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The deubiquitylating enzyme Cops6 regulates different developmental processes during early zebrafish embryogenesis

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ABSTRACT Zebrafish *cops6* encodes a putative deubiquitylating enzyme (DUB) that belongs to the JAMM family. It consists of 297 amino acids and includes the Mov34/MPN/PAD-1 (PF01398) domain. Ubiquitylation is involved in many cellular processes and deconjugation of ubiquitin-modified substrates is important to maintain a sufficient amount of free ubiquitin in the cell. Here, we report our findings regarding the general function of the *cops6* gene, as a continuation of our previous studies involving DUB knockdown screening. We have found that *cops6* plays different roles in early embryonic development in the zebrafish, including dorsoventral patterning, convergent extension movement and brain formation. In addition, our findings indicate that *cops6* plays an anti-apoptotic role during segmentation. Overall, the present study that consolidates our previous work on zebrafish DUB genes, corroborates the hypothesis of multi-functional roles for DUB genes during development.

KEY WORDS: *Cops6, deubiquitylating enzyme, zebrafish, vertebrate development, anti-apoptotic factor*

Introduction

Protein modification by ubiquitin (UBQ) and/or ubiquitin-like (UBL) molecules is an important mechanism in regulating numerous critical cellular processes, such as signal transduction, transcriptional control, protein degradation, epigenetic modification and intracellular trafficking. Deconjugation of UBQ and/or UBL substrates is essential to maintain a sufficient free UBQ/UBL pool within the cell. Deubiquitylating enzymes (DUBs) play a key role in these processes (Hershko and Ciechanover, 1998). Recently, our lab performed an *in silico* genome-wide search and identified more than 90 putative DUB genes in the zebrafish genome (Tse *et al.*, 2009). Here, we report the results of further research on the the general functions of *cops6* (COP9 constitutive photomorphogenic homolog subunit 6) DUB gene, which belongs to the family of JAMM motif proteases (JAMM) family. The JAMM family is the only DUB class that consists of metalloproteases, while all other DUB families (USP, UCHL, OTU and MJD) are cysteine proteases (Nijman *et al.*, 2005). Based on our previous screen, there are 14 JAMM members in the zebrafish genome (Tse *et al.*, 2009). Only a few DUB genes related to disease, such as CYLD (Kovalenko *et al.*, 2003) and ATXN3 (Scheel *et al.*, 2003), have been well studied; the functions of the majority of them remain to be elucidated.

In our previous study, we carried out 85 DUB gene knockdown experiments by means of morpholino (MO) injection and classified the morphants into five groups (GI-GV) according to their *huC* expression patterns. *cops6* belongs to Group III, indicating that morphants exhibit a decreased and disrupted *huC* expression pattern (Tse *et al.*, 2009). Based on the earlier findings, we speculated that the *cops6* gene may play important roles in the early development of the zebrafish. Here, we demonstrate the developmental importance of the *cops6* gene. Having characterized the gene and described its expression pattern in zebrafish, we performed functional knockdown studies. On the basis of these results, we propose that *cops6* is required for dorsoventral patterning, convergent extension movement and brain formation and may act as an anti-apoptotic factor during zebrafish development. To our knowledge, this is the first study that characterizes various developmental functions of *cops6*, thereby providing evidence of the multi-functional roles of zebrafish DUBs.

Abbreviations used in this paper: Cops6, COP9 constitutive photomorphogenic homolog subunit 6; DUB, deubiquitylating enzyme; UBQ, ubiquitin; UBL, ubiquitin-like.

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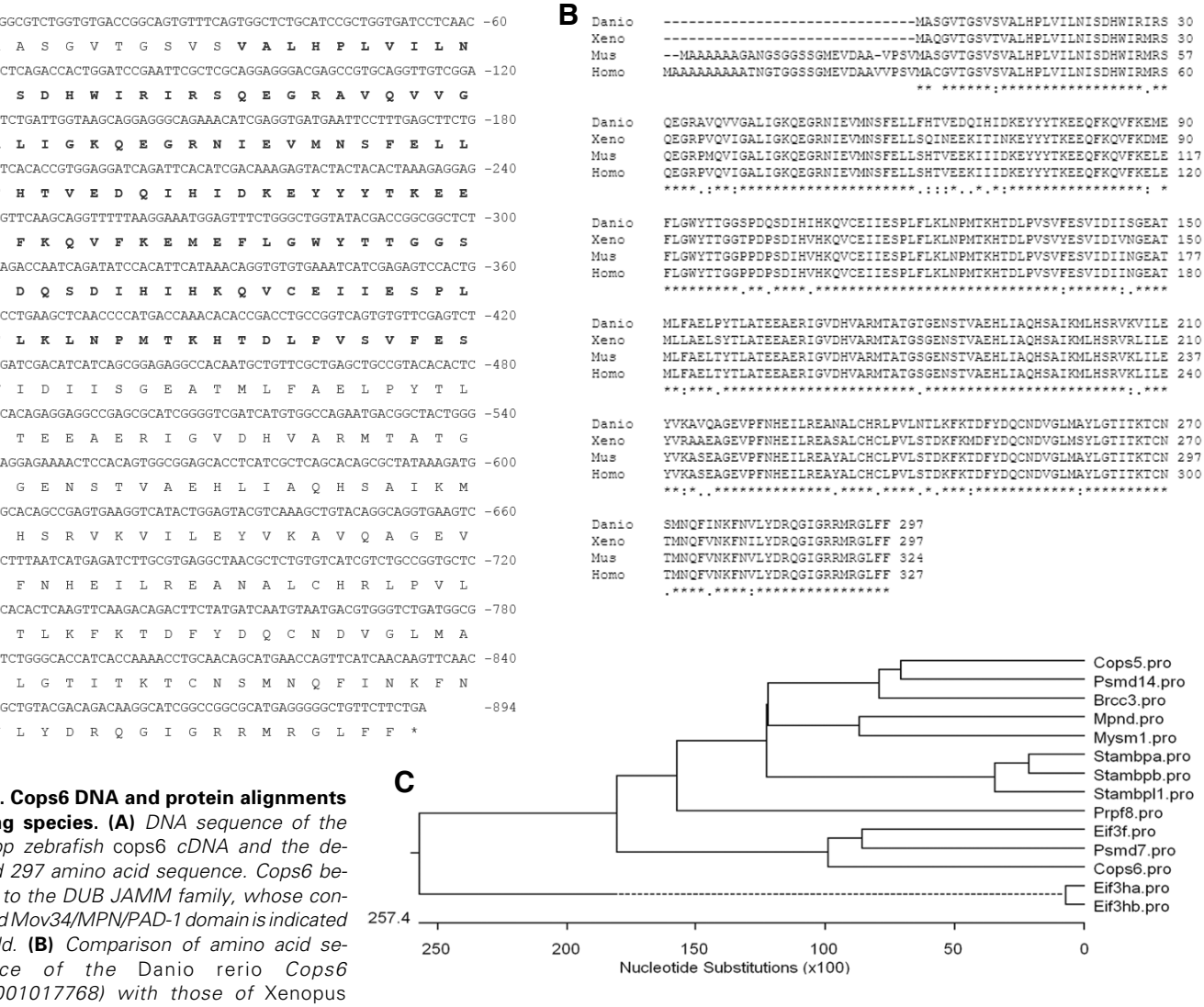


Fig. 1. Cops6 DNA and protein alignments among species. (A) *DNA sequence of the 894 bp zebrafish* cops6 *cDNA and the deduced 297 amino acid sequence. Cops6 belongs to the DUB JAMM family, whose conserved Mov34/MPN/PAD-1 domain is indicated in bold.* **(B)** *Comparison of amino acid sequence of the* Danio rerio *Cops6 (NP_001017768) with those of* Xenopus tropicalis *(NP_001006723),* Mus musculus *(AAH14286) and* Homo sapiens *(NP_006824). The Cops6 sequence is highly conserved (over 85%) among different species. There is an elongation of amino acids in the mouse and human COPS6s. Asterisks represent 100% identity; colons indicate 75% identity, including zebrafish sequence; periods indicate 50-75% identity, excluding the zebrafish sequence.* **(C)** *Protein alignments between Cops6 and 13 other DUB JAMM members in zebrafish. Eif3f (XP_685399) and Psmd7 (NP_956083) showed highest similarity to Cops6. However, they only have about 20% identity with Cops6, which mainly locate in the JAMM domain region. This result suggests that JAMM DUBs may not have evolved in parallel. The analysis was performed by Clustal V method using the Megalign program of Lasergene 6.*

Results

The Cops6 protein contains the conserved JAMM domain

cops6 is a DUB gene that belongs to the JAMM family. It has 297 amino acids, including the Mov34/MPN/PAD-1 (PF01398) domain (Fig. 1A). The domain is evolutionarily conserved among different species. Zebrafish Cops6 exhibits high sequence similarity (over 85% identity) among different species, from frog to human. An elongation of amino acids near the N-terminal occurred during evolution; thus, there are 27 amino acids more in mice and 30 more in humans. Nevertheless, the JAMM domain remains highly conserved (Fig. 1B). Protein alignments among zebrafish Cops6 and other JAMM DUB members revealed that two JAMM members, Psmd7 and Eif3f, share the highest similarity with Cops6 (Fig. 1C).

cops6 *is expressed throughout the body during early zebrafish development*

We examined the expression levels of *cops6* mRNA by RT-PCR and characterized its expression pattern by *in situ* hybridization at different developmental stages. No changes in *cops6* mRNA levels were observed at any of the developmental stages examined (Fig. 2A), suggesting that *cops6* is maternally expressed and zygotically maintained. *In situ* hybridization staining revealed that *cops6* was ubiquitously expressed and not spatially restricted during the early developmental stages, which corresponded to RT-PCR data (Fig. 2 B-F).

cops6 *is required for dorsoventral patterning and convergent extension movement in early zebrafish development*

To investigate the function of *cops6*, we first injected 600 pg

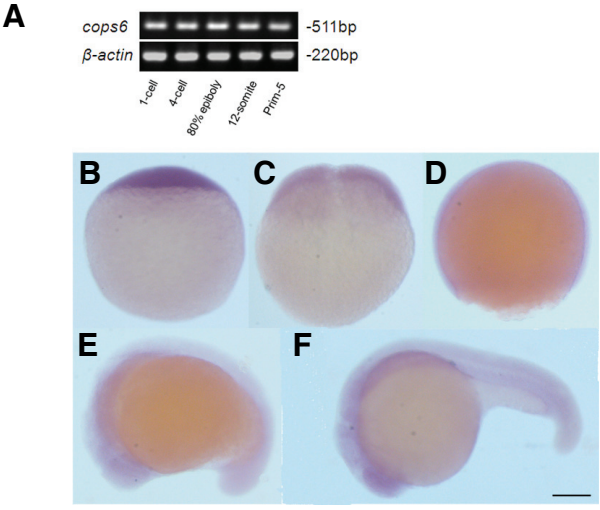


Fig. 2. Zebrafish *cops6* expression at early developmental stages. cops6 *started its expression at the 1-cell stage and was ubiquitously expressed throughout early developmental stages.* **(A)** *RT-PCR results.* **(B-F)** In situ *hybridization results of embryos at different developmental stages:* **(B)** *1-cell;* **(C)** *4-cell;* **(D)** *80% epiboly;* **(E)** *12-somite and* **(F)** *prim-5. Throughout early development,* cops6 *mRNA was expressed at similar levels and no specific expression pattern was observed. Scale bar: 150 μm* (B-F).

cops6 mRNA into single-cell embryos. However, no significant phenotypic changes were observed (Table 1). Next we used MO knockdown to study its loss-of-function effect. Over 80% of *cops6* morphants exhibited C3-C4 dorsalized phenotypes (Table 1), which consisted of the loss of the posterior regions during development (Fig. 3 A-F). In addition, rescue experiments were performed to address MO specificity. The knockdown effect of MO1, which targets ATG region, was rescued by *cops6* mRNA co-injection (Table 1; Fig. 3 A-I).

Phenotypes were further examined and verified by using a panel of *in situ* molecular markers at different stages. At 60-75% epiboly stage, one dorsal marker (*chd*) and one ventral marker (*eve1*) were used. Expression of *chd* is dorsally restricted, while *eve1* is expressed in the ventrolateral marginal cells. *cops6* morphants exhibited an expanded *chd* expression pattern and a contracted *eve1* expression pattern (Fig. 4 A-D). In addition, we examined *ntl* and *dlx3* expression by *in situ* hybridization to identify if knockdown causes aberrant convergent extension (CE) defects (Topczewski *et al.*, 2001). We found that the axial expression of *ntl* became broader at 60-75% epiboly (data not shown), while the *dlx3* expression domain (neural plate boundary) expanded at the 1-4 somite stage (Fig. 4 E-F), indicating that CE movement is also affected.

When the embryo reached the 12-14 somite stage, widening of the somite muscle in morphants became obvious (Fig. 4 G-H). This finding was corroborated using the somite marker, *myoD*. Thus, in morphants, reduced, diffused and laterally-expanded *myoD* expression was detected (Fig. 4 I-J). Using *pax2a*, which labels the presumptive neural region (Krauss *et al.*, 1991), we found that morphants exhibited widening of the trunk with increased lateral distance between otic vesicles and pronephroi (Fig. 4 K-L). Furthermore, shortening of the anteroposterior embryonic axis and a reduction of the longitudinal distance between the mid-hindbrain boundary and otic vesicles were observed in morphants (Fig. 4 K-L).

TABLE 1

PHENOTYPIC FREQUENCY OF RNA AND/OR MO INJECTION EXPERIMENTS

RNA/MO	pg/em (RNA)	pmol (MO)	n ep	n	C5 %	C4 %	C3 %	C2 %	C1 %	WT %
cops6-MO1		1.5	4	152		43	42	15		
cops6-MO2		2.5	2	88		33	32	23	12	
cops6-mRNA	600		2	53						100
cops6-mRNA+ cops6-MO1	600	1.5	3	103					17	83

Two *cops6* MOs were used to test the knockdown specificity. MO1, which targets the ATG site, showed a better knockdown efficiency than MO2 that targets the 5'-UTR. In addition, co-injection of *cops6* mRNA and *cops6*-MO1 could rescue over 80% of the dorsalized phenotype of morphants, indicating that *cops6*-MO1 knockdown is specific. Phenotypic frequency is indicated in the table. C1-C5 phenotypes represent dorsalized phenotypes as described in (Mullins *et al.*, 1996). Abbreviations: em, embryo; n ep, number of experiments; n, number of scored embryos.

cops6 *is critical in early zebrafish brain development*

cops6 has been classified into Group III on the basis of *in situ huC* screening, indicating that its morphants have fewer and unorganized neurons (Tse *et al.*, 2009). In this study, we further investigated its role in brain development. Brain regionalization, which is an important step for brain development, involves the action of a variety of transcription factors such as *Krox20, Otx2, Pax2* and *Eng2* (Joyner and Guillemot, 1994). In addition, the mid-hindbrain region has been suggested to be an organizing center for midbrain patterning and induction (Marin and Puelles, 1994) and its boundary (MHB) is important in restricting cell lineage

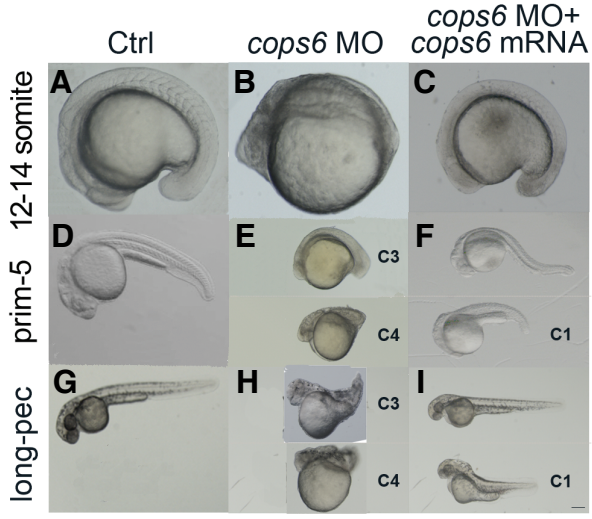
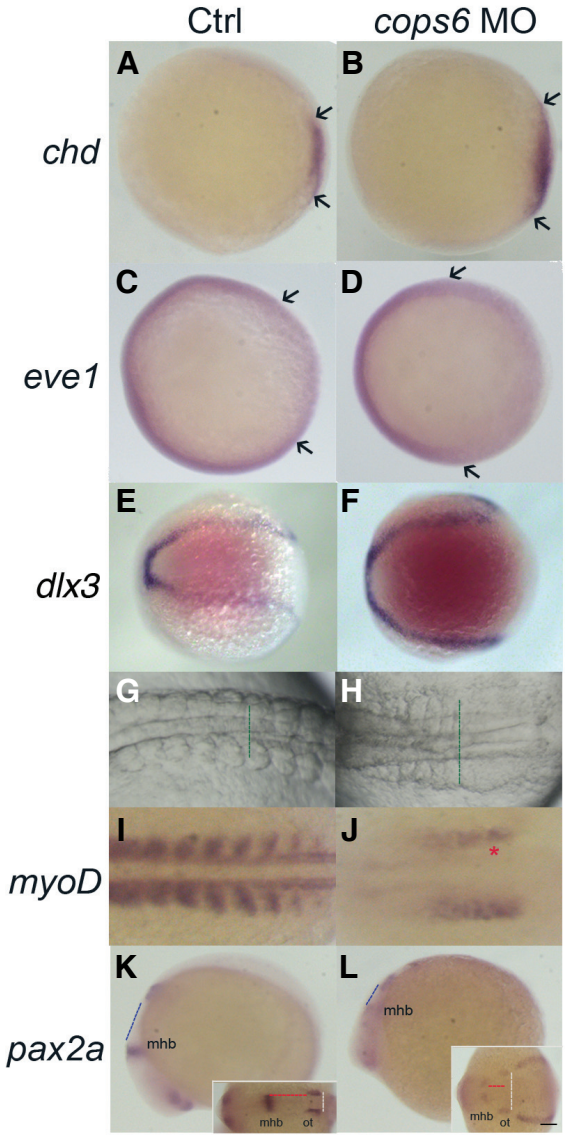


Fig. 3. Phenotypes of *cops6* morphants. cops6 *morphants in an AB wild-type background showed dorsalized phenotypes at different stages:* **(B)** *12-14 somite,* **(E)** *prim-5 and* **(H)** *long-pec. They all showed the reduced development of the posterior part, which is a feature of dorsalization. C3 and C4 dorsalized phenotypes are shown in* **(E,H)**. *The dorsalized phenotype caused by* cops6 *knockdown was rescued by co-injection of* cops6 *mRNA with MO* **(C,F,I)**. *More than 80% of dorsalized embryos were rescued back to a phenotype similar to wild-type, while the remaining ones showed a much milder phenotype, C1, in* **(F,I)**. *Scale bar: 100 μm (A,B), 120 μm (C), 80 μm (D), 150 μm (E,F,I), 90 μm (G) and 135 μm (H).*



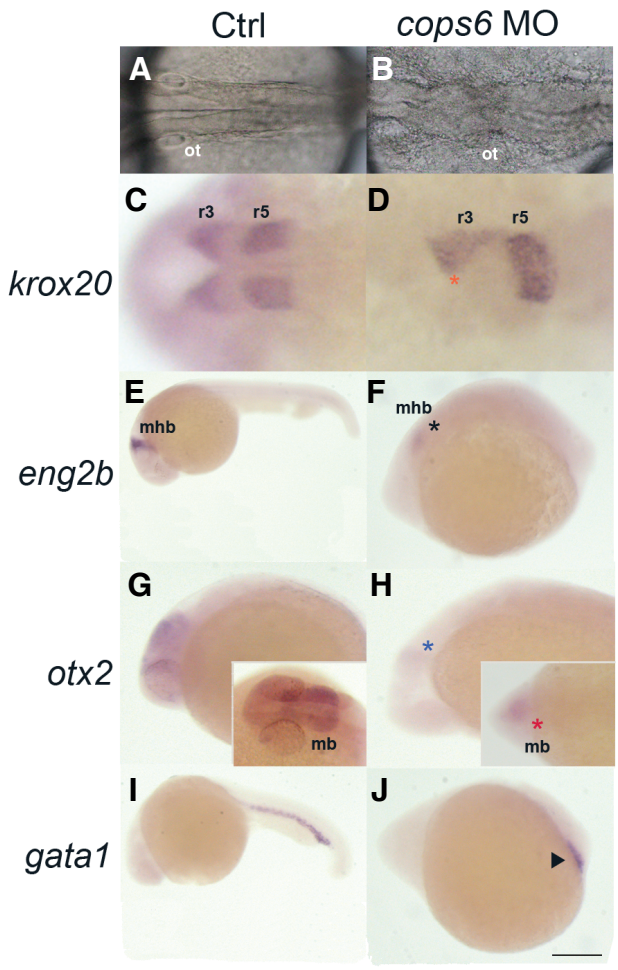


Fig. 4 (Left). Zebrafish *cops6* is required for dorsoventral patterning and convergent extension movement in early development. cops6 *morphants showed a wider expression pattern of* chd, *marked with arrows* **(A,B)**, *but a narrower expression pattern of* eve1, *marked with arrows* **(C,D)**; *animal pole views, dorsal towards the right at 60-75% epiboly stage. The* dlx3 *expression domain in* **(F)** cops6 *morphants was expanded at the 1-4 somite stage when compared to* **(E)** *control. Somite morphology of* **(G)** *wild-type and* **(H)** cops6 *morphants at the 12-14 somite stage. Lateral expansion of somite muscles was apparent (green dot lines). Furthermore,* myoD *expression in* **(J)** cops6 *morphants was widened and severely disrupted (red asterisk) when compared to* **(I)** *control.* pax2a *expression at the 12-14 somite stage* **(K,L)** *lateral view and (K–L, insert) dorsal view. Blue dotted lines represent the distance between the mid-hindbrain boundary and otic vesicles in the lateral view; while red dotted lines represent this distance in the dorsal view. Shortening of the lateral distance was found in* cops6 *morphants. On the other hand, white dotted lines indicated the distance between the two otic vesicles, where lengthening of the ventral distance was found in* cops6 *morphants. mhb: mid-hindbrain boundary; ot: otic vesicle. In all photos, the head is to the left. Scale bar: 70 μm* (A-D *and* G-J), *25 μm* (E,F) *and 150 μm* (K,L).

Fig. 5 (Right). Zebrafish *cops6* plays a role in brain development, but not in blood vessel formation. (A,B) cops6 *morphants in an AB wild-type background showed a brain defect at the prim-5 stage. The hindbrain was not well formed in* **(B)** cops6 *morphants when compared to* **(A)** *control; dorsal view.* **(C,D)** *Expression of* krox20, *a marker of rhombomeres three and five; dorsal view. Reduced size in rhombomere three (orange asterisk) and fused rhombomere five expression due to unfolded hindbrain were found.* **(E,F)** eng2b *expression that indicates the mid-hindbrain boundary (mhb) was reduced in* cops6 *morphants (black asterisk).* **(F)** cops6 *morphants had a smaller mhb when compared to* **(E)** *control; lateral view.* **(G,H)** otx2 *expression was reduced in* cops6 *morphants (blue asterisk in lateral view; red asterisk in dorsal view). The size of the midbrain was decreased in* **(H)** cops6 *morphants in comparison to* **(G)** *control.* **(I,J)** *Presumptive blood marker (*gata1*) did not show any significant differences between* **(I)** *control and* **(J)** cops6 *morphants. They both form a normal blood island (triangle mark), which suggests that* cops6 *is not required for blood development. mb: midbrain; mhb: mid-hindbrain boundary; ot: otic vesicle; r3/r5: rhombomere three/five. All photos are with head to the left. Scale bar: 48 μm* (A,B), *100 μm* (C,D), *265 μm* (E,I), *150 μm* (F,J) *and 170 μm* (G,H).

(Keynes and Krumlauf, 1994). We collected embryos at the prim-5 stage in which the essential brain morphology has been formed in zebrafish.

The hindbrain develops a series of rhombomeres along the anteroposterior axis of the neural tube. This morphological segmentation is visible in the zebrafish at the 18-somite stage (Moens

and Prince, 2002). Knockdown of the *cosp6* gene resulted in abnormal brain morphology (Fig. 5 A-B) and aberrant *krox20* expression (unfolded r5 and reduced r3) at the prim-5 stage (Fig. 5 C-D), indicating a disturbance in the brain development processes. In addition, *eng2b* was used to examine the MHB structure. *cops6* morphants showed a reduced MHB size. *eng2b* was expressed in a much more restricted area in morphants in comparison to controls (Fig. 5 E-F). Abnormal MHB patterning at an early developmental stage (12-14 somite stage) could also be observed by *pax2a* expression, which revealed an obvious gap in the MHB in morphants (Fig. 4 K-L). Finally, in order to verify if the midbrain structure is affected in *cops6* morphants, we examined *otx2* expression. *otx2* expression was greatly reduced in morphants, indicating a loss or reduction of the midbrain region (Fig. 5 G-H). In order to verify if the development of other mesodermal components is altered, we examined *gata1* expression and found no obvious expression change in the presumptive blood region in morphants, suggesting that blood development is not affected in *cops6* morphants (Fig. 5 I-J).

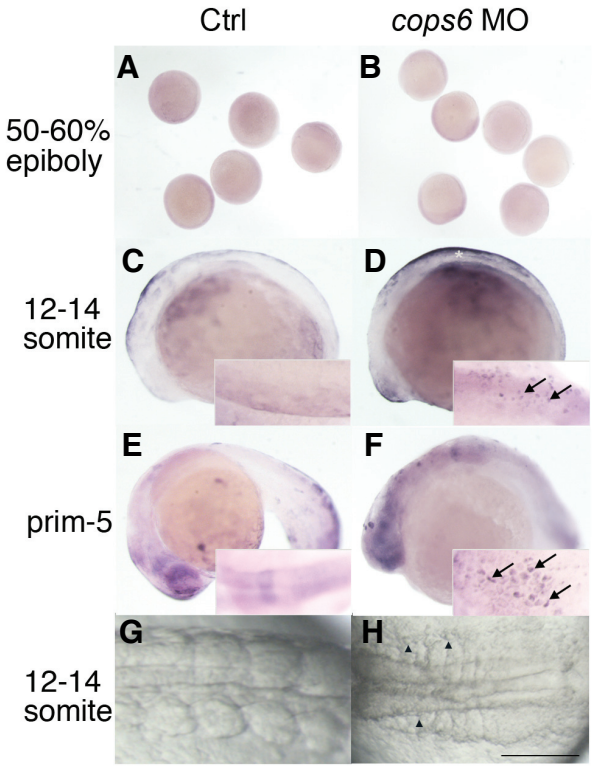


Fig. 6. Zebrafish *cops6* plays an anti-apoptotic role in early development. *TUNEL assay of wild-type embryos and* cops6 *morphants at different developmental stages. There were no significant differences between* **(A)** *control and* **(B)** cops6 *morphants at 50-60% epiboly stage. At the 12-14 somite stage,* **(D)** cosp6 *morphants showed increased positive staining of apoptotic cells in the trunk region (white asterisk) when compared to* **(C)** *control; lateral view. Apoptotic cells (arrows) at the same stage, dorsal view (insert), were found in* **(D)** cops6 *morphants, but not in* **(C)** *control. Afterwards, at* **(E,F)** *prim-5 stage, increased numbers of apoptosis-positive stained cells (arrows) were found only in* **(F)** cops6 *morphants.* **(G,H)** *At the 12-14 somite stage, the apoptotic dying cells, exhibiting a rounded-up cell body (arrowheads), were found in* **(H)** cops6 *morphants. Scale bar: 1250 μm* (A,B)*, 210 μm* (C-F) *and 150 μm* (G,H).

Cops6 plays an anti-apoptotic role during segmentation

On the basis of morphological observations, we suspected that *cops6* morphants may undergo increased apoptosis. During the early stage of development, morphology of *cops6* morphants did not show any significant differences and no obvious apoptotic cells were observed (Fig. 6 A-B). Starting from 12-14 somite stage, we observed an obvious increase in the number of TUNEL-positive apoptotic cells in the trunk region and the number of dying cells was apparently increased at the prim-5 stage (Fig. 6 C-F). Furthermore, rounded-up cells, a feature of dying cells, were found in the trunk region of morphants (Fig. 6 G-H), supporting the idea that Cops6 may have anti-apoptotic functions during early development.

Discussion

Zebrafish deubiquitylating enzyme, Cops6, is highly conserved in evolution. It consists of 297 amino acids with the Mov34/MPN/PAD-1 domain. In this study, we characterize the zebrafish *cops6* gene and provide evidence of its multi-functional roles in early zebrafish embryonic development. Knockdown of *cops6* results in defects in dorsoventral patterning, CE movement and brain development. In addition, our results point to an anti-apoptotic role for this enzyme in the developing embryos. Hence, Cops6 exerts multiple functions during zebrafish development.

Overexpression of *cops6* was not found to cause obvious morphological changes. Cases have been reported that gene overexpression does not lead to any phenotypic changes (Huang *et al.*, 2007). Nevertheless, the co-injection of *cops6* mRNA with its MO rescued MO-induced phenotypes, indicating that *cops6* mRNA is translated and functional (Table 1). This result may supports our previous finding that DUB genes can have redundant roles. Therefore, single knockdown of group IV DUB cannot compensate the effect of *bmp4* overexpression (Tse *et al.*, 2009).

It should be noted that the deubiquitylating activity of zebrafish Cops6 has not been biochemically demonstrated. Nevertheless, Cops6 is generally classified as a DUB member, thanks to its Mov34/MPN/PAD-1 domain, which has been shown to cleave the UBL protein, Nedd8, from the Cul1 subunit of SCF ubiquitin E3 ligases (Cope *et al.*, 2002). Thus, the DUB activity of Cops6 needs to be confirmed, and the substrates of this enzyme also need to be identified. In the light of the fact that its depletion results in multiple defects during zebrafish development, it is conceivable that Cops6 has several substrates that are required for corresponding developmental processes. Furthermore, a genomic search has revealed that the number of ubiquitin E3 ligases is far higher than that of DUBs (Nijman *et al.*, 2005; Tse *et al.*, 2009), which indicates that in general DUBs may have more substrates than E3 ligases. To better understand the function of Cops6 in zebrafish development, it will be necessary to identify its substrates in the future.

Materials and Methods

Fish strains and maintenance

The strain used in this study was the AB wild-type line. They were raised and staged as previously described (Kimmel *et al.*, 1995). All experimental procedures on zebrafish embryos were approved by the Biological Research Centre, A*STAR, Singapore (BRC IACUC No. 080390) and the Institutional Animal Care and Use Committee, National Health Research Institutes, Taiwan (NHRI-IACUC-098018).

Morpholino (MO) sequence site selection, design and specificity

The translation initiation site (TIS) of the Cops6 protein sequence was located by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul *et al.*, 1997), and all potential upstream sequences for MO target oligos were processed with AMOD (Klee *et al.*, 2005). Antisense MOs were selected based on the guidelines from Gene Tools (reviewed by Eisen and Smith, 2008). In addition to MO-1, targeting the ATG site (5'-CGGTCACACCAGACGCCATCACACT-3'), MO-2, targeting 5'-UTR (5'-GGCTCGCTGAACAGAAGAGTGGAGA-3'), was generated to confirm the knockdown phenotypes. Furthermore, *cops6* mRNA rescue experiments were performed to verify the knockdown specificity (Table 1). In addition, the MO-induced phenotypes were not associated with those non-specific effects caused by overdose MO treatment (unpublished observations). Unless specified, all the results were generated by MO-1.

Expression construct generation and mRNA* in situ *probe synthesis

cops6 was amplified with primers:
5'-CCGAATTCATGGCGTCTGGTGTGA-3' and
5'-CCCTCGAGTCAGAAGAACAGCCC-3', containing EcoRI and XhoI restriction sites respectively, from full-length cDNA using *Pfu* DNA polymerase (Stratagene) and ligated into pCS2+ to generate the pCS2+*cops6* expression construct. The construct vector was linearized by NotI; capped RNA was synthesized with the SP6 Message Machine kit (Ambion) and finally dissolved in DEPC-treated water. Antisense RNA probe was synthesized by using digoxigenin RNA labeling mix (Roche) and T7 polymerase (Promega).

Morpholino (MO) and mRNA injection

All MOs were purchased from Gene Tools, re-suspended in distilled water to make a 5 mM stock and stored at -20ºC. Diluted MOs and/or mRNA (amount listed in Table 1) were injected into one- or two-cell stage embryos. Embryos from four different pairs of fish were used for each MO and/or mRNA injection.

Whole-mount* in situ *hybridization (WISH)

Plasmids that were used to make antisense mRNA probes for *in situ* hybridization have been previously described elsewhere (Tse *et al.*, 2009; Ma and Jiang, 2007).

Detection of apoptotic cell death (TUNEL Assay)

Apoptotic cell death in zebrafish was detected according to the manufacturer's protocol (*In situ* Cell Death Detection Kit-AP; Roche).

RNA extraction, reverse transcription and RT-PCR

Embryos at different developmental stages were collected. Their total RNA was extracted by using TRIzol (Invitrogen). Purified RNA with an A260/A280 ratio of 1.8-2.0 was used. Briefly, 0.5 μg of total extracted RNA was reversely transcribed (iScript, Bio-Rad). Our data indicated that the amplification was specific. There was only one PCR product amplified for each individual set of primers. Control amplification was done either without RT or without RNA. RT-PCRs were conducted by using the PCR Core Kit (Roche) in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad). Primers for *β-actin* and *cops6* were

F: 5'-AGATCTGGCATCACACCTTC-3';
R: 5'-TCACCAGAGTCCATCACGAT-3' and
F: 5'-TCTGCATCCGCTGGTGATCC-3';
R: 5'-TCCTGTCCCAGTAGCCGTCA-3', respectively.

Protein alignment

Alignments were generated by the Megalign program of Lasergene 6.

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