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Stat3 and the PI3K regulatory subunits p55α and p50α regulate autophagy *in vivo*

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Abstract

Mammary gland involution involves a process that includes one of the most dramatic examples of cell death in an adult mammalian organism. We have previously shown that Stat3 regulates a lysosomal pathway of cell death in the first 48 hours of involution and induces lysosome leakiness in mammary epithelial cells. Interestingly, Stat3 is associated also with the striking induction of autophagy which occurs concomitantly with cell death, presumably as a transient survival mechanism. The PI3 Kinase regulatory subunits p55α and p50α are dramatically and specifically upregulated at the transcriptional level by Stat3 at the onset of involution. We show here that ablation of either Stat3 or p55α/p50α *in vivo* affects autophagy during involution. We used two different cell culture models, normal mammary epithelial cells and mouse embryonic fibroblasts, to further investigate the role of p55α/p50α in autophagy regulation. Our results demonstrate a direct role for p55α/p50α as inhibitors of autophagy mediated by p85α. Thus Stat3 and its downstream targets p55α/p50α are key regulators of the balance between autophagy and cell death *in vivo*.

Keywords

Stat3; p55α/p50α; autophagy; mammary gland; PI3K

INTRODUCTION

Post-lactational regression, or involution, of the mammary gland is characterised by one of the most dramatic examples of cell death in an adult mammalian organism and occurs with every pregnancy/lactation/involution cycle [1]. Alveolar mammary epithelium, which develops during pregnancy, is removed upon cessation of lactation by a programme of cell death coupled with tissue remodelling that returns the gland to its pre-pregnant state. This provides an ideal experimental system in which to study cell death in a physiological context

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AUTHOR CONTRIBUTIONS

SP planned and performed experiment, analysed the data and wrote the paper; BL-L, TJS and HKR performed experiments and analysed the data; CRK provided the $p55a^{-/-}/p50a^{-/-}$ mice and CJW planned experiments, analysed the data and wrote the paper.

in vivo. We have shown that conditional deletion of the transcription factor Stat3 severely delays involution and abrogates cell death [2].

Although assumed to be apoptosis, recent work from our laboratory has demonstrated that cell death during involution is not classical apoptosis but occurs rather by a lysosomal mediated, and executioner caspase independent, pathway of cell death (LM-PCD) [3].

One intriguing aspect of cell death during involution is that it occurs in two phases; an initial reversible phase that lasts for up to 48h in the mouse and is initiated by LIF mediated activation of Stat3 [4] and a second irreversible phase from 48h that is regulated by oncostatin M (OSM) activation of Stat3 [1, 5]. Interestingly, expression of the receptor for OSM is regulated by Stat3 [6] and conditional deletion of Stat3 extends the reversible phase to at least 6 days [7]. During 1st phase involution, dying cells detach from the alveolar epithelial wall and are shed into the lumen, presumably to maintain the integrity of the alveoli and their functionality. Shed cells are then phagocytosed by the viable epithelium. Cell death during this phase is a stochastic event and it seems likely that a subpopulation of the alveolar cells could use a survival mechanism in order to provide the reversibility of this phase. In 2nd phase involution, the remaining alveolar epithelial cells die rapidly *in situ* in concert with extensive tissue remodelling. A previous study highlighted the induction of autophagy at 24h involution [8] but the mechanism of induction and the role of autophagy are not clear. Here we propose a pro-survival role of autophagy in the 1st phase of involution.

Stat3 has been implicated in regulating autophagy although its precise role is not clear and depends on the localisation of Stat3. While Stat3 phosphorylation in neuroblastoma H4 cells has been shown to inhibit autophagy [9], cytosolic unphosphorylated Stat3 inhibits autophagy by binding to PKR in U20S cells [10]. Thus, the function of Stat3 seems to be context and phosphorylation dependent.

Stat3 regulates the expression of a number of genes during involution and we have demonstrated that unique binding sites for Stat3 are present in the first intron of the *pik3r1* gene which encodes three regulatory subunits of the class IA PI(3)Kinases, p85α, p55α and p50α. The small subunits, p55α and p50α, are strikingly upregulated at 24h involution and mediate cell death [11]. Although Stat3 and Stat5 can bind the same genomic sequences [12] there is no overlap between Stat3 and Stat5 target genes in mammary epithelial cells [6] and Stat5 has recently been shown to regulate only the p85α subunit [13]. These subunits all share a common carboxy-terminal domain which encompasses the p110 catalytic subunit binding site but have unique amino-terminal sequences [14]. While that of p85α comprises a SH3 and a BCR-homology (BH) domain which bind to PTEN, the negative regulator of PI3K, enhancing its lipid phosphatase activity [15] the p55α and p50α subunits have much smaller unique N-terminal sequences of 34aa for p55α and 6aa for p50α. Specific ablation of these subunits by deleting exons IB (p50α) and 1C (p55α) results in improved insulin sensitivity and protection from obesity-induced insulin resistance [16]. However, the function of these subunits is still somewhat enigmatic.

A role for Stat3 in autophagy induction in mammary gland has not been investigated. This, coupled with our previous demonstration of a role for Stat3 as an inducer of p55α and p50α expression specifically at the onset of involution, prompted us to investigate the role of Stat3 and these subunits in autophagy regulation during involution.

RESULTS

Mammary gland involution is accompanied by an early induction of autophagy

Autophagosomes are characterised by double membranes [17] that are evident by electron microscopy (EM). Although evidence that autophagy occurs in mammary gland has been presented previously [8, 18] the mechanism of induction has not been addressed. Initially we confirmed the induction of autophagy at early stages of mammary gland involution, by examining thin sections of 10d lactation and 24h involution mammary tissue by EM and noted a significant increase in the number of autophagosomes in involuting tissue sections (Fig. 1A and B). We next measured changes in RNAs associated with autophagy and observed that *Lc3a*, *Lc3b*, *p62* and *Beclin1* were all upregulated at least 3-fold at 24h involution (Fig. 1C). This is accompanied by appearance of p62 aggregates at 24h involution (Fig. 1D), which could reflect this increase in transcription. The induction of autophagy is further supported by the increase in LC3B lipidation (LC3B II), another marker of autophagy, during the first 48h [17] (Fig. 1E). The decline in p62 aggregates and LC3B II levels from 48h involution, suggests that autophagy has a specific role in the reversible phase of involution. However the high levels of LC3B II, coupled with accumulation of p62 puncta, indicate that although autophagy is induced at the onset of involution, it could be accompanied by a defect in the resolution of the autophagic process possibly due to disruption of lysosomal function, which we have shown to occur during involution [3].

Autophagy is affected by the absence of Stat3

We have previously shown that Stat3 is a master regulator of cell death in mammary gland involution [2] and that cell death occurs by lysosomal membrane permeabilisation (LMP), independently of executioner caspases [3]. Recent work from other laboratories has implicated Stat3 in the regulation of autophagy [9, 10]. To clarify the role of Stat3 in autophagy and to determine if Stat3 is required for the induction of autophagy observed at the onset of involution, we analysed glands from Stat3 controls (*Stat3*$^{fl/fl}$;*BLG-Cre*$^{-}$, hereafter referred to as *Stat3*$^{+/+}$) and Stat3 depleted mice (*Stat3*$^{fl/fl}$;*BLG-Cre*$^{+}$, in which Stat3 is depleted from the epithelium during lactation, hereafter referred to as *Stat3*$^{-/-}$) for autophagy markers. The increase in number of autophagosomes at 24h involution was severely abrogated in the absence of Stat3 (Fig. 1B). The p62 aggregates observed at 24h involution are reduced in both number and size in the *Stat3*$^{-/-}$ glands compared to controls (Fig. 1G) and this could reflect both reduced expression and/or increased autophagic flux. This latter contention is supported by the observation that LC3B lipidation is profoundly diminished in the Stat3 depleted glands (Fig. 1E). Interestingly gene expression was unaffected by Stat3 deletion apart from a small (2-fold) decrease in p62 expression (Fig. 1F). These data highlight a role for Stat3 in regulating autophagy *in vivo* in the mammary gland.

The Stat3 target genes p55α/p50α regulate autophagy during mammary gland involution

We have shown previously that Stat3 upregulates expression of the PI3K regulatory subunits p55α/p50α at the onset of involution [19]. Accordingly, expression of the two small subunits p55α/p50α but not p85α is markedly reduced in *Stat3*$^{-/-}$ glands at the time of autophagy induction (Fig. 2A). We therefore sought to determine if the small subunits play a role in regulating autophagy by analysing *p55α*$^{-/-}$/*p50α*$^{-/-}$ mice [16], which display delayed involution and diminished cell death [11]. Expression of autophagy regulatory genes was investigated and we found that only *Lc3b* was significantly downregulated in the absence of p55α/p50α expression (Fig. 2B). Interestingly, analysis of *p55α*$^{-/-}$/*p50α*$^{-/-}$ mammary tissue revealed a substantial reduction of LC3B lipidation at 24h and 48h of involution with a similar pattern to the *Stat3*$^{-/-}$ tissues (Fig. 2C). This correlates with a striking decrease in the size and number of p62 puncta (Fig. 2D). We have previously shown that p55α/p50α are expressed predominantly in the epithelium and are not expressed in the adipose cells of the mammary gland [11]. Since infiltrating immune cells play a role mostly in the second phase of involution, we suggest that the phenotype observed is a consequence of the absence of the small subunits in the epithelial compartment.

There are two possible explanations for the results observed *in vivo* in the *Stat3*$^{-/-}$ and *p55α*$^{-/-}$/*p50α*$^{-/-}$ glands: in the control glands, accumulation of LC3B II and p62 puncta could indicate induction of autophagy or could result from a failure to clear autophagosomes. We favour the latter interpretation because we have already shown that lysosomes become leaky during involution, which would abrogate the fusion with autophagosomes [3]. This suggests that p55α/p50α block the resolution of autophagy downstream of Stat3. However, it cannot be excluded that both induction of autophagy and a partial block in fusion with lysosomes/late endosomes contribute to the phenotype observed in involution and both could be modulated by the absence of p55α and p50α.

OSM-dependent upregulation of p55α/p50α increases autophagic flux while augmenting the number of p62 aggregates

Since autophagic flux is difficult to address *in vivo* we investigated the role of Stat3 and p55α/p50α in the EpH4 mammary epithelial cell line that we have previously shown accurately mimics LM-PCD mechanisms *in vivo* [3]. While LIF is the physiological inducer of p55α/p50α expression in the mammary gland [20], and other Stat3-activating cytokines could potentially induce the expression of these subunits, we observed that OSM activates Stat3 and induces high levels of p55α and p50α expression in EpH4 cells (Fig. 3A). OSM also induced an increase in LC3B II with levels reaching a maximum at 72h post-treatment (Fig. 3B and C). Interestingly treatment with bafilomycin A1, which blocks the function of the vacuolar ATPase and thus abolishes the ability of the lysosome to fuse with autophagosomes [17], induced rapid accumulation of LC3B II and had an additive effect on OSM treatment (Fig. 3B). This suggests that OSM induces autophagy. OSM treatment also resulted in the accumulation of numerous large puncta of p62 at 24h. Both OSM and vehicle-treated cells showed a similar increase upon bafilomycin A1 treatment (Fig. 3E) although protein levels were mostly unchanged (Fig. 3D). This is indicative of a partial block of lysosome function, a notion supported by our observation that OSM induces leakage of LysoTracker™ in EpH4 cells [3]. Since OSM treatment recapitulates the

autophagy phenotype observed *in vivo*, we can speculate that involution is characterized by an induction of autophagy simultaneously with a partial block of autophagosome/lysosome fusion due to leaky lysosomes. However, we were unable to dissect the direct contribution of the small subunits in EpH4 cells due to an inability to specifically silence their expression in these cells as a consequence of the very short domains of isoform-specific sequence (data not shown).

p55α/p50α inhibit autophagy in primary MEFs by competition with p85α

To investigate a general role of p55α and p50α in the regulation of autophagy in other cell types, we analysed primary mouse embryonic fibroblasts (MEFs) obtained from control and $p55\alpha^{-/-}/p50\alpha^{-/-}$ mice. Starvation of both control and $p55\alpha^{-/-}/p50\alpha^{-/-}$ MEFs resulted in the induction of LC3B lipidation within 2h of treatment, as clearly shown by the accumulation of LC3B II after bafilomycin A1 treatment of starved cells as compared to controls (Fig. 4A). Interestingly however, the levels of LC3B II in the unstarved $p55\alpha^{-/-}/p50\alpha^{-/-}$ MEFs are higher compared to the controls (Fig. 4A and quantification below), demonstrating that the primary role of the small subunits is to inhibit basal autophagy and the higher levels of LC3B II in bafilomycin Al treated starved cells reflect this higher basal level of autophagy in $p55\alpha^{-/-}/p50\alpha^{-/-}$ cells. Analysis of immortalised control and $pik3r1^{-/-}$ MEFs, which are depleted of the longer p85α subunit as well as p55α and p50α, revealed no difference when comparing the two cell lines upon starvation (Fig. 4B). These data point to a role for the small subunits in regulating the extent of starvation-induced autophagy mediated by the longer subunit p85α.

DISCUSSION

Cell death during mammary gland involution is extensive and removes approximately 80% of the mammary epithelium in the space of a few days in the mouse. The programmed cell death occurring at the end of lactation is counteracted by a temporary survival mechanism to allow survival of sufficient alveolar cells that can recommence milk production if offspring are returned to the mother up to 48 hours after separation in the mouse or longer in larger mammals. We propose here that autophagy is such a survival mechanism during the early phase of mammary gland involution and that these two opposing events are co-ordinately regulated by Stat3 (Fig. 4C).

Recent work has demonstrated a role for Stat3 in regulating autophagy [9, 10]. On the one hand LIF-mediated activation of Stat3 in human neuroblastoma H4 cells inhibits LC3B II accumulation in an mTORCl independent manner [9]. On the other hand, genetic deletion of Stat3 in hepatocytes *in vivo* induces autophagy while retention of Stat3 in the cytosol resulted in autophagy suppression [10]. Thus, the function of Stat3 is phosphorylation and context dependent. Interestingly, starvation or rapamycin treatment of HeLa cells have been shown to lead to Stat3 activation, that is necessary for the production of IL-6, which in turn promotes survival and proliferation in cancer cells [21].

We, and others, have shown that autophagy is induced at the onset of involution. Autophagic vesicles are present in the mammary gland as early as 24h of involution (Fig. 1A–C), concomitantly with the appearance of p62 (Fig. 1D) and LC3B puncta [8]. If autophagy

induction in the mammary gland represents a survival mechanism to guarantee the reversibility of involution and a halting of cell death events, it would be expected that disruption of autophagy mediators in the mammary gland would lead to increased cell death. Previous work has shown an accumulation of shed cells in the alveoli of mice carrying a monoallelic deletion of Beclinl or deficient for Atg7 in the first 72h of involution, which the authors interpreted as a compromised ability of mammary cells to phagocytose, and thus clear, dead cells present in the lumen [8]. This is indeed possible although we favour a different interpretation and suggest that the accumulation of dead cells observed in *Beclin1*$^{+/-}$ and *Atg7*-deficient glands indicates an increased rate of cell death in the 1st phase of involution. The class III PI3K Vps34 is involved in autophagy and has been shown to be fundamental in regulating phagosome maturation [22]. In the past few years, however, a number of studies have underlined a function for members of class IA PI3K in autophagy regulation. One catalytic subunit, pll0β, has been shown to positively regulate autophagy both *in vitro* and *in vivo* in liver and heart in a kinase-independent manner, p110β, associates with the Vps34-Vpsl5-Beclin l-Atgl4L complex to increase the generation of PtdIns(3)P, thus promoting autophagy [23]. A different catalytic subunit, p110α, is required for the delivery of the lysosomal membrane proteins LAMP-1 and LAMP-2 to Rab7-positive vesicles during vacuole maturation in human leukemic monocytes, although phagocytosis does not seem to be substantially affected by the absence of p110α in these cells [24]. In addition, degradation of the regulatory subunit p85β, through interaction with the F-box protein FBXL2, inhibits serum starvation-induced autophagy in NHF fibroblasts [25]. Furthermore, the p85α regulatory subunit has been shown to bind Rab5 [26], which is an important component of the vesicle trafficking machinery [27].

Given the role of Stat3 in regulating cell death and expression of the class I PI3K regulatory subunits p55α and p50α during mammary gland involution, we examined both Stat3 and p55α/p50α deficient involuting mammary glands for evidence of altered autophagy. Conditional deletion of Stat3 disrupted autophagy in the first 48h of involution (Fig. 1) and this phenotype was mirrored in the p55α/p50α deficient glands (Fig. 2). Only minor changes in *p62* and *Lc3b* gene expression were detected suggesting that direct transcriptional regulation of these proteins by Stat3 and p55α/p50α is not a major factor in induction of autophagy. In both these strains, cell death is also abrogated [2, 3, 11]. In order to tease apart the mechanism, we utilised EpH4 cells treated with OSM to induce Stat3 activity as a surrogate for involution. Elevated levels of LC3B II were observed upon OSM stimulation, and a corresponding appearance of p62 puncta suggested an induction and/or a block of autophagy (Fig. 3). Since an additive effect was achieved with bafilomycin Al treatment this suggests that at least part of the response to OSM is an induction of autophagy. However, although a considerable number of p62 puncta were apparent upon treatment with OSM, we did not observe a notable difference with the addition of bafilomycin Al plus OSM compared to bafilomycin Al alone, suggesting a considerable block in the resolution of autophagy in the presence of OSM. Moreover, immunoblotting revealed a failed turnover of p62 further supporting an impairment of the later stages of autophagy, as lysosomes become leaky with prolonged treatment with OSM [3].

In order to look more generally at the role of p55α/p50α in autophagy, we investigated starvation- rather than stress-induced autophagy utilising MEFs derived from *p55α*$^{-/-}$/*p50α*$^{-/-}$ mice (Fig. 4). Although starvation is able to induce LC3B lipidation in both control and *p55α*$^{-/-}$/*p50α*$^{-/-}$ MEFs, the level of LC3B II was enhanced in the absence of the subunits, indicating their inhibitory effects on autophagy. As we have previously suggested, one mechanism of action of the small subunits is direct competition with p85α for binding to partner proteins [28]. Thus we utilized MEFs deleted for all three subunits and found that the enhancing effect of deletion of the small subunits is abrogated. This suggests that p55α/p50α compete with, or block the ability of p85α to contribute to autophagy.

Expression of the p55α and p50α regulatory subunits is not limited to mammary gland with variable levels of each expressed in a wide range of tissues (data not shown). This raises the possibility that autophagy in other tissues could be modulated by inducing either p55α or p50α. Given the very small unique N-terminal domains, and our inability to detect any binding partners other than the catalytic subunits (data not shown), we propose that the small subunits could function in autophagy regulation in response to starvation or other stressors that mediate Stat3 activity by competing with p85α. In mammary gland, we propose that Stat3 regulates involution by inhibiting the pro-survival, autophagic process through two mechanisms: inducing the expression of p55α and p50α and disrupting lysosomal function [3].

MATERIALS AND METHODS

Animal husbandry

p55α/p50α and *Stat3* null mice have been generated previously (respectively [16] and [2]). C57BL/6 mice were purchased from Harlan labs (Indianapolis, IN, US). The mice were bred in regular cages with food and water ad libitum. Virgin female mice, 8–14 weeks old, were mated and males were subsequently removed before birth to avoid second pregnancies. Dams were killed at indicated time points. For involution studies, pups were removed at 10 days of lactation. Unless otherwise stated, at least three mice were used for each time point in every experiment. All animals were treated according to local ethical committee and the UK Home Office guidelines and killed through CO2 asphyxiation or dislocation of the neck.

Transmission electron microscopy

Transmission electron microscopy was carried out as previously described [3].

Cell culture

Embryos were dissected 13.5 days post coitum for MEF derivation and primary MEFs were used within the first 5 passages in culture. *pi3kr1*$^{-/-}$ 3T3 MEFs were a kind gift of Dr. David A. Fruman. EpH4, primary MEF and 3T3 MEF cells were grown in DMEM (Gibco, Life Technologies, Carlsbad, CA, US) containing 10% fetal calf serum (FCS, Sigma, St. Louis, MO, US). For protein extraction or immunofluorescence experiments, cells were seeded into 6-well plates containing glass slides as required at a density of 100,000 cells per well in DMEM 10% FCS. The next day media was replaced with DMEM media containing 1% FCS and either 25 ng/ml recombinant mouse OSM (495-MO, R&D Systems,

Minneapolis, MN, US) or vehicle for the indicated times. Bafilomycin A1 (final concentration of 200 nM, Tocris-R&D, 1334) treatment was carried out four hours prior to harvesting cells. Starvation of primary and 3T3 MEFs was carried out in EBSS (24010-043, Life Technologies) for two hours. Bafilomycin Al (Santa Cruz Biotechnology, Dallas, TX, US, final concentration of 200 nM) treatment was carried out four hours prior to harvesting cells.

Immunoblotting

Sample preparation and immunoblotting was carried out as previously described [29]. The following antibodies from Cell Signaling Technologies (Danvers, MA, US) were used: anti-phospho-Stat3 (Tyr705: 9131), anti-total-Stat3 (9132), anti-LC3B (2775) and anti-phospho-p70 S6K (9234). The following antibodies from Abcam (Cambridge, UK) were used: anti-Tubulin (ab6160) and anti-β-actin (ab8227). Other commercial antibodies used were: anti-pan-p85 (Millipore, Danmstadt, Germany, 06-49 6, also detects p50α/p55α subunits), anti-p62/SQSTMl (MBL International, Woburn, MA, US, PM045) and anti-E-Cadherin (BD biosciences, San Jose, CA, US, 610182). All antibodies were used at a standard dilution of 1:1,000. Secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Dako (Ely, UK).

Immunohistochemistry

Tissue sections were prepared as previously described [29]. For tissue-culture samples, EpH4 cells were seeded on glass slides. After OSM treatment, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin in PBS for the p62 staining and ice cold methanol for the LC3B staining, and blocked in 5% normal goat serum (Dako) in PBS, with 0.5% saponin for the p62 staining. The pictures of p62 and LC3B puncta on EpH4 cells were acquired on a Zeiss Axioplan 2 microscope. All the other pictures were acquired on a Zeiss LSM 700 confocal microscope.

Quantitative real-time PCR

RNA extraction, complementary DNA synthesis and quantitative real-time PCR were carried out as previously described [29]. Primers used were: *cyclophilin A* (housekeeping gene) forward, 5′-CCT TGG GCC GCG TCT CCT T-3′, reverse, 5′- CAC CCT GGC ACA TGA ATC CTG-3′; *Lc3α* forward, 5′- GAC CGC TGT AAG GAG GTG C -3′, reverse, 5′- CTT GAC CAA CTC GCT CAT GTT A-3′; *Lc3b* forward, 5′- TTA TAG AGC GAT ACA AGG GGG AG -3′, reverse, 5′- CGC CGT CTG ATT ATC TTG ATG AG -3′; *p62* forward, 5′- AGG ATG GGG ACT TGG TTG C -3′, reverse, 5′- TCA CAG ATC ACA TTG GGG TGC -3′. *Beclin1* forward, 5′- ATG GAG GGG TCT AAG GCG TC -3′, reverse, 5′- TCC TCT CCT GAG TTA GCC TCT -3′. Primers were designed using the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/).

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ABBREVIATIONS

LM-PCD	lysosomal-mediated programmed cell death
PI3Ks	phosphatidylinositol 3-kinases
ctsB	cathepsin B
ctsL	cathepsin L
SH3	Src homology 3
BH	breakpoint cluster region-homology
OSM	oncostatin M
LIF	leukemia inhibitory factor
PTEN	phosphatase and tensin homolog
EM	electron microscopy
Stat3	signal transducer and activator of transcription 3
LMP	lysosomal membrane permeabilisation
MEFs	mouse embryonic fibroblasts

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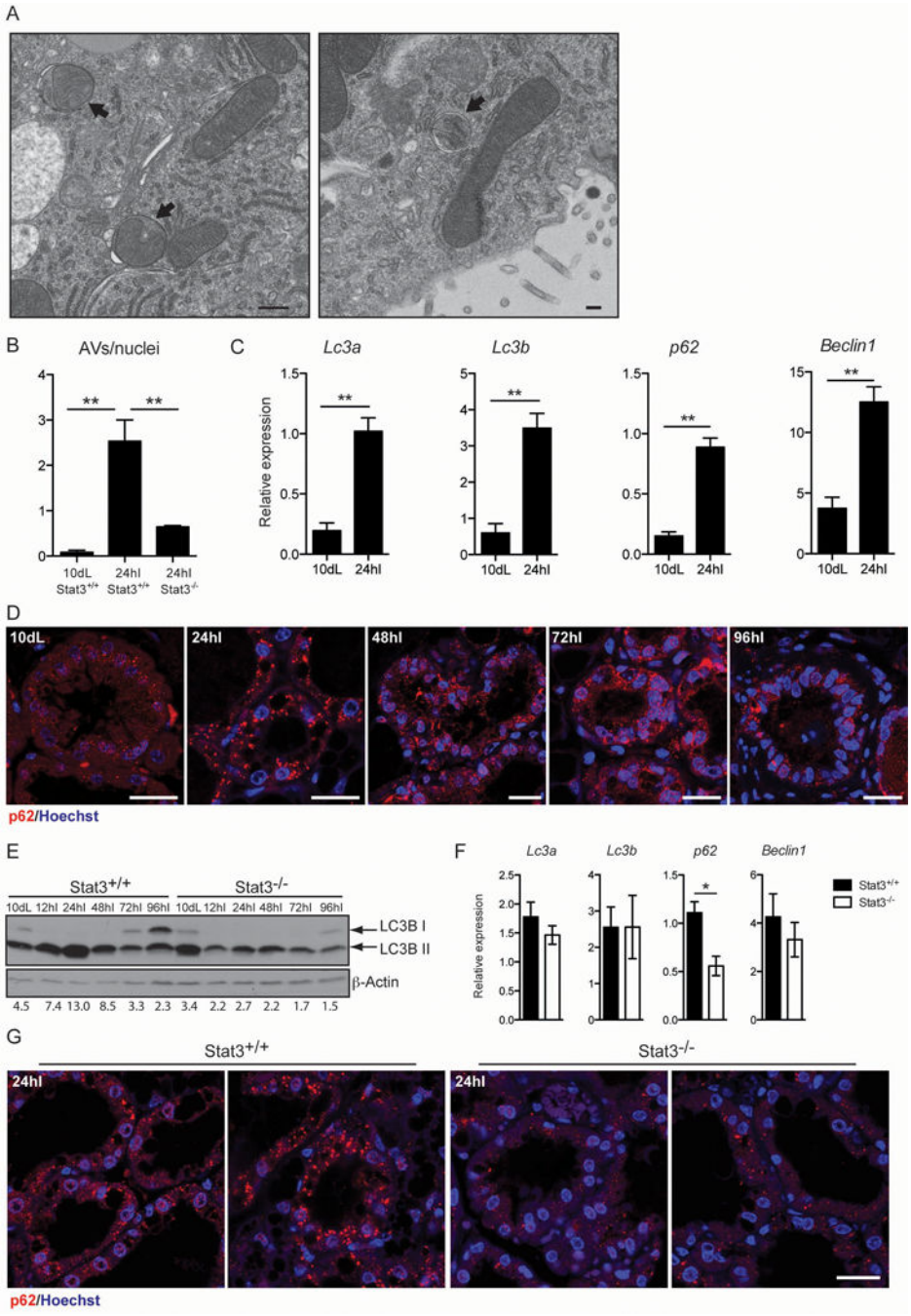

Figure 1.

Mammary gland involution is accompanied by an early induction of autophagy, which is disrupted in the absence of Stat3. (A) Representative transmission electron micrograph of mammary epithelial cells in tissue from 24h involution time point, showing evidence of double membrane autophagic vesicles (black arrows; scale bar, 500 nm). (B) Quantification of autophagic vescicles (AVs) over nuclei as observed by transmission electron microscopy. Quantification has been performed on 3 independent biological replicates per group and is shown as means ± s.e.m. (** $P<0.01$, as determined by one-way ANOVA analysis with

post-hoc Bonferroni′s correction). (C) Quantitative real-time PCR relative to *cyclophilin a* of the following genes: *Lc3a*, microtubule-associated protein 1 light chain 3 alpha; *Lc3b*, microtubule-associated protein 1 light chain 3 beta; *p62*, SQSTM1, sequestosome 1; *Beclin1*. All results are means ± s.e.m. of at least three independent biological repeats. 10dL, 10 days lactation; 24hI, 24 hours involution. (** $P<0.01$, as determined by Student's t—test). (D) Immunohistochemical analysis of p62 (red) of lactation (Lac) and involution (Inv) time points in control mice, as indicated. Nuclei are stained with Hoechst. Scale bar, 20 μm. (E) Immunoblot showing lipidation of LC3B (LC3B II) in control and *Stat3*$^{-/-}$ during involution. dL, days of lactation; hI, hours of involution. β-Actin is the loading control. Quantification of LC3B II over β-Actin is shown below the Immunoblot. (F) Quantitative real-time PCR relative to *cyclophilin a* of the indicated genes at 24 hours involution. All results are means ± s.e.m. of at least four independent biological repeats (*$P<0.05$, as determined by Student's t -test). (G) Reduced size and number of p62 puncta in the *Stat3*$^{-/-}$ glands, as shown by immunohistochemistry of p62 (red). Nuclei are stained with Hoechst. Scale bar, 20 μm.

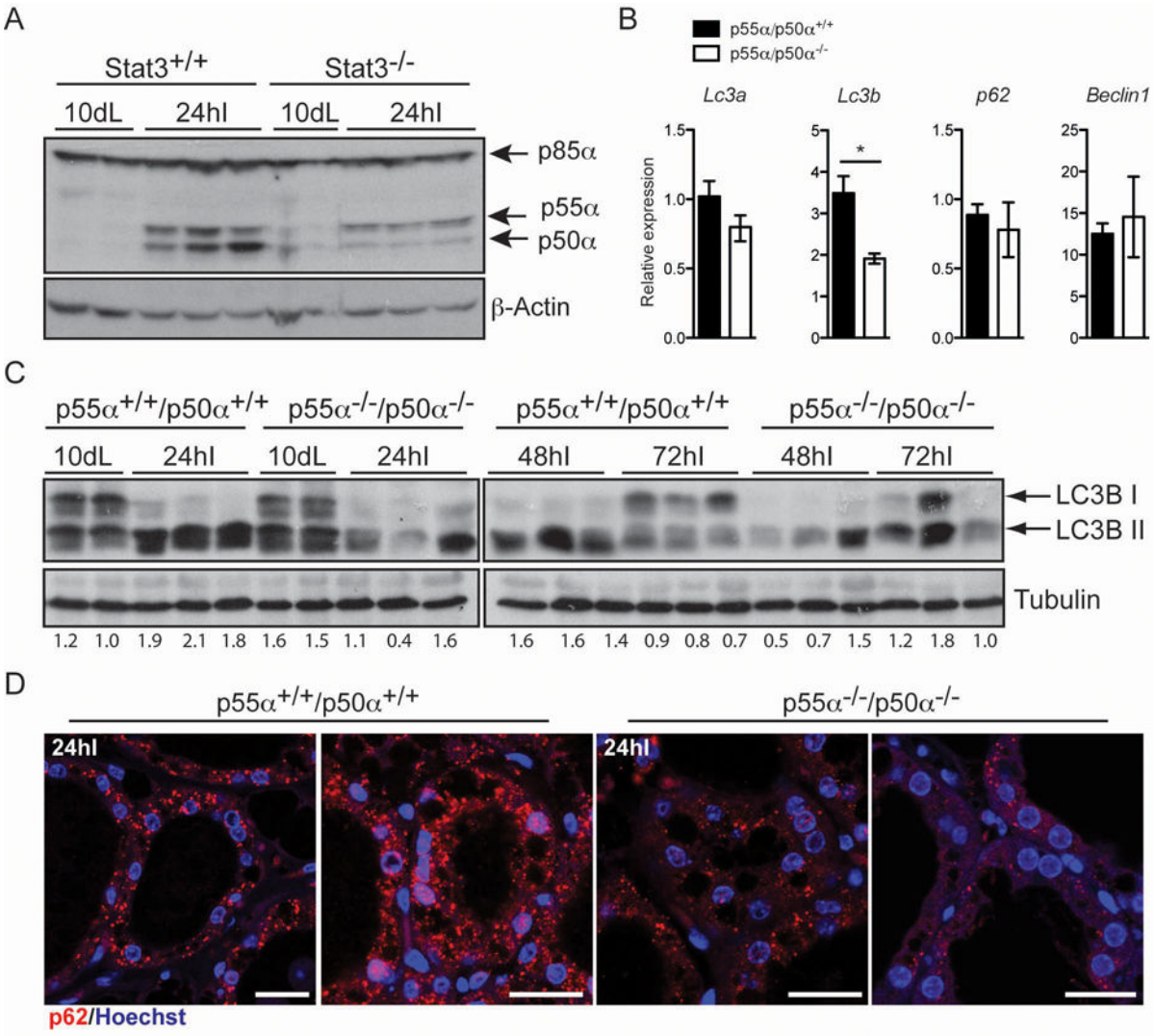

Figure 2.

p55α/p50α deletion disrupts autophagy in the mammary gland. (A) Immunoblot showing reduced levels of p55α/p50α in the *Stat3*$^{-/-}$ glands at 24h of involution. β-Actin is the loading control. L, lactation; I, involution. (B) Quantitative real-time PCR relative to *cyclophilin a* of the indicated genes at 24 hours involution. All results are means ± s.e.m. of at least five independent biological repeats (**P*<0.05, as determined by Student's t -test). (C) Immunoblot showing lipidation of LC3B (LC3B II). Lanes represent independent biological replicates. Tubulin is the loading control. L, lactation; I, involution. Quantification of LC3B II over Tubulin is shown below the Immunoblot. (D) Reduced size and number of p62 puncta in the *p55α*$^{-/-}$/*p50α*$^{-/-}$ glands, as shown by immunohistochemistry of p62 (red). Nuclei are stained with Hoechst. Scale bar, 20 μm.

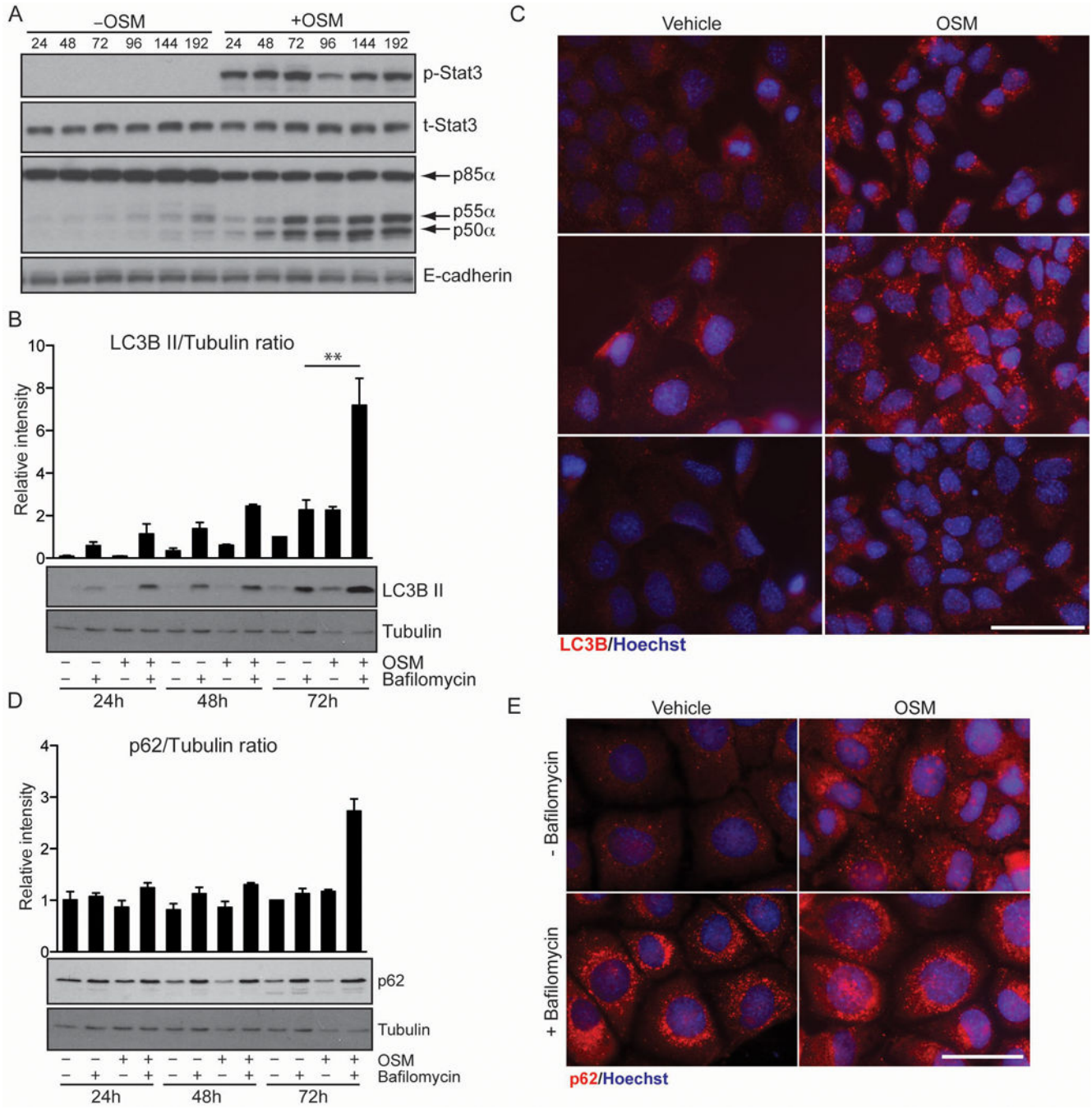

Figure 3.

OSM-dependent upregulation of p55α/p50α increases autophagic flux while augmenting the number of p62 aggregates. (A) EpH4 cells were stimulated with vehicle or OSM for the indicated hours and immunoblotted for the indicated proteins to show the induction of p55α/p50α downstream of OSM. E-cadherin is shown as loading control. (B) Increased LC3B II in cells stimulated with OSM. EpH4 cells were treated with vehicle, OSM and/or bafilomycin Al as indicated. LC3B lipidation was then analysed by immunoblot, one time course representative of three independent experiments is shown. Tubulin is the loading

control. (C) Immunohisotchemistry showing endogenous expression of LC3B (red) in EpH4 cells treated with vehicle or OSM for 48h. Nuclei are stained with Hoechst. Scale bar, 50 μm. (D) p62 protein levels are mostly unchanged in response to OSM. EpH4 cells were treated with vehicle, OSM and/or bafilomycin Al as indicated. p62 was analysed by immunoblot, one time course representative of three independent experiments is shown. Tubulin is the loading control. Graph above (B) and (D) is the quantification of the intensity of the bands of three independent biological replicates shown as means ± s.e.m. (** $P<0.01$, as determined by one-way ANOVA analysis with post-hoc Bonferroni's correction). (E) Immunohistochemistry for p62 (red) in EpH4 cells stimulated with vehicle or OSM for 48h and/or bafilomycin Al showing increased accumulation of p62 puncta in response to OSM with additional increment upon Bafilomycin Al treatment. Scale bar, 50 μm.

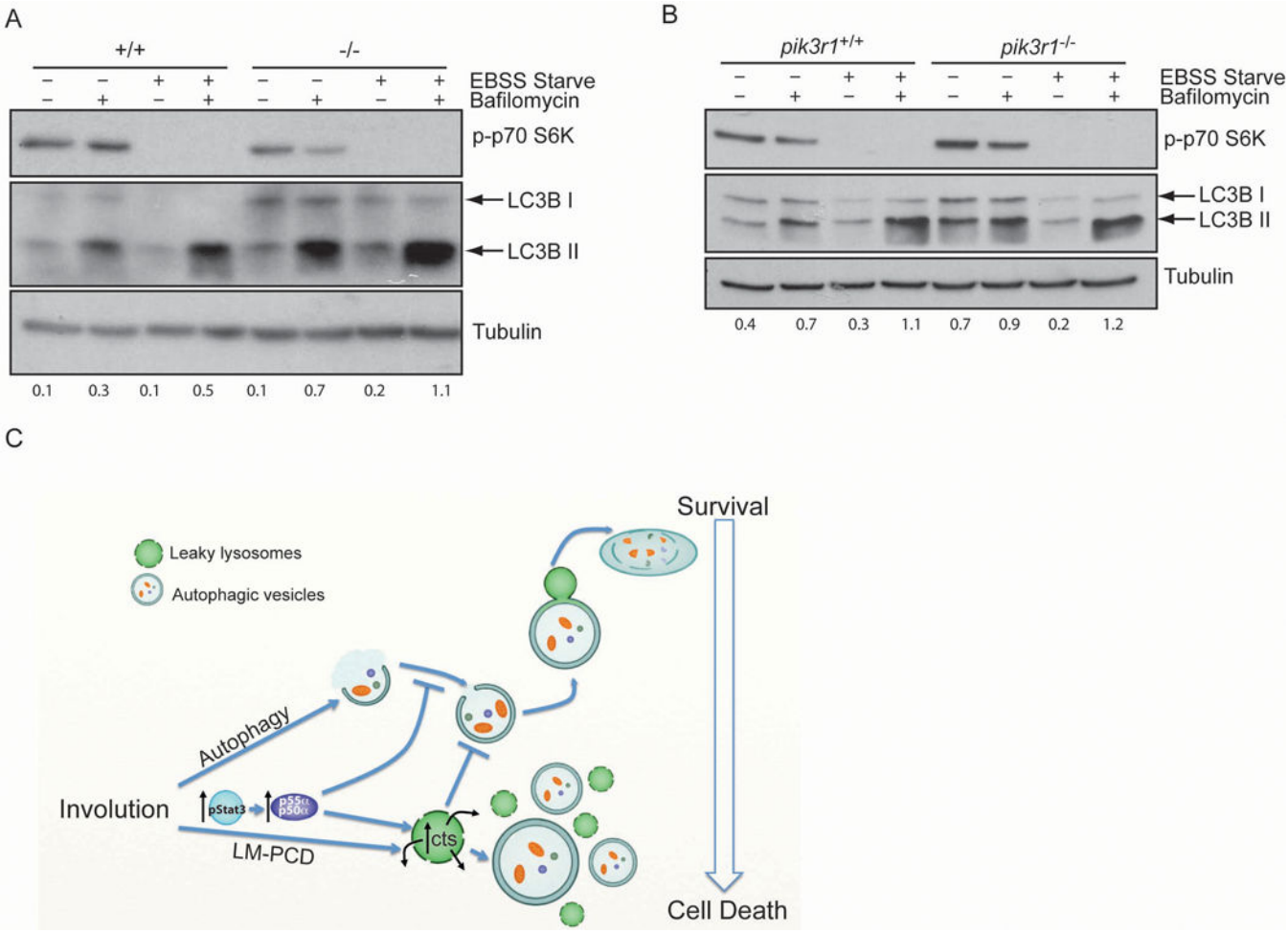

Figure 4.

p55α/p50α inhibit autophagy in primary MEFs. (A, B) Primary MEFs derived from control (+/+) and $p55\alpha^{-/-}/p50\alpha^{-/-}$ mice (−/−) (A) or $pik3r1^{+/+}$ and $pik3r1^{-/-}$ 3T3 MEFs (B) were pre-treated with bafilomycin Al for 2h and then starved with EBSS medium for 2h in the presence of bafilomycin Al as indicated. LC3B lipidation was then analysed by immunoblot. Immunoblot of phospho-p70 S6K shows the efficient inhibition of mTOR upon starvation. Tubulin is the loading control. Quantification of LC3B II over Tubulin is shown below the Immunoblot. (C) Graphical summary of the interplay between autophagy and LM-PCD during mammary gland involution highlighting the roles of Stat3 and p55α/p50α subunits. Our data suggest that autophagy provides a transient survival signal which is overridden by Stat3-induced expression of p55α/p50α. Concomitantly, the induction of lysosomal cathepsins (cts) and lysosomal leakiness further inhibits autophagy by blocking its resolution.