

Expression of the De Novo DNA Methyltransferases (*dnmt3 – dnmt8*) During Zebrafish Lens Development

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Background: De novo DNA methylation is thought to be critical for cellular reprogramming during tissue differentiation and development. Little is known about the roles of de novo DNA methylation during eye development, and particularly during lens development. The lens is composed of lens epithelial (LE) and lens fiber (LF) cells, with proliferative LE cells giving rise to differentiated LFs at the “transition zone.” Given the unique architecture and developmental program of the lens, and the involvement of de novo DNA methylation during differentiation events in other tissues, we sought to identify de novo DNA methyltransferases expressed in the zebrafish lens. **Results:** Zebrafish possess six de novo DNA methyltransferase genes, *dnmt3 – dnmt8*. At 24 hr postfertilization (hpf), all six are expressed ubiquitously throughout the eye. By 72 hpf, *dnmt3* and *dnmt5* become restricted to cells of the retinal ciliary marginal zone (CMZ), *dnmt4* and *dnmt7* to cells of the CMZ and LE, and *dnmt6* and *dnmt8* to ganglion cells and cells of the inner nuclear layer of the retina. **Conclusions:** These data identify regions of the eye where de novo methyltransferases could mediate DNA methylation events during development. Overlapping expression domains also suggest functional redundancy within this gene family in the eye. *Developmental Dynamics* 243:350–356, 2014. © 2013 Wiley Periodicals, Inc.

Key words: de novo DNA methyltransferase; DNA methylation; lens; retina; ciliary marginal zone

Key findings:

- Expression of *dnmt3*-family genes in developing zebrafish eye.
- Unique and overlapping expression domains in lens epithelium, ciliary marginal zone, and inner retina.
- Two *dnmt3*-family transcripts detected in each ocular domain.

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INTRODUCTION

DNA methylation is an epigenetic mechanism by which methyl groups are added to DNA, predominantly at CpG dinucleotides. Genomic regions rich in CpG sites (CpG islands) are often associated with gene promoters, and their methylation statuses are often inversely correlated with gene expression levels (Meissner et al., 2008; Illingworth and Bird, 2009; Deaton and Bird 2011; VanderKraats

et al., 2013). Maintenance methylation, mediated by Dnmt1, copies the methylation pattern from parental to daughter strand after DNA replication, a process that has been associated with the maintenance of proliferative cell identities (e.g., Sen et al., 2010; Tittle et al., 2011). De novo methylation, mediated by Dnmt3a and Dnmt3b in mammals, is a process in which unmethylated CpG sites become methylated, and has been shown to play critical roles dur-

ing development of plants and animals, particularly in tissue differentiation when cells undergo epigenetic reprogramming to adopt new identities (Illingworth et al., 2008; Feng et al., 2010; Merbs et al., 2012; Hu et al., 2012). Defects in, or loss of, de novo methyltransferase function cause a multitude of phenotypes associated with aberrant tissue-specific gene expression, and in a variety of animal model systems (e.g., Okano et al., 1999; Gao et al., 2011;

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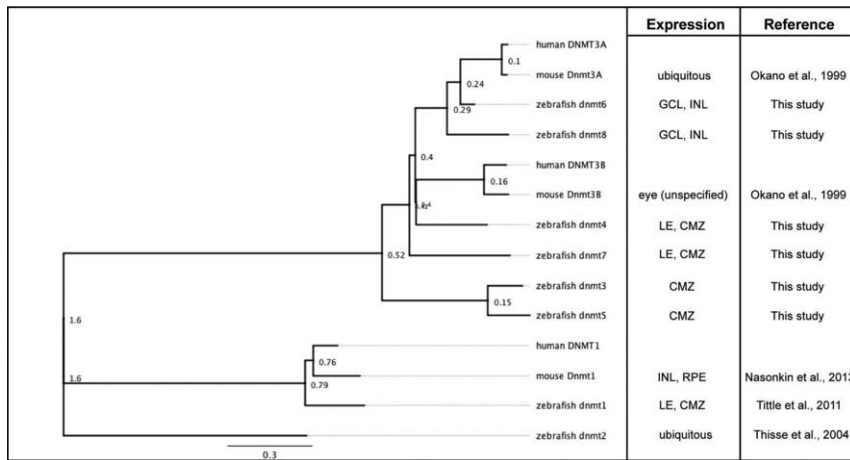


Fig. 1. Phylogenetic relationship between DNA methyltransferases and their corresponding expression patterns. Tree was constructed using Geneious Tree Builder from an alignment of DNA methyltransferase amino acid sequences (zebrafish dnmt1, 3-8; mouse and human Dnmt1, 3A and 3B), with zebrafish RNA methyltransferase (dnmt2) as an outgroup.

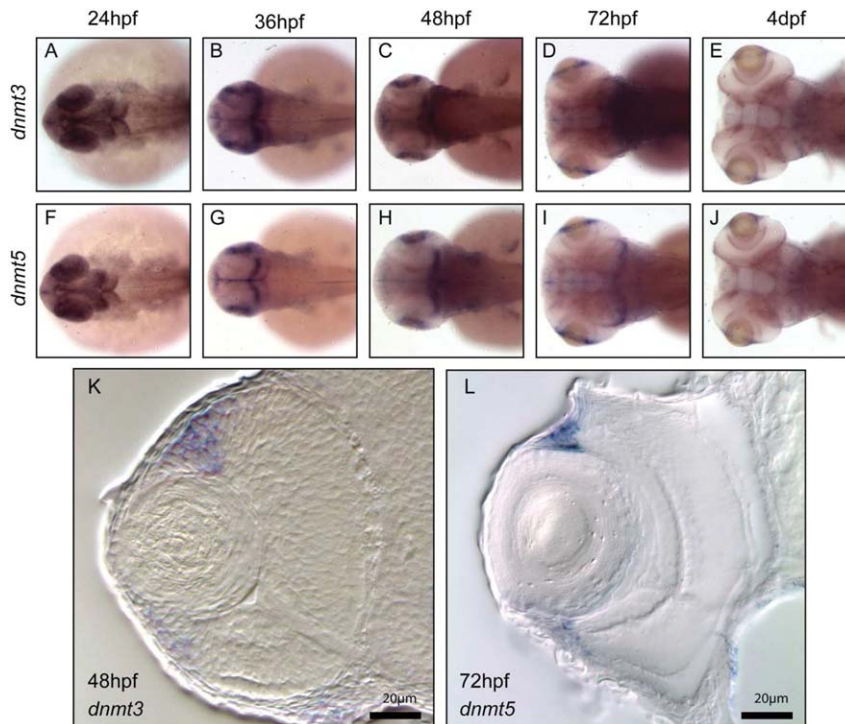


Fig. 2. *dnmt3* and *dnmt5* expression is enriched in the ciliary marginal zone. **A,F:** In situ hybridization shows *dnmt3* and *dnmt5* expression throughout the anterior region of the embryo at 24 hr postfertilization (hpf). **B–D,G–I:** Expression becomes restricted to the CMZ, tectum, pectoral fins, and pharyngeal arches between 36 and 72 hpf. **E,J:** At 4 days postfertilization (dpf), ocular expression is only detected in the CMZ. Transverse sections, dorsal up (K,L).

Wu et al., 2012). Additionally, in humans, somatic mutations in DNMT3A are associated with acute leukemia and certain types of solid cancer (Kim et al., 2013).

Vertebrate lenses are composed of two cell types: lens epithelial (LE) and lens fiber (LF). During develop-

ment and continuously throughout life, proliferative LE cells in the anterior of the lens give rise to differentiated and elongated LFs, which are then packed into the lens cortex to generate the bulk of lens tissue (Greiling and Clark, 2009; Greiling et al., 2010). LFs are transparent,

highly organized and organelle-free. To obtain this biophysical property, LFs undergo substantial morphological changes during differentiation that include a massive elongation and complete elimination of all light-scattering organelles, including the nucleus (Bassnett et al., 2011; Wride, 2011). Failure of LF cells to achieve their final shape and organization have been shown to cause congenital cataracts in human and other model organisms (Santana and Waiswol, 2011; Hejtmancik, 2009). Given its unique architecture and developmental program, the lens is an ideal tissue in which to study how epigenetic regulation affects tissue development and differentiation.

Zebrafish is a powerful vertebrate model system that is highly amenable to genetic and biochemical manipulation. In zebrafish, the lens placode delaminates from the surface ectoderm as a solid mass of cells, rather than as a hollow vesicle as it does in mammals (Greiling and Clark, 2009; Greiling et al., 2010). Beyond this early difference, all other aspects of lens development appear to be identical between zebrafish and mammals, and the overall structure and architecture of zebrafish and mammalian lenses are virtually identical (Bibliowicz et al., 2011). This suggests a common genetic and epigenetic program controlling vertebrate lens development and growth. Given its amenability to forward and reverse genetics (Cade et al., 2012; Bedell et al., 2012; Lee et al., 2012; Hwang et al., 2013), the zebrafish is an attractive model system in which to study the epigenetic regulation of lens development.

Previous work in our laboratory has shown that the functions of *dnmt1* and its recruitment factor, *uhrf1*, are required for normal development and maintenance of the zebrafish lens (Title et al., 2011). Both genes are expressed in an overlapping domain in the lens epithelium that correlates with regions of proliferation, and loss-of-function mutations in either results in altered LE gene expression, reduced proliferation, and loss of tissue integrity in the lens epithelium. While these data support a role for the maintenance methyltransferase *dnmt1*, during lens development, little is

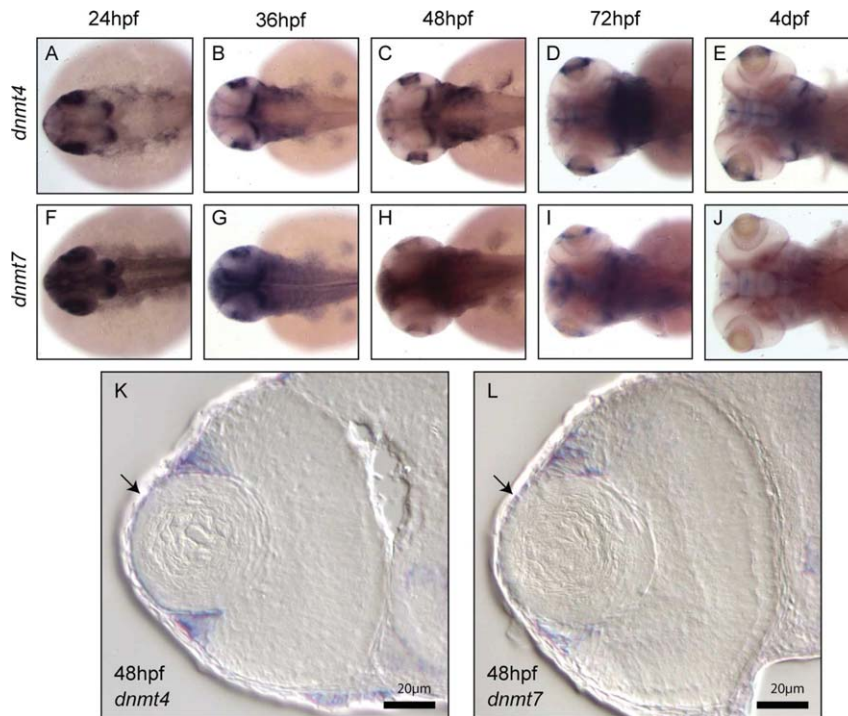


Fig. 3. *dnmt4* and *dnmt7* expression in the lens epithelium and ciliary marginal zone. **A–E,K:** In situ hybridization shows broad *dnmt4* expression in the developing eye at 24 hr postfertilization (hpf) (A), which later becomes localized in the CMZ and lens epithelium (B–E, K, arrow points to LE). *dnmt4* expression is also observed in the tectum, developing pectoral fin, and pharyngeal arches (A–E). **F:** *dnmt7* is expressed ubiquitously at 24 hpf. **G–J,L:** At 36 hpf, expression remains diffuse in the anterior part of the embryo, and surrounding of the eye, while expression within the eye is only observed in the CMZ and LE (L, arrow points to LE). Transverse sections, dorsal up (K,L).

known regarding the roles of de novo DNA methylation during vertebrate lens development.

Zebrafish possess six “dnmt3 family” de novo methyltransferase genes, *dnmt3* – *dnmt8* (reviewed in Goll and Halpern 2011). This group includes both orthologs of mammalian *dnmt3A* and *dnmt3B* as well as fish-specific genes with no mammalian orthologs (Shimoda et al., 2005; Goll and Halpern, 2011; Campos et al., 2012). Despite our understanding of their evolutionary history and their likely functional importance in regulating de novo methylation during organ and tissue development, the tissue-specific expression of these dnmt3 family genes is largely unknown. This is particularly true in the vertebrate eye, where we have a poor understanding of how DNA methylation regulates retina and lens development. To facilitate functional studies of de novo methylation in the lens and retina, we have catalogued the distribution of dnmt3-family genes in the developing zebrafish eye over time. Our data demon-

strate that dnmt3-family genes are expressed in both unique and overlapping patterns, and in several ocular structures, supporting a model in which they likely play functional roles in regulating numerous aspects of normal eye development.

RESULTS

Phylogenetic Analysis of Zebrafish De Novo Methyltransferases

To examine the phylogenetic relationship among DNA methyltransferases in zebrafish and mammals, we constructed a phylogenetic tree using zebrafish, mouse, and human de novo and maintenance DNA methyltransferase proteins. Using dnmt2 RNA methyltransferase as an outgroup, de novo and maintenance methyltransferases cluster into two distinct clades, with all Dnmt1 orthologs in one and all Dnmt3 proteins in the other. Within the dnmt3 clade, dnmt3 and dnmt5 form a distinct branch with no mammalian ortholog,

while dnmt6 and dnmt8 closely cluster with mouse Dnmt3A, consistent with previous studies (Shimoda et al., 2005; Goll and Halpern, 2011; Campos et al., 2012). The rest of the de novo methyltransferase clade includes zebrafish dnmt4, dnmt7, and mouse Dnmt3B. Within this group, dnmt4 branches with mouse Dnmt3B, while dnmt7 does not (Fig. 1). Therefore, zebrafish dnmt3, dnmt5, and dnmt7 appear to be fish-specific with no obvious mammalian orthologs.

Expression of Zebrafish De Novo Methyltransferases

All dnmt3-family genes show ubiquitous expression throughout the anterior region of the embryo at 24 hr postfertilization (hpf), after which, expression patterns begin to resolve into distinct, spatially restricted domains. Within the developing eye, expression domains can be categorized into three general groups, with each group constituted by two dnmt3-family genes (summarized in Fig. 5).

CMZ expression: dnmt3 and dnmt5

At 24 hpf, *dnmt3* and *dnmt5* are expressed in the eye, as well as broadly throughout the embryonic head, and weakly in the posterior part of the embryo (Fig. 2A,F). Beginning at 36 hpf, and lasting through 72 hpf, transcripts of both genes are no longer detected throughout most of the embryo except for the ciliary marginal zone (CMZ) of the retina, where expression remains relatively high (Fig. 2B–D,G–I), and the optic tectum and pectoral fin. Transverse sections through the eyes highlight the CMZ expression of these two genes, and this is most pronounced at 48 hpf (Fig. 2K,L). At 4 days postfertilization (dpf), transcripts are still detected in the CMZ (Fig. 2E,J), but are no longer apparent in other parts of the eye or embryo. *dnmt3* and *dnmt5* transcripts are not detected within the lens.

CMZ and LE expression: dnmt4 and dnmt7

At 24 hpf, *dnmt4* and *dnmt7* expression resembles that of *dnmt3* and

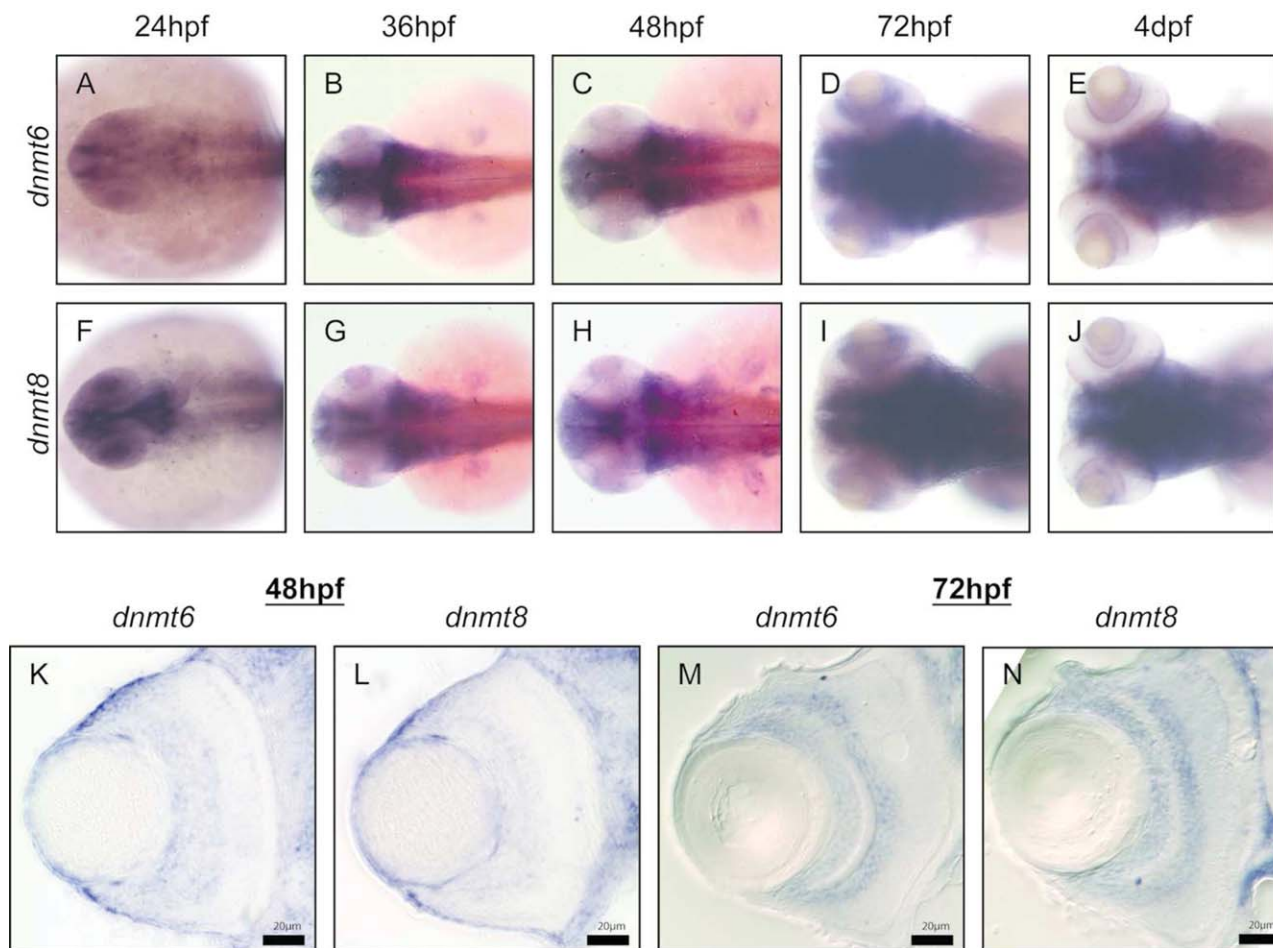


Fig. 4. *dnmt6* and *dnmt8* expression in lens epithelial (LE), ciliary marginal zone (CMZ), ganglion cell layer (GCL), and inner nuclear layer (INL). In situ hybridization shows similar expression patterns between *dnmt6* and *dnmt8*. **A,B,F,G:** At 24–36 hr postfertilization (hpf), both genes are expressed ubiquitously throughout the anterior part of embryo. **C,H,K,L:** At 48 hpf, expression is detected specifically in LE and CMZ, and weakly in the developing GCL and INL. **D,I,M,N:** At 72 hpf, expression is only detected within the GCL and INL. **E,J:** At 4 dpf, expression is faint in the GCL. **K–N:** Transverse sections, dorsal up.

dnmt5, where transcripts are observed broadly throughout the anterior part of the embryo (Fig. 3A,F). In the eye, both genes are expressed within the CMZ, and throughout the lens epithelium (Fig. 3K,L). From 36 hpf to 4 dpf, *dnmt4* is also expressed in the optic tectum, pharyngeal arches, and posterior border of the developing pectoral fins (Fig. 3B–E). From 36 hpf on, outside of the eye, *dnmt7* expression appears to be overlapping, but more diffuse than, that of *dnmt4* (Fig. 3G–J).

Inner Retina (GCL and INL) Expression: *dnmt6* and *dnmt8*

dnmt6 and *dnmt8* share a similar and overlapping expression pattern throughout embryogenesis, with both genes expressed ubiquitously at 24 hpf

(Fig. 4A,F). Expression remains broad throughout the anterior of the embryo outside of the eye up to 4 dpf (Fig. 4B–E,G–J). Within the eye, the expression appears to be dynamic. At 48 hpf, both genes are weakly expressed in the LE, CMZ, and developing ganglion cell layer (GCL) and inner nuclear layer (INL; Fig. 4C,H,K,L). However, at 72 hpf, expression is detected in only the GCL and the INL, and is no longer detected in the lens or CMZ (Fig. 4D,I,M,N).

DISCUSSION

Our analysis reveals dynamic and spatiotemporally restricted patterns of de novo DNA methyltransferase gene expression during zebrafish eye development. These distributions suggest distinct, but possibly redundant, functions for dnmt3 family proteins

during lens and retina development in vertebrates.

De Novo DNA Methyltransferases and Lens Development

The LE-specific expression of *dnmt4/dnmt7* (from 36 hpf onward) and *dnmt6/dnmt8* (at 48 hpf) suggest possible functions for the proteins they encode in 1) maintenance of the continuously proliferating LE cell population and/or 2) during the reprogramming of LE cells to become LF cells in the transition zone, a process that involves genome-wide changes in expression of numerous genes including activation of LF-specific genes and silencing of LE-specific genes (e.g., Cvekl and Duncan 2007; Wride, 2011; Hayes et al., 2012).

For example, delta-crystallin genes (*CRYD1/2*) in the embryonic chicken lens undergoes changes in methylation status and transcription levels as LE cells differentiate into LFs (Sullivan and Grainger, 1986; Sullivan et al., 1989), suggesting that LE and LF cells are epigenetically distinct cell populations. Interestingly, these de novo methyltransferase transcripts are distributed throughout the entire LE, a domain that is broader than that of the maintenance methyltransferase (*dnmt1*), which is expressed solely in the proliferative regions of the LE (Titte et al., 2011). Beyond the regulation of gene expression during the LE to LF transition, de novo DNA methylation machinery could cooperate with chromatin-remodeling enzymes in the LE cells to mediate chromatin compaction in preparation for DNA degradation that occurs during LF terminal

differentiation (Appleby and Modak, 1977; Bassnett et al., 2009; He et al., 2010). Lack of proper DNA degradation causes nuclear cataract in mouse (Nishimoto et al., 2003), and hypermethylation of a LF-specific α A-crystallin (*CRYAA*) contributes to age-related cataract in human (Zhou et al., 2012). These examples also suggest that defects in de novo DNA methylation may play a role in the progression of ocular diseases.

De Novo DNA Methyltransferases and Retina Development

All six members of the *dnmt3* gene family are expressed within the retina in two distinct domains: the CMZ and the GCL/INL layers. Because the CMZ is an area of active cell proliferation and a known niche for retinal stem

cells (Raymond et al., 2006), it is possible that these de novo DNA methyltransferases function in maintaining the proliferative properties of CMZ cells and/or in the reprogramming of retinal precursors destined to exit the CMZ and differentiate into retinal neurons. Consistent with this possibility, a recent study in *Xenopus* demonstrated that the transcripts for all core components of the polycomb repressive complex 2 (PRC2), a chromatin-remodeling complex that catalyzes histone H3 lysine 27 tri-methylation (H3K27me3), are also expressed within the CMZ (Aldiri et al., 2013). These data suggest that the CMZ is an area of highly active epigenetic modifications, and that de novo DNA methylation may cooperate with histone-modifying enzymes to facilitate stem cell maintenance or reprogramming during differentiation.

The distribution of *dnmt6* and *dnmt8* in the GCL and INL of the retina, as well as the absence of detectable *dnmt3* family gene expression in other regions of the retina suggest that GCL and INL cells may undergo additional epigenetic modification before they terminally differentiate. These two layers of the retina are also where 5-hydroxymethyl-cytosine (5-hmC), a mark of active cytosine demethylation (Pastor et al., 2013), is enriched (Almeida et al., 2012), and where *tet3*, a DNA demethylase, is expressed (P.S., unpublished observations, 2013). It will be of interest to determine the function of these enzymes during the specification, differentiation and maintenance of GCL and INL neurons.

Given our current understanding of the evolutionary history of genes in the *dnmt3* family and the overlapping distributions reported here (Fig. 5), it is very likely that the encoded proteins will have redundant roles

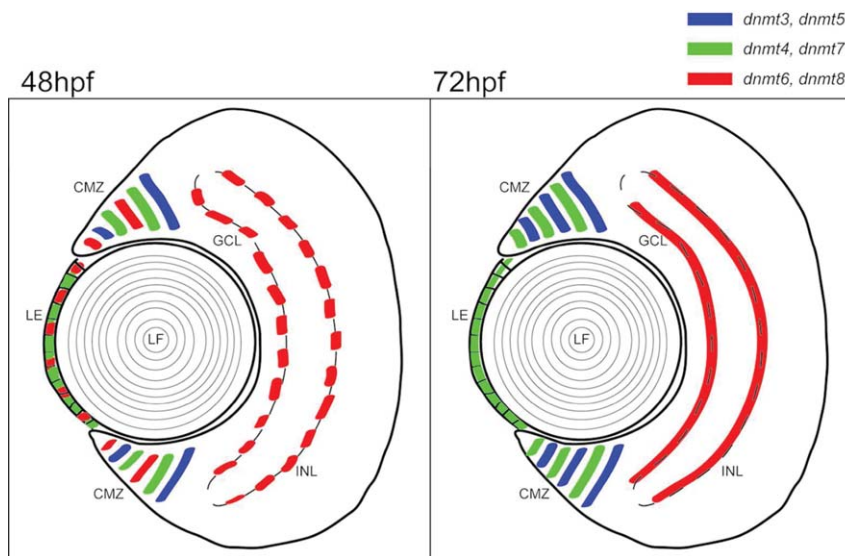


Fig. 5. Summary of *dnmt3* family gene expression in the developing zebrafish eye. Cartoon depicting a transverse section through an eye of a 48–72 hr postfertilization (hpf) embryo. Expression domains are marked in blue for *dnmt3* and *dnmt5*, green for *dnmt4* and *dnmt7*, and red for *dnmt6* and *dnmt8*. LE, lens epithelial cells; LF, lens fibers; CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer.

TABLE 1. Primers Used for Cloning of *dnmt3* Family Genes

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>dnmt3</i>	TGAATCAACACCATTTCACGG	AAAGCCCAACAAGACACTG
<i>dnmt4</i>	CGGCACCTTCAATCTCTGCTAT	GCCAGGGGAAAACACAATACTC
<i>dnmt5</i>	TCCCACTGTTATGGAGAATGGTTG	TCCTGAAAATCACAAGCGGC
<i>dnmt6</i>	TGGGGCAGGAAAAAAGTGAC	TCTCATAGGGCTCCGATTCAGG
<i>dnmt7</i>	AATCCTGATGACAAGTGCAGTCG	CAGCAAGTACCACATGACAGTTGG
<i>dnmt8</i>	ACACACACAGCGACCTAAGAGG	CACAGTGAGGGAACGGTTTACCTG

during zebrafish lens and retinal development, with genes that are most closely related phylogenetically sharing expression domains and possible functions. This overlap in expression also highlights potential difficulties in performing functional experiments due to the possibility of genetic redundancy within this group of genes. In zebrafish, efforts have been made to knockdown individual members of the dnmt3 family with modest success (e.g., Shimoda et al., 2005; Rai et al., 2010). Moving forward, functional analyses will likely necessitate the generation of combinatorial loss-of-function animals where more than one dnmt3-family gene is simultaneously perturbed. Given the amenability of zebrafish to TALEN- and CRISPR-based targeted mutagenesis (Huang et al., 2011; Cade et al., 2012; Bedell et al., 2012), it will not be difficult to generate mutations in each of these genes, and then generate double or even triple mutants to ascertain the roles of de novo DNA methylation during vertebrate lens and retina development.

EXPERIMENTAL PROCEDURES

Phylogenetic Analysis

All amino acid sequences were obtained from NCBI with the following accession numbers: AAI63893.1 (zebrafish dnmt1); NP_001018153.1 (zebrafish dnmt2); NP_571461.1 (zebrafish dnmt3); AAI24099.1 (zebrafish dnmt4); NP_001018315.1 (zebrafish dnmt5); AAI62582.1 (zebrafish dnmt6); AAI63546.1 (zebrafish dnmt7); NP_001018144.1 (zebrafish dnmt8); AAH53047.1 (mouse Dnmt1); NP_001258682.1 (mouse Dnmt3A); NP_001258673.1 (mouse Dnmt3B); AAI26228.1 (human DNMT1); AAH23612.1 (human DNMT3A); NP_008823.1 (human DNMT3B). Sequence alignment and phylogenetic tree construction were performed using Geneious bioinformatics suite (Biomatters) with default parameters.

Animal Husbandry

Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14/10 light/dark cycle and treated in accordance with the University of Texas at Austin pro-

visions governing animal research. Embryos were collected from natural breeding and incubated at 28.5°C in system water with phenothiourea (PTU) to inhibit pigmentation.

Cloning and Probe Synthesis

Reverse transcription - polymerase chain reaction (RT-PCR) was performed using cDNA from 48 hpf embryos and primers specific for each dnmt3-family gene (Table 1). Because all DNA methyltransferases in zebrafish contain a conserved catalytic domain at the 3' end of the gene, in situ probe target regions were specifically selected within 1kb of the most 5' end of the gene to maximize specificity. PCR products were ligated into pGEM-T-easy vector (Promega) and verified by Sanger sequencing. Plasmids containing the correct clones were linearized and used as templates to in vitro transcribe digoxigenin-labeled RNA probes (Roche).

In Situ Hybridization

Whole-mount in situ hybridization was performed essentially as described (Jowett and Lettice, 1994) with the following modification to allow probes to access the lens: (1) Full-length RNA probes were hydrolyzed with a mixture of 0.6 M sodium carbonate and 0.4 M sodium bicarbonate for 20 min. (2) Embryos older than 72 hpf were pretreated with 1 mg/ml Collagenase type IA before proteinase treatment step. Once the appropriate level of staining is achieved, embryos were re-fixed and embedded in tissue-freezing medium and cryosectioned at thickness of 12 µm.

Imaging

All images were obtained using Leica MZ-16F (for whole-mount embryos) and Leica DM-2500 (for sections) and processed using Adobe Photoshop and Illustrator CS5.

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