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

Rachel K. Tittle a,1, Ryan Sze a,1, Anthony Ng a,1, Richard J. Nuckels a, Mary E. Swartz a, Ryan M. Anderson c, Justin Bosch c, Didier Y.R. Stainier c, Johann K. Eberhart a,b, Jeffrey M. Gross a,b,⁎

a Section of Molecular, Cell and Developmental Biology, Institute of Cell and Molecular Biology, The University of Texas at Austin, Austin TX 78712, USA
b Institute for Neuroscience, USA
c Department of Biochemistry and Biophysics, Program in Developmental Biology, Liver Center, Diabetes Center and the Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94158-2324, USA

⁎ Corresponding author. Fax: +1 512 471 3878.
E-mail address: jmgross@mail.utexas.edu (J.M. Gross).

Available online 30 November 2010
Accepted 4 November 2010
Revised 14 October 2010
Received for publication 1 May 2010

Article history:

Keywords:

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 unravelling 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.

© 2010 Elsevier Inc. All rights reserved.

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 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

0012-1606/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2010.11.009
alleles used in this study were of Texas at Austin provisions governing animal use and care. Mutant 10 h dark cycle. Animals were treated in accordance with University Zebra Materials and methods development and maintenance. During lens development. Our results demonstrate that Uhrf1 and Dnmt1 play in DNA methylation during zebra dnmt1 homogenized in 0.1% Triton X100 and protease inhibitors (Roche) in advantage of zebra ment for DNA methylation during lens development, we took utilize the speci important for the development, differentiation, and survival of Zebra Materials and methods development and that Uhrf1 and Dnmt1 are required for lens development. Our results demonstrate that Uhrf1 facilitates DNA methylation in vivo during zebra 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 ubr1f1s20 (Anderson et al., 2004) and dnmt1 (Anderson et al., 2009) to determine what role Ubr1 and Dnmt1 play in DNA methylation during zebrafish embryogenesis and during lens development. Our results demonstrate that Ubr1 facilitates DNA methylation in vivo during zebrafish embryonic development and that Ubr1 and Dnmt1 are required for lens development and maintenance.

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, C9091) to allow probe entry though the lens capsule. A cDNA clone encoding dnmt1 (clone # c983) was purchased from ZIRC (Eugene, OR), ubr1f1 was cloned from 24 hpf cDNA and ligated into pGEM-T Easy, and tgf3 was cloned from cDNA derived from 1 to 4 dpf embryos and ligated into pcDNA3+ (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 (Se11; 1:100, kindly provided by Jim Fadool), Lengsin (1:500; (Harding et al., 2008), kindly 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 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 ddH2O. 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. 1Du si ng the two-tailed t-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 prep 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 uhrf1hi3020 allele is either null or severely hypomorphic

The uhrf1hi3020 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 uhrf1hi3020 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](image-url)

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<sup>hi3020</sup> 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 <sup>dnmt1</sup><sup>s872</sup> (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 <sup>dnmt1</sup> and <sup>uhrf1</sup> 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 <sup>dntm1</sup> and <sup>uhrf1</sup> sibling groups were not significantly different from wild-type levels (Fig. 1D). However, genomic DNA from <sup>dnmt1</sup> or <sup>uhrf1</sup> 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 <sup>dnmt1</sup> and <sup>uhrf1</sup> 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.
The uhrf1 lens phenotype is phenocopied by mutations in dnmt1

Differentiating fi

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<sup>5904</sup>, in which a frameshift 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<sup>5904</sup> also phenocopied the uhrf1 disrupted lens phenotype (Fig. S3). All further experiments were carried out in the dnmt1<sup>s872</sup> 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<sup>−/−</sup>; dnmt1<sup>−/−</sup> 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<sup>−/−</sup>; dnmt1<sup>−/−</sup> compound heterozygous embryos (Fig. 4B). Body morphology in ~50% of uhrf1<sup>−/−</sup>; dnmt1<sup>−/−</sup> 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 embryo 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.
of the single mutants, and range from mild (C) to severe (D). Lens phenotypes to lethal before later aspects of eye formation can be studied (Lei et al., 2010). 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; This et al., 2001; This and This, 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. S5B–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 uhrf1 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

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),
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 quantitative 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 cell cycle defects are a potential mechanism underlying the lens epithelium, as is the case in wild-type zebrafish (Greiling et al., 2010), 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 proliferation was performed using BrdU incorporation assays and phosphohistone H3 (phH3) 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 = 6.2 ± 1.3 s.d.) lenses than in wild-type lenses (Fig. 6J, mean = 6.2 ± 1.3 s.d.) (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, phH3 immunostaining revealed that an average of 1.8 ± 1.0 s.d. cells per lens section were phH3-positive in wild-type lenses (Fig. 6M), while there were zero phH3-positive cells observed in lenses of uhrf1 (Fig. 6N, n = 8) and dnmt1 (Fig. 6O, n = 5).
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 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 dnm1t 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 dnm1t 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.
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

<table>
<thead>
<tr>
<th></th>
<th>Low contribution</th>
<th>Medium contribution</th>
<th>High contribution</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No phenotype</td>
<td>Mild</td>
<td>Severe</td>
<td>WT to WT (uhrf1 siblings)</td>
</tr>
<tr>
<td>Whole embryos</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>WT to WT</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>uhrf1 to WT</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>uhrf1 to uhrf1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT to WT (dnmt1 siblings)</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT to dnmt1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>dnm1 to WT</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>dnm1 to dnm1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Histological verification</td>
<td></td>
<td></td>
<td></td>
<td>WT to WT (uhrf1 siblings)</td>
</tr>
<tr>
<td>WT to WT</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>uhrf1 to WT</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>uhrf1 to uhrf1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT to WT (dnmt1 siblings)</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT to dnmt1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>dnm1 to WT</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>dnm1 to dnm1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
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. 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.

**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; Dunigan et al., 2008; Espada et al., 2004; Karagianian 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 dnm1ts72 allele utilized in the present study) that Dnmt1 activity, presumably maternally provided, remained in the brain of dnm1 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 dnm1 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 dnm1 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- 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 dnm1 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 dnm1 lens defects (de Jongh et al., 2005). However, we do not favor this as the mechanism underlying lens defects in uhrf1 and dnm1 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 CGs 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.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.11.009.

**Acknowledgments**

This work was supported by grants from the NIH (F32-EY020745 to RKT; R01-EY18005 to JMG; R01-DK60322 and R01-DK075032 to DYRS; and R00-DE018088 to JKE), and by grants from the Knights Templar Eye Foundation to JMG and RKT. We are grateful to Paul...

References


