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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Keratin 16 Null Mice Develop Palmoplantar Keratoderma, A Hallmark Feature of Pachyonychia Congenita and Related Disorders

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Keratin 16 (KRT16 in human, Krt16 in mouse), a type I intermediate filament protein, is constitutively expressed in epithelial appendages and is induced in the epidermis upon wounding and other stressors. Mutations altering the coding sequence of KRT16 cause pachyonychia congenita (PC), a rare autosomal dominant disorder characterized by hypertrophic nail dystrophy, oral leukokeratosis, and palmoplantar keratoderma (PPK). PPK associated with PC is extremely painful and compromises patient mobility, making it the most debilitating PC symptom. In this study, we show that, although inherited in a recessive manner, the inactivation of Krt16 in mice consistently causes oral lesions as well as PPK-like hyperkeratotic calluses on Krt16\textsuperscript{−/−} front and hind paws, which severely compromise the animals' ability to walk. Our findings call into question the view that PC-related PPK arises exclusively as a gain-of-function on account of the dominantly acting mutated keratins, and highlight the key role of modifiers in the clinical heterogeneity of PC symptoms.

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INTRODUCTION

Keratin 16 (KRT16 in human, Krt16 in mouse), a type I intermediate filament protein, is constitutively expressed in a variety of epithelial appendages, including the tongue and the hair follicle, and in glabrous skin (Moll et al., 1982; Swensson et al., 1998; Bernot et al., 2002). Upon stressful epithelial stimuli, such as wounding or chronic inflammation, Krt16 and its binding partner Krt6 are selectively induced in the suprabasal layers of the epidermis (Paladini et al., 1996). Mutations in KRT16 are associated with the development of pachyonychia congenita (PC), a rare autosomal dominant disorder characterized by hypertrophic nail dystrophy, palmoplantar keratoderma (PPK), and oral leukokeratosis (McLean et al., 1995). PC symptoms are highly variable in penetrance, age of onset, and severity, even between individuals with the same mutation (Leachman et al., 2005; Liao et al., 2007; Fu et al., 2011). In patients carrying KRT16 mutations, PPK is the most prominent symptom and may sometimes appear as focal PPK (FPPK) with little or no nail involvement (Shamsheer et al., 1995; Leachman et al., 2005; Smith et al., 2000, 2005; Liao et al., 2007). KRT16 mutation-linked PPK is typically non-epidermolytic (McLean et al., 1995; Shamsheer et al., 1995; Liao et al., 2007), although blistering underneath and around PPK calluses has been linked to palmoplantar pain in PC patients (Dahl et al., 1995). Previous mouse models harboring both dominant and recessive mutations in Krt16 and Krt75 recapitulate several PC-like symptoms (Wong et al., 2000, 2005; Wojcik et al., 2001; Chen et al., 2008), but have failed to phenocopy PPK, which is considered to be the most debilitating PC symptom as it is extremely painful and significantly impacts patient mobility and quality of life (Dahl et al., 1995; Leachman et al., 2005). In this study, we show that adult mice lacking Krt16 develop hyperkeratotic calluses on their front and hind paws that are strikingly similar to human PPK and significantly compromise the animals' ability to walk.

RESULTS

Failure to thrive and increased postnatal mortality in Krt16\textsuperscript{−/−} mice

Krt16\textsuperscript{−/−} mice were born alive at approximately Mendelian ratios and were initially visually indistinguishable from wild-type (WT) and Krt16\textsuperscript{+/−} litters (Supplementary Figure S1A online). However, 34% of Krt16\textsuperscript{−/−} mice died within the first 24 hours after birth, as opposed to 6% of WT and 11% of Krt16\textsuperscript{+/−} mice (Figure 1a). Toluidine Blue dye exclusion assays performed on newborn pups ruled out any gross early postnatal skin barrier defects in Krt16\textsuperscript{−/−} (Supplementary Figure S1B online). Newborn Krt16\textsuperscript{−/−} mice

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Abbreviations: FPPK, focal PPK; KRT16, Keratin 16; PC, pachyonychia congenita; PBS, phosphate-buffered saline; PPK, palmoplantar keratoderma; WT, wild type

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Figure 1. Failure to thrive, high postnatal mortality, and posterior tongue lesions in Krt16−/− mice. (a) High postnatal mortality in Krt16−/− mice. Thirty-four percent of Krt16−/− mice die within 24 hours after birth; only a third survive past weaning. (b) Krt16−/− mice weigh significantly less than control littermates. Error bars denote standard error of the mean. (c) P17 Krt16−/− mice are smaller than littermates and a white-yellow plaque covers the posterior dorsal area of their tongues (arrowhead). (d) Hematoxylin and eosin-stained sections of posterior tongues at P0 and P7. Krt16−/− mice show no obvious defects at birth. By P7, the architecture of filiform papillae (fp) is markedly disrupted, and massive hyperkeratosis leads to macroscopically visible plaques. Note the paucity of cell lysis in the suprabasal layer. Dotted line indicates the epithelial/muscle junction. Bar = 50 μm.

weighed less than their littermates (Supplementary Figure S1C online) and subsequently lag behind their littermates in size and in weight (Figure 1b and c). Such phenotypic changes coincided with the persistence of a markedly higher level of postnatal mortality, and over 60% of Krt16−/− mice died before weaning age. The Krt16−/− mice that did survive continued to grow and gained weight, but remained smaller and lighter than their littermate controls (data not shown).

Krt16 is essential for the structural integrity of the dorsal tongue epithelium

Mice lacking the Krt16 binding partners, Krt6a and Krt6b, exhibit a more severe albeit similar growth retardation and early postnatal lethality phenotype (Wong et al., 2000; Wojcik et al., 2001). In Krt6a/b−/− mice, the formation of large hyperplastic lesions on the dorsal posterior tongue (akin to oral leukokeratosis, a PC symptom) is thought to occlude the laryngeal space, impair feeding, and lead to early death due to starvation. Such laryngeal obstruction, although extremely rare, has been reported in pediatric PC patients carrying KRT6a mutations (Smith et al., 2005; Haber and Drummond, 2011), and infants suffering from oral leukokeratosis often have trouble breastfeeding (Leachman et al., 2005).

When we examined the tongues of Krt16−/− mice at different ages, we also detected the presence of hyperplastic lesions, which are fully penetrant and become macroscopically and microscopically visible by P3 (Figure 1d). These lesions were confined to the dorsal midline area of the posterior tongue and were smaller than those observed in Krt6a/b−/− mice (Wong et al., 2000). Surviving Krt16−/− mice
no longer had visible lesions, but the tongue architecture remained severely compromised, showing a thickened epithelium and loss of the characteristic filiform papillae morphology (Supplementary Figure S2A online). Although no epithelial fragility was detectable by routine histology in PO Krt16−/− tongues (Figure 1d), transmission electron microscopy revealed early stages of cell lysis in a subset of posterior filiform papillae (Supplementary Figure S2B online). Interestingly, Krt17 protein, and to a lesser extent Krt17 mRNA, levels were significantly reduced in the tongue epithelium of Krt16−/− mice (Figure 2a and b), and immunofluorescence staining for Krt17 was consistently absent from the anterior column of Krt16−/− filiform papillae, but not the fungiform papillae (Figure 2a). The expression of Krt5, Krt6 and Krt10 was normal in Krt16−/− filiform papillae (Figure 2a and b), suggesting that the loss of Krt17 was not due to an overall change in the differentiation program in anterior column of filiform papillae. The constitutive and inducible expression of Krt17 in other tissues, including skin, was not affected (Figure 2a and 4d). Our findings thus extend the notion that Krt6- and Krt16-containing filaments are essential for the maintenance of dorsal tongue epithelial integrity. Loss of Krt16 and concomitant reduction of Krt17 levels deplete most, but not all, of Krt6's primary binding partners in the tongue epithelium, leading to increased cell fragility and hyperplastic lesion formation, starvation, and a higher chance of postnatal death in Krt16−/− mice. Residual levels of Krt17 present in Krt16−/− tongue epithelium may explain the smaller lesions and lower number of early deaths in Krt16−/− mice as compared with Krt6a/b−/− mice (Wong et al., 2000; Wojcik et al., 2001). Despite their less severe nature, we cannot rule out the possibility that oral lesions in Krt16−/− mice are painful and thus impact feeding behavior.

**Adult Krt16−/− develop PPK**

Starting at 4-6 weeks of age, surviving Krt16−/− mice developed prominent, hyperkeratotic calluses on the glabrous parts of both front and hind paws (Figure 3a-c). The boundaries of these calluses did not directly correlate with the loss of endogenous Krt16, which is expressed in a patchy manner throughout paw pad epithelia, including the nail hyponychium, as shown by whole-mount X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) staining (Figure 3a). Instead, consistent with focal PPK (FPPK) in human PC patients, calluses in Krt16−/− mice were restricted to areas subject to physical pressure, i.e., the heel and the wrist (Figure 3a). Occasionally, we observed generalized hyperkeratosis on the front paw pads as early as at 3 weeks of age (Supplementary Figure S3A online), and focal hyperkeratosis near the base of the tail. Importantly, nail morphology was not affected in Krt16−/− mice (Supplementary Figure S3B online).

The age of onset for callus formation on both front and hind paws was variable, and calluses differed in severity.
between animals (Figure 3a shows two representative examples of callus severity in Krt16−/−). In all 36% of Krt16−/− mice showed no alteration of their hind paw pads at all. Hind paw calluses also consistently formed after front paw pad calluses were already established. Visual changes in Krt16−/− front paw pads correlated with an expanded epithelial compartment (Figure 3b) that correlated with a 2-fold increase in proliferation (Figure 4c) in the absence of apoptotic cell death (Supplementary Figure S3C online). Areas adjacent to the callus showed a thickened epithelium, but no significant increase in proliferation (Figure 3b and 4c). Paw pad epithelia in younger mice with no obvious signs of callus formation were indistinguishable from controls (data not shown). As PPK in humans greatly impairs mobility, we asked whether callus formation in Krt16−/− mice has a negatively impact on the animals' activity level in a behavioral assay. We found that adult Krt16−/− mice are significantly less active than control animals and spend more time resting than walking (Figure 4a, Supplementary Figure S3D online). Several anomalies, including the patchy expression of Krt17 (which is absent from control mouse paw pad epidermis) and focal toluidine blue dye penetration (Figure 4b and d), suggested a disruption of the outside-in epidermal barrier in Krt16−/− front paw calluses, which may
lead to secondary infections as implied by the presence of prominent hyper-pigmentation in those areas (Figure 3a). Krt17 expression is induced in response to barrier breach (McGowan and Coulombe, 1998; DePlanto et al., 2010), and a mutation in the barrier protein filaggrin has been shown to intensify PC-related symptoms in a human patient with a coincident Krt16 mutation (Liao et al., 2007; Gruber et al., 2009). Filaggrin expression is normal in Krt16−/− paw pad epithelia, as reported for PC plantar lesions (Wollina et al., 1991), but is reduced in established front paw calluses (Figure 4d), confirming that Krt16−/− glabrous skin, although initially intact, is eventually unable to fend off the continuous physical pressure generated by walking and/or cleaning behavior, resulting in hyperproliferation and a focal loss of
barrier protection. In humans, PPK is very painful. The cause of the pain is unknown, but clinical observations have noted the formation of blisters underneath or adjacent to calluses (Dahl et al., 1995), as well as secondary infections following fissuring of the hyperkeratotic tissue (Leachman et al., 2005). Alternatively, it is possible that PPK calluses exert an increased pressure on nerve endings in plantar skin, especially while walking. Although an objective assessment of the level and type of pain or its source in Krt16-/- mice is beyond the scope of this study, we hypothesize that Krt16-/- mice experience substantial discomfort as a direct result of palmoplantar lesions and thus exhibit a significant decrease in their overall mobility.

DISCUSSION
Here, we show that the loss of Krt16 function in mice causes the development of prominent calluses on the plantar side of front and hind paws, which significantly compromise mobility and eventually lead to overt loss of barrier properties. Although the molecular mechanism of PPK pathogenesis in PC is still unclear, this symptom is currently thought to develop as a consequence of intermediate filament network disruption by dominantly-acting mutations in relevant keratins (Fu et al., 2011). To our surprise, deletion of Krt16 produced spontaneously arising PPK-like lesions in mice, suggesting that PPK pathogenesis in PC is more complex than previously appreciated and may represent, at least in part, a loss of function phenotype. Almost all human PC patients harboring KRT16 mutations report PPK (Leachman et al., 2005; Liao et al., 2007; Fu et al., 2011). However, many mutations in KRT16 only elicit milder overall PC phenotypes, often diagnosed as FPPK because of the limited and at times absent nail involvement (Shamsheer et al., 1995; Smith et al., 2000, 2005; Liao et al., 2007). In particular, this is seen with small deletions in KRT16, which are thought to cause exclusion of the mutant form of KRT16 from filament assembly (Smith et al., 2000; Wilson et al., 2009; Cao et al., 2011). These findings, together with our data suggest that both the dominant-negative disruption of intermediate filaments as well as the exclusion of keratins from the network can contribute to PPK pathogenesis. Nail dystrophy, on the other hand, was not observed in Krt16-/- mice, and may depend more heavily on the dominant-negative interference of keratin filaments. Accordingly, the deletion of individual or even multiple keratins leads to no or very minor nail defects in mice (Wong et al., 2000, 2005; Wojcik et al., 2001; this study), whereas a dominant mutation in Krt75 elicits the characteristic nail overgrowth in mouse (Chen et al., 2008). In humans, where all known PC-causative keratin mutations are dominant, nail involvement is highly penetrant. These observations in humans, mouse models, and now our findings in Krt16-/- mice showcase how a combination of loss- and gain-of-function phenotypes can contribute to the complex overall clinical presentation of PC. Furthermore, our data substantiate the hypothesis that FPPK and PC can share a common pathogenesis (Wilson et al., 2009; Bowden, 2010), as Krt16-/- mice developed FPPK along with oral lesions, another PC-like symptom. We also noticed differences in disease onset, phenotype severity, and change in barrier permeability in Krt16-/- mice, supporting the idea of keratin, non-keratin, and/or environmental modifiers in PPK pathogenesis (Smith et al., 2000). It will be interesting to examine whether the mechanism(s) by which loss of Krt16 results in the development of PPK overlap(s) with the pathogenesis of other keratodermas, e.g., striate PPK (Desmoplakin) or diffuse PPK (KRT11, Desmoglein).

We only observed occasional and locally restricted areas of cell lysis in established Krt16-/- front paw calluses (Figure 3b, arrowhead and inset), but never in hind paw calluses or uninvolved skin, despite the global expression pattern of Krt16 in glabrous tissue. Expression of other structurally important keratins in the suprabasal layer, such as Krt10 and Krt1, was also generally intact (Figure 4d) and likely contributes to the maintenance of cellular integrity in the absence of Krt16. Thus, we speculate that Krt16 has an additional function in glabrous skin, separate from its role of structural support. Several studies have already implicated Krt16 and other type I keratins, such as Krt10 and Krt17, as significant players in epidermal homeostasis (Takahashi et al., 1994; Paladini et al., 1996; Reichelt and Magin, 2002; Kim et al., 2006; DePianto et al., 2010). A key feature of Krt16 expression in the skin is its selective induction following injury, UV exposure, or in chronic disease states (e.g., psoriasis; Leigh et al., 1995; Paladini et al., 1996; Del Bino et al., 2004). Glabrous skin is a specialized tissue designed to withstand and adapt to significant mechanical trauma (Bowden et al., 1987; Svensson et al., 1998). It will be worth investigating the function of constitutive Krt16 expression in this unique environment, and whether the underlying mechanism bears any relationship to the role(s) fulfilled by Krt16 when induced by injury and other relevant stressors.

MATERIALS AND METHODS

Generation of Krt16-/- mice
C57 Bl/6 ES cells in which the Krt16 coding sequence has been replaced with a lacZ-loxP-Neo4-loxP cassette were created by Velocigene using funds provided by the trans-NIH KOMP (Knock-Out Mouse Project), and obtained from the KOMP repository (supported by the NCRR-NIH). ES cells were injected into C57 Bl/6 f/embryonic albino blastocysts (distributed by the NCI, Frederick, MD). A high coat color male chimera was bred to C57 Bl/6 f/embryonic albino females and 100% germline transmission was observed. Krt16-/- F1 offspring were born at the expected 50:50 ratio and inter-crossed to generate Krt16-/- mice. All experiments involving mice were reviewed and approved by the Johns Hopkins Institutional Animal Care and Use Committee. Mouse lines were maintained under specific pathogen-free conditions, and fed chow and water ad libitum. All experiments were performed using littermate controls (wild-type or Krt16+/-).

Histopathology and immunofluorescence
Whole tongues and paws were fixed overnight in Bouin’s (Sigma) or 4% para-formaldehyde/phosphate-buffered saline (PBS). Tissue was then either rinsed and embedded in Sakura Tissue-Tek OCT. (VWR, Radnor, PA), or dehydrated and processed for routine paraffin

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embedding. Sections were cut at 5 μm and stained with hematoxylin/eosin according to standard protocols. For immunofluorescence, frozen sections or re-hydrated paraffin-embedded sections were washed in PBS, blocked in 5% normal goat serum, 0.1% Triton-X100 for 1 hour at room temperature, incubated in primary antibody solution (2.5% normal goat serum, 0.1% Triton-X100) for 1 hour at room temperature, washed in PBS, incubated in secondary antibody (2.5% NGS) for 1 hour, counterstained with DAPI, mounted, and imaged using an inverted Zeiss fluorescence microscope with ApoTome attachment. Antibodies used were directed against Krt17 (1:1,000; McGowan and Coulombe, 1998), Krt6 (1:250; McGowan and Coulombe, 1998), Krt10 (1:500; Covance, Princeton, NJ), filaggrin (1:500; Covance), K67 (Sp6 clone, 1:200; Thermofisher, Pittsburgh, PA), and AlexaFluor488 (1:1,000; Invitrogen, Carlsbad, CA). TUNEL staining was performed as described (McGowan et al., 2002).

Whole-mount X-gal and barrier function assays
Whole paws were fixed for 1 hour in 4% paraformaldehyde/PBS at 4°C. Paws were permeabilized via 3 × 15-minute washes in PBS containing 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP-40. The tissue was then transferred to scintillation vials containing a 1-mg/ml⁻¹ X-gal solution (30 mM K₄Fe(CN)₆, 30 mM K₃Fe(CN)₆, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, PBS) for 30 minutes at 30°C. Samples were photographed immediately. Toluidine Blue dye staining, to assess outside-in barrier function, was carried out on newborn paws and adult paws as described (Hardman et al., 1998).

Behavioral assays to assess mobility
Individual mice were allowed to acclimate for 30 minutes and then placed into a rectangular plexiglass chamber with a stainless steel floor. To eliminate outside positional cues, the chamber was uniformly illuminated and its sides masked with a cardboard. Mobility was monitored over a time frame of 30 minutes for infrared beam-breaking activity and the data were recorded using the OptiMax software (Columbus Instruments, Columbus, OH). Results represent the average of three separate experiments.

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 https://www.nature.com/jid

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