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C1orf106 is a colitis risk gene that regulates stability of epithelial adherens junctions

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Abstract

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Data and materials availability: Data in this paper are tabulated in the main text and supplementary materials. The original mass spectra can be downloaded from MassIVE (Mass Spectrometry Interactive Virtual Environment; http://massive.ucsd.edu) using the identifier MSV000081941. The data are directly accessible at ftp://massive.ucsd.edu/MSV000081941.

Supplementary Materials
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Polymorphisms in *C1orf106* are associated with increased risk of inflammatory bowel disease (IBD). However, the function of C1orf106 and the consequences of disease-associated polymorphisms are unknown. Here we demonstrate that C1orf106 regulates adherens junction stability by regulating the degradation of cytohesin-1, a guanine nucleotide exchange factor that controls activation of ARF6. By limiting cytohesin-1–dependent ARF6 activation, C1orf106 stabilizes adherens junctions. Consistent with this model, *C1orf106*$^{-/-}$ mice exhibit defects in the intestinal epithelial cell barrier, a phenotype observed in IBD patients that confers increased susceptibility to intestinal pathogens. Furthermore, the IBD risk variant increases C1orf106 ubiquitination and turnover with consequent functional impairments. These findings delineate a mechanism by which a genetic polymorphism fine-tunes intestinal epithelial barrier integrity and elucidate a fundamental mechanism of cellular junctional control.

Intestinal epithelial cells are required for gut homeostasis and are involved in numerous physiologic processes including nutrient absorption, protection against microbes, and intestinal restoration following insult (1). Abnormal intestinal permeability has been observed in patients with inflammatory bowel disease (IBD), a chronic inflammatory condition of the gastrointestinal tract (2). Healthy family members of some IBD patients have been reported to have changes to the intestinal barrier, suggesting that host genetics can underlie cell-intrinsic barrier defects, although the underlying mechanisms are as yet undefined (3). *C1orf106* was identified as an IBD susceptibility gene through genome-wide association studies, and follow-up exome sequencing revealed that a coding variant in *C1orf106* (*333F) increased risk of IBD (4–6). Here we elucidate the function of C1orf106 and find a role for it in epithelial homeostasis. We report a mechanism whereby the *C1orf106* IBD-associated risk variant decreases cellular junctional integrity, suggesting a means by which this variant increases susceptibility to IBD.

C1orf106 is highly expressed in the human intestine and intestinal epithelial cell lines but expressed at low levels in myeloid cells and mouse bone marrow–derived macrophages (fig. S1, A to C). In Caco-2 cells, a human colorectal cell line, C1orf106 protein expression increased as cells differentiated and formed a polarized epithelial monolayer, a characteristic feature of the intestinal epithelium (Fig. 1A). To decipher the function of C1orf106, we sought to identify C1orf106-interacting proteins by tandem mass spectrometry–based affinity proteomics, using epitope-tagged C1orf106 immunoprecipitated from human embryonic kidney (HEK) 293T cells. Cytohesin-1 and cytohesin-2 were two of the top interactors (Fig. 1B, fig. S1D, and table S1). Cytohesin-1 is one of the guanine exchange factors (GEFs) that control the activation of ARF6 guanosine triphosphatase (GTPase) (7). Depending on the GEF involved, ARF6 functions to control the recycling of proteins from the plasma membrane (8). Coimmunoprecipitation experiments confirmed the interaction between C1orf106 and cytohesin-1 and -2 by overexpression in HEK293T cells and with endogenous proteins in Caco-2 cells (Fig. 1, C and D, and fig. S1E). Domain-mapping experiments further indicated that the N-terminal domain of C1orf106 interacts specifically with the N-terminal domain of cytohesin-1 (Fig. 1, C and E).

To investigate the functional interaction between these proteins in a physiologically relevant model, we generated *C1orf106*$^{-/-}$ mice (fig. S2, A and B) and examined the steady-state

levels of cytohesin-1 in this model system. We found that cytohesin-1 protein levels in colon and small intestine epithelial cells isolated from *C1orf106*$^{-/-}$ mice were consistently increased 1.5- to 2-fold compared with those in cells isolated from *C1orf106*$^{+/+}$ mice (Fig. 1F). Consistent with these findings, *C1orf106*$^{-/-}$ epithelial monolayers derived from colonic organoids also exhibited increased levels of cytohesin-1 protein in both membrane and cytosolic protein fractions (Fig. 1G), despite no difference in cytohesin-1 mRNA levels (fig. S3A). These data suggest that the increase in cytohesin-1 is posttranscriptionally regulated and is not due to differential localization of the protein in the membrane versus in the cytoplasmic compartments of the cells. Consistent with this hypothesis, increasing C1orf106 expression significantly decreased the levels of either overexpressed or endogenous cytohesin-1, indicating that C1orf106 expression is sufficient to regulate the steady-state levels of cytohesin-1 (Fig. 1H and fig. S3B). Similar results were observed with cytohesin-2 (fig. S3C). These data suggest that expression of C1orf106 limits the steady-state levels of cytohesins.

We next investigated whether cytohesin-1 levels were regulated by ubiquitination and proteasomal degradation. Treatment of cells with MG132, a proteasome inhibitor, increased the steady-state levels of cytohesin-1, suggesting that cytohesin-1 is degraded by the proteasome (fig. S4A). Overexpression of C1orf106 was sufficient to increase the levels of ubiquitinated cytohesin-1 (Fig. 2A). Analysis of colonic intestinal epithelial cells demonstrated that *C1orf106*$^{-/-}$cells have reduced levels of ubiquitinated cytohesin-1 at steady state (Fig. 2B). These data suggest a model whereby C1orf106 expression limits cytohesin-1 levels through ubiquitin-mediated degradation.

C1orf106 has one putative domain of unknown function, DUF3338, which is predicted to be involved in protein-protein interactions but lacks enzymatic activity. Therefore, we hypothesized that C1orf106 acts as a cofactor for ubiquitin ligases to ubiquitinate cytohesins. To understand the mechanism of C1orf106-mediated control of cytohesin-1 protein levels, we identified C1orf106-binding proteins in our proteomics data that have the potential to mediate ubiquitination. Importantly, each subunit of the SKP1–CUL1–F-box (SCF) E3 ubiquitin ligase complex and two F-box substrate adaptors, BTRC1 and FBXW11, were identified as C1orf106 interactors (Fig. 1B, fig. S1D, and table S1). SCF ubiquitin ligase complexes play important roles in regulating the ubiquitination and subsequent degradation of specific substrate proteins (9, 10). We performed coimmunoprecipitation experiments to determine which proteins from the SCF complex interact specifically with C1orf106 (Fig. 2, C and D, and fig. S4, B and C); we found that the substrate adapters BTRC1 and FBXW11 do so, suggesting that C1orf106 may serve as a substrate cofactor (Fig. 2, C and D).

To test the hypothesis that the SCF complex mediates the ubiquitination of cytohesin-1, we knocked down expression of *BTRC1* and *FBXW11* and evaluated cytohesin-1 expression levels. Cells treated with *FBXW11* small interfering RNA (siRNA) showed significantly increased levels of cytohesin-1 (Fig. 2E and fig. S5), suggesting that the SCF complex containing FBXW11, but not BTRC1, regulates the stability of cytohesin-1. We next tested the effect of MLN4924, a small-molecule inhibitor of a NEDD8-activating enzyme that is required for neddylation and activation of cullin-RING ubiquitin E3 ligases, including the

SCF complex. Treatment of human colon HT-29 cells with MLN4924 resulted in a dose-dependent increase in endogenous levels of cytohesin-1 (Fig. 2F) (11). Taken together, these results indicate that cytohesin-1 levels are dynamically regulated by ubiquitination by the SCF ubiquitin ligase complex and subsequent proteasomal degradation.

We next sought to understand how C1orf106-mediated degradation of cytohesin-1 alters epithelial cell function. Cytohesin-1 acts as a GEF to regulate the activity of ARF6, a GTPase that controls the rate of membrane receptor recycling and mediates signaling pathways that control actin remodeling (12). We therefore hypothesized that increased levels of cytohesin-1 protein in *C1orf106*$^{-/-}$ cells would increase levels of ARF6 activation. To test this hypothesis, we evaluated the levels of activated ARF6 (ARF6-GTP) in organoid-derived intestinal epithelial monolayers, finding that ARF6-GTP levels were 1.5 times as high in *C1orf106*$^{-/-}$ cells as in *C1orf106*$^{+/+}$ cells, despite comparable total levels of ARF6 (Fig. 3A). Given that activated ARF6-GTP localizes to the plasma membrane (8), we next analyzed ARF6 localization in these cells. Immunostaining confirmed increased levels of ARF6 at the plasma membrane in *C1orf106*$^{-/-}$ epithelial monolayers (Fig. 3B). Analysis of insoluble membrane fractions from *C1orf106*$^{+/+}$ and *C1orf106*$^{-/-}$ epithelial monolayers demonstrated increased levels of ARF6 in the membrane fraction in *C1orf106*$^{-/-}$cells, further supporting the finding of increased levels of membrane-associated ARF6-GTP in these cells (fig. S6A).

ARF6 plays a key role in regulating surface levels of critical adherens junction proteins, and ARF6 activation in epithelial cells is known to increase internalization of E-cadherin (8, 13). We therefore hypothesized that increased cytohesin-1 and ARF6-GTP levels in *C1orf106*$^{-/-}$ intestinal epithelial cells would result in decreased surface levels of E-cadherin. As predicted, immunostaining for E-cadherin in *C1orf106*$^{-/-}$ intestinal epithelial monolayers revealed more than a threefold increase in the proportion of cells containing intracellular E-cadherin puncta compared with the proportion among *C1orf106*$^{+/+}$ cells (Fig. 3C). An increase in intracellular E-cadherin puncta was also observed in colonic tissue sections from *C1orf106*$^{-/-}$ mice (Fig. 3D). We detected no differences in the localization of epithelial tight junction proteins occludin, ZO-1, claudin1, or claudin2 and no differences in mRNA or protein levels (Fig. 3, B to D, and fig. S6, B to E). These data confirm that the effect was specific for E-cadherin. The staining pattern of E-cadherin in *C1orf106*$^{-/-}$ colonic organoids was disorganized along the junctions and revealed increased puncta formation in the cytosol (fig. S6F). Moreover, disorganized E-cadherin was also observed after knockdown of *C1orf106* in differentiated human Caco-2 cells (fig. S6G). Additionally, internalized E-cadherin colocalized with intracellular ARF6 puncta, consistent with a role for ARF6 in E-cadherin internalization (fig. S7A). ARF6 is known to regulate actin dynamics. We observed prominent vesicular staining for actin along the inner cell membrane in *C1orf106*$^{-/-}$cells, which further supports a role for altered ARF6 dynamics in these cells (fig. S7B). To confirm decreased localization of E-cadherin along the cell surface, we performed biotinylation of extracellular membrane-bound proteins followed by immunoblot analysis of biotinylated E-cadherin in freshly isolated colonic intestinal epithelial cells and organoid-derived monolayers from *C1orf106*$^{+/+}$ and *C1orf106*$^{-/-}$ mice. Despite similar total expression of E-cadherin, we found more than a twofold decrease in surface E-cadherin in *C1orf106*$^{-/-}$ cells compared with *C1orf106*$^{+/+}$ cells (Fig. 3, E and F). These data suggest a

critical role for C1orf106 in maintaining adherens junctions by limiting ARF6 activation through regulated cytohesin degradation.

Epithelial junction integrity is important in intestinal homeostasis, as well as tissue repair after damage (14). We next monitored epithelial barrier integrity by testing the ability of fluorescently labeled molecules to pass through the intestinal barrier. *C1orf106*$^{-/-}$ and *C1orf106*$^{+/+}$ mice exhibited similar permeability to FITC (fluorescein isothiocyanate)–dextran (4 KDa) (Fig. 3G). However, *C1orf106*$^{-/-}$ colon tissue showed significantly increased permeability to a smaller compound, Lucifer yellow (0.4 KDa) (Fig. 3H). Together, these data suggest that loss of C1orf106 confers increased permeability to smaller solutes (15). To further confirm this finding, we measured transepithelial electrical resistance (TEER) to assess barrier function in *C1orf106*$^{+/+}$ and *C1orf106*$^{-/-}$ monolayers derived from organoids and Caco-2 cells with stable knockdown of *C1orf106*. Maximal TEER was significantly reduced in *C1orf106*-deficient cells compared with control cells, indicating impaired epithelial barrier integrity (fig. S8, A and B).

To test whether changes in E-cadherin recycling altered the ability of *C1orf106*$^{-/-}$ cells to repair epithelial junctions after injury, we subjected organoid-derived monolayers to a calcium switch assay by treating cells with EGTA to disrupt extracellular E-cadherin interactions, followed by treatment with normal media; in this assay, we monitored E-cadherin staining to evaluate the reformation of junctions after 2 hours of recovery time (16). Whereas both *C1orf106*$^{+/+}$ and *C1orf106*$^{-/-}$ monolayers were similarly disrupted by EGTA treatment, *C1orf106*$^{-/-}$ monolayers displayed a lack of reorganization compared with *C1orf106*$^{+/+}$ monolayers after 2 hours of recovery (fig. S8C). TEER was also measured after calcium switch during the recovery phase. *C1orf106*$^{-/-}$ monolayers displayed decreased TEER compared with *C1orf106*$^{+/+}$ monolayers at baseline and during the recovery phase (fig. S8D). Selective knockdown of cytohesin-1 was sufficient to rescue baseline TEER in *C1orf106*$^{-/-}$ monolayers, demonstrating that cytohesin-1 is a key mediator of the observed barrier phenotype in *C1orf106*$^{-/-}$ cells (Fig. 3I and fig. S9, A and B).

In organoid-derived epithelial monolayers, *C1orf106*$^{-/-}$ cells had a significantly increased migratory rate at baseline and during hepatocyte growth factor–induced cell migration compared with *C1orf106*$^{+/+}$ cells (fig. S10). These findings suggest that loss of C1orf106 decreases junctional integrity, resulting in increased cellular migration at steady state, and that growth factor stimulation cannot compensate for this defect.

Increased susceptibility to microbial pathogens and dysbiosis is commonly associated with IBD (17). To determine whether *C1orf106*$^{-/-}$ mice have compromised epithelial barrier integrity resulting in increased bacterial dissemination, we challenged *C1orf106*$^{+/+}$ and *C1orf106*$^{-/-}$ mice with the extracellular intestinal murine pathogen *Citrobacter rodentium*, which induces colonic lesions, similarly to the clinical enteropathogenic *Escherichia coli* strains associated with Crohn's disease (18). Additionally, epithelial defenses are critical in limiting *C. rodentium* early after infection. *C1orf106*$^{-/-}$ mice exhibited significantly increased bacterial loads of *C. rodentium* at day 5 (Fig. 4A). Notably, translocation of *C. rodentium* to the mesenteric lymph nodes and spleen was also significantly increased in *C1orf106*$^{-/-}$ mice at day 5 (Fig. 4A). Although *C1orf106*$^{-/-}$mice were able to control *C.*

rodentium infection by day 12 postinfection, they exhibited significantly shortened colon length compared with *C1orf106*$^{+/+}$ mice and more severe histopathology, including crypt damage (Fig. 4, B to D, and fig. S11A). Cytokine response was not impaired in *C1orf106*$^{-/-}$ mice 12 days postinfection (fig. S11, B and C). Additionally, levels of immune cell types such as T and B lymphocytes, macrophages, dendritic cells, and innate lymphoid cells were unchanged at baseline (fig. S12A). Levels of interleukin-22, lipocalin-2, fecal immunoglobulin A (IgA), fecal albumin, and antimicrobial peptides were also unaltered at baseline, suggesting that these do not contribute to the early impairment in bacterial defense (fig. S12, B to F). *C1orf106*$^{-/-}$ mice also exhibited impaired recovery from dextran sodium sulfate–induced colitis, as evidenced by greater body weight loss, reduced colon length, and more severe histopathology, consistent with an impaired ability to recover from intestinal insults (fig. S13, A to D).

Deep exon sequencing has identified a coding variant in *C1orf106*, *333F, which is associated with increased risk of IBD. Expression of C1orf106 *333F was reproducibly decreased during transient transfection compared with that of wild-type C1orf106 (C1orf106 WT), despite comparable levels of mRNA, suggesting that the risk variant is poorly expressed or unstable (Fig. 4E and fig. S14A). To test whether the decreased levels of C1orf106 *333F protein were due to ubiquitination and degradation by the proteasome, we treated cells with MG132; treatment with this proteasome inhibitor restored C1orf106 *333F protein to WT levels (Fig. 4F). We also observed increased ubiquitination of C1orf106 *333F compared with WT, suggesting that the IBD risk polymorphism increases protein turnover of C1orf106, resulting in decreased expression of functional protein (Fig. 4F). Consistent with these results, we found that C1orf106 *333F had a half-life of 10.2 hours, compared with the C1orf106 WT half-life of almost 17 hours, using a cyclohexamide assay in LS174T cells (fig. S14B). To study the phenotypic effects of the decreased half-life of C1orf106 *333F, *C1orf106*$^{-/-}$ organoids were transduced with either C1orf106 WT or C1orf106 *333F. Expression of C1orf106 *333F was not sufficient to restore WT levels of C1orf106, mediate degradation of cytohesin-1, or increase the TEER in *C1orf106*$^{-/-}$monolayers (Fig. 4, G and H). Expression of C1orf106 *333F disrupted E-cadherin and actin organization and staining in monolayer-derived intestinal epithelial cells and human intestinal cells (Fig. 4, I and J, and fig. S15). Taken together, these data suggest a mechanism by which the *333F polymorphism decreases C1orf106 protein stability and thus confers increased susceptibility to IBD by compromising gut epithelial integrity through impaired turnover and degradation of cytohesin-1.

Our findings define a critical function for C1orf106 in IBD by regulating the integrity of intestinal epithelial cells. We have shown that C1orf106 functions as a molecular rheostat to limit cytohesin levels through SCF complex–dependent degradation and thereby modulates barrier integrity. The finding that C1orf106 regulates the surface levels of E-cadherin is notable given that polymorphisms in both *C1orf106* and *CDH1* (E-cadherin) are associated with increased risk of ulcerative colitis, a form of IBD (19). Increasing the stability of C1orf106 may be a potential therapeutic strategy to increase the integrity of the epithelial barrier for the treatment of IBD.

Supplementary Material

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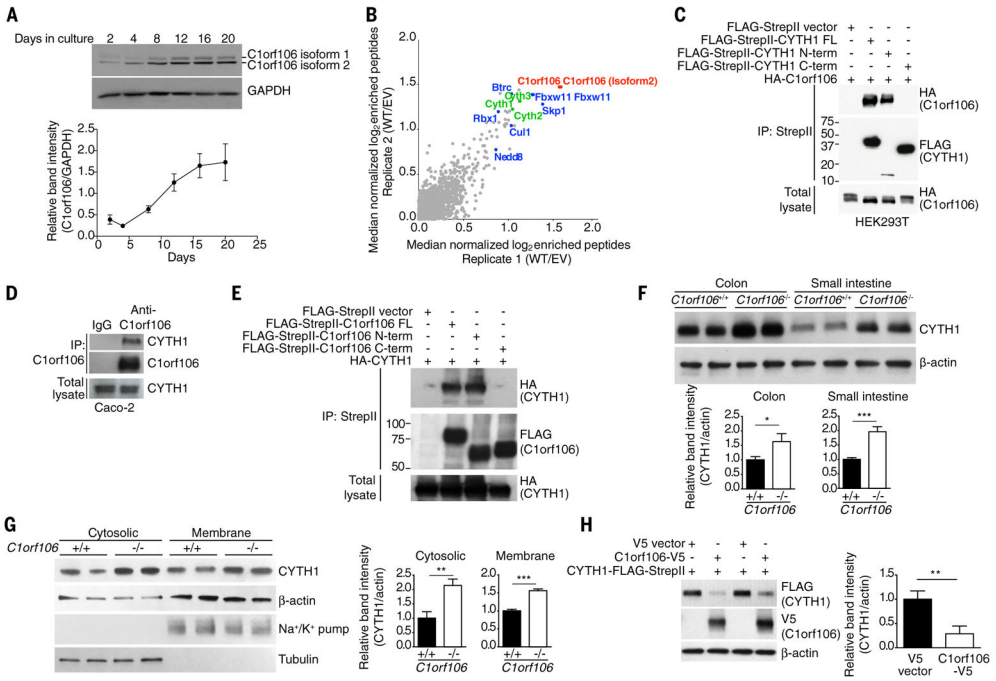

Figure 1.

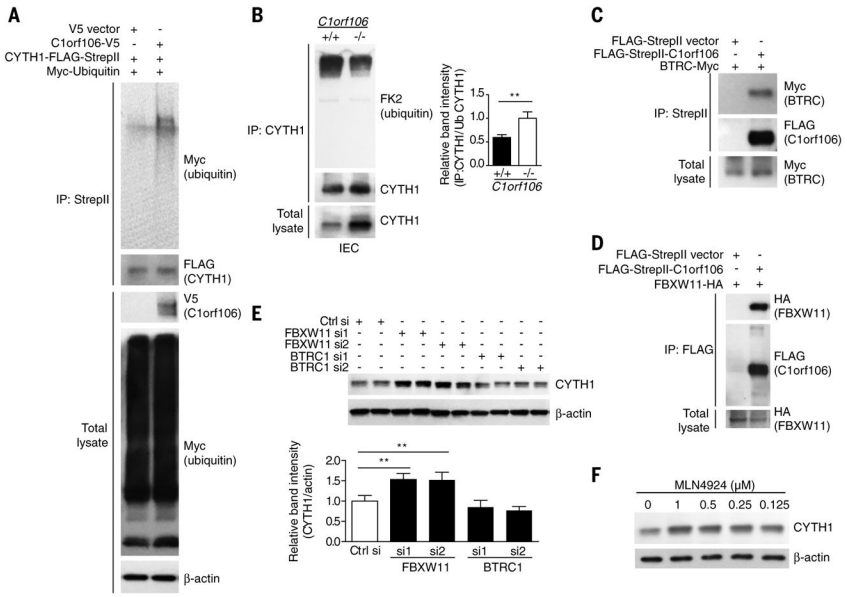

Figure 2.

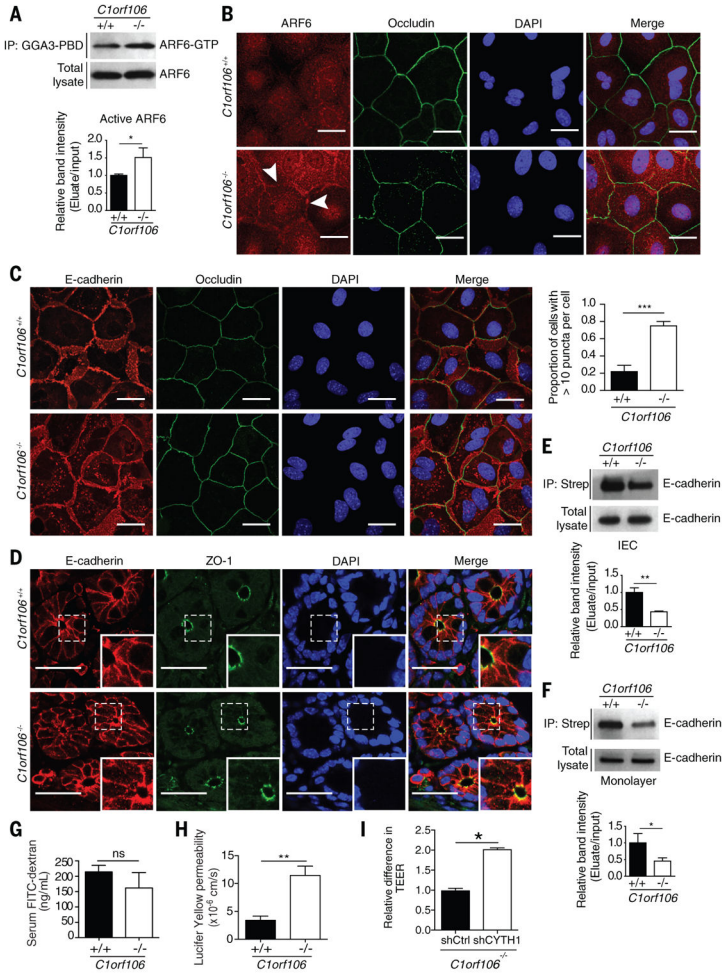

Figure 3.

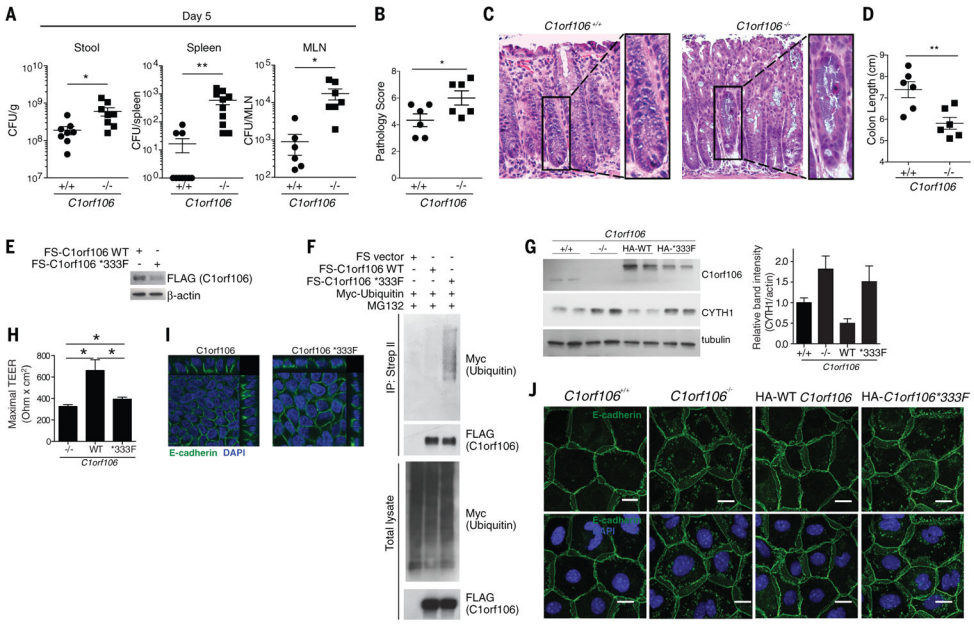

Figure 4.