A direct functional link between the multi-PDZ domain protein GRIP1 and the Fraser syndrome protein Fras1

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Cell adhesion to extracellular matrix (ECM) proteins is crucial for the structural integrity of tissues and epithelial-mesenchymal interactions mediating organ morphogenesis^{1,2}. Here we describe how the loss of a cytoplasmic multi-PDZ scaffolding protein, glutamate receptor interacting protein 1 (GRIP1), leads to the formation of subepidermal hemorrhagic blisters, renal agenesis, syndactyly or polydactyly and permanent fusion of eyelids (cryptophthalmos). Similar malformations are characteristic of individuals with Fraser syndrome and animal models of this human genetic disorder, such as mice carrying the blebbed mutation (*bl*) in the gene encoding the Fras1 ECM protein^{3,4}. GRIP1 can physically interact with Fras1 and is required for the localization of Fras1

to the basal side of cells. In one animal model of Fraser syndrome, the eye-blebs (*eb*) mouse, *Grip1* is disrupted by a deletion of two coding exons. Our data indicate that GRIP1 is required for normal cell-matrix interactions during early embryonic development and that inactivation of *Grip1* causes Fraser syndrome-like defects in mice.

The glutamate receptor interacting proteins GRIP1 and GRIP2 form a small family of cytoplasmic proteins first isolated as interactors of AMPA-type glutamate receptors⁵. Both contain seven PDZ domains, modules of 80–90 amino acids that mediate heterophilic and homophilic protein-protein binding, which typically recognize short peptide sequences at the C terminus of their interaction partners.

Figure 1 *Grip1* mutants phenocopy mice lacking the Fraser syndrome protein Fras1. (a–c) Prominent blebs (arrows) formed over the heads and limbs of E12.5 (a) and E13.5 (b,c) embryos. (d) Transverse section of an E13.5 *Grip1*^{−/−} head with hemorrhaging in a subset of blisters. (e,f) Deformation of embryonic *Grip1*^{−/−} limbs. Epidermal detachment and hemorrhaging in a section through a distal E13.5 forelimb (e) and appearance of variable polydactylous or syndactylous malformations at E16.5 (f). (g,h) Surviving *Grip1* and *Grip1Grip2* mutants showed unilateral cryptophthalmos (arrows), whereas *Grip2* null mice (h) appeared normal. WT, wild-type. (i) Adult *Grip1Grip2* double mutant with syndactylous fusion of digits in the hindlimb.

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Although the biological roles of multi-PDZ proteins are poorly understood, they might control the assembly of signaling complexes and the surface presentation and trafficking of transmembrane proteins^{6–8}. Consistent with this function, GRIP1 associates with the kinesin heavy chain and is involved in synaptic vesicle transport by steering motor proteins to their targets⁹. To study the *in vivo* roles of GRIP1 and GRIP2, we inactivated the corresponding genes in mice (Supplementary Fig. 1 online). Disruption of *Grip1* led to detachment of epithelia in the oral and nasal cavities, formation of large subepidermal hemorrhagic blisters and lethality at midgestation that was similar to observations reported for an independently generated *Grip1* knockout¹⁰ (Fig. 1a–e). Blisters formed underneath the epidermal basement membrane of head, limb and back skin from around embryonic day (E) 11.0 and were initially transparent (Fig. 1a). At later stages of embryonic development, blisters became increasingly hemorrhagic (Fig. 1b–e), and most homozygotes in the original mixed 129 C56Bl/6 background died around E13.5. *Grip1*^{−/−} mice on the C57Bl/6 background, however, developed to term, and this enabled us to study several aspects of the mutant phenotype. The blistered skin recovered during late gestation and only few small hemorrhagic blisters were visible at E16.5. Mutant hindlimbs were affected by variable defects ranging from complete syndactyly of all digits to polydactyly, which suggested that normal morphogenesis of distal limbs might be compromised in areas with epidermal detachment (Fig. 1f). In contrast, *Grip2*^{−/−} mice appeared normal and were born in the expected statistical ratio (Fig. 1h and data not shown). We also obtained a few adult *Grip1* mutants (*n* = 5) and *Grip1 Grip2* double mutants (*n* = 1); these mice were devoid of externally visible blisters but showed permanent fusion of one or both eyelids (Fig. 1g,h). We observed similar fusion malformations in adult autopsies (Fig. 1i).

On examination of the internal organs of *Grip1*^{−/−} embryos, we found that kidneys were completely absent. At E13.5, only unstructured mesenchymal rudiments remained, which were devoid of metanephric tubules and contained large numbers of apoptotic cells (Fig. 2a–d). In the absence of GRIP1, even the earliest steps of metanephric development failed: mesenchymal condensations did not assemble around the ureteric bud and expression of the Wilms' tumor

antigen-1 (encoded by *Wt1*) was not induced (Fig. 2e–h). Consistent with the malformation observed in the mutants, immunofluorescence with the use of antibody to GRIP1 labeled the embryonic epidermis, eyelids, epithelia of oral and nasal cavities and ureter of wild-type mice (Fig. 3a–g).

Defects caused by the loss of GRIP1 were markedly similar to the phenotype of *Fras1*^{−/−} mice, an animal model of the human congenital disease Fraser syndrome, the symptoms of which include blistering of embryonic but not adult skin, renal agenesis and cryptophthalmos^{3,4}. To explore a possible connection between *Fras1* and GRIP1, we compared their spatial distribution patterns by double immunofluorescence and found that expression of the two proteins overlapped in many embryonic tissues, including skin, oral and nasal cavities, ureteric bud and the gastrointestinal system (Fig. 3h,i and data not shown). At higher magnification, we observed substantial colocalization of *Fras1* and GRIP1 at the basal surface of epidermal cells (Fig. 3j).

Fras1 is a putative ECM protein with a large extracellular domain, transmembrane region and a short C-terminal cytoplasmic domain^{3,4}. The primary sequence of the *Fras1* C terminus presents a good consensus for a class I PDZ domain-binding motif (Fig. 4a), raising the possibility of a direct physical interaction with GRIP1. Indeed, we were able to precipitate GRIP1 from HEK293T cells with a fusion protein that contained the *Fras1* cytoplasmic region. Mutating the C-terminal residue (V4010G) to disrupt the PDZ consensus in *Fras1* abolished GRIP1 binding (Fig. 4b). *Fras1* and the unnamed mouse protein BAC27425 share considerable sequence homology (35% identity over 755 residues), suggesting that the two gene products might be a part of the same family. Like *Fras1*, BAC27425 contains a transmembrane region and a short cytoplasmic domain with PDZ-binding motif, which interacts with GRIP1 (Fig. 4a,b). We found that both *Fras1* and BAC27425 can also bind GRIP2 in a PDZ-dependent mode (Fig. 4d). PDZ domains 1–3, but not domains 4–7, of GRIP1 or GRIP2 were sufficient for *Fras1* binding (Fig. 4c,e). Although the biological role of BAC27425, functional similarities to *Fras1* and the question of its relevance for Fraser syndrome have not yet been addressed, our results suggest that both gene products might be connected to GRIP proteins in an analogous fashion.

Figure 2 Loss of *Grip1* leads to renal agenesis. (a,b) Kidneys were absent in E17.5 *Grip1*-deficient embryos, whereas adrenal glands (A) were of normal size. (c,d) Only rudimentary and unstructured metanephric condensations (arrow) with numerous apoptotic cells, as shown by TUNEL staining (red nuclei in inset; all nuclei are stained blue by DAPI), were visible in transverse sections through the E12.5 mutant abdomen (d). Kidneys of control littermates showed extensive epithelial branching and were devoid of apoptotic cells (c). (e–h) Early steps of kidney morphogenesis, such as the formation of mesenchymal condensations around the distal ureter (arrowheads in e,f) and induction of *Wt1* expression (g,h) at E11.5, require *Grip1*. *Grip1*^{−/−} distal ureters are indicated by arrows (f,h).

Consistent with the protein-protein interaction described above and the overlapping expression patterns *in vivo*, GRIP1 and Fras1 colocalized in cytoplasmic vesicular structures of cultured keratinocytes (Fig. 4f–h), indicating that a direct link between the two gene products might be responsible for similar loss-of-function phenotypes.

This was further confirmed by the immunofluorescence on tissue sections from *Grip1*^{−/−} embryos. In normal epidermis, the Fras1 immunofluorescence signal is concentrated at the basal side of keratinocytes and overlaps with ECM proteins, such as laminin- γ 1. Loss of GRIP1 resulted in reduced Fras1 expression and abnormal retention of the protein in the keratinocyte layer (Fig. 5a,b). The defective localization of Fras1 did not affect basement membrane integrity in general, because lamina lucida and densa were visible by electron microscopy (Supplementary Fig. 2 online), and neither did it affect the expression of the ECM proteins collagen IV, laminin- α 1, - α 2, - α 5, - β 1, - γ 1 or - γ 2 (Fig. 5b and data not shown). A central region of Fras1 shows strong homology to the collagen V and VI-binding domain of the membrane-spanning NG2/AN2 chondroitin sulphate proteoglycan (CSPG)^{3,4,11}, which can also interact with GRIP1 through its C-terminal PDZ-binding motif¹².

Considering the potential relevance of this connection, we analyzed the pattern of NG2 expression in embryonic skin. Whereas NG2 was concentrated at the epidermal-dermal junction in normal E13.5 embryos, this accumulation was markedly reduced in *Grip1* knockouts (Fig. 5c,d). The absence of GRIP1 had similar effects on the deposition of collagens V and VI, substrates for NG2 and possibly Fras1, so that the accumulation of both matrix proteins was compromised in the mutant basement membrane region (Fig. 5e,f). We observed comparable changes to expression of Fras1, NG2 and collagens V and VI in other tissues of *Grip1*^{−/−} embryos, which indicated that defective deposition of these molecules is a common factor in areas with disrupted epithelial attachment (Supplementary Fig. 3 online). These findings indicated that GRIP1 activity is necessary for correct Fras1 presentation, which, in turn, might be required for the recruitment of NG2 CSPG and collagens V and VI from the dermis to the basement membrane region. This is also consistent with the accumulation defects seen for collagen VI in *Fras1*-knockout embryos⁴ and for NG2 in *bl* mutants (data not shown). An important role of dermis-derived structural proteins is further supported by the occurrence of blisters at the dermis–basement membrane junction in both *Grip1* (Fig. 5m and Supplementary Fig. 2 online) and *Fras1* knockouts^{3,4}. During later embryonic development, skin of *Grip1* mutants showed an almost complete recovery, leading to the disappearance of blisters in a manner similar to that reported for surviving *Fras1*^{−/−} mice. Our analysis showed that the skin of *Grip1*^{−/−} embryos improved although Fras1 localization remained compromised. In contrast, the accumulation of NG2 and collagens V and VI was largely restored by E17.5 (Supplementary Fig. 4 online). Hence intact CSPG-matrix interactions might be sufficient for adhesion at the basement

membrane–mesenchymal interface, whereas the role of GRIP1 and Fras1 seems to be transient but crucial.

We next examined the distribution of endogenous Fras1 protein in cultured embryonic keratinocytes. Confocal microscopy showed Fras1 immunofluorescence in perinuclear vesicular structures in the apical region of both control and *Grip1*^{−/−} cells (Fig. 5g,j). In basal sections Fras1 was widely distributed, extending almost to the edge of wild-type keratinocytes; in contrast, this peripheral staining was markedly reduced in the absence of GRIP1 (Fig. 5h,i,k,l). Thus, the observed alterations of Fras1 localization in GRIP1-deficient skin and cultured keratinocytes are consistent with an essential role for the multi-PDZ domain protein in trafficking Fras1 to the basal side of cells.

The phenotype of *Grip1* mutants resembles the dystrophic form of epidermolysis bullosa (DEB), a human blistering disease with skin rupturing that results from defects in the gene encoding collagen VII (COL7A1) that affect the anchoring fibrils that link dermis and basement membrane¹⁰. Mice with a targeted inactivation of *Col7a1*, an animal model of DEB, develop skin blisters postnatally in areas exposed to mechanical stress¹³ in a manner that is distinct from GRIP1-deficient embryos. Although collagen VII deposition was compromised in some regions of early *Grip1*^{−/−} embryos, perhaps due to the disrupted adhesive interactions at the basement membrane–dermal interface described above, anchoring fibrils were formed in E17.5 skin (Supplementary Fig. 2 online), arguing against a direct connection with DEB.

Figure 3 The tissue distributions of GRIP1 and Fras1 largely overlap. (a–d) Cryosections of E13.5 skin stained with antibodies to GRIP1. Signals are prominently seen in developing hair follicles (arrow in a), the basal region of interfollicular keratinocytes (b) and in the eyelid (Ey) area near the lens (Le; c). (d) Absence of GRIP1 immunofluorescence in *Grip1*^{−/−} skin (KO; arrowhead). (e–g) GRIP1 staining in the basal oral epithelium of wild-type (e) but not knockout (KO; arrowheads in f) E13.5 embryos. (g) Consistent with a role in early metanephric development, GRIP1 is expressed in the E11.5 ureter. (h–j) GRIP1 (green) and Fras1 (red) signals overlap in E13.5 epidermis (h) and oral epithelium (i). Higher resolution shows colocalization of the GRIP1 and Fras1 signals at the epidermal-dermal interface (j). Epidermis (Ep) and dermis (De) are indicated. Cell nuclei are stained by DAPI (blue).

In contrast, our results indicate that GRIP1 might have a role in Fraser syndrome: GRIP multi-PDZ proteins and Fras1 directly interact and GRIP1 is essential for normal Fras1 localization. *Grip1*^{-/-} embryos and adult survivors show phenotypes that are similar to a number of mutant mouse strains with embryonic blistering (often described as 'blebs'), which have been proposed to be animal models of human Fraser syndrome^{14,15}. One of these strains, called blebbed, carries a premature stop codon in *Fras1* (ref. 3). A different mutant, eye-blebs, has been mapped to mouse chromosome 10 and linked to the marker *D10Mit187* (ref. 16). We found that *D10Mit187* physically overlaps with the *Grip1* locus and that a region of *Grip1* is disrupted in eye-blebs, so that the gene lacks coding exons 10 and 11 (Fig. 5n). There are several consanguineous Fraser syndrome families with heterozygosity for markers at *Fras1*. Human GRIP1 maps to chromosome 12q14.3 and short tandem repeat polymorphisms flanking the gene could be used to assess whether any of the phenotype of these individuals might be secondary to mutations in GRIP1.

Thus, we have provided evidence that GRIP1, a cytoplasmic PDZ domain protein, mediates cell-matrix interactions through a role in the trafficking of cell surface-ECM molecules and that its activity is essential for several morphogenetic processes in the embryo.

METHODS

Generation of *Grip1* and *Grip2* mutant mice. We isolated *Grip1* and *Grip2* clones from 129 genomic phage libraries. We constructed gene targeting vectors

by inserting PGK-*neo* cassettes into *EcoRI* or *BspEI* sites in *Grip1* or *Grip2* DNA, respectively, so that exons corresponding to PDZ domain 1 were disrupted. We electroporated linearized targeting constructs into R1 embryonic stem cells¹⁷ and tested G418-resistant clones for homologous recombination by PCR and Southern-blot analysis. Blastocyst injection produced several chimeric mice, which were backcrossed to C57Bl/6 wild-type mice. The genotype was determined by Southern-blot analysis and then by PCR screening. We confirmed the absence of GRIP proteins in homozygous mutants by Western-blot analysis.

All the mice used had a mixed 129 C56Bl/6 genetic background. After six generations of backcrossing to C56Bl/6 mice, we observed that homozygous *Grip1*^{-/-} embryos survived beyond E13.5 and developed to term. We obtained *Grip2*-deficient mice with the expected mendelian ratios, indicating normal survival of these mutants.

All mouse experiments were done in compliance with the relevant laws and institutional guidelines and were approved by the Johns Hopkins University Animal Care and Use Committee and the Cancer Research UK Animal Ethics Committee.

Staining of tissue sections. We fixed freshly isolated embryos in 4% paraformaldehyde, dehydrated them, embedded them in paraffin and prepared 5- μ m sections. We then removed the paraffin from the sections and analyzed them by staining with hematoxylin and eosin. We detected apoptotic cells with the ApopTag In Situ Apoptosis Detection Kit (Oncor), according to the manufacturer's instructions.

Immunofluorescence staining was done on acetone-fixed 5- μ m cryosections with primary antibodies directed against GRIP1 (ref. 5; BD Pharmingen), Fras1 (ref. 4), NG2 (Chemicon), collagens V and VI (Rockland) and laminin- γ 1 (rat monoclonal 3E10). Secondary antibodies were coupled to Alexa Fluor-488 or Fluor-546 (Molecular Probes).

We generated the digoxigenin-labeled *Wt1* antisense RNA probe by *in vitro* transcription from a linearized DNA template and hybridized it at 70 °C overnight to pretreated 5- μ m tissue sections, which we then washed extensively and incubated with antibody to DIG-AP Fab fragment (Roche). We used NBT/BCIP substrates (Roche) to develop the signal.

Analysis of protein-protein interactions. We constructed GFP-GRIP1 by fusing green fluorescent protein (GFP) to the C terminus of rat *Grip1* cDNA. Similarly, we fused GFP with PDZ domains 1-3, 4-6 and 7. For GRIP1 and GRIP2 expression vectors, we subcloned rat *Grip1* and *Grip2* into pBK-CMV (Stratagene) and pRK-5 (BD Pharmingen), respectively.

We amplified DNA fragments encoding the C terminus of wild-type Fras1 (mFras1), Fras1 TEG mutant and BAC27425 by PCR and fused them to GST by subcloning into the pGEX-4T vector (Amersham Pharmacia Biotech).

Figure 4 PDZ domain-mediated interaction between GRIP1 and Fras1. **(a)** The C terminus of human (hFras1) and mouse Fras1 (mFras1) contains a potential PDZ binding site (shown in green). C-terminal PDZ recognition motifs also occur in the predicted mouse protein BAC27425 and in the sea urchin ECM3 protein (lvECM3). **(b)** Direct physical interaction of Fras1 and BAC27425 proteins with GFP-tagged GRIP1 *in vitro*. The Western blot shows GFP-GRIP1 protein that was precipitated from HEK293T cells with GST-Fras1 and GST-BAC27425 but not GST alone. Mutation of the C-terminal valine in Fras1 abolishes the interaction completely. A GST fusion of GluR2, a known interactor of GRIP1, was used as positive control. Input GST fusion protein levels are visualized by colloidal Coomassie blue (CBB) staining. **(c)** Fras1 binding is mediated by PDZ domains 1, 2 and 3 in the N-terminal region of GRIP1, whereas GST-Fras1 did not immunoprecipitate GFP-PDZ4,5,6 and GFP-PDZ7 proteins. Mutation of the Fras1 C terminus (TEG) abolished the interaction. Input protein levels are shown on the left side. **(d)** Fras1 and BAC27425 also show direct binding to GFP-tagged GRIP2. **(e)** The interaction between the cytoplasmic region of Fras1 and GRIP1 PDZ domains 1, 2 and 3 was confirmed in the yeast two-hybrid assay. Yeast double transformants were selected on double dropout plates (Trp⁻, Leu⁻) and tested for physical interaction of the fusion proteins by growth on triple dropout selection plates (Trp⁻, Leu⁻, His⁻). The GluR2 cytoplasmic region interacts with GRIP1 PDZ domains 4, 5 and 6. Deletion of the C-terminal TEV motif in Fras1 (Fras1 Δ 3) abolished binding to GRIP1. **(f-h)** GFP-GRIP1 (green) and antibody to Fras1 (red) signals colocalize in keratinocytes (a single cell is shown).

We produced GST fusion recombinant proteins in *Escherichia coli* strain BL21 transformed with each GST fusion construct according to standard procedures. We incubated lysates of HEK293T cells transiently transfected with rat GRIP1 or GRIP2 expression constructs with 10 μ g of GST fusion protein attached to glutathione Sepharose beads for 4 h at 4 °C. After GST immunoprecipitation, we processed the samples for Western blotting with antibodies to GRIP1, GRIP2 (ref. 5) or GFP (Molecular Probes).

We used the yeast two-hybrid system to identify the PDZ domains interacting with the C-terminal sequence of Fras1. Fragments encoding GRIP1 PDZ domains, the C terminus of Fras1, a mutant version carrying a deletion of the TEV motif (Δ 3) and the GluR2 C-terminal domain were amplified by PCR and cloned into pPC86 (GAL4 AD) or pPC97 (fusion with GAL4 DB). We cotransformed constructs into yeast strain CG1945 and grew them on double dropout medium (Trp⁻, Leu⁻) to select the double transformants. Growth on triple dropout medium (Trp⁻, Leu⁻, His⁻ + 50 mM 3-aminotriazole) indicated protein-protein interactions, that is formation of the active GAL4 transcriptional activator complex.

Fras1 localization in cultured cells. We isolated primary keratinocytes from *Grip1* knockout and wild-type embryos (E17.5) containing the temperature-sensitive (ts) A58 'immortal' allele¹⁸ and maintained them as reported for neonatal keratinocytes¹⁹. For immortalization, we cultured embryonic keratinocytes at 33 °C in the presence of IFN- γ as described¹⁸. To study the cellular localization of Fras1, we plated keratinocytes on collagen VI-coated coverslips, cultured them overnight, fixed them in acetone-methanol (1:1) and stained

them with primary antibody to Fras1 (1:80 dilution) followed by secondary antibody to rabbit Alexa Fluor-488 (Molecular Probes).

For the GRIP1 colocalization studies, we transfected keratinocytes with the GFP-GRIP1 expression vector or the control EGFP-C1 plasmid (BD Clontech) with the use of Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After 24 h, we stained the transfected cells for Fras1 with secondary antibody to rabbit Alexa-546 (Molecular Probes). The GFP-GRIP1 protein was visualized with Alexa488-conjugated antibody to GFP (Molecular Probes). We acquired optical sections (Z series) and analyzed them with an LSM510 confocal laser scanning microscope (Zeiss).

PCR analysis of the *eb* mutant *Grip1* gene. We amplified individual *Grip1* exons from genomic DNA. Primer sequences and PCR conditions are available on request. Products were directly sequenced and run on an Amersham MegaBACE1000 automated sequencer before analysis with Sequencer software (GeneCodes) or separated by electrophoresis on a 2% w/v agarose gel.

We obtained DNA from the ATEB strain segregating the *eb* mutation from the Jackson laboratories (strain number 000277). The mutation arose spontaneously in a noninbred strain of the hairless mouse and had previously been mapped to 77cM on MMU10 (ref. 20). We designed primers for amplification of individual exons to conduct mutation screening by direct sequencing. Exons 10 and 11 repeatedly could not be amplified from ATEB DNA but could be amplified from C57/Bl6 DNA (Fig. 5n) and from four other strains of mouse (data not shown). All other exons amplified normally and no putative mutations were detected. Our data suggested a deletion of exons 10 and 11,

Figure 5 GRIP1 is required for normal trafficking and biological activity of Fras1. (a–f) Defects at the *Grip1* mutant epidermal-mesenchymal interface. Green fluorescence indicates expression of Fras1 (a,b), NG2 (c,d) and collagen VI (e,f) in *Grip1*^{+/+} (a,c,e) and *Grip1*^{-/-} (b,d,f) skin. Basement membrane is labeled by staining with antibody to laminin- γ 1 (red) and cell nuclei by DAPI (blue). Panels a'–f' show green channels for representative areas from a–f, respectively. Position of the basement membrane is indicated by an arrow in b', d' and f'. Note mislocated Fras1 in the *Grip1*-deficient epidermis (arrowheads in b). (g–l) Cellular distribution of Fras1 in apical (g,i) or basal (h,i,k,l) optical sections of cultured wild-type (WT; g–i) and *Grip1*^{-/-} keratinocytes (j–l). (g,h,j,k) Phase contrast is shown; cell borders are indicated by arrowheads. (m) Model showing GRIP1-dependent transport of Fras1 to the basal epidermis and the proposed interactions with collagens V and VI (green) and NG2. Fras1 localization is abnormal in absence of GRIP1 and the dermis separates from the basement membrane. (n) PCR identified a deletion in *Grip1* in eye-blebs (*eb*) genomic DNA. Diagram of the genomic region containing *Grip1* exons 9–11, not to scale and with exon numbering based on the refGene NM_028736 sequence. Downward-pointing arrows labeled a–k indicate the locations of the corresponding PCR amplifiers shown in the agarose gel; the upward-pointing arrow indicates the position of *D10Mit187*, which was previously reported as failing to amplify in *eb* (ATEB inbred strain) DNA. The approximate position of the deletion (*eb* Δ) is shown. The bottom panels show the amplifiers obtained from control and *eb* DNA after agarose gel electrophoresis. M, 100-bp ladder.

which was corroborated by the failure to amplify a region from intron 10 and four other sequences 5' of exon 10 and 3' of exon 11. The deleted segment is 3.22–5.38 kb in length. Conceptual translation of the deleted *Grip1* gene results in a protein of 353 amino acids, the last six of which (AAHEEP) are novel, as a result of a frame shift and premature termination. Assuming no nonsense-mediated decay, the resultant protein would lack the C-terminal four PDZ domains.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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