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# Transcriptional regulation and O-GlcNAcylation activity of zebrafish OGT during embryogenesis ☆

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**Abstract**

Zebrafish OGT (zOGT) sequence was identified in zebrafish (*Danio rerio*) genome and six different transcriptional variants of zOGT, designated *var1* to *var6*, were isolated. Here we describe the developmental regulation of zOGT variants at transcriptional level and characterization of their OGT activities of protein O-GlcNAcylation. OGT transcriptional variants in zebrafish were differentially generated by alternative splicing and in particular, *var1* and *var2* were contained by 48 bp intron as a novel exon sequence, demonstrating that this form of OGT was not found in mammals. Transcript analysis revealed that *var1* and *var2* were highly expressed at early phase of development including unfertilized egg until dome stage whereas *var3* and *var4* were begins to be expressed at sphere stage until late phase of development. Our data indicate that *var1* and *var2* are likely to be maternal transcripts. The protein expression assay in *Escherichia coli*–p62 system showed that OGT activities of *var3* and *var4* were found to be only active whereas those of other variants were inactive.


*Keywords:* z*O*-GlcNAc transferase; Transcriptional variants; *O*-GlcNAc; Developmental regulation

Nucleocytoplasmic protein O-GlcNAcylation is proceeded by *O*-GlcNAc transferase (OGT) and in mammalian cells, a variety of proteins have been known to be modified by single GlcNAc residue through the unique action of OGT [1–4]. Functional role of OGT has been known to be involved either directly or indirectly in many biological processes, such as dynamic interplay with phosphorylation of signaling factors [5–7], cytoskeletal organizations [8], and hexoseamine biosynthetic pathway [9,10]. OGT has been known to be localized both in cytosol and nucleus [11] either alone or as a complexed form with other molecules [7], and also in mitochondria [12].

Recently, it has been reported that OGT-mediated protein O-GlcNAcylation may function in cellular sensing of nutrients [13], toxic stresses [14], and heat [15]. Moreover, several studies showed that O-GlcNAcylation could be linked to oocyte maturation in xenopus [16,17]. However, the role of OGT during embryo development is poorly understood. During past several years, cDNAs encoding OGT have been cloned in mammalian species including human [4], rat [3], and mouse [18], and also reported in other species such as *Drosophila*, xenopus, and plant [19]. However, little information is available for zebrafish OGT (zOGT).

Zebrafish is now well utilized as a model system of vertebrates to identify gene-function during embryogenesis [20,21]. We searched the whole genome of zebrafish to find out a homolog of the mammalian OGT. Here, we show the zOGT sequence in genomic organization and its six different transcriptional variants, and demonstrate, for the first time, that these transcriptional variants of zOGT are differentially regulated during development. Furthermore, we expressed these transcriptional variants and analyzed their OGT activities in *Escherichia coli*–p62 system.

## Materials and methods

*Bioinformatics.* The zebrafish genome search was initially performed using human OGT sequence (NM_181672) and rat OGT

☆ *Abbreviations:* OGT, *O*-GlcNAc transferase; zOGT, zebrafish *O*-GlcNAc transferase; GlcNAc, *N*-acetyl-glucosamine; hpf, hour post fertilization.

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sequence (NM_017107) as query by BLAST search programs at Zebrafish Genome Browser (www.ensemble.org/Danio_rerio). The genomic sequence (contig BX323828.7.1-206011 on chromosome 14) with the closest similarity to mammalian OGT sequence was finally identified and translated protein sequences were further searched by BLAST X.

*Zebrafish embryo preparation.* Zebrafish embryos were maintained and prepared as previously described in the Zebrafish Book [22]. Briefly, based on morphological criteria of embryos, developmental stages were classified as hour post-fertilization (hpf). After natural spawning, embryos were collected and cultured in fishwater system containing 0.2 mM 1-phenyl-2-thiourea (PTU) to prevent pigmentation and 0.01% methylene blue to prevent fungal growth.

*Gene-specific primer sequences.* Standard PCR amplifications were performed using the following oligonucleotides:

Primer 1: 5′-AAGGATCCACCATGGCGAGCTCGGTGGG-3′
Primer 2: 5′-AACTCGAGTCAGGTGCTCTCGCTGGTCTCC-3′
Primer 3: 5′-GAGCTCGGTGGGGAACGT-3′
Primer 4: 5′-CCAGTGTGCTGAAGTGAGC-3′
Primer 5: 5′-GAGCTCGGTGGGAAACGC-3′
Primer 6: 5′-GCATGGGGTTCTGCTTGG-3′
Primer 7: 5′-ATGGTCAAGAGGTTGCTGAC-3′
Primer 8: 5′-GTCACCACAATGGTTCTGG-3′
Primer 9: 5′-TGTGATGATCAAGTGGCG-3′
Primer 10: 5′-CGGGTGGTCACCACAATT-3′
Primer 11: 5′-TTGTAGTATCTGGGACTGTAG-3′
Primer 12: 5′-TGGCCACCACACAG…ATTAAC-3′
Primer 13: 5′-TGCCAAGCTTTACTGCAAC-3′
Primer 14: 5′-TGCCAAGCTTTACTGCAATG-3′
Primer 15: 5′-AGATCTGGCTGGCCATCC-3′
Primer 16: 5′-GGGAACACCTCCAGTGC-3′
Primer 17: 5′-GAGGAGCACCCCGTCCTGCTCAC-3′
Primer 18: 5′-GATGGCTGGAACAGGGCCTCTGG-3′
Primer 19: 5′-AAAGGATCCATGGCGTCTTCCGTGGGCAAC-3′
Primer 20: 5′-TTTGTCGACTTATGCTGACTCAGTGACTTC-3′
Primer 21: 5′-TTTGTCGACTCAGGCTGACTCAGTGACTTC-3′
Primer 22: 5′-CCGGATCCATGAGTGGGTTTAACTTTGG-3′
Primer 23: 5′-CCCAAGCTTCTAGTCAAAGGCAATGCGCAG-3′

Primer 1 (forward)/primer 2 (reverse) (primer 1/2) is for zOGT full length amplification, primer 3/4 is for zOGT *var1*, *3* (234 bp) and *var2*, *4* (204 bp) amplification, primer 5/6 is for zOGT *var5* (257 bp) and *var6* (227 bp) amplification, primer 7/8 is for zOGT *var1*, *2* (256 bp) and *var3*, *4* (208 bp) amplification, primer 9/10 is for zOGT *var5*, *6* (218 bp) amplification, primer 11/13 is for zOGT *var1*, *2* (546 bp) amplification, primer 12/13 is for zOGT *var3*, *4* (531 bp) amplification, primer 12/14 is for zOGT *var5*, *6* (531 bp) amplification, primer 15/16 is for zOGT all variants (457 bp) amplification, primer 17/18 is for zebrafish β-actin (500 bp) amplification, primer 19/20 is for human OGT (full length) amplification, primer 19/21 is for rat OGT (full length) amplification, and primer 22/23 is for rat p62 (full length) amplification.

*DNA constructions.* Total RNA was purified from zebrafish embryo samples which were collected during 17 developmental stages by RNAzol B (Tel-Test) and cDNA library was synthesized using random hexamers (Takara) by M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Transcriptional variants of zebrafish OGT (zOGT) were cloned from cDNA library by PCR amplification using 50 pmol forward and reverse gene-specific primers, 200 μM dNTPs and 1.25 U exTaq (Takara-Shuzo) in the presence of 10 mM Tris–Cl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was carried out under the condition of 25 cycles of denaturation for 3 min at 94 °C, annealing for 30 s at 58–62 °C, and elongation for 30–40 s at 72 °C. zOGT *var1*, *2*, *5*, and *6* were cloned from cDNA of unfertilized egg, and zOGT *var3* and *4* were cloned from cDNA of 24 hpf embryo. Each variant of zOGT was cloned in pGEM-T easy vector (Promega) and further constructed in pMAL-c2 (New England Biolabs) for protein expression in *E. coli* system. cDNA sequences of human and rat OGT were cloned by RT-PCR using primer 19 (forward)/20 (reverse) with cDNA from H293 cells, and using primer 19/21 with cDNA from PC12 cells [15], respectively.

*Transcript analysis of zOGT variants by RT-PCR.* Six transcriptional variants of zOGT in 17 different developmental stages were analyzed using gene-specific primers by RT-PCR and quantitative RT-PCR. For analysis of total expression levels of zOGT, RT-PCR was performed using cDNAs of 17 stage-specific embryos and primer 15 (forward)/16 (reverse) resulting in 457 bp PCR product. Primer 11/13, and primer 12/13 were used for analysis of expression level of zOGT *var1* and *2* (546 bp), and for zOGT *var3* and *4* (531 bp), respectively. Finally, either primer 12/14 or primer 9/10 was used for analysis of expression level of zOGT *var5* and *6* (531 bp). Expression level of zOGT *var1* and *2* (256 bp), and zOGT *var3* and *4* (208 bp) was further analyzed by quantitative RT-PCR using primer 7/8 (Copy-I specific). Expression of exon 2a in Copy-I, and Copy-II genomic sequence was also analyzed by quantitative RT-PCR using primer 3/4, and primer 5/6, respectively (expected size of 234 and 257 bp in case of presence of exon 2a whereas expected size of 204 and 227 bp in case of absence of exon 2a). Primer 9/10 was used for competitive RT-PCR analysis for presence of exon 19 sequence on Copy-II genomic sequence (expected size of 218 bp in case of absence of exon 19 whereas expected size of 266 bp in case of presence of exon 19). For a control, zebrafish β-actin was amplified using primer 17/18 resulting in 500 bp PCR product.

*Southern blot analysis of genomic DNA.* Genomic DNA was prepared from zebrafish fin and muscle tissues as previously described [22]. Ten micrograms of genomic DNA was digested overnight with 50 U *Bam*HI (Roche) and then, digested DNA was separated on 0.8 % (w/v) agarose gel followed by ethidium bromide staining (0.5 μg/ml). Denatured DNA in gel was transferred to Hybond-$N^+$ nylon membrane (Amersham Biosciences) using a vacuum apparatus (Appligene). Hybridization probe was prepared by PCR using primer 11/13 and zOGT *var1* as a template. Resulting PCR product was digested with *Pst*I and *Kpn*I, and $^{32}$P-labeled using [α-$^{32}$P]dCTP (3000 Ci/mmol) by Ready-To-Go DNA labeling bead (Amersham biosciences) followed by separation on microspin G-50 column (Amersham biosciences). Hybridization using radioactive probe was performed according to standard protocols as described [23]. Hybridization and washing steps were carried out under high stringency conditions. Radioactive signals were visualized with a BAS2000 Image Analyzer (Fuji Photo Film).

*OGT activity assay in E. coli–p62 system.* Assay for OGT activity in *E. coli*–p62 system was newly developed as follows (unpublished). Full-length cDNA of p62 in expression vector of pET28a(+) (Novagen) and variant cDNA of zOGT including human and rat OGT in expression vector of pMAL-c2 (New England Biolabs) were co-transformed to BL21 (DE3) codon plus-RIL cells (Stratagene) according to manufacturer's instructions. Bacterial clone containing two plasmids was selected in presence of both 100 μg/ml ampicillin and 50 μg/ml kanamycin. Bacterial cell lysates were subjected to 10% SDS–PAGE and immunoblot analysis for *O*-GlcNAcylated proteins was carried out using RL2 antibody (Alexis, Switzland) as previously described [15]. Expression levels of OGT and p62 were confirmed by immunoblotting using anti-MBP-OGT antibody (rabbit polyclonal antibody prepared in our laboratory) and T7 tag antibody (Novagen). Immuno-signals were developed on Super RX film (Fuji) using SuperSignal West Pico chemiluminescent substrates (Pierce).

*Phylogenetic tree.* Raw DNA sequences were aligned using CLUSTAL_X [24] and neighbor-joining analysis were carried out using MEGA 3 [25]. Sequences (GenBank Accession No.) available to generate the phylogenetic tree are as follows. *Homo sapiens* transcript variant 1, NM_181672 [26], *H. sapiens* transcript variant 2, NM_181673 [26], *H. sapiens* mitochondrial form, U77413 [27], *Mus musculus*, NM_139144 [28], *Rattus norvegicus*, NM_017107 [3], *Xenopus laevis*, BC082353 [29], *Xenopus tropicalis*, NM_001024576 (direct submission), and *Drosophila melanogaster*, AF217788 (direct submission). The aligned data set are reproducible and significant.

## Results

### *Identification of six different zOGT variants in Copy-I and Copy-II genomic sequences*

During the search of zebrafish whole genome in order to find authentic zOGT sequence, we found two distinct genomic locations designated Copy-I (C-I) and Copy-II (C-II) containing the closest similar sequence to mammalian OGT. We identified C-I and C-II genomic sequence to be localized in the contig BX323828.7.1-206011 on chromosome 14 where C-I is localized at the genomic sequence of 15752823 through 15732518 and C-II begins at 15712498, and there is around 20 kb gap between C-I and C-II (Fig. 1B), suggesting that gene duplication might occur during evolution. Relative location of exons and introns in zOGT sequence of C-I and C-II is illustrated and compared with human OGT (Fig. 1B). These results demonstrate, for the first time that a novel form of zOGT is generated by alternative splicing of 48 bp sized exon 19 that results in insertion of 16 additional amino acids near the COOH-terminus in catalytic domain of zOGT, which is not found in mammalian OGT. Presence of two copies of zOGT in C-I and C-II was further confirmed by Southern blot analysis of *Bam*HI digested genomic DNA (Fig. 1C). Furthermore, transcripts-cDNA analysis of C-I and C-II genomic sequences revealed that six different variants of zOGT, designated *var1* to *var6*, were differentially expressed at transcriptional level (Fig. 1A). *var1* to *var4* were transcribed from the C-I genomic sequence whereas *var5* and *var6* were made from C-II genomic sequence (Fig. 1D), indicating that independent promoter specific to C-I and C-II transcription might be present. cDNA sequences of zOGT in C-I (*var1* to *var4*) and C-II (*var5* and *var6*) were compared in detail (Fig. 2), demonstrating that alternative splicing of exon 19 was uniquely processed in C-I transcription but not in C-II transcription whereas alternative splicing of exon 2a was processed in both C-I and C-II transcription. cDNA sequences of zOGT variants, *var1–var4* have been deposited to GenBank/EMBL database (Accession No.: *var1*, NM_001017359 (AY696178); *var2*, NM_001018105 (AY696179); *var3*, NM_001018106 (AY696180); *var4*, NM_001018107 (AY696181)).

### *Developmental regulation of six different zOGT variants*

To explore the expression level of six zOGT variants during development, RT-PCR and quantitative RT-PCR were performed using gene-specific primers (Fig. 2) and cDNAs from 17 embryonic stage-specific samples including unfertilized egg up to 120-hfp embryos (Fig. 3). To examine total expression patterns of zOGT, RT-PCR was carried out using primer 15 (forward)/16 (reverse), which resulted in 457 bp of PCR product, demonstrating that total expression levels representing the mixed state of six variants appeared not significantly fluctuated (Fig. 3A). Primer 11/13 was used in RT-PCR to detect expression levels of

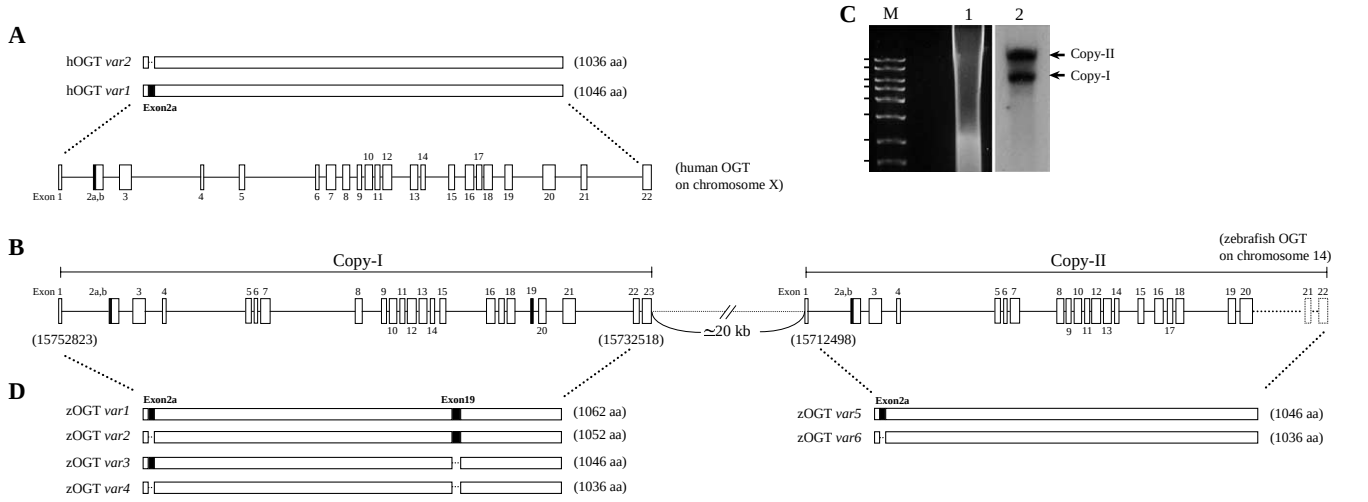


Fig. 1. Genomic organization of zOGT variants and their protein profiles are schematically illustrated. (A) Diagrams of hOGT variants, *var1* and *var2* polypeptide showing that *var1* is contained by exon 2a (indicated by black box) in $NH_2$-terminus whereas *var2* is not. Sizes of hOGT polypeptide of each variant are shown in parentheses. (B) Diagrams of genomic organization of zOGT in Copy-I and Copy-II showing numbers and relative sizes of exons and introns. Genomic sequence containing zOGT is shown to be composed of two distinct regions of Copy-I (beginning at 15752823) and Copy-II (beginning at 15712498) in chromosome 14 and is compared to hOGT in chromosome X. Exons are indicated by box where exon 2a and exon 19 are represented as black box. (C) Southern blot of zebrafish genomic DNA digested with *Bam*HI and probed with radiolabeled DNA fragment of zOGT. Agarose gel was stained with EtBr (lane 1) and hybridized bands were exposed on X-ray film (lane 2). Radioactive bands derived from Copy-I and Copy-II are indicated by arrows. DNA size markers are indicated at left bars (lane M) (10, 8.0, 6.0, 5.0, 4.0, 3.0, 2.0, and 1.5 kb DNA fragments). (D) Diagrams of zOGT polypeptides of *var1*, *var2*, *var3*, and *var4* transcribed from Copy-I genomic sequence, and zOGT polypeptides of *var5* and *var6* transcribed from Copy-II genomic sequence. Insertion or deletion of exon 2a and exon 19 in zOGT polypeptides is indicated by black box, and dotted line, respectively. Sizes of zOGT polypeptide of each variant are shown as amino acid residues in parentheses.

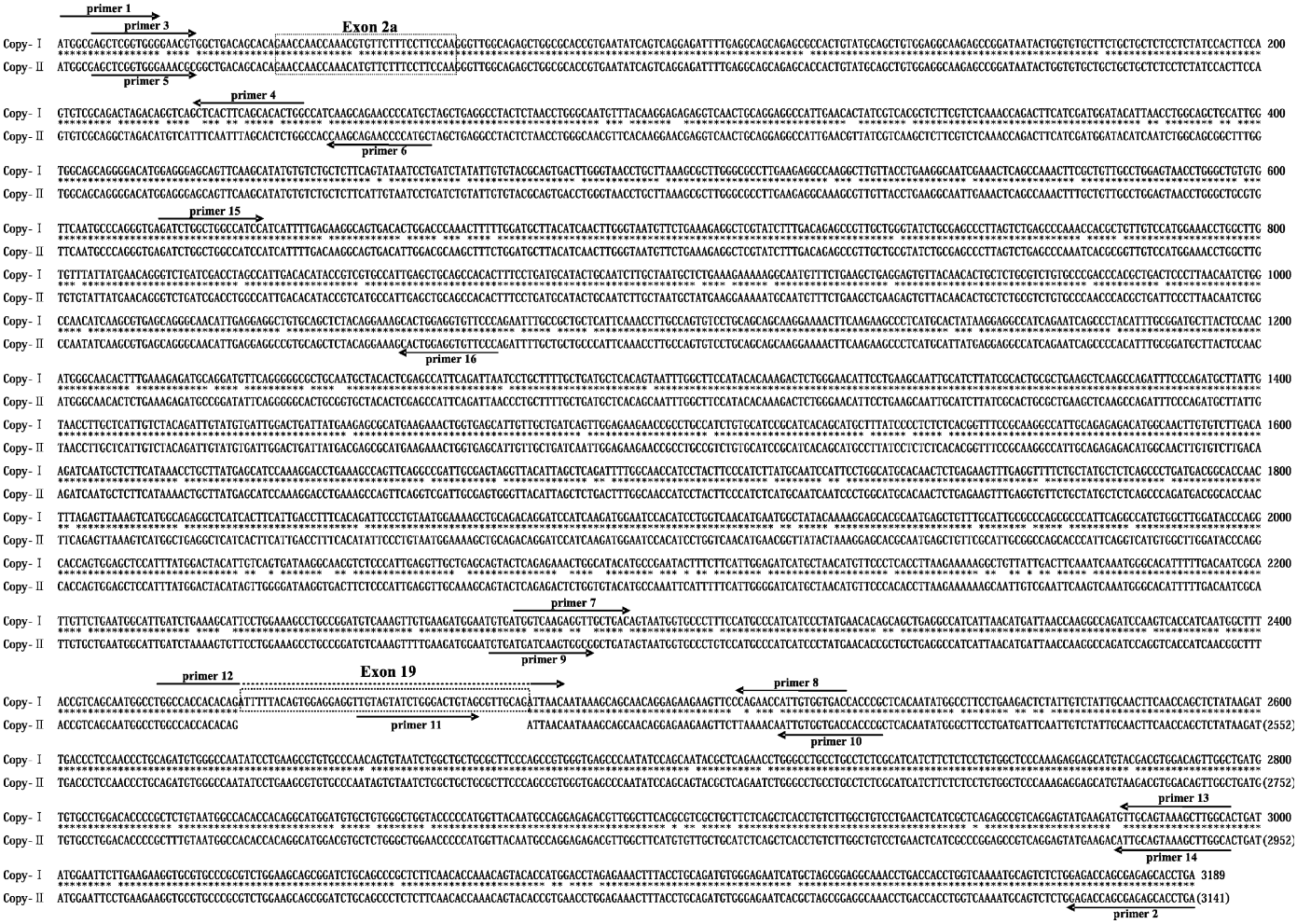


Fig. 2. Comparison of cDNA sequence of zOGT in Copy-I and Copy-II in which numbering starts at ATG codon and primers used in PCR are indicated with sized arrows with own specific numbers. Exon 2a and exon 19 are represented as box with dotted line. Sequence identities are indicated with asterisks (*).

*var1* and *var2*, and interestingly, expression of zOGT *var1* and *var2* was substantially high at early stages including unfertilized egg until dome or sphere stage and gradually repressed in subsequent stages (Fig. 3B). These results strongly indicate that transcripts of *var1* and *var2* could be maternally supplied and processed by a unique alternative splicing resulting in the insertion of a novel exon 19 sequence. In contrast, expression of zOGT *var3* and *var4* by RT-PCR using primer 12/13 showed exactly reversed patterns of *var1* and *var2* (Fig. 3C). We further verified these expression patterns to be consistently occurring by quantitative RT-PCR using primer 7/8 (Fig. 3D). Expression of zOGT *var5* and *var6*, by RT-PCR using primer 12/14, however, turned out to be very gradually decreased during entire stages (Fig. 3F). Next, to determine relative difference of transcription level between *var1* and *var2*, and between *var3* and *var4*, we further performed quantitative RT-PCR using primer 3/4, and primer 5/6, respectively, revealing that *var1*, not *var2*, was exclusively transcribed at early stages, and *var3*, not *var4*, was exclusively transcribed at late stages (Fig. 3E). Together, these findings suggest that expression of six variants of zOGT may be differentially regulated at transcriptional level during embryo development. Expression of β-actin was estimated as a loading control (Fig. 3G).

### *Protein O-GlcNAcylation of zOGT variants in E. coli–p62 system*

To investigate whether six variants of zOGT possess enzyme activity of protein O-GlcNAcylation in vivo, we newly established *E. coli*–p62 system (unpublished). zOGT var1–6, including human and rat OGT cloned in pMAL-c2 expression vector, were co-expressed in p62-transformed *E. coli* and bacterial cell lysates were analyzed by immunoblotting using RL2 antibody [15]. Surprisingly, our data showed that only *var3* and *var4* were functionally active whereas *var1*, *2*, *5*, and *6* were completely inactive. Particularly, these data suggest the possibility that insertion of 16 amino acid residues in *var1* and *2* might be deleterious on the catalytic activity of OGT (Fig. 4A). Furthermore, our data showed that capability

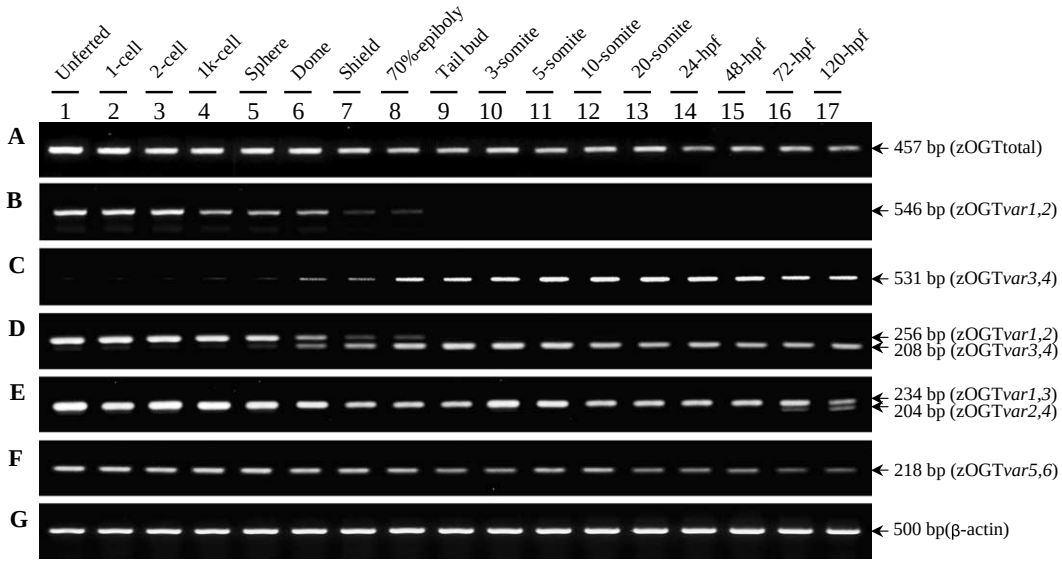


Fig. 3. RT-PCR analyses for expression of zOGT variants at transcriptional level during embryogenesis. (A) Amplifications of mRNAs extracted from zebrafish in 17 specific developmental stages (lanes 1–17) were performed by RT-PCR using primers 15 (forward)/16 (reverse) for analysis of total expression level of zOGT. (B) RT-PCR using primer 11/13 for expression of zOGT *var1* and *var2*. (C) RT-PCR using primer 12/13 for expression of zOGT *var3* and *var4*. (D) Quantitative RT-PCR using primer 7/8 for simultaneous analysis of exon 19 insertion in zOGT *var1* and 2, and *var3* and *4*. (E) Quantitative RT-PCR using primer 3/4 for simultaneous analysis of exon 2a insertion in zOGT *var1* and *2*, and *var3* and *4*. (F) RT-PCR using either primer 9/10 or primer 12/14 for expression of zOGT *var5* and *var6*. (G) RT-PCT using primer17/18 for expression of actin as a control. In each panel, PCR product is indicated by an arrow with expected size.

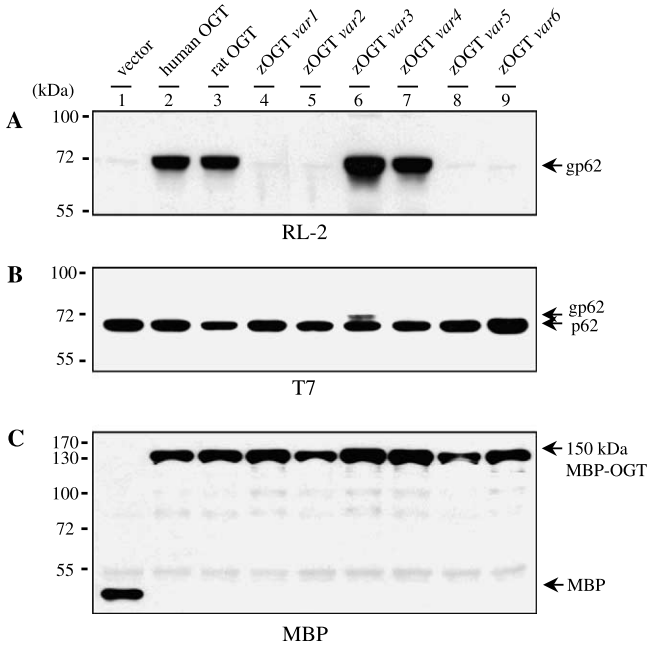


Fig. 4. In vivo OGT activities of transcriptional variants of zOGT. (A) cDNAs for zOGT variants were co-expressed with p62 substrate in *E. coli*–p62 system and GlcNAcylation of p62 was analyzed by immunoblotting using RL2 antibody. Reaction product produced by in vivo OGT action in zOGT transformants is designated gp62 and indicated with an arrow. As a control, OGT activities in transformants of cDNAs of mammalian OGT (human and rat) and mock transformants of vector alone were assayed (lanes 1–3). (B) Expression levels of p62 (T7-tagged) in all OGT transformants were analyzed by immunoblotting using T7 antibody. p62 and gp62 are indicated with arrows. (C) Expression levels of OGT (MBP-fused) in all OGT transformants were analyzed by immunoblotting using MBP antibody. Expressions of OGT and MBP are indicated with arrows.

of protein O-GlcNAcylation toward p62 substrate of *var3* could be more active than that of *var4* (Fig. 4A, lanes 6 and 7). As a control, expression levels of p62 and OGT were determined by immunoblot analysis using T7 antibody for p62 (Fig. 4B) and MBP antibody for OGT, respectively (Fig. 4C), demonstrating that expression levels of substrate and enzyme in vivo were similar in all transformants.

### *Comparison of zOGT protein sequence and phylogenetic analysis*

cDNA translated protein sequences of zOGT in C-I (1062 aa) and C-II (1046 aa) were aligned with human OGT (1046 aa), showing that in C-I, 16 amino acid residues near the COOH-terminus could be inserted or not inserted by alternative splicing to generate a new form of OGT (Fig. 5A). Phylogenetic analyses of OGTs among several different species were performed using CLUSTAL_X (version 1.83) for alignments of amino acid sequences followed by pairwise distance analyses of neighbor-joining method using MEGA 3 (Fig. 5B).

## Discussion

Zebrafish is well known as a mainstream model system for the understanding of vertebrate development and also, it has been used as alternative model species for identification of genetic factors in human diseases [20]. Here we have identified six different transcriptional variants of zOGT sequence, designated *var1* to *var6*, in zebrafish and analyzed

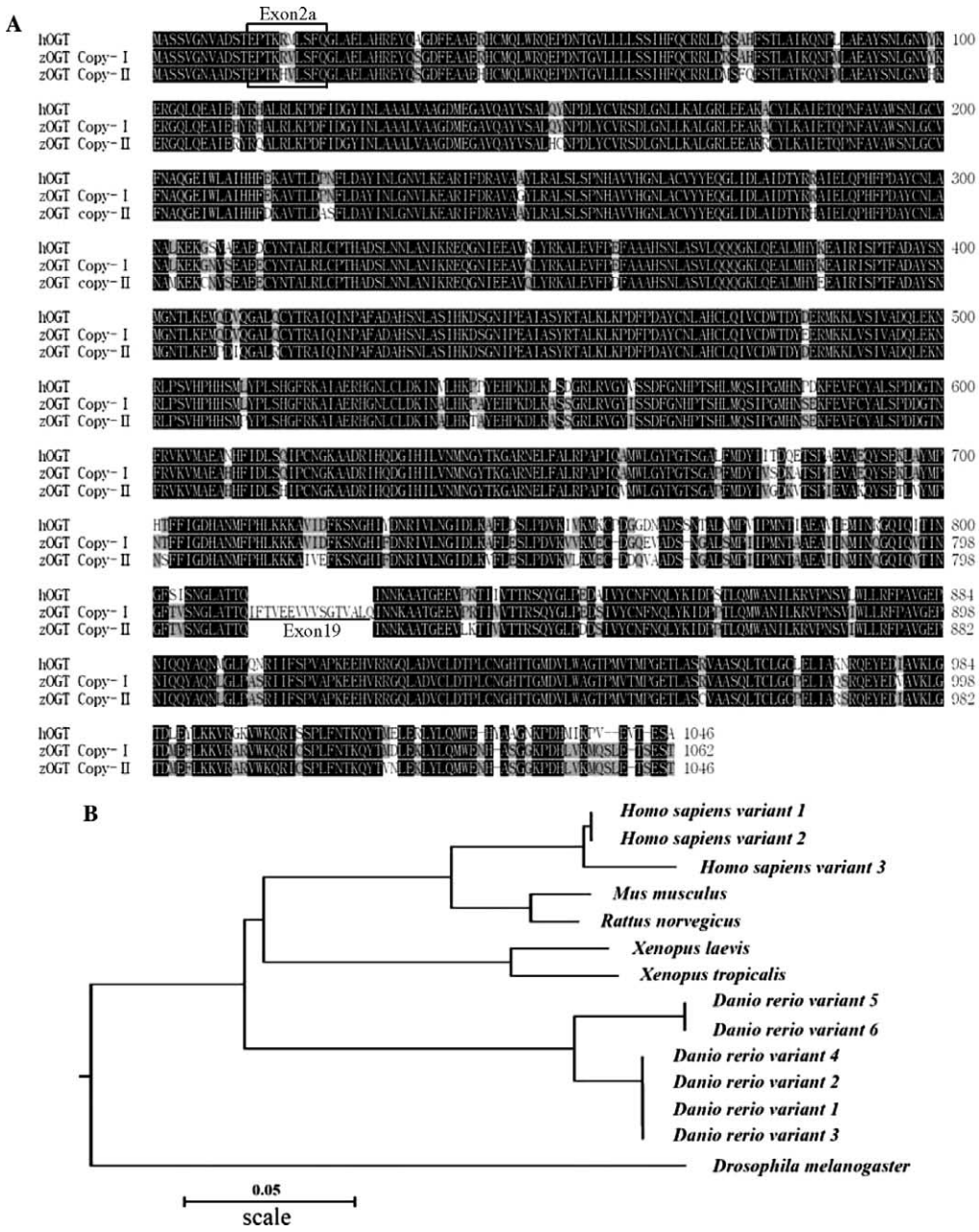


Fig. 5. Alignment of zOGT protein sequences and phylogenetic tree analysis (A) Amino acid sequences of zOGT from Copy-I and Copy-II were compared to that of human OGT. Amino acids that are completely identical in three alignments are shaded in black, and amino acids that are partially identical are shaded in gray. Sequences of exon 2a and exon 19 are represented by box and underline, respectively. Absence of exon 19 motif in human OGT and zOGT of Copy-II is represented as an empty space in the alignment. (B) Amino acid sequences of OGT among species were analyzed by CLUSTAL_X followed by neighbor-joining methods using MEGA 3. The scale bar corresponds to 0.05 substitutions per 100 positions per unit branch. Sequences used to generate the phylogenetic tree are as follows. *H. sapiens* transcript variant 1 (NM_181672), *H. sapiens* transcript variant 2 (NM_181673), *H. sapiens* mitochondrial form (U77413), *M. musculus* (NM_139144), *R. norvegicus* (NM_017107), *X. laevis* (BC082353), *X. tropicalis* (NM_001024576), and *D. melanogaster* (AF217788).

expression patterns of these OGT variants at the RNA level during embryogenesis. Moreover, we have expressed zOGT variants in *E. coli*–p62 system and determined their OGT activities in vivo.

Based on the genome data search of zebrafish (*Danio rerio*), we have found two copies of zOGT genomic sequence, designated C-I and C-II, that are closely localized in chromosome 14. We have further confirmed the presence of two copies of zOGT on C-I and C-II by Southern blot analysis. It is interesting that six zOGT variants are differentially transcribed from C-I and C-II during embryogenesis, respectively, indicating that expression of these zOGT variants may be regulated embryonic stage-specifically during development (Fig. 3). *Var1* through

*var4* turned out to be transcribed from the C-I genomic sequence whereas *var5* and *var6* are generated from the C-II genomic sequence. It seems likely that C-I genomic sequence may be gene-duplicated to produce C-II as a paralog during evolution. In particular, comparison with other mammalian OGT shows that zOGT, such as *var1* and *var2*, contains a novel exon 19 which is not found in mammalian OGT. It is of interest to note that these transcriptional variants appear to be highly expressed at early stage of development including unfertilized egg until at dome stage. Considering the timing of major zygotic transcription [30] and beginning of midblastula transition (MBT) in zebrafish [31], these data strongly suggest a possibility that *var1* and *var2* may function as maternal transcripts to generate a novel form of OGT during early embryogenesis in zebrafish. Particularly, *var1* rather *var2* turned out to be a major transcript by quantitative RT-PCR (Figs. 3B and E). Interestingly, transcription patterns of *var3* and *var4* show exactly reverse fashion of *var1* and *var2*, whereas *var5* and *var6* are almost constantly expressed during entire stages of zebrafish development. And also, *var3*, rather *var4*, turned out to be a major transcript by quantitative RT-PCR. Together, these data suggest that during early development of zebrafish, alternative splicing to insert both exon 2a in $NH_2$-terminus and exon 19 in COOH-terminus might be predominantly activated. At late stages of development, however, alternative splicing to insert only exon 2a in $NH_2$-terminus could be continued, whereas splicing activity for insertion of exon 19 in COOH-terminus might be repressed.

To assess OGT activity in vivo, we have established *E. coli*–p62 system in which p62 is co-expressing with OGT followed by immunoblot analysis of *O*-GlcNAcylated p62 (unpublished). OGT activities of *var1* and *var2* determined in *E. coli*–p62 system are non-functional while those of *var3* and *var4* are normally active as much as human and rat OGT (Fig. 4A), demonstrating that an insertion of sixteen amino acids encoded by exon 19 completely abolishes the conventional OGT activity. Based on sequence comparison with *var3* and *4*, and mammalian OGT, it is very likely that insertion of exon 19 in zOGT may change the enzyme specificity of protein substrates. We are currently investigating this hypothesis. These results also indicate that normal OGT activities of *var3* and *var4* might be more necessary at late stages than at early stages (before dome stage) including unfertilized egg during embryogenesis. To our knowledge, this is the first report to suggest that the expression of OGT can be developmentally regulated at the RNA level. Transcriptions of *var5* and *var6* seem to be almost constantly active during entire stages of development; however, their OGT activities are completely null. Based on the sequence comparison with mammalian OGT, it is very likely that *var5* and *var6* might contain multiple point-mutations in catalytic domain that might be crucial for OGT activity (Fig. 5A). Notably, our data suggest that insertion of exon 2a in *var3* results in more enhanced OGT activity than *var4* in which exon 2a is deleted (Fig. 4A, lanes 6 and 7) although the addition of exon 2a in $NH_2$-terminus of zOGT appears to not greatly affect OGT activity. These observations are further supported by identical levels of OGT and p62 expressions in transformants of *var3* and *var4* (Figs. 4A and C, lanes 6 and 7).

Protein O-GlcNAcylation in vivo by OGT is essential for cell viability in mammals and seems to be tightly linked to embryo stem cell division [18,28]. Our phylogenetic analyses of OGT among various species demonstrate that amino acid sequence of zOGT shows almost more than 90% homology to mammalian OGTs and also, protein domains of OGT are highly conserved among species (Fig. 5A). Therefore, it is very reasonable to consider that zOGT variants may critically be involved in zebrafish embryogenesis as in mammals. In the present study, our data demonstrate that a novel form of zOGT can be expressed by alternative splicing as a maternal transcript in early development of zebrafish including unfertilized egg. These results suggest a possibility that this novel zOGT may be specifically functioning during early embryo-development in zebrafish. Further investigation is necessary to find its function.

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