Intracellular MLCK1 diversion reverses barrier loss to restore mucosal homeostasis

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Epithelial barrier loss is a driver of intestinal and systemic diseases. Myosin light chain kinase (MLCK) is a key effector of barrier dysfunction and a potential therapeutic target, but enzymatic inhibition has unacceptable toxicity. Here, we show that a unique domain within the MLCK splice variant MLCK1 directs perijunctional actomyosin ring (PAMR) recruitment. Using the domain structure and multiple screens, we identify a domain-binding small molecule (divertin) that blocks MLCK1 recruitment without inhibiting enzymatic function. Divertin blocks acute, tumor necrosis factor (TNF)-induced MLCK1 recruitment as well as downstream myosin light chain (MLC) phosphorylation, barrier loss, and diarrhea in vitro and in vivo. Divertin corrects barrier dysfunction and prevents disease development and progression in experimental inflammatory bowel disease. Beyond applications of divertin in gastrointestinal disease, this general approach to enzymatic inhibition by preventing access to specific subcellular sites provides a new paradigm for safely and precisely targeting individual properties of enzymes with multiple functions.

Epithelial barriers are essential for survival. Barrier dysfunction, which characterizes many diseases, can occur by two distinct mechanisms. The first, cellular damage, results in catastrophic barrier loss. A second, more nuanced mechanism reflects increased permeability of the tight junctions that seal the paracellular space1. This selectively permeable seal is regulated by MLCK and MLC phosphorylation within the PAMR2-4. MLCK isoforms expressed in various epithelia and smooth muscle (visceral and vascular) are encoded by a single gene, MYLK, and have identical catalytic and calmodulin-binding regulatory domains (Fig. 1a)4,5. The approximately 210 kDa long MLC expressed in epithelia includes additional 5’ exons that encode six amino-terminal immunoglobulin-like cell adhesion molecule (IgCAM) domains; these are absent in smooth muscle (short) MLCK (Fig. 1a). Intestinal epithelia express two splice variants of long MLCK, MLCK1 and MLCK2 (Fig. 1a), but do not express short MLCK. These long MLCK variants differ by a single exon that is only present in MLCK1 and completes IgCAM3-10.

Long MLCK-deficient mice are viable, develop normally9, and are protected from acute lipopolysaccharide- and ventilator-induced lung injury, acute TNF-induced intestinal barrier loss and diarrhea, and chronic, immune-mediated colitis11,12. In contrast, short MLCK knockout mice die soon after birth13. Thus, although long MLCK could be an attractive therapeutic target, systemic toxicity associated with enzymatic MLCK inhibition limits the utility of this approach.

In this study, we report an alternative strategy for the therapeutic inhibition of long MLCK-dependent barrier loss. We show that MLCK1, but not MLCK2, is recruited to the PAMR in response to pathogenic stimuli. Using an in silico structure-based screen, we have identified a small molecule that binds to IgCAM3 and prevents stimulus-induced MLCK1 recruitment to the PAMR. This molecule, termed divertin because it diverts MLCK1 from the PAMR, does not interfere with MLCK enzymatic activity, epithelial cell wound repair, or smooth muscle contraction. However, it prevents MLCK-mediated intestinal barrier loss in vitro and in vivo, restores barrier function in spontaneous colitis, and attenuates experimental, immune-mediated colitis. We conclude that IgCAM3-dependent MLCK1 recruitment to the PAMR is a viable target for therapeutic preservation of epithelial barrier function in intestinal disease and may also be beneficial in pathophysiology affecting other organs.

Results

Inflammatory stimuli induce long MLCK1 trafficking to the PAMR. In the human small intestine, MLCK1 expression is restricted to villous enterocytes, where it is concentrated at the PAMR and with the apical cytoplasm just beneath the brush border (Fig. 1b). MLCK2 is expressed throughout the crypt-villus axis and comprises the remainder of intestinal epithelial MLCK. The diffusely distributed cytoplasmic pool detected by antibodies against total MLCK, but not MLCK1-specific antisera, suggests that this pool represents MLCK2 (Fig. 1b).

To directly compare MLCK1 and MLCK2 localization, MLCK1-enhanced green fluorescent protein (EGFP) and MLCK2-EGFP were expressed in Caco-2 cells, human intestinal epithelial monolayers. Both splice variants were present in the cytoplasm and in association with the PAMR.

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with lateral membranes (Fig. 1c). However, MLCK1-EGFP demonstrated significantly greater localization within the apical PAMR relative to MLCK2 (Fig. 1d; \( P < 0.05 \)). Moreover, TNF, which activates MLCK-dependent phosphorylation of perijunctional MLC2, markedly increased the PAMR-associated pool of MLCK1 that began within 0.5 h of TNF addition and continued to 4 h (Fig. 1e).

To determine if MLCK1 recruitment to the PAMR occurs in vivo, the intestines of immunodeficient mice with colitis induced by adoptive transfer of CD4+CD45RBhi naïve effector T cells were examined. In colonocytes from these mice, MLCK1 was mostly concentrated within the apical cytoplasm where it formed a distinct line at the level of the PAMR (Fig. 1f; lower panel, arrow). In contrast, MLCK1 was distributed diffusely within the colonocytes of healthy mice (Fig. 1f; upper panel). Thus, MLCK1 is recruited to the PAMR during chronic immune-mediated disease.

Identification and analysis of potential MLCK1 IgCAM3-targeted small molecules. The 69 amino acids encoded by exon 8, which complete IgCAM3, are the only difference between MLCK1 and MLCK2. Therefore, we hypothesized that a small molecule that binds to IgCAM3 might interfere with MLCK1 recruitment to the PAMR. To identify such a molecule, we solved the crystal structure of IgCAM3 to 2.5 Å (Fig. 2a and Supplementary Table 1). Overall, IgCAM3 formed a Greek key \( \beta \)-sandwich that is characteristic of the IgCAM superfamily. The overall structure was also similar to that reported for telokin, which forms the IgCAM9 of long and short MLCK1.

Structural and sequence data from all IgCAMs of human and mouse MLCK1 were used to select a potential drug-binding pocket: (1) was comprised of residues that are unique to IgCAM3 (Fig. 2a); (2) was conserved between mouse and human MLCK1 (Fig. 2b); and (3) had a suitable, surface-accessible, hydrophobic core (Extended Data Fig. 1a). A library of approximately 140,000 molecules was docked in silico and the compounds with the lowest predicted binding energy were obtained for functional testing (Extended Data Fig. 1b).

As an initial, medium-throughput biological screen, we assessed the ability of small molecules to reverse physiological, MLCK-dependent tight junction permeability increases. Activation of Na+-glucose cotransport reduced transepithelial electrical resistance (TER), a sensitive measure of paracellular permeability, in Caco-2BBe monolayers (Fig. 2c). A highly specific MLCK inhibitor, membrane permeant inhibitor of myosin light chain kinase (PIK), reversed the effects of Na+-glucose cotransport on barrier function. Small molecules with very low (left group) and intermediate (right group) predicted \( \Delta G \) of binding to IgCAM3 were tested.

![Fig. 1] Long MLCK1 is specifically recruited to the PAMR in response to inflammatory stimuli. a. Protein domain structure of MYLK gene products. The IgCAM domains are numbered from the amino terminus. Long MLCK1 is expressed as two splice variants, MLCK1 and MLCK2, in intestinal epithelial cells. Short MLCK is expressed in smooth muscle. b. Normal human jejunum stained for long MLCK1 (green), total MLCK (red), and nuclei (blue). Bar, 50 \( \mu \)m. The inset of the boxed region is shown on the bottom right and includes an arrow indicating the PAMR. Bar, 10 \( \mu \)m. Images are representative of more than 10 independent experiments. c. Caco-2BBe monolayers expressing MLCK1-EGFP or MLCK2-EGFP were primed with IFN-\( \gamma \) followed by treatment with TNF for 4 h. Images are representative of more than 12 independent experiments. Bar, 10 \( \mu \)m. d. MLCK1-EGFP or MLCK2-EGFP colocalization with F-actin at the PAMR was determined. For this experiment, which is representative of 4 independent studies, \( n = 8 \) biologically independent samples with 3-5 fields analyzed for each condition. \( * P < 0.05 \); \( ** P < 0.01 \) by Kruskal–Wallis test with Dunn’s multiple comparison test. e. Monolayers were treated with IFN-\( \gamma \) and/or TNF, as indicated, before immunostaining for endogenous MLCK1 and F-actin. PAMR localization of endogenous MLCK1 was determined. The data show the fraction of total MLCK1 localized to the PAMR and are therefore independent of absolute MLCK1 expression, which increases in response to TNF. For this experiment, which is representative of 4 independent studies, \( n = 3 \) biologically independent samples with 3-4 fields analyzed for each condition. \( ** P < 0.01 \) by ANOVA with Dunn’s multiple comparison test. The mean ± s.e.m. is shown. f. Colon sections from healthy mice (control) or those with T cell transfer colitis (colitis) were stained for MLCK1 (green) and nuclei (blue). The intensity of MLCK1 staining in colonocytes from healthy control mice is enhanced to allow direct comparison with diseased colonocytes, which have increased MLCK1 expression. The arrows indicate the position of the PAMR, where a distinct line of MLCK1 can be detected in colonocytes from colitic, but not control, mice. Bar, 5 \( \mu \)m. Images representative of more than 6 independent experiments are shown.
Three molecules from the very low ΔG group, NSC31211, NSC159456, and NSC55937, but none from the intermediate ΔG group, increased the TER of monolayers with active Na⁺-glucose cotransport to an extent similar to PIK (Fig. 2c and Supplementary Table 2). In addition to identifying three molecules that passed this screen, this result suggests that the ΔGs predicted by the in silico model are relevant in vitro.

Enzymatic MLCK inhibition could be a simple explanation for the effects of small molecules on TER. To evaluate this, the effects on intestinal epithelial MLCK activity were tested in a cell-free assay. Several small molecules, including one of the three that increased TER (NSC159456), inhibited MLCK activity (Fig. 2d and Supplementary Table 2). Thus, of the small molecules that enhanced barrier function, only NSC31211 and NSC55937 passed this screen.

As inhibition of MLCK-dependent smooth muscle contraction, which is essential for normal gastrointestinal motility and regulation of vascular tone, would represent an unacceptable toxicity, we assessed the effect of small molecules on the contraction of human smooth muscle cells. Consistent with the observed MLCK enzymatic inhibition, NSC42233 and NSC294786 limited contraction (Fig. 2e and Supplementary Table 2). NSC31211 and NSC487770, which did not inhibit MLCK enzymatic activity, also reduced smooth muscle-dependent gel contraction. Therefore, NSC55937 was the only small molecule that increased TER without inhibiting MLCK enzymatic activity or smooth muscle contraction (Supplementary Table 2).

Further, NSC55937 did not interfere with epithelial wound closure (Fig. 2f), which requires intact MLCK function.

Consistent with our initial hypothesis that a small molecule bound to IgCAM3 might prevent MLCK1 recruitment, NSC55937 markedly reduced the PAMR-associated MLCK1 fraction (Fig. 2g). In contrast, MLCK enzymatic inhibition did not affect MLCK1 localization. Because NSC55937 diverted MLCK1 from the PAMR, it was designated ‘divertin.’

To directly measure divertin binding, we took advantage of the single tryptophan (W447) buried within IgCAM3 (Extended Data Fig. 2a). Divertin caused a dose-dependent red shift in peak 

**Fig. 2 | Identification and preliminary characterization of small molecules that bind to IgCAM3.**

**a.** Crystal structure of human IgCAM3. The colors indicate a low (blue) or high (red) degree of amino acid similarity to other human long MLCK1 IgCAM domains. The box encloses the putative small molecule binding pocket, which includes a hydrophobic region that is poorly conserved between IgCAMs (Extended Data Fig. 1a). **b.** Crystal structure of human IgCAM3. The colors indicate identical (gold) or non-identical (purple) residues relative to mouse MLCK1 IgCAM3. The putative binding pocket is identical in humans and mice. **c.** TER of Caco-2 monolayers treated with 20 μM Y27632, 150 μM PIK, or 250 μM small molecule drugs. **d.** Representative autoradiograms of in vitro MLCK activity shown as γ³₂P-ATP phosphorylation of recombinant MLC in the presence of PIK or small molecules. n = 3 biologically independent samples within this experiment, which is representative of 3 independent experiments. The mean ± s.d. is shown. ***P < 0.001 by ANOVA with Dunn’s multiple comparison test. **e.** Human aortic smooth muscle cells embedded in rat tail collagen were treated with 20 μM Y27632, 150 μM PIK, or 250 μM small molecule drugs. Gel contraction was quantified after 24 h. Data are representative of n = 4 biologically independent samples. *P < 0.05; ***P < 0.001 by ANOVA with Dunn’s multiple comparison test. **f.** Wound recovery in confluent Caco-2 monolayers. Bar, 500 μm. Micrographs shown are of representative n = 5 biologically independent samples in each of 3 independent experiments. The mean ± s.d. is shown. *P < 0.05; ***P < 0.001 by ANOVA with Dunn’s multiple comparison test. **g.** Confuent Caco-2 monolayers treated with 150 μM PIK or 250 μM NSC55937 were stained for endogenous MLCK1 (green) and F-actin (red). Arrows within the images indicate the PAMR. Bar, 5 μm. The fraction of total MLCK1 localized to the PAMR is shown. For this experiment, which is representative of 4 independent studies, n = 8 biologically independent samples with 3–5 fields analyzed for each condition. The mean ± s.d. is shown. ***P < 0.001 by ANOVA with Dunn’s multiple comparison test.
Divertin prevents acute TNF-induced MLC phosphorylation, barrier loss, and diarrhea in vivo. The in silico screen was designed to identify small molecules capable of interacting with both human and mouse MLCK1 to allow the analysis of divertin efficacy in vivo. Mouse jejunal segments were perfused in situ as described previously (Fig. 4a)\(^2\,20\). Intraperitoneal TNF administration reversed the direction of fluid flow to net secretion (Fig. 4b), induced barrier loss (Fig. 4c), and enhanced intestinal epithelial MLC phosphorylation (Fig. 4d). The addition of divertin to the perfusate in a manner topologically analogous to oral administration restored fluid absorption, prevented barrier loss, and blocked TNF-induced increases in MLC phosphorylation. Divertin did not interfere with basal fluid transport or barrier function in the absence of TNF, suggesting that it is unlikely to negatively impact epithelial homeostasis or intestinal transport under basal conditions.

Divertin prevented TNF-induced recruitment of MLCK1 to the PAMR (Fig. 4e,f), although it did not interfere with TNF-induced upregulation of MLCK1 expression\(^21\). Divertin also prevented TNF-induced phosphorylation of perijunctional MLC (Fig. 4g). Previous work has shown that caveolar endocytosis of the tight junction protein occludin is both required for and is a robust marker of TNF-induced MLCK phosphorylation-mediated barrier regulation\(^21\). Consistent with this, divertin prevented TNF-induced occludin endocytosis (Fig. 4h)\(^20\). These data indicate that divertin-mediated inhibition of MLCK1 recruitment can prevent TNF-induced tight junction barrier loss and diarrhea in vivo.

Divertin prevents TNF-induced MLCK1 trafficking, MLC phosphorylation, and tight junction reorganization in human jejunal mucosa ex vivo. To determine if divertin is also effective in the human jejenum, mucosal biopsies from normal human individuals were treated with TNF and divertin (Fig. 4i). As in intact mouse jejunum, divertin prevented TNF-induced MLCK1 recruitment to the PAMR in human jejunal enterocytes (Fig. 4j,k). However, divertin did not interfere with TNF-induced upregulation of MLCK1 expression (Fig. 4k), indicating that divertin did not simply block TNF signaling within intestinal epithelia. Divertin also limited TNF-induced perijunctional MLC phosphorylation (Fig. 4l).

Although barrier function was not measured directly, the ability of divertin to inhibit the endocytosis of tight junction-associated proteins occludin and MLCK1 is consistent with previous work, the effect of divertin reflects regulation of paracellular, rather than transcellular, transport. Finally, divertin reversed TNF-induced MLC phosphorylation (Fig. 3d). Thus, although cell-free assays showed that divertin is not a direct inhibitor of MLCK enzymatic activity, it is as effective as an enzymatic inhibitor in correcting TNF-induced MLC phosphorylation and restoring barrier function. Together, these data suggest that divertin acts by preventing MLCK1 from gaining the physical proximity required for phosphorylation of perijunctional MLC (Fig. 3e).
Doses did not affect survival or behavior but induced weight loss for nine or three days, respectively, had no effect on survival after terms of weight loss or behavior, over 31 d (Fig. 5a). Daily 50 mg kg doses did not affect survival or behavior but induced weight loss (data not shown).

To better characterize potential toxicity, histopathology and epithelial turnover were assessed in the small intestine and colon after divertin treatment. No divertin-induced histopathology was evident on hematoxylin and eosin (H&E)-stained sections. Moreover, divertin had no effect on epithelial turnover; the numbers of 5-ethyl-2'-deoxyuridine (EdU)-labeled cells were similar in the small intestine and colon (Fig. 5b) of mice treated with 12.5 mg kg day or 25 mg kg day of divertin for 31 d. Thus, divertin does not appear to have systemic, mucosal, or epithelial toxicity over this relatively short period of exposure.

Divertin limits the development of experimental inflammatory bowel disease (IBD). These data indicate that divertin can prevent acute inflammatory diarrhea when given before disease onset and is not toxic when given daily at a dose of 25 mg kg. To determine if divertin can also prevent the initiation and subsequent progression of chronic disease, we used a T cell transfer model. We have previously reported that intestinal epithelial MLCK activation accelerates disease progression and, conversely, intestinal epithelial MLCK knockout delays onset and lessens disease severity in this IBD model. Mice were treated with daily intraperitoneal injections of divertin or saline (vehicle) beginning 14 d after adoptive transfer, before the onset of symptoms (Extended Data Fig. 3a). Mice treated with saline developed disease activity and began to lose weight one week later (day 21), but this was delayed by nearly two weeks, to day 33, in mice that received divertin (Extended Data Fig. 3a,b). Divertin also prevented mortality in this colitis model (Extended Data Fig. 3c).

As expected, disease progression in saline-treated mice was associated with increased intestinal permeability to 4 kDa dextran (28 Å diameter), but barrier function was largely preserved in divertin-treated mice (Extended Data Fig. 3d). Consistent with reduced disease, mucosal TNF production was also lower in divertin-treated mice (Extended Data Fig. 3e). Finally, the efficacy of divertin in preventing disease development was evident on gross examination as reduced colonic shortening and thickening (Extended Data Fig. 3f) and on microscopic examination by reduced inflammation and mucosal hyperplasia (Extended Data Fig. 3g,h). Therefore, divertin can limit the initial development of experimental IBD.

Divertin acutely restores barrier function in spontaneous immune-mediated colitis. We used the interleukin-10 (IL-10) knockout mouse model of spontaneous colitis to determine whether divertin could restore intestinal barrier function in chronic disease. Intestinal permeability was assessed in IL-10 knockout mice with mild disease and reassessed one week later in the same mice following a single 25 mg kg dose of divertin. Intestinal permeability to fluorescein, a sensitive marker of tight junction barrier loss due to its small size (hydrodynamic diameter = 8 Å), in IL-10 knockout mice was 2.9 ± 0.3-fold that of wild-type controls, consistent with the presence of disease (Fig. 5c). The recognized variability of disease severity in IL-10 knockout mice is reflected in the significantly greater range of intestinal permeability of IL-10 knockout mice relative to wild-type controls (Fig. 5c).

Divertin collapsed the range of permeabilities and reduced the absolute fluorescein permeabilities in IL-10 knockout mice such that they were similar to wild-type mice (Fig. 5c). Overall, divertin reduced intestinal permeability in 9 of 11 IL-10 knockout mice.
but only 4 of 8 wild-type mice (Fig. 5c). These data indicate that divertin can restore intestinal barrier function in a mechanistically relevant IBD model.

**Divertin limits the severity of experimental chronic IBD.** The disease variability inherent in IL-10 knockout mice makes it difficult to use this model to test the efficacy of divertin as therapy for established disease. Therefore, we returned to the T cell transfer model (Fig. 6a), where disease is relatively uniform in each mouse. Like human disease, this model is responsive to anti-TNF therapy. By day 18 after transfer, all mice that had received T cells displayed softened stool, weight loss, and modest increases in clinical scores.
Discussion

Epithelial barrier loss is a critical component of acute and chronic gastrointestinal diseases, including infectious enterocolitis, food allergy, celiac disease, and IBD. This relationship is further emphasized by the observations that intestinal epithelial barrier loss in healthy individuals is linked to IBD risk alleles and that barrier loss during remission is a prognostic marker of disease reactivation in Crohn's disease. Experimental models further show that intestinal epithelial barrier dysfunction precedes the onset of enterocolitis, that increased intestinal permeability enhances IBD progression, and that barrier restoration limits IBD pathogenesis.

Although the molecular mechanisms that trigger intestinal permeability defects are incompletely defined, non-muscle MLCK activation is a convergence point for many pathophysiological stimuli. MLCK is also implicated in barrier dysfunction in other organ systems and in vascular endothelia; therefore, it represents a potential target for therapeutic intervention.
therapeutic target. However, the catalytic domains of non-muscle and smooth muscle MLCK are identical; thus enzymatic inhibitors that target one will target both. This would be a source of substantial toxicity, since smooth muscle MLCK inhibition results in hypotension, intestinal obstruction, and death. Further, the most widely used non-muscle and smooth muscle MLCK inhibitors, ML-7 and ML-9, inhibit many other kinases, including cardiac and skeletal muscle MLCK, rho-associated protein kinase 2, 5′ AMP-activated protein kinase, and dual specificity tyrosine-phosphorylation-regulated kinase 1, at concentrations that fail to inhibit smooth and non-muscle MLCK completely. Together with recognition that MLCK serves critical roles in epithelial migration and wound repair, among other functions, enzymatic MLCK inhibition is unlikely to be achievable without toxicity.

In the current study, we demonstrate an alternative therapeutic strategy for preventing MLCK-dependent barrier dysregulation in acute and chronic intestinal disease. We show that a specific long MLCK splice variant, MLCK1, is recruited to the PAMR where it mediates TNF-induced intestinal epithelial tight junction regulation. Taking advantage of the unique IgCAM3 within MLCK1, we screened for compounds that bind to this domain. Secondary screens identified molecules that could enhance barrier function and eliminated those that inhibited MLCK enzymatic activity or smooth muscle contraction. One small molecule, termed divertin, binds to this unique IgCAM domain and prevents MLCK1 recruitment to the PAMR without interfering with epithelial migration and wound repair.

To assess the effects of divertin in intact tissue, we used an established in vivo mouse model of TNF-induced, MLCK-dependent diarrhoea and acute TNF-induced MLCK activation leading to tight junction reorganization in human jejunal mucosa ex vivo. In both tissues, divertin prevented MLCK1 recruitment to the PAMR and subsequent phosphorylation of perijunctional MLCK. Further, divertin prevented TNF-induced diarrhoea and restored net fluid absorption in mouse jejunum in vivo. This functional preservation correlated directly with maintenance of occludin at the tight junction pools, that is, inhibition of endocytosis. This data confirm the necessity of depleting occludin from the tight junction to drive TNF-stimulated barrier loss and diarrhoea; previous studies have shown that occludin overexpression, which augments tight junction occludin pools, or direct, genetic, or pharmacological inhibition of occludin endocytosis limits TNF-induced barrier loss and diarrhoea in mice.

Thus, while we were unable to measure transport, the efficacy of divertin in blocking occludin endocytosis in human intestinal biopsies can be taken as de facto evidence that paracellular barrier function and transepithelial fluid transport were preserved. Thus, divertin is equally effective in an in vivo mouse model and an ex vivo human model of acute, TNF-induced tight junction barrier loss. Moreover, these data indicate that divertin has no effect on epithelial morphology, tight junction structure, barrier function, or fluid transport in the absence of stimuli but can prevent the development of TNF-induced barrier loss. Finally, when given before clinical presentation, divertin limited experimental IBD development. These data indicate that divertin might be safely used prophylactically, for example, as a maintenance therapy in IBD.

In vitro, divertin reversed both the morphological and functional sequelae of acute TNF-induced MLCK activation, including perijunctional MLCK phosphorylation, tight junction reorganization, and barrier loss. Divertin had a similar effect in vivo, where a single dose restored intestinal barrier function in colitic IL-10 knockout mice. These data suggest that continuous divertin treatment might be effective in established disease. To assess this, mice received T cell transfer, but therapy was not initiated until day 19, after weight loss, disease activity, cytokine production, and histopathological features of experimental IBD were established. In this model, divertin was compared directly to treatment with anti-TNF antibodies, which have revolutionized therapy in human IBD. Consistent with previous work, anti-TNF treatment provided only partial protection from T cell transfer, colitis-associated weight loss and mortality. In contrast, divertin limited weight loss and mortality. Thus, by these measures, divertin was superior to anti-TNF treatment. Because they have different mechanisms of action, we hypothesized that divertin and anti-TNF treatment might have additive effects. However, combination therapy was not significantly superior to divertin alone by any measure.

The efficacy with which divertin prevents barrier loss and restores function suggests that it may also be useful in other diseases with epithelial and vascular endothelial tight junctions dysfunction, including celiac disease, atopic dermatitis, pulmonary infection and acute respiratory distress syndrome, graft versus host disease, and multiple sclerosis. Moreover, the molecularly targeted approach defined in this study may have much broader relevance since recruitment to specific subcellular locations is essential for the function of proteins involved in diverse cellular activities including mitosis, membrane trafficking, cytokoskeletal regulation, and ion transport. Disruption of site-specific recruitment may allow...
certain processes to be blocked without affecting other essential functions. Therefore, our characterization of divertin demonstrates a novel means of enhancing therapeutic specificity without many of the toxicities that often plague less precisely targeted inhibitors. In summary, we have shown that the long MLCK splice variant MLCK1 is recruited to the intestinal epithelial tight junction in response to inflammatory stimuli. We used a structure-based drug discovery approach to identify a small molecule that blocks MLCK1 recruitment, corrects acute inflammatory barrier loss and diarrhea, and prevents chronic immune-mediated colitis. In addition to the potential of this specific agent, this approach to kinase inhibition by limiting substrate accessibility, rather than enzymatic activity,
represents a new paradigm for enhancing specificity of small-molecule therapies.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41591-019-0393-7.

Received: 19 February 2018; Accepted: 8 February 2019; Published online: 1 April 2019

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Author contributions
J.R.T. conceptualized the study. W.V.G., W.H., A.M.M., J.Z., G.S., H.-S.L., A.B., M.L.D.M.O., Z.-H.J., W.C., H.Z., Yitang Wang, J.G., J.W., H.J.R., Yingmin Wang, and J.R.T. carried out the investigations. S.B.S., D.O., S.C.M., L.W.M., and J.R.T. managed the resources. H.Z., J.G., and D.O. managed the software. W.V.G., W.H., W.C., and J.R.T. carried out the visualization. W.V.G. and J.R.T. wrote the original manuscript draft. All authors reviewed and edited the draft. J.R.T. and W.H. acquired the funding. These pairs of authors contributed equally: W.V.G. and W.H., and A.M.M. and J.Z.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41591-019-0393-7.
Supplementary information is available for this paper at https://doi.org/10.1038/s41591-019-0393-7.
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Methods

Cloning and vector construction. The human MLCK1 IgCAM3 domain sequence was selected based on the previously solved structure of IgCAM9, also known as telokin (Protein Data Bank code: 1tkl)\(^1\). Oligonucleotides were designed based on the human MLCK1 complementary DNA (cDNA) sequence (GenBank accession no. AA290962). The PCR products were ligated into pETBlue-1 (EMD Millipore) and confirmed for sequencing. Amino acid substitution to create mutant IgCAM3 (IgCAM3 Leu449Arg, Gln457Lys, Asp481Val) was done by mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Oligonucleotides were: Leu449Arg sense 5′-TGAAGTGCCGGT GCTGAGCA GAGGGACCC GTGA-3′; Leu449Arg antisense 5′-TCAGG GGGTGCCTT CTCTGAAACA GGCACCTACA-3′; Gln457Lys sense 5′-CCCGCTG AGGAGGAGG AAGAGGCACTG TG-3′; Gln457Lys antisense 5′-CAATGC TGCCCTTCC TTTCCCTCAG GGGG-3′; Asp481Val sense 5′-GAAAG CCGCGGACCC GGGAGAATGG GGCATA CGGCTG-3′; Asp481Val antisense 5′-CAGCTGT ATGTCACCCT ACCTGGTCGCG GGGTCTC-3′. MLCK1-EGFP and MLCK2-EGFP fusion protein constructs were generated using MLCK1 (AY42470.1) and MLCK2 (AY424269.1) cDNA cloned from Caco-2 cells and fused at the C terminal to monomeric EGFP (after removing the stop codon). These were cloned into piggyBAC-TREtight plasmids and stably expressed in Caco-2BBe cells, as described\(^4\). All constructs were verified by direct sequencing.

Recombinant protein expression and purification. Constructs were transformed into BL21-CodonPlus (DE3)-RIPL Competent Escherichia coli (Stratagene) cells for isopropyl β-D-thiogalactopyranoside (IPTG)-induced expression. Cells were grown to an A\(_{600}\) of 0.5−0.9 at 37 °C and IPTG was added to a final concentration of 1 mM. Cultures were grown for an additional 3 h at 37 °C. After induction, bacteria were collected by centrifugation (6,000 × g) and sonicated. SDS–PAGE confirmed expression. Solubilized protein was initially passed through a series of centrifugal filtration devices (Amicon) to remove debris and insoluble protein. These were then cloned into piggyBAC-TREtight plasmids and stably expressed in Caco-2 cells, as described\(^4\). All constructs were verified by direct sequencing.

Crystallization and data collection. The purified MLCK1 IgCAM3 domain was concentrated to 6 mg/ml. Crystals of the MLCK1 IgCAM3 domain were obtained from the NeXtal PEGs screen (Qiagen) condition F7 (200 mM ammonium dihydrogen phosphate (pH 6.0), 5% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol, 4°C). Crystals were mounted in cryoloop and frozen in liquid nitrogen. Data were collected at the Advanced Light Source, beamline 5.2.2, wavelength 1.0 Å, at 100 K. The X-ray structure was determined by molecular replacement using MrBump\(^5\) as implemented in the CCP4 version 7.0.04 program suite\(^6\). Refinement and model building were performed with BUSTER version 2.11.7\(^7\) and Coot version 0.8.8\(^8\), respectively, using data to 2.5 Å, with 98.2% in preferred and allowed regions of the Ramachandran plot. Data collection and refinement statistics appear in Supplementary Table 1.

Molecular docking and acquisition of potential IgCAM3 interacting molecules. The NCI Developmental Therapeutics Program chemical library was filtered for compounds that met the Lipinski’s rule of five. The three-dimensional coordinates for the NCI’s Developmental Therapeutics Program set of 139,735 compounds were downloaded from the ZINC database. All docking calculations were performed with DOCK 6 (University of California, San Francisco) and run by parallel processing on 16 central processing units of a Linux cluster at the University of Florida’s High Performance Computing Center. The general features of DOCK include rigid orientation of ligands to receptor spheres, AMBER energy terms, generalized Born/surface area solvation scoring, contact scoring, internal non-bonded energy scoring, ligand flexibility, and both rigid and torsional simplex minimization. To prepare the site for docking, all water molecules were removed. Protonation of receptor residues was performed with DockPrep in Chimera 1.12 (University of California, San Francisco). The receptor model was created using sets of spheres to describe potential binding pockets using SPHGEN. The number of orientations per molecule was 100. Intermolecular AMBER energy scoring (van der Waals + cumbic), contact scoring, and bump filtering were implemented in DOCK 6. Chimera was used to generate the molecular graphics images. Top scoring non-proprietary compounds and designated compounds were obtained from the Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health. Each compound was solubilized in dimethylsulfoxide at 10 mM and stored at −20 °C.

Divertin synthesis. An aqueous formalin solution (0.2 mol, 37% w/v) was added dropwise to hydrazine (0.75 equiv) at room temperature or 0.15 mol at 0 °C. Stirring was continued for 20 min at room temperature; then, the reaction mixture was left to stand at room temperature for 3 d. The crude product was filtered and washed with hot propan-2-ol to yield the product, octahydro(1,2,4,5)tetrazino(1,2-a)(1,2,4,5)tetrazine, as a white powder. The yield was 18%. The carbon, hydrogen and nitrogen elemental analysis yielded the following: C 32.70%; H 8.28%; N 56.60%. The proton (1H) nuclear magnetic resonance (500 MHz, deuterium oxide) was 3.61 (d, J = 11.5 Hz, 2H) and 3.32 (d, J = 11.5 Hz, 2H).

Epithelial cell culture and TER measurement. Caco-2 cells were grown on collagen-coated polycarbonate membrane Transwell permeable supports (Corning) and used 17–20 d after confluence, as described previously\(^9\). Electrophysiological measurements were made using agar plugs and Ag/AgCl current electrodes. A 50 μA current was passed across the monolayer at a model 558 voltage clamp (University of Iowa Biomedical Engineering) to measure potential differences before and during the application of a 50 μA current. The transepithelial short-circuit current (Isc) and TER were calculated with Ohm’s law, as described\(^10\).

For the drug screens (Fig. 2c), it was not possible to test all drugs in a single experiment. All trials included monolayers with active Na+–glucose cotransport (where MLCK is active)\(^11\) and inhibited Na+–glucose cotransport, which blocks MLCK activation and typically produces an approximately 20% increase in TER\(^12\). After measuring TER, small molecules (250 μM) were added to the apical surface (in the presence of active Na+–glucose cotransport) and the TER was measured after 60 min. The TER values for each monolayer after drug treatment were normalized to those before the drug was added. To control for differences in monolayer responsibility between experiments, a scale was developed using monolayers with active Na+–glucose cotransport (set to 0%) and inhibited Na+–glucose cotransport (set to 100%) for two-point calibration. The enzymatic MLCK inhibitor PIK, which increases TER similarly to inhibition of Na+–glucose cotransport, was included in each experiment. The scaled values shown for active and inhibited Na+–glucose cotransport, PIK treatment, and drug treatments are taken from multiple experiments.

For experiments examining cytokine-treated monolayers (Fig. 3c), IFN-γ (10 ng/ml)\(^13\) and PeproTech was added to the basolateral chamber 1 h before TNF addition to induce TNF receptor expression\(^14\). Preliminary experiments showed that this did not affect TER. TER was then measured and TNF (1 or 2.5 ng/ml)\(^15\) was added to the basolateral chamber (at t = 0) without manipulating the apical media, as described\(^16\). Drugs were added to the apical chamber only. The TER of each monolayer was normalized, at each time point, to the starting TER (before TNF addition) of that monolayer.

Tryptophan fluorescence. N-acyl-L-tryptophanamide (NATA), recombinant IgCAM3, or mutant IgCAM3 were added to the phosphate buffer (10 mM potassium phosphate, pH 6.7, 130 mM NaCl) in a quartz cuvette. Samples were then analyzed on a FluoroMax-3 scanning fluorimeter (Horiba) at 25°C using an excitation wavelength of 296 nm (1-nm bandwidth) and recording the emission spectra from 315 to 400 nm.

Animals. All experiments were performed in an Association of Assessment and Accreditation of Laboratory Animal Care-accredited facility under protocols approved by the University of Chicago, Brigham and Women’s Hospital, Boston Children’s Hospital, and Soocho University Animal Care and Use Committees. All studies are in compliance with all relevant ethical regulations. For the acute studies, 7–10-week-old C57BL/6 mice (The Jackson Laboratory) received intraperitoneal injections of vehicle or 5 mg recombinant mouse TNF as described previously\(^17\). In vivo perfusion analyses were performed as described previously\(^18\). Sections of jejunum were collected and snap-frozen in optimal cutting temperature media.

129S1/SvImJ and 129(B6)-Il10tm1Cgn (The Jackson Laboratory) were used as controls and IL-10 knockout mice, respectively, at 7–10 weeks of age. Mice received intraperitoneal injections of vehicle or divertin (25 mg/kg) in 5% (v/v) Tween 80 with 250 μl of 10 mg/ml fluorescein or 80 μg/ml fluorescein isothiocyanate (FITC)-4KDa dextran solution. Mice were bled from the tail vein 3 h later and recovery was assessed using a fluorescent plate reader. For analysis of epithelial turnover, mice received 0.1 mg EdU\(^19\) 16 h before being killed.

Adoptive transfer colitis was established in 6-week-old C57BL/6 Rag\(^{-/-}\) or C57BL/6 Rag\(^{-/-}\) xollorb\(^{-/-}\) mice (The Jackson Laboratory) by intravenous injection of 500,000 CD4\(^+\)CD25\(^-\) or CD4\(^+\)CD45RB\(^-\) T cells. Disease activity was scored from 0 to 2 each for motor activity, fur texture, posture, and diarrhea (0–8). For the preventative experiments, divertin was given 14 d after T cell transfer and treatment was initiated 4 d after adoptive transfer, mice had reproducible disease characterized by reduced weight (relative to the mice that did not receive T cells), increased stool water, and other clinically evident features of disease. The latter were generally too mild to warrant injections of vehicle or divertin, so inflammation was monitored daily until mice were scheduled to be killed at that time (to confirm disease) or to receive daily 200 μl intraperitoneal injections of saline or drugs beginning on day 19. Drug schedules were 25 mg kg\(^{-1}\).
divertin each day or 150 μg rat anti-mouse TNF clone XT3.11 (catalog no. BP0058, Bio X Cell) every 3 d. Mice receiving only anti-TNF received saline on the other days. For mice receiving combination treatment, divertin and anti-TNF were combined in a single intraperitoneal injection on days when both were given. Cytokines were measured in homogenates of distal colon by multiplex enzyme-linked immunosorbent assay (Quansys Biosciences). Histopathology was scored on well-oriented colonic cross sections stained by H&E and scored semiquantitatively from 0 to 3 each for lymphoid infiltrates, polymorphonuclear infiltrates, mucosal hyperplasia, and ulceration (total 0–12), as described previously.

**Human tissue.** Mucosa was removed from normal small intestine tissue excised during bariatric surgery and incubated in Hank's Balanced Salt Solution with 300 ng ml−1 recombinant human TNF, divertin (250 μM), or both, as indicated, for 60 min. Tissue was then snap-frozen in optimal cutting temperature media. De-identified, dissected human tissues were provided under an exempt protocol that did not require informed consent. All procedures involving human tissues were approved by the University of Chicago institutional review board; all relevant ethical regulations were followed.

**Immunoblotting.** Lysates of Caco-2 Δα5 monolayers or jejunal epithelial cells, isolated as described previously, were separated by SDS–PAGE (Bio-Rad Laboratories), transferred to polyvinylidene difluoride membranes, and blotted using affinity-purified rabbit anti-p-MLC (Ser19) antibody (catalog no. 3671, Cell Signaling Technology) and a monoclonal mouse anti-β-actin clone AC-15 (catalog no. A-178, Sigma-Aldrich) followed by peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and enhanced chemiluminescent detection.

**β-Cell Signaling Technology) and a monoclonal mouse anti-MLCK clone K36 antibody (1 μg ml−1; catalog no. M7905, Sigma-Aldrich), affinity-purified rabbit anti-p-MLC (Ser19) antibody (1 μg ml−1; catalog no. 3671, Cell Signaling Technology), monoclonal mouse anti-occludin clone OC-3F10 (1 μg ml−1; catalog no. 331500, Invitrogen), monoclonal mouse anti-α-adducin clone M168 antibody (2 μg ml−1; catalog no. Ab76055, Abcam), or monoclonal rat anti-CD3 clone CD3-12 antibody (2 μg ml−1; catalog no. 11089, Abcam). Primary antibodies were followed by Alexa Fluor 488- or 594-conjugated, multiply adsorbed, affinity-purified secondary antibodies (Jackson ImmunoResearch) along with Alexa Fluor 594-conjugated phallolidin (Invitrogen) and Hoechst 33342 (Invitrogen). Stained sections were mounted in ProLong Gold Antifade Mountant (Invitrogen).

**Microscopy.** Specimens were imaged using an epifluorescence microscope (DM4000; Leica) equipped with 4.6 diaminobenzo-2 phenylindole, Endow GFP, and Texas red zero-pixel shift filter sets (Chroma Technology), a 63× 1.32 numerical aperture immersion objective, and a camera (CoolSNAP HQ2; Roper Scientific) controlled by MetaMorph version 7. Z-stacks were collected at 0.2 μm intervals and deconvolved using AutoDeblur version X1 (Media Cybernetics) for 10 iterations. Line scans (100 pixels wide) were performed using MetaMorph version 7. For formalin-fixed, paraffin-embedded specimens stained as part of tissues sections were cut and fixed in 1% paraformaldehyde. EdU was detected using the Click-iT RNA Alexa Fluor 488 Imaging Kit (Invitrogen). Immunostaining used affinity-purified rabbit anti-human MLCK1 antibody (2 μg ml−1; clone K36), monoclonal mouse anti-MLC36 clone K36 antibody (1 μg ml−1; catalog no. M7905, Sigma-Aldrich), affinity-purified rabbit anti-p-MLC (Ser19) antibody (1 μg ml−1; catalog no. 3671, Cell Signaling Technology), monoclonal mouse anti-occludin clone OC-3F10 (1 μg ml−1; catalog no. 331500, Invitrogen), monoclonal mouse anti-α-adducin clone M168 antibody (2 μg ml−1; catalog no. Ab76055, Abcam), or monoclonal rat anti-CD3 clone CD3-12 antibody (2 μg ml−1; catalog no. 11089, Abcam). Primary antibodies were followed by Alexa Fluor 488- or 594-conjugated, multiply adsorbed, affinity-purified secondary antibodies (Jackson ImmunoResearch) along with Alexa Fluor 594-conjugated phallolidin (Invitrogen) and Hoechst 33342 (Invitrogen). Stained sections were mounted in ProLong Gold Antifade Mountant (Invitrogen).

**Image analysis.** For the analysis of MLCK1 or MLCK2 localization in cultured monolayers (Figs. 1d, 2g, and 3b), the PAMR was defined using labeled phallolidin. ImageJ was used to generate a region of interest (ROI) around the entire cell (just encompassing the entire cell). Each pixel represents a molecule of DNA present in one monolayer field; n represents the number of independent biopsy fragments or mice analyzed for human or mouse tissues, respectively. MLC phosphorylation was quantified using MetaMorph version 7. The counting of Edu-labeled nuclei and CD3+ T cells was performed manually.

**In vitro kinase assay.** Confluent Caco-2 Δα5 monolayers expressing MLCK1 and MLCK2 were used as the source of MLCK kinase. After dilution in kinase reaction buffer (20 mM 3- phosphopropylphospho-1-sulfonic acid, pH 7.4, 2 mM MgCl2, 0.25 mM CaCl2, and 0.2 μM calmodulin), small molecule inhibitor compounds obtained from the NCI or PK was added to the reactions, as indicated. The reaction was initiated by the addition of γ[32]ATP (ICN) and 5 μM recombinant MLCK and transfigured at 30°C. MLCK phosphorylation was determined by autoradiography after SDS–PAGE. All experiments were performed at least three times, with triplicate or greater samples in each experiment.

**Gel contraction assay.** Human aortic smooth muscle cells (Invitrogen) were maintained in Medium 231 supplemented with smooth muscle growth supplement. Collagen lattices were prepared by mixing, on ice, collagen I (2 mg ml−1) in 0.1% acetic acid with supplemented Medium 231 and 0.1 mM NaOH to neutralize the pH before adding a cell suspension, as described previously. After polymerization (30 min at 37°C), gels were detached and covered with supplemented Medium 231, with or without small molecule inhibitor compounds obtained from the NCI as indicated. After 24 h, lattices were photographed and the percentage gel contraction was calculated using ImageJ.

**Wound closure assay.** Confluent Caco-2 Be monolayers were wounded with a suction pipette, treated with 20 μM Y27632, 150 μM PKI, or 250 μM NSC59397 (divertin) and recovery was measured immediately after injury and after 72 h of recovery. Closure of each wound is shown relative to the initial wound size. As shown previously, inhibition of either rho kinase or MLCK limited repair.

**Statistical analysis.** Statistical significance (P) was determined with a paired t-test, unpaired t-test with Welch’s correction, two-tailed Kruskal–Wallis test, analysis of variance (ANOVA) with Dunst’s multiple comparison test, ANOVA with Bonferroni correction, ANOVA with Tukey’s multiple comparison test, or Gehan-Breslow–Wilcoxon test, as indicated in each figure legend.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All requests for raw and analyzed data and materials are promptly reviewed to verify if the request is subject to any intellectual property or confidentiality obligations. Human participants were de-identified and no further data are available. Any data and materials that can be shared will be released via a Material Transfer Agreement. The crystal structure data are available as Protein Data Bank code: 6C6M (https://www.rcsb.org/structure/6C6M).

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Extended Data Fig. 1 | IgCAM3 drug-binding pocket and in silico candidate identification. a, Crystal structure of human IgCAM3. The colors indicate hydrophobic (blue) or hydrophilic (orange) residues. b, Predicted ΔG scored for the 139,735 molecules docked into the region of small molecule binding. Two groups of molecules with either very low or intermediate ΔG are indicated by symbols that coincide with those in Fig. 2c. The inset shows the IgCAM3 binding pocket (red), with predicted docking of selected small molecules.
Extended Data Fig. 2 | Divertin binds to recombinant IgCAM3.  
a. IgCAM3 shown as a ribbon diagram with the location of tryptophan 447 (W447) and three residues in the putative drug-binding pocket, leucine 449 (L449), glutamine 457 (Q457), and aspartic acid 481 (D481).  
b. Changes in peak fluorescence of wild-type IgCAM3, mutant IgCAM3, and NATA in the presence of increasing concentrations of NSC55937 reveal a shift in the maximum wavelength of wild-type IgCAM3 only.  
c. Fluorescence emission spectrum of wild-type IgCAM3 in the presence of increasing NSC55937 concentrations demonstrates dose-dependent tryptophan fluorescence quenching and a red shift in the maximum emission wavelength, indicative of NSC55937 binding to IgCAM3. NSC55937 was used at 0 μM (red), 10 μM (orange), 33 μM (yellow), 100 μM (green), 333 μM (blue), and 1000 μM (violet).  
d. Mutant IgCAM3 (Leu449Arg, Gln457Lys, and Asp481Val) abolishes the ability of NSC55937 to quench IgCAM3 tryptophan fluorescence across all concentrations of divertin.  
e. Fluorescence emission spectra of the tryptophan analog (1 mM) in the presence of increasing concentrations of NSC55937. No fluorescence quenching occurred. Data are representative of three or more independent experiments with similar results.
Extended Data Fig. 3 | Divertin delays the development of experimental inflammatory bowel disease. a, Fourteen days after T cell transfer, mice were treated with daily intraperitoneal injections of saline (green) or divertin (red). n = 10 independent animals per condition. The mean ± s.e.m. is shown. **P < 0.01 by unpaired, two-sided t-test with Welch’s correction. b, Divertin-treated mice were protected from the weight loss experienced by saline-treated (green) mice. n = 10 independent animals per condition. the mean ± s.e.m. is shown. **P < 0.01 by unpaired, two-sided t-test with Welch’s correction. c, Divertin significantly increased survival during adoptive transfer colitis. n = 10 independent animals per condition. *P < 0.05 by Gehan–Breslow–Wilcoxon test. d, Intestinal permeability on day 56. Data are normalized to recovery from a healthy wild-type mouse. n = 7 (saline), n = 10 (divertin). **P < 0.01 by unpaired, two-sided t-test with Welch’s correction. e, Mucosal TNF on day 56 was significantly reduced by divertin treatment (red). n = 7 (saline), n = 10 (divertin). *P < 0.05 by unpaired, two-sided t-test with Welch’s correction. f, Colon lengths on day 56. Images of representative colons are shown, with their lengths corresponding to the labels on the y axis. n = 7 (saline) or n = 10 (divertin) independent animals per condition. **P < 0.01 by unpaired, two-sided t-test with Welch’s correction. g, Colonic histopathology scores on day 56. n = 7 (saline) or n = 10 (divertin) independent animals per condition. **P < 0.01 by unpaired, two-sided t-test with Welch’s correction. h, Histopathology shows crypt loss (asterisk) and crypt abscesses (arrow) in the mucosa from a saline-treated mouse and partial goblet cell preservation in the mucosa from a divertin-treated mouse (arrowhead). Bar, 50 μm. The insets show complete cross sections of colon, with the boxes indicating the areas shown at higher magnification. Bar, 250 μm. Images are representative of three independent experiments with similar results. The experiment shown in this figure used female mice as T cell donors and immunodeficient recipients. Results were similar in two independent studies that, in combination, included nine saline-treated and nine divertin-treated male mice.
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- A list of figures that have associated raw data
- A description of any restrictions on data availability

All requests for raw and analyzed data and materials are promptly reviewed by to verify if the request is subject to any intellectual property or confidentiality obligations. Human subjects were de-identified and no further data are available. Any data and materials that can be shared will be released via a Material Transfer Agreement. Crystal structure data are available as dataset ID D_1000232147 and PDB ID 6C6M.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

- sample sizes were based on previous experience with these types of experiments. the analysis after each experiment verifies that these were sufficient

**Data exclusions**

- no data were excluded

**Replication**

- All data are representative of two or more independent experiments. Specifics are provided in each figure legend. All attempts at replication were successful.

**Randomization**

- Allocation was random. For Figures 6 and Extended Data 3, mice that received T cell transfer were allocated into treatment groups in order to equalize weight loss between groups.

**Blinding**

- Investigators were blinded to group allocation during data collection and analysis wherever possible. This was not possible during real-time treatment of live animals in mouse studies (Fig 5, 6), as the treatment of each mouse would need to be known to the person handling the mice. Investigators were, however, blinded during subsequent imaging and histological analyses.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | Involved in the study | Involved in the study |
| ☐ Antibodies | ☐ | ☐ ChIP-seq |
| ☐ Eukaryotic cell lines | ☑ | ☑ Flow cytometry |
| ☑ Palaeontology | ☑ | ☑ MRI-based neuroimaging |
| ☑ Animals and other organisms | ☑ | | |
| ☑ Human research participants | ☑ | | |
| ☑ Clinical data | | | |

**Antibodies**

**Antibodies used**

- Polyclonal anti-human MLCK1 were made by us as rabbit anti-peptide antibodies and purified by antigen-affinity chromatography (Clayburgh et al. J Biol Chem, 2005).
- Polyclonal anti-phosphomyosin light chain antibodies were from Cell Signaling Technologies (catalog 3671).
- Monoclonal anti-total myosin light chain kinase (clone K36) was used (Sigma, catalog M7905).
- Monoclonal mouse anti-occludin clone OC-3F10 (Invitrogen, catalog 33-1500).
- Monoclonal mouse anti-occludin clone M168 (Abcam catalog 76055).
- Monoclonal rat anti-CD3 clone CD3-12 (Abcam catalog ab11089).
- Monoclonal anti-actin (clone AC-15) was from Sigma (catalog A5441).

**Validation**

- Anti-MLCK1 was validated by western blot against recombinant MLCK1 and MLCK2 and showing that it only reacts with MLCK1 (Clayburgh et al. J Biol Chem, 2005).
- The commercial phosphoMLC antibodies gave results nearly identical to a polyclonal rabbit anti-phosphomyosin light chain antisera that we made years ago and validated in numerous ways, including direct correlation with P32 incorporation (Berglund et al. 2001).
- K36 has been validated in detail using western blot and by functional depletion of myosin light chain kinase activity from intestinal epithelial cell lysates (Clayburgh et al. J Biol Chem, 2005).
- Occludin antibodies were validated using occludin KD cell lines and occludin KO mice.
- Monoclonal mouse anti-E-cadherin clone M168 (Abcam catalog 76055) was validated by western blot and immunohistochemistry.
- Monoclonal rat anti-CD3 clone CD3-12 (Abcam catalog ab11089) was validated using Rag1 KO mice.
- All other antibodies were validated by western blot or other means (e.g. colocalization with F-actin or other antibody stains).
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | A Caco-2BBe line expressing SGLT1 was used in these studies. This line was initially reported by us in 1996 (Turner et al., J Biol Chem). |
|---------------------|----------------------------------------------------------------------------------------------------------------------------------|

Authentication

| Authentication       | The cell line was authenticated on the basis of morphology, Na-dependent glucose uptake, basal barrier function, and barrier regulation by nutrients, PIK, and cytokines. |
|----------------------|----------------------------------------------------------------------------------------------------------------------------------|

Mycoplasma contamination

| Mycoplasma contamination | Cells were tested for mycoplasma contamination quarterly and were negative throughout the course of these studies. |
|--------------------------|----------------------------------------------------------------------------------------------------------------------------------|

Commonly misidentified lines (See ICLAC register)

| Commonly misidentified lines | None were used. |
|-----------------------------|-----------------|

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Mice were C57BL/6J, 129S1/SvImJ, and 129(B6)-Il10tm1Cgn/J mice, as indicated. All were used at 6 - 10 weeks of age, depending on the experiment, as indicated in the methods. Male and female mice were used in all studies (usually in separate experiments). |
|-------------------|----------------------------------------------------------------------------------------------------------------------------------|

Wild animals

| Wild animals | None used |
|--------------|-----------|

Field-collected samples

| Field-collected samples | None used |
|-------------------------|-----------|

Ethics oversight

| Ethics oversight | All experiments were performed in an Association of Assessment and Accreditation of Laboratory Animal Care–accredited facility under protocols approved by The University of Chicago, Brigham and Women’s Hospital, Boston Children’s Hospital, and Soochow University Animal Care and Use Committees. All studies are in compliance with all relevant ethical regulations. |
|-------------------|----------------------------------------------------------------------------------------------------------------------------------|

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

| Population characteristics | Jejunal mucosa was harvested from de-identified, discarded tissue resected during bariatric surgery. The study used only de-identified, discarded tissue. Other than obesity (the reason for the surgery), no clinical information is available. The subjects were healthy enough to undergo elective surgery which suggests that, with the exception of obesity, these were normal and otherwise healthy individuals. |
|---------------------------|----------------------------------------------------------------------------------------------------------------------------------|

Recruitment

| Recruitment | There was no recruitment. |
|-------------|---------------------------|

Ethics oversight

| Ethics oversight | De-identified, discarded human tissues were used under an exempted protocol that did not require informed consent. All procedures involving human tissues were approved by The University of Chicago Institutional Review Board and all relevant ethical regulations were followed. |
|-----------------|----------------------------------------------------------------------------------------------------------------------------------|

Note that full information on the approval of the study protocol must also be provided in the manuscript.