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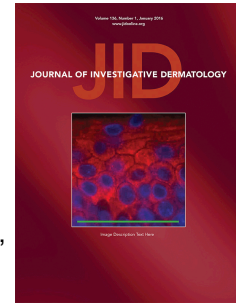
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Constitutive autophagy and nucleophagy during epidermal differentiation

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ABSTRACT

Epidermal keratinocytes migrate through the epidermis up to the granular layer where, upon terminal differentiation they progressively lose organelles and convert into anucleate cells or corneocytes. Our report explores the role of autophagy in ensuring epidermal function providing the first comprehensive profile of autophagy marker expression in developing epidermis. We show that autophagy is constitutively active in epidermal granular layer where by electron microscopy we identified double membrane-autophagosomes. We demonstrate that differentiating keratinocytes undergo a selective form of nucleophagy characterized by accumulation of LC3/LAMP2/p62 positive autolysosomes. These perinuclear vesicles displayed positivity for histone interacting protein HP1 α and localize in proximity with Lamin A and B1 accumulation, while in newborn mice and adult human skin we report LC3 puncta coincident with misshaped nuclei within the granular layer. This process relies on autophagy integrity as confirmed by lack of nucleophagy in differentiating keratinocytes depleted from WIPI1 or ULK1. Final validation into a skin disease model showed that impaired autophagy contributes to the pathogenesis of psoriasis. Lack of LC3 expression in psoriatic skin lesions correlates with parakeratosis and deregulated expression or location of most of the autophagic markers. Our findings may have implications and improve treatment options for patients with epidermal barrier defects.

INTRODUCTION

The epidermis is a multilayered structure continuously renewed by keratinocytes of the basal layer which divide and differentiate to form cells of the spinous, granular and cornified layers. The proliferating basal layer is a heterogeneous population comprising of epidermal stem cells and transit-amplifying cells which have limited self-renewal capacity and undergo differentiation after a few cycles (Watt, 1998). In the granular layer, keratinocytes begin to lose their organelles, express structural proteins characteristic for epidermal terminal differentiation, leading to the flattening, collapse and the eventual death of the cells. These flattened cells, corneocytes, which form the cornified layer, are rich in proteins and are embedded in a lipid matrix, giving the epidermis its water-retaining, chemical and mechanical properties, ensuring effective epidermal barrier function (Blank, 1953; Candi et al., 2005; Proksch et al., 2008).

Autophagy is generally used to describe cellular processes leading to the degradation of cytoplasmic components within lysosomes (Levine and Klionsky, 2004; Shintani and Klionsky, 2004; Mizushima, 2007; Klionsky et al., 2016). Macroautophagy (commonly referred to as autophagy) is a conserved catabolic process characterized by formation of intracellular double-membrane structures which degrade and recycle cytosolic proteins and organelles (Arstila and Trump, 1968; Mizushima, 2007). Specialized forms of autophagy are directed at specific organelles such as mitophagy (mitochondria), nucleophagy (nuclei) (Klionsky et al., 2007; Cecconi and Levine, 2008; Levine and Kroemer, 2008; Park et al., 2009; Mizushima and Levine, 2010; McGee et al., 2011) and more recently 'ER-phagy' (endoplasmic reticulum) (Mochida et al., 2015; Khaminets et al., 2015). Autophagy in keratinocytes so far has been described as a mechanism of senescent cell death (Deruy et al., 2010; Gosselin et al., 2009), stress response leading to expression of early markers of differentiation and eventual cell death (Aymard et al., 2011), as well as a pro-survival mechanism which protects from UV-induced damage (Yang et al., 2012; Zhao et al., 2013; Qiang et al., 2013). However most published work has been performed in monolayer keratinocyte culture which may not fully represents the stratified *in vivo* situation. A key regulator of epidermal development and differentiation is the AKT/mTOR pathway. Downstream of AKT is mTORC1 which regulates anabolic and catabolic processes including autophagy. Hyperactivation of mTORC1 signalling has been associated with a defective epidermal barrier in psoriasis (Kjellerup et al., 2009; Buerger et al., 2013) and vitamin D analogues used to treat epidermal barrier defect diseases such as psoriasis induce autophagy in cultured cells (Wang and Levine, 2011). Here we investigate the role of epidermal autophagy and its link epidermal terminal differentiation.

RESULTS

Autophagy is involved in epidermal granular layer formation.

During foetal development, expression studies are possible because of the temporal separation of epidermal terminal differentiation and skin barrier formation. We examined expression patterns of ULK1, BECN1, WIPI1, ATG5-ATG12 complex and LC3 as key markers of sequential stages of autophagy in the mouse embryo (Fig. 1A). Epidermal barrier formation correlates with development of the cornified layer which occurs between E15.5 and E18.5 in mice 43. In E16.5 mouse foetal skin, filaggrin expression confirmed activation of terminal differentiation and granular layer formation. Filaggrin is also constitutively expressed in the granular layer of adult human skin (Fig. 1B and S1B), and is further increased in foetal granular layers at E17.5 and E18.5, when AKT1 is also expressed, indicating the presence of an intact granular layer. At E15.5, before granular layer development, the epidermis consists of proliferating basal and spinous layers (Fig S1A). At this time point, LC3 as well as other autophagy proteins are present at low levels (Fig.1A). Initiation of granular layer formation at E16.5 is accompanied by upregulation of LC3 in the uppermost layers which correspond to the early granular layers. Levels of ULK1, WIPI1 and granular ATG5-ATG12 are also further increased at E16.5, accompanied by switching-on of BECN1 expression in the basal and upper epidermal layers. This autophagy marker expression pattern is maintained until birth and persists in adult mouse and human skin (Fig. 1A). In summary, expression of autophagy markers is highly upregulated at E16.5 indicating that autophagy occurs during epidermal development and differentiation, and may contribute to granular layer formation.

Detection of epidermal autophagic vesicles in newborn mouse epidermis

We then aimed to establish the presence and localisation of autophagic vesicles in normal epidermis. For this purpose we used three-day-old mouse epidermis since this time point allows a short recovery period after birth to exclude artefacts due to neonatal starvation-induced autophagy (Kuma et al., 2004). We first characterized by immunofluorescence the expression of the main autophagic markers which reveal the same patterns described during mouse epidermal development as well as adult mouse and human skin (Fig.2A). By using transmission electron microscopy (TEM) we investigated the ultrastructure of the epidermal granular layer (Fig. 2B) and we identified autophagic vesicles in both transitional cells and the cornified envelope (Fig. 2C-D). Autophagic vesicles within the epidermal layers varied in shape (from rounded to oval-shaped) with cross-sections within the range of 300-500 nm. These data confirmed previous reports describing epidermal autophagosomes in cultured keratinocytes, and also reveal that the autophagy process is occurring continuously, even in newborn mouse skin, and not only during epidermal development.

mTORC1 regulation of epidermal autophagy.

mTORC1 is a key regulator of autophagy in yeast and mammals (Blommaert et al., 1995; Noda and Ohsumi, 1998; Ravikumar et al., 2004). Under nutrient-rich conditions, mTORC1 activates anabolic processes such as protein synthesis and inhibits catabolic processes such as autophagy. In order to establish whether epidermal autophagy is also regulated by mTORC1 signalling, the effects of mTOR inhibitors rapamycin and torin1 were analysed using a well-established assay for *in vitro* skin differentiation (O'Shaughnessy et al., 2009). Mouse skin explants were harvested from E15.5 fetuses when the granular layer and *stratum corneum* are still absent (Fig. 1A and S1A). After 72h of drug treatment (corresponding to E18.5, the time point just before birth), inhibition of mTORC1 does not have a striking effect on filaggrin expression patterns or on epidermal granular layer formation (Fig 3A and S1C). S6 phosphorylation (S240/244), a down-stream target of mTORC1, is reduced in drug-treated explant epidermis (Fig. 3A) confirming effective mTORC1 inhibition. The autophagosome marker LC3 is mainly expressed in the granular layer of adult epidermis (Fig. 1A) as well as in vehicle-treated explants. mTORC1 inhibition strongly increases epidermal LC3 levels (Fig. 3A) and induces conversion of LC3I to LC3II (Fig. 3B), a measure of autophagic activity. The expression of WIPI1, present as puncta in the upper layers of vehicle-treated foetal explants, is upregulated (Fig. 3A) as is ULK1 expression. Basal-lower spinous layer expression levels of ATG5-ATG12 and BECN1 were also increased (Fig. S1D). Therefore, mTORC1 inhibition increases expression of essential autophagy proteins and up-regulates LC3 processing in the granular layer, consistent with induction of autophagy in the upper epidermis. Treatment in the last 4 hours of mTORC1 inhibition with Bafilomycin A1 (BafA1) - which prevents fusion of lysosomes with autophagosomes (Bowman et al., 1988; Klionsky et al., 2008) - confirmed the integrity of the autophagic flux in the granular layers of these mouse skin explants (Fig. 3C).

To further explore whether mTORC1 inhibition effectively up-regulates autophagy in human epidermis, primary monolayer keratinocyte cultures were treated with rapamycin in the presence and absence of BafA1. Treatment with BafA1 leads to LC3II accumulation mainly in rapamycin-treated cells (Fig. 3D and E), confirming inhibition of autophagic vesicle degradation. Increased LC3 expression upon Rapamycin combined with BafA1 (Fig. 3D), as well as up-regulation of LC3 turnover (Fig. 3E) was mainly evident in differentiated keratinocytes. Rapamycin induces a significantly higher turnover of LC3I to LC3II in differentiated compared to undifferentiated keratinocytes. Rapamycin also strongly down-regulates S6 phosphorylation confirming effective mTORC1 inhibition. These data are consistent with our observations in mouse foetal explant cultures as well as in organotypic models using rat epidermal keratinocytes (REK) (Fig. 3F and S2A) and reveal the integrity of the "epidermal" autophagic flux, but also how sensitivity of autophagy in keratinocytes could be influenced by differentiation status.

Epidermal nucleophagy during keratinocyte terminal differentiation

Having established that keratinocytes from differentiated granular layers are capable of higher levels of autophagy compared to basal proliferating cells, the role of autophagy during epidermal terminal differentiation was further investigated. Immunofluorescence analysis of autophagy proteins showed perinuclear localization of LC3, p62 und ULK1 in differentiated keratinocytes (Fig. S2B). Detailed examination of cell morphology revealed irregular or misshaped nuclei in a significant proportion of differentiated keratinocytes whereas these were rare in undifferentiated cells (Fig. 4A). Regions of degraded nuclear material in differentiated keratinocytes are replaced by LC3 aggregates which are also positive for LAMP2, a lysosomal membrane protein (Fig. 4B and Fig. S3A-B) which labels lysosomes and autolysosomes just before autophagic degradation. DAPI staining is reduced in intensity but is present in these LAMP2/LC3 aggregates, suggestive of active degradation of DNA in these bodies (Fig S3B). The regions of missing nuclear material may therefore be sites of high autophagic activity, a process defined as nucleophagy. p62 acts as an adaptor, recognizing and binding both ubiquitinated autophagic cargo and LC3 within autophagosomal membranes, and is commonly used as a read-out for targeted autophagic degradation of ubiquitinated cargo (Pankiv et al., 2007, Lamark et al., 2009, Johansen and Lamark, 2011, Klionsky et al., 2012). In keratinocyte cultures, nucleophagic regions in the differentiated population are p62 positive (Fig.4C). However, most LC3 vesicles in undifferentiated keratinocytes are negative for p62 indicating that basal autophagy in proliferating keratinocytes is not targeted. These data indicate that nucleophagy is a mechanism of targeted autophagic degradation of cargo by which terminally differentiating keratinocytes might degrade their nuclei.

To confirm whether these autophagic vesicles contain nuclear material, nucleophagic keratinocytes were analysed for expression of a histone interacting protein, Heterochromatin Protein 1 α (HP1 α), a marker of genetically inactive, tightly packed DNA found at the periphery of the nucleus (Maison and Almouzni, 2004). HP1 α is mainly expressed in nuclei of undifferentiated keratinocytes (Fig.4D). However, in differentiated keratinocytes, HP1 α is present in both the nucleus and cytoplasm where it forms perinuclear aggregates in regions of missing nuclear material and overlaps with LAMP2. This observation suggests that nucleophagic vesicles contain nuclear material.

To determine whether nucleophagic vesicles are in contact or coincident with the nucleus, the expression pattern of the nuclear membrane proteins lamin A (LMNA) and lamin B1 (LMNB1) was examined. Lamins are inner nuclear membrane proteins required for the maintenance of nuclear integrity (Hutchison, 2002; Broers et al., 2006). In both undifferentiated and differentiated keratinocytes, LMNA and LMNB1 are expressed at the nuclear membrane. Differentiating keratinocytes display an accumulation of LMNA and LMNB1 at the border between the existing nucleus and the autophagic regions (Fig. 4E-F). This shows

that LAMP2 positive autophagic vesicles at nucleophagic sites are cytosolic (4F) and also confirmed the involvement of Lamins in epidermal autophagic nuclear degradation (Dou Z et al., 2015).

However in differentiated keratinocytes, acetylated histone H3 (Lys14) is only expressed within the intact nuclei and absent in nucleophagic regions (Fig. S3C). Thus, nucleophagy in keratinocytes is a targeted process whereby nuclear material within the nucleophagic regions differs from that within the intact nucleus where gene transcription may still occur. Nucleophagy is not a DNA damage response, as shown by lack of phosphorylated H2AX within nucleophagic regions (Fig. S3D). Moreover no caspase-3 cleavage (Fig. S4A) nor TUNEL staining (unshown data) were detected within nucleophagic regions confirming no apoptotic response is triggered during nucleophagy.

In order to establish whether autophagy is essential for degradation of nuclei during keratinocyte differentiation, a knockdown of key autophagy proteins WIPI1 and ULK1 was performed. WIPI1 interacts with autophagic structures under conditions of starvation or mTORC1 inhibition and physiologically colocalises with LC3 positive puncta; its expression is associated with terminal differentiation, both in culture and in the epidermis (Fig. S4B). WIPI1 knockdown significantly reduced nucleophagic cells in differentiating keratinocytes (~2%) compared to controls (~15%) whereas undifferentiated keratinocytes were unaffected (Fig.4G). Likewise, knockdown of, ULK1, another protein essential for autophagy, also reduced the number of nucleophagic differentiated keratinocytes, although to a lesser extent than with WIPI1 silencing (Fig. S4C), reinforcing the importance of the integrity of the autophagy pathway for nucleophagy in differentiating keratinocytes. Finally, we analyzed newborn mouse and adult human skin in order to validate the above observation in keratinocytes and confirm whether the nucleophagy process also occurs in epidermal models. By performing confocal microscopy analysis at higher magnification of LC3 expression within the granular layer of mice newborn skin we clearly observed that some of the LC3 puncta in the granular layer were coincident with areas of loss of nuclear material, strongly suggesting an active nucleophagy process ongoing within the granular layer (Fig. 5A). Likewise, in upper layers of adult human skin, LC3 puncta were localized to the same regions of misshapen nuclei (Fig. 5B). By further analysing the granular layer ultrastructure of newborn mice skin with electron microscopy we also identified double-membrane vesicles containing heterochromatin material corresponding to perinuclear regions where nuclear material was lost (Fig. 5C-D). These data provide evidence of an epidermal autophagy process targeting nuclear integrity within the terminally differentiated layers of the skin.

Deregulation of granular layer autophagy in psoriatic skin lesions.

Psoriasis is a skin barrier defect characterized by epidermal hyperplasia, abnormal terminal differentiation of keratinocytes, infiltration of immune cells into the dermis and epidermis and increased vascularisation

(Raychaudhuri, 2012). Lesional psoriatic skin has a hyperproliferative, thickened epidermis, elongated rete ridges and a thicker cornified layer with retained nuclei or parakeratosis (Fig. 5A-B) (Wolberink et al., 2011; Donetti et al., 2012; Kim et al., 2011), suggesting incomplete terminal differentiation. However, uninvolved skin in psoriasis has a clearly defined granular layer and corneocytes similar to healthy epidermis. To further investigate our hypothesis that autophagy is a mechanism for organelle degradation during terminal differentiation in epidermis, the expression pattern of several autophagy markers were analysed in skin from 6 patients with psoriasis and compared with several samples from healthy skin. In healthy adult epidermis, LC3 is expressed in all layers of the epidermis with the strongest expression in the granular layer (Fig. 1A; Fig. 5B). In psoriatic lesions, LC3 is completely absent from all layers of the epidermis (Fig. 5B). In non-lesional psoriatic epidermis where cornification occurs, LC3 is present in the granular layer, but its expression is reduced. Therefore in non-lesional psoriatic skin, where keratinocyte terminal differentiation and cornification are intact, granular layer LC3 is present but at lower levels compared to healthy epidermis. In psoriatic lesions where terminal differentiation is impaired leading to nuclear retention and improper cornification, LC3 is absent. WIPI1 is only expressed in the upper layers where cornification can occur, as in non-lesional psoriatic skin and in healthy skin. Its expression is reduced (like LC3), in parakeratotic regions of psoriatic epidermis. ULK1 is expressed in all layers of healthy epidermis with highest expression in the upper layers (Fig. 5B). Its expression significantly increases in supra-basal and granular layers of non-lesional psoriatic epidermis, whereas in lesional psoriatic epidermis, ULK1 is highly expressed everywhere except in the uppermost parakeratotic layers. Likewise BECN1, mainly expressed in the proliferating basal layer of healthy epidermis, shifts to basal and para-basal layers of non-lesional psoriatic epidermis, and supra-basal layers of lesional psoriatic skin (Fig. 5B). ATG5-ATG12 is similar to BECN1 with a strong basal layer expression in healthy epidermis and no changes in non-lesional psoriatic skin, whereas a shift into supra-basal layers is observed in lesional psoriatic epidermis. This data shows deregulation of autophagy markers in both lesional and non-lesional psoriatic epidermis, providing a possible reason for the defective epidermal barrier observed in this disease.

DISCUSSION

In this report we demonstrate that autophagy is an active process playing a critical role in normal epidermal development and differentiation and that impaired autophagy may contribute to the pathogenesis of human disorders of epidermal differentiation such as psoriasis.

An increasing number of publications has shown that keratinocytes are capable of autophagy (Gosselin et al., 2009; Aymard et al., 2011; Deruy et al., 2010; Lee et al., 2011; Chatterjea et al., 2011; Wang and Levine, 2011; Misovic et al., 2013). Most of these reports have used monolayer keratinocyte culture as a

research model, although this does not accurately represent late terminal differentiation *in vivo*. To date, an accurate profile of autophagy marker expression in healthy epidermis has not been established. The epidermal autophagy marker expression pattern suggests that autophagy plays an important role in the terminal differentiation process of the granular layer. We have recently identified a mechanism modulating the autophagy process in keratinocytes mediated by iASPP (Chikh et al., 2014), whose expression is also important for the maintenance of epidermal homeostasis (Chikh et al., 2011). Decreased iASPP expression in keratinocytes not only triggers autophagy by derepression of the ATG5/ATG12–ATG16L1 complex, promoting autophagosome maturation, but also induces terminal differentiation. Likewise, another elegant study has recently highlighted the importance of BNIP3 in inducing autophagy but also terminal differentiation of epidermal keratinocytes (Moriyama et al., 2014).

Here we have shown that induction of autophagy marker expression during foetal development coincides with the initiation of epidermal terminal differentiation. This suggests that autophagy is constitutively active in granular layer keratinocytes and may be important for granular layer formation. TEM analysis of 3-day-old mouse epidermis shows that double-membrane vesicles containing engulfed material are present in basal and granular layers. Another report has recently shown autophagosome-like structures in granular layer of mouse skin (Soma-Nagae et al., 2013). Autophagy is regulated by different cell signaling pathways of which mTORC1 is a central player. In epidermis, the AKT/mTORC1 pathway is a key regulator of epidermal development and differentiation (Calautti et al., 2005; Thrash et al., 2006; O'Shaughnessy et al., 2007; Naeem et al., 2015) and we have previously shown that mTORC1 inhibition upregulates activity of epidermal AKT1, the AKT isoform associated with terminal differentiation (Sully et al., 2013). We were therefore interested to determine whether epidermal autophagy is also regulated by mTORC1. We observed that mTORC1 inhibition in epidermal explant cultures significantly increased the expression levels of most of the autophagy markers. These data support observations made in monolayer cultures where mTORC1 inhibition induces a striking increase in LC3 turn-over in differentiated keratinocytes. Moreover, increased expression of lipidated LC3 was also detected upon block of the autophagic flux, confirming integrity of the autophagy process in epidermal keratinocytes. Our findings strongly suggest that epidermal mTORC1 not only mediates AKT1 activity but also regulates constitutive granular layer autophagy. Interestingly, not all the autophagic markers localized within the granular epidermal layers. It is currently unclear whether inter-dependency exists between basal (BECN1, ATG5-ATG12) epidermal layer autophagy markers and upper layer epidermal markers, or whether different streams of epidermal autophagy occur within the skin. Future experiments would elucidate the role of BECN1 and ATG5-ATG12 in the epidermal basal layer and may explore the function of basal layer epidermal autophagy.

Analysis of autophagy in monolayer cultures revealed that differentiating keratinocytes undergo nucleophagy - autophagic degradation of the nucleus. This differs from micro-nucleophagy in which satellite nuclei are formed due to stress or genome instability and then engulfed by autophagosomes (Krick et al., 2008; Rello-Varona et al., 2012). Recently a novel mechanism of nucleophagy mediated by Atg39, a nuclear envelope receptors which induces autophagic sequestration of part of the nucleus, has been identified in *Saccharomyces cerevisiae* (Mochida et al., 2015). Although we cannot exclude at this stage whether Atg39 could also be involved in our proposed mechanism, in differentiating keratinocytes nucleophagy is characterized by accumulation of LC3/LAMP2/p62 positive autolysosomes also containing HP1 α positive cargo in the regions of missing nuclear material, suggesting selective autophagic degradation is occurring. Lack of DAPI detection within the nucleophagic regions might be due to chromatin conformational changes or partial degradation of the DNA leading to reduced DAPI incorporation into DNA. Our data suggest that nuclear material within the autophagosome may be damaged or modified and bound to HP1 α , which is targeted for autophagic degradation. However, the material within the nucleophagic vesicles is not positive for the marker of DNA double-strand breaks, γ -H2AX, nor for cleaved Caspase-3, suggesting nucleophagy degradation does not trigger an apoptotic response. A similar type of nucleophagy has recently been described in the aging intestinal epithelia of *C. elegans*, probably due to aging-related changes in LMNA, and compromised nuclear integrity (McGee et al., 2011). In our monolayer keratinocyte cultures nucleophagic vesicles are outside the partially degraded nucleus. Another model has just been reported in human primary fibroblasts also based on nuclear LC3 directly interacting with LMNB1. Lamin B1 lysosomal degradation would rely on LC3-LMNB1 interaction as a general mechanism to protect the cells from oncogene-induced senescence and tumorigenesis (Dou Z et al., 2015). Lamin B1 is phosphorylated by AKT1 in the granular layer of the epidermis targeting it for degradation (Naeem et al., 2015). These two models of nucleophagy resemble our data in differentiating keratinocytes where we observe A and B1 Lamins accumulation exactly corresponding to regions of nuclear degradation. The rarity of nucleophagic events *in vitro* and *in vivo* suggests a rapid process which is dependent on the differentiating stage of keratinocytes. Finally we reinforced the importance of the integrity of the epidermal autophagy confirmed by lack of nucleophagy in differentiating keratinocytes depleted from key autophagy proteins WIPI1 or ULK1.

A report recently showed that epidermal ATG7 knock-out mice have no skin phenotype suggesting that autophagy in the epidermis does not require ATG7 (Rossiter et al., 2013). Our observations of strong basal layer expression of BECN1 and ATG5-ATG12 suggest that these proteins may also play other roles in epidermis. It is likely that the precise mechanism of autophagy in epidermis may differ depending on the stage of keratinocyte differentiation and may also vary between mice and humans. It is noteworthy

mentioning that although no cornification defects were revealed by ultrastructural analysis, an increase in corneocyte thickness was identified in the back skin where Atg7 was specifically inactivated (Rossiter et al., 2013). Another group has recently grafted Atg7-deficient skin onto severe combined immunodeficient mice and observed acanthosis, hyperkeratosis, abnormal hair growth and an overall retardation of granular layer differentiation in the autophagy-deficient grafts (Yoshihara et al., 2015). Further investigation will determine and validate alternative Atg5/Atg7-independent autophagy pathways (Nishida et al., 2009) in human epidermis.

Psoriasis was used as a human disease model to validate our hypothesis and observations. Psoriasis is characterized by keratinocyte hyperproliferation, deregulated terminal differentiation and parakeratosis, inflammation and abnormal vascularisation, resulting in the defective epidermal barrier present in psoriatic plaques (Griffiths and Barker, 2007; Raychaudhuri, 2013). We show that LC3 puncta were still present in the granular layer of non-lesional psoriatic epidermis but at a much lower level compared to healthy adult epidermis, whereas in lesional psoriatic skin, LC3 was not expressed at all. Likewise, no expression of ULK1 or WIP1 was detected in the parakeratotic regions of psoriatic skin, whereas a shift of BECN1 and ATG5-ATG12 expression was observed in psoriatic lesions. Therefore, the overall autophagy expression marker profile does only apply to an epidermal model capable of complete terminal differentiation. Moreover, hyperactivation of mTORC1 has been shown in lesional psoriatic skin (Kjellerup et al., 2009; Buerger et al., 2013). High mTORC1 signalling would inhibit autophagy and may explain why LC3 aggregates formation is absent in psoriatic lesions. Previous reports have shown that activation of Toll-Like-Receptor (TLR) signalling in keratinocytes causes hyperproliferation and inflammation and induces p62 expression which further increases TLR signalling. However, in healthy keratinocytes, autophagy keeps epidermal p62 levels in check thereby preventing epidermal inflammation (Lee et al., 2011; Miller, 2008). The absent or reduced LC3, ULK1, WIP1 expression levels in psoriatic skin suggest that autophagy may be blocked or impaired, explaining the high p62 levels and contributing to excessive inflammation (Lee et al., 2011). These data suggest that patients with epidermal barrier defect diseases such as psoriasis may benefit from treatment with mTORC1 inhibitors. Such an approach could restore constitutive epidermal granular layer autophagy which may lead to normalisation of terminal differentiation and barrier formation, and possibly reduce inflammation and hyperproliferation.

In conclusion, our data reveal the extent and location of autophagy in epidermal development and differentiation and highlight its critical importance in ensuring normal epidermal function and its potential role in disease of epidermal barrier function.

MATERIALS AND METHODS

Cultures of primary keratinocytes and skin explants. Human neonatal primary keratinocytes purchased from Invitrogen were expanded in Medium 154 (Invitrogen) with 0.2 mM Calcium chloride on collagen-coated plates (rat-tail collagen, BD Biosciences). More details of this technique are included in the *Suppl. Materials and Methods*

Immunohistochemistry. Tissue specimens were fixed in Bouin's solution and embedded in paraffin. More details of this technique and a list of the antibodies used are included in the *Suppl. Materials and Methods*.

Transmission Electron Microscopy. The processing, embedding, cutting and imaging of the TEM sections were performed by the Pathology Core Facility of Queen Mary University of London. 3 day old dorsal mouse skin from CD1 mice was harvested and cut into strips of 1 mm width. The strips were fixed in 4% Glutaraldehyde in 0.1 M phosphate buffer (3.1 g Sodium phosphate monobasic monohydrate, 10.9 g Sodium phosphate dibasic (pH 7.4) per L) and embedded in LR White.

More Experimental procedures are described in *Suppl. Materials and Methods*.

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FIGURE LEGENDS

Figure 1: *Induction of epidermal terminal differentiation during foetal development is accompanied by activation of autophagy marker expression.* A) Expression of AKT1, epidermal terminal differentiation marker, filaggrin and autophagy proteins LC3, ULK1, WIPI1, BECN1 and ATG5-ATG12 in mouse foetal skin development. Epidermal expression levels of LC3, ULK1 and WIPI1 were quantified in n= 3 foetal mouse samples from E15.5 to E18.5. ANOVA for average intensities from n=3 samples of epidermal LC3 ($p<0.00005$), ULK1 ($p<0.0005$), WIPI1 ($p<0.005$) respectively. B). Expression of epidermal terminal differentiation and autophagy markers in both adult mouse and adult human epidermis. Bar = $20\mu\text{m}$. Dotted line = basement membrane.

Figure 2: *Epidermal autophagic vesicles in epidermal layers of newborn mice skin.* A) Immunofluorescent analysis of d3 mouse back skin for the key autophagic markers LC3, ULK1, ATG5-ATG12 and BECN1. B) Transmission electron microscopy analysis (TEM) of the granular layer of the epidermis shows degrading nuclei (N). The cornified layer in this image is to the right. C) Double membrane-autophagic vesicles are present in the granular layer keratinocytes. D) Autophagic vesicles are present in the cornified layer of the epidermis. E) Another image of a double membrane putative autophagic vesicle in the basal layer of the epidermis. Dotted line (A) indicates the dermal-epidermal boundary. Bars = $50\mu\text{m}$ (A), $2\mu\text{m}$ (B), 500nm (C), 200nm (D and E).

Figure 3: *mTOR inhibition up-regulates constitutive autophagy in terminally differentiating keratinocytes.* A) Expression of epidermal S6 phosphorylation, terminal differentiation and autophagy markers in mouse foetal explants isolated at E15.5 and treated with vehicle, $2.5\mu\text{M}$ Torin1 or $5\mu\text{M}$ rapamycin for 72h. * = Two tailed paired t-test ($P<0.05$) quantified for 4 different fields of view. B) Western blots analysis of the above (A) treated explant cultures. C) Western blots analysis showing autophagic flux of Torin1 ($2.5\mu\text{M}$ for 72h) treated epidermal explant w/o 100 nM BafA1 (in the last 4h). D) Immunofluorescence LC3 staining of

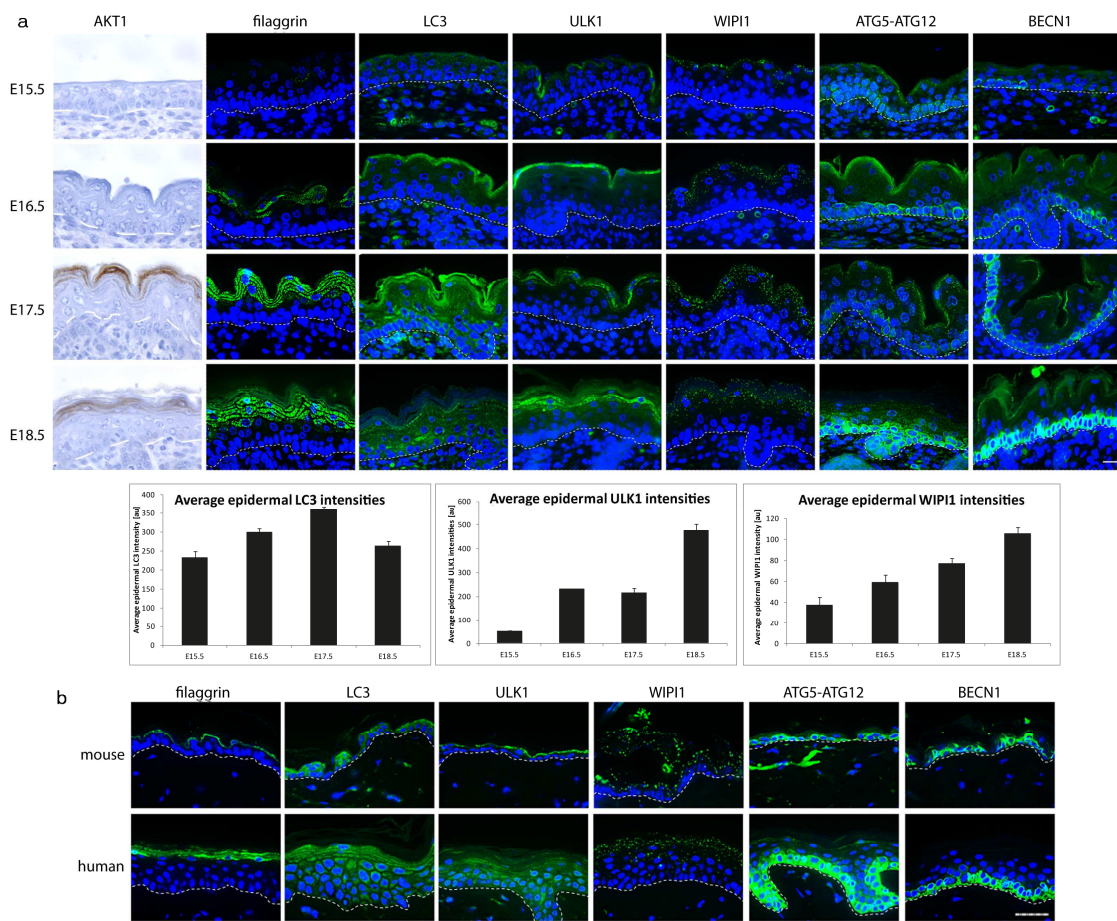
differentiated primary keratinocytes treated with 10nM rapamycin w/o 100 nM BafA1. E) Measurement of autophagic flux by Western blots analysis of rapamycin treated human primary keratinocytes w/o 100 nM BafA1. Actin was used as a loading control. *= Two tailed paired t-test ($P<0.05$) for LC3II/LC3I ratios in differentiated keratinocytes. F) Measurement of autophagic flux by Western blots analysis of 10nM rapamycin treated REK w/o 200 μ M Chloroquine. Bar = 20 μ m. Dotted line = basement membrane.

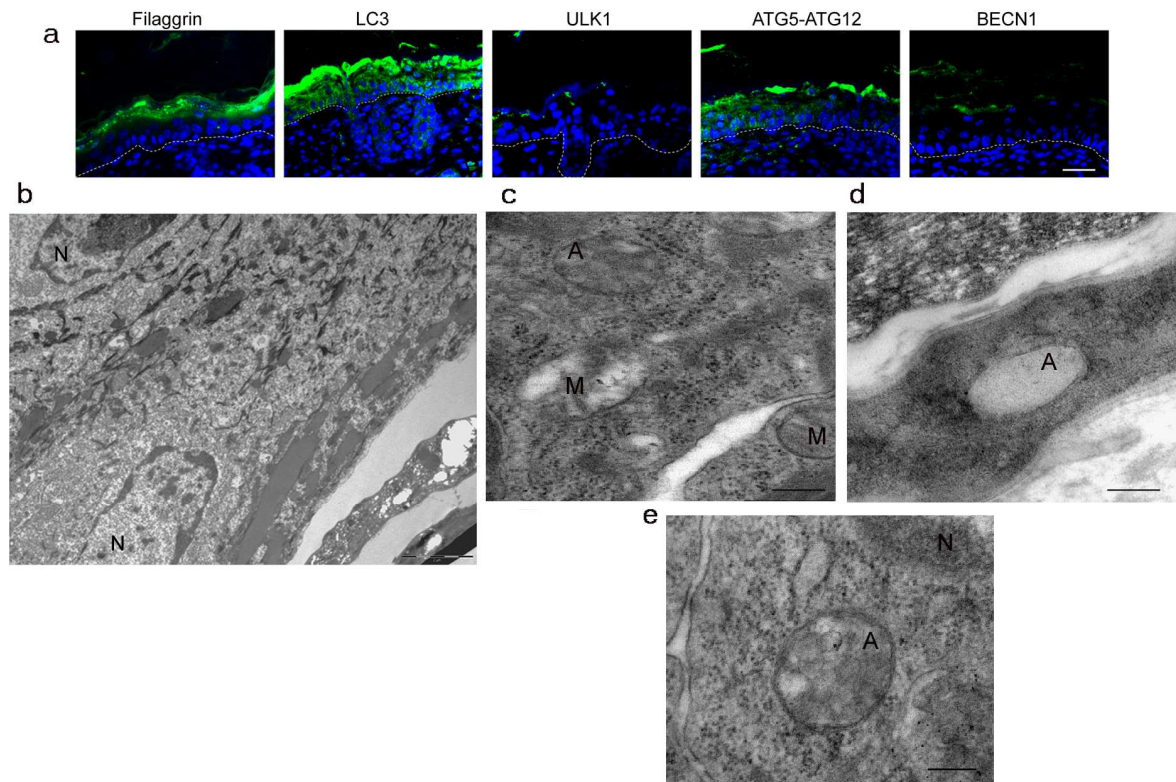
Figure 4: *Terminal differentiation in monolayer keratinocyte cultures is accompanied by targeted autophagic degradation of nuclear material – nucleophagy.* A) DAPI staining analysis reveals presence of misshaped nuclei (red arrows) within differentiated keratinocytes. Statistical significance ($*P<0.05$) was measured by Two tailed paired t-test. Immunofluorescence staining reveals B) co-expression of LC3/LAMP2; C) co-expression of LC3/p62 (D) co-expression HP1 α /LAMP2 specifically within regions of missing nuclear material of primary differentiated keratinocyte cultures. Red arrows indicate HP1 α aggregates. (E) LMNA labelling reveals that nuclear membrane is still present between nucleus and nucleophagic regions. F) Detailed investigation of LMNB1 and LAMP2 expression in calcium-switched human keratinocytes. *Pre*, indicates early events whereby a small amount of nuclear material is exposed. *Early*, indicates a time-point where co-expression of LAMP2, LMNB1 and DAPI is occurring. *Mid*, indicates a time-point where DAPI and LMNB1 expression is lost within LAMP2 positive bodies. *Late*, indicates misshaped nuclei in the absence of LAMP2 positive bodies (like in A). G) Western blots analysis of WIPI1 siRNA silencing in keratinocytes. *=Two tailed paired t-test ($P<0.05$). Bar = 10 μ m. Small red arrows = regions of nuclear deformity (A) and (E) or regions of peri-nuclear protein accumulation (D).

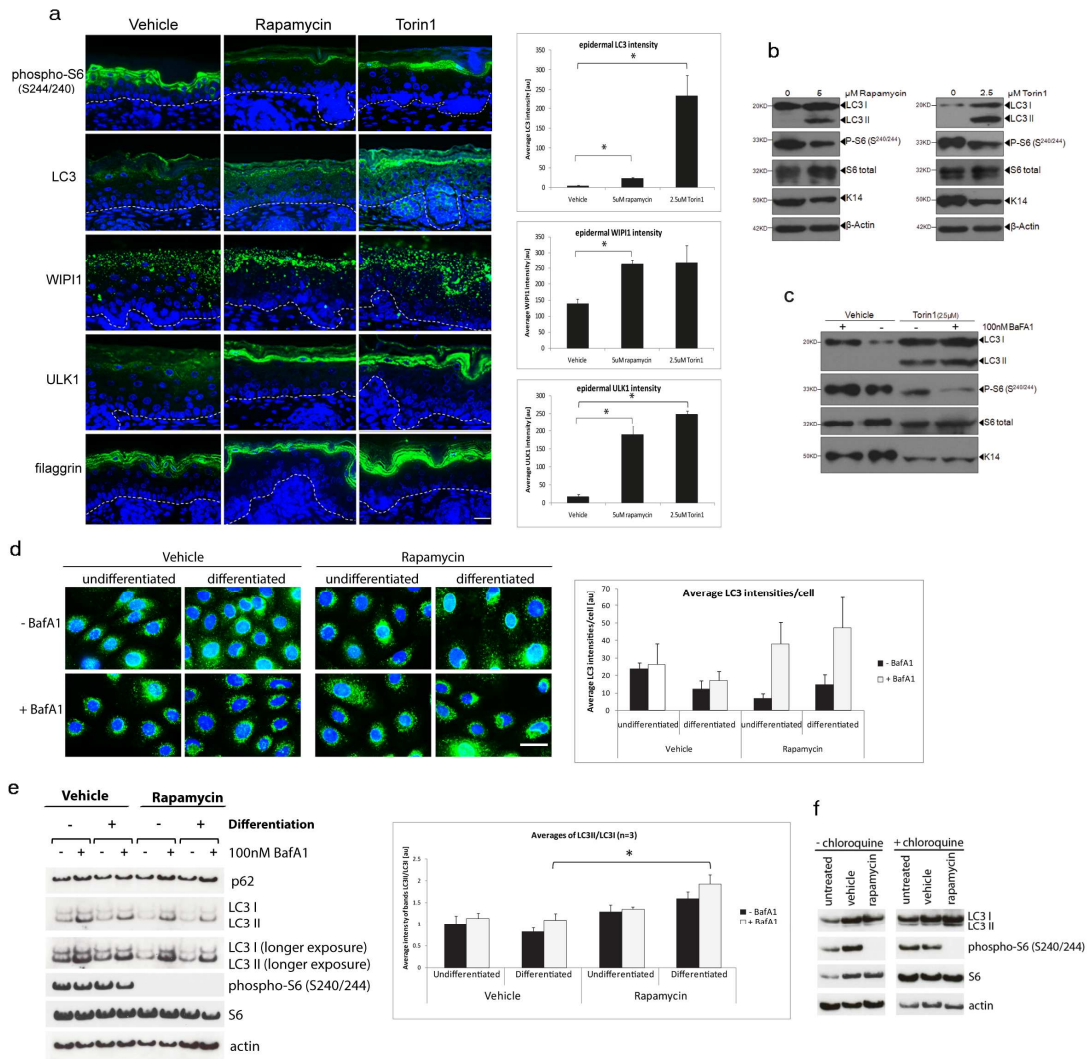
Figure 5: *Nucleophagy in terminal differentiated layers of mouse and human epidermis.* A) Confocal microscopy of LC3 expression in d3 postnatal back skin. Left panel: low magnification of multiple LC3 positive nuclei. Right panel: optical section demonstrates LC3 bodies through nuclear invaginations (magnification 0.5 micron). Bar graph shows percentage of nuclei co-localizing in the granular layer and the rest of the epidermis. B) LC3 accumulation within the granular layer of adult human trunk skin. Insets show 0.5 micron optical sections through one of the nuclei undergoing nucleophagy, with LC3 puncta in an invagination of the nucleus. Bar graph shows percentage of nuclei co-localizing in the granular layer and the rest of the epidermis. C) and D), TEM images of nuclei in the granular layer of d3 postnatal back skin. Arrowheads point to putative double membrane nucleophagic bodies associated with degrading nuclei. M, mitochondrion, N, Nucleus. **, $p<0.001$, Fishers exact test (n=81 mouse, n=419 human). Bars 50 μ m (A and B, low mag) 10 μ m (A and B, High mag). Dotted line (A) indicates the dermal-epidermal boundary.

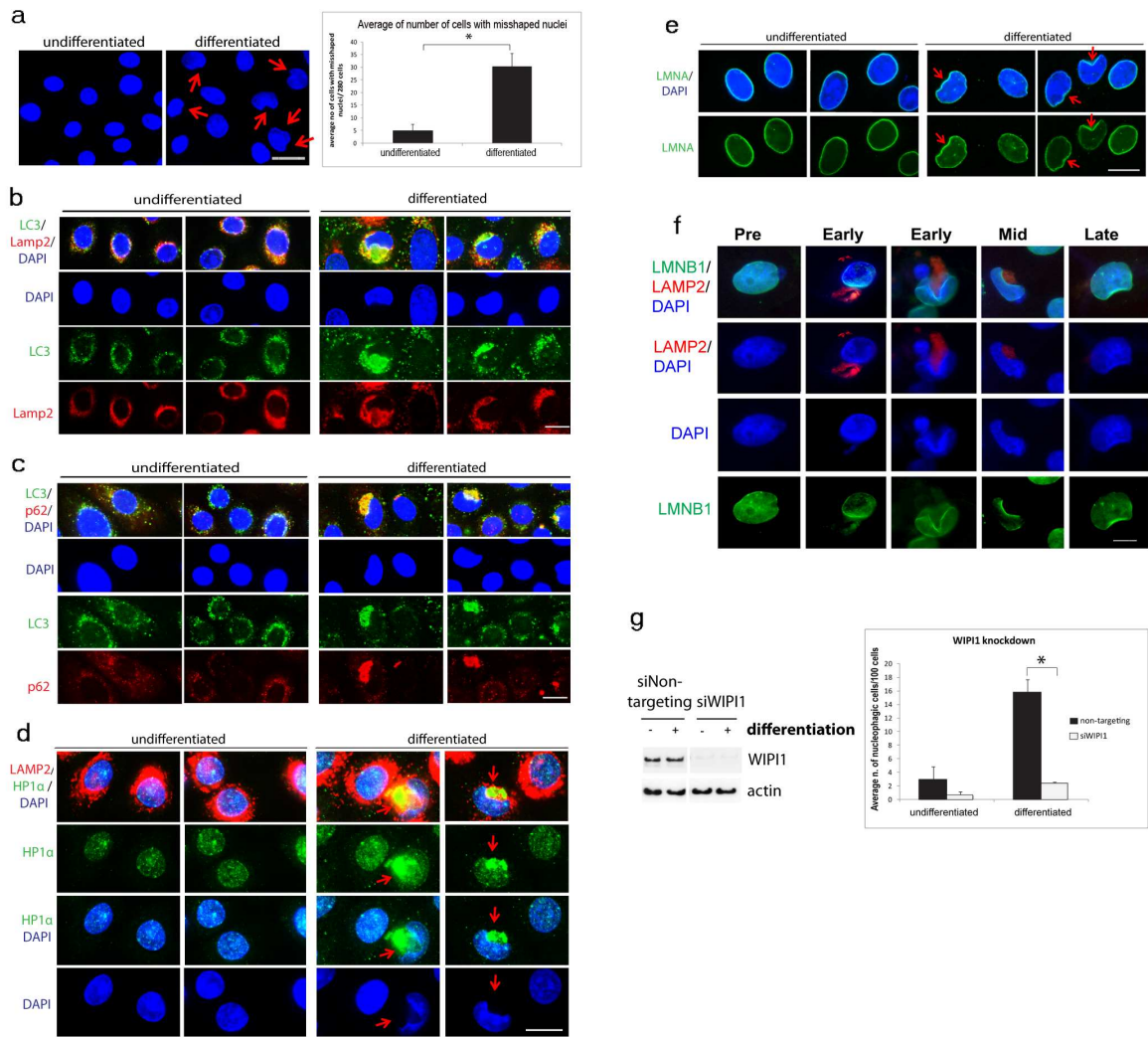
Figure 6: *Constitutive granular layer autophagy is deregulated in psoriasis, an epidermal barrier defect disease.* (A) H&E staining of psoriatic lesion, non-lesional psoriasis and healthy epidermis showing thicker uppermost layer and nuclei retention in psoriatic lesion (blue vertical line), whereas cornified layer of non-

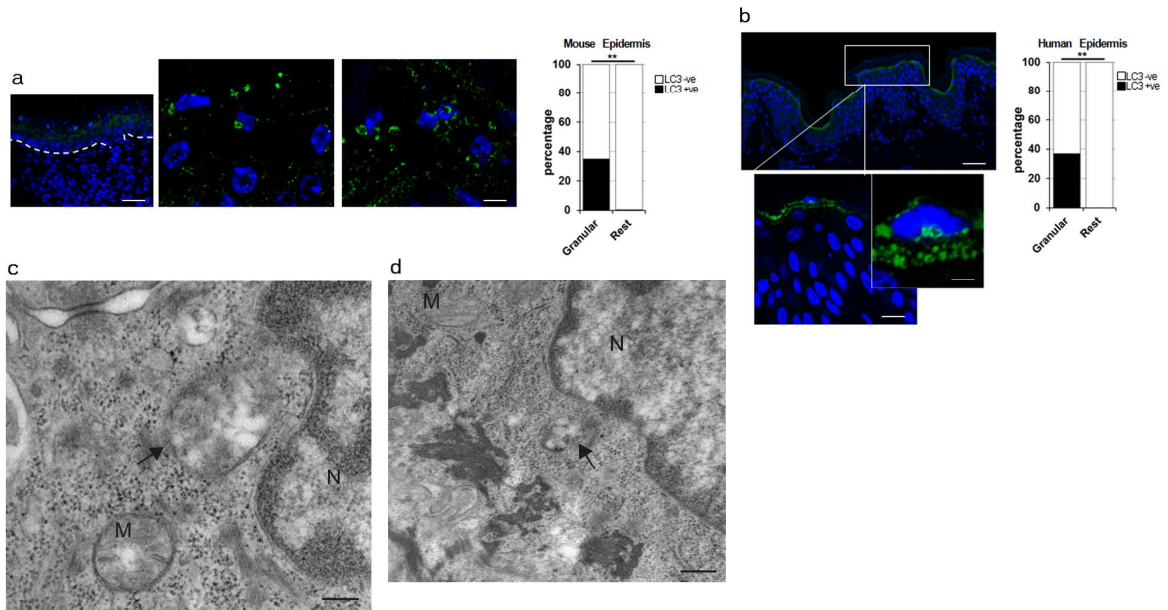
lesional psoriatic skin and healthy epidermis are completely free from nuclei (blue vertical line). In psoriatic lesions, there is no clear granular layer compared to non-lesional psoriatic skin and healthy epidermis (blue arrows). Black bar = 20 μm . (B) Immunofluorescence analysis of autophagy markers on lesional psoriatic skin compared to non-lesional psoriatic and healthy epidermis. Red vertical bars = cornified layers. Black bar = 20 μm . This figure is representative of n=6 samples of psoriatic lesions and n=5 samples of healthy epidermis.











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