Germline Mutation in *EXPH5* Implicates the Rab27B Effector Protein Slac2-b in Inherited Skin Fragility

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The Rab GTPase Rab27B and one of its effector proteins, Slac2-b (also known as EXPH5, exophilin-5), have putative roles in intracellular vesicle trafficking but their relevance to human disease is not known. By using whole-exome sequencing, we identified a homozygous frameshift mutation in *EXPH5* in three siblings with inherited skin fragility born to consanguineous Iraqi parents. All three individuals harbor the mutation c.5786delC (p.Pro1929Leufs*8) in *EXPH5*, which truncates the 1,989 amino acid Slac2-b protein by 52 residues. The clinical features comprised generalized scale-crusts and occasional blisters, mostly induced by trauma, as well as mild diffuse pigmentary mottling on the trunk and proximal limbs. There was no increased bleeding tendency, no neurologic abnormalities, and no increased incidence of infection. Analysis of an affected person's skin showed loss of Slac2-b immunostaining (C-terminal antibody), disruption of keratinocyte adhesion within the lower epidermis, and an increased number of perinuclear vesicles. A role for Slac2-b in keratinocyte biology was supported by findings of cytoskeletal disruption (mainly keratin intermediate filaments) and decreased keratinocyte adhesion in both keratinocytes from an affected subject and after shRNA knockdown of Slac2-b in normal keratinocytes. Slac2-b was also shown to colocalize with Rab27B and β4 integrin to early adhesion initiation sites in spreading normal keratinocytes. Collectively, our findings identify an unexpected role for Slac2-b in inherited skin fragility and expand the clinical spectrum of human disorders of GTPase effector proteins.

Collectively termed epidermolysis bullosa, the inherited skin fragility disorders reflect a clinically and genetically heterogeneous group of conditions that result from mutations in at least 14 genes that mostly encode structural proteins involved in keratinocyte cell-cell or cell-matrix adhesion.[1] Despite considerable progress in the molecular characterization of the different forms of epidermolysis bullosa, some variants thereof remain unclassified. Nevertheless, recent technical innovations in next-generation sequencing now provide new opportunities to identify gene mutations in unresolved cases. In this report, we used whole-exome sequencing to identify an additional cause of inherited skin fragility, namely mutations in *EXPH5* (MIM 612878) encoding the Rab27B GTPase effector protein Slac2-b (also known as exophilin-5).

We investigated an Iraqi family in which three of eight siblings born to first-cousin parents had inherited skin fragility (Figure 1A). The clinical features were noted from early childhood and mainly comprised trauma-induced scale crusts and intermittent skin blistering that was mostly induced by trauma (e.g., direct injury or adhesive tape), although some appeared spontaneously (Figures 1B and 1C; Figure S1 available online). Some of the crusted areas were hemorrhagic and accompanied by occasional bruising. Most lesions cleared over several weeks to leave slightly atrophic scars and moderate postinflammatory hyperpigmentation. Some mild diffuse mottled hyper- and hypopigmentation was noted on the trunk and proximal limbs. There was no increased bleeding tendency (normal platelet counts and coagulation studies demonstrated in individual II-2), no neurologic abnormalities, and no increased incidence of infection. Hair color was normal. No clinical abnormalities were noted in either parent or reported in any other relative.

To investigate the etiology of the blistering, we first assessed by immunohistochemistry and transmission electron microscopy a nonlesional skin biopsy taken from the upper arm of individual II-2 under local anesthetic. The subject's legal guardian provided written and informed consent according to a protocol approved by the St. Thomas' Hospital Ethics Committee (Molecular basis of inherited skin disease: 07/H0802/104). Blood and skin samples (ellipse of skin taken under local anesthesia by 1% lignocaine) were obtained in adherence to the Helsinki guidelines. Light microscopy showed mild acanthosis and hyperkeratosis and an irregular ruffled or jagged appearance at the dermal-epidermal junction (Figure 1D). Immunolabeling showed normal intensity basement membrane zone staining with antibodies to laminin-332, keratin 14, plectin, tetraspanin CD151, collagen IV, collagen VII, collagen XVII, and β4 integrin, although some staining patterns differed from normal control skin (Figure S2). Notably, labeling at the dermal-epidermal junctional in patient skin appeared to have

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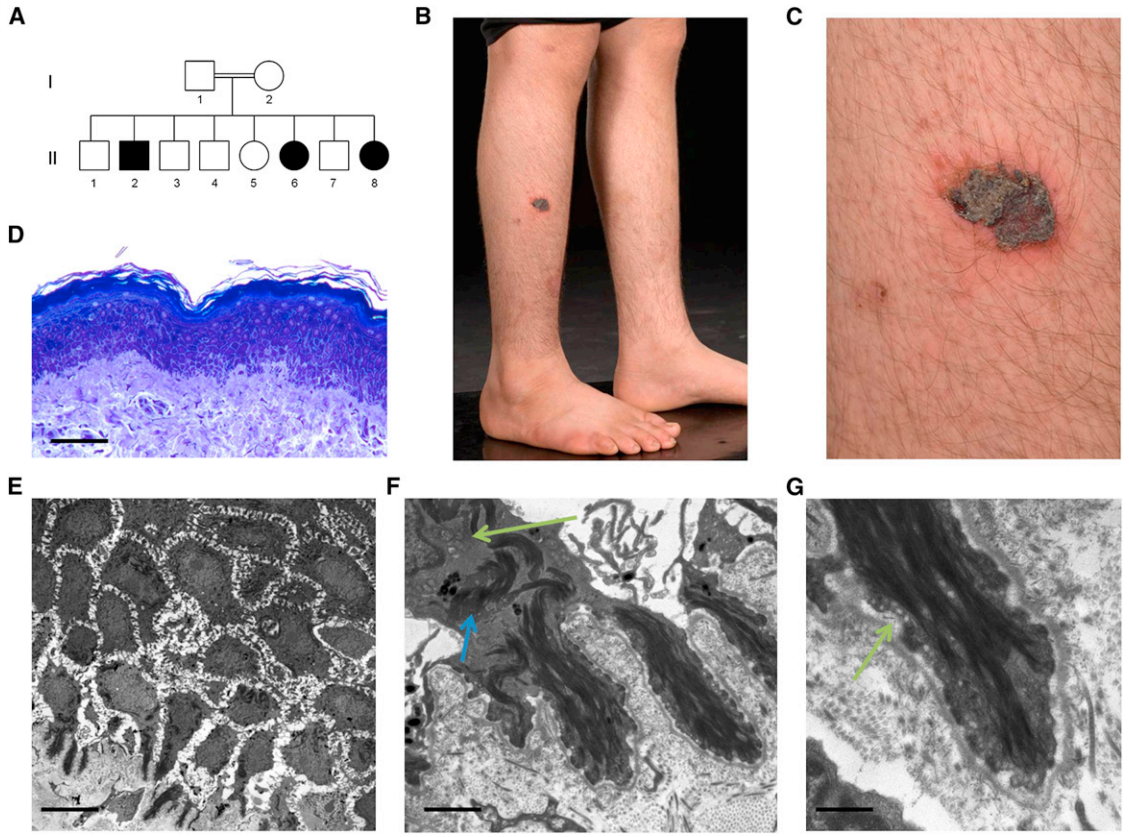

Figure 1. Pedigree and Skin Pathology in This Autosomal-Recessive Skin Fragility Disorder
(A) The family pedigree. Squares denote male family members, and circles female family members; filled-in symbols indicate clinically affected individuals.
(B) Affected individual II-2 with skin crusting at the site of a recent trauma-induced erosion.
(C) Higher magnification of the crusted erosion. Additional clinical images are shown in Figure S1.
(D) Light microscopy of skin sampled from the upper arm reveals mild acanthosis and hyperkeratosis as well as a ruffled appearance to the dermal-epidermal junction (Richardson's stain; scale bar represents 50 μm).
(E) Low-magnification transmission electron micrograph shows widening of spaces between keratinocytes in the lower epidermis with some aggregation of keratin filaments (scale bar represents 3 μm).
(F) Higher-magnification transmission electron micrograph reveals keratin filament disruption (blue arrow) as well as perinuclear accumulation of vesicles (green arrow) (scale bar represents 0.5 μm).
(G) There is also focal accumulation of vesicles close to the plasma membrane (green arrow) (scale bar represents 0.25 μm).

a broader jagged pattern, keratin 14 labeling was more diffuse pan-epidermal, and CD151 staining was confined to the dermal-epidermal junction in contrast to the pericellular pattern seen in control basal keratinocytes (Figure S2). Low-magnification transmission electron microscopy revealed basement membrane keratinocyte disruption within the lower epidermis (Figure 1E). Higher magnification showed aggregated intermediate filaments as well as an increased number of perinuclear vesicles (Figure 1F) and some vesicles clustered near the plasma membrane (Figure 1G). Collectively, however, the clinicopathologic features were not diagnostic for any particular subtype of epidermolysis bullosa.

Next, after obtaining approval from the ethics committee and informed consent from all subjects, we extracted genomic DNA from peripheral blood or saliva samples from ten individuals (all eight siblings and both parents) in compliance with the Declaration of Helsinki Principles. We first excluded a possible diagnosis of epidermolysis bullosa simplex (MIM 131800) by sequencing *KRT5* and *KRT14* (MIM 148040, 148066) that encode keratin 5 and 14, respectively (data not shown). Then, by using DNA from subject II-2, whole-exome capture was performed by in-solution hybridization with the SureSelect All Exon 50 Mb Version 4.0 (Agilent) followed by massively parallel sequencing (Illumina HiSeq2000) with 100 bp paired-end reads. More than 8.6 gigabases of mappable sequence data was generated, such that >90% of the coding bases of the exome defined by the GENCODE Project were represented by at least 20 reads. Variant profiles were generated with our in-house variant-calling pipeline.[2] In brief, reads generated on the Illumina HiSeq2000 platform were aligned to the reference human genome with the Novoalign software package (Novocraft Technologies Sdn Bhd). Duplicate reads, resulting from PCR clonality or optical duplicates, and reads mapping to multiple locations were excluded from downstream analysis. Depth and breadth of sequence coverage was

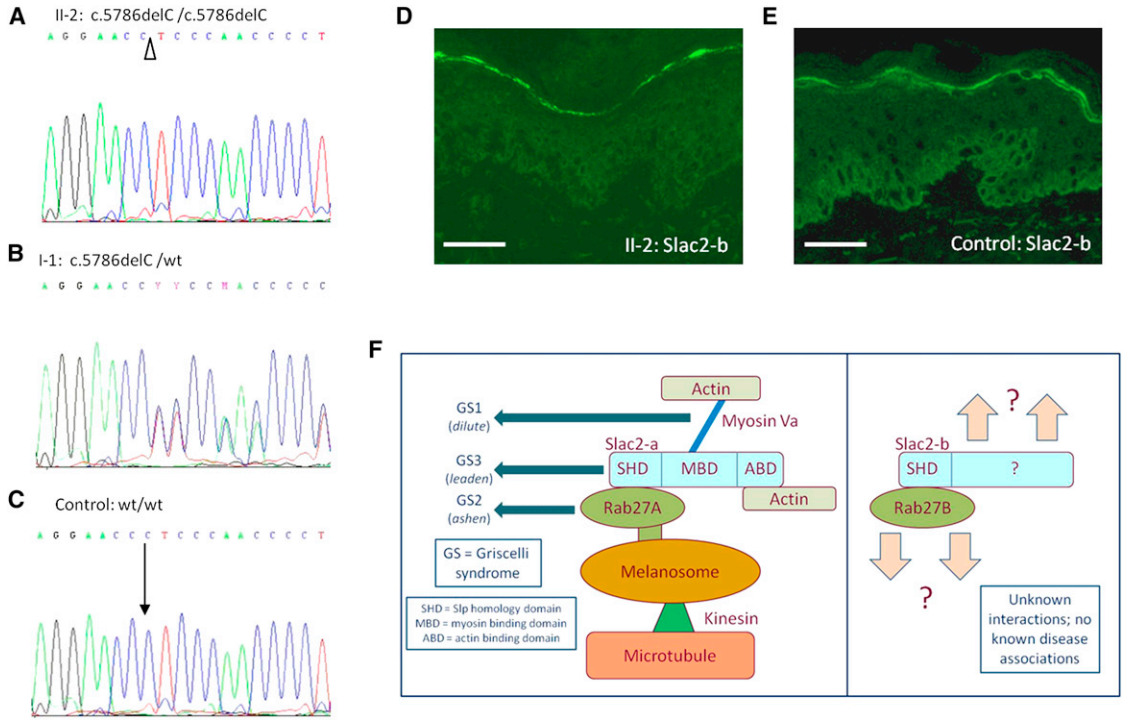

Figure 2. Identification of *EXPH5* Mutations in This Pedigree
(A) Sequencing of genomic DNA from individual II-2 reveals a homozygous 1 bp deletion in *EXPH5*, c.5786delC. Family members II-6 and II-8 were also homozygous for this mutation.
(B) Heterozygosity for this frameshift mutation was also demonstrated in individual I-1 (illustrated), as well as subjects I-2, II-4, and II-5.
(C) Wild-type (WT) sequence is shown for control DNA, which was also demonstrated in family members II-1, II-3, and II-7.
(D) Immunolabeling with a C-terminal antibody to Slac2-b (clone 99021, Abcam, Cambridge UK; working dilution 1:100) shows a complete absence of staining in an affected person's skin (scale bar represents 50 μm).
(E) In contrast, in control skin, there is cytoplasmic staining within basal and some suprabasal keratinocytes (scale bar represents 50 μm).
(F) Schematic representation of the known protein binding partners and disease associations for Slac2-a (also known as Slp4-a) and Slac2-b. Slac2-a contains a N-terminal Slp-homology domain (SHD) that binds to Rab27A, a central myosin-binding domain, and a C-terminal actin-binding domain. In contrast, the protein-protein interactions for Slac2-b are less well defined: there is a Rab-binding N-terminal SHD domain (amino acids 7–57) but no other homology-predicted binding sites and, apart from this report, there are no known disease associations.

calculated with custom scripts and the BedTools package.[3] Single-nucleotide substitutions and small insertion deletions were identified and quality filtered with the SamTools software package and in-house software tools.[4] Variants were annotated with respect to genes and transcripts with the Annovar tool.[5] Identified variants were cross-referenced with publicly available variant data (dbSNP135 and 1000 Genomes Project) and approximately 400 control exome variant profiles generated with the same methodology in our laboratory. The control exomes are predominantly of European ancestry but also include approximately 60 individuals of Middle Eastern origin. We identified 373 previously unobserved variants (25 homozygous, 348 heterozygous). Of these, given the consanguinity in the Iraqi pedigree, we noted a homozygous 1 bp deletion in *EXPH5* (RefSeq accession number NM_015065.2, c.5786del in exon 6, p.Pro1929Leufs*8).

EXPH5 encodes the Rab27B GTPase effector protein Slac2-b (also known as exophilin-5). None of the other homozygous variants represented clear loss-of-function mutations and by using Sanger sequencing we confirmed the presence and segregation of the mutation in the pedigree (Figure 2A). By sequencing, we then excluded this mutation in 200 control chromosomes (mixed Iraqi, Iranian, Kuwaiti, and Omani populations). We also evaluated the significance of the cosegregation of the c.5786delC variant with the disease status in the pedigree by using parametric linkage analysis with the Merlin software package.[6] Analysis was undertaken on the assumption of the parents being first cousins (indicated by the clinical history) with a fully penetrant autosomal-recessive model, an estimate of the disease frequency of 0.00001, and a frequency of 0.001 for the c.5786delC variant. On this basis, a generated LOD score of 3.157 provides evidence of linkage and indicates that the observed cosegregation is unlikely to have occurred by chance.

The frameshift mutation occurs within the Pro1929 codon and results in a premature termination codon eight amino acids downstream that is predicted to truncate the 1,989 amino acid Slac2-b protein by 52 residues. Immunolabeling of skin from an affected family member by a C-terminal Slac2-b antibody (clone ab99021, Abcam;

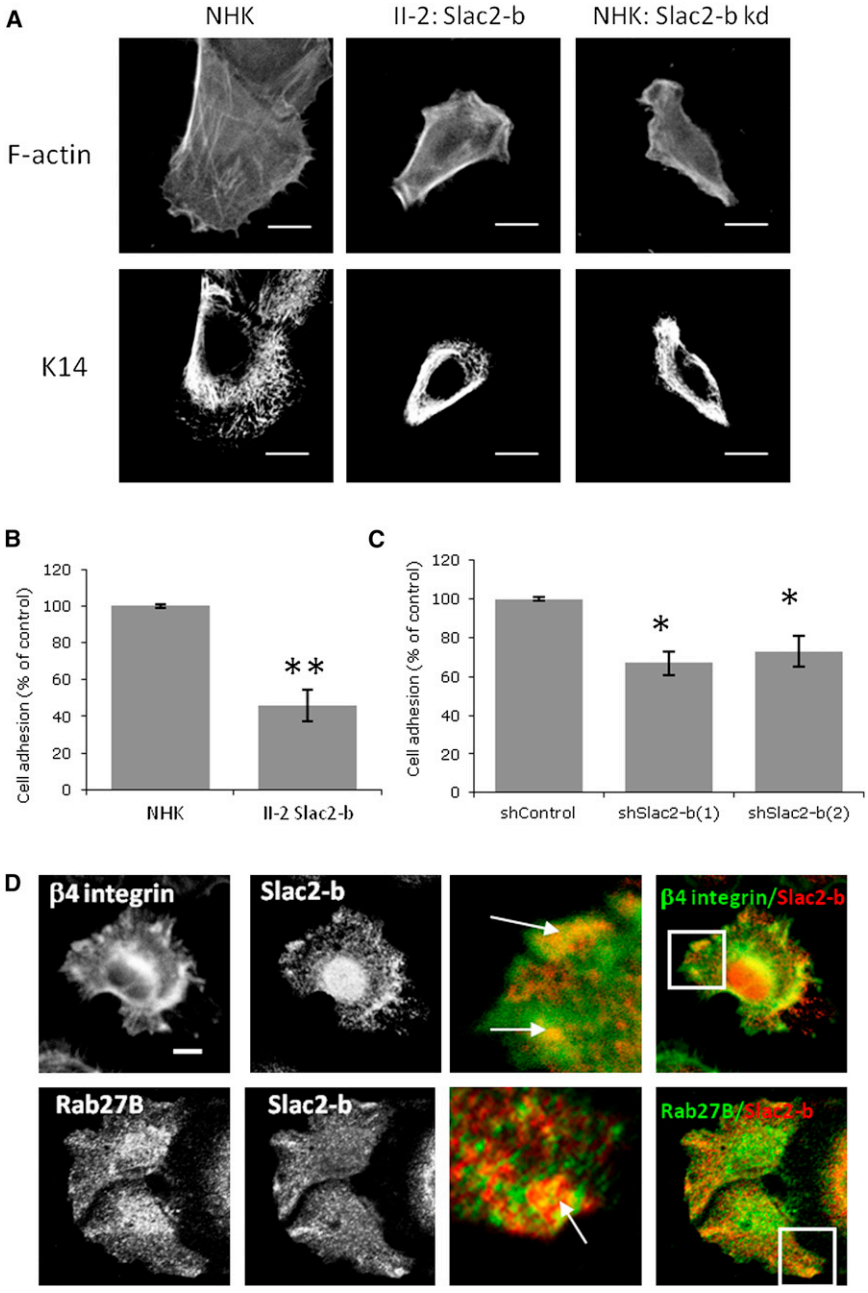

Figure 3. Keratinocyte Cell Biology Associated with *EXPH5* Mutations

(A) Confocal images of normal keratinocytes (NHK) and keratinocytes from an affected individual (II-2 Slac2-b) and in NHK after Slac2-b knockdown (NHK Slac2-b kd) stained with phalloidin (to show F-actin; Phalloidin-Alexa488 and 569, Invitrogen) and antibodies to keratin intermediate filaments (keratin 14, clone LLOO2, Abcam). Images show similar changes in cell morphology and aggregation of the keratin intermediate filament network in affected person cells and knockdown cells (scale bars represent 10 μm).

(B) Keratinocyte adhesion assays demonstrate reduced cell adhesion to the basement membrane extracellular matrix protein laminin-332 for patient cells (II-2 Slac2-b).

(C) Loss of adhesion in these assays is also shown for knockdown cells: two knockdown clones are shown, shSlac2-b(1) and (2).

Bars in (B) and (C) are pooled data from experiments performed in triplicate, normalized to respective controls and representative of three experiments. Error bars are SEM. *$p < 0.01$, **$p < 0.001$.

(D) Confocal microscopy images of normal keratinocytes plated for 45 min on laminin-332, fixed and stained with antibodies to β4 integrin (clone 3E1, Millipore) and Slac2-b (top) and Slac2-b and Rab27B (clone AB76779, Abcam) (bottom). Images indicate that Rab27B is mainly present in a perinuclear distribution, whereas Slac2-b is more localized to intracellular vesicles and at the periphery of spreading cells where some colocalization with Rab27B and β4 is seen (arrows). This finding suggests that Slac2-b colocalizes with Rab27B only in specific subcellular regions, and this is coincident with early adhesion sites (scale bar represents 10 μm).

epitope amino acids 1,907–1,956) showed a complete absence of staining compared to normal control skin (Figures 2D and 2E). The mutation occurs in exon 6 of *EXPH5* close to the 3′ end of the gene and therefore might not be expected to have a major impact on mRNA degradation.[7] By semiquantitative RT-PCR we were able to show expression of *EXPH5* mRNA in affected subject and control skin, as well as obtaining RT-PCR amplification from muscle, heart, and brain. We did not achieve amplification from kidney, liver, or lung (Figure S3).

To determine whether the loss-of-function mutation in *EXPH5* disrupts normal keratinocyte function, we first examined the cytoskeletal architecture in keratinocytes isolated from an affected individual as well as in normal control keratinocytes in which Slac2-b had been knocked down by using retrovirally delivered shRNA (see Supplemental Data for methods). We noted disruption of the keratin filament network and more cortically distributed F-actin in both the patient and Slac2-b knockdown keratinocytes compared to wild-type keratinocytes (Figure 3A). The alteration in the keratin intermediate filaments, in combination with the keratin filament aggregation seen by transmission electron microscopy (Figures 1E–1G), supports a role for Slac2-b in maintaining keratinocyte integrity.

To determine whether Slac2-b-driven changes in cytoskeletal architecture led to altered cell function, we performed keratinocyte adhesion assays to the extracellular

matrix protein, laminin-332. Data demonstrated significantly reduced cell adhesion in both an affected person's cells (Figure 3B) and also the Slac2-b knockdown keratinocytes (Figure 3C) compared to respective controls. These findings support a role for Slac2-b in maintaining adhesion between keratinocytes and basement membrane and provide some insight into why skin fragility occurs in affected family members. We also assessed localization of Slac2-b and Rab27B in human keratinocytes by confocal microscopy. Rab27B was mainly present in a perinuclear distribution, whereas Slac2-b localized more to intracellular vesicles and at the periphery of spreading cells where some colocalization with Rab27B was seen (Figure 3D). This finding suggests that Slac2-b and Rab27B colocalize in certain cell compartments only during keratinocyte adhesion and spreading. We also noted partial colocalization of Slac2-b with β4 integrin at the periphery of spreading cells (Figure 3D). Knockdown of Slac2-b led to a dramatic reduction in levels of the β4 integrin subunit at the plasma membrane as well as the cell surface glycoprotein CD151 (Figure S2 and data not shown). Interestingly, surface levels of β1 integrins were unchanged, suggesting a specific role for Slac2-b in controlling β4 integrin function in keratinocytes (data not shown).

Rab proteins form part of the Ras superfamily of monomeric G proteins and more than 60 Rab proteins have been identified in humans. Rab GTPases regulate many key steps of cell membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion (for review see Stenmark[8]). Rab GTPases control trafficking in endocytic and secretory pathways by recruiting proteins onto membrane surfaces that can (1) drive cargo collection, (2) direct organelle motility, or (3) steer vesicle docking at membranes. Many Rab GTPases are differentially expressed in various tissues and this diversity, in combination with differential expression of their numerous effector proteins (such as sorting adaptors, tethering factors, kinases, phosphatases, motors, etc.), accounts for a broad spectrum of biological functions. Some inherited or acquired disorders of Rab pathways have been determined, the types of conditions encompassing immunodeficiencies, infections, cancer, and neurological disorders.[9–13] With regard to inherited skin diseases and GTPases, mutations in Rab27A and one of its effector proteins, Slac2-a (also known as melanophilin or exophilin-3), as well as Myosin Va, have been shown to underlie the autosomal-recessive disorder Griscelli syndrome (GS2 [MIM 607624]; GS3 [MIM 609227]; GS1 [MIM 214450], respectively)[14–16] (Figure 2F). Similar mutations in mice give rise to the *ashen*, *leaden*, and *dilute* skin coat color abnormalities, respectively. In Griscelli syndrome there is a pigmentary dilution in skin and hair—this is the only phenotypic abnormality in GS3, but in GS1 there are additional neurologic features and in GS2 the syndrome includes neurologic and immunologic defects.[17] Rab27A recruits the adaptor protein Slac2-a to the membranes of melanosomes and connects them to myosin Va.[18] Slac2-a contains a N-terminal Slp-homology domain (SHD) that binds to Rab27A, a central myosin-binding domain, and a C-terminal actin-binding domain.[19] In contrast, the protein-protein interactions for Slac2-b are less well defined: there is a Rab-binding N-terminal SHD domain (amino acids 7–57) but no other homology predicted binding sites apart from a possible PDZ domain at the C terminus (within the part of the protein truncated by the mutation in our pedigree). Slac2-b lacks the C2A/C2B domains seen in other Rab27 effectors, domains that interact with SNARE fusion machinery, and there is no sequence similarity with domains interacting with motor linkers such as Myosin Va, VIIa, and actin.[19] Furthermore, in contrast to Rab27A, Rab27B has a more restricted distribution, being predominantly expressed in platelets, stomach, large intestine, pancreas, pituitary, and bladder.[20] Nevertheless, a mouse model (lacking Rab27b but expressing *lacZ* under the control of the Rab27b promoter) has shown that Rab27b is also expressed in keratinocytes and indeed in a few other cell types engaged in surface protection and mechanical extension.[20] Murine *Rab27b*$^{-/-}$ knockouts (and also double Rab27a/b knockouts) tend to have a mild phenotype, although in one study, Rab27b knockout led to a significant hemorrhagic disease with a reduction in platelet granules and platelet serotonin content, implying a role for Rab27B as a key regulator of dense granule secretion.[21] With regard to our pedigree phenotype, apart from occasional mild bruising, we did not observe any clinical indicator of a significant platelet anomaly. The major abnormality was skin fragility, although the subtle diffuse pigmentary skin mottling noted was similar (but less marked) to the pigment changes seen in Griscelli syndrome, a clinical observation that could indicate some functional overlap/interplay for Slac2-b with Rab27A, Slac2-a, and myosin-Va.

Further clues to the functions of Rab27A and Rab27B have recently been gleaned from knockdown experiments in HeLa cells.[22] Notably, a small-scale shRNA screen identified Rab27A and its effector protein Slac2-a, as well as Rab27B and its effector protein Slac2-b, as key drivers of exosome vesicle release as well as the delivery of other secretory vesicles. Exosomes are small membrane cup-shaped vesicles, 40–100 nm in diameter, that are released from many cell types when multivesicular endosomes fuse with cell surfaces.[23] Exosomes contain proteins involved in lipid rafts, metabolism, adhesion, signaling, T cell interactions, and cytoskeletal integrity.[24] Knockdown of Rab27A or Rab27B (or Slac2-a or Slac2-b) leads to a decrease in the amount of exosomes secreted and more motile multivesicular endosomes although the biochemical composition of the exosomes is unchanged.[22] The knockdown studies showed that Rab27A and Slac2-a are needed for multivesicular endosome docking and fusion, whereas Rab27B and Slac2-b probably link microvesicular endosomes to outward directed motor protein(s).[22]

The similarities in cell biologic abnormalities in both the keratinocytes from an affected person and the Slac2-b knockdown keratinocytes implies that the C-terminal domain of Slac2-b may be functionally significant, perhaps with involvement in a key protein-protein interaction germane to either generating the Slac-2b-Rab27B complex and/or a direct domain interaction with a cytoskeletal or other protein; such data exist for Slac2-a but not Slac2-b.[25] Assessment of the Slac2-b sequence via homology or ab initio protein structure prediction models (including GlobProt, DisoPred, and ANCHOR), however, fails to indicate a model structure or putative interacting proteins. Studies on the C-terminal 100 amino acids of Slac2-a have shown an interaction with the microtubule end-binding EB1 protein[25] but there are currently no data to support similar biology for Slac2-b. Mutations in Slac2-a, Rab27A, and Myosin Va all lead to perinuclear accumulation of melanosomes in melanocytes. In contrast, mutations in Slac2-b result in accumulation of vesicles close to nuclei in keratinocytes. Slac2-b mutations also appear to disrupt the keratin filament cytoskeleton (Figure 3A). Previously, a role for intermediate filaments in organelle/adaptor protein positioning has been demonstrated,[26,27] and our new data support this interaction but from the other perspective, namely that impairment of the Slac2-b vesicle transport protein can lead to disruption of intermediate filaments. This vesicle trafficking/intermediate filament disruption may also help explain the ruffled or jagged appearance at the dermal-epidermal junction on light microcopy and basement membrane immunohistochemistry. Notably, normal transport of exosomes in cells involves physiologic transport and delivery of morphogens and RNA, which can influence cell polarity and developmental patterning of tissues,[28] a process that may be impaired in the presence of Slac2-b mutations.

In summary, we have used whole-exome sequencing to identify a further cause of autosomal-recessive inherited skin fragility. Our data expand the clinicopathological spectrum of the epidermolysis bullosa group of diseases and also demonstrate an unexpected role for Slac2-b in keratinocyte cell biology.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and three figures and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

ANCHOR, http://anchor.enzim.hu/
BLAST Assembled RefSeq Genomes, http://blast.ncbi.nlm.nih.gov/Blast.cgi
DisoPred, http://bioinf.cs.ucl.ac.uk/disopred/
Ensembl Genome Browser, http://www.ensembl.org/index.html
GlobProt, http://globplot.embl.de/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Primer3, http://frodo.wi.mit.edu/primer3/
PubMed, http://www.ncbi.nlm.nih.gov/PubMed/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

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