The extracellular matrix gene *Frem1* is essential for the normal adhesion of the embryonic epidermis

Ian Smyth*†, Xin Du‡, Martin S. Taylor*§, Monica J. Justice¶, Bruce Beutler‡, and Ian J. Jackson*

*Medical Research Council Human Genetics Unit, Crewe Road, Edinburgh EH4 2XU, United Kingdom; ‡Department of Immunology, The Scripps Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; §Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, United Kingdom; and ¶Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030

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Fraser syndrome is a rare recessive disorder characterized by cryptophthalmos, syndactyly, renal defects, and a range of other developmental abnormalities. Because of their extensive phenotypic overlap, the mouse blebbing mutants have been considered models of this disorder, and the recent isolation of mutations in *Fras1* in both the *blebbed* mouse and human Fraser patients confirms this hypothesis. Here we report the identification of mutations in an extracellular matrix gene *Fras1-related extracellular matrix gene 1* (*Frem1*) in both the classic *head blebs* mutant and in an *N*-ethyl-*N*-nitrosourea-induced allele. We show that inactivation of the gene results in the formation of *in utero* epidermal blisters beneath the lamina densa of the basement membrane and also in renal agenesis. *Frem1* is expressed widely in the developing embryo in regions of epithelial/mesenchymal interaction and epidermal remodeling. Furthermore, *Frem1* appears to act as a dermal mediator of basement membrane adhesion, apparently independently of the other known ''blebs'' proteins Fras1 and Grip1. Unlike both *Fras1* and *Grip1* mutants, collagen VI and Fras1 deposition in the basement membrane is normal, indicating that the protein plays an independent role in epidermal differentiation and is required for epidermal adhesion during embryonic development.

Human Fraser syndrome (OMIM no. 219000) is a rare recessive disorder characterized by cryptophthalmos, soft-tissue syndactyly, and a range of other developmental malformations, including renal agenesis, heart defects, reproductive tract anomalies, and deafness (1). Cryptophthalmos, in which skin covers the globe of the eye, is the most common malformation and is apparent in ≈90% of patients. Because of their similar phenotypes, the family of mouse blebbing mutants are considered models of Fraser syndrome (2–4). These mice are characterized by unilateral and bilateral cryptophthalmos, soft-tissue syndactyly, and a range of other defects encompassing abnormalities of the kidney, skin, hair, and CNS. The mutant family comprises four mapped and one umapped loci known as *blebbed*, *eye blebs*, *head blebs* (*heb*), *myelencephalic blebs*, and *fetal hematoma* (2).

All of these mutants are characterized by the formation of epidermal blisters from ≈12 days postcoitum (dpc) and, as the embryo ages, these can become hemorrhagic. The epidermis of adult blebs mice is largely normal, suggesting that the genes mutated in these animals are uniquely required for maintaining epidermal adhesion only during embryonic development. Many defects observed in blebs mice, such as syndactyly and cryptophthalmos, are likely a consequence of epidermal delamination and subsequent disruption of epithelial/mesenchymal interactions required for normal tissue differentiation, although such a mechanism is harder to invoke for defects such as renal agenesis (3). The formation of blisters on the head, limbs, and rump also suggests that the initial separation of the epidermis may be mediated by *in utero* friction.

The recent identification of mutations in the *Fras1* gene in both the *blebbed* (*bl*) mutant and in several Fraser syndrome patients has confirmed that blebs mice are models of the disease (3). *Fras1* encodes a large transmembrane extracellular matrix protein, comprising several domains. These include the C domain of von Willebrand factor, the cysteine-rich domain of the furin proteases, the core protein of NG2 [comprising multiple repeats of a chondroitin sulfate proteoglycan element (CSPG)], and the calcium-binding loop of the Na^+-Ca^+ exchange proteins (CALXb). Mutations of *Fras1* in both Fraser syndrome and the *bl* mouse invariably lead to premature truncation of the protein (3, 5). The presence of multiple motifs involved in the processing of type β transforming growth factor molecules has led to speculation that the protein also plays a role in modulating the action of various growth factors during development (3). Indeed, studies of the structurally related NG2 protein suggest that the CSPG domains are also capable of interacting with basic fibroblast growth factor and platelet-derived growth factor (6).

It has recently emerged that mutation of *Grip1* underlies the *eye bleb* (*eb*) mutant, and that inactivation of the gene gives rise to an *eb*-like phenotype (7, 8). *Grip1* encodes a protein containing seven PDZ domains, which is thought to act as a cytoplasmic scaffold for the assembly and trafficking of transmembrane proteins. Grip1 interacts with Fras1 through a short but evolutionarily conserved hydrophobic motif at its intracellular C terminus, which is similar to class 1 PDZ-binding motifs. *In vivo* studies indicate that one or more of the first three PDZ domains in Grip1 binds to this motif, and studies of *Grip1* null cells indicate that the protein is required for correct localization of Fras1 on the basal side of cultured epithelial cells (7). Studies of both *Fras1*- and *Grip1*- null mice have documented defects in the deposition of collagen VI in the epidermal basement membrane (3, 5, 7). Previous studies of the NG2 protein have shown that the CSPG domains are also capable of interacting with both collagens V and VI (9–11), and a similar role has been proposed for Fras1.

heb is a spontaneous mutation that, like the other blebs mutants, is characterized by absent or malformed eyes, which are often open at birth (12). Cryptophthalmos is noted in all *heb* homozygous animals, as is occasional hindlimb polydactyly. Curiously, the renal dysmorphology and agenesis characteristic of *blebbed*, *myelencephalic blebs*, and *eye blebs* mice were not reported in *heb* animals, and the blebs in *heb* fetuses are more restricted to the head than in other mutants. We report the identification of the gene mutated in *heb* and describe mutations in a second *N*-ethyl-*N*-nitrosourea (ENU)-induced allele. These mutations truncate an extracellular matrix gene that we term

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Abbreviations: *heb*, *head blebs*; dpc, days postcoitum; CSPG, chondroitin sulfate proteoglycan element; ENU, *N*-ethyl-*N*-nitrosourea; Frem1, Fras-related extracellular matrix gene 1; ECM, extracellular matrix.

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†To whom correspondence should be sent at the present address: Keratinocyte Laboratory, London Research Institute, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom. E-mail: ian.smyth@cancer.org.uk.

Fras1-related extracellular matrix gene 1 (*Frem1*). *Frem1* delineates a chordate gene family that encode proteins forming part of an evolutionary clade distinct from Fras1. *Frem1* is expressed predominantly in the dermal rather than epidermal component of developing epithelia, which is required for the maintenance of epidermal adhesion during embryonic development and is a candidate for Fraser syndrome mutations in humans.

Materials and Methods

Identification and Mapping of *bat*. Mutagenesis of C57BL/6J male mice with ENU was performed as described (13). *bat* was mapped by analysis of 217 meioses in an F_2 hybrid back-cross. Within the 3-Mb critical region, three crossovers separated *bat* from the proximal marker (BA_5_13: left, CGT GAT GTA TTG TCA GCA TTT GCA GCT C; and right, TTC AAA TAT GGT GCT GGT CTC AAT AAA CTT G), and two crossovers separated *bat* from the distal marker (BA_6_7: left, CGA AGC TGA TTA AAC AAC CTC TCC ACT GCT AC; and right, AAG TGA ATT GGA GAA AGG TAG TGT GTT CAG TG).

Characterization of Frem1, Frem2, Frem3, and Cspg5 and Computational Analysis. A consensus *Frem1* cDNA was constructed from EST sequences and by RT-PCR amplification from brain and skin RNA. Homologues of *Frem1* were identified by homology searches against sequence databases and using Frem1/Fras1-derived hidden Markov models of the CSPG repeat domains (Fig. 6, which is published as supporting information on the PNAS web site). Transcript and protein sequences from these related genes were generated from ESTs and manual curation of Genewise sequence predictions. CSPG repeat units were initially defined by using PROSPERO (14) and used as seeds for PSIBLAST (15) searches of the nonredundant protein database. Significant ($e < 0.001$) similarity to classic cadherin domains was identified by iteration 6. Boundaries of the CSPG domain were refined by alignment to the *N*-cadherin structure [Protein Data Bank ID code 1NCJ_A (16)]. Comprehensive analysis of posttranslational modification of the Frem proteins is detailed in Fig. 6. Phylogenetic analysis was carried out by using MEGA2 software (17) on alignments of the CSPG repeat regions of proteins. Gapped alignment columns were excluded from analysis, and all analyses were subject to 1,000 bootstrap replicates. Neighbor-joining and minimum-evolution trees were based on *P* distance matrices.

Characterization of the *heb* and *bat* Mutations. The *bat* mutation in intron 25 was identified by direct sequencing of amplified exons. A complete list of primers for all exons of *Frem1* is available upon request. RNA from *bat* mice was isolated (RNAeasy, Promega), and primers in exons 24, 25, and 29 were used to amplify brain RNA by using RT-PCR and to confirm skipping of exon 25 (ex24f, AGC CTG TTC GCT TCA CAA TC; ex25f, CGT ATC ACA CAC TTG CAC TC; and ex29r, GCA TTC ACA GGG GAG TTC AG). RT-PCR was carried out by using Access RT-PCR kits (Promega). To identify the *heb* rearrangement, 5 μg of DNA from *heb* and AKR/J mice (The Jackson Laboratory) was digested with *Bgl*II and Southern blotted onto Zeta-Probe GT genomic membranes (Bio-Rad). A PCR-amplified probe was generated from the distal end of exon 17 for radiolabeling and hybridization (ex17fout, ATT CAT GTT GGC TTC TCA GCC; ex17r, TTT AAA TCC TCA CGC ATG TCC). Proximal and distal insertion boundaries were amplified from 1 μg of circularized *Bgl*II-digested homozygous *heb* DNA by using inverse PCR (18). Gene-specific primers were then used to amplify the 5′ (left, TGT TGA TTG GGA CAC TGC CC; and right, CAC TGT GAG CAT CCT ACC TG) and 3′ (left, CAC AAG TCT GTT ATT TGG GC; and right, CAA TCT CTT GAA CGC ATG TG) breakpoints.

***In Situ* Hybridization and Immunohistochemistry.** A *Frem1* probe was generated by RT-PCR (left, TAC CAT CAG CAA TGG ACT GC; right, GGA TTG TGA AGC GAA CAG G) and hybridized to sections and whole embryos (19). Rabbit α-Fras1 antibodies were a kind gift of Georges Chalepakis (University of Crete, Hevaklion, Greece) and rabbit α-collagen antibodies (Rockland, Gilbertsville, PA) were kindly provided by Ralf Adams (Cancer Research UK, London). Sections (7–10 μm) of paraffin-embedded *bat* and wild-type embryos were subject to citric acid-based antigen retrieval and immunofluorescence using Texas red-labeled anti-rabbit secondary antibodies (Molecular Probes). Imaging of *Frem1* expression in stained embryonic kidneys was undertaken by using optical projection tomography (OPT) (20) and confirmed by cryosectioning of stained tissues. Podocytes were identified by immunofluorescence by using an α-Wilms tumor suppressor 1 monoclonal antibody (6F-H2) (Chemicon).

Results

Identification and Characterization of the *Frem* Gene Family. We identified a gene that shares several structural domains with Fras1 (Fig. 1*A*). These include a conserved signal sequence, the calcium-binding loop of the Na^+-Ca^+ exchange proteins (CALXb), and the CSPG repetitive element initially described in the NG2 core protein (21) and in other proteins in a variety of organisms (22). The protein lacks the furin and the C domain of von Willebrand factor domains present in Fras1 but has an additional C-terminal lectin type C domain. Significantly, there is no evidence of either transmembrane or C-terminal PDZ-binding motifs in the protein sequence. We termed this gene *Frem1*, and database and RT-PCR analysis confirmed that it comprises 36 coding exons encoding a 2,191-aa protein. Using a hidden Markov model derived from the CSPG repeat domains in *Frem1*, we identified two related genes named *Frem2* and *Frem3* in mouse and human, as well as orthologues of these genes in several other species (Fig. 1*A* and Table 1, which is published as supporting information on the PNAS web site). Unlike Frem1 and -3, Frem2 retains a transmembrane domain as well as a conserved C-terminal PDZ-binding motif that has been shown to interact with Grip1 in an *in vitro* assay (7). The CSPG domains are structurally similar to the cadherin protein fold (Fig. 1*B*) and several, but not all, conserve the residues shown to be critical for Ca^{2+} chelation between tandemly arranged cadherin domains (Figs. 1*B* and 6). Several evolutionarily conserved sites for *N*-linked glycosylation were noted in all CSPG-containing proteins, and potential sites for glycosaminoglycan modification were noted in all Frem1 proteins, although they were not well conserved in Frem1. A comprehensive analysis of potential Frem protein posttranslational modification is presented in Fig. 6. Fras1, Frem1, -2, -3, and extracellular matrix 3 (ECM3) all share a core region of 12 consecutive CSPGs, whereas CSPG4 [variously known as MCSP (23), NG2 (21), and AN2 (24)], CSPG5, and dipterian proteins SPTR:Q9VJ82 and SPTR:EAA12258 share 15 repeats of this domain (Fig. 1*A*). A single CSPG domain-containing gene was identified in *Caenorhabditis elegans*. Consistent with their domain organization, phylogenetic analysis of sequence alignments demonstrates that Fras1 and the Frem proteins form a discrete monophyletic clade that has been identified only in chordates, whereas the CSPG4 and CSPG5 genes are more distantly related (Fig. 1*A*). Frem2 and -3 phylogentically cluster with the sea urchin protein ECM3, which mediates mesenchymal cell migration during gastrulation (25), and domain conservation suggests that Frem2 and ECM3 are orthologous (Fig. 1*A*).

Mutation of *Frem1* in *bat* and *heb* Mice. Our attention was drawn to *Frem1* through the identification of an ENU-induced mutation called *bat*, which mapped to a 3-Mb interval encompassing the

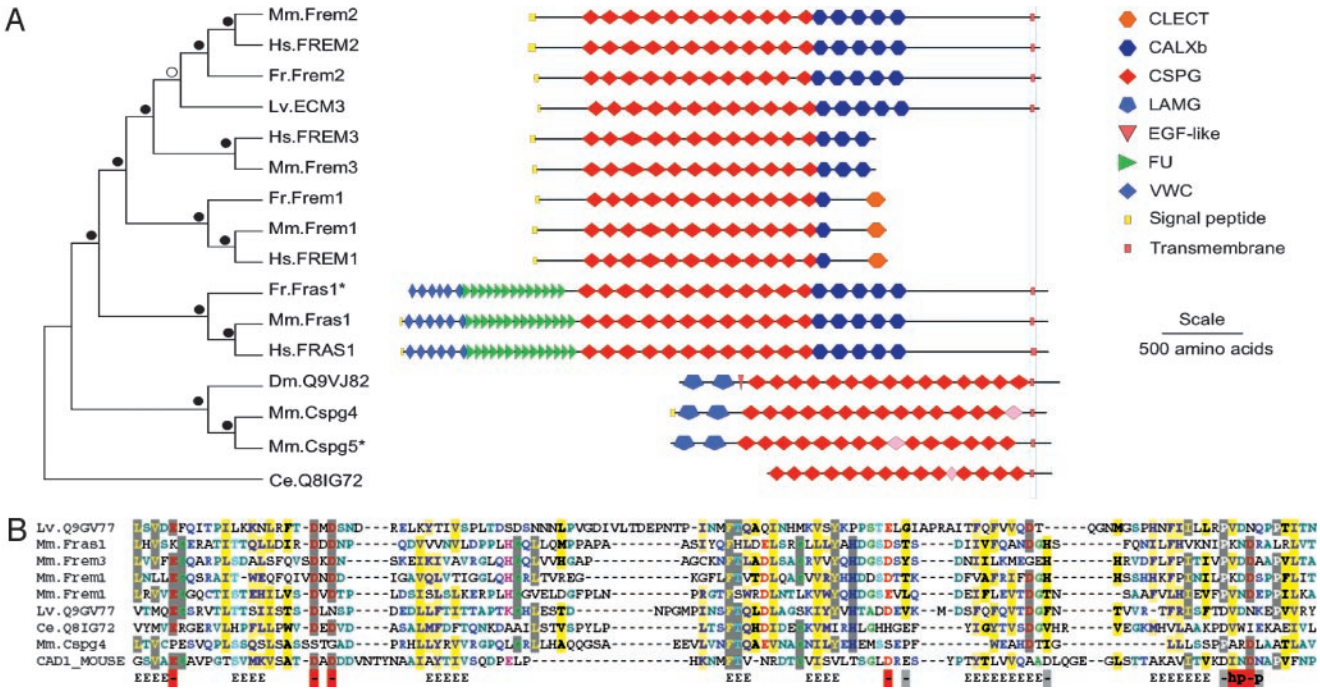

Fig. 1. Phylogenetic analysis and domain organization of CSPG-containing proteins. (*A*) Phylogenetic trees were constructed from aligned CSPG domain-containing regions of each protein by using neighbor-joining (NJ), maximum parsimony (MP), and minimum evolution (ME) methods. (Mm, *Mus musculus*; Hs, *Homo sapiens*; Fr, *Fugu rubripes*; Dm, *Drosophila melanogaster*; Ce, *C. elegans*; and Lv, *Lytechinus variegatus*. Black spots indicate tree nodes with >95% bootstrap support for all three methods, and the gray spot indicates a node supported to 89% by NJ and MP and 60% by ME. The remaining node was not consistently resolved by these methods. Fr.Fras1 and Mm.Cspg5 are incomplete at their N termini (*). The three lightly shaded CSPG domains only partially match the full domains as defined in *B*. (*B*) Multiple sequence alignment of CSPG domains with a cadherin of known structure (16) presented by using CHROMA (33). The line below the alignment shows the consensus structural features of cadherins aligned to the CSPG domains. E indicates β-strand residues. Residues shown to directly interact with chelated Ca^{2+} ions are shaded; red indicates physical–chemical properties that are >60% conserved between cadherins and CSPGs and gray indicates where they are not conserved. The consensus properties are summarized by the symbol − for negative and by the letters p for polar and h for hydrophobic residues.

gene. *bat* mice have a range of phenotypic defects including cryptophthalmos, occasional limb syndactyly, and renal anomalies (Fig. 2*A*). Necropsy revealed unilateral renal agenesis similar to that of Fras1$^{-/-}$ mice in ≈20% of adult *bat* homozygotes (n = 23). We examined the histology of kidneys from adult

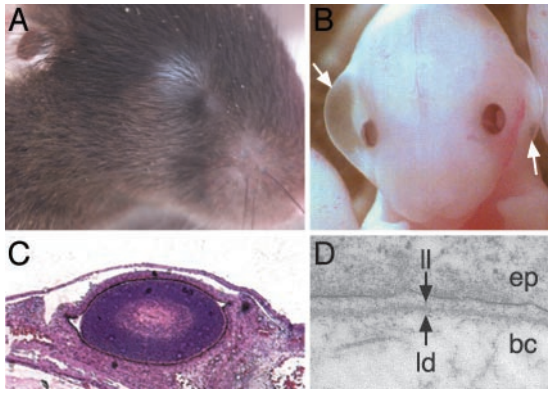

Fig. 2. *bat* mice display cryptophthalmos and embryonic blebbing. (*A* and *B*) *bat* mice display cryptophthalmos in adults and embryonic blebs from ≈13.5 dpc, which invariably affect the developing eyes (*B*, arrowheads). (*C*) Sectioning at 16.5 dpc illustrates the formation of a pseudoblepharon (arrowheads), which severely affects development of the underlying eyelids. (*D*) The epidermis (ep) in the blebs of *bat* embryos separates from the dermis below the lamina lucida (ll) and the lamina densa (ld) of the basement membrane. The resulting blister cavity (bc) contains residual anchoring fibrils on the subepidermal face.

bat mice but observed no gross malformations. From 13.5 dpc, homozygous *bat* embryos develop blebs located predominantly around the eyes and the sides of the head (Fig. 2*B*). Sectioning highlighted defects in the adhesion of the surface ectoderm and corneal stroma, an interaction required for eyelid development (Fig. 2*C*). *bat* mice consequently have no true eyelids, and the closed-eye appearance results from the presence of a pseudoblepharon that can be either unilateral or bilateral [ratio ≈1:1.8 (n = 184)]. Significantly, transmission electron microscopy of *bat* embryos indicated that, like *Fras1* mutants, the plane of epidermal cleavage lies below the lamina densa of the basement membrane, and that the formation of hemidesmosomes appears relatively normal (Fig. 2*D*). The phenotype of *bat* animals was 100% penetrant on a *C57BL/6J* background, although backcrossing to *C3H/NeJ* reduced this to ≈70%. Unlike other blebs mutants, we did not detect any appreciable *in utero* death in *bat* mice. A second ENU-induced recessive mutant termed *bfd* was identified on chromosome 4 and has a phenotype similar to *bat*. This mutant was mapped to a small interval (<300 kb) encompassing *Frem1* by crossing *bfd* mice with animals heterozygous for chromosome deletions (11R30M, 1OZ, and 13R75M) (26). Intercrossing demonstrated that *bat* and *bfd* failed to complement one another and are allelic mutations.

Mutation screening of *Frem1* in *bat* animals identified a T to C transition at position +2 in the splice donor of intron 25, a residue that is highly conserved in mammalian splice sites. RT-PCR analysis indicates that this alteration results in the complete skipping of exon 25 (Fig. 3*A*), and we failed to detect splicing between exons 25 and 26 using a forward primer in exon 25 (data not shown). The misspliced transcript produced leads to

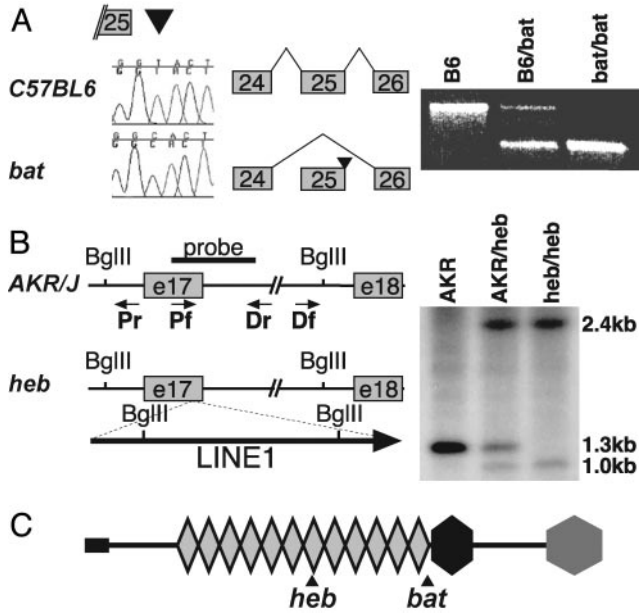

Fig. 3. The *bat* and *heb* phenotypes result from mutation of *Frem1*. (*A*) Sequencing of all 36 *Frem1* coding exons identified an ENU induced T-C mutation in the splice donor site of exon 25 (*Left*). RT-PCR analysis shows that this mutation leads to skipping of exon 25 in *bat* RNA compared with C57BL/6J mice on which the mutation was induced. (*B*) Southern blot analysis of *heb* DNA with a probe spanning exon 17 detected a genomic rearrangement not present in the parental AKR/J strain. Inverse PCR identified the insertion of a LINE1 element 41 bp from the end of the exon. The *Bgl*II sites and primers used for inverse PCR (Pr, Pf, Dr, and Df) are indicated. (*C*) Position of the *heb* and *bat* mutations relative to the protein domains of Frem1 (CSPG, light gray; Calx, black; LectinC, dark gray).

a frame shift and the introduction of a premature stop codon 36 bp into exon 26 and hence truncation of the protein toward the C-terminal end of the CSPG repeat elements (Fig. 3 *A* and *C*). Previous studies have shown that the spontaneous *heb* mutation also maps to this region of chromosome 4 (12), and screening of *Frem1* indicated a rearrangement in exon 17 of the gene. Inverse PCR and Southern blot analysis identified a LINE1 insertion 41 bp from the 3′ end of the exon that was not present in the *AKR/J* background upon which this mutation arose (Fig. 3 *B* and *C*). Sequencing of the allelic *bfd* mutant failed to identify any coding mutations, indicating that the causative mutation might lie in regulatory elements of *Frem1*.

Expression of *Frem1* During Development. To identify a role for this protein in the formation of the epidermis, we examined the expression of *Frem1* during embryonic development. The gene is expressed in many developing epidermal appendages, including the whisker and sensory vibrissae, cranial and trunk hair follicles, meibomian glands, teeth, footpads, eyelash primordia, and invaginating mammary glands (Fig. 4 *A–C*). Limb expression localizes to sheets of dermal cells on the apical and basal surfaces of the digits but, unlike *Fras1*, is excluded from the apical ectodermal ridge (Fig. 4*H*). Examination of sections of stained embryos and *in situ* hybridization of tissue sections established that *Frem1* expression is usually highest in dermal cells underlying the differentiating epithelial components (Fig. 4 *D–G*), especially underlying the epidermis of the head, limbs, and eyelids. Expression in the eyelid dermis was apparent as early as 13 dpc. Postnatal expression of the gene in the skin is limited to the dermal papillae (data not shown). Contrary to the reported phenotype of the *heb* allele (12), ≈20% of *bat* mice present with unilateral renal agenesis. Whole-mount *in situ* hybridization of embryonic kidneys from 11.5 to 14.5 dpc followed by subsequent sectioning and optical projection tomography imaging (20) detected expression of *Frem1* from ≈12.5 dpc in the mesenchyme surrounding the branching ureteric tree (Fig. 4*I*). The highest expression was noted in the more proximal regions of these tubules rather than at the proliferating and branching ends of the ureteric buds. Similar analysis of *Fras1* expression at 12.5 dpc indicated that this expression was complementary to *Frem1*. By 13.5 dpc, *Frem1* expression was also detected in morphologically distinct cells associated with the differentiating nephron, and staining with antibodies to Wilms tumor suppressor 1 (Wt1) confirmed that these were differentiating podocytes (Fig. 4*J*). Surprisingly, Fras1 is coexpressed in the podocytes (Fig. 4*J*), suggesting that, whereas complementary expression of *Fras1* and *Frem1* generally occurs in regions of epithelial/mesenchymal interactions, in podocytes the genes are coexpressed.

Basement Membrane Deposition of Collagen VI and Fras1 Is Normal in *Frem1* Mutants. To determine whether the mechanism of formation of the blebs in *bat* mice was similar to that in *Grip1* and *Fras1* null animals, we examined the basement membrane composition of the epidermis. In both *Fras1*- and *Grip1*-null epidermis, Fras1 and collagen VI are absent from the basement membrane (3, 5, 7). Because CSPG domains in a related protein NG2 have been shown to interact with collagen VI (10), it has been proposed that disruption of epidermal adhesion is the result of loss of deposition of this protein in the basement membrane. Immunohistochemical analysis of *bat* and wild-type embryonic epidermis indicated that collagens V and VI, and Fras1 were all normally localized (Fig. 5). Furthermore, all proteins were detected in the roof of blebs blisters, highlighting the fact that epidermal splitting occurs below the level of the lamina densa.

Discussion

The extracellular matrix is increasingly being recognized as a critical mediator of intercellular interactions that control both tissue development and structural integrity (27). We have identified an ECM protein termed *Frem1* that shares a number of structural domains with the Fraser syndrome protein Fras1 but that notably lacks the transmembrane or intracellular domains that allow Fras1 to interact with Grip1. As a consequence, Frem1 is unlikely to directly interact with Grip1 but is likely to be secreted from the cell and to associate with carbohydrates in the extracellular milieu. The protein is part of a three-gene family, and structural and sequence analysis indicated that the CSPG domains present in these proteins are structurally similar to cadherins. On this basis, we suggest that Ca^{2+} binding is likely to be required for inter- and intramolecular interaction of the CSPG domains. In this respect, it is also notable that in other proteins, the carbohydrate-binding activity of the lectin type C motif present at the C terminus of Frem1 has been shown to be modulated by calcium levels (28). All Frem proteins contain multiple consensus sites for *N*-linked glycosylation, and some contain conserved glycosaminoglycan modification sites, suggesting that the proteins are extensively posttranslationally modified.

We identified two truncating mutations in *Frem1* in both the classic *heb* allele and in an ENU-generated allele termed *bat*. Given that the phenotype of both *bat* and *heb* mutants is very similar, that analogous truncations in *Fras1* give phenotypes indistinguishable from early truncations, and that the number of CSPG repeats in Frem1 is rigorously conserved throughout evolution (Fig. 1*A*), we predict that both *bat* and *heb* alleles represent null mutations of *Frem1*. *bat* epidermis separates from the dermis below the level of the lamina densa in a manner analogous to that observed in patients with dystrophic epidermolysis bullosa (DEB) who carry mutations of type VII collagen (29). Unlike DEB patients or mice carrying mutations in colla-

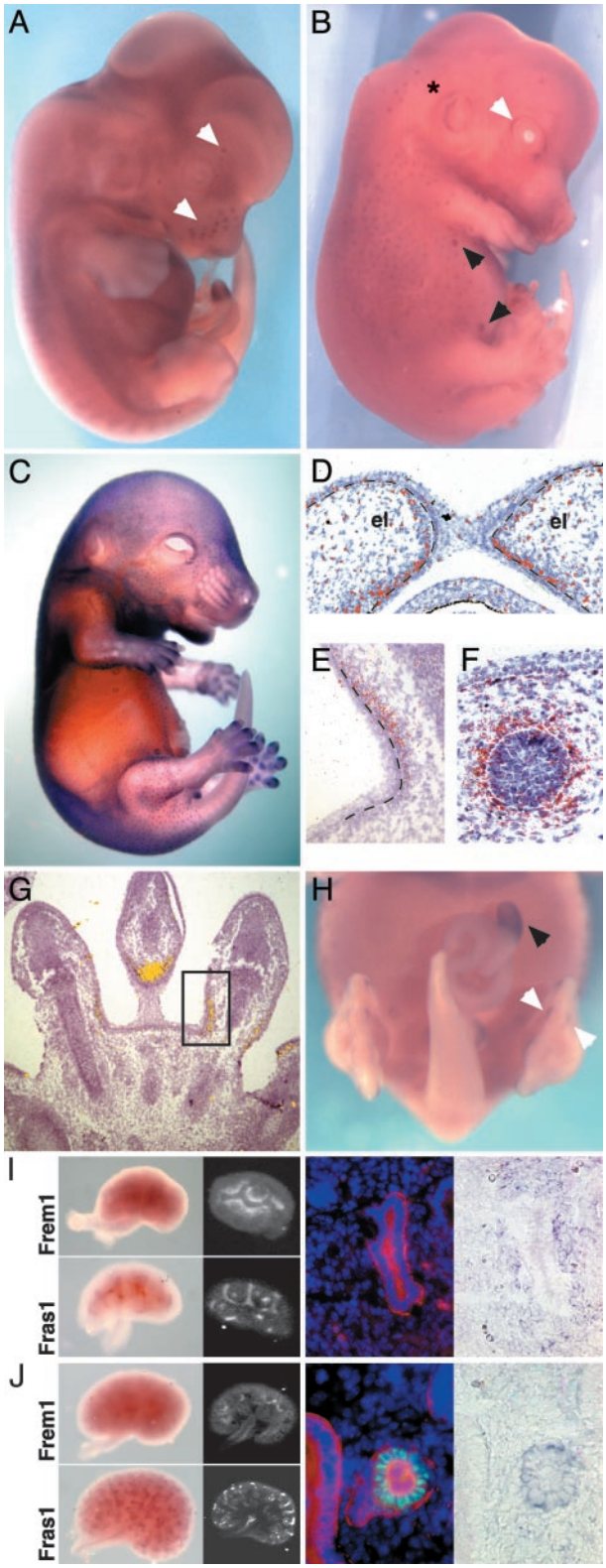

Fig. 4. *Frem1* is expressed dynamically during embryogenesis in regions of epithelial/mesenchymal interaction. (*A*) *Frem1* is expressed from ≈10.5 dpc and becomes pronounced in the differentiating eyelids and sensory vibrissae (white arrowheads) by 12.5 dpc. At this stage, low levels of epithelial expression are noted over the head and trunk. (*B*) By 14.5 dpc, expression is maintained in the vibrissae and eyelids (white arrowhead) and is initiated in the epithelium of the developing ear (*) and in the mammary glands (black arrowheads). Transcripts are also detected in the hair follicle placodes, initiating on the trunk. Follicle expression is initially detected in both epidermis and condensing mesenchyme at ≈14 dpc, but as development progresses, it becomes restricted to cells of the future dermal papillae. (*C*) By 16.5 dpc, *Frem1* is expressed in the differentiating pelage follicles, limbs, and paw pads as well as in the meibomian glands. Section *in situ* hybridization at 15.5 dpc indicated strong dermal/mesenchymal expression of *Frem1* in the developing eyelids (el, *D*), limb (*E* and *G*), and mammary gland (*F*). Hybridization signal is shown in yellow and red. The position of the basement membrane is indicated by a dashed line. (*H*) *Frem1* expression was also detected in the developing caecum (black arrowhead) and is restricted to the mesenchyme (data not shown). At this stage, apical and basal dermal limb expression is detected (white arrowheads). (*I*) *Frem1* is expressed in the kidney at 12.5 dpc in the mesenchyme surrounding the growing ureteric bud in a pattern complementary to Fras1. (*Left*) *In situ* expression and virtual optical projection tomography sections. (*Right*) α-Fras1 immunostaining (red) and *Frem1 in situ* hybridization (bright-field). (*J*) At 13.5 dpc, *Frem1* and Fras1 expression also colocalizes with α-Wilms tumor suppressor 1 (Wt1) immunostaining (green) in the podocytes (as in *I*).

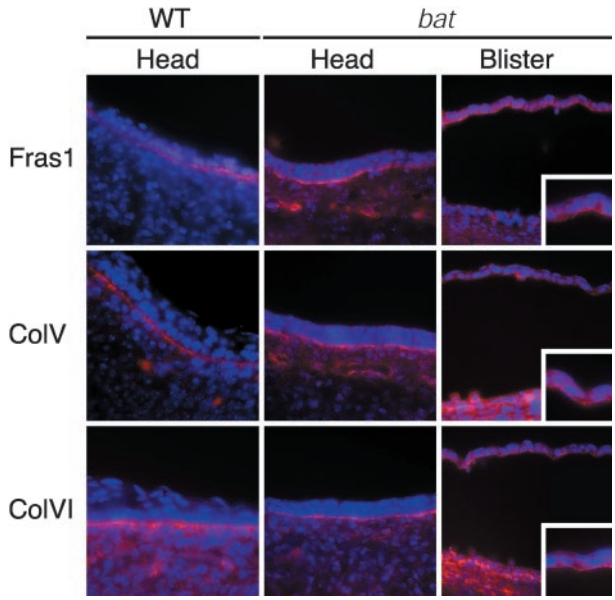

Fig. 5. *bat* mice display normal localization of Fras1 and collagen VI (ColVI) in the epidermal basement membrane. Immunolocalization of Fras1, collagen V (COLV), and collagen in both the head and blister epidermis of *bat* mice appeared indistinguishable from that in wild-type embryos. Staining for different proteins is indicated in red, and sections have been counterstained with 4′,6-diamidino-2-phenylindole (blue). High-magnification images of blister roofs are shown (*Insets*).

gen VII (30), the epidermis of *heb* alleles is apparently normal after birth, highlighting the important role of Frem1 in the initial establishment of epidermal adhesion but not its postnatal maintenance. Indeed, the Frem1/Fras1/Grip1 pathway, to our knowledge, is unique in its specific requirement during embryonic development.

Frem1 is expressed in the developing skin epidermis and in a number of differentiating epidermal structures such as the mammary and meibomian glands, teeth, and hair follicles. It is notable that expression of *Frem1* in many of these organs, including the skin and eyelids, is restricted to the dermis. This contrasts markedly with the expression of both *Fras1* and *Grip1*, which are predominantly detected in the epidermis (3, 5, 7, 8). Given that Frem1 appears incapable of interacting directly with Grip1, and that the expression of the gene is largely complementary to both *Grip1* and *Fras1*, we propose that it provides a dermal complement to other known blebs proteins. The expression of *Frem1* is markedly more restricted than *Fras1* (3, 5),

suggesting that the reduced severity of the *heb/bat* phenotype reflects the more restricted expression pattern of *Frem1*.

Previous studies of *heb* homozygous mice failed to identify any defects in kidney development (12). Our investigation of *bat* mice demonstrates unilateral renal agenesis in a proportion of homozygous offspring. We consider this is unlikely to be an allele-specific effect, given that the *Frem1* mutations in both mutants are in roughly the same region and are both likely to inactivate the protein. Rather, we consider that the difference in phenotype probably relates to differences in genetic background, which we have shown can greatly modulate phenotype penetrance in the *bat* mice. In early renal development, *Frem1* and *Fras1* are expressed in the mesenchyme and epithelia, respectively, although at later developmental stages *Frem1* and *Fras1* are also coexpressed in the developing podocytes. Reciprocal signaling interactions between the epithelial and mesenchymal components of the developing kidney have already been shown to be important for normal renal development (31), and a similar interaction is likely to underlie the agenesis observed in the blebs mutants. Given that the CSPG domains in NG2 can interact with various growth factors (6), it will be interesting to investigate whether the Fras and Frem proteins can modulate their activity in the developing kidney.

We propose that the maintenance of epithelial integrity during development requires at least two components from the epidermis (Fras1 and Grip1) and one from the dermis (Frem1). CSPG domains in NG2 have been shown to interact with collagens V and VI (10), and it is possible that both Fras1 and Frem1 can mediate epithelial adhesion via analogous interactions. However, we observed no defects in deposition of these collagens in *bat* epidermis. Given the significant structural and sequence conservation between CSPG domains and the cadherin protein family (Fig. 1) (22), Fras1 and/or Frem2 might interact directly with Frem1 through these motifs. Studies of NG2 have indicated that its intracellular C terminus is able to transduce extracellular signals upon engagement of the CSPG domains and alter cell motility and spreading (32), and a similar mechanism of Fras1 "activation" upon Frem1 binding is proposed. Given the timing of the appearance of blisters in blebs mice, we suggest that the Frem and Fras proteins are required for very early adhesion of the epidermis. However, the observations that these blisters resolve in late gestation and that the postnatal skin of the blebs mice is normal suggest that other components of the ECM are probably sufficient to mediate normal adhesion at later developmental stages. The identification of mutations in *Frem1* highlights a gene expressed in the future dermis that is necessary for the normal development and adhesion of the epidermis and for renal development. Furthermore, it delineates a family of ECM proteins that present further candidates for mutations in the remaining uncloned blebs mutants and in human Fraser syndrome patients.

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