Autophagy is a tightly regulated catabolic process whereby cells degrade their constituents to dispose of unwanted cytoplasmic elements and recycle nutrients for cellular remodeling. Studies of autophagy in mammals have elicited substantial interest because it is linked to a range of physiologic and pathologic states. In this issue of the JCI, Mariño et al. uncover a role for autophagy in a balance disorder related to inner ear pathologies. Mice lacking the protease autophagy-related 4B (Atg4B, also known as autophagin-1) exhibited a systemic reduction in autophagy and showed defects in the development of ototonia, organic particles that contain calcium carbonate crystals and proteins and that are essential for balance perception (equilibriumception) in mammals. The intriguing aspect of this work is that an autophagy block impairs the secretion and assembly of ototocial proteins, emphasizing a role for autophagy in functions distinct from macromolecule degradation.

The physiological importance of autophagy

Autophagy, a tightly regulated process by which cells consume unwanted cytoplasmic macromolecular constituents and recycle nutrients for cellular remodeling, is mediated by the coordinated activity of autophagy-specific (ATG) genes. There are several forms of autophagy, but here we focus on the best-characterized form, macroautophagy (referred to herein as “autophagy”). During this evolutionarily conserved process, a double membrane known as the isolation membrane wraps around portions of the cytoplasm to form a double-membrane vesicle, the autophagosome. The engulfed cargo, including organelles, is degraded upon autophagosome fusion with late endosomes or lysosomes. Autophagy plays a central role in cancer, neurodegeneration, innate immunity, organellar clearance, the organismal response to starvation, and aging (1). Genetic variations in the autophagy-related genes autophagy-related 16–like 1 (ATG16L1) and immunity-related GTPase family M (IRGM) are linked to susceptibility to Crohn disease, a human chronic inflammatory disorder affecting the gastrointestinal tract (2, 3). Mice exhibit basal and induced forms of autophagy, both of which play important physiological roles (4, 5). The former is involved in the homeostasis of cellular constituents, including organelles, and the degradation of long-lived proteins and protein aggregates (4, 5). However, autophagy can also be induced by physiologic and pathologic conditions, such as nutrient starvation and infection by pathogens (4, 5). Consequently, it should not be surprising that mice incapable of autophagy die perinatally, revealing the necessity of autophagy in surviving neonatal starvation (i.e., after the transplacental nutrient supply is stopped, autophagy becomes essential for maintaining the amino acid supply; ref. 6).

Tissue-specific knockouts of autophagy genes have also revealed diverse pathologies. These include severe hepatomegaly and hepatic hypertrophy caused by liver-specific deficiency of autophagy-related 7 (Atg7) (5); behavioral problems, as indicated by abnormal limb clasping, reduced coordinated movement, and neuronal loss, caused by either Atg5 or Atg7 deficiency in the central nervous system (7); the degeneration of islet cells, reduced glucose tolerance, and insulin secretion caused by Atg7 deficiency in pancreatic β cells (8, 9); and the abnormal morphology and function of intestinal Paneth cells caused by deficiency in either Atg16L1 or Atg5 (10). These studies demonstrate that the physiological phenotypes are caused not only by the loss of...
lysosome-mediated autophagy per se, but also by other effects such as an increase in the cellular concentrations of certain multifunctional signaling proteins (e.g., p62, as discussed in “A testable hypothesis for the link between autophagy and protein secretion”). In this issue of the JCI, Marito et al. expand the list of diverse pathologies linked to autophagy by showing that mice lacking the protein Atg4b exhibit defects in the development of otocoria, minute organic particles that contain calcium carbonate crystals and proteins and that are essential for gravity perception in mammals, and exhibit a balance disorder related to inner ear pathology (11). They also describe a deficiency in secretion of certain proteins associated with otocoria biogenesis, suggesting the possibility of a new link between a block in autophagy and a concomitant reduction in specific protein secretion and vesicle sorting.

**Autophagins activate LC3 family members during autophagy**

Autophagy is an ancient process that is conserved from yeast to humans. More than 20 Atg proteins are required for autophagosome formation in yeast, and a central player is the ubiquitin-like molecule Atg8. There are four mammalian homologs of yeast Atg8 — LC3A–C, GATE-16, GABARAP, and Atg8l — that are specifically cleaved by the Atg4 family of cysteine proteases, resulting in presentation of a C-terminal glycine that is conjugated to PE in the isolation membrane by a ubiquitin-like modification system involving Atg7, Atg3, and the Atg5/Atg12 complex. PE conjugation anchors LC3 proteins to the isolation membrane. Atg4 isoforms also deconjugate LC3s from the cytosolic face of the autophagosome. (B) Mechanism of autophagosome formation. Cytosolic components (e.g., cytosolic pathogens, superfluous or dysfunctional organelles, and cytotoxic protein aggregates) tagged with ubiquitin (Ub) are specifically recognized by p62, which also efficiently binds membrane-conjugated LC3, thus mediating engulfment of the target. Elongation of the isolation membrane results in completion of the autophagosome and deconjugation of LC3 on the cytosolic face of the autophagosome, which ultimately fuses with an early endosome or lysosome to form an autolysosome. Acid hydrolysis leads to degradation of the contents of the autophagosome and recycling of the molecular components.

**Figure 1**

Atg4-dependent molecular mechanisms of the autophagy pathway. (A) Role of Atg4 isoforms in processing of LC3 family members. The four mammalian homologs of yeast Atg8 — LC3A–C, GATE-16, GABARAP, and Atg8l — are specifically cleaved by the Atg4 family of cysteine proteases, resulting in presentation of a C-terminal glycine that is conjugated to PE in the isolation membrane by a ubiquitin-like modification system involving Atg7, Atg3, and the Atg5/Atg12 complex. PE conjugation anchors LC3 proteins to the isolation membrane. Atg4 isoforms also deconjugate LC3s from the cytosolic face of the autophagosome. (B) Mechanism of autophagosome formation. Cytosolic components (e.g., cytosolic pathogens, superfluous or dysfunctional organelles, and cytotoxic protein aggregates) tagged with ubiquitin (Ub) are specifically recognized by p62, which also efficiently binds membrane-conjugated LC3, thus mediating engulfment of the target. Elongation of the isolation membrane results in completion of the autophagosome and deconjugation of LC3 on the cytosolic face of the autophagosome, which ultimately fuses with an early endosome or lysosome to form an autolysosome. Acid hydrolysis leads to degradation of the contents of the autophagosome and recycling of the molecular components.
MEK kinase 3 (MEKK3; reviewed in ref. 15). The p62 molecule also mediates stress response programs dependent on nuclear factor (erythroid-derived 2)-like 2 (Nrf2) via inhibition of Kelch-like ECH-associated protein 1 (Keap1) (16) (see “A testable hypothesis for the link between autophagy and protein secretion”). As a cargo receptor, p62 contributes to the autophagic turnover of ubiquitin aggregates, damaged mitochondria, the mid-body ring (which forms at the site of cytokinesis), peroxisomes, and microbes (15). Among other structural motifs (Figure 2B), p62 contains an N-terminal self-oligomerization domain and a C-terminal ubiquitin-associated (UBA) domain that interacts with ubiquitylated proteins. Ubiquitylated autophagic cargoes, as well as p62 itself, are degraded during autophagy, mediated by interaction of the LC3-interaction region (LIR) of p62 with LC3, which is recruited to the isolation membrane (15). Consistent with this role of p62 in the formation of intracellular inclusions or aggregates of ubiquitylated proteins, p62-deficient mice display a marked reduction in the formation of ubiquitin inclusions in hepatocytes and neurons (17). Conversely, in autophagy-deficient cells, p62 and ubiquitin aggregates accumulate (5).

**Atg4b deficiency knocks mice off balance**

In this issue of the JCI, Mariño et al. report the phenotype of mice lacking Atg4b (11). The impetus for this work was that a previous study by this group had found that another Atg4 isoform (Atg4c) is primarily involved in induced, rather than basal, autophagy in a tissue-specific manner in mice (18). In the current study, Mariño and colleagues characterize levels of p62 and LC3 homologs in different tissues (11). They demonstrate defects consistent with a reduction in autophagic flux in various tissues, with reduced proteolytic processing of all LC3 homologs except GATE-16, reduced PE conjugation of most LC3 homologs, and elevated p62 levels. Surprisingly, mice lacking Atg4b exhibited abnormal behavior characteristic of inner ear pathologies, and this deficiency was traced to defects in the formation of otoconia in the inner ear. Otoconia represent crystalline particles consisting of glycoproteins (collectively referred to as otocinsics) and inorganic calcite (CaCO₃) crystals. By stimulating the vestibular sensory epithelium upon movement, otoconia facilitate perception of gravity and linear acceleration. The otocorial biogenesis defects in the Atg4b⁻/⁻ mice...
observed by Marito et al. caused severe balance disorders, but the penetrance of this phenotype was incomplete (i.e., not all animals displayed the same severity of balance disruption), probably because of functional redundancy between Atg4 isoforms or the presence of unknown modifier loci. Mechanistically, the authors linked the otocochial formation defects to an autophagy defect that resulted in impaired secretion of otocional core proteins into the vestibular lumen. The outcome of this defect was the cytosolic mislocation of these proteins and thus the impairment of otocional assembly. The reduced secretion of otocional proteins was traced to decreased levels of biogenesis of lysosome-related organelles complex 1 (BLOC-1) and adaptor protein complex 3 (AP-3), which function as adaptors in the coat-mediated vesicular trafficking of otocional proteins from the Golgi apparatus to the plasma membrane. To prove that this defect resulted from impaired autophagy, Marito et al. showed that Atg5−/− mice exhibited a similar phenotype (11).

The important question raised by this study is whether the otocional pathologies are caused by a loss of lysosomal proteolysis of some proteins and/or by an elevation in the level of certain proteins, such as p62. The new link described between a block in autophagy and reduced secretion of otocional proteins (11) is particularly intriguing and is reminiscent of the impaired secretion of antimicrobial peptides by intestinal Paneth cells in Atg16l1- and Atg5-hypomorphic mice (10).

A testable hypothesis for the link between autophagy and protein secretion
When considering whether the phenotypes observed in autophagy-deficient mice are the result of loss of lysosome-mediated autophagy per se and/or an elevation in the level of proteins such as p62, it is worth noting that p62 is more than just the receptor that delivers cargo to the autophagic machinery. It is a key signaling regulator, coordinating cell fate decisions and transcriptional programs that allow cells to cope with redox insults (15). These functions are mediated via p62 interaction with cellular partners such as Keap1, TRAF6, MEKK3, and caspase-8 (Figure 2, A and B). Thus, when p62 protein levels increase in cells as a consequence of a block in autophagy, other cellular pathways are also dysregulated. A great example of this is the finding that the liver-specific hepategaly and hypertrophy observed in Atg5- and Atg7-deficient mice is suppressed by the simultaneous depletion of p62 (17).

A recent study on the interaction between p62 and Keap1 suggests a potential avenue worthy of further exploration (16). Keap1 is a Cullin3-type ubiquitin ligase for Nrf2, a transcription factor that regulates the expression of many antioxidant proteins and detoxification enzymes. Interestingly, p62 competes with the Nrf2-binding site on Keap1. Thus, both overexpression of p62 and its accumulation due to reduced levels of autophagy disrupt the interaction between Nrf2 and Keap1, resulting in stabilization of Nrf2 and transcriptional activation of Nrf2 target genes (15). While studies on Nrf2 have focused on its transcriptional activation function (19), it is tempting to speculate that elevated p62 levels could also repress the transcription of genes such as Bloc1 and Ap3, perhaps directly or indirectly involving Nrf2-dependent mechanisms. This might explain why the levels of Bloc1 and Ap3 mRNA and protein are reduced in the Atg4B-deficient cells (11). Consequently, it would be interesting to analyze whether there are Nrf2-responsive antioxidant protein expression upstream of Bloc1 and Ap3. Additionally, would mice lacking Atg4B and either p62 or Nrf2 have no otocional defects? Given the prominent role of BLOC-1 and AP-3 in synaptic vesicle biogenesis (20), these analyses could also further our understanding of how dysregulated autophagy contributes to neuropathologies. Since Nrf2 activation is a response to redox stress, the notion of a block in protein secretion during conditions of elevated cellular stress has a precedent because another form of stress, nutrient starvation, also blocks protein secretion (21). Our hypothesis would predict that other protein secretion defects and pathologies might be caused by a reduction in autophagy while also adding to the repertoire of drug targets for interventions in these cases (22).

Acknowledgments
S. Subramani was supported by NIH grant GM069373 and A. Till by a fellowship from the German Research Foundation (DFG). Address correspondence to: Suresh Subramani, Room 3326 Bonner Hall, UCSD, La Jolla, California 92093, USA. Phone: 858.534.2327; Fax: 858.534.0053; E-mail: ssubramani@ucsd.edu.


