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DNA Methylation in Zebrafish

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Abstract

DNA methylation is crucial for normal development and cellular differentiation in many large-genome eukaryotes. The small tropical freshwater fish *Danio rerio* (zebrafish) has recently emerged as a powerful system for the study of DNA methylation, especially in the context of development. This review summarizes our current knowledge of DNA methylation in zebrafish and provides evidence for the general conservation of this system with mammals. In addition, emerging strategies are highlighted that use the fish model to address some of the key unanswered questions in DNA methylation research.

I. Introduction

DNA methylation is essential for normal development in plants and some animals, but it is absent or found at very low levels in classic genetic models such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (reviewed in Ref. 1). Currently, much of our understanding of DNA methylation has been derived from studies in mouse, the plant *Arabidopsis thaliana*, and the fungus *Neurospora crassa*. While each of these organisms offers advantages, the zebrafish is becoming a compelling alternative for the study of DNA methylation.

On the basis of gene homology and genome-wide profiling, the zebrafish methylation system appears more similar to mammals than to plants or fungi. Therefore, it is an appealing option for probing vertebrate-specific aspects of the DNA methylation machinery and the evolution of methylation in the vertebrate lineage. Moreover, because zebrafish do not require imprinting of genes or sex chromosomes for viability,² as is the case in mammals, they provide a simplified system for exploring the roles of DNA methylation in vertebrate development.

In recent years, zebrafish have become widely accepted as a useful model for studying development. Many of the same attributes that have made them appealing for these studies are also relevant to the study of epigenetic regulation. Zebrafish are easy to maintain in large numbers and each female can lay hundreds of eggs per week. Such large numbers of embryos facilitate quantitation of non-Mendelian inheritance patterns, which frequently arise in epigenetic studies. Zebrafish embryos develop rapidly and externally, which allow access to stages of development that can be difficult to study in mammals. Moreover, haploid and uniparental diploid embryos can be produced readily,² allowing analysis of methylation patterns inherited specifically from the maternal or paternal genome.

Importantly, the developing embryos are transparent, permitting the transcriptional status of fluorescent reporter genes to be monitored *in vivo* and across generations. Finally, zebrafish are highly amenable to forward genetic screens that have the potential to identify new factors not previously implicated in epigenetic regulation. Collectively, these advantages provide the framework for new and unique approaches to the study of DNA methylation.

Despite its advantages, the potential for epigenetic research in zebrafish has not been fully realized. In part, this is due to the belated application of technologies for targeted deletion of specific genes and the reliance on anti-sense morpholino oligonucleotides injected at the one-cell stage. As morpholinos reduce protein by blocking translation or splicing of mRNAs, they are not always effective and rarely result in 100% depletion of protein. In addition, up to 18% of morpholinos appear to have “off-target” effects that include p53-mediated cell death in the central nervous system and somites.^{3,4} Although rescue of phenotypes by coinjection of mRNA can control these secondary effects,⁵ the analysis of morpholino-injected embryos remains challenging. Fortunately, the number of available zebrafish mutants is rapidly growing, and methods for targeted gene mutation such as zinc finger nucleases have now been validated. Thus, while information gained from morpholino-based experiments provides valuable initial insights into DNA methylation in zebrafish, it will be important to revisit these experiments using genetic mutations.

II. Methylation Profile of the Zebrafish Genome

A. Early Evidence for DNA Methylation

The first clues that zebrafish might have an efficient DNA methylation system came from early studies of integrated transgenes. Initial approaches to transgenesis in zebrafish relied on injection of naked, linearized DNA into the one-cell stage embryo.⁶ In a small percentage of embryos, the linearized DNA would integrate randomly into the genome, usually as a large concatemeric array containing many copies of the transgene. Despite having numerous copies, these early zebrafish lines were often reported to have low or variegating transgene expression.^{7–10} Silencing of transgene expression was reminiscent of the DNA methylation and transcriptional silencing that had been described in mouse lines carrying integrated transgene arrays^{11–16} and suggested a similar mechanism might be operating in zebrafish.

The presence of DNA methylation at zebrafish transgene arrays was confirmed by Gibbs et al., using integrated luciferase reporters that were strongly silenced.⁹ High levels of DNA methylation were found at the transgenes by digestion with methylation-sensitive restriction enzymes, and modest increases in luciferase expression were achieved after exposing embryos to the DNA methyltransferase inhibitor 5-azacytidine.⁹ The role of DNA methylation in transgene silencing was further substantiated by Martin and McGowan, who likewise demonstrated the accumulation of DNA methylation at transgene arrays with variegated expression of the chloramphenicol acetyltransferase resistance gene *cat*.^{10,17}

Curiously, methylation levels at the *cat* resistance transgene array appeared to be somewhat plastic, with modest differences in the extent of methylation reported among individuals that depended on genetic background, rearing temperature, sex, and parent of origin.¹⁷ While these early studies provided the first evidence that DNA methylation could play a role in

transcriptional silencing in zebrafish, they revealed little about the overall DNA methylation landscape or how DNA methylation systems in zebrafish might compare to other organisms.

B. Global Methylation Profiles in Fish

Genome-wide sequencing technologies have now provided a wealth of data, enabling comparison of DNA methylation systems in diverse organisms (see Chapter by Eleanor Wong and Chia-Lin Wei). Completely sequenced genomes allow comparison of DNA methyltransferase homologs present in different lineages, while recent high-throughput methods of shotgun bisulfite sequencing allow the methylation profile of many organisms to be characterized at the level of the whole genome.^{18,19}

Bisulfite sequencing analysis of the zebrafish genome has been reported for pooled larvae at 5 days postfertilization (dpf).¹⁸ Similar to mouse embryos, zebrafish larvae have high levels of DNA methylation (80%) at CpG dinucleotides in virtually all sequence contexts, except CpG islands where it is low.¹⁸ Although levels of DNA methylation are otherwise uniformly high, a mild enrichment can nonetheless be detected at repetitive elements and gene bodies.¹⁸ The level and distribution of CpG methylation observed in zebrafish is on par with mouse (74% methylation) and significantly higher than the levels observed in plants (e.g., *Arabidopsis*, 22%) or insects (e.g., honey bee, 0.93%; Table I).¹⁸

While non-CpG methylation in the forms of CpHpG and CpHpH can be readily detected in plant and fungal sequences undergoing *de novo* methylation, its role in mammals is less clear¹ (H = A, C, or T). Similar to mouse, in zebrafish both CpHpG and CpHpH methylation are present at relatively low levels and evenly distributed across the zebrafish genome.¹⁸ The amount of non-CpG methylation in the zebrafish embryo is somewhat higher than that reported for mouse embryos (2.13% compared to 0.59%; Table I), although further analysis will be required to determine whether this difference is significant.¹⁸

So far, dense non-CpG methylation has only been described once in the zebrafish literature, with injected plasmid DNA as the substrate.²² In contrast, a number of other experiments examining specific genomic loci by bisulfite sequencing have reported only background levels of non-CpG methylation.^{23–25} As in mammals, the biological significance of non-CpG methylation in zebrafish has not been determined.

Studies of other species suggest DNA methylation is widespread in fish. DNA methylation has been reported in *Oryzias latipes* (medaka), *Gasterosteus aculeatus* (stickleback), *Tetraodon nigroviridis* (green spotted puffer), and a number of other fish species.^{19,26–28} Detailed analysis of DNA methylation at the level of shotgun bisulfite sequencing has also been performed on DNA from *Tetraodon*, which has a small genome containing very few transposable elements.²⁹ Similar to zebrafish, the *Tetraodon* genome was heavily methylated at CpG dinucleotides (66%) with enrichment of methylation in genes and the few present transposons.¹⁹ Curiously, non-CpG methylation was not detected in the *Tetraodon* genome.¹⁹ However, because zebrafish and *Tetraodon* methylation levels were determined in two different studies, direct comparison is difficult. It is possible that these differences are due to the developmental stage at which methylation was examined. Methylation in zebrafish was examined in larvae, while the status of a more mature juvenile *Tetrodon* was

reported. Given that in mouse, highest levels (3.1%) of non-CpG methylation are present in embryonic stem (ES) cells,³⁰ it may be informative to reexamine non-CpG methylation in zebrafish and *Tetraodon* at the same early and late developmental stages.

III. The Zebrafish Methylation Machinery

Based on sequence similarity in 10 diagnostic amino acid motifs, the zebrafish genome codes for eight DNA methyltransferase orthologs, which include at least one member of the Dnmt1, Dnmt2, and Dnmt3 families found in mammals²² (Fig. 1). Not surprisingly, homologs of the plant-specific chromomethylase and fungal-specific Dim-2 families were not detected in the zebrafish genome. The presence of mammalian-like Dnmt1 and Dnmt3 proteins suggests that pathways involved in maintenance and *de novo* methylation are generally conserved in vertebrate species (see also Chapter by Lakshminarayan M. Iyer *et al.*), and that zebrafish may be well suited for their study.

A. Maintenance of Methylation by Dnmt1 and Uhrf1 Proteins

In order to be maintained over the lifetime of an organism, methylated cytosine residues must be propagated at every cell division. Thus far, two proteins have been identified that act cooperatively to maintain methylation through DNA replication. In mouse, hemimethylated DNA is recognized and bound by a SET- and RING-associated (SRA) domain containing protein called UHRF1 that recruits the DNA methyltransferase DNMT1 to the replication fork^{31,32} (see Chapter by Jafar Sharif and Haruhiko Koseki). A methyl group is then added to the newly synthesized DNA strand, allowing for passive transfer of previously established methylation patterns. A version of this simple, yet elegant mechanism for maintaining DNA methylation appears to be widely used in plants, vertebrates, and possibly some insect lineages (reviewed in Ref. 33).

1. Dnmt1 and Uhrf1 Conservation—Dnmt1 was the first DNA methyltransferase identified in zebrafish, providing an early indication that fish and mammals might share common mechanisms of DNA methylation.³⁴ Zebrafish and human orthologs are 73% identical overall, with 89% identity in the C-terminal catalytic domain. Zebrafish Dnmt1 retains the N-terminal bromo-adjacent homology (BAH) and CXXC zinc finger domains found in mammalian Dnmt1 homologs.

In mouse, the *Dnmt1* locus includes 5′ sex-specific exons that are expressed specifically in either the testes or ovaries. In the ovary, an alternative 5′ exon leads to production of high levels of active DNMT1 protein, which becomes sequestered in the cytoplasm during later stages of oocyte growth.³⁵ This transcript is important for the proper maintenance of imprinted genes during oogenesis.³⁶ In contrast, the spermatocyte-specific 5′ exon interferes with translation and decreases DNMT1 levels during male meiosis.³⁵ A directed search for sex-specific *dnmt1* transcripts has yet to be performed in zebrafish, and available expressed sequence tags (ESTs) do not provide evidence for oocyte-specific exons. However, one EST (BC057461) cloned from an adult zebrafish male does code for a nonfunctional version of Dnmt1 protein, suggesting that alternative splicing could also play a regulatory role in zebrafish gametes. A definitive answer to this question will require careful 5′ RACE studies on dissected gonadal tissues.

As expected, the zebrafish genome also includes an ortholog of *uhfr1* that is 66% identical to the human protein, and contains the SRA, ubiquitin-like, and plant homeodomain (PHD) domains present in the mammalian orthologs.³⁷ Similar to mouse and human, the zebrafish genome also contains a second related gene, *uhfr2*.³⁸ However, data linking Uhrf2 proteins and DNA methylation have yet to be reported in any organism.

2. Dnmt1 and Uhrf1 Are Required for DNA Methylation—As would be predicted for proteins with a generalized role in maintenance of DNA methylation, expression of both *dnmt1* and *uhfr1* appears to be fairly ubiquitous in the developing embryo. Highest levels of expression are observed throughout early development with later widespread expression in zones of proliferation during organogenesis.^{39–42} *Dnmt1* expression has not been examined in adults, but *uhfr1* mRNA was detected in proliferating adult tissues by RT-PCR.³⁷ There is also indirect evidence to suggest that maternal Uhrf1 and Dnmt1 proteins may be maternally deposited in the oocytes.^{23,42,43}

Mutations in the zebrafish *dnmt1* and *uhfr1* genes support a conserved role for this pair of proteins in DNA methylation. When present in homozygous form, null mutations in the zebrafish *dnmt1* gene lead to decreased levels of DNA methylation.⁴² This finding is consistent with an earlier report describing a 40% reduction in DNA methylation following depletion of Dnmt1 protein by injection of translation-blocking antisense morpholinos into the one-cell embryo.⁴⁰ Similarly, zebrafish homozygous for a hypomorphic mutation in the *uhfr1* gene have a 75% reduction in *uhfr1* mRNA, and an approximately ~50% reduction in global DNA cytosine methylation.^{18,37,44} Consistent with a generalized role in maintenance of DNA methylation, methylation in *uhfr1* mutants is decreased throughout the genome including at intergenic regions, exons, and introns.¹⁸

3. Maintenance Methylation Is Essential for Normal Zebrafish Development—

In initial experiments to address the role of DNA methylation in zebrafish development, embryos were exposed to the DNA methyltransferase inhibitor 5-azacytidine.³⁹ Following exposure, a fraction of the developing embryos exhibited defects including a shortened trunk and abnormal development of the somites, notochord, and muscle. However, the timing of administration was critical and only 20–30% of embryos treated during the blastula period showed these phenotypes.³⁹

Somewhat different phenotypes were reported upon depletion of Dnmt1 using antisense morpholinos. At 24 hpf, 40% of injected embryos were dead, while the remaining ones exhibited developmental abnormalities including curled tails, pericardial edema, and jaw defects.⁴⁰ Most strikingly, knockdown of Dnmt1 caused loss of expression of terminal differentiation markers in the intestine, exocrine pancreas, and retina, while markers of the liver and endocrine pancreas appeared normal.⁴⁰

Two alleles of *dnmt1*, identified in a screen for mutations that affect zebrafish liver and pancreas development, recapitulated many of the phenotypes observed in morpholino experiments.⁴² Mutants initially developed normally, but by 4 dpf had a markedly smaller exocrine pancreas as well as liver, eye, and jaw defects. *Dnmt1* mutant zebrafish also showed increased p53-mediated apoptosis similar to that observed for *Dnmt1* mutant mouse

fibroblasts.^{42,45} At the morphological level, *uhrf1* mutants and *dnmt1* mutants have very similar phenotypes,³⁷ supporting the hypothesis that the primary role of Uhrf1 in development is to target Dnmt1 to the replication foci for maintenance of DNA methylation.

While all of these experiments point to a fundamental role for DNA methylation in development, the phenotypes observed following 5-azacytidine treatment are distinct from those in *dnmt1* mutants and morphants. There are several possibilities for the discrepancy. First, treatment with 5-azacytidine is expected to block activity of all DNA methyltransferases, which might cause a more severe phenotype than depletion of Dnmt1 alone. Second, maternal Dnmt1 protein deposited in the yolk would not be depleted in mutant or morphant embryos, but would be inactivated by azacytidine treatment. Therefore, 5-azacytidine phenotypes may be due to earlier loss of Dnmt1 activity. However, 5-azacytidine is also highly unstable in water, raising the potential for nonspecific effects. It is difficult to rule out this possibility because of the crude assay used to measure methylcytosine depletion in this early experiment. Analyses of maternal zygotic *dnmt1* mutants may aid in clarifying the consequence of early inhibition of Dnmt1 activity.

The tissue-specific differences in sensitivity following loss of Dnmt1 are also intriguing. Given that the tissues undergoing the highest levels of proliferation at 3–4 dpf were also the most sensitive to loss of Dnmt1 protein, it is possible that proliferation itself caused susceptibility. Maternal protein stores should be depleted by cell division and, thus, cells undergoing more rounds of division might have increased sensitivity to loss of zygotic Dnmt1. Alternatively, Rai et al. have suggested that differences in tissue responses could indicate a role for DNA methylation in terminal differentiation of some organs but not others. While further studies will be required to distinguish between these two possibilities, characterization of *dnmt1* morpholino-injected embryos and mutants supports the general conclusion that Dnmt1 function is important for growth or maintenance of endodermal organs.

4. Posttranslational Regulation of Dnmt1—Given the widespread conservation of the maintenance methylation machinery, it is not surprising that Dnmt1 and Uhrf1 functions are conserved in zebrafish. However, it is not yet known whether other proteins involved in the regulation of maintenance methylation in mammals have the same function in fish. Recent reports have suggested that regulation of DNMT1 protein is achieved, at least in mammals, via the antagonistic action of a lysine-specific protein methyltransferase and demethylase. The methyltransferase SET7 is thought to lead to protein degradation through direct methylation of specific lysine residues in DNMT1⁴⁶ (see Chapter by Shannon R. Morey Kinney and Sriharsa Pradhan). Conversely, the demethylase LSD1 has been hypothesized to prevent this degradation by removing the methyl mark.⁴⁷ Although methylation of Dnmt1 has not been demonstrated in zebrafish, the N-terminal lysine and flanking amino acid signature targeted by SET7 are conserved, and orthologs for *set7* and *lsd1* are present in the zebrafish genome.

B. Conserved Role for Zebrafish Dnmt2 in RNA Methylation

Dnmt2 proteins represent the most widely conserved family of DNA methyltransferase paralogs in eukaryotes, but their function has been the most controversial. Although several reports have described very low levels of Dnmt2-dependent DNA methylation in *Dictyostelium discoideum* and *Drosophila*,^{48–50} additional reports have failed to identify any DNA methylation activity for this family of proteins.^{48–50} In contrast, Dnmt2 shows robust methyltransferase activity on specific tRNA substrates and this activity is important for protecting RNAs against stress-induced cleavage.^{51–53}

The zebrafish genome includes a single copy of *dnmt2* (Fig. 1), which is ubiquitously expressed and has activity on tRNA^{Asp}.⁵⁴ Surprisingly, while mutations in *dnmt2* in a variety of organisms including mouse, *Drosophila*, *Arabidopsis*, and *Schizosaccharomyces* do not result in any obvious phenotypes, depletion of Dnmt2 in zebrafish embryos using an antisense morpholino affected the development of the retina, liver, and brain.⁵⁴ No detectable changes in DNA methylation were observed and Dnmt2 protein targeted to the cytoplasm, but not the nucleus, could rescue the phenotypes.⁵⁴ Although it is unclear why loss of Dnmt2 should cause developmental abnormalities specifically in zebrafish, these results suggest that Dnmt2-mediated RNA methylation rather than DNA methylation is required for normal development in this organism.⁵⁴

C. Dnmt3 De Novo Methyltransferases

The establishment of DNA methylation has not been extensively explored in zebrafish. To date, *de novo* methylation has been reported at the zebrafish promoter of the *no tail* gene, as well as at newly integrated transgenes.^{9,17,23,55} More comprehensive efforts to identify targets of *de novo* methylation have not been described. In mammals, the closely related proteins DNMT3A and DNMT3B are primarily responsible for the establishment of DNA methylation^{56,57} (see Chapter by Frédéric Chédin). Based on sequence comparisons, the zebrafish genome specifies six likely orthologs of DNMT3A and DNMT3B that could potentially serve as *de novo* methyltransferases.²² Similar to mouse, the zebrafish *dnmt3* family members (*dnmt3*, *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8*) all contain conserved PWWP (pro-trp-trp-pro) and cysteine-rich ADD (ATRX-DNMT3-DNMT3L) motifs near the N-terminus (Figs. 1 and 2).

1. Dnmt3A-Like Proteins—Zebrafish possesses two highly related proteins, Dnmt6 and Dnmt8 (Fig. 1), that are the most related to mouse DNMT3A (Fig. 2). The presence of two DNMT3A orthologs appears to be conserved in many fish species, as two genes specifying Dnmt3a-like proteins are also present in the genomes of *Tetraodon*, fugu, and stickleback (Fig. 2). Dnmt6 and Dnmt8 show 77% identity overall, with 89% identity in the catalytic domain. In mouse, *Dnmt3a* is expressed as two different isoforms, with the short form being highly expressed in germ cells and ES cells. While EST data do not show evidence for alternative splicing of *dnmt6* or *dnmt8* in zebrafish, it is of note that the most N-terminal portions of the proteins are the most divergent, and vary in length by 150 amino acids. It is possible that, instead of employing different isoforms of *Dnmt3a*, fish achieve similar results by using two independent genes. Although high sequence similarity makes gene expression

analyses challenging, knowing whether the zebrafish Dnmt3a homologs show tissue-specific differences in their expression, particularly in germ cells, would be informative.

2. Dnmt3B-Like Proteins—In mammals, DNMT3B is responsible for *de novo* methylation of diverse sequences. Mutations in the mouse gene result in embryonic lethality, while the immune centromere and facial abnormalities (ICF) syndrome is caused by mutations in the human gene. The zebrafish protein Dnmt4 shows highest similarity to mouse DNMT3B, with 68% identity in amino acid sequences (Fig. 2). However, injection of *dnmt4* antisense morpholinos did not cause any developmental phenotypes, and targets of Dnmt4 methylation have not yet been described. Nonetheless, dynamic expression of *dnmt4* in early embryos and tissue-specific expression in larvae imply that this protein may have important roles during zebrafish development.⁴¹

3. Dnmt3 Orthologs Unique to Fish—Fish-specific Dnmt3 family members can also be detected in the genomes of zebrafish, *Tetraodon*, fugu, and stickleback. While these proteins have substantial sequence similarity to mouse DNMT3A and DNMT3B, they do not appear to be any more related to one protein than the other (Fig. 2). Moreover, these additional orthologs appear to be diverging from each other much faster than DNMT3A or DNMT3B family members (Fig. 2). It is most odd that zebrafish Dnmt3 and Dnmt7 both contain an N-terminal calponin homology domain²² (Fig. 1). Calponin homology domains are generally associated with actin binding, and are typically found in cytoskeletal and signal transduction proteins.⁵⁸ Thus far, calponin homology domains have not been reported in any methyltransferase protein outside of fish and their functional significance remains to be determined. Dnmt5 is most closely related to Dnmt3, but lacks the N-terminal calponin domain. *dnmt3* and *dnmt5* reside in a gene cluster with *dnmt4*, suggesting they may have arisen from a recent gene duplication event followed by functional diversification.⁵⁹

4. DNMT3L Orthologs Are Absent in Fish—Mammalian genomes encode for an additional Dnmt3 homolog, DNMT3L, which has lost most catalytic motifs associated with methylation. Despite lacking these motifs, DNMT3L is essential for methylation at mono-allelically expressed imprinted genes in the maternal genome.⁶⁰ Unlike mammals, zebrafish do not require monoallelic expression of imprinted genes for survival, as both androgenetic and parthenogenetic zebrafish are viable and fertile in adulthood.^{2,61} DNMT3L orthologs have not been detected in the zebrafish genome, and it has been hypothesized that DNMT3L may have been acquired during the evolution of imprinting.⁶² However, DNMT3L is also required for the methylation of transposable elements in the paternal mouse genome,⁶³ and methylation has been detected at transposable elements in zebrafish.¹⁸ It will be important to determine whether *de novo* methylation of transposable elements occurs in the zebrafish germ line and, if so, whether any of the fish-specific Dnmt3 orthologs substitute for DNMT3L in this process.

5. Evidence for Methylation by Dnmt3 Family Members—Although all five *dnmt3* genes were shown to be transcribed in 1-day-old embryos by 5'- and 3'-RACE, so far there is only limited functional evidence for their activity.²² Significant effects on global methylation were not observed following injection of antisense morpholinos against each

mRNA.²² At the level of individual genes, injection of antisense morpholino against *dnmt3*, but not other *dnmt3* family members, was reported to decrease CpG methylation at the *ntl* gene. However, embryos developed normally and no differences in *ntl* gene expression could be detected.²² In contrast, another study reported modest decreases in methylation and correlating increases in gene expression at the *no tail* and *lef1* genes using a *dnmt3* antisense morpholino.⁶⁴ It is likely that the zebrafish Dnmt3 family members share at least some overlapping sequence targets and that combinatorial knockdown of Dnmt3 proteins may reveal more pronounced effects on methylation.

6. Phenotypes of Dnmt3 Mutants—Thus far, Dnmt3 is the only member of the zebrafish *de novo* methyltransferase family for which a phenotype has been described. *Dnmt3* morpholino-injected embryos die at 96 hpf with multiple abnormalities including very small brains, defective pharyngeal arch formation, and abnormal retinal neural epithelial differentiation.⁶⁴ Although overall patterning of the developing brain appeared normal, several proneural markers were not expressed. The authors attribute the loss of these makers to decreased DNA methylation and ectopic expression of the *lef1* gene, a transcription factor that is critical for neurogenesis in the hypothalamus.⁶⁵

It is noteworthy that the phenotypes reported for *dnmt3* morphants are very distinct from those observed in *dnmt1* morphants, and that overexpression of either mRNA cannot rescue phenotypes associated with the other.⁶⁴ Given that DNMT1 is typically thought to maintain methylation established by DNMT3s, it is curious that the neuronal defects seen in *dnmt3* morpholino-injected embryos were not observed in *dnmt1* morphants or mutants. However, expression of *dnmt3* mRNA was detected in the brain, eyes, and somites between 1 and 3 dpf⁶⁴ and it is possible that continuous *de novo* methylation by Dnmt3 might perpetuate DNA methylation in the absence of Dnmt1.

D. Conservation of Additional Proteins Associated with Methylation

The zebrafish genome also codes for orthologs of additional proteins that have been associated with DNA methylation in mammals. Although these proteins have not been extensively studied in fish, their apparent presence is consistent with broad conservation of the methylation machinery between fish and mammals.

1. CFP1—In mouse, the CXXC finger protein 1 (CFP1) specifically binds to unmethylated CpG dinucleotides, and has been demonstrated to influence chromatin structure at CpG islands by recruitment of the H3K4 methyltransferase Setd1.⁶⁶ In addition, mouse ES cells lacking CFP1 fail to differentiate, and exhibit a 60–80% reduction in global genomic cytosine methylation.⁶⁷ Although the mechanism by which CFP1 influences global methylation levels has not been fully elucidated, it appears that this role is conserved among vertebrates: as depletion of the zebrafish Cfp1 protein by morpholino injection results in a 60% reduction in genome-wide methylation.⁶⁸

2. Methyl-Binding Proteins—Mammalian proteins that show selective binding to methylated CpG sites have been proposed to inhibit the transcription of methylated promoters (see Chapter by Pierre-Antoine Defossez and Irina Stancheva). Orthologs of the

most prominent of the proteins, MeCP2 and Mbd2, are also found in the zebrafish genome.⁶⁹

3. Piwi Proteins—The mouse Piwi-related proteins MIWI, MIWI2, and MILI are important for the *de novo* methylation of transposons in germ cells and likely mediate this activity through interactions with small RNA molecules called piRNAs (see Chapter by Anton Wutz). The zebrafish genome encodes for two *piwi* homologs: *ziwi* and *zili*.^{70,71} Both proteins and piRNAs with homology to transposons can be found specifically in germ cells, and increased transposon expression was reported in the gonads of *zili* homozygous mutants.⁷¹ However, because Piwi proteins are essential for germ cell development, it has been technically challenging to assess the methylation status of transposable elements in mutants. In contrast to mouse, *Drosophila* Piwi proteins mediate silencing of transposons independent of DNA methylation.⁷² It is therefore of interest to determine whether zebrafish Piwi proteins facilitate transposon constraint through DNA methylation or other mechanisms.

IV. Link Between DNA and Histone Modification in Zebrafish

As the primary protein component of chromatin, histones have been attributed roles in both packaging of DNA into higher order structures and in regulation of transcription, in both cases through modification of their N-terminal tails (see Chapter by Taiping Chen). Despite extensive study, it remains unclear how DNA and histone modifications are targeted to particular regions of the genome. However, emerging data suggest that there is coordination between DNA methylation and histone modifications in many organisms including zebrafish.

While dependence of DNA methylation on histone H3 lysine 9 (H3K9) methylation has been clearly demonstrated at non-CpG dinucleotides in plants and fungi,^{73,74} the relationship between these modifications has been less clear in mammals. Decreased DNA methylation was observed at pericentric satellite DNA from ES cells lacking the H3K9 methyltransferases SUV39h1/SUV39h2, suggesting that in some situations, mammalian DNA methylation is also dependent on H3K9 methylation. In contrast, H3K9 methylation of pericentric satellite DNA was normal in *Dnmt1* mutant ES cells.⁷⁵

Surprisingly, evidence for the opposite relationship has been reported in zebrafish, with H3K9 methylation being dependent on DNA methylation. *Dnmt1* morpholino-injected embryos were reported to have a 70% reduction in H3K9 methylation, whereas *Suv39h1*-depleted embryos had normal levels of cytosine methylation.⁴⁰ Remarkably, developmental defects observed in *dnmt1* morpholino-injected embryos could be rescued by overexpression of *Suv39h1* mRNA.⁴⁰ These results suggest that, in contrast to other systems, zebrafish CpG methylation may direct H3K9 methylation and even more surprisingly, H3K9 methylation may be sufficient for normal development in the absence of *Dnmt1*-mediated DNA methylation.

Additional work in zebrafish has also suggested a link between the euchromatic H3K9 methyltransferase G9a and methylation by *Dnmt3*. Global changes in methylation of DNA

or H3K9 were not detected following injection of morpholinos for either gene. However, both decreased H3K9 methylation and DNA methylation were observed at the *lef1* promoter in Dnmt3 morphant embryos ("morphants" are organisms treated with morpholinos). In addition, injection of G9a mRNA into Dnmt3 morphant embryos was reported to rescue phenotypes associated with *lef1* dysregulation.⁶⁴ Although it will be important to extend these observations to additional genes, this work raises the interesting possibility that distinct pairs of DNA and histone methyltransferases might act upon unique subsets of genes.⁶⁴

V. DNA Methylation Dynamics in Early Development

In mammals, erasure and reestablishment of global methylation patterns have been described during gametogenesis and early embryogenesis.⁷⁶ While the functional significance of these dynamic events has not been confirmed, they are thought to reset gametic methylation patterns, thereby reprogramming the embryonic genome for somatic development.⁷⁷ In mouse, the process of erasure and reestablishment in the early embryo has been well studied. Demethylation of the paternal pronucleus occurs almost immediately after fertilization. Additional stepwise decreases in embryonic methylation then continue until the morula stage when *de novo* methylation is initiated.⁷⁸

Because zebrafish embryos should also need to erase gametic methylation patterns prior to somatic development, one might predict conservation of these early reprogramming events. However, there are conflicting reports on whether a similar phenomenon occurs in zebrafish. Initial studies by Macleod et al. did not detect changes in DNA methylation over the course of development, at DANA repetitive elements or three randomly selected zebrafish genes.²⁴ Similarly, an early study of methylation dynamics in medaka detected constant levels of global methylation between 16-cell and larval stages of development.²⁶

In contrast, other studies have provided evidence for dynamic methylation in the early embryo. Using methyl-sensitive restriction digests of genomic DNA, Mhanni et al. reported very low levels of global methylation in one to two cell stage embryos, compared to blastula stage embryos.⁷⁹ These observations were corroborated by an additional report using a 5-methyl cytosine antibody to monitor methylation in the early embryo. In this study, robust methylation was detected in blastulae by Southwestern blots and immunocytochemistry, but little to no methylation was detected before this stage.⁸⁰

It is difficult to fully reconcile the results of these reports. The study by Macleod et al. omitted very early embryonic stages from analysis, and only examined a selected group of sequences. Thus, demethylation occurring immediately after fertilization or at other unexamined sequences would have been missed. However, while the report from Mhanni et al. described the most severe demethylation in one to two cell embryos, substantial demethylation of both genomic DNA and DANA elements was also described at 2.2 hpf, a time at which Macleod et al. reported DANA elements to be fully methylated.

Perhaps one reason for the discrepancies is the substantial technical hurdles associated with measuring DNA methylation in the early embryo, where small amounts of starting material,

a high yolk to DNA ratio, and abundant stores of maternal RNA complicate analyses. Given that a large amount of high-quality genomic DNA is essential for accurate assessment of DNA methylation by restriction enzyme digestion, alternative methods such as bisulfite sequencing may be better suited to the early embryo. Many different genomic loci will need to be examined to characterize genome-wide methylation dynamics but, given its importance from an evolutionary standpoint, a more comprehensive assessment to clarify methylation dynamics in zebrafish should be well worth the effort involved.

VI. Evidence for Active DNA Demethylation in Zebrafish

If DNA methylation patterns during development are dynamic, a means for demethylation is required. In animals, DNA demethylation has a long and controversial history and, despite the efforts of multiple laboratories, mechanisms for its control remain unclear (see Chapter by Taiping Chen). One mechanism termed “passive demethylation” involves failure to methylate CpG dinucleotides in the newly synthesized DNA strand during replication. Accordingly, after two cycles of replication, 50% of DNA duplexes would be fully demethylated. However, some experiments have detected DNA demethylation in the absence of replication, a scenario that would require “active” enzymatic removal of methylated cytosine. Over the years, many different mechanisms have been proposed and discounted in animals,⁸¹ and to date active demethylation remains a contentious issue.

A. Early Evidence for Active Demethylation in Zebrafish

There is some evidence for active demethylation in zebrafish. Initial studies reported demethylation of *in vitro* methylated plasmid DNA injected into the one-cell stage embryo, even in the presence of the replication inhibiting drug camptothecin.⁸² Supporting the DNA replication-independent nature of the demethylase activity, extracts from embryos collected at 4 hpf were also capable of demethylating plasmid DNA *in vitro*. Surprisingly, this activity was abolished upon treatment with RNase A, suggesting that the activity was RNA dependent. While these early studies favor the presence of an active demethylase in zebrafish embryos, *in vivo* targets of the demethylase activity have not been described nor the mechanism of demethylation identified.

B. Demethylation by Aid and Mbd4

In animals, activation-induced cytosine deaminase (AID) is currently at the epicenter of demethylation research. AID has been implicated in the erasure of DNA methylation in primordial germ cells and in fibroblasts undergoing reprogramming. Recent work suggests that Aid is likewise capable of mediating DNA demethylation in zebrafish.^{25,83,84}

Provocative work in the zebrafish system suggests a coupled mechanism of demethylation, in which Aid-mediated deamination of methylated cytosine residues is followed by base excision through the glycosylase activity of the methyl-binding domain protein Mbd4. Supporting this model, overexpression of *aid* and *mbd4* mRNAs resulted in a 30% reduction in genomic DNA methylation, as assayed by mass spectrometry and *HpaII* digestion.²⁵ In addition, the growth arrest and DNA damage protein Gad45, which had been previously implicated in DNA demethylation, stimulated Mbd4- and Aid-mediated demethylation.²⁵ It

remains to be determined whether the same demethylase system is utilized for demethylation in the early zebrafish embryo.

C. Potential Roles for Aid Demethylase Activity in Development

The Aid demethylase machinery has been implicated in the maintenance of intestinal cells and possibly the progression of colon cancer. At 72 hpf *aid*, *mbd4* and *gadd45* mRNAs were all shown to be upregulated in zebrafish homozygous for mutations in the *adenomatous polyposis coli* (*apc*) tumor suppressor gene.⁸⁵ Genome-wide changes in DNA methylation were not detected in *apc* mutants; however, methylated DNA immunoprecipitation (MeDIP) revealed decreases in DNA methylation at a number of genes many of which are involved in intestinal differentiation and colorectal cancer progression.⁸⁵ In particular, the authors observed high expression and hypomethylation of a marker of colon crypt progenitor cells. Increased expression of the progenitor cell marker correlated with decreased expression of an intestinal differentiation marker. Proper expression of both genes could be partially rescued by injection of an anti-Aid morpholino, implicating Aid-mediated demethylation in their regulation. These observations lead to the hypothesis that APC downregulates the demethylase machinery to facilitate progenitor commitment to differentiation during normal development and, conversely, increased expression of *aid*, *mbd4*, and *gadd45* in *apc* mutants may drive the hypomethylation frequently observed in colon cancer.⁸⁵

The developmental defects described in this study are intriguing although somewhat surprising given that overall development appears normal in *Aid* and *Mbd4* mutant mice.^{83,86} To clarify the roles of Aid in normal zebrafish development, it will be important to examine its direct role independent of the *apc* mutant background. Intriguingly, zebrafish embryos injected with antisense morpholinos against either *aid* or *mbd4* were reported to show reduced expression of the neural marker *neurogenin-1*,²⁵ suggesting AID could have more widespread functions in normal development.

VII. Perspectives and Future Directions

The initial studies presented in this review provide a fundamental understanding of DNA methylation in zebrafish and demonstrate conservation of the basic methylation machinery with mammals. These studies provide a framework for new research that exploits the unique features of the zebrafish system for the study of DNA methylation, particularly in the context of development.

A. DNA Methylation in Development

An important question in the field of methylation research centers around why DNA methylation is required for development in vertebrates, when many other organisms survive without methylated genomes.¹ Mammals require methylation at imprinted genes, whose proper regulation is necessary for viability.¹ Thus, it has been hypothesized that dysregulation of imprinting could explain the lethality observed in mammalian *Dnmt1* mutant embryos. Zebrafish do not require imprinting for normal development, yet similar to mice, *Dnmt1* mutant zebrafish are inviable and show significant levels of p53-mediated apoptosis. These observations suggest that there are other underlying reasons for

hypomethylation-induced lethality.^{40,42} The accessibility of the developing zebrafish embryo, the wide array of characterized developmental markers, and the lack of imprinting should make zebrafish a simple and well-defined system for probing the additional requirements for DNA methylation in vertebrates.

B. *In Vivo* Monitoring of Methylation

To date, much of our understanding of methylation has come from biochemical assays on DNA extracted from whole animals or tissues. However, these snapshots of methylation may overlook dynamic changes during development or differences in methylation among distinct cell populations in heterogeneous tissues. Moreover, biochemical approaches prove challenging for multigenerational studies as animal sacrifice is frequently required for sample collection. The ease of creating transgenic zebrafish, coupled with the clarity of the developing larvae, makes zebrafish an ideal system for *in vivo* monitoring of methylation via fluorescent reporters.

This strategy has recently been validated in transgenic zebrafish carrying the green fluorescent protein (GFP) under the control of 14 copies of the yeast upstream activation sequence (14X UAS). In the absence of DNA methylation, the yeast transcriptional activator Gal4 binds to the 14X UAS to drive transcription of GFP. However, the UAS is rich in CpG dinucleotides and the 14 copies constitute a repetitive sequence, which likely attracts methylation.²³ Over several generations, methylation accumulates at the 14X UAS leading to increasing variegation in GFP labeling and finally complete silencing of the transgene.²³ Consistent with this, reactivation of GFP expression is observed in *dnmt1* and *uhrf1* zebrafish mutants that have reduced methylation.^{18,23} Thus, transgenic lines can be used to continuously monitor methylation in the developing zebrafish at the resolution of single cells.

Somewhat surprisingly, when reactivation of the 14X UAS was examined in *dnmt1* mutant larvae using a brain-specific driver, not all regions of the brain showed equivalent reactivation of GFP expression. While robust labeling was observed in the mid- and forebrain regions, little to no reactivation was observed in the hindbrain.²³ The mechanisms contributing to this difference have not been identified, although it is possible that other DNA methyltransferases compensate for Dnmt1 in the hindbrain. The observation of regional differences in reactivation underscores the importance of monitoring DNA methylation *in situ* and in the context of development.

C. Reprogramming and Cellular Differentiation

Additional transgenic lines may reveal important information about the roles of DNA methylation in cellular differentiation and regeneration.⁸⁷ Transgenic lines carrying GFP reporters that are silenced in adult tissue, but reactivated in dividing cells of the regenerating fin, have recently been described.⁸⁸ The same transgenes were also found to be reexpressed during retinal regeneration and could potentially serve as reporters in a variety of regeneration models.⁸⁵ Intriguingly, DNA methylation may control the expression status of these transgenes.⁸⁸ Although additional research will be required to validate these tools, they offer enormous potential for monitoring dynamic changes in methylation during the

regeneration process.⁸⁵ Given that roles for Dnmt1 and Uhrf1 have recently been described in zebrafish pancreas and liver regeneration,^{37,42} and that Aid-mediated demethylation has been shown to be important for the maintenance of intestinal stem cells, the zebrafish is likely to become a widely used system for understanding the roles of DNA methylation in cellular differentiation and regeneration.

D. Environmental Influences on DNA Methylation

A new and intriguing research direction has emerged, which is focused on the effects of dietary and environmental factors on DNA methylation patterns, and their relevance to the etiology of human disease.⁸⁹ For example, bisphenol A (BPA), an endocrine-disrupting compound used in the production of plastics and resins, causes hypomethylation of several genetic loci in mouse.⁸⁹ Additional studies in rodents and humans have also suggested that exposure to a number of other chemicals including metals, peroxisome proliferators, air pollutants, and endocrine-disrupting toxins may alter methylation patterns.⁹⁰ Rearing adult or larval zebrafish in water containing chemicals would provide a simple way to probe how exposure impacts DNA methylation. Changes in methylation can be assayed by direct examination of harvested genomic DNA, or *in vivo* using transgenic reporters of methylation such as those described above. Preliminary evidence for changes in methylation at several zebrafish genes following exposure to either 17 α -ethinylestradiol or arsenic validates the potential of these approaches.^{91,92}

E. Genetic Screens to Identify Factors Important for Methylation

Despite extensive research, the complete machinery required for the establishment, maintenance, and interpretation of methylation marks has not been fully elucidated in vertebrates. Forward genetic screens in *Arabidopsis* have provided a number of mutations that affect methylation; however, many genes identified from plant screens are not conserved in animals.^{93–95} A screen for dominant modifiers of an epigenetically regulated transgene in mouse has provided mutants demonstrating the importance of animal screens.⁹⁶ However, to date, an unbiased screen for recessive mutations that affect DNA methylation in vertebrates has not been described.

One of the most powerful features of the zebrafish system is its potential for unbiased genetic and chemical screens. Screens for components required for DNA methylation in zebrafish could be performed by direct analysis of genomic DNA, as has been described in plants.⁹⁷ Alternatively, transgenic reporters of methylation such as those described above may offer an efficient means for rapid screening at multiple stages of development. Reactivation of transgenic reporters in zebrafish mutants with hypomethylated genomes provides proof of principle for using genetic screens to identify new mutations that influence gene silencing.

VIII. Conclusions

Although there are nearly 300 million years separating the last common fish and human ancestor, DNA methylation pathways remain well conserved. Given the similarities between DNA methylation in mammals and zebrafish, the zebrafish model holds great promise for

providing new insights into the mechanisms that regulate DNA methylation in vertebrates and for better understanding its roles in normal development and disease.

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Fig. 1.

Schematic of the eight DNA methyltransferase orthologs present in the zebrafish genome (adapted from Ref. 22). All orthologs share a C-terminal catalytic domain containing amino acid motifs that are conserved in all 5mC-generating DNA methyltransferases. In addition, these proteins exhibit distinct motifs in the N-terminus. BAH: bromo-adjacent homology domain, CH: calponin homology, CXXC: cysteine-rich domain, PWWP: conserved proline-tryptophan-tryptophan-proline domain, and ADD: ATRX-DNMT3-DNMT3L domain.

Fig. 2.

Phylogenetic tree of Dnmt1 and Dnmt3 families from mouse, zebrafish, fugu, *Tetraodon*, medaka, and stickleback. Alignments were performed on full-length amino acid sequences via ClustalW and DRAWTREE (<http://mobyle.pasteur.fr/cgi-bin/portal.py>). The multiple alignment parameters were as follows: Gonnet 250 matrix with gap open penalty 10, gap extension penalty 0.2, and gap separation penalty 4 sequences. Mouse and zebrafish protein accessions are as follows: mouse Dnmt1 NP_034196.3, Dnmt3A NP_031898.1, Dnmt3B NP_001003960.1 and zebrafish Dnmt1 NP_571264.1, Dnmt3 NP_571461.1, Dnmt4 NP_001020621.1, Dnmt5 NP_001018315.1, Dnmt6 NP_001018150, Dnmt7 NP_001018312.1, Dnmt8 NP_001018144.1. Fugu, *Tetraodon*, medaka, and stickleback homologs represent predicted proteins identified by homology to zebrafish proteins using BLAT (<http://genome.ucsc.edu>). Ensemble predictions numbers are: Medaka Chr24 ENSORLP00000022087, Chr Un1 ENSORLP00000025108, Chr7 ENSORLP00000018521; *Tetraodon* Chr10 ENSTNIP00000022492, Chr14 ENSTNIP00000022331, Un1 ENSTNIP00000009615, Un2 ENSTNIP00000009617, Chr9 ENSTNIP00000003297, Chr4 ENSTNIP00000003297, Un5 ENSTNIP00000009617; Fugu Un1 ENSTRUP00000024461, Un2 ENSTRUP00000009050, Un3 ENSTRUP000000031152; Stickleback Chr15 ENSGACP00000013579, Chr18 ENSGACP00000009407, Chr12a

ENSGACP00000013579, Chr12b ENSGACP00000015822, Chr12c
ENSGACP00000013567. Un indicates undesignated chromosome.

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TABLE I

Methylation of the Zebrafish Genome Compared to Other Model Organisms

	Zebrafish	Mouse	Honey bee	<i>Arabidopsis</i>	<i>Neurospora</i>
CpG	80.3% ¹⁸	74.2% ¹⁸	0.93% ¹⁸	22.3% ¹⁸	0.37% ²⁰
CpNpG	1.22% ¹⁸	0.30% ¹⁸	0.26% ¹⁸	5.92% ¹⁸	NA
CpHpH	0.91% ¹⁸	0.29% ¹⁸	0.17% ¹⁸	1.51% ¹⁸	1.2% ²⁰
Transposons	Mildly enriched ¹⁸	Mildly enriched ¹⁸	Not enriched ¹⁸	Strongly enriched ¹⁸	Strongly enriched ²¹

NA, not assessed. H, not G (i.e., A, C, or T).