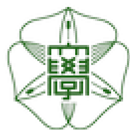

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Type XVII collagen interacts with the aPKC-PAR complex and maintains epidermal cell polarity

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Short title

COL17 interaction with aPKC/PAR3 complex

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Keywords

COL17, atypical PKC, PAR3, cell polarity, epidermal stem cell

Abstract

Type XVII collagen (COL17) is a transmembrane protein expressed in the basal epidermis. COL17 serves as a niche for epidermal stem cells, and although its reduction has been implicated in altering cell polarity and aging of the epidermis, it is unknown how COL17 affects epidermal cell polarity. Here, we uncovered COL17 as a binding partner of the aPKC-PAR complex, which is a key regulating factor of cell polarity. Immunoprecipitation-immunoblot assay and protein-protein binding assay revealed that COL17 interacts with aPKC and PAR3. COL17 deficiency or epidermis-specific aPKCλ deletion destabilized PAR3 distribution in the epidermis, while aPKCζ knockout did not. Asymmetrical cell division was pronounced in COL17-null neonatal paw epidermis. These results show that COL17 is pivotal for maintaining epidermal cell polarity. Our study highlights the previously unrecognized role of COL17 in the basal keratinocytes.

1 INTRODUCTION

The epidermis is a multilayered epithelium that serves as a barrier against external pathogens and prevents water loss from the body.[1] Epidermal stem cells (SCs) undergo symmetrical or asymmetrical cell division (SCD or ACD) to meet the need for tissue development or homeostasis.[2-7] SCD of a progenitor cell gives rise to two daughter progenitor cells. In contrast, ACD gives rise to two daughter cells with different cell fates: one progenitor cell and one differentiated cell. The fine-tuned balance between SCD and ACD maintains the tissue integrity in the epidermis. The key molecules regulating cell polarity include atypical protein kinase C (aPKC) and partitioning-defective (PAR) proteins, which form a complex.[8] The epidermis expresses two aPKC isoforms (aPKCλ and aPKCζ).[9]

Epidermal SCs reside in the basal layer of the epidermis where integrins and type XVII collagen (COL17) are present as SC niche in the basement membrane zone. COL17 is a transmembrane protein located in the hemidesmosomes and non-hemidesmosomal plasma membrane of basal keratinocytes.[10] Non-hemidesmosomal COL17 is reduced with aging.[11] Simultaneously, the aged epidermis is characterized by a skewed cell polarity into ACD, which leads to either thickening (paw) [11] or thinning (tail) [12] of the murine epidermis. COL17 overexpression rejuvenates the aged epidermis,[11,12] suggesting that COL17 maintains cell polarity in the epidermis. However, COL17's mechanism of coordinating cell polarity has not been elucidated.

2 QUESTIONS ADDRESSED

Does COL17 interact with aPKC-PAR complex?

3 EXPERIMENTAL DESIGN

3.1 Animals

C57BL/6 strain mice were obtained from Clea (Tokyo, Japan). *Col17a1*-/- (COL17 KO), K5-Cre;*aPKCλ*$^{\Delta E5/\Delta E5}$ (aPKCλ eKO), and *Prkcz*$^{-/-}$ (aPKCζ KO) mice were generated as previously described.[13-16] aPKCλ$^{\Delta E5/\Delta E5}$ and *Prkcz*$^{+/-}$ mice were used as aPKCλ eKO and aPKCz KO controls, respectively, and littermate *Col17a1*$^{+/-}$ or *Col17a1*$^{+/+}$ mice were used as COL17 KO control. The institutional review board of the Hokkaido University Graduate School of Medicine approved all the animal studies described below.

3.2 Immunoblot and Immunoprecipitation-Immunoblot (IP-IB) assay

HEK293 control cells and HEK293 cells that stably express human COL17 [17] were cultured in DMEM plus fetal bovine serum. The cultured cells were collected and lysed in 1% NP-40-containing buffer. The samples were applied on NuPAGE 4-12% Bis-Tris gel (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and transferred to a PVDF membrane. The membrane was incubated with primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase. The blots were detected using ECL-plus (GE Healthcare, Madison, Wisconsin, USA).

For immunoprecipitation, cell lysates were incubated with anti-FLAG M2 beads (Sigma-Aldrich, St Luis, Missouri, USA) at 4°C overnight. After washing off the beads with PBS containing 0.1% NP-40, bound proteins were eluted by boiling for 5 min with Laemmli's sample buffer. The PAR3 or aPKC antibody was used to perform immunoblotting on the immunoprecipitated samples or the cell lysates mixed with sample buffer, as described above.

3.3 Protein-protein binding (GST-pull down) assay

The protein-protein binding assay was performed using recombinant human COL17, as described previously.[18] Briefly, the full-length recombinant human COL17 with a FLAG-tag on the N-terminus (FLAG-COL17) was prepared and purified from HEK293 cells stably expressing COL17.[19] The original cDNA was gifted by Professor Kim B. Yancey. For the recombinant C-terminus of PAR3 (PAR3, C-term), cDNA encoding His1029 to Ser1356 (NP_062565) of human PAR3 was subcloned into the pGEX6P-1 (GE Healthcare). The predicted molecular weight of GST-tagged PAR3 C-term is around 65.4 kDa. PAR3 C-term construct was transformed into BL21 cells (GE Healthcare). The transformed cells were grown in LB broth at 25°C using the Overnight Express Autoinduction system (Novagen, Madison, Wisconsin, USA). The pellets were lysed with B-PER II (Thermo Fisher Scientific, Carlsbad, California, USA) containing lysozyme (Sigma-Aldrich). After centrifugation, the supernatant was added to glutathione Sepharose 4B (GE Healthcare) and washed with binding buffer (Tris 50 mM, NaCl 150 mM, Triton-X 1%). The Sepharose was incubated with the full-length recombinant COL17 in the binding buffer at 4°C overnight. After washing, the Sepharose was boiled

with Laemmli's sample buffer and centrifuged. The supernatant was separated using gel electrophoresis, as described above, and the gels were stained with CBB or used for subsequent immunoblotting with anti-FLAG antibody.

3.4 Histology

Specimens from murine paw skin at P1 were either fixed in formalin and embedded in paraffin after dehydrating or frozen on dry ice in an optimal cutting temperature (OCT) compound. We employed neonatal paw skin as it lacks hair follicles and is devoid of the influence of hair follicle growth on the epidermal cell polarity. The frozen sections were either stained without fixation or fixed with 4% paraformaldehyde (PFA) or cold acetone. Antigen-retrieval with pH 6.0 (citrate) or pH 9.0 (EDTA) buffer was performed on the deparaffinized sections. After blocking with 0.5% fish skin gelatin, 5% goat serum, and 4% BSA in PBS, the cells were incubated with the primary antibodies overnight at 4°C. After washing in PBS, the sections or cells were incubated with secondary antibodies conjugated with Alexa488 or Alexa568 for 1 h at room temperature (RT). The nucleus was stained with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI), and the stained immunofluorescent samples were observed using a confocal laser scanning microscope [FV1000 (Olympus, Tokyo, Japan) or LSM-710 (Zeiss, Oberkochen, Germany)].

The direction of cell division of the epidermal basal cells was determined using survivin staining as described previously.[11,20] Wheat Germ Agglutinin (Thermo fisher scientific) was used to mark plasma membrane. The angle of the division was evaluated by scaling the angle of the plane transecting two daughter cells relative to the plane of

the basement membrane. The images were analyzed using ImageJ software (NIH, Bethesda, Maryland, USA). When quantifying PAR3 and COL17 intensity in suprabasal epidermis (defined as 22.5 μm above basement membrane zone (BMZ)), COL17-stained images were analyzed using ImageJ. The data were normalized to the staining intensity in basal epidermis (7.5 um above BMZ).

3.5 Antibodies

The following antibodies were used: anti-K10 (DAKO, Santa Clara, California, USA; DE-K10), anti-PAR3 (Millipore, Billerica, Massachusetts, USA; 07-330), anti-PAR3 (Abcam, Cambridge, UK; ab64646), anti-aPKC (Santa Cruz Biotechnology, Dallas, Texas, USA; C-20), anti-COL17 (Abcam; ab186415), anti-COL17 (10C1-1, homemade, antibody to intracellular COL17), anti-COL17 (09040, homemade, antibody to extracellular COL17 [21]), anti-survivin (Cell Signaling Technology, Danvers, Massachusetts, USA; 71G4B7), and anti-FLAG (Sigma-Aldrich; F1804).

3.6 Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA). P values were determined using the Mann-Whitney or Kolmogorov-Smirnov test and indicated as $*0.01<p<0.05$ and $**0.001<p<0.01$. Graphs were prepared using GraphPad Prism or Mathematica (Wolfram Research, Champaign, Illinois, USA).

4 RESULTS

First, we investigated the epidermal distribution of PAR3, a component of the aPKC-PAR complex. PAR3 expression was observed in the cell periphery of the basal epidermis where COL17 was present (**Figure 1a**). Given that the absence of COL17 in the adult tail epidermis favored ACD [12], we hypothesized that COL17 interacts with the aPKC-PAR complex. To test this hypothesis, we performed immunoprecipitation-immunoblot (IP-IB) assays using HEK293 cells that stably express FLAG-tagged human COL17 (FLAG-COL17). The cell lysates of HEK293 cells showed several bands of PAR3, including two major bands that migrated around 180 kDa and 150 kDa (**Figure 1b**, asterisks). The IP-IB assay revealed that only the 180 kDa variant of PAR3 interacted with COL17 (**Figure 1b**). In line with COL17-PAR3 interaction, aPKC also formed a complex with COL17 (**Figure 1c**). There are 11 isoforms of human PAR3 (https://www.uniprot.org/uniprot/Q8TEW0). According to murine PAR3 isoforms,[22] the longer (180 kDa) and shorter (150 kDa) bands of human PAR3 are to correspond to isoforms 1-7 and 11, and 8-10, respectively. The shorter PAR3 isoforms lack the C-terminus of the protein but maintain the aPKC-binding domain. The data that COL17 only interacted with the 180 kDa variant of PAR3 led us to investigate the binding of COL17 to the C-terminus of PAR3. GST-tagged recombinant protein of the C-terminus of PAR3 (a.a.1029-1356 (NP_062565.2), GST-PAR3 (C-term)) and FLAG-COL17 recombinant proteins were subjected to a protein-protein binding assay (**Figure. 1d**). FLAG-COL17 (input) was detected at approximately 180 kDa with CBB staining. GST (~25 kDa) and GST-PAR3 (C-term) proteins (~60 kDa) were also detected using CBB. GST-PAR3 (C-term), but not GST, was bound to FLAG-COL17 (**Figure 1d**). These

results demonstrate that COL17 interacts with the aPKC-PAR complex and can bind to the C-terminus of PAR3.

Next, we examined the consequences of the loss of either COL17 or aPKC-PAR components in the epidermis. In contrast to the controls that mostly showed PAR3 expression in the basal epidermis, COL17 deficiency (*Col17a1*$^{-/-}$, COL17 KO) [16] showed PAR3 expression in both suprabasal and basal epidermis (**Figure 2a, 2b**). This expanded PAR3 distribution was also found in epidermis-specific deletion of *aPKCλ* (K5-Cre;*aPKCλ*$^{\Delta E5/\Delta E5}$, aPKCλ eKO mice) [13,14] (**Figure 2a, 2b**). However, suprabasal PAR3 was not pronounced in *aPKCζ*-null mice (*Prkcz*$^{-/-}$, aPKCζ KO), which shows no apparent skin phenotype [13] (**Figure 2a, 2b**). These results indicate that COL17 and aPKCλ stabilized the aPKC-PAR complex in the epidermis. In line with PAR3 labeling data, suprabasal COL17 expression was increased in aPKCλ eKO mice, while aPKCζ KO mice showed COL17 distribution only in the basal epidermis (**Figure 2c, 2d**). These PAR3+ or COL17+ suprabasal keratinocytes were differentiated cells, as indicated by keratin 10 (K10) labeling (**Supplementary Figure 1a-c**).

Finally, we examined the cell polarity of the COL17 KO epidermis at the neonatal stage, where the ACD/SCD ratio is higher than that of adult skin. Consistent with the COL17-null epidermis of adult tail skin,[12] the axis of cell division of the COL17 KO neonatal paw epidermis was inclined towards ACD (**Figure 2e, Supplementary Figure 1d**), demonstrating that COL17 participates in the maintenance of epidermal cell polarity. This skewed cell polarity can explain the transient hyperproliferation of the COL17 KO neonatal epidermis.[11]

5 DISCUSSION

Previous studies using genetic ablation of the aPKC-PAR complex have demonstrated its role in maintaining stem cells, especially in hair follicles.[14,20,23] Among those, aPKCλ eKO [14,20] shows a prematurely aged phenotype, resembling COL17 KO mice.[16,24,25] Our study has revealed that COL17 helps coordinate epidermal polarity with the aPKC-PAR complex, which accounts for the phenotypic similarity between aPKCλ eKO and COL17 KO. Suprabasal COL17 labeling in aPKCλ eKO epidermis is contrasted by COL17 reduction in the apicolateral portions of basal keratinocytes with pharmacological aPKC inhibition.[11] This discrepancy could be due to the differences in genetic ablation and transient suppression strategy.

COL17 anchors the epidermis to the dermis, and COL17 deficiency leads to the development of junctional epidermolysis bullosa (JEB).[26] JEB patients with *COL17A1* mutations (COL17-JEB) typically present with hair loss and skin atrophy, which are also a hallmark of aged skin. Disturbed epidermal polarity in COL17-null skin might account for the prematurely aged phenotype of COL17-JEB. Besides, COL17-JEB skin tends to show patches of spontaneously healed skin due to revertant mosaicism (RM).[27] Although RM skin, which restores the defective protein expression, is also observed in other diseases such as ichthyosis with confetti (IWC) and loricrin keratoderma (LK),[28] RM spots of COL17-JEB patients are much larger than those of patients with IWC or LK. Our findings, and those of others[12], explain the disadvantage of COL17-negative epidermal basal cells with a lower SCD/ACD ratio in the cell/clone competition, possibly leading to the larger RM spots in COL17-JEB skin.

6 CONCLUSIONS

In addition to playing the role of SC niche and anchoring at the dermo-epidermal junction, COL17 interacts with the aPKC-PAR complex and participates in the regulation of epidermal cell polarity.

Data availability statement

No datasets were generated or analyzed during this study.

Acknowledgments

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Conflict of Interest

The authors have no conflicts of interest to declare.

Author contributions

MW, HS, and KN designed the research. MW, HK, SIO, ST, YW, WN, TO, TH, and KN performed the experiments and assisted with the data analysis. MW and KN wrote and revised the paper. All authors have read and approved the final manuscript.

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Figure legends

Figure 1. COL17 interaction with the aPKC-PAR complex

(a) COL17 and PAR3 labeling of C57BL/6 wild-type skin (P1, paw). Images at lower magnifications are shown in the left panels. The area outlined with a dotted square is shown at higher magnifications in the right panels. The basement membrane zone (BMZ) is indicated with arrowheads. Scale bar: 20 μm. (b) The IP-IB assay showing COL17 and PAR3 interaction. 180 kDa and 150 kDa forms of PAR3 are indicated by asterisks. (c) The IP-IB assay showing COL17 and aPKC interaction. (d) The protein-protein binding (GST pull-down) assay showing COL17 binding to the C-terminus of PAR3. M=molecular weight marker.

Figure 2. COL17 involvement in epidermal cell polarity

(a) PAR3 labeling of COL17 KO, aPKCλ eKO, and aPKCζ KO skin (P1, paw). BMZ is indicated with arrowheads. (b) Quantification of the suprabasal PAR3 intensity of COL17 KO (COL17 KO, n=4; COL17 control, n=4), aPKCλ eKO (aPKCλ eKO, n=5; aPKCλ control, n=4), and aPKCζ KO (aPKCζ KO, n=6; aPKCζ control, n=4) epidermis. *$0.01<p<0.05$. Mann-Whitney test. (c) COL17 labeling of aPKCλ eKO and aPKCζ KO skin (P1, paw). Scale bar: 20 μm. BMZ is indicated with arrowheads. (d) Quantification of the suprabasal COL17 intensity of aPKCλ eKO (aPKCλ eKO, n=6; aPKCλ control, n=6) and aPKCζ KO (aPKCζ KO, n=4; aPKCζ control, n=5) epidermis. *$0.01<p<0.05$. Mann-Whitney test. (e) Radial histograms of the mitotic spindle orientation for control (n=57 from four mice) and COL17 KO (n=49 from four mice) epidermis (P1, paw). **$0.001<p<0.01$. Kolmogorov-Smirnov test.

Figure 1. COL17 interaction with the aPKC-PAR complex

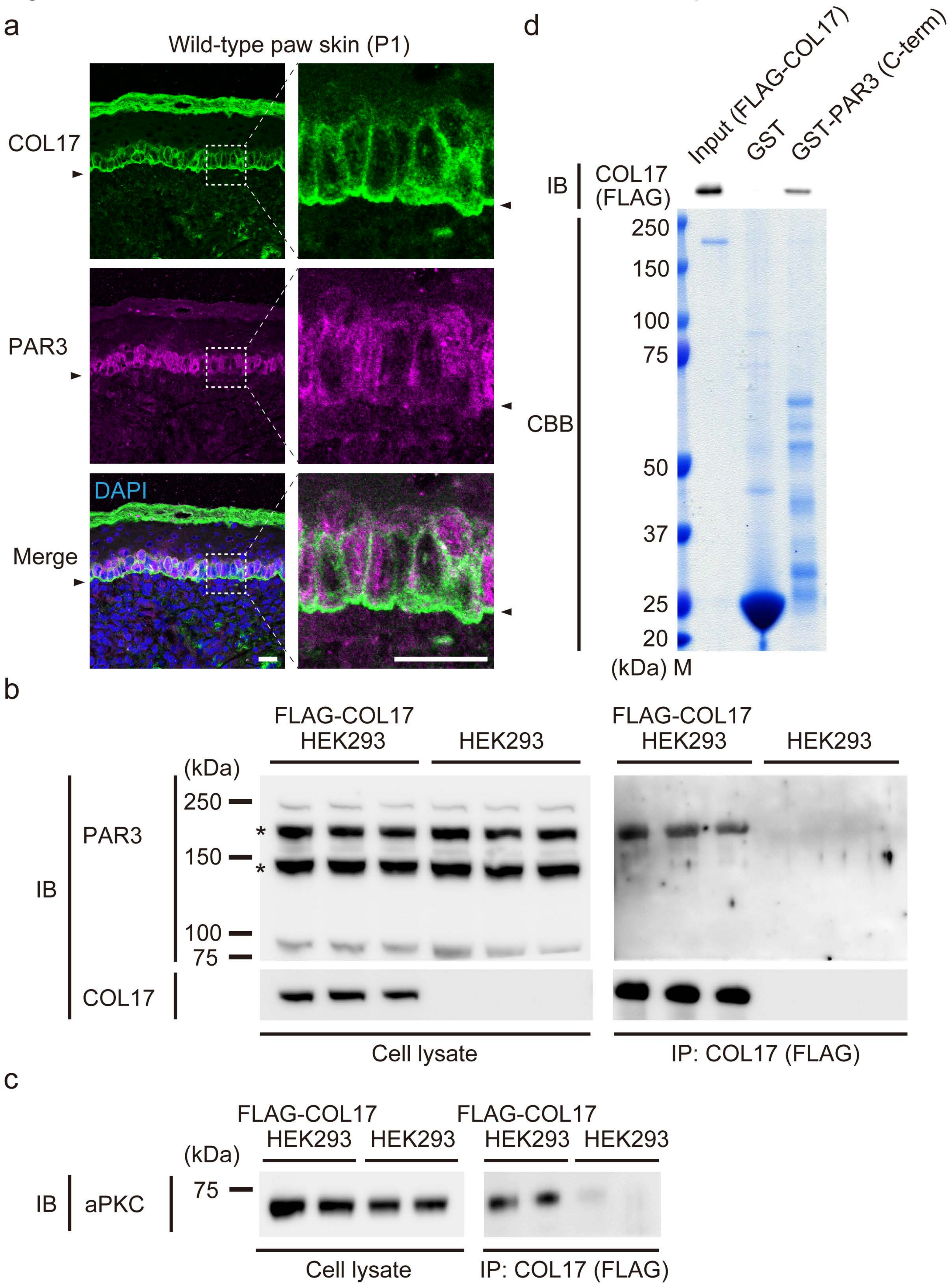
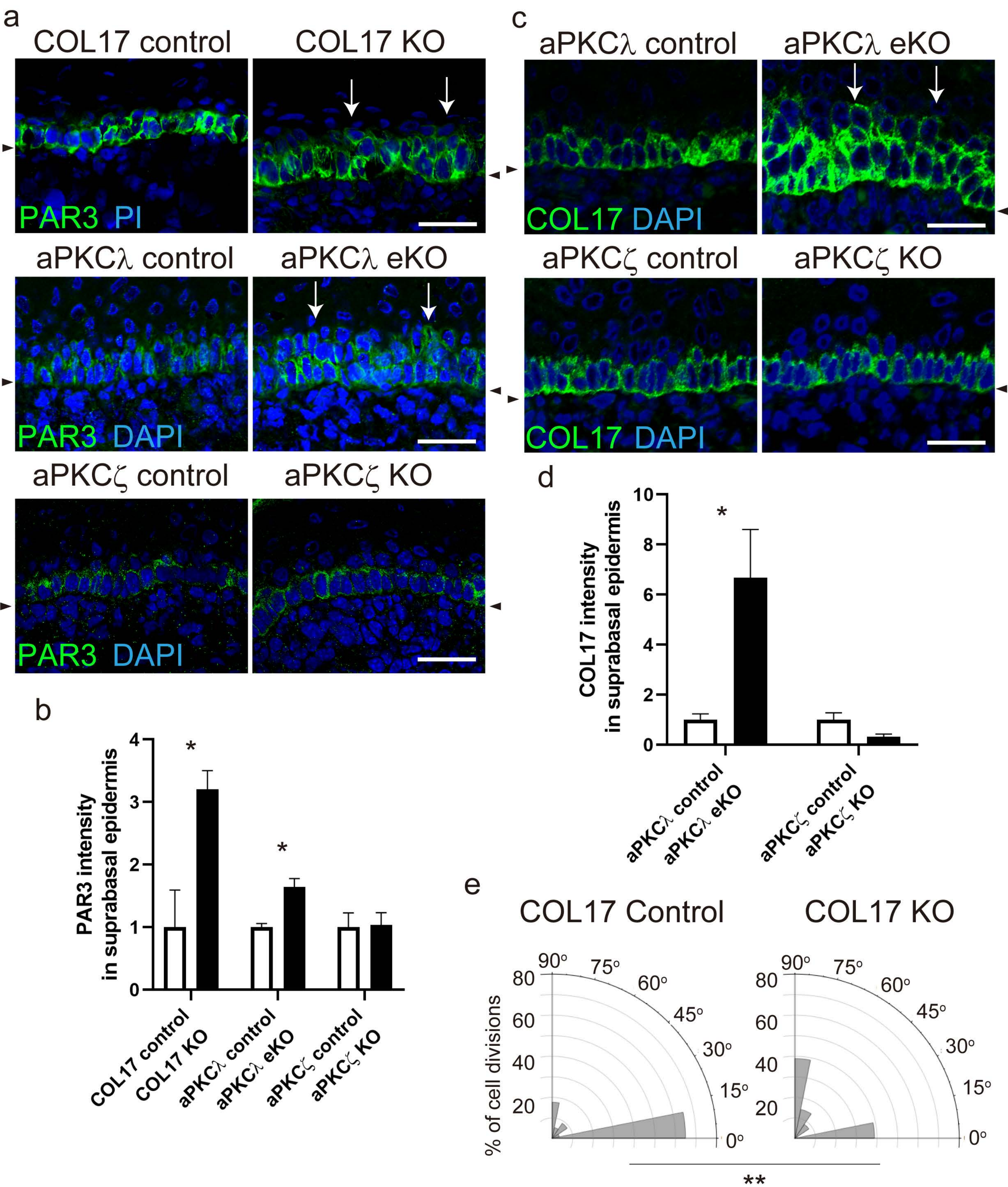
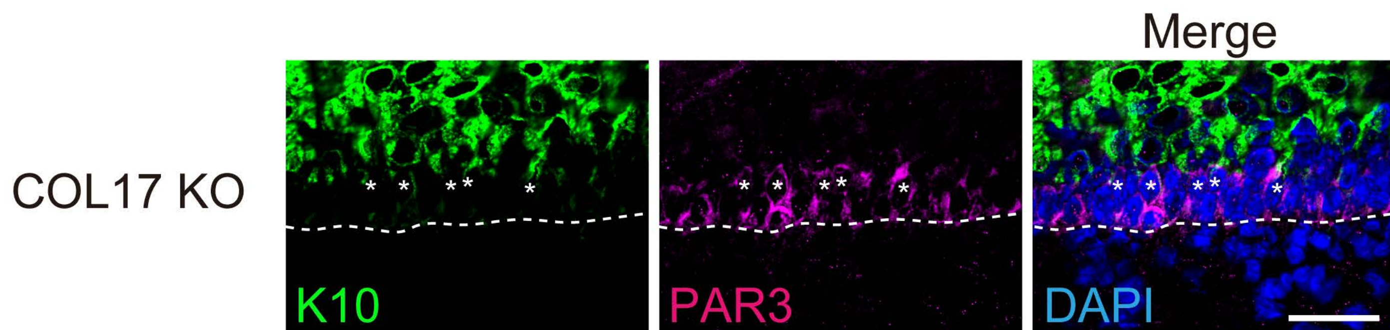
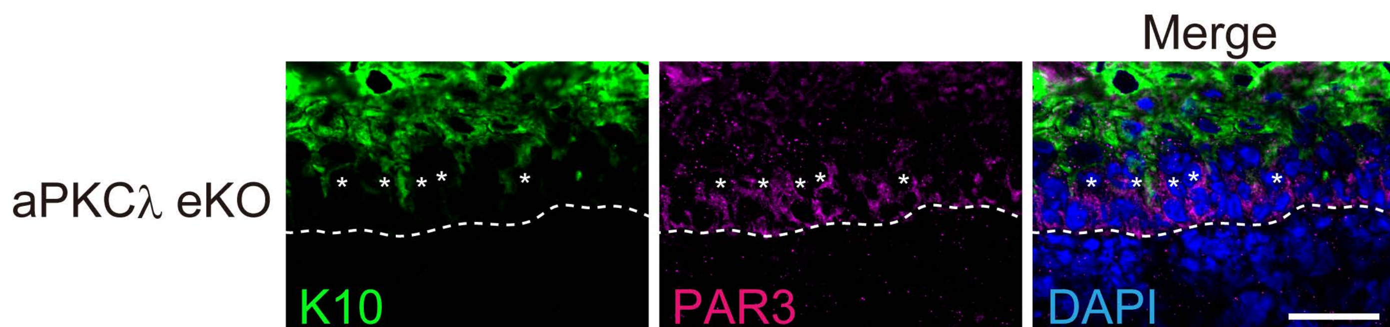


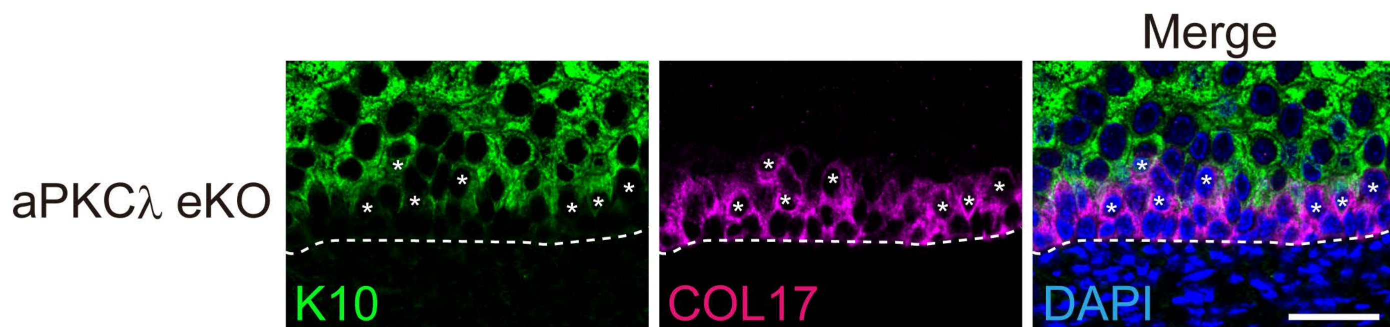
Figure 2. COL17 involvement in epidermal cell polarity

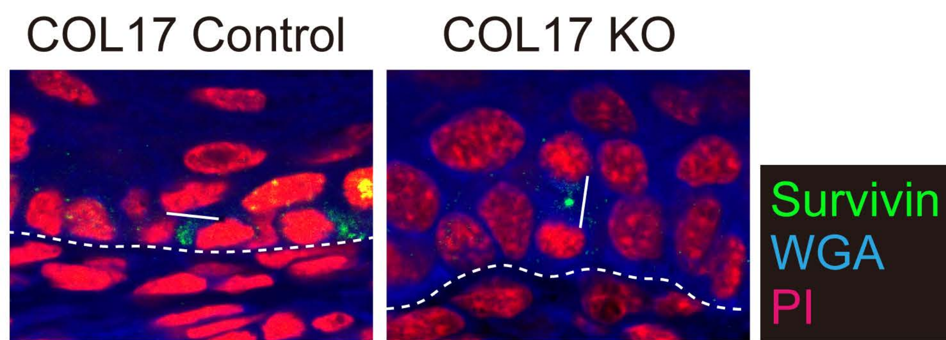


Supplementary Figure 1. COL17 and epidermal cell polarity









Supplementary Figure 1. COL17 and epidermal cell polarity

(a), (b) PAR3 and K10 labeling of COL17 KO (a) and aPKCλ eKO (b) skin (P1, paw). (c) COL17 and K10 labeling of aPKCλ eKO skin. Asterisks indicate PAR3+K10+ or COL17+K10+ suprabasal keratinocytes. BMZ is indicated with dotted lines. Scale bar: 20 μm. (d) Survivin labeling of COL17 KO and control skin (P1, paw). Wheat Germ Agglutinin (WGA) labels cell membrane. The bars indicate the direction of cell division. BMZ is indicated with dotted lines. Scale bar: 10 μm.