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ErbB2 and ErbB3 regulate recovery from dextran sulfate sodium-induced colitis by promoting mouse colon epithelial cell survival

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Abstract

ErbB2 and ErbB3 receptor tyrosine kinases are key regulators of proliferation, migration, differentiation and cell survival; however, their roles in gastrointestinal biology remain poorly defined. We hypothesized that ErbB2 and ErbB3 promote colon epithelial cell survival in the context of the wound healing response following colitis. In this study, mice bearing intestinal epithelial-specific deletion of ErbB2 or ErbB3 were treated with dextran sulfate sodium (DSS). Colon sections were examined for injury, cytokine expression, epithelial cell proliferation and apoptosis. Deletion of epithelial ErbB2 did not affect the extent of intestinal injury in response to DSS, while deletion of ErbB3 slightly increased injury. However, the roles of both receptors were more apparent during recovery from DSS colitis in which ErbB2 or ErbB3 epithelial deletion resulted in greater inflammation and crypt damage during the early reparative period. Moreover, loss of ErbB3 prevented normal epithelial regeneration in the long-term with damage persisting for at least 6 weeks following a single round of DSS. Delayed recovery in mice with epithelial deletion of ErbB2 or ErbB3 was associated with increased colonic expression of TNF- α and increased epithelial apoptosis. Furthermore, epithelial ErbB3 deletion increased apoptosis at baseline and during DSS injury. Additionally, epithelial cell hyperproliferation during recovery was exacerbated by deletion of either ErbB2 or ErbB3. These results suggest that ErbB2 and ErbB3 play important cytoprotective and reparative roles in the colonic epithelium following injury by promoting colon epithelial cell survival.

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Growth factors play significant roles in maintaining epithelial integrity through the regulation of intestinal epithelial cell migration, proliferation and cell survival^{1, 2}. Disruption of intestinal epithelial integrity contributes to the pathogenesis of several gastrointestinal disorders, including inflammatory bowel disease. The ErbB family of receptor tyrosine kinases, consisting of ErbB1 (EGFR), ErbB2 (Her2), ErbB3 (Her3), and ErbB4 (Her4), can be activated by direct interaction with ligands or by forming heterodimers with other ErbBs, leading to increased kinase activity and downstream signal transduction. The ErbB family members are potential therapeutic targets as evidenced by the protective effects of EGF in experimental colitis and in clinical trials with ulcerative colitis^{3, 4}. However, the direct influence of individual ErbBs on intestinal injury and repair *in vivo* is relatively not well defined.

ErbB2 has been extensively studied in many types of cancers⁵; however, determination of its role in intestinal development and epithelial response to injury and inflammation has been hampered by the fact that ErbB2 deletion is embryonic lethal due to both cardiac and neural defects⁶. Although no ligands have been identified for ErbB2, its activation can be induced by heterodimerization with ligand-occupied EGFR, ErbB3 or ErbB4⁷. ErbB2 has been implicated in mediating myoblast cell survival and in inhibiting cancer cell apoptosis^{8, 9}. In addition, our group has shown that ErbB2 is transactivated by tumor necrosis factor alpha (TNF- α), which in turn protects intestinal epithelial cells from TNF- α -induced apoptosis¹⁰.

Interestingly, increased ErbB3 expression has been found in many tumors that overexpress ErbB2^{11, 12}. In contrast to ErbB2, ErbB3 binds numerous ligands including neuregulin; however, ErbB3 lacks intrinsic kinase activity and downstream signal transduction relies on heterodimerization with other ErbBs. For example, the ErbB2-ErbB3 dimer is crucial for ErbB2-mediated proliferation in ErbB2-overexpressing tumors¹³. ErbB3 signaling likely plays an important role in cell survival given that there are six putative PI3 kinase binding sites within its C-terminal domain¹⁴. Indeed, ErbB3 silencing by siRNA promotes apoptosis in lung adenocarcinoma cells¹⁵. However, delineation of the physiological role of ErbB3 has been difficult since, like ErbB2, ErbB3 deletion also causes embryonic lethal cardiac and neural defects¹⁶. ErbB3 deletion in the intestinal epithelium sensitizes the colonic epithelium to dextran sulfate sodium (DSS)-induced colitis¹⁷; however, the mechanism by which ErbB3 protects from injury and the effect of ErbB3 on intestinal epithelial recovery following injury are not known.

Numerous studies have implicated ErbB ligands and EGFR in the protection against DSS-induced colitis^{18–20}. Since EGFR and ErbB2 promote intestinal epithelial cell survival *in vitro* and *in vivo* in the presence of TNF- α , we hypothesized that ErbB2 and ErbB3 protect the intestinal epithelium during injury and inflammation *in vivo*. DSS causes a chemically induced colitis^{21, 22}, with direct cytotoxic insult to the colon epithelium. This results in a well-characterized pattern of increased apoptosis, subsequent disruption of crypt structure, followed by severe inflammation^{22, 23} and extensive proliferation to restore the epithelial

barrier²³. Using this model, we studied the role of ErbB2 and ErbB3 in injury/repair responses using mice with intestinal epithelial cell-specific ErbB2 or ErbB3 deletion. Our data show that intestinal epithelium-specific ErbB3 knockout mice develop worse colitis than controls and that ErbB2 and ErbB3 both play important roles in epithelial recovery following injury. Deletion of ErbB2 or ErbB3 increases colonic tumor necrosis factor alpha (TNF- α) production, epithelial apoptosis, and worsens colitis. In addition, deletion of ErbB3 resulted in histological evidence of sustained colitis for at least six weeks following a single DSS insult. These findings provide important insights into the specific roles of ErbB2 and ErbB3 *in vivo* in the regulation of crypt survival and epithelial recovery following colonic injury.

Materials and Methods

Mice and DSS Treatment

All animal procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee. ErbB2^{flox/flox} (FVB background) or ErbB3^{flox/flox} (129 background) mice were crossed with villin-Cre²⁴ (C57BL/6) mice to generate constitutive intestinal epithelium-specific ErbB2 knockout (ErbB2 KO^{IEC}) or ErbB3 knockout mice (ErbB3 KO^{IEC})^{8, 17}. Littermate ErbB2^{flox/flox} (wild-type) or ErbB3^{flox/flox} (wild-type) mice were used as control mice. ErbB3^{flox/flox} mice were also crossed with villin-Cre ERT2²⁴ (C57BL/6) mice to generate tamoxifen-inducible ErbB3^{flox/flox}/Cre ERT2 mice. Intestinal epithelium-specific ErbB3 knockout mice (ErbB3 KO^{IEI}) were obtained by giving 6–8 week old ErbB3^{flox/flox}/Cre ERT2 mice tamoxifen (1 mg/mouse, i.p., Sigma Chemical Co, St Louis, MO) for five days, as reported²⁴. ErbB3^{flox/flox}/Cre ERT2 mice receiving vehicle only (sunflower oil, Sigma) were used as controls (wild-type) for inducible ErbB3 deletion. Experiments began four weeks following tamoxifen to allow for efficient Cre-mediated recombination of the floxed ErbB3 allele.

To induce colitis, mice received DSS (36–50 kDa; MP Biomedicals, Solon, OH) dissolved in water (3 % w/v) for 4–8 days, as indicated. Control groups received normal drinking water without DSS. Mice were either sacrificed immediately after DSS treatment or allowed to recover for the indicated times on normal drinking water. All groups contained balanced numbers of age-matched male and female mice.

Epithelium Isolation and Western Blot Analysis

Epithelial cell isolation and western blot analysis was performed as previously described²⁵. Colons were cut into 4–5 mm pieces and incubated in Cell Recovery Solution (BD Biosciences, San Jose, CA) at 4 °C overnight followed by manual shaking to release the epithelial fraction; the stromal fraction consisted of the remaining tissue. Western blotting against E-cadherin was used to determine the purity of epithelial and stromal fractions. Tissue lysates were made with RIPA buffer²⁶ (1% Triton X-100, 1mM EDTA, 10mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.2% sodium deoxycholate) with protease and phosphatase inhibitors (Sigma Chemical Co, St. Louis, MO). Cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with milk, incubated with primary antibodies at 4°C overnight, and then incubated with

horseradish peroxidase-conjugated secondary antibodies. Membranes were developed using Western Lighting (Perkin Elmer, Waltham, MA).

Histological Scoring

The extent of injury and inflammation was scored by a gastrointestinal pathologist blinded to genotype and treatment. After the mice were sacrificed, colons were removed, opened longitudinally, rolled from the distal to proximal end and fixed in 10% neutral-buffered formalin; paraffin-embedded sections (5 μ m) were then prepared and H&E stained as previously reported²⁵. Histological scores were evaluated using a protocol described by Dieleman et al²⁷ that quantifies colitis according to the following five categories in which each category has a score range from 0–3: the amount of inflammation, depth of inflammation, percentage of the crypts involved by inflammation, crypt damage, and the percentage of crypts involved by crypt damage. The total injury and inflammation score is the sum of the individual scores assigned to each category according to severity and extent of colitis as we have previously reported²⁵.

Real-Time PCR analysis

Total RNA was isolated from whole colon tissue using a Qiagen RNA isolation kit (Qiagen, Valencia, CA) following the manufacturer's instructions, including DNase digestion. One microgram of RNA sample was then reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster City, CA). Quantitative real-time PCR was performed on an iCycler iQ5™ system (Bio-Rad, Hercules, CA, USA) using SYBR green PCR Master Mix (Bio-Rad, Hercules, CA, USA) for TNF- α , or on an ABI7000 system (Applied Biosystems, Foster City, CA) using a primer and probe set (Assay ID: Mm99999071_m1; Applied Biosystems) for IFN- γ . Actin expression was used as internal reference in all PCR experiments. The PCR primer sequences used were as follows: TNF- α , 5'-CTGTGAAGGGAATGGCTGTT-3' and 5'-GGTCACTGTCCCAGCATCTT-3'; actin, 5'-GAAGCATTTGCGGTGGACGAT-3' and 5'-CCAGGTCATCACCATTGGCAA-3'.

Apoptosis

Apoptotic cells were detected within tissue samples using terminal deoxynucleotidyl transferase-mediated dNTP nick-end labeling (TUNEL) as previously reported²⁸. Briefly, 5 μ m paraffin-embedded colon sections were deparaffinized, rehydrated and then pretreated with proteinase K (20 μ g/ml) for 15 minutes at room temperature, followed by incubation with 3% H₂O₂ for 5 minutes to quench endogenous peroxidase. Apoptotic cells were labeled using a TUNEL assay kit according to the manufacturer's instructions (ApopTag, #S7100; Millipore) and sections were counterstained with hematoxylin. TUNEL positive cells were counted in a blinded fashion and expressed as the number of apoptotic cells per 100 colonic crypts as we have previously reported²⁹.

Immunohistochemistry

Slides were stained using previously described approaches²⁵. Paraffin-embedded colon sections were deparaffinized, rehydrated and subjected to antigen retrieval by boiling in either citrate buffer (Vector Labs) or target solution (DAKO). Sections were then blocked

with 10% goat serum (Zymed, Carlsbad, CA) and stained with rabbit anti ErbB2 (Abcam, Cambridge, MA) or rat anti Ki67 antibody (DAKO, for determining epithelial cell proliferation) as previously reported.²⁵ Slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-rat antibodies and developed with DAB substrate (Sigma Chemical Co, St. Louis, MO). Ki67 positive-staining epithelial cells in 30 crypts from distal colon for each mouse were counted in a blinded fashion and expressed as the number of proliferating cells per crypt.

For colonic expression of ErbB3, 5 μ m frozen colon sections were fixed in methanol (5 minutes at -20°C) and blocked in 1% BSA/0.2% nonfat dry milk in PBS for 20 minutes at room temperature. Sections were stained with antibody against ErbB3 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight and then with rhodamine-conjugated anti-rabbit antibody at room temperature for 30 minutes. Slides were mounted with aqueous solution containing DAPI to counterstain nuclei. ErbB3 expression was visualized with standard fluorescence microscopy.

Statistics

Statistical significance for injury scores, mRNA transcript expression, and proliferative and apoptotic indices were determined using the nonparametric Mann-Whitney test. Body weights were analyzed using unpaired Student's *t* tests. Two-sided *P* values are reported for all tests and *P* values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Results

Deletion of colonic epithelial ErbB2 delays colon epithelial recovery following DSS-induced colitis

ErbB2 mediates cell survival in addition to its more established roles in regulating cell migration, proliferation and differentiation^{8, 10, 15}. Since global deletion of ErbB2 leads to embryonic lethality^{6, 16}, we used intestinal epithelium-specific ErbB2 (ErbB2 KO^{IEC}) knockout mice to test the hypothesis that ErbB2 protects the intestinal epithelium from injury and promotes epithelial recovery following injury in experimental colitis. ErbB2 KO^{IEC} mice were generated by crossing ErbB2^{fl^{ox}/fl^{ox}} (wild-type) mice with mice expressing a constitutively-active Cre recombinase driven by the villin promoter. Intestinal epithelium-specific deletion of ErbB2 was confirmed by western blot analysis of isolated epithelial cells and by immunohistochemistry (Figure 1A); ErbB2 protein was efficiently deleted in the colonic epithelium, but not in the stroma of ErbB2 KO^{IEC} mice. There were no changes in the expression of EGFR or ErbB3 in the ErbB2 KO^{IEC} colon epithelium or stroma (data not shown).

In contrast to global ErbB2 knockout mice, ErbB2 KO^{IEC} mice survived normally and exhibited no obvious phenotypic changes. Furthermore, these mice showed normal architecture of the epithelium in the small intestine (data not shown) and colon (Figure 2A). ErbB2 KO^{IEC} mice also displayed no basal defects in epithelial cell proliferation or migration (data not shown).

Administration of DSS induces superficial mucosal erosions mostly through direct cytotoxic damage to the colonic epithelium leading to mucosal inflammation^{21, 22, 27}, and has been widely used to delineate the requirement of specific gene products and/or signaling pathways in response to tissue damage and inflammation^{25, 30}. Therefore, we used this model to dissect the role of epithelial ErbB2 in intestinal injury and repair following injury. ErbB2 KO^{IEC} mice received either water alone or 3% DSS for 4 d to induce colitis. After DSS treatment, wild-type and ErbB2 KO^{IEC} mice showed a similar degree of colonic injury and inflammation (Figure 2A and Figure 2C). During this 4 d DSS treatment, neither ErbB2-deleted mice nor their wild-type controls exhibited colitis-induced weight loss.

To assess whether loss of ErbB2 alters the response to a more severe injury, we then extended the length of DSS treatment. ErbB2 KO^{IEC} mice treated with DSS for 7 d showed about 12 % body weight loss (Figure 2E), after which treatment was discontinued to prevent extensive morbidity; in contrast, during this period, wild-type controls did not lose any body weight. After 7 d of DSS, wild-type and ErbB2-deleted mice each demonstrated extensive crypt damage and inflammation (Figure 2A and 2C); although there was an apparent increase in the ErbB2 KO^{IEC} injury scores, this difference did not reach statistical significance. However, since the ErbB2 knockout mice lost more body weight than wild-type controls during prolonged DSS treatment, this indicates that epithelial ErbB2 may contribute to protection from colitis that is not reflected in our injury score data.

To determine if epithelial ErbB2 plays a role in epithelial reestablishment following injury, ErbB2 KO^{IEC} mice were allowed to recover on normal drinking water for a 3 d period following 4 d of DSS treatment. Although no histological difference was observed between wild-type and ErbB2 KO^{IEC} mice in 4 d DSS-induced injury (Figure 2A and 2C), in this short-term recovery period, ErbB2 KO^{IEC} mice exhibited exacerbated colitis with significantly increased injury and inflammation scores compared to wild-type controls (Figure 2B and 2D). Indeed, the extent of injury during this recovery period in ErbB2 KO^{IEC} mice was greater and involved most of the mid- and distal colon; in fact, some specimens displayed a complete denudation of the epithelium with full-thickness mucosal inflammation throughout the majority of the length of the colon. In contrast, although wild-type mice during this recovery period also displayed epithelial injury and mucosal inflammation, this occurred with a patchy distribution, sparing much of the mid colon. The percentage of the colon involved with crypt damage and inflammation was significantly higher in recovering ErbB2 KO^{IEC} mice compared to control mice (Supplemental Figure 1). Interestingly, the body weight of ErbB2 knockout mice decreased after DSS withdrawal and was significantly lower during the second and third day of recovery than that of wild-type mice (Figure 2F). These results suggest that epithelial ErbB2 is crucial in the regenerative response of the epithelium following injury, with its absence leading to heightened inflammatory responses during recovery.

Since DSS treatment induced a significant inflammatory response in recovering ErbB2 knockout mice, we determined whether epithelial ErbB2 deletion altered the phenotype of the immune response. However, flow cytometry analysis failed to detect any alterations in immune cell populations in the lamina propria of ErbB2 KO^{IEC} mice when compared to wild-type controls (Supplemental Figure 2).

Furthermore, to rule out the possibility that Cre recombinase expression itself resulted in increased sensitivity to DSS, we treated mice carrying the villin-Cre recombinase alone with 3% DSS for four days and then allowed the mice to recover for three days. No differences were observed in these mice compared to non-Cre expressing control mice during recovery phase as shown in Supplemental Figure 3. These data suggest that ErbB2 expression contributes to epithelial recovery in response to DSS-induced acute injury.

Given this exacerbated injury and inflammation following an initial DSS insult, we determined if this abnormal restitution in ErbB2 epithelial-null mice would lead to chronic changes. Thus, we extended the DSS recovery period to 6 wk in ErbB2 KO^{IEC} mice and wild-type controls following a single insult of 4 d of DSS. Both wild-type and ErbB2 KO^{IEC} mice showed complete weight recovery after DSS withdrawal for six weeks (not shown). Histological analysis indicated that the colons of ErbB2 KO^{IEC} mice and controls had very mild colonic injury, with no differences observed between these two genotypes (Figure 2B and 2D). Thus, although the data suggest that epithelial ErbB2 was critical for short-term recovery from DSS injury, it appears to be dispensable during a longer recovery period.

Deletion of ErbB3 in colonic epithelium exacerbates DSS-induced injury and impairs recovery

Since global deletion of ErbB3 also leads to embryonic lethality^{6, 16}, we used inducible intestinal epithelium-specific ErbB3 (ErbB3 KO^{IEI}) knockout mice to determine the role that ErbB3 plays in DSS-induced colitis. ErbB3 KO^{IEI} mice were generated by crossing ErbB3^{flox/flox} (wild-type) mice with mice expressing a tamoxifen-inducible Cre recombinase under control of the villin promoter. Tamoxifen-induced intestinal epithelium-specific deletion of ErbB3 was confirmed as shown in Figure 1B; there were no changes in the expression of EGFR or ErbB2 (not shown). Deletion of epithelial ErbB3 did not cause defects in architecture of the epithelium in the small intestine (data not shown) or colon (Figure 3A), as well as basal epithelial cell proliferation or migration (data not shown).

For DSS-induced colitis, ErbB3 KO^{IEI} mice received either water alone or 3 % DSS for 4 d to induce injury, 4 wk following tamoxifen-induced ErbB3 deletion. After this DSS treatment, ErbB3 KO^{IEI} mice showed slightly more histological damage compared to controls, although this did not reach statistical significance using conservative nonparametric methods (Figure 3A and 3C). However, ErbB3 KO^{IEI} mice showed increased inflammatory infiltrate, greater depth of inflammation, and a greater distribution of inflammation throughout the colon following acute injury versus wild type controls. Neither genotype lost body weight during 4 d DSS treatment (Figure 3E). To confirm that these results were specific to the role of epithelial ErbB3 in acute DSS injury in mice, we also studied constitutive deletion of epithelial ErbB3 (ErbB3 KO^{IEC}). In agreement with results published by Lee et al.¹⁷, ErbB3 KO^{IEC} mice displayed greater histological injury following 4 d DSS treatment than wild-type controls (Supplemental Figure 4).

As with the ErbB2 KO^{IEC} mice, we also tested the response of ErbB3 KO^{IEI} mice to extended DSS treatment. ErbB3 KO^{IEI} mice and wild-type controls were treated with DSS for 8 d; during this period ErbB3 KO^{IEI} mice lost 10 % of their starting body weight, while wild-type controls maintained normal body weights (Figure 3E). Although both genotypes

displayed extensive colonic injury, there was no difference in their injury scores following prolonged DSS treatment (Figure 3A and 3C).

We then allowed ErbB3 KO^{IEI} mice to recover on normal drinking water for a 3 d period following 4 d of DSS treatment to determine the role of ErbB3 in epithelial recovery after injury. In this short-term recovery period, significantly more severe damage was seen in ErbB3 KO^{IEI} mice when compared to wild-type controls (Figure 3B and 3Di). As with the ErbB2 KO^{IEC} mice, the extent of injury during this recovery period in the ErbB3 KO^{IEI} mice was greater and involved most of the colon with epithelial denudation and full-thickness mucosal inflammation. In addition, ErbB3 KO^{IEI} mice lost significantly more body weight than wild-type mice following DSS withdrawal (Figure 3F). These data provide evidence that epithelial ErbB3 is an important regulator of colonic epithelial repair following injury.

Given this exacerbated injury and inflammation in ErbB3 KO^{IEI} mice during initial repair from a DSS insult, we tested whether ErbB3 regulates long-term recovery. Thus, we allowed wild-type and ErbB3 knockout mice to recover for 6 wk after a single round of 4 d of DSS treatment. To study the long-term effect of ErbB3 deletion on recovery from DSS-induced colitis, we used ErbB3 KO^{IEC} mice. After 6 wk recovery, ErbB3 KO^{IEC} mice had significantly higher injury and inflammation scores when compared to wild-type littermate controls (Figure 3B and 3Dii). Initially, we had also used the inducible ErbB3 knockout (ErbB3 KO^{IEI}) for these studies. Although the tamoxifen-induced ErbB3 KO^{IEI} mice initially exhibited significantly slower weight recovery following DSS withdrawal (Figure 3F), after six weeks, ErbB3 KO^{IEI} mice only had a moderate, but similar degree of colitis to that of wild-type mice (Supplemental Figure 5). Given that inducible loss of ErbB3 caused such an extensive loss of the epithelium during the 3 d recovery period, this raised the possibility that there might be a strong selective pressure for rare ErbB3-expressing epithelial cells (i.e. not recombined during prior tamoxifen treatment) during this 6 wk recovery period. Indeed, Western blot analysis indicated that epithelial ErbB3 expression was partially restored 6 wk following DSS treatment in ErbB3 KO^{IEI} mice (Supplemental Figure 5), whereas water-treated ErbB3 KO^{IEI} mice did not show restoration of epithelial ErbB3 during the same time period. This suggests that selection for epithelial ErbB3-expressing cells may account for normal regeneration in these mice. Taken together, these data show that ErbB3 plays a critical role in regeneration following DSS-induced injury.

Increased cytokine expression ErbB2 KO^{IEC} and ErbB3 KO^{IEI} during recovery from DSS injury

DSS-induced colitis involves the release of proinflammatory cytokines such as TNF- α and IFN γ by infiltrating immune cells in the lamina propria, which in turn further worsen subsequent injury³¹. Therefore, we measured inflammatory cytokines in the colon during recovery in ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice. While mRNA levels of IL-1 β and IL-6 were not changed (data not shown), there were significantly higher mRNA levels of TNF- α and IFN- γ in ErbB2 KO^{IEC} mice following 3 d recovery (Figure 4A and 4B). Furthermore, in ErbB3 KO^{IEI} mice, mRNA levels of TNF- α were also increased compared to controls following this 3 d recovery period (Figure 4C). Both ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice

showed redistribution of the tight junctional protein, ZO-1 in colonic sections during the recovery period from DSS colitis (Supplemental Figure 6). Taken together, these data are consistent with increased histological inflammation seen in recovering ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice.

Colonic epithelium of ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice retain proliferative capacity during recovery from DSS injury

During recovery from acute injury, colon epithelial cells undergo extensive proliferation to reestablish the damaged epithelium²³. We examined crypt proliferation to determine whether epithelial-specific ErbB2- and ErbB3-null colons retained the capacity to repopulate the epithelium during the recovery phase following DSS-induced injury using immunohistochemistry for the proliferative marker Ki67. Deletion of ErbB2 or ErbB3 did not affect basal levels of proliferation in unchallenged mice (Figure 5). In contrast, following 4 d DSS treatment, there was a significant decrease in the level of proliferation in ErbB3 KO^{IEI} compared to wild-type controls (Figure 5D). Despite this fact, this effect was relatively small and the ErbB3-null epithelium retained approximately 75 % the proliferative capacity of wild-type controls. The effect on proliferation was most dramatic during the recovery period, in which loss of either ErbB2 or ErbB3 significantly increased rather than decreased the level of epithelial proliferation (144 % and 145 % increase compared to wild-type controls, respectively). It is likely that this increased proliferation is an effect secondary to increased injury in these mice during recovery. Therefore, although the ErbB2- and ErbB3-null epithelium experiences more extensive damage during recovery from DSS-induced injury, these findings suggest that this is not likely due to an impairment in epithelial proliferation, per se, and moreover raises the possibility that ErbB2 and ErbB3 regulate epithelial regeneration by promoting cell survival.

ErbB2 and ErbB3 are differentially required to prevent colon epithelial cell apoptosis

Since apoptosis is a pathological feature of DSS colitis^{22, 23}, we hypothesized that the increased severity of crypt damage and inflammation in ErbB2- and ErbB3-null mice may result from decreased epithelial cell survival. We therefore measured basal and DSS-induced apoptosis in the remaining intact crypts in ErbB2- and ErbB3-null mice during the injury and recovery period using TUNEL staining. In unchallenged mice, epithelial deletion of ErbB2 had no effect on the level of epithelial apoptosis (Figure 6A and 6C), while ErbB3 deletion increased basal epithelial apoptosis (Figure 6B and 6D) even without DSS injury. Following 4 d DSS injury, all genotypes (ErbB2 KO^{IEC}, ErbB3 KO^{IEI} and their respective control mice) showed an increased rate of apoptosis compared to water-treated mice. However, during this injury period, only ErbB3 expression had an effect on apoptosis with a significantly increased apoptotic rate in the ErbB3-null epithelium compared to wild-type mice controls (327 % increase compared to wild-type controls; Figure 6B and 6D). This appears to be consistent with the more significant effect of ErbB3 deletion on short-term DSS injury compared to that of ErbB2 deletion (Figure 3 and Supplemental Figure 4). Interestingly, apoptotic rates were significantly increased by either ErbB2 or ErbB3 deletion during the recovery period (267 % and 258 % increase compared to controls, respectively; Figure 6A–D). Again, these data are also consistent with histopathologic findings that ErbB2- and ErbB3-null mice had extensive injury specifically during this recovery period

(Figures 2 and 3). These data demonstrate that ErbB2 and ErbB3 protect against colitis through promoting epithelial cell survival, and that ErbB3 regulates epithelial apoptosis basally, during injury and during recovery, while ErbB2 is important only during recovery from injury.

Discussion

In this study, using mice with intestinal epithelium-specific deletion of ErbB2 or ErbB3, we uncovered a direct reparative role for colon epithelial ErbB2 and ErbB3 following DSS-induced injury and a lesser role for ErbB3 in regulating the extent of the injury itself. We show that ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice had markedly reduced recovery following DSS-induced colitis, most likely due to increased epithelial cell apoptosis and increased TNF- α production. Interestingly, a single round of DSS treatment in ErbB3 KO^{IEC} mice resulted in long-term histological alterations, with a failure to resolve inflammation and injury by six weeks following DSS administration. This study is the first to uncover the roles of ErbB2 and ErbB3 as important reparative factors following colonic injury.

Immediately following DSS administration, neither ErbB2 KO^{IEC} nor ErbB3 KO^{IEI} mice exhibited increased injury; however, both genotypes presented with a greater loss of body weight than controls (Figures 2 and 3). However, we did observe a small protective role for epithelial ErbB3 during this injury period since ErbB3 KO^{IEC} mice (constitutive deletion of ErbB3) had greater injury scores (Supplemental Figure 4), which confirms previous findings from Lee *et al*¹⁷. Moreover, deletion of ErbB3 in ErbB3 KO^{IEI} mice (inducible deletion of ErbB3 in adult mice) did result in increased epithelial cell apoptosis and decreased cell proliferation during DSS injury despite there being no differences in injury scores. The reasons for this discrepancy between the two different lines of epithelial ErbB3 knockout may involve strain-specific differences or compensatory changes in other factors resulting from embryonic deletion of ErbB3 in ErbB3 KO^{IEC} mice. Nevertheless, we conclude that epithelial ErbB3, but not epithelial ErbB2, plays a small protective role from colonic injury during DSS administration, likely by promoting epithelial cell survival and the maintenance of cell proliferation.

Importantly, we found that epithelial ErbB2 and ErbB3 are important reparative factors during recovery from injury. For these studies, we made a distinction between short-term (i.e. 3 days) and long-term (i.e. 6 weeks) recovery periods following a single round of DSS administration. Epithelial ErbB2 was required for normal short-term recovery from colitis, and ErbB2 KO^{IEC} mice showed elevated injury scores, cytokine expression, hyperproliferation and epithelial apoptosis during this period. However, the effect of epithelial ErbB2 knockout was confined to the acute period; following 6 weeks of recovery, we found no histological differences between wild type and ErbB2 KO^{IEC} mice. Thus, epithelial ErbB2 appears to be important for optimal early recovery of the epithelium from injury, but may be dispensable for long-term regeneration. However, it is important to note that the model used in this study involved only a single inflammatory insult; upon repeated injury or chronic injury, such as that which occurs in inflammatory bowel disease patients, the role of ErbB2 in ongoing epithelial repair could be more important.

While epithelial ErbB3 appeared to play a small protective role in determining the extent of injury during DSS administration, we found that it was required for optimal regeneration both in the short-term (3 days) and in the long-term (6 weeks). Mice with inducible ErbB3 deletion (ErbB3 KO^{IEI}) had increased colonic injury, TNF- α expression, hyperproliferation and epithelial apoptosis during short-term recovery from DSS. Moreover, the extent of this injury was much greater than wild-type controls and involved much of the colon with areas of complete epithelial ulceration. Although epithelial ErbB2 knockouts showed a similar phenotype during this period, loss of ErbB2 increased TNF- α and IFN- γ transcripts; in contrast ErbB3 deletion only affected TNF- α expression. Interestingly, in the long-term recovery period, we found that expression of epithelial ErbB3 in the tamoxifen-inducible strain (ErbB3 KO^{IEI}) was restored, which we conclude contributed to the observed normal regeneration in these mice (Supplemental Figure 5). Indeed, this type of strong selective pressure for the restoration of wild-type intestinal epithelial cells following inducible deletion has been reported by others³². In contrast, the permanent and constitutive deletion of epithelial ErbB3 (ErbB3 KO^{IEC}) produced substantial long-term changes in epithelial recovery, with injury and inflammation that persisted throughout this 6-week period. These differences emphasize the importance of ErbB3 in long-term regeneration, since it appeared that a strong selective pressure was involved in the reestablishment of ErbB3 expression in the inducible strain.

ErbB2 and ErbB3 are implicated in cell proliferation, particularly in breast tumor cells¹³. During recovery from DSS colitis, epithelial cells undergo extensive hyperproliferation to regenerate damaged crypts and repopulate the denuded epithelium²³. Although we observed a significant decrease in proliferation in ErbB3 KO^{IEI} mice relative to wild-type controls after 4 days DSS administration, this decrease may be related to the greater degree of crypt damage and/or a greater degree of epithelial apoptosis in these mice during injury (Figure 6), indirectly leading to a decrease in epithelial progenitors during this injury phase. For instance, it has been reported that DSS preferentially affects stem cells and progenitor cells in the base of the crypt²⁸; thus this reduction in proliferation may be due to increased apoptosis of progenitor cells caused by DSS in an ErbB3-null epithelium.

Elevated apoptosis is important in the etiology of colon disorders and is seen in both ulcerative colitis patients and in the mouse DSS colitis model^{23, 28, 33}. Indeed, epithelial apoptosis appears to be one of the mechanisms through which DSS induces colitis. Increased apoptosis leads to a disruption of the intestinal barrier and increased exposure of lamina propria immune cells to luminal antigens; in turn, subsequent colonic inflammation further increases epithelial injury³⁴⁻³⁶. Apoptotic rates were comparable between ErbB2 KO^{IEC} and wild-type mice in the unchallenged state and following DSS injury. However, during epithelial recovery, ErbB2 KO^{IEC} mice had higher apoptotic rates than those of littermate controls. In addition, there was a dramatic reduction in the number of surviving crypts that could support proliferation in the recovery phase in ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice compared to their wild-type controls. Thus, during this early recovery period, increased cell death in epithelial cells leads to more severe colitis and early recovery failure in ErbB2 KO^{IEC} mice. Our previous report demonstrated that transactivation of ErbB2 by TNF- α is required for colonic epithelial survival following exposure to TNF- α ¹⁰. Indeed, TNF- α levels are elevated in both DSS-induced colitis and in inflammatory bowel disease

patients; furthermore, anti-TNF- α antibodies reduce colitis progression³⁷. In our study, we confirmed that TNF- α transcripts were increased in recovering ErbB2 and ErbB3 knockout mice. Thus, one potential mechanism for the delayed short-term recovery following DSS withdrawal in ErbB2 KO^{IEC} mice is due to increased TNF- α -induced apoptosis in the absence of ErbB2 transactivation.

Surprisingly, inducible deletion of ErbB3 resulted in increased epithelial cell death in unchallenged mice. In a recent study, Lee *et al.* showed that constitutive deletion of ErbB3 in the colon epithelium did not alter basal intestinal epithelial cell survival¹⁷. Our studies involved inducible deletion of ErbB3 in adult mice with a mature gastrointestinal system. In contrast, constitutive villin-Cre constructs used in their study delete ErbB3 starting from embryonic day 9²⁴. One possibility for this discrepancy is that early embryonic deletion of ErbB3 may lead to developmental compensation by other ErbB family members, which may explain the differences in our respective studies.

Although loss of ErbB3 increased injury during DSS administration, the protective role of epithelial ErbB3 was even more apparent during recovery in which ErbB3 KO^{IEI} colons displayed greatly exaggerated injury and apoptosis. Furthermore, these changes failed to resolve in ErbB3 KO^{IEC} mice over a 6-week period. These data suggest that the ErbB3-deleted intestinal epithelium shows different patterns of DSS-induced toxicity than that with ErbB2 deletion. Although these studies were performed in mice with different genetic backgrounds, when compared to their respective wild-type controls, epithelial ErbB3 appeared to play a greater role in regulating levels of apoptosis basally, and during injury and short- and long-term recovery. A mechanistic reason may be that ErbB3 is a potent activator of PI3 kinase since it possesses multiple PI3 kinase binding sites; since this is known to be an important regulator of cell survival^{5, 14}, this may explain why ErbB3 is more potent than ErbB2 to prevent apoptosis. Another mechanism through which ErbB3 deletion resulted in severe crypt damage and inflammation might be that the ErbB3-deleted intestinal epithelium is more sensitive to TNF- α -induced toxicity compared to that containing wild type ErbB3. Our group has shown that TNF- α can activate EGFR, ErbB2 as well as ErbB4 to promote epithelial cell survival and knock down of any of these ErbBs significantly potentiates TNF- α -induced apoptosis^{10, 38}. Activation of ErbB3 depends on heterodimerization with other ErbBs; thus, it is also possible that increased TNF- α -induced transactivation may also promote ErbB3-regulated signaling pathways. Indeed, the ErbB2-ErbB3 heterodimer is the most potent signaling pair amongst the ErbB heterodimers⁵, and ErbB3 is the favored heterodimerization partner for ErbB2³⁹. Cell death following the loss of ErbB3 may therefore represent a higher sensitivity of the epithelium to TNF- α -induced apoptosis. Therefore, our data suggest that ErbB2 and ErbB3 each contribute complementary yet non-redundant roles in regulating intestinal injury and repair.

In summary, the results of our study demonstrate that ErbB2 and ErbB3 are important regulators of colonic epithelial cell responses playing a critical role in optimizing intestinal recovery from injury. These findings expand the known range of activities of EGFR family receptor tyrosine kinases, suggesting that strategies designed to activate ErbB2 and ErbB3 may be of therapeutic value in promoting recovery following colon injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DSS	dextran sulfate sodium
IFN-γ	interferon gamma
KO^{IEC}	constitutive intestinal epithelium-specific knockout
KO^{IEI}	inducible intestinal epithelium-specific knockout
TNF-α	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase-mediated dNTP nick-end labeling

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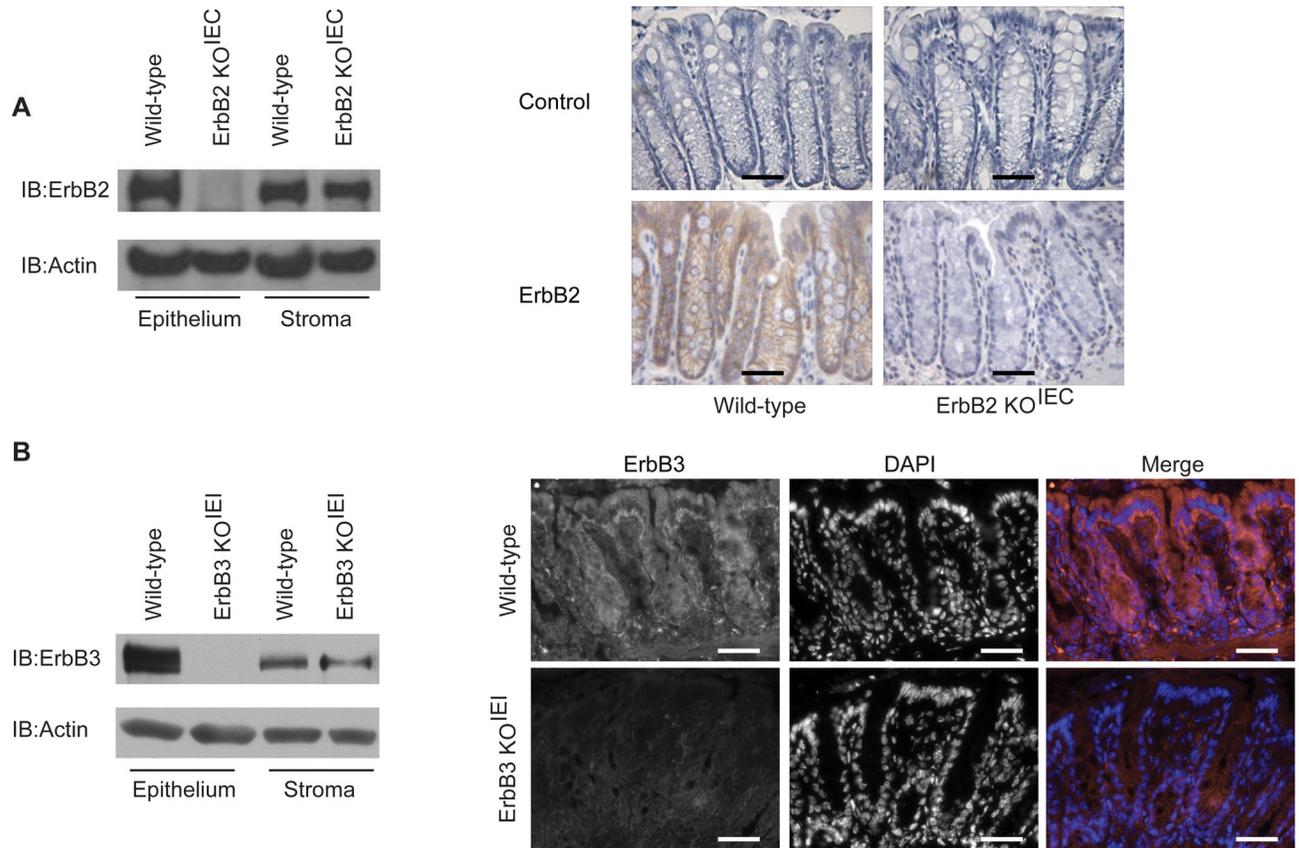


Figure 1. Deletion of colon epithelial ErbB2 or ErbB3 expression in ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice

Epithelial and stromal fractions were isolated from colons of the indicated mice. (A and B) Western blot and immunohistochemical analysis with antibodies against ErbB2 (A) or ErbB3 (B). Omission of primary antibody was used as a control for immunohistochemistry. Actin expression was used as loading control. Brown indicates ErbB2 staining. Red indicates ErbB3 staining, blue indicates DAPI staining. Scale bars, 50 μ m. These results are representative of independent analyses of at least three different mice in each group.

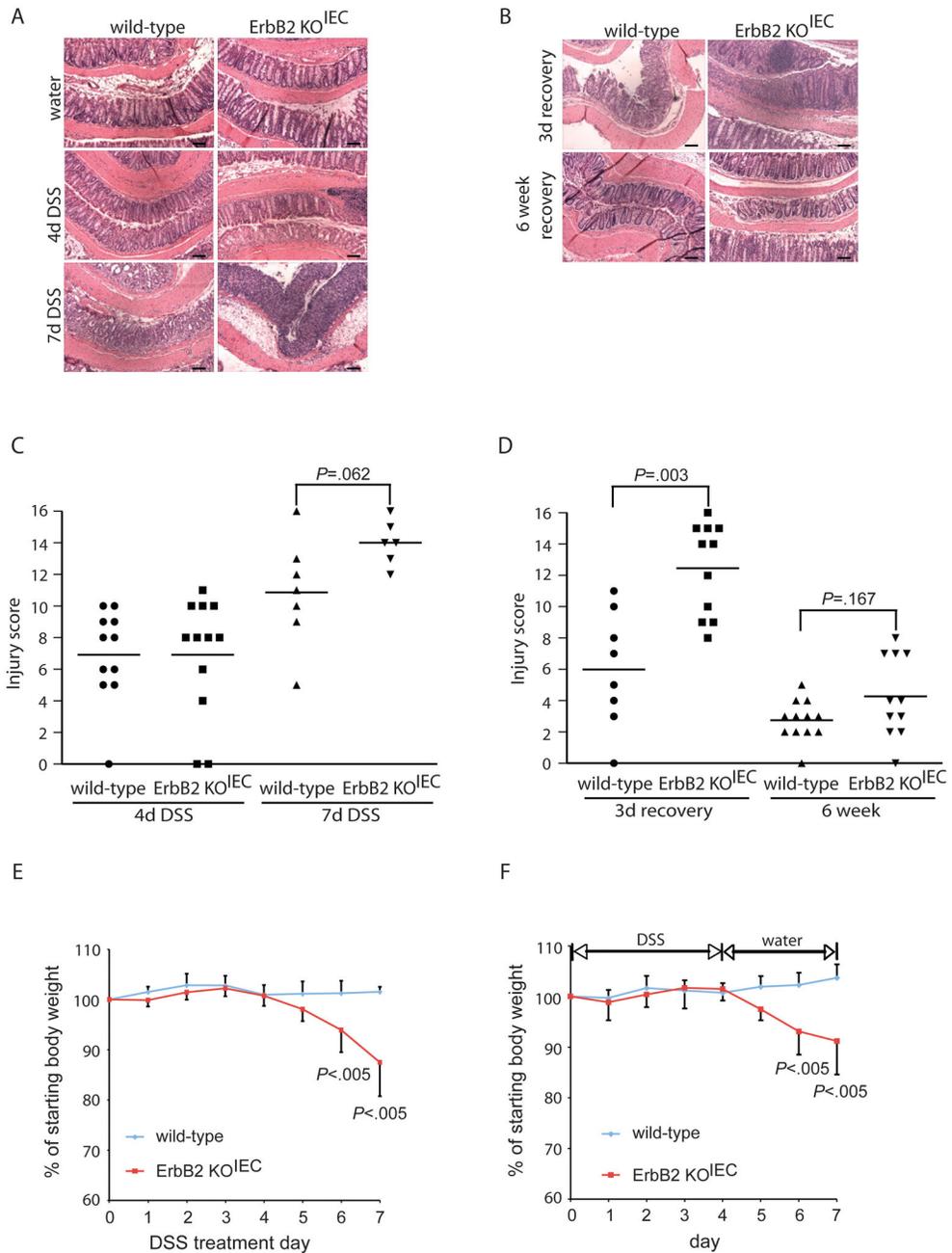


Figure 2. Epithelial ErbB2 is required for short-term recovery from DSS-induced colitis ErbB2 KO^{IEC} and wild-type controls were treated with 3% DSS for 4 or 7 days (A, C, E), or were treated with 3% DSS for 4 days followed by a recovery period of 3 days or 6 weeks on normal water (B, D, F); n=6–12. (A, B) Representative H&E sections of colons from the indicated mice. Scale bars, 100 μ m. (C, D) Colonic injury scores were quantified by a pathologist blinded to the treatment and group. Bars indicate mean value. (E) Body weight loss during DSS administration; values indicate mean \pm SD. (F) Body weight loss during the 3 day recovery period following 4 days of DSS administration; values indicate mean \pm SD.

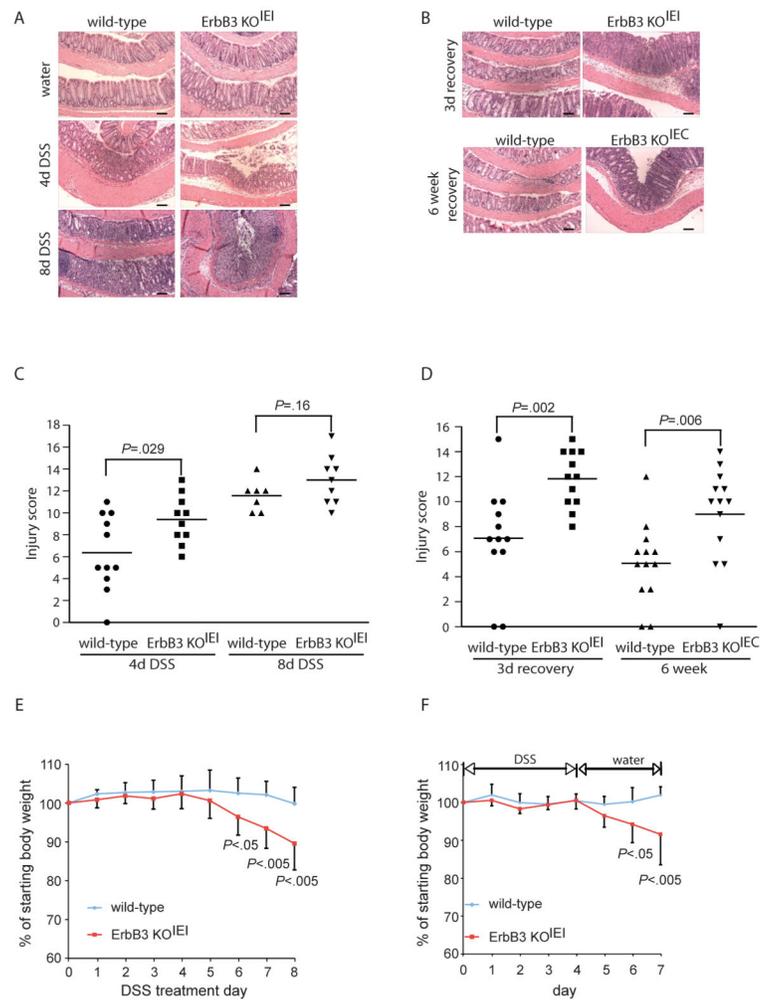


Figure 3. Epithelial ErbB3 is required for short-term and long-term recovery from DSS-induced colitis

ErbB3 KO^{IEI} (A, B, C, Di, E, F) or ErbB3 KO^{IEC} (B, Dii) mice and their respective wild-type controls were treated with 3% DSS for 4 or 8 days (A, C, E), or were treated with 3% DSS for 4 days followed by a recovery period of 3 days or 6 weeks on normal water (B, D, F), as indicated; n=7–13. (A, B) Representative H&E sections of colons from the indicated mice. Scale bars, 100 μ m. (C, D) Colonic injury scores were quantified by a pathologist blinded to the treatment and group. Bars indicate mean value. (E) Body weight loss during DSS administration; values indicate mean \pm SD. (F) Body weight loss during the 3 day recovery period following 4 days of DSS administration; values indicate mean \pm SD.

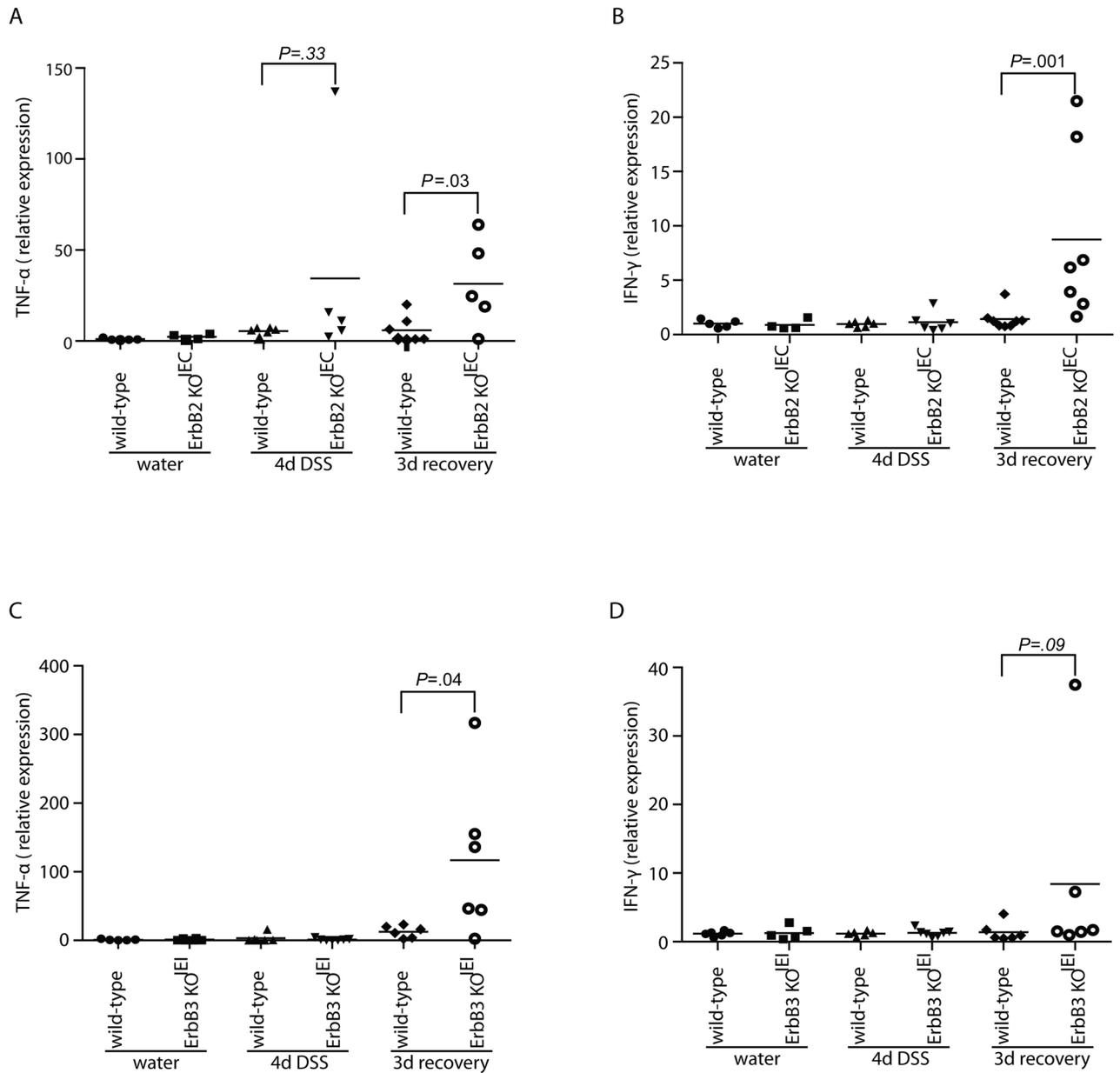


Figure 4. Colonic TNF- α expression is increased in ErbB2 KO^{IEC} and ErbB3 KO^{IEI} mice during recovery from DSS-induced colitis

TNF- α (A, C) and IFN- γ (B, D) mRNA transcript levels were measured by real-time RT-PCR from whole colon RNA of the indicated genotypes and treatment groups. Bars indicate mean expression of transcripts normalized for actin; n=4–7.

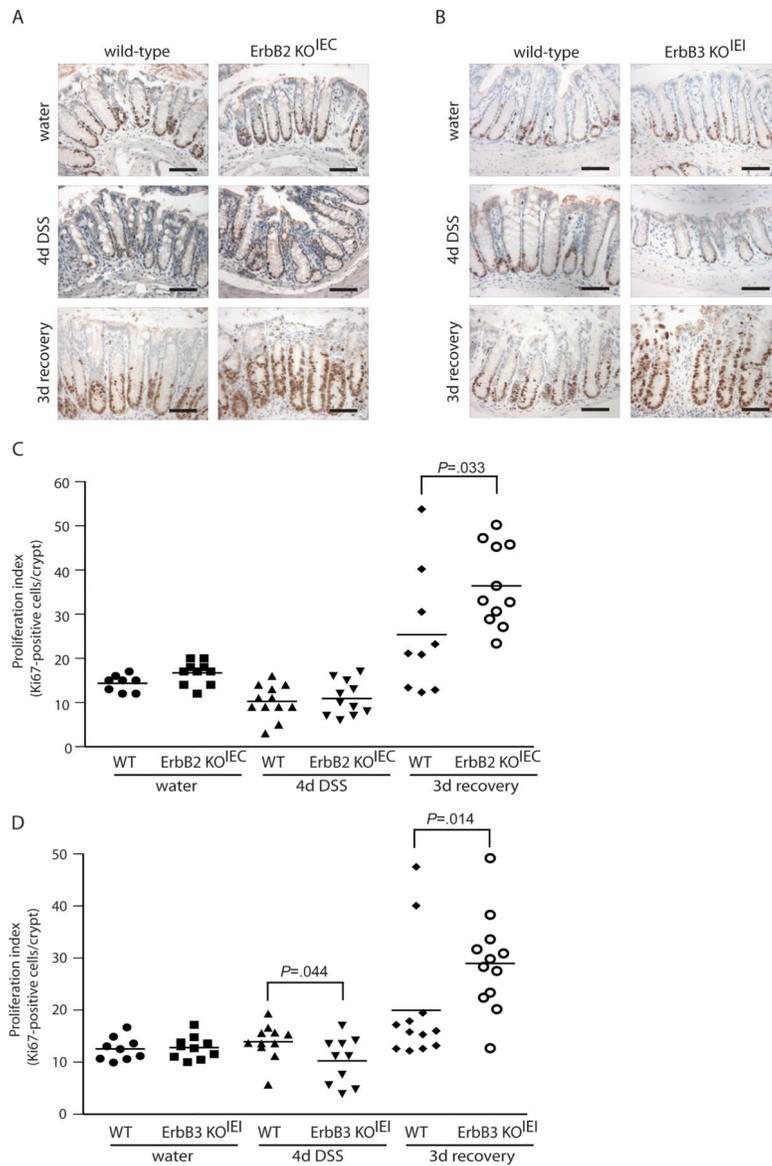


Figure 5. Loss of ErbB2 or ErbB3 exacerbates epithelial hyperproliferative response during recovery from DSS-induced colitis
 (A, B) Immunostaining for the proliferative marker Ki67 (brown nuclei) in colonic sections of ErbB2 KO^{IEC} (A), and ErbB3 KO^{IEI} mice (B) and their respective wild-type controls treated with water, DSS for 4 days or DSS followed by a 3 day recovery period, as indicated. Images are representative of 8–12 mice per group. Scale bars, 100 μ m. (C, D) The number of Ki67-positive cells per crypt were quantified in a blinded manner. Bars indicate mean values.

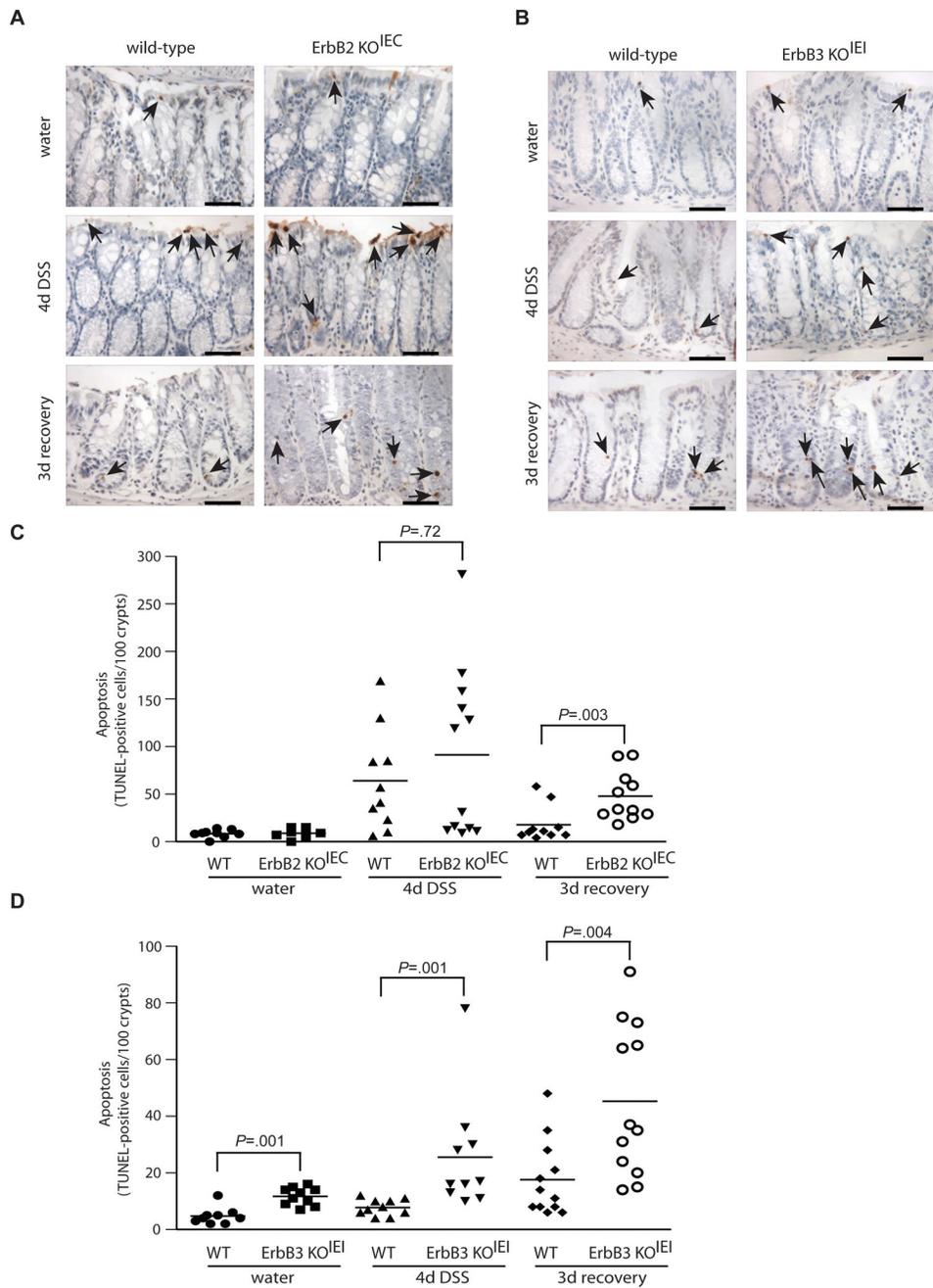


Figure 6. Loss of ErbB2 or ErbB3 increases colonic epithelial apoptosis

(A, B) Apoptotic cells (arrows) in colon sections were detected by TUNEL staining from wild-type or knockout mice treated with water, DSS for 4 days or DSS followed by a 3 day recovery period, as indicated. Images are representative of 8–12 mice per group. Scale bars, 50 μ m. (C, D) The number TUNEL-positive cells per 100 crypts were quantified in a blinded manner. Bars indicate mean values.