The integrated stress response (ISR) is a homeostatic mechanism by which eukaryotic cells sense and respond to stress-inducing signals, such as amino acid starvation. General controlled non-repressed (GCN2) kinase is a key orchestrator of the ISR, and modulates protein synthesis in response to amino acid starvation. Here we demonstrate in mice that GCN2 controls intestinal inflammation by inhibiting inflammasome activation. Enhanced activation of ISR was observed in intestinal antigen presenting cells (APCs) and epithelial cells during amino acid starvation, or intestinal inflammation. Genetic deletion of Gcn2 (also known as Eif2ka4) in CD11c+ APCs or intestinal epithelial cells resulted in enhanced intestinal inflammation and T helper 17 cell (T_h17) responses, owing to enhanced inflammasome activation and interleukin (IL)-1β production. This was caused by reduced autophagy in Gcn2−/− intestinal APCs and epithelial cells, leading to increased reactive oxygen species (ROS), a potent activator of inflammasomes. Thus, conditional ablation of Atp5 or Atp7 in intestinal APCs resulted in enhanced ROS and T_h17 responses. Furthermore, in vivo blockade of ROS and IL-1β resulted in inhibition of T_h17 responses and reduced inflammation in Gcn2−/− mice. Importantly, acute amino acid starvation suppressed intestinal inflammation via a mechanism dependent on GCN2. These results reveal a mechanism that couples amino acid sensing with control of intestinal inflammation via GCN2.

The immune system can sense pathogens through pathogen recognition receptors, but emerging evidence suggests that it can also sense and respond to environmental changes that cause cellular stress. The ISR is an evolutionarily ancient mechanism that enables eukaryotic cells to sense and respond to diverse stress signals, such as amino acid starvation and endoplasmic reticulum stress. The four known sensors of the ISR include: GCN2, protein kinase R (PKR), haem-regulated inhibitor (HRI) and PKR-like endoplasmic reticulum kinase (PERK). GCN2 senses amino acid depletions, PKR senses endoplasmic reticulum stress, and PKR can recognize viral double-stranded RNA. Activation of HRI is induced by haem deficiencies, and is important for the survival of erythroid precursors. Activation of each of these four sensors results in phosphorylation of eukaryotic initiation factor 2α (eIF2α), leading to the initiation of global translational arrest. Recent evidence suggests a crosstalk between the ISR and the immune system. Thus, our recent systems-based analysis of immune responses to the yellow fever vaccine (YF-17D) in humans revealed a correlation between the expression of GCN2 in the blood and the magnitude of the later CD8+ T-cell response. Furthermore, YF-17D induced GCN2 activation in dendritic cells, resulting in enhanced autophagy and antigen presentation. Whether GCN2 can modulate immune responses during conditions of amino acid restriction remains unexplored. This is particularly relevant in the intestine, where the immune system has to endure dynamic changes in nutrient bioavailability. We thus determined whether GCN2 impacts immune homeostasis in the intestine.

Phosphorylated eIF2α was detected in intestinal dendritic cells, macrophages and epithelial cells under steady-state and inflammatory conditions (Extended Data Fig. 1a). Furthermore, expression of phosphorylated PKR, PERK, eIF2α and GCN2 could be detected in tissues from healthy and inflamed human colon (Extended Data Fig. 1b). Analysis of public gene expression databases revealed that the expression of genes encoding GCN2 and other eIF2α kinases was highest in the colon, relative to other organs (Extended Data Fig. 1c). Interestingly, there was a higher expression of genes encoding GCN2, PERK and PKR in ulcerative colitis and Crohn’s disease, relative to healthy controls (Extended Data Fig. 1d).

To investigate the functions of GCN2 in vivo, we analysed the structure and morphology of gut tissue isolated from the Gcn2−/− mice. Ki-67 and chromogranin A staining in small and large intestines were unaffected in Gcn2−/− mice, suggesting that GCN2 is not required for steady-state cell differentiation and proliferation in the intestine (Extended Data Fig. 2a, b, d). Gcn2−/− mice had normal Paneth cell granules, as evident with lysozyme staining (Extended Data Fig. 2c), and did not exhibit any spontaneous gut inflammation up to 45 weeks of age.

We then assessed the impact of GCN2 deficiency on acute colitis by challenging the mice with 2% dextran sodium sulfate (DSS), a chemical irritant that induces inflammation with the clinical and histological features of inflammatory bowel disease in mice. Upon DSS administration, Gcn2−/− mice exhibited enhanced severity of colitis compared with littermates, including greater weight loss, inflammation, T_h17 responses and colon shortening (Fig. 1a–c and Extended Data Fig. 3a–c). Histopathological analysis revealed severe mucosal epithelial erosion, displacement and crypt loss (Extended Data Fig. 3a). Consistent with enhanced gut inflammation, we observed a severely impaired epithelial barrier, evidenced by increased intestinal permeability (Extended Data Fig. 3d). These differences were not due to differences in the expression of antimicrobial defensins between wild-type and Gcn2−/− mice (Extended Data Fig. 3e).

To assess potential roles for APCs versus epithelial cells in mediating the effects of GCN2, we generated mice lacking GCN2 specifically in epithelial cells (Gen2Δθ glo villin creΔθ; referred to as Gen2ΔθAPC hereafter) (Fig. 1d–f and Extended Data Fig. 3a–c), or in CD11c+ APCs (Gen2Δθ ci11 creΔθ; referred to as Gen2ΔθAPC hereafter) (Fig. 1g–i and Extended Data Fig. 3a–c). DSS induced enhanced colitis in both strains, with a higher severity and inflammation score in the Gen2ΔθAPC mice compared with their littermates. Consistent with our in vitro findings, Gcn2ΔθAPC mice had a severely impaired epithelial barrier, evidenced by increased intestinal permeability (Extended Data Fig. 3d). These differences were not due to differences in the expression of antimicrobial defensins between wild-type and Gcn2−/− mice (Extended Data Fig. 3e).
evidenced by weight loss, colon shortening and increased Tg17 responses (a surrogate readout of intestinal inflammation) relative to littermate controls (Fig. 1 and Extended Data Fig. 3b, c). Consistent with a role for GCN2 in APCs, isolated intestinal dendritic cells from Gcn2−/− mice could stimulate enhanced IL-17 production from antigen-specific CD4+ T cells, in vitro (Extended Data Fig. 3f). Collectively, these findings demonstrate that GCN2 deficiency in both epithelial cells and APCs results in enhanced inflammation and DSS-induced colitis.

Since PERK activation by endoplasmic reticulum stress is also known to be an important component of the host ISR14, we generated mice lacking PERK in epithelial cells (PerkΔvillin (d–f) or APCs (Gcn2ΔAPC) (g–i)) to protect mice from DSS-induced colitis. J, LC3–GFP expression and the GFP puncta counts in the crypts (three-dimensional) from Gcn2−/− LC3–GFP and LC3–GFP mice before and 12 h after DSS. WT, wild type. k, Mean fluorescence intensity (MFI) comparison of LC3B and p62 expression with and without chloroquine (Chloro) on APC subsets and epithelial cells by flow cytometry before and after 3% DSS administration. I, Comparison of body weights of Atg5ΔAPC and Atg7ΔAPC mice to littermate controls subjected to acute 2% DSS-induced colitis. Data are representative of three separate experiments (n = 5). ***P < 0.005, ****P < 0.0005. Error bars indicate mean ± standard error of the mean (s.e.m.), two-tailed unpaired Student’s t-test.
We therefore generated Atg5Δ/Δ Cdl1c−/− (Atg5ΔAPC) and Atg7Δ/Δ Cdl1c−/− (Atg7ΔAPC) mice that are conditionally deficient in the autophagy proteins Atg5 and Atg7 in Cdl1c+ APCs (Extended Data Fig. 7). Upon treatment with 2% DSS, both the Atg5ΔAPC and Atg7ΔAPC strains exhibited greater weight loss (Fig. 1a), enhanced shortening of colon length, TH17 responses and immunopathology (Fig. 2a) and small intestine (Extended Data Fig. 8b). In vivo blockade of IL-1β with a neutralizing antibody in Gcn2−/− mice ameliorated the deleterious effects of DSS (Fig. 3c, d and Extended Data Fig. 8c), and significantly reduced intestinal T H17 response (Fig. 3e). However, there were no detectable effects on histopathology, possibly due to incomplete neutralization of IL-1β (Extended Data Fig. 8d). Additionally, we observed that increased inflammation and T H17 responses in the Gcn2−/− mice were negated by the deletion of the inflammasome adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) (Fig. 3f–i), demonstrating a clear role for inflammasome activation in mediating the enhanced inflammation in Gcn2−/− mice.

Given the importance of Gcn2 in sensing amino acid starvation, we hypothesized that mice fed an amino-acid-deprived diet might display enhanced activation of ISR in intestinal cells, resulting in dampened inflammation. Therefore we fed mice a reduced amino acid diet (2% protein/weight versus 16% in control mice) and observed rapid activation of phosphorylated (p)-eIF2α (Fig. 3a). Intestinal cells isolated from wild-type mice that were on a low protein diet rapidly upregulated α- and β-actin (Fig. 3b) and small intestine (Extended Data Fig. 8b). In vivo blockade of IL-1β with a neutralizing antibody in Gcn2−/− mice ameliorated the deleterious effects of DSS (Fig. 3c, d and Extended Data Fig. 8c), and significantly reduced intestinal T H17 response (Fig. 3e). However, there were no detectable effects on histopathology, possibly due to incomplete neutralization of IL-1β (Extended Data Fig. 8d). Additionally, we observed that increased inflammation and T H17 responses in the Gcn2−/− mice were negated by the deletion of the inflammasome adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) (Fig. 3f–i), demonstrating a clear role for inflammasome activation in mediating the enhanced inflammation in Gcn2−/− mice.

Figure 2 | GCN2 suppresses ROS activity and intestinal inflammation.

We therefore generated Atg5Δ/Δ Cdl1c−/− (Atg5ΔAPC) and Atg7Δ/Δ Cdl1c−/− (Atg7ΔAPC) mice that are conditionally deficient in the autophagy proteins Atg5 and Atg7 in Cdl1c+ APCs (Extended Data Fig. 7). Upon treatment with 2% DSS, both the Atg5ΔAPC and Atg7ΔAPC strains exhibited greater weight loss (Fig. 1a), enhanced shortening of colon length, TH17 responses and immunopathology (Fig. 2a) and small intestine (Extended Data Fig. 8b). To determine whether autophagy regulates mitochondrial ROS, we analysed superoxide levels in colonic epithelial cells using MitoSOX, a fluorescent probe that reacts with numerous types of ROS19. After oxidation by ROS, the non-fluorescent MitoSOX is converted to fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), a fluorescent probe that reacts with numerous types of ROS19. After oxidation by ROS, the non-fluorescent H2DCFDA is converted to fluorescent 2′,7′-dichlorofluorescein (DCF)19, which was detected in intestinal APCs and epithelial cells (Fig. 2a and Extended Data Fig. 7a, b) by flow cytometry. Gcn2−/− mice exhibited significantly higher levels of ROS compared with littermate controls, indicating enhanced oxidative stress in the colon (Fig. 2a) and small intestine (Extended Data Fig. 8a, b). We also analysed the levels of mitochondrial ROS in the large and small intestine using MitoSOX, a fluorescent dye that specifically detects mitochondrial superoxide20. Gcn2−/− mucosal cells produced excess mitochondrial ROS in comparison with the littermate controls in the colon (Fig. 2a) and small intestine (Extended Data Fig. 8a, b). To determine whether autophagy regulated mitochondrial ROS, we analysed superoxide levels in colonic epithelial cells isolated from Atg5ΔAPC and Atg7ΔAPC mice after DSS treatment. As in the Gcn2−/− strain, there was higher production of mitochondrial ROS in Atg5ΔAPC and Atg7ΔAPC mice compared with littermate controls (Fig. 2b). Furthermore, blockade of ROS via administration of the antioxidant N-acetyl-l-cysteine (NAC) in vivo led to reduced disease severity, and reduction of T H17 responses in Gcn2−/− mice (Fig. 2c–f). Thus, these data demonstrate a key role for ROS in mediating the enhanced inflammation observed in Gcn2−/−.

Oxidative mitochondrial stress is known to be involved in the activation of the inflammasome pathway1,15. We therefore hypothesized that excess ROS enhanced inflammasome activation in the Gcn2−/− cells under inflammatory conditions. Gcn2−/− dendritic cells produced excess amounts of cleaved IL-1β and cleaved caspases when subjected to amino acid starvation (Fig. 3a). Additionally, there was higher production of pro-IL-1β in the colon macrophages and dendritic cells isolated from DSS-treated Gcn2−/− mice in the large (Fig. 3b) and small intestine (Extended Data Fig. 8b). In vivo blockade of IL-1β with a neutralizing antibody in Gcn2−/− mice ameliorated the deleterious effects of DSS (Fig. 3c, d and Extended Data Fig. 8c), and significantly reduced intestinal T H17 response (Fig. 3e). However, there were no detectable effects on histopathology, possibly due to incomplete neutralization of IL-1β (Extended Data Fig. 8d). Additionally, we observed that increased inflammation and T H17 responses in the Gcn2−/− mice were negated by the deletion of the inflammasome adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) (Fig. 3f–i), demonstrating a clear role for inflammasome activation in mediating the enhanced inflammation in Gcn2−/− mice.
Figure 3 | Enhanced intestinal inflammation in Gcn2−/− mice is dependent on inflammasome activation. a, Western blot analysis of pro–IL-1β, pro-caspase, cleaved IL-1β and cleaved caspase in lysate and culture supernatants of bone marrow dendritic cell cultures from wild-type and Gcn2−/− mice treated with lipopolysaccharide (LPS) alone or LPS plus ATP (potassium efflux agent that triggers inflammasomes) under amino acid starvation conditions. b, Quantification of MFI’s of pro–IL-1β in colon APC subsets in Gcn2−/− and littermates after DSS. Macro, macrophages. c–e, Body weight (c), colon length (d), and colonic T:\it{H}17 responses (e) in DSS-treated Gcn2−/− mice treated with anti–IL-1β antibody or isotype control (iso). f–i, Comparison of body weight (f), colon length (g), histology (h) and T:\it{H}17 frequencies (i) between littermates, Gcn2−/−, Asc−/− (Asc is also known as Pycard) and Gcn2−/− Asc−/− mice subjected to DSS-induced colitis. Data are from one experiment that is representative of three separate experiments (n = 4–5). *P < 0.05; **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.

protein-restricted diets and normal diets had similar weights before DSS (data not shown). After DSS, wild-type mice on protein-restricted diets weighed significantly less than those on control diet (Fig. 4c), but this was not the case in Gcn2−/− mice, indicating that Gcn2 protected against gut inflammation (Fig. 4c). The colon lengths and histopathology (epithelial integrity, cellular infiltration, crypt loss) were similar (Extended Data Fig. 8e and data not shown). By contrast, mice on protein-modified diets showed a reduced incidence of ‘bloody diarrhoea’, compared with control diet mice (Fig. 4d). Remarkably, the frequencies of colonic T:\it{H}17 cells were significantly lower in wild-type mice on modified diets compared with mice on control diets (Fig. 4e). In contrast, there were no significant differences in the T:\it{H}1 responses or regulatory T cells, indicating that amino-acid-restricted diets selectively impair T:\it{H}17 responses (Extended Data Fig. 8f). Notably, there were no differences in the T:\it{H}17 frequencies among Gcn2−/− mice on various diets, indicating that this effect is Gcn2 dependent (Fig. 4e). Together, these data demonstrate that amino acid starvation protects the symptoms of colitis and limits T:\it{H}17 cells via a Gcn2-dependent mechanism.

We demonstrate that Gcn2 suppresses intestinal inflammation and T:\it{H}17 responses via a mechanism dependent on autophagy and sequestration of ROS, which is a trigger for inflammasome activation (Extended Data Fig. 9a). Gcn2−/− mice displayed enhanced ROS and inflammasome activation, leading to increased inflammation and T:\it{H}17 responses (Figs 1–3). Thus, blockade of ROS and IL-1β led to lower inflammation and T:\it{H}17 responses in Gcn2−/− mice (Figs 2 and 3). In addition, Gcn2−/− mice were deficient in autophagy, which sequesters ROS, and consistent with this there was enhanced ROS and T:\it{H}17 inflammation in Atg5ΔAPC and Atg7ΔAPC mice (Fig. 1 and Extended Data Fig. 7). Future studies aimed at the functional reconstitution of a constitutively active autophagy pathway specifically in intestinal APCs and epithelial cells in Gcn2−/− mice should provide greater insight into the extent to which the observed phenotype in Gcn2−/− mice is due to impaired autophagy. Consistent with these results it is known that halofuginone, a compound that activates the amino acid starvation pathway, selectively inhibits mouse and human T:\it{H}17 differentiation24. Remarkably, we observed that a low protein diet, which activates the amino acid starvation response pathway, reduces the symptoms of colitis and colonic T:\it{H}17 responses. Although prolonged protein deficiency impairs critical immune functions5, short-term protein restriction can enhance immunity to pathogens26–28 and cancer29. Also, pharmacological activation of Gcn2 protected mice against ischaemia reperfusion injury30.

It is tempting to speculate on the evolutionary significance of coupling amino acid starvation with control of inflammation. Tissue injury and cell death, which occur during inflammation, inevitably result in tissue regeneration. Tissue regeneration, in turn, is accompanied by protein synthesis, which could lead to amino acid depletion in the cytosol. The consequent activation of Gcn2 will suppress inflammasome activation through the mechanisms described here, in effect representing a negative feedback mechanism that limits the inflammation (Extended Data Fig. 9b). Our results show a role for Gcn2 in protecting mice against intestinal inflammation. Thus, targeting the Gcn2 pathway may provide new strategies for pharmacological intervention for the amelioration of inflammatory bowel disease and other inflammatory disorders.
5.14
21.6

2. Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate
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Figure 4 | Dietary restriction of amino acids can partially protect
against DSS-induced colitis. a. Expression of p-eIF2α on APC subsets
and epithelial cells from wild-type (WT) mice on a 2% protein diet.

b. Kinetics of LC3B–GFP expression after a 2% protein diet in colonic APC
subsets and epithelial cells isolated from
Figure 4 (b) and colonic TH17 responses (c) in 3% DSS-induced wild-type or
Gcn2−/− mice that were on protein-modified diets (2% protein diet or
leucine-deficient diet) compared with control diet (16%). Data are from
two separate experiments that were then pooled. *P < 0.05; **P < 0.005,
***P < 0.0005. Error bars indicate mean ± s.e.m.

Online Content Methods, along with any additional Extended Data display items
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1. Zhou, R., Yazdi, A. S., Menu, P. & Schopp, J. R. A role for mitochondria in NLRP3
inflammasome activation. Nature 469, 221–225 (2011).

2. Kawai, T, & Akira, S. Toll-like receptors and their crosstalk with other innate
receptors in infection and immunity. Immunity 34, 637–650 (2011).

3. Pulendran, B. The varieties of immunological experience: of pathogens, stress,
and dendritic cells. Annu. Rev. Immunol. 33, 563–606 (2015).

4. Donnelly, N., Gorman, A. M., Gupta, S. & Samali, A. The eIF2α kinases: their
structures and functions. Cell. Mol. Life Sci. 70, 3493–3511 (2013).

5. Han, A. P. et al. Heme-regulated eIF2α kinase (HRI) is required for transisational
regulation and survival of erythroid precursors in iron deficiency. EMBO J. 20,
6909-6918 (2001).

6. Quebec, T. D. et al. Systems biology approach predicts immunogenicity
of the yellow fever vaccine proteins. Nature Immunol. 10, 118–125
(2009).

7. Ravindran, R. et al. Vaccine activation of the nutrient sensor Gcn2 in dendritic
cells enhances antigen presentation. Science 343, 313–317 (2014).

8. Funk, B. et al. Functional characterisation of deoxy receptor 3 in Crohn’s
disease. Gut 58, 483–491 (2009).

9. Kugathasan, S. et al. Loci on 20q13 and 21q22 are associated with pediatric-onset
inflammatory bowel disease. Nature Genet. 40, 1211–1215 (2008).

10. Kleene, S. J., Toews, M. L. & Adler, J. Isolation of glutamic acid methyl ester from
an Escherichia coli membrane protein involved in chemotaxis. J. Biol.
Chem. 252, 3214–3218 (1977).

11. Harding, H. P., Zhang, Y. & Ron, D. Protein translation and folding are coupled
by an endoplasmic-reticulum-resident kinase. Nature 397, 271–274 (1999).

12. Back, S, H. et al. Translation attenuation through eIF2α phosphorylation
prevents oxidative stress and maintains the differentiated state in (i) cells.
Cell Metab. 10, 13–26 (2009).

13. Cao, S. S. et al. Phosphorylation of eIF2α is dispensable for differentiation but
required at a posttranscriptional level for panel cell function and intestinal
homeostasis in mice. Inflamm. Bowel Dis. 20, 712–722 (2014).

14. Tattoli, I. et al. Amino acid starvation induced by invasive bacterial pathogens
triggers an innate host defense program. Cell Host Microbe 11, 563–575
(2012).

15. Saitoh, T. et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-
induced IL-1β production. Nature 456, 264–268 (2008).

16. Mizushima, N. & Kuma, A. Autophagosomes in GF-LC3 transgenic mice.
Methods Mol. Biol. 445, 119–124 (2008).

17. Martínez, J. et al. Molecular characterization of LC3-associated phagocytosis
reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nature Cell
Biol. 17, 893–906 (2015).

18. Damiani, C. R. et al. Oxidative stress and metabolism in animal model of colitis
enhanced by dextran sodium sulfate. J. Gastroenterol. Hepatol. 22, 1846–1851
(2007).

19. Brubacher, J. L. & Bolis, N. C. Chemically de-acylated 2,7-dichlorodihydrofluorescein diacetate as a probe of respiratory burst
activity in mononuclear phagocytes. J. Immunol. Methods 251, 81–91 (2001).

20. Julian, D., April, K. L., Patel, S., Stein, J. R. & Wohlgemuth, S. E. Mitochondrial
depolariization following hydrogen sulfide exposure in erythrocytes
from a sulfite-tolerant marine invertebrate. J. Exp. Biol. 208, 4109–4122
(2005).

21. Zhang, P. et al. The Gcn2 eIF2α kinase is required for adaptation to amino acid
deprivation in mice. Mol. Cell. Biol. 22, 6681–6688 (2002).

22. Hao, S. et al. Uncharged tRNA and sensing of amino acid deficiency in
macrophages in vitro. Nature 424, 771–778 (2003).

23. Anthony, T. G. et al. Preservation of liver protein synthesis during dietary
leucine deprivation occurs at the expense of skeletal muscle mass in mice
deleted for eIF2 kinase Gcn2. J. Biol. Chem. 279, 36553–36561 (2004).

24. Sundrud, M. S. et al. Halofuginone inhibits Tp17 cell differentiation by
activating the amino acid starvation response. Science 324, 1334–1338
(2009).

25. Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L. & Gordon, J. I. Human
nutrition, the gut microbiome and the immune system. Nature 474, 327–336
(2011).

26. Hu, J. F. et al. Repression of hepatitis B virus (HBV) transgene and HBV-induced liver injury by low-protein diet. Oncogene 15, 2795–2801 (1997).

27. Arinyasinghe, A. et al. Protection against malaria due to innate immunity
enhancement by low-protein diet. J. Parasitol. 92, 531–538 (2006).

28. Obara, M. et al. Beneficial effects of a low-protein diet on host resistance to
Paracoccidioides brasiliensis in mice. Nutrition 55, 994–963 (2009).

29. Li, W. M. et al. Immunopotential of NKT cells by low-protein diet and the suppressive effect on tumor metastasis. Cell. Immunol. 231, 96–102
(2004).

30. Peng, W. et al. Surgical stress resistance induced by single amino acid
depression requires Gcn2 in mice. Sci. Transl. Med. 4, 118ra11 (2012).

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databases. P.H. and Y.-C.W. genotyped mice. P.S. performed the histology
analysis. J.M. provided critical insight and advice about the autophagy
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METHODS

Mice. Gcn2−/− mice were provided by D. Munn. The Gcn2-floxed mice were obtained from Jackson Laboratories, and bred onsite. Perk-floxed mice were obtained from D. Cavener. To delete specifically GCN2 or PERK in APCs, floxed Gcn2 mice (Gcn2fl/fl) were crossed with Cd11c cre or villin cre mice that express the Cre enzyme under the control of the CD11c promoter or villin promoter, respectively, generating Gcn2ΔcDNA, PerkΔcDNA, PerkΔVillin and PerkΔVillin mice. eIF2αSer51Ala Tg (eIF2α floxed) mice were provided by R.J.C.15, and across the Line 111315 and villin cre mice to obtain the conditional expression of non-phosphorylatable Ser51Ala mutant eIF2α in APCs or intestinal epithelial cells. Successful Cre-mediated deletion was confirmed by PCR and protein expression (fluorescence-activated cell sorting (FACS) or western blot) (Extended Data Fig. 10). LC3−GFP mice were generated by N. Mizushima35 and provided by H. Virgin. These mice were crossed with Gcn2−/− mice to generate Gcn2−/− LC3−GFP mice.

Animal studies were conducted using age-matched littermate controls for each experiment. Both male and female mice were used and were between 8 and 14 weeks of age at the time of experiments. Mice were maintained under specific-pathogen-free conditions in the Emory Vaccine Center vivarium. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments.

Histology. Formalin-fixed and paraffin-embedded murine intestinal tissue was sectioned (4 μm) and standard haematoxylin and eosin (H&E) stains were carried out to assess DSS-induced damage. Paraffin-embedded sections were subjected to deparaffinization in xylene, rehydration in graded series of ethanol, and rinsing with distilled water. The resident pathologist at Yerkes performed all histopathological analyses in a blinded fashion.

Immunohistochemistry was performed by using labelled anti-mouse anti-ribbit/anti-goat biotin antibody followed by streptavidin–alkaline phosphatase using a multistep staining protocol. All reactions were detected by development of the chromogen (Warp Red; Biocare Medical). Appropriate positive and negative controls were run in parallel. After rehydration, slides were treated with a target retrieval solution (Diva decloaker; Biocare Medical) and then steamed for antigen retrieval for 20 min and cooled for 20 min. The following primary antibodies were used for immunohistochemical staining: Ki67 (#12202, Cell Signaling Technology, 1:200), lysozyme (Sc-27956, Santa Cruz, 1:50), chromogranin A (ab15160, Abcam, 1:200). Nuclei were counterstained using Gill’s haematoxylin. Human sections from naive or inflamed colons (Pantomics, catalogue numbers COLO1 and COLO2) were stained in a similar fashion with the following primary antibodies: PERK (#T980, Cell Signaling Technology, 1:200), phospho-GCN2 (ab32036, Abcam, 1:200), PKR (ab32036, Abcam, 1:200), anti-IL-1β (ab9722, Abcam, 1:200).

Isolation of intestinal APCs and lymphocytes. For epithelial and lamina propria (LP) lymphocyte isolation, mice were euthanized and the intestine was washed, cleaned of fat tissue and Peyer's patches, and longitudinally cut and suspended in 1× HBSS with 20 mM HEPES, 1 mM dithiothreitol (DTT) and 5 mM EDTA for 30 min at 37 °C. After washing with 1× HBSS, pieces were digested with collagenase VIII (Sigma) (1 U/ml−1 in RPMI with 2% FCS) for 30 min at 37 °C with shaking (150 r.p.m.). Tissue was processed through a 100-μm cell strainer, and the resulting suspension was pelleted. For lymphocyte isolation, cells derived following collagenase were resuspended in 7 ml of 40% Percoll and layered on top of 2 ml of RPMI complete medium. The culture supernatants were analysed after 72 h and cells were harvested and resuspended for 6 h with plate-bound antibodies against CD3 (10 g/ml−1), 145×1 C1 (from Becton Dickinson) and CD28 (20 g/ml−1, 37.51 from Becton Dickinson) in the presence of brefeldin A (100 ng/ml−1) for intracellular cytokine detection (IL-17A and IFN-γ). For analysis of IL-17A and IFN-γ responses from freshly isolated LP, the Perl-perfused lymphocytes from colon or small intestine were stimulated with PMA (100 ng/ml−1) and ionomycin (1,000 ng/ml−1) for 4 h in the presence of GolgiPlug and then stained for intracellular IL-17A and IFN-γ for FACS analysis.

Phospho-eIF2α staining by flow cytometry. Mice were orally gavaged with 2% DSS in 200 μl volume. The LP preparations were isolated and immediately fixed using the BD fixation solution for 10 min. Fixed cells were stained for intracellular p-eIF2α using a monoclonal antibody from Cell Signalling (3398). Cells were then stained with anti-rabbit IgG-PE (Jackson Laboratories, catalogue number 111-116-144) at a 1:400 dilution.

Gene expression analysis. Total RNA was extracted from gut samples using Qiagen RNeasy mini kit (catalogue number 74104) according to the manufacturer’s recommended protocol with the column DNAse–RNase-free treatment. Extracted RNA was reverse transcribed with SuperScript Vilo cDNA synthesis kit (catalogue number 11754050) according to the manufacturer’s protocol. An aliquot (10 ng) of cDNA was used to quantitate Ang4 (Mm03475554_g1), Lyc (Mm00721783_s1) and Reg3g (Mm0441127_m1) on the ABI7900 system. GAPDH (Mm99999999_g1) was used as the reference gene. The comparative gene expression method was used to determine gene expression relative quantity.

ROS and MitoSOX staining by flow cytometry. For detecting the ROS levels, the LP preparations were incubated with CM-H2DCFDA (10 μM; C6827, Life Technologies), a cell-permeable indicator for ROS. After incubation the levels of ROS were measured by flow cytometry. For MitoSOX staining the LP preparation was incubated with MitoSOX (M36608, Life Technologies) for 15 min (manufacturer’s instructions). Cells were then surface stained and analysed by flow cytometry.

NAC and anti-IL-1β treatment in vivo. For in vivo treatment, NAC (Sigma) was injected into mice intraperitoneally (i.p.; 257 μg g−1) in PBS solution, pH 7.4, every other day at days 1, 3, 5 and 7 after DSS. For in vivo IL-1β neutralizing experiments, 300 μg per mouse of anti-IL-1β (BioXcell, catalogue number BE0246) was injected i.p. on days 1, 3, 5 and 7 after DSS. Control mice received 300 μg isotypric control antibody (Hamster IgG, BioXcell BE0091) i.p.

Detection of LC3–GFP. Mice were euthanized, and intestines were fixed (3.7% formaldehyde for 3 min) and promptly washed with PBS. The tissue was fixed in formalin for an additional 12–18 h. Fixed tissue was embedded in OCT and sectioned on a cryotome into 6-μm sections. Slides were washed with PBS and mounted with Prolong Gold Antifade reagent with 3,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were collected using LSM510 META confocal microscopy (Carl Zeiss). The z-stack images were collected and the GFP signals were analysed through the sections. Each individual crypt was analysed in three dimensions to reveal the number of LC3–GFP-positive crypts using an Imaris 7.3D/4D image processing and analysis software (Bitplane). Multiple crypts (5–7) were chosen from 3 different animals per group and the average mean LC3–GFP counts were quantified per crypt using Imaris software 7 (Bitplane).

In vivo autophagy flux. To study autophagy flux chloroquine was used as a mode of inhibiting degradation of the components of the autophagosomes. Accumulated versus steady-state form of the autophagosomes in wild-type and Gcn2ΔcDNA mice after oral administration of DSS (200 μl of 3% DSS) was compared. Chloroquine (10 mg/kg−1) or saline was administered i.p. 2 h after DSS gavage and mice were killed at 12 and 24 h after DSS treatment. The colons were cleaned, collagenase-treated and the LP preparation was quickly stained for various APC surface markers (CD11c, CD45, Epac, McHC-II, CD11b) and intracellular levels of p62 (1:150) (H00008878-M01PE) and LC3B (1:150) (NB100-220F) to study the levels of intracellular autophagosomes. Intracellular staining was performed using BD perm buffer (BD Bioscience, 554723) after fixation with the BD Cytofix/ Cypermert solution (BD Bioscience, 554714). In some instances the LP was treated with digitonin (200 μg ml−1) for 10 min before undergoing intracellular staining.

ELISA. Briefly, IL-17A and IL-13 in culture supernatants were quantified by ELISA according to product protocol (R&D Systems catalogue number DY42; BD Biosciences catalogue number 559603).

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After the treatment, mice were kept with normal drinking water for 3 more days. Mice were killed and tissues analysed for immune and histological analysis. In addition, mice were orally gavaged with 200 μl of 2% DSS, thus minimizing the variation within a group owing to differences in the consumption of the drinking water. In the experiment involving protein restriction diets, wild-type (littermate) or \textit{Gcn2}^−/− mice were used with 3% DSS to induce colitis in the wild-type mice. Three per cent rather than 2% DSS was used to induce intestinal inflammation in wild-type mice, to be able to detect any effect of a low protein diet in these mice. Low protein mouse diet. The mice were fed with either test diet 5CC7 (1812281), Baker amino-acid-defined diet (16% protein) or diet 5CC7 modified with 2% defined protein or no leucine (Test Diet Land O’Lakes Purina Feed).

**Generation of murine bone-marrow-derived dendritic cells.** Mice tibiae and femurs were flushed with ice-cold PBS through a 70-μm cell strainer. Cells were pelleted and plated at a density of 5–7 × 10^6 bone marrow cells per 10-cm Petri dish in (RPMI complete) in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF; 20 ng ml$^{-1}$, Peprotech) and IL-4 (5 ng ml$^{-1}$). At day 3, the cultures were supplemented with another 10 ml of RPMI complete plus GM-CSF. At day 6, bone-marrow-derived dendritic cells were harvested by gently flushing the cells from the plate.

**In vitro activation of dendritic cells.** The bone-marrow-derived dendritic cells purified from wild-type or \textit{Gcn2}^−/− mouse tibia cultures were plated in 96-well microculture plates (1 × 10^5 cells per well) and primed for 8 h with 100 ng ml$^{-1}$ LPS (InvivoGen, LPS-SM) in RPMI 1640 (US Biological; R8999-04A) plus 1% dialysed FBS (Gibco 26400-036). Cultures were further stimulated for 1 h with ATP (5 mM) (InvivoGen, tlrl-atp). After the stimulation, cell supernatants were collected and assayed for IL-1β by ELISA or western blot. Cell pellets were lysed and assayed for the presence of pro-IL-1β and pro-caspase 1.

**Western blotting.** Purified dendritic cells (1 × 10^6) were lysed with 100-μl protein extraction reagent (89900, Thermo Scientific) containing protease inhibitor (5872S, Cell Signaling). Equal amounts of protein (lysate) or supernatants were run on an SDS–PAGE and transferred onto nitrocellulose membranes after electro blotting. After blocking with 5% fat-free milk, the membranes were incubated at 4°C overnight with the following primary antibodies: anti-mouse pro- and cleaved IL-1β (Cell Signaling, 12507), pro-caspase-1 (Abcam, ab108362), caspase-p20 and 10 (Adipogen, AG-208-0042) and di-p-mannose receptor (CD209) (BioLegend, 158103). Secondary antibodies were conjugated with horseradish peroxidase and visualized with SuperSignal West Pico chemiluminescent substrate (34078, Thermo Scientific).

**Statistical analysis.** To assess the significance of a difference between groups, a two-sample, unpaired t-test was performed using Graph Prism software. A P value less than 0.05 was considered to be significant, a P value less than 0.01 was considered to be very significant, and a P value less than 0.001 was considered to be extremely significant.

31. Harding, H. P. et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell \textbf{6}, 1099–1108 (2000).
32. Zhang, P. et al. The PERK eukaryotic initiation factor 2α kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. Mol. Cell. Biol. \textbf{22}, 3864–3874 (2002).
33. Caton, M. L., Smith-Raska, M. R. & Reizis, B. Notch–RBP-J signaling controls the homeostasis of CD8− dendritic cells in the spleen. J. Exp. Med. \textbf{204}, 1653–1664 (2007).
34. Madison, B. B. \textit{et al.} cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J. Biol. Chem. \textbf{277}, 33275–33283 (2002).
35. Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T. & Ohsumi, Y. \textit{In vivo} analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol. Biol. Cell \textbf{15}, 1101–1111 (2004).
Extended Data Figure 1 | eIF2α kinases are expressed in human and murine gut cells. a, Analysis of p-eIF2α expression in APCs and epithelial cells in large intestine of naive and 2% DSS-treated mice by flow cytometry. DCs, dendritic cells; LI, large intestine. b, Comparison of immunohistological analysis of phosphorylated PKR, PERK, eIF2α and GNC2 in healthy and inflamed human colon tissue (n = 1). c, Expression levels of HRI, PKR, PERK and GCN2 in human organs quantified based on information from a public database (http://www.ebi.ac.uk). d, Expression intensity of various eIF2α kinases plotted from known published microarray data from colonic biopsies of patients with either ulcerative colitis or Crohn's disease compared to healthy controls. Data are from one experiment that is representative of three separate experiments. *P < 0.05, **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
Extended Data Figure 2 | GCN2 deficiency does not affect the proliferation or differentiation of intestinal epithelial cells.

a–d, Immunohistology analysis (a–c) and quantification (d) of colons and jejunums from Gcn2−/− and wild-type (littermate) mice for chromogranin A (a), Ki67 (b) and lysozyme (c). KO, knockout; WT, wild type. Data are from one experiment that is representative of three separate experiments (n = 3). *P < 0.05, **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
Extended Data Figure 3 | GCN2 expression protects mice from DSS-induced colitis. a, H&E staining of colon sections before and after DSS in wild-type versus Gcn2−/− mice, Gcn2fl/fl versus Gcn2Δvillin and Gcn2fl/fl versus Gcn2ΔAPC. b–d, IL-17 levels in large intestinal (b) and small intestinal (c) CD4+ T cells measured by flow cytometry; Gcn2−/− mice show increased intestinal permeability after DSS treatment as evidenced by higher levels of fluorescein isothiocyanate (FITC)-conjugated dextran in the serum (d). SI, small intestine. e, Expression of antimicrobial defensins in wild-type and Gcn2−/− mice via quantitative polymerase chain reaction (qPCR). f, IL-17 production by flow cytometry and enzyme-linked immunosorbent assay (ELISA) of OTII-CD4 T cells after culturing with different large intestinal APC subsets. Data are from one experiment that is representative of three separate experiments (n = 4–5). *P < 0.05, **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
Extended Data Figure 4 | PERK expression in epithelial cells has only a minor role in controlling mucosal homeostasis after DSS challenge. 

a–d. The body weight (a), colon length (b), histology by H&E (c) and T\textsubscript{H}17 responses (d) both in the colon (large intestine; LI) and small intestine (SI) of Perk\textsuperscript{Δ\textsubscript{villin}} and control wild-type littermates treated with DSS. 

e–h. The body weight (e), colon length (f), histology by H&E and histology score (g), and T\textsubscript{H}17 responses (h) both in the colon (LI) and small intestine (SI) of Perk\textsuperscript{Δ\textsubscript{APC}} and control wild-type littermates treated with DSS. Data are representative of two separate experiments (n = 5). *P < 0.05; **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
Extended Data Figure 5 | eIF2α expression in epithelial cells and APCs control weight loss and partially control T H 17 responses after DSS challenge. a–c, The body weight (a), colon length (b), histology by H&E and histology score (c), and T H17 responses in both the colon (LI) (d) and small intestine (SI) (e) of Eif2αΔvillin and control wild-type littermates treated with DSS. f–j, The body weight (f), colon length (g), histology by H&E and histology score (h), and T H17 responses in both the colon (LI) (i) and small intestine (SI) (j) of Eif2αΔAPC and control wild-type littermates treated with DSS. Data are representative of three separate experiments (n = 5). *P < 0.05, **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
Extended Data Figure 6 | Intestinal APCs and epithelial cells reveal high expression of LC3. a, Expression of LC3–GFP in APC subsets and epithelial cells of naive LC3–GFP mice by flow cytometry (n = 3). Data are from a single experiment. b, Kinetic MFI comparison of LC3B and p62 expression with and without chloroquine on individual APC subsets and epithelial cells by flow cytometry after 12 and 24 h of single DSS administration. c, Western blot detection of LC3-I and II on lamina propria APCs before and after digitonin. d, LC3B staining of individual APCs and epithelial cells 12 h after they were treated with DSS. The portion of the lamina propria cells were subjected to digitonin before intracellular staining with the LC3B antibody. Data are representative of two separate experiments (n = 4–5). *P < 0.05, **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
Extended Data Figure 7 | Atg5 and Atg7 expression in APCs partially protects mice from DSS challenge. a–c, Colon length (a), histology by H&E and histology score (b), and TH17 responses (c) in both the colon (LI) and small intestine (SI) of Atg5ΔAPC and control wild-type littermates treated with DSS. d–f, Colon length (d), histology by H&E and histology score (e), and TH17 responses (f) in both the colon (LI) and small intestine (SI) of Atg7ΔAPC and control wild-type littermates treated with DSS. Data are representative of three separate experiments (n = 5). *P < 0.05, **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
Extended Data Figure 8 | GCN2-induced autophagy protects the intestinal tissue from the effects of excess oxidation and inflammation.

a–f, Gcn2−/− and littermate wild-type mice were treated with 2% DSS in the drinking water for 5 days. a, MFIs of ROS and mitochondrial ROS (MitoSOX) in individual APC subsets and epithelial cells of the small intestine isolated from wild-type and Gcn2−/− mice were analysed by flow cytometry (n = 5). b, MFIs of pro-IL-1β in individual APC subsets and epithelial cells of the small intestine isolated from wild-type or Gcn2−/− mice (day 5 after DSS) were analysed by flow cytometry (n = 3). c, Histology analysis of Gcn2−/− mice that were treated with neutralizing antibody. d, Effects of low protein diet on DSS-induced colitis. e, Colon length. f, Frequencies of CD4+ T cells. Data are from one experiment that is representative of two or three separate experiments. *P < 0.05, **P < 0.005, ***P < 0.0005. Error bars indicate mean ± s.e.m.
**Extended Data Figure 9 | Mechanisms by which GCN2 contributes to protection in the gut against acute colitis.**

**a,** Amino acid starvation induced by an inflamed colon activates GCN2, which triggers autophagy, which is important in inhibiting oxidative stress and pro-IL-1β.

**b,** A hypothetical model for the evolutionary significance of coupling amino acid starvation with control of inflammation.

Furthermore, levels of IL-1β dictate the magnitude of IL-17A-producing CD4 T cells in the colon.
Extended Data Figure 10 | Mouse phenotyping by flow cytometry and western blot. a, b, In addition to molecular genotyping via tail DNA, we analysed the protein levels in various mucosal subsets by flow cytometry in various APC-specific (a) and epithelial-specific (b) conditional knockouts. c, Western blot to show selective depletion in APC populations in Atg5ΔAPC and Atg7ΔAPC mice. DC, dendritic cell; Ep, epithelial cell.