TRP Channel Regulates EGFR Signaling in Hair Morphogenesis and Skin Barrier Formation

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SUMMARY

A plethora of growth factors regulate keratinocyte proliferation and differentiation that control hair morphogenesis and skin barrier formation. Wavy hair phenotypes in mice result from naturally occurring loss-of-function mutations in the genes for TGF-α and EGFR. Conversely, excessive activities of TGF-α/EGFR result in hairless phenotypes and skin cancers. Unexpectedly, we found that mice lacking the Trpv3 gene also exhibit wavy hair coat and curly whiskers. Here we show that keratinocyte TRPV3, a member of the transient receptor potential (TRP) family of Ca2+-permeant channels, forms a signaling complex with TGF-α/EGFR. Activation of EGFR leads to increased TRPV3 channel activity, which in turn stimulates TGF-α release. TRPV3 is also required for the formation of the skin barrier by regulating the activities of transglutaminases, a family of Ca2+-dependent crosslinking enzymes essential for keratinocyte cornification. Our results show that a TRP channel plays a role in regulating growth factor signaling by direct complex formation.

INTRODUCTION

Skin and its appendages provide a protective barrier essential for animal survival. Hair morphogenesis and epidermal development are orchestrated by an array of cytokines and growth factors (Fuchs and Raghavan, 2002). Signaling by these diffusible molecules provides spatially and temporally controlled cellular programs for keratinocyte proliferation, differentiation, migration, and finally, terminal differentiation and cornification. TGF-α and epidermal growth factor (EGF) are related autocrine/paracrine growth factors that activate the EGF receptor (EGFR; ErbB1) to regulate the balance between keratinocyte proliferation and differentiation (Schneider et al., 2008). Defective TGF-α/EGFR signaling leads to abnormal hair morphogenesis, manifested by the “wavy hair” and “curly whiskers” phenotypes of spontaneous loss-of-function mouse mutations in TGF-α (named waved-1 or wa1) and in EGFR (named waved-2 or wa2), respectively (Ballaro et al., 2005; Luetteke et al., 1993, 1994; Mann et al., 1993; Murillas et al., 1995; Schneider et al., 2008; Sibilia and Wagner, 1995; Threadgill et al., 1995). Excessive activities of TGF-α/EGFR cause a hairless phenotype and skin cancers (Ferby et al., 2006; Schneider et al., 2008). The mechanisms by which TGF-α/EGFR signaling determines cell fate (proliferation versus differentiation) of follicular and interpilляр (epidermal) keratinocytes are not completely understood.

Accumulated evidence suggests that both negative and positive feedback mechanisms coexist in the TGF-α/EGFR signaling axis. EGF binding triggers rapid degradation of the EGFR through endocytic pathways but also leads to further production and release/shedding of TGF-α/EGF (Coffey et al., 1987; Peschon et al., 1998). This unique autoinduction mechanism may contribute to the effects of TGF-α/EGF on keratinocyte terminal differentiation (Peus et al., 1997; Sakai et al., 1994;
Figure 1. Targeted Deletion of Mouse Trpv3 Abolishes the Response of Keratinocytes to TRPV3 Activators

(A) PCR genotyping of wild-type (WT), V3 KO, and heterozygous (Hets) mice. Two sets of primers were used as described in Experimental Procedures. PCR products for primer set A: WT 800 bp, KO 300 bp. For primer set B: WT 130 bp, KO no product.

(B) Lack of TRPV3 protein expression in the skin of V3 KO mice. TRPV3 was immunoprecipitated and immunoblotted using a TRPV3-specific monoclonal antibody. Cell lysates from HEK293T cells expressing recombinant mouse TRPV3 (V3-HEK) were used as positive controls. β-Tubulin served as a loading control for skin lysates.

(C–E) Lack of agonist-induced V3-like Ca2+ response in V3 KO primary keratinocytes. (C) TRPV3 agonist cocktail (200 μM 2-APB + 500 μM Carvacrol) induced large increases of [Ca2+]i in primary cultured keratinocytes isolated from WT (V3+/+) but not V3 KO (V3−/−) mice. Whereas more than 80% of WT keratinocytes responded strongly to the V3 agonist cocktail, negligible responses were observed for V3 KO keratinocytes. Positive controls: 60%–80% keratinocytes from both genotypes (WT and V3 KO) responded to 4α-PDD (3 μM; agonist of TRPV4). All cells responded to ionomycin (1 μM). (D) Ca2+ responses of two representative WT cells from (C) (arrows; upper panels). One cell responded to both TRPV3 and TRPV4 agonists whereas the other one only responded to the V3 agonist cocktail. (E) Ca2+ responses of two representative V3 KO cells from (C) (arrows; lower panels). One cell responded to the TRPV4 agonist (cell 2); neither cell responded significantly (<0.1 fura-2 ratio) to the V3 agonist cocktail.
lysine isopeptide bonds between proteins, and their activi-

at +80 mV (n = 8), respectively.

The transglutaminases (TGases) primarily form

plex layer of lipids attached to a layer of crosslinked proteins.

Blue dashed line = zero current. (G) Representative ramp current of

and Massague, 1991). The Ca2+ influx pathway under physiolog-

cal conditions, however, has not been identified.

The cornified cell envelope (CE) is a protein-lipid layer that

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Graham, 2003). Cornification-promoting cellular cues may acti-

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and biochemical analyses of TRPV3-deficient mice and isolated

wa1 Xu et al., 2006). In this study, we find that TRPV3-deficient

mice exhibit hair phenotypes similar to wa1 and wa2. Molecular and biochemical analyses of TRPV3-deficient mice and isolated keratinocytes reveal defective TGF-α/EGFR signaling. We propose that TRPV3 is a Ca2+-entry pathway tightly associated with the TGF-α/EGF signaling complex orchestrating keratinocyte terminal differentiation.

Previous studies suggest that TGF-α/EGF regulate keratino-

cyte terminal differentiation likely in a Ca2+-dependent manner

(Denning et al., 2000; Sakai et al., 1994). Intracellular Ca2+ regu-

lates both expression and shedding from the membrane-teth-

ered precursors of EGFR ligands (Denning et al., 2000; Horiiuchii et al., 2007). Ca2+ ionophores are sufficient to induce both production and release of TGF-α (Horiiuchii et al., 2007; Pandiella and Massague, 1991). The Ca2+ influx pathway under physiological conditions, however, has not been identified.

Functional Characterization of TRPV3 Global

and Keratinocyte-Specific KO Mice

We performed functional studies in keratinocytes isolated from

V3 KO and control (wild-type [WT], V3+/+; heterozygous or

Hets, V3+/−) mice. Fura-2 Ca2+ imaging was employed to study the response of keratinocytes to TRPV3 chemical agonists (Xu et al., 2006). Application of most TRPV3 agonists alone, for example, 2-APB (200 μM) or Carvacrol (500 μM), induced a small increase in intracellular Ca2+ ([Ca2+]i) in a subset of cells (30% to 80% of cells; data not shown). However, coapplication of two agonists, for example, 200 μM 2-APB + 500 μM Carvacrol (TRPV3 agonist cocktail), reliably induced a dramatic (ΔF340/ F380 > 1) increase of [Ca2+]i in the majority (>80%) of keratino-

cytes isolated from WT mice (WT keratinocytes; Figures 1C and 1D). Similar results were obtained for other combinations of TRPV3 agonists such as 200 μM 2-APB + 5 mM Camphor; removal of external Ca2+ abolished most of the agonist-induced Ca2+ responses. No significant increase (ΔF340/F380 < 0.1 in [Ca2+]i) was seen in keratinocytes isolated from V3 KO mice (V3 KO keratinocytes; Figures 1C and 1E). In contrast, in both WT and V3 KO cells, large [Ca2+]i increases were evoked by 4x-PDD (3 μM), an agonist of TRPV4 (Watanabe et al., 2002) that is also expressed in keratinocytes. Similar results were
obtained in keratinocytes from V3 cKO mice. These results demonstrate that TRPV3 KO mouse keratinocytes completely lack TRPV3-mediated Ca\(^{2+}\) responses.

Consistent with the Ca\(^{2+}\) imaging results, TRPV3 agonist cocktail evoked a slowly developing, large TRPV3-like current (\(I_{\text{TRPV3}}\)) in most WT keratinocytes (Figures 1F, 1G, and 1J). Ruthenium red (RuR, 5 \(\mu\)M), a nonspecific voltage-dependent blocker of TRPV1-4 channels (Chung et al., 2004; Hu et al., 2004; Ramsey et al., 2006; Xu et al., 2006), almost completely (>99%) inhibited agonist-activated inward \(I_{\text{TRPV3}}\). Similar RuR-sensitive \(I_{\text{TRPV3}}\) was also evoked by other TRPV3 agonists (200 \(\mu\)M 2-APB + heat or 200 \(\mu\)M 2-APB + 5 mM Camphor) in the same cells. In V3 KO keratinocytes, in contrast, no significant current was evoked by the V3 agonist cocktail (Figures 1H, 1I, and 1J). Similar results were obtained in keratinocytes from V3 cKO mice.

TRPV3-Deficient Mice Exhibit Curly Whiskers and Wavy Hair

Although previous animal studies identified TRPV3’s function in temperature sensation (Moqrich et al., 2005), the most obvious phenotypic changes we observed from our V3 KO mice are abnormalities in skin, hair, and whiskers. In contrast with littermate controls (WT and heterozygotes [Hets]), most whiskers of V3 KO mice were characteristically curved or hooked (Figure S2). The curly morphology of whiskers was apparent at birth (Figure S2); newborn V3 KO mice could be identified based on whisker morphology alone. Keratinocyte-specific V3 cKO mice exhibited similar curly whiskers (Figures 2A and 2B), suggesting that the phenotype was caused by specific V3 deficiency in keratinocytes. Whisker curliness grew more obvious with age (Figure 2B and Figure S2). Both the dorsal and ventral coat fur, as well as the tail hair, of V3 KO and cKO mice were wavy (Figure 2B and Figure S2) beginning 1 week after birth but was most apparent once the hair was well formed (~3–4 weeks postnatal), gradually reducing with age. In contrast to a previous study reporting abnormality of ventral hairs in a subset of V3 KO mice (Moqrich et al., 2005), curly whiskers and wavy hair were present throughout the coat at all ages with 100% penetrance for both V3 KO and cKO mice that we have generated, as well as the V3 KO mice reported by Moqrich et al. (H.L., S.M.H., and M.J.C., unpublished data). In comparison, TRPV1 KO mice hair shape and distribution are normal (Figure S2). Consistent with the expression of TRPV3 in follicular keratinocytes of mouse (Peier et al., 2002) and human (Xu et al., 2002), the wavy phenotype correlated with V3 deficiency in keratinocytes.

Skin contains many cell types, including sensory nerves. However, as the K14 promoter drives the expression of Cre recombinase specifically in all keratinocytes (both follicular and interfollicular) in the skin (Coulombe et al., 1989), the results obtained from V3 cKO, V3 fl/fl: K14 Cre mice suggested that the defect in hair morphogenesis was due to the lack of TRPV3 expression in keratinocytes in a cell-autonomous manner. Both hair and whisker phenotypes were independent of pigmentation of the hair and genetic background, as black-coated (C57BL/6) backcrossed (>6 generations) V3 KO mice displayed phenotypes similar to those of mice with mixed genetic background (BL6 and 129sv) (Figure S2). V3 KO pups were born with the expected Mendelian ratio, and body weight was comparable to control mice (Figure S2).

In histological examinations of skin from the mid-dorsal region of mice of different ages, we found that a subset of hair follicles exhibited an obvious but gentle curvature (Figure S2). In Haematoxylin & Eosin (H&E) stained skin sections from control mice (P4), hair follicle shafts were parallel and posed roughly at a 45° angle to the subcutaneous muscle layer (Figure 2C). In contrast, individual hair follicles of V3 cKO mice were, in many cases, gently curved and pointed in different directions with variable.
Defective TGF-α/EGFR Signaling in TRPV3-Deficient Mice

The hair and whisker phenotype of V3 KO mice resembled largely those of wa1 and wa2 mice, as well as other mouse mutations with reduced expression/release/activity in TGF-α and/or EGFR (Ballaro et al., 2005; Du et al., 2004; Luetteke et al., 1993, 1994; Mann et al., 1993; Miettinen et al., 1995; Murillas et al., 1995; Peschon et al., 1998; Schneider et al., 2008; Sibilia and Wagner, 1995; Threadgill et al., 1995) (see Figure S3). Thus we investigated whether TGF-α and/or EGFR signaling was altered in V3 KO mice. Real-time semiquantitative PCR (q-PCR) analysis of the skin of V3 KO pups revealed that the mRNA expression level of TGF-α was half that of WT (P4; Figure 3A). TGF-α mRNA levels of newborn (P0) V3 KO animals, though significantly less than those of the P4 mice, were comparable to those of the littermate controls (P0). Expression (mRNA) levels of several other EGFR ligands and EGFR (Figure S3), however, were not significantly altered in the skin of V3 KO mice. These results suggest that TRPV3 affects the expression level of TGF-α in postnatal skin in vivo.

Both expression and proteolytic shedding of the membrane-tethered TGF-α are known to be Ca²⁺ dependent (Denning et al., 2000; Horiiuchi et al., 2007). We used an ELISA assay optimized for human TGF-α to investigate the role of TRPV3 in TGF-α shedding/release. In the presence of TRPV3 agonist cocktail (100 μM 2-APB + 250 μM Carvacrol; 30 min), normal human epidermal keratinocytes (NHEK) released more than twice the amount of TGF-α into the culture medium. This is comparable to the effect of PMA, a stimulus well known to induce release and expression of TGF-α (Figure 3B). ADAM17, the principal shedding enzyme for TGF-α release (Peschon et al., 1998), was required for V3 agonist-induced TGF-α release/shedding (Figure 3C).

As the level of TGF-α was reduced in V3 KO skin, one would expect that its activated receptor, phosphorylated EGFR (P-EGFR), might also be reduced. By immunoblot analysis of P-EGFR in skin lysates of V3 KO skin, EGFR activity was only about one-third of that of WT controls (Figures 3D and 3E). Interestingly, the expression level of total EGFR was slightly but significantly increased in V3 KO skin (1.8 ± 0.3-fold, n = 6), so that the ratio of P-EGFR/total EGFR was about 5-fold less (0.20 ± 0.03, n = 4) in V3 KO skin. This level of the reduction was comparable to those of the hypofunctional EGFR mutations causing “wavy” phenotypes (Du et al., 2004). Consistent with biochemical results, EGFR staining was more prominent in V3 KO frozen skin sections (Figure S3), whereas P-EGFR immunostaining was weaker. These results are consistent with dramatically reduced EGFR activity in V3 KO mice and suggest that the level of total EGFR was increased as a consequence of reduced activity, tyrosine phosphorylation-dependent endocytotic degradation (Schneider et al., 2008), or other compensatory mechanisms. The reduction of EGFR activity in V3 KO mice was probably due to the loss of the TRPV3 channel activity, as activation of TRPV3 in cultured keratinocytes using TRPV3 agonists resulted in increases in both TGF-α release (Figures 3B and 3C) and EGFR activity (Figure 3F and Figure S3). Notably, the TRPV3-induced increase of EGFR activity in keratinocytes was abolished by a neutralizing TGF-α antibody or ADAM17 shedding inhibitor (Figures 3F and 3G), suggesting that activation of TRPV3 led to an increase in TGF-α release and subsequent EGFR activation.

Regulation of TRPV3 Channel Activity by TGF-α/EGFR Signaling

Several in vitro studies provided evidence that TRP channels are regulated by members of the receptor tyrosine kinase (RTK) family (Li et al., 1999; Ramsey et al., 2006). We hypothesized that TRPV3 channel activity could also be upregulated by EGFR signaling. In serum-starved primary keratinocytes cultured in the absence of TGF-α, Ca²⁺ responses could be induced by a high concentration (100 μM 2-APB + 250 μM Carvacrol), but not by a lower concentration (50 μM 2-APB + 125 μM Carvacrol), of V3 agonist cocktail (Figures 4A and 4C). In the presence of TGF-α (100 ng/ml for 3–5 hr), however, large [Ca²⁺]i increases were recorded even with a low concentration (50 μM 2-APB + 125 μM Carvacrol) of V3 agonist cocktail; responses to a high concentration of V3 agonists were comparable to those without TGF-α treatment (Figures 4B and 4C). Similar results were seen with EGFR (100 ng/ml) pretreatment (Figure S4). TGF-α/EGFR treatment augmented the sensitivity of TRPV3 to keratinocytes to V3 agonists, suggesting that the increased activity was at least partially mediated by increased channel gating, rather than the expression or surface expression of TRPV3 proteins. Consistent with this interpretation, the temperature-induced response (from 22°C to 41°C) was also significantly larger in TGF-α-treated keratinocytes (Figure S4). The sensitizing effect of TGF-α was most likely mediated by EGFR, as an EGFR inhibitor (AG1478) or shRNA knockdown of EGFR expression (Figure S4) completely or largely eliminated its potentiation (Figures 4D and 4E). Because inhibitors of PLC (U73122) and ERK (PD98059) completely or partially blocked potentiation (Figure 4D), these pathways may underlie the sensitizing effect downstream of EGFR receptor activation. In support of this finding, shRNA knockdown of PLC-γ1 expression (Figure S4) significantly decreased the sensitizing effect of TGF-α (Figure 4E). Consistent with our [Ca²⁺]i measurements, Iₘₐₜₚ₃ exhibited a similar dependence on TGF-α (Figure 4F).

The results presented so far raise the possibility that TRPV3 and EGFR might be in a signaling complex. To test this hypothesis, coimmunoprecipitation (co-IP) experiments were first performed in a heterologous expression system. In cells transfected with TRPV3, either alone or together with EGFR, both endogenous (data not shown) and overexpressed EGFR (Figure S4) were found to co-IP with TRPV3. Next, we confirmed this finding...
Figure 3. Reduced Levels of TGF-α and Decreased Activity of EGFR in the Skin of TRPV3-Deficient Mice

(A) mRNA expression levels (q-PCR) of TGF-α were significantly (p < 0.05) lower in V3 KO skin tissues from P4 but not P0 mice.

(B) Short application of V3 agonist cocktail (100 μM 2-APB + 250 μM Carvacrol; 30 min) significantly increased TGF-α release into the culture medium from primary human keratinocytes (NHEK). TGF-α was measured with ELISA; PMA was used as a positive control.

(C) V3 agonist-induced TGF-α release was diminished in the presence of BB2116 (20 μM), an inhibitor of ADAM17 required for the shedding of TGF-α.

(D) Immunoblotting analysis of phosphorylated (active; P-EGFR) and total EGFR expression levels of WT and V3 KO skin lysates. Compared to WT mice, the level of P-EGFR was significantly decreased in V3 KO skin lysates. In contrast, the expression level of total EGFR was slightly but significantly increased in V3 KO skin lysates.

(E) Statistical analyses of EGFR and P-EGFR expression levels.

(F and G) EGFR activity (P-EGFR) was enhanced by V3 agonist cocktail (100 μM 2-APB + 250 μM Carvacrol) for 30 min. The basal activation of EGFR was induced by a minimal concentration of EGF (0.5 ng/ml). The enhancement was abolished in the presence of BB2116 (20 μM) or a neutralizing antibody against TGF-α (1 μg/ml). Data in (A), (B), (C), (E), and (F) are presented as the mean ± SEM. See also Figure S3.
in keratinocytes (Figure 4G) using skin tissues from TRPV3-YFP transgenic mice (Huang et al., 2008). These results suggest that EGFR can directly or indirectly associate with TRPV3 in both heterologous and native systems. Consistent with the close association of these molecules, we found that activation of EGFR with TGF-α resulted in tyrosine phosphorylation of TRPV3 (Figure 4H).

Altered Keratinocyte Differentiation in TRPV3-Deficient Mice

EGFR signaling is known to have at least two distinct functions in epidermis (Schneider et al., 2008). In the basal layer, TGF-α/EGFR signaling promotes keratinocyte proliferation (Schneider et al., 2008). The function of EGFR signaling in suprabasal cells is to promote late terminal differentiation (Ballaro et al., 2005; Dlugosz et al., 1994; Peus et al., 1997; Wakita and Takigawa, 1999). Whereas proliferating keratinocytes in the basal layer express structural keratins K5/K14, differentiating keratinocytes express the structural keratins K1/K10 (Byrne et al., 2003). As keratinocytes move closer to the skin surface, expression of K1/K10 declines and loricrin expression increases (Byrne et al., 2003), as they undergo cornification. Consistent with previous studies (Wakita and Takigawa, 1999), we observed that EGF significantly reduced the expression of the early differentiation marker K1 in suspended keratinocytes (Figure S5).
We reasoned that a reduced rate of autonomous EGFR-dependent proliferation or terminal differentiation would accelerate keratinocyte early differentiation in V3 KO cells. Compared to WT skin sections, V3 KO epidermis exhibited a >2-fold increase in the thickness of the K1-positive layer (Figure 5A), whereas cell sizes and densities were normal (Figure S5). Similar results were reported in transgenic mice with defective TGF-α/EGFR signaling or keratinocytes cultured in the presence of EGFR inhibitors (Ballaro et al., 2005; Peus et al., 1997). No significant change was observed in the K14 layer of V3 KO epidermis (Figure 5B). Loricrin expression was relatively less elevated in V3 KO epidermis (Figure 5C). Consistent with the increased early differentiation in V3 KO epidermis, thickness of K10 (interaction partner of K1) layer also increased in V3 KO animals (Figure 5D). Finally, V3 cKO animals exhibited similar alterations in keratinocyte differentiation (Figure 5E).

Figure 5. Genetic Inactivation of TRPV3 Results in Increased Expression of Early Epidermal Differentiation Markers in Skin
(A–E) Immunofluorescence analyses of frozen skin sections from P4 pups.
(A and A') Compared to WT mice, the immunofluorescence of keratin protein 1 (K1; a keratinocyte structural protein and a marker for the differentiating spinous and granular layers) was elevated in V3 KO skin sections. Integrin α6 antibody labeled the basement membrane, the boundary between epidermis and dermis. DAPI is a nuclear marker. The K1-positive layer was 2-fold thicker in V3 KO epidermis (quantified in the A' panel).
(B and B') Normal immunofluorescence of keratin protein 14 (K14; a keratinocyte structural protein and a marker for the proliferating basal layer).
(C and C') Slightly but significantly elevated loricrin (a marker for the differentiating granular layer) immunofluorescence in V3 KO epidermis.
(D and D') Elevated keratin protein 10 (K10; a keratin protein associated with K1 immunofluorescence in V3 KO epidermis.
(E and E') Elevated K1 immunofluorescence in V3 cKO epidermis.
Data in (A')–(E') are presented as the mean ± SEM. See also Figure S5.
Ca\(^{2+}\) is an important regulator of keratinocyte differentiation both in vitro and in vivo (Yuspa et al., 1989). We next examined Ca\(^{2+}\)-dependent keratinocyte differentiation in vitro using a well-established Ca\(^{2+}\) switch protocol. In these experiments, epidermal basal cells were selectively cultured in 0.05 mM Ca\(^{2+}\) medium and terminal differentiation was induced by raising [Ca\(^{2+}\)]\(_{o}\) to 0.2–1.4 mM (Yuspa et al., 1989). Compared to WT cells, more loricrin was expressed in cultured V3 KO keratinocytes after induction of differentiation (Figure S5). Collectively, these results suggest a role of TRPV3 in keratinocyte differentiation both in vitro and in vivo.

**Figure 6. Defective Barrier Formation and Diminished TGase Activity in the Skin of TRPV3-Deficient Mice**

(A) Compared to newborn (P0) WT mice (on the left), V3 KO skin was dry, reddened, and scaly. (B) Similar dry and scaly skin was also seen in neonatal (P1) cKO mice. (C) Toluidine blue dye exclusion assay of embryonic day 17 (E17) embryos. Staining indicates dye permeability and defective or immature barrier function. The upper and lower halves of the pictures were taken separately but shown in combination for the purpose of illustration. (D and E) Compared to WT littermate pups (P4), the cornified cell envelopes (CEs) of skins of V3 cKO pups were significantly less mature. (F) Compared to WT littermates, reduced TGase activity was detected in the frozen skin sections of neonatal (P1; upper two panels) V3 cKO mice. TGase activity was detected using an immunofluorescence-coupled in situ enzymatic assay. Positive staining was restricted to the granular layer of epidermis. Reduced TGase activity in the P4 (lower two panels) skin of V3 cKO. (G) Expression levels of TGase1 were comparable for both WT and V3 cKO mice. (H) Short (40 min) application of V3 agonist cocktail (50 \(\mu\)M 2-APB + 200 \(\mu\)M Carvacrol) dramatically increased TGase activity in primary cultured keratinocytes from WT but not V3 KO mice. (I) V3 agonist cocktail induced an \(-11\)-fold increase of TGase activity in WT keratinocytes. Data in (E) and (I) are presented as the mean ± SEM. See also Figure S6.

Defective Epidermal Barrier Function and TGase Activity in TRPV3-Deficient Mice

In addition to hair abnormalities, the skin of newborn V3 KO and cKO mice was red in color (erythroderma), dry, and scaly (Figures 6A and 6B), resembling the skin phenotype of mice with defective barrier formation (Koch et al., 2000; Sevilla et al., 2007). Before hair penetration (P0–P3), the skin of V3 KO mice was rougher and shinier than that of WT. To measure skin barrier integrity, we used toluidine blue exclusion. In newborn (P0) V3 WT, KO, and E17 WT mice, dye was almost completely excluded, indicating normal maturation of the skin barrier. In E17 V3 KO embryos, however, dye permeability was significant, particularly in ventral areas (Figure 6C). EGF is known to increase the thickness of stratum corneum (Ponec et al., 1997), and consistent with a role for TRPV3 in barrier formation, the stratum corneum layer of V3 KO (Figure S2) and cKO (Figures 2C and 2D) mice was significantly thinner and more compact than WT littermates. We next examined the morphology of the mature CE. Mature CE in WT skin was symmetrical and smooth, whereas immature CE was irregular and fragile. The density of
mature CE in V3 KO (Figure S6) and cKO mice (Figures 6D and 6E) was only 15%–18% of that in WT mice.

Multiple mechanisms might lead to defective CE formation. TGases are a family of enzymes that crosslink proteins essential for CE formation (Lorand and Graham, 2003). Among them, TGase1 and TGase3 are expressed in the epidermis and are regulated by intracellular Ca²⁺ (Lorand and Graham, 2003). One possibility is that the activity of TGases was reduced in V3 KO mice. EGFr or TGF-α is known to dramatically increase the activity of TGases and CE formation in cultured keratinocytes in suspension, which presumably mimics the conditions of suprabasal keratinocytes (Wakita and Takigawa, 1999). We found that the activity of TGases (Koch et al., 2000; Raghunath et al., 1998) was significantly lower in both newborn (P1) and P4 V3 cKO epidermis (Figure 6F) but not in age-matched V1 and V4 KO epidermis (Figure S6). The expression level of TGase1, however, was comparable for WT and V3 cKO (Figure 6G). When TGase activity was measured in cultured keratinocytes, TGase activity was abnormally low in both WT and V3 KO keratinocytes (Figures 6H and 6I). Application of V3 agonist cocktail (Lorand and Graham, 2003) to cells was comparable for WT and V3 cKO (Figure 6G). When TGase activity was measured in cultured keratinocytes, TGase activity was abnormally low in both WT and V3 KO keratinocytes (Figures 6H and 6I). Application of V3 agonist cocktail (Lorand and Graham, 2003) to cells was comparable for WT and V3 cKO (Figure 6G).

DISCUSSION

Intracellular Ca²⁺ regulates both the production and the release of EGFR ligands (Denda et al., 2010; Dugolo et al., 1994). As TGF-α production/release is an autoinduction process (Coffey et al., 1987), the reduced production of TGF-α might result from reduced EGFR activity. The positive feedback loop (Figure 7) in which TGF-α/EGFR activation potentiates TRPV3-mediated Ca²⁺ entry, which in turn potentiates TGF-α/EGFR signaling, may provide an explanation for its unique property of autoinduction (Coffey et al., 1987). Ca²⁺-induced differentiation dramatically increases TGF-α production, suggesting a nonproliferative role of the TGF-α/EGFR signaling axis (Denning et al., 2000). On the other hand, EGFr/TGF-α itself is known to promote late terminal differentiation both in vitro and in vivo by dramatically increasing TGase activity and CE formation while suppressing the expression of K10 (Dugolo et al., 1994; Wakiita and Takigawa, 1999). Thus, for suprabasal cells, the function of EGFR signaling is to promote late terminal differentiation (Wakiita and Takigawa, 1999). The primary defect in V3 KO mice is late terminal differentiation but not proliferation. Thus, the feedback mechanism described above may contribute to the process of late terminal differentiation.

It is still not clear how TRPV3 is activated to trigger or promote late terminal differentiation. Temperatures in the range of 31°C–39°C activate TRPV3 in heterologous expression systems (Ramsey et al., 2000). Thus temperature may be the primary activator for keratinocyte TRPV3. Consistent with this notion, temperature is known to affect the barrier function and modulate the effect of EGFr/TGF-α on keratinocyte differentiation (Denda et al., 2007; Ponce et al., 1997). At skin temperatures in vivo (∼32°C), TRPV3 is constitutively but weakly active. Thus release of TGF-α may increase the activity of weakly constitutively active TRPV3.

We provide evidence that TRPV3 and TGF-α/EGFR are in the same signaling complex regulating epidermal homeostasis. Whereas loss-of-function in TGF-α/EGFR leads to “wavy hair” (Loetteke et al., 1993; 1994; Mann et al., 1993; Schneider et al., 2008; Sibilia and Wagner, 1995; Threadgill et al., 1995), elevated TGF-α/EGFR activities cause a “hairless” phenotype (Ferby et al., 2006; Schneider et al., 2008; Wang et al., 2006). Interestingly, whereas our V3 KO mice exhibit “wavy hair,” mice carrying a gain-of-function mutation in TRPV3 are hairless.
(Asakawa et al., 2006). It may prove informative to generate transgenic mice with TRPV3 loss of function and concurrent TGF-α/EGFR gain of function, or with TRPV3 gain of function and concurrent TGF-α/EGFR loss of function.

EGFR is the prototype of the RTK family. EGFR signaling is necessary for proper development and tissue homeostasis whereas its dysregulation rapidly results in defects in cellular proliferation and differentiation. The consequences of its malignant function are abnormal hair follicle morphogenesis, impaired wound healing, and tumorigenesis (Schneider et al., 2008). We have identified another key element in this important signaling pathway, the TRPV3 channel. Our studies not only provide the first in vivo evidence in mammals for the close interaction of RTK and TRP channels but also suggest that TRPV3 can be a novel target for hair growth and removal agents as well as in the treatment of skin cancers or other dermatological diseases.

EXPERIMENTAL PROCEDURES

Conditional and Global Disruption of Trpv3 in Mice

Mouse Trpv3 was disrupted either globally or in a keratinocytes-specific manner (see Extended Experimental Procedures in the Supplemental Information).

Real-Time Semiquantitative PCR

After a small piece of back skin was dissolved in TRIzol (Invitrogen, Carlsbad, CA, USA), mRNA was purified using RNeasy columns (Qiagen Inc., Valencia, CA, USA). First-strand cDNA was synthesized using Superscript III RT (Invitrogen) and utilized for Semiquantitative PCR based on intron-spanning primers. A Bio-Rad IQ Cycler was used to measure the expression level of transcripts. The primer sequences are provided in the Extended Experimental Procedures.

Preparation and Culture of Mouse Keratinocytes

Mice (P0–P2) were sacrificed and soaked in 10% povidone-iodine for 5 min. After rinsing in 70% ethanol multiple times, the skin was removed and placed in a Petri dish containing PBS solution with 0.25% trypsin (Invitrogen) for incubating at 4°C overnight. Epidermis was then separated from the subcutaneous tissues. Vortexing dissociated cells and keratinocytes were first plated in a high [Ca2+]i (1.4 mM) minimal essential medium (MEM; Gibco), which was replaced with a low [Ca2+]i (0.05 mM; differentiation-restricted) medium after 6 hr. The keratinocytes were then cultured in MEM containing 8% Chelax-treated (Bio-Rad) FBS with the final [Ca2+]i adjusted to 0.05 mM. Suspension cultures were on polyhydroxyethylmethacrylate (poly-HEMA)-coated plates as described previously (Wakita and Takigawa, 1999).

NHEK Cell Culture

Normal human epidermal keratinocytes were obtained from Invitrogen and cultured in EpiLife Medium supplemented with Human Keratinocyte Growth Supplement (Invitrogen).

Immunoblotting and Immunoprecipitation

Back skin lysates for the immunodetection of EGFR and P-EGFR were prepared as follows: a small piece of back skin was lysed on ice for 30 min with 1 ml of lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 0.25% sodium deoxycholate, and 1 x protease inhibitor cocktail). Immunoprecipitation and immunoblotting were then performed using skin lysates as described in the Extended Experimental Procedures.

Histology and Immunostaining

Tissues were fixed for 3 hr with 4% paraformaldehyde (PFA) at 4°C, embedded in OCT or paraffin. Sections (~4 μm) of paraffin-embedded tissues were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on cryostat sections (~10 μm) using antibody dilutions described in the Extended Experimental Procedures.

Dye Exclusion Assays

Toluidine blue staining of mouse embryos and newborn pups was performed as described in the Extended Experimental Procedures.

In Vivo and In Vitro Transglutaminase Activity Assay

Detection of TGase activity in skin sections (in vivo) and cultured keratinocytes (in vitro) used the amine donor substrate monodansylcadaverine (Molecular Probes) as described in the Extended Experimental Procedures.

Analysis of Cornified Envelopes

A piece of back skin (P4) was isolated and treated as described previously (Koch et al., 2000). Briefly, CEs were prepared by boiling skin for 30–60 min in a buffer consisting of 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT, and 2% SDS. After centrifugation (5,000 g), CEs were washed twice at room temperature with a buffer consisting of 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT, and 0.2% SDS. The density of CE was manually determined using a hemacytometer.

TGF-α ELISA

The medium of near confluent NHEK keratinocytes was harvested for TGF-α measurements using an ELISA kit for human TGF-α (Calbiochem).

Electrophysiology

Whole-cell recordings were performed in primary keratinocytes. Details of recording conditions are described in the Extended Experimental Procedures.

Ca2+ Imaging

Mouse and NHEK primary keratinocytes were loaded with 5 μM Fura-2 AM in culture medium at 37°C for 60 min. Cells were then washed in modified Tyrode’s solution for 10–30 min. Fluorescence at different excitation wavelengths was recorded on an EasyRatiopro system (Photon Technology International, Birmingham, NJ, USA). Fura-2 ratios (F340/F380) recorded changes in [Ca2+]i upon stimulation. Ionomycin (1 μM) was added at the conclusion of all experiments to induce a maximal response for comparison.

Data Analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made using analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2010.03.013.

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EXTENDED EXPERIMENTAL PROCEDURES

Conditional and Global Disruption of Trpv3 in Mice
We targeted exon 13 of mouse Trpv3, located on chromosome 11 B4, to disrupt its function. Deletion of exon 13 was predicted to remove the entire 3rd transmembrane segment (TM3) and part of TM4 and shift the open reading frame thereafter (see Figure S1). Thus, the putative pore region (TM5-TM6) would not be translated in TRPV3 KO mice regardless of whether the resulting transcript was stable. Since global knockout mice can be easily obtained from conditional deletions via a global Cre transgenic, such as Sox2-Cre (Hayashi et al., 2002), we made a construct for conditional disruption of Trpv3 based on a recombineering method (Liu et al., 2003). In this construct, exon 13 of Trpv3 was flanked by two LoxP sites plus an FRT-flanked neomycin resistance cassette (see Figure S1). This modified allele is referred to as Trpv3flneo. Deletion of the FRT-flanked neomycin resistance cassette via the recombinase results in the floxed allele referred to as Trpv3fl. Deletion of the floxed exon 13 results in a null allele, referred to as Trpv3-/-.

For ES cell targeting, the construct was electroporated into J1 embryonic stem cells and cells were selected for neomycin resistance. Positive ES cell clones with correct homologous recombination were identified by Southern analysis. Three positive ES cell clones were obtained, and all three showed the correct targeting event by both Southern analysis and PCR as described in Experimental Procedures. DNA prepared from Agouti pups. The neomycin resistance cassette was removed from the targeted allele by breeding Trpv3flneo/+ mice with transgenic mice expressing the Flp recombinase (Farley et al., 2000) (Jackson Laboratory #003946), resulting in Trpv3flfl mice. Trpv3flfl and Trpv3-/- mice were used for all experiments. All genotypes of Trpv3 KO mice and K14 promoter-driven TRPV3-YFP transgenic mice were determined by PCR as described previously (Huang et al., 2008; Suzuki et al., 2003).

Southern Blot and PCR Genotyping
For Southern blot analysis, 10 μg of genomic DNA was digested overnight with KpnI, fractionated on a 0.7% agarose gel, and transferred to Hybond N+ membrane (Amersham). Southern analysis was performed using a standard non-radioactive labeling protocol with DIG-labeled dTTP (Roche). The probe for identification of the Trpv3flneo allele was confirmed by Southern analysis using tail DNA prepared from Agouti pups. The neomycin resistance cassette was removed from the targeted allele by breeding Trpv3flneo/+ mice with transgenic mice expressing the Flp recombinase (Farley et al., 2000) (Jackson Laboratory #003946), resulting in Trpv3flfl mice. The Trpv3flfl mice were backcrossed with C57BL/6J females for more than 6 generations to obtain a clean genetic background. K14-Cre transgenic (Jackson laboratory #004782) was bred with Trpv3flfl mice to obtain keratinocyte-specific disruption of Trpv3.

Real-Time Semiquantitative PCR
The primer sequences were as follows. For mL32, F: 5'-TGTTGAAGCCCAAGATCGTGC-3'; R: 5'-CTTCTCCGACCCCTGTGTC-3'. For mTGF-α, F: 5'-GGCGCTGGAATATCTTGTTAGC-3'; R: 5'-TTGGAAAACTGCCACTTGTTG-3'. h EGFR F: 5'-CCGGACATAGTCAGCACTGGA-3'; R: 5'-GGGACAGCTGGATGACACATG-3' h PLC-γ1 F: 5'-TGCGGTCAGGAGGAGCTTTAATA-3'; R: 5'-GAGGACGGCCATGGTGGTGTGC-3'. h GADPH F: 5'-GGGACAGCTGTCACATGATC-3'; R: 5'-GGGACAGCTGTCACATGATC-3'. m EGFR F: 5'-GGGGTCGTCCTCGCGTCCCTAT-3'; R: 5'-CCAAATCGCCTTGTTGTTG-3'. m AR F: 5'-CATTCCGCTGTATACACG-3'; R: 5'-AGCGGCTCAGCATGGGAAA-3'. m HBEFG F: 5'-ATCCACGGAGAGCTAGATG-3'; R: 5'-AGCGGCTCAGCATGGGAAA-3'. m EGFR F: 5'-GGGACACCAACCATGACAAA-3'; R: 5'-AGCGGCTCAGCATGGGAAA-3'. For each sample, the expression levels of mTGF-α, mEGF, mAR, mHBEFG, and mEGFR were normalized using that of mL32. The expression levels of hEGFR and hPLC-γ1 were normalized using that of hGADPH.

Reverse Transcriptional-PCR Analysis
Single-stranded cDNA from P0 mouse skin was prepared as described in Experimental Procedures. Primer sequences of TRPV3 were as follows.

Primer set C: forward primer, 5'-CCGGACATAGTCAGCACTGGA-3'; reverse primer 5'-ATCCACGGAGGAGCTTTAATA-3'. Primer set D: forward primer, 5'-TGCGGTCAGGAGGAGCTTTAATA-3'; reverse primer, 5'-GGGACAGCTGGATGACACATG-3'. Primer set D was designed based on sequences within the putative deletion region, exon 13.
Lentiviral pLKO.1-ShRNA Knockdown
A series of Lentiviral pLKO.1-ShRNA constructs against human EGFR and PLC-γ1 were purchased from Sigma and tested using q-PCR in HEK293T cells. The following two ShRNA constructs were chosen to knock down EGFR and PLC-γ1 in human epidermal keratinocytes (NHEK):

- **EGFR**: 5′-CCGGGCTGCTGAATCCTTTACTGAGTAAGAGATTTTCAGAGCGACTTTTTG-3′;
- **PLC-γ1**: 5′-CCGGCCAGATCTACGAAAACCTGAGAATTCAGAGGTTAACCTGCTGCTTTTT-3′.

The ShRNA and pLKO.1 control lentivirus stocks were generated via co-transfection of HEK293T cells with packaging plasmids VSV-G-pMAD.G and pCMVdeltaR8.91. NHEK cells were infected with each lentivirus stock and 3 days post-puromycin (2 μg/ml) selection, were used for Ca2+ imaging experiments.

Immunoblotting and Immunoprecipitation
For the immunodetection of EGFR and P-EGFR, back skin lysates were incubated with 2 μg of EGFR antibody (Upstate Cell Signaling) and rotated for 12 hr at 4 °C. Protein A/G beads (30 μl; Amersham Pharmacia) were added, and after 12 hr incubation, the beads were pulled down and washed 5–6 times with lysis buffer. Bound proteins were eluted from the beads with SDS (1x) sample buffer, vortexed, boiled for 5 min, and analyzed by immunoblotting. The total cell lysate or immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 hr with 5% skim milk in PBST and incubated with the anti-EGFR or P-EGFR antibody (diluted 1:1000) in PBST. Detection was carried out using Peroxidase-conjugated rabbit-antibody secondary antibody with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Co-immunoprecipitation for TRPV3 and EGFR was performed in skin lysates or HEK293T cells transfected with pEGFP-C3, TRPV3-EGFP, and EGFR plasmids. The lysis buffer contained 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, and 20 mM Tris-HCl (pH 8.0). The lysate was stirred on ice for 30 min and then centrifuged. The supernatant was incubated with anti-EGFR (Upstate) or anti-GFP (Covance) at 4 °C overnight. The protein complex was then visualized by western blotting using antibodies against GFP or EGFR (Upstate).

Histology and Immunostaining
Immunohistochemistry was performed on cryostat sections (~10 μm) using antibodies for K14 (1:5000; Covance), K1 (1:2000; Covance), K10 (1:1000; Sigma), Integrin α6 (1:1000; BD Lab), Integrin β4 (1:1000; BD Lab), Loricrin, (1:5000; Abcam), EGFR (1:200; Upstate Biotechnology), and P-EGFR (anti-P-Tyr 1173 EGFR, 1:200; Upstate Biotechnology). Nuclei were counterstained with DAPI reagents. Images were taken using an Olympus (IX 81) microscope and a Leica (TCS SP5) confocal microscope.

Dye Exclusion Assays
Toluidine blue staining of mouse embryos and newborn pups was performed as described previously (Koch et al., 2000; Sevilla et al., 2007). The developmental stage of mouse embryos was determined based on the assumption that fertilization occurred in the middle of the day’s dark cycle before vaginal plugs were identified. Embryos were dehydrated by incubation in 25%, 50%, and 75% methanol solution for 1 min each, washed in PBS, and stained for 10 min in 0.0125% toluidine blue O (Fisher Scientific)/PBS. The embryos were then de-stained in PBS.

In Vivo Transglutaminase Activity Assay
Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C. Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C. Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C. Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C. Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C. Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C. Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C. Detection of TGase activity in skin sections (Raghunath et al., 1998) used the amine donor substrate monodansylcadaverine (Molecular Probes). A solution of 2 mg biotinylated-X-cadaverine in 0.1 N HCl (50 μl) was prepared and then mixed with 394 μl doubly distilled H2O. This 10 mM stock solution was stored at 4°C.
**TGF-α ELISA**
To measure TGF-α release, near confluent NHEK keratinocytes were pretreated with or without the metalloproteinase inhibitor BB-2116 (20 μM; British Biotechnology, Oxford, UK) in the presence of 100 ng/ml EGF or 1 mM Tyrophostin AG 1478 (an EGFR inhibitor; Cell Signaling), which was expected to block EGFR activation and receptor-mediated endocytosis of TGF-α. Cells were then treated with V3 agonist cocktail (100 μM 2-APB + 250 μM Carvacrol) or 1 μM PMA (Cell Signaling) for 30 min in the presence or absence of 20 μM BB-2116. The medium was then harvested for TGF-α measurements using an ELISA kit for human TGF-α (Calbiochem).

**Electrophysiology**
Whole-cell patch-clamp recordings were performed in primary keratinocytes. The pipette solution contained 147 mM Cs, 120 mM methane-sulfonate, 4 mM NaCl, 10 mM EGTA, 2 mM Na₂-ATP, 2 mM MgCl₂, 20 mM HEPES (pH 7.2; free [Ca²⁺]i < 10 nM). Standard extracellular bath solution (modified Tyrode’s solution) contained 153 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 10 mM glucose (pH 7.4). All solutions were applied via a perfusion system to achieve a complete solution exchange within a few seconds. Data were collected using an Axopatch 2A patch clamp amplifier, Digidata 1440, and pClamp 10.0 software (Axon Instruments). Whole-cell currents were digitized at 10 kHz and low pass filtered at 2 kHz. Capacity current was reduced as much as possible using the amplifier circuitry; series resistance compensation was 60%–85%. For heat activation experiments, the perfusate was heated using a Warner TC-325B temperature controller and an SH-27B solution heater as described previously (Xu et al., 2006). All other experiments were conducted at room temperature (21–23°C). All recordings were analyzed with pCLAMP10 (Axon Instruments, Union City, CA, USA) and Origin 7.5 (OriginLab, Northampton, MA, USA).

**SUPPLEMENTAL REFERENCES**
Figure S1. Generation of TRPV3-Deficient Mice, Related to Figure 1

(A) Mouse Trpv3 gene targeting strategy. A construct was designed for conditional disruption of Trpv3 based on a recombineering method (see Experimental Procedures). In this construct, exon 13 of Trpv3 was flanked by two LoxP sites plus an FRT-flanked neomycin resistance cassette. This modified allele is referred to as Trpv3flneo. Deletion of the FRT-flanked neomycin resistance cassette via the Flp recombinase results in the floxed allele referred to as Trpv3fl. Deletion of the floxed exon 13 results in a null allele, referred to as Trpv3−/−.

(B) Keratinocyte-specific targeted deletion of Trpv3. PCR genotyping of TRPV3 K14-Cre conditional KO mice. Primers for genotyping and Cre transgene are described in Extended Experimental Procedures. PCR products for primer set B: fl allele 200 bp; WT 130 bp; Primer set A: WT 800 bp, null allele 300 bp; Cre: 200 bp.

(C) Absence of TRPV3 mRNA expression in the skin of V3 KO mice. TRPV3 mRNA was detected by RT-PCR method using two pairs of primers. Primer set D was designed to span the region to be deleted. The expression level of ribosomal protein L32, a housekeeping gene, served as a loading control.
Figure S2. TRPV3-Deficient Mice Exhibit Curly Whiskers, Wavy Hair, and Misaligned Hair Follicles, Related to Figure 2

(A) Whisker abnormality of newborn TRPV3 global KO mice. Whiskers (vibrissae) of newborn (P1) heterozygous mice were straight; the whiskers of littermate V3 KO mice were visibly kinked.

(B) TRPV3 KO, but not WT or TRPV1 KO mice, exhibited curly whiskers and wavy dorsal coats. Whiskers in an adult WT mouse (P28) were straight; the whiskers of littermate V3 KO mice were distinctively curly and hooked (upper panels). In contrast, the whiskers of TRPV1 KO mice (P42) were straight. Similarly, V3 KO, but not WT or TRPV1 KO mice, exhibited wavy dorsal coats (lower panels).

(C) "Wavy" tail hair of adult V3 KO mice.

(D) Wavy hair phenotype of V3-deficient mice is independent of genetic background and pigmentation. V3 cKO mice in a genetic background of black pigmentation (C57BL/6) exhibited curly whiskers and wavy hair (ventral view).

(E) Comparable body weight for newborn V3 KO and control mice. V3 KO (V3+/−) and control (V3+/+; V3+/−, or V3+++) mice were determined based on whisker morphology and/or PCR genotyping.

(F) Hair follicle abnormality of TRPV3-deficient mice. Misaligned and curved hair follicles (arrow) in the intact skin from V3 KO mice (P4). Littermate heterozygotes served as controls.

(G) Skin and hair follicle abnormalities of V3-deficient mice revealed by H&E staining of dorsal skin sections from WT and V3 KO mice. (Upper panels) H&E stained sections (dorsal skin) from V3 KO mice. Arrows indicated two curved hair follicles of V3 KO mice. Note the thin stratum corneum. Thin and compact stratum corneum was observed in the dorsal (upper panels) and tail (lower panels) skins of neonatal (P0) V3 KO mice.

(H) Normal hair cycle of V3-deficient mice revealed by H&E staining of dorsal skin sections from WT and V3 KO mice.
Figure S3. Decreased Activity of EGFR in the Skin of TRPV3-Deficient Mice, Related to Figure 3

(A) TRPV3-deficient mice exhibit curly whiskers, but not open eyelids at birth. Neonatal (P0) antimorphic EGFRvel mice were born with eyelids open, whereas the eyelids of WT mice or V3 cKO mice remained closed until several days after birth (upper panels). Both EGFRvel and V3 cKO mice exhibited curly whiskers (lower panels).

(B) Normal expression levels of EGF, HB-EGF, and AR in the skin of TRPV3-deficient mice. mRNA expression levels (q-PCR) of EGF, HB-EGF, and AR in the V3 KO skin tissues (P4) were not significantly (p > 0.05) different from WT skin.

(C) Normal mRNA expression level of EGFR in the skin of TRPV3-deficient mice. At P0 or P4, mRNA expression levels (q-PCR) of EGFR in the V3 KO skin tissues were not significantly (p > 0.05) different from WT skin.

(D and E) Reduced EGFR activity in the epidermis of TRPV3-deficient mice. Immunohistochemical staining of frozen skin sections from WT and V3 KO pups (P4). (D) Compared to WT mice, total EGFR staining appeared to be more intense in skin sections from V3 KO mice. (E) In contrast, P-EGFR immunostaining was more prominent in the basal layer of WT skin sections, although weak P-EGFR staining was detected in both basal and suprabasal layers of V3 KO skin sections. Red dotted lines denote dermo-epidermal borders; green dotted lines denote the surface of the epidermis.

(F) Activation of TRPV3 increases the response of EGFR to low concentrations of TGF-α in mouse keratinocytes. TGF-α-induced EGFR activity was measured with P-EGFR immunoreactivity. Wild-type and V3 KO keratinocytes were treated with TGF-α (2 ng/ml) in the presence and absence of V3 agonist cocktail (100 μM 2-APB + 250 μM Carvacrol) for 30 min.
Figure S4. EGFR Associates with TRPV3 and Mediates PLC-Dependent Sensitization of TRPV3 Channel Activity in the Keratinocytes, Related to Figure 4

(A) Sensitizing effect of EGF in mouse primary keratinocytes. The effect of EGF (100 ng/ml) on V3 Ca\textsuperscript{2+} responses in mouse keratinocytes. (B–D) TGF-\alpha sensitizes the heat-induced Ca\textsuperscript{2+} responses of mouse keratinocytes. (B) Increase of bath temperature from 22\degree C to 41\degree C failed to induce significant Ca\textsuperscript{2+} increases in V3 KO keratinocytes, treated with or without TGF-\alpha (100 ng/ml). Temperature-induced responses were measured in the presence of 300 \mu M 2-APB. (C) TGF-\alpha treatment increased the responses of WT keratinocytes to changes in temperature. (D) Average sensitizing effect of TGF-\alpha to the temperature-induced responses of WT keratinocytes.

(E) ShRNA-mediated knockdown of EGFR and PLC-\gamma 1 in primary human keratinocytes. mRNA expression levels (q-PCR) of EGFR and PLC-\gamma 1 were significantly reduced by shRNA treatment of NHEK cells.

(F) Coimmunoprecipitation of TRPV3 and EGFR from HEK293 heterologous expression. HEK293 cells were transiently transfected with the cDNAs indicated (top). Immunoprecipitates (IP) were formed with the indicated antibodies and visualized on western blot. TRPV3-GFP was IP/WB'd by a monoclonal anti-GFP; TRPV3-GFP band indicated by arrowhead. EGFR was IP/WB'd by polyclonal anti-EGFR; EGFR band indicated by arrow. Heavy chain bands (\sim 55 kDa) indicated by asterisks.

(G) Coimmunoprecipitation of TRPV3 and EGFR is not dependent on activation of TRPV3 or EGFR. HEK293 cells were transiently transfected with both V3-GFP and EGFR expression constructs. Cells were treated with V3 agonist cocktail (100 \mu M 2-APB + 250 \mu M Carvacrol) or EGF (100 ng/ml) for 30 min. Immunoprecipitates (IP) were formed with the indicated antibodies and visualized on western blot. TRPV3-GFP was IP'd by a monoclonal anti-GFP antibody. EGFR was IP/WB'd by polyclonal anti-EGFR; EGFR band indicated by arrow. Heavy chain bands (\sim 55 kDa) indicated by asterisks.

(H) TRPV3 does not coimmunoprecipitate FGFR2 in HEK293 heterologous expression system. HEK293 cells were transiently transfected with the cDNAs of V3-GFP and FGFR2-His. TRPV3-GFP was IP/WB'd by a monoclonal anti-GFP antibody; TRPV3-GFP band indicated by arrowhead. FGFR2 was IP/WB'd by an anti-His antibody; FGFR2 band indicated by arrow. Heavy chain bands (\sim 55 kDa) indicated by asterisks.

(I) TRPV3 does not coimmunoprecipitate with ADAM17. HEK293 cells stably expressing ADAM17-HA were transiently transfected with TRPV3-EGFP cDNA. Immunoprecipitates (IP) were formed with the indicated antibodies and visualized on western blot. TRPV3-EGFP was IP'd by a monoclonal anti-GFP antibody (m); TRPV3-EGFP band indicated by arrowhead. ADAM17 was IP/WB'ed by an anti-HA antibody (m); ADAM17-HA band indicated by arrows. Heavy chain bands (\sim 55 kDa) indicated by asterisks.
Figure S5. Expression of Early Epidermal Differentiation Markers Is Reduced by EGF but Increased in TRPV3-Deficient Cells, Related to Figure 5

(A) EGF reduces K1 expression in cell-matrix interaction-disrupted keratinocytes cultured in vitro. Immunofluorescence analyses of K14 and K1 were performed after keratinocytes were cultured for 48 hr in suspension.

(B) Normal cell size in the K1-positive layer of V3 KO epidermis. Cell densities of K1-positive layer in WT and V3 KO mice were similar. Cell densities were estimated from the number of DAPI-stained nuclei divided by the area.

(C and D) Cultured V3 KO primary keratinocytes express more early differentiation markers. (C) Immunoblotting analyses of various differentiation markers in lysates from cultured primary keratinocytes. Differentiation was induced by adding 0.2 mM Ca²⁺ to the culture medium (Ca²⁺ switch) for 1 or 2 days. Expression of loricrin was significantly increased in V3 KO keratinocytes. (D) Average increase of loricrin expression in V3 KO keratinocytes.
Figure S6. Differential Roles of TRPV1, TRPV3, and TRPV4 in Cornified Envelope Formation and Regulation of TGase Activity, Related to Figure 6

(A and B) Defective cornified envelope formation in TRPV3 global KO mice. Compared to WT littermate pups (P4), the cornified cell envelopes (CEs) of skins of V3 KO pups were significantly less mature.

(C) Comparable TGase activity in the frozen skin sections of neonatal (P1 and P4) WT, V1 KO (P1), and V4 KO (P4) mice. TGase activity was detected using an immunofluorescence-coupled in situ enzymatic assay. Positive staining was restricted to the granular layer of the epidermis.

(D and E) Differential roles of TRPV1, TRPV3, and TRPV4 in CE formation. (D) The effects on TGase activity in WT primary cultured keratinocytes by 40 min application of V1 agonist (1 μM Capsaicin), V3 agonist cocktail (50 μM 2-APB + 200 μM Carvacrol), and V4 agonist (10 μM 4α-PDD). (E) V3 agonist cocktail induced an ~20-fold increase of TGase activity in WT keratinocytes. Whereas V1 agonist induced no significant change of TGase activity, V4 agonist induced a modest but significant (~3 fold) increase of TGase activity.