Forced expression of α2,3-sialyltransferase IV rescues impaired heart development in α2,6-sialyltransferase I-deficient medaka

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Abstract:

Sialic acids (Sias) are often linked to galactose (Gal) residues by α2,6- and α2,3-linkages in glycans of glycoproteins. Sias are indispensable for vertebrate development, because organisms deficient in some enzymes in the Sia synthetic pathway are lethal during the development. However, it remains unknown if the difference of Siaα2,6Gal or α2,3Gal linkage has a critical meaning. To find a clue to understand significance of the linkage difference at the organism level, medaka was used as a vertebrate model. In embryos, Siaα2,6Gal epitopes recognized by *Sambucus nigra* lectin (SNA) and Siaα2,3Gal epitopes recognized by *Maackia amurensis* lectin (MAA) were enriched in the blastodisc and the yolk sphere, respectively. When these lectins were injected in the perivitelline space, SNA, but not MAA, impaired embryo body formation at 1 day post-fertilization (dpf). Most Siaα2,6Gal epitopes occurred on *N*-glycans owing to their sensitivity to peptide:*N*-glycanase. Of knockout-medaka (KO) for either of two β-galactoside:α2,6-sialyltransferase genes, *ST6Gal I* and *ST6Gal II*, only *ST6Gal I*-KO showed severe cardiac abnormalities at 7-16 dpf, leading to lethality at 14-18 dpf. Interestingly, however, these cardiac abnormalities of *ST6Gal I*-KO were rescued not only by forced expression of *ST6Gal I*, but also by that of *ST6Gal II* and the β-galactoside:α2,3-sialyltransferase IV gene (*ST3Gal IV*). Taken together, the Siaα2,6Gal linkage synthesized by ST6Gal I are critical in heart development; however, it can be replaced by the linkages synthesized by ST6Gal II and ST3Gal IV. These data suggest that sialylation itself is more important than its particular linkage for the heart development.

Key words: α2,6-sialyltransferase, α2,3-sialyltransferase, heat development, medaka,

Footnote:

[1]Abbreviations: CMP-Sia, cytidine 5'-monophospho-sialic acid; CMAS, CMP-Sia synthetase; dpf, day post-fertilization; Gal, galactose; GalNAc, N-acetylgalactosamine; MAA, *Maackia amurensis* lectin; PVS, perivitelline space; SNA, *Sambucus nigra* lectin; Sia, sialic acid; WT, wild-type.

Introduction

Sialic acids (Sias) are negatively charged nine-carbon sugars that occur at the non-reducing ends of glycans in proteins and lipids on the cell surface [1-3], and are involved in ligand-receptor and cell-cell interactions during fertilization, embryogenesis, development, immune responses, and brain functions [1,4-7]. In particular, Sias have been demonstrated to play an essential role in survival of embryos in mice and medaka fish, because a loss of critical enzymes in the Sia synthetic pathway leads to lethality during development: Mice deficient in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase gene or the CMP-Sia synthetase gene (*CMAS*) gene are lethal during early development [8,9]; Medaka fish with a point-mutated *CMAS* gene died in young fry [10].

One of unique features of Sias is a linkage diversity [1,5,7]. Sias are often linked to galactose (Gal) and *N*-acetylgalactosamine (GalNAc) residues by α2,3- and α2,6-linkages in glycoproteins, whereas in rare cases, they are linked to Sia residues by α2,8- and α2,9-linkages in oligo/polysialic acid chains [5,6]. The linkage diversity has significance in case of specific lectin-ligand interactions in infection and immune systems. For example, the human hemagglutinin of influenza virus A binds with Siaα2,6Gal, but not Siaα2,3Gal, termini of glycans of host cells in the viral infection [11,12]. The human sialic acid-binding immunoglobulin-type lectins (Siglecs) bind with Sia residues in a linkage-specific manner in self/non-self recognition in immune systems [13-15]. However, it remains unknown if the linkage diversity always has some critical meanings. Indeed, sialylation is known to be significant in determining lifetime of blood glycoproteins by recruiting them, immediately after desialylation, to hepatocytes from blood stream by the asialoglycoprotein receptor, a Gal-specific lectin. This happens irrespective of differences of Siaα2,6Gal or Siaα2,3Gal linkage [16,17]. In this case, the linkage difference of Sia does not look significant; however, biological functions of Siaα2,6Gal or Siaα2,3Gal residues in the blood glycoproteins prior to its removal still remain to be elucidated. Furthermore, many studies have been focusing on the effects of a decrease or increase of Siaα2,6Gal linkage in sialoglycoproteins on their activities: Deletion of particular *N*-glycosylation sites in β1-integrin results in the impairment of cell adhesion activity [18], while hypersialylation of β1-integrin up-regulates cell motility [19]; Up-regulation of

ST6Sia I in many cancers facilitates tumor progression [20,21] through prevention of apoptosis by α2,6-sialylation of Fas receptor [21]. However, to best of our knowledge, no study has examined the effects of linkage conversion from Siaα2,6Gal to Siaα2,3Gal at the same sites on phenotypes at the organism level. Thus, our objective is to gain new insights into the significance of the linkage diversity of Sia using medaka, *Oryzias latipes*, as a model organism.

We first investigated the effects of Siaα2,6Gal- or Siaα2,3Gal-specific lectins on the developing embryos. We also generated knockout medaka (KO) for the two β-galactoside:α2,6-sialyltarnsferase genes, *ST6Gal I* and *ST6Gal II*, using CRISPR/Cas 9 system for analyzing the phenotypes. We then performed rescue experiments by forced expression of *ST6Gal I*, *ST6Gal II*, the β-galactoside:α2,3-sialyltarnsferase genes, *ST3Gal IV*, or *ST3Gal V* in the *ST6Gal I*-KO.

Materials and methods

Materials

The Cas9 expression vector with SP6 promoter, pCS2+hSpCas9, and the sgRNA expression vector with a T7 promoter, pDR274, were gifts from Masato Kinoshita (Addgene 51815) [22], and Keith Joung (Addgene 42250) [23], respectively. Human transferrin was purchased from FUJIFILM Wako (Kanagawa, Japan). Molecular weight markers were purchased from Integrale (Tokushima, Japan).

Ethics statement and the ARRIVE guidelines

All procedures for the use of animals were approved by the Animal Care and Use Committee of Nagoya University (Permit Number: BBC2019001 for medaka; BBC2019002 for mouse), and performed under the relevant guidelines and regulations, which are set up based on the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines by the same committee.

Medaka fish

Medaka, *Oryzias latipes*, was used and keeping as previously described [10]. A transgenic medaka strain (TG941) was purchased from NBRP Medaka (Okazaki, Japan) [24].

Preparation of anti-SNA and anti-MAA antibodies

The BALB/c mice was first immunized intraperitoneally with 40 μg of SNA or MAA lectin in Freund's complete adjuvant. Two booster injections were given with 40 μg of SNA or MAA in Freund's incomplete adjuvant at 1-week interval, and the antiserum was collected 3 days after injection with 40 μg of pure SNA or MAA in Freund's incomplete adjuvant.

Plasmid construction

Medaka *ST6Gal I* (XM_011473262.3), *ST6Gal II* (NM_001104772.1), *ST3Gal IV* (XM_023961429.1), and *ST3Gal V* (XM_011480452.2) cDNA were cloned into a pCS2+ plasmid as previously described [25], except that NEBuilder HiFi DNA Assembly Master Mix was used instead of In-fusion HD Cloning Kit. The cloned DNAs for *ST6Gal I* and *II* include silent mutations of c.225A>G and c.1288T>C, respectively. The *DsRed-monomer* cDNA fragments were cloned from the DsRed-monomer-encoded pcDNA3.1 (Takara Bio, Japan) into pCS2+ plasmid as previously described [25]. Medaka *β-actin* (NM_001104808.1) cDNA was cloned into a pGEM-T easy vector (Promega, WI, USA) as previously described [25]. The sequences of all the plasmids were confirmed using the dideoxynucleotide chain termination-based sequencing method.

Whole mount lectin staining

Embryos at 16~32-cell stage were fixed 4% paraformaldegyde, blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS), and immunostained with 1 μg/ml of FITC-SSA or FITC-MAA lectin, followed by observation on fluorescent microscopy (BX-51, OLYMPUS, Japan).

Lectin injection into the perivitelline space

Approximately 2-4 nL of a mixture containing final 250 or 500 μg/mL SNA, or MAM lectin (EY Laboratory, INC, CA, USA) and 5 mg/mL FITC-dextran (10 kDa) (CosmoBio, Japan) were injected into the perivitelline space (PVS) of 1~4-cell-stage embryos of wild type medaka (WT). Mouse IgG (mIgG) antibody was used as the negative control. The microinjection was performed using PV820 Pneumatic PicoPump (WPI, FL, USA) and MZ AP0 microscope

(LEICA, Switzerland). Phenotypes were observed under the stereoscopic microscope (Olympus SZX12 DP80).

Generation of *ST6Gal I*- and *II*-knockout medaka

ST6GalI- and *II*-KO medaka was generated using CRISPR/Cas9 system following the published protocol [25,26].

Genotyping

Genomic DNA was prepared as previously described [25]. The targeted region of *ST6Gal I* gene was amplified by KOD-Plus-Neo DNA polymerase (Toyobo, Japan) using the following primers: TCTTAGGACACGCTGCTAAC (forward 1), CTTCTTCTGCCTCTACTGCC (forward 2), CTCCAGGACGTTCTCACCTG (reverse), and the targeted region of *ST6Gal II* gene was amplified using the following primers: AGTGCTTCTGCTGAAGTCCAC (forward 1), GAGGTTCAGCATGAGGCAGT (forward 2), TCGGCCCTCCTCTCAACTAA (reverse), and analyzed by agarose gel electrophoresis.

Digital video recording and analysis of the heart contraction

The heart contraction of embryos at 7dpf was observed as previously described [10,25]. Contraction rhythms were measured based on the fluorescent intensity in atrium and ventricle of each embryo, and the movies were processed using FIJI software.

Rescue experiments

For rescue experiments, 5'-capped mRNAs of medaka *ST6Gal I*, *ST6Gal II*, *ST3Gal IV*, *ST3Gal V*, and D*sRed-monomer* were synthesized using MEGAscript SP6 Transcription Kit (Thermo Fisher Science, MA, USA). Approximately 2-4 nL of a mixture containing 500 ng/μL of each synthesized RNA was injected into *ST6Gal I*-KO embryos at 1-cell-stage as described above.

Quantitative PCR

Quantitative PCR (qPCR) was performed as described previously [25] except that reverse transcription was performed using ReverTra Ace, and qPCR was performed using

THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) and emitted fluorescence was detected using the CFX Connect Real-Time System (Bio-Rad, CA, USA). The amplification efficiencies of targeted genes were determined by absolute standard curve method and normalized by the amplification efficiencies of medaka *β-actin* gene. All experiments were conducted in triplicate. The primers used were CAGCAGCAGAATGAAGAAGGTG (forward) and CCACTTTACGCTTGAGCTGAG (reverse) for *ST6Gal I*; GATGCCGTTCTACGCTTCAAC (forward) and AACTCATGCTTGGGATTCGC (reverse) for *ST6Gal II*; GATCATCATCCTTGCTGCGTAC (forward); TCTCCCACTTGCCATTGCATTC (reverse) for *ST3Gal IV*; CACCATGTCAGTGCTGAGCAG (forward) and GGAGGCAGGAAGTAAAGACAGC (reverse) for *ST3Gal V*; and CACCATGTCAGTGCTGAGCAG (forward) and GGAGGCAGGAAGTAAAGACAGC (reverse) for *β-actin*.

Lectin blotting and western blotting

SDS-PAGE and western blotting were performed as previously described [10,27]. For sialidase digestion, 2 milliunits *Arthrobacter ureafaciens* sialidase (Nacalai, Japan) or 2 milliunits *Vibrio cholerae* sialidase (Roche, Germany) were added to each lysate (2-4 μg protein) in 50 mM sodium acetate (pH 5.5), and incubated overnight at 37℃. For lectin blotting, the membranes were blocked with PBS containing 0.05 % Tween-20, immunostained with 1 μg/ml of SNA or 1 μg/ml of MAA lectin. The signal was enhanced with 1 μg/ml anti-SNA or 1 μg/ml anti-MAA. The immunostaining of β-actin was used as a loading control, the membrane was blocked with 0.25 % skim milk, and then incubated with 0.2 μg/ml anti β-actin mouse monoclonal antibody (Santa Cruze Biochemistry, TX, USA). For the secondary antibody, peroxidase-conjugated anti-mouse IgG+M (American Qualex, CA, USA) was used. For color development, EzWestLumiOne Western blot detection reagent (ATTO, Japan) was used.

Statistical analysis

All values were expressed as the mean±SE (n is at least three) and p-values were evaluated by the Student’s t-test.

Results

Effects of SNA and MAA treatment on early development of medaka

SNA and MAA are known to recognize Siaα2,6Gal and Siaα2,3Gal linkages, respectively (Fig. 1A). To localize the SNA and MAA epitopes in medaka embryos, 16~32-cell stage embryos (1 dpf) were subjected to whole mount lectin-staining using the FITC-labeled SSA and MAA (Fig. 1B). The SNA and MAA epitopes were mainly localized in the blastodisc and the yolk sphere, respectively, suggesting that Siaα2,6Gal and Siaα2,3Gal structures are differently distributed on the medaka embryo surface. During early development before hatching, embryos are surrounded by the PVS that is formed between embryos and fertilization envelope (FE) at fertilization (Fig. 1C, *upper*). Owing to semi-permeability of FE, 10 kDa FITC-dextran was retained in the PVS at least up to 2 dpf after injected at 1~4-cell stages (Fig. 1C, *lower*). Therefore, the PVS injection to 1~4-cell-stage embryos was performed to investigate the effects of SNA, MAA, and mouse immunoglobulin G (mIgG) (control) on gastrulation progress at 18 h post-injection (Fig. 1D). Although MAA allowed normal embryo body formation like mIgG, SNA induced lethality or abnormal embryo body formation at a dose-dependent manner. These results indicate that Siaα2,6Gal, but not Siaα2,3Gal, linkages are important for early development at 0-1 dpf.

Occurrence of major SNA epitopes in *N*-glycans during early development

To clarify whether Siaα2,6Gal epitopes are expressed on *N*- or *O*-glycans, medaka embryos at 9 dpf were analyzed by SNA lectin blotting with or without PNGaseF treatment (Fig. 2A). The intensity of SNA-positive bands was decreased to 30.6 ± 4.0% (n = 3) after the PNGaseF treatment, indicating that most Siaα2,6Gal epitopes mainly occur in *N*-glycans. It has been shown that Siaα2,6Gal structures in *N*-glycans are synthesized by two β-galactoside:α2,6-sialyltransferases, ST6Gal I and ST6Gal II in vertebrate [26]. Therefore, cDNAs for *ST6Gal I* and *ST6Gal II* were cloned from medaka embryos at stage 10. The expression profiles of *ST6Gal I* and *II* during early development at 0-14 dpf were analyzed by qPCR (Fig. 2B). Both *ST6Gal I* and *II* were expressed throughout the developmental stages. Highest expression levels of both genes at 0 dpf appear to be mostly derived from maternal RNA. Interestingly, the

expression level of *ST6Gal I* changed places with that of *ST6Gal II* after hatching.

Phenotypes of *ST6Gal I*-KO and *ST6Gal II*-KO medaka in early development

To investigate the significance of Siaα2,6Gal in early development, *ST6Gal I*-KO and *ST6Gal II*-KO medaka were generated using CRISPR/Cas9 system (Fig. 3A). Because of an 8-bp insertion in exon 3 for the *ST6Gal I*-KO and a 4-bp deletion in exon 3 for the *ST6Gal II*-KO, the mutated alleles of *ST6Gal I*-KO and *ST6Gal II*-KO encoded 77 and 37 amino-acid proteins, respectively, whereas the WT alleles encoded 499 and 509 amino-acid *ST6Gal I* and *ST6Gal II*, respectively. These results suggest that no functional enzymes were expressed in both KO medaka. The genotypes of *ST6Gal I* and *II* (-/-), (+/-), and (+/+) medaka could successfully be classified by PCR (Fig. 3B). Since *ST6Gal I*-KO medaka strains could not grow into adult fish, the medaka strain with heterozygous genotype, *ST6Gal I* (+/-), was established and subcultured. In contrast, *ST6Gal II*-KO could survive until fully matured adult, and the medaka strain with homozygous genotype, *ST6Gal II* (-/-), was established and subcultured.

Prominently, cardiac abnormalities in morphology were observed in *ST6Gal I*-KO fry at 7 dpf (Fig. 3C, *middle column*), although no such changes were observed WT and *ST6Gal II*-KO (Fig. 3C, left and *right column*). To clearly observe the cardiac abnormalities, both KO medaka were crossed with TG941 medaka, showing strong mCherry expression in the cardiac muscle. In WT and *ST6Gal II*-KO, an atrium and a ventricle were aligned side-by-side in the heart. In contrast, they were vertically lined up in *ST6Gal I*-KO (Fig. 3C, *lower*). The heart beating profiles of atrium and ventricle were also monitored. In WT and *ST6Gal II*-KO, the atrium and ventricle beated periodically and alternatively with each other (Fig. 3D, *left and right*). However, in *ST6Gal I*-KO, their heartbeat was periodical, but synchronous (Fig. 3D, *middle*). These results showed that *ST6Gal I*, but not *ST6Gal II*, was critical for normal heart development.

Rescue experiments of cardiac abnormalities in *ST6Gal I*-KO medaka

(a) Forced expression of *ST6Gal I* and *ST6Gal II* genes in *ST6Gal I*-KO:

To confirm that the cardiac abnormalities were caused by a *ST6Gal I* deficiency, rescue experiments were performed by injecting *ST6Gal I* mRNA in 1-cell stage embryos of *ST6Gal I*-KO medaka (Fig. 4A, B). When *DsRed* mRNA was injected as a control, no normal embryo

was observed: 17% were dead, and 83% showed cardiac abnormalities (Fig. 4A, D). In contrast, 72% of fish recovered to normal by injecting the *ST6Gal I* mRNA, by which increased expression of *ST6Gal I* was observed at 1 dpf (Fig. 4E, *left*), while 14% and 14% showed cardiac abnormalities and severe anomalous morphologies, respectively (Fig. 4B, D). This high recovery rate indicates that the cardiac abnormalities in *ST6Gal I*-KO are caused by a deficiency of *ST6Gal I*.

Like ST6Gal I, ST6Gal II has the activity to synthesize a Siaα2,6Gal linkage, and may compensate the Siaα2,6Gal synthesis in place of ST6Gal I [28]. *ST6Gal I*-KO expressed the *ST6Gal II* gene at 1 dpf (Fig. 4E, *left, DsRed*). The expression level of endogenous *ST6Gal II* had no effect on improvement of the impaired heart development. Interestingly, however, when *ST6Gal II* mRNA was exogenously injected in 1-cell-stage embryos of *ST6Gal I*-KO, the *ST6Gal II* expression level at 1 dpf was 3.9-fold increased (Fig. 4E, *right*), and severe cardiac abnormalities were rescued: 60% of fish were restored to normal, while 20% and 20% showed heart abnormalities and lethality, respectively (Fig. 4C, D). The high recovery rate of the phenotype by *ST6Gal II* mRNA injection suggests that forced expression of *ST6Gal II* can compensate the *ST6Gal I*-deficiency for improving impaired heart development with high frequency.

Expression of Siaα2,6Gal epitopes at 7 dpf in the *ST6Gal I*-KO that were injected with *DsRed*, *ST6Gal I*, or *ST6Gal II* mRNA was analyzed by SNA lectin blotting. SNA epitopes were intensely detected as a long smear in the whole region, and most of them were obviously sialidase-sensitive (Fig. 4F, *upper*). The amount of SNA epitopes did not significantly change between *DsRed* mRNA- and *ST6Gal I* mRNA-injected *ST6Gal I*-KO (Fig. 4F, *lower*). Similar profiles of SNA staining were obtained for *DsRed* mRNA- and *ST6Gal II* mRNA-injected *ST6Gal I*-KO (Fig. 4F, *upper*), and no significant change in the SNA epitope amount was observed between them (Fig. 4F, *lower*). These results show that the amount of ST6Gal I-synthesized SNA epitopes is very low at 7 dpf. Therefore, the lectin-blotting results cannot easily tell anything as to whether the forced expression of *ST6Gal I* and *ST6Gal II* recovered the Siaα2,6Gal epitopes.

(b) Forced expression of *ST3Gal IV* and *ST3Gal V* genes in *ST6Gal I*-KO:

In the previous section, the cardiac abnormalities in the *ST6Gal I*-KO are shown to be restored by forced expression of *ST6Gal I* and *ST6Gal II*, both of which are involved in glycoprotein α2,6-sialylation. In this section, we asked whether this impaired phenotype of the *ST6Gal I*-KO can be rescued by forced expression of the β-galactoside:α2,3-sialyltransferase IV gene (*ST3Gal IV*), whose substrate specificity is to *N*-glycoproteins [29]. To our surprise, forced expression of *ST3Gal IV* mRNA could rescue the cardiac abnormalities of *ST6Gal I*-KO medaka, whereas that of *DsRed* mRNA did not rescue at all (Fig. 5A, B). In case of *DsRed* control mRNA injection, 17% were dead, and 83% showed cardiac abnormalities (Fig. 5A, D). In contrast, in case of *ST3Gal IV* mRNA injection, by which increased expression of *ST3Gal IV* was observed at 1 dpf (Fig. 5E, *left*), 43% of fish recovered to normal, while the rest showed no recovery: 14%, 14% and 29% showed cardiac abnormalities, severe anomalous morphologies, and lethality, respectively (Fig. 5B, D). The fact that the *ST3Gal IV* mRNA injection restored the impaired heart development by 43% suggests that forced expression of *ST3Gal IV* can compensate the *ST6Gal I*-deficiency.

ST3Gal V is an α2,3-sialyltransferase that is different from ST3Gal IV in that it is specifically involved in the synthesis GM3 ganglioside [30]. Thus, when *ST3Gal V* mRNA was injected in 1-cell-stage embryos of *ST6Gal I*-KO medaka (Fig. 5C), the *ST3Gal V* expression level at 1 dpf was 1.3-fold increased (Fig. 5E, *right*). Unlike the *ST3Gal IV* injection, forced expression of *ST3Gal V* did not improve the impaired heart development (Fig. 5C, D): no fish were recovered to normal, and 60%, 20%, and 20% showed heart abnormalities, severe anomalous morphologies, and lethality, respectively (Fig. 5C, D). These results indicate that *ST3Gal V* cannot compensate for the function of *ST6Gal I*, suggesting that α2,3-sialylation on *N*-glycoproteins, but not glycolipids, is useful for the heart development.

Expression of Siaα2,3Gal epitopes at 7 dpf in the *ST6Gal I*-KO that were injected with *DsRed* or *ST6Gal IV* mRNA was analyzed by MAA lectin blotting. MAA epitopes were detected, and most of them were sialidase-sensitive (Fig. 5F, *upper*). The amount of MAA epitopes did not significantly change between *DsRed* mRNA- and *ST3Gal IV* mRNA-injected *ST6Gal I*-KO (Fig. 5F, *lower*). Similar profiles of MAA staining were obtained for *DsRed* mRNA- and *ST6Gal II* mRNA-injected *ST3Gal V*-KO (Fig. 5G, *upper*), and no significant change in the MAA epitope amount was observed between them (Fig. 5G, *lower*). By the same

reason as suggested above, the lectin-blotting results cannot tell anything as to whether the forced expression of *ST3Gal IV* increased the Siaα2,3Gal epitopes. ST3Gal V might not be involved in protein α2,3-sialylation.

Discussion

Medaka embryos at 0-1 dpf express both Siaα2,6Gal and Siaα2,3Gal mainly in the blastodisc and the yolk sphere, respectively (Fig. 1B). To demonstrate which linkage of them was functionally important at this stage, inhibition experiments by lectin injection into PVS were performed, showing that the Siaα2,6Gal-recognizing lectin affected embryonic survival more seriously than the Siaα2,3Gal-recognizing lectin (Fig. 1D). Thus, it is concluded that the Siaα2,6Gal is more important than Siaα2,3Gal for embryonic survival (Fig. 1D). However, it still remained unknown if α2,6-sialylation was indispensable, or if it could be replaced by α2,3-sialylation for the embryonic survival. Therefore, to attain this replacement, we first prepared KO medaka for the *ST6Gal I* and *ST6Gal II* genes, and subsequently, performed forced expression of β-galactoside:α2,3-sialyltransferases in the KO medaka. Of the *ST6Gal I*-KO and *ST6Gal II*-KO, only *ST6Gal I*-KO medaka showed severe cardiac abnormalities in morphology and heart beating at 7-16 dpf, which might cause the lethality observed at 14-18 dpf, whereas *ST6Gal II*-KO showed any obvious phenotypes in life cycle of the fish (Fig. 3). These results suggested that the Siaα2,6Gal linkages synthesized by ST6Gal I, but not ST6Gal II, was essential for normal heart development at 7-16 dpf. Notably, no obvious abnormality was observed in *ST6Gal I*-KO medaka at 0-1 dpf, which might conclude that the Siaα2,6Gal linkages are not essential in the survival of 0-1 dpf embryos. However, in medaka it is difficult to establish the *ST6Gal I*-KO strain, because the KO embryos are lethal in young fish. Therefore, the *ST6Gal I*-KO zygotes could be obtained only by crossing *ST6Gal I* (+/-) parents with each other, and thus always include maternal materials in the zygotes. Indeed, both *ST6Gal I* and *ST6Gal II* are expressed throughout developmental stages in WT with the highest expression level at 0 dpf for both genes (Fig. 2B). Thus, it is technically difficult to conclude something about significance of Siaα2,6Gal synthesized by *ST6Gal I* at 0-1 dpf.

We then performed rescue experiments by forced expression of *ST6Gal I*, *ST6Gal II*,

ST3Gal IV, or *ST3Gal V* in *ST6Gal I*-KO medaka suffering from cardiac abnormalities (Fig. 4, 5). The abnormalities were 72% and 60% rescued by forced expression of *ST6Gal I* and *ST6Gal II*, respectively. Interestingly, they were 43% rescued by that of *ST3Gal IV*; however, no rescue was observed in the case of *ST3Gal V*. These results are consistent with the following lines of evidence: Most Siaα2,6Gal epitopes occur in *N*-glycans in 7 dpf-medaka (Fig. 2A); and ST6Gal I, ST6Gal II, and ST3Gal IV are mainly involved in sialylation of *N*-glycans [28,29], while ST3Gal V is glycolipid-specific, or called ganglioside GM3 synthase [30]. It should be noted that *ST6Gal II* and *ST3Gal IV* cannot rescue the cardiac abnormalities in *ST6Gal I*-KO unless over-expressed (Fig. 4,5). Thus, in developing heart, there might be some *N*-glycoproteins containing ST6Gal I-specifically involving Gal residues that can accept sialylation only by over-expressed ST6Gal II and ST3Gal IV. Thus, masking of these Gal residues by Sia residues in either α2,6- or α2,3-linkage is important for normal heart development.

Here are a few questions emerging from our study. First, does medaka ST6Gal I have its specific *N*-glycoproteins in heart development? Second, what are the acceptor glycan specificities of medaka ST6Gal I and ST6Gal II? The mouse ST6Gal I and ST6Gal II are shown to effectively transfer α2,6-Sia residue to *N*-glycoprotein, preferring Galβ1,4GlcNAc and GalNAcβ1,4GlcNAc structures as acceptors, respectively [28]. Third, how does the Siaα2,6Gal structure work in heart development in medaka? In the future, we want to solve these questions.

Several reports have demonstrated on the significance of α2,6-sialylation of Gal residues in glycoproteins. For example, α2,6-sialylation of β1-integrin, an adhesion protein between cells and extracellular matrix, is demonstrated to be critical for cell adhesion and progression of cancer cells in human [18,19,31-33]. α2,6-Sialylation of the Fas receptor, a death receptor on the cell surface, is known to prevent apoptosis through masking of the Gal residues by galectin-3 binding [21]. These studies have investigated functional importance of Sia at the cell level by deleting terminal Sia residues or by site-specific point-mutation that would delete particular *N*-glycans [31-33]. However, no such experiments that convert the Siaα2,6Gal to Siaα2,3Gal linkages in these sialoglycoproteins have ever been done at the organism level. To best of our knowledge, this study for the first time performed such critical experiments, indicating the significance of masking effect of Sia to Gal residues during early development of medaka [1,4].

In mouse, both *ST6Gal I*-KO and *ST6Gal II*-KO organisms can survive, although *ST6Gal I*-KO exhibits severe immunosuppression phenotypes [34-36]. This is in contrast to our results in medaka that *ST6Gal I*-KO is lethal, while *ST6Gal II*-KO survive. In addition, *ST3Gal IV*-KO mice are known to suffer from dilated cardiomyopathy and stress-induced heart failure [37], suggesting α2,3-sialylation is more important than α2,6-sialylation for heart function in mice. These features indicate that function and significance of Siaα2,6Gal are different between medaka and mice.

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References

[1] Schauer R, Kamerling JP. (2018) Exploration of the sialic acid world. *Adv Carbohydr Chem Biochem*. 75:1-213.
[2] Varki A. (2017) Are humans prone to autoimmunity? Implications from evolutionary changes in hominin sialic acid biology. *J Autoimmun*. 83, 134–142.
[3] Kitajima K. Varki N, Sato C. (2015) Advanced technologies in sialic acid and sialoglycoconjugate analysis. *Top Curr Chem*. 367:75-103.
[4] Schauer R. (2004) Sialic acids: fascinating sugars in higher animals and man. *Zoology (Jena)*. 107, 49-64.
[5] Sato C, Kitajima K. (2019) Sialic acids in neuology. *Adv Carbohydr Chem Biochem*. 76:1-64.
[6] Sato C, Kitajima K. (2021) Polysialylation and disease. *Mol Aspects Med*. 79, 100892.
[7] Angata T, Varki A. (2002) Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. *Chem Rev*. 102, 439-69.
[8] Schwarzkopf M, Knobeloch KP, Rohde E, et al. (2002) Sialylation is essential for early development in mice. *Proc Natl Acad Sci USA*. 99, 5267-5270.
[9] Weinhold B, Sellmeier M, Schaper W, et al. (2012) Deficits in sialylation impair podocyte maturation. *J Am Soc Nephrol*. 23, 1319-1328.
[10] Wu D, Arakawa H, Fujita A, et al. (2021) A point-mutation in the C-domain of CMP-sialic acid synthetase leads to lethality of medaka due to protein insolubility. *Sci Rep*. 11, 23211.
[11] Rogers GN, Paulson JC. (1983) Receptor determinants of human and animal influenza

virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology*, 127, 361–373.
[12] Connor RJ, Kawaoka Y, Webster RG, Paulson JC. (1994) Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology*. 205, 17–23.
[13] Crocker PR, Paulson JC, Varki A. (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol*. 7, 255-66.
[14] Angata T, Varki A. (2022) Discovery, classification, evolution and diversity of Siglecs. *Mol Aspects Med*. 18, 101117.
[15] Yamakawa N, Yasuda Y, Yoshimura A, et al. (2020) Discovery of a new sialic acid binding region that regulates Siglec-7. *Sci Rep*. 10, 8647.
[16] Ellies LG, Ditto D, Levy GG, et al. (2002) Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proc Natl Acad Sci USA*. 99, 10042-7.
[17] Bridges K, Harford J, Ashwell G, Klausner RD. (1982) Fate of receptor and ligand during endocytosis of asialoglycoproteins by isolated hepatocytes. *Proc Natl Acad Sci USA*. 79, 350-354.
[18] Hou S, Hang Q, Isaji T, Lu J, Fukuda T, Gu J. (2016) Importance of membrane-proximal N-glycosylation on integrin β1 in its activation and complex formation. *FASEB J*. 30, 4120-4131.
[19] Seales EC, Jurado GA, Brunson BA, et al. (2005) Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by up-regulating cell motility. *Cancer Res*. 65, 4645-4652.
[20] Recchi MA, Hebbar M, Hornez L, et al. (1998) Multiplex reverse transcription polymerase chain reaction assessment of sialyltransferase expression in human breast cancer. *Cancer Res*. 58, 4066-70.
[21] Swindall AF, Bellis SL. (2022) Sialylation of the Fas Death Receptor by ST6Gal-I Provides Protection against Fas-mediated Apoptosis in Colon Carcinoma Cells. *J Biol Chem*. 286, 22982-22990.
[22] Cong L, Ann Ran F, Cox D, et al. (2013) Multiple genome engineering using CRISPR/Cas Systems. *Science.* 339, 819-823.
[23] Hwang W. Fu Y, Reyon D, et al. (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol*. 31, 227-229.
[24] Shimizu A, Shimizu N. (2013) Dual promoter expression system with insulator ensures a stringent tissue-specific regulation of two reporter genes in the transgenic fish. *Transgenic Res*. 22, 435-444.
[25] Ertunc N, Phitak T, Wu D, et al. (2022) α2,8-sialyltransferase 6 (ST8Sia6) localizes in the ER and enhances the anchorage-independent cell growth in cancer. *Sci Rep*. 12, 12496.
[26] Ansai S, Kinoshita M. (2014) Targeted mutagenesis using CRISPR/Cas system in medaka. *Biol Open*. 3, 362-371.
[27] Hatanaka R, Araki E, Hane M, et al. (2022) Sulfation of sialic acid is ubiquitous and essential for vertebrate development. *Biochem Biophys Res Commun*. 11, 608.
[28] Takashima S, Tsuji S, Tsujimoto M. (2003) Comparison of the Enzymatic Properties of Mouse β-Galactoside α2,6-Sialyltransferases, ST6Gal I and II. *J. Bio Chem*. 134, 287-296.

[29] Kono M, Ohyama Y, Lee Y, et al. (1997) Mouse β-galactoside α2,3-sialyltransferases: comparison of *in vitro* substrate specificities and tissue specific expression. *Glycobiology*. 7, 469-479.

[30] Ishii A, Ohta M, Watanabe Y, et al. (1998) Expression coning and functional characterization of human cDNA for ganglioside GM3 synthase. *J Biol Chem*. 273, 31652–31655.

[31] Stephens LE, Sutherland AE, Klimanskaya IV, et al. (1995) Deletion of B1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev*. 9, 1883–1895.

[32] Chammas R, Veiga SS, Travassos LR, Brentani RR. (1993) Functionally distinct roles for glycosylation of alpha and beta integrin chains in cell-matrix interactions. *Proc Natl Acad Sci USA*. 90, 1795-1799.

[33] Gu J, Taniguchi N. (2004) Regulation of integrin functions by N-glycans. *Glycoconj J*. 21, 9-15.

[34] Hennet T, Chui D, Paulson JC, Marth JD. (1998) Immune regulation by the ST6Gal sialyltransferase. *Proc Natl Acad Sci USA*. 95, 4504-4509.

[35] Grewal PK, Uchiyama S, Ditto D, Varki N, Le DT, Nizet V, Marth JD. (2008) The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nat Med*. 14, 648–655.

[36] Ohmi Y, Nishikaze T, Kitaura Y, et al. (2021) Majority of alpha2,6-sialyated glycans in the adult mouse brain exist in O-glycans: SALSA-MS analysis for knockout mice of alphs2,6-sialyltransferase genes. *Glycobiology*. 31, 557–570.

[37] Deng W, Ednie AR, Qi J, Bennett ES. (2016) Aberrant sialylation causes dilated cardiomyopathy and stress-induced heart failure. *Basic Res Cardiol*. 111, 57.

Legends to figures

Fig. 1. Localization and significance of α2,6- and α2,3-sialosides in medaka embryos at 0-1 dpf. A. Structures of Siaα2,6Gal (*upper*) and Siaα2,3Gal (*lower*). R, other monosaccharides; **B.** Whole mount immunofluorescent staining of 16~32-cell-stage embryos using FITC-SSA and FITC-MAA. DIC, differential interference contrast. A dotted square is enlarged at right panel; **C.** Cartooning of lectins injection into PVS of 1~4-cell embryos (*upper*). Fluorescence stayed at the PVS at 2 dpf (*lower right*), after FITC-dextran was injected there at 0 dpf (*lower left*); **D.** SNA, MAA, and mIgG (control) were PVS-injected in 1~4-cell embryos and the effects were observed at gastrula embryos at 18 h-post-injection. Embryos of dead, abnormal, and normal morphology were counted. n, number of embryos tested.

Fig. 2. Distribution of Siaα2,6Gal epitope and expression profiles of *ST6Gal I* and *ST6Gal II* in WT. A. SNA lectin blotting of 9 dpf embryos before (-) and after (+) PNGase F treatment (LB: SNA). IB: β-actin, loading control; Human transferrin (hTf) was used as a control for the lectin blotting; **B.** Developmental expression of *ST6Gal I* and *ST6Gal II* by qPCR. The expressions were normalized with the β-actin expression. The bars represent standard deviations from the triplicated experiments.

Fig. 3. Phenotypes of the *ST6Gal I*-KO and *ST6Gal II*-KO medaka embryos. A. Diagrams of the target site in the *ST6Gal I* and *ST6Gal II* genes and their base sequences; **B.** Genotyping of the (+/+), (+/-), and (-/-) medaka for *ST6Gal I* (*upper*) and *ST6Gal II* (*lower*) genes. N.C., water instead of the templates; **C.** *Upper*, DIC images of WT (*left*), *ST6Gal I*-KO (*middle*), and *ST6Gal I*-KO (*right*) embryos at 7 dpf. Scale bars, 85 μm. *Lower*, Fluorescent images (mCherry) of Tg941 WT (*left*), *ST6Gal I*-KO (*middle*), and *ST6Gal II*-KO (*right*) embryos at 7 dpf. A, atrium; V, ventricle. Scale bars, 85 μm; **D.** Monitoring of heartbeat of Tg941 WT (*left*), *ST6Gal I*-KO (*middle*), and *ST6Gal II*-KO (*right*) embryos at 7 dpf for 2 s. The heartbeats of atrium (black) and ventricle (gray) were analyzed by the FIJI software. Relative area stands for their sizes, and most dilated ones were set to 1.0.

Fig. 4. Rescue experiments of *ST6Gal I*-KO by forced expression of *ST6Gal I*- and *ST6Gal II*-mRNAs. A, B, C. DIC images of the embryos at 7 dpf that were injected with DsRed mRNA (control, A), *ST6Gal I* mRNA (B), and *ST6Gal II* mRNA (C) at 1-cell embryos. Scale bars, 85 μm; **D.** Summary of the heart phenotypes of *ST6Gal I*-KO embryos at 7 dpf injected with *DsRed*, *ST6Gal I*, and *ST6Gal II* mRNAs. n, number of experiments; Dead, Abnormal, Cardiac abnormality, and Normal stand for lethality, severe anomalous morphologies, cardiac abnormalities, and normal embryos; **E.** *ST6Gal I* (*left*) and *ST6Gal II* (*right*) expression in *DsRed*, *ST6Gal I*, and *ST6Gal II* mRNAs-injected *ST6Gal I*-KO embryos as evaluated by qPCR. The expression level was normalized by that of β-actin. The bars represent standard deviations from the triplicated experiments. **, $p < 0.005$; ***, $p < 0.0005$; **F.** (*upper*)SNA lectin blotting of *DsRed*, *ST6Gal I*, and *ST6Gal II* mRNAs-injected *ST6Gal I*-KO embryos at 7 dpf before (-) and after (+) sialidase treatment (LB: SNA). IB: β-actin, loading control; hTf was used as a control for the lectin blotting. (*Lower*) quantification of SNA epitopes/β-actin values calculated from the above data. The bars represent standard deviations from the triplicated experiments.

Fig. 5. Rescue experiments of *ST6Gal I*-KO by forced expression of *ST3Gal IV*- and *ST3Gal V*-mRNAs. A, B, C. DIC images of the embryos at 7 dpf that were injected with DsRed mRNA (control, A), *ST3Gal IV* mRNA (B), and *ST3Gal V* mRNA (C) at 1-cell embryos. Scale bars, 85 μm; **D.** Summary of the heart phenotypes of *ST6Gal I*-KO embryos at 7 dpf injected with *DsRed*, *ST3Gal IV*, and *ST3Gal V* mRNAs. n, number of experiments; Dead, Abnormal, Cardiac abnormality, and Normal stand for lethality, severe anomalous morphologies, cardiac abnormalities, and normal embryos; **E.** *ST3Gal IV* (*left*) and *ST3Gal V* (*right*) expression in *DsRed*, *ST3Gal IV*, and *ST3Gal V* mRNAs-injected *ST6Gal I*-KO embryos as evaluated by qPCR. The expression level was normalized by that of β-actin. The bars represent standard deviations from the triplicated experiments. *, $p < 0.05$; ***, $p < 0.0005$. **F.** MAA lectin blotting (*upper*) of *DsRed* and *ST3Gal IV* mRNAs-injected *ST6Gal I*-KO embryos at 7 dpf before (-) and after (+) sialidase treatment (LB: MAA). IB: β-actin, loading control; Ft was used as a control for the lectin blotting. (*Lower*) quantification of MAA epitopes/β-actin values. The bars represent standard deviations from the triplicated experiments; **G.** MAA lectin blotting (*upper*)

of *DsRed* and *ST3Gal V* mRNAs-injected *ST6Gal I*-KO embryos at 7 dpf. (*Lower*) quantification of MAA epitopes/β-actin values. For details, see the legend for F.

Fig. 1

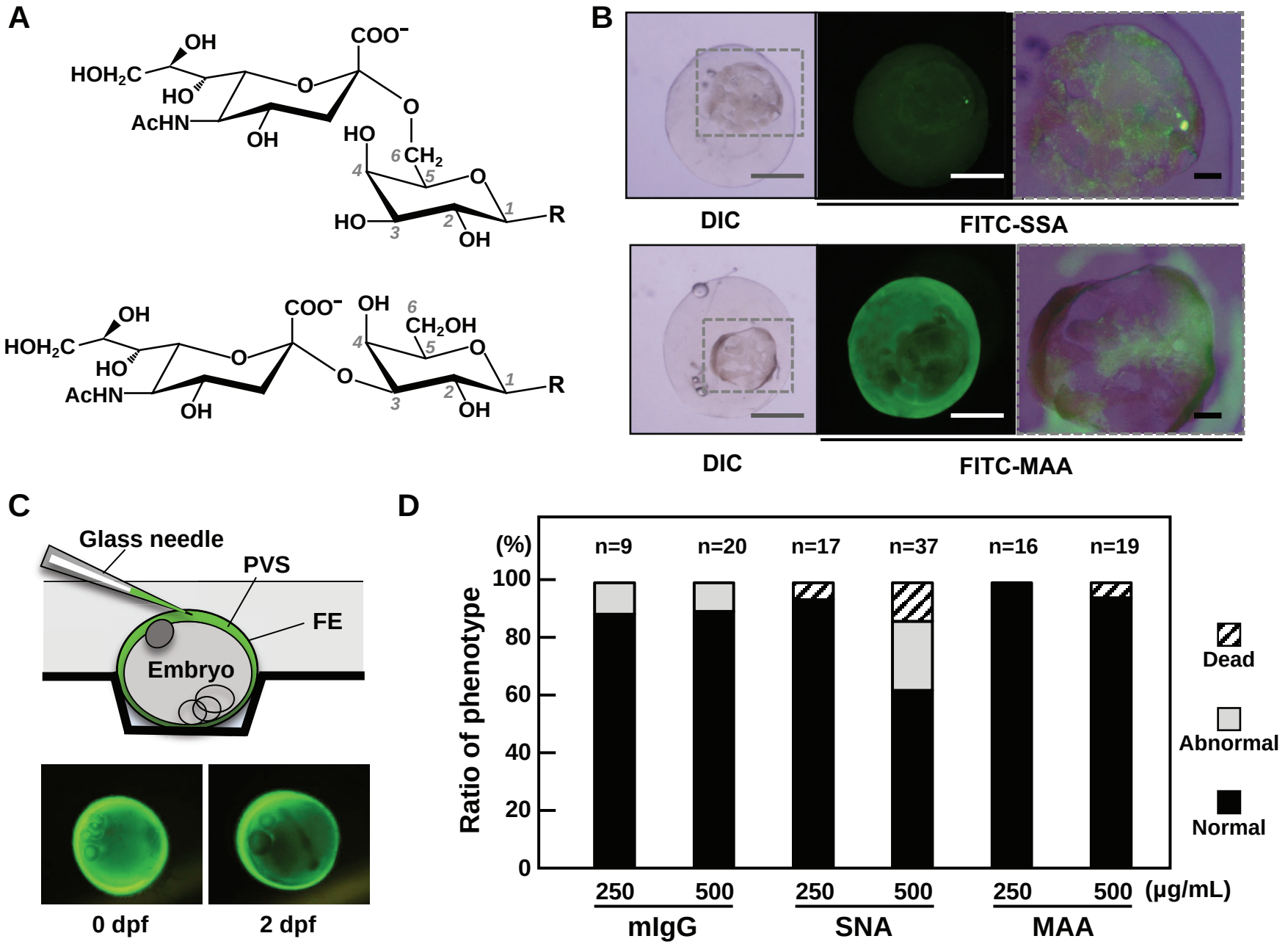

A
B
C
D
DIC
FITC-SSA
DIC
FITC-MAA
Glass needle
PVS
FE
Embryo
0 dpf
2 dpf
Ratio of phenotype
(%)
n=9
n=20
n=17
n=37
n=16
n=19
Dead
Abnormal
Normal
250
500
250
500
250
500
(μg/mL)
mIgG
SNA
MAA

Fig. 2

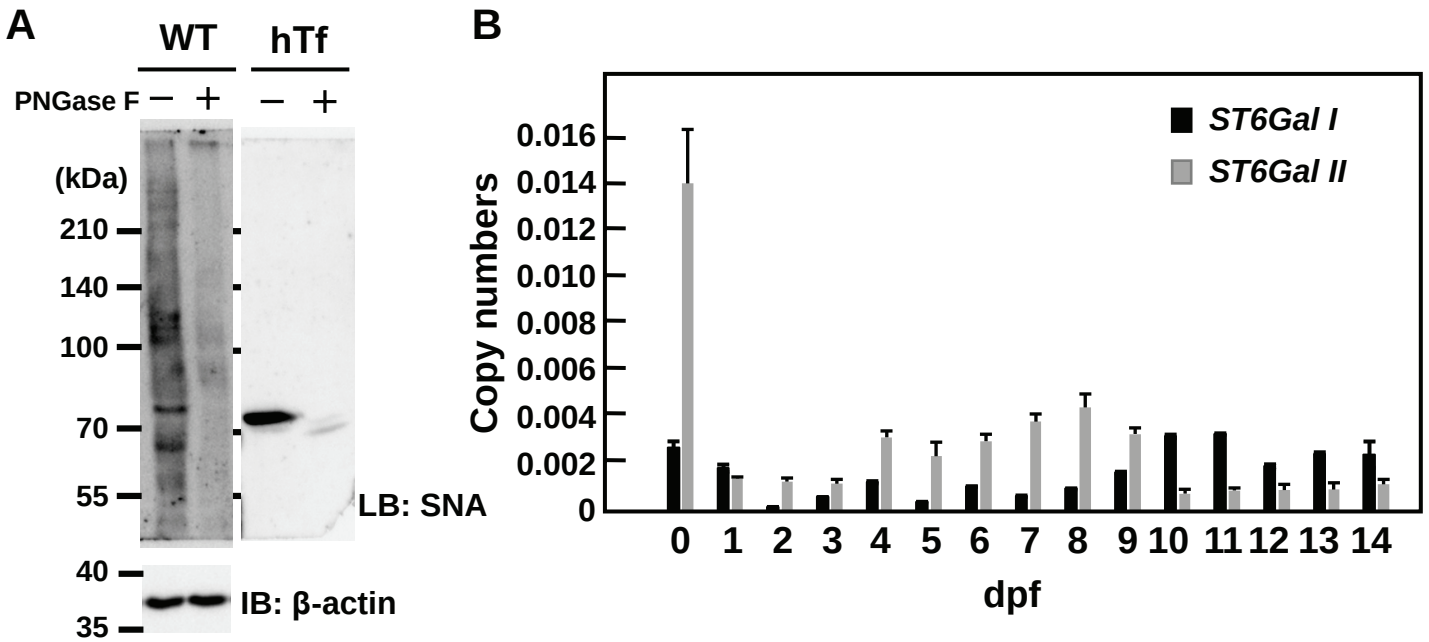

A
WT
hTf
PNGase F − + − +
(kDa)
210
140
100
70
55
40
35
LB: SNA
IB: β-actin
B
Copy numbers
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
ST6Gal I
ST6Gal II
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
dpf

Fig. 3

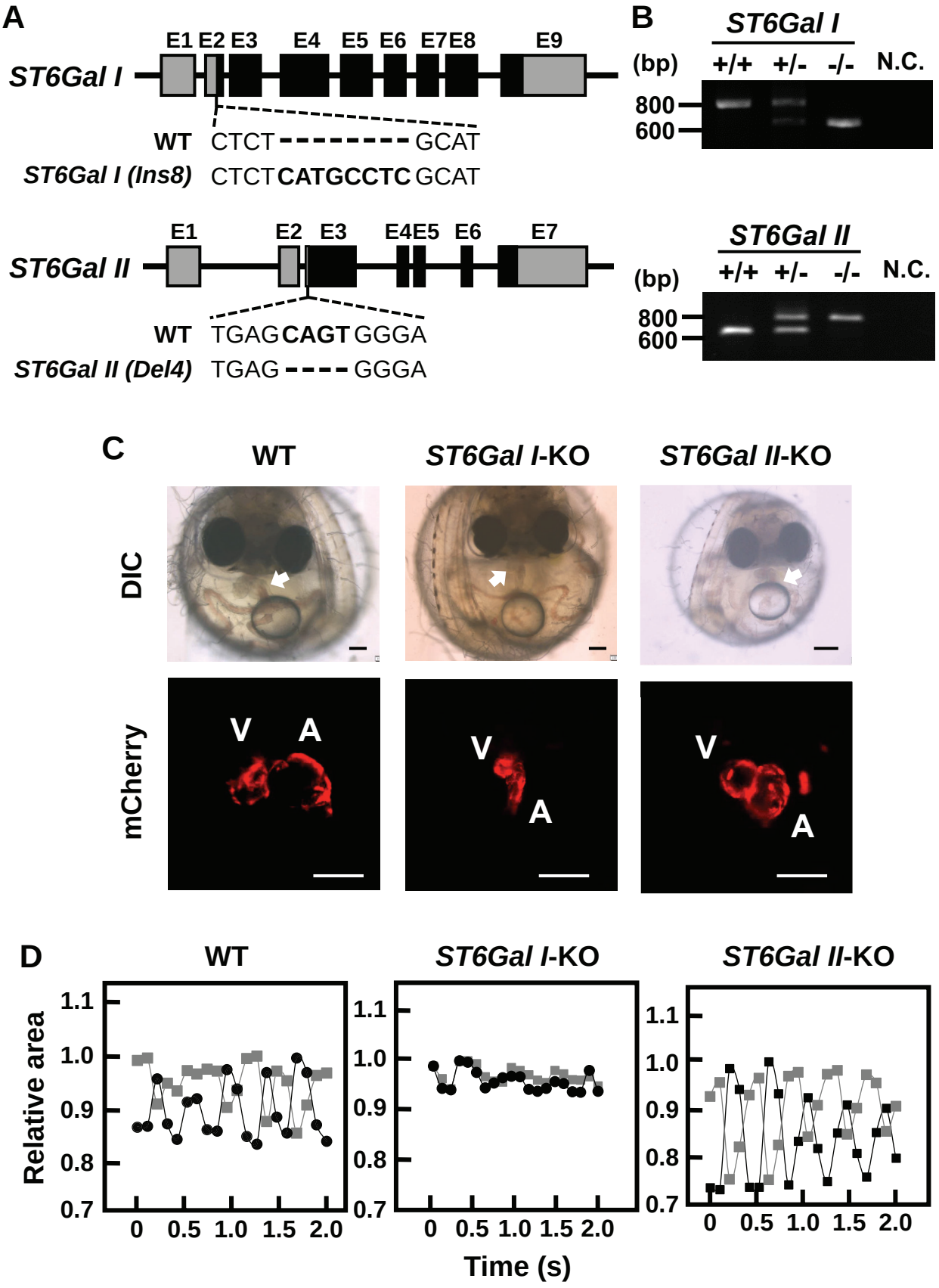

A
ST6Gal I
E1 E2 E3 E4 E5 E6 E7E8 E9
WT CTCT GCAT
ST6Gal I (Ins8) CTCT CATGCCTC GCAT
ST6Gal II
E1 E2 E3 E4E5 E6 E7
WT TGAG CAGT GGGA
ST6Gal II (Del4) TGAG GGGA
B
ST6Gal I
(bp) +/+ +/- -/- N.C.
800
600
ST6Gal II
(bp) +/+ +/- -/- N.C.
800
600
C
WT
ST6Gal I-KO
ST6Gal II-KO
DIC
mCherry
V A
V A
V A
D
WT
ST6Gal I-KO
ST6Gal II-KO
Relative area
1.1
1.0
0.9
0.8
0.7
0 0.5 1.0 1.5 2.0
Time (s)

Fig. 4

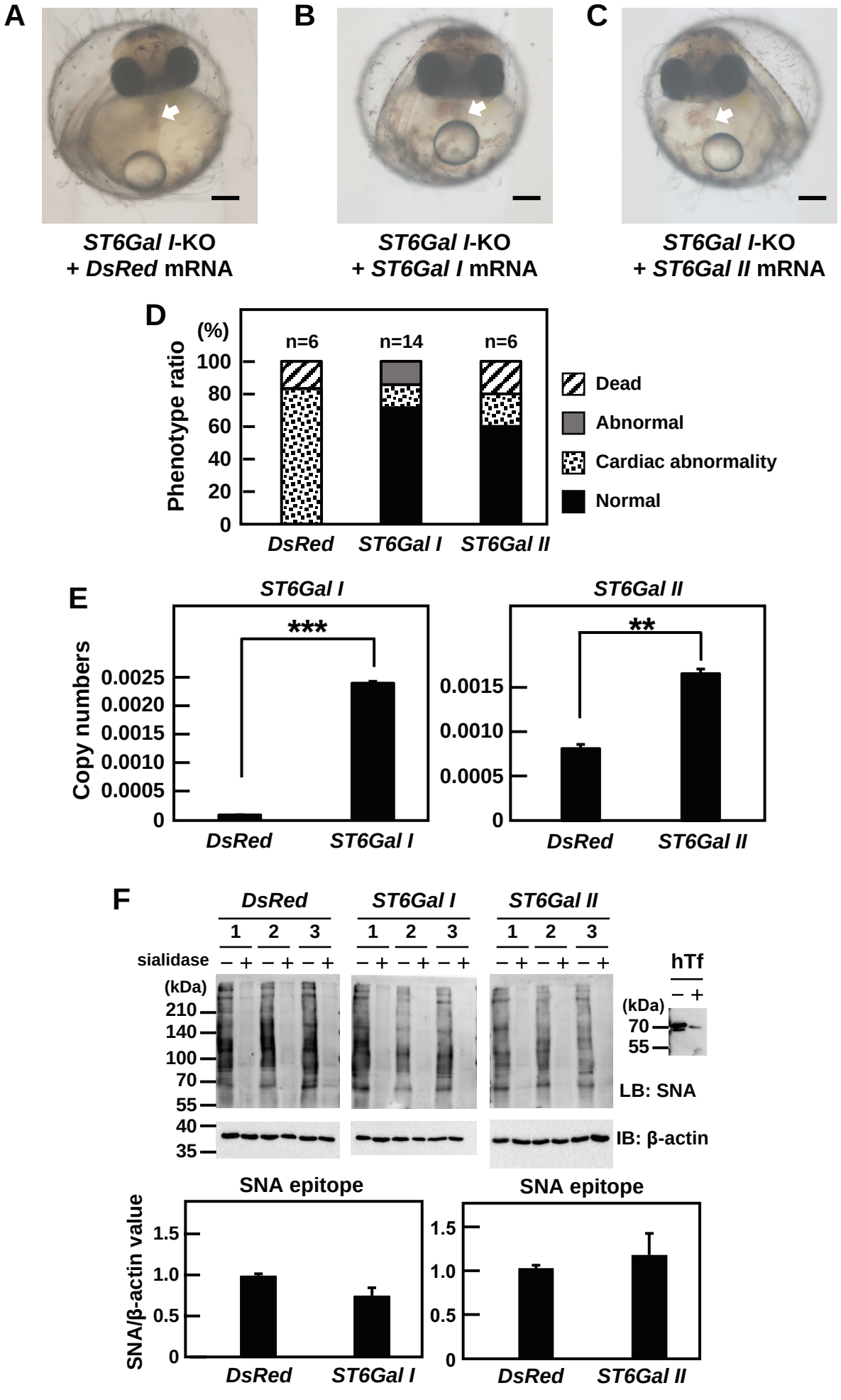
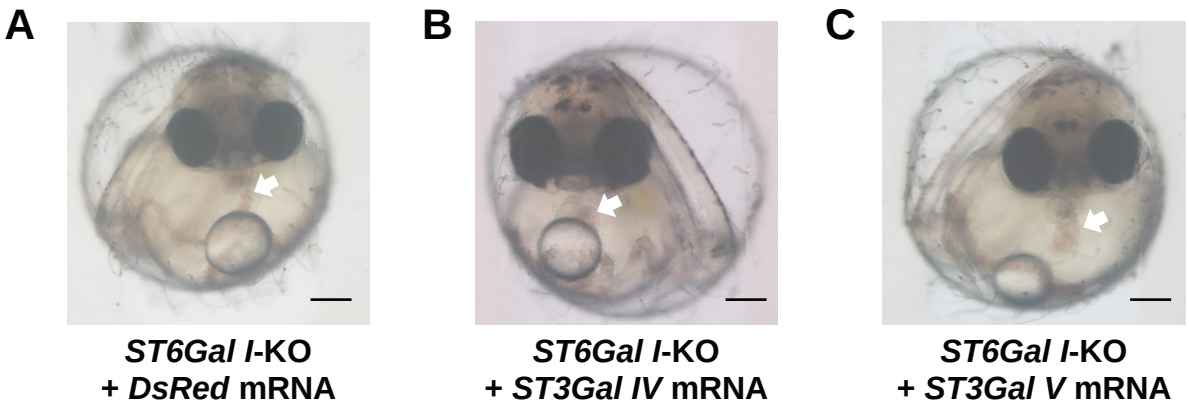
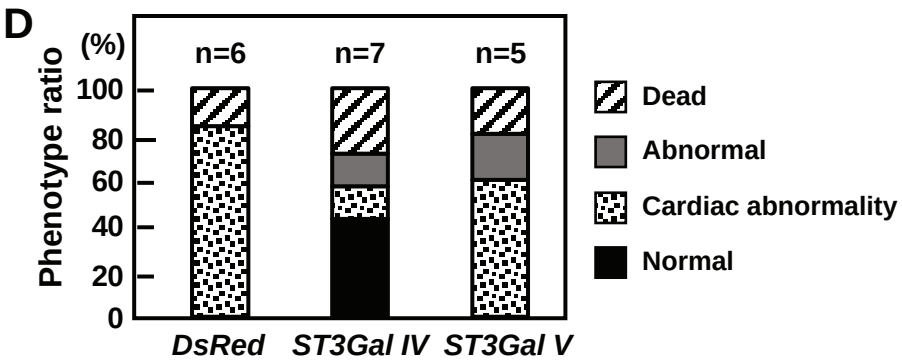
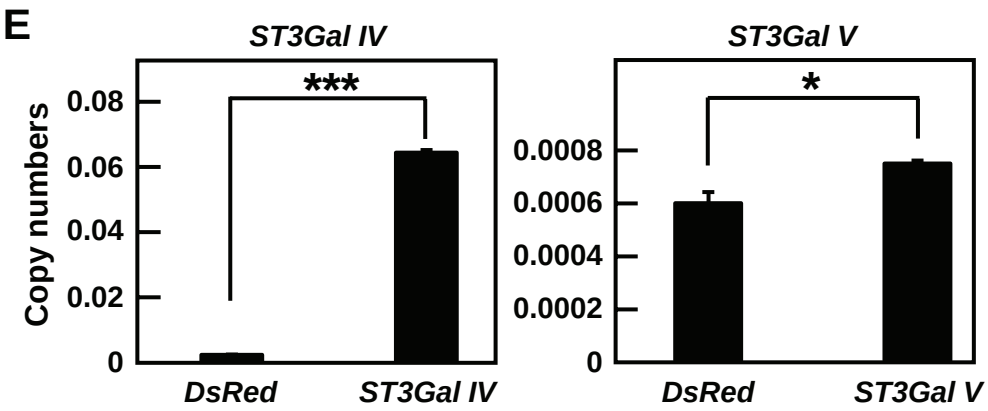

A
B
C
ST6Gal I-KO
+ DsRed mRNA
ST6Gal I-KO
+ ST6Gal I mRNA
ST6Gal I-KO
+ ST6Gal II mRNA
D
Phenotype ratio
(%)
100
80
60
40
20
0
n=6
n=14
n=6
DsRed
ST6Gal I
ST6Gal II
Dead
Abnormal
Cardiac abnormality
Normal
E
ST6Gal I
ST6Gal II
Copy numbers
***
**
0.0025
0.0020
0.0015
0.0010
0.0005
0
DsRed
ST6Gal I
ST6Gal II
F
DsRed
ST6Gal I
ST6Gal II
1 2 3
sialidase
hTf
(kDa)
210
140
100
70
55
40
35
LB: SNA
IB: β-actin
SNA epitope
SNA/β-actin value
1.5
1.0
0.5
0

Fig. 5


A
B
C
ST6Gal I-KO
+ DsRed mRNA
ST6Gal I-KO
+ ST3Gal IV mRNA
ST6Gal I-KO
+ ST3Gal V mRNA


D
Phenotype ratio
(%)
n=6
n=7
n=5
100
80
60
40
20
0
DsRed
ST3Gal IV
ST3Gal V
Dead
Abnormal
Cardiac abnormality
Normal


E
ST3Gal IV
ST3Gal V
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***
*
0.08
0.06
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0
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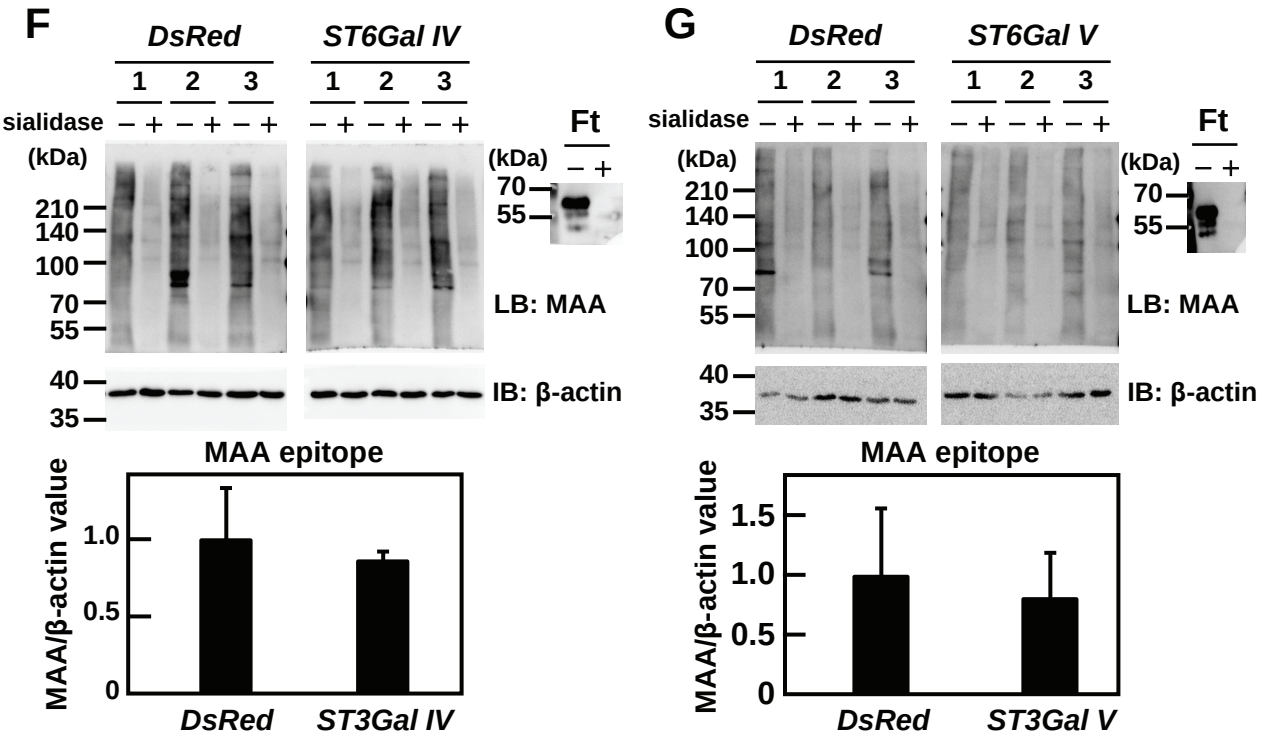
0.0006
0.0004
0.0002
0
DsRed
ST3Gal IV
DsRed
ST3Gal V


F
DsRed
ST6Gal IV
1 2 3 1 2 3
sialidase – + – + – + – + – + – +
(kDa)
210
140
100
70
55
40
35
Ft
(kDa)
70
55
LB: MAA
IB: β-actin
MAA epitope
MAA/β-actin value
1.0
0.5
0
DsRed
ST3Gal IV
G
DsRed
ST6Gal V
1 2 3 1 2 3
sialidase – + – + – + – + – + – +
(kDa)
210
140
100
70
55
40
35
Ft
(kDa)
70
55
LB: MAA
IB: β-actin
MAA epitope
MAA/β-actin value
1.5
1.0
0.5
0
DsRed
ST3Gal V

Conflict of interest

The authors declare no conflicts of interest regarding this manuscript