The lysosomal signaling anchor p18/LAMTOR1 controls epidermal development by regulating lysosome-mediated catabolic processes

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Summary

The lysosomal adaptor protein p18 is an essential anchor of a scaffolding complex for the mTORC1 and MAPK pathways, which play crucial roles in controlling cell growth and energy homeostasis. To elucidate the in vivo function of the p18-mediated pathway, we conditionally ablated p18 in the mouse epidermis. Mutant mice were born with severe defects in formation of the stratum corneum and died within 12 h after birth due to dehydration caused by loss of skin barrier function. Mutant epidermal cells can grow and differentiate into granular cells, but exhibit functional defects in corneocyte maturation. Electron microscopy identified abnormal immature cells, overlying the mutant granular cells, which accumulated autophagosomes, glycogen granules and dead nuclei. Cell culture analysis showed that loss of p18 attenuated lysosome function, resulting in accumulation of immature lysosomes and autophagosomes. Analyses of lysosome behavior revealed that p18 is required for functional interaction between lysosomes and target organelles including autophagosomes. These findings suggest that p18-mediated pathways control lysosome-mediated catabolic processes, which are crucial for the development of mouse epidermis.

Key words: p18, mTORC1, Lysosomes, Autophagy, Epidermis

Introduction

To survive and respond to environmental cues, cells import nutrients and extracellular macromolecules via the endosome system. Endocytosed materials are first delivered to early endosomes, where they are sorted; subsequently they are either recycled to the cell surface or targeted for degradation (Huotari and Helenius, 2011; Luzio et al., 2007). Materials fated for degradation are transported to late endosomes (Falguieres et al., 2008; Falguieres et al., 2009), which subsequently fuse with primary lysosomes, forming late endosome–lysosome hybrids, i.e. mature lysosomes (Luzio et al., 2007). These mature lysosomes accumulate in the perinuclear compartment by associating with microtubules (Harada et al., 1998), and digested materials are either released into the cytoplasm or exocytosed. Under starved conditions, cells degrade intracellular components by autophagy to regenerate metabolic precursors that are recycled for macromolecular synthesis and ATP generation (Mizushima et al., 2011). Autophagosomes that have engulfed cellular components fuse with lysosomes to form autolysosomes, in which components are degraded by lysosomal hydrolases. Many secreting cells contain cell type-specific lysosome-related organelles (LROs), such as melanosomes and lamellar bodies, which contain lysosomal enzymes required for macromolecule processing and secretion (Bonifacino, 2004; Dell’Angelica et al., 2000). Together, these lysosome-related endosome systems are crucial for the maintenance of cell homeostasis, as well as cell type-specific functions such as the processing of secretory macromolecules. However, the regulatory mechanisms underlying lysosome-mediated cellular functions remain elusive.

The endosome system is associated with intracellular signaling (Miaczynska et al., 2004). Components of a branch of the MAPK pathway are recruited to late endosomes via the p14–MP1 complex, which serves as a MEK1-specific scaffold (Teis et al., 2002). Ablation of the p14–MP1 complex results in aberrant subcellular distribution and trafficking of late endosomes (Bohn et al., 2007; Teis et al., 2006), suggesting that the p14–MP1-mediated pathway is involved in the regulation of endosome/lysosome biogenesis. Recently, we identified a novel membrane adaptor protein, p18 (also LAMTOR1), which specifically binds to the p14–MP1 complex (Nada et al., 2009). p18 is exclusively localized to late endosomes/lysosomes and acts as an essential anchor for the p14–MP1 complex. The p18–p14–MP1 complex is highly conserved from yeast to humans (Kogan et al., 2010), indicating that it plays a critical role in eukaryotic cells. p18-deficient mouse embryos die during early developmental stages with severe defects in endosome/lysosome organization and membrane–protein transport in the visceral endoderm (Nada et al., 2009). Analysis of p18-deficient cells suggests that the p18–p14–MP1 complex plays a crucial role in controlling cell growth and endosome dynamics, including membrane–protein transport and lysosome biogenesis.

More recently, the p18–p14–MP1 complex was found to serve as a scaffold (Ragulator) for the Rag GTPase complex (RagAB/CD), which is required for amino acid-dependent activation of
the mammalian target of rapamycin complex 1 (mTORC1) on lysosomes (Sancak et al., 2010; Zoncu et al., 2011a). To respond to amino acid levels, the Ragulator functionally interacts with lysosomal v-ATPase (Zoncu et al., 2011b) and forms a complex with HBXIP and C7orf59 to serve as a GEF for Rag GTPases (Bar-Peled et al., 2012). These findings demonstrate that the p18–p14–MP1–Rag complex is essential for controlling the activity of mTORC1, and indicate that the complex plays a crucial role in controlling cell growth by promoting protein translation. On the other hand, we recently showed that p18–mTORC1 activity is controlling lysosomal maturation (Takahashi et al., 2012). Furthermore, other groups have proposed potential roles of p18 in cellular cholesterol homeostasis (Guillaumot et al., 2010) and p53-dependent apoptosis via aberrant lysosomal activation (Malek et al., 2012). These in vitro observations suggest the importance of the p18-related complex in the regulation of lysosomal function, but its in vivo function and the sites of its action remain unknown.

To elucidate the function of the p18-related complex, we conditionally ablated p18 in mouse epidermis. Mutant epidermis exhibited severe defects in formation of the stratum corneum, accompanied by functional defects in granular cells. Analyses of tissues and keratinocytes revealed that the p18-related complex is required for promoting lysosome-mediated degradation of cellular components, including autophagosomes and endocytosed materials. Our observations of lysosome dynamics further showed that the p18 complex is involved in regulating functional interaction between lysosomes and target organelles, e.g. autolysosome formation. These findings suggest that the p18-related complex controls lysosome-mediated catabolic processes, which is crucial for development of mouse epidermis.

**Results**

**Ablation of p18 in the epidermis**

p18 is expressed ubiquitously (Nada et al., 2009), but it is highly concentrated in epithelial and exocrine tissues, such as the gastrointestinal tract, epidermis and pancreas (data not shown). Therefore, to address the in vivo function of p18, we conditionally ablated p18 in epidermal basal cells. p18flox/flox mice, in which exon 1 of the p18 gene is flanked by loxP sites (Fig. 1A), were crossed with Keratin 5-Cre (K5-Cre) transgenic mice (Tarutani et al., 1997), and F2 offspring or embryos (E17.5) were genotyped by PCR (Fig. 1B). The elimination of p18 protein in the mutant epidermis was confirmed by immunohistochemistry (Fig. 1C). Keratin 5-Cre p18flox/flox [k5p18−/−; knockout (KO)] mice were born at the expected Mendelian ratio, but neonates died within 12 h after birth with dramatic skin defects. The skin of mutant mice was generally very thin and adhesive, and their eyelids were absent. Mutant embryos at E17.5 exhibited additional defects; the skin of heterozygous control k5p18+/− embryos dried soon after caesarean section, whereas the skin of KO k5p18−/− embryos was continuously humid. Mutant KO neonates rapidly died within a couple of hours, before skin dried (Fig. 1D). These results suggest that loss of p18 affects skin barrier function, which protects against leakage of body fluid. Indeed, an epidermal barrier assay based on the permeability of X-gal exhibited a prototype stratified squamous epithelium that was stably covered by the stratum corneum (SC), whereas the mutant epidermis was very thin and consisted of only a few (2–4) cell layers (Fig. 1F). Notably, the mutant epidermis completely lacked the stratum corneum.

**Defects in epidermal organization upon loss of p18**

To elucidate the molecular basis for the epidermal defects resulting from loss of p18, we examined differentiation of the epidermis by immunofluorescence (Fig. 2A). Staining for laminin α3 indicated that formation of the basement membrane is not affected by loss of p18. Keratin 5 was positive in the undifferentiated basal layers of the epidermis in mice of both genotypes. In addition, staining for the cell-proliferation marker Ki67 was almost identical in the basal cell layers of both genotypes. These results indicate that basal cells develop normally even in the absence of p18.

Keratin 1, a component of keratin fibers in the spinous and granular layers of the epidermis, was also detected in the mutant epidermis, although the thickness of the keratin 1-positive cell layers, particularly the granular layers, was greatly reduced in the...
mutant, suggesting that loss of p18 causes some defect in the granular layers. In both wild-type and mutant epidermis, E-cadherin and claudin-1 were detected at cell–cell junctions in the spinous and granular layers. Thus, formation of cell–cell junctions, such as adherens junctions and tight junctions, may not be dramatically affected by loss of p18. These observations indicate that the mutant epidermal cells can grow and differentiate into granular cells. However, loss of p18 impairs some function of granular cells, and this impairment may be linked to defective formation of the stratum corneum in the mutant epidermis.

**Defects in the stratum corneum upon loss of p18**

Based on these findings, we focused our analysis on the effects of loss of p18 on the processes of stratum corneum formation by granular cells. The stratum corneum is the outermost layer of the epidermis, consisting of terminally differentiated corneocytes that lack nuclei and organelles (supplementary material Fig. S1). Corneocytes are hexagonal cells packed mainly with keratin filaments bundled with filaggrin, and are surrounded by a cornified cell envelope composed of involucrin and loricrin, which are cross-linked by transglutaminase 1 (TGM1) and covalently bound to lipids. The inter-corneocyte space is filled mostly with ceramides and cholesterol, as well as several catabolic enzymes that are secreted from granular cells via lamellar bodies (Bouwstra et al., 2003).

To investigate the effects of loss of p18 on the formation of stratum corneum, we determined the contents of the inter-corneocyte space, i.e. hydrolytic enzymes and lipids, which are secreted via lamellar bodies (supplementary material Fig. S1).

One such component, sphingomyelin phosphodiesterase 1 (SMPD1) catalyzes hydrolysis of sphingomyelin to produce ceramides. Immunofluorescence revealed that in control epidermis, most of the SMPD1 was detected in the inter-corneocyte space, whereas it was not detectable in mutant epidermis (Fig. 2B, upper). Cholesterol was also detected in the stratum corneum only in control epidermis (Fig. 2B, lower). Furthermore, analysis of lipids in the epidermis showed that the levels of cholesterol and ceramides were dramatically reduced in the mutant epidermis (Fig. 2C). Due to the loss of lipids, mutant skin sank in phosphate buffer solution, whereas control skin floated on the surface (Fig. 2D). These observations suggest that the function and development of granular cells, i.e. production of the inter-corneocyte components, is impaired upon loss of p18.

**Defects in corneocyte formation upon loss of p18**

To further determine the effects of loss of p18 on the formation of corneocytes, we examined filaggrin proteins. In control epidermis, filaggrin was detected in large keratohyalin granules and adjacent to keratin patterns in the stratum corneum (Fig. 3A). In mutant epidermis, however, filaggrin signals were detected in smaller granules in a single cell layer, and no fibrous staining was detected. Furthermore, electron microscopy revealed that control epidermis contained multiple layers of granular cells that contain large keratohyalin granules, whereas the mutant epidermis consisted of a single layer of granular cells that contain smaller keratohyalin granules (Fig. 3B). These observations suggest that loss of p18 decreases the supply of filaggrin, resulting in defective formation of keratin fibers in the stratum corneum.

Over the course of these observations, we noticed that mutant epidermis contained abnormal corneocyte-like cells (termed...
interference contrast (DIC) images are shown. TUNEL-positive signals and merged images with PI staining and differential.

autophagosomes were accumulated in the outermost layers of the marker of autophagosomes, also revealed that LC3-positive (WT) and mutant (KO) mice were subjected to TUNEL assay. Images of abnormal cells accumulated autophagosome-like structures (Fig. 4A). Immunofluorescence staining for LC3 puncta, a marker of autophagosomes, dramatically

We next characterized the abnormal CLCs in greater detail by higher-resolution electron microscopy. We found that these abnormal cells accumulated autophagosome-like structures (Fig. 4A). Immunofluorescence staining for LC3 puncta, a marker of autophagosomes, also revealed that LC3-positive autophagosomes were accumulated in the outermost layers of the

autophagosome degradation that is mediated by lysosomes.

Defects in lysosomal function upon loss of p18
To further investigate the above possibility, we used p18-deficient mouse embryonic fibroblasts (MEFs) established from p18flox/flox mouse embryos. Autophagy was induced by depleting nutrients, and autophagosomes were detected by staining for LC3 and p62, another marker of autophagosome formation, on the same schedule as used for keratinocytes (Fig. 5A). Mutant fibroblasts also exhibited a significant increase in the number of basal autophagosomes, and retained greater numbers of autophagosomes, even when the cells were returned to nutrient-rich media (Fig. 5B,C). Western blot analysis for LC3-I/II also revealed that degradation of autophagosomes was suppressed by loss of p18 (Fig. 5D). When cells were starved for up to 24 h, degradation of autophagosomes and LC3 were suppressed in cells lacking p18. Under starved conditions, autophagosome formation was greatly induced in both cell types, indicating that the initiation of autophagosome formation is not affected by loss of p18. When cells were returned to nutrient-rich media, autophagosomes rapidly disappeared in wild-type keratinocytes, whereas greater numbers of autophagosomes were retained in mutant keratinocytes (Fig. 4E). Electron microscopy of keratinocytes confirmed the accumulation of autophagosomes in mutant keratinocytes (Fig. 4F). Based on these observations, we hypothesize that loss of p18 suppresses the process of autophagosome degradation that is mediated by lysosomes.

Defects in autophagy upon loss of p18
We next characterized the abnormal CLCs in greater detail by higher-resolution electron microscopy. We found that these abnormal cells accumulated autophagosome-like structures (Fig. 4A). Immunofluorescence staining for LC3 puncta, a marker of autophagosomes, also revealed that LC3-positive autophagosomes were accumulated in the outermost layers of the

mutant epidermis (Fig. 4B). Furthermore, we identified accumulations of unstructured materials, organelles and glycogen granules in the mutant cells (Fig. 4C). These unusual accumulations of undigested materials suggest that loss of p18 suppresses degradation of cellular components via autophagy and/or the endosome system.

To address this possibility, we examined the effects of loss of p18 on autophagy in primary keratinocytes (Fig. 4D). Wild-type and mutant keratinocytes prepared under nutrient-rich conditions were amino acid-starved for 1 h and then the media were replaced with a nutrient-rich medium to allow the cells to recover for up to 40 min. Even under nutrient-rich conditions, the number of LC3-positive puncta was significantly increased in cells lacking p18. Under starved conditions, autophagosome formation was greatly induced in both cell types, indicating that the initiation of autophagosome formation is not affected by loss of p18. When cells were returned to nutrient-rich media, autophagosomes rapidly disappeared in wild-type keratinocytes, whereas greater numbers of autophagosomes were retained in mutant keratinocytes (Fig. 4E). Electron microscopy of keratinocytes confirmed the accumulation of autophagosomes in mutant keratinocytes (Fig. 4F). Based on these observations, we hypothesize that loss of p18 suppresses the process of autophagosome degradation that is mediated by lysosomes.
vacuolar H^+ ATPase (V-ATPase) (Yamamoto et al., 1998). In both wild-type and mutant cells, treatment with BafA1 did not affect the expression of Lamp1 or mature cathepsin D1 (Fig. 6B), indicating that lysosome assembly is not affected by BafA1 treatment. Prior to BafA1 treatment, wild-type cells contained only low levels of LC3-II (Fig. 6B) and autophagosomes (Fig. 6C,D), but BafA1 treatment induced dramatic (~20-fold) accumulation of autophagosomes. This result indicates that in wild-type cells, autophagy is actively induced and autophagosomes are rapidly degraded through lysosomes. On the other hand, mutant cells contained substantial numbers of autophagosomes even before BafA1 treatment; consequently, the drug facilitated accumulation of autophagosomes without BafA1 treatment, even despite the excessive accumulation of immature lysosomes, it is likely that the efficiency of lysosome-mediated degradation of autophagosomes is greatly reduced upon loss of p18. We further verified the defect in lysosome function resulting from loss of p18 by performing a DQ-Red BSA analysis, which revealed that the efficiency of lysosomal degradation of endocytosed materials is also attenuated in mutant cells (supplementary material Fig. S3). Taken together, these findings suggest that loss of p18 inactivates function of lysosomes that target autophagosomes as well as late endosomes.

Defects in functional interaction between lysosomes and autophagosomes upon loss of p18

To address the mechanism of action of the p18 complex, we observed behaviors of organelles positive for p18, Lamp1 and LC3. Time-lapse analyses of cells expressing p18-GFP and Lamp1-mCherry showed that p18 exclusively co-localizes with Lamp1 on lysosomes (supplementary material Fig. S4; Movie 1), indicating that the p18-associated complex acts on lysosomes to regulate their functions. Co-expression analyses of Lamp1-mCherry and LC3-GFP revealed that in wild-type cells, Lamp1-positive lysosomes dynamically extended and retracted tubular branches and moved rapidly along microtubules (Fig. 7A; supplementary material Movies 2–5). We also observed that Lamp1-positive lysosomes occasionally and temporarily interact with LC3-positive autophagosomes, potentially via kiss-and-run interactions (Fig. 7A, insets) that are critical for autolysis (Jahreiss et al., 2008). In contrast, in mutant cells, Lamp1-positive lysosomes were distributed widely throughout the cytoplasm, rarely formed tubular structures and moved slowly, particularly in the peripheral area of the cells (Fig. 7A). The efficiency of inter-vesicular interaction was further analyzed by scoring the incidence and duration of Lamp1-mCherry and LC3-GFP colocalization. For these analyses, LC3-GFP was transiently expressed in p18 KO cells (KO) and p18 re-expressing cells (Rev) that stably express Lamp1-mCherry. The incidences of longer kiss-and-run interactions tended to be reduced in KO cells.
compared with Rev cells (Fig. 7B). It was also shown that inhibition of mTORC1 and the MAPK pathways by rapamycin and PD0325901, respectively, was able to mimic the effects of loss of p18. These findings raise the possibility that the p18 complex mediates the functional interaction between lysosomes and their target autophagosomes.

**Defects in autolysosome formation upon loss of the p18 complex**

To further define the action of the p18 complex, we used BafA1 to temporally accumulate autophagosomes by inhibiting autolysis. p18 KO and Rev cells were incubated with BafA1, and localizations of LC3 and Lamp1 signals were analyzed using Z-stack confocal microscopy (Fig. 8A). In Rev cells, treatment with BafA1 for 4 h induced accumulation of larger sized vacuolar-like vesicles in which LC3 and Lamp1 are mostly co-localized in the lumen of these vesicles. Longer treatment (16 h) further enlarged the double-positive vacuoles. Four hours after BafA1 was washed out to re-activate lysosome function, LC3 autophagosomes were mostly abolished, though Lamp1 vacuoles remained enlarged. These observations suggest that in the presence of p18, functional interaction, i.e. formation of autolysosomes via membrane fusion between lysosomes and autophagosomes or engulfment of autophagosomes by lysosomal vacuoles, can be accomplished even in the presence of BafA1, and autolysis is restarted by the disinhibition of lysosomal hydrolases.

In p18 KO cells, BafA1 treatment induced accumulation of larger Lamp1-positive vacuoles and increased in the number of LC3-positive autophagosomes. However, the enlargement of autophagosomes was more moderate, and some were not co-localized with vacuoles or remained attached to the surface of vacuoles. Even when BafA1 was washed out, most autophagosomes remain undigested. Essentially the same effects were reproducibly observed in other cultures (supplementary material Fig. S5). These findings suggest that loss of p18 interferes with interactions between autophagosomes and lysosomes, such as organelle fusion, consequently suppressing degradation of autophagosomes.

Finally, to assess the contribution of the p18-associated signaling pathways to lysosomal functions, we examined the effects of inhibition of mTORC1 and MAPK pathways on the functional interaction between autophagosomes and lysosomes in BafA1-treated Rev cells (Fig. 8B). In a manner quite similar to the effects observed in p18 KO cells, treatment with rapamycin attenuated formation of Lamp1-positive larger vacuoles and their co-localization with LC3-positive autophagosomes, resulting in impaired degradation of autophagosomes after BafA1 was washed out. This suggests that mTORC1 activity is crucial for executing p18-mediated functions. Furthermore, treatment with PD0325901 induced similar effects, suggesting that the activity of the MAPK pathway is also required for these cellular events. Essentially the same results were reproducibly observed in other sets of cultures (supplementary material Fig. S6).
Taken together, these findings suggested that the p18-associated pathways, mTORC1 and/or MAPK pathways, are involved in promoting functional interactions between lysosomes and target organelles to regulate lysosome-mediated catabolic processes (Fig. 8C) which are crucial for maintenance of cell homeostasis and cell fate determination such as formation of the stratum corneum during epidermal development.

Discussion
To address the in vivo function of the p18 complexes, we examined the effects of loss of p18 on epidermal development. The mutant epidermis exhibited severe defects, particularly in development of the stratum corneum, causing a loss of skin barrier function. Histology revealed that loss of p18 abrogated the formation of corneocytes; in their place appeared immature corneocyte-like cells that contained various undigested components such as dead nuclei, autophagosomes and glycogen granules. In previous work, we showed that wild-type visceral endoderm (VE) cells in normal embryos develop huge lysosome-like vacuoles (‘giant lysosomes’), whereas p18-deficient VE cells fail to form these structures and instead accumulate smaller lysosome-like organelles filled with amorphic materials (Nada et al., 2009). These findings suggest that the p18 complex plays general and critical roles in controlling lysosome-mediated catabolic processes.

We showed that the p18 complex is required for functional interaction between lysosomes and their targets, such as autophagosomes and late endosomes, to promote lysosomal degradation processes. Analyses of BafA1-treated cells revealed that loss of p18 complexes attenuates autolysosome formation potentially by suppressing lysosomal fusion. This raises the possibility that the p18 complex regulates functions of molecules involved in controlling specific vesicular interaction with lysosomes (Bonifacino and Rojas, 2006; Johannes and Wunder, 2011; Wickner and Schekman, 2011; Yu et al., 2010). Lysosomal fusion requires various components, including SNARE proteins, Rab GTPases and SNARE-associated proteins (SM proteins, NSF, SNAP and others) (Luzio et al., 2007). The functions of some components are regulated by phosphorylation (Wickner and...
late endosomes. Functionally interact with their target organelles, such as autophagosomes and complex on lysosomes. Lysosomes conveying the p18 complex can images are shown. (LC3 and Lamp1 signals were analyzed using confocal microscopy. Z-stack images and Z-axis images at the yellow lines are shown.

We observed accumulation of autophagosomes upon loss of p18 in the epidermis, as well as in cultured cells. It is well established that autophagy is regulated by mTORC1 activity (Yang and Klionsky, 2010; Zoncu et al., 2011a). Under normal conditions, active mTORC1 phosphorylates and inactivates ATG proteins to suppress induction of autophagy (Hosokawa et al., 2009). However, we observed that the inhibition of lysosomal function with BafA1 treatment could induce accumulation of autophagosomes even in wild-type cells. This finding suggests that under normal conditions, autophagy is active, but autophagosomes are rapidly degraded by efficient fusion with lysosomes. Because our data indicate that the p18 pathway is involved in the activation of lysosome functions, it is likely that mTORC1 can regulate autophagy not only by suppressing initiation of autophagy but also by facilitating degradation of autophagosomes. If this is indeed the case, the inhibition of mTORC1 by starvation not only induces autophagy, but also inactivates lysosome function. If these events were to continue during starvation, cells would be unable to degrade autophagosomes to regenerate metabolic precursors. This would appear to conflict with the overall purpose of autophagy. However, a recent study demonstrated that mTORC1 signaling is reactivated by prolonged starvation, and this increased mTORC1 activity attenuates autophagy and induces reformation of lysosomes (Yu et al., 2010). In this study, we also observed reactivation of mTORC1 during longer starvation periods, which occurred concomitantly with degradation of autophagosomes (supplementary material Fig. S2). By contrast, there was no reactivation of mTORC1 in p18 KO cells, which have very low levels of mTORC1 activity, resulting in a significant delay in autophagosome degradation. These findings shed new light on the regulatory mechanism of autophagy.

In the mutant epidermis, we observed reduced production of cholesterol, ceramides and SMPD1, all of which are secreted into the stratum corneum via lamellar bodies from the granular cells. The lamellar bodies are categorized as lysosome-related organelles (LRO) that share some features of late endosome/lysosomes (Raposo et al., 2007; Ridsdale et al., 2011), and cholesterol and ceramides are stored and processed by late endosomes (Bouwstra et al., 2003; Ganley and Pfeffer, 2006; Sobo et al., 2007). Thus, the dysfunction of lamellar bodies in the mutant epidermis suggests that the p18 complex is also involved in the regulation of LRO biogenesis. We also observed the accumulation of glycogen granules in mutant epidermal cells,
Carrying the floxed locus by homologous recombination in ES cells (Fig. 1). ES cells of floxed clones identified with anti-S6K and anti-phospho S6K (Cell Signaling). Horseradish peroxidase (HRP)-conjugated secondary antibodies were used to detect endogenous proteins. Immunofluorescence was observed using an Olympus IX81 confocal microscope equipped with a CoolSNAP HQ camera (Roper Scientific, Trenton, NJ) controlled by Metamorph software (Universal Imaging, West Chester, PA). Cell cultures
Skin from newborn mice was treated with 0.05% collagenase A in Epilife Medium (GIBCO) for 24 h at 4°C, and the epidermis was pealed away from the dermis and trypsinized for 15 min at 37°C. Keratinocytes were cultured on collagen type I-coated plates in keratinocyte growth medium (KGM, GIBCO) under low-oxygen conditions (3% O2). Mouse embryonic fibroblasts (MEFs) were established from p18flox/flox embryos by immortalizing with SV40 large T antigen. p18−/− cells (KO) were generated by the expression of the Cre gene, and MEFs re-expressing p18 (Rev) were prepared by introducing p18 cDNA. MEFs, KO cells and Rev cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

Fluorescence analysis
Keratinocytes cultured in 24-well plates were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with Tris-buffered saline containing 0.1% Tween 20 (TTBS), the samples were incubated with 5% BSA/TTBS, followed by incubation with primary antibodies in TTBS overnight at 4°C. After incubation with secondary antibodies for 45 min at room temperature, coverslips were mounted on glass slides. Cell lines were seeded onto fibronectin-coated coverslips and were fixed in 4% formaldehyde, followed by blocking with 1% BSA in PBS. Endogenous proteins were detected by immunofluorescence staining. Fluorescence was observed using an Olympus IX71 confocal microscope controlled by Fluoview FV1000 software. Signal intensities and areas were determined using MetaMorph software (Universal Imaging). To monitor behavior of LC3−positive autophagosomes and Lamp1−positive lysosomes, LC3−GFP and Lamp1−mCherry fusion proteins were expressed in cell lines. pEGFP−LC3 vector was kindly provided by T. Yoshimori (Osaka University) and pmCherry−Lamp1 vector was generated by inserting Lamp1 cDNA into pmCherry vector (Clontech). For live-cell imaging, cells were seeded onto a 35 mm diameter glass-bottom dishes coated with collagen or fibronectin in DMEM (phenol red-free) and monitored using an Olympus IX71 microscope equipped with a CoolSNAP HQ camera (Roper Scientific, Trenton, NJ) controlled by Metamorph software (Universal Imaging, West Chester, PA).

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Author contributions
T.S.-N. designed the experiments and analyzed mutant epimides and keratinocyte cultures; S.N. generated mutant mice and performed in vitro experiments in cell lines; M.K. genotyped the mice and performed histology; Y.T. analyzed lysosomal function; S.M. analyzed expression of lysosomal proteins. C.O. assisted gene transfer experiments; M.O. designed the experiments and wrote the paper.
References


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**Fig. S1.** The mechanism of stratum corneum formation. The stratum corneum is the outermost layer of the epidermis, consisting of terminally differentiated corneocytes that lack nuclei and organelles. Corneocytes are hexagonal-shaped cells packed mainly with keratin filaments bundled with filaggrin, and are surrounded by a cornified cell envelope composed of involucrin and loricrin, which are cross-linked by transglutaminase1 (TGM1) and covalently bound to lipids. The inter-corneocyte space is mostly filled with ceramides and cholesterol, as well as several catabolic enzymes that are secreted from granular cells via lamellar bodies.

**Fig. S2.** Loss of p18 causes autophagosomes to accumulate in fibroblasts. (A) Wild-type (WT) and mutant (KO) MEFs prepared under nutrient-rich conditions were starved by depletion of amino acids, and the formation of autophagosomes was observed for the indicated times by immunofluorescence staining for LC3. (B) Numbers of LC3 puncta were counted for 50 cells, and average numbers ± s.d. in a single cell at the indicated times are shown. ***P<0.001, **P<0.01 by Student’s t-test. (C) Western-blot analyses of LC3, phospho-S6K, and S6K in cells observed in panel A. β-tubulin is an internal control. Re-activation of S6K was observed in wild-type cells. Degradation of autophagosomes was suppressed in the mutant cells.

**Fig. S3.** The p18 pathway is required for lysosomal protein degradation. (A) Lysosome function was analyzed using Alexa Fluor 488 BSA and DQ Red BSA. DQ Red BSA requires enzymatic cleavage in acidic lysosomal compartments to generate a highly fluorescent product. Wild-type (WT), mutant (KO), and mutant MEFs re-expressing p18 (Rev) were cultured in media containing both Alexa Fluor 488 BSA and DQ red BSA (10 μg/ml each) for 30 min, and then the tracers were washed out. After incubation for 0 or 1 h, localization of Alexa Fluor 488 BSA and the fluorescent degradation products of DQ Red BSA were imaged using confocal microscopy. (B) The relative intensities of DQ red BSA to Alexa Fluor 488 BSA are shown. Average value ± s.d. was obtained from more than 10 different fields. ***P<0.001, **P<0.01 by Student’s t-test.

**Fig. S4.** Defects in interaction between lysosomes and autophagosomes upon loss of p18. (A) p18 and Lamp1 are co-localized on lysosomes. p18-GFP and Lamp1-mCherry constructs were transiently co-transfected into wild-type cells, and their behaviors were observed by time-lapse microscopy (supplementary material Movie 1). Initial images of p18-GFP (white) and Lamp1-mCherry (white), as well as the merged image of p18/Lamp1 (green/red), are shown. (B) Cells expressing Lamp1-mCherry and LC3-GFP were time-lapse analysed (Fig. 7A), and frequency of kiss-and-run interactions was scored by manually counting the number of colocalizations of Lamp1-mCherry and LC3-GFP signals and timing the duration of each interaction in 6 independent cells. Total number of interactions was plotted as a function of duration (sec).

**Fig. S5.** Defects in autolysosome formation upon loss of p18. LC3-GFP was transiently expressed in p18 KO cells (KO) and p18 re-expressing cells (Rev) that stably express Lamp1-mCherry. Cells were incubated with Bafilomycin A1 (BafA1) for 4h and 16h, and for additional 4h after BafA1 was washed out by replacing the media. At the indicated time points, localizations of LC3 and Lamp1 signals were analyzed using confocal microscopy. Z-stack images are shown.

**Fig. S6.** Effects of inhibitors of mTORC1 and MAPK pathways on autolysosome formation. LC3-GFP was transiently expressed in p18 re-expressing cells (Rev) that stably express Lamp1-mCherry. Cells were incubated with BafA1 in the absence or presence of Rapamycin (Rapa) or PD0325901 (PD) for 16h, and for additional 4h after BafA1 was washed out. At the indicated time points, localizations of LC3 and Lamp1 signals were analyzed using confocal microscopy. Z-stack images and Z-axis images at the yellow lines are shown.
Figure S1

Stratum corneum

Corneocytes

Terminal differentiation

Cholesterol, ceramide, cathepsin D

Granular cells

Cornified envelopes

Keratohyaline granules (filaggrin)

Keratin-patterns

Lamellar bodies

Organelles

Nuc
Figure S3

A

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</tr>
</thead>
<tbody>
<tr>
<td>Alexa488 BSA</td>
<td>DQ Red BSA</td>
<td>merge</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rev</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Scale bar: 10 µm

B

Average intensity of DQ Red BSA in 488BSA positive area

0 h       1 h

WT       KO       Rev

***       **       ***
Figure S4

Scale bar: 10 µm
Figure S5

KO

+ BafA1 4h  + BafA1 16h  Wash 4h

Rev

LC3/Lamp1

LC3

KO

LC3/Lamp1

Scale bar: 10 µm
Figure S6

+ BafA1 16h

Wash 4h

Rev

LC3

LC3/Lamp1

Rev

+Rapa (50 nM)

LC3

LC3/Lamp1

Rev

+PD (100 nM)

LC3

LC3/Lamp1

Scale bar: 10 µm
Movie 1. Behavior of p18-GFP and Lamp1-mCherry in WT cells (supplementary material Fig. S4).

Movie 2. Behavior of Lamp1-mCherry in WT cells (Fig. 7Aa).

Movie 3. Behavior of LC3-GFP and Lamp1-mCherry in WT cells (Fig. 7Ab).
Movie 4. Behavior of Lamp1-mCherry in p18 KO cells (Fig. 7Ac).

Movie 5. Behavior of LC3-GFP and Lamp1-mCherry in p18 KO cells (Fig. 7Ad).