Abrogation of HMX1 Function Causes Rare Oculoauricular Syndrome Associated With Congenital Cataract, Anterior Segment Dysgenesis, and Retinal Dystrophy


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PURPOSE. To define the phenotypic manifestation, confirm the genetic basis, and delineate the pathogenic mechanisms underlying an oculoauricular syndrome (OAS).

METHODS. Two individuals from a consanguineous family underwent comprehensive clinical phenotyping and electrodiagnostic testing (EDT). Genome-wide microarray analysis and Sanger sequencing of the candidate gene were used to identify the likely causal variant. Protein modelling, Western blotting, and dual luciferase assays were used to assess the pathogenic effect of the variant in vitro.

RESULTS. Complex developmental ocular abnormalities of congenital cataract, anterior segment dysgenesis, iris coloboma, early-onset retinal dystrophy, and abnormal external ear cartilage presented in the affected family members. Genetic analyses identified a homozygous c.650A>C; p.(Gln217Pro) missense mutation within the highly conserved homeodomain of the H6 family homeobox 1 (HMX1) gene. Protein modelling predicts that the variant may have a detrimental effect on protein folding and/or stability. In vitro analyses were able to demonstrate that the mutation has no effect on protein expression but adversely alters function.

CONCLUSIONS. Oculoauricular syndrome is an autosomal recessive condition that has a profound effect on the development of the external ears; anterior segment, and retina, leading to significant visual loss at an early age. This study has delineated the phenotype and confirmed HMX1 as the gene causative of OAS, enabling the description of only the second family with the condition. HMX1 is a key player in ocular development, possibly in both the pathway responsible for lens and retina development, and via the gene network integral to optic fissure closure.

Keywords: congenital cataract, anterior segment dysgenesis, coloboma, early-onset retinal dystrophy, oculoauricular syndrome

In 1945 Franceschetti and Valerio described a 3-year-old girl from a consanguineous Swiss family who had mild microphthalmia, opacified corneas, and increased IOP. She also had an abnormal appearance to her external ears which had dysplastic auricles with a “coloboma” of the lobule. Her brother was similarly affected. In 2008, Schorderet et al. reported the same family, describing the condition as a novel autosomal recessive phenotype, which they termed oculoauricular (OAS) or Schorderet-Munier-Franceschetti syndrome. All affected individuals had complex ocular anomalies, malformed external ears but otherwise normal development. The group identified a causative homozygous c.215_240del mutation in the gene encoding the homeobox protein HMX1 that was predicted to prevent translation of the homeodomain in all affected individuals. This remains the only report to date of a HMX1 mutation causing a human phenotype, although mutations within HMX1 or putative conserved regulatory elements of the gene have been identified in animal models. Accurate replication of the complex human eye phenotype has not been seen in zebrafish, mice, the rat, or cattle.

The HMX genes encode a family of homeodomain-containing transcription factors that are required for sensory organ development and are expressed from early stages of embryogenesis. Homeobox transcription factors contact DNA via a
highly conserved 60 amino acid homeodomain7,8 consisting of three α-helices (I, II, and III) that fold in to a tight globular structure,3 forming a highly conserved helix-turn-helix motif that allows the homeodomain and its N-terminal arm to make specific contacts with DNA and the phosphate backbone.16 The H6 homeobox family consists of HMX1 (NKX5-3), HMX2 (NKX5-2), and HMX3 (NKX5-1), which are highly conserved across orthologs9,12 and share similarity with NKX homeobox genes.13 HMX1 is expressed in discrete regions of the craniofacial mesenchyme, the second branchial arch, peripheral nervous system, and the eye.12 Within the human embryonic eye, HMX1 expression is seen within the anterior lens, ventral retina and optic nerve.2 In the mouse, chick and zebrafish developing eye, HMX1 expression is confined to the lens and retina, with protein expression gradually becoming polarized in the retina at later stages of development.2,12,14,15 HMX1 is believed to have preferential binding for the transcriptional repressor.16

The characterization of HMX1 has been hindered by a lack of knowledge of its exact role in embryogenesis and the anonymity of its gene targets. However, recently Boulling et al.17 were able to successfully predict and verify a number of candidate genes using the GeneDistiller software (in the public domain, http://dna.leeds.ac.uk/autosnpa/). Copy number data was generated using the Affymetrix Genotyping console. Autozygosity analysis was carried out using AutoSNPa (in the public domain, http://www.rcsb.org/pdb/home/home.do). Sequences were aligned with ClustalW19, over the region modelled, human HMX1 and drosophila clawless have 47% sequence identity, suggesting that they have similar structures. Homology models built with Modeller.20 Twenty models were built and the one with the lowest Discrete Optimized Protein Energy score chosen. For analysis of van der Waals interactions, hydrogen atoms were added with Reduce,21 and all-atom contact analysis performed with Probe.22 The p.Q217P mutation was modelled using KI Nob23 with the rotamer with the smallest van der Waals overlap chosen.

Materials and Methods

Patients

The family involved in this study were recruited through a joint pediatric ophthalmology and genetics clinic held at Manchester Centre for Genomic Medicine, St Mary’s Hospital (Manchester, UK). The patients’ ocular abnormalities were diagnosed by an experienced ophthalmologist and a complete systemic and dysmorphic assessment was undertaken by a clinical geneticist. A three generation family history and a full medical history were obtained from the family. Written informed consent was obtained from the parents of each affected individual. Consent was not given for the study of unaffected siblings, whose DNA samples were not made available for testing. This research followed the tenets of the Declaration of Helsinki and ethics committee approval was obtained from the North West Research Ethics Committee (11/NW/0421).

Autozygosity Mapping

Genome-Wide Single Nucleotide Polymorphism (SNP) analysis was carried out using the Affymetrix Genome-Wide SNP6.0 microarray. Genotypes were generated using the Birdseed V2 algorithm with a confidence threshold of 0.01 and copy number data was generated using the SNP 6.0 CN/LOH Algorithm both within the Affymetrix Genotyping console. Autozygosity analysis was carried out using AutoSNPa (in the public domain, http://dna.leeds.ac.uk/autoSNPa/). Copy number results were analyzed using the Affymetrix Chromosome Analysis Suite. Autozygous regions over 2 Mb that were shared between the affected family members were analyzed for candidate genes using the GeneDistiller software (in the public domain, http://mitonet.charite.de/GeneDistiller/).18

Sanger Sequencing

Oligonucleotide primers were designed to flank the coding exons of HMX1 (transcript NM_018942) plus a minimum of 100 bp of surrounding sequence using Primer3 (version 0.4.0) software (in the public domain, http://frodo.wi.mit.edu/primer3/) to generate PCR amplicons no bigger than 900 bp. Polymerase chain reaction products underwent sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit on the ABI3730XL sequencer (Applied Biosystems, Waltham, MA, USA). Sequencing chromatograms were analyzed using the Staden Package 4.1.4b1.

Protein Modelling

The p.Q217P mutation resides within the homeodomain of HMX1. The crystal structure of human HMX1 is yet to be resolved. Amino acids 181 to 265, incorporating the entire homeodomain and flanking residues, were used to establish a comparative model utilizing the homeodomain of drosophila “clawless” protein (RCSB PDB code 3A01, in the public domain, http://www.rcsb.org/pdb/home/home.do). Sequences were aligned with ClustalW19, over the region modelled, human HMX1 and drosophila clawless have 47% sequence identity, suggesting that they have similar structures. Homology models built with Modeller.20 Twenty models were built and the one with the lowest Discrete Optimized Protein Energy score chosen. For analysis of van der Waals interactions, hydrogen atoms were added with Reduce,21 and all-atom contact analysis performed with Probe.22 The p.Q217P mutation was modelled using KI Nob,23 with the rotamer with the smallest van der Waals overlap chosen.

Site Directed Mutagenesis

A full length cDNA clone of human HMX1 in C-terminal Myc-DDK-tagged pCMV6-entry vector (pHMX1W5) was obtained from Origene Technologies, Inc. (Rockville, MD, USA). Site directed mutagenesis (SDM) PCR reactions were conducted using MasterAmp ExtraLong PCR kit with Premix 4 (Epipcentre, Illumina Inc., San Diego, CA, USA), using primers F-CAGC CAGGTCTTCCGCCGTGAATCCACCT and R-AGGTGGAATTCCAGCCGGAGACCTGCTG to make the pHMX1Q217P construct. Polymerase chain reactions underwent DpnI digest and were subsequently transformed in to Alpha-select Bronze Efficiency competent cells (Bioline, London, UK) and purified using the Hi-speed Midiprep Kit (QIAGEN, Venlo, Limburg).

Cell Culture and Transfection

HEK293 EBNA and HeLa cells obtained from ATCC were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) with high glucose (4.5 mg/mL; Sigma-Aldrich Corp., St Louis, MO, USA) supplemented with 10% (vol/vol) foetal bovine serum (Sigma-Aldrich Corp.) and 100 U/mL streptomycin/penicillin (Sigma-Aldrich Corp.) and grown at 37°C, 5% CO₂ in 75 cm² culture flasks (Corning, New York, USA). All transfection experiments were conducted using Lipofectamine2000 (Invitrogen) in Dulbecco’s modified Eagle’s Medium (DMEM) with high glucose (4.5 mg/mL; Sigma-Aldrich Corp.) supplemented with 5% (vol/vol) foetal bovine serum (Sigma-Aldrich Corp.).

Western Blotting

HEK293 EBNA cells were seeded in 6-well plates at a density of 1 × 10⁶ cells per well and incubated under standard cell culture conditions for 24 hours prior to transfection. For one
reaction, cells were transfected using 7.5 µL Lipofectamine2000 mixed with 2500 ng of either the pHMX1 wt, pHMX1 F217P, or no DNA (negative control). Forty-eight hours post transfection, cells were washed in PBS (Sigma-Aldrich Corp.), lysed using 200 µL Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich Corp.) and harvested by scraping. Lysates were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Fifty micrograms of total protein sample was diluted with 5x SDS loading buffer (60 mM Tris-HCl, pH 6.8, 10% [wt/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] beta-mercaptoethanol, 0.05% [wt/vol] bromophenol blue), denatured and electrophoresed on a 12.5% SDS-PAGE gel (Biorad). The proteins of interest were labelled using mouse monoclonal c-Myc antibody (Santa-Cruz Biotechnology, Inc., Santa-Cruz, CA, USA) and mouse monoclonal SOD2 antibody (loading control) and detected to CL-XPosure Film (Thermo Scientific) then developed using Kodak GBX Developer (Sigma-Aldrich Corp.) and fixed in Kodak GBX Fixer (Sigma-Aldrich Corp.). Transfection and Western blotting was performed in triplicate for each plasmid.

Construction of Reporter Plasmids
The pGL3-EPH46, pGL3-Sema4a, pGL3-Pi3k, and pGL3-Segc constructs are detailed in Marcelli et al.24 and Boulling et al.17

Luciferase Assays
For luciferase assays, HeLa cells were seeded in 12-well plates at 5 x 10^3 cells per well 24 hours before transfection. Each well was transfected with 5 µL Lipofectamine 2000 mixed with 600 ng of one pGL3 reporter construct and 200 ng pCMV6-HMX1 wt or pCMV6-HMX1 F217P. A renilla-luc reporter plasmid (pRL-CMV; Promega, Madison, WI, USA) was cotransfected in all experiments as a transfection efficiency control. Forty-eight hours post transfection, cells were lysed with 1X Passive Lysis Buffer (Promega) and transcriptional activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated by division of the luciferase measurement with that of the cotransfected renilla. Three transfections of each reporter were performed in each experiment, and the entire experiment conducted in triplicate. The statistical significance between the luciferase activity of each reporter construct cotransfected with pCMV6-HMX1 wt and pCMV6-HMX1 F217P was assessed using the Student’s t-test.

RESULTS
Detailed Phenotyping Reveals Complex Developmental Ocular Abnormalities
Patient I, a 28-month-old boy (Figs. 1A–D) and his similarly affected 13-year-old male cousin, Patient II, were each born to first cousin parents of Asian origin (Fig. 1E). Each presented with a range of complex ocular developmental abnormalities involving both the anterior segment of the eye and retina (Table).

Patient I was born at full term following an unremarkable pregnancy. His birth weight was 7 lb 4 oz (3.29 kg) but he was unable open his eyes. Upon ophthalmic assessment at 3-days old an inability to fix and follow was noted. He was found to have dense bilateral congenital cataract (CC) requiring lensectomy that was performed at 6 weeks of age. At this time, he was found to have bilateral microcornea (7.5-mm horizontally bilaterally) with high pachymetry measurements (72 µm in the right eye and 64 µm in the left). Short axial lengths were observed (15.9-mm right, 14.9-mm left) but IOP measurements were normal. He displayed a manifest horizontal nystagmus with right divergent strabismus. Developmental abnormalities included colobomatous microphthalmia with inferior iris coloboma, localized sclerocornea in the right eye and posterior embryotoxon in the left. He was also found to have naso-lacrimal duct obstruction. Examination of the posterior segment identified bilateral uveoretinal coloboma and small, dysplastic optic discs. Despite these problems, Patient I went on to develop some useful vision following surgery (0.7 logMAR left eye, 0.9 logMAR right, bilateral 0.6). At 2 years and 4 months of age, he underwent electrodiagnostic testing (EDT). The ERGs from each eye were symmetrical and well developed in the light adapted state; however, dark adaptation revealed attenuated responses, suggestive of early-rod dysfunction. At this time, it was also noticed that he was developing symptoms of loss of peripheral vision.

Patient II was diagnosed with CC at birth following an uneventful pregnancy. Ophthalmic examination revealed bilateral cataracts with a particularly dense, central nuclear component. Significant bilateral microcornea was also observed (5-mm horizontally right; 6.75-mm left). The right cornea was grossly opaque and vascularized. Patient II had bilateral microphthalmia that was more severe in the right eye. Lensectomy was performed in the left eye at 4 months of age. The right, however, remained unoperated due to the severity of malformation and microphthalmia. Other developmental abnormalities included bilateral inferior iris coloboma, left posterior embryotoxon with anterior synchia, and sclerocornea of the right eye. Axial lengths measured 17-mm left and 16.9-mm right, with normal IOPs. Visualization of the posterior segment revealed small, dysplastic optic discs similar to those seen in Patient I. Electrodiagnostic testing was conducted at 5 months of age. Good light-adapted ERG responses were observed, however, on dark adaptation he also showed a reduced light rise but this could not be ruled out as artefactual due to limitations of the pediatric ERG protocol. At 4 years of age, his vision began to deteriorate (0.7 logMAR left eye, 0.8 logMAR right, 660 [1.0 log]). Aged 9 years, further deterioration was seen as his vision dropped to 1.6 logMAR. By the age of 12 years, peripheral corneal opacification had developed in the left eye although his visual axis remained clear. By this time he had also developed high frequency asymmetric horizontal jerk nystagmus, which was worse in his left eye. At 14 years of age, his vision had shown further decline measuring 1.8 in the left eye, with only light perception in the right. His ERG results at 14 years showed grossly attenuated light and dark adapted responses, much worse in the right eye than the left, consistent with a severe generalized retinal dystrophy.

Review of both boys by an experienced dysmorphologist revealed a highly unusual ear phenotype of malformed, low-set pinna with crumpled helix, narrow external acoustic meatus and deficient lobe (Figs. 1A, B, D). The father of Patient I was found to have minor external ear abnormalities and marked bilateral posterior embryotoxon potentially suggestive of carrier manifestations.

GENETIC ANALYSES REVEAL MISSENSE MUTATION IN HMX1 DNA BINDING DOMAIN
Given the history of parental consanguinity, autozygosity mapping was conducted to determine genomic regions identical by descent that were shared between the affected
boys. Two regions of homozygosity were identified (Chr 4: 3,536,175–14,927,828 and Chr 17: 55,607,567–62,377,812; Fig. 1F) that contained a total of 278 genes. Candidate gene analysis using the GeneDistiller software identified HMX1 located within the autozygous region on chromosome 4 at g.8,847,802-8,873,543 (Hg19). Searching for the human phenotype ontology (HPO) terms cataract, coloboma, microcornea, microphthalmos, and sclerocornea, HMX1 provided a 100% match. No other strong candidates were identified within any of the autozygous regions. Thus, HMX1 was a clear candidate for further investigation given the phenotypic features of Patients I and II and those previously reported in association with mutations in HMX1 in both humans and animal models.

Direct Sanger sequencing of the coding region of the HMX1 gene identified a homozygous nonsynonymous missense mutation at c.650A>C in each affected individual (Fig. 1G). The parents of Patient I were confirmed as heterozygous carriers of this variant. Samples from the parents of Patient II were unavailable for carrier testing. This variant results in the substitution of a polar uncharged glutamine residue for a hydrophobic proline residue at amino acid position 217 of the encoded HMX1 protein (p.[Gln217Pro]), causing a moderate alteration in Grantham distance (score: 76). The affected amino acid residue resides within the first helix of the encoded homeodomain and is highly conserved, showing 93% conservation across 30 species (Fig. 2A). In silico analysis tools predicted this change to be pathogenic with high confidence scores: -Polyphen2: probably damaging (0.99), SIFT: deleterious (0), AlignGVGD: most likely to be pathogenic (Class 65), Mutation Taster: disease causing (P value 0.001). Based on the location of the identified mutation, we hypothesized that it is highly likely to be the cause of the ocular phenotype in the two boys. No other changes within the coding region of HMX1 were identified in either affected individual.

**Figure 1.** Phenotypic features, molecular genetic analysis, and computational modelling. Patient I presenting with bilateral microcornea, microphthalmia, coloboma, and congenital cataract (ASD: [A] and close-up in [C]), and unusual external ear morphology ([B] and close-up in [D]) with deficient lobule (arrow). Three generation family pedigree with affected family members represented by black squares (E), father of Patient I with carrier manifestations represented by the white square with black insert. Autozygosity mapping results for Patient I and Patient II (F). Yellow bars indicate regions of the chromosome with heterozygous calls; black bars indicate homozygous calls (autozygous regions); and gray bars represent regions of the chromosome not covered by genetic markers on the Affymetrix Genome-Wide SNP6.0 microarray. HMX1 Sanger sequencing results ([G]). Chromatogram depicting the homozygous HMX1 c.650A>C (p.[Gln217Pro]) sequence change (*) present in Patients I and II that is heterozygous in the parental sample and absent in the control. Structural modelling of HMX1 homeodomain ([H]). Homology model of the HMX1 homeodomain (white) complexed with the DNA double helix (orange backbone with blue and green branches representing nucleotides) (i). The wild-type Gln 127 (green) is not making direct interactions with the DNA molecule. Close up view of Gln 217, showing its interaction with the neighbor region of the protein; small van der Waals clashes (yellow and orange) represent a small degree of coordinate error introduced by the modelling process (ii). Proline (red) substituted at position 217 in the most favorable conformation; substantial van der Waals clashes (red, pink, yellow) indicate that this substitution is predicted to be destabilizing (iii).
<table>
<thead>
<tr>
<th>Eye</th>
<th>Patient I</th>
<th>Patient II</th>
<th>Proband*</th>
<th>Individual 1 (VII.8)*</th>
<th>Individual 2 (VII.9)*</th>
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<td>Anterior segment</td>
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<td>Cornea</td>
<td>Posterior embryotoxon (L)</td>
<td>Posterior embryotoxin (L)</td>
<td>Microcornea</td>
<td>Sclerocornea</td>
<td>Microcornea</td>
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<td>Localized sclerocornea (R)</td>
<td>Gross sclerocornea (opaque and vascularized in [R])</td>
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<td>Microcornea (7 mm [R]; 8 mm [L])</td>
<td>Microcornea (6 mm [R]; 6.75 mm [L])</td>
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<td>Lens</td>
<td>Bilateral dense CC</td>
<td>Dense central &amp; nuclear bilateral CC</td>
<td>Rapidly progressive bilateral CC</td>
<td>Cataract (onset not clear)</td>
<td>Cataract (onset not clear)</td>
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<td>Iris</td>
<td>Inferior iris coloboma</td>
<td>Inferior iris coloboma</td>
<td>Posterior synechiae</td>
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<td>Retina</td>
<td>Bilateral uveoretinal coloboma</td>
<td>Small, tilted, dysplastic optic discs</td>
<td>Bilateral optic nerve dysplasia</td>
<td>Inferior chorioretinal coloboma (R)</td>
<td>Retinal detachment</td>
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<td>Small dysplastic optic discs</td>
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<td>Macular hypoplasia</td>
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<td>Lacunae (L)</td>
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<td>Pigment deposits within the RPE</td>
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<td>Motiled RPE appearance (aged 4 y)</td>
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<td>Equatorial chorioretinal atrophic lacunae</td>
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<td>Thinning of the retinal vessels</td>
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<td>ERG</td>
<td>N/A</td>
<td>Significantly attenuated light &amp; dark ERGs (age 14 y)</td>
<td>Moderately severe, generalized retinal dystrophy, apparent loss of rod photoreceptor function, mild cone abnormality (aged 6 y). Rod-cone dystrophy (aged 7 y)</td>
<td>Almost undetectable rod specific ERG, severe cone ERG abnormalities (aged 12 y)</td>
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<td>Other</td>
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<td>Globe</td>
<td>Colobomatous microphthalmia</td>
<td>Gross microphthalmia (R)</td>
<td>Colobomatous microphthalmia (R)</td>
<td>Microphthalmia</td>
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<td>Short axial length (13.9 mm [R]; 14.9 mm [L])</td>
<td>Slightly short axial length (17 mm [L]; 16.9 mm [R])</td>
<td>Esotropia (R) (aged 4 y)</td>
<td>Increased IOP</td>
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<td>Enophthalmos x-NEW-CELL-x</td>
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<td>Other</td>
<td>Congenital nystagmus</td>
<td>Congenital nystagmus</td>
<td>Nystagmus</td>
<td>(L) eye enucleated due to painful complications of partially calcified phthisis bulbi</td>
<td>N/R</td>
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<td>Visual symptoms</td>
<td>Poor peripheral vision</td>
<td>Poor visual acuity from infancy, marked visual field constriction (aged 9 y)</td>
<td>Vision limited to light perception (aged 6 y). Normal eye tension and some vision maintained through inferior iris coloboma until aged 65 y when cataracts progressed</td>
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<td>Poor dark adaptation (age 2.5 y)</td>
<td>Good light response, poor dark adaptation (age 5 mo)</td>
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found rarely in $\alpha$-helices,\textsuperscript{25} and, where present, introduce a “kink” in the helix of 20° or more.\textsuperscript{26} Thus, introduction of a proline at this position is predicted to either result in steric clashes with the previous turn of the helix, or a substantial conformational change in the helix, which must be accommodated by a range of other structural rearrangements, which may include packing rearrangements in the protein core. Either scenario is likely to destabilize the protein structure in the DNA-binding domain. It should be noted that the cow orthologue of HMX1 has a proline residue at the equivalent position. However, this sequence also has a number of other substitutions as compared with the human protein, including a change from phenylalanine to the smaller isoleucine at the preceding position. We note that no other HMX sequences have been observed with a proline at position 217 that do not also have other substitutions that are close in the protein structure. It is not possible to rule out interactions of the HMX1 homeodomain with other parts of the protein that are not modelled.

**In Vitro Investigations Demonstrate That the p.Q217P Mutation Abrogates HMX1 Function**

Given the predicted detrimental effect of this mutation on protein structure, we investigated the pathogenic effect at the in vitro level. In vitro overexpression of wild-type and mutant HMX1 permitted confirmation of stable and equal expression of each isoform by Western blotting (Fig. 2B). We assessed the ability of the wild-type and Q217P mutant HMX1 isoforms to drive expression of a luciferase reporter located downstream of promoter sequences from genes EPHA6, Sema3f, Ptpro, and Scgc, previously identified as HMX1 targets.\textsuperscript{17} The same pattern of results was obtained for the Scgc, Ptpro, and Sema3f reporters. The overall findings of the dual luciferase assays indicate that in HeLa cells the pHMX1wt expression construct acts as a transcriptional activator of its targets, in contrast to mouse N2A cells where it acts as a repressor. This supports recent suggestions that HMX1 can act as either a transcriptional activator or repressor depending on cellular expression.\textsuperscript{17,24} Importantly, the pHMX1Q217P mutant was shown to be unable to function in this manner; consistently failing to activate the transcription of the reporter constructs (Fig. 2C).

**DISCUSSION**

This study has successfully provided a detailed phenotypic account of two affected family members of a large consanguineous family with Schorderet-Munier-Franceschetti syndrome, an OAS (Table). This further delineates the condition and also a possible milder carrier phenotype. Interestingly, the mother of the first two siblings reported was described as having mild bilateral abnormalities of the earlobes, also supporting carrier manifestations.\textsuperscript{1} Furthermore, this study has identified the molecular basis of the condition using a combination of autozygosity mapping and candidate gene sequencing, confirming HMX1 as the gene causative of OAS. We have described what is to date only the second human pathogenic mutation in HMX1. The affected individuals described by this study presented with a complex developmental ocular phenotype of congenital cataract, coloboma, anterior segment dysgenesis (ASD), and early-onset retinal dystrophy, consequently affecting development of the whole globe of the eye. HMX1, therefore, should be screened in individuals with ASD, since ‘milder’ mutations may underlie milder phenotypes encompassing ASD. Our identification of a mutation in this gene enabled a better understanding of the
visual outcome, contributing to genetic counselling and clinical management. This understanding of the phenotype is complemented by a broadened molecular understanding and underlines the importance of obtaining an early molecular diagnosis of developmental ocular abnormalities such as congenital cataract and ASD particularly where they predict the onset of retinal dystrophy at an early age.

**FIGURE 2.** Conservation and in vitro assessment of p.(Gln217Pro) mutation. Multispecies amino acid sequence alignment of HMX1 (A). Thirty species alignment of a region of HMX1 amino acid sequence containing the homeodomain, which is schematically represented below the sequences. Amino acid residues that are fully conserved are represented by an *asterisk*; conservation between residues with strongly similar properties are represented by a *colon*; conservation between residues with weakly similar properties are represented by a *period symbol.* Asterisk indicates residue mutated in Patients I and II. Western blot of transiently transfected HEK293 EBNA cells demonstrating protein expression by the Q217P mutant (Mut) compared with wild-type HMX1 (Wt) (B). The C-terminal vector encoded Myc epitope was detected and demonstrated a stable product sized at approximately 45 kDa. Levels of expression appeared equal, suggesting that the Q217P mutant construct was being translated and stably expressed in the same way as the wild-type form. Untransfected cells demonstrated no endogenous expression of c-Myc (Neg). Probing for SOD2 confirmed equal loading of the SDS-PAGE gel. Dual-luciferase reporter assay findings for cotransfection of HMX1 expression constructs with four reporter constructs (C). Following normalization to refill (prl-CMV) activity, the bar charts represent the luciferase activity recorded by cotransfection with either HMX1 wild-type (pCMV6-HMX1 WT; blue bar) or HMX1 Q217P mutant (pCMV6-HMX1Q217P; red bar) expression constructs and one of four reporter constructs relative to the measurement recorded for transfection of the empty pCMV6 vector alone (green bar). Error bars denote the SD; *P < 0.001; **P < 0.0001; y-axis represents the fold difference relative to the observed empty vector reading. Wild-type HMX1 consistently activated the transcription of each reporter construct in HeLa cells compared with cells transfected with empty vector. However, the Q217P mutant form of HMX1 was unable to activate the transcription of any of the five reporter constructs. The fold increase in observed reporter activity varies between each of the four constructs, with the greatest level of activation seen for EPHA6 where an approximately 24-fold increase was measured compared to empty vector alone. When transfected with the HMX1 Q217P mutant expression construct, the EPHA6 reporter was activated to barely detectable levels; approximately 0.6-fold that of the empty vector, retaining only 2.5% of wild-type HMX1 activity.

**HMX1** has been implicated in the specification and diversification of neuronal cell fate during neurogenesis, as well as development of the external ear and the eye. Specifically within the eye, HMX1 appears to play an important role in development of the anterior segment, as well as axonal guidance and the establishment of polarity within the retina.2,12,14,15 Clinicians are in a unique position to recognize...
the subtle additional dysmorphic and/or systemic features that may be associated with optic colobomas, such as the abnormal external ear phenotype in patients with OAS, so that genetic testing can be instigated and appropriate counselling regarding expected visual outcome can be provided. The identification of further mutations in novel genes underlying such severe ocular phenotypes would better our understanding of optic fissure closure, establishment of the anterior segment, and development and maintenance of the retina.

Ocular coloboma is a genetically heterogeneous developmental eye defect caused by failure of ectodermal optic vesicle fissure closure. A wide range of genes, from multiple biochemical pathways have been implicated in causing this abnormality and the severity of the ocular and associated systemic abnormalities have been attributed to the pleiotropic, spatial, and temporal expression of the underlying mutated gene.29 It is possible to predict visual prognosis based on the presence or absence of other ocular abnormalities such as microcornea and microphthalmia, however, this prediction is further complicated by the presence of chorioretinal coloboma and/or optic nerve abnormalities. A select group of homeodomain containing transcription factors integral to eye development have been implicated in ocular coloboma, anterior segment abnormalities and retinal dystrophy, including VSX2 (CHX10), SIX3, and PAX6. The identification of a second family with an HMX1 mutation in association with coloboma, severe ASD and early-onset rod-cone dystrophy, suggests that this homeobox gene is part of the same developmental network. Moreover, HMX1 has recently been identified as a target of miR-204.30 a microRNA that has been implicated in optic fissure closure, lens differentiation, optic cup development and subsequent dorso-ventral patterning of the retina by way of Meis2 gene dosage and subsequent misregulation of genes in the Pax6 pathway.31 Further research in to the regulation of HMX1 by miR204 during oculogenesis would be of utmost importance in improving understanding and expanding the gene networks important in eye development.

The mutation underlying OAS in the family reported by this study is a homozygous nonsynonymous missense variant within Helix I of the homeodomain. The striking phenotypic similarity between these affected individuals and those described by Schorderet et al.2 (Table) suggests that the missense mutation identified here has as significant effect on protein function as a 26-bp deletion that prevents translation of missense mutation identified here has a significant effect on protein function as a 26-bp deletion that prevents translation of HMX1. Our results support the recent findings of Marcelli et al.,24 which suggested that although the protein is expressed, Q217P mutation show that although the protein is expressed, Q217P HMX1 function is abolished. The striking phenotypic similarity between these affected individuals and those described by Schorderet et al.2 (Table) suggests that the missense mutation identified here has as significant effect on protein function as a 26-bp deletion that prevents translation of HMX1. Our results support the recent findings of Marcelli et al.,24 which suggested that although the protein is expressed, Q217P HMX1 function is abolished. The striking phenotypic similarity between these affected individuals and those described by Schorderet et al.2 (Table) suggests that the missense mutation identified here has as significant effect on protein function as a 26-bp deletion that prevents translation of HMX1. Our results support the recent findings of Marcelli et al.,24 which suggested that although the protein is expressed, Q217P HMX1 function is abolished.

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References