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Manitoba-oculo-tricho-anal (MOTA) syndrome is caused by mutations in *FREM1*

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Abstract

Background—Manitoba-oculo-tricho-anal (MOTA) syndrome is a rare condition defined by eyelid colobomas, cryptophthalmos and anophthalmia/ microphthalmia, an aberrant hairline, a bifid or broad nasal tip, and gastrointestinal anomalies such as omphalocele and anal stenosis. Autosomal recessive inheritance had been assumed because of consanguinity in the Oji-Cre population of Manitoba and reports of affected siblings, but no locus or cytogenetic aberration had previously been described.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Committee for Human Research, UCSF.

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Methods and results—This study shows that MOTA syndrome is caused by mutations in *FREM1*, a gene previously mutated in bifid nose, renal agenesis, and anorectal malformations (BNAR) syndrome. MOTA syndrome and BNAR syndrome can therefore be considered as part of a phenotypic spectrum that is similar to, but distinct from and less severe than, Fraser syndrome. Re-examination of *Frem1*$^{bat/bat}$ mutant mice found new evidence that *Frem1* is involved in anal and craniofacial development, with anal prolapse, eyelid colobomas, telecanthus, a shortened snout and reduced philtral height present in the mutant mice, similar to the human phenotype in MOTA syndrome.

Conclusions—The milder phenotypes associated with *FREM1* deficiency in humans (MOTA syndrome and BNAR syndrome) compared to that resulting from *FRAS1* and *FREM2* loss of function (Fraser syndrome) are also consistent with the less severe phenotypes resulting from *Frem1* loss of function in mice. Together, Fraser, BNAR and MOTA syndromes constitute a clinically overlapping group of FRAS–FREM complex diseases.

INTRODUCTION

Manitoba-oculo-tricho-anal (MOTA) syndrome (MIM 248450) is rare, but of considerable interest because of clinical overlap with Fraser syndrome (FS) (MIM 219000), a complex multiple malformation syndrome. MOTA syndrome comprises eye defects, typically with upper eyelid colobomas but also with cryptophthalmos and anophthalmia/micro-phthalmia, triangular growths of hair extending from scalp to eyebrow, a bifid or broad nasal tip, and gastrointestinal anomalies including omphalocele and anal atresia and stenosis.[1–3] In contrast, Fraser syndrome exhibits greater pleiotropy and a more severe phenotype, with characteristic findings of cryptophthalmos, syndactyly, ambiguous genitalia, ear malformations, and renal defects.[4–6] The close relationship between FS and MOTA syndrome can be demonstrated by the fact that most of the phenotypic findings present in MOTA syndrome have also been documented in patients with FS, including eyelid colobomas (17.9% of FS patients), cryptophthalmos (88% of FS patients), anophthalmia (6.0% of FS patients), expanded hair growth (34.2% of FS patients), hypertelorism (21.4% of FS patients), bifid nasal tip/midline nasal groove (15.4% of FS patients), anal stenosis (6.8% of FS patients), and imperforate anus (12.8% of FS patients).[4]

Fraser syndrome is genetically heterogeneous, with mutations in *FRAS1* and *FREM2*[7–10] accounting for approximately half of cases.[6] The FRAS1 and FREM2 proteins form a macromolecular ternary complex with a third protein, FREM1.[1112] Although mutations in *FREM1* have not been identified in patients with FS, *FREM1* loss of function was associated with the phenotype of bifid nose, renal agenesis, and anorectal malformations (BNAR) syndrome (MIM 608980).[13]

We present a new family with MOTA syndrome and report loss of function mutations in *FREM1* in four families with MOTA syndrome. We also reexamine and image the soft tissues of *Frem1*$^{bat/bat}$ mutant mice for the anal and ocular findings in MOTA syndrome and show that a significant proportion of the mutant mice have anal prolapse, eyelid defects consistent with coloboma, and craniofacial defects consistent with the dysmorphology seen

in humans. Our findings establish one genetic cause of MOTA syndrome and confirm the close clinical and molecular relationships between MOTA syndrome and FS.

PATIENTS AND METHODS

Array hybridisation and exploration of *FREM1* copy number in patients with MOTA syndrome

All patients studied provided informed written consent. We used Affymetrix genome-wide human single nucleotide polymorphism (SNP) arrays, version 6.0, to look for loss of heterozygosity (LOH) and copy number variants (CNVs) in the affected individuals from families 1 and 2 (Affymetrix, Santa Clara, California, USA). Affymetrix 6.0 CEL files were analysed using the Affymetrix genotyping console 4.0 program with the Birdseed V-2 algorithm. We used the statistical package R to analyse patterns of SNP and CNVs in the samples and for all further analysis (http://www.r-project.org/). Copy number analysis on the data was performed using Nexus software and the SNP-FASST segmentation algorithm (BioDiscovery Inc, http://www.biodiscovery.com).

PCR and genomic sequencing

Genomic sequencing of *FREM1* was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730 machine (Applied Biosystems, Foster City, California, USA).[14] The full length transcript of *FREM1* (NM_144966) was used as reference. Primer sequences are available on request. To amplify the breakpoints of the *FREM1* deletion found in families 1 and 2, we designed sets of primers in IVS7 and IVS23 flanking the intervening deleted exons. These primers were also used for duplex PCR in families 1 and 2 together with the primers used to amplify exon 17 of *FREM1*.

Copy number assay

To assess for copy number variants, a FAM-labelled Taqman probe targeting exon 17 of *FREM1* (Custom TaqMan Copy Number Assays; Applied Biosystems) and a Taqman Copy Number Reference Assay (VIC®-RNaseP; Applied Biosystems) were used according to the manufacturer's instructions.

Reverse transcriptase-PCR

A control fibroblast cell line derived from human foreskin was obtained from the cell culture facility at UCSF (http://www.ccf. ucsf.edu/). Cultured fibroblasts were obtained from patient 3 following a skin biopsy. RNA extraction using Trizol (Invitrogen, Carlsbad, California, USA) and cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen) were performed according to standard methods. We designed reverse transcriptase-PCR (RT-PCR) primers in exons 30 and 32 to amplify and sequence exon 31 in patient 3. We also designed primers to amplify the non-deleted allele from cDNA from patient 3 in three fragments: 5′UTR to exon 8, exon 8 to exon 23, and exon 23 to 3′UTR. We cloned (TOPO TA cloning kit, Invitrogen) and sequenced the products from the exon 23 to 3′UTR fragment in patient 3 and sequenced the remaining cDNA in patient 3 without cloning.

Western blot

Proteins were obtained from skin fibroblasts from a control and from patient 3 using a cell extraction buffer containing protease inhibitors (Pierce, Rockford, Illinois, USA). Determination of protein concentration in the soluble fraction of the whole-cell lysate was performed using a DC protein assay (BioRad, Hercules, California, USA). Approximately 25 μg of total protein extract was separated on a 4–15% gradient SDS-PAGE gel under reducing conditions and transferred to polyvinylidene fluoride membranes (BioRad). Membranes were incubated with anti-FREM1 (1:200; raised in rabbit, a gift from Dr Ian Smyth) or anti-actin (1:1000, raised in mouse, Sigma, St Louis, Missouri, USA) and appropriate secondary antibodies. Immunoreactivity was visualised using chemiluminescence (ECL plus, GE Healthcare, Piscataway, New Jersey, USA).

Examination of *Frem1*$^{bat/bat}$ mutant mice for ocular and anal defects

Frem1$^{bat/bat}$ mutant mice that have splice site mutations in exon 25 of the gene[15] were examined for anal and ocular defects as found in MOTA syndrome. All mice were housed under the same laboratory conditions and were maintained on a uniform inbred (C57BL6) background.

Soft tissue imaging of *Frem1*$^{bat/bat}$ mutant mice for craniofacial defects

For soft tissue imaging and morphometric analysis of craniofacial shape in *Frem1*$^{bat/bat}$ mutant mice, depilated heads of 25, 28-day-old male mice (seven wildtype, 14 Frem1 heterozygotes, and four Frem1 homozygotes) were imaged with a Skyscan 1076 microcomputed tomograph. Scan data were reconstructed using NRecon V1.6.3.3 (Skyscan, Belgium) and then rendered using Drishti V2.0. Seven soft tissue landmarks were collected for each scanned mouse. For assessment of craniofacial shape, Euclidean distance matrix analysis (EDMA) was performed using the WinEDMA package[16] as previously described.[17] Measurements from homozygotes and heterozygotes were combined (group 1) for comparison against controls (group 2). All measurements were corrected for centroid size and Student t tests, under assumptions of identical variance, were used to assess the mean distances between landmarks.

RESULTS

Case reports

A summary of the clinical findings in the families described in this study is provided in table 1.

The first child (patient 1 in this report; V-I in Li *et al*[2]) had hypertelorism, a bifid nose, and a small omphalocele, whereas her more severely affected brother (patient 2 in this report; V-5 in Li *et al*[2]) had a unilateral tongue of hair at his hairline, hypertelorism, a notched nasal tip, a small omphalocele, and an anteriorly placed anus with anal stenosis.

The second Oji-Cre family has not been reported (figure 1). The eldest affected daughter (patient 3) had a bifid nasal tip that was surgically repaired and a V-shaped wedge of hair on her left forehead. The second affected child (patient 4) had anophthalmia of the left eye and

a similar V-shaped wedge of hair. The third affected daughter (patient 5) had a bifid nasal tip with a prominent central vein. A male second cousin (patient 6) had bilateral cryptophthalmos and abnormal ocular globes, frontal extension of his hairline bilaterally, a broad nasal bridge with a mildly widened nasal tip, omphalocele, and anal stenosis. An ultrasound showed mild renal pelviectasis in the newborn period. At 4 years and 8 months of age, he had mild to moderate delays, but was independently ambulant and could speak in sentences. There was no history of consanguinity and the individuals in family 1 and family 2 were not known to be related to one other.

The third patient (patient 7) was born in Belgium to third cousin parents and was published previously as an unclassified syndrome comprising micro-ablepharon of the upper eyelids and vaginal atresia.[18] She had bilateral upper eyelid colobomas and vaginal atresia/agenesis with additional facial features of a high forehead with a frontal upsweep of hair, maxillary hypoplasia, small nasal alae with colobomas and a bifid nasal tip, a short philtrum, a thin upper lip, and relative microstomia.

The next propositus (patient 8) was born to a non-consanguineous Dutch couple and had eyelid colobomas with an absent fornix of the left eye and a hairline that extended down to the left upper eyelid (patient 8 in Li *et al*[2]). There was left corneal clouding, a broad columella and a broad nasal tip with a palpable groove and a small vascular nevus.

The last family has also been described previously.[19] The male propositus (patient 9) had non-consanguineous parents of Greek and Malaysian heritage and was born with colobomas of the upper eyelids, disruption of the middle segment of each eyebrow, a tongue of hair extending towards the colobomas, hyper-telorism, and a broad nasal tip. His second cousin (patient 10) was a female baby with an extensive coloboma of the left upper eyelid, disruption of the left eyebrow, an ipsilateral plunging hairline, and a broad nasal tip.

SNP array hybridisation in families 1 and 2

We used genome-wide SNP arrays to look for loss of heterozygosity (LOH; more strictly, autozygosity) and CNVs in the affected individuals from families 1 and 2. We found 35 893 SNPs with identical genotypes in the three affected daughters from family 2; 16 678 of these SNPs were homozygous in these three women but heterozygous in the unaffected individuals in this family. Only 71 of the SNPs were inherited from heterozygous parents, thus representing likely LOH/autozygosity. The same analysis was then performed in the affected male cousin (patient 6) and his mother (samples from the father were unavailable). We focused on the 71 SNPs above and noted that 44 of them were also present in the affected cousin. Most of the regions were scattered throughout the genome and thus likely represented isolated false discoveries; however, four of the SNPs mapped to the *FREM1* gene.

Exploration of *FREM1* copy number in families 1 and 2

Copy number analysis on the above data showed a specific deletion of *FREM1* in affected individuals from family 2 at chromosome 9p22.3 (chr9:14 780 425–14 840 536; figure 2). All of the four *FREM1* SNPs that showed LOH/autozygosity were within the boundaries of

this deletion. We performed a search in the Database of Genomic Variants (http://www.tcag.ca/) and found no evidence that the *FREM1* deletion was a CNV. To confirm this, we performed CNV analysis on Hapmap 3 Affy SNP6.0 Cel files (supplementary table 1). No deletions in the *FREM1* gene were identified in 756 Hapmap samples of varying ethnicities, thus confirming that the deletion was novel and specific to the MOTA families described in this study.

PCR and genomic sequencing in families 1 and 2

Following identification of the *FREM1* deletion in family 2, we used PCR to determine which exons were deleted. We mapped the deletion breakpoints to 631 bp after the end of exon 7 (IVS7 +631) and 1311 bp before the start of exon 24 (IVS23-1311) using intronic primers on patient 6, who was homozygous for the deletion (figure 2Aiii). PCR with the intronic primers used for patient 6 showed that an identical deletion involving both *FREM1* alleles was also present in patients 1 and 2 (figure 2B) from family 1. The deletion removed amino acids 385–1327 from FREM1, but the frame of the protein was unchanged. Duplex PCR using a primer pair specific for amplification of the allele with the deletion and a primer pair specific for the allele without the deletion confirmed homozygosity for the deleted allele in patients 1, 2, and 6 and heterozygosity for the deleted allele in patients 3, 4, and 5 (figure 2C). A Taqman copy number assay with a probe for exon 17 of *FREM1* also confirmed loss of both copies for exon 17 in patients 1, 2, and 6 compared to patients 3, 4, and 5 (table 2).

We postulate that the MOTA syndrome phenotype in patients 1, 2, and 6 can be explained by homozygosity for the deletion and the resultant *FREM1* deficiency resulting from a protein that is likely to be non-functional.

These results did not explain the pathogenesis of MOTA syndrome in the three affected daughters from family 2, as each girl was heterozygous for the deletion and all carrier parents were phenotypically normal, making haploinsufficiency an unlikely mechanism to explain affected status. We sequenced the coding exons of *FREM1* in patient 4 and found one synonymous, single nucleotide alteration, c.5556A→G, in exon 31 that occurred one bp before the last coding nucleotide of this exon (supplementary table 2; figure 2D; numbering according to NCBI transcript NM_144966; A of start codon=1). The alteration was predicted to fully abolish the donor splice site for this exon (Automated Splice Site Analysis Program: https://splice.cmh.edu)[2021] and was inherited from the mother of the affected children (table 2). Each affected daughter in family 2 therefore had a splice site alteration inherited from their mother and the exon 8–23 deletion inherited from their father (table 2). The splice site alteration was absent from 118 Caucasian control chromosomes, but was present in five chromosomes out of 95 control patients from various ethnic groups (2.6%).

We sequenced exon 30 to exon 32 in cDNA from patient 3 from the same family and found that exon 31 was spliced out of the *FREM1* transcript (figure 2E); we also cloned the non-deleted allele from patient 3′s cDNA using primers located in exon 23 and the 3′UTR and showed that exon 31 was absent from the non-deleted allele (data not shown). However, the same splicing pattern with splicing out of exon 31 was also seen on sequencing cDNA from a control fibroblast cell line (data not shown). In addition, the alteration at the donor splice

site predicted a lack of splicing with 'run-on' into the intron, and this was not observed in the patient cDNA (data not shown). It is therefore likely that the exon 31 sequence variant distinguishes a pathogenic allele, as it is maternally inherited and segregates with the deleted allele and the disease phenotype in family 2, but that this sequence variant itself is not pathogenic. We did not find any other mutations in *FREM1* in genomic DNA in patient 4 or in cDNA from patient 3, and thus the second mutation in the three daughters was undetected. An SNP in exon 34 of *FREM1*, rs4741426, was heterozygous (both 'T' and 'C' alleles) in genomic DNA from patient 3, with the T allele being paternally inherited and the C allele being maternally inherited (data not shown). This SNP showed only the maternally inherited 'C' allele in both cloned cDNA (non-deleted allele only) and uncloned cDNA from patient 3, verifying that the deleted allele is not transcribed and may undergo mRNA decay (data not shown).

Genomic sequencing in families 3–5

We sequenced *FREM1* in patients 7, 8, 9, and 10 from families 3, 4, and 5. Patient 7 was homozygous for a 4 bp deletion in exon 13 of *FREM1*, c.2097_2100delATTA (supplementary figure S1) that was inherited from both parents, who each carried one copy of the 4 bp deletion. This mutation is predicted to cause a frameshift and premature truncation codon (p.K699NfsX10), and is likely to be disease causing and result in a non-functional protein. In patient 8, two sequence variants were identified: c.3971T→G, predicting p.Leu1324Arg (supplementary figure S2A), and c.6271G→A, predicting p.Val2091Ile (supplementary figure S2B). The first alteration affected a highly conserved leucine residue and was predicted to be 'probably damaging' (HumDiv score 1.000, sensitivity: 0.00, specificity: 1.00; Polyphen-2; http://genetics.bwh.harvard.edu/pph2/) and to affect protein function (with a score of 0.00; SIFT; http://sift.jcvi.org/). This alteration was absent from 504 Caucasian control chromosomes. The second sequence variant, p.Val2091Ile, also involved a highly conserved amino acid residue that was located in a motif of the C-type lectin domain and was predicted to be 'probably damaging' by Polyphen-2 (HumDiv score 0.708, sensitivity: 0.83, specificity: 0.89) but predicted to be tolerated with a score of 0.14 by SIFT. However, although valine and isoleucine have similar physicochemical properties, no isoleucine has been observed at the respective position in the homologous proteins from other species and this substitution was absent from 500 Caucasian control chromosomes.

In the last family (patients 9 and 10), no mutations in *FREM1* could be detected in either of the children (data not shown). Segregation analysis based on genotyping of intragenic SNPs indicated that the affected children did not share a *FREM1* allele from one of the common ancestors (data not shown), thus making a causative role of *FREM1* very unlikely in this family.

Western blot

A western blot did not show a smaller size protein in patient 3 compared to control fibroblast protein (supplementary figure S3). This is in keeping with the hypothesis that the deleted allele may undergo decay.

Examination of *Frem1*$^{bat/bat}$ mutant mice for ocular and anal defects

Examination of a colony of *Frem1*$^{bat/bat}$ mutant mice identified a small but statistically significant proportion of homozygous mutant animals with anal prolapse not observed in *Frem1*$^{+/bat}$ or *Frem1*$^{+/+}$ littermates (figure 3A,B; 4/138 homozygotes versus 0/272 heterozygotes and wild type littermates, z=−2.822 two-tailed z-test; >99% CI). Histological analysis revealed protrusion of the rectal epithelia and an immune infiltrate in exposed tissue, but not in the internal mucosa. The musculature of the anal sphincter was present, but misplaced, and gross malformations in musculature in the rectum were not apparent (figure 3B).

Examination of *Frem1*$^{bat/bat}$ mutant eyes at birth showed that, although the majority of animals presented with frank cryptophthalmos, a subset exhibited defects strikingly similar to the eyelid coloboma observed in MOTA (figure 3C), seemingly affecting only one part of the eyelid. Histological analysis demonstrated that these defects were associated with a number of ocular malformations including failure of eyelid formation (figure 3Di), defects in the formation of the conjunctiva (figure 3Dii), and absence of corneal epithelium leading to fibrosis (figure 3Diii).

Soft tissue imaging of *Frem1*$^{bat/bat}$ mutant mice for craniofacial defects

Morphological analysis of craniofacial shape in *Frem1* deficient mice showed that the *Frem1* mutants (homozygotes and heterozygotes combined) were slightly smaller overall than their sex, age and background matched wildtype controls (supplementary figure S4). Direct inter-landmark distance comparisons between each genotype confirmed the reduced snout length in homozygotes when compared to controls (p=0.0382). In addition, homozygotes also presented with a significantly shorter philtrum–columella height (p=0.000407), greater intercanthal distance (p=0.0483), and greater midfacial asymmetry (p=0.0152) when compared to heterozygotes. Notably, reduced snout length was inversely correlated with both cranial width (p=0.288) and intercanthal distance (p=0.303).

DISCUSSION

We have identified *FREM1* mutations in patients from several independent families with MOTA syndrome, including a deletion that removed exons 8 to 23 of *FREM1* in families 1 and 2 and a frameshift mutation predicting premature truncation of the protein at amino acid 708 in family 3 (figure 4). These mutations are likely to lead to complete or near complete loss of FREM1 function and we consider that they are responsible for MOTA syndrome in these families. However, the phenotypic consequences of two other *FREM1* sequence variants found were less obvious. A putative splice site alteration one bp before the end of exon 31, c.5556A→G, was detected in 2.6% of ethnically diverse control chromosomes and the missense alteration, c.6271G→A, predicting p.Val2091Ile, was assessed differently by Polyphen-2 and SIFT. We have not excluded a role for the splicing variant in other non-tested tissues, but it is speculative as to whether it would be relevant in a full length transcript. We did not detect mutations in family 5 and segregation analysis excluded *FREM1* as the causative gene in the kindred, making genetic heterogeneity likely for the MOTA phenotype.

Homozygous, loss-of-function mutations in *FREM1* have previously been described in BNAR syndrome (MIM 608980).[13] The mutations in our patients with MOTA syndrome are also likely to lead to loss of *FREM1* function and thus unite MOTA syndrome and BNAR as allelic conditions. Although eye defects occur consistently in patients with MOTA syndrome, despite the expression of *Frem1* in the developing murine eye,[12] no ocular abnormalities have been associated with BNAR to date. Conversely, renal agenesis appears to be characteristic of BNAR but has not been observed in MOTA. Although these findings enable distinction between MOTA and BNAR for some patients, others exhibit more clinical overlap and could be diagnosed with either syndrome. The predicted loss of function mutations observed in both MOTA and BNAR syndrome and the close phenotypic relationship between the two suggests that there is a spectrum of clinical manifestations associated with *FREM1* deficiency. Both modifying factors and ascertainment bias may have influenced the apparent clustering of BNAR and MOTA phenotypes in these families; as both conditions are very rare, the possibility of other phenotypes associated with *FREM1* loss of function cannot currently be dismissed.

In family 1, the eldest affected child who was homozygous for the exon 8–23 deletion had mild craniofacial features compared to bilateral cryptophthalmos in the most severely affected individual from family 2 who was also homozygous for the same deletion. The interfamilial and intrafamilial variability associated with *FREM1* mutations is considerable, and requires an explanation. In murine research, Fras1, Frem1, and Frem2 form a ternary complex in which Frem2 mediates an interaction between Fras1 and Frem1 to stabilise the basement membrane, implying that the phenotype of the mutant ‘bleb’ mice resulting from deficiencies of either Fras1, Frem1 or Frem2 stems from the loss of all three proteins.[12] Loss of Fras1 or Frem2 from the sublamina densa of epithelial basement membranes has been shown to diminish or abolish the localisation of the remaining two proteins and the formation of the ternary complex.[12] However, Fras1 and Frem2 remain detectable in Frem1 deficient mutants and it has been hypothesised that, since they are not dependent on Frem1, the retained expression and function of Fras1, Frem2 and the resultant Fras/Frem complex confers a milder phenotype in Frem1 mutants.[1222] The same explanation could be advanced to account for the quantitatively milder phenotypes of MOTA syndrome and BNAR associated with FREM1 deficiency in comparison to Fraser syndrome resulting from FRAS1 and FREM2 deficiency in humans. Differential residual levels of the FRAS1 and FREM2 proteins as a result of SNPs, modifier genes, epigenetic or stochastic features may account for the intrafamilial variability associated with *FREM1* mutations. The phenotypic expression of *FREM1* mutations can also be considered to be qualitatively different from that resulting from *FRAS1* and *FREM2* mutations, in that several characteristic clinical manifestations in Fraser syndrome, notably cutaneous syndactyly and ambiguous genitalia, have not so far been observed in either MOTA syndrome or BNAR. In addition, *FREM1* mutations appear compatible with life, compared to the early mortality that is frequently witnessed with Fraser syndrome.[4]

To characterise further the phenotypic parallels between a mouse model of Frem1 deficiency and MOTA patients, we examined a colony of *Frem1*$^{bat/bat}$ mutant mice.[15] Anorectal malformations have not previously been observed in Frem1 mutant mice, but examination of

a large cohort of the *Frem1*$^{bat/bat}$ animals identified a small but statistically significant proportion of homozygous mutant animals with anal prolapse not observed in *Frem1*$^{+/bat}$ or *Frem1*$^{+/+}$ littermates. Colitis[23] or genetic background were not likely causative factors and we found no overt obstruction or disease in the intestinal tracts of our mice. Instead these results, combined with the developmental anorectal defects observed in MOTA patients, suggest that FREM1 plays a role in the structural development of the lower gastrointestinal tract. Defects in the pelvic floor have been associated with the development of such disease in rodents[24] and further study is warranted to examine this possibility in *Frem1* mutant mice.

Optic defects in Frem1 mutant mice have been previously reported[1525] and the animals are typically born either with cryptophthalmos or with their eyelids open at birth. The latter phenotype differs from that of Fras1 and Frem2 mutants ('myelencephalic blebs' and 'blebbed', respectively), where these defects uniformly manifest as cryptophthalmos.[79] Our examination of *Frem1* mutant eyes at birth showed defects similar to the eyelid colobomas observed in MOTA syndrome that were associated with failure of eyelid formation, defective conjunctiva development, and absence of the corneal epithelium, and we propose that defects in the development or adhesion of the corneal epithelium contribute to the opacity apparent in some MOTA patients.[2] Collectively, these results strongly suggest that the blistering and/or failure of eyelid fusion caused by loss of FREM1 can produce phenotypes distinct from those resulting from loss of FREM2 or FRAS1. Notwithstanding the likely impact of genetic background and allele specific effects, the distinct ocular phenotypes observed in Frem1 and Frem2/Fras1 mutants most probably relate either to the extent of the blisters in utero and/or to the previously noted expression of Frem1 in the embryonic periderm.[11] It is notable that we observed blistering of the head epithelium around E12.5 of gestation, far earlier than the normal timeframe of eyelid fusion in the developing mouse (E15.5–E16.5). These differences point to a subtly different role for FREM1 in eyelid development.

We also conducted three dimensional morphometric analysis of the craniofacial soft tissue of Frem1 mutants to investigate whether craniofacial dysmorphology similar to that seen in MOTA syndrome was evident. When compared to age, sex, and genetic background matched controls, we found that homozygotes tended to show reduced midface growth and asymmetry as well as a greater intercanthal distance that was inversely correlated to midface outgrowth. Homozygotes also consistently presented with a significantly shortened philtrum–columella height reminiscent of that seen in some patients with MOTA syndrome (eg, patient 7). Our further analysis of the phenotype of the Frem1 mutant mice shows clear phenotypic overlap with MOTA syndrome features, and identifies this as a useful model in which to study disease aetiology.

We have described a new Oji-Cre family with MOTA syndrome and demonstrated mutations in the *FREM1* gene, establishing *FREM1* deficiency as the cause of this condition. In two Oji-Cre families, three individuals with MOTA syndrome were homozygous for a deletion involving exons 8 to 23 of *FREM1*, and in a Belgian family, one patient was homozygous for a frameshift mutation predicted to cause premature protein termination. Loss of function mutations in *FREM1* have previously been noted in BNAR syndrome, and

our results indicate that MOTA syndrome and BNAR syndrome are allelic conditions that can be considered to be part of a phenotypic spectrum. MOTA syndrome has long been noted to have a close phenotypic relationship to Fraser syndrome, and the finding of *FREM1* deficiency ties the molecular cause of MOTA syndrome closely to the pathogenesis of Fraser syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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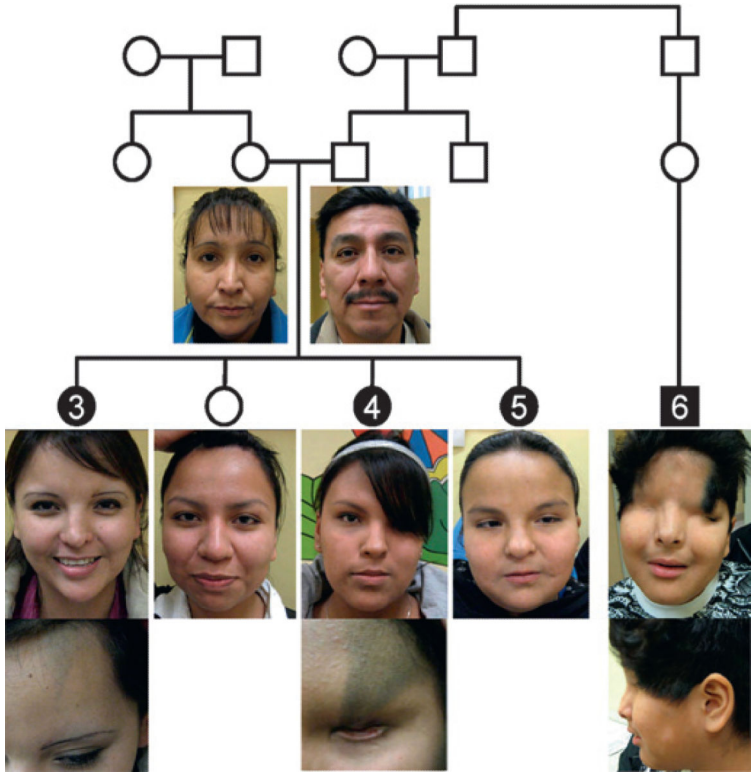

Figure 1.
Pedigree and clinical images from the second family with Manitoba-oculo-tricho-anal (MOTA) syndrome.

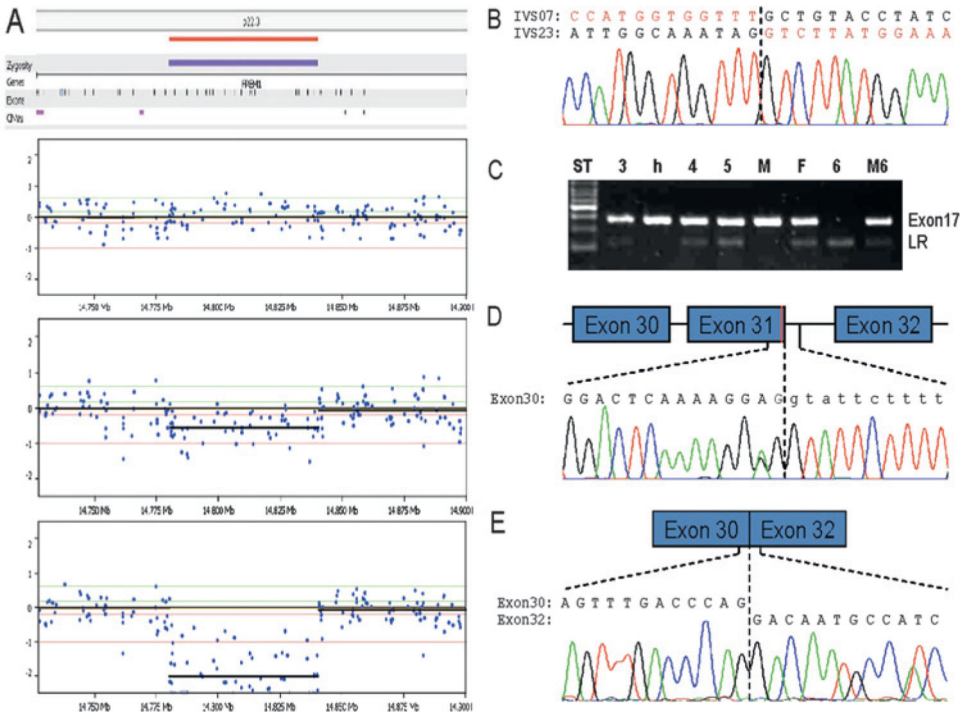

Figure 2.

A homozygous deletion of exons 8–23 in *FREM1* causes Manitoba-oculo-tricho-anal (MOTA) syndrome. (A) Plots of copy number in the region of the *FREM1* gene at chromosome 9p22.3 from Affymetrix 6.0 chips in the second family with MOTA syndrome. (Ai) (top line) shows normal copy number in the region of *FREM1* (mother of patients 3, 4. and 5). (Aii) (middle line) shows heterozygous copy number loss in the region of *FREM1* (patient 5). (Aiii) (bottom line) shows homozygous copy number loss in the region of *FREM1* (patient 6). (B) PCR results showing sequencing across deletion breakpoints in introns 7 and 23 in patient 2 from family 1. The sequence across the breakpoints for these introns that was present in patient 2 is written in red font above the chromatograms as observed, whereas the wildtype sequence for both introns as seen in individuals without the deletion (not observed) is written in black font above the chromatograms. (C) Duplex PCR showing two differently sized bands—*FREM1* exon 17, present only in wildtype allele (expected product size 386 bp), and *FREM1* long range fragment, present only in deleted allele (expected product size 232 bp). Both the normal mother (M) and unaffected sister (h) have two wildtype alleles, whereas the normal father (F) and each affected daughter (3, 4, 5) have one wildtype allele and one deleted allele. In the affected male cousin (6), there is no wildtype allele, whereas his normal mother (M6) has one wildtype allele and one deleted allele. (D) Chromatogram showing c.5556A→G in exon 31 of *FREM1* in patient 4. This sequence alteration was present in the mother of family 2 and in all of the affected daughters. (E) Sequencing traces showing removal of exon 31 from the non-deleted *FREM1* allele from patient 3.

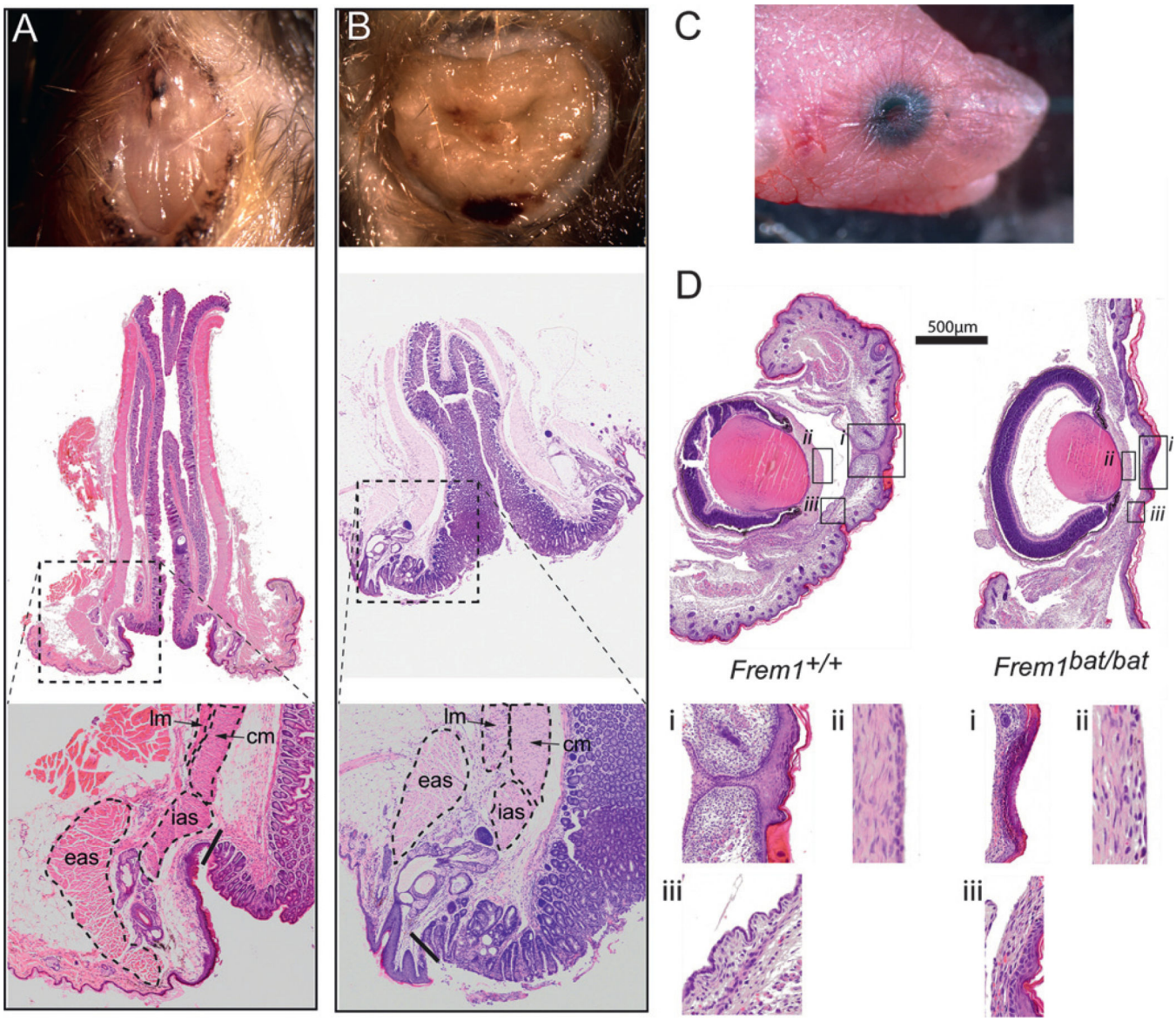

Figure 3.

Rectal prolapse and eye defects in *Frem1*$^{bat/bat}$ mice. (A, B) Rectal prolapse occurs at a low level in *Frem1* mutant mice compared with wild type or heterozygous littermates. Anorectal musculature is similar between wild type (A) and mutant (B) littermates despite displacement of the rectum and anal epithelium. The dentate line at the border between rectal and anal epithelia is indicated in both cases (solid line). eas, external anal sphincter; ias, internal anal sphincter; cm, circular muscle; lm, longitudinal muscle. (C) Eyelid defects at P1 range from complete cryptophthalmos to subtle defects in specific sub-compartments of the eyelid that parallel the eyelid coloboma observed in Manitoba-oculo-tricho-anal (MOTA) patients. (D) At a cellular level eyelid formation is severely compromised (i), conjunctival development is defective (ii) and the corneal epithelium is absent, leading to thinning of the cornea and fibrosis on its external face (iii). Regions i, ii and iii in (D) are shown magnified in the lower panels.

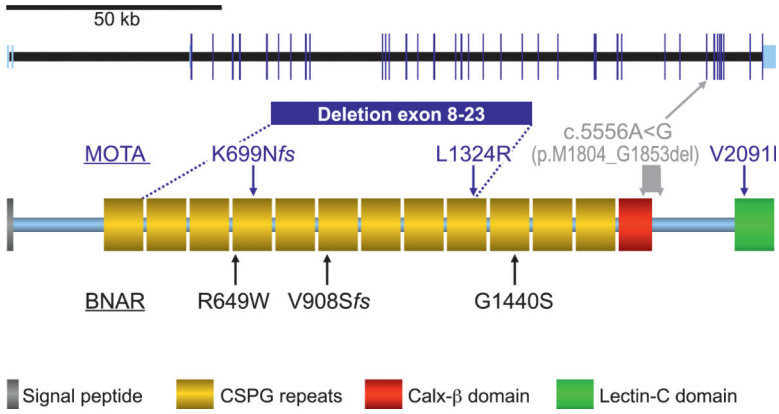

Figure 4.

Schematic drawing of *FREM1* and the location of patient mutations in Manitoba-oculo-tricho-anal (MOTA) syndrome and bifid nose, renal agenesis, and anorectal malformations (BNAR) syndrome.

Table 1

Patient findings in Manitoba-oculo-tricho-anal (MOTA) syndrome

	Patient 1*	Patient 2*	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7*	Patient 8†	Patient 9‡	Patient 10‡
Age at reporting; sex	11 y; F	Not stated; M	26 y; F	15 y; F	10 y; F	8.5 y; M	36 y; F	Not stated; F	6 m; M	2 y; F
Pregnancy	Normal	Not stated	Not stated	Not stated	Not stated	Not stated	Normal	Normal	Oligohydramnios	Normal
Delivery	Term	Not stated	Not stated	Not stated	Not stated	Not stated	Normal	39 w	34 w	Not stated
Eye findings										
Cryptophthalmos	–	–	–	–	–	+; both eyes	–	–	–	–
Anophthalmia	–	–	–	+; left eye	–	–	–	–	–	–
Eyelid coloboma	–	–	–	–	–	–	+; both eyes	+	+	+
Aberrant hairline	–	+	+	+	–	+	Not stated	+	+	+
Hypertelorism	+	+	–	–	–	–	+	–	+	–
Nasal findings										
Bifid nasal tip	+	+; notched	+	–	+	+	+	+; broad	+; broad	+; broad
GIT findings										
Omphalocele	+	+	–	–	–	+	–	–	–	–
Anterior anus	–	+	–	–	–	+	–	–	–	–
Anal stenosis	–	+	–	–	–	+	–	–	–	–
GUT findings										
Renal pelviectasis	Unknown	Unknown	Unknown	Unknown	Unknown	+	–	Not done	–	Unknown
Vaginal atresia	–	NA	–	–	–	NA	+	–	NA	–
Development	Normal	Normal	Normal	Normal	Normal	Delayed	Normal	Normal	Normal	Normal
Karyotype	46,XX	Not done	Not done	Not done	Not done	Not done	46,XX	46,XX	46,XY	Not done
FREM1 results	Homozygous for deletion exons 8–23	Homozygous for deletion exons 8–23	Heterozygous for deletion exons 8–23	Heterozygous for deletion exons 8–23	Heterozygous for deletion exons 8–23	Homozygous for deletion exons 8–23	Homozygous for p.K699NfsX10	p. Leu1324Arg; p.Val2091Ile	No mutations	No mutations

F, female; GIT, gastrointestinal; GUT, genitourinary tract; M, male; m, months; y, years.

*From Li et al, 2007.[2]

†From Fryns, 2001.[18]

‡From Yeung et al, 2009.[19]

Table 2

Copy number for *FREM1* exon 8–23 deletion and *FREM1* genotypes for families 1 and 2

Individual	6.0 Array copies of wildtype *FREM1*	Duplex PCR copies of wildtype *FREM1*	Copy number assay–exon 17 probe Ct	Copy number assay–control probe Ct	*FREM1* C.5556A→G	*FREM1* genotype
Family 1						
Patient 1 (AF)	**0**	**0**	**31.4±0.22**	**26.47±0.17**	**A/A**	**Del/Del**
Patient 2 (AF)	**0**	**0**	**31.1±0.29**	**26.56±0.2**	**A/A**	**Del/Del**
Mother to 1 and 2	1	1	27.26±0.05	26.41±0.16	A/A	Del/A
Family 2						
Patient 3 (AF)	**1**	**1**	**26.84±0.1**	**26.02±0.03**	**A/G**	**Del/G**
Unaffected sister	2	2	26.20±0.08	26.29±0.06	A/G	+/G
Patient 4 (AF)	**1**	**1**	**27.03±0.12**	**26.37±0.23**	**A/G**	**Del/G**
Patient 5 (AF)	**1**	**1**	**26.92±0.13**	**26.26±0.3**	**A/G**	**Del/G**
Father to 3, 4, 5	1	1	27.2±0.23	26.38±0.35	A/A	Del/A
Mother to 3, 4, 5	2	2	26.01±0.06	26.35±0.17	A/G	+/G
Patient 6 (AF)	**0**	**0**	**30.95±0.25**	**26.77±0.24**	**A/A**	**Del/Del**
Mother to 6	1	1	27.18±0.2	26.47±0.23	A/A	Del/A

(AF), affected (in bold font); Ct, cycle threshold value; Del, exon, 8-23 deletion; +, wildtype (non-deleted) allele; A, C.5556A (wildtype allele); G, c.5556G allele (predicted to alter splicing).