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MIR106B and MIR93 Prevent Removal of Bacteria From Epithelial Cells by Disrupting ATG16L1-Mediated Autophagy

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BACKGROUND & AIMS: Variants in genes that regulate autophagy have been associated with Crohn's disease (CD). Defects in autophagy-mediated removal of pathogenic microbes could contribute to the pathogenesis of CD. We investigated the role of the microRNAs (miRs) MIR106B and MIR93 in induction of autophagy and bacterial clearance in human cell lines and the correlation between MIR106B and autophagy-related gene 16L1 (ATG16L1) expression in tissues from patients with CD. **METHODS:** We studied the ability of MIR106B and MIR93 to regulate ATG transcripts in human cancer cell lines (HCT116, SW480, HeLa, and U2OS) using luciferase report assays and bioinformatics analyses; MIR106B and MIR93 mimics and antagonists were transfected into cells to modify levels of miRs. Cells were infected with LF82, a CD-associated adherent-invasive strain of *Escherichia coli*, and monitored by confocal microscopy and for colony-forming units. Colon tissues from 41 healthy subjects (controls), 22 patients with active CD, 16 patients with inactive CD, and 7 patients with chronic inflammation were assessed for levels of MIR106B and ATG16L1 by in situ hybridization and immunohistochemistry. **RESULTS:** Silencing Dicer1, an essential processor of miRs, increased levels of ATG protein and formation of autophagosomes in cells, indicating that miRs regulate autophagy. Luciferase reporter assays indicated that MIR106B and MIR93 targeted *ATG16L1* messenger RNA. MIR106B and MIR93 reduced levels of ATG16L1 and autophagy; these increased after expression of ectopic ATG16L1. In contrast, MIR106B and MIR93 antagonists increased formation of autophagosomes. Levels of MIR106B were increased in intestinal epithelia from patients with active CD, whereas levels of ATG16L1 were reduced compared with controls. Levels of c-Myc were also increased in intestinal epithelia of patients with active CD compared with controls. These alterations could impair removal of CD-associated bacteria by autophagy. **CONCLUSIONS:** In human cell lines, MIR106B and MIR93 reduce levels of ATG16L1 and autophagy and prevent autophagy-dependent eradication of intracellular bacteria. This process also appears to be altered in colon tissues from patients with active CD.

Keywords: Inflammatory Bowel Disease; microRNA; Cell Biology; Infection.

Autophagy is a cellular self-digestion process characterized by sequestration of bulk cytoplasm and organelles in autophagic vesicles and delivery of them to the lysosome, where the materials sequestered in the vesicles are degraded and recycled to general nutrient stores to maintain essential cellular functions under stress conditions such as nutrient starvation.[1,2] Specialized autophagy mediates the degradation, processing, and removal of a variety of intracellular substrates, playing a role in a myriad of cell-autonomous processes. Notably, a number of studies have implicated autophagy in the removal of invading pathogenic microbes (namely xenophagy) as an innate line of defense against viral and bacterial infections.[3,4] In murine intestinal epithelium, autophagy was shown to be important for the biology of Paneth cells, which produce secretory granules containing antimicrobial peptides.[5] In addition, autophagy also functions to facilitate the immune system of the host organism to mount adaptive immune responses against pathogen invasion by modulating antigen presentation.[6] Thus, autophagy may play a multifaceted role in host defense against intracellular pathogens.

Autophagy-related gene (ATG) 16L1 was originally identified as a component of a multiprotein complex containing

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Abbreviations used in this paper: AIEC, adherent-invasive *Escherichia coli*; ATG, autophagy-related gene; Baf, bafilomycin; CD, Crohn's disease; FBS, fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IHC, immunohistochemistry; IRGM1, immunity-related guanosine triphosphatase family M protein 1; LC3, light chain 3; miRNA/miR, microRNA; mRNA, messenger RNA; siRNA, small interfering RNA; UTR, untranslated region.

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ATG5-ATG12 showing a sequence homologue with yeast Apg16L.[7] During autophagy, the ATG16L1 complex acts as an E3-like enzyme to mediate ATG8 (also known as light chain 3 [LC3]) lipidation, which is essential for autophagosome formation.[8] Genome-wide association studies have identified the ATG16L1 polymorphism (T300A) as a risk variant for Crohn's disease (CD), an inflammatory bowel disease that affects 50 to 200 per 100,000 people in the Western world.[9–11] Further studies showed that ATG16L1 T300A is less capable of mediating antibacterial autophagy in a *Salmonella typhimurium* infection model.[12]

Although significant progress has been made in identifying genetic loci linked to CD and ulcerative colitis, a recent meta-analysis of data from genome-wide association studies in inflammatory bowel disease has shown that 163 of the common genetic loci discovered so far account for only a minority of the genetic attributes of the disease.[13] Single gene mutations appear to have limited effects on the development of CD.[14] Thus, additional mechanisms or synthesized action of multiple genes may contribute to the dysfunction of autophagy and the development of CD.

MicroRNAs (miRNAs) are a family of noncoding short RNAs that suppress gene expression by selectively base pairing to the complementary messenger RNAs (mRNAs).[15,16] miRNAs mediate posttranscriptional silencing of target genes by specifically targeting homologous mRNAs for cleavage or inhibition of protein synthesis, regulating a wide spectrum of cellular processes.[15,16] Brest et al reported that the miR-196 family of miRNAs down-regulate the CD protective variant (c.313C) but not the risk-associated allele (c.313T) of immunity-related guanosine triphosphatase family M protein 1 (IRGM1) gene in patients with CD.[17] The subsequent loss of IRGM1 expression compromises the control of intracellular replication of CD-associated adherent-invasive *Escherichia coli* (AIEC) by autophagy.[17] These results emphasize the role of miRNA-mediated gene silencing in the development of CD. In this report, we provide evidence showing that MIR106B and MIR93 suppress autophagy-mediated removal of bacteria in epithelial cells by targeting ATG16L1.

Materials and Methods

Cell Lines

Human cell lines HCT116, SW480 (colon cancer), HeLa (cervical cancer), and U2OS (osteosarcoma) were maintained in the laboratory. HCT116/GFP-LC3, SW480/GFP-LC3, and U2OS/GFP-LC3 were parental cell lines stably transfected with GFP-LC3 and established in the laboratory.[18] HCT116 cells were grown in McCoy's 5A medium with 10% fetal bovine serum (FBS). Other cells were cultured in Dulbecco's modified Eagle medium with L-glutamine and 10% FBS. HeLa/mCherry-LC3 cell line was established by stably transfecting pmCherry-LC3.

Bacterial Strain and Invasion Assay

CD-AIEC strain LF82 was used for invasion assay.[19] Details are described in Supplementary Materials and Methods.

Transfection of Small Interfering RNA, miRNA Precursors and Antagonists, DNA Constructs, and Luciferase Reporter Assay

Detailed information is described in Supplementary Materials and Methods and in Supplementary Table 1.

Fluorescence Microscopy

Fluorescence signals were analyzed with an Olympus IX51 fluorescence microscope (Center Valley, PA) or a Zeiss LSM 710 confocal microscope (Jena, Germany). Details are described in the Supplementary Materials and Methods.

In Situ Hybridization and Immunohistochemistry

The protocol for the application of human tissues was approved by the institutional review board at the University of Alabama at Birmingham (protocol no. N120831003). Colon tissues from 41 healthy subjects, 22 patients with active CD, 16 patients with inactive CD, and 7 patients with chronic inflammation of the colon were provided by the University Hospital and purchased from US Biomax, Inc (BC05002; Rockville, MD). Protocols for in situ hybridization and immunohistochemistry have been previously reported[20,21] and are detailed in the Supplementary Materials and Methods.

Statistics

All data presented are representative of 3 or more experiments, each with similar results. Quantitative data are shown as mean $\pm$ SD. Statistical significance was determined using Student t test if not specially indicated ($^*P < .05$ and $^{**}P < .01$).

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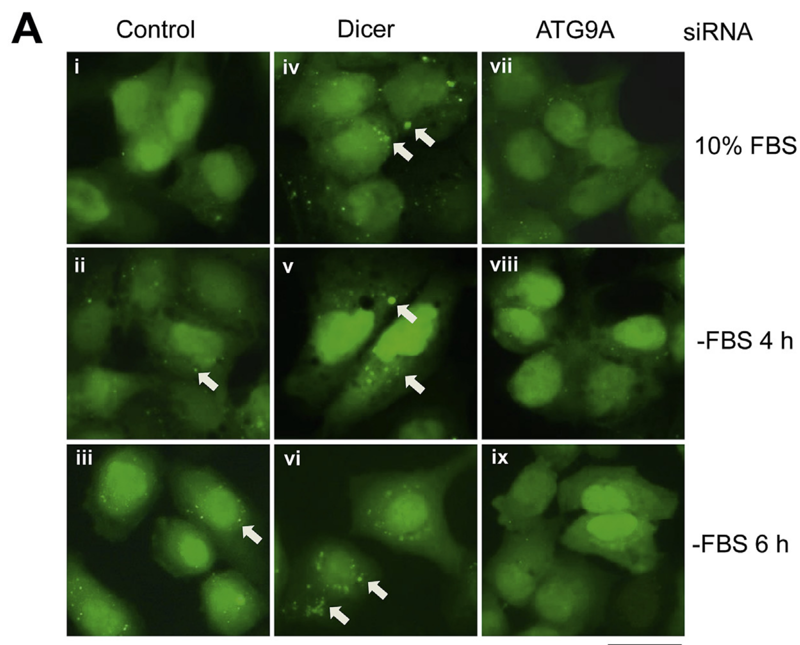
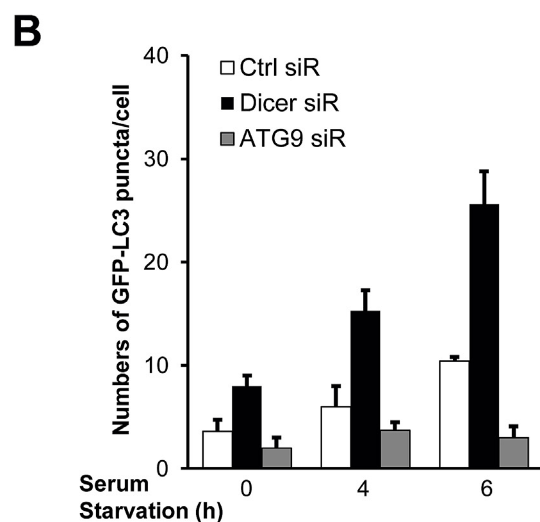
Results

Silencing of Dicer Enhances the Expression of ATGs and Formation of Autophagosomes

To determine whether abrogation of miRNA biogenesis is sufficient to induce ATG expression and autophagy, small interfering RNA (siRNA)-mediated knockdown of Dicer was performed.[22] We transfected Dicer1 siRNA into U2OS/GFP-LC3 cells, a cell line we previously established for monitoring autophagy.[18] On serum starvation, GFP-LC3 in control cells redistributed from a diffuse cytoplasmic location (Figure 1*Ai*) to discrete vesicular structures (Figure 1*Aii* and *iii*), an indication of autophagy.[18] The GFP-LC3 puncta were induced as early as 4 hours after serum starvation, and the number of cells with punctate GFP-LC3 increased in a time-dependent manner (Figure 1*Ai–iii* and *B*). In contrast, silencing of Dicer1 with siRNA induced formation of autophagosomes even in FBS-replete conditions (Figure 1*Aiv*). The number of autophagosomes/autolysosomes further increased on serum starvation (Figure 1*Av* and *vi* and *B*). siRNA-mediated depletion of ATG9 led to a decrease in GFP-LC3 puncta in cells with or without FBS (Figure 1*Avii–ix* and *B*), suggesting that the observed GFP-LC3 puncta represent autophagosomes rather than an artifact of transfection. The formation of autophagosomes was also confirmed biochemically by immunoblot analysis of LC3 (Figure 1*C*),





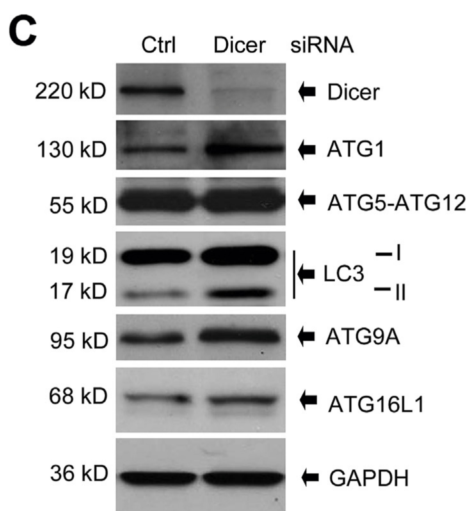


Figure 1. Autophagy flux is elevated after silence of Dicer1. (*A*) Elevated autophagy flux by silencing Dicer1. U2OS/GFP-LC3 cells were transfected with siRNAs against Dicer1, ATG9A, or negative control for 2 days. Cells were then cultured in serum-free medium for 4 or 6 hours. *White arrows* show the punctate GFP-LC3 fluorescence (autolysosomes/autophagosomes). (*B*) Average numbers of GFP-LC3 puncta per cell in *panel A*. (*C*) Expression of ATGs after silence of Dicer1 by Western blot detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control.

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because LC3-II was increased on Dicer1 RNA interference (Figure 1*C*). Depletion of Dicer1 is likely to have a widespread effect on cellular protein expression given its pleiotropism. Thus, to determine whether the autophagy machinery might be targeted by potential miRNAs, we assayed key autophagy-related gene products. Indeed, expression of ATG1, ATG9A, and ATG16L1 was up-regulated after depletion of Dicer1 (Figure 1*C*). Together, our data indicate that miRNAs may be implicated in the regulation of autophagy gene expression and the subsequent formation of autophagosomes/autolysosomes.

MIR106B and MIR93 Inhibit Autophagy Under Physiological and Stress Conditions

The involvement of miRNA biogenesis in regulating ATGs is consistent with our previous findings that miRNAs, including MIR-17-92 and MIR-106B-25 clusters, were down-regulated during differentiation of human monocytes into dendritic cells, during which autophagy was induced.[23] The majority of these miRNAs are predicted to target ATGs, in particular ATG16L1, according to public algorithms for prediction of miRNA (eg, Target Scan, PITA, and miRanda) (Supplementary Table 2). To validate the role of miRNAs in regulation of autophagy, we first assessed MIR106B and MIR93 levels in cells transfected with Dicer1 siRNA. We focused on MIR106B and MIR93 because the bioinformatics prediction revealed that this family of miRNAs possesses the highest number of matched target sites in ATGs (Supplementary Table 2). Indeed, siRNA of Dicer1 efficiently reduced expression of MIR106B and MIR93 (Supplementary Figure 1). We then transfected MIR106B and MIR93 mimics to HCT116/GFP-LC3 and SW480/GFP-LC3 cells followed by treatment with rapamycin to induce autophagy.[18] Cells

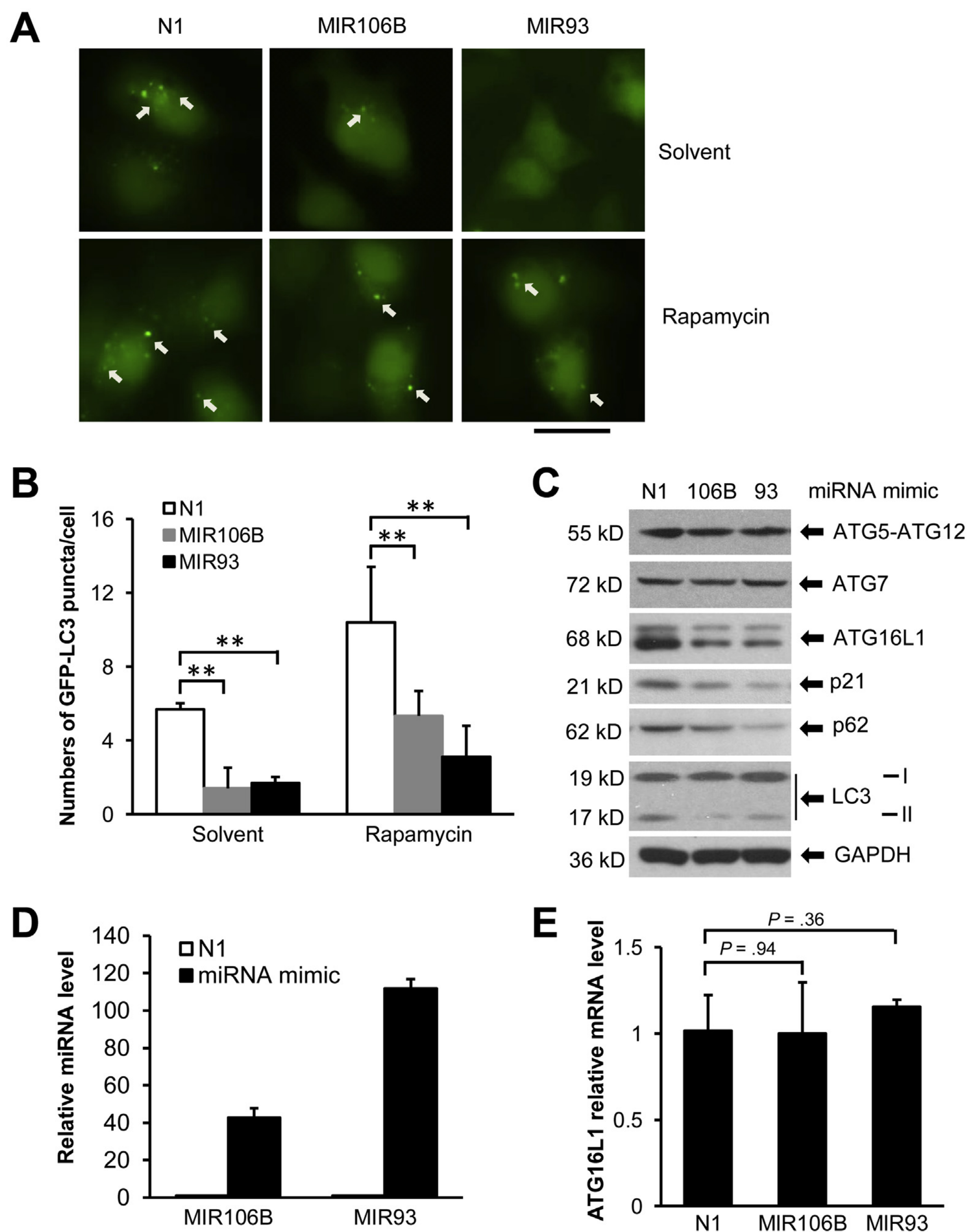

Figure 2. MIR106B and MIR93 down-regulate ATG16L1 and inhibit autophagy. HCT116/GFP-LC3 cells were transfected with MIR106B and MIR93 mimics or negative control (N1) for 48 hours. The cells were then treated with 10 nmol/L rapamycin for an additional 6 hours. (*A*) GFP-LC3 fluorescence in the cells. *White arrows* indicate the punctate GFP-LC3. *Scale bar* = 20 μm. (*B*) Average numbers of GFP-LC3 puncta per cell in *panel A*. $^{**}P < .01$. (*C*) Detection of ATGs in MIR106B and MIR93 mimic-treated cells by Western blot. GAPDH served as a loading control. (*D*) Quantitation of MIR106B and MIR93 in cells transfected with MIR106B and MIR93 mimics by quantitative polymerase chain reaction. (*E*) ATG16L1 mRNA levels in cells transfected with MIR106B and MIR93 mimics.

transfected with control miRNA showed a diffused GFP-LC3 signal with basal levels of autophagosomes/autolysosomes (2–4 puncta/cell) (Figure 2*A* and *B* and Supplementary Figure 2). Treatment with rapamycin led to a substantial increase in autophagy, with an average of 11 to 20 puncta in each cell (Figure 2*A* and *B* and Supplementary Figure 2). In contrast, transfection of either MIR106B or MIR93 dramatically reduced formation of autophagosomes/autolysosomes (2–8 puncta/cell) in either the presence or absence of rapamycin (Figure 2*A* and *B* and Supplementary Figure 2). Consistent with the fluorescence analysis of GFP-LC3, immunoblots showed that transfection of MIR106B and MIR93 reduced lipidation of LC3 (LC3-II isoform) (Figure 2*C* and Supplementary Figure 2). Furthermore, on transduction of MIR106B and MIR93, the protein level of ATG16L1 was markedly reduced although the mRNA level of ATG16L1 was not significantly changed (Figure 2*C*–*E*). In contrast, ATG5-ATG12 and ATG7 displayed only marginal alterations (Figure 2*C*). The action of the miRNA was validated by the down-regulation of p21 (Figure 2*C* and Supplementary Figure 2), a well-characterized target of MIR106B and MIR93.[24]

To assess autophagic flux, we treated the cells with bafilomycin (Baf) A1, an agent that prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes.[2] Treatment with Baf A1

markedly enhanced the accumulation of autophagosomes in cells transduced with control miRNA (Supplementary Figures 3 and 4). In stark contrast, cells stably expressing MIR106B or transiently transduced with MIR106B and MIR93 mimics only slightly responded to Baf A1 and showed substantially reduced amounts of punctate LC3 (Supplementary Figures 3 and 4), suggesting that reduction in the steady-state levels of autophagy indicators caused by MIR106B and MIR93 was not due to an accelerated autophagy flux (substrate degradation) but most probably resulted from a suppressed autophagy activity. Thus, we have shown that MIR106B and MIR93 are active regulators of autophagy, which likely act to target ATG16L1 to inhibit autophagy activity.

Depletion of Endogenous MIR106B and MIR93 Induces Autophagy

To further validate the role of MIR106B and MIR93 in regulating autophagy, we examined the effects of MIR106B and MIR93 inhibitors on autophagy in HCT116/GFP-LC3 and SW480/GFP-LC3 cells. Transduction of a scramble miRNA inhibitor exhibited little effect on the extent of autophagy with only isolated LC3 puncta (Figure 3*A* and *B* and Supplementary Figure 5), whereas transduction of MIR106B and MIR93 inhibitors led to a substantial decrease in endogenous MIR106B and MIR93 and an increase in punctate LC3 (Figure 3*A*, *B*, and *D* and Supplementary Figure 5). Treatment of the cells with rapamycin further enhanced the presence of autophagy structures (Figure 3*A* and *B* and Supplementary Figure 5). As opposed to the miRNA mimics, MIR106B and MIR93 inhibitors increased the expression of p21 and LC3-II (Figure 3*C* and Supplementary Figure 5). Consistently, expression of ATG16L1 was increased in inhibitor-treated cells while ATG16L1 mRNA levels were not altered (Figure 3*C* and *E*). Unexpectedly, autophagy induced by MIR106B and MIR93 inhibitors was not associated with loss of p62 (Figure 3*C* and Supplementary Figure 5), a selective autophagy substrate commonly observed to be degraded during active autophagy.[1,2,25] Instead, p62 levels were modestly increased in MIR106B and MIR93 inhibitor–treated cells (Figure 3*C* and Supplementary Figure 5), which is consistent with a previous report showing that p62 is a direct target of the MIR106 cluster.[25] Thus, p62 levels under the treatment of MIR106B and MIR93 mimics or inhibitors may reflect a balance between autophagy-mediated degradation and the direct action of the miRNAs (Figures 2*C* and 3*C*). Hence, we have shown the role of endogenous MIR106B and MIR93 in induction of autophagy.

MIR106B and MIR93 Target ATG16L1

Both MIR106B and MIR93 mimic and inhibitor assays suggest that ATG16L1 is a target of these miRNAs (Figures 2*C* and 3*C* and Supplementary Figures 2–5). To determine whether ATG16L1 is directly regulated by MIR106B and MIR93, a fragment of the 3′–untranslated region (UTR) of ATG16L1, which contains 2 binding sites for the MIR106B cluster (Figure 4*A*), was subcloned into the pmir-GLO dual luciferase reporter vector at the 3′ end of the coding sequence of firefly luciferase to generate pmir-GLO-ATG16L1-3′-UTR-WT (wild-type) and mutant construct lacking the miRNA binding sites pmir-GLO-ATG16L1-3′-UTR-MUT. Significantly lower luciferase activity was detected in cells transfected with pmir-GLO-ATG16L1-3′-UTR-WT as compared with cells transfected with pmir-GLO-ATG16L1-3′-UTR-MUT (Figure 4*B*), suggesting that repression of the luciferase activity by the miRNAs depends on these specific recognition sequences in the 3′-UTR of the ATG16L1 transcript. To further validate the ATG16L1-targeting activity of the miRNAs, we transfected MIR106B and MIR93 mimics together with either pmir-GLO-ATG16L1-3′-UTR-WT or pmir-GLO-ATG16L1-3′-UTR-MUT. Ectopic MIR106B and MIR93 mimics resulted in attenuation of the luciferase activity derived from pmir-GLO-ATG16L1-3′-UTR-WT but not the mutant 3′-UTR, whereas negative control nucleotides had no effect (Figure 4*C*). Together, our data showed that ATG16L1 is a direct target of MIR106B and MIR93.

Overexpression of ATG16L1 Rescues MIR106B- and MIR93-Induced Suppression of Autophagy

ATG16L1 forms complexes with ATG5-ATG12 conjugates to promote elongation and closure of autophagosomes via an E3-ligase–like role in LC3 lipidation and localization to the autophagosome membrane.[26] Given our data suggesting that MIR106B and MIR93 reduced formation of autophagosomes by targeting 3′-UTR of ATG16L1 (Figures 2*C*, 3*C*, 4*B*, and 4*C*), we expected that overexpression of ATG16L1 would rescue the defects in autophagy caused by MIR106B and MIR93. To assess this postulation, we made FLAG-ATG16L1 expression constructs with 3′-UTR-WT or 3′-UTR-MUT linked to the stop code of ATG16L1 and established ATG16L1 expression cell lines in HCT116/GFP-LC3 cells (Figure 4*D*). The cells were then transfected with MIR106B and MIR93 mimics. MIR106B and MIR93 mimics markedly down-regulated the expression of ATG16L1 construct with 3′-UTR-WT but not construct with 3′-UTR-MUT (Figure 4*D*). Consistently, ATG16L1 construct with 3′-UTR-MUT, but not with 3′-UTR-WT, rescued MIR106B- and MIR93-induced suppression of autophagy and triggered punctate GFP-LC3 redistribution, although fewer autophagosomes were present as compared with control miRNA transduced cells (Figure 4*E*). The incomplete rescue of autophagy formation suggests additional targets of MIR106B and MIR93 in autophagy regulation.[27] Together, these data support the conclusion that ATG16L1 is an important mediator for MIR106B- and MIR93-induced inhibition of autophagy.

MIR106B Impairs the Removal of CD-Associated AIEC in Epithelial Cells

It was reported that dysregulation of ATG16L1 and the resultant deficiency in autophagy play an important role in

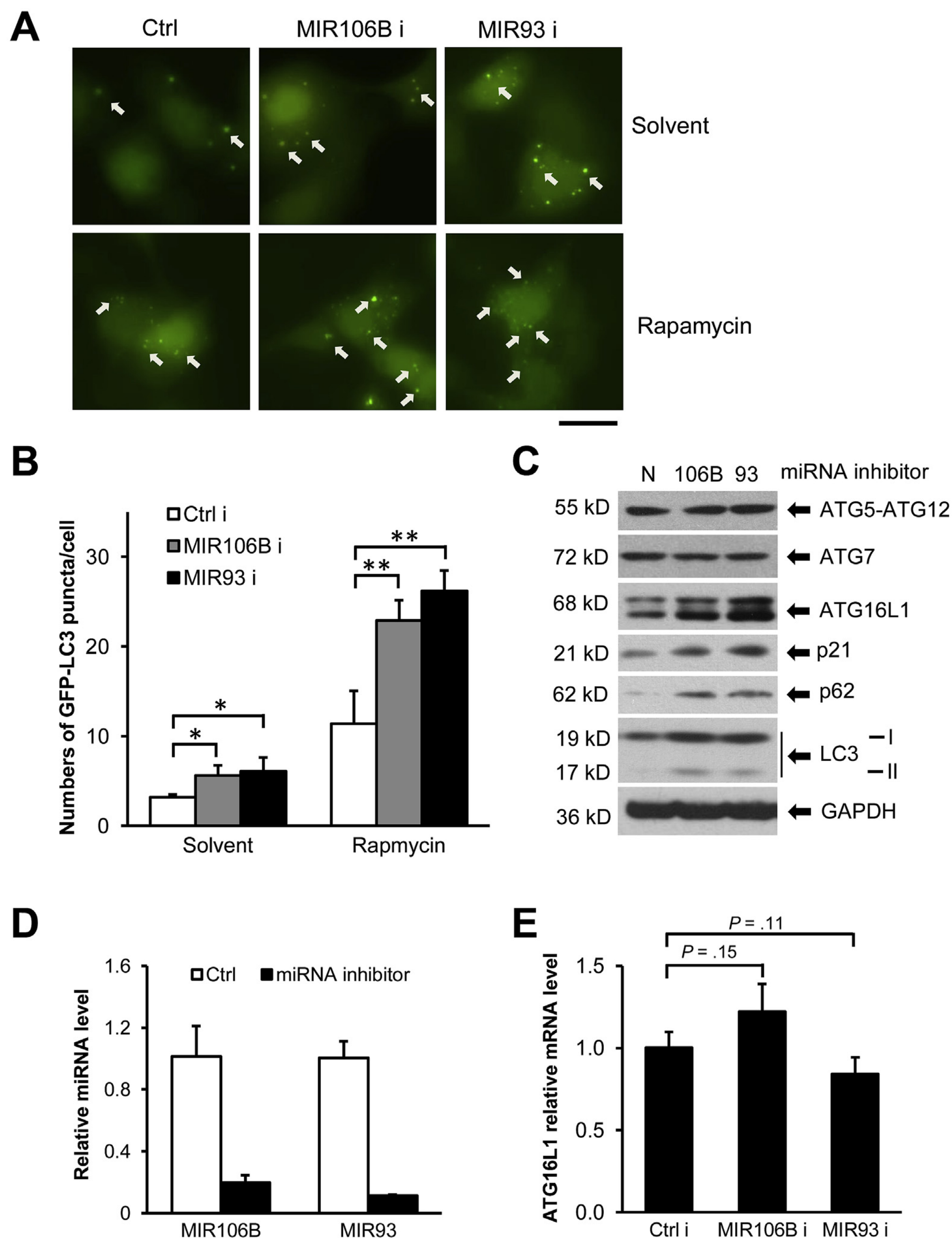

Figure 3. Depletion of endogenous MIR106B and MIR93 induces autophagy. HCT116/GFP-LC3 cells were transfected with MIR106B and MIR93 inhibitors or scramble oligonucleotides (Ctrl) for 48 hours. Cells were then treated with 10 nmol/L rapamycin for an additional 6 hours. (*A*) GFP-LC3 fluorescence in the cells. *White arrows* indicate the punctate GFP-LC3 fluorescence. *Scale bar* = 20 μm. (*B*) Average numbers of GFP-LC3 puncta per cell in *panel A*. **P* < .05 and ***P* < .01. (*C*) Western blot detection of ATGs in MIR106B and MIR93 inhibitor–treated HCT116/GFP-LC3 cells. GAPDH served as a loading control. (*D*) Determination of endogenous MIR106B and MIR93 in cells transfected with MIR106B and MIR93 inhibitors by quantitative polymerase chain reaction. (*E*) ATG16L1 mRNA levels in cells transfected with MIR106B and MIR93 inhibitors.

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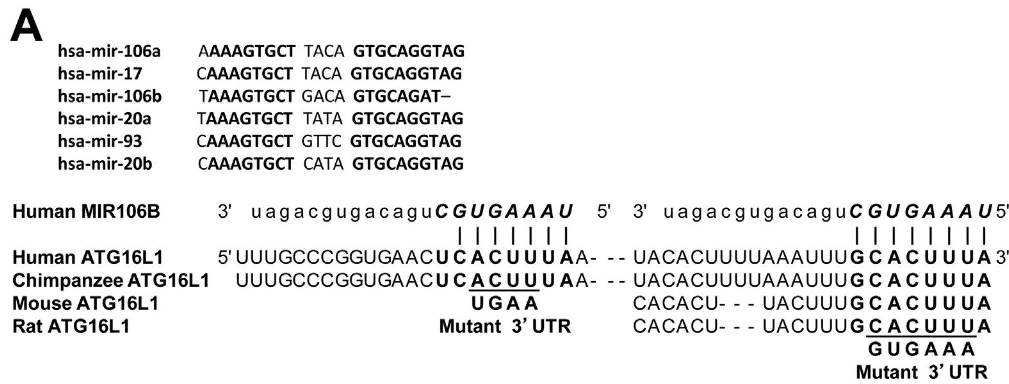
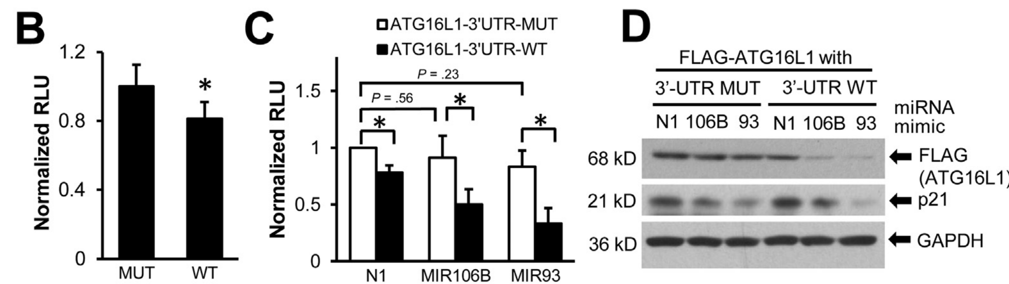
the development of CD.[10,12,28] To determine whether MIR106B- and MIR93-mediated depletion of ATG16L1 would affect acute removal of CD-associated intracellular pathogens, we used HeLa/mCherry-LC3 as host cells. CD-associated AIEC strain LF82, which was originally isolated from a chronic ileal lesion of a patient with CD and was able to invade and proliferate in epithelial cells,[9,19] was used as an infecting pathogen. The bacteria were transformed with pFPV25.1/GFP-mut3 to express green fluorescent protein (GFP). In response to AIEC LF82 infection, autophagy was induced in HeLa/mCherry-LC3 cells (Figure 5*A*) and endogenous MIR106B levels were marginally reduced (Supplementary Figure 6). The cells were transfected with a MIR106B mimic followed by AIEC LF82/pFPV25.1/GFP-mut3 infection. Intracellular bacterial infection prompted formation of autophagy in control negative oligonucleotide (N1) transfected cells (Figure 5*B* and Supplementary Figure 7) and the pathogens partially colocalized with autophagosomes/autolysosomes (Figure 5*C*), a process that may mediate bacterial clearance. In contrast, MIR106B mimic transfection reduced AIEC LF82-induced autophagy (Figure 5*B* and Supplementary Figure 7), with fewer bacteria associated with autophagosomes/autolysosomes (Figure 5*C*). We determined numbers of intracellular





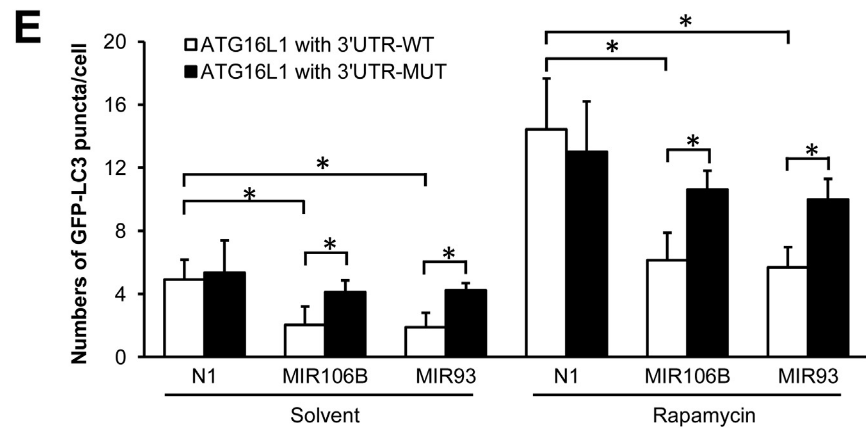


Figure 4. MIR106B and MIR93 target ATG16L1. (*A*) Sequences of mature MIR106 family members and MIR106B sequence aligned with human, chimpanzee, mouse, and rat ATG16L1 3′-UTRs. The mutation of the MIR106B binding site sequence is shown below. (*B* and *C*) Luciferase reporter assays in HCT116 cells. pmir-GLO-ATG16L1-3′-UTR-WT were used to determine targeting of ATG16L1 3′-UTR (*B*) by endogenous miRNAs or (*C*) by transfected MIR106B and MIR93 mimics. (*D* and *E*) HCT116/GFP-LC3 cells stably transfected with FLAG-ATG16L1 with WT or mutant 3′-UTRs were transfected with MIR106B and MIR93 mimics or N1 for 48 hours. (*D*) Western blot analysis of FLAG-ATG16L1. (*E*) After transfection with MIR106B and MIR93 for 48 hours, cells were treated with 10 nmol/L rapamycin for an additional 6 hours. The number of GFP-LC3 puncta per cell was calculated. $^*P < .05$.

bacteria by counting colony-forming units from bacterial cultures of the cell lysates. A significantly higher number of intracellular AIEC LF82 bacteria were identified in cells transfected with MIR106B mimic (Figure 5*D* and *E*). Taken together, our data suggest that MIR106B can disrupt autophagy-mediated elimination of pathogenic bacteria in infected human cells.

MIR106B Is Overexpressed in Intestinal Mucosa With Lower Expression of ATG16L1 in Subjects With CD

To investigate the pathological correlation between MIR106B and ATG16L1 in CD, we analyzed expression of MIR106B and ATG16L1 using in situ hybridization and immunohistochemistry (IHC), respectively, in 86 colon tissue samples consisting of healthy, active CD, inactive CD, and chronic inflammation of colon. We first validated the in situ hybridization detection of MIR106B using U2OS cells stably expressing MIR106B (Supplementary Figure 8). Healthy colon tissues showed low levels of MIR106B with isolated small focuses of expression in intestinal epithelia (Figure 6*A* and *C* and Supplementary Figures 9 and 11). In contrast, approximately 80% of the colon tissues from subjects with active CD exhibited higher levels of MIR106B (Figure 6*A* and *C* and Supplementary Figures 9 and 11). Intestinal epithelia in the actively inflamed mucosae exhibited the highest levels of MIR106B (Figure 6*A* and Supplementary Figure 11). Mucosae from subjects with inactive CD displayed a mild increase in expression of MIR106B with a predominantly epithelial distribution (Supplementary

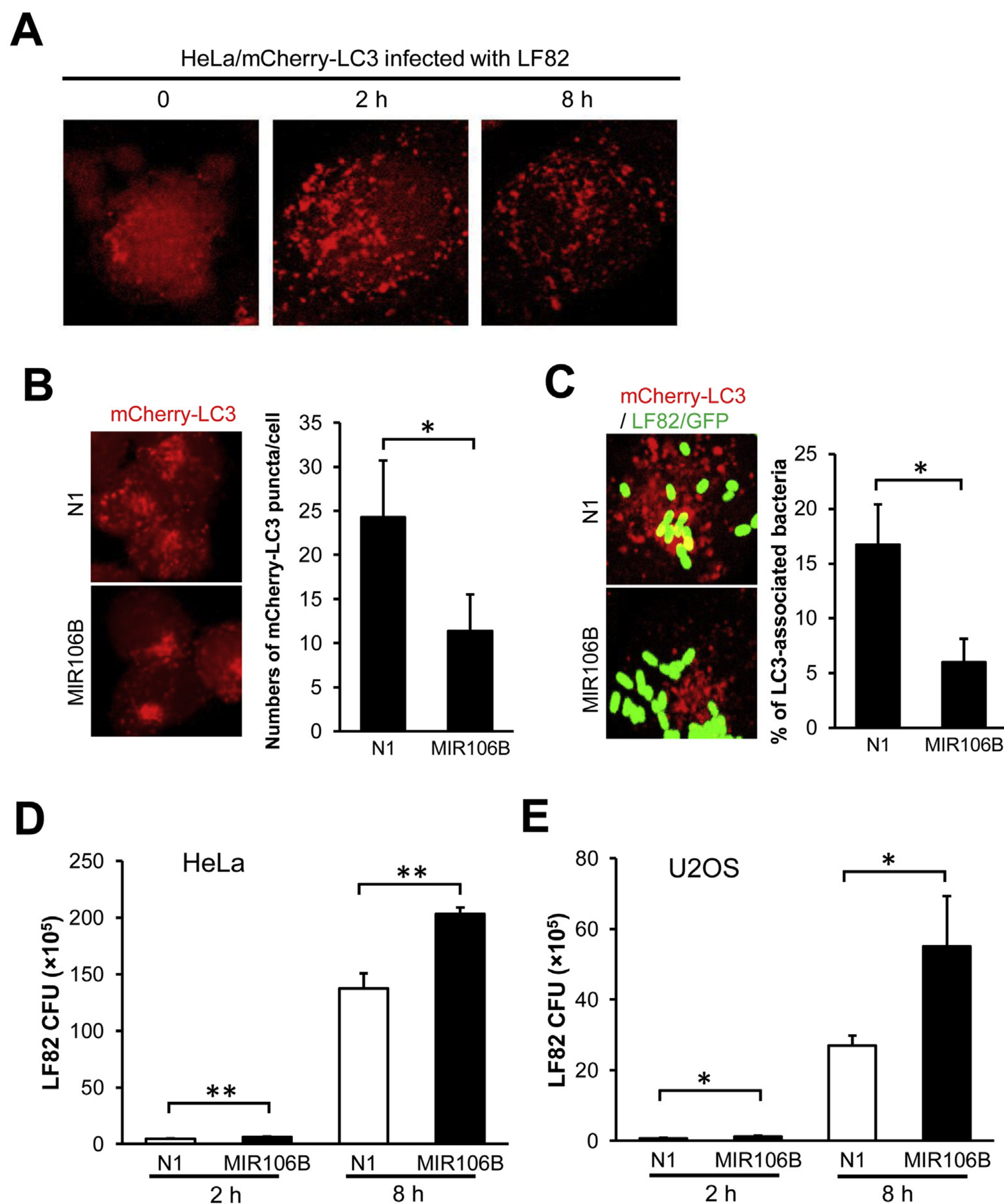


Figure 5. MIR106B impairs the removal of intracellular bacteria in epithelial cells. (*A*) AIEC strain LF82 was used to infect HeLa/mCherry-LC3 cells (multiplicity of infection of 10) for the indicated time. The mCherry-LC3 fluorescence was captured with a Zeiss LSM 710 confocal microscope after treatment. (*B* and *C*) Confocal microscopy examination of (*B*) autophagy flux and (*C*) LC3 (*red*)-associated LF82 bacteria (*green*) in MIR106B overexpressed cells. (*D* and *E*) MIR106B reduces the removal of intracellular LF82 bacteria in HeLa and U2OS cells. Cells in a 6-well plate were transduced with MIR106B mimic or control (N1) for 48 hours and then infected with LF82 (multiplicity of infection of 10) for 2 hours. After the infection, cells were either washed and lysed or cultured in regular medium for an additional 2 or 8 hours before lysis for bacterial colony formation assay. Total colony numbers per well from 2 groups are presented. $^{*}P < .05$ and $^{**}P < .01$.

Figures 9 and 11). Interestingly, comparing MIR106B levels in active, inactive (quiescent), and normal (uninvolved) mucosae in the same patients revealed that MIR106B was consistently elevated in inflamed focuses but only weakly expressed in normal and quiescent sites (Supplementary Figures 9 and 11), suggesting that expression of MIR106B is causally linked to the onset of CD and may serve as an indicator for active CD.

Next, we examined expression of ATG16L1 in the colon tissue samples. In healthy mucosa, ATG16L1 was expressed in epithelia and lamina propria, with the expression stronger in the epithelial compartment (Figure 6*B* and Supplementary Figure 10). In contrast, expression of ATG16L1 was strikingly weak in the intestinal epithelia from subjects with active CD (Figure 6*B* and *C* and Supplementary Figure 10). Consistent with the uneven levels of MIR106B expression (Figure 6*A* and Supplementary Figure 9), ATG16L1 was undetectable in some of the CD intestinal epithelial cells (Figure 6*B* and Supplementary Figures 10 and 11). ATG16L1 in inactive areas of diseased mucosae showed an appreciable weak expression as compared with normal tissues but was stronger than that of active lesions (Supplementary Figure 10). Opposite to the expression pattern of MIR106B in serial tissue sections of the same subjects with CD (Supplementary Figure 9), ATG16L1 staining exhibited a decreasing trend in the order of normal, quiescent, and inflamed mucosae (Supplementary Figures 10 and 11). Together, our findings show that expression of MIR106B in CD correlates with down-regulated ATG16L1 in human intestinal epithelia and active inflammation.

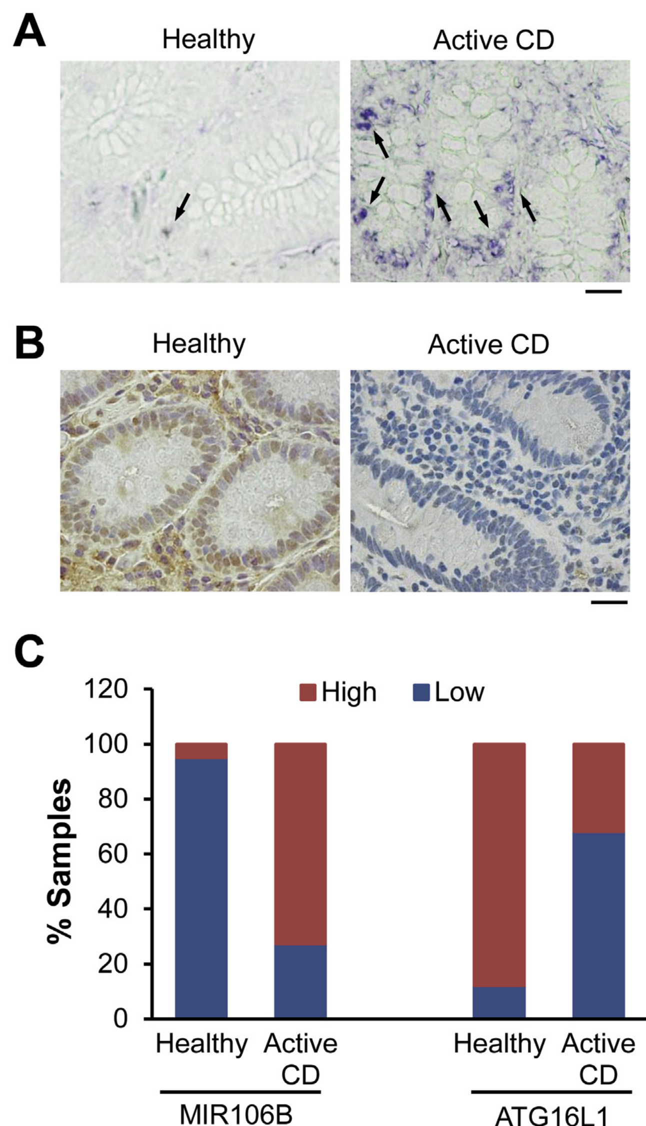


Figure 6. In situ hybridization of MIR106B and IHC of ATG16L1 in intestinal mucosa. (*A*) Representative in situ hybridization of the colon tissues from healthy controls (n = 41) or patients with active CD (n = 22) labeled for MIR106B. *Scale bar* = 20 μm. (*B*) Representative IHC staining of ATG16L1 in serial human colon tissues as detected in *panel A*. *Scale bar* = 20 μm. (*C*) Quantitation of MIR106B and ATG16L1 expression in human colon disease spectrum tissues. Masked reading was performed by 3 different investigators with the same criteria to evaluate the staining (low, overall negative or weak staining; high, overall moderate or strong staining). Pearson χ^2 test was used to analyze the distribution difference of MIR106B or ATG16L1 between healthy and active CD tissues ($P < .05$).

c-Myc, a MIR106B and MIR93 Transcriptional Regulator, Is Highly Expressed in the Intestinal Mucosa of Subjects With CD

c-Myc was reported to be highly expressed in CD, particularly in patients with active CD.[29–31] Recent reports also showed that c-Myc regulates the expression of miRNAs, including MIR106B and MIR93, through a transcriptional mechanism.[15,16,27] Thus, we studied the role of c-Myc in regulating MIR106B and MIR93 levels. Using IHC, we assessed c-Myc in colon tissues from healthy subjects and patients with CD and found that c-Myc levels were markedly higher in intestinal epithelia from patients with active CD compared with those from healthy subjects (Figure 7*A* and *B*). There was an intense staining of c-Myc in the nuclei of epithelial cells from CD samples, whereas the signal in cells from healthy subjects was relatively weak and more cytoplasmic (Figure 7*A*). Expression of c-Myc is positively correlated with MIR106B levels (Figure 7*C*). Further, MIR106B and MIR93 levels were considerably increased in cells transfected with c-Myc as compared with those in cells transfected with vector alone (Figure 7*D* and *E*). Accordingly, overexpression of c-Myc reduced levels of p21 and ATG16L1 in the cells (Figure 7*D*). Thus, our data support a model that increased c-Myc in subjects with CD enhances expression of MIR106B and MIR93, which reduces formation of autophagosomes and intracellular bacterial removal by targeting ATG16L1 (Figure 7*F*).

Discussion

Autophagy has been shown to be essential for the clearance of intracellular microbes in mammalian cells.[1–4,32] However, the underlying mechanism controlling autophagy in these cells remains unknown. We previously reported that the miR-106 cluster was among the miRNAs down-regulated during differentiation of human monocytes into dendritic cells.[23] We also noted that ATG16L1 and ATG9 levels were substantially increased and that autophagy was markedly induced during differentiation of dendritic cells (Lu and Xu, unpublished data, October 2013). In the current study, we further characterized that MIR106B and MIR93 decreased lipidation of LC3 and reduced the number of autophagy puncta in human epithelial cells (Figure 2 and Supplementary Figures 2 and 3). More importantly, expression of ATG16L1 with a mutated 3′-UTR rescued MIR106B- and MIR93-mediated suppression of autophagy (Figure 4*D* and *E*), supporting the notion that MIR106B and MIR93 inhibit autophagy by targeting ATG16L1. In a most recent report, Zhai et al showed that MIR106A and MIR106B regulate ATGs and fine-tune autophagy.[33] Together, current evidence presented in this study and by other groups[27,33] clearly reveals that the MIR106B cluster plays an important role in regulating autophagy.

Mounting evidence suggests the involvement of autophagy defects in the etiology of CD.[3,4,32,34] ATG16L1 and 2 additional genes, NOD2 and IRGM1, whose aberrations are frequently associated with CD, contribute to the removal of the bacteria by regulating autophagy in intestinal epithelial cells and peripheral dendritic cells.[17,28,35] Dysregulation or mutations of these genes disrupt autophagy and delay the removal of intracellular bacteria, leading to the chronic inflammatory pathology in affected subjects.[5,17,28,35] MIR106A was previously found to be up-regulated in patients with CD.[36] Several groups further showed that MIR106A

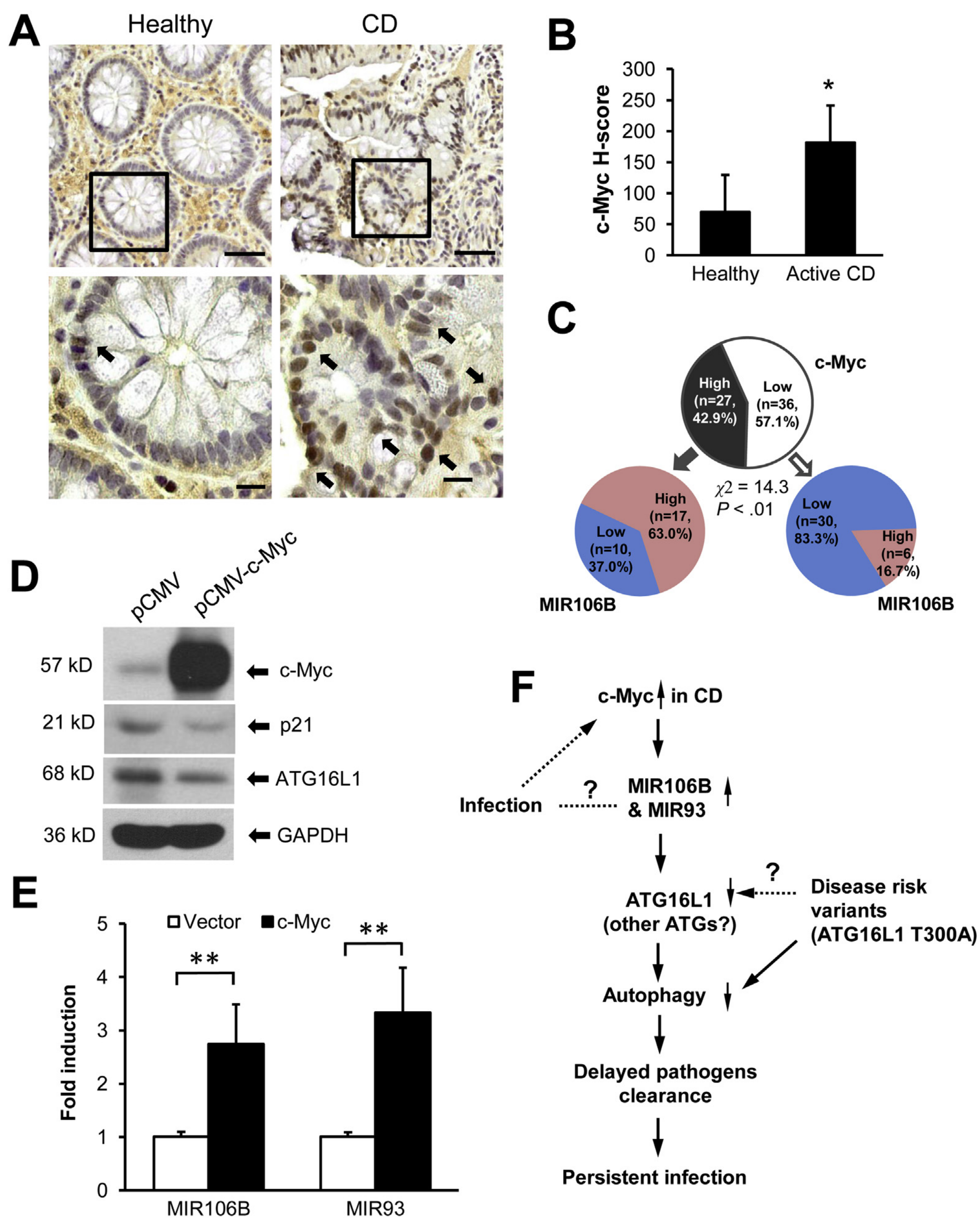


Figure 7. High expression of c-Myc in CD correlates to elevated levels of MIR106B. (*A*) Assessment of c-Myc in colon tissues from healthy subjects (n = 41) and patients with CD (n = 22) by IHC. *Scale bars* = 50 μm (*upper panel*) and 20 μm (*lower panel*). (*B*) H scores of c-Myc in *panel A* (see Supplementary Materials and Methods for an explanation of H scores). (*C*) Correlation between c-Myc and MIR106B in colon tissues from healthy subjects (n = 41) and patients with active CD (n = 22). Pearson χ^2 test was used to analyze the significance of the correlation ($P < .01$). (*D and E*) Overexpression of c-Myc increases MIR106B and MIR93 and reduces ATG16L1. HCT116 cells were transfected with pCMV-c-Myc or empty vector for 48 hours. Whole cell lysates (protein) or total RNA were isolated for (*D*) immunoblot or (*E*) quantitative polymerase chain reaction analysis. (*F*) A schematic model for elevated MIR106B and MIR93 levels in CD. *$P < .05$ and **$P < .01$.

family miRNAs play a role in the onset and/or relapse of inflammation from mucosal tissues in patients with inflammatory bowel disease.[36,37] We found that MIR106B was up-regulated in the colon mucosa of subjects with CD, which correlated with reduced ATG16L1 and active inflammation (Figure 6 and Supplementary Figures 9–11). Further, we established that MIR106B disrupted the removal of CD-LF82 strain bacteria in epithelial cells. Thus, MIR106B serves as a likely pathogenic factor of CD via targeting ATG16L1 and ATG16L1-mediated autophagy. In addition to ATG16L1, MIR106B and MIR93 appear to target p62 directly, independent of autophagy (Figures 2*C* and 3*C*), which is intriguing because p62 has been proposed to be a selective autophagy substrate and a mediator of cargo recognition in autophagy.[1,2,25] The contribution of microRNA-mediated down-regulation of p62 to CD is currently unclear; future studies are needed to determine whether reduced p62 levels may further interfere with autophagy-mediated clearance of pathogens.

Regulation of ATG16L1 and the risk variant ATG16L1 T300A in particular in CD has not been fully understood. The Xavier and Netea groups showed that expression of ATG16L1 responded to intracellular bacterial infection or exposure to bacterial components.[10,38] The T300A risk variant reduced ATG16L1 stability and inflammatory responses, such as the production of interleukin-1β,[38] whereas Hampe et al failed to find marked alteration of ATG16L1 at mRNA and protein levels in a pilot analysis of

samples from healthy subjects and patients with CD.[39] The differences among these findings may derive from patient samples or unclear status of the patients (active or inactive).[38,39] Interestingly, our data showed that instead of the entire epithelial tissue, MIR106B was expressed at a strikingly high level in a small population of CD epithelial cells, which may account for complete loss of ATG16L1 staining in some of the cells. The significance of such a mosaic pattern of MIR106B and ATG16L1 expression is currently unclear. However, it is conceivable that the persistent presence of pathogenic bacteria in even a fraction of cells could potentially contribute to the chronic and episodic nature of the disease. Further studies are needed to validate the expression of the MIR106B cluster in larger numbers of CD intestinal tissue samples, especially in the context of disease remission and relapse.

The MIR106B and MIR93 cluster is located within intron 13 of the *minichromosome maintenance protein 7* (*MCM7*) gene, which is transcriptionally regulated by c-Myc.[27,40] Thus, the MIR106B and MIR93 cluster is frequently amplified with the host gene MCM7 under the promotion of c-Myc.[40,41] Consistently, we found that ectopic expression of c-Myc markedly increased MIR106B and MIR93 expression and decreased the levels of ATG16L1 and p21 (Figure 7*D* and *E*). Interestingly, c-Myc was found to be highly expressed in active CD in this study (Figure 7*A* and *B*) and in previous reports.[29–31] Thus, it seems rational to propose that increased c-Myc in CD enhances expression of MIR106B and MIR93, which reduces formation of autophagosomes by targeting ATG16L1, thereby suppressing intracellular bacterial removal (Figure 7*F*).

CD is poorly understood and refractory to clinical management; therefore, there is an immediate need to identify both genetic and nongenetic risk factors of the disease.[11,32] Based on our data, we speculate that subjects with MIR106B- and MIR93-mediated loss of ATG16L1 expression will manifest an altered antibacterial activity of intestinal epithelial cells and an abnormal presence of CD-associated intracellular bacteria with substantial impact on the outcome of intestinal inflammation. Our findings may eventually contribute to the improvement of CD therapeutics through the use of MIR106B and MIR93 inhibitors and/or autophagy activators.

Supplementary Materials

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.09.006.

References

1. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 2009;43:67–93.
2. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010;140:313–326.
3. Deretic V, Levine B. Autophagy, immunity, and microbial adaptations. Cell Host Microbe 2009;5:527–549.
4. Huang J, Brumell JH. Autophagy in immunity against intracellular bacteria. Curr Top Microbiol Immunol 2009;335:189–215.
5. Cadwell K, Liu JY, Brown SL, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 2008;456:259–263.
6. Crotzer VL, Blum JS. Autophagy and its role in MHC-mediated antigen presentation. J Immunol 2009;182:3335–3341.
7. Mizushima N, Yoshimori T, Ohsumi Y. Role of the Apg12 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 2003;35:553–561.
8. Noda T, Yoshimori T. Molecular basis of canonical and bactericidal autophagy. Int Immunol 2009;21:1199–1204.
9. Lapaquette P, Glasser AL, Huett A, et al. Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. Cell Microbiol 2010;12:99–113.
10. **Kuballa P, Huett A**, Rioux JD, et al. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS One 2008;3:e3391.
11. Cosnes J, Gower-Rousseau C, Seksik P, et al. Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology 2011;140:1785–1794.
12. Rioux JD, Xavier RJ, Taylor KD, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet 2007;39:596–604.
13. **Jostins L, Ripke S**, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119–124.
14. **Franke A, McGovern DP, Barrett JC**, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010;42:1118–1125.
15. **He L, Thomson JM**, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. Nature 2005;435:828–833.
16. O'Donnell KA, Wentzel EA, Zeller KI, et al. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435:839–843.
17. Brest P, Lapaquette P, Souidi M, et al. A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. Nat Genet 2011;43:242–245.
18. Liang J, Shao SH, Xu ZX, et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 2007;9:218–224.
19. Wine E, Ossa JC, Gray-Owen SD, et al. Adherent-invasive Escherichia coli, strain LF82 disrupts apical junctional complexes in polarized epithelia. BMC Microbiol 2009;9:180.
20. Obernosterer G, Martinez J, Alenius M. Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. Nat Protoc 2007;2:1508–1514.
21. Chen J, Yuan K, Mao X, et al. Serum response factor regulates bone formation via IGF-1 and Runx2 signals. J Bone Miner Res 2012;27:1659–1668.

22. **Hutvagner G, McLachlan J**, Pasquinelli AE, et al. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 2001;293:834–838.
23. Lu C, Huang X, Zhang X, et al. miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27kip1, KPC1, and SOCS-1. Blood 2011;117:4293–4303.
24. Ivanovska I, Ball AS, Diaz RL, et al. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol 2008;28:2167–2174.
25. **Meenhuis A, van Veelen PA**, de Looper H, et al. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. Blood 2011;118:916–925.
26. Fujita N, Itoh T, Omori H, et al. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol Biol Cell 2008;19:2092–2100.
27. **Wu H, Wang F, Hu S**, et al. MiR-20a and miR-106b negatively regulate autophagy induced by leucine deprivation via suppression of ULK1 expression in C2C12 myoblasts. Cell Signal 2012;24:2179–2186.
28. Homer CR, Richmond AL, Rebert NA, et al. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. Gastroenterology 2010;139:1630–1641. 1641 e1–2.
29. Ciclitira PJ, Macartney JC, Evan G. Expression of c-myc in non-malignant and pre-malignant gastrointestinal disorders. J Pathol 1987;151:293–296.
30. Alexander RJ, Panja A, Kaplan-Liss E, et al. Expression of protooncogene-encoded mRNA by colonic epithelial cells in inflammatory bowel disease. Dig Dis Sci 1996;41:660–669.
31. Macpherson AJ, Chester KA, Robson L, et al. Increased expression of c-myc proto-oncogene in biopsies of ulcerative colitis and Crohn's colitis. Gut 1992;33:651–656.
32. Kaser A, Blumberg RS. Autophagy, microbial sensing, endoplasmic reticulum stress, and epithelial function in inflammatory bowel disease. Gastroenterology 2011;140:1738–1747.
33. Zhai Z, Wu F, Chuang AY, et al. miR-106b fine tunes ATG16L1 expression and autophagic activity in intestinal epithelial HCT116 cells. Inflamm Bowel Dis 2013;19:2295–2301.
34. **Saitoh T, Fujita N**, Jang MH, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 2008;456:264–268.
35. **Cooney R, Baker J**, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med 2010;16:90–97.
36. **Fasseu M, Treton X**, Guichard C, et al. Identification of restricted subsets of mature microRNA abnormally expressed in inactive colonic mucosa of patients with inflammatory bowel disease. PLoS One 2010;5(10).
37. Archanioti P, Gazouli M, Theodoropoulos G, et al. Micro-RNAs as regulators and possible diagnostic bio-markers in inflammatory bowel disease. J Crohns Colitis 2011;5:520–524.
38. Plantinga TS, Crisan TO, Oosting M, et al. Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. Gut 2011;60:1229–1235.
39. **Hampe J, Franke A**, Rosenstiel P, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 2007;39:207–211.
40. Poliseno L, Salmena L, Riccardi L, et al. Identification of the miR-106b∼25 microRNA cluster as a proto-oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. Sci Signal 2010;3:ra29.
41. Kumar P, Luo Y, Tudela C, et al. The c-Myc-regulated microRNA-17∼92 (miR-17∼92) and miR-106a∼363 clusters target hCYP19A1 and hGCM1 to inhibit human trophoblast differentiation. Mol Cell Biol 2013;33:1782–1796.

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Conflicts of interest
The authors disclose no conflicts.

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