Skin Barrier Development Depends on CGI-58 Protein Expression during Late-Stage Keratinocyte Differentiation

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Adipose triglyceride lipase (ATGL) and its coactivator comparative gene identification-58 (CGI-58) are limiting in cellular triglyceride catabolism. Although ATGL deficiency is compatible with normal skin development, mice globally lacking CGI-58 die postnatally and exhibit a severe epidermal permeability barrier defect, which may originate from epidermal and/or peripheral changes in lipid and energy metabolism. Here, we show that epidermis-specific disruption of CGI-58 is sufficient to provoke a defect in the formation of a functional corneocyte lipid envelope linked to impaired ω-O-acylceramide synthesis. As a result, epidermis-specific CGI-58-deficient mice show severe skin dysfunction, arguing for a tissue autonomous cause of disease development. Defective skin permeability barrier formation in global CGI-58-deficient mice could be reversed via transgenic restoration of CGI-58 expression in differentiated but not basal keratinocytes suggesting that CGI-58 is essential for lipid metabolism in suprabasal epidermal layers. The compatibility of ATGL deficiency with normal epidermal function indicated that CGI-58 may stimulate an epidermal triglyceride lipase beyond ATGL required for the adequate provision of fatty acids as a substrate for ω-O-acylceramide synthesis. Pharmacological inhibition of ATGL enzyme activity similarly reduced triglyceride-hydrolytic activities in wild-type and CGI-58 overexpressing epidermis implicating that CGI-58 participates in ω-O-acylceramide biogenesis independent of its role as a coactivator of epidermal triglyceride catabolism.

INTRODUCTION

Humans carrying mutant alleles of the lipolytic coactivator comparative gene identification-58 (CGI-58), also designated as αβ-hydrolase domain-containing 5, develop neutral lipid storage disease with ichthyosis (NLSDI) (Lefèvre et al., 2001; Schweiger et al., 2009). In mice, the phenotype of CGI-58 deficiency is even more severe leading to premature lethality soon after birth due to a defect in the transepidermal barrier function of the skin (Radner et al., 2010, 2011). CGI-58 is a cofactor required for the stimulation of the enzymatic activity of adipose triglyceride lipase (ATGL), the rate-limiting enzyme in the catabolism of intracellular triglyceride (TG) deposits in most if not all organs of the body (Lass et al., 2006, 2011; Zierler et al., 2014). Remarkably, humans and mice harboring mutant ATGL alleles show normal skin development and function indicating that CGI-58 possesses an ATGL-independent role in the skin. The fact that CGI-58 is critically required for the ATGL-mediated TG catabolism in multiple organs of the body including liver, muscle, and adipose raised the question whether the skin barrier defect and postnatal lethality of mice globally lacking CGI-58 (Cgi-58<sup>−/−</sup>) solely originates from the lack of epidermal CGI-58 or from changes in whole-body TG and energy homeostasis.

To investigate the epidermis-specific role of CGI-58 in skin development, we disrupted CGI-58 expression exclusively in keratinocytes and examined the consequences on epidermal barrier formation and systemic lipid and energy homeostasis. Moreover, we generated CGI-58-deficient mice solely expressing CGI-58 in an early or late stage of keratinocyte differentiation to unravel the temporal and spatial role of CGI-58 in epidermal development. Finally, we examined the role of CGI-58 in ATGL-dependent and -independent...
epidermal TG catabolism to address whether CGI-58 may additionally activate a yet unknown epidermal TG lipase required for efficient fatty acid (FA) supply as a substrate for the synthesis of complex epidermal lipids.

RESULTS

Epidermal CGI-58 is a prerequisite for functional skin barrier formation

To generate mice lacking CGI-58 exclusively in the epidermis, we bred mice expressing the Cre recombinase transgene under the control of the epidermis-specific human keratin 14 (K14) promoter (Vasioukhin et al., 1999) with mice homozygous for the Cgi-58-flox allele (Cgi-58<sup>fl<sup>ox</sup></sup>) (Zierler et al., 2013). Very similar to mice globally lacking CGI-58, Cgi-58<sup>fl<sup>ox</sup></sup> mice heterozygous for the K14-Cre recombinase transgene (Cgi-58<sup>epi</sup>−/−) died within 12 hours after birth and exhibited a glossy thin skin (Figure 1a). Western blotting experiments revealed that CGI-58 protein expression was not detectable in the epidermis of Cgi-58<sup>epi</sup>−/− mice, mildly reduced in the dermis, and unchanged in liver, lung, and heart when compared with levels in Cgi-58<sup>fl<sup>ox</sup></sup> littermates (Supplementary Figure S1 online).

Reduced CGI-58 protein expression in the tongue is in accordance with K14 expression in tongue epithelial cells (Vasioukhin et al., 1999). Morphologically, newborn Cgi-58<sup>epi</sup>−/− mice were smaller and wet weight was reduced (∼25%) compared with Cgi-58<sup>fl<sup>ox</sup></sup> littermates (Figure 1b). Plasma energy substrates including FA, glycerol, TG, and glucose were markedly decreased in Cgi-58<sup>epi</sup>−/− mice (ranging from −47% to −86%) compared with controls (Table 1), which may derive from the absence of suckling in newborn Cgi-58<sup>epi</sup>−/− mice.

The skin of Cgi-58<sup>epi</sup>−/− mice exhibited intense penetration of a toluidine blue solution characteristic for a severe defect in the epidermal water permeability barrier (Figure 1c). Histological analyses of skin sections by light or transmission electron microscopy (TEM) revealed orthoperkeratosis with a strongly condensed stratum corneum (SC) and a relatively thin layer of granular keratinocytes (Figure 1d and e). Consistent with this ichthyosiform skin phenotype, degradation of cornesomesomes was decelerated in Cgi-58<sup>epi</sup>−/− SC indicative of an abnormal desquamation. In fact, TEM revealed retained nonperipheral cornesomesomes up to the upper SC layers of Cgi-58<sup>epi</sup>−/− epidermis (Figure 1f), which are normally degraded during corneocyte maturation (Ishida-Yamamoto and Igawa 2014). Correspondingly, desmoglein 1, a major extracellular component of cornesomesomes, was undetectable in the SC of control mice but preserved throughout the SC of Cgi-58<sup>epi</sup>−/− mice (Figure 1g).

Next, the expression and distribution of proteins representative for basal or hyperproliferative keratinocytes were examined by immunohistochemistry of skin sections. K6 was massively stained in keratinocytes of the upper stratum spinosum and stratum granulosum throughout the interfollicular epidermis of Cgi-58<sup>epi</sup>−/− mice (Figure 1h, upper panel) compared with the unique localization of K6 to hair follicles in Cgi-58<sup>fl<sup>ox</sup></sup> controls. K14 was exclusively present in the basal and lower spinous epidermal layers of controls, whereas the protein was more diffusely distributed reaching keratinocytes of the upper stratum spinosum and stratum granulosum (Figure 1h, middle panel) in Cgi-58<sup>epi</sup>−/− mice. Expression of filaggrin (FLG), a marker for terminally differentiated keratinocytes (Sandilands et al., 2009), was reduced in Cgi-58<sup>epi</sup>−/− epidermis compared with controls (Figure 1h, lower panel), indicating a delay in keratinocyte differentiation. The latter was in line with TEM findings, revealing smaller proFLG containing F-granules in Cgi-58<sup>epi</sup>−/− granular keratinocytes (Figure 1e). Western blot analysis of FLG protein expression further showed insufficient proFLG processing with increased abundance of multimeric, nonprocessed FLG units at the expense of active, monomeric FLG in Cgi-58<sup>epi</sup>−/− compared with Cgi-58<sup>fl<sup>ox</sup></sup> epidermis (Figure 1i). In accordance with delayed keratinocyte differentiation, protein expression of loricrin, the main constituent of the cornified envelope (CE) expressed late during cornification (Candi et al., 2005), was strongly decreased in epidermal extracts of Cgi-58<sup>epi</sup>−/− mice (Figure 1j, upper panel). In contrast, protein expression of involucrin (IVL), which is an early marker in CE formation, was only marginally reduced (Figure 1j, lower panel). Taken together, these findings demonstrate that similar to global CGI-58 deficiency, epidermis-specific deletion of CGI-58 causes a defect in the epidermal permeability barrier that is linked to abnormal desquamation and delayed keratinocyte differentiation. Thus, tissue autonomous alterations in epidermal keratinocyte metabolism are causative for defective epidermal development in global CGI-58-deficient mice.

CGi-58 is essential for embryonic skin barrier formation

Cgi-58<sup>epi</sup>−/− (and Cgi-58−/−) mice show severe ichthyosis already at birth indicating that CGI-58 is critically required for prenatal skin barrier formation. Thus, we explored the role of CGI-58 in embryonic skin development using both an inward skin permeability assay that reveals first stages in skin barrier formation (Hardman et al., 1998) and a gravimetric measurement of the transepidermal water loss (TEWL) (Hanley et al., 1996; Nolte et al., 1993). The epidermal barrier forms late in embryogenesis starting at embryonic day (E) 16.5 of gestational age (Hardman et al., 1998), which is in accordance with the intensive toluidine blue staining of Cgi-58<sup>fl<sup>ox</sup></sup> and Cgi-58<sup>epi</sup>−/− embryos at E15.5 (Figure 2a). At E16.5, both Cgi-58<sup>fl<sup>ox</sup></sup> and Cgi-58<sup>epi</sup>−/− mice showed a reduction in dorsal toluidine blue staining indicating the initiation of barrier acquisition. At this stage, the extent of TEWL was comparable between Cgi-58<sup>fl<sup>ox</sup></sup> and Cgi-58<sup>epi</sup>−/− skin explants (Figure 2b) despite the apparent lack of CGI-58 in the epidermis (Figure 2c). Barrier function was almost complete at E17.5 in Cgi-58<sup>fl<sup>ox</sup></sup> control mice as evident from marginal toluidine blue staining and a drastic reduction in TEWL values (−88%). Curiously, Cgi-58<sup>epi</sup>−/− mice also established a pronounced toluidine blue-resistant barrier at E17.5 (Figure 2a) and exhibited functional proFLG processing (Figure 2d), indicative of proper keratinocyte differentiation at this stage. In line, TEWL values markedly decreased in E17.5 Cgi-58<sup>epi</sup>−/− skin explants (−60%), yet to a lesser extent compared with Cgi-58<sup>fl<sup>ox</sup></sup> controls (Figure 2b). Concomitant with a marked increase in covalently bound ω-hydroxy-ceramides (ω-OH-Cer) (Figure 2e), TEWL decreased to the level of neonates in E18.5 Cgi-58<sup>fl<sup>ox</sup></sup> embryos (Figure 2b) indicating full barrier competence.
Figure 1. Defective skin permeability barrier and ichthyosis in Cgi-58<sup>−/−</sup> mice. (a) Phenotype, (b) body wet weight (n = 10), and (c) skin permeability barrier function analyzed by toluidine blue staining of newborn Cgi-58<sup>fl/fl</sup> and Cgi-58<sup>−/−</sup> mice. (d) Histology (hematoxylin and eosin staining; scale bar = 50 µm) and (e) transmission electron microscopy (TEM; scale bar = 1 µm) show hyperkeratosis, smaller F-granules, and lipid droplets (arrows) throughout the stratum corneum (SC) of Cgi-58<sup>−/−</sup> mice. (f) Impaired degradation of corneodesmosomes (arrows) in Cgi-58<sup>−/−</sup> mice as analyzed by TEM (scale bar = 1 µm). (g) Immunohistochemistry of desmoglein 1/2 (DSG1/2), keratin 6 (K6), K14, and filaggrin (FLG) in skin sections of newborn Cgi-58<sup>fl/fl</sup> and Cgi-58<sup>−/−</sup> mice. Protein levels of (i) FLG or (j) loricrin (LOR) and involucrin (IVL) detected by western blot analysis using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as loading control. ***P < 0.001. CGI-58, comparative gene identification-58; SG, stratum granulosum.
Table 1. Plasma energy metabolites of newborn Cgi-58\textsuperscript{flox/flox} and Cgi-58\textsuperscript{epid−/−} mice\textsuperscript{1}

|                | Cgi-58\textsuperscript{flox/flox} | Cgi-58\textsuperscript{epid−/−} |
|----------------|-------------------------------|-------------------------------|
| FA (mM)        | 0.57 ± 0.12                   | 0.21 ± 0.08\textsuperscript{**} |
| Glycerol (mg/dl) | 39.36 ± 17.67                 | 5.37 ± 2.68\textsuperscript{**} |
| TG (mg/dl)     | 101.52 ± 29.50                | 15.75 ± 3.63\textsuperscript{**} |
| Glucose (mg/dl)| 52.80 ± 9.95                  | 27.89 ± 7.61\textsuperscript{**} |

Abbreviations: FA, free fatty acid; TG, triglyceride.
\textsuperscript{1}Plasma parameters were measured on average 10 h postpartum (n = 13). Data are shown as means ± standard deviations and are representative for three independent measurements.
\textsuperscript{**}p < 0.001.

In contrast, levels of protein-bound ω-OH-Cer remained low in Cgi-58\textsuperscript{epid−/−}/embryos at that stage (Figure 2e) paralleled by high TEWL values (8.4-fold compared with Cgi-58\textsuperscript{flox/flox}), massive toluidine staining, and impaired proFLG processing (Figure 2d). These findings suggest a defect in epidermal barrier acquisition in Cgi-58-deficient embryos that is primarily linked to abnormal corneocyte lipid envelope (CLE) formation. Yet, barrier competence improved between E18.5 and birth in Cgi-58\textsuperscript{flox/flox} neonates, albeit TEWL was still increased 4-fold compared with Cgi-58\textsuperscript{flox/flox} controls. Wet body weights of Cgi-58\textsuperscript{flox/flox} embryos were comparable with Cgi-58\textsuperscript{flox/flox} littermates during different gestational ages but were reduced (−25%) after birth (Figure 2f), most likely the consequence of rapid water loss and absence of suckling.

Epidermal barrier formation depends on CGI-58 expression in suprabasal epidermal layers

Next, we investigated the temporal and spatial role of CGI-58 expression during keratinocyte differentiation and skin barrier formation. We generated transgenic mice expressing CGI-58 either under the control of the human K14 or the IVL promoter, which activates gene expression in basal (Vasilevskikh et al., 1999; Vassar et al., 1989) or differentiated keratinocytes (Carroll and Taichman, 1992; Carroll et al., 1993), respectively. Mice expressing the Cgi-58 transgene under the control of the K14 promoter [Tg(K14-Cgi58\textsuperscript{663Biat}], subsequently designated K14C (Supplementary Figure S2a online), were bred onto a CGI-58-deficient background to generate mice that express CGI-58 exclusively in the basal epidermal layer (Cgi-58\textsuperscript{−/−}/K14C). Cgi-58\textsuperscript{−/−}/K14C mice appeared smaller, exhibited a shiny rigid skin (Figure 3a), and died postnatally similar to Cgi-58\textsuperscript{−/−} and Cgi-58\textsuperscript{epid−/−} mice. Western blot analysis confirmed epidermis-specific expression of CGI-58 (Supplementary Figure S2b). Nevertheless, the epidermis of Cgi-58\textsuperscript{−/−}/K14C mice was strongly permeable for toluidine blue (Figure 3b) and morphologically very similar to that of Cgi-58\textsuperscript{−/−} and Cgi-58\textsuperscript{epid−/−} mice (Figure 3c). Accordingly, restoration of CGI-58 expression solely in basal keratinocytes is not sufficient for the establishment of a functional epidermal barrier.

Next, mice expressing the Cgi-58 transgene under the control of the human IVL promoter [Tg(IVL-Cgi58\textsuperscript{663Biat}], subsequently designated IVLC (Supplementary Figure S2c), were bred onto the CGI-58-deficient background to generate mice that express CGI-58 exclusively in suprabasal epidermal layers (Cgi-58\textsuperscript{−/−}/IVLC). Notably, IVL promoter-driven CGI-58 expression rescued the lethal skin barrier defect of Cgi-58\textsuperscript{−/−} mice, and mutant mice were phenotypically indistinguishable from wild-type (WT) littermates (Figure 3d). This indicates that CGI-58 expression in suprabasal epidermal layers is a prerequisite for the establishment of a functional skin permeability barrier. In epidermal preparations from Cgi-58\textsuperscript{−/−}/IVLC mice, CGI-58 protein levels were 13-fold increased (Supplementary Figure S2d) compared with that of WT mice, whereas in the dermis (except one sample that was presumably contaminated with epidermal proteins), brown adipose tissue, and heart CGI-58 expression was absent (Supplementary Figure S2e). Cgi-58\textsuperscript{−/−}/IVLC mice were protected from toluidine blue staining (Figure 3e) arguing for a functional water permeability barrier in these animals. The SC in Cgi-58\textsuperscript{−/−}/IVLC and WT mice was similar and characterized by the typical basket weave structure (Figure 3f). In contrast to Cgi-58\textsuperscript{−/−} and Cgi-58\textsuperscript{epid−/−} mice, Cgi-58\textsuperscript{−/−}/IVLC transgenic mice survived the suckling period and reached adulthood without apparent hair or skin alterations (Figure 3g). Newborn Cgi-58\textsuperscript{−/−}/IVLC mice were similar to WT mice in terms of body weight and size (Figure 3h and i). Nonetheless, plasma concentrations of FA (−34%), glycerol (−54%), TG (−49%), and glucose (−21%) were reduced in Cgi-58\textsuperscript{−/−}/IVLC compared with WT mice (Supplementary Table S1 online) but markedly higher than in Cgi-58\textsuperscript{−/−} and Cgi-58\textsuperscript{epid−/−} mice (Table 1). Taken together, these data demonstrate that CGI-58 function is essential during late keratinocyte differentiation and the establishment of a functional epidermal permeability barrier.

Epidermal loss of CGI-58 provokes TG deposition in the SC involving impaired ATGL-mediated TG catabolism

Global CGI-58 deficiency significantly increases the epidermal TG content, which is less pronounced in ATGL-deficient mice (Radner et al., 2010). Previously, it has been hypothesized that impaired generation of long chain unsaturated FAs as a substrate for ω-OH-acylceramide (ω-O-AcylCer) synthesis may originate from the lack of CGI-58 as a coactivator of an unknown epidermal TG lipase in Cgi-58\textsuperscript{−/−} mice and patients with NLSDI (Radner et al., 2010; Uchida et al., 2010). Consistent with this hypothesis, the epidermis-specific deletion of CGI-58 strongly increased epidermal TG levels, whereas CGI-58 expression exclusively in suprabasal epidermal layers of Cgi-58\textsuperscript{−/−}/IVLC mice did not interfere with epidermal TG homeostasis (Figure 4a). Ultrastructural analysis of the epidermis by TEM revealed the presence of lipid droplet inclusions within corneocytes of Cgi-58\textsuperscript{epid−/−} mice (Figures 1e and 4b), which is consistent with observations in patients with NLSDI (Akiyama et al., 2003; Demerjian et al., 2006). Furthermore, label-free coherent anti-Stokes Raman scattering microscopy revealed numerous neutral lipid puncta in the SC of Cgi-58\textsuperscript{epid−/−} mice, which was not observed in Cgi-58\textsuperscript{flox/flox} epidermal sections (Figure 4c). These findings suggest that the impairment of epidermal but not global TG metabolism causes lipids accumulation in CGI-58-deficient mice (and patients with NLSDI).

The availability of a small molecule inhibitor of ATGL activity (Atglistatin) (Mayer et al., 2013) allowed us to examine whether CGI-58 stimulates epidermal TG catabolism via an ATGL-independent TG hydrolase. We measured
TG-hydrolytic activities in epidermal preparations of controls (Cgi-58\(^{\text{flox/flox}}\) or WT mice), Cgi-58\(^{\text{epid/C0/C0}}\), and Cgi-58\(^{\text{C0/C0/IVLC}}\) mice in both the absence and presence of recombinant CGI-58 and/or Atglistatin. TG-hydrolytic activities were markedly reduced (\(\sim 55\%\)) in epidermal preparations of Cgi-58\(^{\text{epid/C0/C0}}\) compared with Cgi-58\(^{\text{flox/flox}}\) mice and the presence of recombinant CGI-58 significantly increased the activities in samples of both genotypes (Figure 4d). In line with the established role of CGI-58 as a lipolytic coactivator, TG-hydrolytic activities of Cgi-58\(^{\text{epid/C0/C0}}\) samples reached levels comparable with Cgi-58\(^{\text{flox/flox}}\) epidermal preparations on addition of recombinant CGI-58. The addition of Atglistatin completely abolished this CGI-58-mediated increase in TG-hydrolytic activity, indicating that the CGI-58 effect on TG hydrolysis exclusively depends on ATGL. Notably, in the presence of Atglistatin, substantial TG-hydrolytic activity was measured in epidermal lysates of Cgi-58\(^{\text{flox/flox}}\) and Cgi-58\(^{\text{epid/C0/C0}}\) mice, which is in line with the presence of several potential epidermal TG-lipase(s) (Toulza et al., 2007). Based on our results, it is very unlikely that these TG hydrolases depend on CGI-58 as a coactivator.

Unlike epidermal CGI-58 disruption, transgenic epidermis-specific overexpression of CGI-58 in Cgi-58\(^{\text{C0/C0/InvC}}\) mice led to a 79% increase in TG-hydrolytic activity in epidermal lysates when compared with WT samples (Figure 4e). The addition of recombinant CGI-58 to epidermal preparations of

**Figure 2.** Embryonic skin permeability barrier development in the absence of CGI-58. Skin permeability barrier function of Cgi-58\(^{\text{epid/C0/C0}}\) and Cgi-58\(^{\text{flox/flox}}\) mice (a) assayed by toluidine blue staining between embryonic stages E15.5–E18.5, and (b) measured using a gravimetric TEWL assay at E16.5–E18.5, and P0. (c) CGI-58 and (d) filaggrin (FLG) protein levels analyzed by western blotting at indicated gestational ages in epidermal extracts of Cgi-58\(^{\text{epid/C0/C0}}\) and Cgi-58\(^{\text{flox/flox}}\) mice (loading control = GAPDH). (e) Levels of covalently bound \(\omega\)-hydroxy-ceramides (\(\omega\)-OH-Cer) were determined by UPLC/MS in E16.5, E17.5, E18.5, and P0 epidermis from Cgi-58\(^{\text{epid/C0/C0}}\) and Cgi-58\(^{\text{flox/flox}}\) mice (\(n = 4–6\)). (f) Body wet weight of Cgi-58\(^{\text{epid/C0/C0}}\) and Cgi-58\(^{\text{flox/flox}}\) mice during late embryonic development (E15.5–E18.5) and shortly after birth (P0). Data are presented as means ± SD and are representative for two independent experiments. ***\(p < 0.001\). CGI-58, comparative gene identification-58; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MS, mass spectrometry; SD, standard deviation; TEWL, transepidermal water loss; UPLC, ultra performance liquid chromatography.
WT mice raised the lipase activities to levels measured in epidermal extracts of transgenic Cgi-58<sup>+/−</sup>/InvC mice. Notably, inhibition of ATGL by Atglistatin reduced epidermal TG-hydrolytic activities in Cgi-58<sup>+/−</sup>/InvC mice to those measured in Atglistatin-treated WT samples. This further supports the conclusion that CGI-58 controls epidermal TG catabolism exclusively via activating ATGL. To further verify this assumption, we determined TG-hydrolase activities in epidermal extracts of ATGL-deficient (Atgl<sup>−/−</sup>) mice in the presence of recombinant CGI-58 and/or Atglistatin (Figure 4 f). Consistent with the conclusion that CGI-58 does not activate a “non-ATGL” TG lipase, the presence of recombinant CGI-58 raised TG-hydrolytic activities in WT but not in Atgl<sup>−/−</sup> epidermal extracts.

Defective ω-O-AcylCer formation is reversed in Cgi-58<sup>−/−</sup>/IVLC mice

The water-resistant epidermal barrier is due to the unique architecture of the SC including extracellular lipid bilayers organized in between the CLEs of the corneocytes. To investigate the role of epidermal CGI-58 in the creation of the SC lipid barrier, we first assessed the SC ultrastructure of WT-specific CGI-58-deficient and control mice by TEM using both osmium tetroxide and ruthenium tetroxide postfixation methods. Cgi-58<sup>−/−</sup> epidermis revealed aberrant lamellar body internal structures, inhomogeneous lamellar body secretion with entombed organelles in corneocytes, and impaired postsecretory lipid processing, resulting in disorganized lamellar bilayers as compared with the uniformly organized lamellar bilayers in controls (Figure 5 a). Furthermore, whereas Cgi-58<sup>floxed/floxed</sup> corneocytes were surrounded by a lipid monolayer representing the CLE, this structure was absent in the SC of Cgi-58<sup>−/−</sup>/IVLC mice (Figure 5b). The CE was present in both mouse models, albeit CE appeared slightly thinner in Cgi-58<sup>−/−</sup>/IVLC mice (Figure 5b). Thus, like in patients with NLSDI (Uchida et al., 2010), abnormalities in SC extracellular lipid structures affecting both lamellar bilayers and the CLE might underlie the permeability barrier defect in epidermis-specific CGI-58-deficient mice.

Next, we performed lipid analyses by HPLC/LSD and UPLC/mass spectrometry of epidermal lipid preparations derived from Cgi-58<sup>−/−</sup>/IVLC mice. Levels of cholesterol, FAs, CerNS (representative for nonacylated ceramides), and ω-O-AcylCer, characteristic for extracellular lamellar bilayers, were determined. Although concentrations of cholesterol, FAs, and CerNS were not or only moderately altered, levels of ω-O-(18:2)AcylCer, the most prominent ω-O-AcylCer species in the epidermis, were drastically reduced...
DISCUSSION

In this study, we examined the temporal and spatial role of epidermal CGI-58 in keratinocyte differentiation and the formation of a functional skin barrier. We demonstrate that epidermis-specific disruption of CGI-58 in basal keratinocytes and consequently in all epidermal layers provokes a lethal skin barrier defect almost identical to that observed in mice globally lacking CGI-58 (Radner et al., 2010). Conversely, functional restoration of CGI-58 expression in differentiated keratinocytes (Cgi-58+/IVL/C) was compatible with normal skin barrier function and survival of newborn Cgi-58+/− mice. These findings suggest that the ichthyosiform skin phenotype and systemic changes in TG and energy homeostasis in newborn Cgi-58+/− mice primarily originate from the tissue-specific absence of CGI-58 in the epidermis. This is in contrast to DGAT2-deficient mice, where the lethal skin barrier defect does not predominantly evolve from impaired epidermal TG metabolism (Stone et al., 2004). Yet, it relates to mice lacking elongation of very long chain FA or FA transport protein 4, which exhibit comparable skin barrier abnormalities that can be rescued by expression of the respective protein in suprabasal keratinocytes (McMahon et al., 2011; Moulson et al., 2007). Both proteins are involved in the synthesis of epidermis-specific ultralong chain ceramides that essentially contribute to the SC lipid barrier. In line with a critical role of CGI-58 in the synthetic
Figure 5. Defective \( \omega \)-O-acylceramide and CLE formation caused by global CGI-58-deficiency is reversed on CGI-58 expression in the epidermis. (a) Compared with uniformly organized lamellar bilayers in Cgi-58\textsuperscript{flox/flox}, bilayers show nonlamellar domains (arrows) in Cgi-58\textsuperscript{mid−/−} mice. This can be ascribed to aberrant lamellar body (LB) internal structures (arrowheads), inhomogeneous LB secretion with entombed organelles in corneocytes (asterisks), and impaired postsecretory lipid processing (double arrows). TEM; ruthenium postfixation; scale bars = 100 nm. (b) Absent corneocyte lipid envelopes (arrows) and slightly thinner cornified envelopes (arrowheads) in Cgi-58\textsuperscript{epi−/−} compared with Cgi-58\textsuperscript{flox/flox} epidermis. TEM; pretreatment with pyridine; scale bar = 100 nm. Lipid analyses were performed by HPLC/LSD (cholesterol) or UPLC/MS (FA, ceramides) of indicated epidermal lipid extracts. Levels of (c) cholesterol, (d) FA, CerNS, and \( \omega \)-O-(18:2)AcylCer, of (e) \( \omega \)-O-(18:2)AcylGlcCer, and of (f) covalently bound \( \omega \)-OH-Cer and FA in Cgi-58\textsuperscript{mid−/−} and Cgi-58\textsuperscript{flox/flox} epidermis. (g) Levels of free \( \omega \)-OH-Cer and their glucosylated derivatives in Cgi-58\textsuperscript{mid−/−} and Cgi-58\textsuperscript{flox/flox} epidermis. Values represent means ± SD (n = 5) and are representative for three independent measurements. *P < 0.05; ***P < 0.001. CGI-58, comparative gene identification-58; CLE, corneocyte lipid envelope; F, F-granule; FA, fatty acid; LSD, light-scattering detection; MS, mass spectrometry; N, nucleus; \( \omega \)-O-(18:2)AcylGlcCer, glucosylated \( \omega \)-O-AcylCeramide; \( \omega \)-OH-Cer, \( \omega \)-hydroxy- ceramides; SD, standard deviation; TEM, transmission electron microscopy; UPLC, ultra performance liquid chromatography; WT, wild type.
Skin Function Depends on Epidermal CGI-58

K14 has shown that driven transgene expression. A study by Merrill et al. (2001) suitable for immunohistochemistry, reliable conclusions may epidermis due to the unavailability of a CGI-58 antibody in CGI-58-deficient mice lacking CE and CLE, E17.5, further barrier acquisition was delayed and did not result from lipid barrier dysfunctions. This assumption was further confirmed by the finding that proFLG processing was initially functional at E17.5 even in the absence of CGI-58. On the other hand, epidermal K6 expression, a marker for actively proliferating cells, was significantly increased in Cgi-58epid-/- epidermis, which might relate to a general attempt of the skin to counteract defective epidermal barrier formation via keratinocyte hyperproliferation (Elias et al., 2008).

In agreement with a previous report (Hardman et al., 1998), we found that resistance toward dye penetration emerges shortly before birth. Both barrier patterning and TEWL levels were similar between Cgi-58epid-/- and WT embryos at E16.5, indicating that the initiation of embryonic barrier formation occurs independently of epidermal CGI-58. Although Cgi-58epid-/- mice gained considerable barrier properties at E17.5, further barrier acquisition was delayed and did not progress to full barrier competence. This is in contrast to mice with transglutaminase 1 deficiency, lacking CE and CLE, resulting in the complete absence of barrier development (Kuramoto et al., 2002). The late onset of barrier dysfunction in CGI-58-deficient epidermis might reflect a unique role of CGI-58 in epidermal lipid barrier formation. In fact, WT embryos already exhibited an almost functional CLE at E18.5 characterized by a marked raise in ultralong chain ceramides covalently attached to the CE, which is in accordance with a study by Doering (2002). In parallel, Cgi-58epid-/- mice failed to produce sufficient covalently bound ω-OH-Cer (and ω-OH-C30:0-Cer, data not shown) providing further evidence that the skin barrier defect on CGI-58 deficiency is primarily linked to defective ω-O-AcylCer and CLE formation.

CGI-58 is an established coactivator of ATGL and the absence of either CGI-58 or ATGL strongly impairs TG and energy catabolism in multiple organs (Haemmerle et al., 2006; Lord and Brown, 2012; Zierler et al., 2014). Normal skin development of ATGL-deficient mice has led to the notion that CGI-58 could coactivate a yet unknown epidermal TG hydrolase required for adequate FA supply as energy fuel and/or substrate for the generation of complex epidermal lipids (Radner et al., 2010; Uchida et al., 2010). Experiments with the recently developed ATGL inhibitor Atglistatin (Mayer et al., 2013) argue against this hypothesis. Although merely 30% of the total TG-hydrolase activity was inhibited by Atglistatin and therefore attributable to ATGL, the remaining 70% of “non-ATGL” TG-hydrolase activity was not affected by the presence or absence of CGI-58. Thus, it is unlikely that CGI-58 acts as a coactivator of an epidermal TG lipase other than ATGL. Because ATGL deficiency can be fully compensated by other epidermal lipases and lack of ATGL does not impact epidermal ω-O-AcylCer levels, it may be concluded that the provision of FA from TG for ω-O-AcylCer formation is redundant and that CGI-58-stimulated FA release is not limiting in this process.

ω-O-Acyl(Glc)Cer as integral constituents of extracellular lamellar bilayers and obligatory precursors for CLE formation (Breiden and Sandhoff, 2014; Elias et al., 2014; Rabionet et al., 2014; Uchida and Holleran, 2008) are indispensable for epidermal permeability barrier function. Their biosynthesis requires multiple enzymatic steps most of which are unique to the epidermis and limiting for epidermal barrier formation, as evidenced by the phenotype of mice lacking one of the respective enzymes (Breiden and Sandhoff, 2014; Rabionet et al., 2014). A common feature of these mouse models is the absence of covalently bound ω-OH-Cer. The very low levels of ω-O-Acyl(Glc)Cer and subsequently the deficient CLE in Cgi-58epid-/- mice implicate that CGI-58 is critically involved in the generation of these lipid classes and confirms previous reports (Radner et al., 2010; Uchida et al., 2010). Importantly, Cgi-58epid-/- epidermis accumulates both glucosylated and nonglucosylated free ω-OH-Cer, which is so far unique as compared with other mouse models affecting the synthesis of epidermis-specific ultralong chain ceramides. Furthermore, we show that overexpression of CGI-58 in the epidermis increases levels of ω-O-Acyl(Glc)Cer. These findings strongly suggest that CGI-58 actively participates in the acylation of ω-OH-Cer to generate ω-O-Acyl(Glc)Cer.

Whether CGI-58 exhibits acyltransferase activity (Ghosh et al., 2008; Khatib et al., 2016; McMahon et al., 2014; Montero-Moran et al., 2010) is controversial. Extensive experiments in our laboratory have not provided any evidence for an enzymatic activity of CGI-58 to catalyze the transfer of linoleic acid onto ω-OH-Cer. Alternatively, it is conceivable that CGI-58 acts as a coactivator to mediate acylation of ω-OH-Cer by a currently unknown acyltransferase. Indeed, low levels of covalently bound ω-OH-Cer are present in CGI-58-deficient epidermis suggesting that the formation of the CLE is not entirely absent but rather highly inefficient. However, following the protocol of Takagi et al. (2004), we were unable to measure ω-acyl activity when epidermal preparations of WT and CGI-58-deficient mice were incubated with ω-OH-C30:0-ceramide and radioactive linoleoyl-CoA. We assume that either the epidermal ω-acyl activity is too low to be detectable in our assay system or that linoleoyl-CoA is not the appropriate acyl-group donor in this reaction. It is conceivable, for example, that linoleic acid is transferred to ω-OH-Cer via a
transacylation reaction from a linoleate-containing epidermal lipid species (e.g., TG and/or glycerophospholipids).

To summarize, we show that ω-O-AcylCer and CLE formation critically depend on CGI-58 expression in differentiated keratinocytes. Furthermore, we deliver several lines of evidence that the availability of epidermal CGI-58 is a prerequisite for the formation of ω-O-Acyl(Glc)Cer, which is independent of the role of CGI-58 as a coactivator of epidermal and nonepidermal TG catabolism.

**MATERIALS AND METHODS**

**Animals**

CGI-58<sup>flu<sup>flox</sup></sup>-<sup>−</sup>- mice were generated by breeding Cgi-58 floxed mice (Radner et al., 2010; Zierler et al., 2013) with transgenic mice expressing K14-driven Cre recombinase (Vasioukhin et al., 1999). For transgene constructs, murine Cgi-58 cDNA was cloned downstream of the human K14 (Vassar et al., 1989) or IVL promoter (Carroll et al., 1993; Carroll and Taichman, 1992) thereby replacing the Cre recombinase or the galactosidase insert, respectively. Primer sequences used for cloning are listed in Supplementary Table S2 online. Microinjections were performed according to a standard protocol. All animal studies were approved by the Austrian Federal Ministry for Science and Research and by the ethics committee of the University of Graz and of the University of Veterinary Medicine Vienna.

**Plasma parameters**

Blood sampling and measurement of plasma parameters were performed as described previously (Radner et al., 2010).

**Lipid analyses and in vitro TG-hydrolase activity assays**

Procedures are described in the Supplementary Materials and Methods online.

**Western blot analysis**

Western blots were performed according to standard protocols applying commercially available antibodies listed in Supplementary Table S3 online.

**Skin permeation, gravimetric TEWL assay, histology, immunohistochemistry, and TEM**

Methods are described in the Supplementary Materials and Methods.

**Coherent anti-Stokes Raman scattering microscopy**

Paraformaldehyde-fixed cryo sections (10 µm) of skin samples from newborn mice were used to visualize neutral lipids by coherent anti-Stokes Raman scattering microscopy as described in the Supplementary Materials and Methods.

**Statistics**

Statistical analysis was performed using the unpaired two-tailed Student's t-test.

**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.2016.09.025.

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