Tamaru et al., J Glycomics Lipidomics 2014, 4:1
DOI: 10.4172/2153-0637.1000111

Journal of Glycomics & Lipidomics

Research Article Open Access

Molecular Characterization of Protein *O*-linked Mannose *β*-1,2-*N*-acetylglucosaminyltransferase 1 in Zebrafish

Yutaka Tamaru[1]*, Eriko Avarban Ban[1], Hiroshi Manya[2], Tamao Endo[2] and Shin'ichi Akiyama[3]

[1]*Department of Life Sciences, Graduate School of Bioresources, Mie University, Japan*
[2]*Molecular Glycobiology, Research Team for Mechanism of Aging, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Japan*
[3]*Department of Nephrology, Graduate School of Medicine, Nagoya University, Japan*

Abstract

Muscular dystrophies are genetic diseases characterized by progressive muscle degeneration and muscular weakening. Defects in glycosylation of α-dystroglycan are responsible for certain congenital muscular dystrophies to be called α-dystroglycanopathies. The structure of glycans in α-dystroglycan is Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr and required for binding basal lamina proteins. The first step of *O*-mannosyl glycan synthesis on α-dystroglycan is catalyzed by protein *O*-mannosyltransferases (POMT1 and POMT2), and detect in POMT1 or POMT2 result in Walker-Warburg syndrome one of the α-dystroglycanopathies. Next step is catalyzed by *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) and it is responsible for muscle-eye-brain disease. We have previously reported that protein *O*-mannosylation is necessary for normal embryonic development in zebrafish and revealed that zebrafish is a useful model for α-dystroglycanopathies. In this study, we focused on zebrafish POMGnT1. Zebrafish *POMGnT1* revealed high level of expression in ovary and ubiquitously throughout early developmental stage as well as zebrafish *POMT1* and *POMT2*. Morpholino experiments of zebrafish *POMGnT1* in juvenile zebrafish showed several phenotypes of bended body, small eyes and edematous pericardium. More importantly, morpholino-injected zebrafish had reduction of the reactivity to the monoclonal antibody IIH6 that recognizes a glycosylated α-dystroglycan. Furthermore, phenotypes observed by knockdown of zebrafish *POMGnT1* were similar to zebrafish *POMT2* rather than zebrafish *POMT1*. Finally, in order to measure POMGnT1 activity, we cloned and expressed zebrafish *POMGnT1* in human embryonic kidney 293T cells. As a result, zebrafish POMGnT1 had the enzymatic activity to transfer GlcNAc from UDP-GlcNAc to *O*-mannosyl peptide, indicating that *O*-mannosylation pathway of α-dystroglycan is conserved in zebrafish.

Keywords: Development; Glycosylation; *O*-mannosyl glycan; POMGnT1; Zebrafish

Abbreviations: α-DG: α-Dystroglycan; CMD: Congenital Muscular Dystrophy; DGC: Dystrophin-Glycoprotein Complex; FKRP: Fukutin-related Protein; GlcNAc: *N*-Acetylglucosamine; hpf: Hour Post-fertilization; MEB disease: Muscle-Eye-Brain disease; MO: Morpholino Oligonucleotide; POMGnT1: Protein *O*-linked Mannose β-1,2-*N*-acetylglucosaminyltransferase 1; POMT: Protein *O*-Mannosyltransferase; WWS: Walker-Warburg Syndrome

Introduction

More than half of all proteins are expected to be glycoproteins [1]. Glycans of glycoproteins contribute to solubility and stability of proteins, proper protein folding, mediating cell adhesion, and so on. Glycosylations of glycoproteins are mainly classified into two types: *N*-glycosylation that the addition of glycans can happen at asparagine residues of Asn-X-Ser/Thr sequon in glycoproteins and *O*-glycosylation that glycans are attached to serine or threonine residues of polypeptides. Among *O*-glycosylations, *O*-mannosyl glycan whose main structure is Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr appears to be present on a limited number of proteins in animals [2,3] and few of these proteins have been identified. α-Dystroglycan (α-DG) is one of the well-known *O*-mannosylated proteins and a key component of the dystrophin-glycoprotein complex (DGC), which is a multimeric transmembrane protein complex and links the cytoskeletal actin to laminin in the extracellular matrix. α-DG is central protein of DGC and binds to basal lamina proteins, such as laminin, neurexin, agrin, and pikachurin [3-8], through its glycans.

In mammals, initiation of protein *O*-mannosylation in α-DG is catalyzed by two homologues, protein *O*-mannosyltransferase 1 (POMT1) and POMT2 transferring a mannosyl residue from dolichol phosphate mannose to Ser/Thr residues of α-DG [9,10]. The second step of *O*-mannosyl glycosylation is mediated by protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) adding *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to *O*-mannose protein [11]. In addition to POMT1, POMT2, and POMGnT1, there are three putative glycosyltransferases, Fukutin and Fukutin-related protein (FKRP) those are supposed to associate with modification of *O*-mannosyl glycans [12-14]. Recently, there has been reported that posttranslational modification of α-DG by LARGE is required for it to function as an extracellular matrix receptor [15] and in the research of congenital muscular dystrophy (CMD) models mouse, LARGE overexpression *in vivo* was effective at restoring functional glycosylation of α-DG and interaction between α-DG and pikachurin [8], and rescuing the muscular dystrophy phenotype in deficiency of not only LARGE but also POMGnT1 [16]. However, the biochemical properties of other two proteins are still unclear. Mutations in these glycosyltransferase genes cause hypoglycosylation of α-DG result in various CMDs accompanied by abnormal neuronal migration, cardiomyopathy, and ocular defects, so-called α-dystroglycanopathies, including Fukuyama-type congenital muscular dystrophy (FCMD) [12], Walker-Warburg syndrome (WWS) [17,18], and muscle-eye-brain disease (MEB) [11]. In addition

***Corresponding author:** Yutaka Tamaru, Department of Life Sciences, Graduate School of Bioresources, Mie University, 1577 Kurimamachiya, Tsu, Mie, 514-8507 Japan, Tel/Fax: +81 59 231 9560; E-mail: ytamaru@bio.mie-u.ac.jp

Received January 31, 2014; **Accepted** March 12, 2014; **Published** March 23, 2014

Citation: Tamaru Y, Ban EA, Manya H, Endo T, Akiyama S, et al. (2014) Molecular Characterization of Protein *O*-Linked Mannose β-1,2-*N*-acetylglucosaminyltransferase 1 in Zebrafish. J Glycomics Lipidomics 4: 111. doi:10.4172/2153-0637.1000111

to these genes, β-1,3-*N*-acetylglucosaminyltransferase 1 (B3GNT1) and β-1,3-*N*-acetylgalactosaminyltransferase 2 (B3GALNT2) orthologues were revealed to cause CMD and to reduce α-DG glycosylation in morpholino knockdown zebrafish [19,20]. In the recent study, receptor protein tyrosine phosphatase ζ (RPTPζ) was identified as another substrate for protein *O*-mannosylation in the brain using # POMGnT1 knockout mice, an animal model of MEB [21]. Relationship between protein *O*-mannosylation pathway and CMD has not been fully characterized and clarified.

POMT1 and *POMT2*, which responsible for WWS, are present in both vertebrates [10,22-24] and invertebrates [25-29], on the other hand, *POMGnT1*, which responsible for MEB, is present in vertebrates but absent in invertebrates such as *Drosophila* [27]. Additionally, only non-elongated *O*-linked mannose has been identified in this species [28,29]. Therefore, the structures of *O*-mannosylglycans of invertebrates are different from vertebrates. The genes involved in glycosylation of dystroglycan are all present in zebrafish [30], suggesting that *O*-mannosylation pathway is not confined to mammals. Therefore, it is suggested that *O*-mannosylglycans are likely to exist in zebrafish.

The experiment using mice for genetic diseases is useful but time consuming and expensive. In relation to this *O*-mannosylation pathway, *POMT1* and *Fukutin* knockout mice result in embryonic lethality [24,31], thereby it seems difficult to investigate the function of *O*-mannosylation of α-DG in muscular dystrophies through this approach. The zebrafish is an attractive model for investigation of genes involved in muscle development and degeneration, especially during early embryogenesis. In addition to the similarity of the muscle fibers in vertebrates, the muscle structure and embryonic development has been well studied in zebrafish. Since basal membrane formation is not crucial during organogenesis in zebrafish, defect of basal membrane does not severely affect early embryonic development as in mice [32]. Zebrafish has orthologues of DGC components and glycosyltransferases involved in *O*-mannosylation of α-DG [30, 33-35]. Using morpholino oligonucleotide (MO) against genes of DGC components and glycosyltransferases, the resulting morphants showed aberrant formation of eye and muscle similar to CMD pathology [22,30,32,36,37]. Besides, zebrafish is especially suited for screening of modified genes on a large scale and assay of potential therapeutic reagent [38]. For example, we succeeded to express human POMGnT1 (hPOMGnT1) protein keeping its enzymatic activity in zebrafish embryo as a part of Research and Development of our innovative gene expression system using zebrafish [39]. Trying to obtain transgenic zebrafish which expressing hPOMGnT1 protein in whole body has turned out well.

We have previously isolated full length of cDNAs encoding two zebrafish POMT orthologues, *zPOMT1* and *zPOMT2*, and showed that they have protein *O*-mannosyltransferase activity only when they are co-expressed in human embryonic kidney 293T (HER 293T) cell [22]. In this study, we cloned full-length cDNA encoding zebrafish *POMGnT1* and showed expression pattern of *zPOMGnT1* mRNA. We also characterized the enzymatic property of *zPOMGnT1*, indicating that protein *O*-mannosylation machinery is conserved in zebrafish, and zebrafish would be an excellent model for elucidation of mechanisms that underlie developmental process, genetic disease and so on.

Materials and Methods

Zebrafish and embryos

Zebrafish adults were maintained at 28°C under light condition of 14 h light period and 10 h dark period. Embryos were collected from pair-wise mating of adult and kept in filter-sterilized fresh water at 28°C.

Quantitative PCR analyses

Total RNA was extracted from embryos at 0, 6, 12, 18, 24, 48, 72 and 96 hpf and the tissue samples (brain, heart, liver, kidney, spleen, intestine, muscle, testis and ovary) of either male or female adult zebrafish. One microgram of total RNA was used for cDNA synthesis. First-strand cDNA was synthesized as described in the section of cDNA cloning and sequencing of *zPOMGnT1*. Quantitative PCR was carried out with SYBR Green Realtime PCR Master Mix (TOYOBO Co. LTD., Osaka, Japan). Two microliter of cDNA (0.1 μg/μl of embryonic stage samples and 0.5 μg/μl of adult tissue samples) was used for a template. The primers used to detect the message of *zPOMGnT1* were 5'-ttacaaggatgagctggaacc-3' (forward) and 5'-ataagagcgggaaacatcagg-3' (reverse). *zβ-actin2* and *zCox1* were used as internal control the sequences of primers for *zβ-actin2* and *zCox1* were 5'-agttcagccatggatgatgaaa-3' (forward) and 5'-accatgacaccctgatgtct-3' (reverse), 5'-ttggccacccagaagtctac3' and 5'-gctcgggtgtctacatccat-3', respectively. Annealing temperatures were 65 °C for *zPOMGnT1*, 52°C for *zβ-actin2* and 54°C for *zCox1*. Melting curves were calibrated by LineGene (NIPPON Genetics Co. LTD., Tokyo, Japan).

Whole-mount *in situ* hybridization

Digoxigenin-labeled RNA probes were synthesized by *in vitro* transcription using T7 or SP6 RNA polymerase. Whole-mount *in situ* hybridization was performed as previously described [22].

Knockdown analysis of *zPOMGnT1*

Antisense morpholino oligonucleotide (MO) targeted to interfere with *zPOMGnT1* translation was purchased from Gene Tools LLC (Philomath, OR). The antisense sequence of *zPOMGnT1* gene was designed using the 50 sequence around the putative start of translation of *zPOMGnT1* mRNA (accession no. AB281277). The MO sequences of *zPOMGnT1* and control were 5'-gtttggtgtccaggtgtccatacct-3' and 5'-gtacgtcacacaatttgacgggcag-3', respectively. MOs at a concentration of 0.25, 0.5 or 1.0 mM were injected into embryos at the one- to two-cell stage.

Immunohistochemistry

Embryos were fixed overnight in 4% paraformaldehyde solution, embedded in paraffin and sectioned at 10 μm and mounted on slides. Sections were left to dry for 2 h. After being dewaxed and rehydrated, some sections were stained with hematoxylin and eosin, while others were subjected to immunohistostaining as described in the report by Mulero et al. in 2007 [40]. Anti-glycosylated α-DG IIH6 (Upstate, Millipore, Billerica, MA) was used at a dilution of 1:100 with PBS containing 0.1% Tween 20 and 5% BSA. Slides were washed with PBS containing 0.1% Tween 20 (PBT) and incubated with secondary antibody for 1 h. The secondary antibody used was Alexa Fluor 488 Goat Anti-Mouse IgM (Molecular Probes Invitrogen Life Technologies Corp., Tokyo, Japan) at a dilution of 1:500 with PBT. The embryos were observed using ECLIPSE E600 and mercury lamp (Nikon Corp., Tokyo, Japan).

Assay for POMGnT1 activity

Full length of cDNA encoding zPOMGnT1 gene was cloned by RT-PCR using zebrafish ovary and primers 5'-ggcttcacaacactcgttacc-3' (forward) and 5'-gtaacacaaggcccattatcagc-3' (reverse). The expression plasmid of zPOMGnT1 fused by histidine-tag was constructed by inserting PCR product into *HindIII* and *XbaI* site of pcDNA3.1 Hygro

(+) vector (Life Technologies Corp., Tokyo, Japan). The primer pairs were 5'- aagcttgccgccaccatgggccaccaccaccaccaccacgacacctggac-3' (forward) and 5'-aaaaaatctagactacatctgttccacaggagg-3' (reverse). The expression plasmid was transfected into HEK293T cells, and the cells were cultured for 3 days. The cells were homogenized in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, with protease inhibitor mixture (3 mg/ml pepstatin A, 1 mg/ml leupeptin, 1 mM benzamidine-HCl, 1 mM PMSF). After centrifugation at 900Xg for 10 min, the supernatant was subjected to ultracentrifugation at 100,000Xg for 1 h. The precipitate was used as the microsomal fraction. Protein concentration was determined by BCA assay (Thermo Fisher Scientific Inc., Waltham, MA, USA).

POMGnT1 activity was obtained from the amount of [^{3}H]GlcNAc transferred to an *O*-mannosyl peptide [10]. The reaction buffer containing 140 mM MES buffer (pH 7.0), 0.5 mM UDP-[^{3}H]GlcNAc (450,000 dpm/nmol), 0.5 mM *O*-mannosyl peptide (Ac-AAPT(Man) PVAAP-NH^2), 10 mM MnCl^2, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol and 50 μg microsomal protein was incubated at 37°C for 2-4 h. After moiling for 3 min, the mixture was analyzed by reversed phase HPLC with a Wakopak 5C18-200 column (4.6×250 mm). Solvent A was 0.1% trifluoroacetic acid in distilled water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1-25% solvent B. The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

Western blot analysis

The microsomal fractions (50 μg) were separated by SDS-PAGE (7.5% gel) and proteins were transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.05% Tween 20, incubated with anti-His (OGHis) antibody (Medical & Biological Lab. Co., Nagoya, Japan), and treated with anti-mouse IgG conjugated with horseradish peroxidase (HRP) (GE Healthcare Bio-sciences Corp., Piscataway, NJ). Proteins that bound to the antibody were visualized with an ECL kit (GE Healthcare Bio-sciences).

Results

Gene expression of *zPOMGnT1*

Quantitative PCR of *zPOMGnT1* was performed with adult tissues and developmental stages. In particular, expression level of *zPOMGnT1* in adult tissues was predominant in ovary. Moreover, gene expression of internal organs in female was higher than those in male (Figure 1A). On the other hand, *zPOMGnT1* was highly expressed at 0 (hpf) and its expression level was decreased gradually at 6 to 18 hpf. At 24 hpf, *zPOMGnT1* expression was then increased and became constant after 48 hpf (Figure 1B). Whole-mount *in situ* hybridization indicated that *zPOMGnT1* mRNA was detected predominantly in eyes and muscles at 24 hpf (Figure 1C). Thus, *zPOMGnT1* transcripts were detected during early developmental stages and ubiquitously expressed throughout embryogenesis.

Knockdown analysis of *zPOMGnT1*

Antisense morpholino oligonucleotide against *zPOMGnT1* was injected into the zebrafish embryos at the one- to two-cell stages, and the morphant was compared with untreated and control MO injected embryos at 96 hpf (Figure 2A). Each embryo was categorized as having normal, moderate or severe phenotypes according to morphological characteristics. There were no significant differences between control morphant and *zPOMGnT1* morphant with normal phenotype, on the other hands, moderate phenotype had no swim bladder, curved tail and small eyes, and severe phenotype showed no swim bladder, twisted tail, and significant reduction in the size of eyes and aberrant pericardium. Quantitative analyses of embryos phenotypes were summarized (Table 1). The frequency of severe phenotype was increased with injection of increasing amounts of *zPOMGnT1* MO. To investigate the glycosylation status of α-DG in *zPOMGnT1* morphants, zebrafish embryos at 96 hpf were immunostained with the anti-glycosylated α-DG antibody IIH6 (Figure 2B). Strong signals by IIH6 antibody were detected in the horizontal and vertical myosepta of both untreated and control MO-injected embryos. However, the reactivity was almost completely lost in *zPOMGnT1* morphants.

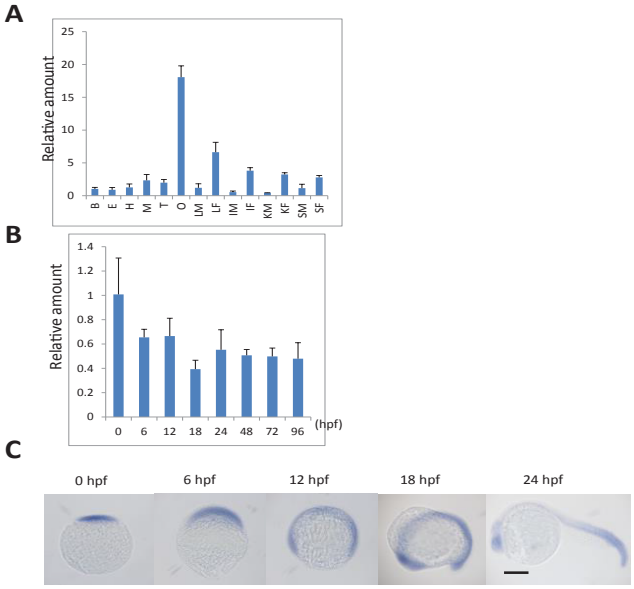

Figure 1: Gene expressions by quantitative PCR analysis and whole-mount *in situ* hybridization for *zPOMGnT1*. (A, B) Quantitative PCR analysis of *zPOMGnT1* mRNA in adult tissues (A) and during early developmental stages (B). *zPOMGnT1* was detected in all tissues predominantly in ovary and throughout early developmental stages. Internal organs in female zebrafish showed higher level of expression than in male ones. *zβ-actin2* and *zCox1* were used as internal controls for quantitative PCR in adult tissues and developmental stages, respectively. B, brain; E, eye; H, heart; M, muscle; T, testis; O, ovary; LM, liver (male); LF, liver (female); IM, intestine (male); IF, intestine (female); KM, kidney (male); KF, kidney (female); SM, spleen (male); SF, spleen (female). All reactions were performed in triplicate, and average values ± SD are shown. (C) Whole-mount *in situ* hybridization for *zPOMGnT1* during early developmental stages. *zPOMGnT1* mRNA was expressed ubiquitously throughout developmental stage. Scale bar = 200 μm.

Enzymatic activity of zPOMGnT1

In order to examine whether zPOMGnT1 had enzymatic activity, microsomal fraction was prepared from HEK293T cells expressing zPOMGnT1. Western blot analysis showed that the recombinant zPOMGnT1 was detected using anti-His antibody in HEK293T cells (Figure 3A). And then microsomal fraction had high level of enzymatic activity by measuring GlcNAc-transfer activities (Figure 3B).

Discussion

α-DG is an important component of DGC and is highly glycosylated to bind to several proteins in the extracellular matrix such as laminin, neurexin, pikachurin, and so on. Six human genes encoding *POMT1*, *POMT2, POMGnT1*, *Fukutin, FKRP,* and *LARGE* are thought to be involved in the *O*-mannosylation process. On the other hand, lack of

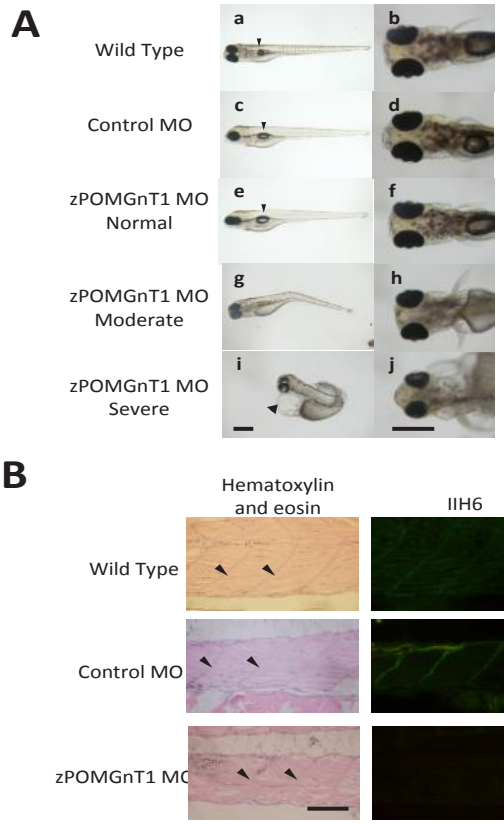

Figure 2: Knockdown analysis of *zPOMGnT1*. (A) Embryos were injected with no sample (a, b), control MO (c, d), and *zPOMGnT1* MO (e-j). In control MO-injected embryos, tail and eye developed normally (c, d). In *zPOMGnT1* MO-injected embryos, normal phenotype showed normally developed tail and eye (e, f), moderate phenotype showed curved tail and small eye (g, h), and severe phenotype showed twisted tail, significant reduction in the size of eye and aberrant pericardium (i, j). Embryos were injected with each MO at the one- to two-cell stages and observed at 96 hpf. Arrowheads and arrow indicate swim bladder and pericardium, respectively. Scale bars = 500 μm. (B) Immunohistochemistry with anti-glycosylated α-DG antibody IIH6 in 96 hpf embryos. Left panels, hematoxylin and eosin staining; right panels, IIH6 staining. IIH6 immunoreactivity was detected in wild type and control morphant but decreased in *zPOMGnT1* morphant. Arrowheads represent vertical myosepta. Scale bar = 50 μm.

	Concentration (mM)	Normal	Moderate	Severe
Wild Type	-	90 (98.9%)	1 (1.1%)	0 (0%)
Control MO	1.00	114 (95.8%)	5 (4.2%)	0 (0%)
zPOMGnT1MO	1.00	38 (58.5%)	1 (1.5%)	26 (40.0%)
	0.50	60 (71.4%)	14 (16.7%)	10 (11.9%)
	0.25	82 (95.3%)	3 (3.5%)	1 (1.2%)

Table 1: Quantification of *zPOMGnT1* morphant phenotypes at 96 hpf. The number of embryos observed for each phenotypic class is shown and also presented as a percentage of the total number of embryos studied for each morpholino. Moderate phenotype showed curved tail and small eyes and severe phenotype showed twisted tail, small eyes and edematous pericardium.

O-mannosylation in α-DG has caused severe CMD once these six genes are mutated. Since zebrafish also has the orthologues of six genes and there are no copies among six genes by gene duplication, furthermore novel causative genes for CMD, B3GNT1 and B3GALNT2, are conserved in zebrafish, this small fish will be a good model and will expect to provide us excellent evidences for muscular dystrophy.

POMGnT1 is a type II transmembrane protein and is localized onto Golgi apparatus, whereas both POMT1 and POMT2 possess multiple transmembrane regions and are localized onto endoplasmic reticulum. So far, several animal models have been used for investigating the molecular, biochemical and developmental pathway that are associated with α-dystroglycanopathies. Although there are no orthologues of *POMGnT1* in *Drosophila* [27], co-expression of *Drosophila POMT1 (dPOMT1)* and *dPOMT2* genes is required for transferase activity *in vitro* and *in vivo* [27,41]. In addition, sugar chain elongated with *O*-linked mannose has not been identified in *Drosophila*. On the other hand, LARGE orthologue, a bifunctional glycosyltransferase with xylosyl- and glucuronyltransferase activity [15], is unevenly present among insect species in the presence of not *Drosophila* but bees [14]. Therefore, human type of *O*-mannosylation in α-DG seems unlikely to be absolutely conserved in invertebrate.

In this study, we demonstrated molecular characterization of zebrafish *POMGnT1 (zPOMGnT1)*. The expression patterns of *zPOMGnT1* in adult tissues and during developmental stages were examined by quantitative RT-PCR and whole-mount *in situ*

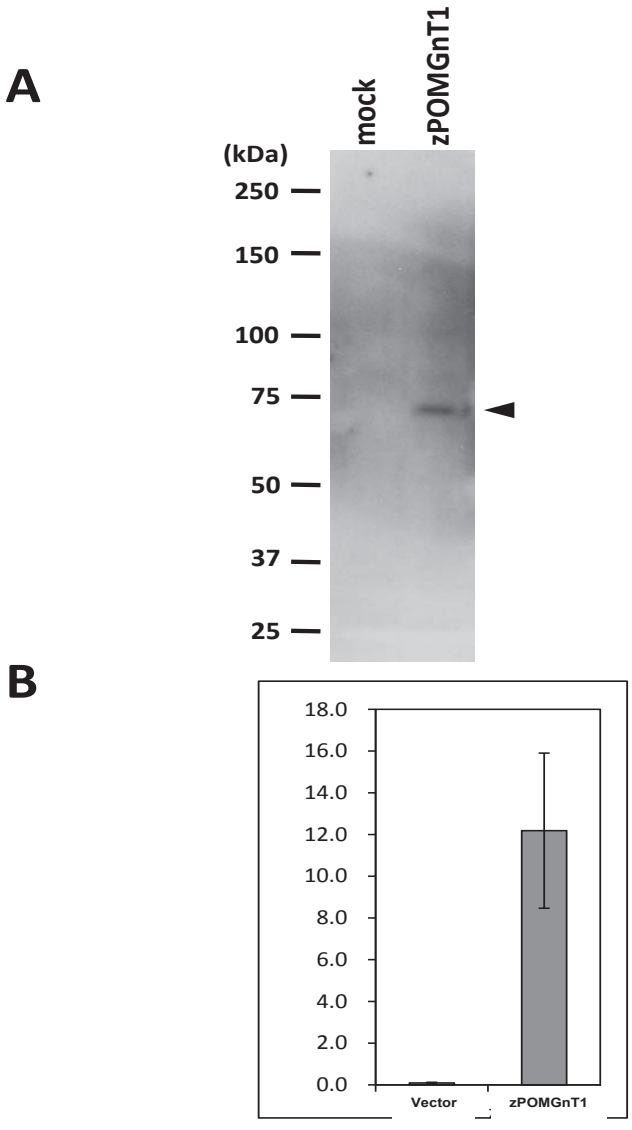

Figure 3: Detection of the recombinant zPOMGnT1 and its enzymatic activity. (A) Western blot analysis of zPOMGnT1 fused to His-tag expressed in HEK293T cells. An arrowhead indicates the expressed zPOMGnT1. (B) Assay of POMGnT1 activity. Specific activity was determined using mannosyl nanopeptide as an acceptor.

hybridization. Among adult tissues, expression level of *zPOMGnT1* was highest in ovary (Figure 1A), whereas expression levels of both *zPOMT1* and *zPOMT2* were highest in ovary [22]. On the other hand, expression levels of *zPOMGnT1* in female tissues were obviously higher than male ones. In case of mammalian *POMTs*, there are two types of *Pomt2* transcripts, somatic *sPomt2* and testis-specific *tPomt2* [42]. Interestingly, expression level of *zPOMT2* mRNA was also high in testis next to ovary, whereas *zPOMGnT1* mRNA in testis was much lower than that in ovary (Figure 1A). The putative promoter region of *zPOMT2* gene contained a putative binding site for transcription factor Tst-1 (Figure S1), which was categorized into class III POU transcription factor and expressed in brain and testis in mammals [43]. The putative promoter region of *zPOMGnT1* gene contained putative binding sites for MyoD and E47 with flanking (Figure S1). MyoD is a master regulator of skeletal muscle development belonging to the family of basic helix-loop-helix (bHLH) myogenic transcriptional factors which induce muscle-specific transcription upon binding to E-box consensus sequences, and acquires high affinity for the muscle E-box upon heterodimerization through the HLH domain with ubiquitous bHLH protein such as E47 so-called E proteins [44]. These results suggest that the function of zPOMGnT1 might be more essential to not only muscle but also female tissues during adulthood. Expression level of *zPOMGnT1* during embryogenesis was gradually decreased from 0 to 18 hpf and was increased at 24 hpf (Figure 1B). Since *zPOMGnT1* mRNA was originally maternal, it seemed that expression pattern of *zPOMGnT1* was completely changed from maternal to somatic mRNA from 18 to 24 hpf. Furthermore, *zPOMGnT1* was expressed during early developmental stages especially in eyes, brain and somite (Figure 1C). In the recent study, the pharyngula stage of vertebrate is most conserved during developmental stages. In other words, gene expression profiles are most highly conserved in vertebrate pharyngula embryos [45]. In addition to highly conserved genes among vertebrates, *zPOMGnT1* was comparatively and constitutively expressed during embryonic developmental stages with fluctuation of expression levels around 18 to 24 hpf. In general, zebrafish embryos at 24 hpf are applicable to the pharyngula stage that achieves characteristic organogenesis such as head, pharyngeal arch, somites and neural tube in vertebrates. To investigate the promoters of the glycosyltransferase genes involved in *O*-mannosylation would reveal fundamental functions of *O*-mannosylglycans or POMT1, POMT2, and POMGnT1 by itself.

zPOMGnT1 morphants revealed variant phenotypes, moderate one had small eyes, bent tail and no swim bladder, and severe one had small eyes, twisted tail, pericardial edema and no swim bladder (Figure 2A), whereas knockdown of *zPOMT1* and *zPOMT2* by MO indicated that phenotypes showed abnormalities in eyes and tails [22]. Ratio of moderate and severe morphants was appeared to shift concentration dependently (Table 1). Therefore, disruption of glycosylation of α-DG was caused by insufficient zPOMGnT1 resulting in pericardial edema at 96 hpf (Figure 2A), suggesting a loss of pronephric osmoregulatory function. In the recent study α-DG was revealed to be highly expressed in the pronephric duct in the recent study [34]. Zebrafish as a freshwater fish has to excrete large amount of water that inflows into body because of its low intracellular osmotic pressure. In addition, pronephros plays an important role in osmoregulatory function and this function is established at around 40 to 48 hpf [46]. Therefore, the water accumulated in the body and might be cause of edema in soft pericardium.

To further elucidate the function of zPOMGnT1, immunohistochemistry with IIH6 antibody was performed to detect the glycosylated α-DG in zebrafish embryos. Knockdown of *zPOMGnT1* by MO indicated there were no signals of glycosylated α-DG in zebrafish embryos at 96 hpf (Figure 2B). These results suggested that functional *O*-mannosylation on α-DG is conserved in zebrafish as well as human. Since the structure of *O*-mannosyl glycans in zebrafish has not been analyzed yet, further studies involved in α-dystroglycanopathies will need to clarify the existence of mammalian type of *O*-mannosylation pathway in zebrafish.

POMGnT1 orthologues are well conserved among teleost including medaka and *Tetraodon*, with highly similarity to human POMGnT1. Furthermore the amino acid residues that have been found in clinical mutants are also well conserved (Figure S2). And biochemical characterization of clinical mutant POMGnT1 has been well studied and the amino acid residues which affect to enzymatic activity were shown [47-55]. The POMGnT1 protein consists of four domains: a cytoplasmic tail (M1-R37), a transmembrane domain (F38-I58), a stem domain (L59-L300) and a catalytic domain (N301-T660) [11]. Teleost POMGnT1 has high similarity to human POMGnT1 in the catalytic domain. And in this study, we revealed that the recombinant zPOMGnT1 showed the enzymatic activity (Figure 3B). Zebrafish POMGnT1 might have the well-conserved three-dimensional protein structure in the substrate-specific region. We had revealed that hPOMGnT1 could be expressed in zebrafish embryo, in the recent study [39], and the transgenic fish has normally grown up implying that genes involved in α-dystroglycanopathies are enable to be replaced by human orthologues and there are potential to produce a genetically humanized zebrafish. R and D of our useful zebrafish expression system is in progress for procuring recombinant protein as well as disclosing biological phenomena and disease mechanisms.

Acknowledgments

This work was supported by grants from the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence and SENTAN, Japan Science and the Technology Agency and the New Energy and Industrial Technology Development Organization (02A09003d), and Grant-in-Aid for Scientific Research (20370053) from the Japan Society for the Promotion of Science, Intramural Research Grant (23-5) for Neurological and Psychiatric Disorders of NCNP, a Comprehensive Research on Disability Health and Welfare from the Ministry of Health (H22-021) and a Grant-in-Aid for Scientific Research on Innovative Areas (No. 23110002, Deciphering sugar chain-based signals regulating integrative neuronal functions) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

References

1. Apweiler R, Hermjakob H, Sharon N (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim Biophys Acta 1473: 4-8.

2. Endo T (1999) O-mannosyl glycans in mammals. Biochim Biophys Acta 1473: 237-246.

3. Ervasti JM, Campbell KP (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J Cell Biol 122: 809-823.

4. Chiba A, Matsumura K, Yamada H, Inazu T, Shimizu T, et al. (1997) Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve alpha-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. J Biol Chem 272: 2156-2162.

5. Sugita S, Saito F, Tang J, Satz J, Campbell K, et al. (2001) A stoichiometric complex of neurexins and dystroglycan in brain. J Cell Biol 154: 435-445.

6. Sugiyama J, Bowen DC, Hall ZW (1994) Dystroglycan binds nerve and muscle agrin. Neuron 13: 103-115.

7. Talts JF, Andac Z, Göhring W, Brancaccio A, Timpl R (1999) Binding of the G domains of laminin alpha1 and alpha2 chains and perlecan to heparin, sulfatides, alpha-dystroglycan and several extracellular matrix proteins. EMBO J 18: 863-870.

8. Hu H, Li J, Zhang Z, Yu M (2011) Pikachurin interaction with dystroglycan

is diminished by defective O-mannosyl glycosylation in congenital muscular dystrophy models and rescued by LARGE overexpression. Neurosci Lett 489: 10-15.

9. Manya H, Chiba A, Yoshida A, Wang X, Chiba Y, et al. (2004) Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. Proc Natl Acad Sci U S A 101: 500-505.
10. Manya H, Chiba A, Margolis RU, Endo T (2006) Molecular cloning and characterization of rat Pomt1 and Pomt2. Glycobiology 16: 863-873.
11. Yoshida A, Kobayashi K, Manya H, Taniguchi K, Kano H, et al. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev Cell 1: 717-724.
12. Kobayashi K, Nakahori Y, Miyake M, Matsumura K, Kondo-Iida E, et al. (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature 394: 388-392.
13. Brockington M, Yuva Y, Prandini P, Brown SC, Torelli S, et al. (2001) Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. Hum Mol Genet 10: 2851-2859.
14. Grewal PK, McLaughlan JM, Moore CJ, Browning CA, Hewitt JE (2005) Characterization of the LARGE family of putative glycosyltransferases associated with dystroglycanopathies. Glycobiology 15: 912-923.
15. Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, et al. (2012) Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. Science 335: 93-96.
16. Yu M, He Y, Wang K, Zhang P, Zhang S, et al. (2013) Adeno-associated viral-mediated LARGE gene therapy rescues the muscular dystrophic phenotype in mouse models of dystroglycanopathy. Hum Gene Ther 24: 317-330.
17. Beltrán-Valero de Bernabé D, Currier S, Steinbrecher A, Celli J, van Beusekom E, et al. (2002) Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. Am J Hum Genet 71: 1033-1043.
18. van Reeuwijk J, Janssen M, van den Elzen C, Beltran-Valero de Bernabé D, Sabatelli P, et al. (2005) POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. J Med Genet 42: 907-912.
19. Buysse K, Riemersma M, Powell G, van Reeuwijk J, Chitayat D, et al. (2013) Missense mutations in β-1,3-N-acetylglucosaminyltransferase 1 (B3GNT1) cause Walker-Warburg syndrome. Hum Mol Genet 22: 1746-1754.
20. Stevens E, Carss KJ, Cirak S, Foley AR, Torelli S, et al. (2013) Mutations in B3GALNT2 cause congenital muscular dystrophy and hypoglycosylation of α-dystroglycan. Am J Hum Genet 92: 354-365.
21. Dwyer CA, Baker E, Hu H, Matthews RT (2012) RPTPζ/phosphacan is abnormally glycosylated in a model of muscle-eye-brain disease lacking functional POMGnT1. Neuroscience 220: 47-61.
22. Avsar-Ban E, Ishikawa H, Manya H, Watanabe M, Akiyama S, et al. (2010) Protein O-mannosylation is necessary for normal embryonic development in zebrafish. Glycobiology 20: 1089-1102.
23. Willer T, Amselgruber W, Deutzmann R, Strahl S (2002) Characterization of POMT2, a novel member of the PMT protein O-mannosyltransferase family specifically localized to the acrosome of mammalian spermatids. Glycobiology 12: 771-783.
24. Willer T, Prados B, Falcón-Pérez JM, Renner-Müller I, Przemeck GK, et al. (2004) Targeted disruption of the Walker-Warburg syndrome gene Pomt1 in mouse results in embryonic lethality. Proc Natl Acad Sci U S A 101: 14126-14131.
25. Jurado LA, Coloma A, Cruces J (1999) Identification of a human homolog of the Drosophila rotated abdomen gene (POMT1) encoding a putative protein O-mannosyl-transferase, and assignment to human chromosome 9q34.1. Genomics 58: 171-180.
26. Martín-Blanco E, García-Bellido A (1996) Mutations in the rotated abdomen locus affect muscle development and reveal an intrinsic asymmetry in Drosophila. Proc Natl Acad Sci U S A 93: 6048-6052.
27. Ichimiya T, Manya H, Ohmae Y, Yoshida H, Takahashi K, et al. (2004) The twisted abdomen phenotype of Drosophila POMT1 and POMT2 mutants coincides with their heterophilic protein O-mannosyltransferase activity. J Biol Chem 279: 42638-42647.
28. Aoki K, Porterfield M, Lee SS, Dong B, Nguyen K, et al. (2008) The diversity of O-linked glycans expressed during Drosophila melanogaster development reflects stage- and tissue-specific requirements for cell signaling. J Biol Chem 283: 30385-30400.
29. Nakamura N, Stalnaker SH, Lyalin D, Lavrova O, Wells L, et al. (2010) Drosophila Dystroglycan is a target of O-mannosyltransferase activity of two protein O-mannosyltransferases, Rotated Abdomen and Twisted. Glycobiology 20: 381-394.
30. Moore CJ, Goh HT, Hewitt JE (2008) Genes required for functional glycosylation of dystroglycan are conserved in zebrafish. Genomics 92: 159-167.
31. Kurahashi H, Taniguchi M, Meno C, Taniguchi Y, Takeda S, et al. (2005) Basement membrane fragility underlies embryonic lethality in fukutin-null mice. Neurobiol Dis 19: 208-217.
32. Bassett DI, Currie PD (2003) The zebrafish as a model for muscular dystrophy and congenital myopathy. Hum Mol Genet 12 Spec No 2: R265-270.
33. Steffen LS, Guyon JR, Vogel ED, Beltre R, Pusack TJ, et al. (2007) Zebrafish orthologs of human muscular dystrophy genes. BMC Genomics 8: 79.
34. Guyon JR, Mosley AN, Zhou Y, O'Brien KF, Sheng X, et al. (2003) The dystrophin associated protein complex in zebrafish. Hum Mol Genet 12: 601-615.
35. Bassett DI, Bryson-Richardson RJ, Daggett DF, Gautier P, Keenan DG, et al. (2003) Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo. Development 130: 5851-5860.
36. Parsons MJ, Campos I, Hirst EM, Stemple DL (2002) Removal of dystroglycan causes severe muscular dystrophy in zebrafish embryos. Development 129: 3505-3512.
37. Thornhill P, Bassett D, Lochmüller H, Bushby K, Straub V (2008) Developmental defects in a zebrafish model for muscular dystrophies associated with the loss of fukutin-related protein (FKRP). Brain 131: 1551-1561.
38. Guyon JR, Steffen LS, Howell MH, Pusack TJ, Lawrence C, et al. (2007) Modeling human muscle disease in zebrafish. Biochim Biophys Acta 1772: 205-215.
39. Avşar-Ban E, Ishikawa H, Akiyama S, Manya H, Endo T, et al. (2012) Functional and heterologous expression of human protein O-linked mannose β-1,2-N-acetylglucosaminyltransferase 1 in zebrafish. J Biosci Bioeng 114: 237-239.
40. Mulero I, Sepulcre MP, Meseguer J, García-Ayala A, Mulero V (2007) Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response. Proc Natl Acad Sci U S A 104: 19434-19439.
41. Wairkar YP, Fradkin LG, Noordermeer JN, DiAntonio A (2008) Synaptic defects in a Drosophila model of congenital muscular dystrophy. J Neurosci 28: 3781-3789.
42. Balci B, Morris-Rosendahl DJ, Celebi A, Talim B, Topaloglu H, et al. (2007) Prenatal diagnosis of muscle-eye-brain disease. Prenat Diagn 27: 51-54.
43. Lommel M, Willer T, Strahl S (2008) POMT2, a key enzyme in Walker-Warburg syndrome: somatic sPOMT2, but not testis-specific tPOMT2, is crucial for mannosyltransferase activity in vivo. Glycobiology 18: 615-625.
44. He X, Treacy MN, Simmons DM, Ingraham HA, Swanson LW, et al. (1989) Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature 340: 35-41.
45. Blackwell TK, Weintraub H (1990) Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250: 1104-1110.
46. Irie N, Kuratani S (2011) Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis. Nat Commun 2: 248.
47. Drummond IA, Majumdar A, Hentschel H, Elger M, Solnica-Krezel L, et al. (1998) Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function. Development 125: 4655-4667.
48. Biancheri R, Bertini E, Falace A, Pedemonte M, Rossi A, et al. (2006) POMGnT1 mutations in congenital muscular dystrophy: genotype-phenotype correlation and expanded clinical spectrum. Arch Neurol 63: 1491-1495.
49. Taniguchi K, Kobayashi K, Saito K, Yamanouchi H, Ohnuma A, et al. (2003) Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease. Hum Mol Genet 12: 527-534.

50. Vervoort VS, Holden KR, Ukadike KC, Collins JS, Saul RA, et al. (2004) POMGnT1 gene alterations in a family with neurological abnormalities. Ann Neurol 56: 143-148.

51. Shenoy AM, Markowitz JA, Bonnemann CG, Krishnamoorthy K, Bossler AD, et al. (2010) Muscle-Eye-Brain disease. J Clin Neuromuscul Dis 11: 124-126.

52. Diesen C, Saarinen A, Pihko H, Rosenlew C, Cormand B, et al. (2004) POMGnT1 mutation and phenotypic spectrum in muscle-eye-brain disease. J Med Genet 41: e115.

53. Godfrey C, Clement E, Mein R, Brockington M, Smith J, et al. (2007) Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. Brain 130: 2725-2735.

54. Hehr U, Uyanik G, Gross C, Walter MC, Bohring A, et al. (2007) Novel POMGnT1 mutations define broader phenotypic spectrum of muscle-eye-brain disease. Neurogenetics 8: 279-288.

55. Balci B, Morris-Rosendahl DJ, Celebi A, Talim B, Topaloglu H, et al. (2007) Prenatal diagnosis of muscle-eye-brain disease. Prenat Diagn 27: 51-54.

56. Topaloğlu H, Cila A, Taşdemir AH, Saatçi I (1995) Congenital muscular dystrophy with eye and brain involvement. The Turkish experience in two cases. Brain Dev 17: 271-275.