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Col4a1 mutation causes endoplasmic reticulum stress and genetically modifiable ocular dysgenesis

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Ocular anterior segment dysgenesis (ASD) is a complex and poorly understood group of conditions. A large proportion of individuals with ASD develop glaucoma, a leading cause of blindness resulting from retinal ganglion cell death. Optic nerve hypoplasia is thought to have distinct causes and is a leading cause of blindness in children. Here, we show that a mutation in the type IV collagen alpha 1 (*Col4a1*) gene can cause both ASD and optic nerve hypoplasia. COL4A1 is a major component of almost all basement membranes. The mutation results in non-secretion of the mutant COL4A1 proteins, which instead accumulate within cells. Basement membrane abnormalities may, therefore, contribute to the phenotype. The mutation also induces endoplasmic reticulum stress and so intracellular stress may contribute to pathogenesis. The overall consequence of the *Col4a1* mutation depends on genetic context. In one genetic context, the mutation causes severe ASD with intraocular pressure abnormalities and optic nerve hypoplasia. In a different genetic context, both the ASD and optic nerve hypoplasia are rescued, and we have identified a single dominant locus that confers the phenotypic modification.

INTRODUCTION

Glaucoma describes a heterogeneous group of neurodegenerative diseases where death of retinal ganglion cells (RGCs) leads to vision loss (1). One of the strongest known risk factors for glaucoma is an elevated intraocular pressure (IOP). However, some patients have normal-tension glaucoma where death of RGCs occurs in the absence of detected IOP elevation (2). Lowering IOP can often slow disease progression, even in normal tension glaucoma patients (3). This suggests that normal tension glaucoma patients have RGCs that are susceptible to pressure-related death even at normal IOPs. Together, this illustrates that glaucoma is a complex disease where factors that influence IOP regulation and factors that determine ganglion cell viability interact to influence the final course of the disease.

IOP is a balance of aqueous humor production, by the ciliary body (CB), and aqueous humor drainage. Aqueous humor drains through the trabecular meshwork and Schlemm's canal in the iridocorneal angle and through the uveoscleral drainage pathway. Dysgenesis of the ocular anterior segment can impede aqueous humor outflow and lead to IOP elevation. Consequently, patients with anterior segment dysgenesis (ASD) are at an elevated risk for developing glaucoma. A number of genes have been identified in which mutations lead to ASD in human patients and in mice, but the precise pathogenic mechanisms remain largely unknown (4). Pathogenic alleles of developmental genes often cause a spectrum of ocular phenotypes that vary in severity (5). It is possible that some of these same genes contribute to age-related, open angle glaucoma, where the ocular drainage structures have abnormalities that are not clinically visible but that cause dysfunction with age. Similarly, genes influencing survival of RGCs during development (where severe mutations might lead to optic nerve aplasia or hypoplasia) may modulate RGC susceptibility to glaucoma. Therefore, continued characterization of factors influencing ocular development and dysgenesis may identify new pathways and processes important

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for age-related ocular diseases. Integration of this information will be important for understanding specific disease processes leading to glaucoma susceptibility.

Phenotype-driven approaches represent an unbiased mechanism to identify new genetic factors and biological pathways underlying disease processes. In a mutagenesis screen performed to identify mice with abnormalities in IOP regulation, we discovered a semi-dominant mutation in type IV collagen alpha 1 (*Col4a1*) (6). The mutation is within the triple helical domain of the COL4A1 protein and leads to an inhibition of secretion of both COL4A1 and its binding partner COL4A2. COL4A1 is the most abundant and ubiquitous basement membrane protein, and the mutation has pleiotropic effects (7). In the eye, COL4A1 is present in the basal lamina of the conjunctiva, corneal epithelium, corneal endothelium, trabecular meshwork, Schlemm's canal, lens, CB, retinal inner limiting membrane (ILM), Bruch's membrane and vascular basement membranes (8–11).

Here, we show that on the C57BL6/J genetic background $Col4a1^{\Delta ex40}$ mice have severe ocular dysgenesis including ASD and optic nerve hypoplasia. IOP in the mutant mice is variable with mice exhibiting both high and low pressures. To begin to understand the pathogenic mechanism(s) and to identify genes that might interact with *Col4a1* in ocular development, we tested other genetic backgrounds to determine if they modify the phenotypes. We found that they do and we mapped a dominant CAST/Ei derived modifier locus to Chromosome 1.

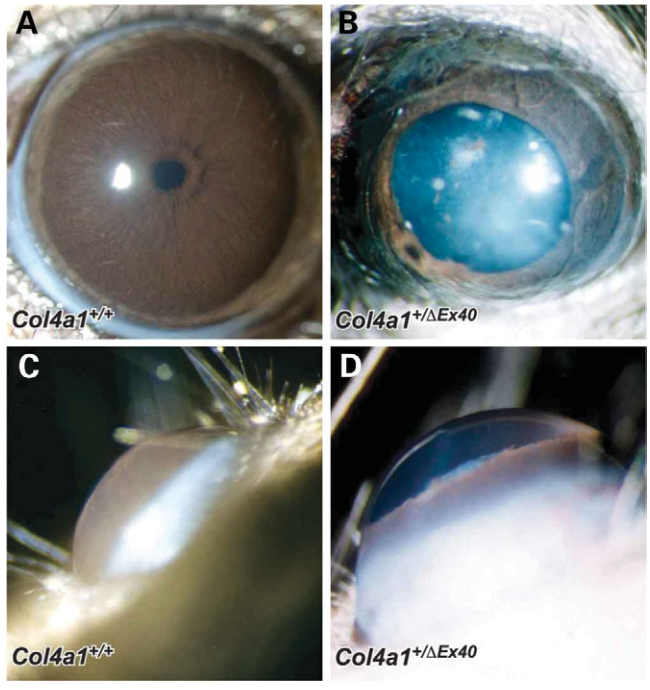

Figure 1. *Col4a1* mutant mice have ASD. Representative images from slit lamp examination of eyes from control (**A,C**) and mutant (**B,D**) mice show severe dysgenesis of the anterior segment in mutant mice including open pupil, cataracts, pigment dispersion, corneal opacification, enlarged and tortuous iris vasculature and buphthalmos (not shown here are iridocorneal attachments).

RESULTS

Mutation of *Col4a1* causes ASD

We performed a random embryonic stem cell mutagenesis screen to identify genes that contribute to glaucoma pathology (12). We identified a mutant lineage with ocular ASD and buphthalmos (enlargement of the eye), and we determined that the causative mutation was in a splice acceptor site of *Col4a1* resulting in the absence of exon 40 from the mature transcript ($Col4a1^{\Delta ex40}$) (6). $Col4a1^{\Delta ex40/\Delta ex40}$ mice are not viable, and $Col4a1^{+/\Delta ex40}$ mice have decreased viability. On the C57BL/6J genetic background, all surviving $Col4a1^{+/\Delta ex40}$ mice have clinically obvious ASD. The phenotype is variable and includes all tissues of the anterior segment. Some combination of buphthalmos, corneal opacification, pigment dispersion, iridocorneal synechiae (attachments of the iris to the cornea), cataracts, persistence of tunica vasculosa lentis and abnormal iris vasculature is present in all mutant mice (Fig. 1).

To understand how $Col4a1^{\Delta ex40}$ caused ASD, we determined the age-at-onset of observed dysgenesis. Histologic analysis of eyes from control and mutant embryos at embryonic day (E) 16.5 revealed anterior hyphema (hemorrhage in the anterior chamber) in five of six eyes from $Col4a1^{+/\Delta ex40}$ mice but not in control mice (Fig. 2). At E18.5, remnants of anterior hyphema were still present. Despite the hyphema, the overall anterior segment morphology, including the iridocorneal angle, was indistinguishable between control and mutant mice until birth. The iridocorneal angle in mice continues to develop postnatally (4,13). Histologic analysis of anterior segments of control and mutant mice at postnatal day (P) 10 and P20 revealed extensive iridocorneal adhesions and severe dysgenesis of the iridocorneal angle (Fig. 2). The CB, the site of aqueous production, also appeared smaller and less foliated than in control mice.

Abnormal IOP in *Col4a1* mutant mice

Buphthalmos and extensive iridocorneal adhesions in $Col4a1^{+/\Delta ex40}$ mice led us to hypothesize that they had ocular hypertension. To test this, we measured IOP in $Col4a1^{+/\Delta ex40}$ and age-matched control littermates at approximately 1.5 and 3.0 months of age—ages when buphthalmos is observed (Fig. 3). Compared with control mice, $Col4a1^{+/\Delta ex40}$ mice showed a broader distribution of IOP values. At 1.5 months, over half of the mutant mice (34/65) had IOPs higher than the highest control. Some mutant mice had very low IOPs. Five mutant mice at 1.5 months and seven mutant mice at 3 months had IOPs lower than the lowest control at each of these ages. The IOP distributions of mutant and control animals were significantly different at both ages (Fig. 3), demonstrating clearly that *Col4a1* genotype influences IOP.

Col4a1 mutation causes ILM abnormalities and optic nerve hypoplasia

Because the *Col4a1* mutation affects IOP, we hypothesized that some mutant mice would develop age-related, glaucomatous RGC loss. Before assessing glaucoma, it was necessary

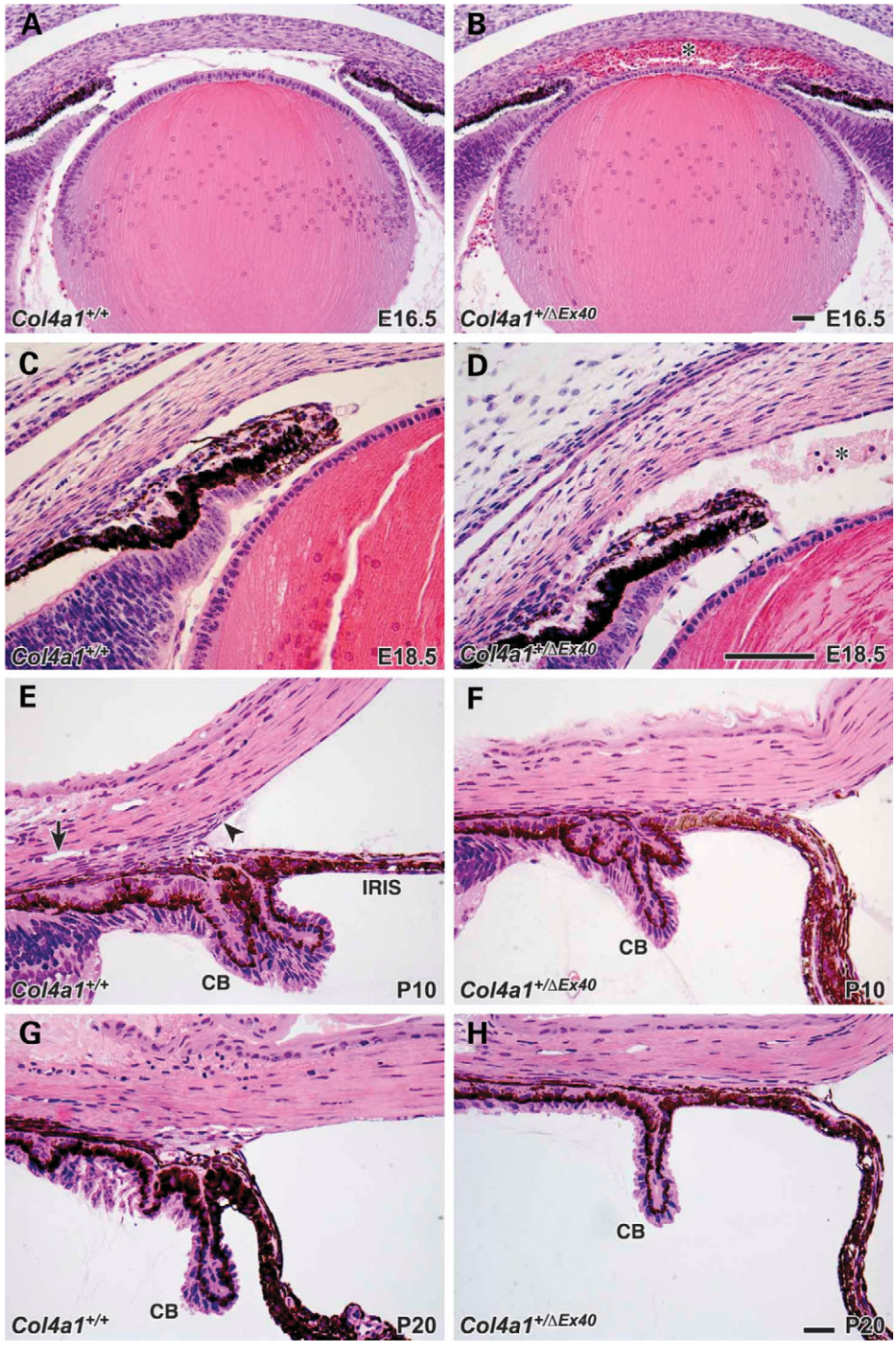

Figure 2. Mutant eyes have anterior hyphema, iridocorneal adhesions and hypoplastic ciliary bodies. (**A–D**) Histological analysis of anterior segment development at E16.5 and E18.5 revealed hyphema (asterisk in B and D) filling the anterior chamber in mutant mice (5 out of 6 eyes) (B,D) that was not present in control mice (A,C). Morphologically, the anterior segment structures were indistinguishable between control and mutant mice at these ages. **(E–H)** All eyes analyzed from P10 and P20 mutant mice showed obvious dysgenesis of the anterior segment. In control eyes (E and G), a robust, foliated CB was present and normal differentiation and remodeling of the iridocorneal angle created identifiable Schlemm's canal (arrow) and trabecular meshwork (arrowhead). In contrast, in mutant eyes, the CB was often small and unfoliated and there were severe and extensive iridocorneal adhesions. Scale bar = 50 μm.

to determine if control and mutant animals had equal numbers of ganglion cells at a young age. Therefore, we counted the cells in the ganglion cell layer of flat mounted retinas from 2-month-old mice. Surprisingly, mutant mice ($n = 6$) had approximately two-thirds the number of cells in the ganglion cell layer compared with control mice (n=4 and $P = 0.0042$; Student's t-test). To confirm that the reduction of cell number involved RGCs, we analyzed optic nerves

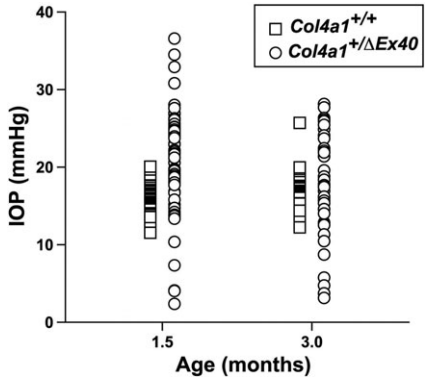

Figure 3. *Col4a1* genotype affects IOP. IOP measurements at 1.5 and 3.0 months revealed that the IOP distribution in mutant mice was substantially increased relative to controls. To determine if the observed differences were significant, we compared the numbers of mice that were below, within and above 2 standard deviations from the mean of control mice. At 1.5 months of age, the numbers of control mice ($n = 82$; mean = 15.5 mmHg; SD = 1.3) in each group were 2, 77 and 3, respectively. Numbers of mutant animals ($n = 65$) in each group were 5, 15 and 45 ($P < 0.001$, χ^2). At 3.0 months of age, the numbers of control mice ($n = 57$; mean = 16.4 mmHg; SD = 1.8) in each group were 1, 55 and 1, respectively. Numbers of mutant animals ($n = 40$) in each group were 9, 17 and 14 ($P < 0.001$, χ^2). There was no correlation between IOP values and the severity of clinically evident ASD in mutant eyes.

and found that mutant mice had a striking reduction of RGC axon number (Fig. 4). Additionally, the optic nerves of mutant mice had cross-sectional areas that were significantly reduced compared with control mice. Further analysis revealed that mutant optic nerves were dysgenic as early as P10, supporting a developmental and not acquired mechanism of hypoplasia.

Col4a1 is not expressed by RGCs but the protein is a component of the adjacent ILM. We hypothesized that RGC viability and optic nerve hypoplasia could be secondary to changes in the ILM. Consistent with our hypothesis, we observed molecular changes in the ILM in mutant animals compared with control animals (Fig. 4). *Col4a1* mutant animals labeled positively for type XII collagen (a fibril-associated collagen not previously seen in the ILM), whereas control animals were negative for this collagen. These data demonstrate that there are molecular differences, either structural or compositional, in the ILM between control and *Col4a1* mutant animals.

Mutant *Col4a1* causes ER stress

We sought to understand the pathogenic processes that lead to ocular dysgenesis caused by $Col4a1^{\Delta ex40}$. Previously, we showed that the *Col4a1* mutation inhibited secretion of COL4A1/COL4A2 heterotrimers into Reichert's membrane of mutant embryos (6). To determine if collagen non-secretion also occurred in eyes, we performed immunohistochemical labeling in eyes of control and mutant mice. We determined that $Col4a1^{+/\Delta ex40}$ mice have an accumulation of non-secreted COL4A1, most notably in the lens epithelial cells (Fig. 5). Consistent with this, we observed molecular differences in two ER resident proteins, heat shock protein 47 (HSP47) and protein disulfide isomerase (PDI). HSP47 is a collagen-specific protein chaperone and PDI is a general oxidoreductase that, as the beta subunit of prolyl-4-hydroxylase, participates in hydroxylation of prolyl residues on collagens. Both HSP47 and PDI show increased labeling in $Col4a1^{+/\Delta ex40}$ eyes compared with $Col4a1^{+/+}$ eyes (Fig. 5). We hypothesized that the accumulation of mis-folded mutant COL4A1 proteins might cause endoplasmic reticulum stress. Indeed, labeling of a marker of ER stress was strongly increased in $Col4a1^{+/\Delta ex40}$ eyes compared with $Col4a1^{+/+}$ eyes (Fig. 5). Thus, our data indicate that $Col4a1^{\Delta ex40}$ inhibits

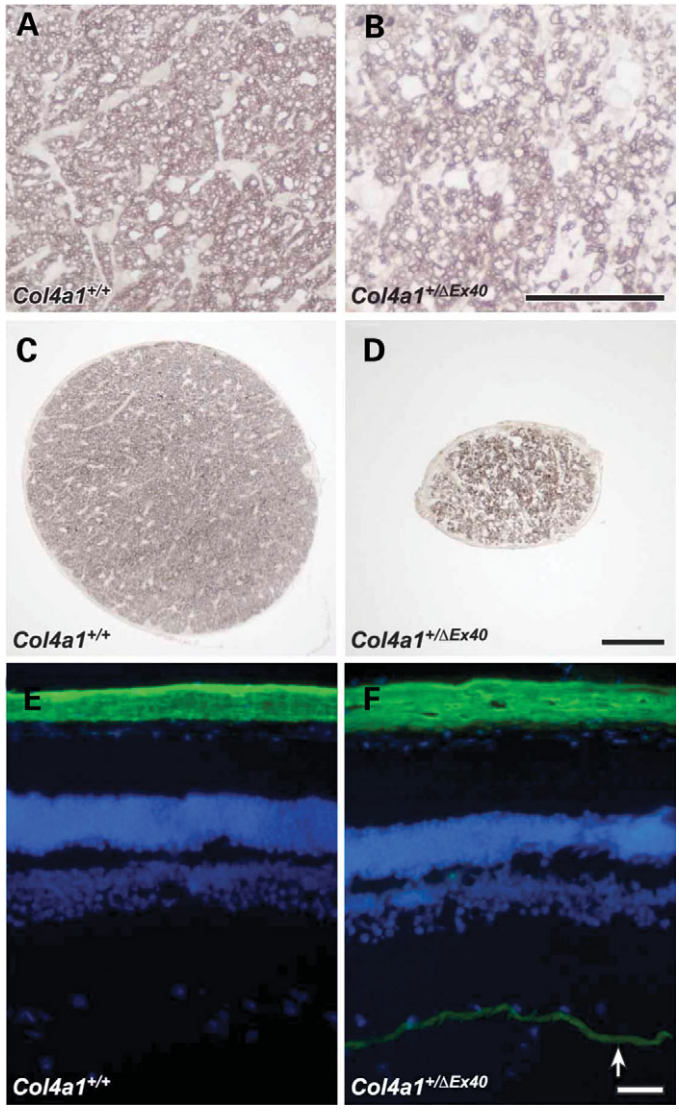

Figure 4. $Col4a1^{\Delta ex40}$ mice have optic nerve hypoplasia. (**A, B**) Histological analysis of optic nerves from control and mutant mice revealed severe axon depletion and (**C,D**) a reduction in cross-sectional area in mutant mice (at each age tested, $P < 0.0001$; Student's *t*-test comparing areas). Mean cross-sectional areas (+/− SEM) for control and mutant mice, respectively, at 1.5, 3.0 and 5 months of age were 0.099 mm^2 (0.002 mm^2, $n = 16$) and 0.065 mm^2 (0.004 mm^2, $n = 18$); 0.121 mm^2 (0.002 mm^2, $n = 14$) and 0.069 mm^2 (0.004 mm^2, $n = 14$); 0.130 mm^2 (0.002 mm^2, $n = 16$) and 0.064 mm^2 (0.009 mm^2, $n = 12$). (**E,F**) Immunohistochemical labeling with an antibody for collagen XII revealed positive labeling of the ILM of the retina (white arrow) in mutant animals that was not detected in eyes from control animals. Scale bar = 50 μm.

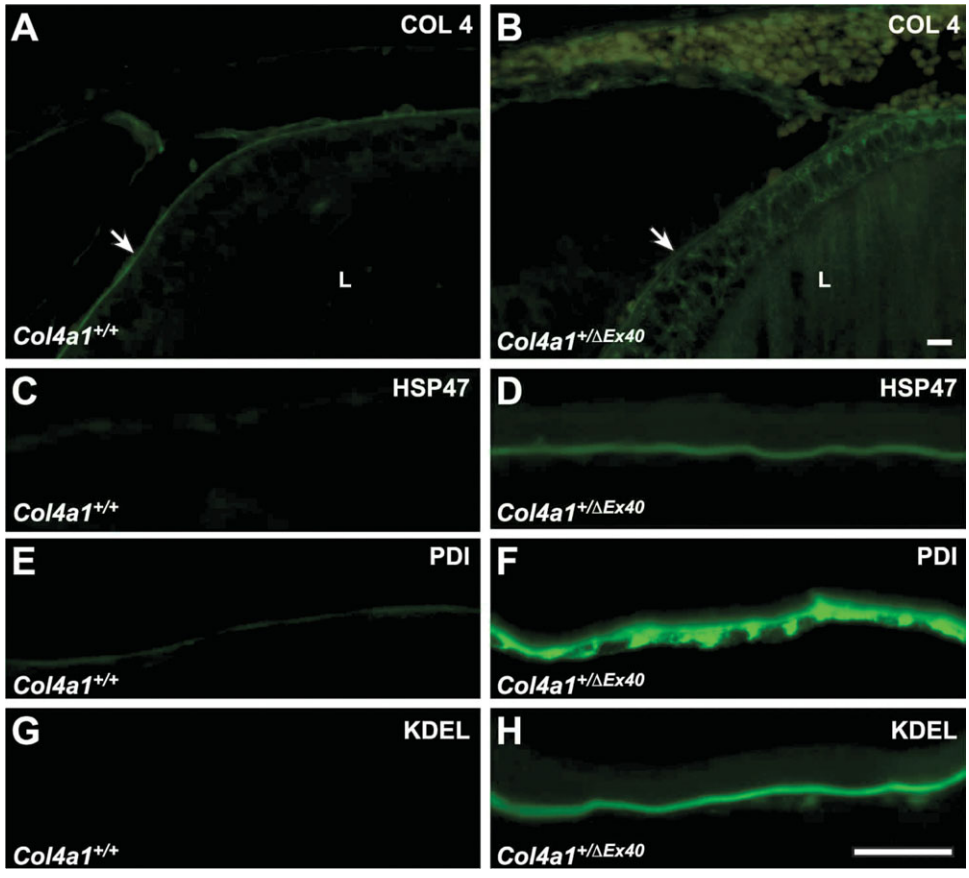

Figure 5. Mutant COL4A1 accumulates in lens epithelium and causes ER stress. (**A,B**) Imunnohistochemical labeling of the developing lens (E16.5) with antibodies for collagen IV showed strong labeling of the lens capsule (arrow) but no other lens (L) structures in control mice (A). In contrast, mutant mice (B) showed strong labeling throughout the lens epithelial cells suggesting a substantial amount of the protein has not been secreted. (**C**–**F**) In 2 month old mice, the collagen-specific chaperone, HSP47 (C,D), and the protein disulfide isomerase/prolyl hydroxylase subunit, PDI (E, F), also show significant differences between mutant and control mice, consistent with an abnormality in secretion of COL4A1. (**G**,**H**) Differential labeling with an antibody for the KDEL epitope indicates presence of ER stress in lens epithelial cells in mutant mice (H) but not in control mice (G). Scale bar = 50 μm.

collagen secretion in the eye and that the observed ASD may be due to decreased collagen secretion and/or ER stress.

Ocular dysgenesis is genetically modifiable

Ocular dysgenesis is genetically complex and the degree to which dysgenesis occurs can vary greatly depending on genetic context (5,13–15). To determine if *Col4a1* mutant phenotypes could be genetically modified, we crossed mice that were congenic for the mutation on a C57BL/6J genetic background to mice of two different inbred strains (129/SvEvTac and CAST/EiJ) and analyzed the F1 progeny. In both cases, the F1 progeny were profoundly modified as determined by slit lamp analysis with the CASTB6F1 mutant mice appearing almost indistinguishable from control mice (Fig. 6). Although appearing largely normal, 129B6F1 and CASTB6F1 mutant mice had mild enlargement of the anterior chamber suggesting that there may be sub-clinical, morphological abnormalities of the iridocorneal angles. Histologic analysis confirmed the presence of a mild degree of iridocorneal angle dysgenesis (Fig. 7). Despite this, preliminary data suggest that IOPs of young mutant 129B6F1 and CASTB6F1 animals are not elevated compared with control animals of each strain (data not shown). To determine if the 129/SvEvTac and CAST/EiJ backgrounds could also rescue optic nerve hypoplasia, we examined cross-sections of the optic nerves of control and mutant 129B6F1 and CASTB6F1 mice. Compared with age-matched littermate controls, mutant mice from each background did not demonstrate differences in optic nerve morphology (Fig. 7).

Clinical and histologic rescue of ASD was more complete in CASTB6F1 mice than in 129B6F1 mice. Therefore, we used CASTB6 mice to test whether there is a strong modifier locus. By using the appropriate crosses and genetic mapping (see Materials and Methods), we identified a single dominant locus on mouse Chromosome 1 that segregated with rescue of ASD (Fig. 8). Genotyping of individual mice for markers throughout the chromosomal region revealed a 38 Mb critical interval between markers D1Mit211 and D1Mit303 that strongly associates with the rescued phenotype and likely contains the modifier gene(s).

DISCUSSION

Here, we show that mutation of a gene, *Col4a1*, that encodes a major component of basement membranes causes genetically

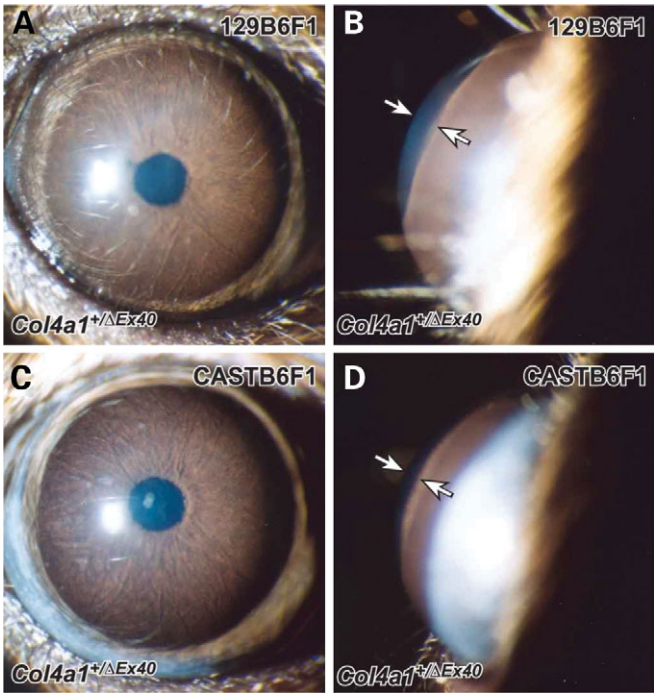

Figure 6. ASD is altered by genetic context. Mutant mice were crossed a single generation to either 129/SvEvTac (**A,B**) or CAST/EiJ (**C,D**) mice. Severe ASD observed on the C57BL/6J genetic background (Fig. 1) was profoundly modified in both of these crosses with the CAST/EiJ strain having a slightly greater rescue. Modified mutant mice from the 129/SvEvTac crosses occasionally had slight corneal haze and persistence of the pupillary membrane, but these phenotypes were too mild to be detected in these photographs. Mice from both crosses also had slight enlargement of the anterior chambers (between arrows in B and D).

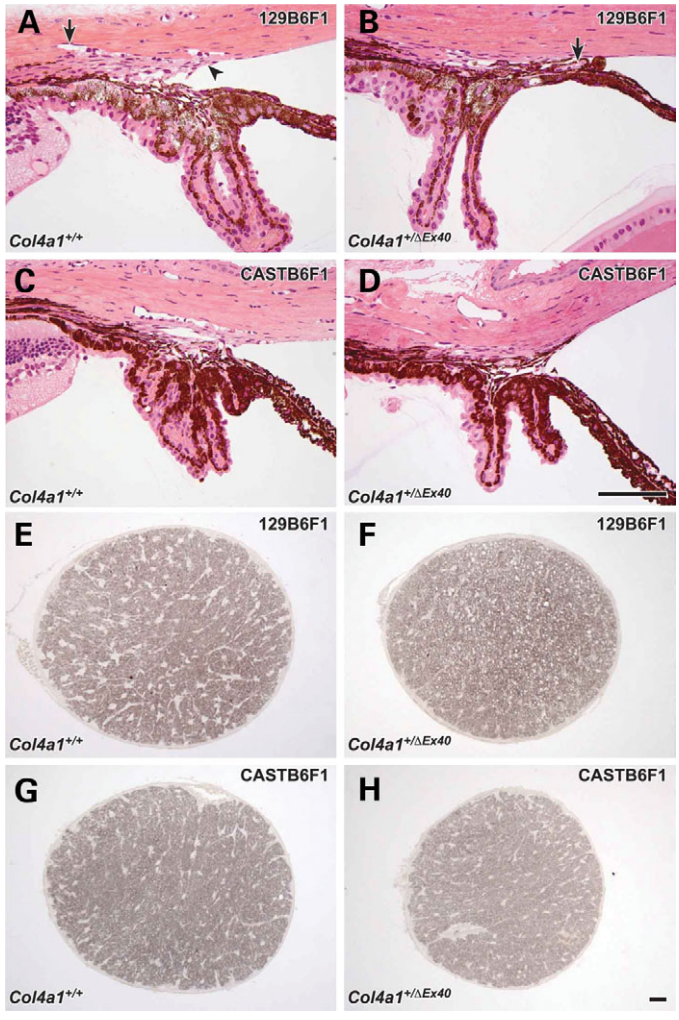

Figure 7. Severe iridocorneal adhesions and optic nerve hypoplasia are genetically modified. The iridocorneal angles of 129B6F1 mice (**A,B**) and CASTB6F1 mice (**C,D**) indicate that iridocorneal angle development is modified by genetic background. 129B6F1 and CASTB6F1 mutant eyes (B and D, respectively) were much more normal in appearance than C57BL/6J mutant mice (compare with Fig. 3). However, they were still not completely normal when compared with strain matched control eyes (A and C, respectively; arrow in A indicates Schlemm's canal, arrowhead in A indicates trabecular meshwork). Mutant eyes had TM that appeared less robust than in control mice and had focal attachments of the iris to the TM and cornea (arrow in B). Optic nerve cross-sections from 129B6F1 (**E,F**) and CASTB6F1 (**G,H**) showed rescue of the optic nerve hypoplasia that was observed in C57BL/6J mice (compare with Fig. 4). Even though some were a little smaller, optic nerves from 129B6F1 (F) and CASTB6F1 (H) mutant mice were very similar to those of age matched control nerves (E and G, respectively). Quantification of optic nerve cross-sectional areas from control ($0.092\ mm^2 \pm 0.004\ mm^2$, $n = 7$) and mutant ($0.086\ mm^2 \pm 0.006\ mm^2$, $n = 9$) CASTB6F1 mice revealed considerable overlap with no significant difference in area ($P = 0.41$ Student's t-test). Scale bar = 50 μm.

complex ocular dysgenesis. The ASD includes extensive and severe iridocorneal adhesions that presumably block aqueous humor drainage from the anterior chamber and lead to elevated IOP in over 50% of mutant mice. This is similar to the proportion of human ASD patients that advance to glaucoma. The *Col4a1* mutation also leads to optic nerve hypoplasia on the C57BL/6J background. Because optic nerves are severely dysgenic on this background, it is difficult to study glaucoma. Although the ASD and optic nerve hypoplasia are genetically complex, we have determined that a single, CAST/EiJ-derived, dominant locus on Chromosome 1 can rescue these phenotypes.

Mutations in a number of genes that lead to ASD have been identified, but the primary pathogenic mechanisms remain largely unknown. Many of the identified ASD-related genes are transcription factors (1,4). Including the current study, another class of molecules is emerging that cause ASD in model systems. Mutations in basement membrane components cause ASD in zebrafish, mice and humans (16–19). We have previously hypothesized that mutations in ASD-causing transcription factor genes might lead to pathogenesis via extracellular matrix molecules (4). For example, PITX2 is known to directly regulate an important collagen-processing enzyme (20) and LMX1B is known to directly regulate basement membrane collagens *Col4a3* and *Col4a4* (21).

Several possible mechanisms may explain the *Col4a1*$^{\Delta ex40}$ mutant phenotype. *Col4a*$^{\Delta ex40}$ might cause ASD as a secondary response to anterior hyphema. Similar to the *Col4a1* mutants, mice with a targeted mutation of the laminin gamma 1 gene also had anterior hyphema (22). However, those mice died perinatally, before morphological abnormalities may have occurred. In our study, anterior hyphema was

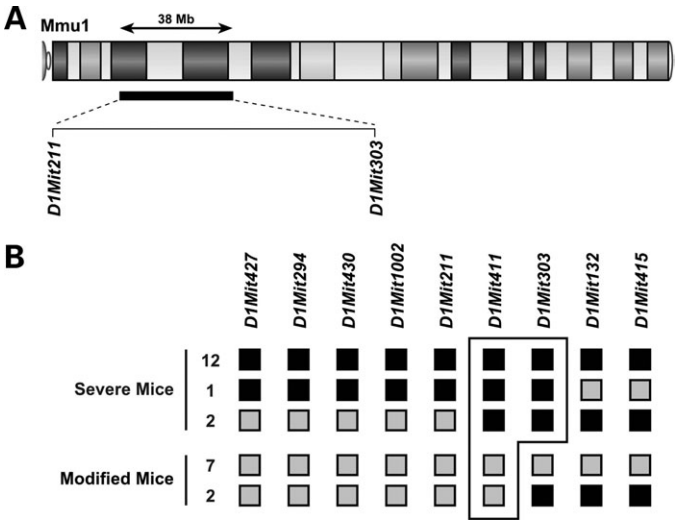

Figure 8. Genetic localization of modifier locus from the CAST/Ei strain. (**A**) Schematic of mouse Chromosome 1 showing the relative location of the modifier locus. (**B**) Marker analysis of 29 individual mice identified a critical interval of 38 Mb between D1Mit211 and D1Mit303. Black boxes indicate homozygosity for C57BL/6J alleles while gray boxes indicate heterozygosity for CAST/EiJ and C57BL/6J alleles. The numbers of mice with each genotype are indicated to the left. Fifteen of 16 severe mice did not inherit the region between D1Mit211 and D1Mit132. This is consistent with a modifier gene(s) at this location. The single severe mouse carrying the modifier suggests that there might be a slight reduction of the modifier effect penetrance. Nine of 13 modified animals were consistent with this same interval and two recombinants refined the interval to between D1Mit211 and D1Mit303. Four mice had a modified phenotype and did not carry this modifier locus suggesting that another modifying factor(s) may still be segregating. Overall, 24 of 29 individually genotyped animals are consistent with a dominant modifier locus between D1Mit211 and D1Mit303.

also observed in some mutant 129B6F1 mice (data not shown). Since ASD is extremely mild in almost all 129B6F1 mice, it is unlikely that anterior hyphema alone causes ASD.

Another potential mechanism for *Col4a1*-induced ASD is that the mutation leads to primary defects within non-vascular ocular tissues. Type IV collagen is a major protein component of the lens capsule (23) and here we have demonstrated that mutant COL4A1 proteins accumulate within the lens epithelial cells and cause ER stress. These data indicate that there are molecular differences in the lens epithelial cells between mutant and control mice. The lens is known to be critical for normal anterior segment development (24) and many ASD-causing genes are involved in proper lens induction or development (reviewed in 4). The observed abnormalities in lens epithelial cells and lens capsule could influence cell migration, the presence or availability of growth factors or the differentiation of specific cell types in the developing anterior segment.

Other affected tissues of the anterior segment are derived largely from neural crest with some contribution from cranial paraxial mesoderm cells. It is possible that the primary pathogenesis directly involves one of these cell populations (25–29). For example, abnormalities in the basal lamina in the neural tube could indelibly change the molecular signature of neural crest cells. Thus, by the time of epithelial to mesenchymal transition, the neuroepithelium/neural crest cells could be destined to produce dysgenic ocular structures. Determining the precise location of primary pathogenesis will require more detailed follow-up experiments.

Optic nerve hypoplasia is a major cause of blindness in children (30,31). Although usually idiopathic, optic nerve hypoplasia has also been associated with central nervous system disorders (32) [including porencephaly (33)], retinal vascular tortuosity (34) and aniridia (35). We have previously reported porencephaly and retinal vascular tortuosity in *Col4a1* mutant mice on the C57BL/6J genetic background (6,7). Here, we show that optic nerve hypoplasia is co-incident with ASD in C57BL/6J mice and both are rescued in 129B6F1 mice and CASTB6F1 mice. Despite this, it is possible that ASD and optic nerve hypoplasia occur via distinct pathogenic mechanisms. Because RGCs do not appear to express *Col4a1* (9,36), pathogenesis must involve a non-cell autonomous mechanism likely involving the ILM. Basement membrane integrity is known to be critical for neural development (37–42). Accordingly, ILM disruptions in chicks and in mice with a mutation in the laminin gamma 1 gene lead to retraction of radial epithelial cells and loss of RGCs (22,43). This is consistent with human studies suggesting that optic nerve hypoplasia is the result of excess loss of RGCs during development rather than a primary failure of differentiation (31). Our data reveal novel expression of type XII collagen in the ILM of the *Col4a1* mutant mice. This collagen has never been observed in the ILM and was visible only in mutant, not control, animals. Further experiments are needed to determine if collagen XII is ectopically produced or if it is a normal ILM component being unmasked in the mutant setting. Regardless of the mechanism, the data demonstrate a structural and/or compositional difference between mutant and control ILMs and suggest that abnormalities of basal lamina components might be an important cause of optic nerve hypoplasia.

Optic nerve hypoplasia in C57BL/6J mutant mice confounds the study of glaucoma. Although we observe morphologically normal optic nerves in young mice on other genetic backgrounds, it is possible the RGCs in these other mice may be predisposed to later death in either a pressure-dependent or pressure-independent manner. The *Col4a1* mutation may alter the ILM creating an abnormal local environment that predisposes to ganglion cell death in adults. Thus, in addition to predisposing to glaucoma by elevating IOP, *Col4a1* mutation might predispose to glaucoma as a susceptibility factor more directly influencing RGC death. Although IOP appears to be rescued in young mice (data not shown), experiments are underway to determine if aged 129B6F1 or CASTB6F1 mice develop elevated IOP and glaucoma. Recently, ASD was reported in mice with different *Col4a1* mutations, and so an important allelic series of mutations is available (16). It was also suggested that those mice developed glaucoma. Although this interpretation may be correct, the presented data do not unambiguously demonstrate glaucoma or rule out developmental optic nerve defects.

The current critical interval for the modifier locus contains 227 genes (according to MGI and NCBI build 36) and so it is premature to speculate about candidates for the modifier gene(s). Diverse functional candidate genes can be hypothesized to have a modifying effect on the $Col4a1^{\Delta ex40}$ phenotype. Among the possible mechanisms for modification are up-regulation of *Col4a1* to overcome the collagen

non-secretion, down regulation of *Col4a1* to alleviate protein accumulation, increased efficiency of protein folding or increased efficiency of mis-folded protein degradation. Recently, modification of kidney disease caused by mutations in *Col4a3* was modified by compensatory up-regulation of the *Col4a6* gene in a strain-specific manner (44). Thus, compensations by other type IV collagens or other basement membrane associated proteins are also possible.

To date, six different *COL4A1* mutant alleles have been reported in human patients (6,7,45). None of these patients had ASD, ocular hypertension or optic nerve hypoplasia. Retinal vascular tortuosity only occurs in *Col4a1* mutant mice of some genetic backgrounds and proteinuria is incompletely penetrant in human patients (7). These observations suggest that the combination of genetic background and possibly the specific mutant allele will determine the phenotype of any particular patient with a *COL4A1* mutation. With this in mind, our study implicates *COL4A1* as an important new candidate gene for optic nerve hypoplasia both with and without other developmental anomalies.

MATERIALS AND METHODS

IOP and clinical examination

Anterior clinical examinations were performed with a slit lamp biomicroscope (Haag-Streit USA, Mason, OH). IOPs were measured as previously described (46,47).

Histological analysis

Embryos were harvested at appropriate ages from timed matings and the eyes were fixed *in situ* with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH = 7.2). Paraffin embedded tissues were sectioned and stained with hematoxylin and eosin (H&E). Postnatally, enucleated eyes were fixed in 4% PFA in 0.1 M phosphate buffer (pH = 7.2), dehydrated in graded ethanol and embedded in fresh Historesin (Leica, Heidelberg, Germany). Twenty-four 1.5 μm sections were analyzed from each of three different ocular regions, with the lens as a landmark (region A, periphery of lens; region B, halfway between lens center and periphery; region C, center of lens and optic nerve head), and stained with H&E. For RGC counts, retinas were flat mounted and Nissl-stained with cresyl violet as described previously (48).

Optic nerves were harvested as follows: immediately after death, the top of the skull and most of the brain were removed, leaving ~1 mm of brain overlying the intact optic nerves. The nerves were fixed in *in situ* with 0.8% PFA and 1.2% glutaraldehyde in 0.1 M phosphate buffer. The nerves were embedded in Embed 812 resin (Electron Microscopy Sciences, Ft. Washington, PA), and 1 μm cross-sections of the orbital end of postorbital-prechiasmal nerve were stained with *p*-phenylenediamine. Cross-sectional areas were determined using Metamorph imaging software.

Immunohistochemistry

For labeling with Goat Anti-Type IV Collagen (Southern Biotechnology Associates Cat# 1340-01), carefully enucleated eyes were fixed in 4% PFA overnight at 4°C and embedded in paraffin. Five-micron sections were treated as follows: slides were deparaffinized in xylene and rehydrated through graded ethanol followed by antigen retrieval with 10× sodium citrate pH = 6.0. Blocking and hybridization steps were all performed in a humid chamber for 1 h at room temperature. All washes were performed three times for 10 min each in phosphate buffered saline (PBS pH = 7.5) at room temperature. Sections were blocked with 5% skim milk in PBS with 0.1% Tween20 for 1 h before primary antibody (1:10) was added to the blocking solution. After washing, a 1:1000 dilution of Alexa Flour 488 Donkey anti Goat (Molecular Probes Cat#A11055) was added. Following the final wash, cover slips were added with Vectashield Hardset Mounting Media with Dapi (Vectashield).

For all other antibodies, carefully enucleated eyes were treated with 4% sucrose/PBS, 15 min followed by 8% sucrose/PBS for an additional 15 min. Tissues were mounted in OCT embedding medium and stored at −20°C. Six-micron sections were cut, washed in PBS and fixed in 4% PFA, 4°C, 5 min. Following PBS washes, sections were treated with 50 mM glycine/50 mM lysine in PBS, 10 min. Sections were blocked with 1%BSA/PBS for 30–60 min and reacted with primary antibodies 2–4 h. Following PBS washes, sections were treated with secondary antibodies 1 h, washed and mounted with Vectashield hardset mounting medium (Vector Laboratories, Inc.). The anti-HSP47 antibody (Cat # SPA-470), anti-KDEL antibody (Cat # SPA-827) and anti-PDI antibody (Cat # SPA-891) are mouse monoclonals obtained from Stressgen Biotechnologies). The anti-type XII collagen antibody is a polyclonal made in rabbit and kindly provided by Dr Manuel Koch. Fluorescent conjugated Alexa Fluor secondary antibodies were obtained from Invitrogen.

Identification of modifier gene(s)

Mutant CASTB6F1 mice were crossed with C57BL/6J background where the mutation typically causes severe ASD. The *Col4a1* mutation was then backcrossed to C57BL/6J. At each generation, we selected for a phenotypically rescued mouse (normal appearance of anterior segment). Thus, we were selecting for the presence of one or more modifier genes from the CAST background. After four generations of backcrossing to C57BL/6J, we produced 70 mutant progeny and divided them into three phenotypic classes; those that were bilaterally severe ($n = 23$), those that were bilaterally modified ($n = 20$) and those that were obviously affected but not severely so ($n = 27$). Equal concentrations of purified genomic DNA from mice with each of the phenotypic extremes (bilaterally severe and bilaterally modified) were separately pooled and a genome-wide scan was performed to identify chromosomal regions that segregated with the modified phenotype. A single locus on Chromosome 1 segregated with phenotypic modification in most animals and individual mice were genotyped with markers across this region.

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Conflict of Interest statement. None declared.

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