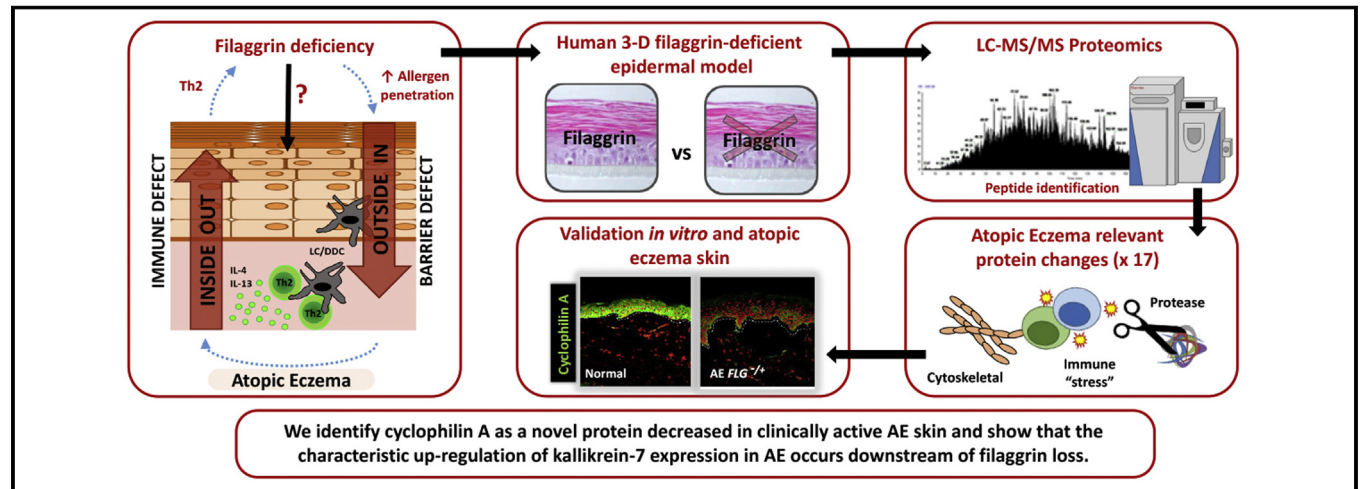


Proteomic analysis of filaggrin deficiency identifies molecular signatures characteristic of atopic eczema

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GRAPHICAL ABSTRACT



Background: Atopic eczema (AE) is characterized by skin barrier and immune dysfunction. Null mutations in filaggrin (FLG), a key epidermal barrier protein, strongly predispose to AE; however, the precise role of FLG deficiency in AE pathogenesis remains incompletely understood.

Objectives: We sought to identify global proteomic changes downstream of FLG deficiency in human epidermal living skin-equivalent (LSE) models and validate findings in skin of patients with AE.

Methods: Differentially expressed proteins from paired control (nontargeting control short hairpin RNA [shNT]) and FLG knockdown (FLG knockdown short hairpin RNA [shFLG]) LSEs were identified by means of proteomic analysis (liquid chromatography–mass spectrometry) and Ingenuity Pathway

Analysis. Expression of key targets was validated in independent LSE samples (quantitative RT-PCR and Western blotting) and in normal and AE skin biopsy specimens (immunofluorescence).

Results: Proteomic analysis identified 17 ($P \leq .05$) differentially expressed proteins after FLG knockdown, including kallikrein-7 (KLK7; 2.2-fold), cyclophilin A (PPIA; 0.9-fold), and cofilin-1 (CFL1, 1.3-fold). Differential protein expression was confirmed in shNT/shFLG LSEs; however, only KLK7 was transcriptionally dysregulated. Molecular pathways overrepresented after FLG knockdown included inflammation, protease activity, cell structure, and stress. Furthermore, KLK7 (1.8-fold) and PPIA (0.65-fold) proteins were differentially expressed in

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lesional biopsy specimens from patients with AE relative to normal skin.

Conclusions: For the first time, we show that loss of FLG in the absence of inflammation is sufficient to alter the expression level of proteins relevant to the pathogenesis of AE. These include proteins regulating inflammatory, proteolytic, and cytoskeletal functions. We identify PPIA as a novel protein with levels that are decreased in clinically active AE skin and show that the characteristic upregulation of KLK7 expression in patients with AE occurs downstream of FLG loss. Importantly, we highlight disconnect between the epidermal proteome and transcriptome, emphasizing the utility of global proteomic studies. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: Atopic eczema, dermatitis, skin, proteomic, filaggrin, kallikrein-7, cyclophilin A

Filaggrin (FLG) is a major constituent of the epidermal barrier, contributing to its structure, function, and hydration.¹ Both single- and double-allele loss-of-function mutations in *FLG* strongly predispose to the development of atopic eczema (AE) and secondary atopic conditions, including asthma and allergic rhinitis.²⁻⁴ To date, more than 40 different population-specific *FLG* mutations have been identified, each resulting in a truncated profilaggrin gene product, which is not processed into functional FLG monomers.³ Up to 50% of cases of moderate-to-severe AE in northern Europe can be attributed, at least in part, to an *FLG*-null mutation,^{5,6} representing the strongest and most consistent genetic risk factors identified for AE to date (overall odds ratio, 3.12-4.78).^{6,7} Furthermore, *FLG* is polymorphic, with common allelic variants encoding a profilaggrin molecule composed of 10, 11, or 12 FLG monomeric repeats. Each additional FLG repeat confers a reduced risk of AE by a factor of 0.88, suggesting that even small increases in *FLG* expression might be therapeutically beneficial.⁸

Both the flaky tail mouse (which carries a natural mutation virtually ablating all FLG expression⁹) and FLG knockout mice exhibit enhanced percutaneous antigen transfer and allergen/irritant-induced AE-like inflammatory responses.^{10,11} These models support a primary role for FLG and highlight the importance of a functional cutaneous barrier in AE disease pathogenesis.

Recent cohort studies have examined global gene expression changes in patients with AE stratified by FLG genotype.^{12,13} Although interesting, their interpretation with regard to the direct role of FLG in AE pathogenesis is confounded by the complex interplay between the epidermal barrier, immune system, and environment.¹⁴ For example, loss of FLG is also observed in patients with *FLG* wild-type AE, likely because of its extrinsic downregulation by the atopic T_H2-polarized microenvironment.¹⁵ Moreover, murine models are additionally limited both by secondary mutations, notably null mutations in *TMEM79/Matt* (Mattrin) in the flaky tail mouse,¹⁶ and interspecies differences. The human and murine *FLG* sequences lack homology,¹⁷ and murine models do not display the heterozygote phenotype typical of most patients with AE.^{10,11} Consequently, the precise molecular changes occurring directly as a result of FLG loss are relatively unknown.

A useful alternative tool for the study of epidermal biology is the 3-dimensional living skin-equivalent (LSE) model derived exclusively from primary human keratinocytes. Although by definition these simplified models do not recapitulate the complexity of human AE skin, they enable the study of epidermal

Abbreviations used

AE:	Atopic eczema
ANXA3:	Annexin A3
CFL1:	Cofilin-1
CTSV:	Cathepsin V
eIF:	Eukaryotic initiation factor
FLG:	Filaggrin
IPA:	Ingenuity Pathway Analysis
KLK7:	Kallikrein-7
LSE:	Living skin equivalent
mTOR:	Mammalian target of rapamycin
PPIA:	Cyclophilin A
RT-qPCR:	Quantitative RT-PCR
shNT:	Nontargeting control short hairpin RNA
shFLG:	Filaggrin knockdown short hairpin RNA
TXN:	Thioredoxin
VIM:	Vimentin

biology in the absence of confounding inflammatory cells.¹⁸ Furthermore, they enable the effect of *FLG* gene silencing to be analyzed in a pairwise manner on a homogeneous genetic background.

Previous LSE studies have largely focused on understanding the ultrastructural and functional consequence of FLG deficiency.¹⁹⁻²¹ In many regards these mirror changes observed in AE skin, supporting their utility as a disease model. However, to the best of our knowledge, no systematic transcriptomic or proteomic analysis has been performed after *FLG* knockdown in human epidermis. The aim of this study was to use FLG knockdown LSE models to investigate the global molecular consequences resulting directly from *FLG* deficiency. A proteomics-based approach was used because protein changes largely represent the functional end point of cell signaling, and recent studies have identified an important disconnect between the transcriptome and proteome, suggesting that significant posttranscriptional regulation occurs.²²

Notably, for the first time, we have identified 17 proteins that are significantly differentially expressed after FLG knockdown in LSE cultures. Bioinformatic analysis was used to categorize and align these to putative regulatory networks and showed that loss of FLG alone is sufficient to induce protein changes relevant to the pathogenesis of AE. Specifically, pathways relating to protease activity, inflammation, cell structure, and stress were overrepresented after FLG knockdown. The expression profile of key targets, namely the AE-relevant protease kallikrein-7 (KLK7), the novel AE immune modulator cyclophilin A (PPIA), and the actin-binding protein cofilin-1 (CFL1), were replicated first in independent FLG knockdown LSEs and then further characterized in skin biopsy specimens from patients with AE.

METHODS

Primary keratinocyte culture

Normal human epidermal keratinocytes extracted from surplus foreskin tissue obtained after informed biobank consent were cultured in low-calcium (0.06 mmol/L) EpiLife supplemented with 1% human keratinocyte growth supplement (Life Technologies, Paisley, United Kingdom), as previously described.²³ All donor subjects had no history of AE.

Lentivirus (short hairpin RNA) production and transduction of primary keratinocytes

Lentivirus production from GIPZ lentiviral shRNAmir vectors (Open Biosystems, Lafayette, Colo) targeting FLG (filaggrin knockdown short hairpin RNA [shFLG], V3LHS_369921) or a nontargeting control sequence (nontargeting control short hairpin RNA [shNT], RHS4346) and keratinocyte transduction were performed, as previously described.²⁴ Further details are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

LSE culture and protein extraction

LSEs were generated from independent biological donors ($n = 10$ for proteomic analysis and a further $n = 3-8$ for validation experiments) and proteins extracted in 8 mol/L urea lysis buffer, as previously described.²⁴ Further details are provided in this article's Online Repository.

Patient samples

Patients with AE (as diagnosed per the United Kingdom–modified Hanifin and Rajka criteria²⁵) were recruited from a tertiary referral dermatology clinic. Patients older than 18 years not receiving systemic immune-modifying drugs were included in the study after written informed consent and a 2-week washout period from potent topical steroids. Five-millimeter punch biopsy specimens were taken from both lesional and nonlesional skin after achievement of local anesthesia. After obtaining informed consent, normal (control) skin was collected after plastic surgery or excision of benign cutaneous lesions. A blood sample was also collected from all patients and control subjects for *FLG* mutational analysis (further details provided in this article's Online Repository). All control subjects reported in the study had no history of AE and were *FLG* wild-type. This study was approved by the Regional Research Ethics Committee and was conducted according to the Declaration of Helsinki principles.

Immunoblotting and densitometry

Immunoblotting was performed by using standard protocols, as previously described.²⁴ Primary antibody conditions were as follows: FLG clone 15C10 (1:200; NCL-FLG; Leica Biosystems, Newcastle upon Tyne, United Kingdom), CFL1 (1:500; PA5-27627, Thermo Scientific, Waltham, Mass), KLK7 (1:1000; AF2624; R&D Systems, Minneapolis, Minn), PPIA (1:300; 39-1100; Life Technologies), and glyceraldehyde-3-phosphate dehydrogenase (1:10,000; #2118; Cell Signaling Technology, Danvers, Mass). Further details are provided in this article's Online Repository.

Proteomic and data analysis

Proteins were prepared by means of in-solution or in-gel digestion and subjected to liquid chromatography/mass spectrometry proteomic analysis. Data analysis was performed with Proteome Discoverer (Thermo Fisher Scientific) and Scaffold (Proteome Software, Portland, Ore). Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides. Further details are provided in this article's Online Repository.

Immunofluorescence

OCT-embedded LSE and skin cryosections (4 μ m) were fixed and stained, as previously described.²⁴ Primary antibody conditions were as follows: FLG (Acetone fix, 1:100; MS-449-P1; Thermo Scientific), CFL1 (paraformaldehyde fix, 1:300; PA5-27627; Thermo Scientific), KLK7 (Methanol fix, 1:250; AF2624; R&D Systems), and PPIA (paraformaldehyde fix, 1:500; 39-1100; Life Technologies). Quantification was performed with ImageJ software (National Institutes of Health, Bethesda, Md). Further details are provided in this article's Online Repository.

Quantitative RT-PCR

Quantitative RT-PCR (RT-qPCR) was performed, as described by Forrester et al,²⁴ with exon-spanning probe-based assays: FLG (Hs.PT.53a.23095403, FAM/TAMRA; Integrated DNA Technologies, Coralville, Iowa), PPIA (forward: ATGCTGGACCCACACAAAT; reverse: TCTTTCACTTTGCCAAACACC; UPL PROBE#48 FAM/NFQ; Roche, Mannheim, Germany), CFL1 (forward: GTGCCCTCTCCTTTTCGTTT; reverse: TTGAACACCTTGATGACACCAT; UPL PROBE#5 FAM/NFQ; Roche), and KLK7 (Hs.PT.58.39819237, FAM/TAMRA; Integrated DNA Technologies). *18S* was used as a housekeeping gene for normalization purposes.²⁶ Further details are provided in this article's Online Repository.

Statistical analysis

Unless otherwise stated in the figure legends, data points represent means $\pm$ 95% CIs. Statistical analysis was performed with Prism 5 software (GraphPad Software, La Jolla, Calif). Further details are provided in this article's Online Repository.

RESULTS

Establishment of an FLG-deficient human LSE model

Reliable and reproducible knockdown of FLG expression in primary keratinocytes was achieved by using a second-generation pGIPZ lentiviral shRNAmir construct targeting the *FLG* mRNA sequence (shFLG). A nontargeting shRNAmir construct was used as a control (shNT). LSEs were established from transduced keratinocytes, and protein and mRNA knockdown was assessed at day 14 of culture, the time point at which LSEs are fully differentiated ([Fig 1](#) and see [Fig E1](#) in this article's Online Repository at www.jacionline.org). Densitometry of immunoblots confirmed significant knockdown in both profilaggrin ($75\% \pm 4.1\%$, $P \leq .0001$) and FLG ($79\% \pm 3.1\%$, $P \leq .0001$) proteins ([Fig 1, A, B, and D](#)). RT-qPCR confirmed a significant reduction in *FLG* mRNA expression ($69\% \pm 4.9\%$, $P \leq .0001$; [Fig 1, E](#)). Histologic examination of LSE sections showed no overt phenotypic changes between nonvirally modified LSEs and the shNT control LSEs (data not shown) or between the shNT and shFLG LSEs ([Fig 1, C](#)). However, quantification with ImageJ analysis software identified a small but significant increase in epidermal thickness after FLG knockdown compared with the shNT control. As such, both a thicker viable cell layer (1.14 ± 0.03 -fold, $P = .0007$) and stratum corneum (1.13 ± 0.06 -fold, $P = .05$) were observed (see [Fig E2](#) in this article's Online Repository at www.jacionline.org).

Seventeen proteins were significantly differentially regulated after FLG knockdown in human epidermal skin equivalent

The soluble protein fraction from paired shNT and shFLG LSE samples generated from 10 independent biological human donors displaying normal patterns of baseline FLG expression (see [Fig E3](#) in this article's Online Repository at www.jacionline.org) were subject to an open proteomic workflow ([Fig 2, A](#)). One thousand six hundred forty unique proteins were identified across all samples, with an average of 634 proteins identified per LSE. Of these, 367 proteins were identified in at least 75% of the shNT and shFLG paired samples (see [Table E1](#) in this article's Online Repository at www.jacionline.org). The unweighted spectrum counts from this subset of proteins were used in subsequent

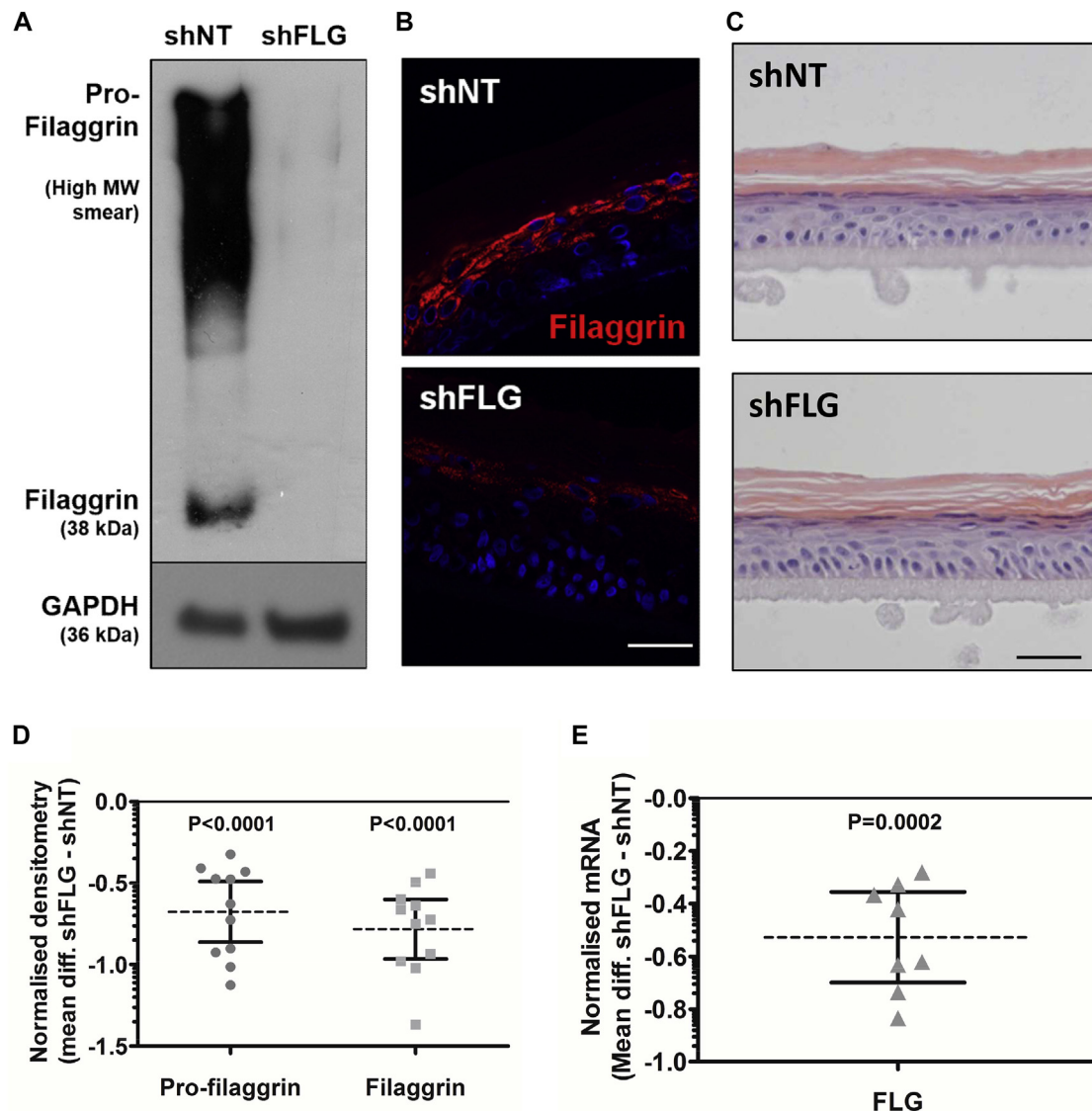


FIG 1. Generation of shFLG and shNT LSE models. **A** and **B**, Representative FLG immunoblot ($n = 11$; Fig 1, A) and immunofluorescence ($\sim 150 \mu\text{m}$, $n = 3$; Fig 1, B). MW, Molecular weight. **C**, Hematoxylin and eosin-stained LSE morphology ($\sim 100 \mu\text{m}$, $n = 10$). **D** and **E**, Normalized FLG densitometry of immunoblots ($n = 11$; Fig 1, D) and TaqMan mRNA expression levels ($n = 8$; Fig 1, E). Scatter plots of $\log_{10}$ -transformed data (means $\pm$ 95% CIs).

analysis. Principal component analysis reported clustering by biological donor and not FLG status (see Fig E4 in this article's Online Repository at www.jacionline.org). To account for the prominent donor effect, $\log_{10}$ differences between shNT and shFLG conditions were calculated for each biological donor (ie, a paired design), and $\log_{10}$ estimated differences in expression profile were used for input for statistical analysis. A summary of the differential expression profile for each biological donor is represented as a heat map (see Fig E5 in this article's Online Repository at www.jacionline.org). Overlaid dendrogram plots depict clustering between protein response profiles.

Notwithstanding the prominent intradonor variability, statistical analysis of the combined data set identified 17 differentially regulated proteins ($P \leq .05$; Fig 3 and see Table E2 in this article's Online Repository at www.jacionline.org). Positive/negative mean differences represent upregulated/downregulated proteins after FLG knockdown, respectively. Differentially expressed

proteins broadly relate to a range of functional classes, including proteases (KLK7, cathepsin V [CTSV], and carboxypeptidase A4 [CPA4]), inflammatory mediators (annexin A3 [ANXA3], lymphocyte antigen 6D [LY6D], PPIA, and thioredoxin [TXN]), and cytoskeletal interactors (FLG, vimentin [VIM], CFL1, T-complex protein 1 subunit zeta [CCT6A], and tripartite motif-containing protein 29 [TRIM29]). The most significantly downregulated protein was FLG ($P = 2.0\text{E-}04$), internally validating the analysis methodology.

FLG-deficient LSEs display expression changes consistent with inflammatory dermatoses

Ingenuity Pathway Analysis (IPA) identified "immunological disease" ($P = 2.14\text{E-}06$ to $3.58\text{E-}02$, 9 proteins), "dermatological disease" ($P = 2.42\text{E-}05$ to $3.99\text{E-}02$, 6 proteins), and "inflammatory disease" ($P = 2.42\text{E-}05$ to $3.66\text{E-}03$, 7 proteins; Fig 4, A, and

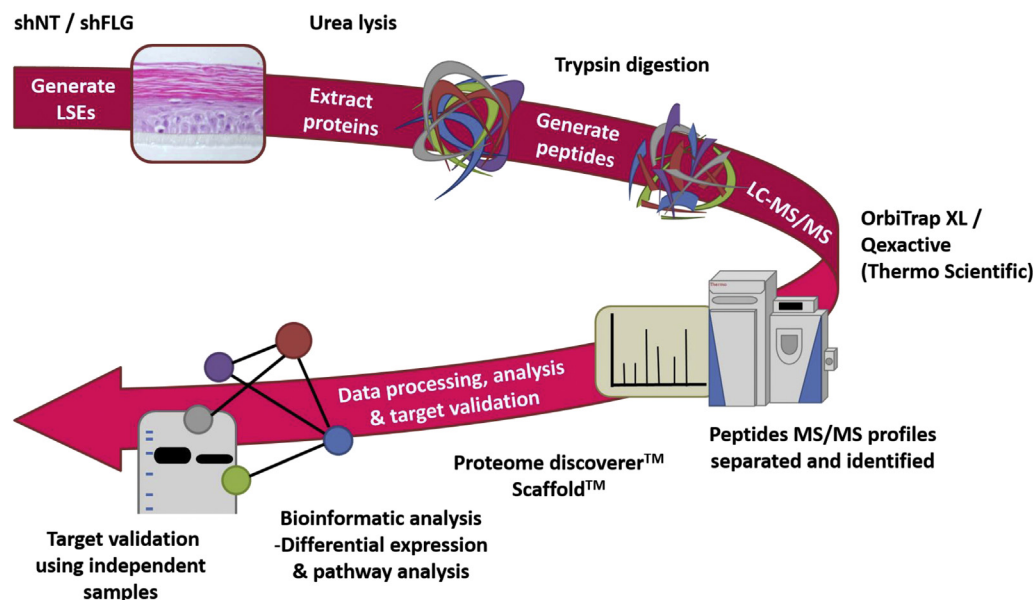


FIG 2. Overview of the open proteomic workflow. Peptides were extracted from shNT and shFLG LSEs and separated by means of liquid chromatography mass spectrometry (LC-MS and LC-MS/MS). Proteins were identified from MS/MS spectra, and unweighted spectrum counts were used to determine relative expression levels. Selected targets were independently validated.

see Table E3 in this article's Online Repository at www.jacionline.org) as the categories most significantly associated with FLG knockdown. Interestingly, within these categories, the most significantly associated functional term was "allergy" ($P = 2.15\text{E-}06$, 6 proteins). A detailed breakdown of the functional terms underlying the top 5 associated disease categories is included in Table E4 in this article's Online Repository at www.jacionline.org.

FLG knockdown in LSEs leads to enrichment in proteins associated with cellular assembly

Further IPA analysis identified that the "molecular and cellular functions" most significantly overrepresented in our data set were "cellular movement" ($P = 3.05\text{E-}04$ to $4.96\text{E-}02$, 7 proteins), "cellular development" ($P = 3.94\text{E-}04$ to $4.47\text{E-}02$, 7 proteins), and "cell growth and proliferation" ($P = 3.94\text{E-}04$ to $4.47\text{E-}02$, 6 proteins; Fig 4, B, and see Table E5 in this article's Online Repository at www.jacionline.org). Significant functional terms included "migration of cells" ($P = 3.06\text{E-}03$, 7 proteins) and "differentiation of cells" ($P = 2.94\text{E-}02$, 6 proteins). Full details of the functional terms associated with the top 5 cellular function categories are provided in Table E4.

Network analysis returned a single putative network linking all of the differentially regulated proteins with an IPA p-score of 45 ($p\text{-score} = -\log_{10} [P \text{ value}]$, see Fig E6 in this article's Online Repository at www.jacionline.org). Consistent with the previous analysis, significant functional terms associated with the network included "hyperplasia of epithelial cells" ($P = 1.02\text{E-}06$, 7 proteins) and "formation of skin" ($P = 3.69\text{E-}06$, 12 proteins). Proteins implicated in dermatological and/or inflammatory disease have been highlighted in pink (see Fig E6).

Proteins involved in cell stress signaling pathways are overrepresented in LSEs after FLG knockdown

The canonical pathways most significantly associated with the differentially expressed proteins included "EIF2 signaling" ($P = 1.51\text{E-}05$, 4 proteins), "regulation of eukaryotic initiation factor (eIF) 4 and p70S6K signaling" ($P = 1.40\text{E-}03$, 2 proteins), and "mammalian target of rapamycin (mTOR) signaling" ($P = 1.08\text{E-}02$, 2 proteins; Fig 4, C, and see Table E6 in this article's Online Repository at www.jacionline.org). The top 5 significantly associated canonical pathways are also overlaid onto the putative molecular network (see Fig E6).

Confirmation of proteomics results in independent LSEs

A number of differentially expressed proteins were selected for independent validation at the protein and mRNA level in independent shNT and shFLG LSEs derived from biological donors distinct from those used in the original study to confirm the findings from the proteomic analysis. The expression profiles of key representatives from a distinct protein class, such as proteases (KLK7), immunomodulatory functions (PPIA), and cytoskeletal interacting proteins (CFL1), were selected based on their low P value, high donor ratio, and relevance to skin morphology/disease.

In line with the proteomic results, all 3 proteins were significantly differentially expressed, as assessed by means of immunoblotting and densitometric analysis in the independent LSEs (Fig 5, A and B). Furthermore, both the relative magnitude and direction of response were preserved: CFL1, 2.1 ± 0.27 -fold ($P = .03$), KLK7, 1.89 ± 0.29 -fold ($P = .007$), and PPIA, 0.52 ± 0.11 -fold ($P = .05$; Fig 5, A and B).

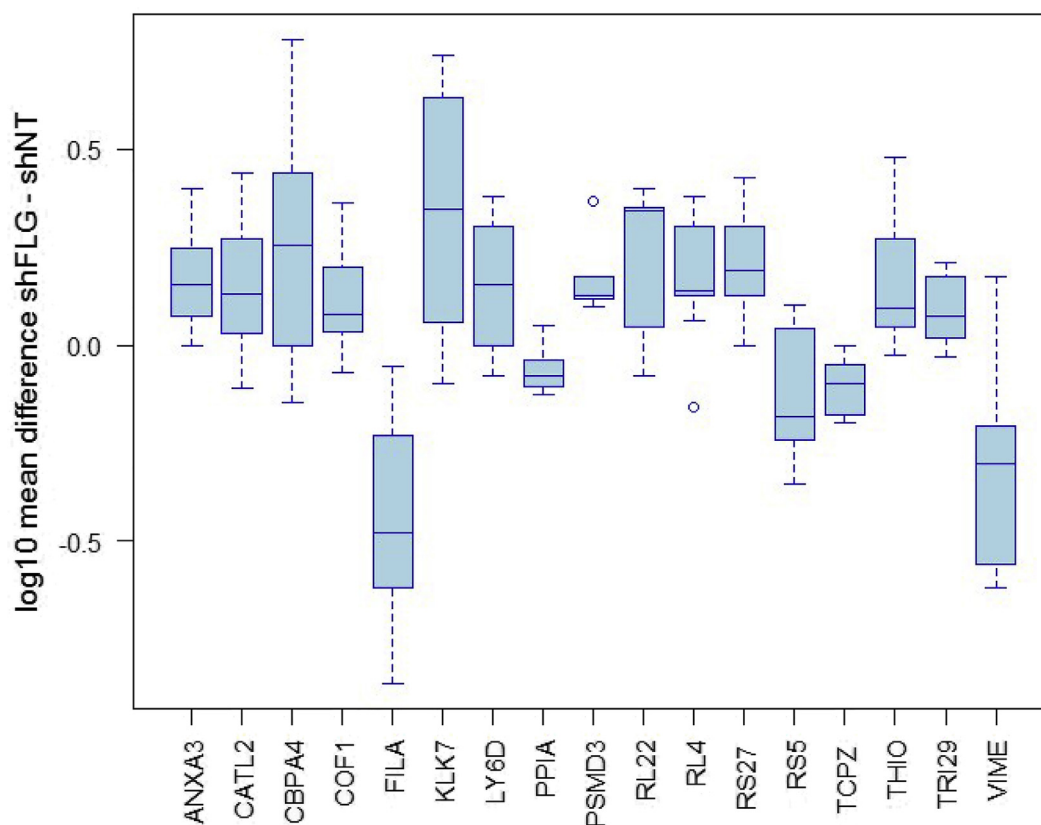


FIG 3. Box plot showing differentially expressed proteins after FLG knockdown. Log₁₀ mean protein differences (shFLG – shNT) were calculated independently for each biological replicate and collated for statistical analysis (n = 10). Displayed proteins were differentially regulated. $P \leq .05$, 1-sample t test. Values are medians and interquartile ranges.

In contrast to the protein data, there was no significant difference in *CFL1* and *PPIA* mRNA expression levels between shNT and shFLG LSEs, as assessed by using probe-based RT-qPCR assays, underscoring the relevance of a proteomics-based approach. However, *KLK7* mRNA expression was increased in the shFLG condition, which is consistent with the protein data (1.2 ± 0.06 -fold, $P = .05$; Fig 5, C).

Replication of FLG knockdown LSE findings in AE skin

Protein expression analysis was performed on human skin from healthy control subjects and patients with AE to determine whether these *in vitro* findings were relevant to the *in vivo* AE disease setting. AE samples were collected from sites of both clinically active eczema (involved skin) and macroscopically normal skin (uninvolved).

Protein expression of FLG, PPIA, KLK7, and CFL1 was assessed by using a combination of immunofluorescence imaging (Fig 6, A) and quantification by means of image analysis (Fig 6, B-E). *FLG* mutational status of all control subjects and patients with AE was determined by using next-generation sequencing. All control subjects reported were *FLG* wild-type, and 31% of subjects with AE carried *FLG* mutations (Fig 6, B-E, and see Table E7 in this article's Online Repository at www.jacionline.org).

Overall FLG expression was reduced in both uninvolved and involved AE skin compared with that in healthy control subjects,

although significance was only observed at sites of clinically active disease (0.6 ± 0.1 -fold, $P \leq .05$; Fig 6, A and B). FLG expression was particularly reduced in *FLG*-heterozygous subjects and almost absent in the *FLG* double-allele mutant subject (Fig 6, A and B), and accordingly, a significant correlation between *FLG* genotype (number of expressed alleles) and expression was observed ($r = 0.48$, $P = .007$) across the whole data set.

The protein abundance of PPIA was significantly reduced in involved AE skin compared with control skin (0.65 ± 0.06 -fold, $P \leq .02$), whereas KLK7 expression was significantly increased in both uninvolved (1.8 ± 0.02 -fold, $P \leq .02$) and involved (1.84 ± 0.02 -fold, $P \leq .009$) AE skin. There was a trend toward correlation between FLG and PPIA expression ($r = 0.31$, $P = .09$) and a significant inverse correlation between FLG and KLK7 expression ($r = -0.38$, $P = .04$). However, in neither instance did protein expression stratify or correlate according to *FLG* mutational status (PPIA: $r = 0.08$, $P = .68$; KLK7: $r = -0.18$, $P = .34$; Fig 6, A-D). In contrast, the abundance of CFL1 was unchanged between normal and AE skin and did not display any correlation with respective FLG expression ($r = -0.12$, $P = .51$; Fig 6, A).

DISCUSSION

In this study we used an *in vitro* human LSE model to investigate the proteomic changes arising from FLG deficiency *per se* in the absence of any confounding systemic inflammation.¹⁹

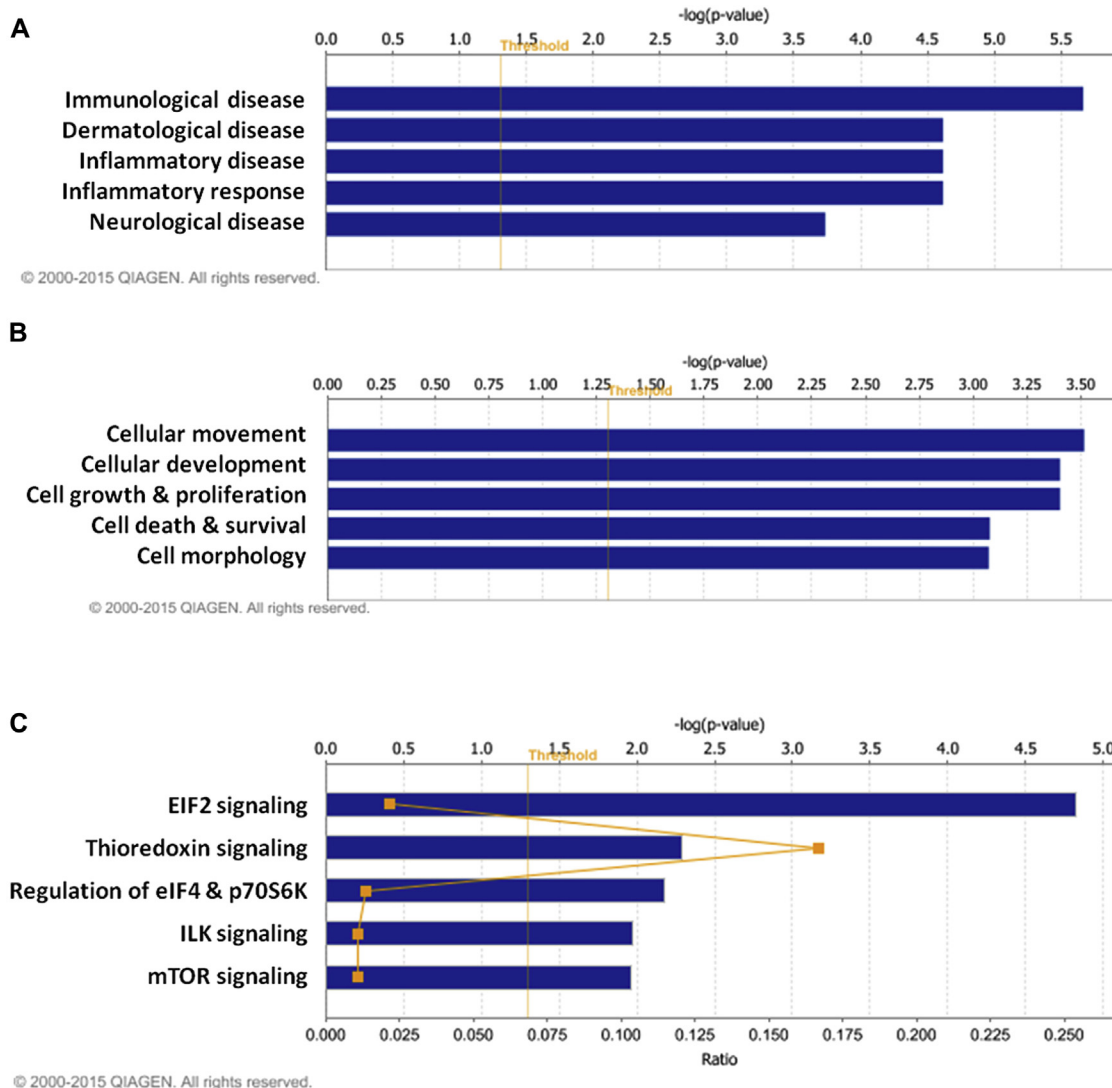


FIG 4. Summary of IPA core functional analysis. $\log_{10}$ mean protein differences (shFLG – shNT) with an adjusted P value of less than .05 (threshold) were considered significantly differentially expressed and used as input for IPA. The top 5 “disease and disorders” (A), “molecular and cellular functions” (B), and “canonical pathways” (C) altered after FLG knockdown are depicted. The orange line depicts the ratio (number of pathway-relevant proteins in the data set divided by the total number of proteins in the pathway).

A global proteomics approach and IPA strikingly revealed that shFLG LSEs displayed a disease profile consistent with inflammatory dermatologic disease and an enrichment in proteins associated with cellular assembly. Significant functional terms included “allergy” (increased: ANXA3, CFL1, CTSV, and KLK7; decreased: FLG and PPIA) and “differentiation of cells” (increased: CFL1, CTSV, and RPL22; decreased: FLG, PPIA, and VIM), suggesting that our FLG knockdown model displays many molecular characteristics typical of AE (Fig 4 and see Tables E3–E6). Similar functional annotations, specifically inflammatory and extracellular matrix dysregulation, have also been described in AE transcriptomic data sets,^{12,13,27} including those from FLG-null and haplosufficient patients with AE,^{12,13} reinforcing the utility of our disease model and underscoring the central role of FLG deficiency in AE pathophysiology.

Carefully controlled protease activity is essential for the maintenance of epidermal homeostasis and barrier function.²⁸

In addition to increased expression of the chymotrypsin-like serine protease KLK7, expression of the cysteine protease CTSV and zinc-dependent protease CPA4 was significantly upregulated after FLG knockdown in the LSE model. KLK7 is highly expressed in skin,²⁹ and its dysregulation has been implicated to AE pathogenesis; protein expression is increased in both involved and uninvolved AE skin and in the epidermis of flaky tail mice.^{29–31} Furthermore, overexpression of human KLK7 in murine models causes severe pruritus, increased epidermal thickness, and dermal inflammation.³⁰ Our findings in AE skin are consistent with these results, demonstrating both increased overall epidermal KLK7 expression and ectopic expression within the lower epidermal compartments compared with those seen in healthy control skin. Importantly our novel *in vitro* data demonstrating increased *KLK7* mRNA and protein expression after FLG knockdown identifies for the first time that KLK7 is transcriptionally regulated, either directly or indirectly downstream

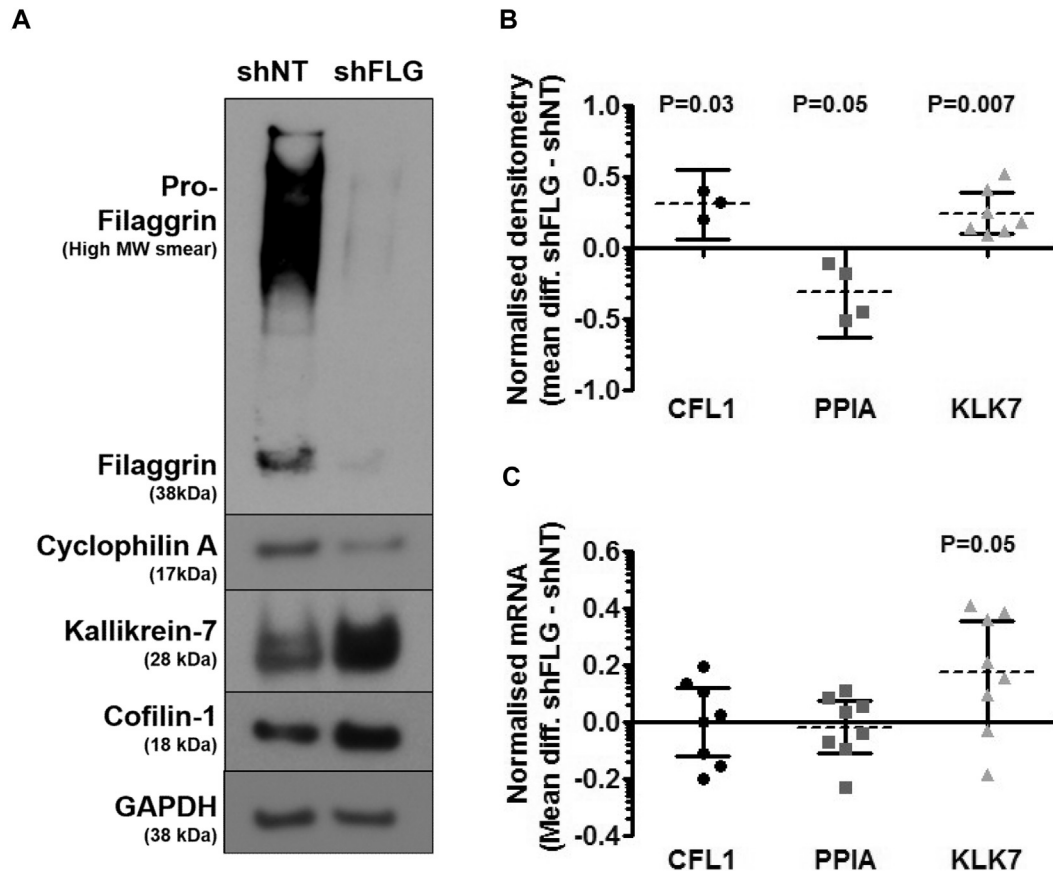


FIG 5. Validation of proteomics findings. Normalized expression levels of CFL1, PPIA, and KLK7 were determined in shNT and shFLG LSEs generated from independent biological samples. **A** and **B**, Representative immunoblots ($n \geq 3$; Fig 5, A) and associated densitometric quantification ($n \geq 3$; Fig 5, B). MW, Molecular weight. **C**, TaqMan mRNA expression levels ($n = 8$). Scatter plots of $\log_{10}$ -transformed data (means $\pm$ 95% CIs).

of FLG (Figs 3 and 5). The combination of increased KLK7 expression and an atopic epidermal environment known to be permissive for protease activation³² has significant implications for barrier homeostasis. In addition to the perturbations in epidermal structure, enhanced KLK activity also drives inflammation both directly through IL-1 activation³³ and indirectly through overexpression of thymic stromal lymphopoietin, a potent inducer and propagator of T_H2 -polarized inflammation downstream of protease activated receptor 2 signaling.³⁴ The mRNA expression of thymic stromal lymphopoietin is increased in lesional AE skin,³⁴ flaky tail mice,³¹ and our LSEs after FLG knockdown (data not shown). This, combined with our observation that proteins of known immunomodulatory function (PPIA, TXN, ANXA3, and LY6D) are significantly perturbed by FLG knockdown, supports the idea that FLG deficiency can contribute to AE inflammation independently of immune cells.

PPIA is an ubiquitously expressed immunophilin family member protein with roles in protein folding, trafficking, and immune modulation.³⁵ For the first time, we show that PPIA is downregulated in the context of FLG deficiency and in AE skin (Figs 3, 5, and 6), suggesting that PPIA might represent a novel disease mechanism downstream of FLG. Interestingly, PPIA is the cytoplasmic binding partner of cyclosporine, an effective therapeutic in patients with AE.³⁶ Cyclosporine regulates gene transcription, growth, and differentiation of human keratinocytes,³⁷

as well as their production of inflammatory cytokines.³⁸ PPIA knockout mice experience spontaneous T_H2 -polarized allergic disease and an immune profile akin to AE, although no dermatitis is reported.³⁹ No concurrent reduction in *PPIA* mRNA expression was observed in shFLG LSEs (Fig 5, C); the reduction in intracellular protein expression might instead reflect an increase in PPIA secretion known to be triggered after inflammatory/stress stimuli in epithelia.³⁵ Extracellular PPIA functions as both a proinflammatory stimuli (autocrine/paracrine) and potent chemoattractant of CD147⁺ immune cells.^{35,40} Stress and inflammatory pathways were overrepresented in our data set after FLG knockdown (Fig 4), and keratinocytes are known to secrete the close family member cyclophilin B and to express CD147 cell-surface receptors,⁴¹ suggesting that similar regulation of PPIA might occur within the epidermis. Furthermore, extracellular PPIA and TXN (increased in shFLG LSEs) have been identified as IgE-reactive self-antigens in patients with AE,^{42,43} and therefore their dysregulation might represent an additional pathogenic mechanism downstream of FLG.

Finally, cyclophilin members are regulated during epithelial terminal differentiation,⁴⁴ suggesting that PPIA dysregulation downstream of FLG might also have implications for epidermal barrier structure and function. Keratinocyte differentiation is associated with extensive keratin and actin remodeling.⁴⁵ We also identified significantly differentially expressed proteins

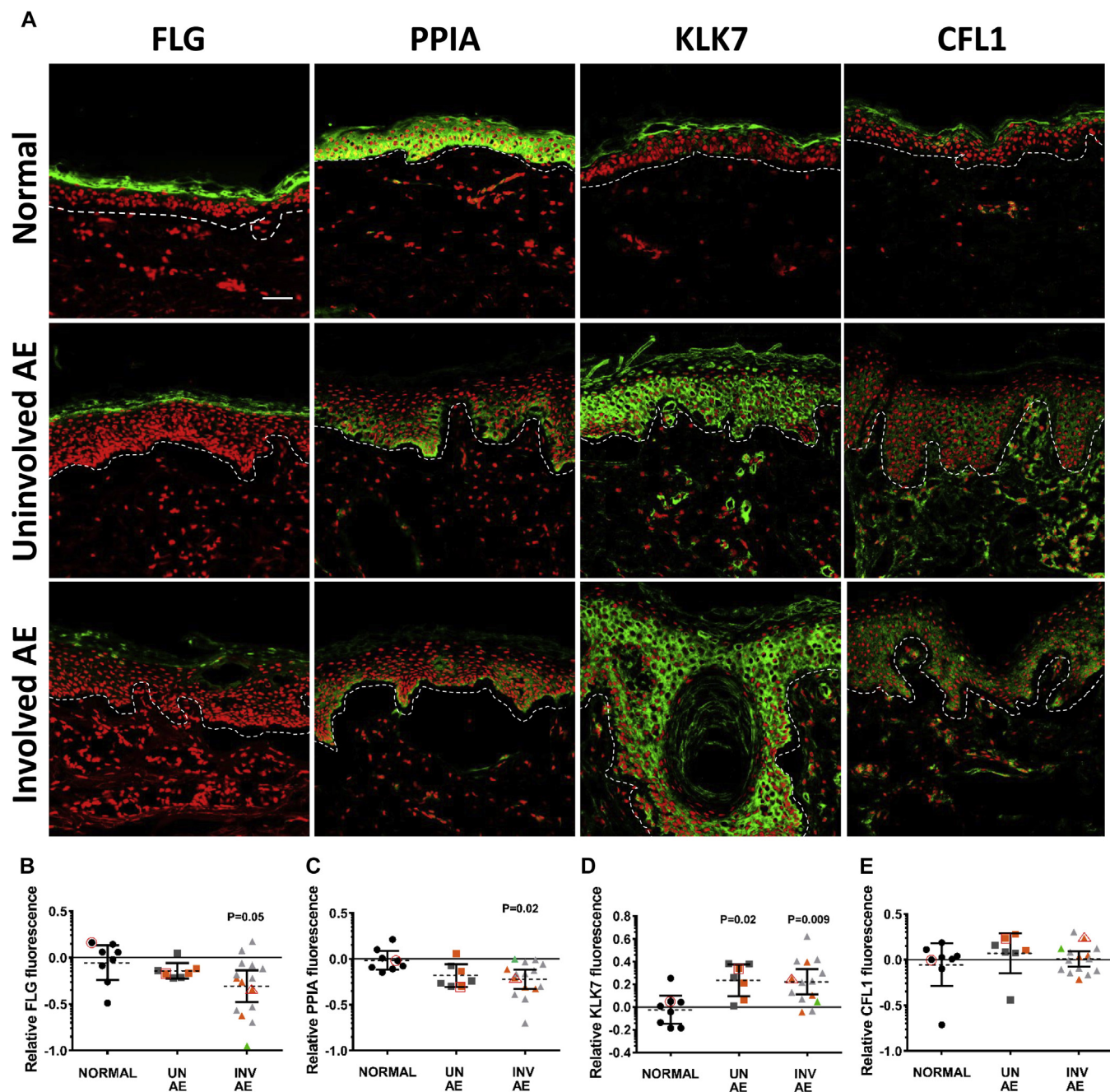


FIG 6. Replication of FLG knockdown LSE findings in AE skin. Representative immunofluorescence images of normal (FLG wild-type), uninvolved (UN AE), and involved (INV AE) AE skin ($n \geq 3$). Green staining depicts FLG, PPIA, KLK7, or CFL1, as indicated. Hoechst 33342 nuclear counterstaining is shown in red ($\sim 50 \mu\text{m}$). Dashed lines represent the epidermal/dermal boundary. **A**, Corresponding quantification values are outlined in red on the bottom scatter plots. **B–E**, Scatter plots of the corresponding protein fluorescence intensities expressed as function of unit area and on a $\log_{10}$ scale (means $\pm$ 95% CIs). All subjects are FLG wild-type unless indicated in orange (FLG heterozygous) or green (FLG homozygous/compound heterozygous).

with known roles in cytoskeletal/actin remodeling (CFL1, CCT6A, and VIM) as novel targets of FLG loss. CFL1 is an essential actin-binding protein that regulates cell motility, growth, division, and differentiation by controlling actin filament assembly and disassembly.⁴⁶ Overexpression of active (unphosphorylated) CFL1 in LSE models reduces corneocyte compaction, whereas partial knockdown increased proliferation, supporting a role for

CFL1 in keratinocyte homeostasis.⁴⁷ In our LSE model CFL1 protein but not mRNA expression was significantly increased after FLG knockdown (Fig 5). Pathways associated with regulation of the actin cytoskeleton have been reported to be transcriptionally overrepresented in AE skin,¹³ and CFL1 mRNA expression is significantly upregulated in the involved skin of patients with AE, psoriasis, and lichen planus,⁴⁸ suggesting that dysregulation

of the actin cytoskeleton might be a contributing pathogenic mechanism. Interestingly, unlike PPIA and KLK7, the protein expression pattern of CFL1 in AE skin did not align to the shFLG LSE model, and we observed no increase in CFL1 protein expression in the patients with AE (Fig E6). The regulation of CFL1 is complex, involving phosphorylation, inhibitor complexes, and cooperation with other actin-binding proteins⁴⁶; understanding its role in patients with AE will require further investigation.

Further IPA analysis significantly associated dysregulation of eIF2 (RPL22, RPL4, RPS27, and RPS5), eIF4 (RPS27 and RPS5), and mTOR (RPS27 and RPS5) signaling with FLG knockdown. Collectively, these pathways cooperate to regulate transcriptional and translational initiation in response to a wide variety of environmental stress stimuli, including infection.^{49,50} Patients with AE have an increased susceptibility to both disseminated viral (eczema herpeticum) and bacterial skin infections,⁵¹ and the risk of both is increased in FLG-deficient subjects.⁵² Consistent with this, stress responses, namely type 1 interferon-mediated anti-pathogenic deference, are overrepresented in the transcriptome of FLG-null and haplosufficient patients with AE,¹² and furthermore, type 1 interferon expression is increased in patients with eczema herpeticum.⁵³ Type 1 interferons are secreted after viral⁵⁴ or bacterial⁵⁵ infection and mediate both immunomodulatory responses and local cell-cycle regulation in part through activation of eIF2,⁵⁶ mTOR, and downstream p70-S6K/eIF4 signaling.⁵⁷ For the first time, our *in vitro* data suggest that the exaggerated but ineffective upregulation of type 1 interferon-mediated defense observed in patients with AE¹² might reflect an inherent defect downstream of FLG rather than/in addition to a deregulated response to pathogen exposure.^{12,53} Therefore dysregulation of these pathways as a result of inherited or acquired FLG deficiency might contribute to the propensity of patients with AE to have bacterial and viral skin infections.

Overall, our data reinforce that loss of FLG is itself an important pathogenic factor in patients with AE, driving the development and exacerbation of barrier and immune dysfunction. Importantly, we have shown that loss of FLG alone in the absence of any systemic immune signals can disrupt epidermal homeostasis resulting in proteomic changes relevant to AE. Our data are consistent with the so-called “outside-to-inside” disease hypothesis and places FLG deficiency firmly upstream of protease, innate inflammatory, and cytoskeletal pathway dysregulation. The observed protein changes in AE skin occurred to a similar degree in FLG-depleted, *FLG* wild-type, and single- and double-allele mutated subjects, suggesting that the mechanism of FLG reduction (whether through genotype, cytokine effects, or a combination thereof) might not be important.

For the first time, we have identified 17 proteins that are significantly differentially expressed in the absence of FLG, including KLK7, PPIA, and CFL1. Confirmation of these results in independent LSE cultures validates the proteomics methodologies, interpretation and the biological reproducibility of our identified targets. Replication of PPIA and KLK7 expression profiles in skin biopsy specimens from patients with AE further underscores the utility of our disease model. To the best of our knowledge, this is the first study to implicate loss of PPIA resulting from FLG deficiency to AE pathogenesis and also to show that the known increase in KLK7 expression observed in AE skin can occur as a consequence of FLG loss. Finally, our pathway analysis suggests a possible role of FLG deficiency in regulating cell stress, including mTOR, eIF4, and eIF2 signaling, which is

broadly consistent with RNA sequencing analysis of uninvolved FLG-deficient AE skin.¹² Lack of correlation between the protein and mRNA expression profiles of CFL1 and PPIA emphasize the value of proteomic studies. Complementary transcriptomic studies will provide further understanding of the signaling mechanisms upstream of our observations. To this end, development of robust bioinformatic platforms for the integration of transcriptomic and proteomic data sets will be hugely valuable.

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Key messages

- Loss of FLG in the absence of inflammation is sufficient to induce molecular changes characteristic of AE, including inflammatory, proteolytic, and cytoskeletal dysregulation.
- The immunophilin PPIA is a novel protein decreased in FLG-deficient LSEs and clinically active AE skin.
- The characteristic upregulation of KLK7 expression in patients with AE occurs downstream of FLG loss.
- Disconnect occurs between the epidermal proteome and transcriptome, highlighting the importance of proteomic studies for the identification of disease mechanisms.

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Supplementary Methods:

Lentivirus (shRNA) production

The HEK293T packaging cell line was used to produce replication incompetent, shRNA expressing, lentivirus. Briefly, cells were cultured in DMEM supplemented with 10% foetal calf serum. GIPZ lentiviral shRNAmir vectors were purchased from Open Biosystems (CO, USA) with a sequence targeting filaggrin (shFLG, V3LHS_369921), or a non-targeting control sequence (shNT, RHS4346, NS). HEK293T cells were co-transfected with pMD2.G envelope plasmid (5 µg), pCMVδ8.91 packaging plasmid (15 µg) and pGIPZ shRNA transfer plasmid (20 µg) using the calcium chloride precipitation method as previously described [1]. The following day media was replaced with 12 ml of enriched DMEM supplemented with 20% FCS and incubated for 2 further days before collecting the lentiviral particle containing supernatant. Lentivirus-containing media was cleared of cell debris by centrifugation (2000 rpm, 30 minutes, 4°C).

Lentiviral Transduction of keratinocytes

Passage 1 keratinocytes were transduced at 5—70% confluence in 6 well plates. Lentiviral aliquots were diluted 1:4 in naive DMEM, supplemented with hexadimethrine bromide (4 µg/ml) and added to wells. Plates were spin transduced at room temperature (600g, 1.5 hrs). Cells were washed and cultured in fresh complete Epilife media for 48 hours, to allow for the induction of transgene expression, before selection in puromycin supplemented media (1 µg/ml). Lentiviral work was carried out in accordance with safety requirements from the Health and Safety Executive.

Living skin equivalent (LSE) culture

LSEs were generated as described previously [1]. Briefly, 1×10^6 NHEKs were seeded into 12mm diameter, 0.4 µM PCF Millicell[®] cell culture inserts (Millipore) and cultured overnight in complete Epilife[®] medium adjusted to 1.5 mM calcium (high calcium Epilife[™]). LSEs were subsequently raised to the air-liquid interface (ALI) and outer media was replaced with fresh high calcium Epilife[®] supplemented with 5 µg/ml ascorbic acid. Outer medium was changed on

alternate days for a total of 14 days post ALI culture. LSEs were harvested on ice in pre-chilled PBS before removal from plastic insert and preparation for subsequent analysis.

LSE protein extraction

LSEs were resuspended in 150 µl of 8M Urea extraction buffer (8 M Urea, 50 mM Tris-HCL pH 8.0, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 0.25% SDS and 1x protease inhibitor cocktail (Sigma Aldrich, UK). The detached polycarbonate insert was removed and the LSE then homogenised on ice by sonication (3 x 10 seconds, 8 Hz). Insoluble material was removed by centrifugation (23,000 g, 20 minutes, 4°C).

Immunoblotting and densitometry

Protein concentration of urea lysates was determined by BCA assay (Pierce, Thermo-Fischer, UK). Protein lysates (20 µg) were resolved under reducing conditions using NOVEX 4-12% Bis-Tris gel system (Life Technologies, UK) and immunoblotted using standard protocols. Primary antibody conditions were: filaggrin clone 15C10, 1:200 (NCL-FLG, Novocastra)(antibody raised against the filaggrin repeat domain), cofilin-1, 1:500 (PA5-27627, Thermo Scientific), kallikrein-7, 1:1000 (AF2624, R&D systems), cyclophilin A, 1:300 (39-1100, Invitrogen), GAPDH, 1:10,000 (#2118, Cell Signaling Technology. Appropriate peroxidase conjugated secondary antibodies (Vectorlabs, UK) were diluted 1:5000. Immunoblots were developed chemoluminescently using film. Densitometry was performed using ImageJ and normalised to GAPDH.

Immunohistochemistry

Paraffin embedded LSE sections (4 µM) were stained with haematoxylin and eosin (H&E) staining as per standard protocols [1]. Images were acquired on a Zeiss Axio-imager 2 microscope (Carl Zeiss Ltd, UK) and processed using Adobe Photoshop CS3 (Adobe Corp.).

Immunofluorescence

OCT-embedded LSE cryo-sections (4 µM) were fixed and stained as described in [2]. Primary antibody conditions were: filaggrin (Acetone fix, 1:100, MS-449-P1, Lab Vision-Neomarkers), cofilin-1 (paraformaldehyde fix, 1:300, PA5-27627, Thermo Scientific), kallikrein-7 (Methanol fix, 1:250, AF2624, R&D systems), cyclophilin A (paraformaldehyde fix, 1:500, 39-1100, Invitrogen). Appropriate fluorescently conjugated secondary antibodies (Alexa Fluor goat 568) were used in

combination with Hoechst 33342 nuclear counterstain (Life Technologies). Slides were imaged under constant conditions using a Nikon A1 confocal microscope (x20 plus an additional 1.54x zoom) (Nikon Instruments Europe B.V, Netherlands). Images were processed using Photoshop CS5 software (Adobe Corp.).

Quantification was performed in image J; the total fluorescence density and area of manually defined regions of interest were calculated. Fluorescence intensity was defined as the fluorescence density per unit area. Background fluorescence was determined from the appropriate IgG control and negated from each calculation.

Filaggrin sequencing

Blood derived total DNA was analysed using Access Array 48.48 IFC (Integrated Fluidic Circuit) technology. 50ng of each DNA sample was enriched and amplified via PCR in the Access Array chip and subject to 48 validated, overlapping *FLG* specific Primer pair assays (500bp amplicons). This provided comprehensive coverage of the FLG coding regions including the extra filaggrin allelic repeats in at position 8 and 10. PCR products were harvested from the IFC array and quantified using an Agilent DNA 1000 Kit. After determining correct range of products was obtained, PCR products were pooled and purified using Agencourt® AMPure® XP Reagent Beads (Beckman Coulter Genomics). This pool was sequenced on Illumina MiSeq platform (2x250bp read mode). The raw sequence files were processed by a custom pipeline implemented in Pipeline Pilot (www.accelrys.com) and input reads were mapped to a filaggrin in house reference sequence containing 12 repeats, introns, 5' and 3' UTRs. The Mapper (version 0.7.10--r789) with the following parameters: gap opening penalty = 8, gap extension, Penalty = 2, mismatch penalty = 4, match score = 1 and minimum score threshold= 100. The Mapped reads are stored as indexed bam files which are then processed with the GATK toolkit (version v3.4-46-gbc02625, Java Version 1.7.0_75). The HaplotypeCaller module was used to calculate SNPs and indels for each sample separately using the following settings: minimum variant quality = 10, minimum pass quality = 50, maximum deletion length =12 and coverage was down sampled to 500 in high coverage regions. The individual gVCF files for each sample were then collectively processed with the Genotype GVCFs module to produce a joint VCF report for multiple sample (same quality cut offs). This approach is Flexible and scalable and increases the sensitivity of

SNP detection. FLG mutational analysis was performed by the Institute of Medical Biology Group (A*STAR Singapore) by contractual agreement. A summary of the sequencing results is provided in Table E1.

Proteomic Analysis

In-gel digest

The whole lane was excised and spilt into 22 bands/ 22 samples cut in small pieces (1x1mm cubes) and the standard reduction, alkylation and trypsin (Promega) digestion protocol was performed as per this Nature methods publication [3].

In-solution digest

The lysates were reduced, alkylated and digested as per [4]. After digestion, the samples were desalted using StageTips before MS analysis [5].

MS analysis

Two different systems were used in the analysis of these samples. The in-gel digested samples were analysed on a LTQ-Orbitrap XL (Thermo Fisher Scientific) via a NanoFlex electrospray ion source (Proxeon) coupled to an easy-nLC 1000 (Thermo Fisher Scientific) using an Acclaim PepMap 100 75umx2cm nanoViper pre-column (Thermo Fisher Scientific) and an Acclaim PepMap 75umx15cm nanoViper C18, 2um, 100A (Thermo Fisher Scientific) chromatography column. The in-solution samples were run on a QExactive (Thermo Fisher Scientific) via an EasySpray nanoelectrospray ion source (Thermo Fisher Scientific) coupled to an easy-nLC 1000 (Thermo Fisher Scientific) using a Acclaim PepMap 100 75umx2cm nanoViper pre-column (Thermo Fisher Scientific) and an Easy PepMap 75umx50cm C18, 2um, 100A chromatography column (Thermo Fisher Scientific). In both cases the mobile phases were 0.1% Formic acid (Thermo Fisher Scientific) in water (Fluka) for the aqueous phase and Acetonitrile (Fluka) for the organic phase. The gradient time was 1hr for the Orbitrap XL run and the instrument was set to operate in data-dependent mode measuring both a full scan MS in the Orbitrap and the top 5 most intense ions in the LTQ after collisionally induced dissociation.

The Qexactive gradient time was 2.5hrs and column temperature was set to 40°C, the MS data was acquired using a Top 10 method using HCD fragmentation to sequence the top 10 ions per full scan spectrum and the instrument was set to 2% underfill ratio, and 3m/z isolation window [6].

Data processing and Analysis

Data analysis was performed with Thermo Proteome discoverer version 1.3 using Mascot (Matrix Science, London, UK) and the uniprot human database and its decoy, the false discovery rate was set to 1% and a label free quantification node, the other parameters were as per [7].

The samples were then further processed using Scaffold (Proteome Software Inc., Portland, OR) version 3.6.1 where proteome discoverer files were imported and reprocessed using X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet [8].

Proteomic analysis of the technical replicates identified 1,640 unique proteins. Given the high number of missing values the dataset was filtered to include only those proteins reported in 15 or more of the 20 sample preparations. Filtering reduced the data suitable for statistical analysis to a subset of 367 unique proteins. Given the classic paired study design (i.e. 10 paired donor samples) no additional data normalisation was implemented. All protein expression values were $\log_{10}$ transformed and the difference between the shFLG and shNT un-weighted spectrum counts for each protein were calculated for each of the 367 proteins. A one sample t-Test was applied to each of the estimated differences, with the null hypothesis that the mean of the differences was zero. Log ratios with an adjusted p-value of <0.05 were considered significantly differentially expressed and used as input for Ingenuity pathway analysis (IPA) and further downstream validation. All statistical analysis was completed using R version 3.1.2.

Ingenuity Pathway Analysis

Ingenuity pathway analysis (IPA) software (Qiagen, Redwood city, USA) was employed to assist with the biological interpretation of the significantly altered data set. Differentially regulated proteins were grouped by cellular function, disease relevance and canonical pathway involvement based on an extensive hand curated IPA knowledge base of human and rodent studies. A right-tailed Fisher's exact test was implemented by IPA to determine if the representation of our overlaid proteins within each group/pathway was considered significant ($P \leq 0.05$). This takes into account both the number of imputed focus proteins that participate within each group/pathway and the respective total number of proteins known to be associated within the reference set (<http://www.ingenuity.com>).

Reverse transcription-qPCR

RNA was extracted from LSEs using the ReliaPrep miniprep system following manufacturer's instructions (Promega). cDNA was prepared from 0.5 - 2 μ g of total RNA using random priming. Moloney murine leukemia virus enzyme (MMLV) (100 units) and buffer system was used in a 20 μ l reaction volume according to manufacturer's instructions with the addition of RNase OUT (5 units) (Life technologies).

The following exon-spanning probe based assays were used: filaggrin (Hs.PT.53a.23095403, FAM/TAMRA, Integrated DNA technologies), cyclophilin A (F: ATGCTGGACCCAACACAAAT, R: TCTTTCACCTTGCCAAACACC, UPL PROBE#48 FAM/NFQ, Roche), cofilin-1 (F: GTGCCCTCTCCTTTTCGTTT, R: TTGAACACCTTGATGACACCAT, UPL PROBE#5 FAM/NFQ, Roche), kallikrein-7 (Hs.PT.58.39819237, FAM/TAMRA, IDT, IA, USA). 18S was used as a housekeeping gene for normalisation purposes [9], in combination with Taqman gene expression mastermix according to manufacturer's instructions (Life Technologies, UK). Assays were run on a StepOne plus qPCR machine (Life Technologies) and fold changes derived via the $2^{-\Delta\Delta C(T)}$ method.

Statistical analysis

Statistical analysis was performed using prism 5 (GraphPad software, San Diego, CA). For paired comparisons between shNT and shFLG LSEs, data was expressed as a $\log_{10}$ fold change relative to shNT and a paired one sample T.Test was performed. For comparisons between normal, AE

uninvolved and AE involved skin, data was expressed as a log₁₀ fold change relative to normal and a one way Anova with Tukey post test was performed.

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Table E1: List of 360 proteins common to $\geq 75\%$ of analysed samples

Uniprot ID	Protein name	Mean difference	P-value
A2ML1_HUMAN	alpha-2-macroglobulin-like 1	-0.02	0.73
ACADV_HUMAN	acyl-CoA dehydrogenase, very long chain	-0.15	0.11
THIC_HUMAN	acetyl-CoA acetyltransferase 2	-0.08	0.48
ACLY_HUMAN	ATP citrate lyase	-0.08	0.55
ACON_HUMAN	aconitase 2, mitochondrial	-0.03	0.79
ACTBL_HUMAN	actin, beta-like 2	-0.06	0.25
ACTN4_HUMAN	actinin, alpha 4	-0.07	0.21
ACTZ_HUMAN	ARP1 actin-related protein 1 homolog A, centractin alpha (yeast)	0.11	0.47
ARP3_HUMAN	ARP3 actin-related protein 3 homolog (yeast)	0.05	0.57
AHNK_HUMAN	AHNAK nucleoprotein	0.12	0.16
AHNK2_HUMAN	AHNAK nucleoprotein 2	0.08	0.47
ALDH2_HUMAN	aldehyde dehydrogenase 2 family (mitochondrial)	0.16	0.14
ALDOA_HUMAN	aldolase A, fructose-bisphosphate	0	0.95
ALDOC_HUMAN	aldolase C, fructose-bisphosphate	0.07	0.58
ANXA1_HUMAN	annexin A1	0.07	0.14
ANXA2_HUMAN	annexin A2	0.04	0.25
ANXA3_HUMAN	annexin A3	0.17	0.02
ANXA4_HUMAN	annexin A4	-0.06	0.44

ANXA5_HUMAN	annexin A5	0.02	0.83
ANXA8_HUMAN	annexin A8-like 1	-0.06	0.56
AP1B1_HUMAN	adaptor-related protein complex 1, beta 1 subunit	0.12	0.28
ACPH_HUMAN	acylaminoacyl-peptide hydrolase	-0.2	0.14
ARF1_HUMAN	ADP-ribosylation factor 1	-0.05	0.46
ARF4_HUMAN	ADP-ribosylation factor 4	0.11	0.34
ARG11_HUMAN	arginase 1	0.03	0.59
ARPC4_HUMAN	actin related protein 2/3 complex, subunit 4, 20kDa	0.03	0.78
APRV1_HUMAN	aspartic peptidase, retroviral-like 1	-0.05	0.59
PUR9_HUMAN	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	-0.08	0.56
AT1A1_HUMAN	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	0.07	0.43
AT2A2_HUMAN	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2	-0.17	0.17
ATPA_HUMAN	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	-0.01	0.80
ATPB_HUMAN	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, beta polypeptide	-0.01	0.89
ATPO_HUMAN	ATP synthase, H ⁺ transporting,	-0.06	0.32

mitochondrial F1 complex, O subunit

VATA_HUMAN	ATPase, H ⁺ transporting, lysosomal 70kDa, V1 subunit A	0.07	0.42
VATB2_HUMAN	ATPase, H ⁺ transporting, lysosomal 56/58kDa, V1 subunit B2	-0.08	0.51
ZA2G_HUMAN	alpha-2-glycoprotein 1, zinc-binding	0.01	0.92
BAP31_HUMAN	B-cell receptor-associated protein 31	0.09	0.42
BLMH_HUMAN	bleomycin hydrolase	0.02	0.83
C1QBP_HUMAN	complement component 1, q subcomponent binding protein	-0.06	0.36
CAH2_HUMAN	carbonic anhydrase II	-0.06	0.59
CALL3_HUMAN	calmodulin-like 3	0.08	0.12
CALL5_HUMAN	calmodulin-like 5	0.05	0.41
CALR_HUMAN	calreticulin	-0.02	0.82
CAND1_HUMAN	cullin-associated and neddylation-dissociated 1	0.13	0.27
CALX_HUMAN	calnexin	0.08	0.28
CAP1_HUMAN	CAP, adenylate cyclase-associated protein 1 (yeast)	0.1	0.08
CAPG_HUMAN	capping protein (actin filament), gelsolin-like	0.11	0.25
CAN1_HUMAN	calpain 1, (mu/I) large subunit	-0.02	0.85
CAZA1_HUMAN	capping protein (actin filament) muscle Z-line, alpha 1	0.04	0.70

CAPZB_HUMAN	capping protein (actin filament) muscle Z-line, beta	0.03	0.74
CASPE_HUMAN	caspase 14, apoptosis-related cysteine peptidase	0.08	0.14
ICAL_HUMAN	calpastatin	0.04	0.75
CATA_HUMAN	catalase	-0.01	0.91
CBR1_HUMAN	carbonyl reductase 1	0.02	0.85
TCPB_HUMAN	chaperonin containing TCP1, subunit 2 (beta)	0.05	0.34
TCPG_HUMAN	chaperonin containing TCP1, subunit 3 (gamma)	0.05	0.65
TCPD_HUMAN	chaperonin containing TCP1, subunit 4 (delta)	0.02	0.87
TCPE_HUMAN	chaperonin containing TCP1, subunit 5 (epsilon)	-0.06	0.44
TCPZ_HUMAN	chaperonin containing TCP1, subunit 6A (zeta 1)	-0.11	0.01
TCPH_HUMAN	chaperonin containing TCP1, subunit 7 (eta)	-0.07	0.21
TCPQ_HUMAN	chaperonin containing TCP1, subunit 8 (theta)	-0.01	0.94
COF1_HUMAN	cofilin 1 (non-muscle)	0.11	0.03
CKAP4_HUMAN	cytoskeleton-associated protein 4	0.06	0.55
CLIC1_HUMAN	chloride intracellular channel 1	0.05	0.59
CLIC3_HUMAN	chloride intracellular channel 3	-0.02	0.87
CLH1_HUMAN	clathrin, heavy chain (Hc)	-0.04	0.60

COHA1_HUMAN	collagen, type XVII, alpha 1	0.13	0.10
COPB2_HUMAN	coatomer protein complex, subunit beta 2 (beta prime)	0.04	0.78
CBPA4_HUMAN	carboxypeptidase A4	0.25	0.04
CISY_HUMAN	citrate synthase	-0.17	0.11
CYTM_HUMAN	cystatin E/M	0.05	0.65
CYTA_HUMAN	cystatin A (stefin A)	0.03	0.72
CYTB_HUMAN	cystatin B (stefin B)	0.04	0.80
CTNA1_HUMAN	catenin (cadherin-associated protein), alpha 1, 102kDa	0.02	0.83
CATC_HUMAN	cathepsin C	0.04	0.66
CATD_HUMAN	cathepsin D	0	0.96
CATL2_HUMAN	cathepsin V	0.15	0.05
SRC8_HUMAN	cortactin	0.03	0.55
DDX3X_HUMAN	DEAD (Asp-Glu-Ala-Asp) box helicase 3, X-linked	-0.04	0.50
DHCR7_HUMAN	7-dehydrocholesterol reductase	0.07	0.67
DHX9_HUMAN	DEAH (Asp-Glu-Ala-His) box helicase 9	0	0.98
DLDH_HUMAN	dihydrolipoamide dehydrogenase	-0.01	0.78
DMKN_HUMAN	dermokine	-0.07	0.44
DSC1_HUMAN	desmocollin 1	0.03	0.55
DSC2_HUMAN	desmocollin 2	0.11	0.38
DSC3_HUMAN	desmocollin 3	0.07	0.26

DSG1_HUMAN	desmoglein 1	0.05	0.45
DSG3_HUMAN	desmoglein 3	0.03	0.70
DESP_HUMAN	desmoplakin	-0.02	0.69
DYHC1_HUMAN	dynein, cytoplasmic 1, heavy chain 1	-0.03	0.85
ECH1_HUMAN	enoyl CoA hydratase 1, peroxisomal	0.1	0.39
EF1A3_HUMAN	eukaryotic translation elongation factor 1 alpha 1 pseudogene 5	-0.12	0.06
EF1B_HUMAN	eukaryotic translation elongation factor 1 beta 2	0.06	0.47
EF1D_HUMAN	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	0.01	0.90
EF1G_HUMAN	eukaryotic translation elongation factor 1 gamma	-0.07	0.44
EF2_HUMAN	eukaryotic translation elongation factor 2	-0.03	0.22
IF2G_HUMAN	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	0.04	0.80
EIF3B_HUMAN	eukaryotic translation initiation factor 3, subunit B	-0.03	0.84
IF4A1_HUMAN	eukaryotic translation initiation factor 4A1	-0.07	0.46
IF4G1_HUMAN	eukaryotic translation initiation factor 4 gamma, 1	-0.05	0.69
IF6_HUMAN	eukaryotic translation initiation factor 6	0.06	0.49
ENOA_HUMAN	enolase 1, (alpha)	-0.02	0.74
EPIPL_HUMAN	epiplakin 1	-0.08	0.24

SYEP_HUMAN	glutamyl-prolyl-tRNA synthetase	-0.03	0.66
ERP29_HUMAN	endoplasmic reticulum protein 29	-0.06	0.55
EVPL_HUMAN	envoplakin	0.06	0.51
EZRI_HUMAN	ezrin	-0.01	0.74
FABP5_HUMAN	fatty acid binding protein 5 (psoriasis-associated)	0.03	0.59
FAS_HUMAN	fatty acid synthase	0.01	0.82
FILA_HUMAN	filaggrin	-0.46	0.00
FILA2_HUMAN	filaggrin family member 2	-0.01	0.92
FLNA_HUMAN	filamin A, alpha	-0.01	0.86
FLNB_HUMAN	filamin B, beta	0	0.98
FSCN1_HUMAN	fascin actin-bundling protein 1	-0.05	0.49
GANAB_HUMAN	glucosidase, alpha; neutral AB	0.11	0.31
G3P_HUMAN	glyceraldehyde-3-phosphate dehydrogenase	-0.05	0.37
SYG_HUMAN	glycyl-tRNA synthetase	0.01	0.86
GDIB_HUMAN	GDP dissociation inhibitor 2	-0.04	0.76
GGCT_HUMAN	gamma-glutamylcyclotransferase	0.07	0.18
GLTP_HUMAN	glycolipid transfer protein	0.06	0.62
DHE3_HUMAN	glutamate dehydrogenase 1	-0.06	0.48
SAP3_HUMAN	GM2 ganglioside activator	0.02	0.75
GBLP_HUMAN	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	0.1	0.12

AATC_HUMAN	glutamic-oxaloacetic transaminase 1, soluble	-0.03	0.76
AATM_HUMAN	glutamic-oxaloacetic transaminase 2, mitochondrial	0.16	0.13
G6PI_HUMAN	glucose-6-phosphate isomerase	-0.04	0.27
GSDMA_HUMAN	gasdermin A	0.02	0.85
GELS_HUMAN	gelsolin	0	0.94
GSTP1_HUMAN	glutathione S-transferase pi 1	-0.07	0.20
H2AY_HUMAN	H2A histone family, member Y	0.08	0.35
H2BFS_HUMAN	H2B histone family, member S (pseudogene)	0.03	0.46
ECHA_HUMAN	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	-0.01	0.91
ECHB_HUMAN	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	0	0.98
HUTH_HUMAN	histidine ammonia-lyase	-0.02	0.80
H13_HUMAN	histone cluster 1, H1d	0.14	0.38
HXK1_HUMAN	hexokinase 1	0.02	0.73
HMOX1_HUMAN	heme oxygenase (decycling) 1	-0.15	0.56
ROA1_HUMAN	heterogeneous nuclear ribonucleoprotein A1	-0.05	0.36
ROA2_HUMAN	heterogeneous nuclear ribonucleoprotein A2/B1	0.09	0.44

HNRPC_HUMAN	heterogeneous nuclear ribonucleoprotein C (C1/C2)	0.06	0.26
HNRH1_HUMAN	heterogeneous nuclear ribonucleoprotein H1 (H)	0	0.99
HNRPK_HUMAN	heterogeneous nuclear ribonucleoprotein K	-0.05	0.48
HNRPM_HUMAN	heterogeneous nuclear ribonucleoprotein M	0.12	0.24
HNRPU_HUMAN	heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	0.06	0.53
HCD2_HUMAN	hydroxysteroid (17-beta) dehydrogenase 10	-0.24	0.07
DHB4_HUMAN	hydroxysteroid (17-beta) dehydrogenase 4	-0.03	0.73
ENPL_HUMAN	heat shock protein 90kDa beta (Grp94), member 1	0.05	0.27
HSP74_HUMAN	heat shock 70kDa protein 4	0.01	0.96
HS74L_HUMAN	heat shock 70kDa protein 4-like	-0.01	0.97
HSP7C_HUMAN	heat shock 70kDa protein 8	0.03	0.51
GRP75_HUMAN	heat shock 70kDa protein 9 (mortalin)	0.1	0.16
HSPB1_HUMAN	heat shock 27kDa protein 1	-0.11	0.05
CH60_HUMAN	heat shock 60kDa protein 1 (chaperonin)	0.02	0.71
CH10_HUMAN	heat shock 10kDa protein 1	-0.1	0.22
HS105_HUMAN	heat shock 105kDa/110kDa protein 1	-0.08	0.46
HTRA1_HUMAN	HtrA serine peptidase 1	0.05	0.66
HYOU1_HUMAN	hypoxia up-regulated 1	-0.05	0.67
IDE_HUMAN	insulin-degrading enzyme	-0.15	0.06

IDHC_HUMAN	isocitrate dehydrogenase 1 (NADP+), soluble	0.01	0.94
IL36G_HUMAN	interleukin 36, gamma	-0.05	0.71
ILF2_HUMAN	interleukin enhancer binding factor 2	-0.08	0.28
IMDH2_HUMAN	IMP (inosine 5'-monophosphate) dehydrogenase 2	0.09	0.49
IQGA1_HUMAN	IQ motif containing GTPase activating protein 1	-0.01	0.93
ITA6_HUMAN	integrin, alpha 6	0.12	0.24
ITB1_HUMAN	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	-0.22	0.11
ITB4_HUMAN	integrin, beta 4	0.02	0.81
INVO_HUMAN	involucrin	0.02	0.55
PLAK_HUMAN	junction plakoglobin	-0.06	0.27
KINH_HUMAN	kinesin family member 5B	0	0.99
KLK10_HUMAN	kallikrein-related peptidase 10	0.06	0.61
KLK7_HUMAN	kallikrein-related peptidase 7	0.34	0.02
IMB1_HUMAN	karyopherin (importin) beta 1	-0.01	0.91
KPRP_HUMAN	keratinocyte proline-rich protein	0.24	0.13
K1C10_HUMAN	keratin 10	-0.07	0.10
K22E_HUMAN	keratin 2	-0.07	0.10
K2C80_HUMAN	keratin 80	-0.07	0.54
K1C9_HUMAN	keratin 9	-0.07	0.58

LAD1_HUMAN	ladinin 1	-0.02	0.73
LAMP1_HUMAN	lysosomal-associated membrane protein 1	0.1	0.39
NGAL_HUMAN	lipocalin 2	-0.02	0.80
LDHA_HUMAN	lactate dehydrogenase A	0.03	0.60
LDHB_HUMAN	lactate dehydrogenase B	0.04	0.55
LG3BP_HUMAN	lectin, galactoside-binding, soluble, 3 binding protein	0.01	0.92
LEG7_HUMAN	lectin, galactoside-binding, soluble, 7	-0.11	0.17
LMNA_HUMAN	lamin A/C	0.05	0.33
LMNB1_HUMAN	lamin B1	-0.04	0.78
LY6D_HUMAN	lymphocyte antigen 6 complex, locus D	0.14	0.04
MAP4_HUMAN	microtubule-associated protein 4	0.06	0.27
MDHC_HUMAN	malate dehydrogenase 1, NAD (soluble)	0.06	0.51
MDHM_HUMAN	malate dehydrogenase 2, NAD (mitochondrial)	0.02	0.65
MAOX_HUMAN	malic enzyme 1, NADP(+)-dependent, cytosolic	0.07	0.50
MVP_HUMAN	major vault protein	0.04	0.62
MX1_HUMAN	MX dynamin-like GTPase 1	-0.04	0.65
MYH9_HUMAN	myosin, heavy chain 9, non-muscle	-0.06	0.16
MYL6_HUMAN	myosin, light chain 6, alkali, smooth muscle and non-muscle	0.04	0.61
NUCL_HUMAN	nucleolin	0.03	0.54

NDKA_HUMAN	NME/NM23 nucleoside diphosphate kinase 1	-0.15	0.27
NPC2_HUMAN	Niemann-Pick disease, type C2	0.11	0.39
PSA_HUMAN	aminopeptidase puromycin sensitive	0.04	0.85
NPM_HUMAN	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	0.07	0.18
PDIA1_HUMAN	prolyl 4-hydroxylase, beta polypeptide	-0.02	0.58
PA2G4_HUMAN	proliferation-associated 2G4, 38kDa	-0.03	0.64
PABP1_HUMAN	poly(A) binding protein, cytoplasmic 1	-0.02	0.85
PARK7_HUMAN	parkinson protein 7	0.06	0.54
PCBP1_HUMAN	poly(rC) binding protein 1	0.06	0.42
PDC6I_HUMAN	programmed cell death 6 interacting protein	0.07	0.33
PDIA3_HUMAN	protein disulfide isomerase family A, member 3	0	0.96
PDIA4_HUMAN	protein disulfide isomerase family A, member 4	0.02	0.77
PDIA6_HUMAN	protein disulfide isomerase family A, member 6	-0.03	0.73
PDLI1_HUMAN	PDZ and LIM domain 1	0.09	0.53
PEBP1_HUMAN	phosphatidylethanolamine binding protein 1	0.05	0.57
PROF1_HUMAN	profilin 1	0	0.96
PGAM1_HUMAN	phosphoglycerate mutase 1 (brain)	0.03	0.61
6PGD_HUMAN	phosphogluconate dehydrogenase	-0.09	0.48
PGK1_HUMAN	phosphoglycerate kinase 1	-0.07	0.37

PHB_HUMAN	prohibitin	0.06	0.43
PHB2_HUMAN	prohibitin 2	-0.04	0.61
SERA_HUMAN	phosphoglycerate dehydrogenase	0	0.97
ELAF_HUMAN	peptidase inhibitor 3, skin-derived	0.1	0.26
KPYM_HUMAN	pyruvate kinase, muscle	-0.02	0.63
PKP1_HUMAN	plakophilin 1	0.02	0.72
PKP3_HUMAN	plakophilin 3	0.04	0.48
PLEC_HUMAN	plectin	-0.05	0.51
PLST_HUMAN	plastin 3	0.02	0.61
PNPH_HUMAN	purine nucleoside phosphorylase	0.04	0.86
POF1B_HUMAN	premature ovarian failure, 1B	-0.01	0.82
PPIA_HUMAN	peptidylprolyl isomerase A (cyclophilin A)	-0.06	0.00
PPIB_HUMAN	peptidylprolyl isomerase B (cyclophilin B)	0.02	0.79
PEPL_HUMAN	periplakin	0.1	0.24
2AAA_HUMAN	protein phosphatase 2, regulatory subunit A, alpha	-0.06	0.46
PRDX1_HUMAN	peroxiredoxin 1	0	0.98
PRDX2_HUMAN	peroxiredoxin 2	-0.01	0.89
PRDX5_HUMAN	peroxiredoxin 5	-0.04	0.78
PRDX6_HUMAN	peroxiredoxin 6	-0.04	0.69
GLU2B_HUMAN	protein kinase C substrate 80K-H	0.03	0.72
PSA1_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 1	0.1	0.35

PSA2_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 2	0.01	0.87
PSA3_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 3	0	0.99
PSA4_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 4	-0.06	0.52
PSA5_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 5	-0.07	0.43
PSA6_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 6	0.05	0.43
PSA7_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 7	0	0.98
PSB6_HUMAN	proteasome (prosome, macropain) subunit, beta type, 6	0.04	0.57
PSMD1_HUMAN	proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	-0.02	0.90
PSMD2_HUMAN	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	0	0.99
PSMD3_HUMAN	proteasome (prosome, macropain) 26S subunit, non-ATPase, 3	0.17	0.00
PSME1_HUMAN	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	0.02	0.91
PTBP1_HUMAN	polypyrimidine tract binding protein 1	-0.07	0.33
ASC_HUMAN	PYD and CARD domain containing	0.04	0.58
RAB10_HUMAN	RAB10, member RAS oncogene family	0.03	0.68

RB11A_HUMAN	RAB11A, member RAS oncogene family	0.13	0.19
RAB5C_HUMAN	RAB5C, member RAS oncogene family	0.12	0.14
RAC1_HUMAN	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	0.08	0.33
RAN_HUMAN	RAN, member RAS oncogene family	-0.02	0.86
SYRC_HUMAN	arginyl-tRNA synthetase	0.14	0.13
RNAS7_HUMAN	ribonuclease, RNase A family, 7	-0.03	0.77
RINI_HUMAN	ribonuclease/angiogenin inhibitor 1	-0.04	0.49
RL10A_HUMAN	ribosomal protein L10a	-0.04	0.71
RL11_HUMAN	ribosomal protein L11	-0.15	0.09
RL12_HUMAN	ribosomal protein L12	-0.04	0.61
RL13_HUMAN	ribosomal protein L13	0.06	0.41
RL17_HUMAN	ribosomal protein L17	0.03	0.74
RL18_HUMAN	ribosomal protein L18	0.01	0.89
RL22_HUMAN	ribosomal protein L22	0.21	0.03
RL23_HUMAN	ribosomal protein L23	0.01	0.82
RL23A_HUMAN	ribosomal protein L23a	0.11	0.21
RL28_HUMAN	ribosomal protein L28	0.1	0.42
RL3_HUMAN	ribosomal protein L3	0.16	0.09
RL30_HUMAN	ribosomal protein L30	0.13	0.25
RL34_HUMAN	ribosomal protein L34	-0.03	0.77
RL4_HUMAN	ribosomal protein L4	0.17	0.01
RL5_HUMAN	ribosomal protein L5	0.02	0.72

RL6_HUMAN	ribosomal protein L6	0.09	0.33
RL7_HUMAN	ribosomal protein L7	0.04	0.53
RL7A_HUMAN	ribosomal protein L7a	-0.05	0.53
RL8_HUMAN	ribosomal protein L8	0.16	0.15
RL9_HUMAN	ribosomal protein L9	0	1.00
RLA0_HUMAN	ribosomal protein, large, P0	0.04	0.29
RLA2_HUMAN	ribosomal protein, large, P2	0	0.97
RPN1_HUMAN	ribophorin I	-0.01	0.91
RS10_HUMAN	ribosomal protein S10	0.01	0.90
RS11_HUMAN	ribosomal protein S11	0.13	0.11
RS13_HUMAN	ribosomal protein S13	-0.13	0.09
RS14_HUMAN	ribosomal protein S14	0.14	0.22
RS15A_HUMAN	ribosomal protein S15a	0.1	0.34
RS16_HUMAN	ribosomal protein S16	-0.06	0.41
RS18_HUMAN	ribosomal protein S18	-0.16	0.15
RS2_HUMAN	ribosomal protein S2	0.05	0.58
RS25_HUMAN	ribosomal protein S25	-0.21	0.07
RS26_HUMAN	ribosomal protein S26	0.07	0.53
RS27_HUMAN	ribosomal protein S27	0.21	0.02
RS27A_HUMAN	ribosomal protein S27a	0.05	0.36
RS3_HUMAN	ribosomal protein S3	-0.03	0.60
RS3A_HUMAN	ribosomal protein S3A	0.03	0.50

RS4X_HUMAN	ribosomal protein S4, X-linked	-0.04	0.39
RS5_HUMAN	ribosomal protein S5	-0.12	0.04
RS6_HUMAN	ribosomal protein S6	0.15	0.10
RS7_HUMAN	ribosomal protein S7	-0.03	0.80
RS8_HUMAN	ribosomal protein S8	0.03	0.49
RS9_HUMAN	ribosomal protein S9	-0.03	0.74
RSSA_HUMAN	ribosomal protein SA	0.04	0.42
S10AA_HUMAN	S100 calcium binding protein A10	-0.06	0.42
S10AB_HUMAN	S100 calcium binding protein A11	0.03	0.66
S10AE_HUMAN	S100 calcium binding protein A14	0.09	0.17
S10AG_HUMAN	S100 calcium binding protein A16	0.05	0.64
S10A2_HUMAN	S100 calcium binding protein A2	0.04	0.61
S10A6_HUMAN	S100 calcium binding protein A6	-0.03	0.71
S10A7_HUMAN	S100 calcium binding protein A7	0.06	0.56
S10A8_HUMAN	S100 calcium binding protein A8	-0.11	0.28
S10A9_HUMAN	S100 calcium binding protein A9	-0.12	0.17
SBSN_HUMAN	suprabasin	-0.02	0.87
SCEL_HUMAN	sciellin	0.04	0.75
SC61B_HUMAN	Sec61 beta subunit	0.13	0.29
PAIRB_HUMAN	SERPINE1 mRNA binding protein 1	0.01	0.92
SPB3_HUMAN	serpin peptidase inhibitor, clade B (ovalbumin), member 3	0.02	0.75
SPB5_HUMAN	serpin peptidase inhibitor, clade B	0.01	0.71

(ovalbumin), member 5

SET_HUMAN	SET nuclear proto-oncogene	-0.03	0.82
1433S_HUMAN	stratifin	0.01	0.77
SFPQ_HUMAN	splicing factor proline/glutamine-rich	-0.01	0.90
ADT2_HUMAN	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	0.02	0.80
4F2_HUMAN	solute carrier family 3 (amino acid transporter heavy chain), member 2	-0.05	0.32
LAT1_HUMAN	solute carrier family 7 (amino acid transporter light chain, L system), member 5	0.17	0.10
SLPI_HUMAN	secretory leukocyte peptidase inhibitor	0.08	0.43
SND1_HUMAN	staphylococcal nuclease and tudor domain containing 1	0.02	0.87
SODC_HUMAN	superoxide dismutase 1, soluble	0.03	0.66
SPR1B_HUMAN	small proline-rich protein 1B	0.04	0.57
SPR2D_HUMAN	small proline-rich protein 2D	0.15	0.16
SPR2F_HUMAN	small proline-rich protein 2F	0.16	0.19
SQRD_HUMAN	sulfide quinone reductase-like (yeast)	0.19	0.07
SSRD_HUMAN	signal sequence receptor, delta	0.04	0.71
STIP1_HUMAN	stress-induced phosphoprotein 1	0.1	0.38
HNRPQ_HUMAN	synaptotagmin binding, cytoplasmic RNA interacting protein	-0.04	0.74
TAGL2_HUMAN	transgelin 2	-0.02	0.75

TALDO_HUMAN	transaldolase 1	0.06	0.41
TCPA_HUMAN	t-complex 1	-0.15	0.21
TGM1_HUMAN	transglutaminase 1	-0.07	0.52
TGM3_HUMAN	transglutaminase 3	-0.07	0.40
TKT_HUMAN	transketolase	-0.02	0.66
TLN1_HUMAN	talin 1	-0.06	0.72
TB182_HUMAN	tankyrase 1 binding protein 1, 182kDa	0.05	0.79
TPIS_HUMAN	triosephosphate isomerase 1	0.02	0.82
TPM4_HUMAN	tropomyosin 4	-0.04	0.61
TRI29_HUMAN	tripartite motif containing 29	0.08	0.02
TBA4B_HUMAN	tubulin, alpha 4b (pseudogene)	-0.03	0.54
TBB5_HUMAN	tubulin, beta class I	-0.09	0.17
EFTU_HUMAN	Tu translation elongation factor, mitochondrial	-0.01	0.96
THIO_HUMAN	thioredoxin	0.16	0.01
TXD17_HUMAN	thioredoxin domain containing 17	0.06	0.48
TYPH_HUMAN	thymidine phosphorylase	0.04	0.59
UBA1_HUMAN	ubiquitin-like modifier activating enzyme 1	-0.06	0.51
QCR2_HUMAN	ubiquinol-cytochrome c reductase core protein II	0.07	0.58
VAT1_HUMAN	vesicle amine transport 1	0.06	0.59
VINC_HUMAN	vinculin	0	0.99
TERA_HUMAN	valosin containing protein	0.03	0.58

VDAC1_HUMAN	voltage-dependent anion channel 1	0.03	0.61
VDAC2_HUMAN	voltage-dependent anion channel 2	0.09	0.16
VIME_HUMAN	vimentin	-0.32	0.02
WDR1_HUMAN	WD repeat domain 1	-0.13	0.47
YBOX1_HUMAN	Y box binding protein 1	0.13	0.38
ZN185_HUMAN	zinc finger protein 185 (LIM domain)	-0.02	0.86

Table E2: List of significantly differentially expressed proteins following filaggrin knockdown

Uniprot ID	Protein name	Symbol	Mean difference (LOG ₁₀)	P-value
ANXA3_HUMAN	Annexin A3	ANXA3	0.17	0.0225
CATL2_HUMAN	Cathepsin L2	CTSV	0.15	0.048
CBPA4_HUMAN	Carboxypeptidase A4	CPA4	0.25	0.0365
COF1_HUMAN	Cofilin-1	CFL1	0.11	0.0331
FILA_HUMAN	Filaggrin	FLG	-0.46	2.00E-04
KLK7_HUMAN	Kallikrein-7	KLK7	0.34	0.02
LY6D_HUMAN	Lymphocyte antigen 6D	LY6D	0.14	0.0374
PPIA_HUMAN	Cyclophilin A	PPIA	-0.06	0.0039
PSMD3_HUMAN	26S proteasome regulatory subunit 3	PSMD3	0.17	0.0031
RL22_HUMAN	60S ribosomal protein L22	RPL22	0.21	0.0312
RL4_HUMAN	60S ribosomal protein L4	RPL4	0.17	0.0137
RS27_HUMAN	40S ribosomal protein S27	RPS27	0.21	0.0201
RS5_HUMAN	40S ribosomal protein S5	RPS5	-0.12	0.0425
TCPZ_HUMAN	T-complex protein 1 subunit zeta	CCT6A	-0.11	0.0143
THIO_HUMAN	Thioredoxin	TXN	0.16	0.0111
TRI29_HUMAN	Tripartite motif-containing protein 29	TRIM29	0.08	0.018
VIME_HUMAN	Vimentin	VIM	-0.32	0.024

Table E3: Top 5 disease and disorders predicted by IPA

IPA Diseases & disorders	(p-value)	Molecules
Immunological disease	2.15E-06 - 3.58E-02	↑ANXA3, ↑CFL1, ↑CSTV, ↓FLG, ↑KLK7, ↓PPIA, ↓VIM, ↑TXN, ↑LY6D
Dermatological disease	2.42E-05 - 3.99E-02	↑CFL1, ↑CSTV, ↓FLG, ↑KLK7, ↑ANXA3, ↓VIM
Inflammatory disease	2.42E-05 - 3.66E-03	↑ANXA3, ↑CFL1, ↑CSTV, ↓FLG, ↑KLK7, ↓PPIA, ↓VIM
Inflammatory response	2.42E-05 - 3.86E-04	↑ANXA3, ↑CFL1, ↑CSTV, ↓FLG, ↑KLK7, ↓PPIA, ↓VIM, ↑TXN
Neurological disease	1.84E-05 - 4.96E-05	↑CSTV, ↓PPIA, ↓VIM, ↑TXN

Ingenuity pathway analysis (IPA)

Table E4: List of functional annotations associated with the top 5 disease and disorder categories

Categories	Function	P-value	Molecules
Cellular movement	migration of smooth muscle cells	3.05E-04	CTSV,PPIA,TXN
Cellular movement	chemotaxis of inflammatory leukocytes	8.46E-04	PPIA
Cellular movement	scattering of neuronal progenitor cells	8.46E-04	CFL1
Cellular movement	cell rolling of squamous cell carcinoma cell lines	1.69E-03	LY6D
Cellular movement	migration of AT2 cells	1.69E-03	VIM
Cellular movement	migration of podocytes	1.69E-03	CTSV
Cellular movement	delay in transmigration of smooth muscle cells	2.54E-03	CTSV

Cellular movement	migration of cells	3.06E-03	ANXA3,CFL1,CTSV,LY6D,PPIA,TXN,VIM
Cellular movement	migration of vascular smooth muscle cells	3.63E-03	PPIA,TXN
Cellular movement	cell movement of eosinophils	4.10E-03	PPIA,TXN
Cellular movement	migration of inflammatory leukocytes	4.22E-03	PPIA
Cellular movement	recruitment of leukemia cell lines	4.22E-03	VIM
Cellular movement	invasion of thyroid tumor cell lines	7.59E-03	VIM
Cellular movement	cell movement of monocytes	1.00E-02	CTSV,PPIA
Cellular movement	chemotaxis of peripheral blood monocytes	1.18E-02	PPIA
Cellular movement	cell movement of embryonic stem cells	1.26E-02	CFL1
Cellular movement	infiltration by mast cells	1.35E-02	PPIA
Cellular movement	cell movement of myeloid cells	1.80E-02	CTSV,PPIA,TXN
Cellular movement	cell movement of phagocytes	1.91E-02	CTSV,PPIA,TXN
Cellular movement	migration of tumor cells	1.98E-02	LY6D,VIM
Cellular movement	chemotaxis of embryonic cell lines	2.01E-02	PPIA
Cellular movement	chemotaxis of epithelial cell lines	2.01E-02	PPIA
Cellular movement	migration of melanoma cells	2.01E-02	VIM
Cellular movement	chemotaxis of kidney cell lines	2.51E-02	PPIA
Cellular movement	migration of phagocytes	2.89E-02	CTSV,TXN
Cellular movement	migration of eosinophils	2.92E-02	TXN
Cellular movement	transmigration of T lymphocytes	3.17E-02	CTSV
Cellular movement	transmigration of monocytes	3.33E-02	CTSV
Cellular movement	migration of endothelial cells	4.06E-02	ANXA3,VIM
Cellular movement	migration of neural crest cells	4.31E-02	CFL1

Cellular movement	chemotaxis of leukocyte cell lines	4.39E-02	TXN
Cellular movement	recruitment of cells	4.76E-02	TXN,VIM
Cellular movement	migration of breast cell lines	4.80E-02	VIM
Cellular movement	chemotaxis of leukocytes	4.86E-02	PPIA,TXN
Cellular movement	infiltration by eosinophils	4.96E-02	PPIA
Cellular Development	differentiation of keratinocytes	8.02E-03	CTSV,FLG
Cellular Development	differentiation of cells	2.94E-02	CFL1,CTSV,FLG,PPIA,RPL22,VIM
Cellular Development	development of astrocytes	1.26E-02	VIM
Cellular Development	differentiation of alpha-beta T lymphocytes	1.93E-02	RPL22
Cellular Development	differentiation of epidermis	1.93E-02	FLG
Cellular Development	differentiation of germ cell tumor cell lines	4.07E-02	PPIA
Cellular Development, growth and proliferation	proliferation of monocytes	3.94E-04	PPIA,TXN
Cellular Development, growth and proliferation	proliferation of basal keratinocytes	8.46E-04	CTSV
Cellular Development, growth and proliferation	proliferation of islet cell tumor	1.69E-03	CTSV
Cellular Development, growth and proliferation	arrest in development of double-negative T lymphocyte	4.22E-03	RPL22
Cellular Development, growth and proliferation	arrest in growth of cervical cancer cell lines	4.22E-03	TXN
Cellular Development, growth and proliferation	proliferation of melanocytes	2.01E-02	CTSV
Cellular Development, growth and proliferation	proliferation of neuronal cells	2.28E-02	CFL1,TXN,VIM

proliferation

Cellular Development, growth and proliferation	proliferation of neuronal progenitor cells	4.47E-02	CFL1
Cellular Development, growth and proliferation	proliferation of vascular smooth muscle cells	8.60E-03	PPIA, TXN
Cell Morphology	morphology of nuclear matrix	8.46E-04	VIM
Cell Morphology	polarization of neural crest cells	8.46E-04	CFL1
Cell Morphology	vacuolation of melanocytes	8.46E-04	CTSV
Cell Morphology	formation of microtentacles	1.69E-03	VIM
Cell Morphology	contraction of fibroblast cell lines	3.38E-03	VIM
Cell Morphology	morphology of thyroid tumor cell lines	5.91E-03	VIM
Cell Morphology	polarization of M2 macrophages	5.91E-03	TXN
Cell Morphology	cell spreading of embryonic cell lines	9.27E-03	VIM
Cell Morphology	polarization of cells	1.01E-02	CFL1, TXN
Cell Morphology	cell spreading of epithelial cell lines	1.26E-02	VIM
Cell Morphology	morphology of carcinoma cell lines	1.68E-02	VIM
Cell Morphology	cell spreading of endothelial cells	1.93E-02	VIM
Cell Morphology	cell spreading of kidney cell lines	1.93E-02	VIM
Cell Morphology	morphology of astrocytes	3.74E-02	VIM
Cell Morphology	cell spreading of fibroblast cell lines	4.23E-02	VIM
Cell Death and Survival	cell death of fibroblast cell lines	8.36E-04	CTSV, TRIM29, TXN, VIM
Cell Death and Survival	cell death of fibrosarcoma cells	8.46E-04	TXN
Cell Death and Survival	cytotoxicity of cyclosporin A	1.69E-03	PPIA
Cell Death and	cytotoxicity of eosinophils	4.22E-03	TXN

Survival			
Cell Death and Survival	apoptosis of lymphoid cells	6.75E-03	TXN
Cell Death and Survival	apoptosis of chronic lymphocytic leukemia B cells	8.43E-03	TXN
Cell Death and Survival	cell death of neuroblastoma cell lines	1.26E-02	CCT6A, TXN
Cell Death and Survival	apoptosis of endothelial cells	1.29E-02	PPIA, TXN
Cell Death and Survival	apoptosis of thymoma cell lines	1.43E-02	TXN
Cell Death and Survival	necrosis of prostate cancer cell lines	1.51E-02	PPIA, TXN
Cell Death and Survival	apoptosis of colon cancer cell lines	2.01E-02	PPIA, TRIM29
Cell Death and Survival	apoptosis of osteoclasts	2.09E-02	VIM
Cell Death and Survival	cytotoxicity	2.17E-02	PPIA, TXN
Cell Death and Survival	cell death of bladder cancer cell lines	2.67E-02	TXN
Cell Death and Survival	apoptosis of cervical cancer cell lines	2.82E-02	PPIA, TXN
Cell Death and Survival	apoptosis of lung cell lines	2.92E-02	TXN
Cell Death and Survival	neurodegeneration of Purkinje cells	3.41E-02	CTSV
Cell Death and Survival	apoptosis of fibroblast cell lines	4.14E-02	TRIM29, VIM
Cell Death and Survival	cell viability of embryonic cell lines	4.96E-02	PPIA
Immunological Disease	allergy	2.15E-06	ANXA3, CFL1, CTSV, FLG, KLK7, PPIA
Immunological Disease	immediate hypersensitivity	1.29E-05	CFL1, CTSV, FLG, KLK7, PPIA
Immunological	systemic autoimmune syndrome	2.51E-02	CTSV, LY6D, TXN, VIM

Disease			
Immunological Disease	hairy-cell leukemia	2.99E-02	CFL1,FLG
Immunological Disease, Inflammatory disease	allergic rhinitis	3.58E-02	FLG
Immunological Disease, Inflammatory response	allergic pulmonary eosinophilia	2.76E-02	ANXA3
Dermatological Disease, Immunological Disease, Inflammatory Disease, Inflammatory Response	atopic dermatitis	1.09E-04	CFL1,CTSV,FLG,KLK7
Dermatological Disease, Immunological Disease, Inflammatory Response	susceptibility to atopic dermatitis type 2	8.46E-04	FLG
Dermatological Disease, Immunological Disease, Inflammatory Response	advanced stage atopic dermatitis	7.59E-03	FLG
Dermatological Disease, Inflammatory Disease, Inflammatory Response	folliculitis	5.07E-03	CTSV
Dermatological Disease, Inflammatory Disease, Inflammatory	inflammation of dermis	2.54E-03	KLK7

Response			
Dermatological Disease, Inflammatory Disease, Inflammatory Response	inflammation of skin component	2.42E-05	CTSV,KLK7
Dermatological Diseases	psoriasis	2.45E-04	ANXA3,CFL1,CTSV,FLG,KLK7
Dermatological Diseases	acanthosis of epithelial tissue	8.46E-04	CTSV
Dermatological Diseases	ichthyosis vulgaris	8.46E-04	FLG
Dermatological Diseases	severe pruritus	8.46E-04	KLK7
Dermatological Diseases	Carney complex type 1	1.69E-03	VIM
Dermatological Diseases	hyperkeratosis	2.87E-03	CTSV,KLK7
Dermatological Diseases	hyperplasia of sebaceous glands	4.22E-03	CTSV
Dermatological Diseases	scratch lesion	5.07E-03	VIM
Dermatological Diseases	active stage psoriasis	1.43E-02	ANXA3
Dermatological Diseases	senile lentigo	3.99E-02	FLG
Inflammatory Disease, Neurological disease	neuroinflammation of brain	5.07E-03	VIM
Inflammatory Disease	blepharitis	1.01E-02	PPIA
Inflammatory Disease,	keratitis	3.09E-02	CTSV
Inflammatory Disease	insulitis	3.66E-02	CTSV

Inflammatory Response	inflammation of organ	1.32E-04	ANXA3,CFL1,CTSV,FLG,KLK7,PPIA,VIM
Inflammatory Response	proliferation of monocytes	3.94E-04	PPIA,TXN
Inflammatory Response	inflammation of secretory structure	8.06E-04	CTSV,PPIA
Inflammatory Response	chemotaxis of inflammatory leukocytes	8.46E-04	PPIA
Inflammatory Response	inflammation of footpad	5.91E-03	PPIA
Inflammatory Response	alternative complement pathway	7.59E-03	TXN
Inflammatory Response	cell movement of monocytes	1.00E-02	CTSV,PPIA
Inflammatory Response	chemotaxis of peripheral blood monocytes	1.18E-02	PPIA
Inflammatory Response	infiltration by mast cells	1.35E-02	PPIA
Inflammatory Response	classical complement pathway	1.43E-02	TXN
Inflammatory Response	inflammation of body region	1.65E-02	ANXA3,CTSV,PPIA,VIM
Inflammatory Response	inflammation of salivary gland	1.85E-02	CTSV
Inflammatory Response	cell movement of phagocytes	1.91E-02	CTSV,PPIA,TXN
Inflammatory Response	inflammation of pancreas	2.34E-02	PPIA
Inflammatory Response	degranulation of neutrophils	2.51E-02	ANXA3
Inflammatory Response	phagocytosis of leukemia cell lines	2.51E-02	VIM
Inflammatory Response	phagocytosis of cells	2.67E-02	ANXA3,VIM
Inflammatory Response	antimicrobial response	2.75E-02	ANXA3,PPIA

Inflammatory Response	migration of phagocytes	2.89E-02	CTSV, TXN
Inflammatory Response	transmigration of monocytes	3.33E-02	CTSV
Inflammatory Response	inflammation of heart	3.50E-02	PPIA
Inflammatory Response	chemotaxis of leukocytes	4.86E-02	PPIA, TXN
Neurological Disease	atrophy of brain	1.84E-04	CTSV, VIM
Neurological Disease	neurodegeneration of cerebellum	7.12E-04	CTSV, VIM
Neurological Disease	enlargement of axons	2.54E-03	CTSV
Neurological Disease	neurodegeneration of thalamus	4.22E-03	VIM
Neurological Disease	reactive astrogliosis	5.91E-03	CTSV
Neurological Disease	dystrophy of neurites	6.75E-03	VIM
Neurological Disease	cerebral amyloid angiopathy	1.35E-02	VIM
Neurological Disease	Movement Disorders	2.77E-02	CTSV, PPIA, TXN, VIM
Neurological Disease	neurodegeneration of hippocampus	3.00E-02	VIM
Neurological Disease	neurodegeneration of Purkinje cells	3.41E-02	CTSV
Neurological Disease	disorder of basal ganglia	4.96E-02	PPIA, TXN, VIM

Table E5: Top 5 cellular functions predicted by IPA

IPA Cellular function	(p-value)	Molecules
Cellular movement	3.05E-04 - 4.96E-02	↑ANXA3, ↑CFL1, ↑CTSV, ↓PPIA, ↓VIM, ↑TXN, ↑LY6D
Cellular development	3.94E-04 - 4.47E-02	↑CFL1, ↑CTSV, ↓PPIA, ↓VIM, ↑TXN, ↑RPL22, ↓FLG
Cell growth & proliferation	3.94E-04 - 4.47E-02	↑CFL1, ↑CTSV, ↓PPIA, ↓VIM, ↑TXN,

		↓RPL22
Cell death & survival	8.36E-04 - 4.96E-02	↑TRIM29, ↑CSTV, ↓PPIA, ↓VIM, ↑TXN, ↓CCT6A
Cell morphology	8.46E-04 - 4.23E-02	↑CFL1, ↓VIM, ↑TXN, ↑CSTV

Ingenuity pathway analysis (IPA)

Table E6: Top 5 canonical pathways predicted by IPA

IPA Canonical pathways	(p-value)	Ratio	Molecules
EIF2 Signaling	1.51E-05	2.20E-02	↑RPL4, ↑RPS27, ↑RPL22, ↓RPS5
Thioredoxin Pathway	5.07E-03	1.67E-01	↑TXN
Regulation of eIF4 and p70S6K Signaling	6.64E-03	1.40E-02	↑RPS27, ↓RPS5
ILK Signaling	1.06E-02	1.10E-02	↑CFL1, ↓VIM
mTOR Signaling	1.08E-02	1.10E-02	↑RPS27, ↓RPS5

Ingenuity pathway analysis (IPA)

Table E7: Summary of *FLG* genotype

Donor ID	FLG genotype		Biopsy site
1	WT	WT	Normal
2	WT	WT	Normal
3	WT	WT	Normal
4	WT	WT	Normal
5	WT	WT	Normal
6	WT	WT	Normal
7	WT	WT	Normal
8	WT	WT	Normal
*9	2282del4	WT	Normal
10	WT	WT	INV AE

11	WT	WT	INV AE
12	WT	WT	UN-INV & INV AE
13	WT	WT	INV AE
14	WT	WT	INV AE
15	WT	WT	UN-INV & INV AE
16	WT	WT	UN INV AE
17	R501X	WT	UN-INV & INV AE
18	WT	WT	INV AE
19	2282del4	WT	INV AE
20	R501X	WT	UN-INV & INV AE
21	R3419X	WT	UN-INV & INV AE
22	WT	WT	INV AE
23	WT	WT	INV AE
24	WT	WT	INV AE
25	R501X	R501X	INV AE

Uninvolved (UN-INV), Involved atopic eczema (INV AE), WT (wild type)

*9 Control subject was excluded from IMF analysis as sequencing revealed a heterozygous *FLG* mutation

Figure E1 – LSE model is fully differentiated and displays filaggrin processing and maximal expression at 14 days. LSE models were grown for up to 14 days at the air liquid interphase and the expression of filaggrin analysed by western blot at day 7, 9, 12 and 14 of culture (D7-D14). (N=3).

Figure E2 – Filaggrin knockdown LSEs have a thicker epidermis. shNT and shFLG LSE models were grown for 14 days and histology determined by h&E staining (figure 1). Thickness of the viable cell layer and stratum corneum was calculated using Image J software. Scatter plots of LOG_{10} transformed data, mean $\pm$ 95% CIs (N=10, 5 measurements per donor).

Figure E3 – Filaggrin knockdown confirmation in each of the 10 LSE donors used in the proteomics analysis. shNT and shFLG LSE models were grown for 14 days and filaggrin expression and knockdown confirmed by immunoblotting. Solid line dictates that the samples were run on the same gel but in a discontinuous manner.

Figure E4 - Principal component analysis of paired (shNT and shFLG) samples from 10 biological donors. Each colour represents an individual biological donor.

Figure E5 – Heat map analysis of paired (shNT and shFLG) samples from 10 biological donors. Heat map depicting the protein expression changes between shNT and shFLG LSEs in each pair of LSEs derived from independent donors (N=10). Red/blue represents increased/decreased expression in the shFLG condition relative to shNT. Dendrogram highlights clustering between proteins.

Figure E6 – Network linking the 17 differentially expressed proteins. Differentially expressed proteins are highlighted in red (upregulated) and green (downregulated). The magnitude of response is dictated by the colour intensity. Proteins highlighted in pink have known roles in inflammatory skin disease.

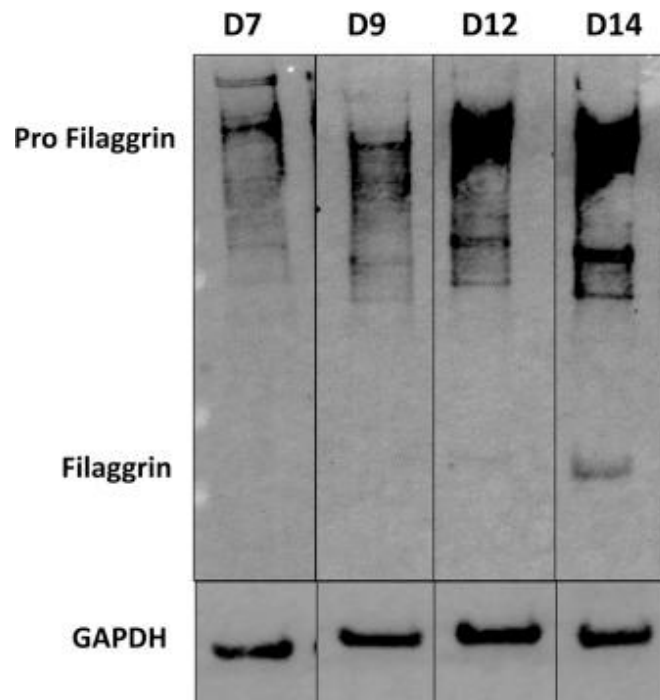


Fig E1.

Martina S. Elias, Heather A. Long, Carla F. Newman, Paul A. Wilson, Andrew West, Paul J. McGill, Keith C. Wu, Michael J. Donaldson, Nick J. Reynolds

Proteomic analysis of filaggrin deficiency identifies molecular signatures characteristic of atopic eczema

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<http://dx.doi.org/10.1016/j.jaci.2017.01.039>

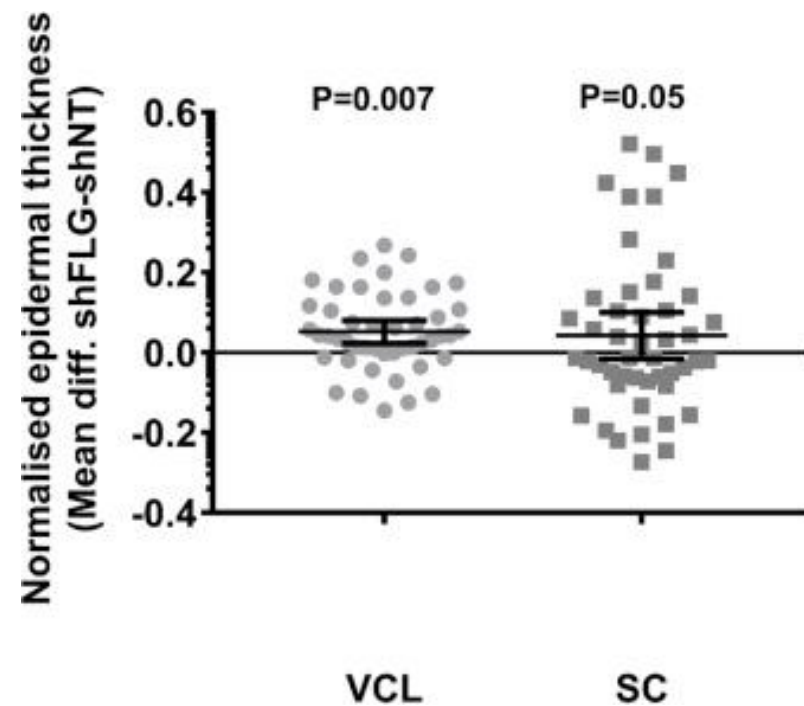


Fig E2.

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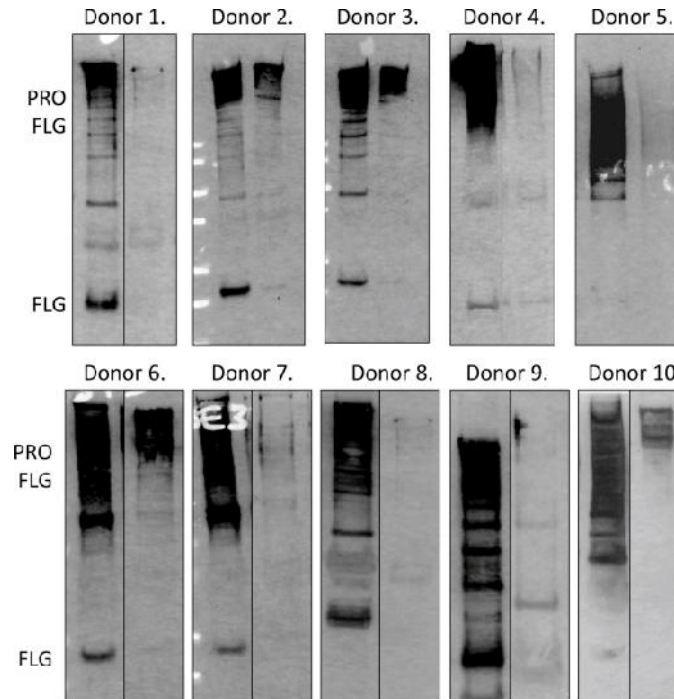


Fig E3.

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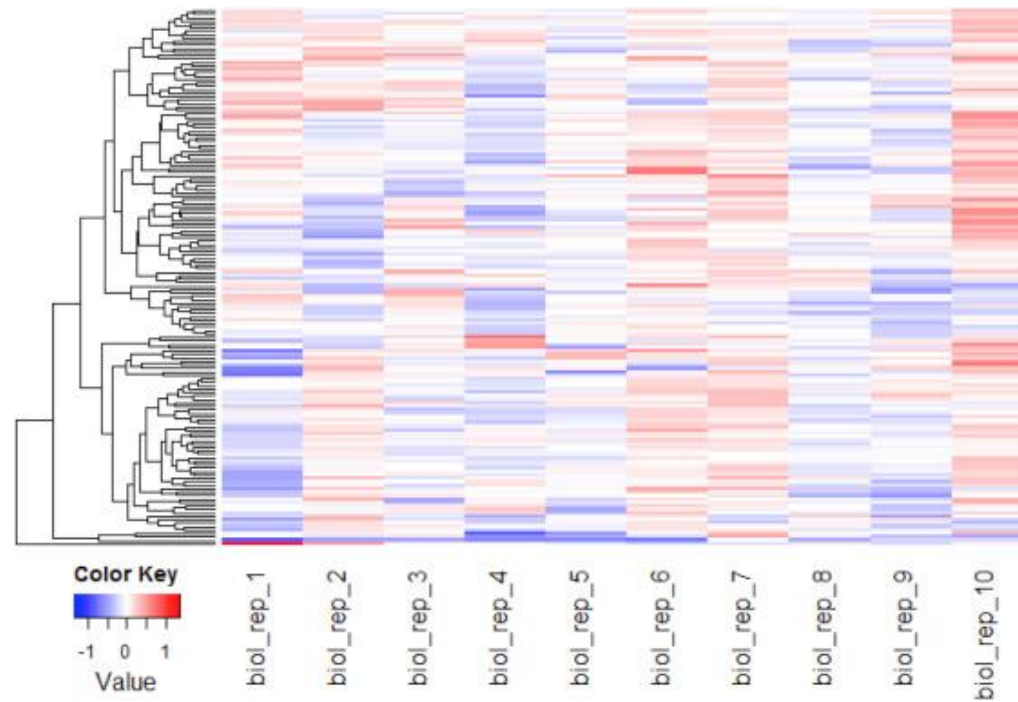


Fig E5.

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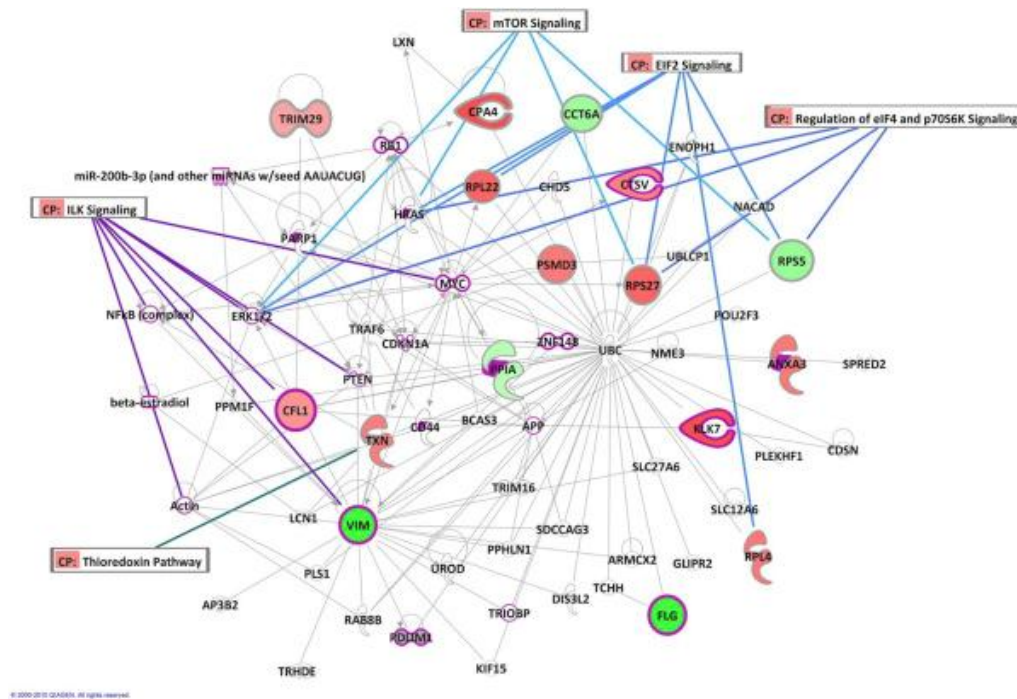


Fig E6.

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