A role for keratins in supporting mitochondrial organization and function in skin keratinocytes

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ABSTRACT Mitochondria fulfill essential roles in ATP production, metabolic regulation, calcium signaling, generation of reactive oxygen species (ROS), and additional determinants of cellular health. Recent studies have highlighted a role for mitochondria during cell differentiation, including in skin epidermis. The observation of oxidative stress in keratinocytes from Krt16 null mouse skin, a model for pachyonychia congenita (PC)–associated palmoplantar keratoderma, prompted us to examine the role of Keratin (K) 16 protein and its partner K6 in regulating the structure and function of mitochondria. Electron microscopy revealed major anomalies in mitochondrial ultrastructure in late stage, E18.5, Krt6a/Krt6b null embryonic mouse skin. Follow-up studies utilizing biochemical, metabolic, and live imaging readouts showed that, relative to controls, skin keratinocytes null for Krt6a/Krt6b or Krt16 exhibit elevated ROS, reduced mitochondrial respiration, intracellular distribution differences, and altered movement of mitochondria within the cell. These findings highlight a novel role for K6 and K16 in regulating mitochondrial morphology, dynamics, and function and shed new light on the causes of oxidative stress observed in PC and related keratin-based skin disorders.

INTRODUCTION Keratinocytes, the primary cell type that constitutes the skin epidermis, must be able to proliferate, move unidirectionally, assemble, and remodel strong adhesive sites as they differentiate and resist mechanical stress. A major family of proteins that impacts all of these functions are the keratin (K) intermediate filaments (IFs), which are regulated in a tissue-type, differentiation-dependent and context-specific manner (Fuchs and Green, 1980; Fuchs, 1995). The human genome features 54 distinct and conserved keratin genes that are partitioned into type I and type II subtypes of IF sequences (Schweizer et al., 2006). Type I and type II keratin genes are expressed in a pairwise manner and their protein products interact obligatorily to form heteropolymeric 10-nm-wide IFs (Fuchs, 1995). Mutations in keratin genes have been linked to a variety of skin disorders exhibiting a broad range of tissue level and cellular phenotypes (McCowan, 1998). One such keratin pair, the type II K6 and type I K16, is primarily expressed in ectoderm-derived epithelial appendages in adult skin under normal conditions (Fuchs, 1995; McCowan, 1998; Bernot et al., 2002). The mature interfollicular epidermis, interestingly, does not express either K6 or K16 unless it experiences injury, UV exposure, or other stresses (McCowan, 1998;
Rotty and Coulombe, 2012; Lessard et al., 2013). Mutations in either KRT6A, KRT6B, KRT6C, or KRT16 (usually, dominantly acting missense alleles) can cause PC (McLean et al., 1995; Leachman et al., 2005; Lehmann et al., 2019). The most clinically significant aspect of PC is palmoplantar keratoderma (PPK), which is acutely painful and presents as thick calluses developing in the palms and especially soles as a result of oxidative stress and misregulated innate immunity and epidermal homeostasis (Lessard et al., 2013; Kerns et al., 2016).

In addition to forming and functioning as filamentous networks, keratins are known to regulate signaling pathways through protein–protein interactions and modulate organelle processes (Nishizawa et al., 2005). For instance, there is an emerging connection between mitochondrial biology and IFs that has the potential to alter the cellular levels of reactive oxygen species (ROS) and metabolic flux (Nishizawa et al., 2005; Tao et al., 2009; Silvander et al., 2017). As mentioned above, our laboratory has previously reported that normal redox balance requires functional K16 via Nrf2 activation and glutathione synthesis. In the case of PC-related PPK, which is acutely painful and presents as thick calluses developing in the palms and especially soles as a result of oxidative stress and misregulated innate immunity and epidermal homeostasis (Lessard et al., 2013; Kerns et al., 2016).

RESULTS AND DISCUSSION
As previously reported, mice homozygous for a Krt6a/Krt6b double-null allele are born with the expected frequency but rapidly develop oral lesions that hamper their postnatal growth and results in their untimely death within a week postbirth (Wong et al., 2000). We used transmission electron microscopy (TEM) to compare the ultrastructural features of epidermis and mitochondria, in particular in late stage (E18.5) embryonic Krt6a/Krt6b null and WT back skin. Low magnification surveys of epoxy-embedded tissue sections show that the epidermis of Krt6a/Krt6b homozygous null mice is intact and shows a normal morphology (Supplemental Figure 1, A and B). In contrast, examinations at higher magnifications reveal that, relative to control, mitochondria in epidermal keratinocytes lacking both keratins 6a and 6b proteins (K6a/K6b) exhibit several anomalies with respect to shape and cristae organization (Figure 1, A–D). These findings suggest that disruption of mitochondrial-keratin interactions, which in turn leads to impaired cellular and redox homeostasis, is related to oxidative stress that precedes PPK lesions.
A and C). To quantify these observations, we categorized mitochondria as intact (cristae running all the way through a single mitochondrion), partially abnormal (some cristae observed but not fully intact), and severely abnormal (no cristae; note: representative images are shown in Supplemental Figure 1, C–E). This confirmed that significantly more mitochondria show a severely abnormal ultrastructure compared with control tissue (Figure 1E). While these anomalies could be the result of inefficient fission and/or fusion leading to disorganized cristae (Figure 1, B and D; Chen and Chan, 2009; Buck et al., 2016; Burman et al., 2017), we did not observe any major expression differences in relevant biomarkers (Mfn1, Mfn2, Opa1, and Drp1) with the exception of a small increase in Mfn2 mRNA in Krt6a/Krt6b null keratinocytes compared with WT cells cultured in primary conditions (Figure 1F). We observed a modest trend toward lower mRNA levels for the mitochondrial markers cytochrome c oxidase subunit IV, succinate dehydrogenase, translocase of the inner membrane (Tim23), complex III subunit 2, and pyruvate dehydrogenase (PDH) in Krt6a/Krt6b null keratinocytes relative to WT, with Tim23 and PDH reaching statistical significance (Figure 1G). However, there were no differences observed at the protein level with these markers (unpublished data), suggesting that fission and fusion processes do not directly contribute to mitochondrial defects. Finally, we did not observe any change in mitophagy markers responsible for mitochondrial turnover (unpublished data), which is consistent with data gathered from keratinocytes of PC patients with K6a mutations (Lehmann et al., 2019; see Results and Discussion). Of note, we previously reported on the occurrence of similar ultrastructural defects in mitochondria from Krt5 null E18.5 mouse epidermis (Alvarado and Coulombe, 2014). The loss of another IF protein, desmin, was also found to induce mitochondrial swelling and matrix disruption in cardiac muscle. Importantly, the mitochondria in desmin-null cardiac muscle display a very similar phenotype to that seen in Krt6a/Krt6b null skin (see Figure 1B and Milner et al., 2000). This suggests that, as is the case for other IFs, the K6/K16 filament network likely plays a direct role in mitochondrial architecture.

Keratin proteins physically interact with several cellular organelles (Tao et al., 2009; Lee et al., 2012; Feng and Coulombe, 2015; Silvander et al., 2017), including mitochondria. We next compared the subcellular distribution of K16, for which we have a high titer (monospecific) antibody (Bernot et al., 2002; Lessard and Coulombe, 2012) to that of PDH using high-resolution confocal microscopy (Airyscan technology) in WT newborn mouse skin keratinocytes in primary culture. The signal for K16 closely aligns with that of PDH, which otherwise is polarized toward the perinuclear space (Figure 2, A–C). When using conventional confocal microscopy, we find that the absolute spatial distribution of the mitochondria (as measured by PDH) is altered when either K6 or K16 is missing. The PDH signal is redistributed to the entire cytoplasm in Krt6a/Krt6b null keratinocytes in primary culture, with a higher PDH signal at the cell periphery compared with the perinuclear localization in WT keratinocyte controls (Figure 2, D and E; quantitation in Figure 2F). This effect was also observed in spontaneously immortalized keratinocytes (SIMEKs) lacking Krt16 compared with WT SIMEKs (unpublished data). These findings suggest that keratin IFs containing K6 and/or K16 may directly or indirectly impact the organization of mitochondria in epidermal keratinocytes. Loss of K6 or K16 leads to a differential dispersion of mitochondria throughout the cell, with the potential to disrupt the physiological signaling capacity of this organelle.

FIGURE 2: Indirect immunofluorescence for K16 and pyruvate dehydrogenase (PDH) in keratinocytes in primary culture. (A–C) Keratinocytes were isolated from P1 WT and Krt16 null mouse strain and cultured in primary conditions. Images were acquired using an LSM 800 Airyscan mode. Bars equal 20 μm. (D, E) Z-stack images of PDH staining in newborn P1 WT and Krt6a/Krt6b null skin keratinocytes in primary culture. Dashed lines depict the cell periphery and nucleus. (F) Graphs depict PDH signal intensity distribution in the cytoplasm, as the intensity fraction of the total intensity with respect to the distance from the nucleus or the distance to the nucleus normalized to the spacing between the nucleus and the cell boundary. The curves are the average intensity over 18 Kr6a/Krt6b null and 34 WT cells. Images were acquired using Z-stack and maximal projection with an LSM 800 confocal mode. Bars equal 20 μm.
Cells rely on the electron transport chain (ETC) of the mitochondria to produce ATP through the reduction and oxidation of ETC protein complexes from electrons donated from the tricarboxylic acid (TCA) cycle (Buck et al., 2016). Efficient operation of this complex process requires that cristae be densely packed inside mitochondria to keep the ETC complexes close together for electron transport (Cogliati et al., 2016; Leveille et al., 2017). If such a continuity is not maintained, electrons can move back into the mitochondrial matrix, reducing overall electron movement across the ETC (Bornhovd et al., 2006; Cogliati et al., 2016). The presence of abnormal mitochondrial ultrastructure led us to hypothesize that the absence of either K6a/K6b or K16 protein leads to imbalances in ROS levels. To test this, we measured the total ROS in the absence of either K6a/K6b or K16 protein.

FIGURE 3: Seahorse analysis of mitochondrial function. Skin keratinocytes (4 × 10⁴) from P1 WT, Krt6a/Krt6b null, and Krt16 null mice were seeded in primary culture. Analyses (XFp96 instrument) were done using the Agilent protocol for mitochondrial stress test assay (cf. Materials and Methods). Samples were normalized by cell density and mitochondrial basal respiration, maximal respiration, and proton leak were determined. (A) Mitochondrial respiration was assessed under baseline conditions to measure basal respiration. Oligomycin treatment (ATP synthase inhibitor) was applied to measure proton leak and mitochondrial-linked respiration, FCCP (fluoro-carbonyl cyanide-4-phenylhydrazone) treatment (proton uncoupler) to measure maximal respiration, and rotenone/antimycin A (complex I and III inhibitor) to measure spare respiratory capacity. The Krt6a/Krt6b null and Krt16 null cells each exhibited lower levels of (B) basal respiration, (C) proton leak, and (D) maximal respiration with no change in ATP production or spare respiratory capacity (unpublished data). n = 10–16 from three independent experiments. Student’s t test was used with significance set at p < 0.05. (E) Skin keratinocytes were isolated from P1 WT and Krt6a/Krt6b null littermates (left) or WT and Krt16 null littermates (right) and cultured in primary conditions. Cells (7 × 10⁴) were seeded in a 96-well plate and labeled with 500 nM TMRE (tetramethylrhodamine ethyl ester perchlorate) for 30 min, and fluorescence was then measured and normalized to the WT controls. Cells were normalized by measuring total DNA using CyQuant dye. n = 5–20 from three independent experiments performed. Student’s t test was used with significance set at p < 0.05.

ROS have been implicated in many forms of cellular dysfunction and can directly damage mitochondria (Guo et al., 2013; Kalogeris et al., 2014; Zorov et al., 2014). To explore how increased ROS affects mitochondrial function in cells lacking either K6a/K6b or K16, we measured mitochondrial respiration using the Seahorse Mito Stress Test Kit (Figure 3A). Keratinocytes isolated from both Krt6a/Krt6b null and Krt16 null mice (P1) showed a significant reduction in basal and maximal respiration compared with WT cells (Figure 3, B and D). Null keratinocytes also displayed a reduced proton leak (Figure 3C), suggesting that there may be an overall reduction in uncoupling protein activity or increased permeability of the inner mitochondrial membrane, reducing overall electron movement across the ETC (Bornhovd et al., 2006; Cogliati et al., 2016). Interestingly, maximal respiration for all cell populations did not exceed 100% of ETC capacity (Figure 3A).

In conclusion, our findings support the hypothesis that abnormal mitochondrial ultrastructure leads to increased ROS levels in the absence of K6a/K6b and K16 proteins.
Keratin proteins play a multifaceted role in keratinocyte homeostasis, and mutations in keratin genes lead to a diverse array of phenotypic outcomes. The K6/K16 keratin pairing, which is robustly wound-inducible, supports and promotes a number of cellular functions including structural integrity (Wong et al., 2000; Wong and Coulombe, 2003; Lessard and Coulombe, 2012), cell migration (Rotty and Coulombe, 2012; Wang et al., 2018), keratinocyte differentiation (Zieman et al., 2019b), regulation of innate immunity (Lessard et al., 2013), and redox homeostasis (Kerns et al., 2016, 2018). Disruption of many of these cellular roles is poised to play a role in the pathophysiology of PC, in particular, oral and palmo-plantar keratoderma lesions (Zieman and Coulombe, 2019a). Mitochondria represent the main cellular protagonist for regulation of ROS, which it achieves mainly via promoting the integrity and efficiency of the ETC. Silvander et al. (Silvander et al., 2017) reported that loss of keratin 8 reduced mitochondrial membrane potential.

Mitochondria are highly dynamic organelles that constantly undergo fission and fusion to achieve an organization that regulates and optimizes several cellular functions in response to exogenous signals (Chen and Chan, 2009, 2017). Respiration and ROS production are regulated by and can impact mitochondrial dynamics (Ott et al., 2007; Anso et al., 2013; Guo et al., 2013; Hamanaka and Soukupova et al., 2017; Logan et al., 2018). This reality suggests that newborn mouse skin keratinocytes in primary cell culture are already maximally respiring even at a resting state. This finding was further supported by reduced membrane potential as measured by tetramethylrhodamine ethyl ester perchlorate (TMRE) fluorescence in both Krt6a/Krt6b null and Krt16 null keratinocytes (Figure 3E). Interestingly, other IFs, namely vimentin, have also been shown to maintain membrane potential, and loss of this interaction alters mitochondrial positioning and physiological activity (Chernoivanenko et al., 2015).

To assess whether and how K6/K16 alter mitochondrial dynamics, we labeled skin keratinocytes in culture (from P1 Krt6a/Krt6b and Krt16 WT and null littermates, respectively) with MitoTracker Red and performed live cell imaging in real time to monitor mitochondrial movement under normal cellular conditions. In keratinocytes null for either keratin, mitochondria showed no alteration in size or circularity but exhibited an increased speed of movement (Samudio et al., 2005; unpublished data and Figure 4). Representative findings can be visualized in Supplemental Figure 3 and Supplemental movies 1–4. A quantitative assessment of the movement of individual mitochondrial profiles, as an overall average (Figure 4, A and B) and as a function of mitochondrial size (Figure 4, C and D), is reported for Krt6a/Krt6b null and Krt16 null newborn keratinocytes relative to their respective WT controls. The increased rate of movement (speed) was unrelated to the size of mitochondrial profiles (represented by the x-axis), although smaller mitochondrial shapes showed a greater rate of movement in the two null genotypes compared with controls (Figure 4, C and D). Further, our data conveys a heterogeneity of mitochondrial populations even within the same cell type and individual cells (Supplemental Figure 4). Mitochondria in Krt6a/Krt6b null and Krt16 null keratinocytes also appeared to move in a disorganized or random manner, but we were unable to reliably quantify this behavior. Overall, these data demonstrate a difference in the movement of mitochondrial profiles in keratinocytes lacking K6a/K6b or K16. While the live imaging performed does not directly measure mitochondrial fission and fusion rates, it extends the findings of disrupted mitochondrial ultrastructure (Figure 1) and reduced respiration (Figure 3), pointing to a state of mitochondrial instability.

Keratin proteins play a multifaceted role in keratinocyte homeostasis, and mutations in keratin genes lead to a diverse array of phenotypic outcomes. The K6/K16 keratin pairing, which is robustly wound-inducible, supports and promotes a number of cellular functions including structural integrity (Wong et al., 2000; Wong and Coulombe, 2003; Lessard and Coulombe, 2012), cell migration (Rotty and Coulombe, 2012; Wang et al., 2018), keratinocyte differentiation (Zieman et al., 2019b), regulation of innate immunity (Lessard et al., 2013), and redox homeostasis (Kerns et al., 2016, 2018). Disruption of many of these cellular roles is poised to play a role in the pathophysiology of PC, in particular, oral and palmo-plantar keratoderma lesions (Zieman and Coulombe, 2019a). Mitochondria represent the main cellular protagonist for regulation of ROS, which it achieves mainly via promoting the integrity and efficiency of the ETC. Silvander et al. (Silvander et al., 2017) reported that loss of keratin 8 reduced mitochondrial membrane potential.
and ATP production in pancreatic β-cells, a novel role that involves an interaction with trichoplein (TCHP). Nishizawa et al. (Nishizawa et al., 2005) provided evidence that K6a/K6b and especially K16 physically interact with TCHP. Here show that K6a/K6b and K16 regulate the organization and function of mitochondria in skin keratinocytes, a novel finding that has potential significance not only for the keratinocyte differentiation (Hamanaka and Chandel, 2013) and epithelial homeostasis (Hamanaka et al., 2013; Rath et al., 2018; Lehmann et al., 2019), but also for the pathophysiology of keratin mutation-based skin epithelial disorders (McLean et al., 1995; McGowan, 1998; Wong and Coulombe, 2003; Kerns et al., 2016; Lehmann et al., 2019). We could not produce reliable evidence for a physical interaction between TCHP and K16 in skin keratinocytes, unfortunately, owing in part to the unavailability of a good antibody to TCHP (unpublished data); addressing this issue awaits the availability of suitable reagents. In addition, the occurrence of markedly reduced levels of K16 protein in Krt6a/Krt6b double-null mouse skin keratinocytes, in both tissue and cell culture settings [7, 16, 41], may help explain the more severe mitochondrial phenotype they exhibit relative to Krt16 null keratinocytes.

ROS levels are significantly higher in keratinocytes null for Krt6a/ Krt6b or Krt16 relative to WT controls. These findings correlate with reductions in both basal and maximal mitochondrial respiration in the Krt6a/Krt6b and Krt16 ablated states, as measured by Seahorse analysis. The latter also indicated that there is reduced proton leak in the two keratin null settings, which is further supported by the reduced membrane potential prevailing in mitochondria. These findings are consistent with the damaged and disorganized cristae observed in mitochondria of Krt6a/Krt6b null epithelial back skin. In addition, mitochondria tend to be localized to the perinuclear cytoplasm in wild-type keratinocytes but show a broader dispersion in keratinocytes lacking K6a/K6b or K16. Of note, keratin filaments themselves readily concentrate to the perinuclear region, particularly in suprabasal keratinocytes of surface epithelia (Lee et al., 2012; Feng and Coulombe, 2015). Accordingly, alterations in keratin filament properties resulting from the loss of either K6a/K6b or K16 may prevent the mitochondria from concentrating near the nucleus. The subcellular localization of mitochondria has a major impact on cell signaling and function, including migration, calcium signaling, and gene expression (Mironov and Symonchuk, 2006; Boldogh and Pon, 2007; Desai et al., 2013; da Silva et al., 2014; Yu et al., 2017).

Alteration of this steady state is an exciting area for future studies to determine the associated effects on keratinocyte function. ROS production and mitochondrial function are also closely linked to the latter’s dynamics and network formation, and indeed differences were measured in mitochondria motility in Krt6a/Krt6b null and Krt16 null keratinocytes relative to WT. These data suggest that keratins help stabilize mitochondria spatially and structurally in keratinocytes, and that disrupting these processes has significant consequences for cell signaling, bioenergetics, and redox homeostasis (Chen and Chan, 2009; Buck et al., 2016; Altieri, 2017; Burman et al., 2017).

Our findings significantly extend a recent study showing that mitophagy turnover is impaired in cultures of immortalized keratinocytes derived from individuals with PC (Lehmann et al., 2019), thus adding to the evidence that anomalies in mitochondria and in redox balance may play a significant role in the pathophysiology of PC-associated PPK. They also add to a growing body of evidence linking keratin (Silvander et al., 2017) and other types of IFs, notably vimentin and desmin, to mitochondrial regulation and function (Milner et al., 2000; Chernoivanoenko et al., 2015; Matveeva et al., 2015; Schwarz and Leube, 2016). Like microtubules and actin filaments, IFs participate in controlling mitochondrial motility, likely through stabilization, which in turn regulates ATP production, calcium signaling, and intermediary metabolism (Straube-West et al., 1996; David et al., 1998; Wagner et al., 2003; Schwarz and Leube, 2016). Many issues remain unresolved, however. First, while others provided evidence that K16 and K6 can bind to trichoplein, a candidate mitochondrial linker protein (Nishizawa et al., 2005; Silvander et al., 2017), there is still a need to definitively identify the mechanism in which keratin filaments interact with the mitochondria. Second, we do not know whether the mitochondrial dysfunction is leading to increased ROS production or whether the damage is a result of an already established oxidative stress. This is significant because activation of oxidative stress pathways precedes PPK lesions in mice (Kerns et al., 2016, 2018). The findings reported here provide a platform to answer these questions along with a deeper understanding of keratins’ active role in regulating mitochondrial function, structure, and dynamics.

**MATERIALS AND METHODS**

**Mouse handling**

All experiments involving mice were reviewed and approved by the Unit for Laboratory Animal Medicine at the University of Michigan. The Krt6a/Krt6b null (Wong et al., 2000) and Krt16 null (Lessor and Coulombe, 2012) mouse strains (C57BL/6 background) were maintained under specific pathogen-free conditions, fed chow and water ad libitum, and bred and genotyped as described. All studies involving E18.5 back skin tissue and newborn skin (P1-P2 pups) keratinocytes in primary culture were performed using littermates with a WT or homozygous null genotype.

**Reagents**

Primary antibodies used include anti-PDH (Abcam), anti-FLAG M2 Magnetic Beads (Sigma-Aldrich), anti-TCHP (abcam), anti-β-actin, anti-K16 (Bernot et al., 2002), and anti-K6 (McGowan, 1998). Mitotracker CMXROS (ThermoFisher) was used for live cell imaging of mitochondria. Secondary antibodies used included HRP-conjugated secondary antibodies (Sigma-Aldrich) and Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647 (ThermoFisher).

**Tissue preparation for sectioning and microscopy**

Krt6a/Krt6b heterozygous mating pairs were set up and E18.5 pups were harvested and genotyped (Wang et al., 2016). Tissue sectioning for immunostaining was performed by submerging back skin into OCT (Sakura Finetek), freezing at −20°C, and preparing 5 μM sections using CryoStarNX50 (Thermo Scientific), and stained as described below. For TEM, E18.5 back skin from WT and Krt6a/ Krt6b null littermates were placed flat at the bottom of a 24-well plate and treated with 1 ml of fixative (2.5% glutaraldehyde, 3% paraformaldehyde [PFA] in Sorenson’s buffer). The samples were then given to the University of Michigan’s Microscopy and Image Analysis Laboratory (MIL) core for thin-sectioning. Sections were imaged using the JEOL 1400 Plus TEM at the MIL.

**Keratinocyte culture**

WT and Krt6a/Krt6b null, and WT and Krt16 null littermates were taken at P1. The skin was removed and left in 0.25% Trypsin overnight at 4°C, and the following day keratinocyte isolation was performed as described (Rotty and Coulombe, 2012). Cells were counted and seeded on collagen I–coated plates in differentiation-promoting mKER media for 2 d unless specified otherwise. Keratinocytes express K6 isoform and K16 proteins under these circumstances [4, 5, 7, 60]. SIMEX cell lines were generated from Krt16 null and WT littermates as described in Reichelt and Haase (2010).
Mitochondrial respiration
Mitochondrial stress test was performed to measure oxygen consumption rate (OCR), using the XF20i Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA; now Agilent Technologies, Santa Clara, CA), as per the manufacturer’s instructions. Briefly, 4 × 10^4 keratinocytes from the back skin of WT and Krt6a/Krt6b null, and WT and Krt16 null littermates were plated in complete mKER media supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum into each well of 96-well Seahorse microplates. Cells were then incubated in 5% CO_2 at 37°C for 24 h. Following incubation, cells were washed twice, incubated (in a non-CO_2 incubator at 37°C), and analyzed in XF assay media (nonbuffered DMEM containing 25 mM glucose, 2 mM l-glutamine, and 1 mM sodium pyruvate, pH 7.4) at 37°C, under basal conditions and in response to 1 μM oligomycin, 2 μM FCCP, and 0.5 μM rotenone + 0.5 μM antimycin from the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies). Data was analyzed by the Seahorse XF Cell Mito Stress Test Report Generator. OCR (pmol O_2/min) values were normalized to the cell count using Hoechst dye in the final prot injection and transferred to the Cytation 5 (BioTek, Winoski, VT).

Measurement of mitochondrial membrane potential
Keratinocytes were isolated from WT and Krt6a/Krt6b null, and WT and Krt16 null littermates. Cells were seeded in the Corning 96-well clear bottom black polystyrene microplates at a cell density of 5 × 10^4. Due to the fast-growing nature of the immortalized cells, 2.5 × 10^4 cells were seeded for the WT and Krt16 null SIMEKs. The protocol from Abcam’s DCFDA cell ROS detection assay kit was used to measure total cellular ROS at baseline and with increasing concentrations of TBHP.

Indirect immunofluorescence and quantitation
Immunostaining (indirect immunofluorescence) was performed on keratinocytes and epidermal back skin tissue sections. Samples were fixed in 4% PFA for 10 min at room temperature, washed in PBS, blocked in 5% normal goat serum/0.1% Triton X-100/ PBS for 1 h at room temperature, incubated in primary antibody solution overnight at 4°C, washed in PBS, incubated in secondary antibody solution for 1 h, incubated with DAPI for 5 min, washed in PBS, and mounted in FluorSave Reagent Mounting Medium (EMD Millipore) before visualization using a Zeiss LSM 800 fluorescence confocal microscope. To quantify the mitochondrial PDH distribution throughout the cell, the nucleus and the cell boundaries were manually segmented with Fiji. Each boundary loop was then smoothed in MATLAB and 100 points on the boundary with even spacing were picked to partition the loop into 100 sections. The points on the cell boundary were linked to corresponding points on the cell nucleus boundary, keeping the circular order of the points and the total distance between paired points at the minimum. In this way, the area between the nucleus and the cell boundary was segmented into 100 regions. For each region (and the associated boundary points) the local distance between the nucleus and the cell boundary was defined as the distance between the middle points of the local nucleus and cell boundary sections. The distance of each pixel in the segmented regions to the nucleus was defined as the distance between the pixel to the middle point of the local nucleus boundary section. As some cells touched other cells, only the regions with cell boundary not touching other cells were manually selected for further analysis. To quantify the average PDH fluorescence intensity with respect to the distance to the nucleus, the distance was binned every 10 pixels (1.6 μm). The average PDH fluorescence intensity of pixels in each distance bin was then calculated.

Biochemical analyses
RNA was isolated from primary keratinocytes using the NucleoSpin RNA kit (Macherey-Nagel) followed by cDNA preparation (iScript cDNA synthesis kit; Bio-Rad). All oligonucleotide primers (see Supplemental Table 1) were designed using the mus musculus RefSeq through the NCBI database. K16-flag protein (C-terminally tagged) was made using the pCDNA™3.1 Directional TOPO™ Expression Kit (ThermoFisher) using primers for the mouse Krt16 gene with flag peptide sequence on the 3’ end (Supplemental Table 1).

Statistical analyses
All statistical analyses, unless indicated elsewhere, were performed using a two-tailed Student’s t test.
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