at −80°C. The embryos were rehydrated in PBS, and hybridization of a DIG-labeled RNA probe was carried out in a hybridization buffer containing 50% formamide, 25% 20×SSC, 1% Tween20, 9 mM citric acid and 1 mg/ml heparin at 65°C for 16 h. Embryos hybridized with a DIG-labeled RNA were incubated with anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) (1:2000) at 4°C for 12 h, stained with BM purple (Roche Diagnostics) at room temperature for 2 h and fixed with 4% PFA-PBS. The embryos were observed using ECLIPSE E600 (Nikon Corp., Tokyo, Japan).

Localization of 3′UTRs in zPOMT1 and zPOMT2 genes

The *EGFP* gene was cloned into pSP72 vector (Promega, Madison, WI). The3′UTRs of *zPOMT1* and *zPOMT2* were fused separately downstream of *EGFP* gene. The 3′UTR of zebrafish *NUDT2* was used as a control. Capped mRNAs of *EGFP-zPOMTs-3′UTR* were synthesized using mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (SP6, Ambion Inc., Austin, TX). Capped mRNAs at a concentration of 0.05 μg/μL were injected into fertilized eggs from one to two cells. The embryos were observed until 24 hpf using ECLIPSE E600 and a mercury lamp (Nikon).

Knockdown analysis of zPOMT1 and zPOMT2

Antisense MOs targeted to interfere with *zPOMT1* and *zPOMT2* translation were purchased from Gene Tools LLC (Philomath, OR). The antisense sequences of *zPOMT1* and *zPOMT2* genes were designed using the 50 sequence around the putative start of translation of *zPOMT1* and *zPOMT2* mRNA (accession nos. AB281275 and AB281276). The morpholino sequences were *zPOMT1*-MO: 5′-gacgggcagtttaacacactgcatg-3′ and *zPOMT2*-MO: 5′-gtccattcttgaagatgaagaggac-3′. The sequence of control MO was 5′-gtacgtcacacaatttgacgggcag-3′. 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

For 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 (Mulero et al. 2007). Anti-glycosylated α-DG IIH6 (Upstate, Millipore, Billerica, MA) was used at a dilution of 1:100 with PBS. Slides were washed 3× 10 min with PBS containing 0.1% Tween (PBSTw) and incubated with secondary antibody for 1 h. The secondary antibody used was Alexa Fluor 488 Goat Anti-Mouse IgM (Molecular Probes Invitrogen Life Technologoies Corp., Tokyo, Japan) at a dilution of 1:500 with PBS. Embryos were fixed in 4% PFA-PBS and transferred into 100% methanol. The embryos were rehydrated in PBS containing 0.1% Tween-20 (PBT) and incubated in PBT containing anti-glycosylated α-DG antibody (IIH6) overnight at 4°C followed by several washes with PBT and incubation with secondary antibody (goat anti-mouse IgM AlexaFluor-488,). The embryos were observed using ECLIPSE E600 and a mercury lamp (Nikon).

Assay for protein O-mannosyltransferase activity

Expression plasmids of zPOMT1 and zPOMT2 were constructed using pcDNA3.1 Hygro (+) vector (Life Technologoies Corp., Tokyo, Japan) and confirmed by the sequencing. The expression plasmids were transfected into HEK293T cells, and the cells were cultured for 3 days in complete medium, harvested, and homogenized. Protein *O*-mannosyltransferase activity was based on the amount of [^{3}H]-mannose transferred from [^{3}H]-mannosylphosphoryldolichol to a glutathione-S-transferase fusion α-DG (GST-α-DG) as described previously (Manya et al. 2004). Approximately 80 μg of microsomal membrane fractions were collected from HEK293T cells coexpressing combinations of *POMT1* and/or *POMT2* genes from either human or zebrafish, suspended in a 20-μL reaction buffer containing 10 μg of GST-α-DG. The reaction mixture was incubated at 22°C for 1 h, and GST-α-DG was purified using glutathione-Sepharose 4B beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The radioactivity adsorbed to the beads was measured by using liquid scintillation counter.

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The nucleotide sequences in this paper have been submitted to the Genbank/EMBL/DDBJ Nucleotide Sequence Database. The accession numbers AB281275 and AB281276 have been assigned to the cDNA sequences of zebrafish POMT1 and POMT2, respectively.

Abbreviations

α-DG, α-dystroglycan; DGC, dystrophin–glycoprotein complex; DIG, Digoxigenin; *EGFP, enhanced green fluorescent protein*; FKRP, fukutin-related protein; HEK293T cells, human embryonic kidney 293T cells; MO, morpholino oligonucleotide; ORF, open reading frame; PBS phosphate-buffered saline; PBT PBS containing 0.1% Tween-20; PFA paraformaldehyde; POMGnT1, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase1; POMT, PMT, protein *O*-mannosyltransferase; RT-PCR, reverse transcriptase-polymease chain reaction; SD, standard deviation; WWS, Walker–Warburg syndrome.

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