Integrated Stress Response and Necroptosis Drive Epithelial Dysfunction in Crohn's Disease: Repurposing Cancer drugs for Permeability Barrier Healing.

Short Title: Repurposed RIPK3 inhibitors for Barrier Healing.

Debasish Halder[1*], Ritwika Biswas Lanford,[1*] Amin Ghazi,[1] Jason Ken Hou,[2] Yaohong Wang,[3] Seema Khurana.[1]

* Both authors contributed equally to this manuscript.

1. Department of Biology and Biochemistry, The University of Houston, Houston, Texas.

2. Section of Gastroenterology and Hepatology, Department of Medicine, Baylor College of Medicine, Houston, Texas.

3. Department of Anatomical Pathology, Division of Pathology-Laboratory Medicine Division, The University of Texas MD Anderson Cancer Center, Houston, Texas.

Corresponding author: Seema Khurana, Ph.D.
Department of Biology and Biochemistry,
369 Science and Research Building SR2, Room 421E,
The University of Houston,
Houston, TX 77204-5001.
E-mail: skhurana@central.uh.edu. Phone: 713-743-2705.

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

Disclosure: Authors disclose no conflict.

Abbreviations used in this paper: AJ, adherens junction; BH, permeability barrier healing; CD, Crohn's disease; DKO, double knockout; DAMPs, damage-associated molecular patterns; ER, endoscopic remission; ERICA, Endoscopic and Barrier Healing in Crohn's Disease Trial; FDA, U.S. Food and Drug Administration; H&E, hematoxylin and eosin; HMGB1, High Mobility Group Box 1; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IHC, immunohistochemistry; IRGM1, immunity-related GTPase family M protein 1; ISC, intestinal stem cell; ISR, integrated stress response; ISRIB, integrated stress response inhibitor; MLKL, mixed lineage kinase domain-like protein; Nec-1, Necrostatin-1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PDE, patient-derived enteroid; PI, propidium iodide; PP1c, protein phosphatase 1 catalytic subunit; RIPK1/3, receptor-interacting protein kinase 1/3; TEER, transepithelial electrical resistance; TJ, tight junction; TNF, tumor necrosis factor; $Tnf^{\Delta ARE/+}$, tumor necrosis factor AU-rich element deletion mutant mouse; WT, wild-type.

Author Contribution: Seema Khurana, Ritwika Lanford, Amin Ghazi and Debasish Halder, conceived the experiments. Seema Khurana, Ritwika Lanford, Debasish Halder, Amin Ghazi, and Yaohong Wang analyzed all histology and immunohistochemistry performed in this study. Ritwika Lanford, Amin Ghazi, and Debasish Halder conducted the experiments. Jason Ken Hou provided human CD tissue samples and provided intellectual input. All authors reviewed the manuscript.

Data Availability: Data, analytic methods, and study materials will be made available to other researchers. Data and analytic methods are included in the published article and supplementary materials. Study materials are available from the corresponding author upon reasonable request.

Summary of study: We show that chronic epithelial-intrinsic integrated stress response activation and RIPK3-mediated necroptosis drive barrier dysfunction in Crohn's disease. Targeting RIPK3 pathways with repurposed cancer drugs restores epithelial regeneration and permeability barrier integrity in murine models and patient-derived enteroids.

Ethical Considerations: All studies were conducted in accordance with ethical standards and approved by the appropriate institutional review boards and animal care and use committees. Human intestinal tissues and patient-derived organoids were obtained with written informed consent and de-identified to protect patient privacy. All animal experiments complied with institutional and national guidelines to minimize pain, distress, and animal use. Experimental design followed principles of rigor, reproducibility, and transparency. No identifiable personal data was used, and all analyses were performed in compliance with relevant ethical, legal, and regulatory requirements.

Abstract.

Background and Aims: Epithelial permeability barrier dysfunction is a central pathogenic driver of Crohn's disease (CD), fueling microbial translocation, chronic inflammation, and progressive tissue injury. While current therapies suppress inflammation, none directly restore epithelial barrier function. Importantly, in CD patients, permeability barrier healing (BH) rather than mucosal healing is associated with long-term remission and a reduced risk of disease complications. Yet BH remains an unaddressed therapeutic target in CD. Here, we investigated whether pharmacologic inhibition of the integrated stress response (ISR) and RIPK3-mediated necroptosis, two convergent pathways of epithelial injury, can promote epithelial viability, regeneration, and barrier integrity in CD.

Methods: We employed villin-1/gelsolin double knockout (DKO) mice with epithelial-intrinsic ISR activation, *Tnf*$^{\Delta ARE/+}$ mice with chronic inflammation, and CD patient-derived enteroids (PDEs). Animals and PDE were treated with ISR inhibitor ISRIB, RIPK3 inhibitor Necrostatin-1 (Nec-1), or FDA-approved cancer drugs pazopanib and ponatinib, repurposed as potent RIPK3 inhibitors. Epithelial survival, regenerative growth (enteroid formation, budding), and barrier function (transepithelial electrical resistance, TEER) were assessed.

Results: Chronic ISR activation and necroptosis were prominent in both murine models and CD PDEs, causing epithelial death, Paneth cell expansion, impaired enteroid survival, and regenerative failure. Pharmacologic inhibition with ISRIB, Nec-1, pazopanib, or ponatinib restored villus architecture, reduced inflammation, enhanced epithelial survival and regeneration, and significantly improved TEER.

Conclusions. ISR activation and RIPK3-mediated necroptosis converge to drive epithelial injury and barrier dysfunction in CD. Repurposing pazopanib and ponatinib offers a potentially translatable approach, to restore barrier integrity in CD.

Keywords: Crohn's disease, permeability barrier healing, integrated stress response, necroptosis, RIPK3 inhibitors.

Synopsis: ISR activation and RIPK3-mediated necroptosis drive epithelial injury in Crohn's disease. Repurposed RIPK3 inhibitors, pazopanib and ponatinib, restore epithelial homeostasis and permeability barrier function, providing a translational strategy to achieve sustained remission in CD.

Introduction.

Crohn's disease (CD) is a chronic, relapsing inflammatory disorder of the gastrointestinal tract characterized by transmural inflammation, progressive mucosal damage, and the development of complications such as strictures, fistulae, and abscesses. Despite major therapeutic advances with biologics and small molecules, most current CD therapies primarily target immune pathways and not re-epithelialization of the damaged gut. Although inflammation control improves symptoms it achieves mucosal healing or more appropriately endoscopic remission (ER), defined as the absence of inflammation, ulcers, or bleeding in the gastrointestinal tract, in a modest 15-20% of CD patients.[1] While endoscopic remission is an important treatment goal in CD associated with improved clinical outcomes, nonetheless a vast majority of CD patients remain vulnerable to disease progression and intestinal complications.[1] More notably, the presence of mucosal healing in CD does not always correspond with clinical remission, or the resolution of a patient's symptoms.[2] Additionally, a proportional clinical benefit is not received by most CD patients and are instead exposed to the risks of potent immunosuppressive therapies.

A defining feature of CD pathogenesis is intestinal permeability barrier dysfunction.[3] Under homeostatic conditions, the intestinal epithelium provides a dynamic and selective permeability barrier that separates luminal microbes from the mucosal immune system. Loss of barrier integrity permits the translocation of commensal and pathogenic bacteria as well as antigens or other foreign substances from the gut lumen into the underlying tissue, triggering an exaggerated and uncontrolled immune response.[4] In CD, this sets in motion a vicious cycle where microbial translocation drives inflammation, inflammation induces further epithelial injury, and the subsequent chronic tissue damage leads to fibrostenosis and/or fistulization, which ultimately increases the need for hospitalization and surgery.[5] Epidemiologic and prospective studies have

demonstrated that permeability barrier dysfunction is not merely a consequence of inflammation but an initiating factor in CD.[3, 6] Specifically, asymptomatic individuals with increased intestinal permeability are at significantly higher risk of developing CD, providing causal evidence that permeability barrier dysfunction contributes to disease initiation.[6] Relatives of CD patients often display increased intestinal permeability even before clinical disease onset, supporting a heritable component of permeability barrier defects as well as identify barrier dysfunction as a primary defect in CD.[7, 8] Together these findings highlight the etiologic and therapeutic importance of epithelial permeability barrier restoration in CD.[5, 9]

Therapeutic goals in CD have historically been framed around symptom control, corticosteroid-free remission, and mucosal healing.[10] Indeed, the STRIDE-II consensus reinforced "deep remission," defined as clinical remission plus ER, as the gold standard treatment target in CD.[11] Achieving ER in CD has been correlated with reduced hospitalization and lower surgical risk.[10, 12] However, emerging evidence shows that compared to ER, permeability barrier healing (BH) is a superior predictor of sustained remission and more favorable long-term outcomes in CD.[13, 14] The prospective ERICa trial demonstrated that BH, outperformed ER and histologic remission in predicting adverse outcomes such as intestinal complications, hospitalization and need for surgery.[2] BH therefore represents a more meaningful therapeutic endpoint in CD. While ER reflects inflammation control and mucosal healing, BH captures restoration of the fundamental epithelial permeability barrier defect that drives CD pathology.[3] Despite this, no approved CD therapy directly targets epithelial permeability barrier repair. This therapeutic gap underscores the urgent need for permeability barrier-focused interventions that complement existing immunosuppressive strategies in CD.

The integrated stress response (ISR) is a highly conserved adaptive pathway that regulates cellular responses to diverse stressors including nutrient deprivation, endoplasmic reticulum stress, mitochondrial dysfunction, microbial and viral infections.[15] Central to ISR signaling is phosphorylation of eukaryotic initiation factor 2α (peIF2α), which transiently attenuates global protein translation while allowing selective translation of stress-adaptive genes.[15] Acute ISR activation is protective, conserves resources and promotes cell survival.[15] In contrast, chronic or unresolved ISR signaling promotes autophagy, or programmed cell death *via* apoptosis or necroptosis, shifting the balance from adaptation to injury.[15, 16] Persistent ISR activation is implicated in the etiology of several human diseases and manipulation of ISR is emerging as a promising therapeutic approach.[17] Resolution of ISR requires dephosphorylation of peIF2α by GADD34-PP1c phosphatase complex, a process sensitive to cellular actin dynamics.[18] Prior work from our group has shown that villin-1 and gelsolin, two intestinal epithelial cell (IEC) actin-severing proteins, regulate PP1c activity.[16] Their loss leads to sustained ISR activation, mitochondrial stress, and IEC death *via* necroptosis.[16] Double knockout (DKO) mice lacking villin-1 and gelsolin develop spontaneous ileocolitis recapitulating human CD pathology due to chronic ISR signaling, induction of the autophagy protein IRGM1, and IRGM1-induced IEC death by necroptosis.[16] Notably, similar molecular defects are seen in CD patients, including reduced villin-1/gelsolin expression, chronic ISR activation, and IRGM1-induced IEC death by necroptosis, underscoring the relevance of this pathway to the pathophysiology of CD.[16] These findings suggest that therapeutic targeting of the ISR could restore epithelial homeostasis in CD.

Necroptosis is a pro-inflammatory form of programmed cell death that is mediated by receptor-interacting protein kinases RIPK1 and RIPK3 and executed by mixed lineage kinase domain like (MLKL).[19, 20] Beyond depleting IECs, necroptosis leads to the release of damage-

associated molecular patterns (DAMPs) which augment intestinal inflammation and epithelial injury.[16] Moreover, independent of its role in necroptosis, RIPK3 is a key regulator of inflammation regulating the activation of NF-kB and the NLRP3 inflammasome.[21, 22] RIPK3 and MLKL levels are increased in inflamed tissues from IBD patients.[23] Additionally, we have shown that CD patients display not only an upregulation of RIPK3 expression, but also higher levels of RIPK3-mediated necroptosis, and increased release of the DAMP, nuclear High Mobility Group Box 1 (HMGB1).[16] The dual roles of RIPK3 in necroptosis and inflammation suggest that pharmacologic inhibition of RIPK3 could attenuate IEC death and pro-inflammatory signaling in CD.

TNF-α is a major contributor to intestinal inflammation that is linked to the pathogenesis of CD.[24] Moreover, genetic studies have identified TNF-α as a CD susceptibility locus while anti-TNF therapy is a mainstay of CD treatment.[24, 25] ISR activation modulates pro-inflammatory cytokine production however it is not known if pro-inflammatory cytokines like TNF-α can act as stressors to activate ISR signaling. In this study, we used two complementary murine models of CD, the villin-1/gelsolin DKO mice, which model epithelial-intrinsic ISR dysregulation, and $Tnf^{\Delta ARE/+}$ mice, which develop chronic inflammation due to TNF-α dysregulation, together with CD patient-derived enteroids (PDEs) to test our hypothesis that ISR activation in response to intrinsic (villin-1/gelsolin loss) or extrinsic (TNF-α) stressors and downstream of it necroptosis, are the main drivers of epithelial dysfunction in CD. Furthermore, using these *in vivo* and *ex vivo* murine and human models of CD, we evaluate our hypothesis that pharmacologic inhibition of ISR and necroptosis can restore epithelial homeostasis and permeability barrier integrity. Our findings establish a mechanistic framework linking ISR and necroptosis to permeability barrier failure in CD and demonstrate the translational potential of

repurposed RIPK3 inhibitors as novel therapies to achieve mucosal and permeability barrier healing in CD.

Methods

Mouse Models of Crohn's Disease. The Institutional Animal Care and Use Committee (IACUC) of the University of Houston approved all animal experiments. Double knockout (DKO) mice lacking villin-1 and gelsolin were generated as previously described.[26] *Tnf*$^{\Delta ARE/+}$ mice, which develop chronic ileitis due to dysregulated TNF-α expression, were obtained from Dr. Fabio Cominelli (Case Western Reserve University).[27] Wild-type (WT) littermates of DKO and the *Tnf*$^{+/+}$ mice served as controls. Both male and female mice were studied at 20 and 35 weeks of age, corresponding to peak spontaneous ileitis in *Tnf*$^{\Delta ARE/+}$ and DKO mice, respectively.[16, 28]

Intestinal Mouse and Human Enteroid Culture. Human CD and non-CD control samples were obtained under protocols approved by the Institutional Review Boards of Baylor College of Medicine and University of Houston. Crypts from murine and human distal ileum were isolated by incubation in 2mM EDTA and enteroids were derived as described before.[29] The organoid forming capacity of stem cell or the organoid forming efficiency (percentage) is defined as a percentage of the number of organoids per number of plated stem cells at day 7, when one cell is added per well.[30] The percentage of budded enteroids was calculated as: (number of enteroids with ≥1 crypt-like bud ÷ total number of enteroids counted) x 100 on day 7.[31] Crypt buds per enteroid, a measure of stem cell-driven proliferation and regeneration, was also quantified as described before.[31] At least 50 enteroids per sample were assessed for all quantitative assays.

Epithelial cell mortality was assessed using Hoechst 33342 or propidium iodide (PI) staining. Imaging was performed on a Fluoview FV2000 laser scanning confocal microscope with a 60X NA 1.35 objective.

***In vivo* and *Ex vivo* Treatments.** Mice received intraperitoneal injections of Necrostatin-1 (Nec-1; 1.65 mg/kg) or ISRIB (0.25 mg/kg) every other day starting at 1 month of age.[32] Following treatment, $Tnf^{\Delta ARE/+}$ mice were euthanized at 6 months of age and DKO at 8 months of age and ileal tissue collected for histology, immunostaining, and protein analysis. Vehicle-treated animals (0.3% DMSO) served as controls. *Ex vivo*, DKO, $Tnf^{\Delta ARE/+}$ mouse enteroids and CD patient-derived enteroids (PDEs) were treated with ISRIB (50 nM, Cayman Chemicals), Necrostatin-1 (10 μM), pazopanib (0.5 μM), or ponatinib (2 nM) for 7 days and compared with vehicle (DMSO) treated controls.

Histology and Immunostaining. Distal ileal specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E) to evaluate villus architecture, crypt morphology, and inflammatory infiltration. Histological evaluation of intestinal inflammation was scored as described before.[33] For immunohistochemistry, sections were deparaffinized, antigen-retrieved, and incubated with antibodies to phospho-eIF2α, IRGM1, or lysozyme. Images were captured with an Olympus BX53 microscope. For immunoblotting, protein lysates from distal ileum were prepared using RIPA buffer with proteases and phosphatase inhibitors. Equal protein amounts were separated on SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against eIF2α, peIF2α, IRGM1, and β-actin. Bands were visualized using chemiluminescence (Thermo Fisher).

Transepithelial Electrical Resistance (TEER). CD PDEs 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. Enteroids were dissociated to single cells using TrypIE™ (Gibco, Thermo Fisher Scientific) for 10 min at 37°C and cultured as monolayers until confluent. TEER was measured using an EVOM2 epithelial voltohmmeter (World Precision Instruments). Each Insert was measured three times; average values were corrected for background and normalized to surface area to calculate net resistance ($\Omega.cm^2$).

Statistical Analysis. All experiments were performed with at least three biological replicates. Data are presented as mean ± standard error of mean (SEM). Statistical analyses were conducted using GraphPad Prism v9. Comparisons between two groups were performed using unpaired two-tailed Student's t-test; comparison of > 2 groups used one-way ANOVA with Tukey's post hoc test. A p-value of <0.05 was considered statistically significant.

Results.

ISR activation is a convergent hallmark of epithelial injury in CD models. To determine whether intrinsic epithelial stress or chronic inflammation (extrinsic stressor) converge on epithelial dysfunction and ISR activation, we examined villin-1/gelsolin double knockout (DKO) mice, which model epithelial-intrinsic ISR dysregulation, and *Tnf*$^{\Delta ARE/+}$ mice, which develop chronic ileitis due to TNF dysregulation.[16, 27] Both strains developed spontaneous ileitis and transmural inflammation resembling CD, as shown before.[16, 27] Histomorphological analysis of distal ileum revealed profound epithelial damage in DKO and *Tnf*$^{\Delta ARE/+}$ mice, including villus blunting, villus fusion, and crypt elongation, accompanied by thickening of the muscularis propria

[Fig 1A-B]. Inflammatory cell infiltration was significantly elevated, as quantified by histological scoring (*P<.0001*, n=6 mice per group). Paneth cell hyperplasia and displacement was observed in both mouse models, mirroring phenotypes characteristic of human CD [Fig 1A-B; *P<.0001*, n=6 mice per group.].[34] These findings are consistent with expansion of the proliferative compartment, a change in IEC lineage, structural compromise of epithelial integrity, and chronic inflammatory remodeling.

Immunoblot analysis of intestinal tissue from $Tnf^{\Delta ARE/+}$ mice revealed a marked increase in phosphorylated eIF2α (peIF2α), a marker of ISR activation, compared to $Tnf^{+/+}$ controls, while total eIF2α remained unchanged [Fig 1C]. Densitometric normalization to total eIF2α and β-actin demonstrated an approximately 2.5-to-3.0-fold increase, respectively, in peIF2α in $Tnf^{\Delta ARE/+}$ mice relative to WT mice. IRGM1 protein expression was also robustly and reproducibly increased in $Tnf^{\Delta ARE/+}$ mice relative to WT, with densitometric analysis indicating an approximately 4-fold induction after normalization to β-actin. The robust induction of IRGM1 in $Tnf^{\Delta ARE/+}$ mice suggests activation of epithelial innate defense/autophagy programs in response to chronic TNF-driven stress, consistent with inflammation-linked remodeling of mucosal homeostasis. These results extend our prior findings in DKO mice and CD patients, demonstrating that despite distinct initiating stressors, epithelial intrinsic *versus* extrinsic and inflammatory, chronic ISR activation and IRGM1 induction are convergent hallmarks of epithelial injury in CD.[16]

Chronic ISR impairs epithelial viability and regenerative capacity in murine and CD patient-derived enteroids. To directly assess epithelial cell-intrinsic responses, we derived enteroids from the distal ileum of DKO and $Tnf^{\Delta ARE/+}$ mice, as well as from ileal pinch biopsies from CD patients

and non-CD controls. Phase contrast imaging and PI staining revealed significantly increased IEC death in DKO and *Tnf*$^{\Delta ARE/+}$ enteroids compared with their respective controls [Fig 2A-B]. Quantification showed markedly elevated PI-positive cells in DKO and *Tnf*$^{\Delta ARE/+}$ enteroids, confirming loss of membrane integrity. Necroptosis inhibition with Nec-1 rescued IEC survival in *Tnf*$^{\Delta ARE/+}$ mouse enteroids, whereas pan-caspase inhibition (Z-VAD-FMK) did not, confirming necroptosis rather than apoptosis as the dominant mode of cell death [Supplementary Fig 1], which is similar to our findings in the DKO mice and CD PDEs.[16] Enteroid forming efficiency, a measure of intestinal stem cell (ISC) regenerative potential, was profoundly reduced in both DKO and *Tnf*$^{\Delta ARE/+}$ mouse enteroids relative to control enteroids ($P<.0001$, n=4 mice per group). Once formed enteroids from these murine CD models exhibited diminished complexity, with fewer crypt-like buds per enteroid and a reduced percentage of budded enteroids [Fig 2A-B]. These findings reflect defects in ISC-driven regeneration and epithelial renewal. Strikingly, CD patient-derived enteroids (PDEs) recapitulated the murine phenotypes, showing impaired survival, decreased enteroid forming efficiency, reduced bud number, and lower proportion of budded enteroids compared to non-CD controls [Fig 2C; $P<.0001$, n=8 patients per group].

Confocal imaging revealed Paneth cell expansion in the DKO and *Tnf*$^{\Delta ARE/+}$ mouse enteroids, consistent with the *in vivo* phenotype [Fig 2D]. In addition, *Tnf*$^{\Delta ARE/+}$ enteroids exhibited sustained peIF2α expression compared to *Tnf*$^{+/+}$ control enteroids [Fig 2E]. Collectively, these results demonstrate that chronic ISR activation compromises epithelial survival, reduces ISC regenerative capacity, and drives Paneth cell lineage expansion across murine and human models of CD. Together with our prior work, these data establish necroptosis as a principal driver of IEC death and chronic ISR as a central regulator of regenerative failure in CD.[16]

<u>Pharmacological inhibition of ISR or necroptosis restores epithelial architecture *in vivo*</u>. We next tested whether targeting ISR or necroptosis could mitigate epithelial pathology *in vivo*. DKO and *Tnf*$^{\Delta ARE/+}$ mice were treated with Nec-1 (RIPK1/3 inhibitor) or ISRIB, a small-molecule inhibitor of ISR that binds to eIF2β and restores protein synthesis even when eIF2α is phosphorylated.[35] Essentially, ISRIB "overrides" the ISR brake on translation.[36] Histological analysis of DKO mouse ileum revealed significant restoration of mucosal architecture in the Nec-1- and ISRIB-treated mice compared to vehicle-treated controls [Fig 3A-B, $P<.0001$, n=6 mice per group.]. Drug-treated mice exhibited reduced villus fusion, normalized crypt length, and decreased muscularis propria thickening [$P<.0001$, n=6 mice per group.]. Importantly, Paneth cell hyperplasia was significantly reduced following Nec-1 or ISRIB treatment of DKO mice, indicating correction of lineage imbalance. Histological scores confirmed marked suppression of intestinal inflammation [$P<.0001$, n=6 mice per group]. We next asked whether ISR inhibition would provide similar benefit in an inflammation-driven model of Crohn's-like ileitis. In *Tnf*$^{\Delta ARE/+}$ mice, treatment with ISRIB significantly reduced histological scores compared with vehicle controls [Fig 3C, $P<.0001$, n=6 mice per group]. Additionally, ISRIB treatment led to significant reduction in villus fusion, crypt elongation, and ileal thickening, thus restoring the ileal tissue architecture [$P<.0001$, n=6 mice per group]. This was accompanied by a significant reduction in the number of Paneth cell counts per crypt, mirroring the effects of ISRIB in DKO mice [$P<.0001$, n=6 mice/group]. Together, these findings demonstrate that chronic ISR activation and necroptotic IEC death are tractable therapeutic vulnerabilities, and that pharmacologic inhibition of either pathway can restore epithelial homeostasis *in vivo*.

Repurposed cancer drugs with RIPK3 inhibitory properties rescue epithelial survival and regeneration in CD. Several FDA-approved cancer drugs including pazopanib, ponatinib, sorafenib, dabrafenib, ibrutinib, quizartinib, and regorafenib, have been found to selectively inhibit RIPK3.[37] Given the translational limitations of ISRIB and Nec-1, we tested FDA-approved cancer drugs pazopanib and ponatinib, both identified as potent RIPK3 inhibitors at sub-oncologic doses.[38] *Ex vivo* treatment of DKO and $Tnf^{\Delta ARE/+}$ mouse enteroids and CD PDEs with ISRIB (150 nM), Necrostatin-1 (10 μM), pazopanib (0.5 μM), or ponatinib (2 nM) markedly restored enteroid-forming efficiency, crypt budding, and increased the number of budded enteroids, while also reducing IEC mortality compared with their respective vehicle (DMSO)-treated controls [Fig 4A-C; $P<.0001$, enteroids were derived from n=4 mice and n=6 patients; Supplementary Fig 2.]. The magnitude of rescue with pazopanib and ponatinib was comparable to that observed with ISRIB and Nec-1, indicating comparable efficacy. These results show that pazopanib and ponatinib can rescue epithelial viability and regenerative capacity in both murine and human CD enteroids, highlighting their potential as clinically translatable mucosal healing therapies in CD.

Repurposed RIPK3 inhibitors restore permeability barrier integrity in CD PDE. Finally, we evaluated whether these pharmacological interventions improved functional permeability barrier integrity, as measured by transepithelial electrical resistance (TEER). Treatment with pazopanib, ponatinib, ISRIB, or Nec-1 significantly improved TEER values compared to vehicle-treated controls [Fig 5, $P<.001$, n=6 patients per group.]. In CD PDE monolayers, pazopanib and ponatinib significantly improved transepithelial electrical resistance (TEER), directly demonstrating functional recovery of permeability barrier integrity. These results directly

demonstrate that repurposed RIPK3 inhibitors not only promote mucosal healing but also promote permeability barrier healing in human CD epithelium.

Across murine models and human CD PDEs, we show that: (1) chronic ISR activation and necroptosis are convergent hallmarks of epithelial dysfunction; (2) these pathways drive IEC death, regenerative failure, IEC lineage defects, and permeability barrier loss; (3) pharmacologic inhibition with ISRIB, Nec-1, pazopanib, or ponatinib rescues epithelial survival, regenerative growth, and permeability barrier integrity. These findings position ISR and RIPK3 inhibition as promising therapeutic strategies to achieve permeability barrier healing in CD.

Discussion.

Our results identify ISR activation as a unifying hallmark of epithelial stress in CD, regardless of whether the initiating insult is intrinsic (loss of villin-1/gelsolin) or extrinsic (TNF-α dysregulation). *Tnf*$^{\Delta ARE/+}$ mice display chronic peIF2α phosphorylation and IRGM1 induction, molecular features we previously identified in the DKO mice and human CD epithelium.[16] By regulating mitochondrial fission and membrane polarization, IRGM1 influences mitochondrial quality control while overexpression of IRGM1 causes mitochondrial depolarization and sensitizes IECs to necroptotic death as shown by us before in DKO mice and CD patients.[16, 39] In acute stress, the ISR restores homeostasis and promotes survival.[15] Our data demonstrate that chronic ISR activation in the gastrointestinal tract is maladaptive, locking epithelial cells in a state of translational arrest and mitochondrial stress that culminates in necroptotic cell death and impairment of epithelial regeneration.[16] Mechanistically, our data supports a model in which chronic ISR activation and RIPK3-mediated necroptosis form a self-reinforcing epithelial injury loop in CD. These shared molecular defects across murine and human models support a

convergent epithelial stress axis driving barrier dysfunction in CD and offer a tractable therapeutic target.

In this study, enteroid assays provided critical functional insights into how ISR and necroptosis compromise epithelial homeostasis and function. Parallel defects in enteroid formation, budding, and regenerative capacity across murine and CD PDEs underscore the role of chronic ISR activation in compromising ISC viability and epithelial renewal. In response to injury, stem cells adopt new fates and lineages to support regeneration of the damaged tissue.[40] While acute ISR activation promotes adaptive epithelial repair, chronic ISR signaling is implicated in defective epithelial regeneration.[41] Our findings demonstrate that in CD, sustained ISR activation maintains IECs into a chronic stress state that inhibits ISC renewal thus contributing directly to defective mucosal and permeability barrier healing. Together with our prior work, in this study we show that aberrant Paneth cell expansion is a conserved feature of epithelial stress response in CD.[16] Paneth cells provide essential niche signals for stem cell maintenance, and antimicrobial peptides and their expansion may represent a compensatory but dysfunctional response to epithelial injury. These findings reinforce the concept that chronic ISR disrupts epithelial homeostasis at multiple levels, by depleting viable IECs but also by inhibiting ISC renewal and disrupting lineage specification. ISR activation has been linked to cellular metabolism and ISR modulates stem cell fate decisions in response to serine metabolism.[40] This raises the intriguing possibility that dietary interventions could synergize with ISR-targeted therapies to promote mucosal and permeability barrier healing in CD. Together with our previous findings, data provided here show that in CD, pathologic IEC death in the intestine is mediated primarily by necroptosis and not apoptosis.[16] In the small intestine, enterocytes and enterocyte progenitor cells are particularly sensitive to necroptosis, a caspase-independent lytic form of

programmed cell death.[16, 42, 43] We postulate that the increased susceptibility of the enterocyte progenitors to necroptosis, contributes to impaired epithelial regeneration identified in the DKO, *Tnf*$^{\Delta ARE/+}$ and CD PDEs.

The link between impaired gut barrier and inflammatory bowel disease (IBD) was established >30 years ago, but the lack of clinical translation is in part due to the lack of identifiable therapeutic targets. Previous studies have shown that growth factors such as EGFR and R-spondin1 can restore the damaged intestinal epithelium, however both are mitogens and could increase the risk of developing colorectal cancer.[44, 45] Larazotide acetate was identified as a BH therapy however, its barrier-protective effects were not recapitulated in clinical trials.[46] A major advance of this work is the demonstration that pharmacologic inhibition of ISR or necroptosis restores epithelial homeostasis by promoting regenerative growth of the epithelium in both murine and human models of CD. *In vivo*, both ISRIB and Necrostatin-1 significantly improved villus architecture, reduced inflammation, and normalized Paneth cell numbers in DKO and *Tnf*$^{\Delta ARE/+}$ mice. *Ex vivo*, ISRIB and Nec-1 improved IEC viability, ISC renewal, and regeneration of the crypt-villus-like enteroid architecture. Notably, ISRIB promotes lung repair and reduces fibrosis by accelerating the differentiation of alveolar epithelial cells after injury.[47] This implies the potential of ISRIB to restore epithelial homeostasis across different tissues. It also indicates that given prophylactically, ISRIB could reduce or prevent fibrostenotic strictures in CD. Treatment of stricturing and fistulizing CD is one of the hardest challenges in CD care.[48] Strictures are caused by chronic inflammation and no approved antifibrotic drug exists yet. Moreover, once fibrosis is established in the gut, treatment has limited impact. While anti-TNF biologics can reduce inflammatory components of a stricture, they do not reverse scar tissue and surgical management remains the only option. Notably, the ERICa trial demonstrated that in CD

patients, permeability barrier healing is associated with a decreased risk of developing intestinal complications.[2] Consistent with that, we propose that inhibition of ISR has the potential to prevent the initiation and development of fibrostricturing CD. ISRIB, though investigational, has shown safety and oral bioavailability in preclinical studies, making it attractive for translational development for CD.[49] Pazopanib and ponatinib are FDA-approved with well-characterized pharmaco-kinetics.[38] While pazopanib and ponatinib have pleiotropic kinase targets at oncologic doses, multiple independent proteomic screens demonstrate RIPK3 selectivity at sub-oncologic concentrations used here, supporting the epithelial-protective mechanism proposed.[38, 50] At micromolar concentrations, ponatinib has been shown to inhibit proliferation and induce apoptosis in cancer cells.[51]. However, it is now clear that at sub-oncologic concentrations ponatinib inhibits necroptosis and not the cell-cycle.[52] We show that at sub-oncologic concentrations, pazopanib and ponatinib effectively restore epithelial viability, epithelial regenerative capacity, and TEER in CD PDEs. Unlike investigational RIPK3 inhibitors like GSK'872 and GSK843, which can paradoxically trigger apoptosis, pazopanib and ponatinib have been shown to suppress both necroptosis and RIPK3-driven inflammatory signaling, making them ideal candidates for CD therapy.[53] We postulate that the dual effect of pazopanib and ponatinib on both necroptosis and inflammation may be critical for effective restoration of permeability barrier integrity in CD.

ER in CD is associated with decreased risk of disease progression and a more favorable long-term disease outcome.[10] Multiple studies have used confocal endomicroscopy to assess the structure and function of the intestinal permeability barrier *in vivo* and demonstrated that impaired barrier integrity in CD patients is linked with persistence of clinical symptoms and relapsing disease behavior.[2, 13, 14, 54] These findings have led to the idea that mucosal healing

or endoscopic remission is not sufficient to improve treatment outcomes and that restoration of permeability barrier is essential to achieve sustained clinical remission and to reduce intestinal complications in CD. Despite that, there are no clinically validated methods for early diagnosis, risk stratification, or targeted intervention based on impaired permeability and there are no BH therapies. Our study introduces a paradigm shift in CD therapy, by focusing on BH, an unaddressed yet decisive driver of clinical outcomes in CD. We demonstrate functional restoration of permeability barrier integrity using TEER. Prior studies have linked RIPK3-mediated necroptosis to alterations in cell-cell junctional proteins (e.g., E-cadherin, occluding, Zonulin-1), but whether RIPK3 inhibition directly stabilizes junctional architecture in CD remains to be determined.[55] Integrating junctional protein imaging and transcriptomics into future PDE assays will clarify how ISR and RIPK3 inhibition mechanistically restore epithelial barrier structure.

Our work also opens several avenues for further investigation. First, development of gut-restricted RIPK3 inhibitors could maximize efficacy while minimizing systemic exposure. Second, incorporating BH as a therapeutic endpoint in clinical trials will be critical for aligning CD therapy with outcomes most predictive of long-term health.[2] Additionally, biomarkers of ISR activation and RIPK3 signaling, such as peIF2α or RIPK3 expression in mucosal biopsies, could be used to stratify patients and guide therapy. Identifying patients with persistent barrier dysfunction will allow targeted use of barrier-restoring agents. Finally, integration of PDE-based assays and TEER measurements into personalized medicine pipelines could enable *ex vivo* prediction of treatment response in CD.

This study has a few limitations. First, while pazopanib and ponatinib rescue epithelial regeneration and barrier integrity, their precise epithelial-intrinsic targets beyond RIPK3 were not dissected here. Second, our models primarily address epithelial-driven pathology and may not

fully capture immune-mediated contributions to CD *in vivo*. Third, we did not directly measure tight junction or adheres junction protein remodeling, which will be essential in future studies to mechanistically link ISR/RIPK3 inhibition to structural barrier repair. Finally, long-term dosing and gut-restricted delivery strategies must be explored to minimize systemic exposure and optimize translational feasibility.

Summary. Our study defines ISR activation and RIPK3-mediated necroptosis as convergent mechanisms driving epithelial dysfunction in CD. By demonstrating that pharmacological inhibition of these pathways restores epithelial regeneration and permeability barrier integrity, we provide proof-of-concept for targeting epithelial stress response and necroptosis as a therapeutic strategy in CD. Repurposing pazopanib and ponatinib offers a potentially translatable approach to achieve permeability barrier healing, a treatment endpoint superior to endoscopic healing for predicting long-term disease outcomes. Incorporating permeability barrier healing into therapeutic targets and clinical trial design has the potential to fundamentally reshape CD management, moving beyond inflammation control toward sustained restoration of epithelial homeostasis.

References.

[1] Peyrin-Biroulet L. Mucosal Healing in Crohn's Disease and Ulcerative Colitis. Gastroenterol Hepatol (N Y) 2020;16(4):206–8.

[2] Rath T, Atreya R, Bodenschatz J, Uter W, Geppert CE, Vitali F, Fischer S, Waldner MJ, Colombel JF, Hartmann A, Neurath MF. Intestinal Barrier Healing Is Superior to Endoscopic and Histologic Remission for Predicting Major Adverse Outcomes in

Inflammatory Bowel Disease: The Prospective ERIca Trial. Gastroenterology 2023;164(2):241–55.

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

[4] Odenwald MA, Turner JR. Intestinal permeability defects: is it time to treat? Clin Gastroenterol Hepatol 2013;11(9):1075–83.

[5] Dunleavy KA, Raffals LE, Camilleri M. Intestinal Barrier Dysfunction in Inflammatory Bowel Disease: Underpinning Pathogenesis and Therapeutics. Dig Dis Sci 2023;68(12):4306–20.

[6] Turpin W, Lee SH, Raygoza Garay JA, Madsen KL, Meddings JB, Bedrani L, Power N, Espin-Garcia O, Xu W, Smith MI, Griffiths AM, Moayyedi P, Turner D, Seidman EG, Steinhart AH, Marshall JK, Jacobson K, Mack D, Huynh H, Bernstein CN, Paterson AD, Crohn's, Colitis Canada Genetic Environmental Microbial Project Research C, Abreu CGPrsdiM, Croitoru K. Increased Intestinal Permeability Is Associated With Later Development of Crohn's Disease. Gastroenterology 2020;159(6):2092–100 e5.

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

[8] Keita AV, Lindqvist CM, Ost A, Magana CDL, Schoultz I, Halfvarson J. Gut Barrier Dysfunction-A Primary Defect in Twins with Crohn's Disease Predominantly Caused by Genetic Predisposition. J Crohns Colitis 2018;12(10):1200–9.

[9] Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke JD, Serino M, Tilg H, Watson A, Wells JM. Intestinal permeability--a new target for disease prevention and therapy. BMC Gastroenterol 2014;14:189.

[10] Shah SC, Colombel JF, Sands BE, Narula N. Systematic review with meta-analysis: mucosal healing is associated with improved long-term outcomes in Crohn's disease. Aliment Pharmacol Ther 2016;43(3):317–33.

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

[12] Baert F, Moortgat L, Van Assche G, Caenepeel P, Vergauwe P, De Vos M, Stokkers P, Hommes D, Rutgeerts P, Vermeire S, D'Haens G, Belgian Inflammatory Bowel Disease Research G, North-Holland Gut C. Mucosal healing predicts sustained clinical remission in patients with early-stage Crohn's disease. Gastroenterology 2010;138(2):463–8; quiz e10–1.

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

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

[15] Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. EMBO Rep 2016;17(10):1374–95.

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

[17] Bond S, Lopez-Lloreda C, Gannon PJ, Akay-Espinoza C, Jordan-Sciutto KL. The Integrated Stress Response and Phosphorylated Eukaryotic Initiation Factor 2alpha in Neurodegeneration. J Neuropathol Exp Neurol 2020;79(2):123–43.

[18] Chambers JE, Dalton LE, Clarke HJ, Malzer E, Dominicus CS, Patel V, Moorhead G, Ron D, Marciniak SJ. Actin dynamics tune the integrated stress response by regulating eukaryotic initiation factor 2alpha dephosphorylation. Elife 2015;4.

[19] Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 2013;38(2):209–23.

[20] Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 2012;148(1-2):213–27.

[21] Najjar M, Saleh D, Zelic M, Nogusa S, Shah S, Tai A, Finger JN, Polykratis A, Gough PJ, Bertin J, Whalen M, Pasparakis M, Balachandran S, Kelliher M, Poltorak A, Degterev A.

RIPK1 and RIPK3 Kinases Promote Cell-Death-Independent Inflammation by Toll-like Receptor 4. Immunity 2016;45(1):46–59.

[22] Rebsamen M, Heinz LX, Meylan E, Michallet MC, Schroder K, Hofmann K, Vazquez J, Benedict CA, Tschopp J. DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. EMBO Rep 2009;10(8):916–22.

[23] Pierdomenico M, Negroni A, Stronati L, Vitali R, Prete E, Bertin J, Gough PJ, Aloi M, Cucchiara S. Necroptosis is active in children with inflammatory bowel disease and contributes to heighten intestinal inflammation. Am J Gastroenterol 2014;109(2):279–87.

[24] Pagnini C, Cominelli F. Tumor Necrosis Factor's Pathway in Crohn's Disease: Potential for Intervention. Int J Mol Sci 2021;22(19).

[25] Yamazaki K, McGovern D, Ragoussis J, Paolucci M, Butler H, Jewell D, Cardon L, Takazoe M, Tanaka T, Ichimori T, Saito S, Sekine A, Iida A, Takahashi A, Tsunoda T, Lathrop M, Nakamura Y. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. Hum Mol Genet 2005;14(22):3499–506.

[26] Wang Y, George SP, Srinivasan K, Patnaik S, Khurana S. Actin reorganization as the molecular basis for the regulation of apoptosis in gastrointestinal epithelial cells. Cell Death Differ 2012;19(9):1514–24.

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

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

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

[30] Fujimichi Y, Otsuka K, Tomita M, Iwasaki T. An Efficient Intestinal Organoid System of Direct Sorting to Evaluate Stem Cell Competition in Vitro. Sci Rep 2019;9(1):20297.

[31] Fuller MK, Faulk DM, Sundaram N, Shroyer NF, Henning SJ, Helmrath MA. Intestinal crypts reproducibly expand in culture. J Surg Res 2012;178(1):48–54.

[32] Hu Z, Yu P, Zhang Y, Yang Y, Zhu M, Qin S, Xu JT, Duan D, Wu Y, Wang D, Rowan MJ, Hu NW. Inhibition of the ISR abrogates mGluR5-dependent long-term depression and spatial memory deficits in a rat model of Alzheimer's disease. Transl Psychiatry 2022;12(1):96.

[33] Erben U, Loddenkemper C, Doerfel K, Spieckermann S, Haller D, Heimesaat MM, Zeitz M, Siegmund B, Kuhl AA. A guide to histomorphological evaluation of intestinal inflammation in mouse models. Int J Clin Exp Pathol 2014;7(8):4557–76.

[34] Baumgart DC, Sandborn WJ. Crohn's disease. Lancet 2012.

[35] Sidrauski C, Acosta-Alvear D, Khoutorsky A, Vedantham P, Hearn BR, Li H, Gamache K, Gallagher CM, Ang KK, Wilson C, Okreglak V, Ashkenazi A, Hann B, Nader K, Arkin MR, Renslo AR, Sonenberg N, Walter P. Pharmacological brake-release of mRNA translation enhances cognitive memory. Elife 2013;2:e00498.

[36] Sidrauski C, McGeachy AM, Ingolia NT, Walter P. The small molecule ISRIB reverses the effects of eIF2alpha phosphorylation on translation and stress granule assembly. Elife 2015;4.

[37] Fulda S. Repurposing anticancer drugs for targeting necroptosis. Cell Cycle 2018;17(7):829–32.

[38] Fauster A, Rebsamen M, Huber KV, Bigenzahn JW, Stukalov A, Lardeau CH, Scorzoni S, Bruckner M, Gridling M, Parapatics K, Colinge J, Bennett KL, Kubicek S, Krautwald S, Linkermann A, Superti-Furga G. A cellular screen identifies ponatinib and pazopanib as inhibitors of necroptosis. Cell Death Dis 2015;6:e1767.

[39] Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science 2006;313(5792):1438–41.

[40] Novak JSS, Polak L, Baksh SC, Barrows DW, Schernthanner M, Jackson BT, Thompson EAN, Gola A, Abdusselamoglu MD, Bonny AR, Gonzales KAU, Brunner JS, Bridgeman AE, Stewart KS, Hidalgo L, Dela Cruz-Racelis J, Luo JD, Gur-Cohen S, Pasolli HA, Carroll TS, Finley LWS, Fuchs E. The integrated stress response fine-tunes stem cell fate decisions upon serine deprivation and tissue injury. Cell Metab 2025;37(8):1715–31 e11.

[41] Han S, Budinger GRS, Gottardi CJ. Alveolar epithelial regeneration in the aging lung. J Clin Invest 2023;133(20).

[42] Patankar JV, Bubeck M, Acera MG, Becker C. Breaking bad: necroptosis in the pathogenesis of gastrointestinal diseases. Front Immunol 2023;14:1203903.

[43] Haber AL, Biton M, Rogel N, Herbst RH, Shekhar K, Smillie C, Burgin G, Delorey TM, Howitt MR, Katz Y, Tirosh I, Beyaz S, Dionne D, Zhang M, Raychowdhury R, Garrett WS, Rozenblatt-Rosen O, Shi HN, Yilmaz O, Xavier RJ, Regev A. A single-cell survey of the small intestinal epithelium. Nature 2017;551(7680):333–9.

[44] Yamaoka T, Yan F, Cao H, Hobbs SS, Dise RS, Tong W, Polk DB. Transactivation of EGF receptor and ErbB2 protects intestinal epithelial cells from TNF-induced apoptosis. Proc Natl Acad Sci U S A 2008;105(33):11772–7.

[45] Zhao J, de Vera J, Narushima S, Beck EX, Palencia S, Shinkawa P, Kim KA, Liu Y, Levy MD, Berg DJ, Abo A, Funk WD. R-spondin1, a novel intestinotrophic mitogen, ameliorates experimental colitis in mice. Gastroenterology 2007;132(4):1331–43.

[46] Leffler DA, Kelly CP, Abdallah HZ, Colatrella AM, Harris LA, Leon F, Arterburn LA, Paterson BM, Lan ZH, Murray JA. A randomized, double-blind study of larazotide acetate to prevent the activation of celiac disease during gluten challenge. Am J Gastroenterol 2012;107(10):1554–62.

[47] Watanabe S, Markov NS, Lu Z, Piseaux Aillon R, Soberanes S, Runyan CE, Ren Z, Grant RA, Maciel M, Abdala-Valencia H, Politanska Y, Nam K, Sichizya L, Kihshen HG, Joshi N, McQuattie-Pimentel AC, Gruner KA, Jain M, Sznajder JI, Morimoto RI, Reyfman PA, Gottardi CJ, Budinger GRS, Misharin AV. Resetting proteostasis with ISRIB promotes epithelial differentiation to attenuate pulmonary fibrosis. Proc Natl Acad Sci U S A 2021;118(20).

[48] Rieder F, Mukherjee PK, Massey WJ, Wang Y, Fiocchi C. Fibrosis in IBD: from pathogenesis to therapeutic targets. Gut 2024;73(5):854–66.

[49] Halliday M, Radford H, Sekine Y, Moreno J, Verity N, le Quesne J, Ortori CA, Barrett DA, Fromont C, Fischer PM, Harding HP, Ron D, Mallucci GR. Partial restoration of protein synthesis rates by the small molecule ISRIB prevents neurodegeneration without pancreatic toxicity. Cell Death Dis 2015;6(3):e1672.

[50] Li JX, Feng JM, Wang Y, Li XH, Chen XX, Su Y, Shen YY, Chen Y, Xiong B, Yang CH, Ding J, Miao ZH. The B-Raf(V600E) inhibitor dabrafenib selectively inhibits RIP3 and alleviates acetaminophen-induced liver injury. Cell Death Dis 2014;5(6):e1278.

[51] Liu C, Mu X, Wang X, Zhang C, Zhang L, Yu B, Sun G. Ponatinib Inhibits Proliferation and Induces Apoptosis of Liver Cancer Cells, but Its Efficacy Is Compromised by Its Activation on PDK1/Akt/mTOR Signaling. Molecules 2019;24(7).

[52] Najjar M, Suebsuwong C, Ray SS, Thapa RJ, Maki JL, Nogusa S, Shah S, Saleh D, Gough PJ, Bertin J, Yuan J, Balachandran S, Cuny GD, Degterev A. Structure guided design of potent and selective ponatinib-based hybrid inhibitors for RIPK1. Cell Rep 2015;10(11):1850–60.

[53] Mandal P, Berger SB, Pillay S, Moriwaki K, Huang C, Guo H, Lich JD, Finger J, Kasparcova V, Votta B, Ouellette M, King BW, Wisnoski D, Lakdawala AS, DeMartino MP, Casillas LN, Haile PA, Sehon CA, Marquis RW, Upton J, Daley-Bauer LP, Roback L, Ramia N, Dovey CM, Carette JE, Chan FK, Bertin J, Gough PJ, Mocarski ES, Kaiser WJ. RIP3 induces apoptosis independent of pronecrotic kinase activity. Mol Cell 2014;56(4):481–95.

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

[55] Negroni A, Colantoni E, Pierdomenico M, Palone F, Costanzo M, Oliva S, Tiberti A, Cucchiara S, Stronati L. RIP3 AND pMLKL promote necroptosis-induced inflammation and alter membrane permeability in intestinal epithelial cells. Dig Liver Dis 2017;49(11):1201–10.

Figure Legends.

Figure 1. ISR activation is a hallmark of epithelial injury in murine models of CD. (A-B) Representative Hematoxylin and eosin (H&E) stained ileal sections from distal ileum of villin-1/gelsolin double knockout (DKO) and *Tnf*$^{\Delta ARE/+}$ mice and their wild-type (WT) littermates which includes *Tnf*$^{+/+}$ mice. DKO and *Tnf*$^{\Delta ARE/+}$ mice show ileal wall thickening, villus blunting, villus fusion, and crypt elongation compared to their respective control mice. Histology scores, measuring intestinal inflammation, were significantly elevated in DKO and *Tnf*$^{\Delta ARE/+}$ mice compared to their control mice. **(C)** Immunohistochemistry and immunoblots show upregulation of phospho-EIF2α (pEIF2α) and IRGM1 expression levels in the *Tnf*$^{\Delta ARE/+}$ mice without any notable change in total eIF2α levels, confirming chronic ISR activation. Densitometric quantification of immunoblots for pEIF2a normalized to total eIF2α and IRGM1 normalized to β-actin is provided in the Results. Data are presented as mean ± SEM from ≥3 independent experiments. Statistical significance was determined using two-tailed Student's t-test, ****$P<.0001$, n=6 mice per group.

Figure 2. Chronic ISR activation impairs epithelial viability, regeneration, and growth in mouse and human models of CD. (A-B) Representative phase-contrast and fluorescence images of PI and Hoechst 33342 labelled enteroids from DKO, *Tnf*$^{\Delta ARE/+}$ and control mice (WT and *Tnf*$^{+/+}$, respectively) show increased intestinal epithelial cells (IEC) mortality in both murine models of CD. (A-B) Enteroids seeded at day 1 and analyzed at day 7 show significant inhibition of enteroid forming efficiency (percentage of plated crypts forming viable enteroids) in Controls *versus* DKO and *Tnf*$^{\Delta ARE/+}$ mice. Quantification of crypt buds per enteroid at Day 7 show reduced budding in

DKO and *Tnf*$^{\Delta ARE/+}$ mouse enteroids compared to their respective controls. Percentage of budded enteroids is significantly decreased in DKO and *Tnf*$^{\Delta ARE/+}$ mice at Day 7 relative to controls. **(C)** CD patient-derived enteroids (PDEs) exhibit significantly increased mortality and inhibition of enteroid forming efficiency, number of enteroids budded, and total number of budded enteroids formed compared to PDEs from non-CD controls. **(D)** Representative confocal images show Paneth cell expansion in DKO and *Tnf*$^{\Delta ARE/+}$ mouse enteroids. Quantification of Paneth cell numbers reveals a significant increase in both DKO and *Tnf*$^{\Delta ARE/+}$ mouse enteroids compared to their respective control mouse enteroids. **(E)** Representative confocal images of enteroids immunoassayed for peIF2α shows significant upregulation of peIF2α expression in the *Tnf*$^{\Delta ARE/+}$ mouse enteroids compared to *Tnf*$^{+/+}$ mouse enteroids. Data obtained from 4 mice per group and 8 patients per group and expressed as mean ± SEM from ≥3 independent experiments. Statistical significance was assessed using one-way ANOVA with Tukey's post-hoc test; $^{**}P<.01$, $^{***}P<.001$, $^{****}P<.0001$. At least 50 enteroids per sample were analyzed for the quantitative analysis.

Figure 3. *In vivo* pharmacologic inhibition of ISR and necroptosis restore epithelial homeostasis in murine models of CD. DKO **(A-B)** and *Tnf*$^{\Delta ARE/+}$ **(C)** mice were administered ISRIB (0.25 mg/kg, i.p.) or Necrostatin-1 (Nec-1; 1.65 mg/kg; i.p.) as described in the Methods. Representative H&E-stained ileal sections show restoration of tissue architecture in the DKO, and *Tnf*$^{\Delta ARE/+}$ mice treated with Nec-1 and ISRIB compared to vehicle-treated controls. Both treatments significantly improved the histologic score showing inhibition of intestinal inflammation. Other indices of tissue histomorphology such as thickening of the muscularis propria, crypt elongation, number of fused villi and total numbers of Paneth cells per crypt, were

also significantly reduced by Nec-1 and ISRIB treatment compared to vehicle-treated controls. Data is presented as mean ± SEM from ≥3 independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test; ****$P<.0001$; n=6 mice per group.

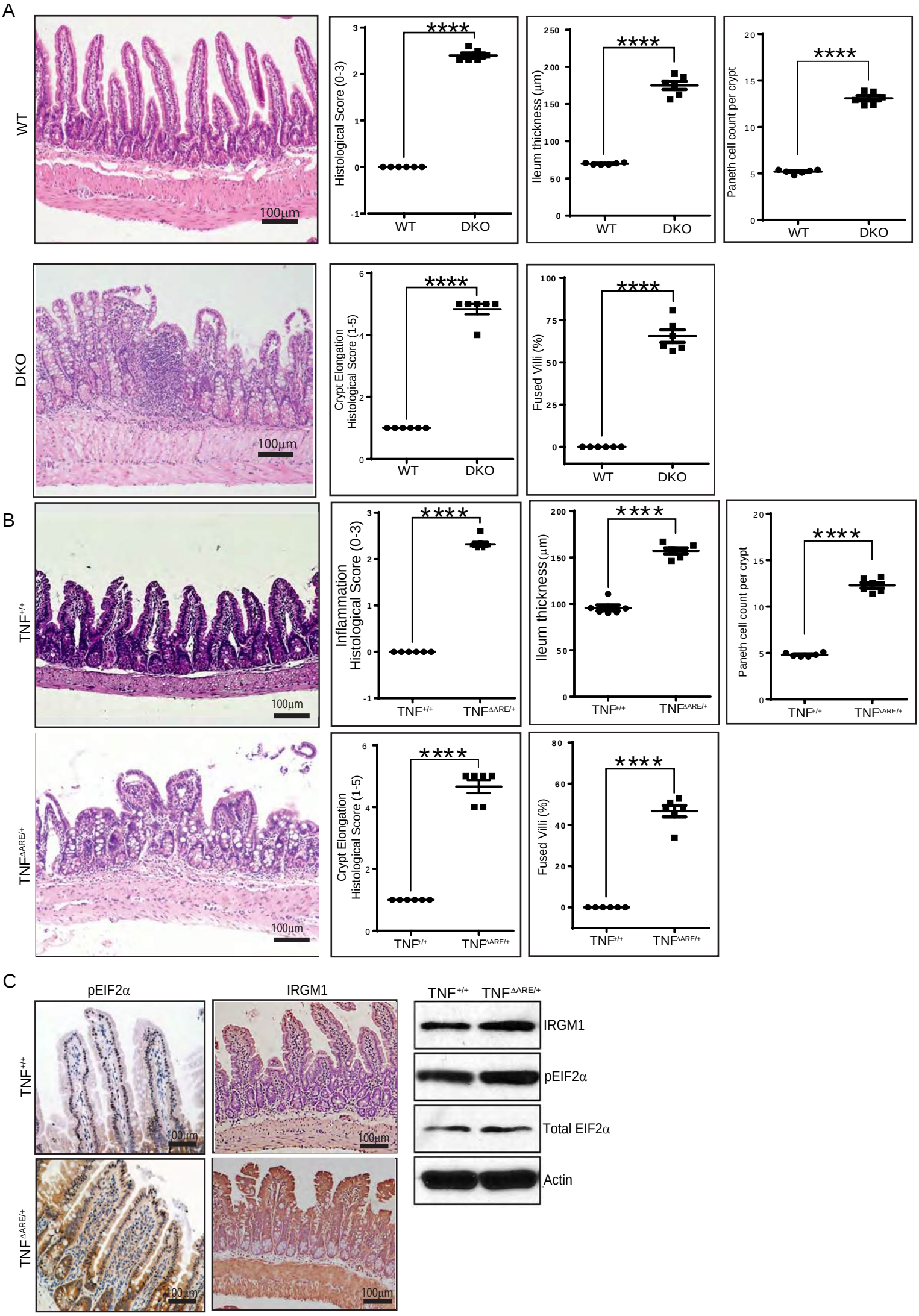
Figure 4. Repurposed cancer drugs with potent RIPK3 inhibiting properties, rescue epithelial viability and regeneration. (A-C) Representative phase-contrast and PI stained-merged images of DKO and *Tnf*$^{\Delta ARE/+}$ and CD PDEs treated with ISRIB, Nec-1, pazopanib, ponatinib, or vehicle (DMSO) show significant inhibition of IEC mortality. Enteroids exogenously treated with these inhibitors also significantly improved enteroid forming efficiency, number of enteroids with buds, and the number of buds per enteroid compared to vehicle-treated control enteroids. Data is from 4 mice per group and 8 patients per group, represented as mean ± SEM from ≥3 independent experiments. Statistical significance was assessed using one-way ANOVA with Tukey's post-hoc test; asterisks (*) represent: **$P<.01$, ***$P<.001$, ****$P<.0001$.

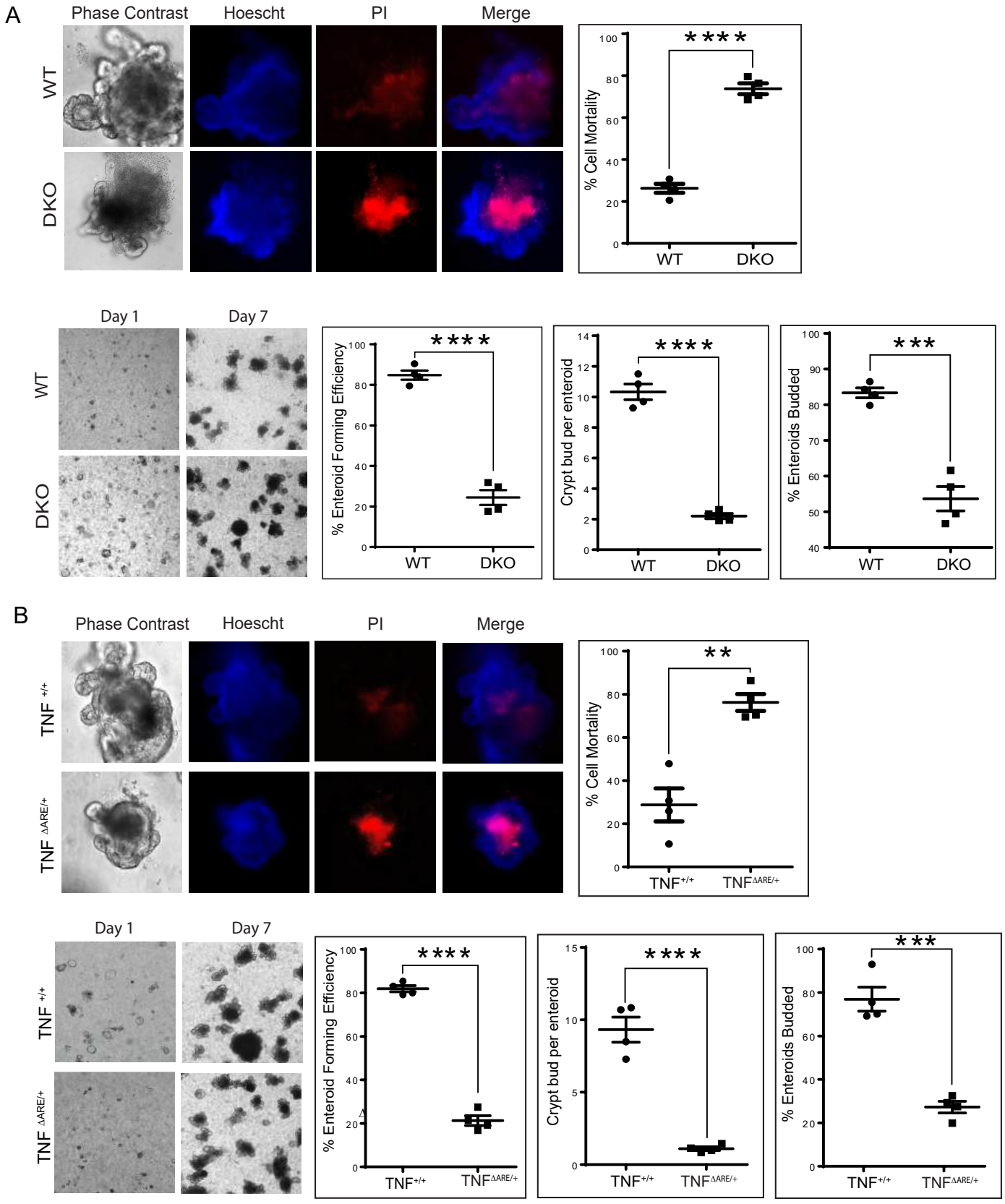
Figure 5. Pharmacologic blockade of ISR, necroptosis, and RIPK3 signaling restore epithelial permeability barrier function in CD patient-derived enteroids. Transepithelial electrical resistance (TEER, $\Omega.cm^2$) was measured in CD PDE treated with vehicle (DMSO), Nec-1, ISRIB, pazopanib, or ponatinib. All agents significantly improved TEER compared with vehicle-treated CD enteroids, indicating restoration of epithelial barrier integrity. Data represent mean ± SEM from ≥3 independent experiments; asterisks (*) denote: ***$P<.001$.

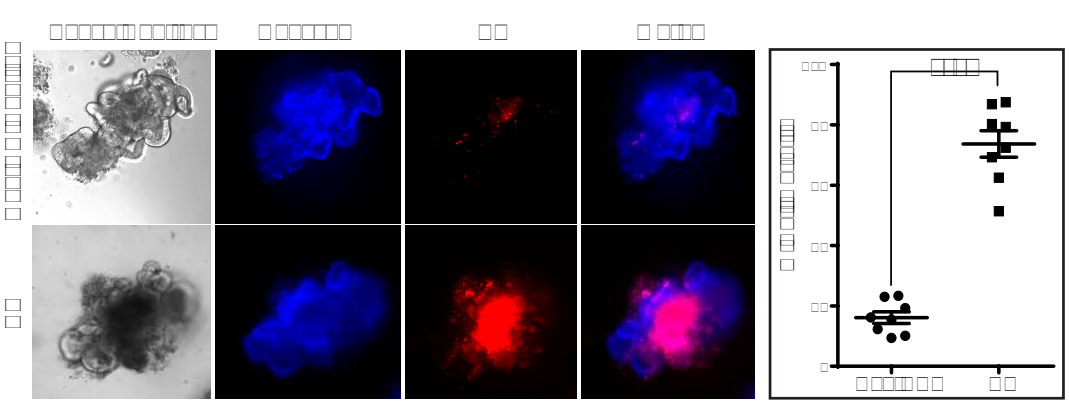
Supplemental Figure 1. Necroptosis inhibition, but not caspase inhibition rescues IEC survival in *Tnf*$^{\Delta ARE/+}$ mouse enteroids. Enteroids were treated with Nec-1 or Z-VAD-FMK. PI

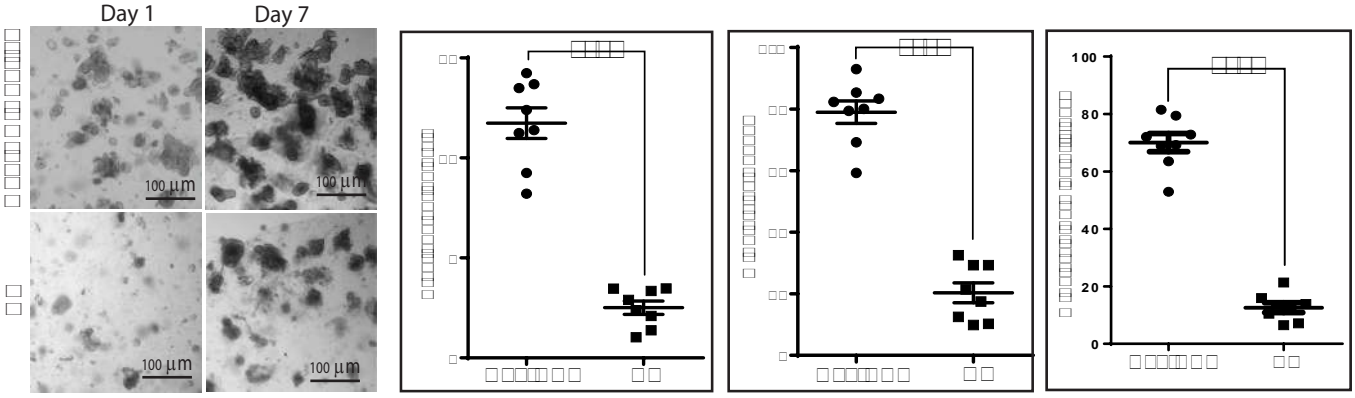
staining demonstrates reduced IEC mortality only with Nec-1 treatment. Quantification shows percent PI-positive cells. Data represent mean ± SEM; n=4 mice per group.

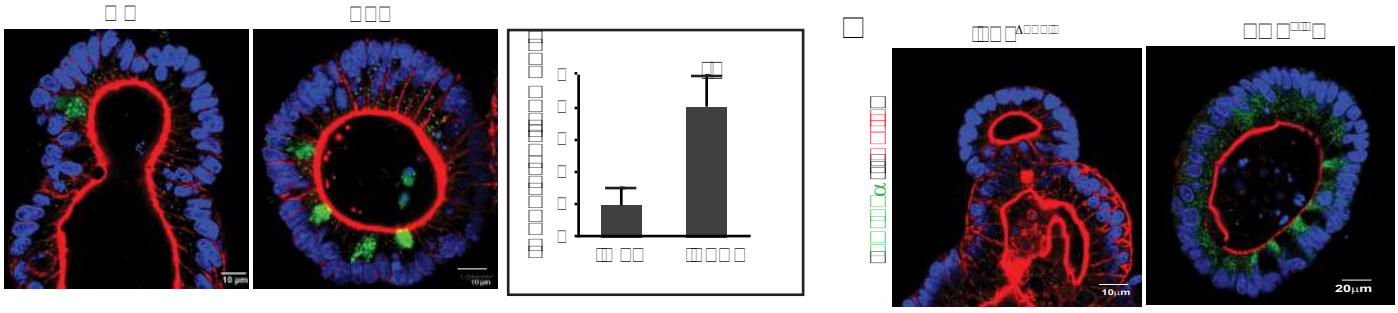
Supplemental Figure 2. Pazopanib and ponatinib restore epithelial survival and regenerative growth in murine and human CD enteroids. Representative images and quantification of enteroid forming efficiency, budding percentage, and crypt buds per enteroid following drug treatment. Data represent mean ± SEM; n=4 mice and n=6 patients per group.

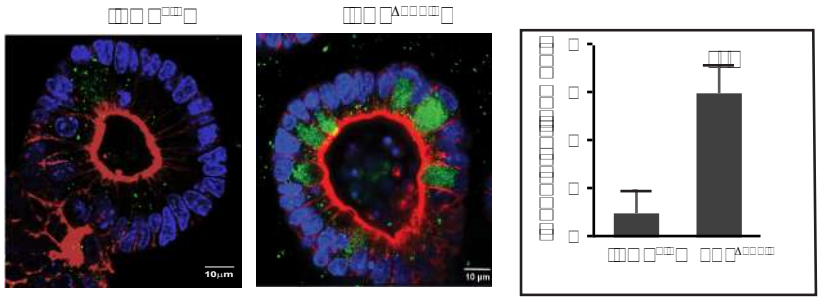

A
WT
DKO
100µm
Histological Score (0-3)
Ileum thickness (µm)
Paneth cell count per crypt
Crypt Elongation Histological Score (1-5)
Fused Villi (%)
****
WT
DKO
B
TNF+/+
TNFΔARE/+
Inflammation Histological Score (0-3)
C
pEIF2α
IRGM1
Total EIF2α
Actin


A
Phase Contrast
Hoescht
PI
Merge
WT
DKO
% Cell Mortality
****
WT
DKO
Day 1
Day 7
WT
DKO
% Enteroid Forming Efficiency
****
WT
DKO
Crypt bud per enteroid
****
WT
DKO
% Enteroids Budded
***
WT
DKO
B
Phase Contrast
Hoescht
PI
Merge
TNF +/+
TNF ΔARE/+
% Cell Mortality
**
TNF+/+
TNFΔARE/+
Day 1
Day 7
TNF +/+
TNF ΔARE/+
% Enteroid Forming Efficiency
****
TNF+/+
TNFΔARE/+
Crypt bud per enteroid
****
TNF+/+
TNFΔARE/+
% Enteroids Budded
***
TNF+/+
TNFΔARE/+

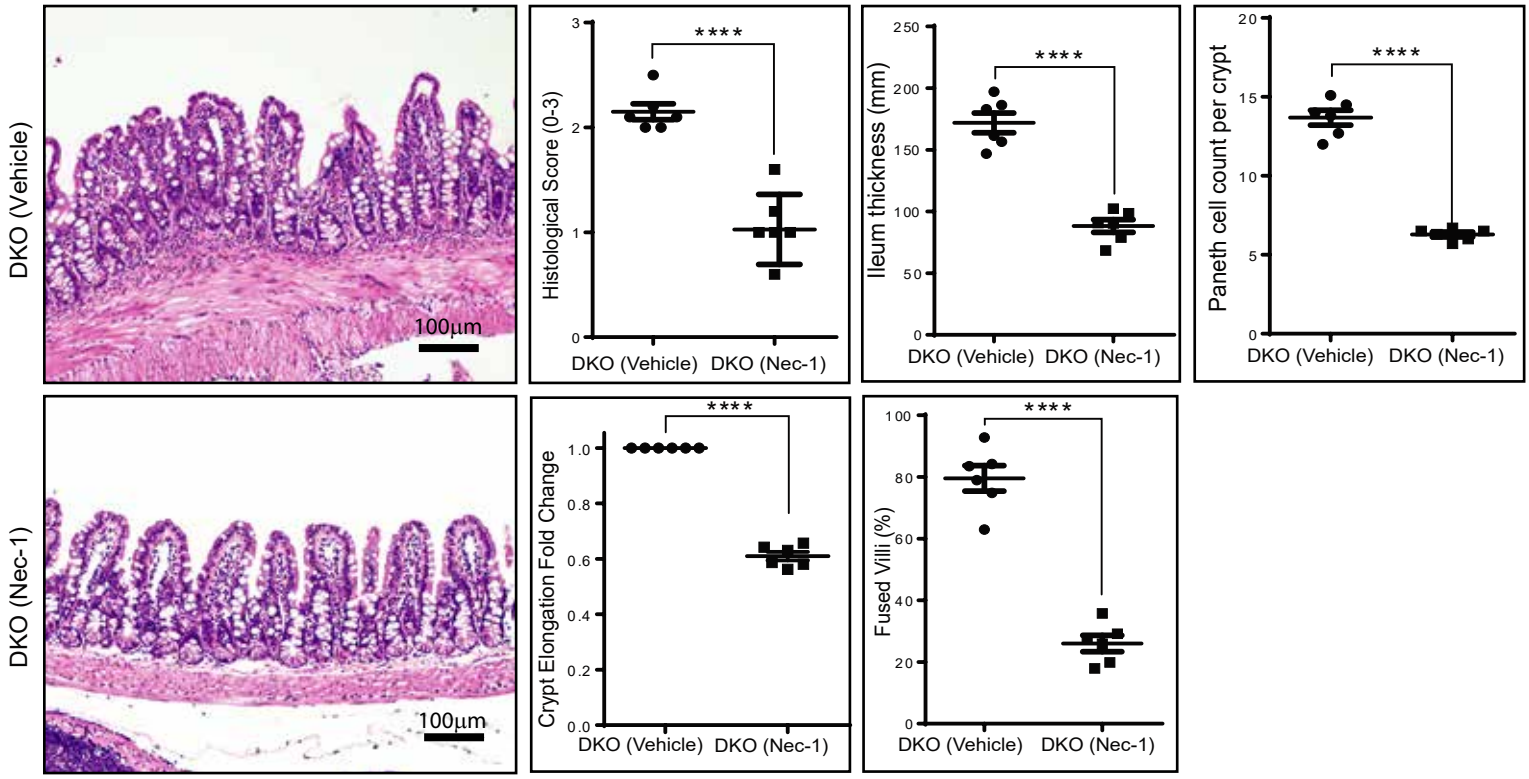



Day 1
Day 7
100 µm
100 µm
100 µm
100 µm
100
80
60
40
20
0

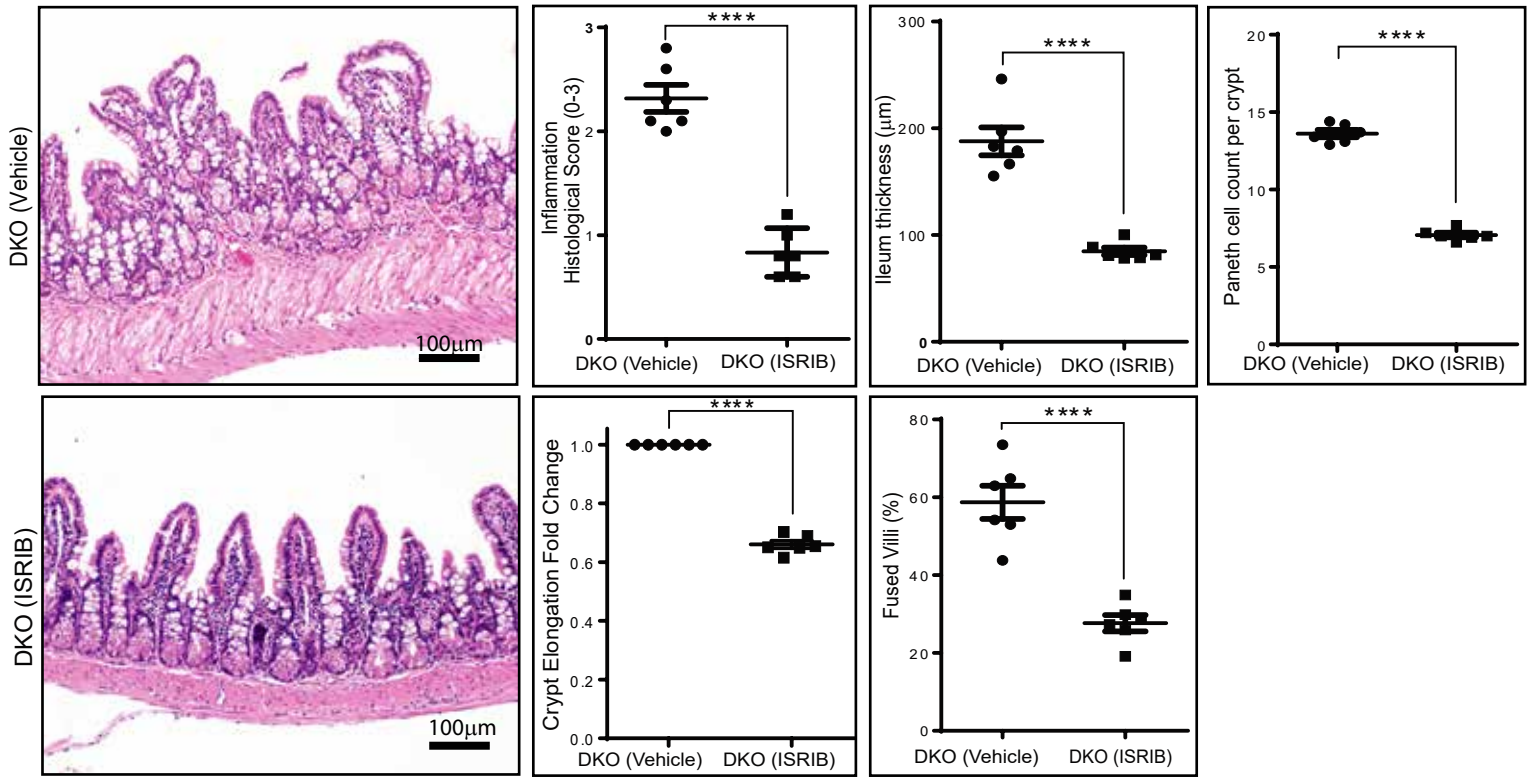

10 µm
10µm
20µm

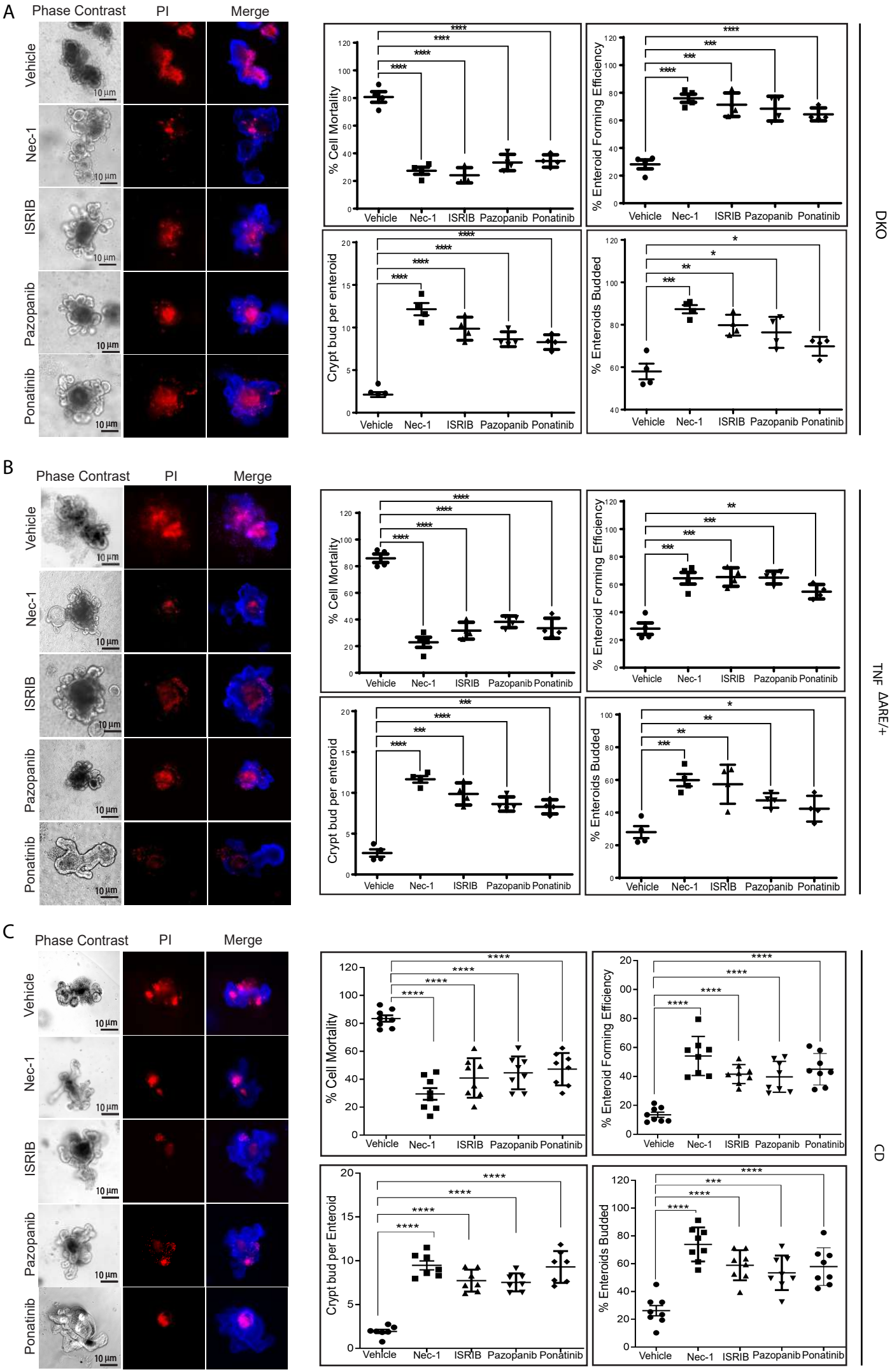

10µm
10 µm

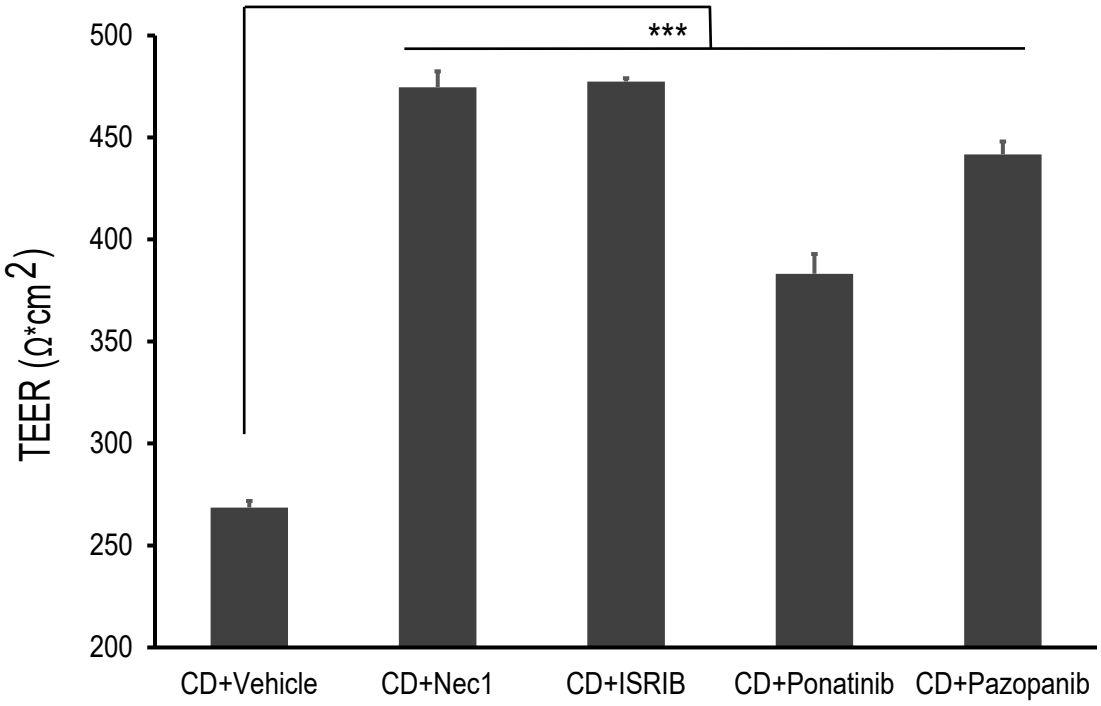
A


DKO (Vehicle)
DKO (Nec-1)
100µm
Histological Score (0-3)
Ileum thickness (mm)
Paneth cell count per crypt
Crypt Elongation Fold Change
Fused Villi (%)
****
DKO (Vehicle)
DKO (Nec-1)

B


DKO (Vehicle)
DKO (ISRIB)
100µm
Inflammation Histological Score (0-3)
Ileum thickness (µm)
Paneth cell count per crypt
Crypt Elongation Fold Change
Fused Villi (%)
****
DKO (Vehicle)
DKO (ISRIB)


A
Phase Contrast
PI
Merge
Vehicle
Nec-1
ISRIB
Pazopanib
Ponatinib
10 µm
% Cell Mortality
% Enteroid Forming Efficiency
Crypt bud per enteroid
% Enteroids Budded
Vehicle
Nec-1
ISRIB
Pazopanib
Ponatinib
DKO
B
TNF ΔARE/+
C
CD


***
500
450
400
350
300
250
200
TEER (Ω*cm2)
CD+Vehicle
CD+Nec1
CD+ISRIB
CD+Ponatinib
CD+Pazopanib