

## A balancing act for autophagin

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### Commentary

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 otoconia, organic particles that contain calcium carbonate crystals and proteins and that are essential for balance perception (equilibrioception) in mammals. The intriguing aspect of this work is that an autophagy block impairs the secretion and assembly of otoconial proteins, emphasizing a role for autophagy in functions distinct from macromolecule degradation.

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1. Bhide SA, Nutting CM. Advances in radiotherapy for head and neck cancer [published online ahead of print April 19, 2010]. *Oral Oncol*. doi:10.1016/j.oraloncology.2010.03.005.
2. Weiss JF, Landauer MR. Protection against ionizing radiation by antioxidant nutrients and phytochemicals. *Toxicology*. 2003;189(1-2):1-20.
3. Komarova EA, et al. Transgenic mice with p53-responsive lacZ: p53 activity varies dramatically during normal development and determines radiation and drug sensitivity in vivo. *EMBO J*. 1997;16(6):1391-1400.
4. Vousden KH, Prives C. Blinded by the Light: The Growing Complexity of p53. *Cell*. 2009;137(3):413-431.
5. Komarov PG, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science*. 1999;285(5434):1733-1737.
6. Strom E, et al. Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. *Nat Chem Biol*. 2006;2(9):474-479.
7. Leonova KI, et al. A small molecule inhibitor of p53 stimulates amplification of hematopoietic stem cells but does not promote tumor development in mice. *Cell Cycle*. 2010;9(7):1434-1443.
8. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature*. 2006;443(7108):214-217.
9. Gudkov AV, Komarova EA. Prospective therapeutic applications of p53 inhibitors. *Biochem Biophys Res Commun*. 2005;331(3):726-736.
10. Komarova EA, et al. Dual effect of p53 on radiation sensitivity in vivo: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice. *Oncogene*. 2004;23(19):3265-3271.
11. Burdelya LG, et al. Inhibition of p53 response in tumor stroma improves efficacy of anticancer treatment by increasing antiangiogenic effects of chemotherapy and radiotherapy in mice. *Cancer Res*. 2006;66(19):9356-9361.
12. Paris F, et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science*. 2001;293(5528):293-297.
13. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006;441(7092):431-436.
14. Herodin F, Bourin P, Mayol JF, Lataillade JJ, Drouot M. Short-term injection of antiapoptotic cytokine combinations soon after lethal gamma-irradiation promotes survival. *Blood*. 2003;101(7):2609-2616.
15. Burdelya LG, et al. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science*. 2008;320(5873):226-230.
16. Johnson SM, et al. Mitigation of hematologic radiation toxicity in mice through pharmacological quiescence induced by CDK4/6 inhibition. *J Clin Invest*. 2010;120(7):2528-2536.
17. Denekamp J. Cell kinetics and radiation biology. *Int J Radiat Biol Relat Stud Phys Chem Med*. 1986;49(2):357-380.
18. Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta*. 2002;1602(1):73-87.
19. Diaz-Padilla I, Siu LL, Duran I. Cyclin-dependent kinase inhibitors as potential targeted anticancer agents. *Invest New Drugs*. 2009;27(6):586-594.
20. Fry DW, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Mol Cancer Ther*. 2004;3(11):1427-1438.
21. Demidenko ZN, Korotchkina LG, Gudkov AV, Blagosklonny MV. Paradoxical suppression of cellular senescence by p53. *Proc Natl Acad Sci U S A*. 2010;107(21):9660-9664.

## A balancing act for autophagin

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**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 otoconia, organic particles that contain calcium carbonate crystals and proteins and that are essential for balance perception (equilibrioception) in mammals. The intriguing aspect of this work is that an autophagy block impairs the secretion and assembly of otoconial 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

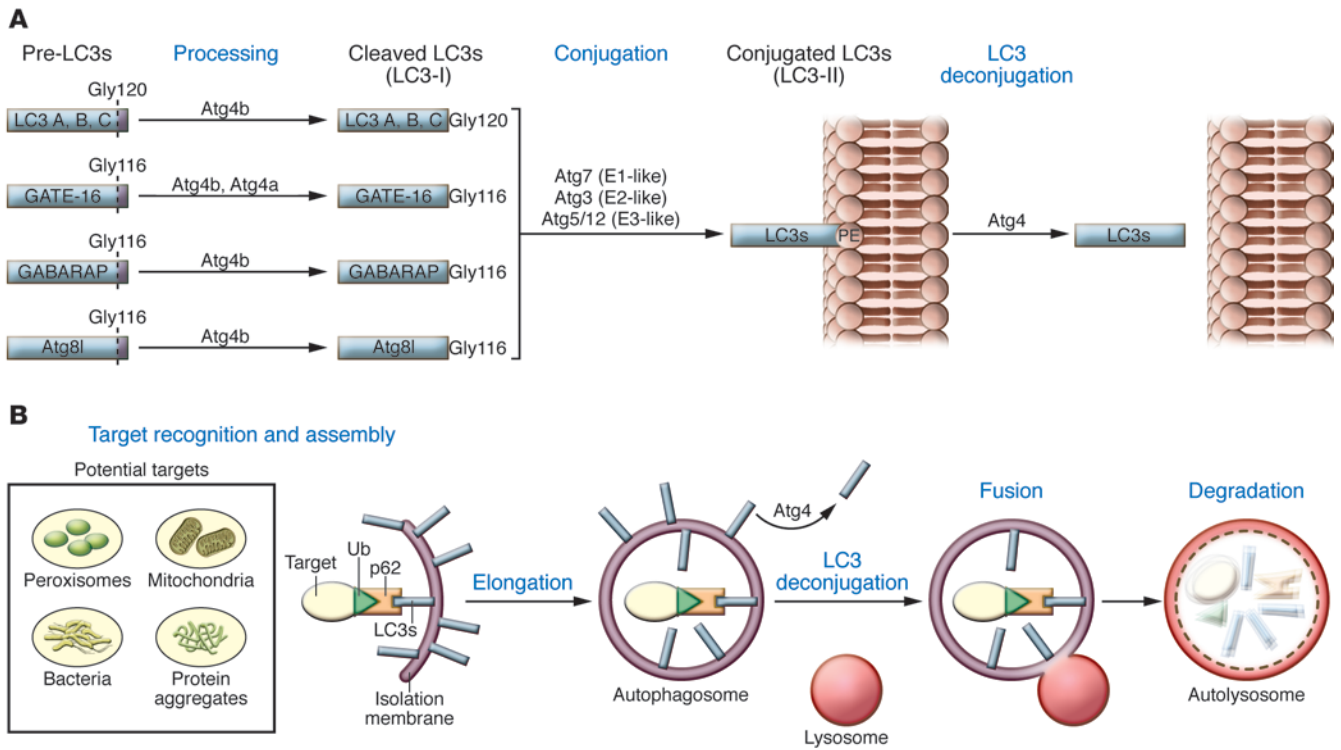
known as the isolation membrane wraps around portions of the cytoplasm to form a double-membrane vesicle, the autophagosome. The engulfed cargo, including organelles, is degraded upon autophagosome fusion with late endosomes or lysosomes.

Autophagy plays a central role in cancer, neurodegeneration, innate immunity, organelle 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  $\beta$  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

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**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.

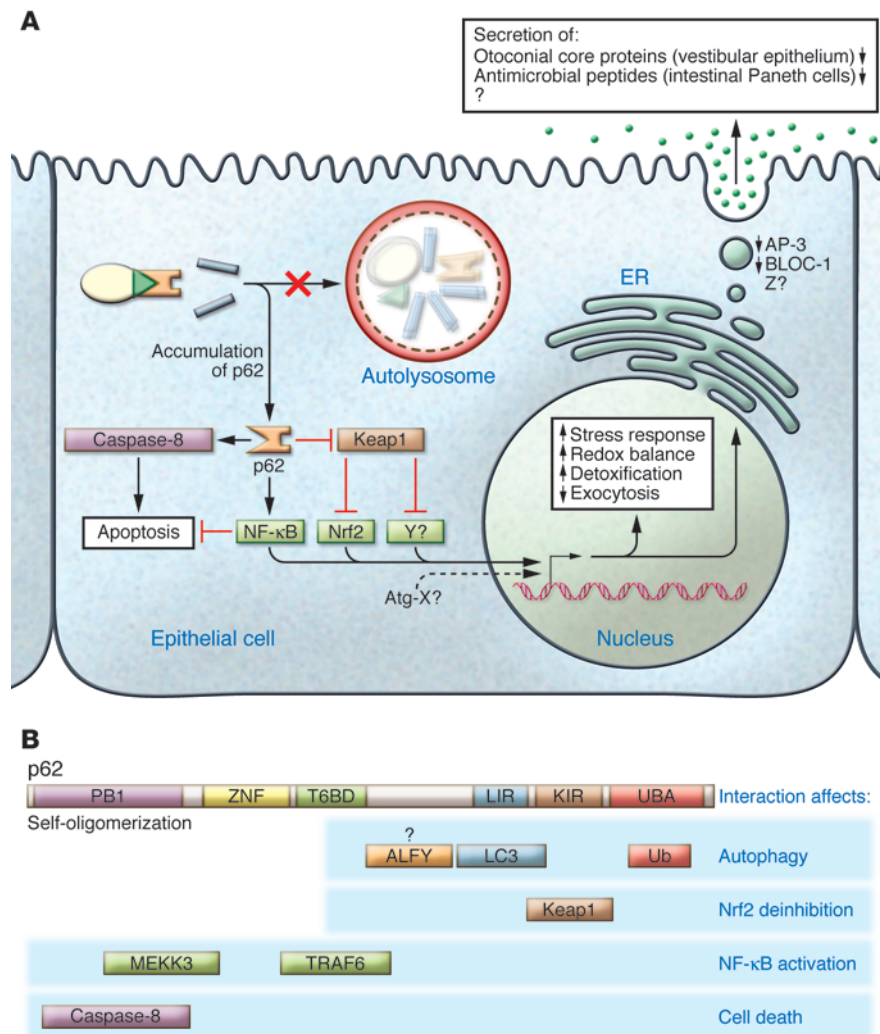
lysosome-mediated autophagy per se, but also by other effects such as an increase in the cellular concentrations of certain multifunctional signaling proteins (e.g., p62, as discussed in “A testable hypothesis for the link between autophagy and protein secretion”). In this issue of the *JCI*, Mariño 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 otoconia, 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 otoconia 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 Atg8: microtubule-associated protein 1 light chain 3 (LC3), of which there are three isoforms (LC3A–C); Golgi-associated ATPase enhancer of 16 kDa (GATE-16, also known as GABARAP-like 2 [GABARAPL2]); GABA(A) receptor-associated protein (GABARAP); and Atg8l. These yeast and mammalian proteins are activated by a proteolytic cleavage event mediated by a family of cysteine proteases dubbed “autophagins” (i.e., the family of Atg4 proteins) (Figure 1A). Four Atg4 isoforms have been described in mammals (Atg4a–d). The Atg4 proteases cleave the LC3 precursors following a conserved glycine residue (12)

that is then conjugated to the amino group of phosphatidylethanolamine (PE) using a canonical ubiquitin-conjugating enzyme cascade (Figure 1A) (13). Atg4 proteases also deconjugate LC3 homologs from the membrane. The conjugated form of LC3 (LC3-II) associates with the isolation membrane, which engulfs autophagic cargoes into the autophagosome (Figure 1B) (13).

In mammalian cells, many autophagic cargoes captured for degradation are polyubiquitylated. These ubiquitylated proteins are bound by the cytosolic receptor sequestosome 1 (SQSTM1; referred to herein as p62) (14). The p62 protein represents a pivotal molecular switch for several signaling pathways (Figure 2A) (15). For example, p62 promotes caspase-8–dependent apoptotic signaling and mediates the activation of the transcription factor NF-κB in response to a variety of stimuli by interacting with TNF receptor-associated factor 6 (TRAF6) and



**Figure 2**  
 Multifaceted role of autophagy. **(A)** Known and potential roles of the autophagy machinery in divergent cellular events. Besides lysosome-dependent degradation of ubiquitin-tagged targets, autophagy also mediates homeostasis of the level of the protein p62. The multifunctional p62 represents a pivotal molecular switch contributing to caspase-8-dependent cell death, NF-κB activation, and regulation of Nrf2. Impairment of autophagy elevates p62 levels, which might activate the transcriptional regulator Nrf2 via effects on Keap1. Nrf2 activates stress response proteins but might also inhibit transcription of *Ap3* and *Bloc1* (directly or indirectly). Alternatively, Keap1 might regulate other transcription factors (Y?) that modulate expression of these genes. Thus, a block in autophagy may cause changes in the composition of the secretion machinery, affecting vesicle sorting and secretion of otoconial proteins. It remains to be elucidated whether additional proteins (represented here as autophagy protein Atg-X, transcription factor Y, and secretory protein Z) are involved in this complex interplay. **(B)** Structural basis of the pleiotropic function of p62. Shown are structural elements of p62 that mediate interaction with key proteins of multiple cellular pathways. Each interaction may influence the outcome of divergent cellular events such as autophagy (via simultaneous binding of LC3 and ubiquitinated targets), deactivation of Nrf2 signaling (via competition for Keap1 binding), NF-κB activation (via association with MEKK3 and TRAF6), and apoptosis (via aggregation of caspase-8). The recently described association between p62 and autophagy-linked FYVE protein (ALFY) (23) has not yet been mapped to a defined structural motif (indicated by question mark). Self-oligomerization of p62 is mediated by the PB1 domain. PB1, Phox and Bem1 domain; ZNF, zinc finger domain; T6BD, TRAF6-binding domain; KIR, Keap1-interacting region.

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 otoconins) and inorganic calcite (CaCO<sub>3</sub>) crystals. By stimulating the vestibular sensory epithelium upon movement, otoconia facilitate perception of gravity and linear acceleration. The otoconial biogenesis defects in the *Atg4b*<sup>-/-</sup> mice





observed by Mariño 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 otoconia formation defects to an autophagy deficiency that resulted in impaired secretion of otoconial core proteins into the vestibular lumen. The outcome of this defect was the cytosolic mislocation of these proteins and thus the impairment of otoconial assembly. The reduced secretion of otoconial 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 otoconial proteins from the Golgi apparatus to the plasma membrane. To prove that this defect resulted from impaired autophagy, Mariño et al. showed that *Atg5*<sup>-/-</sup> mice exhibited a similar phenotype (11).

The important question raised by this study is whether the otoconial 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 otoconial 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 hepatomegaly 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 response elements upstream of *Bloc1* and *Ap3*. Additionally, would mice lacking *Atg4b* and either p62 or Nrf2 have no otoconial 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).

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1. Meijer AJ, Codogno P. Autophagy: regulation and role in disease. *Crit Rev Clin Lab Sci.* 2009; 46(4):210-240.

- Hampe J, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet.* 2007;39(2):207-211.
- Parkes M, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet.* 2007;39(7):830-832.
- Hara T, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature.* 2006;441(7095):885-889.
- Komatsu M, et al. Impairment of starvation-induced and constitutive autophagy in *Atg7*-deficient mice. *J Cell Biol.* 2005;169(3):425-434.
- Kuma A, et al. The role of autophagy during the early neonatal starvation period. *Nature.* 2004;432(7020):1032-1036.
- Komatsu M, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature.* 2006;441(7095):880-884.
- Ebato C, et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab.* 2008;8(4):325-332.
- Jung HS, et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab.* 2008;8(4):318-324.
- Cadwell K, et al. A key role for autophagy and the autophagy gene *Atg16L1* in mouse and human intestinal Paneth cells. *Nature.* 2008; 456(7219):259-263.
- Mariño G, et al. Autophagy is essential for mouse sense of balance. *J Clin Invest.* 2010;120(7):2331-2344.
- Hemelaar J, Lelyveld VS, Kessler BM, Ploegh HL. A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L. *J Biol Chem.* 2003;278(51):51841-51850.
- Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. *Curr Top Microbiol Immunol.* 2009;335:1-32.
- Bjorkoy G, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol.* 2005;171(4):603-614.
- Komatsu M, Ichimura Y. Physiological significance of selective degradation of p62 by autophagy. *FEBS Lett.* 2010;584(7):1374-1378.
- Komatsu M, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol.* 2010;12(3):213-223.
- Komatsu M, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell.* 2007;131(6):1149-1163.
- Marino G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in *Atg4c/autophagin-3*. *J Biol Chem.* 2007;282(25):18573-18583.
- Zhang DD. Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab Rev.* 2006;38(4):769-789.
- Newell-Litwa K, Salazar G, Smith Y, Faundez V. Roles of BLOC-1 and adaptor protein-3 complexes in cargo sorting to synaptic vesicles. *Mol Biol Cell.* 2009;20(5):1441-1453.
- Shorer H, Amar N, Meerson A, Elazar Z. Modulation of N-ethylmaleimide-sensitive factor activity upon amino acid deprivation. *J Biol Chem.* 2005;280(16):16219-16226.
- Yates MS, Kensler TW. Chemopreventive promise of targeting the Nrf2 pathway. *Drug News Perspect.* 2007;20(2):109-117.
- Clausen TH, et al. p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. *Autophagy.* 2010;6(3):330-344.