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Identification of Z-OTU protein during zebrafish oogenesis and early embryogenesis

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Abstract: Zebrafish (*Danio rerio*) Z-OTU, containing OTU and TUDOR domains, was predicted to be a member of OTU-related protease, a family of the deubiquitylating enzymes (DUBs). A previous report from our laboratory clearly describes the expression patterns of *z-otu* mRNA. Here, we characterized the Z-OTU protein during zebrafish oogenesis and early embryogenesis. After prokaryotic expression, the recombinant protein of the OTU domain and GST was purified and injected into rabbits to obtain the polyclonal antibody-anti-Z-OTU, which was used for immunohistochemistry in zebrafish ovaries and embryos. Interestingly, obvious differences existed between the expression patterns of *z-otu* mRNA and its protein during oogenesis and early embryogenesis. In stage I oocytes, *z-otu* mRNA was detected in cytoplasm while its protein existed in the germinal vesicle. In addition, its protein was distributed during entire oogenesis, while mRNA was not detected in oocytes at stage IV or mature oocytes. The *z-otu* mRNA disappeared after midblastula transition (MBT) and its protein gradually decreased after this stage. We inferred that Z-OTU protein, like other OTU-related protease with DUB activity, was required for germinal vesicle breakdown of oocytes during meiosis, germinal vesicle migration, and embryo cleavage maintenance.

Key words: *Danio rerio*; OTU-related protease; DUB; Z-OTU protein; Oogenesis; Embryogenesis

斑马鱼 Z-OTU 蛋白在卵子发生和胚胎发育早期的表达

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摘要：斑马鱼 *z-otu* 基因编码的蛋白可能具有 DUBs 活性, 它包含 OTU 和 TUDOR 结构域, 属于 OTU 相关蛋白酶家族的成员。该研究将原核表达的融合蛋白(OTU 结构域和 GST)纯化后免疫新西兰兔, 获得多克隆抗体 anti-Z-OTU, 并利用该抗体对 Z-OTU 蛋白质在斑马鱼卵子发生和早期胚胎发育过程中的表达进行了分析。根据原位和整体免疫组织化学检测结果并结合以前的研究结论, 分析并比较了 *z-otu* 基因的 mRNA 和蛋白质的分布, 发现在卵子发生和早期胚胎发育过程中, *z-otu* 基因的 mRNA 和蛋白质表达模式存在明显差异：mRNA 仅在卵子发生早期表达, 卵母细胞受精后才重新开始表达, 而其蛋白在卵子发生过程中均表达; 在卵子发生过程中, mRNA 分布于细胞质中, 而蛋白质先分布于细胞核中, 然后向细胞质迁移, 接着又向卵母细胞生发泡(germinal vesicle, GV)集中。推测 Z-OTU 蛋白类似于其他具有去泛素化酶活性的 OTU 相关蛋白酶, 对于卵母细胞减数分裂过程中生发泡破裂、生发泡迁移及维持胚胎的分裂是必需的。

关键词：斑马鱼; OTU 相关蛋白酶; 去泛素化酶; Z-OTU; 卵子发生; 胚胎发育
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The ubiquitin-proteasome pathway (UPP) is important during multiple steps of oocyte meiosis, fertilization, and early embryonic mitosis. Although numerous mammalian UPP studies have been conducted (Ben-Yehoshua Josefsberg et al, 2001; Huo et al, 2004; Sun et al, 2004), understanding of roles and regulatory mechanisms of UPP in zebrafish remains unclear. Deubiquitylating enzymes (DUBs) are important for generating free ubiquitin at various steps of the UPP, for processing inactive ubiquitin precursors, proofreading ubiquitin–protein conjugates, removing ubiquitin from cellular adducts, and keeping 26S proteasome free of inhibitory ubiquitin chains (Wing, 2003).

The OTU-related proteases are members of DUBs and belong to an OTU-like super-family that contains the ovarian tumor domain (OTU). This conserved motif encodes for a presumed catalytic core domain containing conserved Cys, His, and Asp residues (Makarova et al, 2000). Several OTU-related proteases have been identified. For example, Cezanne (Evans et al, 2003), the first demonstration of proteolytic activity in OTU proteins, can cleave ubiquitin monomers from linear or branched synthetic ubiquitin chains and ubiquitinated proteins. Balakirev et al (2003) observed that the OTU domain of otubains contained an active cysteine protease site. While A20, another OTU-related protease containing an N-terminal OTU domain, can cleave ubiquitin monomers from branched polyubiquitin chains linked through Lys48 or Lys63 and bound covalently to a thiol-group reactive ubiquitin-derived probe, with mutation of conserved cysteine residue in the catalytic site (Cys103) able to abolish these activities (Opipari et al, 1992; Evans et al, 2004).

The Z-OTU protein analyzed in this study belongs to the OTU-like super-family and may exhibit activity of DUBs for containing an OTU domain (Mo et al, 2005). Our previous work also determined that the zebrafish ovary-specific gene was expressed at early stages of oogenesis and embryogenesis (Mo et al, 2005). In this study, we investigated the distribution of the Z-OTU protein at different stages of zebrafish oogenesis, fertilization and early embryogenesis using immunohistochemistry and whole mount immunohistochemistry.

1 Materials and Methods

1.1 Preparation of polyclonal anti-Z-OTU antibody

DNA fragments of the OTU domain were amplified using primers of OF (5'-AGCATGGACGAGTACCTGG-3') and OR (5'-ATCCTCAACTCCCAGAACACG-3') (Fig.1) with an *Eco*RI or an *Xho*I restriction site, respectively. The fragments (*otu1*) were cloned into pGEX-6p-1 vector and expressed in the prokaryotic system. The recombinant protein of the OTU domain and GST was purified and injected into rabbits to obtain the polyclonal antibody–anti-Z-OTU. The anti-Z-OTU titer in the serum and the antibody specificity were then checked by western blot analysis (Yang et al, 2010). The immune and pre-immune sera (obtained before stimulation) were stored in aliquots at –20°C until use.

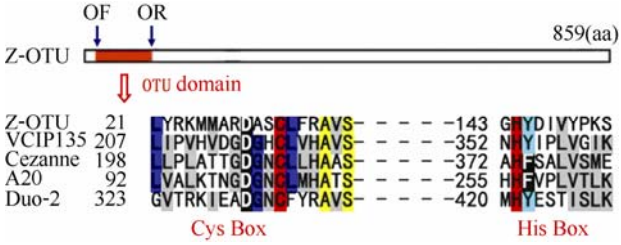

Fig. 1 Locations of primers for amplifying *otu1* and OTU domain alignments of Z-OTU, VCIP135 (Uchiyama et al, 2002), Cezanne (Evans et al, 2003), A20 (Opipari et al, 1992) and duo-2 (Sieburth et al, 2005)

OF: *otu1* forward primer; OR: *otu1* reverse primer; CYS BOX: Cysteine region; HIS BOX: Histidine region.

1.2 Immunohistochemistry

Ovaries from adult zebrafish were collected and embedded in paraffin, then 4 μm sections were cut and treated with 0.3% H_2O_2 in 50 mM PBS for 30 min after dewaxation and rinsed extensively with PBS. These sections were then treated with normal non-immune serum. All antibodies were diluted with 50 mM PBS containing 0.05% Triton X-100 and 5% normal goat serum. Sections were incubated with anti-Z-OTU (1:200) overnight at 4°C, rinsed with PBS and incubated in Biotin-conjugated second antibody for 15 minutes at room temperature, rinsed again with PBS, incubated in streptavidin-peroxidase for 15 minutes at room temperature, rinsed with PBS, and incubated with DAB solution (Maixin Company, Fujian, China) for 5 minutes. Following staining and rinsing with PBS, the sections were stained with hematoxylin and mounted onto glass slides with cover slips and viewed with an Olympus vanox microscope. As a negative control, the first antibody was replaced in diluted preimmune serum.

1.3 Whole-mount immunohistochemistry

The protocol of whole-mount immunohistochemistry was performed as per Braat et al (2000) with a little modification: Embryos were fixed in 4% paraformaldehyde

overnight at 4°C, washed four times for 15 min each in PBT (1% Tween-20 in PBS) and blocked overnight in block buffer (PBT containing 10% heat inactivated sheep serum) at 4°C. Anti-Z-OTU antibodies were diluted in block buffer (1:200) and preabsorbed overnight at 4°C against aceton powder of adult fish from which the gonads had been removed. After discarding the block buffer and adding the antibody, the embryos were incubated overnight at 4°C. After washing in PBT (ten times for 10 min each at room temperature), the embryos were incubated in anti-rabbit IgG-FITC antibody produced in sheep (sigma) diluted in block buffer (1:60) overnight at 4°C. After washing in PBT, the embryos were analyzed on a fluorescence microscope (Olympus).

2 Results

To test Z-OTU protein expression during oogenesis, we generated anti-Z-OTU antibodies for immunohistochemistry to examine the localization of Z-OTU in oocytes and embryos. To determine Z-OTU antibody specificity, western blot analysis showed that Z-OTU antiserum recognized zebrafish Z-OTU (Fig. 2).

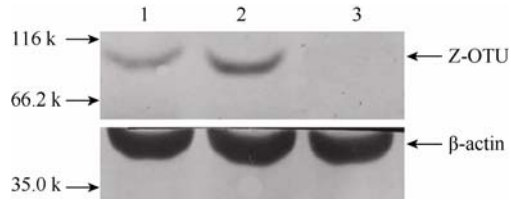

Fig. 2 Results of Z-OTU antibody specificity test and western blotting analyses

The antiserum of Z-OTU recognized a signal in zebrafish ovaries (lane 1) and two-cell embryos (Lane 2); the control used was pre-immune sera (Lane 3). The molecular weight of Z-OTU was 96.5 X 10^3. Equal loading was observed with β-actin antibody.

Our data revealed that Z-OTU protein existed in oocytes throughout oogenesis in adult zebrafish ovaries. In stage I oocytes, the positive signal of Z-OTU was localized in the germinal vesicle (GV) (Fig. 3A); in stage II and stage III oocytes, the Z-OTU protein was present in the perinuclear region, cytoplasm, and cell membrane, and the signal was very obvious (red arrow in Fig. 3B, C). Interestingly, most Z-OTU protein was concentrated in the perinuclear cytoplasm of the vegetative pole of the GV at stage IV oocytes (red arrow in Fig. 3C), while little signal uniformly existed in other cytoplasm and cell membranes. Our results also showed that anti-Z-OTU staining was absent in the cortical alveolus (blue arrow in Fig.3B). The pre-immune rabbit serum did not generate a positive signal (Fig. 3D, E).

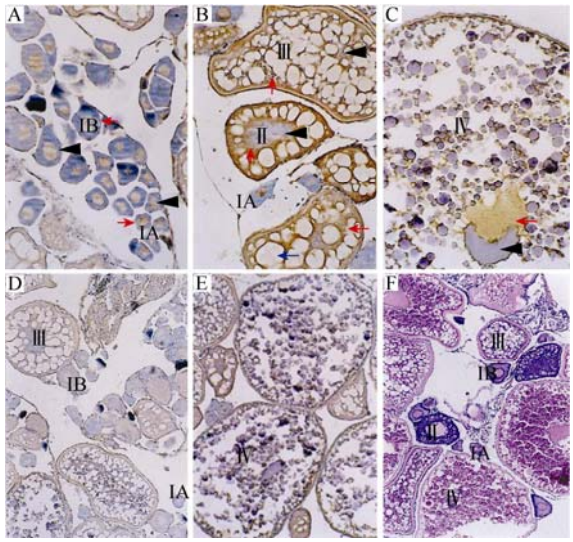

Fig. 3 Immunohistochemical staining of Z-OTU protein in zebrafish oocyte

Ovarian sections were treated with rabbit anti-Z-OTU antibody (A - C) (×40, ×40, and ×100, respectively) or pre-immune antiserum (D and E) (×40). F (×40): H & E stain. IA: the prefollicle stage of the primary growth phase; IB: the follicle stage of the primary growth phase; II: the cortical alveolus stage; III: the vitellogenesis stage; IV: oocyte maturation stage. Black arrowheads show germinal vesicle (GV); Red arrows indicate the regions of positive signal of Z-OTU; Blue arrows indicate the cortical alveolus.

In zebrafish, egg activation and fertilization can initiate cytoplasmic streaming towards the animal pole, which is thought to result in the movement of determinants to the blastoderm (Jesuthasan and Strahle, 1997; Oppenheimer, 1936). To study the role of Z-OTU during this process, we examined its expression in mature oocytes prior to and after egg activation. The results showed that Z-OTU was distributed uniformly through the oocyte cytoplasm in inactivated mature oocytes (Fig. 4A). Upon activation with egg water, however, a dynamic redistribution of Z-OTU was detected (Fig. 4B - E): at 3 min after fertilization, Z-OTU aggregated in clusters throughout the yolk (arrow in Fig. 4B), and a positive signal was also detected in the emerging blastoderm (arrowhead in Fig. 4B); at 10 min post-activation, Z-OTU was detected in the yolk cell as aggregates and in the blastoderm (arrowhead in Fig. 4C); at 20 min post-activation, the aggregates in the yolk became larger (arrow in Fig. 4D) and were closer to the blastoderm (arrowhead in Fig. 4D); and at 30 min post-activation, Z-OTU was detected exclusively in the blastoderm and excluded from the yolk cell (Fig. 4E). Our results also indicated that the positive signal of Z-OTU was highlighted from 1-cell to 1000-cell stage embryos (Fig. 4E-4G). With further embryogenesis, the

signal decreased significantly (arrowhead in Fig. 4H, 4I, 4K). The preimmune rabbit serum did not generate a positive signal (data not shown).

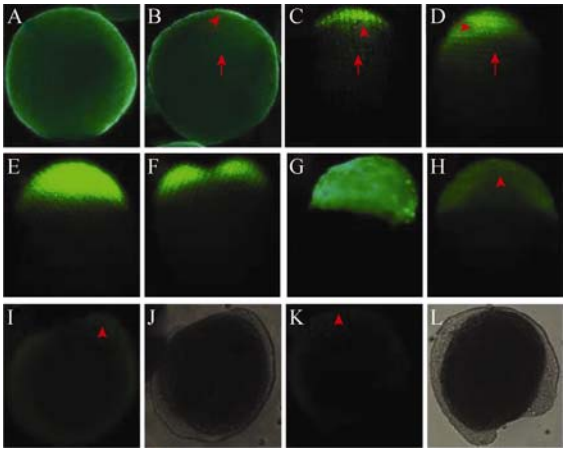

Fig. 4 Immunofluorescent detection of Z-OTU protein at early stages of zebrafish embryogenesis Embryos were incubated with rabbit anti-Z-OTU antibody

A: unfertilized embryo; B: fertilized embryo at 3 minutes; C: fertilized embryo at 10 minutes; D: fertilized embryo at 20 minutes; E: fertilized embryo at 30 minutes; F: 2-cell stage embryo; G: 1000-cell stage embryo; H: 30%-epiboly stage embryo; I: 5-somites stage embryo; J: Phase contrast photomicrograph of I; K: 13-somites stage embryo; L: Phase contrast photomicrograph of K. ×40, applies to A-L.

3 Discussion

We previously characterized the expression pattern of *z-otu* mRNA (Mo et al, 2005). In this study, we determined the locations of Z-OTU protein. According to our results, *z-otu* mRNA and its protein exhibited an overlapping pattern in temporal expression. As *z-otu* mRNA, Z-OTU protein was also expressed at the early stages of oogenesis and embryogenesis, but there existed three obvious differences. First, in stage I oocytes, *z-otu* mRNA was highly expressed in cytoplasm, while its protein had lower expression level in GV. Second, in stage IV oocytes, only Z-OTU protein was detected in the cytoplasm and formed an aggregate undergoing dynamic change with GV migration. This indicated that *z-otu* was not transcribed during this phase of oogenesis, but its protein still persisted. Lastly, *z-otu* mRNA disappeared after midblastula transition(MBT) while Z-OTU protein gradually decreased. This demonstrated that Z-OTU protein was still required for the development of embryos after zygotic transcription was activated. Previous studies have shown that vasa gene mRNAs and their proteins of zebrafish (Braat et al, 2000; Knaut et al, 2000) and Gibel carp (Xu et al, 2005) may have different expression patterns for different biological functions. Whether *z-otu* mRNA and its protein have different roles in zebrafish oogenesis and embryogenesis still requires further investigation.

Vertebrate oocytes are arrested at the diplotene stage of the first meiotic prophase, also termed the germinal vesicle (GV) stage. Previous research has confirmed that the reinitiation of first meiotic prophase requires the inactivation of the M-phase promoting factor (MPF), a complex of the cyclin-dependent kinase containing p34cdc2 and cyclin B (Murray and Kirschner, 1989; Dekel, 1996). Additionally, cyclin B1 degradation is dependent upon UPP (Tokumoto et al, 1997; Huo et al, 2004). We found that Z-OTU was localized in the GV of stage I oocytes and the signal was gradually enhanced from stage IA to stage IB where chromosomes decondensed and acquired a lampbrush appearance, a characteristic of the diplotene stage of first meiotic prophase (Pelegri, 2003). Before GVBD (between zebrafish stage III and stage IV oocytes), the Z-OTU protein was distributed in the cytoplasm of the oocytes (Fig. 3B). Upon meiosis reinitiation, Z-OTU was accumulated at the perinuclear cytoplasm of oocytes and migrated with GV (Fig. 3C). After fertilization, Z-OTU protein was uniformly distributed in the determinants and blastomere. This expression pattern of Z-OTU was similar to that of ubiquitin protein in mouse oogenesis and embryogenesis (Huo et al, 2004), where the ubiquitin protein was accumulated in the GV of GV-stage oocytes and distributed diffusely in whole oocyte shortly after GVBD. With the development of the oocytes, the ubiquitin protein again concentrated around the condensed chromatin and the localization of ubiquitin protein was diffused in the whole oocyte after meiotic resumption of the oocytes. During early embryonic mitosis in mice, the ubiquitin protein mainly accumulated in the nucleus and some staining of the ubiquitin protein was detected in the cytoplasm (Huo et al, 2004). These results suggest Z-OTU might be involved in zebrafish oocyte meiosis, fertilization and early embryonic mitosis. It also indicates that Z-OTU might have DUB activity and regulate UPP.

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