ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation

Tatsuo Hashimoto1,2*, Thomas Perlot1*, Ateequr Rehman3, Jean Trichereau4, Hiroaki Ishiguro2, Magdalena Paolini1, Verena Sigl1, Toshikatsu Hanada1, Reiko Hanada1, Simone Lipinski1, Birgit Wild4, Simone M. R. Camargo2, Dustin Singer2, Andreas Richter2, Keiji Kubad6, Akiyoshi Fukamizu6, Stefan Schreiber3, Hans Clevers8, Francois Verrey5, Philip Rosenstiel3 & Josef M. Penninger1

Malnutrition affects up to one billion people in the world and is a major cause of mortality1,2. In many cases, malnutrition is associated with diarrhea and intestinal inflammation, further contributing to morbidity and death. The mechanisms by which unbalanced dietary nutrients affect intestinal homeostasis are largely unknown. Here we report that deficiency in murine angiotensin 1 converting enzyme (peptidyl-dipeptidase A) 2 (Ace2), which encodes a key regulatory enzyme of the renin-angiotensin system (RAS), results in highly increased susceptibility to intestinal inflammation induced by epithelial damage. The RAS is known to be involved in acute lung failure3, cardiovascular functions4 and SARS infections5. Mechanistically, ACE2 has a RAS-independent function, regulating intestinal amino acid homeostasis, expression of antimicrobial peptides, and the ecology of the gut microbiome. Transplantation of the altered microbiota from Ace2 mutant mice into germ-free wild-type hosts was able to transmit the increased propensity to develop severe colitis. ACE2-dependent changes in epithelial immunity and the gut microbiota can be directly regulated by the dietary amino acid tryptophan. Our results identify ACE2 as a key regulator of dietary amino acid homeostasis, innate immunity, gut microbiological, and transmissible susceptibility to colitis. These results provide a molecular explanation for how amino acid malnutrition can cause intestinal inflammation and diarrhea.

The RAS has now been studied for more than a century. It is regulated by the opposing actions of two key carboxypeptidases, angiotensin converting enzyme (ACE) and ACE2 (refs 4, 6, 7). In addition to its catalytic activity, we and others have recently found that ACE2 associates with the neutral amino acid transporter B0AT1 and that ACE2 is required for expression of this transporter on the luminal surface of intestinal epithelial cells8. Variants in the gene that encodes B0AT1 (SLC6A19) have been identified as a cause of Hartnup’s disease9, a rare autosomal recessive disorder associated with pellagra-like symptoms including diarrhea that manifests under conditions of malnutrition10. However, the in vivo function of ACE2 in the gut epithelium remained to be investigated.

Morphological and ultrastructural analysis of the small and large intestine of Ace2 knockout mice (see below) did not reveal any alterations. When we challenged such mice with dextran sodium sulphate (DSS), an irritant that disrupts the intestinal epithelial barrier and results in colitis11, a profoundly increased inflammatory reaction was observable compared to wild-type littermates. We detected an enhanced infiltration of inflammatory cells, significant shortening of the colon length, increased intestinal bleeding, crypt damage, weight loss and severe diarrhea, resulting in an overall increased disease activity12 (Fig. 1a–c; Supplementary Fig. 1). Similar results were obtained using trinitrobenzene sulphonic acid (TNBS)-induced colitis (Supplementary Fig. 2). Thus, genetic inactivation of the key RAS enzyme ACE2 results in severe colitis following intestinal injury. We note that Ace2 deficiency was investigated in male mice (Ace2+/y, the Ace2 gene being located on the X chromosome) throughout this study, however, we observed a similar phenotype in female Ace2–/y mice (not shown).

Figure 1 | Ace2 deficiency and protein malnutrition worsen DSS-induced colitis. a, Colon histopathology, b, percentage weight loss, and c, diarrhea scores in control and DSS-treated Ace2+/y and Ace2–/y littermates. In a, note crypt damage (arrowheads), ulcerations (arrow), and infiltration of inflammatory cells (asterisks) in DSS-treated Ace2–/y mice. Haematoxylin and eosin staining on day 7 after DSS challenge. Scale bars, 100 μm. d, Colon histopathology (haematoxylin and eosin staining, day 4 after DSS challenge; scale bars, 100 μm), e, percentage weight loss, and f, diarrhea scores of DSS-treated Ace2+/y and Ace2–/y littermates fed either normal chow (Control) or a protein-free diet (PFD; <0.2% protein). All values are mean ± s.e.m. of 5–9 mice per group. *P < 0.05, **P < 0.01 comparing DSS-treated Ace2+/y with Ace2–/y littermates, or Ace2–/y mice on normal diet with those on PFD (paired-t-test).

1MBA, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, 1030 Vienna, Austria. 2The Department of Medical Science and Cardiovascular Medicine, Yokohama City University Graduate School of Medicine and School of Medicine, 2360004 Yokohama, Japan. 3Institute of Chemical Molecular Biology, University of Kiel, Schittenhelmstrasse 12, 24105 Kiel, Germany. 4Department of Chemical Ecology and Ecosystem Research, Center of Ecology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria. 5Institute of Physiology and Center for Integrative Human Physiology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. 6Department of Biological Informatics and Experimental Therapeutics, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. 7Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan. 8Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences and University Medical Center Utrecht, 3584 CT Utrecht, The Netherlands. *Present address: Department of Environmental Health Sciences, University Medical Center, Bresacher Strasse 115b, D-79106 Freiburg, Germany.

*These authors contributed equally to this work.
To test whether the severe colitis phenotype is due to the catalytic activity of ACE2, we treated mice with recombinant soluble ACE2 (rsACE2). We and others have shown that rsACE2 rescues virtually all previously reported in vivo ACE2 functions within the RAS system. As it lacks the transmembrane domain, soluble ACE2 cannot associate with the neutral amino acid transporter B0AT1 and thus cannot stabilize its cell-surface localization. Treatment of mice with rsACE2 did not rescue the severe DSS-induced colitis phenotype of Ace2 mutant mice (Supplementary Fig. 3a–h). One in vivo consequence of Ace2 deletion within the RAS is accumulation of angiotensin II (ANGII), which then primarily acts on the G-protein-coupled AT1a receptor (Agtr1a). We indeed observed increased AngII levels in the colon of DSS-treated Ace2 mutant mice that were reduced to background levels after treatment with rsACE2 (Supplementary Fig. 4), indicating that these effects are independent of the classical RAS system. In addition to cleaving AngII, ACE2 exhibits catalytic activity towards a second peptide system, Apelin. However, DSS-induced colitis was not altered in mice carrying genetic mutations in Apelin (Supplementary Fig. 5) or its receptor Apj (Supplementary Fig. 6). Thus, the catalytic activity of ACE2, essential for its function in the RAS and Apelin cleavage, has no overt role in DSS-induced intestinal inflammation.

It had been reported that the RAS can control immune functions. However, in unchallenged Ace2 mutant mice, we did not observe any apparent differences in immune cell populations of the colon and small intestine (not shown). TLR1–9 mRNA levels in jejunum, ileum, and colon were largely similar in Ace2 deficient and wild-type mice, with the exception of TLR5 and TLR9 in the colon (Supplementary Fig. 7). Transplantation of bone marrow from Ace2 mutant mice (knock-out, KO) into wild-type (WT) mice (KO→WT) did not result in any obvious effect on DSS-induced colitis as compared to WT→WT transplant recipients. By contrast, reconstitution of Ace2 mutant mice with WT bone-marrow (WT→KO) led to an increased severity of DSS-induced colitis that was indistinguishable from the KO→KO control group (Supplementary Fig. 8). Thus, ACE2 exerts its role on intestinal inflammation in non-haematopoietic cells.

ACE2 expression was primarily mapped to the luminal surface of differentiated small intestinal epithelial cells, whereas lower ACE2 expression is observed in crypt cells and the colon (Supplementary Figs 9a, b and 10a). Ace2 inactivation had no overt effect on basal proliferation of small intestinal or colon crypt cells (Supplementary Fig. 10a, b) nor did it affect apoptosis rates of intestinal epithelial cells (Supplementary Fig. 10a, c). ACE levels were slightly, albeit not significantly, increased in the jejunum of Ace2−/− mice (Supplementary Fig. 9c, d). As reported previously, protein expression of the neutral amino acid transporter B0AT1 was absent in the small intestine of Ace2 mutant mice, whereas mRNA expression was not affected (Supplementary Figs 9c, f and 11a). Owing to the lack of intestinal B0AT1 protein expression, serum levels of the neutral amino acids valine (Val), threonine (Thr) and tyrosine (Tyr), and the essential amino acid tryptophan (Trp) were markedly reduced in Ace2−/− mice (Supplementary Fig. 11b). The marked reduction in tryptophan correlates with human data, as patients suffering from Hartnup’s disease caused by Slc6a19 mutations or malnutrition such as in anorexia nervosa exhibit reduced tryptophan serum levels.

ACE2 is a chimaeric protein that emerged from the duplication and inactivation had no overt effect on basal proliferation of small intestinal or colon crypt cells (Supplementary Fig. 10a, b) nor did it affect apoptosis rates of intestinal epithelial cells (Supplementary Fig. 10a, c). ACE levels were slightly, albeit not significantly, increased in the jejunum of Ace2−/− mice (Supplementary Fig. 9c, d). As reported previously, protein expression of the neutral amino acid transporter B0AT1 was absent in the small intestine of Ace2 mutant mice, whereas mRNA expression was not affected (Supplementary Figs 9c, f and 11a). Owing to the lack of intestinal B0AT1 protein expression, serum levels of the neutral amino acids valine (Val), threonine (Thr) and tyrosine (Tyr), and the essential amino acid tryptophan (Trp) were markedly reduced in Ace2−/− mice (Supplementary Fig. 11b). The marked reduction in tryptophan correlates with human data, as patients suffering from Hartnup’s disease caused by Slc6a19 mutations or malnutrition such as in anorexia nervosa exhibit reduced tryptophan serum levels.

ACE2 is a chimaeric protein that emerged from the duplication and fusion of two genes: it is homologous with ACE at the catalytic domain and with collectrin (TMEM27) in the membrane proximal domain. Inactivation of collectrin in mice (Tmem27−/−) results in a near complete downregulation of apical amino acid transporters such as B0AT1 in the kidney, thereby regulating renal amino acid re-absorption. By contrast, ACE2 associates with B0AT1 on the luminal surface of intestinal epithelial cells, that is, ACE2 and B0AT1 regulate the uptake of neutral amino acids in the intestine where collectrin is not expressed (Supplementary Figs 11a, c and 12). In both Tmem27−/− and Ace2−/− mutant mice, tryptophan is reduced in the serum (Supplementary Fig 11b, and ref. 19). To test whether impaired re-absorption of amino acids in the kidney might also result in altered susceptibility to colitis, we challenged Tmem27−/− mice with DSS. However, genetic ablation of both Ace2 or Tmem27 result in a similar deregulation of amino acids in the serum, functionally only the loss of ACE2 affects the susceptibility to intestinal inflammation.

We, therefore, speculated that the function of ACE2 in intestinal uptake of dietary amino acids might underlie the observed phenotype. To test this hypothesis, we fed Ace2 mutant and control mice a protein free-diet (PFD) to eliminate dietary amino acids and challenged these mice with DSS at a dose (1%) that triggers only very mild colitis. Basal weight loss was comparable among control and Ace2 mutant mice during the period of the PFD (Supplementary Fig. 14a). Moreover, average food intake did not show a significant difference between Ace2 deficient and control mice fed PFD or normal chow (Supplementary Fig. 14b). Importantly, PFD markedly worsened DSS-triggered colitis in wild-type mice to levels seen in Ace2 mutants (Fig. 1d–f; Supplementary Fig. 14c–g). Thus, protein malnutrition alters the severity of DSS-induced intestinal inflammation.

The general lack of dietary protein might induce broad effects on several organ systems. We, therefore, set out to define whether a specific amino acid might be responsible for the severe inflammatory phenotype. Because serum tryptophan was markedly decreased in Ace2 mutant mice, we focused on this essential amino acid.

Figure 2 | Rescue of severe colitis with nicotinamide or tryptophan di-peptides. a, Colon histopathology (haematoxylin and eosin, day 10 after DSS challenge; scale bars, 100 μm). b, percentage weight loss, and c, diarrhoea scores of DSS-treated Ace2+/y and Ace2−/− littersmates that received vehicle or nicotinamide (NAM) in their drinking water. Nicotinamide treatment was started 3 days before DSS challenge. d, Colon histopathology (haematoxylin and eosin, day 7; scale bars, 100 μm), e, percentage weight loss, and f, crypt injury scores of Ace2+/y and Ace2−/− mice fed a di-peptidic tryptophan diet (Trp+), or normal chow (Control). Values are mean ± s.e.m. of 3–10 mice per group. *P < 0.05, **P < 0.01 comparing Ace2−/− mice on a normal diet with those on Trp+ diet, or vehicle- versus nicotinamide-treated Ace2−/− mice. #P < 0.01 comparing Ace2−/− versus Ace2+/y mice (paired t-test).
Tryptophan is required for the in vivo generation of nicotinamide (also known as vitamin B3 or niacin),17, and insufficient niacin or tryptophan in the diet is the cause of pellagra, a disease still endemic in many countries with protein malnutrition26. More than 90% of pellagra patients develop colitis19 and for nearly 80 years nicotinamide has been used as treatment for pellagra. In Ace2 mutant mice, nicotinamide almost completely alleviated the severe colitis and diarrhea (Fig. 2a–c; Supplementary Fig. 15). To demonstrate that the severe colitis in Ace2 deficient mice is due to impaired tryptophan uptake, we provided tryptophan in form of a Gly-Trp dipeptide to bypass the loss of the single amino acid transporter B^AT1. Dietary dipeptidic tryptophan (a Trp^+ diet) restored serum tryptophan levels (Supplementary Fig. 16a) and rescued the enhanced DSS-susceptibility of Ace2^-/y mice (Fig. 2d–f; Supplementary Fig. 16b–d). A tryptophan-free (Trp^−) diet resulted in a very marked increase in susceptibility to DSS-induced inflammation (Supplementary Fig. 17a–h). In addition, a Trp^− diet markedly worsened weight loss in wild-type mice challenged with TNBS (Supplementary Fig. 17i–l). Our results show that deficiency of Ace2 causes a critical impairment of local tryptophan homeostasis which alters the susceptibility to intestinal inflammation.

It is conceivable that an altered amino acid availability in the epithelium could lead to decreased regenerative responses and repair mechanisms after epithelial injury21,22. However, proliferation rates and cell death of small intestinal and colon epithelial cells were comparable between control and Ace2 mutant mice following DSS challenge (Supplementary Fig. 18). However, expression of multiple antimicrobial peptides was markedly reduced in isolated gut ileal epithelial cells from unchallenged Ace2 mutant mice (Fig. 3a; Supplementary Fig. 19). A Trp^− diet for wild-type mice also resulted in a significant downregulation of antimicrobial peptides (Fig. 3b). Similar downregulation of antimicrobial peptides was observed on feeding wild-type mice a PFD (Supplementary Fig. 20a). By contrast, dietary nicotinamide (Supplementary Fig. 20b–g) and a Trp^+ diet (Fig. 3c and Supplementary Fig. 20h, i) triggered induction of antimicrobial peptides in intestinal epithelial cells from Ace2 mutant and wild-type mice. Thus, dietary tryptophan controls expression of small intestinal antimicrobial peptides.

Amino acids and nicotinamide can activate mTOR, which is involved in cell proliferation, survival, protein synthesis and transcription23. In epithelial intestinal cells from unchallenged Ace2 mutant mice, we observed markedly impaired p70S6 kinase activity (Supplementary Fig. 21a) as well as reduced S6 phosphorylation (Fig. 3d and Supplementary Fig. 21b), indicative of reduced mTOR activity. A similar reduction of mTOR activity was recently reported in mice lacking B^AT1 (ref. 24). Administration of a Trp^+ diet resulted in increased mTOR activity in the small intestine of Ace2 mutant mice (Fig. 3e). In vivo inhibition of mTOR with rapamycin in wild-type mice resulted in a significant downregulation of antimicrobial peptide expression, an effect that could not be rescued by nicotinamide (Supplementary Fig. 22a). Moreover, administration of rapamycin before the first challenge with DSS increased the severity of colitis (Fig. 3f; Supplementary Fig. 22b–h). Notably, although acute mTOR inhibition may have beneficial effects in murine colitis models25, mTOR blockade has not been proven successful in human clinical trials in inflammatory bowel disease26. Thus, at the molecular level, nicotinamide and dietary tryptophan appear to exert their effects on intestinal antimicrobial peptides and colitis via the mTOR pathway.

How does ACE2 regulated uptake of tryptophan in the small intestine affect DSS-induced inflammation in the colon? It has been shown that α-defensins secreted into the ileum lumen persist in a functional state throughout the entire gut27 and that alterations in antimicrobial peptides can affect the ecology of the small and large bowel microbiota28. We therefore performed deep profiling of the intestinal microbiome of Ace2 mutant and wild-type littermates using 16S rDNA fingerprinting. The luminal ileocaecal microbiome of Ace2 mutant mice was markedly altered, as shown by weighted (Bray–Curtis) and unweighted (Unifrac) (Fig. 4a) and unweighted (Unifrac) (Fig. 4b) analyses. Distinct operational taxonomical units (OTUs) are overrepresented in Ace2 mutant mice (Fig. 4c; Supplementary Tables 1 and 2). Rapamycin treatment resulted in a distinct alteration of the ileocaecal gut microbiome in wild-type animals; but the altered microbiome was more closely related to that found in untreated wild-type animals than that found in untreated Ace2^-/y animals (Supplementary Fig. 23; Supplementary Tables 3 and 4). Rapamycin acts on many different cell types that in addition to the changes in intestinal epithelial cells could alter microbiota composition29. Importantly, both Trp^+ diet and nicotinamide treatment reverted the composition of the intestinal microbiota of Ace2 mutant mice to be more similar to that of untreated wild-type littermates (Fig. 4a–c, Supplementary Fig. 24, Supplementary Tables 1, 2, 5 and 6). Thus, de-regulation of tryptophan amino acid homeostasis in Ace2 mutant mice alters the intestinal microbiome.

Consistent with an alteration in the intestinal microbiome, antibiotic treatment of Ace2 mutant mice alleviated the severe colitis (Supplementary Fig. 25). Finally, we performed gut microbiome transplants from control and Ace2 mutant mice into germ-free hosts followed by DSS challenge. Transplantation of the ileocaecal gut microbiota from Ace2 mutant mice transmitted the inflammatory phenotype to wild-type hosts, which was not the case for mice that were colonized with the microbiota of Ace2^+/y mice (Fig. 4d, e; Supplementary Fig. 26a–f). Expression of antimicrobial peptides in wild-type mice did not change significantly upon transplantation of Ace2^+/y or Ace2^-/y microbiota within the timeframe of analysis (Supplementary Fig. 26g).

We propose the following scenario for how malnutrition and tryptophan deficiency can cause diarrhea and colitis. Dietary tryptophan is primarily absorbed via the B^AT1/ACE2 transport pathway on the luminal surface of small intestinal epithelial cells. This results in the activation of mTOR, either directly through nutrient sensing and/or through the tryptophan-nicotinamide pathway.
transplanted to germ-free hosts. Severity of colitis was monitored as described. Our results provide insights into the intestinal, cachexia, anorexia nervosa, or amino acid malabsorption constitute severe clinical problems. Our results provide information about the role of the intestine, cachexia, anorexia nervosa, or amino acid malabsorption constitute severe clinical problems. Our results provide information about the role of the intestine, cachexia, anorexia nervosa, or amino acid malabsorption constitute severe clinical problems.}

mTOR then regulates expression of antimicrobial peptides, which affect the intestinal composition of the gut microbiota. Whether pathways other than mTOR and antimicrobial peptides also contribute to the altered microbiome needs to be explored. Our results further indicate that under conditions of intestinal injury, such an altered microbiome contributes to the severity of colitis. Importantly, our data indicate that the essential amino acid tryptophan and its metabolite nicotinamide are key regulators of gut microbiota and of propensity to inflammation.

Malnutrition is a major global health burden, affecting up to one billion people in the world and its consequences such as colitis and diarrhoea are often fatal. In addition, chronic inflammatory conditions of the intestine, cachexia, anorexia nervosa, or amino acid malabsorption constitute severe clinical problems. Our results provide novel mechanistic insights into how protein malnutrition can lead to colitis and diarrhoea. Our data also identify a molecular crosstalk between the RAS system and intestinal amino acid homeostasis via ACE2—and provide a direct link between dietary amino acid metabolism and innate immunity; the composition of the intestinal microbiota, and susceptibility to colitis.

**METHODS SUMMARY**

**Colitis models.** Ace2, Tnm27, Atp1, Atp1 and Aplein mutant mice have been described. Mice were given protein-free and tryptophan-free diet, tryptophan dipeptides, nictinamide, rapamycin, or were treated with catalytically active murine collectrin differentially interact with Hartnup mutations. Simpkin correlations and similarity percentage analysis (SIMPER) were performed to determine significant differences between bacterial communities among genotype and diet groups.

**Microbiota sequencing.** Genomic DNA was extracted from the terminal ileum, amplified by PCR, and sequenced using Roche 454 Titanium chemistry. All sequence reads were filtered for quality and length using PANGEA. Sequences were clustered using average neighbour algorithm into species level operational taxonomic units (OTUs). Distance matrices between samples were generated based on weighted (Bray–Curtis similarity) and non-weighted (unweighted UniFrac) algorithms and shown as principal coordinate analysis (PCoA). Spearman correlations and similarity percentage analysis (SIMPER) were performed to determine significant differences between bacterial communities among genotype and diet groups.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

**Figure 4 | Altered gut bacteria from Ace2 mutant mice can confer susceptibility to colitis.** a, b, Principal coordinate analysis plots; a, calculated by Bray–Curtis algorithm and b, based on unweighted UniFrac analysis. Plots show the similarity among ileoceleal bacterial communities in Ace2+/y and Ace2–/y mice fed a Trp+ diet or normal chow (Control) for 10 days. Only the two axes with high R² values are shown (axis 1, R² = 0.335; axis 2, R² = 0.8116). Each dot represents data from an individual animal. c, Comparison of microbial communities in Ace2+/y and Ace2–/y mice fed a Trp+ diet or normal chow (Control). The heat map depicts abundance of the top 25 species level OTUs contributing significantly to the axis shown in the weighted principal coordinate analysis plot (a). d, Diarrhoea scores and e, colon histopathology (haematoxylin and eosin, day 7; scale bars, 100 μm) of DSS challenged germ-free mice that received intestinal microbiota from Ace2+/y or Ace2–/y littermates. Values are mean ± s.e.m. of 4–6 mice per group. *P < 0.05 (paired-t-test).
13. Cooper, H.S., Murthy, S.N., Shah, R.S. & Sedergran, D.J. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab. Invest. 69, 238–249 (1993).

14. Osterreicher, C.H. et al. Angiotensin-converting-enzyme 2 inhibits liver fibrosis in mice. Hepatology 50, 929–938 (2009).

15. Vickers, C. et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J. Biol. Chem. 277, 14838–14843 (2002).

16. Swirski, F.K. et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science 325, 612–616 (2009).

17. Darby, W.J., McNutt, K.W. & Todhunter, E.N. Niacin. Nutr. Rev. 33, 289–297 (1975).

18. Attia, E., Wolk, S., Cooper, T., Glasofer, D. & Walsh, B.T. Plasma tryptophan during weight restoration in patients with anorexia nervosa. Biol. Psychiatry 57, 674–678 (2005).

19. Danilczyk, U. et al. Essential role for collectrin in renal amino acid transport. Nature 444, 1088–1091 (2006).

20. Stratigos, J.D. & Katsambas, A. Pellagra: a still existing disease. Br. J. Dermatol. 96, 99–106 (1977).

21. Nenci, A. et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature 446, 557–561 (2007).

22. Chalaris, A. et al. Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice. J. Exp. Med. 207, 1617–1624 (2010).

23. Ghosh, H.S., McBurney, M. & Robbins, P.D. SIRT1 negatively regulates the mammalian target of rapamycin. PLoS ONE 5, e9199 (2010).

24. Bröer, A. et al. Impaired nutrient signaling and body weight control in a Na+ neutral amino acid cotransporter (Slc6a19)-deficient mouse. J. Biol. Chem. 286, 26638–26651 (2011).

25. Farkas, S. et al. Rapamycin decreases leukocyte migration in vivo and effectively reduces experimentally induced chronic colitis. Int. J. Colorectal Dis. 21, 747–753 (2006).

26. Reinsch, W. et al. A multicenter, randomized, double-blind trial of everolimus versus azathioprine and placebo to maintain steroid-induced remission in patients with moderate-to-severe active Crohn’s disease. Am. J. Gastroenterol. 103, 2284–2292 (2008).

27. Mastroianni, J.R. & Ouellette, A.J. Alpha-defensins in enteric innate immunity: functional Paneth cell alpha-defensins in mouse colonic lumen. J. Biol. Chem. 284, 27848–27856 (2009).

28. Salzman, N.H. et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nature Immunol. 11, 76–83 (2010).

29. Weichhart, T. Mammalian target of rapamycin: a signaling kinase for every aspect of cellular life. Methods Mol. Biol. 821, 1–14 (2012).

30. Abu-Qurshin, R. et al. Crohn’s disease associated with pellagra and increased excretion of 5-hydroxyindolacetic acid. Am. J. Med. Sci. 313, 111–113 (1997).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank all members of our laboratories for discussions. T. Hashimoto was supported by grants from the European Respiratory Society, EuGeneHeart, and SENSHIN. T.P. was supported by a Marie Curie IIF. J.M.P. was supported by grants from IMBA, the Austrian Ministry of Sciences, the Austrian Academy of Sciences, GEN-AU (AustroMouse), an EU ERC Advanced Grant, and the EU network grants EuGeneHeart, ApsSys and INFLA-Care. P.R. was supported by the BMBF Network ‘Systematic genomics of chronic inflammation’, the DFG Cluster of Excellence Inflammation at Interfaces, SPP1399, SFB877, and the DFG project RO1394.

Author Contributions T. Hashimoto and T.P. performed most experiments, together with J.T., H.I., M.P., V.S., R.H. and T. Hanada; A. Rehman, P.R., S.L. and S.S. performed sequencing and analysis of the gut microbiome. S.M.R.C., D.S, F.V. and H.C. analysed collectrin and ACE2 expression in gut and kidney. A. Richter and B.W. performed amino acid analysis in serum. K.K. and A.F. provided essential mouse strains. J.M.P. and P.R. coordinated the project, and together with T. Hashimoto and T.P. wrote the manuscript and designed the experiments.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.M.P. (josef.penninger@imba.oeaw.ac.at) or P.R. (p.rosenstiel@mucosa.de).
transplantation, caecal and ileum extracts were pooled from broad spectrum antibiotics ampicillin (1 g l\textsuperscript{-1}) in drinking water ad libitum for the experimental days 1–7 followed by normal drinking water until the end of the experiment. For mixed C57BL/6 \textit{x} CBA background mice, colitis was induced by feeding mice 5\% (w/v) DSS. The DSS solution was made fresh every day. TNBS colitis was induced by intrarectal administration of 0.1 ml TNBS (5 mg per mouse in 40\% ethanol), through a feeding needle (Fine Science Tools). 3.5 cm proximal to the anal verge as described previously\textsuperscript{34}. Body weight, diarrhoea, and occult blood in stool (Hemoccult test) were determined daily. Diarrhoea scores were: 0, no diarrhoea; 1, mild diarrhoea; 2, severe watery diarrhoea; 3, mild diarrhoea with blood; 4, severe watery diarrhoea with blood. Colon length was determined at the end of the experiments. Crypt scores, inflammation scores, and disease activity indexes were determined as previously described\textsuperscript{13}. For PFD experiment, normal chow (20.8\% protein) and PFD (33\% protein) were obtained from Ssniff GmbH. Mice were fed the protein-free, iso-osmolar diet (Sigma) in their drinking water (0.4 g l\textsuperscript{-1}) until the end of the experiment. For rapamycin (RAPA) treatment, mice were fed the rapamycin (2 mg kg\textsuperscript{-1} d\textsuperscript{-1}; LC Laboratories) or vehicle administered intraperitoneally (i.p.) daily for 10 days with or without rapamycin (Rapa). The solvent for rapamycin was 0.2\% sodium carboxymethylcellulose, 0.25\% polysorbate-80 in water. For tryptophan rescue experiments, standard food was supplemented with glycyl-L-tryptophan (Gly-Trp) hydrate (G0144, TCI Europe) (10 mg per g dry food) daily for 10 days followed by 1.5\% (w/v) DSS treatment. Tryptophan-free diet was purchased from Oriental Yeast and was fed for 18 days before DSS challenge. For antibody treatment, 4-week-old mice were placed on broad spectrum antibiotics ampicillin (1 g l\textsuperscript{-1}) and neomycin (0.5 g l\textsuperscript{-1}) in drinking water for 4 weeks followed by 1.5\% (w/v) DSS treatment. For microbiota transplantation, caecum and ileum extracts were pooled from Ace2 mutant mice and Ace2 expressing littermates, suspended in 3 ml PBS, and administered (0.1 ml per mouse) immediately to sterile-packed 4-week-old Swiss-Webster germ-free mice (Taconic). Transplanted mice were maintained in sterile cages for 2 weeks followed by 5\% (w/v) DSS treatment. For ACE2 reconstitution, mice received intraperitoneal injections of recombinant mouse ACE2 protein (0.1 mg per mouse), and were injected intraperitoneally (i.p.) daily during 1.5\% (w/v) DSS treatment.

**Serum amino acid analyses.** Heparinized serum was mixed with an equal part of a solution containing 20\% (m/v) DSS in their drinking water. Serum amino acid analyses were performed using the automated Ventana system. Sections were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution; Jackson ImmunoResearch) for 20 min, washed three times in TBST, and visualized with enhanced chemiluminescence. Small intestine was collected for visual analysis.

**Western blotting and ELISA.** Western blotting was performed with standard protocols using isolated small intestinal epithelial cells. Primary antibodies reactive to phosphorylated S6 (Ser240/244; Cell Signaling), SLP (Ser10; Cell Signaling), and β-actin (Sigma) were used. Blots were washed three times in TBST for 30 min, incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution; Jackson ImmunoResearch) for 20 min, washed three times in TBST, and visualized with enhanced chemiluminescence. Small intestine was collected for ELISA assays to phosphorylated p70S6K or total p70S6K (DY896, DYC896; R&D) and colonic tissue was collected to determine angiostatin II levels (EK002-12; Phoenix). To analyse membrane proteins, total membranes were prepared using mannitol buffer (200 mM mannitol, 80 mM HEPES, 41 mM KOH, pH 7.5 in the presence of protease inhibitor cocktail, Sigma). After homogenization of the samples using MagNA Lyser green beads (Roche), total membranes were analysed by SDS-PAGE using 12\% (w/v) acrylamide gel and transferred onto nitrocellulose membranes (Millipore). Antibodies were used: anti-Ace2 and anti-Ace (R&D Systems, 1:1,000 dilution; Jackson ImmunoResearch) and α-tubulin (1:10,000; Sigma). Repetitive blots were used: anti-α-tubulin and anti-β-actin (Sigma, 1:10,000). Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrates (Millipore) and chemiluminescence visualized with a Diana III camera (Raytest, Dietikon).

**Histology and immunohistochemistry.** For histological analysis, 2–4-μm-thick sections were stained with haematoxylin and eosin. Immunohistochemistry was performed using the automated Ventana system. Sections were incubated with antibodies against phosphorylated S6 (Cell Signaling), Ace2 (R&D), K67 (Cell Signaling), and cleaved caspase-3 (Cell Signaling). Sections were then incubated in 0.1% trypsin (Quanta Bio) for 5 min, and cleaved caspase-3 staining was performed using a multimarker detection system (UltraMap) at 4 \times 50\% of total magnification. The following primary antibodies were used: anti-Ace2 and anti-Ace (R&D Systems, 1:1,000, anti-BAT1 (Pineda, 1:1,000), anti-mouse β-actin (Sigma, 1:10,000). Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrates (Millipore) and chemiluminescence visualized with a Diana III camera (Raytest, Dietikon).
and a four base key (lower case) were added. The reverse primer (5'- CGTA TGCCCTCCCTCCGCGCA AtcagXXXXXXXXXCATGCTGCCTCCGCTAGGA GT-3') contained the 454 Life Sciences primer A sequence (bold) a unique 10 base multiplex identifier (MIDs designated as XXXXXXXXXX) to tag each PCR product, the broad-range bacterial primer 338R, and a 'CA' linker sequence inserted between the MIDs and the RNA gene primer. In addition, a four base key (lower case) was added before the MIDs as described earlier.\textsuperscript{35} Replicate PCR reactions were performed for each sample and the respective negative controls. Each reaction consisted of 1 μl (10 pmol μl\textsuperscript{-1}) of each primer, 40 ng of DNA, 1 μl of dNTPs mix (10 mM stock), 1.0 units of Phusion Hot Start II High-Fidelity DNA Polymerase, and 10 μl of 5× Phusion HF buffer. The final volume of reaction was adjusted to 50 μl. Amplification was performed using an initial denaturation of 3 min at 98 °C followed by 30 cycles, denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. Final extension was at 72 °C for 10 min. Amplified products were run on an agarose gel, specific bands excised and amplicons purified using the Qiagen gel purification kit (Qiagen). Concentrations of the eluted and purified amplicons were measured using the Quant-IT PicoGreen dsDNA Assay Kit (Invitrogen). Equal amounts of PCR products were mixed in a single tube and sequenced using Roche 454 Titanium chemistry. To generate negative control templates for the PCR reaction, water was taken as a sample during DNA extraction; in all cases, the PCR reactions from these templates were negative.

**Sequence analysis.** After pyrosequencing, all sequence reads were screened and filtered for quality and length using PANGEA.\textsuperscript{36} Sequences were trimmed and homopolymers. Remaining sequences were aligned using the curated SILVA seed database (bacterial reference alignment) as a template with the Needleman-Wunsch algorithm.\textsuperscript{37} Sequences not matching with defined core regions and putative chimeraic sequences were detected by using the uchime\textsuperscript{38} command implemented in Mothur\textsuperscript{39} and were subsequently removed. Sequences were further confirmed to originate from bacteria using the Ribosomal Data base project (RDP) classifier with 60% bootstrap threshold.\textsuperscript{40} For all the downstream analysis, 1,000 sequences per samples were randomly sampled to normalize the number of reads distribution. In order to reduce the overestimation of distances between sequences, leading and trailing periods from aligned files were removed. Pairwise distances between sequences were calculated, gaps of any length were treated as single event or mismatch. Sequences with ≥97% similarity were clustered using average neighbour algorithm in to species level operational taxonomical units (OTUs).\textsuperscript{41} Phylogenetic affiliation of each OTU was performed by using RDP taxonomy and a template file. To visualize the microbial composition and structure in relation on host genotype or treatment, distance matrices between samples were generated based on weighted (Bray–Curtis similarity) and non-weighted (unweighted UniFrac) algorithms and visualized using principal coordinate analysis (PCoA). OTUs contributing for the shifting of the samples along the two axes were ascertained by calculating the Spearman correlation of each OTU with the two axes in the PCoA.\textsuperscript{42} Similarity percentage analysis (SIMPER)\textsuperscript{42} was performed to determine the driving OTUs imparting significant differences among bacterial community composition among genotype and diet groups. The analysis was performed on Bray–Curtis similarity coefficient with the program PAST (Paleontological Statistics, ver. 2.14).\textsuperscript{43}

**Bone marrow transplantation.** Eight week old Ace2 mutant and Ace2 wild-type mice were lethally irradiated with 980 cGy (Gammacell 40). Bone marrow cells were harvested from 8–9-week-old donor Ace2 mutant or Ace2 wild-type mice by flushing the femurs and tibia with DMEM. Recipient mice received 5 × 10\textsuperscript{6} bone marrow cells in 250 μl DMEM from male donor mice by tail vein injection 6 h after irradiation. Six weeks after transplantation, the genotypes of haematopoietic cells were determined by PCR using peripheral blood. The PCR primer set for the mutant Ace2 allele was as follows: 5′-CCAGCCTATTCCCTCCACTC-3′ and 5′-CCGGCTGCTTATTGAGAGGACA-3′. The PCR primer set for wild-type Ace2 was: 5′-CTCTATGGCTCCGTCTTCTTAGC-3′ and 5′-CCGGCTGCTCTTTGAGAGGACA-3′.

**Calorimetry cages.** Average food intake was measured using indirect calorimetry cages from TSE Systems over a period of seven days following 48 h of acclimatization. Data were analysed with proprietary Phenomaster Analysis Software (TSE Systems) and Microsoft Excel.

**Statistical analyses.** All data are shown as mean ± s.e.m. Measurements at single time points were analysed by ANOVA and if significant, further analysed by a two-tailed t-test. P < 0.05 was considered to indicate statistical significance.

31. Ito, M. et al. Regulation of blood pressure by the type 1A angiotensin II receptor gene. Proc. Natl Acad. Sci. USA 92, 3521–3525 (1995).
32. Kuba, K. et al. Impaired heart contractility in Apelin gene-deficient mice associated with aging and pressure overload. Circ. Res. 101, e32-e42 (2007).
33. Ishida, J. et al. Regulatory roles for API, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. J. Biol. Chem. 279, 26274–26279 (2004).
34. McCafferty, D. M., Mlambo, M., Shiota, E., Sharkey, K. A. & Kubbes, P. Role of inducible nitric oxide synthase in trinitrobenzene sulphonic acid induced colitis in mice. Gut 45, 864–873 (1999).
35. Rehman, A. et al. Nod2 is essential for temporal development of intestinal microbial communities. Gut 60, 1354–1362 (2011).
36. Giongo, A. et al. PANGEA: pipeline for analysis of next generation amplicons. ISME J. 4, 852–861 (2010).
37. Puusu, E. et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188–7196 (2007).
38. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200 (2011).
39. Schloss, P. D. et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541 (2009).
40. Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of RNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267 (2007).
41. Schloss, P. D. & Handelsman, J. Status of the microbial census. Microbiol. Mol. Biol. Rev. 68, 686–691 (2004).
42. Clarke, K. R. Nonparametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143 (1993).
43. Hammer, Ø., Harper, D. A. T. & Ryan, P. D. Past: paleontological statistics software package for education and data analysis. Paleontologia Electronica 4, art. 4 (2004).