Uracil-DNA Glycosylase is involved in DNA demethylation and required for embryonic development in the zebrafish embryo

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Running title: Ung in DNA demethylation and zygotic genome activation

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Background: Ung implication in DNA demethylation and embryonic development is poorly understood.

Results: unga knockdown in the zebrafish embryo increases global DNA methylation level, inhibits transcription and causes embryonic lethality while its overexpression produces opposite effects on DNA methylation and transcription.

Conclusion: Unga is involved in postfertilization DNA demethylation and transcription.

Significance: The findings shed new light on Ung function in DNA demethylation and embryonic development.

ABSTRACT

Uracil DNA glycosylase (Ung) is a component of base excision repair process and has the ability to remove uracil from U:G mispairs in DNA. However, its implications in development of vertebrate embryos are poorly understood. In this study, we found that zebrafish uracil-DNA glycosylase a (Unga) is maternally expressed at high levels and accumulated in nuclei during cleavage and blastulation periods. Knockdown of unga in zebrafish embryos causes an increase of the global DNA methylation level concomitantly with a reduction of overall transcriptional activity in the nucleus, ultimately resulting in embryonic lethality during segmentation period. Conversely, unga overexpression is sufficient to reduce the global DNA methylation level, to increase H3K4me3 and H3K27me3 marks and to activate genome transcription. Furthermore, overexpression of unga(D132A) mRNA, encoding a mutant Unga without DNA glycosylase activity, does not affect global DNA methylation level, indicating that its involvement in DNA demethylation is dependent on its glycosylase activity. These results together suggest that Unga is implicated in postfertilization genomic DNA demethylation, zygotic gene transcription and normal embryonic development in zebrafish.
controversial whether parental genomes undergo DNA demethylation immediately after fertilization. Mhanni and Mcgowan disclosed, based on methylation-sensitive restriction enzyme digestion of genomic DNA, that the GDM level of the zygotic genome declines after fertilization and starts to bounce back at blastula stages (9), which was confirmed by immunohistochemistry with an anti-5mC antibody (10). Recently, DNA methylation profiling by whole-genome shotgun bisulfite sequencing also detects a moderate decrease of the GDM level during early cleavage stages in zebrafish embryos (11). However, Jiang et al. reported that postfertilization demethylation of parental genomes may not occur, and that, upon zygotic genome activation (ZGA), many loci in maternal chromosomes of embryos are re-methylated to the state observed in the sperm (12).

One central question is how the parental genomes in an embryo are demethylated to acquire embryonic totipotency and to activate genome transcription. In mouse zygotes, the dioxygenase TET3 (Ten-Eleven Translocation 3) acts to demethylate the paternal genome by converting 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) before the first mitosis (13-16). However, it seems that dioxygenase-catalyzed DNA demethylation may not function in the zebrafish zygote since 5hmC is rarely detected in early zebrafish embryos (11,12).

In this study, we identified uracil-DNA glycosylase a (Unga) as a maternally expressed DNA glycosylase. Ung is a member of uracil DNA glycosylase (UDG) family and participates in base excision repair process by removing uracil from U:G and U:A mispairs in DNA (17). We demonstrate that unga is implicated in postfertilization DNA demethylation and zygotic gene transcription activation and is required for normal embryonic development.

EXPERIMENTAL PROCEDURES

Zebrafish strain and microinjection — Tuebingen strain was used in this study with ethical approval from the Animal Care and Use Committee of Tsinghua University. For producing homogeneous embryos, in vitro fertilization was performed and a fraction of embryos were then fixed at desired time points.

When needed, mRNAs, morpholinos, BrUPT and BrdUPT were individually or in combination microinjected into the yolk or cytoplasm of one-cell stage embryos and collected for analysis at later stages. The dose of BrUPT and BrdUPT was 5 pmol and 1 pmol per embryo, respectively. The mRNAs were injected at a dose of 500 pg per embryo and morpholinos were injected at a dose of 10 ng per embryo unless otherwise stated. For mRNA synthesis, the coding sequence of zebrafish unga (ENSDARG00000042527) was fused to the coding sequence of mcherry, which were together subcloned into pX7 vector. The mutant form of unga, unga(D132A), was modified from other unga-containing vectors. Information of other constructs was described in the other sections. The sequences of unga-MO and cMO were 5'-GCTTTTCTGTCCGATCATTTCCACA-3' and 5'-GGTTTTGTGTGCATGATTCCACA-3' (mutated bases were underlined), respectively. Embryos were incubated in Holtfreter’s solution at 28.5°C and staged according to Kimmel et al. (18).

Whole-mount in situ hybridization — The antisense RNA probe was in vitro synthesized in the presence of digoxigenin-labeled UTP. Whole-mount in situ hybridization was performed using the commonly used protocol.

Immunostaining — Zebrafish embryos at desired stages were fixed by 4% polyformaldehyde for 1 day at 4°C, dechorionated manually and dehydrated with methanol. After being stored at -20°C for 1 h, embryos were rehydrated with 2% PTX (2% Triton-X 100 in PBS), and treated in 2 M HCl for 1 h at room temperature followed by neutralizing in 100 mM Tris-HCl (pH8.5) for 15 min. Embryos were washed with 2% PTX for three times, 5 min each, incubated in the block solution (1% BSA, 10% goat serum, 0.3 M glycine in 2% PTX) for 1 h, and transferred to the block solution containing a primary antibody for incubation overnight at 4°C, followed by washing with 2% PTX for six times, 5 min for the first two times and 30 min for the last four times, at room temperature. Next, embryos were incubated in fluorescence-conjugated secondary antibody overnight at 4°C. After wash with 2% PTX six times, embryos were mounted and observed by confocal microscopy. Confocal laser scanning was done by Zeiss LSM710-3 channel system.
and manipulated by ZEN software. Staining intensity measurement was done by Image J software. The following primary antibodies were used: mouse anti-5mC (Abcam, ab108055, 1:500), mouse anti-5mC (Abcam, ab51552, 1:100), rabbit anti-H3 (Abcam, ab1791, 1:500), mouse anti-H3 (EASYBIO, BE3015, 1:200), mouse anti-BrdU TP (Santa Cruz, sc-32323, 1:100), anti-ssDNA (IBL, JP18731, 1:100), rabbit anti-H3K27me3 (Millipore, 07-449, 1:100), rabbit anti-H3K4me3 (Abcam, ab8580, 1:100) and rabbit anti-H3K27me3 (Millipore, 07-449, 1:100). The secondary antibodies were 488 nm-conjugated anti-rabbit, 543 nm-conjugated anti-mouse, 543 nm-conjugated anti-rabbit, 543 nm-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch, 1:200 diluted for use).

\textit{DNA dot blotting} — Genomic DNA was extracted from 256-cell stage embryos using Animal DNA Kit (DP324, TIANGEN Biotech Co.). All of the purified DNA samples were diluted to a concentration of 50 ng/μl. The diluted samples were denatured at 95°C for 10 min, quenched on ice, and then individually spotted in a desired volume onto a piece of BioTrace™ NT nitrocellulose membrane (Pall Co., T126211), followed by thermal cross-linking at 80°C for 2 h. The membrane was incubated with the block solution (TBS with 0.5% Tween-20, 5% BSA) for 1 h, and then in the block solution containing anti-5mC primary antibody (Abcam, ab108055, 1:1000) overnight at 4°C. The membrane was washed with TBS, 0.5% Tween-20 for 10 min three times, and then incubated in HRP-conjugated anti-mouse secondary antibody overnight at 4°C or 1 h at room temperature. Following wash with TBS, 0.5% Tween-20 for 10 min three times, the membrane was incubated in ECL substrate solution for 1 min and then exposed to a film for 5 min.

Western blotting and cytoplasmic/nuclear fractionation using embryonic lysates — Embryos were dechorionated at a desired stage by pronase treatment and deyolked by pipetting with a 200-μl tip. Embryonic cells were collected after centrifugation at 1,000 rpm for 3 min, and then lysed in TNE buffer (100 μl for 200 embryos). The lysate was centrifuged at 12,000 rpm for 10 min and the supernatant was collected. Following addition of SDS loading buffer, the sample was denatured at 95°C for 10 min and an aliquot (equivalent to 40 embryos) was loaded onto an SDS-PAGE gel. Cytoplasmic and nuclear proteins were fractionated using the Nuclear and Cytoplasmic Extraction Kit (CWBO, CW0199B) according to the manufacturer’s instruction. The used antibodies were: anti-H3 (Abcam, Ab1791, 1:5000), anti-α Tubulin (Sigma, T5168, 1:1000), anti-H3 (EASYBIO, BE3015, 1:10000), anti-Ung (GeneTex, GTX103236, 1:2000), and anti-actin (Santa Cruz, I-19, 1:1000) primary antibodies; HRP-conjugated anti-mouse, HRP-conjugated anti-rabbit and HRP-conjugated anti-goat secondary antibodies (Jackson ImmunoResearch, 1:5000).

\textit{Transcriptome analysis} — Total RNA was isolated from zebrafish embryos at desired stages using the RNaseasy Mini Kit (Qiagen, 74104). The RNAs were sequenced by BGI Tech using Illumina HiSeq™ 2000 Sequencing System. Sequencing quality evaluation, gene expression annotation, and screening of differentially expressed genes were performed by BGI Tech. Then we did more analysis based on results of DEG (Differentially Expressed Genes) screening.

Genes detected by RNA seq in early wild-type zebrafish embryos were categorized into three classes Z, M\textsuperscript{h} and M\textsuperscript{Z}. For a Z gene, its transcript read was less than 2/M (two in a million of total transcripts) in one-cell stage embryos likely due to an absence of its maternal transcripts, and got higher in 512-cell stage embryos presumably due to its transcription after the zygotic genome activation; the transcript read of a M\textsuperscript{h} gene was more than 2/M in one-cell stage embryos and became further higher in 512-cell stage embryos presumably due to its transcription after the zygotic genome activation; the transcript read of a M\textsuperscript{Z} gene was more than 2/M in one-cell stage embryos and became lower in 512-cell stage embryos presumably due to degradation of its maternal transcripts and silence of zygotic transcription.

DNA glycosylase activity assay — Sequence coding for zebrafish Unga or Unga(D132A) was cloned into the expression vector pGEX-6P-1. The recombinant plasmid was transformed into E. coli BL21 cells. The expression of the recombinant protein was induced by IPTG at 1 mM for 4 h at 20°C. The recombinant protein was purified using Ni-NTA spin column. E. coli UDG was purchased from NEB (M0280).

For in vitro assay of DNA glycosylase...
activity, 100 ng DNA oligos and 30-300 ng recombinant protein were incubated in 10 µl of 20 mM Tris-HCl, 1mM EDTA and 1mM dithiothreitol for 30 min at 37°C. The reaction was stopped by adding 90 mM NaOH and 10 mM EDTA and heating at 95°C for 5 min. The product was separated on PAGE gel, followed by ethidium bromide staining.

Statistical analyses—Data averaged from multiple samples were expressed as mean plus standard deviation (SD). Significance of difference between two treatments was analyzed using Student’s t-test. Significance levels were indicated in corresponding context.

RESULTS

The Global DNA Methylation Level Dynamically Changes during Zebrafish Early Embryogenesis—Previous papers reported contradictory conclusions about postfertilization DNA methylation pattern during zebrafish early embryogenesis (9-12). We set out to re-investigate the global DNA methylation (GDM), by co-immunostaining using anti-5mC and anti-Histone 3 (H3) antibodies, during the first 10 cell cycles of zebrafish embryos. The ratio of 5mC/H3 intensities in the nucleus, named relative GDM level, was calculated to allow comparison among embryos at different stages. Results showed that the GDM level tended to decrease gradually from 1-cell (20 min postfertilization) to 8-cell (1.25 hours postfertilization (hpf)) stages, then maintained roughly unchanged until 32-cell (or 64-cell) stage and thereafter rebounded (Fig. 1). These dynamic changes are similar to those previously revealed by the HpaII/MspI end-labeling assay (9) and by whole-genome bisulfite sequencing (see Fig. 1E in Potok et al. 2013)(11). Given that the GDM level showed a decrease far earlier prior to MBT, we speculated that maternal factors might contribute to such a demethylation process.

unga Transcripts Are Maternally Supplied in Zebrafish Early Embryos—To search for maternal factors possibly related to DNA demethylation, we analyzed RNA profiles of unfertilized eggs and embryos at different stages by RNA seq and found 29 maternal genes whose products have been previously reported to involve DNA demethylation in various species (Table 1). Among them, unga, which encodes a uracil-DNA glycosylase superfamily member (19), has the most abundant transcripts in eggs. Its duplicate gene, ungb, did not have transcripts in eggs (data not shown). Unexpectedly, transcripts of tet1-3 and tdg, which are known to be involved in active DNA demethylation during early mouse embryogenesis (13-15,20), were not detected in eggs. Then, our further study had been focused on unga.

We investigated the spatiotemporal expression pattern of unga by whole-mount in situ hybridization. Transcripts of unga were present in immature eggs of different stages and in embryos with uniform distribution during early cleavage period (Fig. 2A). Its expression was reduced to an undetectable level at the 50% epiboly stage (5.3 hpf) and resumed after the bud stage. During segmental period, its transcripts were ubiquitously present with enrichment in the neural tube and tailbud. These results imply that unga may play distinct roles during cleavage period and segmentation period.

Unga Protein Is Located in Nuclei of Blastomeres of Zebrafish Embryos—If Unga functions immediately after fertilization, Unga protein should be present in early embryos. Currently there are no anti-zebrafish Una antibodies available, prompting us to test the utility of an anti-human UNG antibody in fish. To verify the efficacy of this antibody, we designed the antisense morpholino unga-MO to block the translation of endogenous unga mRNA. Injection with unga-MO, but not with the mismatched control MO (cMO), inhibited the expression of the reporter unga-5’UTR-gfp (Fig. 2B), demonstrating an effective blockage. We found that immunostaining signal using anti-human UNG antibody was enhanced by overexpression of unga-mcherry mRNA and decreased by unga knockdown and showed a mutual rescuing effect if both mRNA and MO were injected (Fig. 2, C and D), suggesting that this antibody reacts with endogenous Una protein in zebrafish embryos. By western blotting, we found that Una protein was detectable in embryos from the one-cell stage onward (Fig. 2E). Analysis of nuclear and cytoplasmic fractions indicated that Una protein was predominantly present in nuclei (Fig. 2F). Immunofluorescence assay could detect weak Una signal in nuclei of 4-cell stage embryos (data not shown) and stronger signals in nuclei in embryos at later stages (Fig. 2G). Thus, Una is similar to human UNG2 that is localized in the nucleus (21), suggesting that
Unga functions in the nucleus.

Unga Possesses Uracil Excision Activity—Bacterial and mammalian UDG/Ung proteins have activity to excise uracil from DNA (17,22,23). We tested whether zebrafish Unga possesses similar activity. We found that, like E. coli UDG (22), recombinant Unga efficiently released uracil from mispairing U:G in double-stranded oligos in in vitro assays (Fig. 3, A and B). However, we did not detect excision of other mispairing bases such as T:G, A:G, G:G, C:G or 5mC:G.

We further investigated the uracil excision activity of Unga in zebrafish embryos through dUTP incorporation assay. Embryos were injected at the one-cell stage with BrdUTP in combination with mcherry or unga-mcherry mRNA, cMO or unga-MO, and fixed at 64-cell and sphere stages for co-immunostaining using anti-BrdU and anti-ssDNA antibodies. The relative BrdUTP incorporation level was estimated as the ratio of BrdU signal intensity to ssDNA signal intensity in nuclei. Results showed that unga knockdown enhanced but unga overexpression hindered incorporation of dUTPs into newly synthesized DNA in zebrafish embryos (Fig. 3, C-F), supporting the notion that Unga can function to remove uracil bases from DNA molecules.

unga Knockdown Increases and Overexpression Decreases Global DNA Methylation Level in Zebrafish Pre-MBT Embryos—A recent paper reported a possible involvement of Ung2 in active DNA demethylation in the mouse zygote (24). We asked whether unga was implicated in DNA demethylation during zebrafish early embryogenesis. When unga was knocked down by injecting 10 ng unga-MO, embryos exhibited a significant increase of the relative GDM level as early as the 32-cell stage and at later stages as estimated by immunostaining with anti-5mC antibody (Fig. 4, A and B). The increase in GDM level in unga morphants at the 256-cell stage was also confirmed by dot blotting (Fig. 4C). Conversely, overexpression of unga-mcherry mRNA in zebrafish embryos resulted in a significant decrease of the relative GDM level, as detected by immunostaining at 128-cell and 256-cell stages (Fig. 4, D and E) and by dot blotting at 256-cell stage (Fig. 4F). These results together suggest that Unga is involved in engendering and maintaining low levels of DNA methylation in the embryonic genome during pre-MBT period of zebrafish embryos.

Unga-mediated DNA Demethylation Relies on DNA Glycosylase Activity—Our next question was whether Unga-mediated DNA demethylation depended on its DNA glycosylase activity. Since the aspartate residue in the conserved water-activating loop motif GQDPY of Ung proteins has been shown to be critical for its catalytic activity (25), we generated a presumably inactive mutant of zebrafish Unga, Unga(D132A), which carried a mutation to alanine at position 132 (Fig. 5A). In vitro assay confirmed that Unga(D132A) had much reduced activity to excise uracil bases from double-stranded DNA (Fig. 5B). Injection of unga(D132A) mRNA was unable to reduce the global DNA methylation level at the 256-cell stage, it instead resulted in an increase of the methylation level (Fig. 5, C and D), which might be due to a dominant negative effect. Nevertheless, we conclude that Unga regulation of DNA methylation is dependent on its glycosylase activity.

Unga-mediated DNA Demethylation Facilitates Histone Modifications of Chromatin in Zebrafish Embryos—Methylated histones in zebrafish embryos are observed just before and during MBT, marking chromatin regions for transcription or repression (26,27). We asked whether Unga-mediated DNA demethylation would poise specific chromatin regions for histone modifications. To this end, we injected embryos at the one-cell stage with 500 pg mcherry or unga-mcherry mRNA and examined H3K4me3 and H3K27me3, together with H3, at 256-cell and sphere stages by immunostaining with corresponding antibodies. Compared to mcherry-injected embryos, unga-mcherry-injected embryos exhibited an increase of the relative intensity of both H3K4me3 and H3K27me3, which was normalized to H3 intensity (Fig. 6, A-D). These results indicate that the genome may be unfettered by unga-stimulated hypomethylation for histone modifications.

unga Knockdown Inhibits Yet Its Overexpression Stimulates Nuclear Transcriptional Activity—Given that unga may participate in postfertilization DNA demethylation and regulate histone modifications, we further tested whether its knockdown or overexpression altered global transcriptional activity in the nucleus. We
labeled newly synthesized RNAs by injecting 5-BrUTP together with unga-MO or unga-mcherry mRNA into one-cell stage embryos, and then visualized incorporated BrUTPs by immunostaining embryos at different stages with anti-BrUTP and H3 antibodies. The relative transcriptional activity of the genome was assessed as the ratio of BrUTP immunostaining intensity to H3 intensity in nuclei. Compared to cMO-injected embryos, embryos injected with 10 ng unga-MO exhibited a significant decrease of the relative transcriptional activity at 256-cell, 1k-cell and sphere stages (Fig. 7, A and B). In contrast, unga-mcherry-injected embryos at the 256-cell stage exhibited an increase of overall transcriptional activity in nuclei compared to mcherry-injected embryos (Fig. 7, C and D). These results imply that insufficiency of Unga may repress transcriptional activity of the genome due to higher levels of DNA methylation, and that excess Unga may stimulate nuclear transcriptional activity arising from DNA hypomethylation.

Zygotic Expression of Many Genes Are Altered with Changes of Unga levels in Zebrafish Embryos —To further understand profound impact of Unga-mediated demethylation on zygotic gene transcription, we performed genome-wide mRNA-seq analysis for unga-mcherry-injected embryos at the 256-cell stage and unga morphants at the 1k-cell stage (Fig. 8). For each sample, transcripts of more than 10,000 genes were mapped to the zebrafish genome assembly version Zv9 (Fig. 8, A and D). Compared to mcherry-injected embryos, unga-mcherry-injected embryos at the 256-cell stage had 1,254 up-regulated and 869 down-regulated genes (>1.5 folds with a false discovery rate FDR < 0.005) (Fig. 8B, and Table S2 and S3). Validation of up-regulated genes by RT-PCR analysis revealed that 26 out of 33 genes indeed showed a significant increase of expression by unga-mcherry overexpression (data not shown).

By comparing expression levels in between one-cell and 512-cell stage embryos based on our other RNA seq data (Supplementary information, Table S1), we categorized all genes into three classes: M\textsuperscript{Z1} (with increasing amount of transcripts from the one-cell stage onward), Z (zygotically transcribed) and M\textsuperscript{Z0} (with decreasing amount of transcripts after the one-cell stage). More than 90% of the differentially expressed genes (DEG) identified in this study could be successfully classified. We found that the majority of the DEGs between unga-mcherry- and mcherry-overexpressing embryos were Z- or M\textsuperscript{Z0}-genes, and that differently expressed Z-genes mostly fell within the top 1/3 group as ranked by changing folds (Fig. 8C), supporting the idea that unga-promoted DNA hypomethylation may evoke zygotic transcription. Among the up-regulated genes in unga-mcherry embryos, many are related to nucleotide excision repair, base excision repair or mismatch repair pathways (Table S2), suggesting that ectopic Unga induces a more active DNA repair system; and many are required for general transcription, which is consistent with enhanced genome transcriptional activity. Interestingly, some up-regulated genes could not function normally before cell lineage specification, e.g., kif6, which is normally expressed after the completion of gastrulation and crucial for hematopoiesis (28-30), and gli3, which controls neural induction and patterning (31,32). It is likely that cell lineages could be abnormally specified due to hypomethylation.

Comparison between unga-MO- and cMO-injected embryos at the 1k-cell stage identified 972 down-regulated and 400 up-regulated genes in unga morphants (Fig. 8E, and Tables S4 and S5). Validation of down-regulated genes by RT-PCR analysis revealed that 30 out of 35 genes showed significantly decreased expression levels in unga morphants (data not shown). We noted that the majority of differentially expressed genes were also Z- or M\textsuperscript{Z0}-genes (Fig. 8F). These results suggest that many genes required for early embryonic development are repressed for transcription probably due to hypermethylation of the genome. Among the down-regulated genes in unga morphants, some are also related to transcription, which accords with a repression of general transcriptional activity in the morphants. Interestingly, knockdown of unga led to significant up-regulation of genes related to cell death, e.g., bax, pdec2, pdec5, daxx and dido1, which might be one of the reasons for embryonic lethality of unga morphants (see below).

unga Knockdown Causes Embryonic Lethality during Segmentation Period —We observed morphological changes of embryos depleted of unga during development (Fig. 9A). Like control embryos injected with cMO,
embryos injected with 10 ng unga-MO had no observable defects before 40% epiboly stage (5 hpf) except a slightly retarded epiboly; then, unga morphants continued to show a slower epibolic process and had a thinner germ ring with a smaller embryonic shield around the shield stage; a large proportion of morphants started to deform at the onset of segmentation and none of them could survive beyond 12-somite stage (about 15 hpf) (Fig. 9B). These results imply that Unga is absolutely required for survival of zebrafish embryos. In contrast, embryos injected with 500 pg unga-mcherry mRNA could develop beyond pharyngula period without severe morphological defects (Fig. 9C). It is likely that temporary DNA hypomethylation during early development may not severely disrupt normal developmental programs.

DISCUSSION

In this study, we uncovered an implication of the base excision repair system in reprogramming the embryonic genome through DNA demethylation during zebrafish early embryogenesis. This function is mediated, at least in part, by Unga, a Uracil-DNA glycosylase family member.

Currently, we do not know how Unga participates in DNA demethylation during zebrafish early embryogenesis. Our in vitro DNA glycosylase activity assays indicated that recombinant Unga alone is able to excise uracil base from mispairing U:G in double-stranded DNA, but not other bases (including 5mC) pairing with G (Fig. 3B). In physiological conditions, uracil bases could result from deamination of cytosine bases and misincorporation of dUMPs. There might be several ways for Ung to take part in DNA demethylation. First ly, excision of misincorporated uracil bases by Ung may be followed by excision of a long stretch of adjacent nucleotides including 5mC, resulting in a replacement of 5mC with C during subsequent repair process. This mechanism may not be responsible for the genome-wide demethylation since the genome may not contain many misincorporated U bases.

Secondly, Ung may be involved in deaminase-mediated DNA demethylation since 5-methylcytosine bases can be converted to thymine/cytosine bases through the AID/Apobec2/Gadd45/Mbd4 system (33,34) and cytosine bases can be further deaminated to uracil bases. This mechanism has been recently suggested for active demethylation in mouse zygotes (24). A previous study has indicated that the AID/Apobec2/Gadd45/Mbd4 system is not working in zebrafish embryos before ZGA (33). In our RNA profiles, transcripts for aid, apobec2, dctd, mbd, gadd45 genes were either absent or present in low amount during early embryogenesis (Table 1). It is unknown whether other deaminase may function together with Unga during early embryogenesis.

Thirdly, Ung may be able to excise oxidation/deamination intermediates of 5-methylcytosines. This kind of activity of Ung was not detected in our in vitro glycosylase activity assays. We tried to use embryonic protein extracts from embryos at 4-16 cell stages together with recombinant Unga in these assays, but still failed to detect removal of 5mC and A, T or G mispairing with G (data not shown). We suspected the method we used was not sufficiently sensitive to detect subtle changes in the composition of the substrate DNA. Therefore, more sensitive methods are needed to characterize Ung activity in detail. In addition, identification of oxidation and/or deamination enzymes in early zebrafish embryos will definitely help elucidate mechanisms controlling postfertilization DNA demethylation in this species.

The global DNA methylation level is related to chromatin modifications and genome transcription activity during early embryonic development. Overexpression of unga appears to cause an increase of both H3K4me3 and H3K27me3 marks (Fig. 6) as well as an enhancement of transcriptional activity at the pre-MBT stage (256-cell stage) (Fig. 7, C and D), suggesting that unga-mediated global DNA demethylation facilitates chromatin modifications, thereby poising chromatin for transcription activation or repression. Our transcriptome analysis by RNA seq indicated that the majority of affected genes in unga-overexpressing embryos were up-regulated and the majority in unga knockdown embryos were down-regulated, but still, some genes experienced an opposite change of expression (Fig. 8, and Tables S2-S5). This phenomenon is understandable given that different modifications of chromatin, availability of transcription factors and mutual repression of some factors could all contribute to transcription of a specific gene.

A recent paper reports that mouse zygotes
depleted of cytosine deaminase (AID) or Ung2 have higher levels of 5mC compared to wild-type zygotes (24). It appears that the involvement of the Ung-mediated base excision repair process in active demethylation of zygotes is conserved in vertebrates.

In summary, our findings shed light on the involvement of Ung in postfertilization DNA demethylation and in embryonic survival in zebrafish. Its underlying mechanisms need to be investigated in the future.

REFERENCES


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FOOTENOTES
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The abbreviations used are: Ung, uracil DNA glycosylase; 5mC, 5-methylcytosine; MO, morpholino; hpf, hours postfertilization; GDM, global DNA methylation; MBT, midblastula transition; ZGA, zygotic genome activation; DEG, differentially expressed genes.
Table 1. Identification of zebrafish genes possibly involving DNA demethylation. mRNAs in squeezed eggs and embryos at different stages were sequenced by RNA seq. The expression levels of zebrafish genes, whose homologs have been previously reported to involve DNA demethylation, were shown.

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a Related references were indicated.
Figure legends

**Fig. 1. Dynamic changes of global DNA methylation (GDM) level in early embryos.** A and B, representative confocal images showing 5mC and H3 signals in embryos at different stages. The arrow-indicated nuclei in (A) were imaged at a higher magnification and corresponding pictures were presented in (B). C, the relative GDM (RGDM) level calculated from 5mC and H3 staining signals. The RGDM level was the ratio of 5mC to H3 signal intensity. The data from two independent experiments were shown. For each stage and each experiment, the average was calculated from all nuclei that were clear on the focal plane from at least 3 embryos. Error bars indicated SD.

**Fig. 2. Temporal expression and subcellular localization of Unga.** A, expression of unga in eggs and embryos at different stages, detected by whole-mount in situ hybridization. Egg stages (I - III) and embryonic stages were indicated. B, effect of unga-MO on a reporter expression. The reporter construct unga-5'UTR-gfp was made by ligating 73-bp of 5'UTR and immediate downstream 291-bp coding sequence of unga cDNA to gfp coding region in pEGFP-N3. Embryos were injected with 50 pg of reporter plasmid DNA in combination with 10 ng unga-MO or the control cMO at one-cell stage and observed under a fluorescence stereomicroscope during midgastrulation stages. C and D, anti-human Ung antibody recognized Unga protein in fish embryos. Embryos at the one-cell stage were injected with 10 ng unga-MO, 500 pg unga-mcherry mRNA or 500 pg unga*-mcherry mRNA alone or in different combinations and harvested at the 1k-cell stage for co-immunostaining using anti-UNG and anti-H3 antibodies (C). unga*-mcherry was identical to unga-mcherry except several mutated bases in unga-MO recognizing sequence. The relative immunostaining intensity of Unga, which was normalized to the H3 signal, was shown in the bar graph (D). Ne, number of measured embryos; Nc, total number of measured nuclei. Statistical significances: *, p < 0.1; **, p < 0.05; ***, p < 0.01. E, detection of Unga protein at different developmental stages. Embryos were collected at indicated stages and lysed for western blotting using anti-Ung or anti-histone 3 (H3) antibody. The bottom bar graph showed the average Unga signal level relative to H3 signal intensity. F, detection of subcellular localization of Unga protein. Cell lysates from embryos at different stages were fractionated into nuclear (Nuc) and cytoplasmic (Cyt) fractions, which were then subjected to western blotting using anti-Ung, anti-H3 or anti-Tubulin antibody. Note that the fractions were not very clean due to fast cell division without G1 and G2 phases during cleavage period. G, Unga protein was located in nuclei of embryos at different stages, detected by immunofluorescence with anti-Ung antibody.

**Fig. 3. Unga functions to excise uracil bases from DNA.** A and B, in vitro assay of uracil excision activity of Unga. The sequences of 20-bp double-stranded DNA oligos were shown in (A). DNA substrates were incubated for 30 min at 37°C with 30 ng/μl of recombinant Unga or bacterial UDG, then stopped and separated on a PAGE gel and stained (B). C and D, confocal fluorescence of incorporated BrdU. Embryos were co-injected at the one-cell stage with 1 pmol BrdUTP and 500 pg mcherry (mch) or 500 pg unga-mcherry (unga-mch) mRNA, or 10 ng cMO or 10 ng unga-MO, and fixed at 64-cell and sphere stages for immunostaining with anti-BrdU and anti-ssDNA antibodies. E and F, relative intensities of BrdU signals. The BrdU incorporation level was reflected by the ratio of BrdU/ssDNA intensities in a nucleus. The ratios were shown in a string of crosses for all analyzed nuclei of the same treatment with an average indicated. Ne, number of observed embryos; Nc, total number of analyzed nuclei. ns, not statistically significant (p > 0.1).

**Fig. 4. Effect of unga knockdown or overexpression on the global DNA methylation level during early embryogenesis.** A, B, D and E, immunodetection of 5mC and H3 in nuclei (A and D) and the 5mC/H3 ratio (RGDM) (B and E). Embryos were injected at the one-cell stage with 10 ng cMO, 10 ng unga-MO, 500 pg mcherry (mch) or unga-mcherry (unga-mch) mRNA and fixed at indicated stages for immunostaining. The RGDM was averaged from multiple nuclei and 3-5 embryos for each treatment. Ne, number of measured embryos; Nc, number of measured nuclei. Statistical significances: **, p < 0.05; ***, p < 0.01. C and F, detection of 5mC level by dot blotting using genomic DNA isolated from 256-cell stage embryos and anti-5mC antibody.
Fig. 5. DNA glycosylase activity of Unga is required for DNA demethylation activity. (A) The conserved water-activating loop (red letters) between human (hUNG2) and zebrafish Ung (zUnga). The mutated residue in Unga(D132A) was indicated by an arrow. (B) Comparison of uracil excision activity of Unga and Unga(D132A). Sequence information for DNA oligos was the same as shown in Fig. 3A. The oligo was incubated with the recombinant protein (3 ng/µl) for 30 min at 37°C. C and D, immunofluorescence of injected embryos at the 256-cell stage using anti-5mC and anti-H3 antibodies (C) and the relative global DNA methylation (RGDM) levels were shown in (D). Ne, number of measured embryos; Nc, total number of measured nuclei. Statistical significance: **, p < 0.05; ***, p < 0.01.

Fig. 6. Histone modifications are facilitated by unga overexpression. A and B, representative confocal fluorescence images of embryos following co-immunostaining using anti-H3K4me3 (A) or anti-H3K27me3 (B) together with anti-H3 antibodies. Embryos were injected at the one-cell stage with 500 pg mcherry (mch) or unga-mcherry (unga-mch) mRNA and fixed at 256-cell and sphere stages for immunostaining. C and D, the relative immunostaining intensity of H3K4me3 (C) and H3K27me3 (D) normalized to H3. Ne, number of measured embryos; Nc, total number of measured nuclei. Statistical significance: **, p < 0.05; ***, p < 0.01.

Fig. 7. unga knockdown or overexpression affects nuclear transcriptional activity. Embryos at the one-cell stage were injected with 5 pmol BrUTP in combination with 10 ng cMO, 10 ng unga-MO, 500 pg mcherry (mch) mRNA or 500 pg unga-mcherry (unga-mch) mRNA and fixed at indicated stages for immunostaining with anti-BrUTP and anti-H3 antibodies. The immunostained embryos were imaged by confocal microscopy. The relative transcriptional activity was the ratio of BrUTP to H3 signal intensity in the nucleus. A and B, the representative confocal images (A) and the average relative transcriptional activity (B) in MO-injected embryos. C and D, the representative confocal images (C) and the average relative transcriptional activity (D) in mRNA-injected embryos. In image panels, a selected nucleus (indicated by an arrow) in the representative embryo was enlarged underneath. Ne, number of measured embryos; Nc, total number of measured nuclei. Statistical significance: ***, p < 0.01.

Fig. 8. RNA seq analyses of unga-overexpressing and unga knockdown embryos. A-C, comparison of transcriptional profile between mcherry- and unga-mcherry-injected embryos at 256-cell stage. A, gene numbers mapped to the zebrafish genome assembly version Zv9 in different samples. B, the number of up-regulated or down-regulated genes between two samples (> 1.5 folds with FDR <0.005). DEG, differentially expressed genes. C, the ratios of up- and down-regulated genes in different classes. The DEGs were grouped into three sets (see the Experimental Procedure section for details) based on degrees of fold changes from the highest to the lowest: top 1/3, mid 1/3 and bottom 1/3. D-F, comparison of transcriptional profile between cMO- and unga-MO-injected embryos at the 1k-cell stage. The DEGs were analyzed in ways similar to those in mRNA-injected embryos.

Fig. 9. Effects of unga knockdown and overexpression on embryonic development. A, morphology of embryos. Embryos were injected with 10 ng cMO or unga-MO at the one-cell stage and imaged individually (top panel) or in group (lower panel) at indicated stages. Individual embryos were lateral viewed with dorsal to the right if the dorsal side was distinguishable. The ratio of embryos with representative morphology was indicated in the right corner. The embryonic shield was indicated by an arrow. B, surviving rates of embryos at different time points of development. Data from two independent experiments (I and II) were shown with the number of embryos at 4 hpf in parenthesis. C, morphology of embryos injected with 500 pg mcherry (mch) mRNA or unga-mcherry (unga-mch) mRNA at indicated stages. The ratio of embryos with representative morphology was indicated.
Figure 3

A DNA substrate:

GAGCGTGACXGGAGCTGAAA
CTCGCAGCTGCCCTCGACTTTT

(X = C, M (5mC), A, T, G or U)

B Recombinant Unga E. coli UDG

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-20 nt
-10 nt

C 64c mch unga-mch Sphere mch unga-mch

BrdU

20 µm

ssDNA

Merge

D 64c cMO unga-MO Sphere cMO unga-MO

BrdU

20 µm

ssDNA

Merge

E

BrdU/ssDNA intensity ratios

mch unga-mch mch unga-mch

64c Sphere

Ne = 9 7 10 9
Nc = 36 26 82 62

F

BrdU/ssDNA intensity ratios

cMO unga-MO cMO unga-MO

64c Sphere

Ne = 5 9 10 11
Nc = 14 20 77 99

p < 0.0002

p < 1.6E-08

p < 0.008

0.35 0.47 3.91 5.00
Figure 5

A

hUNG: $^{149}$VILGQDPYHGP$^{159}$
zUnga: $^{127}$VILGQDPYHGP$^{137}$

B

C M A T G U

20 nt

10 nt

Unga

Unga (D132A)

C

mcherry unga-mcherry unga(D132A)

5mC

H3

Merge

D

RGDM level

Ne = 4 6 7
Nc = 37 42 37
Figure 7

A

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C

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D

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**Legend:**
- Ne = number of events
- Nc = number of cells

**Statistical Significance:**
- ***** ***: p < 0.001
Figure 9

A

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<td>500 μm</td>
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<td>77/77</td>
<td>72/75</td>
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B

- I. cMO (136)
- II. cMO (55)
- I. unga-MO (211)
- II. unga-MO (69)

Graph showing percentage of survivors over time.

C

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Uracil-DNA Glycosylase is involved in DNA demethylation and required for embryonic development in the zebrafish embryo

Di Wu, Luxi Chen, Qingrui Sun, Xiaotong Wu, Shunji Jia and Anming Meng

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