Establishment of Two Mouse Models for CEDNIK Syndrome Reveals the Pivotal Role of SNAP29 in Epidermal Differentiation

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Loss-of-function mutations in the synaptosomal-associated protein 29 (SNAP29) gene cause the cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma (CEDNIK) syndrome (OMIM 609528) is a rare autosomal recessive neurocutaneous disorder that is uniformly fatal in childhood (age 5–12 years). It is caused by mutations in the synaptosomal-associated protein 29 (SNAP29) gene (Fuchs-Telem et al., 2011; McDonald-McGinn et al., 2013; Sprecher et al., 2005). SNAP29 belongs to the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins, which mediate membrane fusion between vesicles and target membranes in eukaryotic cells. The human SNAP29 gene is expressed in many tissues and seems to be involved in a variety of intracellular membrane fusion processes (Hohenstein and Roche, 2001; Steegmaier et al., 1998), among others, in endocytotic recycling and cell motility (Rapaport et al., 2010). The pronounced cutaneous phenotype of CEDNIK syndrome suggests a crucial role of SNAP29 in epidermal differentiation and barrier formation (Sprecher et al., 2005).

Genetic causes of ichthyoses can vary widely, including defects in structural proteins (e.g., ichthyosis vulgaris) or enzymes (e.g., recessive X-linked ichthyosis). CEDNIK syndrome belongs to another steadily growing group of cornification disorders associated with abnormal intracellular transport processes. This group includes arthrogryposis, renal dysfunction, and cholestasis syndrome, which is caused by mutations in the VPS33B gene encoding a protein involved in the regulation of soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex formation (Hershkovitz et al., 2008) or the mental retardation, enteropathy, deafness, neuropathy, ichthyosis, and keratoderma syndrome, which is caused by mutations in APT1 encoding a component of adaptor protein complex 1 (Montpetit et al., 2008). Thus, keratinocyte differentiation also depends on transport processes. Lamellar bodies (LBs) serve as transport units to transfer components of the epidermal barrier to the apical surface of keratinocytes in the stratum granulosum (SG) (Odland and Hollbrook, 1981). Abnormalities in LB function can provoke severe cutaneous diseases such as ichthyoses (Chan et al., 2015). Not surprisingly, defective maturation of LBs in the skin of human CEDNIK syndrome patients has been demonstrated (Sprecher et al., 2005). Subsequently, it was shown that SNAP29 deficiency also interferes with LB secretion (Fuchs-Telem et al., 2011).

INTRODUCTION

Cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma (CEDNIK) syndrome (OMIM 609528) is a rare autosomal recessive neurocutaneous disorder that is uniformly fatal in childhood (age 5–12 years). It is caused by mutations in the synaptosomal-associated protein 29 (SNAP29) gene (Fuchs-Telem et al., 2011; McDonald-McGinn et al., 2013; Sprecher et al., 2005). SNAP29 belongs to the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins, which mediate membrane fusion between vesicles and target membranes in eukaryotic cells. The human SNAP29 gene is expressed in many tissues and seems to be involved in a variety of intracellular membrane fusion processes (Hohenstein and Roche, 2001; Steegmaier et al., 1998), among others, in endocytotic recycling and cell motility (Rapaport et al., 2010). The pronounced cutaneous phenotype of CEDNIK syndrome suggests a crucial role of SNAP29 in epidermal differentiation and barrier formation (Sprecher et al., 2005).

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Keratinocyte differentiation also involves organelle clearance and recycling of cellular components. Recent studies revealed that autophagy is directly involved in this process (Belleudi et al., 2014; Moriyama et al., 2014). Interestingly, the Drosophila SNAP29 homolog ubisnap mediates fusion of autophagosomes with endosomes/lysosomes (Takats et al., 2013). This suggests that autophagy is likely affected by SNAP29 deficiency and, therefore, may be required for proper barrier function. Human CEDNIK syndrome patients are very rare, and only three Snap29-deficient nonmammalian animal models have been described to date: Caenorhabditis elegans (Guo et al., 2014; Kang et al., 2011; Sato et al., 2011), Danio rerio (Li et al., 2011), and Drosophila melanogaster (Xu et al., 2014). The complexity and importance of intracellular transport processes in keratinocyte differentiation and barrier formation prompted us to create a mammalian animal model to study the role of SNAP29 in these processes. We generated mice lacking SNAP29 ubiquitously (hereafter referred to as Snap29\(^{+/+}\)/K14-Cre) or exclusively in epidermal keratinocytes (hereafter referred to as Snap29\(^{fl/fl}\)/K14-Cre). Our murine Snap29-deficient models largely replicate the human cutaneous CEDNIK syndrome phenotype.

**RESULTS**

**Total and epidermal knockout of SNAP29**

Total (Snap29\(^{+/+}\) mice) and epidermal (Snap29\(^{fl/fl}\)/K14-Cre mice) knockout of the Snap29 gene was achieved using B6N:B6N-Snap29 \(^{tm1a(EUCOMM)Whi}\) heterozygous knockin mice obtained from the Wellcome Trust Sanger Institute (WTSI) (see Supplementary Materials and Supplementary Figure S1 online). Heterozygous mutant Snap29 pups were phenotypically indistinguishable from wild-type (wt) mice. Snap29\(^{+/+}\) complete knockouts and Snap29\(^{fl/fl}\)/K14-Cre keratinocyte-specific knockouts were largely identical. Hence, we report data for Snap29\(^{fl/fl}\)/K14-Cre mice and refer to the data for Snap29\(^{+/+}\) mice in Supplementary Figures S2–S6 (online). Snap29\(^{fl/fl}\)/K14-Cre mice were born at approximate mendelian ratios but died within a few hours after birth. They exhibited a slightly reduced body size and a distinct ichthyosiform phenotype. None of the mutant mice contained milk in their stomachs. Ablation of SNAP29 protein in Snap29\(^{fl/fl}\)/K14-Cre epidermis was confirmed by immunohistochemistry (Figure 1b).

**Disturbed epidermal barrier due to Snap29 deficiency**

Hematoxylin and eosin staining of Snap29\(^{fl/fl}\)/K14-Cre skin revealed epidermal hyperplasia (acanthosis). The stratum corneum (SC) of mutant mice was thickened, and its structure was condensed (hyperkeratosis). We also observed a reduction of hair follicles in Snap29\(^{fl/fl}\)/K14-Cre mice down to 81% that of wt mice (Figure 1c). In order to test the functional integrity of Snap29\(^{fl/fl}\)/K14-Cre epidermis, we measured dye...
intrusion (inward barrier function) and fluid loss through the epidermis using a dehydration assay (outward barrier function). The loss of weight during a distinct period of time should correspond to the evaporation of water through the skin. The \textit{Snap29} \textit{fl/fl}/K14-Cre newborns showed increased dye intrusion compared to control littermates (Figure 1d). Additionally, \textit{Snap29} \textit{fl/fl}/K14-Cre mice lost more weight than control littermates (Figure 1e). Both experiments indicate severe functional impairment of the epidermal barrier.

Disturbed epidermal differentiation in SNAP29-deficient epidermis

To determine the molecular basis of the acanthotic and hyperkeratotic phenotype of mutant epidermis, we performed immunohistochemical analyses with epidermal differentiation and proliferation markers. In contrast to healthy skin, keratin 14 (K14) expression reached the suprabasal and subcorneal layers of \textit{Snap29} \textit{fl/fl}/K14-Cre epidermis (Figure 2a and b). The levels and distribution of suprabasal keratin 10 (K10) did not change in mutant epidermis (see Supplementary Figure S7a and b online). Involucrin levels were decreased in the cells of the upper stratum spinosum and SG of \textit{Snap29} \textit{fl/fl}/K14-Cre mice epidermis (see Supplementary Figure S7c and d). The epidermal levels of filaggrin and the number of profilaggrin containing keratohyalin granules in the upper SG were decreased in \textit{Snap29} \textit{fl/fl}/K14-Cre epidermis, respectively (Figure 2c and d). The observed epidermal thickening of mutant epidermis prompted us to investigate the effect of SNAP29 deficiency on expression of the hyperproliferation-associated keratin 6.
(K6) and the proliferation marker Ki67. Immunohistochemical staining with anti-K6 antibody showed pronounced expression of K6 in cells of the suprabasal layers of mutant epidermis but not in controls (Figure 2e and f). Staining with an anti-Ki67 antibody revealed 34% positive basal cells in control epidermis compared to 56% in Snap29fl/fl/K14-Cre epidermis (see Supplementary Figure S7e and f). No alteration in apoptotic activity was detected in the epidermis of mutant mice using immunostaining against the activated form of the apoptotic mediator caspase 3 (data not shown).

Reduced LB contents in Snap29fl/fl/K14-Cre epidermis

In order to investigate the influence of Snap29 deficiency on LB function, we examined epidermal levels of secreted LB components in Snap29fl/fl/K14-Cre epidermis compared to control skin. Immunohistochemistry revealed strong reductions of kallikrein 7 (Figure 2g and h) and corneodesmosin (Cdsn) levels, whereas remnants of Cdsn were still found in mutant SC (Figure 2i and j). Nile red staining of Snap29fl/fl/K14-Cre skin showed decreased deposition of neutral lipids in mutant epidermis (Figure 3a). Considerably reduced epidermal distribution of glucosylceramide was also revealed by immunofluorescence (Figure 3b). In contrast to control epidermis, the SC of Snap29fl/fl/K14-Cre mice contained a multitude of lipid droplets, as shown by BODIPY 493/503 staining (Figure 3c). This is suggestive for endoplasmic reticulum (ER) stress. These results indicate pronounced disturbances of LB function and, as a consequence, altered epidermal lipid distribution in mutant mice.

Diminished formation, maturation, and secretion of LBs in Snap29fl/fl/K14-Cre mice

Ultrastructural analyses supported the immunohistochemical findings of altered LB function in Snap29fl/fl/K14-Cre mice epidermis. In agreement with the histological findings, the basket-like structure of the SC was markedly condensed in mutant epidermis as shown by electron microscopy (Figure 3d, I and II). At higher magnification, wt corneocytes appeared homogenously filled, whereas Snap29fl/fl/K14-Cre corneocytes appeared inhomogeneously filled with remnants of non-degraded organelles and electron-lucent vesicle-like structures (Figure 3d, III and IV). In wt epidermis well-formed LBs were detectable in the SG, whereas in mutant epidermis electron-lucent LB-like structures in addition to normal
appearing LBs were present (Figure 3d, V and VI, asterisks). In addition to the observed disturbed maturation of LBs, there was reduced secretion of LB contents in mutant epidermis. The amount of secreted LB contents between SG and SC was strongly reduced in mutant epidermis compared to control epidermis (Figure 3d, V and VI, white arrows).

Altered autophagy and ER function due to Snap29 deficiency
For autophagy assessment, western blot analyses under normal and starvation conditions were performed (Mizushima et al., 2010). Because our data clearly indicate a delay in the degradation of organelles in the SC, we investigated whether Snap29 deficiency also affects autophagy. For that purpose, we quantified the conversion of microtubule associated protein-1 light chain 3, isoform B (LC3B)-I to LC3B-II, which is a marker for the amount of autophagosomes (Kabeya et al., 2000; Wu et al., 2006). In addition, the amount of p62/SQSTM1, an autophagy substrate (Bjorkoy et al., 2005; Pankiv et al., 2007), was investigated. For these experiments, we used protein extracts from Snap29-deficient fibroblasts obtained from Snap29e/e mice. We detected markedly increased LC3B-II levels in Snap29−/− cells under basal as well as starvation conditions (Figure 4a). In contrast, p62/SQSTM1 protein amounts remained largely unchanged in Snap29−/− cells under basal as well as starvation conditions (Figure 4b). Because disturbances in ER homeostasis can also act as inducers of autophagy next to amino acid starvation, we investigated protein levels of the ER stress marker C/EBP homologous protein (CHOP) (Li et al., 2014). Indeed, western blot analysis revealed a strong 5-fold induction of CHOP in mutant fibroblasts compared to wt cells (Figure 4c).

DISCUSSION
SNAP29-deficient mice mimic the human CEDNIK syndrome phenotype
In this study, we report the generation and characterization of two Snap29-deficient mouse lines as models for the rare human CEDNIK syndrome. One mouse strain is completely devoid of Snap29 expression (Snap29e/e); the other is Snap29 deficient exclusively in keratinocytes (Snap29fl/fl/K14-Cre). Both mutant mice showed a distinct ichthyosiform phenotype that resembles the human skin phenotype of CEDNIK syndrome patients. However, the murine skin phenotype is more severe than the human one. Epidermal as well as total Snap29-deficient mice exhibited a congenital ichthyosis with taut skin and ichthyotic lesions accompanied by restricted movement capability and neonatal lethality. The human skin phenotype is characterized by mild and late-onset ichthyosis with ichthyosiform symptoms evolving between 5 and 11 months of age (Sprecher et al., 2005). In contrast to CEDNIK syndrome...
patients, mutant mice exhibited acanthosis in addition to hyperkeratosis (Fuchs-Telem et al., 2011; Sprecher et al., 2005). No thickening of the suprabasal living layers of the epidermis in human patients was visible. In general, such differences may result from different stages of skin development at birth in mice and humans (Eckl et al., 2013). The development of more pronounced phenotypes with rapid desiccation and neonatal death is commonly observed in murine models of human ichthyosiform diseases (Epp et al., 2007; Radner et al., 2010).

Abnormal keratinocyte differentiation and increased proliferation in Snap29-deficient mouse epidermis

Our mutant mice epidermis exhibits considerable changes in the protein levels of keratinocyte differentiation markers and decreased amounts of keratohyalin granules. This finding is supported by organelle remnants in corneocytes as shown by electron microscopy. In addition, expression of the proliferation markers Ki67 and keratin 6 was strongly up-regulated. Hyperproliferation represents a typical mechanism to compensate epidermal barrier defects (Ny and Egelrud, 2004). In conclusion, these findings demonstrate the involvement of SNAP29 in proper epidermal differentiation.

Snap29 deficiency alters the epidermal lipid structure

Under physiological conditions, lipid material delivered by LBs fills up most of the intercellular space in the SC. This is essential for the formation and homeostasis of the epidermal barrier (Wertz, 2000). During histological preparations this material gets lost, causing a fluffy, basket–like appearance of the SC. Our findings of a condensed SC with a reduced number of lacunas indicate a defective formation of lipid lamellae. Glucosylceramides represent essential lipid components of the epidermal barrier and are secreted by LBs (Ishida-Yamamoto et al., 2004). Diminished amounts of glucosylceramide were found in the epidermis of human CEDNIK syndrome patients (Sprecher et al., 2005). Corresponding results were observed in our mice. In contrast, numerous lipid droplets were detected in the SC of our mice.

Ultrastructural analyses also showed a decreased amount of released LB content into the intracellular space between SG and SC in the mouse models. We conclude that Snap29 deficiency provokes malfunctions of LBs and alterations in the intercellular lipid lamellar structure of the SC. This corresponds to the notion that congenital ichthyoses, despite their genetic heterogeneity, can be considered disorders of disturbed epidermal lipid metabolism (Elias et al., 2012).

Snap29 deficiency provokes disturbed LB function

In addition to lipid components, LBs also transport other proteins, proteases, and protease inhibitors that are required for generation and maintenance of the epidermal barrier and for desquamation (Ishida-Yamamoto et al., 2004). Therefore, disturbances in LB function should effect localization and levels of their cargoes such as Cdsn or the Cdsn degrading protease kallikrein 7. In three-dimensional organotypic keratinocyte cell cultures in which human SNAP29 was down-regulated by siRNA, remnants of Cdsn were found in keratinocytes of the SC normally devoid of Cdsn (Fuchs-Telem et al., 2011). Corneocytes of human CEDNIK syndrome patients pathologically harbor abnormal vesicles containing kallikrein 7 (Sprecher et al., 2005). In good agreement with these observations, the keratinocytes of our mouse epidermis showed markedly reduced levels of kallikrein 7 and Cdsn in subcorneal epidermal layers, but Cdsn remnants in the SC. These findings indicate a delay in degradation of Cdsn due to reduced amounts of kallikrein 7. Electron microscopic data also support SNAP29-dependent vesicular transport. The epidermis of CEDNIK patients showed vesicles in the SG as expected, but also in the SC. Many of these vesicles appear normal in shape, but some are electron lucent. The extent to which these electron-lucent vesicles possess normal LB function is unknown. It was hypothesized that the electron-lucent vesicles represent empty or malformed LBs (Sprecher et al., 2005). We detected similar results in Snap29-deficient mouse epidermis. LBs with normal and electron-lucent appearances were present in the SG. Electron lucent vesicle-like structures were also present in the SC. These findings resemble observations not only in humans but also in a Snap29-deficient Danio rerio model, which also showed electron-lucent vesicles in the epidermis (Li et al., 2011).

Analogous observations regarding disturbed cellular transport processes were made for other ichthyosiform diseases. For example, arthrogryposis, renal dysfunction, and cholestasis syndrome is mainly associated with mutations in VPS33B whose gene product is thought to regulate soluble N-ethylmaleimide–sensitive factor attachment protein receptor protein-mediated vesicle fusion. Ultrastructural examinations of skin samples from patients revealed retention of normal-appearing LBs in corneocytes (Hershkovitz et al., 2008). In harlequin ichthyosis, abnormally shaped, reduced, or absent LBs were found (Scott et al., 2013). Harlequin ichthyosis is caused by mutations in the ABCA12 gene, which encodes for the ATP-binding cassette transporter A12, a lipid transporter in LBs (Akiyama, 2014). However, disturbances of LB morphology were also observed in cutaneous diseases affecting structural proteins such as filaggrin. This suggests the presence of additional indirect factors that orchestrate LB function (Mildner et al., 2010). Whether SNAP29 directly or indirectly affects such functions and vesicular trafficking remains to be elucidated.

SNAP29 deficiency affects autophagy and ER function

We observed a relative increase of LC3B-II levels in Snap29-deficient fibroblasts, particularly under amino acid starvation conditions, compared to Snap29-proficient fibroblasts. Accumulation of autophagosomes, and therefore increased LC3B-II levels, may indicate an induction of autophagy or blockage of downstream steps, for example, inhibition of autophagosome-lysosome fusion (Mizushima et al., 2010). Presumably, our experiments point toward an induction of autophagy, because no strikingly enhanced levels of p62/SQSTM1 could be detected. Because ER stress, which is associated with increased expression of CHOP (Li et al., 2014), is involved in autophagy as well as in epidermal differentiation (Sugiura et al., 2009), we investigated CHOP expression in relationship to Snap29 deficiency. Interestingly, our results revealed a marked increase of CHOP levels in Snap29-deficient fibroblasts versus proficient fibroblasts, indicating ER stress. It is known that lipid droplets represent
storage departments of neutral lipids arising from the ER (Wilfiling et al., 2014). They are suggested to be formed in order to store and degrade excessive lipids or unfolded or misfolded proteins to protect the ER (Hapala et al., 2011). Therefore, our observed accumulation of lipid droplets in Snap29-deficient mouse epidermis could also be a consequence of enhanced ER stress. The notion that Snap29 deficiency may promote autophagy by induction of ER stress is intriguing and warrants further investigations.

In summary, we generated two Snap29-deficient mouse lines that resemble the human epidermal CEDNIK syndrome phenotype. The phenotype comprises epidermal differentiation defects, defective LB function, abnormal lipid profiles, and generally impaired vesicular trafficking that impairs proper barrier formation. The exact role of SNAP29 in autophagy and ER stress and their contributions to normal epidermal homeostasis warrant further investigations.

MATERIAL AND METHODS

Generation of Snap29−/− and Snap29fl/fl/K14-Cre mice

All experiments involving animals were performed in accordance with legal and ethical requirements. Snap29−/− and Snap29fl/fl/K14-Cre mice were generated and genotyped as described in the Supplementary Materials.

Analysis of the epidermal barrier

To test the fluid loss across the epidermis, neonate mice were separated from their mothers and kept at 37 °C. Body weight was measured every 15 minutes. Skin permeability was assayed by dye diffusion using toluidine blue as described previously (Epp et al., 2007).

Histopathological, immunohistochemical, and fluorescence analyses

Dorsal skin biopsies were fixed overnight in 4% phosphate-buffered formaldehyde and embedded in paraffin. Sections 3 μm thick were stained with hematoxylin and eosin or subjected to immunohistochemical or immunofluorescence analysis. Biopsies were also embedded in Tissue Tek O.C.T. (Sakura Finetek, Staufen, Germany), and 5-μm cryosections were stained with Nile red or BODIPY 493/503. Details are described in the Supplementary Materials.

Electron microscopy

Skin of sacrificed mice was dissected, followed by immersion into fixation solution consisting of 2.5% glutaraldehyde (EM grade, Science Services, München, Germany) and 4% formaldehyde (Serva, Heidelberg, Germany) in phosphate buffer pH 7.3 according to Karlsson and Schultz (1965) as previously described (Mobius et al., 2010). After postfixation with 2% OsO4 (Science Services) and dehydration with ethanol, isopropanol, and propylene oxide, samples were embedded in Epon (Serva). Ultrathin sections were placed on Formvar-coated grids (Plano, Wetzlar, Germany) and imaged in an EM 900 electron microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) with a 2k side-mounted CCD camera (TRS, Moorwein, Germany).

Autophagy assays and western blot analysis

Primary mouse fibroblasts were grown in DMEM high glucose (Lonza, Cologne, Germany). For induction of autophagy by starvation, the medium was changed to earl’s balanced salt solution (Gibco, Eggenstein, Germany). Western blot analysis was performed as described in the Supplementary Materials. Band intensities were determined using ImageJ software (freeware).

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.2015.12.020.

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