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一、摘要：

蛋白質精胺酸甲基化為一轉譯後修飾，和其相關的細胞功能包括訊息傳遞、蛋白質胞內定位分布、DNA 修補以及轉錄調控等。我們在胚胎發育研究的模式生物斑馬魚中找到這些基因的異種同源基因，因此在本計畫中我們以斑馬魚為模式生物系統來闡析第一型 PRMT。我們選擇最主要表現的 PRMT1，及其同種同源基因 PRMT8 來研究。我們已研究各種 PRMT 於斑馬魚不同發育階段的表達，並已利用 morpholino 寡核苷酸(MO)來抑制 *zprmt1* 及 *zprmt8* 基因表現；我們觀察 *zprmt1* knock-down 胚胎早期變化，可見到其畸形率極嚴重程度隨劑量增加的情形。胚胎畸形包含體型彎曲、體軸變短、脊椎彎曲、卵黃形狀改變、發育遲緩等。斑馬魚 *zprmt1* morphant 的胚胎的確出現 PRMT1 蛋白質表現量下降，以及已知 PRMT1 受質如組蛋白 H4 的 R3 殘基上的精胺酸甲基量降低等情形。此外另一個斑馬魚精胺酸甲基轉移酶 PRMT8 的研究，*zprmt8* knock-down 胚胎早期也有體型彎曲、體軸縮短、心包膜嚴重水腫等變化。我們也已建構好不受 MO knock-down 之突變且可表達 *zprmt1* 及 *zprmt8* 的質體，並表達出各 mRNA，以注射回 mRNA 是否能彌補缺陷，研究這些演化上相關的 PRMT 基因是否功能上可以部分互補。此外甲基接受蛋白基因在斑馬魚系統中分析也配合進行中。本計劃研究結果對這幾種甲基轉移酶提供寶貴資訊。

關鍵詞: 蛋白質精胺酸甲基化，蛋白質精胺酸甲基轉移酶，斑馬魚，胚胎發育

Abstract

Protein arginine methylation has become one of the most intensively studied posttranslational modification for cell signaling, protein localization, DNA repair and transcriptional regulation. Protein arginine methyltransferase 1 (PRMT1) is the predominant type I methyltransferase in the mammalian system responsible for protein arginine methylation involved in various cellular functions. PRMT1 and PRMT8 are highly conserved with more than 90% sequence identity and are likely to be paralogues in vertebrates. We studied the expression of *prmt*1 and *prmt8* genes in zebrafish by RT-PCR, whole-mount in situ hybridization and western blot analyses. We also used morpholino-oliogonucleotides (MO) to specifically inhibit the expression of these *prmt* genes in zebrafish to observe the

effects of specific PRMT knockdowns during early developmental stages. PRMT1 protein level, type I protein arginine methyltransferase activity as well as specific asymmetric arginine methylation were reduced in the *zprmt1* morphants. Detailed marker analyses revealed defective medial-lateral convergence and anterior-posterior extension of the body plan and the abnormalities were even serious at the posterior than the anterior part of the embryo. Furthermore, the *prmt8* morphants showed significant shortened body-length, irregularly bent tails and serious heart edema. We had constructed plasmid to express the mutated MO-resistant *zprmt1* or *prmt8* mRNA. Rescue of the phenotypes by co-injection of different *prmt* mRNA should confirm the specificity and can help investigate whether the putative paralogues can complement each other. We also use the zebrafish system to study the substrate for PRMT. Our results confirm the importance of the methyltransferase in zebrafish early development and provide valuable insights for protein arginine methylation.

Key words:

Zebrafish, PRMT1, PRMT8, protein arginine methylation, antisense morpholino oligo, convergence/extension

二、 研究目的與背景

General introduction to the protein *N*-arginine methyltransferase (PRMT) gene family

Protein arginine methylation is a posttranslational modification involved in various cellular functions such as signal transduction, protein subcellular localization, transcriptional regulation protein–protein interactions and DNA repair (Bedford and Clarke., 2009). Since the identification of the first protein *N*-arginine methyltransferase (PRMT) gene PRMT1 (Lin et al., 1996), by now at least eleven PRMT genes have been identified in the mammalian system and seven of them has direct or indirect evidences to be able to catalyze the transfer of methyl groups from AdoMet to the side chain ω−guanido nitrogens of arginine residues in protein substrates (Krause et al., 2006). The PRMT activity can be further divided into type I and type II, depending on the catalyses of formation of asymmetric di-ω-*N, N*–methylarginines (aDMA) or symmetric di-ω-*N, N'*–methylarginine (sDMA) residues respectively (Gary and Clarke, 1998).

Different mammalian protein arginine methyltransferases are localized in different

subcellular compartments and appear to have their own substrate sets and physiological roles. The type I enzymes in the mammalian system include the predominant PRMT1 (HRMT1L2) (Lin et al., 1996; Pawlak et al., 2000; Tang et al., 2000), the zinc-finger containing PRMT3 (Tang et al., 1998), the coactivator-associated arginine methyltransferase CARM1/PRMT4 (Chen et al., 1999), PRMT6 (Frankel et al., 2002) and PRMT8 (Lee et al., 2005). Direct evidences of the formation of the type I products catalyzed by these methyltransferases. In this project we focus on PRMT1 and its vertebrate paralogue PRMT8. These PRMTs will be described further in later sections.

The first type II PRMT identified is the Janus kinase-binding protein JBP1/PRMT5 (Branscombe et al., 2001; Pollack et al., 1999; Rho et al., 2001). Another PRMT7 gene p82/p77 is a genetic suppressor element mediating cell sensitivity to DNA-damaging agents. Lee at al. showed that PRMT7 is a new type II protein arginine methyltransferase capable for sDMA modification (Lee et al., 2005). PRMT9 (also known as F-box only protein 11, FBOX11), which is not structurally related to PRMT1-8, can symmetrically dimethylate arginine residues (Cook et al., 2006). PRMT 10 identified by homology to PRMT7 and PRMT11 (FBOX10) identified as homologues to PRMT9/FBOX11 have also been suggested (Krause et al., 2006)

Introduction to PRMT1 and PRMT8

PRMT1 is the predominant and most abundant type I methyltransferase in the mammalian system (Lin et al., 1996; Pawlak et al., 2000; Tang et al., 2000). Imunofluorescence analysis localized PRMT1 predominantly to the nucleus of the RAT1 rat fibroblast cells (Tang et al., 1998). GFP-fusions of PRMT1 appeared to be largely localized to the nucleus, but significant fluorescence was also observed in the cytosol. A dynamic study of human PRMT1 *in vivo* showed that PRMT1 is predominantly cytoplasmic and is highly mobile both in the cytoplasmic and the nucleus. Inhibition of methylation leads to a significant nuclear accumulation of PRMT1 (Herrmann *et al.*, 2005). PRMT1 with few N- or C-terminal residues outside the methyltransferase core region is small compared with other members of the PRMT family. However, there are reports on its roles in many different processes and interacting with various proteins. These involved its interaction of TIS21 and BTG1 discover by a yeast-two–hybrid screen when the gene was first identified (Lin et al., 1996). The interaction with the cytoplasmic domain of interferone (INF) α receptor (Abamovich, 1997) and its methylation of STAT1 (Mowen et al., 2001) indicate its role in INF signaling. Even though controversial results of STAT1 methylation have been reported (Meissner et al., 2004; Komyod et al., 2005), its putative involvement in HCV or HBV

infection due to reduced PRMT1 and thus decreased STAT1 methylation and INFα signaling upon the infection had been reported (Christen et al., 2007). In addition, as another PRMT member PRMT4/CARM1 (coactivator-associated arginine methyltransferase) that was first identified as a transcriptional coactivator (Chen et al., 1999), PRMT1 later has been shown to be a coactivator involved in various transcriptional regulation (Strahl et al., 2001; Wang et al., 2001). Its methylation of R3 on histone H4 is critical for transcriptional activation. Early reports about typical PRMT1 substrate specificity indicate that RNA binding proteins with RGG motif (such as many hnRNPs, fibrillarin and Sam 68) (Lee and Bedford, 2002; Lin et al., 1996; Mowen et al., 2001; Tang et al., 1998; Wada et al., 2002) or RXR sequence (Smith et al., 1999) are recognized by PRMT1. However, some recent reports indicate PRMT1 can methylates specific arginines in proteins such as PRMT1 receptor protein 140 (RIP140) (Mostaqul Huq et al., 2006) and HNF4 (Barrero and Malik, 2006) with wide context variation instead of the typical RG sequence. Knock-out mice die early during embryogenesis but PRMT1 deficient ES cell lines are viable (Pawlak et al., 2000).

A PRMT-1-like PRMT gene (PRMT8/HRMT1L3) at chromosome 12p13 with 80% sequence identity with PRMT1 was identified in human (Zhang and Cheng, 2003). In a screen of mammalian neural gene, this gene was identified by microarray analyses to be expressed in developing and mature nervous system (Aubert et al., 2003). As the deduced 2nd amino acid residues of PRMT8 is glycine, N-terminal myristoylation at the glycine residue that should be the N-terminal one after the removal of the initiating methionine was suggested. Transfection experiments confirmed the modification and the association of PRMT8 with the plasma membrane. It has type I PRMT activity, and is tissue-specific, largely expressed in the brain (Lee et al., 2005). However, in a recent report by Kousaka et al. (2009), the subcellular localization of the immunoreactivity was dominantly nuclear in the neurons of CNS. The results thus argue against the plasma membrane localization of PRMT8 via the N-terminal myristoylation. The size of the endogenous protein is more consistent with the translation initiation from the 3rd AUG and thus no myristoylation of PRMT8 might occur.

Analyses of PRMT substrates or arginine methylaccepting proteins

Most of the methylarginines in the proteins identified appear to be N^G–monomethylarginine (MMA) and asymmetric N^G, N^G –dimethylarginines (aDMA) in various RNA binding proteins within the Arg-Gly-Gly context (Najbauer et al., 1993). The arginine methylaccepting substrates were identified and studied through different approaches. Some proteins, for example, fibrillarin (Lischwe et al., 1985), nucleolin (Lapeyre et al., 1986), and hnRNPA1 (Rajpurohit et al., 1994) were identified to contain asymmetric

dimethylarginines through direct biochemical analyses by amino acid analyses or Edman sequencing. More proteins with similar arginine and glycine rich motifs, mostly RNA binding proteins such as EWS (Belyanskaya et al., 2001) were further identified to be methylaccepting substrates. After identification of the PRMT genes, proteins without typical arginine and glycine rich sequences have been found to contain methylarginines. For example, in PABPII protein the methylation site has been identified on the RXR motif (Smith et al., 1999) while the methylarginine in STAT1 is located in the EIRQY context (Mowen et al., 2001); however, arguments on STAT1 methylation has been published (Komyod et al., 2005). The other approach to systematically study methylaccepting proteins is to make cells deficient in methyltransferase activity either by genetic approaches (Frankel and Clarke, 1999; Pawlak et al., 2000; Yadav et al., 2003) or by chemical treatment (Huang et al., 2002; Najbauer et al., 1993). The members on the list of arginine methylaccepting substrates for specific PRMT enzymes as reviewed by Bedford et al. (Bedford and Richard, 2005) are growing very fast.

Questions to be answered about arginine methylation and why use zebrafish to answer the questions

Why so many different methyltransferases are required to modulate different biological functions in the vertebrates and other organisms and how the methyltransferases are evolved are interesting questions. As suggested by the interacting partners and the substrates of various PRMTs, arginine methylation has been implicated to play roles in signal transduction, protein subcellular localization, RNA processing, transcriptional regulation as well as DNA repair (Bedford and Clarke., 2009).

Basically, the genomic structures of PRMT orthologues are conserved from fish to human. The conservation indicates that these genes are conserved in vertebrates and the divergence of these PRMT genes probably occurred before the origin of vertebrates. Other biological systems such as *Drosophila malangaster* or *C. elegans* appear to have different sets of PRMT genes while zebrafish is the biological system with the complete set of PRMT genes as in mammals (Krause et al., 2006). Since the PRMT genes are highly conserved from fish to human, we suggest that zebrafish would be a valuable model system close to human to study protein arginine methylation. Zebrafish is a model organism that is amenable to genetic manipulations and has been widely used in developmental studies. We would like to use this system to investigate their involvements in early embryonic development.

Diversities of the substrates modified by PRMT complicated the understanding of the function of arginine methylation. Only part the PRMT enzyme substrate relationships have been established in cellular systems (Bedford and Clarke., 2009). Thus in this project we will

use zebrafish as a model organism to study some of the interesting issues raised above. Firstly, we will study the most predominant and evolutionally most conserved PRMT1 in zebrafish. As the PRMT1 knock-out mice die early during embryogenesis (Pawlak et al., 2000), we will prepare PRMT1 knock-down in zebrafish. Since the embryos are transparent and can be directly observed under microscope, we can observe the changes in the PRMT1 knockdowns at different early developmental stages. As the PRMT8 gene is so closely related to PRMT1, we will investigate this gene in this proposal parallel to the PRMT1 studies. We will then analyze if PRMT8 can complement (completely or partially) in the PRMT1 knockdown and vice versa. Another type I PRMT we will analyze is PRMT6. We will perform similar analyses as for PRMT1/PRMT8 to study the evolutionarily less conserved PRMT (Hung and Li, 2004). By now there were no suitable models to analyze the over all picture of the PRMT enzyme-substrate relationships and the putative cross-PRMT inter-relationships, we are confident that the zebrafish system we are going to develop will be an excellent system to approach these critical issues.

三、 結果與討論

Ubiquitous expression of *zprmt1* RNA and protein in zebrafish embryonic development

As alternative splicing of mammalian *prmt1* results in various mRNA and protein isoforms (Goulet et al., 2007; Pawlak et al., 2000; Scorilas et al., 2000; Scott et al., 1998), we investigated whether *prmt1* is also alternatively spliced in zebrafish. Ensembl (ENSDARG00000010246) illustrates that z*prmt 1* contains ten exons. No support of putative alternative splicing of the z*prmt1* could be obtained from data base search and the z*prmt1*mRNA appears to be analogous to the v1 form of mammalian *prmt1* mRNA (connecting the 1st exon and the constitutive 102-nt exon, skipping the alternative exons in between)(Fig. 1A).

We thus designed a primer set (ZF1-ASF and ZF1-ASR) that can specifically amplify this putative alternatively spliced region (Fig. 1A). Developmental *zprmt1* expression was analyzed by RT-PCR. Only single RT-PCR product of 138 bp was observed for RNA prepared from different developmental stages (1cell, 1.5, 6, 14, 18, 24, 48 and 72 hpf; Fig. 1B). The results are opposed to alternative splicing at the 5'-end of the *prmt1* gene in zebrafish. The results also showed that zebrafish *prmt1* mRNA was maternally derived during early stages and were further transcribed in the zygotes. Furthermore, the RT-PCR product confirmed the presence of an upstream in-frame ATG with a reasonable Kozak sequence encoding a

full-length protein with 348 amino acids. The ATG is 21-nt upstream of the ATG start site suggested in the NCBI (NM_200650) according to previous incomplete mammalian 5' sequences. The predicted N-terminal amino acid sequence and the lengthened amino acid sequence are indicated (Fig. 1A).

RT-PCR with a primer set amplifying the middle and 3' region of z*prmt1* mRNA that are not alternatively spliced in the homologous mammalian *prmt1*gene also detected the *zprmt1* expression at all developmental stages and ubiquitous expression in various adult tissues such as brain, heart, spleen, swim bladder, gill, testis, ovary and muscle (Fig. 1B).

We further analyzed the zPRMT1 protein expression at different embryonic stages. Single signal at molecular mass about 42 kDa was detected by western blot for all tested stages (Fig. 1C). Therefore PRMT1 is expressed both maternally and zygotically, comparable to the results of RT-PCR.

Spatial and temporal expression pattern of zprmt1 mRNA by whole-mount in situ hybridization (WISH)

Whole-mount *in situ* hybridization of zebrafish embryos was conducted with an anti-sense riboprobe to analyze the *prmt1* spatial and temporal expression pattern. Zebrafish *prmt1* mRNA was expressed as early as one-cell stage and strongly and ubiquitously expressed in embryos through 2- to 4- cell stages (Fig. 2A, B, C). The results further demonstrate the maternal origin and homogeneous distribution of *prmt1* mRNA during the very early cleavages. Continuing homogenous expression at 6 and 12 hpf indicated zygotic transcription from gastrulation to early segmentation period (Fig. 2D, E). At 24 hpf, *prmt1* was strongly expressed in the head regions including eyes, optic tectum, otic vesicle, mesencephalon, hindbrain and middle hind-brain boundary (Fig. 2G). Expression in somites was also detected. As development proceeded, the expression of *prmt 1* continued but decreased in most parts of the brain at 48 or 72 hpf. Expression at blood vessels and somites were observed at these stages (Fig. 2H, I). The signals are specific to *prmt1* since the sense riboprobe did not detect any significant signals (Fig. 2J).

knockdown of zprmt1 with a specific morpholino oligonucleotide affects zebrafish development.

Antisene morpholino oligonucleotides (AMOs) designed to hybridize the 5' region of a target mRNA can selectively block translation for specific gene knock-down (Corey et al., 2001). As two in-frame ATGs separated with 21-nt were present at the 5'-region of *zprmt1*, we synthesized two translational blocking AMOs corresponding to the upstream and downstream ATG (MO1 and MO2 respectively) (Fig. 3A). Injections of *zprmt1* AMO at the

1- to 2-cell stage resulted in *prmt1* morphants. One and a half fold of p53 AMO was co-injected as suggested by Robu *et al*. to avoid apoptosis induced through the off-target activation of p53 by the injected AMO (Robu et al., 2007).

Injection of *zprmt1* MO1 at high dose (8 ng) resulted in serious developmental defects and high death rate at 24 hpf. Injection of mid-dosed MO1 (4 ng) led to high defect rate but very low death rate at 24 hpf (Fig. 3). Dose-dependent phenotypic severity indicates the specificity of *zprmt1* AMO knock-down. Injection of high-dosed MO2 (8 ng) resulted in similar phenotypes as injection of MO1 at mid dose. Basically, abnormal phenotypes were recorded at 48 hr with different degree of body curvature associated with curved or shortened tail. Other abnormalities such as cardiac edema, enlarged and shortened yolk stalk, smaller eyes, and seriously truncated or bended tails were also observed in some morphants (Fig. 3B). Both MO-1 and MO-2 specifically reduced the expression of PRMT1 protein in the injected zebra fish embryos compared to the wild type or control AMO-injected embryos as revealed by western blot analysis (Fig.3C). We thus studied the morphants by injection of MO1 (4 ng) or MO2 (8 ng).

Reduced level of type I PRMT activity and protein arginine methylation in zprmt1 morphants

We showed that both the injected MO-1 and MO-2 indeed blocked the expression of PRMT1 protein in zebrafish embryos effectively and persistently. Correspondingly, type I protein arginine methyltransferase in the morphants was reduced. The result was illustrated by i*n vitro* methylation reaction using fish embryonic extracts as the source of methyltransferase upon a typical PRMT substrate fibrillarin (Fig. 4A).

As PRMT1 protein expression and type I protein arginine methyltransferase activity were specifically reduced by MO injection, we further examined whether protein arginine methylation catalyzed by PRMT1 decreased upon the knock-down. Antibody ASYM24 against asymmetrically dimethylated arginines present in alternate RG sequences (Côté et al., 2003) were used for western blot analyses (Fig. 4B). The antibody recognized dozens of zebrafish embryonic proteins and most of the methylarginine-specific signals reduced significantly in the *zprmt1* morphants.

We then examined specific protein arginine methylation of specific PRMT1 substrates. Histone H4 arginine 3 methylation catalyzed by PRMT1 is abolished in $PRMT^{-/-}$ mouse embryonic stem cells (Wang et al., 2004). We thus determined the H4 R3 methylation with a specific antibody. As shown in Fig. 4C, asymmetric arginine dimethylation at this residue was reduced in the morphants. Detection with another H4-specific antibody confirmed equal

loading of H4 protein. The results showed that the reduction of H4 R3 methylation was not due to decreased expression of H4 protein and should be due to the seduced expression of PRMT1 in the morphants.

Delayed epiboly of the prmt1 morphants

We further examined the morphological defects in the embryos at early stages. At epiboly, rapid intercalation of cells leads to the spreading of the blastomeres over the yolk. Gastulation begins at 50% of epiboly in the wild type embryos. The shortened anterior-posterior axis in the morphants suggested a possible defect in convergence (the narrowing of embryonic tissues mediolaterally) and extensions (elongation anterioposteriorly) at gastrulation. As no apparent differences can be observed at earlier cleavage and blastula periods, we analyze the morphants at gastrulation for the abnormal development.

At 10 hpf of the 100% epiboly, defective epiboly can be observed in the majority of the morphants. Wildtype embryos showed complete blastopore closure while the AMO-injected embryos cannot close the yolk plug and demonstrate varying degrees of open blastopore (Fig. 5A). Staining with notail (*ntl*, expressed in the ring mesoderm and endodermal precursors around the margin) also showed delayed epiboly with shortened but widened notochords in the *prmt1* morphants (Fig. 5B, C). The morphnats are grouped according to the degree of shortening and widening of the notochord. On the other hand, another marker *gsc* (goosecoid, expressed mainly prechordal plate and in ventral neurectoderm) did not reveal clear differences between wildtype and morphants (Fig. 5D). The results suggest that the abnormalities in the *prmt1* morphants are even serious at the posterior than the anterior part of the embryo, as observed at the end of epiboly.

Lateral expansion and shortened anterior-posterior axis in the morphants at early segmentation stage

WISH experiments demonstrated strong *prmt1* RNA expression in the head as well as somites from 24-72 hpf. We thus examined these regions in the morphants by marker gene staining. The mid-hind brain boundary stained by *wnt1* in wild-type or MO-injected 24-hpf embryos was similar. However, somite regions stained by *myoD* revealed lateral extension and shortened anterior-postrior distance in the 24-hpf morphants. Overall phenotype observations also showed curled body with shortened anterior-posterior axis in the *zprmt1* morphants at 24 hpf, suggesting the presence of earlier developmental defects. We thus further analyzed the morphants at segmentation stages with different markers for the abnormalities.

krox20 expression is detected only in the hindbrain region and is restricted to

rhombomeres 3 (r3) and 5 (r5). Even though high level of *krox20* expression in the morphants was detected at r3 and r5, the *krox20* territory appeared to be abnormal at 10-somite stage (Fig. 6A). Generally, both r3 and r5 were laterally extended and the posterior r5 is more extended than r3. The anterior-postrior distance between r3 and r5 was also reduced, and the extent of reduction is related to the lateral extension.

pax2.1 is specifically expressed at anterior retina, midbrain-hindbrain boundary (MHB), otic vesicle, spinal chord neurons and pronephric duct. Analyses of the *pax2.1* expression in the *prmt1* morphants at 14-somite stage revealed that the distance from the otic vesicle to the beginning of the pronephric ducts and the length of the pronephric ducts were reduced while the width between the pronephric ducts was increased. In the *prmt1* morphants, *pax2.1* expression level was unchanged in anterior retina and MHB, however, it was reduced in otic vesicle and increased in the pronephric ducts and tubules (Fig. 6B).

Analysis with a muscle and somite-specific marker *myoD* at 10-somite stage clearly revealed different degrees of abnormal somite development in the *zprmt1* morphants. As shown in Fig. 6C, *myoD* expression in the two straight rows of adaxial cells flanking the notochord was irregularly bended at the posterior end. The width was broadened and the lateral *myoD* expression was greatly extended. The distances between the segments were compressed even though the same number of segments was present in the morphants. Generally, the level of *myoD* expression was also reduced in the morphants. In some morphants, the distance between two rows of posterior adaxial cells are even widened with *myoD* expression diminished at the end of one side, and laterally extended and compressed at the other side.

These marker analyses revealed defective medial-lateral convergence and anterior-posterior extension of the body plan and the abnormalities were even serious at the posterior than the anterior part of the embryo.

Expression analysis of *zprmt8* RNA and protein in zebrafish embryonic development

We deduced the *prmt8* gene in zebrafish by assembly of genomic as well as different EST sequences. The sequence was 85-amino acid longer than the NCBI one (NP_001038507, Fig. 7). RT-PCR and sequencing confirmed the cDNA sequence and revealed no alternative splicing. As expected, the message is only expressed in brain but not other adult tissues (Fig.8). At early embryonic stages, the prmt8 expression can be detected by RT-PCR from 1.5-3 hpf and is highly expressed at 72 hpf (Fig. 8). However, the spaciotempo expression by WISH showed ubiquitous prmt8 expression at very early developmental stages from one cell to 12 hpf. The expression at the head regions continues from 24 to 60 hpf. Surprisingly, the

expression in the brain is gone but moves to the somites at 72 hpf. The signals appear in the midbrain again and fade in the somites at 96 hpf (Fig. 9).

Survial and phenotypes of the zebrafish embryos knocked down of *zprmt8*.

The putative N-terminal sequence of zebrafish PRMT8 we deduced has an initiating methionine followed by a glycine as the second amino acid as those in mouse and human (Lee et al., 2005). We thus designed a translation-blocking MO against this initiating AUG (MO1). However, as recent investigation suggested putative initiation at the 3rd AUG in mammalian prmt8, we thus have another MO designed for this site (MO2) (Fig. 10).

Injection of MO1 at the dose of 8 ng resulted in abnormal phenotypes in about 70% of the injected embryos 48 hpf. The abnormalities include different degree of body curvature, enlarged and swollen yolk and edema. Less than 40 % of the MO1 morphants can survive after 6 dpf (Fig. 10).

Injection of the zprmt8 MO2 led to more severe phenotypes than the injection of MO1 at the same dosage. More than 80% death was observed for the embryos injected with 8 ng of MO2 (Fig.10). Death ratios reduced to 50% and less than 5 % when 4 ng and 2 ng of MO2 were injected respectively. The defect rate of 2 ng-injected morphants is 87% with phenotypes close to the embryos injected with 8 ng of MO1 (Fig. 11).

Analysis of a putative PRMT substrate RNA-binding protein SERBP1 in zebrafish

An RNA binding protein SERBP1 has various names indicating the multiple roles of the proteins. SERBP1 was named PAIRBP1 as a polypeptide bound to the cyclic nucleotide-responsive sequence in the plasminogen activator inhibitor (PAI) mRNA (Heaton et al., 2001). Even though arginine methylation in PAIRBP1 has been reported (Passos et al., 2006), the physiological importance of the modification has not been analyzed in light of the multiple phases of the protein. We thus use zebrafish system to analyze the function of the protein in correlation with arginine methylation.

We first compared the protein sequence as well as the gene structure of the *serbp1* gene in human and zebrafish. Zebrafish SERBP1 protein is most closely related to the isoform4 of the human SERBP1 protein without the two alternatively spiced exons included (Fig. 12A). The mRNA and protein products of serbp1 gene are present as early as one-cell stage and continues through the embryonic stages (Fig.12B).

四、 成果自評

We are glad that we were able to obtain results to contribute to the understanding of

arginine methylation in zebrafish. We have found interesting phenotypes in *zprm1* and *prmt8* morphants. Reduced expression of the enzyme exhibited delayed embryonic development and several morphological defects. The results indicate the importance of protein arginine methylation in zebrafish embryonic development. The results are valuable and provide important insights into the research of this field. The manuscript describing the results of the *zprmt1* study is at the final polishing stage. We are also excited about the progress of zebrafish *prmt8* study which appears to provide general valuable information for some unsettled issues about the protein. Basically, the expression analyses by RT-PCR, western blot and WISH, MO knockdown as well as mRNA rescue experiments of the zebrafish system works well for both the methyltransferases and their substrates.

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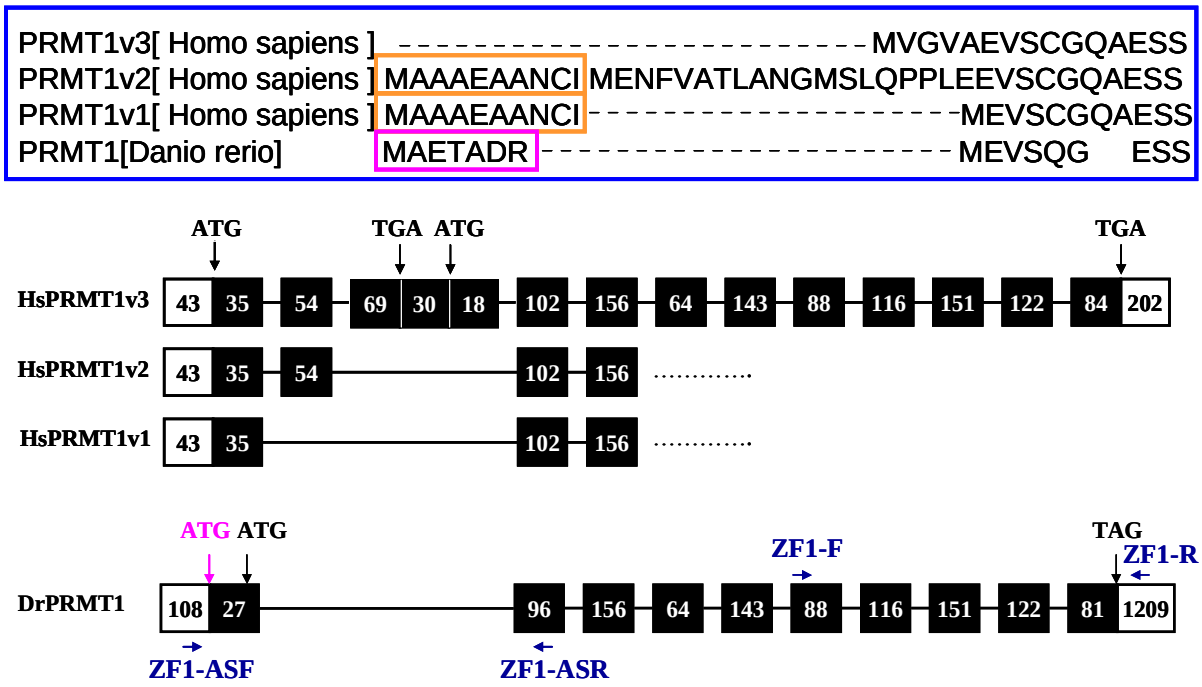
A



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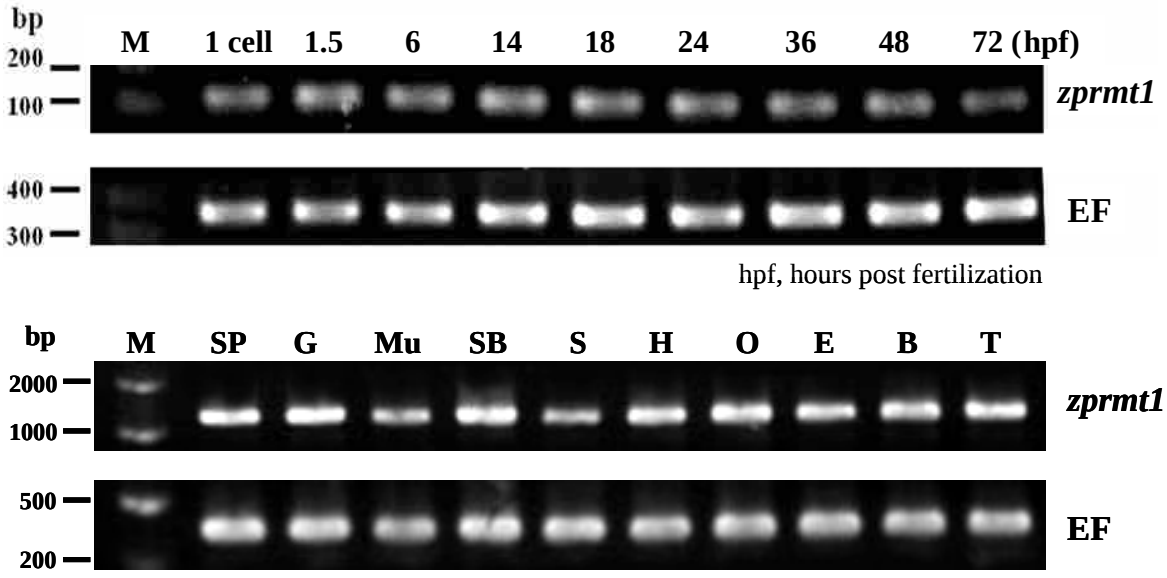


SP: spleen S: skin E: eye
G: gill H: heart B: brain
Mu: muscle O: ovary T: tetsis
SB: swim bladder

C

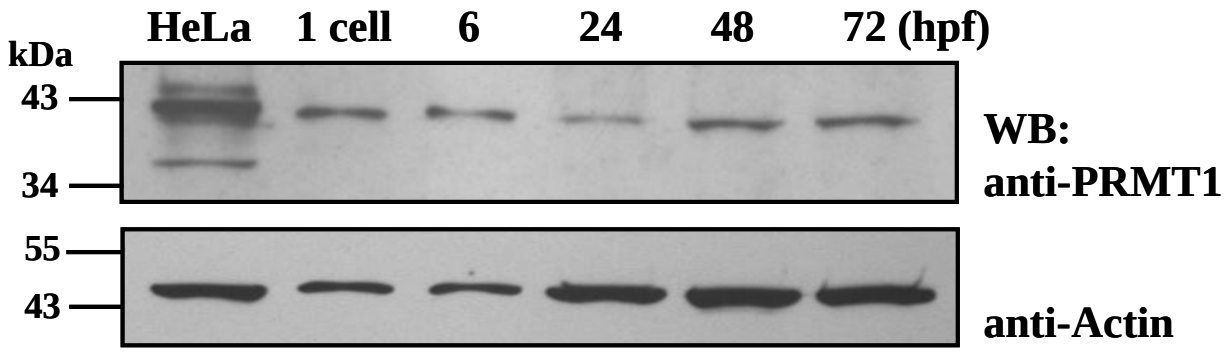
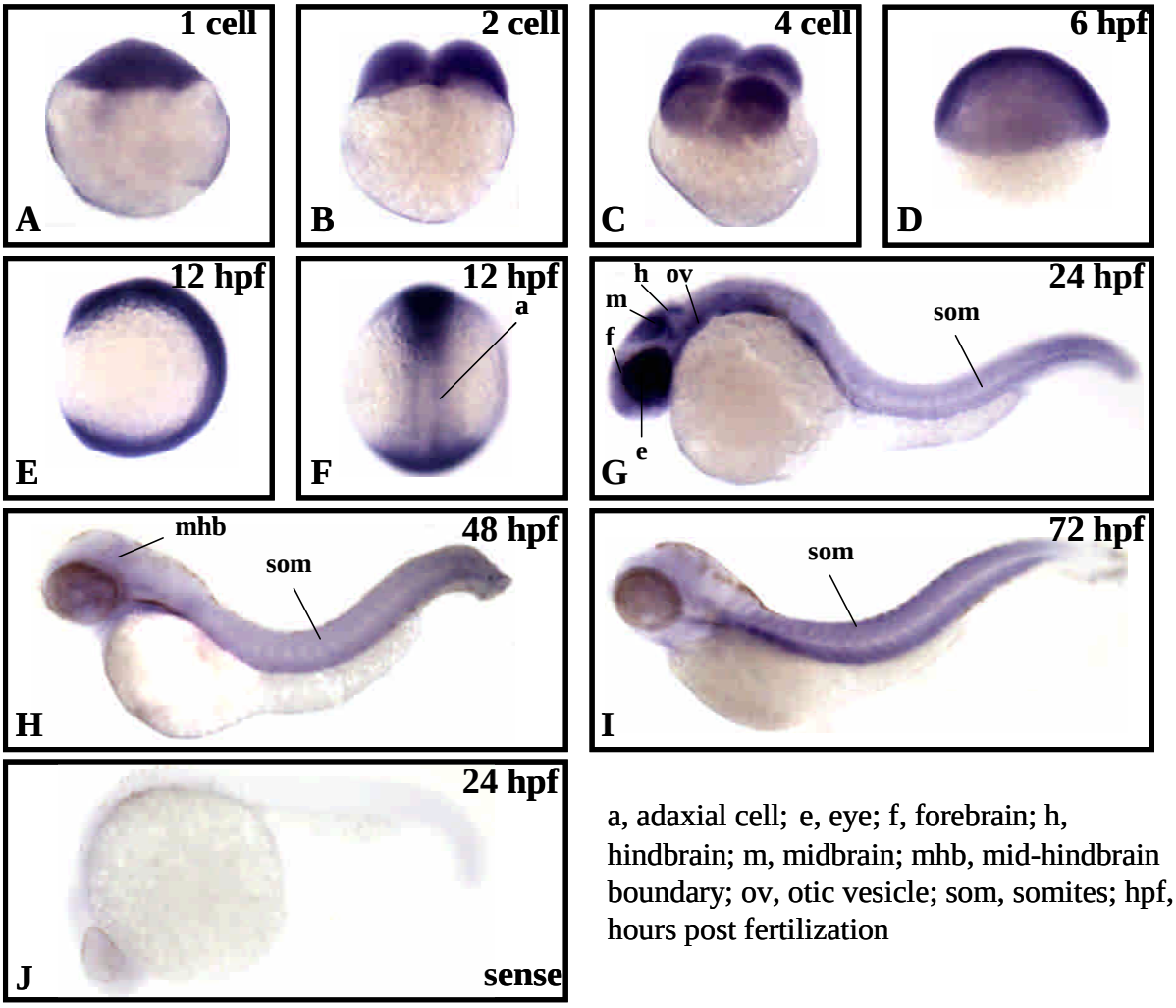


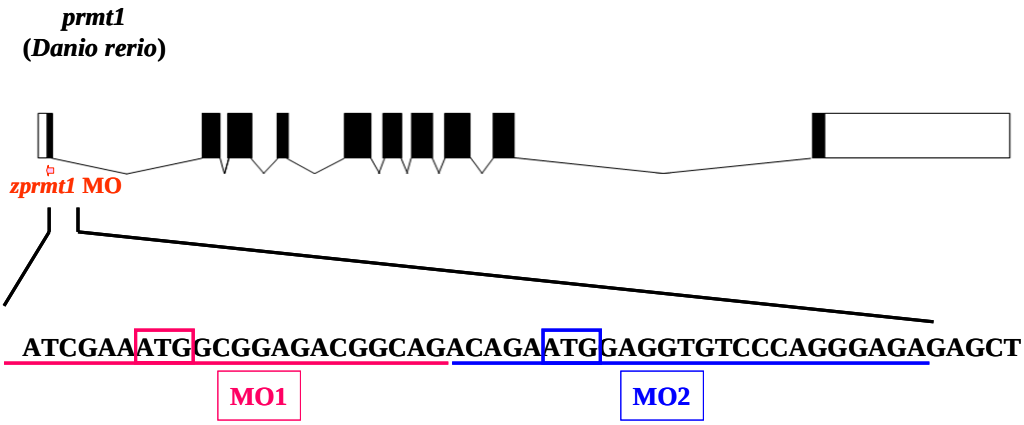
Fig. 1. Genomic structure and partial nucleotide and amino acid sequences of zebrafish *prmt1* gene and expression of *zprmt1* mRNA and protein during early development of zebrafish. (A) Genomic structure of human and zebrafish *prmt1*. Three major human splicing variants (Scorilas, 2000) and the only identified zebrafish slicing form are shown. Exons are represented as boxes and introns by the connecting lines. Numbers in the boxed represent the exon length in base pairs. Arrows indicate the position of the start and stop codons. Filled boxes are coding and open boxes are non-coding regions. The start ATG in human prmt1 were as suggested by Goulet et al., 2007. According to *zprmt1* mRNA sequence, an ATG (red arrow) 21-nucleotide upstream of the previously identified ATG (black arrow, NM_200650) is mostly likely to be the translational start site. HsPRMT1v3 (NP_938075.2), HsPRMT1v2(NP_001527.3), HsPRMT1v1 (NP_938074.2), DrPRMT1(NP_956944.1). Hs, *Homo sapiens*; Dr, *Danio rerio*. Comparison of the N-terminal sequences of human and zebrafish *prmt1*. Multiple sequence alignments showing the N-terminal sequences of PRMT1 from human and zebrafish. RT-PCR primers used to quantify *zprmt1* are shown. (B) Expression of *zprmt1* during different developmental stages analyzed by RT-PCR. EF indicated the RT-PCR product of elongation factor 1A as a loading control. hpf, hours post fertilization; SP: spleen, S: skin, E: eye, G: gill, H: heart, B: brain, Mu: muscle, O: ovary, T: testis, SB: swim bladder. (C) Endogenous PRMT1 protein (approximately 42 kDa) in extracts of different embryonic stages (1 cell, 6, 24, 48 and 72 hpf) was detected by Western blot analysis. Lane 1: HeLa cell extract was included as a positive control. Detection by anti-β-actin was used as a loading control. EF, elongation factor; hpf, hours post fertilization.



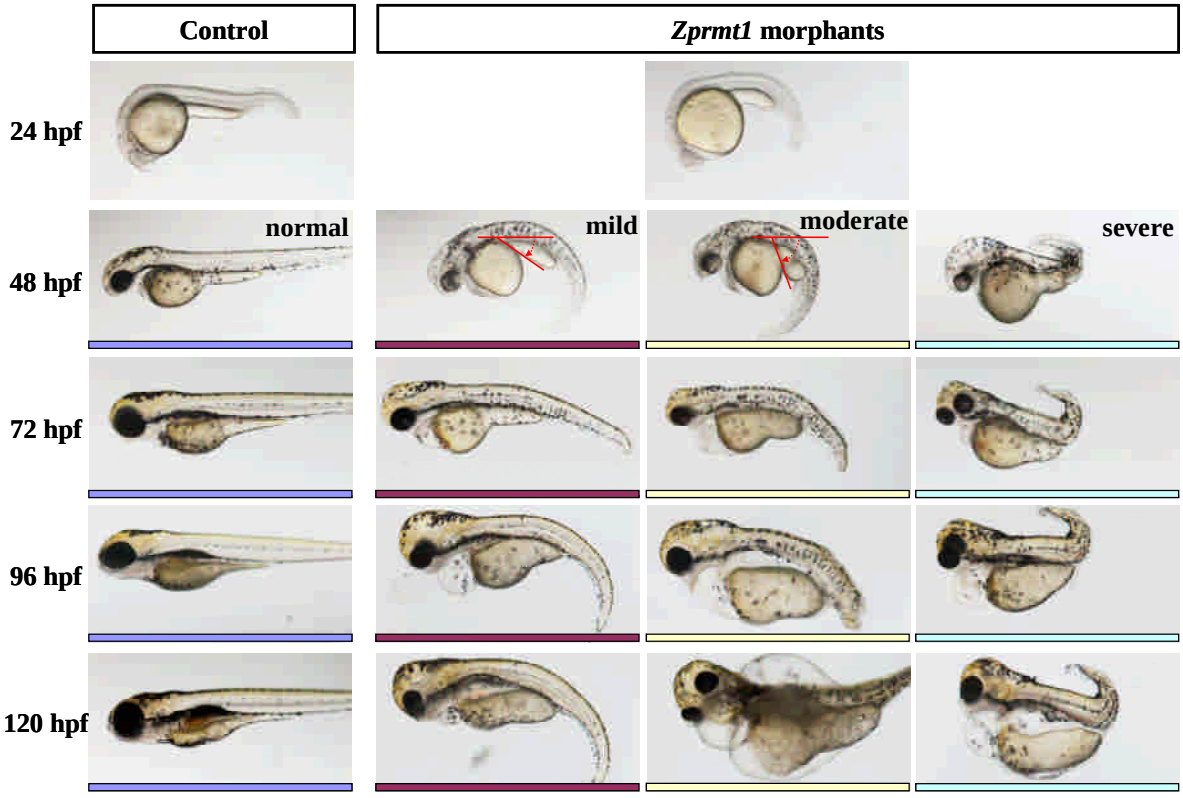
a, adaxial cell; e, eye; f, forebrain; h, hindbrain; m, midbrain; mhb, mid-hindbrain boundary; ov, otic vesicle; som, somites; hpf, hours post fertilization

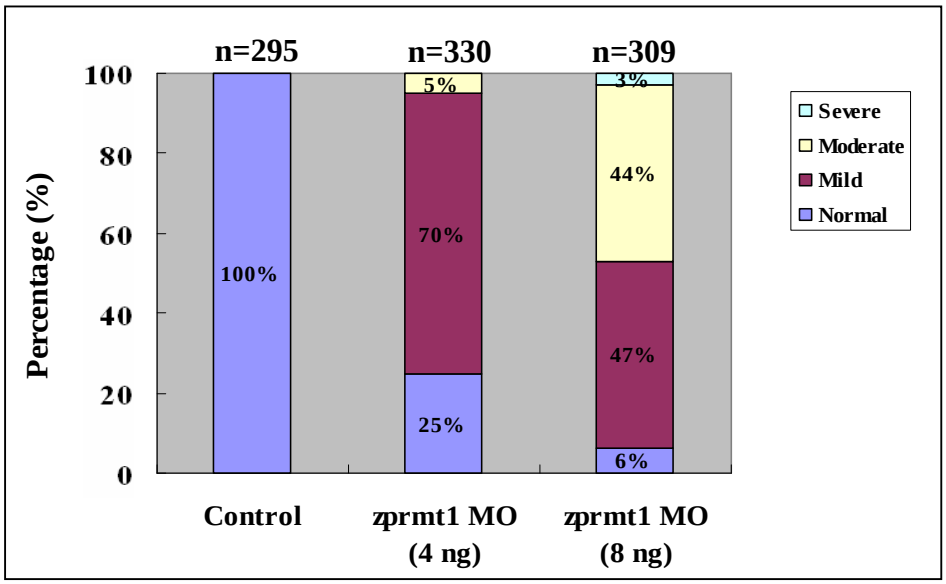
Fig. 2. Spatial and temporal expression of *zprmt1* by whole mount *in situ* hybridization analysis. Zebrafish embryos at 1 cell (A), 2 cell (B), 4 cell (C), 6 hpf (D), 12 hpf (E), 24 hpf (G), 48 hpf (H) and 72 hpf (I) are analyzed. Dorsal view of the 12 hpf is shown (F). WISH with sense riboprobe is shown in (J). a, adaxial cell; e, eye; f, forebrain; h, hindbrain; m, midbrain; mhb, mid-hindbrain boundary; ov, otic vesicle; som, somites; hpf, hours post fertilization.

A


prmt1
(Danio rerio)
zprmt1 MO
ATCGAAATGGCGGAGACGGCAGACAGAATGGAGGTGTCCCAGGGAGAGAGCT
MO1
MO2

B


Control
Zprmt1 morphants
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
normal
mild
moderate
severe



C

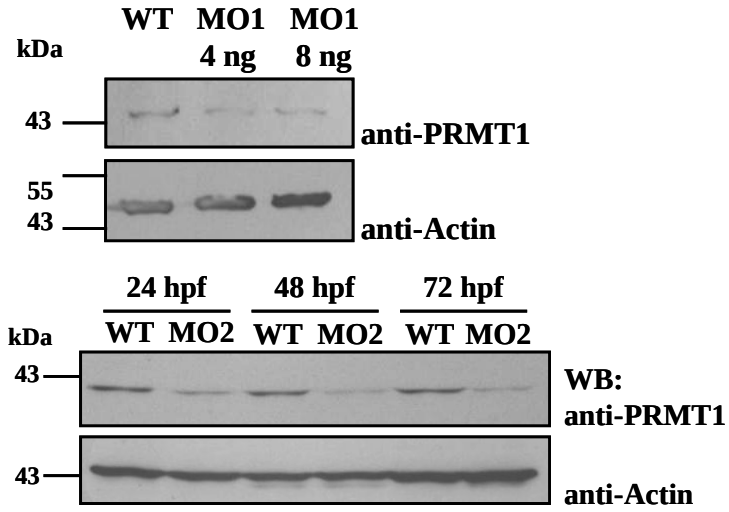


Fig. 3. *Prmt1* knock-down in zebrafish. (A)The DNA sequence around the ATG translational start site of *zprmt1*. The two in-frame ATGs are boxed in red and blue, respectively. MO binding sites complementary to the antisene morpholino oligonucleotide MO1 and MO2 are underlined. (B) Phenotypes of embryos injected with *zprmt1* MO2 at 24, 48, 72, 96 to 120 hpf. The injected embryos are classified according to the phenotypes at 48 hpf. Embryos with normal body axes as un-injected wild-type embryos were classified as "normal". Frequencies of different phenotypes (shown in b) caused by injection of *zprmt1* MO (4, or 8 ng). (C) Proteins were prepared from embryos injected with control MO, *zprmt1* MO-1 or MO-2. Western blot analysis of PRMT1 protein in embryos injected with control MO and 4 ng or 8 ng of *zprmt1* MO-1 at 24 hpf were shown in the upper panel and those of MO-2 at 24, 48 and 72 hpf were shown in the lower panel. Detection by anti-β-actin was used as a loading control.

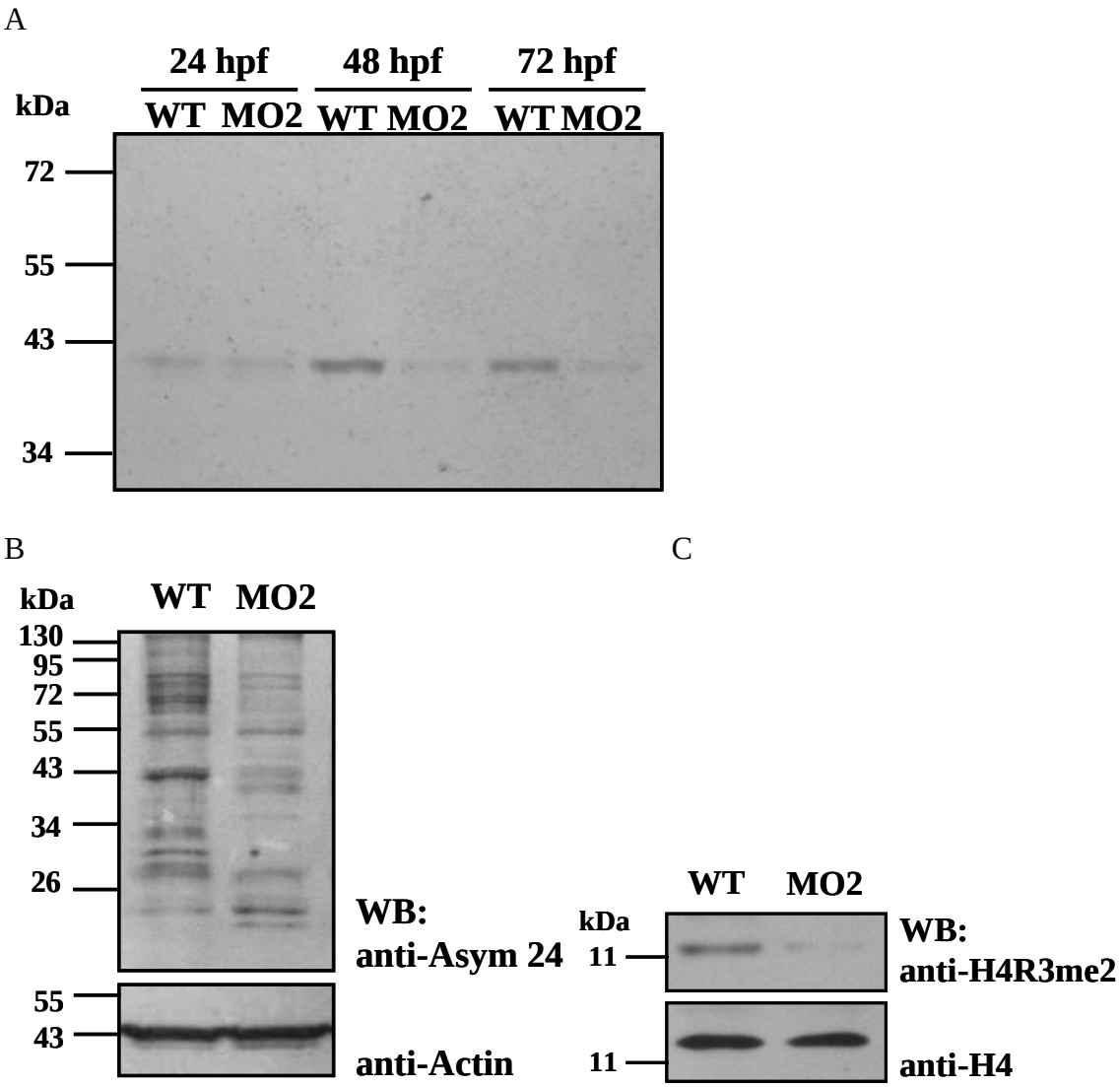


Fig. 4. Reduced type I protein arginine methyltransferase activity and specific protein arginine methylation of *zprmt1* morphants. (A) *In vitro* methylation was conducted with fish embryonic extract from 8ng MO-2 or standard MO injected embryos as the source of protein arginine methyltransferase and recombinant mouse fibrillarin, a standard type I PRMT substrate, as the methylaccepting protein. Methylation reaction was conducted as described in the Materials and Methods. The samples were separated by SDS-PAGE and the methylated proteins were detected by fluorography. (B) Western blot analysis of arginine methylation levels in 48-hpf morphant embryos. Antibody specific for asymmetric dimethyl arginine (Asym24). Detection by anti- β -actin was used as a loading control. (C) Western blot analysis of H4R3me2 levels in 48-hpf morphant embryos. Analysis of histone H4 loading served to normalize levels of H4R3me2 for morphants relative to levels for the wild type.

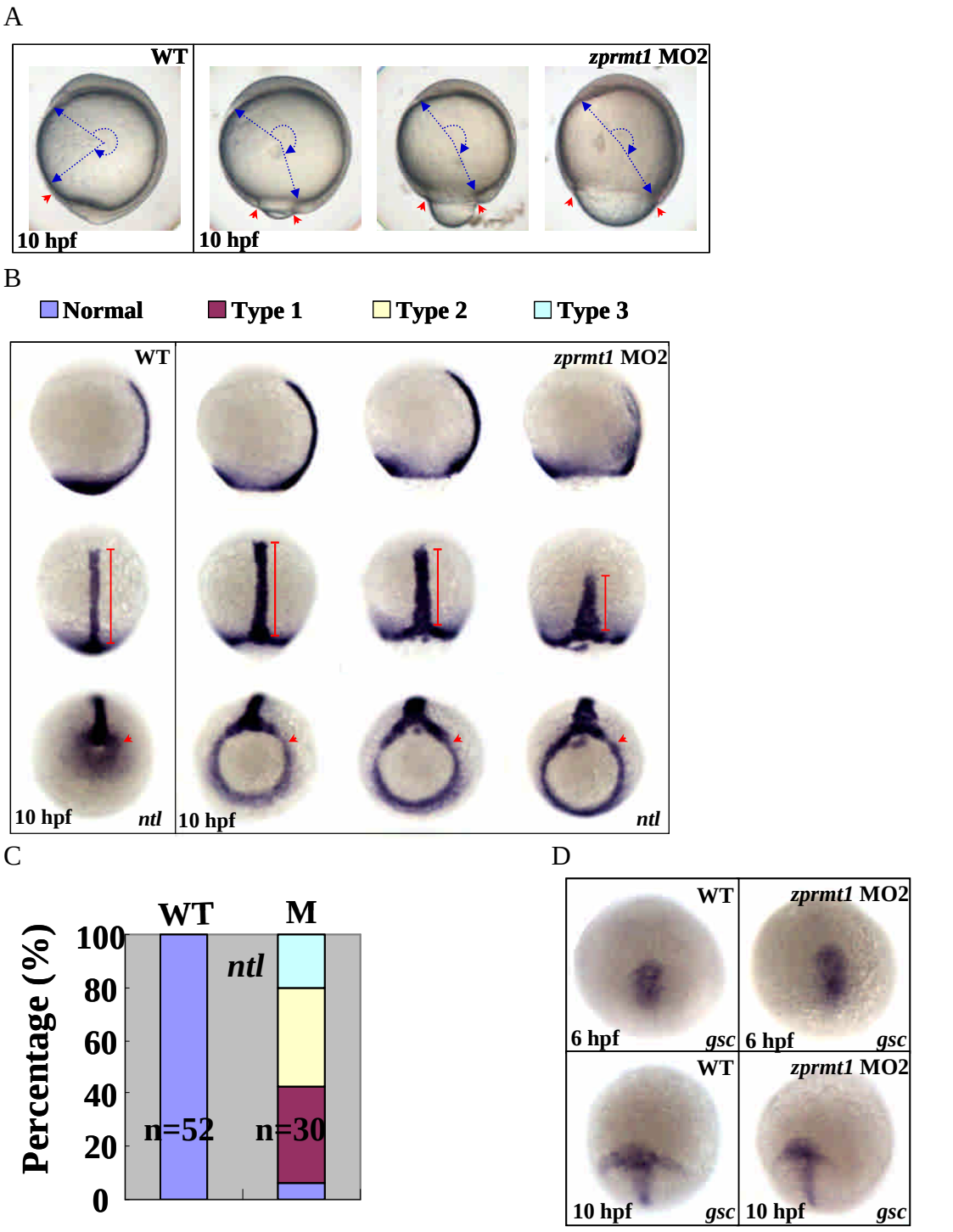
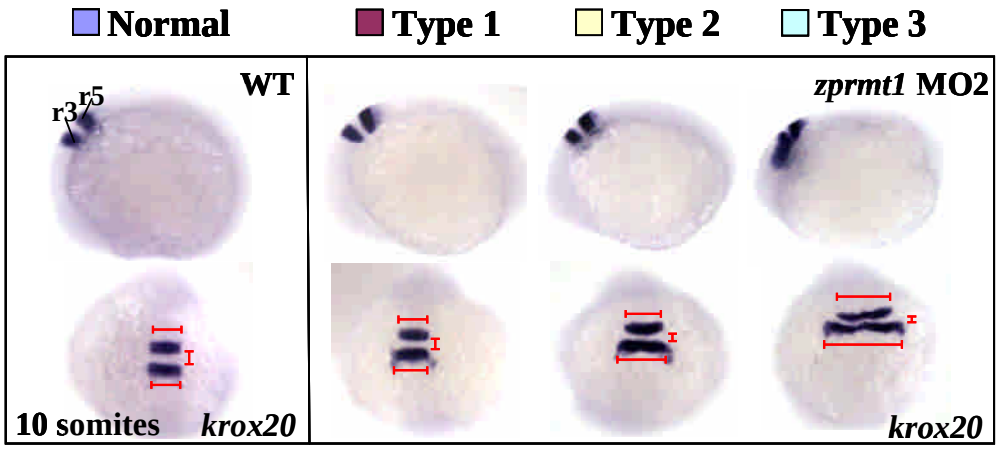
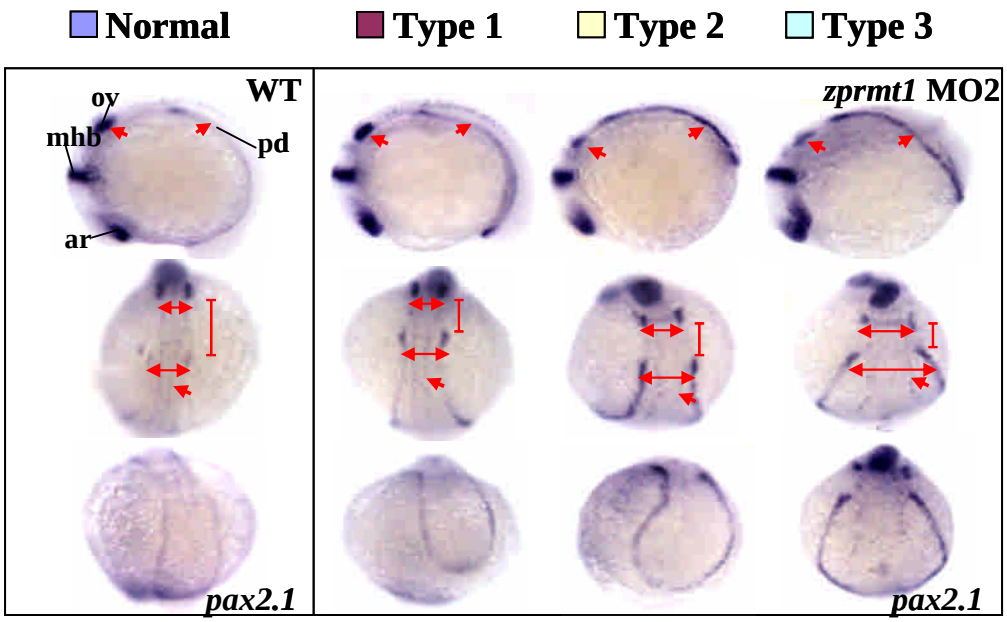


Fig. 5. Prmt1 knockdown induces gastrulation defects. Wild-type and *prmt1* MO injected embryos at 10 hpf (the end of epiboly) were examined. (A) The morpahnts were classified into three phenotype categories according to their degrees of abnormal morphology at the end of epiboly, as the opening of the blastopore. Lateral views, dorsal to the right. (B) *ntl* (staining the forerunner cell group, axial chorda mesoderm) staining at 10 hpf. (C) Percentages of wild-type and morphants embryos within each phenotypic category are shown in the bar graphs. n equals to the total embryos counted. (D) *gsc* staining at 6 and 10 hpf.

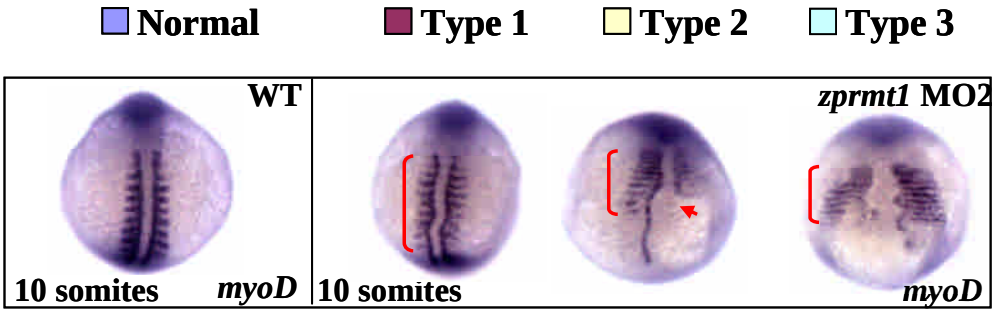
A


Normal
Type 1
Type 2
Type 3
WT
r3
r5
zprmt1 MO2
10 somites
krox20
krox20

B


Normal
Type 1
Type 2
Type 3
WT
oy
mhb
pd
ar
zprmt1 MO2
pax2.1
pax2.1

C


Normal
Type 1
Type 2
Type 3
WT
zprmt1 MO2
10 somites
myoD
10 somites
myoD

D

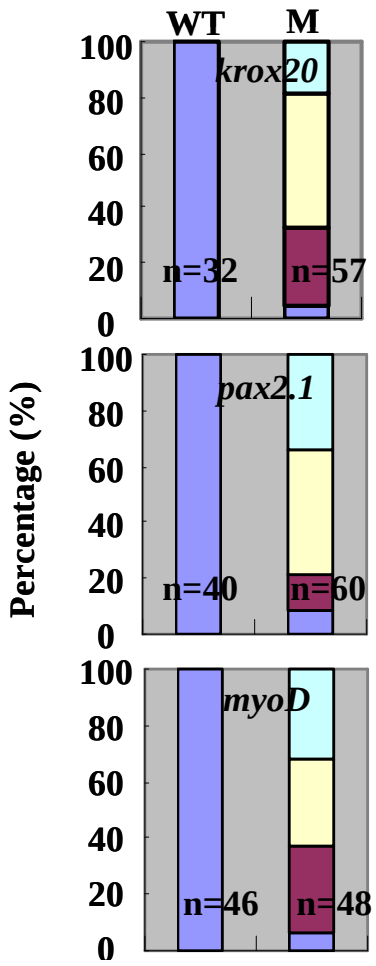


Fig. 6. Whole-mount in situ hybridization analysis for different markers (*knox20, pax2.1*and *myoD*) at segmentation stage for *zprmt1* morphants. The phenotypes were classified according the degree of abnormalties as type 1, 2, and 3. (A) Whole-mount embryos (10-somite stage), lateral view, anterior to the left (upper) and dorsal view (lower) for *knox20* staining of wild-type and *prmt1* morphants. The positions of rhombomeres 3 (r3) and 5 (r5) are indicated. The width of r3 and r5 and the vertical distance between r3 and r5 are indicated. (B) Lateral view, anterior to the left (upper) and dorsal view, animal poles at top (middle) or posterior at top (lower) for *pax2.1* staining of *prmt1* morphants at 14-somite stage. The positions of anterior retina (ar), mid-hindbrain boundary (mhb), otic vesicle (ov), pronephric duct (pd) are indicated. The arrowheads indicate otic vesicles or the anterior beginning of the pronephric ducts. The width between the left and right otic vesicles or the pronephric ducts is shown as double arrow. The vertical distance between the otic vesicles or the anterior beginning of the pronephric ducts is also indicated. (C) Expression of *myoD* at paraxial/adaxial mesoderm in the wild-type and control zebrafish embryos at 10-somite stage were detected by WISH. Dorsal views, anterior at top. (D) Percentages of wild-type and morphants embryos within each phenotypic category are shown in the bar graphs. n equals to the total embryos counted in the experiments.

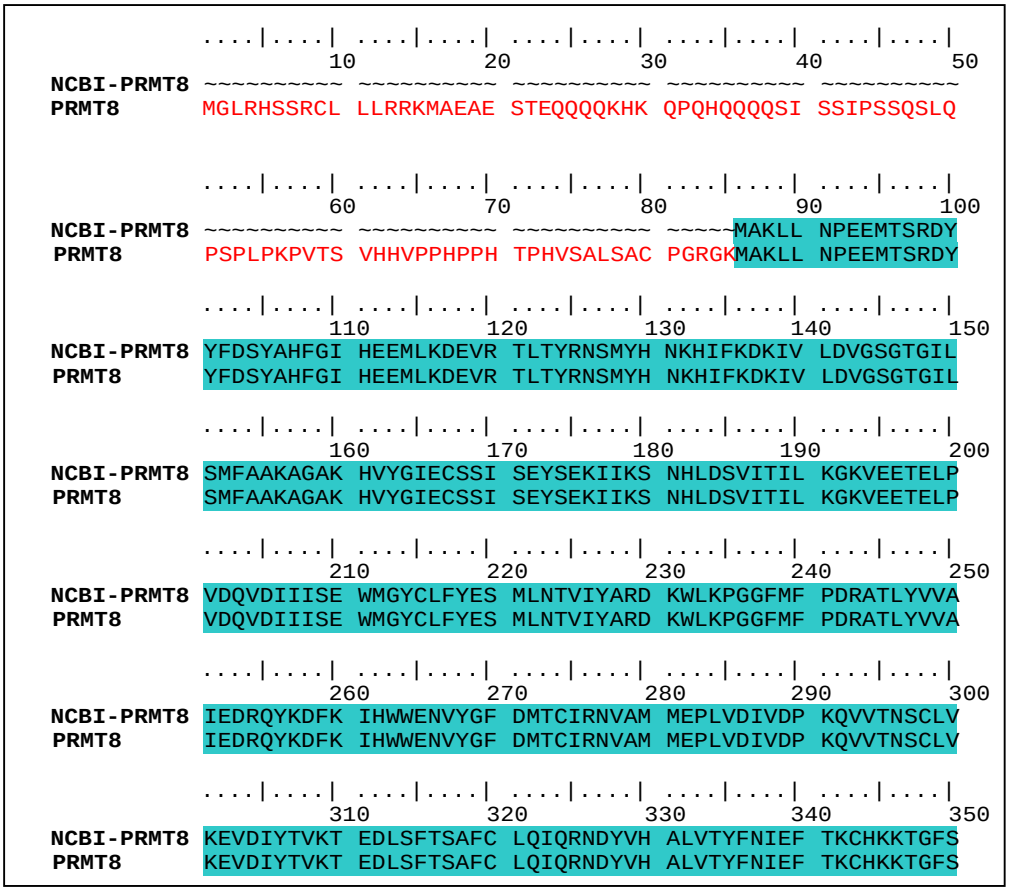
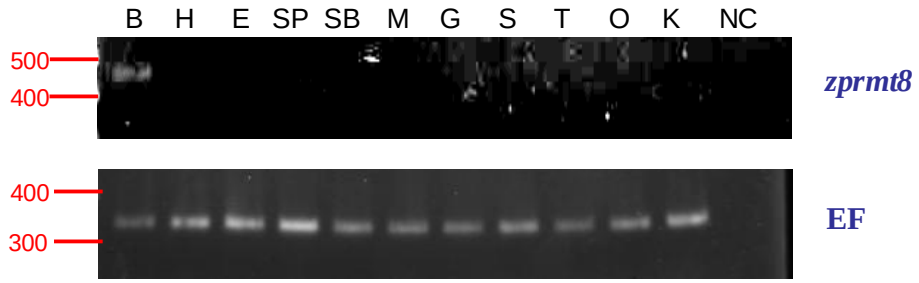


Fig. 7 Comparison of the amino acid sequence of zebrafish PRMT8 deduced and cloned by our laboratory and that from N CBI (NP_001038507).

A.



B.

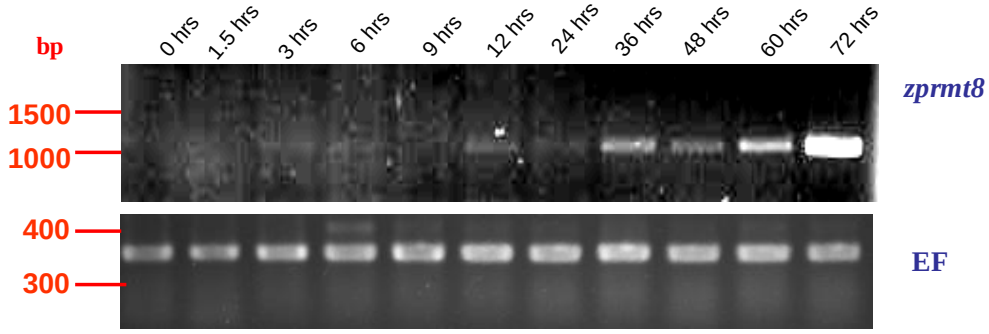


Fig. 8. mRNA expression of zebrafish prmt8. The expression of prmt8 was analyzed by RT-PCR with mRNA from different adult tissues or different embryonic stages.

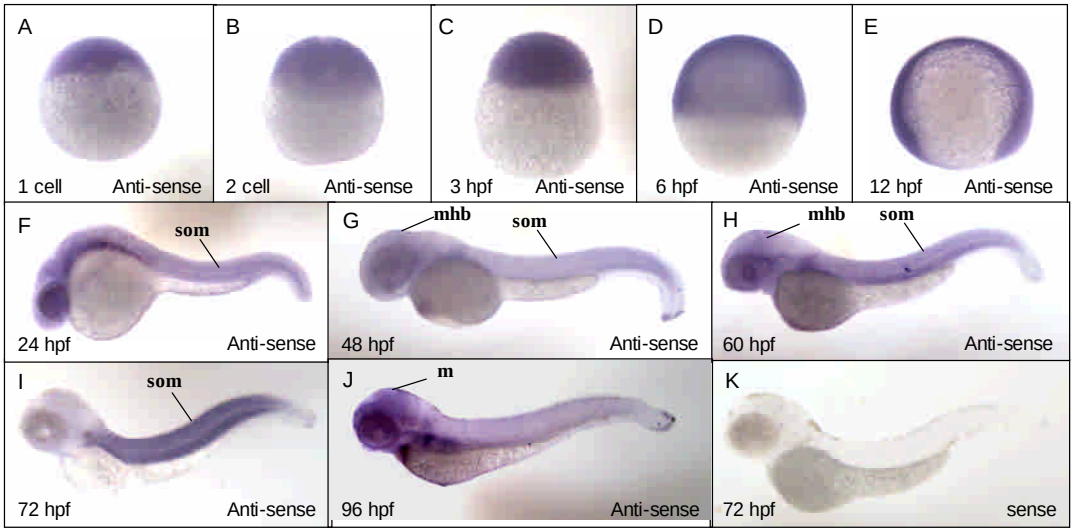
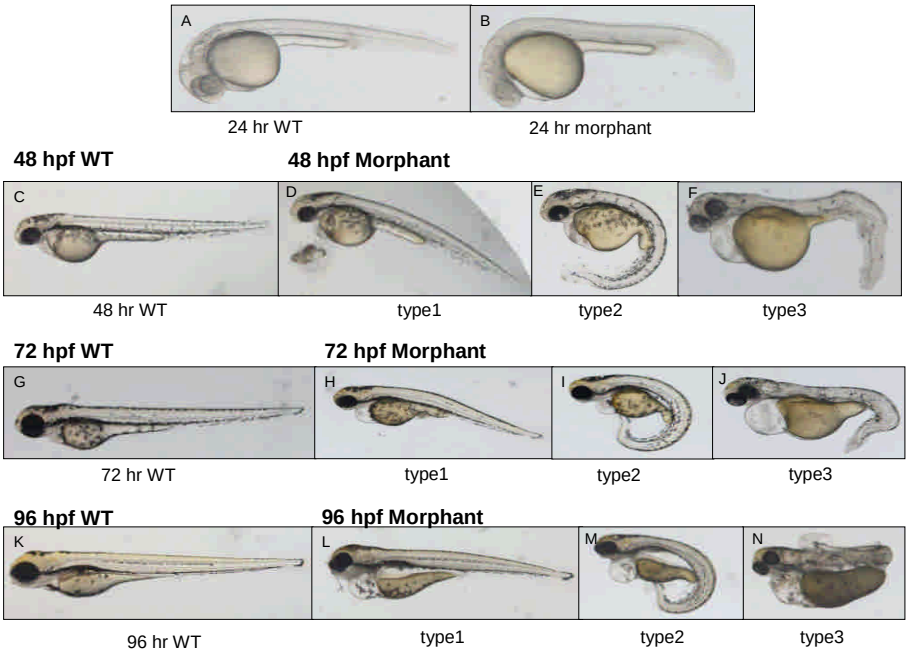
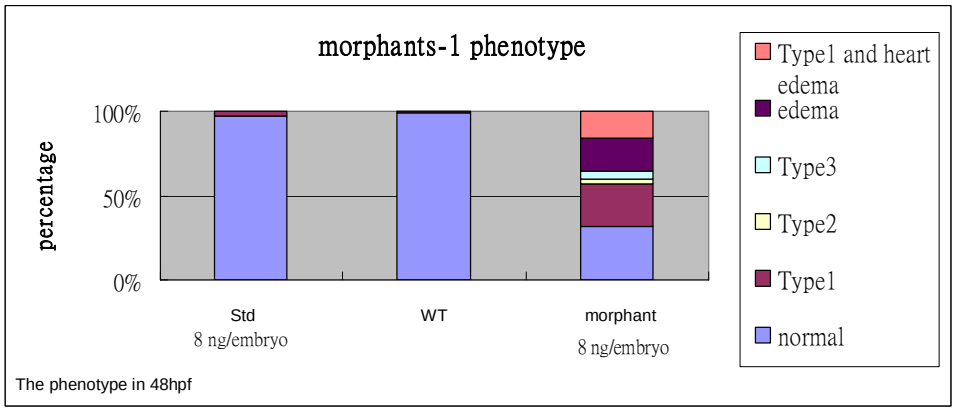


Fig. 9. The spatial and temporal expression of zebrafish prmt8 revealed by whole-mount in situ hybridization. Zebrafish embryos at 1 cell (A), 2 cell (B), 3 hpf (C), 6 hpf (D), 12 hpf (E), 24 hpf (F), 48 hpf (G) 60 hpf (H) 72 hpf (I) and 96 hpf (J). are analyzed. WISH with sense riboprobe is shown in (K). mhb, mid-hind brain boundary; m, mesencephalon; som, somites.

***prmt8* MO sequence**

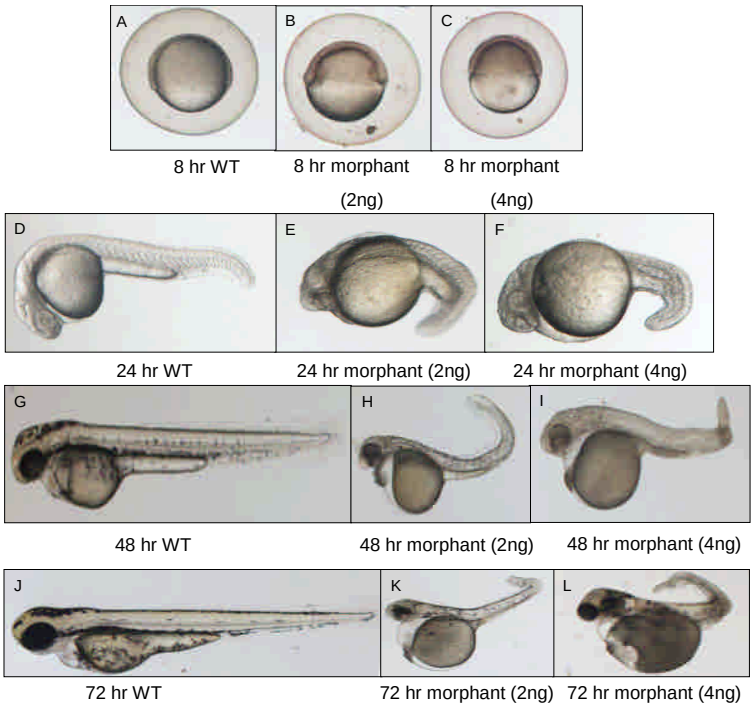
ATGGGACTGA GGCACTCATC GCGGT GTCTG CTCCT
+1 +25 (MO1)
GCGGA GAAAG ATGGC GGAGG CGGAG AGCAC GGAGCAGCAGCAA
+40 +64 (MO2)

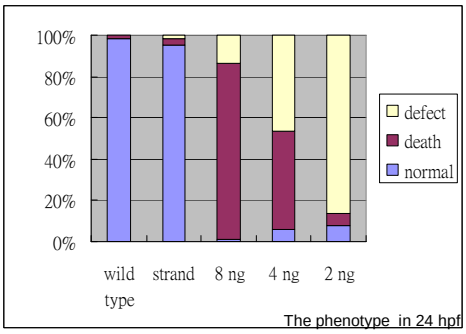




strand	normal	Type1	Type2	Type3	edema	Type1 and heart edema	total
N=	98	3	0	0	0	0	101
percentage	97.03%	3%	0.00%	0%	0%	0%	100%
WT							
N=	107	1	0	0	0	0	108
percentage	99.07%	1%	0.00%	0%	0%	0%	100%
Morphants							
N=	55	43	6	7	34	28	173
percentage	31.79%	25%	3.47%	4%	20%	16%	100%

Fig. 10. *zprmt8* morphants. *zprmt8* MOs were designed as indicated in the upper panel. Phenotypes of wild type embryos (A, C, G, K) and *zprmt8* morphants injected with MO1 (B, D-F, H-J, L-N) are shown. The phenotypes were classified and analyzed in the lower panel.

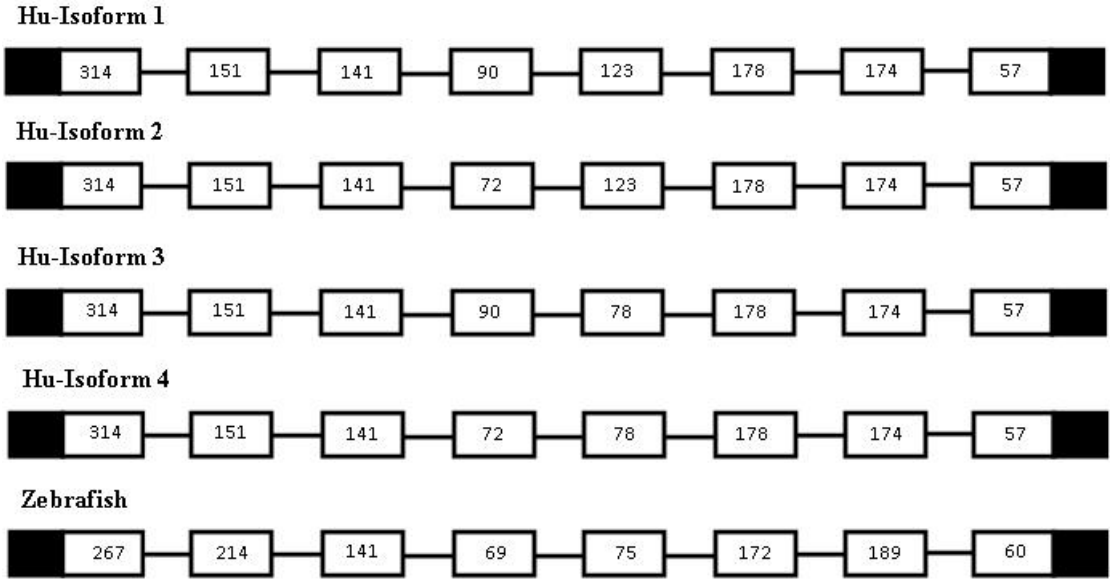




	normal	death	defect	total
wild type	108	2	0	110
	98.18%	1.82%	0.00%	100.00%
strand	120	4	2	126
	95.24%	3.17%	1.59%	100.00%
8 ng	3	177	28	208
	1.44%	85.10%	13.46%	100.00%
4 ng	11	90	88	189
	5.82%	47.62%	46.56%	100.00%
2 ng	20	14	215	249
	8.03%	5.62%	86.35%	100.00%

Fig. 11. Phenotype analyses of zebrafish prmt8 MO2 morphants. Phenotypes of wild type embryos (A, D, G, J) and *zprmt8* morphants injected with MO2 (B, E, H, K) are shown. The phenotypes were classified and analyzed in the lower panel.

A.



B.

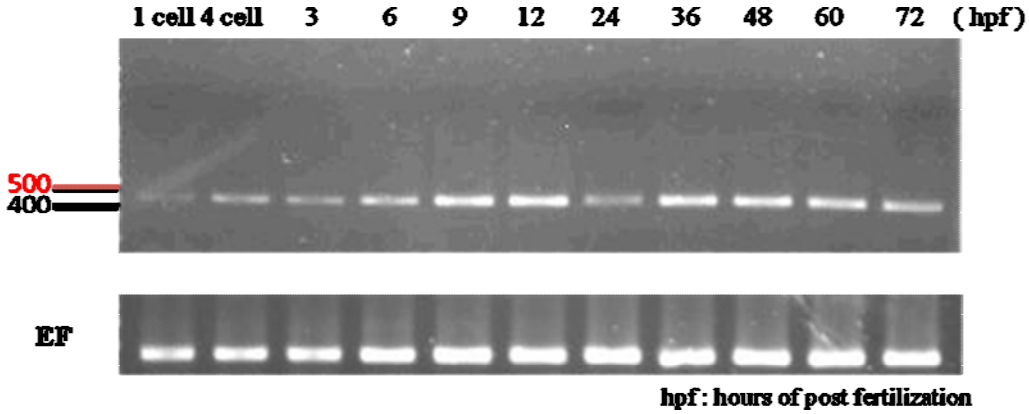


Fig. 12. Comparison of the gene structure of human and zebrafish *serbp1* and the expression of *zebrafish serbp1* at different developmental stages. From the comparison, zebrafish serbp1 appears to be most closely related to human isoform 4. The mRNA form different embryonic stages were prepared and amplified by RT-PCR.