mTORC1 loss impairs epidermal adhesion via TGF-β/Rho kinase activation

Kaushal Asrani, Akshay Sood, Alba Torres, Dan Georgess, Pornima Phatak, Harsimar Kaur, Amber Dubin, C. Conover Talbot Jr., Loubna Elhelu, Andrew J. Ewald, Bo Xiao, Paul Worley, and Tamara L. Lotan

Department for Pathology and Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. Baltimore Veterans Affairs Medical Center, Baltimore, Maryland, USA. Institute for Basic Biomedical Sciences, Department of Oncology, and Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

Introduction

The mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in integrating upstream nutrient inputs to regulate cell proliferation, growth, and metabolism (1). mTOR kinase exists in 2 complexes, of which mTOR complex 1 (mTORC1) is better studied and is classically inhibited by the macrolide rapamycin (1, 2). mTORC1 activation requires the small Ras-related GTPase Rheb (3), as well as an adaptor protein, Raptor (4). Following activation, mTORC1 triggers cell growth, stimulates cap-dependent protein translation, and regulates glucose uptake, de novo lipid synthesis, and mitochondrial activity and biogenesis (2). Despite its well-characterized role in cellular processes that facilitate tumor progression, mTORC1 inhibitors have been ineffective for the treatment of most solid cancers (5). Though this finding has largely been attributed to relief of feedback inhibition that mTORC1 activity exerts on upstream growth factor receptor signaling (6–11), the in vivo sequelae of mTORC1 loss of function have not been well studied in epithelial tissues that give rise to the majority of cancers in humans. Thus, fully characterizing the role of mTORC1 in epithelial biology is critical before we can devise strategies to circumvent tumor resistance to mTORC1 inhibition.

Among epithelial tissues, the skin is arguably among the best-characterized model systems, and is of additional interest with regard to mTOR function, since mTORC1 inhibitor treatment in transplant or cancer patients is associated with impaired wound healing and a variety of dermatologic toxicity ranging from mild to life-threatening (12–14). The epidermis is a self-renewing, stratified epithelium that serves as a physical and permeability barrier. Cell-cell adhesion — via adherens junctions, desmosomes, and tight junctions — as well as terminal differentiation of keratinocytes into a cornified layer, is essential for epidermal barrier formation (15). Desmosomes are calcium-dependent adhesive structures that anchor to tension-bearing keratin intermediate filaments and provide mechanical strength. Desmosomal isotypes are expressed in a differentiation-dependent pattern in the skin (16), and loss of desmosomal components in humans and mice by genetic or autoimmune mechanisms leads to a spectrum of epidermal barrier defects, from mild defects in cell-cell adhesion and suprabasal acantholysis, to biochemical differentiation defects with severe blistering and lethality (17–21).

In addition to intermediate filaments, the actin cytoskeleton is central to cell-cell adhesion. During adherens junction formation, cadherin-catenin clusters form at nascent cell-cell contacts, coupled with maturation of radial actin filaments into cortical actin bundles (22). Adherens junction assembly requires a fine balance in the activities of 2 members of the Rho family of small GTPases, Rac and Rho, and of the downstream effectors of Rho, mDia and Rho kinase (ROCK) (23–26). In turn, actomyosin contractility and ROCK signaling likely regulate desmosomal junction formation at multiple levels (27). While appropriate RhoA localization and actomyosin contractility are required for membrane translocation of desmosomal precursors, sustained Rho/ROCK activity can hamper this process in vitro (28). In addition,
Figure 1. Epidermal-specific mTORC1 loss-of-function models have skin barrier defects and evidence of impaired cell-cell adhesion. 

(A) Immunoblotting of WT and Rheb-cKO P0 epidermis and keratinocyte cultures for markers of mTORC1 (p-S6) and mTORC2 (p-AKT, p-FOXO1, p-GSK-3β) activity. Total AKT, total FOXO1, and total GSK-3β were immunoblotted separately using the same biological replicate. (B) Immunofluorescence of WT and Rheb-cKO E18.5 epidermis for p-S6. (C) Rheb-cKO pups are smaller at P0, with thin, shiny skin (top left), and fail to exclude toluidine blue dye at E18.5 (top right), consistent with an epidermal barrier defect. Grossly visible abrasions (arrow, bottom right) and a prominent vascular pattern are seen in P0 cKO epidermis. (D) Representative histologic back skin sections from Rheb-cKO P0 pups reveal markedly thinned epidermis with loss of the upper keratinizing cell layers (top right) and grossly apparent subcorneal pustules (bottom left), filled with neutrophil debris on histology (bottom right). (E) Immunoblotting of WT and Rptor-cKO P0 epidermis and WT and inducible Rptor-cKO keratinocyte cultures for markers of mTORC1 (p-S6) and mTORC2 (p-AKT) activity. (F) Rptor-cKO pups have visible epidermal blisters at P0 (right, arrow). (G) Rptor-cKO pups have a thinned epidermis by histology (right half of left panel, H&E staining) with visible blistering (denoted by an asterisk). The epidermal blisters in Rptor-cKO P0 pups are suprabasal as demonstrated by p63 immunostaining that labels basal cells left behind after superficial epidermal layers (right panel, arrow) are shed. Scale bars: 30 μm throughout.
cytoskeletal contractility may exert complex effects on downstream gene expression through mechanotransduction, affecting the activity of multiple transcription factors (29). Despite this wealth of in vitro data, relatively little is known about the in vivo requirement of actomyosin contractility for desmosomal adhesion and epidermal barrier formation.

mTOR signaling perturbation in the epidermis has previously been associated with epithelial carcinogenesis (30), epidermal stem cell senescence (31, 32), and delayed wound healing (33). While this article was in preparation, an additional study demonstrated that mTOR kinase and mTORC1 loss of function is associated with impaired epidermal differentiation and barrier formation; however, the molecular mechanism of this finding was not elucidated, and no effects on cell-cell adhesion were described (34). Here, we used 2 separate genetic models to examine the consequences of conditional epidermal mTORC1 loss of function. Unexpectedly, we find that mTORC1 signaling in the epidermis is required for desmosomal cell-cell adhesion and keratinocyte biochemical differentiation, as exemplified by epidermal blistering, defective skin barrier function, and neonatal lethality of the conditional knockout mice. Mechanistically, these findings are a direct consequence of heightened cytoskeletal tension due to increased ROCK activity, resulting in impaired desmosomal gene expression and adherens junction maturation. Probing further upstream, mTORC1 loss leads to marked feedback upregulation of TGF-β signaling and downstream effects on cytoskeletal contractility, cell adhesion, and biochemical differentiation. These studies reveal a novel in vivo role of mTORC1 function in epithelial biology that has relevance for human disease.

Results

We crossed mice transgenic for Krt14-Cre (35) (which diffusely express Cre recombinase under the control of the keratin 14 promoter in the maturing epidermis by E14.5) to Rheb<sup>fl</sup>/<sup>fl</sup> (36) or Rptor<sup>fl</sup>/<sup>fl</sup> (37) mice to generate mice with mTORC1 loss of function in the epidermis, hereafter referred to as Rheb-<sup>-</sup> or Rptor-<sup>-</sup> cKO (conditional knockout) mice. As expected, Rheb- and Rptor-cKO epidermis and cultured keratinocytes showed evidence of mTORC1 loss of function and a well-described reciprocal increase in mTORC2 activity (38) by immunoblotting and/or immunofluorescence (Figure 1, A, B, and E). Pups from both cKO models were born at expected Mendelian ratios, but demonstrated uniform perinatal lethality with no pups surviving beyond P1. The lethality and smaller size of the Rheb-cKO pups compared with WT at P0 but not at E18.5 (Figure 1C) were suggestive of postnatal dehydration due to a defective epidermal barrier. This could be demonstrated by abnormally diffuse epidermal toluidine blue uptake at E18.5 in Rheb-cKO pups (Figure 1C). Moreover, both cKO models were characterized by taut, shiny skin with a prominent vascular pattern on gross examination (Figure 1, C and F), and a markedly thin epidermis with absence of full stratification on histology (Figure 1, D and G). Rheb-cKO pups had small subcorneal skin abrasions (Figure 1C) and blisters filled with neutrophilic debris (Figure 1D), findings seen in mouse models with mild desmosomal cell adhesion defects (17), while Rptor-cKO pups showed frank suprabasal epidermal blisters at birth (Figure 1, F and G).

Epidermal biochemical differentiation and stratification was abnormal in both cKO models. This confirmed the findings of a recent study describing the phenotype of mice with conditional ablation of epidermal mTOR kinase or Rptor, published while this article was in preparation (34). Basal cell markers, including K5, K14, and p63, highlighted a normal to slightly hypertrophic basal layer in both cKO models by immunofluorescence (Supplemental Figure 1, A and C; supplemental material available online with this article; https://doi.org/10.1172/JCI92893DS1) and immunoblotting (Supplemental Figure 1D). In contrast, the spinous (marked by K1, K10) and granular layers (marked by loricrin, filaggrin, involucrin, transglutaminase 1) were dramatically thinned by immunofluorescence (Supplemental Figure 1, A and C) and immunoblotting (Supplemental Figure 1, B and D). Accordingly, for cultured PO keratinocytes, switching from low (0.05 mM) to high (2.0 mM) calcium (calcium switch assay) failed to induce robust suprabasal biochemical differentiation in the Rheb-cKO model (Supplemental Figure 1E), and similar effects were observed when WT keratinocytes were pretreated with the mTORC1 inhibitor rapamycin before the calcium switch (Supplemental Figure 1F). Taken together, these data demonstrate that mTORC1 is required for keratinocyte biochemical differentiation and the generation of a full-thickness squamous epithelium.

mTORC1 controls proliferation in some contexts (39); thus we tested whether epidermal thinning in Rheb-cKO mice might be due to decreased net proliferation. To avoid measuring a reactive increase in proliferation following birth due to the epidermal barrier defect, we examined expression of Ki-67 in the basal proliferative epidermal compartment at E18.5 and found that Ki-67 labeling was visually equivalent between WT and cKO epidermis for both cKO models (Figure 2, A and D). To exclude the possibility of cell cycle arrest in cKO cells labeling with the Ki-67 proliferation antigen, we also performed BrdU labeling and immunofluorescence for both cKO models (Figure 2, A and D). To exclude the possibility of cell cycle arrest in cKO cells labeling with the Ki-67 proliferation antigen, we also performed BrdU labeling and immunofluorescence for the mitotic marker phospho–histone H3 (phH3) in E18.5 epidermis or cultured keratinocytes and found the proportion of S- and M-phase basal cells to be similar between WT and Rheb-cKO mice (Figure 2, B and C). Similar to recent observations by the Eming group (34), in the Rptor-cKO epidermis, there was an indication of a lower BrdU incorporation rate in the basal layer; however, this just missed statistical significance, and there was no indication of decreased phH3 expression (Figure 2E). Cleaved caspase-3 expression (a marker of apoptosis) was negligible in both WT and Rheb-cKO E18.5 epidermis, labeling only rare cells (Figure 2F). Taken together, these data suggest that there may be a subtle proliferation defect in the Rptor-cKO mice contributing to the thin-skin phenotype that is not seen in the Rheb-cKO mice, and may be potentially attributable to previously described mTORC1-independent effects of Rptor on M-phase progression (40).

Since the Rptor-cKO epidermis is more dramatically thinned than the Rheb-cKO epidermis, we cannot exclude a contribution of decreased keratinocyte proliferation to the Rptor-cKO phenotype. However, the apparent lack of a proliferation defect in the Rheb-cKO model suggested the possibility of additional mechanisms contributing to the defective stratification seen in both models with mTORC1 loss of function. By K14 immunostaining, we noted that the basal-layer keratinocytes of the Rheb-cKO epidermis...
Figure 2. mTORC1 loss of function does not significantly alter epidermal proliferation or apoptosis, but is associated with abnormal mitotic polarization. (A) Ki-67 immunohistochemistry in E18.5 Rheb WT and cKO epidermis. Scale bar: 75 μm. (B) BrdU labeling detected by immunofluorescence (top panels) in E18.5 WT or Rheb-cKO epidermis. Scale bar: 60 μm. Immunofluorescence for phospho–histone H3 (pH3), an M-phase marker (bottom panels), in E18.5 WT and Rheb-cKO epidermis. Scale bar: 60 μm. (C) Mean proportion of basal or total epidermal BrdU-labeled (r = 3, n > 20, ×200 fields each) or pH3-positive cells (r = 3, n > 33, ×200 fields each) in WT versus Rheb-cKO E18.5 epidermis (at least 15 ×200 fields quantified for each; error bars represent SEM; P values indicated are by Student’s t test). (D) Ki-67 immunohistochemistry in E18.5 Rptor WT or cKO epidermis. Scale bar: 75 μm. (E) Mean proportion of basal cells labeled with BrdU (left panel) or pH3 (right panel) in WT versus Rptor cKO E18.5 epidermis (r = 8; at least 20 ×200 fields were quantified for each; error bars represent SEM; P values indicated are by Student’s t test). (F) Representative immunofluorescence for cleaved caspase-3 (CC3) in WT and Rheb-cKO E18.5 epidermis. Scale bar: 60 μm. (G) Immunofluorescence for γ-tubulin in WT and Rheb-cKO E18.5 epidermis (top). Scale bar: 60 μm. Angles between centrosomal axis and plane of basement membrane in Rheb WT versus cKO cells (bottom) (r = 3; minimum of 15 ×200 fields quantified for each). (H) Representative immunofluorescence for the spindle midbody marker survivin (green) and K14 (red) in WT versus Rptor-cKO E17.5 epidermis (top panel). Radial histogram of the angle of basal cell division in WT (r = 8, n = 128) versus Rptor-cKO (r = 9, n = 61) (bottom panel, left) or WT (r = 4, n = 77) versus Rheb-cKO (r = 4, n = 66) (bottom panel, right) (P values indicated by χ² test).
appeared poorly polarized and rounded, in contrast to the columnar polarized basal cells in WT epidermis (Figure 2B). Since cell polarity defects can diminish the number of asymmetric mitoses and impair epidermal stratification (41), we examined a marker of cell polarity in cKO keratinocytes by measuring the centrosome position (42, 43). In contrast to WT cells, where the centrosome polarizes to the apical side of the columnar basal cell nucleus, the centrosomes were haphazardly located in Rheb-cKO cells, consistent with an underlying polarity defect (Figure 2G). Potential interactions between centrosome and mitotic spindle position may underlie stratification defects (44). To examine whether the ratio of symmetric and asymmetric mitoses was abnormal in cKO cells, we measured the angle of the mitotic spindle relative to the epidermal-dermal junction in E18.5 Rheb- and Raptor-cKO epidermis compared with controls. While WT mitoses were predominantly oriented perpendicular, with a minority parallel, to the dermal-epidermal junction, cKO mitoses appeared randomly distributed (Figure 2H). Thus, we conclude that impaired polarization and cell stratification likely contribute to the thinned epidermis with mTORC1 loss of function.

Keratinocyte biochemical differentiation and cell-cell adhesion are reciprocally regulated and calcium-dependent processes (45). The subcorneal pustules in Rheb-cKO mice and suprabasal blisters in Raptor-cKO mice were reminiscent of mice with loss of desmosomal cadherins (17, 18, 46) and suggested an underlying defect in cell-cell adhesion after mTORC1 loss of function. Indeed, on transmission electron microscopy (TEM), there were diffuse intercellular gaps in P0 Rheb-cKO epidermis, with a similar phenotype in keratinocyte culture (Figure 3, A and F). Though overall numbers of desmosomes appeared unchanged with epidermal mTORC1 loss of function (Figure 3A), there was prominent keratin tonofilament retraction, with reduced density of keratin filaments inserting on the desmosomal plaques (Figure 3, A, C, and D). In vivo, membranous levels of desmosomal components including desmoplakin (DSP1/2), desmoglein 1 (Dsg1), and DSG3 appeared generally decreased by immunofluorescence (Figure 3B and Supplemental Figure 2A), and desmosomes were significantly shorter both in vivo and in vitro in cKO models by TEM (Figure 3, C and D). In addition, the midline of the desmosomal plaques was poorly formed in cultured keratinocytes from both cKO models (Figure 3D, arrowhead). Taken together, these findings are consistent with failure of in vivo desmosomal expression and maturation in keratinocytes with mTORC1 loss of function.

In vitro, cellular adhesive strength of Rheb-cKO compared with WT keratinocytes was reduced, as measured by dispase dissociation assay (Figure 3E). Compared with WT keratinocytes, there was a marked decrease in in vitro levels of desmosomal components on the cell membrane in cKO cells by immunofluorescence (Figure 3F), surface biotinylation (see below), and keratinocyte membrane lysates (see below). Both in vivo (Figure 3G and Supplemental Figure 2, B and C) and in vitro (Figure 3H and see below), the decreased membrane levels of desmosomes reflected an underlying decrease in total levels of most desmosome proteins in both cKO models, including desmoplakin (DSP1/2), desmoglein 1 (Dsg1), desmocollin 3 (DSC3), plakoglobin 1 (PKP1), plakoglobin 2 (PKP2), and plakophilin 3 (PKP3). Similarly, treatment of WT keratinocytes with rapamycin or mTOR kinase inhibitors (AZD8055 and torin1) resulted in decreased levels of DSP1/2 and DSG1 (see below). A subset of desmosomal proteins undergo differential expression during keratinocyte maturation (47); for example, DSG1 is expressed in the superficial epidermis, while DSG3 is predominantly expressed in basal cells (Figure 3B). Since there was defective keratinocyte differentiation in the cKO models, the decrease in some desmosomal components (such as DSG1) could be exacerbated by this maturation defect. Similarly, total levels of DSG3, predominantly expressed in basal epidermis, were not decreased in Raptor-cKO epidermis (Figure 3G and Supplemental Figure 2B). Lower expression of DSG3 may have been potentially masked by a defect in differentiation in cKO cells or perhaps represented an in vivo compensatory effect not seen in vitro, since DSG3 levels were decreased in cKO keratinocytes in culture (see below). Such a response has previously been described in Netherton syndrome (48). Overall, however, the uniform decrease in most protein levels, including DSP1/2 and PKP3, which are expressed by keratinocytes throughout differentiation (Supplemental Figure 2B and see below), suggested that the failure of desmosome protein expression in keratinocytes with mTORC1 loss of function was at least partly independent of cellular differentiation.

Desmosomal protein expression is regulated at multiple levels, including at the level of gene expression as well as by protein turnover (49). We examined mRNA expression for desmosomal genes in both cKO models. In vivo (Figure 4A) and in vitro in both high (Figure 4, B and C) and low (Supplemental Figure 4J) calcium concentrations, desmosomal gene expression was significantly and fairly uniformly decreased in cKO cells. One exception to this was Dsg3, which was expressed at normal levels in vivo (Figure 4A), though significantly decreased in vitro (Figure 4, B and C, and Supplemental Figure 4, J and K) consistent with DSG3 protein expression described above. If altered gene expression is contributing significantly to regulation of desmosomal adhesion in keratinocytes with mTORC1 loss of function, then transfection with exogenous desmosomal components should be sufficient to rescue the adhesion phenotype in cKO cells. Transient transfection of Raptor-cKO cells with either Dsg1a or Dsg3 significantly increased protein levels of other desmosomal components (Figure 4, D and E), potentially because of protein stabilization in nascent adhesion complexes. Expression of either construct was sufficient to rescue in vitro adhesion in Rheb-cKO cells by dispase dissociation assay (Figure 4, F and G). Because keratinocyte differentiation and cell-cell adhesion are reciprocally regulated and calcium-dependent processes (21, 45), we also tested whether rescue of the cell adhesion defect was sufficient to restore normal biochemical differentiation in vitro. Consistent with our hypothesis, expression of either Dsg1 or Dsg3 resulted in increased expression of involucrin, loricrin, and TGM1, markers of granular and spinous differentiation in keratinocytes (Figure 4, D and E). Taken together, these results indicate that mTORC1 regulates desmosomal adhesion at least in part via gene transcription and that failure of biochemical differentiation with mTORC1 loss of function is largely due to loss of normal desmosomal adhesion.

Next, we examined whether the cell-cell adhesion defect with mTORC1 loss of function was specific to desmosomal junctions. We queried expression and localization of other cell junction proteins in both cKO models. In contrast to desmosomes, adherens
Figure 3. mTORC1 loss of function in the epidermis results in cell-cell adhesion defects. (A) TEM of Rheb-cKO P0 epidermis (first row, E) demonstrates diminished granular layer (G) and intercellular gaps (first row, arrows). Desmosomes (second row, arrowheads) are evident (second row, arrow). Tonofilaments are collapsed (second and third rows, arrows) and not easily visible (third row, arrowheads) in cKO cells. Scale bars: 4 μm (top row), 2 μm (second and third rows). (B) Immunofluorescence for desmosomes (dotted line = dermal-epidermal junction). Representative images (r = 3). Scale bar: 30 μm. (C) Desmosomes in Rptor-cKO P0 epidermis have diminished keratin attachment by TEM (left panels; scale bar: 500 nm). Desmosomal length is quantified at right (bar represents mean length in nanometers; n = 12 and 29; P value by Student’s t test). (D) Desmosomes in Rheb-cKO keratinocytes have less dense cytoplasmic plaques (arrows) and poorly formed midline (arrowhead) (left panels; scale bar: 100 nm). Desmosome length is quantified at right (bar represents mean length in nanometers; n = 7 and 6; P value by Student’s t test). (E) Dispase dissociation for epithelial fragments (arrows) in Rheb WT and cKO keratinocyte monolayers (left panel). Scale bar: 1 cm. Quantification of dispase assay (right panel) (n = 4; error bars represent SEM; P value by Student’s t test). (F) Immunofluorescence for desmosomes in Rheb WT and cKO keratinocytes (top images), with intracellular gaps (arrow). Scale bar: 50 μm (top and middle images), 10 μm (bottom images). The third panel of merged staining is inset from the boxed areas in the top 2 panels. Quantification of desmosome protein border fluorescence intensity (bottom panels; r = 3, n = 58). Error bars represent SEM; P < 0.0001 by Mann-Whitney test. (G) Immunoblotting for junctional proteins in Rptor WT versus cKO P0 epidermis. (H) Immunoblotting for junctional proteins in Rheb WT versus cKO keratinocytes. PKP1 was immunoblotted separately using the same biological replicate.
and tight junction components, such as E-cadherin, β-catenin, α-catenin, afadin, and ZO-1, showed normal or even higher total and membrane levels by immunoblotting and immunofluorescence in the cKO models in vivo (Figure 3G and Supplemental Figure 2E) and in vitro (Figure 3H, Supplemental Figure 2D, and see below) when compared with WT mice. However, immunofluorescence revealed that adherens junctions failed to mature into continuous adhesion belts and remained stalled in punctate adhesion zippers with radially oriented filamentous actin (F-actin) filaments (Figure 5A and Supplemental Figure 2F). Both maturation of adherens junctions (50, 51) and desmoplakin translocation into nascent desmosomes (27, 28) require appropriately regulated actomyosin contractility. We noted that keratinocytes with mTORC1 loss of function showed evidence of abnormally high cytoskeletal tension, with prominent F-actin stress fibers with absence of cortical actin belts (Figure 5A); increased cell size, likely due to cell spreading (Figure 5B); and increased focal adhesion formation (Figure 5, D and E) and basal cell nuclear shape seen in the Rheb-cKO model (Figure 5, D and E), since nuclear shape is regulated in part by cytoplasmic actin contractility (52). Though nuclear area was unchanged in Rheb-cKO basal cells, the nuclear aspect ratio (ratio of nuclear width to nuclear height) was significantly increased in comparison with WT cells (Figure 5E). Collectively, these data suggest that mTORC1 loss of function results in abnormally increased actin contractility in keratinocytes.

Figure 4. mTORC1 loss of function is associated with reduced desmosomal mRNA expression and is compensated by transfection of exogenous desmosomal cadherins. (A–C) Quantitative real-time reverse transcriptase PCR of desmosomal gene transcripts in P0 Rptor WT versus cKO epidermis (A); in WT versus inducible Rptor-cKO keratinocytes grown in high calcium (B); and in Rheb WT versus cKO keratinocytes grown in high calcium (C) (r = 3 for each; error bars represent SEM; P values indicated are by Student’s t test). (D) Immunoblotting of inducible Rptor-cKO keratinocytes transiently transfected with empty vector, DSG1 mouse cDNA ORF, or DSG3 mouse cDNA ORF for desmosomal proteins (DSP, DSG1, DSG3, and PKP3) and markers of biochemical differentiation (TGM1, involucrin, and loricin). (E) Densitometry quantification of representative immunoblot experiments shown in D (r = 3; error bars represent SEM; P values by 1-way ANOVA). (F) Dispase dissociation assay for Rheb-cKO keratinocytes after transient transfection with empty vector, DSG1 mouse cDNA ORF, or DSG3 mouse cDNA ORF. Scale bar: 1 cm. (G) Quantification of representative dispase assay experiments shown in F (r = 4; error bars represent SEM; P values by 1-way ANOVA). EV, empty vector.
Actomyosin contractility is principally orchestrated by small GTPases, including Rho GTPase and its downstream effector ROCK (53). Consistent with the prominent actin stress fibers in cKO keratinocytes, both cKO models also showed evidence of increased keratinocyte ROCK activity in vivo and in vitro, with higher levels of phosphorylated myosin phosphatase (p-MYPT1) and myosin light chain (p-MLC2) in both epidermis (Figure 5F and Supplemental Figure 3C) and keratinocytes (Figure 5G and see below). Increased Rho activity was also evidenced by increased levels of membrane-localized RhoA,B,C and decreased ROCK inhibitor RhoE in cKO keratinocytes (Supplemental Figure 3, D and E). Increased ROCK activity was also seen with pharmacologic mTORC1 inhibition, and the extent of MYPT1 and MLC2 phosphorylation was correlated with the differing efficacy of mTORC1 inhibition documented for these inhibitors (Figure 5H and ref. 5). Notably, both mTORC1 loss-of-function models showed increased levels of MRT2 in cKO keratinocytes (Supplemental Figure 3G). Loss of desmosomal cell adhesion with epidermal mTORC1 loss of function is accompanied by increased cytoskeletal tension and ROCK activity. (A) Immunofluorescence in Rheb and Rptor WT and cKO keratinocytes for desmosomal, focal adhesion (vinculin), and adhesions marker junctions (E-cadherin, phallolidin staining for actin filaments). Second panel of vinculin staining is inset from the boxed areas in the first panel. Scale bar: 50 μm (first panel), 25 μm (second panel), 10 μm (third panel), 50 μm (fourth panel). (B) Distribution of cell perimeter length (r = 6, n = 79) in Rheb WT versus cKO keratinocytes (P value by Mann-Whitney test). (C) Quantification of mean number of focal adhesions per cell (r = 3, n = 50) in Rheb WT versus cKO keratinocytes (P value by Student’s t test). (D) Immunofluorescence in Rheb WT and cKO P0 epidermis for actin (phallolidin) and basal cells (K14). Scale bar: 30 μm. (E) Mean nuclear area and mean nuclear aspect ratio (the ratio of nuclear width to nuclear height) for basal cells in P0 Rheb WT versus cKO epidermis (n > 100 cells each). (F) Immunoblotting of Rptor WT and cKO P0 epidermal lysates for ROCK activation. (G) Immunoblotting of Rheb WT and cKO keratinocyte lysates for ROCK activation. (H) Immunoblotting of WT cultured keratinocytes with or without mTORC1 (rapamycin, 200 nM) or mTORC1/2 inhibitors (AZD8055, 500 nM, and torin1, 1 μM) for desmosomes and ROCK activation (top) p-4EBP1, p-S6, and total S6 were immunoblotted in parallel, contemporaneously with DSP, DSG1, and p-MLC2. p-MYPT1 and total MYPT1 were immunoblotted separately using the same biological replicate. Densitometry quantification of immunoblots (bottom) (r = 3; P values are by Student’s t test). (I) Immunoblotting of Rheb WT and cKO keratinocytes for desmosomes and ROCK activation (top). Densitometry quantification of immunoblots, with additional desmosomal markers DSG3 and PKP1 (bottom) (r > 4; P values indicated are by Student’s t test). Error bars represent SEM.

Loss of desmosomal components such as plakophilins can contribute to actomyosin contractility via RhoA activation (28). However, the increase in ROCK activity could be seen even in cKO keratinocytes cultured in low-calcium media, where desmosomal adhesions are largely unformed (Figure 5I), suggesting that ROCK activation occurs independent of desmosome loss in the context of mTORC1 loss of function. ROCK itself has a well-described role in the regulation of gene transcription networks, and is a key effector of cellular mechanotransduction in this capacity (29). Thus, we hypothesized that decreased desmosomal gene expression in cKO cells could itself be due to increased cytoskeletal tension mediated by ROCK activity. Treatment of cKO cells with ROCK inhibitors (Y27632 or fasudil) restored MYPT1 and MLC2 phosphorylation to normal levels in cKO cells (Figure 6A and Supplemental Figure 3F), eliminated actin stress fiber formation, and restored cortical actin belts in adhesive cells (Figure 6B). Moreover, ROCK inhibitors completely rescued total (Figure 6A and Supplemental Figure 3F) and membrane levels (Figure 6, B and C, and Supplemental Figure 3G) of desmosome proteins in both mTORC1 loss-of-function models. This rescue of desmosomal protein levels by ROCK inhibitors likely occurred at the gene expression level, because treatment with fasudil was sufficient to rescue desmosomal gene expression in Rheb-cKO keratinocytes, increasing levels significantly above those in WT keratinocytes (Supplemental Figure 4, J and K). In addition, Y27632 restored keratin filament insertion to desmosomal plaques (Figure 6B) and desmosomal size (Figure 6B) by TEM and enabled maturation of adherens zippers into continuous adherens junctions (Figure 6B). Finally, functional rescue of cell adhesion upon ROCK inhibition was evidenced by restoration of cKO keratinocyte monolayer integrity in disperse dissociation assays (Figure 6D).

To confirm the specificity of the effects of pharmacologic ROCK inhibition, we also performed siRNA-mediated depletion of ROCK1 in Rheb-cKO keratinocyte cultures. ROCK1 depletion was sufficient to phenocopy the effects of pharmacologic ROCK inhibition, with decreased evidence of ROCK activity by p-MLC2 and p-MYPT1 immunoblotting and increased levels of total desmosomal proteins in Rheb-cKO cells (Figure 6F). We next tested whether rescue of cell adhesion by ROCK inhibition or depletion was sufficient to restore expression of calcium-induced differentiation markers (loricrin, transglutaminase 1, and involucrin) in cKO cells in culture. Indeed, ROCK inhibition by Y27632 or depletion by ROCK1 siRNA rescued Rheb-cKO keratinocyte biochemical differentiation in vitro (Figure 6, E and F, and Supplemental Figure 3H). Interestingly, fasudil treatment (Supplemental Figure 3F) or ROCK1 depletion (Supplemental Figure 3F) in WT keratinocytes also raised desmosomal protein levels and increased expression of markers of biochemical differentiation, suggesting that basal ROCK activity plays a role in regulating desmosomal levels and biochemical differentiation as well. Indeed, this effect of ROCK on biochemical differentiation has been reported previously (55, 56). Taken together, these results indicate that mTORC1 loss of function results in increased cytoskeletal contractility mediated by ROCK, which impairs desmosomal gene expression, cell-cell adhesion, and biochemical differentiation in keratinocytes.

Finally, we sought to elucidate which signaling pathways function upstream of ROCK activation in keratinocytes with mTORC1 loss of function by performing microarray-based differential expression analysis of E18.5 epidermis from Rheb WT and Rheb-cKO mice. Out of 39,601 gene-annotated transcripts we found 1,198 genes significantly (greater than 2 SD log, fold change) upregulated and 815 downregulated in Rheb-cKO compared with
Among the differentially expressed genes, the 2 top upstream regulator pathways predicted to be activated in Rheb-cKO epidermis included MYC (P = 7.80 × 10⁻³²) and TGF-β signaling (P = 9.86 × 10⁻³⁰) (Supplemental Figure 4B). Interestingly, we independently observed before expression analysis that c-MYC protein levels were dramatically increased in the Rheb-cKO epidermis by immunohistochemistry, potentially related to failure of keratinocyte differentiation in Rheb WT epidermis, including downregulation of expected genes involved in keratinocyte maturation (KRT10, filaggrin, involucrin, corneodesmosin) as well as upregulation of those involved in wound healing/migration (KRT6A, KRT16, S100A8, S100A9, MMP3) and barrier loss response (many of the small proline-rich proteins) (Supplemental Table 1). Interestingly, only a subset of desmosomal genes were significantly altered in this analysis, for reasons that remain unclear. We used these genes to identify significantly enriched canonical pathways in the cKO epidermis using Ingenuity Pathway Analysis (Qiagen; https://www.qiagen-bioinformatics.com/products/ingenuity-pathway-analysis/).

Figure 6. ROCK inhibition or depletion rescues adhesion and biochemical differentiation defects in keratinocytes with mTORC1 loss of function. 

(A) Immunoblotting of WT and inducible Rptor-cKO cell lysates with or without ROCK inhibition using Y27632 (2 or 20 μM) or fasudil (5 or 50 μM) for desmosomal components and ROCK activity markers. p-MYPT1/total MYPT1 and PKP2/PKP3 were immunoblotted separately from the upper panel using the same biological replicates. (B) Immunofluorescence for adherens junction components (top panels, left) and DSP1/2 (middle panels, left) in Rheb-cKO keratinocytes following Y27632 (10 μM) treatment. TEM (bottom panels, left) of Rheb-cKO desmosomes with or without Y27632 treatment. Scale bar: 50 μm (top panels, left) and 500 nm (bottom panels, left). Quantification of DSP1/2 immunofluorescence (middle panel, right) and E-cadherin (top panel, right) (n = 3, n = 33 cell borders each) in Rheb-cKO keratinocytes with or without Y27632 (P value by Mann-Whitney test, DSP, or Student’s t test, E-cadherin). Quantification of desmosomal length on TEM for Rheb-cKO keratinocytes with or without Y27632 (bottom panel, right) (bar represents mean length in nanometers, n = 11 and 12, P values by Student’s t test). (C) Immunoblotting of membrane extracts from Rheb WT and cKO keratinocytes with or without Y27632 (20 μM) for desmosomal and adherens junction components. (D) Dispase dissociation (left panel) for Rheb WT or cKO keratinocytes with or without Y27632 (20 μM). Scale bar: 1 cm. Quantification of dispase assay experiments (right panel) (n = 4; P values by 1-way ANOVA). (E) Immunoblotting of Rheb WT or cKO keratinocytes with or without Y27632 (20 μM) for biochemical differentiation markers. (F) Immunoblotting of Rheb-cKO cells with or without ROCK1 siRNA (50 nm) for ROCK1 and markers of ROCK activity, desmosomal and biochemical differentiation markers. ROCK1, total MLC2, and total MYPT1 were immunoblotted in parallel, contemporaneously with the other markers. Error bars represent SEM throughout.
To validate the microarray predictions, we first examined expression of ALK5 (TGF-β receptor I) in Rheb- and Rptor-cKO P0 epidermis by immunoblotting and immunohistochemistry and found ALK5 levels to be increased relative to WT epidermis (Figure 7A and Supplemental Figure 4, C and D). Using cell membrane–enriched protein lysates from Rheb- and Rptor-cKO cells for immunoblotting, we found that membrane levels of ALK5 were also increased in cKO keratinocytes (Figure 7B). Downstream maturation with mTORC1 loss of function (Supplemental Figure 4A). However, the prediction that TGF-β signaling was activated with Rheb cKO was intriguing given that TGF-β is a well-known upstream activator of ROCK signaling (57), and mice and humans with aberrant TGF-β activation in the skin have a thin, translucent epidermis similar to that seen with the Rheb-cKO mice (58, 59). In addition, some previous studies have suggested that mTORC1 may inhibit the related BMP signaling pathway in murine skin (60).
TGF-β signaling was activated with mTORC1 loss of function based on increased nuclear SMAD2 and/or SMAD3 levels in the cKO epidermis (Figure 7C) and keratinocyte cultures (see below in Figure 7F and Supplemental Figure 4G), and increased phosphorylated SMAD2 (p-SMAD2) levels in keratinocyte cultures (Figure 7E and Supplemental Figure 4F). Pharmacologic inhibition of mTORC1 with rapamycin, AZD8055, or torin1 also increased p-SMAD2 levels in WT keratinocytes (Figure 7D). Importantly, TGF-β treatment was sufficient to induce ROCK signaling (measured by p-MYPT1 and p-MLC2 levels) (Supplemental Figure 4E) and suppress desmosomal protein expression (DSP1/2, DSG1, DSG3, and PKP3) in WT keratinocytes (Supplemental Figure 4H).

Finally, we tested whether ALK5/β signaling was required to activate ROCK signaling downstream of mTORC1 loss of function. We used SB431542, a pharmacologic ALK5 inhibitor, the efficacy of which was confirmed by a decrease in p-SMAD2 levels (Figure 7E) and a decrease in nuclear localization of SMAD2/3 (Figure 7F) in cKO keratinocytes. Strikingly, ALK5 inhibition with SB431542 was sufficient to suppress increased ROCK activity in cells with Rheb or Rtor loss, as measured by decreased p-MLC2

Figure 8. TGF-β receptor (ALK) mediates increased ROCK activity and decreased desmosomal cell-cell adhesion. (A) Immunoblotting following surface biotinylation and immunoprecipitation (IP) in WT or inducible Rptor-KO keratinocyte lysates with or without SB431542 treatment (10 μM) for membrane desmosome and adherens junction levels (left panel). Na-K ATPase was used to normalize for membrane protein. Enrichment of cell surface proteins in biotin immunoprecipitates, using Na-K ATPase as a control for membrane protein (middle panel). Densitometry quantification of immunoblot analysis of surface biotinylation experiments demonstrates significantly decreased membrane expression of DSG1 but not E-cadherin in Rptor/+/− keratinocyte cultures treated with adenoviral Cre, compared with those treated with empty adenovirus. Treatment with SB431542 is sufficient to rescue membrane DSG1 expression (n = 3, error bars represent SEM, P values are by 1-way ANOVA). (B) Dispase assay (left) in Rheb WT or cKO keratinocytes with or without SB431542 (10 μM) (scale bar: 1 cm). Quantification of dispase assay experiments (right) (n = 3, error bars represent SEM, P values by 1-way ANOVA). (C) Immunoblotting of WT or inducible Rptor-KO keratinocytes with or without ALKS siRNA for markers of ROCK activity. (D) Immunofluorescence of WT or inducible Rptor-KO keratinocytes with or without ALKS siRNA for desmosomal protein levels and ROCK activity (left panels) (scale bar: 50 μm). Quantification of DSG1 and DSP1/2 border fluorescence (right panels) from experiments on the left (r = 3, n > 24 cells, P values by Kruskal-Wallis test).
levels by immunoblotting (Figure 7E) and decreased F-actin stress fibers (data not shown). In addition, SB431542 rescued total desmosomal protein levels in cKO cells by immunoblotting (Figure 7E) and membranous desmosomal protein levels in cKO cells by immunofluorescence (Figure 7F) and surface biotinylation assays (Figure 8A). Inhibition of ALK5 was also sufficient to rescue expression of biochemical differentiation markers (Figure 7E) in keratinocytes with mTORC1 loss of function. Dispase assays on cKO keratinocyte monolayers demonstrated functional rescue of cell adhesion strength upon inhibition with ALK5 inhibition (Figure 8B). Finally, to verify the specificity of the effects of ALK5 pharmacologic inhibition, we treated cells with ALK5 siRNA. Surprisingly, even relatively modest ALK5 knockdown by siRNA reduced p-MLC2 levels in keratinocytes with mTORC1 loss of function (Figure 8C and Supplemental Figure 5, A and B), as well as actin stress fiber formation (Figure 8D and Supplemental Figure 5D). ALK5 knockdown in cKO cells was sufficient to rescue cell-cell adhesion as evidenced by total (Supplemental Figure 5, A and B) and membranous (Figure 8D and Supplemental Figure 5D) desmosomal protein levels (DSP1/2, DSG1, DSG3, PKP3), and some measures of biochemical differentiation (loricrin, TGM1) were also rescued (Supplemental Figure 5A). Interestingly, similar effects were seen in WT cells with ALK5 inhibition (Supplemental Figure 4I) and ALK5 knockdown (Supplemental Figure 5C), suggesting a role for basal ALK5 signaling in the regulation of adhesion and differentiation. Cumulatively, these data support a model in which mTORC1 silencing leads to tension downstream of ROCK resulting in decreased desmosomal adhesion, cytoskeletal hypercontractility, and TGF-β signaling. In principle, the polarity and stratification defects could result from a parallel pathway downstream of mTORC1 silencing, contributing to the biochemical differentiation defects, and this will be a subject of future study.

Cell-cell contact in response to calcium first initiates adhesions junction assembly, which in turn provides cues for desmosomal assembly. While adhesions junction marker expression was largely normal, we observed a dramatic decrease in total expression levels and membrane localization of all desmosomal isoforms in both mTORC1 loss-of-function models, in vitro and in vivo. This defect correlated with the severity of the phenotype since the degree of blistering and neonatal survival correlated roughly with the extent of desmosomal protein expression in vivo in the 2 models. For reasons potentially attributable to differences in genetic background or non-mTORC1-dependent effects, Rheb-cKO pups exhibited a lesser degree of desmosomal protein loss in vivo, correlating with superficial epidermal pustules, rather than frank suprabasal blistering as seen in the Rptor-cKO pups. However, despite these phenotypic differences, both models showed a profound functional defect in keratinocyte cell adhesion, with smaller desmosomes and poor adhesion in vitro.

It is possible that differences in genetic background of the mouse strains examined also explain why the recent study examining Rptor and mTOR kinase loss of function by the Eming group did not note skin blistering, though profound defects in epidermal differentiation were described (34). Although both cell adhesion and differentiation are calcium-dependent processes, cell adhesion is known to precede biochemical differentiation in vitro (61). Desmosomal proteins have also emerged as important regulators of signaling pathways involved in differentiation (62). Accordingly, we found that rescue of desmosomal protein expression was sufficient to rescue biochemical keratinocyte differentiation in Rptor-cKO keratinocytes in vitro, strongly suggesting that the biochemical differentiation defect is downstream of the cell adhesion abnormality in these mice. Given these data, it is likely that desmosomal levels were decreased in the Eming Rptor loss-of-function mice; however, perhaps because of strain background or environmental differences, there may have been only subclinical evidence of desmosomal dysfunction (i.e., no visible blistering or
very subtle acantholysis). The trauma of birth as a potential contributing factor in blistering/peeling of the skin (19) coupled with the short lifespan of \( \text{Rptor} \)-cKO pups may be an additional reason why subclinical desmosomal dysfunction may remain undetected.

Rho kinases are principal effectors of the small GTpase Rho, and play important roles in regulating actin cytoskeletal organization, cell adhesion, and gene expression via mechanotransduction (29, 63). Previous studies have demonstrated that Rho activity has a complex relationship with adherens junction formation; Rho signaling through mDia promotes peripheral localization of E-cadherin/\(\alpha\)-catenin and maintains stable adherens junctions, while signaling via ROCK disrupts adherens junctions in epithelial cells (24, 25, 64). Lower levels of active Rho may preferentially activate mDia, while higher levels of active Rho favor signaling through ROCK (65). Thus, higher levels of active, membrane-bound Rho might favor ROCK hyperactivity in \( \text{Rheb} \)- and \( \text{Rptor} \)-cKO keratinocytes. In contrast, the role of the actin cytoskeleton and its regulators in desmosomal protein expression, assembly, and dissolution has only recently begun to be elucidated. It is known that DSP incorporation into junctions takes place in 3 distinct phases, with the last phase being cytochalasin-sensitive and actin-dependent (27). Desmosomal components can in turn reciprocally regulate actin dynamics: loss of PKP2 (28) and loss of plakoglobin (66) are associated with a global increase in RhoA activity, actomyosin contractility, loss of cortical actin bundling, and an increase in stress fibers and focal adhesions (67).

Our work adds an additional mechanism by which cytoskeletal regulators may alter desmosomal adhesion: via ROCK-mediated suppression of desmosomal gene expression. Though the downstream transcriptional regulators of this effect remain to be discovered, ROCK activity plays a key role in mechanotransduction, regulating transcription factors such as those involved in Hippo signaling and SRF. It is therefore conceivable that aberrant activation of ROCK may lead to suppression of desmosomal gene expression with or without mislocalization of key desmosomal components, which in turn can exacerbate actomyosin contractility, thereby initiating a cycle of events culminating in profound adhesive dysfunction.

Although the 2 isoforms of ROCK, ROCK1 and ROCK2, share highly conserved kinase domains, they exhibit vastly different biological functionalities. ROCK1 is critical for formation of stress fibers, focal adhesions, and phosphorylation of MLC2, a major substrate involved in actomyosin contractility, while ROCK2 is dispensable for and may even dampen these responses (55, 68–70). In proliferating WT and \( \text{Rheb} \)-cKO keratinocytes grown in low calcium, ROCK1 expression was greater than ROCK2 (data not shown); moreover, ROCK1 knockdown completely inhibited phosphorylation of MLC2 and MYPT1. RhoE is a known negative regulator of ROCK signaling that specifically inhibits ROCK1-induced stress fiber formation (71), and its membrane localization was significantly decreased in \( \text{Rptor} \)-cKO keratinocytes. We therefore conclude that under conditions of mTORC1 inhibition, ROCK1 is likely the principal driver of actomyosin contractility in murine keratinocytes. Accordingly, treatment of \( \text{Rheb} \)- and \( \text{Rptor} \)-cKO cells with ROCK inhibitors or ROCK1 siRNA rescued the expression of desmosomal proteins and increased terminal differentiation markers. In agreement with our findings, it has previously been reported that ROCK1 knockdown promotes terminal differentiation while ROCK2 knockdown inhibits terminal differentiation in human and murine keratinocytes (55, 56).

We examined differentially expressed genes and identified signaling pathways/upstream regulators that were activated following mTORC1 loss of function. One of the top regulators was TGF-\( \beta \) signaling, which was significantly activated in the context of \( \text{Rheb} \) loss. In mouse epidermis, TGF-\( \beta \) signaling regulates wound healing and hair follicle development (72, 73), and can either stimulate or inhibit epidermal proliferation, keratinoocyte differentiation, and tumor formation, depending on the biological context (74–78). Interestingly, induction of active TGF-\( \beta \) during development also results in thinning of the epidermis with a shiny, taut erythemic skin and neonatal lethality (58, 79). A similar phenotype has also been noted in Loeys-Dietz syndrome in humans, characterized by a net increase in TGF-\( \beta \) signaling (59). Notably, mTORC1 inhibition by rapamycin or silencing of \( \text{Rptor} \) can induce phosphorylation of SMAD3 or SMAD1/5/8 in normal or tumorigenic prostate epithelial cells (80, 81) and hair follicle stem cells (60). However, a conclusive mechanism linking mTORC1 loss of function to TGF-\( \beta \)/BMP signaling has been elusive. In validating our microarray results, we found that both total and membrane-localized ALK5 expression was enhanced in epidermis and keratinocytes of \( \text{Rheb} \)- and \( \text{Rptor} \)-cKO mice, with a corresponding increase in SMAD2/3 phosphorylation and nuclear localization. The mechanisms underlying ALK5 upregulation remain unclear and are the subject of further study.

TGF-\( \beta \) signaling regulates Rho/ROCK signaling, actomyosin contractility, and stress fiber formation via multiple mechanisms (82, 83). We tested the hypothesis that TGF-\( \beta \) to ROCK signaling requires ALK5 activity in the context of mTORC1 loss of function, a finding that has been documented in other contexts (84). ALK5 inhibition or knockdown inhibited stress fiber formation and improved cortical organization of actin and phosphorylated MLC2. Importantly, ALK5 inhibition rescued total and membrane-localized desmosomal expression, keratinoocyte differentiation marker expression, and cellular adhesive strength. Significantly, PKP2 loss can also stimulate TGF-\( \beta \) signaling, and this reciprocal regulation may reinforce the effects on both noted in our system (85). TGF-\( \beta \) signaling plays a key role in epithelial-mesenchymal transition, in which SLUG-induced desmosomal disassociation is a necessary and initial step (86). Our findings highlight a potential mechanistic basis for this effect.

Finally, since TGF-\( \beta \) is a known and potent stimulant of PI3K/mTOR signaling, elucidating the effects of mTORC1 inactivation on TGF-\( \beta \) signaling further broadens our understanding of the role of mTORC1 in growth factor receptor feedback suppression. Pharmacologic or genetic mTORC1 silencing in MEFs directly or indirectly stabilizes docking (IRS) (6, 7) and adaptor proteins (GRB10) (10, 11) required for insulin receptor/insulin growth factor receptor signaling and leads to upregulation of platelet-derived growth factor receptor (PDGFR) expression (87). Here, we describe a novel in vivo role for mTORC1 silencing in the regulation of epithelial TGF-\( \beta \) signaling, which could also promote tumor progression. Indeed, via increased TGF-\( \beta \) signaling, mTORC1 inactivation in the normal epidermis is sufficient to substantially inhibit
cell differentiation, compromise desmosomal cell-cell adhesion, and heighten cytoskeletal contractility, cellular processes known to potentiate cell migration or even metastatic spread in epithelial tumors. Though these studies were performed in the mouse skin, their potential relevance to humans is further evidenced by the fact that mTORC1 inhibitor therapy for renal transplantation in humans is associated with delayed wound healing (12) and skin eruptions, sometimes requiring discontinuation of the drug (13). Precisely how mTORC1 silencing modulates TGF-β receptor levels, and whether this promotes tumor progression in carcinogenesis models, are important areas of future study and may be critical to developing strategies to circumvent tumor cell resistance to mTORC1 inhibitors.

Methods

**Mice.** Mice expressing Cre recombinase under control of the human keratin 14 (K14) promoter (Krt14-Cre) [stock no. 004782, STOCK Tg(KRT14-Cre)1Amc/J] (35) and mice carrying loxP sites flanking exon 6 of Rptor (stock no. 013188, B6.Cg-Rptoren1.1J) (37) were from The Jackson Laboratory. Mice with loxP-flanked Rheb alleles were generated in the laboratory of F. Worley (36). Genomic DNA was isolated from tail snips and genotyping performed with primers as described in Supplemental Methods.

**Barrier function assay.** To evaluate the integrity of the epidermal barrier, a toluidine blue penetration assay was performed on E18.5 embryos as described in Supplemental Methods.

**BrdU labeling.** To measure in vivo proliferation, pregnant female mice were injected i.p. with a single dose of BrdU (100 mg/kg body weight) 3 hours prior to sacrificing. BrdU incorporation was detected by immunohistochemistry of paraffin-embedded sections using an anti-BrdU monoclonal antibody (mouse, 1:2,000; Fitzgerald Industries). The number of BrdU-positive cells was plotted as a relative fraction of K14-positive cells in each section.

**Primary mouse keratinocyte cultures.** Primary mouse keratinocytes were isolated from newborn (P0) skin of pups using standard procedures as described in detail in Supplemental Methods. To knock out Rptor, Rptor<sup>lox<sub>fl</sub></sup> keratinocytes were infected with Cre recombinase–expressing or empty adenoviral vectors (Vector Biolabs) before plating. To induce cell-cell contacts, desmosomal maturation, and differentiation, the calcium concentration was elevated to 2 mM.

**Reagents and antibodies.** Sources of primary antibodies are described in Supplemental Methods. The following reagents were used: rapamycin, AZD8055, and fasudil (LC Laboratories); Y27632 (stock no. 013188, B6.Cg-Rptoren1.1J) (37) were from The Jackson Laboratory. Mice with loxP-flanked Rheb alleles were generated in the laboratory of F. Worley (36). Genomic DNA was isolated from tail snips and genotyping performed with primers as described in Supplemental Methods.

**Primary mouse keratinocyte cultures.** Primary mouse keratinocytes were isolated from newborn (P0) skin of pups using standard procedures as described in detail in Supplemental Methods. To knock out Rptor, Rptor<sup>lox<sub>fl</sub></sup> keratinocytes were infected with Cre recombinase–expressing or empty adenoviral vectors (Vector Biolabs) before plating. To induce cell-cell contacts, desmosomal maturation, and differentiation, the calcium concentration was elevated to 2 mM.

**Histology and immunostaining.** Mouse skins were fixed in 10% neutral buffered formalin (Sigma-Aldrich), embedded in paraffin, sectioned at 4 mm, and used for immunohistochemistry. Immunostaining or immunofluorescence was performed as previously described (88) and is detailed in Supplemental Methods.

**Protein lysate preparation and immunoblotting.** Epidermis was separated from the dermis following incubation of P0 pup skin with 3.8% ammonium thiocyanate for 10 minutes at room temperature and lysates prepared and immunoblotted as described previously (88) and in Supplemental Methods. Membrane lysates were prepared using the Subcel-

lular Protein Fractionation kit for Cultured Cells (78840; Thermo Fisher Scientific). Cell surface biotinylation was performed using the Pierce Cell Surface Protein Isolation Kit (89881; Thermo Fisher Scientific).

**siRNA-mediated gene silencing.** siRNAs (Silencer Select) for ROCK1, ALK5, and negative control were purchased from Thermo Fisher Scientific. Primary keratinocytes were transfected with 50 nm siRNA using Lipofectamine RNAiMAX reagent according to the transfection guidelines.

**Plasmid transfection.** Rheb- and Rptor<sup>cKO</sup> cells were transiently transfected with 2 µg of empty vector cDNA, mouse DSG1a cDNA (MR217075; Origene), or mouse DSG3 cDNA (MM10132-21691952; Dharmacon) in 6-well plates, using Lipofectamine 3000 (L3000008; Thermo Fisher Scientific), according to transfection guidelines.

**RNA isolation and quantitative real-time reverse transcriptase PCR.** Total RNA was extracted using TRIzol (15596026; Invitrogen) for epidermis or RNeasy (74104; Qiagen) for keratinocytes according to guidelines. RNA was converted to cDNA using SuperScript III (18080051; Thermo Fisher Scientific) according to guidelines. mRNA levels were quantified using an ABI Prism 7900HT Real-Time PCR system (Applied Biosystems) with primers and probes detailed in Supplemental Methods. Threshold cycle (Ct) was obtained from PCR reaction curves and mRNA levels quantitated using the comparative Ct method with actin serving as reference.

**Immunocytochemistry.** Primary keratinocytes were seeded on cover-slips coated with fibronectin. Cells were fixed in 100% methanol at −20°C for 30 minutes or 4% paraformaldehyde for 15 minutes at room temperature, according to antibody specifications and immunocytochemistry performed using standard protocols (Supplemental Methods), and visualized using an Olympus BX41 epifluorescence microscope. Image analysis for border intensity, cell perimeter, nuclear immunostaining, and quantification was done in ImageJ (NIH) as detailed in Supplemental Methods.

**Transmission electron microscopy.** Following standard fixation and embedding conditions detailed in Supplemental Methods, ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Hitachi 7600, equipped with Dual AMT CCD camera system).

**Disparate dissociation assays.** Rheb WT and cKO cells were grown on uncoated 6-well plates. Confluent keratinocyte monolayers were pretreated with inhibitors for 24 hours prior to switch in 1.2 mM calcium for 18 hours. At assay, cells were rinsed with PBS and incubated in 2.5 U/ml dispase solution (Sigma-Aldrich) in PBS for 15 minutes at 37°C. Detached sheets were transferred to 15-mL conical tubes in PBS and stressed by mechanical rotations. Resulting fragments were visualized and images captured.

**Microarray analysis.** Microarray analysis was performed as detailed in Supplemental Methods (GEO accession number GSE102026).

**Statistics.** For image analysis, normal distribution was assessed using the D’Agostino and Pearson normality test. If normally distributed, statistical significance was determined with Student’s t test when comparing 2 experimental groups, or with 1-way ANOVA with Dunnett’s correction when comparing 3 or more experimental groups. If not normally distributed, statistical significance was determined with the Mann-Whitney test when comparing 2 experimental groups, or with the Kruskal-Wallis test with Dunn’s correction when comparing 3 or more experimental groups. For RNA and protein quantification, cell proliferation experiments, and dispase assays, statistical analysis was performed using unpaired Student’s t test or 1-way ANOVA with
Tukey’s test for multiple comparisons. All tests assumed a 2-tailed deviation and were performed in Prism 7 (GraphPad). Where data are represented as a scatter plot or histogram, bars represent mean ± SEM. P values less than 0.05 were considered statistically significant.

Study approval. All animal studies were carried out following protocol approval from the Johns Hopkins Animal Care and Use Committee.

Author contributions
Conception and design were performed by KA, TLL, BX, and PW. Data acquisition was performed by KA, PP, AD, LE, AJE, CCT, TLL, AS, AT, and HK. Data analysis was performed by KA, TLL, CCT, AD, DG, HK, AS, and AT. Drafting of the manuscript was performed by KA, TLL, AJE, and PW.

Acknowledgments
The authors thank Barbara Smith and Tyler Stephens for their assistance with the transmission electron microscopy. Funding for this research was provided in part by the Tuberous Sclerosis Research Program of the Congressionally Directed Medical Research Programs (CDMRP) (W81XWH-12-1-0186) (to TLL), and the National Cancer Institute (R01CA200858) and National Institute on Drug Abuse (DA00266).

Address correspondence to: Tamara L. Lotan, Department of Pathology and Department of Oncology, Johns Hopkins University School of Medicine, 1550 Orleans Street, Baltimore, Maryland 21231, USA. Phone: 410.614.9196; Email: tlotan1@jhmi.edu.


44. Lechler T, Fuchs E. Asymmetric cell divisions controls the actin cytoskeleton and is rapamycin sensitive of bone morphogenetic protein-6 (BMP-6) expression. *FEBS Lett*. 2008;582(14):2051-2065.


55. Moustakas A, Heldin CH. Dynamic control of