Regulation of PDGFC signalling and extracellular matrix composition by FREM1

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Summary

The Fras1 related extracellular matrix protein 1 (FREM1) is required for epidermal adhesion during embryogenesis and mice lacking the gene develop fetal skin blisters and a range of other developmental defects. Mutations in the FRAS/FREM gene family cause diseases of the Fraser Syndrome spectrum. Embryonic epidermal blistering is also observed in mice lacking PdgfC and its receptor PDGFRα. In this report we show that FREM1 binds to PDGFC and that this interaction regulates signalling downstream of PDGFRα. Fibroblasts from Frem1-mutant mice respond to PDGFC stimulation, but with a shorter duration and amplitude than wild type cells. Significantly, PDGFC-stimulated expression of the metalloproteinase inhibitor Timp1 is reduced in cells with Frem1 mutations, leading to reduced basement membrane collagen I deposition. These results show that the physical interaction of FREM1 with PDGFC can regulate remodelling of the extracellular matrix downstream of PDGFRα. We propose that loss of FREM1 function promotes epidermal blistering in Fraser Syndrome as a consequence of reduced PDGFC activity, in addition to its stabilising role in the basement membrane.
Introduction

The FRAS/FREM extracellular matrix (ECM) proteins (FRAS1, FREM1 and FREM2) mediate adhesion between the epidermal basement membrane and the underlying dermis during embryonic development (reviewed in (Short et al., 2007; Petrou et al., 2008)). Consequently, their mutation results in the formation of fetal epidermal blisters in a number of human conditions including Fraser syndrome (FS; FREM2 and FRAS1 mutations), Mannitoba oculotrichoanal Syndrome (MOTA) and Bifid Nose/Anorectal and Renal anomalies Syndrome (BNAR) (both caused by mutations in FREM1) (McGregor et al., 2003; Vrontou et al., 2003; Jadeja et al., 2005; Alazami et al., 2009; Vissers et al., 2011). These conditions encompass a wide range of developmental defects of which cryptophthalmos, syndactyly, renal agenesis, ambiguous genitalia and respiratory tract defects are prominent. The FS-spectrum diseases are phenocopied by the “blebs” family of mouse mutants whose causative mutations lie in the mouse homologues of these genes (McGregor et al., 2003; Vrontou et al., 2003; Smyth et al., 2004; Jadeja et al., 2005). FRAS1 and FREM2 are expressed exclusively by epidermal cells, while FREM1 is expressed in both the dermis and epidermis (Petrou et al., 2007; Short et al., 2007). FRAS1, FREM1 and FREM2 are then thought to interact in a complex to stabilize and cross-link epidermal basement membrane attachment to the developing dermis (Kiyozumi et al., 2006).

The FRAS/FREM family of proteins share characteristic chondroitin sulphate proteoglycan (CSPG) core repeats similar to those found in the NG2 proteoglycan (Stallcup, 2002). In this protein, they directly bind platelet-derived growth factor A (PDGFA), fibroblast growth factor FGF2 and collagens V and VI (Goretzki et al., 1999). Epidermal blistering defects like those observed in FS syndrome and the blebs mice are also observed in mice lacking either the growth factor PDGFC or its receptor PDGFRα (Soriano, 1997; Tallquist and Soriano, 2003). PDGFC is expressed in a number of developing epithelia
including the epidermis, with complimentary expression of PDGFRα observed in the associated underlying mesenchyme (Ding et al., 2000; Aase et al., 2002). PDGFC signalling acts upstream to drive expression of matrix metalloproteinase1 (MMP1) and its inhibitor, tissue metalloproteinase inhibitor 1 (TIMP1), in vitro and in transgenic over-expression models (Campbell et al., 2005; Jinnin et al., 2005).

Based on the observation that CSPG domains can interact with PDGF proteins and that epidermal blistering caused by Fras/Frem gene mutations in developing blebs mice is ultra-structurally, spatially and temporally similar to that observed in PdgfC mutants, we hypothesised that FREM1 might regulate PDGFC’s capacity to regulate downstream remodelling of the extracellular matrix (ECM). We show that FREM1 binds to PDGFC in vitro and in vivo and demonstrate in mouse embryonic fibroblasts (MEFs), that wild-type (WT) FREM1 maintains the normal duration and amplitude of PI3-kinase/AKT and MAPK activation following PDGFC stimulation. We further demonstrate that this interaction regulates expression of metalloproteinase inhibitor Timp1 and collagen I deposition. We therefore propose that FREM1 potentiates PDGFC signalling which in turn shapes ECM processing and composition during development. These observations provide a mechanistic basis for basement membrane fragility that leads to epidermal blistering in FS-spectrum diseases and in the blebs mutant mice which model them.

Results

FREM1 interacts with PDGFC.

FREM1 is a multi-domain protein and in mice homozygous for the FREM1 bat mutation, a single DNA base change abolishes an intron splicing site. This aberrant intron inclusion leads to a frame shift and premature stop codon within the 12th CSPG domain, thereby removing the C-terminal CalXβ and C-lectin domains (Smyth et al., 2004) (Fig. 1A).
FREM1 expression has been previously established in both epidermal and dermis cells and localises to the basement membrane separating the two populations (Petrou et al., 2007; Short et al., 2007). PDGFC is expressed by epidermal cells and diffuses to the underlying mesenchyme (Ding et al., 2000; Aase et al., 2002). To determine if PDGFC and FREM1 co-localise, paraffin head skin tissue sections from E13.5 embryos were immunostained with rat anti-FREM1 and anti-PDGFC antibodies. Consistent with the position of the Frem1 mutation in the bat mice we observed FREM1 localization in the epidermis and basement membrane as previously reported, but additionally saw FREM1 in the ECM surrounding dermal fibroblasts (Fig. 1B). FREM1 bat mutant mice showed no changes in expression or localization of the FREM1 mutant protein. Immunostaining with rat non-immune antibody was performed as a control and confirmed the specificity of the rat anti-FREM1 signal (data not shown).

Consistent with reported literature, we also observed PDGFC enriched in the epidermis and weakly in the dermis, however most significantly, we observed PDGFC on the basement membrane (Fig. 1B). Thus there is significant overlap in the localisation of FREM1 and PDGFC within keratinocytes, on the basement membrane and within local ECM surrounding dermal fibroblasts. We did not see a change in the distribution of PDGFC in bat mice (Fig. 1B).

To further explore potential interactions between the proteins, NIH3T3 cells were transfected with plasmids constructs encoding full-length FREM1-FLAG (Fig. 1A) and PDGFC-V5. Detection of extracellular deposits of either factor was rare, however, FREM1-FLAG localised with secretory vesicles marked by the ECM component, Laminin γ1. PDGFC-V5 showed a broad intracellular distribution but was also enriched in Laminin γ1 positive vesicles, where it co-localized with FREM1-FLAG (Fig. 1C, see white box). This indicates FREM1 and PDGFC can co-localize intracellularly, as well as in ECM components in the absence of a basement membrane in vitro.
To test for a physical interaction between FREM1 and PDGFC, HEK293 cells were transfected with constructs full-length FREM1-FLAG and PDGFC-V5 or empty vector controls. Immunoprecipitation using a mouse V5 antibody revealed the presence of FREM1-FLAG in the PDGFC-V5 immunoprecipitate, but not in the V5 control pull-down (Fig. 1D). FREM1-FLAG and PDGFC-V5 ran as a 260-kDa and 55-kDa species respectively as demonstrated in earlier studies (Li et al., 2000; Kiyozumi et al., 2006). To determine which domain mediates binding to PDGFC, MYC-tagged FREM1 sub-domains (outlined in Fig. 1A) were co-expressed with PDGFC-V5. Interactions with NV, CSPG, NV-CSPG, and CalXβ-C-lectin were identified (Fig. 1E,F). We consistently observed a ~75kDa band in the NV samples suggesting potential dimerization of the NV domain. Using the rabbit anti-FREM1 polyclonal antibody, we performed reciprocal experiments to pull down full-length FREM1, CSPG or NV-CSPG domains and detected PDGFC in all immunoprecipitates except the empty vector (negative control) (Fig. 1E). To confirm the physiological significance of these interactions we co-immunoprecipitated FREM1 and PDGFC from embryonic protein extracts (Fig. 1G). Co-immunoprecipitation experiments using the unrelated microtubule-associated E3 ubiquitin ligase MID1, tagged with GFP (Short and Cox, 2006) were undertaken as negative controls (data not shown). Collectively this data demonstrates that FREM1 and PDGFC can co-localize and physically interact through multiple domains, in a physiologically relevant manner.

Full-length PDGFC exists in a latent form from which the N-terminal CUB domain is cleaved by plasmin or tissue plasminogen activator (tPA) to release the catalytic growth factor domain (GFD) (Li et al., 2000; Fredriksson et al., 2004). To explore how FREM1 might affect PDGFC further, we next examined processing of PDGFC. However, conditioned media from cells co-expressing tPA, PDGFC-V5 and FREM1-FLAG did not have increased levels of GFD (data not shown), suggesting FREM1 does not affect processing. As FREM1
influences the cellular response to the mature processed PDGFC (the homodimer of PDGFC) when supplied in recombinant form (see below), we propose that principal functional interaction between the proteins occurs after processing and dimerization.

**FREM1 augments PDGFC signalling through PDGFRα.**

The interaction of NG2 and PDGFA is thought to regulate signalling downstream of PDGFRα (Grako and Stallcup, 1995). To investigate the significance of FREM1/PDGFC interactions in a similar context, we profiled analogous receptor activation. Mouse embryonic fibroblasts (MEFs) form an attractive *in vitro* model as they originate from mesenchyme, secrete ECM and express endogenous FREM1 and PDGFRα, analogous to dermal fibroblasts. Binding of PDGFC to PDGFRα stimulates auto-phosphorylation of the receptor which leads to signalling through PI3-kinase and MAPK activation pathways. Whilst stimulation of serum starved MEFs derived from either *Frem1*+/+ or *Frem1*bat/bat embryos for 10 minutes with 100 ng/ml PDGFC resulted in upregulation of AKT phosphorylation, this was dramatically reduced in *Frem1*bat/bat cells at 10, 60 and 120 minute time points (Fig. 2A,B). This difference was also reflected in ERK1/2 activation. Similar experiments using epidermal growth factor (EGF) as an agonist did not result in any differences in AKT or ERK1/2 phosphorylation between different genotypes (results not shown).

We wondered if the overall decrease in AKT activation upon PDGFC stimulation in *Frem1*bat/bat cells is due to impaired PDGFRα activation. MEFs were therefore stimulated with PDGFC for 60 minutes and the levels of PDGFRα phosphorylation analysed by first immunoprecipitating total PDGFRα then immunoblotting with a phospho-specific antibody. We observed a reduction in phosphorylated PDGFRα in PDGFC-stimulated *Frem1*bat/bat MEFs compared to WT cells when adjusted to total PDGFRα levels (Fig. 2C). To determine
where the PDGFc and FREM1 interaction could potentially regulate a PDGF signal, paraffin head skin tissue sections from WT E13.5 embryos were immunostained for PDGFc and PDGFRα (Fig. 2D). PDGFRα was detected in dermal fibroblasts and was strongest on their outer surface, providing for overlap with PDGFc in the dermis. As FREM1 and PDGFc also co-localize here, the interaction might influence PDGFRα signalling globally throughout the dermis. However, the dermal fibroblasts immediately underlying the basement membrane are those most exposed to PDGFCC produced in the epidermis and hence are the most likely to be influenced by the association of FREM1 and PDGFc. The enrichment of all factors on the outside or outer surface of fibroblasts suggests that FREM1 may be involved in facilitating the presentation of PDGFc to PDGFRα.

**FREM1 regulates Timp1 transcription downstream of PDGFCC through a mechanism dependent on PDGFRα, PI3K and MAPK activation.**

In addition to its role as a mitogen, numerous studies suggest PDGFc controls the composition of the ECM in a number of developmental and disease contexts. In particular, in human dermal fibroblasts PDGFCC stimulation induces the synthesis and secretion of tissue inhibitor of metalloproteinase-1 (TIMP1) (Jinnin et al., 2005), which is a key regulator of ECM processing and of basement membrane composition (reviewed in (Singer and Clark, 1999)). To investigate whether the interaction between FREM1 and PDGFRα might also play a role in regulating TIMP1 expression, MEFs were serum starved overnight and incubated with PDGFCC as previously described. Profiling Timp1 expression in these cells showed that the gene is up-regulated by 60 minutes in wild-type cells, but that the levels in _Frem1^bat/bat_ cells were essentially unchanged in response to PDGFCC (Fig. 3A). Increased phosphorylation of AKT and ERK1/2 50 minutes after exposure to growth factor (Fig. 3B) preceded increases in _Timp1_ gene expression and secreted protein levels at 60 and 120 min.
respectively, in WT but not \textit{Frem1}{}\textsuperscript{bat/bat} MEFs (Fig. 3B,C). The mechanism by which PDGFCC regulates \textit{Timp1} transcription in the dermis is unclear. Studies using EGF as an agonist showed that secretion of both TIMP1 and MMP9 in trophoblasts was downstream of the PI3-kinase and MAPK pathways (Qiu et al., 2004). To investigate this process, WT MEFs were pre-treated with the PI3-kinase inhibitor LY294002, AKT1/2 inhibitor or the MAPK inhibitor UO126 prior to PDGFCC stimulation (Fig. 3D). Treatment of cells with LY294002 or AKT1/2 inhibitor abolished AKT phosphorylation, whilst UO126-treated cells remained unchanged. Phosphorylation of ERK1/2 was ablated in UO126-treated cells, but remained unaffected in LY294002 and AKT1/2 inhibitor-treated groups. Analysis of \textit{Timp1} mRNA expression by qRT-PCR found that inhibition of the PI3-kinase/AKT or MAPK pathways by these compounds blocked \textit{Timp1} mRNA transcription upon PDGFCC stimulation (Fig. 3E). Taken together, these results indicate that a consequence of FREM1 mutation in the developing embryo is a reduction in TIMP1 activation downstream of the PDGF receptor in a pathway mediated by PI3-kinase/AKT and MAPK.

COLLAGEN I (COL1) is a fibrillar collagen present in the dermis and basement membrane and its skin deposition was most recently shown to be specifically regulated by TIMP1 activity (Yokose et al., 2012). In the absence of a reliable antibody to directly detect murine TIMP1, as a proxy for TIMP1 activity \textit{in vivo}, we examined basement membrane COL1 deposition in fetal head skin expression in mice at E13.5, with an antibody to native COL1 (Fig. 3F). We noted firstly that at this developmental stage there were significant amounts of COL1 in the basement membrane (in addition to general dermal and epidermal expression) and that FREM1 mutation leads to a specific reduction in presence of the protein at this site. We quantified the fluorescence intensity of COL1 in the epidermis over Keratin 14 (K14) and this confirmed a significant reduction in COL1 in \textit{Frem1}{}\textsuperscript{bat} mutants (Figure 3G). On the basis of our \textit{in vitro} and \textit{in vivo} studies we propose that the loss of FREM1 has a
dual impact on the basement membrane. Firstly, ablation of FREM1 results in a destabilised FRAS/FREM complex, thereby removing a physical cross-link. However, secondly, the loss of FREM1 has a knock on effect of reducing PDGFRα signal transduction, which lowers expression of Timp1 and thereby permits MMP-mediated erosion of COLLAGEN I and potentially other ECM and BM components.
Discussion

A signature feature of Fraser Syndrome and its related diseases is cryptophthalmia, which is thought to arise as a consequence of the formation of large epidermal blisters during embryonic development. While the identity of the *FRAS* and *FREM* genes mutated in these conditions have been known for many years, the mechanisms by which they mediate epidermal blistering remain unclear. Studies of the blebs mice suggest that the proteins form a mutually stabilising complex which acts at the interface between the basement membrane lamina densa and its underlying dermis that appears to be critical for epidermal consolidation.

However, our findings suggest that the importance of this complex and the presence of its individual components extend far beyond a simple physical stabilisation. In particular, when considered as a complex, the FREM and FRAS proteins are comprised of nearly 100 recognisable and highly post-translationally modified domains, many of which might be expected to interact with other molecules in the extracellular milieu. Our studies indicate that PDGFCC is one such protein.

During cutaneous development PDGF, produced in the epidermis, signals to PDGFRα in the underlying cells of the dermis. We provide evidence that FREM1 binds PDGF both in culture and in developing embryos (Fig. 1D-G) and co-localizes with PDGF in close proximity to PDGFRα in dermal ECM and the basement membrane (Fig. 1B, 2D, modelled in 4A). Each domain of FREM1 can separately interact with PDGF, however full length FREM1 does so with the strongest affinity (Fig. 1F,G). The multiple domain interaction of FREM1 to PDGF is reminiscent of the interaction of PDGF with other ECM components. In the case of NG2 and PDGFA for example, both the CSPG-containing domain 2 and juxtamembrane domain 3 can bind to PDGFA (Goretzki et al., 1999). Similarly, multiple PDGF binding sites contribute to its interactions with HSPG-2/perlecan (Gohring et al., 1998; Goretzki et al., 1999). The presence of CSPG domains in
other basement membrane proteins implies that they may act in a similar fashion to FREM1 and warrants further investigation. This may also explain why no changes in PDGFC distribution were observed in bat embryos (Fig. 1B) despite FREM1 mutation.

Our study has demonstrated that the FREM1/PDGFC interaction regulates downstream signalling through PDGFRα to promote TIMP1 expression and thus regulate ECM remodelling. Interestingly, TIMP1 activity regulates COL1 deposition in the skin (Yokose et al., 2012) and transgenic mice over-expressing PDGFC in the liver develop fibrosis with increased COL1 content (Lai et al., 2011), while PDGFC and PDGFRα knockout mice develop skin blistering (Soriano, 1997; Tallquist and Soriano, 2003). Here we demonstrate FREM1 bat mutants also exhibit reduced TIMP1 expression and lowered basement membrane COL1 deposition (Fig. 3). We cannot completely exclude the possibility that COL1 changes in bat mutants reflects a non-specific reduction of ECM molecules as a consequence of FRAS1/FREM-complex disruption, however the reduced COL1 deposition observed is consistent with the associations between COL1 and both PDGFC and TIMP1 reported in the literature (Lai et al., 2011; Yokose et al., 2012). Thus we propose FREM1 mutation alters its association with PDGFC, alters the cascade of downstream PDGFRα signalling events, changing the expression profile of proteases and inhibitors (such as TIMP1) to aberrantly impact ECM deposition.

How this interaction more broadly shapes receptor activation remains to be determined, however we can rule out influences on PDGFC’s proteolytic processing and dimerization. As this interaction occurs on an ECM scaffold in close proximity of PDGFRα, perhaps wild type FREM1 facilitates the presentation of PDGFC to PDGFRα on the surface of dermal fibroblasts contacting the ECM and in particular, contacting the basement membrane. This model is consistent with our core findings that FREM1 is not absolutely required for PDGFC signalling but instead alters the amplitude and duration. This study
provides an additional mechanistic insight into how FREM1 regulates adhesion of the developing epidermis and identifies a key interaction with the PDGFC growth factor. We therefore propose that FREM1 mutation contributes to basement membrane fragility and blistering, in part via reduced PDGFC signalling leading to impaired ECM deposition.

**Materials and methods.**

*Expression constructs.*

Truncated *Frem1* constructs expressing the NV, NV-CSPG, CSPG and CalXβ-Lectin domains were amplified from a full length *Frem1* cDNA (*pcDNA3.1-Frem1-Flag* a gift from Kiyotoshi Sekiguchi, Osaka University, Japan (Kiyozumi et al., 2006)), subcloned into *pENTR/D-TOPO* and recombined into *pcDNA-DEST40* (Invitrogen, Carlsbad, CA, USA) which had been modified to incorporate a signal sequence from mouse Igκ chain and a triple MYC tag at the N-terminus. A mouse *PdgfC* cDNA (Gift from David Loebel, CMRI, Australia) cloned into *pENTR/D-TOPO* and recombined into *pcDNA-Dest40* generated a C-terminal V5 fusion protein.

*Cell culture and transfection.*

NIH3T3 fibroblasts (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% foetal calf serum, 100 units/ml penicillin and 0.1% streptomycin and passaged before reaching confluence. NIH3T3 transfection was performed using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). Briefly, 1 x 10^5 fibroblasts were seeded onto 22x22 mm Collagen I-coated, 1% BSA blocked coverslips in six-well plates, cultured for 24-48 hours, then each well transfected with a total of 2.5μg of plasmid DNA (prepared with Qiagen plasmid purification kits), 10μl of lipofectamine LTX and 2.5μl of Plus reagent. Wells were provided with fresh media and transfection mix prepared in 0.5ml Optimem was added with
gentle mixing. After 4 hours, wells were washed once with PBS and media replaced. 48 hrs after transfection, fibroblasts were harvested for immunofluorescence analysis.

HEK293 cells were transfected using Fugene HD reagent (Roche, Indianapolis, IN, USA) according to manufacturer’s instructions. Primary mouse embryonic fibroblasts (MEFs) were harvested from E13.5 embryos as previously described (Ivetac et al., 2005) and maintained to a maximum of 5 passages. Prior to growth factor stimulation, cells were serum starved overnight. PDGFCC stimulation was performed with 100ng/ml recombinant protein (R&D Systems, Minneapolis, MN, USA).

**Antibodies to FREM1.**

Rat monoclonal (clone 17A6) and rabbit polyclonal antibodies to FREM1 were raised against amino acid positions 1500-1637 corresponding to most of the CSPG repeat 11 and a few residues into repeat 12, as described previously (Petrou et al., 2007) using the Monash Antibody Technology Facility (MATF) and Millipore Custom Antibody production facility (Millipore, Temecula, CA, USA), respectively. Both antibodies were validated by western blotting.

**Additional antibodies.**

Antibodies used and application (Immunofluorescence –IF, Immunoprecipitation -IP, Western Blotting –WB); rabbit anti-total AKT #9272 -WB, rabbit anti-pAKT (Ser473) 193H12 #4058 -WB, rabbit anti-pAKT (Thr308) #9275 -WB, rabbit anti-DYKDDDDDK (FLAG-tag) #2368 –IF, rabbit anti-p44/42 MAPK (ERK1/2) 137F5 #4695 -WB, rabbit anti-p-p44/42 MAPK (ERK1/2) D13.14.4E XP #4370 –WB, rabbit anti-MYC-tag 71D10 #2278 -IF, mouse anti-MYC-tag 9B11 #2276 –WB, rat non-Immune IgG bs-0293P-IF (Bioss Inc, Woburn, MA, USA), rabbit anti-PDGFRα D1E1E #3174 -IF/IP/WB, mouse anti-Phospho-Y
Immunoprecipitation (IP).

For immunoprecipitation experiments, supernatants from HEK293 cell lysates were pre-cleared by incubating with protein A or G-sepharose, incubated with primary antibody overnight and pulled down by the addition of 25 μl 50% slurry of Protein A sepharose or protein G sepharose. Washed beads were analyzed by SDS-PAGE and Western immunoblotting. PDGFRα was immunoprecipitated (Cell Signalling Technology, Beverley, MA, USA) in cells harvested in 20 mM Tris pH8.0, 100 mM EDTA, 1% Triton X-100, 1 mM Na3VO4, and protease inhibitors. Endogenous FREM1 was immunoprecipitated using rabbit anti-FREM1 or pre-immune serum from 2mg of total embryo protein lysate (at E12.5).

Western blotting (WB).

Phospho-antibody blots used antibodies at 1:1000 and were stripped using Gentle Review Buffer (Amresco, Solon, OH, USA) prior to incubation with total-AKT or total-ERK1/2 antibodies, also at 1:1000. For phospho-AKT densitometry analysis, films were
scanned and relative signal intensities of phosphorylated AKT levels (normalised over total AKT) from WT MEFs stimulated for 10 minutes with PDGFCC were defined as 1, and all remaining samples standardised relative to this value.

For AKT and MAPK inhibition experiments, serum-starved cells were pre-treated with 50 μM inhibitors of PI3-kinase (LY294002, Sigma-Aldrich, St Louis, MO, USA), MAPK (UO126, Cell Signalling Technology, Beverly, MA, USA), AKT1/2 (AKT1/2 kinase inhibitor, Sigma-Aldrich, St Louis, MO, USA), or equivalent amount of vehicle control DMSO for 1 hour at 37°C, prior to PDGFCC stimulation for 1 hour at 37°C. For phospho-AKT densitometry analysis, relative signal intensities of phosphorylated AKT levels (normalised over total AKT) from unstimulated WT MEFs were defined as 1, and all remaining samples standardised relative to this value.

For TIMP1 secretion analysis, media samples were concentrated 10-fold through a 10-kDa MWCO Nanosep columns (PALL Corporation, Cheltenham, VIC, Australia) and immunoblotted using goat TIMP1 antibody at 1:1000.

**Immunofluorescence (IF).**

Cells grown on coverslips were fixed, permeabilised, blocked and incubated with the nuclear stain 40-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA) and antibodies indicated following the protocol previously described for C2C12 myoblasts (Cottle et al., 2007). Embryonic tissues for paraffin sections were fixed in 4% paraformaldehyde, with standard xylene dewaxing and immunostained as described in (Cottle et al., 2013), except antigen retrieval was performed with a Dako PT Link according to manufacturer’s protocol. Staining was performed in a modified blocking buffer consisting of 3% BSA in PBS with 1 drop of fish scale gelatin per 20ml. Due to low titre, fluorescent FREM1 detection in paraffin sections using rat monoclonal antibody required tertiary signal
amplification first using rabbit anti-rat-AlexaFluor488 followed by donkey anti-rabbit-AlexaFluor488 and donkey anti-rat- AlexaFluor488. Non-Immune rat IgG staining was performed in parallel and confirmed rat anti-FREM1 staining was distinctive. All Fluorescent images were acquired on an Olympus Fluoroview 500 confocal scanning microscope and processed using ImageJ.

**Quantitative real time-PCR (qRT-PCR).**

RNA was extracted from MEF samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and qRT-PCR performed using the TIMP1 primer set 5’caactggacctgtcataa-3’ and 5’ acagagctttcatgactg-3’ and Power SYBR green PCR master mix (Applied Biosystems, Mulgrave, VIC, Australia). Triplicate samples were run through a standard 2-step reaction using a Stratagene Mx3000 qPCR machine (Agilent Technologies, Santa Clara, CA, USA).

**Mice.**

All studies were performed using the *Frem1<sup>bat</sup> “bat”* mouse allele (Smyth et al., 2004) in accordance with the regulatory statutes set out by the Monash University Animal Welfare Committee and Legislation of the Australian and Victorian governments relating to the use of experimental animals. A minimum of 3 mice per genotype were analysed.

**Statistical Analysis.**

All statistical analysis was performed using the student’s t-test. P values of less than 0.05 were considered significant.
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Competing Interests Statement

The authors declare no competing interests

Author contributions

F.W., D.L.C. and L.J. performed the experiments, F.W. and I.S. conceived and designed the experiments and analysed the data, I.S., D.L.C. and F.W. wrote the paper.

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References


Figure Legends.

Figure 1. Interaction between FREM1 and PDGFC

A) Structural representations of the full-length Frem1 and truncated constructs; NV alone, NV-CSPG, CSPG and CalXβ-C-Lectin domains with the tags indicated. The MYC tag shown refers to a 3xMYC tag + Igκ secretion signal. B) E13.5, WT and bat embryo head skin sections stained for FREM1 (Green), PDGFC (Red), and nuclear dye DAPI (Blue). C) NIH3T3 fibroblasts expressing FREM1-FLAG and PDGFC-V5 and immunostained as indicated. D) Co-immunoprecipitation of FREM1-FLAG and PDGFC-V5 in transfected HEK293 cells. E,F) Co-immunoprecipitation of PDGFC-V5 with MYC-tagged FREM1 sub-domains. G) Co-immunoprecipitation of endogenous FREM1 and PDGFC from embryo extracts at E12.5. A rabbit pre-immune serum was included as a control. * IgG heavy chain, IP = immunoprecipitation antibody, WB = Western blotting antibody. Scalebars as indicated.

Figure 2. FREM1 regulates activation of AKT and MAPK upon PDGFC stimulation.

A) Representative western blotting of phosphorylation of AKT and MAPK ERK1/2 in mouse embryonic fibroblasts from WT and bat mouse embryos stimulated with PDGFCC for the indicated time periods. B) Relative quantification of AKT phosphorylation levels. WT cells 10 minutes after stimulation were assigned a value of 1 and all other samples are standardized against this value. Graph represents average of up to 9 WT and 16 bat samples, performed across 4 independent experiments from at least 3 different cell lines for each genotype, black bars: WT, white bars: bat mutant. C). FREM1 mutation in bat mutation, reduces phosphorylation of PDGFRα in response to the addition of exogenous PDGFCC. IP = immunoprecipitation antibody, WB = Western blotting antibody. D) E13.5, WT embryo head skin sections stained for PDGFC (Green), PDGFRα (Red), and nuclear dye DAPI (Blue). Errorbars represent standard error of the mean (s.e.m.), *p<0.05, **p<0.01, ***p<0.005. Scalebars as indicated.
Figure 3. FREM1 regulates Timp1 transcription downstream of PDGFCC, dependent on PDGFRα, PI3K and MAPK activation.

A) qRT-PCR analysis of Timp1 mRNA levels in WT and bat mouse embryonic fibroblasts following PDGFCC stimulation at the indicated time points. Data was obtained from four independent experiments from at least 3 different cell lines for each genotype, and presented as fold-increase relative to unstimulated WT cells. black bars = WT, white bars = bat mutant.

B) Alterations in phosphorylation of AKT and ERK1/2 precede changes in Timp1 expression.

C) TIMP1 protein secretion is reduced in stimulated FREM1 bat mutant MEF cultures.

D) WT cells were pre-treated with either the PI 3-kinase inhibitor LY294002, AKT1/2 inhibitor, MAPK inhibitor U0126 or DMSO vehicle control prior to PDGFCC stimulation and analysed by immunoblotting with anti-phospho-AKT, anti-phospho-ERK1/2, total AKT or total ERK1/2. E) qRT-PCR for Timp1 mRNA was performed on the same cells. Levels of Timp1 mRNA were presented as a fold-increase relative to unstimulated cells. Experiments were performed three times using three different cell lines.

F) E13.5, WT and bat embryo head skin sections stained for Collagen Type 1 (COL1-Green), Keratin 14 (K14-Red), and nuclear dye DAPI (Blue). Errorbars represent s.e.m., *p<0.05, **p<0.01. Scalebars as indicated.

Figure 4. Summary model of FREM1 bat mutant basement membrane fragility.

A) In WT mice, FREM1 forms a stabilising complex (1) with FRAS/FREM proteins to cross-link the Lamina Densa with the underlaying dermis. Additionally, FREM1 binds keratinocyte secreted PDGFC, which is presented to PDGFRα in the adjacent dermal fibroblasts, potentiating PDGFRα signalling and promoting ECM modelling events, including TIMP1 upregulation and Collagen Type 1 deposition (2). In bat mice, FREM1 mutation removes the
structural cross-link of the FRAS/FREM complex (1), but also reduces PDGFRα signalling, leading to lowered TIMP1 and diminished Collagen Type 1 deposition (2), thereby further weakening part of the foundation of the basement membrane.
Translational Impact

(1) **Clinical Issue:** Fraser Syndrome and its related diseases are caused by mutations in members of the FRAS and FREM extracellular matrix proteins. These molecules localise to the basement membrane of many different developing epithelia and their mutation in FS patients (or in the blebs family of mouse mutants which phenocopy the disorder) leads to defects in epidermal adhesion and the formation of large blisters *in utero*. These blisters are thought to contribute to the formation of a number of craniofacial and soft tissue malformations, principal amongst which is cryptophthalmia, in which skin covers the globe of the eye.

(2) **Results:** The FRAS and FREM proteins are thought to function collectively and cooperatively to structurally cross-link components of the basement membrane. Wiradjaja et al, provide some of the first evidence that these proteins bind to other factors in the extracellular milieu. They show that FREM1 binds to PDGFC and potentiates signalling through its receptor, PDGFRα. This signalling controls expression of the matrix metalloproteinase inhibitor, TIMP1, which in turns plays an active developmental role in regulating ECM deposition, including collagen I, part of the foundation of the basement membrane.

(3) **Implications and future directions:** In establishing a link between FREM1, PDGFC growth factor activity and the downstream regulation of molecules which themselves shape extracellular matrix remodelling, Wiradjaja et al propose a novel mechanism for the development of disease in Fraser Syndrome and its related diseases and in the blebs mouse models which model these conditions. Further studies that expand on these findings focussed on the interaction of these critical proteins and other ECM components will lead to a better understanding of these diseases.
**A**

<table>
<thead>
<tr>
<th>Frem1 +/+</th>
<th>Frem1 bat/bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- p-AKT
- Total AKT
- p-ERK1/2
- Total ERK1/2

**B**

<table>
<thead>
<tr>
<th>PDGFCC stimulation (mins)</th>
<th>0</th>
<th>10</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT phosphorylation (arbitrary units)</td>
<td>1.2</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*** P < 0.0001
** P < 0.01
* P < 0.05

**C**

- IP: anti-PDGFRA
- WB: anti-phospho-Y

- IP: anti-PDGFRA
- WB: anti-PDGFRA

**D**

- PDGFC
- PDGFRα
- DAPI
- Merged

Scale bar: 50 µm
A: Graph showing the fold-increase of Timp1 mRNA relative to unstimulated wild-type.

B: Western blot images showing expression levels of Akt and Erk1/2 under different conditions.

C: Graph showing the fold-increase in Timp1 mRNA under different conditions.

D: Western blot images showing the effects of various treatments on p-Akt, Akt, p-Erk1/2, and Erk1/2.

E: Bar graph showing quantitative data from the Western blots.

F: Confocal microscopy images of DAPI and COL1 staining in different groups.

G: Bar graph showing relative collagen 1 basement membrane intensity between Frem1+/- and Frem1 bat/bat.
A Baseline Membrane

Frem1 +/+ vs. Frem1 bat/bat

- 1. Reduced TIMP1
- 2. Reduced COLLAGEN1