Protein Arginine Methyltransferase 6 (Prmt6) Is Essential for Early Zebrafish Development through the Direct Suppression of gadd45αa Stress Sensor Gene*

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Histone lysine methylation is important in early zebrafish development; however, the role of histone arginine methylation in this process remains unclear. H3R2me2a, generated by protein arginine methyltransferase 6 (Prmt6), is a repressive mark. To explore the role of Prmt6 and H3R2me2a during zebrafish embryogenesis, we identified the maternal characteristic of prmt6 and designed two prmt6-specific morpholino-oligos (MOs) to study its importance in early development, application of which led to early epiboly defects and significantly reduced the level of H3R2me2a marks. prmt6 mRNA could rescue the epiboly defects and the H3R2me2a reduction in the prmt6 morphants. Functionally, microarray data demonstrated that growth arrest and DNA damage-inducible, α, a (gadd45αa) was a significantly up-regulated gene in MO-treated embryos, the activity of which was linked to the activation of the p38/JNK pathway and apoptosis. Importantly, gadd45αa MO and p38/JNK inhibitors could partially rescue the defect of prmt6 morphants, the downstream targets of Prmt6, and the apoptosis ratios of the prmt6 morphants. Moreover, the results of ChIP quantitative real time PCR and luciferase reporter assay indicated that gadd45αa is a repressive target of Prmt6. Taken together, these results suggest that maternal Prmt6 is essential to early zebrafish development by directly repressing gadd45αa.

During embryogenesis, the embryo undergoes dramatic developmental changes. After fertilization, the embryo is engaged in a rapid and synchronous series of cleavages, which are absent of gene transcription and instead regulated by maternal mRNA and proteins. At a certain critical period thereafter, the cell cycle becomes longer and asynchronous, zygotic genome activation starts, and maternal materials begin to degrade (1, 2). This process is known as the maternal-zygotic transition in all animals, paralleling the midblastula transition (MBT) of zebrafish. At the end of this transition, epiboly, which is the spreading and thinning of blastomere cells to enclose the yolk cell and which has been reported to be controlled by many maternal factors (3, 4), is initiated and plays an essential role in the progress of gastrulation. Therefore, maternal factors drive early embryogenesis, and a long standing issue in developmental biology is the identity of the maternal transcripts that are vital for early embryogenesis (5–7).

Early embryo development comes down to transcriptional activation and repression, which are precisely controlled by the state of the local chromatin (9). In this respect, histone methylation, including lysine and arginine methylation, might be an important post-translational modification involved in transcriptional regulation during early embryogenesis. In animal sperm, developmental genes are marked by histone lysine methylation, such as H3K4me2/3 and H3K27me3 in human and mouse spermatozoa (10) and H3K4me2/3, H3K27me3, H3K36me3, and H3K9me3 in zebrafish sperm (11). The modified chromatin in sperm could be transmitted to embryo through fertilization (8, 11, 12). Before the MBT, H3K4me3, H3K9me3, and H3K27me3 mark the zebrafish embryonic genome; they are transcriptionally silent and predict a propensity for transcriptional activation after the MBT (8). After the MBT, H3K4me3 levels increase and mark more than 80% of genes, including many inactive developmental regulatory genes that are also marked by H3K27me3, poised genes for their transcription (13).

Histone arginine methylations are generated by protein arginine methyltransferases (Prmts) (14). It is known that H3R2me2a is methylated primarily by Prmt6, preventing the binding of the SET1 methyltransferase complex or WDR5 (15, 16). Therefore, H3R2me2a mutually antagonizes H3K4me3 (17) and is known as a transcription-repressive mark. Prmt6 has been reported to regulate numerous biological process, including transcription (18–21), DNA repair (22), DNA replication (23), and signal transduction (24). These functions agree with the nuclear localization of Prmt6 (25). To date, many target genes of Prmt6 have been reported, including HoxA2 (17), thrombospondin-1 (26), p21 (20), p27 (27), p53 (21), Oct4 and Nanog (28), CD41 (29), and IL-6 (24).

The literature discussed above highlights the importance of histone lysine methylation for embryonic development; however, the roles of histone arginine methylation in early embryo-
genesis remain unknown. The goal of this study was to explore the role of Prmt6 and H3R2me2a during zebrafish embryogenesis. Our results indicate that Prmt6 is essential for early zebrafish development and acts by repressing growth arrest and DNA damage-inducible, α, a (gadd45αa).

**Experimental Procedures**

**Zebrafish Maintenance and Injection**—Zebrafish (Danio rerio) of the AB strain were provided by the Zebrafish Core Facility at the Shanghai Institute of Biochemistry and Cell Biology, and all experimental protocols were approved by the Institutional Animal Care and Use Committee. The embryos were harvested from the breeding tanks using a sieve and used for microinjection at the one- to two-cell stage (3 nl/embryo).

**Antibodies and Other Reagents**—The following antibodies were used for Western blotting: anti-H3R2me2a (1:500; 04-808, Millipore), anti-H3K4me3 (1:2,000; ab8580, Abcam), anti-H3 (1:5,000; p30266, Abmart), anti-Tp53 Z-FISH® (1:500; AS-55925s, AnaSpec), anti-PhosphoPlus® c-Jun (Ser-73) (1:1000; Duet 8222, Cell Signaling Technology), anti-β-actin (1:10,000; KC-5A08, KangChen Bio-tech), and horseradish peroxidase-conjugated secondary antibody (1:4,000; goat anti-rabbit; sc2030, Santa Cruz Biotechnology). The following antibodies (2 μg of each) were used for the ChIP experiments: anti-Myc (M20002, Abmart), anti-H3 (p30266, Abmart), anti-H3R2me2a and anti-H3K4me3 from Dr. Degui Chen at our institution, and anti-H3K4me3 (1:2,000; ab8580, Abcam), anti-H3 (1:5,000; 04-808, Millipore), anti-H3R2me2a (1:500; 04-808, Millipore). The following antibodies were used for the ChIP-qPCR experiments: anti-H3 (1:1000; Duet 8222, Cell Signaling Technology), anti-β-actin (1:10,000; KC-5A08, KangChen Bio-tech), and anti-H3K4me3 (1:2,000; ab8580, Abcam). The following antibodies were used for Western blotting: anti-H3R2me2a (1:500; 04-808, Millipore), anti-H3 (1:1000; Duet 8222, Cell Signaling Technology), anti-β-actin (1:10,000; KC-5A08, KangChen Bio-tech), and horseradish peroxidase-conjugated secondary antibody (1:4,000; goat anti-rabbit; sc2030, Santa Cruz Biotechnology).

**Mutagenesis and Other Reagents**—The following antibodies were used for Western blotting: anti-H3R2me2a (1:500; 04-808, Millipore), anti-H3K4me3 (1:2,000; ab8580, Abcam), anti-H3 (1:5,000; p30266, Abmart), anti-Tp53 Z-FISH® (1:500; AS-55925s, AnaSpec), anti-PhosphoPlus® c-Jun (Ser-73) (1:1000; Duet 8222, Cell Signaling Technology), anti-β-actin (1:10,000; KC-5A08, KangChen Bio-tech), and horseradish peroxidase-conjugated secondary antibody (1:4,000; goat anti-rabbit; sc2030, Santa Cruz Biotechnology). The following antibodies (2 μg of each) were used for the ChIP experiments: anti-Myc (M20002, Abmart), anti-H3 (p30266, Abmart), anti-H3R2me2a and anti-H3K4me3 from Dr. Degui Chen at our institution, and anti-H3K4me3 (1:2,000; ab8580, Abcam), anti-H3 (1:5,000; 04-808, Millipore), anti-H3R2me2a (1:500; 04-808, Millipore). The following antibodies were used for Western blotting: anti-H3R2me2a (1:500; 04-808, Millipore), anti-H3 (1:1000; Duet 8222, Cell Signaling Technology), anti-β-actin (1:10,000; KC-5A08, KangChen Bio-tech), and horseradish peroxidase-conjugated secondary antibody (1:4,000; goat anti-rabbit; sc2030, Santa Cruz Biotechnology).

**Plasmid Construction**—The coding sequences of zebrafish prmt6 and gadd45αa were subcloned into the EcoRI and XbaI sites in the pCS2+ vector. The catalytically inactive Prmt6 (VLD to KLA) was generated by the Fast Mutagenesis System (VLD to KLA) was generated by the Fast Mutagenesis System (FM111-01, TransGen Biotech), and radioimmune precipitation assay lysis buffer (PL005, Sangon Biotech), Fast Immuno Precipitation Assay Lysis Buffer (with 1% PMSF and protease inhibitors), and horseradish peroxidase-conjugated secondary antibody (1:4,000; goat anti-rabbit; sc2030, Santa Cruz Biotechnology). The following antibodies (2 μg of each) were used for the ChIP experiments: anti-Myc (M20002, Abmart), anti-H3 (p30266, Abmart), anti-H3R2me2a and anti-H3K4me3 from Dr. Degui Chen at our institution, and anti-H3K4me3 (1:2,000; ab8580, Abcam), anti-H3 (1:5,000; 04-808, Millipore), anti-H3R2me2a (1:500; 04-808, Millipore). The following antibodies were used for Western blotting: anti-H3R2me2a (1:500; 04-808, Millipore), anti-H3 (1:1000; Duet 8222, Cell Signaling Technology), anti-β-actin (1:10,000; KC-5A08, KangChen Bio-tech), and horseradish peroxidase-conjugated secondary antibody (1:4,000; goat anti-rabbit; sc2030, Santa Cruz Biotechnology).

**RNA Extraction, RT-PCR, and Quantitative Real Time PCR (qPCR)**—Total RNA was isolated from embryos using TRIzol (15596-018, Invitrogen/Life Technologies), and cDNAs were prepared using a ReverTra Ace qPCR-RT kit. RT-PCRs were performed with 2X Taq PCR Master Mix (PT102-02, Shanghai Lifegene Biotechnology) with the following conditions: 95 °C for 5 min followed by 26 cycles of 95 °C for 20 s, 58/62 °C (β-actin/prmt6) for 20 s, 72 °C for 30 s, and a final extension step of 72 °C for 5 min. β-Actin was used as a reference gene. The PCR products were visualized using a 2% agarose gel electrophoresis. For qPCR, an Applied Biosystems 7500 Fast Real-Time PCR System was used using UltraSYBR Mixture (with ROX) following the manufacturer’s instructions. eef1a1III was used for gene expression normalization, and the primer sequences are shown in Table 1. All experimental results are representative of three replicates.

**Whole Mount in Situ Hybridization**—A fragment sequence targeting bases 656–1407 of prmt6 mRNA was subcloned with pGEM®.T Easy Vector Systems using the primers listed in Table 1. Digoxigenin-UTP-labeled antisense RNA probes were synthesized using the DIG RNA labeling kit (11 175 025 910, Roche Applied Science) after linearization with NotI, and whole mount in situ hybridization was performed as described (30).

**Western Blotting Analysis**—For Western blotting analysis, embryos collected at the indicated times were lysed in radioimmune precipitation assay lysis buffer (with 1% PMSF and protease inhibitor mixture) and sonicated 10 times using a Bioruptor UCD-200 (Diagenode; high, 30 s with a 30-s interval). The supernatants of the lysate were denatured by boiling for 10 min in 1% SDS after centrifugation (13,200 rpm, 4 °C, 10 min). The proteins were then resolved using 15% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk or 1% BSA, according to the instructions for each antibody, for 1 h at room temperature and subsequently incubated with the corresponding antibodies overnight at 4 °C. The next day, the blots were washed three times with 1× TBS-Tween 20 (5 min/wash) and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antibody binding was then detected using enhanced chemiluminescence.

**Apoptosis Assay**—To assess apoptosis, embryos were dechorionated at 6 h postfertilization (hpf) and disaggregated into single cell suspensions in 500 μl of DMEM with 10% FCS. Next, the samples were washed and prepared for analysis of apoptosis using an Annexin V-FITC/PI Apoptosis Detection kit according to the manufacturer’s instructions. Approximately 20 embryos were analyzed from each treatment group.

**Microarray Analysis**—RNAs were extracted by TRIzol, and to ensure the RNA integrity, the RNA integrity number was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Qualified RNAs were then purified using
**Prmt6 in Zebrafish Development**

**Table 1.** The sequences of MOs and primers

| Name               | Sequence (5′−3′)                                                                 | Amplicon length (bp) | Analysis              |
|--------------------|---------------------------------------------------------------------------------|----------------------|-----------------------|
| prmt6-MO1          | ggcctagctgcttttagttttccccac                                                    | 752                  | RT-PCR                |
| prmt6-control MO1  | ggacatccctctatttcttcgac                                                      | 256                  | RT-PCR                |
| prmt6-MO2          | caagtcggcctcttttgtgcgac                                                      | 752                  | RT-PCR                |
| prmt6-control MO2  | ggacatccctctatttcttcgac                                                      | 256                  | RT-PCR                |
| hsp70-MO           | tcagctgctctttttggagaaacgac                                                    | 1068                 | RT-PCR                |
| pS35-MO            | gcgcactcttttctgttcgac                                                      | 262                  | RT-PCR                |
| gadd45saa-MO       | gttctctcttttgcctgtagtgcgac                                                   | 1068                 | RT-PCR                |
| thpl2-MO           | tgaagcatctctttttctcacaag                                                  | 492                  | RT-PCR                |
| Full prmt6 F       | atggccacacctttgctttaactgca                                                    | 1053                 | RT-PCR                |
| Full prmt6 R       | tctatttacctcattcttttctgca                                                   | 150                  | RT-PCR                |
| Mutational prmt6 F | gaggagagaagttgaagctgtggagccccgcgtacc                                             |                       |                       |
| Mutational prmt6 R | gttacgccgccttccacagctgtctttccccctc                                             |                       |                       |
| Probe of prmt6 F   | agagcagacgtgctgtagca                                                        | 102                  | RT-PCR                |
| Probe of prmt6 R   | cccacacaccttattcttaactccttaacc                                                | 102                  | RT-PCR                |
| prmt6s-5′-UTR F    | gctttctctaaacgagagcgctc                                                    | 167                  | qPCR                  |
| prmt6s-5′-UTR R    | cgggtctccacactcagaacc                                                        | 118                  | qPCR                  |
| gadd45saa F        | gadd45saa F                                                                    |                       |                       |
| gadd45saa R        | gadd45saa R                                                                    |                       |                       |
| hsp70 F            | cagagctcagccagctttgca                                                        | 120                  | qPCR                  |
| hsp70 R            | gaggagagaagttgaagctgtggagccccgcgtacc                                             |                       |                       |
| thpl2 F            | gaggagagaagttgaagctgtggagccccgcgtacc                                             |                       |                       |
| thpl2 R            | gaggagagaagttgaagctgtggagccccgcgtacc                                             |                       |                       |
| rx5 F              | gcgtgtctccctccctgtagtgcgac                                                   | 82                   | qPCR                  |
| rx5 R              | gcgtgtctccctccctgtagtgcgac                                                   | 91                   | qPCR                  |
| gadd45saa (−107) F | gadd45saa (−107) F                                                            |                       |                       |
| gadd45saa (−107) R | gadd45saa (−107) F                                                            |                       |                       |
| gadd45saa (−887) F | gadd45saa (−887) F                                                            |                       |                       |
| gadd45saa (−887) R | gadd45saa (−887) F                                                            |                       |                       |
| thpl2 (−1107) F    | thpl2 (−1107) F                                                                |                       |                       |
| thpl2 (−1107) R    | thpl2 (−1107) F                                                                |                       |                       |
| thpl2 (−172) F     | thpl2 (−172) F                                                                 |                       |                       |
| thpl2 (−172) R     | thpl2 (−172) F                                                                 |                       |                       |
| ef1a11 F           | taccctctcttttctgcttgcc                                                      | 108                  | qPCR                  |
| ef1a11 R           | tttttgaccttttgcctgcagc                                                      |                      |                       |

a RNEasy Micro kit (74004, Qiagen) and an RNase-Free DNase Set (79254, Qiagen). Biotin-labeled cRNAs were obtained using a GeneChip 3′IVT Express kit (901229, Affymetrix, Santa Clara, CA). Array hybridization and washing were carried out using a GeneChip® Hybridization, Wash and Stain kit (900720, Affymetrix) in Hybridization Oven 645 (00-0331-220V, Affymetrix) and Fluidics Station 450 (00-0079, Affymetrix) following the manufacturer’s instructions. Scanning of slides was performed with a GeneChip Scanner 3000 (00-00212, Affymetrix) and Command Console Software 3.1 (Affymetrix). The raw data were normalized using an MAS 5.0 algorithm with Gene Spring Software 11.0 (Agilent Technologies). A total of 15,502 probe sets were used for statistical analysis (p < 0.05, -fold change >2), and heat maps were generated using Cluster 3.0 (Michael Eisen). Gene ontology analysis was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (31) v6.7.

**ChIP-qPCR**—A six-repeat myc epitope-coding sequence was tagged at the 5′-end of the prmt6 sequence. The myc-prmt6 mRNA was injected into one- to two-cell embryos (25 pg/embryo). At 5.3 hpf, 50 embryos were collected for the ChIP experiment as described previously (32). Cross-linking of DNA and proteins was conducted by addition of 16% formaldehyde to a final concentration of 1% for 8 min at 37 °C and then quenched by addition of 10× glycerol for 5 min on ice. The samples were then sonicated using a Bioruptor UCD-200 (high, 25 times, 10 s each with 40-s interval) on ice. The ChIP procedure was performed using EZ ChIP kit 22 assays following the manufacturer’s instructions. The enriched DNA samples were used for qPCR analysis using UltraSYBR Mixture (with ROX), and the primer sequences are listed in Table 1. There are only two regions on the promoter of gadd45saa that were suitable for qPCR primer design and analyzed by qPCR. The results are presented as percent input (for anti-Myc and IgG) and over anti-H3 (for anti-H3R2me2a and anti-H3K4me3). ChIP experiments were repeated three times, and the data are shown as average values ± S.D.

**Luciferase Reporter Assay**—The 1503-bp promoter of gadd45saa was obtained using the primers listed in Table 1 and
Prmt6 Is Essential for Early Zebrafish Development—Having established that prmt6 is detectable in the developing zebrafish embryo, we decided to examine its physiological role using a morpholino-mediated gene knockdown strategy. Two pairs of prmt6-specific MOs were designed to block prmt6 translation as follows: one targeted the prmt6 ATG site (MO1) and its 5-base mismatch control (cMO1); the other targeted the prmt6 5′-UTR region (MO2) and its 5-base mismatch control (cMO2) (Fig. 2A). First, to test the effectiveness of the prmt6 MOs, the pCS2+−prmt6-5′-UTR-GFP was constructed and injected alone or co-injected with the prmt6 MOs/cMOs. Using this approach, we observed GFP fluorescence at the late gastrulation stage of embryos injected with the pCS2+−prmt6-5′-UTR-GFP that was unaffected by the prmt6 cMO1 and cMO2 but blocked by the prmt6 MO1 and MO2 (Fig. 2A). These data indicated that the prmt6 MOs could efficiently block the translation of prmt6.

Next, the prmt6 MO1 was injected into one- to two-cell embryos at doses ranging between 0.075 and 0.6 mm. Compared with normal embryos, prmt6 MO1/cMO1-treated embryos had no obvious phenotype before and during the MBT (data not shown). However, after the MBT, the epiboly of embryos injected with prmt6 MO1 was severely compromised (Fig. 2B). We classified this effect into three subtypes (normal, mild, and severe) based on the degree of epiboly at 6 hpf. The amount and severity of the defective epiboly positively correlated with the administered dose of prmt6 MO1 (Fig. 2C). Embryos with severe epiboly arrest died between 8 and 10 hpf, whereas the survival percentage of the mildly affected embryos was 66.7, 41.5, 38.7, and 32.7% with injection of 0.075, 0.15, 0.3, and 0.6 mm prmt6 MO1, respectively. As predicted, prmt6 MO2 injection led to phenotypes similar to those of prmt6 MO1 (Fig. 2C). From these data, we determined that 0.3 mm prmt6 MOs was the optimal dose for generating an effective phenotype. The severe phenotype following MO treatment suggests that Prmt6 has an important role in early zebrafish development.

Co-injection of Prmt6 Messenger RNA Rescues the Prmt6 Morphants—To confirm that the effect of the prmt6 MOs was specific, embryos were co-injected with myc-prmt6 mRNA (containing only the coding sequence), which had only 6 bases matching those of prmt6 MO1 and was not targeted by prmt6 MO2. This experiment showed that prmt6 mRNA could rescue the prmt6 morphants and that the extent of the rescue effect was dose-dependent (Fig. 2D). Furthermore, to determine whether Prmt6 function was enzyme-dependent, a rescue experiment was performed using myc-prmt6 mRNA (contain-
ing only the coding sequence) mutated to a catalytically inactive form (VLD to KLA) by site-specific mutagenesis. This mutated mRNA was expressed at the same level as the WT myc-prmt6 mRNA as shown by Myc tag (Fig. 2E) but could not rescue the prmt6 morphants (Fig. 2D). To further study the effect of prmt6 MOs, the level of H3R2me2a marks was measured in the prmt6 morphants at 6 hpf, showing a reduction over 80% compared with that of cMO treatment that could be rescued by myc-prmt6 mRNA but not by the mutated mRNA (Fig. 2F). Besides, potential off-target effects of MO were of concern (33). To exclude this possibility, embryos were co-injected with p53 MO and prmt6 MO1/MO2. These embryos showed a decrease of p53 protein compared with the prmt6 morphants but displayed a phenotype similar to that of the prmt6 morphants (Fig. 2G). These results indicate that the phenotype of prmt6 morphants is specific and depends on the enzymatic activity of Prmt6. 

Gadd45a Is Up-regulated and Acts as a Mediator in the Prmt6 Morphants—Based on the remarkable decrease of H3R2me2a in the prmt6 morphants, we speculated that the genome of these embryos might be dramatically changed. To verify this hypothesis, a gene microarray was performed in control embryos and in the prmt6 morphants with normal, mild, and severe phenotypes. Compared with the cMO1-injected embryos, microarray analysis of the prmt6 morphant with

**FIGURE 2. Prmt6 is essential for early zebrafish development.** A, successful suppression of prmt6 expression was confirmed in embryos injected with the plasmid pCS2 + prmt6-5′-UTR-GFP and prmt6 MO1/MO2. B, knockdown of Prmt6 expression using the prmt6 MOs resulted in a normal, mild, or severe phenotype characterized by defective epiboly as shown at 6 hpf. The single enlarged embryo is shown from lateral views with the animal pole on the top. C, a quantification of the relative distribution of the phenotype of prmt6 morphants at 6 hpf. D, a quantification of the relative distribution of the phenotype of prmt6 morphants rescued with myc-prmt6 mRNA (containing only the coding sequence) and mutated myc-prmt6 mRNA (containing only the coding sequence) that codes an inactive form of Prmt6 (VLD to KLA). E, a representative Western blot showing the protein expression level of the rescue constructs by Myc tag. -Fold of basal, the Myc ratios were determined by the densitometric value of each construct relative to that of the control (myc-prmt6 mRNA) after normalizing to the β-actin densitometric values. myc-prmt6 mRNA/myc-prmt6 mutated mRNA, 200/200 pg. F, a representative Western blot showing the effect of the prmt6 MOs on the H3R2me2a level. cMOs/MOs, 0.3/0.3 mM; myc-prmt6 mRNA/myc-prmt6 mutated mRNA, 200/200 pg. G, a quantification of the relative distribution of the phenotype of prmt6 morphants and a representative Western blot showing the p53 protein level rescued with p53 MO. Protein loads per lane for E, F, and G, 37.5 μg. In C, D, and G, the number of embryos in each group is indicated above the relevant column. Scale bar in A and B, 200 μm.
A severe phenotype identified 1,878 misregulated probe sets (824 up-regulated and 1,054 down-regulated, \( p < 0.05, \text{fold change} > 2 \)), which accounted for 12% of the total 15,502 probe sets. These differential probe sets mapped to 724 up-regulated and 858 down-regulated genes (supplemental Table 1A). The differentially expressed genes were enriched for the following biological processes: pattern specification process, regionalization, embryonic morphogenesis, dorsal/ventral pattern formation, gastrulation, heart development, digestive tract morphogenesis, gut development, cell migration involved in gastrulation, sensory organ development, and some others (supplemental Table 1B). This indicates that prmt6 MO1 altered global gene expression and development processes in the prmt6 severe morphant embryo.

To explore the Prmt6 candidate targets, the top 10 up-regulated and down-regulated genes (see supplemental Table 1A) were studied using the three prmt6 subtypes (Fig. 3A). To verify the microarray data, four genes were selected from the top 10 up-regulated list, namely gadd45a, hsp70, tbpl2, and rx3, and two genes were selected from the top 10 down-regulated list, namely eve1 and sox2. Our RT-qPCR analysis of these genes confirmed the microarray data with the exception of hsp70 in the severe phenotype group (Fig. 3B). These data suggest a relationship between the phenotype of the
**Prmt6 in Zebrafish Development**

*prmt6* morphants and the degree of dysregulation of the top *prmt6* targets.

Given the reported suppressive function of Prmt6 (19–21) and the decrease of repressive modifications in the *prmt6* morphants (Fig. 2F), we hypothesized that the *prmt6* morphants might be a result of up-regulation of Prmt6 target genes. Interestingly, *gadd45a*, a member of the Gadd45 stress sensor genes involved in activation of the p38/JNK pathway and apoptosis via activation of MEKK4 (34–36), was the top up-regulated gene in the list (Fig. 3, A and B). Besides, some reports have demonstrated that Prmt6 is involved with proliferation and apoptosis (19–21). Based on this, we hypothesized that the up-regulation of *gadd45a* might be responsible for the *prmt6* morphants. To test this hypothesis, we designed a *gadd45a* translation-blocking MO, which was validated using the plasmid pCS2*-gadd45a*-5'-UTR-GFP (Fig. 3C). It was used to rescue the defects of the *prmt6* MOs, showing that the *gadd45a* MO could partially rescue the *prmt6* morphants in a dose-dependent manner (Fig. 3E). We further analyzed the roles of *hsp70* and the maternal and zygotic *tbpl2* in the top 10 up-regulated list using a designed and validated *tbpl2* MO (Fig. 3D) and a reported *hsp70* MO (37, 38). However, neither *tbpl2* MO nor *hsp70* MO could rescue the *prmt6* morphants (Fig. 3F). We also found that one-to-two-cell embryos injected with *gadd45a* mRNA for a gain of function analysis displayed a phenotype similar to that of *prmt6* MO-treated embryos in a dose-dependent manner (Fig. 3G). These data suggest that the up-regulation of *gadd45a* is responsible for the *prmt6* morphants.

The Up-regulation of Gadd45a in the Prmt6 Morphants Is Associated with Activation of the p38/JNK Pathway and Apoptosis—To test whether the up-regulation of stress-inducible *gadd45a* in the *prmt6* morphants could lead to the activation of the p38/JNK pathway, qPCR analysis of p38/JNK targets was carried out, showing that the transcription of *c-jun*, *p53*, and *p21* were up-regulated in the *prmt6* morphants (Fig. 4A). Furthermore, analysis of apoptosis showed that early apoptosis (Annexin V+/H11001) increased in the *prmt6* morphants, particularly in the severe phenotype group, with an apoptosis ratio of 60% versus 18.6% in cMO1-injected embryos (Fig. 4B). Therefore, *prmt6* MO1 might activate the p38/JNK pathway through up-regulation of *gadd45a*, thereby causing apoptosis.

Based on the above data, we performed a series of experiments to determine the importance of the p38/JNK signaling pathway in mediating the effect of the *prmt6* MOs. To confirm the importance of the p38/JNK pathway in this scenario, embryos were treated with the p38/JNK inhibitors SB203580/SP600125 (39, 40). The efficacy of SP600125 was validated by analyzing the phosphorylation level of *c-jun* (Fig. 4C). However, the efficacy of SB203580 could not be tested because of a lack of an effective antibody. As expected, p38/JNK inhibitors partially rescued the *prmt6* morphants with the two inhibitors having a mild additive effect (Fig. 4C).

To detect whether the Prmt6 downstream targets could be rescued, *gadd45a*, *c-jun*, *p53*, and *p21* were analyzed in the embryos of rescue experiments using qPCR. This showed that the *prmt6* mRNA could rescue the expression level of *gadd45a*, *c-jun*, *p53*, and *p21* and that the *gadd45a* MO and the p38/JNK inhibitors could rescue the expression level of *c-jun*, *p53*, and *p21* (Fig. 4, D and E). Accordingly, analysis of apoptosis showed that early apoptosis increased in the *prmt6* MO1 morphants with an apoptosis ratio of 41.3% versus 13.9% in cMO1-injected embryos. This could be rescued by the *prmt6* mRNA, the *gadd45a* MO, and the p38/JNK inhibitors to 18.1, 20.2, and 21.7%, respectively, but not by the *prmt6* mutated mRNA or the *p53* MO (Fig. 4F). The analysis of apoptosis in the rescue experiments showed a similar trend using *prmt6* MO1 and MO2 (Fig. 4F). Taken together, these data show that activation of the p38/JNK pathway by up-regulation of *gadd45a* is responsible for the phenotype of *prmt6* morphants.

*Gadd45a Is a Repressive Target of Prmt6—*To check whether Prmt6 directly regulates *gadd45a* expression, we performed a ChIP analysis with 5.3-hpf embryos. The myc-*prmt6* mRNA was injected into one- to two-cell embryos (25 pg/embryo) without abnormal phenotypes, and the embryos were collected for the ChIP experiment. The myc-*prmt6* translation could be detected by Western blotting from 2 to 5.3 hpf (Fig. 5A). The data show that Prmt6 binds to the *gadd45a* promoter (Fig. 5B). As a control, the maternal and zygotic *tbpl2* gene was also assayed but was found to display no significant enrichment at the *tbpl2* promoter (Fig. 5C).

H3R2me2a is generated by Prmt6 and antagonizes H3K4me3 (15, 16). We analyzed the changes of H3R2me2a and H3K4me3 in the *gadd45a* promoter of the *prmt6* severe morphant using antibodies for H3R2me2a, H3K4me3, and H3. The results showed that the *prmt6* MO1 remarkably reduced H3R2me2a levels and significantly increased H3K4me3 levels on the promoter of *gadd45a* (Fig. 5, D and E). To confirm the direct regulation of the promoter of *gadd45a* by Prmt6, the pGL3-*gadd45a* promoter vector was constructed for the luciferase reporter assay, which showed that *prmt6* MO1 significantly enhanced the activity of the promoter of *gadd45a*, whereas *prmt6* mRNA markedly suppressed the activity of the promoter of *gadd45a* (Fig. 5F).

Based on the above data, we tried to explore the effects on the functional transcription of *gadd45a* of environmental stress caused by 5 mM H2O2 treatment. The data from three time points (2.25, 4, and 6 hpf) showed that the transcription level of *gadd45a* during early zebrafish development is low (Fig. 5G). Under the H2O2 treatment, the transcription of *gadd45a* was induced, and the JNK pathway was activated at 4 and 6 hpf (Fig. 5G). Furthermore, we also observed that the H3R2me2a level at *gadd45a* promoter, which is higher at 4 and 6 hpf than at 2.25 hpf during normal development, was decreased at 4 and 6 hpf by the H2O2 treatment (Fig. 5H). These data suggest that the response of *gadd45a* to stress might be through the modification of H3R2me2a levels at *gadd45a* promoter. Taken together, and as summarized in Fig. 5I, these data indicate that *gadd45a* is directly suppressed through the modification of H3R2me2a generated by Prmt6 to prevent apoptosis induced by the activation of the p38/JNK pathway during early zebrafish development.

**Discussion**

In this study, knockdown of Prmt6 using MO decreased the global level of H3R2me2a and led to early epiboly arrest, highlighting an important role for maternal Prmt6 in early zebrafish development. Our mechanistic studies revealed that the *prmt6*

**408 JOURNAL OF BIOLOGICAL CHEMISTRY**
MO directly up-regulated gadd45a and subsequently activated p38/JNK pathway signaling and induced apoptosis, thereby explaining the phenotype of \textit{prmt6} morphants.

Previous studies have demonstrated that Prmt6 plays important roles in numerous biological processes (19–21, 26, 29). However, the importance of the H3R2me2a and Prmt6 in embryogenesis remains unknown. In this study, we initially detected the existence of the H3R2me2a mark during early zebrafish development (Fig. 1A). This result is consistent with a previous report that four-cell-stage mouse embryos are characterized by high levels of H3R2me2a (41). In addition, we generated a zebrafish catalytically inactive Prmt6 form (VLD to KLA) by means of the reported human methylase-inactive PRMT6 mutant (23). This mutant could not increase the level of H3R2me2a in the \textit{prmt6} morphants as expected (Fig. 2F). This is evidence of the conservation of Prmt6 between zebrafish and human (14).

Many direct targets of Prmt6 have been reported (19–21, 26). In this study, we determined the misregulated genes in the \textit{prmt6} morphants through microarray analysis and proved...
that the up-regulation of gadd45a (Fig. 3E), not the up-regulation of hsp70 and tpbl2 (Fig. 3F), is responsible for the prmt6 morphants. Although the Gadd45a protein level could not be detected because of a lack of an effective antibody, the results from qPCR (Fig. 3B), quantitative ChIP (Fig. 5, B, D, and E), and the luciferase reporter assay (Fig. 5F) confirmed that gadd45a is directly regulated by Prmt6. The up-regulation of hsp70 might be a response to the activation of the p38/JNK pathway induced by the up-regulation of gadd45a, and the up-regulation of tpbl2 might be the result of the failure of degradation of maternal mRNA. This needs to be confirmed by further studies.

Of note, gadd45a MO could only partially rescue the prmt6 morphants, suggesting that additional targets of Prmt6 exist. Interestingly, there were 858 down-regulated genes in the prmt6 severe morphant that included zygotic genes and demonstrated the failure of zygotic genome activation. Therefore, whether Prmt6 directly contributes to zygotic genome activation through generating active modifications, such as H4R3me2a and H3R42me2a (14, 18), deserves to be investigated. Additional ChIP-sequencing studies are required to determine whether the misregulated genes (see supplemental Table 1A) are directly regulated by Prmt6.

The timing and the mediators involved at the start of apoptosis in different animals have been identified (42–44). In zebrafish, the apoptotic response is activated during development at 8 hpf (45) as zBik, a key factor in regulating apoptosis, is not expressed until 8 hpf (46). In this study, apoptosis was observed at 6 hpf in the prmt6 morphants (Fig. 4, B and F) that was the result of the up-regulation of gadd45a by knockdown...
of Prmt6. These results reveal that the high maternal level of Prmt6 might be one of the reasons for the absence of apoptosis in early zebrafish embryos.

Given the specificities of SP600125 (47), JNK-IN-8, another JNK inhibitor, was also used and found to partially rescue the prmt6 morphants with results similar to SP600125 (data not shown). A high mortality rate of embryos incubated in 0.8 μM SP600125 has been reported (48). However, this was not seen in our experiment (Fig. 4C), which suggests that the amount of SP600125 injected (320 μM; 3 nl/embryo) was low. These results indicated that the rescue by SP600125 is specific.

In conclusion, this study identified that maternal Prmt6 is required for early embryogenesis by preventing apoptosis through direct suppression of gadd45αααα. Other reports demonstrate that PRMT6 is up-regulated in diverse cancer types, promoting growth and suppressing apoptosis, which is of potential significance for cancer therapeutics (19, 20, 26). These studies suggest that the balance of Prmt6 plays an important role in proliferation and apoptosis and that further study of this protein may prove of importance to developmental biology and anticancer research.

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