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Ken Cadwell

Washington University School of Medicine in St. Louis

Khushbu K. Patel

Washington University School of Medicine in St. Louis

Masaaki Komatsu

Tokyo Metropolitan Institute of Medical Science

Herbert W. Virgin IV

Washington University School of Medicine in St. Louis

Thaddeus S. Stappenbeck

Washington University School of Medicine in St. Louis

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Article Addendum

A common role for Atg16L1, Atg5 and Atg7 in small intestinal Paneth cells and Crohn disease

Ken Cadwell,¹ Khushbu K. Patel,¹ Masaaki Komatsu,³ Herbert W. Virgin IV^{1,2} and Thaddeus S. Stappenbeck^{1,*}

¹Department of Pathology and Immunology, and ²Department of Molecular Microbiology; Washington University School of Medicine; St. Louis, Missouri USA; ³Laboratory of Frontier Science; Tokyo Metropolitan Institute of Medical Science; Bunkyo-ku, Tokyo, Japan

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Recently identified genetic determinants for enhanced susceptibility to Crohn disease (CD) included polymorphisms in the *ATG16L1* and *IRGM1* loci suggesting that the autophagy pathway plays a role in the pathogenesis of this disease. We have generated and analyzed three mouse models with diminished expression of autophagy proteins and show how the loss of function of various autophagy components contributes to CD pathogenesis. In the mouse small intestine, one common cellular target of Atg16L1, Atg5 and Atg7 is the Paneth cell, a specialized epithelial cell whose main function is the delivery of antimicrobial factors into the intestinal lumen by production and secretion of its characteristic cytoplasmic granules. Autophagy-deficient Paneth cells exhibited a striking loss of function in this granule exocytosis pathway. Transcriptional analysis revealed a gain of function whereby the gene expression associated with inflammatory responses was increased in autophagy-deficient Paneth cells. Importantly, we validated these findings by analyzing intestinal tissues from CD patients. Similar Paneth cell abnormalities were observed in CD patients homozygous for the *ATG16L1* risk allele. Thus, one role for the autophagy pathway in CD pathogenesis is through selective effects on the biology and specialized properties of Paneth cells.

Atg16L1 Mutant Mice Display Reduced Autophagy and Paneth Cell Abnormalities

More than 30 genetic loci are now associated with increased susceptibility to CD.¹ This major type of inflammatory bowel disease is most commonly characterized by uncontrolled, transmural inflammation in the distal ileum (small intestine), though the colon can

be involved as well. One CD susceptibility allele is in the predicted autophagy gene *ATG16L1*.²⁻⁵ To determine the role of Atg16L1 in the intestine, we generated two mouse lines with gene trap-mediated disruptions of the *Atg16L1* locus, Atg16L1^{HM1} and Atg16L1^{HM2} (collectively referred to as Atg16L1^{HM}).⁶ These mice displayed reduced (hypomorphic, HM) expression of Atg16L1 protein, and both fibroblasts and intestinal tissue from these mice were compromised in autophagy activity indicating that Atg16L1, like yeast Atg16, is a bona fide autophagy protein.

The intestinal epithelium is constantly exposed to a wide and diverse population of commensal microbes (microbiota)⁷ and is sporadically exposed to enteric pathogens. Mice deficient in *Nod2*, another CD susceptibility gene, displayed increased sensitivity to oral infection by *Listeria Monocytogenes*.⁸ Thus, one potential mechanism by which autophagy contributes to intestinal homeostasis is through clearance of intracellular pathogens, a well-documented function of autophagy.⁹⁻¹¹ However, we did not detect increased susceptibility to oral *L. monocytogenes* infection in Atg16L1^{HM} mice, suggesting that there may be differences in the roles of *Nod2* and Atg16L1 in the small intestine. Instead, in the absence of exogenous bacterial infection, we found striking morphological abnormalities in Paneth cells that are a highly specialized epithelial cell located at the base of epithelial invaginations called crypts of Lieberkühn. Paneth cells likely shape the intestinal microbiota via secretion of granule contents including antimicrobial peptides and lysozyme.¹² By light and ultrastructural analysis, Paneth cells in Atg16L1^{HM} mice displayed a reduction in the number of granules, dramatically increased cytoplasmic vesicles, and abnormal mitochondria. Furthermore, lysozyme, which is normally packaged efficiently into the granules, displayed striking defects in its distribution including diffuse cytoplasmic expression.

To understand the nature and potential etiology of these morphological abnormalities, we examined the transcriptional profile of Atg16L1-deficient Paneth cells. Microarray analysis comparing ileal crypt base cells (which are primarily Paneth cells)¹³ procured by laser capture microdissection from Atg16L1^{HM} mice and controls revealed an increase in transcripts associated with PPAR signaling, acute phase reactants, adipocytokine signaling and lipid metabolism in the Atg16L1^{HM} mice. Many of these genes are directly implicated in inflammation, and two of these transcripts, leptin and adiponectin, are known to be increased in CD patients.^{14,15} Thus,

*Correspondence to: Thaddeus S. Stappenbeck; Department of Pathology and Immunology; Washington University School of Medicine; 660 S. Euclid Ave; Box 8118; St. Louis, Missouri 63110 USA; Tel.: 314.362.4214; Fax: 314.362.7487; Email: stappenb@wustl.edu

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in addition to its role in maintaining the granule exocytosis pathway, Atg16L1 is an important brake for the expression of proinflammatory genes in Paneth cells.

Identification of a Novel Pathological Hallmark of CD Associated with *ATG16L1*

Since Atg16L1 is important for the biology of Paneth cells in mice, we examined the role of the human *ATG16L1* CD risk allele. Compared to control ileal samples from CD patients that did not carry the risk alleles, ileal specimens from CD patients homozygous for the *ATG16L1* risk allele displayed Paneth cell abnormalities remarkably similar to the Atg16L1^{HM} mice. In addition to the morphological abnormalities, in comparison with controls, human Paneth cells with the CD risk polymorphism of *ATG16L1* were more likely to overexpress leptin protein, an observation that was prompted by the transcriptional analysis in mice. Since the role of NOD2 is controversial in both secretion as well as production of antimicrobial peptides by Paneth cells,¹⁶⁻²¹ our analysis only included samples from patients that did not carry the *NOD2* CD risk alleles. In the future, it will be interesting to examine the effect of polymorphisms in *NOD2* and other CD susceptibility genes using the same assays.

The Role of the Autophagy Pathway in Paneth Cell Abnormalities

The *ATG16L1* variant associated with CD encodes a threonine to alanine substitution that may result in reduced protein expression,²² but Atg16L1 may have roles outside of autophagy. To further examine the potential role of the autophagy pathway in Paneth cell and CD, we generated mice that were deficient in Atg5 in the intestinal epithelium by breeding Atg5^{flox/flox} mice²³ with villin-Cre mice that express the Cre recombinase specifically in the intestinal epithelium including Paneth cells²⁴ (Atg5^{flox/flox}villin-Cre). Remarkably, these mice display morphological abnormalities in their Paneth cells that are indistinguishable from the Atg16L1-deficient mice. Moreover, like the Atg16L1^{HM} mice, Atg5^{flox/flox}villin-Cre mice do not display obvious morphological abnormalities outside the Paneth cells within the ileal epithelium. Subsequently, we have generated mice with a conditional deletion of Atg7 in the intestinal epithelium (Atg7^{flox/flox}villin-Cre). By histological and immunohistochemical characterization, the ileal pathology in these mice is indistinguishable from that observed in Atg16L1^{HM} and Atg5^{flox/flox}villin-Cre mice (Fig. 1). Thus, these results suggest that a defect in the autophagy pathway in the intestinal epithelium is responsible for the Paneth cell pathology.

How does an autophagy defect lead to such specific effects on Paneth cells, while other small intestinal epithelial lineages and stem cells do not exhibit obvious morphological abnormalities? Paneth cells, in contrast to other intestinal epithelial cells, contain abundant endoplasmic reticulum (ER). Loss of key autophagy proteins may disrupt autophagy-mediated organelle turnover thus disrupting ER homeostasis. Studies examining the intestinal role of Xbp1, a transcription factor important in ER homeostasis, provide evidence that Paneth cells are particularly sensitive to ER stress.²⁵ Indeed, electron microscopy of Atg16L1-deficient Paneth cells reveals degeneration of mitochondria and replacement of ER with excess vesicular structures.

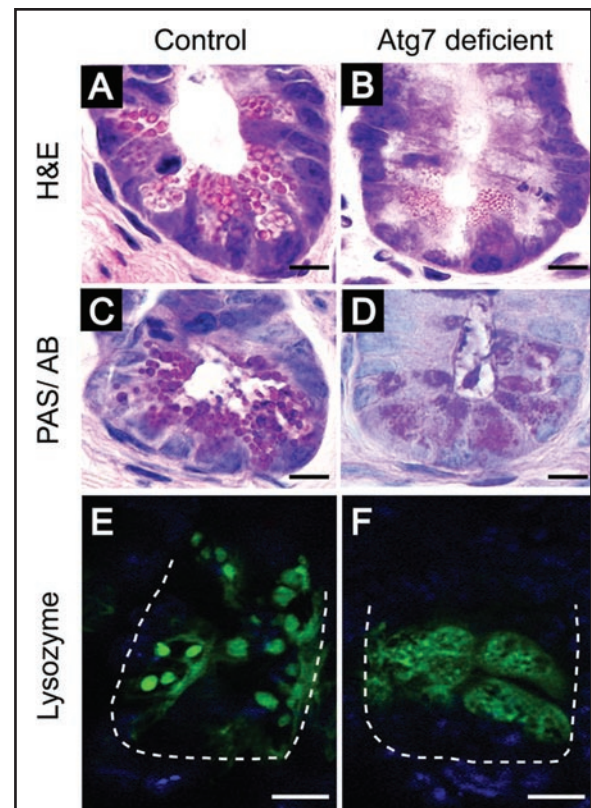


Figure 1. Loss of *Atg7* in the intestinal epithelium leads to Paneth cell abnormalities similar to those associated with loss of function for both *Atg16L1* and *Atg5*. Atg7^{flox/flox} mice³⁰ were bred with mice expressing the Cre recombinase from the villin promoter (villin-Cre)²⁴ to generate mice with conditional deletion of *Atg7* in the intestinal epithelium (Atg7^{flox/flox}villin-Cre). (A–D) Histological examination of the distal (ileal) small intestine reveals morphologically aberrant Paneth granules in mice with loss of *Atg7*. Ileal sections from Atg7^{flox/flox} control (A, C and E) or Atg7^{flox/flox}villin-Cre mice (B, D and F) were stained with hematoxylin and eosin (A and B), PAS/alcian blue (C and D), or goat-antilysozyme antisera and Alexaflour 488-labeled donkey anti-goat Ig (E and F). Mice with loss of *Atg7* in the intestinal epithelium contain Paneth cell granules that are abnormal in size, morphology and number. Instead of labeling granules as in Atg7^{flox/flox} control Paneth cells, lysozyme staining displayed abnormal diffuse cytoplasmic distribution in Atg7^{flox/flox}villin-Cre samples. Green represents lysozyme, blue represents nuclei, and the dashed line denotes the crypt unit. Bars = 10 μ m.

However, loss of Xbp1 does not completely mirror autophagy deficiency, as Paneth cells die in the former model but not in the latter. Another possible explanation for the vesicle formation is that there are autophagosome-independent roles for autophagy proteins as has been previously reported.^{11,26-29}

Conclusion

Using mice with mutations in Atg16L1 and Atg5, and now reported here in Atg7, we have described a novel role for the autophagy pathway in Paneth cells that we confirmed in human CD samples. One consequence of this lineage-specific role of autophagy is that future studies will have to investigate the function of Atg16L1 in relevant cell-types *in vivo*. For instance, to examine the precise effect of the *ATG16L1* polymorphism, a knock-in mouse model in which the endogenous *Atg16L1* is replaced by the CD risk allele of *ATG16L1* would be particularly useful. It may also be informative to

cross Atg16L1^{HM} mice to other models for CD susceptibility such as *Nod2* mutant mice. Moreover, in addition to a clear genetic component, there is also an ill-defined environmental component of CD. Therefore, it will be critical to determine what factors contribute to the observed Paneth cell defect and if environmental damage such as enteric pathogens leads to pathological changes in Atg16L1-deficient mice. Rarely are individual pathological hallmarks of a complicated disease associated with a specific gene, and thus, Atg16L1^{HM} mice provide a unique opportunity to advance our understanding of a complex and debilitating disease.

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