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BASIC RESEARCH PAPER

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Activation of the EIF2AK4-EIF2A/eIF2 α -ATF4 pathway triggers autophagy response to Crohn disease-associated adherent-invasive *Escherichia coli* infection

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ABSTRACT

The intestinal mucosa of Crohn disease (CD) patients is abnormally colonized by adherent-invasive *E. coli* (AIEC). Upon AIEC infection, autophagy is induced in host cells to restrain bacterial intracellular replication. The underlying mechanism, however, remains unknown. Here, we investigated the role of the EIF2AK4-EIF2A/eIF2 α -ATF4 pathway in the autophagic response to AIEC infection. We showed that infection of human intestinal epithelial T84 cells with the AIEC reference strain LF82 activated the EIF2AK4-EIF2A-ATF4 pathway, as evidenced by increased phospho-EIF2AK4, phospho-EIF2A and ATF4 levels. EIF2AK4 depletion inhibited autophagy activation in response to LF82 infection, leading to increased LF82 intracellular replication and elevated pro-inflammatory cytokine production. Mechanistically, EIF2AK4 depletion suppressed the LF82-induced ATF4 binding to promoters of several autophagy genes including MAP1LC3B, BECN1, SQSTM1, ATG3 and ATG7, and this subsequently inhibited transcription of these genes. LF82 infection of wild-type (WT), but not *eif2ak4*^{-/-} mice activated the EIF2AK4-EIF2A-ATF4 pathway, inducing autophagy gene transcription and autophagy response in enterocytes. Consequently, *eif2ak4*^{-/-} mice exhibited increased intestinal colonization by LF82 bacteria and aggravated inflammation compared to WT mice. Activation of the EIF2AK4-EIF2A-ATF4 pathway was observed in ileal biopsies from patients with noninflamed CD, and this was suppressed in inflamed CD, suggesting that a defect in the activation of this pathway could be one of the mechanisms contributing to active disease. In conclusion, we show that activation of the EIF2AK4-EIF2A-ATF4 pathway upon AIEC infection serves as a host defense mechanism to induce functional autophagy to control AIEC intracellular replication.

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AIEC infection; autophagy; autophagy gene transcription; Crohn disease; EIF2AK4

Introduction

Evidence has suggested that the etiology of inflammatory bowel disease (IBD), including Crohn disease (CD) and ulcerative colitis, involves environmental, genetic, immunological and infectious factors.¹ An alteration in the intestinal microbiota, called dysbiosis, has been associated with CD, characterized by a decrease in *Firmicutes*, in particular *Faecalibacterium prausnitzii*, and an increase in *Enterobacteriaceae*, especially *Escherichia coli*.²

Our group and others have shown a high prevalence of the adherent-invasive *Escherichia coli* (AIEC) pathotype in the ileal mucosa of CD patients.^{3–5} Characterization of the AIEC strains show they are able to adhere to and to invade human intestinal epithelial cells (IECs),^{4,6} survive and replicate within macrophages,^{7,8} induce high production of proinflammatory cytokines and chemokines,^{7,9,10} and exacerbate intestinal inflammation in a genetically susceptible mouse model.⁹ Our

recent studies showed that autophagy is induced in host cells upon AIEC infection, and a functional autophagy is required to restrain the intracellular replication of AIEC.^{6,8,10,11} However, the mechanism involved in autophagy induction in response to AIEC infection remains largely unknown.

Autophagy is a tightly regulated homeostatic process responsible for the elimination of damaged cytoplasmic components via the lysosomal pathway.¹² This process plays a key role in the regulation of numerous physiological functions including cell development, differentiation and survival, and in the control of the innate and adaptive immune responses. In response to multiple stresses such as nutrient starvation, damaged organelles and unfolded protein aggregation, or infection with pathogens, autophagy is induced, and this is a crucial host defense mechanism. Dysregulated autophagy has been linked to numerous human pathologies including CD.¹³ Genome-wide association studies have discovered the association with

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CD susceptibility of polymorphisms in the genes involved in autophagy, such as *ATG16L1* (autophagy-related 16 like 1), *IRGM* (immunity-related GTPase family, M), *XBP1* (X-box binding protein 1), *LRRK2* (leucine-rich repeat kinase 2), *PTPN2* (protein tyrosine phosphatase, non-receptor type 2) and *ULK1* (unc-51 like autophagy activating kinase 1).¹⁴ The known CD-associated NOD2 (nucleotide-binding oligomerization domain-containing 2) mutations have been linked to autophagy via its physical interaction with ATG16L1 in response to bacterial infection.^{15–17} Recently, significant advances have been achieved in understanding the functional consequences of the autophagy-related gene defects in CD. Evidence has shown that the autophagy-related risk polymorphisms lead to impaired sensing and handling of intracellular bacteria by innate immunity, in particular the inappropriate stimulation of antimicrobial and inflammasome pathways eventually result in uncontrolled inflammation.^{14,18} These studies bring autophagy to light as a key regulator with the capacity to integrate several aspects of CD pathogenesis.

Activation of the EIF2AK4-EIF2A-ATF4 pathway is necessary to activate autophagy gene expression in response to cellular stress.¹⁹ EIF2AK4 (eukaryotic translation initiation factor 2 alpha kinase 4) belongs to a family of protein kinases that phosphorylate the α subunit of EIF2 (eukaryotic translation initiation factor 2) in response to various stress stimuli. Phosphorylation of EIF2A in response to EIF2AK4 activation subsequently increases translation of specific mRNAs that are important for stress remediation such as ATF4 (activating transcription factor 4).²⁰ Recent evidence demonstrates that the EIF2AK4-EIF2A-ATF4 pathway is activated in epithelial cells upon infection with *Shigella* and *Salmonella*.²¹ However, to our knowledge, no data are available on the link between this pathway and CD and its involvement in host responses to infection with pathogenic bacteria associated with the disease.

Here, we aimed at investigating (i) the ability of AIEC to modulate the EIF2AK4-EIF2A-ATF4 pathway and (ii) the role of this pathway in regulating autophagy to control the intracellular multiplication of AIEC.

Results

AIEC infection induces activation of the EIF2AK4-EIF2A-ATF4 pathway in IECs

We first investigated activation of the EIF2AK4-EIF2A-ATF4 pathway in human intestinal epithelial T84 cells in response to infection with the AIEC LF82 reference strain. Western blot analysis showed that LF82 infection increased the levels of phospho-EIF2AK4, phospho-EIF2A and ATF4 in T84 cells (Fig. 1A). This effect was almost as profound as when cells were incubated for 1 h with amino acid-starvation Krebs-Ringer buffer (KRB; Fig. 1A). In contrast, infection with the nonpathogenic *E. coli* K12 MG1655 or the commensal *E. coli* HS strain did not significantly affect the levels of phospho-EIF2AK4, phospho-EIF2A and ATF4 (Fig. 1B). qRT-PCR analysis showed that mRNA expression levels of the known target genes of ATF4, including *TRIB3* (tribbles pseudokinase 3), *ATF3* (activating transcription factor 3), *DDIT3* (DNA damage inducible transcript 3) and *ASNS* (asparagine synthetase), were significantly increased in LF82-infected cells

compared to uninfected cells or cells infected with either the K12 or HS strain (Fig. 1C); mRNA expression levels of these genes were not significantly changed upon infection with the K12 or HS strain. These results demonstrate that the EIF2AK4-EIF2A-ATF4 pathway is activated upon AIEC infection in host IECs.

EIF2AK4 is essential to restrain AIEC intracellular replication and AIEC-induced inflammation

To explore the role of the EIF2AK4-EIF2A-ATF4 pathway in host response to AIEC infection, mouse embryonic fibroblasts (MEFs) harboring a deletion for the *Eif2ak4* gene (*eif2ak4*^{-/-} MEFs) were used. Infection with AIEC LF82 induced activation of EIF2AK4 and EIF2A, enhanced ATF4 protein levels, and increased mRNA expression levels of ATF4 target genes in wild-type (WT) MEFs (Fig. 2A and B). These results were not observed in *eif2ak4*^{-/-} MEFs (Fig. 2A and B). Incubation of WT MEFs with KRB for 4 h, used as a positive control, also activated the EIF2AK4-EIF2A-ATF4 pathway in WT MEFs but not in *eif2ak4*^{-/-} MEFs (Fig. 2A). To examine whether the EIF2AK4-EIF2A-ATF4 pathway plays a role in the control of AIEC intracellular replication, WT and *eif2ak4*^{-/-} MEFs were infected with AIEC LF82, and the number of intracellular bacteria was counted on Luria-Bertani (LB) agar plates. At different time points post-infection, there was a marked increase in the number of intracellular LF82 bacteria in *eif2ak4*^{-/-} MEFs compared to WT MEFs (Fig. 2C). Confocal microscopy analysis of MEFs infected with a LF82-GFP strain consistently showed that invalidation of *Eif2ak4* led to an increased number of intracellular LF82 (Fig. 2D). This was accompanied by an increase in AIEC-induced production of the pro-inflammatory cytokine IL6 (interleukin 6) and chemokine CXCL1 (chemokine [C-X-C motif] ligand 1), respectively, in *eif2ak4*^{-/-} MEFs compared to WT MEFs (Fig. 2E). These results show that activation of the EIF2AK4-EIF2A-ATF4 pathway in response to AIEC infection is essential to limit AIEC intracellular replication and AIEC-induced inflammation.

EIF2AK4 is necessary for autophagy activation in response to AIEC infection

Given that a functional autophagy is required to restrain AIEC intracellular replication,^{6,8,10,11} the potential involvement of the EIF2AK4-EIF2A-ATF4 pathway in autophagy response to AIEC infection was next examined. Analysis for the shift of LC3-I (microtubule associated protein 1 light chain 3; the free cytosolic form) to LC3-II (the phagophore and autophagosomal form) by western blot, an indication of autophagy induction, showed that autophagy was induced in WT MEFs upon LF82 infection (Fig. 3A). Induction of a functional and degradative autophagy flux in LF82-infected WT MEFs was confirmed by the concomitant decrease in SQSTM1 (sequestosome 1), a receptor protein incorporated into the autophagosome and degraded inside autolysosomes (Fig. 3A). In contrast, autophagy was not induced in LF82-infected *eif2ak4*^{-/-} MEFs (Fig. 3A). However, treatment of both WT and *eif2ak4*^{-/-} MEFs with 40 μ g/ml rapamycin, an inducer of autophagy and used as a positive control, led to increased LC3-II levels and SQSTM1 degradation (Fig. 3A).

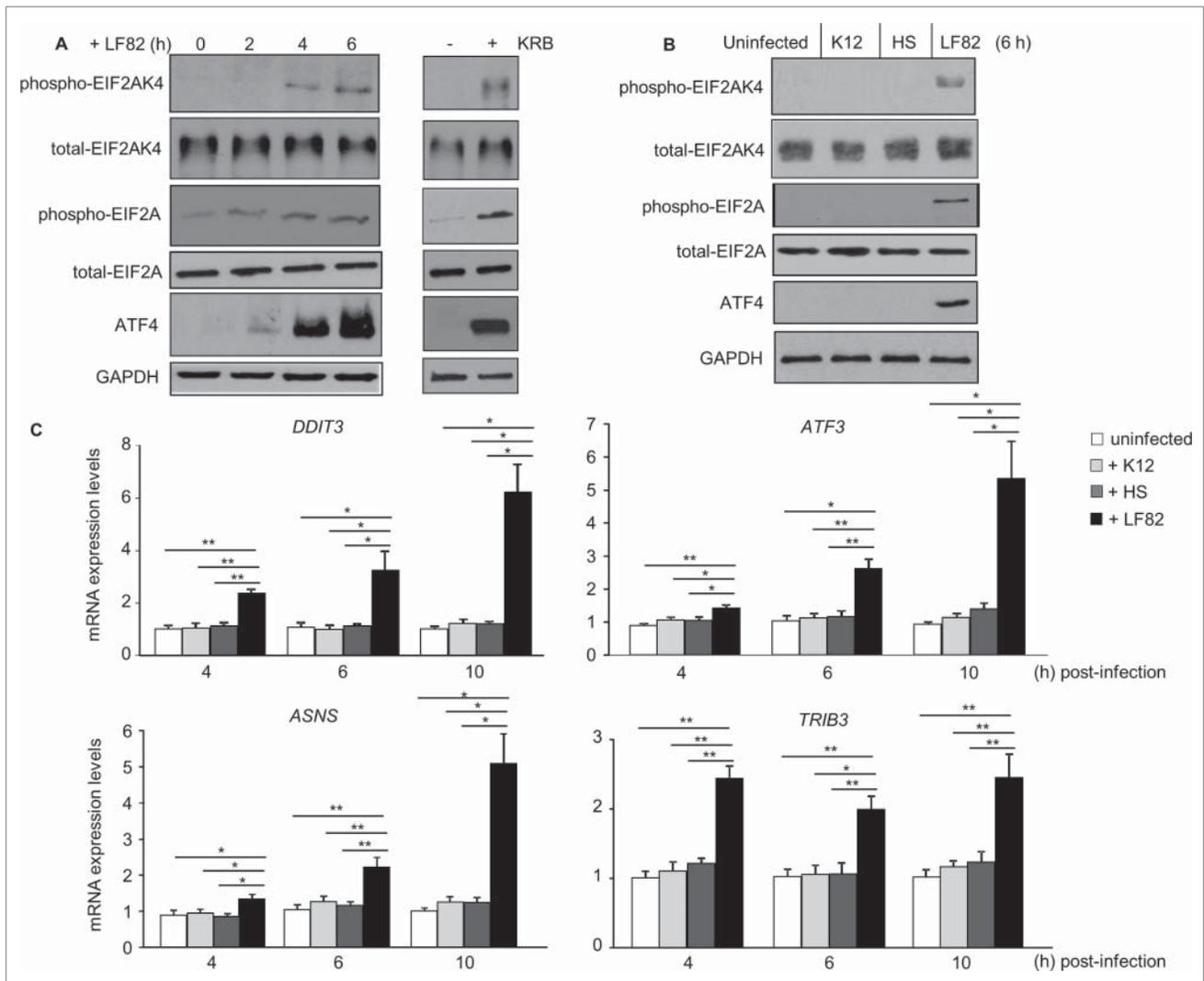


Figure 1. AIEC infection induces activation of the EIF2AK4-EIF2A-ATF4 pathway in T84 cells. T84 cells were infected with AIEC LF82 strain or the *E. coli* K12 MG1655 and the commensal *E. coli* HS strain. In parallel, cells were treated with KRB for 1 h. (A, B) Western blot analysis for the activation of the EIF2AK4-EIF2A-ATF4 pathway. (C) qRT-PCR analysis for mRNA expression levels of known ATF4 target genes, including *TRIB3*, *ATF3*, *DDIT3* and *ASNS*. Data are means \pm SEM of 6 replicates and are representative of 3 independent experiments. *, $P < 0.05$; **, $P \leq 0.005$.

Confocal microscopy analysis showed that depletion of *Eif2ak4* suppressed LC3 aggregation and therefore autophagic response to LF82 infection (Fig. 3B). WT, *eif2ak4*^{-/-} and *atg5*^{-/-} MEFs were transfected with a pEGFP-LC3 plasmid and infected with LF82, and the numbers of puncta per cell, which allow estimation of autophagosome formation, were quantified. As shown in Fig. 3C, the numbers of puncta were markedly increased in WT MEFs compared to *eif2ak4*^{-/-} and *atg5*^{-/-} MEFs upon LF82 infection. Rapamycin treatment, used as a positive control, increased the numbers of puncta in both WT and *eif2ak4*^{-/-} MEFs, but not in *atg5*^{-/-} MEFs (Fig. 3C). These data show that EIF2AK4 is necessary for autophagy induction in response to AIEC infection.

Activation of the EIF2AK4-EIF2A-ATF4 pathway in response to AIEC infection is essential for ATF4-mediated transcription of autophagy genes

It has been recently shown that activation of the EIF2AK4-EIF2A-ATF4 pathway is required for stress-induced transcription of several autophagy genes.¹⁹ In an effort to demonstrate the mechanism underlying the involvement of this pathway in

autophagy response to AIEC infection, we analyzed the transcriptional levels of several genes that are involved in autophagy. Among the autophagy genes tested, mRNA expression levels of *Sqstm1*, *Map1lc3b* (microtubule-associated protein 1 light chain 3 beta), *Becn1/Beclin 1*, *Atg3* (autophagy-related 3) and *Atg7* (autophagy-related 7) were significantly increased in response to AIEC infection in WT MEFs, and this was inhibited in *eif2ak4*^{-/-} MEFs (Fig. 3D).

To investigate whether the transcription factor ATF4, activated in response to AIEC infection, can bind to promoters of these autophagy genes, a ChIP assay was performed using an anti-ATF4 antibody and a negative control IgG. In WT MEFs, AIEC infection induced binding of ATF4 to the promoter of the *Sqstm1*, *Map1lc3b*, *Becn1*, *Atg3* and *Atg7* genes as analyzed by semi-quantitative (Fig. 3E) and quantitative PCR (Fig. 3F). This was not observed in *eif2ak4*^{-/-} MEFs (Fig. 3E and F). No binding was detected for the negative control IgG, indicating specificity of the ChIP assay (Fig. 3E).

Together, these data show that EIF2AK4 is essential to induce transcription of autophagy genes via ATF4 binding, and thereby activation of autophagy in response to AIEC infection.

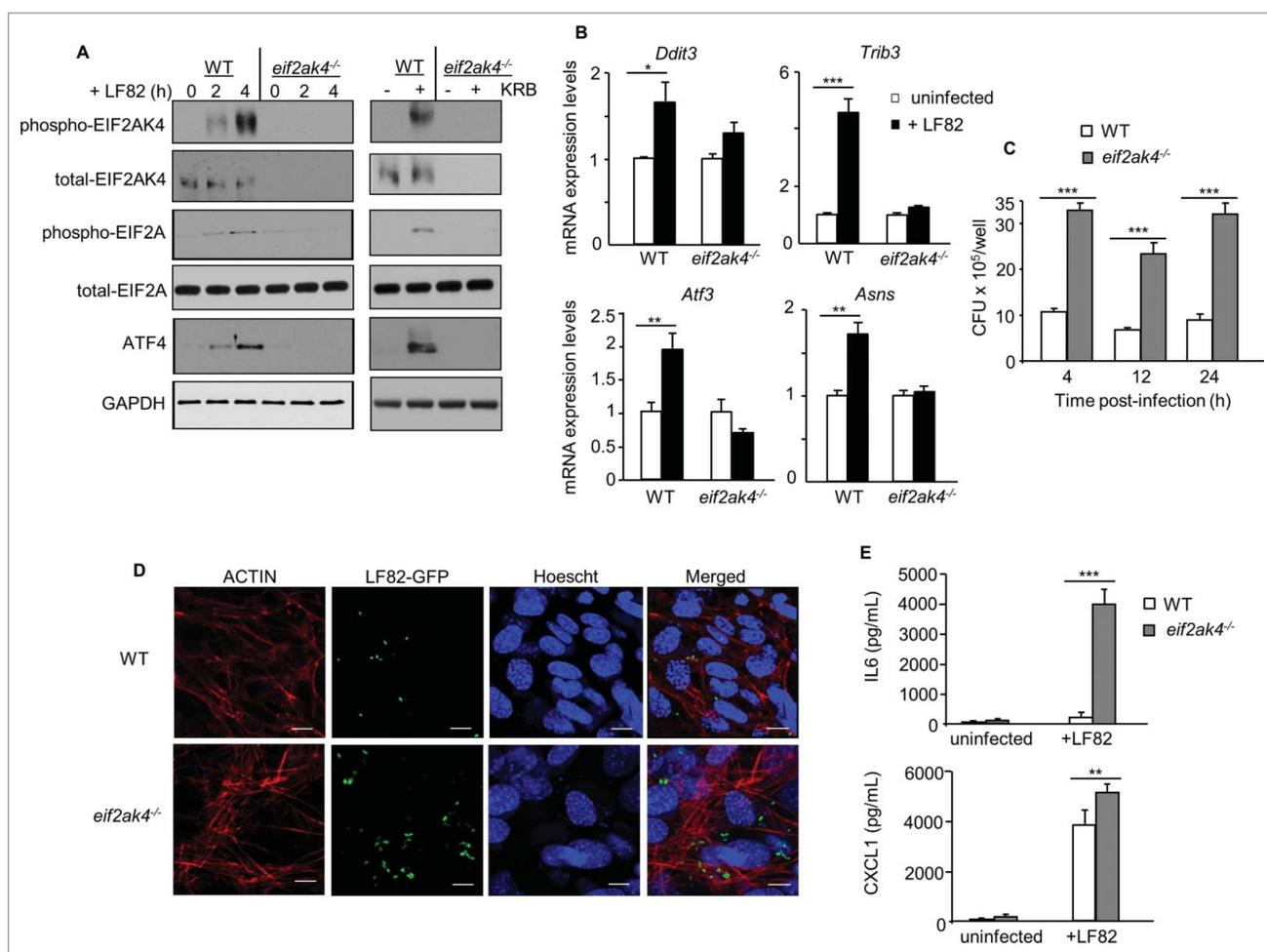


Figure 2. *Eif2ak4* depletion results in increased AIEC intracellular replication and enhanced AIEC-induced production of cytokines and chemokines. Wild-type (WT) MEFs and MEFs depleted for *Eif2ak4* (*eif2ak4*^{-/-}) were infected with AIEC LF82 for 6 h (B), 12 h (D), 24 h (E) or the indicated time (A, C). In parallel, MEFs were treated with KRB for 4 h. (A) Western blot analysis for the activation of the EIF2AK4-EIF2A-ATF4 pathway. (B) qRT-PCR analysis for mRNA expression levels of ATF4 target genes, including *Trib3*, *Atf3*, *Ddit3* and *Asns*. (C) Intracellular LF82 number counted on LB agar plates. (D) Representative confocal micrographs of cells infected with LF82-GFP. Actin cytoskeleton was stained with Rhodamine-phalloidin (red). Nuclei were stained with Hoechst (blue). Bars: 5 μ m. (E) Secreted IL6 and CXCL1 amounts in cell culture supernatant quantified by ELISA. Data are means \pm SEM of 6 replicates and are representative of 3 independent experiments. *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$.

siRNA-mediated depletion of EIF2AK4 in IECs inhibits ATF4-mediated transcription of autophagy genes and autophagy activation in response to AIEC infection, increasing AIEC intracellular replication and AIEC-induced inflammation

To verify the results obtained with *eif2ak4*^{-/-} MEFs, we examined the effect of depleting *EIF2AK4* expression in T84 cells on autophagy-mediated control of AIEC intracellular replication and AIEC-induced inflammation. Transfection of T84 cells with *EIF2AK4* siRNA inhibited *EIF2AK4* and *EIF2A* activation, and *ATF4* expression induced by LF82 infection compared with control siRNA (Fig. 4A). KRB treatment, used as a positive control, also activated the *EIF2AK4*-*EIF2A*-*ATF4* pathway in control siRNA-transfected cells, and this was not observed in *EIF2AK4* siRNA-transfected cells (Fig. 4A).

In control siRNA-transfected cells, autophagy was induced upon LF82 infection as shown by increased LC3-II levels and *SQSTM1* protein degradation (Fig. 4B). This was not observed in *EIF2AK4* siRNA-transfected cells upon LF82 infection (Fig. 4B). Rapamycin treatment, however, activated autophagy in both control siRNA- and *EIF2AK4* siRNA-transfected cells (Fig. 4B). The

suppression of autophagy activation in *EIF2AK4* siRNA-transfected cells was correlated with the inhibition of LF82-induced transcription of the *SQSTM1*, *MAP1LC3B*, *BECN1*, *ATG3* and *ATG7* genes (Fig. 4C). The defect in autophagy response to LF82 infection in *EIF2AK4* siRNA-transfected T84 cells consequently led to an increased number of intracellular LF82 (Fig. 4D) and enhanced IL8 (interleukin 8) production (Fig. 4E). Rapamycin treatment prevented the increased number of intracellular LF82 bacteria in both control siRNA- and *EIF2AK4* siRNA-transfected cells (Fig. 4D). These results, which are consistent with the results obtained with *eif2ak4*^{-/-} MEFs, show that *EIF2AK4* is essential to induce a functional autophagy necessary to control AIEC intracellular replication and AIEC-induced inflammation.

The EIF2AK4-EIF2A-ATF4 pathway is activated in IECs from AIEC-infected mice, inducing transcription of autophagy genes and thereby the autophagy process

To gain insights into the role of the *EIF2AK4*-*EIF2A*-*ATF4* pathway in autophagy response to in vivo infection, we used

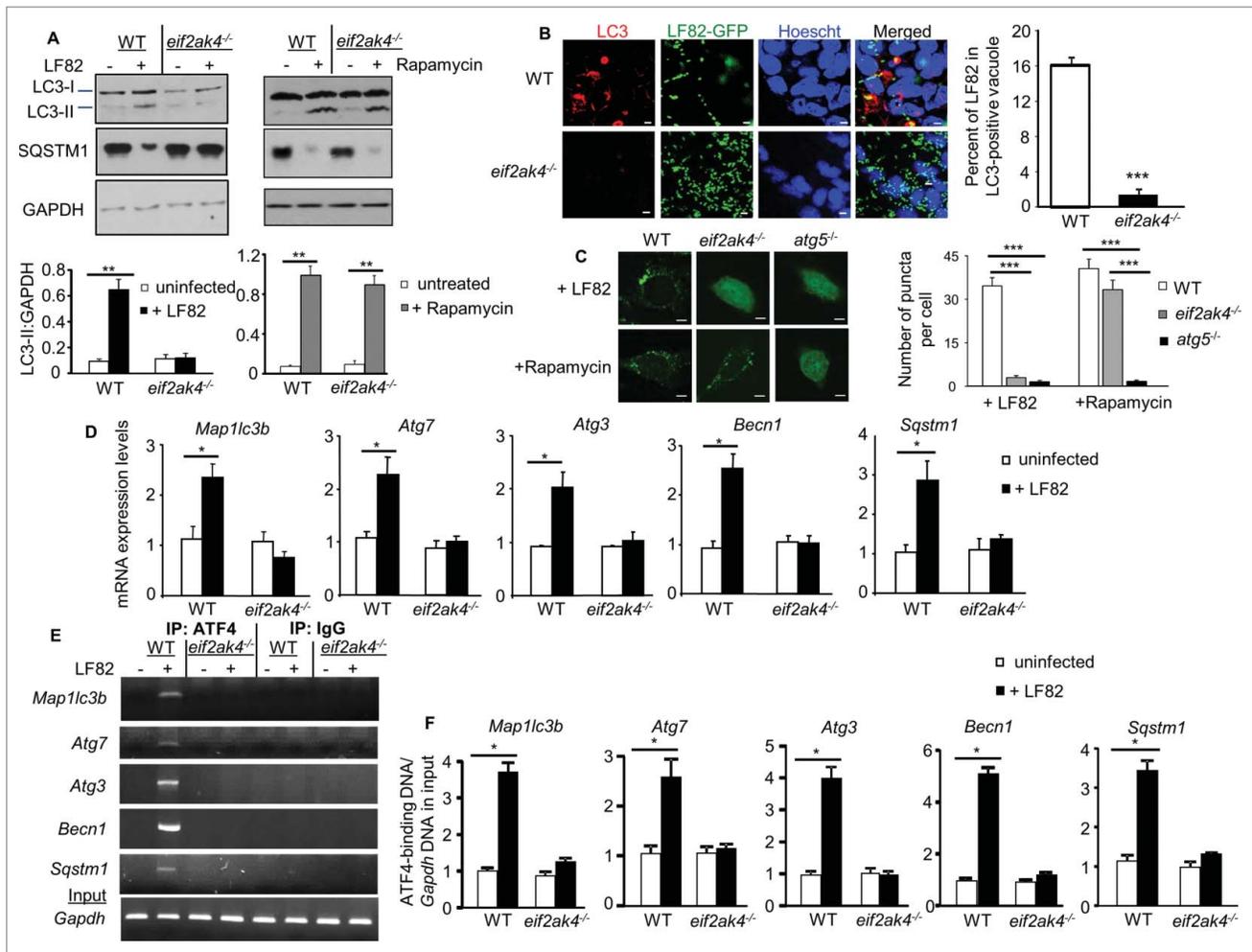


Figure 3. *Eif2ak4* depletion blocks autophagy activation in response to AIEC infection by inhibiting the ATF4-mediated transcription of autophagy genes. Wild-type (WT) MEFs and MEFs depleted for *Eif2ak4* (*eif2ak4*^{-/-}) were infected with AIEC LF82 or treated with 40 μ g/ml rapamycin for 6 h. (A) Representative western blot analysis for LC3 and SQSTM1 levels. Quantification of band intensity of LC3-II relative to GAPDH from 3 independent blots is shown in the bottom panel. Values represent means \pm SEM **, $P \leq 0.005$. (B) Representative confocal micrographs of cells infected with LF82-GFP (green) and immunolabeled for LC3 (red). Nuclei were stained with Hoechst (blue). Bars: 5 μ m. The graph to the right shows the percentage of LF82-GFP in LC3-positive vacuoles determined from 3 independent experiments, counting 20 cells/experiment. Values represent means \pm SEM ***, $P \leq 0.001$. (C) Representative confocal micrographs of WT, *eif2ak4*^{-/-} and *atg5*^{-/-} MEFs pre-transfected with the pEGFP-LC3 plasmid and infected with LF82 or treated with rapamycin. Bars: 5 μ m. The graph to the right shows the number of GFP-LC3 puncta per cell determined from 3 independent experiments, counting 10 cells/experiment. Values represent means \pm SEM ***, $P \leq 0.001$. (D) qRT-PCR analysis for mRNA expression levels of the autophagy genes *Map1lc3b*, *Atg7*, *Atg3*, *Becn1* and *Sqstm1*. Data are means \pm SEM of 6 replicates and are representative of 3 independent experiments. *, $P < 0.05$. (E, F) ChIP analysis for the binding of ATF4 to the autophagy gene promoters. The protein-DNA complexes were immunoprecipitated with anti-ATF4 or a negative control IgG. (E) Representative agarose gels for the ATF4-binding regions in the autophagy gene promoters or *Gapdh* DNA in the input amplified by semiquantitative PCR. (F) Increase in the binding of ATF4 to the autophagy gene promoters, normalized to *Gapdh* DNA in the input, upon LF82 infection analyzed by qPCR. Data are means \pm SEM of 4 replicates and are representative of 2 independent experiments. *, $P < 0.05$.

eif2ak4^{-/-} mice and a model of AIEC LF82 infection. In agreement with the in vitro data, LF82 infection of WT mice activated the EIF2AK4-EIF2A-ATF4 pathway in ileal epithelial cells as shown by increased phospho-EIF2AK4, phospho-EIF2A and ATF4 protein levels (Fig. 5A), as well as enhanced transcriptional levels of ATF4 target genes *Trib3*, *Atf3*, *Ddit3* and *Asns* (Fig. S1). In *eif2ak4*^{-/-} mice, AIEC infection did not affect the levels of phospho-EIF2AK4, phospho-EIF2A or ATF4, as well as the transcriptional levels of the ATF4 target genes (Fig. 5A and S1), indicating the important role of EIF2AK4 in activation of the EIF2A-ATF4 axis in response to AIEC infection. In contrast, this pathway was not activated in the ileum of mice infected with the K12 MG1655 strain (Fig. S2). As the EIF2AK4-EIF2A-ATF4 pathway is activated in IECs in response to AIEC infection, we questioned whether

this could be a consequence of inflammation. We then investigated whether intestinal inflammation induces activation of this pathway using a mouse model of dextran sodium sulfate (DSS)-induced colitis. As shown in Figure S3, DSS treatment did not increase the levels of intestinal phospho-EIF2AK4 in WT mice. Together, these data suggest that activation of the EIF2AK4-EIF2A-ATF4 pathway is specific to AIEC infection and is not a consequence of inflammation.

In agreement with the in vitro data, autophagy was induced in response to LF82 infection in ileal epithelial cells from WT mice, but not *eif2ak4*^{-/-} mice as shown by increased LC3-II levels and SQSTM1 degradation (Fig. 5B). LF82 infection of WT mice increased the transcriptional levels of the autophagy genes *Sqstm1*, *Map1lc3b*, *Becn1*, *Atg3* and *Atg7* in IECs (Fig. 5C). Amplification of ATF4-binding regions in the promoters of these

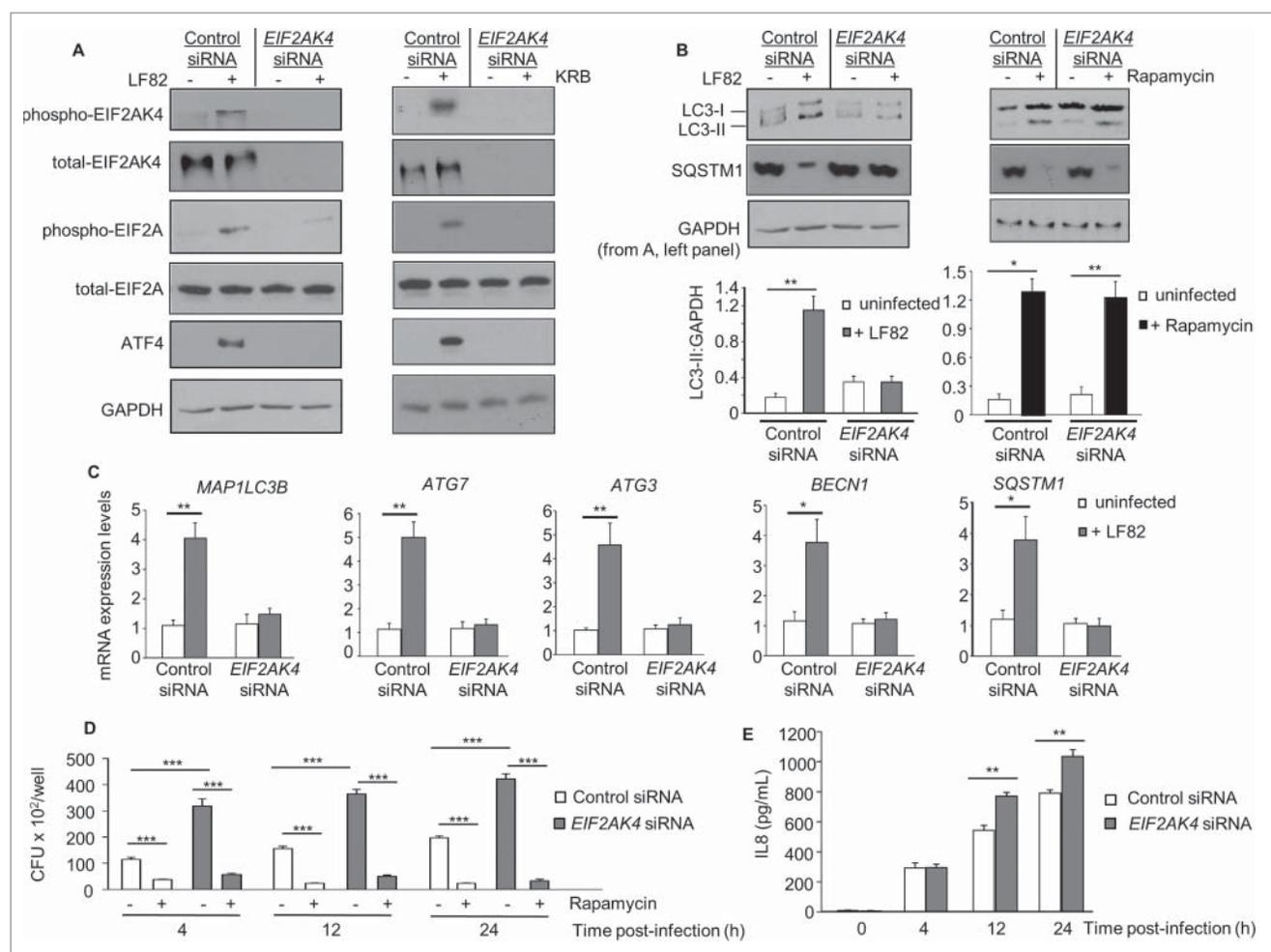


Figure 4. siRNA-mediated depletion of *EIF2AK4* in T84 cells inhibits autophagy activation in response to AIEC infection, increasing AIEC intracellular replication and AIEC-induced inflammation. T84 cells were transfected with control or *EIF2AK4*-specific siRNA for 36 h and then infected or not with the AIEC LF82 strain for 6 h or incubated with KRB for 1 h (A) or treated with 40 μ g/ml rapamycin for 6 h (B). (A) Western blot analysis for the activation of the EIF2AK4-EIF2A-ATF4 pathway. (B) Representative western blot analysis for LC3 and SQSTM1 levels. Quantification of band intensity of LC3-II relative to GAPDH from 3 independent blots was shown in the bottom panel. Values are means \pm SEM. *, $P < 0.05$; **, $P \leq 0.005$. (C) qRT-PCR analysis for mRNA expression levels of the autophagy genes *MAP1LC3B*, *ATG3*, *ATG7*, *BECN1* and *SQSTM1*. (D) Cells were infected with AIEC LF82 in the presence or absence of 40 μ g/ml rapamycin. Intracellular LF82 number was counted on LB agar plates. (E) Secreted IL8 amounts in cell culture supernatant quantified by ELISA. Data are means \pm SEM of 6 replicates and are representative of 3 independent experiments. *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$ (C-E).

genes by semi-quantitative and quantitative PCR showed that LF82 infection induced binding of ATF4 to the promoters of the *Sqstm1*, *Map1lc3b*, *Becn1*, *Atg3* and *Atg7* genes in IECs from WT mice (Fig. 5D and S4). No binding was detected for the negative control IgG, indicating specificity of ATF4 binding (Fig. 5D). Invalidation of *Eif2ak4* suppressed the LF82-induced transcription of these genes in IECs (Fig. 5C) by inhibiting ATF4 binding to their promoters (Figs. 5D and S4).

Together, these data show that activation of the EIF2AK4-EIF2A-ATF4 pathway in mouse enterocytes is essential for ATF4-mediated transcription of autophagy genes, thereby activating autophagy in response to AIEC infection.

EIF2AK4 is essential to restrain AIEC intestinal colonization and AIEC-induced inflammation in mice

We next investigated the role of the EIF2AK4-EIF2A-ATF4 pathway in the control of AIEC colonization of the gut. LF82 persistence in the gut of *eif2ak4*^{-/-} mice was increased

compared to WT mice as shown by marked increased LF82 number counted in the feces at different days post-infection (Fig. 6A). No difference in the number of K12 in the feces between WT and *eif2ak4*^{-/-} mice was observed (Fig. 6A). At day of sacrifice, the presence of LF82 bacteria was observed only in the ileum of *eif2ak4*^{-/-} mice (Fig. 6B). No K12 bacteria were detected in the ileal mucosa from either WT or *eif2ak4*^{-/-} mice (Fig. 6B). Confocal microscopy analysis using rabbit antiserum against *E. coli* lipopolysaccharide O83 consistently showed a huge number of LF82 bacteria (green) associated with the ileal mucosa of *eif2ak4*^{-/-} mice, but not WT mice (Fig. 6C). This was accompanied by increased production of the pro-inflammatory cytokine IL6 and chemokine CXCL1 at mRNA and protein levels in the intestine of *eif2ak4*^{-/-} mice compared to WT mice (Fig. 6D and E). These data, which are in agreement with the in vitro data, confirm the role of the EIF2AK4-EIF2A-ATF4 pathway in the control of AIEC replication and AIEC-induced inflammation.

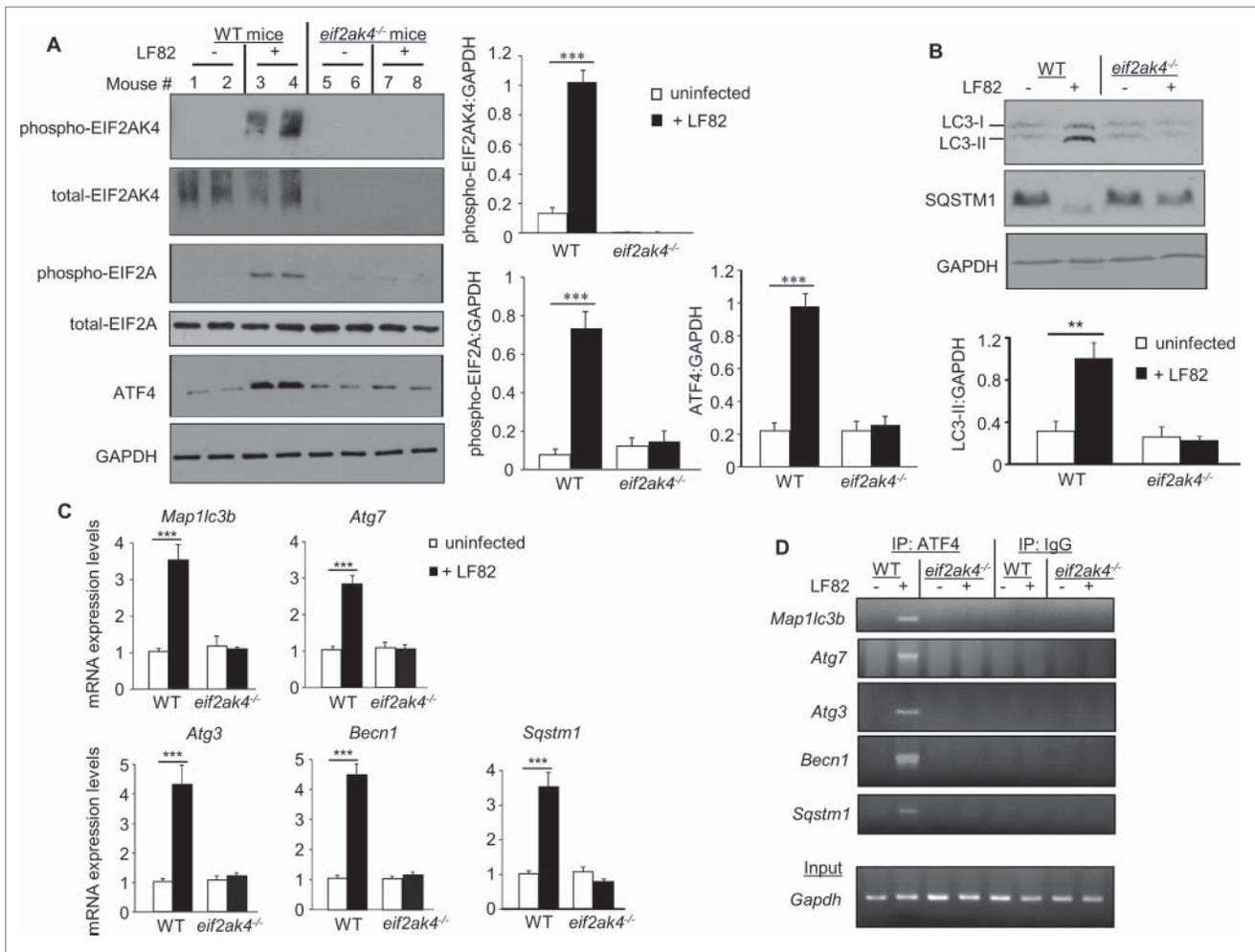


Figure 5. Activation of the EIF2AK4-EIF2A-ATF4 pathway in IECs from AIEC-infected mice, inducing an autophagy response. Wild-type (WT) and *eif2ak4*^{-/-} mice were infected with LF82 by gavage and ileal enterocytes were extracted as described in Materials and methods. (A) Representative immunoblots for the analysis of the EIF2AK4-EIF2A-ATF4 pathway. The graphs to the right represent quantification of immunoblots of phospho-EIF2AK4, phospho-EIF2A and ATF4 relative to GAPDH from $n = 9$ mice/group. (B) Representative western blot analysis for LC3 and SQSTM1 levels. Quantification of band intensity of LC3-II relative to GAPDH from $n = 9$ mice/group was shown in the bottom panel. (C) qRT-PCR analysis for mRNA expression levels of the autophagy genes *Map1lc3b*, *Atg3*, *Atg7*, *Becn1* and *Sqstm1*. (D) ChIP analysis for the binding of ATF4 to the autophagy gene promoters. The protein-DNA complexes were immunoprecipitated with anti-ATF4 antibody or a negative control IgG. Representative agarose gels for the ATF4-binding regions in the autophagy gene promoters or *Gapdh* DNA in the input amplified by semiquantitative PCR are shown. Data are means \pm SEM from $n = 9$ mice/group. **, $P \leq 0.005$; ***, $P \leq 0.001$.

We then sought to determine whether the increase in AIEC-induced intestinal inflammation in *eif2ak4*^{-/-} mice compared to WT mice is because EIF2AK4 plays a role in inflammation or because its role is specific to autophagy response during AIEC infection. For that, we assessed the effect of *Eif2ak4* depletion on intestinal inflammation induced by DSS treatment in mice. During the course of DSS treatment, both WT and *eif2ak4*^{-/-} mice displayed severe body weight loss, rectal bleeding, and diarrhea with no significant difference in clinical score (Fig. S5A and B). In addition, after DSS treatment, WT and *eif2ak4*^{-/-} mice exhibited severe inflammation with loss of entire crypts and surface epithelium in the colon and massive infiltration of inflammatory cells into the mucosa and submucosa with similar levels (Fig. S5C). As shown in Fig. S5D, WT and *eif2ak4*^{-/-} mice showed a similar increase in DSS-induced colonic production of proinflammatory cytokines TNF (tumor necrosis factor), IL1B (interleukin 1 beta), and IL6 and chemokine CXCL1. These results demonstrate that depletion of

Eif2ak4 did not affect susceptibility of mice to DSS-induced colitis.

Together, these data suggest that the role of the EIF2AK4-EIF2A-ATF4 pathway is specific to autophagy modulation during AIEC infection but not to inflammatory responses.

The EIF2AK4-EIF2A-ATF4 pathway is activated in noninflamed CD and deactivated in inflamed CD

We finally investigated whether there is a link between the EIF2AK4-EIF2A-ATF4 pathway and CD. Western blot analysis showed increased levels of phospho-EIF2AK4, phospho-EIF2A and ATF4, indicating activation of this pathway, in ileal biopsies from patients with noninflamed CD compared with the normal control group (Fig. 7A). However, compared to the noninflamed CD group, activation of this pathway was suppressed in inflamed CD. In addition, mRNA expression levels of the known target genes of ATF4 (*TRIB3*, *ATF3*, *DDIT3* and

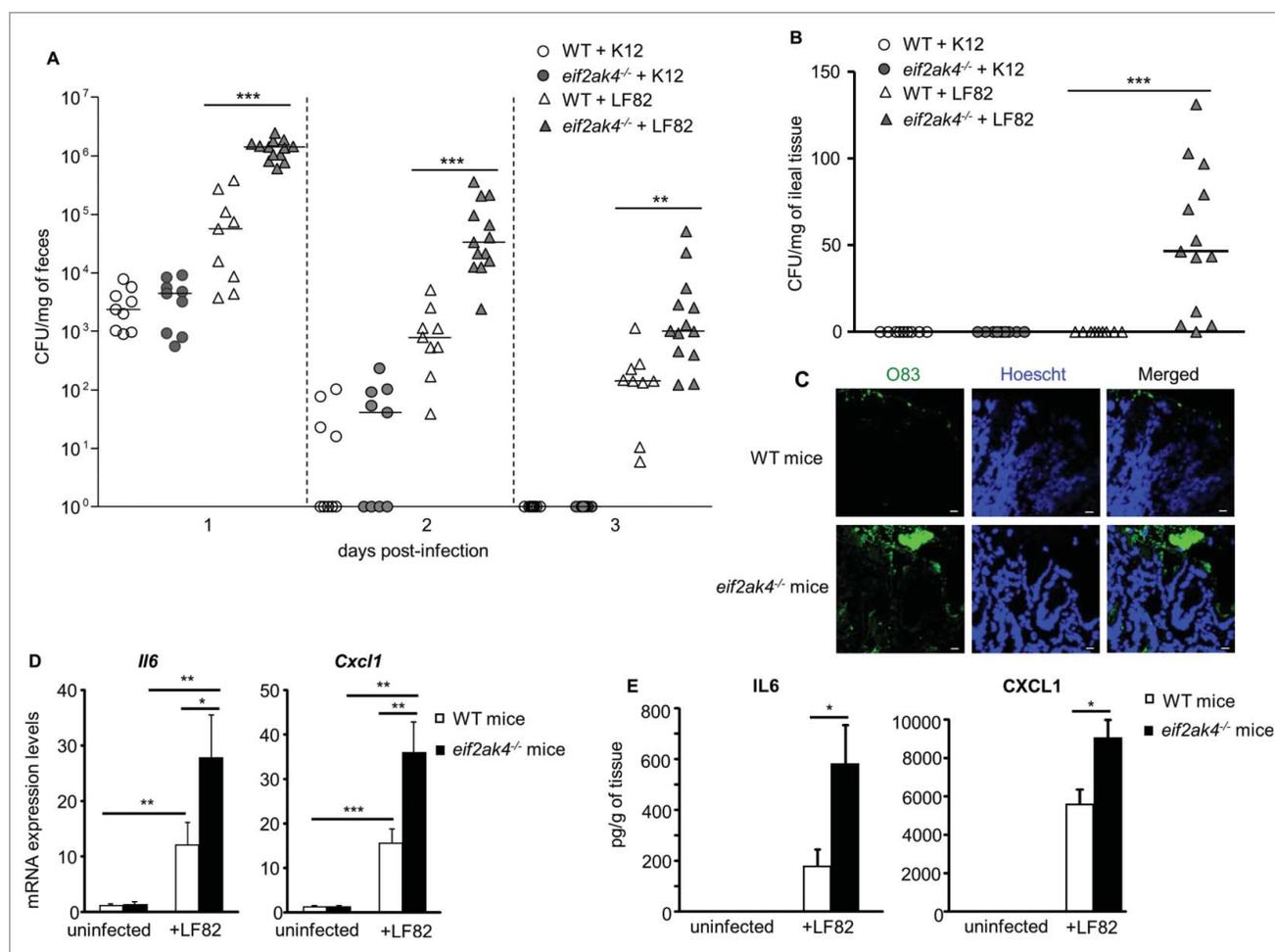


Figure 6. *Eif2ak4* depletion increases AIEC intestinal colonization and AIEC-induced inflammation in mice. Wild type (WT) and *eif2ak4*^{-/-} mice were infected with 10⁹ CFU of LF82 bacteria or the *E. coli* K12 MG1655 strain by gavage for 3 d. Quantification of LF82 and K12 bacteria in the feces collected every day post-infection (A) or associated with the intestinal mucosa determined at day of sacrifice (B). The quantification for each mouse (symbols) and the median (bars) are shown (A, B). (C) Representative confocal micrographs of ileal sections immunolabeled for *E. coli* lipopolysaccharide O83 (green) from LF82-infected WT and *eif2ak4*^{-/-} mice. Nuclei were stained with Hoechst (blue). Bars: 10 μ m. (D) mRNA expression levels of *Cxcl1* and *Il6* in mouse ileal tissues were assessed by qRT-PCR. (E) Secreted CXCL1 and IL6 amount in ileal tissue culture supernatant were quantified by ELISA. Data are means \pm SEM from n = 9 mice/group. *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$.

ASNS) and of the autophagy genes shown in this study to be regulated by ATF4 (*SQSTM1*, *MAP1LC3B*, *BECN1*, *ATG3* and *ATG7*) were markedly increased in the noninflamed CD group, and were suppressed in the inflamed CD group (Fig. 7B). These data suggest that the EIF2AK4-EIF2A-ATF4 pathway is activated in the pre-onset of CD, and a defect in the activation of this pathway could be one of the mechanisms contributing to active disease.

Discussion

Exciting recent progress has been made in understanding the mechanistic links between defects in autophagy-mediated pathogen handling, innate immune activation, and CD pathogenesis. A specific pathogenic group of *E. coli*, called adherent-invasive *E. coli* (AIEC), which abnormally colonize the intestinal mucosa of CD patients,³⁻⁵ has been extensively used to investigate the role of autophagy in CD etiology. AIEC bacteria are able to adhere to and invade human intestinal epithelial cells (IECs),^{4,6} survive and replicate within macrophages,^{7,8} induce high production of proinflammatory cytokines and chemokines,^{7,9,10} and exacerbate

intestinal inflammation in a genetically susceptible mouse model.⁹ AIEC bacteria can also target M cells to interact with Peyer patches and macrophages within the lamina propria.²² We have shown that in response to AIEC infection, autophagy is induced in host cells to control the AIEC bacterial intracellular replication.^{6,8,10,11} CD-associated risk alleles in autophagy genes *ATG16L1* and *IRGM* result in impaired efficacy of autophagy defense with a substantial abnormal persistence of AIEC and intestinal inflammation.^{6,11} The signaling pathways implicated in the induction of autophagy in host cells infected with AIEC remain to be investigated.

Evidence has suggested that the EIF2A-ATF4 axis could be implicated in autophagy regulation. The transcription factor ATF4 plays a key role in regulating transcription of the essential autophagy gene *MAP1LC3B* in response to endoplasmic reticulum stress and severe hypoxia.^{23,24} The EIF2AK4-EIF2A-ATF4 pathway was reported to be required to induce autophagy in yeast and in mammals in response to nutrient deprivation.^{25,26} We recently demonstrated that activation of the EIF2AK4-EIF2A-ATF4 pathway in response to cellular stress is essential for autophagy induction by increasing the

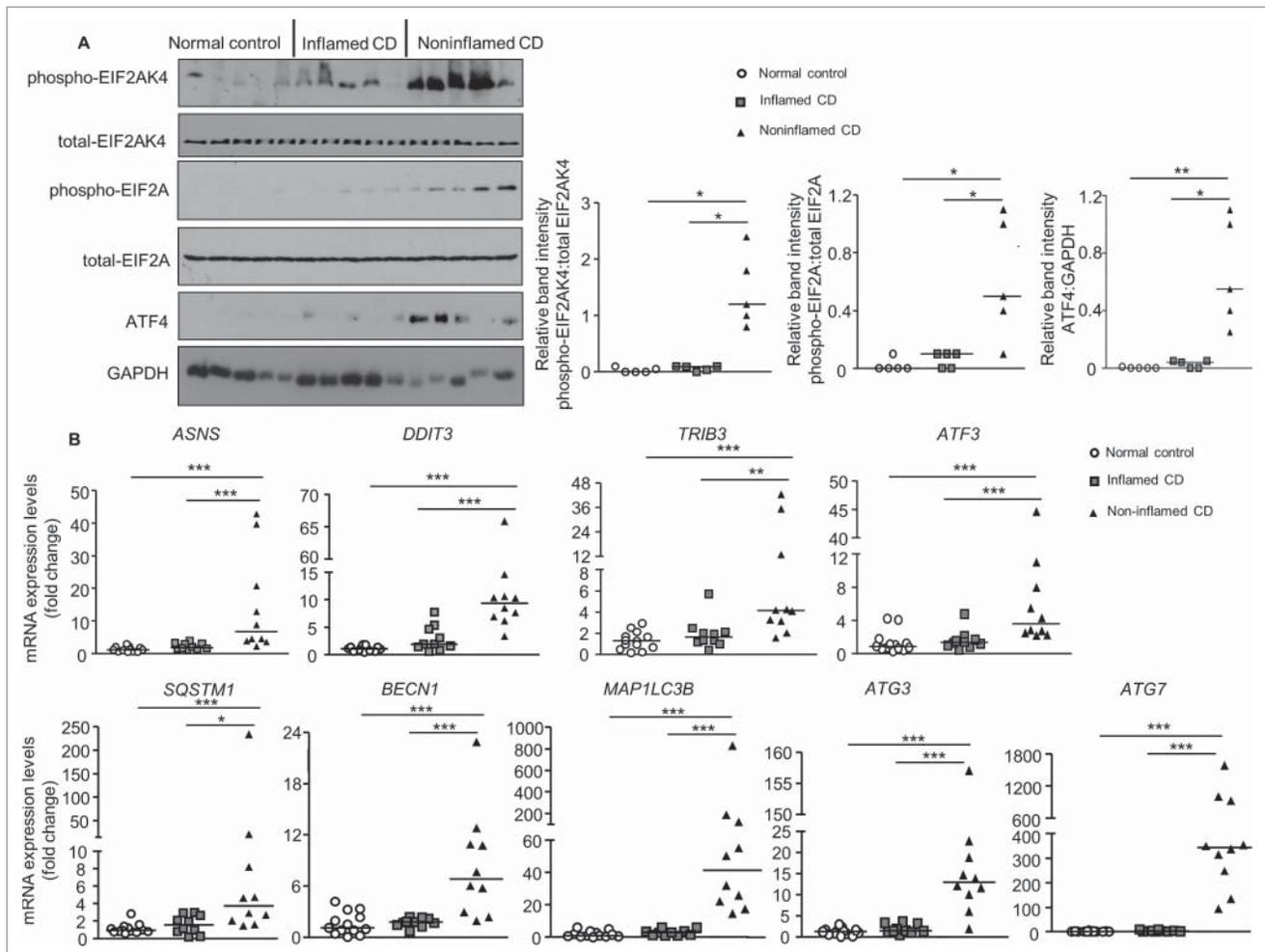


Figure 7. The EIF2AK4-EIF2A-ATF4 pathway is activated in noninflamed CD and deactivated in inflamed CD. Total proteins and RNA were extracted from the terminal ileal biopsy specimens from normal controls or patients with CD (with normal appearance [noninflamed CD] or with macroscopic inflammation [inflamed CD]). (A) Western blot analysis for the activation of the EIF2AK4-EIF2A-ATF4 pathway is shown in the left panel. Quantification of band intensity is shown in the right graphs. (B) qRT-PCR analysis for mRNA expression levels of the known ATF4 target genes (*TRIB3*, *ATF3*, *DDIT3* and *ASNS*) and the autophagy genes shown to be regulated by ATF4 in this study (*SQSTM1*, *MAP1LC3B*, *BECN1*, *ATG3* and *ATG7*). Data are represented as scatter dot plots; each dot shows one case number and the bars show the median. Data were analyzed by the nonparametric Mann-Whitney test. *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$.

transcription of a set of genes implicated in the formation, elongation and function of the phagophore and/or autophagosome.¹⁹ This pathway is also induced in host cells upon infection with the intracellular bacterial pathogens *Shigella* and *Salmonella*.²¹ However, to our knowledge, no data are available on the link between this pathway and CD and its involvement in host responses to infection with pathogenic bacteria associated with the disease.

In the present study, we investigated whether this pathway is involved in autophagy activation in host cells in response to AIEC infection. We show that activation of the EIF2AK4-EIF2A-ATF4 pathway in IECs in response to AIEC infection induces binding of the transcription factor ATF4 to promoters of several autophagy genes including *MAP1LC3B*, *BECN1*, *SQSTM1*, *ATG3* and *ATG7*, activating their transcription. This confers host cells with highly active autophagy to efficiently restrain AIEC intracellular multiplication and to suppress subsequent inflammatory responses. B'Chir et al. reported that transcription of the *ATG5* and *ATG16L1* genes is also activated by the EIF2A-ATF4 signaling pathway in response to cellular stress.¹⁹ However, under AIEC LF82 infection, we previously

reported that mRNA and protein expression levels of *ATG5* and *ATG16L1* are decreased since a post-transcriptional regulation mediated by microRNAs is implicated.¹⁰ We reason that AIEC can upregulate the levels of several host miRNAs to subvert autophagy, while host cells induce a functional autophagy as a defense mechanism to control bacterial replication. These are 2 arms of host-AIEC interaction, and depending on the physio-pathological status, one could dominate the other. We hypothesize that host cells are normally able to control the intracellular replication of AIEC by inducing an autophagy response. However, high levels of *MIR30C* and *MIR130A* under pathological conditions as observed in CD ileal mucosa¹⁰ could impair the autophagy response of the host by inhibiting expression of *ATG5* and *ATG16L1*.

Depletion of *EIF2AK4* blocks the autophagy response to AIEC infection, leading to abnormal AIEC persistence in host cells and aggravated inflammation. The in vivo results using *eif2ak4*^{-/-} mice were consistent with the in vitro data showing an important role for the EIF2AK4-mediated autophagy response to AIEC infection. Importantly, we demonstrate that the EIF2AK4-EIF2A-ATF4 pathway was not activated in IECs

in a mouse model of DSS-induced colitis, and that depletion of *Eif2ak4* did not affect susceptibility of mice to DSS treatment. This suggests that the role of the EIF2AK4-EIF2A-ATF4 pathway is specific to autophagy modulation during AIEC infection but not to inflammatory responses.

Importantly, we showed that the EIF2AK4-EIF2A-ATF4 pathway was activated in noninflamed CD compared with the normal control group, and was suppressed in inflamed CD. These data suggest that in the pre-onset of CD, the EIF2AK4-EIF2A-ATF4 pathway is activated, and a defect in the activation of this pathway could be one of the mechanisms contributing to the active disease. This is the first study showing a potential implication of the EIF2AK4-EIF2A-ATF4 pathway in CD etiology. We propose that one of the crucial events in the pathogenesis of CD might be the loss of tightly regulated autophagy caused by dysregulation of autophagy-regulating signaling pathways.

In summary, we have uncovered the critical role of the EIF2AK4-EIF2A-ATF4 signaling pathway in host autophagy response to CD-associated AIEC infection. The EIF2AK4-EIF2A-ATF4 pathway is activated in host cells upon AIEC infection, inducing transcription of autophagy genes and thereby a functional autophagy. This serves as a host defense mechanism to eliminate intracellular AIEC and to inhibit AIEC-induced intestinal inflammation (Fig. 8). This study highlights how the modulation of host metabolic pathways affects the cellular innate defense against CD-associated bacteria. More generally, this study should provide a better

understanding of the dialogue between AIEC infection and host autophagy response, and therefore may contribute to the development of therapeutic strategies to control CD-associated intracellular bacterial pathogens.

Materials and methods

Cell culture

The intestinal epithelial cell line T84 (ATCC, CCL-248) was maintained in an atmosphere containing 5% CO₂ at 37°C in the culture medium recommended by ATCC. *Eif2ak4*-deficient (*eif2ak4*^{-/-}) and the corresponding wild-type MEFs²⁷ were a gift from Dr. David Ron (Institute of Metabolic Science, Cambridge, UK). *atg5*^{-/-} MEFs²⁸ were kindly provided by Dr. Noboru Mizushima (Graduate School of Medicine, The University of Tokyo, Tokyo, Japan). MEFs were cultured in DMEM (Gibco, 11960-044) supplemented with 10% fetal bovine serum (Dominique Dutscher, S1900-500), 2 mM L-glutamine (Gibco, 25030-024), 1 mM sodium pyruvate (PAA laboratories, SH30239.01) and 1% MEM nonessential amino acids (Dominique Dutscher, X0556-100).

Bacterial strains

The AIEC reference strain LF82 isolated from a chronic ileal lesion of a CD patient,²⁹ the nonpathogenic *E. coli* K12 MG1655 strain and the commensal *E. coli* HS strain were used.

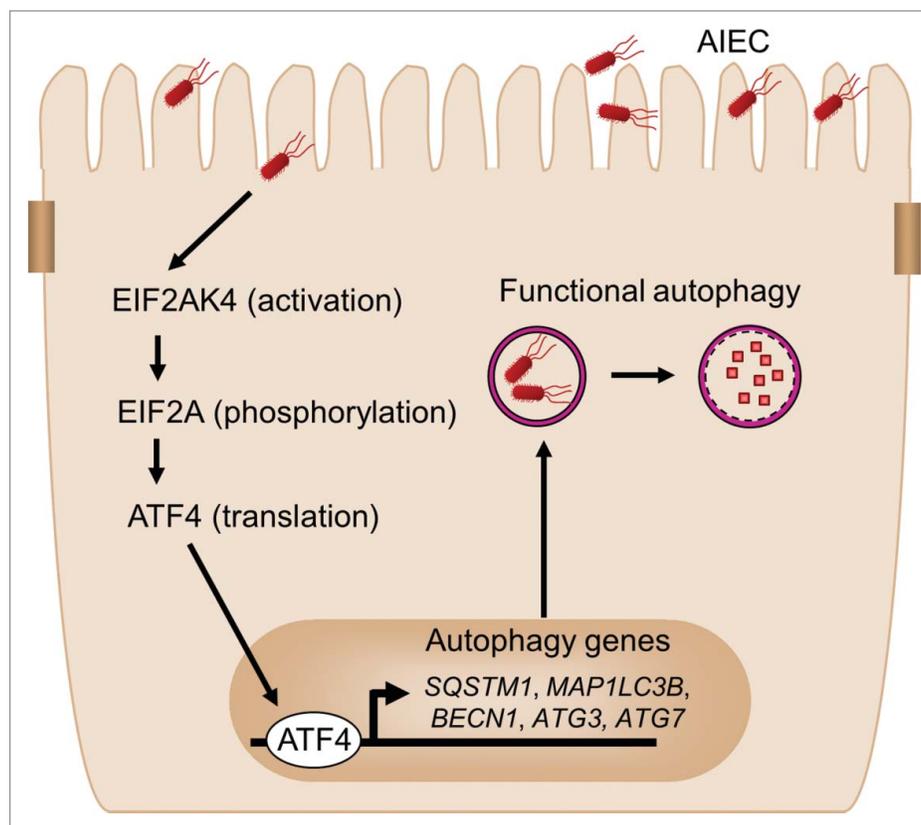


Figure 8. Proposed model for the involvement of the EIF2AK4-EIF2A-ATF4 pathway in autophagy response to AIEC infection in intestinal epithelial cells. Upon AIEC infection, the EIF2AK4-EIF2A-ATF4 signaling pathway is activated in host cells, inducing transcription of several autophagy genes including *SQSTM1*, *MAP1LC3B*, *BECN1*, *ATG3* and *ATG7* and thereby a functional autophagic response. This serves as a host defense mechanism to eliminate intracellular AIEC and to inhibit AIEC-induced intestinal inflammation.

The plasmid pFPV25.1, which harbors the green fluorescent protein (GFP), was used to visualize the LF82 bacteria by confocal microscopy (LF82-GFP strain). Bacteria were grown in Luria-Bertani (LB) broth (Laboratorios Conda, 1551.0) or on LB agar (Conda, 1800.00) plates overnight at 37°C.

Human samples

Biopsies from the terminal ileum of patients with CD (with normal appearance [noninflamed CD] or with macroscopic inflammation [inflamed CD]) and normal controls were collected during endoscopic examination. Non-IBD individuals that had surveillance colonoscopies were considered “normal controls.” Biopsies were immediately soaked in RNAlater (Qiagen, 76104) and stored at -80°C until further processing. Characteristics of human biopsies are presented in Table S1.

siRNA, plamid and in vitro transfection

Control siRNA-A (Ambion, sc-37007) and *EIF2AK4* siRNA (Ambion, sc-45644) were used. The pEGFP-LC3 plasmid was kindly provided by Dr. Tamotsu Yoshimori (Osaka University, Osaka, Japan).³⁰ T84 cells cultured on 12-well and 24-well plastic plates were transfected with 50 nM of *EIF2AK4* siRNA or control siRNA using Lipofectamine 2000 (Invitrogen, 11668027) and Opti-MEM I reduced serum medium (Invitrogen, 319885062). WT, *eif2ak4*^{-/-} and *atg5*^{-/-} MEFs seeded on coverslips were transfected with 500 ng of pEGFP-LC3 plasmid using Lipofectamine 2000 and Opti-MEM I reduced serum medium.

In vitro infection and invasion assay

Cells were seeded on 24-well plates and infected at a multiplicity of infection (MOI) of 10 bacteria per cell. Invasion assays were performed as previously described.⁶ Briefly, after 3 h of incubation in the culture medium without antibiotics, cells were washed with phosphate-buffered saline (PBS; Gibco, 14190-094). To determine the numbers of intracellular bacteria, culture medium containing 100 µg/ml gentamicin was added for the indicated time. Cells were lysed with 1% Triton X-100 (Sigma-Aldrich, G-1264) in deionized water. Samples were serially diluted and plated onto LB agar plates, and the colony-forming units (CFU) were determined.

Buffer for amino acid starvation

Cells were amino acid-starved by incubation with Krebs Ringer buffer (KRB; 118.5 mM NaCl [Euromedex, 1112-A], 4.74 mM KCl [Euromedex, P017-A], 1.18 mM KH₂PO₄ [Euromedex, 2018], 23.4 mM NaHCO₃ [Euromedex, 6885-1], 5 mM glucose [Sigma-Aldrich, G8270], 2.5 mM CaCl₂ [Euromedex, T885], and 1.18 mM MgSO₄ [Euromedex, P027-A], adjusted to pH 7.6 by titration with 1 N NaOH [Euromedex, EU2-9183-5]) for the indicated time.

Colitis induction

Colitis was induced in age- and sex-matched wild-type (WT) and *eif2ak4*^{-/-} littermates by oral administration of 3% (w/v) DSS (36-50 kDa; MP Biomedicals, 160110) in the drinking

water. *eif2ak4*^{-/-} mice²⁷ were kindly provided by Dr. D. Ron (Institute of Metabolic Science, Cambridge, UK). Mice were weighed daily. Clinical score was assessed at the end of DSS treatment as described previously.³¹

In vivo infection and bacterial colonization evaluation

WT and *eif2ak4*^{-/-} littermates were treated with an antibiotic cocktail containing 500 mg/l metronidazole (Sigma-Aldrich, M3761), 500 mg/l vancomycin (Sigma-Aldrich, 861987), 1 g/l neomycin (Sigma-Aldrich, N6386) and 1 g/l ampicillin (Euromedex, EU0400-D) for 7 d as described,³² and 24 h later were orally challenged with 10⁹ CFU of LF82 or K12 MG1655 bacteria for 3 d. Fresh fecal samples (100-200 mg) were collected from individual mice every day post-infection and resuspended in PBS to determine bacterial persistence in the gut. At d 3 post-infection, mice were sacrificed, and 2-cm segments of ileal tissues were removed, opened longitudinally and homogenized in PBS to determine the number of bacteria associated with the intestinal mucosa. After serial dilution, fecal or ileal samples were plated on LB agar plates containing 50 µg/ml ampicillin and 20 µg/ml erythromycin (Euromedex, E002) to isolate LF82 bacteria, or containing 300 µg/ml rifampicin (Euromedex, 1059-B) to isolate K12 MG1655 bacteria, and incubated at 37°C overnight.

Isolation of intestinal epithelial cells (IECs)

Ileal epithelial cells were extracted as described previously.¹⁰ Briefly, mouse ileum was dissected and flushed with a solution containing 154 mM NaCl and 1 mM DTT (Euromedex, EU0006-B) to remove fecal contents. The ileal segments were ligated, filled with PBS, and incubated in PBS at 37°C. After 15 min, the PBS was substituted with PBS supplemented with 1.5 mM EDTA (Euromedex, EU0084-A) and 0.5 mM DTT. After 30 min at 37°C, one ligature was removed and contents were collected. The recovered IECs were washed twice in PBS by centrifugation at 1,300 rpm for 5 min and were used to examine activation of the EIF2AK4-EIF2A-ATF4 pathway and autophagic activity.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the Pierce Agarose ChIP kit following the manufacturer's instructions (Pierce, 1862281). Briefly, 2 × 10⁶ uninfected T84 cells or cells infected with LF82 bacteria at a MOI of 10 for 4 h were fixed with 1% formaldehyde, washed with cold PBS and lysed. Nuclei were digested with micrococcal nuclease (supplied in the kit) to shear crosslinked chromatin, and the lysates were pelleted and precleared. The protein-DNA complexes were incubated with 4 µg of rabbit anti-ATF4 antibody (Cell Signaling Technology, 11845) or a nonspecific negative control rabbit IgG (supplied in the kit) overnight and then incubated with protein A/G agarose resin (supplied in the kit) followed by an elution, and cross-links were reversed. After recovery, DNA was subjected to semi-quantitative or quantitative PCR analysis. Specific primers used to amplify the ATF4-binding region in the promoters of autophagy genes are indicated in Table S2. The inputs were analyzed in parallel using ChIP control primers, which amplify a region of the *GAPDH*

promoter, supplied in the Pierce CHIP kit. Fold increase in the binding of ATF4 to the autophagy gene promoter quantified by quantitative PCR was calculated using the *Ct* method as follows: $\Delta\Delta Ct = (Ct_{ATF4\text{-binding DNA}^-} - Ct_{GAPDH\text{ DNA in input}})_{\text{infected}} - (Ct_{ATF4\text{-binding DNA}^-} - Ct_{GAPDH\text{ DNA in input}})_{\text{uninfected}}$ and the final data were derived from $2^{-\Delta\Delta Ct}$.

Protein extraction and western blot analysis

T84 and MEF cells and human biopsies were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 0.5% sodium deoxycholate [Sigma-Aldrich, D6750], 50 mM Tris-HCl [Euromedex, EU0011-C] pH 8, 0.1% SDS [Euromedex, EU0660-A], 0.1% Nonidet P-40 [Fluka BioChemeka, 74385]) supplemented with protease inhibitors (Roche, 11836145001). Enterocytes extracted from mouse ileum were homogenized in the same buffer and cell suspensions were sonicated. The homogenates were centrifuged at $16,000 \times g$ for 20 min at 4°C. Proteins were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and blocked with 5% nonfat milk in PBS containing 0.1% Tween-20 (Euromedex, 2001-A). Membranes were then probed with the relevant primary antibodies: anti-phospho-EIF2AK4 (Abcam, ab75836), anti-EIF2AK4 (Cell Signaling Technology, 3302), anti-phospho-EIF2A/eIF2 α (Cell Signaling Technology, 3597), anti-EIF2A/eIF2 α (Cell Signaling Technology, 9722), anti-ATF4 (Cell Signaling Technology, 11845), anti-LC3 (Sigma-Aldrich, L8918), anti-SQSTM1 (Santa Cruz Biotechnology, sc-28359) and anti-GAPDH (Cell Signaling Technology, 2118). After washes, membranes were incubated with HRP-conjugated goat anti-rabbit (Cell Signaling Technology, 7074) or rabbit anti-mouse (Cell Signaling Technology, 7076) secondary antibody, and blots were detected using the Enhanced Chemiluminescence Detection kit (Amersham Biosciences, RPN2108).

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNAs were extracted using TRIzol reagent (Invitrogen, 15-596-026) or the RNeasy Mini Kit (Qiagen, 74104), and were reverse transcribed using the PrimeScript™ RT reagent kit (Takara Bio, RR037A) to quantify mRNA expression levels. qRT-PCR was performed using SYBR Green qPCR Master Mix (Takara Bio, RR820A) on a Mastercycler Realplex (Eppendorf, Montesson, France) using the specific primers indicated in Table S3. Human *RNA18S* and mouse *Rplp0/36B4* were used as internal controls. Fold-induction was calculated using the *Ct* method as follows: $\Delta\Delta Ct = (Ct_{\text{target gene}^-} - Ct_{\text{housekeeping gene}})_{\text{treatment}} - (Ct_{\text{target gene}^-} - Ct_{\text{housekeeping gene}})_{\text{nontreatment}}$ and the final data were derived from $2^{-\Delta\Delta Ct}$.

Fluorescence microscopy

T84 cells seeded on coverslips were fixed with 4% paraformaldehyde (Thermo Fisher scientific, 28906), permeabilized with 0.5% Triton X-100 for 20 min, blocked with PBS containing 0.025% Triton X-100, 3% BSA (ID-Bio, 1005-70) and 5% fetal bovine serum. Mouse ileal tissues were embedded in OCT (Tissue Tek, 4583) and cut into 5- μ m sections at -20°C using a cryostat. Cryostat sections were then fixed, permeabilized and

blocked similarly. Cells or cryostat sections were immunostained at room temperature for 1 h or overnight at 4°C, respectively, with the indicated primary antibodies: rabbit antiserum against *E. coli* lipopolysaccharide O83 generously provided by Dr. Lothar Beutin (Department of Biological Safety, Robert Koch Institut, Berlin, Germany), or anti-LC3 (MBL, M115-3). After washes with PBS, slides were incubated with Alexa Fluor 488 donkey anti-rabbit (Molecular Probes, A21206) or Cy3 goat anti-mouse (Molecular Probes, A10521) secondary antibody. Actin cytoskeleton was stained for 15 min using TRITC-phalloidin (Sigma-Aldrich, P1951) or FITC-phalloidin (Sigma-Aldrich, P8582). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich, B2261). Coverslips and tissue sections were mounted with a Mowiol solution (Calbiochem, 475904) and Mountex Mounting Medium (Cell Path, SEA0504-00A), respectively. Slides were examined with a Zeiss LSM 510 Meta confocal microscope. Each confocal microscopy image is representative of 3 independent experiments.

Enzyme-linked immunosorbent assays (ELISA)

The amount of IL8, CXCL1, IL6, IL1B and TNF secreted in the supernatants from cell culture or mouse ileal tissues cultured for 24 h in RPMI medium (Gibco, 31870-025) containing penicillin/streptomycin (Hyclone, SV30079.01) and 100 μ g/ml gentamicin (Euromedex, EU0540-A) in an atmosphere containing 5% CO₂ at 37°C, respectively, was determined by ELISA (R&D Systems, human CXCL8/IL-8 Quantikine ELISA kit, D8000C; Mouse CXCL1/KC DuoSet ELISA, DY453; Mouse IL6 DuoSet ELISA, DY406; Mouse IL1 β DuoSet ELISA, DY401; Mouse TNF-alpha DuoSet ELISA, DY410) according to the manufacturer's instructions.

Ethics statement

Animal protocols were approved by the Ethics Committee CEMEEA (CEMEA CE16-09, Clermont-Ferrand, France). Protocols with human biopsies were approved by the local ethics commission of the Canton of Bern, Switzerland.

Statistical analysis

Results are presented as means \pm SEM or median. Statistical analysis was performed using the paired t tests for in vitro data and the nonparametric Mann-Whitney test for in vivo data with GraphPad Prism version 5.01 software (GraphPad Software). A P value less than 0.05 was considered statistically significant. *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$.

ABBREVIATIONS

AIEC	adherent-invasive Escherichia coli
ASNS	asparagine synthetase (glutamine-hydrolyzing)
ATF3	activating transcription factor 3
ATF4	activating transcription factor 4
ATG16L1	autophagy-related 16 like 1
ATG3	autophagy-related 3
ATG5	autophagy-related 5
ATG7	autophagy-related 7
BECN1	Beclin 1, autophagy-related

CD	Crohn disease
CFU	colony-forming units
ChIP	chromatin immunoprecipitation
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
DDIT3	DNA damage inducible transcript 3
DSS	dextran sodium sulfate
EIF2	eukaryotic translation initiation factor 2
EIF2AK4	eukaryotic translation initiation factor 2 alpha kinase 4
ELISA	enzyme-linked immunosorbent assay
IBD	inflammatory bowel diseases
IEC	intestinal epithelial cell
IL	interleukin
IL1B	interleukin 1 beta
IRGM	immunity-related GTPase family, M
KRB	Krebs-Ringer buffer
LRRK2	leucine-rich repeat kinase 2
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta
MOI	multiplicity of infection
mRNA	messenger RNA
PTPN2	protein tyrosine phosphatase, non-receptor type 2
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
SQSTM1	sequestosome 1
TNF	tumor necrosis factor
TRIB3	tribbles pseudokinase 3
ULK1	unc-51 like autophagy activating kinase 1
WT	wild type
XBP1	X-box binding protein 1

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We dedicate this article to the memory of Prof. Arlette Darfeuille-Michaud, who sadly passed away on June 28, 2014. The authors thank the Imagerie Confocale Clermont-Ferrand (ICCF) platform (IFR79 Santé Université d'Auvergne, Clermont-Ferrand, France) for confocal microscopy and the Centre Imagerie Cellulaire Santé (Facultés de Médecine et Pharmacie) for tissue embedding and sectioning. We thank Drs. Emilie Vazeille, Marie-Agnès Bringer and Pierre Lapaquette for valuable discussion.

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