Chapter 6

Altering Autophagy: Mouse Models of Human Disease

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Additional information is available at the end of the chapter

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1. Introduction

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.

2. 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 [1]. 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 [2]. 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 [3]. 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 GFP-GABARAP is low, yet visible, ameliorating many of the potential effects of highly expressing fluorescent proteins. In podocytes, GFP-GABARAP co-localized with p62 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 [4].

2.1. 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

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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 [5]. Disruptions in pro-autophagy genes *Becn1* [6], *UVRAG* [7], and *Bif-1*[8] resulted in increased frequencies of lymphomas and mammary neoplasias 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 [9, 10]. 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 [11]. 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 [12]. This study provides primarily *in vitro* evidence of a role for autophagy induction in supporting tumor growth.

2.2. 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 [13]. Conversely, autophagy appears to be protective during permanent occlusion events. Long term ischemia causes relief of

mammalian 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 [14]. Two recent mouse models of heart disease provide evidence supporting a protective role of autophagy for the prevention of CHD [15]. 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 [16].

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 [17]. 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 [18]. 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.

2.3. 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 multi-protein 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 [19]. It seems that autophagy is used as a compensatory mechanism to relieve the

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toxic effects of these mutant protein aggregates in the cell, serving a protective role. Neuronal-specific conditional deletion of *FIP200* (*FIP200*$^{fl/fl}$; 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 *FIP200*$^{-/-}$ phenotypes were more severe, present at an earlier age, and resulted in premature death. Unique to the *FIP200*$^{-/-}$ 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 [20]. 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 [21, 22]. 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 [23]. In a similar experiment, deletion of *Atg5* in PCs (*Atg5*$^{fl/fl}$; pcp2-Cre) also resulted in axonal swelling and neurodegeneration [24]. 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.

2.4. 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 [25]. CR can improve heart function through autophagy, as long-term CR preserved cardiac contractile function with improved cardiomyocyte function and lessened cardiac remodeling [26]. 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 [27]. 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 [28]. 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 [29]. 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. Spermi-

dine, 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 [30].

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 [31]. Progerin, the truncated form of lamin A protein, was found to co-localize with the autophagic adapter protein p62 and the autophagy linked FYVE protein, ALFY [32]. Moreover, rapamycin decreased progerin protein levels through autophagy induction, which rescued the progeria phenotype in HGPS fibroblasts [33]. 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 [34]. An oxidative environment potentially plays a crucial role in the aging process, as $p62^{-/-}$ mice exhibited accelerated aging phenotypes and tissues displayed elevated oxidative stress due to defective mitochondrial electron transport [35]. Likewise, *Cisd2*-null mice exhibited nerve and muscle degeneration and a premature aging phenotype [36]. 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 [37]. 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 [38]. 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.

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2.5. 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 [39].

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 [40].

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 [41]. Neurons had increased LC3-II expression and failed to clear LC3-II, once again indicating a functional autophagic block that led to the pathogenesis [42].

2.6. 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 lcsB, 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 [43, 44].

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 [45]. 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 [46]. 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).

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Additionally, in peripheral circulation Atg5 was required to maintain normal numbers of B-1a B cell populations but not B-2 B cells [47].

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 [48]. In another study, a SNP in the coding region of IRGM was identified that affected a microRNA binding site [49]. 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 [50]. 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 [51].

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 [52]. 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.

2.7. 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 [53].

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 [54].

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 [55].

2.8. 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 in [56]. 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 [57]. 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-

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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 [58]. 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 [59]. 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.

2.9. 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 [60]. 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 [61]. These experiments together suggested that β–cells depended on autophagy to clear damaged organelles and toxic intracellular protein aggregates.

2.10. 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 [62]. 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 [63]. 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 [64]. Also, autophagy induction in stallion sperm, as measured by LC3I to LC3II conversation, was important for the survival of sperm post ejaculation [65]. In mice, an *Atg5* sperm-specific conditional deletion has been generated and males developed an infertility phenotype at approximately 15 weeks due to accumulation of abnormal structures in the seminiferous tubules as well as abnormal sperm. Tsukamoto et al. suggested that autophagy may be essential for normal spermiogenesis in mice in order to effect cytoplasmic reduction [66].

In 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.

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