Inactivation of autophagy leads to changes in sebaceous gland morphology and function

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Abstract
We have reported recently that inactivation of the essential autophagy-related gene 7 (Atg7) in keratinocytes has little or no impact on morphology and function of the epidermal barrier in experimental animals. When these mice aged, mutant males, (Atg7 ΔKC), developed an oily coat. As the keratin 14 promoter driven cre/LoxP system inactivates floxed Atg7 in all keratin 14 (K14) expressing cells, including sebocytes, we investigated whether the oily hair phenotype was the consequence of changes in function of the skin sebaceous glands. Using an antibody to the GFP-LC3 fusion protein, autophagosomes were detected at the border of sebocyte disintegration in control but not in mutant animals, suggesting that autophagy was (a) active in normal sebaceous glands and (b) was inactivated in the mutant mice. Detailed analysis established that dorsal sebaceous glands were about twice as large in all Atg7 ΔKC mice compared to those of controls (Atg7 F/F), and their rate of sebocyte proliferation was increased. In addition, male mutant mice yielded twice as much lipid per unit hair as age-matched controls. Analysis of sebum lipids by thin layer chromatography revealed a 40% reduction in the proportion of free fatty acids (FFA) and cholesterol, and a 5-fold increase in the proportion of fatty acid methyl esters (FAME). In addition, the most common diester wax species (58-60 carbon atoms) were increased, while shorter species (54-55 carbon atoms) were under-represented in mutant sebum. Our data show that autophagy contributes to sebaceous gland function and to the control of sebum composition.

KEYWORDS
autophagy, lipid, mouse, sebaceous glands, sebum

1 | INTRODUCTION

Autophagy is an intracellular degradation mechanism, by which macromolecules and organelles are broken down within double-membraned structures, termed autolysosomes and lysosomes. Three main forms of autophagy have been described (reviewed in Refs [1,2]) but here we focus on macro-autophagy, which is generally referred to as “autophagy.” Autophagy is a tightly regulated process involving at least

Abbreviations: Atg7, autophagy-related gene 7; C, cholesterol; CE, cholesterol ester; ELOVL, elongation of very long chain fatty acids; FAME, fatty acid methyl ester; FFA, free fatty acids; GED, glyceryl ether diester; GFP, green fluorescent protein; K14, keratin 14; KC, keratinocyte; LC3, microtubule-associated protein 1A/1B-light chain 3; PG, preputial gland; Plin2, perilipin 2; SG, skin sebaceous gland; TG, triglycerides; WE, wax ester.

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32 different autophagy-related genes (Atg). Molecules and organelles targeted for autophagy associate with specialised carrier molecules such as p62/sequestosome-1 (SQSTM1) which interacts with LC3 II, a lipilated protein situated on the developing autophagosome membrane. Together they are sequestered into autophagosomes, a process for which the autophagy-related gene, Atg7, is essential. The autophagosomes subsequently fuse with lysosomes, thereby exposing their contents to degradative enzymes. Autophagy was initially described as a nonspecific response mechanism to nutrient deprivation, but is now recognised to also specifically target damaged proteins and subcellular organelles including mitochondria (mitophagy), endoplasmic reticulum (ERphagy), peroxisomes (pexophagy) and lipid droplets (lipophagy) (reviewed in Refs 6–10).

Lipophagy plays a major role in many tissues and cell types. Thus for instance, in cultured hepatocytes and mouse liver, it contributes to lipid droplet mobilisation in response to lipid stimulus, and in its absence, hepatic lipids accumulate, resulting in hepatomegaly. In addition, autophagy-mediated lipolysis is involved in cholesterol ester release from lipid droplets in macrophage foam cells, and constitutive lipophagy has been demonstrated in cells not normally noted for lipid storage, such as neurons, fibroblasts and many others. In contrast, in adipose tissue, autophagy regulates the differentiation of adipocytes and enhances lipid deposition. In this case, tissue-specific inactivation of autophagy leads to an accumulation of brown at the expense of white adipose tissue, and a concomitant increase in β-oxidation and decrease in stored lipids.

Sebaceous glands (SG) are a lipid secreting, sac-like appendage of the hair follicle (HF), from which they develop during late embryogenesis. The glands consist of a layer of undifferentiated proliferative peripheral cells, contiguous with the lining of the hair canal. In the process of maturation, the sebocytes detach from the basement membrane, differentiate and accumulate lipids in their cytoplasm. The nucleus and subcellular organelles are subsequently degraded and the cytoplasmic content is released into the hair canal by holocrine secretion. Rather than regulating lipid stores in response to environmental stimuli, such as the tissues and cells mentioned above, the main function of SG is the synthesis and secretion of lipids, which are thought to provide insulation, lubrication of the hair shaft as it grows out of the hair canal, anti-microbial protection and pheromone production. In humans, SG enlargement, excessive sebum production and changes in sebump composition are associated with acne vulgaris, a prevalent inflammatory skin condition of young adults living in developed countries. Sebocytes are found not only in SG of HFs, but also in Meibomian glands near the eye, and in preputial glands (PG) surrounding the penis.

We have shown previously that suppression of autophagy causes premature degradation of nuclei during sebocyte differentiation and aberrant eosinophilic staining in histology of autophagy-deficient PG. During these investigations, we noticed that Atg7 deficient male mice display an oily and scruffy pelage. The objectives of this study were thus to determine whether autophagy affects a major function of SG, that is, the production of lipids.

## 2 MATERIALS AND METHODS

### 2.1 Mice

The mouse line with the Atg7 gene deleted specifically in keratinocytes (Atg7 ΔKC) as well as the Atg7 ΔKC GFP-LC3 reporter lines have been described previously (Appendix S1). Genomic DNA preparation and genotyping to identify K14/cre and LoxP bearing mice was carried out exactly as before, while primers used for the identification of the GFP-LC3 construct were: GFP1 (5′-TCTGCTGGAGTTGATGACC-3′) and LC3 (5′-GAATTCTCGACCCGCTTCATCTCGACG-3′) using the Red Hot DNA polymerase (Thermo Fisher Scientific, Vienna, Austria) with 35 cycles of 1 minute at 94°C, 1 minute at 56°C and 1 minute at 72°C, preceded and followed by 1 minute at 94°C and 10 minutes at 72°C, respectively. All SG analyses reported here were performed on 9- to 14-month-old mice, unless otherwise stated. To circumvent any possible effects of hair cycle stage on SG size, all samples were obtained from mice in the telogen (resting) phase of the hair cycle, as judged by the pink skin colour. As PG in mutant mice undergo atrophy from an age of 8 months onwards, tissue for histology and Western blotting were obtained from 4-5 months old mice.

### 2.2 Western blotting

Preputial glands were excised, cleaned of fat, transferred to tubes containing Precellys beads and SDS lysis buffer, and frozen immediately. The detailed method for Western blotting has been reported previously. Briefly, the samples were separated on 8%-18% gradient gels (Amersham Pharmacia Biotech, Uppsala, Sweden), transferred to nitrocellulose membranes and were developed with ECL substrate (both from BioRad, Hercules, CA, USA). LC3 and p62 bands were quantified using a BioRad ChemiDoc™ XRS+ Molecular Image® with Image Lab™ Software. Antibodies used for both Western blotting are described in the Appendix S1.

### 2.3 Histology

All immunological staining were performed on 7.5% formalin-fixed, paraffin-embedded 4-μm-thick sections, incubating with the antibodies at 4°C overnight, and after washing, for 30 minutes with secondary antibodies. Double staining with the lipid dye, BODIPY 493/503 (0.05 mmol/L), and Plin2 antibody was performed on whole mounts of ear and tail skin or on 6-μm-thick sections of frozen back skin, fixed in formalin (7.5%) for 20 minutes at room temperature and embedded in Optimal Cutting Temperature (O.C.T.) Compound (Scigen, Gardena, USA). For all fluorescent labelling, nuclei were counterstained with 2 μg/mL Hoechst-33258. Antibodies used for immunohistochemistry are listed in the Appendix S1.
Volume measurements were calculated from confocal microscope 3D stacks of BODIPY 493/503 stained whole mount preparations of epi-dermis, or of 100-μm-thick sections of back skin embedded in O.C.T. using equipment as described previously, and the subsequent application of an ImageJ Plugin (3D Manager). The longitudinal section areas of skin SG were determined from digital photographs using Metamorph software, while Ki67 positive cells, total peripheral nuclei and sebocytes were counted manually from photomicrographs. Ki67 positive cells were calculated both as a percentage of SG perimeter, and of total gland cell number (=peripheral cells+sebocytes).

2.5 | Preparation and analysis of lipids from mouse hair

Hair (80-100 mg of hair, or as much as was available from Atg7 ΔKC animals) was obtained by shaving the backs of old, individually held mice, and stored at −20°C to −80°C. Lipids were extracted as previously described, dissolved in chloroform at 10 mg hair equivalent/100 μL chloroform, and stored under argon at −20°C until analysis. To determine the weight of lipid per unit of hair, lipid extracts from known weights of hair were prepared as above, but dried down to about 200 μL under argon. The extracts were then transferred to flat-bottomed sodalime glass tubes (10 × 10 × 0.6 mm, Hilgenberg, Malsfeld, Germany) in a 48 well Costar plastic plate (Corning, USA), and dried down completely under argon. The lipid samples were weighed on a Sartorius Ultramicrobalance SE 2, which is accurate to 0.1 μg.

For initial characterisation of hair lipids (which are largely derived from sebum Ref. [28]), samples were separated by thin layer chromatography (TLC), using the method of Lin et al., which resolves nonpolar lipids only. See Appendix S1 for details.

2.6 | MALDI-MS analysis of mouse hair lipids

Based on previously established protocols, matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) was used to analyse wax esters (WE), the major lipid component of mouse sebum (see Appendix S1 for details). MALDI-MS data were processed by the instrument software version Launchpad 2.9.3 (Shimadzu, Manchester, UK) provided by the manufacturer and exported to Microsoft Excel 2007 for further analysis. Peak assignment was done by calculations based on WE structures and their fatty acid composition known to be present in rodents. Individual peaks were grouped into WE species with 0, 1, and 2 double bonds and plotted against their relative signal intensity from the mass spectra (Table S1).

2.7 | Statistical analysis

Student’s 2-tailed t test was applied to groups to be compared, except for testing the significance of differences between WE frequency at different total carbon atom lengths (Figure 4E and Figure S6), for which the ANOVA Holm-Sidak post-test for multiple comparisons was used. P values of <0.05 were taken as significant.

3 | RESULTS

3.1 | Lack of autophagy leads to oily pelage and enlarged sebaceous glands in aged Atg7 F/F ΔKC male mice

From an age of 8 months onwards, male Atg7 F/F ΔKC mice displayed an oily and scruffy coat (Figure 1A). Male Atg7 F/F ΔKC mice older than 1 year developed rarefication of nuchal hair to a significantly higher degree than autophagy-competent control mice (Figure S1). Younger male and female mice of both genotypes had normal hair growth (not shown). Old male Atg7 F/F ΔKC mice yielded considerably more acetone extractable lipids per unit hair than age-matched controls (Figure 1B). Prompted by these
observations, we evaluated SG of control and mutant mice for changes in morphology and size.

Volume measurements on 100-μm-thick sections of aged mouse back skin or whole mounts of ear and tail skin stained with BODIPY revealed that mutant SG of back, but not ear or tail skin, were significantly larger than controls (Figure 1C,D and Figure S2A). In confirmation of this observation, the areas of mutant glands measured from H&E stains were larger in back skin only (Figure S2B). Notably, SG was also enlarged in young mutant males and mutant females of all ages, although these did not display the oily phenotype at the macroscopic level, nor did they yield higher amounts of lipid per unit hair (not shown). Furthermore, the points of entry of the SG duct into the hair canal were wider in Atg7 F/F ΔKC mice than in control mice (Figure S2C, “lysis zone”). SG from both nape and ear skin showed significantly elevated numbers of proliferating sebocytes per length of gland perimeter, as well as per total SG cell number, as assessed by staining for Ki67 (Figure 1E,F, Figure S2D). In contrast, the thickness of the interfollicular epidermis was neither significantly increased, nor did it display increased basal cell proliferation (Figure S2E).

In addition, mutant sebocyte nuclei and cytoplasm became eosinophilic just prior to lysis (Figure S2B, arrows), similar to, but not as pronounced, as has been observed in differentiating autophagy-deficient PG sebocytes. Thus, in old male mice, lack of epithelial autophagy is associated with oily hair and enlarged SG on trunk skin, and with increased sebocyte proliferation.

3.2 Autophagy is active in skin sebaceous glands and preputial glands, and is abrogated by K14/cre deletion of Atg7

Epithelia of both SG and PG (Figure S3) express keratin 14 (K14). K14/cre driven inactivation of Atg7-dependent autophagy therefore is expected to occur in these cells, leading to autophagy-deficient daughter cells, that is, the maturing and differentiating sebocytes. To confirm the ablation of Atg7 protein and inactivation of autophagy in Atg7 F/F ΔKC sebocytes, we subjected lysates from PG (representing an enriched source of sebocytes) to Western blot analysis. Atg7 protein formation was indeed efficiently reduced in Atg7 F/F ΔKC PG (Figure 2A, upper panel), compared to controls, while robust autophagic flux, as evidenced by copious LC3 II protein in the control lysates was detected. In the absence of autophagosome formation, the nonlipidated, cytosolic precursor of LC3 II, LC3 I, cannot be incorporated into the autophagosome membrane, and thus accumulates (Figure 2A). Similarly, the cargo carrier, p62/SQSTM1, accumulates because of the absence of its binding partner, LC3 II (Figure 2A). Together these observations confirm that constitutive autophagy takes place in both SG and preputial gland sebocytes, and is inactivated in Atg7 F/F ΔKC mice.

To visualise autophagy in situ, we employed K14/cre Atg7 F/F mice into which the GFP-LC3 transgene had been introduced. In these mice, green fluorescent protein (GFP) coupled to LC3 is incorporated into the autophagosome membrane, forming microscopic puncta, which can be detected by fluorescence microscopy. We readily found such puncta in differentiated sebocytes of SG (Figure 3A, left 2 panels, back skin, Figure S4, left 2 panels, ear skin; arrows). GFP positive puncta were not detectable in mutant SG, instead there was a marked accumulation of GFP in the lumina of the glands (Figure 3A, right 2 panels, back skin, Figure S4, right 2 panels, Figure 2B).
Intracellular lipids are contained in lipid droplets, sequestered from the cytoplasm by a layer of structural proteins, the perilipins, which control lipid droplet turnover and metabolism by protecting the lipid core from lipolytic enzymes and references therein. We thus used perilipin 2 (Plin2), which is expressed throughout the differentiating layers, but not in the peripheral layer of skin sebaceous glands. In accordance with this study, we also detected Plin2 expression adjacent to the peripheral cell layers, and it was degraded before the entry of the sebaceous ducts into the hair canal in both control and mutant mice (Figure 3A, Figure S4, central row of panels). Double staining for Plin2 and LC3-GFP demonstrated that autophagic puncta were present just before the disappearance of Plin2 staining in control mice. In contrast, in the mutant glands, the massive accumulation of GFP noted before was observed in areas distal to Plin2 breakdown (Figure 3A, right hand 2 panels, Figure S4, right hand 2 panels). The co-localisation of Plin2 and autophagic puncta in control animals, and the absence of these puncta, together with the accumulation of GFP in mutant animals, suggest that autophagy is active in the final stages of holocrine secretion in SG.

As sebocytes mature, they accumulate lipids prior to cell rupture (see for example Ref. [15]). To the authors’ knowledge, no direct examination of the relationship between Plin2 expression and lipid production in SG in vivo has been published. Thus, to investigate the role of autophagy in the breakdown of Plin2 and lipid secretion, we stained frozen sections of trunk skin or whole mounts of ear skin with the neutral lipid dye, BODIPY. Plin2 staining became weaker concomitantly with the increasing abundance of BODIPY-positive lipids and no differences in BODIPY staining between control and mutant mice were observed (Figures 55A-C). To determine whether general cell lysis was compromised in the mutant glands, we analysed K14 expression, as a marker for cytoskeleton integrity, and compared this to the presence of nuclei and Plin2 expression. K14 was detectable throughout the glands of both strains. In control animals, either all of the K14 was broken down at the same time as Plin2 (Figure 3B, left-hand panels, arrows) or only small areas of reactivity remained. In contrast, appreciable amounts of K14 reactivity were retained after Plin2 degradation in mutant mice (Figure 3B, right-hand panels, arrows). Nuclei were usually, but not always, degraded together with Plin2 in both mouse lines. Figure 3C shows the results of the measurements of remnant K14 areas as a percentage of the sum of K14 and Plin2 positive areas. Thus, in autophagy-deficient SG either Plin2 is degraded prematurely compared to the cytoskeleton, or the breakdown of the cytoskeleton is retarded during sebocyte disintegration. In either case, our results strongly suggest that the absence of autophagy affects the final steps of sebocyte differentiation. Together these data demonstrate that abrogation of autophagy in sebocytes results in changes in the morphology of the glands and the breakdown of sebocytes.

3.4 Lack of autophagy in skin epithelia affects the proportions of sebum lipid classes and the chain length of the most common fatty acyl species in wax esters

Hair lipids are largely derived from sebum, and are composed almost exclusively of neutral lipids and references therein. We thus used...
thin layer chromatography (TLC) to compare hair lipids obtained from both groups of mice by acetone extraction using a solvent system which separates neutral lipids only. In good agreement with these two earlier studies, the main lipid classes detected by this TLC solvent system were cholesterol esters (CE) and wax esters (WE) (Figure 4A). However, we also detected a large band migrating between the fatty acid methyl esters (FAME) and triglycerides (TG), which, by analogy with Lin et al. we propose to be glyceryl ether diesters (GED). In general, similar proportions of CE, WE, GED and triglycerides (TG) were present in both groups of mice but mutant male sebum consistently harboured significantly higher proportions of FAME, and lower proportions of free fatty acids (FFA) and cholesterol (Figures 4A-D).

Oily hair has been reported to correlate with the percentage of WE and CE in sebum lipids. However, as these did not differ between control and mutant mice, we reasoned that differences in chain length and saturation of the esterified fatty acids in the major lipid classes such as WE, could contribute to the oily appearance of hair. The MALDI-MS analysis shown in Figures 4E and Figure S6A-C demonstrates that the general lipid profiles of WE is in good agreement with those described in a comparable analysis in that species with 56-58 total carbon atoms (typically corresponding to acyl moieties of C18-C20) are the most common. However, in sebum of our mutant mice, WEs with these chain lengths were significantly more abundant compared to control sebum (Figure 4E). Conversely, WE with shorter chain lengths (C54 and C55, acyl moieties of C16-C18 carbon atoms) were significantly less abundant in mutant sebum. This pattern was also observed when the WE moieties were separated into saturated, mono- and bi-unsaturated chains (Figure S6A-C, Table S1), but in these cases the differences, although apparent, did not reach statistical significance. Comparison of the weighted means of the WE chain lengths for the species of C54-C60 carbon atoms, which comprise about 80% of the total lipids, showed that mutant WE chains were slightly, but significantly, longer (57.37 ± 0.15 compared to 56.98 ± 0.19 carbon atoms), even though the C54-C55 species were under-represented in the mutant hair lipids. Thus, the lipid analyses reveal a proportionate decrease in free fatty acids and cholesterol in mutant sebum lipids, an increase in FAME, and a shift to longer chain lengths in the most common WE lipid species.

4 | DISCUSSION

Here, we show that autophagy is active in SG and that its disruption impacts SG morphology and function. Autophagic flux in sebocytes was demonstrated by the presence of autophagosomes, appearing as GFP-LC3+ puncta, in glands of wild-type mice and their loss after inactivation of the essential autophagy gene \( \text{Atg7} \). As expected, both GFP and p62/SQSTM1 accumulated in autophagy-deficient glands. Finally, we have demonstrated inactivation of the autophagic machinery by showing that LC3 I and p62/SQSTM1 accumulate in sebocytes of preputial glands.

SG in the nape region of \( \text{Atg7} \Delta \text{KC} \) animals were twice as large as those in wild-type animals and their loss after inactivation of the essential autophagy gene \( \text{Atg7} \). As expected, both GFP and p62/SQSTM1 accumulated in autophagy-deficient glands. Finally, we have demonstrated inactivation of the autophagic machinery by showing that LC3 I and p62/SQSTM1 accumulate in sebocytes of preputial glands.
are well established. To ascertain whether such site-specific effects had been described in other reports, we searched for studies of mouse mutations which have affected SG size. [42] This revealed that most laboratories restrict their observations to either tail or back skin. However, some studies, using the K5 promoter, report that either the effects on epidermis, SG size and lipid production are similar in back and tail skin, or that they are more pronounced in the latter. [43–46] These authors attribute the enhanced effects in tail skin to a greater activity there of the K5 promoter. [47] This is obviously not the case in our mouse model. In the face of enhanced proliferation without increase in size in ear and tail SG, it is tempting to speculate that there is also an increased sebocyte differentiation rate at these sites due to environmental factors, such as decreased temperature or humidity because of the sparser hair covering as compared to trunk skin. Confirmation of this idea must await further studies.

Adding to the many mutations which affect SG size, [42] we have recently shown that increased activity of nuclear factor erythroid-derived 2, like 2 (Nrf2) results in SG hyperplasia and "chloracne/malignant acquired dioxin-induced skin hamartomas" (MADISH) like skin manifestations. [46] As we have furthermore shown that lack of autophagy activates the Nrf2 pathway in KC, [48,49] we suggest that a similar mechanism is responsible for the SG hyperplasia we observed. The finding that p62/SQSTM1 was increased in autophagy-deficient SG supports this suggestion, as p62/SQSTM1 is known to activate Nrf2, thereby promoting transcription of its target genes. [50,51] Apart from other growth factor-mediated mechanisms which lead to SG enlargement, such as direct effects of Epigen, an Nrf2 target gene, [43,46] EGFR [52] or amphiregulin, [44] alterations of lipolysis, lipogenesis and/or lipid metabolism are also possible pathways, and autophagy has been implicated in the first two of these processes, as well as in lipoprotein trafficking (reviewed recently in Ref. [53]). In addition, Pparγ, a lipid-activated nuclear transcription factor, plays an important role in sebum production, sebaceous gland differentiation and lipid metabolism. [54–56] However, our analysis of 31-day-old mouse skin by quantitative RT-PCR showed no difference in expression levels or either Pparγ, or its target gene Pnin2 (not shown). Neither could we find differences in expression levels fatty acid transport protein 4 (FATP4) [29] and Acyl-CoA Binding Protein (Acbp) [57] (not shown). Future studies on the exact role of autophagy during sebocyte differentiation, autolysis and its contribution to lipid composition will have to include the inactivation of autophagy in either isolated sebocytes or sebocyte cell lines, [58] thereby obviating potential effects of the other cell types present in skin.

As we have reported earlier, autophagy deficiency of preputial gland sebocytes results in premature degradation of the nucleus and aberrant eosinophilia in the secretory cells. [19,20] We show here that this observation is also true for SG and also find a defect in the breakdown of keratin 14. Whether these alterations of terminal differentiation of sebocytes are linked to the increased proliferation or whether they represent separate phenomena remains to be determined.

Despite the enlargement of SG in both sexes, oily hair was distinctly noticeable only in older mutant male mice, and the amount of acetone extractable lipid per unit hair was significantly increased only in these animals. We do not have an explanation for these sex differences. However, as androgens are well known to increase sebum production, and androgen receptor levels differ between male and female mice (Ref. [59] and references therein), it is likely that they play a role in the observed sex differences. Although SG biology in mice and humans share many similarities, skin affections comparable to human acne have not been described in mice. Therefore, we abstain from drawing far-reaching conclusions from our model with regard to human SG dysfunction. Nevertheless, it is of note that, mTORC, a potent inhibitor of autophagy, [60] and itself a regulator of lipid synthesis [61] is upregulated in acne vulgaris lesions, [42] potentially linking inhibition of autophagy to increased sebum production also in human sebaceous gland biology. We thus suggest that in older male mice, the combination of prolonged lack of autophagy and increased presence of androgens enhances sebogenesis so that an excess of sebum is evident on the skin surface and manifests itself as oily hair.

We have observed a significant decrease in free cholesterol and free fatty acids, and an increase in fatty acid methyl esters in the sebum of mutant animals compared to controls. Autophagy has been implicated in lipolysis in other cell types, [11] and we suggest that the change in lipid composition is caused by blockade of autophagy-dependent lipolysis in Atg7−/− Kc mouse sebaceous glands, shifting the balance between esterified and non-esterified lipid precursors towards the former.

In addition to a change in the lipid class species in mutant sebum, we found an increase in the abundance of 56-58 carbon atom length WE fatty acid moieties (corresponding to chain lengths of C18-C20) and a decrease of 54-56 carbon atom moieties (C16-C18 chain lengths), especially those containing one and two double bonds. We thus used back skin of 31-day-old control and mutant mice, and adult tail epidermis to examine the expression levels of two members of the elongase family of enzymes, which extend fatty acid chain lengths, [63,64] namely Elongation of very long chain fatty acid 6, (ELOVL6), which has been shown to be upregulated in cultured Atg7−/− deficient KC [38] and ELOVL3, [65] which regulates SG size and sebum lipid composition. However, we could detect no difference between mutant and controls (not shown). We also examined expression levels of stearoyl CoA desaturase 1 (Scd-1), which generates mono-unsaturated fatty acids from saturated fatty acids, and which plays a role in the regulation of autophagy, [66] but found no difference in the expression of this enzyme either. However, we cannot at present exclude that the activity of these enzymes is altered in the mutant sebocytes. Interestingly, the lipid profile of cultured Atg7−/− deficient keratinoocytes is also altered compared controls, [38] but the change in the proportions of the lipid classes examined in that study (sterols, FFA, and TG) is quite different to those which we find in sebum. This once again demonstrates that the effects of autophagy inhibition with regard to lipid metabolism is very context dependent, as...
has been demonstrated on numerous occasions (see for instance Refs [11,13,53]).

A plausible scenario for the increase in unsaturated acyl species that we find in wax esters is that firstly unsaturated fatty acids are found in high proportions in autophagic vacuoles.\[67\] Secondly, the assembly of the preautophagosomal structure (PAS) requires either scd-1, or the unsaturated fatty acid, oleic acid (C18:1).\[68\] In addition, oxidation products of unsaturated lipids are substrates for LC3 lipidation,\[69\] an essential process in autophagosome formation. Thus, any disturbance of autophagosome membrane assembly, such as occurs in the Atg7 deficient cells, could result in an increase in unsaturated fatty acids available for incorporation into other lipid classes, independently of autophagosomal lipolysis and scd1 or elongase activity.

In summary, inactivation of autophagy in sebaceous glands results in oily pelage in old male mice, larger glands, an increase of proliferation in SG epithelia, as well as a change in lipid class composition of sebum and fatty acyl chain length of wax esters.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

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Figure S2. Sebaceous glands are larger in back skin, but not ear or tail skin. (A) Whole mounts of PFA fixed, frozen ear and tail skin, stained with BODIPY, with the corresponding volume measurements. N = 2 to 3/group. Bar: 100 μm. (B) H+E stain of formalin fixed, paraffin embedded sections with measurements of gland area. N = 6 to 8/group for ears, 7-14/group for tails and 19-21 for backs. Rectangles mark the enlarged areas. Bar: 50 μm. *P < 0.005 (C) H+E stain of formalin fixed, paraffin embedded sections with measurements of gland infundibulum diameter. N = 14-41 glands/7-10 mice. Bar: 50 μm. **P < 0.01. (D) Frequency of Ki67 positive cells in the sebaceous gland perimeter (left hand graph) and expressed as a percentage of the total sebaceous gland cells (right hand graph) in formalin fixed, paraffin embedded ear sebaceous glands. Only weakly labelled cells are visible in the Atg7 F/F mouse. N = 5/group. Bar: 50 μm. *P<0.02. **P<0.05. (E) Frequency of Ki67 positive cells in epithelium of ear and nape. N = 5 to 7 mice/group. Bar: 25 μm. n.s.: non-significant.

Figure S3. Keratin14 is expressed in sebocytes of the preputial gland. Preputial glands were fixed with formalin, embedded in paraffin, and sections were stained with K14 antibody (A) and isotype control antibody (B). Bars: 100 μm (A) 50 μm (B). Photomicrographs are representative of 7 mice/group.

Figure S4. GFP-LC3 puncta are present near the border of Plin2 degradation in SG of Atg7 F/F mice, but GFP accumulate in Atg7 ΔKC mice. Sections of formalin fixed, paraffin embedded ear skin were stained with GFP-LC3 and perilipin2 antibodies. Boxes designate the enlarged areas. Bar = 50 μm and 10 μm for originals and enlargements respectively. Arrows point to puncta in Atg7 F/F mouse. Note the accumulation of GFP in SG ducts of mutant mice. N = 3 mice/group.

Figure S5. Plin2 is degraded at the same time as BODIPY stainable lipids accumulate in both Atg7 F/F and Atg7 ΔKC SG. (A) Sections of frozen back skin embedded in O.C.T. medium were fixed in 7.5% buffered formalin for 20 min at RT, and stained as indicated. Rectangles mark the areas enlarged, and lines the outlines of the glands. N = 2 mice for Atg7 F/F, 3 mice for Atg7 ΔKC). (B) PFA (3%) fixed whole mount preparations from ear and (C) tail skin stained as indicated. N = 3 for per group. The lipid droplets outside the SG are BODIPY dependent artefacts. Bar = 50 μm for back and ears, 100 μm for tails.

Figure S6. Mean relative abundance of mono-unsaturated wax di-ester species with acyl chains of 58 and 60 carbon atoms has a tendency to be higher in K14 ΔKC mice compared to controls. (A) Saturated fatty acyl species (B) mono-unsaturated fatty acyl species (C) di-unsaturated fatty acyl species. For all double bond numbers mean abundances are higher for 58-60 carbon atom species (solid line ovals), and lower for 54-56 carbon atom species (dotted line ovals), but do not reach statistical significance using the Holm-Sidak
multiple comparisons post test, due to high individual variation. Male hair lipids were separated by TLC and subjected to MALDI-MS analysis. C18:1, C18:0 etc.: estimated fatty acyl composition of each WE species. N = 6 mice/group.

Table S1. Relative abundance of wax di-ester species with 0, 1, and 2 double bonds in the sebum of Atg7 WT and Atg7 ΔKC mice.

Appendix S1. Supplementary Materials and Methods.