2014

ANALYSIS OF THE ROLE OF TWO AUTOPHAGY PATHWAY RELATED GENES, BECN1 AND TSC1, IN MURINE MAMMARY GLAND DEVELOPMENT AND DIFFERENTIATION

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DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By

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Lexington, Kentucky

2014

ABSTRACT OF DISSERTATION

ANALYSIS OF THE ROLE OF TWO AUTOPHAGY PATHWAY RELATED GENES, *BECN1* AND *TSC1*, IN MURINE MAMMARY GLAND DEVELOPMENT AND DIFFERENTIATION

The mammary gland is a dynamic organ that undergoes the majority of its development in the postnatal period in four stages; mature virgin, pregnancy, lactation, and involution. Every stage relies on tightly regulated cellular proliferation, programmed cell death, and tissue remodeling mechanisms. Misregulation of autophagy, an intracellular catabolic process to maintain energy stores, has long been associated with mammary tumorigenesis and other pathologies. We hypothesize that appropriate regulation and execution of autophagy are necessary for proper development of the mammary ductal tree and maintenance of the secretory epithelia during late pregnancy and lactation. To test this hypothesis we examined the role of two genes during development of the mammary gland.

Beclin1 (Becn1) is an essential autophagy gene. Since the *Becn1* knockout model is embryonic lethal, we have generated a *Becn1* conditional knockout (cKO). We used two discrete mammary gland-specific Cre transgenic lines to interrogate the role of BECN1 during development. We report that MMTV-CreD; $Becn1^{fl/fl}$ mice have a hyper-branching phenotype and WAP-Cre; $Becn1^{fl/-}$ mice are unable to sustain a lactation phase. *Becn1* mutants exhibit abnormal glandular morphology during pregnancy and after parturition. Moreover, when autophagy is chemically inhibited *in vitro*, mammary epithelial cells have an increased mean number of lipid droplets per cell.

MTOR inhibits autophagy upstream of BECN1; we looked higher in the regulatory pathway for regulatory candidates. It has been well characterized that Tuberous sclerosis complex 1 (TSC1), in a heterodimer with its primary binding partner TSC2, inhibits MTOR signaling via inhibition of RHEB. Using the *Tsc1* floxed model we generated a mammary gland specific *Tsc1* cKO and found that these mice phenocopy the *Becn1* cKO mice, including a gross lactation failure. *Tsc1* cKO glands have altered morphology, retained lipid droplets in secretory epithelia, and an overall increase in

MTOR signaling. We show that TSC1 and BECN1 are interacting partners, and that the interaction is nutrient responsive.

These results suggest that *Becn1* and *Tsc1* are necessary for proper mammary gland development and differentiation. Furthermore, we have demonstrated a novel murine protein-protein interaction and an important link between regulation of MTOR pathway and regulation of autophagy in a developmental context.

KEYWORDS: Autophagy, Bax, Beclin1, mammary gland, Tuberous Sclerosis Complex 1

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March 13, 2014
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This work is dedicated to my father, Joseph Hale, who will always be with me.

Acknowledgements

I first want to thank my mentor Dr. Edmund Rucker. He is the one who encouraged me to pursue research and has been the driving force behind the projects that are presented here. He has been an unwavering supporter and has helped me become a well-rounded scientist. Many thanks go to my lab mates Dan Ledbetter and Tom Gawriluk, who are collaborators and friends. My committee, Dr. Vincent Cassone, Dr. Philip Bonner, and Dr. Vivek Rangnekar has been an asset. I would like to recognize Dr. Kay-Uwe Wagner who has been a collaborator and scientific influence. The Forum in Reproductive Sciences and Women's Health has been an avenue for me to interact with other reproductive biologists and gain exposure to a broader perspective on women's health issues. I want to extend my thanks to Dr. Tom Curry, Dr. Michael Kilgore, and Dr. Misung Jo who have been supporters. Dr. Morris Grubbs was instrumental in my effort to earn the Graduate Certificate in College Teaching and Learning. My thanks go to the faculty, staff, and fellow graduate students of the Department of Biology. Specifically, Dr. Brian Rymond, Dr. Kellum, Beverly Taulbee, and Jacqueline Burke have been particularly helpful. I have had the opportunity to mentor many undergraduate students. They have been wonderful to teach, have aided in projects, and had projects of their own. I am proud of all they have and will accomplish.

I want to acknowledge and thank my immediate and extended family who are incredibly supportive. My sincerest thanks go to David Keesling, who is my partner, rock, and source of sanity. You make every day a happy one.

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Chapter One: Autophagy: Regulation and Role in Development

Overview

This chapter has been published and is included here for completeness of the dissertation.[1]

Mammalian autophagy refers to three cellular processes: chaperone-mediated autophagy, microautophagy, and macroautophagy. This review will focus on the latter, macroautophagy, hereafter referred to simply as "autophagy." Autophagy proceeds in successive stages to form a mature autophagosome, which include: 1) *de novo* formation of a double membrane bound structure or *phagophore*, 2) elongation of these lipid-based membranes, and 3) encapsulation of intracellular cargo to form the mature autophagosome. Autolysosomes, formed from the fusion of autophagosomes and lysosomes, then degrade and recycle the macromolecule components in order to maintain energetic homeostasis in the cell. Autophagy is an ancient process that is highly conserved among eukaryotes. The initial characterization of the autophagy pathway was worked out in yeast [2-4] with the identification of approximately 30 autophagy-related (ATG) genes, and many mammalian genetic homologs have been identified. Autophagy has been implicated in longevity/lifespan extension, disease prevention and promotion, as well as mammalian development (reviewed in: Autophagy and longevity: lessons from *C. elegans*,[5] Autophagy in the pathogenesis of disease,[6] Autophagy and disease: always two sides to a problem,[7] The Role of Autophagy in Mammalian Development: Cell Makeover Rather than Cell Death.[8]) This review will concentrate on the process and regulation of autophagy, as well as the role for autophagy in murine development.

Core machinery involved in autophagosome formation

Mechanistic (mammalian) target of rapamycin (MTOR), upstream of autophagy induction, senses cellular nutritional levels and regulates cell growth and survival and directly inhibits autophagy. MTOR is a cellular survival pathway that is the crux of many interconnected cellular pathways and integrates information to modulate cellular growth, metabolism and survival. MTOR is active in nutrient rich conditions, inhibiting autophagy and protein degradation. While in nutrient poor conditions MTOR is inactive and autophagy is induced by alleviating MTOR phosphorylation on unc-51 like kinase 1 (ULK1) and ATG13. When MTOR is inactivated by starvation conditions, dephosphorylated ULK1 dissociates from the MTOR complex and phosphorylates ATG13 and RB1CC1 (RB1-inducible coiled-coil 1, also known as FIP200) to induce the nucleation phase (**Figure 1.1A**).[9] Autophagosome nucleation is a highly orchestrated process relying on the PtdIns3K/VPS34 (phosphoinositide-3-kinase, class 3) complex that serves to phosphorylate phosphatidylinositol to phosphatidylinositol 3-phosphate (PtdIns3P) (**Figure 1.1B**). This modified lipid targets membranes to be recruited into the autophagosome. The PIK3 complex, anchored by the interaction between core members PtdIns3K and Beclin1 (BECN1), exhibits different functions depending upon the composition of ancillary proteins in the complex. UVRAG (UV irradiation resistance-associated gene) and ATG14 are found in BECN1 complexes in a mutually exclusive manner. For example, the complex of ATG14 (proposed mammalian homolog of yeast Atg14, or Barkor), PIK3R4/VPS15, PtdIns3K and BECN1 positively regulate

autophagosome formation at the nucleation step. In contrast, a complex including UVRAG, BIF1, PtdIns3K, PIK3R4, and BECN1 reportedly controls autophagosome maturation and RAB7 (GTPase)-dependent lysosomal fusion.[10] Autophagy regulation may be influenced through additional protein-protein interactions to either promote or inhibit autophagy. BIF1 directly associates with UVRAG and is a positive regulator of autophagy; similarly, AMBRA1 (autophagy/Beclin1 regulator 1) directly binds BECN1 to stimulate autophagy. In contrast, Rubicon (RUN domain and cysteine-rich domain containing, Beclin1-interacting protein) also directly binds BECN1 but acts as a negative regulator of autophagy.[11]

Elongation of the autophagosome membranes is accomplished by two ubiquitin like conjugation systems (**Figure 1.1C**). An ATG12–ATG5-ATG16L1 complex associates on the forming membrane. ATG12 is first activated by an ATP dependent reaction with ATG7, an E1-like enzyme. ATG12 is then conjugated to ATG5 by ATG10, an E2-like enzyme. ATG16L1 then interacts with the ATG12–ATG5 conjugate, forming a multimeric complex. Components of the complex dissociate from the autophagosome and return to the cytoplasm when elongation is complete. The second ubiquitin like conjugation that contributes to membrane elongation modifies MAP1 LC3, the mammalian homolog of yeast protein Atg8. ATG4B, one of the four mammalian ATG4 homologs, cleaves the C-terminal 22 residues of precursor LC3 (proLC3) producing LC3-I. Cytoplasmic LC3-I is then conjugated with phosphatidylethanolamine (PE) by ATG7 and ATG3, an E2-like enzyme. Lipidated LC3 (LC3-II) is selectively incorporated into the forming autophagosomal membrane. LC3-II remains associated with the autophagosome until

fusion with the lysosome, when LC3-II associated with the outer membrane dissociates and LC3-II associated with the inner membrane is degraded by lysosomal proteases along with the autophagosomal cargo (**Figure 1.1D**). This specific association of LC3-II makes it an attractive autophagy marker and will be discussed in further detail in later sections. The autophagosome fuses with a lysosome, forming the autolysosome (or autophagolysosome). Fusion with the lysosome results in breakdown of the inner autophagosomal membrane and cargo by lysosomal proteases and recycling of macromolecules. Soluble NSF attachment protein receptors (SNAREs), RAB7 and the homotypic vacuole fusion protein sorting (HOPS) complex likely are primary regulators of autophagosome/lysosome fusion.[12, 13]

Sources of Autophagosomal Membranes

The main lipid sources that are recruited in the formation of autophagosomes are thought to emanate from the endoplasmic reticulum (ER), mitochondria, Golgi apparatus, or the plasma membrane (PM). Integral membrane proteins normally found in the rough ER have been localized to the inner and outer autophagosome membranes by immunostaining.[14] This was also supported by electron microscopy (EM)-3D tomograms that identified points of contact between the rough ER and the nascent phagophore, which indicated that the growing isolation membrane might be a subdomain of the rough ER.[15, 16] Moreover, ER regions enriched in PtdIns3P have also shown to be the sites of emerging omegasomes. The ER protein ZFYVE1 can be found in omegasomes extending out from the ER by EM-3D tomography, and the PI3K-complex protein ATG14 is localized at the ER surface.[17] Contrary to the ER-based models, Hailey

et al[18] have proposed a novel model suggesting that the mitochondrial outer membrane may provide a membrane source for the biogenesis of autophagosomes. Following amino acid starvation, investigators observed by fluorescence microscopy that fluorescently labeled LC3 and ATG5 transiently localized to the outer mitochondrial membrane (OMM) and NBD–PE (7-nitro-2-1,3-benzoxadiazol-4-yl-phosphoserine; loaded into cells as NBD–PS and converted to NBD–PE in the mitochondria) could be detected in autophagosomes. Further, loss of the mitochondrial protein Mitofusin2 caused a dramatic depletion in starvation-induced autophagy. Other recent observations have suggested that cytoplasmic vesicles derived from the Golgi apparatus may act as a membrane source for forming autophagosomes in an ATG9-dependent manner. In yeast overexpressing Atg9, EM confirmed that under starvation conditions, post-Golgi ATG9-positive vesicles were transported to the vacuole and could be seen forming large tubulo-vesicular structures.[19, 20] Recent reports have described findings indicating that exocytic and endosomal SNARE proteins may mediate fusion of post-Golgi Atg9-positive vesicles and remodeling of tubulo-vesicle clusters in phagophore assembly.[21] Exocytic Q/t-SNAREs Sso1/2 and Sec9 were shown to be essential for homotypic fusion of Atg9-positive vesicles in starvation-induced autophagy in yeast. As well, endosomal Q/t-SNARE Tlg2 and R/v-SNAREs Ykt6 and Sec22 were found to interact with Q/t-SNAREs Sso1/2 and Sec9 and are essential for trafficking of post-Golgi Atg9-positive vesicles to the vacuole and during phagophore assembly. Finally, a number of studies have also indicated that the PM is capable of directly contributing membrane to forming isolation membranes. An analysis by Ravikumar et al[22] described interaction of

the clathrin heavy chain (associated with endocytic vesicles) with ATG16L1 at the extending phagophore and later confirmed that disruption of the interaction resulted in decreased autophagosome formation.[23] Recently, experimental data has also suggested that the action of PM SNAREs in complexes with tethering proteins may be essential for fusion of membranes in the phagophore. In HeLa cells, it was shown that the membrane protein LC3 is sufficient to tether PM-integral PE, potentially facilitating the activity of PM SNAREs.[24]

Cellular Stress Responses

Although the core machinery for autophagy was initially identified in yeast, seminal experiments defining the induction of autophagy were performed in a mammalian cell- the hepatocyte. These studies defined the importance of hormonal regulation, energy status, and nutrient levels as key modulators of autophagy. Initially, the presence of double-membrane organelles were found to be induced in rat hepatocytes exposed to glucagon;[25] these organelles were eventually demonstrated to be autophagosomes years later.[26] Conversely, treatment of hepatocytes with insulin gave the opposite result, which invariably defined autophagy as a catabolic, energy-generating mechanism for the cell.[27] The dependence of the cell on autophagy for energy homeostasis was confirmed the same year by showing that amino acid supplementation inhibited autophagy in the rat liver.[27] A final, important mechanistic link between autophagy and nutrient-energy sensing was established when rapamycin, an inhibitor of the energy sensor MTOR, was found to induce autophagy in

hepatocytes.[28] Thus, cellular stress became a focal point upon which to understand how autophagy could be regulated.

In addition to nutrient status and hormonal regulation, environmental stressors such as: hypoxia,[29] heat stress,[30] and reactive oxygen species (ROS) accumulation can also induce autophagy (Reviewed in: Autophagy signaling through reactive oxygen species).[31] Additionally, ER stress is also a potent inducer. The ER is a highly active and tightly regulated organelle responsible for protein folding fidelity, biogenesis of membrane structures, metabolism and a veritable menagerie of cellular processes. If the delicate microenvironment of the ER is disrupted, for instance by accumulation of unfolded (or misfolded) proteins, then the unfolded protein response (UPR) will be activated. There are three main pathways activated by the UPR: ATF6 (activating transcription factor-6), IRE1 (inositol requiring enzyme 1), and PERK (PKR-like eIF2α kinase, also known as EIF2AK3). All of these proteins are typically bound to an inactivating chaperone molecule, BIP/Grp78, and released/activated in response to unfolded proteins. It has been reported that autophagy is activated in response to ER stress and UPR by both PERK and ATF6 pathways, perhaps in an effort to ameliorate the accumulation and aggregation of misfolded proteins. Interestingly, IRE1 activation inhibits autophagy in some systems and is required for autophagy induction in others; however, more systematic work is needed to define these pathways in mammalian systems.[32] Although evidence is supportive of ER stress inhibiting MTOR- and Akt-mediated cell survival pathways, which would alleviate autophagy inhibition

independently of the UPR, additional studies are needed to elucidate this process. (Reviewed in: Autophagy and the Integrated Stress Response).[33]

Autophagy may also be induced as a survival mechanism in response to hypoxic conditions in normal and tumor cells.[34] Induction of autophagy can proceed through different pathways depending on the severity of hypoxia and cell type.[35] The best characterized means of hypoxia-induced autophagy is by activation of the transcription factor HIF1 (Hypoxia-inducible factor 1).[36] HIF1 is capable of transcriptional activation of a variety of target genes involved in offsetting the damaging effects of hypoxia including erythropoiesis, angiogenesis, and autophagy. The HIF1-target gene *Bnip3* (BCL2/adenovirus E1B 19kDa interacting protein 3) encodes a putative BH3-only (*Bcl2* homology domain-only) protein that is necessary and sufficient to induce autophagy by competitively binding BCL2 (B cell leukemia/lymphoma 2) and disrupting the BCL2-BECN1 interaction.[37, 38] *Bnip3* has also been identified as a target gene of the transcription factor E2F, which can be activated by inhibition of the RB1 (retinoblastoma 1) protein via severe hypoxia.[39] This suggests that BNIP3 can trigger autophagy by HIF1-dependent and HIF1-independent mechanisms. Severe hypoxia/anoxia (<0.1% oxygen) can also induce autophagy by AMP-activated protein kinase (AMPK)-mediated MTOR inhibition as well as protein kinase C activation of MAPK8 (mitogen-activated protein kinase 8) and BECN1.[40 41 42]

The ubiquitin proteasome system (UPS) and autophagy act as the major pathways for cellular catabolism and were initially thought to function independently of

one another. However, new observations suggest that the two degradation pathways function in a highly coordinated manner to maintain cellular homeostasis. The UPS pathway specifically targets soluble proteins in the nucleus or cytoplasm, which are labeled for proteasomal degradation by the addition of the small peptide ubiquitin at various lysine residues. Through the action of three classes of enzymes, E1 ubiquitin-activating, E2 ubiquitin-conjugating, and E3 ubiquitin-ligases, the UPS pathway can ensure high levels of specificity in labeling protein targets for degradation. The most well-known limitations of the UPS pathway result from the small size and cylindrical structure of the 26S proteasome to which poly-ubiquitinated-proteins must enter for degradation by a series of peptidases. It appears that proteins may need to be partially denatured and monomeric in order to be degraded within the proteasome, greatly limiting the UPS pathway for clearance of aggregated proteins or large multimeric complexes.[43] In contrast, autophagy is limited in its activity to the cytoplasm and can efficiently degrade soluble proteins, protein aggregates, and organelles by engulfment by the isolation membrane followed by lysosomal fusion. Previously, autophagy was viewed as a non-specific degradation pathway for cellular recycling; however, it is clear that several proteins and common aggregates are selectively degraded in the autolysosome, rather than the proteasome.[44, 45 46] It has been observed that ubiquitinated proteins could be selectively degraded by autophagy.[45] Suppression of the UPS pathway by siRNA is offset by an increase in autophagy.[47] However, inhibition of autophagy results in inhibited degradation of UPS substrates.[48, 49] As a result, current models for the autophagy and UPS pathways suggest not only overlapping roles for the

two systems, but a more dynamic and coordinated approach than previously described.(Reviewed in: Mechanisms of crosstalk between the ubiquitin-proteasome and autophagy-lysosome systems).[50]

SQSTM1 (Sequestosome 1, also known at p62) is a scaffolding protein with several known functions in various tissues and is the most well-known target of selective autophagy.[44] As SQSTM1 is constitutively expressed, it shows a consistent turnover and normally forms aggregates in the cytoplasm, which are selectively degraded by autophagy. These characteristics are easily observed by immunohistochemistry and have been used to monitor successful autophagy *in vitro*. Tissue-specific ablation of autophagy results in accumulation of SQSTM1 aggregates and proteins carrying poly-ubiquitinated Lys-63 residues.[51] The SQSTM1 protein contains both an ubiquitin-binding domain as well as an LC3-interacting domain, which has led to its implication as a central link between autophagy and the UPS pathway. Current models describe SQSTM1 as a cargo receptor for autophagic degradation of various proteins.[52, 53]

Autophagy can also be pharmacologically stimulated or inhibited in a number of ways; each method is accompanied by its own set of advantages and disadvantages (**Table 1.1**). Autophagy can be stimulated indirectly through inhibiting the UPS pathway, inducing an ER stress response, reducing intracellular calcium levels, or modifying the acetyl proteome. Use of proteasomal inhibitors MG132 or bortezomib in human cancer cell lines induces both the accumulation of the lipidated form of LC3B and the localization of a green fluorescent protein (GFP) tagged reporter (GFP-LC3) to

autophagosomes.[54] Tunicamycin, an inhibitor of N-acetylglucosamine phosphotransferase, is used widely in the literature to induce ER stress.[55] Tunicamycin treatment *in vitro* increases autophagosomes and autolysosomes as detected by EM and GFP-LC3 puncta formation, a fluorescent marker of autophagy. Although thapsigargin acts as an ER stressor to inhibit the sarco/endoplasmic reticulum Ca^{2+} ATPase (ATP2A1/SERCA1), it can also act directly by blocking the fusion of autophagosomes with lysosomes by preventing recruitment of RAB7.[56] Intracellular calcium can activate CAPN-1 (calpain 1), a protease that targets ATG5 for degradation, thus inhibiting autophagy.[57] Fluspirilene induces autophagy through reducing calcium stores, which leads to a deactivation of CAPN-1 and stabilization of the ATG5–ATG12 conjugate. Lastly, resveratrol and spermidine are being studied for their ability to induce autophagy *in vivo* in order to promote longevity.[58, 59] The apparent mechanism though which they act is through modification of the acetylproteome, principally through cytoplasmic deacetylation reactions mediated by the sirtuins.[60]

Rapamycin is a highly specific inhibitor of MTOR and is commonly used both *in vitro* and *in vivo*.[61-63] In contrast, lithium chloride is an MTOR-independent inducer of autophagy that inhibits inositol monophosphatase, leading to a decrease in inositol availability. Inositol monophosphatase inhibition results in increasing cellular levels of PtdIns3P, thus inducing autophagy.[64] L-690,330 also inhibits inositol monophosphatase and is more potent, but has a reduced ability to permeate the PM and blood-brain barrier.[65] Carbamazepine also works by a similar mechanism to lithium, and has been used *in vivo* to ameliorate proteinopathies in mice either suffering from mutant

SERPINA1 (α1-antitrypsin) Z or TAR DNA binding protein (TARDBP) accumulations.[66, 67] A broader range of pharmacological agents used to induce autophagy in the treatment of proteinopathies has been previously reported.[68] The xestospongin family of natural compounds purified from marine sponges, xestospongin B (XeB) and xestospongin C (XeC), are inducers of autophagy. XeC inhibits both the inositol 1,4,5-trisphosphate receptor 1 (ITPR1) and ER Ca^{2+} receptors indiscriminately, thus altering Ca^{2+} flux.[69] XeB was originally identified as an inhibitor of inositol 1,4,5-trisphosphate-mediated Ca^{2+} signaling[70] and has more recently been shown to act as an antagonist of ITPR1 on the ER membrane. Evidence supports a mechanism wherein ITPR1 facilitates BCL2-mediated sequestration of BECN1 to inhibit autophagy. XeB would rapidly induce autophagy by interfering with these protein-protein interactions, freeing BECN1 in an organelle-specific manner.[71] The induction of autophagy by XeB appears to be independent of steady state Ca^{2+} levels in the ER or cytoplasm.[72]

For autophagy inhibition, 3-methyladenine (3-MA) inhibits autophagy in nutrient poor conditions by inhibiting PI3K and autophagosome formation. However, 3-MA inhibits both PIK3C1 and PI3CK3 indiscriminately which could lead to pleiotropic cellular effects. Interestingly, in complete media 3-MA is pro-autophagic, leading to an accumulation of autophagic markers and increased conversion of LC3-I to LC3-II. Wu and colleagues were able to show that the increase in autophagic markers is a result of increased autophagic flux rather than an effect of autophagosome accumulation and seems to be cell line independent. Therefore, use of 3-MA as an autophagic inhibitor is well supported and substantiated when used in starvation conditions and at relevant

concentrations.[73] Another PI3K inhibitor, wortmannin, functions in a similar way but does not show pro-autophagic effects in complete media. This difference is attributed to wortmannin more stably inhibiting PtdIns3K compared to 3-MA, which has a transient effect on PtdIns3K but a stable effect on PIK3C1.[74] The second group of inhibitors either target PI3K stabilization or autophagosome-lysosome interaction. A recently identified inhibitor, spautin-1 (specific and potent autophagy inhibitor-1), blocks the deubiquitinase activities of USP10 and USP13, leading to the UPS-mediated degradation of the PtdIns3K component of the PI3K complex.[75] Vacuolar ATPase is an enzyme that resides on the lysosomal membrane and regulates the lysosomal acidification. Bafilomycin A1 (BafA1) is a vacuolar ATPase inhibitor. BafA1 inhibits fusion of autophagosomes with lysosomes and leads to an accumulation of autophagosomes in the cell as well as a reduction of protein degradation, another indication that autophagy is impaired.[76] The fusion blockage seems to be an effect secondary to the reduced acidification of the lysosome.[77] Conflicting reports in the literature imply that both cell type and length of drug treatment affect the degree of inhibition as well as more general effects. It is important to note here that interpretation of commonly used autophagic activity and flux assays may be altered when using drugs affecting lysosomal acidification. In a short communication, Klionsky et al[78] discuss the complications that may arise such as reduced LC3-II degradation at early time points and the difficulty in discriminating autophagosomes from autolysosomes in this system due to GFP persistence in the less acidified lysosomes.

Types of Autophagy

Aggrephagy

Aggrephagy refers to the autophagic process of degrading proteins that are assembled into large protein aggregates, which are less toxic to the cell than more numerous small protein aggregates. Although this is seen as a companion system to the UPS to promote protein degradation, there are subtle distinctions as to the intertwining of these pathways. For example, proteins targeted for degradation by this means can be ubiquitinated but do not necessarily have to be. Histone deacetylase 6- (HDAC6) mediated dynein transport of proteins along microtubules occurs preferentially with proteins that have K-63 linked polyubiquitin chains. Conversely, BAG3-mediated aggresome formation, which also uses dynein transport along microtubules, does not require such protein modifications. The forming aggresome requires K-63 linked polyubiquitination to recruit autophagy receptors SQSTM1, NBR1, and WDFY3. These receptors provide a physical link to ATG8, found in the developing phagophore, to ultimately envelope the aggresomes.[79]

Allophagy, crinophagy, and zymophagy

Allophagy refers to the autophagic degradation of paternally derived mitochondria upon fertilization in the zygote. As such, this mechanism is a developmental-specific form of mitophagy. Sperm mitochondria, located in the mid-piece region, are initially tagged with K63-linked ubiquitin prior to fertilization. This labeling increases immediately after fertilization, presumably to insure their quick and

successful degradation prior to fusion of the male and female pronuclei.[80] Historically, crinophagy refers to the process by which secretory granules containing hormones are directly routed to lysosomes without contribution from autophagy. However, a parallel mechanism involving encapsulating these granules within autophagosomes does occur, as demonstrated within Paneth cells and pancreatic β-cells.[81, 82] This provides a turnover mechanism to regulate appropriate granule numbers in these cells. Autophagy is also responsible for the regulation of neurotransmitter vesicle levels, as dopamine responses are substantially increased in the dorsal striatum of *Atg7*-deficient mice.[83] Conversely, induction of autophagy through rapamycin administration attenuates dopamine responses. Although it has not been demonstrated, these processes are most likely ubiquitin-dependent. For example, the closely related process of zymophagy allows for the autophagic degradation of activated zymogen granules,[84] which is dependent on VMP1 (vacuole membrane protein 1), SQSTM1 and the ubiquitin protease USP9X.[85] This mechanism prevents acute pancreatitis from occurring by removing potentially harmful activated zymogen granules.

Exophagy

Autophagy is also associated with non-degradative processes involved in protein secretion known as exophagy. Deretic et al[86] review the roles of autophagy in conventional (regulated and constitutive) and unconventional secretion. Conventional secretion pathways normally route through the Golgi complex or occasionally directly from the ER; however, the TOR-autophagy spatial coupling compartment[87] is a newly identified region that is responsible for constitutive secretion of IL-6 and IL-8. Regulated

secretion examples include lysozyme release by Paneth cells and cathepsin K by osteoclasts. Autophagy-based unconventional secretion, or autosecretion, involves omegasome formation at the ER to secrete proinflammatory factors IL-1β and HMGB1 in mammalian cells.

Heterophagy and endosomal microautophagy

Heterophagy is distinguished from autophagy in the sense that it is a process devoted to degrade extracellular material that has been internalized within the cell, in contrast to the degradation of pre-existing intracellular material. Upon endocytosis, proteins are routed into early endosomes and late endosomes/multivesicular bodies (MVBs) for fusion with lysosomes. However, there is synergy with the endosomal system and autophagy, as early endosomes and MVBs can both fuse with autophagosomes to form amphisomes, which in turn fuse with lysosomes. These amphisomes have been shown to carry protein markers early endosome antigen 1 (EEA1) and mannose 6-phosphate receptor (M6PR), present in early and late endosomes, respectively.[88] These fusion events are driven by GTPases, as MVB-autophagosome fusion is RAB11-dependent and amphisome-lysosome fusion is RAB7-dependent.[89] In contrast to internalized materials, cytosolic proteins can be routed into MVBs through endosomal microautophagy either hsc70-mediated or through a non-specific mechanism. This process occurs during MVB formation and requires the ESCRT I and ESCRT III protein machinery.[90]

Immunophagy

More broadly, autophagy plays a larger role in both innate and adaptive immunity in a process termed immunophagy.[91] As recently reviewed, immunophagy is subdivided into three types: Type I, Type II, and Type III.[92, 93] Type I immunophagy involves the processing of foreign or endogenous immunologically active molecules. This would include: xenophagy, the autophagic activation of macrophages, pattern recognition receptor activation, MHC II endogenous antigen presentation, and thymic selection. Type II immunophagy regulates cell viability and immune cell function. Specific roles for this type include: T/B cell homeostasis, T cell maturation, and Paneth cell maintenance. Type III immunophagy utilizes specific ATG proteins but doesn't require the entire process of autophagy to occur. Examples in this class include: inhibition of RIG-I-like receptor signaling by ATG5–ATG12, and the negative regulation of TBK1 signaling for type I interferon secretion by ATG9.

Lipophagy

Lipophagy involves the metabolic regulation of lipids through degradation of lipid droplets (LDs) by autophagy. Ultimately, fusion with lysosomes contributes to lipolysis of LDs, or the breakdown of triglycerides into free fatty acids. In addition to lipophagy, cytosolic lipases promote free fatty acids from lipolysis in an autophagy-independent fashion. Although these cytosolic lipases are well characterized, the autophagy proteins involved in the detection and mobilization of LDs are not known. This field has recently expanded due to the pathologies associated with autophagy-deficient mice. Liver-specific ablation of *Atg7* results in LD accumulation known as liver

steatosis (fatty liver).[94] Similar studies have shown that lipophagy is a sensor for appetite regulation in hypothalamic cells,[95] whereas reduced lipolysis in macrophages causes their premature conversion into foam cells to promote atherosclerosis.[96]

Mitophagy

Mitophagy is the selective degradation of mitochondria through autophagy, although the process may be cell specific within mammals. For example, reticulocytes lose their mitochondria as they mature into red blood cells, an interaction-dependent process driven by BNIP3L (BCl2/adenovirus E1B 19kDa interacting protein 3-like) located on mitochondria and LC3 found on phagophores.[97] Within other cells, a PINK1 (PTEN-induced putative kinase protein 1)-Parkin system seems to regulate mitophagy.[98, 99] PINK1 localization at the OMM of damaged mitochondria recruits the E3 ubiquitin ligase Parkin to cause K63-ubiquitination of three known mammalian proteins: mitofusin1, mitofusin2 and voltage-dependent anion selective channel protein 1 (VDAC1). Autophagy can subsequently be directed by SQSTM1- and HDAC6-dependent mechanisms. Given the enrichment of Parkin within skeletal muscle, brain, heart, and liver, it is possible that other tissues may use distinct E3 ubiquitin ligases for selective removal of mitochondria. A recent genomic mammalian screen revealed 96 proteins required for Parkin-mediated mitophagy, underscoring the complexity and uncertainty of this mechanism.[100]

Nucleophagy

Although yeast undergo piecemeal microautophagy of the nucleus, where portions of the yeast nuclear membrane and nucleoplasm are invaginated into a vacuole for degradation,[101] mammalian cells can exhibit complete encapsulation of the nucleus known as nucleophagy. This was initially demonstrated in murine models that exhibit nuclear envelopathies from mutations in nuclear membrane associated proteins such as lamin A and emerin.[102] Murine embryonic fibroblasts (MEFs) isolated from these mutant mice demonstrated decreased cell viability and increased nuclear abnormalities when pharmacological inhibitors of autophagy were used. Wild-type MEFs also exhibited signs of nucleophagy, although at lower levels than mutant MEFs, suggesting a control mechanism when nuclear damage occurs. This notion is strengthened by findings that anti-cancer drugs that elicit DNA damage can trigger nucleophagy.[103] Topoisomerase inhibitors (e.g. camptothecin, etoposide), DNA intercalating agents (e.g. cisplatin), and oxidative stress damage (e.g. vanadyl (IV)) can induce nucleophagy in cancer cells. It is possible that basal piecemeal nucleophagy occurs to maintain nuclear architecture, energy production, and nucleotide stores for DNA repair enzymes.

Pexophagy

Pexophagy, or degradation of peroxisomes through autophagy, is probably the most utilized of the three known mechanisms to eliminate superfluous peroxisomes. Analysis of liver-specific *Atg7*-deficient mice showed pexophagy contributed to about 70-80% of the turnover, compared to the remainder linked to both a LON protease-mediated mechanism and 15-LOX-mediated autolysis.[104] The only known mammalian

peroxisome receptor involved in pexophagy is PEX14, which can associate with LC3 on the phagophore membrane.[105] An LC3-RAB7-FYCO1-kinesin complex is responsible for the transport of engulfed peroxisomes along microtubules to the lysosome. Although ubiquitination of a distinct membrane protein of the peroxisome allows for SQSTM1-mediated autophagy to occur, the identity of this protein is currently not known.[106]

Reticulophagy and Ribophagy

Degradation of the ER through reticulophagy occurs in response to ER stress, and is seen as an additional stress coping mechanism like the UPR and ER-associated degradation.[107, 108] The trigger for all three mechanisms lies in the accumulation of unfolded protein aggregates within the ER lumen. Activation of the resident ER membrane protein PERK occurs when chaperones dissociate from the luminal side of PERK to assist in protein folding. This causes dimerization of PERK, phosphorylation of EIF2A, and activation of ATG12, thus triggering the autophagic response via ATG12–ATG5-ATG16L1 complex formation. Ribophagy, the selective elimination of free ribosomes in the cytosol, is also linked to the ER stress response similar to reticulophagy. ER stress leads to reduced translation levels, via phosphorylated EIF2A, to avoid the additional burdening of chaperone recruitment to nascent peptides.[109] In modeling neurodegenerative disorders in Purkinje cells, polyribosomes were disassembled into non-translational monosomes, which became associated with autophagosomes.[110] However, it is not known which ATG proteins mediate the recognition and sequestration of ribosomes and ER fragments into autophagosomes.

Xenophagy

Viruses, bacteria, and parasites can be eliminated in an autophagic process involved in innate immunity defense termed xenophagy, which has been previously reviewed.[93, 111] Invading bacteria can generally be classified as vacuolar (e.g. *Salmonella*) or cytosolic (e.g. *Listeria*, *Shigella*). Cytosolic bacteria can undergo ubiquitin-dependent and ubiquitin-independent mechanisms for autophagosomal envelopment followed by translocation to lysosomes. Vacuolar bacteria can be routed into autophagosomes, or in the instance of *Mycobacteria*, autophagy proteins can resume the maturation of the vacuole and promote fusion with the lysosome.[112] The main recognition receptors that link detection and autophagy induction include the membrane TLRs (Toll-like receptors) and the cytoplasmic nucleotide-binding oligomerization domains (NOD)-like receptors (NLRs). The receptors can recognize the lipopolysaccharides and peptidylglycans of Gram-negative bacteria. Microbial interference with autophagy can occur due to the adaptive nature of bacteria. For example, *Shigella flexneri* secretes the protein IcsB, which prevents ATG5-induced autophagy at the bacterial surface.[113] *Yersinia pseudotuberculosis* resides within arrested autophagosomes in macrophages, since it can inhibit the fusion process with lysosomes.[114]

Transcriptional Regulation

A multitude of studies have focused on discovering the transcription factors responsible for controlling ATG genes. Thus far these studies have culminated in nine transcription factors that orchestrate the expression of autophagy-related genes in

mammals. We have learned that autophagy is under the regulatory control of circadian, metabolic, inflammatory and cell death factors.

Circadian Regulation

As might be expected, autophagy genes are transcribed with a circadian rhythm under control of the biological clock due to metabolic demands. A circadian rhythm refers to the oscillation of a biochemical process (e.g. transcription, translation, phosphorylation, etc.) that goes through a cycle roughly every 24 hours. The circadian clock is a transcription/translation feedback loop between the positive transcription factors CLOCK and ARNTL/BMAL1 and the negative transcription factors PER1 and PER2. These signals originate in two clusters of hypothalamic neurons of the suprachaismatic nucleus (SCN), which is reset by a light-dark cycle. The oscillation in the SCN is considered the "master pacemaker" and is responsible for coordinating "subordinate pacemakers" throughout an organism, giving rise to circadian rhythms in every tissue. Autophagy is under circadian control likely because metabolism demands change throughout the day, an idea recently reviewed.[115] While only ~10% of transcripts are globally rhythmically expressed,[116-119] research using mice has shown that the mRNA expression of many autophagy genes such as *Becn1*, and *Map1lc3b* are rhythmic in the distal colon,[120] liver, kidney, heart and skeletal muscle.[121] In addition, the conversion of LC3-I to LC3-II is rhythmic in the liver of mice.[121] The expression of the transcription factor CEBPβ, known to be controlled by the clock, was found to coordinate autophagy gene rhythms by directly binding to the promoters of autophagy genes, such as, *Bnip3*, *Ctsl* and gamma-aminobutyric acid receptor-associated protein (*Gabarap)*. Additionally,

CEBPβ has been identified as the transcription factor responsible for rhythmic autophagy gene expression in the mouse liver.[121] Furthermore, this rhythm was abolished in *Arntl* knockout livers, proving that autophagy rhythms from CEBPβ are dependent on the clock and thus, are circadian.[121] It remains untested if CEBPβ or another component of the clock controls autophagy rhythmicity in other tissues such as the distal colon, but logic would dictate this to be the case. While there have not been any direct links to disease caused by interrupting the link between autophagy and the circadian clock, there are several lines of research suggesting this link exists. Metabolic disorders like diabetes have been shown to be similarly exasperated independently by both autophagy and CLOCK deficiencies.[122, 123] It is surely to be found that the link between autophagy and the biological clock is stronger than we currently understand.

FoxO Family

The FoxO (forkhead box, class O) family is comprised of four proteins in mammals: FOXO1, FOXO3, FOXO4 and FOXO6. FOXO proteins are transcription factors known to be involved in metabolism, longevity, oxidative-stress resistance, apoptosis and autophagy. Once FOXO proteins are activated in the cytosol, FOXO proteins are shuttled into the nucleus where they bind to promoters to initiate transcription. Their role in controlling autophagy gene transcription comes from studies on skeletal muscle,[124-126] neurons,[127] ischemic insults on the heart[128] and human cancer cell lines.[129] Upon starvation or denervation of skeletal or heart muscles, both autophagy and FOXO activity are increased suggesting a transcriptional control link. These studies have shown that FOXO1 and FOXO3 can induce the expression of *Gabarapl1* (GABARAP-like

1), *LC3b, Atg12l, Atg4B, PtdIns3K, Ulk2, Becn1, Bnip3, and Bnip3l*. Further analysis by ChIP has found FOXO proteins bound to the consensus sequence (T/C/G) (G/A/T) AAA (C/A) A found in the promoters of several autophagy genes including *Becn1, Gabarapl1, LC3b, Atg12* and *Bnip3*.[128] The activation of autophagy by FOXO is one major arm of autophagy activation as FOXO is independent of MTORC1 signaling. FOXO1 can be activated in neurons through loss of *Mapk8*.[127] Studying *Mapk8*$^{-/-}$ mouse brains and neurons it is clear that MAPK8 is a negative regulator of autophagy by inhibiting FOXO1. *Mapk8*$^{-/-}$ neurons have increased levels of dephosphorylated, nuclear-localized, activated FOXO1 and greater autophagic flux. In cardiomyocytes, Sirtuin 1 (SIRT1) and nuclear protein transcription regulator 1 (NUPR1 also known as P8) play crucial roles in regulating autophagy through modification of FOXO1 and FOXO3.[130, 131] SIRT1 regulates the activation of autophagy by directly deacetylating and activating FOXO1 during glucose deprivation (GD).[132] Knockdown of either *Sirt1* or *Foxo1* leads to no increase in expression of ATG genes and failure of autophagy induction during GD.[131] Utilizing a mutated FOXO1 (3A/LXXAA), which cannot be recognized by SIRT1, leads to increased acetylation of FOXO1 and failure to induce of autophagy after GD.[131] Additionally, *Foxo1* conditional KO or *Foxo1* (3A/LXXAA) overexpression in cardiomyocytes of mice significantly deteriorates cardiac function after GD proposed to be a direct cause of the significant decrease in autophagic flux. Knockdown of *Nupr1*, a transcriptional corepressor of *Foxo3*, leads to activation and nuclear localization of FOXO3 where it associates more with target gene promoters such as *Bnip3*, which in turn have increased mRNA and protein expression. Subsequently, *Nupr1* knockout mice have increased

Bnip3 expression and increased autophagic flux in cardiac tissue, likely due to the increased FOXO3 activity.[130] In primary skeletal muscle myotubules, AMPK is a potent activator of autophagy through both activation of FOXO3 and inhibition of MTORC1.[126] While more regulators of FOXO proteins are likely to be found, this research shows a clear link between FOXO activation and autophagy induction, through the increased expression of ATG-genes in several cell types.

Trp53

Transformation related protein 53 (TRP53 or TP53/p53) is a tumor-suppressor protein with well-known roles in regulating both cell cycle progression and the cell survival/death axis. Recently, it has also been shown to provide both positive and negative regulation of autophagy. In normal cells, TRP53 remains in the cytoplasm at low levels, which are maintained by the E3-Ubiquitin ligase MDM2 and the UPS. Upon activation by post-translational modifications, TRP53 translocates to the nucleus where it functions to transactivate a number of target genes involved in cell survival, death, and cell cycle regulation. Initial observations that TRP53 inhibition resulted in increased autophagy were complicated by the discovery that nuclear TRP53 was able to induce autophagy by transactivation of specific target genes.[133] The current model for TRP53 regulation of autophagy describes distinct, independent roles for cytoplasmic TRP53 and nuclear TRP53. Analysis *in vitro* in a variety of cell types and *in vivo* in various organisms has shown that cytoplasmic TRP53 functions as a repressor of autophagy. *Trp53* knockout (in enucleated cells) as well as inhibition or inactivation of cytoplasmic TRP53 results in increased levels of autophagy, similar to that of cells following induction by

starvation or treatment with rapamycin.[134] As well, induction of autophagy by starvation or rapamycin results in rapid depletion of cytoplasmic TRP53 by UPS degradation. Mutant TRP53, lacking an ubiquitylation site, has been shown to strongly inhibit the induction of autophagy *in vitro.*[134, 135] In contrast, nuclear TRP53 has shown to transactivate several genes known to stimulate autophagy following genotoxic stress. The TRP53 target gene, *Dram1*, encodes a highly conserved lysosomal protein whose function is unclear but has shown to strongly induce autophagy in a variety of cell types.[133] Other nuclear TRP53 transactivation targets include *Sesn2*, *Tsc2*, and genes encoding AMPK α/β subunits, which function together to stimulate autophagy during nutrient deprivation. AMPK can activate the TSC1/2 complex, which functions as a potent and sensitive inhibitor of MTOR, thereby de-repressing autophagy. The AMPK activator SESN2 functions to amplify the TRP53-directed autophagic response.[136, 137]

While it is widely accepted that TRP53 levels in normal cells are maintained by MDM2 ubiquitylation and proteasomal degradation,[138] it is also well demonstrated that tumor cells commonly carry various TRP53 mutants capable of escaping the regulation by MDM2 and proteolytic degradation.[139-141] Accumulation of mutant TRP53/TP53 in tumor cells is associated with metastases and resistance to chemotherapeutic agents. It has also previously been shown that various post-translational modifications can stabilize and destabilize cytoplasmic TRP53.[142] Recent analyses have shown that glucose restriction of tumor cells results in deacetylation and subsequent autophagy-dependent degradation of mutant TRP53, followed by apoptotic cell death. Inhibition of autophagy prevented degradation of the mutant TRP53 and promoted tumor cell survival during

glucose restriction.[143] This finding presents an interesting link in regulation of TRP53 and the cell death/survival axis by selective autophagy because cytoplasmic TRP53 has classically been characterized as a negative regulator of autophagy.

RB1-E2F1

The Rb1 – E2F pathway is a well-characterized regulator of the G1/S cell cycle transition. Under normal conditions, RB1 directly inhibits E2F so that the cell remains in G1. When RB1 is phosphorylated by CCND1−CDK4, RB1 disassociates from E2F proteins, which allows them to bind to promoters of several genes including *Ccne1* and virtually all initiators of the pre-replication complex necessary for entrance into S-phase. As this pathway is the major pathway augmented to increase proliferation in many cancer models, *Rb1* and E2F have become targets of many therapies. E2F1, a member of the E2F family of eukaryotic transcription factors, is unique in that when DNA damage is sensed E2F1 can directly promote apoptosis through expression of TRP73 and indirectly through TRP53 accumulation by expression of CDKN2A/p19^{arf} (Reviewed in p53 and E2f: partners in life and death.[144]) Evidence for how RB1-E2F regulates autophagy is confusing, as both RB1 and E2F1 can induce autophagy separately and often in an inverse relationship to each other. E2F1 indirectly increases the expression of autophagy related genes after E2F activation in U2-OS cells.[145] A comprehensive analysis of ATG-gene promoters in the presence of E2F1 reveals that E2F1 promotes the expression of *Ulk2*, *Atg4B*, *Atg4D*, *Atg7*, *Gabarap2*, *LC3a*, *LC3b*, *Atg9*, *Atg10*, *Atg12* and *Dram1.*[146] More recently, E2F1 overexpression in SK-MEL2 and MEFs induces autophagic flux by LC3-II conversion and GFP-LC3, irrespective of a functional

transactivation domain.[147] While the E2F2 transactivation domain contains the RB1 binding site, autophagy induction cannot be linked to E2F1-RB1 interaction since the expression of RB1 compared to E2F1 was not investigated. This data also does not address whether E2F1tr (deletion of transactivation domain) can still induce expression of ATG genes. As might be expected, removal of RB1 a negative regulator of E2F1, increases autophagic flux and expression of ATG genes in myoblasts.[148] Together these data suggest that regulation of autophagy by RB1-E2F is dependent on active E2F to promote transcription. However, when *Rb1* is overexpressed in several human cancer cell lines (U2-O2, Saos-2, Hep3b and U-87MG) autophagic flux is dramatically increased by detection through LC3-II conversion, GFP-LC3 localization and transmission EM.[149] Furthermore, when mutant RB1 that cannot bind to E2F family members is overexpressed in these same cell lines, autophagy is not induced which suggests that the repression of E2F by RB1 is necessary for autophagy induction. To corroborate these findings, knockdown of E2F1 in the same cells increases autophagy, suggesting that the regulation of autophagy by RB1-E2F is dependent on RB1 inhibiting E2F. To best determine if the control of autophagy by RB1-E2F is physiological, more studies conducted in non-cancer cell lines are needed since discrepancies could be due to the models used. Either way, RB1-E2F is clearly a transcriptional regulator of ATG-genes and could be involved in post-transcriptional regulation.

TFEB

The transcription factor EB (TFEB) is a well-characterized master regulator of lysosome biogenesis. TFEB directly promotes the transcription of several lysosomal

specific genes. Recent work in several labs has shown that when TFEB is phosphorylated it remains inactive at the lysosome membrane and does not translocate to the nucleus. Along these lines, it has been elucidated that MTORC1 at the lysosomal membrane is responsible for the inhibiting phosphorylation at Ser142 of TFEB.[150-152] Further, the inhibiting activity of MTORC1 is blocked during nutrient starvation, which is strikingly similar to MTORC1's inhibition of ULK1. When *TFEB* is overexpressed in HeLa cells, ATG genes *UVRAG*, *WIPI1*, *LC3b*, *SQSTM1*, *VPS11*, *VPS18*, and *ATG9b* are upregulated along with lysosomal genes.[153] Analysis of these same cells showed that autophagic flux is increased as well, indicating a parallel pathway to induce autophagic flux due to starvation. It is interesting that TFEB, a lysosomal master switch, is implicated in the induction of ATG genes post-starvation and that it is regulated through the starvation sensor MTORC1.

Autophagy-Apoptosis Crosstalk

Beclin 1-Bcl2/X_L Interaction

Classically, crosstalk between autophagy and apoptosis has focused primarily on the interaction of the pro-autophagic protein BECN1 and the anti-apoptotic protein BCL2.[154, 155] While the mechanisms behind the regulation of autophagy remain elusive and highly debated, the most widely accepted theory describes BCL2 as a BECN1 antagonist, through direct interaction with the BH3 domain found on BECN1, preventing activation of the PI3K complex and induction of autophagy.[156-158] Further analyses have identified additional components of regulation in that only ER-localized BCL2 or BCL2L1 (BCL2-like 1/BCL X) in complex with the ITPR1 complex and the NAF1 protein can

sufficiently inhibit the induction of autophagy under starvation conditions.[159] It has been stated that BCL2 and BCL2L1 in complex with BECN1 retain their anti-apoptotic capabilities; however, this data can be misleading as the interaction with BECN1 is dependent upon ER-localization of BCL2 and BCL2L1 while the regulation of intrinsic apoptosis occurs primarily at the mitochondria.[160] This may suggest multiple independent roles of the BCL2 and BCL2L1 proteins, dependent upon sub-cellular localization; however, it is unknown what drives the specific distribution of ER- and mitochondrial-BCL2/BCL2L1 proteins and whether or not redistribution may occur under starvation conditions as a functional link between apoptosis and autophagy. Recent data suggests that BCL2 may indirectly regulate BECN1 by interaction with AMBRA1, a positive regulator of BECN1 and autophagy. BCL2 interaction with AMBRA1 is disrupted upon autophagic stimuli, at which point AMBRA1 competes with ER-localized BCL2 for interaction with BECN1. Interestingly, AMBRA1 is preferentially bound by the mitochondrial fraction of cellular BCL2, rather than ER-localized BCL2, further linking the regulation of apoptosis and autophagy.[161]

Becn1- BCL2L11 Interaction

In contrast to the previous model, a novel interaction has been observed that suggests that the pro-apoptotic protein BCL2L11 (also known as BIM) can inhibit autophagy by recruitment of BECN1. BCL2L11 is a BH3-only *Bcl2* family protein that has been previously shown to function in an active, phosphorylated state or an inactive, dephosphorylated state. Phosphorylated BCL2L11 induces mitochondrial pore formation and apoptosis by activating pro-apoptotic BAX/BAK1 proteins, while the

dephosphorylated BCL2L11 is found in complex with the dynein light chain 1/LC8 protein. Luo et al[162] recently described the complex formed by BCL2L11 bridging the BECN1-LC8 interaction, which appears to sequester and inhibit BECN1 at the dynein motor complex. Upon knockdown or knockout of *Bcl2l11*, cells in culture exhibit an increase in autophagosome formation, which is reversible by overexpression of *Bcl2l11* (mutants unable to induce apoptosis). While this interaction appears sufficient to reduce levels of autophagy in nutrient-rich conditions, starvation-induced phosphorylation of BCL2L11 eliminates its interaction with BECN1, suggesting that inactive BCL2L11 may act to repress BECN1 until autophagy is induced by nutrient deprivation. This model also proposes an interesting link between autophagy and apoptosis in that BCL2L11 phosphorylation potentially enables BECN1-dependent autophagosome formation and also activates pro-apoptotic BAX/BAK1 proteins, inducing mitochondrial apoptosis.

Extrinsic apoptosis and autophagosomes

While the models mentioned above primarily involve interactions between BECN1 and the BCL2 family proteins, a recent study has described autophagosomes as potential platforms for formation of the death-inducing signaling complex (DISC) for the activation of caspase 8 (CASP8). Classically, the DISC forms near the PM off of adapter proteins bound to the cytoplasmic domain of various death receptors, activated by extracellular apoptotic signaling. CASP8 is recruited to the DISC as a monomer, which then self-associates and cross activates by cleavage of the pro domain. Active CASP8 cleaves and activates caspase 3 (CASP3) triggering the apoptotic cascade. It has been

previously reported that CASP8 requires SQSTM1 for efficient self-association; however, Young et al has described complex formation between ATG5 and CASP8, as well as ATG5 and the adapter protein FADD at the autophagosomal membrane. *Atg5* knockdown is associated with reduced CASP8 activation upon induction of apoptosis.[163] While other amplification mechanisms have been described in the apoptotic machinery, this appears to be the first that is dependent upon autophagy/autophagosome formation.

Transgenic Models for Autophagy Detection

GFP-LC3

The best characterized and most widely used detection model is the GFP-LC3 transgenic mouse generated by Mizushima and colleagues. This robustly expressing transgenic mouse, in which LC3 is driven by a constitutive CAG promoter, displays punctate GFP fluorescence that corresponds to LC3 positive phagophores and autophagosomes.[164] With this transgenic model quantitation of autophagosomes and phagophores is feasible using a high-resolution fluorescent microscope. This reporter line has been crossed into many of the knockout and floxed autophagy models generated in the field. For example, *Atg5*$^{-/-}$ mice are autophagy deficient and *Atg5*$^{-/-}$*;* GFP-LC3 mice do not exhibit the punctate fluorescence indicative of autophagosome formation.[165] (Protocols for use are widely available and published references are helpful, for detailed information see Deretic, V, Autophagosome and phagosome, Methods in Molecular Biology, 2008).[166] This model is limited in that only phagophore and autophagosome number, not autophagic flux can be evaluated. In basal conditions, lysosomal degradation clears the autophagosome and contents from the cell, to

maintain a "balance" of autophagosome formation and degradation. An accumulation of LC3 positive structures may represent either an increase in formation or a decrease in fusion events. Ferreting out these differences is relevant for proper data interpretation, especially when using chemical autophagy inhibitors and inducers. Measuring autophagic flux *in vivo* has been problematic to date and the field is in need of an appropriate reporter model; currently, tandem fluorescent-tagged autophagy proteins are a valuable *in vitro* tool.[167] Due to its chemical nature, GFP is quenched by the low pH of the autolysosome; however, red fluorescent proteins are more pH-stable, thus will retain their fluorescence in the lower pH of the autolysosome. The need for better detection mechanisms with regard to cardiac autophagy has led to the generation of a double transgenic reporter. A cardiac muscle specific alpha myosin heavy chain (αMyHC) promoter was used to drive expression of a mCherry-LC3 construct. These mice were crossed with the GFP-LC3 model previously described to produce a double label, which allows for the detection and evaluation of autophagic flux.[165] GFP-LC3 will still function for visualization of LC3 positive phagophores and autophagosomes, while mCherry-LC3 puncta will mark phagophores, autophagosomes and autolysosomes. Any double-labeled puncta are indicative of phagophores or autophagosomes, while red-only puncta correspond to autolysosomes. The comparison of double labeled structures to red fluorescent only labeled structures is informative to distinguish an increase in formation and a decrease of fusion events. Although this model is cardiac-specific, a similar strategy could be used to target other tissues or to generate a global transgenic model.

GFP-GABARAP

GFP-GABARAP transgenic mice were originally generated to address the question of the role of GABARAP in podocytes. Since GABARAP was reported to be highly expressed in podocytes, a pCAG-GFP-GABARAP transgenic mouse was produced in order to examine subcellular localization in this specialized cell type.[168] The expression level of GFP-GABARAP is low, yet visible, ameliorating many of the potential effects of highly expressing fluorescent proteins. In podocytes, GFP-GABARAP merged with SQSTM1 aggregates but not LC3-II. Though it was shown that GABARAP was not the preferred Atg8 ortholog for conjugation in podocytes this is a valuable reporter model for use across the field as differences in Atg8 orthologs are ferreted out.

Autophagy and development

Since the seminal genetic studies conducted in yeast, there has been an explosion in global and tissue-specific mouse knockout models produced to ascertain the role of autophagy in mammalian development and disease. Although this section stresses development over disease, the pathologies that arise from induced mutations frequently lead to diseased states. More commonly, mouse models are generated to profile and to uncover treatment regimens to address human diseases. The models that we present are segregated into 1 of 4 distinct complexes that the encoded protein primarily functions in rather than by resultant phenotype, these being the: 1) ULK1 complex, 2) PI3K complex, 3) ATG9 complex, and 4) ATG12 conjugation/LC3-lipidation system. Lastly, we include a section on induced mutations that impact autophagy more indirectly beyond these complexes.

ULK1 complex

ULK1 is the namesake member of the ULK kinase complex, and was initially identified as the mammalian homolog of yeast Atg1. ULK1 kinase complex functions early in the autophagy pathway, during the induction phase, and consists of ULK1, ATG13, RB1CC1 and ATG101/C12orf44. ULK1 and ATG13 are phosphorylated by activated MTORC1 in nutrient rich conditions; however, during stress MTORC1 is inactivated and the ULK1 complex is liberated to induce autophagy. In this sense, ULK1 is a direct link to nutrient sensing in the cell. Contrary to expectations based on other autophagy component knockouts, the ULK1 conventional knockout mice are viable and have no overt developmental defects. It is noted by the authors that *Ulk1* knockout mice exhibit LC3 conversion at the same rate as wild-type controls, indicating that starvation-induced autophagy is not impaired in these mutants. Under closer inspection *Ulk1* knockout mice exhibit specific red blood cell lineage populations retaining mitochondria and ribosomes, as well as delayed mitochondria elimination in reticulocytes and increased reticulocyte numbers.[169] It is evident from these initial studies that ULK1 is important for organelle clearance during erythrocyte maturation but not necessarily for starvation-induced autophagy. *Ulk2* knockout mice, like *Ulk1* knockouts, are phenotypically normal and viable, which is not surprising since there is most likely some degree of functional redundancy in the mammalian system.[170] To investigate this possibility, the authors generated *Ulk1/Ulk2* double knockout mice, which die shortly after birth. This result suggests that elimination of ULK function yields a similar phenotype to the other autophagy deficient models. *Ulk1*$^{-/-}$*/Ulk2*$^{-/-}$ MEFs are

specifically impaired in response to amino acid deprivation, though they have a similar response to glucose deprivation as wild type MEFs.[170]

RB1CC1, the mammalian functional counterpart of yeast Atg17, is a direct interacting partner of ULK1 and a member of the ULK kinase induction phase complex.[171, 172] *Rb1cc1* knockout mice die *in utero* at approximately E14.5-E15.5, with the lethality attributed to massive liver and cardiac cell death from increased levels of apoptosis. At E14.5, *Rb1cc1* knockout embryos exhibit ventricular abnormalities: the left ventricular wall is lacking in trabeculation, contains fewer cells, and is thinner that control littermates.[173] Autophagy may also impact the differentiation of hematopoietic stem cells (HSCs) as revealed in the conditional knockout of *Rb1cc1*. Tie2-Cre; *Rb1cc1* $^{fl/fl}$ mice die perinatally from severe erythroblastic anemia and have 6-fold fewer HSCs than control mice. There is not a proliferation defect, as apoptosis rates are similar between groups and proliferation is actually slightly higher in the conditional knockout (35% compared to 25%). However, a 4-fold increase in myeloid cells is found in the livers of CKO fetuses at E14.5, possibly explaining the depletion of fetal HSCs.[174, 175] Eight proteins have been identified as binding partners of RB1CC1, including TSC1, a tumor suppressor gene, which is also a negative regulator of MTOR. This interaction has been shown to mediate cell size and growth. As a compliment to the *Rb1cc1* KO studies, *Tsc1* knockout embryos also show defects in the heart and liver; however, the heart defects consist of thickened, rather than thinned, ventricular walls.[176]

As part of the ULK1 kinase complex ATG13 functions during the induction phase. ATG13, the mammalian homolog of yeast Atg13, is directly phosphorylated by both MTORC1 and ULK1/2 and has been characterized as an adapter protein. At this time there is not an *Atg13* knockout mouse, though intriguing *in vitro* studies indicate that Atg13 is essential for autophagy induction. Interestingly, when researchers simultaneously knockout both *Ulk1* and *Ulk2*, RB1CC1 and ATG13 are able to induce autophagy, suggesting that ATG13 and RB1CC1 are functioning independently of MTORC1 input to ULK1/2.[177] This higher order autophagy regulation network is deserving of further attention.

PI3K complex

AMBRA1 promotes the positive association of PtdIns3K and BECN1 to form the core multi-protein autophagy induction complex. Cecconi and colleagues initially reported that *Ambra1* knockout mice, generated by using a gene trap strategy, are non-viable.[178] The majority of *Ambra1* null embryos exhibit severe neural tube defects and spina bifida at E10 through E14.5. Additionally, a myriad of autophagy-deficient effects are seen including cell cycle abnormalities, accumulation of ubiquitinated proteins and increased apoptotic cell death. AMBRA1 is expressed specifically in the developing nervous system and appears to serve a role in controlling neuron survival. Like the *Becn1* knockout model, the phenotype of the *Ambra1* knockout is particularly severe and strongly affects the developing embryo. Interestingly, new evidence suggests that AMBRA1, like its binding partner BECN1, is a regulator of the autophagy-apoptosis crosstalk. Pagliarini et al. report that AMBRA1 is selectively and irreversibly degraded by

caspases and calpains.[179] This would help shift the cell from a survival program into an apoptotic program by destabilizing the autophagy promotion complex (BECN1/AMBRA1/PtdIns3K). To support this hypothesis, a non-cleavable mutant of *Ambra1* introduced into a 2F cell line shows a delay and partial prevention of apoptosis by extending the pro-survival effect of activated autophagy.[178] It has been widely reported in the literature that Parkin, an E3 ubiquitin ligase involved in the pathogenicity of Parkinson's disease, translocates from the cytosol preferentially to depolarized mitochondria and prompts their selective degradation via mitophagy.[180] Emerging evidence shows that AMBRA1 directly interacts with Parkin, although it is not a target for ubiquination. Additionally, *Ambra1* overexpression leads to an increase of mitophagy but only in the presence of Parkin. These findings suggest a novel mechanism whereby recruitment of AMBRA1, by Parkin, to the area of depolarized mitochondria elicits the nucleation of a phagophore.

BECN1 is a core protein component of the PI3K complex needed for the nucleation phase of autophagy, and serves as a scaffolding protein whereby a dynamic grouping of autophagy-related proteins hub. BECN1 is unique among the autophagy related proteins in that it contains an N-terminal BH3, which can be bound and inhibited by BCL2 and family member BCL2L1. [181] This inhibition is primarily disrupted by phosphorylation of BCL2/BCL2L1.[182] BECN1 contains a central coiled-coil interaction domain, which affords it the ability to oligomerize.[183] Interestingly, the other domains of BECN1 are non-essential for self-oligomerization, and self-oligomerization is not affected by starvation, rapamycin or overexpression of *Bcl2l1*, *Uvrag*, or *PtdIns3K*.

Higher-level understanding of this property has yet to be elucidated. The evolutionary conserved domain is responsible for autophagic function. BECN1 interacts with PtdIns3K directly to induce autophagosome nucleation and promote elongation. Perhaps attributed to its unique juxtaposition between two programmed cell death pathways or autophagy-independent functions, *Becn1* knockout mice have a more severe phenotype than other autophagy-related genes. *Becn1*$^{-/-}$ mice die *in utero* around E7.5 days, with the embryonic lethality ascribed to a developmental failure to close the pro-amniotic canal.[184] As a classical tumor suppressor, heterozygous disruption of *Becn1* results in an increase in tumor incidence.[184, 185] Additional induced mutant models for *Becn1* demonstrate a developmental-specific role for autophagy in blood cell lineages. *Becn1*-deficient *Rag1*$^{-/-}$ chimeras have a dramatic reduction in early marrow-derived thymocytes and B cells, while displaying normal levels of peripheral B and T cells. Thus, autophagy is required for undifferentiated lymphocyte progenitor maintenance and is not needed for the peripheral T and B cell compartments.[186] Naive T-cell homoeostasis is crucial for protective immunity against infection and it appears that autophagy is necessary for this process. A T-cell specific deletion of *PtdIns3K* shows that T-cell development does not require autophagy; however, naïve T cell survival is contingent upon autophagy to effectively remove damaged mitochondria via mitophagy.[187]

PtdIns3K is a highly conserved ancient kinase, and the only PI3K identified in yeast. PtdIns3K phosphorylates phosphatidylinositol to generate phosphatidylinositol 3-phosphate, which is used in the elongating autophagosomal membrane. A global

knockout of *PtdIns3K* was generated by crossing a conditional *PtdIns3K* allele with a Meox-Cre transgenic strain, for conversion to a null allele early in development. As with several other autophagy knockout models, the hemizygous mouse is viable and exhibits no obvious phenotype. In stark contrast to the hemizygous state, the homozygous *PtdIns3K* knockout is lethal in the early embryonic stages (E7.5). The embryos fail to form a recognizable mesoderm and both the endosomal trafficking and autophagosomal pathways are disrupted.[188] In a liver-specific knockout of *PtdIns3K* (Alb-Cre; *PtdIns3K* $^{fl/fl}$), mice are smaller and have enlarged, pale livers. At the molecular level, hepatocytes have reduced levels of autophagy nucleation complex and an increase in intracellular lipid droplet formation. Mitochondria are smaller, despite fed conditions, indicating a potential mitochondrial fusion defect. Additionally, when challenged with a 24-hour starvation, there are no observable GFP-LC3 puncta formed in the mutants, indicating a cessation of autophagic flux in the *PtdIns3K* liver-specific knockout mice.[189] A cardiomyocyte-specific knockout (Mck-Cre; *PtdIns3K* $^{fl/fl}$) results in mice with cardiomegaly, with an increased left ventricular wall thickness, decreased cardiac contractility and reduced cardiac output.[189] Although these mutants appear to be healthy at birth, they die between 5 and 13 weeks of age. Consistent with the liver-specific knockout, small and deformed mitochondria are observed, and autophagic flux is disrupted. It is evident that PtdIns3K is essential for autophagy regulation as well as autophagy-independent functions such as endosomal trafficking.

UVRAG is a coiled-coil, BECN1 interacting protein. It has been characterized as a positive regulator of autophagy acting in a complex with requisite autophagy proteins

BECN1 and PtdIns3K. UVRAG has tumor suppressor activity and a negative effect on tumor cell proliferation, as *UVRAG* is recurrently monoallelically mutated in human colon cancers.[190] Although *Becn1* serves a host of autophagy-independent functions, UVRAG may have autophagy-independent roles as well. Liang et al. elegantly showed that a UVRAG- PtdIns3K complex coordinates both late endosome fusion and trafficking, independently of its BECN1-dependent role in mediating autophagosomal formation and maturation.[10] Although neither UVRAG knockout nor conditional knockout mice currently exist, the generation of a model would be of great value to the field, aiding in ascribing autophagy-dependent and independent functions to each member of the induction complex.

Atg9 complex

ATG9 is the only multi membrane spanning autophagy related protein identified to date. Proposed to function in membrane trafficking, it dynamically shuttles between organelles during starvation conditions. There are 2 mammalian homologs: *Atg9a*, with a global expression profile, and *Atg9b*, with a restricted expression in the placenta and pituitary gland.[191] Not surprisingly, *Atg9a*$^{-/-}$ mice, like *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice, die as neonates. Thus, several autophagy-deficient mouse strains are unable to survive the transition from placenta-derived nutrition without an intact autophagic response. ATG9a is necessary for LC3 to conjugate PE, which is essential for autophagy function. This study reveals that *Atg9a* is an essential autophagy gene. In addition to autophagy related functions, Saitoh and colleagues show that Atg9a regulates double stranded DNA innate immune response.[192]

Atg12-conjugation and LC3-lipidation

ATG3 functions as the E2-like enzyme acting with LC3 in the LC3 ubiquitin-like conjugation system. Similar to *Atg5*$^{-/-}$, *Atg7*$^{-/-}$, and *Atg9a*$^{-/-}$ mice, *Atg3*$^{-/-}$ neonates die within one day of parturition, as amino acid levels are reduced and the overall energy homeostasis is disrupted after termination of the placental nutrient supply. The Atg12–Atg5 conjugate system is affected as well as the ATG8 conjugation system,[193] suggesting cooperation between the two distinct conjugation complexes during elongation. *Atg3*$^{-/-}$ cells contained small autophagosome-like, ATG16L1 positive structures, disorganized isolation membranes, and altered levels of GABARAP and GABARAPL2/GATE-16.

In yeast, a single Atg4 cysteine protease is responsible for cleaving Atg8 to expose an essential, terminal glycine residue. In humans and mice, four *Atg4* homologs (also referred to as autophagins) have been identified: *Atg4a*, *Atg4b*, *Atg4c*, and *Atg4d*. Of the *Atg4* homologs, *Atg4b* and *Atg4c* knockout mouse models have been generated to date. *Atg4c* is the most widely expressed Atg4 homolog in human tissues, although *Atg4b* is the most efficient and broadly active family member in vitro.[194] *Atg4b*$^{-/-}$ mice are viable but have a depletion of ATG8-like proteins in normal and nutrient poor conditions. These mice have balance and coordination deficits resulting from defective development in the vestibular system. The otoconia within the inner ear do not develop normally, resulting in abnormal, giant, or missing otoconia, a phenotype recapitulated in the *Atg5*$^{-/-}$ model.[195] In contrast, *Atg4c*$^{-/-}$ mice have normal levels of basal autophagy, but are less responsive to starvation-induced autophagy in the diaphragm. This role in ameliorating cell stress is highlighted in this model's increased susceptible to methyl

cholanthrene (MCA)-induced fibrosarcoma formation.[195] Given the functional redundancy of *Atg4* homologs, double knockout models should be highly informative.

Atg16L1 binds to ATG5 to regulate the localization of the Atg12–Atg5 conjugate to the nascent autophagosomal membrane. *Atg16L1*$^{-/-}$ neonates do not survive the perinatal starvation period and have a disruption of LC3-PE conjugation.[196] Crohn's disease, a chronic inflammatory bowel disease, is strongly linked genetically to the autophagy gene *ATG16L1* in humans,[197] and hypomorphic *Atg16l1* mice show altered Paneth cell morphology and decreased intestinal antimicrobial protein secretion.[198] Immunological synapse dynamics between dendritic cells (DCs) and T cells seems to be autophagy-dependent, as RNAi knockdown of ATG16L1 in DCs stabilizes DC-T cell interaction and hyperstimulates the T cell response.[199] This "hyperstability" is also found in DCs from Crohn's disease patients with an *ATG16L1* risk allele, which suggests that normally autophagy is activated at the synapse to negatively regulate T-cell activation. In addition, *Atg16l1*$^{-/-}$ mice may help to model an autophagy-inflammasome connection, since they have an induced expression of inflammatory cytokines including IL-1β.[196]

Atg5$^{-/-}$ mice die perinatally, suggesting that autophagy is not essential for embryonic development;[200] however, maternal mRNAs and proteins persist in the early embryo and may permit autophagic activity during the preimplantation period. Recent studies demonstrate that autophagy is essential for the oocyte-to-embryo transition. Analysis of GFP-LC3 embryos and oocytes show that autophagy is selectively upregulated in fertilized embryos from the one cell to the four cell stage.[201] Very little

evidence of autophagy is detectable in mature oocytes or ovulated, unfertilized oocytes, indicating that fertilization stimulates the induction of autophagy. Mating of oocyte-specific *Atg5*-deficient females (Zp3-Cre*; Atg5*$^{fl/fl}$) with *Atg5*$^{+/-}$ males, leads to embryos devoid of maternally inherited autophagic protein that fail to develop.[202] Autophagy may function to eliminate maternally inherited proteins or to catabolize cellular components for energy homeostasis. Since *Atg5*$^{-/-}$ neonates die from starvation-induced amino acid deprivation,[200] conditional gene deletion models have been developed to characterize autophagy later in development. From these models, autophagy has been shown to play a pivotal role in the prevention of neurodegeneration and the onset of age-related neurological diseases and cardiomyopathies. Nestin-Cre; *Atg5*$^{fl/fl}$ mice are viable and do not exhibit the suckling defect comparable found with *Atg5*- or *Atg7*-deficient neonates.[203] After three weeks of age, progressive motor and behavioral defects become apparent including: growth retardation, ataxia, poor motor coordination, and failed clasping reflex. High levels of neurodegeneration are found in the cerebellar Purkinje cells, and ubiquinated protein accumulations are observed across several anatomical regions of the brain.[203] A Purkinje-cell specific deletion strategy (pcp-Cre; *Atg5*$^{fl/fl}$ model) shows a later onset of motor defect around 10 months of age compared to the more regional nestin-Cre.[204] Autophagy-dependent cardiomyopathies may have a developmental component, as cardiac-specific *Atg5* deletions in young and adult mice have different pathologies.[205] Inducible deletion of *Atg5* in adult mice leads to cardiac myopathy, contractile dysfunction, altered sarcomere structure, and aberrant mitochondrial organization.

Conversely, cardiac-specific *Atg5* deletion during gestation does not result in these abnormalities, which points to adaptability of cardiomyocytes during early development.

Similar to *Atg5*, *Atg7* is necessary for the survival of neonates through amino acid pool maintenance as shown in the traditional knockout model.[200] Neuronal-specific $Atg7^{-/-}$ mice share many of the Atg5 knockout phenotypes including: abnormal behavior, altered limb-clasping response and reduced motor coordination. The nestin-Cre; $Atg7^{fl/fl}$ model shows high levels of neurodegeneration in the cerebellar and cerebral cortex with death occurring by 28 weeks of age.[206] Remaining neurons, which demonstrate normal proteasome function, accumulate poly-ubiquinated proteins and harbor inclusion bodies that increase with age. The nestin-Cre; $Vps18^{fl/fl}$ model exhibits a more severe neurodegeneration phenotype and results in lethality by postnatal day 12,[207] perhaps due to VPS18 being part of a protein tethering complex at the lysosome needed for its fusion with autophagosomes or endosomes. Ablation of *Atg7* within dopaminergic neurons of the substantia nigra has been performed to model Parkinson's disease.[208] Dopamine transporter (DAT)-Cre; $Atg7^{fl/fl}$ and engrailed-1-Cre; $Atg7^{fl/fl}$ adult mice have ubiquitinated protein aggregates and show a 40% to 60% reduction in dopaminergic neurons, respectively, and a corresponding decline in dopamine by 55% to 65%. Although DAT-Cre; $Atg7^{fl/fl}$ mice do not display locomotive deficits, engrailed-1-Cre; $Atg7^{fl/fl}$ mice exhibit an ataxic gait, presumably from additional *Atg7* ablation within the hindbrain, cerebellum, and midbrain. Using a tyrosine hydroxylase-specific Cre mouse (TH-IRES-Cre) with the $Atg7^{fl/fl}$ model, there is a 40% reduction in dopaminergic

neurons compared to controls and an accumulation of α-synuclein protein.[209] Seizure activity, in human TSC patients, is believed to occur due to a hyperstimulated MTOR pathway.[210] Inhibition of autophagy is demonstrated in CaMKIIα-Cre; *Pten*$^{fl/fl}$ mice, CaMKIIα-Cre; *Tsc1*$^{fl/fl}$ mice, and brains of TSC patients.[211] Moreover, CaMKIIα-Cre; *Atg7*$^{fl/fl}$ mice are prone to spontaneous tremors, as 75% of the mice develop symptoms by 6-7 weeks of age. A liver-specific *Atg7* deletion results in hepatomegaly and an increase in hepatocyte size due to an accumulation of peroxisomes and deformed mitochondria.[212] Insulin-producing pancreatic β-cells use autophagy to maintain homeostasis, as β-cell-specific RIP-Cre; *Atg7*$^{fl/fl}$ mice show β-cell mass reduction and exhibit hypoinsulinemia and hyperglycemia.[213] *Atg7*-deficient islet cells have reduced numbers of insulin granules, increased mitochondrial swelling, and distended rough ER and Golgi complex. Interestingly, these mice do not become diabetic until bred with *ob/ob* mice to trigger an obesity-induced ER stress response. Obesity induces the UPR in β-cells, which in the absence of autophagy leads to an increase in β-cell death from an increase in reactive oxygen species.[213, 214]

Since there are several Atg8 mammalian homologs (e.g. GABARAP, LC3s, GABARAPL1, and GABARAPL2), it is not surprising to find that *Lc3b*$^{-/-}$ and *Gabarap*$^{-/-}$ mice are viable, have normal lifespans, and exhibit normal autophagy levels in fed and starved conditions.[215, 216] While the subcellular re-localization of LC3 is useful in monitoring autophagy, LC3 has been recently implicated in mitophagy. BNIP3L, an outer mitochondrial membrane spanning protein, has a cytoplasmic LC3 interaction region (LIR) domain through which it can recruit mitochondria to the autophagosome;

ablation of this interaction severely affects mitochondrial clearance.[97] Although SQSTM1 interacts with all of the LC3 orthologs, recent studies have revealed that only lipidated LC3 selectively binds to SQSTM1 and directs it to the autophagosome.[164] Thus, closer examination of the *Lc3b*$^{-/-}$ model may reveal defects in mitophagy or aggresome clearance in a cell-specific fashion.

Autophagy-modifying models

Activation of autophagy, through the ectopic overexpression of peroxisome proliferative activated receptor gamma, coactivator 1 α (PPARGC1A, also known as PGC-1α), results in the degradation of HTT/huntingtin protein aggregates and ameliorates the phenotype of Huntington's disease mice.[217] This PPARGC1A-mediated proteolysis occurs by activation of TFEB, a known master regulator of autophagy. Induction of autophagy through rapamycin administration can reverse protein plaque formation in a murine model for Gerstmann–Sträussler–Scheinker disease, a prion disease that results in ataxia from extracellular PrP amyloid plaques.[218] Rapamycin treatment eliminates amyloid plaque formation, reduces symptoms, and extends the survival time of these mice.[219] Within the hippocampus, the region devoted to learning and memory, an increase in autophagosomes in aged mice has been correlated with short-term memory deficits.[220] This suggests an impairment of productive autophagosome-lysosome fusion in aged hippocampal cells. A genetic affirmation of this mechanism is shown in a rescue of the TgCRND8 mouse model for Alzheimer's disease.[221] Ablation of cystatin B, a lysosomal cysteine protease inhibitor, reverses this defect through an increase in productive autophagy and reduction of amyloid protein deposition. The *Sqstm1*

knockout mouse displays an Alzheimer's-like phenotype, characterized by accumulations of cytotoxic aggregates of ubiquitinated proteins in neurons and other tissues.[222] Autophagy mitigates ROS-mediated cellular damage through productive mitophagy. The E3 ubiquitin ligase PARKIN initially was found to be necessary for mitophagy in neurons,[223] and *Parkin* mutations are associated with juvenile Parkinsonism.[224]

However, PARKIN-mediated mitophagy is also important for increased myocardial survival after induced infarction. Fatalities from myocardial infarction increase from 20% in control mice to 60% in *Parkin*$^{-/-}$ mice during the first week after insult.[225] Mitophagy may be a necessary process after injury to eliminate damaged mitochondria to avoid an inflammatory response. Lysosomal DNase II functions to degrade mitochondrial DNA upon autophagy-mediated trafficking of mitochondria to the lysosome. Upon pressure overload, mice with DNase II-deficient hearts exhibit myocarditis from an inflammatory response and dilated cardiomyopathy.[226] Autophagy could impact cell differentiation by restructuring the cell through the elimination of organelles, proteins, and structures while providing necessary 'building block' metabolites. FGF signaling inhibits the transition from cardiac precursor cells to differentiated cardiomyocytes. Abrogation of FGF signaling through the conditional deletion of floxed FGFR1/2 or FRS2α causes premature cardiomyocyte differentiation through precocious autophagy activation.[227] A potential regulatory mechanism for autophagy induction comes from a recent study that examined potent miRNAs that are expressed upon hypertrophic stimuli.[228] *miRNA-212/132*$^{-/-}$ mice are protected from

transaortic constriction-induced hypertrophy and exhibit increased basal levels of autophagy. Transgenic overexpression of miRNA-212/132 causes a decrease in the pro-autophagic transcription factor FOXO3, impairment of starvation-induce autophagy, and leads to hypertrophy and heart failure. Lastly, blocking the fusion of autophagosomes and lysosomes in cardiomyocytes through a cardiac-specific deletion of *Mitofusin 2* (*Mfn2*) leads to increased sensitivity to ischemia and late onset cardiac dysfunction.[229]

Sonic hedgehog (SHH) is a crucial morphogen that promotes angiogenesis during development[230] and after ischemia in adults.[231] Ligation of mouse common carotid arteries induces neointimal lesion formation due to SHH-mediated, autophagy-dependent smooth muscle cell proliferation.[232] Inhibition of autophagy with 3-MA or BafA1 inhibits cell proliferation and neointima formation. This potentially signifies an angiogenic role for autophagy in SHH-mediated smooth muscle cell proliferation during development as well. A reduced level of autophagy is associated with arterial endothelial cell ageing and dysfunction in older humans, as autophagy markers are decreased by 50% and endothelium-dependent dilatation (EDD) is lowered by 30%.[233] In addition, aged mice have a 40% decrease in autophagy markers and a 25% reduction in EDD due to suppressed nitric oxide generation and an increased inflammatory response. Induction of autophagy with trehalose reverses the EDD through normalizing both nitric oxide levels and inflammatory cytokine expression. The dependence of cell differentiation on autophagy is probably cell specific, since autophagy may inhibit skeletal muscle differentiation from myoblasts. TGF-β1, an inhibitor of muscle differentiation, induces autophagy through phosphoprotein enriched in

diabetes/phosphoprotein enriched in astrocytes (PED/PEA-15) activation.[234] Ectopic overexpression of PED/PEA-15 in transgenic mice activates autophagy leading to atrophic fibers accumulation in skeletal muscle.

Summary

Autophagy is a complex process with many constituent players and is highly regulated. Autophagic degradation of cellular contents and turnover of organelles is crucial for cell vitality and for the fidelity of organismal development. The concerted effort of specific types of autophagy is essential for cellular homeostasis and immune function. Transgenic mouse models of autophagic deficiency, both global and tissue/temporal specific, have revealed the importance of autophagy in the oocyte-to-embryo transition, post-natal survival, development, differentiation, and aging. A synopsis of the induced genetic models discussed in this review is provided in **Table 1.2**. From the collection of phenotypes of these models, a common thread emerges to assert that autophagy may not be essential for embryogenesis. Six models (*Atg3*$^{-/-}$*; Atg5*$^{-/-}$*; Atg7*$^{-/-}$*; Atg9a*$^{-/-}$*; Atg16L1*$^{-/-}$*; Ulk1*$^{-/-}$*Ulk2*$^{-/-}$) exhibit a perinatal lethality partly based on an inability for the neonates to maintain a pool of amino acids after parturition. In contrast, a separate cluster of mutations, three within the PI3K complex (*Ambra1*$^{-/-}$; *Becn1*$^{-/-}$; *PtdIns3K*$^{-/-}$) and one in the ULK complex (*Rbc1cc1*$^{-/-}$), demonstrate early to mid-gestation lethality in the respective knockout models. Reconciliation of these phenotypic differences may be argued by functional redundancies or alternative mechanisms (e.g. cell-specific *Atg5/7*-independent autophagy) found within the former group, or additional autophagy-independent roles for members of the latter group.

With regards to the ULK complex, a recent study showed that both *Atg13*- and *Rbc1cc1*-deficient avian DT40 cells had impaired autophagy, which was independent of Ulk1/Ulk2.[177] Generation of an *Atg13*-deficient mouse model should help in discerning the developmental consequences of ULK1/2-independent autophagy. In addition to this ULK1/2-independent activation, ULK3 can also contribute to the induction of autophagy as well.[235] This affirms Einstein's notion that a problem, when looked at in the right context, becomes increasingly more complicated. It also illustrates that there is considerable work to be done in this area, especially as our methods of detection improve.

Our understanding of autophagy has increased tremendously in the past decade and continues to do so as the research effort in this area continues to accelerate. A more productive growth will be greatly benefitted with the advent of more specific pharmacological agents to induce or inhibit autophagy both *in vitro* and *in vivo*, as well as the generation of a global reporter model to monitor autophagic flux in currently existing induced mutant strains. The interconnectedness of many cell regulation pathways (e.g. Akt, MTOR, apoptosis regulation) and autophagy implicitly underscore the importance of these processes to cellular function. It is conservative to predict that as the field continues to mature the tenuous nature of each of these interactions will become clearer.

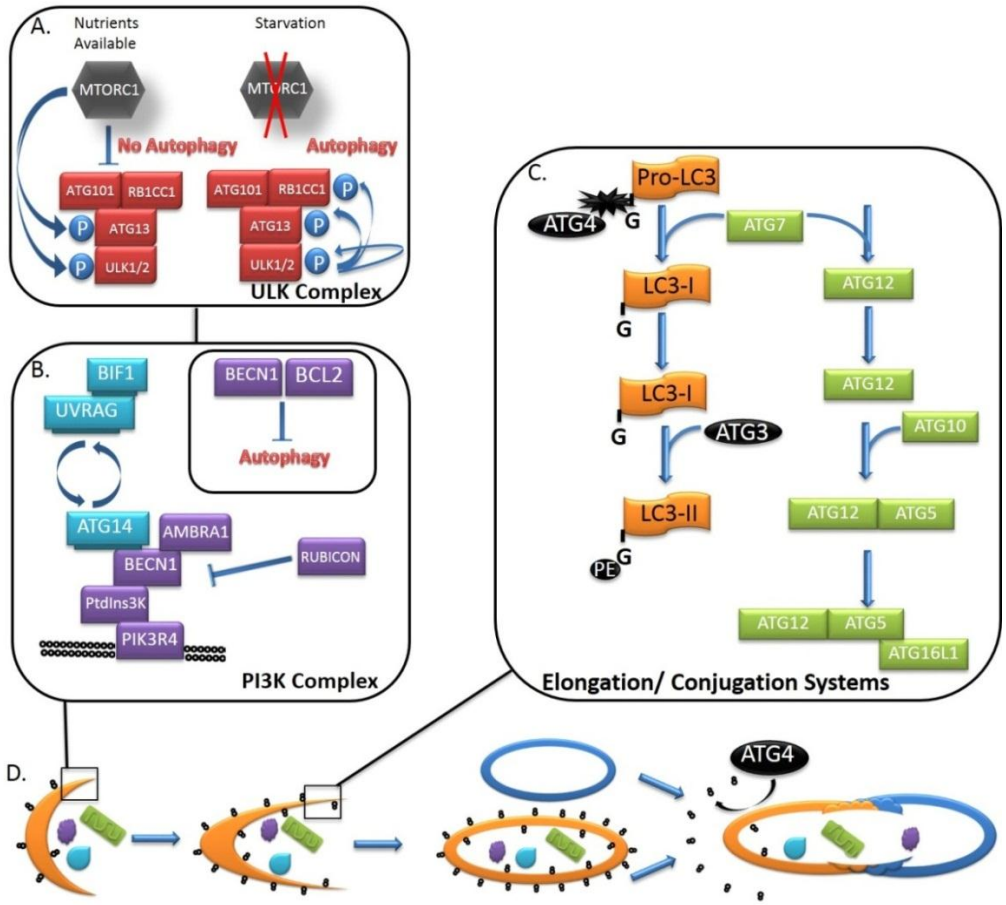

Figure 1.1 The core machinery of autophagy. Autophagy is a complex degradation process in which general cytoplasm or organelles are engulfed by a double membrane bound structure and degraded and recycled following fusion with a lysosome. (**A**) Dephosphorylated ULK1 dissociates from the MTOR complex and phosphorylates itself, ATG13 and RB1CC1 to induce the nucleation phase. (**B**) The PI3K complex is assembled at the site of the nascent autophagosomal membrane. UVRAG and ATG14 are found in BECN1 complexes in a mutually exclusive manner. BECN1 is inhibited when bound by anti-apoptotic BCL2, which results in downregulated autophagy. (**C**) The two ubiquitin-like conjugation systems essential for membrane elongation are outlined schematically. (**D**) The autophagosomal membrane (orange crescent) is studded with ATG8 (stylized in

black). The membrane elongation is dependent on the ATG12–ATG5-ATG16L1 conjugation system. During fusion with the lysosome (blue oval) ATG8 associated with the outer membrane is cleaved and recycled by ATG4 while ATG8 associated with the inner-membrane is degraded by lysosomal proteases along with the cargo of the autophagosome.

Table 1.1 Pharmacological inducers and inhibitors of autophagy

Autophagy Inducer	Action	Reference
MG132	Inhibit 26S Proteasome	54
Bortezomib	Inhibit 26S Proteasome	54
Tunicamycin	Induce ER stress by inhibiting N-acetylglucosamine phosphotransferase	55
Thapsigargin	Induce ER stress via SERCA inhibition Prevent autophagosome/lysosomal fusion by inhibiting Rab7 recruitment	56
Fluspirilene	Stabilize ATG5–ATG12 by preventing Ca^{2+}-mediated CALPAIN-1 activation	57
Resveratrol	Sirtuin-mediated deacetylation of cytoplasmic proteins	58, 60
Spermidine	Sirtuin-mediated deacetylation of cytoplasmic proteins	59, 60
Rapamycin	Inhibit MTOR	61-63
Lithium chloride	Increase PI3P levels by inhibition of IMPase	64
L-690,330	Increase PI3P levels by inhibition of IMPase	65
Carbamazepine	Increase PI3P levels by inhibition of IMPase	66, 67
Xestospongin B	Antagonize IP3R (and IP3R-mediated BCL2-BECN1 interaction)	70-72
Xestospongin C	Inhibit IP3R and ER Ca2+ receptors	
Autophagy Inhibitor	**Action**	**Reference**
3-Methyladenine	Inhibit PI3K (PI3KC1 and PI3KC3)	73
Wortmannin	Inhibit PI3K (PI3KC1 only)	74
Bafilomycin-A1	Inhibit vacuolar ATPase	76
Spautin-1	Inhibit USP10 and USP13 deubiquitinase activity	75

Table 1.2 Atg knockout and conditional knockout mouse phenotypes

Gene	Genotype	Phenotype	Reference
Ulk1	*Ulk1*$^{-/-}$	Viable; No developmental defects or impairment to starvation-induced autophagy; Delayed mitochondria elimination from reticulocytes	169
Ulk2	*Ulk2*$^{-/-}$	Viable; No overt phenotype/fertility defects	170
	Ulk1$^{-/-}$; *Ulk2*$^{-/-}$	Neonatal lethal; impaired response to amino acid deprivation	170
Rb1cc1	*Rb1cc1*$^{-/-}$	Embryonic lethal at E14.5-15.5; Excessive liver and cardiac apoptosis; Thin left ventricular wall, lacking trabeculation	171-173
	Tie2-Cre; *Rb1cc1*$^{fl/fl}$	Perinatal lethal; Severe erythroblastic anemia; 6-fold decrease in HSCs; Increased number of myeloid cells in liver	174, 175
Atg13	Currently no KO mouse		
Atg101	Currently no KO mouse		
Ambra1	*Ambra1*$^{-/-}$	Non-viable; Embryos show neural tube defects, spina bifida at E10-E14.5, accumulation of ubiquitinated proteins, and increased apoptosis in various tissues	178
Becn1	*Becn1*$^{-/-}$	Embryonic lethal at E7.5; Failure to close pro-amniotic canal	184
	Becn1$^{+/-}$	Increased tumor incidence in various tissues	185
	Becn1$^{-/-}$; *Rag1*$^{-/-}$	Reduced levels of early marrow-derived thymocytes and B cells	186
PtdIns3k	Meox-Cre; *PtdIns3k*$^{fl/fl}$	Embryonic lethal at E7.5; Embryos fail to form recognizable mesoderm	188
	Alb-Cre; *PtdIns3k*$^{fl/fl}$	Smaller adult mice; Enlarged, pale liver. In hepatocytes: Reduced levels of autophagy nucleation complex; Arrested autophagic flux; Increased intracellular lipid droplet formation; Reduced size of mitochondria	189
	Mck-Cre; *PtdIns3k*$^{fl/fl}$	Lethal between 5-13 weeks; Cardiomegaly with increased thickness of left ventricular wall, decreased cardiac contractility/reduced cardiac output; Arrested autophagic flux; Reduced size of mitochondria	189
Uvrag	Currently no KO mouse		
Atg9a	*Atg9a*$^{-/-}$	Neonatal lethal during perinatal starvation	192
Atg9b	Currently no KO mouse		
Atg3	*Atg3*$^{-/-}$	Neonatal lethal at P1; Many cells contain small ATG16L1-positive structures and scattered isolation membranes	193

Table 1.2 Continued

Gene	Genotype	Phenotype	Reference
Atg4a	Currently no KO mouse		
Atg4b	*Atg4b*$^{-/-}$	Viable; Inner ear developmental defects and associated defects in balance/coordination	195
Atg4c	*Atg4c*$^{-/-}$	Viable; No overt phenotype/fertility defects; Decreased starvation-induced autophagy in diaphragm	195
Atg4d	Currently no KO mouse		
Atg16l1	*Atg16l1*$^{-/-}$	Neonatal lethal during perinatal starvation; Induced expression of IL-1β	196
Atg5	*Atg5*$^{-/-}$	Neonatal lethal during perinatal starvation	236
	Nestin-Cre; *Atg5*$^{fl/fl}$	Viable; Neurological defects appear after 3 weeks of age: growth retardation, ataxia, poor motor coordination, failed clasping response; Increased accumulations of ubiquinated proteins; Abundant neurodegeneration of cerebellar Purkinje cells	203
	Pcp-Cre; *Atg5*$^{fl/fl}$	Viable; Neurological defects appear after 10 months of age	204
Atg7	*Atg7*$^{-/-}$	Viable; Neurological defects appear after 10 months of age	237
	Nestin-Cre; *Atg7*$^{fl/fl}$	Lethal by 28 weeks of age; Abundant neurodegeneration/neuron death in cerebrum and cerebellum, ubiquitinated protein accumulations in remaining neurons; Motor and clasping defects	206
	DAT-Cre; *Atg7*$^{fl/fl}$	Viable; 40% reduction of dopaminergic neurons and 55% reduction in dopamine; ubiquitinated protein accumulations in remaining neurons	208
	En1-Cre; *Atg7*$^{fl/fl}$	Viable; 60% reduction of dopaminergic neurons and 65% reduction in dopamine; ubiquitinated protein accumulations in remaining neurons; Ataxic gait	208
	TH-IRES-Cre; *Atg7*$^{fl/fl}$	Viable; 40% reduction of dopaminergic neurons; accumulation of α-synuclein protein	209
	CaMKIIαCre;*Atg7*$^{fl/fl}$	Viable; Spontaneous tremors in 75% of mice by 6-7 weeks of age	211

Table 1.2 Continued

Gene	Genotype	Phenotype	Reference
	Alb-Cre; *Atg7*$^{fl/fl}$	Viable; Enlarged hepatocytes; Hepatomegaly; Accumulation of peroxisomes and mitochondria	212
	RIP-Cre; *Atg7*$^{fl/fl}$	Viable; Hypoinsulinemia and hyperglycemia; Reduced number of insulin granules; Swelling of mitochondria, rough ER, and Golgi	213
Lc3b	*Lc3b*$^{-/-}$	Viable; No overt phenotype/fertility defects	215
Gabarap	*Gabarap*$^{-/-}$	Viable; No overt phenotype/fertility defects	216
Sqstm1	*Sqstm1*$^{-/-}$	Alzheimer's-like phenotype; Accumulation of ubiquitinated proteins in neurons and various tissues	222
Parkin	*Parkin*$^{-/-}$	40% increase in fatalities from induced myocardial infarction due to mitophagy defect	225
Mfn2	*Mfn2*$^{-/-}$ *(cardiac sp.)*	Increased sensitivity to ischemia and late onset cardiac dysfunction	229

Chapter Two: Altering Autophagy: Mouse Models of Human Disease

Introduction

This chapter has been published and is included here for completeness of the dissertation.[238]

Since the advent of knockout technologies using mouse embryonic stem cells in the late 1980s, there has been an explosion of murine models to profile human diseases. The understanding of the genetic contribution to these diseases has been further enhanced with the incorporation of tissue-specific gene deletion strategies through the use of the Cre-lox and FLP-FRT site-specific recombination systems. Autophagy, a crucial regulator of cell energy homeostasis, is also a companion process to the ubiquitin-proteasome system to assist in the turnover of proteins. Two distinct types of mouse models have been engineered to characterize autophagy. The first type is based on the reporter model system to both detect and quantitate the *in vivo* levels of autophagy in all tissues and organs. The second type is based on genomic modification to perform global or tissue-specific gene deletions for generation of pathological disease conditions. A wide array of human diseases and conditions have been shown to be intimately linked to alterations in autophagy and include: 1) cancer, 2) heart disease, 3) neurodegenerative diseases (e.g. Alzheimer's and Parkinson's disease), 4) aging, 5) lysosomal storage disorders, 6) infectious disease and immunity (e.g. Crohn's disease), 7) muscle atrophy, 8) stroke, 9) type 2 diabetes, and 10) reproductive infertility. This article will address the role of autophagy in human disease progression by reviewing the strengths and weaknesses of current murine models, as well as discussing their utility as therapeutic models for disease prevention and amelioration.

Transgenic models for autophagy detection

GFP-LC3

The best characterized and most widely used detection model is the GFP-LC3 transgenic mouse generated by Mizushima and colleagues.[164] This robustly expressing transgenic mouse, in which LC3 is driven by a constitutive CAG promoter, displays punctate GFP fluorescence that corresponds to autophagosomes. With this transgenic model, quantitation of autophagosomes is feasible using a high resolution fluorescence microscopy. This reporter has been crossed into many of the knockout and floxed autophagy models generated in the field. For example, *Atg5* $^{-/-}$ mice are autophagy deficient and *Atg5* $^{-/-}$*;* GFP-LC3 mice do not exhibit the punctate fluorescence indicative of autophagosomes. Protocols for use are widely available and published references are helpful, for detailed information see reference.[239] This model is limited in that only autophagosome numbers, not autophagic flux, can be evaluated.

In basal conditions, lysosomal degradation clears the autophagosome and contents from the cell, thus maintaining a "balance" of autophagosome formation and degradation. An accumulation of autophagosomes could either represent an increase in formation or a decrease in fusion events. Ferreting out these differences is relevant for proper data interpretation especially when using chemical autophagy inhibitors and inducers. Measuring autophagic flux *in vivo* has been problematic to date and the field is in need of an appropriate reporter model; currently, tandem fluorescent-tagged autophagy proteins are a valuable *in vitro* tool. An increase in GFP puncta (denoting

labeled autophagosomes) could indicate an increase in autophagosome formation or a decrease in vesicular fusion with the lysosome. Due to its chemical nature, GFP is quenched by the low pH of the autolysosome. Since red fluorescent proteins are more pH stable, they have been utilized in assays designed to monitor autophagic flux. The need for better detection mechanisms with regard to cardiac autophagy has led to the generation of a double transgenic reporter. A cardiac muscle specific alpha myosin heavy chain (αMyHC) promoter was used to drive expression of a mCherry-LC3 construct. These mice were crossed with the GFP-LC3 model previously described to produce a double reporter which allowed for the monitoring of autophagic flux; autophagosomes were identified by GFP and mCherry co-localized puncta, while autolysosomes were tracked by mCherry-only puncta.[165] Although this model is cardiac-specific, a similar strategy could be used to target other tissues. Additionally, a dual labeled RFP-GFP-LC3 construct could be used to generate a ubiquitous transgenic model to quantitate autophagic flux.

GFP-GABARAP

GFP-GABARAP transgenic mice were originally generated to address the question of the role of GABARAP in podocytes. Since GABARAP was reported to be highly expressed in podocytes, a pCAG-GFP-GABARAP transgenic mouse was produced in order to examine subcellular localization in this specialized cell type. The expression level of GFPGABARAP is low, yet visible, ameliorating many of the potential effects of highly expressing fluorescent proteins. In podocytes, GFP-GABARAP co-localized with SQSTM1 aggregates but not LC3II. Although it was shown that GABARAP was not the

preferred Atg8 ortholog for conjugation in podocytes, this may prove to be a valuable reporter model in other tissues.[168]

Cancer

As we will see as a common thread in many of the disease models featured in this chapter, there is no consensus in the literature as to whether autophagy is protective or maladaptive. The contribution of autophagy to cancer development is the most demonstrable example of the Janus-like role of autophagy in disease pathogenesis, and perhaps the most thoroughly investigated. Autophagy is hypothecated to contribute to tumor progression through two distinct mechanisms. Tumor growth is initially restricted when the centrally-located cells undergo nutrient deprivation, hypoxia, and if prolonged, subsequently undergo necrosis. This metabolic stress induces autophagy to maintain energy homeostasis and prevent necrosis until neo-vascularization occurs. Angiogenesis is potentially able to restore a nutrient supply throughout the tumor, promoting tumor survival and facilitating growth and metastasis. Additionally, transformed cells that harbor defects in apoptosis and autophagy pathways tumor cells undergo chronic necrosis, which foments vascularization of the necrotic area, thus promoting tumorigenesis. Conversely, intact autophagy can be cytoprotective by eliminating damaged or aged organelles and degrading aggregated or misfolded proteins, thus preventing accumulation of tumorigenic and/or cytotoxic cellular inclusions.

These two scenarios are manifested in mouse models of autophagy disruption. When *Beclin1* (*Becn1*) was heterozygously disrupted (*Becn1*$^{+/-}$), these mice exhibited an increase in tumor formation when compared to control littermates.[185] Disruptions in pro-autophagy genes *Becn1* [184], *UVRAG* [240], and *Bif-1*[241]resulted in increased frequencies of lymphomas and mammary neoplasia through an increase in genetic instability. *TSC1/TSC2*-deficient mice, which were unable to effectively suppress MTOR, had reduced autophagy and a subsequent increase in tumor occurrence.[242, 243] These models all bolster the stance that autophagy is tumor suppressive. The embryonic or neonatal lethality of many autophagy knockout models is prohibitive for studying diseases, such as cancer, that are associated with age. Tissue-specific knockout studies will provide more insights in elucidating gene-specific mechanisms for tumor suppression. Evidence is forthcoming from cancer cell lines and primary tumor profiling that autophagy is permissive for tumor growth and enhanced in primary tumors. In a pancreatic cancer cell line (8988T cells), inhibition of autophagy by *Atg5* siRNA resulted in an inhibition of soft agar growth, a measure of tumorigenic potential.[244] In cell lines with activated *Ras*, a strong oncogene, autophagy was necessary for the quick growth and high metabolic needs of tumor cells. These cancer cells were described as "addicted to autophagy," and the phenomenon was consistent amongst several Ras-activated cell lines (e.g. T14, H1299, and CHT116). These data suggest that in quickly growing tumors, autophagy inhibition may sensitize the cells to death thus enhancing available treatment options.[245] This study provides primarily *in vitro* evidence of a role for autophagy induction in supporting tumor growth.

Heart disease

Coronary heart disease (CHD) is the leading cause of death of men and women in the United States. CHD is caused by narrowing of the arteries that supply the heart. In mouse models a standard treatment is to induce an ischemic event by surgically occluding one or more coronary artery. This occlusion may be relieved at a later time (ischemia/reperfusion) or remain (permanent occlusion). Interestingly, autophagy is induced in both ischemia/reperfusion (I/R) events and permanent occlusion events but the outcomes are differential. During I/R events autophagy is activated, however in *Becn1*$^{+/-}$ mice damage was reduced indicating that induction of autophagy was maladaptive during reperfusion.[246] Conversely, autophagy appears to be protective during permanent occlusion events. Long term ischemia causes relief of mechanistic target of rapamycin (MTOR) inhibition of autophagy, thus leading to autophagy induction. In concordance with this, expression of a dominant negative MTOR regulator resulted in a reduction of autophagy and subsequent increase in cardiac damage.[247] Two recent mouse models of heart disease provide evidence supporting a protective role of autophagy for the prevention of CHD.[248] Razani et al provided evidence that autophagy prevented cholesterol crystal-induced inflammation that normally can lead to atherosclerosis. Since high fat diets have been shown to inhibit autophagic flux, competent autophagic flux may be necessary for the prevention of CHD. [249]

In addition to CAD, congenital mutations and other pathological conditions (e.g. type 2 diabetes) my result in cardiomyopathies. In a recent study, Choi et al showed that in a cardiomyopathy model resulting from a mutation in A-type lamins (A/C) resulted in

active MTOR in cardiomyocytes, which inhibited autophagy activation.[250] This lack of autophagy activation was proposed to lead to an energy deficit and was detrimental to the survival of the cardiomyocytes and resulted in disease progression. Further evidence of the protective role of autophagy was obtained when autophagy was reactivated by the MTOR-inhibiting drug rapamycin. In mice mutant for A-type lamins, rapamycin treatment attenuated the cardiomyopathy phenotype. In another approach, a mouse model of diabetic cardiomyopathy, generated by diet alteration to include a surplus of saturated fatty acids, has revealed that autophagy was activated in response to pressure increase resultant from cardiac hypertrophy. Autophagy induction was measured by increased Becn1 and LC3B mRNA, as well as increased GFP-LC3 puncta in the diabetic hypertrophy model. In this system, isolated cardiomyocytes that were autophagy impaired did not develop hypertrophy, indicating that increased autophagy was required for hypertrophy. Furthermore, isolated cardiomyocytes treated with myristate led to an increase in BECN1 and ATG7 expression through a ceramide-dependent mechanism.[251] The role of autophagy is complex and seems to be highly context specific with regard to heart disease. It is apparent that the method by which autophagy is inhibited or induced and in which type of model differentially dictates whether autophagy will result in a positive or negative outcome.

Neurodegenerative diseases (e.g. Alzheimer's and Parkinson's disease)

Post-mitotic neurons rely heavily on basal autophagy to clear old, damaged organelles and potentially harmful protein aggregates. Several neurodegenerative disorders result from expansions of poly-glutamine or poly-alanine stretches, including

Huntington's disease, Parkinson's disease, spinocerebral ataxias, and fronto-temporal dementia. Mutant proteins involved in these diseases have an increased propensity to aggregate and poison the cell; accordingly, the disease progression is directly related to the amount of protein aggregates formed in these patients. In normal physiology, neurons rely on both the proteasomal degradation system and autophagy to maintain protein and energy homeostasis. It has been shown that autophagy is used preferentially to remove large aggregated proteins or multiprotein plaques. In a rat model of Machado-Joseph disease (spinocerebral ataxia type 3) overexpression of BECN1 reduced both mutant ataxin-3 accumulation and ubiquitin-positive inclusions.[252] It seems that autophagy is used as a compensatory mechanism to relieve the toxic effects of these mutant protein aggregates in the cell, serving a protective role. Neuronal-specific conditional deletion of *Rb1cc1* (*Rb1cc1*$^{fl/fl}$; nestin-Cre) resulted in dystrophy (axonal swelling), neurodegeneration, accumulation of polyubiquitinated proteins, damaged mitochondria, and neuronal death. When compared to *Atg5/Atg7* neuronal conditional deletion models, the *Rb1cc1*$^{-/-}$ phenotypes were more severe, present at an earlier age, and resulted in premature death. Unique to the *Rb1cc1*$^{-/-}$ conditional knockout mice is the development of diffuse brain spongiosis, observed as early as 2 weeks of age, and associated with ubiquitin positive inclusions, indicative of impaired clearance of cytotoxic proteins by autophagy.[253] Targeted deletions of either *Atg5* or *Atg7* resulted in the accumulation of polyubiquitinated proteins and sensitized neurons to degeneration; supporting the hypothesis that autophagy is neuroprotective.[203, 206] Purkinje cells (PC) reside within the gray matter at the interface

between the molecular and the granular layers of the cerebellar cortex and are important for signal integration, balance, and motor coordination. PC-specific conditional deletion models of *Atg7* (*Atg7*$^{fl/fl}$; pcp2-Cre*)* resulted in PC dystrophy and subsequent axon terminal degeneration. PC degeneration was followed by PC death and behavioral changes in the mutant mice.[237] In a similar experiment, deletion of *Atg5* in PCs (*Atg5* $^{fl/fl}$; pcp2-Cre) also resulted in axonal swelling and neurodegeneration.[204] Although both the nestin-Cre and pcp2-Cre have been employed as useful tools to investigate the result of tissue-specific autophagy loss, one important difference is that nestin-Cre is also expressed in some astrocyte populations as well as other CNS structures, while pcp2-Cre is restricted to PCs. This difference may alter the experimental designs, as nestin-Cre is more appropriate for diffuse CNS ablation studies and pcp2-Cre is more Purkinje cell-specific.

Aging

Autophagy and its association with aging have been explored in two distinct contexts: 1) the impact of autophagy on increasing lifespan or longevity, and 2) the role of autophagy in age-related disease states. Longevity‐promoting regimens, including caloric restriction (CR) and inhibition of TOR with rapamycin, resveratrol or the natural polyamine spermidine, have been associated with autophagy induction.[254] CR can improve heart function through autophagy, as long-term CR preserved cardiac contractile function with improved cardiomyocyte function and lessened cardiac remodeling.[255] Rapamycin prolonged median and maximal lifespan of both male and female mice when fed beginning at 600 days of age; this supplementation led to a life

span increase of 14% for females and 9% for males. In addition, rapamycin-treated mice beginning at 270 days of age also increased survival in both males and females.[256] Lifelong administration of rapamycin extended the lifespan of female 129/Sv mice, as 22.9% of rapamycin-treated mice survived the age of death of the last control mouse. Rapamycin also inhibited age-related weight gain, decreased aging rate, and delayed spontaneous cancer formation.[257] Although rapamycin and caloric restriction both increase the life span of mice, they probably do not occur through similar mechanisms. Dietary restricted mice (40% food restriction) and rapamycin-treated mice both exhibited increased levels of autophagy.[258] The fat mass was similar between control and rapamycin-treated mice, but lower for the caloric restricted mice. There were also striking differences in insulin sensitivity and expression of cell cycle and sirtuin genes in mice fed rapamycin compared with dietary restriction. Spermidine, a natural polyamine whose intracellular concentration declines during human aging, extended the lifespan of yeast, flies and worms, and human immune cells. In addition, spermidine administration potently inhibited oxidative stress in aging mice.[58]

Basal autophagy helps to reduce the deleterious effects from oxidative stress, heat stress and cytoplasmic protein aggregates. During the aging process, basal autophagy levels gradually decline so that the cell is not equipped to deal with these stressors. Since many age-related diseases correlate with a decline in basal autophagy, a targeted therapeutic strategy would be to increase the levels of productive autophagy to reduce the severity of the disease. However, it is important to keep in mind that a tight regulation of basal autophagy levels is important, as too much autophagy could

have a negative outcome. For example, in the *Zmpste24*-null progeroid mice, which model the human laminopathy Hutchinson-Gilford Progeria Syndrome (HPGS), there was an increase in autophagy instead of the anticipated reduction that occurs during normal aging. Although autophagy levels were similar to those found associated with caloric restriction and prolonged lifespan, in this instance autophagy was linked to having a potential role in the premature aging phenotype. However, these mice also have several metabolic alterations including changes in circulating hormones (e.g. leptin, insulin, adiponectin) and glucose levels that probably impact additional contributing cellular processes.[259] Progerin, the truncated form of lamin A protein, was found to co-localize with the autophagic adapter protein SQSTM1 and the autophagy linked FYVE protein, ALFY.[260] Moreover, rapamycin decreased Progerin protein levels through autophagy induction, which rescued the Progeria phenotype in HGPS fibroblasts.[261] Rapamycin-induced autophagy has therapeutic implications for other types of laminopathies as well. For example, *Lmna-null* (lamin A-deficient) mice exhibited skeletal muscle dystrophy and cardiac hypertrophy; these pathologies were improved through rapamycin administration.[262] An oxidative environment potentially plays a crucial role in the aging process, as *Sqstm1*$^{-/-}$ mice exhibited accelerated aging phenotypes and tissues displayed elevated oxidative stress due to defective mitochondrial electron transport.[263] Likewise, *Cisd2*-null mice exhibited nerve and muscle degeneration and a premature aging phenotype.[264] CISD2, the gene responsible for Wolfram syndrome 2 (WFS2), encodes for a mitochondrial protein involved in mammalian life-span control. Although mitochondrial degeneration was exacerbated

with age with a concomitant elevation of autophagy, this elevation was most likely due to a cellular response of mitophagy to clear damaged mitochondria. In addition to induced-mutation mouse models, there have been several different naturally occurring strains of senescence-prone (9 lines) and senescence-resistant (3 lines) mice that have been developed since the 1970s at Kyoto University in Japan.[265] These mice have been important to model aging, senile dementia, and Alzheimer's disease. By 12 months of age, the senescence accelerated mouse prone 8 (SAMP8) mice demonstrated a decline in cognitive ability that corresponded to increased levels of ubiquitinated proteins and autophagic vacuoles (AV) in hippocampal neurons, and decreased expression levels of Becn1.[266] In contract, the senescence-resistant strain did not show an accumulation of these autophagic vacuoles. In the future, it would be interesting to examine whether calorie restriction or rapamycin administration could reduce the accumulation of ubiquitinated proteins and improve learning and memory in the senescence-prone model.

Lysosomal storage disorders

The group of degenerative disorders included in umbrella term lysosomal storage disorders (LSDs) is a heterogeneous and emerging list of diseases that commonly present with an inability to metabolize a normal cellular substrate. The metabolic defect may reside with the ability of the lysosome to degrade the substrate, or a blockade of autophagic flux, most often inhibiting fusion of the autophagosome and lysosome. A reduction in autophagic flux may result in an increase in autophagosome like structures in the cytoplasm as well as uncharacteristically large autophagosome like

structures being formed. Consequently, failure to eliminate/recycle the autophagosomal contents induces cellular stress and may result in death.

Pompe disease, the first LSD to be characterized, is caused by an inability to synthesize acid α-glucosidase (GAA), a lysosomal enzyme needed to breakdown glycogen. Pompe mice (GAA KO) phenocopied the human condition, and abnormal autophagosomal and autolysosomal structures were seen intracellularly. When Pompe mice were crossed with *Atg5/7* muscle-specific conditional knockouts to inhibit autophagy, mice metabolized glycogen more efficiently than the Pompe mice and had a more positive prognosis. In this model system, autophagy was contributing to the pathology of the disease and inhibition of autophagy was been shown to be a useful therapeutic intervention. A side effect of muscle-specific autophagy inhibition, i.e. muscular atrophy, will be discussed in a later section.

Multiple sulfatase deficiency (MSD) is a disease where affected individuals have a reduction in the activity of all sulfatases due to mutations in Sulfatase Modifying Factor 1 (SUMF1), an enzyme responsible for post-translational modification of all sulfatases. A s*umf1* knockout mouse model shared characteristic manifestations of MSD including: skeletal abnormalities, kyphosis, and growth retardation. Impaired autophagosome-lysosome fusion was implicated, as a build-up of undigested material was detrimental to cellular homeostasis and led to death. Though models have exhibited that MSD is accompanied by defective autophagic flux, whether autophagy is protective or detrimental to the pathogenesis of the disease is unclear.

Mucopolysaccharidosis type VI (MPS VI) is caused by a specific sulfatase deficiency (N-acetylgalactosamine-4-sulfate) and patients may be short in stature and suffer from joint stiffness and destruction, cardiac valve abnormalities and corneal clouding. In a rat model of MPS VI, an increased number of autophagic structures were identified by electron microscopy.[267]

Niemann Pick type C disease is a metabolic disorder characterized by the accumulation of lipids in late endosomes/lysosomes. The vast majority of cases are due to mutations in the *NPC1* gene. *Npc1*$^{-/-}$ mice had higher levels of autophagy proteins (LC3II) than controls, and PCs were preferentially affected exhibiting an increase in autophagic vesicles by electron microscopy. *Npc*$^{-/-}$ mice had the ability to form autophagosomes but were defective in autophagosome- lysosome fusion, which resulted in a functional autophagic block and inability to metabolize cargo.[268]

Mucolipidosis type IV disease (MLIV) is caused by mutations in the MCOLN1 gene which encodes a lysosomal cation channel. Affected patients suffer from psychomotor delays and multiple ophthalmic pathologies. The *Mcoln1*$^{-/-}$ mouse model recapitulated most of the symptoms observed in patients with the exception of corneal clouding. In *Mcoln1*$^{-/-}$ brains, lysosomal inclusions were observed in several anatomical areas and cell types.[269] Neurons had increased LC3-II expression and failed to clear LC3-II, once again indicating a functional autophagic block that led to the pathogenesis.[270]

Infectious disease and immunity (e.g. Crohn's disease)

The innate immune system is the first line of defense against pathogens; it is evolutionarily more ancient than the adaptive immune system and is deployed quickly and effectively despite its lack of pathogen specificity or memory. Viruses, bacteria, and parasites can be eliminated in an autophagic process involved in innate immunity defense termed 'xenophagy.' Invading bacteria can generally be classified as vacuolar (e.g. *Salmonella*) or cytosolic (e.g. *Listeria, Shigella*). Cytosolic bacteria can undergo ubiquitin-dependent and ubiquitin-independent mechanisms for autophagosomal envelopment followed by translocation to lysosomes. Vacuolar bacteria can be routed into autophagosomes, or in the instance of *Mycobacteria*, autophagy proteins can resume the maturation of the vacuole and promoter fusion with the lysosome. The main recognition receptors that link detection and autophagy induction include the membrane TLRs (Toll-like receptors) and the cytoplasmic nucleotide-binding oligomerization domains (NOD)-like receptors (NLRs). The receptors can recognize the lipopolysaccharides (LPS) and peptidylglycans of Gram-negative bacteria. Microbial interference with autophagy can occur due to the adaptive nature of bacteria. For example, *Shigella flexneri* secretes the protein IcsB, which prevented ATG5-induced autophagy at the bacterial surface. *Yersinia pseudotuberculosis* resides within arrested autophagosomes in macrophages, since it can inhibit the fusion process with lysosomes.[92, 111]

The more complicated adaptive immune system also relies on autophagy in many capacities. Studies inducing ablation of autophagy proteins have revealed an

essential role for autophagy in maintaining normal numbers of B cells, T cells and hematopoietic stem cell survival and function. *Atg5*- and *Atg7*- deficient models have shown that autophagy was important for T cell survival and maintenance of mitochondria. An increase in mitochondrial mass has been correlated with T cell death in circulating T cells, indicating a potential mechanism of action.[271] Thymic epithelial cells have a high rate of basal autophagy compared to other cell types. During T cell selection thymic epithelial cells display decorations of "self" and "non-self" antigens, aiding in this process autophagy is proposed to facilitate ligand (MHC-II molecule) loading. When autophagy was depleted specifically in thymic epithelial cells, the mature T cell repertoire was diminished due to alterations in positive and negative T cell selection processes. Interestingly, severe colitis, patches of flakey skin, atrophy of uterus, absence of fat pads and enlargement of lymph nodes were observed in many cases. Inflammation was observed in the colon, uterus, lung and Harderian gland of recipient mice. These manifestations are indicative of autoimmune diseases and this model provides a clear linkage between autophagy and autoimmune/inflammatory diseases.[272] A B cell-specific ablation of *Atg5* was achieved by either a Cre-LoxP approach ($Atg5^{fl/fl}$; CD19-Cre) or by repopulating irradiated mice with progenitor cells derived from an $Atg5^{-/-}$ fetal liver. In these experiments, autophagy was found to be essential for the survival of pre-B cells (after the pro-B cell to pre-B cell transition). Additionally, in peripheral circulation Atg5 was required to maintain normal numbers of B-1a B cell populations but not B-2 B cells.[273]

Genome wide association studies of Crohn's Disease identified two autophagy associated genes, Atg16L and IRGM. A naturally occurring insertion/deletion mutation was identified in the 5'UTR (untranslated region) of IRGM (immunity-related GTPase family, M) which disrupted a transcription factor binding site.[274] In another study, a SNP in the coding region of IRGM was identified that affected a microRNA binding site.[275] These identified mutations suggested that IRGM expression level changes were associated with Crohn's disease in humans. A mouse knockout model of *Irgm1* (a.k.a. LRG-47) has been developed and does not display any overt phenotype, including development of the immune system. However, when challenged with infection, $Irgm^{-/-}$ mice were unable to control the replication of intracellular pathogens.[276] Unfortunately, the *Irgm1*-knockout mice have not been investigated specifically in the context of autophagy function in immunity to date. However in parallel with *in vitro* data, IRGM1 has recently been shown to induce autophagy in a mouse model of stroke. The promotion of autophagy, most likely at the level of LC3I to LC3II conversion, was generally protective.[277]

The relationship between Crohn's disease and the autophagic process is more developed in terms of investigating the *Atg16L* risk allele association by using two *Atg16L* gene trap models and an intestinal epithelium-specific *Atg5* knockout ($Atg5^{fl/fl}$; villin-Cre). Both *Atg16L* gene trap models (HM1 and HM2) result in a hypomorphic expression of Atg16L protein. Interestingly, in the Atg16L mutants Paneth cells exhibited abnormal morphology including decreased granule number and disorganization of granules. Researchers concluded that autophagy was required to maintain fidelity of the

Paneth cell granule exocytosis pathway. When challenged with infection, the *Atg16L* hypomorphs performed similarly to controls. In *Atg5*$^{fl/fl}$; villin-Cre ileum, abnormal Paneth cells were identified which paralleled to those identified in the *ATG16L* mutants. Human ileum samples from at risk patients were examined and also exhibited abnormal Paneth cell morphology.[198] It is noteworthy at this point to mention that genome wide association studies for ulcerative colitis did not identify either of these autophagy genes, nor others. This suggests that *Atg16L* and IRMG are specific for the physiopathology of Crohn's disease, not inflammatory bowel diseases generally.

Muscle atrophy

Muscle atrophy is a symptom of a multitude of pathological states including but not limited to fasting conditions, denervation, inactivity, cancer, cardiac failure, and diabetes. Autophagy has been shown to be active in muscular atrophy and other myopathies however due to the nature of the methods used it cannot be said with certainty whether autophagy is promoting atrophy or is activated as a cytoprotective mechanism and coincides with pathology.

In a mouse tissue-specific mouse model generated to investigate the effect of superoxide dismutase 1 (SOD1) ablation on skeletal muscle, it was found that mutants developed muscle atrophy, reduction in contractile force and abnormal mitochondria. Significant upregulation of the mitophagy (specific and selective form of autophagy wherein mitochondria are preferentially enveloped and degraded) gene *Bnip3*, as well as, autophagosome marker LC3 were detected by RT-PCR, most likely as a result of

activation of transcription factor FoxO3. Oxidant accumulation in *SOD1*$^{-/-}$ mice resulted in muscular atrophy through autophagy; this phenotype was rescued by depletion of LC3 by siRNA knockdown suggesting that autophagy was the driving pathway of atrophy. These findings imply that autophagy inhibition is a potential therapeutic target for acute and chronic muscular atrophy.[278]

Muscle-specific *Atg7* conditional knockout mice were autophagy incompetent and morphologically diverged from wild type control littermates beginning at 40 days of age. Muscles of the knockout mice exhibited degenerative changes and a decrease in myofiber size; these abnormal changes were concurrent with the loss of muscle contractile force which further decreased with increasing age. Even more telling may be the ultrastructural changes associated with loss of autophagy in the muscles, abnormally large mitochondria, centrally located nuclei and dilated sarcoplasmic reticulum all observed via electron microscopy.[279]

As discussed previously, a muscle-specific *Atg5* knockout mouse was generated and bred onto a glycogen-degrading enzyme acid-alpha glucosidase knockout background (*GAA*-KO) to interrogate the nature autophagic degradation of glycogen in the pathogenesis of Pompe disease. This study provided additional evidence that autophagy functioned to prevent muscular wasting. Muscle-specific *GAA*$^{-/-}$*; Atg5*$^{-/-}$ mice developed progressive muscular weakness and eventual paralysis beginning earlier (2-3 months of age) and progressing more rapidly than autophagy-competent *GAA* KO mice. Ubiquitin-positive structures accumulated in both *GAA* KO and *GAA*$^{-/-}$*; Atg5*$^{-/-}$ mice with a

differing distribution. In *GAA* KO myocytes, autophagic vesicles built up in the cell and ubiquitin-positive structures associated with the autophagosome. In *GAA*$^{-/-}$*; Atg5* $^{-/-}$ myocytes, ubiquitin-positive structures were distributed throughout the cell and appeared to associate with lysosomes, though were not membrane bound. These data indicated that the disruption of functional autophagy and accumulation of toxic ubiquitin-positive structures promoted muscular myopathy.[280]

Stroke

Cerebral ischemia is achieved in mice most often by surgical intervention and results in global, restricted, or cerebral directed ischemia depending on the method selected. Furthermore in some models ischemia is reversed, allowing reperfusion of the cerebral tissue. Autophagy is induced by hypoxia/ischemia events; however it is unclear whether autophagy is protective or maladaptive in stroke models. Responsibility of much of the dissenting opinions may be attributed to the variety of techniques used to induce ischemia events, these have been reviewed at length by Hossmann, see reference.[281] Neonatal mice subjected to hypoxic/ischemic (H/I) brain injury responded with a robust autophagic response in neurons and hippocampal neuron death. *Atg7*$^{-/-}$ neonates, which are autophagy incompetent, were protected from hippocampal neuron death when subjected to identical (H/I) brain injury as control mice, indicating a neurotoxic role for autophagy induction.[282] Conversely, in when wild type mice were subjected to H/I brain injury and reperfusion, damage was mitigated by intraperitoneal injection of NAD^+. NAD^+ administration inhibited autophagy induction. In the NAD^+ treated group, a reduction of autophagy was correlated with a decrease in neuronal

damage. To further investigate this link, researchers subjected mice to H/I injury and treated them with 3- methyladenine (3-MA), an autophagy inhibitor, and an amelioration of neuronal damage was observed. These data indicated that in adults, H/I brain injury followed by reperfusion autophagy was maladaptive; furthermore, inhibition of autophagy at the time of reperfusion was neuroprotective [283]. Moreover, in a rat ischemia model, inhibition of autophagy by *Becn1*- directed shRNA or 3-MA treatment led to a reduction in damage and neuronal loss in the ipsilateral thalamus. This study supported the hypothesis that autophagy induction increased damage when activated following an ischemia/reperfusion event.[284] The variance amongst the model systems could account for much of the disparity seen in outcomes. The age of the individual, duration of ischemic event, and presence or absence of reperfusion is all potential modulators autophagic response.

Type 2 diabetes

Type 2 diabetes (T2D) is a complex disease that manifests in tissues, especially adipose, muscles, and liver, becoming resistant to insulin signaling and causing hyperglycemia. Pancreatic β–cells initially respond by increasing their production of insulin, but prolonged insulin resistance results in atresia of β–cells and a marked reduction in insulin production. Due to the high metabolic demands placed on β–cells it follows that autophagy would be play an important role in the pathophysiology of this chronic disease. In wild type C57BL/6 mice β–cells, unlike most organs, autophagosomes are sparingly observed after a period of starvation. However, when mice were fed high fat diets for 12 weeks, autophagosomes were readily observed. These results were

confirmed *in vitro* by treating INS-1 β–cells with free fatty acid (FFA), glucose, or tolbutamide. Cells treated with FFA had increased LC3II levels and observable autophagosomes while cells treated with glucose or tolbutamide (a drug regularly prescribed for T2D) did not show any significant change in LC3 conversion or autophagosome formation. To further investigate the link between autophagy and β–cells, a β–cell specific *Atg7*$^{-/-}$ mutant mice was generated (*Atg7*$^{fl/fl}$; Rip-Cre). As early as 4 weeks, and degenerating in an age-dependent manner, enlarged cells with pale staining cytoplasm were identified near the periphery of *Atg7*$^{fl/fl}$; Rip-Cre islets. Inclusion bodies were observed at a high frequency in the enlarged cells the presence of inclusion bodies increased with age, and deformed mitochondria were also observed in these enlarged cells. Resting blood glucose levels were higher and insulin secretion was reduced in *Atg7*$^{fl/fl}$; Rip-Cre mice when compared to control *Atg7*$^{fl/fl}$ mice: these differences were amplified when control and *Atg7*$^{fl/fl}$; Rip-Cre mice were fed high fat diets.[285] In a related model, Marsh et al generated β–cells with a defective secretory pathway in *Rab3A*$^{-/-}$ mice. Although increased intracellular insulin levels were expected, this was not observed. Increased autophagy of the peptide hormone maintained the levels of insulin and prevented accumulation of potentially toxic cellular substrates.[82] These experiments together suggested that β–cells depended on autophagy to clear damaged organelles and toxic intracellular protein aggregates.

Reproductive infertility

The role of autophagy during folliculogenesis is a comparatively new topic of study. In rats, LC3II expression was characterized in follicles of varying developmental

stages. Primordial follicles exhibited only weak expression, but antral follicles exhibited robust expression restricted to granulosa cells. Staining was not observed in the oocyte proper or theca cells in any stage follicle.[286] In mice, expression studies also indicate a role for autophagy during folliculogenesis. An expression profile of *Becn1* mRNA revealed significantly higher expression in primordial follicles than in other stages. Immunohistochemistry for BECN1 confirmed this result and consistent staining of granulose cells, theca cells and oocyte cytoplasm was seen in all follicular stages examined.[287] Interestingly, when *Becn1* or *Atg7* is ablated specifically in the female germline (MMTV-CreA; $Becn1^{fl/fl}$) or globally ($Atg7^{-/-}$), fewer primordial follicles were present in the perinatal ovary. These results indicated that autophagy may be vital for survival of the primordial follicle pool, and be active during folliculogenesis and follicular atresia. In males it is well accepted that autophagy is responsible for post fertilization paternal mitochondrial clearance to prevent paternal mitochondrial DNA transmission; however, the role of autophagy during spermatogenesis is a field in its nascence. It has been shown in *Arabidopsis* that *Becn1* was essential for pollen development.[288] Also, autophagy induction in stallion sperm, as measured by LC3I to LC3II conversation, was important for the survival of sperm post ejaculation.[289]

Conclusion

A variety of mouse models have been established and interrogated to understand the implications of autophagy in human disease. These genetic-based models are primarily either reliant upon: 1) the generation of an autophagy-defective mouse to characterize a given disease state, or 2) the characterization of autophagy

within a pre-established murine model. As this review has shown, conditional knockout models have been extremely useful in disease profiling. The next wave of studies will invariably utilize inducible-based systems for conditional knockouts, genetic-based rescue experiments of disease models, or pharmaceutical-based modification of autophagy. A prevailing theme in the field is that autophagy can either be beneficial or deleterious depending on the disease and its progression state, a theme which must be addressed in designing and implementing appropriate treatment regimes.

Chapter Three: Post-pubertal mammary gland development: morphology and mechanisms

Introduction

This chapter has been published and is included here for completeness of the dissertation.[290]

Interest in the mammary gland, the defining characteristic of the class Mammalia, has stretched across centuries. The primary function of the mammary gland across mammals is the nourishment of young; however additional functions, such as the transmission of immune factors to infants, are important in many species. It has been postulated that mammary glands arose evolutionarily from modified apocrine glands recently in evolutionary history, approximately 125-190 million years ago.[291] Obtaining evidence for these theories has been technically challenging, since the soft tissue of mammary glands is not well preserved in the fossil record. However, we can gain insight from Monotremata, an order of ancient egg laying mammals that represents an interesting link to the reptilian past. The platypus and echidna are extant egg laying species that have functional mammary glands that undergo a true lactation phase. This lends support to the postulation that early apocrine secretions served some beneficial role to protect and/or nourish eggs via semi-permeable shells, thus selecting for the retention of these glands.[291] While the evolutionary origin of the mammary gland is still being investigated and debated, developmental and molecular biologists have made astounding progress in elucidating the mechanisms controlling the development of the mammary gland in its current incarnation.

The mature mammary gland is an arboreal structure with ductal branches extending from a nipple area, colonizing a mammary fat pad, and finally branching and terminating into alveolar units. Mammary gland development is divided into four stages that align with the overall growth, sexual maturation and reproductive-associated changes of mammals, which are: embryonic, pre-pubertal, pubertal, and post-pubertal. The initiation of mammary development in embryos occurs during gestation with the establishment of the anlagen; however, the majority of development occurs in the adult animal. Branching and elongation of the ductal epithelium ensues at the time of puberty, with the expansion of the alveolar epithelium proceeding in response to pregnancy. However, terminal differentiation is not achieved until lactation. This periodic developmental profile is unique amongst tissues and lends the mammary gland as an excellent model to study branching morphogenesis and other developmental paradigms in an adult animal. Furthermore, the accessory nature of the mammary gland is ideal since it allows researchers to make otherwise deleterious mutations in this relatively large, and easily accessible tissue. Classically, post-pubertal development has been separated into four main stages: virgin, pregnant, lactation, and involution (schematically represented in **Figure 3.1** and whole mounts in **Figure 3.2**). These stages will be discussed individually following a description of embryonic and pre-pubertal development.

Fetal Development and Pre-Pubertal Development

Although the focus of this chapter is post-pubertal development, it would be incomplete without a brief overview of embryonic and pre-pubertal mammary

development. In the interest of continuity, the mouse will be the primary focus of this chapter and only major developmental discrepancies will be addressed. In most studied mammalian species mammary lines, ridges of multi-layer ectoderm become visible on the ventral surface of the embryo running parallel to the anterior-posterior axis of the embryo between the limbs. The mammary lines, as anatomically evident structures, are controversial in the mouse; however, specific gene expression patterning corresponding to proposed mammary line location has been observed. *Wnt10b* can be detected in a restricted pattern and *Wnt6* in a broader area just antecedent to the anatomical detection of anlagen presence.[292] Histological analysis revealed a change in epithelia co-localizing with the *Wnt10b* expression, specifically a change to multi-layered cuboidal epithelia from the surrounding squamous epithelia. In addition, the mammary line also has a characteristic dense dermis below it.[292] In the mouse, the first visible surface feature of mammary development is five symmetrical pairs of mammary placodes at E11.5. Interestingly, unlike other models, the mouse placodes do not follow an anterior-posterior developmental pattern. Instead, they proceed pairwise in the order, numbered anterior-posterior: 3, 4, 1 and 5, then 2. Mammary placodes retain their expression of *Wnt10b*, *Wnt6*, fibroblast growth factor (*Fgf10*), and Lymphoid enhancer binding factor 1 (*Lef1)*, a WNT pathway effector. *Lef1*$^{-/-}$ embryos fail to develop placodes by E13.5, a phenotype shared by overexpression of *Dkk1*, a WNT pathway inhibitor.[293, 294] Studies on placode development by Mailleux et al revealed distinct signaling dependence. The thoracic placodes (1, 2, and 3) and the number 5 inguinal placode are dependent on FGF10/FGFR2b while placode 4 is independent of these signals.[295] Of late,

bone morphogenic protein 4 (*Bmp4*) and T-box protein 3 (*Tbx3*) have also been shown to work synergistically to determine positioning of the mammary buds. For a more detailed review of signaling pathways involved in morphogenesis and associated mutants see references.[296, 297]

After the buds become visible at E13.5, there is a brief resting phase during which very few cells are mitotically active. Beginning again at approximately E15.5 and persisting through gestation, each bud extends a small sprout and invades the mammary fat pad precursor. The sprouts are completed by the formation of a lumen and by E18.5 each duct has undergone branching and resembles a small ductal tree. The interaction of parathyroid hormone related peptide (PTHrP) and its receptor PTHR1 drives the rudimentary ductal tree to branch. At the time of parturition, the immature mammary gland is comprised of approximately 10-15 small branches near the nipple.

Puberty

Mammary gland growth is isometric with the individual from approximately E18.5 until puberty, wherein the development will resume under the control of hormones. The ductal tree is primarily established during puberty under the control of circulating hormones. These circulating hormones bind to receptors located in the pituitary gland and throughout the female reproductive system to initiate changes in gene expression. Estrogen receptor (EsR) and progesterone receptor (PR) are instrumental during this period and will be discussed in depth in sections that follow.

At approximately 3-4 weeks of age the ducts become capped with mitotically active, club-shaped structures termed terminal end buds (TEBs). TEBs are composed of inner

"body" cells and outer, undifferentiated "cap" cells. The body cells differentiate into mammary epithelial cells. The cap cells ultimately differentiate into myoepithelial cells. The innermost layer of body cells undergoes apoptosis to form the hollow lumen. The programmed cell death rate in the presumed lumen is very high, with a rate far greater than that detected during involution when the entire gland undergoes remodeling.[298] TEB formation is dependent on the paracrine production of IGF-1 by stromal cells which respond to growth hormone, reviewed in reference.[299] The TEBs vary both among glands within an animal and between animals at the same time point. The number 2 and 3 glands have larger number and size of TEBs when compared to the number 4 inguinal mammary gland. The size difference of the TEBs is attributed to a larger number of cap cells rather than cell hypertrophy.[300] Circulating estrogen (E2) stimulates allometric ductal elongation via TEB proliferation which terminates when the TEBs reaches the limit of the fat pad. Concomitantly, secondary ductal branches are formed off of the primary ducts by TEB division. After expansion of the ductal tree during puberty, TEBs are permanently replaced by terminal end ducts (TEDs) which are not mitotically active. TEDs are then surrounded by stroma with the center cells undergoing apoptosis. The resultant structure is a single layer of epithelia cells resting on a basement membrane. The gland remains in this mature virgin state until stimulation by pregnancy.

Pregnancy and Lactation

Pregnancy hormones stimulate further branching of the ductal tree, the development of the lobuloalveolar compartment, and the terminal differentiation of the secretory epithelia. The ducts are composed of three major layers; the most apical is a

single layer of cuboidal epithelial surrounding a hollow lumen, this layer is referred to as "luminal epithelia." Basal to the luminal epithelia, specialized myoepithelial cell layer encircles the luminal epithelia; these cells contain smooth muscle actin and assist with ductal contraction and movement of milk during lactation. Beyond the myoepithelial cells, a tough laminin-enriched basement membrane separates the epithelia and the surrounding connective tissue with a layer of extracellular matrix (ECM) proteins including proteoglycans and glycoproteins.

The epithelium of the alveoli is a single layer of squamous epithelium which is discontinuously surrounded by myoepithelial cells then finally a basement membrane. As pregnancy progresses, the myoepithelia becomes more discontinuous and the alveolar epithelium may contact the basement membrane directly. During late pregnancy, approximately pregnancy day 18.5, the alveolar epithelial cells produce milk proteins and lipids which are retained in the cells, causing them to appear more cuboidal or pyramidal due to the increased cytoplasmic volume. After the initiation of lactation, milk proteins and lipids are released into the alveolar lumen and the cells once again appear morphologically flattened. This is achieved in part by the contraction of the myoepithelial cells lining the alveoli in response to oxytocin. At this point, large milk fat droplets can be seen in the lumens. The alveoli continue to expand throughout lactation until the gland is completely filled. During the height of lactation, very few adipocytes can be visualized and the ratio of parenchyma to stroma is heavily weighted toward the parenchyma. The lactation phase will continue until the pups are weaned or gradually taper off as the pups begin to gain nourishment from other sources.[301] The

pregnancy associated changes: ductal branching, lobuloalveolar development, proliferation, and differentiation are highly regulated processed. The signaling cascades responsible will be discussed at length in later sections.

Involution

Cessation of suckling, due to natural or acute weaning and milk stasis, results in the mammary gland remodeling to again resemble a resting, "virgin-like" state. Involution proceeds in two stages: the reversible stage and irreversible stage. The early, reversible stage is characterized by waves of apoptosis-mediated cell death. Transcriptional profiling studies have detected changes within hours of involution.[302-304] Histologically, secretory epithelia cells are sloughed and seen in alveoli in greater numbers during early involution than in any other stage. Many of these cells are terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive, a marker for apoptosis that detects DNA fragmentation.[305] It is estimated that during involution 80% of epithelial cells will die via apoptosis, a catastrophic event unparalleled in normal adult tissues. Many induced mutant mouse models generated exhibit involution phenotypes, see Radisky and Hartmann for review and references.[306]

The second phase of involution is initiated approximately 72 hours post-lactation and is more drastic in nature. Once the gland has entered this phase, lactation cannot be re-initiated even if the pups are returned to the dam. The superfluous secretory alveoli collapse, the ECM is remodeled, and adipocytes increase in number. The irreversible phase is characterized by and dependent upon on a temporal increase in matrix metalloproteinase activity (MMPs) to stimulate ECM remodeling. Invasion of

immune cells, primarily phagocytic cells such as macrophages, helps to clear cellular debris.

Autophagy most likely plays a role during mammary involution though at present this is poorly described in murine models. Autophagy, Greek for "self-eating," is a highly-conserved catabolic process initiated in response to cellular stress. In bovine, autophagy is observed morphologically and detected with molecular methods during involution. Additionally, pro-autophagic protein BECLIN1 has been shown to play an important role in mediating involution. It is clear that more work needs to be done to elucidate the role of autophagy during this stage in murine models.[307-309]

Signaling Pathways

Msx Family

The class II homeobox, msh-like (Msx) genes, *Msx1*, *Msx2* and *Msx3*, are common to vertebrates and often define mesenchymal-epithelia interaction sites. *Msx3* expression is restricted to the dorsal neural tube[310], while *Msx1* and *Msx2* are more broadly expressed during embryogenesis. *Msx1* and *Msx2,* but not *Msx3*, are expressed at varying levels during every stage of mammary development except for pregnancy. During embryonic development *Msx2* expression is detectable both in the epithelia and the mesenchymal cells, while after parturition expression is restricted to the mesenchymal cells in close proximity to mammary ducts. *Msx2* expression is dependent on reciprocal interactions with epithelial cells. In cleared fat pads *Msx2* is undetectable, indicating an essential epithelia interaction compartment for normal expression. *Msx2*

expression, but not *Msx1*, is regulated by E2 and progesterone (P4), indirectly via the PR and directly as a transcriptional target respectively.[311]

Msx2 is implicated in the regulation of branching, as the *in vitro* overexpression of *Msx2* is sufficient to drive branching. In addition, *in vivo* approximately one third of *Msx2* null mice have arrested embryonic mammary gland development with failed ductal branching.[312] *Msx1* null neonates do not exhibit a mammary defect, which is not surprising since homeobox genes typically exhibit high levels of functional redundancy.[313] However, double knockout *Msx1/2* mice fail to form mammary anlagen with a complete penetrance of the phenotype.[312] Mice that misexpress *Msx1* in the mammary gland exhibit a reduction in pregnancy-induced differentiation and an increase of *Cyclin D1* (C*cnd1*) expression, and an increase in cell proliferation. This model supports *in vitro* experiments with *Msx-1* overexpressing HC11 mammary epithelial cells which upregulate *Ccnd1* expression and are unable to undergo functional differentiation in response to lactogenic hormones. The authors propose that *Msx1* prevents exit from the cell cycle, which drives an upregulation of *Ccnd1* expression; ultimately, this proliferative cell fate prevents the terminal differentiation of the epithelial cells.[314] Although *Ccnd1* is the only verified target of *Msx1* in the mammary gland identified to date, it can also be induced by *Msx2* most likely in response to hormonal signaling by E2 and P4. Interestingly, expression profiles of *Bmp2* and *Bmp4* closely resemble the profiles of *Msx1* and *Msx2*, respectively. It has been suggested that this indicates a regulatory relationship, though the nature of this relationship is yet to be determined owing to the early embryonic lethality of *Bmp2* and *Bmp4* null mice.[315]

Lef1

Lef1 is a nuclear target of the Wnt signaling pathway. In the presence of Wnt signaling, dephosphorylated β-catenin enters the nucleus and engages *Lef1* to act as a transcriptional activator. Therefore, in the absence of Wnt signaling, *Lef1* is a transcriptional repressor. *Lef1* null neonates lack teeth, whiskers, and hair in addition to lacking mammary glands. Closer inspection of E13.5 embryos revealed a reduced number of mammary buds, indicating that the mammary rudiment develops improperly, arrests, and then regresses by the time of parturition. Since other visceral organs were unaffected, the defect seems to be specific to organs requiring epithelia of ectodermal origin to interact with surrounding mesenchyme.[293]

Hormones

Through both endocrine ablation studies and targeted mouse transgenic models it has been elucidated that E2, P4, and prolactin (PRL) drive the pregnancy-induced growth and differentiation of the mature mammary gland. P4 is an ovarian hormone secreted by a temporary endocrine structure within the ovary, the corpus luteum. PRL is a peptide hormone that signals through PRL receptors (two isoforms, PRLRA and PRLRB). Together, these hormones are responsible for initiating complex, and sometimes intersecting, gene pathways responsible for branching and alveologenesis during pregnancy. Transgenic mouse models have illuminated many of the signaling pathways that will be discussed in this chapter. For reference, a table has been included with a synopsis of the models discussed, see **Table 3.1**.

E2/EsR

E2 is a steroid hormone with two characterized receptors, EsRα and EsRβ, which are members of the nuclear receptor superfamily. The canonical method of activation is: 1) ligand binding, 2) conformational change of receptor, 3) co-activator recruitment, and 4) dimerization and binding of receptor to estrogen responsive elements (EREs) in gene regulatory regions. Several ligand-independent, non-canonical pathways of EsR activation have been described but are beyond the scope of this chapter. The expression patterns of the two EsRs are distinct but overlapping, and EsRα is the dominant receptor type expressed in the mammary gland. Three knockout mouse models have been generated to study the importance of EsR in the overall growth and development of the organism. EsRαKO, EsRβKO, and EsRα/EsRβ double knockout (EsRαEsRβKO) mouse models are all viable and progress to adulthood without defects.[316-318] EsRαKO, EsRβKO, and double knockout (EsRαEsRβKO) do not show defects in embryonic or pre-pubertal mammary gland development, but after puberty females exhibit ovulation failure resulting in infertility or subfertility (EsRβ). EsRαKO mice do not undergo the expected ductal elongation and expansion during puberty. The ductal tree remains sparse with no lobuloalveolar development occurring.[319] Transplantation experiments of EsRαKO epithelia into wild-type cleared fat pads developed without defect to puberty as expected, but failed to undergo any development in response to pregnancy. This effect is epithelia intrinsic since the converse experiment; EsRαKO fat pad grafted into a wild-type host with wild-type mammary epithelial cells grows normally and responds to pregnancy hormones.[320]

EsRβKO females are sub-fertile, yet develop normal mammary glands and are able to undergo a normal lactation phase.[317] EsRαEsRβKO mammary glands phenocopy EsRαKO mammary glands, further underscoring the importance of EsRα in this tissue. EsR ablation causes defects in many reproductive tissues beyond the mammary gland and ovary, see reference for review.[321]

The detection of residual expression from the EsRαKO necessitated the generation of a true null allele. To address this, the Cre-LoxP system was used to generate an EsRα floxed model for tissue-specific deletion strategies. Initially, a global Cre-mediated deletion was analyzed and the results phenocopied the EsRαKO model.[322] An EsRβ conditional knockout based on the Cre-LoxP system has also been generated using a similar targeting strategy as was used to generate the conditional EsRαKO allele. LoxP sites were inserted flanking exon three of the EsRβ gene.[323] Exon three was chosen to ensure a true null allele and effectually reconcile conflicting reports of phenotypes of the EsRβKO mice due to varying generation and housing conditions. In the context of the mammary gland, these models will be important to define the temporal requirement, if any, of EsR in pregnancy-induced mammary gland development. Using any one of the current mammary directed Cre transgenic models, EsR could be effectively attenuated at any point in the pregnancy cycle.

As a supplement to many of the previously mentioned loss-of-function models, an inducible, EsRα model has also been generated using the tetracycline system. In this MMTV-tTA/tet-op-mEsRα model overexpressed EsRα, and administration of doxycycline turns off expression of EsRα.[324] These recent models, will be invaluable in further

characterizing the role of EsR in reproductive development and in oncogenesis when used combination with current cancer models.

P4/PR

P4 is a steroid hormone produced primarily in the ovary and adrenal gland of females. Its activity oscillates with the estrous cycle and is required for the establishment and maintenance of pregnancy. P4 is a ligand for the PR, a nuclear receptor superfamily member. Once the ligand is bound the ligand/receptor complex translocates to the nucleus and affects transcriptional activity of target genes either directly or indirectly. The PR has two receptor isoforms, PR alpha and beta (PRA/PRB), which are splice variants of the gene *Pgr.*[325, 326] The two receptors are structurally similar with the exception of an amino terminal extension of PRB.[326, 327]

Since PR is under control of E2, a targeted approach to sort out the function of P4 independent of E2 was needed. The PR knockout mouse (PRKO) is viable and shows no overt defects through embryogenesis or adulthood. As expected, female mice are infertile, due to the well characterized role of P4 in establishment and maintenance of pregnancy. However, a surprising role for P4 in the mammary gland was revealed. PRKO females produced a rudimentary ductal structure, indicating the P4 was not essential for ductal elongation, but failed to proliferate and undergo normal lobuloalveolar development and ductal side-branching in response to hormonal stimulation.[328] This original characterization established a role for PR in normal mammary development and during pregnancy, but was insufficient to distinguish between systemic and local effects. To this end, several approaches have been taken to dissect the effect of PR in the

mammary gland. Transplantation experiments wherein mammary epithelia from PRKO mice were transplanted into wild-type recipients and vice versa revealed that intrinsic PR expression was essential in the epithelia for ductal outgrowth in response to circulating hormones and for lobuloalveolar development during pregnancy.[329-332] A PR-LacZ reporter helped to clarify the PR expression in both spatial and developmental contexts. During development, the activity is restricted to the epithelia; however, at puberty the activity is high in the body cells of the TEBs and ductal luminal cells. The localization pattern changed in the adult where the activity is clustered around the terminal ductal side branches, and all activity is reduced toward the end of pregnancy at the time of terminal differentiation.[333] Interestingly, in the juvenile PR expression is uniform, but in the mature gland only sub-populations of mammary epithelial cells are PR positive, confirming earlier studies.[334]

Isoform specific function was addressed elegantly with the generation of PRA and PRB-specific knockout alleles (PRAKO, PRKOB). The PRAKO did not show abnormal effects in the mammary gland, indicating that PRB was of greater importance in this tissue.[335] This was confirmed with the PRBKO model that exhibited reduced ductal side branching and lobuloalveolar development during pregnancy.[336] Importantly, this study also revealed an isoform specific activation of receptor activator of nuclear factor κβ ligand (RANKL) by PRB, the importance of RANKL in mammary morphogenesis will be addressed in another section focusing on receptor activator of nuclear factor κβ (RANK) signaling.

Two Cre-LoxP-based conditional PR knockout models have been produced allowing for greater spatial and temporal control of PR ablation. The models differ by targeting strategy, either exon 1 or exon 2 is flanked by LoxP sites, yet both produce homozygous null animals that phenocopy the original PRKO.[337, 338] Additionally, a knock-in of the reverse tetracycline-dependent transactivator (rtTA) at the PR locus (driven by the endogenous PR promoter) has been helpful in identifying target genes of PR signaling.[339] These mice ($PR^{rtTA/rtTA}$) phenocopy the PRKO model, and when treated with doxycycline PR promoter driven rtTA can induce expression of PR target genes in P4 responsive cells. This model combined with a reporter transgene allowed for the precise identification of PR responsive cells. In concordance with earlier studies, this model has confirmed the non-uniform pattern of PR expression in mature mammary epithelial cells, and has importantly clarified the role of RANKL as an effector of PR dependent side branching and alveologenesis.[334, 339, 340] These models will be useful in separating tissue intrinsic functions from systemic effects of PR deficiency and are applicable to many studies of reproductive biology beyond the mammary gland.

RANKL/RANK

RANK is present on mammary epithelial cells and when activated by its ligand, RANKL, is a mammary PR effector which is essential for mammary proliferation and morphogenesis during pregnancy.[341] In wild-type animals, RANKL is upregulated in response to P4 only in PR-positive cells, and PR-positive/RANKL cells cause proliferation in a paracrine fashion. This regulation was tested *in vivo* in a PRKO background with a conditional induction of RANKL targeted only to cells that would normally express EsR,

PR, and RANKL. With induction of RANKL by drug administration, the mammary defect observed in PRKO mice can be partially rescued.[340] This study highlighted the importance of the spatial organization of PR positive cells and showed that when RANKL was induced in PR-negative and PR-positive cells, the ductal outgrowth and alveolar development was disorganized. RANKL/RANK leads to nuclear factor κB (NFκB) dependent transcriptional activation of *Ccnd1,* and disruption of RANKL or the components of this pathway result in a defect in alveolar proliferation.[342] These studies are supported by the *Ccnd1* knockout mouse which has reduced lobuloalveolar development during pregnancy and fails to lactate.[343]

Recent evidence has revealed that RANKL serves as an intersection point between P4 and PRL signaling. Srivastava et al. suggest that RANKL is induced by both P4 and PRL signaling and is dependent on Janus kinase 2 (JAK2)/ signal transducers and activators of transcription 5a (STAT5a) signaling.[344] Other points of intersection of P4 and PRL signaling and their potential consequences are reviewed in reference.[345] RANKL regulates CCAAT-enhancer binding protein (C/EBP), to which we will now turn our attention.

C/EBP

Of the six C/EBP transcription factor family members, C/EBPβ's three protein isoforms seem to be the most important for mammary gland development, though other family members are expressed.[346] C/EBPβ is expressed throughout mammary gland development but is lowest in the virgin, increases during pregnancy, is reduced modestly during lactation, and then is increased again during the reversible phase of

involution.[347] Ablation of C/EBPβ causes defects in ductal outgrowth manifested by enlarged ducts and decreased branching.[348, 349] During pregnancy (transplantation or hormone supplementation experiments were required due to infertility of C/EBPβ$^{-/-}$ mice), ductal outgrowth occurred but lobuloalveolar development is negatively affected.

Another family member, C/EBPδ, has an important function in involution. The C/EBPδ knockout mice exhibit a delayed involution phase characterized by a delay in the appearance of apoptotic cells, indicating a role in early involution, a time when C/EBPδ expression is highest in wild-type glands. Expression analysis revealed a reduction in the levels of pro-apoptotic BAX and BAK, and an increase in anti-apoptotic Bcl2l1. STAT3 is responsible for activating C/EBPδ at the initiation of involution. These results position C/EBPδ as a regulator of involution-induced cell death.[350]

Cytokine Signaling, Stat6, and Alveolar Commitment

STAT6 is unique amongst STATs in the mammary gland as it is important early in gestation and is not directly downstream of PRL signaling (discussed below). Classically, *Stat6* has been characterized as a vital signaling molecule in the T helper (Th) cell lineage, specifically in the differentiation of Th2 cells. In this context, interleukin (IL) IL-4/IL-13 binds its cognate receptor leading to activation of JAK1 and TYK2 which in turn facilitate recruitment and activation of STAT6. STAT6 forms homodimers which translocate to the nucleus activating or repressing IL-4/IL-13 target genes including GATA binding protein, 3 (GATA3), a transcription factor, reviewed in references.[296, 351] More recently, however, a new role for *Stat6* regulating mammary alveolar cell fate has emerged. In two transgenic models interrogating this pathway, concomitant IL-4/IL-13

knockout or *Stat6* knockout, resulted in curtailed alveolar development when compared to controls. Furthermore, in glands lacking SOCS5, a STAT6 inhibitor, accelerated development of the alveoli is observed.[352]

Further support for this pathway's importance in mammary gland proliferation and differentiation is provided by two studies centering on GATA3, a transcription factor and target of IL-4/IL-13/STAT6 signaling. In mice with a conditional deletion of GATA3 before the onset of puberty and continuously thereafter (MMTV-Cre; *Gata3*$^{fl/fl}$), TEBs fail to develop punctually and the ductal epithelia does not invade the fat pad. At 8 weeks of age, a few TEBs are observed but these are unable to invade the fat pad. Additionally, the ducts that are present show a myriad of defects including structural defects and irregular ductal epithelia. Due to clonal expansion of GATA3-positive cells, the authors generated an acute, drug inducible *Gata3* model wherein the rtTA-Cre is driven by the whey acidic protein (WAP) promoter (WAP-rtTA-Cre; *Gata3*$^{fl/fl}$). These mice were administered doxycycline at 12 weeks of age and the drug was given for 14 days. By the fifth day of drug administration, defects in luminal epithelia were observed, reminiscent of those observed in the MMTV-Cre; *Gata3*$^{fl/fl}$ model. Importantly, in the GATA3-deficient glands, luminal epithelia cells lost markers of differentiation and proliferated at a higher rate. Longer term deficiency led to cell death and defective lactation.[353] A second study also reports similar phenotypes for GATA3 loss in the pre-pubertal gland. Two additional models are included in this study, a model of GATA3 deficiency in the mammary primordia (keratin14-Cre; *Gata3* $^{fl/fl}$), and one that causes loss GATA3 during gestation (WAP-Cre; *Gata3* $^{fl/fl}$). Embryonic loss of GATA3 in the mammary primordia

results in absent mammary placodes (one or more) and ablation of *Lef1* expression in the expected areas of placode formation, though any placodes formed in the mutant animals also expressed *Lef1*. Loss of GATA3 during gestation results in defective lobuloalveolar development and lactation.[354] These studies have indicated a clear role for GATA3 in formation of the mammary placodes in the embryo, ductal morphogenesis, maintenance of luminal cell differentiation in mature glands, pregnancy-stimulated lobuloalveolar development, and lactation. Based on these studies, the importance of the Th2 producing signaling pathway is important for mammary development, and adds more complexity to the tightly regulated PRL and P4 cell signaling that has been previously characterized.

PRL and JAK/STAT Signaling

PRL is a small peptide hormone produced primarily in the anterior pituitary, but may also be produced in other peripheral tissues. PRL historically has been characterized as a factor necessary for pregnancy-induced mammary alveolar development and lactation, for historical references see reference.[355] PRL binds to the PRLR to initiate JAK/STAT signaling. PRLR dimerization causes activation of JAK2. JAK2 phosphorylates three target STATs depending on the stage of mammary gland development: STAT1, STAT3 and STAT5. Canonically, phosphorylated STAT forms dimers and then translocates to the nucleus. Dimers bind conserved, palindromic sequences (TTCNNNGAA) termed γ-interferon activation sites (GAS), in gene promoter/regulatory regions to regulate the initiation of target gene activity.

PRL knockout mice (*Prl*$^{-/-}$) are viable, but females are infertile and mammary gland development is defective, manifesting with a fully developed ductal tree devoid of lobuloalveolar units. Since the females are infertile, stages beyond the mature virgin gland were not analyzed.[356] PRLR knockout mice fail to lactate and the glands appear poorly developed with few branches at maturity. Interestingly, multiparous females that are hemizygous for the PRLR knockout allele (*Prlr*$^{+/-}$) regain mammary function, indicating a response to repeated hormone stimulation.[357, 358] The next component in the cascade, JAK2, also has pleiotropic roles in both embryonic and post-natal development.

Since the traditional *Jak2* knockout mice (*Jak2* $^{-/-}$) die during embryogenesis due to defective hematopoiesis leading to severe anemia, a conditional knockout approach to study *Jak2* in the post-pubertal mammary gland was warranted.[359, 360] Wagner and colleagues developed a conditional deletion *Jak2* model (*Jak2*$^{fl/fl}$), and using two Cre transgenic strains (MMTV-Cre and WAP-Cre) showed that JAK2 signaling is necessary for both development of alveolar progenitor cells in mature virgin glands, as well as maintenance and survival of the secretory epithelium in pregnant mice.[361] These functions are non-redundant and data indicates that STAT5 is activated only in a JAK2-dependent fashion.

The transcription factor STAT5 is produced by both *Stat5a* and *Stat5b*, two highly related genes derived from a recent gene duplication event.[362] In mammary tissue, *Stat5a* is the more abundant and is responsible for 70% of STAT5 present.[363] During late pregnancy and lactation, phosphorylated STAT5 increases precipitously, most likely due

to its activation of β-casein and WAP, genes that increase dramatically in preparation for lactation. Knockout studies have revealed independent and overlapping roles of *Stat5a* and *Stat5b*. Mammary glands from Stat5a knockout females (*Stat5a* $^{-/-}$) fail to develop characteristic lobuloalveolar structure and exhibit a complete lactation failure during the first pregnancy.[364] It is important to note that even though *Stat5a* and *Stat5b* share 96% similarity, *Stat5b* does not compensate, indicating either an independent function or independent regulation.[363] After multiple rounds of pregnancy, lactation phase is sustained, which may be attributed to a late compensatory increase in *Stat5b.*[365] *Stat5b* knockout mice are viable and fertile, the mammary glands appear normal, and the primary reported defects are ablation of sexually dimorphic growth differences and liver-specific gene expression.[366] *Stat5a/b* double knockout mice are infertile due ovarian dysfunction, specifically defective corpus luteum formation. Owing to the aforementioned infertility phenotype, Teglund et al. were unable to study the effects on mammary gland development but were able to conclude that either individually or cooperatively *Stat5a* and *Stat5b* mediate essentially all PRL and growth hormone dependent development.[367] In effort to investigate *Stat5a/b* null mammary development, *Stat5a/b* null glands were transplanted into wild-type recipients. These glands developed a ductal tree but did not undergo alveolar morphogenesis, indicating that STAT5 is necessary for the proliferation and differentiation of mammary alveoli.[368]

The role of STAT3 in the mammary gland is best characterized as a harbinger of death, as phosphorylated STAT3 increases dramatically at the onset of involution (within 12 hours) and precedes the wave of post lactation apoptosis and alveolar collapse.[369, 370]

The involution-associated cell death is dependent only on local factors such as mechanical stress due to milk stasis.[370] STAT3 activation is dependent on several secreted factors, namely leukemia inhibitory factor (LIF)[371, 372], serotonin [373] and TGF-β3.[374] *Stat3* null animals die during early embryogenesis (E7.5), with the lethality attributed to a defect in visceral endoderm function.[375] Conditional deletion of *Stat3* in the mammary gland, driven by either WAP-Cre or β-lactoglobulin- Cre (BLG-Cre), both result in delayed involution and an extension of the reversible phase.[376, 377]

Of late, the mechanism of cell death during early involution is being revisited due to the observed delay in TUNEL positivity of shed cells, and cell morphology that does not align with classical apoptotic condensation, fragmentation, and membrane blebbing. Recent work from the lab of Christine Watson suggests that the cell death is a STAT3-driven, cathepsin-dependent and executioner caspase-independent, lysosomal-mediated programmed cells death.[378, 379] This study necessitates re-evaluation of foundational literature and warrants further and more directed attention.

The level of STAT1 remains constant throughout the pregnancy cycle; however, active STAT1 is detected in the virgin gland and in the late involution gland. This pattern of pSTAT1 is a unique amongst STATs and the inverse of the pSTAT5 profile.[370, 377] No clear role has been defined for Stat1 in mammary development, and *Stat1*$^{-/-}$ mice are viable showing no overt developmental or mammary gland defects.[380]

Cell Death Signaling

It is currently thought that of the cells that die during involution, the vast majority die by the classical apoptotic pathway. Apoptosis is a highly regulated, well described

process wherein a cascade of intracellular signaling is initiated in response to an extrinsic or intrinsic signal. It is thought that a balance of pro-apoptotic and anti-apoptotic factors determines cell fate. The Bcl2 protein family namesake, anti-apoptotic *Bcl2,* is not expressed during lactation or early involution, but its expression increases in late (>10 days) involution. Based on this expression profile it is not surprising that *Bcl2* knockout mice do not exhibit a gross mammary defect. Global ablation of *Bcl2 (Bcl2*$^{-/-}$) is not lethal, resulting in small, hypopigmented mice with polycystic kidney disease.[381] Overexpression of *Bcl2* driven by the WAP promoter results in a delayed involution.[382] Studies have revealed that *Bcl2l1* is expressed at higher levels than *Bcl2* in many tissues during development and in the adult, including the mammary gland.[383, 384] The traditional knockout (*Bcl2l1*$^{-/-}$) is not viable and dies embryonically due to excessive apoptosis of immature erythroid cells in the fetal liver.[385] This model provided valuable information regarding the role of *Bcl2l1* during development, but the embryonic lethality was prohibitive to study the role of *Bcl2l1* in mature tissues. As a result two conditional, mammary specific *Bcl2l1* knockout mouse models (WAP-Cre; *Bcl2l1*$^{fl/fl}$, MMTV-Cre; *Bcl2l1*$^{fl/fl}$) were generated. *Bcl2l1* deficient mammary epithelia tissue exhibited an accelerated involution, a phenotype characterized by an increase in apoptosis, indicating that *Bcl2l1* is necessary for cell survival during early involution.[386]

Pro-apoptotic factors include Bcl2 family members *Bax*, *Bad*, and *Bak* among others. *Bax* mRNA is highest at the lactation to involution transition and three splicing products are produced, however the protein level is unchanged.[387] *Bax* deficient mice intuitively exhibit hyperplasia in some cell lineages (e.g. B cells, thymocytes); however,

counterintuitively males are infertile due to the absence of mature sperm and abnormal mammary development has also been reported.[388, 389] Loss of *Bax* delays involution, while overexpression of BAX in the mammary secretory epithelia in a WAP-Bax model results in a lactation defect as well as forced involution based on an increase in apoptotic cells and precocious *Stat3* activation.[387, 390] *Bak* and *Bad* are present throughout pregnancy and remain expressed highly through involution. It is hypothesized that the mammary gland is persistently poised to trigger involution and it is the balance of pro-apoptotic and anti-apoptotic factors determines lactation to involution transition.[387, 391]

The nature of cell death signaling that occurs throughout the developmental cycle of the mature mammary gland is complex. In many cases canonical functions of proteins are observed, but many surprising roles have been elucidated using transgenic technologies.

Conclusion

The mammary gland is a dynamic organ that is reliant upon hormones, growth factors, and epithelial-stromal interactions to regulate well-established cell signaling pathways that orchestrate highly regulated developmental programs. These events span from mid-gestation into adolescence and through adulthood, when terminal differentiation occurs within a cyclical pattern of growth and regression that is linked to reproduction. Due to the accessory nature of the gland, ease of accessibility, and transplantation potential, the mammary gland is a powerful model to characterize the genetics behind organ development and cell differentiation. In addition to its relevance

in modeling normal development, the mammary gland is also essential in profiling breast cancer, neonate immunity, ageing and stem-cell based regeneration. The blossoming of mouse transgenic technologies over the past twenty years has led to breakthroughs in the understanding of the underlying mechanisms controlling mammary gland development. The early endocrine ablation studies that revealed the pituitary and ovarian control of mammary gland development eventually led to genetics-based approaches to further characterize these processes. Genetic engineering has advanced from gain-of-function studies to knockout strategies, giving way to the current state of conditional (Cre-LoxP, Flp-FRT) and inducible (tet-on, tet-off) models. This increased specificity for inducing mutations has allowed researchers to investigate gene and signaling pathway contributions at restricted developmental stages. Although the genetic tools will continue to expand and increase the array if potential applications, technology is but a tool to address the basic biological questions that remain in the field. What is the nature of the crosstalk between the hormonal receptors? Which, if any, of the other immune cell lineage pathways are relevant for mammary cell lineage commitment? What are the specific regulatory mechanisms at work regulating the upregulation of target genes controlled by multiple transcription factors? How do microRNAs regulate the developmental program of the mammary gland? How do apoptosis and autophagy interact to promote regression during involution? How does the methylation state of DNA and histones impact development? These questions and many others will continue to propel this field in the coming years.

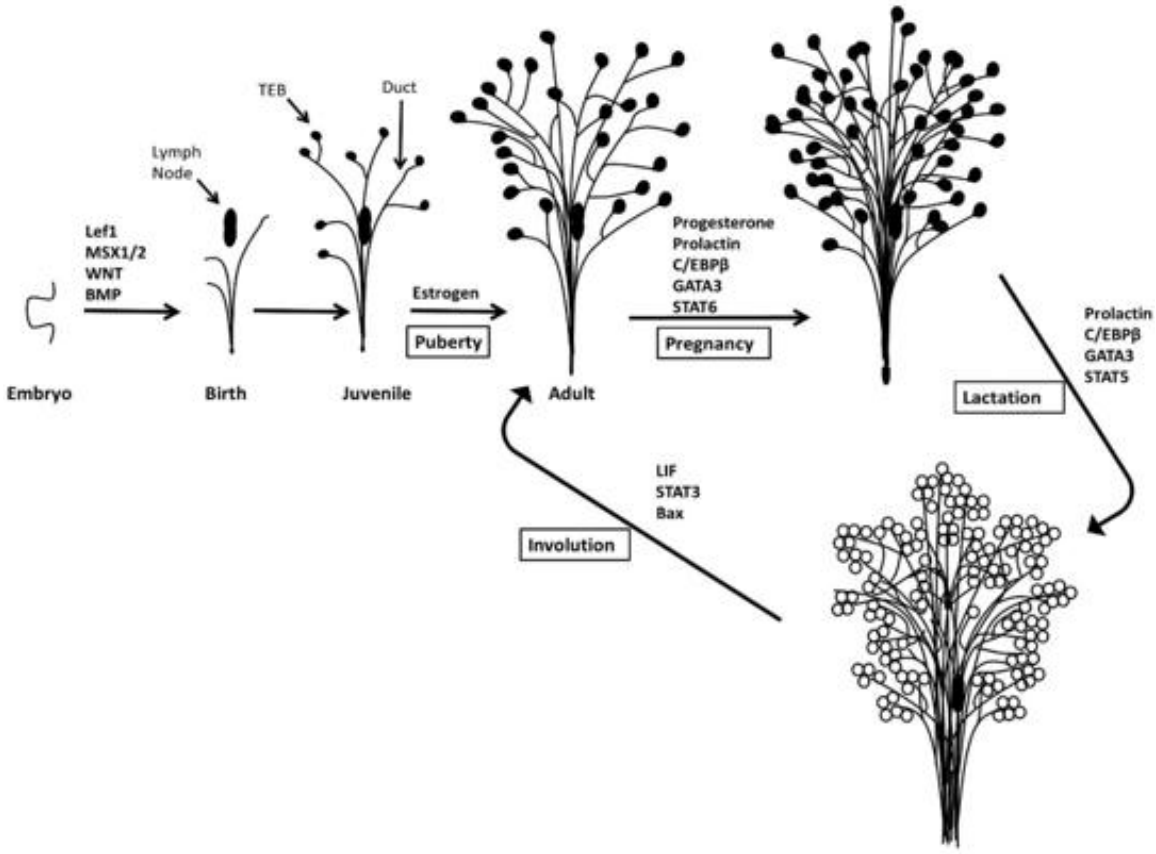

Figure 3.1 Schematic overview of mouse mammary gland development and major influencing factors. Mammary gland development is typically divided into embryonic, perinatal, puberty and adult stages. After sexual maturity pregnancy induced changes drive the terminal differentiation of the gland. These stages are pictured; pregnancy, lactation and involution. Many of the influential molecules discussed in this chapter are included in the figure above the developmental stage/process to which they are integral. Major developmental stages are depicted schematically; stage labels are below mammary glands and process labels are below arrows.

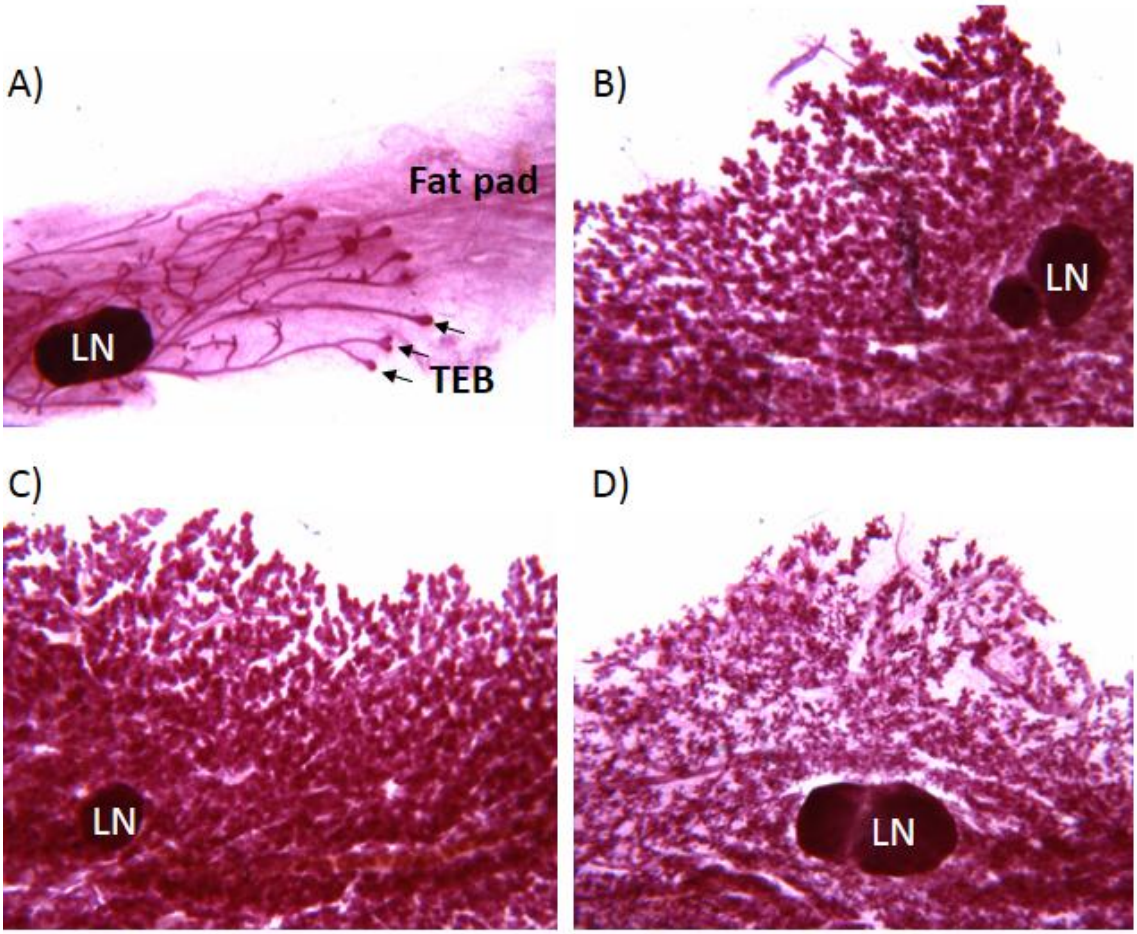

Figure 3.2 Anatomic overview of mouse mammary gland development at four stages. Whole-mounts were taken of mouse mammary glands at four distinct developmental stages: A) virgin 5-week-old glands; B) pregnancy day 14; C) lactation day 1; and D) involution. Epithelial cells stain with the dye carmine alum, whereas the surrounding fat pad is cleared. Notice in the virgin gland the formation of the ductal tree, as the epithelia migrate into the surrounding fat pad. Representative terminal end buds (TEBs) are shown with arrows. Lymph nodes (LN) are shown for orientation in all figures. Images were taken on an Olympus SZ-PT using 6.7X magnification and the imaging software QCapture Pro 6.0.

Table 3.1 Transgenic mouse models and mammary phenotypes observed

	Genotype	Mammary phenotype	Reference
Embryo			
Lef1	*lef1* $^{-/-}$	No placode formation	293
Dkk1	K5-rtTA/tetO-Dkk1 (overexpression)	No placode formation	294
Msx1	*msx1* $^{-/-}$	No phenotype	313
Msx2	*msx2* $^{-/-}$	1/3 of embryos arrest mammary development before ductal branching/perinatal lethality	312
Msx1/Msx2	*msx1/msx2* $^{-/-}$	Anlagen fail to form	312
Puberty			
Esr1	EsRα $^{-/-}$	No ductal side-branching or lobuloalveolar development	316, 319
	deleter-Cre; EsRα $^{f/f}$	No data	322
	MMTV-tTA/tet-op-mEsRα	Toolbox/kinetics study	324
Esr2	EsRβ $^{-/-}$	No phenotype	319,317
	CMV-Cre; EsRβ $^{f/f}$	No phenotype	323
Esr1/ Esr2	EsRα $^{-/-}$; EsRβ $^{-/-}$	same as EsRα $^{-/-}$	318
Cebpb (C/EBPβ)	*cebpb* $^{-/-}$	Enlarged ducts/decreased side-branching	348,349
Pregnancy/Lactation			
Msx1	(overexpression)	Reduction in differentiation/increase in proliferation	314
Pgr	PR $^{-/-}$	No ductal side-branching or lobuloalveolar development in response to hormonal stimulation	328
	PRA $^{-/-}$	No phenotype	335
	PRB $^{-/-}$	Reduced ductal side-branching and lobuloalveolar development	336
	ZP3-Cre;PR $^{f/f}$	Same as PR $^{-/-}$	337
	EIIa-Cre; PR $^{f/f}$	Same as PR $^{-/-}$	338
Cebpb (C/EBPβ)	*cebpb* $^{-/-}$	Reduced lobuloalveolar development in response to hormonal stimulation	348, 349

Table 3.1 Continued

	Genotype	Mammary phenotype	Reference
Ccnd1 (Cyclin D1)	*ccnd1*$^{-/-}$	Reduced lobuloalveolar development/ lactation failure	343
Stat6	*stat6*$^{-/-}$	Reduced lobuloalveolar development	352
Il4/Il13 (IL-4/IL-14)	*IL-4*$^{-/-}$*;IL-14*$^{-/-}$	Reduced lobuloalveolar development	352
Gata3	MMTV-Cre*; gata*$^{f/f}$	Perinatal lethality/ in survivors TEBs do not form on-time/ductal epithelia does not invade the fat pad/irregular ductal epithelia	354
	WAP-rtTA-Cre*; gata* $^{f/f}$	Similar to MMTV-Cre*; gata*$^{f/f}$/ increased proliferation/cell death/ failure of lactation	353
	Keratin14-Cre*;gata*$^{f/f}$	Embryonic death, absent placodes in survivors	354
	WAP-Cre*; gata*$^{f/f}$	Reduced lobuloalveolar development/ defective lactation	354
Prl	PRL$^{-/-}$	Reduced lobuloalveolar development	356
Prlr	PRLR$^{-/-}$	Reduced ductal side-branching /lactation failure	357,358
Jak2	*jak2*$^{-/-}$	No data -Embryonic lethal	359,360
	MMTV-Cre;*jak2* $^{f/f}$	Defect in establishment of alveolar progenitor cells	361
	WAP-Cre;*jak2* $^{f/f}$	Reduced survival of secretory epithelia	361
Stat5a	*stat5a*$^{-/-}$	Defective lobuloalveolar development/lactation failure	364
Stat5b	*stat5b*$^{-/-}$	No phenotype	366
Stat5a/Stat5b	*stat5a*$^{-/-}$*;stat5a*$^{-/-}$	Transplant studies reveal failure of alveolar morphogenesis	367, 368
Stat1	*stat1*$^{-/-}$	No phenotype	380
Involution			
Stat3	*stat3*$^{-/-}$	No data -Embryonic lethal	375
	WAP-Cre;*stat3* $^{f/f}$	Delayed involution	376
	BLG-Cre;*stat3* $^{f/f}$	Delayed involution	377

Table 3.1 Continued

	Genotype	Mammary phenotype	Reference
Bcl2	*bcl2*$^{-/-}$	No phenotype	381
	WAP- *bcl2* (overexpression)	Delayed involution	382
Bcl2l1	*Bcl2l1*$^{-/-}$	No data -Embryonic lethal	385
	WAP-Cre; *bcl2l1*$^{f/f}$	Accelerated involution	386
	MMTV-Cre; *bcl2l1*$^{f/f}$	Accelerated involution	386
Bax	*bax*$^{-/-}$	Delayed involution	388
	WAP- *bax* (overexpression)	Lactation defect/forced involution	390

Chapter Four: Forced Involution of the Functionally Differentiated Mammary Gland by Overexpression of the Pro-Apoptotic Protein BAX

Introduction

This chapter has been published and is included here for completeness of the dissertation.[390]

Mammary gland development is classically compartmentalized into four distinct phases: virgin, pregnancy, lactation, and involution. The contributions of hormones, signaling pathways, and genes that give rise to the mammary gland, from the progression of the rudimentary anlagen during embryonic development through the post-natal stages described above, has been intensely studied.[392] Although estrogen and progesterone are the predominant hormones responsible for development of the gland in the virgin and early pregnancy phases, prolactin directs the proliferation and differentiation of the epithelia during mid-pregnancy through the prolactin receptor PRL-R/JAK2/STAT5 signaling pathway. JAK2-STAT5 signaling is persistent through the lactation phase for transcriptional activation of milk protein genes, and drops at the lactation-involution transition phase. Involution proceeds through: (1) an initial reversible phase marked by programmed cell death of secretory epithelium without remodeling of the lobuloalveolar structure, followed by (2) an irreversible phase characterized by remodeling of the gland by proteases (e.g., matrix metalloproteinases, MMPs) and macrophages.[393]

Forced involution, initiated at lactation day 10, triggers apoptosis in response to milk stasis within the alveolar lumen and the precipitous drop in lactogenic hormones

(e.g., prolactin).[394] The lactation–involution transition is regulated, in part, by the contrasting activation of STAT5 and STAT3. STAT5 phosphorylation is maintained through lactation but drops precipitously at the onset of involution, while STAT3 phosphorylation is initiated as the gland enters involution.[369, 395, 396] Conditional deletion of *Stat5* results in the loss of mammary epithelium during mid-pregnancy and lactation, suggesting that this transcription factor is required for cell survival. In contrast, transgenic overexpression of *Stat5* or gene ablation of *Stat3* delays the involution process.[377, 397] Similarly, loss of *Socs3*, a negative regulator of the JAK-STAT pathway, leads to an increased rate of involution via increased STAT3 activity.[398] In addition to the JAK-STAT pathway, genetic modifications within the IGF and AKT1 signaling pathways reveal their importance. Transgenic IGF-1 or IGF-2 mice have delayed involution, while IGFBP5 overexpressors have increased apoptosis.[399-401] Downstream in the IGF signaling pathway, overexpression of PTEN or *Akt1* in transgenic models displayed accelerated or delayed involution phases, respectively.[402-404] Recently, STAT5 has been demonstrated to transcriptionally regulate *Akt1*, and conditional expression of *Stat5* leads to persistent *Akt1* activation and delayed involution.[405] In addition, PTEN ablation increased the survival of alveolar cells similar to the *Akt1* overexpression. [406]

Programmed cell death (PCD), an important physiological process that is necessary for development, is classified either as either: Type I (apoptosis), Type II (autophagy), or Type III (non-lysosomal vesiculate degradation). Apoptosis can be generally stratified into a three-tiered molecular cascade system consisting of ‘‘elicitor’’ death receptors (e.g., TNFR1, TNFR2, FAS)[407, 408], *Bcl2* family member ‘‘mediators’’ (e.g.,

BCL2, BCL2L1$_L$, BCL2L1$_S$, BAX, BCL2L2)[409-411], and the caspase ‘‘executioners.’’[412-414] The mammary gland is an advantageous organ to profile the stages of involution, as a forced involution allows for the synchronization of the biochemical processes. In the context of cell death programs, the involution phase exhibits one of the most dramatic physiological responses, with around 80% of the mammary epithelial cells undergoing programmed cell death.[415] The cohort of signaling pathways and cell death genes necessary for this transitional period has been reviewed extensively.[416-418] Although microarray and proteome analyses have revealed a predicted cast of proteins and highlighted biochemical pathways involved in involution, the most revealing studies have stemmed from mouse models. [303, 304]

Bcl2 family members are considered the gatekeepers of the mitochondrial-mediated apoptosis, with the fate of the cell dependent upon the relative levels of the individual proteins. *Bcl2*-like proteins can be subdivided into two distinct groups, those that are pro-apoptotic (e.g., BAX, BCL2L1$_S$, BID, and BAD) and anti-apoptotic (e.g., BCL2, BCL2L1$_L$, and BCL2L2). BCL2L1$_L$ and BAX are the two most prominent *Bcl2* family members expressed in mammary tissue. *Bcl2l1$_L$* mRNA levels are low in virgin mammary tissue, but increase during pregnancy in parallel with STAT5 activation and cell differentiation. *Bcl2l1$_L$* mRNA levels decrease during lactation, but increase again sharply within 48 h of involution. Similarly, *Bax* mRNA levels increase with the onset of involution.[389] Within the *Bcl2* family, several models highlight their influence on the maintenance of the secretory epithelium. We have shown previously that the mammary gland-specific deletion of *Bcl2l1$_L$* resulted in an increase in apoptosis and hastened

involution.[386] In the *Bax*-deficient mammary gland, involution is initially delayed but resembles the wild-type gland at involution day 10 after remodeling is completed.[391] Although *Bax*-deficient mammary glands have a delayed early involution, the loss of *Bax* does not rescue the accelerated cell death in *Bcl2l1*$_L$ -null mammary epithelium in our conditional knockout of *Bcl2l1*$_L$.[386] These studies show a cell- and developmental-specific dependence on BAX-mediated apoptosis. Ablation of *Bax* or enforced expression of *Bcl2* (WAP-*Bcl2* transgenic) delays but does not prevent involution, and promotes mammary tumor development.[382, 419 387, 420]

To date, it has not been addressed whether upregulation of a single pro-apoptotic factor is sufficient to initiate involution and remodeling of the functionally differentiated mammary epithelium. To address this issue, we generated transgenic mice that express *Bax* under a mammary gland-specific promoter that is upregulated during secretory differentiation. WAP-*Bax* transgenic females display a lactation defect due to an increase in cell death and premature involution from ectopic STAT3 activation. This suggests that upregulation of BAX can override the pro-survival signaling functions of STAT5 and AKT1 in the lactating mammary gland.

Results

WAP-*Bax* Transgenic Mice Exhibit Impaired Alveologenesis

Co-injection of the WAP-*Bax* transgene (**Figure 4.1A**) and a F1 K14-agouti construct gave five independent founder lines out of 72 progeny as determined by PCR analysis (**Figure 4.1B**). Previous characterization of co-injected transgenes with the K-14

agouti demonstrated 95% co-integration, which allowed for visual determination of transgene inheritance.[421] All WAP-*Bax* lines also carried the K-14 agouti transgene, making it possible to distinguish bi-transgenic mice by a phaeomelanin (yellow) coat color (**Figure 4.1C, D**). Q-PCR was performed to establish copy number of the WAP-*Bax* transgenic lines, with a range of 1–3 copies of the transgene per line. Upon establishing germline transmission of the WAP-*Bax* transgene, F1 females were mated with control C57 males to determine effects on mammary gland development. Out of the five lines, two lines that demonstrated protein expression (IR5, IR15) could not support litters at lactation day 1 (L1). Neonates from WAP-*Bax* dams did not have milk ‘‘spots,’’ but could be fostered onto control lactating females (**Figure 4.1E, F**). Therefore, the defect was not a suckling defect with the neonates but the transgenic dams. Whole mount analysis showed that control mammary glands at lactation day 1 predominantly consisted of secretory epithelia (**Figure 4.2A**). In addition, there was a distinct ‘‘rounded’’ morphology to the alveoli. In contrast, WAP-*Bax* mammary glands demonstrated a reduced epithelial compartment and altered ‘‘star-shaped’’ alveoli (**Figure 4.2B, C**). Hematoxylin-eosin staining of paraffin-embedded mammary glands showed normal, uniform secretory epithelia in the control glands, but reduced epithelia with altered morphology in the postpartum WAP-*Bax* transgenic glands (**Figure4.3**). Epithelia in the mutant glands displayed increased signs of apoptosis; condensed cells had pyknotic nuclei with many being released into the lumen (**Figure 4.3D**).

Activation of an Apoptotic Program in Response to Expression of BAX

To determine the activation of the apoptotic program, a series of immunohistochemistry (IHC) experiments were performed. For these experiments, the IR15 line, which showed the highest expression and greatest loss of epithelia, was used. At lactation day 1, expression of BAX was confined to the epithelial compartment of WAP-*Bax* mammary glands, but not detected in the controls (**Figure 4.4**). Upon BAX-mediated permeabilization of the mitochondria outer membrane, it was expected that cytochrome C is released, culminating in the activation of Caspase 3 downstream of the apoptosome. Using IHC, we detected cytochrome C, ApaF1 localization, and active Caspase 3 within the cytosol of mammary epithelial cells of WAP-*Bax* transgenic females (**Figure 4.5**). This clearly suggests that overexpression of BAX is sufficient to elicits a classical mitochondria-mediated apoptotic program of functionally differentiated mammary epithelial cells.

Secretory Mammary Epithelial Cells in WAP-*Bax* Females are Lost due to Apoptosis

Upregulation of BAX normally coincides with the first phase of involution, which precedes the actual remodeling process 2–3 days later.[389, 391] To confirm that continuous expression of exogenous BAX is sufficient to induce the second and terminal stage of apoptosis, we performed TUNEL staining (**Figure 4.6**). As expected, mammary glands of postpartum control females did not reveal apoptotic cells. In contrast, mammary glands of WAP-*Bax* transgenic mice had high levels of TUNEL-positive cells in the epithelial compartment. Transgenic lines IR62 (**Figure 4.6B**) and IR32 (**Figure 4.6F**)

which expressed less BAX had also fewer TUNEL-positive cells compared to lines IR5 and IR15 (**Figure 4.6C–E**).

Clusterin is a glycoprotein that is found to be expressed at high levels during pregnancy and involution, but is suppressed during lactation.[422] Because of its expression profile, it is a marker for the lactation-involution transition. Clusterin was found by IHC in the WAP-*Bax* mammary glands and not in the control glands (**Figure 4.7**). More importantly, nuclear pSTAT3 was not present in control glands but was found in transgenic glands (**Figure 4.8**). pSTAT5 was expressed in control and transgenic glands as well. In line IR15, where most of the epithelia was already lost, pSTAT3 and pSTAT5 was not detected. Overall, these data suggest that the lactation defect in WAP-*Bax* females is due to a ‘‘forced involution’’ process and loss of the secretory epithelia due to classical pSTAT3-mediated apoptosis.

Discussion

Programmed cell death (PCD) is a requisite cellular mechanism for development and homeostasis. Apoptosis, Type I PCD, can proceed via extrinsic or intrinsic pathways. Extrinsic pathways rely on apoptosis initiation by receptors at the cell surface, while intrinsic pathways from internal signals such as cellular damage.[423] Both pathways can converge on the mitochondria to mediate the cell death program. BAX, a pro-apoptotic protein from the *Bcl2* protein family, can induce pore formation in the mitochondria that ultimately leads to mitochondria outer membrane permeabilization (MOMP). Once MOMP occurs, cytochrome C is released from the damaged mitochondria, thus

triggering apoptosome formation, caspase activation, and DNA degradation. Alternatively, release of additional mitochondrial proteins (e.g., AIF, OMI, and EndoG) can lead to necrosis through caspase-independent events.[423, 424]

Other genetic studies from mouse models have revealed roles for BAX in regulating apoptosis in reproductive tissues. *Bax*-null mice have phenotypes within the testis and ovary. Males are sterile from the developmental failure of primary spermatocytes to mature into secondary spermatocytes, thus prompting a wave of apoptosis in the adolescent testis.[388] Females are endowed with three times the population of primordial follicles compared to wild-type females. In addition, the reproductive lifespan of *Bax*$^{-/-}$ females is longer due to reduced atresia.[425] BAX is also involved with primordial germ cell (PGC) development in the fetus. BAX ensures the apoptotic death of ectopic PGCs that fail to arrive at the indifferent gonad.[426] Moreover, once PGCs colonize the fetal gonads, we have shown that BAX and BCL2L1$_L$ regulate the survival of the germ cells.[427] Reduction of BCL2L1$_L$ in PGCs leads to a sterile ‘‘Sertoli cell only’’ phenotype in the male, and a 30-fold reduction in primordial follicle in the male. The loss of germ cell populations is restored by the concomitant loss of BAX in PGCs. Previously we have shown that the secretory epithelia of the mammary gland are also impacted by the loss of BAX or BCL2L1$_L$. These studies show a cell- and developmental-specific dependence on BAX-mediated apoptosis.

Our WAP-*Bax* transgenic model demonstrated a ‘‘forced involution’’ and premature cessation of lactation at parturition. The cell death we find is due to a Caspase-dependent induction of apoptosis, as determined by active Caspase-3 IHC and

TUNEL assay. Although STAT5 and AKT1 promote cell survival in the mammary gland during pregnancy and lactation, the ectopic overexpression of BAX overcomes these signals. BAX is regulated by AKT1 phosphorylation at Ser184, which serves to maintain the cytosolic localization of BAX and prevent its mitochondrial targeting.[428] Since AKT1, BCL2, and $BCL2L1_L$ are all cleaved by Caspases, initial Caspase-3 activation would provide a positive feedback loop to inhibit these pro-survival proteins.[429-432] It is unclear as to the mechanism of pSTAT3 activation, although either induction of C-Src or Caspase-mediated degradation of SOCS3 could lead to premature STAT3 phosphorylation. An additional cell survival mechanism that the mammary gland would utilize is autophagy. Autophagy is active in the L1 murine mammary glands, which normally would serve to sequester damaged mitochondria into autophagosomes for turnover (unpublished observations EBR, ANH).[433] Apparently, the 2 transgenic WAP-*Bax* lines with lactation defects have overcome the normal capacity of autophagy as a survival pathway within the epithelia. Interestingly, PUMA-dependent activation of BAX has been shown to concurrently induce autophagy of mitochondria (mitophagy) and a pro-apoptotic response.[434] Thus, our WAP-*Bax* model may not only have an increase in BAX mediated apoptosis but also BAX-dependent autophagy. Quantitation of autophagosomes in the WAP-*Bax* model with the transgenic GFP-LC3 autophagy reporter line would establish whether both of these processes are coordinately activated.[164] Some cells could be lost from autophagic cell death, although the abundance of pyknotic nuclei and TUNEL-positive cells argue for apoptosis as the central programmed cell death mechanism. There are two classical stages of mammary gland involution: the programmed cell

death-driven reversible phase and the remodeling irreversible phase. However, gene expression studies of the early phase has demarcated seven signature profiles: (1) a 12-h peak, (2) a 24- h peak, (3) a 24-h increase with persistent expression, (4) a 24-h peak with a slow decrease, (5) a 72- to 96-h peak, (6) a delayed expression for 48 h, and (7) a delayed expression with peak after 96 h.[416] *Bax* mRNA is found in the fourth group with a 24-h peak and slow decrease. As previously stated, this is the developmental window when BAX has an effect on involution. From our study, BAX also has a dramatic effect on the survival of the secretory epithelia around parturition. This highlights the dynamic plasticity of the mammary gland; epithelia poised with the requisite proteome to carry out programmed cell death given the proper internal signal or environmental cue. In summary, overexpression of the pro-apoptotic protein BAX initiates programmed cell death of functionally differentiated mammary epithelial cells *in vivo*.

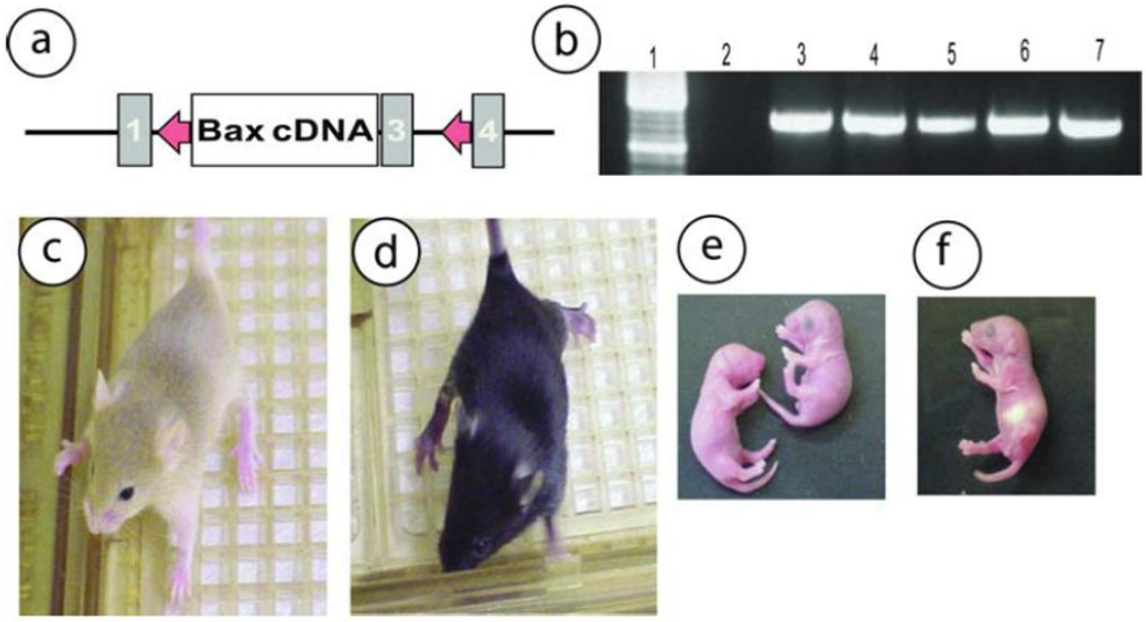

Figure 4.1 Generation of WAP-Bax transgenic lines. WAP-*Bax* transgenic mice were produced by pronuclear injection of the WAP-*Bax* and K14-agouti constructs. (**a**) Schematic of WAP-*Bax* transgene, showing the *Bax* cDNA cloned into a WAP promoter-based plasmid. The WAP promoter, portions of Exons 1 and 3, Intron 3, 4, and downstream sequence are present. (**b**) Gel electrophoresis of Bax PCR products from negative control or transgenic WAP-*Bax* lines IR5, IR15, IR20, IR32, and IR62. Lanes: 1, 100 bp ladder; 2, wild-type control; 3, line IR5; 4, line IR15; 5, line IR20; 6, line IR32; 7, line IR62. (**c**) Coat color of bi-transgenic WAP-*Bax*; K14-agouti mice. (**d**) Coat color of non-transgenic littermate. (**e**) Neonates (Day 1 of lactation) from WAP-*Bax* IR15 dam, which have failed to recover milk after suckling. (**f**) Neonates from WAP-*Bax* IR15 dam fostered onto control lactating dam.

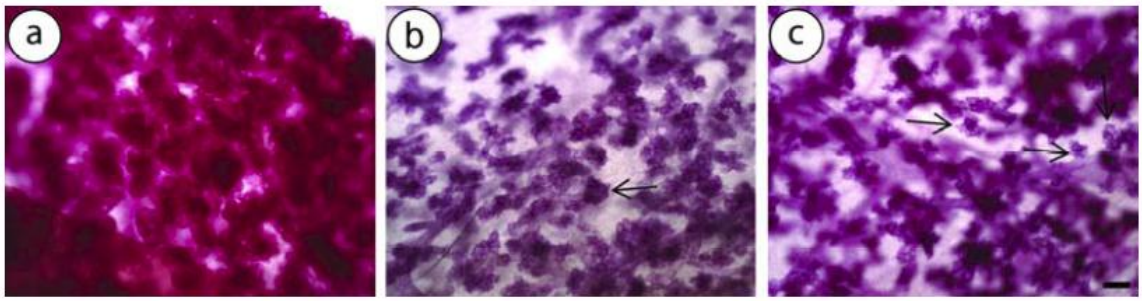

Figure 4.2 Expression of Bax under regulation of the WAP promoter results in reduced epithelial content in the postpartum mammary gland. Whole mounts from lactation day 1 control (**a**) and WAP-*Bax* transgenic mice (**b**, **c**) show reduced epithelial compartments in the transgenic mice. In addition, the wild-type alveoli have a rounded morphology compared to patches of collapsed, ‘‘star-shaped’’ alveoli in the transgenic mice (denoted by arrows). Fp = fat pad. Bar = 0.5 mm.

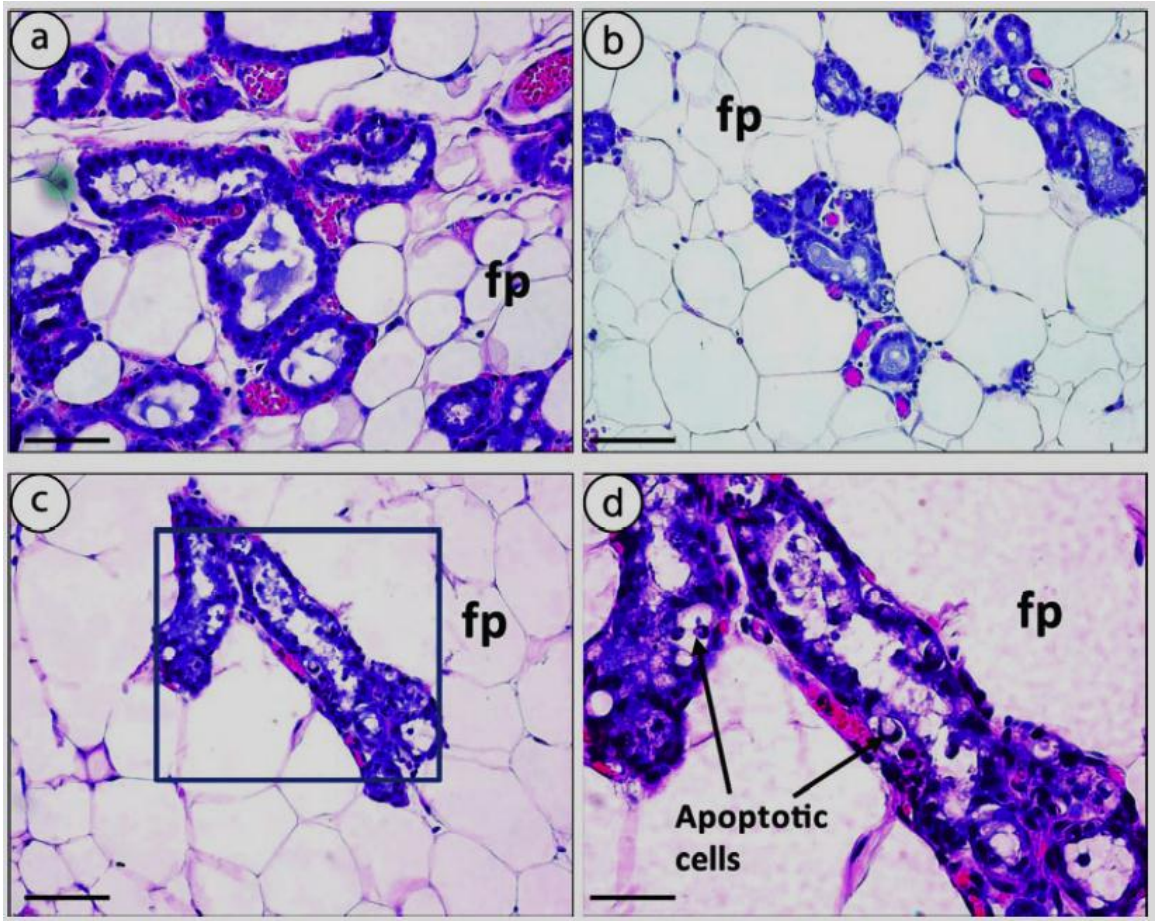

Figure 4.3 Presence of apoptotic cells in mammary glands of WAP-Bax transgenic females at lactation day 1. Hematoxylin-eosin staining of lactation day 1 mammary glands was performed on control (**a**) and WAP-*Bax* mice (**b–d**). (**a**) Control glands show uniform epithelial morphology and large lipid droplets in the lumens. (**b**) WAP-*Bax* glands show reduced epithelia and increased fat pad, and lumens have smaller lipid droplets. (**c**) Altered morphology is apparent in the epithelium and at higher magnification (**d**). Apoptotic cells are present as characterized by pyknotic nuclei (arrows). Fp = fat pad. Bar (**a–c**) = 50 µM. Bar (**d**) = 25 µM.

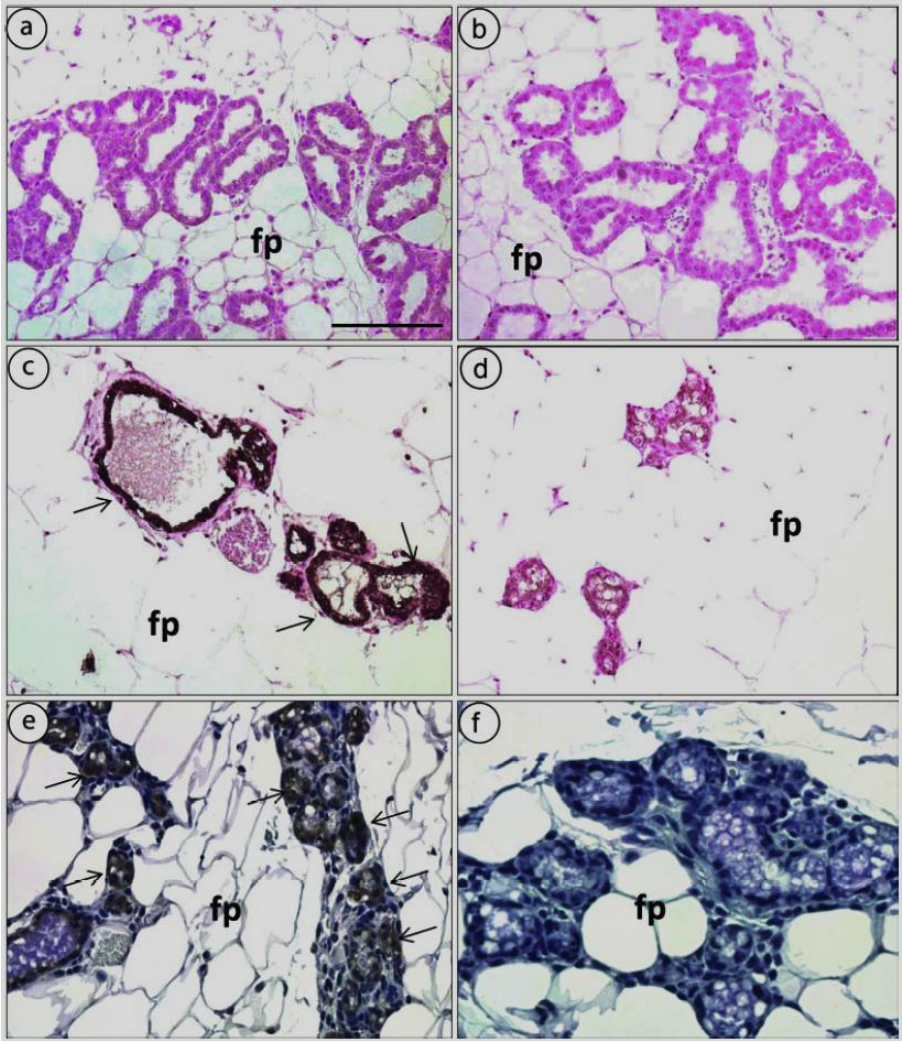

Figure 4.4 Expression of Bax under regulation of the Wap promoter is confined to the epithelial compartment of the developing mammary gland in postpartum females.

BAX IHC shows localized expression in WAP-*Bax* but not control lactation day 1 mammary glands. (**a**) BAX protein is not found to be expressed in the control gland using IHC; (**b**) negative control for wild-type gland. (**c**) and (**e**) reveal BAX confined to the epithelial compartment in WAP-*Bax* glands; (**d**) and (**f**) represent negative IHC controls. Counterstain was performed with nuclear fast red (**a**–**d**) or hematoxylin (**e**, **f**). Arrows point to positive cells/alveoli. Fp = fat pad. Bar = 100 µM.

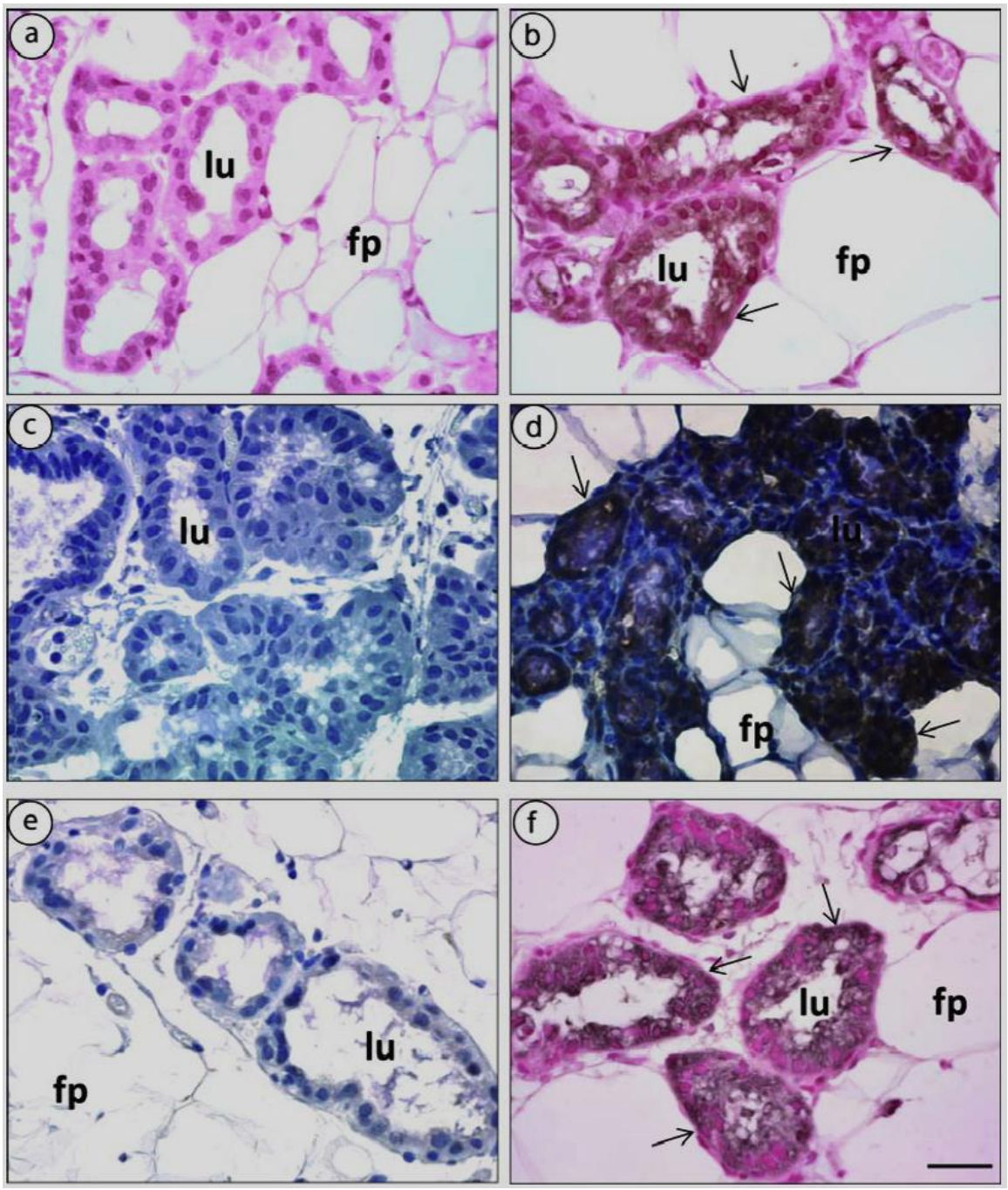
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Figure 4.5 Misexpression of Bax in the secretory mammary epithelium causes mitochondria-triggered Caspase activation. WAP-*Bax* glands showed IHC staining for cytochrome C (**b**), apaF1 (**d**), and active Caspase 3 (**e, f**), whereas control glands did not show cytochrome C (**a**) or apaF1 staining (**c**). (**e**) WAP-*Bax* gland revealed active Caspase-3 IHC staining in the alveoli (denoted with arrow) but not in the duct (du). Counterstain was performed with nuclear fast red (**a, b, f**) or hematoxylin (**c, d, e**). Arrows point to positive cells/alveoli. Fp = fat pad; du = duct; lu = lumen. Bar (**a, b, e, f**) = 25 μM. Bar (**c, d**) = 50 μM.

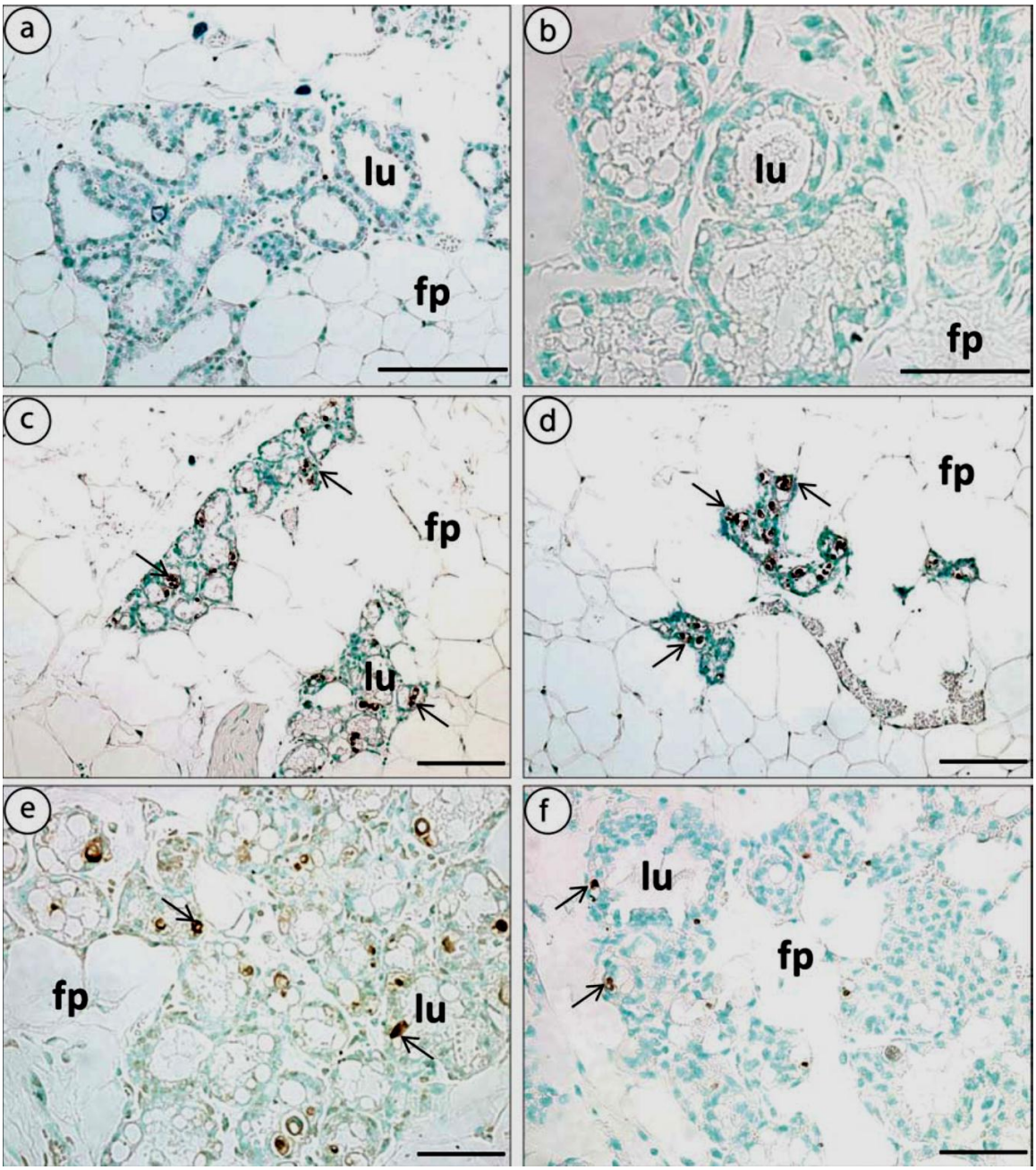
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Figure 4.6 Premature involution of secretory mammary epithelial cells in postpartum WAP-Bax transgenic females. WAP-*Bax* glands showed increased levels of apoptosis compared to control gland. (**a**) Wild-type lactation day 1 mammary gland with no TUNEL-positive cells. (**b**) WAP-*Bax* line IR62. (**c, d**) WAP-*Bax* line IR15. (**e**) WAP-*Bax* line IR5. (**f**) WAP-*Bax* line IR32. Lines IR5 and IR15 (**c–e**), which have lactation defects, have increased levels of TUNEL-positive cells compared to lines IR62 and IR32 (**b** and **f**). Counterstain was performed with methyl green. Arrows point to positive cells. Fp = fat pad; lu = lumen. Bar = 100 µM.

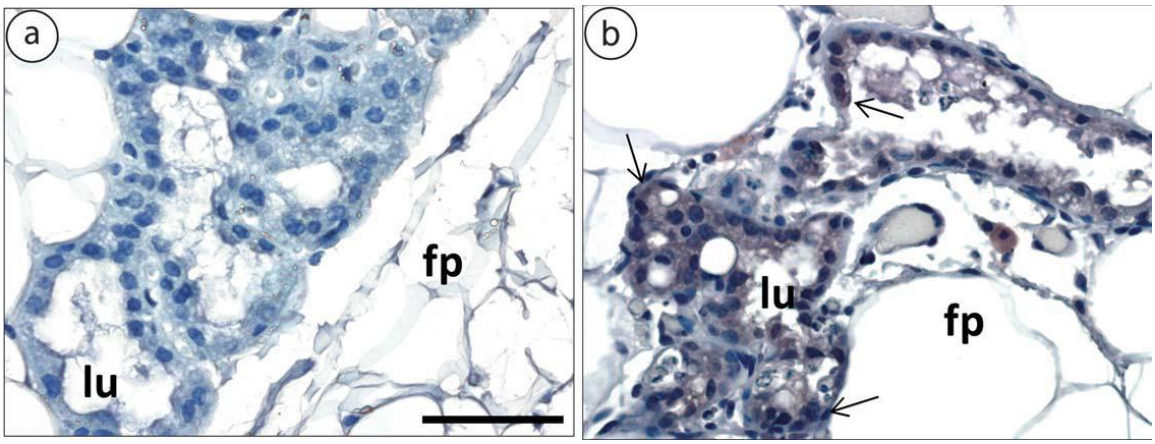

Figure 4.7 Clusterin expression in the lactating mammary gland. Clusterin expression, a marker for involution, was not found in the control lactation day 1 gland (**a**) but was present in the WAP-*Bax* gland (**b**). Counterstain was performed with hematoxylin. Arrows point to positive cells/alveoli. Fp=fat pad; Lu=lumen. Bar=50 µM.

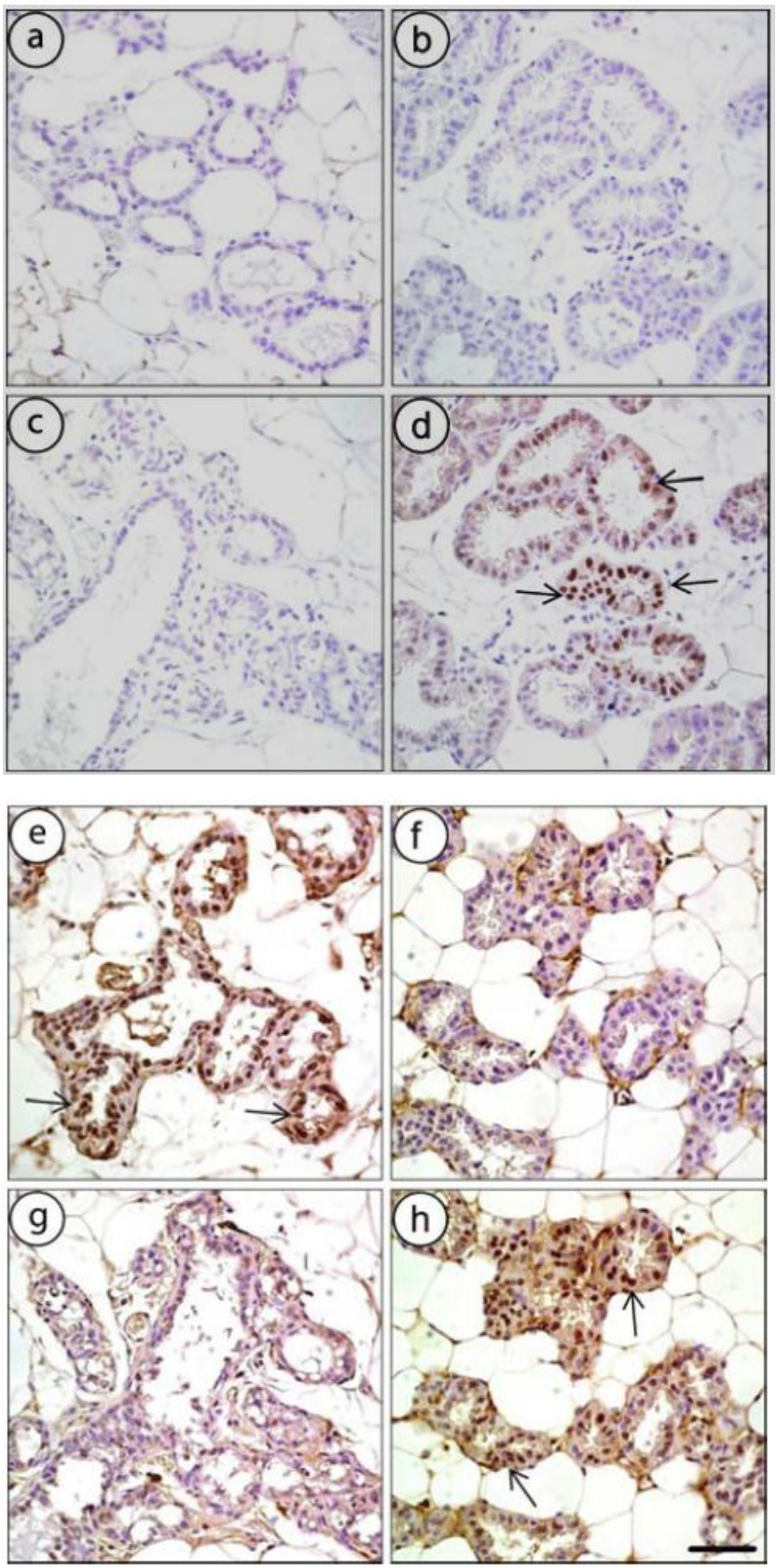
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Figure 4.8 pSTAT3 and pSTAT5 expression in the WAP-Bax mammary gland. pSTAT3 (**a–d**) and pSTAT5 (**e–h**) immunolocalization in wild-type (**a, e**), IR15 (**c, g**), and IR20 (**d, h**) glands at L1. Negative control (no primary Ab) is shown for pSTAT3 (**b**) and pSTAT5 (**f**). Arrows show nuclear localization of pSTAT5 and pSTAT3. Counterstain was performed with hematoxylin. Bar=50 μM.

Chapter Five: An essential, autophagy-dependent role for Beclin1 in murine mammary gland development

Introduction

Macroautophagy, the process by which cytoplasmic cargo and/or organelles are enveloped by a double membrane structure and trafficked to the lysosome for fusion, degradation, and recycling, was once thought to be primarily a type of programmed cell death; however, it has since been shown to be indispensable for normal development and cell survival (reviewed in reference [1]). Macroautophagy, hereafter referred to simply as autophagy, proceeds through four main steps: initiation, elongation, fusion, and degradation. The process is dependent on *de novo* formation of a double membrane structure, termed the autophagosome, which fuses with the lysosome to degrade and recycle cargo to maintain energy homeostasis and prevent cellular damage. BECN1 is an essential autophagy protein that functions during both the initiation and fusion steps.[435] The traditional *Becn1* knockout mice die during fetal development at embryonic day (E) 7.5 due to a failure to close the pro-amniotic canal, thus investigating the subsequent contribution of BECN1 to both normal growth and development as well as under pathologic conditions has been challenging.[184, 185] Our lab has generated a *Becn1* conditional knockout model to remedy this rift in our foundational knowledge.[287] We wanted to explore the contribution and necessity of autophagy in the growth and development of the mammary gland, an organ that is accessory in nature and undergoes the majority of its development in the postnatal period. Additionally, since it has been reported that *Becn1* is a tumor suppressor, owing to the observation that *Becn1* hemizygous mice are prone to sporadic tumorigenesis

and that *Becn1* is mutated or silenced in a majority of examined human breast cancers, the mammary gland is an important, clinically-relevant tissue to investigate in this context.[184, 185, 436,437,438]

The majority of mammary gland development occurs at puberty and in association with reproduction. Briefly, at the time of birth the mouse mammary gland exists as a rudimentary structure and lengthens proportionally to the postnatal individual (i.e. isometric growth) until the onset of puberty, at approximately three to four weeks of age. Under the control of circulating hormones, most notably estrogen, the ducts then begin to lengthen disproportionately to the individual's growth (i.e. allometric growth) and the ductal tree invades the fat pad and branches. After reproductive maturity is attained the gland will remain quiescent unless stimulated by pregnancy hormones.

During the pregnancy cycle, mammary gland development is divided into four stages: mature virgin (resting), pregnancy, lactation, and involution. During the pregnancy to lactation transition, the secretory epithelia must terminally differentiate. The lobulo-alveolar structures mature and prepare for upregulation of *do novo* synthesis of nutrients, both protein and lipid, and the massive secretory burden of lactation. Terminal differentiation of the secretory epithelia is dependent on pregnancy hormones (e.g. progesterone and prolactin). Prior to the day of parturition the alveolar lumena enlarge and the secretory epithelia flatten against the basement membrane. After pups are weaned, the massive programmed cell death and remodeling that characterize the

involution stage return the gland to a resting like state. The majority of the programmed cell death is ascribed to apoptosis; however, some evidence in the literature supports a role for autophagy in turnover and phagocytosis. Data from bovine studies show that *Becn1* expression and autophagy are upregulated during the dry period (i.e. involution) and similar results from were found using mammary epithelial cells (MECs).[439, 440] In mice, only *Becn1*$^{+/-}$ and *Atg7*-deficient glands have been examined by other groups. *Atg7*, important for elongation of the autophagosome, is important for gland remodeling during involution. *Atg7*-deficient glands exhibit delayed involution and fail at mitigating inflammation resulting in persistent inflammation partially due to impaired efferocytosis.[433]

In this study, we pursued a Cre-loxP based strategy centering on a *Becn1* floxed model and two discrete mammary gland-specific Cre transgenic models to interrogate the role of *Becn1* in mammary gland development. We report two distinct and novel roles for *Becn1* in mammary gland biology: the regulation of branching morphogenesis at puberty, and the control of terminal differentiation of the secretory alveoli during pregnancy and lactation. We show that autophagy is reduced concurrent with phenotypic differences in *Becn1* cKO glands, and that the phenotype can be recapitulated *in vitro* using the HC11 cell line. We suggest that *Becn1* is important for properly regulated pubertal development and critical for terminal differentiation of the mammary gland due to its role in proper secretion of milk fat globules.

Results

Mammary gland directed *Becn1* cKO is specific and efficient

It has been postulated that programmed cell death may be essential for mammary gland development and lumen formation. We hypothesized that autophagy was required for proper ductal tree formation. To test this hypothesis, we used our floxed *Becn1* model and a transgenic Cre strain active in the developing mammary gland, MMTV-CreD.[441, 442] Since the description of the MMTV-CreD transgenic did not include all time points we were interested in collecting, it was imperative to verify temporal specificity of the recombination. We generated bi-transgenic reporter mice which express a red fluorescent protein variant, DsRed, in cells that Cre mediated recombination has occurred to examine the efficiency and localization of Cre expression in the MMTV-CreD transgenic line.[443] Nulliparous bi-transgenic females (MMTV-CreD; $DsRed^{+}$) and littermate controls were sacrificed and mammary glands as well as other selected organs (data not shown) were collected at five and six weeks of age. Glands were either prepared for fluorescent whole mount analysis or sectioned. The reporter females indicate high expression was evident in the ducts (**Figure 5.1B-D**) as well as in the terminal end buds (TEBs) (**Figure 5.1C**) but not in the stroma. We identified little aberrant expression in the mammary gland with exception of the lymph node, which was previously reported (**Figure 5.1E**). We concluded that MMTV-CreD transgenic mice have efficient recombination and specificity in the mammary ducts and TEBs.

Becn1 cKO mammary glands exhibit hyper-branching phenotype

The MMTV-CreD; *Becn1*$^{fl/fl}$ mice are viable and fertile, with no evident gross functional mammary defect. Mutant individuals are able to maintain a typical gestation length and sustain litters for three or more pregnancy cycles (data not shown). Mutant females (MMTV-CreD; *Becn1*$^{fl/fl}$) were collected and examined at 3, 5, 8, and 10 week time points. Since whole mounts collected from five week old individuals consistently appeared to have increased branching (**Figure 5.2A, B**), we decided to analyze the branching network of mature nulliparous mice. We reasoned that since some variability exists in maturing glands between four and six weeks, all glands should be in a mature state by ten weeks of age. We found that secondary, tertiary, quaternary, and total branching was significantly increased in mutant glands when compared to sibling controls (*Becn1*$^{fl/fl}$) (**Figure 5.2C**). Mutants exhibit a 1.71-fold increase in secondary branching, a 1.98-fold increase in tertiary branching, a 2.06-fold increase in quaternary branching and 1.94-fold increase in total number of branching (n=5 controls, n=9 mutants). Mutant females were otherwise normal. Females are able to sustain pregnancy and normal lactation. Aged nulliparous or primiparous females did not show any sign mammary tumors, females were observed up to 10 months of age.

Becn1 cKO mammary glands have lactation defect

In this series of experiments, we examined the effect of ablating BECN1 expression in the mammary gland persistently from mid-pregnancy forward, specifically in secretory epithelia cells. This strategy functionally separates the effects of pubertal development from those of pregnancy related development, a necessity since *Becn1*

compromised ductal trees exhibit a hyper-branching phenotype. To examine pregnancy related development and terminal differentiation, we crossed our *Becn1*$^{fl/fl}$ model with a second Cre transgenic line using the Whey-Acidic-Protein promoter to drive Cre expression, hereafter referred to as WAP-Cre.[441] Mammary development was analyzed from pregnancy day (P) 14.5 until 48 hours post parturition. In order to maximize the ablation of BECN1 in the mammary gland, we chose to use one germline null allele and one floxed allele in combination with the Cre transgene (WAP-Cre; *Becn1*$^{fl/-}$). This strategy results in the production of an animal that is globally hemizygous for *Becn1* and the expression from the remaining allele will be recombined by expression of Cre specifically in the secretory epithelial cells in the mammary gland. The presence of a null allele does not have a developmental consequence in the mammary gland, as *Becn1*$^{+/-}$ females exhibit normal pregnancy and lactation stages. The WAP-Cre transgene becomes active beginning at P12.5 and continues to increase throughout pregnancy and remains high for the duration of lactation.[441] Effective recombination of the remaining *Becn1* floxed allele is observable by BECN1 immunohistochemistry as early as P14.5, with further reduction at P16.5 (**Figure 5.3A**). Reduction in total protein is evidenced by western blot conducted on whole glands, sans associated lymph nodes, harvested on the day of parturition (**Figure 5.3E**). In mutant glands, BECN1 expression is reduced 25-fold when compared to control glands. WAP-Cre; *Becn1*$^{fl/-}$ dams are unable to sustain litters and all the pups succumb to starvation within 24 hours of parturition. Pups nursing on mutant dams did not have observable milk spots indicative of failure of nutrient transfer from the dam. This phenotype is a result of a lactation failure in the

dam, as the pups exhibit normal suckling response and are competent when fostered by a wild type dam.

WAP-Cre; *Becn1*$^{fl/-}$ mammary glands are autophagy impaired and structurally compromised

Since BECN1 is an essential autophagy protein, we examined autophagy function relative to control animals using several methods. A chief responsibility of autophagy is the clearance of poly-ubiquinated protein aggregates that are too large to be degraded by the proteasome. An ubiquitin binding protein, SQSTM1 (commonly known as p62), serves as an adaptor molecule for the autophagic engulfment of aggregates.[44] SQSTM1 is a useful marker of autophagic function because when autophagy is impaired SQSTM1 tagged aggregates accumulate in the cell. In the *Becn1* cKO mutant glands autophagy is compromised as determined by changes in SQSTM1. SQSTM1 total protein is increased 1.61 fold relative to control on the day of parturition (**Figure 5.4C**), and SQSTM1 intracellular aggregates can be detected by IHC (**Figure 5.4A, B**). In addition, TEM revealed the accumulation of enlarged autophagosomes with altered morphologies, many with identifiable undigested cargo (**Fig 5.4E, F**) in the mutants while few are observed in the control (**Figure 5.4D**). The loss of normal tissue architecture and morphology changes is increasingly evident as pregnancy reaches term. On the day of parturition, morphologic analysis reveals a loss of the functional, secretory unit organization and a reduction in overall epithelial content and volume. Control animals at 48 hours post parturition have readily identifiable secretory units and lumena full of milk fat globules (**Figure 5.5A**), while mutant glands have milk fat globules retained in

the cytoplasm, epithelial cells are morphologically cuboidal, and have collapsed alveolar structure (**Figure 5.5B**). At the ultrastructure level, control glands have an organized typical secretory structure (**Figure 5.5C, D**) and mutant females lack organized secretory units. In the rarely identifiable secretory units, evidence of improper lipid secretion and enveloping can be seen (**Figure 5.5E**), as well as atypical lysosome associations with milk fat globules (**Figure 5.5F**).

Canonical mammary signaling pathways are intact in *Becn1* mutant glands

Classically, JAK/STAT signaling has been viewed as the intrinsic regulator of mammary function and involution. *Stat5a* KO, *Stat5a/b* double KO, and expectedly the prolactin receptor KO (*Prlr* $^{-/-}$) do not undergo a functional lactation stage.[357, 358, 364, 367, 368] Despite the lack of direct evidence linking BECN1 or autophagy with JAK/STAT regulation, we wanted to investigate the possibility that JAK/STAT signaling was altered in *Becn1* mutant glands. Antibodies recognizing phosphorylated STAT5 (pSTAT5) and Cyclin D1 (CCND1), a transcriptional target of STAT5 and cell cycle regulator, were used to perform IHC on control and mutant glands. *Ccnd1* $^{-/-}$ animals do not lactate and have reduced lobulo-alveolar development.[343] We found that the expression of pSTAT5 and CCND1 in *Becn1* cKO glands was indistinguishable from control glands (**Figure 5.6**), indicating that extrinsic (i.e. progesterone-mediated) signaling is unaffected.

Autophagy inhibition *in vitro* results in retained lipid droplets and exocytosis of autophagosomes

To determine if the lipid accumulation in secretory epithelial cells is autophagy dependent or strictly *Becn1* dependent, we used an *in vitro* system in parallel with our mouse model. We chose to model our *in vivo* system with a mammary epithelial cell line, HC11, derived from a mid-pregnant BALB/c mouse.[444-446] These cells can be coaxed into a lactogenic state by treatment with dexamethasone, insulin, and prolactin (DIP) via media supplementation, and are used in the field extensively to model lactation stage glands. To examine the effect of autophagy inhibition during lactogenic differentiation and during lactogenic fate, we used the chemical autophagy inhibitor BafilomycinA1 (BafA1). BafA1 inhibits fusion of the autophagosome with the lysosome for cargo degradation at early time points but also prevents acidification of the lysosome at later time points (>2 hours of treatment). Since we are examining 24 and 48 hour time points and using a relatively low treatment dose (200nM), it is important to note that we will expect an increase in autophagosomes, amphisomes, and autolysosomes containing undigested cargo to verify autophagy inhibition (**Figure 5.7C**). We analyzed the differentiated HC11 cells at the 24 and 48 hour time points, time 0 indicating the addition of both DIP media and 200nm BafA1. After 48 hours of treatment, TEM analysis confirmed an inhibition of autophagy, as well as large autolysosomes containing undigested cargo in the BafA1 treated lactogenic cells. We identified several cases of direct exocytosis of autophagosome and autolysosomes in BafA1 treated cells (**Figure 5.7C**). Since our *Becn1* cKO model exhibited an increase in retained intracellular lipid

droplets we investigated if this was also the case in our autophagy-inhibited, differentiated HC11 cells. We used BODIPY 493/503 to stain lipid droplets and counterstained with DAPI. BafA1 treated cells accumulated significantly ($p< 3.25E-08$) more lipid droplets than the untreated lactogenic cells, with a mean number of lipid droplets per cell of 23 (SEM ± 1.3) and 14 (SEM ± 0.81) respectively (**Figure 5.7A, B**).

Discussion

We investigated the contribution of *Becn1* to the formation of the mammary ductal tree and the terminal differentiation of secretory epithelial cells. The MMTV-CreD; *Becn1*$^{fl/fl}$ mouse is the first in the field to genetically ablate an essential autophagy protein in the ductal epithelia of the postnatal mouse mammary gland prior to puberty and was a necessary experiment to answer a long-standing questions of whether autophagy is necessary for ductal lumen formation. We found that *Becn1* is not essential for lumen formation during ductal outgrowth. We report *Becn1* cKO glands exhibit a significant increase in higher order branching in the mature nulliparous gland. This effect is likely a result of removal of cell cycle repression by *Becn1*, as has been suggested in the literature by others.[185] We have also shown that *Becn1* is critically important for mammary gland development and terminal differentiation. Interestingly, developmental activation of autophagy increases during late pregnancy and lactation in wild type individuals and is highest during involution, as shown by our lab and others.[433] This confirms several studies that indicate canonical autophagy proteins have high and dynamic expression during pregnancy, lactation, and involution.[440, 447] Our work indicates that canonical BECN1 function and the autophagic machinery is essential for

mammary gland development, despite the energy surplus conditions. This finding is novel and counterintuitive to prevailing opinions. The lactation failure exhibited by WAP-Cre; *Becn1*$^{fl/-}$ dams is completely penetrant in primiparous females and intact JAK/STAT signaling indicates that the glands are receiving extrinsic signaling necessary for lactation and prevention of involution. Thus the phenotype is distinct from the previously published work interrogating this pathway.[363, 376, 377]

SQSTM1 IHC and western blot, as well as ultrastructure analysis by TEM, independently confirm an impairment of autophagy in the mutant glands; however, we can only conclude that autophagy reduction is correlated with, but not necessarily causative, with regard to the phenotype of *Becn1* compromised mammary glands. The increased severity and advanced timing of the *Becn1* cKO phenotype when compared to the *Atg7* cKO phenotype also suggests that *Becn1* is important for cellular activities beyond canonical autophagy, or autophagy in the mammary gland during pregnancy and lactation is ATG7-independent (i.e. non-canonical autophagy). One key difference between the studies is that in our study one allele is a global null and the other is recombined in the mammary gland by WAP-Cre (WAP-Cre; *Becn1*$^{fl/-}$) while Teplova et al. uses a WAP-Cre; *Atg7*$^{fl/fl}$. This difference may be important since WAP-Cre; *Becn1*$^{fl/fl}$ females are able to sustain litters. Since the embryonic lethality of the traditional Becn1 KO mouse is a more severe phenotype than the other autophagy related protein KO models, our study and the results gleaned from the traditional *Becn1* KO together indicate a higher echelon for *Becn1* in the hierarchy of autophagy proteins. The

possibility remains that the observed phenotypes, may be a result of both autophagy-dependent and autophagy-independent functions of BECN1.

Our *in vitro* experiments using BafA1 in lactogenic HC11 cells recapitulate the *in vivo* lipid droplet retention phenotype. Lactogenic, BafA1 treated HC11 cells retained significantly higher numbers of lipid droplets. This result suggests that the lipid droplet retention is an effect of autophagy inhibition and not an autophagy- independent role of BECN1. Although the autophagy complex II (BECN1-UVRAG) is important for autophagosome fusion with the lysosome, other studies have shown that these autophagy proteins also function in endocytosis and exocytosis pathways.[435] TEMs reveal that BafA1 treated cells contain both large, undigested autophagosomes and de-acidified autolysosome remnants. Since direct autophagosome exocytosis has been shown in several model systems, there is a strong likelihood that autophagic proteins (including BECN1) are important for lipid droplet secretion in the lactating mammary gland.[448, 449]

The implication of autophagy proteins and process associated with lipid secretion and lipid metabolism has been strengthened by recent work. It has been recognized for several years that autophagy traffics intracellular lipids to the lysosome for β-oxidation, termed macrolipophagy. In *Atg7* cKO mice, lipids accumulate in large intracellular lipid droplets in the liver; furthermore, the inhibition of autophagy in other tissues has yielded similar results leading to the general conclusion that the most tissues utilize autophagy and/or autophagy proteins to regulate lipid metabolism.[94]

Additionally, acute lipid stimulation can inhibit autophagy, while a chronic increase in lipid stimulates autophagy, suggesting there are multiple regulatory mechanisms involved depending on the type and duration of intracellular lipid content.[450] *Becn1* expression is upregulated in response to an increase in lipid and cholesterol inclusions in models of Neimann-Pick C disease, a lipid storage disorder.[268, 451, 452] Taken together it is apparent that the interplay of lipids and *Becn1* is of paramount importance in development and misregulation is antecedent to numerous clinically-relevant pathologies.

In future studies, our lab will use the tetracycline inducible system to perform BECN1 ablation and rescue experiments to understand the role of BECN1 during involution, a strategy developed and described by Creamer et al.[453] This strategy will give us a broader and clearer picture of the roles for BECN1 throughout mammary gland development.

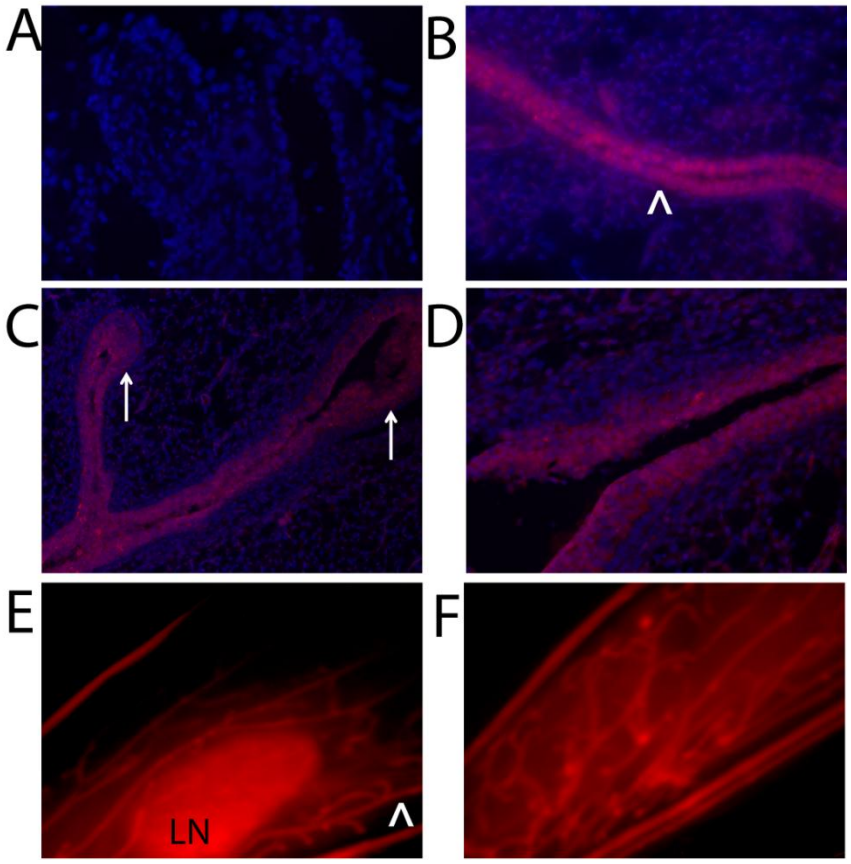


Figure 5.1 MMTV-CreD is a specific and effective driver for mammary specific recombination in nulliparous mice.

(**A**) Control animals labeled with DAPI do not exhibit any red fluorescence of ducts or stroma while reporter females (**B, C**, **D**) exhibit red fluorescence that is highly restricted to mammary ducts and TEBs. Fluorescent whole mounts of inguinal mammary glands from reporter females (**E**, **F**) exhibit red fluorescence in primary and secondary ducts as well as TEBs visible at 5 weeks of age. The lymph node is also targeted by the transgene for recombination (**E**). A, B, D (200X magnification), C (100Xmagnification), arrowhead indicates mammary duct in longitudinal section, arrows indicate TEBs, LN- lymph node.

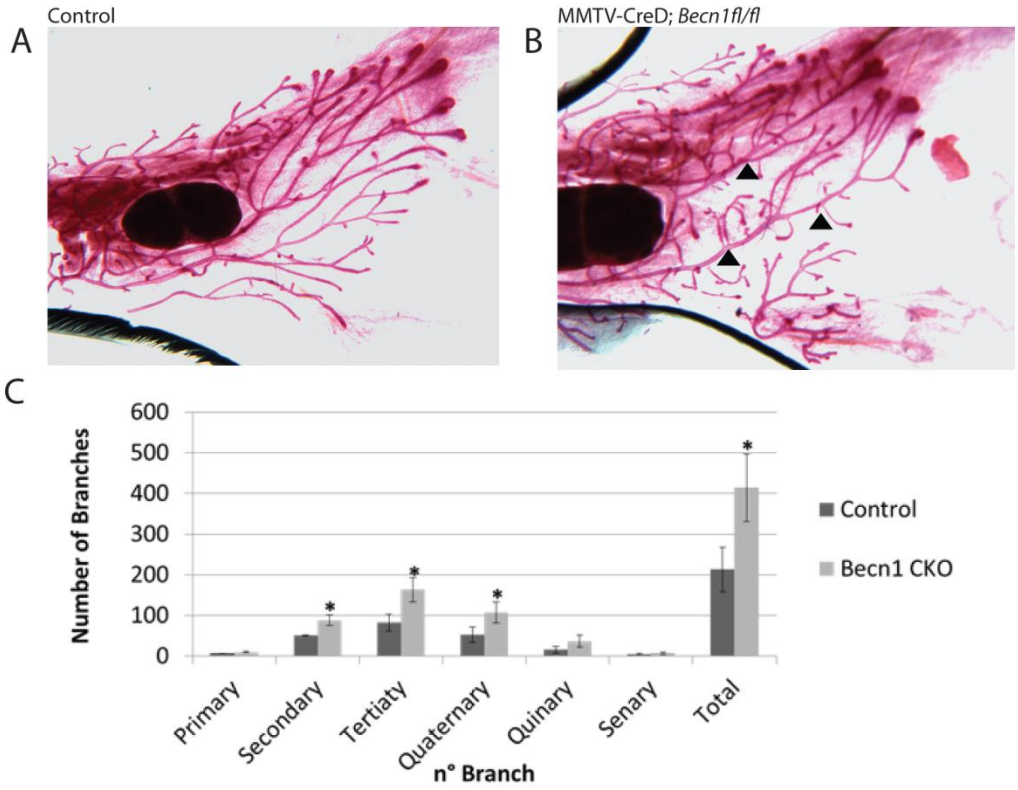
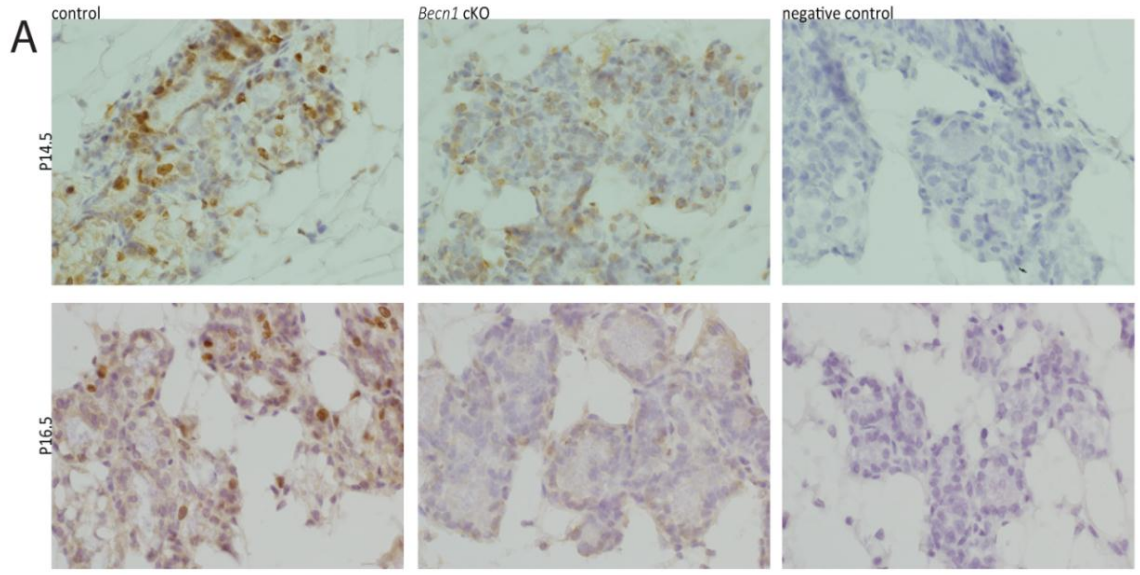


Figure 5.2 MMTV-CreD; *Becn1*$^{fl/fl}$ females exhibit hyper-branching phenotype.

Whole mount analysis of inguinal mammary glands harvested from 5 week old females revealed signs of hyper-branching in the MMTV-CreD;*Becn1*$^{fl/fl}$ glands when compared to control (**A**), as indicated by arrowheads (**B**). Quantitative analysis of branching in 10 week old females (**C**) confirmed these observations indicating a significant increase in secondary, tertiary, quaternary, and total number of branches, n=5 control and n=9 mutants (*P < 0.05, Student t-test).


A
control
Becn1 cKO
negative control
P14.5
P16.5

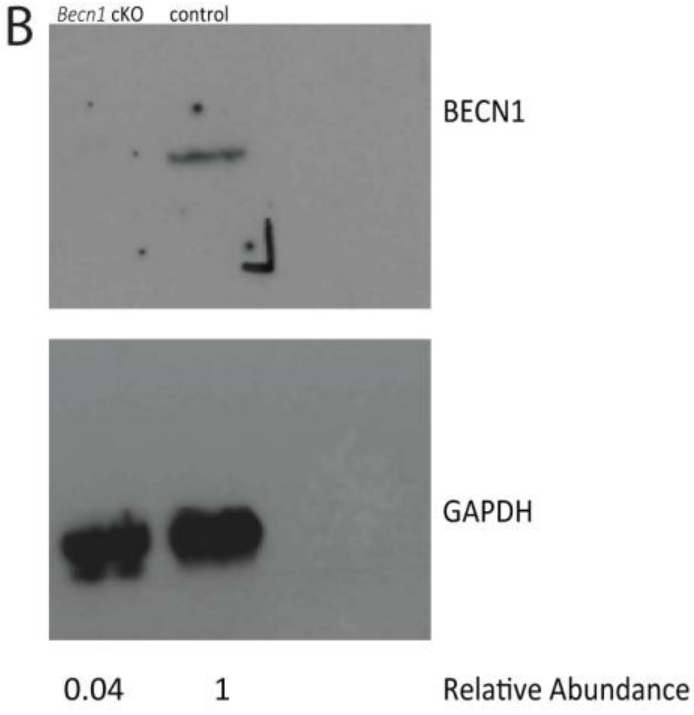

B
Becn1 cKO
control
BECN1
GAPDH
0.04
1
Relative Abundance

Figure 5.3 WAP-Cre; *Becn1*$^{fl/-}$ mammary glands have reduced BECN1 expression in the mammary gland from mid-gestation.

IHC of BECN1 on P14.5 and P16.5 mammary glands (**A**). Glands harvested on the day of parturition exhibit a marked loss in total BECN1 protein abundance when compared to control animals by western blot (**B**).

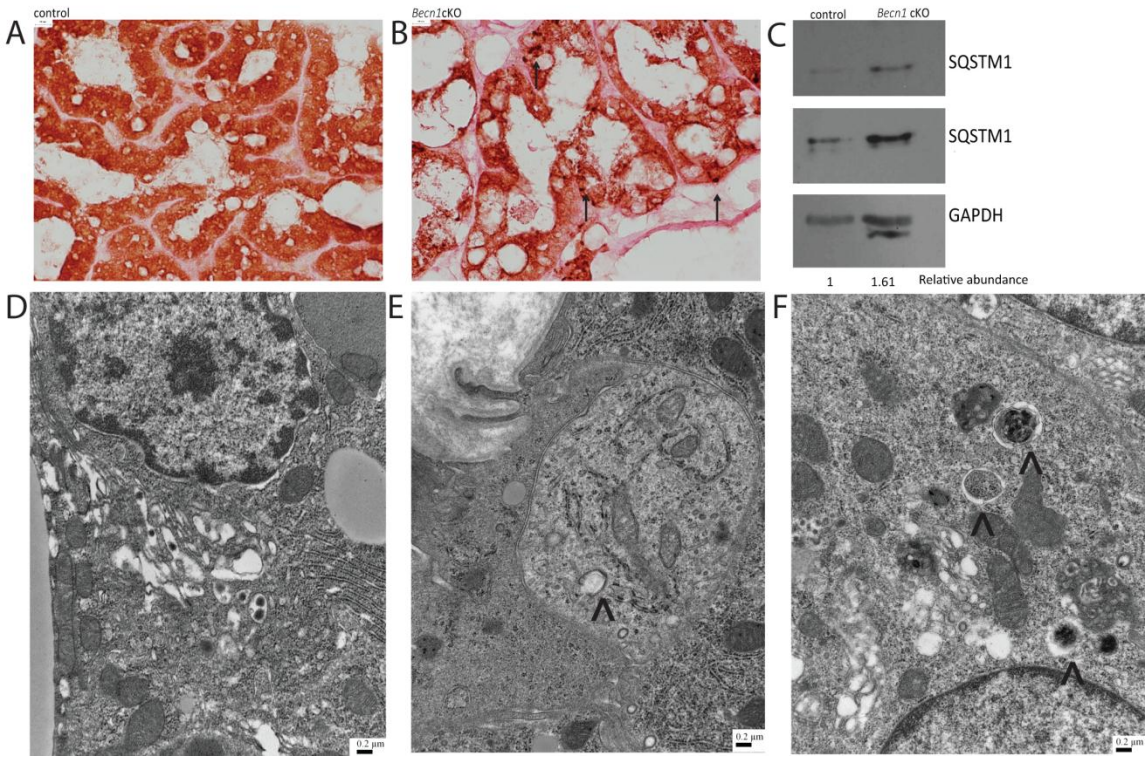


Figure 5.4 WAP-Cre; *Becn1*$^{fl/-}$ mammary glands have impaired autophagy clearance of SQSTM1.

SQSTM1 IHC of control (**A**) and *Becn1* cKO (**B**) inguinal mammary glands on the day of parturition imaged at 400X magnification. Scale bars are 10μm. SQSTM1 protein is increased in *Becn1* cKO mammary glands when compared to controls and normalized to GAPDH (**C**). TEM of control (**D**) and mutant glands (**E, F**) 48 hours after parturition, scale bars as indicated. Arrows indicate SQSTM1 positive aggregates, open arrowheads indicate autophagosomes

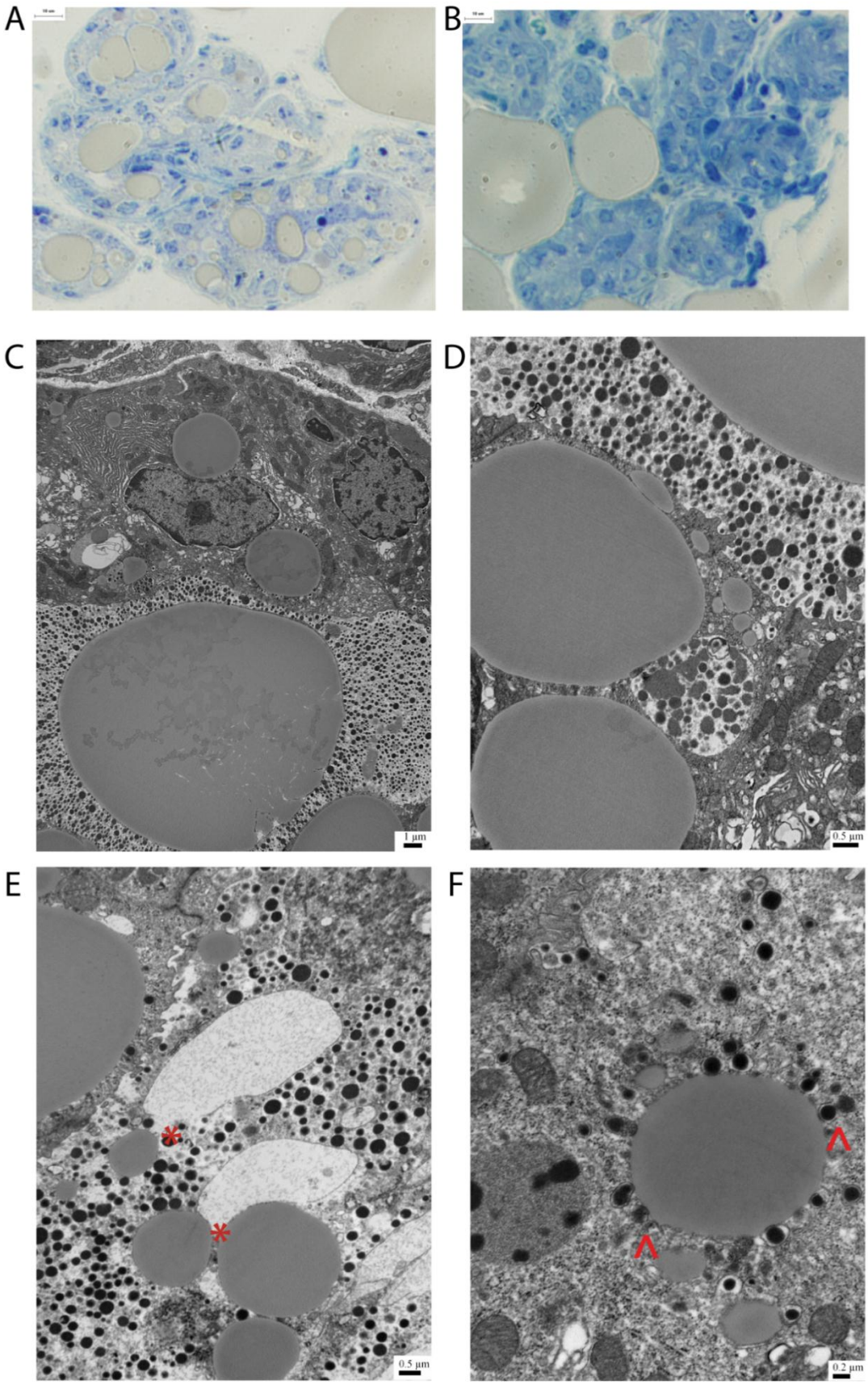

A
B
C
1 μm
D
0.5 μm
E
0.5 μm
F
0.2 μm

Figure 5.5 WAP-Cre; *Becn1*$^{fl/-}$ mammary glands show defects in gland organization and abnormal milk fat globule processing.

Semi-thin sections of resin-embedded control (**A**) and WAP-Cre; *Becn1*$^{fl/-}$ glands (**B**) 48 hours post parturition. Note the lack of milk fat globules in the lumens of (**B**). TEM of control (**C, D**) and mutant (**E, F**) glands, scale as indicated. (**E**) Lipid enveloping and secretion defects are noted with asterisks and arrowheads highlight lysosome abnormalities (**F**).

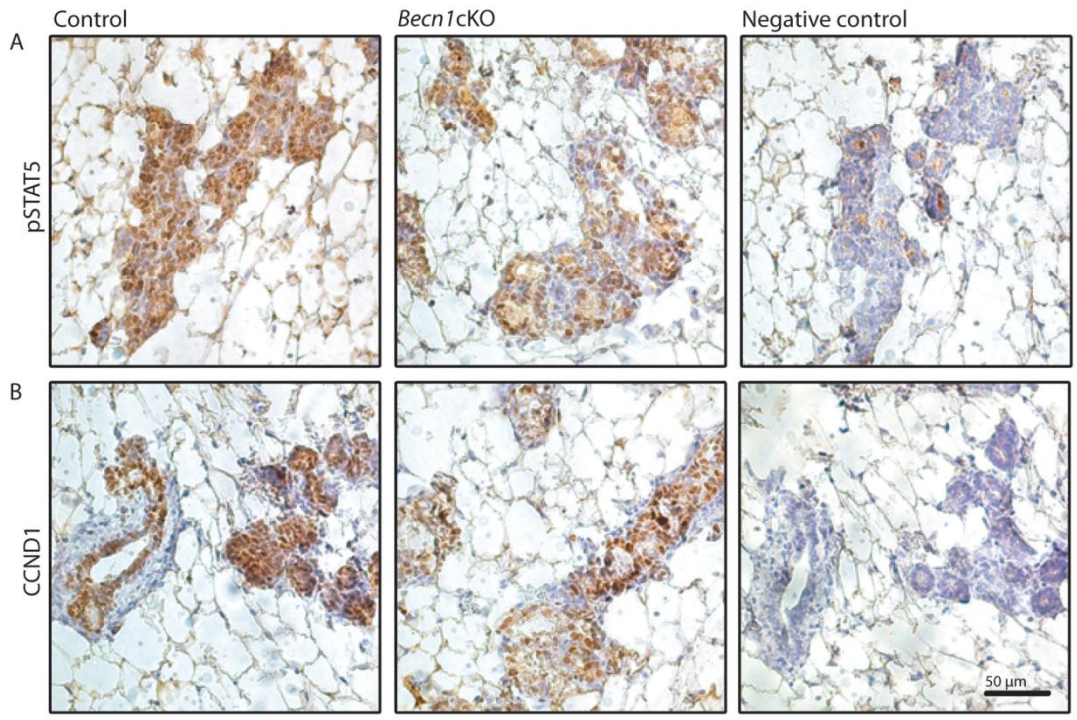


Figure 5.6 WAP-Cre; *Becn1*$^{fl/-}$ mammary glands receive typical extrinsically mediated signaling and CCND1.

Phospho-STAT5 expression, as measured by IHC, is similar in both control and mutant glands (**A**). CCND1 expression is indistinguishable between control and mutant glands (**B**). All glands are inguinal mammary glands harvested on the day of parturition.

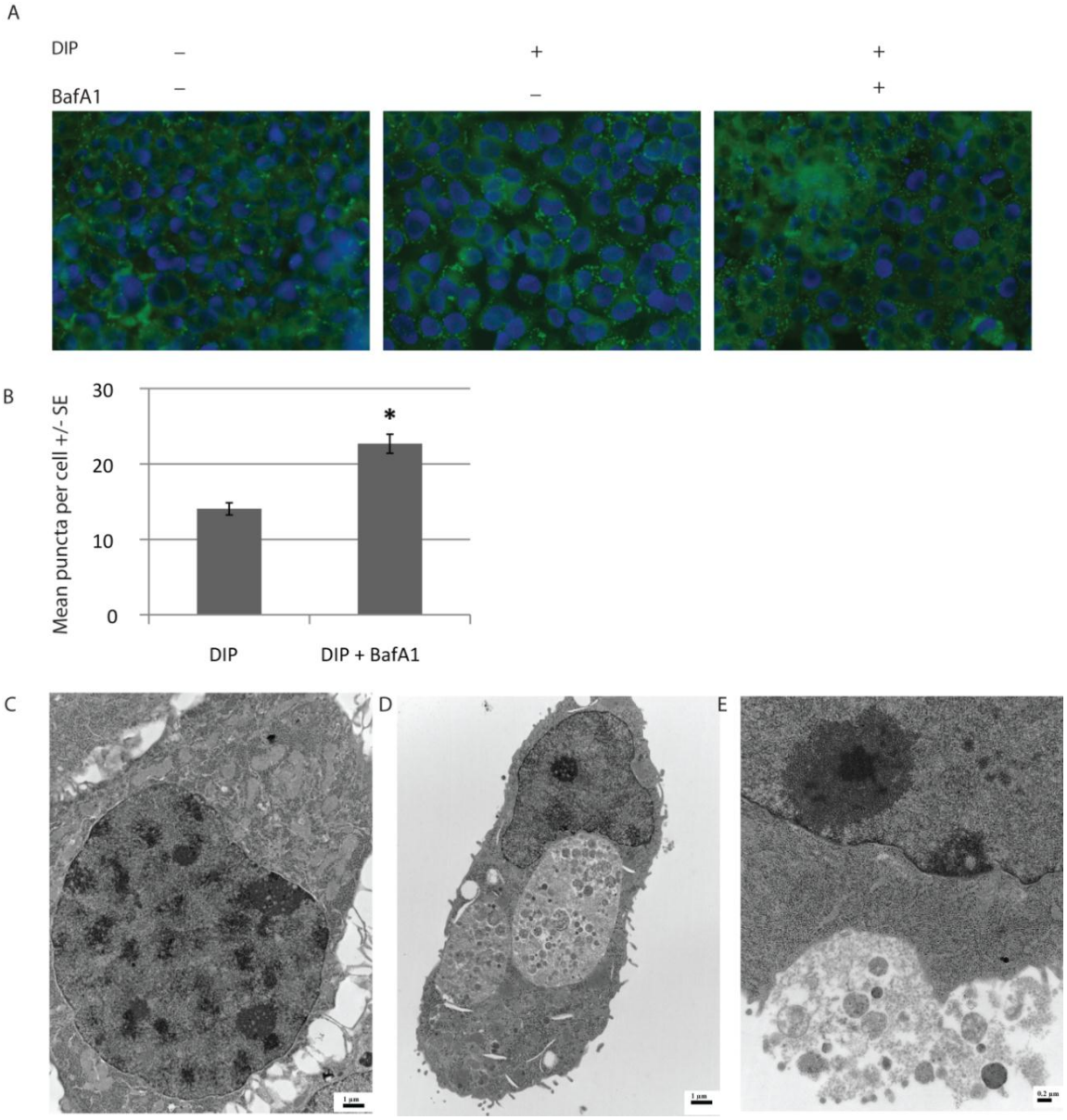

A
DIP
–
+
+
BafA1
–
–
+
B
Mean puncta per cell +/- SE
30
20
10
0
*
DIP
DIP + BafA1
C
D
E

Figure 5.7 Lactogenic HC11 cells recapitulate the lipid accumulation observed in primiparous *Becn1* cKO mammary glands.

HC11 cells were kept in growth media, differentiated (DIP), or differentiated and treated with BafA1 (DIP + BafA1) then all groups were stained with BODIPY 493/503, counterstained with DAPI and imaged (**A**). Mean lipid droplets per cell at 24 hours post treatment time point were calculated for each group (**B**) (*$P < 0.05$, Student t-test). TEM of HC11 cells from DIP (**C**), and DIP + BafA1 treated group (**D**) highlight the accumulation of autolysosomes in the DIP + BafA1 group as well as direct exocytosis of abnormal autophagosomes with undigested cargo (**E**). Scales as indicated.

Chapter Six: TSC1 is a Novel Interacting Partner of Beclin1 and Essential for Terminal Differentiation of the Mammary Gland

Introduction

Tuberous sclerosis 1 (*Tsc1*) and *Tsc2* produce the gene products TSC1/2, also known as hamartin and tuberin respectively. The TSC1/2 heterodimer is a well characterized inhibitor of MTOR signaling. Typically TSC2, when bound to TSC1 for stability, uses its GTPase activating protein domain (GAP) to inhibit Rheb, a GTPase, which inhibits mechanistic target of rapamycin 1 (MTORC1) signaling. *Tsc1* and *Tsc2* are the causal genes of the disorder tuberous sclerosis complex (TSC), a diverse multi-symptomatic autosomal dominant disease characterized by hamartoma formation that affects ~1 in 6,000 births to varying degrees.[454] The *Tsc1* knockout (KO) mouse model dies embryonically between E9 and E13.5 of liver hypoplasia and anemia.[176] Notably the phenotype of the *Tsc1* KO was milder than the *Tsc2* KO where exencephaly was reported in addition to other developmental defects.[243] Embryonic lethality prompted generation of the *Tsc1* floxed model which has been used to ablate TSC1 in a myriad of cell types, tissues, and organs.[455] For a table of published tissue-specific deletions see **Table 6.1**.

Autophagy, an intracellular catabolic process enlisted to envelope and degrade damaged organelles and long-lived proteins, is essential for organismal vitality, growth, and development. Productive autophagy is characterized by four steps: initiation, elongation of a double membrane structure termed the autophagosome around cargo, fusion of autophagosome with a lysosome, and degradation of cargo by lysosomal

enzymes for recycling of macromolecules for energetic homeostasis. Canonically, MTORC inhibits autophagy at the level of the autophagy pre-initiation complex, which is a regulatory complex upstream of the initiation step, and impaired autophagy has been reported in several systems that compromise TSC1 function or expression.[63, 456-459] Beclin1 (BECN1) is an essential, pro-autophagic protein that serves as a scaffolding protein during the initiation stage of autophagy. Interestingly, human BECN1 was identified as a TSC1 binding partner in 293T cells by a high-throughput affinity capture experiment.[460] This finding creates a link between two tumor suppressor genes, as well as suggests the potential existence of an uncharacterized regulatory loop with significant clinical relevance.

Tsc1 and *Tsc2* have been studied extensively in the context of inherited tumorigenic disease, and have been characterized as tumor suppressor genes. Fewer studies have examined *Tsc1* in the mammary gland or in breast cancer. A single nucleotide polymorphism (SNP) analysis of *Tsc1* and *Tsc2* identified a single variant that associated with a significantly later age at diagnosis of estrogen receptor (EsR) positive ductal carcinoma.[461] Additionally, TSC1 and TSC2 expression is reduced in invasive breast cancer and the TSC1 promoter is heavily methylated in several breast cancer cell lines and in most breast tumors while is only rarely methylated in normal tissue. This report also concluded that low expression of TSC1 is correlated with unfavorable clinical outcomes.[462] Inducible *Tsc1* deletion in primary mammary tumor cells results in hyper-activation of MTORC, accelerated breast cancer progression, and changes in autophagy levels.[463] *Becn1* is a well-characterized tumor suppressor. *Becn1* hemizygous mice are

prone to spontaneous tumors, and, in humans, *Becn1* is mutated or silenced in a majority of breast cancers investigated. [184, 185, 436]

In this study we report the interaction of mouse TSC1 and BECN1 *in vitro* and examine the *in vivo* consequences of ablating TSC1 expression in the mouse mammary gland. We hypothesize that TSC1 and BECN1 interact to complete a regulatory feedback loop and this interaction affects autophagy function and the MTORC signaling axis, thus cell growth and differentiation.

Results

TSC1 and BECN1 interact *in vitro*

Since the initial report that TSC1 and BECN1 are interacting partners used a human BECN1 expressed at supraphysiological levels it was important to determine if TSC1 and BECN1 interacted when both are expressed at endogenous levels. We used protein-protein interaction assay based rolling circle amplification. Briefly, cells were cultured as recommended then fixed and blocked. Primary antibodies raised in different species were applied. Secondary antibodies conjugated with oligo probes were applied and incubated. A ligation reaction and polymerase based amplification make physical interactions visible as a fluorescent puncta. Analysis is conducted using fluorescent microscopy (**Figure 6.1A**). We found that a significant 59.3% of NIH3T3 cells were positive for the TSC1 and BECN1 interaction (**Figure 6.1B, C**). On average, across all cells assayed, 1.14 TSC1-BECN1 associations per cell were counted (**Figure 6.1D**). Since we know that levels of TSC1 can affect autophagy dynamically in response to changes in

nutrient status we wanted to investigate the nature of the TSC1-BECN1 interaction under serum-starvation conditions. We subjected established cells to a prolonged serum starvation protocol (**Figure 6.1E**). Following an eight hour serum starvation the percentage of positive cells was reduced significantly from 29.6 % with complete media to 17.7% after serum-starvation (**Figure 6.1F**), suggesting that the TSC1-BECN1 interaction is also nutrient responsive.

TSC1 is important for mammary gland development

The mammary gland is a highly dynamic organ that undergoes the majority of its development in the adult animal. During pregnancy, estrogen and other circulating hormones stimulate the cell proliferation, growth, and branching of an arboreal ductal structure. Nearing the time of parturition secretory epithelia, positioned at the terminal points of branches, prepare for secretory activation and lactation. During lactation the epithelia must produce copious quantities of protein and lipid and secrete these into the ductal lumens for neonate support. After weaning the gland undergoes a bipartite involution stage where programmed cell death and efferocytosis remodel the gland into a resting-like state (**Figure 6.2A**).

Relatively few studies have been conducted investigating the expression of *Tsc1* in the normal developing mammary gland. Studies in bovine have revealed that *Tsc1* expression is dynamic across pregnancy and lactation, notably *Tsc1* expression increases significantly during mid-lactation (120 days post parturition in bovine), relative to late-pregnancy levels.[464] We assayed the expression of TSC1 in mouse mammary glands by

western blot across five stages of development; virgin, pregnancy (P) 14.5, P18.5, L1, Involution (I)2. We cannot directly compare our data with the bovine expression data but we corroborate the dynamic nature of TSC1 at the protein level across developmental time points. In mice, TSC1 expression is very low at the virgin stage so we compared protein expression relative to P14.5 (set to 1). TSC1 protein abundance increases 8.4-fold at P18.5, 6.6-fold at L1, and 4.7-fold at I2 **(Figure 6.2B, C**). Generally, expression is low in the resting gland, increases to a peak during late pregnancy, around the time of secretory activation, and begins to taper off as lactation and involution ensue.

TSC1 is necessary for secretory activation of the mammary gland and lactation

Since both TSC1 and BECN1 have been implicated in breast cancer and we know from previous studies that BECN1 is essential for mammary gland terminal differentiation we wanted to examine the consequences of deleting TSC1 in the mammary gland during pregnancy. We used a Cre transgenic in which the Whey Acidic Protein (WAP) promoter is used to drive Cre expression. The WAP-Cre transgenic line in combination with the *Tsc1* floxed model that carries one germline recombined allele ($Tsc1^{fl/-}$) resulted in an animal globally hemizygous for *Tsc1* and recombination of the remaining floxed *Tsc1* allele in the mammary gland from mid -pregnancy (P12.5) forward.[441, 442]

On the day of parturition the *Tsc1* cKO females were unable to sustain litters. Pups did not have milk spots, indicating failure of nutrient transfer, but were

successfully fostered on control dams. IHC against TSC1 revealed a reduction in expression levels on the day of parturition in *Tsc1* cKO glands when compared to control levels detected (**Figure 6.3**).

The *Tsc1* cKO dams have a lactation defect. Whole mount analysis of inguinal glands harvested from *Tsc1* cKO animals on the day of parturition revealed abnormal swollen and engorged alveoli (**Figure 6.4A- D**). Histologically, retained milk fat globules in secretory epithelial cells and abnormal gland architecture could be observed in *Tsc1* cKO glands. Several lysed secretory cells can be identified. Overall reduction in secretory epithelia is observed. These histological abnormalities indicate a failure of secretory activation (**Figure 6.5A-D**).

TSC1 cKO glands have altered MTORC signaling and reduced autophagy

As discussed above, TSC1 is a known MTOR inhibitor and we investigated the implication of TSC1 ablation on the MTOR signaling pathway in the mammary gland. We found that total MTOR is increased 3.6-fold in *Tsc1* cKO glands as compared to controls and normalized to GAPDH, as expected (**Figure 6.6A**). Downstream MTOR effector p4EBP1 expression was increased 1.7-fold in *Tsc1* cKO glands as compared to controls and normalized to GAPDH (**Figure 6.6B**). Since autophagy is inhibited by MTOR we decided to examine autophagy by looking at Sequestosome 1 (SQSTM1/p62) expression. SQSTM1 is selectively metabolized by the autophagic pathway and, when autophagy is inhibited or impaired SQSTM1, intracellular levels increase. *Tsc1* cKO glands have a 2-fold increase in SQSTM1 over control glands and normalized to GAPDH, indicating a

reduction in autophagy (**Figure 6.6C**). We did not detect any significant difference in cell proliferation rates as measured by phospho-histone H3 (pH3) staining (data not shown).

TSC1 and BECN1 expression in breast cancer

To examine the changes that occur during tumorigenesis we used a tumor tissue microarray to examine TSC1 expression in a variety of breast cancers. Each tissue core was scored 0-4, zero being no expression and 4 being the highest level of expression. In normal human breast tissue TSC1 is evenly distributed in the epithelia (**Figure 6.7A**). We found that 100% of scored breast cancer samples expressed TSC1 to some degree (n=34) and 41.2% had low TSC1 expression (**Figure 6.7C, D**). The remaining cores, 58.8%, had high expression (rated 3 or 4) (**Figure 6.7B**). Typically epithelia stained more dramatically for TSC1 than stromal tissue which stained to varying degrees. TSC1 protein was localized to the cytoplasm in a diffuse pattern and spread throughout positively staining cells, even in the case of polarized secretory cells (**Figure 6.7E, F**). Amongst a group of breast ductal adenocarcinomas the level of TSC1 staining was similar to the level BECN1 staining in 81.3% of samples assayed (n=32), though in some cases the distribution was different. In this ductal adenocarcinoma pictured BECN1 staining is punctate and basally located in polarized cells while TSC1 is diffuse in the cytoplasm (**Figure 6.7G, H**). BECN1 staining is also more prevalent in the non-luminal cells.

Discussion

Using both *in vitro* and *in vivo* approaches we were able to investigate a novel protein-protein interaction and its implications in the development of an organ. We

report the novel interaction of murine TSC1 and BECN1 *in vitro* at endogenous expression levels. Furthermore, we show that the interaction is dependent upon nutrient condition. After serum-starvation the observed TSC1-BECN1 interactions were reduced, indicating that the interaction is nutrient-responsive. *In vivo,* mammary glands of *Tsc1* cKO mice exhibit failed lactation, impaired secretory activation characterized by abnormal morphology at the organ level and the histological level. Engorged and expended alveoli are easily identified in *Tsc1* cKO glands indicating retained lipids. At the histologic level, lipid droplets can be seen centrally located in the secretory epithelia cells and many cells have an uncharacteristic cuboidal appearance. As expected, MTOR expression was increased in the *Tsc1* cKO mammary glands and autophagy was compromised.

This work begins to unravel the complex and likely ephemeral interaction between two tumor suppressor proteins; TSC1 and BECN1. Both traditional KO models are embryonic lethal very early in development and both have been studied intensively as tumor suppressors. We report here that ablation of *Tsc1* in the developing mammary gland results in a lactation defect similar to *Becn1* cKO mammary glands. It is interesting to note that others have reported that in *Tsc1* cKO cells and *Tsc1* cKO mammary tumor cells amino acid-starvation induced autophagy is decreased, as expected since MTORC is hyperactive, but in TSC1 cKO mammary tumor cells glucose-starvation induced autophagy is surprisingly increased.[463] This differential response is important since autophagy has long been characterized as a “double edged sword” in cancer. Autophagic response is typically cytoprotective, allowing cells to survive stress and

protect cells from misfolded proteins or cytotoxic protein aggregates; however, after a tumor is established activation of autophagy can be permissive for tumor progression.[465] Another tumor suppressor, RB1-inducible coiled-coil 1 (RB1CC1), has been reported to interact with TSC1 and is a known member of the autophagy pre-initiation complex.[466] The ULK1/2-ATG13- ATG101-RB1CC1 autophagy pre-initiation complex is directly downstream of MTOR, subject to its regulation, and upstream of the BECN1 anchored autophagy initiation complex.[61] The interaction between RB1CC1 and TSC1 has been shown to regulate cell size, but not proliferation.[466, 467] The regulation of these players, involved in nutrient responsive pathways as well as tumorigenesis, is more complex than has previously been appreciated. Thus, what we are proposing is a nutrient-sensitive, regulatory feedback loop that encompasses TSC1/2 complex inhibition of MTOR and regulation of autophagy and has consequence for normal development and possibly tumor initiation. This work lays the foundation for a specific and directed interrogation of the role of TSC1 in mammary tumor establishment and progression.

Table 6.1 Tsc1ckO produced and corresponding phenotypes

Central Nervous System			
Genotype	**Tissue**	**Phenotype**	**Reference**
Emx1-Cre; *Tsc1$^{fl/fl}$*	Embryonic neural progenitors	Defective cortical lamination; enlarged abnormal astrocytes; decreased myelination	468
Nestin-CreEsR^{T2}; Tsc1$^{fl/fl}$ (tamoxifen induced)	Neuronal precursors and astrocytes	Olfactory nodules; abnormal neuronal infiltration	469
Gbx2-CreEsR; Tsc1$^{fl/fl}$	Thalamus	Enlarged thalamic neurons, disorganized circuitry; seizures; compulsive grooming behavior	470
Pomc-Cre; Tsc1$^{fl/fl}$	Pomc neurons of hypothalamus	dysregulation of Pomc neurons and hyperphagic obesity	471
CaMKIIα-Cre; Tsc1$^{fl/fl}$	Neurons within the cortex and hippocampus	Seizure activity; increased mortality; impaired autophagy;	211
SynI-Cre; Tsc1$^{fl/fl}$	Post mitotic neurons	Seizures; abnormal/enlarged/ectopic neurons; myelination delay	472
Synapsin1-Cre; Tsc1$^{fl/fl}$	Neurons	Increased excitability and a pro-epileptogenic circuit	473
GFAP-Cre; *Tsc1$^{fl/fl}$*	Glia	Seizures; increased astrocyte number; disorganized hippocampal neurons	455
GFAP-Cre; Tsc1$^{fl/fl}$	Glia	Impaired astrocytic gap junction coupling and potassium buffering	474
GFAP-Cre; Tsc1$^{fl/fl}$	Glia	Elevated glutamate levels in hippocampi; increased neuronal death in hippocampus and neocortex; impaired learning and memory	475
Cag-CreER^{T1}; Tsc1$^{fl/fl}$ or Cag-CreERT1; Tsc1$^{fl/-}$ (tamoxifen induced)	Global	Seizures; epileptogenesis	476

Table 6.1 Continued

Peripheral Organs			
Genotype	**Tissue**	**Phenotype**	**Reference**
Fabp4-Cre;Tsc1$^{fl/fl}$	Lung(pulmonary epithelial cells), heart, skin and kidney, and in liver and brain at a lower level	Smaller; lung hemorrhages; reduced levels of surfactant proteins A and B	477
SPC-Cre-EsR^{T2}; Tsc1$^{fl/fl}$ (tamoxifen induced)	Lung alveolar epithelial cells	No data, only recombination efficiency reported	478
SM22-Cre; Tsc1$^{fl/fl}$	Cardiovascular tissue	Cardiac hypertrophy; reduced left ventricular end-diastolic diameter	479
Alb-Cre; Tsc1$^{fl/fl}$	Liver	Hepatocellular carcinomas	480
Alb-Cre;Tsc1$^{fl/fl}$	Liver	Protection from hepatic steatosis; defect in SREBP1c activation	481
Rip2-Cre; Tsc1$^{fl/fl}$	β-cells of pancreas, hypothalamus	Improved glycemic control; obesity (off target effect)	482
Rip2-Cre; Tsc1$^{fl/fl}$	β-cells of pancreas, hypothalamus	hyperphagia and obesity; enlarged neuron cell size in a number of hypothalamic populations	471
Emx1-Cre*; Tsc1$^{fl/fl}$*	Distal convoluted tubule	Cystogenesis; elongated primary cilia	483
Nse-Cre; Tsc1$^{fl/fl}$	Subset of renal tubular cells	polycystic kidney disease	457
Tie2-Cre;Tsc1$^{fl/fl}$	Vasculature	Embryonic lethality; cardiovascular defect; disorganization and failed sprouting of vasculature; defective mitochondrial and endoplasmic reticular morphology	484
K14-Cre; Tsc1$^{fl/fl}$	Epithelia	Accelerated wound healing	485

Table 6.1 Continued

Reproductive System			
Genotype	**Tissue**	**Phenotype**	**Reference**
PR-Cre; Tsc1$^{fl/fl}$	Uterus: epithelium, stroma and myometrium	Infertility; oviductal hyperplasia; retention of embryos in the oviduct; implantation failure; epithelial hyperplasia; embryo development was disrupted	486
Amhr2-Cre; Tsc1$^{fl/fl}$	Uterus: stroma and myometrium	As above with exception: embryo development was unaffected	
Amhr2-Cre; *Tsc1$^{fl/fl}$*	Granulosa cells of ovary; stroma of oviduct and uterus	Poor oocyte quality, compromised implantation, infertility	487
Cyp19-Cre; Tsc1$^{fl/fl}$	Granulosa cells of ovary	Hyperfertility; accumulation of corpus lutea	488
PB-Cre4; Tsc1$^{fl/fl}$	Prostate epithelium	Neoplasia of prostatic epithelia progressing to more advanced lesions with increased age	489
Hematopoietic System			
Genotype	**Tissue**	**Phenotype**	**Reference**
Lck-Cre; Tsc1$^{fl/fl}$ *CD4-Cre; Tsc1$^{fl/fl}$*	T-Cells	Loss of quiescence; reduced number of T-cells	490
CD19-Cre; Tsc1$^{fl/fl}$	B-cells	partial block in B-cell maturation; reduction in the marginal zone population; defective immune response	491
MX-1-Cre; Tsc1$^{fl/fl}$ (polyinosine-polycytidine induced)	Hematopoietic stem cells	Increased mitochondrial biogenesis; increased ROS; reduced hematopoiesis; reduced self-renewal	492
MX-1-Cre; Tsc1$^{fl/fl}$ (polyinosine-polycytidine induced)	Hematopoietic stem cells	Premature aging of HSCs	493
Rosa26-CreEsR^{T2}; Tsc1$^{fl/fl}$ (tamoxifen induced)	Global (hematopoietic organs)	Polycystic kidney disease; failure to thrive; reduced HSCs; loss of quiescence; reduced self-renewal	494

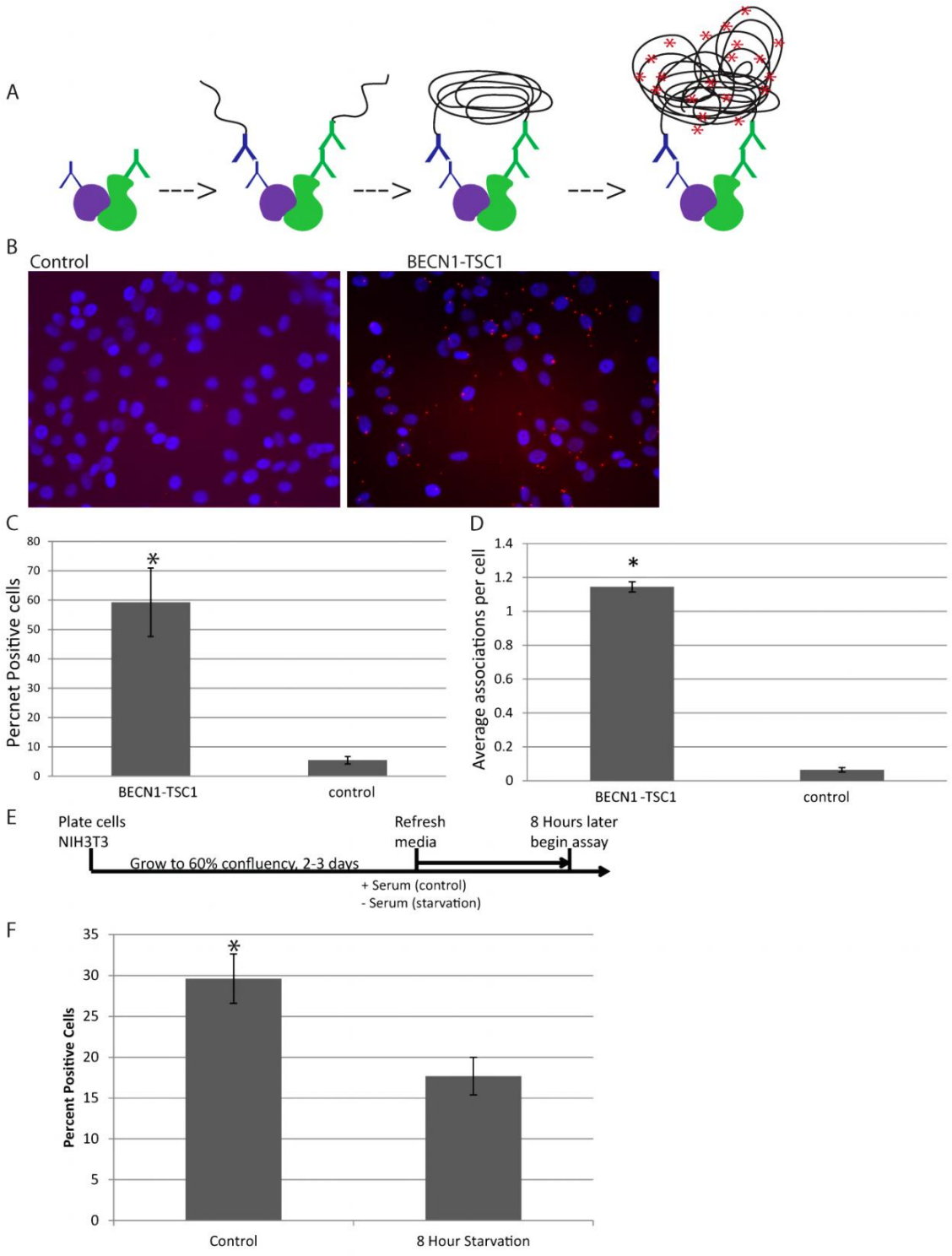

A
B
Control
BECN1-TSC1
C
Percnet Positive cells
80
70
60
50
40
30
20
10
0
*
BECN1-TSC1
control
D
Average associations per cell
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
BECN1 -TSC1
control
E
Plate cells
NIH3T3
Grow to 60% confluency, 2-3 days
Refresh
media
8 Hours later
begin assay
+ Serum (control)
- Serum (starvation)
F
Percent Positive Cells
35
30
25
20
15
10
5
0
*
Control
8 Hour Starvation

Figure 6.1 BECN1 and TSC1 interact *in vitro*.

BECN1 and TSC1 are identified as interacting partners. (**A**) Schematic representation of protein-protein interaction assay depicts two primary and secondary antibodies binding to targets, ligation of conjugated oligos, and rolling circle amplification for visualization. (**B**) The control has very few interaction puncta while the BECN1-TSC1 panel has many puncta, cells are counterstained with DAPI. Overall 59.3% of cell are positive for the interaction (**C**), and on average, cells have 1.14 interactions each (**D**). When cell are subjected to a prolonged serum-starvation protocol (**E**), the number of interaction-positive cells decreases significantly (**F**). *= statistically significant and refers to a p-value<.05

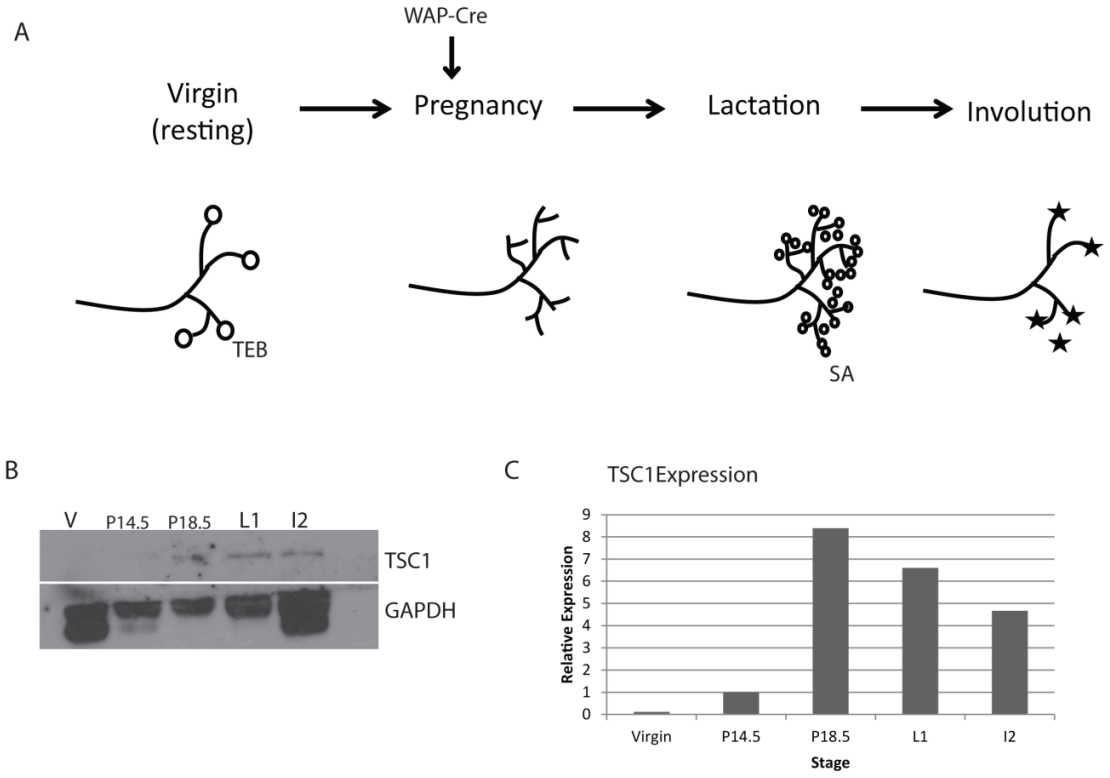


Figure 6.2 TSC1 is expressed during normal pregnancy, lactation, and involution.

Schematic representation of a pregnancy cycle is depicted (**A**). The mammary gland progresses through four phases: mature virgin (resting) which is quiescent, pregnancy, lactation, and involution. The depictions are stylized ductal structures and representative growth of the secretory epithelia as pregnancy and lactation progress. TSC1 is expressed at very low levels in the mature virgin gland and increases to a peak in late pregnancy. Expression remains high during lactation and during the early involution phases (**B, C**).

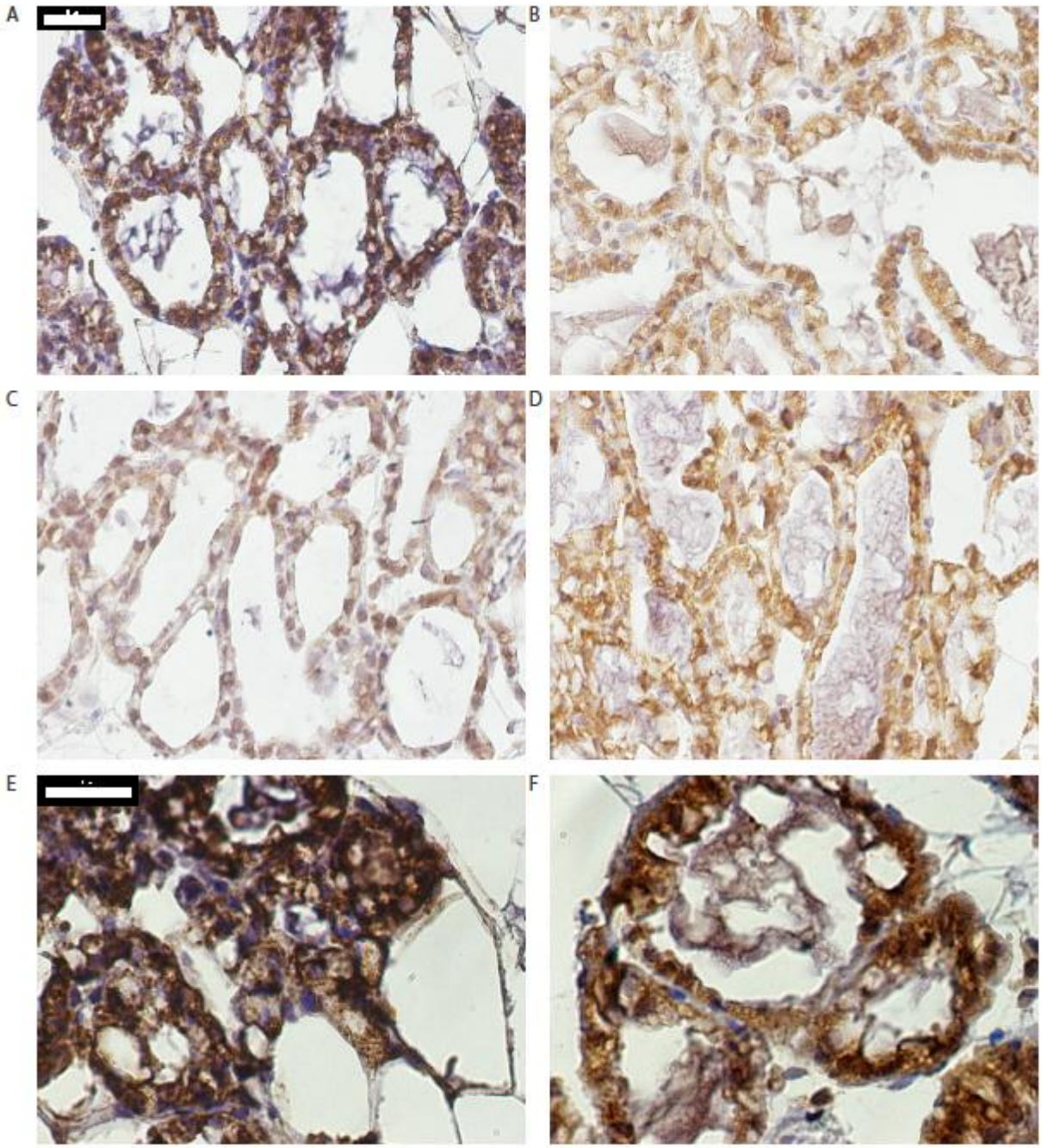

A
B
C
D
E
F

Figure 6.3 TSC1 protein is reduced in *Tsc1* cKO glands at L1.

Glands harvested from L1 controls have strong TSC1 staining in the secretory epithelia (**A**, **E**) as detected by IHC. In *Tsc1* cKO glands (**B**, **C**, **D**, **F**) overall staining in the secretory epithelia is reduced. A-D 400X magnification; E, F 600X magnification; bar=25µm

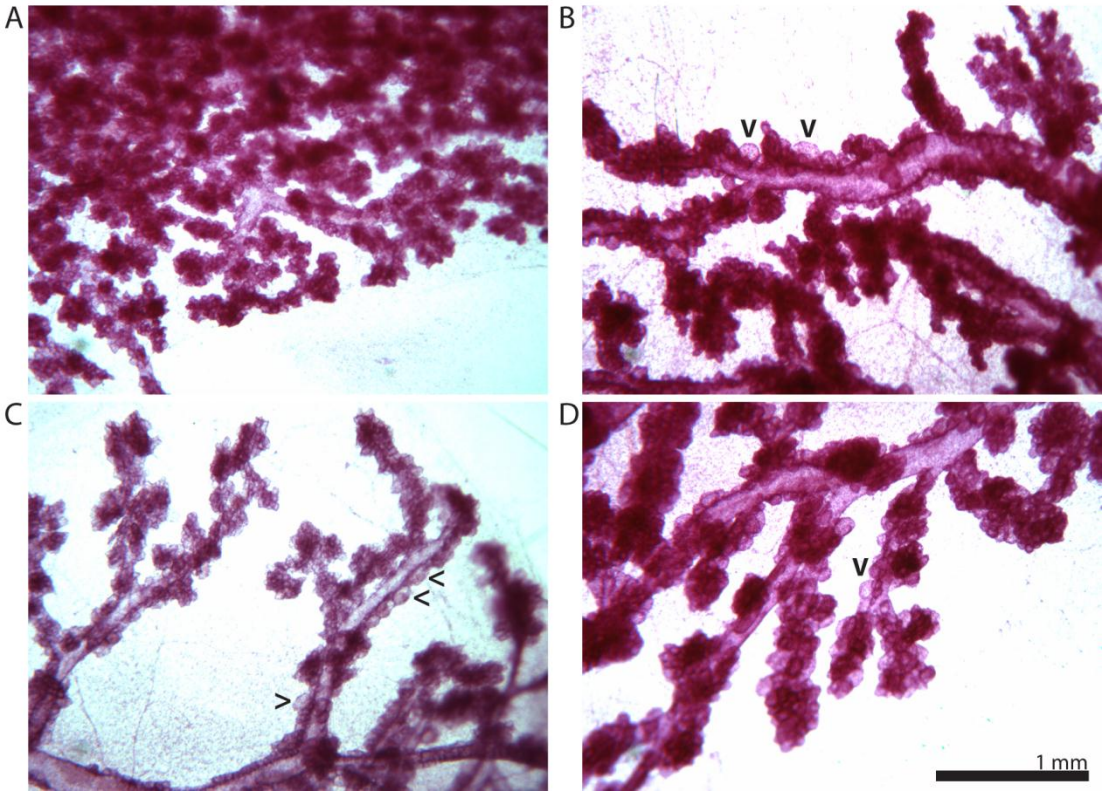


Figure 6.4 *Tsc1* cKO whole mount mammary glands are less dense and have abnormal secretory alveoli.

Mammary glands collected on the day of parturition typically appear dense and differentiated as the control gland (**A**). Mutant glands (**B-D**) appear less dense and present with less secretory alveoli. In many cases secretory alveoli appear engorged and abnormal alveoli can be seen budding off of branches (open arrowheads). Bar= 1mm

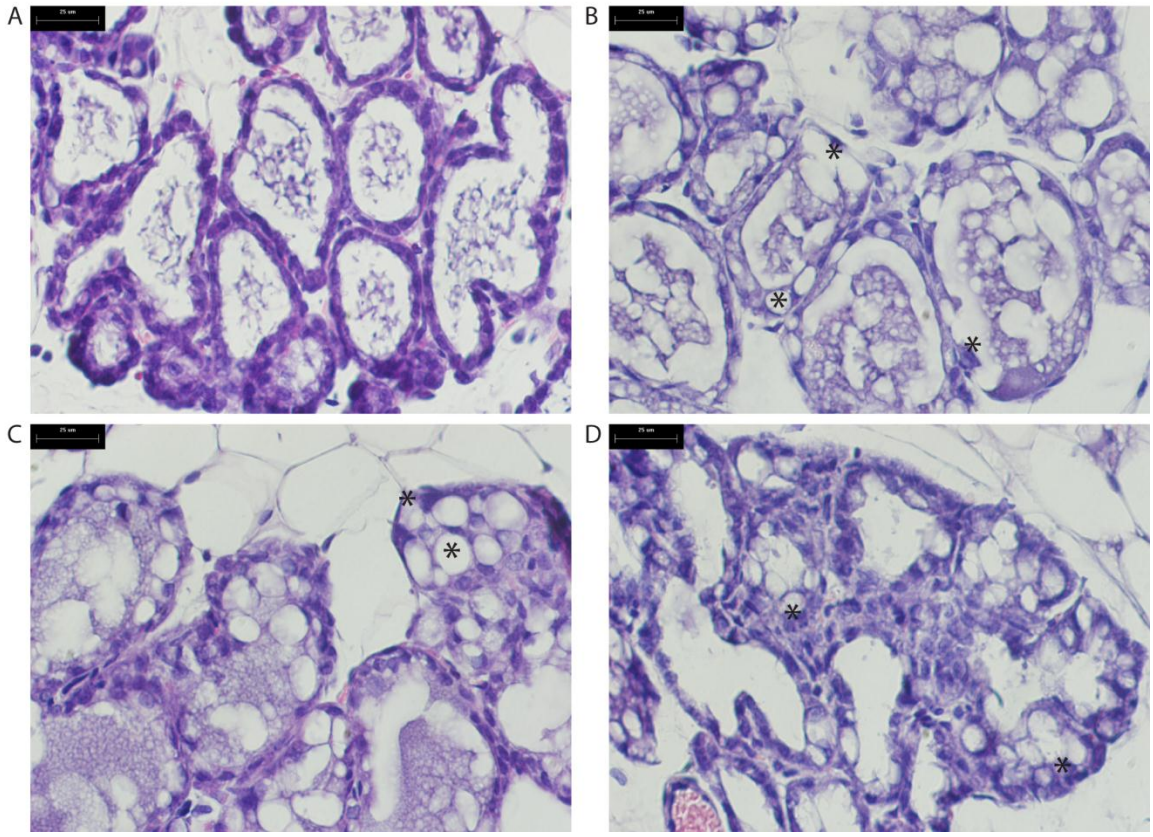


Figure 6.5 Histologic analyses of *Tsc1* cKO mammary glands.

Control mammary glands collected on the day of parturition have undergone secretory activation and epithelia appear squamous, lipid droplets are secreted into large lumena (**A**). Mutant glands (**B-D**) contain secretory epithelia that appear cuboidal and many lipid droplets can be observed retained in the cytoplasm (asterisks). In many cases secretory epithelia has been lysed (asterisks).Bar=25nm

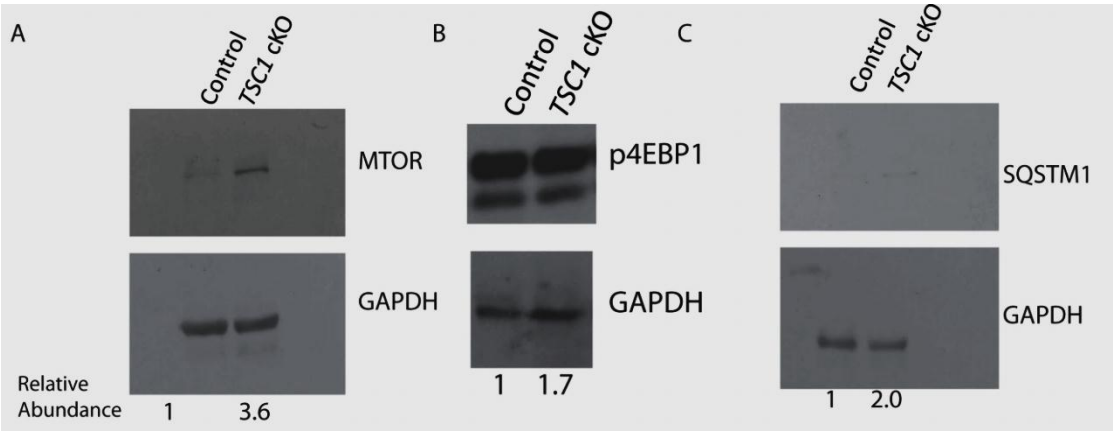


Figure 6.6 *Tsc1* cKO mammary glands have increase MTOR pathway signaling and decreased autophagy.

Total MTOR abundance in increased 3.6-fold in *Tsc1*CKO mammary glands (**A**) and downstream target p4EBP1 is increased 1.7-fold (**B**). SQSTM1 is increased 2-fold in *Tsc1* cKO mammary glands indicating that autophagy is reduced (**C**).

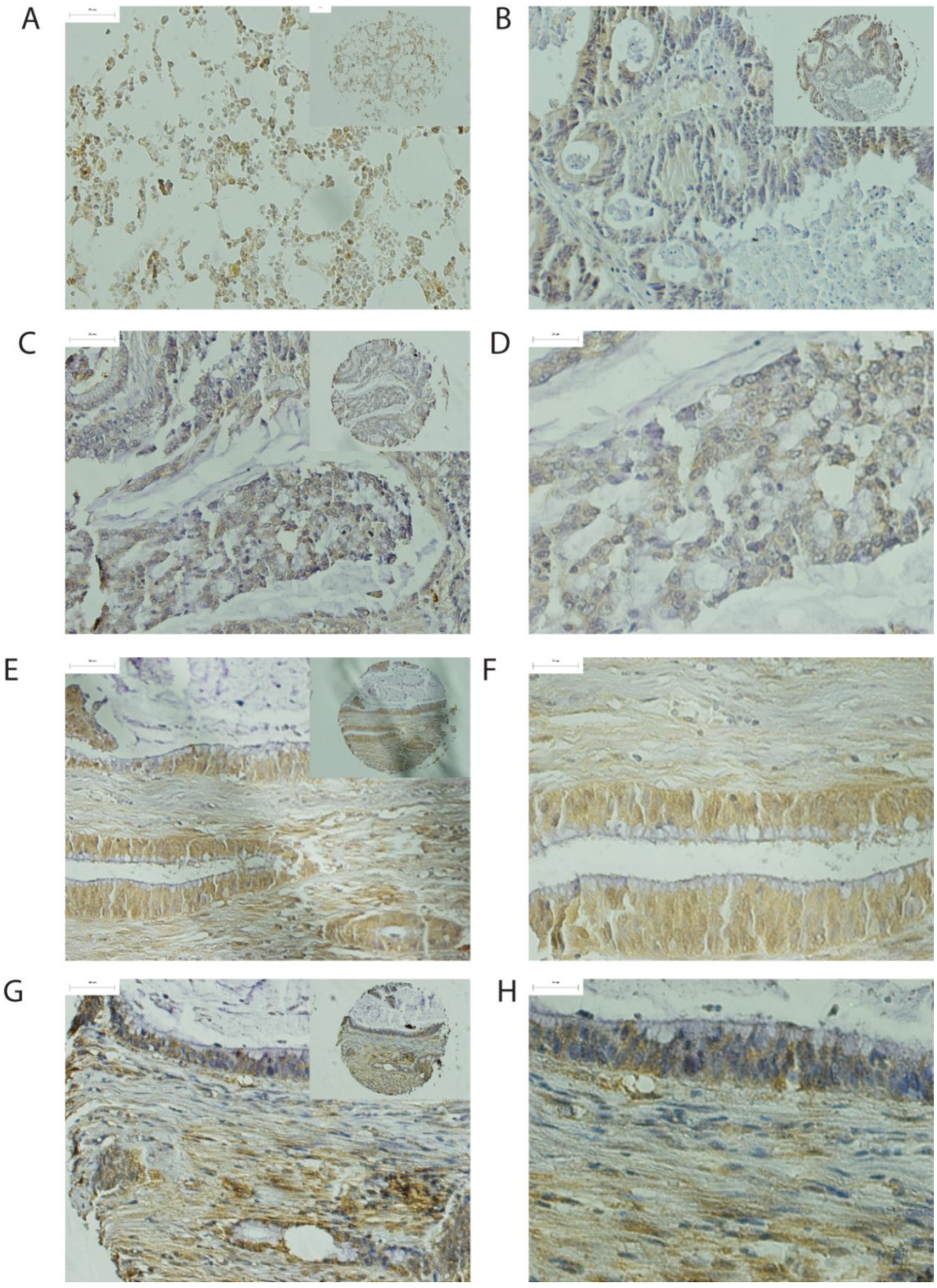

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Figure 6.7 TSC1 expression in breast cancer is variable and typically trends with BECN1 expression.

In normal breast tissue TSC1 is expressed in a majority of cells' cytoplasm (**A**). A panel of breast cancer cores was examined. In a breast ductal adenocarcinoma high expression can be seen in epithelia (**B**), and in similarly graded core expression is overall reduced (**C-D**). TSC1 exhibits high expression (**E-F**) in a restricted patter and BECN1 also exhibits a high expression level but has a slightly different pattern of localization (**G-H**). **A**, **B**, **C**, **E**, **G** 200X Bar=25nm; inset images **A**, **B**, **C**, **E**, **G** 100X; **D**, **F**, **H** 400X, Bar=50nm

Chapter Seven: Conclusions and Future Directions

Introduction

Through these studies we have explored the contribution of apoptosis and autophagy to mammary gland development and differentiation. Combined, they have focused our attention on the importance of balance and timing during dynamic remodeling and shifts in tissue function and physiologic demand. Perturbation of this system highlights the fragility of establishing and maintaining lactation. The WAP-Bax mice showed that overexpression of a single pro-apoptotic protein at an improper time leads to forced involution and lactation failure. Ablation of *Becn1*, viewed primarily as a cell survival protein, during pregnancy causes a similar gross outcome; lactation failure. Reconciliation of the delicate balance of pro-apoptotic and pro-survival signals is central to further our understanding of normal mammary development as well identifying and predicting pathologies, including cancer, which arise in this dynamic tissue. Furthermore, TSC1, an MTOR regulator and tumor suppressor never before examined in the mammary gland, also proved to be essential for lactation. Our continued exploration of the function of *Tsc1 in vitro* has revealed a novel protein-protein interaction between TSC1 and BECN1. This work has left us with many observations that push the boundaries of our knowledge regarding the regulation and function of autophagy pathway related proteins and the regulation of mammary gland development.

Since I have spent considerable time discussing the status of the field and our research findings, I intend to use this space for discussion, speculation, and to expound on experiments to further this line of thinking.

BECN1- More than an autophagy protein

Much has been published regarding the canonical function of *Becn1*, that is to say, the autophagy-promoting, initiation complex I-centric function. The studies described in this compilation push against this presumption and highlight alternative and non-canonical functions of BECN1, on which I wish to elaborate here.

By TEM, an accumulation of autophagosomes, both completed and incompletely closed, was identified in the *Becn1* cKO model. This finding suggests that the engrained notion of BECN1 acting primarily in the autophagosome initiation complex is too simplistic. After *Becn1* ablation we do not see an effect on autophagosome production, but we can identify a defect in functional autophagy, so three possibilities are feasible: 1) the residual BECN1 is sufficient for autophagosome formation, 2) in the mammary gland non-canonical BECN1-independent autophagy is most common, or 3) BECN1 is unnecessary for autophagosome formation in this model system.

We cannot completely discount the first possibility since there is no information regarding the stability of BECN1 *in vivo*; however we have shown that we significantly reduced BECN1 protein levels by both IHC and western blot. Since we are able to detect that functional autophagy is reduced by SQSTM1 accumulation, it is likely that autophagy in the mammary gland is impacted by BECN1 reduction and leads me to discount the second possibility. The third possibility holds water since we observe failure of autophagosomes to productively fuse with the lysosome. This supports an important and less-studied function of BECN1, its inclusion in “complex II” which

mediates fusion of the autophagosome with the lysosome. Interestingly, our lab as well as a collaborator's lab, have identified similar accumulation of autophagosomes in at least two additional tissues (personal communication Rucker, Gawriluk, Wang) suggesting that this phenomenon is not restricted to the mammary gland. This working model is depicted schematically in **Figure 7.1A**.

BECN1 and Lipid Droplets

My data also illuminate a novel role for BECN1 in regulating lipid trafficking and secretion. *In vivo*, lipid retention in the secretory epithelia is easily identified by histologic analysis in late pregnancy and early lactation phases. We then moved to an *in vitro* system to evaluate lipid retention in lactogenic mammary epithelial cells and found that when cells were treated with the autophagy inhibitor BafA1, an inhibitor that targets autophagosome-lysosome fusion and prevents lysosomal re-acidification, lipid droplets were, again, retained. Tellingly, when I repeated this experiment with the inhibitor wortmannin, which inhibits autophagy specifically at the level of the initiation complex (complex I), I found no significant difference in lipid droplet accumulation in the treated vs. untreated cells (**Figure 7.2**). This difference is important as it also supports the hypothesis that BECN1, in a complex that mediates membrane fusion (complex II), is important for lipid trafficking and/or secretion. At this time I am proposing a working model in which BECN1 participates in at least two distinct complexes, and some complex components are shared between the roles of autophagosome-lysosome fusion, and lipid droplet regulation.

The idea that autophagy proteins are important for lipid droplet synthesis, fusion, and trafficking is supported by studies in other model systems. Mammalian Atg2 homologs, ATG2A and ATG2B, are important for lipid droplet size regulation and distribution. Additionally, silencing of *Atg2A* and *Atg2B* together results in a block in autophagic flux and accumulation of unclosed autophagic structures.[495] In hepatocytes and cardiac myocytes LC3-II localizes to the surface of lipid droplets as well as autophagosomes under starvation conditions, and in *Atg7*-deficient livers lipid droplets were reduced and overall reduced in sized compared to controls.[496] This work laid the foundation for the involvement of the ATG7 conjugation system in lipid droplet formation, and in further studies, LC3 was directly associated with lipid droplet formation.[497] It seems that many of the classic autophagy related proteins have roles outside of autophagy and there is significant overlap with lipid droplet formation, regulation, and trafficking.

Similarities can also be pulled from studies focusing on lipid droplet formation and secretion in the mammary gland. The lipid droplet is thought to be formed at the ER and gain membrane material from many sources, similar to the autophagosome, before it is secreted (**Figure 7.1D, II-IV**). Droplets often fuse together in the cytoplasm with other lipid droplets and sometimes secretory vesicles before secretion (**Figure 7.1D, III**). The process of two distinct organelles fusing in the cytoplasm without any known control mechanisms or higher regulation is reminiscent of the fusion of autophagosomes and lysosomes, for which we have shown *Becn1* is important. Interestingly, xanthine oxidase is a constitutive component of the milk lipid globule

membrane and in mice the knockout has a similar phenotype to the *Becn1* cKO with regard to lipid retention and lactation failure.[498] It would be interesting to look at potential BECN1 localization at sites of inception or fusion of lipid droplets. This technically challenging experiment would be best conducted using live imaging of cells to track a likely short and dynamic association.

Together these studies indicate that much of the machinery that has been classically describe as autophagy machinery is essential for lipid droplet formation and size regulation. Furthermore the nature of formation, elongation, and fusion are in many ways parallel between lipid droplet formation and secretion and autophagy. It is therefore reasonable that BECN1 is a member of a complex, potentially complex II with UVRAG or other fusion proteins, which functions in lipid droplet formation, size regulation, trafficking, and fusion in addition to autophagic initiation and fusion.

What About Unconventional Protein Secretion?

There have been many reports of autophagy related proteins being important for unconventional protein secretion, defined as a method for secretion that bypasses the ER-Golgi route and instead leaves the ER and may use a vesicular structure to be exocytosed. A simplistic schematic is depicted in **Figure 7.1D, I**. In yeast, Acyl coenzyme A (CoA)–binding protein (Acb1) is secreted in an unconventional process that is autophagosome and Atg11 dependent.[499, 500] In mammalian cells, pro-inflammatory cytokine IL-1β depends on ATG5 and LC3 co-localizes in the cytoplasm, implicating an autophagic vesicle is required for its transport.[501]

While my data yield no indication that protein secretion is impaired in *Becn1* cKO mammary glands, as casein can readily be seen in any identifiable lumena, we did not specifically measure levels of secreted protein, thus cannot be certain. In our work we used total mammary gland for analysis, removing only the lymph node. A milk protein analysis from whole gland lysate did not reveal any difference between control and cKO mice across pregnancy time points (**Figure 7.3**). The caveat is that any proteins retained within the cells and not secreted would still contribute to the milk protein abundance represented on the gel. For studies focusing on the potential role of BECN1 in unconventional protein secretion, milk protein will need to be analyzed separate from the stromal and epithelial compartments of the mammary gland.

In sparse and old reports, a marrying of unconventional protein secretion and lipid secretion has been described.[502] In short, secretory vesicles leaving the Golgi surround a lipid droplet in the cytoplasm and form a syncytial layer around the droplet (**Figure 7.1D, IV**). The outer membrane, which is now composed of secretory vesicles and cargo, fuses with the plasma membrane and is exocytosed to the lumen. I have identified some of these structures in control animals on the day of parturition by TEM (**Figure 7.4 A, B**), one of only a handful of reported examples of this phenomenon. When lipid droplets are enveloped with plasma membrane (**Figure 7.1D, III**) and secreted, chunks of plasma membrane sometimes remain associated with the globule in the lumen (**7.4C, D**). These enveloping defects occur in the first 24 hours of lactation in controls and are very rarely observed at later time points.

When examining *Becn1* ckO glands on the day of parturition, defining phenotypic differences based on errors in fusion and secretion can be ambiguous since control glands are also likely to make errors at this time. Many sections and individuals must be included in such a study. Preliminary, qualitative results gathered from *Becn1* cKO glands harvested on the day of parturition are discussed here. In *Becn1* cKO glands globules are readily identified in lumena. Incidences of secretory vesicles surrounding and depressing lipid droplets are seen (**Figure 7.5A, B**). In some cases it appears that membrane integrity has been compromised (**Figure 7.5 C, D**). These results are brief but further investigation is needed. Quantification of lipid droplet size would be valuable but would require at least six animals of each genotype for comparison. This is a fascinating, understudied, and technically challenging area; as a result, much of the basic biology of lipid droplet secretion and mammary milk fat globules is still unknown.

BECN1 During Involution

When we initially embarked on this journey we were interested in answering the open question of whether or not autophagy is important during mammary gland involution. Since the WAP-Cre; *Becn1*$^{fl/-}$ mice exhibit a lactation defect and precocious involution, a proper investigation of the role of *Becn1* during involution was prohibited. Future studies using the MMTV-CreD; *Becn1*$^{fl/fl}$ model would be valuable to better examine this process. Involution would be forced at L10 by removal of the litter in mutant and control animals. Tissue would be assayed for proliferation index, apoptosis index, and examined for morphologic differences. Clusterin and pSTAT3 would be used as markers for involution stage by IHC and western blot.

BECN1, Tumor Suppressor?

Since *Becn1* is purported to be a tumor suppressor gene, and our studies to date have not identified tumors in mammary-specific cKO animals, we have designed a study to more directly address the role of *Becn1* in breast cancer. We are using the established MMTV-neu transgenic mouse model in combination with our mammary-specific *Becn1* cKO with WAP-Cre mediated deletion. This project necessitates backcrossing onto the tumor permissive FVB background (6 generations). Focal mammary tumors arise in MMTV-neu female mice beginning at 4 months with a median incidence of 205 days. In this model we can ablate *Becn1* expression in primiparous mice and compare median tumor incidence to transgenic primiparous controls with full *Becn1* compliment. This targeted experiment, conducted on a tumor-permissive background will support either 1) the widely regarded notion that Becn1 is a tumor suppressor, or 2) the model that tumors are addicted to autophagy and ablation of *Becn1* will delay tumor onset and/or retard growth. If *Becn1* is a tumor suppressor we will expect that tumors arise earlier in the *Becn1*-deficient animals. This scenario would be quantified by a shorter median incidence or that tumors arise with similar incidence but grow at a more rapid rate. Conversely, if autophagy promotes tumor incidence and growth by allowing them to survive sub-optimal nutrient and hypoxic conditions, we would expect that the *Becn1* cKO animals present with a longer median incidence and/or tumors that arise do not grow as rapidly as control animals. In essence, either outcome will be important to the research community and clarify the role of BECN1 in tumorigenesis.

High Energy, High Autophagy?

Physiologically it is interesting that both *Becn1* and *Tsc1* are important for proper function of the mammary gland since cell survival pathways including MTOR and AKT, which inhibit autophagy and TSC1/2 complex respectively, have been shown to be active during this time. It has been proposed by others that in certain cell types which are either undergoing senescence, or highly metabolically active (e.g. podocytes), it is beneficial for autophagy (i.e. catabolic process) and protein synthesis (i.e. anabolic process) to be concomitantly operating and spatially linked. The authors propose that in cells under high demand to produce bulk synthesis of secretory products that the catabolic and anabolic machinery are spatially coupled for efficiency.[503] The finding by our lab of yet another example of this and another level of interconnectedness between the MTOR pathway and the autophagic pathway in metabolically active and highly secretory cells beseeches more experiments be conducted teasing out this mechanism. A thorough understanding of the interplay between TSC1, BECN1, and MTOR is paramount.

TSC1-BECN1 Interaction

The interaction between BECN1 and TSC1 complicates the idea of MTOR pathway regulation, and reinforces the importance of multiple nutrient-sensitive regulatory levels. It has been well characterized that TSC1 is necessary for the stability of its binding partner TSC2, and in *Tsc1* cKO animals TSC2 levels are undetectable at the protein level due to rapid degradation by the proteasome. The TSC1-BECN1 interaction could mediate the pool of BECN1 in the cytoplasm that is available for rapid response to

cellular insult (**Figure 7.1B**). Under this model the stability of BECN1 in the *Tsc1* cKO would be compromised, thus BECN1 would be less available to perform its cellular roles leading to a similar, though milder phenotype, to the *Becn1* cKO (**Figure 7.1C**). This is precisely the results we have observed. Moving forward it is important to characterize the domains involved in this interaction, though it is probable that since both proteins contain a coiled-coil domain that this is the point of interaction. Protein-protein interaction assays (e.g. Duo-Link or co-immunoprecipitation) using constructs with targeted domain deletions and truncations will be useful for making this determination.

Summary

In closing, with the experiments that have been described as a foundation, the perceptions and boundaries of at least two fields have been challenged. It is clear that BECN1 is far more that an autophagy protein and the extent of its importance during development is still being revealed. Our conditional *Becn1* model will be a fruit-bearing asset to the autophagy community for many years. We have shown two new proteins that are essential for terminal differentiation in the mammary gland and proposed a mechanism based on a murine protein-protein interaction detected for the first time. In this chapter I have put forth ideas which are a result of an amalgamation of published literature and our findings. We have challenged the long-held characterization of BECN1 as a tumor suppressor and will continue to directly address this question in the future.

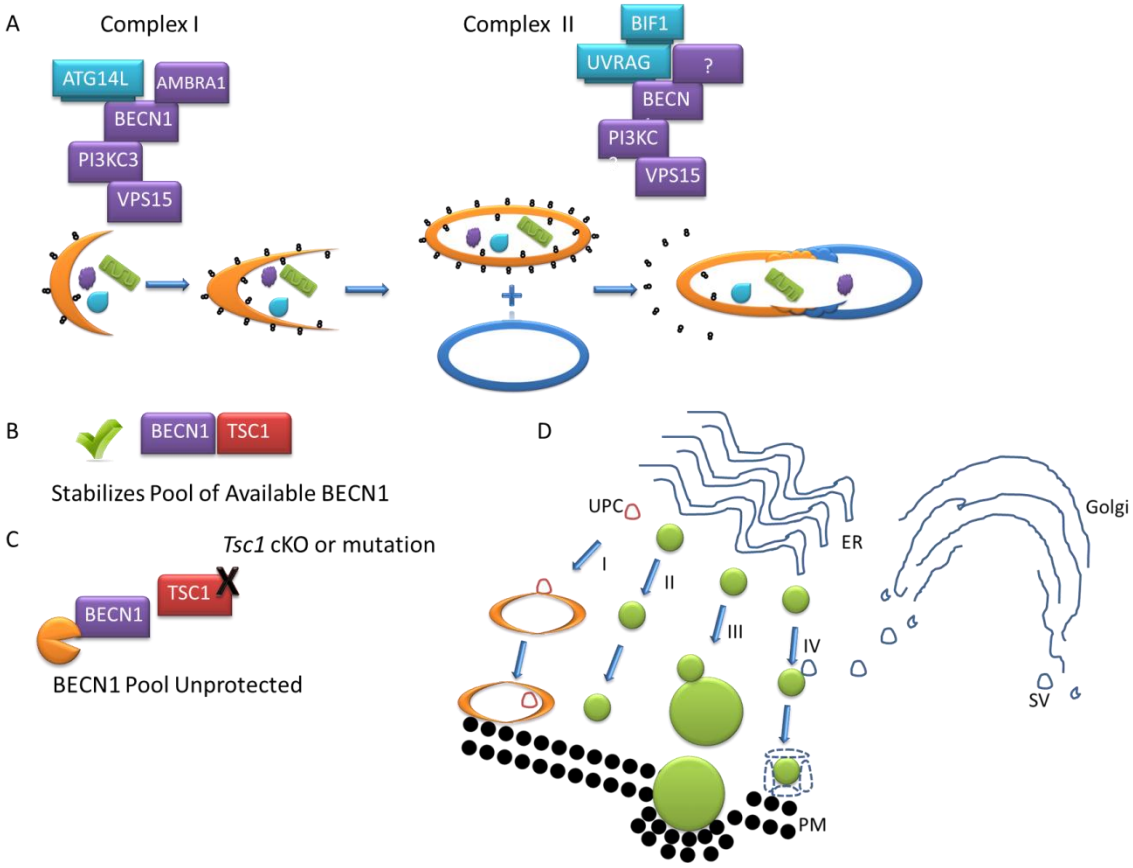

A
Complex I
ATG14L
AMBRA1
BECN1
PI3KC3
VPS15
Complex II
BIF1
UVRAG
?
BECN
PI3KC
VPS15
B
BECN1
TSC1
Stabilizes Pool of Available BECN1
C
Tsc1 cKO or mutation
TSC1
BECN1
BECN1 Pool Unprotected
D
UPC
ER
Golgi
I
II
III
IV
SV
PM

Figure 7.1 BECN1 Working Model

BECN1 is a member of two distinct complexes, complex I, which acts at the initiation stage and complex II, which functions at the autophagosome-lysosome fusion step. The two roles, depicted schematically in **(A)**, have mutually exclusive binding partners and may include uncharacterized components. TSC1-BECN1 interaction stabilizes the available pool of BECN1 in cytoplasm and protects BECN1 from degradation **(B)**, when the association is disrupted **(C)** either by knockout of *Tsc1* or endogenous mutation, BECN1 is left unprotected and destabilized resulting in impairment of BECN1-dependent functions. In **(D)**, autophagosome-dependent unconventional protein secretion **(I)** and several mechanisms of lipid secretion in the mammary gland **(II, III, and IV)** are depicted. Autophagosomes are pictured as orange vesicles; Lysosomes are pictured as blue vesicles; Green spheres represent lipid droplets; UPC, unconventional protein cargo; ER, endoplasmic reticulum; PM, plasma membrane; SV, secretory vesicle

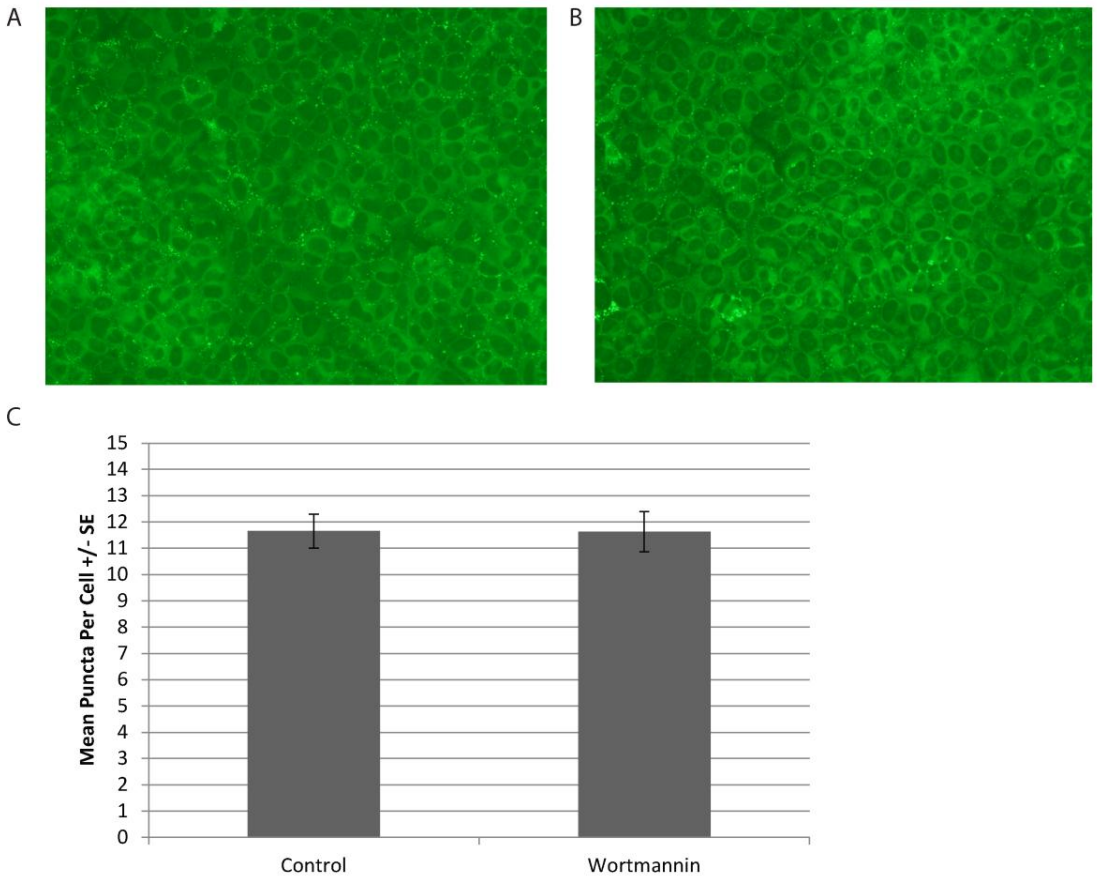


Figure 7.2 Autophagy inhibition with wortmannin does not affect lipid droplet retention *in vitro*.

HC11 cells were driven to lactogenic differentiation by supplementation with DIP (**A, B**). The treatment group (**B**) was concomitantly treated with wortmannin (100 nM). Both groups were stained with BODIPY 493/503 after 24 hours of treatment and cellular lipid droplets counted. The experiment was conducted in triplicate. No significant difference in lipid content was detected between groups by student's t-test (**C**), p-value=.985.

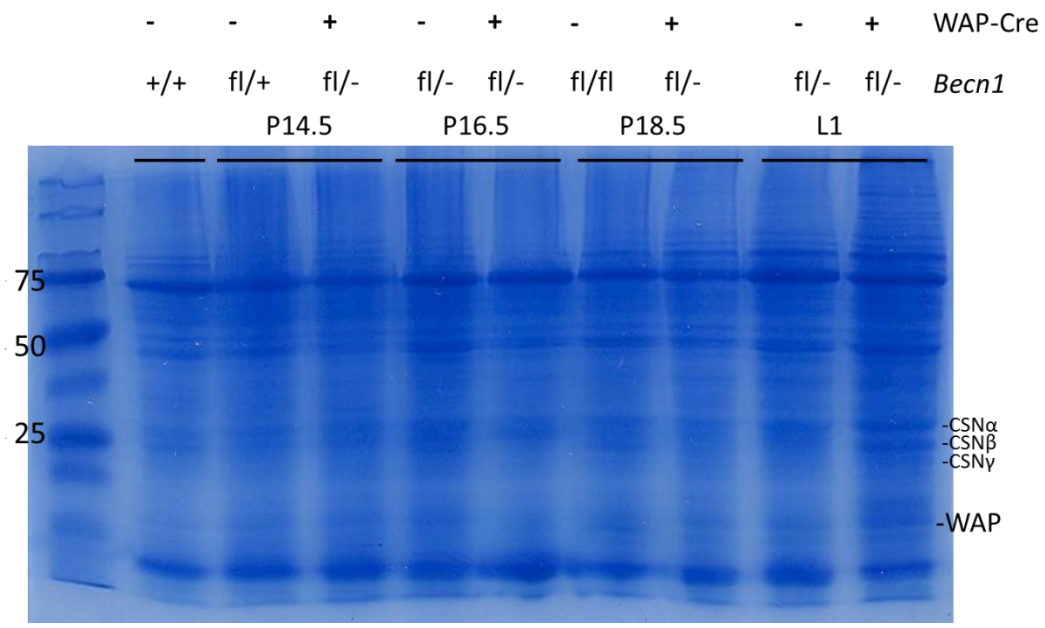
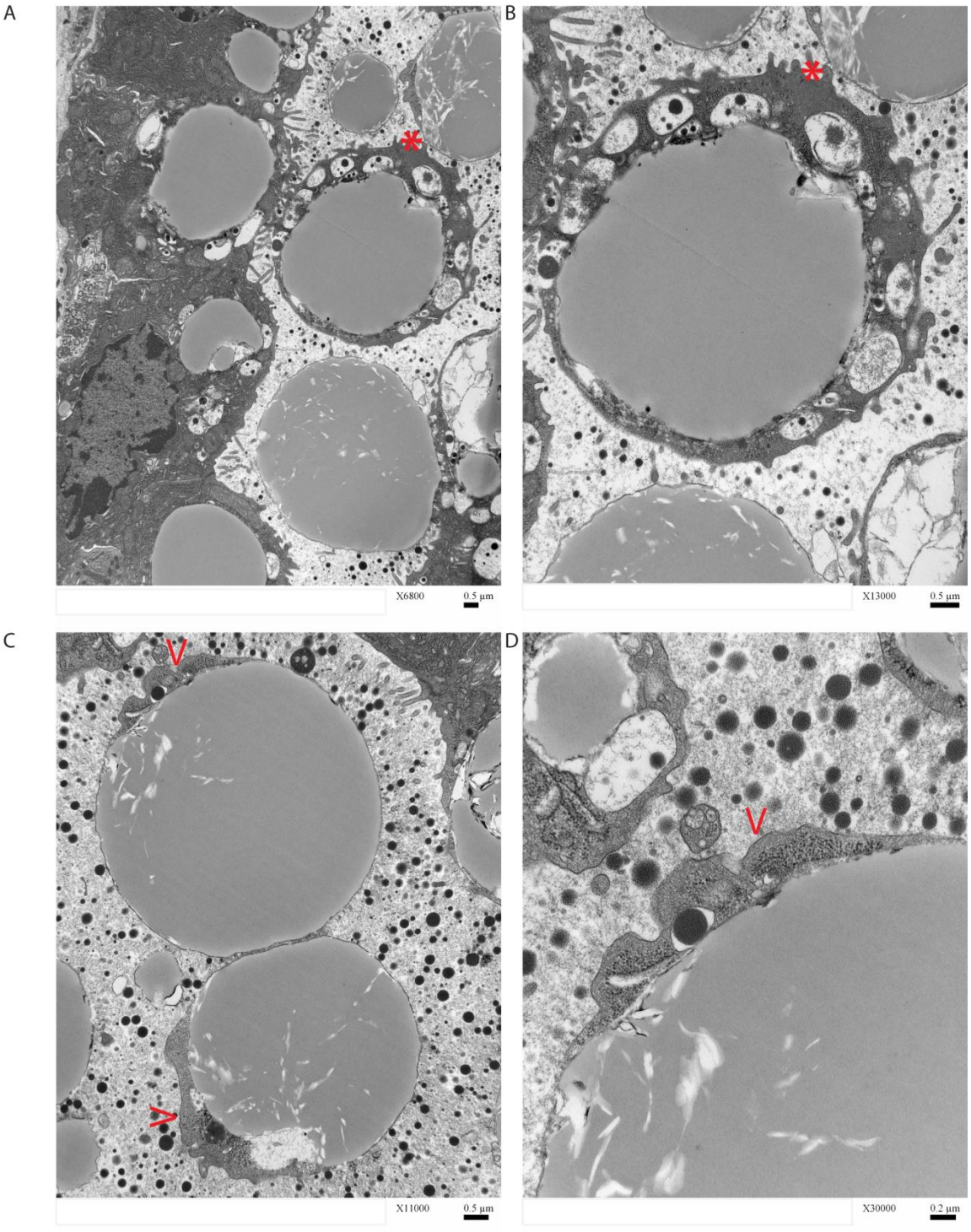


Figure 7.3 Milk protein abundance is unchanged in *Becn1* cKO mice

Secretory activation in the mammary gland precedes milk production. Total protein was extracted from whole mammary glands of control and mutant primiparous females, from which the lymph nodes were removed. Coomassie brilliant blue stain was used to visualize the characteristic milk protein banding patterns. No difference in total protein abundance was detected between control and *Becn1* cKO mice. WAP, whey acidic protein; CSNα, casein alpha; CSNβ, casein beta; CSNγ, casein gamma.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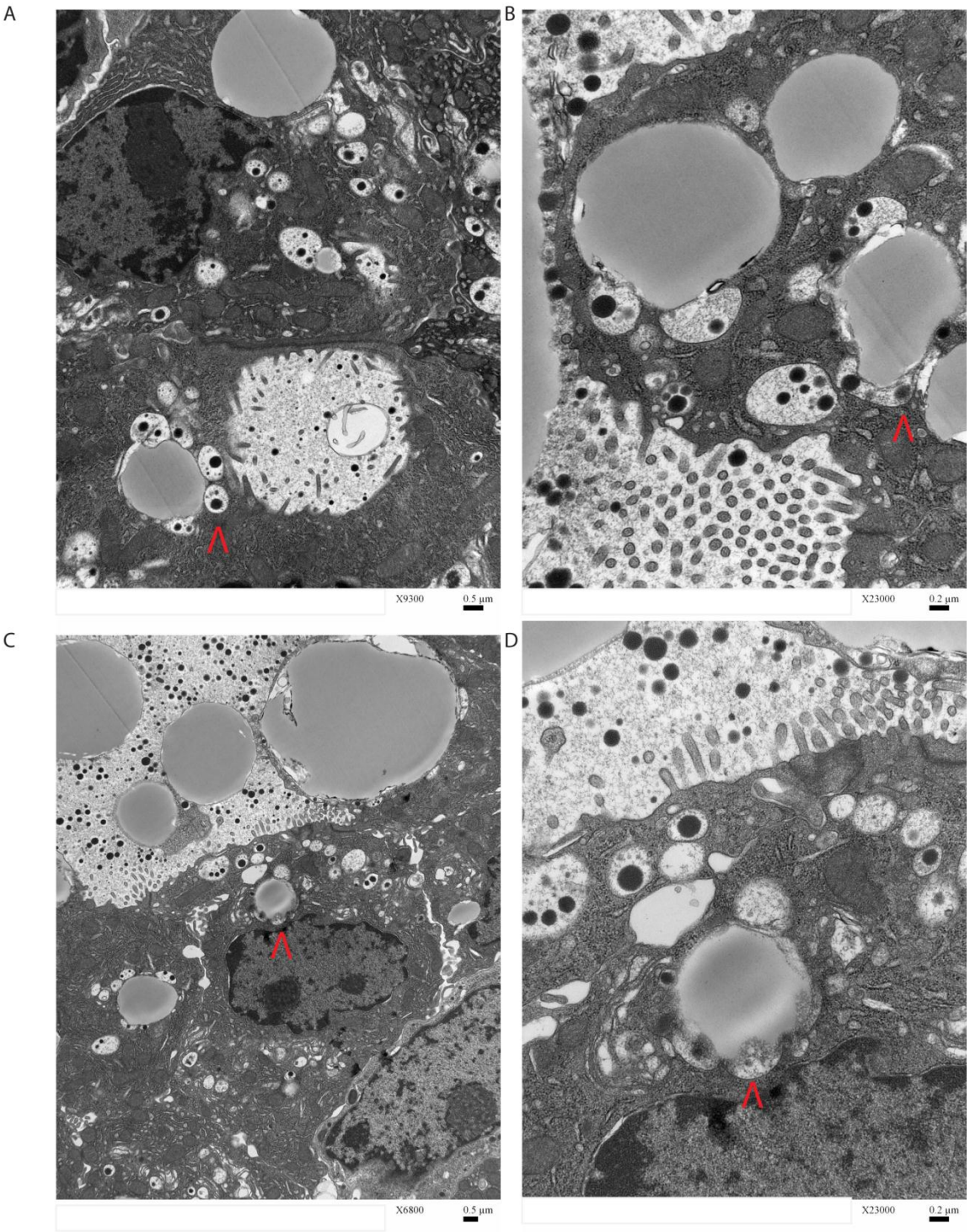
X6800
0.5 µm
X13000
0.5 µm
C
D
X11000
0.5 µm
X30000
0.2 µm

Figure 7.4 Secretory vesicles surround and plasma membrane envelopes lipid droplets in control mammary glands on the day of parturition.

In control glands, units of secretory vesicles surrounding milk fat globules can be seen in the lumen on the day of parturition (**A**, **B**). Asterisks indicate the area around the milk fat globule (secreted lipid droplet) that is encapsulated by secretory vesicles. Note that not every globule is affected. In (**C**), and at higher magnification in (**D**), there is plasma membrane and one secretory vesicle surrounding the globule. Errors in enveloping, the most common method of droplet secretion, are common in controls in the first 24 hours of lactation but are rare after lactation is established. Open arrowheads indicate areas where large amounts of plasma membrane remain on secreted lipid droplets. Scale bars and magnification are as shown below each panel.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X9300
0.5 µm
B
X23000
0.2 µm
C
X6800
0.5 µm
D
X23000
0.2 µm

Figure 7.5 *Becn1* cKO glands may have difficulty fusing membranes

Becn1 cKO glands were harvested on the day of parturition and examined by TEM. Secretory vesicles, probably containing casein, can be seen surrounding lipid droplets (**A**, **B**). In some areas with secretory vesicle-lipid droplet contact the droplet appears distorted. Instances of secretory vesicle membrane fusion failure can also be identified (**C**, **D**). Open arrowheads point to areas of potential fusion failures. Scale bars and magnification are as shown below each panel.

Chapter Eight: Materials and Methods

Generation of WAP-Bax Transgenic Mice

The WAP-Bax construct was digested with NotI and gel purified with the Qiagen Gel Extraction Kit. The keratin 14 (K14)-agouti transgene [421] was digested with ClaI and also gel purified. Transgenes were co- injected at a final concentration of 2 μg/ml^{-1} into pronuclear-staged FVB embryos. Pups (3 weeks old) were genotyped for the Wap-Bax transgene with the following PCR primers and reaction conditions: forward (5'-TAG AGC TGT GCC AGC CTC TTC-3'); reverse (5'-GAC ACA GTC GAC TCA GAA CAT CTT CTT CCA G-3'); cycling conditions of 94°C for 5 min (1 cycle), 94°C for 30 s, 58°C for 30 s, 72°C for 1 min (32 cycles), and a final step of 72°C for 5 min. The product of 650 base pairs was resolved on a 1.5% agarose gel. Founders were backcrossed with C57/BL6 mice for five generations. WAP-Bax transgene copy number was determined using exon 3 and 4 specific primers paired with an exon 3-specific TaqMan probe (5'-6FAM ATG CGT CCA AGA AGC TGA GCG TAMRA-3' at 200 nM final) with cycling conditions of 50°C for 2 min (one cycle), 95°C for 10 min, 95°C 15 s, 60°C for 1 min (40 cycles) on an ABI Prism 7700 thermocycler. Copy number was normalized to the endogenous *Bcl2l1* gene (two copies).

Gene targeting and generation of *Becn1* cKO mouse

For the targeting vector, a three loxP plasmid vector (ploxP3–NeoTK) with phosphoglycerol kinase (PGK)-neomycin and PGK–thymidine kinase cassettes for positive–negative selection was used. Genomic DNA isolated from 129SvEv mice was used to amplify three regions of homology for the targeting arms: a 2.7 kb *Becn1* arm #1

(*Becn1* promoter sequence), a 3.0 kb *Becn1* arm #2 (promoter, exon 1, intron 1, exon 2, and a portion of intron 2), and a 2.0 kb *Becn1* arm #3 (intron 2 sequence). For the amplification, the AccuPrime Pfx was used according to manufacturer's directions to generate blunt PCR fragments that were gel purified (Gel extraction kit, Qiagen) and cloned into pBlunt vector (Invitrogen). For the electroporations, 25 μg NotI-linearized DNA was resuspended in 25 μl electroporation buffer (Chemicon, Billerica, MA, USA) and electroporated into 1×10^7 129SvEv ES cells using the GenePulser II (250 V and 500 μFd; Bio-Rad) with 0.4 cm cuvettes. G418 selection (200 μg/ml) was started on day 1 after electroporation and continued thereafter; ganciclovir selection (2 μM) was performed on days 4–7 after electroporation. On day 12, 3× 96-well plates were picked and expanded for cell stocks and DNA isolation. Clones were initially screened by pooled PCRs using LaTaq (Takara) with a 5′-flanking reverse primer (5′-CCC TAG CTG GCC TGG AAC TCA GAA ATC T-3′) and neomycin-specific reverse primer (5′-TAC CGG TGG ATG TGG AAT GTG TGC GA-3′) set. The presence of the third loxP site was confirmed using flanking PCR primers (forward: 5′-CAG GAG AAG TGC CAT GGT GCA TCC TCT T-3′; reverse: 5′-CAA AGC CAA GGT TTC CAT GCT AAT GCC-3′). Individual clones were subjected to PCR confirmation from positive pools. Positive clones were confirmed by Southern blot diagnostics with an external 5′ probe. Targeted mouse embryonic stem (ES) cells were expanded and used for blastocyst injections at the Transgenic Animal Core facility at Texas A&M University (College Station, Texas).

Generation of mammary gland specific *Becn1* cKO mice

Becn1$^{fl/+}$ mice were generated by gene targeting in 129SvEv ES cells as described in previous section. A germline transmissible *Becn1* null allele (*Becn1*$^{-}$) was generated by breeding hemizygous *Becn1*$^{fl/+}$ with MMTV-CreA mice. Presence of the null allele was confirmed by PCR analysis (sense: 5'-ATG GAA GGA AGG AAG GTT AG-3'; antisense: 5'-CAA AGC CAA GGT TTC CAT GCT AAT GCC-3'). Mice carrying homozygous floxed alleles and neomycin cassettes were confirmed by PCR of tail-snipped DNA. True floxed alleles were generated by crossing *Becn1*$^{flneo/+}$ and Ella-Cre mice. Deletion of the neo cassette and production of "true floxed" allele was confirmed by PCR analysis (sense: 5'-ATG GAA GGA AGG AAG GTT AG-3'; antisense: 5'-GGT TGC ATG TTA AGT CTT TGT CGA-3'). WAP-Cre; *Becn1*$^{fl/-}$ mice were generated by crossing WAP-Cre; *Becn1*$^{fl/-}$ males with *Becn1*$^{fl/fl}$ females. Presence of the WAP-Cre transgene was confirmed using PCR (sense: 5'- CAG ACA CTC AGA CAG CCA TCA GTC-3'; antisense: 5'- CCA TGA GTG AAC GAA CCT GGT CG-3') All animal work was conducted using protocols approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Generation of mammary gland specific *Tsc1* cKO mice

Tsc1$^{fl/+}$ mice (*Tsc1*^{tm1Djk}/J) were crossed with Vasa-Cre transgenic line (FVB-Tg(Ddx4-cre)1Dcas/J) to produce a germline transmissible *Tsc1* null allele (*Tsc1*$^{-}$). Presence of the null allele was confirmed by PCR analysis (sense: 5'- AGG AGG CCT CTT CTG CTA CC-3'; antisense1: 5'- CAG CTC CGA CCA TGA AGT G-3'; antisense2: 5'- AGC CGG CTA ACG TTA ACA AC-3'). Presence of the floxed allele was confirmed by PCR analysis (sense: 5'-GTC ACG ACC GTA GGA GAA GC-3'; antisense: 5'-GAA TCA ACC CCA CAG AGC AT-3'). WAP-

Cre; *Tsc1*$^{fl/-}$ mice were generated by crossing WAP-Cre; *Tsc1*$^{fl/-}$ males with *Tsc1*$^{fl/fl}$ females. Presence of the WAP-Cre transgene was confirmed using PCR (sense: 5'- CAG ACA CTC AGA CAG CCA TCA GTC-3'; antisense: 5'- CCA TGA GTG AAC GAA CCT GGT CG-3'). All animal work was conducted using protocols approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Mammary gland collections

Mutant and control animals were sacrificed at designated time points according to the experimental design. An incision at the midline, but not piercing the peritoneum of the abdominal cavity, is carried to the distal rib. A second incision is made to separate the fourth and fifth mammary glands, parallel to the femoral artery. The number four mammary gland is removed carefully and used for whole mount and histological analysis. RNA and protein samples are taken from the contralateral inguinal mammary gland, but exclude the inguinal lymph node. In some cases a thoracic gland is also removed in a similar manner, but never used for RNA or protein analysis due to its skeletal muscle component.

Histology and Immunohistochemistry

Inguinal mammary glands were fixed in 4% (w/v) paraformaldehyde or 10% neutral buffered formalin solution (WAP-Bax experiments) at 4° C overnight, then placed in 70% ethanol and stored at 4° C. Paraffin embedding was performed at the Histology Laboratory of the University of Kentucky Imaging Facility. Processed glands were sectioned (5-7 µm) and mounted on charged glass slides. Slides were then

deparaffinized and hydrated for immunohistochemistry (IHC) or deparaffinized and hydrated, stained with methyl green, nuclear fast red, hematoxylin, and/or eosin, and dehydrated and mounted. For slides undergoing IHC, antigen unmasking was performed in citrate antigen unmasking buffer (Vector H-3300) followed by endogenous peroxidase quenching with 3% H_2O_2 diluted in methanol. Slides were blocked in 2.5% normal horse serum (Vector S-2012) for 20 minutes at room temperature. Primary antibodies against Bax (1:200; B.D. PharMingen 13686E), BECN1 (1:100, Santa Cruz Biotechnology sc-11427), Active Caspase-3 (1:50, B.D. PharMingen 559565), Caspase-3 (1:50, Santa Cruz 556425), Cytochrome C (1:50, B.D. Phar- Mingen SC7159), SQSTM1 (1:250, Sigma-Aldrich, P0067), TSC1 (1:100, Novus Biologicals, NB 100-80855), antibodies were diluted to indicated titers and incubated overnight at 4°C. ImmPRESS Reagent Kit-Anti-Rabbit IgG (Vector Labs, MP-7401) and ImmPACT DAB (Vector Labs, SK-4105) were used according to manufacturer's directions. Sections were counterstained with Hematoxylin QS (Vector Labs, H-3404) or eosin (Fisher Scientific, E-511) and visualized using an Olympus IX71 microscope (Olympus Corporation of the Americas). pSTAT5 and CCND1 IHC was performed at Epply Institute for Cancer and Allied Diseases, Omaha, Nebraska by our collaborators Dr. Kay-Uwe Wagner and Dr. Kazuhito Sakamoto.

Tissue Array

Tissue microarray slides were obtained from the Cooperative Human Tissue Network and the Tissue Array Research Program (TARP) of the National Cancer Institute, National Institutes of Health, Bethesda, MD, USA. The array analyzed in this study is the T-MTA-6A.

Measurement of Apoptosis in WAP-Bax Mammary Gland Epithelial Cells

For quantitation of apoptotic cells, the TUNEL-based Apoptag assay was performed according to the manufacturer on paraffin-embedded sections (Intergen). Briefly described, mammary gland tissue sections were deparaffinized, quenched in 3% hydrogen peroxidase and incubated with terminal transferase. After applying the anti-digoxigenin conjugate, the color was developed in DAB peroxidase substrate for 6 min. Samples were counterstained with methyl green (Vector Labs).

Mammary Gland Whole Mounts

Inguinal mammary glands (#4) or thoracic (#3) mammary glands were removed and spread on a charged glass microscope slide. Glands were fixed with Carnoy's fixative overnight (6 parts 100 % EtOH, 3 parts CHCl3, 1 part glacial acetic acid). Glands were washed in 70% ethanol for one hour then gradually hydrated. Glands were stained with Carmine Alum, prepared by placing 1 g carmine (Sigma C1022) and 2.5 g aluminum potassium sulfate (Sigma A7167) in 500 ml distilled water and boiling for 20 min. The final volume was adjusted to 500 ml with ddH_2O and filtered before use, and kept at 4° C overnight or until a desirable stain was attained. Glands were then dehydrated with a series of graded ethanol and transferred to xylenes to clear. Glands were left in xylene until sufficiently cleared then mounted with Permount (Fisher Scientific SP15-500). The protocol is described at http://mammary.nih.gov/tools/histological/Histology/index.html. The processed glands were imaged using an Olympus SZ-PT dissecting microscope. Fluorescent Whole Mounts

were prepared as described in Landua et al. (Method 1, Protocol B).[504] They were visualized using an Olympus IX71 microscope (Olympus Corporation of the Americas).

Immunoblotting

Tissues were lysed in RIPA and protease inhibitors (Santa Cruz Biotechnology sc-24948). An equal amount of protein from each cell lysate was resolved on a SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad 162-0094). Nonspecific binding was blocked with 5% nonfat dry milk powder in TBST for 60 min. The blots were incubated overnight at 4°C with primary antibody followed by washing with TBST and incubation with anti-rabbit-HRP secondary antibody in 5% nonfat dry milk powder in TBST for 2 hours. After washing in TBST, the immunoblots were developed with enhanced chemiluminescence detection system (Amersham RPN-2108) as per the manufacturer's instructions. GAPDH was used as loading control. Primary antibodies used: BECN1 (Santa Cruz Biotechnology, sc-11427), MTOR (Cell Signaling Technologies, 2983P), p4EBP1 (Cell Signaling Technologies, 2855P) SQSTM1 (Sigma-Aldrich, P0067), and TSC1 (Novus Biologicals, NB 100-80855).

HC11 cell culture

HC11 cells (a generous gift from Dr. Kay-Uwe Wagner, University of Nebraska) were cultured on coverslips in growth media (RPMI, 10% FBS, 5 µg/mL insulin, 10 ng/mL EGF, 50 µg/mL gentamycin, 100 U/mL pen/strep) until confluent (at least 6 days). Induction media (RPMI, 10% FBS, 5 µg/mL insulin, 5 µg/mL prolactin, 0.1 µM dexamethasone, 50 µg/mL gentamycin, 100 U/mL pen/strep) was added and the cells were maintained for

24 or 48 hours. At appropriate time points cells were prepared for light and fluorescent imaging as follows. Cells were washed three times with PBS, cells were fixed with 4% paraformaldehyde for 15 minutes, and then washed three times with ice cold PBS. To permeabilize cells, samples were incubated in PBS containing 0.25% Triton X-100 for 10 minutes. Cells were washed three times with PBS, five minutes per wash. Coverslip were mounted onto microscope slides using VectaShield mounting medium for fluorescence with DAPI (Vector Laboratories Inc., H-1200). Slides were viewed using an Olympus IX71 microscope (Olympus Corporation of the Americas)

NIH3T3 Cell culture and Protein-Protein Interaction Assay

NIH3T3 cells were cultured on coverslips or using chamber slides (Nunc, 154526) in growth media (DMEM supplemented with 10% bovine calf serum, and 100 U/ml penicillin and streptomycin) in a 5% CO_2 incubator until confluent. Serum-free medium lacks the bovine calf serum. At designated time points, as dictated by experimental design, cells were prepared for protein-protein interaction assay according to manufacturer's directions, using recommended protocol and wash buffers (Omega Bioscience, DUO-LINK II). TSC1 (Novus Biologicals, NB 100-80855) and BECN1 (Santa Cruz Biotechnology, sc-48381) antibodies were employed. Coverslip were mounted onto microscope slides using VectaShield mounting medium for fluorescence with DAPI (Vector Laboratories Inc., H-1200). Slides were viewed using an Olympus IX71 microscope (Olympus Corporation of the Americas).

Transmission Electron Microscopy (TEM)

All TEM experiments were conducted under the supervision of the University of Kentucky Imaging Core. Briefly, mammary glands were harvested and minced into $1mm^2$ pieces in cold fixative (3.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4) for 1.5 hours at 4°C. Glands were washed in 0.1M cacodylate buffer with 5% sucrose (4 times, 15 minutes each). Samples were post fixed with 1% osmium tetroxide for 1.5 hours at 4°C then washed in cacodylate buffer. Tissues were dehydrated in graded ethanol, 10 minutes each and transferred to propylene oxide (2 changes, 15 minutes each). Tissues were infiltrated overnight with resin/propylene oxide overnight then put in 100% resin (2 changes, 1 hour each). Tissue was embedded in resin for 48 hours at 60°C, thin sectioned using Reichert Ultracut E, and imaged using a Philips Tecnai Biotwin 12 transmission electron microscope.

Appendix: Abbreviations

3-MA, 3-methyladenine; ATG, autophagy related; BafA1;bafilomycin A1; Bmp, bone morphogenic protein; BH3, Bcl2 homology domain; cKO, conditional knockout; DAPI, 4', 6-Diamidino-2-Phenylindole, Dihydrochloride; DC, dendritic cell; DISC, death-inducing signaling complex; E, embryo(nic); E2, estrogen; ECM, extracellular matrix; EDD, endothelium-dependent dilatation; EM, electron microscopy; ER, endoplasmic reticulum; ERE, estrogen responsive element; ESCRTI/ESCRTIII, endosomal sorting complex required for transport complex I and III; EsR, estrogen receptor; FFA, free fatty acids; FGF, fibroblast growth factor; GAS, γ- interferon activation sites; GD, glucose deprivation; GFP, green fluorescent protein; H/I, hypoxic/ischemic; HOPS, homotypic vacuole fusion protein sorting complex; HSC, hematopoietic stem cell; IL, interleukin; ITPR, Inositol trisphosphate receptor; JAK, Janus kinase; KO, knockout; LD, lipid droplet; LIF, leukemia inhibitory factor; MEC, mammary epithelial cell; MEF, murine embryonic fibroblast; MMP, metalloproteinase; MOMP, mitochondria outer membrane permeabilization; Msx, homeobox, msh-like; MVB, multivesicular body; NBD-PS, 7-nitro-2-1,3-benzoxadiazol-4-yl-phosphoserine; OMM, outer mitochondrial membrane, P, pregnancy; P4, progesterone; PCD, programmed cell death; PE, phosphatidylethanolamine; PGC, primordial germ cell; PM, plasma membrane; PR, progesterone receptor; PRL, prolactin; PRLR, prolactin receptor; PtdIns3P-phosphatidylinositol 3-phosphate; PThrP, parathyroid hormone related peptide; RANK, receptor activator of nuclear factor κβ; RANKL, RANK ligand ROS- reactive oxygen species; SCN, suprachiasmatic nucleus; SNARES, Soluble NSF attachment protein

receptors; SNP, single nucleotide polymorphism; STAT, signal transducers and activators of transcription; T2D, type 2 diabetes; Tbx, T-box protein; TEB, Terminal End Bud; TED, terminal end duct; TEM, transmission electron microscopy; Th, T helper; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; UPR, unfolded protein response; UPS, ubiquitin proteasome system; WAP, Whey acidic protein; XeB/XeC, xestospongin B/C

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Hale A., Rucker, E., (2013) Post-Pubertal Mammary Gland Development: Morphology and Mechanisms, The Mammary Gland. Dr. Edmund Rucker (Ed.), ISBN: 978-1629488530, Nova Science Pub Inc

Poster Presentations:

Hale, A., Rucker, E., “TSC1 is a novel interacting partner of Beclin1 and essential for terminal differentiation of the mammary gland.” Women’s Health & Reproductive

Sciences Symposium with CCTS spring conference, University of Kentucky, Lexington, KY 2014

Hale, A., Rucker, E., "Defining the role of *Beclin1* and autophagy using transgenic mouse models." Biology Graduate Student Association Research Symposium, University of Kentucky, Lexington, KY 2014

Hale, A., Rucker, E., "Beclin1 is an Important Regulator of Post-Pubertal Mammary Gland Development and Lactation." Women's Health & Reproductive Sciences Symposium, University of Kentucky, Lexington, KY 2013

Hale, A., Rucker, E., "Beclin1 is an Important Regulator of Post-Pubertal Mammary Gland Development and Lactation." Keystone Autophagy, inflammation, and Immunity, Montreal, QC Canada 2013

Hale, A., Rucker, E., "Beclin1 is an important regulator of post-pubertal mammary gland development and lactation." Society for the Study of Reproduction Annual Meeting, University of Pennsylvania, State College, PA 2012

Hale, A., Rucker, E., "Beclin1 is an important regulator of post-pubertal mammary gland development and lactation." Women's Health & Reproductive Sciences Symposium, University of Kentucky, Lexington, KY 2012

Hale, A., Rucker, E., "Forced involution of the functionally differentiated mammary gland by overexpression of the pro-apoptotic protein Bax." Women's Health & Reproductive Sciences Symposium, University of Kentucky, Lexington, KY 2011

Hale, A., Rucker, E., "Determining the Role of *Beclin1* in Mammary Gland Development." Women's Health & Reproductive Sciences Symposium, University of Kentucky, Lexington, KY 2010

Hale, A., Rucker, E., "Defining the role of *Beclin1* and autophagy using transgenic mouse models." Biology Graduate Student Association Research Symposium, University of Kentucky, Lexington, KY 2009

Hale, A., Rucker, E., "Defining the role of *Beclin1* and autophagy using transgenic mouse models." Apoptosis and Cancer: The Bcl-2 Family of Proteins, Dartmouth College, Hanover, NH 2009

Hale, A., Rucker, E., "*Beclin1* Conditional Knockout Mouse Model." Keystone Cell Death Conference, Whistler, British Columbia Canada 2009

Hale, A., Rucker, E., "*Beclin1* Conditional Knockout Mouse Models." Cell Development and Differentiation Conference, Marshall University, Huntington, West Virginia 2008