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Aberrant neurodevelopment caused by GD3 synthase deficiency and its restoration of sea urchin ganglioside: insights from a CRISPR/Cas9-mediated *st8sial* knockout zebrafish

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ABSTRACT: Ganglioside is a sialic acid-containing sphingolipid with multiple biological functions in neurodevelopment and neurodegeneration. The *st8sial*-encoding GD3 synthase (GD3S) is involved in the biosynthesis of gangliosides GD3 and GD2, which are highly expressed in the developmental brain. However, the effect of exogenous ganglioside and endogenous glycosyltransferases GD3S on neurodevelopment still need to be determined due to the lack of *in vivo* ganglioside-deficient model. This study generated *st8sial* knockout zebrafish using CRISPR/Cas9-mediated gene editing. A *st8sial* guide-RNA was designed to target exon 1 in zebrafish and generate the homozygote with a 13-bp frameshift deletion, which caused the loss of GD3S function and disturbed the patterns of gangliosides. The *st8sial* mutant showed delayed development, impaired locomotor behavior, and neurodevelopmental defects represented by increased apoptosis of brain cells and small brain size. Further RNA-Seq analysis revealed that most regulated genes were enriched in axonal and cellular developmental, neurogenesis, and neuroactive ligand-receptor interaction pathways. Notably, the application of exogenous GM4(1S) and GD3 could improve the phenotypes and behavioral activity of *st8sial* knockout zebrafish, as well as restore the abnormal neurodevelopment with the underlying mechanism via upregulating the *nestin*, *bdnf*, *map2*, *islet1*, and *elavl3* gene expressions. This study established a genetic model to elucidate the function of GD3S and exogenous ganglioside intervention in neurodevelopment. It provides the potential for high-throughput bioactive screening for brain nutrition.

Keywords: ganglioside; sea urchin; CRISPR/Cas9; GD3 synthase; neurodevelopment

1. Introduction

Gangliosides, sialic acid-containing sphingolipids, are enriched in the nervous tissues and essential components of biological membranes[1], and abundant in marine-derived foods and animal-derived foods such as meat, fish, egg yolk, and dairy products [2]. Gangliosides has been found play critical role in neurodevelopment, and their expression pattern and content in the brain change consistently throughout

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development [3], which are regulated by the activity and gene expressions of glycosyltransferases. The mutations of these glycosyltransferases usually lead to disordered ganglioside profiles and severe neurological impairment [4]. In earlier developmental stages of murine E14 embryonic stem cells, the ganglioside GD3 and GM3 are predominated, whereas more complex species, particularly GM1, GD1a, GD1b, and GT1b, are the most abundant gangliosides in the mature nervous system [5]. Especially, GD3 is enriched in mouse neural stem cells (NSC) and is present in approximately 80% of these cells *in vitro* [6]. The biosynthesis of GD3 is controlled by GD3-synthase (GD3S), which is highly expressed in the early developmental brain, including the retina, hippocampus, and cerebellum [7]. The knockout of GD3S can disturb the biosynthesis of b-series gangliosides and impaired neurogenesis, probably due to decreased NSC population in mammalian postnatal and adult life [8,9]. Thus, GD3S may be a key target in investigating the role of gangliosides on neurodevelopment.

GD3S is encoded by the *st8sial* gene, also called *siat8a1*, localized on chromosome 12 and composed of five coding exons and spans about 135 kilobases in humans [10]. The expression and activity of GD3S can directly synthesize GD3 by transferring sialic acid residues from CMP-NeuAc to GM3 and link to the biosynthesis of b- and c-series gangliosides [11]. The expression of the *st8sial* gene has been described in human [11], rat [12], and mouse [13]. Zebrafish, a species with high genetic homology to humans [14], have been regarded as a powerful model for studying brain development and behavior due to their high fertility, low cost of rearing, and transparency during larval stages [15]. Fortunately, a homolog of *st8sial* is also found in zebrafish, related to the biosynthesis of the gangliosides during zebrafish development [16,17]. Similarly, disorders of ganglioside biosynthesis, such as the knockdown of sialidase neu, can lead to severe embryonic defects and defective locomotor behavior in zebrafish [18]. CRISPR/Cas9-knockout of sialidase Neu1 (Neu1-KO) showed abnormal phenotypes, including smaller body length and weight than the wild-type fish, slight abnormal embryogenesis with curvature of the vertebra, and no embryonic lethality. The morphological and molecular phenotypes of NEU1-KO fish are rather similar to those detectable in Neu1-KO mice and patients affected by sialidases [19]. However, the elimination of the *st8sial* gene in zebrafish has yet to be achieved. Previous analyses of GD3S have only been conducted in transient knockdown *in vivo* using the morpholino method or in CRISPR knockout tumor cells. The most commonly used morpholino oligonucleotide tool in zebrafish may cause inconsistent phenotypes due to its neurotoxicity or possible genetic compensation responses during gene editing [20]. Therefore, CRISPR/Cas9 knockout of zebrafish *st8sial* urgently needs to be established to understand the function of the GD3S and ganglioside deficiency in biological processes of morphological development neurodevelopment.

In addition, the ganglioside-deficient models are useful tools for verifying the neural efficacy of exogenous gangliosides. The administration of exogenous GD3 and GM1 through intracerebroventricular injection or intranasal infusion promoted neurogenesis and restored functional neurons in Parkinson's disease models [21]. We previously found that dietary supplements of sea urchin gangliosides could promote

the differentiation of neuronal cells and the formation of synapse function in embryonic and early postnatal brain. Especially, sea urchin GM4(1S) exhibited an outstanding effect on promoting neural differentiation [22,23]. These studies have preliminarily found the neurotrophic and neuroprotective functions of exogenous gangliosides, but their efficacy remains controversial. It needs to be verified in ganglioside-deficient models. Various lines of *st8sia1* transgenic mice are generated by the lentiviral-mediated shRNA knockdown or morpholino oligonucleotide-mediated method [24]. Impaired neurogenesis occurs in neuronal cells or GD3S-KO mice, leading to depression-like behaviors [25]. However, these analyses of GD3S were only conducted in transient knockdown *in vivo* using the morpholino method or in CRISPR knockout tumor cellular models. Thus, applying CRISPR/Cas9-mediated *st8sia1* knockout zebrafish would provide studying evidence for the neural functions of exogenous gangliosides.

In the present study, we aimed to investigate the effect of GD3S deficiency and altered ganglioside composition on the zebrafish development caused by the CRISPR/Cas9-knock out *st8sia1* gene. This model was applied to explore the potential effects on the neural efficacy of sea urchin GM4(1S) and GD3. This model will enable a comprehensive study of a mechanistic link between ganglioside and development.

2. Material and methods

2.1 Exogenous gangliosides preparation

GM4(1S), a sulfated ganglioside shown in Fig. S1A, was isolated from sea urchin (*Strongylocentrotus nudus*) using our previous method [23,26]. Briefly, the lyophilized powder of sea urchin ovaries was extracted using the chloroform (C)–methanol (M)–water (W) system. Then, the glycolipid fraction was applied to a DEAE-Sephadex A25 column (GE Healthcare Bio-sciences AB, Sweden) to remove neutral lipids and collect the GLSs fraction. GLSs were further applied to a DEAE-TOYOPEAL 650 column to get GM4(1S), which was identified using the electrospray ionization tandem mass spectrometry (ESI-MS) method of Cong et al. [26]. GD3 ganglioside (purity > 90%, 860060) was purchased from Avanti Polar Lipids (Alabama, USA).

2.2 Zebrafish strains and maintenance

The AB strain zebrafish (*Danio rerio*) were maintained at $(28 \pm 1)^{\circ}\text{C}$ following a standard protocol under a 14:10 light-dark cycle. Embryos were cultured in E3 medium (0.17 mmol/L KCl, 5 mmol/L NaCl, 0.33 mmol/L CaCl_2 , and 0.33 mmol/L MgSO_4). The development of 24 hpf – 7 dpf wild-type (WT), *st8sia1*^{+/+}, and *st8sia1*^{-/-} zebrafish were evaluated in this study. All experimental zebrafish were treated in accordance with the guidelines issued by the Animal Committee of the Ocean University of China (permit number SD2021336).

2.3 CRISPR/Cas9 for *st8sia1* mutants

2.3.1 CRISPR design, synthesis, and microinjections

The *st8sial1* (NM 553210) mutants were generated by CRISPR/Cas9 technology as previously described [27]. A specific guide RNA (gRNA) targeting exon 1 of *st8sial1* was designed using the Crisprscan website (<https://www.crisprscan.org/sequence/>), and the targeting site of *st8sial1* was 5'-GGAGATGTAGCTGGAACCAT-3'. The *st8sial1* single gRNA was cloned into template plasmid and then transcribed with a T7 RNA synthesis kit (New England Biolabs) (*st8sial1*-CRISPR-F: 5'-GAAATTAATACGACTCACTATA-3', *st8sial1*-CRISPR-R: 5'-GGAGATGTAGCTGGAACCAT-3'). Cas9 protein was donated from the lab of Dr. Li [28]. 200 ng/mL Cas9 protein was co-injected with 100 ng/mL *st8sial1*-gRNA into one-cell embryos. The injected embryos were grown in an E3 medium.

2.3.2 Analysis of CRISPR-targeted mutation of *st8sial1*

Genomic DNA was extracted single 48 hpf embryos. The potential CRISPR-induced mutations were identified by PCR amplify, and the primers for the gene targeting site are *st8sial1*-F: 5'-AGCCCTACTCATCCTTTGAGG-3'; *st8sial1*-R: 5'-TCAACCTTGTCTACGTGACCATA-3'. PCR amplification system: PCR Taq Mix, 25 µl; forward and reverse primers, 2.5 µL; Genomic DNA, 10 µL (2 µg). Then, the PCR products were analyzed using Sanger sequencing to determine the knockout efficiency of the target site.

2.3.3 Generation of the *st8sial1*^{-/-} zebrafish mutant line

The injected fish at 1-month post-fertilization (mpf) were anesthetized with 250 mg/L 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222; A5040, Sigma, USA), and their tail fins were cut for DNA extraction. Genomic DNA of injected fish was analyzed using the *st8sial1*-F and *st8sial1*-R primers to identify the F0 founder with frameshift mutations. F0 zebrafish were raised to 3 mpf and then crossed with wild-type (WT) adults to generate F1 progeny. To determine the heritable stability, the F1 embryos were raised to adulthood and genomic DNA was extracted from their tail fins. Then, the genomic DNA was amplified using PCR and the products were performed Sanger sequencing (performed by Sangon Biotech Co., Ltd, Shanghai). Male and female heterozygous F1 (*st8sial1*^{+/-}) with the same mutation site were screened and crossed to generate F2. F2 genotypes were identified using the same method, obtaining the *st8sial1*^{-/-} homozygotes (also named GD3S-KO). Mutant lines, *st8sial1*^{+/-} and *st8sial1*^{-/-} zebrafish were eventually group to investigate the efficacy of ganglioside deficiency.

2.4 Ganglioside analysis

The preparation of zebrafish gangliosides was adjusted to the Svennerholm method [29]. 6 dpf zebrafish were freeze-dried and prepared to 10% tissue homogenate using a freezing grinder. The protein content of each group was determined by the BCA method. Gangliosides were extracted from 1 mg protein of zebrafish homogenate using our previous method [30] and analyzed by high-performance liquid chromatography (HPLC)-mass spectrum (MS)/MS system.

HILIC separations were performed on Agilent 1290 Infinity UPLC system and separated on a Waters ACQUITY UPLC BEH amide column (2.1 mm × 100 mm, 1.7 µm) (Milford, MA, USA) using acetonitrile

as mobile phase A and 5 mmol/L aqueous ammonium acetate as mobile phase B. The flow rate was 0.3 mL/min, and elution conditions were applied with a linear gradient as follows: (0–2) min, 80% A; (2–20) min, 80% A–30% A; (20–25) min, 30% A–90% A; (25–30) min, 80% A. The MS data acquisition was performed by the Q-Exactive (Thermo Fisher Scientific, Rockford, IL, USA) system. For semi-quantitative analysis, d3-GM3 was used as the internal reference standard, and an external standard curve was carried out using the mixed-GLS reference standard (GM1、GD1、GT1、GQ1) according to our previous studies [30,31].

2.5 Exogenous GLSs treatments

Ganglioside GD3 and GM4(1S) were dissolved in water at a stock concentration of 5 mg/mL and then diluted to the intended concentration of 200 µg/mL in an E3 embryo medium. Preliminary experiments have shown that GD3 and GM4(1S) (up to 100 µg/ml and 200 µg/mL) have no embryotoxic effect. Thus, 10 hpf WT and GD3S-KO zebrafish were randomly divided into three groups. The 200 µg/mL GM4(1S) and GD3 were microinjected into embryonic yolk sac cells as following groups: WT, WT + GD3, WT + GM4(1S); GD3S-KO, GD3S-KO + GD3, GD3S-KO+ GM4(1S) (n = 24 embryos per group). All treated zebrafish were cultured for further analysis.

2.6 Morphological analysis

We measured morphological changes of *st8sia1*^{+/+}, *st8sia1*^{+/-}, and *st8sia1*^{-/-} zebrafish to examine the consequences of *st8sia1* deficiency during zebrafish development. Mortality and embryo morphology were monitored and recorded daily in 48 hpf–72 hpf. In each group, 60 embryos (20 embryos in each replicate) were sampled randomly from the three replicates. The mortality (Eq.1) and the rate of deformity and melanin reduction in the eye were calculated at 72 hpf (Eq.2) based on the images obtained from a microscope.

Eq.1 Calculation of mortality:

$$Y = \frac{x_i}{x_0} \times 100\%$$

Y-mortality

x_i -number of dead embryos

x_0 -total number of exposed embryos

Eq.2 Calculation of the rate of deformity and melanin reduction in the eye:

$$Z = \frac{x_j}{x_0} \times 100\%$$

Z-deformity rate

x_j -number of abnormal embryos or melanin reduction in eye

x_0 -total number of exposed embryos

2.7 RNA extraction and RT-qPCR

Total RNA was isolated from experimental zebrafish using Total RNA Kit I (Omega Bio-Tek, USA). 2 µg RNA was transcribed to cDNA using a 5X All-In-One RT MasterMix kit (ABM, Canada). Then, the relative expressions of target genes involved in GD3 synthesis and neurodevelopment were evaluated by PCR

instrument (iCycler iQ5, Bio-Rad, USA). RT-qPCR reaction conditions were as follows: 95°C for 15s, followed by 40 cycles of 95°C for 5s, 60°C for 15s, and 72°C for 35s. Each experiment was replicated three times. The primer sequences designed using NCBI software were shown in Supplementary Table 1, and *gapdh* was used as the internal reference gene. The relative expression of each gene was calculated according to the $2^{-\Delta\Delta C_t}$ method.

2.8 Transcriptome sequencing analysis (RNA-seq)

For transcriptome analysis, three samples (20 embryos of 48 hpf pooled per sample) were collected in the *st8sia1*^{+/+} and *st8sia1*^{-/-} groups. Total RNA was extracted using TRIzol reagent, and its quality was determined using 5300 Bioanalyzer (Agilent). Reverse transcription, library construction, and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China) with the NoveSeq X Plus platform. The data were analyzed online using the I-Sanger Cloud Platform. Differentially expressed genes (DEGs) were divided into up- and down-regulated gene lists with $|\log_2 \text{Fold Change}| \geq 1$ and $\text{FDR} < 0.05$ (DESeq2). The DEGs were used to analyze the functional categories of gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology at Bonferroni-corrected P -value < 0.05 compared with the whole-transcriptome background. Validation and analysis of quantitative RT-qPCR were provided in Supplementary Table 1.

2.9 Reactive oxygen species (ROS) analysis

ROS production was estimated using 2',7'-dichloro-fluorescein diacetate (DCFH-DA; Sigma-Aldrich, D6883). 3 dpf larvae exposed to GD3 and GM4(1S) were placed in 24-well plates and were incubated with 5 $\mu\text{g/mL}$ (0.5% DMSO) of DCFH-DA for 1 hour in the dark. Then, embryos were washed thrice with PBS. 24 larvae (8 larvae in each replicate) were performed in each group. Before observation, zebrafish larvae were narcotized with 0.016% MS-222 (Tricaine methanesulfonate, Sigma) and observed using a fluorescence microscope (Ti2-E, Nikon Corporation, Japan). The fluorescence intensities were analyzed using ImageJ software.

2.10 Locomotion behavior assessment

Spontaneous locomotion activity: To characterize swimming behavior following *st8sia1* knockout, the locomotion of larvae aged at 5 dpf was analyzed according to the method of Park et.al [32]. Individual zebrafish larva was transferred into 24-well plates with fresh E3 medium. During the experiments, blinding was achieved by using new investigators blinded to genotype and experimental group. Larvae were tracked for 5 mins in the Daniovision observation chamber (Noldus, Holland) after an adaptation period of 30 min. Video-tracking of swimming activity and further statistical analysis were performed using the EthoVision™ XT10 software. Behavioral data were shown as total swim distance, average velocity (cm/s), and cumulative duration time (s).

Light/dark tests: To further analyze larval activity in the light/dark test, zebrafish larvae are exposed to alternating light and dark periods, and individuals with a functional visual system should respond by

showing specific swimming patterns. Each larva was recorded for 2 min of light/dark change (1 min of light and 1 min of dark).

2.11 Cell apoptosis

Cell apoptosis was measured using the acridine orange hydrochloride (AO) staining (Solarbio Life Science, Beijing, China). Fifteen embryos per group (*st8sia1*^{+/+}, *st8sia1*^{+/-}, *st8sia1*^{-/-}) at 48 hpf were stained with AO solution diluted to 5 µg/mL using embryo medium under dark condition. After 1 hour, the AO solution was removed, and zebrafish were washed thrice with E3 for 5 min. The region of AO-positive cells in the brain was observed with a fluorescent upright microscope using a FITC light source (ECLIPSE Ti2-E, Nikon Corporation, Japan). The area of the AO-positive signal was measured using Image J software (NIH).

2.12 Histopathological evaluation for brain

H&E staining was performed for histological analysis of brain development. 7 dpf zebrafish in WT and GD3S-KO groups were anesthetized with 0.1% MS-222, euthanized, and immediately fixed in 4% paraformaldehyde. Larvae were embedded in paraffin and then prepared to serial transverse sections. Sections (5-µm thick) were stained with hematoxylin and eosin (H&E) as described [33]. Images were taken by an optical microscope (Nikon, Kanagawa, Japan).

2.13 Statistical analysis

All data were statistically analyzed using GraphPad 7.0 software. The data were expressed as mean ± SD. A two-sample, two-tailed Student's t-test was performed for comparisons between the two groups. The statistical difference among multiple groups was analyzed by one-way ANOVA followed by the post-hoc Tukey's HSD test. *P* value < 0.05 was considered as statistically significant, respectively marked as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns (no significant difference).

3. Results

3.1 CRISPR Cas9-mediated *st8sia1* knockout disturbs the biosynthesis of gangliosides

We aimed to create a homozygous knockout for *st8sia1*^{-/-} using CRISPR/Cas9 to disturb GD3S expression (Fig. S1B). We designed gRNA targeting exon 1 (E1) of *st8sia1* gene and injected the *st8sia1*-gRNA together with Cas9 protein at the one-cell stage of zebrafish (Fig. 1A, Fig. S2A and S2B). Then, we identified the genotype of F0 heterozygotes with a deletion of 13-base pairs (bp). F0 zebrafish were outcrossed with WT zebrafish to generate F1. To guarantee genetic stability, we analyzed the genotypes of the F1 generation. The F1 heterozygote showed a deletion of 13bp, 4bp, and 1bp, as indicated by the onset of double peaks per base pair position near the designed target site (Fig. S2C). These three frameshift mutations were selected for F2 generation. The F2 showed a 13bp deletion in the same location as the F1 generation from 340 to 360 bp, indicating the heritable stability of *st8sia1* knockout (Fig. 1B). This *st8sia1*^{-/-} mutation resulted in the early termination of translation in the CDS region and a frameshift mutation of CDS at ~300bp, which disrupted the functional domains of the GD3S protein (Fig. S2D). These results indicated a transgenic zebrafish line of *st8sia1*^{-/-} mutant (GD3S-KO) was successfully generated.

Further RT-qPCR analysis confirmed that knockout of the *st8sia1* gene resulted in reduced mRNA level (-77% , $P < 0.001$; Fig. 1C). Importantly, this knockout had a significant impact on the ganglioside profile of *st8sia1*^{-/-} zebrafish, as revealed by HPLC-MS/MS analysis (Fig. 1D and 1E). The levels of GD3, GD2, and GD1b in *st8sia1*^{-/-} zebrafish were significantly reduced, while the levels of GM2 and GD1a were significantly elevated compared to WT zebrafish. This suggested that *st8sia1* knockout disturbed the biosynthesis of gangliosides and caused a significant endogenous ganglioside deficiency.

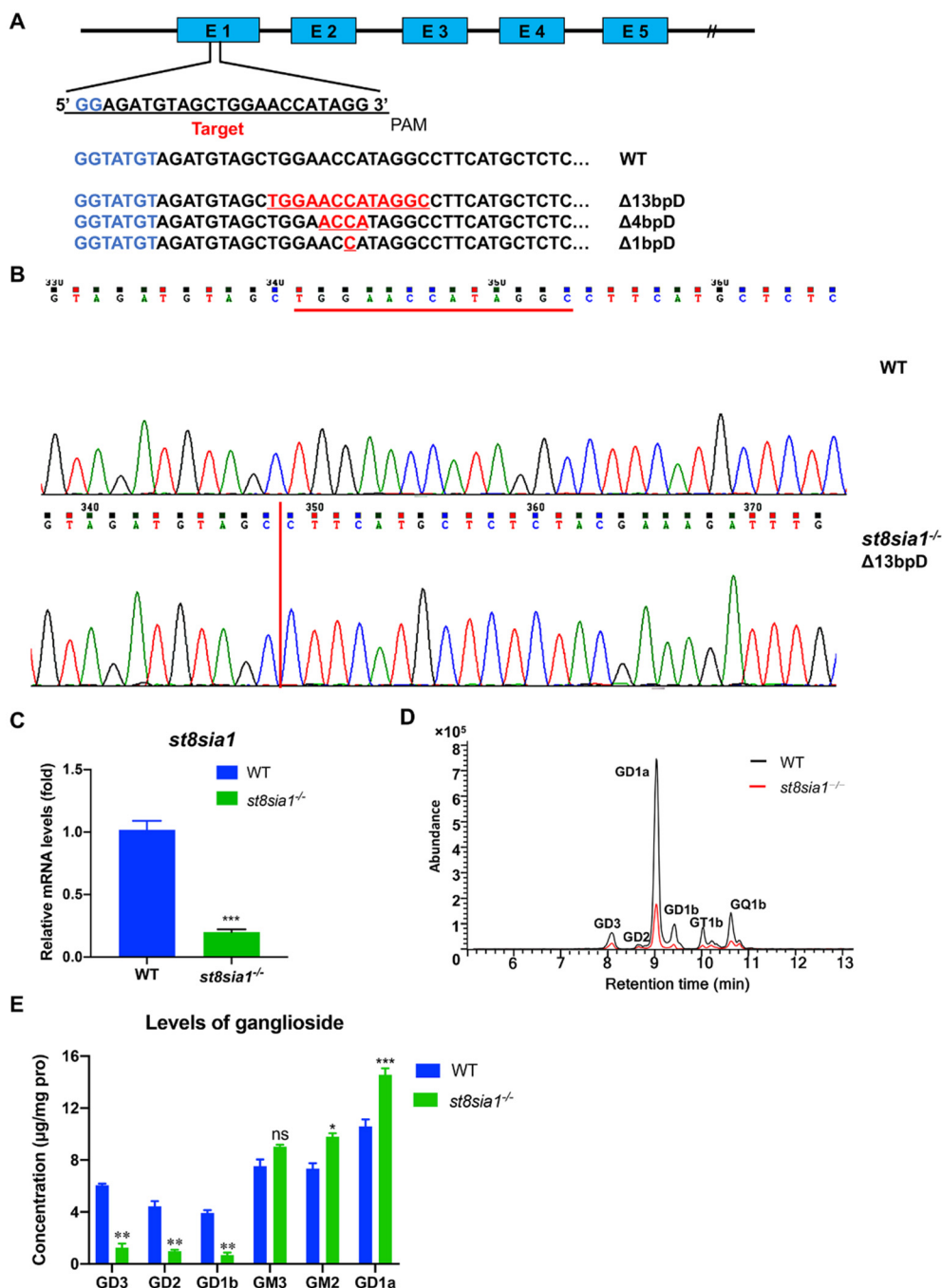


Fig. 1. Generation of *st8sia1* mutation in zebrafish by CRISPR-Cas9 gene editing. (A) Schematic depiction of the target for *st8sia1* gene. The CRISPR target site and protospacer adjacent motif (PAM) sequence are visualized on an excerpt of exon 1 (E1). Three types are shown in annotated *st8sia1* mutant sequences. (B) Exemplary chromatogram results for the frameshift mutations. The target site is highlighted by the red underlining. (C) The *st8sia1* mRNA expression of 5 dpf zebrafish. (D) Extracted ion chromatogram and (E) content analysis of gangliosides in 5 dpf zebrafish from WT and *st8sia1*^{-/-} groups. The ns represent not significance between WT and *st8sia1*^{-/-} zebrafish; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate a significant difference compared to WT zebrafish.

3.2 Ganglioside deficiency affects the morphological development of zebrafish larvae

To investigate the effect of ganglioside deficiency on the embryo's development, we analyzed the morphological development of zebrafish larvae. As shown in Fig. 2A, the 48 hpf WT embryo developed well to the segmentation period, whereas both homozygous and heterozygous embryos generally showed obvious delayed development. The aberrant morphological development was showed as significantly increased death rate (+13%, $P = 0.009$; +7%, $P = 0.01$), retarded development (+11%, $P = 0.01$; +4%, $P = 0.003$), and a reduction of melanin content in the eye (+72%, $P < 0.001$; +36%, $P < 0.001$) in *st8sia1*^{-/-} versus WT zebrafish (Fig. 2C). Especially the ratio of less melanin in the eyes in homozygous embryos was as high as 80%, whereas that of the wild type was only 7.9% ($P < 0.001$, Fig. 2B and 2C). During subsequent development (at 72 hpf), the difference in the general phenotypes gradually became less noticeable ($p = 0.652$; Fig. 2D and 2E). Thus, the *st8sia1* knockout-induced gangliosides deficiency caused developmental delay and significant phenotype differences in zebrafish embryo and larva.

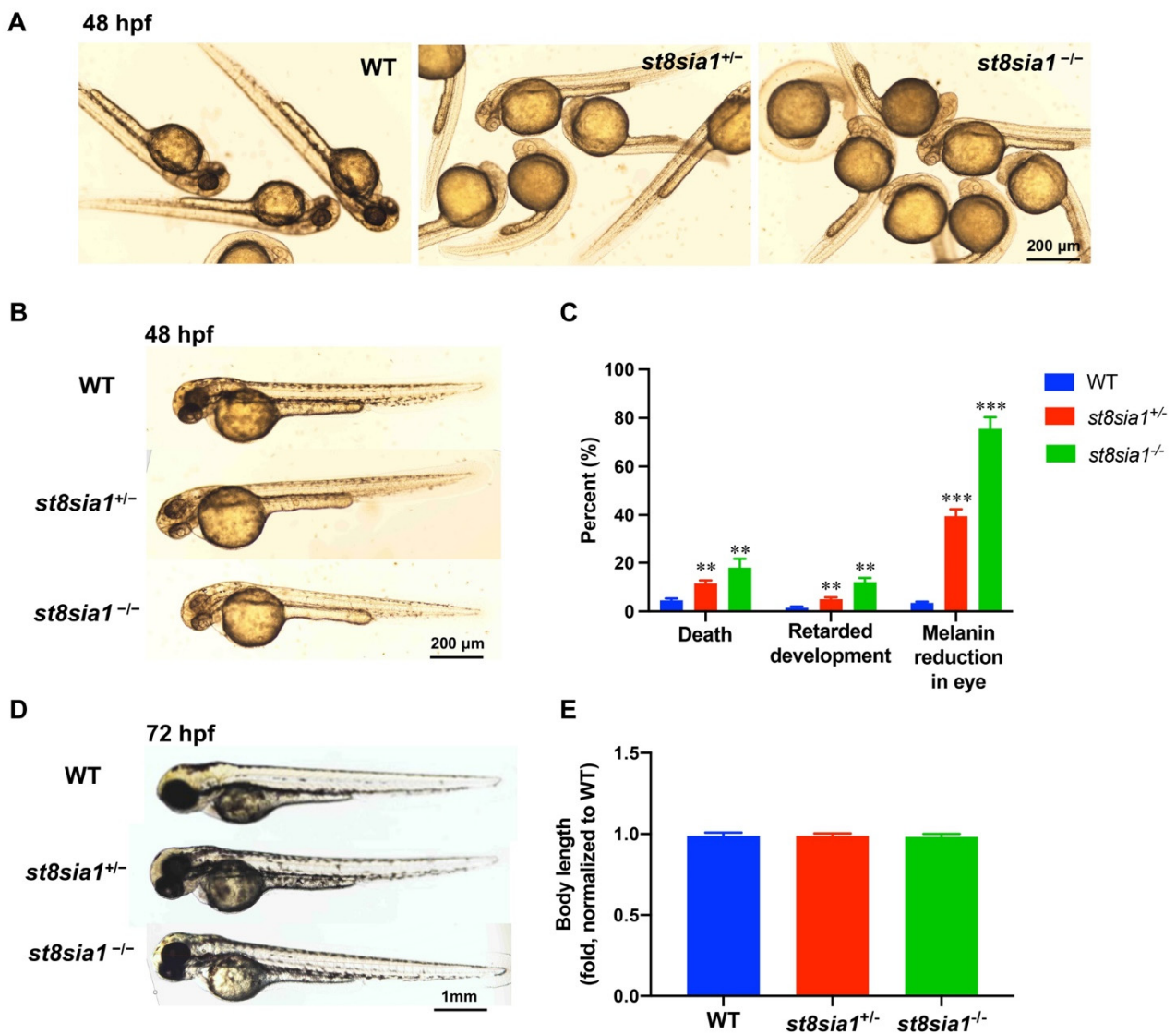


Fig. 2. Morphological development of the wild-type zebrafish and *st8sia1* mutants. (A) Gross morphological development of larvae and (B) single larvae at 48 hpf. Scale bar = 200 μ m. (C) Abnormal morphological analysis of larvae at 48 hpf. (D) Representative developmental images of zebrafish at 72 dpf and (E) their body length quantitative analysis, scale bar = 1mm.

3.3 Ganglioside deficiency impairs locomotor behavior

To determine whether gangliosides deficiency affects the larval behaviors, the spontaneous locomotion of zebrafish upon reaching 5 dpf. As shown in Fig. 3A and 3B, WT zebrafish exhibited regular, spontaneous wall-hugging locomotor behavior, whereas the *st8sia1*^{+/-} heterozygote exhibited rigid turning movements and disoriented movement trajectories. The *st8sia1*^{-/-} homozygote performed significantly worse behavior activity, showing completely irregular movement, repeated small circles around, and reduced swimming distance (-65%, $P < 0.001$) and speed (-65%, $P < 0.001$). We further examined the responses of zebrafish evoked by light changes (light/dark switch). Activity changes were measured as the distance traveled by zebrafish. Under the light stimulation, *st8sia1*^{+/-} and *st8sia1*^{-/-} zebrafish traveled significantly less compared to *st8sia1*^{+/+} zebrafish (Fig. 3C). Light-to-dark transitions elicited a sudden decrease in total distance traveled, whereas *st8sia1*^{-/-} zebrafish showed fewer responses to changes in illumination. Thus, the lack of *st8sia1* impaired the locomotor behavior of zebrafish larvae.

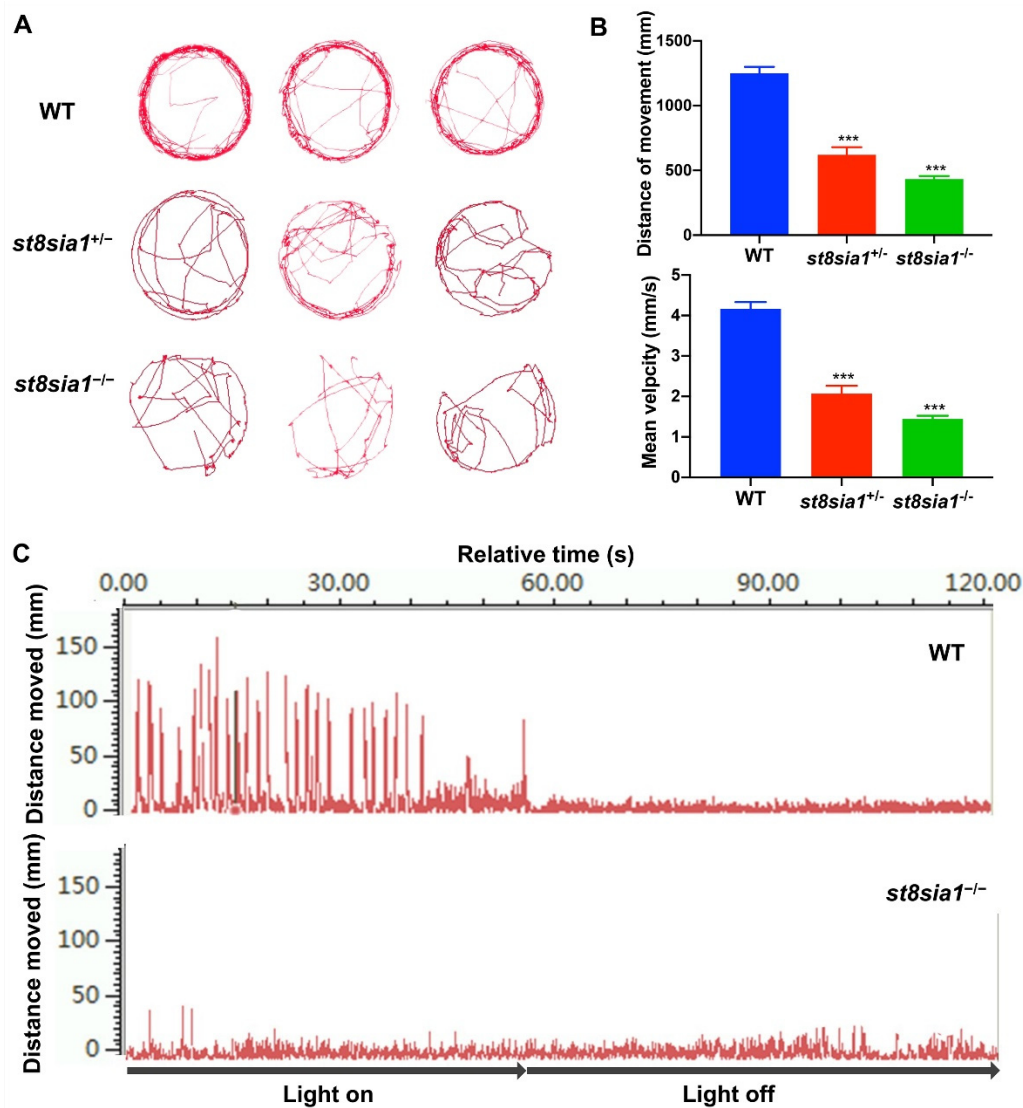
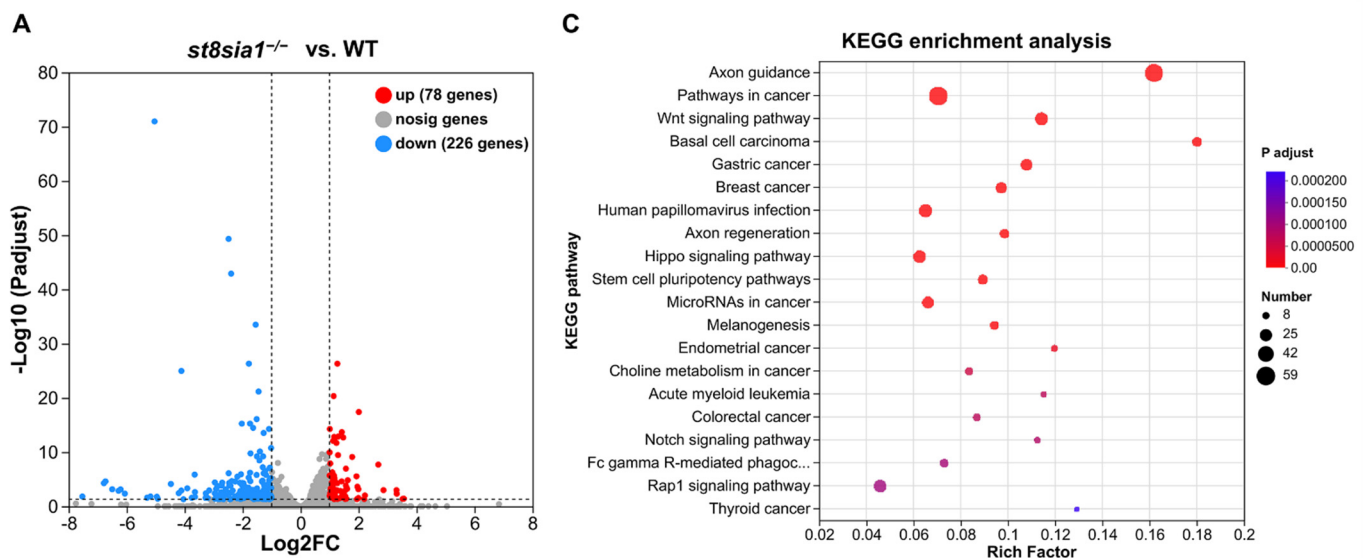


Fig. 3. Changes of locomotor activity in wild-type zebrafish and *st8sia1* mutants. (A) Representative image of spontaneous movement trajectory of WT, *st8sia1*^{+/-}, and *st8sia1*^{-/-} zebrafish larvae at 5 dpf zebrafish. (B) Total distance of movement and the mean velocity of experimental zebrafish. (C) Measurements of the distance swam per 60-second period after light and dark of 5 dpf WT and *st8sia1*^{-/-} zebrafish. $n = 18$ per group. *** $P < 0.001$ indicates a significant difference compared to WT zebrafish.

3.4 Loss function of *GD3S* alters zebrafish transcriptome especially in the neural aspects

In an attempt to determine the involved functional and molecular pathway of *st8sia1* encoded GD3S in zebrafish development, we performed a transcriptomics study using bulk RNA-sequencing in zebrafish embryos of WT and *st8sia1*^{-/-}. Based on 2 selection criteria (fold change > 2 and *P*-adjust < 0.05), 78 upregulated genes and 226 downregulated genes were identified as transcriptionally altered in *st8sia1* knockout zebrafish when compared to their WT siblings (Fig. 4A; Supplementary Table 2). To identify the critical functions affected by *st8sia1* knockout, we mapped all the DEGs to three categories based on GO analysis and classified them into six categories using KEGG functional annotation analysis (Fig. 4B; Fig. S2). These results showed significant enrichment in the cellular, biological, and developmental processes associated with lipid metabolism, signal transduction, cell growth and death, and neurodegenerative disease. Notably, the top 20 KEGG enrichment analysis from DEGs found that axon guidance was noted in addition to the cancer-related pathways (Fig. 4C). The Wnt signaling pathway and axon regeneration were also significantly enriched pathways in the comparison between the WT and *st8sia1*^{-/-} zebrafish. These pathways were highly regulated in the neurodevelopmental process. Thus, we further analyzed the involvement of the *st8sia1* deficiency in zebrafish neurodevelopment.



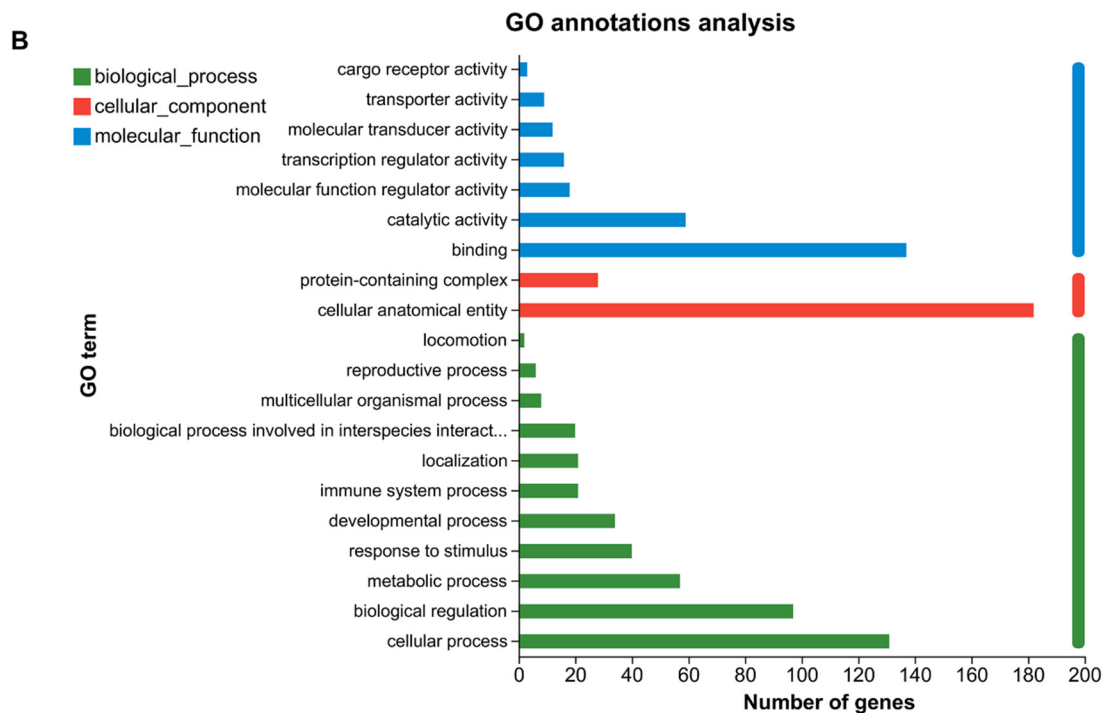


Fig. 4. Analysis of differentially expressed genes in transcriptome between WT and *st8sia1*^{-/-} zebrafish. (A) Volcano plot of the differentially expressed genes (DEGs). (B) Enriched GO terms of biological processes, molecular functions, and cellular components. (C) The ranking of the top 20 KEGG pathway enrichment of DEGs.

3.5 Ganglioside deficiency damages the neurodevelopment of zebrafish larvae

The histopathological analysis of the zebrafish brain was performed to analyze the neurodevelopment of the *st8sia1* mutant. H&E-staining found that 7 dpf *st8sia1*^{+/-} and *st8sia1*^{-/-} zebrafish showed smaller brain size than WT (*st8sia1*^{+/+}) zebrafish (Fig. 5A) and exhibited defective organization in the telencephalon, showing sparse and not tightly arranged granular cells in the brain. Poorly organized retina and underdeveloped eye lens were also found in the *st8sia1* mutant. These damages are more severe in homozygotes than heterozygotes, indicating that gangliosides deficiency significantly impaired brain development. In addition, AO staining showed that significant brain cell apoptosis (increased fluorescence signal of AO) was detected in the brain of ganglioside deficient zebrafish compared to WT zebrafish (-16%, $p = 0.098$; -58%, $P < 0.001$; Fig. 5B and 5C). Moreover, after the transcriptome screening, the differential genes associated with neurodevelopment were further validated by RT-qPCR analysis (Fig. 5D and 5E). We found that significantly differentially expressed genes in neuroactive ligand-receptor (*gpr84* and *nmb*), potassium or sodium ion channel activity (*kcnn4*, *kcna6a*, *slc6a14*), and neuromorphic growth-related genes (*btbd3a*, *smcr8b*, *pik3r3a*) between WT and *st8sia1*^{-/-} zebrafish. These gene expressions are closely associated with neurogenesis, developmental delay, neuroactive signaling, and axon guidance. Therefore, we confirmed that ganglioside deficiency resulted in aberrant development.

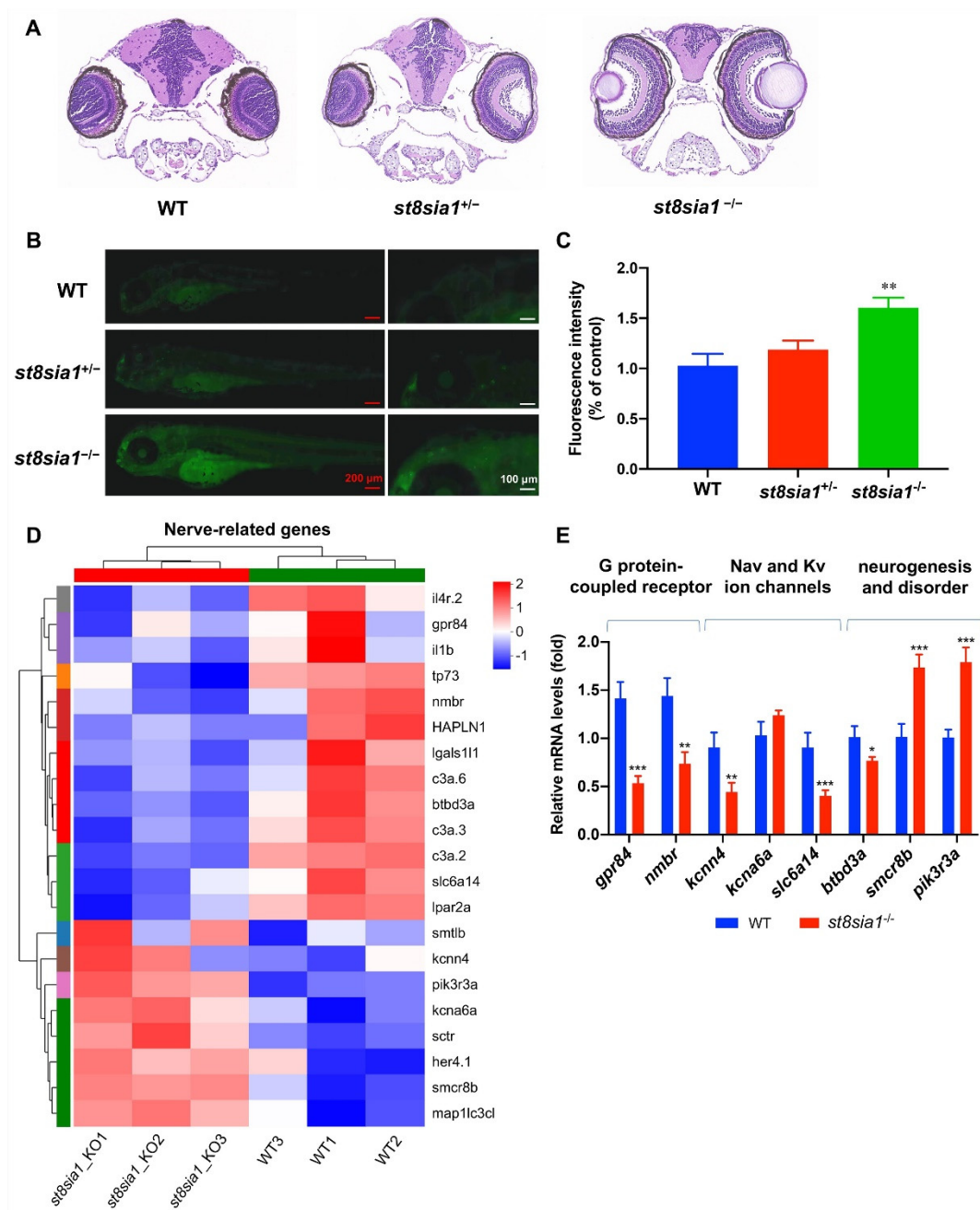


Fig. 5. Gangliosides deficiency altered the neurodevelopment in *st8sia1* mutant zebrafish larvae. (A) H&E-staining for the whole brain of 7 dpf zebrafish. (B) Cell apoptosis was measured using the acridine orange fluorescent dye, scale bar = 200 μ m (left panel) or 100 μ m (right panel). (C) The quantitative analysis of fluorescence signal in the brain region. $n = 15$ per group. (D) Heatmap showing the top upregulated and downregulated neural-related genes based on RNA-Seq analysis in WT vs *st8sia1*^{-/-} group. (E) The expressions of genes related to G protein-coupled receptor, voltage-gated sodium (Nav) and potassium (Kv) ion channels, and neurogenesis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significant differences compared to WT zebrafish.

3.6 Sea urchin ganglioside improves morphological development and locomotor behavior of *st8sia1* knockout zebrafish

We previously found the promoting neural deafferentation functions of sea urchin ganglioside GM4(1S) in wild type cell and animal models. Herein, the *st8sia1* knockout zebrafish was applied to verify the neural function of exogenous ganglioside intervention. Sea urchin GM4(1S) and GD3 could significantly reduce the rate of mortality, the developmental delay, and the melanin reduction in eye of *st8sia1* knockout zebrafish (Fig. 6A). Increased oxidative stress, such as the increased levels of ROS and antioxidant reduced

enzyme activities, always caused neurobehavioral and neurodevelopmental disorders in zebrafish[34]. The *st8sia1* knockout increased the fluorescence of DCFH-DA staining for ROS compared to WT zebrafish. GD3 and GM4(1S) intervention could alleviate ROS accumulation in *st8sia1*^{-/-} zebrafish embryos (-17%, $P = 0.043$ of GD3; -57%, $P < 0.001$ of GM4(1S); Fig. 6B and 6C). Notably, GM4(1S) improved these aspects more effectively than GD3. Moreover, for the *st8sia1*^{-/-} zebrafish larvae, GM4(1S) and GD3 significantly improved their locomotion behavior, although this improvement was not significant in the wild-type groups (Fig. 6D and 6E). These results suggested that exogenous GM4(1S) and GD3 could improve the phenotypes' development and behavioral activity of ganglioside deficient zebrafish.

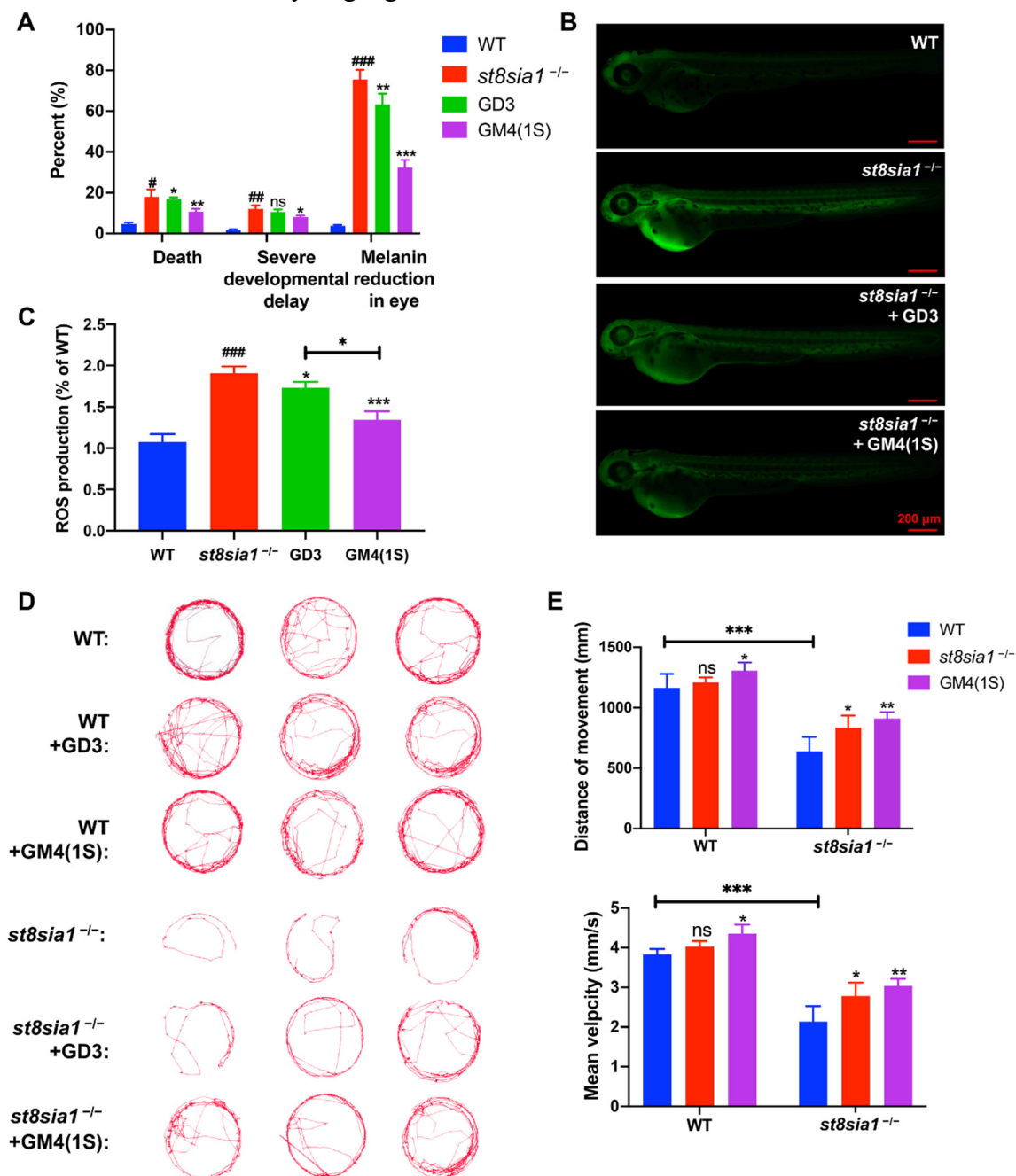


Fig. 6. Effect of exogenous GM4(1S) and GD3 on the phenotypic development and locomotion behavior of WT and *st8sia1*^{-/-} zebrafish. (A) Analysis of the abnormal morphological characteristics in 48 hpf zebrafish (n = 50 per group). (B) Fluorescent images of ROS in zebrafish larvae and (C) its quantitative analysis, scale bar = 200 μm. (n = 15 per group) (D) Representative image of spontaneous movement trajectory. (E) Total distance of movement and the mean velocity of 5dpf zebrafish larvae. (n = 18 per group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate a significant difference compared to the WT group (Blue bar); ns represents no significant difference compared to the WT group.

3.7 Sea urchin ganglioside promotes the neurodevelopment of *st8sia1* knockout zebrafish

To further investigate the neural improvement of sea urchin ganglioside, the brain histopathology was performed in WT and *st8sia1*^{-/-} zebrafish. As shown in Fig. 7A, the poorly organized brain of *st8sia1*^{-/-} zebrafish was alleviated by the intervention of GM4(1S) and GD3, although it did not recover to the normal levels in WT zebrafish. Mechanistically, the expression of genes associated with neurogenesis confirmed the neural function of ganglioside. GM4(1S) treatments led to a more significant up-regulation in the expressions of *nestin*, *bdnf*, *map2*, *elavl3*, and *islet1* in *st8sia1*^{-/-} zebrafish than WT zebrafish (Fig. 7B-F). This improving function of GM4(1S) on neurodevelopment was more potent than that of GD3. These findings verified that sea urchin ganglioside intervention could improve the neurodevelopment of zebrafish embryo and larva by up-regulating the neural gene expression.

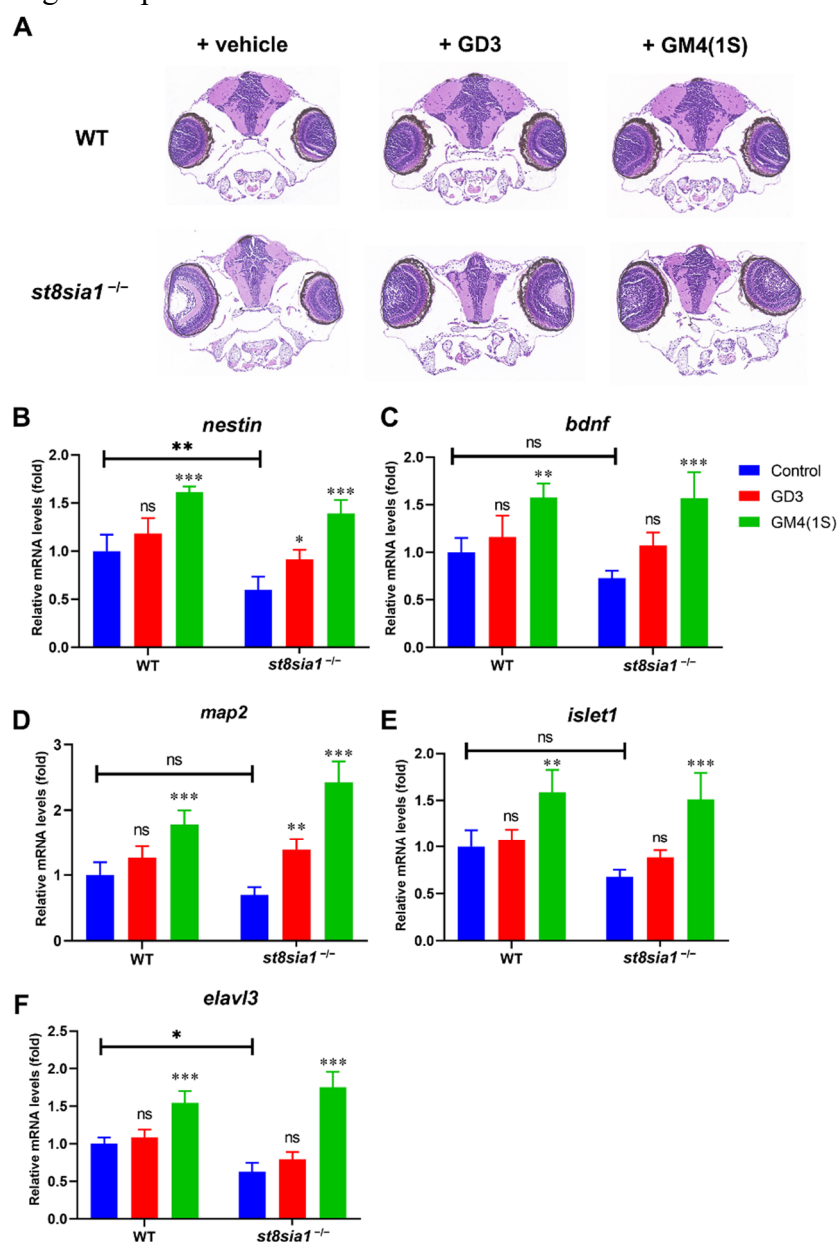


Fig. 7. Effect of exogenous GM4(1S) and GD3 on the neurodevelopment of WT and *st8sia1*^{-/-} zebrafish. (A) H&E staining for the whole brain of 7 dpf zebrafish. Relative mRNA expression of neurodevelopmental genes, including (B) *nestin*, (C) *bdnf*, (D) *map2*, (E) *islet1*, (F) *elavl3*. Four replicates per group (6 larvae per replicate). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate a significant difference compared to the WT group (Blue bar); ns represents no significant difference compared to the WT group.

4. Discussion

Gangliosides exhibit prominent functions in neurogenesis and neurodegeneration, as well as related to the process of malignant phenotypes in cancer [2]. Herein, we generated the *in vivo* CRISPR/Cas9-engineered *st8sial* knockout zebrafish for the first time. This *st8sial* gene deficiency disrupted the GD3S expression and ganglioside biosynthesis, altered zebrafish morphological development, and delayed the neurodevelopment of zebrafish embryo and larva. Additionally, this is the first report to determine the exogenous ganglioside-enhancing neurodevelopment in the ganglioside-deficient model.

Gangliosides are abundant in the brain, including GM1, GD1a, GD1b, GD3, and GT1b, which may be required for neurogenesis [35]. GD3 is mainly expressed in the nervous system of humans. It has primarily been regarded as a marker of neuroprogenitor cells due to its dominant expression in neuronal cells at E12-14 days in mice [36]. GD3S is an ST8SIA1 glycosyltransferase encoded by the *st8sial* gene, controlling the biosynthesis of gangliosides from the b- and c-series [11]. Previous studies have found the critical role of ganglioside GD3 and GD3S expression in the neurodevelopment and progression of neurodegeneration [8,37]. However, these results were not verified in the GD3 deficiency model. Despite the targeted mutation of GD3S in mice has been reported by the morpholino method of homologous recombination [38,39], a CRISPR/Cas9-derived GD3S knockout *in vivo* model has not been successfully established. Until recently, CRISPR/cas9-based *st8sial* gene knockout cell line was generated in human prostate and breast cancer cells rather than neuronal cells [40–42]. Herein, we established the GD3S-deficient zebrafish for the first time, which can be used to investigate the effects of ganglioside deficiency on neurodevelopment *in vivo* and to verify the effects of exogenous ganglioside intervention.

The deficiency of GD3S in *st8sial* knockout mice and cells leads to the altered ganglioside composition and regulated cancer progression [39,43]. We also found the changed ganglioside content, indicated by significantly decreased GD3, GD2, and downstream GD1b in *st8sial*^{-/-} zebrafish compared to WT zebrafish. The upstream ganglioside GM3, GM2, and GD1a levels showed a corresponding increase in *st8sial* mutant. Consistently, the reported data suggest that *st8sial* knockout blocks GD2 expression, and the upstream gangliosides accumulate on the cell surface [40]. Notably, we found that GD3S deficiency showed characteristic morphological abnormalities in the embryonic and larval zebrafish, although the difference in body length gradually decreased with the development between WT and mutants. These abnormal developments are more severe in *st8sial*^{-/-} than in *st8sial*^{+/-} zebrafish, suggesting that *st8sial* knockout caused aberrant morphological development.

Moreover, the behavioral responses of zebrafish larvae are driven by complex neural circuits, including exploratory, locomotor abilities, and sickness behavior [44,45]. The locomotor activity of zebrafish larvae begins in the early stages at 17 hpf, presents coordinated swimming at 24-48 hpf, and expresses more robust behaviors at 96-120 hpf [46]. Herein, a series of behaviors in *st8sial*^{-/-} zebrafish larvae showed damaged reflection, indicated by the regular circling behavior at 120 hpf, a reflexive response to touch. The *st8sial* mutants at the same developmental stage present decreased swimming performance. Additionally, the

visuomotor response is impaired when stimulated by developmental toxicology [47]. The *st8sia1*^{-/-} larvae presented no response in behavioral paradigms when meeting an abrupt transition from light to dark, whereas WT zebrafish larvae showed increased motor activity at light onset and then returned to the baseline level of locomotion at light off. This behavior is due to the diurnal characteristics of zebrafish, always presenting a phototactic behavior and escaping from dark areas [48]. Consistently, the *st8sia1* knockout mice showed increased depression-like behaviors, such as the lack of escape-related behaviors [49]. These behavioral deficits of *st8sia1*^{-/-} zebrafish may related to their neurodevelopmental physiology.

GD3S is required for the self-renewal capacity, outgrowth, and maintenance of neural stem cells by comparing *st8sia1* knockout and WT mice in the postnatal and adult stages [25]. The knockdown of ST3GAL5 (GM3 synthase) also increases neural apoptosis and disturbs the normal embryonic development of zebrafish [50]. We actually found that axon guidance was noted in the top 20 KEGG enrichment by transcriptomics analysis, as well as the poorly organized brain and excessive apoptosis in the brain of *st8sia1*^{-/-} zebrafish. Additionally, the G protein-coupled receptor was downregulated by the GD3S mutant. Its activation played critical roles in synaptic transmission and plasticity [51]. Voltage-gated sodium (Nav) and potassium (Kv) channels are especially crucial in regulating neuronal membrane excitability [52]. GD3S deficiency inhibited the Nav and Kv channel activity and decreased *btbd3a* gene in dendrite morphogenesis. In contrast, the *smcr8b* gene expression was increased, directly related to the spine density in the *smcr8* knockout neurons [53]. These findings confirmed the importance of GD3S and complex gangliosides expressions in normal neural development and function.

In addition, the CRISPR Cas9-mediated *st8sia1* knockout zebrafish was applied to verify the neural effect of exogenous ganglioside intervention. Previously, exogenous gangliosides GD3 and GM1 showed the ability to restore the self-renewal ability of NSC and promote neurogenesis in adult mouse brains [54]. Sea urchin GM4(1S) exhibited an outstanding neurodevelopmental function than mammalian-derived gangliosides in wild-type neuronal cells and postnatal 15-day mice [22,23]. These preliminary findings were worthy of verifying the CRISPR Cas9-mediated *st8sia1*^{-/-} zebrafish model. Exogenous GD3 treatment improved the morphological and brain development of *st8sia1*^{-/-} zebrafish but not WT zebrafish. In line with our results, intracerebroventricular injection of GD3 in adult GD3S-KO mice shows the restoration of the NSC population [55]. Notably, sea urchin GM4(1S) could promote the morphological and neural development of both *st8sia1*^{-/-} and WT zebrafish. These results indicated that GM4(1S) was more effective than GD3 in promoting neurodevelopment, possibly due to their different glycosylated structures [23,56]. Compared to the GD3 structure, GM4(1S) contains the characteristic sulfated residues. The sulfated residues of glycosphingolipids have been found to play an important role in maintaining myelinating axon integrity and myelin stability [57]. However, it remains to be determined whether this structural-selective effect is related to the GM4(1S) metabolism and further synthesis of more complex gangliosides.. Mechanically, sea urchin GM4(1S) was more potent than GD3 in improving neural gene expressions involved in WT and ganglioside-deficient zebrafish, including *nestin*, *bdnf*, and *map2*. This improvement of exogenous

gangliosides is closely related to the survival of neuronal precursor cells, morphological change (head and eyes), and memory consolidation of zebrafish development [58]. GM4(1S) additionally significantly up-regulated *islet1* and *elavl3* expressions, which are required to form zebrafish motoneurons[59]. The *elavl3* deficient mice exhibit dendritic morphology deficits in cortical neurons and slowly progressive motor deficits [60]. These results confirmed the neurodevelopmental effects of sea urchin GM4(1S) intervention in the ganglioside deficient model, primarily by promoting the expression of genes associated with embryonal and larval neurodevelopment.

The present study generated the first *in vivo* CRISPR/Cas9-mediated *st8sia1* mutant, and found that *st8sia1* encoding GD3S is relevant to the synthesis of gangliosides and closely related to neurodevelopment of zebrafish embryo and larva. The ganglioside deficiency damaged zebrafish behavioral activity and neurodevelopment. Notably, exogenous GM4(1S) is more effective than GD3 in improving neurodevelopment, with the upregulating expressions of neural genes. This finding provides clear evidence for the contribution of exogenous ganglioside to neurodevelopment. The versatility of GD3S-KO (*st8sia1*^{-/-}) zebrafish conducts to the study of ganglioside function and the screening of neuroactive substances in the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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