# Cross-Linking of SPINK6 by Transglutaminases Protects from Epidermal Proteases

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Extracellular kallikrein-related peptidases (KLKs) are involved in the desquamation process and the initiation of epidermal inflammation by different mechanisms. Their action is tightly controlled by specific protease inhibitors. Recently, we have identified the serine protease inhibitor of Kazal-type (SPINK) 6 as a selective inhibitor of KLKs in human *stratum corneum* extracts. As SPINK6 is expressed in the same localization as transglutaminases (TGM) and contains TGM substrate motifs, SPINK6 was tested to be cross-linked in the epidermis. Recombinant SPINK6 was shown to be cross-linked to fibronectin (FN) by TGM1 by western blot analyses. Moreover, SPINK6 was cross-linked in epidermal extracts and cultured keratinocytes by immunoblotting analyses. The use of TGM1 and TGM3 resulted in different immunoreactivities in western blot analyses of SPINK6 and epidermal extracts, suggesting substrate specifities of different TGMs for SPINK6 cross-linking in the epidermis. Conjugated SPINK6 exhibited protease inhibitory activity in keratinocytes and *stratum corneum* extracts; cross-linked SPINK6 protected FN from KLK5-mediated cleavage, whereas a lower KLK-inhibiting SPINK6-GM mutation did not. In conclusion, we demonstrated that SPINK6 is cross-linked in keratinocytes and human epidermis and remains inhibitory active. Thus, cross-linked SPINK6 might protect specific substrates such as FN from KLK cleavage and contributes to the regulation of proteases in the epidermis.

## INTRODUCTION

Epidermal proteases have an important role in epidermal barrier function (Meyer-Hoffert, 2009). Kallikrein-related peptidases (KLKs) belong to a group of 15 serine proteases with trypsin-like or chymotrypsin-like activity, whose genes are clustered at chromosome 19q13.4 (Yousef and Diamandis, 2001). In the skin, multiple KLKs are expressed and secreted by lamellar bodies in the extracellular space of the *stratum granulosum* (Ishida-Yamamoto et al., 2005; Komatsu et al., 2005; Shaw and Diamandis, 2007). In the *stratum corneum*, some KLKs are responsible for the degradation of intercellular adhesions molecules such as desmoglein and thus for the desquamation process (Ekholm *et al.*, 2000; Caubet *et al.*, 2004; Borgono *et al.*, 2007). In addition, KLKs are capable of degrading adhesion molecules of the extracellular matrix, and therefore aberrant KLK expression has been implicated in tumor invasion and metastasis (Klucky *et al.*, 2007; Bayani and Diamandis, 2011; Lose *et al.*, 2011).

In skin diseases such as rosacea, psoriasis, and atopic dermatitis, elevated KLK levels have been observed. They contribute to the inflammation reactions by several mechanisms including processing of biologically inactive cathelicidin to inflammatory active fragments by KLK5 (Yamasaki *et al.*, 2007). Moreover, KLK7 and KLK13 are capable of cleaving pro-IL-1β (Nylander-Lundqvist and Egelrud, 1997; Yao *et al.*, 2006). KLKs can activate the proteinase-activating receptor-2, resulting in the production of the proallergic thymic stromal lymphopoetin (Briot *et al.*, 2009). TSLP causes, synergistically with IL1β and TNFα, a Th2-mediated immune response, which has an essential role in atopic dermatitis. The activity of KLKs is controlled by proteinase inhibitors preferentially of the serine protease inhibitor of Kazal-type (SPINK) family. Mutations in the *SPINK5* gene result in Netherton's disease (Chavanas *et al.*, 2000), an inherited inflammatory skin disease with enhanced epidermal KLKs activity, severe congenital erythroderma, bamboo hair defects, and atopic diathesis.

Our work group has purified previously unreported members of the SPINK family, namely SPINK9 and SPINK6, from *stratum corneum* extracts. SPINK6 and SPINK9 contain both a single Kazal-type domain. SPINK9 is expressed at palmoplantar sites and its inhibition is restricted to KLK5, whereas SPINK6 is expressed at the entire body and efficiently inhibits several KLKs (Brattsand *et al.*, 2009; Meyer-Hoffert *et al.*, 2009, 2010; Kantyka *et al.*, 2011; Lu *et al.*, 2012). SPINK6 inhibits desquamation in an *ex vivo* model and might contribute to the epidermal barrier function of the *stratum corneum*. The *stratum corneum* consists mainly of insoluble proteins and lipids. Epidermal transglutaminases (TGM) contribute to the formation of this protective shield by

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Abbreviations: FN, fibronectin; KLK, kallikrein-related peptidase; NHEKs, normal human epidermal keratinocytes; SPINK, serine protease inhibitor of Kazal type; TGM, transglutaminase

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cross-linking between glutamine and lysine residues of different polypeptides and sphingolipids (Nemes and Steinert, 1999). TGMs and SPINK6 are expressed in the same localizations in the epidermis. SPINK6 contains Lys and Glu residues, which could act as substrates for TGM that may enable SPINK6 to be cross-linked to extracellular matrix proteins, thus anchoring the inhibitor at its site of action. Therefore, we investigated whether SPINK6 is a substrate of TGMs in the epidermis and whether this affects the regulation of KLKs.

## RESULTS

### SPINK6 is a substrate for TGM-mediated cross-linking

Fluorescence staining of SPINK6 and TGM1 and TGM3 revealed colocalization of these proteins in the *stratum granulosum* and *stratum corneum* (Figure 1), which suggests possible interaction of SPINK6 with these epidermal TGM.

Taken that fibronectin (FN) is a well-known substrate for TGMs, SPINK6 was incubated together with FN and TGM1. Western blot analyses showed that SPINK6 is cross-linked to FN *in vitro* (Figure 2a). At ~95 kDa, the intermediate product of the recombinant TGM1 (90 kDa) and SPINK6 (6 kDa) was detected.

To test whether cross-linking of SPINK6 occurs with natural epidermal TGM partners, epidermal extracts were incubated with or without SPINK6 and TGM1 and TGM3, which resulted both in multiple higher bands (Figure 2b). The staining pattern was different, depending on the transglutaminase used. For TGM1, a strong signal between 52 and 72 kDa was observed, whereas a high-molecular-weight signal (>250 kDa) was seen after treating the epidermis extract with SPINK6 together with TGM3, suggesting that these TGM exhibit diverse substrate specifities.

Western blot analyses of differentiated normal human epidermal keratinocyte (NHEK) extracts showed distinct higher molecular bands (Figure 2c). The addition of recombinant SPINK6 resulted in an enhancement of these bands, which was diminished by the TGM inhibitor Z-DON. Z-DON is a small peptide-based transglutaminase inhibitor with a

DON (6-diazo-5-oxo-norleucine) core, which is similar to glutamine and is alkylated to the active center cysteine of TGM in an irreversible manner. Interestingly, the addition of Z-DON alone to NHEK cells resulted in weaker SPINK6 immunoreactive bands. The addition of biotinylated SPINK6 to cultured NHEKs resulted in additional bands and a higher background, supporting the fact (Figure 2d) that SPINK6 is covalently bound to NHEKs.

Next, cryosections of healthy human skin were incubated with SPINK6 together with or without Z-DON, followed by rigorous washing to release unbound material. Subsequent fluorescent staining using affinity-purified SPINK6 antibodies resulted in an increase of SPINK6 immunoreactivity in the epidermis after SPINK6 was added (Figure 3). The presence of Z-DON partially inhibited this effect.

### Cross-linked SPINK6 exhibits proteolytic inhibitory activity and protects FN from KLK-mediated cleavage

To clarify whether conjugated SPINK6 still acts as a protease inhibitor, FN was immobilized onto high-adsorption ELISA plates, and after washing 50 nM SPINK6 was conjugated by TGM1. Unbound SPINK6 was removed by washing, and subsequently 0.8 nM KLK14 and the KLK14-substrate GPR-AMC were added. KLK14 activity was significantly reduced when SPINK6 was bound to FN (Figure 4a).

Next, we questioned whether SPINK6 cross-linked to NHEK exhibits protease inhibitory activity. After 24 hours of incubation of NHEKs with 0.6 μM SPINK6 and subsequent washing of the trypsin substrate, GPR-AMC was added and trypsin-like activity was monitored for 6 hours (Figure 4b). In differentiated keratinocytes, the addition of SPINK6 resulted in a significantly reduced trypsin-like activity of NHEKs. In contrast, in undifferentiated NHEKs, when TGM are known to be not expressed (Steinert *et al.*, 1996), no reduction of trypsin-like activity after treatment with SPINK6 was observed, even when KLK14 was added in order to exclude that no proteases inhibited by SPINK6 were present.
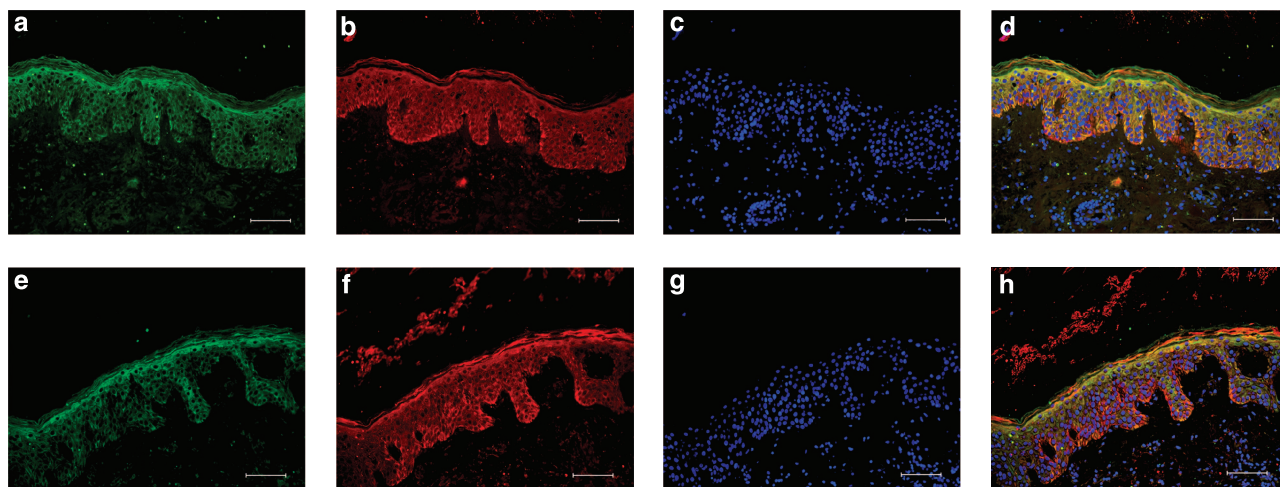


**Figure 1. Serine protease inhibitor of Kazal-type 6 (SPINK6) colocalizes to epidermal transglutaminases (TGM).** Paraffin-embedded skin sections were stained with fluorescent antibodies against SPINK6 (green, **a**, **e**); TGM1 (red, **b**); TGM3 (red, **f**); and Hoechst 33258 (blue **c**, **g**). Merged image of (**a**–**c**) is shown in (**d**) and merged image of (**e**–**g**,) is shown in (**h**). Colocalization of SPINK6 and TGMs result in yellow. Bar = 100 μm.
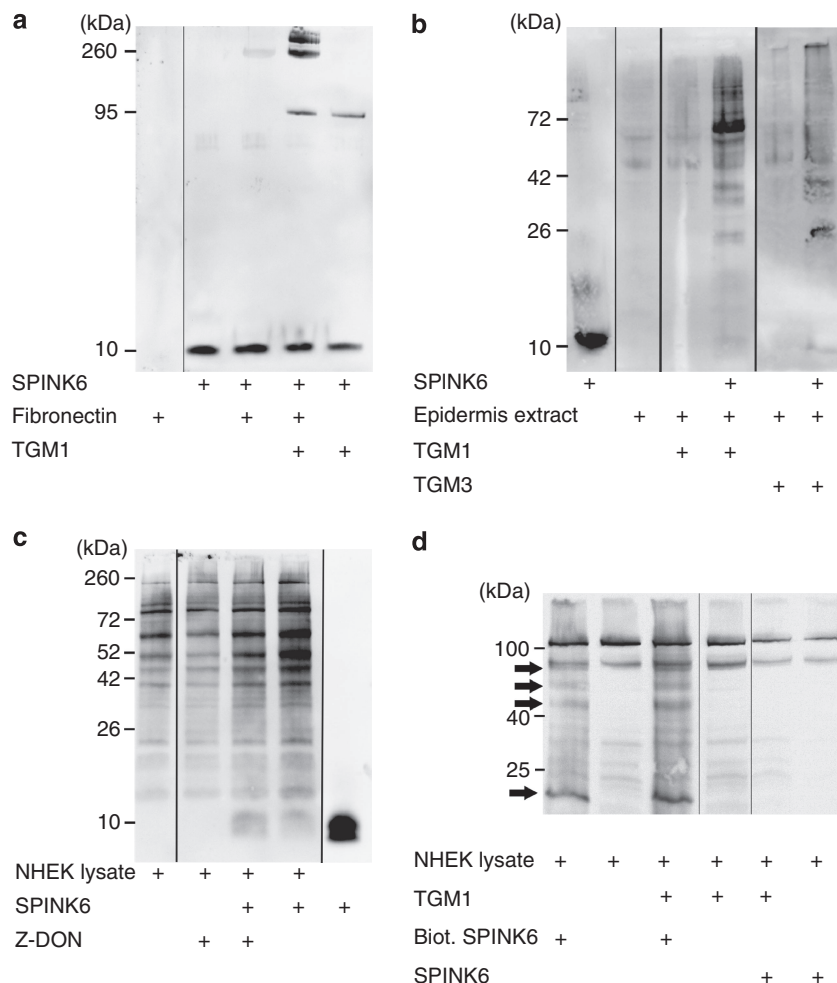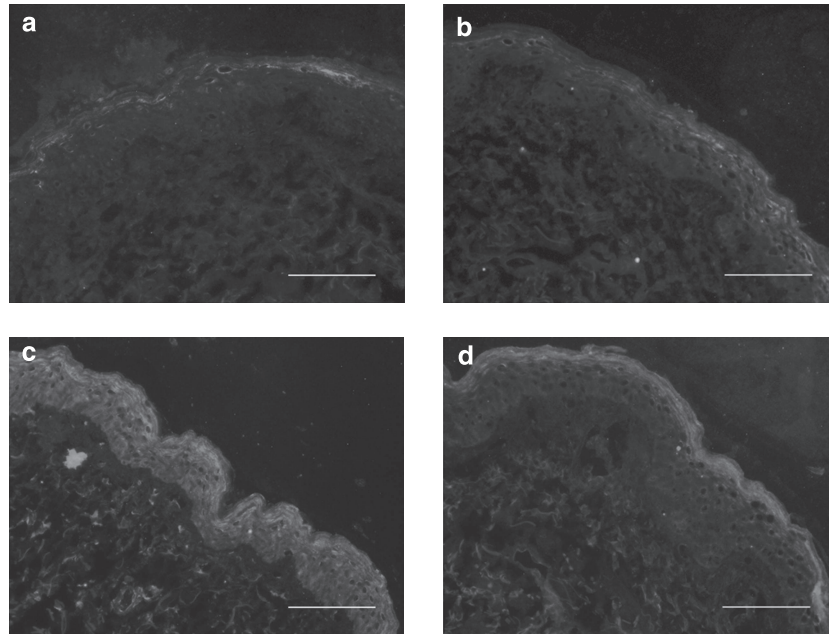
**Figure 2. Serine protease inhibitor of Kazal-type 6 (SPINK6) is a substrate for transglutaminase (TGM)-mediated cross-linking.** (**a**) α-SPINK6 western blot of different incubations of SPINK6, TGM1, and fibronectin (FN) in cross-linking buffer *in vitro* for 3 hours at 37 °C. (**b**) Human epidermis extracts were incubated in the absence or presence of 150 ng SPINK6 in cross-linking buffer either with TGM1 or TGM3 and applied to an α-SPINK6 western blot. (**c**) Differentiated normal human epidermal keratinocytes (NHEKs) were preincubated with or without the cross-linking inhibitor Z-DON for 20 minutes before adding SPINK6 for 8 hours. After washing, cells were lysed for 20 minutes in lysis buffer. Relative protein concentrations determined with nanodrop were normalized with lysis buffer, and 20 μl was applied to the western blot. For the control, 135 ng of SPINK6 was used. (**d**) Biotinylated SPINK6 was incubated with differentiated NHEKs and TGM1 as in described in (**c**) and applied to a western blot. A streptavidin–peroxidase conjugate was used to stain the biotinylated SPINK6.

To further test whether cross-linking of SPINK6 occurs under physiological conditions, human callus extracts were incubated with SPINK6 in the absence or presence of TGM1 (Figure 4c). Western blot analyses revealed that soluble SPINK6 (6 kDa) totally disappeared, even when TGM1 was not added, and a strong signal higher than 260 kDa appeared. This effect was dependent on the $Ca^{2+}$ concentration and was reversed by the addition of EDTA (Supplementary Figure S1 online), indicating that SPINK6 was cross-linked by active calcium-dependent TGM. The addition of TGM1 to callus extract and SPINK6 resulted in no detectable free SPINK6 and no enhancement of higher bands. This might be caused by conjugation of SPINK6 to very large proteins, which did not enter the SDS–PAGE. The samples were diluted in assay buffer. KLK14 (1 nM) and subsequently 0.025 mM GPR-AMC were added to measure the proteolytic activity (Figure 4d). The

callus extract inhibited KLK14 activity by 15% under the given condition, indicating that the inhibition of KLK14 by callus inhibitors is greater than the remaining trypsin-like protease activity. The addition of 10 nM SPINK6 resulted in a significant decrease of trypsin-like activity. The presence of TGM1 had no further influence on these effects. The results suggest that SPINK6 was linked to callus proteins and remained at least partially active as a protease inhibitor. Thus, the disappearance of free SPINK6 in the immunoblot does not result from unspecific proteolytic degradation and might come from high-molecular-weight binding partners not entering the SDS–PAGE.

After FN was bound to SPINK6 and unbound SPINK6 was subsequently removed by ultrafiltration, KLK5 was added (Figure 5). FN was cleaved by KLK5 in a time-dependent manner. An anti-FN western blot resulted in a weaker signal at

**Figure 3. Serine protease inhibitor of Kazal-type 6 (SPINK6) is cross-linked to epidermal substrates *ex vivo*.** Immunofluorescent staining of cryosections using anti-SPINK6 antibodies is shown. Skin cryosections were incubated with buffer control (**a**), with the transglutaminase inhibitor Z-DON (**b**), with recombinant SPINK6 (**c**), and recombinant SPINK6 together with Z-DON (**d**) overnight. After rigorous washing (three times for 5 minutes in buffer solution), sections were stained against SPINK6. Bar = 100 μm.

260 kDa and two main fragments at ∼70 and 40 kDa. No fragments appeared when SPINK6 was cross-linked to FN, indicating that cross-linked SPINK6 effectively protects FN from KLK5 cleavage. In order to test whether the protection of FN is due to the proteolytic inhibitory activity and not due to steric protection from cleavage sites, a low KLK-inhibiting SPINK6 mutation, SPINK6-GM ($K_i = 82$ nM), was created and cross-linked to FN in the same manner. SPINK6-GM did not protect FN from KLK5-mediated cleavage after cross-linking.

## DISCUSSION

In this study, we demonstrate that SPINK6 is a substrate for epidermal TGM. Protein–protein cross-linking by TGMs is an important event in the formation of the *stratum corneum* but also occurs in many other regions of the body. During the first reaction step of the TGM-mediated cross-linking, the γ-carboxamide group of a glutamine residue forms a thiol ester with an active site cysteine of the TGM. In the second deacylation step of the transglutamination reaction, the activated thioester usually undergoes an acyl transfer to an ε-amino group of a second substrate lysine residue. By using only SPINK6 together with TGM1, we only observed a single immunoreactive product, which fits to the size of SPINK6 together with TGM1 (96 kDa) but no other multimere products of SPINK6. This suggests that there is only one cross-linking reaction between SPINK6 and TGM1. After incubating SPINK6 and epidermal extracts with TGM1 or TGM3, the resulting different band patterns indicate that SPINK6 is conjugated to diverse substrates with different preferences for both TGM. In contrast to the *in vitro* cross-linking with FN,

nearly all SPINK6 was bound in the presence of epidermal extracts under comparable conditions, indicating a better specificity toward other epidermal substrates. Different substrate specificities for TGM1 and TGM3 were mentioned in the literature (Candi *et al.*, 1999), although no distinct consensus sequences for different TGM were identified so far. The nature of the glutamine and the neighboring residues are important for the cross-linking reaction, as a positively charged residue next to the glutamine residue dampens the cross-linking reaction (Coussons *et al.*, 1992), whereas other studies have shown the importance of charged amino acids around the glutamine residue (Aeschlimann *et al.*, 1992; Sugimura *et al.*, 2008; Yamane *et al.*, 2010). In SPINK6, three out of four glutamines (Q4, Q11, and Q30) follow a QxxΦ motif (where x is any amino acid and Φ a hydrophobic acid). This motif was also observed for the cross-linked protease inhibitors trappin-2 and secretory leukocyte protease inhibitor (Guyot *et al.*, 2005; Baranger *et al.*, 2011). Our data support the hypothesis that a hydrophobic residue + 3 to glutamine is a common motif for TGMs.

Taking cryosections, we demonstrated that recombinant SPINK6 is bound to the epidermis *ex vivo*. Fluorescent microscopy revealed that the expression of SPINK6 depended on the differentiation status of the keratinocytes with a maximum in the *stratum granulosum*. TGM1 and TGM3 were shown to be expressed intracellularly (Iizuka *et al.*, 2003), and here they colocalized with SPINK6, suggesting that cross-linking starts in the living keratinocytes. Colocalization was also observed in the *stratum granulosum* and *stratum corneum*, indicating that cross-linking of SPINK6 might
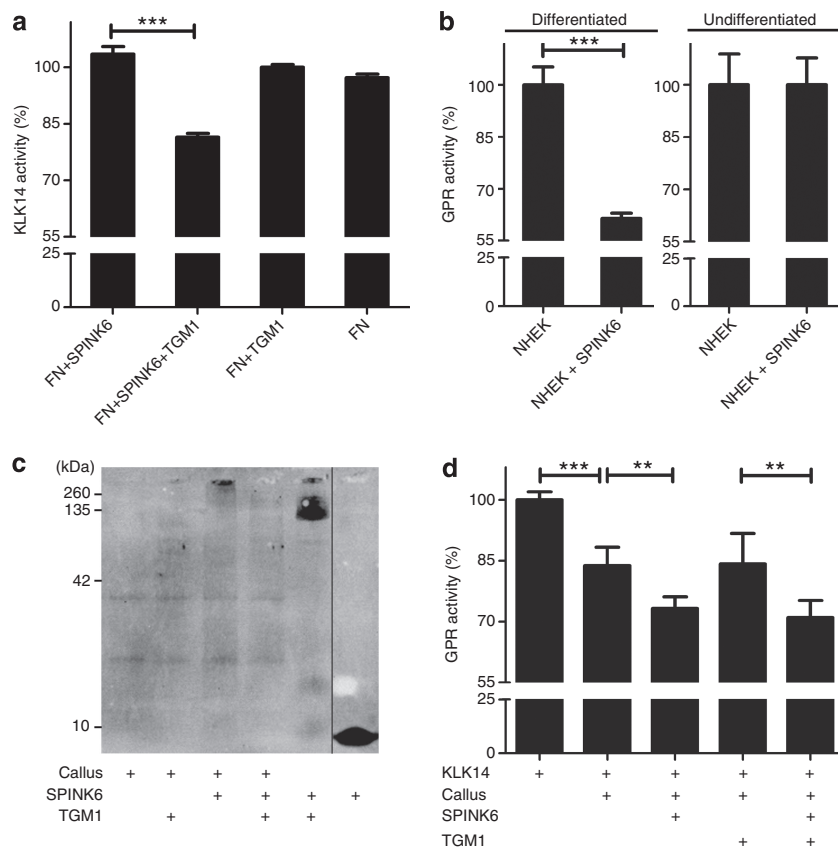
Figure 4. **Cross-linked serine protease inhibitor of Kazal-type 6 (SPINK6) inhibits trypsin-like proteases.** (**a**) Fibronectin (FN) was coated on a 96-well plate and incubated with 100 nM SPINK6 or SPINK6 with transglutaminase (TGM)1 ($n = 6$). Kallikrein-related peptidase 14 (KLK14) next to GPR-AMC was added and activity was measured. As controls, only FN or FN with TGM1 was used. (**b**) Normal human epidermal keratinocytes (NHEKs) were differentiated by the addition of 1 mM $Ca^{2+}$ to the medium or not. After a 24-hour treatment with or without 0.6 μM SPINK6 ($n = 6$), cells were washed thoroughly and trypsin-like activity was measured after the addition of 2 nM KLK14 (undifferentiated cells only) and 0.05 mM GPR-AMC. (**c, d**) Human callus extracts were incubated with or without SPINK6 in the absence or presence of TGM1 for 4 hours at 37 °C. One part of the sample was applied to a α-SPINK6 western blot (**c**), and the other part was given to 1 nM KLK14 and 0.025 mM GPR-AMC activity for activity measurement. The experiment was repeated twice with different callus extracts in triplicates. The mean activities were plotted with SD (**a, b, d**).
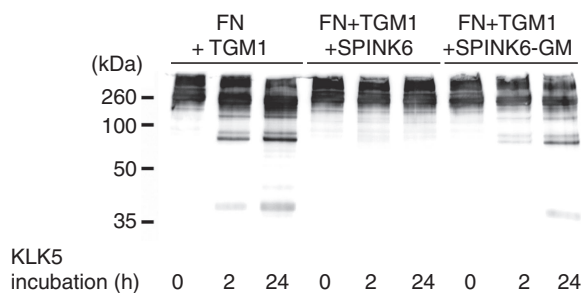


Figure 5. **Protein–protein cross-linking with serine protease inhibitor of Kazal type 6 (SPINK6) protects fibronectin (FN) from kallikrein-related peptidase 5 (KLK5) cleavage.** Anti-fibronectin western blot is shown. After FN was cross-linked to SPINK6 and the low KLK5-inhibiting SPINK6-GM, unbound SPINK6 was subsequently removed by ultrafiltration. KLK5 was added and incubated for the indicated time points. A representative experiment out of three independent ones is shown.

occur in different epidermal areas. This might be modulated by the TGM activation, e.g., by calcium availability and others. An increased fluorescent signal especially in the suprabasal layers was detected in cryosections of human

skin after incubation with recombinant SPINK6. This signal was partially blocked by adding of the small peptide-based transglutaminase inhibitor Z-DON (Figure 3), which is able to enter cells and shows inhibition of different TGMs with different efficiency (McConoughey *et al.*, 2010; Schaertl *et al.*, 2010). Western blot analysis from differentiating cultured keratinocytes showed immunoreactive bands at higher-molecular-weight levels. The intensity of the bands increased after the addition of SPINK6 to cultured keratinocytes and was reduced by the addition of Z-DON, suggesting that SPINK6 was cross-linked to specific keratinocyte substrates, which was further supported by cross-linking biotinylated SPINK6 to NHEK cells. As SPINK6 was purified from *stratum corneum* extracts in its unconjugated form (Meyer-Hoffert *et al.*, 2010), this cross-linking is not complete *in vivo*. So far, other SPINK members were not shown to be cross-linked by TGMs. The same or similar extraction methods of *stratum corneum extracts* did not reveal higher-molecular-weight immunobands for SPINK9 (Meyer-Hoffert *et al.*, 2009) and lympho-epithelial Kazal type–related inhibitor (Fortugno *et al.*, 2011), suggesting

dissimilar functions of SPINK6 compared with SPINK9 and lympho-epithelial Kazal type–related inhibitor in human skin and might explain the ostensible redundancy of KLK inhibitors in human epidermis.

The cross-linking of SPINK6 might be important for the protection of distinct proteins from KLK5-mediated cleavage. Functional analysis of cross-linked SPINK6 to FN revealed inhibitory activity toward KLK14 and protection from KLK5-mediated cleavage. Even when SPINK6 is cross-linked to various interaction partners in callus extracts and cultured keratinocytes, it still exhibits inhibitory activity (Figure 4). To our knowledge, this is a previously unreported description that a protein is protected from proteolytic cleavage by TGM-mediated cross-linking. Cross-linked secretory leukocyte protease inhibitor and trappin-2 inhibit proteases such as elastase and proteinase 3 as well (Guyot *et al.*, 2005; Baranger *et al.*, 2011). Moreover, cystatin alpha is covalently bound to the *stratum corneum* by transglutaminase (Takahashi *et al.*, 1994), suggesting that cross-linkage of protease inhibitors might be a general mechanism in human skin to control proteolytic enzymes. The uppermost skin is frequently in contact with water, and hence covalently bound SPINK6 would not be washed away. Moreover, bound SPINK6 protects specific target proteins from proteolytic cleavage. This might be particularly important in the *stratum corneum*, where active proteases such as KLKs are present and proteins cannot be renewed by transcription.

In conclusion, we demonstrated that SPINK6 is cross-linked in human epidermis and that conjugated SPINK6 is still inhibitory active. Bound SPINK6 protects its conjugation partners such as FN from KLK-mediated cleavage, which might be relevant for epidermal barrier function.

## MATERIALS AND METHODS

SPINK6 was recombinantly expressed in *Escherichia coli*, and polyclonal goat anti-SPINK6 antibodies were produced and purified as previously described (Meyer-Hoffert *et al.*, 2010). For SPINK6-GM mutation, the existing pSUMO3-SPINK6 was used as a template for the following PCR reactions using TaqE polymerase (genaxxon bioscience GmbH, Ulm, Germany): One forward primer with the mutations $R_{42}G$ and $E_{43}M$ (ctg cac tgg aa tgt cta acc cac act gtg g) was used with the existing reversed primer, and in a second reaction one reversed primer with the mutations $R_{42}G$ and $E_{43}M$ (gtt aga cat tcc agt gca gta gac ctt ggt g) was used with the existing forward primer. After gel purification, another PCR reaction was performed using both products as templates and existing SPINK6-pSUMO3 forward and reverse primers. The gel-purified product was inserted in the pSUMO3 vector as previously described (Meyer-Hoffert *et al.*, 2010). The correct sequence was confirmed by sequencing and the expected protein mass after expression was confirmed by mass spectrometry. SPINK6-GM exhibited a $K_i$ of 82 nM against KLK5. To biotinylate SPINK6, 1.6 mmol D-biotin-*N*-hydroxysuccinimidester (biotin-NHS, Roche, Basel, Switzerland) was incubated with 0.17 mmol SPINK6 in 40 µl of phosphate buffer, pH 7.0, for 6 hours at room temperature. After purifying biotinylated SPINK6 by reversed phase chromatography, the sample was lyophilized and stored at −20 °C before use. All proteolytic activity assays were performed in a Twinkle LB 970 reader (Berthold Technologies; Bad Wildbad,

Germany) at 355/460 nm ex/em wavelength. TGM1 and TGM3 were purchased from Zedira (Darmstadt, Germany). Skin samples were taken from healthy volunteers. The study was approved by the authors' Institutional Review Board University Kiel, Germany. The Declaration of Helsinki protocols was followed and patients gave their written and informed consent.

### Cross-linking assays

Equimolar amounts (0.45 µM) of native SPINK6 and FN were incubated in cross-linking buffer (20 mM Tris pH 7.6, 50 mM NaCl, 10 mM $Ca^{2+}$, 1 mM EDTA) in the presence or absence of 2 U transglutaminase (TGM)1 at 37 °C for 4 hours in a total volume of 25 µl and subsequently stopped using 20 mM EDTA. Samples were reduced with 5 mM DTT in the sample buffer before performing SDS–PAGE/western blot.

For KLK5 degradation of SPINK6-bound FN, 413 µM FN together with or without 825 µM SPINK6 or SPINK6-GM was incubated in cross-linking buffer with 20 U TGM1 at 30 °C overnight. To remove unbound SPINK6/SPINK6-GM, samples were applied to a vivaspin ultrafiltration tube (30,000 Da, Sartorius AG, Goettingen, Germany) and centrifuged at 14,000 g for 4 minutes three times by adding 300 µl of 0.1 M Tris, pH 8, 5 mM EDTA, 0.15 M NaCl, and 0.05% Tween to each tube between each step. Each sample was adjusted to 300 µl and 100 ng KLK5 (R&D Systems, Wiesbaden, Germany) was applied at 37 °C. Samples were collected at different time points and stored at −20 °C. The SDS–PAGE was run under reducing conditions, and for the western blot a polyclonal rabbit anti-FN antibody (Sigma-Aldrich Biochemie, Hamburg; Germany) was used. Western blots were detected using Lumi-Light[PLUS] (Roche Diagnostics Deutschland GmbH, Mannheim, Germany).

The epidermis from human skin was scratched from the dermis after 4 hours of incubation in Tris-EDTA buffer. After dissolving the material in 7 M urea and 2 M thiourea, it was homogenized in a SpeedMill PLUS Homogenisator (Analytik Jena AG, Jena, Germany) four times for 30 seconds, centrifuged at 14,000 g, and the supernatant was stored at −20 °C. Four microliter of epidermis extract was incubated in cross-linking buffer with or without SPINK6 in the absence or presence of either TGM1 or TGM3 at 37 °C for 4 hours. A concentration of 1 mM DTT in the sample buffer was added before loading to SDS–PAGE. SDS–PAGE and western blot analyses were performed with equal volumes of samples under reducing conditions in the sample buffer.

NHEKs were isolated from human foreskin and cultivated in collagen-coated 12-well plates (NUNC, Roskilde, Denmark). After reaching 100% confluence, 1 mM $Ca^{2+}$ was added to the used EpiLife medium (Invitrogen, Darmstadt, Germany) for 6 days. 100 µM Z-DON diluted in DMSO was preincubated 20 minutes before 4 µg ml$^{-1}$ SPINK6 was added with the same DMSO concentration. For the control experiments, only DMSO was used. For a second experiment, 4 µg ml$^{-1}$ SPINK6 or biotinylated SPINK6 was given with or without 3 U TGM1 to differentiated NHEK cells. After incubation for 6 hours, the cells were washed three times with PBS buffer. Subsequently, they were incubated in 150 µl of lysis buffer (50 mM HEPES, pH 7.4, 1% TritonX100, 0.1% SDS, 150 mM NaCl, 10 mM EDTA, Roche complete protease inhibitor) for 20 minutes at room temperature. After freezing, the cells were centrifuged for 5 minutes at 14,000 g, and relative concentrations of the supernatant were adjusted with lysis buffer after nanodrop measuring. Western blot analyses using actin antibodies

were performed as internal controls (data not shown). In the second experiment, a streptavidin–peroxidase conjugate (Roche) was used at 1:10,000 to stain biotinylated SPINK6.

Cryosections of healthy skin were incubated with $18 \mu g \, ml^{-1}$ recombinant SPINK6 with or without $200 \mu M$ Z-DON in cross-linking buffer. After washing for 5 minutes three times, sections were fixated with 4% formaldehyde for 30 minutes. SPINK6 was stained using polyclonal goat anti-SPINK6 antibodies followed by chicken anti-goat Alexa Fluor 488 antibodies (Invitrogen), and sections were visualized using an Olympus IX50 fluorescent microscope and the software ''analySIS getIT'' (Olympus, Hamburg, Germany).

## Protease activity assays

A volume of 50 μl of binding buffer (20 mM Tris, pH 7.4, 50 mM NaCl) containing 200 nM FN (Sigma-Aldrich) was coated onto a 96-well plate (NUNC Maxisorp) at 37 °C for 1 hour. After washing twice, 50 μl of cross-linking buffer (20 mM Tris pH 7.6, 50 mM NaCl, 10 mM $Ca^{2+}$) was added with or without 100 nM SPINK6 and with or without 2 U TGM1 at 37 °C for 2 hours. After washing three times, a total volume of 100 μl of assay buffer (20 mM Tris pH 7.6, 50 mM NaCl, 5 mM EDTA) together with 0.8 nM human KLK14 (R&D Systems) and subsequently 0.05 mM GPR-AMC (Bachem Distribution Services GmbH, Weil am Rhein, Germany) were added. Cleavage of GPR-AMC was monitored for 7 hours. Initial velocities were determined with linear regression and plotted with SD ($n = 6$).

NHEKs were cultivated in 24-well plates (NUNC) and grown in EpiLife medium (Invitrogen). To induce differentiation, 1 mM $Ca^{2+}$ was added to the culture medium for 6 days. Cells were treated with or without $4 \mu g \, ml^{-1}$ SPINK6 for 16 hours ($n = 6$) in cross-linking buffer. The supernatant was collected and cells were washed three times with PBS buffer before 0.05 mM GPR-AMC was added. Activity was measured for 6 hours at 25 different positions in each well. The mean was taken as one data point for an activity curve for each well. Initial velocities were determined by linear regression and plotted with their SD.

Total proteins from plantar callus were extracted as described (Meyer-Hoffert *et al.*, 2009). Callus extract (final concentration $0.36 \mu g \, \mu l^{-1}$) was incubated with or without $5 \, ng \, \mu l^{-1}$ SPINK6 in cross-linking buffer and/or 2 U TGM1 at 37 °C for 4 hours. For western blot analyses, 9 μg of callus extract was loaded to each lane, with a total of 135 ng of SPINK6 in each lane. For activity assay, $0.0137 \mu g \, \mu l^{-1}$ callus extract with or without 10 nM SPINK6 was diluted in assay buffer (0.1 M Tris pH 7.6, 5 mM EDTA, 150 mM NaCl, 0.05% Tween) together with 1 nM KLK14. Finally, 0.025 mM GPR-AMC was added, and its cleavage was monitored for 12 hours. Initial velocities were determined by linear regression and plotted with their SD.

Distribution was calculated using the Shapiro–Wilk test, resulting in normal distribution of data sets in all statistical analyses. Student's *t*-test was used to calculate significance levels, taking $P < 0.05$ as significant *a priori*.

## Fluorescent microscopy

Paraffin sections (5 μm) of tissue samples were deparaffinized and rehydrated before heat-induced antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0). Slides were blocked with 10% bovine serum albumin. Staining was performed at 4 °C overnight using an affinity-purified polyclonal goat antibody against SPINK6, $2 \mu g \, ml^{-1}$,

and subsequently a rabbit anti-TGM1 or anti-TGM3 antibody (Zedira, 1:100). After washing, slides were incubated with a mixture of secondary antibodies (chicken anti-goat Alexa Fluor 488, 1:5,000 and a donkey anti-rabbit Alexa Fluor 546, 1:5,000; Invitrogen) for 1 hour at room temperature. Sections were counterstained with the DNA-selective bisbenzimide dye (Hoechst 33258). To exclude artificial autofluorescence secondary to the preparation of the sections, control sections were stained without primary antibodies and no unspecific labeling was observed following incubation with secondary antibodies (data not shown). Fluorescent pictures were taken using a Keyence BZ9000 microscope and the automated Z-stack algorithm (Keyence, Neu-Isenburg, Germany).

## SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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