FREM1 Mutations Cause Bifid Nose, Renal Agenesis, and Anorectal Malformations Syndrome

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An autosomal-recessive syndrome of bifid nose and anorectal and renal anomalies (BNAR) was previously reported in a consanguineous Egyptian sibship. Here, we report the results of linkage analysis, on this family and on two other families with a similar phenotype, which identified a shared region of homozygosity on chromosome 9p22.2-p23. Candidate-gene analysis revealed homozygous frameshift and missense mutations in *FREM1*, which encodes an extracellular matrix component of basement membranes. In situ hybridization experiments demonstrated gene expression of *Frem1* in the midline of E11.5 mouse embryos, in agreement with the observed cleft nose phenotype of our patients. *FREM1* is part of a ternary complex that includes *FRAS1* and *FREM2*, and mutations of the latter two genes have been reported to cause Fraser syndrome in mice and humans. The phenotypic variability previously reported for different *Frem1* mouse mutants suggests that the apparently distinct phenotype of BNAR in humans may represent a previously unrecognized variant of Fraser syndrome.

In 2002, Al-Gazali et al. reported a second-cousin consanguineous Egyptian family in which four siblings had bifid nose associated with anorectal and renal abnormalities (BNAR [MIM 608980]).[1] One of the children was born with bilateral renal agenesis and died within the first hour of life. The surviving three presented with unilateral renal agenesis, low-pitched crying, short and thick oral frenula, incurved fifth toe, anteriorly placed anus, and stenosis of the anal opening. Importantly, the bifid nose with bulbous nasal tip was not associated with hypertelorism. This therefore represented an apparently distinct autosomal-recessive phenotype. We have also observed this rare disorder in two consanguineous families of Afghani and Pakistani origin (Figure 1A), in which the phenotype is essentially similar although kidney involvement is more variable (Table 1).

The apparent autosomal-recessive mode of inheritance suggested that autozygosity mapping would determine the disease locus in these consanguineous families. After obtaining written and informed consent from all patients or their legal guardians (in accordance with a protocol approved by the King Faisal Specialist Hospital institutional review board, protocol no. 2080006), we implemented genome-wide multipoint parametric linkage analysis, using the Affymetrix 250K *Sty*I GeneChip platform (Affymetrix, Santa Clara, CA, USA). Resulting genotyping data were analyzed with the EasyLinkage software package, assuming fully penetrant autosomal-recessive inheritance and a disease allele frequency of 0.0001, with a consanguinity loop utilized for each family. Analysis of all surviving affected individuals yielded a maximum LOD score of 6.62 between SNP markers rs10124106 and rs10963391, a 4.4 Mb interval on 9p22.2–p23 encompassing a total of 28 annotated genes (Figure S1, available online). This was in agreement with data obtained with the use of microsatellite markers and signified that a single locus, but on different haplotypes, was the likely cause of the syndrome in all three families. Candidate-gene selection was prioritized according to expression levels in the developing kidney and assessment of the mouse knockout phenotype, if known. The most promising candidate gene that met our criteria was *FRAS1-related extracellular matrix protein 1* (*FREM1* [MIM 608944]), which encodes a basement membrane protein. Deficiency of the orthologous Frem1 in mouse results in a phenotype that includes renal agenesis.[2,3] Primers were therefore designed to flank all known *FREM1* exons, as identified on the UCSC and Ensembl websites, and were directly sequenced with the dideoxy chain-termination method (Amersham ET Dye Termination Sequencing Kit). Samples were processed on a MegaBACE 1000 (Molecular Dynamics, Sunnyvale, CA, USA).

Sequence analysis with SeqMan II (DNASTAR, Madison, WI, USA) revealed a single basepair (bp) deletion in exon 17 of *FREM1* in the Egyptian family (c.2721delG; NM_144966), predicting a frameshift at amino acid 908 and a premature truncation 17 residues downstream (p.V908S*fs*X17; Figure 1B). In the Afghani sibship, we identified a transition in exon 12, predicted to cause an Arg-to-Trp change (c.1945C>T [p.R649W]), whereas the Pakistani

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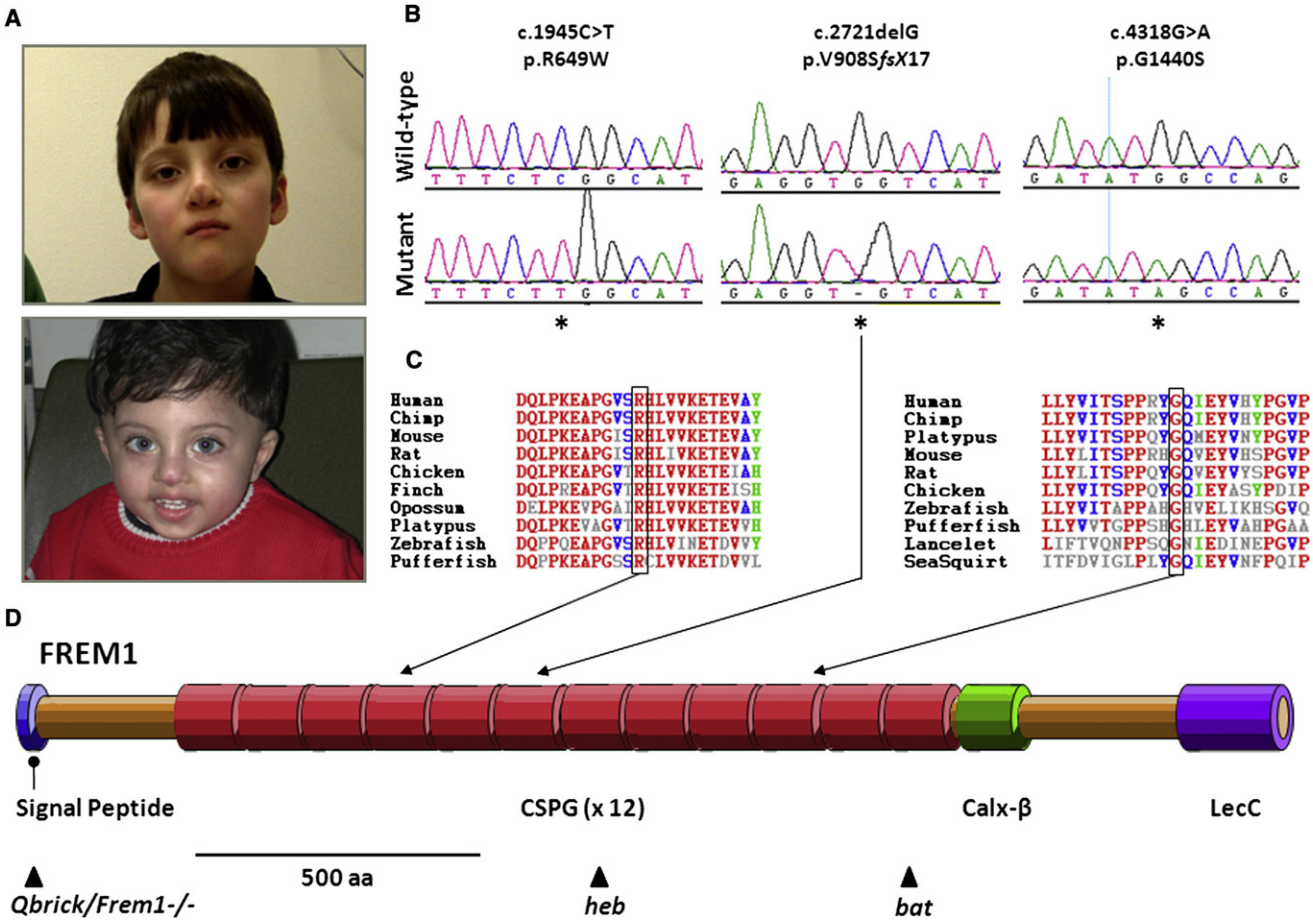

Figure 1. Mutations in FREM1 are Responsible for Bifid Nose, Renal Agenesis, and Anorectal Malformations Syndrome
(A) A representative BNAR patient from each of the two new families described in this report (note the discoloration at the tip of the nose that was observed in a subset of patients).
(B) Chromatograms of the three mutations showing control sequences on top, with the mutation sites denoted by asterisks.
(C) Multiple sequence alignments of the FREM1 protein reveal that the amino acids perturbed by the two missense mutations are highly conserved across species.
(D) Schematic of the FREM1 protein indicating conserved domains and the location of mutations given in (B) (arrows), as well as truncating mutations that have been reported in mice (arrowheads). *CSPG*, chondroitin sulfate proteoglycan; *LecC*, type C lectin-like domain.

family harbored a different transition in exon 25, which predicted a Gly-to-Ser alteration (c.4318G>A [p.G1440S]) (Figure 1B). All mutations segregated with the disease state and were confirmed via bidirectional sequencing, and all patients were homozygous for their respective mutations.

To validate the two missense mutations, we examined 121 Afghani and 97 Indian subcontinental normal control individuals, respectively. No trace of either mutation was found, indicating that these sequence variants are not common polymorphisms within the two populations. Furthermore, protein sequence alignment of FREM1 orthologs demonstrated that the affected residues are highly conserved across species, from human to fugu and for the G1440S alteration, even down to the Florida lancelet and the *Ciona intestinalis* sea squirt (Figure 1C), lending further credence to the pathogenicity of these amino acid substitutions.

Frem1 is part of a ternary complex that includes two other extracellular matrix proteins, Fras1 (MIM 607830) and Frem2 (MIM 608945).[2] To a large extent, these three proteins are functionally interdependent. They exhibit strict colocalization in the epithelial basement membrane of several organs within the developing mouse,[2,4,5] and although one *Frem1* mutant did display normal Fras1 localization,[3] compromising the expression of any one of these three proteins generally leads to reduced deposition of the other two.[2,5,6] Indeed, mutations of either *FRAS1* or *FREM2* lead to Fraser syndrome (FS [MIM 219000]),[7–9] an autosomal-recessive condition characterized most notably by cryptophthalmos (due to failure of palpebral fissure development) in ~90% of human cases, syndactyly in ~60% of cases, as well as renal agenesis and abnormal or ambiguous genitalia (Table 1).[10]

A set of four spontaneous mouse mutants, known as bleb mutants, each mapping to a different chromosome, have long been considered to represent mouse models of FS.[11] The hallmark of these bleb mutants is the formation of subepidermal blisters, appearing at midgestation, which

Table 1. Comparison of Frequency of Key Clinical Features between BNAR and Fraser Syndrome

	BNAR				Fraser Syndrome
	c.2721delG	c.1945C>T	c.4318G>A	Overall	Overall
Cryptophthalmos	0/4	0/3	0/2	0%	85–88%
Syndactyly	0/3	0/3	0/3	0%	62–95%
Abnormal genitalia	0/3	0/3	0/3	0%	40–66%
Bifid nose	4/4	3/3	2/2	100%	15%
Ear malformation	0/3	0/3	0/3	0%	59–75%
Airway malformation	0/3	0/3	2/2	22%	31–58%
Anorectal malformation	2/4	0/3	0/2	22%	16–32%
Renal agenesis	4/4	1/3	1/2	66%	45–77%

Fraser syndrome data are based on Slavotinek and Tifft[10] and van Haelst et al.[21]

then become hemorrhagic, possibly as a result of in utero friction.[3,9,12] Although this often leads to embryonic lethality, mice that are born at term do not display further blistering, an indication that this defect is temporally restricted.[3,7,8] In addition, most bleb mice also exhibit cryptophthalmos, syndactyly, and renal malformation, traits that link them to human FS.[11] The classic bleb mutants *blebbed*, *myelencephalic blebs*, and *eye blebs* are now known to harbor mutations in, respectively, *Fras1*, *Frem2*, and *Grip1* (which encodes a scaffolding protein required for basolateral targeting of Fras1 and Frem2) (MIM 604597).[7–9,13] The fourth classic mutant, *head blebs* (*heb*), though it was similarly found to contain a truncating mutation in Frem1,[3] bears a significantly milder phenotype. None of the homozygous *heb* embryos or adults in the original report was found to exhibit cryptophthalmos, a characteristic finding in the other bleb mutants. Syndactyly also was not present, although polydactyly was.[14] In fact, 97% of E17 embryos demonstrated an "open eyelids" phenotype that is in marked contrast to *Fras1*$^{-/-}$ mice, for example, in which adults were reported to show 20% bilateral and 75% unilateral cryptophthalmos.[9]

In the course of normal mammalian development, the eyelids expand across the corneal surface, temporarily fusing then subsequently reopening. In mice, this temporary closure transpires between E15.5 and E16.5 and remains in place until roughly two weeks after birth, when complete separation occurs.[15] Due to the failure of eyelid fusion in *heb*, the eyes suffer from in utero mechanical damage and reduced eyeball growth. As adults, these mice keep their eyelids closed as a means of coping with their atrophic eyes.[14] The temporary epithelial fusion of both halves of the eyelid involves participation of cells of the periderm layer.[16] Interestingly, whereas Fras1 remains restricted to the basement membrane of the advancing eyelid, Frem1 is also found in the rounded periderm cells that lie along the plane of eyelid fusion, which may help explain the unique "open eyes at birth" phenotype of the *heb* mutants.[5]

Two further *Frem1* mutants have also been described. The first, labeled *bat*, also displays an "open eyes at birth" phenotype, though cryptophthalmos was also observed.[3] In addition, 20% of homozygous *bat* mutants revealed unilateral renal agenesis, whereas the original *heb* mutants were described as having normal kidneys.[14] Occasional syndactyly was also reported in *bat*. The second, *Qbrick/Frem1*$^{-/-}$, exhibits cryptophthalmos, syndactyly, and renal agenesis.[2] This phenotypic variability may be due to differences in genetic background (which in the case of *bat*, for example, was shown to significantly modulate phenotype penetrance),[3] or they may be caused by allelic differences between the three mutants. This latter possibility is further supported by the fact that although both *bat* and *Qbrick/Frem1*$^{-/-}$ utilize C57BL/6 mice, the deposition of Fras1 in the embryonic epidermis is unaffected in *bat*, whereas in *Qbrick/Frem1*$^{-/-}$ the deposition is substantially reduced.

Both human FREM1 and its mouse ortholog are composed of a putative signal sequence, 12 chondroitin sulfate proteoglycan (CSPG) repeats,[17] a Calx-β domain, and a C-terminal type C lectin-like domain (Figure 1D). On the basis of previous delineation of the CSPG repeats,[18] the Afghani missense mutation lies in the fourth CSPG repeat, the truncating frameshift mutation in the sixth, and the Pakistani missense mutation in the tenth repeat. The original mouse *heb* mutant incorporates a LINE1 insertion in the seventh CSPG repeat, and *bat* contains a donor splice site mutation leading to frameshift in the twelfth repeat.[3] *Qbrick/Frem1*$^{-/-}$ knocks out the entire gene by replacing the initiation codon.[2] Correlating our patient phenotypes to the mouse models on the basis of the site of mutation is not straightforward. BNAR appears to be a mixture of the *heb*'s lack of cryptophthalmos and the *bat* and *Qbrick/Frem1*$^{-/-}$ mutants' presence of renal agenesis.

Temporal and spatial expression data have been published on *Frem1* previously.[3] However, the expression pattern of this gene in midline is generally lacking. Therefore, and given the highly penetrant bifid nose component of BNAR, we decided to assay *Frem1* expression in the

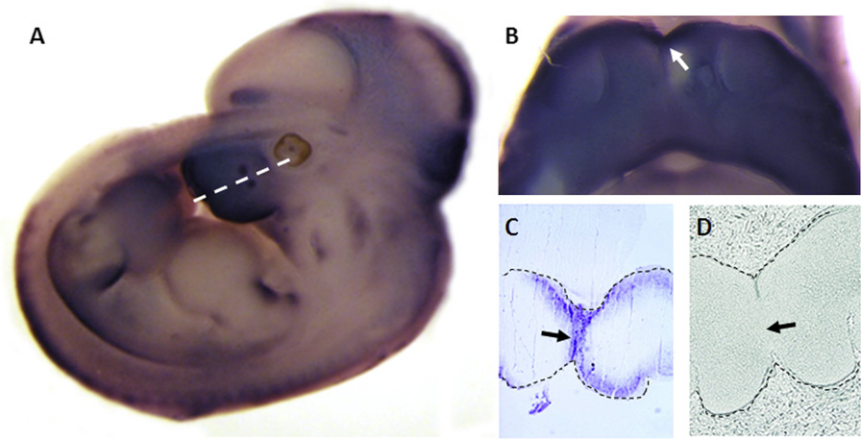

Figure 2. *Frem1* Is Expressed in the Developing Nose
(A) RNA in situ hybridization experiment showing strong expression in the snout region of an E11.5 mouse.
(B) Inferior view along the plane of the dotted line in (A) showing strong expression at midline (x8).
(C and D) Section of (A) showing strong expression at the area of fusion of the two medial nasal processes (x20) (C), and the sense control for comparison (D).

developing nose of E11.5 whole-mount embryos. Two *Frem1* probes were utilized, one spanning c.4446–4852[3] and the other spanning c.2070–2854 (NM_144966). SP6- and T7-tagged primers were used for generating, respectively, sense and antisense digoxygenin-labeled RNA probes with the MaxiScript Kit (Ambion, Austin, TX, USA) and Roche's DIG RNA Labeling Mix (Indianapolis, IN, USA). Embryos were permeabilized with proteinase K (10 μg/ml) at 37°C for 4 min, and in situ hybridization was performed with the InsituPro VSi (Intavis AG, Koeln, Germany) in accordance with a manufacturer-recommended protocol. Both antisense probes demonstrated strong and specific staining in the snout, as well as in the midline, where the two medial nasal processes fuse (Figures 2A and 2B). After in situ hybridization, examination of 5 μm sections of paraffin-embedded embryos revealed that *Frem1* expression in the developing nose was mainly in the epithelial-mesenchymal transitional region at the midline (Figures 2C and 2D). Localization of *Frem1* in this area, coupled with the consistent bifid nose phenotype of our patients, strongly argues for an important role by *Frem1* in the fusion of the medial nasal processes during gestation.

Among the Fras1/Frem proteins, Frem1 is unique in several aspects. It is expressed predominantly from the underlying mesenchyme, although to a lesser extent also from the epithelia,[3,5] and is targeted with the use of a Grip1-independent pathway. Fras1 and Frem2 are strictly secreted from the epiderm and require Grip1 for correct basolateral targeting.[2,13] Although all three overlap in the basement membrane, Frem1 expression in the ocular region is slightly delayed in comparison to the others.[4] Ablation of Fras1 or Frem2 causes complete loss of the ternary complex, but in the *bat* mutant of Frem1, deposition of Fras1 is unaffected,[3] whereas in *Qbrick/Frem1*$^{-/-}$, only residual levels of Fras1 and Frem2 are detectable in the skin basement membrane.[2] Even more intriguing is the supplementary role of Frem1 in periderm cells, a role that is first apparent at E16.5 and becomes almost exclusive by E18.5. Notably, in the adult mouse, Frem1 is not required for complex stabilization, because, unlike Fras1 and Frem2, no appreciable levels of Frem1 are present in the P30 kidney, gut, testes, or esophagus.[19] Finally, unlike the phenotypic consistency observed in mice bearing *Fras1*, *Frem2*, and *Grip1* mutations, the range of phenotype is considerably wider with *Frem1* mutants. Reassessment of the literature demonstrates that at least some of these mutants fall outside the scope of FS. These observations may explain why our patient phenotype differs from that of classic FS, given that we have shown in this study that nonsense and missense mutations in *FREM1* cause BNAR, a disorder with features that are both overlapping and distinct from FS. Previous studies have failed to identify pathogenic *FREM1* mutations in patients with classic FS,[20] and this suggests that *FREM1* mutations are exceedingly rare in this context or that they lead to BNAR or to other phenotypes that are not recognized clinically as FS. On the other hand, the remarkable phenotypic variability observed in murine *Frem1* mutants argues for the classification of BNAR as an atypical FS subtype. Future studies on the genetics of FS should include "atypical" cases for examination of the contribution of *FREM1* to the genetics of this syndrome.

In summary, this report further underscores the differences between Frem1 and Fras1/Frem2 and establishes, for the first time to our knowledge, a role for FREM1 in human craniofacial and renal development.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/AJHG.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org/index.html
Human Genome Browser Gateway (UCSC), http://genome.ucsc.edu/cgi-bin/hgGateway
Multalin (multiple sequence alignment), http://www-archbac.u-psud.fr/genomics/multalin.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim/
UCSC Genome Browser, http://genome.ucsc.edu/

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