Loss-of-Function Variants in SERPINA12 Underlie Autosomal Recessive Palmoplantar Keratoderma

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Inherited palmoplantar keratodermas refer to a large and heterogeneous group of conditions resulting from abnormal epidermal differentiation and featuring thickening of the skin of the palms and soles. Here, we aimed at delineating the genetic basis of an autosomal recessive form of palmoplantar keratoderma manifesting with erythematous hyperkeratotic plaques over the palms and soles, extending to non-palmoplantar areas. Whole-exome sequencing in affected individuals revealed homozygous nonsense variants in the SERPINA12 gene. SERPINA12 encodes the visceral adipose tissue-derived serpin A12, a serine protease inhibitor. The pathogenic variants were found to result in reduced visceral adipose tissue-derived serpin A12 expression in patients’ skin biopsies in comparison to healthy controls. In addition, SERPINA12 downregulation in three-dimensional skin equivalents was associated with marked epidermal acanthosis and hyperkeratosis, replicating the human phenotype. Moreover, decreased SERPINA12 expression resulted in reduced visceral adipose tissue-derived serpin A12-mediated inhibition of kallikrein 7 activity as well as decreased levels of desmoglein-1 and cornodesmosin, two known kallikrein 7 substrates, which are required for normal epidermal differentiation. The present data, taken collectively, demarcate a unique type of autosomal recessive palmoplantar keratoderma, attributable to visceral adipose tissue-derived serpin A12 a role in skin biology, and emphasize the importance of mechanisms regulating proteolytic activity for normal epidermal differentiation.

Abbreviations: CDSN, cornodesmosin; DSG1, desmoglein-1; PPK, Palmoplantar keratoderma; vaspin, visceral adipose tissue-derived serpin A12

INTRODUCTION
Palmoplantar keratodermas (PPKs) form a large and heterogeneous group of hereditary disorders that result from abnormal epidermal differentiation (Guerra et al., 2018a, 2018b). PPKs manifest with diffuse, focal, striate, or punctate thickening of the skin of the palms and soles (Sakiyama and Kubo, 2016). They are usually inherited in an autosomal dominant or recessive fashion (although X-linked and mitochondrial inheritances have been described) (Guerra et al., 2018a, 2018b). PPKs can present as isolated skin conditions or in association with extracutaneous features. Skin hyperkeratosis can occasionally be associated with mutilating features (i.e., auto-amputation) and extend to contiguous (PPK transgrediens) as well as non-contiguous skin areas such as elbows, knees, and periorificial regions (Guerra et al., 2018a, 2018b). More than 20 different genes encoding structural proteins as well as enzymes, their inhibitors, and adhesion proteins, have been implicated in the pathogenesis of non-syndromic PPKs (Guerra et al., 2018a). However, the genetic basis of a substantial fraction of inherited PPKs has yet to be deciphered (Greiner et al., 1983; Guerra et al., 2018a; Khan et al., 2010; Nazik et al., 2016).

In the present study, we demonstrate that nonsense variants in SERPINA12, coding for the visceral adipose tissue-derived serpin A12 (vaspin), underlie an autosomal recessive non-mutilating diffuse form of PPK.

RESULTS
Clinical and histopathological features
We studied two individuals who were referred for investigation because of PPK (Figures 1 and 2a). Patient 1 (individual II-2, family A) is a 6-year-old child who was born to healthy first-degree cousins of Muslim Arab descent (Figure 2a). Apart from a brother with atopic dermatitis, family history was unremarkable. The patient herself had been diagnosed with asthma since early childhood. The patient was observed soon after birth to display erythematous plaques over the distal parts of her fingers and toes. Over the years, her parents noticed the progressive proximal expansion of the lesions as well as thickening and yellowish discoloration of the skin, up to the involvement of the entire palmoplantar surface (Figure 1a and b) with extension to the Achilles tendon region (Figure 1b) and to the anterior aspect of the leg (Figure 1c). The patient occasionally displayed slight peeling over the cheeks (not...
Figure 1. Clinical and histopathological features. Affected individuals presented with (a, b) erythematous diffuse hyperkeratosis of the palms and soles with peripheral peeling, extending to the Achilles tendon area and to (c) the anterior part of the leg in individual II-2, Family A, and (d) involving the groin area in individual II-1, Family B; (e) Histopathological examination of palmar skin biopsy demonstrated marked orthohyperkeratosis, acanthosis and intact granular layer (H&E, Bar = 50 μm). The patient’s guardians consented to the publication of the images.
shown). Patient 2 (individual II-1, family B) is a 2-year-old child who was born to healthy individuals of Jewish descent (Figure 2a). Although her parents denied any familial relation, they both originate from the Indian Jewish community of Mumbai (Behar et al., 2010). Since the age of 6 months, the patient displayed erythematous and hyperkeratotic palms and soles associated with peripheral peeling of the skin and hyperkeratotic well-demarcated plaques over the groin areas (Figure 1d). Her condition worsened in the summer and after exposure to water. Ophthalmological, cardiac, and auditory examinations were unremarkable.

Histopathological examination of skin biopsies obtained from the patients (family A, II-2, and family B, II-1) revealed a thickened orthohyperkeratotic keratin layer along with an intact granular layer (Figure 1e). In addition, widening of intercellular spaces was observed in patient 1 (individual II-2, family A).

**Mutation analysis**

DNA samples obtained from individuals I-1 and II-2 from family A (Figure 2a), were subjected to whole-exome sequencing (Supplementary Table S1). A homozygous transition in SERPINA12 (NM_173850.4) was identified in individual II-2, family A (left upper panel) as well as a homozygous G > T transversion (arrow) at position c.1051 of the cDNA sequence in individual II-1, family 2 (right upper panel). The wild-type sequences are given for comparison (lower panels). The location of the two mutations is depicted along with a schematic representation of the SERPINA12 gene and vaspin protein and its domains. Serpins exhibit a conserved structure comprising a C-terminal β domain which includes the reactive center loop which forms the initial interaction with the target protease during inhibition; (d) Skin biopsies from a healthy individual (control) and individual II-2, family A, and individual II-1, family B were stained with an anti-vaspin specific antibody (upper panel). Immunostaining was significantly reduced in affected vs. normal skin (lower panel) (Bar = 50 μm). Results are expressed as the percentage of fluorescence intensity relative to the intensity in control biopsies ± standard error. Results represent the mean of three different measurements (one-way ANOVA; *P < 0.05, **P < 0.01). Vaspin, visceral adipose tissue-derived serpin A12; WT, wild type.

The two SERPINA12 variants were found to co-segregate with the disease’s phenotype in both families (Figure 2a) (Supplementary Figure S1). Both variants result in a premature termination codon (p.Arg211Ter and p.Glu351Ter) (Figure 2c) and are predicted to be pathogenic according to bioinformatics prediction tools (Supplementary Table S2). In addition, previous studies have demonstrated that the p.Arg211Ter variant is unstable in vitro, and that heterozygous carriers of this variant display reduced circulating vaspin levels (Breitfeld et al., 2013). Moreover, vaspin protein expression was significantly reduced in patients’ skin as compared with normal skin (Figure 2d).

Of note, the c.631C > T, p.Arg211Ter variant is relatively common in the general population (minor allele frequency = 0.005120) and is reported in six individuals in a homozygous state in the Genome Aggregation Database. These data suggest the possibility that the non-painful and non-mutilating form of PPK shown by the patients (Figure 1) is either partially penetrant or clinically underdiagnosed as previously described in the context of other palmoplantar pathologies (Has, 2018; van der Velden et al., 2012; Wilson et al., 2010). In contrast with p.Arg211Ter, the c.1051G > T, p.Glu351Ter variant is very rare, being present in a heterozygous state in 2 of 125,341 individuals in the Genome Aggregation Database.

**Consequences of c.631C>T or c.1051G>T variants in SERPINA12**

To ascertain the consequences of the two variants in eukaryotic cells, we transfected HaCaT cells with expression vectors encoding either wild type or mutant (harboring
c.631C>T or c.1051G>T SERPINA12 and quantified vaspin expression. Despite the fact that the three vectors were found to be expressed at similar levels using RT-qPCR (Figure 3a), the variants-carrying vectors were found to result in a dramatic decrease in vaspin protein expression levels as assessed by ELISA (Figure 3b and c), suggesting that the two variants affect protein stability as previously found in the case of p.Arg211Ter in a bacterial expression system (Breitfeld et al., 2013). Those results were validated using Western blotting (Figure 3d and e).

Figure 3. Expression of PPK-associated pathogenic SERPINA12 variants. HaCaT cells were transfected with either an empty plasmid or with plasmids expressing either wild-type SERPINA12 cDNA (wild type) or SERPINA12 cDNA harboring c.631C>T or c.1051G>T mutations. (a) SERPINA12 mRNA levels were quantified using RT-qPCR. Results were normalized to GAPDH; represent the mean of three experiments and are expressed as a percentage of RNA expression relative to expression in control samples ± SEM (one-way ANOVA: *P < 0.05); (b,c) Vaspin expression was measured by ELISA in (b) cell lysate or (c) supernatant as detailed in Materials and Methods. Results are expressed as a percentage of wild-type vaspin expression. Results represent the mean of three independent experiments ± standard error (one-way ANOVA; **P < 0.01, ***P < 0.005, ****P < 0.001); (d, e) Vaspin protein expression was ascertained using immunoblotting (upper panels) and protein extracted from (d) cell lysates or (e) culture supernatants. β-actin served as a loading control. Protein levels were quantified by ImageJ software (National Institutes of Health, Bethesda, MD) and data was normalized to levels observed in wild-type SERPINA12-transfected HaCaT cells (one-way ANOVA: *P < 0.05, **P < 0.01, ***P < 0.001). EV, empty plasmid; vaspin, visceral adipose tissue-derived serpin A12; WT, wild type.
Functional consequences of vaspin deficiency

The role of vaspin in skin diseases is poorly understood. Vaspin has been hypothesized to play a role in the pathogenesis of inflammatory cutaneous conditions such as psoriasis (Saalbach et al., 2016; Sayed et al., 2019) and systemic sclerosis (Zółkiewicz et al., 2019). In addition, it was found to inhibit kallikrein 7 and 14, two proteases previously shown to play a critical role during skin desquamation (de Veer et al., 2017; Stefansson et al., 2006), but it is unclear whether it is necessary for proper epidermal differentiation. As both PPK-associated SERPINA12 variants are likely to result in decreased vaspin expression and activity, we ascertained epidermal development in three-dimensional organotypic cell cultures generated from keratinocytes expressing normal and reduced levels of SERPINA12 (Supplementary Figure S2).

SERPINA12 downregulation resulted in a significant 2-fold increase in epidermal thickness (Figure 4), reminiscent of the acanthosis and hyperkeratosis seen in patients carrying pathogenic variants in SERPINA12 (Figure 1e).

As mentioned above, vaspin is known to inhibit kallikrein 7 (Heiker et al., 2013). To ascertain the functional consequences of the two SERPINA12 variants, we measured kallikrein 7 activity in cells transfected with wild-type SERPINA12 or vectors expressing the two pathogenic SERPINA12 variants. Kallikrein 7 activity was markedly increased upon transfection of both SERPINA12 variant-encoding vectors as compared with wild-type vector, indicating that SERPINA12 PPK-causing variants result in loss of vaspin inhibitory activity (Figure 5a). To validate these data, we then ascertained the effect of the two variants on the expression levels of desmoglein-1 (DSG1) and corneodesmosin (CDSN). DSG1 and CDSN are known substrates of kallikrein 7 (Borgoño et al., 2007; Iga et al., 2017). In agreement with the protease activity assay results, human keratinocytes transfected with either mutated SERPINA12-expressing vector displayed significantly lower levels of DSG1 and CDSN as compared with keratinocytes transfected with wild-type vector (Figure 5b and c).

Vaspin was previously reported to inhibit apoptosis in several human and non-human tissues (Ke et al., 2018; Kurkowska et al., 2019; Qi et al., 2017; Zhu et al., 2013). We ascertained apoptotic activity using the TUNEL assay on skin biopsies obtained from healthy individuals and patients. In agreement with these previous reports, the TUNEL assay demonstrated a strong increase in apoptosis in patients’ skin as compared to normal skin (Supplementary Figure S3).

DISCUSSION

The present data indicate that loss-of-function variants in SERPINA12 cause a recessive form of diffuse PPK, and thereby implicate vaspin in the maintenance of normal epidermal differentiation. Vaspin was originally isolated from the visceral adipose tissue of the Otsuka Long-Evans Tokushima fatty rat, an experimental model for type 2 diabetes mellitus (Hida et al., 2005). Consequently, its function has mostly been studied in the context of obesity and insulin resistance (Breitfeld et al., 2019; Youn et al., 2008). Inhibition
of kallikrein 7 was found to be essential for vaspin-mediated improved glucose tolerance in mice prone to diabetes mellitus (Heiker et al., 2013). The relevance of these data to humans is unclear; polymorphic variants at the SERPINA12 locus have been associated with metabolic syndrome-related traits in some but not all genetic studies (Breitfeld et al., 2013). In contrast, vaspin might protect against insulin resistance (Hida et al., 2005) and elevated vaspin serum levels may possibly exert a compensatory effect in human subjects with insulin resistance (Feng et al., 2014; Teshigawara et al., 2012; Youn et al., 2008). Of interest, various conditions suggestive of impaired epidermal differentiation have been reported to be common in diabetes mellitus (Azizian et al., 2019; Huntley, 1986). Our patients did not show any signs of obesity nor had been diagnosed with insulin resistance.

Figure 5. Effects of PPK-causing variants on kallikrein 7 activity and adhesion protein expression. (a) Visceral adipose tissue-derived serpin A12 inhibitory activity on kallikrein 7 activity was measured in HaCaT cells transfected with wild-type or mutated SERPINA12 using an enzyme-based assay as detailed in Supplementary Materials and Methods. Fluorescence data were normalized using the ELISA values and expressed as RFU/µg/ml. Results are expressed as a percentage of normalized fluorescence levels relative to wild-type SERPINA12-transfected samples ± standard error. Results represent the mean of at least two independent experiments (one-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.005); (b) Desmoglein-1 and corneodesmosin protein expression was assessed in keratinocytes transfected with wild-type or mutated SERPINA12 using immunoblotting with anti-desmoglein-1 and anti-corneodesmosin antibodies (β-actin served as a loading control); (c) Protein levels were quantified by ImageJ software (National Institutes of Health, Bethesda, MD) and normalized to levels observed in wild-type SERPINA12-transfected keratinocytes. Results were normalized to β-actin expression. Results are expressed as the percentage of protein expression relative to expression in wild-type SERPINA12-transfected samples ± standard error. Results represent the mean of at least two independent experiments (one-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.005). RFU, relative fluorescence unit; WT, wild type.
From a clinical point of view, the present study delineates the genetic basis of a form of autosomal recessive PPK transgrediens and, more broadly, supports the existence of a subgroup of inherited palmoplantar skin disorders associated with abnormal balance between proteolytic and anti-proteolytic activity in the epidermis. Indeed, excessive proteolytic activity seems to underlie keratolytic winter erythema (OMIM: #148370) because of increased activity of cathepsin B (OMIM: #116810) (Ngcungcu et al., 2017). In contrast, decreased protease inhibitor activity result in Nagashima-type palmoplantar keratosis (OMIM: #615598) (Kubo et al., 2013); exfoliative ichthyosis (OMIM: #617115) due to mutations in SERPINB8 (NM_198833.1) (Pigors et al., 2016); and acral peeling skin syndrome (OMIM: #607936) due to decreased cystatin A activity in some cases (OMIM: #184600) (Blaydon et al., 2011; Krunic et al., 2013). Taken collectively, these data tend to suggest that increased proteolytic activity particularly interferes with normal epidermal differentiation in areas exposed to mechanical stress.

Of note, topical treatment with gentamicin was recently found to improve the hyperkeratosis phenotype of patients with Nagashima-type palmoplantar keratosis associated with nonsense variants in SERPINB7 (Ohguchi et al., 2018). This work implicates that a similar readthrough activity-promoting therapy approach may also benefit other PPK patients with premature termination codon pathogenic variants, including in SERPINA12.

Our results and previous data (Breitfeld et al., 2013) suggest that nonsense PPK-causing variants in SERPINA12 abolish vaspin-mediated inhibition of kallikrein 7 activity. Kallikrein 7 overexpression in mice was found to cause increased epidermal thickness and hyperkeratosis (Hansson et al., 2002). Kallikrein 7 mediates the proteolytic degradation of DSG1 and CDSN (Borgoño et al., 2007; Igawa et al., 2017). Accordingly, the SERPINA12 PPK-causing variants were found to be associated with decreased DSG1 and CDSN expression. Of interest, DSG1 and CDSN are required for normal cornification and cell-cell adhesion (Ishida-Yamamoto et al., 2011). In addition, DSG1 and CDSN deficiencies have been associated with PPK (Rickman et al., 1999) and peeling skin (Mohamad et al., 2018), respectively, which may have contributed to the phenotypes displayed by the patients (Figure 6).

In summary, the present data delineate to our knowledge previously unreported a form of autosomal recessive PPK associated with decreased protease inhibitor activity and attribute to vaspin a role in epidermal differentiation.

**MATERIALS AND METHODS**

**Patients**

All affected and healthy family members or their legal guardian provided written and informed consent according to a protocol approved by our institutional review board and by Israel National Committee for Genetic Studies in adherence to Helsinki principles. The patient’s guardians consented to the publication of the images.

**DNA extraction**

Genomic DNA was extracted from peripheral blood leukocytes using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

**Exome sequencing**

Exome sequencing was performed by Otogenetics (Atlanta, GA). Whole-exome capture was carried out by in-solution hybridization with SureSelect Human All Exon Version 5.0 (Agilent, Santa Clara, CA) followed by massively parallel sequencing (Illumina HiSeq2500) with 125-base pair paired-end reads. Reads were aligned to the Genome Reference Consortium Human Build 37 using Burrows-Wheeler (Li and Durbin, 2010). Duplicate reads, resulting from PCR clonality or optical duplicates, and reads mapping to multiple locations were excluded from downstream analysis. Reads mapping to a region of known or detected insertions or deletions were re-aligned to minimize alignment errors. Single-nucleotide substitutions and small insertion deletions were identified, and quality filtered using the Genome Analysis Tool Kit (McKenna et al., 2010). Rare variants were identified by ANNOVAR.
software (Wang et al., 2010) and filtered using data from dbSNP142, the 1000 Genomes Project. Variants were classified by predicted protein effects using PolyPhen2 (Adzhubei et al., 2010) and Sorting Intolerant from Tolerant algorithm (Kumar et al., 2009). Validation and co-segregation of the disease phenotype with the mutation were verified using Sanger sequencing. Supplementary Table S1 summarizes exome sequencing details.

Mutation analysis
Genomic DNA was PCR-amplified using oligonucleotide primer pairs spanning the entire coding sequence as well as intron-exon boundaries of SERPINA12 (Supplementary Table S3) with Taq polymerase (Qiagen, Hilden, Germany). Cycling conditions in the case of the SERPINA12 gene were as follows: 94 °C, 2 minutes; 94 °C, 40 seconds; 64 °C, 40 seconds; 72 °C 60 seconds, for three cycles, 94 °C, 40 seconds; 62 °C, 40 seconds; 72 °C 60 seconds, for three cycles, 94 °C, 40 seconds; 60 °C, 40 seconds; 72 °C 60 seconds, for 34 cycles. Gel-purified (QIAquick gel extraction kit, QIAGEN, Hilden, Germany) amplicons were subjected to bidirectional DNA sequencing with the BigDye terminator system on an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, NY) using oligonucleotides used for PCR amplification.

Quantitative RT-PCR
For quantitative real-time PCR, cDNA was synthesized from 1,000 ng of total RNA using qScript kit (Quanta Biosciences, Gaithersburg, MD, USA). cDNA PCR amplification was carried out with the PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD, USA). cDNA PCR amplification was carried out with the PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD) on a StepOnePlus system (Applied Biosystems, Waltham, MA) using gene-specific intron-crossing oligonucleotides (Supplementary Table S4). Cycling conditions were as follows: 95 °C, 20 seconds, and then 95 °C, 3 seconds; 60 °C, 30 seconds for 40 cycles. Each sample was analyzed in triplicates. For each set of primers, standard curves were obtained with serially diluted cDNAs. Results were normalized to GAPDH mRNA levels.

Cell cultures and reagents
Primary keratinocytes and fibroblasts were isolated from adult skin obtained from plastic surgery specimens after having received written informed consent from the donors according to a protocol reviewed and approved by our institutional review board as previously described (Samuelov et al., 2013). Primary keratinocytes were maintained in keratinocytes growth medium (Lonza, Walkersville, MD). Fibroblasts were cultured in DMEM supplemented with 20% fetal calf serum (Biological Industries Israel, Beit-Haemek, Israel).

HaCaT cells, a spontaneously immortalized human skin keratinocyte cell line, were maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 20% fetal calf serum (Biochrom, Berlin, Germany), 50 U/ml penicillin and 50 U/ml streptomycin. Cells were grown to 70% confluency and then transfected with either human pCDNA3.1 or wild-type SERPINA12 or SERPINA12 harboring c.631C > T or c.1051G > T, using Lipofectamine 2000 (Invitrogen). The medium was replaced 6 hours after transfection, and the cells were then maintained for a total of 48 hours. Proteins were extracted from cells lysates using Cellytic MT (Sigma-Aldrich, St. Louis, MO) in the presence of a protease inhibitor mix, including 1 mM phenylmethanesulfonyl fluoride, and 1 mg/ml aprotinin and leupeptin (Sigma-Aldrich). Cell lysates and supernatant were collected 48 hours after transfection, and the proteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were blocked using Odyssey Blocking Buffer (LI-COR) and then incubated with primary antibodies for 1 hour at room temperature. Blots were washed and incubated with secondary antibodies conjugated with IRDye 700CW or IRDye 800CW (LI-COR). Images were captured using Odyssey Fc (LI-COR).

Expression vectors
HaCaT cells were seeded into 6-well plates, at 37 °C in 5% CO2 in a humidified incubator, adhered overnight, washed, and incubated in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 50 U/ml penicillin and streptomycin. Cells were grown to 70% confluency and then transfected with either human pCDNA3.1 or wild-type SERPINA12 or SERPINA12 harboring c.631C > T or c.1051G > T, using Lipofectamine 2000 (Invitrogen). The medium was replaced 6 hours after transfection, and the cells were then maintained for a total of 48 hours. Proteins were extracted from cells lysates using Cellytic MT (Sigma-Aldrich, St. Louis, MO) in the presence of a protease inhibitor mix, including 1 mM phenylmethanesulfonyl fluoride, and 1 mg/ml aprotinin and leupeptin (Sigma-Aldrich). Cell lysates and supernatant were collected, aliquoted, and kept at −80 °C until analyzed. Protein levels of vasiin secreted into the cell medium or present in cell lysates were measured using an ELISA assay (AdipoGen, Seoul, Korea) according to the manufacturer’s protocol (Yoon et al., 2008). Each experiment was repeated three times, and ELISA measurements were done in duplicates.

Human kallikrein 7 protease activity assay
Cell lysates and supernatant were collected 48 hours after transfection as described previously. Protein levels were determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Activity assay for human recombinant kallikrein 7 (R&D Systems, Minneapolis, MN) was conducted according to the manufacturer’s instructions using the fluorogenic peptide substrate E5OS2 (kallikrein 7) (R&D Systems). Fixed recombinant kallikrein 7 was activated by thermolysin (R&D Systems) before the activity assay. Cleavage of the molecule by kallikrein 7 increases fluorescence. Kallikrein 7 activity

Organotypic cell cultures
Organotypic cell culture generation was modified from the protocol of Mildner et al. (2006), using 0.25 × 10^6 fibroblasts per ml of type I Bovine Collagen matrix (Advanced BioMatrix; PureCol; San Diego, CA) and 4 × 10^6 keratinocytes per cm^2 growth area in 3-μm filter tissue culture inserts (BD, Franklin Lakes, NJ). Human fibroblasts were trypsinized, counted, and resuspended in DMEM medium containing 20% fetal calf serum (Biological Industries) 48 hours after transfection. Bovine Collagen I, fibroblasts, and serum were mixed, and 2.5 ml of this solution was poured into each filter insert and allowed to gel for 2 hours at 37 °C in a humidified atmosphere. The gels were then equilibrated with keratinocyte growth medium (Lonza) for 2 hours. Keratinocytes were seeded onto the matrix in a total volume of 2 ml medium per insert. After 24 hours, the system was raised to air-liquid interface. Models were grown for 10 days, and the medium was changed every second day. For each set of experiments, keratinocytes and fibroblasts were derived from the same donor and used at the third passage. Punch biopsies were obtained from organotypic cell cultures and fixed in 4% paraformaldehyde. Paraffin-embedded sections of thickness 5 μm were processed for H&E staining or immunostaining.
was monitored for 90 minutes at 25 °C by the Tecan Infinite M200 Reader (excitation filter 485 nm, emission filter 535 nm). Results of activity assay were normalized to the ELISA results.

Western blotting
Cells were homogenized in Cellytic MT (Sigma-Aldrich) and a protease inhibitor mix, including 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml aprotonin and leupeptin (Sigma-Aldrich). Following centrifugation, at 10,000 g for 10 minutes at 4 °C, proteins were electrophoresed through a 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Trans-Blot, Bio-Rad, Hercules, CA). After blocking for 1 hour using 1 × Tris-buffered saline with Tween-20 (50 mM Tris, 150 mM NaCl, 0.01% Tween 20) with 3% BSA, blots were incubated overnight at 4 °C with primary polyclonal rabbit anti-human vaspin (diluted 1:2,500; Leica Biosystems, Newcastle Upon Tyne, United Kingdom) or monoclonal mouse anti-human desmoglein-1 (diluted 1:250; P124; Progen Biotechnik, Heidelberg, Germany) or monoclonal mouse anti-human corneodesmosin (diluted 1:500; Abnova, Walnut, CA). The blots were washed five times for 5 minutes each with 1 × Tris-buffered saline, 0.1% Tween 20 with 1.5% BSA. After incubation with secondary horseradish peroxidase-conjugated anti-rabbit antibody (diluted 1:5,000; Sigma-Aldrich) or horseradish peroxidase-conjugated goat anti-mouse antibody (diluted 1:10,000; Jackson Immuno Research Laboratories, West Grove, PA), and subsequent washings (five times, 5 minutes each with 1 × Tris-buffered saline Tween-20), proteins were detected using the EZ-ECL chemiluminescence detection kit (Biological Industries). To compare the amount of protein in different samples, we reprobed the blots using a mouse monoclonal antibody to β-actin (Sigma-Aldrich). Protein levels were quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence analysis
Polyclonal rabbit anti-vaspin (diluted 1:100; Sigma-Aldrich) was used for immunofluorescence analysis of skin biopsies. Antigen retrieval was performed using 0.01 M citrate buffer, pH 6.0 (Invitrogen) in a microwave for 25 minutes. Then, protein block was performed with 2% BSA in PBS for 30 minutes at room temperature followed by incubation of the skin biopsies with anti-vaspin antibody overnight, washing with PBS three times for 5 minutes and incubation with a secondary antibody for 45 minutes. Finally, coverslips were mounted with DAPI Fluoromount-G (Southern Biotechnologies, Birmingham, AL). Positive (slides treated with recombinant DNase I; Roche Diagnostics, Basel, Switzerland) and negative controls were performed as recommended by the manufacturer.

Data availability statement
Exome sequencing datasets related to this article are hosted at the NCBI Sequence Read Archive under accession code PRJNA605633.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS
Conceptualization: ES, OS, JM; Data Curation: OS; Formal Analysis: ES, JM, OS, LM; Funding Acquisition: ES; Investigation: ES, LS, MP, TM, JM, OS, TR, NS, SA, KM, Esh, DD; Project Administration: ES; Resources: ES, LS; Software: OS; Supervision: ES, OS; Validation: ES, JM; Writing - Original Draft Preparation: JM, OS, ES; Writing - Review and Editing: ES

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SUPPLEMENTARY MATERIAL
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