Epidermal mammalian target of rapamycin complex 2 controls lipid synthesis and filaggrin processing in epidermal barrier formation

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2020-01

Journal Pre-proof

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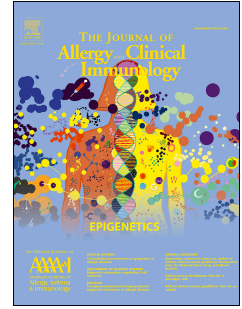
Please cite this article as: Ding X, Willenborg S, Bloch W, Wickström SA, Wagle P, Brodesser S, Roers A, Jais A, Brüning JC, Hall MN, Rüegg MA, Eming SA, Epidermal mTORC2 controls lipid synthesis and filaggrin processing in epidermal barrier formation, *Journal of Allergy and Clinical Immunology* (2019), doi: https://doi.org/10.1016/j.jaci.2019.07.033.

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Epidermal mTORC2 Activity Controls Skin Barrier Function
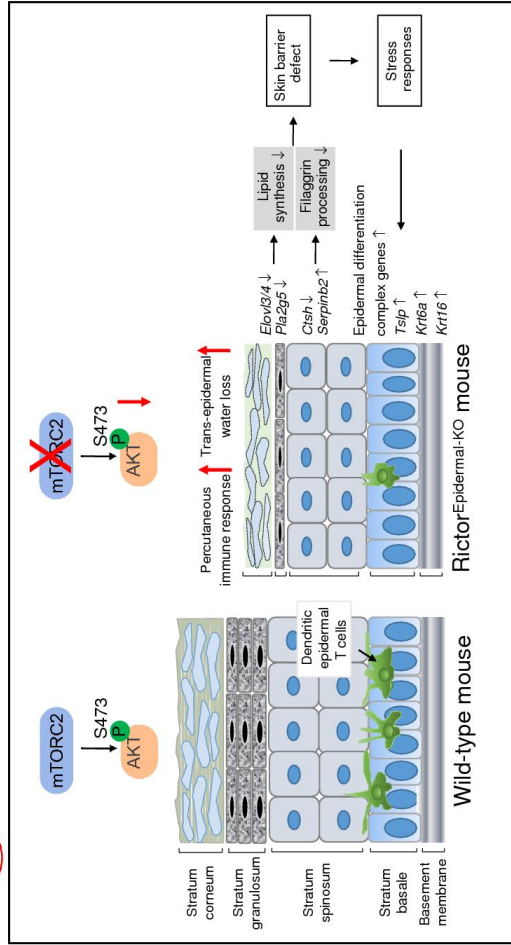
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2 barrier formation

3

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40   Conflict of interest statement:

41   The authors have declared that no conflict of interest exists.

Abstract

Background: Perturbation of epidermal barrier formation will profoundly compromise overall skin function, leading to a dry and scaly, ichthyosis-like skin phenotype, which is the hallmark of a broad range of skin diseases, including ichthyosis, atopic dermatitis, and a multitude of clinical eczema variants. An overarching molecular mechanism that orchestrates the multitude of factors controlling epidermal barrier formation and homeostasis remains to be elucidated.

Objective: Here we highlight a specific role of mammalian target of rapamycin complex 2 (mTORC2) signaling in epidermal barrier formation.

Methods: Epidermal mTORC2 signaling was specifically disrupted by deleting *Rictor*, encoding an essential subunit of mTORC2 in mouse epidermis (Ric^{EKO}). Epidermal structure and barrier function were investigated by a combination of gene expression, biochemical, morphological and functional analysis in Ric^{EKO} and control mice.

Results: Ric^{EKO} newborns displayed an ichthyosis-like phenotype characterized by dysregulated epidermal *de novo* lipid synthesis, altered lipid lamellae structure, and aberrant filaggrin processing. Despite a compensatory transcriptional epidermal repair response, the protective epidermal function was impaired in Ric^{EKO} mice as revealed by increased transepidermal water loss, enhanced corneocyte fragility, decreased dendritic epidermal T cells, and an exaggerated percutaneous immune response. Restoration of Akt-Ser473 phosphorylation in mTORC2-deficient keratinocytes by expression of constitutive Akt rescued filaggrin processing.

Conclusion: Our findings reveal a critical metabolic signaling relay of barrier formation where epidermal mTORC2 activity controls filaggrin processing and *de novo* epidermal

65 lipid synthesis during cornification. Our findings provide novel mechanistic insights into

66 epidermal barrier formation and could open up new therapeutic opportunities to restore

67 defective epidermal barrier conditions.

68

69 **Key words**: Epidermal barrier, mTORC2, rictor, ichthyosis, filaggrin, epidermal lipid

70 synthesis

Abbreviations used	
ACD:	Allergic contact dermatitis
AD:	Atopic dermatitis
CTSH:	Cathepsin H
DETCs:	Dendritic epidermal T cells
EDC:	Epidermal differentiation complex
FLG:	Filaggrin gene
FFA:	Free fatty acids
γδ-TCR:	γδ-T cell receptor
GSEA:	Gene set enrichment analysis
HPTLC:	High performance thin layer chromatography
IV:	Ichthyosis vulgaris
ICD	Irritant contact dermatitis
Krt:	Keratin
LB:	Lamellar body
Lor:	Loricrin
mTORC1/2:	Mammalian target of rapamycin complex 1/2
myr-AKT:	Myristoylated Akt1
Rictor:	Rapamycin-insensitive companion of TOR
Ric^{EKO}:	Epidermis-specific homozygous Rictor deletion
RNA-seq:	RNA-Sequencing
SB:	Stratum basale
SC:	Stratum corneum
SG:	Stratum granulosum
SS:	Stratum spinosum
TEM:	Transmission electron microscopy
TSLP:	Thymic stromal lymphopoietin
TEWL:	Transepidermal water loss

71

72 **Capsule Summary (35 words)**

73 An overarching molecular mechanism that orchestrates the multitude of factors

74 controlling epidermal barrier formation remains to be elucidated. Here, we highlight a

75 specific role of mTORC2 signaling in epidermal barrier function.

76

77 **Key Messages**

78 ● The Ric^{EKO} mouse develops an ichthyosis-like phenotype and might serve as a

79   new preclinical disease model for studying epidermal barrier defects at the

80   molecular level.

81 ● Altered mTORC2 activity may represent a predisposing factor for skin disorders

82   associated with disrupted epidermal barrier function.

83

84

Introduction

The epidermis is a stratified squamous epithelium that provides a life-sustaining permeability barrier preventing water loss and protecting the body from a plethora of physical and chemical insults as well as pathogenic microorganisms [1]. Perturbation of epidermal barrier function will profoundly compromise overall skin barrier function, frequently leading to a dry and scaly, ichthyosis-like skin phenotype, which is the hallmark of a broad range of skin diseases, including ichthyosis, atopic dermatitis (AD), and a multitude of clinical eczema variants associated for example with diabetes mellitus or aging [2-4]. Quality of life is frequently negatively affected and patients are also at high risk for associated morbidity and mortality [5]. The molecular mechanisms regulating proper barrier function and maintenance are not yet understood, and were the aim of this study.

The epidermal barrier function is acquired and maintained by a tightly regulated keratinocyte differentiation process, known as cornification. The innermost mitotically active cell layer, the stratum basale (SB), undergoes continuous proliferation and differentiation, providing cells to the above stratum spinosum (SS), stratum granulosum (SG), and the stratum corneum (SC). The outermost SC makes up most of the permeability barrier. The SC consists of flattened, anuclear and keratin-filled corneocytes embedded in an intercellular lipid-rich matrix composed of ceramides, cholesterol, and free fatty acids (FFA) in an acidic environment [2, 6]. Perturbation of cornification will profoundly compromise skin barrier function [2, 6].

Genetic analyses have established a strong link between genetic defects and skin barrier disorders in both humans and mice, providing the basis for the current

108   understanding of molecular networks orchestrating cornification [7]. Of particularly

109   importance was the discovery of the loss-of-function mutations in the filaggrin (*FLG*)

110   gene, the cause of the most common genodermatosis, namely ichthyosis vulgaris, which

111   is characterized by dry, scaly skin [8-9]. Ichthyosis vulgaris has been identified as a

112   predisposing factor for the development of AD, the most common chronic inflammatory

113   skin disease, affecting up to 20% of children and 7-10% of adults [10]. However, a

114   significant number of AD patients have no *FLG* mutations and recent human genetic

115   studies suggest that also a reduction of profilaggrin at the protein level can lead to skin

116   barrier defects suggesting that other mechanisms and candidate molecules may regulate

117   profilaggrin expression and proteolytic processing [9, 11]. Further, the identification of

118   mutations in diverse *Elovl* genes in mouse and pediatric AD patients, encoding very-long-

119   chain fatty acid elongases revealed a central role for *de novo* ceramide synthesis in

120   cornification and proper epidermal barrier function [1,10, 12-14]. In addition, *Pla2g5* activity

121   catalyzes FFA synthesis, and has been shown to be critical in epidermal barrier formation

122   [15]. However, the overarching molecular mechanisms that orchestrate the multitude of

123   factors controlling keratinocyte fate and cornification are not known [16-17].

124   Recently, we identified the serine/threonine protein kinase mammalian (also

125   known as mechanistic) target of rapamycin (mTOR) as an essential regulator of

126   epidermal differentiation and barrier formation in embryonic development [18-19]. Here we

127   hypothesized that mTORC2 plays a critical role in postnatal epidermal barrier assembly

128   and maintenance. mTOR is a central regulator of cell growth and metabolism, and is

129   evolutionarily highly conserved [20-21]. mTOR assembles in two structurally and

130   functionally distinct, multi-protein complexes, referred to as mTORC1 and mTORC2 [20-21].

131   The Rictor (rapamycin-insensitive companion of TOR) protein is an essential and specific

132   subunit of mTORC2. Growth factors are able to stimulate mTORC2 kinase activity, and

133   activated mTORC2 phosphorylates several members of the AGC kinase family, including

134   AKT, specifically at Ser473 (AKT-pSer473) to control cell metabolism and cytoskeleton

135   organization [22-23]. In keratinocyte differentiation, AKT activity is induced, regulating

136   filaggrin expression and processing [24-25]. Interestingly, a recent study showed that

137   increased Raptor expression, the regulatory associated protein of mTORC1 correlates

138   with reduced AKT activity and filaggrin expression in the skin of AD patients [26]. In this

139   study we assessed the role of epidermal mTORC2 in postnatal skin barrier function by

140   inactivating *Rictor* in the epidermis in mice. We show that mTORC2 promotes non-

141   redundant functions for proper cornification via regulation of epidermal lipid metabolism

142   and filaggrin processing. Our findings could suggest a new therapeutic strategy to

143   regulate epidermal barrier function and to prevent complications in patients with dry skin

144   diseases.

145

146 **Methods**

147 **Mice**

148 Epidermal-specific *Rictor* knockout mice (C57BL/6) were generated by crossing mice

149 carrying loxP-flanked *Rictor* alleles with a Cre-transgenic strain expressing Cre

150 recombinase under control of the human K14 promoter (Supplemental Figure 1A) [18].

151 Littermates that either lacked Cre or expressed Cre but carried a heterozygous loxP-

152 flanked *Rictor* allele served as controls. Genotyping was performed as previously

153 described [18]. Mice were maintained and bred under standard pathogen-free conditions.

154 All procedures were approved by the North Rhine-Westphalian State Agency for Nature,

155 Environment, and Consumer Protection and the University of Cologne.

156

157 **Histological analysis**

158 Skin tissues were fixed in 4% PFA, embedded in paraffin, and sectioned (10 µm).

159 Hematoxylin and eosin staining was performed following a standard procedure [27]. Images

160 were analyzed using a light microscope (Leica DM4000B, Leica Microsystems, Wetzlar,

161 Germany).

162

163 **Separation of epidermis from mouse back skin**

164 The subcutaneous fat tissue was mechanically dissected from excised back skin. The

165 epidermis was separated from the dermis after floating skin biopsies in 0.5 M ammonium

166 thiocyanate (NH_4SCN) in phosphate buffer, pH 6.8 (0.1 M NH_2HPO_4, 0.1 M KH_2PO_4) for

167 30 min on ice, epidermis side up. The epidermis was used for either RNA isolation,

168 biochemical analysis, or immunostaining.

169

Immunostaining

For immunohistochemical and immunofluorescence staining, cryosections from Optimal

Cutting Temperature compound (OCT, Tissue Tek) embedded tissues were fixed (4%

PFA or in methanol), blocked (10% normal goat serum in PBS), and incubated with

primary antibodies (diluted in blocking buffer) overnight at 4°C. Bound primary antibody

was detected by incubation with peroxidase-conjugated (EnVision System, Dako)

secondary antibody followed by incubation with peroxidase substrate (Sigma) or Alexa-

Fluor 488- or Alexa Fluor 594-conjugated antibodies (Invitrogen). Nuclei were

counterstained with hematoxylin or 4,6-diamidino-2-phenylindole (DAPI, Invitrogen).

After washing, slides were mounted in mounting medium. For whole-mount staining,

epidermis was separated from ears as described above. Epidermal sheets were fixed by

acetone and stained with anti-γδ-TCR antibody. Images were taken with a Zeiss Meta 710

Confocal Microscope or KEYENCE Fluorescence Microscope (BZ-9000). Antibody

information can be found in Supplemental Table 1. For quantification of pAkt-S473

staining, three representative HPFs/section were quantified using ImageJ software

(https://imagej.nih.gov/ij/index.html) as described previously[27]. Findings are expressed as

the percentage of pAkt-S473 stained areas within the ear epidermis. Numbers of

inflammatory cells were determined by counting positively stained cells in five

representative HPFs (400x)/section. Analyses were performed in a blinded manner by

two independent investigators.

Transmission electron microscopy

192   Skin tissues were isolated and fixed in buffer (2% paraformaldehyde, 2% glutaraldehyde,

193   0.1M cacodylate buffer at pH 7.35) and postfixed with ruthenium tetroxide. Tissue

194   embeeding and ultrathin section preparation were done as previously described [28].

195

196   **Flow cytometry and cell sorting**

197   For analysis of immune cells, single-cell suspensions of skin from control and Ric[EKO]

198   were prepared by a combination of enzymatic digestion (Liberase Blendzyme, Roche

199   Applied Science) and mechanical disruption (Medimachine System, BD Biosciences) as

200   previously described [28]. For flow cytometry analysis, cells were stained as described

201   previously [28]. Briefly, cells were passed through a 40 µm cell strainer, washed with PBS,

202   and incubated with the following antibodies: eFluor® 450- or APC-eFluor® 780-

203   conjugated anti-CD45 (clone 30-F11, eBiosciences), APC-Cy7-conjugated anti-CD4

204   (clone GK1.5, BD Pharmingen™), PE- or PE-Cy7-conjugated anti-CD3e (clone 145-

205   2C11, eBiosciences), PE-conjugated anti-Siglec-F (clone E50-2440, BD Pharmingen™),

206   FITC- or BV421-conjugated anti-γδ T-Cell Receptor (clone GL3, BD Pharmingen™),

207   FITC-conjugated anti-Vγ3 TCR (clone 536, BD Pharmingen™), FITC-conjugated anti-

208   F4/80 (clone Cl:A3-1, Bio-Rad) or APC-Cy7-conjugated Ly-6G (clone E50-2440, BD

209   Pharmingen™) in FACS buffer. Dead cells were excluded using 7-amino-actinomycin D

210   (7-AAD) (BD Biosciences). Cells were analyzed on a FACSCanto II flow cytometer

211   (BD), which was equipped with FACSDiva software (BD). For flow cytometry sorting,

212   GFP[+] keratinocytes were sorted by FACSAria cell sorting system (BD).

213

214   **RNA transcriptomics and GSEA**

215 Total RNA was extracted from separated epidermal tissue using RNeasy Minikit (Qiagen)

216 and RNA quality was determined using an Agilent 2200 TapeStation. Preparation of

217 amplification reactions of complementary DNA (cDNA) was performed at the Cologne

218 Center for Genomics (CCG) using Ovation RNA-Seq System V2 (NuGen) and library

219 was prepared using the Nextera XT library preparation kit (Illumina). RNA sequencing

220 was carried out on Illumina HiSeq2000 machines using the 2_100-bp protocol and V3

221 chemistry. After quality control, adapter sequences were removed by flexbar63. Reads

222 mapping to ribosomal RNA-related genes were filtered out using a custom ribosomal

223 RNA-only reference. After pre-processing, reads were mapped to the *Mus musculus*

224 reference genome (build GRCm38_79), followed by differential gene expression analysis

225 using the DESeq2 R library (version 1.6.3). Hierarchical clustering was performed in *R*.

226 Transcripts regulated greater than 1.5 fold with a *p* value of < 0.05 were used in GO term

227 analysis (DAVID Bioinformatics Resources 6.8) to identify enriched functional

228 annotations. Gene set enrichment analysis was performed on whole gene list and

229 compared to the Broad Institute Molecular Signatures Database collection of chemical

230 and genetic perturbations (C2) using the Web-based tool available from the Broad

231 Institute [29]. Enrichments with an FDR value < 0.05 were considered significant.

232

233 **CE isolation for microscopy**

234 A defined area of dorsal mouse skin (25 mm^2) was boiled in isolation buffer (20 mM

235 Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT, and 2% SDS) under vigorous shaking for

236 40 min. After centrifugation, the CEs were washed twice with isolation buffer and were

237 analyzed using a hemocytometer [30].

238

Lipid analysis

240 For the analysis of SC lipids, the SC was separated from the back skin of newborn mice

241 by floating the skin with the dermis side down on 0.5% trypsin in phosphate buffered

242 saline (PBS) for 24 h at 37 °C [31]. The SC was washed in PBS, lyophilized and weighed.

243 Lyophilized SC was homogenized in 0.5 ml of water using the Precellys 24

244 Homogenisator (Peqlab, Erlangen, Germany) at 6,500 rpm for 30 sec. After the addition

245 of 0.5 ml of water and 2 ml of chloroform/methanol 1:2 (v/v), freely extractable SC lipids

246 were extracted for 24 h at 37 °C and purified using a modification of the Bligh-Dyer

247 procedure as previously described [32]. For lipid quantification, the equivalent of 1.1 mg of

248 lyophilized SC was loaded on 20×10 cm HPTLC plates which were developed twice in

249 chloroform/methanol/glacial acetic acid 190:9:1 (v/v/v). Quantitative analytical TLC

250 determination was performed as previously described [33]. Nile red staining was performed

251 on cryo-sections for 2 min with 0.2 mg/ml Nile red in 75% glycerol; the fluorescence

252 intensity of Nile red stained epidermis was determined by integrated density using

253 ImageJ software.

254

Trans-epidermal water loss (TEWL) measurements

256 TEWL measurements were carried out as described previously [18] using a Tewameter

257 (Courage and Khazaka Electronic GmbH, Cologne, Germany) and the measurements

258 were performed according to the manufacturer's instructions.

259

Irritant and allergic contact dermatitis

13

261 To assess irritant contact dermatitis (ICD), the inner and outer surface of right ear was

262 treated with 5 µl 1% croton oil in acetone/olive oil (4:1). The left ear was treated with

263 acetone/olive oil only and taken as a control. Ear thickness was measured 4, 8, and 24 h

264 after the treatment using a digital caliper. Ear swelling was determined by calculating the

265 changes of ear thickness between challenged ear (right) and control ear (left). To evaluate

266 hapten-induced allergic contact dermatitis (ACD), mice were sensitized by painting 25 µl

267 of 1% 1-fluoro-2,4-dinitrobenzene (DNFB) (Sigma) in acetone/olive oil (4:1) onto the

268 shaved abdominal skin at day 0. At day 6, mice were challenged by applying 5 µl 0.4%

269 DNFB in acetone/olive oil on the inner and outer surface of right ear, respectively. The

270 left ear was treated with acetone/olive oil only and taken as a control. Ear thickness was

271 measured 8, 24, and 48 h after the treatment using a digital caliper. Ear swelling was

272 determined by calculating the changes of ear thickness between challenged ear (right) and

273 control ear (left).

274

275 **Cell culture**

276 Keratinocytes were isolated from newborns and cultured in low Ca^{2+} medium as

277 described previously [27]. Briefly, skin was floated, dermal side down, on 0.25% trypsin

278 solution (Gibco) for 16 h at 4°C. Epidermis was separated from the dermis and minced

279 with a scalpel. Cell and epidermal tissue were suspended, shaken for 30 min at 37°C, and

280 seeded on collagen-coated 6-well plates with mitomycin C- (Sigma) treated 3T3

281 fibroblast feeder layer cells. For the induction of keratinocyte differentiation, the medium

282 was supplemented with 0.2 mM $CaCl_2$ (high Ca^{2+} medium).

283

284 **Plasmids, virus preparation, and keratinocyte infection**

285 MSCV-IRES-green fluorescent protein (GFP) retrovirus vector with myr-AKT was used

286 as described previously [34]. myr-AKT-IRES-GFP and IRES-GFP retroviral supernatants

287 were produced in 293T cells as previously described [35]. Briefly, 293T cells were

288 transfected with vector constructs and packaging plasmids (pVPack-GP and pVPack-Eco;

289 Stratagene). Forty-eight hours after transfection, virus supernatant was harvested and

290 concentrated by precipitation with chondroitin 6-sulfate (CSC) (Sigma) and polybrene

291 (Sigma), and centrifuged. Keratinocytes (P2) were exposed to concentrated viruses for 24

292 h and GPF$^+$ cells were sorted by flow cytometry.

293

294 **qRT-PCR analysis**

295 RNA from epidermis and skin was extracted using an RNA isolation kit (Qiagen)

296 according to the manufacturer's instructions. Reverse transcription of isolated RNA was

297 performed using the High Capacity cDNA RT Kit (Applied Biosystems). Amplification

298 reactions were performed with PowerSYBR Green PCR Master Mix (Applied

299 Biosystems) using a 7300 Real Time PCRsystem (Applied Biosystems). The comparative

300 method of relative quantification (2-$^{\Delta\Delta}$Ct) was used to calculate the expression level of

301 the target gene normalized to GAPDH. Primer sequence information can be found in

302 Supplemental Table 2.

303

304 **Western blot analysis**

305 Epidermis was separated from the dermis and dissociated with a MixerMill. For analysis

306 of filaggrin, loricrin and keratin epidermis was lyzed in a 4% SDS lysis buffer.

15

307   Alternatively, cells or tissues were lyzed in radio immunoprecipitation assay (RIPA)

308   buffer, containing protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Roche).

309   Protein concentration was determined by Micro BCA Protein Assay Kit (Thermo

310   Scientific) and 20 mg protein per sample was subjected to SDS-PAGE (Invitrogen).

311   Subsequently, protein was blotted to PVDF membranes. After blocking (5% non-fat milk

312   in TBST buffer), membranes were incubated with primary antibodies. Antibody

313   information can be found in Supplemental Table 1. For densitometry analysis, enhanced

314   chemiluminescence exposed film was scanned and quantified with ImageJ software.

315   Briefly, images were convered to grayscale and the background was removed. The

316   densitometry was achieved by Gel Analysis method with ImageJ software. The ratio of

317   filaggrin monomer and total filaggrin, pro-filaggrin and β-actin were determined with

318   control set to 1 or the relative intensity was calculated after normalizing to a loading

319   control (β-actin).

320

321   **Statistical analysis.**

322   Data are presented as mean ± s.e.m. and statistics was performed using PRISM software

323   (GraphPad, version 5.0a). Statistical significance of difference was determined using

324   unpaired 2-tailed Student's *t*-test or ANOVA one-way test analysis with Bonferroni

325   multiple comparison test. *P* value <0.05 was considered to be statistically significant.

326

Results

Ric^{EKO} mice display a transient ichthyosis-like phenotype

To explore the role of epidermal mTORC2 signaling, we knocked out *Rictor* specifically in the epidermis as previously described [18]. Briefly, mice carrying a loxP-flanked *Rictor* allele were crossed with a transgenic mouse expressing Cre recombinase under the control of the human keratin 14 (K14) promoter [36-37], leading to epidermis-specific deletion of *Rictor* (Ric^{EKO}) in all basal cells and their progeny (Supplemental Figure 1A). Epidermis-specific Cre-mediated recombination of floxed *Rictor* alleles, verified by PCR analysis of genomic DNA extracted from different organs including the epidermis, resulted in effective epidermal loss of Rictor protein expression as confirmed by Western blot analysis and immunohistochemical staining (Figure 1A and Supplemental Figure 1B and C). Immediately after birth, Ric^{EKO} mice were clearly distinguishable from their wildtype littermates, exhibiting shiny, translucent and fragile skin. Occasionally, Ric^{EKO} newborns showed small erosions at the trunk, which might have occurred due to mechanical stress of the skin during birth. This skin fragility may have led to the observed death of approximately 20% of the mutant mice before or after weaning (Supplemental Figure 1D). By day 7 (P7), a prominent phenotype of dry and scaly, ichthyosis-like skin was apparent, often beginning with scaling of the ventral side already around P3 (Figure 1B). Histological analysis revealed a stratified epidermis but significantly reduced epidermal thickness in Ric^{EKO} pups at P4 (Figure 1C). Epidermal atrophy was primarily due to reduced granular cell layers and thinner SC. In addition, the SC in Ric^{EKO} mice displayed structural defects including poor organization of upper SC layers and densely packing of lower ones compared with the well-defined basket weave-

17

350   like structure in controls (Figure 1C). Although less pronounced, the epidermal atrophy

351   remained detectable in adults (P70) (Supplemental Figure 1E). Morphological alterations

352   in the epidermis of Ric^{EKO} newborns were paralleled by a transient impairment of

353   epidermal barrier function as revealed by a significant increase in trans-epidermal water

354   loss (TEWL) in P4 mutants (Figure 1D, Supplemental Figure 1F). As assessed by

355   immunofluorescent staining for multiple tight/adherent junction proteins and components

356   of desmosomal plaques, intercellular junctions appeared to be similar in control and

357   Ric^{EKO} epidermis (Supplemental Figure 1G). Ric^{EKO} mice were born at the expected

358   Mendelian ratio and exhibited normal body weight at birth, yet their weight by P5

359   decreased by 20% compared with littermate controls; the smaller body size persisted

360   throughout adulthood (Figure 1E, Supplemental Figure 1H).

361

362   Ric^{EKO} mice display increased corneocyte fragility

363   To test whether epidermal atrophy and mechanical fragility in young Ric^{EKO} mice was

364   associated with decreased physical resistance of the SC, cornified envelopes (CE) were

365   prepared from the back skin of control and Ric^{EKO} mice at P5. Notably, 80% of CE

366   isolated from control mice remained in a uniformly rigid, large and intact, polygonal

367   shape, whereas more than 60% of Ric^{EKO} CE were ruptured, irregular, and fragmented,

368   which is indicative of significant fragility and structural defects (Figure 1F).

369   Ultrastructural analysis of P5 epidermis by transmission electron microscopy (TEM)

370   showed that the inner portion of Ric^{EKO} epidermis, including basement membrane,

371   stratum basale and stratum spinosum, as well as desmosomal plaques were not grossly

372   affected (Supplemental Figure 1I). However, detailed inspection revealed that keratin

373   intermediate filaments in the mutant mice were not condensed and were more loosely

374   packed compared to controls (Figure 1G), which may contribute to the reduced resistance

375   to mechanical stress in Ric^{EKO} CE. Together, our findings demonstrate major structural

376   defects in the formation of the SG and SC in the absence of epidermal mTORC2 activity.

377

378   **Epidermal mTORC2 deficiency induces a compensatory transcriptional epidermal**

379   **repair program in Ric^{EKO} mice**

380   To identify specific genes and pathways mediating mTORC2 signaling and orchestrating

381   the SG to SC transition, we performed a RNA-sequencing (RNA-seq) transcriptome

382   analysis of epidermis from mutant and control embryos at E19.5, when epidermal

383   maturation nears completion and epidermal gene expression is not influenced by

384   exposure to environmental insults that could mask primary mechanistic effects.

385   A one-way analysis of variance was performed to calculate the most variable and

386   differentially expressed genes between both sets of epidermal tissue (fold change >2 and

387   with P value < 0.05). Analysis revealed 231 and 249 genes down- and up-regulated,

388   respectively, in Ric^{EKO} epidermis versus controls (Figure 2A). To identify biological

389   processes underlying the observed gene expression alterations, we performed Gene

390   Ontology Enrichment analysis (GO) and Pathway Enrichment analysis of the

391   differentially expressed genes at the 1.5-fold cut-offs using DAVID 6.8. The upregulated

392   gene set was particularly enriched in genes involved in peptide cross-linking (e.g.

393   *Lce3a/b*, *Sprr1a,* and *Sprr2h*), keratinization (e.g. *Krt6a, Krt16*), keratinocyte

394   differentiation (e.g. *Sfn, Sprr1a, Lce3a*), and wound repair (e.g. *Krt6a, Krt16, S100a8*)

395   among others. The downregulated gene set was highly enriched for immune regulators

19

396   (e.g. *Oas1f*) and lipid metabolism (e.g. *Elovl3/4, Pnpla5, Pla2g5*) (FDR < 0.05) (Figure

397   2A and B). One of the most striking features of the RNA-seq data was the upregulation of

398   genes for late keratinocyte differentiation and SC components, including small proline-

399   rich proteins (Sprr), late cornified envelope proteins (Lce), and S100 proteins (Figure 2A).

400   Several of these gene families comprise the epidermal differentiation complex (EDC),

401   encoding a dense cluster of genes whose protein products have been identified as major

402   molecular markers and important functional regulators for terminal differentiation in the

403   stratified epidermis (Figure 2C) [38]. A panel of differentially regulated genes was

404   confirmed by qRT-PCR-analysis, validating the RNA-seq data (Figure 2D). Further, gene

405   set enrichment analysis (GSEA) revealed an upregulation of stress-response signatures in

406   Ric[EKO] epidermis compared to control, including epidermal barrier stress response

407   regulators (e.g. *Krt6a, Krt16*) [39] and UVB-induced genes in human epidermis (e.g.

408   *S100a8*, *Tnfrsf10b*, *Il24, Mmp3*) (Figure 2D and E) [40]. Collectively, these findings reveal

409   profound alterations in mTORC2 deficient epidermis in gene regulatory networks

410   controlling terminal differentiation and barrier formation, and implicate a critical role for

411   mTORC2 activity in the formation of a protective epidermal barrier.

412

413   **mTORC2 controls epidermal lipid metabolism**

414   Keratinocytes of the granular layer contribute to epidermal lipid synthesis which is

415   essential for effective skin barrier function [6, 41]. During cornification, lipids are packed in

416   intracellular lamellar bodies (LB) which then transport and release the lipids into the

417   intercellular space of corneocytes to form a lipid bilayer. The lipid bilayer mainly

418   consists of ceramides, cholesterol, and FFA [6]. To examine whether the structural and

419 functional SC defects in Ric^{EKO} mice resulted from altered lipid composition, we further

420 analyzed the SC lipid composition. Nile red, a fluorescent lipophilic dye, revealed that

421 the lipid content was markedly diminished in Ric^{EKO} SC (Figure 3A). To quantify SC

422 lipid composition, the levels of ceramides, cholesterol, and FFA in the SC were

423 determined using high performance thin layer chromatography (HPTLC). The analysis

424 revealed a decrease of all three main SC lipid classes in Ric^{EKO} epidermis. In particular,

425 levels of Cer (NS) in Ric^{EKO} epidermis were decreased by more than 40% compared to

426 controls (Figure 3B).

427 Notably, consistent with the reduced SC lipid composition, the GSEA of RNA-seq data

428 revealed a panel of lipid metabolism-associated genes which were significantly

429 downregulated in Ric^{EKO} epidermis. These genes include *Elovl3/4*, *Psap*, *Lipn*, *Pla2g3/5*,

430 *Abhd5* (Figure 3C and D). Of note is that most of these genes have been shown to play a

431 critical role in lipid metabolism and epidermal barrier formation in mice and patients with

432 compromised skin barrier function [42].

433 At the interface between the SG and SC, lipid lamellae are released into the intercellular

434 space. We further examined the organization of LB. In line with the altered epidermal

435 lipid composition, ultrastructural analysis by TEM revealed impaired lipid lamellae

436 structure in the LB of Ric^{EKO} epidermis. In addition, in Ric^{EKO} epidermis, the lipid layer

437 between SC and the SG was disrupted (Figure 3E). Taken together, these findings reveal

438 multiple quantitative defects in SG lipid synthesis that lead to altered lipid composition

439 and LB structure in mTORC2-deficient epidermis, potentially contributing to the

440 compromised epidermal barrier function in Ric^{EKO} mice.

441

Reduced proteolytic activity is paralleled by impaired filaggrin processing in mTORC2 deficient epidermis

Intriguingly, further pathway enrichment analysis using the KEGG database uncovered highly deregulated lysosomal transcripts in Ric[EKO] epidermis (Figure 4A). Genetic skin diseases and conditions with disturbed barrier function are often characterized by a disturbed balance of epidermal protease and antiprotease activities [43]. We thus hypothesized that attenuated proteolytic activity in Ric[EKO] epidermis might contribute to the disturbed barrier formation and function. Transcripts of several genes encoding members of diverse protease families, such as lysosomal cysteine, aspartic acid, and serine proteinases, were downregulated, including *cathepsin H* (*Ctsh*) and *cathepsin D* (*Ctsd*) (Figure 4B, C). Of interest is that *CTSH* and *CTSD* mutations have recently been shown to cause impaired filaggrin processing and disturbed SC formation [26, 44]. Conversely, Serpins, which encode potent protease inhibitors, were upregulated, including *Serpinb2,* which encodes an ovalbumin-like serine protease inhibitor and has been linked to the pathogenesis of certain cornification disorders [45] (Figure 4B, C). Moreover, expression of the gene encoding secretory leukocyte peptidase inhibitor (*Slpi*) was markedly increased in Ric[EKO] epidermis (Figure 4D). *Slpi* encodes an inhibitor of kallikrein 7, a protease required for corneodesmosome cleavage. Interestingly, TEM revealed that corneodesmosomes were retained in Ric[EKO] epidermis with an increase in both number and size (Figure 4E), thereby further corroborating an attenuated corneodesmosome degradation in the Ric[EKO] SC, potentially due to reduced Slpi.

Further, proteolytic processing of the CE reinforcement protein profilaggrin is an essential process during proper keratinocyte terminal differentiation [9]. Having found that

465   *Ctsh* and *Ctsd*, encoding two proteases critically involved in profilaggrin processing, are

466   downregulated in Ric[EKO] epidermis, we analyzed profilaggrin protein expression and

467   processing in extracts prepared from control and Ric[EKO] epidermis by Western blot

468   analysis. Notably, while filaggrin mRNA and profilaggrin protein expression levels were

469   comparable between control and Ric[EKO] epidermis at P5, the level of filaggrin monomer

470   was markedly reduced in mutant epidermis, suggesting that decreased epidermal protease

471   activity might affect filaggrin processing in Ric[EKO] epidermis (Figure 4F and 4G).

472

473   **A keratinocyte-autonomous mTORC2-Akt axis controls filaggrin processing**

474   To tackle the molecular mechanism underlying decreased filaggrin processing in Ric[EKO]

475   epidermis, we isolated primary keratinocytes from control and Ric[EKO] newborn mice and

476   analyzed downstream targets of mTOR pathways during terminal differentiation triggered

477   by Ca^{2+} treatment [46-47]. Ca^{2+} triggered activation of both mTORC1 (pS6-Ser240/244) and

478   mTORC2 (pAkt-Ser473) in wild-type keratinocytes (Figure 5A). As expected, the

479   absence of mTORC2 in Ric[EKO] keratinocytes abolished phosphorylation of Akt-Ser473 in

480   response to Ca^{2+} exposure (Figure 5A). Earlier studies had demonstrated that Akt is a

481   critical regulator of profilaggrin processing during keratinocyte differentiation [24-25].

482   Notably, Western blot analysis revealed a pronounced reduction of processed trimer and

483   monomer filaggrin in Ric[EKO] keratinocytes. Signals for other markers of keratinocyte

484   differentiation, including loricrin and keratin 10 (K10), appeared unchanged in the

485   knockout mice (Figure 5B).

486   To better define the role of mTORC2-mediated filaggrin processing, we retrovirally

487   reconstituted Ric[EKO] keratinocytes with constitutively active myristoylated Akt1 (myr-

488   AKT) [34] and examined filaggrin processing. Empty pMSCV-IRES-GFP vector

489   transfection served as control. Infection efficiencies were determined by analysis of

490   IRES-driven GFP expression. GFP$^+$ cells were sorted by flow cytometry (FACS),

491   expanded *in vitro*, and subjected to high Ca^{2+}-induced differentiation. Western blot

492   analysis confirmed abundant myr-AKT protein expression and increased Akt-Ser473

493   phosphorylation signal in myr-AKT transduced keratinocytes (Figure 5C and D). In line

494   with previous studies[24-25], enforced myr-Akt expression increased filaggrin monomer

495   expression in control keratinocytes upon calcium-triggered differentiation (Figure 5C).

496   Importantly, restoration of Akt signaling in Ric^{EKO} keratinocytes resulted in a significant

497   increase in the amount of filaggrin monomer (Figure 5D), indicating that restored Akt-

498   Ser473 phosphorylation enhanced filaggrin processing in Ric^{EKO} cells. Taken together,

499   our findings implicate mTORC2-mediated activation of Akt-Ser473 as a critical factor in

500   effective filaggrin processing in keratinocyte terminal differentiation.

501

502   **Epidermal mTORC2 determines immune cell composition in the skin**

503   Epidermal barrier defects are often associated with altered immune responses and

504   inflammatory skin diseases [48]. To assess the consequences of epidermal mTORC2-

505   deficiency and the resulting barrier defect on the immune status, we examined

506   inflammation mediator expression in prenatal E19.5 epidermis (RNA-seq analysis) and in

507   P5 Ric^{EKO} epidermis by qRT-PCR. Notably, whereas the RNA-seq analysis in prenatal

508   E19.5 epidermis revealed downregulation of inflammation mediators when compared to

509   controls (Figure 2B; Supplemental Table 3), postnatal (P5) expression of thymic stromal

510   lymphopoietin (*Tslp*) and interleukin-24 (*Il24*) were markedly increased in Ric^{EKO}

511 epidermis compared to littermate controls (Figure 6A). In contrast, transcripts for other

512 potent inflammatory factors, including interleukin-1α (*Il1α*), interleukin-1β (*Il1β*),

513 interleukin-33 (*Il33*), chemokine ligand (*Ccl2*), and tumor necrosis factor-α (*Tnfα*), were

514 not obviously altered in P5 Ric^{EKO} epidermis (Figure 6A). To investigate further the

515 impact of epidermal mTORC2 activity on cutaneous immune cell composition, we also

516 performed immunohistochemical and fluorescent analyses in back skin of controls and

517 Ric^{EKO} mice at P5. While no major difference was detected in numbers of toluidine blue

518 stained mast cells and dermal F4/80$^+$ macrophages, the number of dermal CD4$^+$ T cells

519 was increased in Ric^{EKO} mice (Figure 6B; Supplemental Figure 2A). The tendency

520 towards an increase in CD4$^+$ cells in Ric^{EKO} skin was corroborated by FACS analysis.

521 Unexpectedly, these analyses further revealed that the percentage of CD3$^+$γδ-TCR$^+$ cells

522 (Gate: 7-AAD$^-$CD45$^+$) was markedly reduced from 5.314±0.708% to 0.350±0.085% in

523 Ric^{EKO} skin (Figure 6C; Supplemental Figure 2B).

524 Dendritic epidermal T cells (DETCs) constitute a subset of T cells, which highly express

525 the γδ-T cell receptor (γδ-TCR) and specifically localize to the epidermis [49-50]. Consistent

526 with the FACS analysis, immunofluorescent staining for the pan-T cell marker CD3

527 revealed a pronounced reduction of epidermal T cells in Ric^{EKO} mice as compared to

528 controls (Figure 7A). Further, epidermal CD3 and γδ-TCR co-staining revealed

529 significantly reduced DETCs in Ric^{EKO} epidermis (Figure 7A). Intriguingly, RNA-seq

530 analysis of E19.5 Ric^{EKO} epidermis revealed a significant downregulation of several

531 members of the *Skint* family genes when compared to controls (Figure 7B). γδ-TCR

532 ligand *Skint* genes are expressed by epidermal keratinocyte and play a critical role in

533 maintaining the DETC pool in the epidermis and in regulating γδ-TCR cell homeostasis

534   [51]. Downregulated expression of *Skint1* and *Skint4* was confirmed by qRT-PCR analysis

535   in control und mutant epidermis at P0 (Figure 7C).

536   To answer the question whether epidermal mTORC2 activity is important to maintain the

537   homeostasis of DETCs throughout adulthood, we analyzed whole mounts from ear skin

538   in adult mice at P70. Immunofluorescent analysis and FACS analysis revealed that the

539   number of $CD45^+CD3e^+\gamma\delta$-$TCR^{high}V\gamma3^+$ DETCs was also markedly reduced in adult

540   Ric^{EKO} epidermal sheets, whereas the proportion of $CD45^+CD3e^+\gamma\delta$-$TCR^{low}V\gamma3^-$ dermal

541    $\gamma\delta$ T cells was similar in Ric^{EKO} mice and controls (Figure 7D, E; Supplemental Figure

542   2C). Interestingly, the majority of DETCs in Ric^{EKO} ears displayed an activated

543   phenotype characterized by a rounded morphology and reduced dendritic spines as

544   compared to DETCs in controls (Figure 7D). Reduced DETC number was paralleled by

545   significantly reduced *Skint1* and *Skint4 transcripts* (Figure 7F). Collectively, these

546   findings reveal a critical role of epidermal mTORC2 activity in determining the

547   composition of T cell subpopulations in naïve skin.

548

549   **DNFB-mediated percutaneous immune response is enhanced in Ric^{EKO} mice**

550   Barrier disruption and continuous percutaneous exposure to allergens presumably initiate

551   and drive a variety of inflammatory skin diseases, including AD [52]. In fact, long-term

552   observation under specific pathogen free (SPF) conditions revealed mildly dry eyes and

553   perioral dermatitis in Ric^{EKO} mice, to varying extent among individual mutants

554   (Supplemental Figure 3A). Numbers of mast cells, $CD4^+$ T cells, and $F4/80^+$ myeloid

555   cells and transcripts of proinflammatory mediators including *Il6*, *Tnfα*, *Il1β,* and *Tslp*

556   showed a tendency towards increase, yet, the difference did not reach statistical

557   significantly in unchallenged back skin of control and mutants at 10 weeks (Supplemental

558   Figure 3B and C). In addition, although serum levels of circulating total IgE were

559   increased in Ric^{EKO} mice at 10 weeks, the difference did not reach statistical significance

560   (Supplemental Figure 3D). To quantify the functional impact of epidermal mTORC2

561   activity in percutaneous immune responses to exogenous substances, 10 week-old control

562   and Ric^{EKO} mice were subjected to established models of irritant (ICD) or allergic (ACD)

563   contact dermatitis. Whereas no difference in ICD was observed between control and

564   mutants (Supplemental Figure 3E), ACD in response to the hapten DNFB was

565   significantly pronounced in mutants versus controls (Figure 8A). In controls the positive

566   DNFB response was paralleled by a significant increase in epidermal phosphorylation of

567   Akt-S473, an alteration of the cellular immune cell infiltrate with significant increase in

568   numbers of F4/80^{+} myeloid cells and a significant decline in DETCs (Figure 8C and

569   Supplemental Figure 4). In contrast, the attenuated DNFB response in Ric^{EKO} epidermis

570   was associated with a significant reduction of epidermal Akt-S473 phosphorylation, an

571   alteration of the cellular immune cell infiltrate with significant increase in numbers of

572   granulocytes, F4/80^{+} myeloid and CD4^{+} T cells, and virtually absence of DETCs;

573   differences in F4/80^{+} myeloid cell, CD4^{+} T cell and DETC cell infiltrate were

574   significantly different in Ric^{EKO} versus control mice (Figure 8C and Supplemental Figure

575   4). In addition, transcripts of stress response genes including *Krt6b*, *S100a8* and *Sprr2d*,

576   and proinflammatory mediators such as *Il6*, *Tnfα* and *Il24,* were significantly increased in

577   DNFB challenged ear skin in Ric^{EKO} versus controls (Supplemental Figure 5).

578 Taken together, these findings strongly suggest that the impaired epidermal barrier in

579 Ric^{EKO} mice allows an increased hapten penetration, which in turn leads to the

580 exaggerated stress and percutaneous immune responses.

581

28

Discussion

In the present study, we describe epidermal mTORC2 as central hub orchestrating the complex multicellular crosstalk and signaling required for postnatal epidermal barrier integrity and function. Specifically, we uncover a key role for epidermal mTORC2 activity in the assembly of a protective SC (Figure 8D).

A prominent phenotype of Ric[EKO] mice is the dry, scaly, and ichthyosis-like appearance of skin. A similar phenotype is present in various mouse mutants and human skin diseases with epidermal barrier defects including ichthyosis, AD and multiple clinical eczema variants [16, 53]. Yet, an overarching mechanism that controls the multiple molecular factors in epidermal barrier formation is not resolved. Here, we propose the Ric[EKO] mouse as a novel preclinical disease model to advance our incomplete understanding of the molecular regulation of SC assembly and epidermal barrier function. Ric[EKO] mice displayed multiple skin symptoms which are also hallmarks in patients with compromised epidermal barrier function, including increased SC fragility and increased TEWL [6]. Ric[EKO] epidermis also showed dysbalanced protease/antiprotease activities combined with attenuated filaggrin processing, both previously reported to be associated with skin barrier defects in patients [26, 44, 54-55]. Thus, here we identified mTORC2 as a critical regulator of protease-mediated epidermal terminal differentiation.

In addition, a striking feature of *Rictor*-deficient epidermis is disturbed lipid composition. Specifically, we observed quantitative alterations in lipid content and structural alterations in lipid lamellae formation in Ric[EKO] epidermis. Consistently, expression of multiple genes previously reported to be essential in epidermal lipid synthesis and epidermal function in humans were reduced in Ric[EKO] epidermis emphasizing the utility

29

605   of Ric[EKO] mice as an attractive preclinical disease model for barrier defects [10, 12-15].

606   Recent reports describing a regulatory role for mTORC2 in *de novo* lipid synthesis are

607   limited to hepatic and adipose tissues [56-58]. Here, we provide the first evidence of a role

608   for mTORC2 in epidermal lipid metabolism. However, although it is widely accepted that

609   epidermal lipids are integral components driving the formation and maintenance of the

610   epidermal permeability barrier, the exact mechanistic link how epidermal lipids regulate

611   epidermal barrier function and/or vice versa, is not entirely resolved [59,60]. Therefore,

612   although our findings in Ric[EKO] mice suggest that the quantitative and qualitative

613   perturbations in lipid composition is causative for the observed skin barrier defect in

614   Ric[EKO] mice, at this stage we cannot exclude the possibility that perturbed epidermal

615   architecture contributes to perturbed lipid synthesis [59, 60]. It will be of interest to

616   investigate in future studies additional downstream mediators of mTORC2 signaling to

617   understand how epidermal *de novo* lipogenesis and SC lipid homeostasis is regulated by

618   mTORC2.

619   Intriguingly, the number of epidermal DETCs was remarkably reduced in Ric[EKO] mice.

620   DETCs are profoundly reduced in patients with AD as well as in mouse models lacking

621   key SC proteins [30,61]. Consistent with a reduced DETC number in Ric[EKO] epidermis, the

622   expression of several members of *Skint* family genes was downregulated in Rictor-

623   deficient epidermis. *Skint* genes have been identified as essential regulators of DETC cell

624   development [50]. Hence, our findings indicate that epidermal mTORC2 activity is critical

625   for anchoring DETCs in the epithelial niche and maintaining the DETC repertoire,

626   potentially mediated via *Skint* genes. However, the reduced epidermal DETC number

627   could be caused by a combination of multiple factors including attenuated expression of

628   *Skint* genes, perturbed epidermal architecture and/or disturbed interplay between lipids

629   and/or additional factors and remains to be further investigated in future studies. In

630   addition to the reduced number of epidermal DETCs, the morphology of DETCs was

631   significantly altered in Ric^EKO epidermis. DETCs in Ric^EKO epidermal sheets lead to a

632   phenotype associated with activation and a protective role in injured epidermis [50]. Thus, it

633   is tempting to speculate that DETCs in Ric^EKO epidermis compensate for a defective

634   epidermal barrier. Together, our findings suggest an important function of epidermal

635   mTORC2 in maintaining and shaping DETC homeostasis, and thus, highlight a

636   previously unrecognized function of mTORC2 in the epithelial-immune crosstalk

637   regulating skin barrier function. Along these lines, increased infiltration of CD4$^+$ T cells

638   and enhanced expression of *Tslp* were also observed in the skin of Ric^EKO pups, both of

639   which are hallmarks in epidermis and keratinocytes of AD patients [62]. Although, the adult

640   Ric^EKO mouse skin displayed no spontaneous pathological phenotypes, including skin

641   lesions or signs of skin inflammation, adult mutants exhibited exaggerated percutaneous

642   immune responses. AD patients have an increased risk of sensitization to allergens, as

643   well as asthma, rhinitis, and food allergy. Consistently, *FLG* deficiency in mice facilitates

644   and permits increased percutaneous sensitization with protein allergens, irritants, and

645   haptens [63-64]. Collectively, our findings highlight a direct link between epidermal

646   mTORC2 deficiency and paradigmatic symptoms of patients with defective epidermal

647   barrier conditions such as ichthyosis or AD.

648   An important question is what could be the mechanistic explanation for the various

649   processes and symptoms observed in Ric^EKO mice? A well-defined function of mTORC2

650   is the phosphorylation of Akt at Ser473, which contributes to Akt-Thr308

651   phosphorylation by the phosphoinositide-dependent kinase 1 (PDK1), leading to full Akt

652   activation [20, 65]. Akt activity is elevated at the onset of keratinocyte differentiation and has

653   been shown to regulate epidermal terminal differentiation [18,24-25]. However, the signaling

654   events triggering epidermal Akt activation in epidermal differentiation still remain

655   elusive. We propose a non-redundant role for mTORC2 in Akt-Ser473 activation during

656   epidermal stratification and cornification. It is likely that epidermal mTORC2 acts

657   through Akt in a PDK1-dependent manner since depletion of either component leads to

658   an epidermal barrier phenotype comparable to that in Ric[EKO] mice [66-67]. Several *in vitro*

659   and *in vivo* studies suggest that proper filaggrin processing requires Akt activation [24-26].

660   Accordingly, we observed that Akt-Ser473 phosphorylation was significantly attenuated

661   and filaggrin processing was impaired in Ric[EKO] keratinocytes *in vitro*. Together, our

662   findings suggest that the mTORC2-Akt signaling axis is activated and has mTORC1-

663   independent functions upon terminal differentiation to trigger filaggrin processing and

664   barrier function. Future studies are needed to determine whether induction of epidermal

665   protease activity and/or lipid metabolism is a direct or indirect downstream target of

666   mTORC2-Akt signaling.

667   Finally, prenatal Ric[EKO] epidermis presented a robust upregulation of a stress-response

668   signature characterized by prominent expression of epidermal barrier stress-induced

669   intermediate filament genes as well as UVB-induced genes. We hypothesize that this

670   induced stress response supports a compensatory, protective mechanism in response to

671   the barrier defect, thereby ensuring survival. This hypothesis is further based on the

672   finding that the cytoprotective transcription factor *Nrf2* and several well known Nrf2-

673   target genes, including *Krt6, Krt16, Sprr2h,* and *S100a* were all transcriptionally

674   upregulated in Ric[EKO] epidermis [68]. Along these lines, a Nrf2-regulated compensatory

675   response has been shown to be activated in embryonic epidermis lacking loricrin [69].

676   Therefore, our findings support the idea that the activation of a stress-related homeostatis

677   program is a general feature of a functionally compromised epidermal barrier. Our

678   discovery that the druggable mTORC2 complex orchestrates this generalized stress

679   response opens interesting avenues for exploring therapeutic boosting of barrier function.

680   In conclusion, we have demonstrated that postnatal epidermal mTORC2 activity

681   orchestrates epidermal barrier formation and maintenance via regulation of lipid synthesis

682   and filaggrin processing. We speculate that altered mTORC2 activity may represent

683   another predisposing factor for skin disorders associated with disrupted barrier function.

684   Our findings might be of clinical relevance by, for example, normalizing epidermal

685   mTORC2 activity in patients with defective epidermal barrier conditions by

686   pharmacotherapy.

687

Author Contributions

688

689 XD and SAE conceptualized the study, performed experiments, analyzed the data, and

690 drafted the manuscript; WB performed TEM analysis; SW helped with flow cytometry

691 and experimental animal work; PW performed the bio-informatic analyses; SB performed

692 the lipid analysis; MNH and MAR provided *Rictor* floxed mouse lines; AJ, JCB, SAW,

693 AR, MNH, MAR have made substantial contributions to the analysis and interpretation of

694 data and critical revising of the drafted manuscript.

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901

902

903 **Figure legends**

904 **Figure 1. Ric^{EKO} mice display a transient ichthyosis-like phenotype.**

905 (A) Western blot analysis in epidermal isolates at P5. (B) Macroscopic appearance of

906 littermates; boxed areas are shown at higher magnifications. (C) HE-stained back skin

907 sections at P4 and quantification of epidermal thickness; e, epidermis; d, dermis; SC,

908 stratum corneum; white dashed line indicates basal membrane, scale bar, 25 µm. (D)

909 Quantification of TEWL at P4, each dot or square represents one mouse. (E) Body weight

910 at P5. (F) Left: micrographs of isolated CE from back skin at P4. Right: quantification of

911 the intact CE, each dot or square represents isolates from one mouse. (G) TEM image

912 shows poorly condensed SC in Ric^{EKO} mice, note the presence of cell organelles in C1 of

913 Ric^{EKO} mice (indicated by arrow). SG, stratum granulosum; C1, cornified layer 1, scale

914 bar, 0.5 µm. Data are expressed as the mean; unpaired t-test was used to calculate P value;

915 $**P < 0.01$; $***P < 0.001$.

916

917 **Figure 2. Epidermal mTORC2 deficiency induces a compensatory transcriptional**

918 **epidermal repair program in Ric^{EKO} mice.**

919 (A) Volcano plot of differentially regulated transcripts between control and Ric^{EKO}

920 epidermis at E19.5 (n=3 per genotype). Colored data points meet the thresholds of a $\log_2$

921 fold change of higher than 1 or lower than -1 and an adjusted P value of lower than 0.05.

922 (B) GO of differentially regulated transcripts from Ric^{EKO} vs. control comparison using

923 DAVID 6.8 online software. Only genes that have fold change >1.5 and a P value < 0.05

924 were used. (C) $\log_2$ fold changes of EDC gene expression in the epidermis of Ric^{EKO}

925 skin. EDC genes with an alteration of fold change > 2 and and P value < 0.05 are shown.

926   (D) qRT-PCR analysis validation of differentially expressed genes from RNA-seq data in

927   P0 epidermis (*n*=5/genotype). (E) Left: epidermal UV-response gene set enrichment plot

928   in Ric[EKO] samples. Right: heat map shows the stress-response genes with positive

929   enrichment in Ric[EKO] samples. All data are presented as mean ± s.e.m; unpaired t-test was

930   used to calculate the *P* value; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

931

932   **Figure 3. mTORC2 controls epidermal lipid metabolism.**

933   (A) Fluorescent dye Nile red staining and quantification of back skin sections at P1(*n*=4);

934   counterstained with DAPI; e, epidermis; d, dermis; white dashed line indicates basal

935   membrane; scale bar, 25 µm. (B) Analysis of SC lipids by HPTLC (*n*=3 per genotype).

936   Cer, ceramide; FFA, free fatty acid; Chol, cholesterol. (C) Left: GSEA of differentially

937   regulated genes between control and Ric[EKO] epidermis identifies enrichment of lipid and

938   lipoprotein metabolism genes. Right: hierarchically clustered heat map illustration shows

939   differential expression of lipid metabolism-related genes from RNA-seq data. (D) qRT-

940   PCR analysis of *Elovl3* and *Pla2g5* in epidermis at P0 (*n*=5 per genotype). (E)

941   Ultrastructural analysis of the interface between SG and SC at P5. Ric[EKO] epidermis

942   shows a disorganized interface (red arrows) between SG and SC, and displayed LB

943   abnormalities, including disoriented lamellae and reduced lamellae number. In contrast,

944   the LB of control showed regular lamellae orientation with equal distances lamellae (red

945   bars). SG, stratum granulosum; C1, cornified layer 1; C2, cornified layer 2; LB, lamellar

946   body, scale bar, 200 nm. All data are presented as mean ± s.e.m; unpaired t-test was used

947   to calculate the *P* value; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

948

949 **Figure 4. Reduced proteolytic activity and impaired filaggrin processing in Ric^EKO**

950 **epidermis.**

951 (A) GSEA of RNA-seq data identifies enrichment of the (KEGG) lysosome gene set. (B)

952 Hierarchically clustered heat map shows altered expression of protease and protease

953 inhibitor genes. (C) qRT-PCR analysis of indicated genes at P0 (*n*=5). (D) qRT-PCR

954 analysis shows downregulated *Slpi* expression in Ric^EKO epidermis (*n*=5). (E) TEM

955 analysis shows delayed corneodesmosome degradation in Ric^EKO epidermis at P5.

956 Corneocyte layers (C) were numbered, arrows point to corneodesmosomes. SG, stratum

957 granulosum; scale bar, 200nm. (F) qRT-PCR analysis of epidermal filaggrin mRNA

958 expression at P5 (*n*=5). (G) Left, Western blot analysis at P5; Profilaggrin (Pro-FLG),

959 trimer (3F), dimer (2F) and monomer (1F) filaggrin are indicated. Right, quantified ratio

960 of filaggrin monomer (1F) and total filaggrin, Pro-filaggrin and β-actin with control set to

961 1 (*n*=4). All data are presented as mean ± s.e.m; unpaired t-test was used to calculate the

962 P value; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

963

964 **Figure 5. A keratinocyte-autonomous mTORC2-Akt axis controls filaggrin**

965 **processing.**

966 (A) Western blot analysis in cultured control and Ric^EKO keratinocytes, stimulated by

967 high concentration Ca^{2+} (0.2 mM). (B) Left, representative Western blot analysis in

968 keratinocytes; right, graph of relative densitometry of filaggrin trimer (3F), dimer (2F)

969 and monomer (1F). (C) Western blot analysis of filaggrin and Akt protein expression in

970 empty vector (control) and myr-Akt retrovirus infected control keratinocytes. (D) Left,

971 Western blot analysis in empty vector (control) and myr-Akt retrovirus infected Ric^EKO

972   keratinocytes; right, graph of relative densitometry of filaggrin trimer (3F), dimer (2F)

973   and monomer (1F). All data are presented as mean ± s.e.m; unpaired t-test was used to

974   calculate the *P* value; *$P < 0.05$; **$P < 0.01$.

975

976   **Figure 6. Epidermal mTORC2 determines immune cell composition in the skin.**

977   (A) qRT-PCR profile in epidermis at P5 (*n*=8 per genotype). (B) Left: CD4

978   immunostaining on back skin sections at P5. Right: quantification of CD4$^+$ cells at high-

979   power field (HPF). e, epidermis; d, dermis; white dashed line indicates basal membrane;

980   scale bar, 25 µm. (C) Left: flow cytometry profile of γδ-TCR$^+$ CD3$^+$ cells at P5; right,

981   percentage of different cell populations are shown at P5 (*n*=6 per genotype); cells were

982   gated on 7-AAD$^-$ CD45$^+$ skin cells. All data are presented as mean ± s.e.m; unpaired t-

983   test was used to calculate the *P* value; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

984

985   **Figure 7. DETC number is reduced in Ric^{EKO} epidermis.**

986   (A) Left: CD3 and γδ-TCR immunostaining on back skin at P5; Right: quantification of

987   epidermal CD3$^+$ and γδ-TCR$^+$ cells per 500 µm of basement membrane; white dashed

988   line indicates basal membrane; scale bar, 25 µm. (B) Hierarchically clustered heat map

989   shows altered expression of *skint* family genes in Ric^{EKO} epidermis from RNA-seq data.

990   (C) qRT-PCR analysis validates reduced *Skint1* and *Skint4* expression in Ric^{EKO}

991   epidermis at P0 (*n*=5 per genotype). (D) Left: DETCs in epidermis of ear sheet at P70;

992   separated epidermis was stained against γδ-TCR. Right: quantification of γδ-TCR$^+$ cells

993   in the epidermal sheets. Scale bar, 25 µm. (E) Left: flow cytometry profile of single cell

994   suspensions of ear skin at P70. 7-AAD$^-$CD45$^+$CD3e$^+$ cells were gated and analyzed for

995 expression of γδ-TCR and Vγ3-TCR, (right) quantification of γδ-TCR^{low}Vγ3-TCR^{-}

996 dermal γδ T cells and γδ-TCR^{high}Vγ3-TCR^{+} DETCs. (F) qRT-PCR analysis of *Skint1* and

997 *Skint4* expression in adult epidermis at P70 (*n*=5 per genotype). Each dot or square

998 represents one mouse. Data are presented as mean or as mean ± s.e.m; unpaired t-test was

999 used to calculate the *P* value; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

1000

1001 **Figure 8. Enhanced percutaneous immune response in Ric^{EKO} mice.**

1002 (A) DNFB-induced ACD response; right, H&E-staining of DNFB-treated ears. e,

1003 epidermis; d, dermis; c, cartilage, *$P < 0.05$; ***$P < 0.001$. (B) pAkt-S473

1004 immunofluorescent staining and morphometric quantification of pAkt-S473 stained

1005 unchallenged (0 h) and challenged (48 h) ear skin sections. (C) The number of

1006 eosinophils (Siglec F), mast cells (Giemsa), neutrophils (Gr1), macrophages (F4/80) and

1007 T cells (CD4 and γδ-TCR) per high power field (HPF, 400x) in unchallenged (0 h) and

1008 challenged ear (48 h) ear tissues. (D) Schematic diagram illustrating the proposed

1009 function of mTORC2 in regulating epidermal barrier function. Each dot or square

1010 represents one mouse. Data are presented as mean or as mean ± s.e.m; unpaired t-test or

1011 ANOVA one-way test analysis with Bonferroni multiple comparison test was used to

1012 calculate the *P* value; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

1013

Figure 1

A

Rictor

β-actin

Control   Ric^EKO   kDa

-250

-35

B

Control   Ric^EKO

P3

P7

Control

Ric^EKO

C

Control   Ric^EKO

P4

SC

e

d

SC

e

d

Back skin

• Control
■ Ric^EKO

Epidermal thickness (μm)

***

P4

D

• Control
■ Ric^EKO

TEWL (g/m²/h)

**

P4

E

• Control
■ Ric^EKO

Body weight (g)

**

P5

F

CE (P4)

Control

Ric^EKO

• Control
■ Ric^EKO

Intact CE (%)

***

P4

G

Control   Ric^EKO

C1

SG

C1

SG

Figure 2

Figure 3

A

B

C

D

E

Figure 4

A

KEGG: lysosome

P=0.000, NES=-2.035

Enrichment score (ES)

Signal to noise ratio

Rank in ordered data set

B

Ctsl
Ctsa
Ctsb
Ctsh
Ctsc
Ctss
Ctsf
Ctsz
Ctse
Ctsw
Ctso
Ctsk
Ctsd

Color key

Serpinb2
Serpinb10
Serpina3b
Serpinba3j
Serpinb11
Serpinb1a
Serpine1
Serpin3n
Serpinb6c

Color key

C

■ Control
□ Ric^EKO

Ctsh   *Ctsd*   *Serpinb2*

rel. mRNA level

Epidermis (P0)

D

■ Control
□ Ric^EKO

Slpi

rel. mRNA level

Epidermis (P0)

E

Control   Ric^EKO

F

Filaggrin

■ Control
□ Ric^EKO

rel. mRNA level

Epidermis (P5)

G

kDa   Control Ric^EKO

Pro-FLG

250
130
100   3F
70
55   2F

35   1F
25

β-actin

■ Control
□ Ric^EKO

Relative levels (1F/ total FLG)

Epidermis (P5)

Relative levels (Pro-FLG/β-actin)

Epidermis (P5)

Figure 5

Figure 6

A

B

CD4

C

Gated on 7AAD⁻ CD45⁺

Figure 7

A

CD3   γδ-TCR

P5

Control
e   e
d   d

Ric^EKO
e   e
d   d

• Control
■ Ric^EKO

CD3+ cells /500 μm BM
Back skin

γδ-TCR+ cells /500 μm BM
Back skin

B

Control Control Control Ric^EKO Ric^EKO Ric^EKO

Skint9
Skint4
Skint6
Skint2
Skint10
Skint3
Skint5
Skint7
Skint11
Skint8
Skint1

-2   0   2
Color key

C

■ Control
■ Ric^EKO

rel. mRNA level

Skint1   Skint4

D

• Control
■ Ric^EKO

Control   Ric^EKO

P70

γδ-TCR

γδ-TCR+ cells/hpf
Ear epidermis

E

Gated on 7AAD−

Control

Ric^EKO

CD45

CD3e

20.4%
37.4%

3.7%
43.8%

γδ-TCR

Vγ3-TCR

Gated on
7AAD− CD45+CD3e+

• Control
■ Ric^EKO

(%)

γδ-TCR^low   γδ-TCR^high
Vγ3-TCR−   Vγ3-TCR+

F

■ Control
■ Ric^EKO

rel. mRNA level

Skint1   Skint4
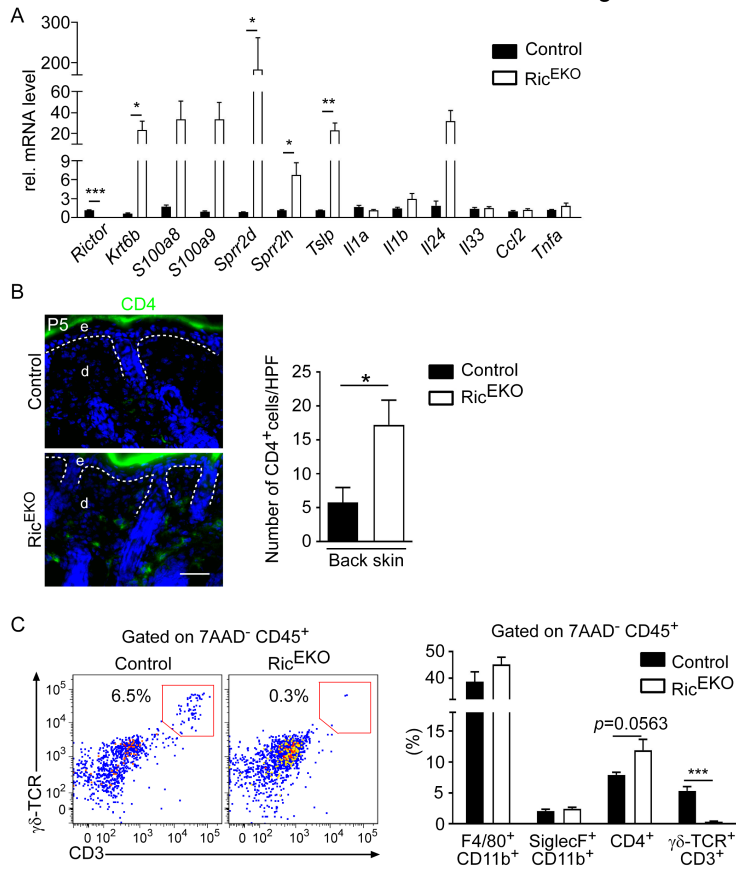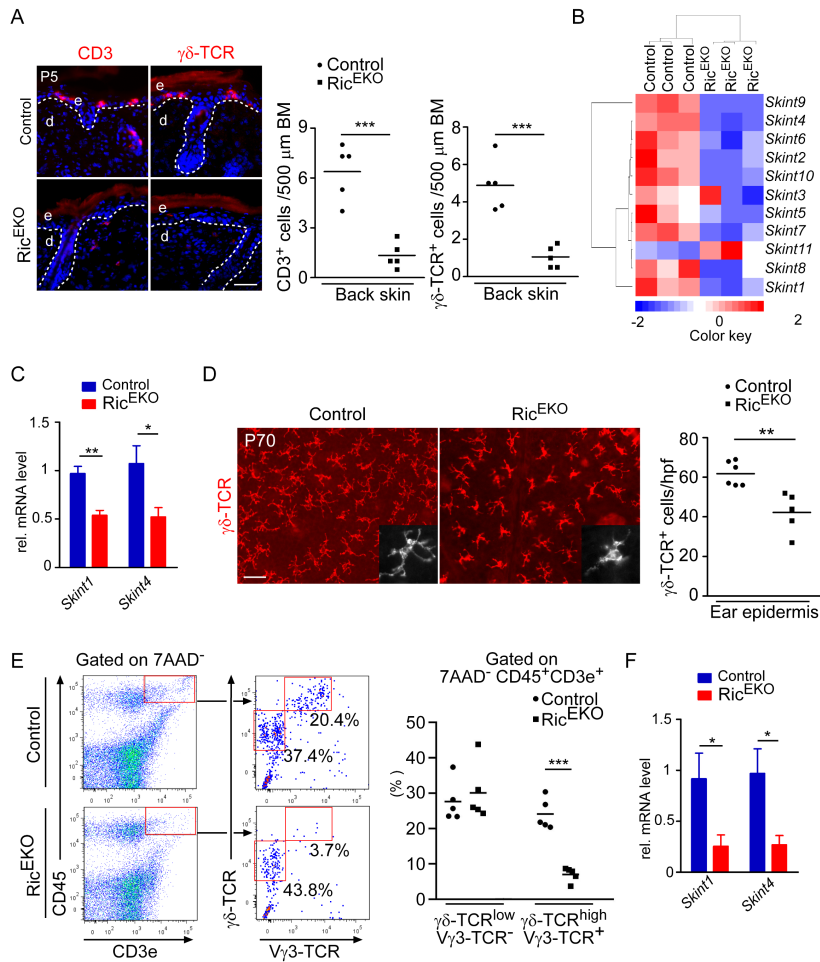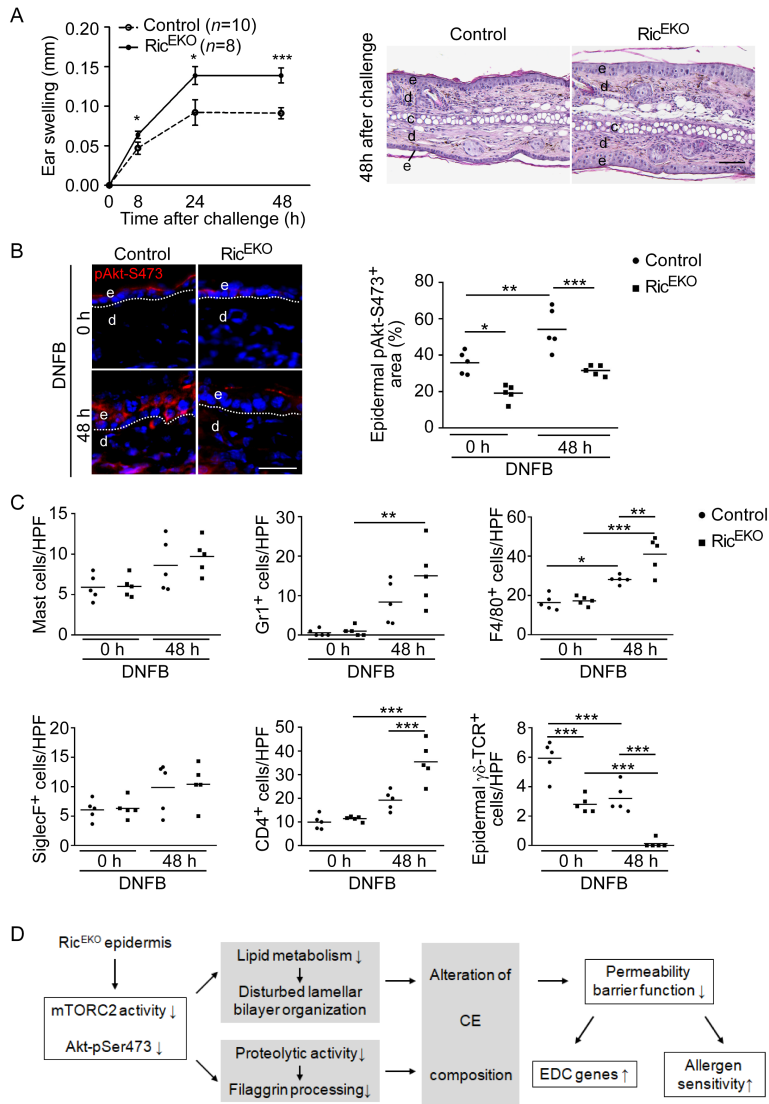
Figure 8

Online Repository

Epidermal mTORC2 controls lipid synthesis and filaggrin processing in epidermal barrier formation

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Supplemental Materials

Supplemental Figure 1. Conditional targeting *Rictor* gene in epidermal keratinocytes.

Supplemental Figure 2. Immune cell composition and gating strategies for FACS analysis of cell suspensions isolated from back skin and ear.

Supplemental Figure 3. Mild skin inflammation in naïve Ric^{EKO} mice under SPF conditions.

Supplemental Figure 4. Immune cell infiltration in ear skin tissue.

Supplemental Figure 5. Gene expression analysis in ear skin tissue.

Supplemental Table 1: Antibodies used for immunostaining and Western blot analysis

Supplemental Table 2: Primer sequence used for qRT-PCR analysis

Supplemental Table 3: Genes in biological processes (BP) identified by GO term analysis.

Supplemental Figure legends

Supplemental Figure 1. Conditional targeting *Rictor* gene in epidermal keratinocytes.

(A) Scheme illustrating the *Rictor* gene construct, the 2 loxP sites flanking exons, and the PCR fragment length before and after recombination. (B) PCR product of genomic DNA isolated from various tissues. (C) Phospho-Akt (Akt-pS473) immunostaining of back skin of control and Ric^{EKO} at P5; scale bar, 25 µm. (D) Survival curve of control (*n*=16) and Ric^{EKO} (*n*=15) mice. Mantel-Cox test was used to calculate the P value. (E) Representative H&E-stained back skin sections from control and Ric^{EKO} mice at P70. Scale bar, 25 µm. (F) TEWL in Ric^{EKO} and control mice. (G) Representative immunofluorescence staining at P5 for ZO-1, E-cadherin (E-cad), Desmoplakin1/2 (DSP1/2), Desmoglein1/2 (DSG1/2), β4-integrin (red); white dashed lines indicates basement membrane; nuclei are visualized with DAPI (blue); scale bar, 20 µm. (H) Body weight of control (*n*=8) and Ric^{EKO} (*n*=7) mice at different ages. (I) Ultrastructural analysis of back skin epidermis of control and Ric^{EKO} mice at P5. Lower panel: higher magnification images show desmosome (de) structure. e, epidermis; d, dermis; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale, scale bar, 5 µm (upper panel) and 100 nm (lower panel). Each dot or square represents one mouse. Data are presented as mean or as mean ± s.e.m; unpaired t-test was used to calculate the *P* value; **P < 0.01; ***P < 0.001.

Supplemental Figure 2. Immune cell composition and gating strategies for FACS analysis of cell suspensions isolated from back skin and ear.

(A) Left: Representative Giemsa staining and F4/80 immunostaining with back skin sections from control and Ric^{EKO} mice at P5. Right: quantification of mast cells and F4/80$^+$ macrophages cells per high-power field (HPF) (*n*=5 per genotype). Scale bar, 25 µm; data are presented as as mean ± s.e.m. (B) Representative FACS analysis of single cell suspensions of back skin in control and Ric^{EKO} mice (P5). Upper panel: single cells were gated (G1-3) and analyzed for 7-AAD staining and expression of CD45 (G4). Lower panel: 7-AAD$^-$CD45$^+$ cells (G4) were analyzed for expression of (from left to right) CD3 and γδ-TCR, CD4, CD11b and F4/80, and CD11b and Siglec F. (C) Representative FACS analysis of single cell suspensions of ear skin in control and Ric^{EKO} mice (P70). Single cells (G1-3) and 7-AAD$^-$ cells (G4) were gated. Red polygons indicate the gate and percentages indicate the proportion of gated cells. FSC-A, forward scatter area; FSC-H, forward scatter height; G, gate; SSC-A, sideward scatter area; SSC-H, sideward scatter height.

Supplemental Figure 3. Mild skin inflammation in naïve Ric^{EKO} mice under SPF conditions.

(A) Representative macroscopic appearance of control and Ric^{EKO} mice at P70. (B) Quantification of CD4$^+$, mast cells (MCs), and F4/80$^+$ macrophages in back skin sections from control and Ric^{EKO} mice at P70 (*n*=5 per genotype). (C) qRT-PCR analysis of *Il6, Tnfα, Il1b,* and *Tslp* expression in Ric^{EKO} and control skin at P70 (*n*=5 per genotype). (D) Serum IgE amount in Ric^{EKO} and control mice were determined by ELISA (*n*=6 per

genotype) at P70. (E) Croton oil-induced irritated contact dermatitis (ICD) response. Ear swelling of control and Ric^{EKO} animals after topical application of croton oil was calculated and shown (n=5 per genotype). Data are presented as mean ± s.e.m.

Supplemental Figure 4. Immune cell infiltration in ear skin tissue. Representative Giemsa stainings and immunohistochemical stainings for Gr1, F4/80, Siglec F, CD4 and γδ-TCR of untreated (DNFB, 0 h) and treated (DNFB, 48 h) ear sections from control and Ric^{EKO} mice (n=5). Arrows indicate mast cells (Giemsa stain, purple) or polymorphonuclear cells (Gr1 stain, brown) and dashed line indicates the border between epidermis and dermis. e, epidermis; d, dermis; scale bar, 25 µm.

Supplemental Figure 5. Gene expression analysis in ear skin tissue.

qRT-PCR analysis of stress response gene (*Krt6b*, *S100a8* and *Sprr2d*) and inflammatory mediator (*Il6*, *Tnfa* and *Il24*) expression in untreated (DNFB, 0 h) and treated (DNFB, 24 h) ear skin tissues from control and Ric^{EKO} mice (n=6 per genotype). Gene expression in untreated control ear skin was referred as 1 and relative fold changes were analyzed and shown. Data are presented as mean ± s.e.m; ANOVA one-way test analysis with Bonferroni multiple comparison test was used; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Supplemental Table 1: Antibodies used for immunostaining and Western blot analysis

Name	Catalog number	Source
Primary antibodies used for immunostaining		
Akt-pS473	9271	Cell Signaling, Beverly, MA
CD4	14-0042-85	Thermo Fisher Scientific
Ly-6G and Ly-6C (Gr1)	550291	BD Biosciences
Siglec F	552126	BD Biosciences
CD3	MCA1477	AbD Serotec
F4/80	MOF4F (V500)	Dianova
ZO-1	(hybridoma) Rat, Clone R26.4C Stevenson BR et al., 1986	
Desmoglein1/2	61002	Progen
Desmoplakin1/2	61003	Progen
β4-integrin	555719	BD Biosciences
E-cadherin	610182	BD Biosciences
γδ T-Cell Receptor	553175	BD Biosciences
Secondary antibodies used for immunostaining		
Anti-rabbit IgG Alexa Fluor® 488	A-32731	Thermo Fisher Scientific
Anti-rabbit IgG Alexa Fluor® 568	A-11011	Thermo Fisher Scientific
Anti-rat IgG Alexa Fluor® 488	A-11006	Thermo Fisher Scientific
Anti-mouse Alexa Fluor® 594	A-11032	Thermo Fisher Scientific
Anti-rabbit IgG HRP	K4003	DAKO

Primary antibodies used for Western blot		
S6-pS240/244	5364	Cell Signaling, Beverly, MA
Rictor	2140	Cell Signaling, Beverly, MA
Akt-pS473	9271	Cell Signaling, Beverly, MA
Loricrin	PRB-145P	Covance, Princeton, New York
Filaggrin	PRB-417P	Covance, Princeton, New York
Akt	9272	Cell Signaling, Beverly, MA
Keratin 10	PRB-159P	Covance, Princeton, New York
β-actin (C4)	MAB1501	Sigma-Aldrich
α-Tubulin (B-5-1-2)	T6074	Sigma-Aldrich
Secondary antibodies used for Western blot		
Anti-rabbit-IgG-HRP	P0448	DAKO
Anti-mouse-IgG-HRP	P0161	DAKO

Supplemental Table 2: Primer sequence used for qRT-PCR analysis

Gene	Forward primer	Reverse primer
GAPDH	CATGTTTGTGATGGGTGTGA	AATGCCAAAGTTGTCATGGA
Nrf2	CCATTCCCGAATTACAGTGTCTTA	CGCCAAAATCTGTGTTTAAGGTG
Sfn	CCGAACGGTATGAAGACATGG	CGGTACTTTCACCTCGGG
Slc7a11	CAACAAAGATCGGGACTGCT	GCTGGCTGGTTTTACCTCAA
Srxn1	CGGTGCACAACGTACCAAT	TTGATCCAGAGGACGTCGAT
Sprr2d	CTGGTACTCAAGGCCGAGAC	CAGGGCACTTTGGTGGAG
Sprr2e	CAGGTCCTAGGCTACTTTGGAG	ACTGTGGATGAGGACAAGGC
Sprr2h	GACACTTGGTACTCAAGCTCTGG	TGCACTGCTGCTGTTGGTAA
Rptn	TCCTGCCTCTTCTGCTCATT	AGCGCCTACCCCATGATATT
Lce3f	TCCTGGCTCTTCCTGTTCTC	CCCAGGCAGTTATCAAAAGC
S100a8	GCCGTCTGAACTGGAGAAG	GTGAGATGCCACACCCACTT
Keratin6b	TGCAGACGAGATCAACTTCC	TGCAGACGAGATCAACTTCC
Elovl3	CTGTTGCTCATCGTTGTTGG	GCTTGAGGCCCACTGTAAAC
Pla2g5	CTCACACTGGCTTGGTTCCT	CATAACAACGGTCGTGCATC
Ctsh	CACGGAGACGGAGTTACCAG	GTGGCCATTACACTCCTGCT

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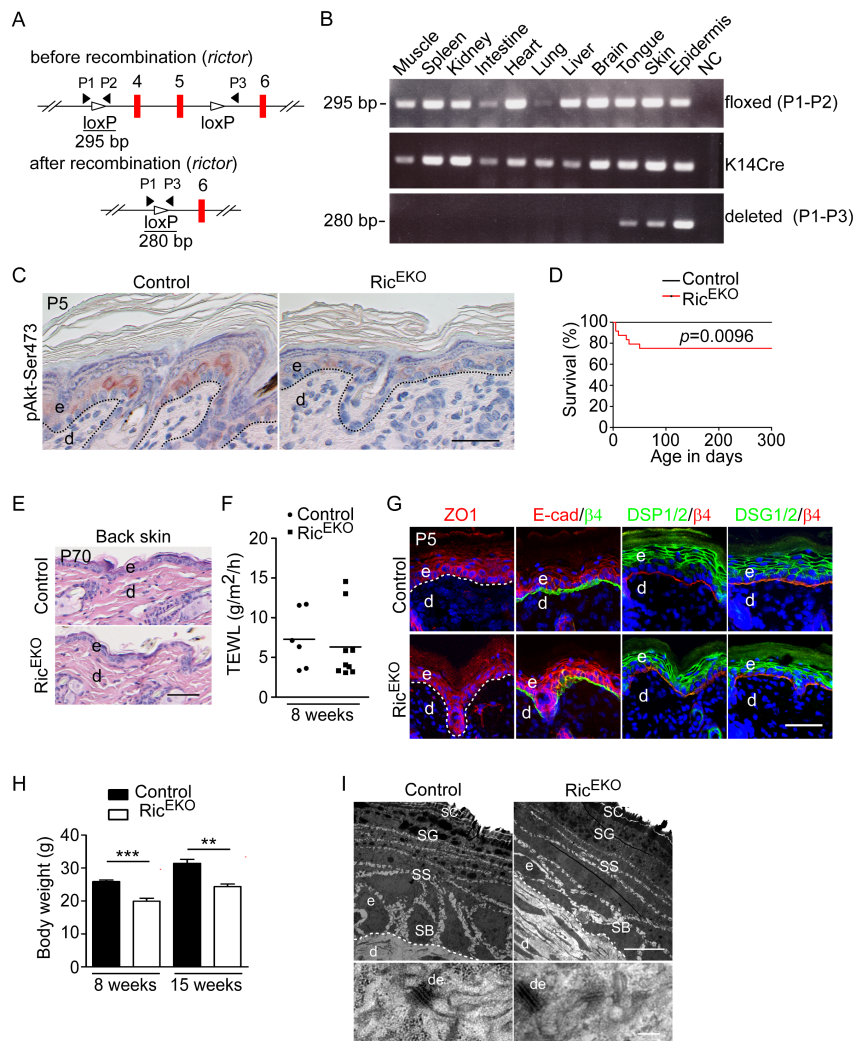| *Ctsd*	AATCCCTCTGCGCAAGTTCA	CACTGGCTCCGTGGTCTTAG
Slpi	CGGCAAATACAAGTGCTGTG	CCTGGGAGCAGGGAAGTAGT
Serpinb2	TGCCAGCTTTCCAAGAAGCATT	AGATTGAGGGCAAACATGGTG
Rictor	GAGAAAGCTGGGCCATCTGA	AACCCGGCTGCTCTTACTTC
Tslp	TCTCAGGAGCCTCTTCATCCT	CTCACAGTCCTCGATTTGCTC
Il-1α	GTCGGGAGGAGACGACTCTA	TGGTCACAAACAGTGGGAGG
Il-1β	GGACCCCAAAAGATGAAGGGCTGC	GCTCTTGTTGATGTGCTGCTGCG
Il-24	TGCCAAGTGACAGGGGTGGTTCT	CAGCACCCGAGACATTCCGCAG
Il-33	TGATCAAAGAGGCCGGGAAC	ACGCAGATTCCGCCTTTACA
Ccl2	TCCACGTGTTGGCTCAGCCAG	CCAGCCTACTCATTGGGATCATCTT
Tnf-α	GACCCTCACACTCAGATCATCTTCT	CCTCCACTTGGTGGTTTGCT

Supplemental Table 3. Related to Figure 3. Differentially expressed genes in GO term analysis identified biological processes (BP). Gene ontology Enrichment analysis (GO) of differentially regulated transcripts from Ric^{EKO} vs. control comparison using DAVID 6.8 online software. Only genes that have fold change >1.5 and a *P* value < 0.05 were used.

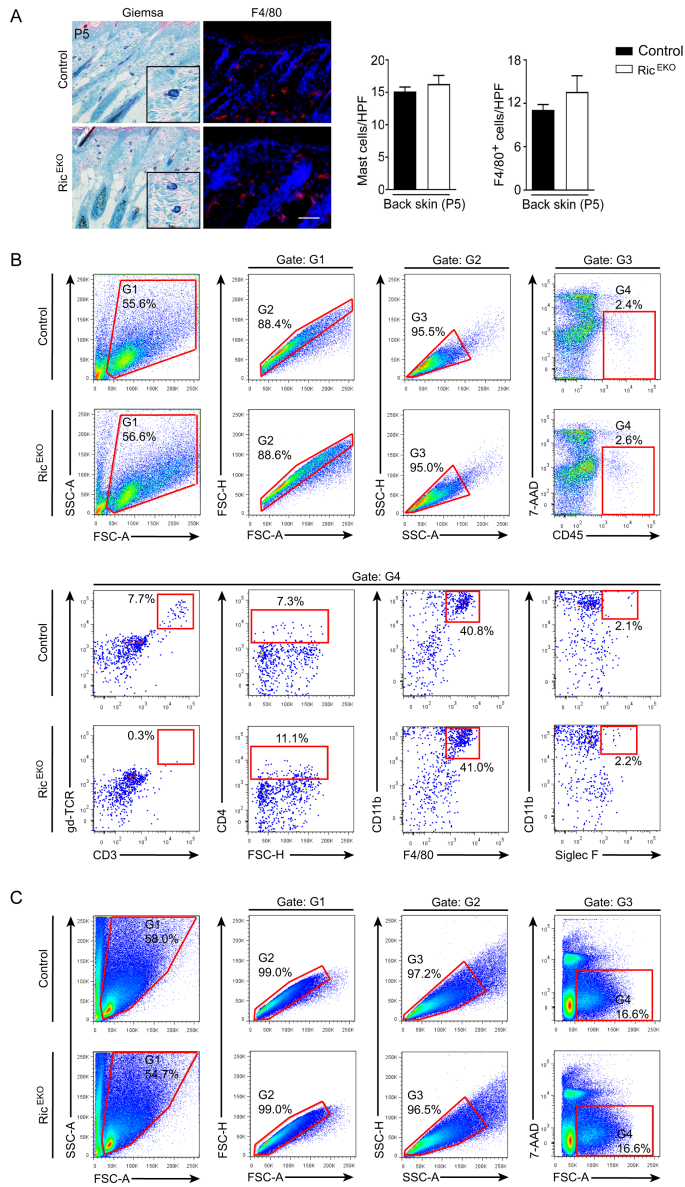| BP	Up-regulated genes in Ric^{EKO} epidermis
Peptide cross-linking	LCE3A, LCE3B, LCE3C, COL3A1, LCE3D, SPRR2H, SPRR2F, SPRR2E, SPRR2K, SPRR2I, LCE1J, SPRR2D, SPRR1A, SPRR2A3, SPRR1B, SPRR2B, TGM1, SPRR2A2, SPRR3, LCE3F, LCE3E, THBS1, LCE1K
Keratinization	KRT6A, KRT6B, SPRR2H, SPRR2F, SFN, SPRR2E, SPRR2K, SPRR2I, KRT17, KRT16, SPRR2D, SPRR1A, SPRR2A3, SPRR1B, CNFN, SPRR2B, TGM1, SPRR2A2, SPRR3
Keratinocyte differentiation	PTGS2, LCE3A, LCE3B, LCE3C, LCE3D, SPRR2H, SPRR2F, SFN, SPRR2E, SPRR2K, SPRR2I, LCE1J, KRT16, SPRR2D, SPRR1A, SPRR2A3, SPRR1B, SPRR2B, TGM1, SPRR2A2, SPRR3, LCE3F, LCE3E, LCE1K
Wound healing	DCBLD2, KRT6A, NOG, S100A8, TNC, COL3A1, CXCL2, TGFBR2, IL24, ELK3, AQP1, TPM1, MMP12, TIMP1, MACF1, CCL20, SERPINE1, SERPINB2, TGFA, CNN2, LOX, COL1A1, NBEAL2
Cell adhesion	CADM4, VCL, NOV, CGREF1, COL12A1, CYR61, KIRREL3, BYSL, CDHR1, ADGRE5, FBLIM1, THY1, TNFAIP6, BVES, HAS1, VCAN, LAMC2, MFAP4, TNFRSF12A, TNC, ITGB4, ITGB1, DCHS1, PTK2B, ITGB6, COL6A2, COL6A1, THBS1, THBS2, THBS3, DPT, COL18A1, PODXL, ITGA3, TINAGL1, PCDH17, COL5A1, LYVE1, COL19A1, CASS4, ITGA6, ITGA5, DSG3, PKP4, ITGA7, SULF1, DSC2, ABL2, NTM, MYH10

BP	Down-regulated genes in Ric^{EKO} epidermis
Immune system process	LY86, TLR1, LY9, C1QC, SKAP1, ISG20, TLR9, OASL2, OASL1, CLEC4A2, CD300C2, CD3E, PRG2, PIK3CD, PADI4, TRAT1, CD84, C1QA, C1QB, H2-AA, CD300LF, TXK, RNF135, TNFAIP8L2, KLRK1, UNC93B1, SP110, OAS2, IL34, RNF125, NAIP5,

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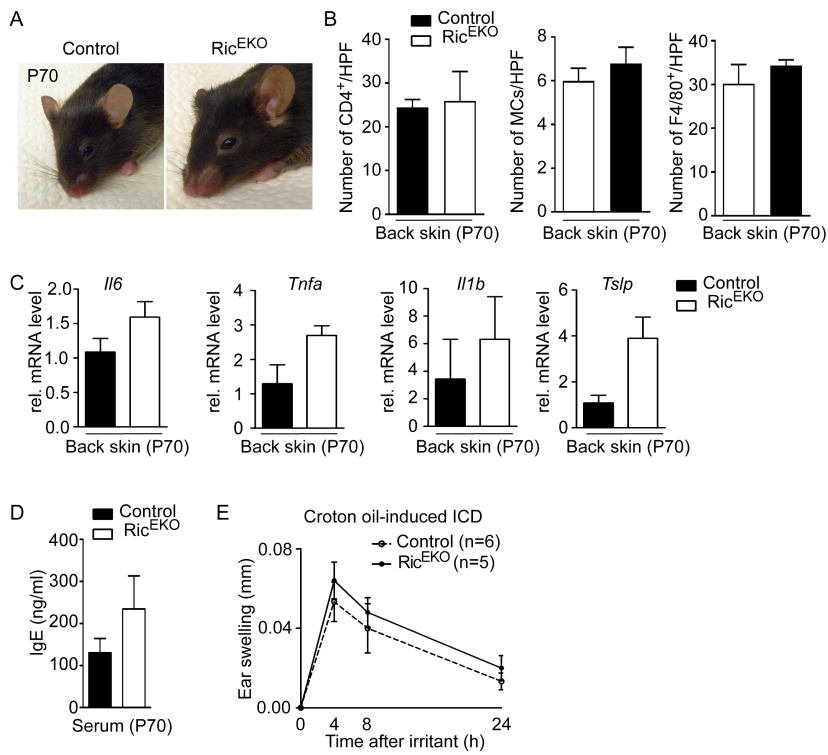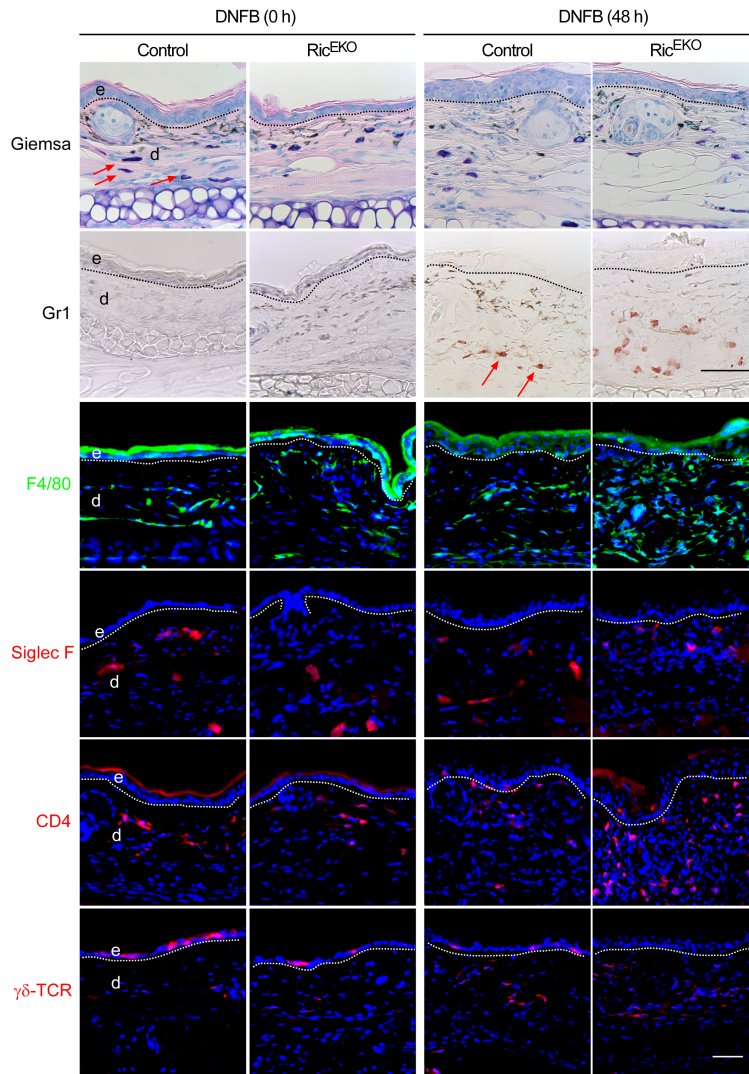|	
	PSTPIP1, ZAP70, MR1, CSF1R, CD7, ZBP1, ITK, CARD9, THEMIS, HCK, TNFRSF13C, CTLA4, TRIL, FCGR1, PSMB9, IFIT3, LAT, IFIT2, H2-EB1, C1RL, THEMIS2
Lipid metabolic process	ACOX2, PPARA, ALOX12E, HINT2, BSCL2, 4833423E24RIK, GPCPD1, ACOX3, FAR2, INSIG2, APOE, ELOVL3, NPC1L1, CES1D, FA2H, PLD4, DECR1, LPCAT2, PNPLA5, HSD11B1, NEU3, THEM5, AKR1D1, PLA2G5, HACL1, ECH1, HSD17B2, ABHD3, CERS4, ACSBG1, PLIN5, ACSL4, ACAA1B, GAL3ST1, ACSL6, SCD1, SOAT1, PLA2G16, SCD3, A4GALT, PLB1, CYP46A1, ACER2, ACER1, FADS3, SPTSSB, FAM213B, ACACB, CRAT, FADS6, GDPD1, AWAT2, ACSM3, AWAT1, ACSM2, ACSM1, LIPC
Metabolic process	ACOX2, ARSB, ALAD, ARSG, HEXB, ECHDC1, GM436, ACOX3, UGT1A7C, UGT1A6B, PHOSPHO1, 9430007A20RIK, ACOXL, NEIL2, FBP1, ALDH3B2, LPCAT2, UGT1A1, PNPLA5, AADAC, CAMK1, NEU3, ALPL, ECH1, GM5538, ISOC2B, HSD3B6, UGT3A1, UGT3A2, ACSBG1, C130079G13RIK, ALDH1A1, ALDH1A7, ACSL4, ACAA1B, FAHD2A, ACSL6, GSTA2, GSTA3, ACY3, PM20D1, NPL, GM13124, ACACB, ENGASE, CPS1, ACSM3, ACSM2, ACSM1, LYG2, ARSA, GM13178, GM13177, ACAD10
Fatty acid metabolic process	ACOX2, PPARA, ECH1, ALOX12E, 4833423E24RIK, ACSBG1, ACOX3, CRYL1, ELOVL3, ACSL4, ACAA1B, ACSL6, SCD1, SCD3, ACOXL, FA2H, FADS3, FAM213B, ACACB, DECR1, CRAT, FADS6, ACSM3, ACSM2, ACSM1, LIPC, THEM5
Innate immune response	TNFAIP8L2, LY86, TLR1, KLRK1, UNC93B1, SP110, TRIM15, OAS2, LY9, IL34, C1QC, TLR9, ISG20, OASL2, NAIP5, OASL1, ZAP70, PSTPIP1, FCER1G, CLEC4A2, MR1, CSF1R, TYROBP, ZBP1, ITK, CARD9, HCK, PIK3CD, PADI4, TRIL, FCGR1, IFIT3, CD84, C1QA, IFIT2, C1QB, CYBB, C1RL, TXK, TREM2, RNF135

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Supplemental Figure 1

Supplemental Figure 2

A

B

C

Supplemental Figure 3

A

Control   Ric^EKO

P70

B

Control
Ric^EKO

Number of CD4⁺/HPF
Back skin (P70)

Number of MCs/HPF
Back skin (P70)

Number of F4/80⁺/HPF
Back skin (P70)

C

Control
Ric^EKO

Il6

Tnfa

Il1b

Tslp

rel. mRNA level
Back skin (P70)

D

Control
Ric^EKO

IgE (ng/ml)
Serum (P70)

E

Croton oil-induced ICD

Control (n=6)
Ric^EKO (n=5)

Ear swelling (mm)
Time after irritant (h)

Supplemental Figure 4

Supplemental Figure 5