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Transglutaminase 3 Protects against Photodamage

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TO THE EDITOR

Transglutaminase (TG) 3 belongs to an important family of protein cross-linking enzymes that includes nine members, three of which (TG1, TG3, and TG5) are expressed in the epidermis (Candi et al., 2005; Pitolli et al., 2017; Terrinoni et al., 2012). TGs are responsible for the formation of a specialized structure: the cornified cell envelope (CE) (Amelio et al., 2012, 2013; Candi et al., 1998, 2002, 2004, 2005; Kalinin et al., 2002). TG3 expression is restricted almost exclusively to the differentiated granular layer of the epidermis and to hair follicles, and it is important in the reinforcement step of CE assembly that occurs in the granular layer (Eckert et al., 2005; Hitomi, 2005; Hitomi et al., 2001). Interestingly, ablation of the TG3 enzyme in mice (John et al., 2012) impaired hair development, with minor defects in skin formation and no gross barrier function defects, indicating that TG3 plays a crucial role in hair development. A deeper analysis of the barrier function has been performed in further studies, in which transglutaminase 3 knockout (TG3KO) mice epidermis was investigated using the T helper type 1 mouse model of contact hypersensitivity induced by the hapten fluorescein isothiocyanate. Fluorescein isothiocyanate penetration and sensitization at the skin surface was found to be more invasive in TG3KO mice, indicating that TG3 contributes to the functionally intact cutaneous barrier (Bognar et al., 2014). One important function of the skin is to protect organisms against environmental stress; in particular, protection against UVB irradiation is of major relevance for life (Kulms and Schwarz, 2000). Because TG3-depleted mice are less efficient in preventing hapten penetration and the TG3 gene has been indicated as a candidate tumor suppressor gene in human head and neck cancer (Wu et al., 2013), we sought to determine whether the UVB-filtering capacity of the TG3KO epidermis is compromised. For this reason, we irradiated newborn TG3KO mice with UVB and analyzed their phenotype. As an experimental model, we used a different TG3 knockout mouse model, obtained by a gene-trap approach (see Supplementary Materials and Methods online), presenting the same phenotype of the previous generated one (John et al., 2012; Supplementary Figures S1 and S2 online). All the experimental procedures were approved by the Animal Ethics Committee of the University of Rome "Tor Vergata." Animal care was in compliance with Italian (D.L. 116/92) and European Council (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. The back skins of 5.5-day-old neonatal wild-type (WT) and TG3KO mice were irradiated with 250 mJ cm^{-2} of UVB. Sections of TG3KO skin displayed significantly more tissue damage than WT skin, indicated by the massive enlargement and ballooning of cells in the basal, spinous, and granular layers as well as in derma (Figure 1a). To quantify cellular and tissue damage, we performed a TUNEL assay (Figure 1b). TUNEL-positive cells were detected in both WT and TG3KO skins; however, the signal was very intense in TG3KO compared with WT. It was also present deeper in the derma, indicating that in the absence of TG3 the CE is less efficient in protecting the organism from UVB-induced damage. Quantification of the epidermal TUNEL-positive cells indicated a three-fold increase of TUNEL-positive cells in TG3KO compared with WT skin (Figure 1c). Accordingly, cleaved caspase 3 is

Abbreviations: CE, cornified cell envelope; TG, transglutaminase; TG3KO, transglutaminase 3 knockout mice; WT, wild type

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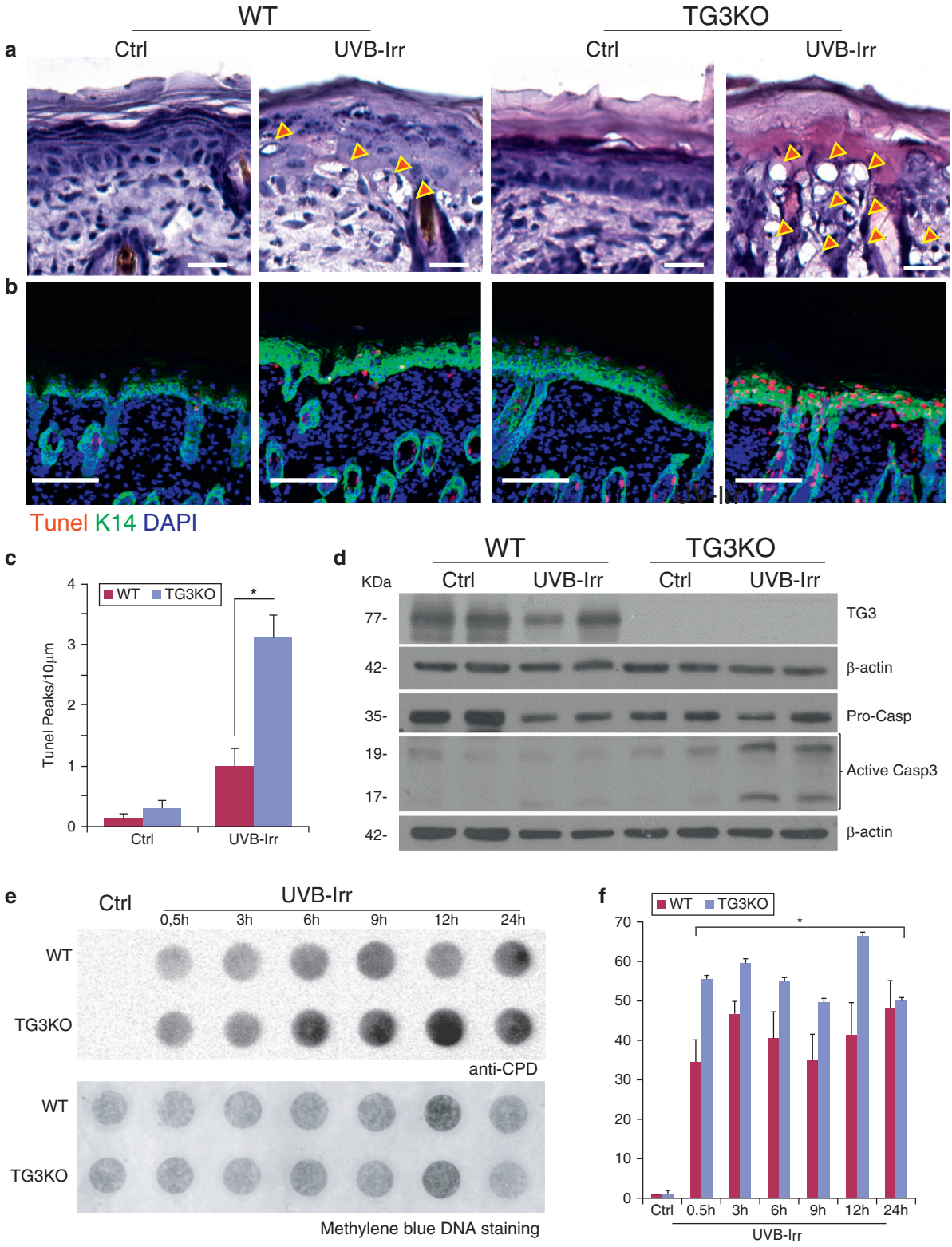

Figure 1. TG3KO mice epidermis is highly sensitive to UVB-induced apoptosis. (**a**) Hematoxylin-eosin stained epidermis of UVB-irradiated and not irradiated 5.5-day-old mice. Arrows indicate tissue damaged. Back skin of mice was analyzed 24 hours after irradiation, bars = 15 μm. (**b**) TUNEL staining (red) of the section as described in (**a**). Nuclei were stained with DAPI (blue) and the basal layer of the epidermis is shown using the anti-K14 antibody (green). (**c**) TUNEL-positive cells were quantified only in the interfollicular epidermis counting positive cells over a linear distance of 1.5 mm. The results shown are from a representative experiment (bars = 15 μm). The quantification was performed using three independent experiments, three mice each. Data are the mean ± SD of total experiments, $*P < 0.005$. (**d**) Western blot showing cleaved caspase 3. The anti-TG3 antibody was included. Beta-actin is the control. One representative experiment of the three is shown. (**e**) Southwestern dot blot using the anti-CPD antibody on genomic DNA from WT and TG3KO epidermis irradiated with UVB at the indicated time points. Methylene blue DNA staining is the loading control. One representative of the three independent experiments is shown. (**f**) Average densitometries (three experiments) are shown as means ± SD, $*P < 0.001$. Description is given in Supplementary Materials and Methods. CPD, cyclobutane pyrimidine dimer; SD, standard deviation; TG3KO, transglutaminase 3 knockout mice; WT, wild type.

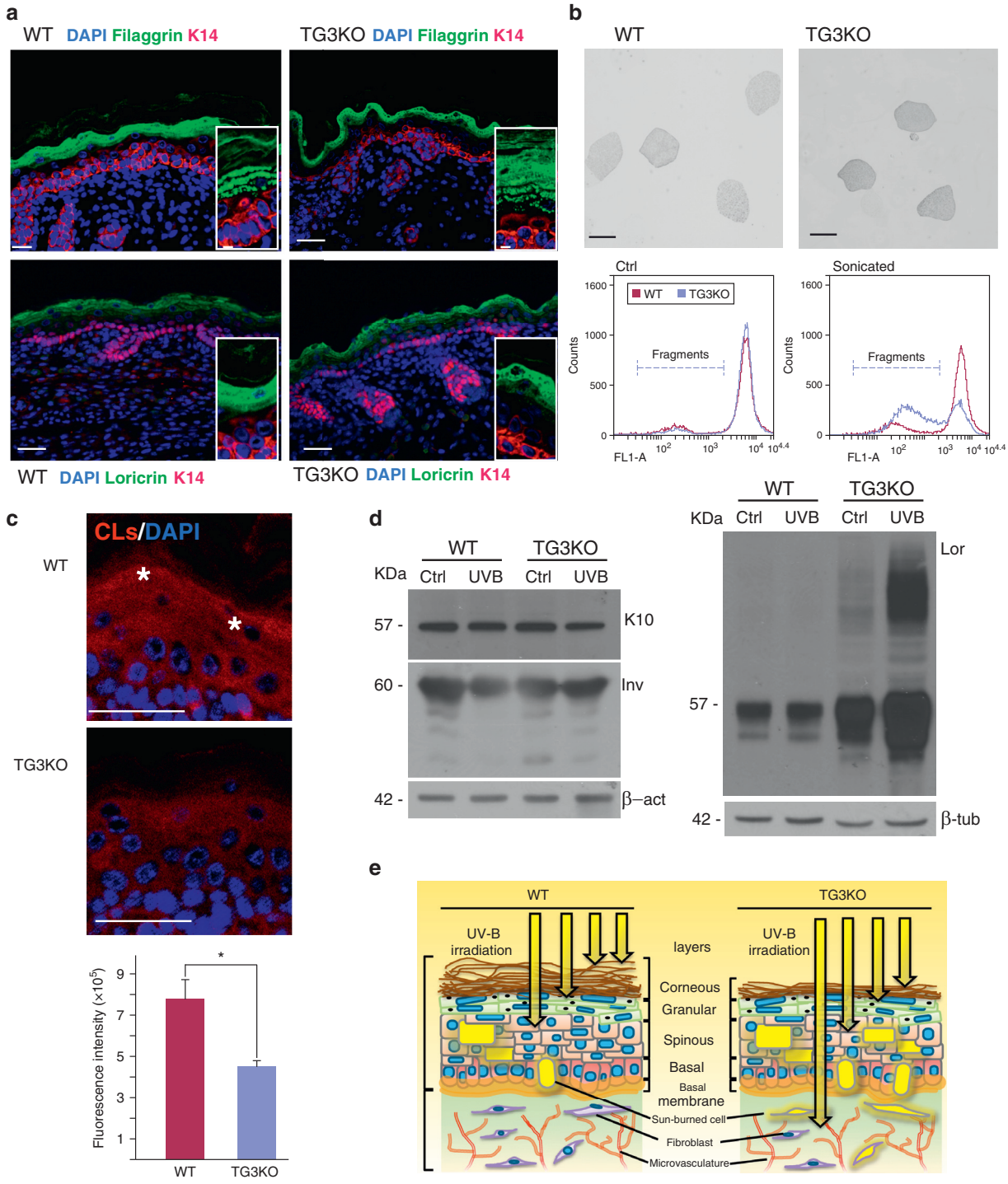

Figure 2. Protein extractability and cross-linking in WT and TG3KO epidermis. (**a**) The expression of two late differentiation markers (filaggrin and loricrin) was mildly altered. Morphometric analysis did not show a difference in thickness of different layers (not shown). The results shown are representative of three independent experiments. Sections were taken from 5.5-day-old mice. Bars = 15 μm. (**b**) CEs isolated from the epidermis of WT and TG3KO mice are apparently identical to wild-type CEs (bars = 60 μm). TG3KO CEs were more susceptible to lysis by sonication. Quantification of the TG3KO fragments in comparison with WT indicated a significant increase (41% in TG3KO and 15% in WT, $P < 0.05$). Quantification by FACS analysis is shown in the lower panels. One representative image of the three independent experiments is shown. (**c**) Evaluation of cross-links using a specific anti-Nε-(γ-glytamyl)lysine antibody in WT and TG3KO epidermis. Stars indicated the granular layer, bars = 30 μm. Quantification of the fluorescent signal is shown in the lower panel. Data are indicated as mean ± SD, $^*P < 0.05$. (**d**) Proteins from the epidermis were extracted in normal conditions or on UVB irradiation from WT and TG3KO skins. Western blot for K10, involucrin, and loricrin was performed. Actin and tubulin were included as loading controls. One representative experiment of the three is shown. (**e**) TG3 protects skin from UVB irradiation (left). In the absence of TG3 (right), the corneus layer presents altered composition and stability, which led to reduced UVB-filtering capability. UVB radiation penetrated more deeply through the skin surface of TG3KO mice, causing high levels of DNA damage. The consequent apoptosis of the damaged sun-burned cells (in yellow) and their increased number was detectable not only in the basal and suprabasal layer but also in the underlying dermis. Description is given in Supplementary Materials and Methods. CE, cornified cell envelope; SD, standard deviation; TG3KO, transglutaminase 3 knockout mice; WT, wild type.

detected by western blot in TG3KO only (Figure 1d). UVB irradiation induces direct formation of cyclobutane pyrimidine dimers, which are crucial for triggering UVB-induced apoptosis (Kulms and Schwarz, 2000). Indeed, in irradiated TG3KO back skin, cyclobutane pyrimidine dimer signal detected by southern dot blots was significantly higher than in WT, from 0.5 to 12 hours after irradiation ($P < 0.05$, Figure 1e and f). Interestingly, isolated WT and TG3KO keratinocytes irradiated with different doses of UVB (75, 150, and 250 mJ cm^{-2}) did not show any difference in cyclobutane pyrimidine dimer levels (Supplementary Figure S3 online), indicating that TG3KO keratinocytes were not more sensitive due to partial, incomplete differentiation capabilities. Taken together, these results demonstrated that TG3 is involved in the biochemical process required to protect skin from UVB-induced apoptosis. To better evaluate the effect of TG3 ablations in skin, we evaluated the expression of TG3 substrates. Confocal analysis indicated that K14 (Figure 2a), K10, and involucrin (not shown) are normally expressed when comparing WT and TG3KO epidermis. Interestingly, differences were observed in filaggrin and loricrin expression; both proteins are expressed mainly in the granular layer, where TG3 is normally present and active; nevertheless, morphometric analysis did not show a difference in thickness of different layers (not shown). Filaggrin staining was stronger than in the WT; in contrast, loricrin staining was less intense and thinner. These differences were not detected at mRNA levels, as indicated by reverse transcriptase-PCR (Supplementary Figure S4 online). Filaggrin and loricrin were also described as important components of the CEs that replace the plasma membrane in corneocytes (Candi et al., 2005). Therefore, CEs were isolated from TG3KO and WT epidermis: the CEs were undistinguishable in shape and size (Figure 2b, upper panels). To assay for their stability, they were subjected to mild serial sonication. After sonication, the TG3KO CEs were more fragmented in comparison with WT (41% with respect to 15%, respectively, $P < 0.05$), indicating that they are more fragile due to partial cross-linking. Furthermore, using the specific anti-Nε-(γ-glytamyl)lysine antibody, we detected a less intense staining in TG3KO skin (Figure 2c). The quantification of the fluorescent signals (Nis Elements AR 3.0; Nikon Instruments Spa, Florence, Italy) normalizing the intensity of fluorescence versus the number of nuclei revealed a significant decrease ($P < 0.01$) in the TG3KO epidermis (fluorescence region of interest was 78×10^5 in WT with respect to 45×10^5 in TG3KO). To confirm that the CE precursors were less cross-linked, we extracted them under reducing conditions from an equal amount of epidermis. Loricrin, involucrin, and K10 were analyzed by western blot. Loricrin showed an increased extractability (Figure 2d and e, compare lanes 1 and 3) both as a monomer and oligomer, indicating that the missing TG3 enzymatic activity affects both the initiation and reinforcement steps of CE assembly. Notably, loricrin extractability increased further on UVB irradiation, possibly due to the higher tissue damage in the TG3KO epidermis compared with WT epidermis. Interestingly, K10 and involucrin, early CE precursor proteins, were not affected by the lack of TG3 activity (Figure 2d), further confirming that these two proteins are not the main TG3 substrates in vivo. In conclusion, we showed that in the absence of TG3, CE is biochemically different, leading to reduced UVB-filtering capacity (Figure 2e). As a result of the reduced UVB-filtering capacity shown in this study and the increased penetration and sensitization at the skin surface shown by Bognar et al. (2014), we believe that TG3 inactivation could cause mild but chronic defects in the barrier function, resulting in an increased propensity for cancer initiation and development as well as for inflammatory diseases. Further investigations are necessary to clarify the role of TG3 as prognostic biomarker for skin diseases.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.02.982.

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Ablation of *Ctip2/Bcl11b* in Adult Epidermis Enhances TPA/UV-Induced Proliferation and Increases Susceptibility to DMBA/TPA-Induced Epidermal Carcinogenesis

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TO THE EDITOR

Transcriptional factor CTIP2-interacting protein 2, also known as BCL11B, is a C_2H_2 zinc finger protein that is highly expressed in mouse epidermis and hair follicles from the embryonic to adult stages (Avram et al., 2000; Ganguli-Indra et al., 2009a). Loss of CTIP2 in the developing epidermis induces disruption of keratinocyte differentiation, depletion of hair follicle niche, prolonged inflammation, and dysregulation of cutaneous wound repair (Bhattacharya et al., 2015; Golonzhka et al., 2009; Liang et al., 2012; Wang et al., 2012). CTIP2 also acts as a tumor suppressor in association with human hematological malignancies, whereas its expression in human head and neck squamous cell carcinoma has been linked to poorly differentiated tumor status (Ganguli-Indra et al., 2009b; Gutierrez et al., 2011). Here, we determined whether deletion of *Ctip2* in adult keratinocytes affects susceptibility to tumor initiators and promoters to clarify the role of this transcription factors in carcinogenesis. The Oregon State University Institutional Animal Care and Use Committee approved all the animal studies.

To determine the function of CTIP2 in stress response and in skin carcinogenesis, we used a tamoxifen inducible Cre-LoxP strategy for selective ablation of *Ctip2* in the epidermis of adult mice. Two weeks after tamoxifen administration, excision of *Ctip2* was verified by PCR of genomic DNA isolated from $Ctip2^{L2/L2}$ (control) and $Ctip2^{ep-/-i}$ mice (lacking *Ctip2* in the adult epidermis) (Figure 1a). By immunofluorescence and immunoblotting analysis, CTIP2 was undetectable in the epidermis of $Ctip2^{ep-/-i}$ mice (Figure 1b and 1c). Abrogation of *Ctip2* in the adult epidermis led to increased transepidermal water loss, suggesting an impaired barrier homeostasis (Figure 1d). Approximately 16 weeks after tamoxifen administration, 30% of $Ctip2^{ep-/-i}$ mice developed spontaneous skin lesions predominantly on the dorsal skin, the neck region, and on the face (Figure 1e). $Ctip2^{ep-/-i}$ mice also showed signs of epidermal hyperplasia (Figure 1f and 1g) and an increase in epidermal proliferation (Figure 1h and 1i). Elevated levels of the early differentiation marker keratin 10 and late differentiation marker loricrin identified in $Ctip2^{ep-/-i}$ dorsal skin illustrate the irregularities in keratinocyte differentiation (Figure 1j and 1k). Altogether, these results indicate an important role for CTIP2 in maintaining epidermal homeostasis and skin barrier permeability in the adult skin.

Epidermal barrier plays an important role in regulation of skin recovery after any environmental insult (Stalder et al., 2014). To investigate whether barrier perturbation in $Ctip2^{ep-/-i}$ skin has led to skin sensitivity to acute insults, control and $Ctip2^{ep-/-i}$ mice (n = 6/group) were treated with single doses of 12-O-tetradecanoyl phorbol-13-acetate, UVB, or a combination of both. We observed an increase in $BrdU^+$ proliferating basal keratinocytes in the dorsal skin of $Ctip2^{ep-/-i}$ mice with all three TPA/UVB perturbations (Figure 1l and m). It has been reported previously that single low-dose TPA treatment has little effect on apoptosis in the skin of wild-type mice (Cataisson et al., 2003), whereas UVB exposure causes DNA damage, which triggers formation of apoptotic keratinocytes (Schwarz et al., 1995). Therefore, TUNEL assay was performed in $Ctip2^{L2/L2}$ and $Ctip2^{ep-/-i}$ mice skin to detect apoptotic keratinocytes after a single dose of UVB or after a combination of TPA and UVB treatment. A significant decrease in apoptosis was observed in the epidermis of $Ctip2^{ep-/-i}$ skin for both treatments (Figure 1n and o). In addition, we also performed a single treatment with 9,10-dimethyl-1,2-benzanthracene (DMBA) to investigate the role of CTIP2 in the promotion of DMBA-initiated cell death. After DMBA treatment, no significant difference in number of proliferating $Ki67^+$ cells or

Abbreviations: DMBA, 9,10-dimethyl-1,2-benzanthracene; TPA, 12-O-tetradecanoyl phorbol-13-acetate

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