Stat3 controls cell death during mammary gland involution by regulating uptake of milk fat globules and lysosomal membrane permeabilization

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We have previously demonstrated that Stat3 regulates lysosomal-mediated programmed cell death (LM-PCD) during mouse mammary gland involution in vivo. However, the mechanism that controls the release of lysosomal cathepsins to initiate cell death in this context has not been elucidated. We show here that Stat3 regulates the formation of large lysosomal vacuoles that contain triglyceride. Furthermore, we demonstrate that milk fat globules (MFGs) are toxic to epithelial cells and that, when applied to purified lysosomes, the MFG hydrolysate oleic acid potently induces lysosomal leakiness. Additionally, uptake of secreted MFGs coated in butyrophilin 1A1 is diminished in Stat3-ablated mammary glands and loss of the phagocytosis bridging molecule MFG-E8 results in reduced leakage of cathepsins in vivo. We propose that Stat3 regulates LM-PCD in mouse mammary gland by switching cellular function from secretion to uptake of MFGs. Thereafter, perturbation of lysosomal vesicle membranes by high levels of free fatty acids results in controlled leakage of cathepsins culminating in cell death.

Lysosomes are key eukaryotic organelles that are essential for the turnover of cellular macromolecules and organelles that are delivered by the endocytic, autophagic and phagocytic membrane trafficking pathways¹. Although previously considered simply as the final destination for cargo intended for breakdown it is becoming increasingly apparent that lysosomes, and their acid hydrolase contents, possess many other specific functions. Notably, this includes the initiation or enhancement of cell death programmes as a result of permeabilization of lysosomal membranes and the subsequent leakage of cathepsins and other constituents into the cytosol, where they can act as executioner proteases¹–³. Aberrant lysosomal function is associated with ageing and neurodegenerative disorders such as Parkinson’s disease and lysosomal storage disorders⁴,⁵. Furthermore, cellular transformation is characterized by lysosomal modifications, including changes to their size, localization and composition and enhanced secretion of their contents to the extracellular space, promoting tumour progression, invasion and metastasis⁶–⁸. Conversely, apoptosis or lysosomal-mediated cell death can be induced by the intracellular release of lysosomal hydrolases, providing a rationale for using anti-cancer drugs that destabilize lysosomal membranes⁷,⁹.

An exquisite example of controlled physiological cell death is the post-lactational regression (involution) of the mammary gland, one of the main cell death events to occur in the adult mammalian organism¹⁰,¹¹. During pregnancy, alveolar epithelial progenitor cells are stimulated to proliferate and differentiate in response to progesterone and prolactin to provide mammary tissue with the secretory cells necessary for milk production during lactation¹². This includes secretion of milk proteins as well as MFGs by a unique mechanism that requires butyrophilin 1A1 (BTN) and xanthine dehydrogenase/oxidoreductase¹³ (XDH). A complex and highly regulated programme of cell death and tissue remodelling is initiated on cessation of lactation to remove alveolar mammary epithelium and return the gland to its pre-pregnant state¹¹. This process occurs in two phases, with the first phase (0–48 h in the mouse) being reversible such that reintroduction of the pups can re-initiate lactation¹⁴. Prolactin-induced Stat5 signalling is abrogated, and LIF-induced Stat3 signalling is initiated, on induction of involution. Stat3 is indispensable for this process as mice with a mammary epithelial-specific knockout (KO) of Stat3 exhibit diminished cell death and a severe delay in post-lactational regression of the gland¹⁵,¹⁶.

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We have shown previously that cell death in the regressing mammary gland occurs independently of caspases, relying instead on lysosomal membrane permeabilization (LMP) and the release of cathepsin proteases to the cytosol\(^\text{17}\). Intriguingly, this mode of cell death is unlikely to be restricted to the mammary gland. Cell death during hormonoprivic involution of the prostate gland\(^\text{18}\) and in the labial gland of the tobacco hornworm bear striking similarities to LMP in mammary epithelial cells during involution\(^\text{19}\). Stat3 regulates this process by stimulating the expression of cathepsins B and L, in addition to concurrently suppressing expression of the endogenous cathepsin inhibitor Spi2A (ref.\(^\text{17}\)). This latter event leaves mammary epithelial cells particularly susceptible to cell death from the amplified activity of cathepsins that have leaked from lysosomal structures. A role for LMP in mediating a physiological cell death program, rather than in pathological conditions or as a back-up mechanism for refractory apoptosis, had not been previously described\(^\text{20}\).

A number of mechanisms have been proposed for LMP in different cellular contexts in vitro including lysosomotropic detergents, toxins, reactive oxygen species, and translocation of Bax to the lysosomal membrane\(^\text{21–23}\). However, a definitive mechanism driving LMP in vivo, and the specific role of Stat3 in this process, has yet to be elucidated. We reasoned that Stat3 drives a series of biological events in the regressing mammary gland that amplifies the lysosomal system at a time of increased demand and stress, while concurrently exploiting this to mediate lysosomal-dependent cell death. We have explored this notion by investigating the micro-architecture of mammary epithelial cells in lactating and involuting mammary glands and discovered that Stat3 mediates the formation and fusion of large lysosomal-like vacuoles, many of which contain triglyceride. This is subsequently metabolized to free fatty acids, including oleic acid, that can distort membranes and result in leakage of cathepsins from lysosomes. We also reveal that Stat3 promotes a phenotypic switch from secretion to phagocytosis of MFGs, the latter function delivering triglyceride to vacuoles with the ensuing consequences of LMP and cell death.

**RESULTS**

**Large vacuolar structures appear during involution**

Our initial approach to investigate the hypothesis that Stat3 both enhances, and exploits, the lysosomal system to mediate cell death was to examine the lysosomal compartment during mammary gland regression in more detail. We noted the appearance of large cathepsin D-positive vacuoles on initiation of involution (Fig. 1a). Significantly, similar vacuoles also appear on induction of involution in the uterus and prostate (Fig. 1a), and were often larger than nuclei (>5 μm). Importantly, such a marked increase in size is a factor that could, of itself, sensitize lysosomes to become leaky\(^\text{24}\). Notably, the morphology of dead cells in all three tissues was similar, being atypical for either classical apoptosis or necrosis (Fig. 1b), and characterized by hypercondensed nuclei and a complete absence of membrane blebbing\(^\text{18}\). This suggests a common mechanism of cell death during regression of hormone-dependent tissues.

We then focused our studies on these presumptive lysosomal vesicles in the regressing mammary gland. Cathepsin D is coordinately upregulated with cathepsins B and L at 24–48 h involution\(^\text{17,25,26}\) (Supplementary Fig. 1a,b), with large cathepsin D structures also staining positively for the lysosomal membrane marker LAMP2 (Fig. 1c). Several very large LAMP2-positive lysosomal vacuoles were frequently seen within individual epithelial cells in the involuting, but not the lactating, mammary gland (Fig. 1d). These structures were much less apparent in tissue from Stat3\(^{39/β};BLG-Cre\) (Stat3 KO) mice that are specifically deleted for Stat3 in the luminal epithelium, compared with Stat3\(^{39/β}\) control mice (Fig. 1e). Quantification of the number of LAMP2-positive structures per nucleus showed a ninefold decrease in the Stat3 KO epithelium (Fig. 1f). This effect was observed only during the first phase of involution. At 48 h and 72 h involution, Stat3 ablation had a diminished effect on the presence of lysosomal vacuoles (Supplementary Fig. 1c), although expression of cathepsins B and L is markedly reduced at these time points in Stat3 KO glands\(^\text{17}\). On further characterization, triglyceride lipid was found to be a significant constituent of the lysosomal system in involuting mammary gland (Fig. 1g and Supplementary Fig. 1d). Thus, these large structures contain lysosomal proteins and triglyceride showing that fusion of lysosomes with either unsecreted or reabsorbed MFGs has taken place.

**Ultrastructural analysis of vacuoles and their contents**

To analyse these structures in greater detail, we performed transmission electron microscopy (TEM) on perfusion-fixed mammary tissue from day 10 lactation and 24 h involution. This revealed the presence of strikingly large, membrane-bound vesicles, many of which were at least the size of nuclei with some occupying most of the cell (Fig. 2a). These have been observed also by TEM at day 3 involution\(^\text{27}\). Notably, these were present only during involution and not at day 10 lactation (Fig. 2a). Once again, in agreement with our observations on LAMP2-positive vacuoles, these large vacuoles were much less abundant in Stat3 KO tissue (Fig. 2a). Additionally, we noted that mitochondria acquire an elongated morphology on the switch from lactation to involution that occurred regardless of Stat3 status (Fig. 2a). This has previously been described as an adaptive response to a highly autophagic environment\(^\text{28}\). The presence of morphologically sound mitochondria, which appeared similar in both control and Stat3-deficient mammary tissue at 24 h involution, further supports our previous data showing that cell death at this time is caspase independent\(^\text{17}\). Confirming results in Fig. 1a,c, immunogold staining for cathepsin D showed this lysosomal enzyme to be focally associated with degrading material contained within vacuoles (Fig. 2b), revealing their lysosomal origin. Vacuoles containing autophagic contents (Fig. 2c), milk protein (Fig. 2d,e) and triglyceride (Fig. 2f–i) were frequently observed to be in the process of fusion with other membrane-bound structures (Fig. 2c,e,f), clearly demonstrating an active process of vesicle biogenesis. Furthermore, lipid was observed inside lysosomal structures that also contained electron-dense lipofuscin-like material (Fig. 2h,i), confirming hydrolysis of bulk triglyceride from lipid droplets in the lysosomal compartment.

**Vesicles are induced by Stat3 in mammary epithelial cells in vitro**

Intrigued by the association of large lysosomal vacuoles with the initiation of cell death, and their absence in the Stat3 KO, we sought to determine a mechanistic involvement of these structures in the execution of cell death. To address this question, we used oncostatin
Figure 1  Enlargement of the lysosomal compartment and digestion of triglyceride in the regressing mammary gland. (a) Large secondary lysosomes are shown by cathepsin D staining (red, arrowheads) in different involuting tissues. The images show mouse uterus at 24 h post-partum and during late pregnancy; prostate gland at 7 d post-castration and uncastrated; and mammary gland at 24 h involution and 10 d lactation. Tissue was stained for cathepsin D (red) and E-cadherin (green). (b) Hoescht staining showing dead cells (arrowheads) in the involuting mouse uterus, prostate and mammary gland. Three independent biological repeats per condition for prostate and mammary glands, one biological repeat per condition for uterus. (c) Dual staining for LAMP2 and cathepsin D shows cathepsin D (red) localizing to the inside of a LAMP2-positive (green) vacuole. Three independent biological repeats were assessed. (d) LAMP2 (red) and E-cadherin (green) staining in 10 d lactating and 24 h involuting mammary glands. Six independent biological repeats per condition analysed. (e) LAMP2-positive vacuoles (red) are found in the control but not the Stat3 KO mammary gland at 24 h involution. (f) Staining for LAMP2 at 24 h involution in control and Stat3 KO animals was quantified. Bars represent means ± s.e.m. of n = 3 mice per genotype with 12–13 fields counted per mouse (*P < 0.05; Student’s t-test). For statistics source data, see the associated worksheet in Supplementary Table 3. (g) Confocal images showing that cathepsin D-positive vacuoles (arrowhead) contain lipid droplets (Lipidtox staining, green) in the 24 h involuting mammary gland. Three independent biological repeats per condition analysed. Nuclei are visualized by Hoescht stain (blue). Scale bars, 20 μm (a, d, e, g) and 10 μm (b, c).
Figure 2 Ultrastructural analysis of lysosomal vacuoles and cargo delivery. (a) TEM of wild-type 10 d lactation, 24 h involution and Stat3 KO glands at 24 h involution. (b) Immunogold staining for cathepsin D within large vacuoles at 24 h involution co-localizing with degrading material (arrowheads). (c) Autophagic vesicles fusing with large vacuoles (arrowheads). (d, e) Large macropinosomes full of milk inside epithelial cells and milk-containing vesicles (d) are seen to fuse with larger vacuoles (e). (f-i) Membrane-bound lipid fuses with vacuoles and can be observed inside lysosomal structures (arrowheads) that contain lipofuscin-like material (arrows). Mammary tissue from three animals assessed for all conditions except for cathepsin D-immunogold staining, where one animal was analysed. Scale bars, 2 \( \mu \text{m} \) (a (epithelial cells)); 500 nm (a (mitochondria), b (low and high magnification), c (high magnification)); 1 \( \mu \text{m} \) (c (low magnification), d-i).

M (OSM) stimulation of the EpH4 mammary epithelial cell line, which we have previously shown to mimic Stat3-induced LM-PCD (ref. 17). We observed a number of vacuoles/vesicles in unstimulated EpH4 cells presumably generated by autophagy and by pinocytosis of the culture medium (Fig. 3a). We then determined whether activation of Stat3 by OSM resulted in a stimulation of the biogenesis of such vesicles. This was indeed the case with OSM treatment resulting in a 1.7-fold increase in the volume fraction of degradative vesicles after 72 h treatment as determined by TEM (Fig. 3b–d).

Milk-derived lipid and fatty acids are toxic to mammary epithelial cells

Having confirmed that EpH4 cells can take up substances from the culture medium we incubated cells with freshly collected mouse milk, which resulted in lipid accumulation (Fig. 4a) and induction of cell death, as measured by phase-contrast microscopy (Fig. 4a) and cell viability analysis (Fig. 4b).

Immunofluorescence imaging of milk-fed EpH4 cells revealed lipid localizing to lysosomal compartments (Supplementary Fig. 2a).
Figure 3 Vesicular biogenesis in EpH4 cells. (a) Vehicle-treated EpH4 cells contain phagosomes/pinosomes (arrowheads) and lysosomes (arrows). (b,c) EpH4 cells treated with OSM (25 ng ml$^{-1}$) for 72 h have 53% more degradative vesicles. For c, n = 4 individual wells of EpH4 cells seeded on one occasion. (d) In some cases, as a result of OSM treatment, lysosomal vesicles occupied more than half of the cytosolic volume. Phagolysosomes are marked by an asterisk. Scale bars, 1 μm. For statistics source data for c, see the corresponding worksheet in Supplementary Table 3.

Co-treating with bafilomycin A1, a potent and selective inhibitor of the vacuolar-type H$^+$ ATPase (ref. 29) that inhibits lysosomal acidification and vesicular fusion, resulted in a partial rescue of milk-induced cytotoxicity (Fig. 4c,d), suggesting that lysosomal lipid accumulation induces cell death.

The toxicity of milk could be due to several components as it contains protein, triglyceride, carbohydrates, minerals and antibodies. Triglycerides are the primary constituent of MFGs (ref. 30) that are degraded in the lysosome during involution, yielding potentially high local concentrations of free fatty acids. Thin-layer chromatography (TLC) analysis (Fig. 4e) revealed that free fatty acids are present in the mammary gland during involution, but are not detectable during lactation. Free fatty acid concentration as measured by TLC, using oleic acid standards, averaged 4.8 μg/20 mg tissue (Fig. 4f,g), which corresponds to a tissue level of approximately 850 μM free fatty acid (Methods). It should be noted that only unsaturated free fatty acid could be detected using this system, implying that 850 μM is a conservative estimate.

The fatty acids found in milk triglycerides are summarized in Supplementary Table 1, with oleic (CH$_3$(CH$_2$)$_7$CH=CH(CH$_2$)$_2$CO$_2$H) and palmitic (CH$_3$(CH$_2$)$_14$CO$_2$H) acids being the most abundant$^{31}$ and hence chosen for further analysis in vitro. Stearic acid (CH$_3$(CH$_2$)$_16$CO$_2$H) was also used, as it has the same number of carbon atoms as oleic acid but lacks the double bond.

The role of free fatty acids in lysosome permeabilization and cell death was then investigated using EpH4 cells. Treatment with physiological levels of oleic and palmitic acids (as determined in Fig. 4e-g) resulted in lipid accumulation and toxicity, as observed by phase-contrast microscopy and cell viability analysis (Fig. 4h,i and Supplementary Fig. 2b,c). Notably, oleic acid treatment resulted in more staining for triglyceride than palmitic acid. Stearic acid treatment resulted in less lipid accumulation, but no cytotoxicity was observed at equivalent concentrations to oleic and palmitic acids (Fig. 4i). The ability of oleic acid to elicit more triglyceride synthesis is well documented$^{32}$ and is considered a protective mechanism from the toxic effects of oleic acid exposure.

Cathepsin activity assays and immunoblot analysis on digitonin-extracted cytosolic fractions (optimized for digitonin concentration to avoid damage to lysosomes; Supplementary Fig. 3a) of fatty-acid-treated EpH4 cells revealed that oleic acid caused a significant release of cathepsins to the cytosol (Fig. 5a). Immunoblot analysis of these samples further demonstrated that only oleic acid induces significant release of cathepsin L to the cytosol (Fig. 5b). In contrast, palmitic acid treatment resulted in limited release of cathepsins to the cytosol, which was not significant. Importantly, stearic acid, the saturated carbon chain counterpart of oleic acid, also had no effect on cathepsin release as measured by cytosolic cathepsin activity assays and immunoblot (Fig. 5a,b). Staining with the lysosomotropic
Figure 4 Free fatty acids are increased during involution and can cause death in vitro. (a) Fluorescence and bright-field microscopy shows milk-derived lipid accumulation (Lipidtox, green) and cell death of EpH4 cells incubated in milk overnight. Scale bars, 10 μm (Lipidtox staining panels) and 100 μm (phase images). Nuclei are visualized by Hoechst (blue). (b) Milk-induced cell death assessed by trypan blue positivity. Means ± s.e.m. from n=5 independent experiments with 3 technical replicates performed per experiment (*P < 0.05; Mann–Whitney U test). (c) Bafilomycin A1 treatment partially rescues milk-induced cell death as assessed by bright-field microscopy and trypan blue positivity. Scale bars, 250 μm. (d) Milk-induced cell death assessed by trypan blue positivity. Means ± s.e.m. from n=4 independent experiments with 3–4 technical replicates performed per experiment (***P < 0.01; Kruskal–Wallis test, Dunn’s multiple comparison post-test). Cells were pre-treated with bafilomycin A1 for 1 h before milk addition for a further 8 h. (e) TLC was used to demonstrate the presence of free fatty acids in mammary gland extracts. Free fatty acids were seen in force-involved mammary glands but not in lactating mammary glands (arrowhead). TG: triglyceride, PA: palmitic acid. (f) A concentration curve of oleic acid was quantified using densitometry. (g) Quantification of the free fatty acid band showed a significant difference between lactating and involuting mammary gland (*P < 0.05, Student’s t-test, n=4 animals). (h) Fluorescence and bright-field microscopy showing lipid accumulation (Lipidtox, green) and cell death of EpH4 cells treated with 500 μM oleic acid (OA), palmitic acid (PA) or stearic acid (SA) overnight. Scale bars, 10 μm (Lipidtox staining panels) and 100 μm (phase images). (i) Fatty-acid-induced cell death assessed by trypan blue positivity. Means ± s.e.m. from n=4 independent experiments with 2–3 technical replicates performed per experiment (**P < 0.01 versus ethanol control; one-way ANOVA, Dunnett’s multiple comparison post-test). For statistics source data, see the corresponding worksheets in Supplementary Table 3.
Figure 5 Free fatty acids induce LMP in vitro. (a) Cathepsin activity assay on EpH4 cytosolic extracts treated with 500 μM fatty acids for 16 h demonstrating release of cathepsins to the cytosol by oleic acid (OA). PA: palmitic acid, SA: stearic acid. Means ± s.e.m. of n = 4 independent experiments (*P < 0.05; Kruskal–Wallis test, Dunn’s multiple comparison post-test). (b) A representative sample from a was analysed by western blot for cathepsin L in total and cytosolic EpH4 extracts, showing release of cathepsin L to the cytosol with 500 μM oleic acid. M denotes marker lane. (c) Schematic of iron nanoparticle lysosomal purification protocol. (d) Optimization of iron-nanoparticle-mediated purification of lysosomes from EpH4 cells. Cells were labelled and chased as indicated followed by extraction and immunoblotting. LAMP2, COXIV, RAB5 and RAB7 are shown as markers for lysosomes, mitochondria, early endosomes and late endosomes respectively. Time-course optimization performed on one occasion. pns: post nuclear supernatant, sn: post magnetic supernatant, mp: magnetic pellet. (e) Cathepsin L immunoblot showing extent of leakiness from the magnetic lysosomal pellet (P) into the supernatant (S) at 30 min with 70 μM oleic acid or palmitic acid. Representative blot from three independent experiments. LAMP2 was used as a lysosomal marker and COXIV was used to show undetectable mitochondrial contamination. (f) Structures of the free fatty acids used in this study. For statistics source data, see the corresponding worksheet in Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 5.

Dye LysoTracker red showed that both oleic and palmitic acid treatment resulted in an appearance of a population of cells with low LysoTracker intensities, indicative of lysosomal de-acidification (Supplementary Fig. 3b,c). Of note, fatty acid treatment did not seem to induce the translocation of the pore-forming protein Bax to lysosomes in EpH4 cells (Supplementary Fig. 4). Therefore, these data clearly demonstrate that lysosomal membranes can be permeabilized efficiently by oleic acid. However, an effect of palmitic acid cannot be completely excluded.

Oleic acid treatment permeabilizes purified lysosomes
It has been observed that palmitic, but not oleic acid, induces cell death by reactive oxygen species (ROS)-mediated mitochondrial disruption. To exclude potential indirect effects such as mitochondrial...
ROS-induced perturbations upstream of lysosomal permeability\(^\text{35}\) we isolated lysosomes from EpH4 cells using a magnetic nanoparticle separation protocol (Fig. 5c), which produces pure lysosomal preparations uncontaminated by mitochondria (Fig. 5d). Addition of fatty acids to purified lysosomes for up to 30 min followed by immunoblot analysis showed leakage of cathepsin L in the presence of oleic acid. In comparison, lysosomes were essentially intact in the presence of palmitic acid (Fig. 5e). Thus, oleic acid is a potent lysosomal permeabilization agent at concentrations below that present locally in the mammary gland during involution, with palmitic acid being unable to permeabilize membranes within the time frame studied. Oleic acid possesses an unsaturated cis-bond and adopts a kinked conformation that has been shown to distort synthetic membranes and leads to their destabilization on insertion\(^\text{35}\) (Fig. 5f). In contrast, palmitic and stearic acids are linear in conformation as they lack a double bond. Thus, the increased potency of oleic acid is likely to be due to the kink in its structure. The oleic acid-bovine \(\alpha\)-lactalbumin complex, BAMLET, has been suggested to cause LM-PCD by interaction with the lysosomal membrane\(^\text{36}\). However, it has since emerged that cell death caused by BAMLET, and its human counterpart HAMLET, is due solely to the function of \(\alpha\)-lactalbumin as a carrier for oleic acid\(^\text{17}\). Our data support this observation and emphasize the potency of oleic acid as a phospholipid membrane destabilizer. As this leak of cathepsins mimics our observations in vivo\(^\text{17}\), we suggest that insertion of MFG-derived fatty acids, and in particular oleic acid, into the limiting membrane of these lysosomal vesicles produces membrane distortions resulting in leakage of cathepsins into the cytosol. This posit is further supported by the slow leak of cathepsins\(^\text{17}\) and the essentially intact lysosomal/vacuolar membranes observed during involution in vivo (Fig. 2).

**Stat3 mediates uptake of BTN-coated MFGs**

In the process of being secreted, MFGs become coated with BTN, which is essential for their secretion\(^\text{38}\). BTN can be visualized as a thin ring surrounding MFGs secreted into the alveolar lumen at day 10 lactation (Fig. 6a). Notably, unsecreted lipid droplets within the mammary epithelium do not stain for BTN (ref. 38). At 24 h involution, the appearance of MFGs coated with BTN becomes strikingly evident within the mammary epithelium (Fig. 6a). This is Stat3 dependent as considerably fewer MFGs coated with BTN are evident in Stat3\(^\text{fl}\) Virgin;BLG\(^\text{Cre}\) glands at 24 h involution (Fig. 6b), indicating reduced uptake of MFGs. Interestingly, microarray analysis indicates that expression of BTN and XDH is suppressed at the onset of involution\(^\text{59}\) (Fig. 6c; www.path.cam.ac.uk/~madgroup). Quantitative PCR analysis shows that these genes are upregulated in Stat3 KO mammary gland at 24 h involution, suggesting that Stat3 represses XDH and BTN transcription (Supplementary Table 2). Immunoblot of Stat3 KO and control mammary tissue corroborates the role of Stat3 in suppressing BTN expression (Fig. 6d).

**Figure 6** MFGs are endocytosed by mammary epithelial cells. (a) Staining for the MFG membrane protein BTN (red) in 10 d lactating and 24 h involuting mammary gland. (b) Number of butyrophilin-positive structures per nucleus quantified in control and Stat3 KO 24 h involuting mammary glands. Bars represent means ± s.e.m. of \(n = 3\) mice per genotype, with 34 alveoli counted per animal (\(^\ast \) \(P < 0.05\); Student’s \(t\)-test). For statistics source data, see the associated worksheet in Supplementary Table 3. E-cadherin staining is green. Images are representative of three independent biological repeats. (c) Microarray analysis of 12 different time points during the mammary gland cycle showing expression of BTN and XDH—‘Mammary Gland Pregnancy Cycle Data (xls)’ (www.path.cam.ac.uk/~madgroup/microarraysummary.shtml). (d) Western blot for BTN (M. 70 K) showing BTN downregulation at 24 h involution in control but not in Stat3 KO samples. The asterisk indicates a spurious band. Lanes show samples from independent biological repeats. Scale bars, 20 \(\mu\)m (a (low magnification), b, and 10 \(\mu\)m (a, high magnification). Uncropped images of blots are shown in Supplementary Fig. 5.
Figure 7 Uptake of MFGs occurs by macropinocytosis and phagocytosis. (a) MFGs are seen in the alveolar lumen (arrowheads). The cytosolic ring bound by milk fat globule membrane is inherited by the fat droplet as it is secreted through the apical plasmalemma (arrows). (b) Ruffles on the apical plasma membrane are indicative of macropinocytosis (arrowheads). (c) Lipid droplets (arrowhead) are present within milk-laden macropinosomes in alveolar epithelium. (d) Phagocytosed milk fat droplets can be identified within epithelium by the presence of MFG-associated cytosol and milk fat globule membrane (arrowheads). (e) Another example of a large phagocytosed MFG is shown by arrowheads. (f) Enlargement of the area outlined in e to show the phagosomal membrane (arrowheads) in close apposition to the milk fat globule membrane (arrows). (g) The highly phagocytic nature of alveolar epithelium was demonstrated using cathepsin L KO (Ctsl KO) mice that were force-involuting for 72 h. In comparison with wild-type controls, cathepsin L KO mammary glands showed accumulation of dead cells within phagosomes (arrowhead). This phagosome is fusing with other vacuoles (arrow). n: nucleus. (h) Staining for the phagocytosis-bridging molecule MFG-E8 (red) and E-cadherin (green) in MFG-E8 heterozygous (control) and KO mice. Images are representative of three independent biological repeats. (i) Cytosolic cathepsin activity in MFG-E8 control and KO mice at 24 h involution. Box and whisker plot of n = 12 and n = 11 cytoplasmic fractions (mice) for control and KO groups respectively, with 2 technical replicates performed per fraction (P = 0.0173, Student’s t-test). For statistics source data, see the associated worksheet in Supplementary Table 3. Scale bars, 1 μm (a–d), 4 μm (e), 500 nm (f), 2 μm (g) and 20 μm (h).

MFGs are taken up by mammary epithelial cells by means of phagocytosis

Having firmly established the presence of triglyceride lipid in secondary lysosomes, we sought to define how lipid was trafficked to these large lysosomal vacuoles. Intracellular lipid can be delivered to the lysosome by macroautophagy (lipophagy)40. However, having shown that BTN-coated secreted lipid droplets were being taken up by the alveolar epithelium, we looked for further evidence of endocytic uptake.

As demonstrated by BTN staining in Fig. 6, MFGs observed in the alveolar lumen with TEM are surrounded by a thin rim of plasma membrane-encased cytosol and the MFG membrane (Fig. 7a). This latter structure is acquired by the lipid droplet during the secretion process from the apical surface of the epithelium13 and is direct evidence that intracellular lipid droplets are taken up by an endocytic pathway. An indication of macroautophagy is seen at the plasmalemma, where membranous ruffles capture luminal fluid (Fig. 7b). In agreement with this, macroautophagosomes, some containing lipid droplets, are seen within the epithelium (Fig. 7c). However, large MFGs that have come from the alveolar lumen are also taken up by phagocytosis. MFGs are seen inside tightly fitting phagosomes within the epithelium (Fig. 7d–f). The luminal origin of fat droplets (that can measure 8 μm in diameter) is confirmed by the presence of the MFG membrane that is found in close proximity to the limiting phagosomal membrane (Fig. 7f). The highly phagocytic nature of involuting mammary gland epithelium is seen clearly in cathepsin L KO mice that, in contrast to control animals, cannot efficiently degrade cell corpses within the phagolysosome, thereby allowing...
Figure 8 A schematic model illustrating the role of Stat3 in regulating the transition from a secretory to a phagocytic cell phenotype at the onset of involution. Subsequent cell death ensues due to LMP caused by an increase in lysosomal size and generation of free fatty acids from hydrolysis of phagocytosed MFGs within lysosomal vacuoles and leakage of cathepsins into a Spi2a cathepsin inhibitor-depleted cytosol.

Figure 8

**DISCUSSION**

Cell death during involution of the mammary gland is a genetically controlled process that is both dramatic in scope and efficient in execution. Although assumed to be an apoptotic process, our previous work suggested a non-apoptotic mechanism that required both Stat3 and leakage of cathepsins from lysosomes through LMP (ref. 17). Many mechanisms of LMP in different cell types have been described in vitro, often with conflicting results and no consensus has been reached. We have thus focused on deciphering the mechanism of LMP in the in vivo context of mammary gland involution where cell death is a developmentally regulated physiological process.

On examination of involuting mammary tissue, we noted the association of cell death with the formation and fusion of large cytosolic vacuoles, many of which contained triglyceride, milk protein and degraded membranes. Furthermore, the appearance of these vacuoles requires Stat3, suggesting that this is a transcriptionally regulated event. Importantly, we show here that involution of other tissues such as prostate and uterus is characterized by similar histological hallmarks suggesting that formation of vacuoles and their fusion with lysosomes, leading to leakage of their cathepsin protease contents, could be a conserved mechanism of developmental cell death in hormone-dependent tissues.

The presence of vacuoles has previously been described in some forms of caspase-independent cell death in a variety of organisms. One of the earliest descriptions is of cells dying during metamorphosis of insect intersegmental muscle cells. The role of vacuoles in plant cell death, during the formation and elimination
of tissue, is well established and is associated with release of hydrolases from collapsed lytic vacuoles \(^{47,48}\), and vacuolization is a prominent feature of dying Dictyostelium discoideum cells \(^{49}\). More recently, an activated form of the H-Ras oncoprotein was shown to induce extensive vacuolization by macropinocytosis with subsequent cell death in cultured carcinoma cells. This process was named methuosis \(^{50}\). Furthermore, a small molecule named Vacquolin-1 was shown to induce catastrophic vacuolization in glioblastoma cells \(^{51}\) highlighting the utility of such an approach for killing apoptosis-resistant cancers.

Interestingly, although vacuolization of itself can kill, delivery of triglyceride to lysosomes accelerates this process. Eph4 mammary epithelial cells die 72 h after the induction of extensive vacuolization. By comparison, the addition of milk to these cells can induce cell death within 24 h. In vivo, dying mammary cells are shed into the alveolar lumen within 12 h of milk stasis and extensive cell death is apparent by 24 h involution. Thus, we suggest that the genetic control of vacuolization, coupled with regulated uptake of triglyceride, is a potent inducer of cell death in mammary tissue. Furthermore, many diseases such as non-alcoholic liver disease, obesity and diabetes are characterized by cellular uptake of lipid \(^{52}\). Thus, this mechanism of cell death may be used more widely.

This work delineates a fundamental mechanism for LMP in vivo. The role of vacuolization in this process is intriguing and may signify that the late evolving mammary gland has hijacked an ancient mechanism embraced by social amoebae and the plant kingdom for regulated cell death. Last, changes to the lysosomal compartment during mammary gland involution are reminiscent of changes in cancer cells, many of which are refractory to apoptosis and frequently harbour constitutively active Stat3 (ref. 53). Thus, understanding the mechanism of LMP, and the critical role of Stat3 in this process, will provide new approaches to cancer therapy.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

T.J.S. and B.L-L. carried out most of the experiments, H.K.R. contributed the cathepsin L \(^{-/-}\) tissue samples, A.R-M. provided the prostate samples, J.S. carried out the TEM and immunogold analysis and assisted in data interpretation. T.J.S., B.L-L. and C.J.W. designed the work, analysed the data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Animal husbandry. C57BL/6J wild-type mice were used for Lipidostained sections, lipid analysis by thin-layer chromatography (TLC) and in electron microscopy experiments. Stat3fl/fl (control) or Stat3fl/fl.BlgCre (Stat3fl/fl) KO mice are on a C57L/6J background and were generated by crossing mice expressing loxP-flanked stat3 (ref. 54) with mice containing a β-lactoglobulin-promoter-driven Cre gene55. Other mouse strains were used: cathepsin L KO mice46 and MEGF-E8 KO mice expressing a β-galactosidase-containing fusion protein under the control of the endogenous Mfge8 promoter47 (both strains on a C57L/6J background). Prostate sections were taken from NOD scid gamma mice (males aged between 4 and 6 months). Uterus samples were from 129 (involuting) or MF1 (control) background strain female mice which were between 4 and 6 months old. The mice were bred in regularly individually ventilated cages with food and water ad libitum and environmental enrichment. For mammary gland studies, virgin female mice between the ages of 7–10 weeks old were mated and males subsequently removed before birth to avoid second pregnancies. For invasion studies, pups were removed at 10 days of lactation and dams killed at indicated time points. At least three mice were used for each time point in every experiment, with precise numbers indicated in figure legends. Animals were randomized between the groups and within cohorts of the same genotype. No statistical method was used to predetermine sample size. The investigators were blinded where possible, including the histological counting undertaken for Fig. 6b. All animal experimentation was carried out in accordance with Animal (Scientific Procedures) Act 1986 and local ethical committee approval. Mouse were euthanized by dislocation of the neck, or terminal anaesthesia for TEM analysis.

Cell culture. EpH4 cells were maintained in DMEM (Life Technologies) media supplemented with 10% FCS (Sigma) at 37 °C in a humidified atmosphere of 5% CO2. Milk collected from 24 h involluting mammary glands was incubated on cells diluted to 10% in media. Four hundred microlitres was used in a 4 cm2 well. Fatty acids purchased from Sigma-Aldrich (stearate, S3381, palmitate, P9767 and oleate O7501) were dissolved in ethanol and used at the concentrations indicated in the figure legends. Cells were treated with milk and fatty acids for 8 h or overnight as indicated. Cytotoxicity was assayed by trypan blue exclusion. For OSM stimulation, cells were stimulated at 50% confluency with a final concentration of 25 ng ml−1 recombinant mouse oncostatin M (495-MO, R&D Systems) or carrier (0.0001% BSA in PBS). Medium was renewed every 48 h. Cells were pre-treated with 200 nM bafilomycin A1 (Sigma) for 1 h before milk addition for a further 8 h.

Immunohistochemistry. For histological analysis on paraffin-embedded tissues, abdominal glands were fixed in 4% formaldehyde in PBS overnight at room temperature. Glands were transferred to 70% ethanol and stored at −20°C until embedding in wax and sectioned at 5 μm. Perfuse-fixed glands were embedded in wax or OCT for cryosections. Paraffin-embedded sections were de-paraffinized in xylene, dehydrated and antigen retrieval was performed by boiling in 10 mM tri-sodium citrate embedding in wax and sectioned at 5 μ. Perfuse-fixed, cryosectioned tissues at 1:200. Nuclei were counterstained with Hoechst 33342 (Merk Millipore) in a Synergy HT Multi-Detection Microplate Reader (excitation 380 nM, emission 442 nM; Bio-TEK). For assessment of cytosolic cathepsin activity was measured after 10 min on ice with intermitent vortexing. The samples were then spun down (90 s, 13,000 r.p.m., 4°C) and the supernatant was quickly transferred to a new tube to be used in downstream cathepsin activity assays. For each condition 175,000 cells were also extracted in 0.1% Triton X-100 as above to obtain total cell lysates for assessing total cathepsin activity.

Cathepsin activity assay. To measure cytosolic cathepsin activity subcellular fractionation was carried out on mammary glands as described above. Protein levels were assessed with the BCA Protein Assay (Thermo Fisher Scientific) and equal amounts of protein were separated on a total of 200 μl cathepsin reaction buffer (sodium acetate 50 mM, EDTA 8 mM, dithiothreitol 8 mM and Pefabloc subcellular fractionation buffer 1 mM, at pH 7.4). Cathepsin B+L activity was measured after incubation (30 min, 37°C) with the fluorescent substrate Z-Phe-Arg-AMC (50 μM; Merck Millipore) in a Synergy HT Multi-Detection Microplate Reader (excitation 380 nM, emission 442 nM; Bio-TEK). For assessment of cytosolic cathepsin activity in EpH4 cells treated with free fatty acids 10 μl of digitoxin-extracted lysates was added to a total of 200 μl cathepsin reaction buffer (sodium acetate 50 mM, EDTA 8 mM, dithiothreitol 8 mM and Pefabloc subcellular fractionation buffer 1 mM, at pH 7.4). Cathepsin B+L activity was measured after incubation (30 min, 37°C) with the fluorescent substrate Z-Phe-Arg-AMC (50 μM; Merck Millipore). Amounts of free fatty acids, as determined by an oleic acid standard (Sigma), were quantified by using area under the curve analysis in ImageJ software. Amounts of free fatty acid were converted to tissue concentration using the following calculations: average micrograms of free fatty acid on TLC plate = 4.79 μg; mg of tissue was loaded on each lane of the TLC plate; therefore, there was 4.79 μg per 20 mg or 0.24 μg per milligram of tissue (0.24 μg mg−1); as 1 mg of tissue approximates 1 μl of volume, 0.24 μg mg−1 = 0.24 μg μl−1; 0.24 μg μl−1 × 10 = 24.00 μg ml−1 or 0.24 μl−1; oleic acid Mw = 282 g mol−1; 0.24 μl−1/282 g mol−1 = 0.85 μl M.

Subcellular fractionation of mammary gland tissues. Lymph node dissected four number glands were minced using scalpels on ice before homogenization in a tight-fitting handheld homogenizer (12 strokes) in 1 ml of subcellular fractionation buffer (HEPES-KOH 20 mM, sucrose 250 mM, KCl 10 mM, MgCl2, 1.5 mM, EDTA 1 mM, EGTA 1 mM, dithiothreitol 8 mM, Pefabloc 1 ml (Fluka), at pH 7.5). Debris and nuclei were pelleted in a tabletop centrifuge (Heraeus Fresco-17 centrifuge) at 750g (3,500 r.p.m.) for 10 min, 4°C. The supernatant was spun at 100,00g (12,900 r.p.m.) for 15 min at 4°C, to pellet organelles. The supernatant, containing cytosolic and extracellular components was spun at 100,000g (40,000 r.p.m.) for 1 h at 4°C in a Beckman Coulter Optima L-100 XP Ultracentrifuge to remove microsomes and collected as the cytosolic fraction.

Digitoxin extraction of EpH4 cells. Cytosolic extraction using digitoxin was performed as previously described48 with the exception that digitoxin was used at 25 μg ml−1. Briefly, cells were seeded in six-well Nunc Round Bottom Plates (Nunc, 150,000 cells per well) in DMEM, 10% FCS (Gibco/Sigma) and the next day treated with free fatty acids as indicated. Cells were collected and counted, with 175,000 cells pelleted and extracted with 300 μl of 25 μg ml−1 Digitoxin (300410, Calbiochem) in subcellular fractionation buffer (HEPES-KOH 20 mM, sucrose 250 mM, KCl 10 mM, MgCl2, 1.5 mM, EDTA 1 mM, EGTA 1 mM, dithiothreitol 8 mM, Pefabloc 1 ml (Fluka), at pH 7.5), a concentration that had been determined to be optimal (concentrations ranging from 12.5 to 50 μg ml−1 were tested and 25 μg ml−1 showed extraction with minimal lysosomal damage). Cells were incubated for 10 min on ice with intermitent vortexing. The samples were then spun down (90 s, 13,000 r.p.m., 4°C) and the supernatant was quickly transferred to a new tube to be used in downstream cathepsin activity assays. For each condition 175,000 cells were also extracted in 0.1% Triton X-100 as above to obtain total cell lysates for assessing total cathepsin activity.

Lysosomal purification and leakiness assay. Lysosomes from EpH4 cells were purified using magnetic iron nanoparticles (EMG-508, Ferrotec) as previously described49. Briefly, EpH4 cells were seeded at a density of 3 × 105 in 15 cm tissue culture plates (168381, Nunc). The following day cells were labelled for 4 h in iron-nanoparticle-containing media (1:100) followed by a 2 h chase period in clean media. Cells were then scraped off in PBS, pelleted in a tabletop centrifuge (300 r.p.m., 4°C, 3 min) and the pellet was homogenized in a tight-fitting handheld homogenizer (5 strokes) in 700 μl subcellular fractionation buffer (HEPES-KOH 20 mM, sucrose 250 mM, KCl 10 mM, MgCl2, 1.5 mM, EDTA 1 mM, EGTA...
1 mM, dithiothreitol 8 mM, Complete protease inhibitor (Roche) at pH 7.5). The homogenate was spun at 750g (3,500 r.p.m., 4 °C, 10 min) to remove nuclei and debris, followed by a second 750g spin to ensure complete removal of contaminating heavy membranes. The resulting supernatant (pns; post nuclear supernatant) was transferred into a clean tube, loaded onto a magnetic rack and incubated for 1 h at 4 °C on a rocker. A 50 µl sample of pns was retained for analysis. Following incubation tubes were left on the magnet as the supernatant (sn) was removed (retaining 50 µl for analysis). Tubes were washed with 1 ml of subcellular fraction buffer three times. Following addition of the last wash tubes were removed and spun at 12,000 r.p.m. (13,800g, 4 °C, 15 min) to pellet magenta-containing lysosomes. Pellets were recombined in subcellular fractionation buffer and equal amounts (30 µl) were aliquoted into individual tubes. Fatty acids (or ethanol control) were added as indicated to a final concentration of 70 µM in a final volume of 60 µl. The lowest concentration of oleic acid that induced reproducible cathepsin leakage was 70 µM. Samples were either re-pelleted immediately (t = 0) at 12,000 r.p.m. (13,800g, 4 °C, 15 min) or incubated for 30 min (t = 30) at 37 °C under gentle agitation. Subsequently, the lysosomes were re-pelleted as above. The supernatant was removed and snap frozen. The pellet was resuspended in 60 µl RIPA buffer (50 mM Tris, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA and Complete protease inhibitor), and lysosomes were lysed on ice for 30 min with intermittent vortexing. Membranes were pelleted at 12,000 r.p.m. (13,800g, 4 °C, 15 min), the supernatant was removed and equal amounts of the resuspended pellet and the supernatant were analysed by western blot. pns and sn samples were pelleted at 12,000 r.p.m. (13,800g, 4 °C, 15 min) with the pellet resuspended in 60 µl RIPA buffer and processed similarly to above.

Immunoblotting. Lymph node dissected mammary tissue was snap frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Protein from mammary gland powder was extracted in RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA and Complete protease inhibitor (Roche), 1 mM Na,VO₃, and 1 mM NaF). Protein concentration was assessed with the BCA Protein Assay (Thermo Fisher Scientific) and equal amounts of protein (20 µg) were denatured and resolved on SDS-polyacrylamide gels. Immunoblotting was performed using standard techniques and antibody detection was achieved with enhanced chemiluminescence reagent (ECL, GE Healthcare). The following primary antibodies were used: rat anti-LAMP2 (ab13524, Abcam, 1:1,000), goat anti-cathepsin L (AF1029, R&D Systems, 1:1,000) and rabbit anti-BTN1A1 (a gift from I. Mather, 1:5,000).

LysoTracker red staining and flow cytometry. EphC4 cells were seeded on six-well Nunclon Delta Surface plates (Nunc, 150,000 cells per well) in DMEM, 10% FCS (Gibco/Sigma). Cells were treated with the indicated concentration of free fatty acids overnight and subsequently collected and resuspended in 500 µl culture medium. LysoTracker red DND-99 (Life Technologies, 100 nM) was added to the suspension and incubated at 37 °C in the dark for 30 min. Single-colour flow cytometry was carried out on a CyAn ADP flow cytometer (DakoCytomation), and the data were analysed using Summit 4.3 software (DakoCytomation).

Quantitative real-time PCR. Lymph node dissected mammary tissue was snap frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. RNA was extracted from tissue using TRIzol (Life Technologies) according to the manufacturer’s guidelines. RNA was quantified using a NanoDrop ND-1000 (NanoDrop Technologies) and cDNA was synthesized from up to 2 µg of RNA using the SuperScript III First-Strand Synthesis System (Life Technologies) and random hexamers (Life Technologies). SYBR green JumpStart TaqReadyMix (Sigma) was used for qRT-PCR, with reactions run in a CFX Connect Real Time PCR detection system (BioRad).

Delta Ct values were calculated by subtracting Ct values for cyclophilin A and relative expression was derived using the delta delta Ct method. Primers used were: cyclophilin A (housekeeping gene) forward, 5'-CTTTGGCCGCGGTCTCCCTT-3', reverse, 5'-CACCCCTGGCAGATGATCCTG-3'; Bmi1 forward, 5'-GCAGGGGAAGGAGTAGAG-3', reverse, 5'-ATGGACCCAATGGTGAGAAA-3'; Xdh forward, 5'-TGCAAGCCCTGAACACAC-3', reverse, 5'-AAGGCGGCTCA TACTTGAGA-3'.

Data analysis and statistics. Experimental repeats are detailed in the figure legends and in Supplementary Table 3. For electron microscopy on animal tissue samples, vesicular fusion events were rare, consistent with their transient nature. In all experiments there were no exclusion criteria, and no data were excluded. All normality and statistical tests were undertaken using GraphPad Prism v4.0a.
**Supplementary Figure 1** Cathepsin D and LAMP2 staining in the involuting mammary gland. (a) Staining for cathepsin D (red) in the lactating and involuting gland. Three animals were assessed per condition. (b) The pro-form of cathepsin D was detected at approximately 46 kDa and higher levels were present at 24 h involution compared to lactating mammary glands. There was no difference between control (C) and Stat3 knockout (KO). Lanes represent independent biological samples. (c) LAMP2 staining (red) is detected lining large vacuolar structures in the control 24 h involuting mammary gland but not in the lactating mammary gland. This becomes more apparent at 48 h and 72 h. In the Stat3 KO mammary gland, LAMP2-positive vacuoles are only seen from 48 h onwards. One animal per condition was analysed. (d) Confocal images displaying immunostaining for cathepsin D (red) is shown in grey-scale and merged with staining for triglyceride (lipidtox, green). Arrowheads show lipid droplets inside lysosomal vesicles. Three animals were used. Nuclei are visualised by Hoechst (blue). Scale bars = 20µm.
**Supplementary Figure 2** (a) Milk induces lysosomal lipid accumulation. Confocal images show Lysotracker red staining overlapping with that for triglyceride (lipidtox, green) (colocalisation shown by arrowheads). Four representative images from two independent experiments displayed. Nuclei are stained with Hoechst (blue). Scale bars first three rows = 1 \( \mu m \), 4th row = 2 \( \mu m \).

(b) Free fatty acids induce cell death. Staining for triglyceride (lipidtox, green) in EpH4 cells treated overnight with 1 mM oleic acid (OA) and palmitic acid (PA) showing lipid accumulation in EpH4 cells. Nuclei are stained with Hoechst (blue); Scale bars = 10 \( \mu m \). Brightfield images showing cytotoxicity in fatty acid treated EpH4 cells; Scale bars = 100 \( \mu m \). (c) Fatty acid induced cell death was assessed by trypan blue positivity. Means +/- s.e.m. from n = three independent experiments with 2-3 technical replicates performed per experiment shown (*\( p \)<0.05, one-way ANOVA, Dunnett’s Multiple Comparison post-test). Raw values can be found in the corresponding worksheet in Supplementary Table 3.
Supplementary Figure 3 (a) Optimisation of digitonin cytosol extraction assay. EpH4 cells were extracted with increasing concentrations of digitonin and cathepsin activity assayed over time with the synthetic substrate Z-Phe-Arg-AMC. Total activity was measured by extraction with 0.1% TritonX-100. A digitonin concentration of 25 µg/ml was selected for cytosol extraction assays. All data is plotted, optimisation performed on one occasion. (b) Fatty acids induce deacidification of the lysosomal compartment. A population of low Lysotracker® Red staining (region R8) is induced with 1 mM OA or PA, indicative of de-acidification of lysosomes. Cells treated with 1 mM PA also display a population with higher lysotracker red staining. Quantification of n = four independent experiments as described in (b). Means +/- s.e.m. are shown, associated statistics source data can be found in the corresponding worksheet in Supplementary Table 3. (c) OA and PA (500 µM) treated cells showing populations of Lysotracker® Red fluorescence (R13), with low levels indicative of de-acidification of lysosomes (n = 1 (PA) and 2 (OA) independent experiments raw values can be found in the corresponding worksheet in Supplementary Table 3).
**Supplementary Figure 4** Fatty acid treatment does not result in Bax translocation to lysosomes. (a) EpH4 cells were transfected with GFP-Bax (green) and treated with ethanol, 500 μM oleic acid (OA), palmitic acid (PA) overnight prior to fixation and LAMP2 immunostaining (red). Cells were treated with 30 ng/ml TNFα and 10 μg/ml cycloheximide for 6.5 h in serum free conditions as a control. No obvious lysosomal co-localisation was observed under these conditions. Two representative examples from all conditions displayed, experiment performed once. (b) GFP-Bax transfected EpH4 cells treated with TNFα and cycloheximide or 1 μM staurosporine as indicated for 6.5 h were fixed and immunostained for AIF (red) and show mitochondrial localisation of Bax under these conditions. Nuclei are stained with Hoechst (blue). Scale bars = 10 μm.
Supplementary Figure 5 Uncropped blots and TLC plates showing experiments that appeared in figures as well as biological replicates that were not shown in main figures.
Supplementary Table 1

| Fatty acid common name | No. of Carbon Atoms: No. double bonds | Molar % |
|------------------------|---------------------------------------|---------|
| Caprylic acid          | 8:0                                   | 0.4     |
| Capric acid            | 10:0                                  | 7.5     |
| Lauric acid            | 12:0                                  | 11.9    |
| Myristic acid          | 14:0                                  | 15.8    |
| Palmitic acid          | 16:0                                  | 32.6    |
| Palmitoleic acid       | 16:1                                  | 3.7     |
| Stearic acid           | 18:0                                  | 1.8     |
| Oleic acid             | 18:1                                  | 17.8    |
| Linoleic acid          | 18:2                                  | 8.5     |

Data taken from Smith, J. et al (1968)

Ref: (28) Smith, S., Watts, R. & Dils, R. Quantitative gas-liquid chromatographic analysis of rodent milk triglycerides. J. Lipid Research 9, 52-57 (1968).
Supplementary Table 2

|                | Average dCt values | Fold change in expression between Control and Stat3 KO |
|----------------|--------------------|--------------------------------------------------------|
|                | Btn1a1 Xdh         | Btn1a1 Xdh                                             |
| Control        | 2.5879813 0.4488334 | 2.58 0.45                                               |
| Stat3 KO       | 1.2946839 -0.849463 | 1.29 -0.85                                               |

Supplementary table 2 Quantitative PCR analysis of Btn and Xdh. QPCR was performed on control and Stat3 KO mammary glands at 24 h involution. Btn and Xdh were upregulated in Stat3 KO tissue 2.45 and 2.46 fold (P<0.03 and P<0.02, Student's t-test), respectively. n = 4 control and 5 Stat3 KO animals. Delta Ct values were calculated by subtracting Ct values for Cyclophilin A. Statistics source data can be found in the corresponding worksheet in Supplementary Table 3.
Supplementary Table 3  Statistics Source data. All raw numerical data from experiments are summarised in sheets corresponding to their associated figures. N numbers are provided in addition to the data and statistics derived from the indicated number of biological replicates.