Combined suppression of HNF4α and HNF4γ Drives a Reversible Epithelial-Intrinsic Barrier Defect in Crohn's Disease.

Short Title: HNF4 paralogs and barrier dysfunction in Crohn's disease.

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Abbreviations used in this paper: BH, intestinal barrier healing; CD, Crohn's disease; CLDN, claudin; CLE, confocal laser endomicroscopy; DAPI, 4',6-diamidino-2-phenylindole; DKO, double knockout; DMSO, dimethyl sulfoxide; ER, endoscopic remission; FITC, fluorescein isothiocyanate; GWAS, genome-wide association study; HNF, hepatocyte nuclear factor; HNF4αγ, HNF4α and HNF4γ; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; KO, knockout; MH, mucosal healing; NCT, N-trans-caffeoyltyramine; NFT, N-trans-feruloyltyramine; PDO, patient-derived organoid; TEER, transepithelial electrical resistance; TJ, tight junction; TNF, tumor necrosis factor; WT, wild-type.

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

Abstract.

Background and aims. Persistent intestinal permeability dysfunction is a defining feature of Crohn's Disease (CD) that precedes clinical disease onset, persists despite endoscopic remission (ER), occurs in non-inflamed mucosa, and strongly predicts relapse and disease progression. However, the molecular mechanisms governing inflammation-independent permeability defects in CD remain poorly defined. We investigated whether the epithelial transcription factors hepatocyte nuclear factors 4α (HNF4α) and its paralog HNF4γ cooperatively regulate tight junction (TJ) architecture and epithelial barrier function in CD.

Methods. Transcriptomic profiling of small intestinal and colonic epithelial cells from *Hnf4αγ* double knockout (DKO) mice was integrated with functional permeability analyses in genetic ($Hnf4\alpha\gamma^{DKO}$) and inflammatory ($Tnf^{\Delta ARE/+}$) mouse models, CD patient tissues, and patient-derived organoids (PDOs). Barrier function was assessed using FITC-dextran permeability assay and transepithelial electrical resistance (TEER). Pharmacologic activation of HNF4 signaling was used to determine whether restoration of HNF4 activity rescues epithelial barrier defects.

Results. Combined loss of *Hnf4α* and *Hnf4γ* caused broad downregulation of TJ-associated gene networks in the small intestine but not the colon, and produced size-selective permeability defects, demonstrating segment-specific, transcriptional regulation of intestinal barrier integrity. HNF4α and HNF4γ proteins and their downstream TJ targets were markedly reduced in ileal tissue and PDOs derived from non-inflamed CD mucosa, establishing the presence of an epithelial-intrinsic

barrier defect independent of active inflammation. Chronic TNF signaling phenocopied suppression of HNF4 paralogs and their downstream junctional proteins, by stabilizing injury repair responses in the *Tnf*$^{\Delta ARE/+}$ mice. Importantly, pharmacologic activation of HNF4 signaling restored TJ gene expression and rescued barrier function in CD PDOs and *Tnf*$^{\Delta ARE/+}$ mouse organoids.

Conclusion. Downregulation of HNF4α and HNF4γ defines an inflammation-independent transcriptional program driving epithelial barrier dysfunction in CD. Restoration of HNF4 signaling reverses permeability defects, identifying HNF4-dependent transcriptional programs as candidate biomarkers of intestinal permeability, and establishing epithelial-directed barrier restoration as a potential therapeutic strategy.

Key words: Crohn's disease; epithelial barrier dysfunction; intestinal permeability, tight junction regulation, claudins; HNF4α/γ paralogs; barrier restoration/reversibility.

Introduction.

Disruption of intestinal epithelial barrier function is increasingly recognized as a central feature of CD that is present in non-inflamed mucosa, persists despite clinical remission, and strongly predicts disease relapse and progression.[1-4] Importantly, increased intestinal permeability precedes clinical disease onset in asymptomatic first-degree relatives of CD patients, providing compelling evidence that barrier dysfunction represents a primary determinant of disease susceptibility rather than a consequence of inflammatory injury.[5-7] Endoscopic remission (ER), defined as the absence of mucosal ulceration, is the current therapeutic goal in CD because it

correlates with improved clinical outcomes.[8] However, a substantial proportion of patients in ER exhibit persistent permeability defects that are strongly associated with relapse and progression to penetrating or fibrostricturing complications.[1,3,4,9-11] Consistent with these findings, restoration of epithelial barrier integrity, termed barrier healing (BH), has emerged as a more accurate predictor of durable remission and long-term disease modification than ER alone.[1] Despite its clinical significance, no therapies directly target BH, and barrier dysfunction is not routinely assessed in CD management because currently available assays lack sensitivity, reproducibility, or scalability. Functional tests like lactulose-mannitol ratio show high variability and do not localize barrier defects.[12] Serum markers including albumin, C-reactive protein, and zonulin, do not reliably correlate with BH.[1] Confocal laser endomicroscopy enables real-time *in vivo* visualization of epithelial barrier defects in CD patients, but its high cost, technical complexity, and limited access to the distal ileum restricts widespread clinical use.[1,13] Together, these limitations highlight the need for scalable and reliable approaches to identify and monitor epithelial barrier dysfunction in CD.

Traditional models of CD pathogenesis attribute barrier dysfunction primarily to inflammatory cytokine-mediated disruption of TJ architecture. Experimental studies demonstrate that cytokines such as TNF-α, IL-1β, and IL-13 induce cytoskeletal remodeling and alter the localization or expression of key junctional proteins such as occludin and ZO-1, thereby impairing epithelial barrier integtrity.[14-16] However, multiple *in vivo* studies in CD patients have demonstrated increased intestinal permeability in macroscopically normal, non-inflamed mucosa, including endoscopically unaffected epithelium.[1,9-11,17-19] These observations indicate that barrier dysfunction in CD cannot be fully explained by inflammatory injury alone and instead support the presence of epithelial-intrinsic defects independent of overt inflammation. Furthermore, genetic

deletion studies demonstrate that individual TJ proteins previously thought to be essential for barrier integrity, such as occludin and ZO-1, are dispensable for baseline intestinal permeability regulation *in vivo*.[14,20-24] Ultrastructural analyses of CD patient tissue have revealed that TJ architecture is severely altered, accompanied by altered expression of select claudins (CLDNs), including *CLDN3*, *CLDN5*, and *CLDN8*.[17] Nevertheless, deletion of individual *CLDNs* does not produce baseline permeability defects *in vivo*, suggesting that permeability barrier dysfunction arises from coordinated remodeling of TJ architecture rather than loss of single junctional components. Collectively, these observations indicate that intestinal permeability defects in CD arise from disruption of transcriptional programs governing epithelial TJ organization; however, the transcriptional mechanisms coordinating these processes remain poorly defined.

HNF4 transcription factors, HNF4α and HNF4γ (collectively referred to here as HNF4αγ), are epithelium-restricted paralogs that serve as key regulators of intestinal epithelial homeostasis.[25] These paralogs function cooperatively to maintain epithelial identity and function, and exhibit significant functional redundancy.[25-27] *In vivo* deletion of either paralog alone produces minimal intestinal abnormalities due to compensatory activity of the remaining paralog.[26,27] Accordingly, studies implicating *Hnf4α* in intestinal permeability regulation based on single-gene knockout (KO) models must be interpretated in the context of paralog compensation. Although *Hnf4α* KO mice exhibit altered permeability in the small intestine, barrier defects are not observed in the colon, where *Hnf4α* is the predominant paralog.[25,26,28-34] Conversely, the small intestine co-express high levels of *Hnf4γ*, which should preserve intestinal permeability barrier programs following *Hnf4α* loss.[25,26,28-34] These findings highlight the importance of paralog-specific expression patterns and limit interpretation of single-gene KO studies linking HNF4α to regulation of intestinal permeability. In contrast, simultaneous deletion of *Hnf4α* and *Hnf4γ* unmasks their

essential cooperative functions and results in severe epithelial dysfunction *in vivo*.[25,26,35] Although transcriptomic data from *Hnf4αγ*^{DKO} intestinal epithelium have been reported, their relevance to regulation of intestinal permeability has not been examined. Genome-wide association studies have identified *HNF4A* as a susceptibility locus for childhood-onset CD, and reduced *HNF4A* expression has been reported in intestinal tissue from CD patients.[28,36] However, whether disease-associated downregulation of *HNF4* paralogs occur at the protein level and contribute to permeability dysfunction in CD patients remains unknown.

In this study, we investigated whether cooperative activity of the epithelial transcription factors HNF4α and HNF4γ regulates transcriptional programs that maintain intestinal permeability barrier integrity and whether disruption of these programs contributes to permeability dysfunction in CD. Using complementary genetic mouse models, an inflammatory model of ileitis, human CD tissue, and PDOs from non-inflamed mucosa, we examined the role of HNF4 paralogs in regulating TJ gene networks and epithelial barrier integrity. We further evaluated whether inflammatory signaling inhibits HNF4 paralog activity and whether restoration of HNF4 signaling restores epithelial barrier function. These studies identify HNF4-dependent transcriptional programs as key regulators of epithelial barrier integrity and establish loss of HNF4 paralog activity as a mechanism underlying inflammation-independent barrier dysfunction in CD.

Materials and Methods.

Human Tissue. Intestinal mucosal samples were obtained from ileal CD patients and non-IBD control individuals undergoing endoscopy under institutional Review Board-approved protocols at Baylor College of Medicine. Non-inflamed mucosa was used for epithelial isolation and generation of patient-derived organoids where indicated.

Human Patient-derived Intestinal Organoids. Intestinal crypts were isolated from human mucosal samples and embedded in Matrigel. Organoids were cultured in defined growth medium supplemented with epithelial niche factors.[37] Organoid were expanded before analysis, and experiments were performed using organoids between passages 3-8. Quantification was performed on ≥50 organoids per condition derived from independent patient-derived organoid cultures.

Mouse Strains and Intestinal Organoids. *Villin-Cre*^{ERT2}, *Hnf4α*$^{f/f}$, *Hnf4γ*$^{Crispr/Crispr}$, and *Tnf*$^{\Delta ARE/+}$ mice were maintained under institutional animal care guidelines at Rutgers University or the University of Houston. Intestinal tissues were collected for histologic and immunofluorescence analyses, and intestinal crypts were isolated from mouse small intestine for organoid culture under conditions like those used for human organoids.[35]

Organoid-derived epithelial monolayers and barrier assays. Intestinal organoids were dissociated into single cells and seeded onto collagen-coated Transwell inserts (20 μg/ml collagen type 1 (rat tail) in 0.01% acetic acid; 0.4 μm pore size, Corning) to generate organoid monolayers. Barrier integrity was assessed using transepithelial electrical resistance (TEER) measurements with an EVOM2 epithelial voltohmmeter. Each insert was measured three times, and values were corrected for background resistance and normalized to surface area.

Organoid Permeability Assay. *Hnf4αγ*^{DKO} and wild-type (WT) mouse organoids were incubated with fluorescent FITC-dextran tracers of 4-, 10-, or 70-kDa size for 15, 30, or 60 minutes, and luminal fluorescence accumulation was quantified as described before.[38] Each tracer was applied

independently. Luminal accumulation was quantified in 15 randomly selected organoids per genotype.

Pharmacologic Treatment. Organoids or epithelial monolayers were treated with vehicle (DMSO) or the HNF4 agonists N-trans-caffeoyltyramine (NCT) and N-trans-feruloyltyramine (NFT) at 80 μM for 12 hours prior to confocal imaging and barrier function analyses.

RNA Sequencing and Transcriptomic Analysis. RNA sequencing was performed on intestinal epithelial cells isolated from mouse colon, as described previously.[39] RNA was extracted using the Qiagen RNeasy Micro Kit. Transcript abundance was quantified using Kallisto (v0.44.0), and differential expression analysis was performed using DESeq2 (1.48.2) in R (v4.5.1/RStudio) Bioconductor version 3.2.1. Experiments included three biological replicates per genotype.

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.[40] Images were captured using an Olympus BX53 microscope. Organoids were fixed, permeabilized, and stained with antibodies against HNF4α, HNF4γ, or CLDN15. Fluorescent images were acquired using a Fluoview FV2000 confocal microscope (60x objective) and analyzed using ImageJ.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism or R. Data are presented as mean ± SEM from ≥ 6 independent experiments. Statistical significance was

determined using unpaired two-tailed Student's t-test, Mann-Whitney test, or Kruskal-Wallis test followed by post-hoc Dunn's test as appropriate.

Results.

HNF4 paralogs regulate tight junction gene networks and epithelial barrier function. To determine whether HNF4 paralogs regulate transcriptional programs regulating intestinal epithelial barrier integrity, we analyzed previously reported transcriptomic data from small intestinal epithelial cells isolated from *Hnf4α*^{KO}, *Hnf4γ*^{KO}, and *Hnf4αγ*^{DKO} mice generated by the Verzi group. Combined loss of *Hnf4α* and *Hnf4γ* but not deletion of either paralog alone, produced widespread transcriptional remodeling of genes associated with TJ organization and epithelial barrier function in the small intestine.[26] Loss of *Hnf4* paralogs resulted in pronounced transcriptional changes in small intestinal epithelium, where 102 TJ-associated genes were significantly downregulated and one was significantly upregulated. These included reduced expression of multiple genes regulating TJ assembly and membrane organization, including *F11r* (which encodes for JAM-A), *Magi3* (MAGI3), *Tjap1*, *Marveld3*, *and Pals1* (MPP5), as well as genes linking TJs to polarity complexes and the actin cytoskeleton such as *Tjp2* (ZO-2), *Pard6b*, *Prkcz* (aPKCζ), *and Apc*. Genes regulating junctional assembly and actin dynamics including *Pak1*, *Myo1e*, *Palld*, and *Zyx,* were also significantly reduced. Consistent with disruption of epithelial barrier programs, expression of barrier-associated claudins *Cldn4*, *Cldn7, and Cldn15* was markedly decreased, whereas expression of permeability-associated claudin, *Cldn2* was significantly increased in the small intestine of *Hnf4αγ*^{DKO} mice.[17,26,41] In contrast, only seven TJ-associated genes (*Ocln*, *Tjp3*, *Marveld2*, *Marveld3*, *Cldn1*, *Cldn2*, *Cldn3*) were significantly altered in the colonic epithelium. Reanalysis of published ileal CD transcriptomic datasets

demonstrated dysregulation of *HNF4A*, *HNF4G*, and multiple TJ-associated genes, including reduced expression of *CLDN4*, *CLDN7*, *CLDN15*, *MAGI3*, *F11R* and *TJAP2*, together with increased *CLDN2* expression.[28,42,43] Several of these genes represent direct or predicted HNF4 transcriptional targets, supporting the conclusion that suppression of HNF4 paralogs disrupts coordinated epithelial barrier programs in CD.

To determine whether these transcriptional changes produce functional barrier defects, we assessed paracellular permeability in intestinal organoids derived from the small intestine of WT and Hnf*4αγ*^{DKO} mice using FITC-dextran tracers of defined molecular sizes (4-, 10-, and 70-kDa). WT organoids excluded FITC-dextran tracers for up to 30 minutes, consistent with slow diffusion across intact TJs [Figure 1]. In contrast, *Hnf4αγ*^{DKO} organoids exhibited a size-selective increase in paracellular permeability to 4- and 10-kDa tracers, whereas permeability to 70-kDa dextran remained unchanged [Figure 1]. Organoid morphology remained intact but displayed smaller epithelial buds, consistent with previous observations in the *Hnf4αγ*-deficient organoids.[35]

Among the TJ-associated genes altered in *Hnf4αγ*^{DKO} mice, *Cldn15* was selected as a representative readout of HNF4-dependent transcriptional activity for subsequent analyses. *Cldn15* is a small intestine-restricted direct transcriptional target of *Hnf4α* and plays a key physiological role in regulating paracellular Na^+ transport in the intestinal epithelium.[30,44,45] Consistent with these findings, *CLDN15* mRNA expression is markedly reduced in ileal tissue of CD patients, supporting the use of CLDN15 as a representative marker of HNF4-dependent TJ gene regulation in the small intestine.[42]

HNF4α and HNF4γ suppression defines an epithelial-intrinsic defect in CD. Although genetic and transcriptomic studies implicate *HNF4A* in IBD pathogenesis, the roles of HNF4 paralogs

HNF4α and HNF4γ in regulating intestinal epithelial dysfunction remain poorly understood.[28] In particular, whether disease-associated alterations in HNF4 paralog expression occurs at the protein level in the intestinal epithelium of CD patients has not been demonstrated. To address this question, we examined HNF4α and HNF4γ protein expression in intestinal biopsies from CD patients and non-CD controls. In non-CD control ileal epithelium, HNF4α exhibits strong nuclear localization in both the proximal and distal crypt, demonstrating that both P1 and P2 isoforms of HNF4α are expressed in the normal ileum [Figure 2A].[25] On the other hand, 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]. Remarkably, HNF4γ protein was also strongly downregulated in the same tissue samples, confirming the combined loss of both paralogs in the disease epithelium [Figure 2A]. Similarly, HNF4α expression was also 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 that HNF4γ is also downregulated, a change not captured by earlier transcriptomic data from CD patient samples.[28] Together, these results establish pan-intestinal HNF4α downregulation and HNF4γ silencing in the small intestine as features of the CD epithelium.

To determine whether these alterations reflect epithelial-intrinsic changes rather than secondary effects of inflammation, we examined PDOs from non-inflamed ileal mucosa form CD patients. 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 change was accompanied by reduced expression of CLDN15, the canonical HNF4 target protein

used here as a representative marker of HNF4 transcriptional activity and TJ remodeling.[26] HNF4γ protein was nearly undetectable in CD PDOs compared with robust nuclear staining in control organoids ($p<0.0001$, n=8 patients per group) [Figure 3B]. The magnitude of HN4αγ loss in the PDOs closely mirrored findings in CD patient tissues, demonstrating that suppression of these transcriptional factors persists independently of inflammatory or stromal cues. Together, these results support the conclusion that the combined downregulation of HNF4αγ is associated with TJ-associated protein dysregulation in CD patient-derived epithelium. Morphologically, CD PDOs exhibited a spheroid phenotype similar to that observed in organoids derived from the small intestine of *Hnf4αγ*^{DKO} mice and consistent with previously reported CD organoid phenotypes.[46,47] Because TJs contribute to epithelial polarity and epithelial differentiation, disruption of TJ architecture can alter epithelial morphology even in the absence of primary defects in differentiation pathways.[48] To determine whether inflammatory injury contributes to suppression of HNF4 paralogs *in vivo*, we examined distal ileal epithelium in *Tnf*$^{\Delta ARE/+}$ mice, a well-established model of chronic TNF-driven ileitis.

Chronic inflammation suppresses HNF4 paralogs and epithelial TJ programs. In response to acute liver injury, HNF4α is transiently downregulated to permit hepatocyte proliferation and regeneration, followed by re-expression of HNF4α to restore differentiated hepatocyte function and tissue homeostasis.[49] Based on this paradigm, we hypothesized that chronic intestinal inflammation may lead to sustained suppression of HNF4αγ expression, thereby contributing to persistent epithelial barrier dysfunction *in vivo*. To test this, we examined distal ileal epithelium from *Tnf*$^{\Delta ARE/+}$ mice, a well-established model of TNF-driven ileitis. In control *Tnf*$^{+/+}$ mice, *Hnf4α* exhibited strong nuclear localization, while *Hnf4γ* was detected in both nuclear and cytoplasmic

compartments [Figure 4A]. In contrast, distal ileal epithelium from *Tnf*$^{\Delta ARE/+}$ mice demonstrated marked reduction of *Hnf4α* nuclear staining. *Hnf4γ* was similarly reduced in the distal ileum compared with *Tnf*$^{+/+}$ controls [Figure 4A], indicating combined inhibition of both paralogs in chronically inflamed intestinal epithelium. To determine whether inflammatory signaling induces stable epithelial changes independent of immune or stromal factors, we analyzed organoids derived from *Tnf*$^{\Delta ARE/+}$ mice. Compared with *Tnf*$^{+/+}$ controls, *Tnf*$^{\Delta ARE/+}$ organoids exhibited significantly reduced HNF4α and CLDN15 expression ($p<0.0001$, n=6 mice per genotype) [Figure 4B]. HNF4γ expression was also markedly reduced in the *Tnf*$^{\Delta ARE/+}$ mouse organoids ($p<0.0001$, n=6 mice per genotype) [Figure 4C]. Because these organoids were cultured *ex vivo* in the absence of inflammatory signaling, these findings indicate that chronic inflammatory injury can induce persistent suppression of HNF4αγ and downstream TJ-associated proteins.

Pharmacologic activation of HNF4 restores epithelial barrier function. Because HNF4αγ are ligand-responsive nuclear receptors that maintain their expression through autoregulatory transcriptional feedback, we tested whether pharmacologic activation could restore HNF4-dependent epithelial programs.[50] NCT and NFT are plant-derived ligands that bind HNF4α and activate HNF4-dependent transcriptional programs.[51-53] Both compounds increase *HNF4A* expression and transcriptional activity in the liver and intestinal epithelium.[51,52] To determine whether pharmacologic activation of HNF4 signaling restores HNF4-dependent transcriptional activity, CD PDOs were treated with NCT or NFT (80 μ for 12 h). Both compounds significantly increased the expression of HNF4α and CLDN15, to levels comparable to controls ($p<0.0001$, n=8 patients per group) [Figure 5A]. Notably, HNF4γ expression was also restored following treatment with NCT or NFT [Figure 5B], suggesting coordinated reactivation of HNF4-dependent

transcriptional programs. The high sequence identity and nearly superimposable ligand-binding domains of HNF4α and HNF4γ explain the parallel responsiveness of both paralogs to pharmacologic activation by NCT and NFT.[54] Similar effects were observed in *Tnf*$^{\Delta ARE/+}$ mouse organoids, where NCT and NFT restored HNF4α and CLDN15 expression compared to controls ($p<0.0001$, n=6 mice per genotype) [Figure 5C]. These findings demonstrate that HNF4-dependent permeability barrier programs remain pharmacologically reversible, even in chronically inflamed epithelium. To determine whether restoration of HNF4 expression rescues epithelial permeability barrier function, TEER was measured in CD PDO monolayers treated with vehicle (DMSO), NCT (80 μM), or NFT (80μM) for 12 h. Both NCT and NFT significantly increased TEER, restoring barrier function to near-control levels ($p<0.0001$, n=8 patients per group) [Figure 5D]. These TEER values are consistent with physiologically relevant barrier resistance observed in human ileum.[17,55-58] Together, these findings show that pharmacologic activation of HNF4 signaling restores HNF4-dependent TJ gene expression and epithelial barrier function.

Discussion.

Intestinal permeability dysfunction is strongly associated with disease relapse and progression in CD.[1,3,4,9-11] Yet, whether epithelial barrier defects reflect primary epithelial abnormalities or secondary inflammatory injury has remained unresolved. Here, we demonstrate that coordinated activity of HNF4α and HNF4γ maintains epithelial barrier integrity and that loss of these paralogs disrupts TJ transcriptional programs and produces an epithelial-intrinsic barrier defect that persists independently of active inflammatory signaling. Chronic inflammation further suppresses HNF4-dependent TJ transcriptional programs and exacerbates barrier dysfunction.

Importantly, pharmacologic activation of HNF4 restores barrier integrity, demonstrating that epithelial barrier dysfunction in CD represents a reversible transcriptional state.

Our findings provide mechanistic insight into how HNF4 paralogs regulate epithelial barrier function. Transcriptomic analysis of intestinal epithelial cells demonstrated that combined loss of *Hnf4α* and *Hnf4γ* profoundly alters transcriptional programs governing TJ organization in the small intestine. This included reduced expression of several barrier-associated claudins, including *Cldn4*, *Cldn7*, *Cldn15*, together with increased expression of the permeability-associated claudin, *Cldn2*. Unlike *Cldn2*, *Cldn15* plays a key physiological role in regulating paracellular Na^+ transport in the small intestine, and its loss may therefore contribute to Na^+-deficiency and glucose malabsorption observed in CD.[59,60] Consistent with these transcriptional changes, *Hnf4αγ*-deficient mouse organoids exhibited increased paracellular permeability to small molecular tracers without overt disruption of epithelial architecture, indicating selective impairment of TJ barrier function. These findings support a model in which HNF4 paralogs coordinate transcriptional programs required for assembly, maintenance, and turnover of epithelial junctional complexes and barrier integrity in the small intestine. Previous studies have implicated Hnf4α in regulation of intestinal epithelial barrier integrity; however, interpretation of these findings has been complicated by functional redundancy between HNF4 paralogs. By examining the combined loss of HNF4α and HNF4γ, our results demonstrate that these paralogs function cooperatively to maintain epithelial junctional organization and barrier integrity in the small bowel.

These observations have important implications for understanding epithelial barrier dysfunction in CD. Persistent intestinal permeability defects are strongly associated with disease relapse and progression, even among patients who achieve ER. Chronic inflammatory signaling is known to disrupt epithelial barrier integrity through cytokine-mediated effects on TJ proteins and

epithelial turnover. Previous studies of barrier dysfunction in CD have largely focused on individual TJ components or claudins, such as occludin, ZO-1, myosin light chain kinase, and CLDN2. The *Tnf*$^{\Delta ARE/+}$ mouse develop chronic TNF-driven ileitis that recapitulates key features of human ileal CD, including epithelial barrier dysfunction and transmural inflammation. In this model, we show that suppression of HNF4-dependent epithelial programs was associated with disruption of TJ gene networks. Importantly, pharmacologic activation of HNF4 restored these gene networks in *Tnf*$^{\Delta ARE/+}$ mouse organoids, demonstrating that inflammatory suppression of HNF4-dependent barrier programs is reversible. These findings also suggest that mechanisms described for HNF4-dependent epithelial repair in the liver may also operate in the intestinal epithelium during chronic inflammatory injury in ileal CD, and that persistent inflammation may impair epithelial recovery and contribute to ongoing barrier dysfunction.

These findings also have important implications for disease stratification and therapeutic development in CD. CD exhibits substantial regional heterogeneity, with ileal and colonic disease representing biologically distinct subtypes.[61,62] Notably, HNF4γ is highly enriched in the distal ileum, whereas HNF4α is expressed more broadly throughout the intestinal epithelium.[25] Consistent with this regional distribution, we did not observe significant alterations in TJ-associated gene networks in the colon of the *Hnf4αγ*^{DKO} mice, supporting a region-specific role for HNF4 paralogs in regulating epithelial barrier integrity. Cooperative regulation by these paralogs suggests that their combined downregulation disproportionately affect distal ileal barrier stability, consistent with the high prevalence of distal ileal involvement in CD and the increased incidence of penetrating and fibrostricturing disease phenotypes in ileal CD.[1,63] Restoration of epithelial barrier integrity therefore represents an attractive therapeutic strategy to prevent disease complications. In this study, pharmacologic activation of HNF4 signaling using the highly

selective small molecules NCT and NFT restored epithelial barrier function in CD organoids models. These findings demonstrate that epithelial barrier defects associated with HNF4 suppression are reversible and support the potential for epithelial-directed therapeutic strategies to complement existing immune-targeted treatments in CD.

Summary: In summary, our findings identify HNF4 paralogs as central regulators of epithelial programs that maintain intestinal barrier integrity. Cooperative activity of HNF4α and HNF4γ coordinates transcriptional networks required for TJ organization and epithelial barrier stability. Loss of these programs produce an epithelial-intrinsic barrier defects that persist independently of active inflammatory signaling, while chronic inflammation further suppresses HNF4-dependent epithelial programs and exacerbates barrier dysfunction. Importantly, pharmacologic activation of HNF4 restored epithelial barrier integrity in epithelial organoid models, demonstrating that both epithelial-intrinsic and inflammation-associated barrier defects are reversible. Together, these findings highlight epithelial transcriptional regulation as a critical component of intestinal barrier homeostasis and suggest that restoration of HNF4-dependent epithelial programs may represent a promising strategy to improve long-term outcomes in CD.

Limitations: This study does not suggest that suppression of HNF4αγ is the sole determinant of epithelial barrier dysfunction in CD, which arises from complex interactions among inflammatory signaling, microbiota, and immune-epithelial crosstalk. Rather, our data identify HNF4αγ-dependent transcriptional regulation as a key epithelial mechanism contributing to loss of barrier integrity, including in patients who have achieved ER. Pharmacologic rescue experiments were performed *ex vivo* to isolate epithelial-specific effects and therefore do not address systemic

pharmacokinetics, dosing parameters, or *in vivo* therapeutic efficacy. Future studies in whole-animal models will be necessary to evaluate epithelial-directed HNF4 modulation within the full inflammatory and physiological context and to define its translational potential.

Ethical Considerations: All human studies were conducted in accordance with institutional guidelines and approved by the appropriate Institutional Review Board (IRBs). Human intestinal tissue was obtained with written informed consent and were de-identified prior to analysis. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and performed in compliance with institutional and national guidelines for the care and use of laboratory animals.

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Figure Legends.

Figure 1. *In vivo* loss of HNF4αγ increases size-selective paracellular permeability in the small intestine. Representative bright-field, FITC fluorescence, and merged images of small

intestinal organoids derived from wild-type (WT) and *Hnf4αγ*^{DKO} mice incubated with FITC-dextran tracers of defined molecular sizes (4-, 10-, and 70-kDa). Organoids were cultured in medium supplemented with 25 mM acetate to compensate for acetyl-CoA deficiency in *Hnf4αγ*^{DKO} organoids.[35] WT organoids exclude FITC-dextran from the lumen, indicating intact epithelial barrier function. In contrast, *Hnf4αγ*^{DKO} organoids show luminal accumulation of 4-kDa and 10-kDa FITC-dextran, indicating increased paracellular permeability, while 70-kDa dextran is excluded, demonstrating preserved epithelial integrity. Right panel shows quantification of luminal FITC fluorescence intensity normalized to WT controls. *Hnf4αγ*^{DKO} organoids exhibit significantly increased permeability to 4-kDa and 10-kDa dextran, but not 70-kDa dextran. Data represent mean ± SEM (n=15 organoids per sample). Asterisks (***) denote $p<0.0001$. Scale bar, 20 μm.

Figure 2A. Downregulation of HNF4α and HNF4γ in distal ileal epithelium of 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 ileal Crohn's disease (CD). In non-CD ileal epithelium, HNF4α exhibits strong nuclear localization along the crypt-villus axis, while HNF4γ is detected in both nuclear and cytoplasmic compartments of epithelial cells. In contrast, ileal tissue from CD patients shows markedly reduced HNF4α nuclear staining and reduced HNF4γ expression, indicating suppression of HNF4 paralog activity in the disease epithelium. Images are representative of n=6 CD patients, n=4 non-CD controls. Scale bars, 100 μm

Figure 2B. Reduced HNF4α expression in colonic epithelium of Crohn's disease patients. Representative immunohistochemical staining for HNF4α in paraffin-embedded sections from transverse colon (TVC), descending colon (DC), and rectum (REC) of patients with ileal or ileocolonic Crohn's disease (CD). A non-CD colon control is shown for comparison. In control tissue, HNF4α exhibits strong nuclear localization in epithelial cells along the colon crypts, whereas CD tissues display markedly reduced epithelial nuclear HNF4α staining across multiple colonic regions. Dashed boxes indicate areas shown at higher magnification in adjacent panels. This is a representative of n=11 CD patients, n=4 non-CD controls. Scale bars, 50 μm

Figure 3A. Reduced HNF4α and claudin-15 expression in Crohn's disease patient-derived organoids. Representative confocal immunofluorescence images of patient-derived intestinal organoids (PDOs) generated from ileal Crohn's disease (CD) patients and non-CD controls, stained for HNF4α (red) and claudin-15 (CLDN15, green). Nuclei were counterstained with DAPI (blue). In non-CD organoids, HNF4α shows strong nuclear localization and CLDN15 is prominently expressed along the apical epithelial membrane. In contrast, CD PDOs display markedly reduced HNF4α and CLDN15 staining, indicating suppression of HNF4-dependent tight junction programs in CD epithelium. Right panel shows quantification of mean fluorescence intensity for HNF4α and CLDN15 in control and CD PDOs. Data represent mean ± SEM from PDOs derived from n = 8 patients per group. Asterisks (***) denote $p<0.0001$. Scale bar, 10 μm.

Figure 3B. Reduced HNF4γ expression in Crohn's disease patient-derived organoids. Representative confocal immunofluorescence images of patient-derived intestinal organoids (PDOs) generated from ileal Crohn's disease (CD) patients and non-CD controls, stained for

HNF4γ (green). Nuclei were counterstained with DAPI (blue). In non-CD PDOs, HNF4γ is abundantly expressed in epithelial cells, whereas CD PDOs show markedly reduced HNF4γ fluorescence, indicating suppression of HNF4γ expression in CD epithelium. Right panel shows quantification of mean fluorescence intensity of HNF4γ in non-CD and CD PDOs. Data represent mean ± SEM from PDOs derived from n = 8 patients per group. Asterisks (***) denote $p<0.0001$. Scale bar, 20 μm.

Figure 4A. Reduced epithelial HNF4α and HNF4γ expression in the inflamed ileum of Tnf$^{\Delta ARE/+}$ mice. Representative immunohistochemical staining for HNF4α and HNF4γ in distal ileal paraffin-embedded sections from *Tnf*$^{\Delta ARE/+}$ and *Tnf*$^{+/+}$ mice. In *Tnf*$^{+/+}$ control mice, HNF4α and HNF4γ exhibit strong epithelial staining along the crypt-villus axis. In contrast, ileal epithelium from *Tnf*$^{\Delta ARE/+}$ mice show markedly reduced HNF4α and HNF4γ staining, indicating suppression of HNF4 paralog expression in the inflamed intestinal epithelium. Images are representative of n=6 mice per genotype. Scale bars, 100 μm

Figure 4B. Reduced epithelial HNF4α and claudin-15 expression in intestinal organoids derived from *Tnf*$^{\Delta ARE/+}$ mice. Representative confocal immunofluorescence images of intestinal organoids derived from distal ileum of control *Tnf*$^{+/+}$ and *Tnf*$^{\Delta ARE/+}$ mice stained, stained for HNF4α (red) and claudin-15 (CLDN15, green). Nuclei were counterstained with DAPI (blue). Organoids derived from *Tnf*$^{+/+}$ mice exhibit strong nuclear HNF4α staining and prominent CLDN15 expression along the apical membrane, whereas organoids derived from *Tnf*$^{\Delta ARE/+}$ mice show markedly reduced HNF4α and CLDN15 fluorescence, indicating suppression of HNF4-dependent epithelial barrier programs. CLDN15 immunostaining shows elevated background due

to cross-reactivity of the anti-human antibody in mouse tissue; however, specific apical CLDN15 signal is visibly reduced in *Tnf*$^{\Delta ARE/+}$ mouse organoids. Right panel shows quantification of mean fluorescence intensity for HNF4α and CLDN15 in *Tnf*$^{+/+}$ and *Tnf*$^{\Delta ARE/+}$ mouse organoids. Data represent mean ± SEM (n=6 mice per genotype). Asterisks (***) denote p<0.0001. Scale bar, 15 μm.

Figure 4C. Reduced HNF4γ expression in intestinal organoids derived from *Tnf*$^{\Delta ARE/+}$ mice. 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). Organoids derived from *Tnf*$^{+/+}$ mice show abundant epithelial HNF4γ expression, whereas organoids from *Tnf*$^{\Delta ARE/+}$ mice display markedly reduced HNF4γ fluorescence, indicating suppression of HNF4γ expression in inflammatory ileitis model. Right panel shows quantification of mean fluorescence intensity of HNF4γ in *Tnf*$^{+/+}$ and *Tnf*$^{\Delta ARE/+}$ mouse organoids. Data represent mean ± SEM (n=6 mice per genotype). Asterisks (***) denote p<0.0001. Scale bar, 20 μm.

Figure 5A. Pharmacologic activation of HNF4 signaling restores HNF4α and claudin-15 expression in Crohn's disease patient-derived organoids. Representative confocal immunofluorescence images of patient-derived organoids (PDOs) from ileal Crohn's disease (CD) and non-CD controls treated with vehicle (Veh; DMSO) or the HNF4 agonists NCT or NFT, stained for HNF4α (red) and claudin-15 (CLDN15, green). Nuclei were counterstained with DAPI (blue). Non-CD PDOs exhibit strong nuclear HNF4α staining and apical CLDN15 localization, whereas CD PDOs treated with vehicle show markedly reduced HNF4α and CLDN15 expression. Treatment with NCT or NFT restores HNF4α and CLDN15 expression in CD PDOs, consistent

with reactivation of HNF4-dependent epithelial barrier program. Bottom panel shows quantification of mean fluorescence intensity of HNF4α and CLDN14 across treatment groups. Data represent mean ± SEM from PDOs derived from n = 6 patients per group. Asterisks (***) denote $p<0.0001$. Scale bar, 20 μm.

Figure 5B. Pharmacologic activation of HNF4 signaling restores HNF4γ expression in Crohn's disease patient-derived organoids. Representative confocal immunofluorescence images of patient-derived organoids (PDOs) from ileal Crohn's disease (CD) and non-CD controls treated with vehicle (Veh; DMSO), NCT, or NFT, stained for HNF4γ (red). Nuclei were counterstained with DAPI (blue). Non-CD PDOs show robust epithelial HNF4γ expression, whereas CD PDOs treated with vehicle display markedly reduced HNF4γ fluorescence. Treatment with NCT or NFT restores HNF4γ expression in CD PDOs, consistent with pharmacologic reactivation of HNF4 signaling. Right panel shows quantification of mean fluorescence intensity of HNF4γ across treatment groups. Data represent mean ± SEM from PDOs derived from n = 6 patients per group. Asterisks (***) denote $p<0.0001$. Scale bar, 20 μm.

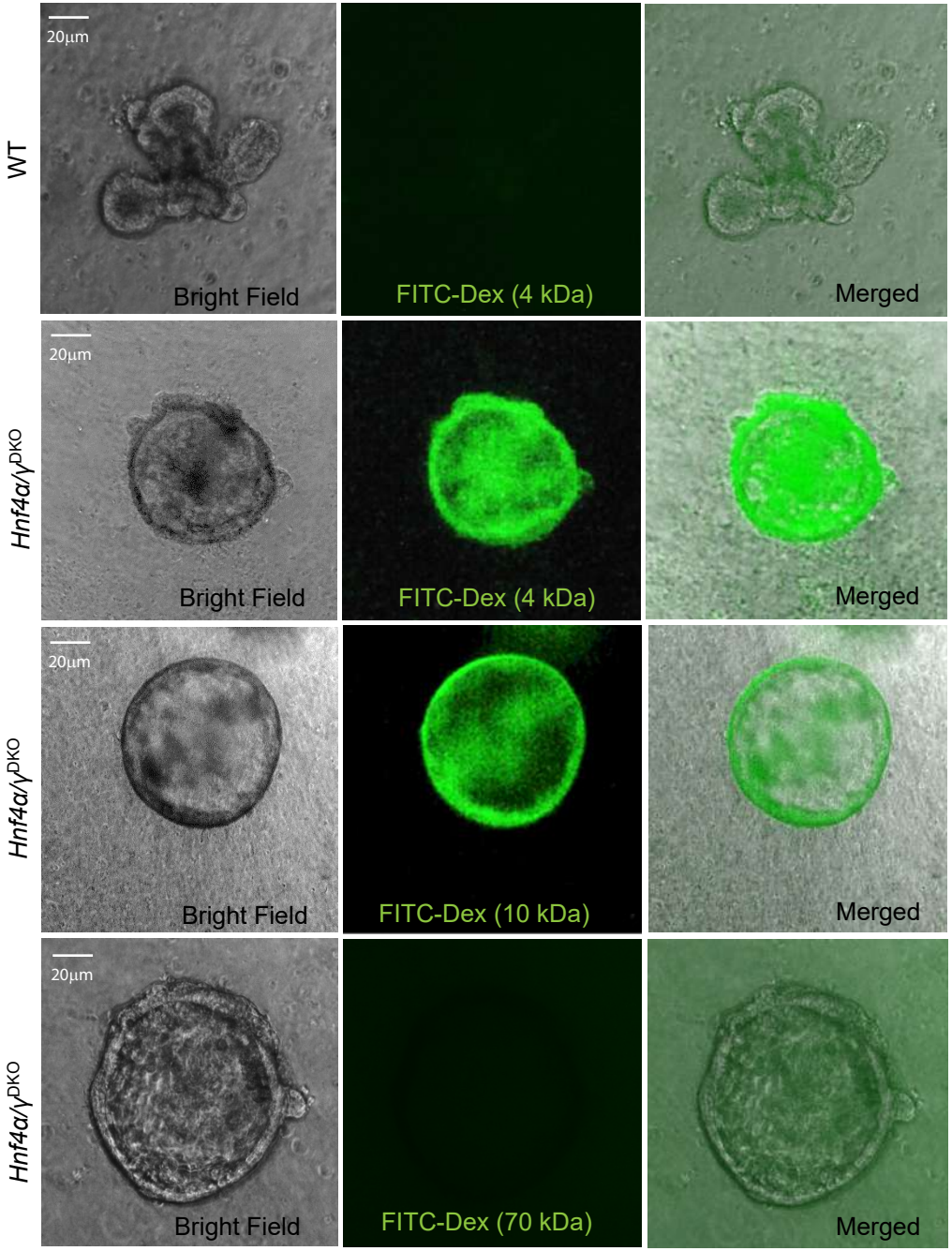
Figure 5C. Pharmacologic activation of HNF4 signaling restores HNF4α and claudin-15 expression in intestinal organoids from *Tnf*$^{\Delta ARE/+}$ mice. Representative confocal immunofluorescence images of intestinal organoids derived from *Tnf*$^{+/+}$ and *Tnf*$^{\Delta ARE/+}$ mice, stained for HNF4α (red) and claudin-15 (CLDN, green). Nuclei were counterstained with DAPI (blue). Organoids derived from *Tnf*$^{+/+}$ mice exhibit strong nuclear HNF4α and CLDN15 localization, whereas *Tnf*$^{\Delta ARE/+}$ mouse organoids treated with vehicle show markedly reduced HNF4α and CLDN15 expression. Treatment of *Tnf*$^{\Delta ARE/+}$ mouse organoids with the HNF4 agonists

NCT or NFT restores epithelial HNF4α and CLDN15 expression, indicating pharmacologic reactivation of HNF4-dependent epithelial barrier programs. The right panel shows quantification of mean fluorescence intensity of HNF4α and CLDN15 across experimental groups. Data represent mean ± SEM (n = 6 mice per genotype). Asterisks (***) denote $p<0.0001$. Scale bar, 20 μm.

Figure 5D. Pharmacologic activation of HNF4 signaling restores epithelial barrier function in Crohn's disease patient-derived organoid monolayers. Transepithelial electrical resistance (TEER) measurement of patient-derived organoid (PDO) monolayers generated from ileal Crohn's disease (CD) and non-CD controls treated with vehicle (Veh, DMSO) or the HNF4 agonists NCT or NFT. CD monolayers exhibit reduced TEER compared with non-CD controls, indicating impaired epithelial barrier integrity. Treatment with NCT or NFT significantly increases TEER in CD monolayers, consistent with restoration of epithelial barrier function following pharmacologic activation of HNF4 signaling. Data represent mean ± SEM from PDOs derived from n = 6 patients per group. Asterisks (***) denote $p<0.001$.

Supplementary Table 1. Differentially expressed genes in *Hnf4αγ*^{DKO} mouse colonic epithelial cells. Differential gene expression analysis comparing colonic epithelial cells isolated from *Hnf4αγ*^{DKO} mice and wild-type (WT) controls. Genes meeting significance criteria (adjusted $P < 0.01$) are shown and categorized as upregulated ($\log_2$ fold change > 1) or downregulated ($\log_2$ fold change < -1) in *Hnf4αγ*-deficient colon epithelium. Columns include Ensembl gene ID, mean normalized expression (baseMean), $\log_2$ fold change, standard error, Wald statistic, raw P value,

adjusted P value (Benjamini-Hochberg correction), and corresponding gene symbol. These results identify transcriptional programs regulated by *Hnf4* paralogs in mouse colonic epithelial cells.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Hnf4α/γDKO
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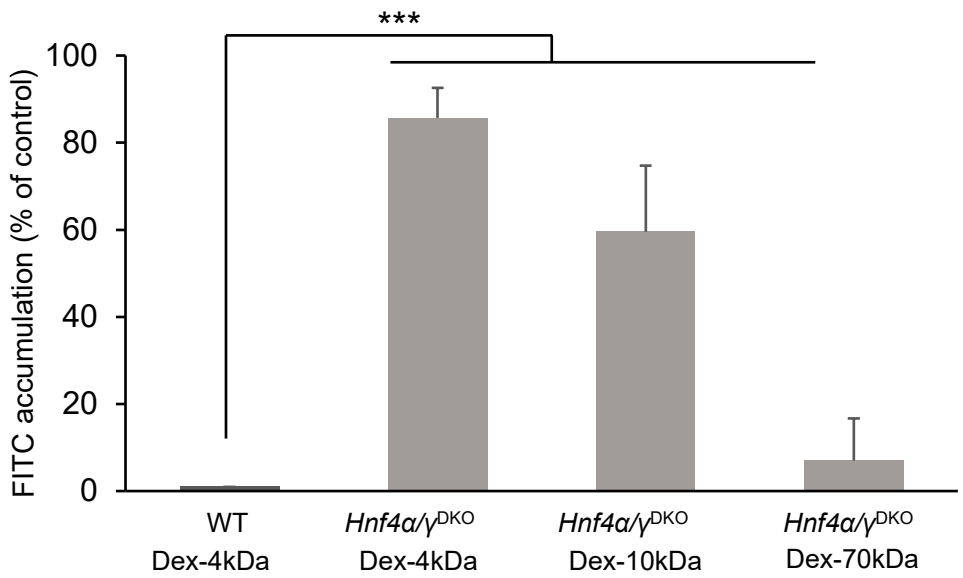
Merged
FITC-Dex (10 kDa)
FITC-Dex (70 kDa)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FITC accumulation (% of control)
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80
60
40
20
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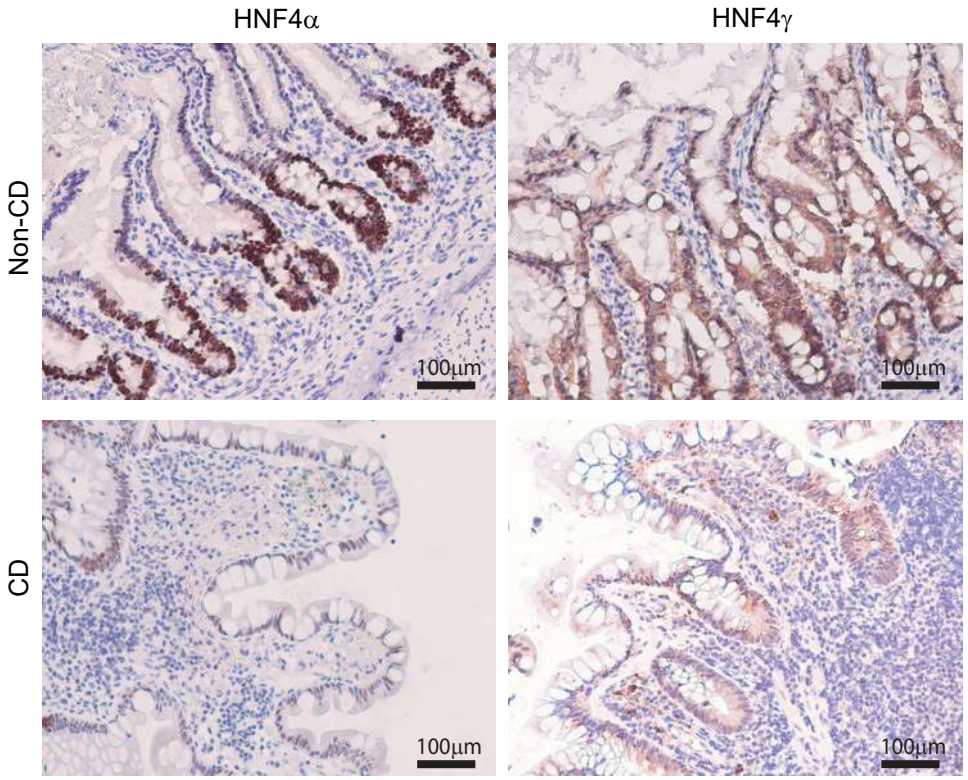
Hnf4α/γDKO Dex-4kDa
Hnf4α/γDKO Dex-10kDa
Hnf4α/γDKO Dex-70kDa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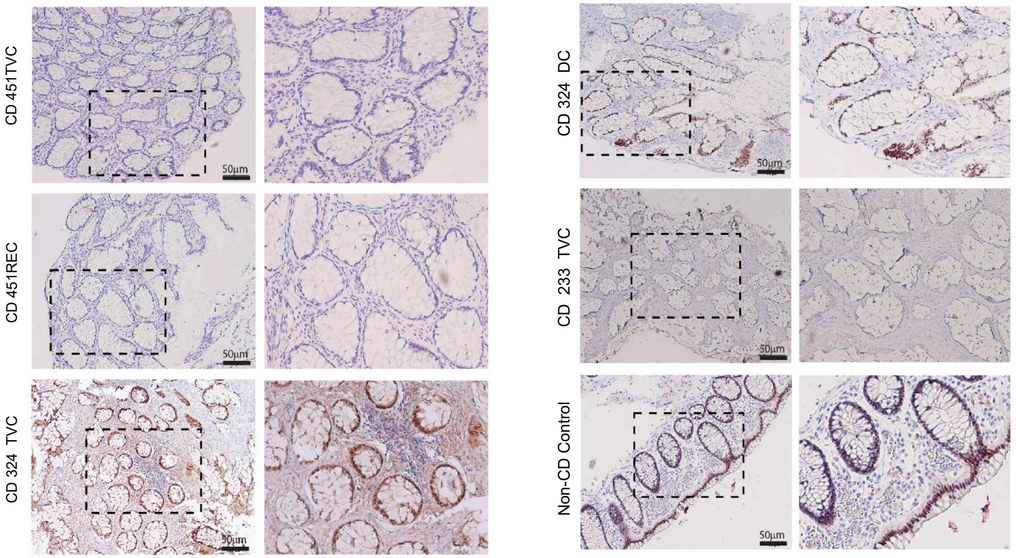
HNF4γ
Non-CD
CD
100μm
100μm
100μm
100μm

HNF4α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CD 451REC
CD 324 TVC
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CD 233 TVC
Non-CD Control
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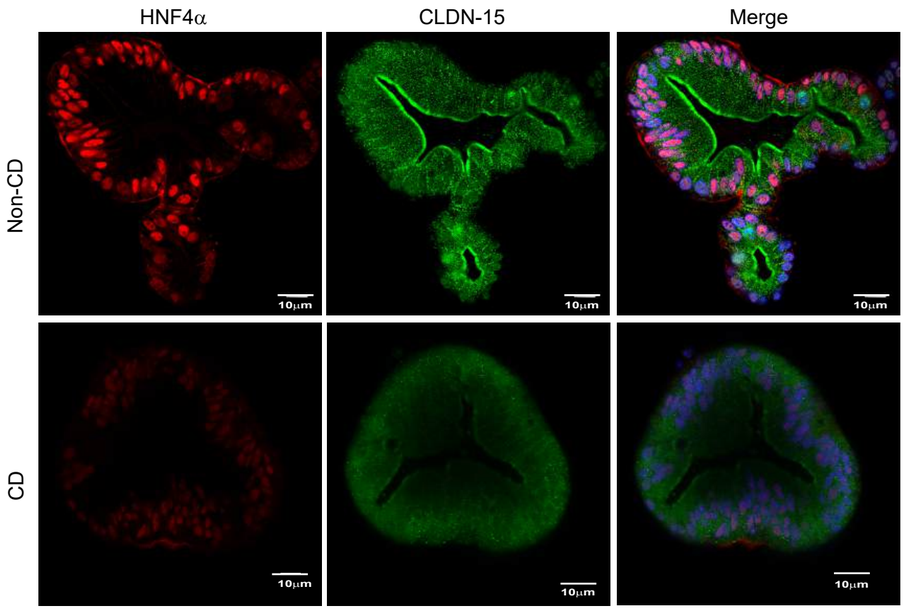
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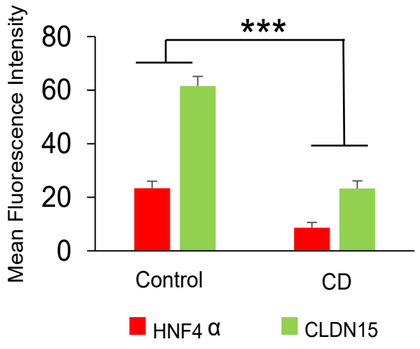
CLDN-15
Merge
Non-CD
CD
10μm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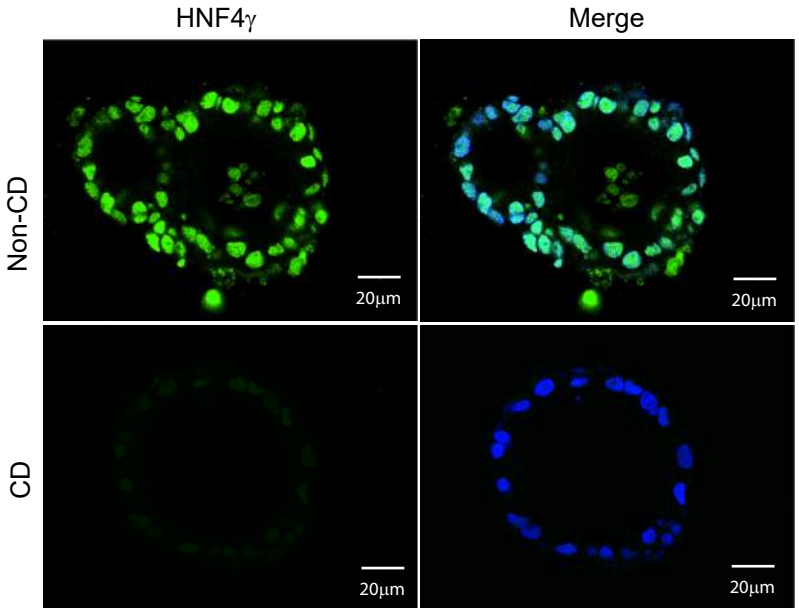
Control
CD
HNF4 α
CLDN15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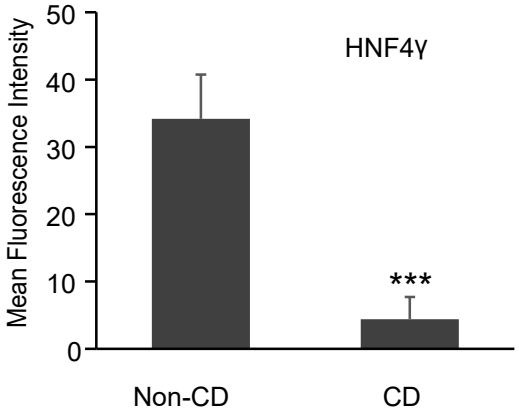
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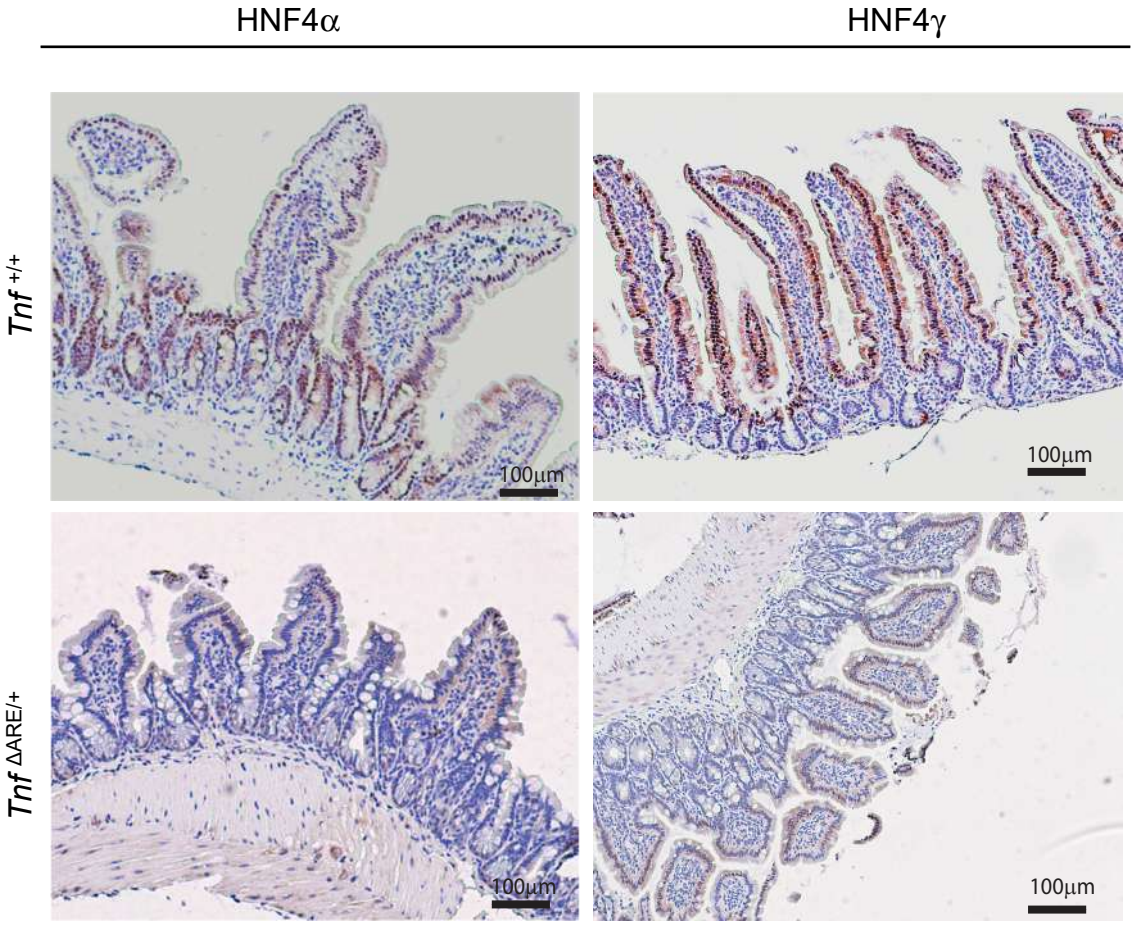
Non-CD
CD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HNF4γ
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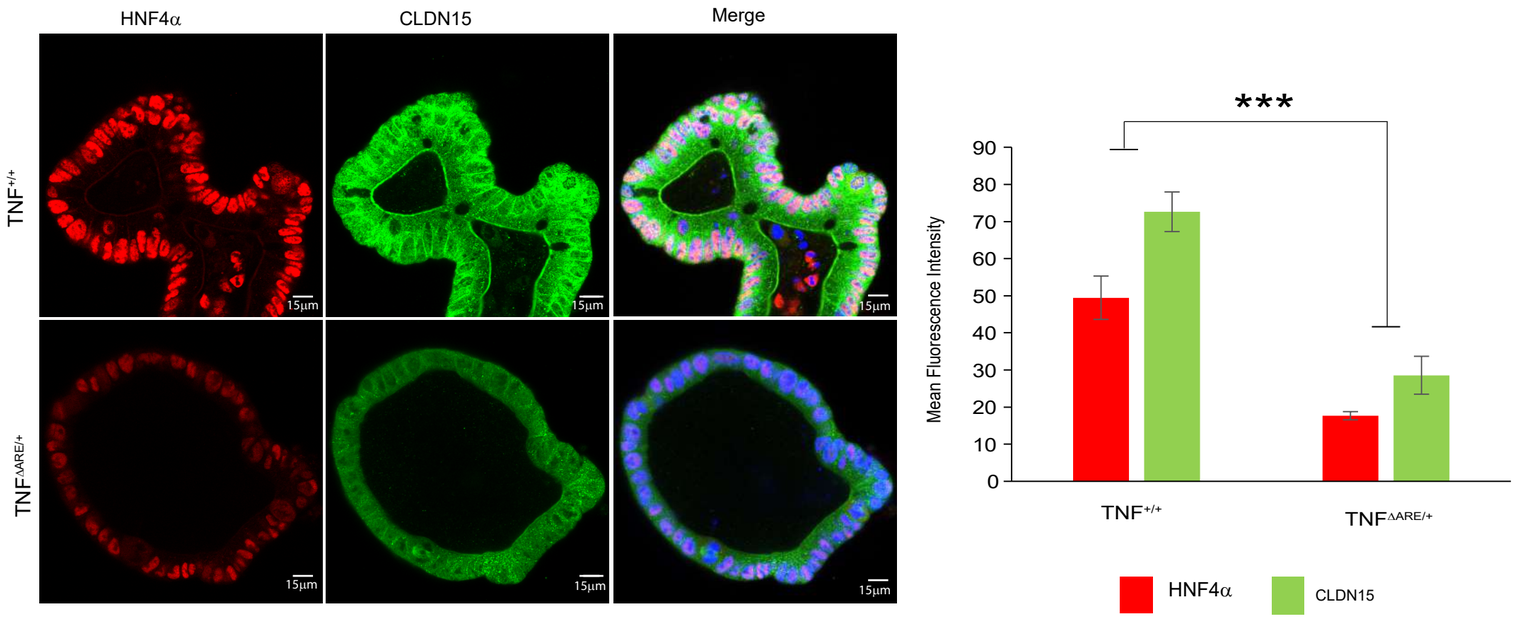
Tnf ΔARE/+
100μm
100μm
100μm
100μm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CLDN15
Merge
TNF+/+
TNFΔARE/+
15µm
***
Mean Fluorescence Intensity
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50
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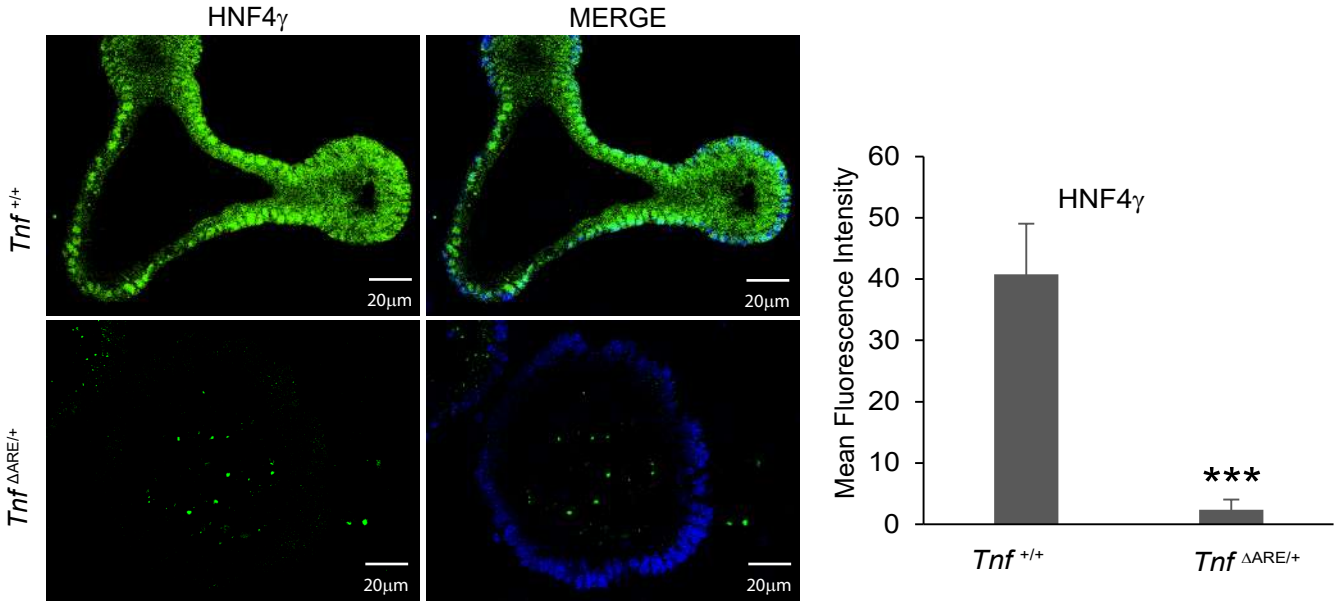
10
0
TNF+/+
TNFΔARE/+
HNF4α
CLDN15


HNF4γ
MERGE
Tnf +/+
Tnf ΔARE/+
20μm
20μm
20μm
20μm
Mean Fluorescence Intensity
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HNF4γ
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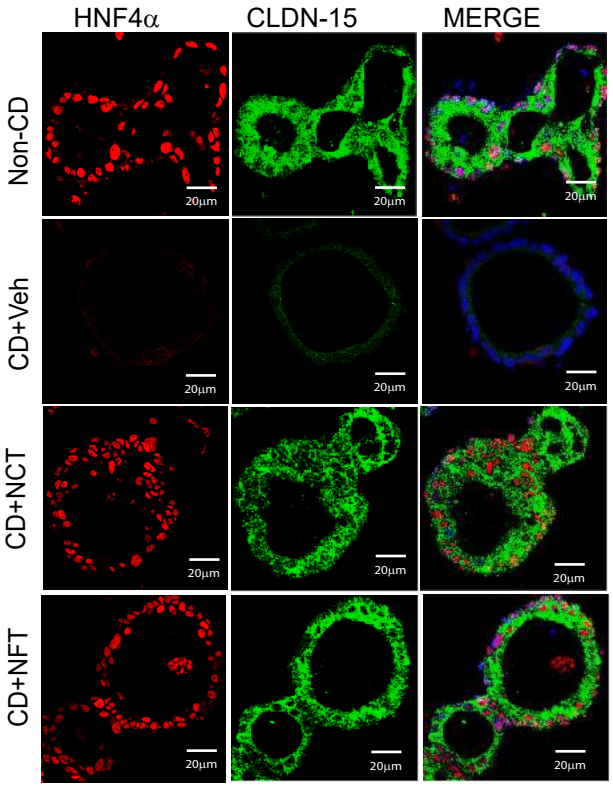
Tnf +/+
Tnf ΔARE/+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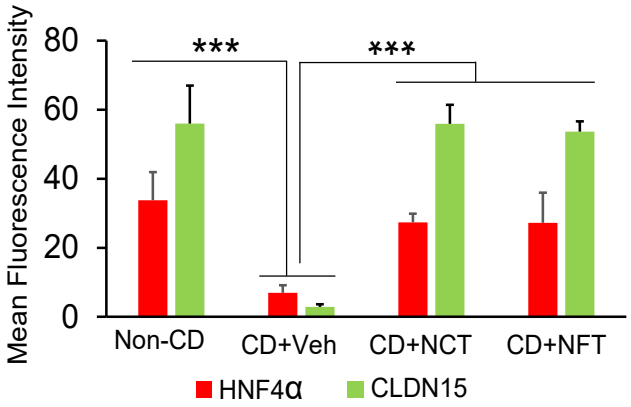
Mean Fluorescence Intensity
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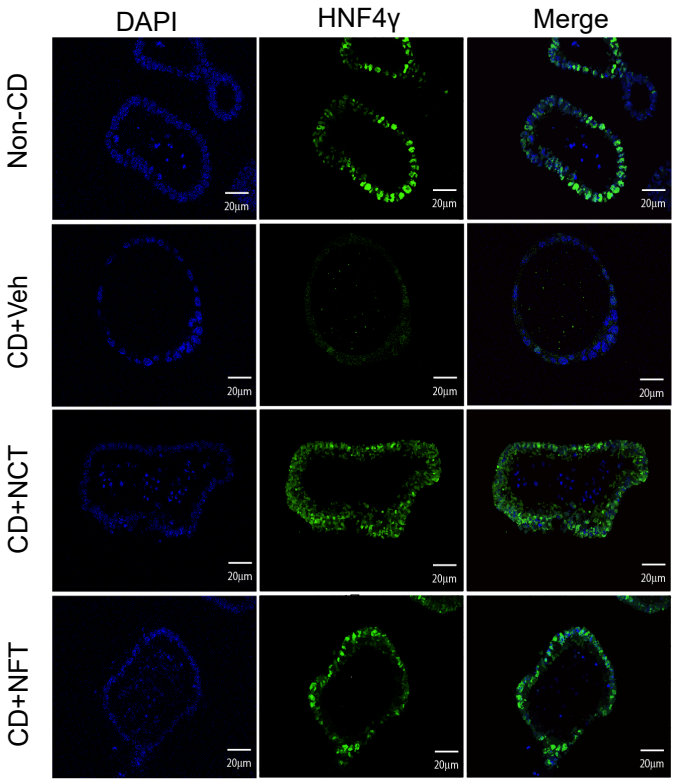
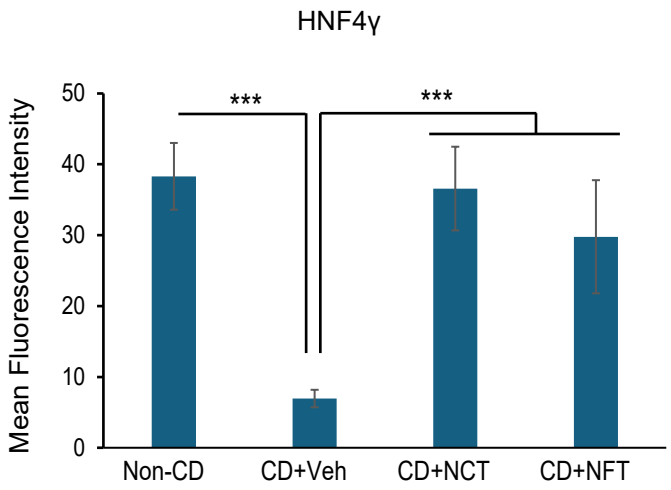
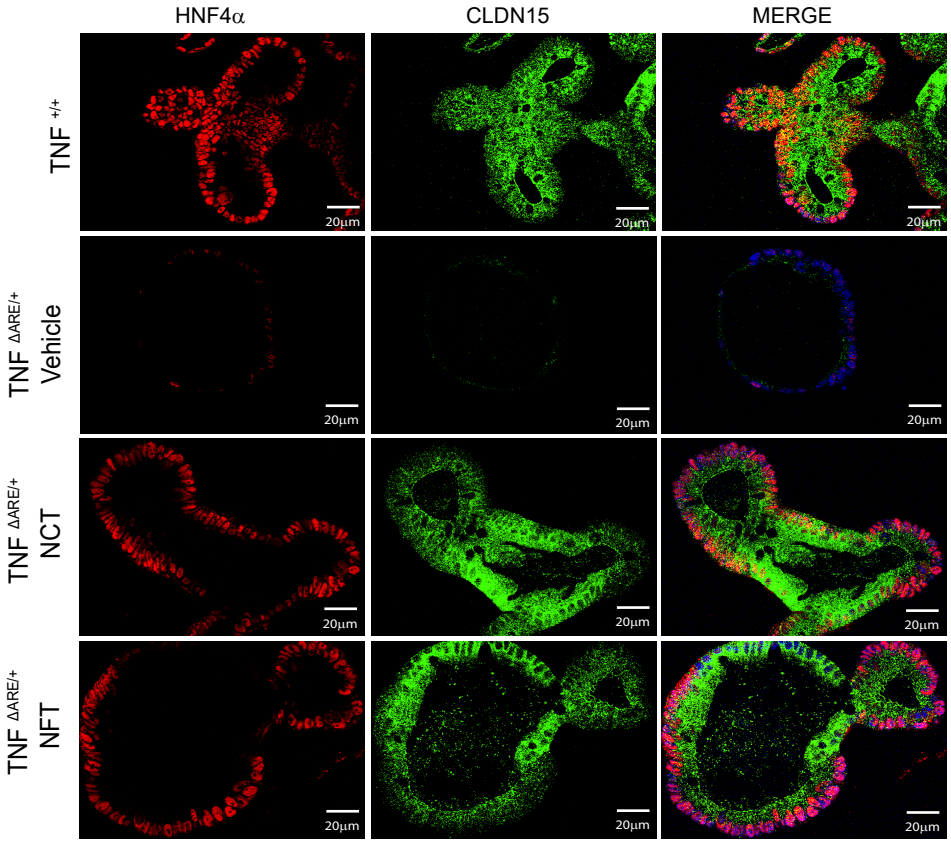
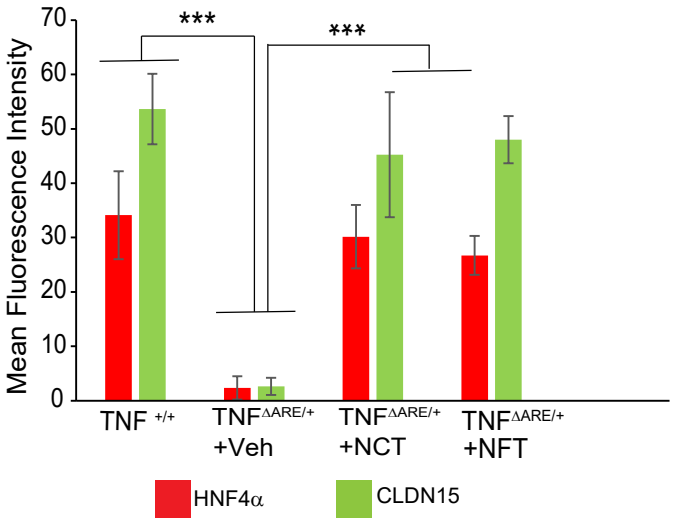
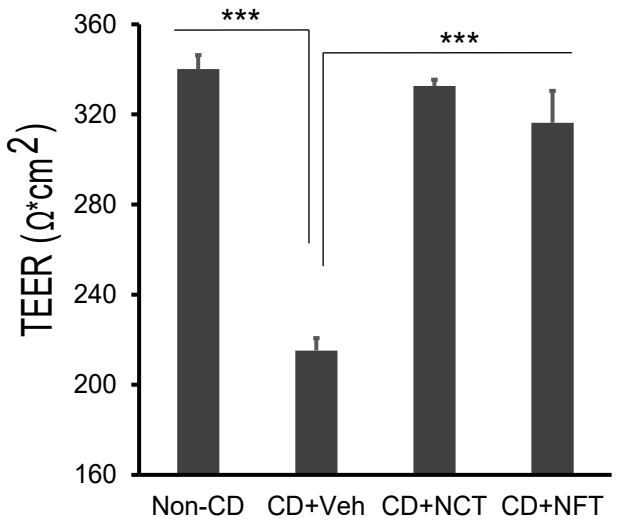
60
40
20
0
***
***
Non-CD
CD+Veh
CD+NCT
CD+NFT
HNF4α
CLDN15


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


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


***
***
360
320
280
240
200
160
TEER (Ω*cm2)
Non-CD
CD+Veh
CD+NCT
CD+NFT