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Vacuolating Cytotoxin and Variants in *Atg16L1* that Disrupt Autophagy Promote *Helicobacter pylori* Infection in Humans

Short Title: *H. pylori* disrupts autophagy

D. Raju[1], S. Hussey[1,2], M. Ang[1], M.R. Terebiznik[3], M. Sibony[1,11], E. Galindo-Mata[1],V. Gupta[4], S.R. Blanke[4], A. Delgado[5], J. Romero-Gallo[5], M. Ramjeet[6], H. Mascarenhas[1,2], R.M. Peek[5], P. Correa[5], C. Streutker[7], G. Hold[8], E. Kunstmann[9], T. Yoshimori[10], M. S. Silverberg[11], S.E. Girardin[6], D.J. Philpott[2], E. El Omar[8], N.L. Jones[1]*

[1] Cell Biology Program, Research Institute, Hospital for Sick Children, Toronto, ON, Canada, Departments of Paediatrics and Physiology, University of Toronto, Toronto, ON, Canada

[2]Department of Immunology, University of Toronto, Toronto, ON Canada

[3]Department of Cell and Systems Biology, University of Toronto, Scarborough, ON, Canada

[4]Department of Microbiology and Institute for Genomic Biology, University of Illinois, Urbana, IL, USA.

[5]Division of Gastroenterology, Hepatology and Nutrition, Vanderbilt University Medical Centre, Nashville, TN

[6]Department of Laboratory Medicine and Pathobiology, and the Li Ka Shing Knowledge Institute University of Toronto, Toronto, ON

[7]Department of Laboratory Medicine, St. Michaels's Hospital, Toronto, ON

[8]Institute of Medical Sciences, School of Medicine and Dentistry, Aberdeen University, Foresterhill, Aberdeen

[9] Praxis fur Humangenetik, Theodore-Boveri-Weg, University Wuerzburg, Germany

[10]Department of Cellular Regulation, Research institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

[11]Zane Cohen Centre for Digestive Diseases, IBD group, Mount Sinai Hospital, Toronto, ON

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***Corresponding Author**

Dr. Nicola Jones

Departments of Paediatrics and Physiology, University of Toronto

Cell Biology Program, Hospital for Sick Children

555, University Avenue

Toronto, ON M5G1X8

Phone no: 416-813-7072

Email: Nicola.jones@sickkids.ca

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Author Contributions:

DR, MRT and MA performed experiments and data analysis. MR, MS, EGM, HM, JRG and GH performed experiments and sample preparation.

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VG, SRB, TY, RMP, PC, MSS provided reagents. CS helped with data analysis. NLJ, DR, MT, SH planned the experiments. NLJ, DR, SH, MT, RMP, EEO, SG, DP wrote the manuscript. NLJ conceived of and supervised the study.

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Abstract

Background & Aims: The *Helicobacter pylori* toxin vacuolating cytotoxin (VacA) promotes gastric colonization and its presence (VacA+) is associated with more-severe disease. The exact mechanisms by which VacA contributes to infection are unclear. We previously found that limited exposure to VacA induces autophagy of gastric cells, which eliminates the toxin; we investigated whether autophagy serves as a defense mechanism against *H pylori* infection.

Methods: We investigated the effect of VacA on autophagy in human gastric epithelial cells (AGS) and primary gastric cells from mice. Expression of p62, a marker of autophagy, was also assessed in gastric tissues from patients infected with toxigenic (VacA+) or nontoxigenic strains. We analyzed the effect of VacA on autophagy in peripheral blood monocytes obtained from subjects with different genotypes of *ATG16L1*, which regulates autophagy. We performed genotyping for *ATG16L1* in two cohorts of infected and uninfected subjects.

Results: Prolonged exposure of AGS and mouse gastric cells to VacA disrupted induction of autophagy in response to the toxin, because the cells lacked cathepsin-D in autophagosomes. Loss of autophagy resulted in the accumulation of p62 and reactive oxygen species. Gastric biopsies samples from patients infected with VacA+, but not nontoxigenic strains of *H pylori*, had increased levels of p62. Peripheral blood monocytes isolated from individuals with polymorphisms in *ATG16L1* that increase susceptibility to

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Crohn's disease had reduced induction of autophagy in response to VacA+ compared to cells from individuals that did not have these polymorphisms. The presence of the *ATG16L1* Crohn's disease risk variant increased susceptibility to *H pylori* infection in 2 separate cohorts.

Conclusions: Autophagy protects against infection with *H pylori*; the toxin VacA disrupts autophagy to promote infection, which could contribute to inflammation and eventual carcinogenesis.

Keywords: stomach cancer, genetic, bacteria toxin, tumor

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Introduction:

Helicobacter pylori has been classified as a Class I carcinogen by the World Health Organization [1]. Chronic *H. pylori* infection is a risk for the onset of serious gastric disease, including peptic ulcer disease, mucosa lymphoid tissue (MALT) lymphoma and gastric cancer, the second leading cause of cancer deaths worldwide [1]. The mechanisms responsible for initial susceptibility to infection and subsequent chronic infection involve a complex interplay between host and bacterial factors. One specific virulence factor important for colonization and disease outcome is the vacuolating cytotoxin (VacA) [2]. The exact mechanisms by which VacA contributes to colonization and disease outcome remain unclear.

Autophagy is an evolutionarily conserved process that results in the sequestration of cytosolic components within double membrane compartments called autophagosomes. These compartments fuse with lysosomes to become autophagolysosomes, which degrade vesicle contents through the action of lysosomal hydrolases. Although initially recognized to occur in response to cellular stresses such as nutrient starvation, current evidence indicates that autophagy plays a critical role in modulating host immunity and inflammatory responses. Importantly autophagy is thought to serve as an innate defense mechanism against infection. Autophagy is stimulated in response to the invasion of intracellular pathogens and the presence of bacterial toxins [3]. In addition, studies in *Caenorohabditis elegans*, *Drosophila* and mice suggest that autophagy is a critical pathway for controlling infection. For example, inactivation of an autophagy dependent gene in *C. elegans* increases intracellular replication of *Salmonella typhimurium* [4]. In mice, disruption of a bacterial-selective autophagy pathway promotes intracellular

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replication of *Shigella* [5]. However, a number of pathogens evade or utilize the autophagy pathway for enhanced survival and persistence in host cells [6,7].

While an increasing number of pathogens have been demonstrated to subvert autophagic pathways to promote intracellular survival, much less is known about the potential consequences of pathogen-mediated disruption of autophagic pathways or, alternatively, the influence of autophagic defects within host cells for controlling infection in humans and influencing disease pathology. Recent studies from our lab revealed that within gastric epithelial cells *in vitro*, autophagic signaling is induced in response to infection with *H. pylori* in a VacA-dependent manner [8]. Furthermore, autophagy eliminates VacA. Here, we specifically evaluated the biologic significance of the autophagy pathway during *H. pylori* infection *in vitro* and *in vivo* in human subjects, and assessed the influence of both pathogen and host genetic factors.

Methods and Materials:

Cells, bacteria and antibodies:

Culture conditions for human gastric epithelial cells (AGS) and murine primary gastric cells were as described previously [9,10]. Growing conditions for wild-type *H. pylori* strain 60190 (ATCC 49503; *cagA*+ *cagE*+ VacA+) and its isogenic *vacA* mutant strain (provided by Dr. R. Peek) were as described previously [11,12]. Rabbit polyclonal p62 antibodies were from Santacruz Biotechnology, CA. Rabbit anti-*H. pylori* antibodies were from DAKO (Denmark). Mouse anti-human Lamp1 antibodies were purchased from (Developmental Studies Hybridoma Bank, Iowa City, IA). Cathepsin D antibodies

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were from Upstate (Lake Placid, NY). Rabbit anti-VacA antibodies were a kind gift from Dr. S. R. Blanke. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Detection of reactive oxygen species and cell death using FACS:

For the detection of reactive oxygen species, a redox-sensitive dye 5-(and-6)-chloromethyl-20,70-dichlorodihydrofluorescein diacetate, acetyl ester(CM-H_2DCFDA) was used. Fluorescence was measured using a FACSCalibur flow cytometer.

Cell death analysis was performed using 3μM propidium iodide (Invitrogen) in PBS buffer and assessed by fluorescent-activated cell sorting FACS (FACSCalibur, BD Biosciences). The percentage of stained cells was determined and compared with appropriate negative controls.

Isolation of Peripheral blood monocytes (PBMC):

Venous blood was drawn from healthy genotyped volunteers in sterile EDTA coated tubes (BD Vacutainer,) diluted 1:1 with pyrogen-free saline and layered over Ficoll-Paque (GE Healthcare Sciences, Canada). Approval for the study was provided by the local ethics board (Approval No. MSH REB#02-0234-E).

Cells were spun at 400g for 30min to obtain the mononuclear cell fractionation via density centrifugation, washed and suspended in culture medium. Cells were counted and plated in 6-well plates where they were treated with various stimuli: muramyl dipeptide (MDP, 10μg/ml, InvivoGen, San Diego), and concentrated culture supernatants of *H. pylori* strain 60190 (200μl/ml) for 24 hours with bafilomycin (50μg/ml, Sigma) and

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rapamycin (100ng/ml, Enzo Life Sciences International, USA) used for the last 4 hours of incubation. Collected cells were then lysed and used for immunoblots.

Densitometric analysis was performed using FlourChem FCII software. All blots from each independent experiment were used. Densities of the LC3 II and actin bands were measured for each treatment and expressed as a ratio of LC3II/actin, normalized to each subjects control.

Statistical analysis:

Analysis of variance (ANOVA), unpaired T test and other statistical analysis of the results were performed utilizing GraphPad Prism 4 for Macintosh V 4.0b.

Other assays:

Immunofluorescence, immunohistochemistry, immunoblotting, bacterial survival, genotyping and other assays were performed as previously described and are outlined in detail in the supplementary materials section.

Results:

Prolonged exposure to VacA toxin causes accumulation of defective autophagosomes:

Previous studies have shown that limited exposure to *H. pylori* VacA for 6h triggers autophagy in AGS cells, resulting in degradation of the toxin in autophagosomes suggesting that autophagy serves as a protective host response during infection [8]. To further assess the biologic significance of the pathway during infection we exposed

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gastric cells to VacA toxin for prolonged periods to mimic the setting of chronic infection and utilized the LC3-eGFP-mRFP tandem construct to follow the maturation of autophagosomes [13]. After formation, autophagosomes fuse with lysosomes and acquire cathepsins and acid phosphatases to become mature autolysosomes [6,14]. Modified LC3 remains associated with autophagosomes until hydrolysis at the autolysosomal stage. During autophagosome maturation the mRFP-LC3 signal persists longer than the GFP-LC3 signal since mRFP is resistant to autolysosomal proteases and has a lower pKa [13]. As we had previously shown that VacA was necessary and sufficient to induce autophagy and that autophagy did not occur during infection with isogenic *vacA*- mutants we focused our experiments on VacA. When AGS cells transfected with the tandem construct were treated with culture supernatants from VacA+ *H. pylori* (CCMS), both the GFP-LC3 and mRFP-LC3 signals persisted (Figure 1B,D) in a comparable manner to cells incubated with vinblastine (Figure 1C,D), which prevents autophagosome-lysosome fusion. These findings were recapitulated in VacA+ CCMS treated primary murine gastric epithelial cells indicating the effect was not related to cancerous transformation (Supplementary figure 1).

Next we measured the hydrolysis of long half-life proteins, whose turnover depends on autophagy, in VacA+ CCMS treated cells [15,16]. Figure 1E shows that the degradation of long half-life proteins in VacA+ CCMS treated cells was lower than in cells undergoing autophagy triggered by rapamycin treatment. Taken together, these findings suggest that the autophagosomes formed by VacA are defective and have reduced catalytic activity.

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VacA protects cells from rapamycin-mediated cell death:

Autophagy is considered cytoprotective but in certain settings excessive autophagy can cause cell death [17,18]. For instance, overexpression of Atg1 in *Drosophila melanogaster* salivary glands triggers an autophagic phenotype followed by cell loss which is prevented by inhibiting autophagy [19]. Similarly prolonged treatment with rapamycin, which triggers autophagy, induces death of mammalian cells [20]. Therefore, we next assessed the effect of VacA mediated changes in autophagy on rapamycin-induced cell death. Similar to ATG12 siRNA treated cells, pre-incubation of cells with VacA + CCMS attenuated rapamycin-triggered cell death (Figure 1F, Supplementary figure 2). These findings indicate that VacA effects on autophagy can promote increased cell viability in response to rapamycin comparable to siRNA-mediated disruption of autophagy (Supplementary figure 2).

VacA induced autophagosomes lack Cathepsin-D:

We next determined if VacA+CCMS-triggered autophagosomes failed to mature due to lack of fusion with lysosomes. VacA+CCMS-induced autophagosomes acquired the late endosomal lysosomal marker Lamp1 (Figure 2) and co localized with lysosensor probes (Supplementary figure 3) indicating that autophagosome-lysosome fusion and acidification were not affected.

Previous reports indicate that VacA alters the degradative properties of the endocytic pathway by subverting the sorting and activation of cathepsin enzymes [11,21]. We had shown that in VacA positive but not isogenic VacA mutant infected cells, lysosomes lack cathepsin D, a key hydrolase, which in turn affects their degradative properties [11,21].

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Therefore, we determined if cathepsin D was present in autophagosomes in VacA+CCMS treated cells. As shown in Figure 2 A-E and Supplementary figure 4, VacA+CCMS treated cells displayed a reduction in cathepsin D over time in comparison with control cells. Furthermore, in comparison with rapamycin, starvation, and vinblastine treated cells, negligible amounts of cathepsin D were found in Lamp1-labeled late endosomes in CCMS-treated cells. These findings indicate that VacA -induced autophagosomes fuse with lysosomes and acquire an acidic pH but lack cathepsin D and thus the catalytic activity required to complete the autophagic degradative process [22].

VacA induces formation of p62 aggregates *in vitro* and *in vivo*:

Dysfunctional autophagy leads to an accumulation of p62, which has been implicated in promoting tumorigenesis [23,24]. Therefore, we determined the levels of p62 in AGS cells treated with VacA+CCMS. AGS cells treated with VacA+CCMS showed accumulation of p62, which increased over time and co-localized with GFP-LC3 (Figure 3A-E). The accumulation of p62 in VacA+CCMS-treated cells was similar to the p62 aggregates in cells where autophagy was suppressed using ATG12 siRNA (data not shown).

To extend our *in vitro* findings to the bacterial niche in humans, we next looked at p62 expression in human gastric biopsies. All *H. pylori* strains carry the VacA gene and can be divided into toxigenic, expressing a functional VacA toxin, or nontoxigenic based on their VacA genotype (*s1m1 vs s2m2*). Gastric tissue sections from patients infected with toxigenic *s1m1* VacA-producing strains showed a significantly higher accumulation of p62 in the foveolar cells of the gastric epithelium when compared to tissue sections

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obtained from patients infected with a nonfunctional VacA genotype (*s2m2*) strain (Figure 3F-G). These *in vivo* observations were concordant with our *in vitro* findings. Furthermore, the expression level of p62 was independent of the degree of gastric inflammation indicating that p62 accumulation was likely due to a bacterial factor (data not shown)

In addition to increasing p62 aggregation, disrupted autophagy increases levels of reactive oxygen species (ROS) in tumor cells[23] . Therefore we next assessed the effect of VacA treatment on ROS expression in gastric cells by flow cytometry. As seen in Fig 4 (A-B), treatment with purified active VacA toxin increased the levels of ROS comparable to cells treated with hydrogen peroxide. In contrast levels of ROS in inactive VacA toxin-treated cells were comparable to untreated control cells. The VacA-mediated increased ROS was ameliorated by pre treatment with the anti-oxidant N- acetyl cysteine (Fig 4B). These findings indicate that VacA treatment increases ROS expression, which could be mediated by VacA's effect on autophagy.

Autophagy restricts the growth of intracellular VacA+ *H. pylori*

Autophagy can inhibit the growth of intracellular bacteria and our results suggest that prolonged exposure to VacA disarms autophagosomes thereby inhibiting their anti-bacterial function. To conclusively determine the effect of autophagy on intracellular survival of *H. pylori*, we employed wildtype and autophagy deficient *Atg5-/-* MEFs. Similar to mutant *Shigella* whose growth is restricted by autophagy[25], survival of VacA+ *H. pylori* was increased in autophagy disrupted *Atg5-/-* MEFs in comparison with

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wildtype MEFs (Figure 5). These findings indicate that autophagy restricts the growth of VacA+ *H. pylori*.

Crohn's disease autophagy risk allele reduces autophagy in response to VacA toxin: Our previous studies indicated that while autophagy targets VacA to eliminate the toxin and restrict the growth of the bacteria, during prolonged exposure to VacA the toxin can counteract this effect by disrupting autophagy. Therefore, we next asked whether alterations in the host genetic autophagy machinery could modulate infection in the human setting. We took advantage of the recent genome wide association studies that identified a SNP in the autophagy gene, *ATG16L1* (ATG16L1T300A-rs2241880), as a causal risk variant for Crohn's disease [26,27]. Although the functional relevance of this variant remains unclear, it is speculated that the *ATG16L1* CD risk allele may result in an unstable ATG16L1 protein and lead to impaired cytokine responses and anti-microbial autophagy, though without affecting basal autophagy function [28-30].

We first assessed if ATG16L1 was required for VacA-mediated autophagy. In ATG16L1 siRNA-treated cells a lack of LC3 II conversion in response to CCMS was detected indicating requirement for ATG16L1 (Supplementary figure 5). To determine if the *ATG16L1* genotype influenced VacA mediated autophagy, we reconstituted the wildtype or CD allele in ATG16L1 knockdown cells. However, we could only rescue the autophagy phenotype by overexpressing ATG16L1 (Supplementary figure 5). Therefore, we assessed the induction of autophagy in response to VacA or CCMS in peripheral blood monocytes (PBMCs). As previous studies indicate the effects of the toxin may differ in immune cells compared to epithelial cells we first characterized the autophagic

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responses to VacA in PBMCs. The response to VacA in PBMCs recapitulated the findings observed in gastric epithelial cells as demonstrated by increased conversion of LC3II and p62 expression (Supplementary Figure 6). We then utilized PBMCs obtained from healthy volunteers who had been genotyped for *ATG16L1*. As we had shown previously that Nod2fs and ATG16L1 interact in the autophagic pathway we also genotyped these individuals for *NOD2fs*. None of the subjects harbored the *NOD2fs* mutation. Induction of autophagy and autophagic flux was determined by assessing conversion of LC3 I to LC3 II in control and CCMS-treated cells treated with bafilomycin. The LC3II/LC3I ratios of PBMC's harboring the risk 300A allele were expressed as ratios of LC3II/LC3I in PBMC's harboring the wild type protective 300T allele to indicate the difference between the two genotypes. In PBMCs with the protective 300T allele, VacA+ CCMS increased LC3 II conversion (Figure 6A,B) comparable to the Nod2 ligand MDP (data not shown), which is known to induce autophagy [31]. In PBMCs from individuals harboring the 300A "risk" allele, the response to VacA+CCMS was reduced when compared to the 300T allele-harboring PBMCs (Figure 6 A, B).

Variants in the host autophagy gene, *ATG16L1*, increase susceptibility to infection:

These *in vitro* findings suggested that induction of autophagy in response to VacA is inefficient in the presence of the CD ATG16L1 risk allele. Since our studies indicate that autophagy restricts the intracellular survival of VacA+ *H. pylori* we next determined if the presence of this ATG16L1 variant had any impact on susceptibility to infection in humans. We initially studied Scottish first degree relatives of patients with gastric cancer, to minimize potential confounders such as variants in dietary and environmental

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exposures, housing standards etc. since potential exposure to infection would be the same [32]. All subjects were genotyped for ATG16L1 and NOD2 and underwent upper endoscopy and biopsies, including culture for *H. pylori*, to diagnose and characterize the extent of *H. pylori* infection and related gastritis phenotypes. Genotyped population controls were derived locally from umbilical cord blood sampling. Odds ratios (OR) for *H. pylori* infection were then calculated. There was a significantly increased OR for *H. pylori* infection among subjects who were homozygous for the 300A allele (GG genotype) compared with those homozygous for the 'protective' 300T allele (AA genotype) (Table 1) but no correlation with the *NOD2* genotype (Data not shown) Since induction of autophagy was entirely dependent on the presence of toxigenic VacA, we then assessed the correlation between VacA genotype of the infecting strain and *ATG16L1* genotype [33]. Among patients harboring the toxigenic s1m1 strains, 38% had the GG genotype. In contrast, of those harboring the less toxigenic s1m2 strain, only 17% had the GG genotype. Taken together, these findings indicate that there was a positive correlation between the *ATG16L1* genotype and susceptibility to infection with the toxigenic strain of *H. pylori.*

We replicated our study in a cohort of German subjects who similarly underwent genotyping and upper endoscopy and biopsies, including culture for *H. pylori*. Once again, subjects homozygous for the risk 300A allele had a significantly higher OR of *H. pylori* infection in comparison to those harboring the 300A allele (Table 1). These results confirm that the 300A allele in *ATG16L1* confers a modest but significant host genetic risk of *H. pylori* infection. This is the first study to show that variants in an autophagy

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gene, *ATG16L1*, increase susceptibility to *H. pylori* acquisition and chronic infection in Caucasian populations.

Discussion:

Current evidence indicates that autophagy can be induced in cells as an innate defense mechanism to eliminate bacteria or toxins during infection [6,7]. However, a number of pathogens are able to subvert this pathway as a strategy for their increased intracellular survival [6,34]. Here we show that although autophagy is initially induced in gastric epithelial cells (AGS) in response to VacA and serves a cytoprotective role, prolonged exposure to VacA causes disrupted autophagy, as determined by i) persistence of both the GFP and RFP signal when the tandem LC3-eGFP-mRFP construct was used, ii) reduced degradation of long-lived proteins, accumulation of p62 and elevated ROS levels iii) protection of cells from rapamycin-induced autophagic cell death and iv) lack of cathepsin D within autophagosomes in VacA treated cells. In addition, our *in vitro* findings were recapitulated in the natural setting of infection in human subjects as evidenced by higher levels of endogenous p62 in gastric tissues from patients infected with toxigenic *H. pylori*.

Our discovery that *H. pylori* infection is increased in hosts harboring the *ATG16L1* 300A polymorphism provides support for the importance of the pathway in mediating resistance to infection in humans. The 300A polymorphism is relatively common in Caucasian populations and the implications of research to date in Crohn's disease would suggest that it facilitates chronic inflammation. That the resultant protein may result in reduced autophagic responses to VacA and increased susceptibility to infection with an

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enteric microbe in humans is intriguing. We propose a model whereby during initial exposure to infection where bacterial load and therefore levels of VacA may be low, host cell autophagy plays an important role in reducing the effects of the toxin, and clearance of bacteria (Figure 6C). In individuals with the *ATG16L1* risk allele, a reduced host autophagic response could result in enhanced toxigenic effects, thereby increasing susceptibility to acquisition of infection. In contrast to the setting of initial exposure to infection, during chronic infection, persistent exposure of VacA could disrupt the autophagic pathway thereby resulting in a failure to clear bacteria and accumulation of genotoxic material in cells (p62, ROS). Since *H. pylori* possesses a variety of antioxidant strategies [35] accumulation of ROS would likely not impact bacterial survival. However increased ROS and genotoxic materials in epithelial cells may ultimately promote carcinogenesis (Figure 6C).

Autophagy plays dual roles, both as a tumor suppressor and tumor enhancer. The inability to eliminate protein aggregates and damaged organelles that produce genotoxic free radicals could increase the mutation rate within the genome, promoting cellular changes associated with oncogenesis. Tumor cells induce autophagy to handle metabolic stress and promote survival. However, defects in autophagy within tumor cells could also lead to cell death, inflammation and genetic instability. This in turn might create a microenvironment that predisposes to cancer [36,37]. Mice lacking *Beclin 1* accumulate p62, ROS and DNA damage in the liver leading to tissue damage, inflammation and ultimately hepatocellular carcinoma [23,24,37]. Thus our finding that VacA-mediated disrupted autophagy increases known risk factors for tumorigenesis including ROS *in*

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vitro and p62 *in vivo* in human subjects suggests that alterations in this pathway may be highly relevant for disease outcome during *H. pylori* infection. Thus infection with *H. pylori* may serve as a unique model system to determine the role of autophagy disruption in microbial-mediated carcinogenesis.

To our knowledge this is the first study to provide evidence that the Crohn's disease variant of ATG16L1 alters susceptibility to infection with an enteric microbe in human subjects at the population level. These findings support a role for altered autophagy in regulating the host response to enteric microbes in Crohn's disease pathogenesis. Of interest recent studies indicate that infection with *H. pylori* may actually serve a protective role against inflammatory bowel disease [38,39]. It is interesting to speculate that due to increased susceptibility to infection, early exposure and acquisition of *H. pylori* in individuals with the ATG16L risk allele may decrease their risk for the subsequent development of inflammatory bowel disease. However, further studies are needed to confirm this contention.

In summary, our studies have uncovered previously unappreciated roles of autophagy in host susceptibility to *H. pylori* infection. We are the first group to identify an autophagy gene, *ATG16L1*, as a host candidate gene for susceptibility to *H. pylori* infection. Our novel *in vitro* and *in vivo* translational studies delineate mechanisms by which *H. pylori* usurps the autophagy pathway which in turn leads to accumulation of ROS and p62. Rather than eliminating the bacteria and the toxin, this sequence of events predisposes to chronic *H. pylori* infection, and ultimately may promote gastric cancer. Thus, the

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relationship between host autophagy genotype and VacA toxin-mediated induction of disarmed autophagosomes is an elegant example of the complex manner in which host factors and bacterial virulence factors modulate host cell responses and disease susceptibility.

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Table 1:

	Number of Cases/Controls Odds Ratio[a] (95% Confidence Interval); *p*-value				
	AA	AG	GG	Recessive	Dominant
German Study	57/57 1.0 (ref)	132/107 1.2 (0.8-1.9); 0.51	84/46 1.8 (1.1-3.1); 0.03	1.7 (1.0-2.8); 0.03	1.4 (0.9-2.1); 0.13
Scottish Study	26/18 1.0 (ref)	39/24 1.2 (0.6-2.5); 0.67	42/13 2.3 (1.0-4.9); 0.05	2.3 (1.0-4.3); 0.05	1.5 (0.8-3.0); 0.18

Table 1. Association of the rs2241880 polymorphism with risk of *H pylori* infection in the German and Scottish studies. The Scottish study data were generated by comparing the two *H pylori*-infected subgroups (n=107) vs. the uninfected group (n=55); 4 samples (1 from infected and 3 from uninfected group) failed. The German study data were generated by comparing *H pylori*-infected subjects (n=273) vs. uninfected subjects (n=210); 3 samples failed (two from uninfected and 1 from infected group). Odds ratios, confidence intervals and *p*-values are given for per-genotype, dominant and recessive models.

[a]For the Scottish study, odds ratios were adjusted for age, sex and within-family sampling. For the German study, odds ratios were adjusted for age and sex. The alleles at the rs2241880 polymorphic locus were in Hardy-Weinberg equilibrium, with non-significant χ^2 values, in all control populations.

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Figure Legends:

Figure 1: Effects of prolonged VacA treatment on autophagy. The confocal micrographs in panels **A-C** show accumulation of LC3-eGFP-mRFP in AGS cells incubated in nutrient starvation conditions (**A**), treated with wild type CCMS (**B**) or vinblastine (**C**) at different time points (2h, 6h and 24h). **Panel D** shows the comparison of the percent co-localization of the GFP and mRFP signals under the different conditions at 2h and 6h. * $p < 0.001$, as analyzed by Student's T-test. Experiments were performed twice, each time in duplicate and at least 10 cells were used to calculate the Pearson's co-localization coefficient using the Volocity acquisition software.

Panel E: The graph shows the quantitation of the hydrolysis of long half-life proteins in control, CCMS treated and rapamycin treated cells. AGS cells were grown for 72h in the presence of [^{3}H]leucine and exposed to CCMS or rapamycin for 8h. [^{3}H]leucine release due to protein hydrolysis was calculated as a percentage of the total radioactivity present in the medium normalized by control cell data. **Panel F**: Quantitation of cell viability using FACS analysis of propidium iodide staining of AGS cells incubated with CCMS (black bars) or standard culture media (white bars) for 12h prior to treatment with the indicated concentrations of rapamycin for an additional 12h. Graphs depict results (expressed as the mean± SEM) of 3 independent experiments performed in triplicate. * represents $p<0.05$

Figure 2: VacA reduces Cathepsin D sorting to autophagosomes. The micrographs in **panels A-C** show 3D projections of de-convolved confocal z-stack slices obtained each

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0.25 μm from (**A**) control GFP-LC3 (green) expressing AGS cells, or GFP-LC3 (green) expressing AGS cells treated with (**B**) rapamycin, (**C**) starvation, (**D**) vinblastine or (**E**) with VacA +CCMS for12h. After treatment cells were immunolabeled for cathepsin D (blue) and Lamp-1 (Red). The insets in panels **A-C** show details of the framed area in the main panels. The top insets show the merge of GFP-LC3 and cathepsin D. The middle insets show the merge of GFP-LC3 and Lamp-1. The bottom insets show the merge of GFP-LC3, cathepsin D and Lamp-1. The scale bars indicate 10μm. Micrographs are representative of 100 cells observed in three independent experiments.

Figure 3: VacA induces p62 aggregation both *in vitro* and *in vivo:* The accumulation of p62 in AGS cells (**Panel A-C**) and in gastric biopsies from patients infected with VacA (+) *H. pylori* or VacA(-) *H. pylori* is shown in these representative images (**Panel D-E**). Panel A shows control AGS cells transfected with GFP-LC3 and treated with CCMS either from wild type *H. pylori* (CCMS +) or VacA mutant *H. pylori* (CCMS-). Cells were fixed and immunostained for p62 (Red). Insets show the colocalization of GFP-LC3 and p62. Scale bars indicate 10μm.

Panel B shows the accumulation of p62 in CCMS treated cells by Western blot with p62 antibody. Cells were left untreated or treated with CCMS from wild type *H. pylori* or VacA mutant *H. pylori* for 24 h. Lysates were then collected and Western blot was performed. β-actin was used as loading control. The blots shown are a representative of three independent experiments. **Panel C** shows the quantitation of p62 by densitometry for three different western blots. Cells treated with CCMS from wild type *H. pylori* showed significant accumulation of p62 compared to control cells. Statistical analysis

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was performed using a one way ANOVA on the Graph Pad Prism program. (P value=0.0133).

Panel D shows immunohistochemistry with p62 antibody on gastric biopsies from patients infected with *cagA (+) vacA s1m1* and *cagA(-) vacA s2m2* strains of *H. pylori* and quantitation in corresponding graph. Arrows indicate increased p62 staining in the gastric epithelial cells. Statistical analysis was performed using a Student's T-test. * indicates P value = 0.0453

Figure 4: VacA increases production of reactive oxygen species.

AGS cells were incubated with activated VacA pure toxin or inactive toxin for 4h. Where indicated, the anti-oxidant NAC (5mM) was utilized alone, or with pure toxin. Cells incubated with H2O2 (200μM, 30min) served as a positive control. **Panel A** shows ROS production measured by using a redox-sensitive dye (CM-H2DCFDA) on live cells, followed by flow cytometry analysis. Overlaid curves indicate fluorescence curve for condition stated (green) versus control curve (red) with corresponding numbers representing the percentage of ROS + cells assessed within the gated region. **In Panel B**, the graph shows quantification of ROS experiments. Fold increase is expressed as ratio of the %ROS+ cells in each condition/control. Columns, means; bars, SE; *, $P < 0.05$, **, $P< 0.01$, using one-way ANOVA (n=3). The overall P value = 0.001.

Figure 5: Disrupting autophagy during infection with VacA+ *H. pylori* enhances intracellular survival:

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Panel A, B: The graphs show the quantitation of the intracellular survival of *Shigella ΔiscB* or VacA+ *H. pylori* in wildtype or *Atg5-/-* MEFs. Cells were infected with *Shigella ΔiscB* or VacA+ *H. pylori* under gentamicin assay conditions (see Materials and Methods). At the indicated invasion times, intracellular bacteria were retrieved from cells utilizing 1% saponin in PBS buffer and plated on Brucella agar (*H. pylori*) or LB agar (*Shigella*) for enumeration of colony formation units (CFU). Data is expressed as percent of bacteria quantitated at specificied time point compared to initial CFU of invasive bacteria detected at 6hrs for VacA+ *H. pylori* and 2hrs for *Shigella ΔiscB* . *P<0.001

Panel C shows wildtype and *Atg5-/-* MEF's transfected with LC3-GFP and infected with wild type VacA+ *H. pylori*. Cells were fixed and observed under a spinning disk confocal microscope. Scale bars = 10μm.

Figure 6: Autophagy response is diminished in PBMC's from individuals harboring the Crohn's disease variant (T300A) of ATG16L1:

Panel A shows a representative western blot image using PBMC lysates. Lysates were run on an SDS-PAGE gel and Western blotting performed using anti-LC3 antibody. The blot shows the formation of LC3 II in PBMC lysates from individuals with the WT and CD allele in response to VacA+ CCMS. β-actin was used as loading control in both experiments. In **Panel B**, graphs depict densitometric analysis of LC3 II:LC3 I/actin ratio from PBMCs obtained from volunteers with either the WT or CD variant of ATG16L1. Cells were either untreated (C) or treated with CCMS for 24 hrs and with bafilomycin (B) added during the last 4 hrs of treatment. The ratios are normalized to the control or CCMS plus bafilomycin treated wild type control, respectively. Statistical analysis was

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performed using a one way ANOVA with a post test to compare each column to its control, * in Panel B indicates P value<0.0001.

Panel C shows the model depicting the effect of acute and chronic infection with *H. pylori* on the autophagy pathway in hosts with either wild type ATG16L1 or the CD risk allele.

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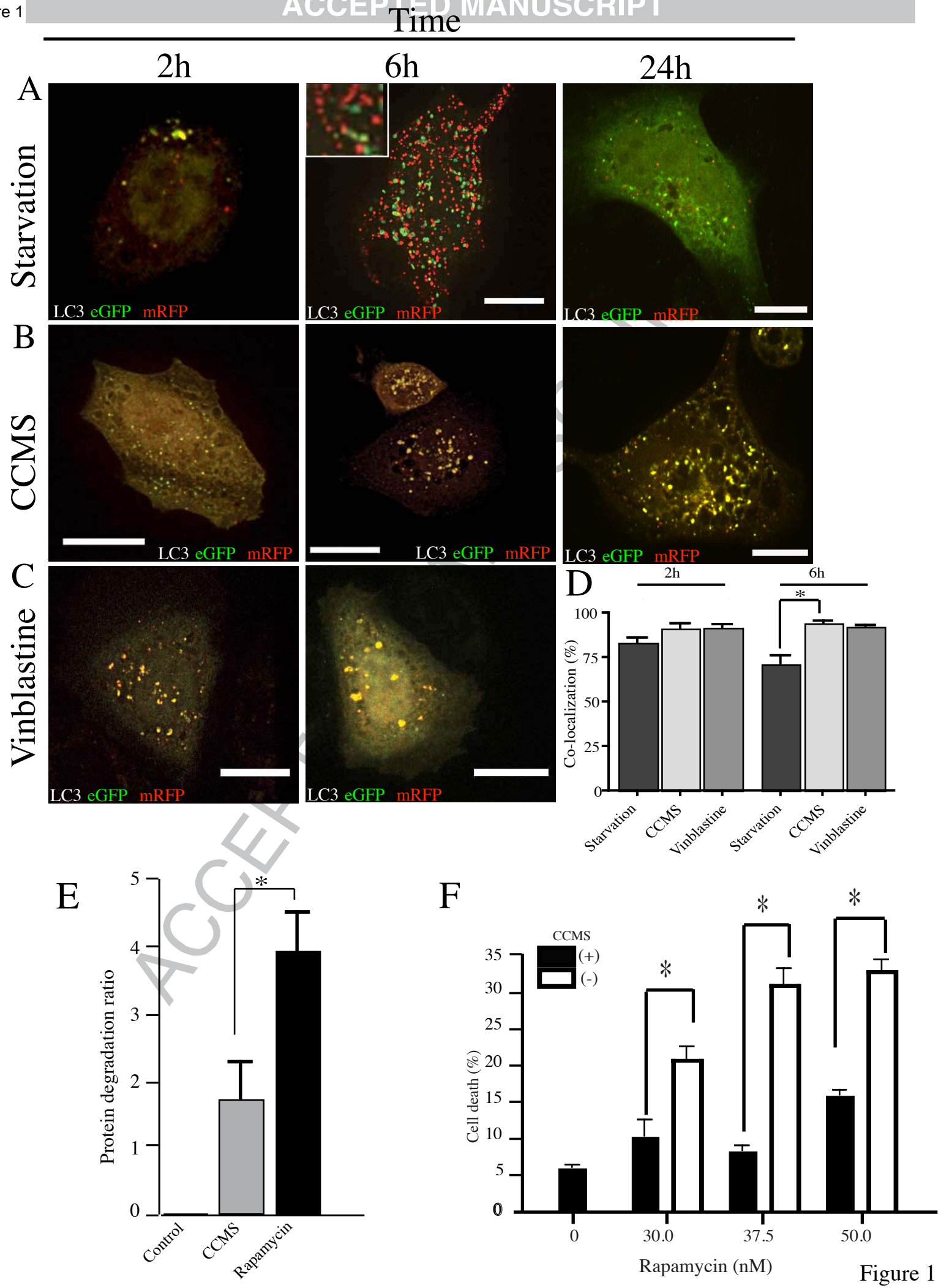

Figure 1

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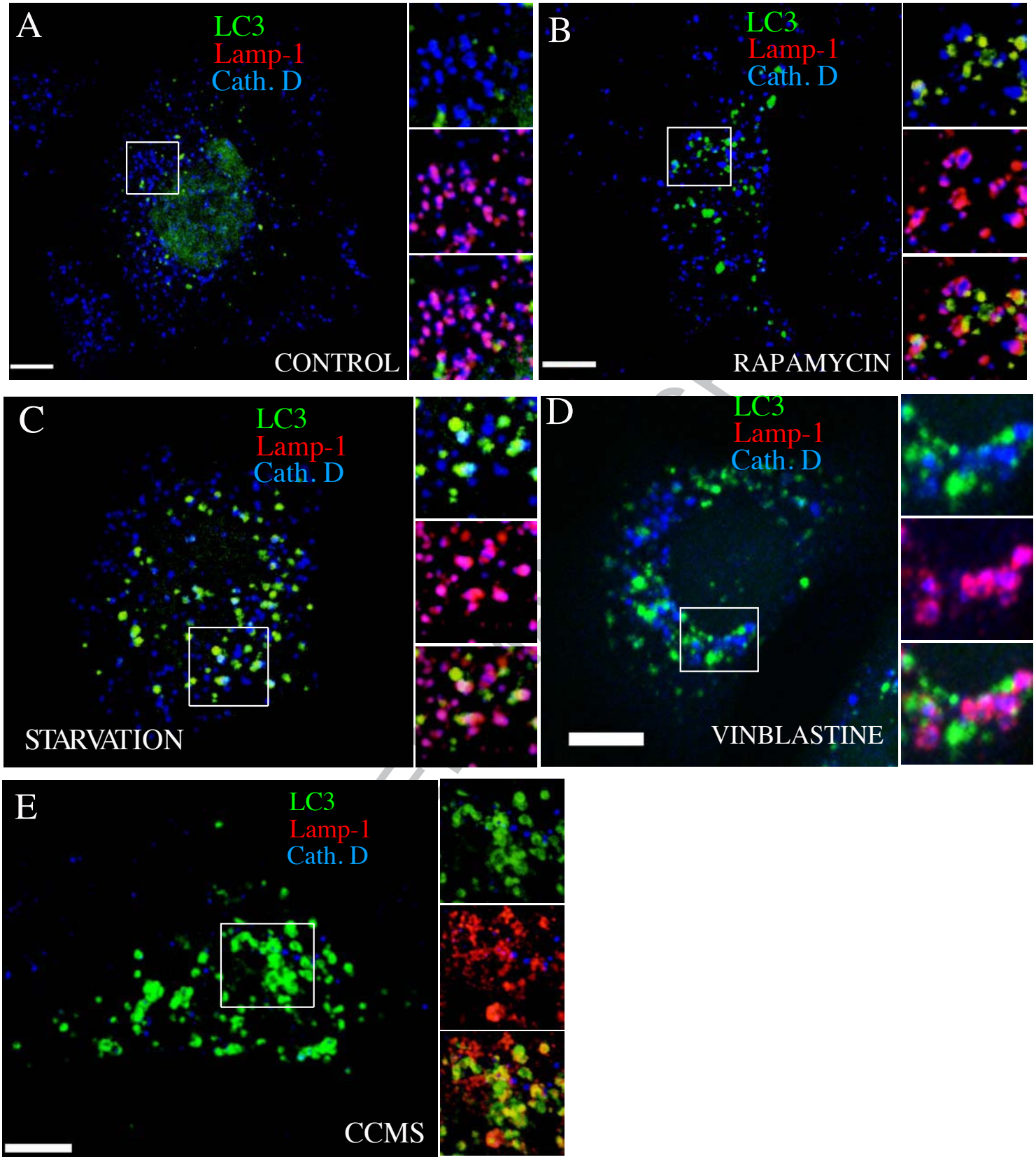

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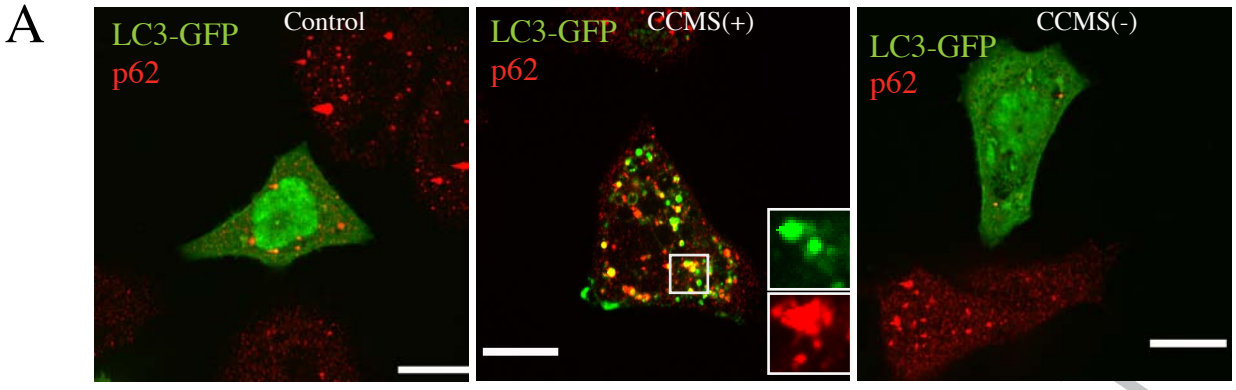

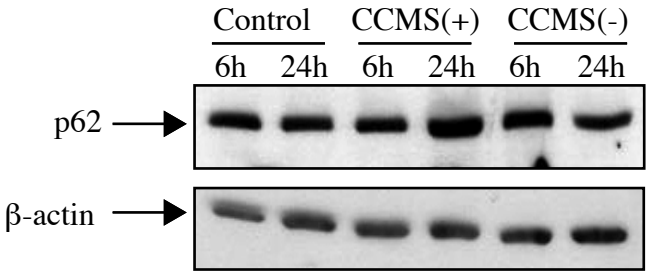

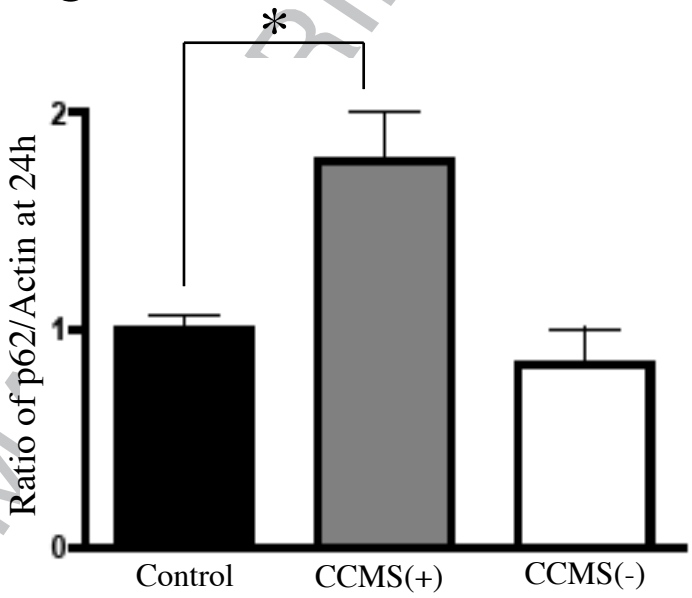

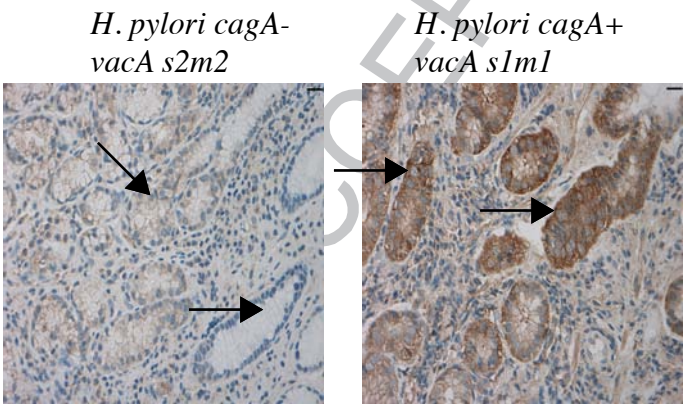

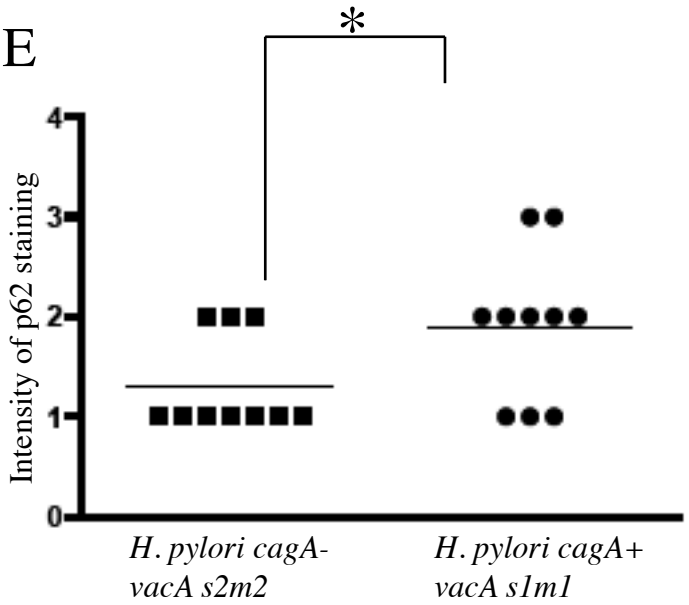

Figure 3

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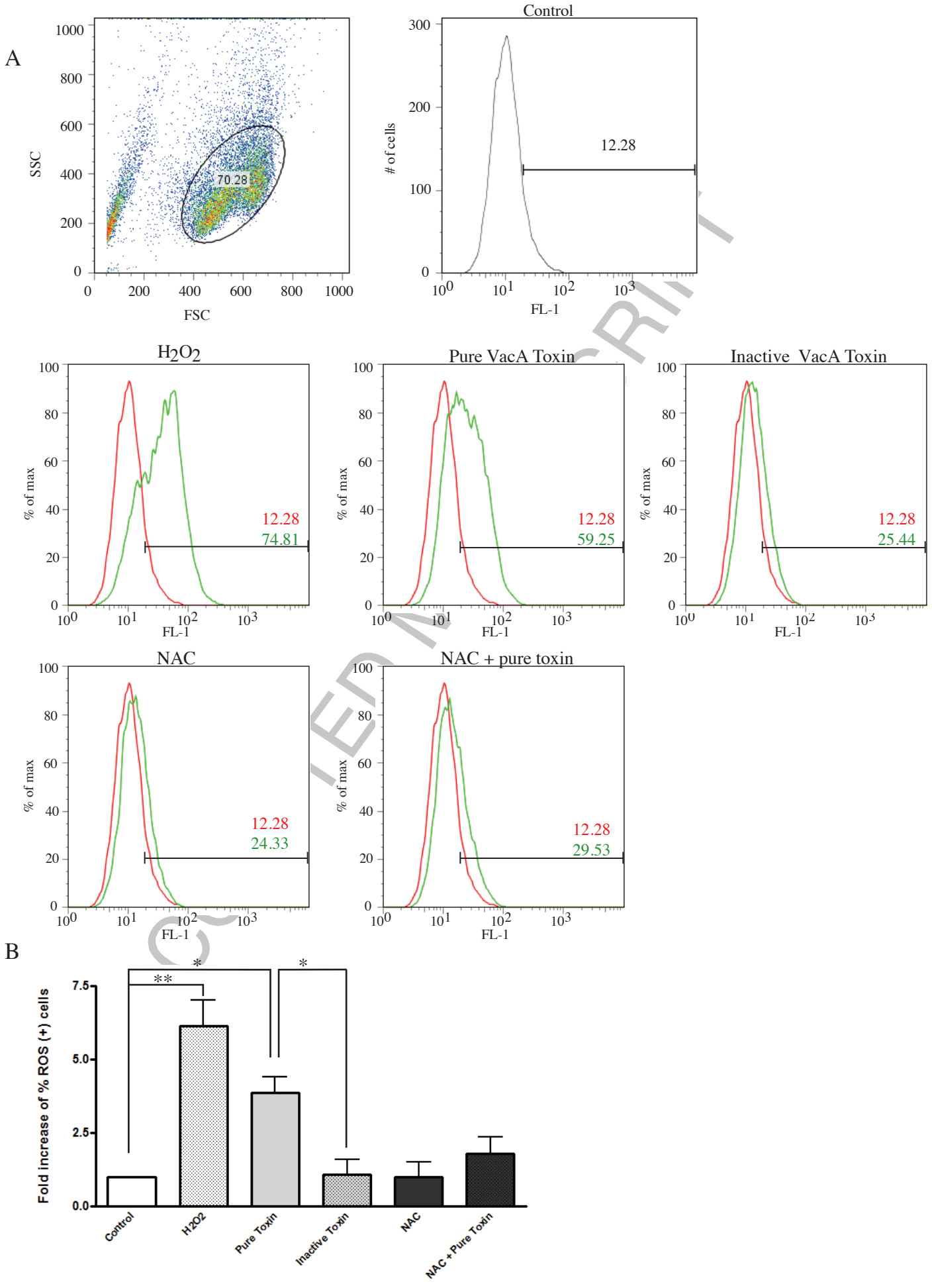

Figure 4

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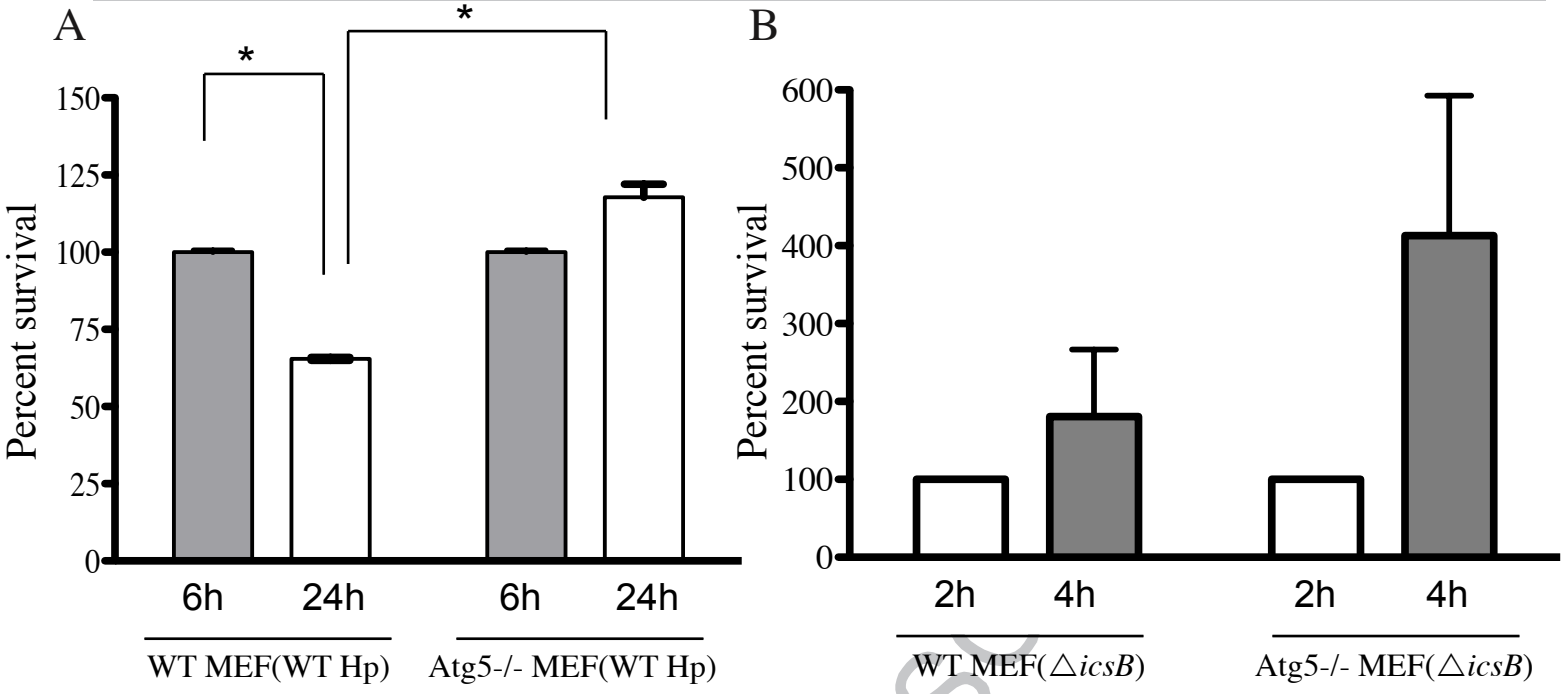

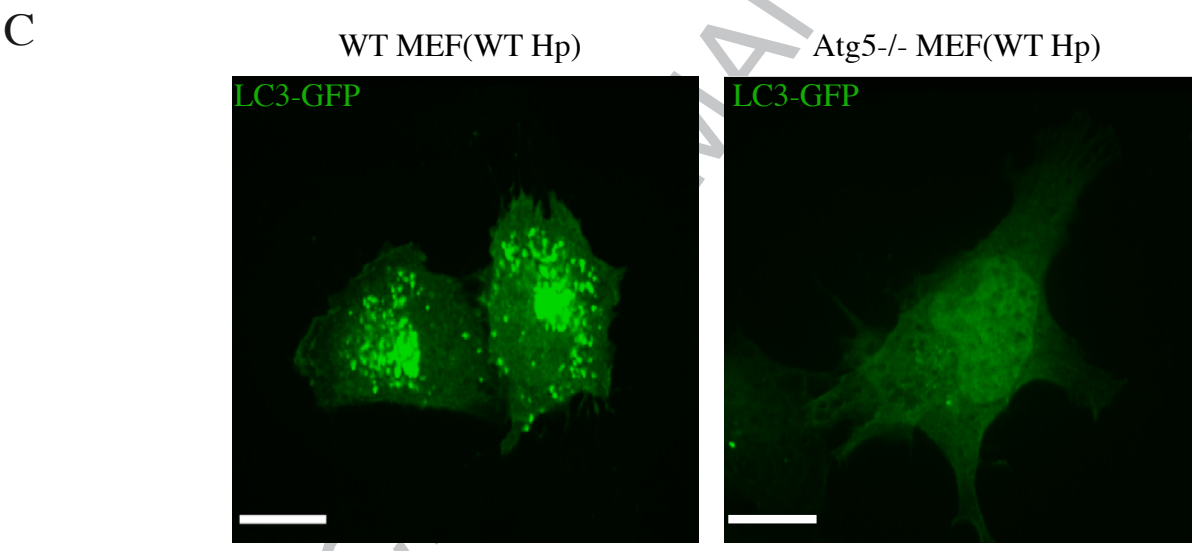

Figure 5

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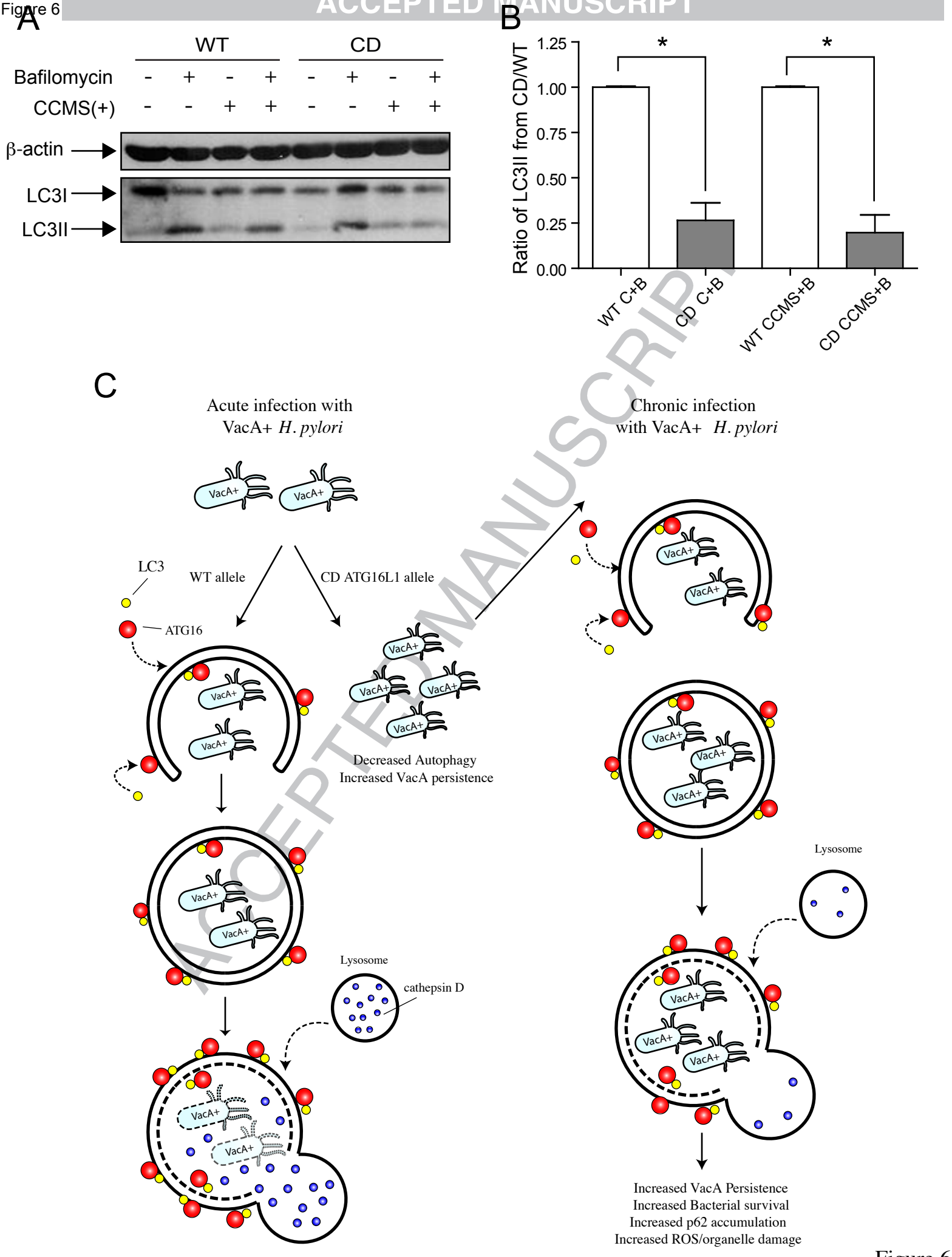

Figure 6