厚生労働科学研究費補助金

障害者対策総合研究事業（神経・筋疾患分野）

中枢神経症状を伴う筋疾患α-ジストログリカノパチーの分子病態と治療法開発に関する研究

平成２２年度　総括研究報告書

研究代表者　萬谷　博

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研究代表者 萬谷　博、 地方独立行政法人東京都健康長寿医療センター、研究員

研究要旨　　遺伝性の神経疾患や筋疾患は進行性で極めて難治性であり、その代表的疾患として筋ジストロフィーがある。筋ジストロフィーは一般的に単一遺伝子の異常によるもので、これまでに多くの原因遺伝子が発見され、発症メカニズムの解明や治療法開発に期待が寄せられている。我々はこれまでに、先天性筋ジストロフィーに分類される muscle-eye-brain 病（MEB）と Walker-Warburg syndrome（WWS）の原因遺伝子産物 POMGnT1 と POMT1、POMT2 が *O*-マンノース型糖鎖を合成する糖転移酵素あることを示し、MEB と WWS が *O*-マンノース型糖鎖不全を起因とする疾患であることを明らかにしている。これらの知見は *O*-マンノース型糖鎖が神経や筋組織の正常な発生や機能に重要な役割を果たしていること示している。本年度は筋ジストロフィーモデルとしてのゼブラフィッシュの可能性を検討するため、ゼブラフィッシュの *O*-マンノース転移酵素 zPOMT1、zPOMT2 を同定し、ヒトと同様の複合体形成による活性発現機構を明らかにした。また、POMGnT1 の基質特異性について検討した。

A. 研究目的

福山型先天性筋ジストロフィー症（FCMD）、Muscle-eye-brain 病（MEB）、Walker-Warburg 症候群（WWS）は中枢神経系の障害を伴う先天性筋ジストロフィー症である。これらの疾患はジストロフィン糖蛋白質複合体の構成分子であるα-ジストログリカンの *O*-マンノース型糖鎖不全を起因とし、α-ジストログリカノパチーと総称される。本研究では、α-ジストログリカノパチーの原因遺伝子産物及び *O*-マンノース型糖鎖の機能を解明することで、病態解明から診断・治療法への応用を目指している。

我々はこれまでに、MEB と WWS の原因遺伝子産物 Protein *O*-linked mannose β1,2-*N*-acetyl glucosaminyltransferase 1（POMGnT1）と protein *O*-mannosyltransferase 1 (POMT1)及び POMT2 が *O*-マンノース型糖鎖（Siaα2-3Galβ1-4 GlcNAcβ1-2Man-Thr/Ser）の生合成に関わる糖転移酵素であることを明らかにしている。

O-マンノース転移酵素（POMT）は蛋白質の Ser/Thr にマンノースを転移する酵素であり、酵素活性の発現に POMT1-POMT2 複合体の形成が必要である。ゼブラフィッシュは、遺伝子操作が容易である、繁殖しやすい、表現型を解析しやすい、脊椎動物である、ことなどから、近年、遺伝子の機能解析や疾患モデル動物として用いられている。特に薬剤を投与しやすく、化合物のスクリーニングなど治療法開発を目指す上での利用価値は高い。そこで、ゼブラフィッシュの *O*-マンノース転移酵素 zPOMT1、 zPOMT2 を解析し、筋ジストロフィー症モデルとしての可能性について検討した。

また、POMGnT1 は *O*-マンノース型糖鎖合成においてマンノースに *N*-アセチルグルコサミンを転移する酵素である。哺乳類の *O*-マンノース型糖鎖はα-ジストログリカンなどの限られた

蛋白質でしか検出されないことから、特定のアミノ酸配列などに特異的な修飾である可能性がある。近年 *O*-マンノース型糖鎖構造の多様性が報告され、糖鎖修飾部位の解析が盛んに行われるなど、部位特異的な糖鎖修飾が注目されている。*O*-マンノース型糖鎖合成の開始酵素は POMT であるので、マンノース修飾の特異性は主に POMT に依存すると考えられるが、POMGnT1 は *N*-アセチルグルコサミン以降の構造の部位特異性に関与する可能性がある。そこで、POMGnT1 の基質特異性を検討した。

B. 研究方法

①ゼブラフィッシュを用いたα-ジストログリカノパチーモデルの開発

ゼブラフィッシュ初期胚から zPOMT1 および zPOMT2 遺伝子をクローニングし、HEK293 細胞を用いて両タンパク質を単独あるいは同時に発現させて酵素活性を測定した。また、定量 RT-PCR 法および whole-mount in situ hybridization (WISH) 法により両遺伝子の発現解析を行った。さらに、両遺伝子に対するアンチセンスモルフォリノオリゴ (MO) を用いたノックダウン解析および免疫組織染色による解析を行った。

②POMTnT1 の基質特異性の解析

α-ジストログリカンのアミノ酸配列を基に Man-O-Thr を含むペプチドを有機科学的に合成した。アミノ酸残基数やアミノ酸組成の異なる複数の合成ペプチドを基質として POMGnT1 の反応速度論的解析を行った。POMGnT1 は膜貫通領域を除いた可溶型蛋白質としてバキュロウイルス発現系により作製し、アフィニティーカラムにて精製したものを使用した。

(倫理面への配慮)

実験動物の使用に関しては、「動物愛護管理法」および「動物実験に関する指針」に基づいて動物愛護に十分配慮するとともに、所内実験動物委員会の承認を受けている。放射性同位元素の使用関しては「放射線障害防止法関連法令」および「所内規定」に基づき安全を確保する。組換え DNA 実験に関しては、「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律」に基づくとともに所内組換え DNA 実験安全委員会の承認を受けている。

C. 研究結果

①ゼブラフィッシュを用いたα-ジストログリカノパチーモデルの開発

zPOMT1 および zPOMT2 はそれぞれ 720 アミノ酸および 756 アミノ酸をコードしていた。活性測定を行った結果、両タンパク質を共発現させた場合に高い POMT 活性が検出された。定量 RT-PCR 法および WISH 法の結果より、両遺伝子は受精直後より発現しており、発生過程を通してほぼ全身で発現していることが確認された。一方、アンチセンス MO による遺伝子ノックダウンの結果、*zPOMT1* ノックダウン体では心膜の浮腫や尾部の形成異常、*zPOMT2* ノックダウン体では心膜の浮腫や尾部の形成異常、眼の色素異常がみられた。また、いずれの表現型においてもα-ジストログリカンの *O*-マンノース型糖鎖修飾の著しい減少が確認された。

②POMTnT1 の基質特異性の解析

1) Man 単糖および Man-O-Thr (アミノ酸残基

数 1）は基質として認識されなかった。2）アミノ酸残基数 3〜8 ではペプチドの伸長に伴って K_m 値は減少、V_{max} 値は増加した。また、Man-O-Thr の N 末端側のアミノ酸が C 末端側よりも長い場合に良い基質となる傾向を示した。3)合成ペプチドのアミノ酸組成を変えることにより K_m 値、V_{max} 値が変化した。

D. 考察

①ゼブラフィッシュを用いたα-ジストログリカノパチーモデルの開発

POMT 活性の検出に zPOMT1 と zPOMT2 を共発現する必要があることから、酵素活性の発現に zPOMT1-zPOMT2 複合体の形成が必要であるという、哺乳類の POMT と同様のメカニズムがあることが明らかとなった。*zPOMT1* および *zPOMT2* 遺伝子のノックダウン体では、ヒトの WWS と同様にα-ジストログリカンの *O*-マンノース型糖鎖異常と、筋（心臓、尾部）および眼の発生異常が観察された。この結果は、ゼブラフィッシュにおいても神経や筋の発生に *O*-マンノース型糖鎖が重要であることを示しており、α-ジストログリカノパチーの病態モデルとしてゼブラフィッシュが有用であることを示している。

②POMTnT1 の基質特異性の解析

アミノ酸残基数により K_m 値と V_{max} 値が影響を受けること、合成ペプチド配列のアミノ酸置換により K_m 値、V_{max} 値が変化することから、POMGnT1 がアミノ酸配列をある程度認識している可能性が考えられる。

E. 結論

ゼブラフィッシュの *O*-マンノース転移酵素 zPOMT1、zPOMT2 を同定し、複合体形成による活性発現機構が種を越えて保存されていることを明らかにした。α-ジストログリカノパチーの病態モデルとしてのゼブラフィッシュの有用性を明らかにした。POMGnT1 がアミノ酸配列を認識している可能性を示した。

F. 研究発表

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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Avşar-Ban E, Ishikawa H, Manya H, Watanabe M, Akiyama S, Miyake H, Endo T, Tamaru Y	Protein *O*-mannosylation is necessary for normal embryonic development in zebrafish.	Glycobiology	20 巻 9 号	1089-1102,	2010

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Protein *O*-mannosylation is necessary for normal embryonic development in zebrafish

Eriko Avşar-Ban[2,*], Hisayoshi Ishikawa[2,*], Hiroshi Manya[3], Masatoki Watanabe[2], Shinichi Akiyama[2,5], Hideo Miyake[2,4], Tamao Endo[3], and Yutaka Tamaru[1,2,4]

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Two distinct cDNAs corresponding to two zebrafish protein *O*-mannosyltransferase genes, *zPOMT1* and *zPOMT2*, were cloned from early developmental embryos. Gene expression analysis revealed that *zPOMT1* and *zPOMT2* were expressed in similar patterns during early embryonic development and in all adult tissues. To study the regulation of *zPOMT1* and *zPOMT2* mRNA distribution during zebrafish embryogenesis, we injected *enhanced green fluorescent protein* (*EGFP*) mRNA fused to the 3′untranslated regions of each *zPOMT* gene. The distribution of EGFP resulting from the two constructs was similar. Injection of antisense morpholino oligonucleotides of *zPOMT1* and *zPOMT2* resulted in several severe phenotypes—including bended body, edematous pericaridium and abnormal eye pigmentation. Immunohistochemistry using anti-glycosylated α-dystroglycan antibody (IIH6) and morphological analysis revealed that the phenotypes of *zPOMT2* knockdown were more severe than those of *zPOMT1* knockdown, even though the IIH6 reactivity was lost in both *zPOMT1* and *zPOMT2* morphants. Finally, only when both *zPOMT1* and *zPOMT2* were expressed in human embryonic kidney 293T cells were high levels of protein *O*-mannosyltransferase activity detected, indicating that both zPOMT1 and zPOMT2 were required for full enzymatic activity. Moreover, either heterologous combination, *zPOMT1* and *human POMT2* (*hPOMT2*) or *hPOMT1* and *zPOMT2*, resulted in enzymatic activity in cultured cells. These results indicate that the protein *O*-mannosyltransferase machinery in zebrafish and humans is conserved and suggest that zebrafish may be useful for functional studies of protein *O*-mannosylation.

Keywords: development/glycosylation/POMT1 and POMT2/protein *O*-mannosyltransferase activity/zebrafish

Introduction

Posttranslational modification of proteins by glycosylation has critical biological functions at both the cellular and organismal levels (Haltiwanger and Lowe 2004; Ohtsubo and Marth 2006). In addition to the generally observed types of glycosylation such as *N*-glycosylation and mucin-type *O*-glycosylation, several unique glycans have recently been found to play important roles in a variety of biological processes. According to current knowledge, protein *O*-mannosylation in mammals is found on a relatively small number of proteins in the brain, nerves and skeletal muscle (Krusius et al. 1986; Chiba et al. 1997; Sasaki et al. 1998; Endo 1999). In contrast to yeast cells, where *O*-mannose is elongated by neutral, linear oligomannose chains (Strahl-Bolsinger and Tanner 1991), the mannose residue of mammalian *O*-mannosylglycans is extended with complex glycans terminating with sialic acid, sulfate or fucose (Endo 1999). The structure Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr is required for binding between α-dystroglycan (α-DG) and laminin G domain (Chiba et al. 1997; Endo 1999; Montanaro and Carbonetto 2003).

Muscular dystrophies are genetic diseases characterized by progressive muscle degeneration and muscular weakening. They can be classified into a number of disease types, and some causative genes have been identified (Burton and Davies 2002). For example, dystrophin forms a dystrophin–glycoprotein complex (DGC), and Duchenne muscular dystrophy is caused by mutations in the gene encoding dystrophin. Mutations in other components of DGC are involved in other muscular dystrophies. Defects in glycosylation of α-DG, one of the DGC components, are responsible for certain congenital muscular dystrophies (Endo and Toda 2003; Michele and Campbell 2003). These kinds of muscular dystrophies, including diseases such as Walker–Warburg syndrome (WWS) and muscle–eye–brain disease, are called α-dystroglycanopathies. Protein *O*-mannosyltransferases (POMT1 and POMT2) catalyze the first step in *O*-mannosyl glycan synthesis (Manya et al. 2004), and defects in human POMT1 (hPOMT1) or hPOMT2 result in WWS, an autosomal recessive disorder associated with severe congenital muscular dystrophy, abnormal

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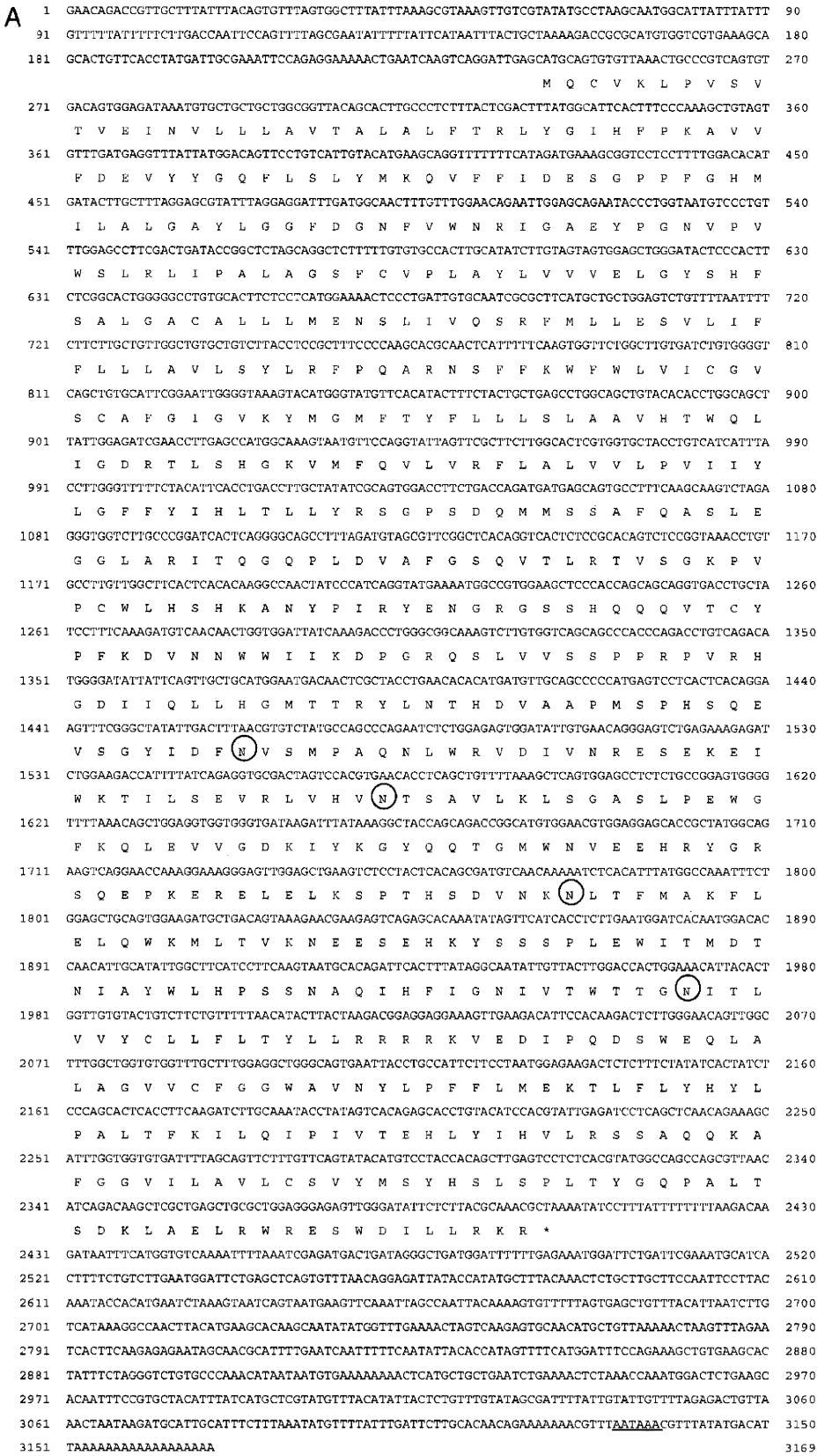
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1351 TGGGGATATTATTCAGTTGCTGCATGGAATGACAACTCGCTACCTGAACACACATGATGTTGCAGCCCCCATGAGTCCTCACTCACAGGA 1440
G D I I Q L L H G M T T R Y L N T H D V A A P M S P H S Q E
1441 AGTTTCGGGCTATATTGACTTTAACGTGTCTATGCCAGCCCAGAATCTCTGGAGAGTGGATATTGTGAACAGGGAGTCTGAGAAAGAGAT 1530
V S G Y I D F (N) V S M P A Q N L W R V D I V N R E S E K E I
1531 CTGGAAGACCATTTTATCAGAGGTGCGACTAGTCCACGTGAACACCTCAGCTGTTTTAAAGCTCAGTGGAGCCTCTCTGCCGGAGTGGGG 1620
W K T I L S E V R L V H V (N) T S A V L K L S G A S L P E W G
1621 TTTTAAACAGCTGGAGGTGGTGGGTGATAAGATTTATAAAGGCTACCAGCAGACCGGCATGTGGAACGTGGAGGAGCACCGCTATGGCAG 1710
F K Q L E V V G D K I Y K G Y Q Q T G M W N V E E H R Y G R
1711 AAGTCAGGAACCAAAGGAAAGGGAGTTGGAGCTGAAGTCTCCTACTCACAGCGATGTCAACAAAAATCTCACATTTATGGCCAAATTTCT 1800
S Q E P K E R E L E L K S P T H S D V N K (N) L T F M A K F L
1801 GGAGCTGCAGTGGAAGATGCTGACAGTAAAGAACGAAGAGTCAGAGCACAAATATAGTTCATCACCTCTTGAATGGATCACAATGGACAC 1890
E L Q W K M L T V K N E E S E H K Y S S S P L E W I T M D T
1891 CAACATTGCATATTGGCTTCATCCTTCAAGTAATGCACAGATTCACTTTATAGGCAATATTGTTACTTGGACCACTGGAAACATTACACT 1980
N I A Y W L H P S S N A Q I H F I G N I V T W T T G (N) I T L
1981 GGTTGTGTACTGTCTTCTGTTTTTAACATACTTACTAAGACGGAGGAGGAAAGTTGAAGACATTCCACAAGACTCTTGGGAACAGTTGGC 2070
V V Y C L L F L T Y L L R R R R K V E D I P Q D S W E Q L A
2071 TTTGGCTGGTGTGGTTTGCTTTGGAGGCTGGGCAGTGAATTACCTGCCATTCTTCCTAATGGAGAAGACTCTCTTTCTATATCACTATCT 2160
L A G V V C F G G W A V N Y L P F F L M E K T L F L Y H Y L
2161 CCCAGCACTCACCTTCAAGATCTTGCAAATACCTATAGTCACAGAGCACCTGTACATCCACGTATTGAGATCCTCAGCTCAACAGAAAGC 2250
P A L T F K I L Q I P I V T E H L Y I H V L R S S A Q Q K A
2251 ATTTGGTGGTGTGATTTTAGCAGTTCTTTGTTCAGTATACATGTCCTACCACAGCTTGAGTCCTCTCACGTATGGCCAGCCAGCGTTAAC 2340
F G G V I L A V L C S V Y M S Y H S L S P L T Y G Q P A L T
2341 ATCAGACAAGCTCGCTGAGCTGCGCTGGAGGGAGAGTTGGGATATTCTCTTACGCAAACGCTAAAATATCCTTTATTTTTTTTAAGACAA 2430
S D K L A E L R W R E S W D I L L R K R *
2431 GATAATTTCATGGTGTCAAAATTTTAAATCGAGATGACTGATAGGGCTGATGGATTTTTTGAGAAATGGATTCTGATTCGAAATGCATCA 2520
2521 CTTTTCTGTCTTGAATGGATTCTGAGCTCAGTGTTTAACAGGAGATTATACCATATGCTTTACAAACTCTGCTTGCTTCCAATTCCTTAC 2610
2611 AAATACCACATGAATCTAAAGTAATCAGTAATGAAGTTCAAATTAGCCAATTACAAAAGTGTTTTTAGTGAGCTGTTTACATTAATCTTG 2700
2701 TCATAAAGGCCAACTTACATGAAGCACAAGCAATATATGGTTTGAAAACTAGTCAAGAGTGCAACATGCTGTTAAAAACTAAGTTTAGAA 2790
2791 TCACTTCAAGAGAGAATAGCAACGCATTTTGAATCAATTTTTCAATATTACACCATAGTTTTCATGGATTTCCAGAAAGCTGTGAAGCAC 2880
2881 TATTTCTAGGGTCTGTGCCCAAACATAATAATGTGAAAAAAAACTCATGCTGCTGAATCTGAAAACTCTAAACCAAATGGACTCTGAAGC 2970
2971 ACAATTTCCGTGCTACATTTATCATGCTCGTATGTTTACATATTACTCTGTTTGTATAGCGATTTTATTGTATTGTTTTAGAGACTGTTA 3060
3061 AACTAATAAGATGCATTGCATTTCTTTAAATATGTTTTATTTGATTCTTGCACAACAGAAAAAAACGTTTAATAAACGTTTATATGACAT 3150
3151 TAAAAAAAAAAAAAAAAA 3169

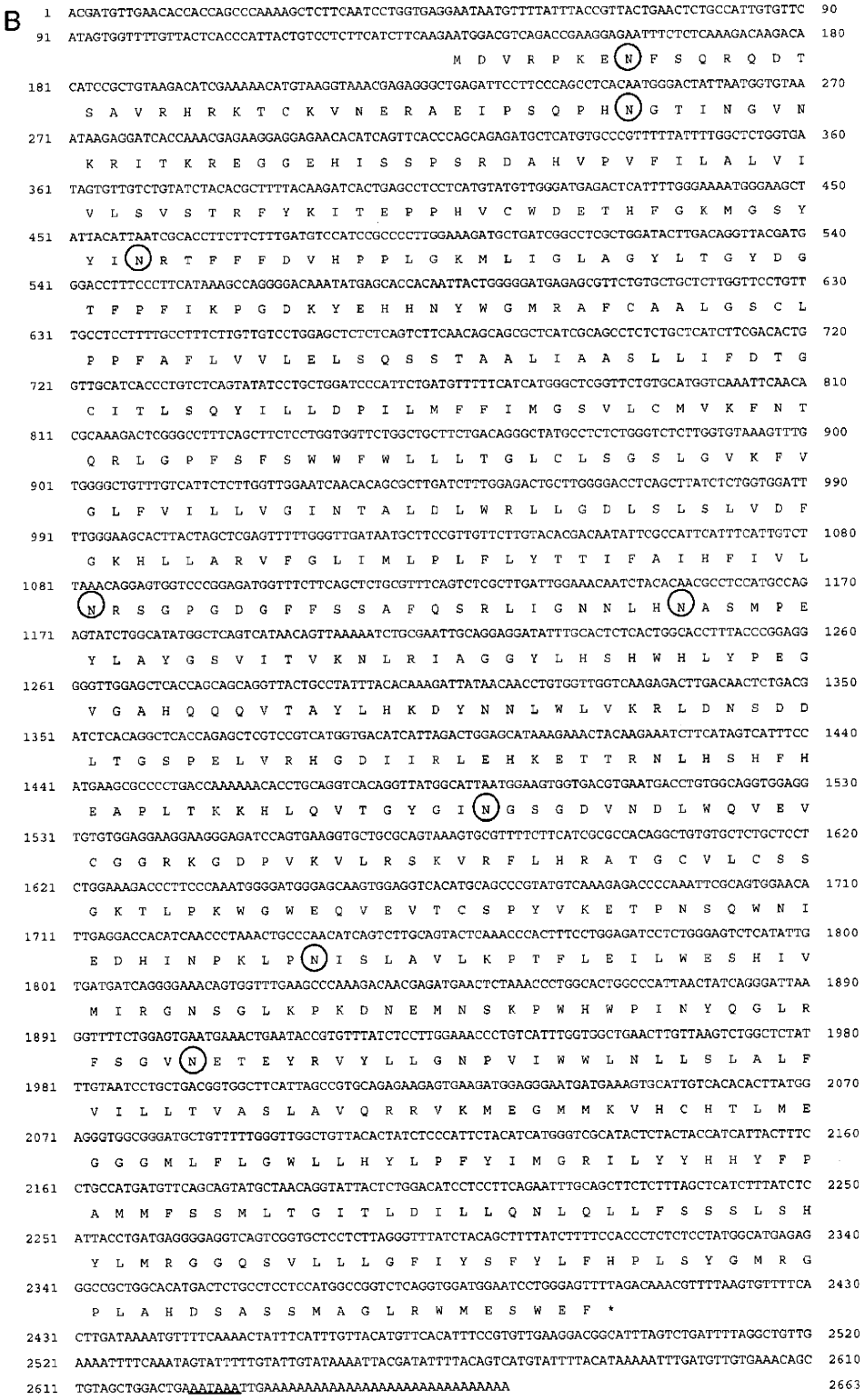
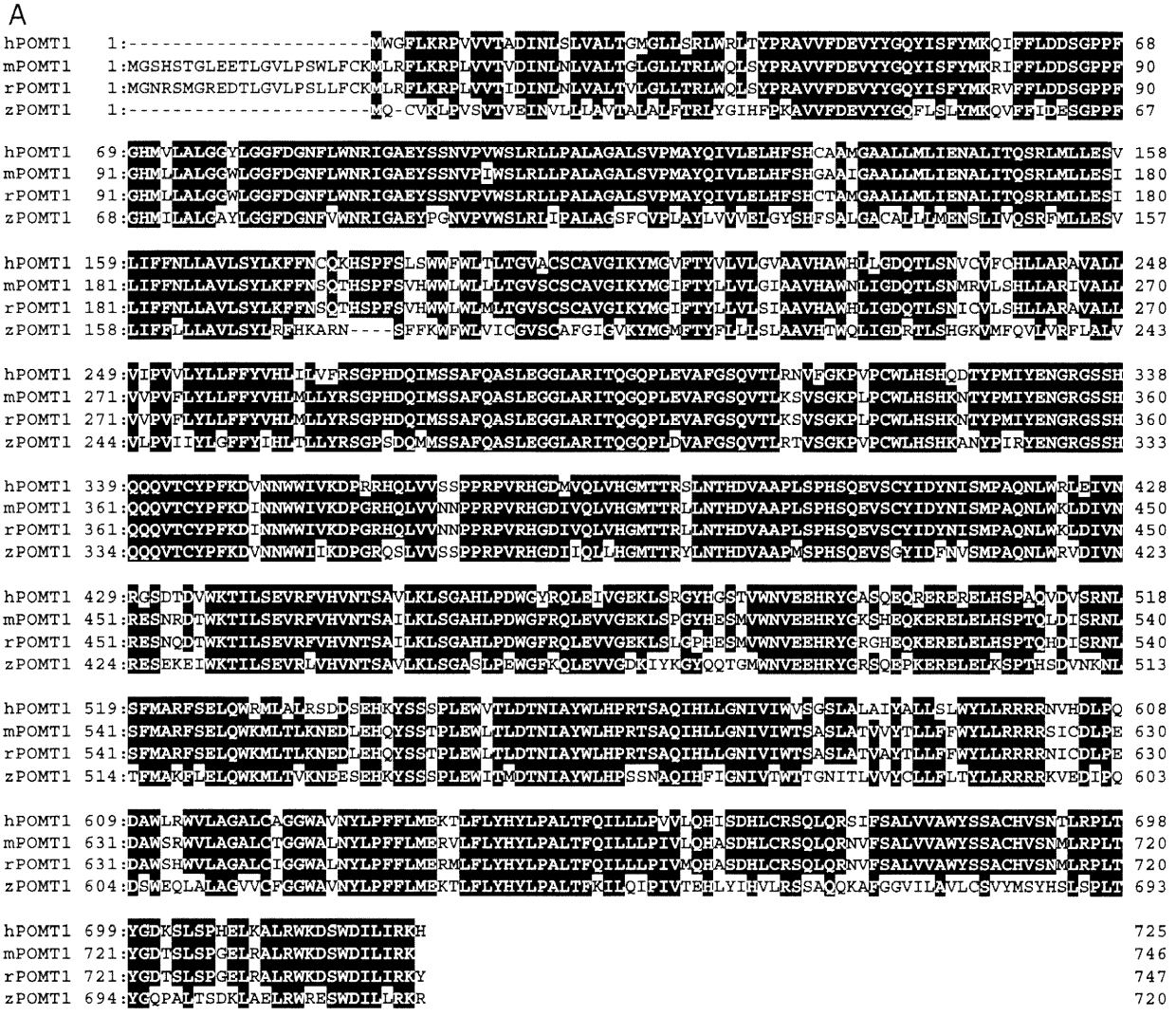


Fig. 1. Nucleotide sequences and deduced amino acid sequences of *zPOMT1* and *zPOMT2*. The cDNA sequences of zebrafish *POMT1* (**A**) and *POMT2* (**B**) genes are presented on the upper line. Deduced amino acid sequences are indicated by the single-letter amino acid codes. Potential *N*-glycosylation sites are indicated by circles. Consensus polyadenylation sites are underlined.

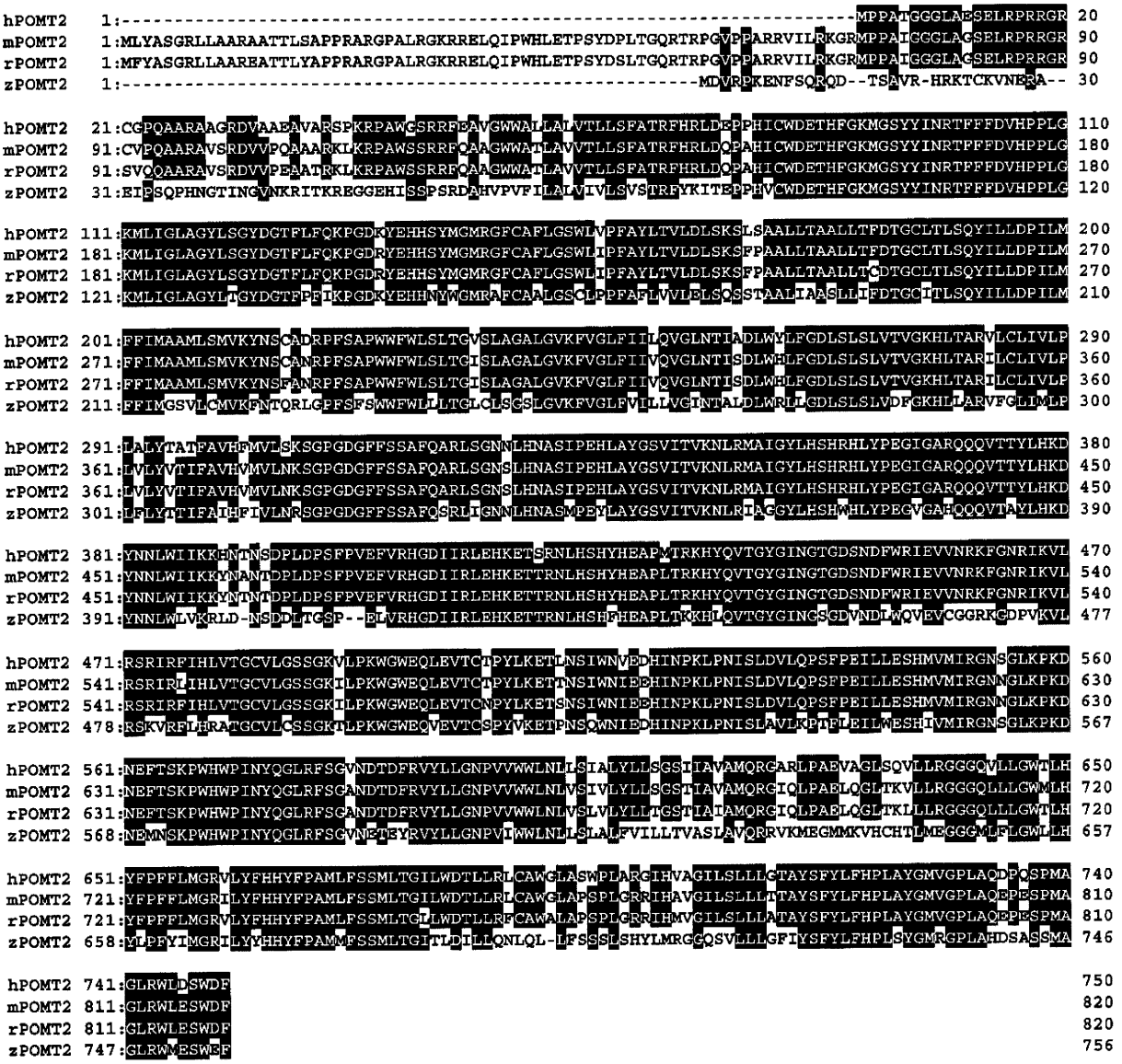
neuronal migration and eye anomalies (Beltran-Valero de Bernabe et al. 2002; van Reeuwijk et al. 2005). POMT orthologs have been identified in many animals, including *Drosophila* (Martín-Blanco and García-Bellido 1996; Willer et al. 2002), mouse (Willer et al. 2002; Willer et al. 2004), rat (Manya et al. 2006) and humans (Jurado et al. 1999; Willer et al. 2002). *Drosophila*, rat and mouse have orthologs of both of human *POMT* genes, and their products have protein *O*-mannosyltransferase activity when only they are co-expressed (Ichimiya et al. 2004; Manya et al. 2006; Lommel et al. 2008). In contrast, *Drosophila* does not have orthologs to human or murine protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase1 (POMGnT1) (Ichimiya et al. 2004). POMGnT1 catalyzes the transfer of GlcNAc from UDP-GlcNAc to the protein *O*-mannosyl residue (Yoshida et al. 2001; Liu et al. 2006; Miyagoe-Suzuki et al. 2009). Therefore, it seems that the structures of *O*-mannosylglycans of invertebrates are quite different from those of vertebrates. Moreover, it has been reported that hPOMT1 and hPOMT2 must form a heterocomplex for protein *O*-mannosyltransferase activity (Akasaka-Manya et al. 2006).

The zebrafish (*Danio rerio*) provides a readily accessible model for human muscle diseases such as muscular dystrophies (Bassett and Currie 2003). Muscle specification and differentiation follow a well-characterized time course and allow detailed analysis with single-cell resolution (Devoto et al. 1996). Zebrafish orthologs of proteins in the human DGC have been implicated in muscle development, and some zebrafish DGC orthologs have uses as models to study human muscular dystrophy and congenital myopathy (Parsons et al. 2002; Bassett and Currie 2003; Guyon et al. 2003). More recently, it has been reported that the zebrafish has orthologs of *POMT1*, *POMT2*, *POMGnT1* and other putative glycosyltransferases expected to contribute to the synthesis of mammalian-type *O*-mannosylglycan (Steffen et al. 2007; Moore et al. 2008). Taken together, the structures of *O*-mannosylglycans are thought to be similar in diverse vertebrates. Therefore, zebrafish may be a useful model for analyses of the biosynthetic

A



B



pathway of *O*-mannosylglycans in vertebrates, the mechanisms of muscular dystrophies, and myogenesis.

In this study, we isolated and cloned full-length cDNAs encoding two zebrafish *POMT* genes, *zPOMT1* and *zPOMT2*, and examined whether they have protein *O*-mannosyltransferase activity. We also investigated the expression patterns of both genes during embryogenesis and in adult tissues. Furthermore, we analyzed the distribution and localization of a protein expressed from constructs containing the 3′untranslated region (3′UTR) of *zPOMT1* or *zPOMT2*. Finally, knockdown analysis using antisense morpholino oligonucleotides (MOs) was performed to assess the function of protein *O*-mannosylation during zebrafish development.

Results

cDNA cloning and sequencing of zPOMT1 and zPOMT2

The full-length cDNAs encoding *zPOMT1* and *zPOMT2* genes were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) using zebrafish embryos. The complete cDNAs and deduced amino acid sequences of *zPOMT1* and *zPOMT2* are shown in Figure 1 (GenBank accession nos. AB281275 and AB281276, respectively). *zPOMT1* consisted of an open reading frame (ORF) of 2160 bases encoding a conceptual translation product of 720 amino acids with a predicted molecular mass of 82,036 Da (Figure 1A). *zPOMT2* consisted of an ORF of 2268 bases encoding conceptual translation product of 756 residues with a predicted molecular mass of 85,710 Da

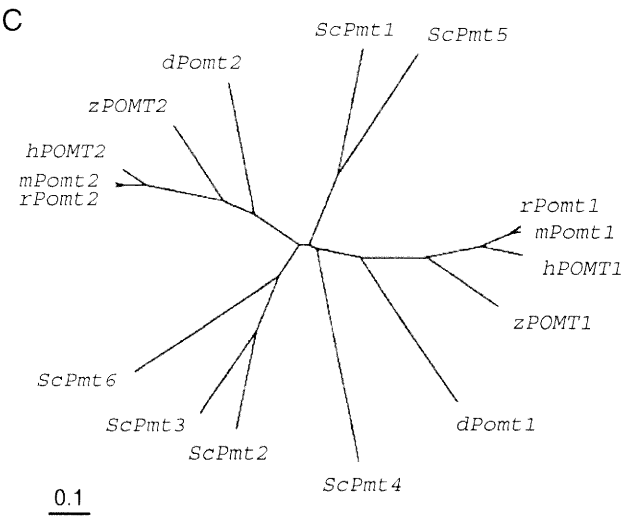


Fig. 2. Comparisons of human, mouse, rat and zebrafish POMTs. ClustalW alignments of human (h), mouse (m), rat (r) and zebrafish (z) POMT1 (**A**) and POMT2 (**B**) amino acid sequences are indicated by single-letter amino acid codes, respectively. Conserved amino acids are highlighted. (**C**) ClustalW phylogenic tree of human, mouse, rat, zebrafish and *Drosophila* (d) POMTs and *S. cerevisiae* (Sc) Pmts. The amino acid sequence of hPOMT1 is a major type that was used for assay of protein *O*-mannosyltransferase activity in this study. The amino acid sequences of mPOMT2 and rPOMT2 belonged to the testis form. The branch lengths indicate amino acid substitutions per site.

(Figure 1B). A consensus polyadenylation site (AATAAA) was located downstream of the translation termination codon in both *zPOMT1* and *zPOMT2*. As shown in Figure 2, the deduced amino acid sequences in both zPOMT1 and zPOMT2 were similar to those of mammals such as human, mouse and rat (Jurado et al. 1999; Willer et al. 2002; Willer et al. 2004; Manya et al. 2006). zPOMT1 had 66% identity to hPOMT1 (Figure 2A), whereas zPOMT2 showed 70% identity to hPOMT2 (Figure 2B). A phylogenetic analysis of 16 proteins—six *Saccharomyces cerevisiae* Pmts (ScPmt1-6) (Willer et al. 2002), two human (hPOMT1 and hPOMT2), two mouse (mPOMT1 and mPOMT2), two rat (rPOMT1 and rPOMT2), two *Drosophila* (dPOMT1 and dPOMT2) and two zebrafish (zPOMT1 and zPOMT2)—indicates that zPOMT1 is in the pmt4 subfamily and zPOMT2 is in the pmt2 subfamily (Figure 2C).

Gene expression of zPOMT1 and zPOMT2

Quantitative PCR was performed with early developmental stages (Figure 3A) and all adult tissues (Figure 3B). There were significant differences in the levels of *zPOMT1* and *zPOMT2* expression during embryogenesis. At 0 h post fertilization (hpf), both genes, *zPOMT1* and *zPOMT2*, were highly expressed. While *zPOMT1* expression decreased after 6 hpf, *zPOMT2* expression increased at 6 hpf and then decreased at 12 hpf. Furthermore, *zPOMT2* expression increased again around 24 hpf. There were no significant differences in *zPOMT1* and *zPOMT2* expression levels in males and females in any adult tissue except for the liver. Interestingly, *zPOMT1* and *zPOMT2* were highly expressed in both testis and ovary (Figure 3B). By means of whole-mount in situ hybridization, mRNAs of *zPOMT1* and *zPOMT2* were detected during early developmental stages. Both *zPOMT1* (Figure 3C) and *zPOMT2* (Figure 3D) transcripts were ubiquitously expressed throughout the gastrulation, tailbud and somite stages. At 24 hpf, both *zPOMT1* and *zPOMT2* mRNAs were detected predominantly in eyes and somites.

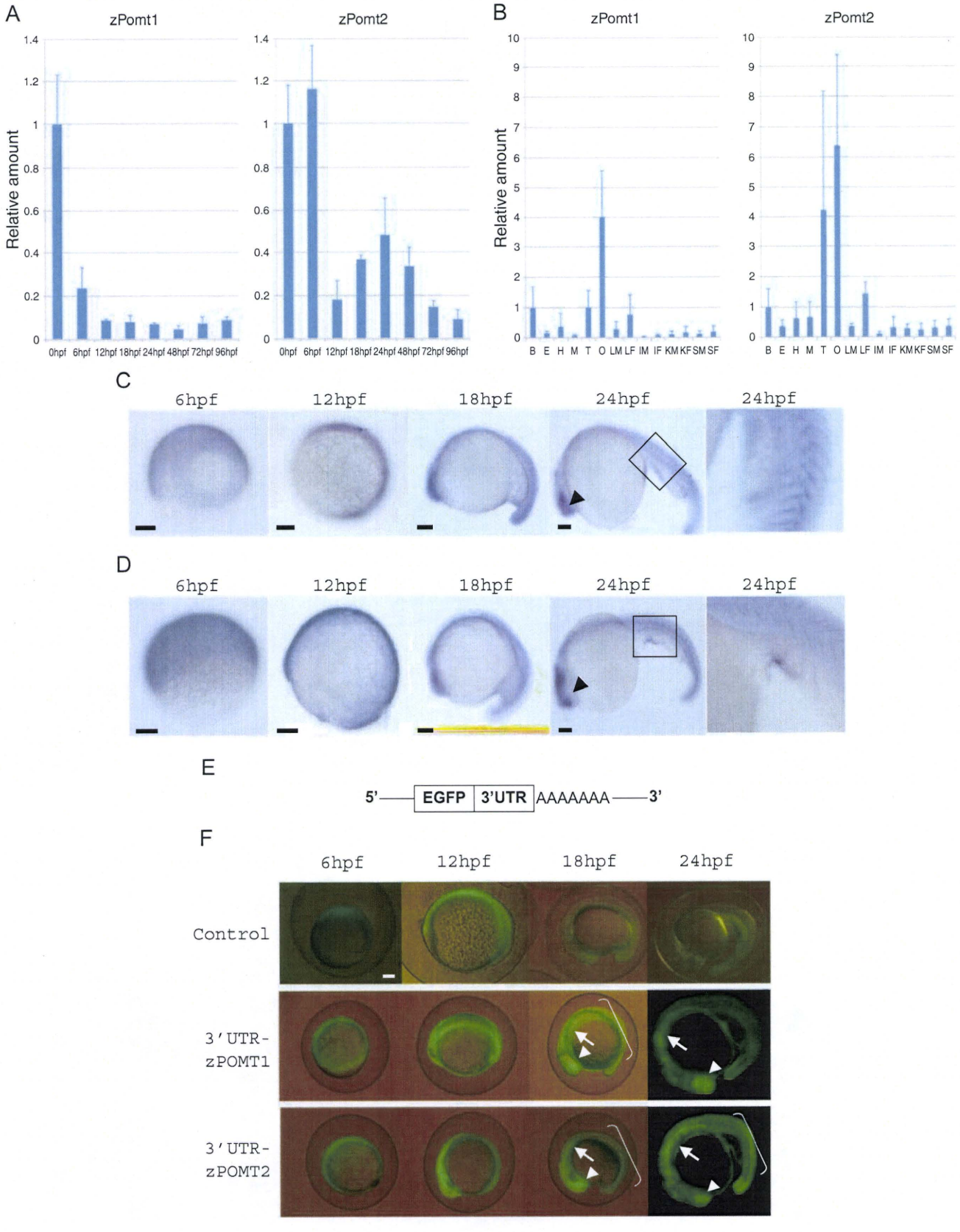
Localization of 3′UTRs in zPOMT1 and zPOMT2

The 3′UTR of an mRNA can affect the expression and/or localization of the mRNA during development within particular cells, including primary germ cells (Hashimoto et al. 2009). Therefore, to investigate the function of 3′ UTRs of *zPOMT1* and *zPOMT2*, we fused the 3′UTR of each *zPOMT* to the *enhanced green fluorescent protein* (*EGFP*) gene (Figure 3E). Capped mRNAs of *EGFP*-3′UTR of *zPOMT1* and *zPOMT2* were synthesized and injected into embryos at the one- to two-cell stage. With both constructs, EGFP was distributed throughout the whole body at 6 hpf, and EGFP was highly expressed in the eye, hindbrain and somite from 18 to 24 hpf (Figure 3F).

Knockdown analysis of zPOMT1 and zPOMT2

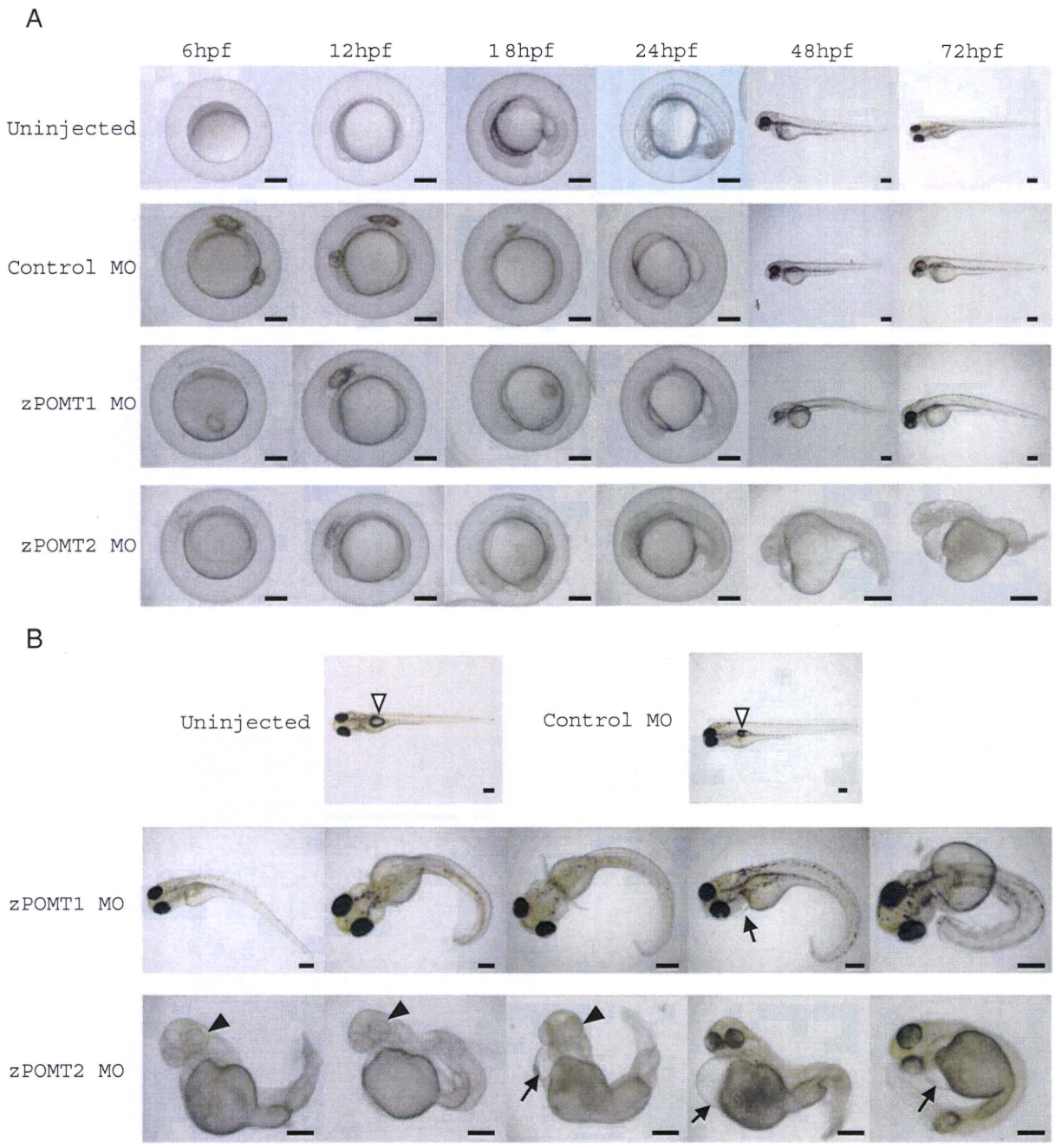
Antisense MOs against *zPOMT1* and *zPOMT2* were injected into the zebrafish embryos at the one- to two-cell stage, and the developmental progress of the morphants was compared with that of uninjected and control MO embryos (Figure 4). There were no significant differences between control MO and morphant embryos until 12 hpf. At 18 hpf, both *zPOMT1* MO and *zPOMT2* MO embryos were developmentally delayed in comparison with control MO (Figure 4A) and uninjected (data not shown) embryos. At 48 and 72 hpf, *zPOMT1* morphants showed slightly curved tails and curvature of the somite boundaries. In contrast, *zPOMT2* morphants at the same stage showed more severe phenotypes—including twisted tails, aberrant pericardium and abnormal eye pigmentation—than did the *zPOMT1* morphants. Quantitative analyses of embryos from all treatment groups were performed at 96 hpf. Each

Fig. 3. Gene expressions and whole-mount in situ hybridization for *zPOMT1* and *zPOMT2*. Quantitative PCR analyses of *zPOMT1* and *zPOMT2* mRNAs during early developmental stages (**A**) and in adult tissues (**B**). PCR products of *zPOMT1* and *zPOMT2* were detected throughout early developmental stages and in all tissues predominantly in gonads. *zβ-actin2* and *zCox1* were used as internal controls for quantitative PCR in early developmental stages and adult tissues, 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. *zPOMT1* (**C**) and *zPOMT2* (**D**) mRNAs were detected. Both genes were expressed ubiquitously throughout early developmental stage, and high levels of expression were detected at 24 hpf. Arrowheads and boxes shown in the right columns at 24 hpf indicate eyes and the central parts of the somites, respectively. Boxes in 24 hpf were enlarged and shown in the far right panels. Scale bars = 100 μm. Illustration of capped mRNA structure (**E**) and localization of *EGFP*-3′UTR of control (upper panel), *zPOMT1* (middle panel) and *zPOMT2* (lower panel) (**F**). Arrows and arrowheads indicate the proteins corresponding to EGFP-*zPOMTs* 3′UTR. At 24 hpf, EGFP-3′UTR of *zPOMT1* was located predominately to the eye (arrowhead), whereas EGFP-3′UTR of *zPOMT2* was expressed highly in eye (arrowhead), hindbrain (arrow), and somite (bracket). Scale bars = 100 μm.


A
zPomt1
zPomt2
Relative amount
0hpf 6hpf 12hpf 18hpf 24hpf 48hpf 72hpf 96hpf
B
zPomt1
zPomt2
Relative amount
B E H M T O LM LF IM IF KM KF SM SF
C
6hpf
12hpf
18hpf
24hpf
24hpf
D
6hpf
12hpf
18hpf
24hpf
24hpf
E
5'
EGFP
3'UTR
AAAAAAA
3'
F
6hpf
12hpf
18hpf
24hpf
Control
3'UTR-zPOMT1
3'UTR-zPOMT2

embryo with or without MO treatment was categorized as having normal, moderate or severe phenotypes according to morphological characteristics (Table I). The frequency of moderate and severe phenotypes increased with injection of increasing amounts of *zPOMT1* and *zPOMT2* MOs. The *zPOMT2* MO embryos had more severe deformities than did the *zPOMT1* MO embryos (Figure 4B, Table I). For example, aberrant eye pigmentation was observed only in *zPOMT2* MO embryos. Finally, swim bladders were not completely formed in *zPOMT1* and *zPOMT2* morphant embryos in comparison with uninjected or control MO embryos (Figure 4B).

To investigate the glycosylation status of α-DG in *zPOMT1* and *zPOMT2* morphant embryos, we immunostained embryos at 48 hpf with the anti-glycosylated α-DG antibody IIH6 (Figure 4C). Strong signals were detected with IIH6 in untreated embryos. Moreover, IIH6 reactivity in control MO embryos was also detected in the horizontal and vertical myosepta. However, the reactivity was almost completely lost in *zPOMT1* and *zPOMT2* morphants.



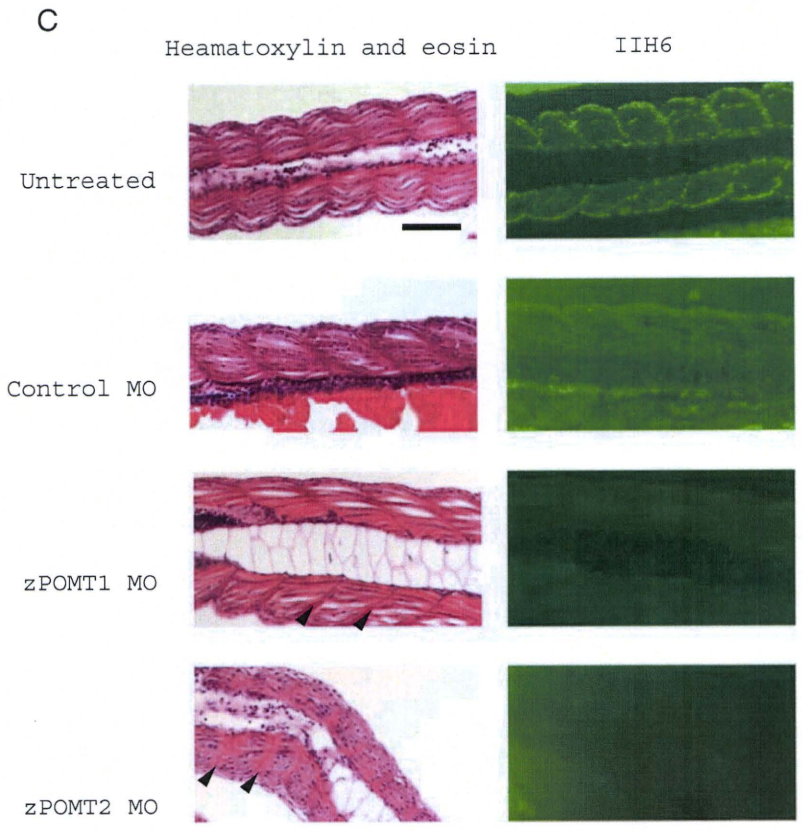


Fig. 4. Knockdown analysis of *zPOMT1* and *zPOMT2*. (**A**) Sequential changes during early developmental stages (6–72 hpf) of uninjected embryos (top panel), embryos injected with control MO (middle upper panel), embryos injected with *zPOMT1* MO (middle lower panel) and embryos injected with *zPOMT2* MO (bottom panel). Zebrafish embryos were injected with each MO at the one- to two-cell stage and were observed at 6, 12, 18, 24, 48 and 72 hpf. Scale bars = 200 μm. (**B**) 96 hpf morphants. Top panel: uninjected embryo (left side) and injected control MO (right side), embryos injected *zPOMT1* MO (middle panel), and embryos injected *zPOMT2* MO (bottom panel). Zebrafish embryos were injected with each MO at the one- to two-cell stages and were observed at 96 hpf. White arrowheads indicate swim bladder. Both morphant embryos revealed curved tail, and some had abnormal pericardium (arrows). Some of the *zPOMT2* morphants showed aberrant eye pigmentation (arrowheads). Scale bars = 200 μm. (**C**) Whole-mount immunohistochemistry with anti-glycosylated α-DG antibody IIH6 in 48-hpf embryos. Left panels, hematoxylin and eosin staining; right panels, IIH6 staining. IIH6 immunoreactivity was detected in uninjected and control MO but decreased in *zPOMT1* and *zPOMT2* morphants. Arrowheads represent vertical myosepta. Scale bars = 50 μm.

Protein O-mannosyltransferase activity of zPOMT1 and zPOMT2

To analyze the protein *O*-mannosyltransferase activity of zPOMT1 and zPOMT2, the cDNAs were cloned into an expression vector. The resulting expression constructs were transfected into human embryonic kidney 293T (HEK293T) cells, and microsomal membranes were used for enzyme assay. High levels of protein *O*-mannosyltransferase activity was observed only when the *zPOMT1* and *zPOMT2* genes were co-expressed in HEK293T cells (Figure 5). Jack bean α-mannosidase digestion showed that the mannosyl residue was linked to GST-α-DG by α-linkage (data not shown), as reported previously (Manya et al. 2004). Although a single transfection of *zPOMT1* in HEK293T cells did not show any enzymatic activity, transfection of *zPOMT2* alone did result in low levels of activity. Cells co-transfected with *zPOMT1* and *hPOMT2* and cells co-transfected with *hPOMT1* and *zPOMT2* showed robust levels of *O*-mannosyltransferase activity. In contrast, cells co-transfected with *zPOMT1* and *hPOMT1* and cells co-transfected with *zPOMT2* and *hPOMT2* did not show any enzymatic activity (data not shown). These results indicated that POMT1 and POMT2 from zebrafish and humans are interchangeable and that POMT1 and POMT2 have different functional roles in POMT enzymatic activity.

Table I. Quantification of *zPOMT* morphant phenotypes at 96 hpf

	Concentration (mM)	Normal	Moderate	Severe
Uninjected	-	97 (100.0%)	0 (0%)	0 (0%)
Control MO	1.0	65 (95.6%)	3 (4.4%)	0 (0%)
zPOMT1 MO	1.0	65 (79.3%)	14 (17.1%)	3 (3.6%)
	0.5	72 (93.5%)	4 (5.2%)	1 (1.3%)
	0.25	72 (98.6%)	0 (0%)	1 (1.4%)
zPOMT2 MO	1.0	33 (35.5%)	31 (33.3%)	29 (31.2%)
	0.5	55 (61.1%)	26 (28.9%)	9 (10.0%)
	0.25	79 (86.8%)	5 (5.5%)	7 (7.7%)

The phenotypic data shown were obtained from three independent experiments. 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 (MO). Moderate (curved tails and curvature of the somite boundaries); Severe (twisted tail and aberrant pericardium).

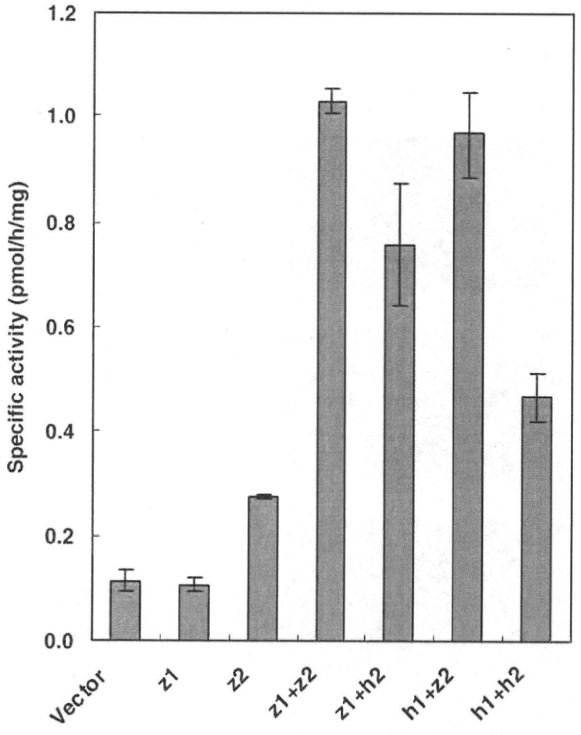


Fig. 5. Protein *O*-mannosyltransferase activities of *zPOMTs*. Protein *O*-mannosyltransferase activity was based on the rate of mannose transfer from mannosylphosphoryldolichol to a GST-α-DG. Vector, cells transfected with vector only; z1, cells transfected with *zPOMT1*; z2, cells transfected with *zPOMT2*; z1+z2, cells co-transfected with *zPOMT1* and *zPOMT2*; z1+h2, cells co-transfected with *zPOMT1* and *hPOMT2*; h1+z2, cells co-transfected with hPOMT1 and *zPOMT2*; h1+h2, cells co-transfected with *hPOMT1* and *hPOMT2*. Average values ± SD of three independent experiments are shown.

Discussion

Zebrafish have been useful for the study of human muscular dystrophies and congenital myopathies (Parsons et al. 2002; Bassett and Currie 2003; Bassett et al. 2003; Guyon et al. 2003) because zebrafish have orthologs of genes implicated in human muscular dystrophies, including *POMT1*, *POMT2*, *POMGnT1*, *dystrophin*, *fukutin* and fukutin-related protein (*FKRP*) (Steffen et al. 2007; Moore et al. 2008). Recently, it has been reported that knockdown analysis of *FKRP*, one of the causative genes in α-dystroglycanopathies, resulted in morphants that showed a pathological spectrum similar to those of human muscular dystrophies associated with mutations in *FKRP* (Thornhill et al. 2008). However, the function of FKRP is not clear yet (Esapa et al. 2002; Esapa et al. 2005; Matsumoto et al. 2004; Dolatshad et al. 2005; Torelli et al. 2005; Beedle et al. 2007). In contrast, POMTs are known to be protein *O*-mannosyltransferases. *FKRP* MO and *zPOMTs* morphant embryos showed a reduction in the glycosylated α-DG staining, indicating that FKRP may affect the biosynthetic pathway of *O*-mannosylglycans (Thornhill et al. 2008).

In mammals, two protein *O*-mannosyltransferase (POMTs) family members, POMT1 and POMT2, are known to exist. hPOMT1 and hPOMT2 catalyze protein *O*-mannosyl transfer to α-DG, which serves as a protein substrate, and mutations in the *hPOMT1* and *hPOMT2* genes result in WWS, a severe muscular dystrophy that also results in structural alterations in eyes and brain malformations, such as cobblestone lissencephaly. In this study, we have identified, cloned and expressed the full-length cDNAs of the *zPOMT1* and *zPOMT2* genes, and our results suggest that high levels of protein *O*-mannosyltransferase activity depends on expression of both genes. Co-expression of both *zPOMT1* and *zPOMT2* genes showed high protein *O*-mannosyltransferase activity similar to results from analyses of hPOMTs. Although transfection of *zPOMT1* alone did not show any enzymatic activity, transfection of *zPOMT2* alone showed slight activity. These results suggest that zPOMT2 itself has enzymatic activity, or zPOMT2 may form a complex with endogenous hPOMT1 resulting in low levels of enzymatic activity. Two heterologous protein combinations, zPOMT1 and hPOMT2 or zPOMT2 and hPOMT1, resulted in robust levels of enzymatic activity (Figure 5). This result suggests that a single mechanism of *O*-mannosylation is common to humans and zebrafish. On the other hand, cells co-transfected with *zPOMT1* and *hPOMT1* and cells co-transfected with *zPOMT2* and *hPOMT2* did not show any enzymatic activity, indicating clearly that POMT1 and POMT2 have different functional roles in POMT enzymatic activity. It is not clear why protein *O*-mannosyltransferase activity requires co-expression of POMT1 and POMT2 (Ichimiya et al. 2004; Manya et al. 2006; Manya et al. 2004); this study). One possibility is that POMT1 is a catalytic molecule and POMT2 is a regulatory molecule or vice versa. Another possibility is that assembly of POMT1 and POMT2 forms a catalytic domain. To further understand the mechanism of protein *O*-mannosylation, it is necessary to perform a structural study of a complex formed by POMT1 and POMT2.

Remarkably, overlapping expression patterns of *zPOMT1* and *zPOMT2* were observed by whole-mount in situ hybridization (Figure 3C). Such overlapping pattern suggests that the two proteins may collaborate functionally in vivo. This expression data are consistent with data suggesting that simultaneous expression of zPOMT1 and zPOMT2 is required for POMT enzymatic activity. Interestingly, similar co-expression of *POMTs* was observed in various tissues and during different developmental stages of embryogenesis in mouse and *Drosophila* (Ichimiya et al. 2004; Lyalin et al. 2006; Lommel et al. 2008). These results suggest that both protein *O*-mannosylation machinery and biological importance of protein *O*-mannosylation may have been conserved during metazoan evolution, although further analyses are necessary to understand the molecular mechanisms of protein *O*-mannosylation and its evolution.

The expression levels of the two *POMTs* genes differed at various developmental stages and in specific tissues of mouse, *Drosophila* and zebrafish. For example, while mouse *POMT2* was highly expressed in testis (Willer et al. 2002), *zPOMT1* and *zPOMT2* were highly expressed in ovary and testis (Figure 3B). Since *POMT1* knockout in mice results in embryonic lethality, protein *O*-mannosylation is necessary for normal development (Willer et al. 2004). In the case of *Drosophila POMTs*, the expression level of *dPOMT1* was higher than that of *dPOMT2* from 0 to 2 h in the embryo (Ichimiya et al. 2004), whereas the expression level of *zPOMT2* was higher than that of *zPOMT1* at from 0 to 6 hpf. It was assumed that both *zPOMT1* and *zPOMT2* mRNAs at these stages were derived

from maternal expression. Furthermore, the expression level of *zPOMT1* decreased from 0 to 6 hpf, whereas *zPOMT2* expression was high from 0 to 6 hpf (Figure 3A). These results suggest that the expression of *zPOMT1* and *zPOMT2* may be regulated differently.

In humans, defects of protein *O*-mannosylation lead to WWS (Manya et al. 2003; Akasaka-Manya et al. 2004). To understand the importance of protein *O*-mannosylation in zebrafish development, we carried out the knockdown analysis of antisense MOs against *zPOMT1* and *zPOMT2*. As a result, *zPOMT1* and *zPOMT2* morphant embryos showed curved tail, and some had edematous pericardium (Figure 4B). Since both *zPOMT1* and *zPOMT2* morphants showed these phenotypic aberrations, they could not swim straight or feed at all, and they eventually died. At 96 hpf, the phenotypes of *zPOMT2* morphant embryos showed a higher incidence of more severe phenotypes than the *zPOMT1* morphant embryos did (Table I), yet immunoreactivity of IIH6 in *zPOMT1* and *zPOMT2* morphants was similar (Figure 4C). We predicted that the phenotypes of zebrafish embryos injected with MOs against *zPOMT1* and *zPOMT2* would be the same or similar because the expression patterns of the two genes were similar before 24 hpf (Figure 3). Therefore, the difference of phenotypes observed between *zPOMT1* and *zPOMT2* morphants in early development of the zebrafish might be explained by the variance of knockdown efficiency or by another function of zPOMT2 in addition to enzymatic activity. It may be consistent with severe phenotypes observed in *zPOMT2* embryos that only *zPOMT2* morphant embryos showed aberrant pigmentation in eyes. Fukutin-deficient chimeric mice revealed abnormalities in eyes, indicating that corneal opacification with vascular infiltration and eye abnormality was quite remarkable according to their aberrant pigmentation (Takeda et al. 2003). Further studies are necessary to reveal the role of *O*-mannosylglycans in the pathogenesis of eye abnormalities.

Here, we demonstrated that zebrafish POMTs possess protein *O*-mannosyltransferase activity when co-expressed in HEK293T cells. This result suggested that the protein *O*-mannosylation machinery is conserved in mammals and zebrafish. Therefore, to elucidate whether the function and mechanism of protein *O*-mannosylation related to *POMTs* are evolutionarily conserved in the vertebrates, the zebrafish should be a useful model. It was also suggested that the zebrafish may be a useful model for understanding the functions of glycans in the whole body. In the knockdown analyses of *zPOMT1* and *zPOMT2* by MOs, no glycosylated α-DG was detected in 48 hpf embryos (Figure 4C). Therefore, it appears that the enzymatic activity was completely lost. Furthermore, zebrafish α-dystroglycanopathy models may be useful to search for chemicals that treat α-dystroglycanopathies; the simple addition of candidate molecules to water could be developed as assays for theraputic effectiveness.

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 adults and kept in filter-sterilized fresh water at 28°C.

Cloning and sequencing of the full-length cDNAs

Total RNAs were purified from 24 and 48 hpf embryos by using QIAzol (Qiagen, Hilden, Germany), and cDNA fragments were generated by RT-PCR using oligo dT primer and Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Degenerated oligonucleotide primers were designed by mRNA sequence of zebrafish POMT1 (zPOMT1) (accession no. NM_001048067.2). zPOMT2 gene (accession no. AB281276) was cloned in a zebrafish embryonic cDNA library that was synthesized with a SMART cDNA Library Construction Kit (Clontech, Mountain View, CA). Both zPOMT1 and zPOMT2 genes were amplified with the forward primers, 5′-atgcagtgtgttaaactgcccgtcagtgt-3′ and 5′-atggatgtcagaccgaaggagaatttc-3′, and the reverse primers, 5′-ttagcgtttgcgtaagagaatatcccaactctc-3′ and 5′-ctaaaactcccaggattccatccacc-3′, respectively. The amplified cDNA fragments were cloned into pT7Blue vector (Novagen, Madison, WI), and the sequences were confirmed by CEQ^{TM} 2000 DNA Analysis System (Beckman Coulter, Inc., Fullerton, CA) with a DTCS Quick Start kit (Beckman Coulter, Inc.). The nucleotide sequence was subjected to the basic local alignment with a BLAST search provided by the National Center for Biotechnology Information. The sequences were obtained from GenBank and aligned using CLUSTAL W (Thompson et al. 1994). A phylogenetic tree was generated using the neighbor-joining method. TREEVIEW software generated visual representations of clusters (Page 1996).

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 zPOMT1 and zPOMT2*. Quantitative PCR was carried out with SYBR Green Realtime PCR Master Mix (TOYOBO Co. LTD., Osaka, Japan). Two microliters of cDNA (0.1 μg/μL) was used for a template. The primers used to detect the messages of *zPOMT1* and *zPOMT2* were 5′-tgttggctgtgctgtcttacc-3′ (forward) and 5′-catggctcaaggttcgatctc-3′ (reverse), 5′-cctcatgtatgttgggatgagac-3′ (forward) and 5′-gaaccaagagcagcacagaac-3′ (reverse), respectively. The primers for *zβ-actin2* and *zCox1*—5′-agttcagccatggatgatgaaa-3′ (forward) and 5′-accatgacaccctgatgtct-3′ (reverse), 5′-ttggccacccagaagtctac-3′ (forward) and 5′-gctcgggtgtctacatccat-3′ (reverse), respectively—were used as internal controls. Annealing temperatures were 63°C for *zPOMT1* and *zPOMT2*, 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

Antisense probe synthesis was performed using a Digoxigenin (DIG) RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland). Zebrafish embryos were collected after spawning and maintained at 28°C. Embryos at 0, 6, 12, 18, 24, 48, 72 and 96 hpf were fixed with 4% paraformaldehyde (PFA)-phosphate-buffered saline (PBS), dehydrated and kept in methanol