kb from *NEU1*, while *RDBP* and *SKIV2L* are on the centromeric side at approximately 90 kb (Fig. 1A). Several genes are present between *NEU1* and the above mentioned genes that cannot be found in the teleost genomes adjacent to *neu1*. This is not surprising considering that *NEU1* lies within the Major Histocompatibility Complex (MHC), a gene-dense region on chromosome 6p21.3 that comprises a group of genes that are functionally involved in the adaptive and innate immune system [28].

The zebrafish chromosome 21 sialidase cluster is flanked on the *neu3.1* side by genes homologous to human *XRR1*, *CHRD2* and *POLD3*. The same genes are found adjacent to the fugu *neu3* sequence. Interestingly, the human *NEU3* gene on chromosome 11q13.4 is similarly flanked on the centromeric side by *XRR1*, *CHRD2* and *POLD3* genes. There is no evidence of synteny conservation between human and zebrafish on the other side of the cluster (Fig. 1B).

Finally, *neu4* is flanked on one side, both in zebrafish and fugu, by sequences homologous to *SRPRB* and *WD53* human genes mapping to chromosome 3q in man. The presence of sequence gaps in the other side of the *neu4* locus did not allow to study synteny in this region. Although both the exon-intron organization and amino acid sequence analysis suggest that *neu4* is closely related to human *NEU4*, we could not detect synteny with human chromosome 2q37 where *NEU4* and *NEU2* both lie (Fig. 1C).

Zebrafish sialidase proteins analysis

A multiple alignment of the amino acid sequences of the human and zebrafish sialidases further points out the high degree of amino acid sequence identity between zebrafish and human sialidases, with a large number of residue blocks highly conserved in topologically equivalent positions along the primary structure (Fig. 3). As expected, the Y(F)RIP motif, typical of sialidases [29] and occurring near the N-terminus of these enzymes, is also present in all the sialidases from *Danio rerio*. In addition, zebrafish Neu polypeptides contain two well conserved Asp blocks (consensus sequence **SxDxGxTW**) [30] and, apart from *NEU1* and its putative zebrafish ortholog *Neu1*, they also present one less conserved Asp box

characterized by the $S_x(D/N/S)_xG_x(D)F$ extended consensus sequence. Noteworthy, a remarkable conservation of the amino acids that are involved in the formation of the active site of the human cytosolic sialidase NEU2 [31] is observed in zebrafish sialidases (Fig. 3), suggesting a high conservation of the active site architecture during the vertebrate evolution. Interestingly, Neu1 of zebrafish origin shows a shorter N-terminus compared to the human enzyme in which the first 45 residues represent the signal peptide [32]. Moreover, the $Yxx\Phi$ motif (where Φ stands for non-polar, aliphatic or aromatic residues) present at the C-terminus of mammalian NEU1 [32] as well as in several lysosome-associated membrane proteins [33], is poorly conserved in the zebrafish ortholog, where the carboxy terminal residue is a positively charged K.

Concerning zebrafish Neu4, it lacks the approximately 80 amino acid stretch located in the last third of the protein that appears to be exclusively present in NEU4 sialidases of mammalian origin [11].

A phylogenetic tree of human, mouse, chicken, fugu and zebrafish sialidases is shown in Fig. 4. This analysis further indicates that *neu1* represents the zebrafish ortholog of the human *NEU1* gene, while *neu4* is more similar to *NEU4*. On the contrary, Neu3.1 - Neu3.5 proteins are highly similar among them and they all have the mammalian plasma membrane-associated sialidase NEU3 as the closest relative.

The general molecular features of zebrafish Neu polypeptides are reported in Table 1. The differences in molecular weight are modest, from 42 kDa in case of Neu3.2 to 48.6 kDa in case of Neu4. The isoelectric points have values below 7, with the exception of Neu3.2. As already reported in the case of the mammalian enzymes [2], all zebrafish sialidases but Neu3.4 show predicted N-glycosylation sites, and all but Neu4 show a number of predicted O- β -GlcNAc attachment sites for glycosylation. Moreover, all the polypeptides have a great number of amino acid residues that could be covalently modified by phosphorylation, suggesting that such a kind of modification could be of relevance in the regulation of their biological behaviors. The Kyte-Doolittle hydrophobicity plots of the zebrafish Neu proteins (Supplementary Fig. S1) do not show typical transmembrane domains as already observed for the mammalian counterparts [2].

Zebrafish sialidases expression analysis

To find out at which stage of zebrafish development sialidases are transcribed, we analyzed their temporal expression by RT-PCR at 2, 4, 8, 12, 18 and 24 hours post-fertilization (hpf)

and 2, 3, 4, 5 days post-fertilization (dpf), as well as in the adult fish. As shown in Fig. 5, *neu3.1* is detected from early hours of development (2 hpf), *neu3.3* and *neu4* mRNAs are detected starting from 12 and 8 hpf, respectively, while *neu3.2* and *neu3.5* transcripts are detected from 24 hpf. All genes mentioned above are expressed in the later stages of development we tested, as well as in the adult fish (data not shown). No RT-PCR amplification is observed for *neu3.4* at any stage of development tested, even using different sets of oligonucleotide primers (data not shown). In agreement with our observations, the absence in the public databases of EST sequences corresponding to *neu3.4* transcripts further suggests that this gene is either expressed at very low level or not expressed at all. To detect mRNA in specific embryonic tissues we performed whole-mount *in situ* hybridizations. The high level of identity among *neu3.2*, *neu3.3*, *neu3.4* and *neu3.5* nucleotide sequences (data not shown) did not allow the synthesis of antisense riboprobes specific for all these gene transcripts. Thus, the whole mount *in situ* hybridization studies were carried out only for *neu3.1* and *neu4*, for whom we could generate riboprobes showing approx. 70% sequence identity to the other gene transcripts. The two sialidases genes studied showed spatially localized expression in different tissues. In particular, *neu3.1* transcripts were detected in the gut in embryos at 24 hpf until 6 dpf (Fig. 6 A and B). Expression of *neu4* is detected in the lens of 21 hpf, 24 hpf, 2 and 3 dpf embryos (Fig. 6 C-F). Hybridization of sense strand cRNA was performed as control and did not give hybridization signals above background (Supplementary Fig. S2).

Expression of zebrafish Neu1, Neu3.1, Neu3.2, Neu3.3 and Neu4 sialidases in COS7 cells

Biochemical characterization was carried out only for proteins encoded by *neu* genes for whom a full-coding IMAGE cDNA clone was available, thus excluding Neu3.4 and Neu3.5 from this analysis.

Zebrafish *Neu1* was expressed in COS7 cells and sialidase activity was tested in a range of pH from 2.2 to 7.0 (data not shown). Only a negligible increase of the enzymatic activity was detected at pH 2.8 compared to mock transfected cells (Fig. S3). This is not surprising considering that Neu1 likely represents the zebrafish ortholog of the human lysosomal sialidase NEU1. Interestingly, the zebrafish genome contains the putative orthologs of human cathepsin A (GenBank accession number XM_001331869, chromosome 7) and β -galactosidase (GenBank accession number NM_001017547, chromosome 1), suggesting that also in this teleost Neu1 forms the catalytically active supramolecular complex described in mammals [2]. Since these peculiar features set NEU1 apart from the other sialidases

identified in mammals, we decided to focus our studies on the other members of the gene family.

The complete ORFs of *neu3.1*, *neu3.2*, *neu3.3* and *neu4* genes were amplified by PCR using the corresponding IMAGE cDNA clones as template (IMAGE clones 7036960, 7257445 and 7275043, respectively). The PCR products were subcloned in pcDNA3.1/Myc-His and pMT21 mammalian expression vectors. The resulting recombinant vectors, encoding for chimeric proteins bearing at the C-terminus the Myc/His epitopes in case of Neu3.1, Neu3.2 and Neu3.3 or the Myc epitope alone in case of Neu4, were used to transiently transfect COS7 cells. Crude homogenates from transfected cells were tested for sialidase activity using the artificial substrate 4-MU-NeuAc. Transient transfection experiments with the different zebrafish cDNAs lead to significant increase of the sialidase activity. Neu4 expression leads to the highest rate of hydrolysis whereas, in the case of Neu3.1, Neu3.2 and Neu3.3 expressing cells, the sialidase activity detected is lower (Fig. S3). The measurement of the sialidase activity at different pH values, from 2.2 to 7.0, reveals that the enzymes have peculiar pH optima. Neu3.1, Neu3.3 and Neu4 show a very acidic optimal pH that is 2.6, 2.8 and 2.6, respectively (Fig. 7A, C and D), whereas Neu3.2 shows a less acidic value, corresponding to pH 5.6 (Fig. 7B).

To analyze the subcellular localization of the different zebrafish sialidase enzymes, a fractionation into soluble and particulate cell materials was carried out by ultracentrifugation of the crude homogenate of COS7 cells transfected with the various recombinant vectors. As shown in Fig. 7C and D, more than 80% of the Neu3.3 and Neu4 enzymatic activity detectable in the crude homogenates is recovered in the rough particulate fraction, demonstrating the association of these enzymes with membranous material. In the case of Neu3.1, the recovery in the particulate fraction is lower (46%) but the association to the rough particulate fraction still evident (Fig. 7A). Conversely, Neu3.2 clearly appears to be a soluble enzyme, its activity being recruited in the soluble cell compartments after ultracentrifugation of the total cell lysate (Fig. 7B). These results have been further confirmed by Western-blot analysis using an anti-Myc monoclonal antibody. A 47.5 kDa and a 49.8 kDa bands, corresponding to Neu3.3- and Neu4-tagged proteins, respectively, are associated to the 100,000 *g* rough particulate fraction. In contrast, in the case of Neu3.2 expressing cells, the Western-blot analysis shows a band of 44.5 kDa, corresponding to the tagged Neu3.2, that is highly enriched in the soluble fraction (Fig. 7B). Finally, in the case of Neu3.1, the Western-blot analysis reveals in the total cell extract a major band with the expected molecular weight of 48.6 kDa, together with two additional minor protein bands of

46 and 44 KDa. Surprisingly, after ultracentrifugation the same bands are detectable in both the soluble and rough particulate membrane fractions, with an enrichment of the higher molecular weight protein band in the supernatant compared to the pelleted material, as would be expected from the enzyme activity distribution. A possible explanation of this result could be that the protein, detached from the membranes, is somehow less active and/or lacks some co-factor essential for catalysis, as suggested from the low recovery of the enzyme activity after cell fractionation.

Zebrafish Neu activity in living COS7 cells

To assess the possible sialidase activity of Neu3.1, Neu3.2, Neu3.3 and Neu4, toward gangliosides, COS7 cells were pulsed with [$1\text{-}^3\text{H}$]sphingosine and transiently transfected with the recombinant plasmids carrying the zebrafish cDNAs. The incorporation of the radiolabeled precursor leads to an extensive labeling of the cell sphingolipids [34] and the comparison of the lipid pattern detectable in mock and transfected cells are related to the effects that the enzyme expression exerts on sphingolipid compartment of intact cells. After 2 h pulse with tritiated sphingosine followed by 20 to 40 h chase, free sphingosine was hardly detectable within the radioactive lipid mixtures and stable radioactive lipid patterns were observed in mock transfected cells (not shown). In the case of COS7 cells expressing Neu3.1, Neu3.2 and Neu4, the sphingolipid patterns show significant variations (Fig. 8), whereas no differences are detectable in Neu3.3 transfected cells (data not shown). In particular, Neu3.1 and Neu3.3 expression for 40 h induces roughly a 37% and 46% decrease of the ganglioside GM3 and a 29% and 27% increase of ganglioside GM1 cell content, respectively (Fig. 8A, B). In case of Neu4 expressing cells, the effects detectable on ganglioside pattern are more pronounced (Fig. 8C). In fact, after only 24 h of expression, roughly a 23% and 21% decrease of ganglioside GM3 and GD1a relative content, respectively, and a 26% increase of ganglioside GM1 content are detectable. In addition, in Neu4 expressing cells a 11% increase of lactosylceramide relative content is detectable (data not shown). No other statistically significant differences are observed between lipid pattern of the organic phase from mock, *neu3.1*, *neu3.2*, *neu3.3* and *neu4* transfected cells (not shown).

Subcellular localization of Neu3.1, Neu3.2, Neu3.3 and Neu4

The subcellular distribution of Neu3.1, Neu3.2, Neu3.3 and Neu4 zebrafish enzymes has been studied by indirect immunofluorescence. Laser confocal analysis further confirms the results obtained with cellular fractionation and Western-blot techniques. Neu3.1 shows a

diffused intracellular labeling pattern and localizes to the endoplasmic reticulum, as shown by its restricted distribution to calnexin- (Fig. 9A) and PDI-positive structures (Supplementary Fig. S4). Neu3.2 is detected as a cytosolic protein (Fig. 9B) with a subcellular distribution roughly superimposable to the one observed in the case of cells expressing the human cytosolic sialidase NEU2 [26]. Neu3.3 shows a typical membranous distribution, with an extensive labeling at the cell surface and in a well-defined membrane network inside the cells (Fig. 9C). Double labeling experiments using endoplasmic reticulum (calnexin and PDI), mitochondrial (cytochrome c) and Golgi apparatus (GM130) markers do not reveal any significant colocalization (data not shown), whereas a partial colocalization is detectable in case of LAMP1-positive vesicles, corresponding to the late endosomes-lysosomes compartment (Fig. 9C). Finally, Neu4 labeling resembles the one observed in case of Neu3.1, with a consistent, although less complete, co-localization with calnexin-positive endocellular compartment (Fig. 9D).

DISCUSSION

In this paper we describe the sialidase gene family in zebrafish. In mammals, four genes are known to encode sialidase enzymes with distinct subcellular localization and physiological roles [2]. In zebrafish the picture is more complex, with 7 genes homologous to human sialidases. We have carried out several bioinformatic analyses to unravel the phylogenetic origin of the various members of the sialidase gene family in zebrafish. Our results indicate that a single ortholog exists for the mammalian *NEU1* gene, corresponding to *neu1*, as also supported by the presence of partial synteny between the genomic sequences containing the orthologous genes. Similarly *neu4* appears to be highly related to human *NEU4*, although no synteny can be detected between zebrafish and mammals. The remaining genes (*neu3.1-5*) are arranged in a cluster on chromosome 21 and, although with different degrees of sequence identities, they all are related to human *NEU3* gene. This finding is further supported by the conserved synteny detectable between the chromosome 21 cluster in *Danio rerio* and chromosome 11q13.4 region harboring *NEU3* in *Homo sapiens*.

To shed light if the redundancy of *NEU3*-like genes is common to other teleosts, we extended our analysis to *Takifugu rubripes* (fugu) and *Tetraodon nigroviridis*. Interestingly, only one *NEU3*-like gene is present in both species, suggesting that the redundancy observed in zebrafish might be the result of an independent gene duplication event. Similar findings have

been described for other duplicate genes in zebrafish [35] and other teleosts [36]. In this perspective, the gene showing the highest level of nucleotide sequence identity to fugu *neu3* is zebrafish *neu3.1* suggesting it may represent the ancestor of *neu3.2*, *neu3.3*, *neu3.4* and *neu3.5* paralogs on chromosome 21 cluster, and they all originated by multiple tandem duplication events. It is intriguing to note that in fugu and *Tetraodon nigroviridis* only three sialidase genes exist representing the putative orthologs of *NEU1*, *NEU3* and *NEU4*, while in chicken and mammals a fourth gene, *NEU2*, is also present. In teleosts, a *NEU2* ortholog could not be necessary or, alternatively, *NEU3*- and *NEU4*-like proteins could play the biological role of a cytosolic sialidase.

The expression of *neu3.1*, *neu3.2*, *neu3.3* and *neu4* genes in COS7 cells demonstrates that they encode enzymatically active sialidases. All but Neu3.2 show an extremely acidic pH optimum, with values below 3.0, which are lower than the corresponding values detectable in the higher vertebrate sialidases characterized so far [2, 10-13]. Based on these data, Neu3.1, Neu3.3 and Neu4 behave as higher vertebrate *NEU3* and *NEU4* proteins, all characterized by very low pH optima, whereas Neu3.2, with a pH optimum of 5.6, behaves as the cytosolic sialidase *NEU2*. Rough cell fractionation experiments further confirm this picture, with the group of the extremely acidic sialidases associated to the particulate membranous fraction while Neu3.2 is recovered as a soluble enzyme. Immunofluorescence localizations of the zebrafish tagged enzymes are in agreement with the subcellular fractionation data: Neu3.1 and Neu4 show a significant colocalization with the calnexin-positive cell membranes, whereas Neu3.3, localizes both at the cell surface and inner membranous structures, with only a partial colocalization with LAMP1-positive vesicles. Finally, Neu3.2 shows a distribution largely superimposable with that of the human cytosolic sialidase *NEU2*. To study the possible activity of zebrafish sialidase enzymes toward ganglioside substrates inserted in the lipid bilayer of living cells, we expressed their cDNAs in sphingosine-metabolically labeled COS7 cells and their ganglioside content was analyzed. The expression of all zebrafish Neus studied but Neu3.2 induce a significant decrease of GM3 and an increase of GM1 content in comparison with mock-transfected cells and, in the case of cells expressing Neu4, a significant decrease of GD1a relative content was detectable too. Similar results have been obtained in the case of the mammalian plasma membrane-associated sialidase *NEU3* [6], suggesting that the overall substrate specificity, as well as the action of the enzymes in living cells, is roughly similar despite the evolutionary distance between teleosts and mammals. These biochemical data further support the classification inferred by genomic analysis: Neu3.1 and Neu3.3 belong to the group of the membrane-associated

NEU3-like enzymes, whereas Neu4 belongs to the NEU4-like enzymes. Rather puzzling is the biochemical behavior of Neu3.2 that, despite its high sequence identity to mammalian NEU3 and its localization in the Neu3-like cluster on Chr 21, is a soluble protein with a pH optimum typical of the mammalian NEU2 cytosolic sialidases cloned so far [26, 37, 38]. On the other hand, as already described for mammalian membrane-associated sialidases, the hydrophobicity plot of the membrane-associated Neu3.1, Neu3.2 and Neu4 sialidases do not show any canonical hydrophobic membrane-spanning domain(s), as well as any typical amino acid motifs involved in post-translational mechanisms of anchorage to the lipid bilayer and, overall, they closely resemble Neu3.2. Based on these findings, Neu3.2 is more likely a NEU3 paralog that has lost the capability to interact with the lipid bilayer, thus becoming a soluble enzyme.

Another intriguing aspect is the redundancy of sialidase enzymes in zebrafish compared to mammals. Interestingly, such a phenomenon appears to be confined to NEU3-like proteins only. Besides *neu3.4*, transcripts for NEU3-like genes are detectable, although with different time-frames during embryogenesis, as well as in adult tissues, as demonstrated by RT-PCR experiments. These results support the biological relevance of these enzymes in tissue differentiation and maintenance. *in situ* hybridization experiments indicate that *neu3.1* and *neu4* genes show temporal and spatially localized expression areas, corresponding to gut (*neu3.1*) and lens (*neu4*). The RT-PCR data on *neu4* indicate that the gene transcript is present in developmental stages where no significant expression is detectable by *in situ* hybridization technique. These discrepancies may be due to the limitation of the latter technique in detecting low but ubiquitous expression pattern.

No *in situ* expression data are available for mammalian sialidases, to date. Based on RT-PCR and Northern-blot analysis, apart from NEU2 that appears to be expressed at very low levels mainly in skeletal muscle [39], the other members of the family, namely *NEU1*, *NEU3* and *NEU4* show a quite ubiquitous expression pattern [11-13, 32, 40, 41]. Thus, at least in the case of zebrafish *neu3.1* and *neu4*, the expression patterns appear to be very peculiar and tissue specific. This could be explained based on the notion that duplicated or redundant genes in zebrafish are not redundant in function, but rather subdivide the function of the ancestral gene [42], or evolved new functions particularly during development [43].

Other proteins involved in sialoglyconjugate biology have been already described in zebrafish. Among them, the sialic acid-binding protein siglec-4 shows binding features very similar to the human ortholog [44]. In addition, putative genes encoding sialyltransferases have been also identified in zebrafish [45]. More recently, overexpression of GM3 synthase

in zebrafish embryos resulted in neuronal cell death in the central nervous system [46]. This result strongly supports the great relevance of gangliosides in the biology of fishes. In addition, a glycomic survey map of zebrafish has been published, revealing unique sialylation features as well as variations during embryogenesis [47].

Overall, these studies in zebrafish suggest the presence of a complex regulation pattern for the expression of the enzymes involved in sialoglycoconjugates synthesis and degradation. In this picture, sialidases play a pivotal role in the degradation and fine regulation of these compounds [1].

The study of the different members of the sialidase gene family in this model organism represents a novel and attractive field of glycobiology. In particular, the developmental roles of sialidases can now be tested in the zebrafish using overexpression and Morpholino loss-of-function approaches. We are confident that the study of these enzymes in zebrafish will help to give a comprehensive picture of the biological roles of sialidase in vertebrates.

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REFERENCES

- 1 Saito, M. Y., R.K. (1995) Biochemistry and function of Sialidases. In *Biology of the Sialic Acids* (Rosenberg, A., ed.). pp. 261-313, Plenum Press, New York
- 2 Monti, E., Preti, A., Venerando, B. and Borsani, G. (2002) Recent development in mammalian sialidase molecular biology. *Neurochem Res.* **27**, 649-663
- 3 d'Azzo, A., Andria, G., Strisciuglio, P. and Galjaard, H. (2001) Galactosialidosis. In *The metabolic and molecular bases of inherited disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.). pp. 3811-3826, Mc-Graw-Hill, Inc, New York
- 4 Sato, K. and Miyagi, T. (1996) Involvement of an endogenous sialidase in skeletal muscle cell differentiation. *Biochem Biophys Res Commun.* **221**, 826-830
- 5 Meuillet, E. J., Kroes, R., Yamamoto, H., Warner, T. G., Ferrari, J., Mania-Farnell, B., George, D., Rebbaa, A., Moskal, J. R. and Bremer, E. G. (1999) Sialidase gene transfection enhances epidermal growth factor receptor activity in an epidermoid carcinoma cell line, A431. *Cancer Res.* **59**, 234-240
- 6 Papini, N., Anastasia, L., Tringali, C., Croci, G., Bresciani, R., Yamaguchi, K., Miyagi, T., Preti, A., Prinetti, A., Prioni, S., Sonnino, S., Tettamanti, G., Venerando, B. and Monti, E. (2004) The plasma membrane-associated sialidase MmNEU3 modifies the ganglioside pattern of adjacent cells supporting its involvement in cell-to-cell interactions. *J Biol Chem.* **279**, 16989-16995
- 7 Wang, Y., Yamaguchi, K., Wada, T., Hata, K., Zhao, X., Fujimoto, T. and Miyagi, T. (2002) A close association of the ganglioside-specific sialidase Neu3 with caveolin in membrane microdomains. *J Biol Chem.* **277**, 26252-26259
- 8 Da Silva, J. S., Hasegawa, T., Miyagi, T., Dotti, C. G. and Abad-Rodriguez, J. (2005) Asymmetric membrane ganglioside sialidase activity specifies axonal fate. *Nat Neurosci.* **8**, 606-615
- 9 Kakugawa, Y., Wada, T., Yamaguchi, K., Yamanami, H., Ouchi, K., Sato, I. and Miyagi, T. (2002) Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression. *Proc Natl Acad Sci U S A.* **99**, 10718-10723
- 10 Comelli, E. M., Amado, M., Lustig, S. R. and Paulson, J. C. (2003) Identification and expression of Neu4, a novel murine sialidase. *Gene.* **321**, 155-161
- 11 Monti, E., Bassi, M. T., Bresciani, R., Civini, S., Croci, G. L., Papini, N., Riboni, M., Zanchetti, G., Ballabio, A., Preti, A., Tettamanti, G., Venerando, B. and Borsani, G. (2004)

- Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family. *Genomics*. **83**, 445-453
- 12 Yamaguchi, K., Hata, K., Koseki, K., Shiozaki, K., Akita, H., Wada, T., Moriya, S. and Miyagi, T. (2005) Evidence for mitochondrial localization of a novel human sialidase (NEU4). *Biochem J*. **390**, 85-93
- 13 Seyrantepe, V., Landry, K., Trudel, S., Hassan, J. A., Morales, C. R. and Pshezhetsky, A. V. (2004) Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells. *J Biol Chem*. **279**, 37021-37029
- 14 Sambrook, J. and Russell, D. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 15 Marck, C. (1988) 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic acids research*. **16**, 1829-1836
- 16 Karolchik, D., Baertsch, R., Diekhans, M., Furey, T. S., Hinrichs, A., Lu, Y. T., Roskin, K. M., Schwartz, M., Sugnet, C. W., Thomas, D. J., Weber, R. J., Haussler, D. and Kent, W. J. (2003) The UCSC Genome Browser Database. *Nucleic Acids Res*. **31**, 51-54
- 17 Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J. M., Dehal, P., Christoffels, A., Rash, S., Hoon, S., Smit, A., Gelpke, M. D., Roach, J., Oh, T., Ho, I. Y., Wong, M., Detter, C., Verhoef, F., Predki, P., Tay, A., Lucas, S., Richardson, P., Smith, S. F., Clark, M. S., Edwards, Y. J., Doggett, N., Zharkikh, A., Tavtigian, S. V., Pruss, D., Barnstead, M., Evans, C., Baden, H., Powell, J., Glusman, G., Rowen, L., Hood, L., Tan, Y. H., Elgar, G., Hawkins, T., Venkatesh, B., Rokhsar, D. and Brenner, S. (2002) Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science*. **297**, 1301-1310
- 18 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. **22**, 4673-4680
- 19 Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. **32**, 1792-1797
- 20 Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol*. **17**, 540-552
- 21 Guindon, S. and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*. **52**, 696-704
- 22 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *J Mol Biol*. **215**, 403-410

- 23 Westerfield, M. (2000) The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). . Univ. of Oregon Press, Eugene, OR
- 24 Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993) Structure of the zebrafish *snail1* gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development*. **119**, 1203-1215
- 25 Kolodkin, A. L., Leventgood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J. and Ginty, D. D. (1997) Neuropilin is a semaphorin III receptor. *Cell*. **90**, 753-762
- 26 Monti, E., Preti, A., Nesti, C., Ballabio, A. and Borsani, G. (1999) Expression of a novel human sialidase encoded by the NEU2 gene. *Glycobiology*. **9**, 1313-1321
- 27 Tringali, C., Papini, N., Fusi, P., Croci, G., Borsani, G., Preti, A., Tortora, P., Tettamanti, G., Venerando, B. and Monti, E. (2004) Properties of recombinant human cytosolic sialidase HsNEU2. The enzyme hydrolyzes monomerically dispersed GM1 ganglioside molecules. *J Biol Chem*. **279**, 3169-3179
- 28 Sambrook, J. G., Figueroa, F. and Beck, S. (2005) A genome-wide survey of Major Histocompatibility Complex (MHC) genes and their paralogues in zebrafish. *BMC Genomics*. **6**, 152
- 29 Roggentin, P., Rothe, B., Kaper, J. B., Galen, J., Lawrisuk, L., Vimr, E. R. and Schauer, R. (1989) Conserved sequences in bacterial and viral sialidases. *Glycoconj J*. **6**, 349-353
- 30 Copley, R. R., Russell, R. B. and Ponting, C. P. (2001) Sialidase-like Asp-boxes: sequence-similar structures within different protein folds. *Protein Sci*. **10**, 285-292
- 31 Chavas, L. M., Tringali, C., Fusi, P., Venerando, B., Tettamanti, G., Kato, R., Monti, E. and Wakatsuki, S. (2005) Crystal structure of the human cytosolic sialidase Neu2. Evidence for the dynamic nature of substrate recognition. *J Biol Chem*. **280**, 469-475
- 32 Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G. and d'Azzo, A. (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes Dev*. **10**, 3156-3169
- 33 Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y. and August, J. T. (1993) The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein 1. *J Biol Chem*. **268**, 1941-1946
- 34 Chigorno, V., Riva, C., Valsecchi, M., Nicolini, M., Brocca, P. and Sonnino, S. (1997) Metabolic processing of gangliosides by human fibroblasts in culture-- formation and recycling of separate pools of sphingosine. *Eur J Biochem*. **250**, 661-669.

- 35 Taylor, J. S., Braasch, I., Frickey, T., Meyer, A. and Van de Peer, Y. (2003) Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res.* **13**, 382-390
- 36 Robinson-Rechavi, M., Marchand, O., Escriva, H., Bardet, P. L., Zelus, D., Hughes, S. and Laudet, V. (2001) Euteleost fish genomes are characterized by expansion of gene families. *Genome Res.* **11**, 781-788
- 37 Miyagi, T., Konno, K., Emori, Y., Kawasaki, H., Suzuki, K., Yasui, A. and Tsuik, S. (1993) Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase. *J Biol Chem.* **268**, 26435-26440
- 38 Ferrari, J., Harris, R. and Warner, T. G. (1994) Cloning and expression of a soluble sialidase from Chinese hamster ovary cells: sequence alignment similarities to bacterial sialidases. *Glycobiology.* **4**, 367-373
- 39 Monti, E., Preti, A., Rossi, E., Ballabio, A. and Borsani, G. (1999) Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. *Genomics.* **57**, 137-143.
- 40 Hasegawa, T., Feijoo Carnero, C., Wada, T., Itoyama, Y. and Miyagi, T. (2001) Differential expression of three sialidase genes in rat development. *Biochem Biophys Res Commun.* **280**, 726-732
- 41 Monti, E., Bassi, M. T., Papini, N., Riboni, M., Manzoni, M., Venerando, B., Croci, G., Preti, A., Ballabio, A., Tettamanti, G. and Borsani, G. (2000) Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane. *Biochem J.* **349**, 343-351
- 42 Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L. and Postlethwait, J. (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics.* **151**, 1531-1545
- 43 Meyer, A. and Schartl, M. (1999) Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol.* **11**, 699-704
- 44 Lehmann, F., Gathje, H., Kelm, S. and Dietz, F. (2004) Evolution of sialic acid-binding proteins: molecular cloning and expression of fish siglec-4. *Glycobiology.* **14**, 959-968
- 45 Harduin-Lepers, A., Mollicone, R., Delannoy, P. and Oriol, R. (2005) The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. *Glycobiology.* **15**, 805-817

- 46 Sohn, H., Kim, Y. S., Kim, H. T., Kim, C. H., Cho, E. W., Kang, H. Y., Kim, N. S., Kim, C. H., Ryu, S. E., Lee, J. H. and Ko, J. H. (2006) Ganglioside GM3 is involved in neuronal cell death. *Faseb J.* **20**, 1248-1250
- 47 Guerardel, Y., Chang, L. Y., Maes, E., Huang, C. J. and Khoo, K. H. (2006) Glycomic survey mapping of zebrafish identifies unique sialylation pattern. *Glycobiology.* **16**, 244-257

Stage 2(a) POST-PRINT

FIGURE LEGENDS

Figure 1 Sialidase gene organization in zebrafish (*Dr*), fugu (*Tr*) and man (*Hs*).

The arrows indicate the direction of gene transcription within each chromosome. Sialidase genes are depicted with gray arrows, while adjacent genes in syntenic regions are represented by black arrows. The size of the arrows is not proportional to that of the genes and the distances between genes are arbitrary. The white arrow indicates the human *SPCS2* gene that appears to be absent in zebrafish and fugu genomes.

Figure 2 Exon–intron organization of human (*Hs*) and zebrafish (*Dr*) sialidase genes.

Introns are depicted by thin lines and their size is arbitrary. Exons are represented by gray boxes and their sizes are indicated by the number of encoded amino acids. Only coding exons have been considered. In the case of *Hs* NEU4, the 484 aa protein isoform (GenBank accession number CAC81904) is reported.

Figure 3 Multiple sequence alignment of sialidases.

Multiple alignment of the amino acid sequences of zebrafish (*Dr* Neu1, *Dr* Neu3.1, *Dr* Neu3.2, *Dr* Neu3.3, *Dr* Neu3.4, *Dr* Neu3.5 and *Dr* Neu4) and human (*Hs* NEU1, *Hs* NEU2, *Hs* NEU3 and *Hs* NEU4) sialidases. The alignment was performed using ClustalW algorithm (see Materials and Methods). Residues that are identical are shown on dark grey background; on light grey background are boxed the conservative substitutions. The active amino acid residues derived from the crystallographic data of the human cytosolic sialidase NEU2 are indicated by asterisks (*) above the sequence. The conserved Y(F)RIP box is underlined with a grey bar under the sequence. Continuous or dotted black lines under the sequence indicate the canonical and poorly conserved Asp boxes, respectively.

Figure 4 Unrooted phylogenetic tree depicting the evolutionary relationship of teleost, avian and mammalian sialidases.

The tree was generated as described in the Materials and Methods section using the amino acid sequences of human (*Hs*), mouse (*Mm*), chicken (*Gg*), zebrafish (*Dr*), and fugu (*Tr*) sialidase polypeptides. It was not possible to identify a putative chicken ortholog of NEU1 in the 2.1 build of the *Gallus gallus* genomic sequence. The horizontal bar represents a distance of 0,5 substitutions per site.

Figure 5 Temporal expression of *neu3.1*, *neu3.2*, *neu3.3*, *neu3.4* and *neu4* genes. RT-PCR was performed on equal amounts of total RNA isolated from embryos at different developmental stages. Time points are expressed in hours post-fertilization (*hpf*). The dimension of the expected PCR products are reported on the left of the figure. β -actin was used as a positive RT-PCR control.

Figure 6 *In situ* hybridization (expression pattern) of zebrafish sialidases *neu3.1* and *neu4*. A and B, *neu3.1* expression in the gut of 2 dpf and 4 dpf embryos. li = liver; pa = pancreatic area; in = intestine; nt = neural tube; nc = notochord; gt = gut tube. C-F, *neu4* expression in the eye region (lens placode) of a 25-somite (21 hpf) embryo (C) and in the lens of 24 hpf (D), 2 dpf (E) and 3 dpf (F) embryos. In the case of F panel, a higher magnification is shown. lp = lens placode; oc = optic cup; re = retina; ls = lens; le = lens epithelium. Embryos are in ventral (A), dorsal (C, D) and lateral (E, F) views with anterior to the left. B is a 40 μ m transversal vibratome section.

Figure 7 Biochemical characterization of zebrafish sialidases.

Neu3.1- (A), Neu3.2- (B) and Neu3.3- (C) and Neu4-Myc (D) fusion proteins were expressed in COS7 cells. *Left column*. The specific activity of different *Dr* sialidases toward 4MU-Neu3.1c over the pH range 2.2 to 7.0 are reported. The enzymatic assay was carried out using Tris/glycine buffer from pH 2.2 to 3.0 (black bar) and sodium citrate/phosphate buffer from pH 2.6 to 7 (white circle). Variations of the values are indicated by the error bar (n=4). *Middle column*. Sialidase activity of the supernatant (SN) and pellet (P) obtained by ultracentrifugation of the total lysates at 100,000 g. The rate of hydrolysis of 4MU-Neu3.1c are expressed as percentage of the value detectable in the total lysates. Variations of the values are indicated by the error bar (n=3). *Right column*. Western-blot analysis of protein sample (20 μ g) of total cell lysate (T) and supernatant (SN) and pellet (P) obtained by ultracentrifugation. Detection of the fusion proteins was performed using anti-Myc antibody and secondary peroxidase-conjugated isotype-specific antibody, followed by chemiluminescence developing reagents. Protein standard position is reported on the left.

Figure 8 Ganglioside pattern of COS7 cell expressing zebrafish sialidase Neu3.1, Neu3.3 and Neu4.

Cells transfected with the different zebrafish sialidases were pulsed for 2 h with [$1\text{-}^3\text{H}$] sphingosine, 3×10^{-8} M final concentration. After 40 h (Neu3.1 and Neu3.3) and 20 h chase (Neu4), cells were harvested and treated for lipid analysis (see Materials and Methods). The ganglioside relative content are compared to the one observed in mock transfected cells. Variations in the ganglioside relative contents are indicated by the error bar ($n=6$). Significance according to Student's t test: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

Figure 9 Subcellular localization of zebrafish sialidase Neu3.1, Neu3.2, Neu3.3 and Neu4. COS7 cells were grown on glass coverslips, transfected with the recombinant vectors encoding the the different Myc epitope-tagged zebrafish sialidases and processed for double indirect immunofluorescence. *Left column* shows the subcellular distribution of Neu3.1 (A), Neu3.2 (B), Neu3.3 (C) and Neu4 (D). *Middle column* shows the labeling of endogenous intracellular markers such as calnexin and LAMP1, as well as the distribution of the human cytosolic sialidase NEU2 detectable in a double transfection experiment. *Right column* shows the colocalization (merge). The different primary antibodies were detected using isotype specific secondary antibodies conjugated with Alexa fluor 488 (green) and Alexa fluor 555 (red). Specimens were analysed using a Zeiss Axiovert 200 microscope equipped with Confocal Laser System LSM 510 META.

Figure 1

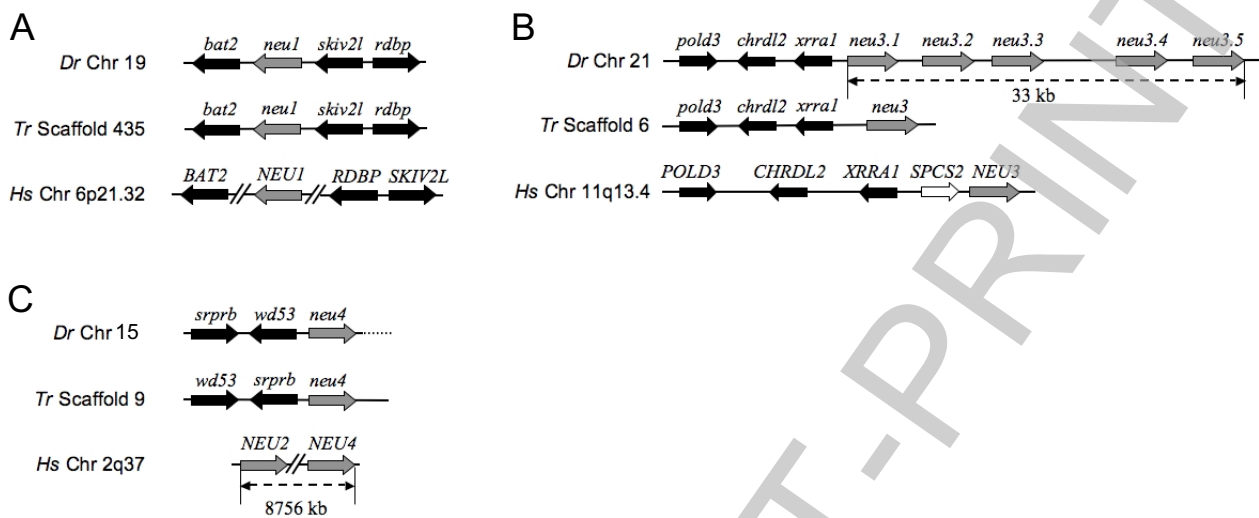


Figure 2

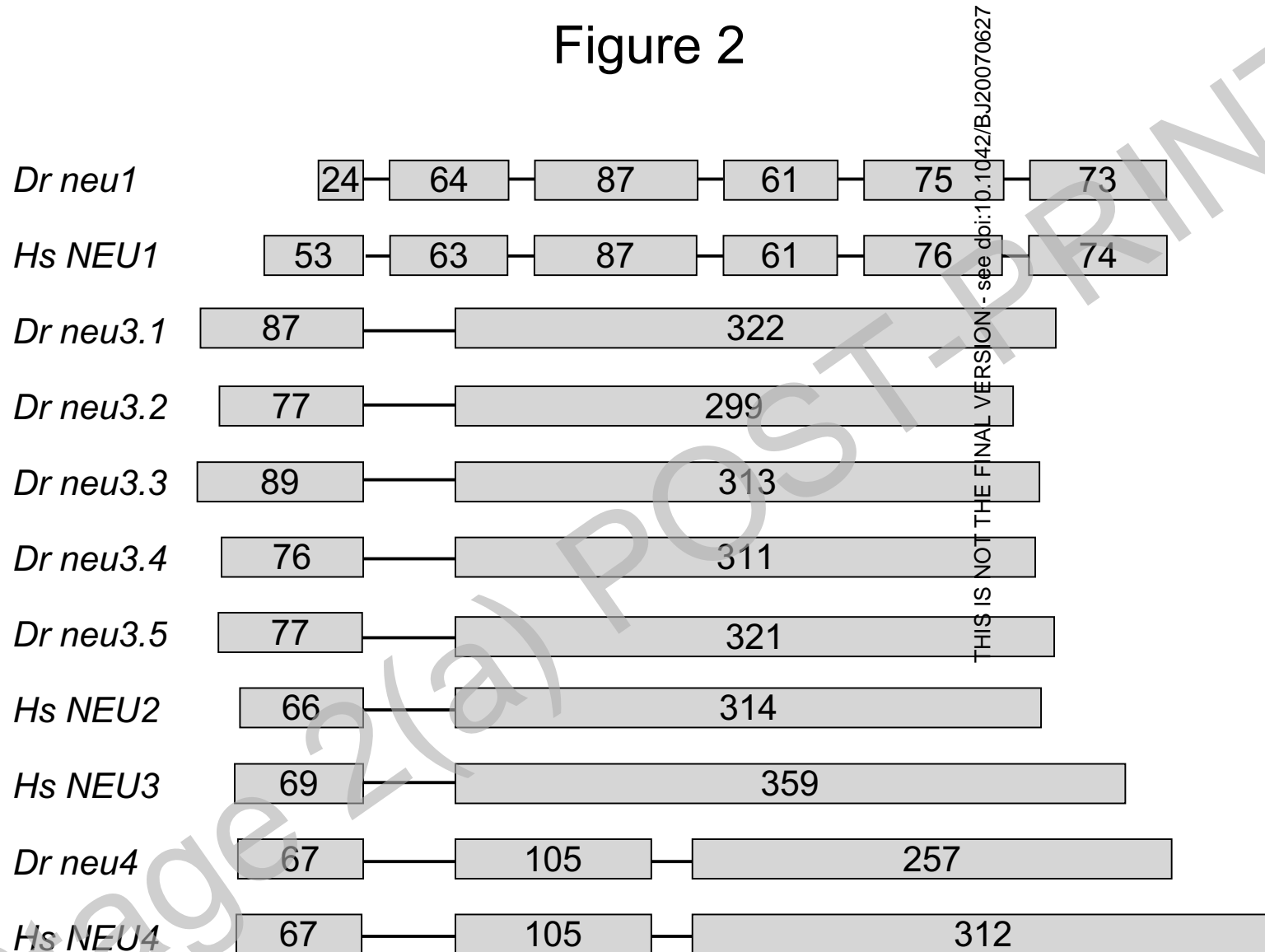
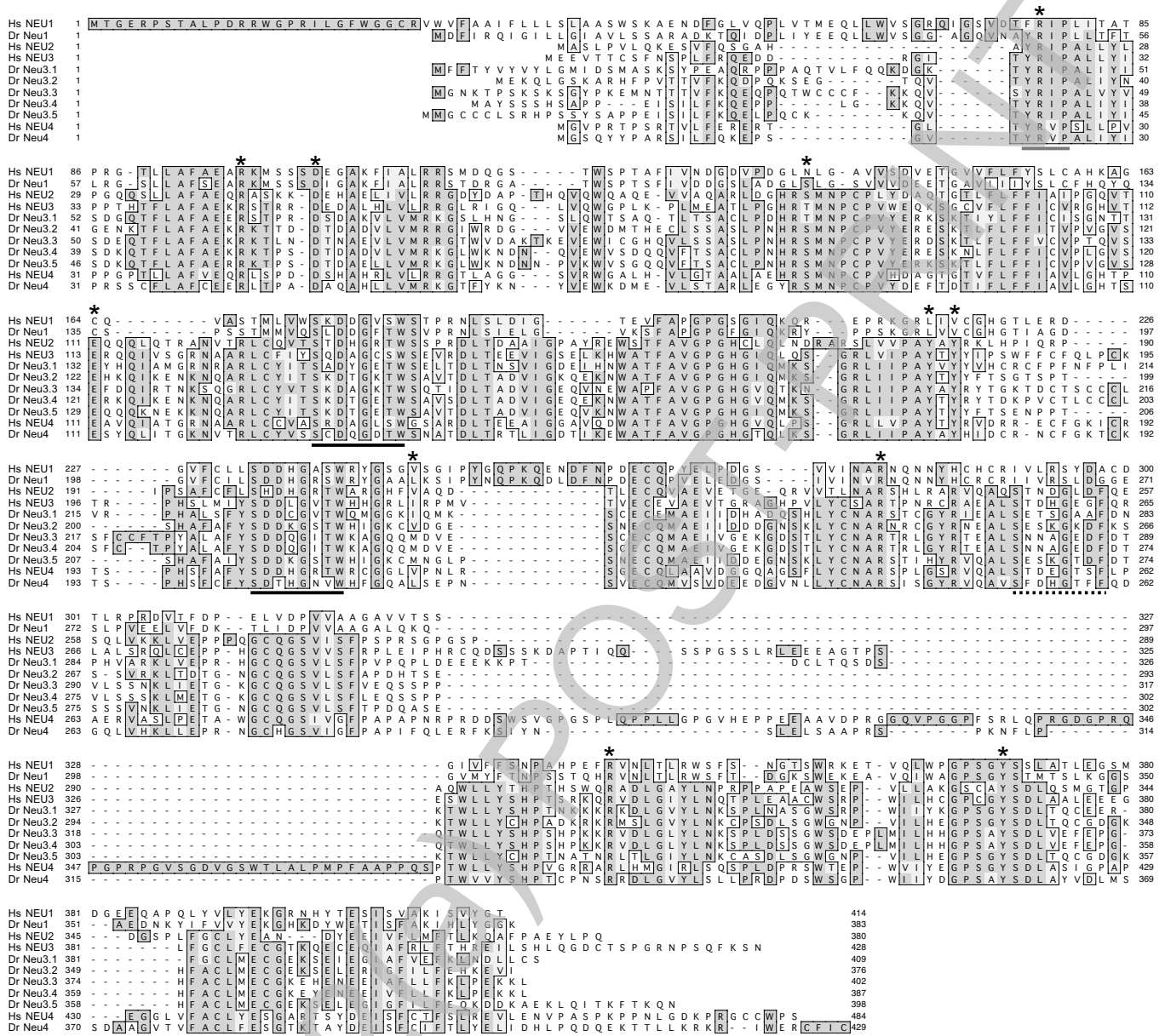


Figure 3



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Figure 4

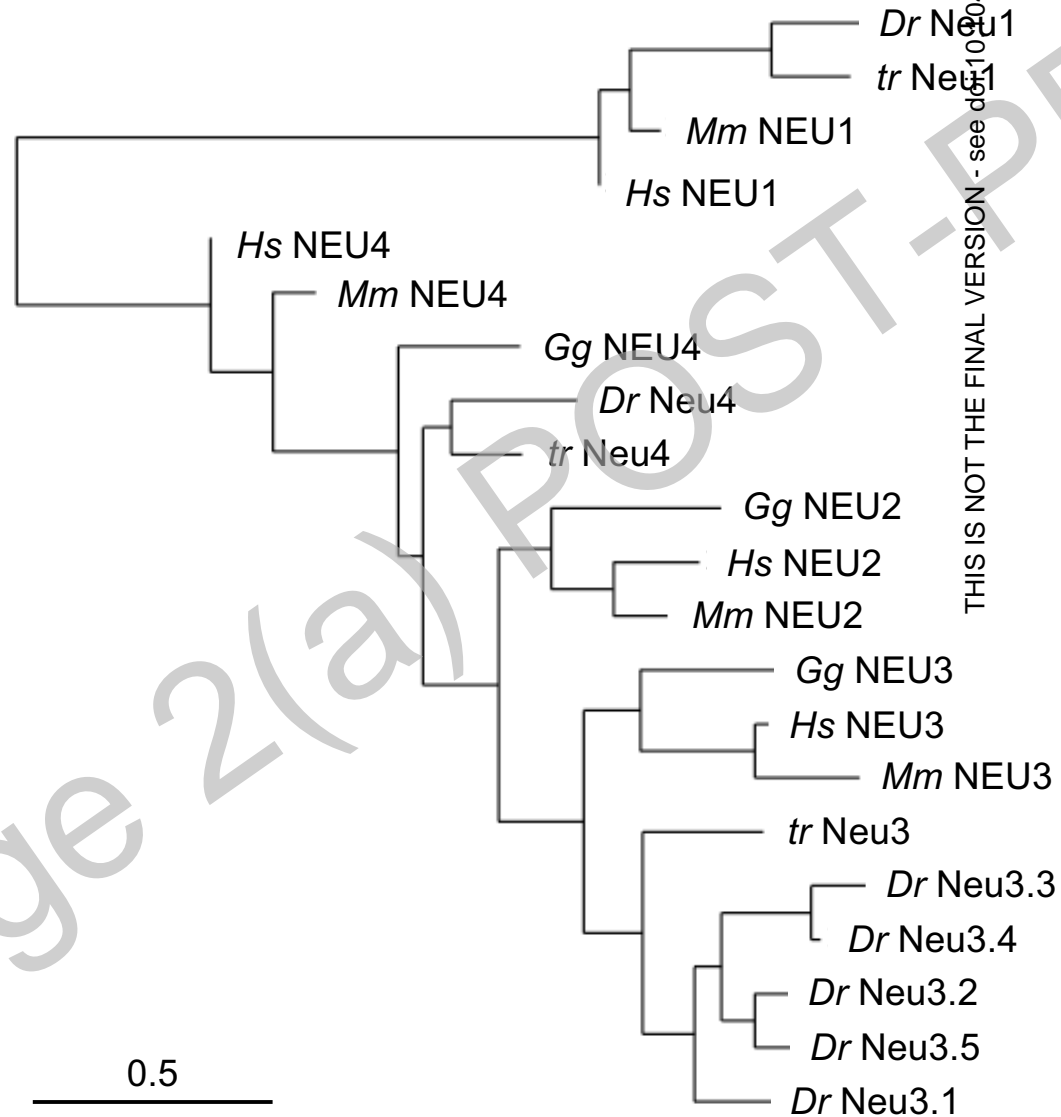


Figure 5

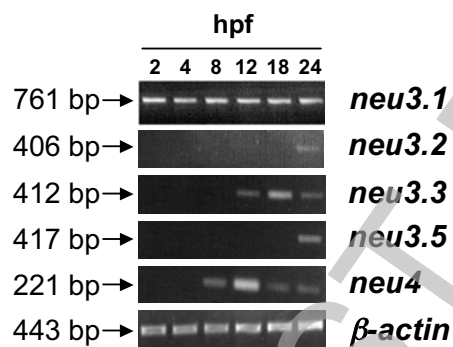
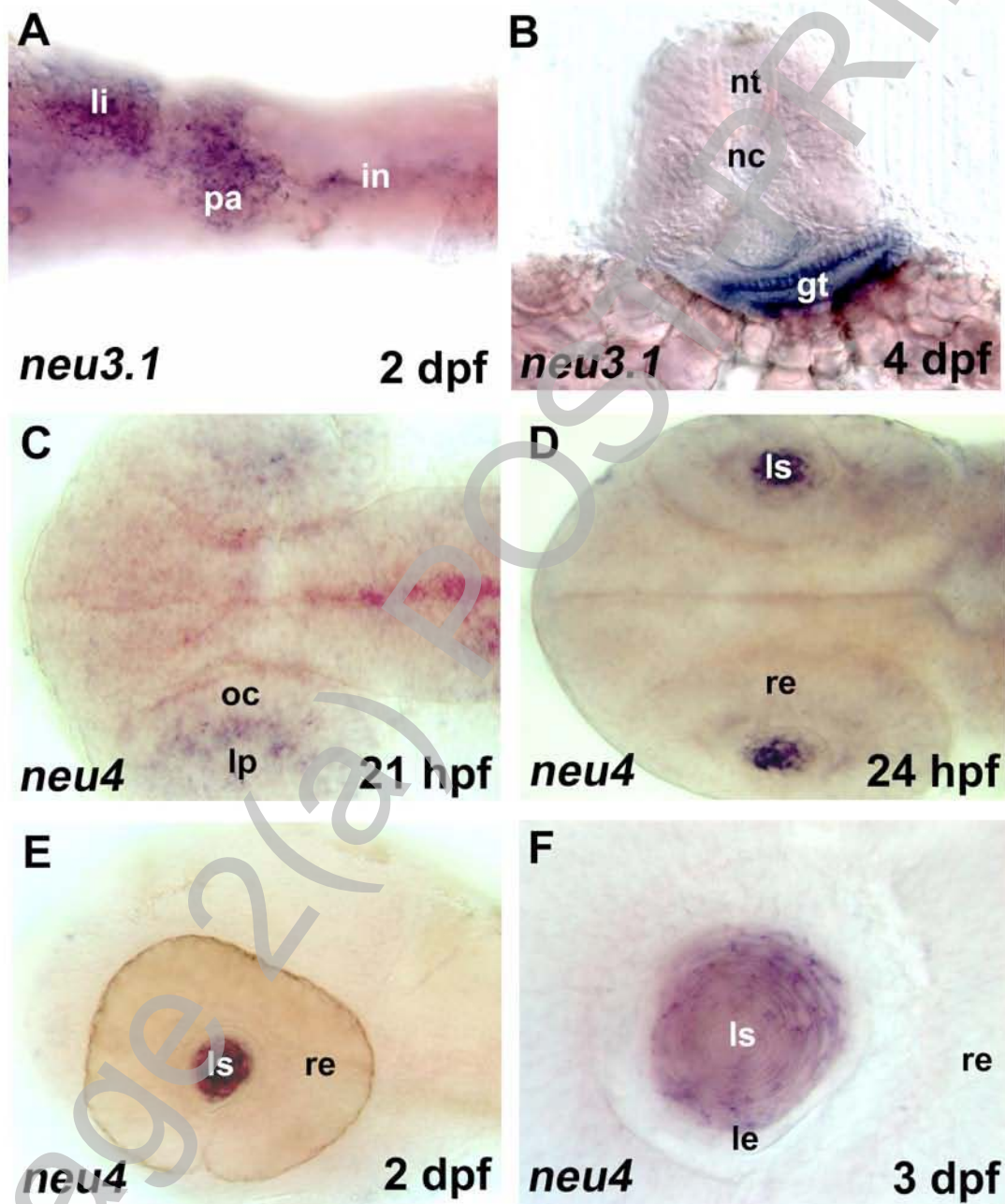
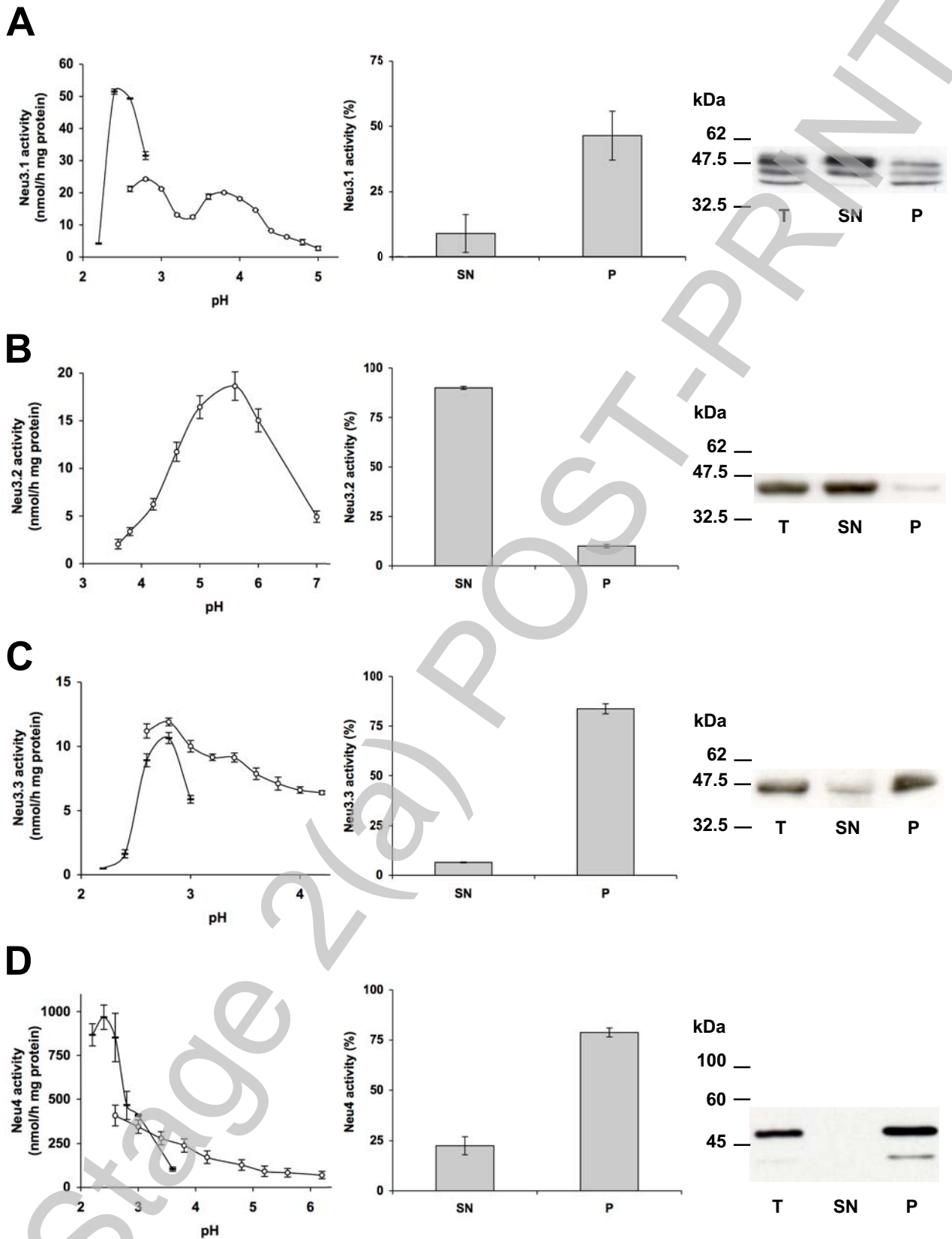


Figure 6



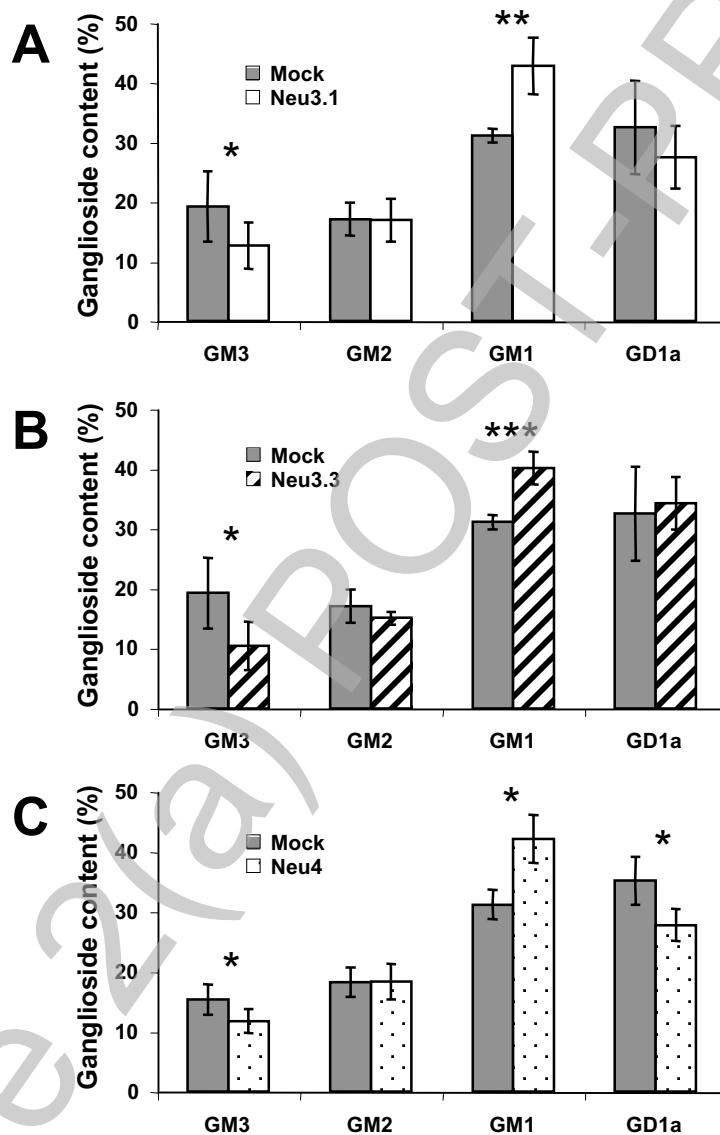
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Figure 7



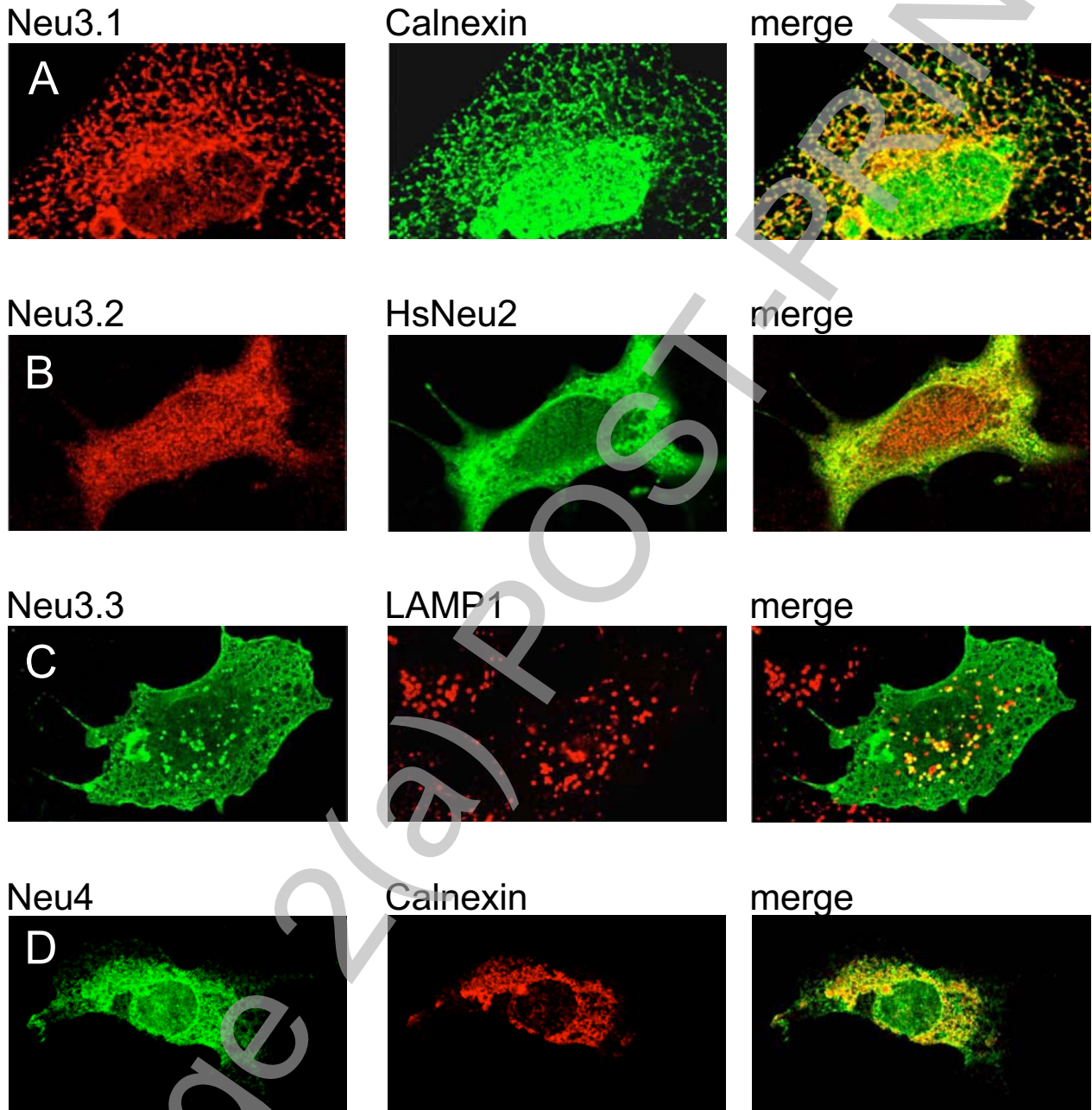
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Figure 8



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Figure 9



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Table 1

Comparison of the Molecular Properties of *D. rerio* Sialidases.

The number of target amino acids for N-Glycosylation, O-glycosylation and phosphorylation are indicated with the single letter code.

<i>D. rerio</i> Sialidases	Amino acids	Molecular mass (kDa)	Theoretical pI	Potential N-glycosylation sites	Potential O- β -GlcNAc attachment sites	Potential phosphorylation sites
Neu1	383	42.14	6.01	N: 3	S: 2	S: 15 T: 4 Y: 1
Neu3.1	409	46.2	6.63	N: 2	T: 1	S: 13 T: 4 Y: 5
Neu3.2	376	42	7.15	N: 3	S: 2 T: 4	S: 12 T: 4 Y: 3
Neu3.3	402	45.12	6.04	N: 3	S: 2 T: 1	S: 16 T: 7 Y: 3
Neu3.4	387	43.48	5.6	—	S: 4	S: 11 T: 4 Y: 4
Neu3.5	398	44.42	6.75	N: 1	S: 3 T: 2	S: 12 T: 5 Y: 2
Neu4	429	48.6	5.96	N: 2	-	S: 15 T: 6 Y: 7