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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Abbreviations used in this paper: AJ, adherens junction; 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.

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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 defining feature of Crohn's disease (CD) that precedes inflammation, persists during remission, and predicts relapse. Despite its clinical importance, no validated biomarkers or therapies directly target epithelial barrier restoration. 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 function.

Methods. We integrated our previously defined HNF4α/γ-regulated epithelial transcriptome with 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, with 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 inflammation independent. In contrast, chronic TNF overexpression in the $Tnf^{\Delta ARE/+}$ mice phenocopied this

suppression through a normally transient epithelial injury response that became pathologically stabilized under sustained cytokine signaling. 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 master transcriptional regulators of epithelial TJ integrity whose loss defines a primary barrier defect in CD. Chronic inflammation can mimic but does not initiate this intrinsic defect. 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 disease complication.[1, 2] Despite its central role in pathogenesis, no current diagnostic or therapeutic approaches directly assess or restore epithelial barrier function in CD. Endoscopic remission (ER), defined as the absence or resolution of epithelial erosions, ulcers, and luminal strictures, is the current clinical target in CD 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] Furthermore, 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 is available to assess 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 epithelial barrier dysfunction is causally linked to CD pathogenesis.[11, 12] Thus, identifying epithelial biomarkers and therapeutic targets for BH 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 yet appears inflammation-dependent in experimental models of IBD. *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 prejunctional actomyosin ring (circumferential actin belt).[13-16] Increased 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 is not restricted to TJs, and *Ocln*$^{-/-}$ mice exhibit morphologically and electrophysiologically normal TJs, indicating that occludin is dispensable for TJ formation and

barrier function.[17] Similarly, ZO-1 is not required for intestinal permeability barrier maintenance *in vivo*, and primarily modulates actomyosin tension at adherens junctions (AJs) rather than TJ sealing, suggesting that additional transcriptional and structural regulators govern epithelial barrier integrity.[18] In contrast, claudin (CLDN) family proteins directly determine paracellular ion selectivity and permeability.[19] Altered CLDN expression including downregulation of CLDN4, CLDN7 and upregulation of CLDN2, has been reported in CD patients, but causal links to functional barrier defects remain undefined.[15, 20, 21] Furthermore, CLE-based imaging studies have demonstrated increased epithelial permeability *in vivo* in CD patients, even in endoscopically and histologically non-inflamed mucosa, independent of erosions or ulcerations, confirming that barrier dysfunction in CD occurs independently of active inflammation.[4-6, 22-24] This distinction suggests that chronic barrier dysfunction in CD reflects an epithelial reprogramming event rather than an inflammatory byproduct.

Hepatocyte nuclear factor 4 (HNF4) the epithelial-enriched transcription factors play pivotal roles in epithelial differentiation, metabolism, and regeneration. The two mammalian paralogs of HNF4, HNF4α and HNF4γ, display distinct spatial expression patterns along the GI tract. HNF4α is broadly expressed in the intestine, whereas HNF4γ is enriched in the distal ileum, the predominant site of CD pathology.[25, 26] Genome-wide association studies (GWAS) have identified several single nucleotide polymorphisms (SNPs) in HNF4A associated with ulcerative colitis (UC) and childhood-onset CD, whereas no SNPs have been described in HNF4G.[26-29] Although HNF4A mRNA is downregulated in IBD, it remains unclear whether this correlates with loss of HNF4α and HNF4γ protein expression or with measurable barrier defects. Transcriptomic analyses of our *Hnf4α/γ* double knockout (DKO) mice reveal broad downregulation of TJ genes, including *Cldn4*, *Cldn7*, *Cldn15*, *Tjp2* (ZO-2), *F11r* (JAM-A) and *Marveld3*, with concurrent

upregulation of the pore-forming *Cldn2*.[30] These findings indicate that HNF4α and HNF4γ orchestrate a transcriptional program essential for TJ assembly and paracellular barrier maintenance. However, whether their loss directly alters epithelial permeability, whether these mechanisms operate in human IBD, and whether HNF4 activation can therapeutically restore barrier function have not been determined.

In this study, using our transcriptomic analysis of HNF4α/γ-deficient intestinal epithelium together with CD patient-derived tissue and organoids we identify a broad HNF4α/γ-dependent TJ gene network that governs epithelial barrier sealing and paracellular selectivity. We further show that loss of HNF4α/γ defines the primary epithelial defect underlying CD, whereas chronic inflammation can phenocopy this suppression through a physiological epithelial injury response. Finally, pharmacologic activation of HNF4α/γ using plant-derived, non-toxic, translationally ready 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.

Mouse Model of Crohn's Disease. The Institutional Animal Care and Use Committee (IACUC) of the University of Houston approved all animal experiments. *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).[31] Both male and female mice were studied at 20 weeks of age, corresponding to peak spontaneous ileitis in *Tnf*$^{\Delta ARE/+}$ mice.[32]

Human Tissue Collection. Human CD and non-CD control samples were obtained under protocols approved by the Institutional Research Board of Baylor College Medicine (Houston, TX) and The University of Houston (Houston, TX).

Intestinal Mouse and Human Organoid Culture. *Hnf4α/γ*^{DKO} and control mouse organoids were derived from the small intestine as described before.[33] Crypts from distal ileum of *Tnf*$^{\Delta ARE/+}$ and *Tnf*$^{+/+}$ mice were isolated by incubation in 2 mM EDTA and organoids were derived as described before.[34] Distal ileal pinch biopsies collected from non-CD controls and non-inflamed sections of the distal ileum from patients with active ileal and ileocolonic CD, were similarly incubated with EDTA to isolate the intestinal stem cells and derive organoids. At least 50 organoids per sample were assessed for all quantitative assays.

***Ex vivo* Treatment of Mouse and Human Organoids.** *Ex vivo*, murine, and human CD patient-derived organoids (PDOs) were treated with NCT (80 μM, Sigma-Aldrich) or NFT (80 μM, Sigma-Aldrich) for 12 hours and compared with vehicle (DMSO) controls.

Immunohistochemistry. Immunohistochemistry for HNF4α and HNF4γ was performed on paraffin embedded distal ileum, colon, and rectal tissue sections obtained from CD patients with ileal or ileocolonic CD and non-CD controls as described earlier.[35] Images were captured with an Olympus BX53 microscope.

Immunofluorescence analysis of organoids. Organoids were fixed and permeabilized for 30 min with 3.7% paraformaldehyde and 0.3% Triton X-100 dissolved in PBS. Fixed organoids were

treated with primary antibodies in a blocking solution (PBS containing 5% BSA) overnight at 4ºC. After washing with PBS, the organoids were incubated with secondary antibodies. Organoids were imaged on a Fluoview FV2000 laser scanning confocal microscope with a 60X NA 1.35 objective. Fluorescence intensity was generated using ImageJ. Unless otherwise indicated, all data was obtained by performing at least 6 independent experiments.

Epithelial barrier permeability assay using FITC-dextran. The epithelial barrier function was assessed using FITC-dextran 4, 10, or 70 kDa at a final concentration of 1.25 μM, as described before.[36] Briefly, mouse organoids were incubated under normal growth conditions in media containing FITC-dextran for 15, 30, or 60 minutes. Following this incubation, organoids were washed 10 times with PBS to remove non-specific binding of FITC-dextran. Luminal accumulation of FITC-dextran was evaluated in 15 organoids per group selected randomly.

Transepithelial Electrical Resistance (TEER). CD PDOs were 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. CD and non-CD control organoids were dissociated to single cells using TrypIE™ (Gibco, Thermo Fisher Scientific) for 10 min at 37ºC and cultured as monolayers until confluent. 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 and normalized to surface area to calculate net resistance ($\Omega.cm^2$).

Results.

HNF4α/γ Are Essential For Tight Junction Integrity and Intestinal Permeability Barrier Function. Previous RNA-sequencing of intestinal epithelium from *Hnf4α*$^{f/f}$, *Hnf4γ*$^{Crispr/Crispr}$, and the *Hnf4α/γ*^{DKO} mice revealed that only combined deletion of both paralogs of HNF4 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.[33] *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 both the pore and 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 Proteins are Markedly Reduced in the Intestinal Epithelium of Crohn's Disease Patients. 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.

HNF4 Dysregulation in CD Is Independent of Gastrointestinal Inflammation. 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.[30] 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]

Inflammation-Driven TNF Signaling Also Suppresses HNF4α/γ. 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.

HNF4α Agonists Restore Structure and Function of TJs. HNF4 is expressed in the liver, intestine, pancreas, and kidney. In the liver, where it is best studied, N-trans caffeoyltyramine (NCT) and N-trans-feruloyltyramine (NFT) were identified as potent HNF4α agonists and *in vivo* NCT treatment increased intestinal HNF4α protein levels.[43, 44] 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.

HNF4α agonists restore Functional Barrier Integrity in CD. 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.

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.[45]

Here we define hepatocyte nuclear factors HNF4α and HNF4γ as principal regulators of epithelial TJ architecture and paracellular barrier integrity. Combined loss of both paralogs disrupts TJ organization, activates both pore- and leak-pathway permeability, and compromises epithelial resistance. In CD patient tissues, HNF4α and HNF4γ proteins are markedly reduced throughout the intestinal epithelium compared with non-CD controls. Importantly, suppression of both paralogs persists in PDOs from non-inflamed mucosa, establishing that this defect is epithelial-intrinsic and not dependent on inflammatory or stromal cues.

Mechanistically, integration of our transcriptomic dataset with permeability measurements in *Hnf4α/γ*^{DKO} mice revealed that HNF4α/γ orchestrate a broad transcriptional program governing epithelial polarity and TJ integrity, including *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.[30] Notably, ZO-1 (*Tjp1*) remains unchanged in the *Hnf4α/γ*^{DKO} mouse intestine, whereas ZO-2 (*Tjp2*) was selectively repressed, consistent with direct transcriptional control of ZO-2 by HNF4α/γ. Because ZO-1 primarily modulates actomyosin tension at AJs, while ZO-2 primarily regulates actomyosin tension at TJs, selective ZO-2 loss leads to TJ strand fragmentation and decreased TEER, explaining the increased

parcellular permeability in the *Hnf4α/γ*^{DKO} mice and decreased TEER in CD PDOs.[46, 47] Concomitant downregulation of polarity regulators (*Pard6b*, *Prkcz*, *Tjap1*, *Amotl2*) and cytoskeletal scaffold genes *(Vapa, Mpp5, Arhgap17)* 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.[48, 49] 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*) impairs actin remodeling required for TJ assembly, repair, and restitution, linking HNF4α/γ loss to defective junctional remodeling and sustained permeability defects.[30, 50, 51] Together, these data delineate an HNF4α/γ-TJ regulatory axis that maintains claudin-based strand continuity and barrier sealing, whose disruption underlies the increased permeability observed in *Hnf4α/γ*^{DKO} mice and is predicted to drive similar TJ defects in CD epithelium.

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, 30, 52] Simultaneous depletion of *Cldn7*, which stabilizes TJ strands and reseals transient breaks during cell extrusion and peristaltic stress, prevents closure of leak pathway. [50, 53] Downregulation of Cldn15 likely reduces sodium-coupled glucose absorption, consistent with impaired nutrient transport in IBD.[30, 54, 55] Collectively, these findings establish HNF4α/γ as master regulators of paracellular selectivity, TJ strand density, and stability.

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. 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. Consequently, 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 aligns with the higher incidence of penetrating and fibrostricturing disease phenotypes in ileal *versus* colonic CD.[1, 56]

Although 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, 57] In contrast, barrier defects in CD are epithelial-intrinsic, focal, and persistent, often detectable in endoscopically and histologically non-inflamed mucosa, and strongly predictive of relapse and disease progression.[5, 7, 12] Increased permeability is even observed in healthy first-degree relatives of CD patients, underscoring an epithelial-intrinsic basis.[12, 58] The combined down-regulation of HNF4α and HNF4γ unique to CD, disrupts the entire TJ transcriptional program, resulting in chronic, inflammation-independent permeability defects. 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.[27, 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.

Together, our findings reconcile a long-standing discrepancy between human and experimental IBD models namely, why barrier loss in CD is inflammation-independent, whereas in murine colitis it is inflammation-dependent and reversible. In CD our data show that loss of HNF4α/γ represents a primary, epithelial-intrinsic transcriptional defect that persists in PDOs established from non-inflamed mucosa, demonstrating that permeability defect is not secondary to cytokine exposure or mucosal injury. In contrast, in *Tnf*$^{\Delta ARE/+}$ mice, chronic TNF overexpression phenocopies this repression through a physiological epithelial injury response, a normally transient protective mechanism that becomes pathologically stabilized under persistent inflammatory signaling in the *Tnf*$\Delta^{ARE/+}$ mice. During acute infection or cytokine exposure, hepatic HNF4α is transiently suppressed by TNF-α, IL-1β, and NF-kB signaling, triggering dedifferentiation and regeneration.[59, 60] In *Tnf*$^{\Delta ARE/+}$ mice HNF4α/γ and TJ genes remain chronically suppressed, converting this adaptive epithelial response into a self-sustaining barrier defect.

A key translational insight from these findings is that the HNF4-dependent epithelial barrier program is pharmacologically reversible. We demonstrate that the naturally occurring hydroxycinnamic acid amides, NCT and NFT, potent, non-toxic HNF4 agonists, reactivate both HNF4α and HNF4γ expression, restoring transcription of TJ genes and re-establishing barrier integrity.[61-63] 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.[64] By reinstating this transcriptional network, NCT and NFT normalize TJ composition and rescue TEER to near-control levels, demonstrating that HNF4 repression in CD is not

irreversible but therapeutically correctable. 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 disease persistence.

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.[65] 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.

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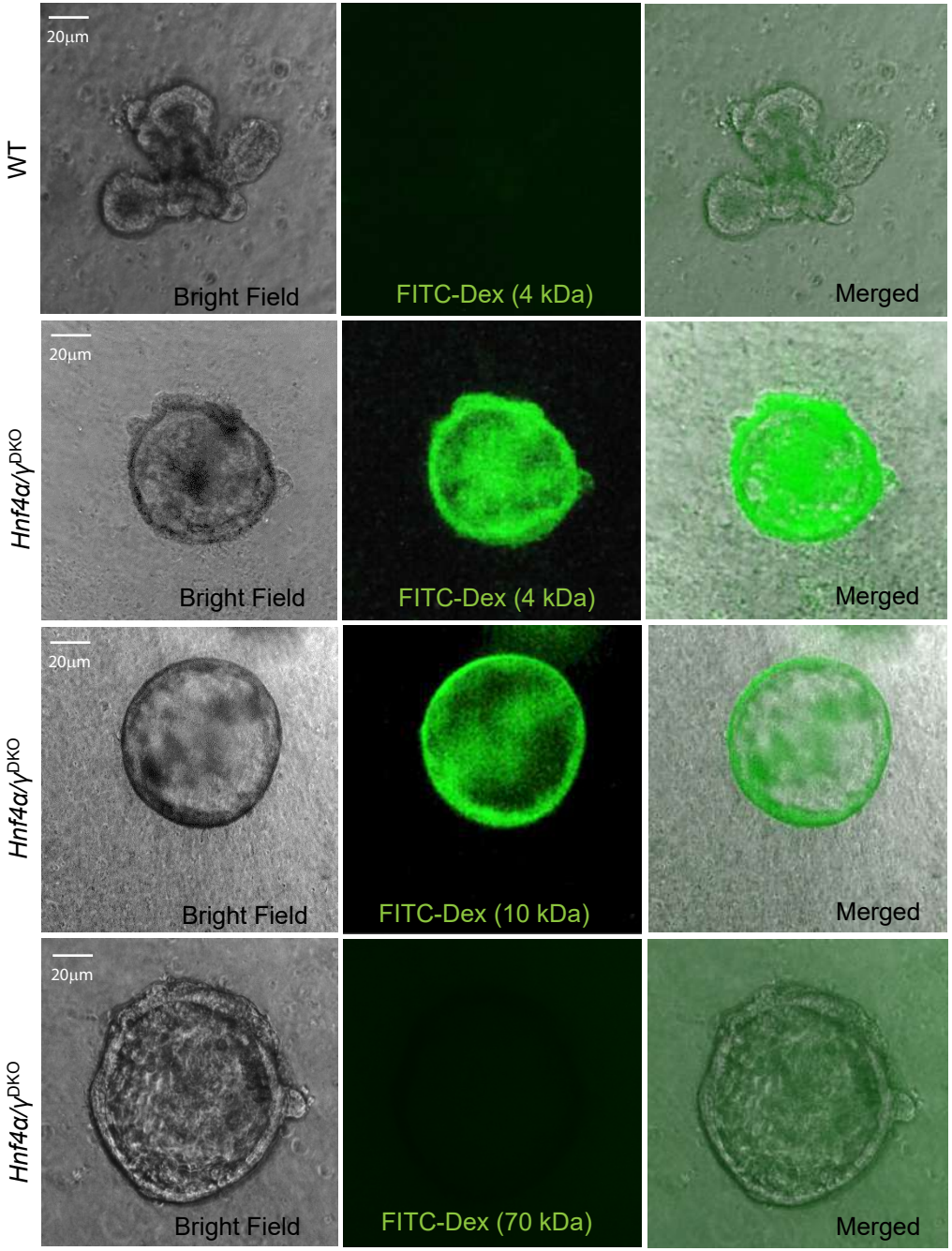
Figure 1. Loss of HNF4α/γ activates both pore and leak pathway permeability in intestinal organoids. Representative phase-contrast and confocal images of FITC-dextran (4, 10, 70 kDa) diffusion into the lumen of wild-type (WT) and *Hnf4α/γ*^{DKO} mouse intestinal organoids. FITC-dextran was applied to 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)

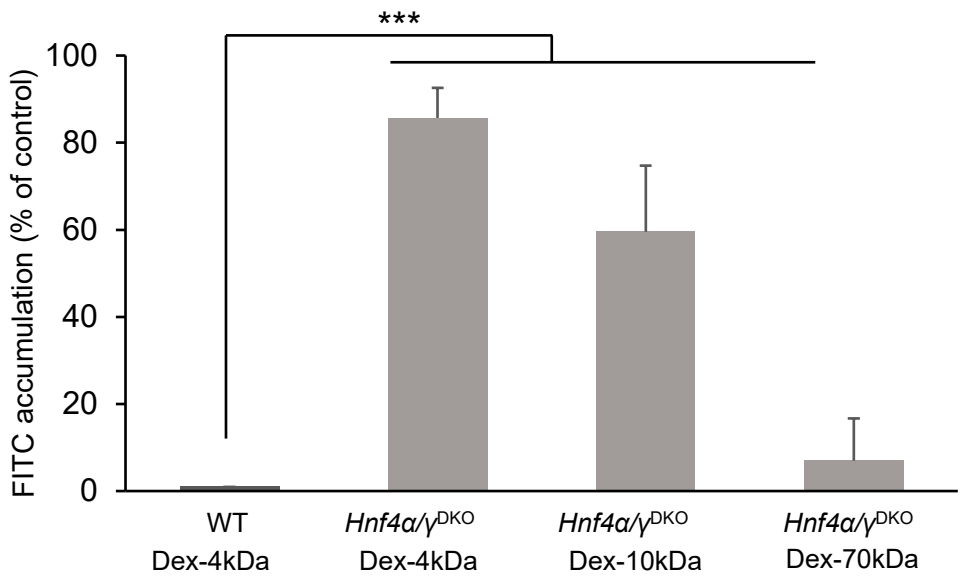

***
FITC accumulation (% of control)
100
80
60
40
20
0
WT Dex-4kDa
Hnf4α/γDKO Dex-4kDa
Hnf4α/γDKO Dex-10kDa
Hnf4α/γDKO Dex-70kDa

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

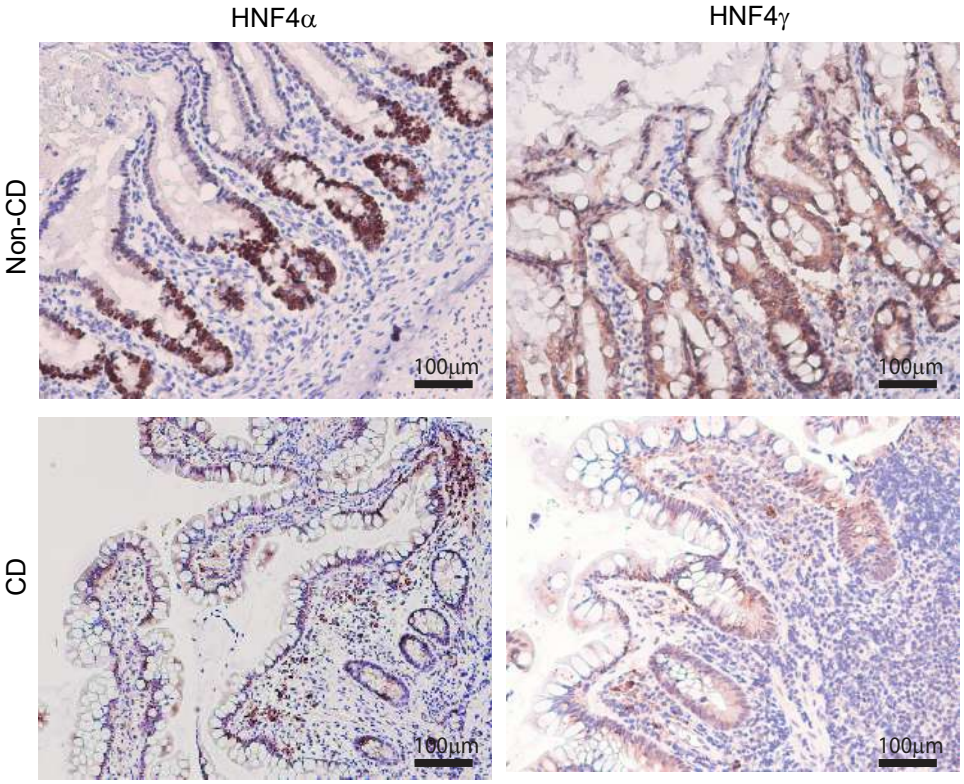

HNF4α
HNF4γ
Non-CD
CD
100μm
100μm
100μm
100μm

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

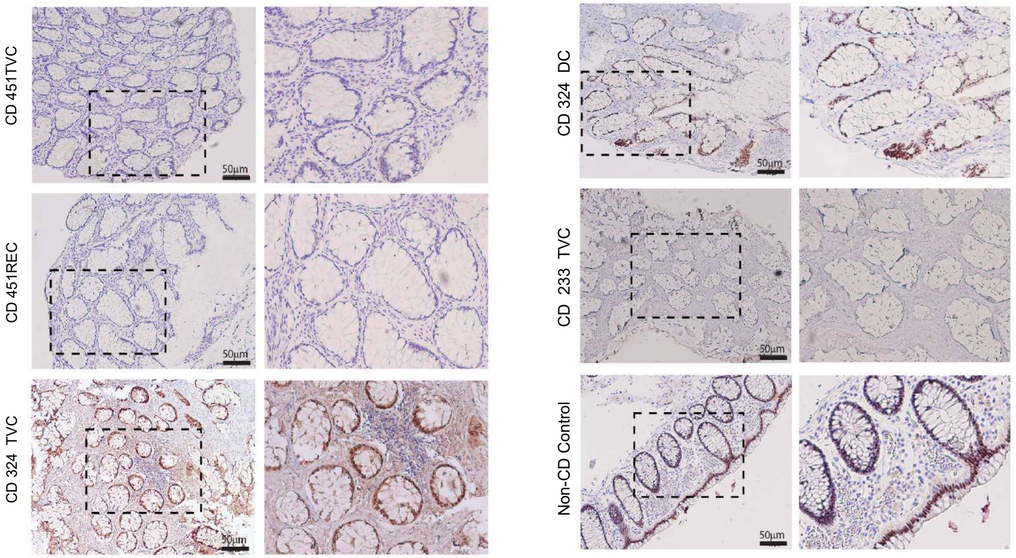
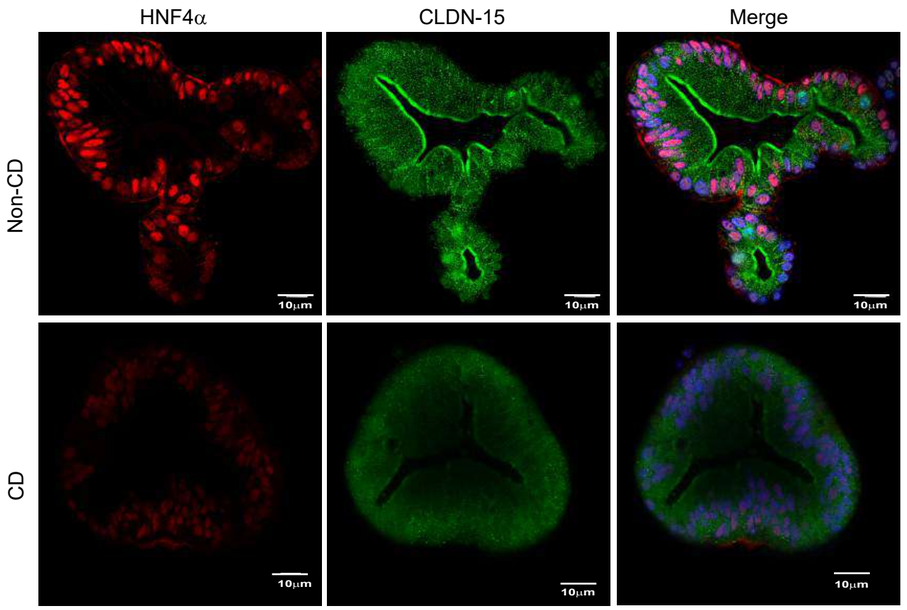

CD 451TVC
CD 451REC
CD 324 TVC
CD 324 DC
CD 233 TVC
Non-CD Control
50μm

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

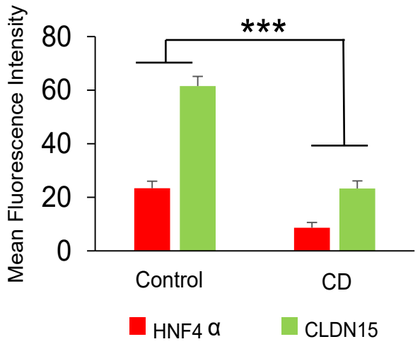
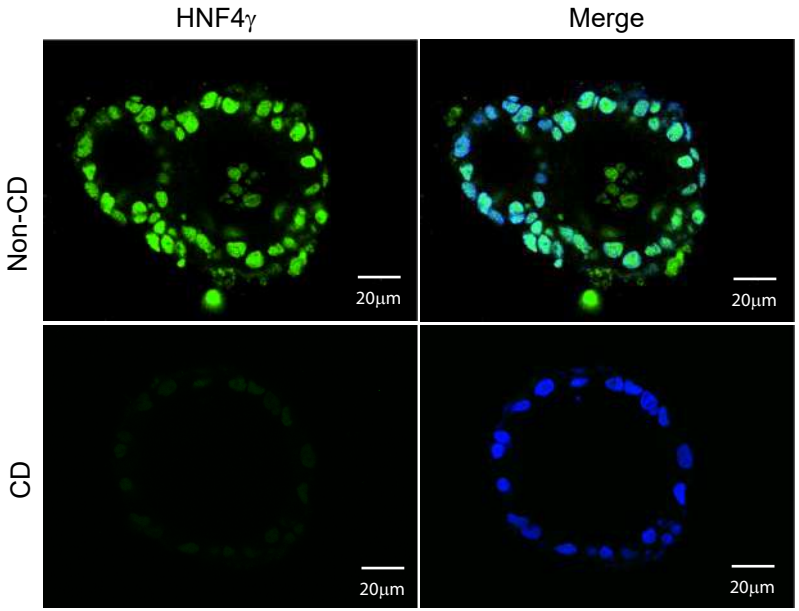

Mean Fluorescence Intensity
80
60
40
20
0
***
Control
CD
HNF4 α
CLDN15

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

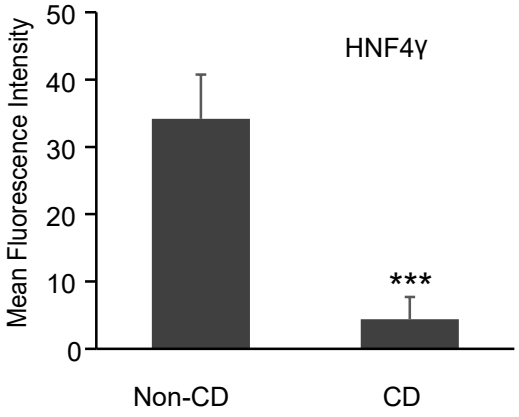

HNF4γ
Mean Fluorescence Intensity
50
40
30
20
10
0
***
Non-CD
CD

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

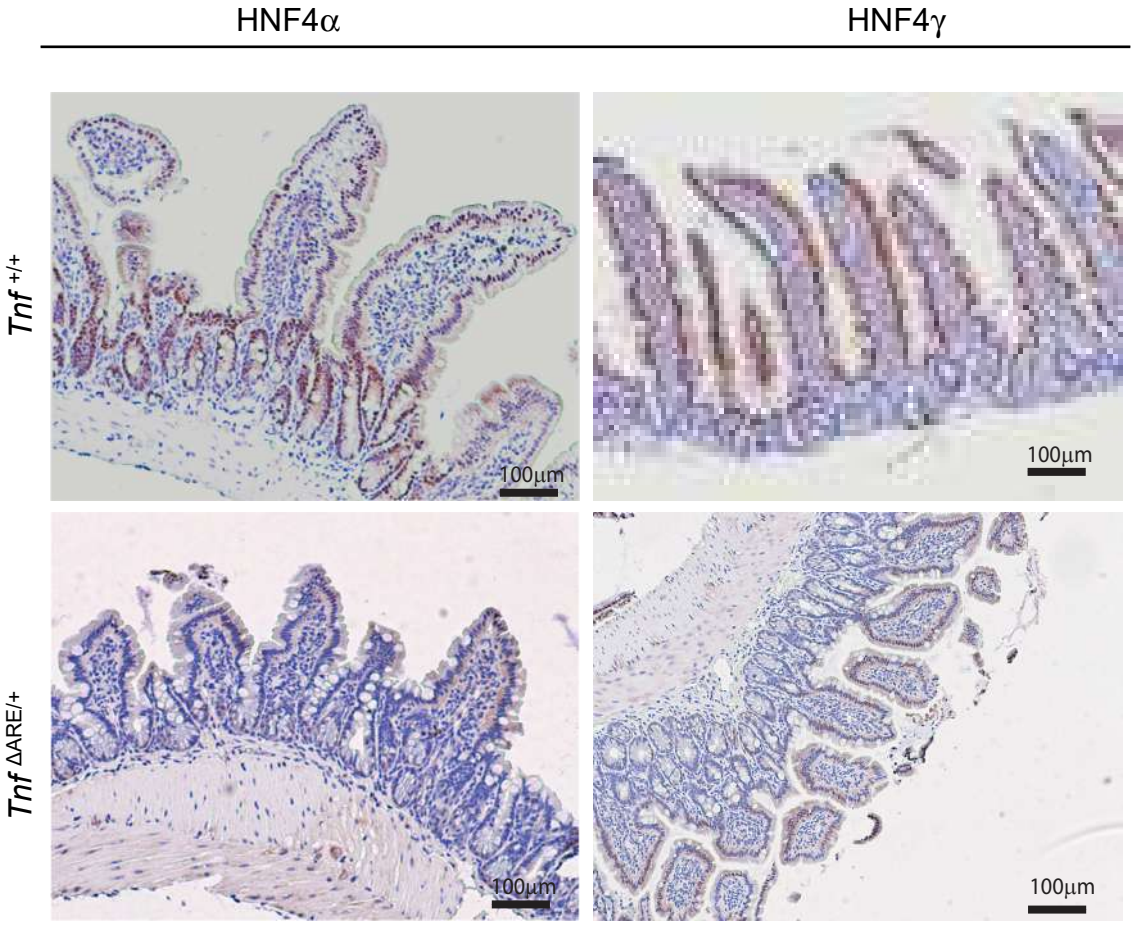

HNF4α
HNF4γ
Tnf +/+
Tnf ΔARE/+
100μm
100μm
100μm
100μm

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.

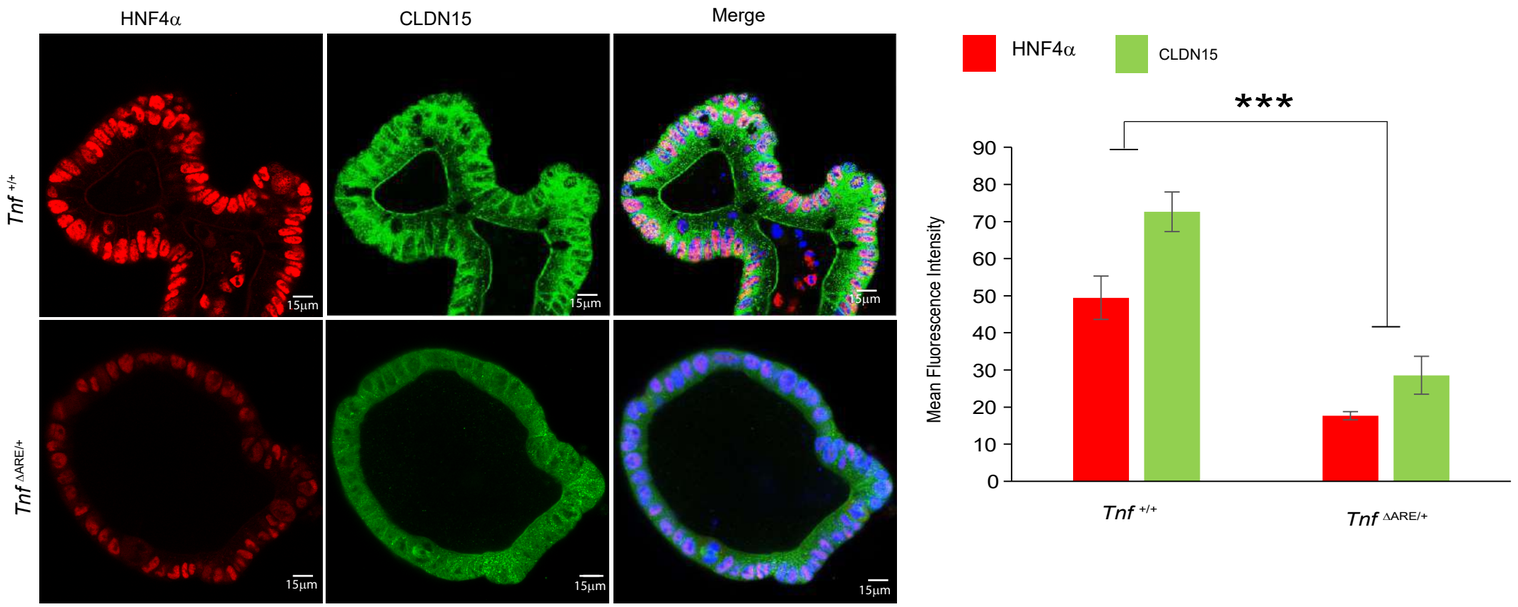

HNF4α
CLDN15
Merge
Tnf +/+
Tnf ΔARE/+
15µm
HNF4α
CLDN15
***
Mean Fluorescence Intensity
90
80
70
60
50
40
30
20
10
0
Tnf +/+
Tnf ΔARE/+

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.

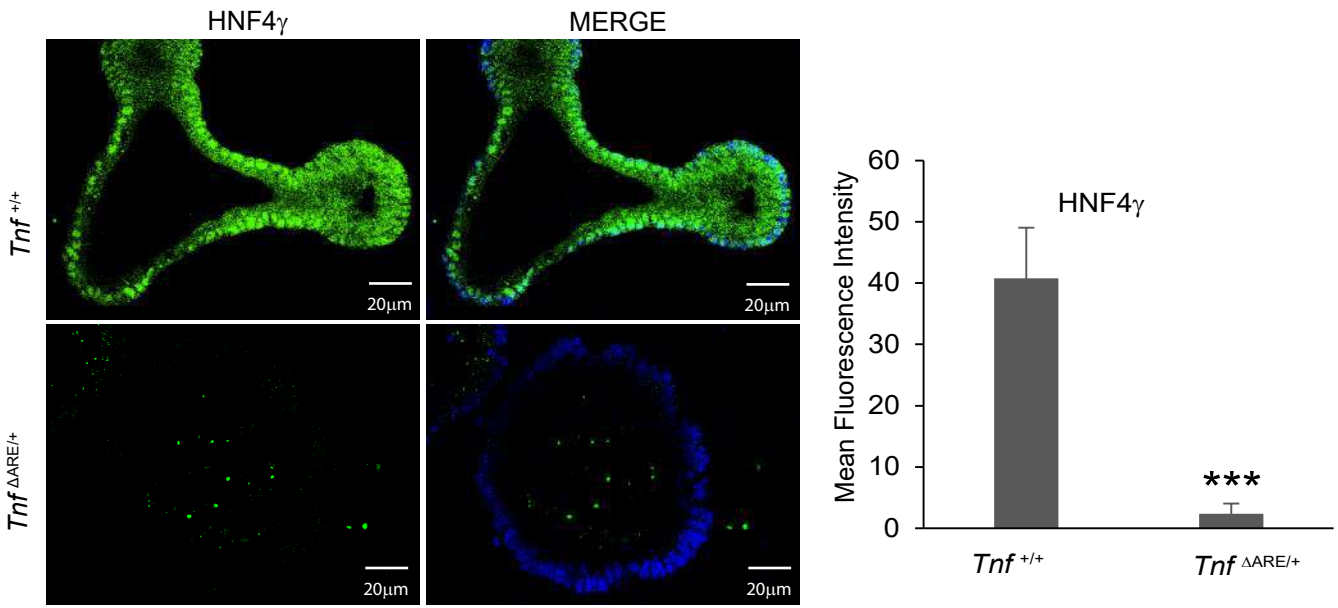
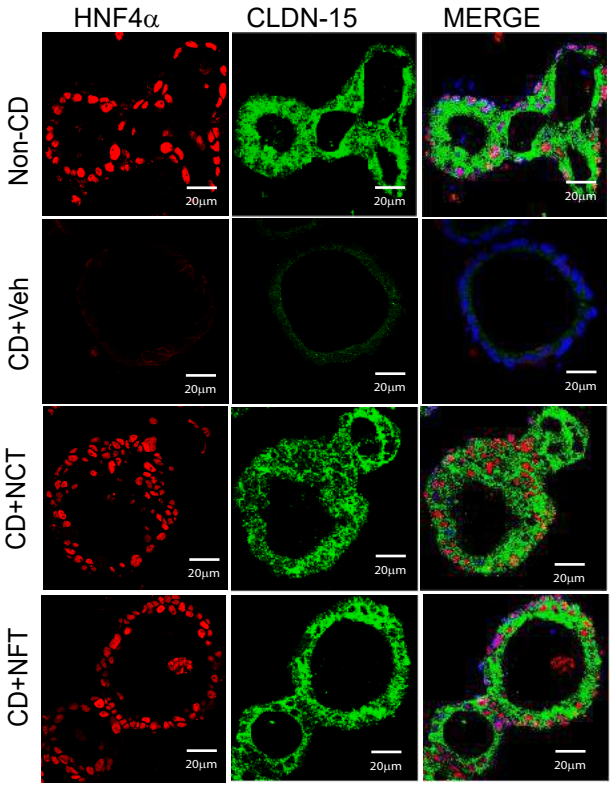

HNF4γ
MERGE
Tnf +/+
Tnf ΔARE/+
20μm
20μm
20μm
20μm
Mean Fluorescence Intensity
60
50
40
30
20
10
0
HNF4γ
***
Tnf +/+
Tnf ΔARE/+

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

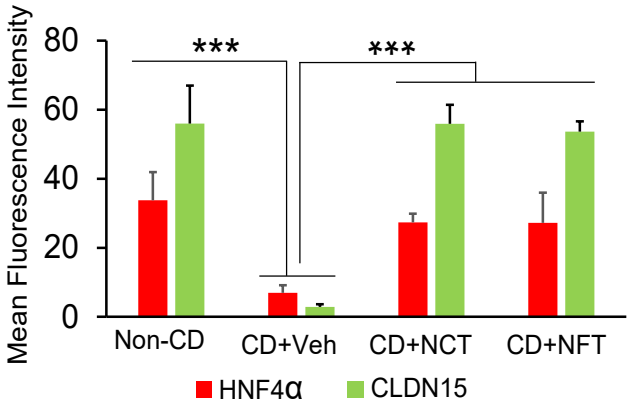
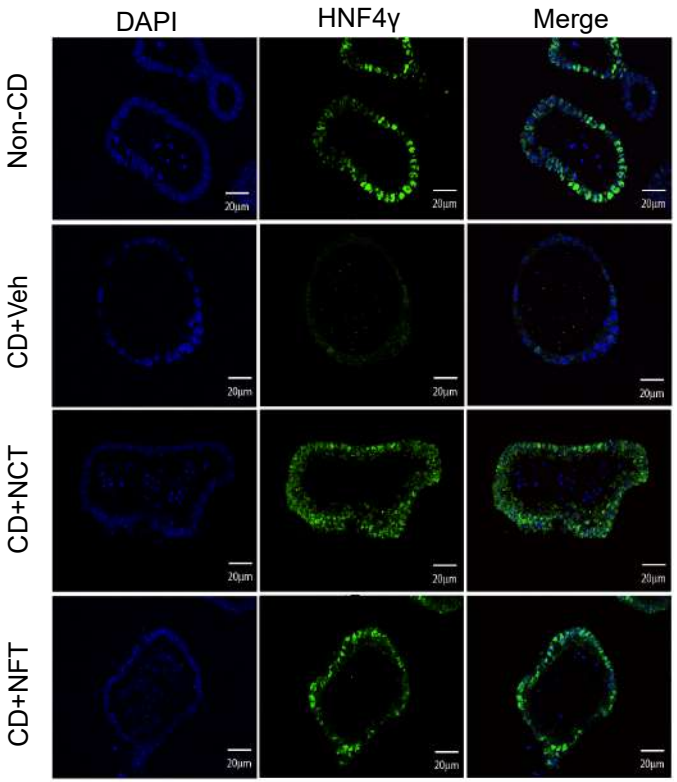

Mean Fluorescence Intensity
80
60
40
20
0
***
***
Non-CD
CD+Veh
CD+NCT
CD+NFT
HNF4α
CLDN15

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

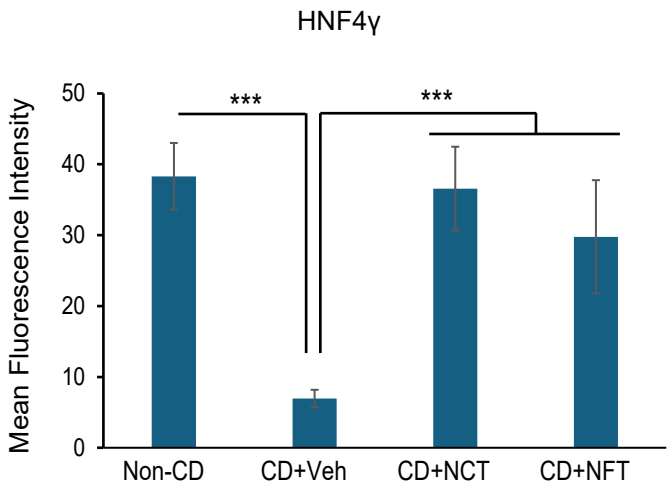
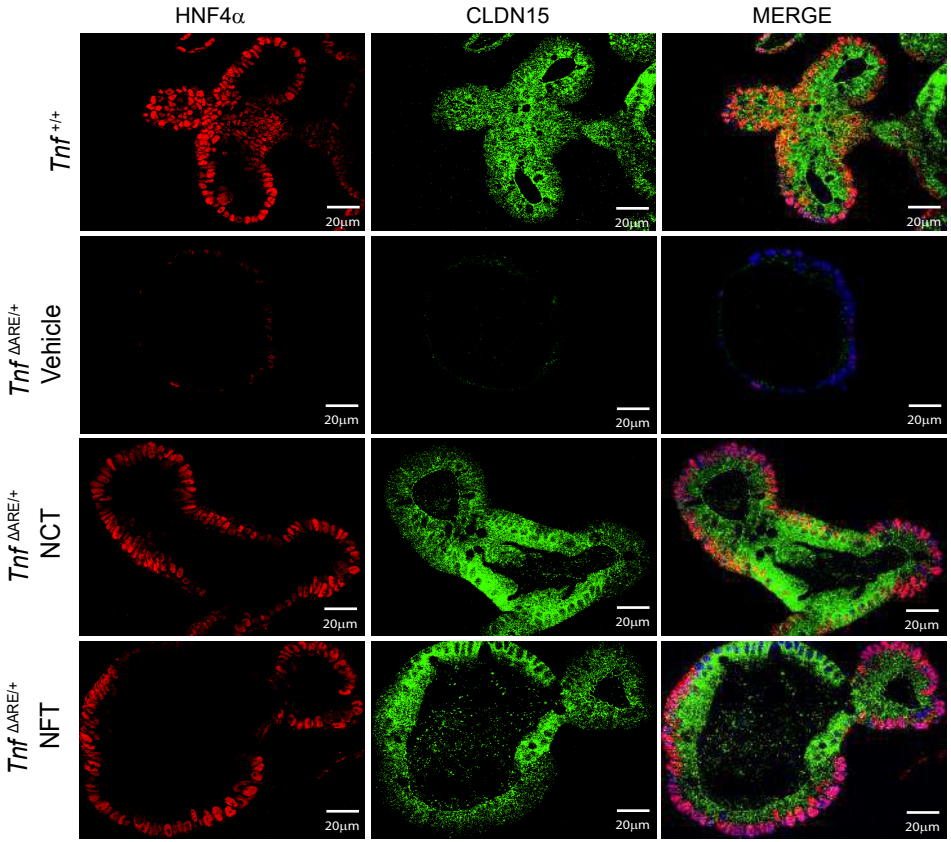

HNF4γ
Mean Fluorescence Intensity
50
40
30
20
10
0
***
***
Non-CD
CD+Veh
CD+NCT
CD+NFT

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

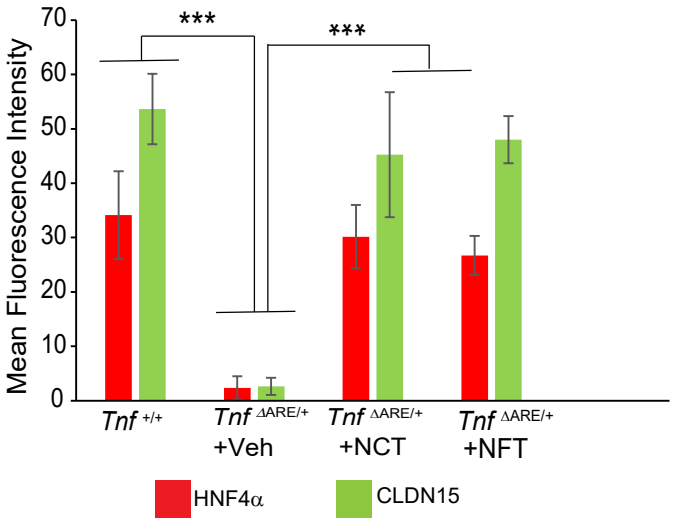
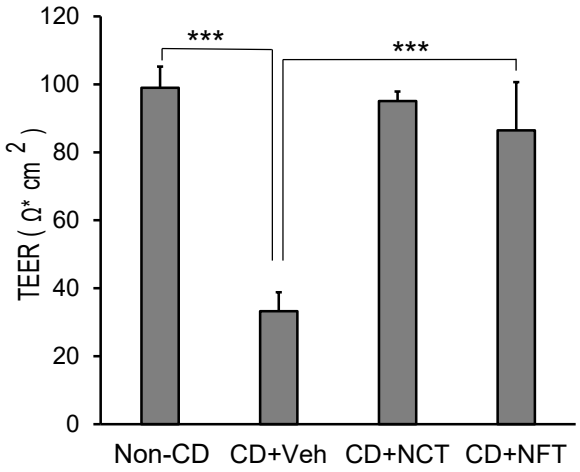

Mean Fluorescence Intensity
70
60
50
40
30
20
10
0
***
***
Tnf +/+
Tnf ΔARE/+ +Veh
Tnf ΔARE/+ +NCT
Tnf ΔARE/+ +NFT
HNF4α
CLDN15

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