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ORIGINAL ARTICLE

The role of FREM2 and FRAS1 in the development of congenital diaphragmatic hernia

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Abstract

Congenital diaphragmatic hernia (CDH) has been reported twice in individuals with a clinical diagnosis of Fraser syndrome, a genetic disorder that can be caused by recessive mutations affecting *FREM2* and *FRAS1*. In the extracellular matrix, FREM2 and FRAS1 form a self-stabilizing complex with FREM1, a protein whose deficiency causes sac CDH in humans and mice. By sequencing *FREM2* and *FRAS1* in a CDH cohort, and searching online databases, we identified five individuals who carried recessive or double heterozygous, putatively deleterious variants in these genes which may represent susceptibility alleles. Three of these alleles were significantly enriched in our CDH cohort compared with ethnically matched controls. We subsequently demonstrated that 8% of *Frem2*$^{ne/ne}$ and 1% of *Fras1*$^{Q1263*/Q1263*}$ mice develop the same type of anterior sac CDH seen in FREM1-deficient mice. We went on to show that development of sac hernias in FREM1-deficient mice is preceded by failure of anterior mesothelial fold progression resulting in the persistence of an amuscular, poorly vascularized anterior diaphragm that is abnormally adherent to the underlying liver. Herniation occurs in the perinatal period when the expanding liver protrudes through this amuscular region of the anterior diaphragm that is juxtaposed to areas of muscular diaphragm.

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Based on these data, we conclude that deficiency of FREM2, and possibly FRAS1, are associated with an increased risk of developing CDH and that loss of the FREM1/FREM2/FRAS1 complex, or its function, leads to anterior sac CDH development through its effects on mesothelial fold progression.

Introduction

Congenital diaphragmatic hernia (CDH) is a life-threatening birth defect that is seen in approximately 1 in 4000 newborns and accounts for 8% of all major congenital anomalies (1,2). In approximately 20% of CDH cases, the herniated abdominal organs are covered by a membranous sheet of tissue referred to as a hernial sac (3). CDH can occur as an isolated defect, but in 30–40% of cases, additional non-hernia-related anomalies are present (4–6). Some individuals with non-isolated CDH (CDH+) are ultimately diagnosed with a genetic syndrome.

CDH has been described in two individuals with a clinical diagnosis of Fraser syndrome [Online Mendelian Inheritance in Man (OMIM, https://omim.org/; date last accessed February 26, 2018): 219000, 617666], an autosomal recessive disorder characterized by cryptophthalmos, syndactyly, renal defects and genital anomalies (7,8). At the time these cases were published, the genes that cause Fraser syndrome, which include *FREM2* (OMIM: 608945) and *FRAS1* (OMIM: 607830), had not been identified (9–11). Since a molecular diagnosis could not be made, it remained unclear whether deficiency of FREM2 and/or FRAS1 could cause CDH in humans.

In the extracellular matrix, FREM2 and FRAS1 form a mutually stabilizing ternary complex with a related extracellular matrix protein, FREM1 (12). This complex plays an important role in cell adhesion and intercellular signaling (12–14). Recessive loss-of-function mutations affecting *FREM1* have been shown to cause isolated sac CDH in humans and anterior sac CDH has been documented in up to 47% (15/32) of *Frem1*$^{eyes2/eyes2}$ mice that are homozygous for a c.2477T>A, p.Lys826* (NM_177863.4) stop-gain variant in *Frem1* (15,16).

Additional evidence for the potential role of FREM2 deficiency in the development of CDH has come from the National Heart, Lung, and Blood Institute (NHLBI) Cardiovascular Development Consortium, Bench to Bassinet Program. Information submitted directly to the Mouse Genome Information database (MGI, http://www.informatics.jax.org/) by this program indicates that mice that are homozygous for a c.6739T>G, p.Phe2247Val (NM_172862.3) variant in *Frem2* develop CDH. However, further details about the diaphragmatic hernias identified in these mice were not provided.

These observations led us to hypothesize that deficiency of FREM2 and FRAS1 can contribute to the development of CDH in humans and mice. To test this hypothesis, we screened a CDH cohort and searched online databases for individuals who carry deleterious, homozygous or compound heterozygous variants in *FREM2* or *FRAS1*, or double heterozygous variants at these two loci. We also examined *Frem2*$^{ne/ne}$ and *Fras1*$^{Q1263*/Q1263*}$ mice and embryos for evidence of CDH and diaphragmatic defects. In addition, we used the FREM1-deficient *Frem1*$^{eyes2/eyes2}$ mouse model to determine the morphogenetic defects associated with FREM1/FREM2/FRAS1-related anterior sac CDH.

Results

Identification of putatively deleterious, recessive *FREM2* changes in individuals with CDH

To determine if deleterious changes in *FREM2* and *FRAS1* can predispose individuals to develop CDH, we screened a cohort of 69 individuals with CDH for putatively deleterious homozygous and compound heterozygous variants in these genes. We identified a European American male (Subject 1) with a large, left-sided CDH who carried two rare (allele frequency < 1%), putatively deleterious, sequence changes in *FREM2*: a maternally inherited c.4031G>A, p.Arg1344His change and a paternally inherited c.4558C>T, p.Arg1520Trp change. In a search of the DECIPHER database, we also identified a 3-year-old female with CDH (Subject 2; DECIPHER 259497) who was homozygous for a c.5938_5940delCTT, p.Leu1980del change in *FREM2* (17).

Clinical and molecular data on Subjects 1 and 2 are summarized in Tables 1 and 2, and Supplementary Material, Figure S1. Detailed clinical summaries are available in the Supplementary Material. We did not identify any individuals with CDH who carried putatively deleterious homozygous or compound heterozygous changes in *FRAS1* in our cohort or in online databases.

Identification of putatively deleterious, double heterozygous changes in *FREM2* and *FRAS1* in individuals with CDH

FREM1, FREM2 and FRAS1 have been shown to function together in a mutually stabilizing ternary complex that plays an important role in cell adhesion and intercellular signaling (12–14). In addition, a synergistic interaction has been demonstrated between *frem2a* and *fras1* in zebrafish embryonic fin development (18). This suggests that individuals harboring deleterious changes affecting two or more of these proteins may also have an increased risk of developing CDH.

With this in mind, we also looked for individuals who carried rare, double heterozygous changes in these genes which were inherited from different parents. We identified three such individuals: a European American female (Subject 3) with a right-sided diaphragmatic eventration and a left-sided sac CDH, a European American male (Subject 4) with a left-sided sac CDH and a Filipino male (Subject 5) with a left-sided CDH whose sister died of CDH at 3 days of age. Molecular and clinical data from these subjects are summarized in Tables 1 and 2, and Supplementary Material, Figure S1. Detailed clinical summaries are available in the Supplemental Material.

Recognizing that the *FREM2* and *FRAS1* changes we identified in these individuals might not be sufficient to cause CDH, we looked for other deleterious changes in CDH-related genes. This search revealed two heterozygous changes in CDH-related genes (Table 2, Supplementary Material, Fig. S1). The first was a *GLI3* c.2726C>G, p.Ala909Gly (OMIM: 165240; NM_000168.5) variant in Subject 3 (19,20). This change was considered probably damaging and possibly damaging by PolyPhen-2 HumDiv and HumVar respectively, damaging by SIFT and disease causing by MutationTaster. It is not seen in the NHLBI Exome Variant Server but is seen in 1/61162 (0.0016%) alleles in non-Finnish Europeans in the Exome Aggregation Consortium (ExAC) Database and in 2/105678 (0.0019%) alleles in non-Finnish Europeans in the Genome Aggregation Database (gnomAD).

The second variant was a heterozygous *ROBO4* c.569G>C, p.Gly190Ala (OMIM: 607528; NM_019055.5) variant in Subject 5 (21). This change is predicted to be possibly damaging by

Table 1. In silico analysis results and allele frequencies of putatively damaging *FREM2* and *FRAS1* alleles

Allele (*FREM2* = NM_207361.5 *FRAS1* = NM_025074.6)	PolyPhen-2 HumDiv and HumVar, SIFT, MutationTaster	Ethnically-matched allele frequency			
		CDH cohort	NHLBI Exome Variant Server	ExAC Database	gnomAD
FREM2 c.4031G>A, p.Arg1344His	P, B, T, DC	European American 3/60 (5%)	European American 23/8600 (0.27%), Homo = 0, P < 0.001	Non-Finnish Europeans 240/66714 (0.36%); Homo = 1, P < 0.0001 Finnish Europeans 37/6614 (0.56%), Homo = 0, P < 0.01	Non-Finnish Europeans 494/126670 (0.39%), Homo = 1, P < 0.01 Finnish Europeans 140/25786 (0.54%), Homo = 1, P < 0.01
FREM2 c.4558C>T, p.Arg1520Trp	PD, PD, D, DC	European American 1/60 (1.67%)	European American 5/8600 (0.058%), Homo = 0, P < 0.05	Non-Finnish Europeans 2/66634 (0.003%), Homo = 0, P <0.0001 Finnish Europeans 0/6614	Non-Finnish Europeans 11/125910 (0.009%), Homo = 0, P < 0.01 Finnish Europeans 0/25790
FREM2 c.5938_5940delCTT, p.Leu1981del	NA, NA, NA, DC	NA	Allele not detected in any ethnic group	Allele not detected in any ethnic group	South Asian 7/30778 (0.023%), Homo = 0
FREM2 c.4994C>T, p.Ser1665Phe	P, P, T, P	Filipino 1/2 (50%)	Allele not detected in any ethnic group	Allele not detected in any ethnic group	Allele not detected in any ethnic group
FRAS1 c.2389G>A, p.Glu797Lys	P, B, T, P	European Americans 1/60 (1.67%)	Allele not detected in European Americans	Non-Finnish European 1/66724 (0.0015%), Homo = 0, P < 0.0001 Finnish European 0/6614	Non-Finnish Europeans 6/111578 (0.005%), Homo = 0, P < 0.01 Finnish European 0/22296
FRAS1 c.9806G>A, p.Arg3269Gln	PD, PD, D, DC	European Americans 1/60 (1.67%)	European Americans 83/8458 (0.98%), Homo = 0, P = 0.45	Non-Finnish European 584/65864 (0.83%), Homo = 3, P = 0.415 Finnish European 30/6614 (0.45%), Homo = 0, P = 0.245	Non-Finnish European 1188/125176 (0.95%), Homo = 8, P = 0.325 Finnish European 99/25568 (0.39%), Homo = 0, P = 0.186
FRAS1 c.6323A>T, p.Asp2108Val	P, B, D, DC	Filipino 1/2 (50%)	Allele not detected in any ethnic group	Allele not detected in any ethnic group	Allele not detected in any ethnic group

B, benign; D, damaging; DC, disease causing; NA, not applicable; P, possibly damaging; PD, probably damaging; T, tolerated.

Table 2. Molecular and clinical summaries of subjects carrying putatively damaging compound and double heterozygous changes in *FREM2* and *FRAS1* inherited from different parents

Subject	Subject 1	Subject 2 (DECIPHER 259497)	Subject 3	Subject 4	Subject 5
Maternal allele[a]	*FREM2* c.4031G>A, p.Arg1344His	*FREM2* c.5938_5940delCTT, p.Leu1980del	*FRAS1* c.2389G>A, p.Glu797Lys	*FRAS1* c.9806G>A, p.Arg3269Gln	*FREM2* c.4994C>T, p.Ser1665Phe
Paternal allele[a]	*FREM2* c.4558C>T, p.Arg1520Trp	*FREM2* c.5938_5940delCTT, p.Leu1980del	*FREM2* c.4031G>A, p.Arg1344His	*FREM2* c.4031G>A, p.Arg1344His	*FRAS1* c.6323A>T, p.Asp2108Val
Other putatively-damaging alleles in CDH-related genes[a]	None	None reported	*GLI3* c.2726C>G, p.Ala909Gly (maternal)	None	*ROBO4* c.569G>C, p.Gly190Ala (maternal)
Age, sex and ethnicity	6-month-old male, European descent	3-year-old female	6-month-old female, European descent	8-year–9-month-old male, European descent	7-year–2-month-old male, Filipino descent
Family history of CDH	None	None reported	None	None	Sister died of CDH
Diaphragm defect	Large, left-sided CDH	CDH	Right-sided diaphragmatic eventration, left-sided Bochdalek-type sac hernia	Left-sided sac hernia	Large, left-sided posterolateral CDH
Non CDH-related defects	Bilateral hydronephrosis	Global developmental delay, microcephaly, malformation of the heart and great vessels, PDA	Small umbilical hernia. congenital scoliosis[b]; bilateral hip dislocation[b] and joint contractures[b]	Muscular VSD, PDA, mild pectus excavatum, bilateral hydronephrosis, cryptorchidism, inguinal hernia	

[a]*FREM2*, NM_207361.5; *FRAS1*, NM_025074.6; *GLI3*, NM_000168.5; *ROBO4*, NM_019055.5; *RYR1*, NM_000540.

[b]Likely secondary to central core disease caused by a de novo, known pathogenic *RYR1* c.14581C>T, p.Arg4861Cys variant[a] (48).

PDA, patent ductus arteriosus; VSD, ventricular septal defect.

PolyPhen-2 HumDiv and HumVar, damaging by SIFT, and disease causing by MutationTaster. This variant is not seen in the NHLBI Exome Variant Server, the ExAC Database or gnomAD.

Comparisons of *FREM2* and *FRAS1* variant allele frequencies in patients with CDH and ethnically matched controls

The *FREM2* c.4031G>A, p.Arg1344His allele was seen in the heterozygous state in 10% (3/30) of European Americans in our CDH cohort (Subjects 1, 3 and 4) and was always associated with the inheritance of another rare, putatively deleterious allele from the other unaffected parent. The allele frequency of this variant among European Americans in our CDH cohort (3/60, 5%) is significantly higher than its corresponding allele frequency among European Americans in the NHLBI Exome Variant Server, and among Finnish and Non-Finnish Europeans reported in the ExAC database and gnomAD (Table 1) (22). No individuals were homozygous for the c.4031G>A, p.Arg1344His allele in the NHLBI Exome Variant Server. However, 17 homozygotes were reported in the ExAC database and gnomAD: 2 non-Finnish Europeans, 1 Finnish European, 1 Latino, 1 individual whose ancestry was not described and 12 individuals from south Asia. The combined allele frequency among south Asians in the ExAC database and gnomAD is 444/47294 (0.939%). This represents the highest allele frequency of any population group, but is still significantly lower than that seen in our CDH cohort ($P < 0.05$). This suggests that carrying the *FREM2* c.4031G>A, p.Arg1344His allele in trans with another, putatively deleterious allele may be associated with an increased risk for developing CDH, but being homozygous for this allele does not always lead to the development of CDH.

The *FREM2* c.4558C>T, p.Arg1520Trp change seen in Subject 1 and the *FRAS1* c.2389G>A, p.Glu797Lys change seen in Subject 3 were not recurrently seen in our CDH cohort. However, their allele frequencies among European Americans in our CDH cohort are significantly higher than the corresponding allele frequencies seen among ethnically matched individuals from the NHLBI Exome Variant Server, the ExAC database and gnomAD (Table 1). No individuals in these databases were reported to be homozygous for either of these alleles.

Anterior sac CDH in FREM2- and FRAS1-deficient mice

Using diaphragm sparing necropsy techniques, we evaluated the diaphragms of $Frem2^{ne/ne}$ mice that are homozygous for a c.6479C>T, p.Ala2160Val (NM_172862.3) change in *Frem2* (23,24). On a mixed CAST/EiJ/B6 background, we found that ~8.2% (5/61) of $Frem2^{ne/ne}$ mice evaluated between P28 and adulthood had anterior sac CDH (Fig. 1, Supplementary Material, Fig. S2). These hernias were indistinguishable from those previously documented in FREM1-deficient mice (16). In all cases, the hernias were located in the midline behind the sternum in a region of the diaphragm that is typically muscularized. Each of these hernias contained the gallbladder and a pedunculated mass of liver tissue surrounded by a thin membranous sac. This hernial sac was devoid of muscle tissue. In some cases, the gallbladder was found to be abnormally fused to the hernial sac.

To determine if FRAS1 deficiency can also cause CDH in mice, we evaluated the diaphragms of mice that are homozygous for a c.3787C>T, p.Q1263* (NM_175473.3) change in *Fras1* (25). In 102 $Fras1^{Q1263*/Q1263*}$ mice on a B6/FVB background, we identified one that had an anterior sac hernia located in the

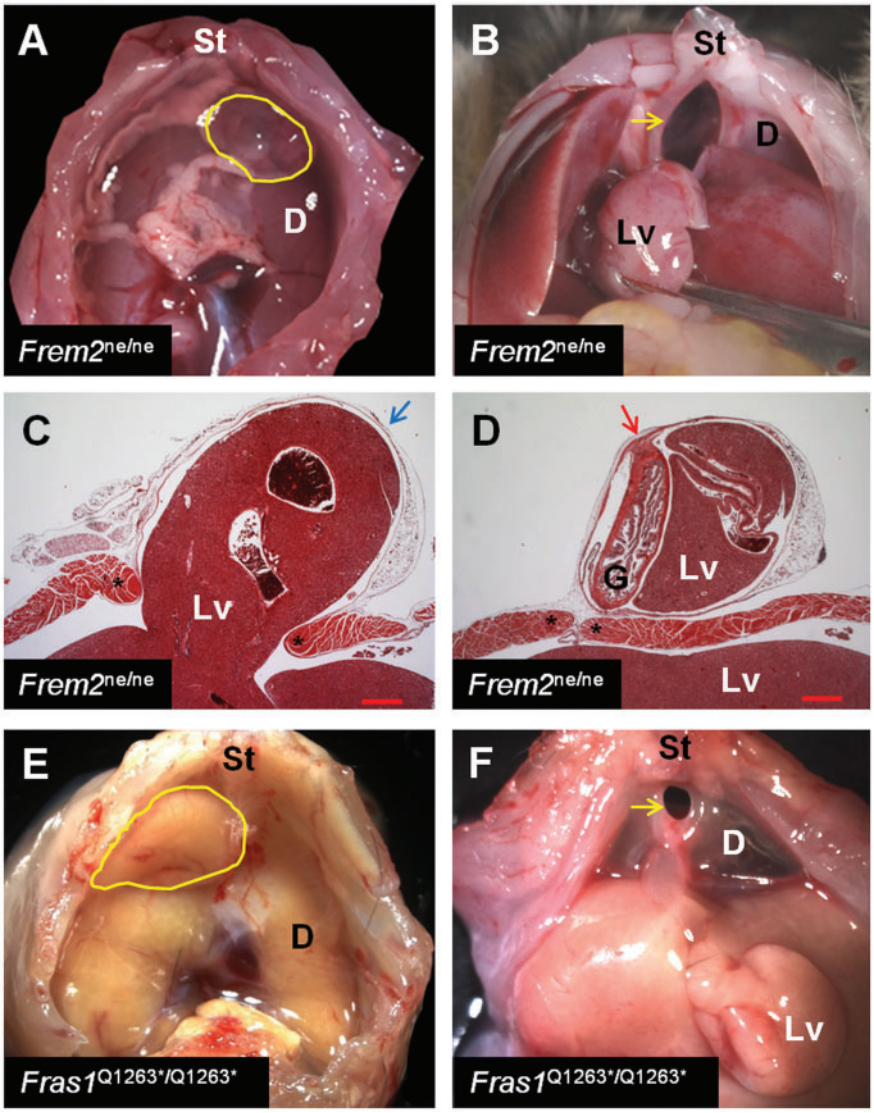

Figure 1. Deficiency of FREM2 and FRAS1 cause anterior sac hernias in mice. (**A**) A retrosternal diaphragmatic hernia in a FREM2-deficient *Frem2*$^{ne/ne}$ mouse as viewed from the thorax. The herniated viscera are covered by a membranous sac (yellow outline). (**B**) A retrosternal diaphragmatic hernia (yellow arrow) in a *Frem2*$^{ne/ne}$ mouse as viewed from the abdomen. (**C, D**) H&E-stained coronal sections through the hernial sac of a *Frem2*$^{ne/ne}$ mouse reveal herniated liver tissue (Lv) and the gallbladder (G, red arrow) surrounded by a thin membranous sac. There is a rapid transition from the muscularized diaphragm (*) to a thin amuscular sac (blue arrow). (**E**) A retrosternal diaphragmatic hernia in a FRAS1-deficient *Fras1*$^{Q1263*/Q1263*}$ mouse as viewed from the thorax. The herniated viscera are covered by a membranous sac (yellow outline). (**F**) The same retrosternal diaphragmatic hernia (yellow arrow) shown in (E) but viewed from the abdomen after reduction of the gallbladder and a pedunculated liver mass (Lv). D, diaphragm; St, sternum.

midline behind the sternum (Fig. 1, Supplementary Material, Fig. S3). As seen previously in FREM1-deficient and *Frem2*$^{ne/ne}$ mice, the gallbladder was fused to the hernial sac which also contained a pedunculated mass of liver tissue (16).

Abnormal mesothelial fold progression prior to anterior sac hernia formation

While studying *Slit3*-null mice, Yuan *et al.* hypothesized that the primary cause of central sac CDH was failure of the mesothelial fold progression—the process by which the liver and diaphragm separate and the neuromuscular elements of the diaphragm migrate through the amuscular diaphragm to their final position at the anterior midline (26). To determine if this same process might underlie the development of anterior sac CDH seen in FREM1/FREM2/FRAS1-deficient mice, we dissected the diaphragms of E16.5 *Frem1*$^{eyes2/eyes2}$ embryos on an inbred B6Brd/129S6 background on which the CDH frequency is ~47% (16). In 100% (9/9) of these embryos, we found a persistent region of amuscular diaphragm directly behind the sternum that was contiguous with the central tendon. In contrast, 100% (9/9) of the diaphragms of wild-type embryos on a mixed B6Brd/129S6 background showed complete muscularization of the anterior diaphragm at E16.5 (Fig. 2A and B). Among diaphragms harvested from *Frem1*$^{eyes2/eyes2}$ mice at P3–P4, 27% (3/11) had frank herniation with a sac, 45% (5/11) had a persistent region of amuscular diaphragm directly behind the sternum that was continuous with the central tendon, and 27% (3/11) had complete muscularization of the anterior diaphragm (Fig. 2C and D).

To determine if the liver was abnormally adherent to the anterior diaphragm in FREM1-deficienct embryos, we analyzed coronal sections from E16.5 *Frem1*$^{eyes2/eyes2}$ embryos on an

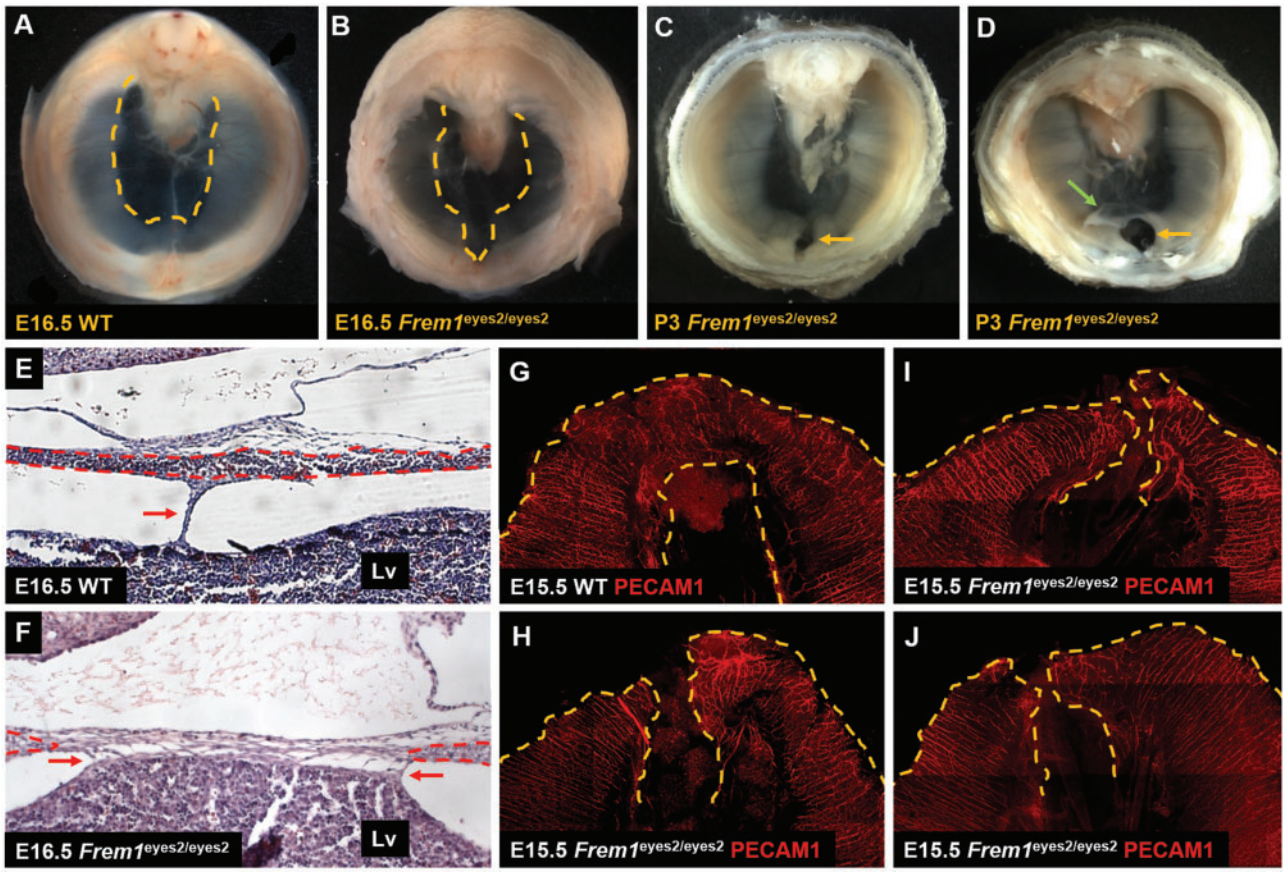

Figure 2. Morphogenetic abnormalities associated with anterior sac hernia development in FREM1-deficient mice. (**A**, **B**) By E16.5 wild-type embryos on a mixed B6Brd/129S6 background showed complete muscularization of the anterior diaphragm. In contrast, *Frem1*$^{eyes2/eyes2}$ embryos on an inbred B6Brd/129S6 background have a persistent region of amuscular diaphragm directly behind the sternum. Dashed yellow lines mark the boundary between amuscular and muscular regions of the diaphragm. (**C**, **D**) At P3 and P4, 45% (5/11) of *Frem1*$^{eyes2/eyes2}$ embryos on an inbred B6Brd/129S6 background had a persistent region of amuscular diaphragm directly behind the sternum (yellow arrow in panel C) and 27% (3/11) had frank herniation with a sac. The hernia contents have been reduced in the embryo pictured in (D) leaving a hernia sac (green arrow) and a circular defect in the diaphragm (yellow arrow) that is completely surrounded by muscular diaphragm. (**E**, **F**) Coronal sections through a wild-type E16.5 embryo on a mixed B6Brd/129S6 background, reveal complete muscularization of the anterior diaphragm (dashed red lines). In these embryos, the liver was attached to the anterior diaphragm only by the falciform ligament (red arrow). In contrast, the muscular components of the diaphragm (dashed red lines) fail to meet in the midline in a subset of E16.5 *Frem1*$^{eyes2/eyes2}$ embryos on an inbred B6Brd/129S6 background. In these embryos, the liver is also abnormally attached to the anterior diaphragm (red arrows). (**G**–**J**) Whole mount immunohistochemistry using a PECAM1 antibody, reveals a network of blood vessels surrounding the central tendon in wild-type E15.5 embryos on a mixed B6Brd/129S6 background (yellow dashed lines). In contrast, a poorly vascularized region of the anterior diaphragm (yellow dashed lines) was identified in 3/5 (60%) of *Frem1*$^{eyes2/eyes2}$ embryos on an inbred B6Brd/129S6 background at E15.5.

inbred B6Brd/129S6 background. In ~29% (2/7) of these embryos, the amuscular region of the anterior diaphragm was abnormally adherent to the liver (Fig. 2E and F). In contrast, in 100% (6/6) of wild-type embryos on a mixed B6Brd/129S6 background, the liver was attached to the diaphragm only by the falciform ligament.

Abnormal vascularization of the anterior diaphragm prior to sac hernia formation

After studying *Slit3*-null mice and mice in which the *Ndst1* gene has been ablated in the endothelium (*Ndst1*$^{flox/flox}$;Tie2-Cre), Zhang *et al.* contended that abnormal vascular development was the primary defect leading to central sac CDH in these models (21,27). They specifically demonstrated that the diaphragms of *Slit3*-null and *Ndst1*$^{flox/flox}$;Tie2-Cre E15.5-E16.5 embryos had regions of disrupted angiogenesis leading to development or persistence of a poorly vascularized region at the anterior edge of the central tendon (21,27).

To determine if FREM1/FREM2/FRAS1 deficiency could lead to a similar defect in vascular development, we performed whole-mount immunohistochemistry for PECAM1 on diaphragms harvested from E15.5 *Frem1*$^{eyes2/eyes2}$ embryos and E15.5 wild-type B6Brd/129S6 controls. In 3/5 (60%) of *Frem1*$^{eyes2/eyes2}$ embryos, we found a midline region of poorly vascularized diaphragm. These regions of decreased vascularization were continuous with the central tendon. In contrast, the vasculature of 3/3 (100%) wild-type controls completely surrounded the central tendon (Fig. 2G–J).

Discussion

CDH is a relatively common, life-threatening birth defect. Several lines of evidence from both human data and mouse models suggest that genetic factors play an important role in the development of CDH. These lines of evidence include: 1) the descriptions of genetic syndromes in which CDH is either a defining characteristic or a recurrent feature, 2) the association

of CDH with deletions and duplications of specific chromosomal regions, 3) the association of CDH with deleterious sequence changes in specific genes, 4) the existence of families in which multiple individuals are affected by CDH, and 5) identification of CDH in transgenic animal models in which specific genes have been disrupted (6,28–38). Here we use both human data and mouse models to test the hypothesis that deficiency of FREM2 and FRAS1 can contribute to the development of sac CDH. We also identify morphogenetic defects associated with the development of anterior sac hernias in FREM1-deficient mice.

FREM2 and FRAS1 deficiency in the development of CDH in humans

CDH has been described in two individuals with a clinical diagnosis of Fraser syndrome which can be caused by autosomal recessive mutations affecting *FREM2* and *FRAS1* (9–11). Although this suggests that FREM2 and/or FRAS1 deficiency may predispose individuals to the development of CDH, a clear conclusion could not be drawn from these two clinical cases, since a molecular diagnosis was not possible. Here, we have identified two individuals who carry homozygous or compound heterozygous, putatively deleterious sequence changes in *FREM2* and three individuals who are double heterozygous for putatively deleterious sequence changes in *FREM2* and *FRAS1*. One of these individuals (Subject 4) had sac CDH and another had a sac CDH and a diaphragmatic eventration (Subject 3). Two individuals (Subjects 1 and 5) had large, left-sided hernias without hernial sacs that required a patch repair. This suggests that loss of *FREM2* and *FRAS1* function may contribute to the development of diaphragmatic eventrations, sac CDH and non-sac CDH. Deficiencies of other proteins have previously been shown to cause a variety of diaphragmatic defects (31,39).

Subjects 1, 3 and 4 carried a *FREM2* c.4031G>A, p.Arg1344His allele in combination with another rare, putatively deleterious sequence variant. The frequency of this allele was found to be statistically over-represented among European Americans within our CDH cohort when compared with ethnically matched controls. However, this allele has also been seen in the homozygous state in control individuals. This suggests that the *FREM2* p.Arg1344His allele, and possibly the other alleles seen in Subjects 1–5, may be acting as susceptibility alleles with the penetrance of CDH in homozygotes, compound heterozygotes and double heterozygotes being determined by the presence of other genetic and/or environmental factors in an oligogenic or multifactorial model of inheritance. This mode of inheritance has long been suspected due to the low recurrence risk (~2%) associated with isolated CDH (40–42).

A role for both genetic and environmental/stochastic factors is also consistent with the incomplete penetrance for CDH seen among the FREM2- and FRAS1-deficient mice in this study and the background-dependent CDH penetrance previously documented in FREM1-deficient mice (16).

The exact nature of the genetic factors that may increase the likelihood of developing CDH in the setting of FREM2 or FRAS1 deficiency is unclear. However, we note that Subject 3 is heterozygous for a putatively deleterious c.2726C>G, p.Ala909Gly change in *GLI3* and Subject 5 is heterozygous for a putatively deleterious c.569G>C, p.Gly190Ala change in *ROBO4*. Although *GLI3* has not been clearly associated with the development of CDH in humans, *Gli3*-null mice develop CDH (19,20). Similarly, mutations of *ROBO4* have not been clearly associated with the development of CDH in humans, but *ROBO4* is located in a region of chromosome 11q that is recurrently duplicated in individuals with CDH (43). We also note that ROBO4 is a cell surface receptor of SLIT3, a secreted protein that plays a role in cell migration and angiogenesis, whose deficiency causes central sac CDH in mice (26,27,44). ROBO4 deficiency also increases the CDH penetrance in mice in which the *Ndst1* gene, which encodes a heparan sulfate biosynthetic enzyme, has been ablated in the endothelium (21).

FREM2 and FRAS1 deficiency in the development of CDH in mice

Information submitted directly to the MGI database (http://www.informatics.jax.org/) by the NHLBI Cardiovascular Development Consortium, Bench to Bassinet Program indicates that mice that are homozygous for a c.6739T>G, p.Phe2247Val (NM_172862.3) variant in *Frem2* develop diaphragmatic hernias. Here, we have shown that ~8% of $Frem2^{ne/ne}$ mice on a mixed CAST/EiJ/B6 background develop anterior sac CDH. The identification of CDH in two different FREM2-deficient mouse lines provides strong evidence that FREM2 deficiency can cause CDH in mice.

The evidence that FRAS1 deficiency causes CDH in mice is less compelling. CDH has not been documented previously in FRAS1-deficient mice and in a screen of 102 $Fras1^{Q1263*/Q1263*}$ mice on a B6/FVB background, we found only one with sac CDH. If FRAS1 deficiency is associated with the development of CDH in mice, the low penetrance of CDH in $Fras1^{Q1263*/Q1263*}$ mice could suggest that loss of FRAS1 is less detrimental to diaphragm development than the loss of FREM1 or FREM2. However, it is also possible that the reduced penetrance in this model is the result of differences in genetic background. We note that a statistically significant difference was observed in the penetrance of CDH previously documented in $Frem1^{eyes2/eyes2}$ mice on an inbred B6Brd/129S6 background (15/32, 46.9%) when compared to those on a congenic C57BL/6J background (6/73, 8.2%; $P < 0.0001$) (16).

The sac hernias documented in $Frem1^{eyes2/eyes2}$, $Frem2^{ne/ne}$ and $Fras1^{Q1263*/Q1263*}$ mice are indistinguishable. Specifically, all of them are anterior sac hernias that form at the midline behind the sternum, in a region of the diaphragm that is typically muscularized. These hernias are covered by a thin membrane that is devoid of muscle and contain a pedunculated mass of liver tissue. The gallbladder is often herniated along with the liver and is sometimes abnormally fused to the hernial sac. The similarities seen in the type of CDH documented in these mice is consistent with the observation that FREM1, FREM2 and FRAS1 form a mutually stabilizing complex in the extracellular matrix and that loss of one component leads to reduced expression and mislocalization of the other components (12).

Formation of anterior sac hernias in FREM1/FREM2/FRAS1-deficient mice

The low penetrance level of CDH in $Frem2^{ne/ne}$ and $Fras1^{Q1263*/Q1263*}$ mice, makes them ill-suited for studies aimed at identifying the morphogenetic processes associated with anterior sac hernia formation. In contrast, $Frem1^{eyes2/eyes2}$ mice on an inbred B6Brd/129S6 background have a relatively high rate of CDH (16). Since the sac hernias of $Frem1^{eyes2/eyes2}$, $Frem2^{ne/ne}$ and $Fras1^{Q1263*/Q1263*}$ mice are indistinguishable, and these proteins are known to function together in a complex (12), it is likely that the same abnormal morphogenetic processes lead to the development of anterior sac CDH in each of these models.

All of the herniated viscera documented in *Frem1*$^{eyes2/eyes2}$, *Frem2*$^{ne/ne}$ and *Fras1*$^{Q1263*/Q1263*}$ mice were covered by a membranous sac. This suggests that a loss of FREM1, FREM2 or FRAS1 function does not lead to failure of the formation of the amuscular diaphragm which is derived, at least in part, from the non-myogenic cells of the pleuroperitoneal folds (PPFs) (45). Instead, we observed that frank herniation in *Frem1*$^{eyes2/eyes2}$ mice is preceded by failure of mesothelial fold progression—the process by which the liver and diaphragm separate and the neuromuscular elements of the diaphragm migrate through the amuscular diaphragm to their final position at the anterior midline (26). Specifically, we observed failure of the muscularization of the anterior diaphragm at E16.5 in *Frem1*$^{eyes2/eyes2}$ embryos, a time point when the central tendon is completely surrounded by muscular diaphragm in wild-type B6Brd/129S6 embryos.

While studying *Gata4*$^{-/flox}$;Prx1-Cre mice that develop sac hernias randomly throughout the diaphragm, Merrell *et al.* observed that herniation occurred only when amuscular regions of the diaphragm develop in juxtaposition with muscular regions (45). Based on their observations and finite element modeling, they proposed that herniation occurs at lower pressures when the amuscular region is weaker and more compliant than the tissues surrounding it. In keeping with their findings, sac hernias in *Frem1*$^{eyes2/eyes2}$, *Frem2*$^{ne/ne}$ and *Fras1*$^{Q1263*/Q1263*}$ mice are consistently located in the anterior diaphragm where an amuscular region of the diaphragm is juxtaposed on both sides by regions of muscular diaphragm.

Sections through the hernia sacs of both *Frem1*$^{eyes2/eyes2}$ and *Frem2*$^{ne/ne}$ reveal an abrupt transition between the muscular diaphragm and the membranous, amuscular hernial sac. This provides further evidence that herniation occurs through an amuscular region of the anterior diaphragm. In the *Frem1*$^{eyes2/eyes2}$ model, frank herniation of the abdominal viscera occurs in the perinatal period with 27% (3/11) of mice at P3–P4 having frank herniation with a sac, and 45% (5/11) still having a persistent region of amuscular diaphragm directly behind the sternum.

In a subset of *Frem1*$^{eyes2/eyes2}$ embryos at E16.5, the amuscular diaphragm was abnormally adherent to the underlying liver. In these embryos, no muscular elements were observed over the region of adherence. Hence, it is possible that delayed separation of the diaphragm and liver impedes muscularization of the anterior diaphragm. Since the adherent amuscular diaphragm is contiguous with the central tendon, it is possible that the same processes which impedes muscularization of the central tendon are at work in the anterior diaphragms of *Frem1*$^{eyes2/eyes2}$ embryos. Alternatively, failure of migration could be caused by a lack of a positive migratory factor. Further studies are needed to distinguish between these possibilities.

Adhesion of the liver to the hernial sacs of *Frem1*$^{eyes2/eyes2}$, *Frem2*$^{ne/ne}$ and *Fras1*$^{Q1263*/Q1263*}$ mice was never observed (16). In keeping with this finding, we note that in all cases, the herniated liver appeared to be covered by a fibrous capsule. This suggests that while separation of the diaphragm and liver may be delayed in some embryos, separation is ultimately completed, even in cases in which there is frank herniation of the liver. In contrast to the liver, the gallbladder was often found to be abnormally fused to the hernia sac.

In a subset of E15.5 *Frem1*$^{eyes2/eyes2}$ embryos, we observed a poorly vascularized region of the anterior diaphragms that was not seen in E15.5 wild-type embryos. This poorly vascularized region was similar in size and location to the amuscular regions of the diaphragm we identified in *Frem1*$^{eyes2/eyes2}$ embryos. Hence, muscularization and vascularization of the anterior diaphragm appear to coincide.

Although it is possible that poor vascularization could lead to hypoxia-induced histopathologic changes that could weaken the anterior diaphragms of *Frem1*$^{eyes2/eyes2}$ embryos, we have previously shown that there are no differences in the levels of cell proliferation or apoptosis in the anterior diaphragms of *Frem1*$^{eyes2/eyes2}$ and wild-type embryos at E14.5 (16). However, in the same study, we observed a significant decrease in cell proliferation in the mid-diaphragm underlying the heart. While this region corresponds to the central tendon region of the mature diaphragm, which does not undergo herniation, we cannot rule out the possibility that decreased cell proliferation in this region places additional stress on the anterior diaphragm and contributes, secondarily, to the development of anterior sac CDH.

Based on these findings, we propose a model of FREM1/FREM2/FRAS1-related anterior sac hernia development in which a primary failure of mesothelial fold progression leads to persistence of a weak, poorly vascularized, amuscular anterior diaphragm that is juxtaposed by stronger regions of muscular diaphragm. In the perinatal period, frank herniation occurs through this amuscular region, leading to the formation of a membranous, amuscular sac which encapsulates the herniated liver and gallbladder.

Interspecies variation in the location of FREM1/FREM2/FRAS1-related CDH

As previously noted, the sac hernias found in *Frem1*$^{eyes2/eyes2}$, *Frem2*$^{ne/ne}$ and *Fras1*$^{Q1263*/Q1263*}$ mice consistently form in the anterior regions of the diaphragm. In contrast, the location of the CDH in humans with FREM1 deficiency (16) and Fraser syndrome (7,8), and in Subjects 1–5, are more variable.

Such differences are not unique to FREM1/FREM2/FRAS1-deficient mice. For example, haploinsufficiency of *GATA4* and *SOX7* are thought to underlie the development of CDH associated with recurrent microdeletions of chromosome 8p23.1 (36,38). While *Gata4*$^{+/-}$ and *Sox7*$^{+/-}$ mice develop anterior sac CDH, the majority of hernias associated with 8p23.1 microdeletions in humans are posterior (36,38,46).

In contrast to *Gata4*$^{+/-}$ mice that develop only anterior sac hernias, *Gata4*$^{-/flox}$; Prx1-Cre mice develop sac hernias randomly throughout the diaphragm (45). This suggests that more severe perturbations of the same pathway may lead to a greater variation in the location of sac hernia development.

Although we cannot provide a clear explanation for the interspecies variation in the location of FREM1/FREM2/FRAS1-related CDH, possibilities include; 1) intrinsic differences in the developing diaphragms of humans and mice that make specific regions more susceptible to herniation, 2) differences in the timing and magnitude of the forces acting on human and mouse diaphragms at different stages of development, 3) differences in the molecular role of the FREM1/FREM2/FRAS1 complex between humans and mice, and 4) differences in the level of molecular redundancy between humans and mice.

Loss of the FREM1/FREM2/FRAS1 complex predisposes to the development of CDH

At E14.5, *Frem1* transcripts are detectable in the mesenchymal cells of the anterior diaphragm (16). At the same time point, *Frem2* and *Fras1* transcripts are detectable in the mesothelial layer of the diaphragm (16). This expression pattern parallels

the expression of these genes in other organs including the skin, where FREM1 is secreted by mesenchymal cells in the dermis and forms a mutually stabilizing ternary complex in the basement membrane with FRAS1 and FREM2, which are secreted from the epidermis (12). Since *Frem1*$^{eyes2/eyes2}$, *Frem2*$^{ne/ne}$ and *Fras1*$^{Q1263*/Q1263*}$ mice develop indistinguishable forms of anterior sac CDH, it is reasonable to assume that disruption of this ternary complex, or its function, underlies the development of CDH in their respective mouse models.

The FREM1/FREM2/FRAS1 complex has been shown to play a role in both cell adhesion and intercellular signaling (12–14). Further studies will be required to determine how loss of this complex leads to failure of mesothelial fold progression in the developing diaphragm.

Materials and Methods

Patient accrual, DNA preparation and sequencing

Informed consent was obtained from study participants in accordance with institutional review board (IRB)-approved protocols. DNA extracted from whole blood and lymphoblastic cell lines were used for next generation sequencing studies (47). The CDH cohort consisted of 40 males and 29 females—30 European Americans, 25 Hispanics, 5 African Americans, 1 Asian, 1 Asian Indian, 1 Filipino, 1 Middle Easterner, 1 European American/Hispanic and 4 individuals of undeclared ancestry. None of the individuals in this cohort are known to be related. At the time of their accrual, the molecular cause of their CDH had not been determined. Since that time, a deleterious sequence change in *FBN1* has been shown to be the cause of CDH in one European American from this cohort (47).

Exome sequencing and variant annotation were performed in the Human Genome Sequencing Center at Baylor College of Medicine as described previously (47). All variants reported were confirmed by Sanger sequencing and have been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/; date last accessed February 26, 2018). In silico analyses were performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/; date last accessed February 26, 2018), SIFT (http://sift.jcvi.org/; date last accessed February 26, 2018) and MutationTaster (http://www.mutationtaster.org/; date last accessed February 26, 2018). Allele frequencies for putatively damaging *FREM2* and *FRAS1* alleles were acquired using the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS/; date last accessed February 26, 2018), ExAC Browser (http://exac.broadinstitute.org/; date last accessed February 26, 2018) and the Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org/; date last accessed February 26, 2018).

Exome sequencing data from Subjects 1, 3, 4 and 5 were also screened for rare, putatively damaging sequence changes in the following genes that have been implicated in the development of CDH in humans and/or mice and could be acting as modifiers of CDH penetrance: *ARRDC4*, *CHAT*, *CHD7*, *COL20A1*, *CTBP2*, *DISP1*, *DLL3*, *DNASE2*, *DSEL*, *EFEMP2*, *ELAC2*, *EYA2*, *FBN1*, *FGFR2*, *FGFRL1*, *FOXC1*, *FOXF2*, *FREM1*, *FYB*, *FZD2*, *GATA4*, *GATA6*, *GLI2*, *GLI3*, *GPR125*, *GRIP1*, *HLX*, *HOXB4*, *IGF1R*, *ILF3*, *KIF7*, *LRP2*, *LTBP4*, *MEF2A*, *MET*, *MLL2*, *MMP14*, *MPP2*, *MSC*, *MYH10*, *NEDD4*, *NEIL2*, *NIPBL*, *NR2F2*, *PAEP*, *PAX3*, *PBX1*, *PDGFRA*, *PORCN*, *PTPN13*, *RARA*, *RARB*, *RC3H1*, *ROBO1*, *ROBO2*, *ROBO4*, *RUNX1*, *SIX4*, *SLIT3*, *SMARCC1*, *SOX2*, *SOX7*, *STK36*, *STRA6*, *TBX6*, *TCF21*, *TGIF1*, *TWIST*, *WT1*, *ZEB1*, *ZFHX4*, and *ZFPM2*.

Statistical analyses

The prevalence of sequence variants in *FREM2* and *FRAS1* were compared between populations using Fisher's exact test. These tests were performed using Simple Interactive Statistical Analysis (SISA) software (http://www.quantitativeskills.com/sisa/; date last accessed February 26, 2018).

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 of 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).

All efforts were made to minimize suffering. Euthanasia was carried out using methods consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association and included carbon dioxide (CO_2) inhalation or an overdose of an inhaled anesthetic, such as isoflurane, in an appropriate enclosure followed by a physical method to ensure that the animals were euthanized.

Phenotypic analysis of FREM2-, FRAS1- and FREM1-deficient embryos and mice

To determine the frequencies of CDH in FREM2- and FRAS1-deficient mice, we performed necropsies using diaphragm-sparing techniques (16). In all cases, mice were evaluated between P28 and adulthood.

FREM1-deficient and wild-type embryos and mice were harvested at E16.5 and between P3 and P4. After fixation in Formalde-Fresh solution (Fisher Scientific, Pittsburgh, PA, USA) at room temperature for 48 h, diaphragms were isolated to identify morphogenetic changes associated with the development of anterior sac hernias.

Histological analyses

Hernial sacs from FREM2-deficient mice were dissected and fixed with Formalde-Fresh solution at room temperature. Specimens were trimmed, washed in 1× phosphate-buffered saline (PBS), dehydrated in ethanol, embedded in paraffin and sectioned coronally at 8 μm with an RM2155 microtome (Leica Biosystems GmbH, Nussloch, Germany). Representative sections were then stained with hematoxylin and eosin and imaged using an Axiocam MRc5 camera (Carl Zeiss Microscopy GmbH, Gena, Germany) attached to an Axioplan 2 microscope (Carl Zeiss Microscopy GmbH, Gena, Germany).

Whole mount immunohistochemistry

Whole diaphragms were harvested from FREM1-deficient embryos at E16.5. Embryonic diaphragms were placed in cold 4% PFA fixative for 30 min at room temperature and then placed at 4°C for 2 days. Diaphragms were then washed with 1× PBS (pH 7.3), incubated for 1 h in 0.1 M glycine in 1× PBS and rinsed

with 0.5% Triton X-100 in 1× PBS. Subsequently, diaphragms were blocked in blocking buffer (2.5% BSA and 5% donkey serum in 0.5% Triton X-100 in 1× PBS) overnight at 4°C. Diaphragms were then incubated with primary antibody (CD-31/PECAM1, 1:500, 550274, BD Pharmingen, San Jose, CA) in blocking buffer overnight at 4°C. Following primary incubation, diaphragms were rinsed three times for 1 h each with phosphate buffered saline with Tween-20 (PBS-T) and incubated overnight with Alexa Fluor 594 conjugated anti-rat IgG (1:1000, A21209, Thermo Fisher Scientific, Waltham, MA) at 4°C. Stained diaphragms were rinsed three times for 1 h with 0.5% Triton X-100 in PBS, rinsed once with 1× PBS and mounted with VECTASHIELD HardSet Antifade Mounting Medium (H-1400, Vector Laboratories, Burlingame, CA, USA). Images were obtained using a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy GmbH, Gena, Germany).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. D.A.S. is a member of the Clinical Advisory Board of Baylor Genetics. J.R.L. has stock ownership in 23andMe, is a paid consultant for Regeneron Pharmaceuticals, has stock options in Lasergen, Inc., is a member of the Scientific Advisory Board of Baylor Genetics and is a co-inventor on US and European patents related to molecular diagnostics for inherited neuropathies, eye diseases and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from the genetic tests offered by Baylor Genetics. The other authors declare no conflict of interest.

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