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Title
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Permalink
https://escholarship.org/uc/item/5762t4fp

Journal
Journal of Proteome Research, 15(8)

ISSN
1535-3893

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Publication Date
2016-08-05

DOI
10.1021/acs.jproteome.6b00108

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Proteomic Analysis of Loricrin Knockout Mouse Epidermis

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KEYWORDS. Corneocyte, Cross-linked envelopes, Filaggrin, Ichthyosis, Isopeptide bonding, Keratinocyte, Keratin, Label-free estimation, Protein-protein interactions, Transglutaminase

ABSTRACT: The cross-linked envelope of the mammalian epidermal corneocyte serves as a scaffold for assembly of the lipid barrier of the epidermis. Thus, deficient envelope cross-linking by keratinocyte transglutaminase (TGM1) is a major cause of the human autosomal recessive congenital ichthyoses, characterized by barrier defects. Expectations that loss of some envelope

protein components would also confer an ichthyosis phenotype have been difficult to demonstrate. To help rationalize this observation, the protein profile of epidermis from loricrin knockout mice has been compared to that of wild type. Despite the mild phenotype of the knockout, some 40 proteins were incorporated into envelope material to significantly different extents compared to wild type. Nearly half were also incorporated to similarly altered extents into the disulfide bonded keratin network of the corneocyte. The results suggest that loss of loricrin alters their incorporation into envelopes as a consequence of protein-protein interactions during cell maturation. Mass spectrometric protein profiling revealed that keratin 1, keratin 10 and loricrin are prominent envelope components and that dozens of other proteins are also components. This finding helps rationalize the potential formation of functional envelopes, despite loss of a single component, due to availability of many alternative transglutaminase substrates.

INTRODUCTION

Mammalian epidermal corneocytes consist primarily of a keratin network cross-linked by disulfide bonds, a structure that accounts for the lack of protein extractable by denaturants in the absence of reducing agent and for the physical barrier the epidermis presents to the environment.[1] Over half a century ago, however, corneocytes were found to be endowed with a chemically resistant protein structure or “envelope” visible at the cell surface.[2,3] Much effort has since been devoted to identifying the components of this structure and how it is assembled. Findings that hair proteins exhibit ε-(γ-glutamyl)lysine isopeptide bonds[4] and that hair follicles express transglutaminase activity[5,6] provided a conceptual framework for understanding the cohesiveness of these structures and their resistance to solubilization. Subsequent observation that epidermal cells in culture can synthesize envelopes[7] displaying extensive isopeptide

bonding[8] permitted more facile analysis of the biosynthetic process, including transglutaminase labeling of putative precursors with fluorescent or radioactive aliphatic amines.[9] Some proteins, such as involucrin (Ivl), were identified in this way and confirmed immunochemically to be envelope constituents.[10,11] Immunochemical studies have shown that desmosomal proteins[12] as well as keratin proteins[13,14] are incorporated into cross-linked envelopes.

Direct identification of proteins derived from purified envelope preparations has presented difficulties due to the inability to reverse isopeptide cross-linking so as to recover the constituent participants. Nevertheless, isolation and sequencing of individual peptides from proteolytic digests of the complex envelope structure has proven possible, and sites of cross-linking have been deduced from peptides exhibiting more than a single amino terminus.[15] In this way, some proteins were identified as envelope components from human epidermis[16] and cultured human epidermal cells[17], and the presence of most has been confirmed immunochemically.[18] Such work indicated that loricrin (Lor) is a major constituent of envelopes,[19,20] and investigation of its properties has led to elegant models of envelope formation.[21]

Inasmuch as deficiency in Tgm1-mediated cross-linking can induce lamellar ichthyosis,[22,23] intact envelopes appear critical for assembly of the epidermal lipid barrier to the environment. This realization suggested that loss of envelope constituents could also have severe effects on this epidermal barrier function. However, the finding that ablating the Lor gene had only a mild effect on epidermal appearance in the mouse was surprising[24] and led to the conclusion that alternate proteins must be available to create functional envelope structures.[25] Similarly, ablation of Ivl alone produced little visible effect on mouse skin,[26] although ablation of an additional two genes encoding envelope components did display barrier defects.[27] Such observations prompted the present effort to identify more comprehensively the various protein components of envelopes

in normal mouse epidermis and to describe how loss of Lor is compensated. Protein profiling by mass spectrometry has revealed some 40 proteins whose incorporation into envelopes is significantly altered.

METHODS

Sample origin and preparation

Maintained under specific pathogen-free conditions, the mice were obtained from colonies used in previous work.[24] Experiments were approved by the Institutional Animal Use and Care committee and conducted in compliance with university, state and federal regulations. Epidermis from mice sacrificed 1 or 4 days after birth was dissected from dermis after immersion in water at 65°C and then ice-cold phosphate buffered saline.[28] To analyze envelopes, samples (3 from Lor knockout mice and 4 from control mice) were each rinsed 5 times in 2% sodium dodecyl sulfate – 0.1 M sodium phosphate buffer (pH 7.8), which removed lipid material forming a white lipid layer at the top of the supernatant after centrifugation. The insoluble material in the pellets was then extracted 4 times with 2% sodium dodecyl sulfate – 25 mM dithioerythritol - 0.1 M sodium phosphate, pH 7.8,[29] alkylated with iodoacetamide, rinsed with 67% ethanol and digested with stabilized bovine trypsin[30] in 0.1 M ammonium bicarbonate - 10% acetonitrile. In a separate experiment, the total cross-linked material was isolated from samples (4 each Lor knockout and control mice) by extracting the isolated epidermis as above with the sodium dodecyl sulfate - sodium phosphate buffer, then reducing, alkylating and digesting the insoluble material as above. Peptide counts are comparable for a given protein within these samples but not to the envelope or solubilized fractions.

Mass spectrometry

Peptides were analyzed using a Thermo-Finnigan LTQ iontrap mass spectrometer and analyzed essentially as previously described.[31] X! Tandem was set up to search a March 10, 2014 Uniprot mouse database (87,012 proteins), appended to an identical but reversed database for calculating false discovery rates. Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications for further analysis, based on a minimum of two peptides found in at least 3 envelope samples, were accepted if they could be established at >99% probability (protein false discovery rate 0.8% using decoy database). Proteins sharing significant peptide evidence were grouped into clusters, where spectral counts were adjusted for shared peptides. Spectral counts of exclusive peptides (unique to only one protein) were compiled and compared to the weighted counts (adjusted according to the number of other proteins sharing the same peptide sequence) to permit removal of a small fraction of the entries with many more weighted than exclusive counts and thus not certain to be present. Intensity based absolute quantification (iBAQ) values of samples analyzed with a Q-Exactive Plus mass spectrometer were used to calculate relative molar amount of each protein.[32] iBAQ values were calculated by importing MaxQuant (version 1.5.3.8) MS1 intensity values and identification results (protein and peptide false discovery rate of 1%) into Scaffold and having Scaffold calculate the iBAQ values. To this end, average calculated values for each protein were normalized to the total values for a given genotype and day after birth. Proteins are represented by their gene abbreviations except the proteins encoded by BC117090 (Stfa1l) and 2310050C09Rik, the latter termed here Ssp32l to signify Skin specific protein 32-like. Ssp32l is 70% identical in amino acid sequence to Ssp32.

Statistical analysis

Proteins or clusters with an average count less than 1 across samples were filtered out prior to analysis. Count data were transformed using a variance stabilizing transformation for negative binomial data, which takes the form $f\theta(x) = \ln[x + (x^2 + x/\theta)^{0.5} + (2\theta)^{0.5}]$. This transformation, when θ is selected to minimize the correlation between the variance and standard deviation of the transformed data, removes mean-variance dependency from the data so that they may be analyzed using methods that assume constant variance across the range of the data. Data were then analyzed using the Bioconductor package for gene expression analysis limma,[33] which fits linear models to each protein separately then applies empirical Bayes shrinkage to the estimated variances to increase power. The application of the above transformation to RNA-Seq data is discussed in Rocke et al. (http://biorxiv.org/content/biorxiv/early/2015/06/11/020784.full.pdf). The overall analysis approach is similar to that called "limma-trans" in Sonenson and DeLorenzi,[34] which employs the variance stabilizing transformation from the DESeq RNA-Seq analysis package.[35] Analyses were conducted using R, version 3.2.1.[36] Multidimensional scaling plots of the transformed data, a dimension-reduced visualization similar to principal components, were generated using a leading log fold change distance matrix (computed in limma from the average of the largest absolute log fold changes between samples) with classical multidimensional scaling.[37] These showed that data from the samples at each day and for each genotype formed distinct groups that were well separated from each other (Supplementary Figure S1).

RESULTS

Protein profile

A total of 155 proteins were detected in the insoluble isopeptide cross-linked envelope samples after extensive SDS extraction of epidermis under reducing conditions. Of these, 42 differed

significantly in relative amount in the wild type (WT) and Lor knockout (KO) samples at both day 1 and day 4 time points. As anticipated, this material exhibited substantial Lor in the wild type (WT) mice but none in the KO mice. Since the results at these two time points were quite similar, data are illustrated at day 1 for simplicity. (Corresponding day 4 results are illustrated in Supplementary Figures S2 and S3.) In addition to the envelope fraction, samples were also analyzed from the solubilized fraction after removal of the envelopes and, in a separate experiment, from the total cross-linked fraction (isopeptide and disulfide bonded) isolated by extraction with SDS but without reducing agent.

Among the numerous proteins in the envelope fraction, 11 of those that differed in relative amount in the two genotypes were detected at lower levels in the KO and are illustrated in Figure 1. Among these, in addition to Lor, were the major components Flg, Krt1 and Krt10. Other such proteins were Krt77, Npl, Serpinb3, Ggct, Pepd, Casp14 and Csta. As also illustrated, only Lor was present at higher levels in the protein solubilized from epidermis of the WT versus KO mice. Thus, their paucity in the envelope fraction appeared not to be due to reduced expression in KO epidermis. Incorporation into the fraction with both disulfide and isopeptide cross-links (Total XL) appeared lower in the KO for Flg, Npl, Serpinb3, Ggct, Casp14 and Csta, but not for Krt1, Krt10 or Krt77 or Pepd.

Among the 31 proteins that were increased in relative amounts in the KO mouse epidermis were the 6 known envelope components Kprp, Ivl, Anxa1, Serpinb2, Lce1 and S100a11. As shown in Figure 2, these also were increased in parallel in the total cross-linked fraction, but they were not detected or were found at essentially the same levels in the solubilized protein from KO and WT samples. Other proteins increased in the KO samples included 6 junctional proteins Dsg1a, Ppl, Dsp, Jup, Pkp1 and Evpl (known envelope components) and another 6 not previously reported in

envelopes (Ahnak, Ssp32l, Ecm1, Eps8l1, Stfa, Pof1b). Eps8l2 (not shown) was also in this last group. The levels were parallel in the total cross-linked fraction, and in no case could this difference in incorporation be attributed to a difference in the expression level in the epidermis as judged by results from the solubilized fraction.

Differential isopeptide cross-linking in these samples could not be attributed to different levels of transglutaminase expression (Figure 3). Levels of Tgm1 and Tgm3 were essentially the same in WT and KO envelope and total cross-linked fractions, although Tgm3 was marginally (but not significantly) higher in the KO solubilized samples compared to WT.

Relative ranking

The results demonstrate that a large number of different proteins are envelope constituents and that keratins and Flg are prominent among them. Although spectral count data provide accurate measurements of a given protein in parallel samples, they cannot easily give the relative amounts of different proteins directly in view of large differences in protein molecular weights and different numbers of theoretical tryptic peptides. However, label free estimation of these amounts is possible by several methods.[38,39] The numbers generated in this case are useful for estimating the relative importance of the various participants. Table 1 lists the top 16 for each genotype at days 1 and 4, accounting for some 70-90% of the total protein in each. An inability to identify cross-linked peptides by database searching is assumed not to bias the calculations unduly, but is an obvious source of possible inaccuracy. Those proteins with altered envelope participation consistent with the observed changes in spectral counts are indicated. It is clear that many of the proteins available in the cell are incorporated into envelopes, as has also been observed for hair shaft and nail plate. Reflecting their variable use as transglutaminase substrates, incorporation is not in direct proportion to relative total amount. Lor appears to be a major envelope component,

but previous higher estimates of its content were based on the small group of envelope constituents known at that time,[20] in contrast to the complexity of the composition revealed now. Too low in relative amount to appear in Table 1, values for Sprr2d, Sprr2h and Rptn were each an order of magnitude higher in the KO than in the normal mouse samples, consistent with previous observations.[24]

DISCUSSION

This work has focused on changes in mouse epidermal cross-linked envelope proteins as a result of Lor KO. As shown by proteomic profiling, Lor loss in the KO mice had a striking influence on the relative levels of numerous other constituents. Notable among these were the marked decreases in incorporation of the major components Krt1, Krt10 and Flg and several other less prominent components. Compensating for the loss were increases in known envelope proteins, junctional proteins and some others. A net mild effect on skin appearance evidently reflects a low degree of disruption of barrier function.[40] Nevertheless, altered organization of corneocyte protein interactions might still occur. This phenomenon was indeed observed in the total cross-linked fraction, where alterations in protein levels largely paralleled effects seen in the envelope fraction.

The basis for lack of incorporation of some proteins and preferential incorporation of others into envelopes is uncertain but in this case does not reflect relative availability, since changes in overall expression levels were not evident in analysis of the solubilized proteins. Moreover, small changes observed in Tgm1 and Tgm3 levels as indicators of changes in transglutaminase substrate preference could not rationalize these findings. In at least some cases, however, the difference in incorporation could reflect interactions among proteins before envelope formation occurs. Proteins preferentially incorporated into envelopes were similarly incorporated into total

cross-linked material (isopeptide plus disulfide bonding), raising the possibility their availability for cross-linking was enhanced by proximity to proteins suitably positioned for it. This rationale is consistent with lower incorporation of some of the proteins, such as Flg, Npl, Serpinb3 and Casp14, inasmuch as they were also lower in the total cross-linked fraction, but appears not applicable to Krt 1 and Krt 10. A simple model is that Lor interaction with Flg and some of the other proteins enhances their cross-linking, a process that excludes or shields incorporation of those proteins whose incorporation is enhanced by its absence. Beyond being passively incorporated into envelopes, Lor is known to interact with other prominent proteins such as Flg and thus potentially to influence envelope formation.[41]

The high content of keratin in the envelope fraction is reminiscent of that in the hair shaft[42] and nail plate.[43] In hair shaft, the large content of keratin may reflect the large fraction of amorphous material not extracted from cells of the medulla and the resistance of cytoplasmic material in the cuticle cells to extraction.[44] The inside of nail plate cells is not completely empty but often contains amorphous material and remnant nuclei while, in the case of epidermal keratinocyte envelopes, the cytoplasmic space is often clear but in some instances structures are visible.[45] Present analyses reflect the variety of proteins comprising such structures and illustrate the utility of employing available proteins to stabilize the corneocyte as a physical barrier to the environment. A recent study of keratin type I or type II ablation, which induces severe envelope defects, emphasizes the importance of keratins for proper envelope function.[46]

The data offer an updated perspective on the ability of the epidermis to compensate for missing envelope proteins. A major conclusion is that envelopes have many constituent proteins. Thus the degree of compensation for loss of a single component is less than might have been expected in the prevailing model of envelopes comprised of a small number of constituents, of which

loricrin was 70-80% of the total protein.[20] Loricrin now appears to be a smaller fraction, and many other proteins are available to assist in the compensation. This finding helps rationalize the potential formation of functional envelopes, despite loss of a single component, due to availability of many alternative transglutaminase substrates, consistent with the hypothesis that functional envelopes may be constructed with different components.[25]

FIGURES

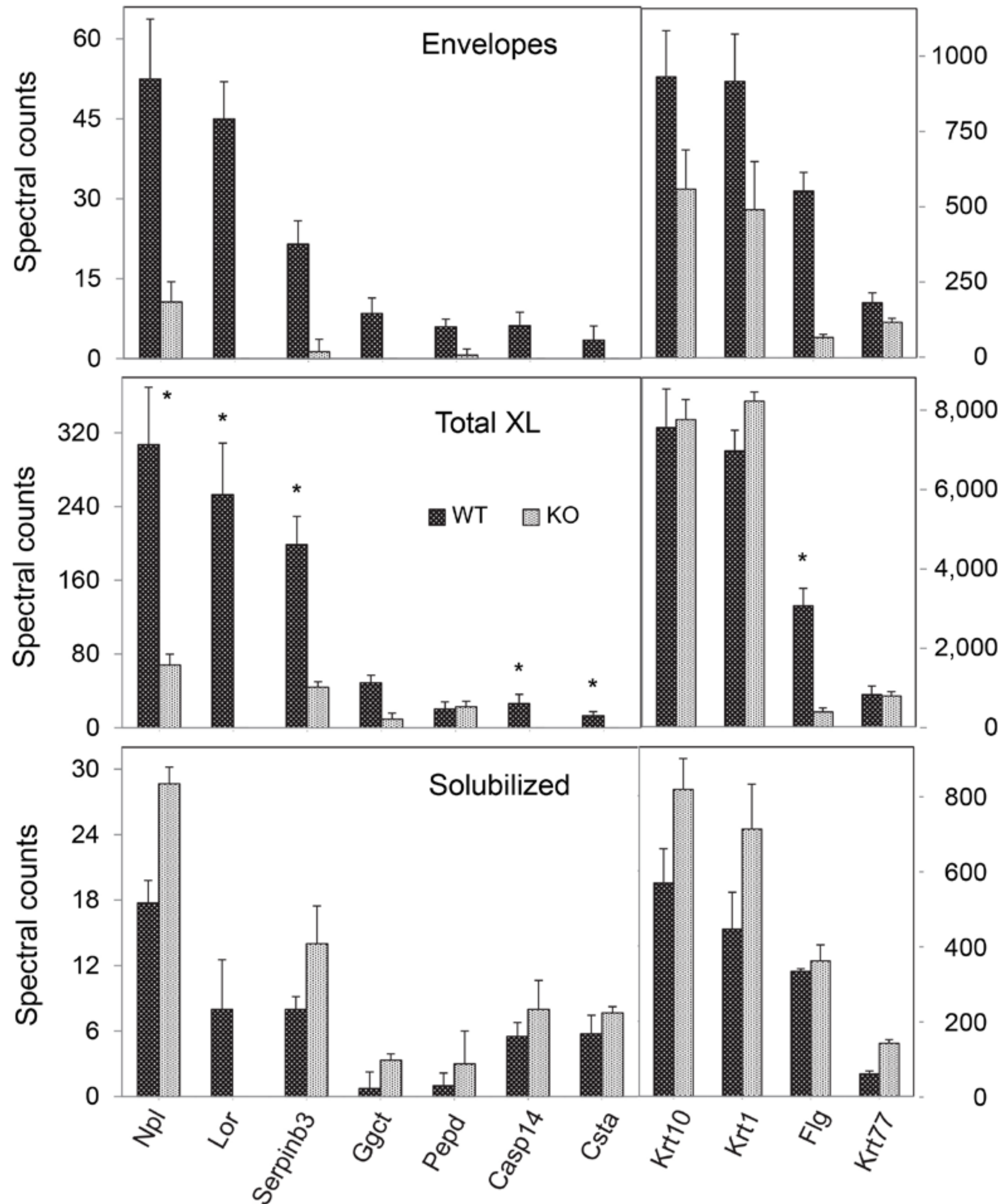

Figure 1. Proteins with reduced incorporation into envelopes in Lor KO samples harvested 1 day after birth. The top panel shows envelope proteins with significant differences in relative level in wild type (WT) and knockout (KO) epidermis. The middle panel gives the relative protein levels in equivalent epidermal samples extracted without reducing disulfide bonds (Total XL), where significant differences are indicated by asterisks (*). The bottom panel gives relative protein levels in the protein fraction solubilized with SDS and reducing agent.

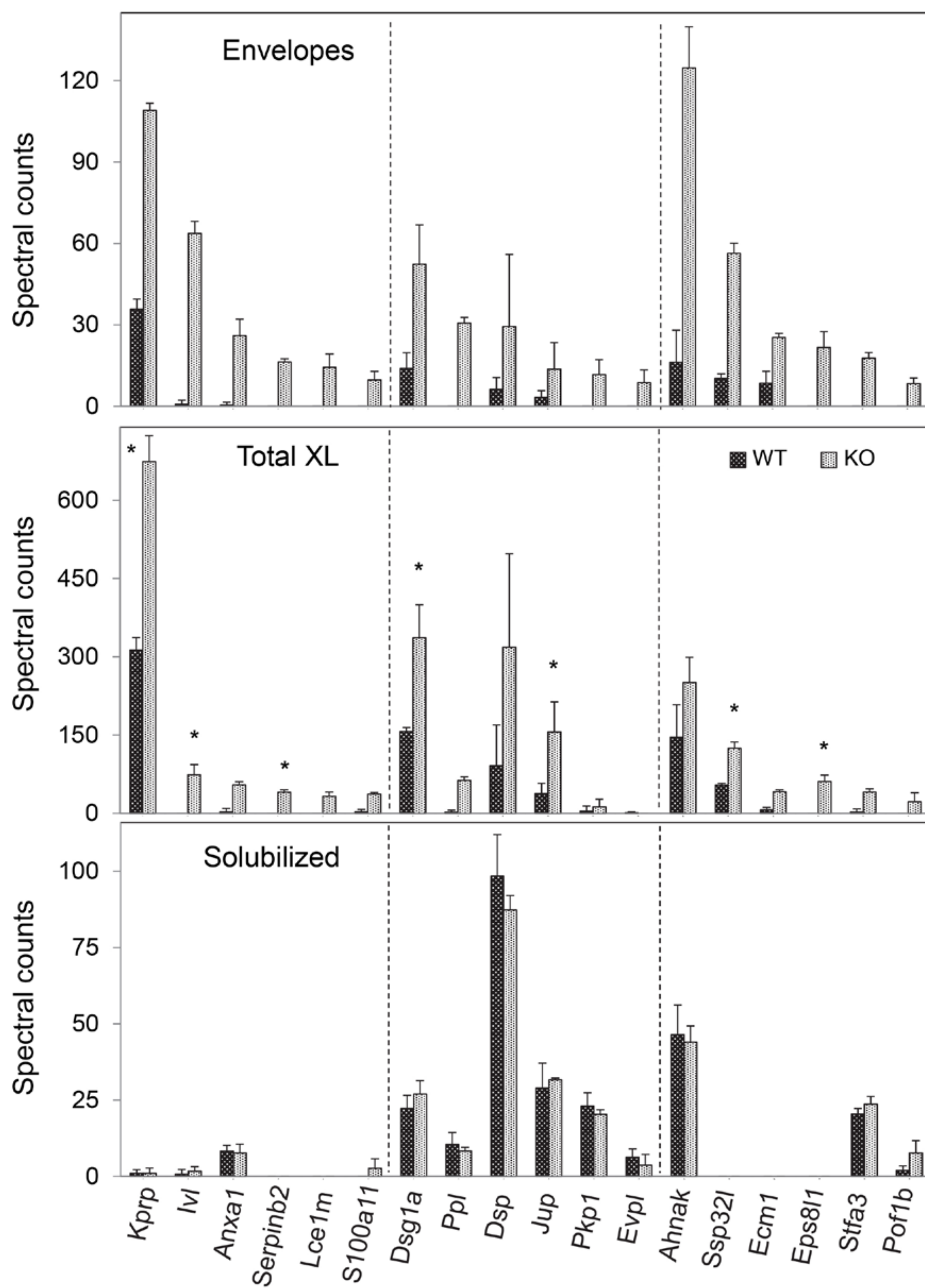
Envelopes
Total XL
Solubilized
Spectral counts
WT
KO
0
30
60
90
120
150
300
450
600
25
50
75
100
*
Kprp
Ivl
Anxa1
Serpinb2
Lce1m
S100a11
Dsg1a
Ppl
Dsp
Jup
Pkp1
Evpl
Ahnak
Ssp32l
Ecm1
Eps8l1
Stfa3
Pof1b

Figure 2. Proteins with increased incorporation into envelopes in Lor KO samples harvested 1 day after birth. The top panel shows envelope proteins with significant differences in relative level in wild type (WT) and knockout (KO) epidermis. The middle panel gives the relative protein levels in equivalent epidermal samples extracted without reducing disulfide bonds (Total XL), where significant differences are indicated by asterisks (*). The bottom panel gives relative protein levels in the protein fraction solubilized with SDS and reducing agent from epidermis of WT or KO animals. From the left are illustrated 6 known envelope proteins, then 6 junctional proteins, and finally 6 others not previously reported to be incorporated into envelopes.

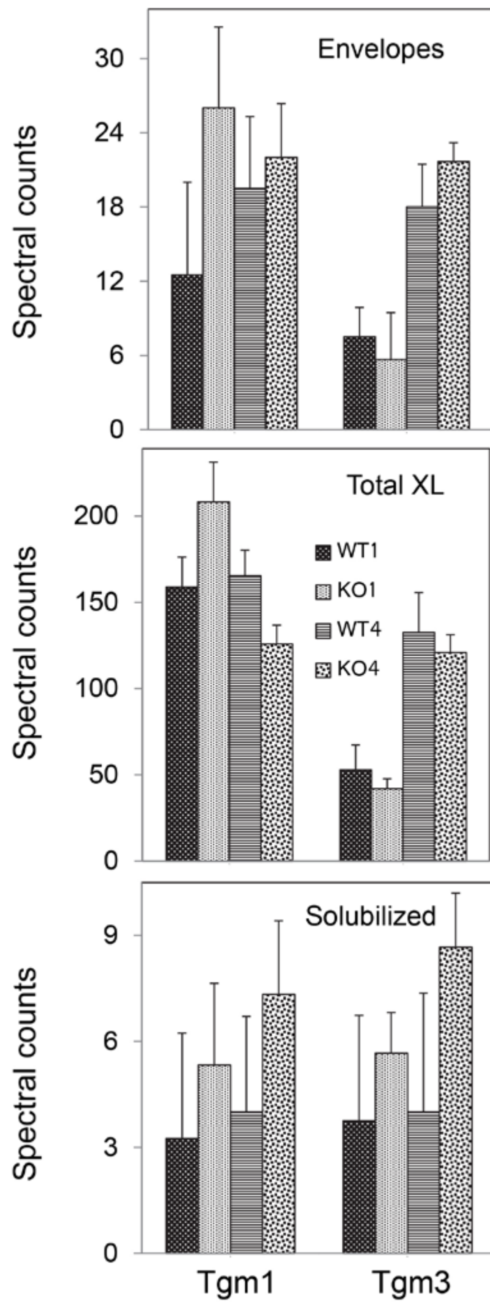

Figure 3. Relative levels of TGM1 and TGM3 in epidermal samples harvested 4 d after birth. The top panel compares relative TGM protein levels in envelopes from wild type (WT) and knockout (KO) epidermis. The middle panel gives the relative protein levels in equivalent epidermal samples extracted without reducing disulfide bonds (Total XL). The bottom panel gives relative protein levels in the protein fraction solubilized with SDS and reducing agent.

TABLE

Table 1. Proteins identified in envelope fractions of extracts from wild type (WT) and knockout (KO) mouse skin 1 or 4 days after birth[a]

WT1		**KO1**		**WT4**		**KO4**	
Krt1	22.1	***Krt1***	18.3	***Krt10***	21.6	***Krt1***	15.5
Krt10	21.7	***Krt10***	16.3	***Krt1***	19.8	***Krt10***	14.5
Lor	21.5	Flg2	7.3	***Lor***	11.8	Krt71	5.2
Krt77	4.1	***Kprp***	6.5	Flg2	3.3	***Kprp***	4.2
Hrnr	4.0	Hrnr	6.3	Krt28	3.1	Krt5	3.9
Flg2	2.7	Krt77	5.0	Hrnr	2.9	Hrnr	3.9
Krt28	2.4	***Ssp32l***	3.5	Krt77	2.8	Flg2	3.6
Krt5	2.0	S100a16	2.9	Krt5	2.5	Krt27	3.1
Flg	1.8	Krt5	1.7	Krt16	2.2	***Ssp32l***	2.7
Krt79	1.4	Krt71	1.6	Krt27	2.2	Sprr1a	2.7
Blmh	1.3	***Stfa3***	1.6	Krt25	2.1	Krt77	2.5
Kprp	0.9	Krt28	1.4	***Flg***	1.8	Krt25	2.4
Krt16	0.8	Krt79	1.4	Krt71	1.8	Krt16	2.2
Hist1h4a	0.8	***Ivl***	1.3	***Kprp***	1.5	Krt28	2.2
Krt27	0.8	Krtap13	1.2	Csta	1.1	Krt79	2.0
Krt25	0.8	Krt16	1.2	Krt79	1.1	***Stfa3***	1.8

[a]The proteins are listed in order of their calculated relative abundance (iBAQ). Those envelope proteins found to be altered in relative amounts between WT and KO in day 1 are shown in bold italic font as are those that differed in day 4.

ASSOCIATED CONTENT

Supporting Information

Figure S1. Multidimensional scaling plots for the envelope and total cross-linked samples.

Figure S2. Proteins with reduced incorporation into envelopes in Lor KO samples harvested 4 days after birth.

Figure S3. Proteins with increased incorporation into envelopes in Lor KO samples harvested 4 days after birth.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifiers PXD003532 and PXD003576.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was supported by NIEHS Superfund grant ES 2 P42 ES04699, the National Center for Advancing Translational Sciences (NIH) grant #UL1 TR000002 and NIAMS grant AR47898.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

Flg, filaggrin; iBAQ, intensity based absolute quantification; Ivl, involucrin; KO, knockout; Lor, loricrin; keratinocyte translglutaminase, Tgm1; WT, wild type. To minimize ambiguity, proteins are identified by symbols of the genes encoding them.

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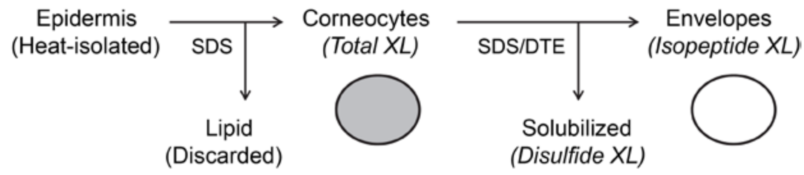

Proteomic Analysis of Loricrin Knockout Mouse Epidermis

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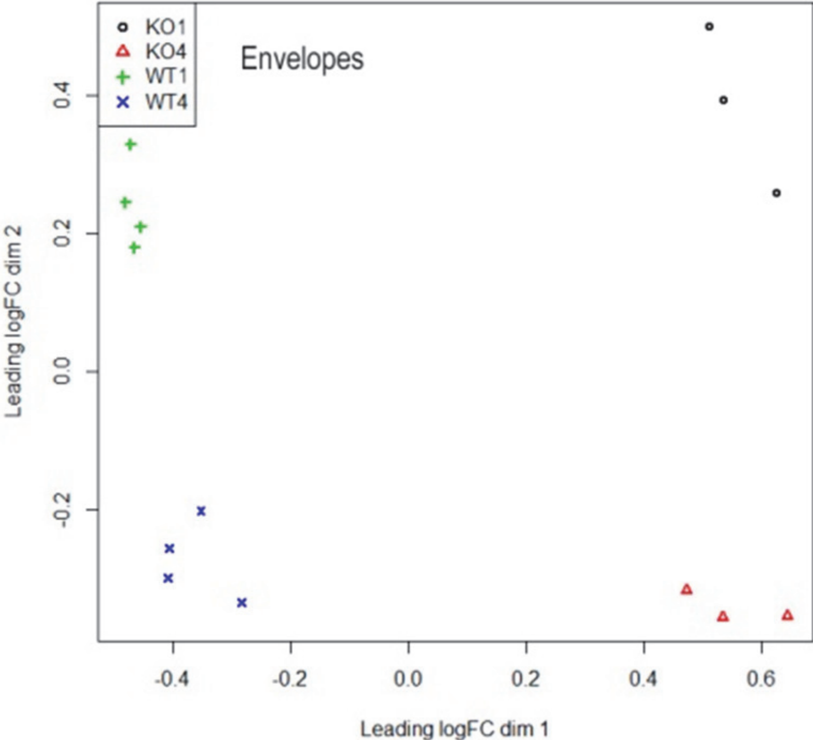
Figure S1. Multidimensional scaling plots for the envelope and total cross-linked samples. KO1, KO4 = samples from Lor knockout mice 1 or 4 days after birth, respectively. WT1, WT4 = samples from wild type mice 1 or 4 days after birth, respectively.



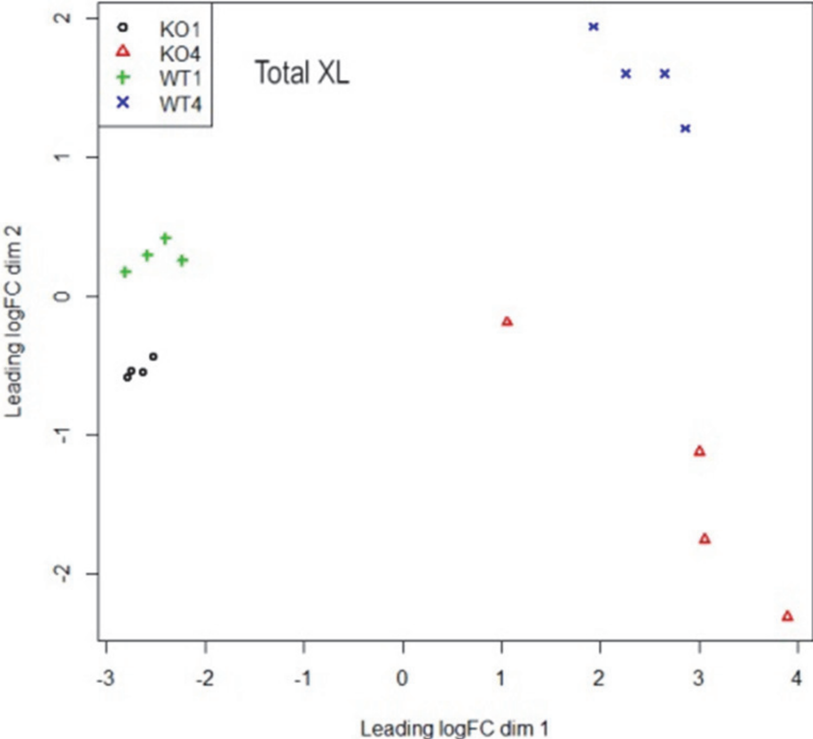


Figure S2. Proteins with reduced incorporation into envelopes in Lor KO samples harvested 4 days after birth.

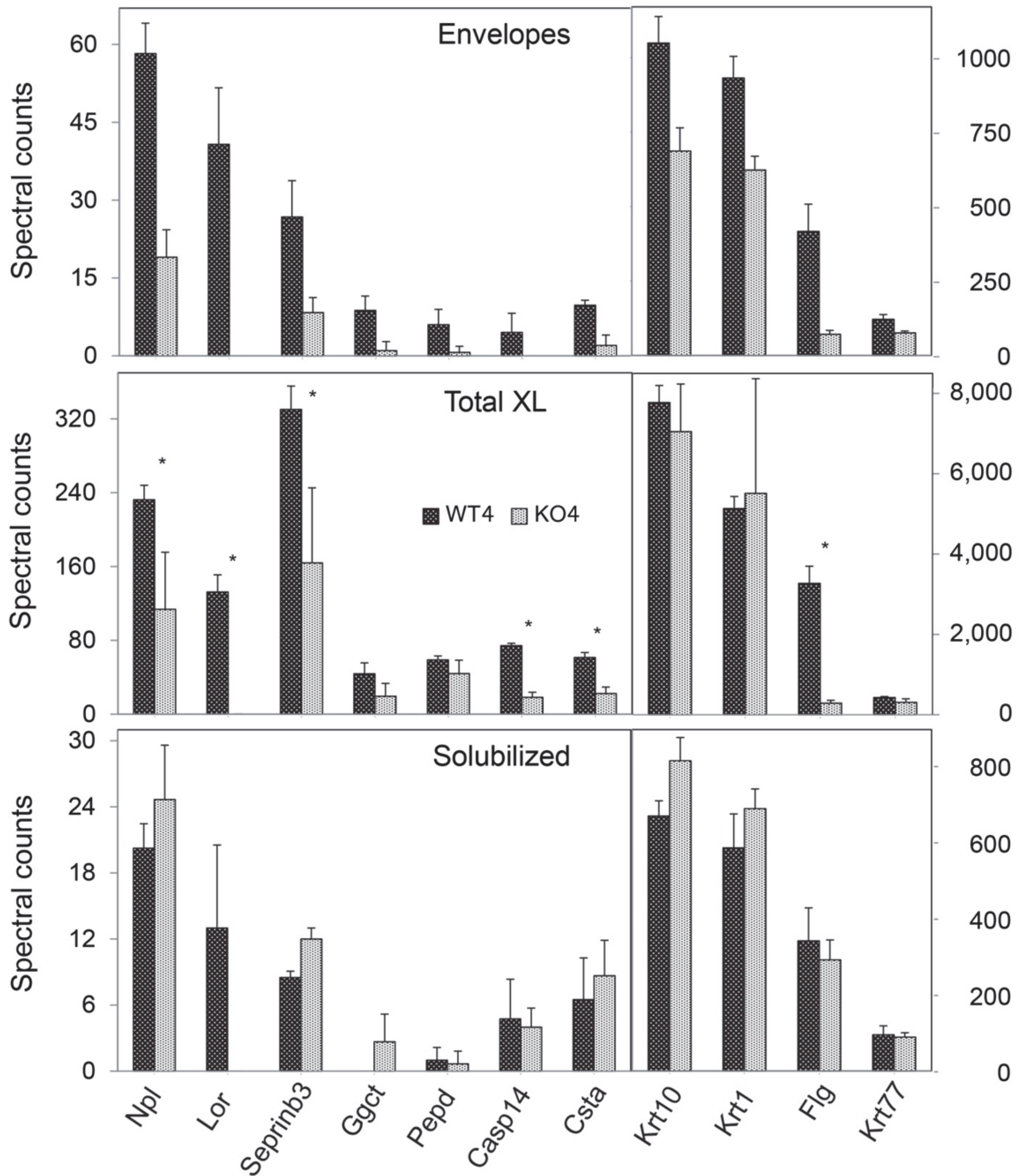


Figure S3. Proteins with increased incorporation into envelopes in Lor KO samples harvested 4 days after birth.