An ENU Mutagenesis Screen in Zebrafish for Visual System Mutants Identifies a Novel Splice-Acceptor Site Mutation in *patched2* that Results in Colobomas

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PURPOSE. To identify recessive mutations affecting development and/or maintenance of the zebrafish visual system.

METHODS. A three-generation ENU (N-Nitroso-N-ethylurea)-based forward genetic screen was performed. F3 embryos were screened visually from 1 to 5 days postfertilization (dpf) for ocular abnormalities, and 5 dpf embryos were fixed and processed for cryosectioning, after which eye sections were screened for defects in cellular organization within the retina, lens, and cornea. A combination of PCR and DNA sequencing, in situ hybridization, and pharmacological treatments were used to clone and characterize a coloboma mutant.

RESULTS. A total of 126 F2 families were screened, and, from these, 18 recessive mutations were identified that affected eye development. Phenotypes included lens malformations and cataracts, photoreceptor defects, oculocutaneous albinism, microphthalmia, and colobomas. Analysis of one such coloboma mutant, *uta1*, identified a splice-acceptor mutation in the *patched2* gene that resulted in an in-frame deletion of 19 amino acids that are predicted to contribute to the first extracellular loop of Patched2. *ptch2uta1* mutants possessed elevated Hedgehog (Hh) pathway activity, and blocking the Hh pathway with cyclopamine prevented colobomas in *ptch2uta1* mutant embryos.

CONCLUSIONS. We have identified 18 recessive mutations affecting development of the zebrafish visual system and we have characterized a novel splice-acceptor site mutation in *patched2* that results in enhanced Hh pathway activity and colobomas. (*Invest Ophthalmol Vis Sci.* 2012;53:8214–8221) DOI:10.1167/iovs.12-11061

The zebrafish, *Danio rerio*, has been increasingly used over the past 3 decades as a model system in which to study visual system development, ocular physiology, and visual system function, as well as one in which to study the molecular and cellular underpinnings of ocular diseases.[1–5] Indeed, capitalizing on the ability to perform large-scale forward genetic screens in the zebrafish system, researchers have identified numerous mutations that result in phenotypes similar to those in a variety of human ocular disorders (reviewed in Bibliowicz et al.[1]). Analyses of these mutations have contributed to a better understanding of ocular diseases and processes that affect nearly every part of the eye, highlighting the efficacy of the approach, and the importance of the zebrafish model system in ocular research.

Although forward genetic screens have been performed using a variety of mutagens, the saturation of these screens has been low, and multiple alleles have been identified for only a few loci, supporting the notion that further screening is likely to identify additional mutations that affect the visual system, as well as additional alleles that will be useful in biochemical analyses of the proteins encoded by the affected loci. Moreover, given the recent advances in whole-genome sequencing, it is now possible to rapidly and cost-effectively identify the affected loci in mutant lines, enabling one to quickly target relevant mutations in ocular disease-causing genes for further study.[6–9] With this in mind, we have recently completed an ENU (N-Nitroso-N-ethylurea)-based forward genetic screen to identify recessive mutations that affect eye morphology in zebrafish embryos up to 5 days postfertilization (dpf). Herein, we report the results of this screen and the identification and characterization of a new mutant allele of the zebrafish *patched2* gene.

MATERIALS AND METHODS

Animals and Maintenance

Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14-hour light/10-hour dark cycle. Embryos were obtained from the natural spawning of heterozygous carriers set up in pairwise crosses. Embryos were collected and raised at 28.5°C after Westerfield,[10] and were staged according to Kimmel et al.[11] All animals were treated in accordance with provisions established at the University of Texas at Austin governing animal use and care, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

ENU Mutagenesis

An ENU mutagenesis protocol was modified from Solnica-Krezel et al.[12] Briefly, 18 AB males were exposed to a freshly made 3-mM solution of ENU (Sigma ISOPAC, St. Louis, MO), buffered in a 1-mM sodium phosphate solution. Fish were exposed at a density 3 males/1 L of ENU solution, maintained at approximately 21°C, and they remained in the solution for 1 hour. Fish were removed from the ENU solution, placed into fresh fish water, and maintained overnight in a

dark, quiet area to recover. The following morning, they were placed back onto the aquatic system. ENU exposures were repeated weekly for 3 additional weeks, after which 9 mutagenized F0 males remained. These males were bred once to release any nonmutagenized sperm, and then they were outcrossed to AB females to generate F1s. F1s were either intercrossed, or outcrossed to wild-type ABs, to generate F2 families; 1 to 12 pairs of F2 fish per family were mated to generate F3 embryos for screening.

F3 Mutant Screening

F3 embryos were examined daily through 5 dpf under a dissecting microscope (Nikon SMZ800; Nikon, Melville, NY) to identify embryos with ocular defects. At 5 dpf, embryos were fixed and processed for cryosectioning after Uribe and Gross[13]; 12-µm sections were taken from at least 5 embryos and stained with either hematoxylin and eosin (Sigma, St. Louis, MO) or Sytox-Green (Molecular Probes, Eugene, OR) to examine retinal and lens morphology and cellular organization. Putative F2 carriers were rescreened, and those that produced embryos resulting in reproducible ocular phenotypes were outcrossed to AB fish. Heterozygous carriers were then identified in these F3 lines and phenotypes verified, and then an F3 line was given an allele number and the mutation was considered "recovered" in the screen.

Riboprobes and In Situ Hybridization

Hybridizations were performed essentially as described by Jowett and Lettice[14] using digoxigenin-labeled antisense RNA probes. *ptch2* cDNA and probes are described in Lee et al.[15] cDNA for *pax2a* was provided by Bruce Riley (Texas A&M University).

Cyclopamine Treatments

Cyclopamine (Sigma) was resuspended at 10 mg/mL in 100% ethanol and diluted into fish water for exposures; 100% ethanol was used for vehicle controls. Embryos derived from heterozygous *uta¹* incrosses were used for all exposures. Embryos were removed from cyclopamine at defined times and washed into fish water for further culturing. Cyclopamine rescue data were analyzed by Fisher's exact test for statistical significance.

RESULTS

F3 Mutant Screen to Identify Recessive Ocular Mutations

To identify recessive zebrafish mutants with morphological defects in eye formation, a three-generation ENU-based forward genetic screen was performed (Fig. 1). F3 embryos were screened under a dissecting microscope to identify those that possessed obvious defects in optic cup or lens morphology, eye size, RPE pigmentation, and/or lens transparency. Observations were also made with regard to the overall health of the F3 embryos, to eliminate F2 families that produced embryos that possessed significant nonocular defects, generalized growth defects, or increased levels of cell death, as ocular phenotypes in these lines would likely be secondary to more systemwide defects. At 5 dpf, a subset of F3 embryos from each F2 pair was fixed and processed for cryosectioning and imaging following either immunostaining with Sytox-Green (a DNA marker) or hematoxylin and eosin staining. Eye sections from these embryos were analyzed for defects in retinal lamination, the presence and organization of retinal cell types, and overall lens and cornea morphology. From the 126 F2 families screened, 18 mutants were isolated that displayed ocular defects (Table). Mutants were identified with colobomas (Figs. 2A, 2A', 2B, 2B'), photoreceptor defects (Figs. 2C, 2C'),
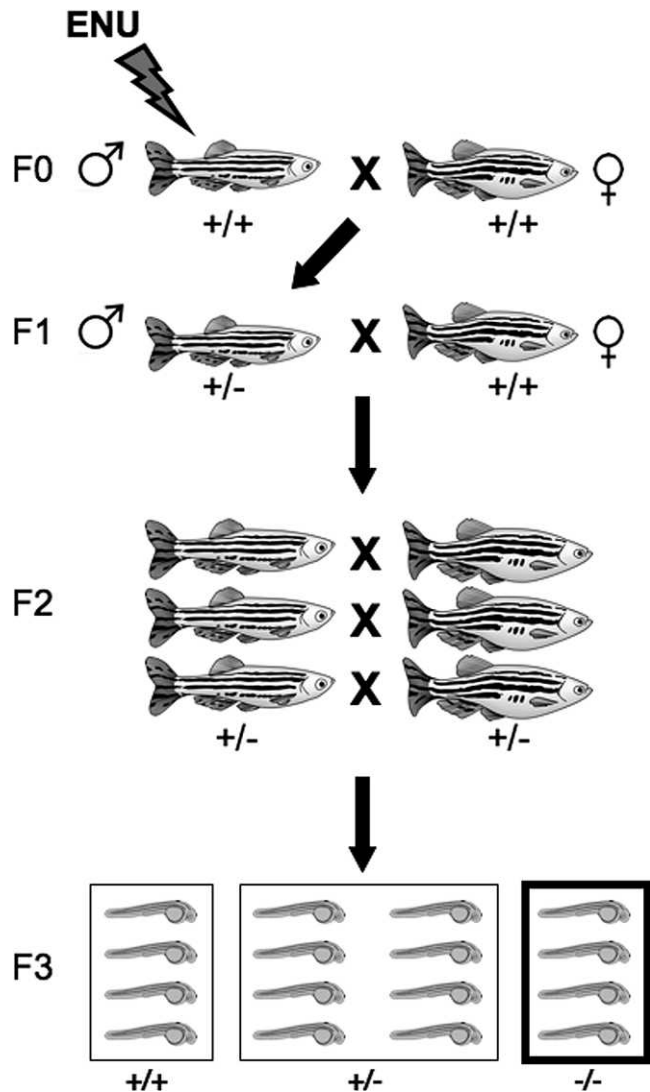

FIGURE 1. Forward genetic screening in zebrafish. Schematic diagram of a typical ENU-based forward genetic screen in zebrafish (modified from Bibliowicz et al.[1]). Male founders (F0) are mutagenized with ENU for several weeks and then outcrossed to wild-type females. Progeny of this cross (F1) are raised and either outcrossed to wild-type, or incrossed to generate F2 families. Fish from F2 families are incrossed and F3 progeny are collected and screened for phenotypes of interest (recessive screen).

cataracts (Figs. 2D, 2D'), and defects in lens morphology (Figs. 2E, 2E').

uta¹ Mutants Possess Colobomas

We were particularly interested in identifying mutants with defects in choroid fissure closure, and, thus, those mutants that presented with colobomas.[16] Several coloboma mutants were identified in our primary screen. After further analysis, only two of these lines presented without additional, systemic defects and, thus, represented loci encoding gene products with potentially limited functions outside of the eye: *uta¹* and *uta³*. Coloboma phenotypes in these mutants are similar at a gross histological level, although colobomas in *uta¹* are more severe. *uta¹* was of particular interest to us given that it did not appear to have any other obvious embryonic defects outside of the eye (Fig. 3). In *uta¹* mutants, the choroid

TABLE.   Mutants Identified in the Screen

Mutant	Ocular Phenotype	Additional Phenotypes
uta1	Coloboma	
uta2	Abnormal proliferation and differentiation in retina	Bifurcated tail, enlarged brain
uta3	Coloboma	
uta4	Cataract/lens degeneration, anterior segment defects, microphthalmia	
uta5	Photoreceptor defects, microphthalmia	Tail curl
uta6	Photoreceptor defects, microphthalmia	No iridophores
uta7	Cataract	
uta8	Lens morphology, lens dysplasia	
uta9	Cataract	Pericardial edema
uta10	Small lens, flattened optic cup	
uta13	Oculocutaneous albinism (moderate)	
uta16	Microphthalmia, lens morphology/lens dysplasia	
uta17	Microphthalmia, small pupil	
uta18	Oculocutaneous albinism (severe)	
uta19	Cataract, microphthalmia	Small head, pericardial edema
uta20	Cataract, microphthalmia	Small head, pericardial edema
uta21	Cataract	No pectoral fin
uta22	Cataract, microphthalmia	Small head, pericardial edema

fissure remained open and, as a result, retinal and RPE tissue are not contained within the eyecup (Figs. 3C–H). The severity of the phenotype is relatively invariant between homozygous embryos, with almost all presenting with severe, bilateral colobomas (Fig. 3H). The incidence of colobomas ranges from 22% to 25% of total embryos in any given clutch derived from heterozygous parents, and this Mendelian ratio is consistent with a single gene mutation. Within the eye, beyond colobomas, other aspects of development and patterning appear overtly normal, although the eye is microphthalmic when viewed laterally, but this could be due to the presence of the coloboma.

Optic Vesicle Patterning Is Abnormal in *uta1*

The overall phenotype of *uta1* resembled a coloboma mutant previously studied in our laboratory, *blowout*, which possesses a recessive mutation in the *patched2* gene (*ptch2tc294z*, formerly known as *patched1* in zebrafish[15,17]). *ptch2tc294z* mutants present with a visible expansion of the optic stalk at 1 dpf, and obvious colobomas at 2 dpf. Expansion of the optic stalk in *ptch2tc294z* correlated with an expansion of the expression domain of the distal optic vesicle marker, *pax2a*, and optic stalk tissue resided within the choroid fissure, possibly preventing apposition of the lateral edges of the fissure, leading to colobomas in *ptch2tc294z* mutants.[15] To determine if colobomas in *uta1* mutants also correlated with defects in optic vesicle patterning, we examined *pax2a* expression (Fig. 4). Indeed, the domain of *pax2a* was substantially expanded relative to that in sibling control embryos at 30 hpf (Figs. 4A, 4B). Moreover, at 48 hpf, concomitant with optic stalk differentiation, *pax2a* expression was almost absent from wild-type siblings, whereas in *uta1* mutants, expression was main-
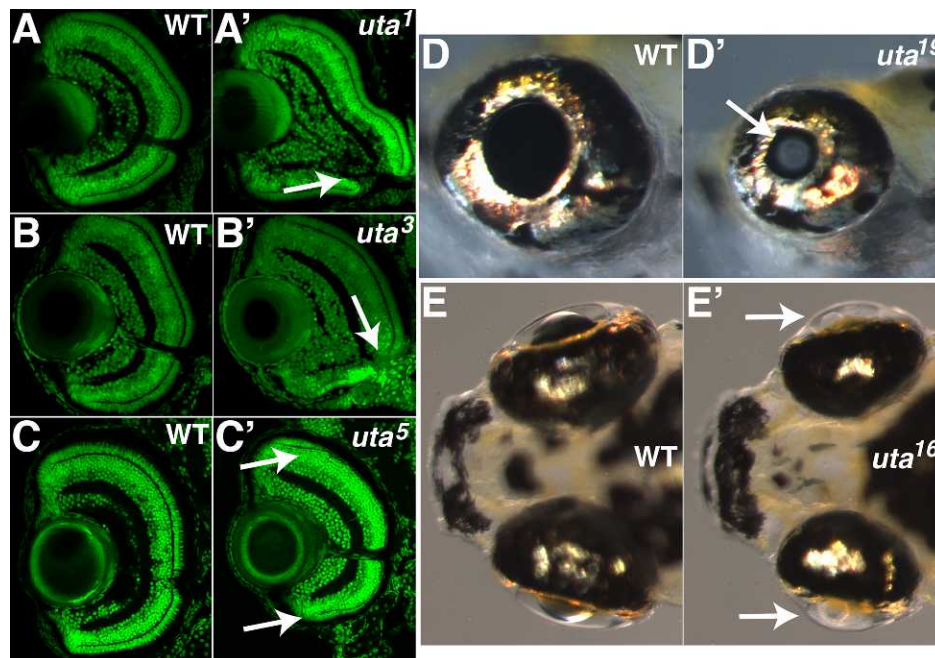

FIGURE 2.   Examples of ocular mutants identified in the screen. *uta1* (A') and *uta3* mutants (B') display colobomas at 3 dpf when compared with phenotypically wild-type siblings ([A, B], respectively). *Arrows* in (A') and (B') point to the open choroid fissure in the mutants. *uta5* mutants (C') lack photoreceptors in the central retina at 7 dpf when compared with phenotypically wild-type siblings (C). Photoreceptors are detected at the peripheral retina, adjacent to the ciliary marginal zones in mutant embryos (*arrows* in [C']). *uta19* mutants (D') possess visible cataracts at 6 dpf when compared with phenotypically wild-type siblings (D). *Arrow* in (D') highlights cataract in mutant lens. *uta16* mutants (E') possess severe defects in lens morphology at 7 dpf when compared with phenotypically wild-type siblings (E). *Arrows* in (E') highlight mutant lenses.
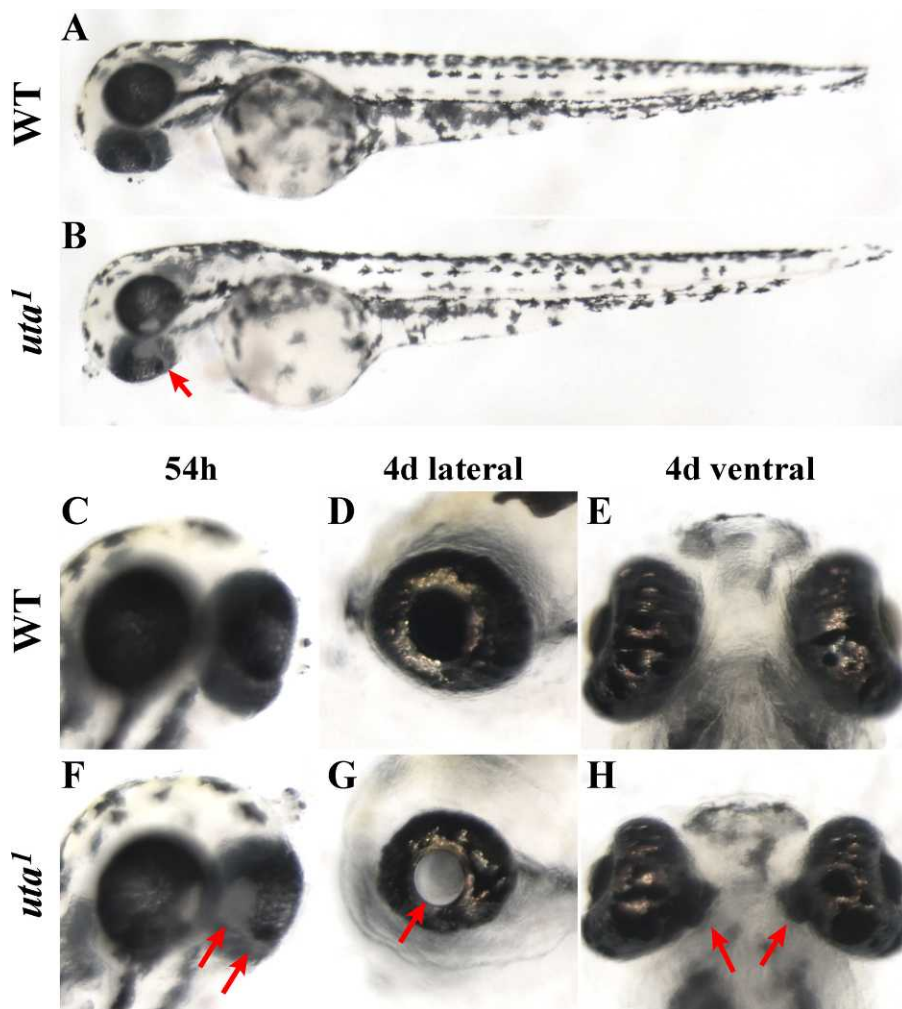
FIGURE 3. *uta¹* mutants present with colobomas. (**A**, **C–E**) Wild-type and (**B**, **F–H**) *uta¹* mutant embryos at 54 hpf (**A–C**, **F**) and 4 dpf (**D**, **E**, **G**, **H**). Colobomas are evident at 54 hpf (*arrows* in [**B**, **F**]). By 4 dpf, an obvious nonpigmented "hole" is present in the posterior of the mutant eye, when imaged through the lens (*arrow* in [**G**]), and retinal and RPE tissues are extruded into the forebrain (*arrows* in [**H**]).

tained and also appears to extend into the retina (Figs. 4C, 4D)

uta¹ Mutants Possess a Mutation in the *patched2* Gene

ptch2^tc294z mutants possess a recessive mutation, *ptch2^W1040X*, that truncates the Ptch2 protein at amino acid 1039 in the C-terminus, just after the eighth transmembrane segment.[15,17] Given the similarity in coloboma phenotypes and *pax2a* expression between *uta¹* and *ptch2^tc294z* mutants, we next determined if *uta¹* was a *ptch2* allele. Complementation crosses between *uta¹* and *ptch2^tc294z* resulted in embryos with colobomas, supporting the possibility that they were alleles of the same gene (data not shown). Cloning and sequencing of the *ptch2* gene from cDNA derived from *uta¹* mutants and wild-type siblings revealed a 57-bp internal deletion in the *ptch2* open-reading frame that resulted in an in-frame deletion of 19 amino acids from the Ptch2 protein (Fig. 5A). Cloning and sequencing of this region of the *ptch2* gene from genomic DNA isolated from *uta¹* mutants and wild-type siblings revealed a G → A mutation at the intron 4 splice-acceptor site (Fig. 5B). Reanalysis of cDNA sequences from exon 5 of the *ptch2* gene identified a cryptic, in-frame splice-

acceptor site whose use results in the removal of the first 57 bp of exon 5 (Fig. 5A). The 19 amino acids absent from the Ptch2^uta1 protein are highly conserved between zebrafish, human, and mouse Ptch2 proteins, as well as with Ptch1 orthologs in each of these systems (Fig. 5C). Based on the predicted structure/topology of the Ptch2 protein,[18] these 19 amino acids are predicted to contribute to the first extracellular loop of the protein (Fig. 5D). Hereafter, we refer to *uta¹* as *ptch2^uta1* in accordance with zebrafish nomenclature guidelines.

Hh Pathway Activity Is Expanded in *ptch2^uta1* Mutants

Patched2 is a negative regulator of the Hedgehog (Hh) pathway, functioning to inhibit Smoothened and thereby block Hh pathway activity in the absence of Hh ligands. When Patched function is deficient, Hh pathway activity is constitutively active in a ligand-independent manner, and Hh target gene expression is expanded.[19] To test this model in *ptch2^uta1* mutants, *ptch2* expression was examined, as this is a useful readout for Hh pathway activity.[19-23] In wild-type embryos, *ptch2* is expressed in the optic stalk at 28 hpf (Figs. 6A, 6C), as well as throughout the ventral brain and the somites[20] (data
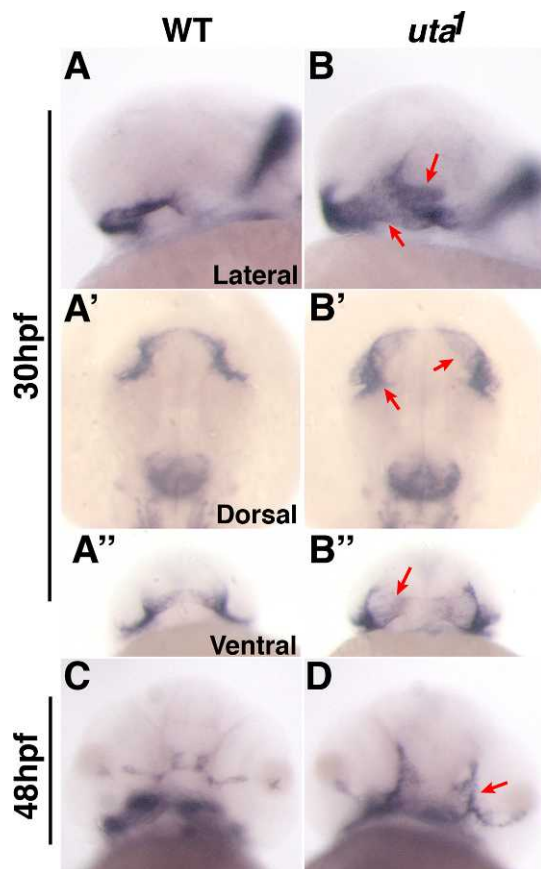
FIGURE 4. Optic stalk formation is disrupted in *uta¹* mutants. *Pax2a* expression marks the optic stalk at (**A**, **B**) 30 hpf and (**C**, **D**) 48 hpf in (**A**, **C**) wild-type and (**B**, **D**) *uta¹* mutant embryos. (**B**) *pax2a* expression in *uta¹* mutants is expanded within the optic stalk region at 30 hpf (*arrows*). **A'** and **B'**) are dorsal views, **A"** and **B"** are ventral views. (**D**) *pax2a* expression is maintained at 48 hpf and extends into the retina (*arrow*) when compared with wild-type siblings (**C**).

not shown). In *ptch2^uta1* mutants, however, *ptch2* expression appears in a substantially broader domain of expression in each of these regions (Figs. 6B, 6D). These data support a model in which Hh pathway activity is expanded as a result of loss of Ptch2 function in *ptch2^uta1* mutants.

To directly test the hypothesis that upregulation of Hh pathway activity is the molecular mechanism underlying colobomas in *ptch2^uta1* mutants, we used cyclopamine, a pharmacological inhibitor of the Hh pathway that acts on Smoothened, downstream of the Patched receptor,[24,25] and examined whether low doses of cyclopamine were capable of suppressing colobomas in *ptch2^uta1* mutants, an assay that has been performed successfully with *ptch2^tc294z* mutants,[15] as well as with mutations in other negative regulators of the Hh pathway (e.g., *ptch1/lep* and *Hip/uki*[26]). Low levels of cyclopamine (4 μM) did not lead to overt defects in eye development (Figs. 6E, 6F); however, exposure of *ptch2^uta1* mutants to 4 μM of cyclopamine between 5.5 hpf and 24 hpf was completely effective in suppressing colobomas (Fig. 6G). In vehicle-treated controls, 24.4% ± 1.3% possessed colobomas, whereas treatment with 4 μM cyclopamine completely suppressed the incidence of colobomas in mutant embryos (0%; $n = 3$ biological replicates, $P < 0.005$). These data indicate that disrupted Hh signaling pathway activity likely underlies coloboma formation in *ptch2^uta1* mutants.

DISCUSSION

We have performed a three-generation ENU mutagenesis screen in zebrafish to identify recessive mutations affecting development of the eye. Our screen identified 18 mutations that affected a variety of ocular tissues, and these mutants will be useful in better understanding the development and maintenance of these tissues, as well as the mechanistic underpinnings of congenital ocular diseases that affect them. The most prevalent class of mutations identified in the screen was those that affected the formation of the lens and/or resulted in cataracts, with 10 such mutants recovered. Several detailed descriptions of zebrafish lens morphogenesis and the establishment of cell fates within the zebrafish lens have been published in the past few years,[27–29] and these, in combination with mutagenesis screens targeting lens mutants, highlight the utility of zebrafish in furthering our understanding of the mechanisms facilitating vertebrate lens development and cataractogenesis. Indeed, recent studies using zebrafish mutants have begun to elucidate roles for DNA methyltransferase activity during lens development,[30] and in vivo roles for the ubiquitin proteasome system during lens epithelial cell proliferation and lens fiber differentiation in zebrafish,[31] highlighting the utility of zebrafish in identifying novel components required for normal lens development and maintenance in vivo.

In addition to lens/cataract mutants, numerous other mutants with ocular phenotypes of interest were identified in the screen (i.e., microphthalmic mutants, mutants with photoreceptor defects, coloboma mutants, and oculocutaneous albinos). Each of these lines will be useful in modeling human congenital ocular disorders and thereby gaining mechanistic insight into the underlying cellular and molecular underpinnings of these diseases. Recent technological advances in zebrafish, using whole-genome sequencing and single nucleotide polymorphism mapping algorithms, have enabled the rapid mapping and cloning of recessive ENU mutations, eliminating in many cases the need for more time-consuming PCR-based positional cloning strategies.[6–9] Historically, identification of the affected locus in zebrafish mutants has been the rate-limiting step in postscreen analysis of mutants; therefore, these recent advances in cloning techniques also promise to rapidly increase the number of cloned mutations, and thus the number of zebrafish ocular disease models. These advances will undoubtedly contribute to identifying mutations in zebrafish orthologs of previously unidentified disease-causing genes, but also to identifying additional alleles of existing mutations, which will facilitate in vivo structure-function analyses of the affected proteins.

With this latter point in mind, we have identified a new *patched2* mutant allele, *ptch2^uta1*, in which a splice-acceptor site mutation leads to an in-frame deletion of 19 amino acids from the Ptch2 protein. The deleted region of the protein is predicted to contribute to the first extracellular loop of Ptch2, and this loop is thought to function, in conjunction with the second extracellular loop, in binding of Hh ligands[32] (Fig. 5D). In the absence of Hh ligands, Patched proteins normally function to repress Hh pathway activity by inhibiting the Smoothened protein. On binding of the Hh ligand to Patched, the inhibition on Smoothened is relieved and the Hh pathway becomes activated.

Hh signaling regulates a number of diverse aspects of eye development,[33,34] including two key developmental roles during early eye development. The first of these is in segregating the eye field into two bilateral optic vesicles (OVs),[35,36] and the second is in promoting proximal OV fates (optic stalk, choroid fissure) at the expense of distal OV fates (retina, RPE, lens). Loss of Hh signaling results in holopro-
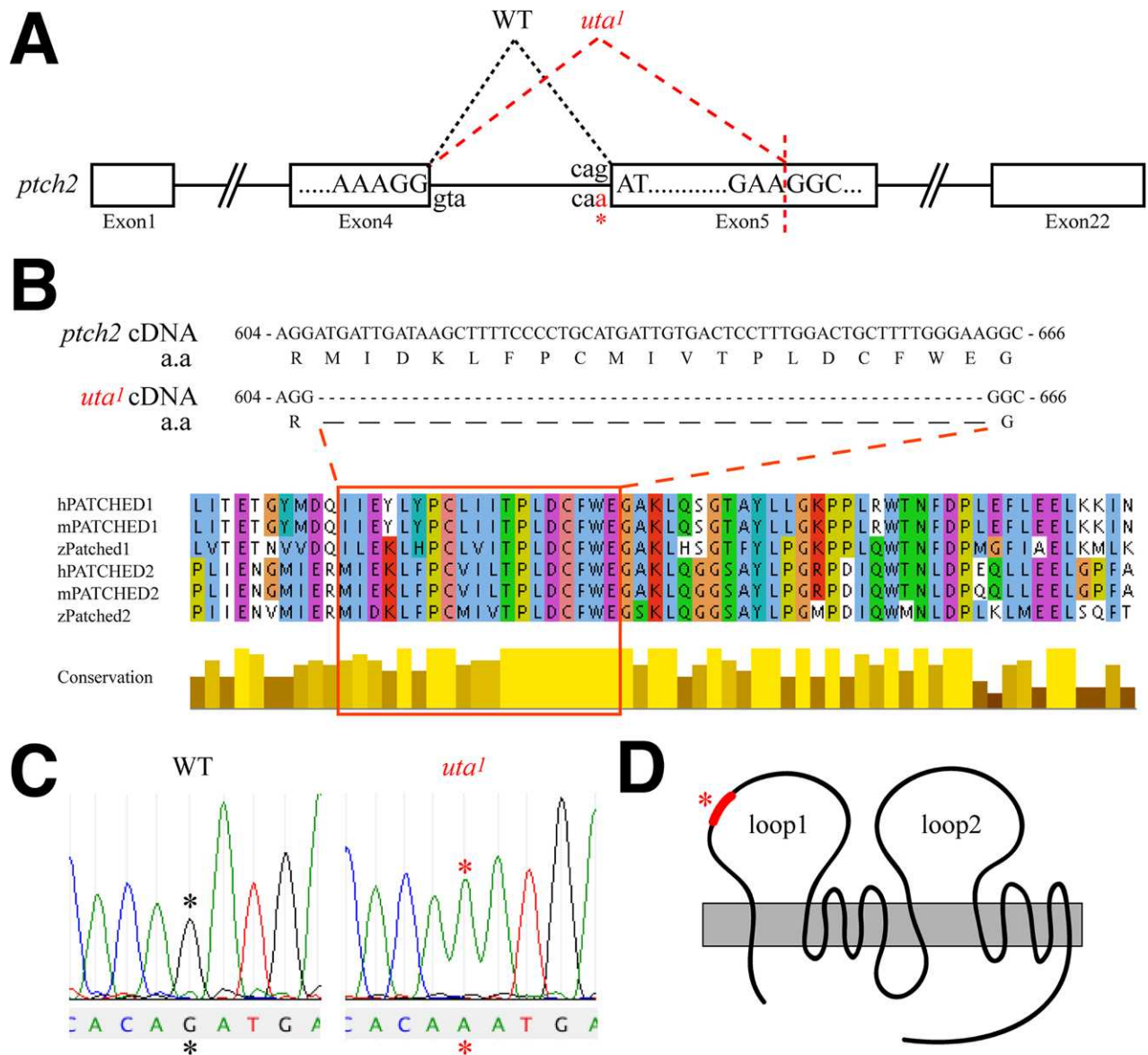
FIGURE 5. *uta¹* mutants possess a splice-acceptor site mutation in *patched2*. (**A**) Schematic of the *ptch2* genomic locus. *uta¹* mutants possess a G → A mutation at the Intron 4 splice-acceptor site (*asterisk*), and a cryptic splice site is recognized in exon 5. (**B**) Mis-splicing of *ptch2^uta¹* results in the excision of 57 bp of *ptch2*. These excised bases encode 19 amino acids that are highly conserved between *ptch1* and *ptch2* orthologs in human (h), mouse (m), and zebrafish (z). (**C**) Genomic sequence traces from wild-type and *uta¹* mutants indicating the Intron 4 mutation (*asterisk*). (**D**) The 19 amino acids absent from ptch2^uta¹ are predicted to contribute to the first extracellular loop of ptch2 (modified from Briscoe et al.[18]).

sencephaly in human patients and animal models,[37,38] whereas overexpression of Shh in a number of model systems results in expansion of proximal OV fates at the expense of distal ones.[39–43] *ptch2^uta¹* mutants possess an expansion of proximal fates (Fig. 4) and expanded Hh pathway gene expression (Fig. 6), supporting a model in which the *ptch2^uta¹* mutation leads to upregulated Hh pathway activity. Indeed, suppression of colobomas in these mutants by cyclopamine supports such a mechanism (Fig. 6G). Two other *ptch2* alleles have been identified in zebrafish, *ptch2^tc294z* and *ptch2^bu1602*, and both of these also show elevated Hh pathway activity.[15,17] In *ptch2^uta¹* and *ptch2^bu1602* mutants, colobomas are highly penetrant, averaging 21% to 25% per clutch, whereas *ptch2^tc294z* is thought to be a hypomorphic allele due to the low penetrance of the colobomas, averaging 3% to 5% per clutch.[15,17]

    Given the unique mutations in each of these alleles, and what has been learned from a number of biochemical studies

on the Patched proteins, it is interesting to speculate on how these mutations might affect Ptch2 structure and function, and thereby influence Patched-dependent Hh pathway regulation. As discussed above, *ptch2^tc294z* mutants have a truncation of the Ptch2 protein just after the eighth transmembrane domain. The C-terminus of Patched mediates multimerization with other Patched subunits, with a trimeric complex of Patched thought to be the plasma membrane localized and physiological state of the receptor.[44] C-terminal deletions of Patched result in ligand-independent activation of the Hh pathway,[15,17,19] despite their ability to bind to Hh proteins,[19] supporting a model in which the C-terminus of Patched is required to inhibit Smoothened activity. The results of these studies suggest a model in which the low penetrance and hypomorphic nature of the *ptch2^tc294z* allele may be a result of the mutant protein only partially impairing the ability of the remaining subunits of the trimeric complex to interact with
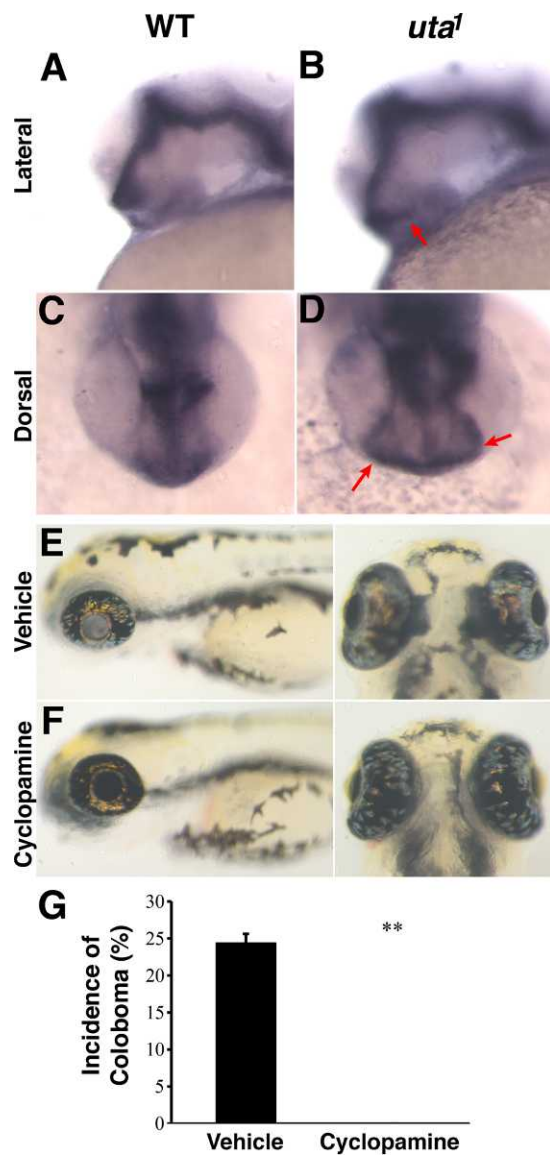
WT uta1

A Lateral
B
C Dorsal
D
E Vehicle
F Cyclopamine
G

FIGURE 6. Hh pathway activity is upregulated in *ptch2^uta1* mutants and results in colobomas. (**A–D**) *ptch2* expression serves as a marker of regions of Hh pathway activity. (**A**, **C**) At 28 hpf, *ptch2* is expressed in the optic stalk and regions of the brain. (**B**, **D**) Expression in *ptch2^uta1* mutants is expanded in each of these regions (*arrows* in [**B**, **D**] – optic stalk). (**E**, **F**) *ptch2^uta1* vehicle-treated (EtOH) control mutants possess colobomas (**E**), whereas those treated with 4 μM cyclopamine display normal choroid fissure closure and eye development (**F**). (**G**) 24.4% ± 1.3% of vehicle-treated (EtOH) controls possessed colobomas, while treatment with 4 μM cyclopamine completely suppressed the incidence of colobomas in mutant embryos (0%; **$P < 0.005$) ($n = 3$ biological replicates, aggregate data: 27/110 embryos with colobomas in vehicle-treated controls, 0/99 embryos with colobomas in 4 μM cyclopamine-treated group).

and inhibit Smoothened, or the emergence of phenotypes either when the trimeric complex contains two or three mutant subunits, or when a threshold number of trimeric complexes contain mutant subunits is surpassed, thereby preventing Smoothened inhibition in the absence of ligand.

ptch2^uta1 and *ptch2^bu1602* mutants, on the other hand, must behave in an entirely different mechanism, as the mutations are not predicted to disrupt the ability of the Ptch2 protein to interact with Smoothened. The *ptch2^uta1* mutation removes a portion of the protein predicted to constitute part of the first

extracellular loop that facilitates Hh binding, whereas the *ptch2^bu1602* mutation inserts 27 amino acids into the protein, possibly also residing in this Hh-binding loop.[17] Deletion of either of the extracellular loops prevents Hh binding[32]; moreover, deletion of most of the second loop of Patched (PtchΔ^Loop2) results in a cell-autonomous dominant-negative blockade of Hh pathway activity in expressing cells.[18] Phenotypes in *ptch2^uta1* and *ptch2^bu1602* mutants represent a gain-of-function in Hh pathway activity (i.e., an expansion of *pax2a* and *ptch2* expression, phenotypic rescue by cyclopamine exposure), however. Mechanistically, it is possible that the *ptch2^uta1* encoded mRNA or protein is unstable, and that the *ptch2^uta1* allele is a protein null. Our data do not support the former of these, as *ptch2^uta1* mRNA is detected in mutant embryos by RT-PCR (Fig. 5). No cross-reacting antisera have been identified that recognize zebrafish Ptch2 and therefore the latter of these possibilities cannot be ruled out. Alternatively, the *ptch2^uta1* and *ptch2^bu1602* mutations could be activating mutations, mimicking Hh binding, and relieving Patched-mediated inhibition of Smoothened activity in the absence of ligand. This would be interesting, as it would be informative in understanding how Hh binding is transduced through Patched to effect downstream signaling events in the Hh pathway. A detailed biochemical analysis of the mutant proteins will be required to address these possibilities in the future.

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