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

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Abstract

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

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CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

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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 α/β-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*$^{-/-}$) 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-floxed* allele (*Cgi-58*$^{flox/flox}$) (Zierler et al., 2013). Very similar to mice globally lacking CGI-58, *Cgi-58*$^{flox/flox}$ mice heterozygous for the *K14-Cre* recombinase transgene (*Cgi-58*$^{epid-/-}$) died within 12 hours after birth and exhibited a glossy tight skin (Figure 1a). Western blotting experiments revealed that CGI-58 protein expression was not detectable in the

epidermis of *Cgi-58*$^{epid-/-}$ mice, mildly reduced in the dermis, and unchanged in liver, lung, and heart when compared with levels in *Cgi-58*$^{flox/flox}$ controls (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*$^{epid-/-}$ mice were smaller and wet weight was reduced (−25%) compared with *Cgi-58*$^{flox/flox}$ littermates (Figure 1b). Plasma energy substrates including FA, glycerol, TG, and glucose were markedly decreased in *Cgi-58*$^{epid-/-}$ mice (ranging from −47% to −86%) compared with controls (Table 1), which may derive from the absence of suckling in newborn *Cgi-58*$^{epid-/-}$ mice.

The skin of *Cgi-58*$^{epid-/-}$ 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 orthohyperkeratosis 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 corneodesmosomes was decelerated in *Cgi-58*$^{epid-/-}$ SC indicative of an abnormal desquamation. In fact, TEM revealed retained nonperipheral corneodesmosomes up to the upper SC layers of *Cgi-58*$^{epid-/-}$ epidermis (Figure 1f), which are normally degraded during corneocyte maturation (Ishida-Yamamoto and Igawa 2014). Correspondingly, desmoglein 1, a major extracellular component of corneodesmosomes, was undetectable in the SC of control mice but preserved throughout the SC of *Cgi-58*$^{epid-/-}$ 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*$^{epid-/-}$ mice (Figure 1h, upper panel) compared with the unique localization of K6 to hair follicles in *Cgi-58*$^{flox/flox}$ 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*$^{epid-/-}$ mice. Expression of filaggrin (FLG), a marker for terminally differentiated keratinocytes (Sandilands et al., 2009), was reduced in *Cgi-58*$^{epid-/-}$ 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*$^{epid-/-}$ 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*$^{epid-/-}$ compared with *Cgi-58*$^{flox/flox}$ 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*$^{epid-/-}$ 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$^{epid-/-}$ (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*$^{flox/flox}$ and *Cgi-58*$^{epid-/-}$ embryos at E15.5 (Figure 2a). At E16.5, both *Cgi-58*$^{flox/flox}$ and *Cgi-58*$^{epid-/-}$ 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*$^{flox/flox}$ and *Cgi-58*$^{epid-/-}$ 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*$^{flox/flox}$ control mice as evident from marginal toluidine blue staining and a drastic reduction in TEWL values (−88%). Curiously, *Cgi-58*$^{epid-/-}$ 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 levels markedly decreased in E17.5 *Cgi-58*$^{epid-/-}$ skin explants (−60%), yet to a lesser extent compared with *Cgi-58*$^{flox/flox}$ 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*$^{flox/flox}$ embryos (Figure 2b) indicating full barrier competence. In contrast, levels of protein-bound ω-OH-Cer remained low in *Cgi-58*$^{epid-/-}$ embryos at that stage (Figure 2e) paralleled by high TEWL values (8.4-fold compared with *Cgi-58*$^{flox/flox}$), massive toluidine staining, and impaired proFLG processing (Figure 2d). These findings suggest a defect in epidermal barrier acquisition in CGI-58-deficient epidermis that is primarily linked to abnormal corneocyte lipid envelope (CLE) formation. Yet, barrier competence improved between E18.5 and birth in *Cgi-58*$^{epid-/-}$ neonates, albeit TEWL was still increased 4-fold compared with *Cgi-58*$^{flox/flox}$ controls. Wet body weights of *Cgi-58*$^{epid-/-}$ embryos were comparable with *Cgi-58*$^{flox/flox}$ 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 (Vasioukhin 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)604Biat*], 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*$^{-/-}$/*K14C*). *Cgi-58*$^{-/-}$/*K14C* mice appeared smaller, exhibited a shiny rigid skin (Figure 3a), and died postnatally similar to *Cgi-58*$^{-/-}$ and *Cgi-58*$^{epid-/-}$ mice. Western blot analysis confirmed epidermis-specific expression of CGI-58 (Supplementary Figure S2b). Nevertheless, the epidermis of *Cgi-58*$^{-/-}$*/K14C* mice was strongly permeable for toluidine blue (Figure 3b) and morphologically very similar to that of *Cgi-58*$^{-/-}$ and *Cgi-58*$^{epid-/-}$ 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)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*$^{-/-}$/*IVLC*). Notably, *IVL* promoter-driven CGI-58 expression rescued the lethal skin barrier defect of *Cgi-58*$^{-/-}$ 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*$^{-/-}$/*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*$^{-/-}$*/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*$^{-/-}$/*IVLC* and WT mice was similar and characterized by the typical basket weave structure (Figure 3f). In contrast to *Cgi-58*$^{-/-}$ and *Cgi-58*$^{epid-/-}$ mice, *Cgi-58*$^{-/-}$ *IVLC* transgenic mice survived the suckling period and reached adulthood without apparent hair or skin alterations (Figure 3g). Newborn *Cgi-58*$^{-/-}$/*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*$^{-/-}$*/IVLC* compared with WT mice (Supplementary Table S1 online) but markedly higher than in *Cgi-58*$^{-/-}$ and *Cgi-58*$^{epid-/-}$ 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-O-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*$^{-/-}$ 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*$^{-/-}$*/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*$^{epid-/-}$ 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*$^{epid-/-}$ mice, which was not observed in *Cgi-58*$^{flox/flox}$ epidermal sections (Figure 4c). These findings suggest that the impairment of epidermal but not global TG metabolism causes lipid 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*$^{flox/flox}$ or WT mice), *Cgi-58*$^{epid-/-}$, and *Cgi-58*$^{-/-}$*/IVLC* mice in both the absence and presence of recombinant CGI-58 and/or Atglistatin. TG-hydrolase activities were markedly reduced (−55%) in epidermal preparations of *Cgi-58*$^{epid-/-}$ compared with *Cgi-58*$^{flox/flox}$ 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*$^{epid-/-}$ samples reached levels comparable with *Cgi-58*$^{flox/flox}$ 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*$^{flox/flox}$ and *Cgi-58*$^{epid-/-}$ 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*$^{-/-}$*/InvC* mice led to a 79% increase in TG-hydrolase activity in epidermal lysates when compared with WT samples (Figure 4e). The addition of recombinant CGI-58 to epidermal preparations of WT mice raised the lipase activities to levels measured in epidermal extracts of transgenic *Cgi-58*$^{-/-}$*/InvC* mice. Notably, inhibition of ATGL by Atglistatin reduced epidermal TG-hydrolytic activities in *Cgi-58*$^{-/-}$*/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*$^{-/-}$) mice in the presence of recombinant CGI-58 and/or Atglistatin (Figure 4f). 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*$^{-/-}$ epidermal extracts.

Defective ω-O-AcylCer formation is reversed in *Cgi-58*$^{-/-}$*/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 epidermis-specific CGI-58-deficient and control mice by TEM using both

osmium tetroxide and ruthenium tetroxide postfixation methods. *Cgi-58*$^{epid-/-}$ 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 5a). Furthermore, whereas *Cgi-58*$^{flox/flox}$ corneocytes were surrounded by a lipid monolayer representing the CLE, this structure was absent in the SC of *Cgi-58*$^{epid-/-}$ mice (Figure 5b). The CE was present in both mouse models, albeit CE appeared slightly thinner in *Cgi-58*$^{epid-/-}$ 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*$^{epid-/-}$ and *Cgi-58*$^{flox/flox}$ 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 (−85%) in CGI-58-deficient compared with control samples (Figure 5c). Analysis of these lipid species in isolated SC preparations resulted in comparable results (data not shown). Glucosylated ω-O-AcylCer serve as essential precursors for the covalent linkage of ω-OH-Cer to CE proteins for the formation of the CLE (Breiden and Sandhoff, 2014; Elias et al., 2014; Rabionet et al., 2014; Uchida and Holleran, 2008). Consistent with a marked reduction in glucosylated ω-O-AcylCer (−88%) (Figure 5d), levels of covalently bound ω-OH-Cer as well as protein-bound ultralong chain FAs (Figure 5e) were drastically reduced in the epidermis of *Cgi-58*$^{epid-/-}$ mice (−60 and −75%, respectively). Importantly, the marked decline in−ω-O-(18:2)Acyl(Glc)Cer levels together with the prominent reduction in covalently bound ω-OH-Cer content was completely restored on *IVL*-driven overexpression of the CGI-58 transgene (Figure 5f) in *Cgi-58*$^{-/-}$*/IVLC* mice. In fact, CGI-58 overexpression led to increased levels of ω-O-(18:2)Acyl(Glc)Cer in *Cgi-58*$^{-/-}$*/IVLC* (+23%) or WT/*IVLC* (+34%) compared with WT mice, indicating that CGI-58 plays a prominent role in the biosynthesis of ω-O-(18:2)Acyl(Glc)Cer. This is in line with strongly increased levels of free extractable ω-OH-Cer and their glucosylated derivatives (5.2- and 13.6-fold, respectively) in *Cgi-58*$^{epid-/-}$ epidermis (Figure 5g), which both might serve as a substrate for ω-acylation with linoleic acid.

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*$^{-/-}$*/IVLC*) 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 pathway of essential barrier lipids during late stages of keratinocyte differentiation together with its predominant expression in granular keratinocytes (Akiyama et al., 2008), gain of CGI-58 function solely in basal keratinocytes (*Cgi-58*$^{-/-}$/*K14C*) did not prevent defective skin barrier formation. Although the absence of CGI-58 protein expression in *stratum granulosum* could not be verified in *Cgi-58*$^{-/-}$/*K14C* epidermis due to the unavailability of a CGI-58 antibody suitable for immunohistochemistry, reliable conclusions may be drawn from other mouse models applying *K14* promoter-driven transgene expression. A study by Merrill et al. (2001) has shown that *K14* promoter-driven expression of myc-tagged Tcf3 or Lef1 was targeted to basal keratinocytes and persisted in some suprabasal cells. In agreement with an essential role of CGI-58 in late-stage keratinocyte differentiation, levels of loricrin and monomeric processed FLG, as a measure of advanced cornification (Candi et al., 2005; Sandilands et al., 2009), were markedly reduced in *Cgi-58*$^{epid-/-}$ epidermis. Consistently, nonlateral corneodesmosomes persisted in outermost SC layers preventing shedding of corneocytes further emphasizing a defect in the cornification process. Similar abnormalities in late corneocyte maturation were also reported in ceramide synthase 3, ATP-binding cassette subfamily A, or glucosylceramide synthase mutant mice (Amen et al., 2013; Jennemann et al., 2012; Zuo et al., 2008), suggesting that these defects might generally 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-58*$^{epid-/-}$ 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-58*$^{epid-/-}$ and WT embryos at E16.5, indicating that the initiation of embryonic barrier formation occurs independently of epidermal CGI-58. Although *Cgi-58*$^{epid-/-}$ 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-58*$^{epid-/-}$ mice failed to produce sufficient covalently bound ω-OH-Cer (and ω-

O-(18:2)AcylCer, 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-58*$^{epid-/-}$ 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-58*$^{epid-/-}$ 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-acylation 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-acylation 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$^{epid-/-}$ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATGL	adipose triglyceride lipase
CE	cornified envelope
CGI-58	comparative gene identification-58
CLE	corneocyte lipid envelope
En	embryonic day n
FA	fatty acid
FLG	filaggrin
IVL	involucrin
Kn	keratin n
NLSDI	neutral lipid storage disease with ichthyosis
ω-O-AcylCer	ω-O-acylceramide
ω-OH-Cers	ω-hydroxy-ceramides
SC	stratum corneum
TEM	transmission electron microscopy
TEWL	transepidermal water loss
TG	triglyceride
WT	wild type

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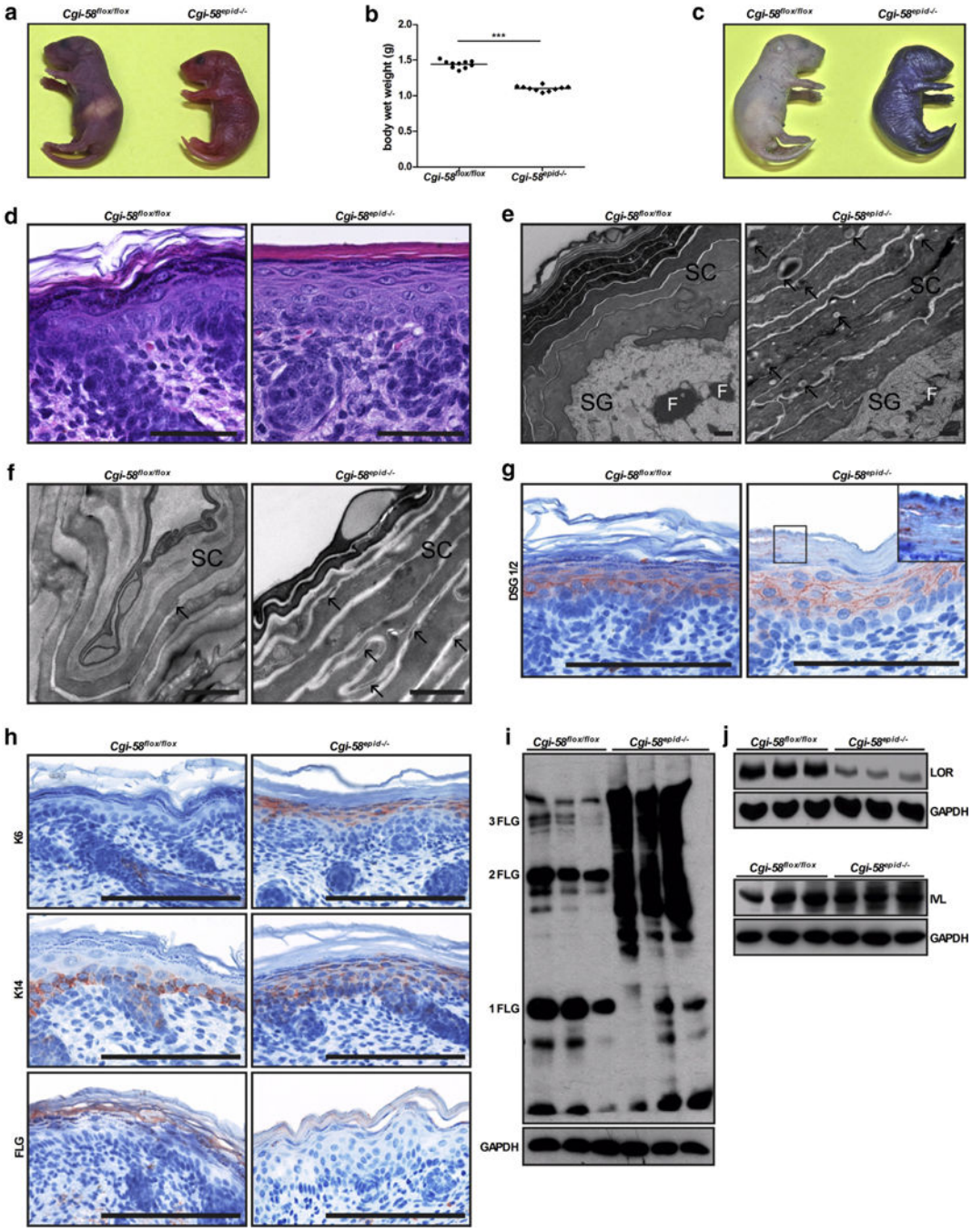

Figure 1. Defective skin permeability barrier and ichthyosis in *Cgi-58*$^{epid-/-}$ mice

(**a**) Phenotype, (**b**) body wet weight (n = 10), and (**c**) skin permeability barrier function analyzed by toluidine blue staining of newborn *Cgi-58*$^{flox/flox}$ and *Cgi-58*$^{epid-/-}$ 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*$^{epid-/-}$ mice. (**f**) Impaired degradation of corneodesmosomes (arrows) in *Cgi-58*$^{epid-/-}$ mice as analyzed by TEM (scale bar = 1 μm). (**g**) Immunohistochemistry of desmoglein 1/2 (DSG1/2), (**h**) keratin 6

(K6), K14, and filaggrin (FLG) in skin sections of newborn *Cgi-58*$^{flox/flox}$ and *Cgi-58*$^{epid-/-}$ 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*.

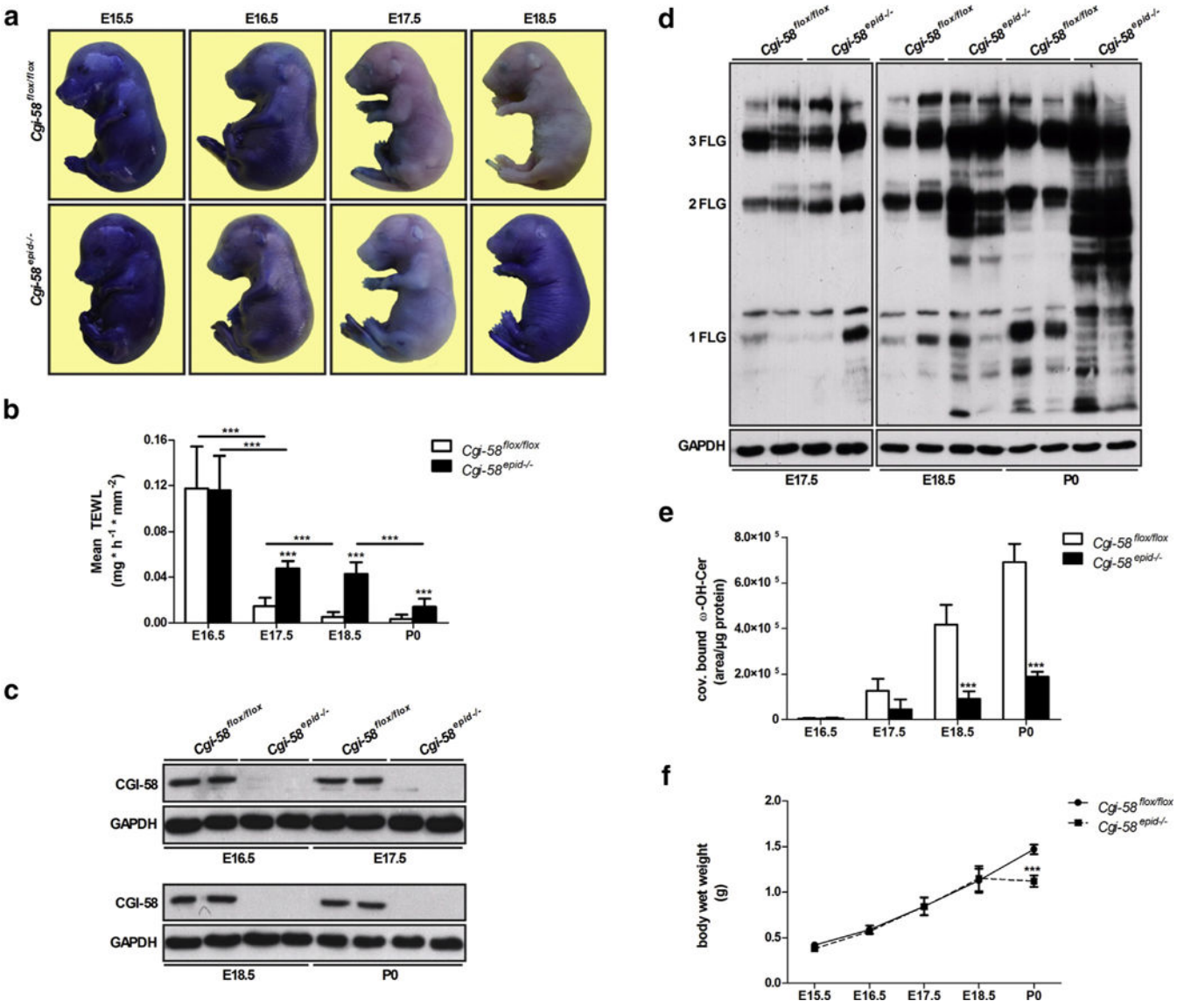

Figure 2. Embryonic skin permeability barrier development in the absence of CGI-58

Skin permeability barrier function of *Cgi-58*$^{epid-/-}$ and *Cgi-58*$^{flox/flox}$ 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*$^{epid-/-}$ and *Cgi-58*$^{flox/flox}$ mice (loading control = GAPDH). (**e**) Levels of covalently bound ω-hydroxy-ceramides (ω-OH-Cer) were determined by UPLC/MS in E16.5, E17.5, E18.5, and P0 epidermis from *Cgi-58*$^{epid-/-}$ and *Cgi-58*$^{flox/flox}$ mice (n = 4–6). (**f**) Body wet weight of *Cgi-58*$^{epid-/-}$ and *Cgi-58*$^{flox/flox}$ 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.

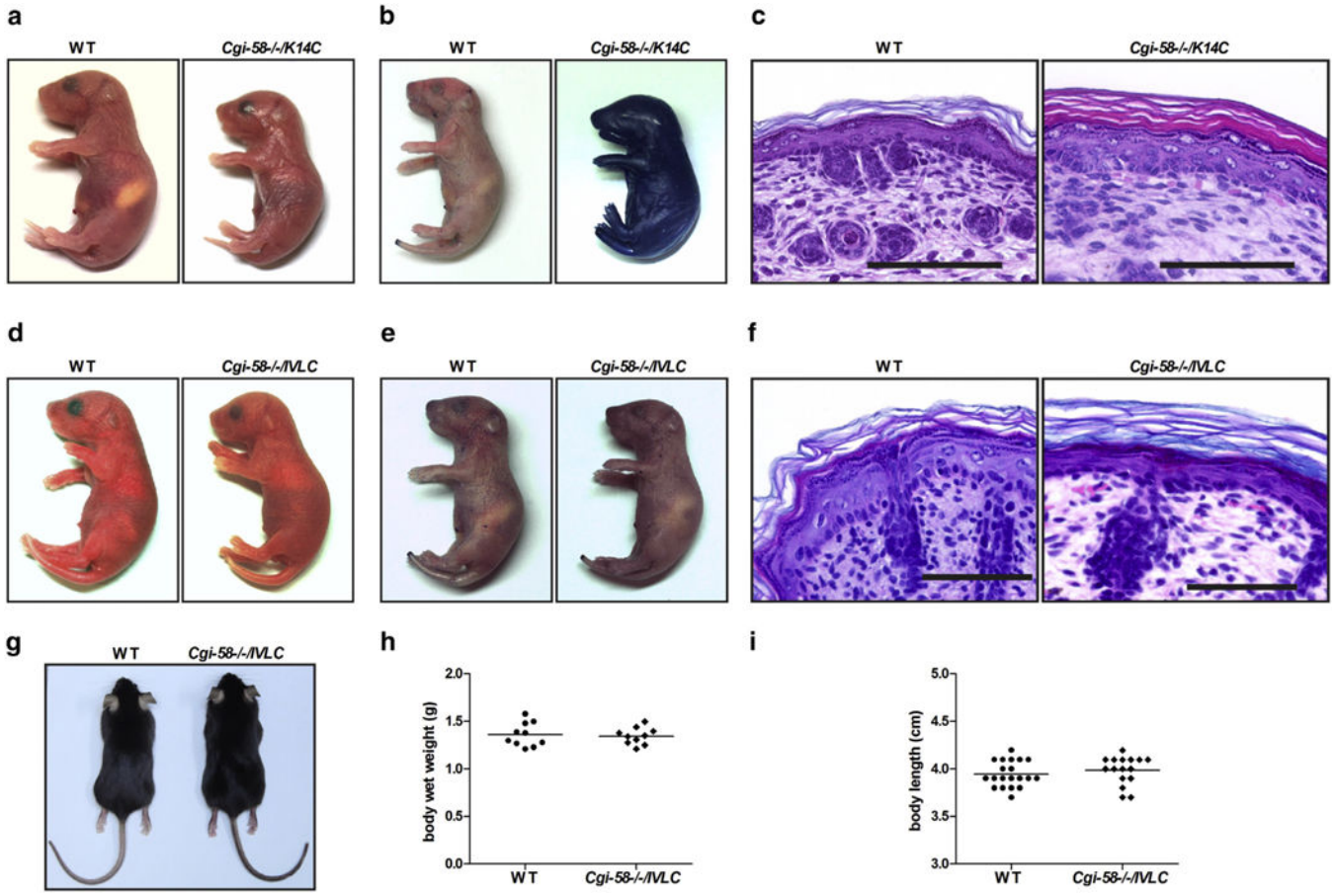

Figure 3. Restoration of CGI-58 expression in differentiated but not basal keratinocytes rescues the lethal skin barrier defect of *Cgi-58*$^{-/-}$ mice

Phenotypic characterization including gross appearance, skin permeability (assayed by toluidine blue penetration), and hematoxylin and eosin stain of skin sections (scale bar = 70 μm) of newborn (**a**–**c**) *Cgi-58*$^{-/-}$/*K14C* and (**d**–**f**) *Cgi-58*$^{-/-}$/*IVLC* mice compared with WT. (**g**) Phenotypes of 12-week-old female WT and *Cgi-58*$^{-/-}$/*IVLC* mice. (**h**) Body wet weight and (**i**) body length of newborn WT and *Cgi-58*$^{-/-}$/*IVLC* mice. CGI-58, comparative gene identification-58; K, keratin; WT, wild type.

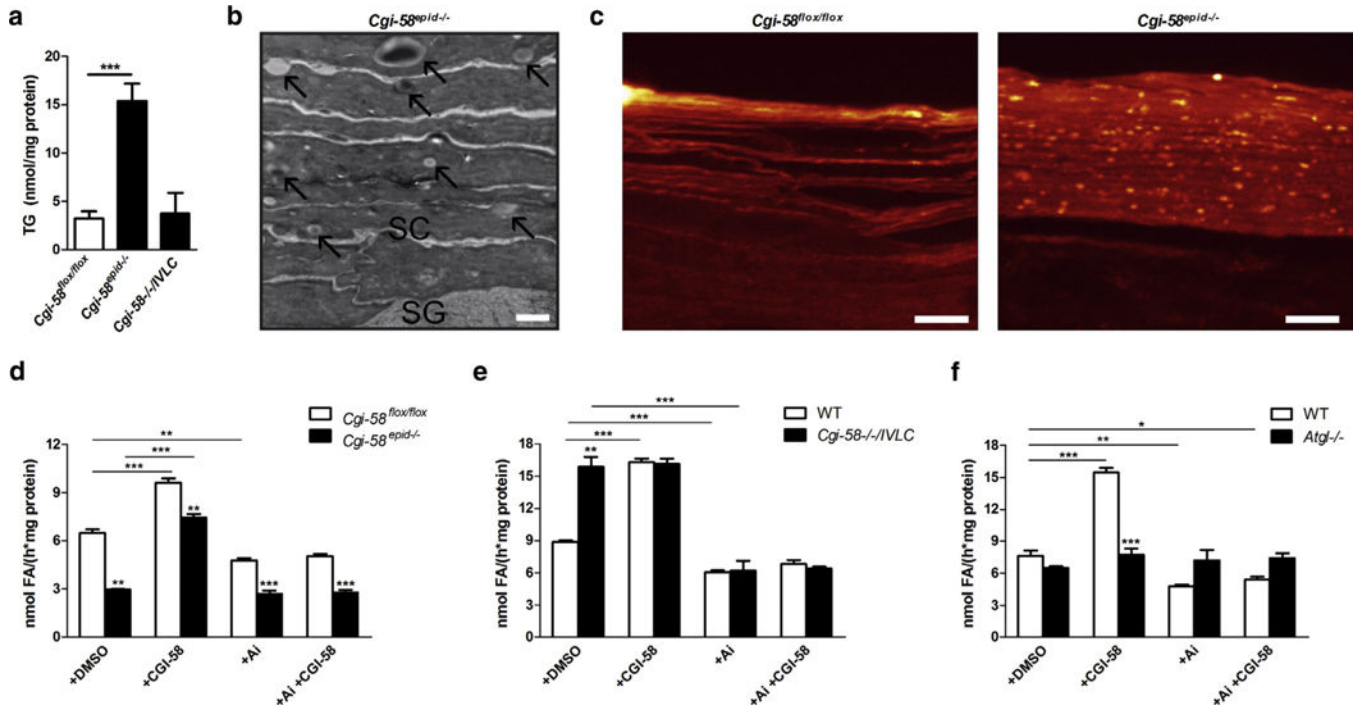

Figure 4. Epidermal TG accumulation in the absence of CGI-58 involves impaired ATGL-mediated TG catabolism in the epidermis

(**a**) TG contents of epidermal lipid extracts derived from *Cgi-58*$^{flox/flox}$, *Cgi-58*$^{epid-/-}$, and *Cgi-58*$^{-/-}$/*IVLC* mice were quantified by HPLC/LSD. Skin sections of newborn *Cgi-58*$^{epid-/-}$ mice show (**b**) lipid droplets (arrows) throughout the *stratum corneum* (SC) as analyzed by TEM (scale bar = 1 μm), and (**c**) numerous neutral lipid puncta as detected by label-free coherent anti-Stokes Raman scattering (CARS) microscopy (scale bar = 5 μm). Measurement of TG-hydrolase activities in epidermal preparations derived from (**d**) *Cgi-58*$^{epid-/-}$, (**e**) *Cgi-58*$^{-/-}$/*IVLC*, (**f**) *Atgl*$^{-/-}$ mice, and respective control mice (*Cgi-58*$^{flox/flox}$ and WT, respectively) in the absence and/or presence of recombinant CGI-58 and the ATGL-specific inhibitor Atglistatin (Ai), or a combination of both (n = pool of 4–7). Values represent means ± SD and are representative for three independent measurements. *P < 0.05; **P < 0.01; ***P < 0.001. ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification-58; FA, fatty acid; LSD, light-scattering detection; SD, standard deviation; SG, *stratum granulosum*; TEM, transmission electron microscopy; TG, triglyceride; WT, wild type.

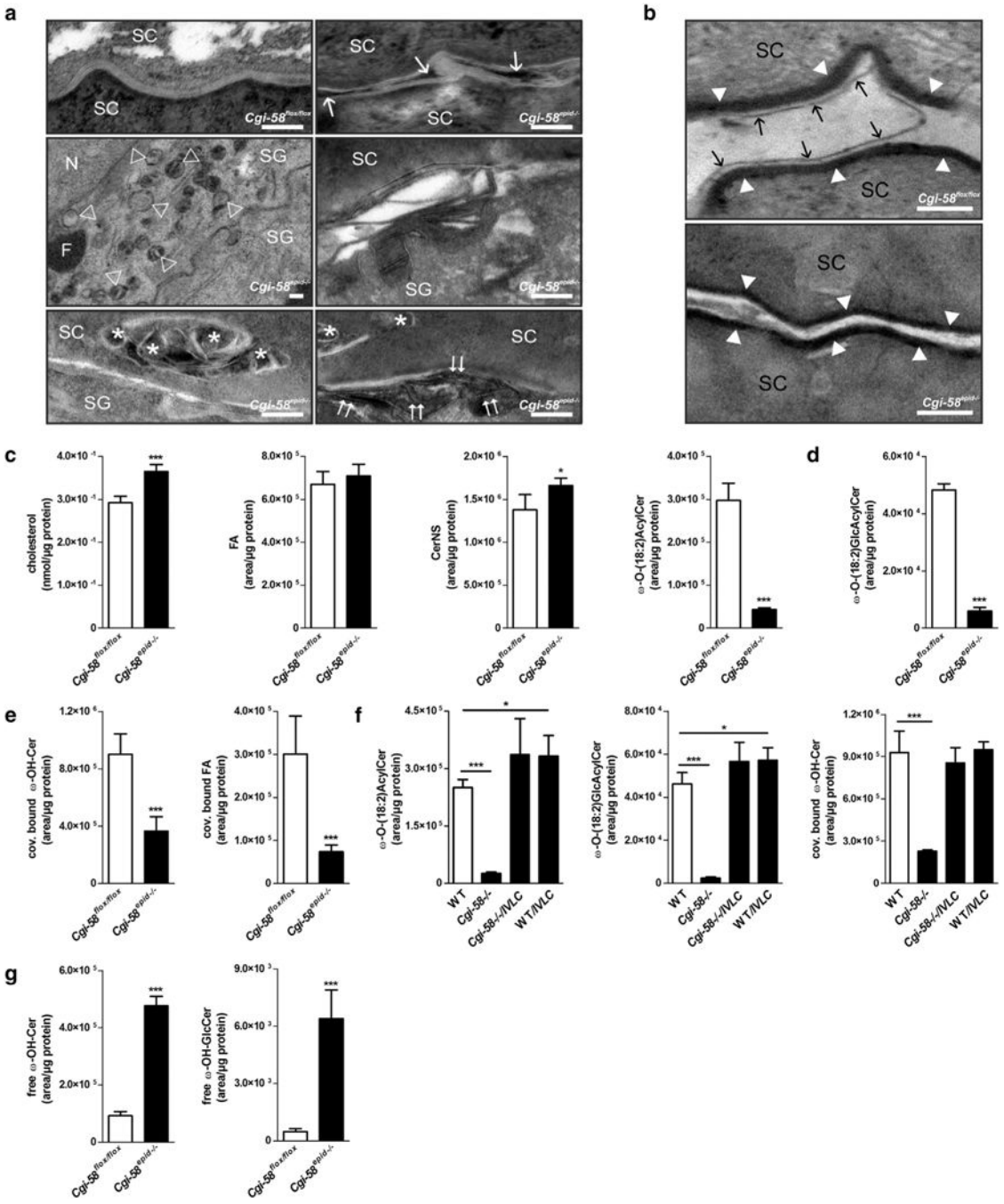

Figure 5. Defective ω-O-acylceramide and CLE formation caused by global CGI-58-deficiency is reversed on CGI-58 expression in the epidermis

(**a**) Compared with uniformly organized lamellar bilayers in *Cgi-58*$^{flox/flox}$, bilayers show nonlamellar domains (arrows) in *Cgi-58*$^{epid-/-}$ 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$^{epid-/-}$ compared with *Cgi-58*$^{flox/flox}$ 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, FA, CerNS, and ω-O-(18:2)-AcylCer, of (**d**) ω-O-(18:2)AcylGlcCer, and of (**e**) covalently bound ω-OH-Cer and FA in *Cgi-58*$^{epid-/-}$ and *Cgi-58*$^{flox/flox}$ epidermis. (**f**) ω-O-(18:2)-AcylCer, ω-O-(18:2)AcylGlcCer, and covalently bound ω-OH-Cer levels in WT, *Cgi-58*$^{-/-}$, *Cgi-58*$^{-/-}$/*IVLC*, and WT/*IVLC* epidermis. (**g**) Levels of free ω-OH-Cer and their glucosylated derivatives in *Cgi-58*$^{epid-/-}$ and *Cgi-58*$^{flox/flox}$ 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; ω-O-(18:2)AcylGlcCer, glucosylated ω-O-AcylCeramide; ω-OH-Cer, ω-hydroxy-ceramides; SD, standard deviation; TEM, transmission electron microscopy; UPLC, ultra performance liquid chromatography; WT, wild type.

Table 1

Plasma energy metabolites of newborn *Cgi-58*$^{flox/flox}$ and *Cgi-58*$^{epid-/-}$ mice[1]

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

Abbreviations: FA, free fatty acid; TG, triglyceride.

[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.

*** $P < 0.001$.