Exophilin-5 supports lysosome-mediated trafficking required for epidermal differentiation

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TO THE EDITOR

Germline mutations in *EXPH5* are associated with a recessive form of epidermolysis bullosa (EB), with skin fragility that cannot be attributed to mutations in *KRT5*, *KRT14*, or other established EB-related genes (McGrath et al., 2012, Pigors et al., 2014). Clinically identified mutations in *EXPH5* result in premature truncations in the encoded protein Exophilin-5 (also referred to as Slac2-b). This is associated with mild blistering, which in some cases is accompanied by mottled hypopigmentation. EXPH5 knockout mice are not available. The normal physiologic role of Exophilin-5 in epidermis, and the mechanism by which Exophilin-5 loss contributes to skin disease are unclear.

Exophilin-5, an effector of Rab27, is implicated in intracellular vesicular trafficking and secretion (Ostrowski et al., 2010). Therefore, we hypothesized that Exophilin-5 may be required in keratinocytes for the normal vesicular trafficking of lamellar bodies (LBs) that extrude lipids into the extracellular space during normal epidermal differentiation. As LBs

Conflict of Interest
The authors have no conflict of interest.

contain enzymes and membrane features associated with lysosomes, these secretory vesicles are also classified as Lysosome Related Organelles (LROs) (Eckhart et al., 2013, Raymond et al., 2008).

To define the role of Exophiln-5 and lysosomal exocytosis in human epidermal homeostasis, we inhibited the LRO exocytosis trafficking pathway both pharmacologically and genetically, in three-dimensional human organotypic skin cultures (OTCs) engineered using primary keratinocytes and devitalized human dermis (Ridky et al., 2010). Control tissues stratified and differentiated normally, as evidenced by the coordinated expression of keratin-10 and filaggrin (Figure 1a). However, epidermal differentiation was inhibited in the presence of vacuolin, a small molecule that blocks the fusion of secretory LROs with the plasma membrane (Figure 1a) (Supplementary Figure S1). To specifically define the role of EXPH5 in lysosomal trafficking in keratinocytes, and to test whether EXPH5 is required for normal epidermal homeostasis, we genetically depleted EXPH5 using two different shRNAs (Supplementary Figure S2). In contrast to the well-differentiated epidermis observed in the Non-Silenced (NS) shRNA control, tissues with EXPH5 depletion (EXPH5i) were poorly differentiated, as evidenced by loss of keratin-10 and filaggrin (Figure 1b, Supplementary Figure S2). Consistent with the skin fragility phenotype associated with EXPH5 germline mutation, organotypic epidermis lacking EXPH5 was also hypoproliferative, and lacked the uniform, peripheral distribution of Desmoglein-3 seen throughout the keratinocyte plasma membranes in normal control samples. Normal expression and localization of collagen VII and β1 integrin demonstrates that architecture at the dermal-epidermal junction was grossly intact in EXPH5- depleted tissue. Together, these data demonstrate that normal epidermal differentiation and proliferation depend on EXPH5, and delivery of LROs to the keratinocyte plasma membrane.

To determine whether secreted LROs from differentiating keratinocytes communicate with adjacent cells to promote differentiation, we assembled mosaic OTCs containing and equal mixture of EXPH5i and Non-Silenced (NS) keratinocytes. Epidermal differentiation was rescued in EXPH5i keratinocytes when they were co-cultured with NS keratinocytes (tagged with K14- HA), as evidenced by the expression of both early and late differentiation proteins including keratin-10, filaggrin, and loricrin (Figure 2, Supplementary Figure S4). To test whether this ability of normal keratinocytes to rescue differentiation *in trans* was specific to EHPH5 deficiency, we pharmacologically inhibited epidermal differentiation using Lys05, a lysosome inhibitor that we previously determined inhibits differentiation (Monteleon et al., 2018). As we observed with EXPH5 depletion, differentiation was also restored in Lys05 treated keratinocytes when co-cultured with an equal number of normal keratinocytes (Supplementary Figure S5).

LRO-lamellar bodies contain complex cargo, and it is unlikely that rescue *in trans* results from trafficking of a single factor. Lysosome-associated enzymes contribute to the biosynthesis of ceramide, formation of the cornified envelope, and the proteolytic degradation that facilitates corneocyte desquamation (Egberts et al., 2004). It has also been demonstrated that secretory cargo initiates signaling back to keratinocytes through pathways that are not well understood (Appelqvist et al., 2013, Conus et al., 2012, Kovalenko et al., 2009). Interestingly, the signals that stimulate lysosomes to traffic as exocytic vesicles

(Appelqvist et al., 2013, Jans et al., 2004), principally the sustained elevation of cytoplasmic calcium and increased oxidative stress, are the same factors responsible for initiating keratinocyte differentiation, and therefore may be inextricably linked.

Many different physical, chemical, and genetic factors can lead to dysfunction in LB/LRO assembly or trafficking in keratinocytes. Disruption of normal LB secretion compromises the lipid barrier, which renders skin more susceptible to dehydration, mechanical stress, and infiltration by microbes, and contributes to functional barrier defects in skin disorders including eczema and ichthyosis (Elias and Wakefield, 2014, Milner et al., 1992, Rizzo et al., 2010, Werner et al., 1986). LROs are also necessary for packaging and trafficking of melanin, which may be partially responsible for the mottled pigmentation in patients with *EXPH5*-EB (Turcan et al., 2016).

This current work furthers our understanding of epidermal homeostasis, and suggests that keratinocyte differentiation within epidermis may not be a purely cell-autonomous process. Specifically, cell-cell communication via lysosome-mediated exocytosis may contribute to both early and late differentiation. The capacity for normal keratinocytes to rescue epidermal differentiation *in trans* in adjacent keratinocytes with defective lysosomal exocytosis suggests that future gene therapy approaches for some genetic skin disorders may require correction of only a subset of keratinocytes, rather than the entire epidermal keratinocyte population.

MATERIALS AND METHODS

Cell culture

All experiments were conducted using primary keratinocytes. Cells were isolated from normal human skin by previously described methods (Ridky et al., 2010). Keratinocytes were cultured in a 1:1 mixture of Gibco Keratinocytes-SFM medium + L-glutamine + EGF + BPE and Gibco Cascade Biologics 154 medium with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Transduced keratinocytes were fully puromycin selected before the commencement of each experiment. Lys05 (Gift from R. Amavaradi at U. of Pennsylvania) was used at 2 μM. Vacuolin (Sigma, St. Louis, MO) was used at 2 μM. Ionomycin (Sigma) was used at 30 μM. LysoSensor (Molecular Probes, Carlsbad, CA) was used at 1 μM.

Lentiviral and Retroviral Production and Transduction

293T or 293T phoenix cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% FBS containing Antibiotic/Antimycotic. Lentiviral shRNA (OpenBiosystems, Lafayette, CO) particles were generated according to Thermoscientific specifications and as described previously (Ridky et al., 2010). For the production of viral particles, lentiviral constructs were co-transfected with viral packaging plasmids pCMV R8.91 and pUC-MDG into 293T cells using Fugene 6 Transfection Reagent (Promega, Fitchburg, WI). Retroviral particles, made from a phoenix cells carrying a LZRS viral vector expressing human K14 with a c-terminal HA tag, were used to transduce keratinocytes in order to label with HA.

Organotypic cultures

Organotypic skin cultures were established using parental or genetically engineered keratinocytes. For each culture, between 8.0×10^5 and 1.0×10^6 keratinocytes were suspended in 80 μL KGM or high calcium (1.2uM CaCl^2) growth media, and seeded onto devitalized human dermis, according to previously established methods (Ridky et al., 2010). Unless otherwise indicated, small molecule and other chemical treatments were begun at seeding. OTCs were maintained at 37 °C at an air-liquid interface for 4–12 days.

Immunofluorescence microscopy

Whole mount cryosections were prepared for immunofluorescence microscopy as previously described (Ridky et al., 2010). In short, slides were fixed in 4% paraformaldehyde or −20°C methanol, permeabilized as required and blocked with 10% horse serum/PBS, followed by incubation with primary antibodies and secondary antibodies conjugated to fluorophores. Slides were mounted with Prolong Gold Antifade Reagent with DAPI (Life Technologies, Grand Island, NY). The primary antibodies used in this study were collagen-VII (Millipore, Burlington, MA), ß1-integrin (Abcam, Cambridge, MA), ki67, desmoglein-3 (Thermo Fisher Scientific, Carlsbad, CA), HA, keratin-10, keratin-5, loricrin, and filaggrin (Covance, Dedham, MA). To quantify differentiation, the ratio of the area in pixels of keratin-10 positive epidermis to area in pixels of total epidermis was measured as a percentage in ImageJ. This analysis was based previously reported methods (Billings et al., 2015, Natale et al., 2018). Results are the mean of at least 3 technical replicates across at least three biologic replicates from individual donors (± s.d.). Significance was assessed by Welch's t-test across the biological replicates.

Quantitative RT/PCR

RNA was extracted from cells and tissues according to the RNeasy Mini Kit protocol (Qiagen, Valencia, CA), and reverse transcribed to cDNA using the High Capacity RNA-to cDNA kit (Applied Biosystems, Grand Island, NY). Quantitative PCR of resulting cDNA was conducted using Power SYBR Green Master Mix (Applied Biosystems, Grand Island, NY) and gene-specific primers, with three technical replicates on a ViiA 7 Real-Time PCR System (Life Technologies, Grand Island, NY). Relative expression was determined using the 2-[delta][delta] Ct method. Results are the average from at least two individual donors (± s.d.).

DATA AVAILABILITY STATEMENT

Datasets related to this article can be found at http://dx.doi.org/10.17632/82zk6kdx6c.1, hosted at Mendeley (Lee, In Young (2019), "Role of Exophilin-5 in keratinocyte differentiation", Mendeley Data, v1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations:

EB	Epidermolysis Bullosa
EXPH5	Exophilin-5
LBs	lamellar bodies
OTC	Organotypic Culture
NS	Non-silenced
LRO	Lysosome Related Organelle

REFERENCES

Appelqvist H, Wäster P, Eriksson I, Rosdahl I, Öllinger K. Lysosomal exocytosis and caspase-8-mediated apoptosis in UVA-irradiated keratinocytes. J Cell Sci 2013;126(24):5578–84. [PubMed: 24127565]

Conus S, Pop C, Snipas SJ, Salvesen GS, Simon H-U. Cathepsin D primes caspase-8 activation by multiple intra-chain proteolysis. Journal of Biological Chemistry 2012;287(25):21142–51. [PubMed: 22528489]

Eckhart L, Lippens S, Tschachler E, Declercq W. Cell death by cornification. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 2013;1833(12):3471–80. [PubMed: 23792051]

Egberts F, Heinrich M, Jensen JM, Winoto-Morbach S, Pfeiffer S, Wickel M, et al. Cathepsin D is involved in the regulation of transglutaminase 1 and epidermal differentiation. J Cell Sci 2004;117(Pt 11):2295–307. [PubMed: 15126630]

Elias PM, Wakefield JS. Mechanisms of abnormal lamellar body secretion and the dysfunctional skin barrier in patients with atopic dermatitis. J Allergy Clin Immunol 2014;134(4):781–91 e1. [PubMed: 25131691]

Jans R, Sartor M, Jadot M, Poumay Y. Calcium entry into keratinocytes induces exocytosis of lysosomes. Archives of dermatological research 2004;296(1):30–41. [PubMed: 15127211]

Kovalenko A, Kim JC, Kang TB, Rajput A, Bogdanov K, Dittrich-Breiholz O, et al. Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. The Journal of experimental medicine 2009;206(10):2161–77. [PubMed: 19720838]

McGrath JA, Stone KL, Begum R, Simpson MA, Dopping-Hepenstal PJ, Liu L, et al. Germline mutation in EXPH5 implicates the Rab27B effector protein Slac2-b in inherited skin fragility. The American Journal of Human Genetics 2012;91(6):1115–21. [PubMed: 23176819]

Milner ME, O'Guin WM, Holbrook KA, Dale BA. Abnormal lamellar granules in harlequin ichthyosis. J Invest Dermatol 1992;99(6):824–9. [PubMed: 1281866]

Monteleon CL, Agnihotri T, Dahal A, Liu M, Rebecca VW, Beatty GL, et al. Lysosomes Support the Degradation, Signaling, and Mitochondrial Metabolism Necessary for Human Epidermal Differentiation. J Invest Dermatol 2018.

Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nature cell biology 2010;12(1):19–30. [PubMed: 19966785]

Pigors M, Schwieger-Briel A, Leppert J, Kiritsi D, Kohlhase J, Bruckner-Tuderman L, et al. Molecular heterogeneity of epidermolysis bullosa simplex: contribution of EXPH5 mutations. Journal of Investigative Dermatology 2014;134(3):842–5. [PubMed: 24005056]

Raymond A-A, de Peredo AG, Stella A, Ishida-Yamamoto A, Bouyssie D, Serre G, et al. Lamellar Bodies of Human Epidermis Proteomics Characterization by High Throughput Mass Spectrometry and Possible Involvement of CLIP-170 in their Trafficking/Secretion. Molecular & Cellular Proteomics 2008;7(11):2151–75. [PubMed: 18622020]

Ridky TW, Chow JM, Wong DJ, Khavari PA. Invasive three-dimensional organotypic neoplasia from multiple normal human epithelia. Nat Med 2010;16(12):1450–5. [PubMed: 21102459]

Rizzo WB, S'Aulis D, Jennings MA, Crumrine DA, Williams ML, Elias PM. Ichthyosis in Sjogren-Larsson syndrome reflects defective barrier function due to abnormal lamellar body structure and secretion. Arch Dermatol Res 2010;302(6):443–51. [PubMed: 20049467]

Turcan I, Pasmooij AM, Van den Akker PC, Lemmink H, Sinke RJ, Jonkman MF. Association of Epidermolysis Bullosa Simplex With Mottled Pigmentation and EXPH5 Mutations. JAMA Dermatol 2016;152(10):1137–41. [PubMed: 27384765]

Werner Y, Lindberg M, Forslind B. Membrane-coating granules in" dry" non-eczematous skin of patients with atopic dermatitis. A quantitative electron microscopic study. Acta dermato-venereologica 1986;67(5):385–90.
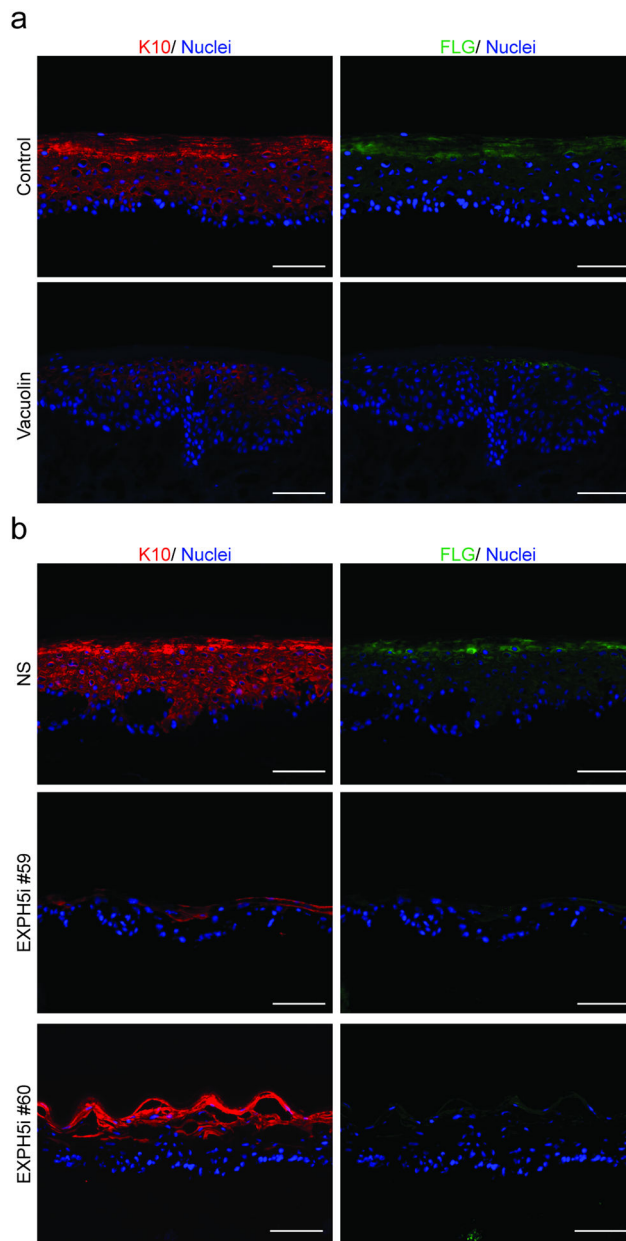
Figure 1: Delivery of Lysosome Related Organelles to the Plasma Membrane is essential for normal keratinocyte differentiation.

(**a**) Control organotypic human epidermis differentiated properly, including the spatially coordinated expression of keratin-10 (red) and filaggrin (green), nuclei (blue). Differentiation was inhibited when lysosome-mediated trafficking was blocked with Vacuolin (10 µM) (**b**) shRNA knockdown of Exophilin-5 (EXPH5i) resulted in similarly diminished epidermal differentiation, keratin-10 (red) and filaggrin (green), nuclei (blue). (Scale Bars = 100um).
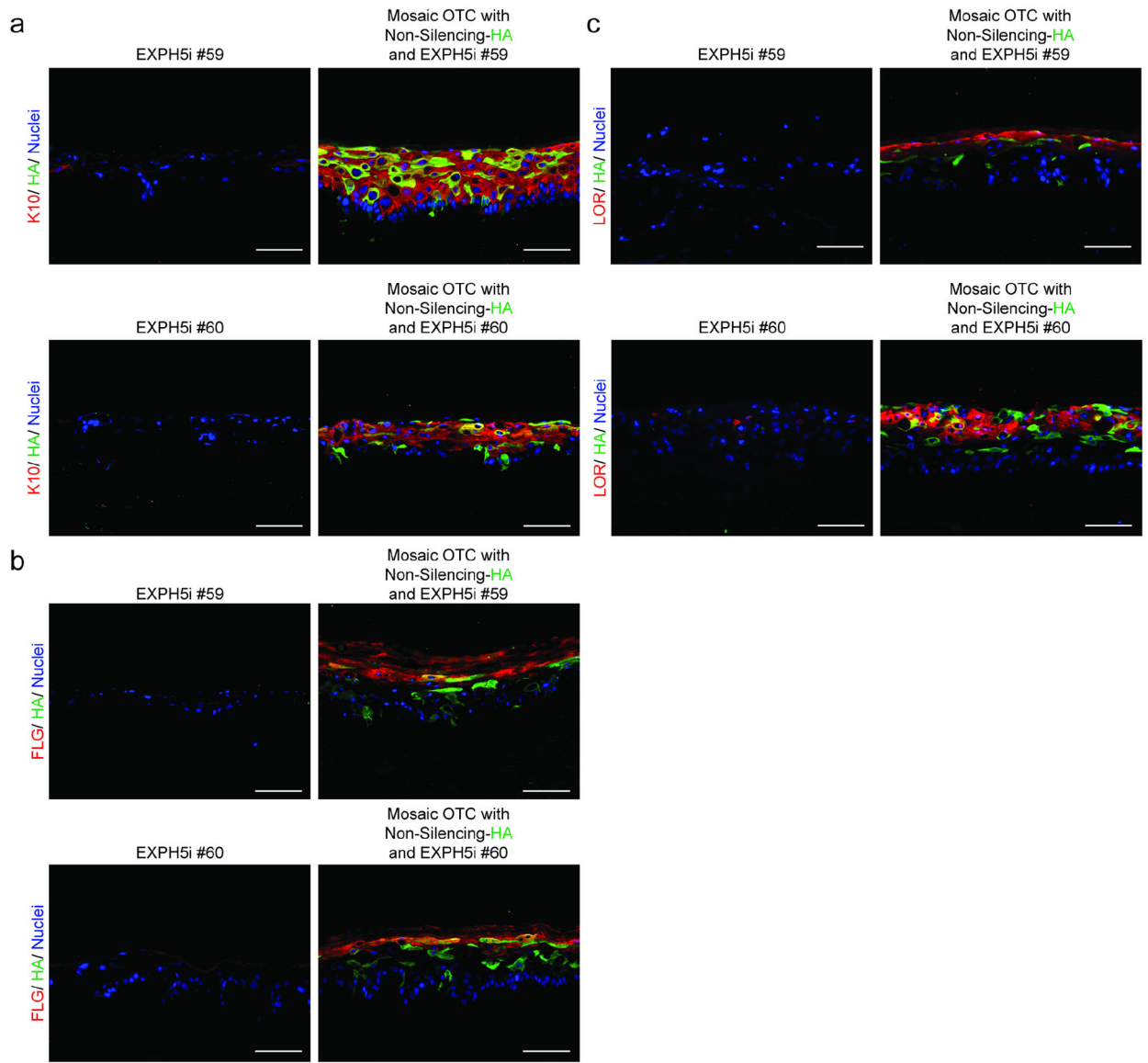
Figure 2: The differentiation defect in keratinocytes with lysosomal defects is rescued in trans by co-culture with normal keratinocytes.
(**a**) Organotypic epidermis engineered with EXPH5i keratinocytes yielded undifferentiated tissue lacking keratin 10 (red). In contrast, mosaic tissue engineered using an equal mixture of EXPH5i keratinocytes and control NS keratinocytes (tagged with K14-HA, green) expressed keratin 10 (red) in both normal and EXPH5i keratinocytes. (**b, c**) Expressions of filaggrin and loricrin are also restored in EXPH5i- keratinocytes when co-cultured with NS-keratinocytes. (Scale Bars = 100um).