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Uhrf1 and Dnmt1 are required for development and maintenance of the zebrafish lens

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ABSTRACT

DNA methylation is one of the key mechanisms underlying the epigenetic regulation of gene expression. During DNA replication, the methylation pattern of the parent strand is maintained on the replicated strand through the action of Dnmt1 (DNA Methyltransferase 1). In mammals, Dnmt1 is recruited to hemimethylated replication foci by Uhrf1 (Ubiquitin-like, Containing PHD and RING Finger Domains 1). Here we show that Uhrf1 is required for DNA methylation *in vivo* during zebrafish embryogenesis. Due in part to the early embryonic lethality of *Dnmt1* and *Uhrf1* knockout mice, roles for these proteins during lens development have yet to be reported. We show that zebrafish mutants in *uhrf1* and *dnmt1* have defects in lens development and maintenance. *uhrf1* and *dnmt1* are expressed in the lens epithelium, and in the absence of Uhrf1 or of catalytically active Dnmt1, lens epithelial cells have altered gene expression and reduced proliferation in both mutant backgrounds. This is correlated with a wave of apoptosis in the epithelial layer, which is followed by apoptosis and unraveling of secondary lens fibers. Despite these disruptions in the lens fiber region, lens fibers express appropriate differentiation markers. The results of lens transplant experiments demonstrate that Uhrf1 and Dnmt1 functions are required lens-autonomously, but perhaps not cell-autonomously, during lens development in zebrafish. These data provide the first evidence that Uhrf1 and Dnmt1 function is required for vertebrate lens development and maintenance.

Introduction

In mammals and other vertebrates, the majority of CpG sequences in the genome are methylated at cytosine residues (Suzuki and Bird, 2008). The exception to this is CpG islands (CGIs), which are stretches of typically unmethylated CpG sequences which often correspond to gene transcription start sites (Illingworth and Bird, 2009). After replication, the DNA daughter strand must be methylated in accordance with the parent strand to maintain CpG methylation information in the daughter cell. Among the proteins required for "maintenance methylation" in mammals are DNA Methyltransferase 1 (Dnmt1), which catalyzes the methylation reaction (Bestor, 2000; Yoder et al., 1997), and Ubiquitin-like, Containing PHD and RING Finger Domains 1 (Uhrf1), which recruits Dnmt1 to hemimethylated replication foci (Bostick et al., 2007; Sharif et al., 2007). Hypermethylation of promoter CGIs (or of flanking regions known as "shores") correlates with reduced gene transcription, and a subset of these regions are differentially methylated according to tissue and cell type (Bird, 2002; Illingworth and Bird, 2009; Irizarry et al., 2009).

Studies identifying tissue-specific roles for DNA maintenance methylation during vertebrate embryonic development and organogenesis, such as in the eye, have been limited, owing largely to the early lethality of *Uhrf1* and *Dnmt1* knockout mice (Lei et al., 1996; Li et al., 1992; Muto et al., 2002; Sharif et al., 2007). Mouse conditional knockout studies have revealed an essential requirement for Dnmt1 in hematopoiesis (Broske et al., 2009; Trowbridge et al., 2009) and in neuronal differentiation and function (Fan et al., 2001; Feng et al., 2010; Golshani et al., 2005; Hutnick et al., 2009). Mouse $Dnmt1^{-/-}$ embryonic stem (ES) cells tolerate DNA hypomethylation until they are induced to differentiate (Lei et al., 1996; Li et al., 1992), and mouse $Dnmt1^{-/-}$ embryonic fibroblasts express inappropriate genes, including some specific for placental and germline lineages, before undergoing apoptosis (Jackson-Grusby et al., 2001). In *Xenopus*, reduction of Dnmt1 results in ectopic gene expression, and in p53-mediated apoptosis of ectodermal cells attempting to differentiate into mesodermal or neural tissues (Stancheva et al., 2001; Stancheva

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and Meehan, 2000); interestingly, a portion of the repressor function of Dnmt1 in this context was found to be independent of its role as a methyltransferase (Dunican et al., 2008). Morpholino knock-down of zebrafish *dnmt1* results in ~40% embryonic lethality; in surviving embryos, defective terminal differentiation was observed in the retina, exocrine pancreas, and intestine (Rai et al., 2006). A recent study of mutant zebrafish with catalytically inactive Dnmt1 demonstrated that Dnmt1 is required for survival of pancreatic acinar cells, and that it may play a role in pancreas cell fate decisions during regeneration (Anderson et al., 2009). Knockdown experiments in a human epidermal system have demonstrated that Dnmt1 and Uhrf1 are necessary to maintain proliferation of epidermal progenitors and to prevent premature differentiation (Sen et al., 2010). Uhrf1 has also been shown to function during liver development and regeneration (Sadler et al., 2005; Sadler et al., 2007).

Collectively, these studies suggest that DNA methylation is important for the development, differentiation, and survival of specific vertebrate organs and tissues, but much remains to be learned. With an interest in this process, and specifically the requirement for DNA methylation during lens development, we took advantage of zebrafish mutations in *uhrf1* (Amsterdam et al., 2004) and *dnmt1* (Anderson et al., 2009) to determine what role Uhrf1 and Dnmt1 play in DNA methylation during zebrafish embryogenesis and during lens development. Our results demonstrate that Uhrf1 facilitates DNA methylation *in vivo* during zebrafish embryonic development and that Uhrf1 and Dnmt1 are required for lens development and maintenance.

Materials and methods

Zebrafish maintenance

Zebrafish (*Danio rerio*) were maintained at 28.5 °C on a 14 h light/10 h dark cycle. Animals were treated in accordance with University of Texas at Austin provisions governing animal use and care. Mutant alleles used in this study were *uhrf1*^{hi3020}, *dnmt1*^{s872}, and *dnmt1*^{s904}. Unless otherwise stated, all experiments involving *dnmt1* mutants utilize the *dnmt1*^{s872} allele. Transgenic *Tg(beta actin2:mCherry-CAAX)* zebrafish were constructed as described (Kwan et al., 2007) using a construct generously provided by Kristen Kwan and Chi-Bin Chien, University of Utah, Salt Lake City.

RT-PCR

10–20 embryos were homogenized in Trizol Reagent (Invitrogen) using a 25-gauge needle and syringe. Total RNA was purified by chloroform extraction and isopropanol precipitation. Using 500 ng of total RNA, cDNA was synthesized with an iScript cDNA synthesis kit (BioRad). PCR was performed using 1.25 μL of the resulting cDNA. Primer sequences are available upon request.

Antibody generation

Two rabbits were immunized with a KLH-conjugated peptide derived from amino acids 222–240 of zebrafish Uhrf1 (DDPKERGYWYDAEIQRKRE; Open Biosystems). Rabbits were immunized with 0.25 mg of peptide emulsified with Freund's complete adjuvant and boosted at Days 14, 42, 56 and 113 with 0.10 mg peptide emulsified with Freund's incomplete. Animals were euthanized, bled and serum isolated. Anti-Uhrf1 was purified from serum using repeat affinity purification.

Western blots

Ten embryos were collected at 5 days-post-fertilization (dpf) and homogenized in 0.1% Triton X100 and protease inhibitors (Roche) in PBS. Samples were mixed with NuPAGE Sample Reducing Agent and NuPAGE LDS sample buffer (Invitrogen), heated at 70 °C for 10 min, and then centrifuged at 13,000 rpm for 10 min. Samples were separated on a NuPAGE 7% Tris–Acetate Gel with Tris–Acetate SDS Running Buffer (Invitrogen). Proteins were transferred to nitrocellulose and the membrane was blocked with 5% milk/0.2% Tween-20 in TBS for 3 h at RT. Blots were probed overnight at 4 °C with anti-Uhrf1 (1:100) and anti-Hdac1 (1:4000; Abcam). The membrane was washed 4× for 30 min in TBS/0.1% Tween-20 (TBST) and exposed to anti-rabbit-HRP secondary antibody (1:10,000; Jackson ImmunoResearch) for 1–2 h at RT, washed in TBST and developed using an ECL Detection/Blocking Agent (Amersham Biosciences), and CL-XPosure Film (Thermo Science — Pierce).

Riboprobes and in situ hybridization

Hybridizations using digoxigenin labeled antisense RNA probes were performed essentially as described (Jowett and Lettice, 1994), except that embryos over 2 dpf were pre-incubated with 1 mg/mL Collagenase type 1A (Sigma, C9891) to allow probe entry though the lens capsule. A cDNA clone encoding *dnmt1* (clone # cb983) was purchased from ZIRC (Eugene, OR), *uhrf1* was cloned from 24 hpf cDNA and ligated into pGEM-T Easy, and *tgfB3* was cloned from cDNA derived from 1 to 4 dpf embryos and ligated into pCS2+ (cloning details available upon request).

Histology and transmission electron microscopy (TEM)

Histology and TEM were performed as described in Lee and Gross (2007) and Nuckels and Gross (2007).

Immunohistochemistry

Immunohistochemistry was performed as described in Uribe and Gross (2007) except for anti-Lengsin staining, where Harding et al. (2008) was followed, and anti-Crystallin AlphaA staining, where Shi et al. (2006) was followed. The following antibodies and dilutions were used: red/green cones (zpr1; 1:200), rods (zpr3; 1:200), ganglion cells (zn8; 1:100), amacrine cells (5e11; 1:100, kindly provided by Jim Fadool), Lengsin (1:500; (Harding et al., 2008), kindly provided by David Hyde), Crystallin AlphaA (1:500; (Shi et al., 2006), provided by David Hyde), aquaporin 0 (1:500; Chemicon ab3071), phosphohistone H3 (1:200; Millipore), Goat anti-mouse and anti-rabbit Cy3 secondary (1:200; Jackson ImmunoResearch) and nuclei were counterstained with Sytox Green (1:10,000; Molecular Probes). mCherry was visualized using anti-dsRed (Clontech (632496) 1:150). Alexa Fluor-555 Phalloidin (1:50, Molecular Probes) was used to visualize F-actin. Imaging was performed on a Zeiss LSM Pascal laser scanning confocal microscope. 3–5 1 μm optical sections were collected and projected using Zeiss software.

BrdU assays

BrdU incorporation assays were performed as in (Nuckels et al., 2009). Anti-BrdU antibody (Abcam) was used to detect BrdU^{+} nuclei on cryosections.

TUNEL assays

TUNEL assays were performed on cryosections using a TMR-Red labeled *in situ* cell death detection kit (Roche) per manufacturer's instructions and were imaged by confocal microscopy.

Methylation assays

The SouthWestern Blot was based on MacKay et al. (2007). 5 dpf embryos were homogenized in extraction buffer (10 mM Tris pH 8, 100 mM EDTA pH 8, 0.5% SDS) and sheared with a 25-gauge needle. 200 µg/mL proteinase K was then added and the homogenate was incubated at 55 °C overnight. This was followed by Phenol-Chloroform extraction, ethanol precipitation, incubation with RNAse at 5 µg/mL, a second phenol-chloroform extraction, ethanol precipitation and final resuspension in ddH_2O. DNA concentrations were measured by Nanodrop and equal quantities of DNA were loaded onto nylon membranes (Amersham Hybond N+, GE Healthcare) by a slot blotter. DNA was crosslinked to the membrane using a UV stratalinker 1800 (Stratagene). The membrane was blocked with 3% milk/TBST (Block) and incubated with mouse anti-5-methylcytosine (Calbiochem) at 2 µg/ml in 3% milk/PBST overnight at 4 °C. The membrane was washed four times in Block and incubated with a horseradish peroxidase-conjugated anti mouse antibody (Jackson ImmunoResearch) diluted 1:3333 in Block for 1.5 h at RT. The membrane was washed 4× in Block, rinsed in TBST, then overlaid with chemiluminescence reagent and exposed to X-ray film. The film was developed, and analysis of the blot was performed using Adobe Photoshop. Band densitometry values relative to wild-type are compared in Fig. 1D using the two-tailed *t*-test function of Microsoft Excel.

For enzymatic analysis of DNA methylation, genomic DNA was isolated using a Genomic DNA Extraction Kit (Zymo Research). 750 ng of genomic DNA was digested with either HpaII, MspI (New England Biolabs), or a buffer-only control overnight, separated on a 1% agarose gel containing ethidium bromide and imaged.

Mosaic lens analyses

Shield stage transplants were performed as described (Eberhart et al., 2006). Donor embryos were injected with Alexa Fluor 488 dextran (10 kDa) (Molecular Probes) in 0.2 M KCL. At 6 hpf cells were removed from one donor embryo and placed into each of three host embryos, targeting the lens-fated region immediately adjacent to the oral ectoderm precursors. At 34 hpf, the percent contribution was determined in each host as the estimated amount of fluorescent cells present by volume in the lens. At 5 dpf, donor and host embryos were phenotyped and imaged before being prepped for histology. Other shield stage transplants were performed with *Tg(beta actin2:mCherry-CAAX)* as donors and *uhrf1* mutants and siblings as hosts, and immunohistochemistry was performed at 4 dpf with an anti-dsRed antibody to detect *beta actin2*:mCherry-expressing donor cells in the host lens.

Larval lens transplants

Lens transplants were performed at 37 hpf essentially as described in (Yamamoto and Jeffery, 2002).

Results

The uhrf1^{hi3020} allele is either null or severely hypomorphic

The *uhrf1*^{hi3020} mutant was identified in an insertional mutagenesis screen for morphological defects in eye formation (Amsterdam et al., 2004; Gross et al., 2005). The proviral insert in *uhrf1*^{hi3020} mutants is located upstream of exon 2, the first coding exon of *uhrf1* (Fig. S1A). To determine the effect of the proviral insertion on expression of *uhrf1*, RT-PCR was performed on RNA extracted from wild-type, *uhrf1* mutants, and phenotypically wild-type sibling embryos (Fig. S1B). No transcripts were detected in *uhrf1* mutants when assayed by several different primer sets. To analyze Uhrf1 levels, a rabbit polyclonal antibody was raised against zebrafish Uhrf1. Anti-Uhrf1 antibodies recognized a single band of expected molecular

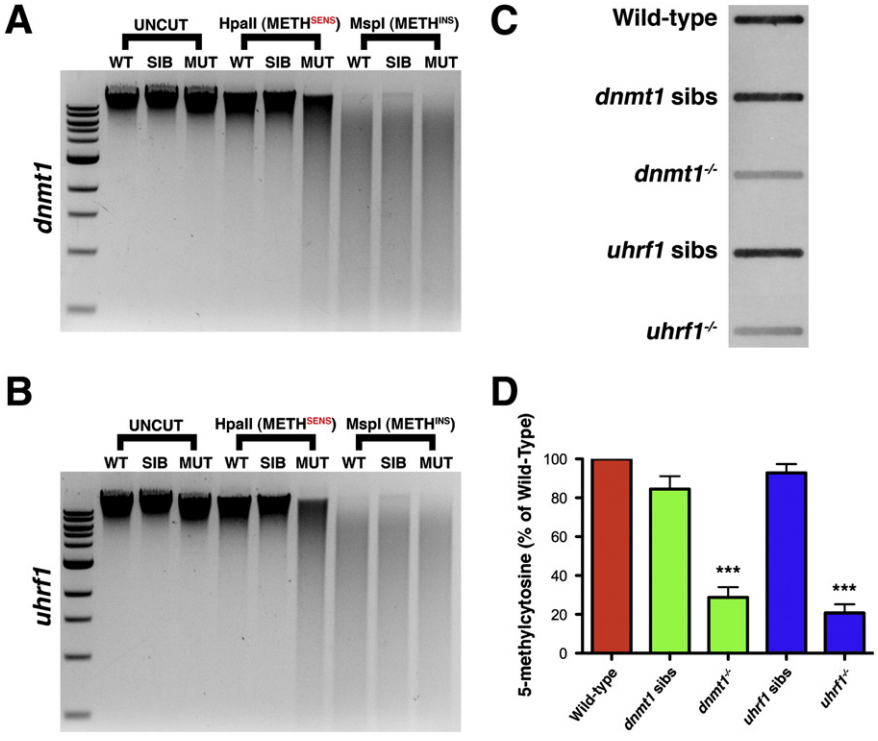


Fig. 1. *uhrf1* mutants possess hypomethylated genomic DNA. Genomic DNA methylation assay in which 750 ng of genomic DNA is digested by a methylation-sensitive enzyme (HpaII), a methylation-insensitive enzyme (MspI), or a mock digestion with no enzyme present. (A) Genomic DNA isolated from Wild-type AB, *dnmt1* siblings or *dnmt1* mutant embryos demonstrates the efficacy of the assay. Genomic DNA is digested by the methylation-sensitive HpaII to a greater extent in the mutant embryos than in siblings or wild-types. (B) Genomic DNA isolated from Wild-type AB, *uhrf1* siblings or *uhrf1* mutant embryos; *uhrf1* mutant DNA is digested by the methylation sensitive HpaII to a greater extent than sibling or wild-type DNA. (C) SouthWestern assay to quantify 5-methylcytosine levels on genomic DNA. 2 µg of genomic DNA is extracted from the indicated group of embryos, loaded onto a membrane and probed with anti-5-methylcytosine antibody. (D) Quantification of 5-methylcytosine levels ($n=8$ trials; *** $p<0.00002$). Error bars represent s.e.m.

mass (~85 kDa) by Western blot in wild-type samples, and this band was absent in *uhrf1* mutants (Fig. S1C). From these data we consider the *uhrf1*^{hi3020} allele to be either null or severely hypomorphic.

DNA methylation in zebrafish requires uhrf1

Uhrf1 recruits Dnmt1 to hemimethylated DNA (Bostick et al., 2007; Sharif et al., 2007), which facilitates maintenance methylation of cytosine residues after DNA replication. The absence of either Uhrf1 or Dnmt1 in mouse embryos or embryonic stem cells results in severely hypomethylated genomic DNA (Bostick et al., 2007; Jackson et al., 2004; Lei et al., 1996; Li et al., 1992; Sharif et al., 2007). Similarly, the zebrafish mutant *dnmt1*^{s872} (in which Dnmt1 contains a point mutation expected to render the methyltransferase domain catalytically inactive) has reduced global methylation of genomic DNA (Anderson et al., 2009; Goll et al., 2009). To determine whether Uhrf1 is also required for DNA methylation in zebrafish embryos, two genomic DNA methylation assays were performed. In the first assay, genomic DNA from 5 dpf embryos was digested by either the methylation-sensitive restriction enzyme HpaII or its methylation-insensitive isochizomer MspI. While the methylation-insensitive restriction enzyme MspI digested DNA of all genotypes to an equal degree, methylation-sensitive HpaII digested *dnmt1* and *uhrf1* mutant genomic DNA to a greater degree than wild-type genomic DNA (Figs. 1A,B). To quantify differences in methylated cytosine levels, a second assay was performed in which genomic DNA from 5 dpf embryos was loaded onto a membrane using a slot blotter and probed with an antibody against 5-methylcytosine (SouthWestern assay; (MacKay et al., 2007)) (Fig. 1C). Methylation levels in phenotypically wild-type *dnmt1* and *uhrf1* sibling groups were not significantly different from wild-type levels (Fig. 1D). However, genomic DNA from *dnmt1* or *uhrf1* mutants was hypomethylated, with levels of 5-methylcytosine at 29% (±15% s.d.) and 21% (±13% s.d.) of wild-type, respectively (Fig. 1D). These data demonstrate that Uhrf1 function is required in zebrafish for DNA methylation, indicating that Uhrf1's role in DNA methylation is likely conserved throughout vertebrates. Moreover, the fact that relative methylation of DNA between *dnmt1* and *uhrf1* mutants is not significantly different is consistent with a functional interaction between Uhrf1 and Dnmt1 during zebrafish development.

Lens morphology is abnormal in uhrf1 mutants

The vertebrate lens is a transparent sphere of tightly packed lens fibers which acts to focus light onto the retina. Through the life of the organism, proliferating epithelial cells at the anterior periphery of the lens undergo terminal differentiation to become lens fibers (Lovicu and Robinson, 2004). In this process, epithelial cells exit the cell cycle (Griep, 2006), elongate, express genes required for lens fiber differentiation, and finally degrade their light-scattering organelles

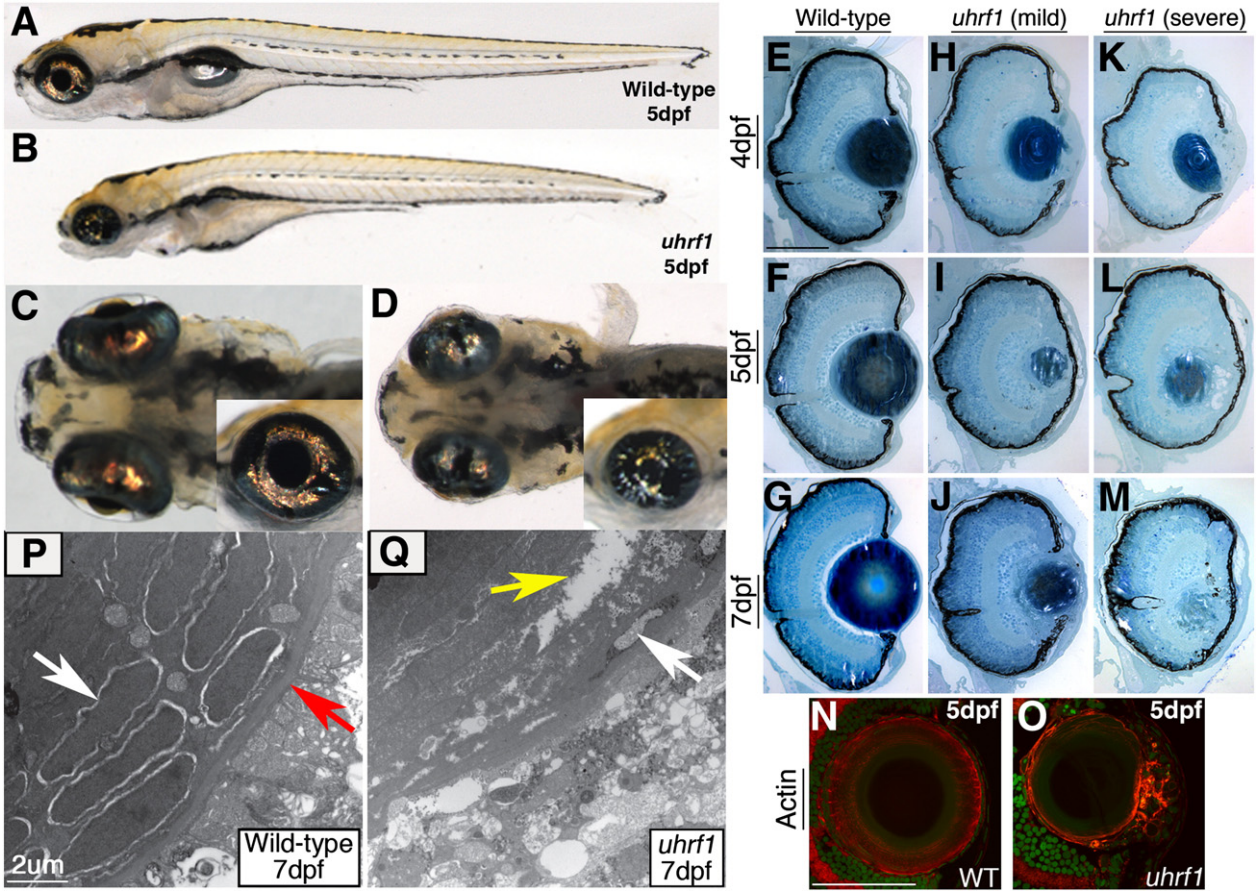


Fig. 2. *uhrf1* mutants possess abnormal lenses and cataracts. (A,C) Wild-type sibling and (B,D) *uhrf1* mutant embryos at 5 dpf. Mutants possess defects in lens formation and cataracts. Pupils are smaller (inset in C,D) and lenses are malformed. (E–M) Transverse histology from wild-type, (H–J) "mild" and (K–M) "severe" *uhrf1* mutants at (E,H,K) 4 dpf, (F,I,L) 5 dpf, and (G,J,M) 7 dpf. (H–J) Mild phenotypes include smaller lenses, anterior opacifications and some unraveling of lens fibers at the anterior and/or posterior of the lens. (K–M) Severe phenotypes include substantial opacifications throughout the lens, lens dysplasias, peripheral fiber unraveling from the core of differentiated fibers and lens degeneration. (N,O) Wild-type (N) and mild *uhrf1* mutant (O) cryosections stained for F-actin. The *uhrf1* anterior lenses contain disorganized, nucleated cells often in excess of the normal wild-type lens epithelial monolayer. (P,Q) TEM analyses of the lens sub-equatorial region in 7 dpf wild-type embryos (P) reveal early differentiating fibers (which still contain nuclei, white arrow) surrounded by the lens capsule (red arrow). In *uhrf1* mutants (Q) the lens capsule is absent, and apoptotic lens fiber nuclei (white arrow) and intracellular gaps or tears in the mutant fibers (yellow arrow) are observed. Scale bars are 80 μm.

(Bassnett and Beebe, 2004). Eye development appears normal in *uhrf1* mutants until 4–5 dpf, at which point they develop morphologically abnormal lenses and cataracts (Figs. 2A–D,N,O). At 3 dpf, *uhrf1* mutant eyes are phenotypically indistinguishable from wild-type embryos (Figs. S2A, B). The *uhrf1* lens phenotype is homozygous recessive and the mutation is embryonic lethal by 10 dpf. Heterozygotes have no ocular phenotype.

The *uhrf1* mutation is fully penetrant, but the severity of the lens phenotype is variable. Mildly affected *uhrf1* mutant lenses (~50%) show anterior lens opacifications (Figs. 2H–J). When 5 dpf mild mutant lenses are stained for F-actin, the anterior lens contains disorganized, nucleated cells often in excess of the normal lens epithelial monolayer (Figs. 2N,O). In more severely affected mutants (~50%) the lens often ruptures through the lens capsule and becomes ectopically localized within the retina (Figs. 2L,M), or it ruptures through the cornea and remains tethered to the anterior of the eye (data not shown). In these severe mutant lenses, secondary fibers appear to unravel from the primary core of the lens, and the fiber cells are often disorganized and vacuolated (Figs. 2K–M). TEM analyses of the lens sub-equatorial region in 7 dpf wild-type embryos reveal early differentiating fibers (which still contain nuclei) surrounded by the lens capsule (Fig. 2P). In *uhrf1* mutants, severe ultrastructural defects are observed in which fiber morphologies are abnormal, apoptotic nuclei are present in the region of differentiating fibers, and the lens capsule is absent (Fig. 2Q). Differentiating fibers of all *uhrf1* mutant lenses examined also possessed intracellular gaps or tears (Fig. 2Q and data not shown).

The uhrf1 lens phenotype is phenocopied by mutations in dnmt1

Uhrf1 recruits Dnmt1 to hemimethylated DNA (Bostick et al., 2007; Sharif et al., 2007), and this facilitates CpG maintenance methylation after DNA replication. Therefore, if defective DNA methylation leads to the lens defects observed in *uhrf1* mutants, one would expect similar lens defects in *dnmt1* mutants. Indeed, this is the case; *dnmt1* mutants also possess abnormal lenses and cataracts (Fig. 3B). As in *uhrf1* mutants, the anterior region of mild 5 dpf *dnmt1* mutant lenses contains many disorganized nucleated cells, which do not resemble the cuboidal structure of the wild-type lens epithelial monolayer (Figs. 3A′,B′). Histological examination reveals unraveled and disorganized fibers similar to those observed in *uhrf1* mutants (Figs. 3D,F). As in *uhrf1* mutants, the lenses of *dnmt1* mutants also often rupture through the lens capsule and are found either within the retina or emerging from the cornea (Fig. 3D). Also like the *uhrf1* mutants, the *dnmt1* lens phenotype, though fully penetrant, varies in severity between mild and severe. There is no observable phenotype in *dnmt1* mutants before 4 dpf (Fig. S2C).

To further explore the role of Dnmt1 in zebrafish lens formation, a second *dnmt1* allele was examined: $dnmt1^{s904}$, in which a frame-shift mutation leads to predicted protein truncation and total loss of the C-terminal CXXC, BAH1, BAH2 and DNA methyltransferase domains (Anderson et al., 2009). $dnmt1^{s904}$ also phenocopied the *uhrf1* disrupted lens phenotype (Fig. S3). All further experiments were carried out in the $dnmt1^{s872}$ allele.

Given the similarity in lens phenotype between *uhrf1* and *dnmt1* mutants, and the fact that the proteins functionally interact in mammalian systems (Achour et al., 2008; Bostick et al., 2007; Sharif et al., 2007), $uhrf1^{-/-}$; $dnmt1^{-/-}$ double mutants were generated and analyzed for lens defects to genetically test whether *uhrf1* and *dnmt1* also interact during zebrafish lens development (Fig. 4). There is no overt eye phenotype in $uhrf1^{+/-}$; $dnmt1^{+/-}$ compound heterozygous embryos (Fig. 4B). Body morphology in ~50% of $uhrf1^{-/-}$; $dnmt1^{-/-}$ double mutants was much more severe than in single mutants. These embryos were edemic with morphological abnormalities in axial development (data not shown), suggesting that Uhrf1 and Dnmt1

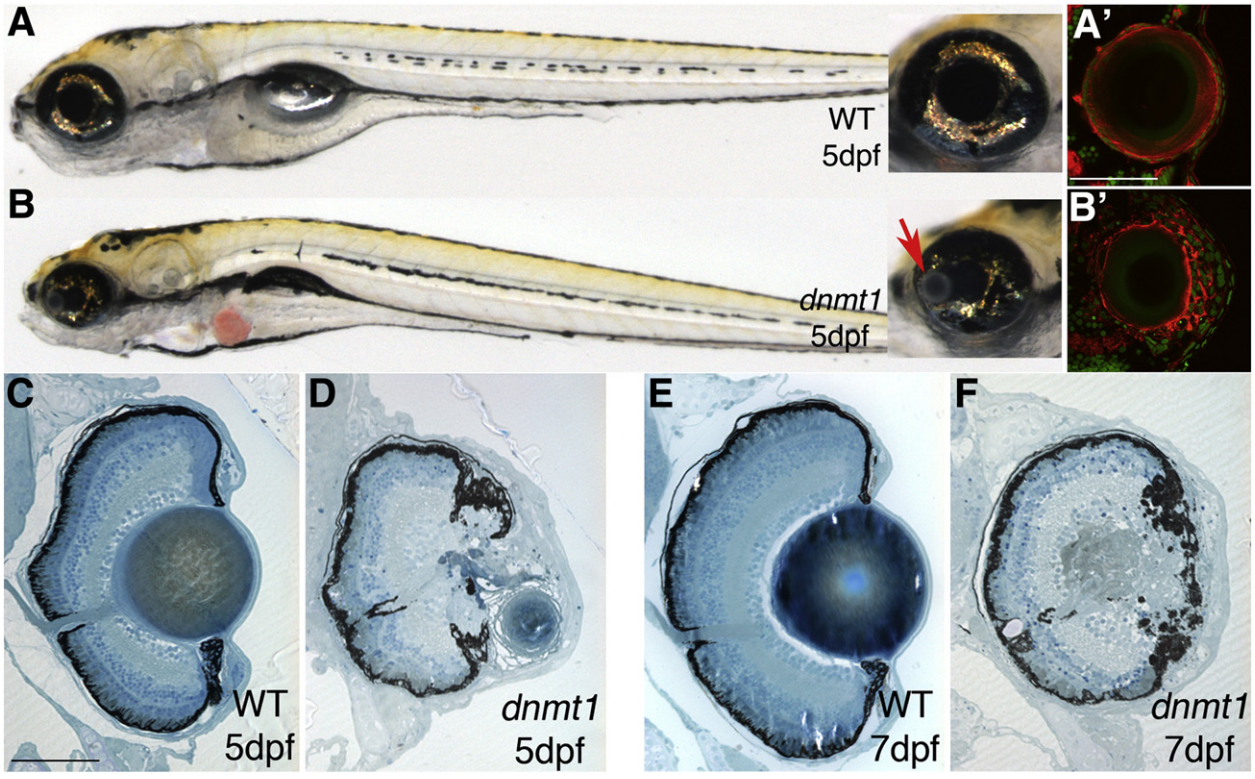


Fig. 3. *dnmt1* mutants also possess abnormal lenses and cataracts. (A) Wild-type and (B) *dnmt1* mutant embryos at 5 dpf. Mutants display obvious lens defects and in the more severely affected embryos lens dysplasias are observed (arrow in B). F-actin staining of wild-type (A′) and mild *dnmt1* (B′) eye cryosections show that the anterior region of *dnmt1* mutant lenses contain many disorganized nucleated cells that do not resemble the cuboidal structure of the wild-type lens epithelial monolayer. (C–F) Transverse histology from (C,E) wild-type and (D,F) *dnmt1* mutant embryos at (C,D) 5 dpf and (E,F) 7 dpf. Mutants display lens dysplasias, peripheral fiber unraveling from the core of differentiated fibers and lens degeneration. Scale bars are 80 μm.

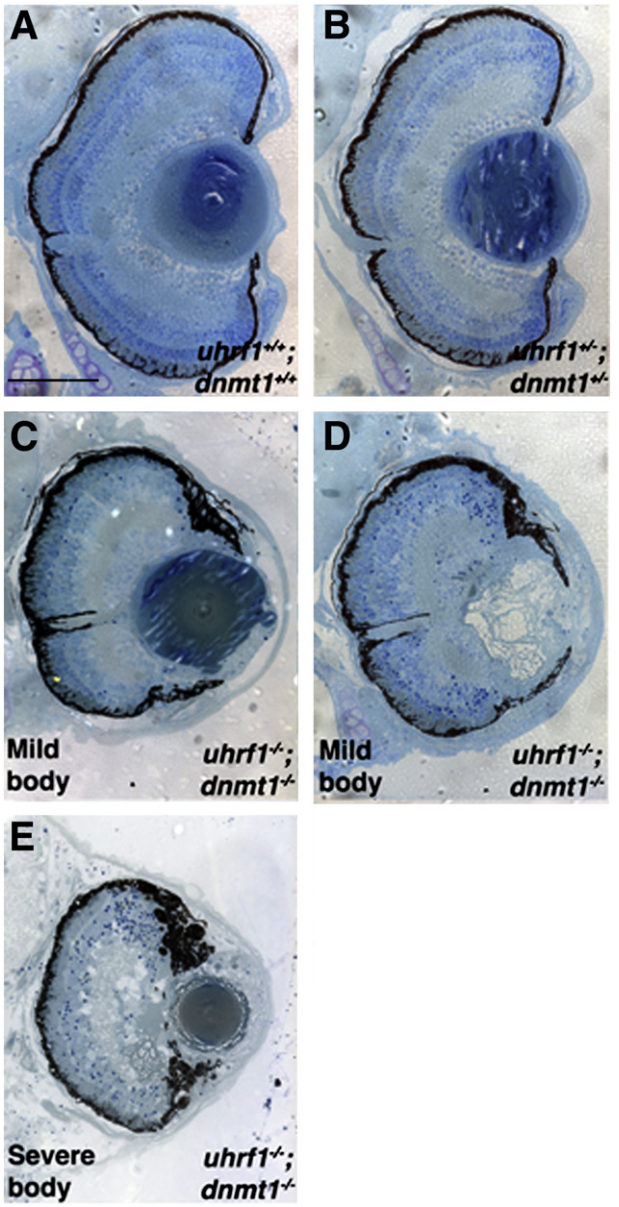


Fig. 4. Genetic interaction between *uhrf1* and *dnmt1* during lens development. (A) 5 dpf histology from *uhrf1*$^{+/+}$; *dnmt1*$^{+/+}$, (B) *uhrf1*$^{+/-}$; *dnmt1*$^{+/-}$, and (C–E) *uhrf1*$^{-/-}$; *dnmt1*$^{-/-}$ embryos. No compound heterozygous eye phenotype is observed (B). Lens defects in *uhrf1*$^{-/-}$; *dnmt1*$^{-/-}$ double mutants are no more severe than those in either of the single mutants, and range from mild (C) to severe (D). *uhrf1*$^{-/-}$; *dnmt1*$^{-/-}$ double mutants with severe body morphology (E) do not have more severe lens phenotypes. Scale bars are 80 μm.

may have separate roles outside of the lens. However, consistent with a functional interaction between Uhrf1 and Dnmt1 during lens development, all *uhrf1*$^{-/-}$; *dnmt1*$^{-/-}$ double mutants have similar lens phenotypes to *uhrf1* and *dnmt1* single mutants, and are not more severely affected (Figs. 4C–E). As in the single mutants, the severity of *uhrf1*$^{-/-}$; *dnmt1*$^{-/-}$ lens defects between embryos varies from mild (Fig. 4C) to severe (Figs. 4D,E). These data provide genetic support for a model in which Uhrf1 and Dnmt1 interact during lens development in zebrafish.

Differentiated retinal cell types are present in uhrf1 and dnmt1 mutants

While Dnmt1- and Uhrf1-deficient mouse embryos are embryonic lethal before later aspects of eye formation can be studied (Lei et al., 1996; Li et al., 1992; Muto et al., 2002; Sharif et al., 2007), morpholino-mediated knock-down of *dnmt1* in zebrafish results in retinal defects (Rai et al., 2006). Specifically, the authors observed defective lamination of the retina as well as loss of dorsal retinal pigmented epithelium (RPE) and no expression of an mRNA marker of photoreceptor and RPE terminal differentiation. However, no lens phenotype was reported at 4 dpf. At 5 dpf, the present study shows that *uhrf1* and *dnmt1* mutants are microphthalmic and possess defects in lens formation, but the laminar organization of their retinas appears largely normal, and the RPE remains intact (Figs. 2 and 3). Given these differences between mutant and morpholino-induced ocular phenotypes, immunohistochemical analyses were performed to better assess retinal neuron differentiation and laminar organization of the retina in *uhrf1* and *dnmt1* mutants.

At 5 dpf, differentiated retinal ganglion cells, amacrine cells, red/green cones, and rods were all present and in appropriate laminar positions in *uhrf1* and *dnmt1* mutant retinas (Fig. S4). Despite correct localization, both red/green cones and rods have a distorted morphology in *uhrf1* and *dnmt1* mutant retinas (Figs. S4H′,I′,K′,L′), the severity of which correlated with the severity of lens phenotype. Therefore, it appears that zygotic mutations in *uhrf1* and *dnmt1* are less disruptive to retinal neuron differentiation than injection of a translation-blocking morpholino targeting *dnmt1*, at least through 5 dpf.

This difference in retinal phenotype may be explained by the expected time at which Dnmt1 function is lost in the two systems. Maternally provided Dnmt1 transcript or protein is believed to account for Dnmt1 activity in *dnmt1* mutant embryos which remains through the end of 1 dpf (Goll et al., 2009), while a translation-blocking morpholino would be expected to knock down expression of both maternal and zygotic Dnmt1 much earlier in development. Similarly, the fact that no lens phenotype was observed in 4 dpf *dnmt1* morphant embryos may be explained by the steady increase in Dnmt1 expression that would be expected as the morpholino is titrated out over time.

uhrf1 and dnmt1 are expressed in proliferative regions of the lens and retina

uhrf1 and *dnmt1* have previously been shown to be expressed in the zebrafish eye (Rai et al., 2006; Sadler et al., 2007; Thisse et al., 2001; Thisse and Thisse, 2004), but precise expression domains therein have not been reported. *In situ* hybridizations of *uhrf1* and *dnmt1* in wild-type embryos demonstrate that both genes are expressed in the lens and retina during the time of mutant phenotype onset (4 and 5 dpf) (Figs. 5 and S5). Both genes are also expressed in the lens and retina earlier in development (data not shown).

At 4 and 5 dpf, *uhrf1* and *dnmt1* are expressed in the continually proliferative ciliary marginal zones (CMZs) of the retina, as well as in a ring of cells in the lens epithelium consistent with the proliferative germinative zone (Greiling et al., 2010) (Figs. 5B–D,F–H and S5B,C,E,F). This expression pattern is also consistent with a likely role of Uhrf1 and Dnmt1 in maintenance methylation, which occurs in conjunction with DNA replication, as well as with their established expression domains in proliferating cells (including adult somatic stem and progenitor cells) in other systems (Hopfner et al., 2000; Suetake et al., 2001; Trowbridge and Orkin, 2010). Consistent with our genetic interaction data, the distribution of *dnmt1* transcript is also remarkably similar to that of *uhrf1*. These results indicate that both *uhrf1* and *dnmt1* are normally expressed in the lens during the time of the disrupted lens phenotype in *uhrf1* and *dnmt1* mutants.

The lens epithelium is affected in urhf1 and dnmt1 mutants

The lens is made up of two cell types: lens epithelial cells and lens fibers (Lovicu and Robinson, 2004). Because *uhrf1* and *dnmt1* are normally expressed in a subset of lens epithelial cells at the time of

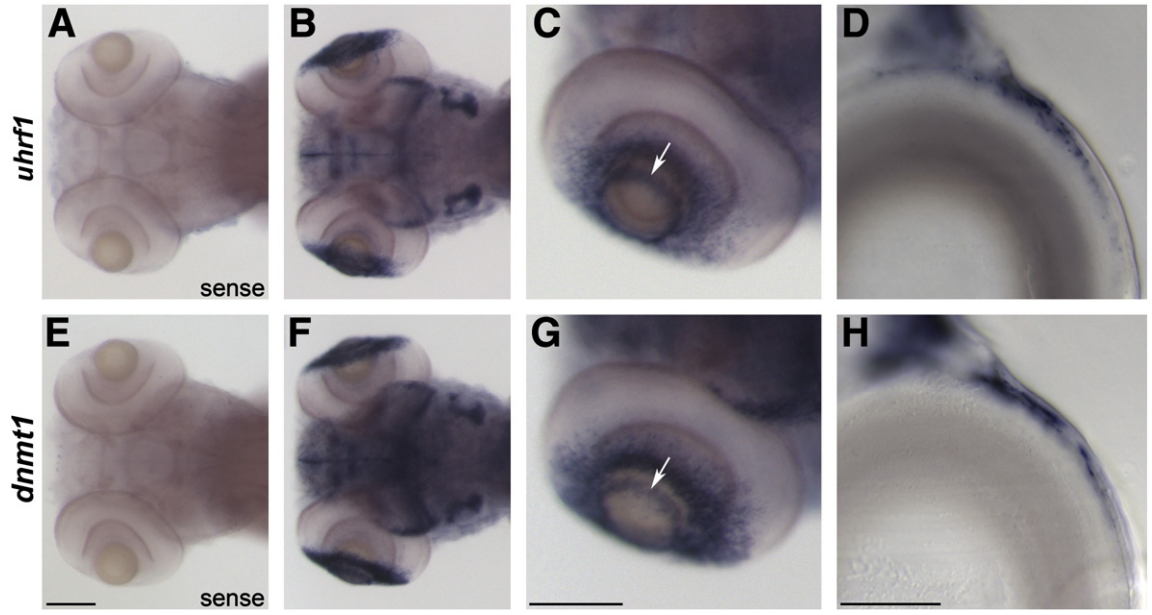


Fig. 5. *uhrf1* and *dnmt1* are expressed in proliferative regions of the lens and retina at the time of phenotype onset. *In situ* hybridization of *uhrf1* sense (A) and antisense (B–D) and *dnmt1* sense (E) and antisense (F–H) riboprobe on wild-type embryos at 4 dpf. No expression was detected in sense controls (A,E). Dorsal (B,F) and lateral (C,G) whole mount images demonstrate that *uhrf1* and *dnmt1* transcripts are expressed in both the ciliary marginal zone of the retina and in the proliferative germinative zone of the lens epithelium (which resembles a ring around the anterior lens when viewed in whole mount, arrows in C and G). Sectioned images of the lens (D, H) confirm the restriction of *uhrf1* and *dnmt1* expression to distinct regions of the epithelium (anterior is right, dorsal is up). Scale bars in A–C and E–G are 100 μm, and scale bars in D and H are 20 μm.

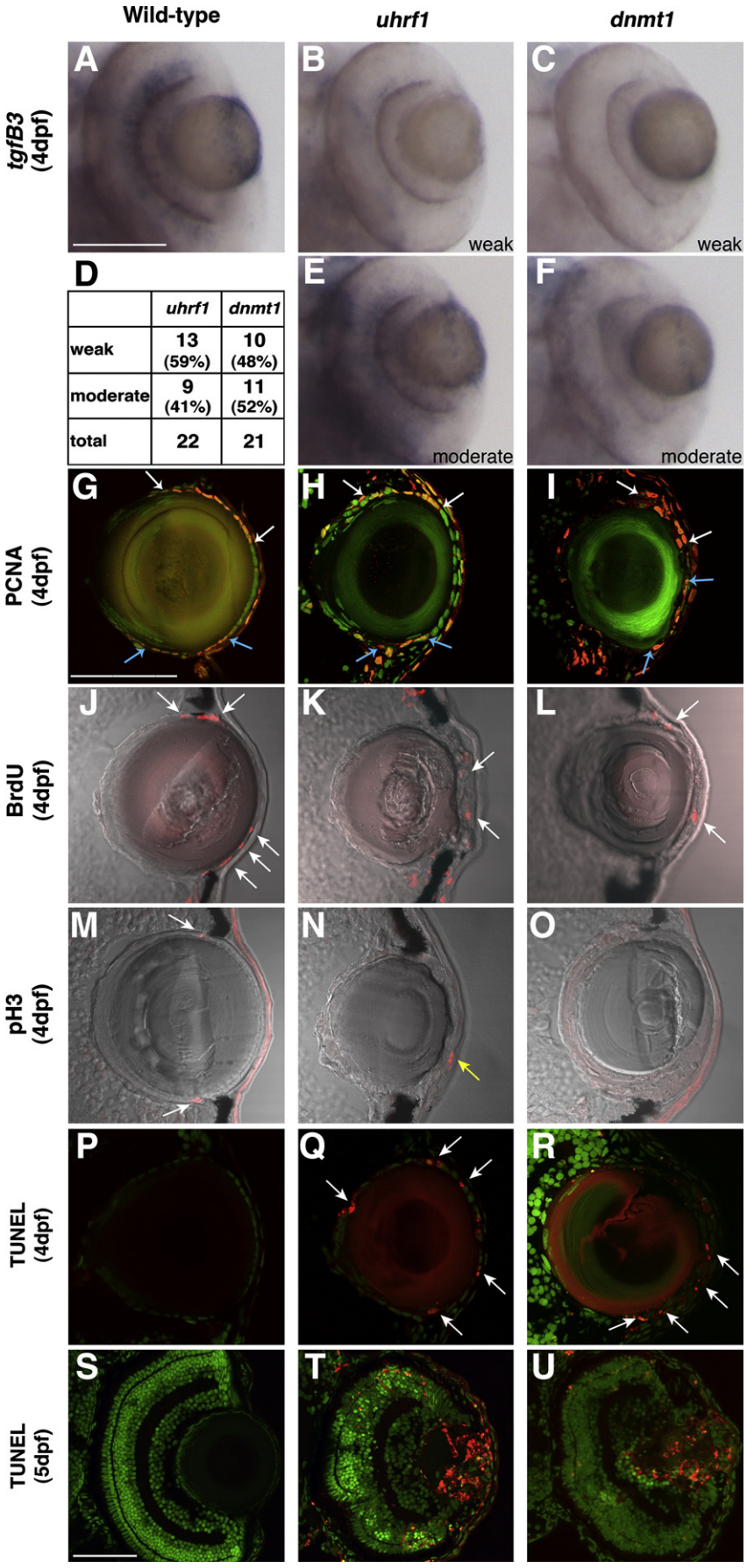
phenotypic onset in mutant embryos (Figs. 5 and S5), we sought to determine whether epithelial marker gene expression was affected. Members of the TGF-β family are expressed in the lens (Gordon-Thomson et al., 1998), and *tgfB3* serves as a lens epithelial marker in zebrafish (Cheah et al., 2005)(Fig. 6A). Compared to wild-type siblings, staining was essentially absent in approximately 50% of *uhrf1* and *dnmt1* mutant embryos (defined as 'weak'; Fig. 6B–D), and it was much reduced in intensity in the remainder of mutant embryos (defined as 'moderate' Fig. 6D–F).

Although *in situ* hybridization is not a quantitive assay, the essential loss of lens staining in approximately half the mutant embryos, and the reduction in staining in the remainder suggests that expression of *tgfb3* is reduced in *uhrf1* and *dnmt1* mutant lenses.

The bulk of the vertebrate lens is made up of lens fibers, which continue to accumulate throughout the life of the organism due to the proliferation of lens epithelial cells (Lovicu and Robinson, 2004), which exit the cell cycle and differentiate (Griep, 2006). Uhrf1 and Dnmt1 have been implicated in cell cycle regulation in other contexts (Arima et al., 2004; Chen et al., 2007; Jeanblanc et al., 2005), and thus, cell cycle defects are a potential mechanism underlying the lens phenotype in *uhrf1* and *dnmt1* mutants. To explore this possibility, immunohistochemistry with an antibody against Proliferating Cell Nuclear Antigen (PCNA) was performed on 4 dpf lens cryosections, at which time the disrupted lens phenotype is relatively mild and the lens epithelial layer is still intact in *uhrf1* and *dnmt1* mutants. In the wild-type lens, PCNA-positive cells were positioned in the lateral epithelium (likely in the 'germinative zone' of the lens epithelium (Greiling et al., 2010)) (Fig. 6G). Cells in similar regions of the mutant epithelial layer also stained positively for PCNA (Figs. 6H,I), suggesting that cells within the epithelial region of mutant lenses maintain their proliferative capacity. Although PCNA expression in wild-type lens corresponded to the proliferative 'germinative zone' (Fig. 6G), PCNA is also involved in the process of DNA repair (Kelman, 1997). Because DNA damage is associated with reduction of Uhrf1 or Dnmt1 (Chen et al., 2007; Muto et al., 2002), quantification of epithelial cell proliferation was performed using BrdU incorporation assays and phosphohistone H3 (pH3) immunostaining (Figs. 6J–O).

Fewer BrdU-incorporating cells were observed in the *uhrf1* (Fig. 6K, mean = 2.9 ± 1.6 s.d. per section) and *dnmt1* (Fig. 6L, mean = 2.6 ± 1.5 s.d.) lenses than in wild-type lenses (Fig. 6J, mean = 6.2 ± 1.3) ($p < 0.005$). Additionally, the position of BrdU-incorporating cells within the *uhrf1* and *dnmt1* lens epithelium was not restricted to the lateral epithelium, as is the case in wild-type zebrafish ((Greiling et al., 2010), Fig. 6G). These data demonstrate that fewer epithelial cells in mutant lenses are in S-phase. Similarly, pH3 immunostaining revealed that an average of 1.8 (±1.0 s.d.) cells per lens section were pH3-positive in wild-type lenses (Fig. 6M), while there were zero pH3-positive cells observed in lenses of *uhrf1* (Fig. 6N, $n = 8$) and *dnmt1* (Fig. 6O, $n = 5$)

Fig. 6. Lens epithelium gene expression, cell proliferation, and apoptosis is altered in *uhrf1* and *dnmt1* mutant lenses. (A–C,E,F) Whole mount dorsal images of the retina and lens of *tgfB3* antisense *in situ* hybridizations on wild-type (A), *uhrf1* (B,E) and *dnmt1* (C,F) mutant embryos at 4 dpf. (A) Wild-type lenses showed strong staining for *tgfB3*, while staining in mutant lenses was classified as either weak (B,C) or moderate (E,F) (summarized in the table (D)). (G–I) Cryosections from 4 dpf embryos immunostained for PCNA (red). PCNA-positive cells are present in the lens epithelium of phenotypically wild-type (G) embryos as well as *uhrf1* (H) and *dnmt1* (I) mutants. In each panel, arrows of the same color indicate the dorsal and ventral proliferative zones within the lens epithelium, as defined by PCNA staining. (J–L) Cryosections through 4 dpf phenotypically wild-type (J), *uhrf1* (K), or *dnmt1* (L) lenses stained for incorporation of BrdU (applied between 96 and 98 hpf). White arrows point to lens cells which have incorporated BrdU. Fewer BrdU-incorporating cells are labeled in the *uhrf1* and *dnmt1* mutant lenses than in wild-type sibling lenses, and mutant cells labeled with BrdU are not positioned exclusively in the lateral epithelium as they are in the wild-type lens. (M–O) Phosphohistone H3 (pH3) immunostaining of 4 dpf (M) phenotypically wild-type, (N) *uhrf1* mutant and (O) *dnmt1* mutant embryos. White arrows in panel M show two cells in the wild-type lens epithelium stained positively for pH3. (N,O) No pH3 positive cells are observed in mutant lens epithelium. The yellow arrow in (N) indicates a pH3 positive cell in the cornea. Some sections have diffuse background staining in the cornea which is not localized to nuclei. (P–U) TUNEL immunostaining (red) of 4 dpf (P–R) and 5 dpf (S–U) phenotypically wild-type (P,S), *uhrf1* (Q,T) and *dnmt1* (R,U) mutant cryosections. No apoptotic cells are observed in the lens of wild-type embryos at 4 dpf (P), but numerous TUNEL-positive cells are observed in the still-nucleated epithelial and early fiber regions of the *uhrf1* and *dnmt1* mutant lenses (white arrows in Q, R). At 5 dpf, TUNEL-positive cells are rare in the wild-type eye (S), however, *uhrf1* and *dnmt1* mutant lenses contain numerous TUNEL-positive cells in the lens, and some in the cornea and retina (T,U). In all 4 dpf panels, mild mutants were selected for analysis. Sections in panels A–C and J–O are counter-stained with Sytox-Green (green). Anterior is to the right in all panels. Scale bars are 100 μm in A–C and E–F, 70 μm in G–R, and 80 μm in S–O.



	uhrf1	dnmt1
weak	13 (59%)	10 (48%)
moderate	9 (41%)	11 (52%)
total	22	21

mutant embryos at 4 dpf. Therefore, a reduced number of lens epithelial cells are in either S or M phase in mutant lenses.

Apoptosis is elevated in uhrf1 and dnmt1 mutant lenses

Apoptosis is elevated in proliferating somatic cells and differentiated cells in the absence of Dnmt1 (Jackson-Grusby et al., 2001; Latham et al., 2008; Li et al., 1992; Stancheva et al., 2001), suggesting that apoptosis could be elevated in *uhrf1* and *dnmt1* mutant lenses. To test this possibility, TUNEL immunostaining was performed to positively identify apoptotic cells in the mutant lenses. At 4 dpf, numerous TUNEL-positive cells are observed in the still-nucleated epithelial and early fiber regions of *uhrf1* and *dnmt1* mutant lenses, although no apoptotic nuclei are observed in wild-type lenses (Figs. 6P–R). Apoptosis of epithelial cells may therefore partially explain why fewer mutant epithelial cells expressed S and M phase cell cycle markers at 4 dpf (Figs. 6K,L,N,O). At 5 dpf, there are rare TUNEL-positive cells in the wild-type lens (Fig. 6S), however, *uhrf1* and *dnmt1* mutant lenses contain numerous TUNEL-positive cells, and moreover, isolated TUNEL-positive cells are also present in the cornea and retina of mutant eyes (Figs. 6T,U).

Lens fibers express markers of terminal differentiation in uhrf1 and dnmt1 mutants

Methylation of gene promoters is associated with reduced transcriptional activity (Bird, 2002), and methylation may be a mechanism to regulate cell type-specific gene expression patterns during development and differentiation (Illingworth and Bird, 2009). At 5 dpf, *uhrf1* and *dnmt1* mutant lenses are characterized by the presence of disorganized peripheral fibers, suggesting that terminal differentiation may be disrupted in these cells. To analyze this possibility, the expression of lens fiber differentiation markers was compared between wild-type and *uhrf1* and *dnmt1* mutant embryos. Crystallins are upregulated during fiber cell differentiation (Bassnett and Beebe, 2004), and in wild-type zebrafish Crystallin AlphaA is expressed in cortical fibers at 5 dpf (Fig. 7A; (Shi et al., 2006)). In *uhrf1* and *dnmt1* mutants, Crystallin AlphaA expression is still observed in the disorganized fibers, indicating that these cells have initiated differentiation (Figs. 7B,C). Furthermore, Lengsin is expressed in the subpopulation of early differentiating fiber cells which are not yet denucleated (Fig. 7D; (Harding et al., 2008; Wyatt et al., 2008)). In *uhrf1* and *dnmt1* mutants, Lengsin is expressed in the disorganized cells that make up the mutant lens periphery (Figs. 7E,F). Finally, Aquaporin 0 is expressed in fibers of the lens (Shiels and Bassnett, 1996; Shiels et al., 2001) (Fig. 7G). This marker is also observed in disorganized fibers of the *uhrf1* and *dnmt1* mutant lenses (Figs. 7H, I). Therefore, these disorganized cells express markers appropriate for early differentiating lens fibers.

Uhrf1 and Dnmt1 are required lens-autonomously for lens maintenance

Lens development requires a precise interplay between the retina and the lens (Lang and McAvoy, 2004). Indeed, lens fiber differentiation requires proteins synthesized within the lens as well as signaling molecules released from the retina (Bassnett and Beebe, 2004). The strong expression of *uhrf1* and *dnmt1* in the retina (Figs. 5, S5), and the role of DNA methylation in silencing genes (Bird, 2002), raised the possibility that lens defects in *uhrf1* and *dnmt1* mutants resulted from a non-autonomous, retina-dependent process.

To address this possibility, mosaic embryos were generated in which gene function could be limited to entire tissues, or groups of cells (Carmany-Rampey and Moens, 2006; Yamamoto and Jeffery, 2002). In the first set of experiments, shield-stage transplants were utilized to generate lenses that were mosaic for wild-type and mutant cells (Eberhart et al., 2006). Transplanted cells were dextran-labeled and percent contribution in the mosaic lens was quantified at 34 hpf as either low (≤30% of the lens was donor-derived), medium (30–70%), or high (≥70%) (Fig. S6). Rare host embryos with any donor cell contamination in the retina were discarded. At 5 dpf, lens phenotypes were assayed in whole mount to identify the genotype of the donor and host embryos, and only transplants with severe *uhrf1* or *dnmt1* mutant donors or hosts were used for subsequent analyses. Mosaic embryos were scored by whole-mount imaging, and a subset of these was verified through histology. Summary results from all mosaic lens combinations are presented in Table 1, and Fig. 8 shows representative whole mount and histological sections for each mosaic combination. Control transplants are presented in Fig. S7 and data from all mosaics that were verified by histology are presented in Figs. S8–S11.

Wild-type cells transplanted into either *uhrf1* (Figs. 8A–C) or *dnmt1* (Figs. 8G–I) mutants rescued the mutant lens, even at low contributions. Slightly imperfect lenses (classified as mild in Table 1) were observed in some mosaics, but in all cases the phenotype of the mosaic lens was drastically improved from that of the mutant donor. These data indicate that mutant retinas do not induce a mutant lens phenotype when the lens contains some wild-type cells and suggest that each gene is required lens-autonomously for lens maintenance. In reciprocal transplants, wild-type embryos receiving either *uhrf1* (Figs. 8D–F) or *dnmt1* (Figs. 8J–L) mutant cells also appeared normal, even with high contributions of mutant cells. This result suggests either that there is a cell non-autonomous function for Uhrf1 and Dnmt1 within the lens, or that the wild-type retina is able to non-autonomously support normal development of a lens, even when it is composed of 70% or more mutant cells.

To ensure that wild-type cells are not simply out-competing mutant cells in the lens epithelium (which might lead to an epithelium made up entirely of wild-type cells by the time of mutant phenotype onset), we performed an experiment to visualize the donor and host contribution to lens cells at the time of phenotype onset (Fig. S12). Because the dextran used to label donor cells was no longer detectable at 4–5 dpf, transplants were performed in which all donor cells were derived from transgenic zebrafish expressing mCherry driven by the *beta actin2* promoter. As in the previous experiment, percent contribution was quantified at 34 hpf, and any mosaic embryos with donor cell contamination in the retina were discarded. Confocal microscopy of control (wild-type donors and wild-type hosts; Figs. S12A,B) or experimental (wild-type donors and *uhrf1* mutant hosts; Figs. S12C,D) mosaic lenses was performed at 4 dpf. In both cases, the lens epithelium contained a mixture of donor- and host-derived cells, demonstrating that rescue of the mutant lens phenotype, at least in the case of *uhrf1*, is not mediated by wild-type cells simply replacing mutant cells in the lens epithelium.

Finally, to distinguish between the possibility that there is a cell non-autonomous function for Uhrf1 and Dnmt1 within the lens, or that the wild-type retina can non-autonomously support normal development of a lens composed of greater than 70% mutant cells, we transplanted entire lenses between wild-type and mutant embryos (Yamamoto and Jeffery, 2002). Unilateral transplants were performed at 37 hpf, a time well prior to any visible lens phenotype in the mutant eye (Fig. S2). In all of these late-stage transplants the lens phenotype was also lens-autonomous. Transplantation of either a *dnmt1* or a *uhrf1* mutant lens into a wild-type sibling embryo resulted in a mutant lens (Fig. 9B and *data not shown*) indicating that, at least post-37 hpf, the wild-type retina is not able to rescue a mutant lens and enable its normal development. In reciprocal transplants, wild-type lenses transplanted into *dnmt1* or *uhrf1* mutant retinas resulted in a wild-type lens (Fig. 9D and *data not shown*), indicating, as in the mosaics above, that loss of Dnmt1 or Uhrf1 in the retina does not underlie the lens defects in the mutant eye. Combined, these early and late-stage transplant data support a model in which Uhrf1 and Dnmt1 are required lens-autonomously, but perhaps not cell autonomously, for lens development and maintenance.

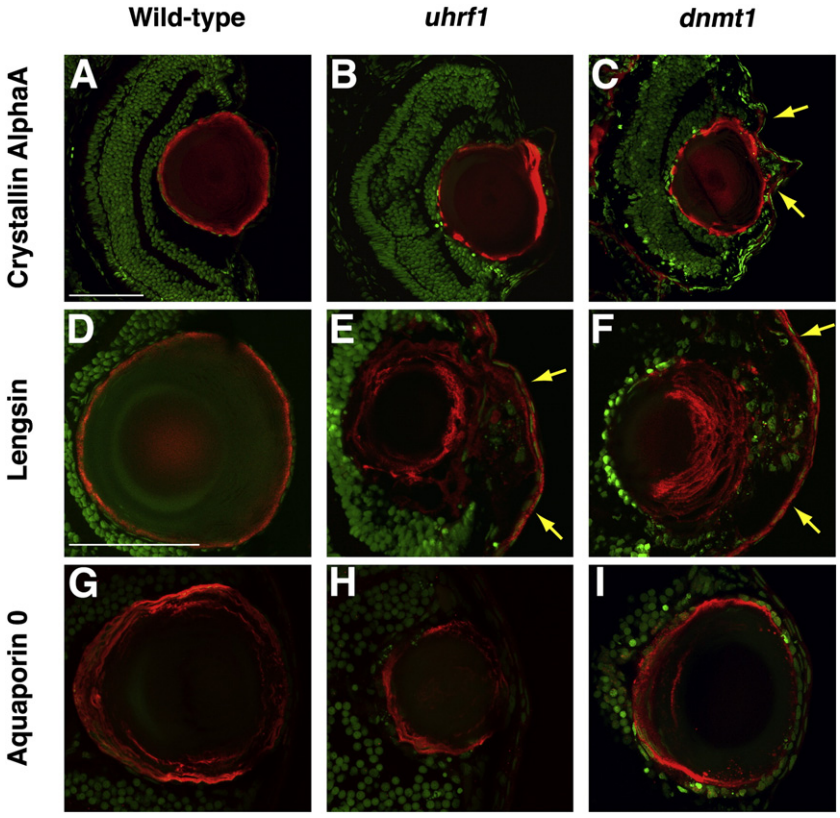


Fig. 7. Lens fibers express markers of terminal differentiation in *uhrf1* and *dnmt1* mutants. (A–I) Cryosections from 5 dpf embryos immunostained for the lens fiber markers Crystallin AlphaA (A–C, red), Lengsin (D–F, red), or Aquaporin 0 (G–I, red). In all sections, nuclei are counterstained with Sytox-Green (green). *uhrf1* (B,E,H) and *dnmt1* (C,F,I) fibers express these markers, indicating that the disorganized secondary fibers in the mutant lenses still express markers of terminal differentiation. Yellow arrows in panels C, E, and F indicate regions of the mutant cornea which ectopically express lens fiber genes. In all panels, mild mutants were selected for analysis. Anterior is to the right in all panels. Scale bars are 80 μm.

Discussion

Uhrf1 interacts with Dnmt1 in mammalian cells (Achour et al., 2008; Bostick et al., 2007; Sharif et al., 2007), and this interaction facilitates maintenance methylation (Bostick et al., 2007; Sharif et al., 2007). The present study demonstrates that Uhrf1 is required for DNA methylation in zebrafish, and that DNA methylation is reduced to a similar degree when either Uhrf1 or the catalytic function of Dnmt1 is lost. Knockout of Dnmt1 or Uhrf1 in the mouse results in early embryonic lethality, which has precluded an analysis of their roles in later aspects of organogenesis (Lei et al., 1996; Li et al., 1992; Muto et al., 2002; Sharif et al., 2007). Mouse conditional knockout studies have shown that Dnmt1 is required in hematopoiesis (Broske et al., 2009; Trowbridge et al., 2009) as well as in neuronal differentiation and function (Fan et al., 2001; Feng et al., 2010; Golshani et al., 2005; Hutnick et al., 2009). The fact that zebrafish *uhrf1* and *dnmt1* mutants survive to late embryonic stages enabled us to identify, for the first time, crucial roles for Uhrf1 and Dnmt1 in lens development and

Table 1

	Low contribution			Medium contribution			High contribution			*N*
	No phenotype	Mild	Severe	No phenotype	Mild	Severe	No phenotype	Mild	Severe	
Whole embryos										
WT to WT (*uhrf1* siblings)	2	0	0	13	0	0	15	0	0	30
WT to *uhrf1*	1	2	0	4	0	0	3	0	0	10
uhrf1 to WT	4	0	0	8	0	0	5	4	0	21
uhrf1 to *uhrf1*	0	0	3	0	0	2	0	0	1	6
WT to WT (*dnmt1* siblings)	0	0	0	1	0	0	4	0	0	5
WT to *dnmt1*	0	1	0	1	2	0	0	0	0	4
dnmt1 to WT	2	0	0	3	0	0	1	0	0	6
dnmt1 to *dnmt1*	0	0	0	0	0	0	0	0	0	0
Histological verification										
WT to WT (*uhrf1* siblings)	0	0	0	0	0	0	1	0	0	1
WT to *uhrf1*	0	2	0	2	0	0	1	0	0	5
uhrf1 to WT	0	0	0	7	0	0	1	2	0	10
uhrf1 to *uhrf1*	0	0	0	0	0	1	0	0	1	2
WT to WT (*dnmt1* siblings)	0	0	0	1	0	0	2	0	0	3
WT to *dnmt1*	0	1	0	1	2	0	0	0	0	4
dnmt1 to WT	1	0	0	3	0	0	1	0	0	5
dnmt1 to *dnmt1*	0	0	0	0	0	0	0	0	0	0

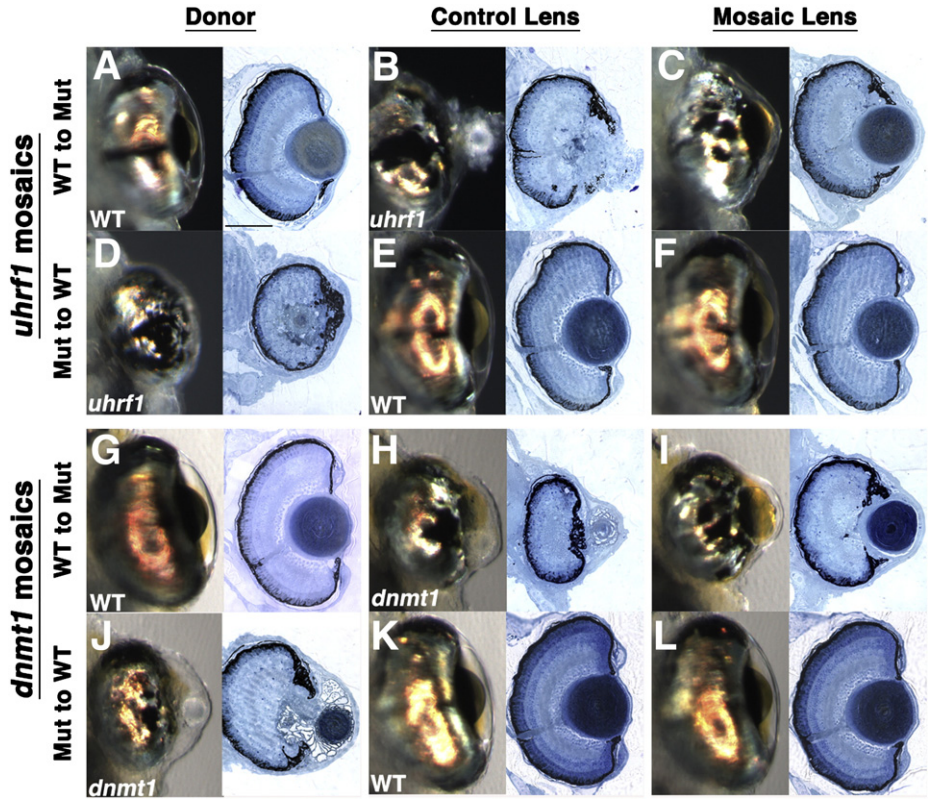


Fig. 8. Uhrf1 and Dnmt1 are required lens-autonomously for normal lens development. Shield-stage transplants were performed as described in Fig. S6. (A–F) Representative transplants between wild-type embryos and *uhrf1* mutants and (G–L) wild-type embryos and *dnmt1* mutants. All images are from 5 dpf embryos and each pair of whole-mount and histology images is derived from the same embryo and eye. For consistency, all data presented are taken from embryos with medium (30–70%) contribution of cells in the mosaic host lens. (A,D,G,J) whole-mount and histology images of eyes formed from donor embryos, (B,E,H,K) whole-mount and histology images of eyes formed from the non-mosaic side of host embryos and (C,F,I,L) whole-mount and histology images of eyes formed from the mosaic lens. Mutant to wild-type (A–C,G–I) transplants and wild-type to mutant transplants (D–F,J–L) both yielded normal lens formation in the mosaic host lens indicating that Uhrf1 and Dnmt1 function is required lens autonomously for lens development. Scale bars are 80 μm.

maintenance. Moreover, comparison of lens defects in single *uhrf1* and *dnmt1* mutants with those in *uhrf1*$^{-/-}$; *dnmt1*$^{-/-}$ double mutants provides genetic support for a functional interaction between these proteins in the lens.

We have shown that *uhrf1* and *dnmt1* are normally expressed in proliferative cells of the zebrafish lens epithelium at the time of mutant lens phenotype onset, and that the requirement for wild-type *uhrf1* and *dnmt1* is lens-autonomous. In the absence of either Uhrf1 or of Dnmt1 catalytic function, secondary lens fibers continue to express differentiation markers. However, lens epithelial cells, which are proliferative in the wild-type lens, show reduced expression of *tgfB3*, a zebrafish epithelial marker, reduced BrdU incorporation, and reduced

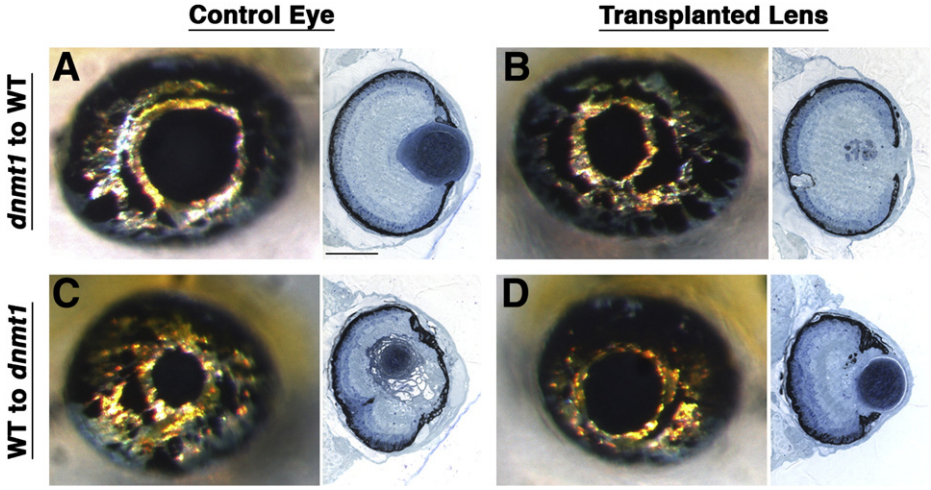


Fig. 9. Wild-type retinas cannot rescue development of a mutant lens. Lens transplants performed at 37 hpf (Yamamoto and Jeffery, 2002) demonstrate that mutant lenses placed in wild-type retinas retain the mutant phenotype. (A–D) Representative 5 dpf whole-mount and histology data for lens transplants between wild-type embryos and *dnmt1* mutants. Each pair of whole-mount and histology images is derived from the same embryo and eye, and transplants were unilateral so control and transplanted lenses are from the same fish. (A,B) *dnmt1* lenses transplanted to wild-type host eyes resulted in the mutant phenotype ($n=5$). (C,D) Wild-type lenses transplanted to *dnmt1* mutant hosts resulted in wild-type lens formation ($n=5$). Similar results were obtained from corresponding transplants between *uhrf1* mutant and wild-type embryos (data not shown). Scale bars are 80 μm.

phospho-Histone H3 staining in both mutant backgrounds. This is correlated with a wave of apoptosis in the epithelial layer, which is followed by apoptosis and unraveling of secondary lens fibers. Many distinct cellular roles for Uhrf1 and Dnmt1 have been published (Achour et al., 2009; Chen et al., 2007; Dunican et al., 2008; Espada et al., 2004; Karagianni et al., 2008; Papait et al., 2007; Rottach et al., 2010), including a role for Uhrf1 in Dnmt1-independent gene silencing and methylation in conjunction with the *de novo* (non-maintenance) methyltransferases Dnmt3a and Dnmt3b (Meilinger et al., 2009). We have demonstrated that a process which requires both Uhrf1 and the catalytic function of Dnmt1 within cells of the lens is required for lens development and maintenance. We propose that this function is likely to be DNA maintenance methylation, which is known to require both genes (Bestor, 2000; Bostick et al., 2007; Sharif et al., 2007; Yoder et al., 1997). We have shown that overall levels of cytosine methylation are disrupted to the same degree in both mutant lines (Fig. 1); however, we cannot exclude the possibility that another process which requires both proteins is responsible for the lens phenotypes in mutant embryos.

The onset of the disrupted lens phenotype in *uhrf1* and *dnmt1* mutants in this study is relatively late in zebrafish eye development: 4 to 5 dpf. For comparison, the zebrafish lens appropriately focuses light onto the plane of retinal photoreceptors at 72 hpf, by which time zebrafish embryos exhibit visual function (Easter and Nicola, 1996). Goll and colleagues recently demonstrated (with the same *dnmt1*^{s872} allele utilized in the present study) that Dnmt1 activity, presumably maternally provided, remained in the brain of *dnmt1* mutant embryos through 1 dpf (Goll et al., 2009). Maternally provided Dnmt1 and Uhrf1 (transcript or protein) may therefore explain the relatively late onset of the disrupted lens phenotype in *dnmt1* and *uhrf1* mutants. This maternal contribution would presumably titrate out with each cell division, and the timing of complete loss of gene function in mutant tissues should vary according to the number of cell divisions in a particular tissue.

DNA methylation and lens development

The results of lens transplant experiments demonstrate that Uhrf1 and Dnmt1 functions are required lens-autonomously during lens development in zebrafish. Additionally, the results of mosaic lens experiments demonstrate that the downstream function of Uhrf1 and Dnmt1 is cell non-autonomous within the lens, as even a low concentration of wild-type lens cells can rescue the *uhrf1* or *dnmt1* mutant lens phenotype.

Because abundant mutant host cells are still present in the lens epithelium at 4 dpf (Fig. S12), it is possible that a gene product produced within wild-type lens cells could non-autonomously rescue the mutant lens cells. Indeed, signaling molecules (such as FGFs), which are involved in fiber cell differentiation, are expressed within the lens (Lang and McAvoy, 2004). If these are deficient in mutant lenses, they may potentially mediate a non-autonomous rescue of the mutant phenotype when provided by wild-type cells. Furthermore, mouse lens fibers form a "stratified syncytium" with other fibers of the same age, thereby forming concentric shells of interconnected cells and enabling the passage of macromolecules between cells; a similar syncytium has been shown in the chicken lens (Shestopalov and Bassnett, 2000, 2003; Shi et al., 2009). If early fibers in the zebrafish lens are similarly connected, cytoplasmic and membrane proteins would be expected to diffuse between wild-type and mutant fibers in mosaics, and enable rescue of the mutant phenotype. In this case, the result of our mosaic lens experiments would be more correctly interpreted as either cell non-autonomous or as "syncytium-autonomous". Although much further work is needed to determine whether the zebrafish lens forms a syncytium, the results of our *beta actin2*:mCherry lens mosaics are consistent with this possibility (Fig. S12). Specifically, in both wild-type to wild-type and wild-type to mutant transplants, we observed only occasional *beta actin2*:mCherry-expressing donor cells in the lens epithelium and transition zone, while membranes of mature lens fibers appeared to be uniformly red.

Another possible cause of the disrupted lens phenotype in *uhrf1* and *dnmt1* mutants is ectopic epithelial–mesenchymal transition (EMT), a process which results in cataracts in both mouse mutants and in human patients, and which superficially resembles the early stages of *uhrf1* and *dnmt1* lens defects (de Iongh et al., 2005). However, we do not favor this as the mechanism underlying lens defects in *uhrf1* and *dnmt1* mutant zebrafish because transcripts for the EMT marker *alpha-smooth muscle actin* were not detected in mutant lenses at either 4 or 5 dpf (RKT, unpublished observations).

Generally, hypermethylation of gene promoter CGIs is associated with reduced gene transcription (Bird, 2002), and this is a potential mechanism by which cell type-specific gene expression patterns are set during differentiation (Illingworth and Bird, 2009). The vertebrate lens consists of an anterior monolayer of proliferative epithelial cells which give rise to terminally differentiated lens fibers (Lovicu and Robinson, 2004). Recent studies have shed light on the role of Dnmt1 in other populations of self-renewing progenitors (Broske et al., 2009; Sen et al., 2010; Trowbridge et al., 2009). In epidermis, depletion of *dnmt1* or *uhrf1* leads to a reduction of self-renewal and to premature differentiation of proliferative progenitors (Sen et al., 2010). Results by Sen et al. indicate that Dnmt1 maintains methylation of transcriptionally repressed differentiation genes in epidermal progenitors, and that these genes are demethylated during terminal differentiation by an active process which involves Gadd45. It is possible that a similar role for Dnmt1-mediated methylation occurs in the proliferative epithelial cells of the vertebrate lens. Although few specific roles for DNA methylation during lens development have yet been identified, it is known in rat lens that transcription of the lens fiber-specific gene *gamma D crystallin* is regulated in part by demethylation of its promoter (Dirks et al., 1996; Klok et al., 1998; Peek et al., 1991). The *gamma D crystallin* gene promoter is both heavily methylated and untranscribed in rat lens epithelial cells, but during lens fiber differentiation, *gamma D crystallin* is demethylated by an active process which is necessary for its subsequent transcription in lens fibers. Future genome-wide experiments to examine changes in gene methylation and transcription during lens development may shed additional light on gene regulation by DNA methylation in the lens.

In summary, this study demonstrates that Uhrf1 is required for DNA methylation *in vivo* during zebrafish embryogenesis. Due in part to the early embryonic lethality of *Dnmt1* and *Uhrf1* knockout mice (Lei et al., 1996; Li et al., 1992; Muto et al., 2002; Sharif et al., 2007), roles for these proteins during lens development have yet to be reported. In the absence of either Uhrf1, or of catalytically active Dnmt1, zebrafish secondary lens fibers continue to express differentiation markers. However, lens epithelial cells show reduced expression of a zebrafish epithelial marker, *tgfB3*, reduced BrdU incorporation, and reduced phospho-Histone H3 staining in both mutant backgrounds. This is correlated with a wave of apoptosis in the epithelial layer, which is followed by apoptosis and unraveling of secondary lens fibers. Uhrf1 and Dnmt1 functions are required lens-autonomously, but perhaps not cell-autonomously, during lens development in zebrafish. Combined with expression of these genes within lens epithelial cells and the fact that lens defects in mutants begin in the epithelium, these data support a model in which Uhrf1 and Dnmt1 function is required within cells of the lens epithelium for lens development and maintenance.

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