Pharmacologic activation of HNF4α/γ Restores Epithelial Barrier Function in Crohn's disease.

Short Title: Targeting HNF4 restores barrier function in Crohn's disease.

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Grant Support: Supported by NIDDK (DK-98120, DK-117476 to S.K.) and the Public Health Service (DK-056338).

Disclosure: The authors declare no conflicts of interest.

Abbreviations used in this paper: BH, permeability barrier healing; CD, Crohn's disease; CLDN, claudin; CLE, confocal laser endomicroscopy; DKO, double knockout; ER, endoscopic remission; GWAS, genome-wide association study; HNF, hepatocyte nuclear factor; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; MH, mucosal healing; PDO, patient-derived organoid; SNP, single-nucleotide polymorphism; TEER, transepithelial electrical resistance; TJ, tight junction; TNF, tumor necrosis factor; UC, ulcerative colitis; WT, wild-type.

Word Count: 7000

Author Contributions: Seema Khurana, Amin Ghazi and Debasish Halder, conceived the experiments. Amin Ghazi and Debasish Halder conducted the experiments. Jason Ken Hou provided human tissue samples and his clinical expertise in IBD. Michael P. Verzi, provided the *Hnf4α/γ*^{DKO} and control mouse organoids and provided both technical and intellectual support. Yaohong Wang, a gastrointestinal pathologist with expertise in IBD analyzed the immunohistochemistry data and provided intellectual support. All authors reviewed the manuscript.

Data Transparency: Data, analytic methods, and study material will be made available to other researchers.

Abstract.

Background and aims. Intestinal permeability barrier dysfunction is a hallmark of Crohn's disease (CD) that occurs before inflammation, persists during remission, and predicts relapse. Despite its clinical importance, no validated biomarkers or therapies directly target epithelial barrier restoration in CD. We investigated whether hepatocyte nuclear factors HNF4α and HNF4γ regulate tight junction (TJ) organization and barrier integrity in CD, and whether pharmacologic HNF4 activation can restore epithelial barrier function.

Methods. To investigate the role of HNF4α/γ in TJ regulation and CD pathogenesis, we integrated transcriptomic data from the small intestinal epithelium of *Hnf4α/γ* double knockout (DKO) mice, with functional analyses of genetic (*Hnf4α/γ* double knockout) and inflammatory ($Tnf^{\Delta ARE/+}$) mouse models, human CD tissues, and patient derived organoids (PDOs). Barrier function was assessed by FITC-dextran flux and transepithelial electrical resistance (TEER). Two plant-derived, non-toxic HNF4 agonists, N-trans-caffeoyltyramine (NCT) and N-trans-feruloyltyramine (NFT), were tested for therapeutic rescue of barrier function in CD.

Results. Combined loss of *Hnf4α/γ* impaired epithelial barrier function by activating both pore and leak pathway, resulting in selective permeability to 4-10 kDa tracers. Integration with our transcriptomic data from the *Hnf4α/γ* double knockout (DKO) mice revealed broad repression of TJ-associated genes, including *Tjp2* (encoding ZO-2), *F11r* (encoding JAM-A), *MarvelD3*, *Cldn4*, *Cldn7*, and *Cldn15*, together with induction of the pore-forming gene *Cldn2*. HNFα/γ proteins were markedly downregulated in CD intestinal epithelium and in PDOs established from non-inflamed mucosa, establishing that the barrier defect is epithelial-intrinsic and independent of

inflammation. In contrast, chronic TNF overexpression phenocopied this suppression through a normally transient epithelial injury response that became pathologically stabilized under sustained cytokine signaling in *Tnf*$^{\Delta ARE/+}$ mice. Treatment of both CD PDOs and *Tnf*$^{\Delta ARE/+}$ mouse organoids with NCT or NFT reactivated HNF4α/γ expression, normalized TJ gene profile, and restored TEER to near-control levels.

Conclusion. HNF4α/γ are primary transcriptional regulators of epithelial TJ integrity whose loss defines a primary, inflammation-independent barrier defect in CD. Chronic inflammation can mimic but does not initiate this intrinsic epithelial dysfunction. Pharmacologic activation of HNF4α/γ with plant-derived, non-toxic, translationally ready agonists (NCT and NFT) restore barrier function, establishing the first barrier-healing therapy for CD.

Key words: Crohn's disease; epithelial barrier; tight junction, claudins; HNF4α/γ; permeability barrier healing.

Introduction.

Intestinal permeability barrier dysfunction is a defining feature of Crohn's disease (CD) pathogenesis that precedes inflammation, persists during remission, and predicts disease relapse and complication.[1, 2] Despite its central role in CD pathogenesis, no current diagnostic or therapeutic approaches directly assess or restore epithelial barrier function in CD. Endoscopic remission (ER) in CD, defined as the absence or resolution of epithelial erosions, ulcers, and luminal strictures, is the current clinical goal because of its association with improved outcomes.[3] However, more than 70% of CD patients in ER exhibit persistent intestinal

permeability defects that strongly predict relapse, hospitalization, and surgery.[1, 4-8] Indeed, permeability barrier healing (BH), rather than endoscopic or histological remission, is the most accurate predictor of long-term outcomes in CD, including the risk of developing penetrating and fibrostenotic complications.[1] Despite this, no reliable methods exist to monitor or therapeutically restore epithelial barrier integrity, and no validated biomarker accurately measures barrier loss *in vivo*. Current diagnostic tools such as lactulose mannitol ratio lack specificity, exhibit high inter-individual variability, fail to measure colonic permeability, and provide no spatial resolution of barrier defects.[9] Confocal laser endomicroscopy (CLE) provides spatially resolved, real-time assessment of mucosal barrier function but is costly, technically complex, and limited by restricted sampling areas that do not encompass the entire small intestine.[1, 10] Serum biomarkers such as albumin, zonulin, or C-reactive protein do not correlate with intestinal permeability in IBD.[1] Importantly, increased intestinal permeability precedes clinical disease onset in asymptomatic first-degree relatives of CD patients, demonstrating that barrier dysfunction is causally linked to CD pathogenesis.[11, 12] Identifying epithelial biomarkers and therapeutic targets for BH therefore represents a critical unmet need.

A major unresolved question in the field is why epithelial barrier loss in CD occurs independently of inflammation in patients but appears inflammation-dependent in experimental models. *In vitro* and in murine models, inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, and IL-13 activate non-muscle myosin light chain kinase (MLCK) which reorganizes the perijunctional actomyosin ring (circumferential actin belt).[13-16] Increased perijunctional actomyosin contraction promotes endocytosis of occludin and redistribution of ZO-1, a scaffolding protein that links TJs to the actin cytoskeleton, resulting in enhanced paracellular flux.[13-16] However, occludin and ZO-1, long regarded as canonical TJ components, are dispensable for

barrier formation and maintenance *in vivo*, suggesting that additional transcriptional and/or structural regulators control epithelial barrier integrity.[17, 18] In contrast, claudin (CLDN) proteins directly determine paracellular ion selectivity and permeability.[19] Altered CLDN expression, particularly downregulation of CLDN4, CLDN7 and upregulation of CLDN2, has been reported in CD patients, yet causal links to barrier defects remain undefined.[15, 20, 21] CLE imaging confirms increased epithelial permeability in CD patients, in endoscopically and histologically non-inflamed mucosa, independent of erosions or ulcerations, supporting the concept that barrier dysfunction in CD reflects an epithelial reprogramming event rather than a byproduct of inflammation.[4-6, 22-24]

Hepatocyte nuclear factors 4 (HNF4) are epithelial-enriched transcription factors critical for differentiation, metabolism, and regeneration. The two mammalian paralogs, HNF4α and HNF4γ, exhibit distinct spatial expression pattern namely, HNF4α is broadly expressed throughout the intestine, whereas HNF4γ is enriched in the distal ileum, the predominant site of CD pathology.[25, 26] In the mouse intestine, HNF4A knockout (KO) does not increase the expression of HNF4G because they are largely independent.[27] Genome-wide association studies (GWAS) have identified HNF4A variants associated with ulcerative colitis (UC) and childhood-onset CD, though no single nucleotide polymorphisms (SNPs) have been described in HNF4G.[26, 28-30] While HNF4A mRNA is reduced in IBD, it remains unclear whether this translates to protein loss or functional barrier defects. Transcriptomic analyses of our *Hnf4α/γ* double knockout (DKO) mice reveal broad repression of TJ genes, including *Cldn4*, *Cldn7*, *Cldn15*, *Tjp2* (ZO-2), *F11r* (JAM-A), and *Marveld3*, with concurrent upregulation of the pore-forming *Cldn2*.[31] These data suggest that HNF4α and HNF4γ orchestrate a transcriptional program essential for TJ assembly and barrier maintenance.

Here, by integrating transcriptomic analyses of *Hnf4α/γ*-deficient epithelium with CD patient tissues and organoids, we define a broad HNF4α/γ-dependent network governing TJ organization and paracellular sealing. We further show that loss of HNF4α/γ constitutes the primary epithelial defect underlying CD, while chronic inflammation can phenocopy this loss through transient epithelial injury response. Finally, we demonstrate that pharmacologic activation of HNF4α/γ using the non-toxic, plant-derived small molecules N-trans-caffeoyltyramine (NCT) and N-trans-feruloyltyramine (NFT) fully restores barrier integrity, establishing HNF4 activation as the first epithelial-targeted, barrier-healing therapy for CD.

Methods.

Study Design. To investigate the role of HNF4α and HNF4γ in TJ regulation and CD pathogenesis, we integrated transcriptomic data from small intestinal epithelium of *Hnf4α/γ* double knockout (DKO) mice with functional analyses of genetic ($Hnf4\alpha/\gamma^{DKO}$) and inflammatory ($Tnf^{\Delta ARE/+}$) mouse models, human CD tissues, and CD PDOs. These complementary models enabled evaluation of HNF4α/γ-dependent transcriptional networks, epithelial permeability, and pharmacologic rescue of barrier function.

Mouse Model of Crohn's Disease. All animal studies were approved by the University of Houston Institutional Animal Care and Use Committee (IACUC). $Tnf^{\Delta ARE/+}$ mice, which develop CD-like ileitis due to dysregulated TNF-α expression, were obtained from Dr. Fabio Cominelli (Case Western Reserve University) and analyzed at 20 weeks of age, corresponding to peak inflammation.[32, 33] Intestinal organoids from distal ileum of $Tnf^{\Delta ARE/+}$ and littermate ($Tnf^{+/+}$) mice were used to assess the role of HNF4 paralogs in the presence of intestinal inflammation.

Intestinal organoids from *Hnf4α/γ*^{DKO} mice and littermate controls were used to assess epithelial-intrinsic role of HNF4 paralogs independent of inflammation.[27]

Human Tissue and Organoid Culture. Distal ileal biopsies were obtained from non-CD controls and from non-inflamed mucosa of patients with active ileal or ileocolonic CD under IRB-approved protocols (Baylor College of Medicine and University of Houston). Crypts were isolated by EDTA chelation and cultured in Matrigel-based medium to generate PDOs as described before.[34] These PDOs were used to assess the functional contribution of HNF4α/γ to epithelial barrier regulation and to test the effects of pharmacologic activation. At least 50 organoids per sample were analyzed for each quantitative assay.

Barrier Function Assays. Epithelial permeability was assessed using FITC-dextran flux and transepithelial electrical resistance (TEER). Mouse organoids were incubated with FITC-dextrans of 4, 10, or 70 kDa for 15, 30, or 60 minutes, and luminal fluorescence accumulation was quantified as a measure of paracellular permeability, as described before.[35] The FITC-dextrans of increasing molecular weights were added to the organoids individually. Luminal accumulation of FITC-dextran was evaluated in 15 organoids per group selected randomly. TEER was measured in non-CD and CD PDOs seeded on Transwell inserts (0.4 μm pore size, Corning) coated with 20 μg/ml collagen type 1 (rat tail) in 0.01% acetic acid. TEER was measured using an EVOM2 epithelial voltohmmeter (World Precision Instruments). Each Insert was measured three times, and the average values were corrected for background resistance and normalized to surface area.

Pharmacologic Activation of HNF4α/γ. Two plant-derived, non-toxic small molecules, NCT and NFT, were used as HNF4 agonists. Murine and human CD organoids were incubated *ex vivo* with NCT or NFT (80 μM, 12 h) and compared with vehicle (DMSO) controls. HNF4α/γ expression, TJ gene profile, and barrier function were subsequently evaluated.

Immunohistochemistry and Immunofluorescence. Immunohistochemistry for HNF4α and HNF4γ was performed on paraffin embedded distal ileum, colon, and rectal tissue sections from CD patients with ileal or ileocolonic CD and non-CD controls as described before.[36] Images were captured with an Olympus BX53 microscope. Mouse and human organoids were fixed and permeabilized with 3.7% paraformaldehyde and 0.3% Triton X-100 followed by incubation with primary and secondary antibodies. The organoids were imaged on a Fluoview FV2000 laser scanning confocal microscope with a 60X NA 1.35 objective. Fluorescence intensity was generated using ImageJ.

Statistical Analysis. All data are presented as mean ± SEM from at least 6 independent experiments. Statistical significance was determined using unpaired two-tailed Student's t-test. $P < 0.05$ was considered significant.

Results.

HNF4α/γ are essential for tight junction integrity and epithelial barrier function. RNA-sequencing of intestinal epithelium from *Hnf4α*$^{f/f}$, *Hnf4γ*$^{Crispr/Crispr}$, and the *Hnf4α/γ*^{DKO} mice revealed that only the combined loss of both paralogs triggers broad transcriptional remodeling of TJ genes.[37] This expression pattern is consistent with increased paracellular flux and suggests

that HNF4α/γ coordinate a transcriptional program essential for epithelial barrier maintenance. To directly assess their functional contribution, we performed size-selective FITC-dextran permeability assays in 3D intestinal organoids derived from the small intestine of wild-type (WT) and *Hnf4α/γ*^{DKO} mice. Because HNF4α/γ regulate fatty acid oxidation, an essential pathway for intestinal stem cell renewal, *Hnf4α/γ*^{DKO} mouse organoids were cultured in medium supplemented with 25 mM acetate to compensate for acetyl-CoA deficiency.[27] *Hnf4α/γ*^{DKO} mouse organoids were then incubated with FITC-dextrans of increasing molecular weights (4, 10, and 70 kDa), and luminal fluorescence accumulation was quantified as a measure of paracellular penetration. In wild-type (WT) mouse organoids, FITC-dextran was excluded from the lumen up to 30 min, consistent with slow diffusion across intact epithelial TJs [Figure 1]. In contrast, *Hnf4α/γ*-deficient organoids displayed early and robust luminal accumulation of the 4 kDa and 10 kDa tracers, indicating increased permeability through the leak pathways. Importantly, 70 kDa FITC-dextran remained excluded, confirming that the increased permeability in *Hnf4α/γ*^{DKO} mice was size-selective rather than due to gross epithelial disruption [Figure 1]. These findings demonstrate that HNF4α and HNF4γ are essential for maintaining TJ integrity and small intestinal barrier function in the absence of inflammatory signaling. Consistent with the expression pattern of these transcription factors, both paralogs are co-expressed in the small intestine but not in the colon. Because permeability assays were performed using small intestinal organoids, our data specifically identify HNF4α/γ as essential regulators of small intestinal barrier integrity.

HNF4α/γ downregulation defines an epithelial-intrinsic defect in Crohn's Disease. Although HNF4 transcription factors have been implicated in IBD pathogenesis, only one prior RNA-sequencing study reported reduced HNF4A mRNA expression in ulcerative colitis (UC) and

CD.[38] Moreover, only one study has shown downregulation of HNF4G mRNA in UC patients, even though HNF4γ is undetectable in the normal adult colon.[39] To determine whether HNF4α and HNF4γ protein levels are altered in CD, we performed immunohistochemistry (IHC) analysis on biopsy specimens from the distal ileum, colon, and rectum of CD patients compared with non-CD controls. In non-CD control ileal epithelium, HNF4α exhibited strong nuclear localization in both the proximal crypt and the distal crypt , indicative of expression of both P1 and P2 isoforms of HNF4α [Figure 2A].[26] Whereas HNF4γ was detected in both the nuclei and the cytoplasm in non-CD controls [Figure 2A]. In contrast, ileal epithelium from CD patients showed a marked reduction in HNF4α nuclear staining [Figure 2A]. Strikingly, HNF4γ protein was also strongly downregulated in the same tissue samples, confirming combined loss of both paralogs in the disease epithelium [Figure 2A]. Similarly, HNF4α expression was significantly decreased in colonic and rectal biopsies from patients with ileal and ileocolonic CD compared to non-CD controls [Figure 2B]. These findings provide the first demonstration that previously reported HNF4A mRNA reduction corresponds to broad protein-level loss across intestinal regions in CD, and further reveal that HNF4γ is also suppressed, a change not captured by earlier transcriptomic data.[38] Together, these results establish pan-intestinal HNF4α downregulation and HNF4γ silencing as hallmarks of CD epithelium.

To determine whether HNF4 loss is driven by inflammation or represents an intrinsic epithelial defect, we analyzed patient-derived organoids (PDOs) generated from non-inflamed tissue of patients with active ileal or ileocolonic CD. Immunofluorescence staining revealed significantly reduced HNF4α protein levels in CD PDOs compared with non-CD controls ($p<0.0001$, n=8 patients per group) [Figure 3A]. This was accompanied by reduced CLDN15 expression, a direct target of HNF4α/γ, indicating impaired activation of HNF4-dependent TJ

program.[31] Similarly, HNF4γ protein was nearly undetectable in CD PDOs compared with robust nuclear staining in controls ($p<0.0001$, n=8 patients per group) [Figure 3B]. The magnitude of HN4α/γ loss closely mirrored findings in CD patient tissues, demonstrating that suppression of these transcriptional factors persists independently of inflammatory or stromal cues. Morphologically, CD PDOs exhibited a spheroid phenotype similar to *Hnf4α/γ*^{DKO} mouse organoids, consistent with previously reported changes in CD organoid morphology.[40, 41] Given that TJs are key regulators of epithelial differentiation, these morphological alterations likely reflect downstream consequences of TJ disruption.[42]

Chronic inflammation phenocopies HNF4α/γ suppression through a stabilized injury response. To determine whether inflammatory signaling can recapitulate HNF4 suppression, we performed immunohistochemistry (IHC) analysis on tissue from the distal ileum of *Tnf*$^{\Delta ARE/+}$ mice, which develop spontaneous Crohn's-like ileitis due to systemic TNF overexpression. In *Tnf*$^{+/+}$ control mice, HNF4α exhibited strong nuclear localization, whereas HNF4γ was detected in both the nuclei and the cytoplasm [Figure 4A]. In contrast, ileal epithelium from the *Tnf*$^{\Delta ARE/+}$ mice showed a marked reduction in HNF4α nuclear staining. Notably, HNF4γ protein was also strongly downregulated in the *Tnf*$^{\Delta ARE/+}$ mouse intestine compared to the control *Tnf*$^{+/+}$ mice [Fig 4A]. To determine whether inflammatory signaling can recapitulate HNF4 suppression *ex vivo*, we examined intestinal organoids derived from *Tnf*$^{\Delta ARE/+}$ and their control *Tnf*$^{+/+}$mice. Compared with *Tnf*$^{+/+}$ controls, *Tnf*$^{\Delta ARE/+}$ mouse organoids displayed significantly reduced HNF4α and CLDN15 protein levels ($p<0.0001$, n=6 mice per group) [Figure 4B]. HNF4γ was also markedly reduced in the *Tnf*$^{\Delta ARE/+}$ mouse organoids compared to the *Tnf*$^{+/+}$ controls ($p<0.0001$, n=6 mice per group) [Figure 4C]. Because these organoids are cultured *ex vivo* in the absence of immune and stromal

components, this persistent suppression of HNF4α/γ and their downstream TJ targets indicates that TNF-driven inflammatory signaling can imprint a stable epithelial phenotype characterized by HNF4α/γ suppression and loss of TJ proteins. However, the presence of the same defect in non-inflamed PDOs from CD patients demonstrate that HNF4α/γ downregulation and barrier dysfunction occur independently of active inflammation. Together, these data suggest that while inflammatory cytokines can initiate or exacerbate HNF4α/γ suppression, the resulting epithelial defect is self-sustaining and defines an intrinsic, inflammation-independent hallmark of CD.

Pharmacologic activation of HNF4α/γ restores TJ integrity and epithelial barrier function. HNF4α is a ligand-dependent nuclear receptor that maintains its own expression *via* autoregulatory promoter binding.[43] N-trans caffeoyltyramine (NCT) and N-trans-feruloyltyramine (NFT) are potent HNF4α agonists, that bind to the ligand-binding site of HNF4α which activates HNF4α protein and leads to an increase in HNF4α expression as shown *in vivo* in the liver and the intestine.[44, 45] Thus NCT and NFT can be used to pharmacologically modulate HNF4α expression and activity. Based on that, we elected to treat CD PDOs with NCT and NFT and evaluated HNF4α and CLDN15 expression. Both NCT and NFT significantly increased HNF4α and CLDN15 to near-control levels ($p<0.0001$, n=8 patients per group) [Figure 5A]. Most notably, even though NCT and NFT are identified as HNF4α agonists, both compounds also restored HNF4γ levels in CD PDOs [Figure 5B]. Next, we elected to treat $Tnf^{\Delta ARE/+}$ mouse organoids with NCT and NFT and determine their effect on HNF4α and CLDN-15 expression [Figure 6]. Similar rescue effects were observed in the $Tnf^{\Delta ARE/+}$ mouse organoids treated with either NCT or NFT ($p<0.0001$, n=6 mice per group) [Figure 6]. These results show that HNF4

agonists overcome both intrinsic and inflammation-induced repression of HNF4α/γ and restore expression of its downstream TJ targets.

Finally, we examined whether restoring HNF4α/γ expression is sufficient to reestablish barrier function. Transepithelial Electrical Resistance (TEER) was measured in CD PDO monolayers cultured on Transwell permeable supports and treated with vehicle (DMSO), NCT (80 μM), or NFT (80 μM) for 12 h. Treatment with NCT or NFT produced a robust TEER increase, restoring barrier function to near-control levels (**$p<0.0001$, n=8 patients per group) [Figure 7]. These data demonstrate that HNF4 agonists reverse transcriptional and structural TJ defects and re-establish epithelial permeability barrier integrity, supporting their potential as the first BH therapies for CD.

Summary: Collectively, these results define HNF4α/γ as primary transcriptional regulators of TJ architecture and paracellular barrier function. Their loss represents the primary epithelial defect underlying CD, while chronic inflammation phenocopies this repression through a stabilized injury response. Pharmacologic HNF4 activation with NCT and NFT reverses transcriptional and functional defects, establishing the first barrier-healing therapeutic strategy for CD.

Discussion.

Intestinal permeability barrier dysfunction is a defining feature of CD pathogenesis, yet whether this breakdown reflects a secondary consequence of inflammation or a primary epithelial defect has remained unresolved. While endoscopic mucosal healing (MH) is the current therapeutic goal in CD, it does not necessarily reflect restoration of epithelial barrier function.[1] Persistent permeability defects strongly predict relapse, hospitalization, and the development of

fibrostenotic or penetrating complications despite apparent MH.[1] This dissociation underscores that conventional ER fails to capture the underlying epithelial dysfunction driving disease recurrence. Our findings provide mechanistic insight into this phenomenon by identifying loss of HNF4α/γ as a central epithelial defect that underlies sustained barrier failure in CD, independent of inflammation or visible mucosal injury.[1] In this context, it is noteworthy that barrier function of the epithelium is preserved even in response to extensive epithelial cell loss, such as that triggered by UV radiation, demonstrating that mucosal injury itself does not necessarily induce barrier breakdown.[46]

This study identifies HNF4α and HNF4γ as master transcriptional regulators of epithelial TJ architecture and paracellular barrier integrity, establishing their loss as the primary epithelial defect underlying CD. By integrating transcriptomic and functional data from *Hnf4α/γ*^{DKO} mice with analyses of *Tnf*$^{\Delta ARE/+}$ mice, CD patient tissues, and PDOs, we define a conserved HNF4α/γ-dependent TJ gene network that maintains epithelial sealing and paracellular selectivity. Pharmacologic activation of HNF4α/γ with NCT or NFT restores barrier function across species and disease models, providing the first proof-of-concept for a barrier-healing therapy in CD.

Mechanistically, integration of our transcriptomic dataset with permeability measurements in *Hnf4α/γ*^{DKO} mice revealed that HNF4α/γ orchestrate a broad transcriptional network controlling TJ composition and integrity. Transcriptomic analysis of *Hnf4α/γ*^{DKO} mice revealed coordinated repression of *Pard6b*, *Prkcz*, *F11r*, *Arhgef2*, *Mag13*, *Rap2c*, *Cldn2*, *Cldn4*, *Cldn7*, *Vapa*, *Cyth1*, *Mpp5*, *Arhgap17*, *Amotl2*, *Ubn1*, *Tjap1*, *Cldn15*, *Ddx58*, *Marveld3*, *Rapgef2*, *Tjp2*, *Atp7b*, *Pals1*, *Wnk1*, *Yes1*, *Plec*, *Amotl2*, and *Apc*, genes that collectively maintain TJ organization and barrier sealing.[31] Notably, ZO-1 (*Tjp1*) remains unchanged in the *Hnf4α/γ*^{DKO} mouse intestine, whereas ZO-2 (*Tjp2*) is selectively repressed.[31] Because ZO-1 primarily modulates actomyosin

tension at adherens junctions, while ZO-2 primarily regulates actomyosin tension at TJs, selective ZO-2 loss which is linked to TJ strand fragmentation and decreased TEER, explains the increased paracellular permeability in the *Hnf4α/γ*^{DKO} mice and decreased TEER in CD PDOs.[47, 48] Concomitant downregulation of polarity regulators (*Pard6b*, *Prkcz*, *Tjap1*, *Amotl2*) and cytoskeletal scaffold genes *(Vapa, Mpp5, Arhgap17)* in the *Hnf4α/γ*^{DKO} mouse intestinal epithelium would be expected to destabilize apical-lateral polarity complexes, causing mislocalization of ZO-1, occludin, and claudins, and resulting in discontinuous TJ strands and enhanced paracellular leak.[49, 50] Reduced JAM-A (*F11r*) in the Hnf4α/γ^{DKO} mouse intestine further weakens cell-cell adhesion and junctional cohesion.[14] In parallel, suppression of small GTPase and Rho-pathway regulators (*Arhgef2, Arhgap17, Rap2c, Rapgef2, Vav2*, *Vav3*, *Bcr*, *Arhgef26*, *Arhgef18*, *Arhgef37*, *Arhgef19*, *Arhgef5*, *Arhgef16*, *Itsn1*, *Arhgef11*, *Plekhg2*, *Sos1*, *Sos2*, *Fgd4*, *Net1*, *Arhgdib*) likely impairs actin remodeling required for TJ assembly, repair, and restitution, linking HNF4α/γ loss to defective junctional remodeling and sustained permeability defects.[31, 51, 52] These results position HNF4α/γ as central regulators of TJ gene architecture and barrier sealing, linking their transcriptional activity directly to epithelial barrier function.

Functional assays confirmed that loss of HNF4α/γ activates both pore and leak pathways, resulting in size-selective permeability to 4-10 kDa solutes. HNF4α/γ deficiency also alters claudin composition in a pattern consistent with combined pore- and leak-pathway permeability. Sealing claudins (*Cldn4, Cldn7*) are repressed, while cation-selective *Cldn2* is upregulated, promoting paracellular ion leak through the pore pathway. [20, 31, 53] Simultaneous depletion of *Cldn7*, which stabilizes claudin strands and reseals transient breaks during cell extrusion and peristaltic stress, prevents closure of leak pathway. [51, 54] Downregulation of Cldn15 likely reduces sodium-coupled glucose absorption, consistent with impaired nutrient transport in IBD.[31, 55,

56] Collectively, these findings establish HNF4α/γ as principal regulators of paracellular selectivity, claudin strand density, and stability.

A key conceptual advance from this study is that barrier dysfunction in CD originates as an epithelial-intrinsic defect, rather than a consequence of inflammation or mucosal injury. Both HNF4α and HNF4γ were markedly reduced in non-inflamed CD mucosa and in PDOs derived from these regions, indicating that HNF4 loss precedes histological inflammation. These findings reconcile long-standing discrepancies between human and murine models, explaining why permeability defects persist during clinical remission and predict relapse in CD. Notably, transient repression of HNF4 is a well-characterized feature of epithelial injury and repair programs, as demonstrated in the liver, where HNF4α downregulation in response to TNF-α, IL-1β, and NF-kB promotes proliferation during regeneration, followed by reactivation to restore differentiation and homeostasis.[57, 58] However, chronic TNF-α overexpression in the *Tnf*$^{\Delta ARE/+}$ mice locks this reversible state into a sustained, pathologic repression, phenocopying the epithelial signature in CD. Thus, persistent inflammatory signaling converts an adaptive regenerative mechanism into a stable epithelial barrier defect in the *Tnf*$^{\Delta ARE/+}$ mouse model of CD. The spatial distribution of HNF4 paralogs provides a mechanistic basis for the regional localization of barrier dysfunction in CD. HNF4γ is highly enriched in the distal small intestine, particularly the terminal ileum, whereas HNF4α is expressed throughout the intestinal epithelium. Because both paralogs cooperatively maintain TJ architecture, their combined loss is required to produce a severe permeability defect, consistent with the pronounced barrier failure observed in the *Hnf4α/γ*^{DKO} mice and the predominant involvement of the ileum in CD. Dual depletion of HNF4α and HNF4γ in the ileum leads to a synergistic collapse of the TJ transcriptional network, defining the regional vulnerability

of the distal small intestine in CD. This mechanism aligns with the higher incidence of penetrating and fibrostricturing disease phenotypes in ileal *versus* colonic CD.[1, 59]

Increased permeability is also observed in healthy first-degree relatives of CD patients, underscoring an epithelial-intrinsic basis.[12, 60] The combined down-regulation of HNF4α and HNF4γ, unique to CD, disrupts the entire TJ transcriptional program, resulting in chronic, inflammation-independent permeability defects. Although HNF4γ is absent from the colon, the colonic epithelium relies primarily on HNF4α-driven programs and may engage in additional lineage-defining transcriptional factors such as GATA4, CDX2, or HNF1β to preserve epithelial identify and mitigate barrier disruption.

While epithelial barrier dysfunction also occurs in UC, its character and persistence differ markedly from CD. In UC, TJ disruption is largely inflammation-dependent and confined to actively inflamed mucosa, typically normalizing with MH.[20, 61] In contrast, barrier defects in CD are epithelial-intrinsic, focal, and persistent, detectable in endoscopically and histologically non-inflamed mucosa, and strongly predictive of relapse and disease progression. [5, 7, 12] In UC, where HNF4γ is absent from the colon, only partial loss of HNF4α occurs, producing a milder, cytokine-driven barrier defect that resolves with the attenuation of inflammation.[28, 38] This paralog-specific regulation thus provides a molecular explanation for the greater severity, chronicity, and inflammation-independence of barrier dysfunction in CD compared to UC.

Our demonstration that NCT and NFT reactivated HNF4α/γ expression and restored TJ architecture across CD PDOs and *Tnf*$^{\Delta ARE/+}$ mouse organoids provides compelling preclinical evidence that HNF4 activation is sufficient to reverse established barrier defects. Both compounds are naturally occurring hydroxycinnamic acid amides, potent HNF4 agonists, non-toxic and structurally amenable to oral delivery, supporting their translational potential.[62-64] The high

sequence identity (>80%) and nearly superimposable ligand-binding domains of HNF4α and HNF4γ explain why both paralogs responds to these agonists with parallel efficacy.[65] The plant-derived, well-tolerated nature of these compounds supports their feasibility for chronic adjunctive use as the first barrier-directed therapies that complement immune modulation and directly target the epithelial defect underlying CD persistence. Notably both agonists have been studied in animal models of liver disease and sepsis noting no evidence of toxicity from long-term oral administration.[66] Current CD therapies primarily target immune suppression and MH, yet the vast majority of patients in ER retain abnormal permeability that predicts relapse. Our findings redefine barrier healing as a measurable and targetable endpoint, with HNF4α/γ serving as both biomarkers and effectors of epithelial barrier recovery.

Clinically, loss of HNF4α/γ or their downstream TJ targets offers a new framework for epithelial biomarker development and patient stratification in CD. Quantifying HNF4-dependent transcriptional signatures could identify patients with subclinical barrier dysfunction who remain at high risk for relapses or complications despite endoscopic remission. Such stratification would enable precision application of barrier-restorative therapy alongside immune modulation. Because persistent permeability defects are strongly predictive of fibrostenotic and penetrating disease phenotypes, prophylactic activation of HNF4α/γ with NCT or NFT may prevent long-term complications by stabilizing the epithelial barrier early in disease course. Beyond CD, restoration of HNF4 function may have broad relevance for other disorders of epithelial barrier failure, including UC, graft-*versus*-host disease, celiac disease, food allergy, and systemic inflammatory conditions characterized by mucosal leak.[67] Together, these data clarify that while inflammation can transiently mimic HNF4α/γ repression, CD barrier dysfunction is fundamentally

epithelial-intrinsic, and that restoring HNF4α/γ function converts this chronic, self-sustaining defect into a reversible, therapeutically correctable phenotype.

References.

[1] Rath T, Atreya R, Bodenschatz J, Uter W, Geppert CE, Vitali F, Fischer S, Waldner MJ, Colombel JF, Hartmann A, Neurath MF. Intestinal Barrier Healing Is Superior to Endoscopic and Histologic Remission for Predicting Major Adverse Outcomes in Inflammatory Bowel Disease: The Prospective ERIca Trial. Gastroenterology 2023;164(2):241–55.

[2] Pearson AD, Eastham EJ, Laker MF, Craft AW, Nelson R. Intestinal permeability in children with Crohn's disease and coeliac disease. Br Med J (Clin Res Ed) 1982;285(6334):20–1.

[3] Turner D, Ricciuto A, Lewis A, D'Amico F, Dhaliwal J, Griffiths AM, Bettenworth D, Sandborn WJ, Sands BE, Reinisch W, Scholmerich J, Bemelman W, Danese S, Mary JY, Rubin D, Colombel JF, Peyrin-Biroulet L, Dotan I, Abreu MT, Dignass A, International Organization for the Study of IBD. STRIDE-II: An Update on the Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE) Initiative of the International Organization for the Study of IBD (IOIBD): Determining Therapeutic Goals for Treat-to-Target strategies in IBD. Gastroenterology 2021;160(5):1570–83.

[4] Karstensen JG, Saftoiu A, Brynskov J, Hendel J, Klausen P, Cartana T, Klausen TW, Riis LB, Vilmann P. Confocal laser endomicroscopy: a novel method for prediction of relapse in Crohn's disease. Endoscopy 2016;48(4):364–72.

[5] Kiesslich R, Duckworth CA, Moussata D, Gloeckner A, Lim LG, Goetz M, Pritchard DM, Galle PR, Neurath MF, Watson AJ. Local barrier dysfunction identified by confocal laser endomicroscopy predicts relapse in inflammatory bowel disease. Gut 2012;61(8):1146–53.

[6] Chang J, Leong RW, Wasinger VC, Ip M, Yang M, Phan TG. Impaired Intestinal Permeability Contributes to Ongoing Bowel Symptoms in Patients With Inflammatory Bowel Disease and Mucosal Healing. Gastroenterology 2017;153(3):723–31 e1.

[7] Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H. Intestinal Permeability and the Prediction of Relapse in Crohns-Disease. Lancet 1993;341(8858):1437–9.

[8] D'Inca R, Di Leo V, Corrao G, Martines D, D'Odorico A, Mestriner C, Venturi C, Longo G, Sturniolo GC. Intestinal permeability test as a predictor of clinical course in Crohn's disease. Am J Gastroenterol 1999;94(10):2956–60.

[9] Gan J, Nazarian S, Teare J, Darzi A, Ashrafian H, Thompson AJ. A case for improved assessment of gut permeability: a meta-analysis quantifying the lactulose:mannitol ratio in coeliac and Crohn's disease. BMC Gastroenterol 2022;22(1):16.

[10] Neumann H, Vieth M, Atreya R, Grauer M, Siebler J, Bernatik T, Neurath MF, Mudter J. Assessment of Crohn's disease activity by confocal laser endomicroscopy. Inflamm Bowel Dis 2012;18(12):2261–9.

[11] Mehandru S, Colombel JF. The intestinal barrier, an arbitrator turned provocateur in IBD. Nat Rev Gastroenterol Hepatol 2021;18(2):83–4.

[12] Turpin W, Lee SH, Raygoza Garay JA, Madsen KL, Meddings JB, Bedrani L, Power N, Espin-Garcia O, Xu W, Smith MI, Griffiths AM, Moayyedi P, Turner D, Seidman EG, Steinhart AH, Marshall JK, Jacobson K, Mack D, Huynh H, Bernstein CN, Paterson AD, Crohn's, Colitis Canada Genetic Environmental Microbial Project Research C, Abreu

CGPrsdiM, Croitoru K. Increased Intestinal Permeability Is Associated With Later Development of Crohn's Disease. Gastroenterology 2020;159(6):2092–100 e5.

[13] Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol 2005;166(2):409–19.

[14] Laukoetter MG, Nava P, Lee WY, Severson EA, Capaldo CT, Babbin BA, Williams IR, Koval M, Peatman E, Campbell JA, Dermody TS, Nusrat A, Parkos CA. JAM-A regulates permeability and inflammation in the intestine in vivo. J Exp Med 2007;204(13):3067–76.

[15] Vetrano S, Rescigno M, Cera MR, Correale C, Rumio C, Doni A, Fantini M, Sturm A, Borroni E, Repici A, Locati M, Malesci A, Dejana E, Danese S. Unique role of junctional adhesion molecule-a in maintaining mucosal homeostasis in inflammatory bowel disease. Gastroenterology 2008;135(1):173–84.

[16] Shen L, Black ED, Witkowski ED, Lencer WI, Guerriero V, Schneeberger EE, Turner JR. Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. J Cell Sci 2006;119(Pt 10):2095–106.

[17] Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, Tsukita S. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell 2000;11(12):4131–42.

[18] Kuo WT, Zuo L, Odenwald MA, Madha S, Singh G, Gurniak CB, Abraham C, Turner JR. The Tight Junction Protein ZO-1 Is Dispensable for Barrier Function but Critical for Effective Mucosal Repair. Gastroenterology 2021;161(6):1924–39.

[19] Gunzel D, Yu AS. Claudins and the modulation of tight junction permeability. Physiol Rev 2013;93(2):525–69.

[20] Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U, Kroesen AJ, Zeitz M, Fromm M, Schulzke JD. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut 2007;56(1):61–72.

[21] Weber CR, Nalle SC, Tretiakova M, Rubin DT, Turner JR. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. Lab Invest 2008;88(10):1110–20.

[22] Buda A, Hatem G, Neumann H, D'Inca R, Mescoli C, Piselli P, Jackson J, Bruno M, Sturniolo GC. Confocal laser endomicroscopy for prediction of disease relapse in ulcerative colitis: a pilot study. J Crohns Colitis 2014;8(4):304–11.

[23] Soderholm JD, Peterson KH, Olaison G, Franzen LE, Westrom B, Magnusson KE, Sjodahl R. Epithelial permeability to proteins in the noninflamed ileum of Crohn's disease? Gastroenterology 1999;117(1):65–72.

[24] Soderholm JD, Streutker C, Yang PC, Paterson C, Singh PK, McKay DM, Sherman PM, Croitoru K, Perdue MH. Increased epithelial uptake of protein antigens in the ileum of Crohn's disease mediated by tumour necrosis factor alpha. Gut 2004;53(12):1817–24.

[25] Sasaki S, Urabe M, Maeda T, Suzuki J, Irie R, Suzuki M, Tomaru Y, Sakaguchi M, Gonzalez FJ, Inoue Y. Induction of Hepatic Metabolic Functions by a Novel Variant of Hepatocyte Nuclear Factor 4gamma. Mol Cell Biol 2018;38(24).

[26] Vemuri K, Radi SH, Sladek FM, Verzi MP. Multiple roles and regulatory mechanisms of the transcription factor HNF4 in the intestine. Front Endocrinol (Lausanne) 2023;14:1232569.

[27] Chen L, Vasoya RP, Toke NH, Parthasarathy A, Luo S, Chiles E, Flores J, Gao N, Bonder EM, Su X, Verzi MP. HNF4 Regulates Fatty Acid Oxidation and Is Required for Renewal of Intestinal Stem Cells in Mice. Gastroenterology 2020;158(4):985–99 e9.

[28] Marcil V, Sinnett D, Seidman E, Boudreau F, Gendron FP, Beaulieu JF, Menard D, Lambert M, Bitton A, Sanchez R, Amre D, Levy E. Association between genetic variants in the HNF4A gene and childhood-onset Crohn's disease. Genes Immun 2012;13(7):556–65.

[29] Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature 2011;474(7351):307–17.

[30] Barrett JC, Lee JC, Lees CW, Prescott NJ, Anderson CA, Phillips A, Wesley E, Parnell K, Zhang H, Drummond H, Nimmo ER, Massey D, Blaszczyk K, Elliott T, Cotterill L, Dallal H, Lobo AJ, Mowat C, Sanderson JD, Jewell DP, Newman WG, Edwards C, Ahmad T, Mansfield JC, Satsangi J, Parkes M, Mathew CG, Donnelly P, Peltonen L, Blackwell JM, Bramon E, Brown MA, Casas JP, Corvin A, Craddock N, Deloukas P, Duncanson A, Jankowski J, Markus HS, Mathew CG, McCarthy MI, Palmer CNA, Plomin R, Rautanen A, Sawcer SJ, Samani N, Trembath RC, Viswanathan AC, Wood N, Spencer CCA, Barrett JC, Bellenguez C, Davison D, Freeman C, Strange A, Donnelly P, Langford C, Hunt SE, Edkins S, Gwilliam R, Blackburn H, Bumpstead SJ, Dronov S, Gillman M, Gray E, Hammond N, Jayakumar A, McCann OT, Liddle J, Perez ML, Potter SC, Ravindrarajah R, Ricketts M, Waller M, Weston P, Widaa S, Whittaker P, Deloukas P, Peltonen L, Mathew CG, Blackwell JM, Brown MA, Corvin A, McCarthy MI, Spencer CCA, Attwood AP, Stephens J, Sambrook J, Ouwehand WH, McArdle WL, Ring SM, Strachan DP, The UKIBDGC, The Wellcome Trust Case Control C, Management C, Data, Analysis G, Dna GDQC, Informatics G, Publications C, Controls UKBS, Birth Cohort C. Genome-wide

association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. Nature Genetics 2009;41(12):1330–4.

[31] Chen L, Toke NH, Luo S, Vasoya RP, Fullem RL, Parthasarathy A, Perekatt AO, Verzi MP. A reinforcing HNF4-SMAD4 feed-forward module stabilizes enterocyte identity. Nat Genet 2019;51(5):777–85.

[32] Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity 1999;10(3):387–98.

[33] Bamias G, Dahman MI, Arseneau KO, Guanzon M, Gruska D, Pizarro TT, Cominelli F. Intestinal-specific TNFalpha overexpression induces Crohn's-like ileitis in mice. PLoS One 2013;8(8):e72594.

[34] Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersema PD, Clevers H. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 2011;141(5):1762–72.

[35] Horcas-Nieto JM, Versloot CJ, Langelaar-Makkinje M, Gerding A, Blokzijl T, Koster MH, Baanstra M, Martini IA, Coppes RP, Bourdon C, van Ijzendoorn SCD, Kim P, Bandsma RHJ, Bakker BM. Organoids as a model to study intestinal and liver dysfunction in severe malnutrition. Biochim Biophys Acta Mol Basis Dis 2023;1869(3):166635.

[36] Roy S, Esmaeilniakooshkghazi A, Patnaik S, Wang Y, George SP, Ahrorov A, Hou JK, Herron AJ, Sesaki H, Khurana S. Villin-1 and Gelsolin Regulate Changes in Actin Dynamics That Affect Cell Survival Signaling Pathways and Intestinal Inflammation. Gastroenterology 2018;154(5):1405–20 e2.

[37] Chen L, Toke NH, Luo S, Vasoya RP, Aita R, Parthasarathy A, Tsai YH, Spence JR, Verzi MP. HNF4 factors control chromatin accessibility and are redundantly required for maturation of the fetal intestine. Development 2019;146(19).

[38] Ahn SH, Shah YM, Inoue J, Morimura K, Kim I, Yim S, Lambert G, Kurotani R, Nagashima K, Gonzalez FJ, Inoue Y. Hepatocyte nuclear factor 4alpha in the intestinal epithelial cells protects against inflammatory bowel disease. Inflamm Bowel Dis 2008;14(7):908–20.

[39] Bueno-Hernandez N, Sanchez-Munoz F, Barreto-Zuniga R, Dominguez-Lopez A, Yamamoto-Furusho JK. Expression of HNF4gamma is downregulated in patients with active ulcerative colitis (UC) compared to UC patients in remission and healthy controls. Inflamm Bowel Dis 2011;17(8):E91.

[40] Kobayashi S, Ogasawara N, Watanabe S, Yoneyama Y, Kirino S, Hiraguri Y, Inoue M, Nagata S, Okamoto-Uchida Y, Kofuji S, Shimizu H, Ito G, Mizutani T, Yamauchi S, Kinugasa Y, Kano Y, Nemoto Y, Watanabe M, Tsuchiya K, Nishina H, Okamoto R, Yui S. Collagen type I-mediated mechanotransduction controls epithelial cell fate conversion during intestinal inflammation. Inflamm Regen 2022;42(1):49.

[41] Lee C, Hong SN, Kim ER, Chang DK, Kim YH. Epithelial Regeneration Ability of Crohn's Disease Assessed Using Patient-Derived Intestinal Organoids. Int J Mol Sci 2021;22(11).

[42] Otani T, Nguyen TP, Tokuda S, Sugihara K, Sugawara T, Furuse K, Miura T, Ebnet K, Furuse M. Claudins and JAM-A coordinately regulate tight junction formation and epithelial polarity. J Cell Biol 2019;218(10):3372–96.

[43] Boj SF, Parrizas M, Maestro MA, Ferrer J. A transcription factor regulatory circuit in differentiated pancreatic cells. Proc Natl Acad Sci U S A 2001;98(25):14481–6.

[44] Lee SH, Veeriah V, Levine F. Liver fat storage is controlled by HNF4alpha through induction of lipophagy and is reversed by a potent HNF4alpha agonist. Cell Death Dis 2021;12(6):603.

[45] Lee SH, Veeriah V, Levine F. A potent HNF4alpha agonist reveals that HNF4alpha controls genes important in inflammatory bowel disease and Paneth cells. PLoS One 2022;17(4):e0266066.

[46] Rosenblatt J, Raff MC, Cramer LP. An epithelial cell destined for apoptosis signals its neighbors to extrude it by an actin- and myosin-dependent mechanism. Curr Biol 2001;11(23):1847–57.

[47] Fanning AS, Van Itallie CM, Anderson JM. Zonula occludens-1 and -2 regulate apical cell structure and the zonula adherens cytoskeleton in polarized epithelia. Mol Biol Cell 2012;23(4):577–90.

[48] Gonzalez-Mariscal L, Gallego-Gutierrez H, Gonzalez-Gonzalez L, Hernandez-Guzman C. ZO-2 Is a Master Regulator of Gene Expression, Cell Proliferation, Cytoarchitecture, and Cell Size. Int J Mol Sci 2019;20(17).

[49] Alarcon VB. Cell polarity regulator PARD6B is essential for trophectoderm formation in the preimplantation mouse embryo. Biol Reprod 2010;83(3):347–58.

[50] Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M, Tsukita S. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell 2006;126(4):741–54.

[51] Stephenson RE, Higashi T, Erofeev IS, Arnold TR, Leda M, Goryachev AB, Miller AL. Rho Flares Repair Local Tight Junction Leaks. Dev Cell 2019;48(4):445–59 e5.

[52] Jou TS, Schneeberger EE, Nelson WJ. Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. J Cell Biol 1998;142(1):101–15.

[53] Yu AS, Cheng MH, Angelow S, Gunzel D, Kanzawa SA, Schneeberger EE, Fromm M, Coalson RD. Molecular basis for cation selectivity in claudin-2-based paracellular pores: identification of an electrostatic interaction site. J Gen Physiol 2009;133(1):111–27.

[54] Varadarajan S, Stephenson RE, Miller AL. Multiscale dynamics of tight junction remodeling. J Cell Sci 2019;132(22).

[55] Chatterjee I, Zhang Y, Zhang J, Lu R, Xia Y, Sun J. Overexpression of Vitamin D Receptor in Intestinal Epithelia Protects Against Colitis via Upregulating Tight Junction Protein Claudin 15. J Crohns Colitis 2021;15(10):1720–36.

[56] Prasad H, Visweswariah SS. Impaired Intestinal Sodium Transport in Inflammatory Bowel Disease: From the Passenger to the Driver's Seat. Cell Mol Gastroenterol Hepatol 2021;12(1):277–92.

[57] Ehle C, Iyer-Bierhoff A, Wu Y, Xing S, Kiehntopf M, Mosig AS, Godmann M, Heinzel T. Downregulation of HNF4A enables transcriptomic reprogramming during the hepatic acute-phase response. Commun Biol 2024;7(1):589.

[58] Nishikawa Y, Sone M, Nagahama Y, Kumagai E, Doi Y, Omori Y, Yoshioka T, Tokairin T, Yoshida M, Yamamoto Y, Ito A, Sugiyama T, Enomoto K. Tumor necrosis factor-alpha promotes bile ductular transdifferentiation of mature rat hepatocytes in vitro. J Cell Biochem 2013;114(4):831–43.

[59] Cleynen I, Boucher G, Jostins L, Schumm LP, Zeissig S, Ahmad T, Andersen V, Andrews JM, Annese V, Brand S, Brant SR, Cho JH, Daly MJ, Dubinsky M, Duerr RH, Ferguson LR, Franke A, Gearry RB, Goyette P, Hakonarson H, Halfvarson J, Hov JR, Huang H,

Kennedy NA, Kupcinskas L, Lawrance IC, Lee JC, Satsangi J, Schreiber S, Theatre E, van der Meulen-de Jong AE, Weersma RK, Wilson DC, International Inflammatory Bowel Disease Genetics C, Parkes M, Vermeire S, Rioux JD, Mansfield J, Silverberg MS, Radford-Smith G, McGovern DP, Barrett JC, Lees CW. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study. Lancet 2016;387(10014):156–67.

[60] Hollander D, Vadheim CM, Brettholz E, Petersen GM, Delahunty T, Rotter JI. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. Ann Intern Med 1986;105(6):883–5.

[61] Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. Gut 2006;55(10):1512–20.

[62] Leonard W, Zhang P, Ying D, Fang Z. Tyramine-derived hydroxycinnamic acid amides in plant foods: sources, synthesis, health effects and potential applications in food industry. Crit Rev Food Sci Nutr 2022;62(6):1608–25.

[63] Tang J, Dunshea FR, Suleria HAR. LC-ESI-QTOF/MS Characterization of Phenolic Compounds from Medicinal Plants (Hops and Juniper Berries) and Their Antioxidant Activity. Foods 2019;9(1).

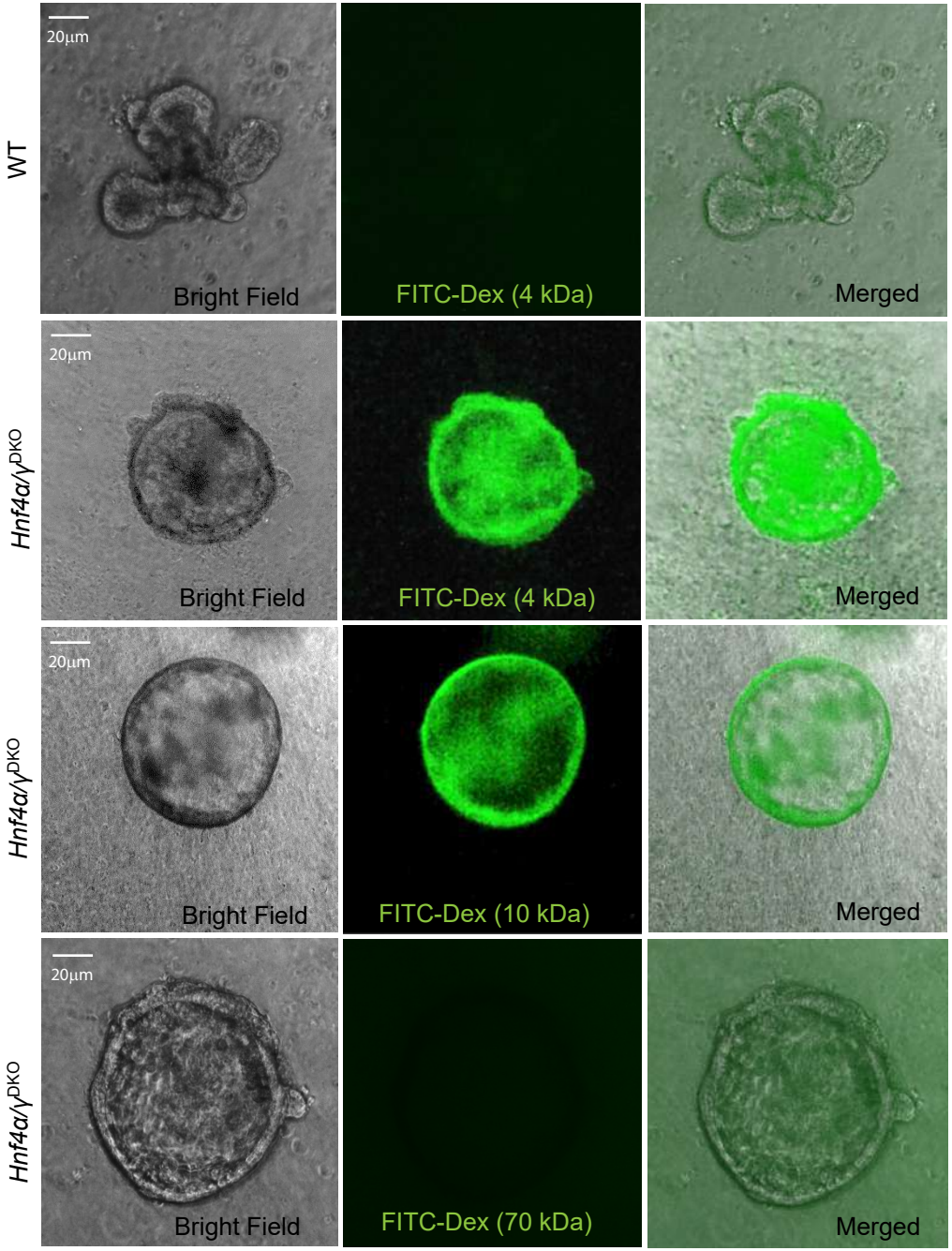
[64] Jiang Y, Yu L, Wang MH. N-trans-feruloyltyramine inhibits LPS-induced NO and PGE2 production in RAW 264.7 macrophages: Involvement of AP-1 and MAP kinase signalling pathways. Chem Biol Interact 2015;235:56–62.

[65] Beinsteiner B, Billas IML, Moras D. Structural insights into the HNF4 biology. Front Endocrinol (Lausanne) 2023;14:1197063.

[66] Veeriah V, Lee SH, Levine F. Long-term oral administration of an HNF4alpha agonist prevents weight gain and hepatic steatosis by promoting increased mitochondrial mass and function. Cell Death Dis 2022;13(1):89.

[67] Horowitz A, Chanez-Paredes SD, Haest X, Turner JR. Paracellular permeability and tight junction regulation in gut health and disease. Nat Rev Gastroenterol Hepatol 2023;20(7):417–32.

Figure 1. Loss of HNF4α/γ activates both pore and leak pathway permeability in intestinal organoids. Representative phase-contrast and confocal images of FITC-dextran (4, 10, 70 kDa) diffusion into the lumen of wild-type (WT) and *Hnf4α/γ*^{DKO} mouse intestinal organoids. FITC-dextran was applied to the basolateral medium for 30 minutes before imaging. Scale bars, 20 μM. Quantification of luminal fluorescence intensity (mean ± SEM; normalized to WT) reveals a size-selective increase in permeability in *Hnf4α/γ*^{DKO} organoids. Uptake of small 4 kDa dextran reflects activation of the pore pathway, mediated by upregulation of cation-selective *Cldn2*, whereas increased entry of larger 10 kDa dextran indicates disruption of the leak pathway, consistent with downregulation of sealing claudins (*Cldn4, Cldn7*) and junctional scaffolds (*Tjp2*, *Tjap1*) in the *Hnf4α/γ*^{DKO} mouse small intestine. Absence of luminal accumulation of 70 kDa dextran confirms that overall membrane integrity and epithelial viability remain intact, indicating that barrier defects in *Hnf4α/γ*^{DKO} mouse organoids are paracellular rather than due to mucosal injury. Asterisks (***) denote $p<0.0001$, n=15 organoids per sample. Scale bar, 20 μm.


WT
Hnf4α/γDKO
Hnf4α/γDKO
Hnf4α/γDKO
20μm
Bright Field
FITC-Dex (4 kDa)
Merged
FITC-Dex (10 kDa)
FITC-Dex (70 kDa)

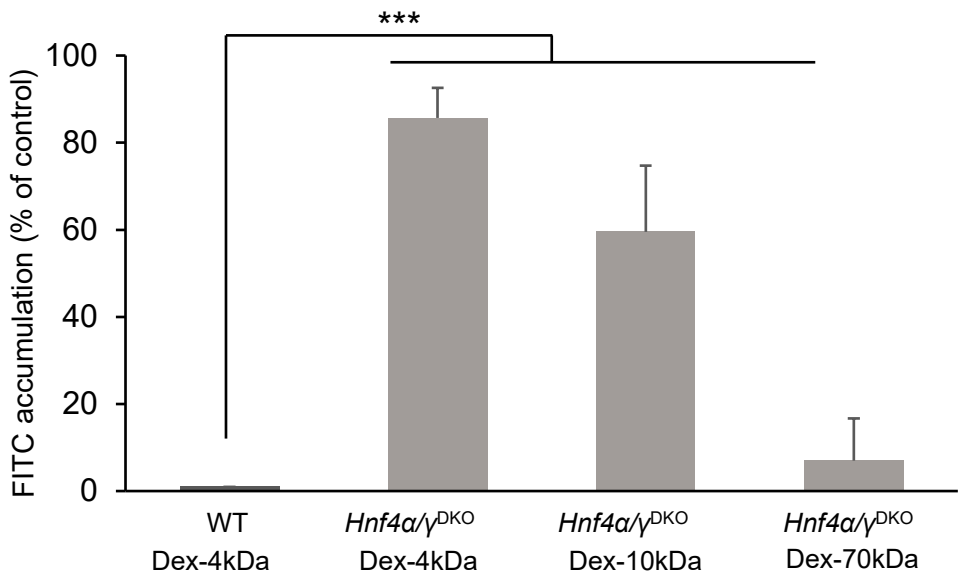

***
FITC accumulation (% of control)
100
80
60
40
20
0
WT Dex-4kDa
Hnf4α/γDKO Dex-4kDa
Hnf4α/γDKO Dex-10kDa
Hnf4α/γDKO Dex-70kDa

Figure 2A. Regional downregulation of HNF4α and HNF4γ in intestinal tissue from Crohn's disease patients. Representative immunohistochemical staining for HNF4α and HNF4γ in paraffin-embedded sections of distal ileum from non-CD controls and patients with Crohn's disease (CD). In control tissues, HNF4α shows strong nuclear staining along the crypt-villus axis. In the same tissue samples, HNF4γ shows nuclear and cytoplasmic staining along the crypt-villus axis. In CD patients nuclear HNF4α and HNF4γ are markedly reduced, indicating attenuated HNF4 signaling in the small intestine. This is a representative of n=6 CD patients, n=4 non-CD controls. Scale bars, 100 μm

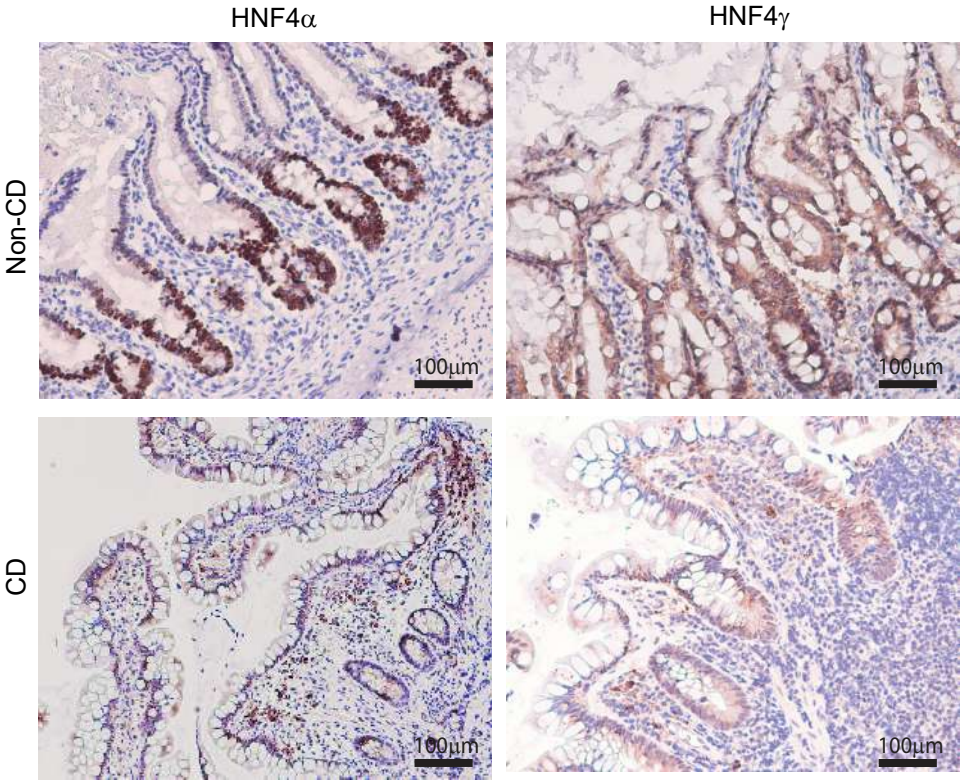

HNF4α
HNF4γ
Non-CD
CD
100μm
100μm
100μm
100μm

Figure 2B. Regional downregulation of HNF4α and HNF4γ in intestinal tissue from Crohn's disease patients. Representative immunohistochemical staining for HNF4α in paraffin-embedded sections of transverse colon (TVC), descending colon (DC), and rectum (REC) from patients with Crohn's disease (CD), and transverse colon from non-CD controls. In control tissue, HNF4a exhibits uniform nuclear localization throughout the crypt epithelium and in the surface colonocytes, whereas CD tissue shows variably reduced staining intensity, consistent with region-wide downregulation of HNF4α transcriptional activity. HNF4γ is undetectable in colon tissue from non-CD controls (data not shown). This is a representative of n=11 CD patients, n=4 non-CD controls. Scale bars, 100 μm

HNF4α

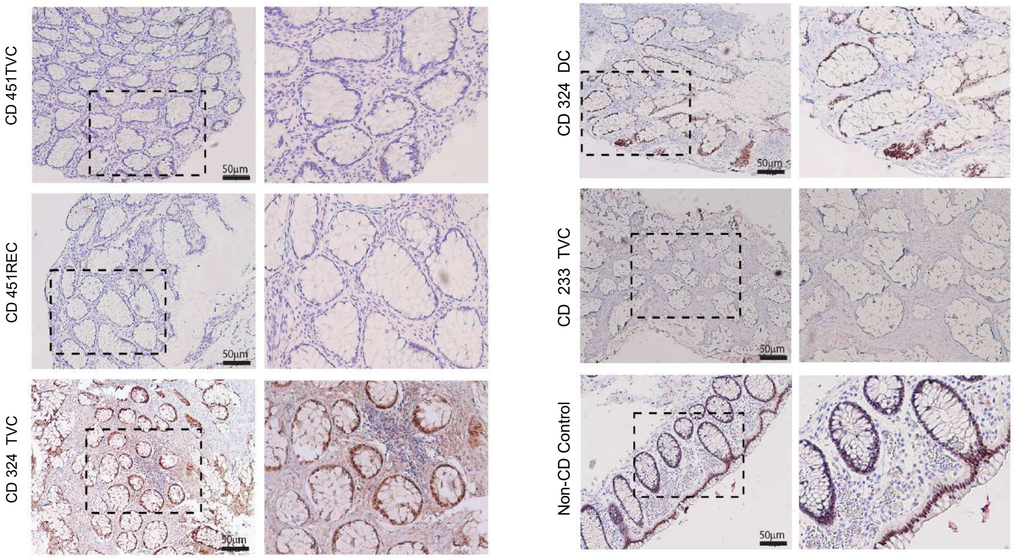
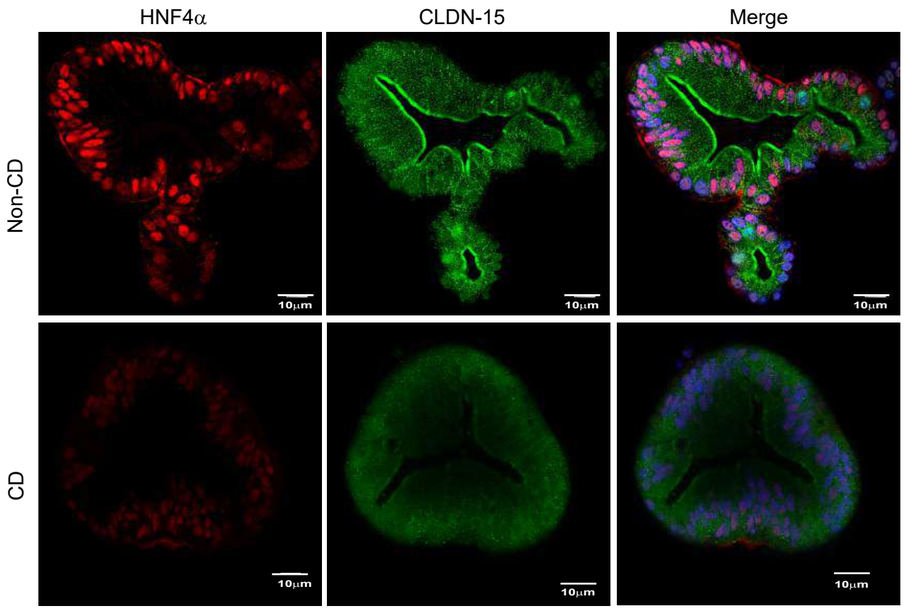

CD 451TVC
CD 451REC
CD 324 TVC
CD 324 DC
CD 233 TVC
Non-CD Control
50μm

Figure 3A. HNF4α/γ and their downstream target tight junction protein Claudin-15 are downregulated in Crohn's Disease ileal organoids. Representative confocal images of patient-derived intestinal organoids generated from non-inflamed distal ileal biopsies of non-CD controls and patients with active CD, immunoassayed for HNF4α (red) and Claudin-15 (CLDN15, green). Nuclei were counterstained with DAPI (blue). In control organoids, HNF4α shows strong nuclear localization, and CLDN15 is abundantly expressed along the apical membrane. In CD organoids, nuclear HNF4α and CLDN15 expression are markedly reduced, indicating transcriptional downregulation of Cldn15 secondary to loss of HNF4α activity, independent of inflammation. Quantification of mean fluorescence intensity (mean ± SEM; normalized to non-CD controls) demonstrates a significant reduction of both HNF4α and CLDN15 in CD organoids. Asterisks (***) denote $p<0.0001$, n=8 patients per group. Student's t test. Scale bar, 100 μm.


HNF4α
CLDN-15
Merge
Non-CD
CD
10μm

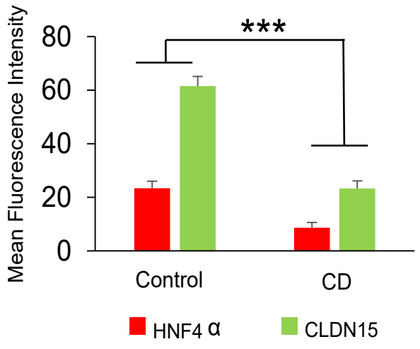
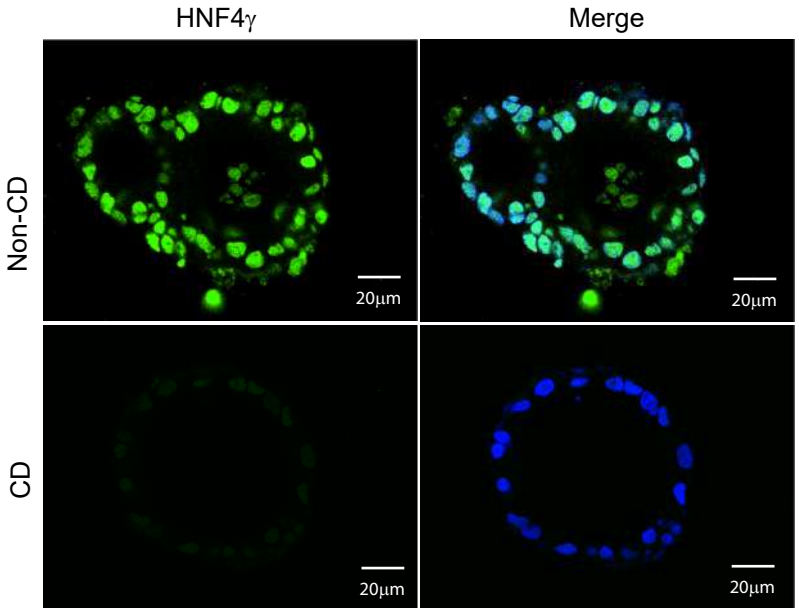

Mean Fluorescence Intensity
80
60
40
20
0
***
Control
CD
HNF4 α
CLDN15

Figure 3B. HNF4α/γ and their downstream target tight junction protein Claudin-15 are downregulated in Crohn's Disease ileal organoids. Representative confocal images of patient-derived intestinal organoids generated from non-inflamed distal ileal biopsies of non-CD controls and patients with active CD, immunoassayed for HNF4γ (green). Nuclei were counterstained with DAPI (blue). In control organoids, HNF4γ shows strong nuclear localization and is abundantly expressed. In CD organoids, nuclear HNF4γ expression is markedly reduced, indicating loss of HNF4γ activity, independent of inflammation. Quantification of mean fluorescence intensity (mean ± SEM; normalized to non-CD controls) demonstrates a significant reduction of HNF4γ in CD organoids. Asterisks (***) denote $p<0.0001$, n=8 patients per group. Student's t test. Scale bar, 20 μm.


HNF4γ
Merge
Non-CD
CD
20μm
20μm
20μm
20μm

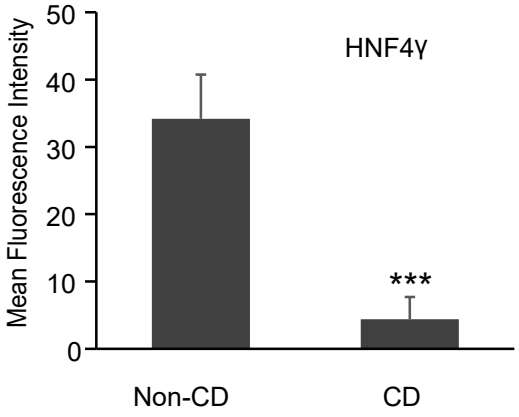

HNF4γ
Mean Fluorescence Intensity
50
40
30
20
10
0
***
Non-CD
CD

Figure 4A. TNF-driven inflammation suppresses HNF4α/γ expression in the intestinal epithelium. Representative immunohistochemical staining for HNF4α and HNF4γ in paraffin-embedded sections of distal ileum from *Tnf*$^{\Delta ARE/+}$ and *Tnf*$^{+/+}$ mice. In *Tnf*$^{+/+}$ mouse tissues, HNF4α shows strong nuclear staining along the crypt-villus axis. In the same tissue samples, HNF4γ shows nuclear and cytoplasmic staining along the crypt-villus axis. In *Tnf*$^{\Delta ARE/+}$ mice, nuclear HNF4α and HNF4γ are markedly reduced, indicating attenuated HNF4 signaling in the small intestine. This is a representative of n=6 mice per group. Scale bars, 100 μm

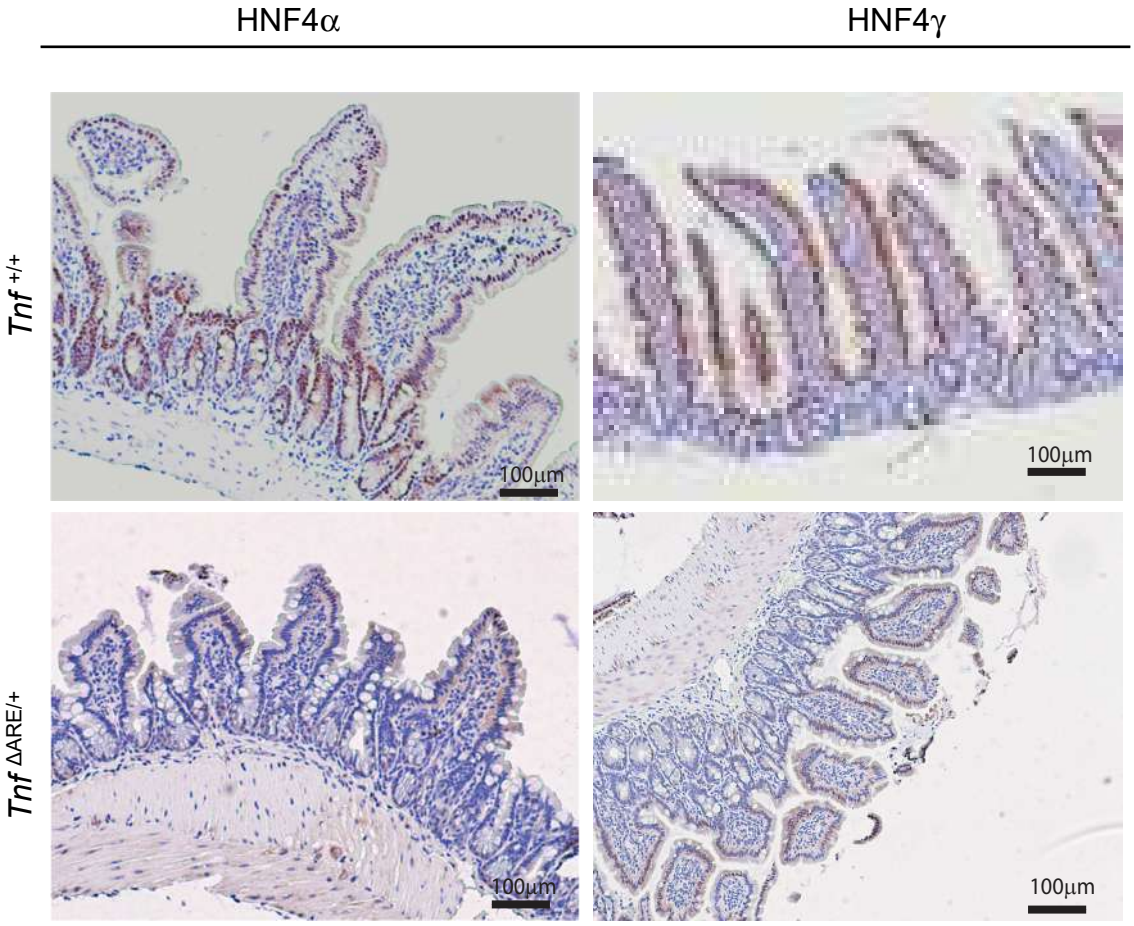

HNF4α
HNF4γ
Tnf +/+
Tnf ΔARE/+
100μm
100μm
100μm
100μm

Figure 4B. TNF-driven inflammation suppresses HNF4α/γ expression in intestinal organoids. Representative confocal immunofluorescence images of intestinal organoids derived from distal ileum of *Tnf*$^{+/+}$ and *Tnf*$^{\Delta ARE/+}$ mice stained for HNF4α (red) and claudin-15 (CLDN15, green). Nuclei were counterstained with DAPI (blue). In control (*Tnf*$^{+/+}$) mouse organoids, HNF4α shows strong nuclear localization accompanied by abundant apical CLDN15 expression. Organoids from *Tnf*$^{\Delta ARE/+}$ mice display markedly reduced HNF4α nuclear staining and loss of apical CLDN15, indicating inflammation-associated repression of HNF4α and its downstream target TJ protein, CLDN15. CLDN15 immunostaining shows high background due to cross-reactivity of the anti-human antibody in mouse tissue, however, apical ClDN15 signal intensity is reduced in *Tnf*$^{\Delta ARE/+}$ mouse organoids. Quantification of mean fluorescence intensity (mean ± SEM; normalized to *Tnf*$^{+/+}$ controls) demonstrates significant reduction of HNF4α and CLDN15 in *Tnf*$^{\Delta ARE/+}$ mouse organoids. Asterisks (***) denote $p<0.0001$ by unpaired Student's t test; n=6 mice per group. Scale bar, 15 μm.

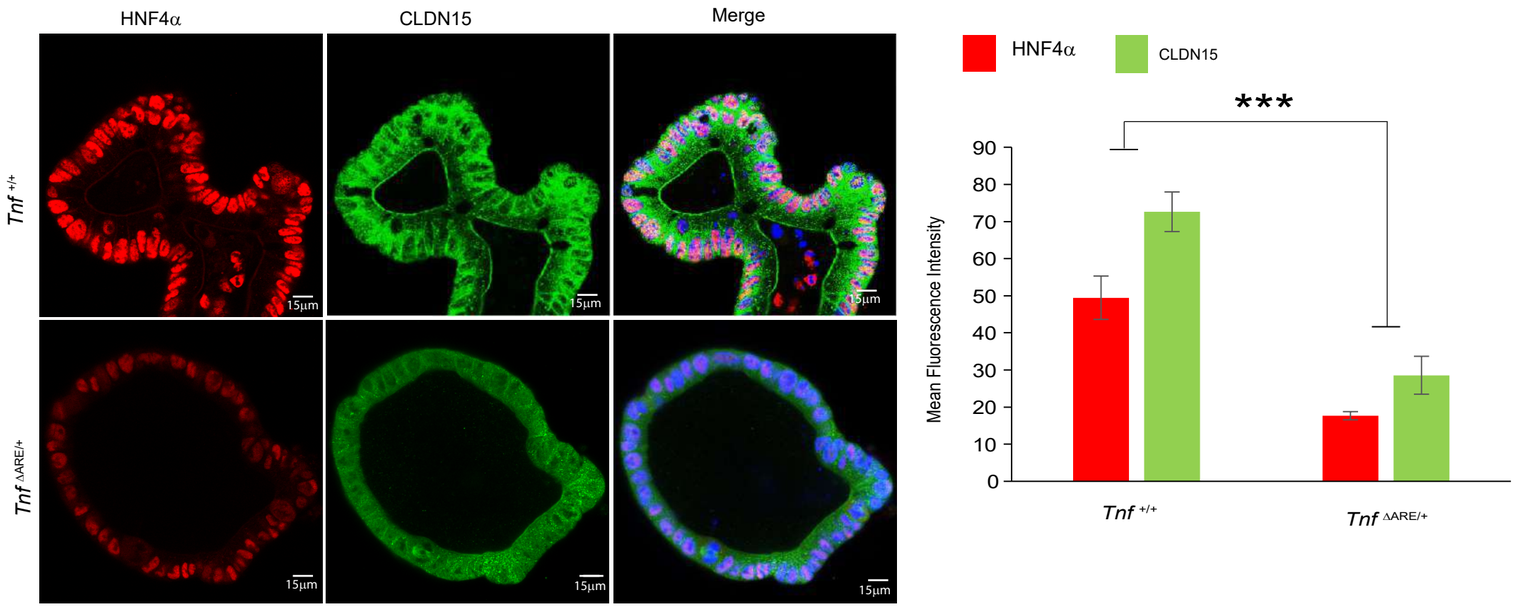

HNF4α
CLDN15
Merge
Tnf +/+
Tnf ΔARE/+
15µm
HNF4α
CLDN15
***
Mean Fluorescence Intensity
90
80
70
60
50
40
30
20
10
0
Tnf +/+
Tnf ΔARE/+

Figure 4C. TNF-driven inflammation suppresses HNF4α/γ expression in intestinal organoids. Representative confocal immunofluorescence images of intestinal organoids derived from distal ileum of *Tnf*$^{+/+}$ and *Tnf*$^{\Delta ARE/+}$ mice stained for HNF4γ (green). Nuclei were counterstained with DAPI (blue). In control (*Tnf*$^{+/+}$) mouse organoids, HNF4γ shows strong nuclear and cytoplasmic localization. Organoids from *Tnf*$^{\Delta ARE/+}$ mice display almost complete loss of HNF4γ staining, indicating inflammation-associated repression of HNF4γ. Quantification of mean fluorescence intensity (mean ± SEM; normalized to *Tnf*$^{+/+}$ controls) demonstrates significant reduction of HNF4γ and CLDN15 in *Tnf*$^{\Delta ARE/+}$ mouse organoids. Asterisks (***) denote $p<0.0001$ by unpaired Student's t test; n=6 mice per group. Scale bar, 20 μm.

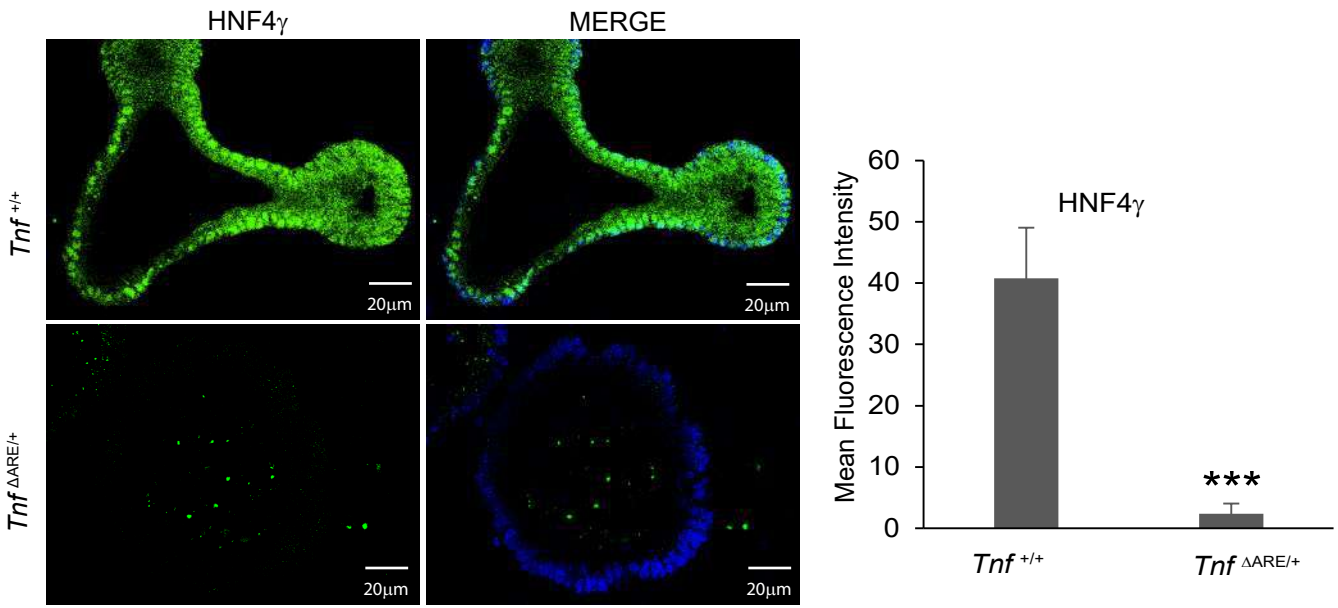
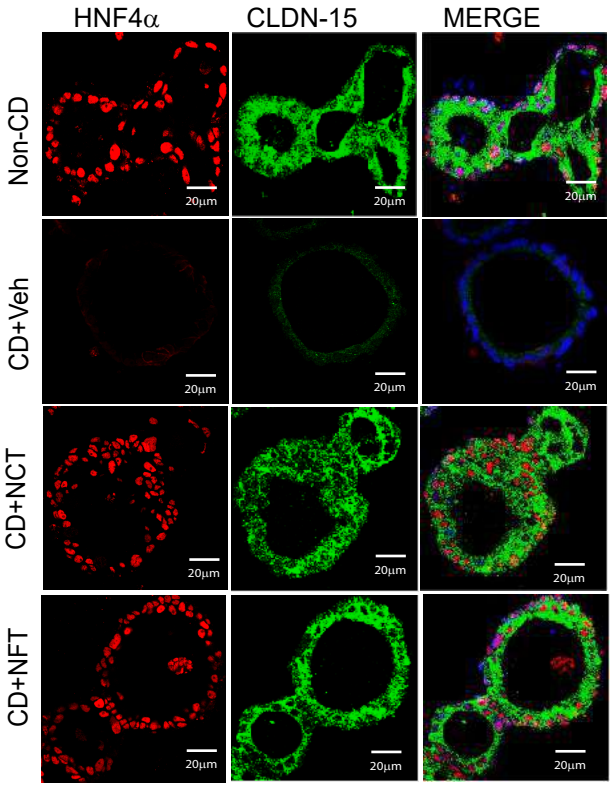

HNF4γ
MERGE
Tnf +/+
Tnf ΔARE/+
20μm
20μm
20μm
20μm
Mean Fluorescence Intensity
60
50
40
30
20
10
0
HNF4γ
***
Tnf +/+
Tnf ΔARE/+

Figure 5A. Treatment of CD patient-derived organoids with the HNF4 agonists NCT and NFT restored the expression of HNF4α, HNF4γ and their downstream target TJ target protein Claudin-15. Representative confocal immunofluorescence of CD patient-derived organoids (PDOs) treated with vehicle (DMSO), NCT (80 μM, 12 h), or NFT (80 μM, 12h), stained for HNF4α (red) and CLDN15 (green). Nuclei were counterstained with DAPI (blue). Vehicle (Veh) treated CD PDOs show markedly reduced nuclear HNF4α and CLDN15 expression compared with non-CD control organoids. Treatment with NCT or NFT restored HNF4α and CLDN15 staining to levels comparable to non-CD controls. Quantification of mean fluorescence intensity (mean ± SEM; normalized to non-CD controls) demonstrates significant upregulation of HNF4α and CLDN15 following treatment with NCT or NFT compared to vehicle-treated CD organoids. Asterisks (***) denote $p<0.0001$ by Student's t test. Scale bar, 20 μm.


HNF4α
CLDN-15
MERGE
Non-CD
CD+Veh
CD+NCT
CD+NFT
20μm

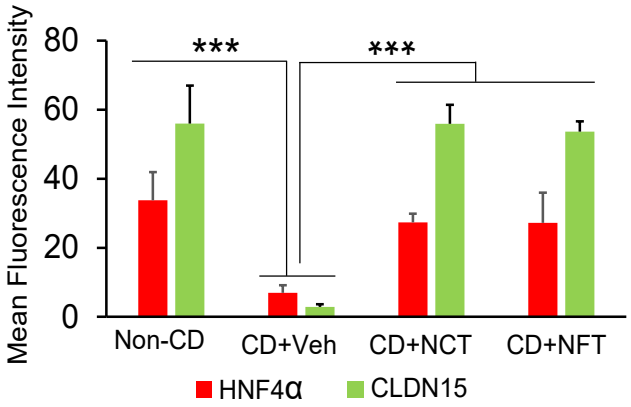
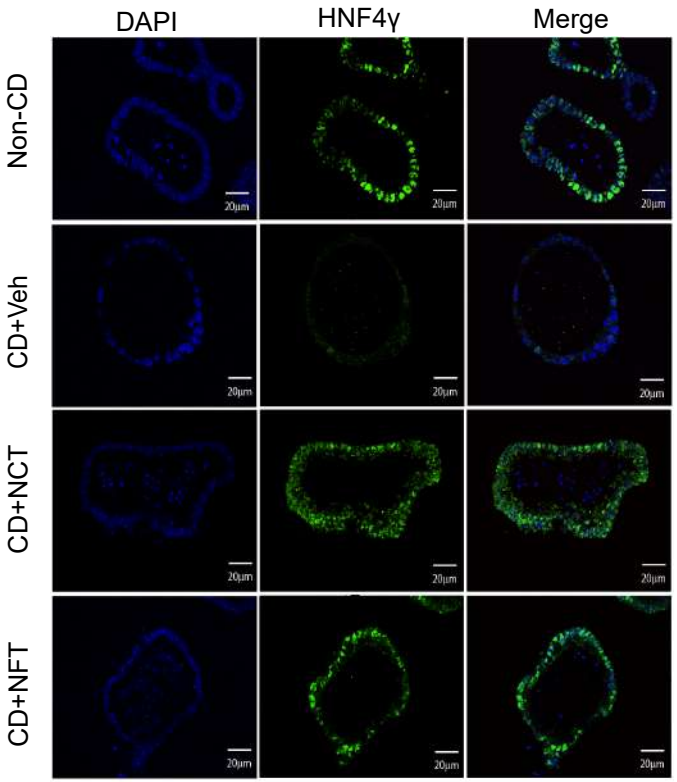

Mean Fluorescence Intensity
80
60
40
20
0
***
***
Non-CD
CD+Veh
CD+NCT
CD+NFT
HNF4α
CLDN15

Figure 5B. Treatment of CD patient-derived organoids with the HNF4 agonists NCT and NFT restored the expression of HNF4α, HNF4γ and their downstream target TJ target protein Claudin-15. Representative confocal immunofluorescence images of matched CD patient-derived organoids (PDOs) following vehicle (DMSO), NCT (80 μM, 12 h), or NFT (80 μM, 12h) treatment. HNF4γ (red) nuclear staining is diminished in vehicle-treated CD PDOs but restored by NCT or NFT, indicating reactivation of *Hnf4γ* expression by HNF4 agonists. Nuclei were counterstained with DAPI (blue). Quantification of mean fluorescence intensity (mean ± SEM; normalized to non-CD controls) demonstrates significant upregulation of HNF4γ following treatment with NCT or NFT compared to vehicle-treated CD organoids. Asterisks (***) denote $p<0.0001$ by Student's t test. Scale bar, 20 μm.


DAPI
HNF4γ
Merge
Non-CD
CD+Veh
CD+NCT
CD+NFT
20μm

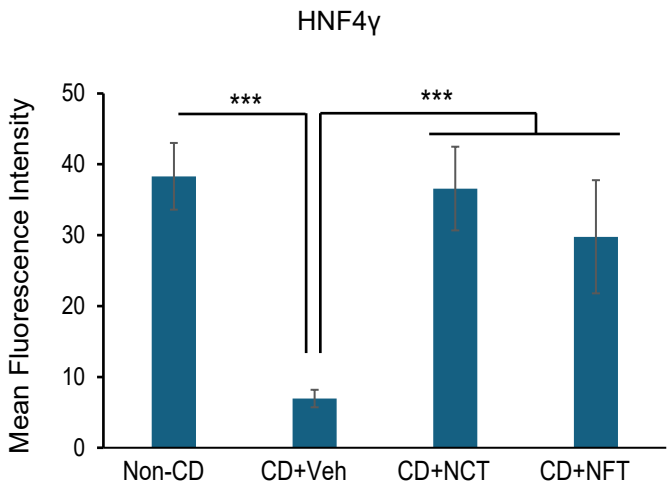
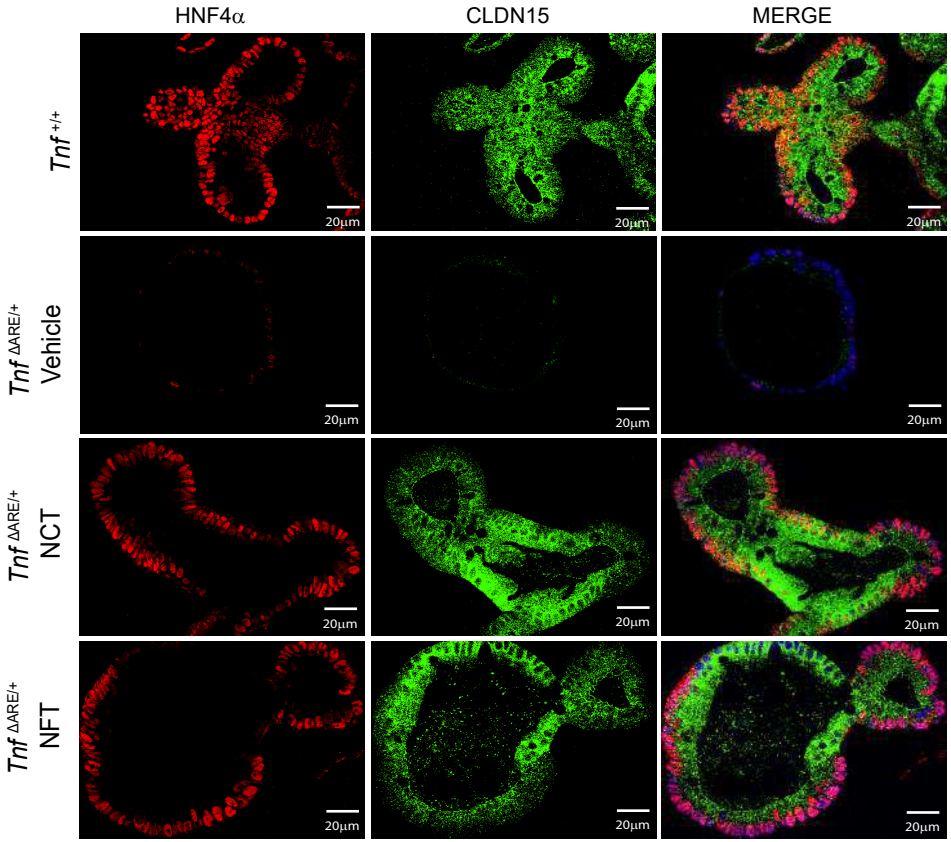

HNF4γ
Mean Fluorescence Intensity
50
40
30
20
10
0
***
***
Non-CD
CD+Veh
CD+NCT
CD+NFT

Figure 6. Inflammation-induced suppression of HNF4α and Claudin-15 in the *Tnf*$^{\Delta ARE/+}$ mouse organoids is reversed by the HNF4α agonists NCT and NFT. Representative confocal immunofluorescence images of intestinal organoids derived from *Tnf*$^{+/+}$ and *Tnf*$^{\Delta ARE/+}$ mice treated with vehicle (DMSO), NCT (80 mM, 12h), or NFT (80 mM, 12h). Organoids were stained for HNF4α (red) and CLDN15 (green). Nuclei were counterstained with DAPI (blue). In *Tnf*$^{+/+}$ mouse organoids, HNF4α shows strong nuclear localization and CLDN15 is expressed along apical membranes. In contrast, *Tnf*$^{\Delta ARE/+}$ mouse organoids exhibit markedly reduced nuclear HNF4α and apical CLDN15 expression, indicating inflammation-induced repression of *Hnf4α* and its downstream target tight junction genes. Treatment with NCT or NFT restores both HNF4α and apical CLDN15 staining to levels comparable to *Tnf*$^{+/+}$ control organoids. Quantification of mean fluorescence intensity (mean ± SEM; normalized to *Tnf*$^{+/+}$ controls) demonstrates significant upregulation of HNF4α and CLDN15 following treatment with NCT or NFT compared to vehicle-treated CD organoids. Asterisks (***) denote $p<0.0001$ by Student's t test. Scale bar, 20 μm.


HNF4α
CLDN15
MERGE
Tnf +/+
Tnf ΔARE/+ Vehicle
Tnf ΔARE/+ NCT
Tnf ΔARE/+ NFT
20μm

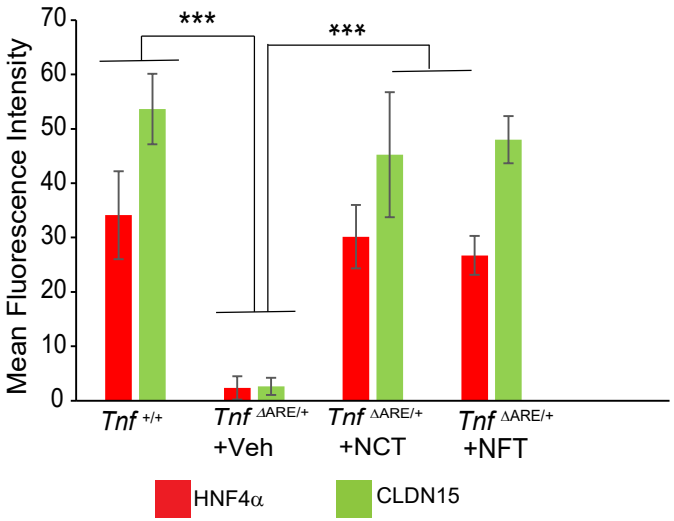
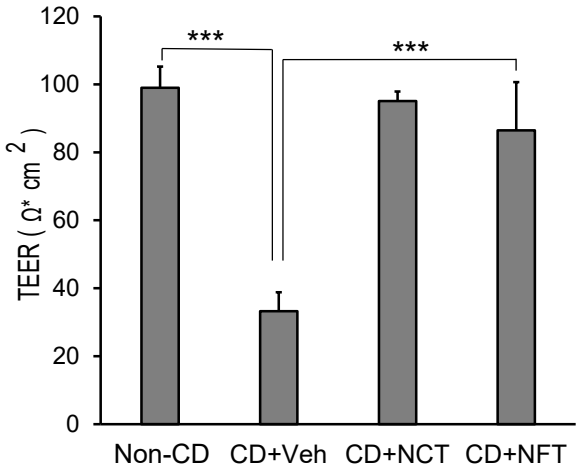

Mean Fluorescence Intensity
70
60
50
40
30
20
10
0
***
***
Tnf +/+
Tnf ΔARE/+ +Veh
Tnf ΔARE/+ +NCT
Tnf ΔARE/+ +NFT
HNF4α
CLDN15

Figure 7. HNF4 agonists NCT and NFT restore epithelia barrier function in Crohn's disease patient-derived intestinal organoids. Transepithelial electrical resistance (TEER) measurement of intestinal organoid monolayers derived from non-CD controls and Crohn's disease (CD) patients treated with vehicle (DMSO), NCT (80 μM, 12h), or NFT (80 μM, 12h). CD organoids exhibit significantly reduced TEER compared to non-CD controls, indicating impaired epithelial permeability barrier integrity. Treatment with either NCT or NFT markedly increased TEER in CD PDOs, restoring TEER to near-control levels. Quantification of TEER (mean ± SEM) normalized to non-CD controls demonstrates significant recovery of barrier integrity following NCT or NFT treatment compared with vehicle-treated CD PDOs. Asterisks (***) denote $p<0.001$ by unpaired Student's t test; n=8 patients per group.


120
100
80
60
40
20
0
TEER (Ω* cm 2)
***
***
Non-CD
CD+Veh
CD+NCT
CD+NFT