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**Slac2-b co-ordinates extracellular vesicle secretion to regulate keratinocyte adhesion
and migration**

Yonis Bare¹, Grace K. Chan¹, Thomas Hayday¹, John A. McGrath², Maddy Parsons^{1,3}

¹ Randall Centre for Cell and Molecular Biophysics, King's College London, Guy's Campus, London, SE1 1UL, UK.

² St Johns Institute of Dermatology, King's College London, Guys Campus, London, SE1 9RT, UK.

³ To whom correspondence should be addressed: Randall Centre for Cell and Molecular Biophysics, King's College London, Guy's Campus, London, SE1 1UL, UK. Tel: +44 207 848 8164. Email: maddy.parsons@kcl.ac.uk

RUNNING TITLE: Slac2-b co-ordinates keratinocyte secretion and adhesion

ABBREVIATIONS: EBS – epidermolysis bullosa simplex, ECM – extracellular matrix, EVs – extracellular vesicles, FN – fibronectin, MVB – multivesicular bodies, WT - wildtype

ABSTRACT

Slac2-b, also known as exophilin-5, is a Rab27b effector protein with a role in exosome transport and is encoded by the exophilin-5 (*EXPH5*) gene. We previously described biallelic loss-of-function mutations in *EXPH5* in an autosomal recessive form of epidermolysis bullosa simplex. However, how loss of Slac2-b expression leads to skin fragility and erosions is unknown. Here, we demonstrate that keratinocytes isolated from two different individuals with mutations in *EXPH5* have significant defects in cell-matrix adhesion. *EXPH5* mutant keratinocytes also showed increased perinuclear accumulation and significantly reduced trafficking of CD63⁺ vesicles. These phenotypes were also seen in Slac2-b deficient keratinocytes. This was coincident with a reduction in Rab27a protein expression in Slac2-b mutant keratinocytes, as well as reduced secretion of extracellular vesicles containing extracellular matrix proteins. Live imaging analysis revealed a strong correlation between CD63⁺ vesicle trafficking to the plasma membrane and focal adhesion dynamics. These findings support a role for Slac2-b in regulating local focal adhesion dynamics to support effective keratinocyte adhesion and provide insight into the underlying pathophysiology of inherited skin blistering.

INTRODUCTION

Epidermolysis bullosa (EB) is a clinically and genetically heterogeneous group of inherited skin fragility disorders resulting from mutations in 21 distinct genes (Uitto, 2019). EB simplex (EBS) is the most common subtype of EB in which blistering occurs within the epidermis. Most affected individuals have autosomal dominant disease resulting from heterozygous missense mutations in *KRT5* or *KRT14* encoding the intermediate filament proteins keratin-5 and -14, respectively, but at least 12 other genes can be implicated in a range of dominant and recessive forms of EBS and related disorders. Within this collection of genes, biallelic mutations in *EXPH5* have been shown to result in an autosomal recessive form of EBS (Liu et al., 2014, Malchin et al., 2016, McGrath et al., 2012, Pigors et al., 2014, Rashidghamat et al., 2016, Turcan et al., 2016).

EXPH5 encodes for Slac2-b, also known as exophilin-5, that is a known effector protein for the GTPase Rab27b with current functional data suggesting a role in extracellular vesicle (also known as exosomes) secretion (Ostrowski et al., 2010). Slac2-b is distinct from other Rab27b effector proteins as it lacks the SNARE fusion protein-interaction C2A/B domains and other domains which interact with myosin-Va and actin (Fukuda, 2013). Our previous study reported the initial EBS patient with an *EXPH5* mutation that was predicted to truncate the final 52 amino acids of the C-terminal region of Slac2-b. This mutation resulted in reduced staining of Slac2-b in patient skin and disruption of keratinocyte adhesion and F-actin architecture *in vitro* (McGrath et al., 2012). We further showed that Slac2-b localizes to early adhesion sites in spreading keratinocytes (McGrath et al., 2012) and subsequent studies have reported additional pathogenic mutations in *EXPH5*, with all the reported mutations located in exon 6 of the gene leading to premature stop codons and the majority being homozygous with some compound heterozygous loss-of-function mutations also reported (Liu et al., 2014, Pigors et al., 2014).

Clinically, affected individuals have trauma-induced erosions and skin crusting although more extensive blisters and erosions can occur in some individuals, despite a similar nature and location of mutations in *EXPH5* (Pigors et al., 2014). Ultra-structurally, there are some features similar to EBS resulting from keratin gene mutations with intermediate filament aggregation, clumping and cytolysis, but with additional findings of increased intracellular vesicles, especially around keratinocyte nuclei (Malchin et al., 2016, McGrath et al., 2012, Turcan et al., 2016, Vahidnezhad et al., 2018).

In the current study, we aimed to analyze the underlying mechanisms by which Slac2-b contributes to keratinocyte adhesion. Data revealed that cells derived from two separate individuals with very similar clinical phenotypes and loss-of-function mutations in *EXPH5* showed significant defects in cell-matrix adhesion and collective cell migration, with an increased number of nascent focal complexes observed after initial adhesion to ECM. Furthermore, Slac2-b mutant keratinocytes displayed reduced Rab27a protein expression, defective CD63⁺ vesicle trafficking and a reduction in extracellular vesicle release. Slac2-b-depleted keratinocytes exhibited similar phenotypes to patient cells, indicating the different mutations likely result in loss of function, despite retaining *EXPH5* mRNA expression. Finally, we observed a strong correlation between focal adhesion turnover and CD63⁺ vesicle trafficking. Together, our findings provide novel insight into Slac2-b-dependent co-ordination of vesicle transport and release to facilitate keratinocyte adhesion and migration.

RESULTS

Mutations in Slac2-b leads to accumulation of perinuclear CD63⁺ vesicles

To determine whether the mutations in *EXPH5* led to nonsense-mediated mRNA decay, we extracted total RNA from keratinocytes taken from healthy donors (wild-type [WT]) or from two EBS patients, denoted as EB52 and EB63, containing mutations in *EXPH5* that are predicted to truncate the resulting protein Slac2-b by 59 and 494 residues, respectively (Figure 1a). Using primers directed against the start and end of exon 6, *EXPH5* mRNA expression was detected in all 3 cell lines with no detectable differences between them (Figure 1b), but reduced levels of full-length protein were detected in the Slac2-b mutant cell lines (Figure 1c).

Previous studies have demonstrated that Slac2-b plays a role in exosome secretion by facilitating the transport of multivesicular bodies (MVBs) to the plasma membrane (Ostrowski et al., 2010). Electron micrograph images of skin taken from EBS patients with mutations in *EXPH5* have shown a unique phenotype of aggregated cytoplasmic vesicles (Rashidghamat et al., 2016). To assess the localisation of cytoplasmic vesicles, immunostaining was performed using CD63 as a marker of MVBs. Resulting confocal images (Figure 1d) and quantification of localisation with respect to the cell edge or peri-nuclear regions (Figures 1e,f) demonstrated that EB52 and EB63 cells displayed an increase in perinuclear accumulation of CD63⁺ vesicles compared to WT cells. As an alternative means to demonstrate the perinuclear clustering of CD63⁺ vesicles, cells were also co-stained with LAMP1, a marker for late endosomes/lysosomes, revealing increased perinuclear accumulation in the Slac2-b mutant keratinocytes compared to WT cells (Figure 1g) and a significant increase in colocalisation of LAMP1⁺ vesicles with CD63⁺ vesicles in Slac2-b mutant cells (Figure 1h).

Slac2-b is a known effector protein for the GTPases Rab27a and Rab27b (Fukuda, 2013), and interacts with the activated GTPases via an N-terminal Rab-binding domain (Kuroda et al., 2002). Rab27 binding to effectors has previously been proposed to stabilize the protein. To assess whether the Slac2-b mutations affected Rab27 expression, we analyzed Rab27a protein levels in Slac2-b WT and mutant keratinocytes. To determine whether the phenotypes observed in Slac2-b mutant cells also mimicked a loss of Slac2-b, CRISPR-Cas9 gene editing was used to generate Slac2-b depleted keratinocytes (CRISPR; Figure 1i,j). Western blot analysis demonstrated a significant reduction in Rab27a expression in both Slac2-b mutant cell lines compared to WT cells (Figure 1k) and a further reduction was also observed in Slac2-b CRISPR keratinocytes (Figure 1l), suggesting a loss of interaction between Slac2-b and Rab27a leading to degradation of the latter.

Mutations in Slac2-b perturb CD63⁺ vesicle trafficking lead to aberrant EV secretion in keratinocytes

To explore the CD63 vesicle redistribution phenotype further, Slac2-b WT and mutant keratinocytes were transiently transfected with GFP-CD63 and analyzed by live fluorescence time-lapse microscopy to observe CD63⁺ vesicle dynamics over time. Resulting analysis of vesicle movement revealed CD63⁺ vesicles had slower velocity and reduced displacement in the Slac2-b mutant cells compared to WT controls (Figures 2a-c), suggesting defective traffic from intracellular compartments may be occurring in these cells. A similar phenotype was also observed in Slac2-b-depleted keratinocytes (Figures 2a-c).

Slac2-b is known to have an important role in extracellular vesicle (EV) secretion by transporting MVBs to the plasma membrane via interactions with Rab27a/b (McGrath et al., 2012, Ostrowski et al., 2010). To assess whether mutations in Slac2-b affect its function in EV

secretion, cells were transiently transfected with CD63pHluorin, a pH-sensitive fluorescently tagged CD63 protein. When this reporter is present in intraluminal vesicles (ILVs) within the MVBs, its fluorescence is quenched due to the acidic environment. Upon fusion with the plasma membrane, the ILVs are released as EVs in a neutral environment, resulting in visible fluorescence (Figure 2d) (Verweij et al., 2018). Analysis revealed no change in the average number of CD63⁺ fusion events between cells (Figure 2e, left panel), but a significant increase in the signal duration was seen in EB63 mutant cells compared to Slac2-b WT keratinocytes (Figure 2e, right panel), suggesting that this mutation may have a greater effect on the role of Slac2-b in EV secretion.

To further explore this, EVs were isolated from cell culture by serial ultracentrifugation. Equal protein levels of whole cell lysates (WCLs) and EV pellets were analyzed by western blot for CD63 (an EV marker) and Calnexin (an endoplasmic reticulum marker, absent from EV's). The resulting blots demonstrated little to no Calnexin detected in the EV pellets compared to WCL, whereas significantly lower levels of CD63 were observed in EV pellets from both Slac2-b mutant cell lines (Figure 2f). To further determine whether EV release was compromised in these cells, EV pellets were subjected to analysis on a NanoSight instrument. Resulting data demonstrated a significantly lower concentration of EVs in samples from EB52 and EB63 cells (Figure 2g), coupled with significantly higher EV size in the upper ~50% percentile of the EV population (Figure 2h). These data collectively demonstrate that mutations in Slac2-b leads to defective EV secretion in basal keratinocytes.

Mutating or depleting Slac2-b disrupts keratinocyte adhesion and migration

Previous published reports have demonstrated that the EB52 mutation (c.5786delC) in *EXPH5* leads to reduced cell-matrix adhesion in keratinocytes (McGrath et al., 2012). To determine

whether EB63 and *EXPH5* CRISPR cells showed a similar phenotype, adhesion assays were performed by plating the cell lines onto Matrigel-coated wells. Data demonstrated that compared to Slac2-b WT keratinocytes, both Slac2-b mutant and CRISPR cells exhibited significantly reduced adhesion (Figure 3a) and increased spread area (Supplementary Figures 1a-c), confirming an important role for Slac2-b in regulating keratinocyte cell-matrix attachment. To explore this in more detail, keratinocytes were fixed 2h post-adhesion and immunostained with antibodies against vinculin and $\beta 4$ integrin as markers for focal adhesions and hemidesmosomes, respectively. Images (Figure 3b) demonstrated that both Slac2-b mutant cell lines assembled more focal adhesions, but hemidesmosome coverage was unaffected (Figure 3c). Slac2-b CRISPR cells demonstrated a similar phenotype to mutant cells, which was rescued following re-expression of GFP-Slac2-b (Supplementary Figures 1c-e). To determine whether the observed differences in adhesion number were due to differences in focal adhesion dynamics, cells were transfected with GFP-Talin (as a marker of focal adhesions) and analyzed by time-lapse confocal microscopy. Analysis of resulting movies demonstrated significantly slower rates of adhesion assembly and disassembly in Slac2-b mutant cells compared to WT (Figure 3d). Imaging of Slac2-b mutant CRISPR cells also revealed disorganized Keratin-14 staining in agreement with our previous findings (Supplementary Figure 1f; (McGrath et al., 2012)).

EBS is associated with defects in cell-cell adhesion via adherens junctions and desmosomes, and previous EM images of skin taken from patients with Slac2-b mutations showed widened spaces between neighboring keratinocytes highlighting cell-cell adhesion defects (Pigors et al., 2014). To determine whether mutations in Slac2-b affect the ability of keratinocytes to form an intact monolayer in vitro, Slac2-b WT and mutant keratinocytes were grown for 24 h in the presence of 2 mM calcium to enhance cell-cell adhesion formation and then fixed and stained

for the adherens junction (AJ) markers, E-cadherin and β -catenin (Figure 3e). Resulting confocal images showed that AJs formed in Slac2-b mutant keratinocytes, but some cell-cell junctions display a ‘jagged’ appearance suggesting a potential weakness in the monolayer and defective cell-cell adhesion. Total levels of AJ proteins did not change between cell lines (not shown). Analysis of monolayer permeability using dextran assays further revealed a significant increase in permeability in Slac2-b mutant cells (Figure 3f), suggesting compromised cell-cell adhesion integrity in these cells.

In order to determine whether the defects in cell-matrix adhesion resulted in functional changes to migratory behavior, time-lapse analysis of scratch-wound healing assays was performed on Slac2-b WT and mutant keratinocytes over a 12 h period (Figure 3g). The resulting analysis showed that WT keratinocytes migrated to close the wound within the 12h period whereas Slac2-b mutant and CRISPR keratinocytes showed a ~75% reduction in closure of the wound compared to WT cells (Figure 3g,h). Collectively, these data demonstrate that truncation or loss of Slac2-b leads to defective adhesion resulting in reduced keratinocyte migration.

Focal adhesion dynamics are correlated with CD63⁺ vesicle trafficking

Previous studies have suggested a link between focal adhesion dynamics and EV secretion (Sung et al., 2015), and we hypothesized that EV secretion may act as a local trigger to control keratinocyte adhesion dynamics. To assess the relationship between vesicle trafficking and adhesion dynamics, WT cells stably expressing Talin-mCherry (a marker for focal adhesions) were transiently transfected with GFP-CD63 and imaged using fluorescence time-lapse microscopy (Figure 4a). Resulting movies were analyzed by isolating regions focal adhesion assembly/disassembly and quantification of the fluorescence intensity of GFP/mCherry channels over time. Data revealed that at regions of adhesion assembly (increasing Talin

intensity), CD63-GFP intensity increased over time (Figure 4b). However, during focal adhesion disassembly (decreasing Talin intensity), CD63 intensity was maintained (Figure 4c) suggesting that sites of EV release may correspond to sites of new adhesion assembly. Further analysis demonstrated a strong positive correlation ($r = 0.9379$) between CD63 and Talin intensity during focal adhesion assembly (Figure 4d); conversely, a negative correlation between the two was seen during focal adhesion disassembly (-0.7623) (Figure 4e). Taken together, these data demonstrate that CD63⁺ vesicle trafficking to the membrane correlates with the assembly of focal adhesions, but this signal is maintained during disassembly, suggesting that the delivery and positioning of CD63⁺ vesicles to adhesion complexes at the plasma membrane is important to control assembly and subsequent adhesion dynamics.

Previous reports have shown that EVs play a key role in local delivery of matrix proteins to promote cell adhesion and migration (Sung et al., 2015, Sung and Weaver, 2017). To determine whether Slac2-b-dependent EV secretion may alter the extracellular levels of ligand for integrin-mediated cell-matrix adhesion in basal keratinocytes, western blot analysis was performed on whole cell lysates (WCLs) and EV pellets to probe for Fibronectin (FN) and Laminin-332 (LN332). Resulting data showed reduced levels of both ECM proteins detected in the EVs secreted from Slac2-b mutant keratinocytes compared to WT cells (Figure 4f). Analysis of images from cells stained for these ECM proteins also showed a significant reduction in FN and LN332 deposition in Slac2-b mutant cells compared to WT cells (Figure 4g,h). These data collectively demonstrate that EV targeting to the membrane correlates with adhesion assembly and that mutation or loss of Slac2-b leads to reduced release of EVs containing ECM proteins that are required for efficient adhesion dynamics and keratinocyte migration.

DISCUSSION

Our analysis of Slac2-b mutant and depleted keratinocytes has revealed a previously unidentified role for Slac2-b in regulating keratinocyte intracellular vesicle trafficking, adhesion and migration. Mutations in Slac2-b, or depletion of Slac2-b, leads to reduced Rab27a levels and an increased number of focal adhesions which are more stable compared to WT keratinocytes. Furthermore, CD63⁺ vesicle trafficking and EV release was reduced in Slac2-b mutant keratinocytes, and CD63⁺ vesicles localised at focal adhesions, suggesting a potential role in Slac2-b-dependent vesicle trafficking in regulating focal adhesion dynamics and maturation. Altogether, our findings suggest a novel role for Slac2-b in regulating keratinocyte adhesion and migration via co-ordination of intracellular vesicle trafficking and local EV secretion (Figure 4i).

EM images of skin sections taken from EBS patients with mutations in Slac2-b showed increased cytoplasmic vesicles aggregating around the nucleus and the cell membrane (Liu et al., 2014, McGrath et al., 2012). Silencing of Rab27a in HeLa cells led to an increase in size of MVBs whilst silencing of Rab27b led to increased perinuclear accumulation of these vesicles (Ostrowski et al., 2010). However, the functional interactions between Slac2-b and the Rab27 isoforms are still poorly understood. Here, we show mutations in Slac2-b, or depleting Slac2-b protein lead to a significant reduction in Rab27a protein expression in keratinocytes. A similar phenotype was observed in a recent study, where the knockdown of KIBRA, an adaptor protein for Rab27a, lead to the reduction in Rab27a protein expression (Song et al., 2019), as KIBRA interacts with Rab27a, stabilizing the protein and preventing its degradation via the ubiquitin-proteasome pathway. Another study demonstrated that Rab27a knockdown in melanocytes led to reduced levels of the effector protein Melanophilin due to an increase in proteasomal degradation (Park et al., 2019). This has also been shown *in vivo* using Rab27a-

deficient mice (Wu et al., 2002), suggesting that the interactions between Rab27 isoforms and their effectors are essential to the stability of each other. The reduced Rab27a in Slac2-b mutant cells is somewhat surprising as both mutations are predicted to truncate Slac2-b from its C-terminal region, leaving the Rab-binding domain at the N-terminus intact. The lack of any current structural information describing Slac2-b conformation precludes any definitive determination of why loss of C-terminal domains may alter N-terminal interactions. However, our findings do suggest that Slac2-b may be conformationally regulated and that this controls binding partners and function.

In this report, we observed an increased perinuclear accumulation of enlarged CD63⁺ vesicles in Slac2-b mutant keratinocytes, coupled with a significant decrease in CD63⁺ vesicle movement. This is in agreement with the phenotypes observed in *EXPH5* mutant patient skin (Liu et al., 2014, Pigors et al., 2014). HeLa cells silenced for Rab27a also show larger MVBs, whereas Rab27b-deficient cells exhibited increased perinuclear accumulation of MVBs (Ostrowski et al., 2010). This finding suggests both Rab27a/b may function via parallel routes, with Slac2-b interacting with each Rab protein to perform specific roles. Our analysis further demonstrated that EVs released from Slac2-b mutant cells are larger than those from WT cells. Whether Slac2-b plays a direct role in regulating the size of MVBs or whether the disruption in Slac2-b-dependent vesicle transport leads to a delay of trafficking MVBs and subsequent swelling remains unclear. Slac2-b mutant cell lines also showed a similar number of CD63⁺ fusion events to WT cells, and EVs are still secreted from both Slac2-b mutant cell lines albeit at a lower concentration than WT cells. Rab27a/b, Rabs 7, 11 and 35 are also involved in the transport of MVBs to the plasma membrane in a number of cell types (Baietti et al., 2012, Fruhbeis et al., 2013, Ostrowski et al., 2010, Stuffers et al., 2009) suggesting this may be facilitated by different Rab GTPases depending on cell type. Our data further shows that

CD63+ fusion events are significantly longer in EB63-mutant cells. EVs are released into the extracellular environment upon fusion of MVBs with the plasma membrane (Hessvik and Llorente, 2018). Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), tethering factors, Rabs and other small GTPases are all involved in membrane fusion (Pfeffer, 2007) and Rab27a and its effector, Slp4-a, are involved in the docking and fusion of MVBs with the plasma membrane in HeLa cells (Ostrowski et al., 2010, Pfeffer, 2010). Our data demonstrating Rab27a protein expression is significantly reduced in Slac2-b mutant keratinocytes may provide an explanation as to why those cells show longer fusion events compared to WT cells.

Our data further confirm that mutation or loss of Slac2-b leads to reduced cell adhesion as previously described (McGrath et al., 2012) and we additionally observed an increase in the number of focal adhesions in Slac2-b mutant keratinocytes, as well as slower adhesion assembly and even slower disassembly rates. These experiments were performed under different timings post-plating, allowing us to observe initial attachment defects and then subsequent homeostatic focal adhesion dynamics. Slower focal adhesion assembly seen in Slac2-b mutant keratinocytes may explain the lack of initial adhesion to ECM. The putative PDZ motif identified at the extreme C-terminus of Slac2-b (KESEL) exists in three proteins in the mammalian genome: two of which are α -actinin-1 and α -actinin-4, which are known to interact with the PDZ-LIM class of proteins through PDZ-binding motifs. α -actinins have been shown to be vital in a number of cytoskeletal functions such as cell adhesion, migration and contractility (Hamill et al., 2013, Le Clainche and Carrier, 2008) suggesting similar roles for Slac2-b. Altogether, the phenotypes displayed by Slac2-b mutant and Slac2-b depleted cells suggest that the C-terminal region, or possibly the PDZ motif alone, is vital for correct Slac2-b function. However, further work is required to elucidate how this region regulates function.

We observed a strong correlation between increasing CD63 intensity at sites of focal adhesion assembly in basal keratinocytes, suggesting that CD63⁺ vesicles are trafficked towards these sites. This could be required to regulate the levels of adhesion and/or ECM proteins that are secreted to support focal adhesion dynamics. A similar phenomenon has been previously reported in HT1080 cells, where a role of Rab27a was shown in docking and fusion MVBs that release EVs containing adhesion complexes to regulate nascent adhesions and promote cell motility (Sung and Weaver, 2011, Sung et al., 2011). Furthermore, we observed that CD63 intensity was maintained at sites of focal adhesion disassembly, suggesting that these points could be sites of EV secretion. CD63 is known to interact with a number of integrins at the cell surface (Rubinstein et al., 1996, van Niel et al., 2011). It is plausible that CD63 may influence integrin activity and stability through interactions in the tetraspanin-enriched domains (TEMs), generated by post-translational modifications of tetraspanins moving to the plasma membrane (Hemler, 2005). Interestingly, our data also suggests that the predominant effect of Slac2-b mutations is on focal adhesions, with some concomitant loss of barrier integrity. This is in agreement with the mild skin fragility observed in other types of EB with altered focal adhesion dynamics (Michael et al., 2014).

In summary, our findings identify a new role for Slac2-b in regulating epidermal homeostasis via co-ordination of intracellular vesicle trafficking to facilitate the dynamics of integrin-mediated adhesions to ECM proteins in basal keratinocytes. The exact mechanisms by which Slac2-b functions in keratinocytes to facilitate cell-cell and cell-matrix adhesions and trafficking of CD63⁺ vesicles to specific sites is still not fully understood. Further work will be required to identify any associated binding partners for Slac2-b and determine how these interactions enable Slac2-b to control keratinocyte adhesion, migration and secretion.

MATERIALS AND METHODS

Plasmids

The GFP-tagged CRISPR constructs, containing a sgRNA sequence directed against a coding region in exon 4 of human *EXPH5*, were purchased from GenScript (New Jersey, USA). The GFP-CD63 construct was provided by Fedor Beditchevski (University of Birmingham, UK). The CD63pHluorin construct was provided by Frederik Vermeij (Insitut Curie, Paris, France (Verweij et al. 2018)). Talin-mCherry was a gift from Gareth Jones (King's College London, UK).

Antibodies

Primary antibodies used were β -Catenin (Santa Cruz), β 4 integrin (R&D Systems), Calnexin (Abcam), CD63 (Developmental Studies Hybridoma Bank), Collagen I (Abcam), E-cadherin (Abcam), Fibronectin (Sigma Aldrich), Laminin-332 (γ 2 chain; Merckmillipore), Keratin-14 (Abcam), HSC70 (Santa Cruz Biotechnology, Dallas, TX), LAMP1 (Cell Signaling Technology), Rab27a (Abcam), Vinculin (Sigma Aldrich). Anti-species HRP-conjugated secondary antibodies (Dako, Carpinteria, CA), AlexaFluor-conjugated antibodies and Phalloidin (Thermofisher), Hoechst (Sigma Aldrich).

EV preparation

Cells were seeded onto 15 cm² dishes and cultured to confluency in normal growth media. Cells were then washed with PBS and incubated in 17 ml of growth media containing 2% fetal bovine serum over a 24h period. Cultured media was then collected into 50 ml falcon tubes and centrifuged at 300 x g for 10 minutes. The supernatant was transferred into a 50 ml falcon tube, centrifuged at 12,200 x g for 45 minutes, transferred to polycarbonate ultracentrifugation tubes (Beckmann Coulter, Indianapolis, USA) and centrifuged at 100,000 x g for 2h. The

supernatant was discarded and pellets were resuspended in 1 ml of PBS. The resuspended volume was transferred into 1.5ml polyallomer tubes (ThermoFisher) and centrifuged at 100,000 x g for 2 h. The supernatant was discarded and the pellet resuspended in 25 ul of PBS and stored at -20°C.

Junctional line scan analysis

Cells were seeded onto coverslips in 24-well plates and grown overnight. Cells were then fixed and stained for antibodies against β -catenin, E-cadherin and phalloidin. Images were then acquired by confocal fluorescence microscopy (Nikon A1R) and analysed on ImageJ. Lines 20 μ m in length and 1 μ m in width were drawn across junctions identified via E-cadherin localisation. Lines were aligned so that the 10 μ m point was at the centre of junctions. The intensity profile of each channel was exported and the individual data values for each cell were normalised to control cell values.

Wound healing assay

Cells were seeded onto 12-well plates and grown overnight. A wound was generated using a pipette tip and wound closure monitored using time-lapse phase contrast microscopy. Data was analysed in ImageJ and wound closure calculated over time.

Data availability

No externally accessible datasets were generated or analyzed during the current study.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: MP, JAM

Investigation: YB, TH, GC

Formal Analysis: YB, TH, GC

Funding acquisition: MP, JAM

Methodology: JB, TH, MP, JAM, GC

Resources: MP, JAM

Supervision: MP, JAM

Writing original draft: YB, MP

Writing review & editing: MP, JAM

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FIGURE LEGENDS

Figure 1: Mutation or loss of Slac2-b results in CD63 perinuclear accumulation

- (a) Predicted effects of mutations on Slac2-b protein structure. RBD – Rab-binding domain, Magenta box – putative PDZ binding motif.
- (b) RT-PCR analysis of *EXPH5* mRNA in WT and Slac2-b mutant keratinocytes. HSC70 was used as a loading control.
- (c) Western blot of Slac2-b (~270kDa) in WT and Slac2-b mutant keratinocytes.
- (d) Immunofluorescence of cells stained with CD63 and Phalloidin-568.
- (e) Quantification of CD63+ vesicle distribution within the cell. Perinuclear region is specified within dotted lines.
- (f) Quantification of CD63 intensity within the perinuclear region (dotted lines in (e)).
- (g) Immunofluorescence of CD63 and LAMP1 distribution in WT and Slac2-b mutant keratinocytes. White lines in merged images denote cell periphery.
- (h) Co-localization analysis of cells stained for CD63 and LAMP1.
- (i) RT-PCR analysis of *EXPH5* mRNA expression in WT and Slac2-b CRISPR cells. Quantification of *EXPH5* mRNA levels shown below.
- (j) Western blot of Slac2-b in WT and Slac2-b CRISPR cells. HSC70 was used as a loading control. Graph beneath shows quantification of Slac2-b protein levels.
- (k) Western blot of Rab27a in WT and Slac2-b mutant cells. HSC70 was used as a loading control. Graph shows quantification of Rab27a protein levels.
- (l) Western blot of Rab27a in WT and Slac2-b CRISPR cells. HSC70 was used as a loading control. Graph shows quantification of Rab27a protein levels.

Data shown in (f) and (h) are representative graphs of three independent experiments with a total of 30 cells per cell line analyzed, (i-l) are pooled from 3 independent experiments and plotted as mean $\pm$ s.e.m. Significant values assessed by Students T-

test (i-l) or one-way ANOVA with Dunnetts multiple comparisons test (f,h) are * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.001$. Scale bars: 10 μm .

Figure 2: Mutation or loss of Slac2-b leads to reduced extracellular vesicle release

- (a) Live cell confocal imaging of WT and Slac2-b mutant or CRISPR keratinocytes transfected with CD63-GFP. Top panels: Images of CD63-GFP localization in stills from movies. Middle panels: Speed tracks of individual CD63+ vesicles taken from TrackMate analysis. Bottom panels: track plots of CD63 vesicles. Color-coded bar below represents speed of individual tracks (denoted by pseudocolor table on right side).
- (b) Quantification of mean track speed from experiments as in (a).
- (c) Quantification of mean track displacement from experiments as in (a).
- (d) Representative still images from CD63+ fusion event in a WT keratinocyte expressing CD63-pHluorin. Inset shows MVB-PM fusion event.
- (e) Quantification of number of fusion events and signal duration from movies as in (d). 30 cells per cell line were analyzed,
- (f) Western blot showing CD63 (~60kDa) and Calnexin (~67kDa) protein levels in whole cell lysates (WCL) and extracellular vesicle (EV) pellets from WT and Slac2-b mutant keratinocytes. Quantification from n=4 experiments beneath+/-SEM.
- (g) Quantification of concentration of EV release (as particles/ml) from all cells.
- (h) Distribution of EV size as a function of percentile within each cell line.

Data shown in (b), (c), (e), (g) and (h) are from three independent experiments with 30 cells per cell line analyzed and plotted as mean $\pm$ s.e.m. Significance values assessed by

one-way ANOVA with Dunnetts multiple comparisons test are $\ast=p<0.05$, $\ast\ast = p < 0.01$, $\ast\ast\ast\ast = p < 0.001$. Scale bar: 10 μm .

Figure 3: Mutation of Slac2-b leads to reduced keratinocyte adhesion and migration

- (a) Adhesion assays of WT, Slac2-b mutant and Slac2-b CRISPR keratinocytes (bottom) plated onto Matrigel.
- (b) Immunofluorescence of WT and Slac2-b mutant keratinocytes stained with vinculin (red) and $\beta 4$ integrin (green).
- (c) Quantification of number of focal adhesions and hemidesmosomal (HD) coverage per cell area.
- (d) Quantification of adhesion assembly and disassembly rates from time-lapse movies of WT and Slac2-b mutant keratinocytes expressing GFP-Talin.
- (e) Immunofluorescence of monolayers of WT and Slac2-b mutant keratinocytes stained with E-cadherin, β -catenin and Phalloidin-647.
- (f) Analysis of monolayer permeability using TRITC-dextran.
- (g) Representative images of wound closure over a period of 12 hours in Slac2-b mutant, CRISPR and WT keratinocytes. Scale bar – 100 μm .
- (h) Quantification of the percentage of wound closure.

Data shown in (a), (c), (d), (f) and (h) are representative graphs of three independent experiments (10-15 cells per condition per experiment) and plotted as mean $\pm$ s.e.m.

Significance values assessed by one-way ANOVA with Dunnetts multiple comparisons test are $\ast=p<0.05$, $\ast\ast=p<0.01$, $\ast\ast\ast = p < 0.005$, $\ast\ast\ast\ast = p < 0.001$. Scale bars – 10 μm .

Figure 4: Extracellular vesicle traffic is coordinated with focal adhesion assembly

- (a) Representative still images taken from movies of WT keratinocytes stably expressing Talin-mCherry and transfected with CD63-GFP. Scale bars: 10 μ m. Highlighted region in white box of merged panel shown on far right.
- (b) Graph showing normalized fluorescence intensity of CD63 (green) and assembly of Talin positive focal adhesions. Movies were generated imaging single cells for 10 mins with a frame taken every 30 seconds. Data are plotted as mean $\pm$ s.e.m.
- (c) Graph showing normalized fluorescence intensity of CD63 (green) and disassembly of Talin FAs. Movies were generated imaging single cells for 10 mins with a frame taken every 30 seconds. Data are plotted as mean $\pm$ s.e.m.
- (d) Graphs showing correlation between FA assembly and CD63.
- (e) Graphs showing correlation between FA disassembly and CD63.
- (f) Western blots of whole cell lysates (WCL) and extracellular vesicle (EV) pellets from WT and Slac2-b mutant keratinocytes probed for Fibronectin (~220kDa) and LN332 (~150kDa).
- (g) Immunofluorescence of WT and Slac2-b mutant keratinocytes stained for Laminin332 (red) and Fibronectin (green).
- (h) Graphs showing quantification of FN and LN332 deposition.
- (i) Summary model of Slac2-b functions; see Discussion for further details.

Data shown in (b), (c), (d), (e) and (h) are representative of three independent experiments from a total of 30 cells and plotted as mean $\pm$ s.e.m. Significant values assessed by one-way ANOVA with Dunnetts multiple comparisons test are $*=p<0.05$, $**=p<0.01$, $*** = p < 0.005$.

Figure 1

Figure 2

Figure 3

Figure 4

Supplementary information

MATERIALS AND METHODS

Cell culture and transfections

Immortalised WT keratinocytes and cells taken from *EXPH5* mutant patients harbouring the c.5786delC/p.Pro1929Leufs*11 mutation (EB52) have been previously described (McGrath et al. 2012). Cells carrying the *EXPH5* c.4483delA/p.Met1495fs*0 mutation (EB63) were isolated from a 7 year old British Pakistani male. The original study in which the cells were isolated was conducted according to the principles of the Declaration of Helsinki. All cells were obtained under the St. Thomas Hospital Ethics Committee-approved project ‘Molecular Basis of Inherited Skin Disease’ (07/H0802/104) after participating individuals gave written, informed consent. All cell lines were grown in high glucose DMEM (Sigma Aldrich) and Ham’s F12 Nutrient Mix (at a 3:1 ratio) containing 10% fetal bovine serum (FBS, Hyclone Industries) and supplemented with penicillin and streptomycin, L-glutamine and RM+ supplement containing EGF (10 ng/ml), Insulin (5 µg/ml), Transferrin (5 µg/ml), Hydrocortisone (40 ng/ml), Cholera toxin (8.4 ng/ml) and Liothyronine (13 ng/ml).

CRISPR-Cas9 and GFP Slac2-b plasmids

Immortalised WT keratinocytes were transfected with CRISPR plasmids (GenScript, New Jersey, USA) containing GFP, the coding sequence for Cas9 enzyme from *S. pyogenes* and single guide RNAs (sgRNAs) directed against exon 4 of *EXPH5* (CAGTTTCTTAAATGACGAAG). FACS analysis was then performed to enrich the GFP⁺ population and cells were then expanded for analysis. Gene expression of resulting cell populations was assessed by RT-PCR analysis and targeting verified by sequencing. Full-length Slac2-b sequence was purchased from Transomics and cloned into eGFP between

*Bam*HI and *Not*I sites. To ensure that the Slac2-b coding sequence was in frame with the open reading frame of the promoter and fluorescent tag, the Q5 Site-Directed Mutagenesis kit (NEB) and the following primers were used: 5'-AAGAAAATGACGAAGTTCCTCC-3' and 5'-CTCGGTACCAAGCTTGGC-3'. The construct was verified by sequencing.

Western blotting

Cells were lysed in cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 200 mmol/L NaCl, 1% NP-40, 2 mmol/L MgCl₂, 10% glycerol) containing protease inhibitors, and the lysates were cleared by centrifugation. An equal volume of 2x SDS-PAGE sample buffer (60 mmol/L Tris-HCl, pH 6.8; 25% glycerol; 2.5% SDS; 0.02% bromophenol blue) containing 2-mercaptoethanol was added to the lysate and boiled for 10 minutes. All cell lysates were resolved on 6%, 10% and 12% SDS-PAGE gels at 150V for 1.5h. Proteins were then transferred onto nitrocellulose western blot membranes at 100V for 1h. Membranes were then blocked for 1h at room temperature in 5% bovine serum albumin/PBS and then probed with primary antibodies diluted in 5% bovine serum albumin/PBS overnight at 4°C. Blots were then washed three times with TBS containing 0.1% Tween-20 (TBST) and incubated with secondary antibodies diluted in 5% bovine serum albumin/PBS for 1h at room temperature. Finally, blots were washed three times in TBST and proteins were detected by using a ECL chemiluminescence kit (BioRad) and directly imaged using a BioRad imager. Blots were analysed using the BioRad Image Lab software (v5.2.1).

Reverse Transcriptase PCR

RNA extraction from cells was performed using RNAeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcription of RNA was carried out using LunaScript RT SuperMix Kit (NEB, Ipswich, MA), according to the manufacturer's instructions. Reverse transcriptase-

PCR primers sequences are as follows: *GAPDH* (forward 5'-AGAAGGCTGGGGCTCATTTG-3' , reverse 5'-AGGGGCCATCCACAGTCTTC-3'); *EXPH5* start of exon 6 (forward 5'-CATTGAGGAATGGGCCACCT-3', reverse 5'-GGAGGCAGCCATTTTACCCT-3'); *EXPH5* end of exon 6 (forward 5'-CAGATCTTTTCTGAACTCCCC-3', reverse 5'-GCACATGTACACCTTAGTTCCA-3').

Dextran permeability assays

Cells were seeded in 6.5 mm collagen coated Transwell chambers (Corning) with 8.0 µm pores. Wells were reviewed after 24 hrs to ensure an even covering of cells with a stable complete monolayer. 10 µl of FITC-dextran (20 KDa; Sigma) solution was added to the upper chamber of all wells. 100 µl of the media was then collected from the lower chamber at 30 minutes and one hour after addition of FITC-dextran. The collected samples were then compared for relative FITC-dextran concentration on a Fluostar Omega fluorescence plate reader (BMG). The system compared light absorption from each sample to provide a comparative reading of light transmission between the samples.

Focal adhesion and spread area analysis

To calculate spread cell area and focal adhesion/HD number post-adhesion, images of cells were thresholded in ImageJ and area (phalloidin) or focal adhesion size/no. per cell (vinculin) calculated. Mean FA size was normalised for cell area. For live and fixed analysis of focal adhesions, keratinocytes expressing Talin-GFP were plated onto 8 well imaging chambers (Ibidi) pre-coated with fibronectin and incubated for 5 hours at 37 °C and 5% CO₂. The time-lapse images were then uploaded onto the Focal Adhesion Analysis Server (FAAS - <https://faas.bme.unc.edu>) to generate threshold values of the focal adhesion for subsequent analysis. The dynamic properties, such as the rates of focal adhesion assembly and

disassembly, were calculated by tracking the changes in fluorescence intensity from single adhesions through the subsequent time frames. For correlation analysis of CD63 and focal adhesions, a region of interest (ROI) was drawn around areas containing 1- 2 focal adhesions and the stack was duplicated. The channels were then split and the fluorescence intensities over time were quantified using ImageJ (Stacks > Plot Z-axis profile). Values were then normalised as a function over time, whereby the intensity was measured as 1 at $t = 0$.

Time-lapse imaging and analysis of CD63

Images of live CD63-pHluorin or GFP-CD63 expressing cells were acquired on an Eclipse Ti-E TIRF inverted microscope (Nikon) or Nikon A1R inverted confocal microscope with an environmental chamber maintained at 37 °C and a DU-897 X-3430 camera (Andor) using a 60x oil immersion objective with a numerical aperture of 1.4. Excitation wavelengths of 488 nm and 561 nm were used. Images were acquired using NIS-Elements imaging software (v4) and were saved in the .nd2 format. Image processing and analysis was performed using ImageJ. For CD63 positioning analysis in fixed cells, a region of interest (ROI) was drawn around the cell area identified by phalloidin staining. The channels were then split and the image corresponding to the CD63 channel was binarised. The Region Scaler macro (gift from Dr. Mark Dodding, University of Bristol) was then applied, quantifying the fluorescence intensity of a series of 10 regions at 10% decrements from the original ROI. These values were then normalised against the total fluorescence intensity of the entire cell and plotted against the distance from the cell periphery to the cell centre. To quantify CD63- positive vesicle trafficking in live cells, images were analysed using the TrackMate plug-in (ImageJ). The LoG (Laplacian of Gaussian) detector was applied and the spot diameter and threshold values were set according to each image to filter out spurious spots. Then to track the spots over time, a simple LAP (Linear Assignment Problem) tracker was used. The maximal

linking and gap-closing distances were set to 4 μm for optimal particle tracking. The colours of each track were changed to represent their mean velocities to better visualise their trafficking. Once the analysis was run, the data corresponding to the mean velocity and displacement of all the tracks per cell were plotted for each cell. To quantify CD63 membrane fusion events, an ROI was drawn around the individual fusion events and the fluorescence intensity over time of each ROI was plotted in ImageJ (Stacks > Z-Plot Z-axis profile). The number of fusion events and the duration taken for the fluorescence intensity to return back to plasma membrane levels were plotted for each cell line.

Statistical analysis

All statistical tests were performed using either student's T test or analysis of variance in GraphPad Prism (GraphPad, La Jolla, CA). All data represent at least three independent experiments. Statistically significant results were taken as $p < 0.05$, and significance values were assigned in specific figures/experiments as shown.

Supplementary Figure 1

(a) Slac2-b WT and mutant keratinocytes plated on Collagen I- or Matrigel-coated plates and stained with Phalloidin. Scale bar – 25 μm .

(b) Quantification of cell area.

(c) Confocal image of Slac2-b CRISPR cells re-expressing GFP or GFP-Slac2-b. Scale bar – 10 μm

(d) Quantification of spread area in Slac2-b CRISPR cells expressing GFP or GFP-Slac2-b

(e) Quantification of focal adhesion no./cell in Slac2-b CRISPR cells expressing GFP or GFP-Slac2-b

(f) Confocal images of WT, Slac2-b mutant and Slac2-b CRISPR keratinocytes monolayers stained for keratin14 (green), F-actin (red) and nuclei (blue). Scale bar – 10 μm .

All data is representative of three independent experiments and is plotted as mean $\pm$ s.e.m. Significance values assessed by Students T-test (d,e) or one-way ANOVA with Dunnetts multiple comparisons test (b) are values are **= $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.001$.