Université des Sciences et Technologies de Lille
Ecole doctorale Biologie Santé
et
Institute of Biochemical Science,
National Taiwan University

THESE
en cotutelle

présentée par

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Pour l'obtention du grade de
Docteur de l'Université
Sciences de la vie et de la santé
Spécialité : Biochimie

Developmentally regulated oligosialylation pattern in Zebrafish embryogenesis

Etude de la régulation des sialo-conjugués au cours de l'embryogenèse du poisson zèbre

Soutenue le 19 novembre 2008 devant la commission d'examen :

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博士論文
Institute of Biochemical Science, College of Life Science,
National Taiwan University &
Unité de glycobiologie structurale et fonctionnelle,
Université des Sciences et Technologies de Lille
PhD Thesis

斑馬魚多重唾液酸化醣質體在胚胎發育過程的表現調控與結構分析

Developmentally regulated oligosialylation pattern in Zebrafish embryogenesis

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中華民國 97 年 11 月

Nov, 2008

謝辭

故事的緣起，要從一個細菌的糖蛋白把我帶到中研院生化所邱繼輝老師的實驗室開始，然後我就跟酷老師和醣生物還有質譜結了緣。因緣巧合下參與了實驗室的法國博士後 Yann 的斑馬魚胚胎醣質體計畫，並在酷老師和 Yann 共同指導下，開始了這段橫跨台灣與法國的學習旅程。過程中最困難的地方，對我而言不只是技術層面，也是跨領域與跨國與跨實驗室的學習，學習在不同思考方式下，和不同語言間，把與資源、數據和資料作整合。每個人來攻讀博士的理由都不同，而我當初只是簡單地想知道自己適不適合唸博班，也許過程已經結束了我卻還沒找到答案，也可能已經找到，因為答案似乎延伸成另一個問題，變成是"我想不想"而不是"我能不能"。而在找答案的過程中，我學到了好多，也得到了更多。

能完成這本論文，內心其實充滿了感謝，因為我很幸運遇到了好多照顧我提攜我的好人。首先要感謝的是邱繼輝老師，台灣的指導老師，引領我進入美麗的醣生物世界的心靈導師，尤其要感激老師牽成，圓了我的留學夢; 另外一位貴人是 Yann，我的法國導師，這個跨國雙學位計畫的複雜程序大多是靠他張羅搞定的! 感激 Yann 開啟我的國際觀，以及在這個計畫對我的支持和鼓勵，也跟我分享許多法國文化之美。還有十分感謝 Anne，另一位法國的指導老師，她在我分生實驗上的細心指導以及經常互動性的討論。另外也感謝黃銓珍老師，宜瑾，宜群，和盧叔叔在斑馬魚胚胎處理的協助，和 Vasta 提供斑馬魚胚胎 cDNA，Hamed Hafiz 提供斑馬魚腎臟 cDNA library, Thisse 夫婦提供斑馬魚及胚胎以及幫忙，吳世雄老師友情提供重組 endo-N 的表現系統，Kitajima 和 Sato 在分析唾液酸的協助。Sandrine, Monti 和 Li YT 在唾液酸水解酵素活性分析的經驗分享，還有 group leader Philippe 先生對唾液酸傳遞酶的宏觀指教。當然也要感謝陪伴我在中研院生化所 603 一同成長的大夥兒，精妹學姊、鳥、泓翔、Jimy、智蕾、阿妹、小龜，還有駿逸、心宜、祁宏、佳葳、思緯、慧鐘、耀云、怡昀、穗華、瑩徹、胖達、平福。以及在法國里爾的 UGSF (Unite de Glycobiologie structurale et fonctionnelle) 陪伴我度過旅法美妙的兩年的 Estelle, Nao, Ying, Beatrice, Sophie, Marie-Ange, Sylvain, Manu, Xavier, Bernard。尤其是和我同月同日生的 Estelle，不但與我分享豐富的法國學生生活，還幫忙處理許多複雜的行政事務; Nao，同時抵法的有緣日本麻吉，一起探索與分享法國生活的新奇與感動; Ying，在異鄉同文同種的好友，時常互相加油打氣。最後，我想感謝我的家人和鎮邦，有你們一路的支持鼓勵和體諒，才有今天的我!

我想把這本論文獻給所有幫助過及關心我的人，以及需要它的人。希望我的這小片磚瓦，可以幫助正在蓋房子的人更看清楚一點這棟建築的輪廓。也獻給所有正在追尋自我的你，要把握機會實現夢想，人生的每個過程都是學習的機會和奇妙的體驗，而且凡走過必留下痕跡。最後，分享這幾年自己的領悟 "你從哪裡來，決定了你的個性，而你的決定，則決定了你往哪裡去"，並用 "成功增魅力、挫折增智慧" 再次勉勵我自己也獻給所有在學術領域默默耕耘的研究人。

Abstract

The nine-carbon sugar, sialic acid, plays important roles as the recognition epitopes in cell-cell communications. Its regulated expression often reflects the status of a cell, and has been reported as stage-specific antigen for tumorigenesis or embryogenesis. In this thesis work, the biological functions of developmentally regulated sialylation, particularly in relation to the spatial and temporal regulated expression of α2-8 sialyltransferases (ST8sia) during the embryogenesis of Zebrafish, was investigated using a combination of chemical and mass spectrometry (MS) analyses. Glycomic survey mapping followed by advanced glycan sequencing were first performed across the different developmental stages of fertilized Zebrafish eggs. Among the unusual structures identified are the complex type N-glycans that carry β4-galactosylated sialyl Lewis X, the disialylated O-glycans, Fucα1-3GalNAcβ1-4NeuAc/NeuGcα2-3Galβ1-3GalNAc, and the oligosialylated glycolipids. The stage-specific, distinctive Neu5Ac/Neu5Gc sialylation patterns observed then prompted a more precise quantification of the respective oligosialylation by fluorescent chromatography after chemical derivatization. Interestingly, the oligosialic acids of glycoproteins were found to decrease whereas those of glycolipids increased along the embryogenesis. To determine if the observed profiles are due to the regulation of biosynthesis or catabolism, or both, the transcripts of all inferred Zebrafish ST8Sia genes responsible for the biosynthesis of oligosialylation were quantitatively mapped by real-time PCR, and the activity of endogenous sialidases were assayed, for each of the developmental stages. The presence of sialidases and an increased level of expression of all the ST8Sia genes led to a working model, which proposes that the oligosialic acids from glycoproteins are maternally inherited (synthesized before fertilization) and exocytosis out of the embryo into the perivitelline space where it undergoes catabolism, whereas oligosialic acids on the glycolipids are de novo synthesized by the increasingly expressed ST8Sia in the embryos. Furthermore, through whole mount in-situ hybridization, most of the ST8Sia genes were shown to be expressed in the nervous system during early embryogenesis, with different onset and locations. In summary, the precise MS and MS/MS-based glycomic profiles, together with detailed structural determination, quantitative analysis of the developmentally regulated oligosialylation pattern, and the spatial and temporal expression profiling of ST8Sia of Zebrafish, collectively provides new sialoglycobiology insights for neuroscientists at the molecular level and highlighted the significance and complicated regulation of oligosialylation in early neuronal development.

Keywords: sialic acid, oligosialylation, Zebrafish, glycomic, developmental, mass spectrometry, sialyltransferase, ST8Sia.

中文摘要

九碳糖唾液酸是位於生物體醣質末端的修飾分子，它是細胞與細胞間的溝通橋樑，也是傳達訊息的細胞表面辨識分子。位於細胞表面的醣分子常常可以反應細胞的生理狀態，因此也被當作腫瘤生成或是胚胎發育時期的階段性指標抗原。在本篇論文，我們以斑馬魚為模式動物，去探討負責合成α2,8 鍵結的多重唾液酸鏈的多重唾液酸傳遞酶(oligo/polysialyltransferase)家族(ST8Sia)其基因表現的部位和時間，如何在胚胎發育過程中被影響和調控，並且結合化學方法和質譜儀分析去定出唾液酸化的醣質結構。首先，利用先進的質譜作醣質分子定序，我們比較不同胚胎發育階段的斑馬魚之受精卵醣質，這些唾液酸化的醣質有別於一般在哺乳類的醣質結構，像是連接在天門冬素(Asparagine)的氮分子上的唾液酸化複合醣(sialylated complex type N-glycans)為 4-galactosylated sialyl Lewis X; 連在絲氨酸(Serine)或羥丁氨酸(Threonine)的氧分子上的醣(O-glycans)為雙唾液酸化的 O-glycans，Fucα1-3GalNAcβ1-4NeuAc/NeuGcα2-3Galβ1-3GalNAc，及多重唾液酸化的醣脂質。以質譜觀察到多重唾液酸可能只出現在特定發育階段之後，我們進一步用螢光標示液象層析去更精準的偵測及量化不同發育階段的多重唾液酸。值得注意的是，在醣蛋白上的多重唾液酸，不論在量上或鏈長都會隨著胚胎發育而漸減，而在醣脂質上的反而會漸增。為了進一步去了解多重唾液酸化的消長現象是如何發生，我們利用定量及時聚合酶連鎖反應(quantitative real-time PCR)去檢視多重唾液酸傳遞酶的基因表現層次，和內源性唾液酸水解酶的活性在不同發育階段的調控情形。結果所有的 ST8Sia 的表現，皆隨著胚胎發育而增加。內源性的唾液酸水解酶活性，為醣蛋白上多重唾液酸的降解提供了一個可能的解釋，即大多數的多重唾液酸化的醣蛋白是來自母體，他們在授精前就已經存在於卵子中，而在受精後被釋放到胚胎與卵膜的間隙，再被內源性的唾液酸水解酶降解。進一步分離授精卵的卵膜、胚胎、和介於兩著之間的間質液，並分析這三部分的多重唾液酸的鏈長和含量，以及唾液酸水解酶活性，我們初步證實了大多數重唾液酸化的醣蛋白的確是來自母體。而位於醣脂質上的多重唾液酸因為在胚胎發育後期才逐漸出現，與負責其生合成的多重唾液酸傳遞酶表現量成正相關，因此推測是由胚胎新合成，而非源自母體。經由核糖核酸整體原位雜交技術(whole-mount in situ hybridization)，顯示多重唾液酸傳遞酶在發育早期都表現在胎兒的神經系統。總結本篇論文，我們利用高感度的串聯式質譜，解開了斑馬魚受精卵醣質在發育過程的全貌及改變，以及精確量化多重唾液酸化醣質的結構的消長，並且分析其調控因子，其中包括多重唾液酸傳遞酶的基因表現量和唾液酸水解梅的活性。這些數據交叉證實了多重唾液酸化在胚胎發育期間複雜的調控機制，也進一步為神經發育學家提供了唾液酸醣生物學層面的研究線索與觀點。

關鍵字：唾液酸，斑馬魚，胚胎發育，質譜儀，唾液酸傳遞酶。

Table of content

List of figures and tables

1. Introduction

1.1 Functional glycomics and glycobiology

From the discovery of DNA to the sequenced human genome, the template-dependent formation of biological molecules from gene, RNA, to proteins has been the central dogma for biology. Unlike the genome and proteome, the glycome and lipidome, which participate in many biological functions and the pathogenesis of diseases, are not directly encoded by DNA. Carbohydrates can be assembled into oligo/polysaccharides, also called glycans, which are covalently linked to lipids or proteins. In general, their structural architecture may be viewed as comprising various well defined core structures, variably elongated with and without branching, and capped by terminal glycosylation, as illustrated in Fig. 1-1. They participate in cell-cell adhesion, recognition and signaling events and therefore their spatial-temporal presence and the expression of glycosyltransferases and glycosidases responsible for their biosynthesis often reflect the status of the bio-organisms in embryogenesis, cancers, and the activation of immune responses (Hakomori, S. 2001, Haltiwanger, R.S. and Lowe, J.B. 2004, Rudd, P.M., et al. 2001).

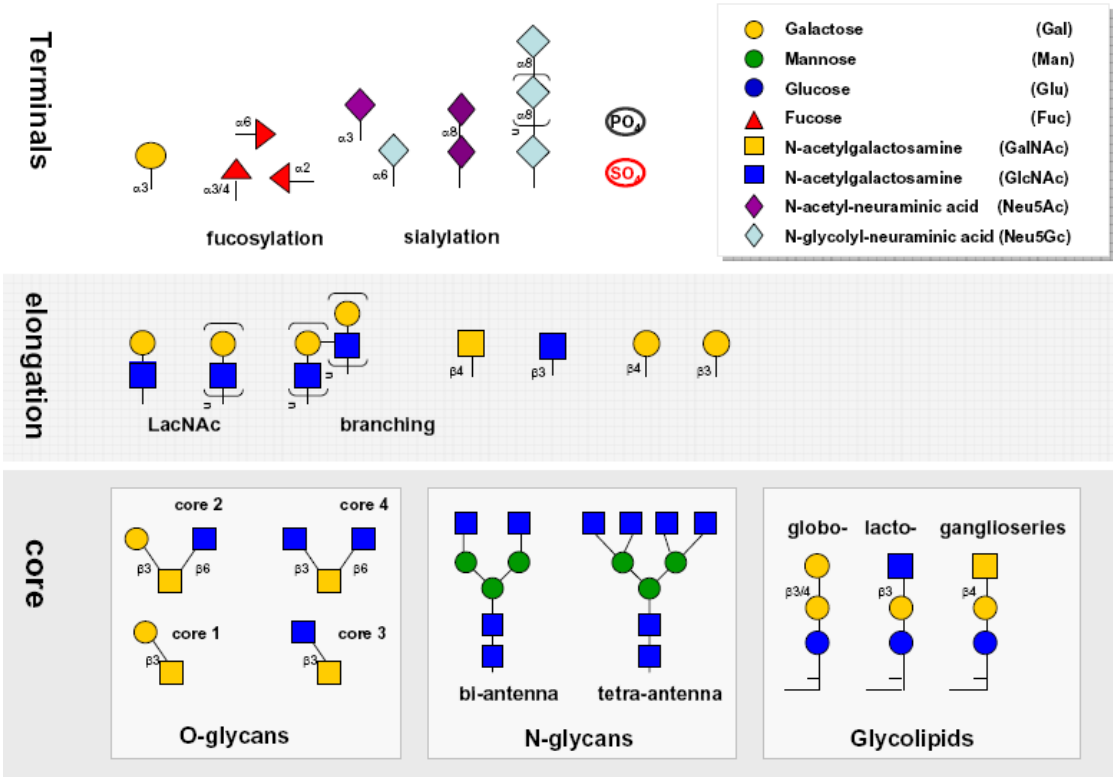

Fig. 1-1. Common glycan structures found in vertebrate. Glycolipids, N-glycans, and O-glycans are assembled mainly by core, elongating chain, and terminal modifications. Each kind of sugar is represented by the symbols listed on the right.

In essence, glycomics is to investigate the whole glycome in its entirety, including glycans on glycosphingolipids and glycoproteins. In turn, functional glycomics aims to decipher the biological functions of the constituents of a glycome by mapping their regulated expression at any particular physiological stages to interactions with their associated molecules. These conceptual ideas emerging at the turn of the century have given birth to the Consortium for Functional Glycomics (CFG), a large international research initiative aiming to elucidate the roles of carbohydrate-protein interactions in cell-cell communications at the cell surface. They integrate the research groups of glyco-gene microarray analysis, novel knockout mouse strains, glycan microarray analysis, glycan analysis, mouse phenotyping and provide the specialty databases for glycan-binding proteins, glycan structures, and glycosyltransferases.

It is worth to note that the dysfunction or mislocation of a single glycosyltransferase may affect the modifications of multiple proteins or lipids. Thus to understand how glycosylation assumes a specific cellular function, we need to combine the knowledge of regulated glycan synthesis with the phenotypes observed in intact organisms bearing that particular glycosylation defects. In general, the biological functions of a particular glycan structure can be approached by either identifying its biosynthetic pathways or its cognate lectin that would act to translate its functions, or both. The former can be done by modifying the corresponding glycosyltransferases expressions in cells or animal models. Then, the possible function can be speculated by examining the difference in morphology or behavior after such genetic manipulations. Likewise, the latter involves comparing the different behavior by knocking down/out the counter molecule or competitively inhibiting the interaction of target-and-counter molecule in living organisms. Either way, animal models and/or cell lines are indispensable in the pursue of functional glycomics.

1.2 Animal models for embryogenesis and developmental glycobiology

Descriptive studies in mice and also in lower metazoans (*Caenorhabditis elegans*, *Drosophila melanogaster*, *Arbacia punctulata* (sea urchin) etc.) have indicated that glycan structural diversity is characteristic of developmental event in these model animals. To understand the biological significance of these specific glycan structures, which appear in spatial and temporal specific patterns throughout the development, genetic approaches in conjunction with structural studies have been employed. Zebrafish, the newest model among the vertebrate model animals, has risen to prominence because both genetic and experimental embryological methods can be easily applied (Grunwald, D.J. and Eisen, J.S. 2002). Due to its external fertilization with huge quantity of transparent eggs, it provides an easy observation and accessibility at very early embryonic stages compared with mouse. Moreover, the full genome database of Zebrafish is available, as well as an established fine platform for genetic and

molecular manipulation including targeted gene knockdown by Morpholino (Nasevicius, A. and Ekker, S.C. 2000). All these make Zebrafish an excellent model system to study developmental biology and developmentally regulated glycosylation.

The developmentally regulated glycoconjugates have been studied from as early as the 70's when glycan-based biomolecular complexities were recognized by applications of plant lectins to red blood cells (Morgan, W.T. and Watkins, W.M. 1953). This work established that the glycans-based ABO blood group antigens are expressed in a dynamic manner during human embryogenesis (Szulman, A.E. 1964, Szulman, A.E. 1971, Szulman, A.E. and Marcus, D.M. 1973). As lectin-based immunohistochemistry expanded, similar observations in humans, rodents, and other mammals demonstrated that these organisms elaborate a wide array of glycan structures and control the amount and structure of such glycans with temporal and lineage specificity during development and differentiation (Oppenheimer, S.B. 1977). This orchestrated modulation of glycan diversity during development implicates important functions for these molecules and similar cell surface molecules whose expression are restricted to specific stages early in embryogenesis were gradually identified.

Among the earlier examples, Solter and Knowles obtained monoclonal antibodies reacting with mouse embryonic carcinoma cells, by immunizing mouse with mouse teratocarcinoma cells, F9. They discovered the first stage-specific antigen (SSEA-1) which can be detected on blastomere of 8-cell stage mouse embryo (Solter, D. and Knowles, B.B. 1978). The antigenic epitope of SSEA-1 was later characterized as Galβ1-4 (Fucα1-3)GlcNAc, corresponding to Lewis x antigen (Gooi, H.C., et al. 1981). Using similar strategy, Kannagi et al isolated SSEA-3 and SSEA-4 antigens from glycolipids of human teratocarcinoma cells (Kannagi, R., et al. 1983). At the same period, Dietert et al found that the chicken fetal antigens, lost during chick development, reappear as blood group antigens (Dietert, R.R. and Sanders, B.G. 1977). Mouse early embryonic cells have also been identified to contain abundant branched poly-N-acetyl-lactosaminoglycans (Muramatsu, H., et al. 1983, Muramatsu, T., et al. 1978), commonly referred to as the I antigen in contrast with the i antigen of linear structures. This class of glycan carries various carbohydrate markers such as Le^x and an α-galactosyl antigen, ECMA-2, which are expressed on early embryonic cells, thus called embryoglycan (Muramatsu, T., et al. 1979, Suganuma, T., et al. 1987). Interestingly, I antigen is intensely expressed through the preimplantation and early postimplantation of embryogenesis, whereas i antigen first appears in parietal and visceral endoderm of the early postimplantation embryos, and then expressed only at some parts of the embryos (Pennington, J.E., et al. 1985). It is also expressed on fetal erythrocytes but replaced later by I antigen in the adult erythrocytes (Kapadia, A., et al. 1981).

Notably among the embryonic antigens reported, sialylated structures did not receive as much attention as they deserved in early days. This is probably due to partial desialylation of those

immunogens during preparation or to the poor immunogenicity of sialyl-epitopes (Finne, J., et al. 1987). Nonetheless, the biological significance of sialic acid in fertilization and development was increasingly being recognized. For example, the sialylated glycans on sea urchin eggs were reported to increase the intracellular pH and potentiate the acrosome reaction of sperm (Hirohashi, N. and Vacquier, V.D. 2002), and that the inactivation of a key enzyme of sialic acid biosynthesis, UDP-GlcNAc 2-epimerase, causes early embryonic lethality in mice (Schwarzkopf, M., et al. 2002).

1.3 Sialoglycoconjugates: structures, biosynthesis and catabolism

Sialic acids are nine-carbon sugars and constituted of more than 50 different compounds (Angata, T. and Varki, A. 2002). It has special structural features including the amino group at position 5 and the carboxyl group at position 1 that confers a negative charge on the molecule under physiological conditions which make it a strong organic acid (Traving, C. and Schauer, R. 1998). The original definition of sialic acids was “neuraminic acid, 5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid, and its derivatives” (Blix, F.G., et al. 1957). This definition was expanded by the discovery of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (or 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid, KDN), which has a hydroxyl group in place of the amino group at the C5 position. N-Glycolylneuraminic acid (Neu5Gc) is derived from CMP-Neu5Ac by replacing the hydrogen atom (-H) with a hydroxyl group (-OH) on the N-acetyl group, catalyzed by CMP-Neu5Ac hydroxylase (CMAH), as shown in Fig. 1-2. Other currently recognized sialic acids are biosynthetic derivatives of either N-acetylneuraminic acid (Neu5Ac) or KDN with substitutions at the 4, 5, 7, 8 and 9 positions by various O-acetyltransferases and O-methyltransferases to generate the diversity of sialic acids found in nature (Kamerling, R.S.a.J.P. 1997, Varki, N.M. and Varki, A. 2007).

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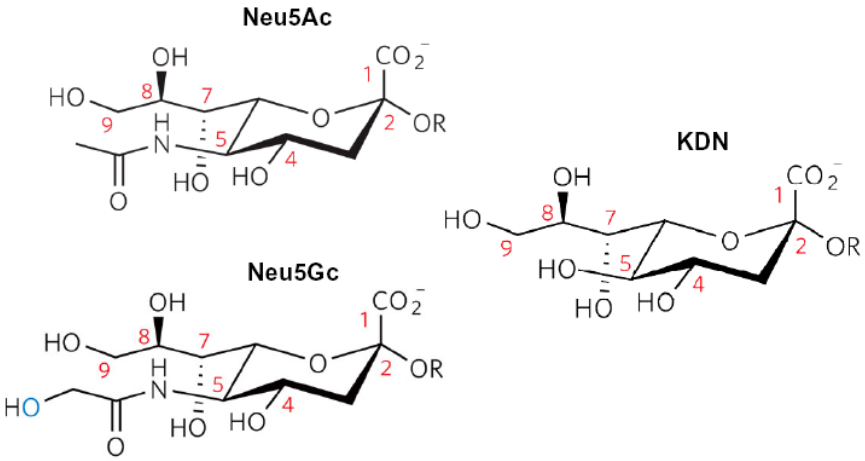

Fig. 1-2 Diverse structures of sialic acids (modified from (Varki, A. 2007)

Sialoconjugates are synthesized by sialylatransferases, which transfer sialic acid from CMP-Neu5Ac to nonreducing terminal positions of the glycan moiety of glycoproteins and glycolipids. Like all other glycosyltransferases, the animal sialyltransferases are Golgi type II transmembrane proteins and share four conserved peptide motifs L (large), S (small), motif III, and motif VS (very small)] which are hallmarks for sialyltransferase identification (Harduin-Lepers, A., et al. 2001). More than 20 different animal sialyltransferases have been identified and classified into four families (ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia) according to their abilities in catalyzing the formation of different linkages (α2–3, α2–6, and α2–8) and their diverse acceptor specificities (Harduin-Lepers, A., et al. 2005, Harduin-Lepers, A., et al. 1995). Although the *in vitro* specificity of each individual sialyltransferase can be readily determined, the physiological meaning of these data remains to be demonstrated *in vivo*.

In reverse to sialyltransferases, sialidases, also called neuraminidases, belong to a family of exoglycosidases that catalyze the hydrolysis of the nonreducing terminal sialic acid linkage in glycoproteins, glycolipids, gangliosides, and polysaccharides. Mammalian sialidases are present in several tissues, organs and cells with a typical subcellular distribution: namely the lysosomal (Neu1), cytosolic (Neu2), plasma membrane–associated (Neu3), and intracellular-membrane-associated sialidase (Neu4) (Monti, E., et al. 2002). The human lysosomal sialidase NEU1 is part of a multienzyme complex containing *β*-galactosidase and the protective protein cathepsin A and is implicated in the severe lysosomal storage disorders sialidosis and galactosialidosis (Zhou, X.Y., et al. 1996). 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. The plasma membrane-associated sialidase NEU3 is the most extensively studied member of the family and characterized by a high degree of specificity towards ganglioside substrates (Monti, E., et al. 2002). Intriguingly, NEU4 has been reported to associate with the mitochondrial membranes (Yamaguchi, K., et al. 2005) and to localize in the lysosomal lumen (Seyrantepe, V., et al. 2004). Recently, the corresponding orthologs were found in Zebrafish, which facilitated the studies of the biological function of vertebrate sialidases (Manzoni, M., et al. 2007). While sialylated glycotopes remain under-explored in the context of embryogenesis across different animal models and human, much evidence has been accumulated to support instead a role for oligo- and/or polysialylation in developmental biology.

1.4 Occurrence and functional implications of oligo- and polysialylation

The homopolymers of *α*2-8-linked residues are named according to the degree of

polymerization (DP), namely di-(DP = 2), oligo-(2 < DP < 7) or poly-sialic acid (DP > 7) chains (Sato, C., et al. 1999). As early as 70's, researchers who worked on neuron and developmental biology have discovered a neural cell adhesion molecule (NCAM) (Thiery, J.P., et al. 1977) which contains an amazingly high content of α2-8 linked polysialosyl units (Finne, J., et al. 1983). Subsequently through biochemical approach, Fukuda et al. reported an embryonal lactosaminoglycan with a disialyl structure in human embryonal carcinoma cell (Fukuda, M.N., et al. 1985). Another large body of early work on the occurrence and structural details of poly- and oligosialylation was in fact those seminal works from Inoue's laboratory on the fish glycoconjugates (Inoue, S.a.I., Y 1997). Before these breakthroughs, polysialic acids were mostly known and structurally characterized as a virulence factor on the capsule of pathogenic bacteria, such as *Streptococcus meningitis* and *E.coli* K1 (Finne, J., et al. 1987), which contributed to several useful antibodies for functional and structural studies in mammals and other animal models.

Polysialylated glycoproteins (PGSP) were first identified in fish by Inoue's group from mature unfertilized eggs of rainbow trout (Inoue, S. and Iwasaki, M. 1978). Subsequently, similar PGSP containing α2,8-linked oligo-Neu5Gc attached to the O-linked glycan core were found in many other fish species (Inoue, S. and Iwasaki, M. 1980, Iwasaki, M., et al. 1984, Kanamori, A., et al. 1990, Kitajima, K., et al. 1989, Kitajima, K., et al. 1986, Shimamura, M., et al. 1984). The unfertilized fish eggs contain an outer vitelline layer that coats the plasma membrane of the gamete. Right after fertilization, the fertilized eggs undergo cortical reaction in which the components in the Golgi-derived vesicles (alveoli) are exocytosized into perivitelline space. Due to increased osmolarity, water comes into the peri-vitelline space, and the outer protein layer is transformed into a fertilization envelope, which is elevated and subsequently harden by cross-linking the proteins in the peri-vitelline space to prevent to polyspermy (Fig. 1-3). Based on the immunostaining of antibody against polysialic acids, PGSP was localized in the cortical alveoli of unfertilized eggs and shown to be released out to the eggs peri-vitelline space after fertilization. The protein moiety is then digested into peptides. Among the fishes PSGP, there is a variation in sialic acids compositions and strictly species-specific oligo/poly-Sia chains length and linkage pattern (Sato, C., et al. 1993). For example, the PSGP from *Oncorhynchus* contain exclusively Neu5Gc, whereas those from *Salmo* and *Salvelinus* species contain both Neu5Ac and Neu5Gc. In those species, polysialic acids chains can either be poly-Neu5Ac, poly-Neu5Gc or hybrid type in α2-5, α2-8, α2-9, and α2-8/α2-9 linkage. Furthermore, a novel deaminoneuraminic acid (3-deoxy-D-*glycero*-D-*galacto*-nononic acid, Kdn) was found to present as a cap on most of the polysialic acid chains.

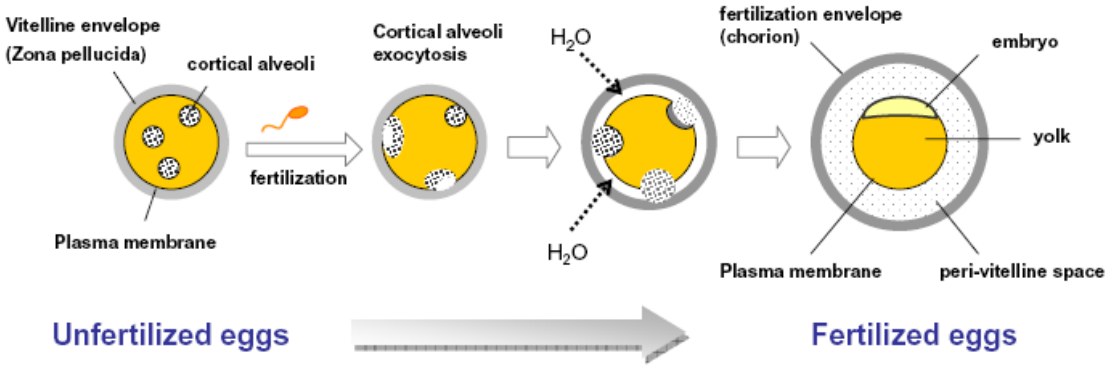

Fig. 1-3 Basic components of unfertilized and fertilized fish eggs

In mammals, polysialylated NCAM was cloned and characterized (Chuong, C.M., et al. 1982) with the help of existing antibody against bacterial polysialic acid. It was shown that polysialic acids (PSA) on NCAM was only present in embryo but not in adult (Inoue, S., et al. 2000, Sunshine, J., et al. 1987). The NCAM knockout mice had reduced olfactory bulb, and an almost total absence of sialic acid (Cremer, H., et al. 1994). During embryonic development, the polysialylated embryonic NCAM is restricted to specific migrating cell types (Fredette, B., et al. 1993). Interestingly, it was shown that the removal of polysialic acid from the NCAM influence motor neuron projections in the embryonic tissues (Tang, J., et al. 1994). The NCAM knock-out mice pointed out a defect in spatial learning and memory, due to an anomaly of the olfactory bulb and hippocampus (Cremer, H., et al. 1994), where PSA is continuously expressed in adult brain (Key, B. and Akeson, R.A. 1991, Theodosis, D.T., et al. 1991). Altogether, these results strongly suggest that polysialylation regulates the function of NCAM. It has been suggested that the role of PSA in the neonatal nervous system is in the rostral migration of large numbers of progenitor cells from their birthplace in the subventricular zone (SVZ) along a pathway to the olfactory bulb, where they become interneurons (Rutishauser, U. 2008). Although the expression of PSA is diminished in the majority of tissues in the adult, some tumors are known to re-express PSA. Thus, PSA may also represent an onco-developmental antigen.

1.5 α2,8-Sialyltransferases (ST8Sia) and polysialylation

The enzymes responsible for PSA biosynthesis were identified as α-2,8-polysialyltransferases ST8Sia II and ST8Sia IV (Nakayama, J., et al. 1995, Ong, E., et al. 1998, Tsuji, S., et al. 1996). The α2,8-sialyltransferases (ST8Sia), a subset of the animal sialyltransferases (Cazy GT-family # 29 (Coutinho, P.M., et al. 2003)), catalyze the transfer of one or several sialic acid residues onto another sialic acid residue found on glycolipids or glycoproteins.

In mammals, ST8Sia I and ST8Sia V are implicated in the biosynthesis of gangliosides. ST8Sia I (G_{D3} synthase) shows a strict specificity towards G_{M3} resulting in the formation of G_{D3}. ST8Sia V (G_{T3} synthase) sialylates different gangliosides such as G_{D3}, G_{M1b}, G_{D1a} and G_{T1b}

(Harduin-Lepers, A., et al. 2001). The human ST8Sia VI catalyzes the transfer of a single sialic acid residue on sialylated *O*-glycans of glycoproteins, leading to the formation of diSia motifs (Teintenier-Lelievre, M., et al. 2005). ST8Sia III catalyzes the transfer of one or several sialic acid residues either on glycoproteins or glycolipids (Yu, R.K., et al. 1988) and is implicated in the biosynthesis of G_{T3} (Yu, R.K., et al. 1988) and diSia motif on CD-166 (Sato, C., et al. 2002). Finally, poly-*α*2,8-sialyltransferases refer to the vertebrate ST8Sia II and ST8Sia IV. Both enzymes are expressed in the nervous system of most vertebrates where they catalyze the transfer of hundreds of sialic acid residues mainly to the *N*-glycans of N-CAM (reviewed in (Nakayama, J., et al. 1998)) resulting in an increased neuronal plasticity and migration in embryonic vertebrate embryos (Hildebrandt, H., et al. 2007). The mice deficient in these α2,8-sialyltransferases showed severe defects in the migration and differentiation of neural precursor cells in embryonic brains (Angata, K., et al. 2007).

While each mammalian ST8Sia has been studied, in fish, only ST8Sia II and ST8Sia IV (Marx, M., et al. 2007) responsible for polysialic acid synthesis, and ST8Sia III (Bentrop, J., et al. 2008, Tsuji, S., et al. 1996) have been addressed so far. Both ST8Sia II and ST8Sia IV have been reported to be expressed in the fish ovary, which suggests their possible involvement in the biosynthesis of polysialic acid chain on O-linked glycoprotein in rainbow trout (Asahina, S., et al. 2006). ST8Sia II expression seems to be restricted to the early development stages among vertebrates, while ST8Sia IV has a more extended expression, from later development stages to adulthood in mammals. More recently, the animal ortholog of human ST8Sia, including Zebrafish, was identified (Harduin-Lepers, A., et al. 2005), which paves the way to concerted functional and structural investigation of Zebrafish sialobiology.

1.6 Glycomics and glycobiology of Zebrafish

A survey of the literature prior to this thesis work revealed that very little is known about Zebrafish glycome. Using monoclonal antibody, the glycoepitope HNK-1, sulfoglucuronyl carbohydrate linked to neolactotetraose on glycolipids, has been reported to be expressed in the nervous system of early development (Becker, T., et al. 2001, Jungalwala, F.B. 1994, Metcalfe, W.K., et al. 1990, Pradel, G., et al. 1999). The occurrence of N-glycans in the chorion of fertilized eggs of Zebrafish was first suggested by lectin blot analysis. Based on positive binding of concanavalin agglutinin (Con A), Galanthus nivalis agglutinin (GNA), Sambucus nigra bark agglutinin (SNA) and Ricinus communis agglutinin (RCA 120), a 116 kDa and a 50 kDa proteins were considered to be N-linked glycoproteins (Bonsignorio, D., et al. 1996). The possible presence of Lewis X epitope can be inferred from a report on the isolation of two fucosyltransferase genes from embryonic Zebrafish (Kageyama, N., et al. 1999). Other than that, in general, very limited structural and functional studies of glycoconjugates have been focused on very specific forms of glycosylation although several classes of glycoconjugates

have been implicated roles in embryogenesis. For example, the glycosaminoglycans (GAG) were shown to express a wide range of functions during Zebrafish embryogenesis, including central nervous system (Becker, C.G. and Becker, T. 2002, Bernhardt, R.R. and Schachner, M. 2000) and muscle development (Bink, R.J., et al. 2003). The involvement of chitin oligosaccharides during Zebrafish embryogenesis was strongly suggested by the inhibition of their biosynthesis (Bakkers, J., et al. 1997) (Semino, C.E. and Allende, M.L. 2000, Semino, C.E., et al. 1998). More recently, the existence of a specific receptor for chitin tetrasaccharide that would link its activity to the Raf, MEK, and ERK pathway in Zebrafish cells was demonstrated (Snaar-Jagalska, B.E., et al. 2003).

Through PSA-specific antibodies, whole-mount immunocytochemistry, and endo-neuraminidase, PSA was first demonstrated to be expressed in the central nervous system of Zebrafish during embryogenesis, while PSA-removal affects axon growth and pathfinding (Marx, M., et al. 2001). These studies are supported by recent identification of human orthologs of sialyltransferases in Zebrafish although their actual activities and specificities remain to be demonstrated, which, in turn, needs to be supported by actual identification of sialylated glycans with their fine structures defined at the molecular level.

Recognizing this structural need and the relevance of Zebrafish as a model system for developmental glycobiology, we have initiated a mass spectrometry (MS)-based glycomic profiling of Zebrafish glycoconjugates at different developmental stages shown in Fig. 1-4, aiming to define the structures of major glycoprotein-derived glycans and glycolipids expressed by this organism (Yann et al., 2006), from which the endogenous activity of glycosylation-related enzymes, including glycosyltransferases, can be inferred.

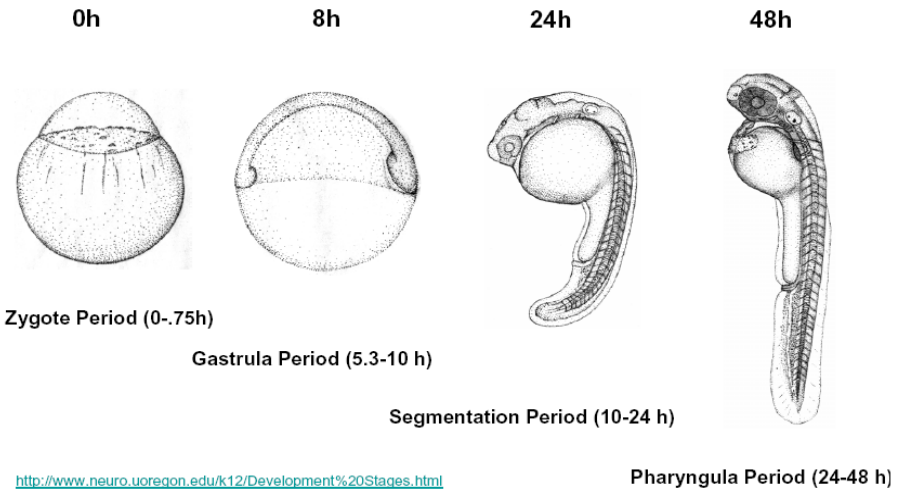

Fig. 1-4. 4 embryonic stages and the corresponding developmental periods of Zebrafish used for glycomic survey mapping (Kimmel, C.B., et al. 1995).

Among the salient findings (summarized in Fig. 1-5) which led directly to this thesis work are

1) oligosialylated lactosylceramides were found to occur only in the later stages of development;
2) mono- and disialylated mucin type O-glycans structures with novel sequence were identified, in which the disialylated O-glycans were found only in the early stages, in direct contrast with the occurrence of oligosialylated glycolipids. Based on further MS/MS and 2D NMR analyses, the detailed structures of the isolated major O-glycans were determined to be Fucα1-3GalNAcβ1-4(Neu5Gc/Acα2-3)Galβ1-3GalNAc-itol. Curiously, for the disialylated structures, MS/MS analyses showed that the inner sialic acid is always Neu5Gc, suggesting that only Neu5Gc but not Neu5Ac can be extended further;
3) the N-glycans are predominantly of high-mannose type, with very little amount of sialylated, fucosylated complex-types N-glycans also detected by MS analysis but awaiting confirmation and further structural characterization.

1.7 Specific Aims:

To complete the systematic investigation of Zebrafish as a model of developmental glycobiology, the emerging picture gleaned from the initial MS-based glycomic profiling described above needs to be substantiated from both structural and biosynthetic aspects. It is our overall goals to complete the analysis of all novel structures identified, particularly the N-glycans, and proceed to define the sialylation pattern of Zebrafish embryos, analyze the developmentally regulated oligosialylation, and characterize the corresponding α2,8-sialyltransferases, in order to understand the biological significance of sialylation during embryogenesis. Specifically, this thesis work aims to achieve the followings:

1) Further detailed structural analysis of the detected complex type N-glycans by first optimizing their recovery and focusing on defining their terminal sialylated and fucosylated epitopes (Section 3.1);

2) Molecular cloning of α2,8-sialyltransferases and enzymatic characterization of ST8Sia VI, the human ortholog sialytransferase responsible for the biosynthesis of di-sialylated O-glycans (Section 3.2);

3) Structural and biosynthetic studies of the developmentally regulated oligosialylation, focusing on analyzing the chemical nature of the oligosialic acids and to correlate their expression pattern with that of the corresponding α2,8-sialyltransferases (Section 3.3);

4) Characterization of the spatial-temporal expression pattern of the α2,8-sialyltransferases in the developing nervous system by whole-mount in situ

hybridization (Section 3.4).

2. Materials and methods

2.1 Materials

The molecular biology kits RNeasy Midi and Plasmid extraction were obtained from Qiagen (Chatsworth, CA, U.S.A.), the DMEM (Dulbecco's modified Eagle's medium) containing 4.5 g/l glucose without glutamine was from BioWhittaker Europe. TC100 medium, MEM (minimal essential medium), L-glutamine, antibiotics, geneticin G418, FCS (foetal calf serum) used in cell culture, LIPOFECTAMINETM Plus reagent and TOPO TA-cloning kit was from Invitrogen (Cergy Pontoise, France), the NucleoSpin ® RNA II kit was from Macherey-Nagel (Düren, Germany). The oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium), Sybr Green Brilliant Q-PCR master mix, and 8-well strip tubes and the MX-4000 Quantitative PCR System were from Stratagene (La Jolla, CA, USA). The first strand cDNA synthesis kit was from Amersham Pharmacia Biotech (Little Chalfont, U.K.). The experion ARN Std Sens Analysis kit was from Biorad (Marnes-la Coquette, France). Taq polymerase, 4-methylumbelliferone (4-MU) and 2'-(4methylumbelliferyl)-d-N-acetylneuraminic acid (4-MU-Neu5Ac) were from Sigma (St Louis, MO, USA). CMP-^{14}C-Neu5Ac (CMP-^{14}C-N-acetylneuraminic acid, 10.7 GBq/mmol) and First Strand cDNA Synthesis kit were from Amersham Pharmacia Biotech (Little Chalfont, U.K.). PGNase F (peptide N-glycosidase F) were from Roche (Meylan, France). HiSpeed Plasmid Midi kit was from Qiagen (Courtaboeuf, France). CMPNeu5Ac, α1-acid glycoprotein, fetuin, pFLAG-CMV-1, p3xFLAG-CMV-9 and p3xFLAG-CMV-10 and DMB (1,2-diamino-4,5-methylene dioxybenzene) dihydrochloride, aryl glycosides and Triton CF-54 were from Sigma-Aldrich (St Louis, MO, U.S.A.). Glyco® Sialidase S, Glyco® Sialidase C and Glyco® Sialidase ATM were from Glyko Inc. (Novato, CA, U.S.A.). PA-sugar chain: Neu5Gcα2-3Galβ1-4Glc-PA, Neu5Acα2-3Galβ1-4Glc-PA, and Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-PA were from Takara (Shiga, Japan). The cDNA Kidney library was kindly provided by L. Zon (ZFIN, Oregon). Embryonic cDNA of Zebrafish were generously given by Dr Gerardo Vasta (Baltimore, US) and Dr Thisse Christine (Strasbourg, France). Zebrafish embryos for glycomic survey mapping were kindly provided by Dr Huang (Taipei, Taiwan) and embryos for total RNA extraction and real-time PCR analysis were kindly provided by Dr Thisse.

2.2 Extraction and preparation of glycoconjugates

Embryos were suspended in 200 μl of water and homogenised by sonication on ice. The resulting material was dried and then sequentially extracted three times by chloroform/methanol (2:1, v/v) and three times by chloroform/methanol/water (1:2:0.8, v/v/v). Supernatants from the latter extractions were pooled, dried and subjected to a mild saponification in 0.1 M sodium hydroxide in methanol at 37°C for 3 h, and then evaporated to dryness (Schnaar, R.L. 1994). Sample was reconstituted in methanol/water (1:1, v/v) and applied to a C18 Sep-Pak cartridge (Waters) equilibrated in the same solvent system. After washing with 5 volumes of methanol/water (1:1, v/v), glycosylceramides were eluted by 5 volumes of methanol and 5 volumes of chloroform/methanol (2:1, v/v). Delipidated pellet from chloroform/methanol/water extraction was re-suspended in a solution of 6M guanidinium chloride and 5 mM EDTA in 0.1M Tris/HCl, pH 8, and agitated for 4 h at 4 °C. Dithiothreitol was then added to a final concentration of 20 mM and incubated for 5 h at 37 °C, followed by addition of iodoacetamide to a final concentration of 50 mM and further incubated overnight in the dark at room temperature. Reduced/alkylated sample was dialysed against water at 4°C for three days and lyophilized. The recovered protein samples were then sequentially digested by TPCK treated trypsin for 5 h and chymotrypsin overnight at 37°C, in 50 mM ammonium bicarbonate buffer, pH 8.4. Crude peptide fraction was separated from hydrophilic components on a C18 Sep-Pak cartridge (Waters) equilibrated in 5% acetic acid by extensive washing in the same solvent and eluted with a step gradient of 20, 40 and 60% 1-propanol in 5% acetic acid. Pooled propan-1-ol fraction was dried and subjected to N-glycosidase F (Roche) digestion in 50 mM ammonium bicarbonate buffer pH 8.4, overnight at 37°C. Alternatively, both the chloroform/methanol/water extraction and the C18 Sep-Pak purification step following tryptic digestion may be omitted to increase the yield of the sialylated N-glycans. Omission of the latter step would however increase the content of contaminant Hex polymers and possibly other free glycans. The released N-glycans were separated from peptides using the same C18 Sep-Pak procedure as described above. To liberate O-glycans, retained peptide fraction from C18 Sep-Pak was submitted to alkaline reductive elimination in 100 mM NaOH containing 1.0 M sodium borohydride at 37°C for 72 h. The reaction was stopped by addition of Dowex 50X8 cationexchange resin (25–50 mesh, H+ form) at 4°C until pH 6.5 and, after evaporation to dryness, boric acid was distilled as methyl ester in the presence of methanol. Total material was then submitted to cation-exchange chromatography on a Dowex 50X2 column (200–400 mesh, H+ form) to remove residual peptides.

2.3 Chromatographic separation of glycans

The released N-glycans were either analyzed directly or after separation into neutral and sialylated fractions on a weak anion exchanger, DEAE Sephadex A-25 column (Amersham). Samples were dissolved in 20 mM Tris/HCl, pH 8 for loading onto a column equilibrated in the same buffer. Non-binding neutral glycans were recovered in the washed through fractions while sialylated glycans were eluted in a single fraction by a 0.8 M NaCl solution in 20 mM

Tris/HCl, pH 8. Both fractions were desalted by passage through a Bio-Gel P2 column (Bio-rad) equilibrated in water. To remove the contaminating neutral N-glycans and to enrich for the sialylated components, the O-glycans were dissolved in water and fractionated on a strong anion exchanger Dowex 1x2 (200-400 mesh, HCOO- form) column pre-equilibrated in water. Neutral glycans were washed off by water while mono- and oligo-sialylated compounds were recovered by a step wise elution at 0.1 and 2 M pyridine acetate, pH 5.5, respectively. High salt fractions were desalted by passage through a Bio-Gel P2 column (Bio-Rad) equilibrated in water.

2.4 Exoglycosidase digestions

The N-glycans were digested with 20 mU of neuraminidase from *Arthrobacter ureafaciens* (Roche) in 100 μl of 50 mM sodium acetate buffer, pH 5.5, at 37°C for 16-18 h. Desialylated N-glycans were further treated with 3 mU of β1–4 galactosidase from *Streptococcus pneumoniae* (Calbiochem) in 100 μl of 50 mM sodium acetate buffer, pH 5.5, at 37°C for 12 h, before and after chemical defucosylation by 48% aqueous hydrofluoric acid at 4°C for 48 h.

2.5 Chemical derivatization

Monosaccharide compositions were determined by gas chromatography (GC)-mass spectrometry (MS) analysis as either per-hepta-fluorobutyryl (Zanetta, J.P., et al. 1999) or alditol acetate derivatives. For alditol acetates analysis, glycan samples were hydrolysed in 4 M trifluoroacetic acid (TFA) for 4 h at 100°C and then reduced with sodium borohydride in 0.05 M NaOH for 4 h. Reduction was stopped by dropwise addition of acetic acid until pH 6 was reached and borate salts were co-distilled by repetitive evaporation in dry methanol. Peracetylation was performed in acetic anhydride at 100°C for 2 h. To determine the chemical nature of sialic acids, intact sialic acids were liberated directly by mild hydrolysis in 0.01 N TFA at 50 °C and reacted with a volume of DMB reagent at 50 °C for 2 h 30 min (Hara, S., et al. 1987). The monomeric DMB-sialic acid derivatives were separated isocratically on a C18 reverse phase HPLC column (250 x 4.6mm, 5 micron, Vydac) by a solvent mixture of acetonitrile/methanol/water (7:9:84) and identified by referring to the elution positions of standard NeuAc and NeuGc derivatives. For MALDI-MS analyses, the glycan samples were permethylated using the NaOH/dimethyl sulfoxide slurry method as described by Dell *et al* (Dell, A., et al. 1994). The permethyl derivatives were then extracted in chloroform and repeatedly washed with water. GC-MS linkage analysis was performed as described previously (Suzuki, N., et al. 2003).

2.6 Analysis of oligo-sialylated sequences

In order to minimize internal fragmentation of polysialylated sequences, sialylated glycan samples were directly coupled to 1,2-diamino-4,5-methylenedioxybenzene (DMB) without prior mild hydrolysis (Sato, C., et al. 1999). Samples were incubated for 2.5 h at 50°C in 50 µl of a DMB reagent solution (2.7 mM DMB, 9 mM sodium hydrosulfite, and 0.5 mM

β–mercapto-ethanol in 20 mM TFA). 10 µl of 1 M NaOH was then added and the reaction mixtures further incubated in the dark at room temperature for 1 h. Samples were stored at 4°C before analysis. DMB-derivatized sialic acid oligomers were separated on a HPLC apparatus fitted with either an anion exchanger column, mono-Q (Amersham-Biosciences), or a CarboPac PA-100 column (Dionex). For mono-Q column, the sample was loaded and eluted with a flow rate of 0.5 ml/min with 20 mM Tris–HCl (pH 8.0), followed by a NaCl gradient (0-10 min, 0 M; 10-60 min, 0 to 0.6 M; 60-65 min, 0.80 M) in 20mMTris-HCl (pH 8.0). CarboPac column was eluted at 1 ml/min with a concentration gradient of 2 to 32 % of 1M NaNO3 in water. In both systems, elution was monitored by an on line fluorescence detector set at wavelengths of 373 nm for excitation and 448 nm for emission. Periodate oxidation and C7/C9 analyses for oligosialyl linkage determination were performed essentially as described by Sato *et al.* (Sato, C., et al. 1998). Briefly, samples were dissolved in a mixture of 25 µl of 40 mM sodium acetate buffer (pH 5.5) and 2 µl of 0.25 M sodium metaperiodate and left at 0°C for 45 min in the dark. 5 µl of 5% glycerol was then added and allowed to react for another 40 min at 0°C, followed by 32 µl of 0.2 M sodium borohydride in 0.2 M sodium borate buffer (pH 8.0) and left overnight at 0°C. Finally, TFA was added to a final concentration of 1 M and incubated at 80 °C for 1 h before subjected to DMB derivatization. To determine the chemical nature of sialic acids, intact sialic acids were liberated directly by mild hydrolysis in 0.01 N TFA at 50 °C and reacted with a volume of DMB reagent at 50 °C for 2 h 30 min. The monomeric DMB-sialic acid derivatives were separated isocratically on a C18 reverse phase HPLC column (250 x 4.6mm, 5 micron, Vydac) by a solvent mixture of acetonitrile/methanol/ water (7:9:84) and identified by referring to the elution positions of standard Neu5Ac and Neu5Gc derivatives. For additional MS analysis, the DMB-derivatives were separated instead with a gradient of acetonitrile/methanol/water (7:9:84) mixture in water (0-10 min, 10%; 10-40 min, 10 to 100%). Fluorescence-detected peaks were individually collected on ice and immediately freeze dried. Samples were then reconstituted in 10 µl of water and analysed by nanoESI-MS and MS/MS on an LCQ DK XP+ ion trap (Thermo Finnigan).

2.7 Mass spectrometry analyses of glycans and glycolipids

For MALDI-TOF MS glycan profiling, the permethyl derivatives in acetonitrile were mixed 1:1 with 2,5-dihydroxybenzoic acid (DHB) matrix (10 mg/ml in acetonitrile), spotted on the target plate, air-dried and recrystallized on-plate with ethanol whenever necessary. Data acquisition 20 was performed manually on a benchtop M@LDI LR system (Micromass) operated in the reflectron mode. For DHB matrix, the coarse laser energy control was set at high and fine adjusted using the % slider according to sample amount and spectra quality. Laser shots (5 Hz, 10 shots/spectrum) were accumulated until a satisfactory signal to noise ratio was achieved when combined and smoothed. Glycan mass profiling was also performed on a dedicated Q-Tof Ultima MALDI instrument (Micromass) in which case the permethylated samples in acetonitrile were mixed 1:1 with α–cyano-4-hydrocinnamic acid matrix (in acetonitrile:0.1% TFA, 99:1 v:v) for spotting onto the target plate. The nitrogen UV laser (337 nm wavelength)

was operated at a repetition rate of 10 Hz under full power (300 μJ/pulse). MS survey data were manually acquired and decision to switch over to CID MS/MS acquisition mode for a particular parent ion was made on-the-fly upon examination of the summed spectra. Argon was used as the collision gas with a collision energy manually adjusted (between 50~200 V) to achieve optimum degree of fragmentation for the parent ions under investigation. Data acquisition on 4700 Proteomic Analyzer (MALDI-TOFTOF) instrument was performed using the 2,5-dihydroxybenzoic acid (DHB) matrix (10 mg/ml in water, mixed 1:1 with sample dissolved in acetonitrile) . A typical data acquisition comprises a total of 20 sub-spectra of 125 laser shots each with laser energy normally set at 4500 (Yu, S.Y., et al. 2006). The potential difference between the source acceleration voltage and the collision cell was set at 2 kV to obtain the desirable high energy CID fragmentation pattern. The indicated collision cell pressure was normally increased from 3.0×10^{-8} torr (no collision gas) to 5.0×10^{-7} torr (argon).

2.8 RNA extraction and cDNA synthesis

Danio rerio unfertilized eggs and embryos (0, 6, 14, 24 and 36 phf) kindly provided by the Thisses' Lab were sampled (200 embryos) and snap frozen in liquid nitrogen. Total cellular RNA was extracted from embryos at various developmental stages using the RNeasy Midi kit according to the manufacturer's instructions. Total RNA purity was established by calculating the ratio of the absorbance readings at 260 and 280 nm and quantified using the NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). The integrity and purity of the extracted RNA was also analyzed by means of gel electrophoresis on a bioanalyzer (Experion, Biorad). Total RNA (1.2 μg) was reverse transcribed using the first strand cDNA synthesis kit in 33 μl following the manufacturer's instruction. RNA samples were tested for genomic DNA contamination by PCR amplification of Zebrafish β-actin (GenBank accession number AF025305 (Suzuki, N., et al. 2003), using oligonucleotide primers designed in two distinct exons (Table 1) and aerosol contamination by including no template controls (NTC). Another set of developmental stages cDNAs were kindly prepared by H. Ahmed and G. Vasta as before (Ahmed, H., et al. 2004).

2.9 Real-time PCR of ST8Sia genes during development in Zebrafish

Primers used for quantitative Q-PCR (Table 1) were designed in the coding region of previously identified Zebrafish ST8Sia genes (Harduin-Lepers, A., et al. 2005) using the Primer Premier version 31.1 software (Primer Premier, Biosoft International, Palo Alto, USA). Each primer pair was carefully selected so to give rise to an amplified DNA fragment of about 300 bp and such that their Tm values were very close (around 51°C). The suitability of the primers for their uniqueness to amplify a single PCR product was assured by regular end-point PCR (Denaturation step at 94°C for 2 min followed by 38 cycles at 95 °C 1 min; 50 °C 1 min; 72 °C 1 min and an elongation step at 72°C for 10 min) using cDNA kidney library provided by L. Zon. The amplified products were subsequently run on an agarose gel, sub-cloned in TOPO TA cloning vector and finally, fully sequenced (Genoscreen, Lille). The TOPO plasmids containing

the amplified regions of the targeted genes were amplified, purified and quantified by nanodrop and used for the establishment of a standard curve for absolute quantification. Efficiency of target amplification for each primer set (ST8Sia I, ST8Sia II, ST8Sia III, ST8Sia IV, ST8Sia V, and ST8Sia VI in Table 1) was optimized by real-time PCR performed in a Stratagene MX4000 by trialing several final primer concentrations. Each 25 µl Q-PCR master mix contained 12.5 µl 2X Master Mix (Brilliant® SYBR® Green Q-PCR Master Mix (Stratagene, CA)), 150 nM of each primer, and 5µl of diluted cDNA (equivalent to 100 ng total RNA) extracted from 0, 6, 14, 24 hpf embryos and the real-time quantitative PCR were the thermal cycling program consisted of 10 min at 95°C followed by 45 cycles of 30 sec at 95°C, 1 min at 50°C and 30 sec at 72°C and this was followed by a melting step consisting of heating from 50 °C to 95°C at an increment of 1°C per 30 sec to check the specificity of the amplified product. PCR for all the samples were carried out in triplicate in 8-well strip tubes and data were expressed as means +/- SD. The reactions were quantified by selecting the amplification cycle when the PCR product of interest was detected (threshold cycle, Ct). Calibration curves were generated by 10-fold serial dilution of *Hind* III linearized TOPO plasmids containing the amplified regions of the targeted genes (from 2 x 10^5 copies to 2x 10^1 copies). The same PCR master mix and thermocycler conditions as described above were used and plasmid standard curve equations were used to calculate the absolute copy number of each gene. The amplification efficiencies of each calibrator were found to be between 95.9% and 100.5%. We used absolute quantification relying on the serial diluted DNA fragment with known concentration, called calibrators, which were amplified from cDNA of 24-hpf embryos with the same primers.

Table I. Primers of *ST8Sia* genes for real-time PCR. Primer nucleotide sequences, expected amplicon size, Accession number in GeneBank for the identified sialyltransferases and actin sequences are indicated.

ST8Sia	Primer sequence	Accession number (GenBank)	PCR Product size (bp)
ST8Sia I	Forward 5'-TTGCGGTTACTAAGGAGA Reverse 5'-ACGAAAGATTTGCGGGAC	AJ715535	346
ST8Sia II	Forward 5'-GACTCGCACGACTTTGTT Reverse 5'-TGGTTGGTCAGCCAGTAA	AY055462	335
ST8Sia III	Forward 5'-AACAACCTGCTGACCATCC Reverse 5'-ATGATACGGCAGCTCCTT	AJ715543	354
ST8Sia IV	Forward 5'-TCTTGACTTGGGAGTTGG Reverse 5'-TCTGACCGCAATCCTACA	AJ715545	366
ST8Sia V	Forward 5'-AAATAAGGAGGAGACGGATAA Reverse 5'-AAAGTCAGAAGCGTCAAT	AJ715546	291
ST8Sia VI	Forward 5'-TGTCTATGATGGCGAAAG Reverse 5'-TGACCGTATGAATGAAGG	AJ715551	333
β-actin	Forward 5'-GTTGGTATGGGACAGAAAGA Reverse 5'-GGCGTAACCCTCGTAGAT	AF025305	378

2.10 Sialidases assays

Danio rerio embryos (0, 8, 24 and 48 hpf) and unfertilized eggs were sampled (500 embryos) and snap frozen in liquid nitrogen. The eggs were homogenized in 500 µl of distilled water, then different amount of total cell lysate corresponding to 1 to 60 eggs, were mixed with 0.2 mM 4-MU-Neu5Ac in 50 mM sodium acetate buffer (Chang, L.Y., et al. 2008 6 and 7) in a final volume of 250 µl. Protein concentration used were determined using the micro BCA TM protein assay reagent kit (Thermo Scientific Pierce, Rockford, USA). The incubation was performed at 37^{o}C. At 0.5, 1, 2 and 4 h, 30 µl of the reaction mixture was taken back, and the reaction was quenched by adding 120 µl 0.5M Na_2CO_3. The released 4-methyl-umbeliferone (Finne, J., et al.) was measured and quantified by fluorescence detector at 360 nm for excitation, 460 nm for excitation. Sialidase activity was calculated according to a MUN standard curve. For the assay of different compartments of fertilized eggs, first put 40 eggs in 4 ml 50 mM sodium acetate buffer (pH 5.5), and the fertilization envelope (chorion) was partially ripped (enable the contact of peri-vitelline content of 4-MU-Neu5Ac) with tweezers under anatomic microscopy and centrifuged. The endogenous sialidase activities were assayed with 3 sets, 1) the supernatant after centrifugation as “peri-vitelline content”, 2) the supernatant together with pellet named “peri-vitelline content plus chorion” and 3) the whole egg homogenate as “peri-vitelline content, chorion, and embryo”, at the final volume of 500 µl. 100 µl of the each reaction mixture was taken out at 0.5, 1, 2, 4h, and added 150 µl 0.5M Na_2CO_3 to stop the reaction. The released 4-methyl-umbeliferone (Varki, N.M. and Varki, A.) was measured and quantified by

fluorescence detector at 360 nm for excitation, 460 nm for excitation.

2.11 Cloning, expression, and transfection of ST8Sia VI

Full length ST8Sia VI was amplified from a kidney cDNA library in ZAP kindly which was provided by L. Zon (Boston Children Hospital, BOSTON, USA) with the primers for full length ST8Sia VI (sense: 5'- AGAGCGGCAGCAGCATCTG, and anti-sense: 5'-CATTTCCCACCAGC CTCGT) and N-terminal truncated ST8Sia VI (sense: 5'-CATCTCCAAGAATTCTGTAATCCCT CATCCTGC and, anti-sense: 5'-TCCCACCAGGTACCTGTTTCATCTATGAGCGG) cloned into pCR®2.1-TOPO® vector (Invitrogen) and fully sequenced (Genoscreen) to confirm its sequence. Excised by EcoRI and KpnI, N-terminal truncated ST8Sia VI was subsequently cloned to the expression vectors, pFlag-CMV™-1, p3xFlag-CMV™-9, and p3XFLAG-CMV™-10, while the full length ST8Sia VI was cloned to p3xFlag-CMV™-9 and p3XFLAG-CMV™-10 (Sigma). pFlag-CMV or p3XFlag-CMV vectors (5 μg) with or without full-length or N-terminal truncated ST8Sia VI were transiently transfected into COS-7 cells in 100 mm diameter dishes using LIPOFECTAMINETM Plus reagent, according to the manufacturer's instructions. 10mM sodium butyrate was added into the medium to activate the production of recombinant ST8Sia VI 16h before harvesting the medium. The medium was harvested 48 h after transfection and the recombinant protein expressed in the medium was used as the enzyme source for sialyltransferases assays. The expressions of recombinant ST8Sia VI in the culture medium and cell lysates, with or without PNGaseF digestion, were confirmed by western blot by monoclonal antibody M2 against Flag tag (Invitrogen).

2.12 Enzymatic assay of ST8Sia VI

Sialyltransferase assays were performed in 100 mM cacodylate buffer (pH 6.2) containing 10 mM MnCl2, 0.2% Triton CF-54, 40 μM CMP-[^{14}C]Neu5Ac (1.94 KBq) and one of the acceptor substrates (2 mg/ml for glycoproteins, 5 to 10 pM for PA-sialyllactose) and 23 μl of the enzyme source in a total volume of 50 μl. Unless stated otherwise, the reactions were performed at 32◦C for overnight. For glycoproteins, the reactions were stopped by addition of 2.5×SDS sample buffer and the reaction products were separated by SDS/PAGE. After transfer onto nitrocellulose membrane (Biotrace, Pall Corporation) the radioactive products were detected and quantified by radio-imaging using a PersonalMolecular Imager FX (Bio-Rad, France). For linkage analysis of sialic acids, [^{14}C] Neu5Ac-labelled sialylated fetuin was treated with specific sialidases at 37◦C for 1 h, according to the manufacturer's instructions (Glyko Inc.). For analysis of the glycan acceptor, sialylated fetuin was incubated with PGNase F at 37◦C for 2h according to the manufacturer's instructions. Further examination of the degree of polymerization sialic acid of the reaction product and colominic acids were subsequently labeled with DMB by incubated for 2.5 h at 50◦C in 50 μl of DMB reagent, then separated by HPLC with different column [Amide, CarboPac PA 200 (Dionex), mono-Q (Amersham-Pharmacia)] and detected as described above. The samples were automatically collected and subsequently counted for radioactivity.

2.13 Molecular cloning of full-length Zebrafish ST8Sia

Zebrafish ST8Sia full-length open reading frame were cloned using a PCR based protocol from kidney cDNA library (ST8Sia I, ST8Sia II, ST8Sia VI), from total RNA isolated from 36 hpf embryos (ST8Sia III) or from adult brain (ST8Sia IV and ST8Sia V) (Table II). Based on the gene nucleotide sequences determined in silico, primers (Eurogentec, Herstal, Belgium) were designed to the 5´- and 3´-UTR of each ST8Sia gene (Table 1). Additional primers within the previously described open reading frame (data not shown) were necessary to amplify overlapping regions within the coding sequences of ST8Sia IV and ST8Sia V and the resulting overlapping PCR products were subsequently annealed. PCR amplifications were carried out with hot-star Qiagen Taq polymerase (Qiagen, Courtaboeuf, France) using the buffer solution provided by the manufacturer. Annealing temperatures varied from 50 to 52°C. Fulllength sequences were subcloned in the pCR(R)2.1-TOPO vector (TOPO TA Cloning, Invitrogen, Cergy Pontoise, France) and nucleotide sequence was confirmed by sequencing (Genoscreen, Lille, France).

2.14 RNA probes

The cDNA fragments were then cloned in pGEM-T vector (Promega, Charbonnières-les-Bains, France). Linear templates were generated from this construct by performing a PCR amplification of the cDNA using the following primers: 5'-GGATCCATTAACCCTCACTAAAGG GAATTTAGGTGACACTATA (that contains a T3 RNA polymerase promoter) and 5'-TAATAC GACTCACTATAGGG. After purification these templates have been used to generate the RNA probes. Both antisense- and sensedigoxigenin-labeled RNA probes were obtained using T7 or T3 RNA polymerase. After synthesis, RNA probes were purified with an RNA purification kit (Macherey-Nagel, Hoerdt, France) and checked for purity by denaturing agarose gel electrophoresis. Synthesized control sense probes gave no staining after whole-mount in situ hybridization (ISH).

2.15 Whole mount mRNA in situ hybridization

In situ hybridization was performed as described (Thisse, B., et al. 2004, Thisse, C., Thisse, B 1998, Thisse, C. and Thisse, B. 2008). Embryos from AB/TU fish (a strain generated from crosses of two wild-type lines, AB and TU) are collected, dechorionated by pronase treatment, allowed to develop at 28.5°C until the appropriate stage and then fixed by incubation over night in 4% paraformaldehyde at 4°C. Embryos older than 24 hpf are incubated in 0.3× Danieau medium supplemented with 1-phenyl-2-thiourea (PTU, 0.003%) to prevent accumulation of pigment. After fixation, embryos are dehydrated and stored at −20°C in 100% methanol prior to ISH. The labeling reaction is monitored under a dissecting microscope and the reaction is stopped with 1× PBS at pH 5.5. Embryos are then mounted under a coverslip in 100% glycerol and incubated at least 24 h in the dark at room temperature prior to observation. Pictures are taken using a color CCD camera (Roper Scientific, Coolsnap) mounted on a

dissecting microscope (Leica, M420) or on a compound microscope (Leica, DM RA2HC or Nikon, FXA).

Table 1 Primer nucleotide sequences used for amplification of full-length open reading frame of zebrafish ST8Sias

	Human				Zebrafish			
ST8Sia	GB Acc. #	Chromosome	Primer	Sequence	cDNA source	Product (bp)	GB Acc. #	Chromosome
ST8Sia I	L32867, D26360, X77922	12p12	Forward	5′-TTTCTTGCAAT ACATCGGCGG	Kidney	1,095	AJ715535	4
			Reverse	5′-GTATTCTCATGC CTGTTGCAG				
ST8Sia II	U91641	15q26	Forward	5′-TTGCCCTG CGTTAGGAACCA	Kidney	1,271	AY055462	18
			Reverse	5′-TGTGTGTGTA AGGTCCAGTG				
ST8Sia III	AF004668	18q21	Forward	5′-CTGAAAGGAT GCGGGTTTCC	36 h embryos	1,252	AJ715543	24
			Reverse	5′-CATTAGTGCA ACCGAGACTC				
ST8Sia IV	L41680	5q21	Forward	5′-AGATGAGATGG GTGGTTATG	Brain	1,209	AJ715545	10
			Reverse	5′-ACAATGCCA AATTGGTCGAC				
ST8Sia V	U91641	18q12	Forward	5′-CCCGTGAGA ACGGTAAACAC	Brain	1,214	AJ715546[a], AM287263, AM287264	21
			Reverse	5′-TAAAGCACCT GCGACTAGCG				
ST8Sia VI	AJ621583	10p12	Forward	5′-AGAGCGGCA GCAGCATCTG	Kidney	1,183	AJ715551	3
			Reverse	5′-CATTTCCCACC AGCCTCGT				
ST8Sia VIIA	Not identified	Not identified	Forward	5′-TTTCCTGGTG GTCCTGAT	Ovary	1,101	AM287257	23
			Reverse	5′-CCTGTAATGTG CTCTCACATG			AM287258	23

Specific ST8Sia primer sets used for RT-PCR amplification of the full-length ORF of each zebrafish ST8Sia from various cDNA sources. Human ST8Sia cDNA and their zebrafish counterparts are indicated with corresponding GenBank accession numbers (GB Acc. #). In zebrafish, ST8Sia VII gene is duplicated on chromosome 23 (ST8Sia VIIA and ST8Sia VIIB) and ST8Sia VIIB is not transcribed in the zebrafish tissues studied

[a] The three accession numbers of zebrafish ST8Sia V correspond to splice variants of the transcript in the region encoding the stem region of the protein

3. Result

3.1 Glycomic survey mapping of Zebrafish identified unique sialylation pattern

Our overall glycomic survey mapping strategy involved sequential extraction of glycolipids and glycoproteins and the subsequent sequential release of *N*- and *O*-glycans from the proteolytic-digested peptides/glycopeptides mixtures, for matrix-assisted laser-desorption ionization-MS (MALDI-MS) and MS/MS analyses. The released glycans were permethylated to allow more informative MS/MS sequencing, but native glycans were also analyzed where sample amount permitted. Such approach, in general, gives a good representative profile of the glycome but does not optimize for the yield of any particular class of glycoconjugates. It provides the first picture, uncovers any novel structural features, and facilitates subsequent more detailed investigations. Typically, the fertilized eggs at five distinctive developmental stages, 0.5, 8, 24, 45, and 48 h, were analyzed to allow a fair assessment of possible developmental regulation from first cell stage to hatching. Any significant differences were noted while common features were reported without distinguishing the origin of sample stage. The O-glycans and GSL were further characterized by Yann et al (Guerardel, Y., et al. 2006) and the major findings have been described in the introduction (Section 1.5). Only the N-glycans work will be described in detail here. However, all results will be discussed in Section 3.1.2 together to give a complete picture.

3.1.1 Identification of the major N-*glycans*

Typically, the total N-glycans were obtained after three steps: 1) the fertilized Zebrafish eggs were homogenized and the glycolipids were extracted out with organic solvent, 2) the delipidated protein pellet were solubilized in guanidinium chloride and then digested into peptides after reduction/alkylation and dialysis, and 3) the N-glycans were released by PNGaseF and separated from the peptides by Seppak C18. As shown in Fig. 3-3-1, such a protocol typically resulted in substantial amout of contaminating poly-Hex peaks (labeled as G) being detected. This can be rid of by first passing the tryptic digest through Seppak C18 as the hydrophilic poly-Hex could be collected into the unbound and washed fractions. However, it was subsequently found that this additional step actually resulted poorer recovery of the sialylated glycans of interest, probably due to non-binding of the highly hydrophilic glycopeptides. Consequently, this step was omitted in getting the result presented below. Aiming to confirm the glycan sequence, the recovery of sialylated N-glycans were further optimized by 1) omitting to clean glycopeptides by passing through seppak C18 cartridge after tryptic digestion in order to eliminate the loss of negatively charged glycopeptides and 2) enriching sialylated complex-type N-glycans by amine cartridge through which the neutral (high mannose-type and polyHex contaminants) and charged (sialylated) glycans can be fractionated.

MALDI-MS profiling of the permethyl derivatives of *N*-glycans released from the total Zebrafish embryo extracts afforded five major peaks at *m*/*z* 1579, 1783, 1988, 2193, and 2397, corresponding respectively to sodiated molecular ions, $[M + Na]^+$, of the composition $Hex_{5-9}HexNAc_2$ (Fig. 3-1-1A). Further collision-induced dissociation (CID)-MS/MS analysis and treatment with α-mannosidase (data not shown) demonstrated that these major signals are indeed the common high–mannose-type structures. In addition, several signals of lower intensity were visibly present among which a prominent cluster at *m*/*z* 3551, 3581, and 3611 could be tentatively assigned as $[M + Na]^+$ of $Neu5Ac_2Fuc_2Hex_7HexNAc_4$, $Neu5Ac1Neu5Gc_1$ $Fuc_2Hex_7HexNAc_4$, and $Neu5Gc_2Fuc_2$ Hex_7 $HexNAc_4$, respectively. These unusual compositions were shown by MALDI-MS/MS analyses to be biantennary complex-type structures with monosialylated Hex_2(Fuc)HexNAc sequence on both antennae (Fig. 3-1-2). Both parent ions at *m*/*z* 3551 and 3611 afforded similar consecutive losses of terminal sialic acid residues and monosialylated antennae. Importantly, after losing both sialylated antennae, a common fragment ion at *m*/*z* 1143 corresponds to the sodiated trimannosyl core, $Man_3GlcNAc_2$, containing two free OH groups and thus confirming their biantennary nature.

For the $Neu5Ac_2$-containing parent (*m*/*z* 3551), a primary sodiated b ion, $Neu5Ac_1Hex_2$(Fuc) HexNAc, was detected at *m*/*z* 1225, accompanied by a $Neu5Ac^+$ oxonium ion at *m*/*z* 376 and a sodiated c ion, Neu5Ac-Hex2-OH, at *m*/*z* 824 (Fig. 3-1-2B). In comparison, the $Neu5Gc_2$-containing parent (*m*/*z* 3611) afforded a sodiated b ion, $Neu5Gc_1Hex_2$(Fuc)HexNAc, at *m*/*z* 1255, a Neu5Gc+ ion at *m*/*z* 406, and a sodiated c ion, Neu5Gc-Hex_2-OH, at *m*/*z* 854 (Fig. 3-1-2C). Further loss of the distinguishing Neu5Ac/Neu5Gc residue from the respective primary b and c ions degenerated the mass difference and yielded the common secondary fragment ions at *m*/*z* 850 and 449, corresponding to $(HO)_1Hex_2$-$(Fuc)_1$HexNAc and $(HO)_1Hex_2$-OH, respectively. The mass difference of 60 u between the two parents could thus be unambiguously attributed to a Neu5Ac and Neu5Gc difference (30 u) on each of the two monosialylated antennae. It could be further deduced that the third molecular ion signal (*m*/*z* 3581) in between the $Neu5Ac_2$-and $Neu5Gc_2$-containing parents corresponds to a similar biantennary complex-type structure carrying a Neu5Acand a Neu5Gc-sialylated antennae.

Higher in mass (Fig. 3-1-1A) and of even lower abundance was another cluster of molecular ion signals which could be assigned as trisialylated triantennary complextype structures with similar monosialylated terminal sequence carrying the Neu5Gc/Neu5Ac heterogeneity. Thus the signal at *m*/*z* 4830 corresponds to species with all three Hex_2(Fuc)HexNAc antennae sialylated by Neu5Gc, whereas the one at *m*/*z* 4740 carries only Neu5Ac-Hex_2(Fuc)HexNAc antenna. Supporting data were obtained when after desialylation by neuraminidase, a peak at *m*/*z* 3656 was detected which corresponds to [M +Na]+ of a triantennary complex-type structure with three Hex_2-$(Fuc)_1$HexNAc antennae. The corresponding desialylated biantennary structure was observed as a major sodiated molecular ion at *m*/*z* 2827 (Fig. 3-1-1B). Interestingly, after an overnight (>12 h) digestion, Neu5Ac desialylation appeared to

be more complete than removal of Neu5Gc. Additional molecular ions corresponding to incompletely digested mono-Neu5Gc sialylated bi- and triantennary structures were detected at *m*/*z* 3218 and 4048, respectively (Fig. 3-1-1B), but not their mono-Neu5Ac-sialylated counterparts which could only be observed if the neuraminidase digestion was kept to a shorter period (data not shown).

In accordance with MS/MS sequencing of the sialylated counterparts described above, MALDI-MS/MS on the desialylated biantennary structure yielded prominent nonreducing terminal primary fragment ions at *m*/*z* 463 and 864 (Fig. 3-1-2A), corresponding to sodiated c ion, Hex_2-OH, and b ion, Hex_2-(Fuc)HexNAc, respectively. The Fuc substitution could be deduced as 3-linked to the HexNAc based on the detection of the secondary ions produced through the elimination of the Fuc (minus 206 mass units from the parent and other major primary fragment ions), whereas the 246 elimination of ±Neu5Ac/Neu5Gc-Hex_2 was not observed. Further confirmation was obtained when the desialylated structures were digested with β4-galactosidase. MALDI-MS and MS/MS analyses demonstrated that one Hex was removed from each of the nonreducing termini, whereas prior defucosylation with aqueous hydrofluoric acid afforded bi- and triantennary structures with Hex_2-HexNAc termini (Fig. 3-1-1C) which could then be completely degalactosylated by β4-galactosidase (data not shown). The failure to remove the internal Gal attached to a fucosylated GlcNAc is consistent with the well-known selectivity of the β4-galactosidase acting on a Gal-(Fuc)GlcNAc unit. Linkage analysis on the isolated sialylated structures (see *Materials and methods*) further showed that the amount of terminal Gal relative to 2-linked Man or 3,6-linked Man did not change significantly before and after desialylation. Moreover, mono-substituted Gal residue was not detected. Instead, 3,4-linked Gal was quantitatively converted to 4-linked Gal after desialylation, therefore indicating that the sialic acid was attached to the 3-position of an internal 4-linked Gal and not to the terminal Gal.

Taken together, the data unambiguously defined the common monosialylated terminal sequence on each antenna of the major bi- and triantennary complex-type. *N*-glycan structures as Galβ1-4(Neu5Gc/Neu5Acα2-3)Galβ1-4(Fucα1-3)GlcNAc, namely an internal Lewis x unit which was further galactosylated and sialylated with Neu5Gc or Neu5Ac. A very small amount of incomplete sialylation could be detected (Fig. 3-1-1A) as monosialylated bi- (*m*/*z* 3189/3219) and disialylated triantennary structures (*m*/*z* 4439), as well as species that lack both sialic acid and Gal residues on one of the antennae (*m*/*z* 2985/3015; 4235). Curiously, the major disialylated biantennary structures were found to occur also as minor species lacking the reducing terminal GlcNAc, giving sodiated molecular ion signals at *m*/*z* 3305/3335/3365 (Fig. 3-1-1A). CID MS/MS analysis of its desialylated counterpart (*m*/*z* 2582; Figure 3-1-1B) firmly established that the same antennary sequence is carried on the implicated $Hex_3HexNAc_1$ core in place of the usual $Man_3GlcNAc_2$ for *N*-glycans (data not shown).

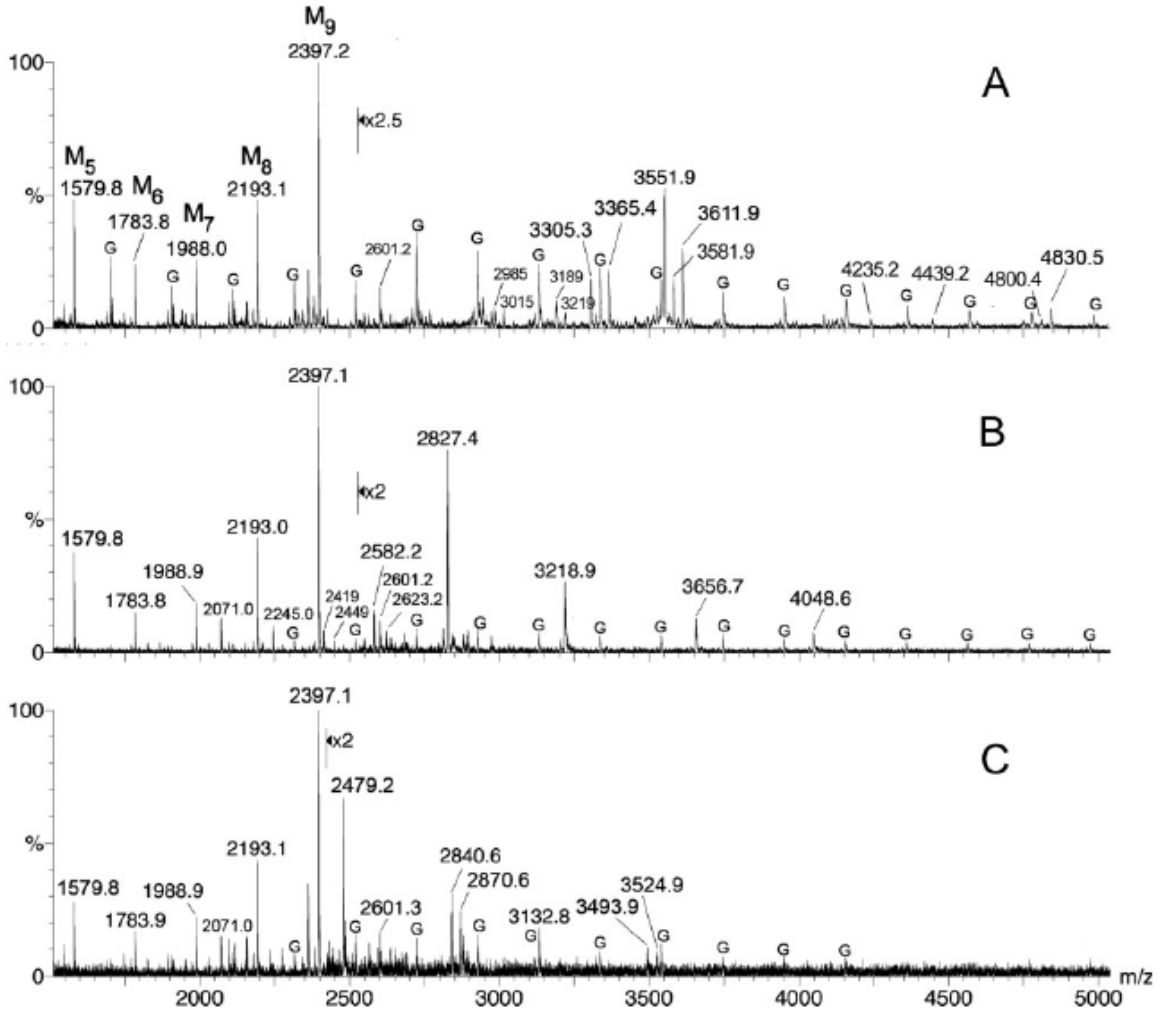

Fig. 3-1-1. MALDI-MS profiles of the permethylated *N*-glycans from Zebrafish embryos before (**A**) and after neuraminidase (**B**) or after aqueous hydrofluoric acid (**C**) treatment. High–mannose-type structures were labeled M_5–M_9 in (**A**), representing $Man_{5\text{-}9}GlcNAc_2$ structures. Signal at *m*/*z* 2601 most likely corresponds to $Glc_1Man_9GlcNAc_2$. Glucose oligomer contaminants were labeled G. In panel **B**, additional minor signals corresponding to nonsialylated biantennary structures with incomplete fucosylation and/or galactosylation were also detected (*m*/*z* 2071, 2245, 2419, 2449, 2623), the smallest of which at *m*/*z* 2071 could be assigned as nonfucosylated biantennary *N*-glycan with simple Gal-GlcNAc termini. In panel **C**, complete defucosylation and desialylation produced the bi- (*m*/*z* 2479) and triantennary (*m*/*z* 3132) structures carrying $Hex_2HexNAc$ termini, accompanied by their mono-Neu5Ac/Neu5Gc sialylated counterparts at *m*/*z* 2840/2870 and 3492/3524, respectively. Under the conditions employed, aqueous hydrofluoric acid would remove α2,3,4-Fuc almost completely but sialic acid only partially. For simplicity, other minor products corresponding to a combination of incomplete galactosylation and desialylation were not labeled.

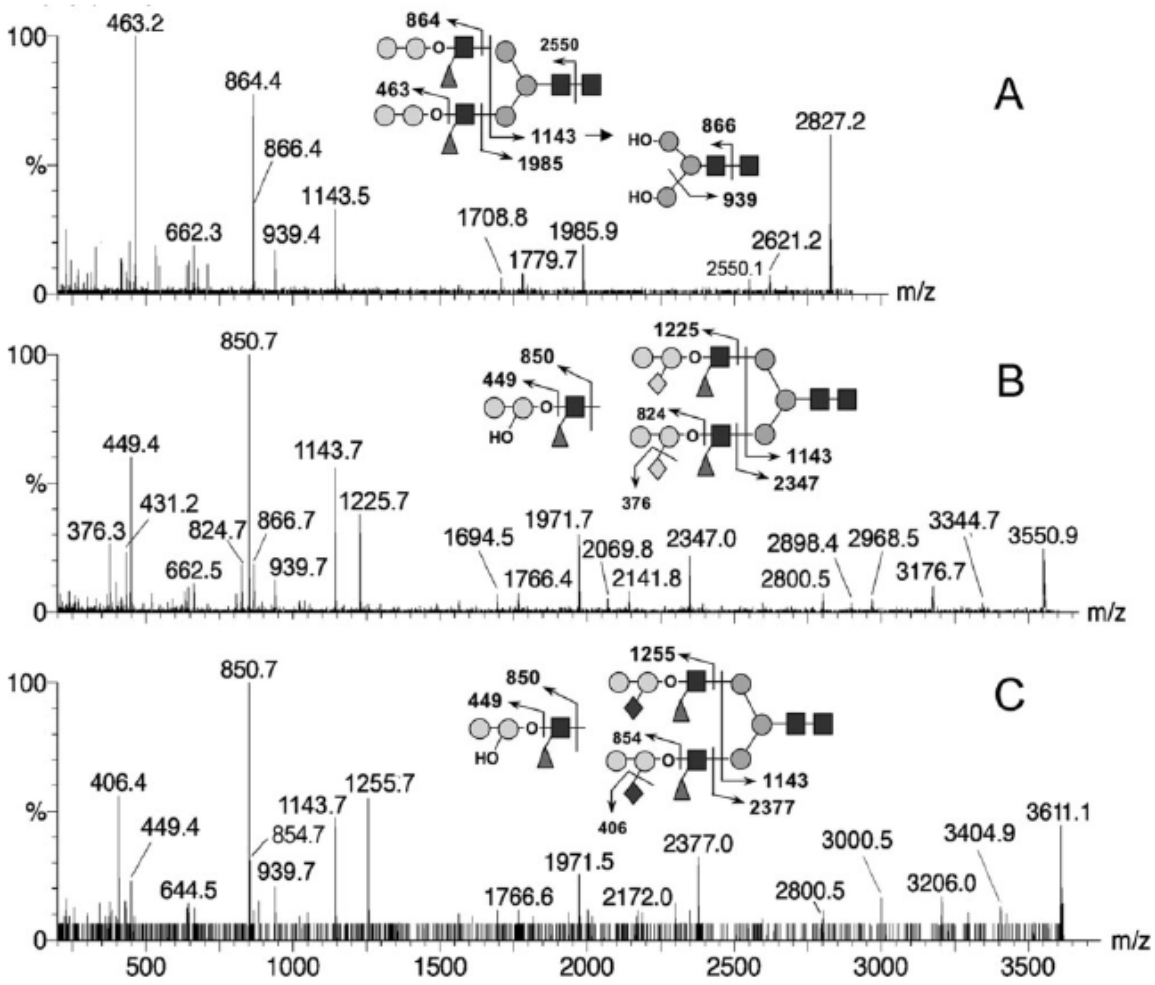

Fig. 3-1-2. MALDI-MS/MS sequencing of the biantennary complex-type *N*-glycans from Zebrafish embryos. (**A**) MS/MS on the desialylated parent at *m*/*z* 2827; (**B**) MS/MS on the Neu5Ac-disialylated parent ion at *m*/*z* 3551; (**C**) MS/MS on the Neu5Gc-disialylated parent ion at *m*/*z* 3611. Major fragmentation pattern is as indicated schematically. Cleavage ions are mostly b and y ions except when indicated with an oxygen atom which correspond to c ions. All three gave common trimannosyl core ions at *m*/*z* 1143 as shown in panel **A** which yielded additional common fragment ions (*m*/*z* 939, 866, 662). Elimination of Fuc (minus 206 u) from parent and primary fragment ions are commonly observed, whereas loss of terminal Neu5Ac yielded signals at *m*/*z* 3176, 2800 (from 3550), and 1971 (from 2347) in panel **B**; loss of terminal Neu5Gc gave signals at *m*/*z* 3206, 2800 (from 3611), and 1971 (from 2377) in panel **C**. Symbols used are square, HexNAc; circle, Hex; diamond, Neu5Gc (dark) and Neu5Ac (light); triangle, Fuc. OH denotes exposed hydroxyl group because of cleavage on the permethyl derivatives.

3.1.2 Discussion

A MS-based glycomic analysis provides several advantages in defining the glycosylation patterns when applied to lower organism such as Zebrafish whose glycobiology is under

investigation. Of prime consideration, the detection and tentative compositional assignment, including *de novo* sequencing, is not dependent on standard references and hence more conducive to identification of novel structures than any other methods. In this context, MALDI-MS mapping coupled with facile CID MS/MS sequencing on the permethyl derivatives is by far the most informative and sensitive analytical strategy. As a first attempt, we have successfully derived an overall picture of Zebrafish glycome, as presented on both the glycoproteins and the glycolipids (Fig. 3-1-3 upper), but have excluded analysis on the GAG or the chitin oligosaccharides. Our collective results show a diverse oligosialylation pattern which appears to be developmentally regulated (Fig. 3-1-3 lower).

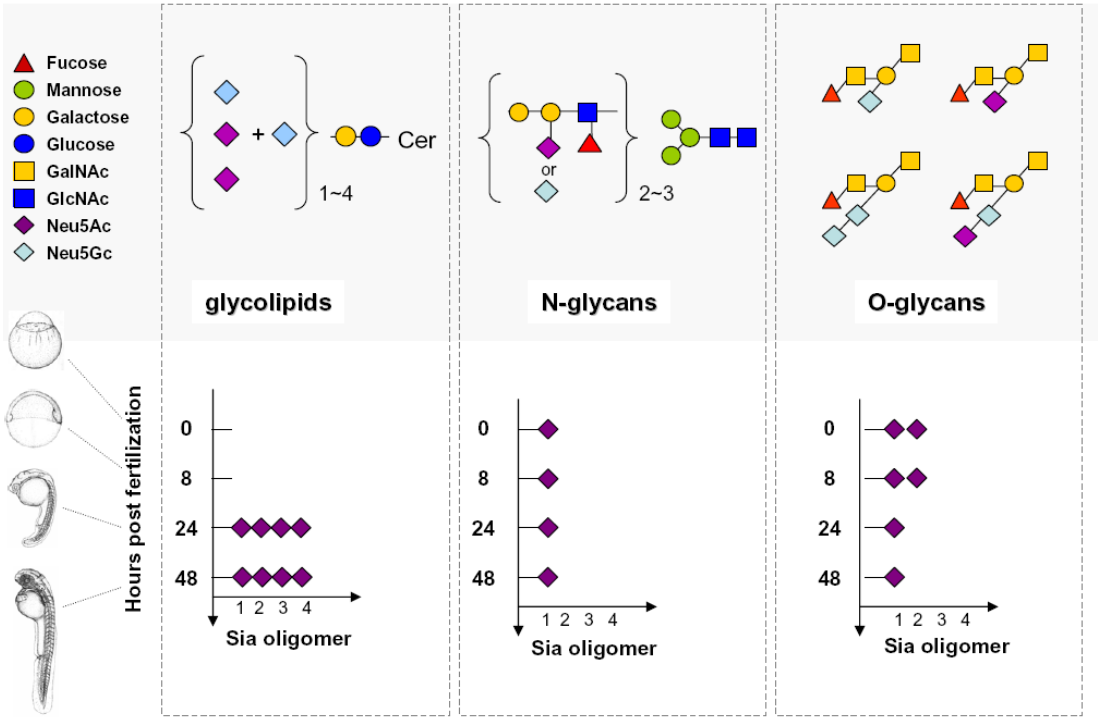

Fig. 3-1-3. Major sialylated glycans and the corresponding degree of polymerization of sialylation identified by mass spectrometry survey mapping

The high–mannose-type *N*-glycans are the only nonsialylated population of the glycome that occurs at any abundance. Otherwise, both the complex-type *N*-glycans and the *O*-glycans are each predominantly represented by a single unique terminal sequence, monosialylated with either Neu5Ac or Neu5Gc. For the *N*-glycans, the Galβ1-4(Neu5Gc/Neu5Acα2-3) Galβ1-4(Fucα1-3)GlcNAc nonreducing terminal sequence constitutes the antenna of the major bi- and triantennary structures, along with some minor degrees of incomplete sialylation and/or galactosylation on the internal Lewis x epitope. For the *O*-glycans, a core 1-type structure was identified which carries a Fucα1-3GalNAcβ1-4(Neu5Gc/Neu5Acα2-3)Galβ1-3 GalNAc sequence. In both cases, α2-3-sialylation was found on an internal β-Gal. Zebrafish sequences can be distinguished from those of more commonly found mammalian type by

virtue of either an additional β4-Gal extension on a sialyl Lewis x or an α3-fucosylated β4-GalNAc extension on a sialylated core 1 *O*-glycan. On the other hand, they bear much similarity to other characterized fish glycans (reviewed in Inoue and Inoue, 1997). The Galβ1-4Galβ1-4GlcNAc motif was first identified on the complex-type free sialoglycans released from the glycophosphoproteins of unfertilized eggs of *Tribolodon hakonensis* and *Oryzia latipes* (Inoue, S., et al. 1989, Iwasaki, M., et al. 1992). α2-3-Neu5Ac monosialylation was found to occur on either the terminal or the internal Gal, giving monosialylated antenna for the predominantly bi- and triantennary structures. Such epitope was also identified on the bulky multiantennary *N*-glycans isolated from cortical alveolus glycoproteins (hyosophorins) of fertilized fish eggs which carry species-specific, highly branched poly-*N*-acetyllactosaminoglycans (Taguchi, T., et al. 1996, Taguchi, T., et al. 1995, Taguchi, T., et al. 1993, Taguchi, T., et al. 1994). Of all the structural variants determined, only those presented by the hyosophorins of medaka fish, *O. latipes*, also contain the fucosylated version of the α2-3-Neu5Ac sialylated Galβ1-4Galβ1-4GlcNAc motif, identical to that currently identified on Zebrafish *N*-glycans. Interestingly, the Fucα1-3GalNAcβ1 epitope as found on Zebrafish *O*-glycans has also been identified on the *N*-glycans of hysophorin of flounder (Seko, A., et al. 1989), but not sialylated and is attached to the same -3Galβ1-4Galβ1-4GlcNAc sequence. Thus, a Galβ1-4Galβ1-4GlcNAc unit, occurring either at the terminal or as an internal unit, with and without further α2-3Neu5Ac sialylation on the β4-Gal and/or α3-fucosylation on the β4-GlcNAc appears to be a shared feature among the *N*-glycans from several fish eggs characterized to date. A slightly different version with an additional α4-Gal capping instead of sialylation or fucosylation has also been recently identified on the pigeon serum immunoglobulin G (Suzuki, N., et al. 2003), suggesting a possible wider occurrence on nonmammalian vertebrates.

Despite similarity to previously characterized glycans structures of fish eggs, Zebrafish glycans are unique and novel in many additional aspects. First, the hyosophorin *N*-glycans are bulky, and those of *O. latipes* have been shown to be exclusively pentaantennary, whereas the much simpler bi- and triantennary *N*-glycans from the fish glycophosphoproteins characterized to date do not have fucosylation. Second, whereas sialylated core 1 *O*-glycans with terminal Fucα1-3GalNAc epitope have also been identified in fish eggs (Inoue, S.a.l., Y 1997), none carries the exact sequence as defined here. Oligo- or polysialylation, when occurs, extends from the C6 of the reducing end GalNAc or nonreducing terminal GalNAc. In contrast, Zebrafish Neu5Ac-Neu5Gc or Neu5Gc-Neu5Gc disialyl unit extends from an internal Gal of the *O*-glycans. Further work is needed to confirm the tentatively defined structures for the disialylated core 2-type *O*-glycans which appear to carry one to several units of Lewis x on its 6-arm.

Notably then, we have shown that Neu5Ac and Neu5Gc sialylation were not evenly distributed. Most Neu5Ac is located at the nonreducing terminal position and, mostly, if not, exclusively as

monosialylated motif. Neu5Gc occurs as both terminal and internal residues. For the *O*-glycans, our MS/MS data indicated that only the species sialylated with Neu5Gc can be further sialylated with another Neu5Ac or Neu5Gc residue. Likewise, although both Neu5Ac and Neu5Gc monosialylated antennae could be detected for the *N*-glycans, oligosialylation analysis with the more sensitive 1,2-diamino-4,5-methylene-dioxybenzene high pressure liquid chromatography (DMB-HPLC) fluorescent detection method indicated that only a Neu5Gcα2-8Neu5Gc-DMB derivative could be additionally derived from the *N*-glycans (see Section 3.3.1.1). These data strongly suggest the occurrence of a certain form of donor and acceptor substrate selectivity in the differential transfer of Neu5Ac and Neu5Gc on glycoprotein-type glycans of Zebrafish or a strict intra-/extracellular compartmentalization of sialyltransferase activities.

The concentration of CMP-Neu5Gc in the cytosol has been suggested to play the most important role in regulating the level of Neu5Gc sialylation because neither the CMP-sialic acid antiporter nor the sialyltransferases examined so far seem to exhibit a preference for CMP-Neu5Ac or CMP-Neu5Gc (Higa, H.H. and Paulson, J.C. 1985, Lepers, A., et al. 1989, Lepers, A., et al. 1990). In contrast, different donor substrate specificities have been observed for enzymes involved in the elongation of oligo-/poly-sialylated chains. Thus, whereas rainbow trout polysialyltransferase (polyST) can use both CMP-Neu5Ac and CMP-Neu5Gc as activated sialyl donors, chick brain polyST was shown to not recognize CMP-Neu5Gc (Kitazume, S., et al. 1994, Sevigny, M.B., et al. 1998). However, to our knowledge, nothing is presently known on the possible specificity of these enzymes toward their acceptor substrates for Neu5Ac/Neu5Gc composition that may explain the absence of polysialyl elongation from Neu5Ac residues in Zebrafish *O*-glycans. Further studies of Zebrafish α2,8-sialyltransferases, especially ST8Sia VI, are discussed in Section 3.2.

Contrary to the glycoproteins, synthesis of oligosialyl sequences in glycolipids did not seem to be affected by the same biosynthetic restrictions. The major sialylated glycolipids detected conform to a family of lactosylceramides extended by up to four sialic acids which can be further elongated by a Hex-HexNAc unit to form either sialylated ganglio-tetraglycosylceramides or sialylated (neo) lactotetraglycosylceramides, with up to five sialic acids. Both series of glycolipids have previously been identified in other fishes (Ando, S. and Yu, R.K. 1979, DeGasperi, R., et al. 1987, Nakamura, K., et al. 1997). However, to our knowledge, tetrasialylated lactosylceramides have not been observed previously in any model system. Such a compound, that would be named GQ3 according to used nomenclature, does not fit into accepted ganglioside synthesis pathway model in which GT3 is the biosynthetic precursor of the so called c-series (including GT2, GT1c, GQ1c, and GP1c) and is not further elongated by sialic acids (Freischutz, B., et al. 1995). The sialylated moieties of all observed glycolipids are made up by heterogeneous mixtures of Neu5Ac and Neu5Gc residues in all possible combinations. The presence of polymerized Neu5Ac sequences distinguishes their

sialylation pattern from those of *N*- and *O*-glycans. Furthermore, homogeneous Neu5Ac-sialylated glycolipids are the major forms compared with Neu5Gc-containing glycolipids. Altogether, these data demonstrate that although the glycoprotein glycans and glycolipids are both highly sialylated, the biosynthesis of their respective oligosialylated moieties are differently regulated, and the sialylation pattern changes as the embryos develop.

In particular, the disialylated *O*-glycans were exclusively observed in the very first stages of development, before 24 h after the fertilization, whereas, surprisingly, the pattern of oligosialylation in glycolipids seems to follow the opposite trend with the oligosialylated glycolipids being exclusively observed in later developmental stages. We could detect glycolipids of even higher degree of sialylation which collectively represent a complete shift in the glycolipid profile from the very early stage that contained a range of very different, highly heterogeneous neutral glycolipids (see Section 3.3.1.3). By furnishing the structural data pertaining to the glycome of *D. rerio*, our studies reported here provide a solid basis for further functional investigations into the specificity of glyco-related enzymes and, by extension, the role of glycosylation during development. Preliminary screening of gene data banks revealed that Zebrafish genome not only contains orthologs of human polysialyltransferase genes, ST8Sia II and ST8Sia IV, but also other members of ST8Sia family (Harduin-Lepers, A., et al. 2005). Further characterization of the developmentally regulated oligosialylation and the responsible sialyltransferases are described in Section 3.3.

3.2 Molecular cloning of Zebrafish α2, 8-sialyltransferases (DreST8Sia) and enzymatic characterization of DreST8Sia VI

The first embryonic glycomic survey mapping leads to the discovery of the unusual monosialylated O-glycans, Fucα1-3GalNAcβ1-4(Neu5Gcα2-3)Galβ1-3GalNAc-itol. Noteworthily, it is only the Neu5Gc and not Neu5Ac that can be further extended with an α2,8-linked sialic acid (Fig. 3-2-1), which is transferred by α2,8-sialyltransferases.

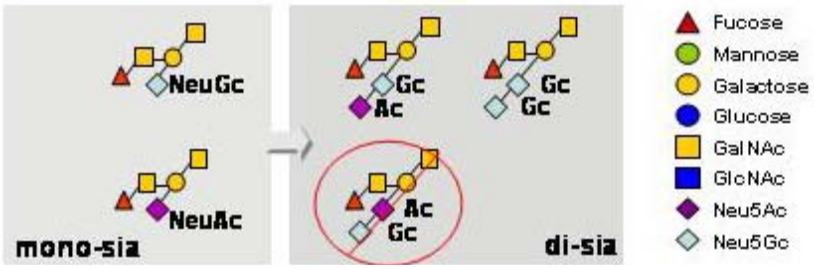

Fig 3-2-1. Mono- and di-sialylated O-glycans found in Zebrafish embryos

It has been reported from *in vitro* enzymatic assays that ST8Sia VI may be responsible for the biosynthesis of di-sialylated O-glycosylproteins in mouse (Takashima, S., et al. 2002) and human (Teintenier-Lelievre, M., et al. 2005). Two questions arise after this structural study: **1)** whether the α2,8-linked sialic acid found on the di-sialylated O-glycans are transferred by DreST8Sia VI? **2)** If so, does DreST8Sia VI prefer the Neu5c-bearing O-glycan instead of Neu5c-bearing one? In order to further investigate the enzymatic specificity of DreST8Sia VI, the first step was to identify Zebrafish ortholog of the mammalian ST8Sia gene, followed by cloning, expression, and characterization of its enzymatic activity.

The α2,8-sialyltransferase (ST8Sia) genes in Zebrafish genome, *Danio rerio* EST and genomic sequence databases were queried by BLAST search by Harduin-Lepers et al and sequences showing similarities to human ST8Sia genes have been identified (Harduin-Lepers, A., et al. 2005). Through 1) sequence comparisons, 2) exon–intron organization of the genes, and 3) phylogenetic analysis, the conserved genetic feature and phylogenic relations between Zebrafish ST8Sia was analyzed.

3.2.1 In silico identification, molecular cloning and sequencing of ST8Sia

Zebrafish ST8Sia genes were identified as single gene orthologues (Fig. 3-2-2a), with the exception of two ST8Sia related genes found tandemly duplicated on chromosome 23 in Zebrafish genome that we named ST8Sia VIIA and ST8Sia VIIB. All the identified Zebrafish ST8Sia sequences retained the same genomic organization predicted for human genes and the deduced protein sequences contained the sialylmotifs and family-motifs characteristic of

the sialyltransferases and α2,8-sialyltransferases, respectively (Fig. 3-2-2b). To verify the actual expressed DNA sequences, primer pairs were designed in the 5′- and 3′-UTR of each sequence in order to amplify the full-length ORF and each of Zebrafish ST8Sia transcripts from various Zebrafish tissues were amplified using RT-PCR. Amplification products were subcloned and sequenced confirming the sequences of the predicted transcript and protein (Harduin-Lepers, A., et al. 2005). Deduced protein sequence comparison indicates that ST8Sia III is highly conserved from Zebrafish to human (78% amino acid identity), whereas ST8Sia VI is less conserved, Zebrafish and human sequences sharing only 37% identity (Table III).

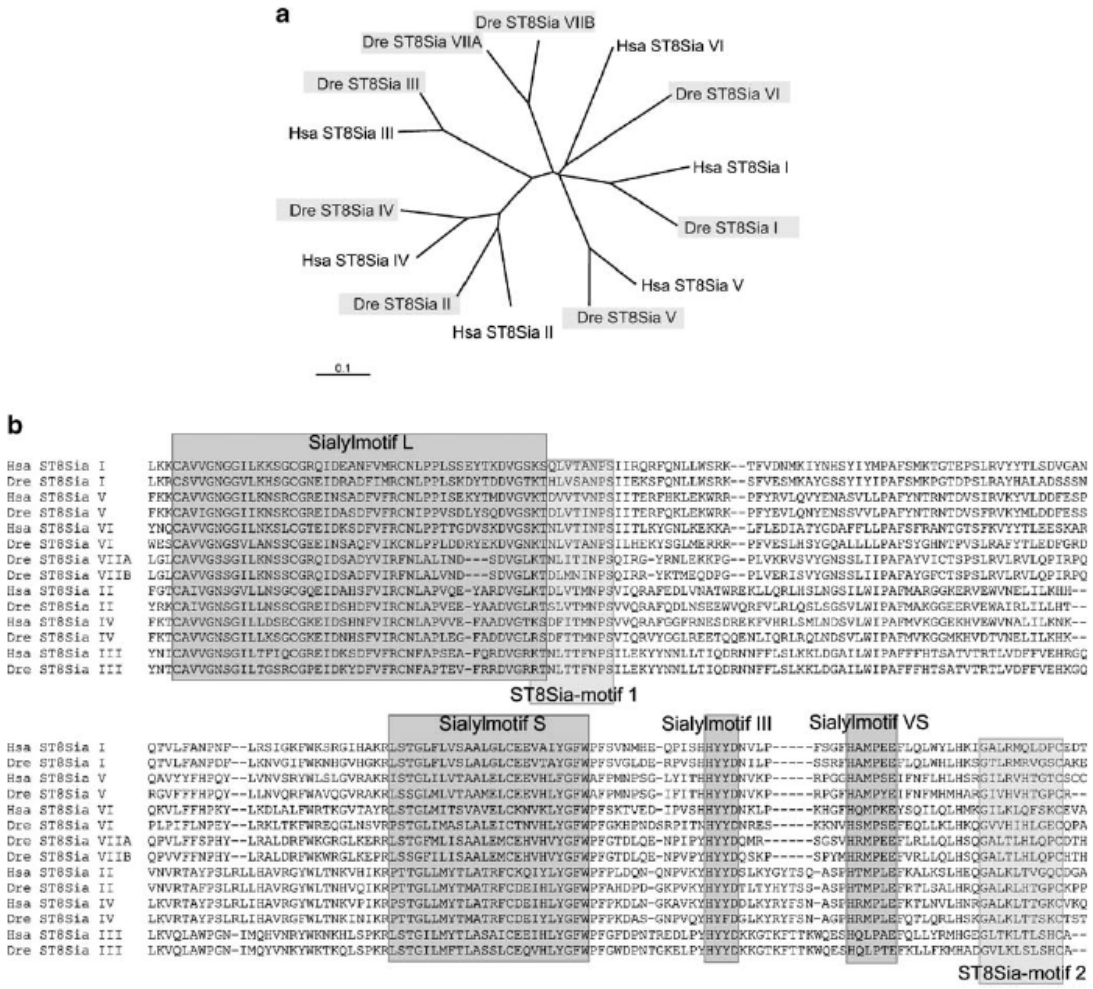

Fig. 3-2-2. A Unrooted phylogenetic analysis of Zebrafish (Dre) and human (Hsa) ST8Sia sequences. Zebrafish and human ST8Sia protein sequences were aligned with clustal W at the PBIL. The aligned sequences were used for the construction of a tree using the maximum parcimony method with mega 3.1. This unrooted tree indicates the relationship of Zebrafish ST8Sia sequences to their human counterparts. b Comparison of the deduced amino acid sequences of Zebrafish and human ST8Sia catalytic domain. The sequences were aligned with Clustal W (PBIL) from the third amino acid residue upstream the sialylmotif L to the third amino acid residue downstream the ST8Sia-motif 2. The sialylmotifs L, S, III and VS above sequences are shaded in dark grey. The ST8Sia-family specific motifs 1 and 2 (Patel, R.Y. and Balaji, P.V. 2006) below sequences are represented in light grey boxes. The last

one is the C-term motif recently described (Harduin-Lepers, A., et al. 2008).

TableIII Zebrafish α2,8-sialyltransferases

	ST8Sia	Structures synthesized	Human protein size (AA)	Zebrafish protein size (AA)	Protein sequence identity
Poly α2,8-sialyltransferase	ST8Sia II (STX)	(Neu5Acα2–8)$_n$Neu5Acα2–3/6Galβ1–4GlcNAc- [a]	375	381	62%
	ST8Sia IV (PST)	(Neu5Acα2–8)$_n$Neu5Acα2–3/6Galβ1–4GlcNAc- [a]	359	358	69%
Oligo α2,8-sialyltransferase	ST8Sia III	Neu5Acα2–8Neu5Acα2–3Galβ1–4GlcNAc-(Neu5Aca2–8)$_n$ Neu5Acα2–3Galβ1–4GlcNAc- [b]	380	374	78%
Mono α2,8-sialyltransferase	ST8Sia I (G_{D3} synthase)	Neu5Acα2–8Neu5Acα2–3Galβ1–4Glc-Cer	356	339	60%
	ST8Sia V(G_{T3} synthase)	G_{D3}, G_{T3}, G_{D1c}, G_{T1a}, G_{Q1b}	376	374	72%
	ST8Sia VI	Neu5Acα2–8Neu5Acα2–3Galβ1–3GalNAc-0-Ser/Thr Neu5Acα2–8Neu5Acα2–6GalNAc-0-Ser/Thr	398	358	37%
	ST8Sia VII	Unknown	Not identified	358	–

Alternative sialyltransferase names are indicated in brackets. The sialylated compound formed, diSialylated motif (DP=2), oligosialylated motif (2<DP<7) or polysialylated motif (DP>7) [2] correspond the biosynthetic activity of mono-, oligo- or poly-α2,8-sialyltransferases, respectively. ST8Sia VII has no orthologous counterpart in the human genome

[a] $n>7$

[b] $2<n<7$

3.2.2 human ST8Sia VI ortholog in Zebrafish

The nucleotide sequence of the DreST8Sia Vi gene was assembled and found to be similar to the genomic organization of its human counterpart. Construction of phylogenetic tress using the predicted amino acids sequence corresponding to the DreST8Sia VI gene demonstrated the orthology of the human sequence and Zebrafish ST8Sia VI sequence (Fig 2-3A), (Harduin-Lepers, A., et al. 2005). In addition, analysis of neighbor-joining phylogenetic tree showed that ST8Sia VI belongs to one of the two main branches, which contains three subfamilies of mono-α2,8-sialyltransferases: ST8Sia I, ST8Sia V, and ST8Sia VI. The second branch contains oligo- and poly-α2,8-sialyltransferases namely, ST8Sia II, ST8Sia III, and ST8Sia IV (Fig. 3-2-3B).

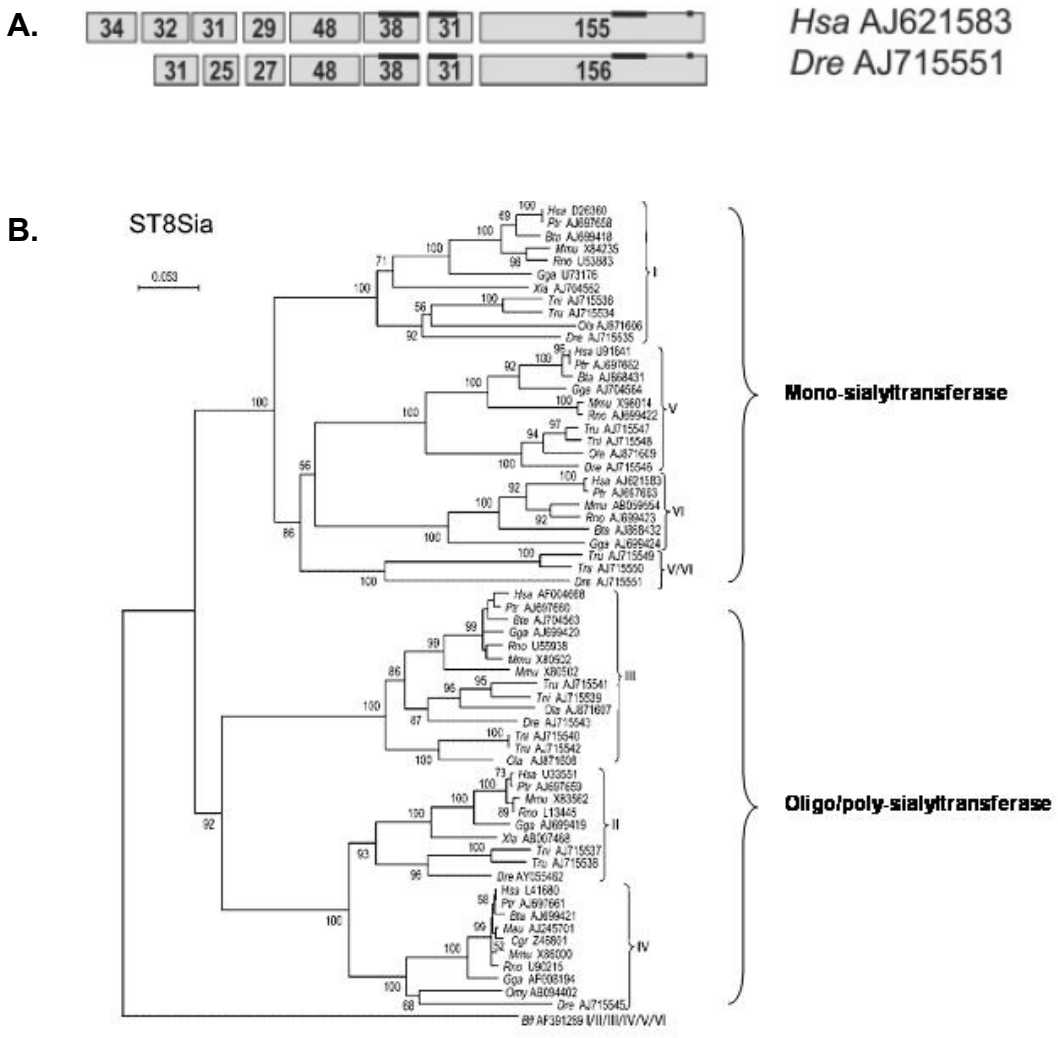

Fig. 3-2-3. Zebrafish ST8Sia VI. A. comparison of genomic organization of human and Zebrafish ST8Sia VI. B. Neighbor-joining phylogenic tree of α2,8-sialyltransferases (modified from Harduin-Lepers 2005).

3.2.3 Screening the expression of DreST8Sia VI

To confirm the actual expression of the Dre*ST8Sia6* gene, we extracted total RNA from various adult tissues of Zebrafish and 36 hpf embryo, which were kindly provided by the Dr. Thisse (Strasbourg, France) and screened the expression of ST8Sia VI by amplifying a 300 base pair DNA fragment inside its coding region using RT-PCR (Fig. 3-2-4). DNA fragment amplified were subcloned in TOPO and sequenced (Genoscreen, Lille). The result indicated the actual expression of the *ST8Sia6* gene in the 36h embryo, ovaries, intestine, brain, and in kidney, but not in liver. This demonstrates the Dre*ST6Sia6* gene is expressed in almost all the tissues tested, which is similar to mouse and human genes.

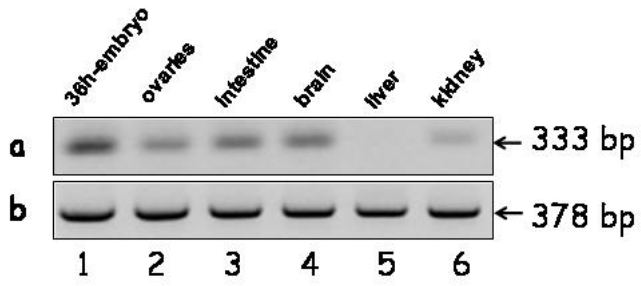

Fig. 3-2-4. Zebrafish ST8Sia VI is expressed in different tissues.
(a) ST8Sia VI and (b) β-actin are amplified from cDNA of 36h-embryo, ovaries, intestine, brain, and kidney.

Further demonstration of the expression of *ST8Sia6* gene in various embryonic stages has done by real-time PCR with various sources of staged embryos or adult tissue cDNAs. We first analyzed staged embryo cDNAs from Dr Gerardo Vasta (Baltimore, USA) and from B. and C. Thisse (Strasbourg, France), then we extracted by ourselves total RNAs from the staged embryos provided by Thisse and reverse transcribed to cDNA. It is shown that *ST8Sia6* gene is not expressed at 0 hpf but starts to be expressed after 14h (Fig. 3-2-5) from late gastrula stage. This was confirmed by preliminary whole mount *in situ* hybridization assays performed by Thisse (Section 3.4). The preliminary results show general expression of *ST8Sia6* gene starting 10 hpf refining to the rhombencephale at the larval stage (5 dpf).

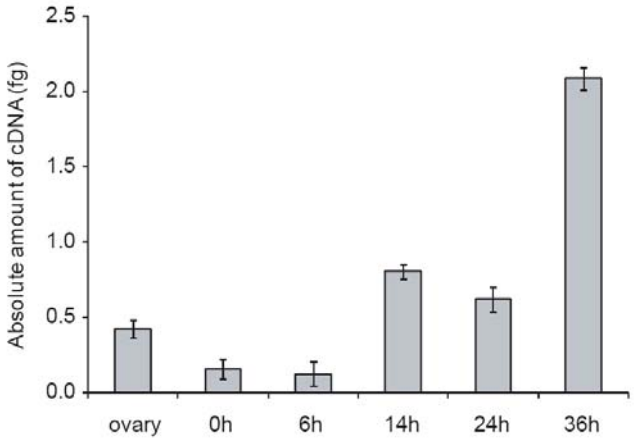

Fig. 3-2-5. Real-time PCR quantification analysis of the expression of Zebrafish ST8Sia VI in the ovary and during embryogenesis.

3.2.4 Molecular cloning and expression of ST8Sia VI

We had a kidney cDNA library in ZAP kindly provided by L. Zon (Boston Children Hospital, BOSTON, USA). The full length ORF was cloned from this library into TOPO (Invitrogen) and fully sequenced (Genoscreen) to check sequence. A hydrophobic domain in N-terminal region of the deduced protein sequence, which may represent the transmembrane stem region was found by analyzing the amino acids sequence of ST8Sia VI on the website TMpred http://www.ch.embnet.org/software/TMPRED_form.html. In order to characterize the enzymatic activity of DreST8Sia VI, we first produced a soluble recombinant active protein with N-terminal truncation and constructed in an expression vector p3xFlag-CMV (sigma) and transiently transfected in COS-7 cells. It is a soluble form enzyme, secreted in the culture medium, bearing proper N-glycosylation as confirmed by PNGase F digestion (Fig. 3-2-6). The molecular mass of the recombinant p3xFlag-ST8Sia VI protein produced is calculated to be 42 kDa, which correspond to the molecular weight of the band of western blot (detected by M2 monoclonal antibody) after PNGaseF digestion.

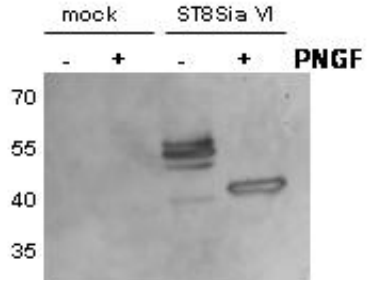

Fig. 3-2-6. Western Blot of pFlag-CMV mock and ST8Sia VI transfected COS-7 cell lysates with or without prior PNGaseF de-N-glycosylation.

3.2.5 Enzymatic characterization of ST8Sia VI

In vitro enzymatic assays were carried out using the culture medium of transfected COS-7 cell as the enzyme source. Data obtained showed that the secreted Dre ST8Sia VI catalyzes the transfer of [^{14}C] labeled sialic acid residues onto the sialylated-glycans of native fetuin, which originally possess 3 O-glycans and 3 N-glycans (Fig. 3-2-7). The preference of O-glycans rather than N-glycans as the acceptors substrate has been demonstrated in human ST8Sia VI (Teintenier-Lelievre, M., et al. 2005). [^{14}C] labeled fetuin obtained after incubation of Dre ST8Sia VI with native fetuin was treated with PNGase F. Fig 2-7A shows that removal of N-glycans of fetuin led to a shift of the molecular mass of the detected band but did not change its signal intensity. In order to define the linkage of incorporated [^{14}C] sialic acid on fetuin, [^{14}C] labeled fetuin was treated with various sialidases: Nanase I cleaves α2,3 linked sialic acid, (lane 2), Nanase II cleaves α2,3 and α2,6 linkages, (lane 3), and Nanase III cleaves α2,3, α2,6, α2,8 and α2-9-linkages (lane 3). Our data show that the Dre ST8Sia VI catalyzes the formation of α2-8 linkages on fetuin.

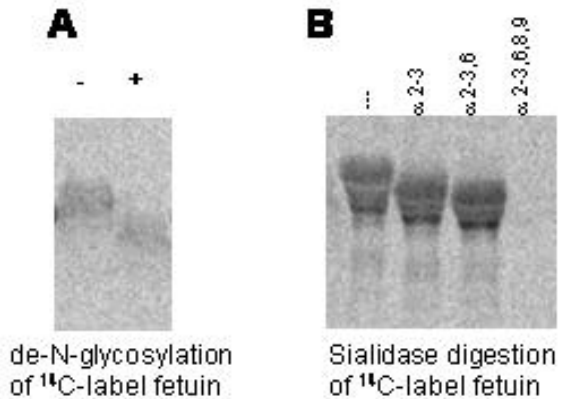

Fig. 3-2-7. De-N-glycosylation and desialylation of ST8Sia VI-sialylated fetuin.
[^{14}C]Neu5Ac-labelled fetuin was produced using soluble recombinant Dre ST8Sia VI and was subjected to A) PNGase F or B) sialidase digestions to confirm the linkage of incorporated sialic acid. The resulting products were separated by SDS/PAGE and detected by phosphorimaging.

Further analysis of the actual presence of α2,8-linked sialic acids from the enzymatic reaction mixture has been done by HPLC separation of reaction products and isolated the radioactive peak(s) correspond to di-, oligo-, or polysialylated glycans. We have carried out various enzymatic assays using different acceptor substrates (fetuin, polysialoglycoproteins prepared from unfertilized Zebrafish eggs, and PA-tagged NeuAc/NeuGc-lactose then co-injected the reaction product mixture with DMB-labeled colominic acid separated on different columns (Amide, Dionex-PA200, and mono-Q) but failed to detect the radioactivity at any place that could correspond to di- or oligosialic acids.

3.2.6 Discussion

Phylogenetic analyses have shown that the ST8Sia gene family can be divided into two main groups of genes Fig 2-3B. In the first group, *ST8Sia II, ST8Sia III* and ST8Sia *IV* are known as oligo- or poly-sialyltransferases involved in the elongation of linear chains of sialic acids found mainly in glycoproteins. ST8Sia II and IV were clearly shown to be responsible for the elongation of polysialylated chains of N-CAM (Kojima, N., et al. 1996, Kojima, N., et al. 1997). The second group includes ST8Sia I, ST8Sia V and ST8Sia VI. ST8Sia I (GD3 synthase) and ST8Sia V(GT3 synthase) are acting as di-sialyltransferases, transferring only one sialic acid residue onto GM3 or GD3 to convert these gangliosides into GD3 or GT3, respectively.

The occurrence of diSia motifs in animal tissues was suggested a long time ago by Finne and her colleague (Finne, J., et al. 1977) in various tissues of rat and by Inoue (Inoue, S. and Iwasaki, M. 1978) in fish eggs. These diSia motifs also occur in several embryonic and adult pig brain glycoproteins (Sato, C., et al. 2000), in the murine CD-166 (Sato, C., et al. 2002), on the N-glycans of Band 3 of human erythrocytes (Fukuda, M., et al. 1984), on the O-glycans of human erythrocyte glycophorin (Fukuda, M., et al. 1987), bovine adipo-Q (Sato, C., et al. 2001) and chromogranin (Kiang, W.L., et al. 1982). It has been suggested that the biosynthesis of these diSia residues on glycoproteins might be catalysed by α2,8-sialyltransferases distinct from the known polysialyltransferases ST8Sia II and ST8Sia IV and it was shown that a recombinant ST8Sia III could sialylate purified bovine adipo-Q (Sato, C., et al. 2001). It has been predicted that the enzymatic properties of mouse ST8Sia VI would be those of a di-sialyltransferase. However, the previous work done by Teintenier-Lelievre has clearly demonstrated in *in vitro* and *in vivo* assays that hST8Sia VI is highly specific for the synthesis of the diSia epitope found on O-glycosylproteins. Therefore, the finding of both the actual presence of di-sialylated O-glycans in Zebrafish as well as its corresponding sialyltransferases ST8Sia VI prompted us to pursue its enzymatic specificity in Zebrafish.

In this work, Zebrafish ST8Sia VI was successfully identified in various adult tissue and developing embryos, and cloned from kidney. The over-expression of N-terminal truncated soluble form of ST8Sia VI showed that it was N-glycosylated and secreted in the culture medium, which suggests it may be properly produced with correct folding. We have investigated its enzymatic activity using native bovine fetuin as an acceptor substrate. However, we failed to demonstrate any enzymatic activity of the recombinant Dre ST8Sia VI produced by transiently transfected COS-7 using other acceptor substrates such as Neu5Ac-lacto-PA, Neu5Gc-lacto-PA, and $(Neu5Ac)_2$-lacto-PA. These first observations suggest that we might not have used the proper acceptor substrate since Zebrafish sialylated O-glycans are fucosylated. Therefore, we also tried to assay with the purified endogenous sialylated glycoproteins from Zebrafish, but still failed to detect the radio-activity. It is also possible that the sialylated O-glycans, which are the substrate for Dre ST8Sia VI, need to be born by a particular glycoprotein, which is not yet identified. Future proteomic studies will aim to identify

in vivo substrate(s) of DreST8Sia VI. Another possibility is the presence of fucose and/or NeuGc residue on the acceptor substrate, which would be required for full enzyme activity.

A difficulty we have encountered is to prove the presence of di- or oligoSia on sialylated fetuin. This problem can be caused by several reasons: **1)** the recombinant enzyme produced in the cell culture medium by transfected COS-7 cells is not very active, therefore the sialylated products are formed in too little amount to be detected. **2)** there are too many proteins in culture medium, which we took as the ST8Sia VI enzyme source, leading to columns (Amide, Carbopack PA-100, and Mono-Q) overloading and inability to properly separate the oligo/polysialic acid. Pre-cleaning the enzymatic product on a C18 seppak followed by separation on mono-Q column, did not seem to help either. **3)** The fetuin from the culture medium, used as the enzyme source, could serve as a competitive acceptor for the exogenous substrate added. To overcome this difficulty, we have tried to immunoprecipitate the recombinant ST8Sia VI produced with anti-Flag antibody prior enzymatic assays with the purified ST8Sia VI. However, no sialylated product could be observed. Since glycosyltransferase might require interactions with other cell components (GTs form heterodimers in gangliosides biosynthetic pathways, for example), future experiments will be conducted using the full-length Dre ST8Sia VI produced within the transfected COS-7 cells and sialyltransferase assays will be achieved using cell lysates as the enzyme source. **4)** Find right acceptors: try fucosylated or endogenous substrate. It is noteworthy that the unique disialylated O-glycan sequence, Fucα1-3GalNAcβ1-4(Neu5Gc/Neu5Acα2-3)Galβ1-3GalNAc of Zebrafish, implies also the possibility of the substrate specificity of ST8Sia VI for the fucosylated O-glycans. That means if the biosynthetic order of the terminal modification of this glycan is first fucosylation, the disialylation by ST8Sia VI, it is expectable to see no reactivity by using unsuitable substrates like fetuin or sialyl-lactose-PA sugars. For that, we will extract monosialylated O-glycans from Zebrafish tissue and develop transfections in other Zebrafish cell lines, where the natural acceptor substrate of ST8Sia VI will be present.

3.3. Developmental regulation of oligosialylation in Zebrafish

In contrast to the unusual di-sialylated O-glycans described previously (in Section 3.2), which are down regulated during embryonic development, our glycomic mapping (in Section 3.1) revealed an increased level of oligosialylated GSL being expressed. On the other hand, any changes in the expressions of sialylated complex type N-glycans is less obvious. To further investigate the expression pattern of these sialylated glycoconjugates, which are potentially regulated along Zebrafish development, a concerted approach combining structural, biochemical and molecular biology analyses were employed and the result described in this Section. As a first step, different structures of oligoSia sequences associated to different types of glycoconjugates were established. Subsequently, the implication of different glycogenes, including α2,8-sialyltransferases (ST8Sias) and sialidases in the early developmental regulation of the α2-8-linked sialic acids were evaluated.

3.3.1 Glycans from embryos contain oligosialic acid chains

3.3.1.1 Oligosialylation on N-Glycans

Zebrafish complex type *N*-glycans as identified by MS in Section 3.1 are characterized by the presence of Neu5Ac/Neu5Gc monosialylated Lewis x motifs further substituted by a β4-Gal residue. A 10% sialic acid content of the total *N*-glycan fraction (Fig. 3-3-1) nonetheless implied that a much larger proportion of sialylated *N*-glycans than that detectable by MS might be present.

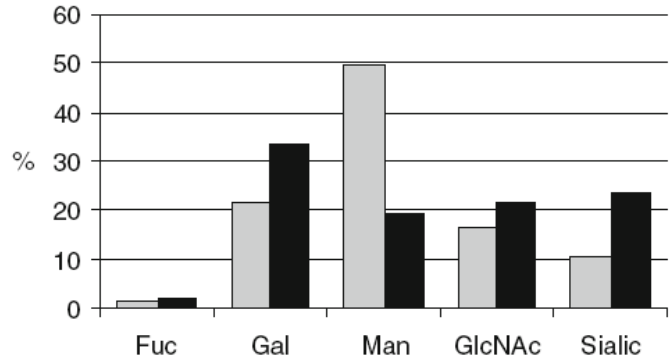

Fig. 3-3-1. Relative monosaccharide compositions of *N*-glycans. Monosaccharide compositions of total (in grey) and acidic (in black) N-glycans liberated from 1 hpf embryos were analyzed by gas-chromatography. Results are expressed in percentage of total monosaccharides.

In fact, after purification of sialylated *N*-glycans by anion exchange chromatography, the proportion of sialic acids in sialylated *N*-glycans increased sharply up to 23 % of total monosaccharides, which represents an average of three to four sialic acid residues per *N*-glycan. As demonstrated by reverse phase (RP)-HPLC analysis of sialic acid-DMB

derivatives, the Neu5Gc:Neu5Ac ratio, which ranges between 2:1 and 4:1 depending on the sample batch, was also somewhat inconsistent with a prevalence of Neu5Ac over Neu5Gc implicated by MALDI-MS profiling of the *N*-glycans. These discrepancies between the MS and sialic acid composition data indicated that some additional oligosialylated *N*-glycans may be refractory to MALDI-MS detection

To gain a better picture of the sialylation, the well established DMB-tagging and HPLC analytical method (Inoue, S., et al. 2001) was further employed to identify possible presence of oligo- or polysialyl motifs. We first conducted structural analyses of glycoproteins associated oligosialylation on 1hpf embryos, and then established that oligosialylation was qualitatively identical in other developmental stages. The *N*-glycan sample was incubated in the acidic derivatization reaction mixtures without prior acidic liberation to minimize internal fragmentation of polysialic acid chain. The resulting tagged products were then separated on anion exchange HPLC columns (MonoQ and CarboPac PA-100) according to their degree of polymerization (DP) and detected with a fluorescent detector (FD). Under the experimental conditions employed, a monoQ column permits a ready detection of polymeric sialic acid chains from DP 2 up to DP 50 (data not shown), whereas the CarboPac PA-100 column also allows detection of Neu5Ac monomer. Since the FD response per mol of $(Neu5Ac)_n$-DMB remains constant for low DPs, integration of peak areas therefore provides a good estimation of the relative abundance of various Sia_n units. On MonoQ column, total *N*-glycans fraction was found to yield at most six peaks with retention times corresponding to $Sia[(\alpha 2\text{-}8)Sia]_n$-DMB standards of DP 2 to DP 7 (Fig. 3-3-2a).

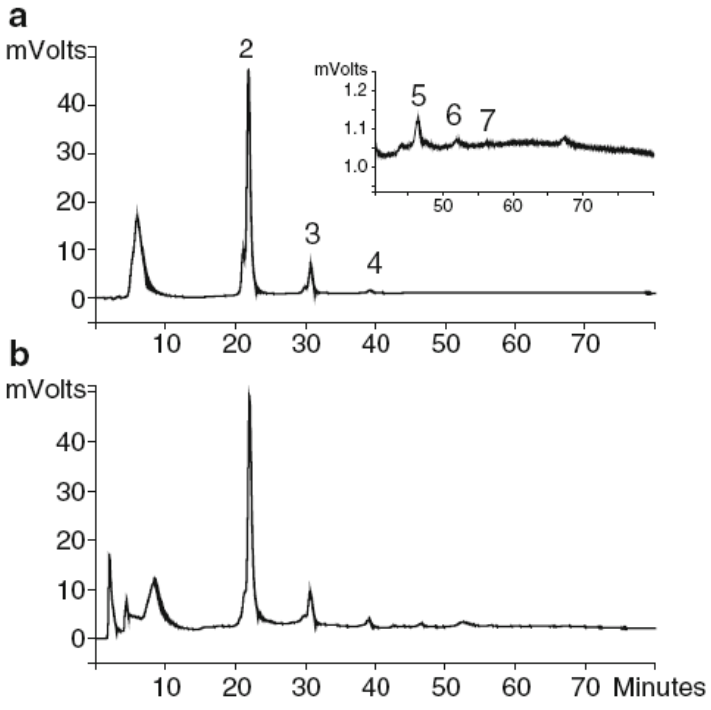

Fig. 3-3-2. Profiles of oligosialylated sequences on *O*- and *N*-glycans. OligoSia

sequences were released from glycans isolated from 1hpf embryos, tagged with DMB and separated by HPLC-FD on an anion exchange column. OligoSia profiles from (a) *N*-glycans including the inset in upper panel, and (b) *O*-glycans. Peaks are labeled according to the DP values as established by comparison with authentic standards.

Identical result was obtained with acidic *N*-glycans obtained after purification by anion exchange chromatography (data not shown). A sharp drop in the relative intensities of peaks occurred from DP 2 onwards. On PA-100 column, it could be estimated that DP 1 and DP 2 constitute 59 and 38% respectively of the total content with higher oligosialyl chains contributing to less than 3% in total (data not shown). As expected, the observed peaks are sensitive to the action of exoneuraminidase (data not shown). Closer examination of the chromatograms showed that standard [-8)Neu5Ac(α2-]$_n$ and [-8)Neu5Gc(α2-]$_n$ exhibited slightly different retention times, in particular for DP 2 and DP 3 (Fig. 3-3-3a). Accordingly, chromatographic behaviours of DMB-tagged oligoSia from *N*-glycans suggest that Sia_2 and Sia_3 are exclusively composed of Neu5Gc residues. As shown in Fig. 3-3c, standard $Neu5Gc_2$ peak co-migrates with DP 2, whereas $Neu5Ac_2$ peak exhibits a clear time shift compared to DP 2 (Fig. 3-3-3b).

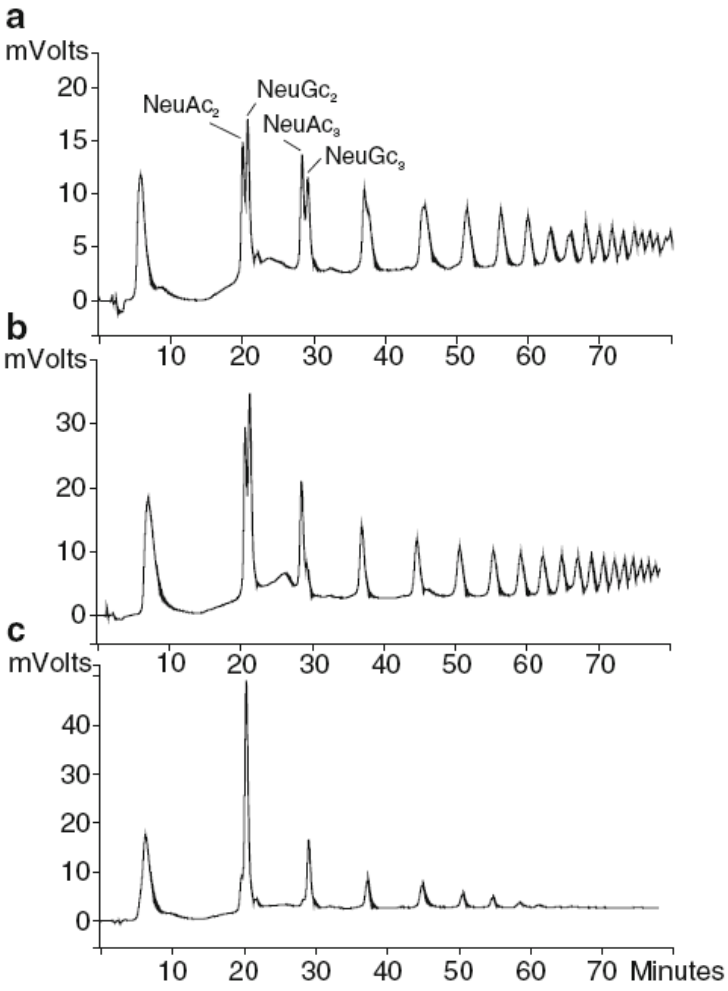

Fig. 3-3-3. Identification of oligosialylation on *N*-glycans by anion exchange DMB/HPLC-FD. Chromatographic profiles of co-injected DMB-derivatized (a) [-8)Neu5Ac (α2-]$_n$ and [-8)Neu5Gc(α2-]$_n$ standards, (b) [-8)Neu5Ac(α2-]$_n$ standard and oligo-Sia from Zebrafish 1hpf embryos *N*-glycans, (c) [8)Neu5Gc(α2-]$_n$ standard and oligo-Sia from Zebrafish 1hpf embryos *N*-glycans, showing that the diSia (DP=2) peak from Zebrafish *N*-glycans co-migrate exclusively with Neu5Gc(α2-8)Neu5Gc.

To ascertain the identity of the major dimeric peak, the same DMB derivative mixtures were subjected to RP-HPLC in order to purify DP 2. The elution position of dimeric sialic acid-DMB was inferred from standard Neu5Ac$_{1-3}$-DMB mixtures. A single major dimeric peak was detected at a retention time similar to that of standard Neu5Ac$_2$-DMB (data not shown) and was collected for MS and MS/MS analyses. As shown in Fig. 3-3-4a, ESI-MS analysis of a standard Neu5Ac$_2$-DMB afforded 3 molecular ion signals in positive ion mode, corresponding to $[M+H]^+$, $[M+Na]^+$ and $[M-H+2Na]^+$ at *m/z* 717, 739 and 761, respectively. Further CID-MS/MS on the mono-sodiated parent ion (Fig. 3-3-4b) yielded a major y ion at *m/z* 448 due to facile loss of the non-reducing terminal Neu5Ac residue. In contrast, similar ESI-MS analysis on the collected dimeric peak from the sample gave the corresponding molecular ions at *m/z* 749, 771 and 793 (Fig. 3-3-4c), which differ from those afforded by Neu5Ac$_2$-DMB dimer by 32 mass units and are consistent with a Neu5Gc$_2$-DMB composition. This is supported by CID-MS/MS on the candidate mono-sodiated parent ion at *m/z* 771 (Fig. 3-3-4d), which afforded a major y ion at *m/z* 464, corresponding to loss of a non-reducing terminal Neu5Gc. Further confirmation was then sought by referring to the CID MS/MS spectrum of an authentic Neu5Gc$_2$-DMB standard which was found to co-elute with Neu5Ac$_2$-DMB standard at the same retention time under the HPLC conditions employed. In contrast, a putative mono-sodiated Neu5Ac$_1$Neu5Gc$_1$-DMB peak at *m/z* 755 did not afford either a loss of Neu5Ac or Neu5Gc and was subsequently shown to be a prominent ESI-MS contaminant peak commonly observed when sample amount was low. Thus, our innovative MS and MS/MS approaches have provided unambiguous evidence for the presence of a Neu5Gc-Neu5Gc dimer, and not a Neu5Ac$_2$ or Neu5Ac$_1$Neu5Gc$_1$ dimer, as a major oligosialyl motif on the *N*-glycans.

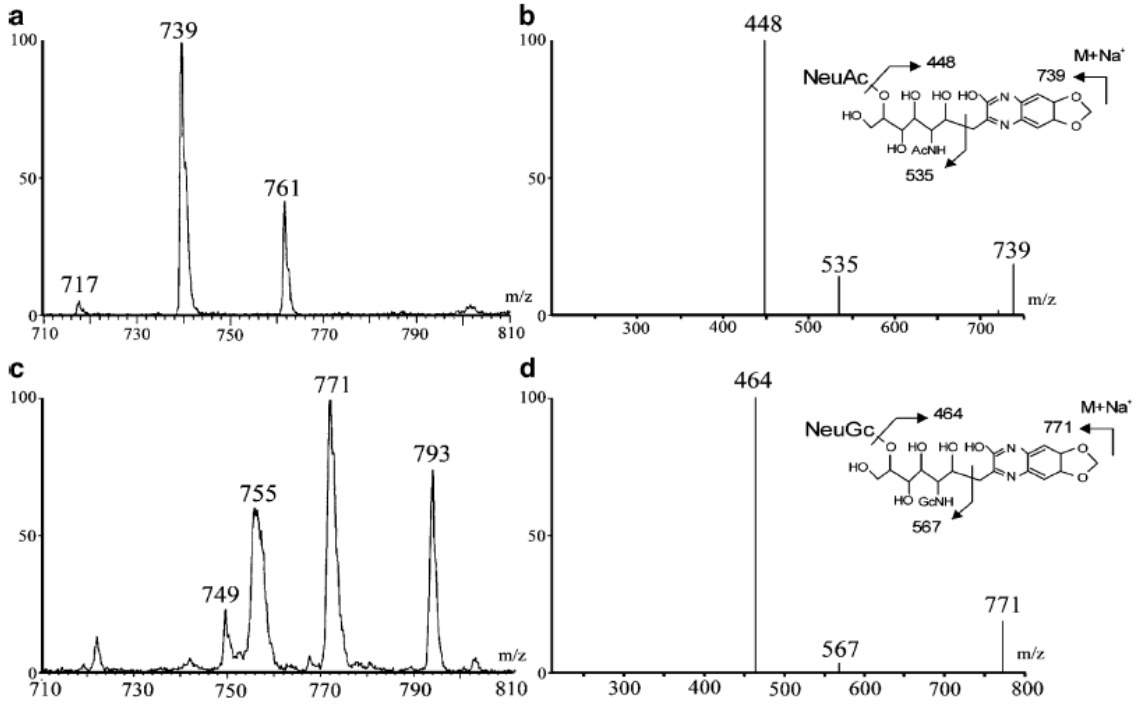

Fig. 3-3-4. Structural determination of the disialylated sequences on *N*-glycans by CID-MS/MS of DMB-derivatives. (**a**) ESI-MS profile of a Neu5Ac$_2$-DMB standard isolated by C18 HPLC fractionation. The most abundant molecular ion at *m/z* 739 corresponds to a monosodiated species which was selected for CID MS/MS sequencing as shown in (**b**). (**c**) ESI-MS analysis of the putative dimeric sialic acid-DMB peak afforded by Zebrafish embryonic *N*-glycans and similarly isolated by C18 HPLC, followed by CID MS/MS on the most abundant molecular ion at *m/z* 771 (**d**) which established its identity as monosodiated Neu5Gc$_2$-DMB derivative. In both MS/MS, loss of 204 u corresponds to loss of the common DMB moiety through cleavage at C3-C4 of the derivatized, reducing end Neu5Ac/Neu5Gc, as shown schematically.

Exclusive presence of Neu5Gc in oligosialylated sequences was further assessed by mild periodate oxidation followed by hydrolysis and DMB-labelling. Applied to the sialylated *N*-glycans, it cleaves the non-substituted side chains of Neu5Ac/Neu5Gc at the C_7-C_8 bond which are identified by RP-HPLC as DMB-labelling C7/C9 analogues (Sato, C., et al. 1998). This demonstrated that all 4 expected products, namely C_9(Neu5Gc)-DMB, C_7(Neu5Gc)-DMB, C_9(Neu5Ac)-DMB and C_7(Neu5Ac)-DMB could be detected at increasing retention time (Fig. 3-3-5a), and quantified as representing 28, 51, 2 and 18% of the total sialic acid content, respectively on an *N*-glycan sample with a Neu5Gc to Neu5Ac ratio of 4:1. Assuming the mild periodate oxidation of sialic acid has proceeded to completion and not hampered by any undetected non-saccharide substitution on the side chain, the recovery of intact C_9 Neu5Ac/Neu5Gc-DMB derivatives (about 30% of total) is normally indicative of an internal 2,8-linked sialyl motif since terminal or α2-9 linked sialic acids would be cleaved to C7

analogues. Strikingly, there was 14 fold more α2,8-linked Neu5Gc than Neu5Ac which suggests that where oligosialylation may occur, it preferentially extends from Neu5Gc and not from Neu5Ac. This conclusion is consistent with the observation that Neu5Gc dimer and trimers are the major oligosialyl motifs. Moreover, after subtracting the proportion of terminal Neu5Gc that was α2-8 linked to internal Neu5Gc/Neu5Ac in the dimer, the amount of the remaining C_7-Neu5Gc (21%) was roughly the same as that of C_7-Neu5Ac (18%). This figure is in good agreement with the MALDI-MS analysis which detected complex type *N*-glycans with antenna monosialylated by approximately equal amount of terminal Neu5Gc and Neu5Ac in previous work in Section 3.1.

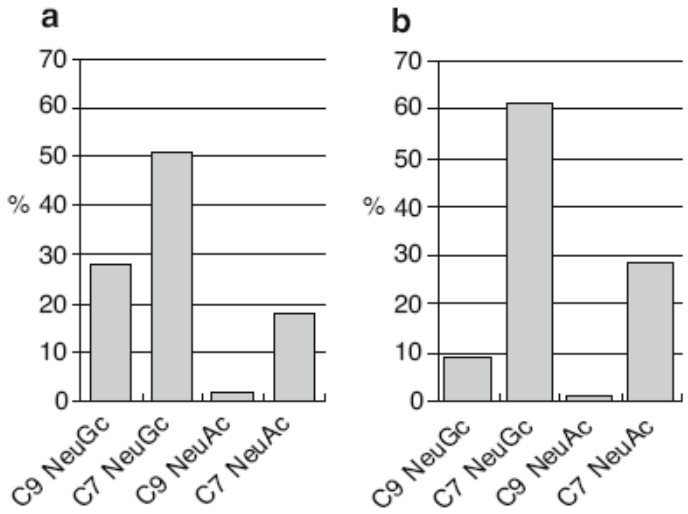

Fig. 3-3-5. Relative quantification of internal and external non-reducing sialic acids in glycoproteins oligosialylated motifs. Periodate oxidized Neu5Ac/Neu5Gc-DMB monomers from Zebrafish embryonic glycans were resolved on RP-HPLC to distinguish the respective C7/C9 products by referring to the elution positions of authentic standards. Chromatographic profiles of (a) *N*-glycans and (b) *O*-glycans from 1 hpf embryos. Results are expressed in percentage of total oxidized derivatives and are representative of two independent experiments.

3.3.1.2 Oligosialylation on O-Glycans

In contrast to *N*-glycans, disialylated motifs on *O*-glycans could be directly identified by MS analysis owing to lower molecular mass of *O*-glycans compared with *N*-glycans. In particular, we identified Neu5Gc-Neu5Gc as well as Neu5Ac-Neu5Gc motifs, but could not observe Neu5Gc-Neu5Ac and Neu5Ac-Neu5Ac, suggesting again the existence of exquisite specificity in the synthesis of α2,8-sialylated epitopes. The extent of α2,8-sialylation on *O*-glycans was evaluated using an identical experimental approach identical to that for *N*-glycans and showed very similar results. *O*-glycans are substituted by oligosialylated motifs including up to 7 residues, as determined by DMB/HPLC-FD (Fig. 3-3-2b). As observed for *N*-glycans, slight

shifts in the retentions times compared with Neu5Ac[(α2-8)Neu5Ac]$_n$-DMB suggest the prevalence of Neu5Gc containing oligoSia over Neu5Ac (Fig. 3-3-6). Separation of periodate oxidised compound by RP-HPLC confirmed also the absence of internal α2,8-linked Neu5Ac residues in the molecule, as observed on *N*-glycans (Fig. 3-3-5b). However, *O*-glycans differed from N-glycans by a lower C_9(Neu5Gc) to C_7(Neu5Gc) ratio which suggests that the proportion of oligosialylation is lower in *O*-glycans.

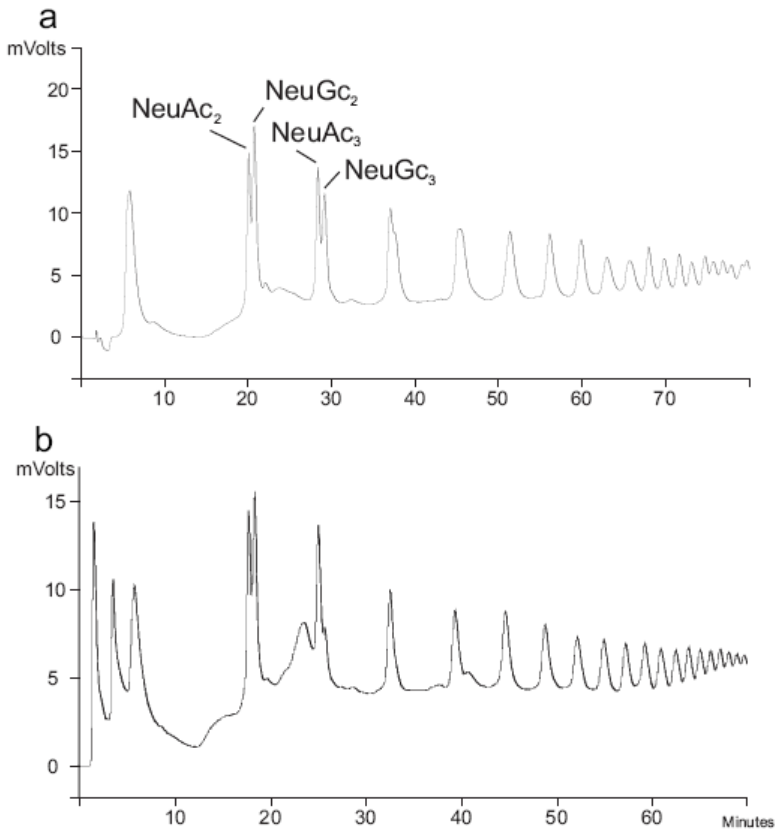

Fig. 3-3-6. Identification of oligosialylation on *O*-glycans by anion exchange DMB/ HPLC-FD. Chromatographic profiles of co-injected DMB-derivatized (a) [-8) Neu5Ac(α2-]n and [-8)Neu5Gc(α2-]n standards, (b) [-8)Neu5Ac(α2-]n standard and oligo-Sia from Zebrafish 1hpf embryos *O*-glycans, showing that the diSia (DP=2) peak from Zebrafish *O*-glycans does not co-migrate with Neu5Ac(α2-8)Neu5Ac.

Collectively, the data presented show that both *O*- and *N*-glycans are substituted by Neu5Gc containing oligosialylated sequences which exhibit similar overall patterns. Although Neu5Ac has been identified along Neu5Gc in *O*- and *N*-glycans, it seems to be restricted to monosialylated compounds or in non-reducing terminal position of oligosialylated sequences.

3.3.1.3 Oligosialylation on glycolipids

Direct MALDI-MS-mapping of the acidic glycolipids demonstrated the presence of

oligosialylated glycolipids substituted by up to 5 sialic acids (Fig. 3-3-7b). The major tri-sialylated components were previously shown to be substituted by a mixture of Neu5Ac and Neu5Gc residues in all possible combinations in project I. In contrast to *N*- and *O*-glycans, the presence of polymerized Neu5Ac indicates that no restriction seems to prevail in the synthesis of oligosialylated motifs in glycolipids. Accordingly, characterization of sialic acids by DMB/RP-HPLC demonstrated the prevalence of a molar ratio of 1.5:1 for Neu5Ac:Neu5Gc, indicating that sialylation patterns of glycolipids differ from those of glycoproteins in which Neu5Gc prevails over Neu5Ac. In agreement with direct observation of sialylated glycolipids by MS, DMB/HPLC-FD analysis shows the presence of oligosialylated motifs up to DP 6 (data not shown).

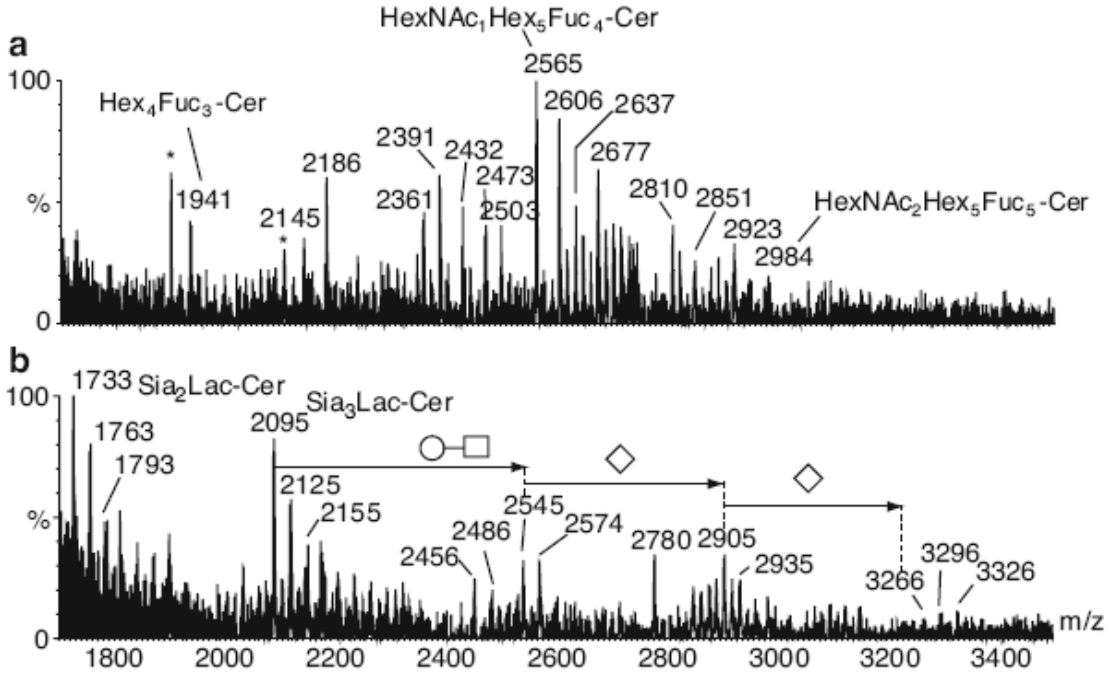

Fig. 3-3-7. MALDI-MS analyses of permethylated glycolipids from Zebrafish embryos. Glycolipid profiles of high molecular mass glycolipids from (a) 1 hpf and (b) 48 hpf embryos. No sialylated glycolipids were observed at 1hpf, whereas a complex mixture of oligo-sialylated glycolipids containing from 2 to 5 sialic acids were detected at the later stages. Symbols used: circle, Hex; square, HexNAc; diamond, sialic acid; Cer, ceramide.

3.3.2 The sialic acid content changes along development

The presence of α2,8-sialylation on glycoproteins and glycolipids was assessed along the development timeline from 0 to 48 hpf. For *O*-glycans, presence of di-sialylated glycans could be directly assessed by MS profiling. MALDI-TOF analysis of permethylated glycans after separation of mono- and disialylated compounds shows that previously identified di-sialylated *O*-glycans can be observed exclusively in the earlier stages of developments (0 and 8 hpf) as

signals at m/z 1706 (Fucα–3GalNAcβ1-4(Neu5Ac-Neu5Gcα2-3)Galβ1-3GalNAc-itol) and at m/z 1736 (Fucα–3GalNAcβ1-4(Neu5Gc-Neu5Gcα2-3)Galβ1-3GalNAc-itol), but never in the later stages (Fig. 3-4-8). Although mass spectrometry does not provide quantitative information, it suggests a disappearance of oligosialylation on *O*-glycans along development.

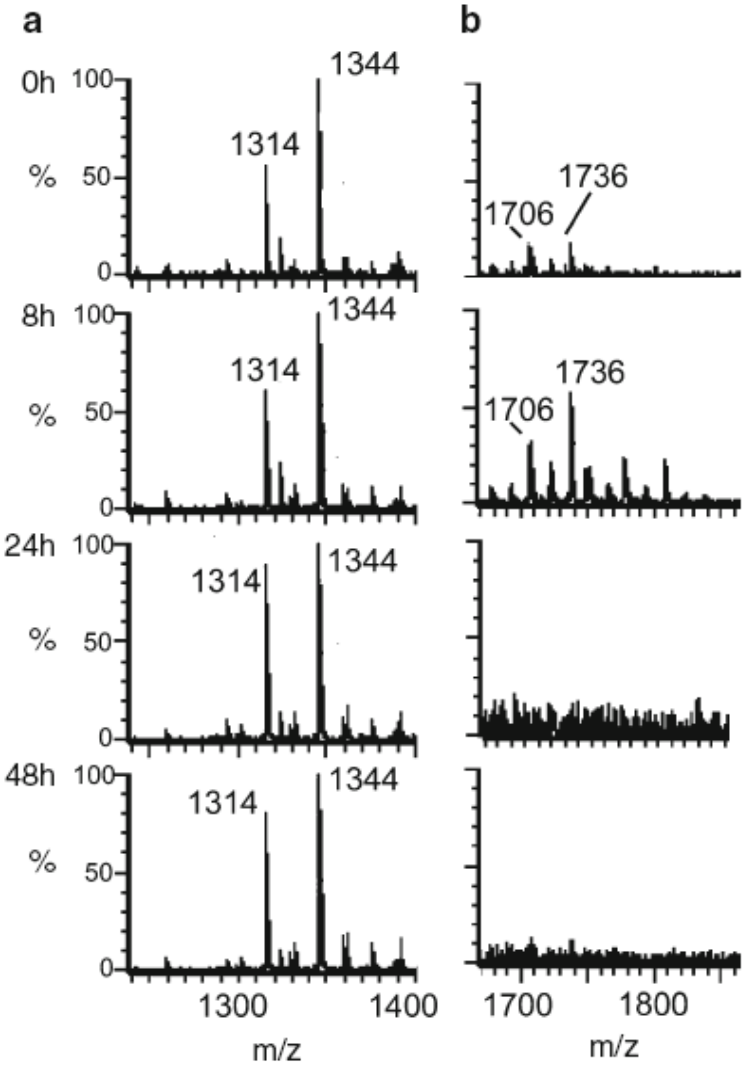

Fig. 3-3-8. MALDI-MS profiling of permethylated O-glycans from Zebrafish embryos. The presence of (a) mono-sialylated *O*-glycans Fucα1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-3 GalNAc-itol at m/z 1314 and Fucα1-3GalNAcβ1-4(Neu5Gcα2-3)Galβ1-3GalNAc-itol at m/z 1344 and (b) di-sialylated *O*-glycans Fucα–3GalNAcβ1-4(Neu5Ac-Neu5Gcα2-3)Galβ1-3 GalNAc-itol) at m/z 1706 and Fucα–3GalNAcβ1-4(Neu5Gc-Neu5Gcα2-3)Galβ1-3GalNAc-itol) at m/z 1736 was checked by MALDI-MS of the four embryonic stages from 0 to 48 hpf.

DMB/HPLC-FD analysis provided a semi-quantitative comparison of the oligosialylation content of total *O*-Glycan fractions purified from identical numbers of embryos at each development stage. Each fraction presented a very similar pattern of $(Sia)_n$-DMB with $1<n<6-7$, and thus did not show qualitative variation in the extent of α2,8-sialylation. However, a clear decrease in the quantity of each oligomer was observed as shown by integration of chromatographic peaks for di-, tri and tetra-sialyl components (Fig. 3-3-9a), confirming the rapid decrease of oligosialylated *O*-glycans during embryonic development. Indeed, after 8 hpf, quantity of disialylated motif dropped by more than 60 % and less than 5 % of the initial di-sialylated motif could be observed prior to hatching (48 hpf). Similarly, the content of tri-sialylated motifs was reduced by more than 20 fold in 48 h. Identical methodology was applied to *N*-glycans and showed a similar trend of rapid clearance of oligosialylation along development (Fig. 3-3-9b).

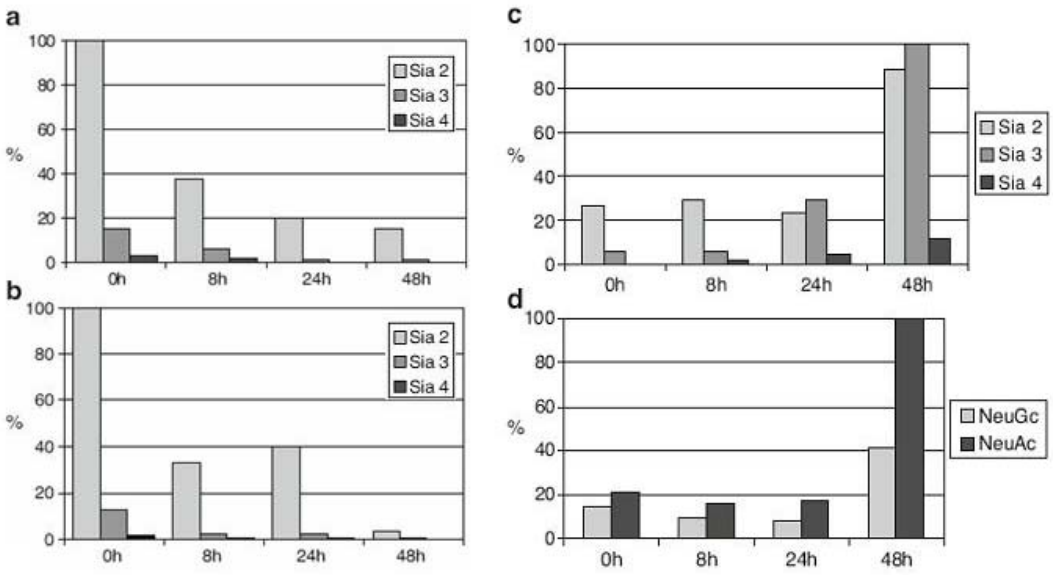

Fig. 3-3-9. Relative quantification of oligosialylation along embryos development. Proportions of Sia 2, Sia 3 and Sia 4 associated to (a) *O*-glycans, (b) *N*-glycans and (c) glycolipids were compared from 0 to 48 hpf by anion exchange DMB/HPLC-FD. Relative quantities of Neu5Ac and Neu5Gc from glycolipids were also followed along development by RP-HPLC after total release of sialic acids (d). Results are representative of three independent experiments.

In contrast to glycoprotein glycosylation, several lines of evidence demonstrated that oligosialylation in glycolipids increases along development. First, direct observation of sialylated glycolipids by MS was only possible in later stages of development (24 and 48 hpf) as previously reported. Indeed, MS profiling of total glycolipid from 0 and 8 hpf embryos exclusively showed a complex pattern of neutral fucosylated glycolipids (Table IV), but no sialylated compounds (Fig. 3-3-10).

Table IV. Monosaccharide composition of neutral glycolipids calculated from MALDI-MS analysis of permethyl derivatives

m/z $[M+Na]^+$	Composition		
	HexNAc	Hex	dHex
1,941	0	4	3
2,145	0	5	3
2,186	1	4	3
2,361	1	4	4
2,391	1	5	3
2,432	2	4	3
2,473	3	3	3
2,503	3	4	2
2,565	1	5	4
2,606	2	4	4
2,637	2	5	4
2,677	3	4	3
2,810	2	5	4
2,851	3	4	4
2,923	4	4	3
2,984	2	5	5

Repeated attempts to purify acidic compounds from early stages embryos failed to provide any evidence for their presence. These results were confirmed by comparing endoceramidase treated total glycolipid fractions from embryos at 0 and 48 hpf. Both samples show overall similar profiles characterized by complex mixtures of identical neutral glycans (Fig. 3-3-10). Later stage sample shows the presence of additional major signals at m/z 838.6, 868.6, 1083.8 and 1113.8 attributed to $Neu5Ac_1Hex_2$, $Neu5Gc_1Hex_2$, $Neu5Ac_1Hex_2HN_1$ and $Neu5Gc_1Hex_2HN_1$, respectively.

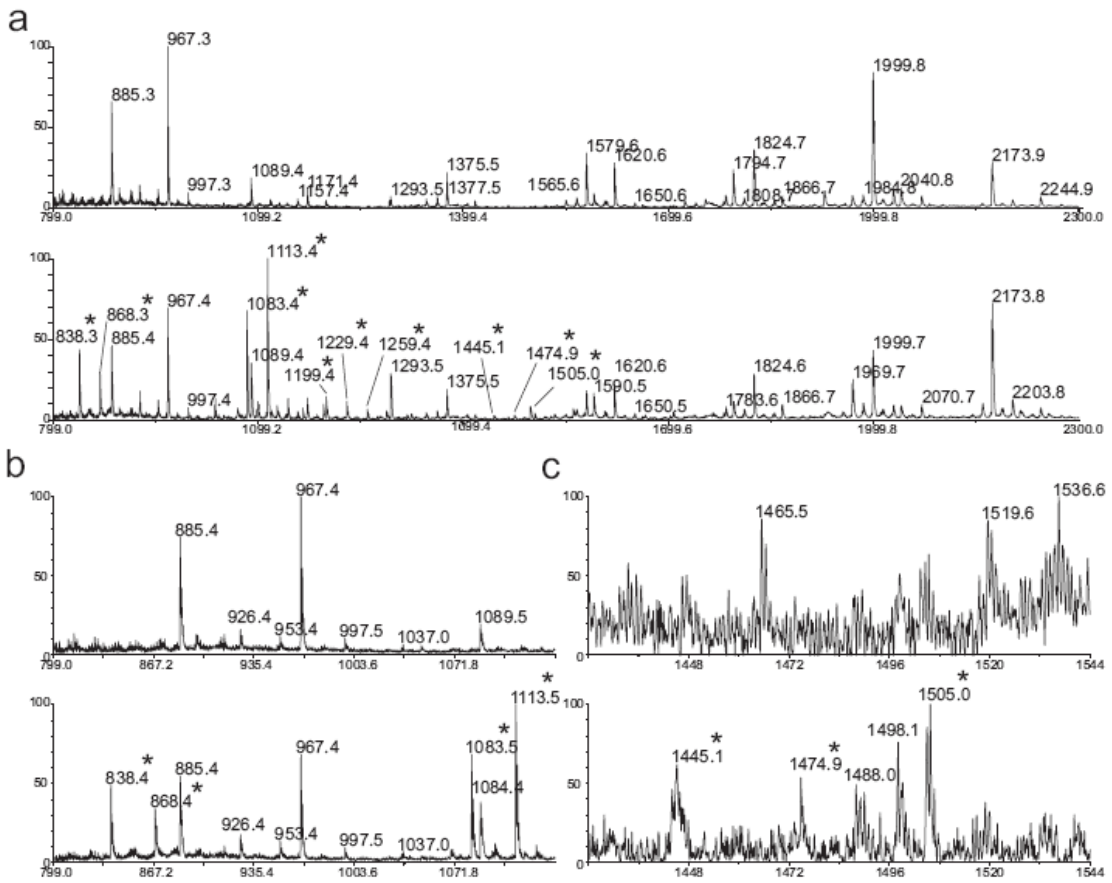

Fig. 3-3-10. Comparison of glycolipid profiles of embryos along development.
MALDI-MS profiles permethylated derivatives of endoceramidase treated glycolipids from (upper panels) 1hpf embryos and (lower panels) 48hpf embryos. (a) Spectra m/z 800 to 2300, (b) close up of spectra m/z 800 to 1150 and (c) close up of spectra m/z 1425 to 1545. Values labeled with an asterisk where attributed to sialylated components exclusively observed in 48 hpf embryos. m/z 838.6, $Neu5Ac_1Hex_2$; 868.6, $Neu5Gc_1Hex_2$; 1083.8, $Neu5Ac_1Hex2HN_1$; 1113.8, $Neu5Gc_1Hex_2HN_1$; 1199.9, $Neu5Ac_2Hex_2$; 1229.9, $Neu5Ac_1Neu5Gc_1Hex_2$; 1259.9, $Neu5Gc_2Hex_2$; 1145.0, $Neu5Ac_2Hex_2HN_1$; 1475.1, $Neu5Ac_1Neu5Gc_1Hex_2HN_1$; 1505.1, $Neu5Gc_2Hex_2HN_1$.

Furthermore, careful analysis of MS spectra reveals the presence of additional minor signals exclusively in later stage at m/z 1199.9, 1229.9 and 1259.9 attributed to $Neu5Ac_2Hex_2$, $Neu5Ac_1Neu5Gc_1Hex_2$ and $Neu5Gc_2Hex_2$ as well as m/z 1145.0, 1475.1 and 1505.1 attributed to $Neu5Ac_2Hex_2HN_1$, $Neu5Ac_1Neu5Gc_1Hex_2HN_1$ and $Neu5Gc_2Hex_2HN_1$. The chemical nature of oligosialylated motifs in endoceramidase treated glycolipids was confirmed by MS/MS sequencing of their permethylated derivatives. Indeed, fragmentation of molecular ions at m/z 1199, 1229 and 1259 showed the presence of $Neu5Ac_2$, $Neu5Ac_1Neu5Gc_1$ and $Neu5Gc_2$ sequences owing to the presence of B/Y ion pairs at m/z 760/463, 790/463 and 820/463, respectively (Fig. 3-3-11).

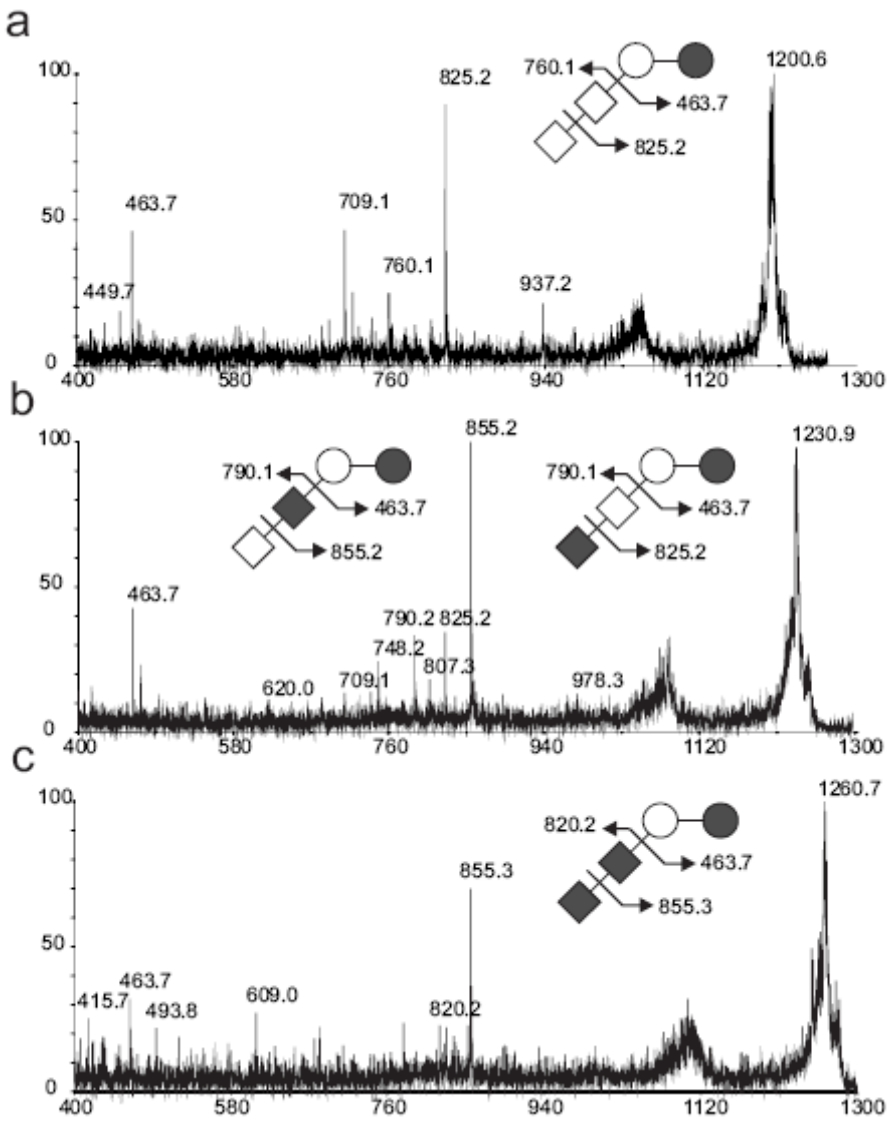

Fig. 3-3-11. MALDI-MS/MS sequencing of endoceramidase treated glycolipids from Zebrafish embryos. MS/MS analyses of permethylated derivatives of (a) $Neu5Ac_2Hex_2$ at m/z 1200, (b) $Neu5Ac_1Neu5Gc_1Hex_2$ at m/z 1230 and (c) $Neu5Gc_2Hex_2$ at m/z 1260. Fragmentation pattern of parent ion at m/z 1230 established the presence of two isomers differing by the respective positions of Neu5Ac and Neu5Gc within the diSia sequence. Sequences of these compounds are in agreement with their assignment as derivatives of disialylated lactose. Symbol used: dark circle, Glc; white circle, Gal; dark diamond, Neu5Gc; white diamond, Neu5Ac.

The presence of both Y ions at m/z 825 and 855 established that compound $Neu5Ac_1Neu5Gc_1Hex_2$ at m/z 1229 is a mixture of the two isobaric structures Neu5Gc-Neu5Ac-Hex-Hex and Neu5Ac-Neu5Gc-Hex-Hex. In agreement with MS analysis, profiling by DMB/HPLC-FD demonstrated that oligosialylation increased along development, with a sharp

increase between 24 and 48 hpf (Fig. 3-3-9c). Indeed, as compared with embryos at 0 hpf, quantity of diSia increased by 3.5 fold and triSia by 17 fold in 48 hpf embryos. Accordingly, the total amount of sialic acid at 48 hpf is 4 to 6 higher than in other stage (Fig. 3-3-9). These data clearly demonstrate that the amount of oligosialylation in embryos varies along development. Surprisingly, comparative profiling of glycoconjugates established that the overall content of sialylation decreases for glycoproteins along embryogenesis, but increases for glycolipids.

3.3.3 Distinctive correlation with the expression of ST8Sias and sialidase activity.

3.3.3.1 Positive correlation with ST8Sia mRNA level

To investigate whether ST8Sia genes may participate in the di-, oligo- and polysialylation of major glycoconjugates during Zebrafish development, we examined the mRNA levels of the α2,8-sialyltransferase genes (ST8Sia I, ST8Sia II, ST8Sia III, ST8Sia IV, ST8Sia V and ST8Sia VI) in different developmental stages by real time PCR. As described in Section 3.2, six human ST8Sia orthologs could be identified in Zebrafish genome (Harduin-Lepers, A., et al. 2005). These genes were molecularly cloned from various Zebrafish organs by RT-PCR and sequenced (the detail procedure will be mentioned in the Section 3.4). Since β-actin showed variable level of expression among the various developmental stages with the same amount of total cDNA (data not shown), we have chosen absolute quantification to quantify ST8Sia. Except for ST8Sia II, all the ST8Sia genes showed very low level of expression at 0 hpf (Fig. 3-3-12). They all increase along embryogenesis from 0 to 36 hpf but at very different rates (Fig. 3-3-12a). Indeed, expression levels of ST8Sia I and ST8Sia VI sharply increase in the first 14 hours of development whereas other ST8Sia exhibited either more modest or delayed increase (Fig. 3-3-12a). Then, ST8Sia I and ST8Sia III reach maximum sustained expression levels at 14 hpf whereas the others show a gradual increase in expression level up to 36 hpf. Interestingly, ST8Sia I and ST8Sia V genes, the human orthologs of which are known to be involved in glycolipids sialylation, are both dramatically up regulated along development by a factor of 300 and 165, respectively. This result is in agreement with the observation of an increase of sialylation associated to glycolipids. On the contrary, the modest increased expression levels of ST8Sia II and ST8Sia IV, which are responsible for the biosynthesis of polysialic acid, and of ST8Sia III also involved in the α2,8-sialylation of glycoproteins are still slightly inconsistent with the decreased oligosialylation status observed on glycoproteins. More surprisingly is the 4000 fold increase of ST8Sia VI gene expression that is not directly correlated with an increase of Sia_2 motifs synthesis on glycoproteins.

a

	ST8Sia I	ST8Sia II	ST8Sia III	ST8Sia IV	ST8Sia V	ST8Sia VI
unf	0.15	6.38	3.91	1.82	2.79	21.65
0h	1.00	1.00	1.00	1.00	1.00	1.00
14h	266.78	0.91	4.51	1.19	0.46	173.39
24h	92.78	0.97	5.77	1.55	13.00	75.18
36h	303.74	6.31	5.53	4.58	165.56	4059.40

b

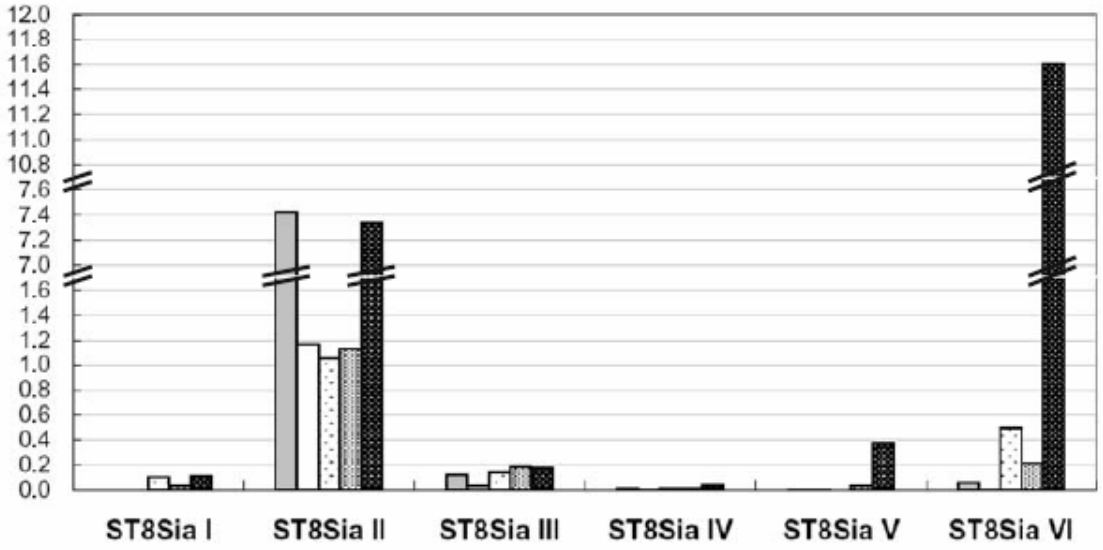

Fig. 3-3-12. Absolute quantification of *Dre* ST8Sia genes expression by real-time PCR.
a) "Fold of increase" represents the relative expression quantity of each ST8Sia in different developmental stages compared with expression at 0 hpf. **b)** The different expression levels of all the ST8Sia genes were analyzed with cDNA from 0 hpf (empty square), 14 hpf ⊡, 24 hpf ▨, 36 hpf ▩ eggs and ovary (filled square in grey) by quantitative real-time PCR. The absolute amount of transcripts was quantified according to the same DNA fragment amplified and cloned in the plasmid. Values are the mean of triplicate points.

3.3.3.2 Positive correlation with sialidase activities

To check whether Zebrafish sialidases might be involved in these α2,8-sialoglycoprotein metabolism, the endogenous sialidase activity were assayed by using a fluorescenct substrate, 4-methyl-umbelliferyl-Neu5Ac (4MU-Neu5Ac), at various pH for fertilized eggs at different embryonic stages (0, 8, 24, and 48 hpf) and unfertilized eggs. Our preliminary data showed the existence of intense sialidase activities associated to embryos. Survey of sialidase activities showed dramatic differences of intensities depending on the pH and along development (Fig. 3-3-13). The fact that the evolution of total sialidase activities measured at different pH follows different trends strongly suggest the presence of different enzymes, as recently demonstrated by the identification of several genes coding for potential sialidases in Zebrafish (Manzoni, M.,

et al. 2007). Indeed, the sialidase activities detected at pH 5 sharply decreased along Zebrafish development whereas sialidase activities detected at pH 4 increased slightly along Zebrafish development. These data demonstrate a tight regulation of sialidase activities along development and suggest a possible involvement of sialidases in the α2,8-sialylation status of sialoglycoproteins, which may explain the discrepancies observed between the regulation of ST8Sia genes involved in the glycoproteins biosynthesis and the actual synthesis of oligosialylated motifs on glycoproteins.

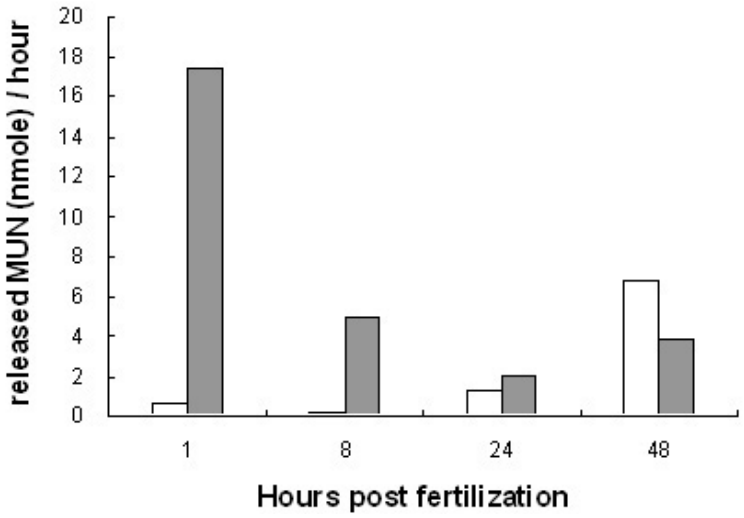

Fig. 3-3-13. Endogenous sialidase activities in fertilized Zebrafish eggs. Sialidase activities were defined by the amount of 4-methyl-umbelliferone (MUN) released from 4-methyl-umbelliferyl-NeuAc (4MUN-NeuAc) within one hour. 0, 8, 24, and 48 hours post-fertilized eggs were assayed at pH 4 (white bar) and pH 5 (grey bar).

3.3.3.3 Oligosialylation patterns and sialidase activities in different compartments of eggs

To clarify if the decreased and increased oligosialylation from glycoproteins and glycolipids are indeed differentially originated from mother and embryo, we have assessed the oligosialylation patterns of glycoproteins in different compartments of the fertilized eggs: embryo tissue, chorion and perivitelline space (Fig. 3-3-14). Semi-quantification analysis by DMB-HPLC revealed that about 94% of the total oligoSia in 1 hpf fertilized oocyte was associated with soluble glycoconjugates in the perivitelline space, 5% in the chorion and less than 1% in the embryonic tissue (data not shown). Surprisingly, the extent of oligosialylation distributed within each of these fractions was very different. The perivitelline space associated components show a very short DP distribution dominated by DP 2, reminiscent of the one observed on total glycoprotein fraction, whereas the chorion and embryonic tissues exhibit more evenly distributed patterns with up to DP 10. Each fraction was further differentiated by their sialic acids content, as embryo associated glycoproteins was almost exclusively composed of Neu5Gc (Neu5Gc/Neu5Ac 14:1) whereas perivitelline space associated glycoproteins of

Neu5Gc and Neu5Ac in a 4:1 ratio (data not shown).

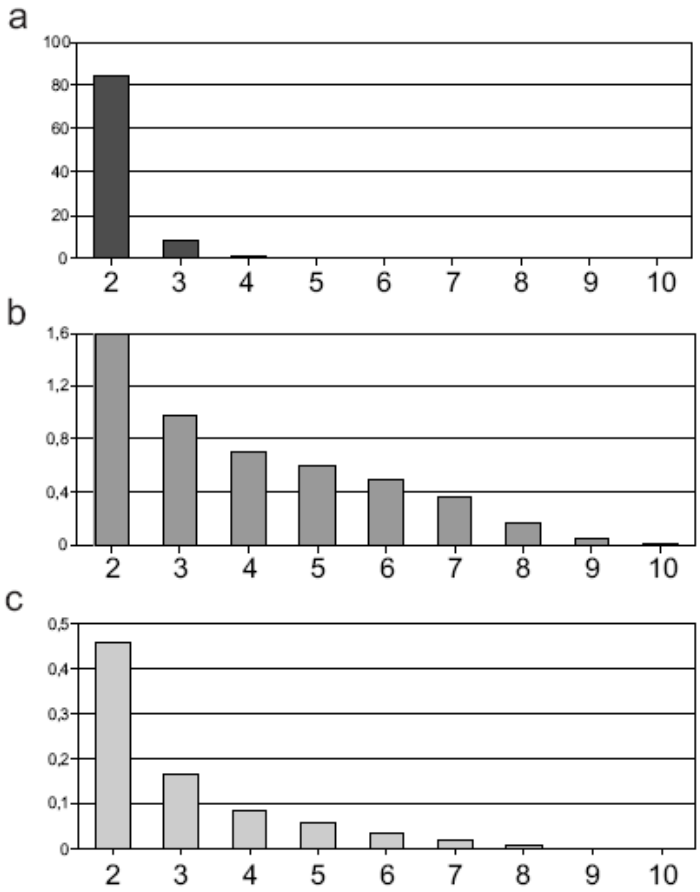

Fig. 3-3-14. Profiles of glycoprotein associated oligosialic acids from different locations in the fertilized eggs. Proteins associated oligoSia were purified from (a) perivitelline space, (b) chorion and (c) embryonic tissue, and analyzed by anion exchange DMB/HPLC-FD. X-axis represents the DP from 2 to 10; Y-axis represents the intensity of each DP is plotted in percentage, 100% being the sum of oligoSia from all fractions.

The presence of huge amount of di-sialylated glycoproteins in peri-vitelline space and their subsequent disappearance prompted us to further investigate whether the sialidase activities are also present between the fertilization envelope and plasma membrane of embryo. The endogenous sialidase assay was performed with 4MU-NeuAc for the different compartments of fertilized eggs. To detect the sialidase activity, pronase digestion was avoided. Instead the fertilization envelopes were partially ripped with needles in the buffer for later assay. The reason that the envelope was not completely removed is due to the technical limitation that the embryos within the first few hours after fertilization are too fragile to be separated from the envelope physically. Consequently, the perivitelline content was obtained from the supernatant after centrifugation of the envelope-ripped eggs. It is expected that under such gentle ripping,

the enclosed embryos remain intact and the intracellular content would not be accessible for the sialidase assay. This sample, namely one without cebtrifugation and removal of supernatant was referred to as “peri-vitelline + chorion”. On the other hand, the whole egg homogenates were taken as “peri-vitelline + chorion + embryo” (Fig 3-3-15). The peri-vitelline content alone (blue diamond), already showed nearly half the activity of whole egg homogenate (yellow triangle). The sialidase activities of the “perivitelline + chorion”, including the soluble form sialidases and the plasma membrane-bound sialidases, appeared to higher activity than the peri-vitelline alone (pink square). Thus the actual presence of endogenous sialidase activities in the peri-vitelline space may account for the dramatically decreased di-sialyl content in early developmental stages (Fig. 3-3-9 a and b).

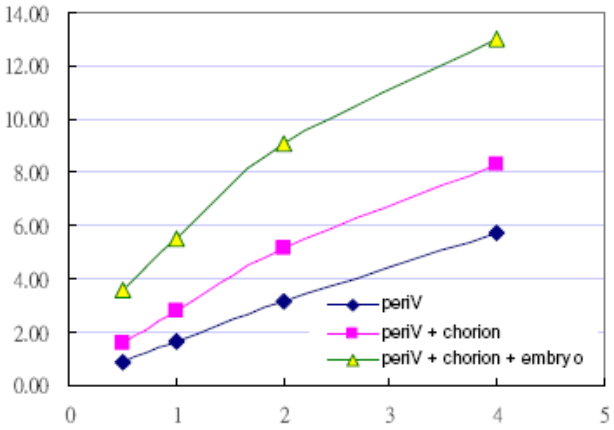

Fig. 3-3-15. Compartmentalized sialidase activities of Zebrafish eggs 0hpf at pH 5.5. Endogenous sialidase activities from different compartments were defined by the amount of 4-methyl-umbelliferone (MUN) released from 4-methyl-umbelliferyl-NeuAc (4MUN-NeuAc) at pH 5. Yellow triangles represent sialidase from whole egg lysates which includes embryo, chorion, and peri-vitelline content; Pink squares represent sialidase from partially damaged fertilization envelope which includes the activity of fertilization envelope and peri-vitelline space; Blue diamonds represent the sialidase activities from peri-vitelline space after centrifugation the fertilized eggs with partially-opened envelope.

3.3.4 Discussion

This study extended our knowledge from the initial glycomic survey (Section 3.1) those fine structures of sialylated glycans synthesized by Zebrafish along embryonic development by focusing on the α2,8-sialylation pattern in quality and quantity. Our data clearly established that both glycoproteins and glycolipids were α2,8-sialylated. Surprisingly, fine structural analysis demonstrated that the major glycolipids and glycoproteins presented different patterns of oligosialylation. Whilst the α2,8-sialylated glycolipids may be substituted by mixtures of oligo(Neu5Ac), oligo(Neu5Gc) and hybrid type oligo(Neu5Ac, Neu5Gc) sequences,

glycoproteins are mainly substituted by oligo(Neu5Gc). Indeed, although Neu5Ac and Neu5Gc are present in similar proportions on mono-sialylated motifs of N-glycans (Guérardel, Y., et al. 2006), we have now demonstrated that only Neu5Gc is further elongated by other sialic acids to form oligo(Neu5Gc) sequences. Identical biosynthetic restrictions seems to prevail during the extension of oligosialic acids associated with *O*-glycans that prevent formation of oligo(Neu5Ac). These differences between glycoproteins and glycolipids were also reflected in their respective contents of sialic acids: two to four times more Neu5Gc than Neu5Ac in glycoproteins and about twice as more Neu5Ac than Neu5Gc for glycolipids. As previously reported, polysialic acid structures of fish egg glycoproteins are exquisitely species specific, differing by their extent, composition, acetylation and sequences (Sato, C., et al. 1993). However, to our knowledge, Zebrafish represents the only documented example of the preferential use of Neu5Gc over Neu5Ac for oligosialic acid elongation found on glycoproteins. This suggests an α2,8-sialyltransferase activity dedicated to α2,8-sialylation of glycoproteins, such as ST8Sia II, ST8Sia III, ST8Sia IV or ST8Sia VI, that would exhibit a preference for CMP-Neu5Gc over CMP-Neu5Ac.

In mammals, ST8Sia II and ST8Sia IV are known to be involved primarily in the polysialylation of the N-glycans of N-CAM in Zebrafish and on PSGP *O*-glycans in rainbow trout, which suggest that these enzymes might have multiple substrates acceptors in various animal species (Asahina, S., et al. 2006, Marx, M., et al. 2001). ST8Sia III catalyzes the transfer of one to seven sialic acid residues onto glycoproteins or glycolipids, whereas ST8Sia VI catalyzes the transfer of a unique sialic acid residue on the O-glycans of glycoproteins as reported (Harduin-Lepers, A., et al. 2001, Teintenier-Lelievre, M., et al. 2005). In this context, it is also interesting to note that Zebrafish ST8Sia IV shows very low capability to transfer Neu5Ac from CMP-Neu5Ac onto N-CAM compared to Zebrafish ST8Sia II (Marx, M., et al. 2007). This last observation might reflect a preference of Zebrafish ST8Sia IV for CMP-Neu5Gc over CMP-Neu5Ac. Also, it is noteworthy that the major glycoprotein associated α2,8-sialyl motifs in Zebrafish exhibit a significantly lower DP compared to those previously identified on the polysialylated glycoprotein (PSGP) of other fish species. Indeed, while salmonids eggs contain polysialyl units with chain length up to 20 residues (Inoue, S. and Iwasaki, M. 1980, Inoue, S.a.I., Y 1997), the major sialylated glycoproteins of Zebrafish eggs are mainly substituted by diSia (DP=2) motif as well as minute amounts of oligoSia (3<DP<6).

In addition, expression of oligosialylation on glycolipids and glycoproteins is differentially regulated along embryonic development. Indeed, the extent of α2,8-sialylation on major glycoproteins sharply decreases whereas that of glycolipid increases along development. Surveys of sialylation by MS analyses of intact and endoceramidase treated glycolipids, as well as the quantification of sialic acids and oligosialic acids, all indicated that significant sialylation specifically and reproducibly appears around 24 hpf, which strongly suggests that glycolipid associated α2,8-sialylation is triggered during early development. Accordingly, we

failed to detect significant glycolipid associated sialylation in mature ovaries before spawning (data not shown). It is noteworthy that the complex neutral glycolipids observed in all development stages are apparently not the substrates for sialylation events occurring in later developmental stages. MS analyses demonstrated that the sialylated glycolipids of later stages comprised mainly Neu5Ac/Neu5Gc substituted $(Sia)_{1-4}$LacCer, GM2 and GD2 with no sialylated equivalents of the fucosylated neutral glycolipids (Table IV), suggesting that these two families of compounds are independently synthesized.

The appearance of glycolipids associated α2,8-sialylation in later stages of embryonic development positively correlates with the up-regulation of genes coding for α2,8-sialyl-transferases ST8Sia I, ST8Sia III and ST8Sia V (Fig. 3-3-12). The human recombinant enzymes have been shown to be involved in the biosynthesis of gangliosides GD3, GT3, GD1a, GT1b and GQ1c (Bink, R.J., et al. 2003, Daniotti, J.L., et al. 1997, Fukuda, M., et al. 1987, Haraguchi, M., et al. 1994, Harduin-Lepers, A., et al. 2001, Nara, K., et al. 1994, Nikonova, E.Y., et al. 2004). The onset expression of these genes starts around 10 hpf and is essentially located in the developing brain which will be discussed in next Section 3.4. Up-regulation of oligo-sialylation along Zebrafish development is in agreement with previous observations made on *Xenopus laevis* showing by *in vitro* assays that ST8Sia I (SAT-2) and ST8Sia V (SAT-4) activities were dramatically increased along the early development, with a maximum activity at day 4 (Gornati, R., et al. 1997). However, whereas quantities of both neutral glycolipids and gangliosides sharply increase in *X. laevis*, only gangliosides appear to be up-regulated in *D. rerio* (Gornati, R., et al. 1997).

In contrast to glycolipids, evolution of glycoproteins associated α2,8-sialylation does not correlate with the temporal expression and the general increase of mRNAs of ST8Sia II, ST8Sia IV and ST8Sia VI from 10 hpf along the early stages of Zebrafish development, therefore implicating a different kind of regulation. An up-regulation of the ST8Sia II gene expression along embryonic development of Zebrafish has been previously reported in the context of an increased synthesis of polysialic acid chains (PSA) on the *N*-glycans of the neural cell adhesion molecule (N-CAM), which reaches a maximum around 27-40 hpf (Marx, M., et al. 2001). Our present study focused instead on the global α2,8-sialylation status of the different classes of glycoconjugates along embryonic Zebrafish development and has identified a rapid decrease of glycoproteins associated α2,8-sialylation content. This might be explained by the large quantities of PSGP synthesized in the cortical alveoli during oogenesis in fishes (Kitazume, S., et al. 1994) compared to the natural low abundance and restricted localization of PSA-N-CAM. It is most probable that the N-CAM polysialylation pattern cannot be discriminated from the one of PSGP or other polysialylated glycoproteins by a global approach. It is noted that the ST8Sia II, ST8Sia IV and ST8Sia VI genes are also found to be expressed in the ovaries (Fig. 3-3-12), suggesting that the α2,8-sialylated glycoproteins of Zebrafish embryo detected at very early developmental stages well before the onset zygotic

expression of these ST8Sia (around 10 hpf), might be inherited from the mother. We hypothesize that these inherited α2,8-sialylated glycoproteins could be subsequently degraded in the embryos by endogenous sialidases.

The disappearance of oligo-sialic acid on glycoproteins during development can be a result of decrease in its biosynthesis (as the matter of glycosyltransferases) and/or the presence of endogenous sialidases. A recent study of Manzoni *et al.* (Manzoni, M., et al. 2007) reported the identification of seven Zebrafish sialidase genes homologous to three of the four known human genes (NEU1, NEU2, NEU3, NEU4) Zebrafish neu3.1, neu3.3 and neu4 were shown to be active towards gangliosides at very low pH (2-3) and the corresponding genes were found to be expressed differentially along the embryonic development. To substantiate this hypothesis, we have assayed the sialidase activities at various pH (3, 4, 5, 6, 7) with unfertilized eggs. Since the higher and lower activities were obtained at pH 5 and 4 respectively, we then assayed the sialidase activities at pH 4 and 5 for the various developmental stages of interest. Our data showed that sialidase activities found at pH 4 increased along Zebrafish development, whereas sialidase activities found at pH 5 decreased, which suggested the presence of different sialidase activities. When the sialidase activities were assayed with whole egg lysates, it reflects the net activity of total sialidases, which were located in different compartments in normal physical conditions, at a particular pH. It is therefore necessary to characterize the enzyme kinetics of each sialidase before reaching a conclusion on which sialidase contributed to the activity at pH4 and pH5. As an alternative approach, so as to distinguish the locations (inside embryo, peri-vitelline space, or fertilization envelope) and the origins of the sialidases (from mothers or embryos), another sialidase assay was carried out with different parts of fertilized eggs, in particular the activities residing in the perivitelline space only. Our result positively indicated the presence of soluble maternal sialidases in the peri-vitelline space also involve along embryogenesis (Fig. 3-3-15).

The presence of large quantities of oligosialylated soluble glycoproteins in perivitelline space of Zebrafish is in good agreement with previous reports of the of polysialylated peptides originating from a fertilization induced proteolysis of cortical alveoli PSGP in several other species of fish, including trouts, salmonids and medaka fish (Kitazume, S., et al. 1994, Sato, C., et al. 1993, Taguchi, T., et al. 1993). Accordingly, we observed large quantities of protein associated oligoSia chains in Zebrafish mature ovaries that exhibit a distribution pattern and a composition identical to that of the soluble glycoproteins of fertilized oocytes (data not shown). However, despite the postulated conservation of this phenomenon among fishes and the large quantity of excreted PSGP, the destiny and the function of these compounds are still largely unknown.

Altogether, our data established that the vast majority of oligosialylated glycoproteins observed in fertilized oocytes are synthesized prior to embryogenesis in the mother ovary and then degraded along embryonic development for a yet unknown purpose. These results presented here establish the structural bases for the investigation of the fine enzymatic specificities of the different ST8Sia identified from Zebrafish genome (Harduin-Lepers, A., et al. 2005). To further investigate the possible function of ST8Sia during embryogenesis, whole mount in situ hybridization was performed to delineate its spatial and temporal expression profile, as reported in the next section (Section 3.4).

3.4 Characterization of the spatial-temporal expression pattern of Zebrafish α2, 8-sialyltransferases in the developing nervous system

Decades of research have uncovered essential roles for both *α*2-8-sialylated glycolipids and glycoproteins in the central nervous system (CNS) (Troy, F.A., 2nd 1992). Gangliosides are a heterogeneous group of sialylated glycosphingolipids present in the outer leaflet of cell membranes, which contain highly antigenic carbohydrate moieties facilitating the production of antibodies and immunolocalization studies in the developing CNS (Schwarz, A. and Futerman, A.H. 1996). DiSialylated gangliosides are known to play a role in neurite outgrowth and cell adhesion (Rauvala, H. 1984), differentiation and signal transduction. Although there are large differences between animal species and tissues, an increase in the content of gangliosides and changes in their degree of sialylation are observed along with the development of mammalian embryonic brain (Ngamukote, S., et al. 2007). A shift from the simplest gangliosides of the a- and b-series (G_{M3} and G_{D3}) in mid-embryonic brains to more complex gangliosides such as G_{D1a}, G_{T1b} or polysialogangliosides of the c-series at later developmental stages has been reported (Ngamukote, S., et al. 2007, Yu, R.K., et al. 1988) for mouse and rat. Cichlid fish brain was shown to be enriched with c-series gangliosides including G_{T3} and polysialo-species, suggesting a tissue-specific regulation of the gangliosides biosynthesis in brain (Freischutz, B., et al. 1994).

As described in Section 3.1 and 3.3, MS and fluorescence-HPLC analyses of Zebrafish glycolipids extracted at different embryonic stages have shown that the major sialylated glycolipids contained up to four polymerized sialic acid residues. The expression pattern of the corresponding Zebrafish ST8Sia genes revealed that all the human ST8Sia orthologs were zygotically expressed during Zebrafish embryo development and correlated with the oligosialylated glycolipids. It was further shown that, except for the ST8Sia II gene, ST8Sia genes lack maternal expression in the embryos (no PCR amplification detected at 0 hpf) and start to be expressed approximately at the first stage of segmentation (10 hpf). To further address the biological implications of oligosialylations during embryogenesis, we chose to map the spatial-temporally transcriptional profile of ST8Sia by whole mount in situ hybridization.

3.4.1 Spatial-temporal expression of Zebrafish ST8Sia mRNA

In order to assess the topographical distribution of Zebrafish ST8Sia transcript at early stages of Zebrafish development, we used specific anti-sense digoxigeninlabelled RNA probes with no homology to other Zebrafish sialyltransferases and whole-mount in situ transcript hybridization (Thisse, B., et al. 2004, Thisse, C. and Thisse, B. 2008). Negative controls using sense ST8Sia probes did not produce detectable signal (data not shown). No expression of Zebrafish ST8Sia III gene was detected in embryo until the early pharyngula period (24 hpf), when we detected the ST8Sia III gene in the forebrain marginal zone (telencephalon and diencephalon), in olfactory placodes, in cranial ganglia and spinal cord neurons (Fig. 3-4-1A, E). At 36 hpf, Zebrafish ST8Sia III gene was expressed in the forebrain and midbrain marginal zone, in the epiphysis and ventral

part of the hindbrain (Fig. 3-4-1B, F) and from 48 hpf to 5 days, it was found in the brain marginal zone.

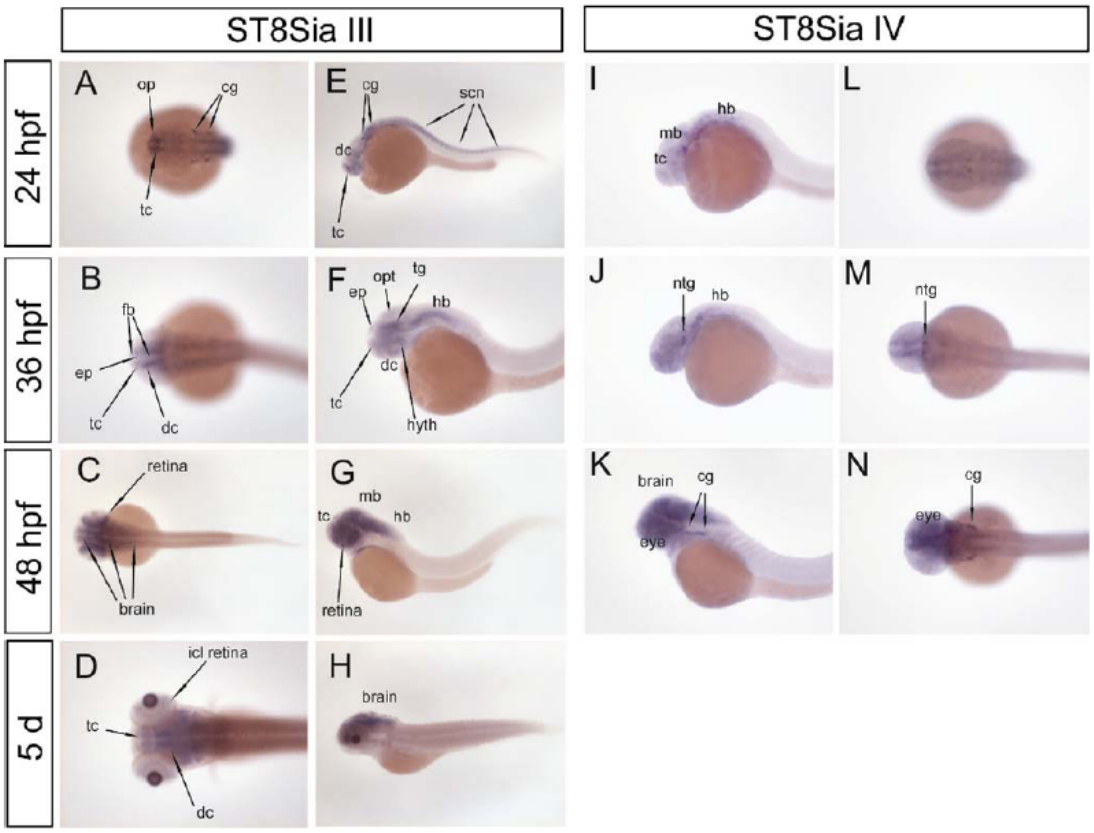

Fig. 3-4-1. Expression of the oligo-α2,8-sialyltransferases ST8Sia III gene and of the poly-α2,8-sialyltransferase ST8Sia IV gene in the differentiating central nervous system during embryonic and larval development of Zebrafish. On the left side of the figure, panels A, B, C, D show dorsal and E, F, G, H show lateral views of the ST8Sia III gene expression (anterior is to the left). From pharyngula stage (24 hpf: A, E) to larval stage (5 dpf: D, H), expression of the ST8Sia III gene is mainly detected in forebrain marginal zone (telencephalon and diencephalon), in the olfactory placodes, cranial ganglia and spinal cord neurons (A, E). At 36 hpf, it is expressed in the epiphysis and ventral part of the hindbrain (B, F). At 48 hpf, expression of Zebrafish ST8Sia III is found in the retina and at 5 dpf, it is restricted to the inner cell layer in the retina. On the right side of the figure, panels I, J, K show lateral and L, M, N show dorsal views of the pattern of expression of the ST8Sia IV gene (anterior is to the left). From 24 hpf (I, L) to 36 hpf (J, M) strong basal level of expression of the ST8Sia IV gene is observed in the ventral part of the hindbrain and in a nucleus in tegmentum. At 48 hpf (K, N), ST8Sia IV gene is expressed in the whole brain and in cranial ganglia. cg Cranial ganglia, dc diencephalons, ep epiphysis, fb forebrain, hb hindbrain, hyth hypothalamus, mb midbrain, ntg nucleus in tegmentum, op olfactory placodes, opt optic tectum, scn spinal cord neurons, tc telencephalon, tg tegmentum.

Zebrafish ST8Sia III mRNA was also localized in the retina of the developing eye at 48 hpf and this expression was restricted to the inner cell layer in the retina at the larval stage (5 dpf) (3-4-1C, D, G, H). No expression of Zebrafish ST8Sia III gene could be detected in the trunk. In this study, we did not observe ST8Sia IV gene expression in embryos until 24 hpf (Fig. 3-4-1) and the expression level of ST8Sia IV gene was generally weaker in the CNS throughout early developmental stages when compared to the one of ST8Sia II, previously reported. We have also observed strong basal level of expression of ST8Sia IV gene in the differentiating Zebrafish brain, in a nucleus in tegmentum and in the ventral part of the brain from 24 hpf to 36 hpf (Fig. 3-4-1I, J, L, M). At 48 hpf, ST8Sia IV gene is expressed all over the brain and cranial ganglia (Fig. 3-4-1K, N).

There was no detectable expression of the zygotic ST8Sia I, ST8Sia V and ST8Sia VI genes before the early somitogenesis stage (10 hpf). In addition, Zebrafish ST8Sia VII gene was not expressed during embryonic development and the ST8Sia VI gene had a dispersed pattern of expression, not restricted to the NS (data not shown). At 10 hpf, low levels of ST8Sia I mRNA were first evident in the forebrain (telencephalon and diencephalon), but not in the ventricular zone as well as in the anterior part of the spinal cord and in one presumptive cranial ganglia (Fig. 3-4-2A). Later in somitogenesis (16 hpf), the ST8Sia I gene showed a similar pattern of expression in forebrain, in several cranial ganglia and in spinal cord neurons (Fig. 3-4-2D). By 24 hpf, the expression of the ST8Sia I mRNA extended to the whole CNS and shows a higher expression level in telencephalon with the exception of the ventricular zone (Fig. 3-4-2B), and a strong level of expression in the anterior and posterior lateral line of cranial ganglions (trigeminal ganglion, gV). Expression of the ST8Sia I gene was also detected in the midbrain (tegmentum) and in the ventral part of the hindbrain; no labelling was detected in optic tectum and weak labelling was observed in the corpuscle of Stannius, the fish organ responsible for the homeostasis of calcium (Fig. 3-4-2E). Peak expression of the ST8Sia I gene was reached by 36 hpf overall in the CNS and strong expression was detected in telencephalon, especially in olfactory bulb and in the peripheral nervous system (posterior and anterior lateral line ganglia). Weak expression was found in optic tectum and the corpuscle of Stannius was no longer detectable (Fig. 3-4-2C, F). At 48 hpf, the ST8Sia I gene was mainly detected in the brain excluded from the ventricular zone. A strong hybridization was observed in the diencephalon subventricular zone as well as in ventral diencephalon, near the neurohypophysis (Fig. 3-4-2G) and in the hindbrain, two nuclei in rhombomere 3 expressed the ST8Sia I gene more strongly. A weak labelling was observed in the retina ganglion cell layer and the ST8Sia I gene expression was lost in cranial ganglia. At 5 dpf, expression of the ST8Sia I gene was weak and restricted to telencephalon and hindbrain (Fig. 3-4-2H). We did not observe ST8Sia V gene ISH in the developing Zebrafish embryo until the middle of somitogenesis stage (22 hpf), when it was expressed in trigeminal ganglion and in telencephalon (Fig. 3-4-2I). By 24 hpf, the ST8Sia V mRNA was predominantly and highly expressed in olfactory vesicles, olfactory bulb, diencephalon marginal zone, in the lateral part of the hindbrain, tegmentum, cranial ganglia and in neurons of the dorsal part of the spinal cord (Fig.

3-4-2J, N). At 36 hpf, a high level of expression was detected in the CNS with the exception of the ventricular zone, in all cranial ganglia and in the ganglion cell layer of the retina (Fig. 3-4-2K). Levels of the ST8Sia V gene remained consistently high during pharyngula stage (24–36 hpf) and peak expression was reached by hatching period at 48 hpf (Fig. 3-4-2L, O). At that stage, we observed the same pattern of strong expression in the CNS and PNS. In addition, we detected a weak expression in the inner cell layer of retina for both ganglion and inner cell layer and no expression was found in the proliferative zone. At the larval stage (5 dpf), the level of expression of the ST8Sia V gene was still very high in the brain and in all cranial ganglia and no expression was detected in the ventricular zone of the brain (Fig. 3-4-2M, P).

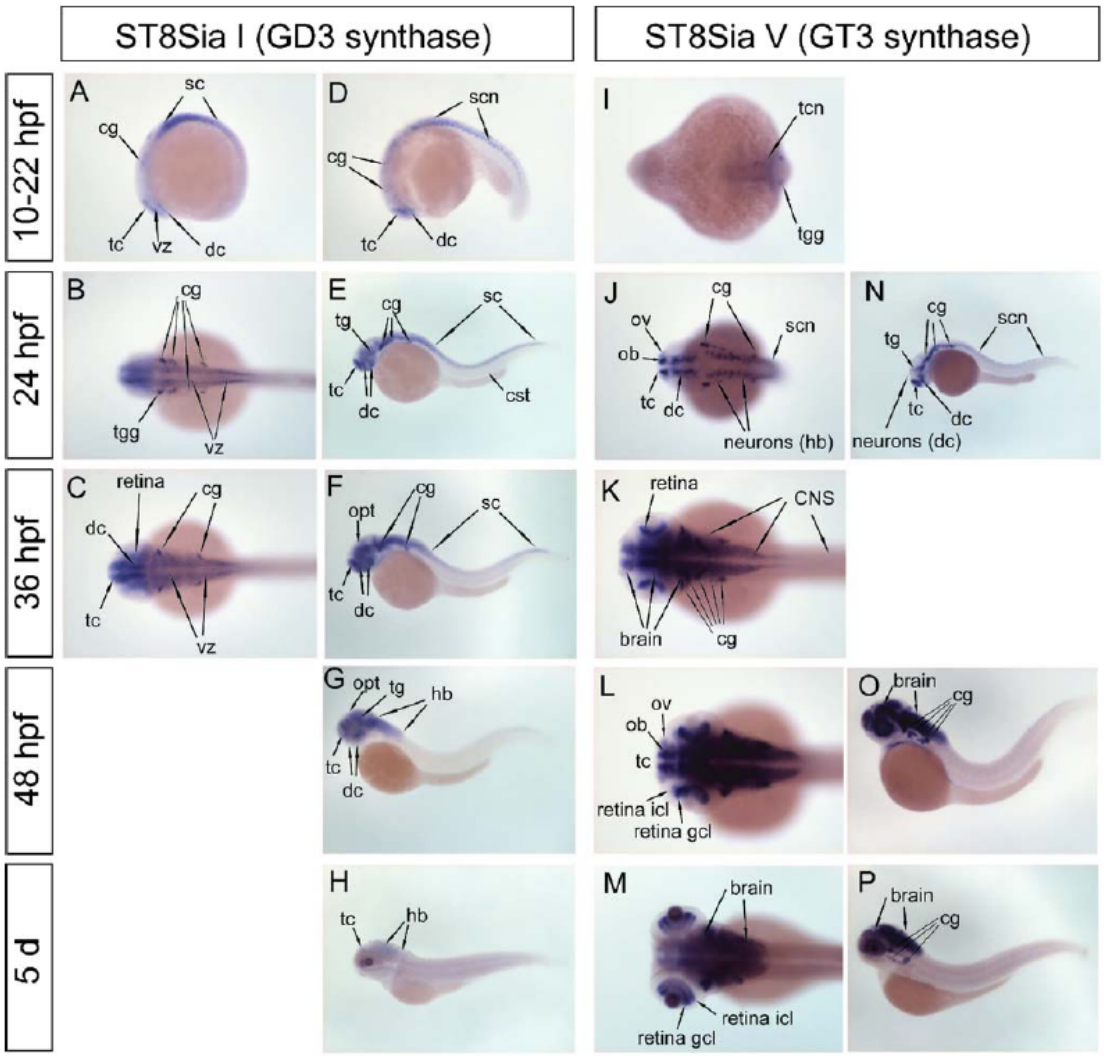

Fig. 3-4-2. Expression of the mono-α2,8-sialyltransferases ST8Sia I (GD3 synthase) and ST8Sia V (GT3 synthase) gene in the differentiating central nervous system during embryonic and larval development of Zebrafish. Panels B, C, I, J, K, L and M show dorsal and A, D, E, F, G, H, N, O, P show lateral views (anterior is to the left). At early somitogenesis (10 hpf), ST8Sia I gene is expressed in the anterior part of spinal cord, in one presumptive cranial ganglion

and in forebrain, but not in the ventricular zone (A) later on, at the middle of somitogenesis (18 hpf) it shows similar pattern of expression in forebrain, several cranial ganglia and spinal cord neurons (D). At 24 hpf, the staining denotes an ST8Sia I gene expression in the whole CNS, in midbrain and ventral part of hindbrain, stronger in telencephalon, with the exception of the ventricular zone. A strong expression is found also in cranial ganglia and a weak labelling in the corpuscles of Stannius (B, E). At 36 hpf, same observations are made and ST8Sia I gene expression is higher than at 24 hpf especially in telencephalon (olfactory bulb) and in the posterior and anterior lateral line ganglia. A weak ST8Sia I expression is detected in optic tectum and there is a small expression spot at the tip of the tail. The corpuscle of Stannius is no longer detectable (C, H). At 48 hpf, expression of the ST8Sia I gene is found in brain with the exception of the ventricular zone and strong expression in the diencephalon subventricular zone as well as in ventral diencephalon (near the neurohypophysis), no more expression was detected in cranial ganglia (G). At 5 dpf, expression of ST8Sia I gene is weak and restricted to the telencephalon and to the brain (H). Expression of the ST8Sia V gene starts in the middle of somitogenesis with strong labelling of trigeminal ganglion and telencephalon (I). At 24 hpf, expression of the ST8Sia V gene locates in the olfactory vesicle, olfactory bulb marginal zone of diencephalon, tegmentum, lateral part of hindbrain, in cranial ganglia and in neurons of the dorsal part of the spinal cord (J, N). A very strong level of expression in the CNS (non ventricular), in all cranial ganglia and in the ganglion cell layer of the retina appeared at 36 hpf (K) and at 48 hpf (L, O). In addition, there is a weak expression of the ST8Sia V gene in the inner cell layer and no expression in the proliferative zone (L, O). At 5 days, the ST8Sia V gene expression is very strong in brain (non ventricular zone) and in all cranial ganglia (M, P). cg cranial ganglia, cst corpuscle of Stannius, dc diencephalons, ep epiphysis, fb forebrain, hb hindbrain, hyth hypothalamus, mb midbrain, ntg nucleus in tegmentum, op olfactory placodes, opt optic tectum, retina gcl retina ganglion cell layer, retina icl retina inner cell layer, sc spinal cord, scn spinal cord neurons, tc telencephalon, tg tegmentum, tgg trigeminal ganglia, vz ventricular zone.

3.4.2 Discussion

α2-8-sialylated glycoconjugates are ubiquitously expressed in many tissues of vertebrates and are differentially distributed in the NS. In particular, they are enriched in brain and spinal cord and they are known to undergo both qualitative and quantitative changes in sialic acid content during NS development (Hildebrandt, H., et al. 2007, Ngamukote, S., et al. 2007). However, the regulatory mechanisms underlying these changes remain largely unknown. Among these mechanisms, which account for the variable expression of α2-8-sialylated glycoconjugates, there is the regulated expression the α2,8-sialyltransferases (ST8Sia) genes implicated in their biosynthesis. In a previous study, we identified several homologues of the known human ST8Sia in various vertebrate and invertebrate genomes that have evolutionary conserved genomic organizations and protein domain structures, and we found that the six orthologous ST8Sia genes are shared by Zebrafish and mammals.

In this study we analyze the spatio-temporal pattern of expression of the ST8Sia genes implicated in the biosynthesis of α2-8- sialylated glycoconjugates. Zebrafish is a simple model organism for assessing gene expression in the developing vertebrate NS (Key, B. and Devine, C.A. 2003) and offers a number of advantages over invertebrates such as Drosophila melanogaster or mammalian systems such as mouse. Zebrafish fertilization is external; the embryos develop rapidly in vitro and are transparent, which enabled us to visualize the early expression of the putative ST8Sia genes in the developing nervous system. Invertebrate models are also extremely powerful, but do not have α2-8-sialylated glycoconjugates, while higher vertebrate embryos have a greater complexity of NS and are not amenable to genetic analysis as Zebrafish. The classical vertebrate model for genetic analysis has been mouse and single knock-out mice for ST8Sia I and for each of the two polysialyltransferases (ST8Sia II and ST8Sia IV) have been generated and characterized (Angata, K., et al. 2004, Eckhardt, M., et al. 2000, Handa, Y., et al. 2005, Hildebrandt, H., et al. 2007). Various phenotypes were observed corresponding to subtle changes in adult physiology. Double mutant mice for both ST8Sia II and ST8Sia IV resulted in complete loss of PSA in the perinatal brain and displayed severe defects in anatomical organization of the forebrain and wiring defects, progressive hydrocephalus, postnatal growth retardation and precocious death (Angata, K., et al. 2007, Hildebrandt, H., et al. 2007, Weinhold, B., et al. 2005). ST8Sia I gene knockout mice exhibited increased sensory responses to thermal and mechanical stimuli suggesting that the b- and c-series of gangliosides might be critical in the development and/or maintenance of the sensory NS (Handa, Y., et al. 2005). Mice deficient in ST8Sia III, ST8Sia V and ST8Sia VI have not been created yet, but will provide insights into the role played by α2-8-mono- and oligo-sialylation during development, provided that these defects do not lead to embryonic lethality because these stages of development are not easily accessible in mammals.

The oligo-α2,8-sialyltransferase ST8Sia III catalyzes the transfer of one to several sialic acid residues either on glycoproteins or glycolipids (Harduin-Lepers, A., et al. 2001). Even though its function remains largely unknown, it is thought to be implicated in the biosynthesis of the gangliosides GT3 (Yu, R.K., et al. 1988) and the diSialylated motif of CD-166 (Sato, C., et al. 2002). Our ISH experiment in the developing Zebrafish embryo lacks expression of Zebrafish ST8Sia III gene until 24 hpf, which is in contrast to another recent study showing early expression of the ST8Sia III gene along the anterior–posterior axis of the developing embryo at the end of gastrulation (12 hpf) (Bentrop, J., et al. 2008) and correlates with exact mRNA quantification by real time PCR data (Section 3.3. Late induction of the ST8Sia III gene is also in agreement with the fact that oligosialylated glycolipids are exclusively detected in embryonic tissues from 24 hpf onwards (Section 3.3). At 24 hpf we detected the ST8Sia III gene in the forebrain marginal zone (telencephalon and diencephalon), in olfactory placodes, in cranial ganglia and spinal cord neurons, at 36 hpf it is found in the forebrain and midbrain marginal zone, in the epiphysis and ventral part of the hindbrain and from 48 hpf to 5 dpf, it is found in the brain marginal zone (Fig. 4-1). Accordingly, structural analysis of glycolipids purified from these stages identified

oligosialylated lactoceramides (Guerardel, Y., et al. 2006), some of which were described in adult cod brain (Avrova, N.F., et al. 1979). As mentioned previously by Bentrop et al.(Bentrop, J., et al. 2008), the ST8Sia III mRNA was also localized in the retina of the developing eye at 48 hpf and this expression is restricted to the inner cell layer of retina at the larval stage (5 dpf). Several sialylated glycoproteins such as PSA N-CAM or the sialoprotein associated with cones and rods (SPACR) expressed in the retina of fish (Marx, M., et al. 2001, Rieger, S., et al. 2008), rodents (Bartsch, U., et al. 1990, Sawaguchi, A., et al. 1999) or birds (Zako, M., et al. 2002) might be the acceptor substrates for the ST8Sia III activity. Finally, no expression of Zebrafish ST8Sia III gene could be detected in the trunk, which is in contrast to the observation made by Bentrop et al (Bentrop, J., et al. 2008) of an intense expression of this gene between 20 hpf and 32 hpf in the fast muscle fibres of the entire myotome. The discrepancies might be explained by the use of different experimental setups.

Poly-α2,8-sialyltransferases refer to the two vertebrate sialyltransferases known as ST8Sia II (STX) and ST8Sia IV (PST), which are expressed in the CNS of vertebrates, where they catalyze the biosynthesis of PSA on N-CAM enabling the transfer of hundreds of sialic acid residues mainly on the N-glycans of N-CAM (Harduin-Lepers, A., et al. 2001, Nakayama, J., et al. 1998). This is related to their role in increasing neuronal plasticity and migration in embryonic vertebrates (Hildebrandt, H., et al. 2007). The ST8Sia II gene seems to have conserved the early development stage specific expression among vertebrates, while the ST8Sia IV gene has a more extended one, from later stages to adulthood in mammals, but at low level whatever the developmental stage in Zebrafish (Marx, M., et al. 2007, Rieger, S., et al. 2008). Expression of an orthologous ST8Sia II gene in Zebrafish has been previously described in a high-throughput analysis (accessible on line, (ZFIN database at http://zfin.org) (Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q., Thisse, C 2001)) and thus ST8Sia II ISH was not repeated in these data set. It was shown that the onset of ST8Sia II gene expression occurred at about 10 hpf and remained relatively ubiquitous in the CNS until 48 hpf (Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q., Thisse, C 2001). More recently, the molecular cloning and expression of Zebrafish ST8Sia II and ST8Sia IV homologues were reported by two other groups and both Zebrafish polysialyltransferases were shown to be able to drive the expression of PSA on N-CAM (Marx, M., et al. 2007, Rieger, S., et al. 2008). Marx et al. described very similar pattern of expression of the ST8Sia II gene in the central nervous system, forebrain, midbrain, neural tube and spinal cord at 22 hpf. Rieger et al. have studied the expression of ST8Sia II gene at later developmental stages and adult brain showing that, at 48 hpf, the ST8Sia II gene expression remains high along the dorsal hindbrain ventricle, in the differentiating cerebellum and in some cranial motor neurons, but was low in the telencephalon, diencephalon, tectum and anterior spinal cord. At 96 hpf, when migration ceases in Zebrafish NS, ST8Sia II gene expression levels declined in these regions and only faint expression remained in the cerebellum and along the ventricles. We did not observe ST8Sia IV gene expression in ovary (data not shown) and embryos until 24

hpf as previously described (Marx, M., et al. 2007, Rieger, S., et al. 2008), expression of this polysialyltransferase was generally weaker in the CNS throughout development when compared to ST8Sia II. Almost no expression of the ST8Sia IV gene could be detected at early embryonic stages by Marx et al. 2007(Marx, M., et al. 2007, Rieger, S., et al. 2008), whereas we could observe strong basal level of expression of ST8Sia IV gene in the differentiating Zebrafish brain, in one nucleus in tegmentum and in the ventral part of the brain from 24 hpf to 36 hpf (Fig. 3-4-1), which is in agreement with the observation made by Rieger et al. 2008. These slight differences in ISH observations could simply result from different experimental setups. At 48 hpf, ST8Sia IV gene is expressed overall in brain and cranial ganglia (Fig. 3-4-1). Interestingly, no expression of the polysialyltransferase genes was detected in the developing nor in the adult Zebrafish retina, even though PSA N-CAM was described in adult rodent retina (Bartsch, U., et al. 1990, Sawaguchi, A., et al. 1999), in the retinotectal pathway of adult goldfish (Bastmeyer, M., et al. 1990), and in the developing Zebrafish retina (Marx, M., et al. 2001, Rieger, S., et al. 2008) suggesting that another ST8Sia gene expressed might be implicated in the biosynthesis of PSA N-CAM in the retina.

Mammalian ST8Sia I, ST8Sia V and ST8Sia VI are considered as mono-α2,8-sialyltransferases catalyzing the transfer of a single sialic acid residue on another sialic acid residue (Table 2). The human ST8Sia VI catalyzes the transfer of single sialic acid residues on sialylated O-glycans of glycoproteins leading to the formation of diSia motifs on O-glycans and does not use glycolipids as acceptor substrates (Teintenier-Lelievre, M., et al. 2005). Our in situ analysis illustrates a dispersed pattern of expression of the ST8Sia VI gene, that is not restricted to the NS (data not shown), suggesting a potential implication of α2-8-sialylated O-glycosylproteins in brain development. ST8Sia I and ST8Sia V are known in mammals to be implicated in the biosynthesis of gangliosides (Harduin-Lepers, A., et al. 2001). ST8Sia I (GD3 synthase) presents a strict specificity towards GM3 resulting in the formation of GD3, whereas ST8Sia V (GT3 synthase) is able to sialylate different gangliosides such as GD3, and also GM1b, GD1a and GT1b. ST8Sia I, ST8Sia III and ST8Sia V genes were analyzed by RT-PCR using specific primer sets in developing mouse brain, and no significant difference in the expression pattern during mouse brain development was observed (Ngamukote, S., et al. 2007). Our ISH experiment in the developing Zebrafish embryo did not show any detectable expression of the zygotic ST8Sia I and ST8Sia V before the early somitogenesis stage (10 hpf), which is in accordance with the quantification of ST8Sia and ST8Sia V mRNAs by real time PCR (Section 3.3). The ST8Sia I mRNAwas detected in the CNS, mainly in the forebrain with the exception of the ventricular zone, in spinal cord and also in the cranial ganglia. The ST8Sia I gene expression in the NS peaks at 24 hpf and was restricted to the telencephalon and hindbrain at 5 dpf (Fig. 3-4-2). Similar pattern of expressions have been reported in the early developmental stages of mouse brain using northern blot, RT-PCR, and ISH (Yamamoto, A., et al. 1996, Yamamoto, A., et al. 1996) and more recently, Luque et al. reported on a very similar temporal and spatial pattern of expression of the Xenopus laevis ST8Sia I gene (Luque, M.E., et al. 2008). In a previous study, Sohn et al. examined the

expression of Zebrafish ST3Gal V (GM3 synthase) gene using whole mount ISH and showed an ubiquitous pattern of expression with a high level in the CNS. Overexpression of the ST3Gal V transcript in the developing Zebrafish led to neuronal cell death particularly apparent in the forebrain, midbrain and mid/hindbrain boundary indicating that these brain regions are sensitive to GM3 over expression (Sohn, H., et al. 2006). Overlapping expression pattern in the same brain regions of the ST3Gal V and ST8Sia I genes is also indicative of a regulated pathway of gangliosides use in brain. The ST8Sia V gene had a similar pattern of expression in the developing CNS and PNS, with unusual high level of expression in cranial ganglia. The peak expression was reached by hatching period at 48 hpf. In addition, high expression level of the ST8Sia V gene was reached at hatching (48 hpf) in the ganglion cell and inner nuclear layers of the neural retina. This overlapping expression pattern in the retinal neuroepithelial cells of the ST8Sia I, ST8Sia Vand ST8Sia III genes implicated in the gangliosides biosynthesis is in good agreement with high ganglioside concentrations described in retina (Holm, M., et al. 1972) and suggests an important role of gangliosides in retina development (Pujic, Z., et al. 2006). In this reverse genetic approach, we aimed to determine the spatio-temporal pattern of expression of each human ST8Sia genes ortholog during Zebrafish embryonic development. Our studies with whole mount ISH during early Zebrafish embryogenesis illustrate a predominant expression of the ST8Sia genes in the CNS with the notable exception of the ST8Sia VI gene, which exhibited an ubiquitous pattern of expression (Chang, L.Y., et al. 2008). In addition, we observed overlapping pattern of expression among ST8Sia genes mainly in the developing NS and retina suggestive of common functions of the ST8Sia genes and uncovering potential functions of α2-8-sialylated glycoconjugates during CNS and retina formation.

4. Conclusion and Discussion

Taking advantage of the genetic underpinnings of Zebrafish as an excellent model system, a multifaceted study of sialylation during embryonic development was initiated and undertaken in this thesis work. Through a systematic glycomic survey mapping of the developmentally regulated glycoconjugates, we have identified a β4-galactosylated, Neu5Ac/Neu5Gc monosialylated Lewis X terminal epitope on N-glycans, similar to that found in medaka fish (Taguchi, T., et al. 1994), and a novel O-glycan structure, Fucα1-3GalNAcβ1-4(Neu5Ac/Neu5Gcα2-3)Galβ1-3GalNAc, which can be further α2,8-sialylated, possibly by the human α2,8-sialyl-transferase VI ortholog cloned and characterized in this work. The developmentally regulated oligosialylation and their distinctive expression patterns on glycoproteins and glycolipids were further demonstrated by fine structural analysis and profiling of the expression of α2,8-sialyltransferases and endogenous sialidases across different developmental stages. It appears that a large amount of oligosialic acids on glycoproteins observed are synthesized during oogenesis and subsequently catabolized in the perivitelline space after fertilization by endogenous sialidase, while oligosialylated glycolipids are synthesized *de novo* by the embryonic α2,8-sialyltransferases. The spatial and temporal expression of these α2,8-sialyltransferases further indicates the biological relevance of oligosialylation for neural development in embryogenesis. Apart from these generalized conclusions, several issues of glycobiology significance are additionally noted, as discussed below.

4.1. SSEA-1 in Zebrafish

The presence of the Lewis x-type α3-fucosylation on N-glycans as characterized in this work is in agreement with the identification of two Zebrafish α1,3-fucosyltransferases capable of synthesizing Lewis x from lacto-*N*-neotetraose *in vitro* (Kageyama, N., et al. 1999). Takemoto et al. from Hase's group first reported the major N-glycans in embryonic Zebrafish as biantennary complex-type, with and without fucose and/or bisecting *N*-acetylglucosamine residues (Takemoto, T., et al. 2005). More recently, and subsequent to our work (Guerardel, Y., et al. 2006), they further reported such Lewis x carrying *N*-glycans appeared from segmentation period (18 h) onward (Moriguchi, K., et al. 2007). While the major N-glycan structures identified are consistent with our findings, our MS-based profiling did not reveal a significant increase in the relative abundance of the nonsialylated complex-type *N*-glycans following segmentation. This discrepancy may be attributed to different experimental approaches taken in performing the structural mapping including 1) 2D-HPLC analysis of PA-tagged glycans versus MALDI-MS mapping of permethylated glycans without fractionation; 2) N-glycan release by hydrazinolysis and prior desialylation for further analysis by Hase's group; and 3) removal of chorion and yolk before releasing the N-glycans from

acetone-precipitated proteins by Hase's group, compared to analysis of total N- and O-glycans from the whole delipidated egg adopted here. It is possible that the sialylated glycoproteins from perivitelline space might have masked minute changes of other glycans.

Interestingly, Hase's group has also identified a significant proportion of biantennary *N*-glycans with the reducing end GlcNAc missing, namely with a trimannosyl GlcNAc1 core instead of the expected di-*N*-acetylchitobiose, and attributed the findings to elevated endo-β-*N*-acetyl-glucosaminidase activity (Natsuka *et al*., 2005). Although the activity of peptide:*N*-glycanase or glycoamidase has been convincingly demonstrated in the early embryos of medaka fish (Seko *et al*., 1991) and elsewhere in other animals, an endoglycosidase F or a chitobiase-type activity has not been previously identified in fish. It is nevertheless conceivable that mammalian-type stepwise action of the lysosomal aspartylglucosaminidase and chitobiase (Michalski *et al*., 1977; Strecker *et al*., 1988) could lead to intracellular generation of such free sialylated complex-type *N*-glycans in Zebrafish embryos which were not completely removed from our glycoprotein sample preparation because of the use of a low molecular weight cutoff dialysis (3500 Da), coupled with subsequent omission of C18 Sep-Pak step after tryptic digestion to increase the yield of oligosialylated *N*-glycans. Chemical degradation during permethylation was deemed unlikely as similar structures were also observed with peracetylation. Furthermore, none of the more abundant high–mannose-type *N*-glycans was found to exhibit similar degradation which seems to be restricted to the complex-type subset. The origin of these atypical "*N*-glycans" remains a moot point and may also represent a novel linkage or form of glycosylation merit further investigations.

4.2. Unique Neu5Ac/Gcα2-8Neu5Gc disialylated O-Glycans

The sialyltransferase for the biosynthesis of disialylated O-glycans is ST8Sia VI in mouse and human cell line. Whether ST8Sia VI is the only sialytransferase for the unique di-sialylated O-glycan remains to be investigated, especially when there is an extra ST8Sia gene, ST8Sia VII, identified in Zebrafish genome and highly expressed in the ovary. Intriguingly, our MS/MS data demonstrated that only O-glycans with Neu5Gc and not those of Neu5Ac are further α2-8-sialylated. Thus, besides trying to identify and characterize the sialyltransferase responsible for the synthesis of this disialyl motif on Zebrafish O-glycans (Section 3.2), we have also taken the experimental approach to block the biosynthesis of CMP-Neu5Gc by knocking down the key enzyme cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) in Zebrafish. The idea is to see if preventing the addition of the first Neu5Gc by depleting the available CMP-Neu5Gc pool would prevent further disialylation.

It was found that, through in-situ hybridization, CMAH seems to be expressed ubiquitously at

the beginning of embryogenesis and then gradually focused at the hindbrain and tail at late developmental stage. Abnormal phenotype such as the swelling of hindbrain and curved tail were observed for the embryos with CMAH gene knockdown by morphorlino (Lin CH and Huang CJ, unpublished work). However, no significant difference in total glycans profiles and oligosialylation status could be detected between wild type and CMAH-knockdown embryos, which could probably be masked by maternally inherited oligosialic acids from glycoproteins. Further work is in progress to delineate the underlying mechanism for the observed phenotypic manifestation of defects in *de novo* CMP-Neu5Gc synthesis. In parallel, collaboration has been initiated with Dr Vasta (Baltimore, US) to investigate the possible phenotypic defect of ST8Sia VI knockdown by Morpholino. In relation to this, functional studies of other Zebrafish ST8Sias have also been undertaken by other groups. Among them, the knockdown of ST8Sia II, but not ST8Sia IV, was shown to affect PSA synthesis, producing defects in axonal growth and guidance in embryo (Marx, M., et al. 2007). On other hand, ST8Sia III knockdown leads to anomalous somite morphologies, suggesting a non-neuronal function (Bentrop, J., et al. 2008); while the GD3 synthase, ST8Sia I, is being investigated by Kim's group (Daejeon, Korea).

4.3. Developmental stage-specific oligosialylation

The ganglioseries, globoseries and lactoseries of glycosphingolipids are well studied and documented (Hakomori, S. 2000, Hakomori, S.I. 2008, Ngamukote, S., et al. 2007). In mouse, the globoseries and lactoseries appear at the beginning of embryogenesis, around E0 to E4, and disappeared afterward. Ganglioseries appear after E7 in mice and are observed till birth. However, the ganglioside pattern changes along development. In E12-E14, the major compound is GD2, but shifts to GD1a and GT1b from E16 till adult. In Zebrafish, through the use of MS and HPLC, we observed a pattern of glycosphingolipids regulation very similar to that in mice. From molecular probing with real-time PCR and whole mount in situ hybridization, the expression pattern of α2,8-sialyltransferases, ST8Sia I (GD3 synthase) and ST8Sia V (GT3 synthase), could be positively correlated with the presence of oligosialylated glycolipids, namely not detected at early developmental stages but appeared afterward (Chang, L.Y., et al. 2008, Guerardel, Y., et al. 2006).

The interesting phenomenon of oligosialylation, which decreases in glycoproteins but increases in glycolipids, was further investigated by dissecting the fertilized Zebrafish eggs, and subtle difference in the oligosialylation patterns of embryo, chorion, and peri-vitelline space was found. While the longer oligosialic chain from chorion and embryo might be synthesized by ST8Sia II and ST8Sia IV during oogenesis (Asahina, S., et al. 2006), the huge amount of diSia in the peri-vitelline space might be synthesized either by ST8Sia VI or the newly identified ST8Sia VII, which was found to be highly expressed in the ovary (personal

communication from Dr. Harduin-Lepers, Lille, France). To prove this, much work still has to be done, including precise characterization of ST8Sia VII acceptor specificity, and its spatial and temporal expression pattern. Indeed, this new enzyme might represent an extra or alternative way for the biosynthesis of diSia and oligoSia in the ovary.

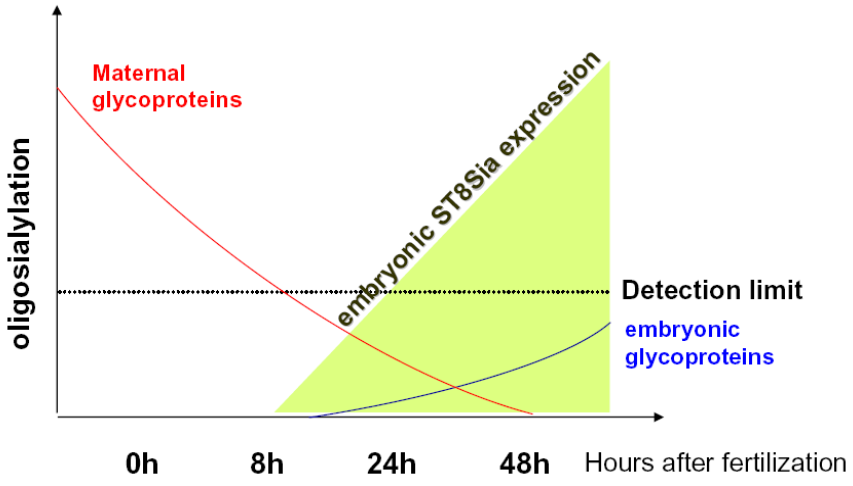

Fig. 4-1. Concerted regulation of oligosialylation on glycoproteins during Zebrafish embryogenesis.

In addition, the presence of endogenous exo-sialidase and the decreased Neu5Gc content in the peri-vitelline (data not shown) implicate the dynamics of oligosialylated proteins in the fertilized eggs. It is possible that huge amount of sialic acids (especially Neu5Gc) from the di- and oligosialylated glycopeptides may be catabolized by sialidases and the released sialic acids be taken up by the embryo and serve as the donor substrate for the *de novo* synthesis of sialylated glycoconjugates by α2,8-sialyltransferases during embryogenesis. If so, chances are the newly synthesized glycoconjugates may still bear Neu5Gc, with unchanged glycomic profiles and oligosialylation patterns in Zebrafish embryos despite CMAH gene knockdown. The hypothesis of sialic acid-uptake (either actively transported or passively diffused) from perivitelline space requires further studies, possibly with metabolic labeling (Laughlin, S.T., et al. 2008) by soaking dechorionated embryos in the solution of unnatural sialic acids or radio-active CMP-Neu5Ac and tracing these sialic acids to see if they are incorporated in the newly synthesized oligosialylated glycoproteins and glycolipids.

4.4. Biological implications of developmentally regulated oligosialylation

The successful development of an embryo requires the health of both the embryo and mother since embryogenesis is dependent on developmentally regulated molecules from both. The glycoprotein-bound oligosialic acids isolated from the perivitelline space are likely constituents

of cortical alveoli (also called cortical granules or cortical vesicles) synthesized during oogenesis, long before fertilization. These oligosialylated proteins, along with other proteins, and some catalytic enzymes located in the cortical alveoli are discharged into perivitelline space soon after fertilization. Subsequently, proteolytic peptides /glycopeptides in the peri-vitelline space are cross-linked to the protein-coat outside the plasma membrane of embryo, and transform it into the thicker and hardened fertilization envelope to block polyspermy, which is lethal for the embryo.

The cortical reaction, in which the cortical granule exocytosized, is a conserved mechanism to prevent polyspermy at fertilization and many vertebrates and invertebrates share this mechanism. In sea urchin, it includes a transformation of vitelline layer into fertilization envelope against polyspermy through a trypsin-like protease which digests the proteins connecting the vitelline envelope and embryonic plasma membrane, bindin (sperm receptor), and any sperm attached (Hirohashi, N. and Vacquier, V.D. 2002). Finally, another cortical component, hyaline, forms a coating around the embryo (Gilbert, S.F. 2006). The mucopolysaccharides (glycosaminoglycans) as well as oligo/polysialyloglycoproteins were speculated to produce an osmotic gradient that forces water to rush into the perivitelline space in order to make the fertilization envelope elevated. However, are there other unknown, overlooked, and to be proven biological functions of polysialylation on glycoproteins?

According to the finding of Kitazume et al, the presence of di- or polysialylated glycoproteins in rainbow trout during oogenesis is consistent with the expression profile of α2,8-sialyltransferases, with α2,8-monosialyltransferase earlier and α2,8-polysialyltransferase later, prior to ovulation (Kitazume, S., et al. 1994). As disialylated O-glycans were found to be located mainly in perivitelline space, while the longer oligosialic acids was found on chorion/fertilization envelope (Section 3.3), these differential sialylation pattern may thus be attributed to activities of different α2,8-polysialyltransferases during oogenesis. A possible role of oligosialic acids is to serve as a shield to protect the oligosialylated proteins from proteolytic degradation in cortical alveoli (yolk vesicles) during vitellogenesis when most of the vitellogenin (yolk proteins precursors) are cleaved into yolk proteins. Those protease-resistant oligosialylated glycoproteins would remain assembled inside the cortical vesicles, along with catalytic enzymes including proteases and possibly sialidases, which are inactive due to inappropriate pH. Right after cortical reaction, when all the components have been discharged into perivitelline space, the sialidases may be activated in the less acidic environment and hydrolyze the oligosialic acids on the glycoproteins. Some of the disialylated proteins may be cleaved into peptides later on and cross-linked to the inner part of fertilization envelope, while others remain soluble in perivitelline space. Whether oligo/polysialylation is a common and requisite characteristic for the maternal organisms to qualify the mature oocyte before

ovulation, or the negative charge of oligosialic acid provides an insulated and silent environment for early development of the embryos, is worth further pursuing among other animal models.

Apart from polyspermy blockage, it is as yet unclear whether these components also play other roles during embryogenesis, after the cortical reaction. The reported components of cortical alveoli include hyaline (McClay, D.R. and Fink, R.D. 1982) and β-1,3-glucanase (Wessel, G.M., et al. 1987) in sea urchin, and hyosophorin (Kitajima, K., et al. 1989) and metalloproteinase (alveolin) in medaka (Shibata, Y., et al. 2000) inside the oocyte. In addition to blocking polyspermy, the hyaline layer was reported to function as a substrate for cell adhesion through early development (Wessel, G.M., et al. 1998). It is known that most of the embryos begin to use their own products of zygotic genes after midblastula transition. Therefore, most process that occurred before zygotic gene expression must rely on maternal factors stored in the eggs (Pelegri, F. 2004). In our work, oligosialylation of Zebrafish glycoproteins was found to decrease dramatically after fertilization possibly due to sialidase activity present in perivitelline space from the beginning of fertilization. What remained unknown are which sialidases participate and their respective enzymatic activities. According to the sialidases identified recently by Manzoni et al (Manzoni, M., et al. 2007), there are lysosomal, cytosolic, and plasma membrane-bound sialidases in Zebrafish. Would it be the lysosomal sialidase (neu1) or cytosolic sialidase from oocyte, or the maternal or zygotic plasma membrane-bound sialidases (neu3)? Further studies to define the enzymatic activity and specificity of each sialidase and their spatial-temporal expression profiles will help to clarify their functional roles. Moreover, whether the liberated free sialic acids, as a gift from the mother, will be taken up by the embryo to synthesize its new glycoconjugates merits further investigation.

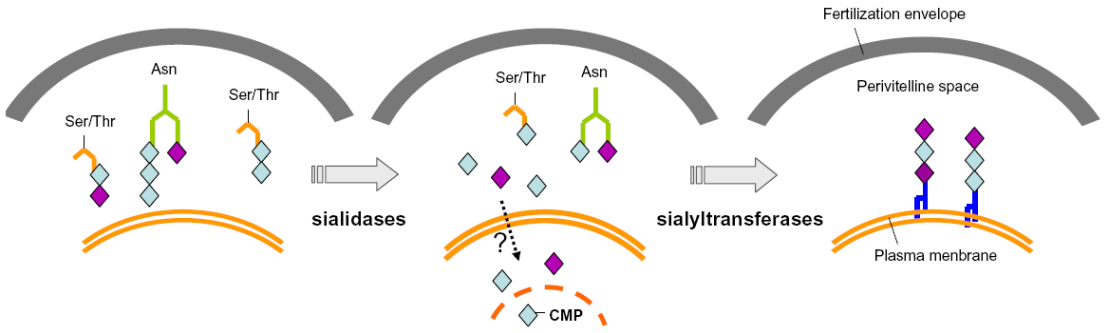

Fig. 4-2. Hypothetical model for the metabolism of oligosialylated glycoconjugates during early embryogenesis of Zebrafish. Maternal oligosialic acids on glycoteins in the perivitelline space could be cleaved by sialidases and served as new acceptors for newly synthesized oligosialylated glycolipids by α2,8-sialyltransferases.

To conclude, this thesis work has evolved from an initial glycomic survey to unraveling the molecular basis of developmentally regulated sialylation and its functional implications during fertilization and embryogenesis, using Zebrafish model. A thorough understanding of the underlying mechanisms regulating the observed changes in the sialo-glycome in conjunction with the functional genomic delineation of the implicated sialyltransferases is anticipated to enable the identification of sialylation-related genetic defects in development, as well as maternal environmental factors that may influence the physiological vitality of the embryos.

5. Reference

Ahmed, H., Du, S.J., O'Leary, N. and Vasta, G.R. (2004) Biochemical and molecular characterization of galectins from zebrafish (Danio rerio): notochord-specific expression of a prototype galectin during early embryogenesis. Glycobiology, 14, 219-232.

Ando, S. and Yu, R.K. (1979) Isolation and characterization of two isomers of brain tetrasialogangliosides. J Biol Chem, 254, 12224-12229.

Angata, K., Huckaby, V., Ranscht, B., Terskikh, A., Marth, J.D. and Fukuda, M. (2007) Polysialic acid-directed migration and differentiation of neural precursors are essential for mouse brain development. Mol Cell Biol, 27, 6659-6668.

Angata, K., Long, J.M., Bukalo, O., Lee, W., Dityatev, A., Wynshaw-Boris, A., Schachner, M., Fukuda, M. and Marth, J.D. (2004) Sialyltransferase ST8Sia-II assembles a subset of polysialic acid that directs hippocampal axonal targeting and promotes fear behavior. J Biol Chem, 279, 32603-32613.

Angata, T. and Varki, A. (2002) Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. Chem Rev, 102, 439-469.

Asahina, S., Sato, C., Matsuno, M., Matsuda, T., Colley, K. and Kitajima, K. (2006) Involvement of the alpha2,8-polysialyltransferases II/STX and IV/PST in the biosynthesis of polysialic acid chains on the O-linked glycoproteins in rainbow trout ovary. J Biochem, 140, 687-701.

Avrova, N.F., Li, Y.T. and Obukhova, E.L. (1979) On the composition and structure of individual gangliosides from the brain of elasmobranches. J Neurochem, 32, 1807-1815.

Bakkers, J., Semino, C.E., Stroband, H., Kijne, J.W., Robbins, P.W. and Spaink, H.P. (1997) An important developmental role for oligosaccharides during early embryogenesis of cyprinid fish. Proc Natl Acad Sci U S A, 94, 7982-7986.

Bartsch, U., Kirchhoff, F. and Schachner, M. (1990) Highly sialylated N-CAM is expressed in adult mouse optic nerve and retina. J Neurocytol, 19, 550-565.

Bastmeyer, M., Schlosshauer, B. and Stuermer, C.A. (1990) The spatiotemporal distribution of N-CAM in the retinotectal pathway of adult goldfish detected by the monoclonal antibody D3. Development, 108, 299-311.

Becker, C.G. and Becker, T. (2002) Repellent guidance of regenerating optic axons by chondroitin sulfate glycosaminoglycans in zebrafish. J Neurosci, 22, 842-853.

Becker, T., Becker, C.G., Schachner, M. and Bernhardt, R.R. (2001) Antibody to the HNK-1 glycoepitope affects fasciculation and axonal pathfinding in the developing posterior lateral line nerve of embryonic zebrafish. Mech Dev, 109, 37-49.

Bentrop, J., Marx, M., Schattschneider, S., Rivera-Milla, E. and Bastmeyer, M. (2008) Molecular evolution and expression of zebrafish St8SiaIII, an alpha-2,8-sialyltransferase involved in myotome development. Dev Dyn, 237, 808-818.

Bernhardt, R.R. and Schachner, M. (2000) Chondroitin sulfates affect the formation of the segmental motor nerves in zebrafish embryos. Dev Biol, 221, 206-219.

Bink, R.J., Habuchi, H., Lele, Z., Dolk, E., Joore, J., Rauch, G.J., Geisler, R., Wilson, S.W., den Hertog, J., Kimata, K. and Zivkovic, D. (2003) Heparan sulfate 6-o-sulfotransferase is essential for muscle development in zebrafish. J Biol Chem, 278, 31118-31127.

Blix, F.G., Gottschalk, A. and Klenk, E. (1957) Proposed nomenclature in the field of neuraminic and

sialic acids. Nature, 179, 1088.

Bonsignorio, D., Perego, L., Del Giacco, L. and Cotelli, F. (1996) Structure and macromolecular composition of the zebrafish egg chorion. Zygote, 4, 101-108.

Chang, L.Y., Harduin-Lepers, A., Kitajima, K., Sato, C., Huang, C.J., Khoo, K.H. and Guerardel, Y. (2008) Developmental regulation of oligosialylation in zebrafish. Glycoconj J.

Chang, L.Y., Mir, A.M., Thisse, C., Guerardel, Y., Delannoy, P., Thisse, B. and Harduin-Lepers, A. (2008) Molecular cloning and characterization of the expression pattern of the zebrafish alpha2, 8-sialyltransferases (ST8Sia) in the developing nervous system. Glycoconj J.

Chuong, C.M., McClain, D.A., Streit, P. and Edelman, G.M. (1982) Neural cell adhesion molecules in rodent brains isolated by monoclonal antibodies with cross-species reactivity. Proc Natl Acad Sci U S A, 79, 4234-4238.

Coutinho, P.M., Deleury, E., Davies, G.J. and Henrissat, B. (2003) An evolving hierarchical family classification for glycosyltransferases. J Mol Biol, 328, 307-317.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S. and et al. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature, 367, 455-459.

Daniotti, J.L., Rosales Fritz, V., Kunda, P., Nishi, T. and Maccioni, H.J. (1997) Cloning, characterization and developmental expression of alpha2,8 sialyltransferase (GD3 synthase, ST8Sia I) gene in chick brain and retina. Int J Dev Neurosci, 15, 767-776.

DeGasperi, R., Koerner, T.A., Quarles, R.H., Ilyas, A.A., Ishikawa, Y., Li, S.C. and Li, Y.T. (1987) Isolation and characterization of gangliosides with hybrid neolacto-ganglio-type sugar chains. J Biol Chem, 262, 17149-17155.

Dell, A., Reason, A.J., Khoo, K.H., Panico, M., McDowell, R.A. and Morris, H.R. (1994) Mass spectrometry of carbohydrate-containing biopolymers. Methods Enzymol, 230, 108-132.

Dietert, R.R. and Sanders, B.G. (1977) Evidence for multiple cell surface chicken fetal-leukemic antigens (CFA) in the developing chick and other avian species. J Exp Zool, 202, 171-177.

Eckhardt, M., Bukalo, O., Chazal, G., Wang, L., Goridis, C., Schachner, M., Gerardy-Schahn, R., Cremer, H. and Dityatev, A. (2000) Mice deficient in the polysialyltransferase ST8SiaIV/PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. J Neurosci, 20, 5234-5244.

Finne, J., Bitter-Suermann, D., Goridis, C. and Finne, U. (1987) An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. J Immunol, 138, 4402-4407.

Finne, J., Finne, U., Deagostini-Bazin, H. and Goridis, C. (1983) Occurrence of alpha 2-8 linked polysialosyl units in a neural cell adhesion molecule. Biochem Biophys Res Commun, 112, 482-487.

Finne, J., Krusius, T., Rauvala, H. and Hemminki, K. (1977) The disialosyl group of glycoproteins. Occurrence in different tissues and cellular membranes. Eur J Biochem, 77, 319-323.

Fredette, B., Rutishauser, U. and Landmesser, L. (1993) Regulation and activity-dependence of N-cadherin, NCAM isoforms, and polysialic acid on chick myotubes during development. J Cell Biol, 123, 1867-1888.

Freischutz, B., Saito, M., Rahmann, H. and Yu, R.K. (1994) Activities of five different sialyltransferases in fish and rat brains. J Neurochem, 62, 1965-1973.

Freischutz, B., Saito, M., Rahmann, H. and Yu, R.K. (1995) Characterization of sialyltransferase-IV activity and its involvement in the c-pathway of brain ganglioside metabolism. J Neurochem, 64, 385-393.

Fukuda, M., Dell, A. and Fukuda, M.N. (1984) Structure of fetal lactosaminoglycan. The carbohydrate moiety of Band 3 isolated from human umbilical cord erythrocytes. J Biol Chem, 259, 4782-4791.

Fukuda, M., Lauffenburger, M., Sasaki, H., Rogers, M.E. and Dell, A. (1987) Structures of novel sialylated O-linked oligosaccharides isolated from human erythrocyte glycophorins. J Biol Chem, 262, 11952-11957.

Fukuda, M.N., Dell, A., Oates, J.E. and Fukuda, M. (1985) Embryonal lactosaminoglycan. The structure of branched lactosaminoglycans with novel disialosyl (sialyl alpha 2----9 sialyl) terminals isolated from PA1 human embryonal carcinoma cells. J Biol Chem, 260, 6623-6631.

Gilbert, S.F. (2006) Developmental Biology. Sinauer.

Gooi, H.C., Feizi, T., Kapadia, A., Knowles, B.B., Solter, D. and Evans, M.J. (1981) Stage-specific embryonic antigen involves alpha 1 goes to 3 fucosylated type 2 blood group chains. Nature, 292, 156-158.

Gornati, R., Basu, S., Bernardini, G., Rizzo, A.M., Rossi, F. and Berra, B. (1997) Activities of glycolipid glycosyltransferases and sialidases during the early development of Xenopus laevis. Mol Cell Biochem, 166, 117-124.

Grunwald, D.J. and Eisen, J.S. (2002) Headwaters of the zebrafish -- emergence of a new model vertebrate. Nat Rev Genet, 3, 717-724.

Guérardel, 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.

Hakomori, S. (2000) Traveling for the glycosphingolipid path. Glycoconj J, 17, 627-647.

Hakomori, S. (2001) Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol, 491, 369-402.

Hakomori, S.I. (2008) Structure and function of glycosphingolipids and sphingolipids: recollections and future trends. Biochim Biophys Acta, 1780, 325-346.

Haltiwanger, R.S. and Lowe, J.B. (2004) Role of glycosylation in development. Annu Rev Biochem, 73, 491-537.

Handa, Y., Ozaki, N., Honda, T., Furukawa, K., Tomita, Y., Inoue, M., Furukawa, K., Okada, M. and Sugiura, Y. (2005) GD3 synthase gene knockout mice exhibit thermal hyperalgesia and mechanical allodynia but decreased response to formalin-induced prolonged noxious stimulation. Pain, 117, 271-279.

Hara, S., Takemori, Y., Yamaguchi, M., Nakamura, M. and Ohkura, Y. (1987) Fluorometric high-performance liquid chromatography of N-acetyl- and N-glycolylneuraminic acids and its application to their microdetermination in human and animal sera, glycoproteins, and glycolipids. Anal Biochem, 164, 138-145.

Haraguchi, M., Yamashiro, S., Yamamoto, A., Furukawa, K., Takamiya, K., Lloyd, K.O., Shiku, H. and Furukawa, K. (1994) Isolation of GD3 synthase gene by expression cloning of GM3 alpha-2,8-sialyltransferase cDNA using anti-GD2 monoclonal antibody. Proc Natl Acad Sci U S A, 91, 10455-10459.

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.

Harduin-Lepers, A., Petit, D., Mollicone, R., Delannoy, P., Petit, J.M. and Oriol, R. (2008) Evolutionary history of the alpha2,8-sialyltransferase (ST8Sia) gene family: tandem duplications in early deuterostomes explain most of the diversity found in the vertebrate ST8Sia genes. BMC Evol Biol, 8, 258.

Harduin-Lepers, A., Recchi, M.A. and Delannoy, P. (1995) 1994, the year of sialyltransferases. Glycobiology, 5, 741-758.

Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M.A., Samyn-Petit, B., Julien, S. and Delannoy, P. (2001) The human sialyltransferase family. Biochimie, 83, 727-737.

Higa, H.H. and Paulson, J.C. (1985) Sialylation of glycoprotein oligosaccharides with N-acetyl-, N-glycolyl-, and N-O-diacetylneuraminic acids. J Biol Chem, 260, 8838-8849.

Hildebrandt, H., Muhlenhoff, M., Weinhold, B. and Gerardy-Schahn, R. (2007) Dissecting polysialic acid and NCAM functions in brain development. J Neurochem, 103 Suppl 1, 56-64.

Hirohashi, N. and Vacquier, V.D. (2002) Egg sialoglycans increase intracellular pH and potentiate the acrosome reaction of sea urchin sperm. J Biol Chem, 277, 8041-8047.

Holm, M., Mansson, J.E., Vanier, M.T. and Svennerholm, L. (1972) Gangliosides of human, bovine and rabbit retina. Biochim Biophys Acta, 280, 356-364.

Inoue, S. and Iwasaki, M. (1978) Isolation of a novel glycoprotein from the eggs of rainbow trout: occurrence of disialosyl groups on all carbohydrate chains. Biochem Biophys Res Commun, 83, 1018-1023.

Inoue, S. and Iwasaki, M. (1980) Sialoglycoproteins from the eggs of Pacific herring. Isolation and characterization. Eur J Biochem, 111, 131-135.

Inoue, S., Iwasaki, M., Ishii, K., Kitajima, K. and Inoue, Y. (1989) Isolation and structures of glycoprotein-derived free sialooligosaccharides from the unfertilized eggs of Tribolodon hakonensis, a dace. Intracellular accumulation of a novel class of biantennary disialooligosaccharides. J Biol Chem, 264, 18520-18526.

Inoue, S., Lin, S.L. and Inoue, Y. (2000) Chemical analysis of the developmental pattern of polysialylation in chicken brain. Expression of only an extended form of polysialyl chains during embryogenesis and the presence of disialyl residues in both embryonic and adult chicken brains. J Biol Chem, 275, 29968-29979.

Inoue, S., Lin, S.L., Lee, Y.C. and Inoue, Y. (2001) An ultrasensitive chemical method for polysialic acid analysis. Glycobiology, 11, 759-767.

Inoue, S.a.I., Y (1997) Fish glycoproteins. Elsevier.

Iwasaki, M., Inoue, S., Kitajima, K., Nomoto, H. and Inoue, Y. (1984) Novel oligosaccharide chains on polysialoglycoproteins isolated from rainbow trout eggs. A unique carbohydrate sequence with a sialidase-resistant sialyl group, DGa1NAc beta 1 leads to 4(NeuGc2 leads to 3)DGa1NAc. Biochemistry, 23, 305-310.

Iwasaki, M., Seko, A., Kitajima, K., Inoue, Y. and Inoue, S. (1992) Fish egg glycophosphoproteins have species-specific N-linked glycan units previously found in a storage pool of free glycan chains. J Biol Chem, 267, 24287-24296.

Jungalwala, F.B. (1994) Expression and biological functions of sulfoglucuronyl glycolipids (SGGLs) in the nervous system--a review. Neurochem Res, 19, 945-957.

Kageyama, N., Natsuka, S. and Hase, S. (1999) Molecular cloning and characterization of two

zebrafish alpha(1,3)fucosyltransferase genes developmentally regulated in embryogenesis. J Biochem, 125, 838-845.

Kamerling, R.S.a.J.P. (1997) Chemistry, biochemistry and biology of sialic acids. Elsevier, Amsterdam.

Kanamori, A., Inoue, S., Iwasaki, M., Kitajima, K., Kawai, G., Yokoyama, S. and Inoue, Y. (1990) Deaminated neuraminic acid-rich glycoprotein of rainbow trout egg vitelline envelope. Occurrence of a novel alpha-2,8-linked oligo(deaminated neuraminic acid) structure in O-linked glycan chains. J Biol Chem, 265, 21811-21819.

Kannagi, R., Cochran, N.A., Ishigami, F., Hakomori, S., Andrews, P.W., Knowles, B.B. and Solter, D. (1983) Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. Embo J, 2, 2355-2361.

Kapadia, A., Feizi, T. and Evans, M.J. (1981) Changes in the expression and polarization of blood group I and i antigens in post-implantation embryos and teratocarcinomas of mouse associated with cell differentiation. Exp Cell Res, 131, 185-195.

Key, B. and Akeson, R.A. (1991) Delineation of olfactory pathways in the frog nervous system by unique glycoconjugates and N-CAM glycoforms. Neuron, 6, 381-396.

Key, B. and Devine, C.A. (2003) Zebrafish as an experimental model: strategies for developmental and molecular neurobiology studies. Methods Cell Sci, 25, 1-6.

Kiang, W.L., Krusius, T., Finne, J., Margolis, R.U. and Margolis, R.K. (1982) Glycoproteins and proteoglycans of the chromaffin granule matrix. J Biol Chem, 257, 1651-1659.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. and Schilling, T.F. (1995) Stages of embryonic development of the zebrafish. Dev Dyn, 203, 253-310.

Kitajima, K., Inoue, S. and Inoue, Y. (1989) Isolation and characterization of a novel type of sialoglycoproteins (hyosophorin) from the eggs of medaka, Oryzias latipes: nonapeptide with a large N-linked glycan chain as a tandem repeat unit. Dev Biol, 132, 544-553.

Kitajima, K., Inoue, Y. and Inoue, S. (1986) Polysialoglycoproteins of Salmonidae fish eggs. Complete structure of 200-kDa polysialoglycoprotein from the unfertilized eggs of rainbow trout (Salmo gairdneri). J Biol Chem, 261, 5262-5269.

Kitazume, S., Kitajima, K., Inoue, S., Troy, F.A., 2nd, Cho, J.W., Lennarz, W.J. and Inoue, Y. (1994) Identification of polysialic acid-containing glycoprotein in the jelly coat of sea urchin eggs. Occurrence of a novel type of polysialic acid structure. J Biol Chem, 269, 22712-22718.

Kojima, N., Kono, M., Yoshida, Y., Tachida, Y., Nakafuku, M. and Tsuji, S. (1996) Biosynthesis and expression of polysialic acid on the neural cell adhesion molecule is predominantly directed by ST8Sia II/STX during in vitro neuronal differentiation. J Biol Chem, 271, 22058-22062.

Kojima, N., Tachida, Y. and Tsuji, S. (1997) Two polysialic acid synthases, mouse ST8Sia II and IV, synthesize different degrees of polysialic acids on different substrate glycoproteins in mouse neuroblastoma Neuro2a cells. J Biochem, 122, 1265-1273.

Laughlin, S.T., Baskin, J.M., Amacher, S.L. and Bertozzi, C.R. (2008) In vivo imaging of membrane-associated glycans in developing zebrafish. Science, 320, 664-667.

Lepers, A., Shaw, L., Cacan, R., Schauer, R., Montreuil, J. and Verbert, A. (1989) Transport of CMP-N-glycoloylneuraminic acid into mouse liver Golgi vesicles. FEBS Lett, 250, 245-250.

Lepers, A., Shaw, L., Schneckenburger, P., Cacan, R., Verbert, A. and Schauer, R. (1990) A study on the regulation of N-glycoloylneuraminic acid biosynthesis and utilization in rat and mouse liver. Eur J Biochem, 193, 715-723.

Luque, M.E., Crespo, P.M., Monaco, M.E., Aybar, M.J., Daniotti, J.L. and Sanchez, S.S. (2008) Cloning and functional characterization of two key enzymes of glycosphingolipid biosynthesis in the amphibian Xenopus laevis. Dev Dyn, 237, 112-123.

Manzoni, M., Colombi, P., Papini, N., Rubaga, L., Tiso, N., Preti, A., Venerando, B., Tettamanti, G., Bresciani, R., Argenton, F., Borsani, G. and Monti, E. (2007) Molecular cloning and biochemical characterization of sialidases from zebrafish (Danio rerio). Biochem J, 408, 395-406.

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

Marx, M., Rutishauser, U. and Bastmeyer, M. (2001) Dual function of polysialic acid during zebrafish central nervous system development. Development, 128, 4949-4958.

McClay, D.R. and Fink, R.D. (1982) Sea urchin hyalin: appearance and function in development. Dev Biol, 92, 285-293.

Metcalfe, W.K., Myers, P.Z., Trevarrow, B., Bass, M.B. and Kimmel, C.B. (1990) Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish. Development, 110, 491-504.

Monti, E., Preti, A., Venerando, B. and Borsani, G. (2002) Recent development in mammalian sialidase molecular biology. Neurochem Res, 27, 649-663.

Morgan, W.T. and Watkins, W.M. (1953) The inhibition of the haemagglutinins in plant seeds by human blood group substances and simple sugars. Br J Exp Pathol, 34, 94-103.

Moriguchi, K., Takemoto, T., Aoki, T., Nakakita, S., Natsuka, S. and Hase, S. (2007) Free oligosaccharides with Lewis x structure expressed in the segmentation period of zebrafish embryo. J Biochem, 142, 213-227.

Muramatsu, H., Ishihara, H., Miyauchi, T., Gachelin, G., Fujisaki, T., Tejima, S. and Muramatsu, T. (1983) Glycoprotein-bound large carbohydrates of early embryonic cells: structural characteristic of the glycan isolated from F9 embryonal carcinoma cells. J Biochem, 94, 799-810.

Muramatsu, T., Gachelin, G., Damonneville, M., Delarbre, C. and Jacob, F. (1979) Cell surface carbohydrates of embryonal carcinoma cells: polysaccharidic side chains of F9 antigens and of receptors to two lectins, FBP and PNA. Cell, 18, 183-191.

Muramatsu, T., Gachelin, G., Nicolas, J.F., Condamine, H., Jakob, H. and Jacob, F. (1978) Carbohydrate structure and cell differentitation: unique properties of fucosyl-glycopeptides isolated from embryonal carcinoma cells. Proc Natl Acad Sci U S A, 75, 2315-2319.

Nakamura, K., Tamai, Y. and Kasama, T. (1997) Gangliosides of dogfish (Squalus acanthias) brain. Neurochem Int, 30, 593-604.

Nakayama, J., Angata, K., Ong, E., Katsuyama, T. and Fukuda, M. (1998) Polysialic acid, a unique glycan that is developmentally regulated by two polysialyltransferases, PST and STX, in the central nervous system: from biosynthesis to function. Pathol Int, 48, 665-677.

Nakayama, J., Fukuda, M.N., Fredette, B., Ranscht, B. and Fukuda, M. (1995) Expression cloning of a human polysialyltransferase that forms the polysialylated neural cell adhesion molecule present in embryonic brain. Proc Natl Acad Sci U S A, 92, 7031-7035.

Nara, K., Watanabe, Y., Maruyama, K., Kasahara, K., Nagai, Y. and Sanai, Y. (1994) Expression

cloning of a CMP-NeuAc:NeuAc alpha 2-3Gal beta 1-4Glc beta 1-1'Cer alpha 2,8-sialyltransferase (GD3 synthase) from human melanoma cells. Proc Natl Acad Sci U S A, 91, 7952-7956.

Nasevicius, A. and Ekker, S.C. (2000) Effective targeted gene 'knockdown' in zebrafish. Nat Genet, 26, 216-220.

Ngamukote, S., Yanagisawa, M., Ariga, T., Ando, S. and Yu, R.K. (2007) Developmental changes of glycosphingolipids and expression of glycogenes in mouse brains. J Neurochem, 103, 2327-2341.

Nikonova, E.Y., Tertov, V.V., Sato, C., Kitajima, K. and Bovin, N.V. (2004) Specificity of human trans-sialidase as probed with gangliosides. Bioorg Med Chem Lett, 14, 5161-5164.

Ong, E., Nakayama, J., Angata, K., Reyes, L., Katsuyama, T., Arai, Y. and Fukuda, M. (1998) Developmental regulation of polysialic acid synthesis in mouse directed by two polysialyltransferases, PST and STX. Glycobiology, 8, 415-424.

Oppenheimer, S.B. (1977) Interactions of lectins with embryonic cell surfaces. Curr Top Dev Biol, 11, 1-16.

Patel, R.Y. and Balaji, P.V. (2006) Identification of linkage-specific sequence motifs in sialyltransferases. Glycobiology, 16, 108-116.

Pelegri, F. (2004) The role of maternal factors in early Zebrafish development.

Pennington, J.E., Rastan, S., Roelcke, D. and Feizi, T. (1985) Saccharide structures of the mouse embryo during the first eight days of development. Inferences from immunocytochemical studies using monoclonal antibodies in conjunction with glycosidases. J Embryol Exp Morphol, 90, 335-361.

Pradel, G., Schachner, M. and Schmidt, R. (1999) Inhibition of memory consolidation by antibodies against cell adhesion molecules after active avoidance conditioning in zebrafish. J Neurobiol, 39, 197-206.

Pujic, Z., Omori, Y., Tsujikawa, M., Thisse, B., Thisse, C. and Malicki, J. (2006) Reverse genetic analysis of neurogenesis in the zebrafish retina. Dev Biol, 293, 330-347.

Rauvala, H. (1984) Neurite outgrowth of neuroblastoma cells: dependence on adhesion surface--cell surface interactions. J Cell Biol, 98, 1010-1016.

Rieger, S., Volkmann, K. and Koster, R.W. (2008) Polysialyltransferase expression is linked to neuronal migration in the developing and adult zebrafish. Dev Dyn, 237, 276-285.

Rudd, P.M., Elliott, T., Cresswell, P., Wilson, I.A. and Dwek, R.A. (2001) Glycosylation and the immune system. Science, 291, 2370-2376.

Rutishauser, U. (2008) Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. Nat Rev Neurosci, 9, 26-35.

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

Sato, C., Inoue, S., Matsuda, T. and Kitajima, K. (1998) Development of a highly sensitive chemical method for detecting alpha2-->8-linked oligo/polysialic acid residues in glycoproteins blotted on the membrane. Anal Biochem, 261, 191-197.

Sato, C., Inoue, S., Matsuda, T. and Kitajima, K. (1999) Fluorescent-assisted detection of oligosialyl units in glycoconjugates. Anal Biochem, 266, 102-109.

Sato, C., Kitajima, K., Tazawa, I., Inoue, Y., Inoue, S. and Troy, F.A., 2nd (1993) Structural diversity in the alpha 2-->8-linked polysialic acid chains in salmonid fish egg glycoproteins. Occurrence of poly(Neu5Ac), poly(Neu5Gc), poly(Neu5Ac, Neu5Gc), poly(KDN), and their partially acetylated forms. J Biol Chem, 268, 23675-23684.

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

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

Sawaguchi, A., Idate, Y., Ide, S., Kawano, J., Nagaike, R., Oinuma, T. and Suganuma, T. (1999) Multistratified expression of polysialic acid and its relationship to VAChT-containing neurons in the inner plexiform layer of adult rat retina. J Histochem Cytochem, 47, 919-928.

Schnaar, R.L. (1994) Isolation of glycosphingolipids. Methods Enzymol, 230, 348-370.

Schwarz, A. and Futerman, A.H. (1996) The localization of gangliosides in neurons of the central nervous system: the use of anti-ganglioside antibodies. Biochim Biophys Acta, 1286, 247-267.

Schwarzkopf, M., Knobeloch, K.P., Rohde, E., Hinderlich, S., Wiechens, N., Lucka, L., Horak, I., Reutter, W. and Horstkorte, R. (2002) Sialylation is essential for early development in mice. Proc Natl Acad Sci U S A, 99, 5267-5270.

Seko, A., Kitajima, K., Iwasaki, M., Inoue, S. and Inoue, Y. (1989) Structural studies of fertilization-associated carbohydrate-rich glycoproteins (hyosophorin) isolated from the fertilized and unfertilized eggs of flounder, Paralichthys olivaceus. Presence of a novel penta-antennary N-linked glycan chain in the tandem repeating glycopeptide unit of hyosophorin. J Biol Chem, 264, 15922-15929.

Semino, C.E. and Allende, M.L. (2000) Chitin oligosaccharides as candidate patterning agents in zebrafish embryogenesis. Int J Dev Biol, 44, 183-193.

Semino, C.E., Allende, M.L., Bakkers, J., Spaink, H.P. and Robbins, P.P. (1998) Expression of Rhizobium chitin oligosaccharide fucosyltransferase in zebrafish embryos disrupts normal development. Ann N Y Acad Sci, 842, 49-54.

Sevigny, M.B., Ye, J., Kitazume-Kawaguchi, S. and Troy, F.A., 2nd (1998) Developmental expression and characterization of the alpha2,8-polysialyltransferase activity in embryonic chick brain. Glycobiology, 8, 857-867.

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.

Shibata, Y., Iwamatsu, T., Oba, Y., Kobayashi, D., Tanaka, M., Nagahama, Y., Suzuki, N. and Yoshikuni, M. (2000) Identification and cDNA cloning of alveolin, an extracellular metalloproteinase, which induces chorion hardening of medaka (Oryzias latipes) eggs upon fertilization. J Biol Chem, 275, 8349-8354.

Shimamura, M., Endo, T., Inoue, Y., Inoue, S. and Kambara, H. (1984) Fish egg polysialoglycoproteins: structures of new sialooligosaccharide chains isolated from the eggs of Oncorhynchus keta

(Walbaum). Fucose-containing units with oligosialyl groups. Biochemistry, 23, 317-322.

Snaar-Jagalska, B.E., Krens, S.F., Robina, I., Wang, L.X. and Spaink, H.P. (2003) Specific activation of ERK pathways by chitin oligosaccharides in embryonic zebrafish cell lines. Glycobiology, 13, 725-732.

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.

Solter, D. and Knowles, B.B. (1978) Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci U S A, 75, 5565-5569.

Suganuma, T., Muramatsu, H., Murata, F. and Muramatsu, T. (1987) Purification and properties of N-acetylglucosaminide beta 1----4 galactosyltransferase from embryonal carcinoma cells. J Biochem, 102, 665-671.

Sunshine, J., Balak, K., Rutishauser, U. and Jacobson, M. (1987) Changes in neural cell adhesion molecule (NCAM) structure during vertebrate neural development. Proc Natl Acad Sci U S A, 84, 5986-5990.

Suzuki, N., Khoo, K.H., Chen, C.M., Chen, H.C. and Lee, Y.C. (2003) N-glycan structures of pigeon IgG: a major serum glycoprotein containing Galalpha1-4 Gal termini. J Biol Chem, 278, 46293-46306.

Szulman, A.E. (1964) The Histological Histological Distribution of the Blood Group Substances in Man as Disclosed by Immunofluorescence. Iii. the a, B, and H Antigens in Embryos and Fetuses from 18 Mm in Length. J Exp Med, 119, 503-516.

Szulman, A.E. (1971) The histological distribution of the blood group substances in man as disclosed by immunofluorescence. IV. The ABH antigens in embryos at the fifth week post fertilization. Hum Pathol, 2, 575-585.

Szulman, A.E. and Marcus, D.M. (1973) The histologic distribution of the blood group substances in man as disclosed by immunofluorescence. VI. The Le and Le antigens during fetal development. Lab Invest, 28, 565-574.

Taguchi, T., Iwasaki, M., Muto, Y., Kitajima, K., Inoue, S., Khoo, K.H., Morris, H.R., Dell, A. and Inoue, Y. (1996) Occurrence and structural analysis of highly sulfated multiantennary N-linked glycan chains derived from a fertilization-associated carbohydrate-rich glycoprotein in unfertilized eggs of Tribolodon hakonensis. Eur J Biochem, 238, 357-367.

Taguchi, T., Kitajima, K., Muto, Y., Inoue, S., Khoo, K.H., Morris, H.R., Dell, A., Wallace, R.A., Selman, K. and Inoue, Y. (1995) A precise structural analysis of a fertilization-associated carbohydrate-rich glycopeptide isolated from the fertilized eggs of euryhaline killi fish (Fundulus heteroclitus). Novel penta-antennary N-glycan chains with a bisecting N-acetylglucosaminyl residue. Glycobiology, 5, 611-624.

Taguchi, T., Seko, A., Kitajima, K., Inoue, S., Iwamatsu, T., Khoo, K.H., Morris, H.R., Dell, A. and Inoue, Y. (1993) Structural studies of a novel type of tetraantennary sialoglycan unit in a carbohydrate-rich glycopeptide isolated from the fertilized eggs of Indian Medaka fish, Oryzias melastigma. J Biol Chem, 268, 2353-2362.

Taguchi, T., Seko, A., Kitajima, K., Muto, Y., Inoue, S., Khoo, K.H., Morris, H.R., Dell, A. and Inoue, Y. (1994) Structural studies of a novel type of pentaantennary large glycan unit in the fertilization-associated carbohydrate-rich glycopeptide isolated from the fertilized eggs of

Oryzias latipes. J Biol Chem, 269, 8762-8771.

Takashima, S., Ishida, H.K., Inazu, T., Ando, T., Ishida, H., Kiso, M., Tsuji, S. and Tsujimoto, M. (2002) Molecular cloning and expression of a sixth type of alpha 2,8-sialyltransferase (ST8Sia VI) that sialylates O-glycans. J Biol Chem, 277, 24030-24038.

Takemoto, T., Natsuka, S., Nakakita, S. and Hase, S. (2005) Expression of complex-type N-glycans in developmental periods of zebrafish embryo. Glycoconj J, 22, 21-26.

Tang, J., Rutishauser, U. and Landmesser, L. (1994) Polysialic acid regulates growth cone behavior during sorting of motor axons in the plexus region. Neuron, 13, 405-414.

Teintenier-Lelievre, M., Julien, S., Juliant, S., Guerardel, Y., Duonor-Cerutti, M., Delannoy, P. and Harduin-Lepers, A. (2005) Molecular cloning and expression of a human hST8Sia VI (alpha2,8-sialyltransferase) responsible for the synthesis of the diSia motif on O-glycosylproteins. Biochem J, 392, 665-674.

Theodosis, D.T., Rougon, G. and Poulain, D.A. (1991) Retention of embryonic features by an adult neuronal system capable of plasticity: polysialylated neural cell adhesion molecule in the hypothalamo-neurohypophysial system. Proc Natl Acad Sci U S A, 88, 5494-5498.

Thiery, J.P., Brackenbury, R., Rutishauser, U. and Edelman, G.M. (1977) Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. J Biol Chem, 252, 6841-6845.

Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., Kirchner, J., Parkhill, J.P. and Thisse, C. (2004) Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. Methods Cell Biol, 77, 505-519.

Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q., Thisse, C (2001) Expression of the zebrafish genome during embryogenesis.

Thisse, C. and Thisse, B. (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc, 3, 59-69.

Thisse, C., Thisse, B (1998) High resolution whole-mount in situ hybridization.

Traving, C. and Schauer, R. (1998) Structure, function and metabolism of sialic acids. Cell Mol Life Sci, 54, 1330-1349.

Troy, F.A., 2nd (1992) Polysialylation: from bacteria to brains. Glycobiology, 2, 5-23.

Tsuji, S., Datta, A.K. and Paulson, J.C. (1996) Systematic nomenclature for sialyltransferases. Glycobiology, 6, v-vii.

Varki, A. (2007) Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature, 446, 1023-1029.

Varki, N.M. and Varki, A. (2007) Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest, 87, 851-857.

Weinhold, B., Seidenfaden, R., Rockle, I., Muhlenhoff, M., Schertzinger, F., Conzelmann, S., Marth, J.D., Gerardy-Schahn, R. and Hildebrandt, H. (2005) Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. J Biol Chem, 280, 42971-42977.

Wessel, G.M., Berg, L., Adelson, D.L., Cannon, G. and McClay, D.R. (1998) A molecular analysis of hyalin--a substrate for cell adhesion in the hyaline layer of the sea urchin embryo. Dev Biol, 193, 115-126.

Wessel, G.M., Truschel, M.R., Chambers, S.A. and McClay, D.R. (1987) A cortical granule-specific enzyme, B-1,3-glucanase, in sea urchin eggs. Gamete Res, 18, 339-348.

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.

Yamamoto, A., Haraguchi, M., Yamashiro, S., Fukumoto, S., Furukawa, K., Takamiya, K., Atsuta, M., Shiku, H. and Furukawa, K. (1996) Heterogeneity in the expression pattern of two ganglioside synthase genes during mouse brain development. J Neurochem, 66, 26-34.

Yamamoto, A., Yamashiro, S., Fukumoto, S., Haraguchi, M., Atsuta, M., Shiku, H. and Furukawa, K. (1996) Site restricted and neuron dominant expression of alpha 2,8sialyltransferase gene in the adult mouse brain and retina. Glycoconj J, 13, 471-480.

Yu, R.K., Macala, L.J., Taki, T., Weinfield, H.M. and Yu, F.S. (1988) Developmental changes in ganglioside composition and synthesis in embryonic rat brain. J Neurochem, 50, 1825-1829.

Yu, S.Y., Wu, S.W. and Khoo, K.H. (2006) Distinctive characteristics of MALDI-Q/TOF and TOF/TOF tandem mass spectrometry for sequencing of permethylated complex type N-glycans. Glycoconj J, 23, 355-369.

Zako, M., Iwaki, M., Yoneda, M., Miyaishi, O., Zhao, J., Suzuki, Y., Takeuchi, M., Miyake, G., Ikagawa, H. and Kimata, K. (2002) Molecular cloning and characterization of chick sialoprotein associated with cones and rods, a developmentally regulated glycoprotein of interphotoreceptor matrix. J Biol Chem, 277, 25592-25600.

Zanetta, J.P., Timmerman, P. and Leroy, Y. (1999) Determination of constituents of sulphated proteoglycans using a methanolysis procedure and gas chromatography/mass spectrometry of heptafluorobutyrate derivatives. Glycoconj J, 16, 617-627.

Zhou, X.Y., van der Spoel, A., Rottier, R., Hale, G., Willemsen, R., Berry, G.T., Strisciuglio, P., Morrone, A., Zammarchi, E., Andria, G. and d'Azzo, A. (1996) Molecular and biochemical analysis of protective protein/cathepsin A mutations: correlation with clinical severity in galactosialidosis. Hum Mol Genet, 5, 1977-1987.

6. Abbreviations

(KDN)n, oligo/polyKDN;

(Neu5Ac)n, oligo/polyNeu5Ac;

(Neu5Gc)n, oligo/polyNeu5Gc;

α2,5-Oglycolyl-linked polyNeu5Gc or a2,5-Oglycolyl-linked (Neu5Gc)n;

α2,8-linked oligo/polyKDN, a2,8-linked oligo- and poly-KDN;

CID, collision induced dissociation;

CNS, central nervous system;

COSY, correlation spectroscopy;

DHB, 2,5-dihydroxybenzoic acid;

DHB, 2,5-dihydroxybenzoic acid;

diSia, diSialic acid;

DMB-(Neu5Ac)n, DMB-tagged (Neu5Ac)n;

DMB, 1,2-diamino-4,5-methylenedioxybenzene;

DMB/HPLC-FD, HPLC with fluorescence detection of ulosonates derivatized with DMB

DP, degree of polymerization;

dpf, days post fertilization;

Dre, Danio rerio;

Endo-N, endo-N-acylneuraminidase;

GC, gas chromatography;

Hex, hexose;

HexNAc, N-acetyl hexosamine;

HexNAcitol, reduced N-acetyl hexosaminitol;

HMQC, heteronuclear multiple quantum coherence;

hpf, hours post fertilization;

HPLC, high performance liquid chromatography;

Hsa, Homo sapiens;

ISH, in situ hybridization;

LacCer, lactosylceramide;

MALDI, matrix-assisted laser-desorption ionization;

MS, mass spectrometry;

NCAM, neural cell adhesion molecule;

Neu5Ac, N-acetylneuraminic acid or 2-keto-3,5-dideoxy-5-acetylamino-D-glycero-D-galacto-nononic acid;

Neu5Gc, N-glycolylneuraminic acid or 2-keto-3,5-dideoxy-5-glycolylamino-Dglycero-D-galacto-nononic acid;

KDN, 2-keto-3-deoxy-Dglycero-D-galacto-nononic acid;

NMR, nuclear magnetic resonance;

NOE, nuclear Overhauser effect;

NS, nervous system;

oligo/polyNeu5Gc, oligo- and polyNeu5Gc;

ORF, open reading frame;

PNS, peripheral nervous system;

polyNeu5Ac, a2,8-linked poly-N-acylneuraminic acid;

PSA, polysialic acid;

PSGP, poly-sialoglycoprotein;

ROESY, rotating frame Overhauser enhancement spectroscopy;

RP, reverse phase;

Sia, sialic acid;

ST8Sia, α2,8-sialyltransferase;

TFA, trifluoroacetic acid;

TLC, thin layer chromatography;

TOF, time-of-flight.

triSia, trisalylmotif;