Deficiency of FRAS1-related extracellular matrix 1 (FREM1) causes congenital diaphragmatic hernia in humans and mice

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Congenital diaphragmatic hernia (CDH) is a common life-threatening birth defect. Recessive mutations in the FRAS1-related extracellular matrix 1 (FREM1) gene have been shown to cause bifid nose with or without anorectal and renal anomalies (BNAR) syndrome and Manitoba oculotrichoanal (MOTA) syndrome, but have not been previously implicated in the development of CDH. We have identified a female child with an isolated left-sided posterolateral CDH covered by a membranous sac who had no features suggestive of BNAR or MOTA syndromes. This child carries a maternally-inherited ~86 kb *FREM1* deletion that affects the expression of *FREM1*'s full-length transcripts and a paternally-inherited splice site mutation that causes activation of a cryptic splice site, leading to a shift in the reading frame and premature termination of all forms of the *FREM1* protein. This suggests that recessive *FREM1* mutations can cause isolated CDH in humans. Further evidence for the role of *FREM1* in the development of CDH comes from an *N*-ethyl-*N*-nitrosourea-derived mouse strain, *eyes2*, which has a homozygous truncating mutation in *Frem1*. *Frem1*^{eyes2} mice have eye defects, renal agenesis and develop retrosternal diaphragmatic hernias which are covered by a membranous sac. We confirmed that *Frem1* is expressed in the anterior portion of the developing diaphragm and found that *Frem1*^{eyes2} embryos had decreased levels of cell proliferation in their developing diaphragms when compared to wild-type embryos. We conclude that *FREM1* plays a critical role in the development of the diaphragm and that *FREM1* deficiency can cause CDH in both humans and mice.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a life-threatening birth defect in which the abdominal viscera protrude into the thorax through an abnormal opening or defect in the diaphragm that is present at birth. In some cases, the herniated

viscera are encapsulated in a membranous sac. CDH has an incidence of approximately 1 per 2500 births and accounts for approximately 8% of all major congenital anomalies (1,2). Although CDH can present as an isolated defect, other congenital anomalies are present in approximately 30–40% of

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cases (3–5). Some individuals with non-isolated or complex CDH can be diagnosed with a specific genetic syndrome based on their clinical features and/or molecular testing (5,6). In most of these genetic syndromes, the CDH phenotype is incompletely penetrant and, in some cases, a propensity to develop CDH was not recognized when the syndrome was first characterized (6–8). In such cases, the identification of CDH in a mouse model can help to confirm that the association between the syndrome and CDH is more than coincidental.

The FRAS1-related extracellular matrix 1 (*FREM1*) gene—formerly called *QBRICK*—encodes an extracellular matrix protein that is expressed in a variety of tissues including the skin, lungs, kidneys, and intestines (9,10). During epidermal development, *FREM1* is secreted by mesenchymal cells in the dermis and forms a ternary complex in the basement membrane with *FRAS1* and *FREM2*, which are secreted from the epidermis with the help of the cytosolic adapter protein *GRIP1* (11). In mice, recessive mutations in *Frem1*, *Fras1*, *Frem2* or *Grip1* cause a loss of epidermal integrity leading to the development of large fluid-filled blisters over the eyes and/or digits between E10.5 and E12.5. As a result, these mouse strains are collectively referred to as ‘bleb’ mutants (12–18). At birth, these mice display a constellation of findings which can include cryptophthalmos and syndactyly—which are likely associated with blister formation—and renal agenesis. Several *Frem1*-deficient mouse strains have been described that have these features (11,13,16,19,20).

In humans, recessive mutations in *FRAS1*, *FREM2*, and *GRIP1* cause Fraser syndrome (OMIM #219000), which is characterized by cryptophthalmos, syndactyly, renal defects, and genital anomalies (17,21,22). Recessive mutations in *FREM1* have not been described in association with Fraser syndrome but cause two rare syndromes—bifid nose with or without anorectal and renal anomalies syndrome (BNAR; OMIM #608980) and Manitoba oculotrichoanal syndrome (MOTA; OMIM #248450)—whose phenotypic characteristics overlap those seen in individuals with Fraser syndrome (23–29).

Three consanguineous families with BNAR syndrome have been described to date (23,24). All affected individuals had median nose clefts. Anorectal malformations—including anteriorly placed anus with stenosis and anal atresia associated with rectovaginal fistula—and renal agenesis were found in only a subset of individuals. MOTA syndrome was first described among individuals from the Oji-Cre population in Manitoba, Canada (26). Individuals with MOTA syndrome can have the bifid or broad nasal tips and anal stenosis described in BNAR syndrome, but have also exhibited other features not described in BNAR syndrome, including eye anomalies—eyelid colobomas, cryptophthalmos, microphthalmia, anophthalmia—aberrant hair lines extending toward the eye, and omphalocele (25–29). While renal anomalies are common in BNAR syndrome, they have not been described in individuals with MOTA syndrome, underscoring the phenotypic variability that can be seen in individuals with *FREM1* deficiency.

Although *FREM1* clearly plays a role in multiple developmental processes, mutations in *FREM1* have never been associated with the development of CDH in either humans or mice. Here, we describe a female child with an isolated left-sided

posterolateral CDH covered by a membranous sac, who carries a maternally-inherited ~86 kb *FREM1* deletion that affects the expression of *FREM1*’s full-length transcripts and a paternally-inherited splice site mutation that causes activation of a cryptic splice site, leading to a shift in the reading frame and premature termination of all forms of the *FREM1* protein. We also describe retrosternal CDH in the *eyes2* mouse strain—which we identified in an *N*-ethyl-*N*-nitrosourea (ENU)-based screen (30). This phenotype is caused by a homozygous nonsense mutation (c.2477T > A, p.Lys826*) in *Frem1* and is associated with decreased levels of cell proliferation in the developing diaphragm. These results demonstrate that *FREM1* plays a critical role in the development of the diaphragm and that *FREM1* deficiency can cause CDH in both humans and mice.

RESULTS

Deficiency of *FREM1* can cause CDH in humans

A left-sided posterolateral CDH was diagnosed prenatally in a female fetus of a couple from India. No other anomalies were identified on ultrasound examinations. An amniocentesis was performed in the third trimester and the fetus was found to have a normal 46, XX chromosomal complement. FISH analysis of the 15q26 region, which is recurrently deleted in individuals with CDH, was also normal (31–33). At 34 6/7 weeks, the lung-to-head ratio was 2:1, suggesting a good prognosis (34,35).

The pregnancy was otherwise uncomplicated and labor was induced at 38 6/7 weeks gestation. The infant weighed ~2.2 kg at birth (first centile for a term baby). After delivery, the only abnormal physical feature identified on examination was mild 2–3 toe syndactyly. Specifically, the patient’s nose was documented to be normal and there was no evidence of an aberrant hairline or obvious eye defects. Imaging studies of the renal system were also normal. As a result, the infant was diagnosed with isolated CDH.

Surgical correction of her CDH was performed on the 9th day of life. The CDH was located in the left posterolateral region of the diaphragm and measured ~3 cm (anterior-posterior) by ~4 cm (lateral). A rim of diaphragm was present in all directions around the CDH and the herniated viscera were covered by a membranous sac that was attached to the lower lobe of the left lung. At a one-year follow-up visit, her motor, speech and cognitive development were appropriate for her age.

Since both isolated and non-isolated CDH can be caused by genomic alterations, all children born with CDH at the Erasmus Medical Center in Rotterdam, the Netherlands are screened on a clinical basis for deleterious copy number variations (7,36). The copy-number analysis performed on the proband’s DNA revealed a ~86 kb deletion on chromosome 9p22.3 (minimal deleted region chr9:14,892,957–14,941,672; maximal deleted region chr9:14 869 861–14 955 988; hg19). The minimal deleted region encompassed the first non-coding exon(s) of all of *FREM1*’s protein-coding full-length transcripts, their transcriptional start sites and >30 kb of downstream sequence that includes a number of putative transcription factor binding sites (Fig. 1A and C; <http://genome.ucsc.edu/>). This deletion

Figure 1. Recessive changes affecting *FREM1* are responsible for the development of CDH in the proband. (A) Array data from the proband showing the ~86 kb *FREM1* deletion inherited from her unaffected mother. The minimal and maximal deleted regions are represented by a black (minimal) and white (maximal) bar. The approximate locations of *FREM1* and *LOC389705* are represented by open block arrows. The approximate location of fosmid clone G248p8100A4, which was used for FISH confirmation, is represented by a blue bar. (B) Chromatograms show the heterozygous point mutation in an invariant base of the splice donor site of *FREM1* intron 28 (c.5334 + 1G > A) that the proband inherited from her unaffected father. (C) A schematic showing the approximate locations of the *FREM1* deletion (black and white bar) and the 5334 + 1G > A point mutation in relation to the exons (vertical bars) and introns (horizontal bars) of each of the protein-coding transcripts of *FREM1* (www.ensembl.org). (D) Sequencing analysis of cDNA made from EBV-transformed lymphocytes from the proband (P) and a control individual (C) reveals the activation of a cryptic splice site in the patient's sample and deletion of 8 bp from the end of exon 28 (based on *FREM1* transcript 001). No wild-type sequence is seen in the proband's sample, indicating that *FREM1* transcripts originating from the maternal allele are below the level of detection in the proband's lymphocytes. (E) The amino acid sequences of a normal human *FREM1* protein (top) and the predicted amino acid sequence of the *FREM1* protein translated from the paternal allele (bottom) are shown with variant amino acids italicized and underlined. The 5334 + 1G > A point mutation causes a shift in the reading frame leading to an altered amino acid sequence starting at position 1777 and premature termination at amino acid 1793 which affects all of *FREM1*'s protein products. L, ladder.

does not include the start codon of *FREMI* transcripts 001, 201 and 202 and does not directly affect *FREMI* transcript 002 or other genes in this region. The deletion was confirmed by FISH (Supplementary Material, Fig. S1) and was found to have been inherited from the proband's unaffected mother (data not shown). Genomic alterations affecting *FREMI* were not found in copy number analyses of 171 other individuals with CDH.

The coding sequence and intron–exon boundaries of the proband's *FREMI* gene were then screened for deleterious changes. A heterozygous point mutation (c.5334 + 1G > A) was identified in an invariant base of the splice donor site of *FREMI* intron 28 (based on *FREMI* transcript 001, which will be used as a reference throughout this report) (Fig. 1B and C). This variant was neither found in the SNP database (www.ncbi.nlm.nih.gov/snp) nor in data from the 1000 genomes project (www.1000genomes.org). Since the proband was of north Asian Indian origin, we screened 199 individuals of north Indian origin (398 chromosomes) for the presence of the c.5334 + 1G > A variant, using an allele-specific mismatch PCR assay (Supplementary Material, Fig. S2). None of these individuals was found to bear the variant. Sequence analysis, and data from the allele-specific mismatch PCR assay, showed that the c.5334 + 1G > A variant was inherited from the proband's unaffected father (Fig. 1B; Supplementary Material, Fig. S2). This change is predicted to affect all of *FREMI*'s protein-coding transcripts (Fig. 1C).

To determine the effects of the maternal deletion and the paternal c.5334 + 1G > A splice site mutation, we used PCR to amplify cDNA samples created from the proband's lymphocytes and control lymphocytes using primers in exons 27 and 30 whose amplification product spans multiple introns. PCR products from both cDNA samples were indistinguishable by gel electrophoresis (Fig. 1D). Sequence analysis of PCR products from the control lymphocytes revealed the expected wild-type sequence with normal splicing of exon 28 to exon 29 (Fig. 1D). However, sequence analysis of PCR products from the patient's cDNA sample revealed an 8 bp deletion at the end of exon 28 caused by activation of a cryptic splice site within that exon (Fig. 1D). This deletion leads to a shift in the reading frame which is predicted to cause an altered amino acid sequence starting at position 1777 and premature termination at amino acid 1793 (Fig. 1E). These changes are predicted to affect all *FREMI* proteins with disruption of the Calx-beta domain (amino acids 1743–1829) and the loss of the C-type lectin domain (amino acids 2063–2177; Fig. 1E; Supplementary Material, Fig. S3).

No evidence of a wild-type transcript was seen in sequence data from the proband's cDNA sample. This suggests that *FREMI* transcripts originating from the maternal allele are below the level of detection in the proband's lymphocytes. This decrease in transcript level is most likely due to the proband's maternally-inherited *FREMI* deletion, which affects all of *FREMI*'s full-length transcripts, combined with very low levels of lymphocytic expression of *FREMI* transcript 002—which is not directly affected by the maternal deletion.

To confirm decreased expression from the proband's maternal allele, we took advantage of a synonymous SNP

(rs10738380) in *FREMI* exon 20. The proband and her father are heterozygous (A/G) at this locus but her mother is homozygous (G/G). This allowed us to use Sanger sequencing to determine the ratio at which the paternal allele (A) and maternal allele (G) are being transcribed. When this locus was interrogated in the proband's lymphocyte cDNA sample, the sequence appeared homozygous for the paternal allele (A/A) (Supplementary Material, Fig. S4). Similar results were obtained from a second synonymous SNP (rs17219005) in *FREMI* exon 26 with the proband and her father being heterozygous (A/C), her mother being homozygous (C/C) and the proband's lymphocyte cDNA sample appearing homozygous for the paternal allele (A/A) (Supplementary Material, Fig. S4). These results confirm that the majority of *FREMI* transcripts in the proband's lymphocytes are being transcribed from her paternally-inherited allele, and that expression from her maternally-inherited allele is severely compromised.

Recessive *Frem1* mutations in *eyes2* mice

In a recessive ENU mutagenesis screen, we identified a mouse strain with unilateral and bilateral microphthalmia and/or cryptophthalmos. The strain was named *eyes2* (MGI: 3038748, Mouse Genomic Informatics at the Jackson Laboratory, <http://www.informatics.jax.org/>) based on its ophthalmologic abnormalities (30). After several generations of backcrossing to 129S6/SvEvTac mice, the *eyes2* phenotype was linked to a region of mouse chromosome 4. Additional mapping revealed that the causative gene was located in a ~19.7 Mb region between rs13477765 (chr4:71 225 531–71 226 031; GRCm38/mm10) and rs6396816 (chr4: 90 906 056–90 906 556; GRCm38/mm10).

Of the >100 genes in this region, we concluded that the *Frem1* gene was the most likely candidate based on phylogenetic profiling and previously published reports of autosomal recessive *Frem1* mutations causing cryptophthalmos secondary to *in utero* bleb formation (11,13,16,19). Similar blebs were subsequently identified over the eyes—but not the limbs—of *eyes2* embryos harvested at E13.5 (data not shown). Sequencing of the *Frem1* coding region and intron–exon boundaries revealed a homozygous c.2477T > A, p.Lys826* change in DNA samples from *eyes2* mice which was not found in DNA from C57BL/6Br^{Tyr} and 129S6/SvEvTac control mice (Fig. 2A). This change is predicted to cause truncation of the FREM1 protein with the loss of all or a portion of CSPG motifs 5–12, the Calx-beta motif and the C-type lectin domain (Fig. 2B).

In addition to eye anomalies, *eyes2* mice—phenotyped between P28 and adulthood on a mixed B6Brd/129S6 background—had unilateral kidney agenesis and a propensity to develop anal prolapse as adults (Fig. 2C–E). Both of these phenotypes have been previously described in FREM1-deficient mouse strains (19,25). Fewer than expected *FREMI*^{eyes2/eyes2} progeny were obtained from heterozygous *FREMI*^{eyes2/+} crosses at P28 (data not shown). Embryonic lethality has been previously documented in FREM1-deficient mice and in other bleb mutants and is thought to occur as a result of blister formation with subsequent hemorrhage (13,16,19,24).

Figure 2. A homozygous truncating mutation in *Frem1* is responsible for the eye, kidney, anal, and diaphragmatic defects seen in *eyes2* mice. (A) Chromatograms of a wild-type mouse (top) and an *eyes2* mouse (bottom). The *eyes2* mouse carries a homozygous c.2477T > A point mutation which creates a premature stop codon (p.Lys826*). (B) Schematic representation of the mouse FREM1 protein showing the approximate location of the p.Lys826* change in relation to various protein motifs. (C–E) *Frem1*^{eyes2} mice have anomalies previously described in other FREM1-deficient mouse strains including cryptophthalmos (C), unilateral kidney agenesis (D) and a propensity to develop anal prolapse in adulthood (E). (F) A retrosternal diaphragmatic hernia in a *Frem1*^{eyes2} mouse as viewed from the thorax. The herniated viscera are covered by a membranous sac (yellow outline). The liver (Lv) and stomach (Stm) are visible through the transparent diaphragm. (G) A retrosternal diaphragmatic hernia (yellow arrow) in a *Frem1*^{eyes2} mouse as viewed from the abdomen. The gallbladder (green arrow) is visible along with a mass of liver tissue (black arrows) which has been reduced into the abdomen. In this example, the gallbladder is abnormally attached to the hernial sac which has not been reduced into the abdomen, (H and I) H&E-stained sections through hernial sacs revealed herniated liver tissue (Lv) and the gallbladder (Gb) surrounded by a thin membrane (red arrows). There is a sharp demarcation between the diaphragmatic musculature and the membrane (black arrow) and evidence of muscular thickening at the edge of the diaphragmatic defect (H, *). In one case, the gallbladder was abnormally fused to the hernia sac (I, blue arrow). Gb, gallbladder; Lv, liver; Lg, lung; Sp, spine; Stm, stomach; Str, sternum.

FREM1 deficiency causes anterior CDH in *eyes2* mice

Anterior CDH was seen in ~3% (1/39) of *Frem1*^{eyes2} mice analyzed between P28 and adulthood in our initial phenotyping cohort. However, the penetrance of this phenotype varied among different *Frem1*^{eyes2} breeding pairs on a mixed B6Brd/129S6 background, with the progeny of one inbred line showing up to 46.9% (15/32) penetrance. As another means of determining the effect of genetic background on the penetrance of the CDH phenotype, we backcrossed *Frem1*^{eyes2} mice for eight generations onto a pure C57BL/6J background. We found the CDH penetrance in *Frem1*^{eyes2} mice on this C57BL/6J background to be 8.2% (6/73), which is significantly different than the penetrance observed in the progeny of the inbred strain of *Frem1*^{eyes2} mice on a mixed B6Brd/129S6 background ($P < 0.0001$). The penetrance of CDH, eye anomalies and kidney agenesis on both genetic backgrounds is summarized in Table 1.

Regardless of genetic background, diaphragmatic hernias found in *Frem1*^{eyes2} mice were always located in the anterior midline directly behind the sternum, in a region that would typically be muscularized (Fig. 2F and G; Supplementary Material, Fig. S5). In severe cases, these retrosternal diaphragmatic hernias consisted of a lobulated mass of herniated liver tissue, and sometimes the gallbladder, covered by a membranous sac (Fig. 2F–I; Supplementary Material, Fig. S5). In less severe cases, a retrosternal opening in the diaphragm was identified, but without evidence of frank visceral herniation (data not shown). Gross and histological analyses showed that the gallbladder was sometimes abnormally fused to the membranous sac covering the herniated viscera (Fig. 2I; Supplementary Material, Fig. S5). In contrast, the liver was never found to be fused to the membranous sac.

***Frem1* is expressed in the developing mouse diaphragm**

Previous studies have shown that FREM1 is expressed in the skin, kidney, lung and intestine, but no information has been published about FREM1's expression in the developing diaphragm (10). To determine whether FREM1 is expressed in the developing mouse diaphragm, we looked for evidence of *Frem1* expression by *in situ* hybridization in midline sagittal sections from wild-type embryos at E14.5. We found that *Frem1* transcripts were present in the inner, mesenchymal cell layers of the anterior and mid-diaphragm (Fig. 3A–C). Since FREM1 is known to form a complex with FRAS1 and FREM2, we also looked for the expression of *Fras1* and *Frem2* in the anterior and mid-diaphragm at the same time point. Although *Fras1* and *Frem2* transcripts were detected in the anterior and mid-diaphragm, they were located only in cells lining the thoracic cavity (Fig. 3D–I). *Fras1* and *Frem2* transcripts were also detected in cells lining the thoracic cavity behind the sternum. This suggests that *Fras1* and *Frem2* are expressed in the mesothelial cell layer of the diaphragm. These patterns of expression are similar to those observed in the skin, where FREM1 is secreted by mesenchymal cells in the dermis and forms a ternary complex in the basement membrane with FRAS1 and FREM2, which are secreted from the epidermis (11).

Table 1. Strain-dependent variations in the prevalence of *Frem1*^{eyes2} phenotypes

Phenotype	Strain background		<i>P</i> -value
	Inbred B6Brd/129S6	Congenic C57BL/6J	
Eye defects (%)	18/32 (56.3)	62/73 (84.9)	$P = 0.0015$
Diaphragmatic hernia (%)	15/32 (46.9)	6/73 (8.2)	$P < 0.0001$
Renal agenesis (%)	5/32 (15.6)	9/73 (12.3)	$P = 0.6476$

Histopathological changes in the diaphragms of *eyes2* mice

To determine whether *Frem1* deficiency causes histopathological changes that could predispose to the development of CDH, we compared the prevalence of cell proliferation and apoptosis in the anterior and mid-diaphragms of wild-type and *Frem1*^{eyes2} embryos at E14.5 by staining for Phospho-Histone H3 and cleaved-Caspase 3, respectively, in midline sagittal sections. Although no differences were observed in the prevalence of cell proliferation in the anterior diaphragms of wild-type and *Frem1*^{eyes2} embryos (Fig. 4A), a significant difference was detected in the level of cell proliferation in the mid-diaphragm (Fig. 4B). We found that the number of cells/ μm^2 undergoing apoptosis in the anterior and mid-diaphragms of *Frem1*^{eyes2} embryos was not significantly different from that of wild-type littermates (Fig. 4C and 4D).

The thickness of the anterior diaphragm at E14.5 varies considerably from its anterior to its posterior aspect, making it difficult to accurately compare the thickness of the anterior diaphragm between embryos. However, no difference in the thickness of the mid-diaphragm—which has a relatively uniform thickness within any given embryo—was observed between wild-type and *Frem1*^{eyes2} embryos at E14.5 (Fig. 4E).

DISCUSSION**FREM1 deficiency causes CDH in humans and mice**

The clinical phenotypes associated with recessive *FREM1* mutations have only recently been identified. Alazami *et al.* (24) identified autosomal recessive *FREM1* mutations in families with BNAR syndrome in 2009. At the time, FREM1 deficiency was not known to cause eye anomalies in humans, since ophthalmologic anomalies had not been documented in any of the published families with BNAR syndrome (23,24). It was only in 2011, when Slavotinek *et al.* showed that recessive mutations in *FREM1* were the cause of MOTA syndrome, that it became apparent that FREM1 deficiency could also cause eye anomalies in humans, even though ophthalmologic anomalies were among the most prominent and penetrant phenotypes seen in FREM1-deficient mice (11,13,16,19,25).

In this report, we describe a female child with an isolated left-sided posterolateral CDH covered by a membranous sac. Copy number and sequence analyses showed that she is a compound heterozygote for two deleterious *FREM1* changes: a maternally-inherited ~86 kb deletion involving only *FREM1* and a paternally-inherited splice junction mutation in *FREM1* (c.5334 + 1G > A). The maternal deletion does not include the start codons of any of *FREM1*'s transcripts. However, it removes the first non-coding exon(s) of all of

Figure 3. *Frem1*, *Frem2* and *Fras1* are expressed in the mid-diaphragm and anterior diaphragm at E14.5. The expression of *Frem1*, *Frem2* and *Fras1* was verified in sagittal sections from E14.5 embryos using *in situ* hybridization. In each case, a sense probe was used as a negative control. Dashed yellow lines outline the diaphragm in all panels. (A–C) Expression of *Frem1* is seen primarily in the inner, mesenchymal cell layers of the diaphragm (black arrows) and is not detected in the upper, mesothelial layer of the diaphragm (red arrows). (D–I) *Frem2* (D–F) and *Fras1* (G–I) are not expressed in the inner, mesenchymal cell layers of the diaphragm (black arrows) but are expressed in the upper, mesothelial layer of the diaphragm (red arrows) and in the mesothelial lining of the thorax (blue arrows). H, heart; D, diaphragm; L, liver.

FREMI's full-length protein-coding transcripts, their transcriptional start sites, and >30 kb of downstream sequence which includes a number of putative transcription factor binding sites. This deletion leads to a dramatic decrease in the expression of these transcripts, but is not expected to have a direct effect on *FREMI* transcript 002, which is independently regulated through its own 5'-UTR and an initiator codon within a unique exon (37,38). The paternally-inherited splice junction mutation causes activation of a cryptic splice site, which leads to a shift in the reading frame. This shift is predicted to cause premature truncation of all forms of the *FREMI* protein, with disruption of the Calx-beta domain and the loss of the C-type lectin domain.

Since CDH is a relatively common birth defect, one could question whether the diaphragmatic hernia seen in the

proband might have occurred simply by chance in the setting of *FREMI* deficiency. However, our identification of anterior CDH in *eyes2* mice, which are homozygous for a deleterious nonsense mutation (c.2477T > A, p.Lys826*) in *Frem1*, provides additional evidence that *FREMI* deficiency is the underlying cause of the CDH seen in the proband. Indeed, these results lead us to conclude that *FREMI* plays a critical role in the development of the diaphragm and that *FREMI* deficiency can cause CDH in both humans and mice.

Our patient had none of the phenotypes previously associated with BNAR or MOTA syndromes. One could speculate that her atypical presentation was due to retained expression of *FREMI* transcript 002 from the maternal allele. However, the majority of individuals with BNAR or MOTA syndrome have mutations that are not expected to affect this transcript (Table 2), which

Figure 4. *Frem1*^{eyes2} embryos have decreased levels of cell proliferation in the mid-diaphragm at E14.5. (A and B) The level of cell proliferation in the developing diaphragms of wild-type and *eyes2* embryos was measured by calculating the number of Phospho-Histone H3-positive cells/μm² in midline sagittal sections of the anterior (A) and mid-diaphragm (B). While the level of cell proliferation was similar between wild-type and *eyes2* embryos in the anterior diaphragm ($P = 0.729$), decreased levels of cell proliferation were seen in the mid-diaphragms of *eyes2* embryos ($P = 0.006$). (C and D) The level of apoptosis in the developing diaphragms of wild-type and *eyes2* embryos was measured by calculating the number of cleaved-Caspase 3-positive cells/μm² in midline sagittal sections of the anterior (C) and mid-diaphragm (D). No differences were seen in the level of apoptosis in the anterior diaphragms ($P = 0.703$) or mid-diaphragms ($P = 0.292$) between wild-type and *eyes2* embryos. (E) No differences were seen in the average thickness of the mid-diaphragms of wild-type and *eyes2* embryos ($P = 0.221$).

is thought to play a role in the regulation of inflammatory responses (37,38). Since considerable variability has already been reported in the phenotypes associated with recessive *FREMI* mutations in humans—and some *Frem1*^{eyes2} mice have no other identifiable defects beside CDH—it is likely that our patient's lack of features suggestive of BNAR or MOTA syndromes is due to variations in genetic, environmental and/or stochastic factors rather than a unique effect of her *FREMI* genotype.

Since our patient's presentation was unlike previously reported cases, a *FREMI*-related disorder was only considered as a possible diagnosis after her *FREMI* deletion was identified by copy number analysis. With the expanded use of copy number analysis and whole exome/whole genome sequencing on a clinical basis, it is possible that other individuals with both isolated and non-isolated forms of CDH will be found to have autosomal recessive mutations in *FREMI*.

The identification of recessive *FREMI* mutations in a patient with CDH is clinically important for several reasons.

This finding should alert physicians to the possibility of unidentified anomalies—particularly renal anomalies which are likely to remain undiagnosed without dedicated studies. Identification of recessive *FREMI* mutations can dramatically change the recurrence risk estimation for siblings from the <2% typically quoted for isolated cases of CDH to as high as 25% if the mutations are inherited (6). Families should also be counseled about the spectrum of anomalies that have been associated with *FREMI* deficiency and how phenotypes may differ between affected siblings.

The role of other factors on the penetrance of CDH associated with *FREMI* deficiency

Similar to most—if not all—CDH-related syndromes, the CDH phenotype associated with *FREMI* deficiency shows incomplete penetrance (6). Although one could speculate that CDH was seen uniquely in our patient due to the severity of

Table 2. Phenotypic features found in patients with *FREMI* mutations

<i>FREMI</i> mutations	Zygosity	Associated phenotypes	Diagnosis	Reference
c.2721delG p.V908SfsX17	Homozygous	Unilateral or bilateral renal agenesis, low-pitched crying, short and thick oral frenula, incurved fifth toe, anteriorly placed anus, anal stenosis	BNAR	Al-Gazali <i>et al.</i> (23); Alazami <i>et al.</i> (24)
c.1945C > T p.Arg649Trp	Homozygous	Bifid nose, renal agenesis	BNAR	Alazami <i>et al.</i> (24)
c.4318G > A p.Gly1440Ser	Homozygous	Bifid nose, airway malformation, renal agenesis	BNAR	Alazami <i>et al.</i> (24)
Identity by descent (IBD) upstream of <i>FREMI</i>	Homozygous	Bifid or dimpled nose, eyelid-corneal fusion, aberrant hairline	MOTA	Mateo <i>et al.</i> (28)
~60.1 kb deletion of exons 8–23	Homozygous	Anophthalmia, aberrant hairline, bifid nasal tip	MOTA	Slavotinek <i>et al.</i> (25)
	Heterozygous (second mutation not identified)	Cryptophthalmos, aberrant hairline, hypertelorism, bifid nasal tip, omphalocele, anteriorly placed anus, anal stenosis	MOTA	Slavotinek <i>et al.</i> (25)
c.2097delATTA p.Lys699AsnfsX10	Homozygous	Eyelid coloboma, hypertelorism, bifid nasal tip, vaginal atresia	MOTA	Li <i>et al.</i> (27); Slavotinek <i>et al.</i> (25)
c.3971T > G p.Leu1324Arg; c.6271G > A p.Val2091Ile	Compound heterozygous	Eyelid coloboma, aberrant hairline, broad nasal tip	MOTA	Fryns <i>et al.</i> (29); Slavotinek <i>et al.</i> (25)
Deletion of exon 1 ^a ; c.5334 + 1G > A	Compound heterozygous	Congenital diaphragmatic hernia, mild 2–3 toe syndactyly	Isolated CDH	Current report

^aBased on *FREMI* transcript 001.

the alterations in her *FREMI* alleles, we note that homozygosity for truncating mutations in *FREMI*—including a 1 bp deletion in exon 16 (c.2721delG), an ~60.1 kb in-frame deletion involving exons 8 through 23, and a 4 bp deletion in exon 13 (c.2097delATTA)—have previously been reported in individuals with BNAR or MOTA syndrome without CDH (Table 2) (24,25). This suggests that other genetic, environmental and/or stochastic factors influence the development of CDH in humans with *FREMI* deficiency.

Incomplete penetrance for CDH was also seen in *Frem1*^{eyes2} mice. The existence of genetic factors that influence the penetrance of CDH in these mice was specifically demonstrated by the variation in the incidence of CDH seen in the offspring of *Frem1*^{eyes2} breeding pairs on different genetic backgrounds. Similar strain-dependent variations in CDH penetrance have been seen in other mouse models of anterior CDH including those caused by deficiencies of *SOX7* and *GATA4* (39,40). This variation could prove useful in future studies involving *Frem1*^{eyes2} mice aimed at identifying genes which modulate the development of CDH.

Interspecies variation in the location of *FREMI*-related CDH

The anterior CDH seen in *Frem1*^{eyes2} mice is always located in the ventral midline, directly behind the sternum, in a region of the diaphragm that is typically muscularized. In the most severe cases, herniated liver tissue and the gallbladder were found encapsulated in a membranous hernial sac. The diaphragmatic hernia seen in the proband was also covered by a membranous sac that was attached to the lower lobe of the left lung. However, her CDH was located in the left posterolateral region of the diaphragm, where the majority of CDH occurs in humans (5).

As additional cases are identified, we may find that *FREMI*-related CDH in humans occurs predominantly in the anterior

diaphragm as it does in mice. However, similar differences in the location of the CDH are seen between individuals with recurrent 8p23.1 deletions—which include the transcription factor encoding genes *GATA4* and *SOX7*—and *GATA4*- and *SOX7*-deficient mice. In humans, all but one of the reported cases of CDH caused by 8p23.1 deletions have been posterolateral (41). In contrast, both *GATA4*- and *SOX7*-deficient mice consistently have anterior retrosternal diaphragmatic hernias that are grossly indistinguishable from those seen in our *FREMI*-deficient mice (39,40).

It is possible that variations in location of CDH caused by *FREMI*, *GATA4* and *SOX7* deficiency are primarily due to intrinsic differences in the developing diaphragms of humans and mice that make specific regions more susceptible to herniation. Alternatively, the underlying biological and/or molecular processes that cause CDH in humans and mice may vary, even in cases in which the same gene is affected. In either case, it would be imprudent to assume that similar genetic changes will always result in the development of the same diaphragmatic phenotype in both humans and mice.

Histopathologic changes associated with anterior CDH

At E14.5, *Frem1*^{eyes2} embryos were found to have a decreased level of cell proliferation in the mid-diaphragm compared with wild-type embryos. At this time in development, the mid-diaphragm is part of the non-muscularized central tendon, the pleuroperitoneal canals have closed (typically between E12.5 and E13.5) and the liver is undergoing accelerated growth as it is vascularized and colonized by hematopoietic cells to become the major fetal hematopoietic organ (42). It is possible that decreased cell proliferation in the mid-diaphragm, in conjunction with accelerated liver growth, places additional stress on the anterior diaphragm and contributes to the development of the retrosternal CDH seen in *Frem1*^{eyes2} mice.

Interestingly, a similar pattern of decreased cell proliferation in the central tendon region of the diaphragm has been documented in mice that lack another basement membrane protein, SLIT3 (43). However, unlike *Frem1*^{eyes2} mice that exhibit retrosternal CDH, *Slit3*-null mice have a central form of CDH in which the diaphragmatic defect forms in the anterior portion of the central tendon next to the muscular diaphragm (43,44). It is possible that this difference in CDH type can be traced to other variances seen between *Slit3*-null mice and *Frem1*^{eyes2} mice. For example, the central tendon of *Slit3*-null embryos was found to be abnormally thin and never separates from the underlying liver (43). In contrast, the thickness of the mid-diaphragm was indistinguishable between wild-type and *Frem1*^{eyes2} embryos, and abnormal fusions of the central tendon and the liver were not seen in *FREM1*-deficient mice.

Anterior hernias are not always associated with decreased levels of cell proliferation. As previously mentioned, retrosternal CDH has been described in mice that are haploinsufficient for the transcription factor-encoding gene *Gata4* (39,40). Although *Frem1* and *Gata4* are both expressed in the mesenchymal cells of the developing diaphragm, *Gata4*^{+/-Δex2} embryos have increased levels of apoptosis in the anterior central tendon but normal levels of cell proliferation (39). This suggests that distinct processes which lead to a decrease in the number of viable cells in the mid-diaphragm/central tendon region of the developing diaphragm may predispose to the development of retrosternal CDH in mice.

Since the retrosternal hernias described in *FREM1*-deficient mice were identified between P28 and adulthood, it is possible that postnatal muscular or connective tissue weakness may also be contributing to the development of CDH in this mouse model.

Potential role of a *FREM1*/*FRAS1*/*FREM2* complex in the development of CDH

During epidermal development, *FREM1* is secreted by mesenchymal cells in the dermis and forms a ternary complex in the basement membrane with *FRAS1* and *FREM2*, which are secreted from the epidermis with the help of the cytosolic adapter protein *GRIP1* (11). Although we have shown that *Frem1*, *Fras1* and *Frem2* are all expressed in the diaphragm, additional studies will be needed to determine whether they form a similar ternary complex in the diaphragm and if this complex plays a role in the normal development of the diaphragm.

If failure to form a *FREM1*/*FRAS1*/*FREM2* complex is responsible for the development of CDH, one would expect that mice that are deficient for *FRAS1*, *FREM2* or *GRIP1* and individuals with Fraser syndrome should be predisposed to the development of CDH. Although CDH has never been documented in *FRAS1*-, *FREM2*- or *GRIP1*-deficient mice, two reports describe individuals with CDH and a clinical diagnosis of Fraser syndrome—one with a Bochdalek-type (posterior) hernia and one in which the location of the CDH was not described (45,46). While these reports suggest that failure to form a *FREM1*/*FRAS1*/*FREM2* complex may predispose to the development of CDH, a lack of molecular testing in these cases makes it impossible to clearly link deficiency of *FRAS1*, *FREM2* or *GRIP1* with the development of CDH at this time.

MATERIALS AND METHODS

Patient accrual and DNA preparation

Informed consent was obtained from the proband and her parents in accordance with IRB-approved protocols. DNA extracted from whole blood was used for copy number variant analysis and sequencing studies.

Copy number variant and FISH analyses

Copy number variant analysis was performed on a clinical basis using Illumina cyto-SNP bead chips version 12.2 (Illumina, San Diego, CA, USA). Nexus Copy Number Version 5 software (BioDiscovery, El Segundo, CA, USA) was used for filtering, normalization and data analysis as previously described (47). FISH analyses of the *FREM1* deletion were carried out using the fosmid clone G248p8100A4 (W12-548A7; chr9:14,891,722-14,932,320, hg19) obtained from the BACPAC Resource Center (Oakland, CA, USA) as previously described (47,48).

Sequencing of *FREM1*

The coding sequence and intron–exon boundaries of *FREM1* (based on *FREM1* transcript 001) were amplified from DNA using primers listed in Supplementary Material, Table S1 (25). Sequence changes in PCR-amplified products were identified in samples obtained from the proband and her parents using the Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Screening for the c.5334 + 1G > A variant in ethnically matched controls

An allele-specific mismatch PCR assay was designed to screen control individuals of north Asian Indian origin for the presence of the c.5334 + 1G > A variant. In this assay, the forward primer PP2556, 5'-GTGTAGGTTGATCAACCACG-3', and the reverse primer PP2557, 5'-TGAAAGACACCAAAGAACAACATAAGTTTGTAGCTTGATAATTTACATTTTCAGGAT-CATTA-3', were used to amplify a 295 bp product containing the variant residue. Two bases—a C and a T residue located 7 bp and 8 bp 3' to the G–A variant, respectively—were changed to a T and a C, respectively, in the reverse primer in order to create a novel *BsaBI* site in conjunction with the c.5334 + 1G > A variant. These bases are bolded and underlined within the PP2557 sequence above. When PCR was performed using a template that bore the variant residue A, the resulting amplified product of 295 bp was cleaved by the enzyme *BsaBI* into 238 bp and 57 bp fragments.

cDNA synthesis, amplification and analysis

cDNA was synthesized from total RNA extracted from the proband's EBV-transformed lymphocytes and lymphocytes from a control individual with no personal or family history of CDH using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) or Superscript III (Invitrogen, Carlsbad, CA, USA), respectively, according to manufacturer's recommendations. cDNA was then PCR amplified

using primers 5'-ATAAACCCATCTTTGGAAGTAAAT-3' and 5'-TTCCTCTAATCCGTCATAGGTAAT-3' which amplify a 339 bp product from normal human cDNA and have a predicted genomic product size of >19 000 bp. The resulting PCR products were gel purified using a Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA), followed by reamplification and Sanger sequencing using the same primers. Sequences were analyzed using the Sequencher 4.7 software (Gene Codes Corporation).

Mouse studies

All experiments using mouse models were conducted in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. The associated protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (Animal Welfare Assurance #A3832-01).

N-ethyl-N-nitrosourea mutagenesis and generation of *eyes2* mice

ENU mutagenesis was carried out using 8- to 12-week male C57BL/6Brd^{Tyr} mice as previously described (49). These mice were then bred and intercrossed to screen for viable recessive phenotypes. The *eyes2* strain (MGI: 3038748, Mouse Genomic Informatics at the Jackson Laboratory, <http://www.informatics.jax.org/>) was identified based on the presence of unilateral and bilateral microphthalmia and/or cryptophthalmos (30).

Mapping and cloning of the *eyes2* allele

Mice from the *eyes2* strain were backcrossed to 129S6/SvEvTac mice. The progeny of these crosses were intercrossed to identify mice carrying the *eyes2* allele. After several generations of backcrossing, *eyes2* mice were genotyped using single nucleotide polymorphism markers that discriminate between C57BL/6Brd^{Tyr} and 129S6/SvEvTac strains and linkage analysis was performed as previously described (50). The *eyes2* allele was found to be linked to markers on mouse chromosome 4. Additional backcrosses were carried out and *eyes2* mice were genotyped by amplifying and sequencing regions of the genome which harbored SNPs known to vary between the C57BL/6Brd^{Tyr} and 129S6/SvEvTac strains.

Candidate genes from the *eyes2* interval—including *Frem1*—were selected for further analysis based, in part, on the results of phylogenetic profiling carried out using the method previously described by Chiang *et al.* (51). Briefly, two gene sets—the first composed of candidate genes from the linkage interval and the second composed of the mouse orthologs of the genes previously shown to cause eye structural defects in humans [*Eya1*, *Eya2*, *Six3*, *Gli3*, *Pax6*, *Mks1*, *Tmem67* (*Mks3*), *Pax2*, *Casp3*, *Sox2*, *Shh*, *Bcor*, *Chx10*, *Sox10*, *Otx2*, *Rax*, *Hesx1*, *Ikbkg*, *Ptch1*, *Pomt1*, RP23-464N23.1 (*Chd7*), *Gmn*, *Mitf*, *Ndph*, *Cryba4*, *Crybb2*] were compared against 14 proteomes representing different branches of the phylogenetic tree. Candidate and control gene sets were compared with proteomes using BLASTP 2.2.14. The e-values were analyzed with a custom written Perl script (ActivePerl 5.8.8 build 817). The enriched gene set was further prioritized

based on the known or predicted role of each gene in organogenesis through comprehensive GO profiling (<http://www.geneontology.org/>).

The coding region and associated intron–exon junctions of *Frem1* were amplified by PCR and the resulting amplification products were sequenced. Primer sequences are available on request. Sequence traces were analyzed using the Sequencher 4.7 software (Gene Codes Corporation).

Phenotypic analysis of *eyes2* mutant mice

Necropsies were performed on *Frem1*^{eyes2} mice to determine the penetrance of various *Frem1*-associated phenotypes on a mixed B6Brd/129S6 background and a derivative line that had been backcrossed to C57BL6/J mice for at least eight generations. A chi-square analysis was performed using the Opus12 chi-square calculator (http://www.opus12.org/Chi-Square_Calculator.html) to assess strain-dependent differences in the penetrance of individual phenotypes.

Histological analyses

Histological analyses of diaphragmatic hernias from *eyes2* mice—including the diaphragm, herniated viscera and the associated membranous covering and liver—were performed as previously described (40).

In situ hybridization

In situ probes for *Frem1*, *Frem2* and *Fras1* were generated by PCR amplification using cDNA from C57BL6/J mice as template. PCR primers for the *Frem1* probe (5'-GTACAAGCTTGATGTCATCTCAGGGGCTGT-3' and 5'-ATGCAAGCTTAATCTTCTCTCTGGGCGAGTC-3') were designed using the Sequencher 4.7 software (Gene Codes Corporation) and amplified a 1079 bp product. PCR primers for the *Frem2* probe (5'-ACTGACTGAAGCTTATGTGACCATCCTCACA GACAG-3' and 5'-ACTGACTGAAGCTTAGGAATGTG ACAGTGGAAACA-3') and *Fras1* probe (5'-AGCTAGCTA AGCTTAAGCACACTTTTAGGTGGGTGT-3' and 5'-AGCTA GCTAAGCTTTCTCATCCAGAGTTCACAACGA-3') generated 611 bp and 653 bp products, respectively, and were based on *in situ* probe designs from the Euxpress database (<http://www.euxpress.org/ee/>; *Frem2* template ID T38972; *Fras1* template ID T36392). All PCR primers included 5' *Hind*III linkers. PCR products were gel purified, cut with *Hind*III and cloned into pBlueScript SK(–). *In situ* studies were performed by the IDDRC RNA *In Situ* Hybridization core at Baylor College of Medicine, on sagittal sections of E14.5, C57BL6/J embryos as previously described (52).

Immunohistochemistry

Immunohistochemistry was performed as previously described using 1:200 dilutions of the following antibodies: anti-Phospho-Histone H3 (#9701S, Cell Signaling Technology, Danvers, MA, USA), anti-cleaved-Caspase 3 (#9664S, Cell Signaling Technology) and Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG (#711-065-152, Jackson ImmunoResearch, Newmarket, Suffolk, UK) (40).

Phospho-Histone H3- and cleaved-Caspase 3-positive cells were counted in regions of the anterior or mid-diaphragm in midline sagittal sections of E14.5 embryos. To standardize the region of the diaphragm selected for interrogation, a 10 033 μm^2 rectangle was placed over images of the anterior diaphragm starting at the intersection between the diaphragm and the sternum or over a non-overlapping region of the mid-diaphragm located underneath the heart. The number of positive cells within the selected region of the diaphragm were counted and normalized to the diaphragmatic area calculated using the AxioVision Release 4.6.1.0 software (Carl Zeiss AG). This software was also used to measure the thickness of the mid-diaphragm at five representative points within the selected region. Three embryos of each genotype were used, with measurements being averaged over three to seven sections per embryo. Results were analyzed using one-way analysis of variance performed using the IBM SPSS Statistics software (IBM, Armonk, NY, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors have no conflicts of interest to declare.

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