Breakdown of the reciprocal stabilization of QBRICK/Frem1, Fras1, and Frem2 at the basement membrane provokes Fraser syndrome-like defects

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An emerging family of extracellular matrix proteins characterized by 12 consecutive CSPG repeats and the presence of Calx-β motif(s) includes Fras1, QBRICK/Frem1, and Frem2. Mutations in the genes encoding these proteins have been associated with mouse models of Fraser syndrome, which is characterized by subepidermal blistering, cryptophthalmos, syndactyly, and renal dysmorphogenesis. Here, we report that all of these proteins are localized to the basement membrane, and that their basement membrane localization is simultaneously impaired in Fraser syndrome model mice. In *Frem2* mutant mice, not only Frem2 but Fras1 and QBRICK/Frem1 were depleted from the basement membrane zone. This coordinated reduction in basement membrane deposition was also observed in another Fraser syndrome model mouse, in which GRIP1, a Fras1- and Frem2-interacting adaptor protein, is primarily affected. Targeted disruption of *Qbrick/Frem1* also resulted in diminished expression of Fras1 and Frem2 at the epidermal basement membrane, confirming the reciprocal stabilization of QBRICK/Frem1, Fras1, and Frem2 in this location. When expressed and secreted by transfected cells, these proteins formed a ternary complex, raising the possibility that their reciprocal stabilization at the basement membrane is due to complex formation. Given the close association of Fraser syndrome phenotypes with defective epidermal–dermal interactions, the coordinated assembly of three Fraser syndrome-associated proteins at the basement membrane appears to be instrumental in epidermal–dermal interactions during morphogenetic processes.

epithelial–mesenchymal interaction | gene targeting | morphogenesis

The extracellular matrix (ECM) is an insoluble supramolecular complex surrounding metazoan cells that is often fibrous or sheet-like. The ECM functions in the control of cellular behaviors, including migration, proliferation, and differentiation, and mediates intercellular communication, as in epithelial–mesenchymal interactions; both of these functions are critical during development. Because individual ECM components often function in combination with other ECM components and soluble factors, genetic disorders of the ECM are often linked to severe developmental abnormalities.

Fraser syndrome is a recessive multiorgan disorder characterized by cryptophthalmos, syndactyly, renal agenesis, and a variety of morphogenetic defects (1). Approximately 45% of human cases are stillborn or die within the first year, primarily because of pulmonary and/or renal complications (2). The phenotypic similarities between these patients and five mouse "blebbing" mutants, *blebbed* (*bl*), *myelencephalic blebs* (*my*), *eye blebs* (*eb*), *head blebs* (*heb*), and *fetal haematoma*, suggested these mutant mice represent animal models of Fraser syndrome (3). The developmental defects observed in Fraser syndrome and the associated mouse models suggest that these defects arise from disruption of the epithelial–mesenchymal interactions required for normal morphogenetic processes. Recently, two novel genes, *FRAS1* and *FREM2*, have been identified as the causative genes in human Fraser syndrome, and *Fras1*, *Frem2*, *Grip1*, and *Qbrick/Frem1* have been recognized as the genes mutated in *bl*, *my*, *eb*, and *heb* mice, respectively (4–9). *Grip1* encodes an intracellular adaptor protein containing multiple PDZ domains, whereas *Fras1*, *Qbrick/Frem1*, and *Frem2* encode members of a novel family of ECM proteins characterized by 12 consecutive CSPG repeats and a varying number of Calx-β domains (refs. 8 and 10; Fig. 1*A*).

The observation of overlapping developmental defects in the four blebbing mutant mice (i.e., *bl*, *my*, *eb*, and *heb*) implies that the deficits underlying Fraser syndrome-like phenotypes all affect a common process or pathway, in which the four Fraser syndrome-associated proteins function cooperatively. Indeed, a functional linkage was shown between GRIP1 and Fras1; GRIP1 binds to the PDZ-binding motif of Fras1, which is necessary for the extracellular localization of Fras1 to the basal surface of epidermal cells (7). Despite conspicuous features in their domain structures and extracellular localization, the functions of three Fraser syndrome-associated ECM proteins, Fras1, Frem2, and QBRICK/Frem1, are poorly understood. It remains unclear whether three ECM proteins function cooperatively.

In this study, we examined the cooperativity between the three Fraser syndrome-associated proteins at the basement membrane. Using immunohistochemical analyses of three Fraser syndrome model mice, including a strain of *Qbrick/Frem1*−/− mice, we demonstrated that these ECM proteins fail to assemble into the basement membrane when their simultaneous expression is compromised; the reciprocal expression of Fras1, Frem2, and QBRICK/Frem1 is required for their stable localization at the basement membrane. Failure of cooperativity between these proteins at the basement membrane explains the overlapping phenotypes observed in Fraser syndrome model mice.

Results

Diminished Expression of Frem2 in *my* Mutant Mice. We first examined whether Frem2 localizes to the basement membrane in a manner similar to Fras1 and QBRICK/Frem1. Using an antibody specific to the Frem2 ectodomain, we found that Frem2 immunoreactivity colocalized at the epidermal basement membrane with that of laminin-γ1, a ubiquitous marker of the basement membrane (Fig. 1*B*). An antibody specific for the cytoplasmic tail of Frem2 failed to detect the protein *in situ* (Fig. 1*C*), likely because of loss of the epitope after ectodomain shedding. In support of this possibility, Frem2 and Fras1 were both secreted into the medium when expressed in mammalian cells; the secreted Frem2 lacked a cytoplasmic tail (Fig. 1 *D* and

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Abbreviations: ECM, extracellular matrix; E*n*, embryonic day *n*.

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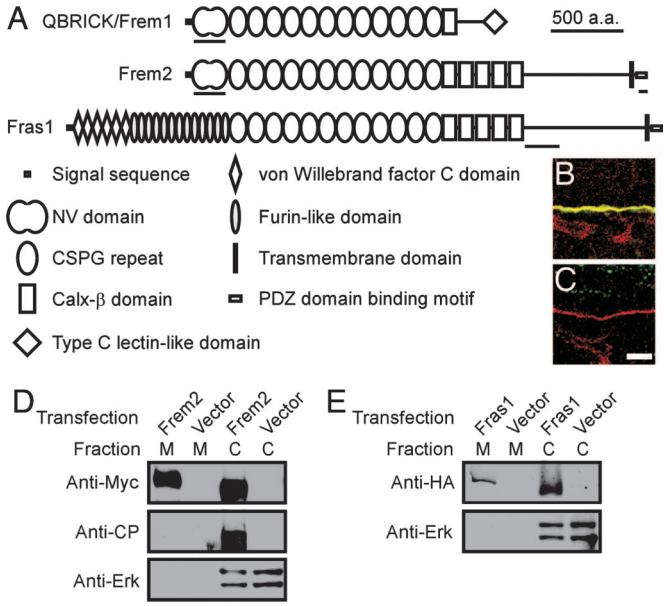

Fig. 1. Localization of Frem2 at the basement membrane zone. (*A*) Schematic view of the protein family that contains 12 CSPG repeats and variable Calx-β motifs. Underlined are the regions used as immunogenic epitopes. (*B* and *C*) Immunolocalization of Frem2 in the skin of E17.5 mice. In the dorsal epidermal basement membrane, which is counterstained for laminin-γ1 (red), Frem2 (green) was detected by antibodies recognizing the ectodomain (*B*) but not the cytoplasmic tail (*C*). (Scale bar, 20 μm.) (*D* and *E*) Secretion of Frem2 and Fras1 after expression in 293F cells. 293F cells were transfected with Frem2 expression vector or empty vector, followed by immunoblotting of the conditioned medium (M) and cell lysates (C) with an anti-Myc antibody (to detect Frem2 containing an N-terminal 3xMyc tag) or an antibody against the cytoplasmic tail of Frem2 (CP). Adequate separation of the medium and the cell fraction was monitored by measuring immunoreactivity to cellular Erk (*D*). Similarly, 293F cells were transfected with Fras1 expression vector or empty vector, followed by immunoblotting with anti-HA (to detect Fras1 containing an N-terminal 3xHA tag) and anti-Erk (*E*).

E). These results demonstrate that Frem2 is also a basement membrane protein.

Next, we examined whether Frem2 expression is impaired in the *my* mutant, a mouse model of Fraser syndrome. Although disruption of Frem2 is reported in *my*^{KST} and *my*^{UCL} mutants, both of which are thought to be allelic to *my*, the original *my* allele has not been fully investigated (5, 9, 11). In *my*/*my* mutant embryos and newborns, Frem2 immunoreactivity was reduced in the epidermal basement membrane zone when compared with *my*/+ animals (Fig. 2 *B–D*). Expression of other ECM proteins, including laminin-γ1, collagen-VI, NG2, collagen-IV, and perlecan, at the epidermal basement membrane zone was unaffected (Fig. 2 *H–K*). Consistent with the reduction of Frem2 at the basement membrane, the expression levels of the *Frem2* transcript were greatly reduced in *my*/*my* mice from those seen in wild-type mice, although the expression of genes adjacent to *Frem2* was unaffected (Fig. 2*A*; see also Fig. 7, which is published as supporting information on the PNAS web site). These results are consistent with recent reports that Frem2 is dysfunctional in mice bearing the *my*^{KST} and *my*^{F11} alleles, both of which cause Fraser syndrome-like phenotypes similar to those observed in *my* mice (5, 9). Because we failed to detect any deletion or missense/nonsense mutation in the exons encoding the Frem2 protein (see Table 1, which is published as supporting information on the PNAS web site), the reduced expression of Frem2 may result from either mutation(s) affecting the activity of cis-transcriptional elements or the stability of *Frem2* transcripts.

Impaired Basement Membrane Localization of Fras1 and Frem2 in *my* and *eb* Mice. Of the proteins associated with Fraser syndrome, both Fras1 and Frem2 contain a transmembrane domain and a PDZ domain-binding motif at their C termini (Fig. 1*A*); in addition, both are expressed by embryonic epidermal cells (refs. 5, 6, and 9; see also Fig. 8, which is published as supporting information on the PNAS web site). Coordinated expression of Fras1 and Frem2 in epidermal cells suggests they are deposited concomitantly, potentially functioning in the basement membrane cooperatively. In support of this possibility, Fras1 expression at the epidermal basement membrane zone was decreased in *my* mutant embryos and newborns (Fig. 2 *E–G*). The levels of *Fras1* transcript appeared unaffected in *my*/*my* embryos (Fig. 2*A*), suggesting that the reduction in Fras1 basement membrane expression observed in *my* mice occurs posttranslationally. The expression of GRIP1, which is necessary for the extracellular localization of Fras1 at the basal surface of epidermal cells, was unaffected in *my*/*my* mice (Fig. 2*L*).

We further investigated the coordinated deposition of Fras1 and Frem2 at the basement membrane in *eb* mutant mice, another model of Fraser syndrome in which GRIP1 is disrupted (7). In embryonic day (E)14.5 embryos and newborn *eb*/*eb* mice, expression of both Fras1 and Frem2 was diminished compared with *eb*/+ animals at the epidermal basement membrane (Fig. 3 *A–D*). The expression of other basement membrane proteins, including laminin-γ1, perlecan, and collagen-IV, was unaffected

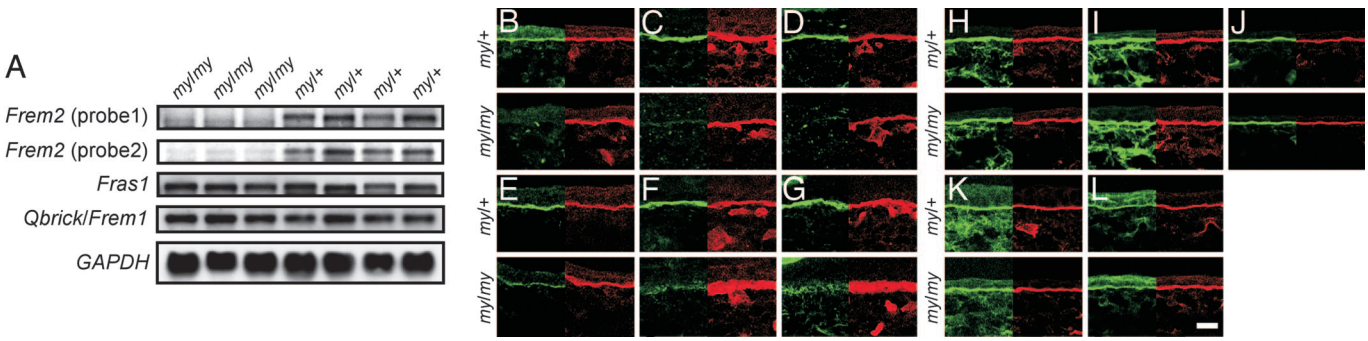

Fig. 2. Expression profiles of Fraser syndrome-associated proteins in *my* mutant mice. (*A*) Northern blot analyses of *Frem2*, *Fras1*, *Qbrick*/*Frem1*, and *GAPDH* expression. Two independent probes were used to detect *Frem2* transcripts. (*B–L*) Comparative immunohistochemical analyses of dorsal skin cryosections. In each image, *Upper* and *Lower* represent representative immunofluorescence seen in *my*/+ and *my*/*my* littermates, respectively. Basement membranes were counterstained (red) with antibodies against laminin-γ1 chain (*B–J*, *L*) or EHS-laminin (*K*). (*B–D*) Frem2 immunofluorescence (green) in E13.5 (*B*), E17.5 (*C*), and newborn (*D*) *my*/+ and *my*/*my* mice. Frem2 immunoreactivity was scarce in *my*/*my* animals. (*E–G*) Fras1 immunofluorescence (green) in E13.5 (*E*), E17.5 (*F*), and newborn (*G*) *my*/+ and *my*/*my* mice. Fras1 immunoreactivity was considerably reduced in *my*/*my* animals. Collagen-VI (*H*), NG2 (*I*), collagen-IV (*J*), perlecan (*K*), and GRIP1 (*L*) were expressed at equal levels in *my*/+ and *my*/*my* animals at E13.5 (green). (Scale bar, 20 μm.)

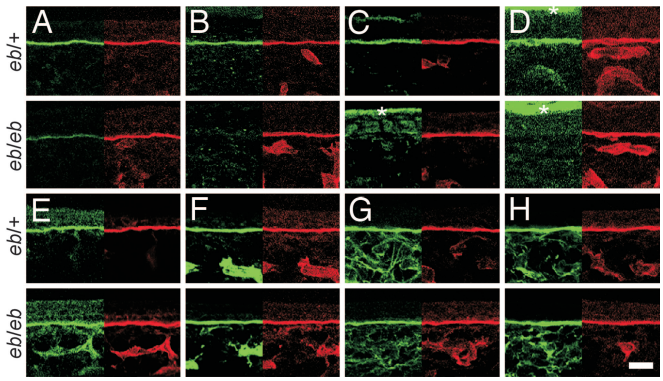

Fig. 3. Impaired expression of Frem2 and Fras1 in *eb* mutant mice. Frem2 (*A* and *B*), Fras1 (*C* and *D*), and other ECM proteins (*E*, perlecan; *F*, collagen-IV; *G*, collagen-VI; and *H*, NG2) were localized by immunofluoresccence (green) in cryosections of dorsal skin isolated from E14.5 (*A*, *C*, *E–H*) and newborn (*B* and *D*) *eb* mutant mice. Basement membranes were counterstained (red) with antibodies against laminin-γ1 (*A–D*, *F–H*) or EHS-laminin (*E*). In each image, *Upper* and *Lower* were taken from *eb*/+ and *eb*/*eb* animals, respectively. In *eb*/*eb* animals, Frem2 immunoreactivity was reduced at E14.5; this reduction became more prominent in newborn mice. Fras1 immunoreactivity is almost absent in *eb*/*eb* animals. Asterisks indicate nonspecific binding of antibodies to the cornified epithelium. (Scale bar, 20 μm.)

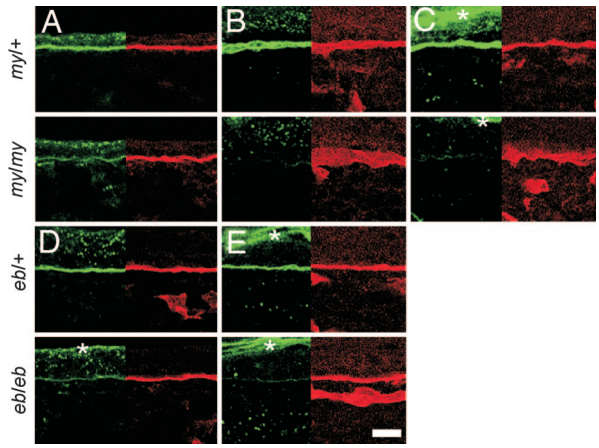

Fig. 4. Impaired expression of QBRICK/Frem1 in *my* and *eb* mutant mice. (*A–C*) QBRICK/Frem1 immunofluorescence (green) in *my*/*my* and *my*/+ mice at E13.5 (*A*), E17.5 (*B*), and at birth (*C*). QBRICK/Frem1 immunoreactivity is reduced in *my*/*my* mice at E13.5; this reduction becomes more prominent at later developmental stages. (*D* and *E*) QBRICK/Frem1 immunofluorescence (green) in E14.5 (*D*) and newborn (*E*) *eb*/*eb* and *eb*/+ mice. The immunoreactivity was reduced at E14.5 and almost absent at birth. Asterisks represent nonspecific binding of antibodies to the cornified epithelium. (Scale bar, 20 μm.)

(Fig. 3 *E* and *F*), confirming specific depletion of Fras1 and Frem2 from the basement membrane in *eb*/*eb* mice. These results indicate that GRIP1 is required for the deposition of both Fras1 and Frem2 on the basement membrane, possibly through interactions with the cytoplasmic tails of these proteins (7), and support the hypothesis that disruption of the coordinated basement membrane deposition of Fras1 and Frem2 leads to the Fraser syndrome-like phenotypes seen in *my* and *eb* mutant mice. Unlike in *Grip1*−/− mice (7), expression of collagen-VI and NG2 at the epidermal basement membrane was not compromised in *eb*/*eb* mice (Fig. 3 *G* and *H*). This discrepancy may be due to the expression in *eb*/*eb* mice of a mutant GRIP1 protein bearing a deletion of amino acids 389–451 (see Fig. 9, which is published as supporting information on the PNAS web site).

Requirement of Fras1 and Frem2 for the Stable Localization of QBRICK/Frem1 at the Basement Membrane. QBRICK/Frem1, another member of the 12 CSPG-containing protein family, lacks a cytoplasmic tail that would be capable of interacting with GRIP1 (Fig. 1*A*). This protein is predominantly expressed in mesenchymal cells (ref. 8; see also Fig. 8), in striking contrast to Fras1 and Frem2, which are both expressed in epidermal cells. Because two *Qbrick*/*Frem1* mutant mice *heb* and *bat* also exhibit Fraser syndrome-like phenotypes (8, 12), we investigated whether the expression of QBRICK/Frem1 was affected in *my* and *eb* mutant mice. Although the levels of *Qbrick*/*Frem1* transcript were unaffected (Fig. 2*A*), deposition of QBRICK/Frem1 at the basement membrane was greatly diminished in *my*/*my* mice (Fig. 4 *A–C*). Similarly, the basement membrane expression of QBRICK/Frem1 was reduced in *eb*/*eb* embryos and newborns (Fig. 4 *D* and *E*), indicating that the localization of QBRICK/Frem1 to the basement membrane depends upon intact expression of Fras1 and Frem2, both of which are produced by epidermal cells and deposited at the basement membrane zone in a GRIP1-dependent manner. These results raise the possibility that these three Fraser syndrome-associated proteins are deposited to the basement membrane of the epidermal–dermal interface together, each requiring the others to assemble at the basement membrane in an active form.

Reciprocal Requirement of QBRICK/Frem1 for the Stable Basement Membrane Deposition of Fras1 and Frem2. To explore cooperativity in the basement membrane deposition of the three Fraser syndrome-associated proteins, we examined whether QBRICK/Frem1 is required for the basement membrane localization of Fras1 and Frem2. We generated *Qbrick*/*Frem1* knockout mice (Fig. 5 *A* and *B*) and confirmed that the mice lacked QBRICK/Frem1 protein expression at the basement membrane zone (Fig. 5*G*). These mice also exhibited a Fraser syndrome-like phenotype, including cryptophthalmos, syndactyly, subepidermal blistering occurring below the lamina densa (Fig. 5 *C–F*), and renal agenesis (data not shown). These results are consistent with previous observations made with *heb* and *bat* mutant mice (8, 12). Although the transcriptional levels of *Fras1* and *Frem2* remained unaffected (data not shown), the localization of Fras1 and Frem2 proteins to the epidermal basement membrane was significantly reduced in *Qbrick*/*Frem1*−/− mice at E14.5 (Fig. 5 *H* and *J*) and at birth (Fig. 5 *I* and *K*). These results support a role for QBRICK/Frem1 in the basement membrane deposition of Fras1 and Frem2. The basement membrane deposition of other ECM proteins, including perlecan, collagen-IV, NG2, and collagen-VI, remained unaffected in *Qbrick*/*Frem1*−/− embryos (Fig. 5 *L–O*).

Molecular Complex Formation by Fras1, Frem2, and QBRICK/Frem1. The reciprocal stabilization of Fras1, Frem2, and QBRICK/Frem1 suggests these three proteins may interact and form a macromolecular complex. To address this possibility, we cocultured 293F cells transfected with QBRICK/Frem1 with cells transfected with Fras1 and Frem2 or Fras1 alone, and we then immunoprecipitated Fras1 from the conditioned medium. QBRICK/Frem1 was coprecipitated with Fras1 only in the presence of Frem2 (Fig. 6*A*), supporting ternary complex formation by these proteins. Furthermore, when we further examined interactions between individual proteins by immunoprecipitation, QBRICK/Frem1 and Fras1 were coprecipitated with Frem2 but failed to coprecipitate each other (see Fig. 10, which is published as supporting information on the PNAS web site). These results indicate that Frem2 serves as a mediator of ternary complex formation. Immunoelectron microscopic analyses provided further evidence that Fras1 was located in close proximity to QBRICK/Frem1 and Frem2 (see Fig. 11, which is published as supporting information on the PNAS web site), consistent with the ternary complex formation by these proteins.

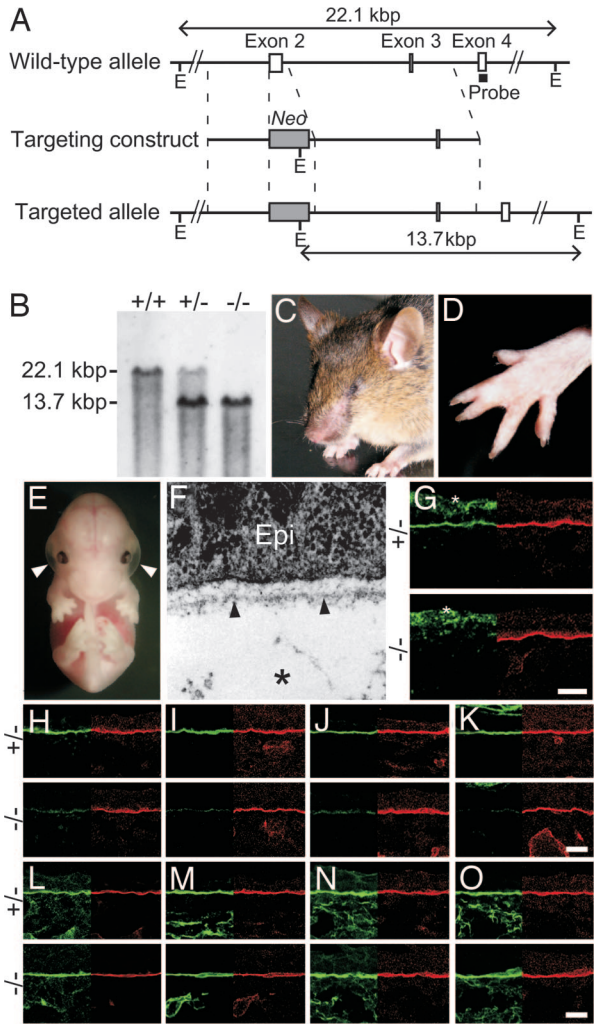

Fig. 5. Reduced expression of Frem2 and Fras1 in *Qbrick/Frem1*−/− mice. (*A*) Schematic representation of the targeted disruption of *Qbrick/Frem1*. Open boxes represent exons. The targeting construct was designed to replace exon 2, which contains the initiation codon, with a neomycin-resistance gene (shaded box). The probe used for Southern blotting in *B* is indicated as a bold line. E, EcoRV restriction site. (*B*) Southern blot analysis of genomic DNA from wild-type, heterozygous, and homozygous offspring after digestion with EcoRV. Detection of a 13.7-kbp fragment indicates targeted disruption of the *Qbrick/Frem1* gene. (*C–E*) *Qbrick/Frem1*−/− animals exhibit cryptophthalmos (*C*), syndactyly in the hindlimb (*D*), and subepidermal blistering at E14.5 (arrowheads in *E*). (*F*) Electron microscopic observation of the blebs of *Qbrick/Frem1*−/− embryos demonstrated that blistering occurred between the lamina densa of the basement membrane (arrowheads) and the underlying dermis. A blister cavity is indicated by an asterisk. Epi, epidermal cell. (*G–M*) Immunohistochemistry of dorsal skin cryosections of *Qbrick/Frem1*+/− and *Qbrick/Frem1*−/− animals taken at E14.5 (*G*, *H*, *J*, and *L–O*) and at birth (*I* and *K*). Basement membranes were counterstained (red) with antibodies against laminin-γ1 (*G–K*, *M–O*) or EHS laminin (*L*). In addition to the absence of QBRICK/Frem1 immunoreactivity at the epidermal BM in *Qbrick/Frem1*−/− animals (*G*), Frem2 (*H* and *I*) and Fras1 (*J* and *K*) immunoreactivities (green) were reduced in comparison to heterozygotes, whereas staining for laminin-γ1 (red) remained constant in the same area. Perlecan (*L*), collagen-IV (*M*), NG2 (*N*), and collagen-VI (*O*) were expressed at equal levels in *Qbrick/Frem1*+/− and *Qbrick/Frem1*−/− animals at E14.5 (green). Asterisks indicate nonspecific binding of antibodies to the cornified epithelium. (Scale bar, 20 μm.)

Discussion

Four mouse models of Fraser syndrome, bearing mutations in *Fras1*, *Frem2*, *Qbrick/Frem1*, and *Grip1*, exhibit closely overlapping phenotypes of subepidermal blistering, cryptophthalmos, syndactyly, and renal agenesis (4–9, 11–13). The similarities in their phenotypes and the expression patterns of their causative genes (4–9) imply that the protein products of these genes function cooperatively, although little has been known about such cooperativity, except that the intracellular adaptor protein GRIP1 is required for the transport of Fras1 (7). Our results clearly show that the three Fraser syndrome-associated ECM proteins, Fras1, Frem2, and QBRICK/Frem1, each characterized by the presence of 12 CSPG repeats and variable Calx-β motif(s), exhibit mutually dependent basement membrane deposition; their basement membrane localization strongly depends on intact expression of all three proteins. Cooperativity in their basement membrane assembly explains both why *Qbrick/Frem1*, *Fras1*, *Frem2*, and *Grip1* mutant mice exhibit similar phenotypes, and why *Fras1* and *Frem2* double mutant mice do not exhibit increased severity of the Fraser syndrome-like phenotype in comparison to the single *Frem2* mutant (5).

It seems likely that the Fraser syndrome-associated ECM proteins become fully functional only when assembled into the basement membrane together. Reciprocal stabilization of proteins at the basement membrane is likely due to macromolecular complex formation through direct protein–protein interactions, because recombinant QBRICK/Frem1 was coprecipitated with Fras1 and Frem2 *in vitro*, and coprecipitation of QBRICK/Frem1 with Fras1 was abolished in the absence of Frem2. As shown in Fig. 6*B*, Fras1 and Frem2 are shed from epithelial cells as a complex, and this complex is stabilized in the basement membrane through the formation of a ternary complex with QBRICK/Frem1 that is secreted by mesenchymal cells. Incomplete assembly of this complex secondary to impaired expression of any component at the basement membrane renders the complex comprised of the remaining proteins unstable and/or susceptible to rapid turnover (Fig. 6*C*). Complex formation by these proteins *in vivo* was further supported by immunoelectron microscopic data showing Fras1 in close proximity to QBRICK/Frem1 and Frem2.

In contrast to the reduced expression of Fras1 in *Qbrick/Frem1*−/− mice, Fras1 expression is unaffected in *bat* mutant mice (8). This discrepancy may be due to differences in the mutations; *bat* mutant mice appear to express a truncated QBRICK/Frem1 protein lacking the Calx-β and type C lectin-like domains (8). The expression of this truncated form may minimize the effect of QBRICK/Frem1 deficiency on Fras1 deposition at the epidermal basement membrane. Because reduced expression of Fras1, Frem2, and QBRICK/Frem1 in *my/my*, *eb/eb*, and *Qbrick/Frem1*−/− mice becomes increasingly prominent with embryonic development (Figs. 2 *E–G*, 3 *A–D*, 4, and 5 *H–K*), it will be interesting to examine the expression levels of these 12 CSPG proteins in *bat* mice at later developmental stages.

Subepidermal blistering occurs at the interface between epidermal basement membrane and the underlying dermal mesenchyme in blebbing mice (Fig. 5*F*; also refs. 4–8 and 13). Because electron microscopic analyses demonstrated that the lamina densa and lamina lucida of the basement membrane at the blister remained undisturbed (Fig. 5*F*; also refs. 4–8 and 13), the blister formation could be due to the defects in the anchoring structures connecting the epidermal basement membrane to the dermis. In *Fras1* and *Grip1* mutant mice, loss of collagen-VI and NG2 has been thought to be part of the mechanism underlying epidermal blistering (6, 7). However, no reduction in collagen-VI and NG2 expression was observed in *my/my*, *eb/eb*, or *Qbrick/Frem1*−/− mice, opposing the explanation of collagen-VI and NG2 loss as a major cause of epidermal blistering. Although such a loss may enhance the severity of blistering, our results suggest that a ternary complex of Fras1, Frem2, and QBRICK/Frem1 serves as a linkage between the epidermal basement membrane and the underlying mesenchyme in embryonic skin. This possibility was

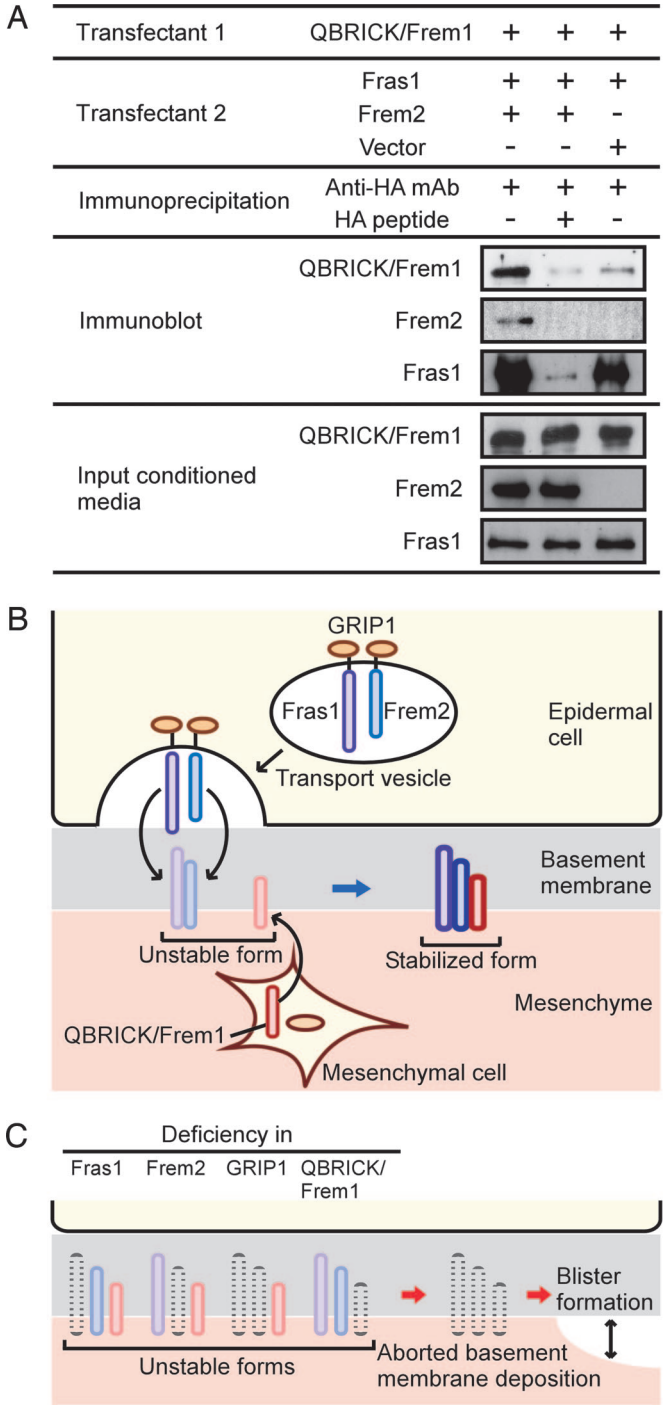

Fig. 6. A model for the reciprocal stabilization of the Fras1, Frem2, and QBRICK/Frem1 through complex formation at the epidermal basement membrane. (*A*) HA-tagged Fras1 protein was immunoprecipitated by using anti-HA mAb in the absence or presence of HA peptide. The absence of Frem2 resulted in a failure of QBRICK/Frem1 to coprecipitate with Fras1. Coprecipitation of QBRICK/Frem1 with Fras1 was abolished in the presence of the antigenic peptide, confirming the specificity of immunoprecipitation. (*B*) Fras1 and Frem2, expressed in epidermal cells, are transported to the plasma membrane in a GRIP1-dependent manner and shed from the cell surface by proteolytic processing. The secreted Fras1/Frem2 complex is deposited in the basement membrane at the epidermal–dermal interface after secretion of QBRICK/Frem1 from dermal mesenchymal cells. Stable basement membrane deposition of QBRICK/Frem1 also depends on the secretion of Fras1 and Frem2 from epidermal cells. This reciprocal stabilization of the three Fraser syndrome-associated proteins is due to their macromolecular complex formation, which secures the stable anchoring of the dermis to the basement membrane. (*C*) Abortion of the interactions between the three Fraser syndrome-associated proteins, which can arise from deficiency or mutation in any individual gene, results in their failure to stably deposit at the basement membrane, leading to impaired anchoring of the dermis to the basement membrane and subsequent blister formation.

supported by the immunoelectron microscopic localization of these three proteins not only to the basal lamina but also to the region of the mesenchyme underneath the basal lamina (ref. 14; see also Fig. 11).

Dysmorphogenesis of the eye, digit, and kidney in Fraser syndrome model mice implies that colocalization of the three Fraser syndrome-associated proteins is required for the establishment of the proper epithelial–mesenchymal interactions governing morphogenetic processes. Given the Arg-Gly-Asp motif-dependent interaction of QBRICK/Frem1 with integrins such as $\alpha 8\beta 1$ (10), the stable deposition of QBRICK/Frem1 at the basement membrane may be instrumental in mediating epithelial–mesenchymal interactions. In support of this possibility, renal agenesis has been reported in integrin $\alpha 8$-deficient mice (15). Alternatively, the stable deposition of QBRICK/Frem1, Fras1, and Frem2 in the basement membrane may be necessary for the propagation of morphogenetic factor signals, as seen in the requirement of heparan sulfate chains by FGF for effective signal transduction through the FGF receptor (16). Because the CSPG repeats of NG2 bind PDGF-AA and basic FGF (17), these Fraser syndrome-associated 12-CSPG-containing proteins may bind soluble ligands and present them to their receptors on adjacent cells, facilitating epithelial–mesenchymal interactions. The wide spectrum of dysmorphogenesis observed in Fraser syndrome model mice suggests that QBRICK/Frem1, Fras1, and Frem2 function not as individual ligands in a specific signaling pathway but as part of the fundamental machinery governing epithelial–mesenchymal interactions by orchestrating intercellular signaling pathways.

In summary, we present evidence that a breakdown in the reciprocal stabilization of QBRICK/Frem1, Fras1, and Frem2 at the basement membrane is closely associated with the development of a Fraser syndrome-like phenotype. Our data suggest that the coordinated assembly of these 12-CSPG-containing proteins into the basement membrane acts as a functional extracellular unit that ensures epithelial–mesenchymal interactions during organogenesis. Further investigation of these 12-CSPG-containing proteins should provide insight into the pathogenesis of Fraser syndrome, as well as the molecular basis of epithelial–mesenchymal interactions during development.

Materials and Methods

Antibody Production. Rabbits were immunized with a GST-fusion protein containing amino acids 3,132–3,369 of Fras1. Immune serum was serially adsorbed onto beads conjugated with GST alone and those conjugated with a GST-fusion protein containing amino acids 2,332–2,531 of Frem2, followed by affinity purification using antigen-conjugated beads. To generate a Frem2-specific antiserum, rabbits were immunized with a GST-fusion protein containing the NV domain of Frem2 (amino acids 63–287). Immune serum was serially adsorbed onto beads conjugated with GST alone and those conjugated with a maltose-binding protein fusion containing the NV domain of QBRICK/Frem1 (amino acids 23–280), followed by affinity purification using antigen-conjugated beads. To produce polyclonal antibodies against the cytoplasmic tail of Frem2, rabbits were immunized with the synthetic peptide CMMSPQSHY-NDSSEV (amino acids 3,147–3,160) conjugated to keyhole limpet hemocyanin. The antibody was affinity-purified by using the antigenic peptide conjugated to Sulfolink coupling gel (Pierce).

Immunohistochemistry. Embryos or newborn mice were embedded in OCT compound for cryosectioning. Ten-micrometer sections

were fixed in absolute ethanol for 20 min at −20°C, then rehydrated with PBS. After incubation overnight with primary antibodies against Fras1, Frem2, QBRICK/Frem1 (10), GRIP1 (Upstate Biotechnology), laminin-γ1 (rat monoclonal; Chemicon), collagen-IV (Rockland), collagen-VI (LSL), NG2 (Chemicon), perlecan (Chemicon), or EHS laminin (Sigma) at 4°C, specimens were incubated with Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) and rhodamine-conjugated goat anti-rat IgG (Biomeda) secondary antibodies. Specimens were mounted in PermaFluor (Thermo Shandon) and visualized by using an LSM510 laser confocal microscope (Zeiss). To enable comparative analysis by immunofluorescence, paired homozygous and heterozygous *my*, *eb*, and *Qbrick/Frem1* mutant littermates were processed by using identical immunohistochemical procedures.

Electron Microscopy. Embryos were sequentially fixed in 1% glutaraldehyde/4% paraformaldehyde in PBS, 2% glutaraldehyde in PBS, and 2% osmium tetraoxide in 0.1 M cacodylate buffer. After dehydration, samples were embedded in an epoxy resin. Uranyl acetate and lead citrate were used for poststaining of ultrathin sections. Images were acquired on a JEM2000EX electron microscope (JEOL), operated at 80 kV.

Northern Blotting. Total RNA was isolated from embryos by using an RNeasy-mini kit (Qiagen). Digoxigenin-labeled antisense RNA probes directed against *Frem2* (1–525 and 9,466–9,965 of AB236662 for probes 1 and 2, respectively), *Fras1* (12,892–13,391 of AJ489280), *Qbrick/Frem1* (10), and *GAPDH* (120–672 of BC083065) were used to probe Northern blots of 10 μg of total RNA isolated from *my/+* and *my/my* embryos at E13.5. Signals were detected with a DIG Luminescent Detection kit (Roche).

Animals. The MY/HuLeJ and ATEB/LeJ mouse strains were obtained from The Jackson Laboratory. The original MY/HuLeJ animals are *my/my* homozygotes. The male MY/HuLeJ animals were outbred with female C57BL/6 animals; the resulting *my/+* females were backcrossed to MY/HuLeJ males to obtain the *my/my* and *my/+* littermates used for histological and Northern blot analyses.

The ATEB/LeJ strain maintains *eb* and *at* in repulsion phase. Male ATEB/LeJ animals were outbred with female C57BL/6 animals; the resulting *eb/+* females were backcrossed to ATEB/LeJ males to obtain *eb/eb* males. The *eb/+* females and *eb/eb* males were crossed to obtain the *eb/eb* and *eb/+* littermates used for histological analyses. The genotype of the *at* locus of these mice should be +/+, unless recombination occurred between the *eb* and *at* loci.

To generate *Qbrick/Frem1−/−* mice, we created a targeting construct in which the 2.0-kb upstream and 5.8-kp downstream *Qbrick/Frem1* genomic sequences flanked a neomycin resistance gene (see Fig. 5*A*). We introduced the targeting vector into 129-strain mouse embryonic stem cells. Two targeted clones, identified by PCR and Southern blotting, were injected into C57BL/6 blastocysts. We identified two 30% chimeric male offspring that transmitted the inactivated gene through the germ line, as determined by both Southern blotting and PCR. After two generations of outbreeding with C57BL/6 mice, the resulting F_2 heterozygotes were crossed to obtain littermates that were heterozygous or homozygous for the null mutation; these animals were used for immunohistochemical analysis.

All mouse experiments were performed in compliance with institutional guidelines and were approved by the Animal Care Committee of Aichi Medical University.

Protein Expression in Mammalian Cells. We first constructed a cDNA encoding a recombinant Fras1 protein; this protein contained the Ig κ-chain signal sequence followed by the 3xHA epitope tag at the N terminus. Similarly, we generated a cDNA encoding a recombinant Frem2 protein containing the Ig signal sequence followed by the 3xMyc epitope tag at its N terminus. These constructs were each subcloned into pcDNA3.1(+) (Invitrogen) and transfected into Freestyle 293F cells (Invitrogen) by using 293Fectin (Invitrogen). After culturing the transfectants for 3 days, the conditioned medium was recovered by centrifugation, and proteins were precipitated with cold acetone. Cells and acetone precipitates were dissolved in SDS/PAGE sample buffer and subjected to SDS/PAGE and subsequent immunoblotting with anti-Frem2 directed to the cytoplasmic tail, anti-Myc (Sigma), anti-HA (Covance), and anti-Erk (Cell Signaling Technology) antibodies.

Immunoprecipitation Assays. We subcloned a cDNA encoding a recombinant QBRICK/Frem1 protein with the 3xFLAG epitope tag at its C terminus into pcDNA3.1(+). We transfected the FLAG-tagged QBRICK/Frem1 cDNA into 293F cells and washed the cells twice with fresh medium 5 h after transfection to remove the cDNA, followed by coculture with 293F cells cotransfected with HA-tagged Fras1 and Myc-tagged Frem2 for 3 days. The conditioned medium was recovered by centrifugation, and 12 ml of the conditioned medium was mixed with 200 μl of monoclonal anti-HA conjugated to agarose beads (Sigma) at 4°C for 1 h in the absence or presence of 50 μg of HA peptide (Sigma). The beads were then washed with 20 mM Tris·HCl, pH 7.4, containing 150 mM NaCl. Proteins bound to the beads were eluted with 0.1 M glycine·HCl, pH 2.5, and subjected to SDS/PAGE and subsequent immunoblotting with anti-Myc, anti-HA, and anti-FLAG (Sigma) antibodies.

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