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## ZBP-89 Regulates Expression of *Tryptophan Hydroxylase I* and Mucosal Defense Against *Salmonella Typhimurium* in Mice

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### Abstract

**Background & Aims**—ZBP-89 (also ZNF148 or Zfp148) is a butyrate-inducible zinc finger transcription factor that binds to GC-rich DNA elements. Deletion of the N-terminal domain is sufficient to increase mucosal susceptibility to chemical injury and inflammation. We investigated whether conditional deletion of ZBP-89 from the intestinal and colonic epithelium of mice increases their susceptibility to pathogens such as *Salmonella typhimurium*.

**Methods**—We generated mice with a conditional null allele of *Zfp148* (ZBP-89<sup>FL/FL</sup>), using homologous recombination to flank *Zfp148* with LoxP sites (ZBP-89<sup>FL/FL</sup>), and then breeding the resulting mice with those that express VillinCre. We used microarray analysis to compare gene expression patterns in colonic mucosa between ZBP-89<sup>FL/FL</sup> and C57BL/6 wild-type mice (controls). Mice were gavaged with 2 isogenic strains of *S typhimurium* after administration of streptomycin.

**Results**—Microarray analysis revealed that the colonic mucosa of ZBP-89<sup>FL/FL</sup> mice had reduced levels of *tryptophan hydroxylase 1* (*Tph1*) mRNA, encoding the rate-limiting enzyme in enterochromaffin cell serotonin (5HT) biosynthesis. DNA affinity precipitation demonstrated direct binding of ZBP-89 to the mouse *Tph1* promoter, which was required for its basal and butyrate-inducible expression. ZBP-89<sup>FL/FL</sup> mice did not increase mucosal levels of 5HT in response to *S typhimurium* infection and succumbed to the infection 2 days before control mice. The  $\Delta$ hilA isogenic mutant of *S typhimurium* lacks this butyrate-regulated locus and stimulated, rather than suppressed, expression of *Tph1* approximately 50-fold in control, but not ZBP-89<sup>FL/FL</sup> mice, correlating with fecal levels of butyrate.

**Conclusions**—ZBP-89 is required for butyrate-induced expression of the *Tph1* gene and subsequent production of 5HT in response to bacterial infection in mice. Reductions in epithelial ZBP-89 increase susceptibility to colitis and sepsis following infection with *S typhimurium*, partly

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The authors have all declared that no conflict of interest exists.

**Website for Microarray data:** file:///Microarray%20ZBP-89%2010.09%20results/VillinCre%20xZBP-89%20v%20WT%20colon.html

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due to reduced induction of 5HT production in response to butyrate and decreased secretion of anti-microbial peptides.

## Keywords

butyrate; 5HT; cryptdins; *Δhila*

## Introduction

ZBP-89 (ZNF148, Zfp148) is a zinc finger transcription factor induced by the short chain fatty acid (SCFA) butyrate<sup>1-3</sup>. Butyrate also induces the interaction of ZBP-89 with the tumor suppressor ataxia telangiectasia mutated and this protein-protein interaction is important in mucosal protection during dextran sulfate-induced colitis<sup>4</sup>. Commensal bacterial species, e.g., *Bifidobacteria* and *Lactobacillus*, generate SCFAs (acetate, butyrate, propionate) from dietary fiber<sup>5,6</sup>. Although several beneficial effects are attributed to SCFAs in the colon, the most prominent effect ascribed to butyrate is its ability to inhibit histone deacetylases (HDACi)<sup>7</sup>. Consistent with its HDACi activity, butyrate maintains the differentiated state of the colon and is a potent anti-inflammatory and anti-cancer agent<sup>8,9</sup>. Several studies have shown that butyrate stimulates the production of antimicrobial peptides and that this function contributes to the elimination of bacterial pathogens<sup>10-12</sup>.

The food industry has effectively applied the medicinal effects of SCFAs by providing butyrate-laced feed to livestock to reduce *Salmonella* colonization and ostensibly food born illnesses<sup>13,14</sup>. In addition to stimulation of the host innate immune system<sup>15-17</sup>, SCFAs inhibit genes within the *Salmonella Pathogenicity Island 1 (SPI-1)* that regulate bacterial invasion, e.g., *Hyperinvasion locus hila*<sup>18-20</sup>. Moreover, there is evidence that specifically butyrate suppresses expression of the *SPI-1* gene locus<sup>19,21</sup>.

Pluripotent stem cell progenitors differentiate into enterocytes, goblet cells, hormone-producing enteroendocrine cells (EECs) and Paneth cells, which synthesize antimicrobial peptides such as defensins<sup>22</sup>. IECs express Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs), and participate in mucosal defense<sup>23,24</sup>. Like IECs, enteroendocrine cells of both the small and large intestine express TLRs<sup>25,26</sup>. Specifically, the enteroendocrine cell line STC-1 expresses 5-hydroxytryptamine (5HT, serotonin) and the same spectrum of TLRs as primary enteroendocrine cells<sup>25,27</sup>. Moreover, bacterial flagellin and CpG DNA motifs induce hormone secretion from STC-1 cells through TLR activation<sup>27</sup>.

*Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is the most common bacterial cause of food borne gastroenteritis<sup>28</sup>. Symptoms of nausea, vomiting, diarrhea and crampy abdominal pain characteristic of pathogen-induced 5HT release appear within 8 hours of ingesting contaminated food or water<sup>29,30</sup>. 5HT is the primary bioamine secreted from gut enterochromaffin (EC) cells<sup>31</sup>. When released from EC cells, platelets, mast cells and neurons, 5HT stimulates fluid secretion from enterocytes and activates submucosal ganglia to stimulate peristalsis and flush the GI tract of ingested pathogens. Residual 5HT secretion is a probable cause of post-infectious irritable bowel syndrome (IBS) following *Salmonella* gastroenteritis<sup>32,33</sup>.

We report here the generation of a mouse conditionally null for ZBP-89 in the intestine. A microarray analysis was used to identify genes modulated in the colon after ZBP-89 gene deletion and revealed suppression of *tryptophan hydroxylase 1 (Tph1)* mRNA, which encodes the rate-limiting enzyme in 5HT biosynthesis. Therefore, we tested the hypothesis that ZBP-89 is essential for an effective innate mucosal defense against colonic pathogens such as *S. typhimurium*.

## Methods and Materials

### Targeting Vector

The mouse *ZBP-89* locus (*Zfp148*) spans a genomic distance of ~130 kb preventing efficient deletion of the entire locus. Exons 8 and 9 encode greater than 60% of the protein coding domain and when deleted causes mRNA instability and subsequently an absence of ZBP-89 protein<sup>34</sup>. High fidelity long range PCR was used to amplify the targeting arms from a mouse BAC clone #452J12 generated from a 129SvJ library (Incyte, Wilmington, DE) then sequenced and assembled into plasmid pBmZBP-89 (Genomatix, Cincinnati, OH). The left targeting arm consisted of the distal 3.1 kb of intron 7. The right targeting arm contained 5.6 kb of the 3' untranslated region. Using the Vector pLM10-iKO-FinalTV, the neomycin resistance cassette driven by the phosphotransferase gene (PGK) promoter was in the reverse orientation and flanked by FRT sites. A single LoxP site was inserted into intron 7 upstream of the neomycin cassette and the second LoxP site was placed downstream of the poly A termination signal located within exon 9.

### Generation of ZBP-89<sup>ΔInt</sup> mice

The University of Michigan Animal Care and Use Committee, which maintains an American Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) facility, approved all procedures used in this study. The University of Michigan Transgenic Animal Model Core electroporated Bruce4 (C57BL/6 derived) embryonic stem (ES) cells with the pLM10-iKO-FinalTV targeting vector (Intrexon Corporation, Blacksburg, VA). G418 was used to select ES cells expressing the targeting vector. Homologous recombination occurred in 18 of 480 ES clones (3.75% success rate). All animals were housed in microisolator polycarbonate cages under SPF conditions. The neomycin cassette was removed by breeding the F1 mice to the *Flpe* deleter mouse strain in which the *Flpe recombinase* was expressed from the human beta actin promoter (Jackson Laboratory, Bar Harbor, ME)<sup>35, 36</sup>. Mice expressing the targeted *ZBP-89* locus with flanking LoxP sites (ZBP-89<sup>FL/FL</sup>) were maintained on the C57BL/6 genetic background. The mouse colons analyzed from the ZBP-89<sup>FL/FL</sup> x VillinCre (from D. Gumucio<sup>37</sup>) cross were designated ZBP-89<sup>ΔInt</sup> for deleted in the small and large intestinal mucosa.

### Microarray Analysis

Mucosa was scraped from the colons of WT and ZBP-89<sup>ΔInt</sup> mice. Total RNA was prepared using TRIzol followed by RNA clean-up using the RNEasy Microkit (Qiagen, Valencia, CA), and the quality was assessed on an Agilent nucleic acid analyzer using the Mouse Genome 430 2.0 Perfect Match Peg Array (Affymetrix). The Microarray Core Facility at the University of Michigan performed the gene chip analysis.

### Statistics

Data were analyzed by unpaired 2-tailed Student's *t* test, non-parametric Mann-Whitney U test (quantitative cultures), or ANOVA for multiple comparisons. For survival cures, the log-rank/Mantel-Cox test was used to determine significance. Results were presented as means ± SEM using GraphPad Prism version 5 (San Diego, CA). P-values of < 0.05 were considered significant.

## Results

### Characterization of ZBP-89 null allele in the colon

ZBP-89 is a ubiquitous transcription factor regulated by butyrate that inhibits cell growth when ectopically expressed in cells lines<sup>1, 3, 38</sup>. However, the in vivo function of ZBP-89

has been difficult to study since homozygous disruption of the locus in mice was previously shown to be non-viable<sup>34</sup>. Therefore, we generated a floxed *ZBP-89* allele with the goal of generating a conditional knockout of the gene in intestinal and colonic mucosa when bred to the VillinCre-expressing mouse line (Figure 1A,B). We focused on the colon due to our prior study implicating ZBP-89 in colonic mucosal protection<sup>4</sup>. A Western blot confirmed reduced levels of ZBP-89 protein in the homozygous conditionally null colons (*ZBP-89<sup>ΔInt</sup>*, Figure 1C). ZBP-89 protein expression in WT colons was ubiquitous and located in the nuclei of epithelial, lamina propria and smooth muscle cells (Figure 1D). Since VillinCre expression is exclusively epithelial, there was residual staining of the lamina propria and smooth muscle cells in the conditional null colons (*ZBP-89<sup>ΔInt</sup>*) (Figure 1D), which is consistent with the known expression of ZBP-89 in T lymphocytes, myeloid and smooth muscle cells<sup>39</sup>.

Despite significant reduction of ZBP-89 in the epithelium, there was no spontaneous phenotype in either the small intestine or colon. Therefore we performed a genome-wide analysis of the colons from these mice (Figure 2A). Of interest was the decrease in *Tph1*, *defensin 5 (Defcr5)*, *defensin 22 (Defcr22)* and *chromogranin A (ChgA)* on the microarray that was confirmed by RT-qPCR (Figure 2B). Although we did not observe a change in *Tph2* on the microarray, we also measured the mRNA levels of this *Tph* isoform in the total colon extracts (Figure 2C) and extracts in which the mucosa was separated from the mesenchyme (Figure 2D,E). Although *Tph2* mRNA is generally expressed in neural tissues<sup>50</sup>, apparently some *Tph2* is expressed in the epithelium and was reduced in the *ZBP-89<sup>ΔInt</sup>* mice (Figure 2D, E).

*Tph1* is expressed in a subset of enteroendocrine cells that also express *ChgA*<sup>40</sup>. Therefore, we used confocal microscopy analysis to confirm that ZBP-89 protein co-localizes to colonic 5HT-expressing EC cells (Figure 3A,B). Enumerating the number of EC cells in the colons of WT and *ZBP-89<sup>ΔInt</sup>* mice revealed a greater than 50% reduction in the number of 5HT-staining cells (Figure 3C). Moreover, plasma 5HT levels were significantly reduced in these mice (Figure 3D). *ChgA* protein is a neuroendocrine marker whose expression was also reduced in the *ZBP-89<sup>ΔInt</sup>* mice (Figure 3E,F). Thus we concluded that ZBP-89 expression modulates *Tph1* and *Tph2* gene expression consistent with reduced 5HT in the circulation. However since *ChgA* levels were also reduced, the effect was likely due to an overall decrease in the enteroendocrine cell lineage, suggesting that ZBP-89 contributes to EC cell development.

### ***Tph1* is a direct target of transcription factor ZBP-89**

We identified several potential ZBP-89 consensus sites within the proximal promoter (Figure 4A). To demonstrate that ZBP-89 bound to a *mTph1* DNA element, DNA affinity precipitation (DAPA) was performed with nuclear extracts from the 5HT-producing mouse STC-1 cell line using a putative ZBP-89 binding site at -123 bp upstream from the cap site. The DAPA showed that ZBP-89 is indeed present at the *mTph1* promoter and that a 2 bp mutation within the GC-rich site did not compete effectively for ZBP-89 protein binding (Figure 4B). Ectopic expression of ZBP-89 induced endogenous *mTph1* mRNA (Figure 4C). ZBP-89 is a transcription factor induced by butyrate, one of the major fatty acids produced by bacterial fermentation of dietary fiber<sup>41</sup>. Butyrate induced ZBP-89 protein then *Tph1* mRNA and protein in the mouse 5HT-producing STC-1 cell line (Figure 4D,E). Moreover, a knockdown of ZBP-89 with siRNA oligos (Figure 4E) reduced basal expression of *Tph1* mRNA as well as *Tph1* mRNA induced by butyrate (Figure 4E). A western blot confirmed that transfection of siRNA oligonucleotides decreased while ectopic expression increased ZBP-89 protein levels consistent with parallel changes in *Tph1* protein levels (Figure 4F). Therefore we concluded that ZBP-89 binds the *mTph1* promoter and is required for basal promoter as well as butyrate-inducible *mTph1* gene expression.

## ZBP-89<sup>ΔInt</sup> mice are more susceptible to *S. typhimurium* infection

Prior studies have suggested that gut EC cells contribute to the innate immune response during enteric infections<sup>42, 43</sup>. However, there is little known regarding how EC cells sense the luminal content then subsequently increase production and release of 5HT to stimulate fluid secretion and peristalsis. Therefore to understand the significance of *Tph1* gene regulation by ZBP-89 in vivo, we challenged the ZBP-89<sup>ΔInt</sup> mice with the enteric pathogen *S. typhimurium* SL1344.

Both WT and ZBP-89<sup>ΔInt</sup> mice lost 7% and 10% of their body weight over time and eventually required euthanization by day 8 and 6 respectively (Figure 5A,B). Although ZBP-89<sup>ΔInt</sup> mice exhibited slightly more inflammation at baseline (Figure 5C), both groups reached about the same level of inflammation at the time of euthanization and as documented by the colitis score, myeloperoxidase activity (MPO) and number of leukocytes (Figure 5C–F, Suppl. Figure 1A). However, the ZBP-89 mice consistently succumbed to their infection 2 days before the WT mice. Since both groups are on a C57/BL6 genetic background, they are null for the natural resistance-associated macrophage protein 1 (Nramp1), which is required to control bacterial replication within the phagosome<sup>44, 45</sup>. Confocal microscopy of 5HT+ EC cells after infection with GFP-labeled *S. typhimurium* SL1344 revealed fewer bacteria in the lumen of the wild type compared to the ZBP-89<sup>ΔInt</sup> mice (Figure 6A,B) and was consistent with the higher bacterial loads in the null mice (Figure 6C, Suppl. Figure 1B,C). ZBP-89<sup>ΔInt</sup> mice also exhibited significantly higher systemic levels of bacteria (Figure 6D–E), which correlated with reduced survival.

To demonstrate that the 2-day difference in survival was due to lower levels of Tph1 enzyme and subsequent EC cell production of 5HT, we treated both WT and ZBP-89<sup>ΔInt</sup> mice with 5-hydroxytryptophan (5-HTP), the direct product of the Tph1 reaction to compensate for the ostensibly reduced EC cell 5HT levels. We observed a delay in weight loss that correlated with a 2-day improvement in the survival of both groups of mice (Suppl. Figure 2A,B). Histological examination of the colons and determination of the bacterial load demonstrated that the mice still exhibited colitis but had reduced bacterial loads in both the bowel lumen (data not shown) and systemically (Suppl. Figure 2C,D). At the time of euthanasia, both groups had lost about half of their blood volumes with hematocrits of ~20% (Suppl. Figure 2E), and positive fecal blood within two days of the gavage (data not shown). Thus as expected, the mice die from the combination of severe colitis resulting in anemia due to fecal blood loss and subsequent systemic infection. Since the mice are on a pure C57BL/6 background, which is NRAMP negative, mortality was ultimately observed in both groups. ELISAs confirmed the expected boost in tissue 5HT levels after 5HTP treatment (Suppl. Figure 2F). Therefore we concluded that the 2-day shift in survival was related to higher 5HT levels. Despite fewer 5HT positive EC cells, the basal and infection-induced tissue 5HT levels were similar in the colons of both the WT and ZBP-89<sup>ΔInt</sup> mice (Suppl. Figure 2F) and did not correlate with plasma levels (Figure 3). Thus other sources of 5HT such as platelets, which store 5HT and neurons which exclusively express *Tph2*, contributed to the observed tissue levels prior to and during infection<sup>31, 46, 47</sup>.

## 5HT induces inflammatory cytokines

Ghia et al. demonstrated that 5HT enhances dextran sulfate-induced colitis by stimulating macrophage recruitment and secretion of IL-1 $\beta$ , TNF $\alpha$  and IL-6<sup>48</sup>. Sustained colitis in the absence of a bona fide bacterial infection possibly contributes to inflammatory bowel disease or irritable bowel syndrome<sup>46</sup>. Indeed, the infection induced several cytokine pathways (Th1, Th2 and Th17) to the same extent in both groups (Suppl. Table 1). Thus, differences in the 5HT levels of both the WT and ZBP-89<sup>ΔInt</sup> mice did not differentially affect the cytokine response to the bacterial infection.

## 5HT and 5HTP induce intestinal crypt killing activity

Since ZBP-89<sup>ΔInt</sup> mice also exhibited impaired *defensin* (Suppl. Figure 3A,B) and *Tph1* gene expression and consequently increased susceptibility to *Salmonella*, we examined whether the ZBP-89 gene deletion decreased bacterial crypt killing activity. Crypts isolated from the colon of both WT and ZBP-89<sup>ΔInt</sup> mice were stimulated with LPS, 5HT, 5HTP, muramyl dipeptide (MDP) or inactive chiral MDP isomers (MDP<sub>DD</sub>) revealed reduced bacterial killing from colonic crypts isolated from ZBP-89<sup>ΔInt</sup> compared to wild type mice (Suppl. Figure 3D). MDP is a Nod2 activator<sup>49</sup>, known to induce Paneth cell secretion of antimicrobial peptides, while the inactive isomer MDP<sub>DD</sub> does not (Suppl. Figure 3D). A pan-anti-defensin antibody blocked LPS induction of the crypt killing activity. Thus we concluded that 5HT-induced secretions contain antimicrobial peptides.

## *S. typhimurium* inhibits *Tph1* gene expression

Due to low levels of 5HT generated in the infected WT mice, we examined *Salmonella*'s effect on *Tph1* gene expression in WT mice and were surprised to find a decrease in *Tph1* and *defensin 5/22* mRNA expression within 3 days (Suppl. Figures 4 and 3A,B). The significantly lower *Tph1* mRNA levels were nearly equivalent to the levels in ZBP-89<sup>ΔInt</sup> mice (Figure 7A). Moreover, *S. typhimurium* suppressed other putative ZBP-89 targets, e.g., mRNA for *defensin 5* and *defensin 22* (Figure 7B,C). Although pathogens typically induce 5HT production via TLRs<sup>50</sup>, *Tph1* suppression by *S. typhimurium* suggested an additional mechanism. Therefore we examined whether bacterial invasion genes encoded by the *SPI-1* pathogenicity island were responsible. Indeed, the suppression was due to bacterial effectors encoded by the *SPI-1* locus, since infecting WT mice with the isogenic *hilA SPI-1* mutant *S. typhimurium* strain (ST<sup>Δ*hilA*</sup>) induced rather than suppressed both *Tph1* and *defensin 5/22* mRNAs (Figure 7A–C). The induction required ZBP-89 since *Tph1* and *defensin 5* were not significantly induced in the ZBP-89<sup>ΔInt</sup> mice infected with ST<sup>Δ*hilA*</sup>. Moreover, *ZBP-89* mRNA levels were induced by the ST<sup>Δ*hilA*</sup> isogenic mutant strain (Figure 7D). Therefore bacterial effectors encoded by the *SPI-1 Salmonella* locus modulated *ZBP-89* gene expression, which is ultimately required for butyrate induction of *Tph1* gene expression and subsequently secretion of 5HT and antimicrobial peptides. In particular, we found that butyrate and total SCFA levels in the colonic lumen were nearly absent in mice infected with the WT<sup>SL1344</sup> but not the ST<sup>Δ*hilA*</sup> mutant strain (Figure 7E, F).

Both bacterial strains induced *Tph1* and *βcatenin* gene expression ex vivo when co-cultured with STC-1 cells (Suppl. Figure 5A). Moreover, the mutant ST<sup>Δ*hilA*</sup> strain induced significant levels of *ZBP-89* mRNA and subsequently downstream targets *Tph1* and *defensin 5* mRNA compared to the wild type strain (Figure 7A,B,D). Considering the known presence of TLRs on EECs and that TLR signaling is required for *Salmonella* virulence<sup>50</sup>, we concluded that the mutant strain elicits the expected cell response by inducing *Tph1*. *Salmonella* activation of TLRs increases *βcatenin*<sup>51</sup>. Indeed cotransfection of WT *βcatenin* with ZBP-89 cooperated to induce *Tph1* protein and *Tph1* mRNA in STC-1 cells (Suppl. Figure 5B,C). However, reduced luminal butyrate levels correlated with reduced *ZBP-89* gene expression (Figure 7E) and presumably its ability to cooperate with TLR induction of mucosal defense gene targets, e.g., *βcatenin*. TLRs have been shown to increase transcriptional activation of *βcatenin* and its nuclear accumulation in response to bacteria<sup>52</sup>. In particular, *Salmonella* induce host epithelial defense mechanisms through the activation of the Wnt/*βcatenin* pathway<sup>53</sup>, which includes epithelial cell proliferation, maintenance of tight junctions and antimicrobial peptide expression<sup>26</sup>. Thus gut homeostasis is a balance between cell damage due to the collateral effects of bacteria killing and epithelial repair<sup>54</sup>.

## Discussion

The goal of this study was to identify the transcriptional targets of ZBP-89 that mediate mucosal protection. A microarray analysis of colonic mucosa from WT versus ZBP-89 $\Delta$ Int mice revealed several genes encoding antimicrobial peptides and *Tph1* as potential ZBP-89 gene targets. We show here that ZBP-89 was essential for the production of 5HT in colonic EC cells through its ability to directly regulate *Tph1* gene expression. Moreover, we demonstrated that butyrate strongly induces *Tph1* gene expression in vitro and that the induction requires ZBP-89. Targeted ZBP-89 deficiency revealed that an important consequence is decreased survival to infection from the clinically-relevant pathogen *S. typhimurium*. Unexpectedly, *S. typhimurium* infection suppressed colonic butyrate levels, which also reduced ZBP-89 and *Tph1* gene expression acutely in WT mice. However removing the butyrate-regulated *hila* locus abolished this suppressive effect resulting in robust induction of *Tph1* and *defensin* expression. *Salmonella* induction of epithelial proliferation through  $\beta$ catenin stabilization has been previously reported<sup>51, 55, 56</sup>. Indeed, we also found that *Salmonella* induces  $\beta$ catenin and synergizes with ZBP-89 to induce *Tph1*. Therefore, we concluded that ZBP-89 regulates several essential mucosal defense genes activated during bacterial pathogenesis (Suppl. Fig. 6, Model). Moreover consistent with its in vitro regulation by butyrate, ZBP-89 gene expression in vivo correlated with fecal SCFA levels, which were also modulated by the *Salmonella* infection.

The overall levels of 5HT at both baseline and during colonic injury vary widely due to multiple mechanisms impacting the tissue levels such as its reuptake mediated by SERT, storage in platelets, in addition to production by myeloid cells and enteric neurons<sup>31</sup>. 5HT has been implicated in the inflammation generated after chemical injury to the colon, and as such has been an important therapeutic target considered in the treatment of both inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)<sup>57</sup>. However, the results here underscore the importance of 5HT in the colon's initial defense against an invading pathogen. 5HT targets multiple processes in mucosal defense, e.g., as a regulator of immune cells, the enteric nervous system and fluid secretion by colonocytes. Indeed, we show here that reduced serotonin levels compromised mucosal defenses since increasing tissue serotonin levels with 5HTP administration dramatically reduced the bacterial load and subsequently improved survival. An underappreciated result observed with the burst of 5HT included the secretion of antimicrobial peptides, e.g., defensins. Regulated production of antimicrobial peptides has been examined in studies focused on suppressing pathogen colonization of livestock<sup>10-12</sup>, but has not been extensively studied as a mechanism by which susceptible mammalian hosts mount a defense against food borne pathogens. Moreover, there are multiple examples implicating IBD and IBS as multigenic disorders developing surreptitiously after a distant episode of bacterial gastroenteritis<sup>32, 58, 59</sup>.

Studying the acute mucosal defense response to *S. typhimurium* in the ZBP-89 mutant colons uncovered the potential role of SCFAs. SCFAs especially butyrate are required to maintain homeostasis of the normal colon. The millimolar butyrate concentration generated from commensal colonic bacteria is purported to also exhibit anti-inflammatory properties<sup>5</sup>. Despite the intense focus regarding how butyrate induces apoptosis, cell cycle arrest and epigenetic modifications in mammalian cells, there is little mechanistic understanding regarding its actual antimicrobial effects in vivo<sup>60</sup>. Moreover, any antimicrobial activity mediated by butyrate has only been studied in livestock<sup>61</sup>.

Considering both the biochemical and in vivo studies reported here, we conclude that a plausible mechanism by which *Tph1* enzyme levels and therefore 5HT production are modulated by commensal effectors and their byproducts (e.g., butyrate) is through the transcriptional regulatory protein ZBP-89. Our studies are consistent with reports showing

that butyrate suppresses the *S. typhimurium* regulatory locus *hila*<sup>18, 19</sup>. In addition, we found that deletion of this bacterial locus permits induction of *ZBP-89* gene expression and downstream targets such as *Tph1* and *defensin 5*. Since infection by *S. typhimurium* apparently must occur in a butyrate-poor environment<sup>62, 63</sup>, it appears that the pathogen might be capable of suppressing bacteria generating SCFAs. In that way, *Salmonella* could partially inhibit butyrate-responsive genes like *ZBP-89* and its downstream targets such as *Tph1* and the antimicrobial peptides secreted during colonic mucosal defense.

In summary, a conditional null *ZBP-89* allele reduced innate mucosal defenses against a bacterial pathogen in the colon. The mechanism is in part due to the requirement of this transcription factor to induce butyrate-dependent target genes, e.g., *Tph1* and subsequently to generate optimal amounts of 5HT and antimicrobial peptides.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Biographies

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**Milena Saqui-Salces:** acquisition of data; technical and material support

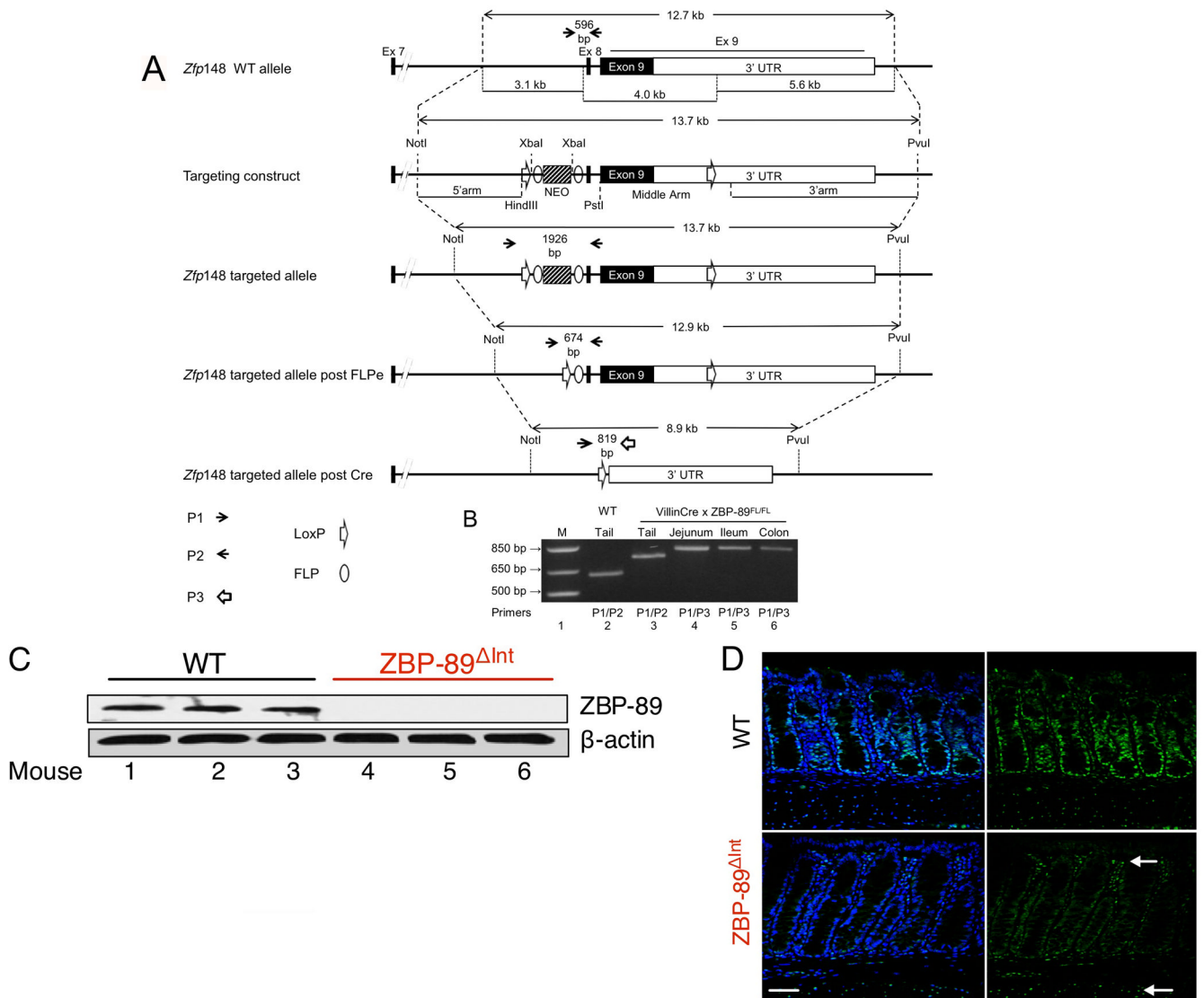
**Mohamad El-Zaatari:** acquisition of data; technical and material support

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**Michael M. Hayes:** experimental design; acquisition of data; technical and material support

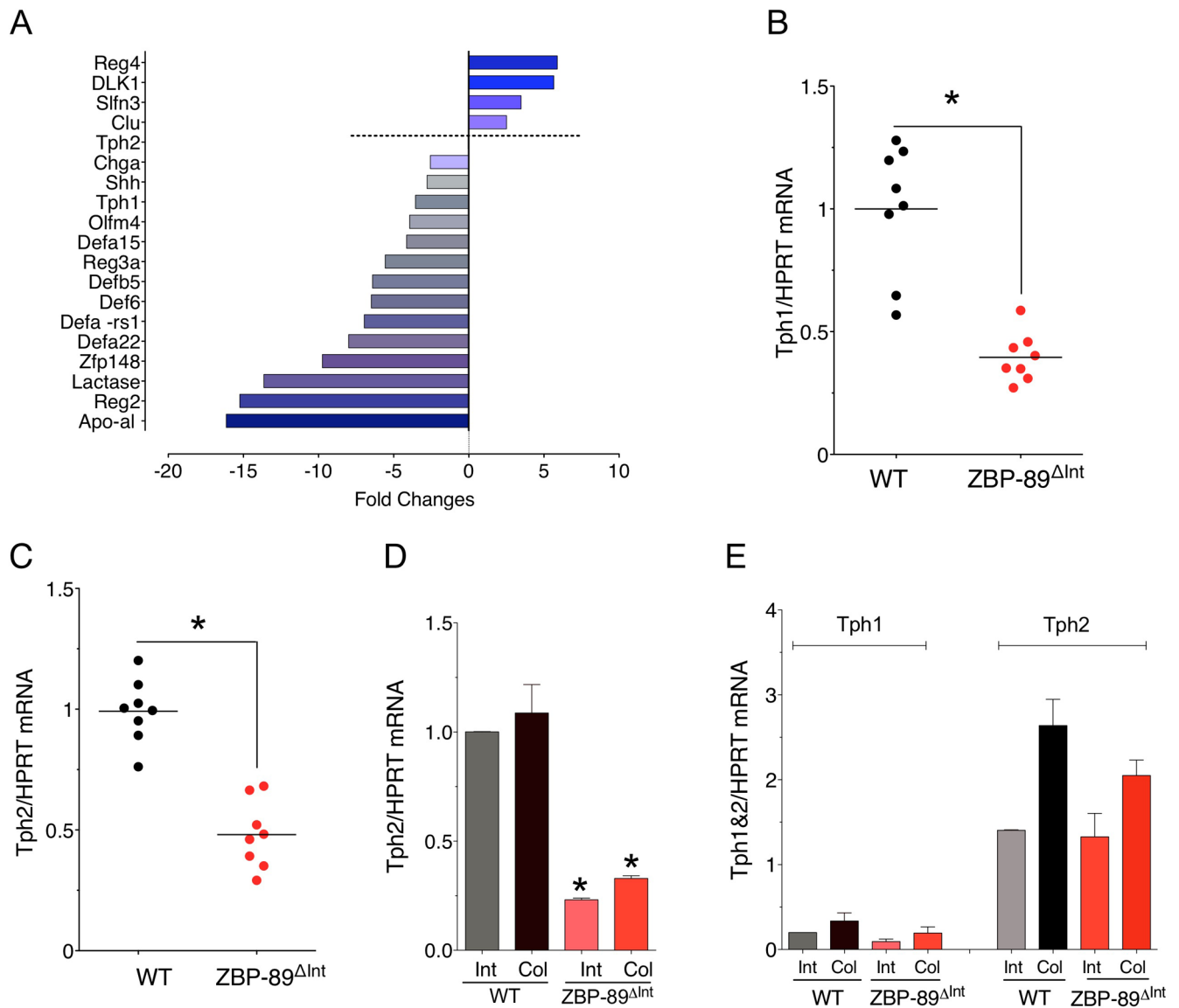
**Alexander C. Yang:** technical and material support

**Juanita L. Merchant:** study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained funding; study supervision



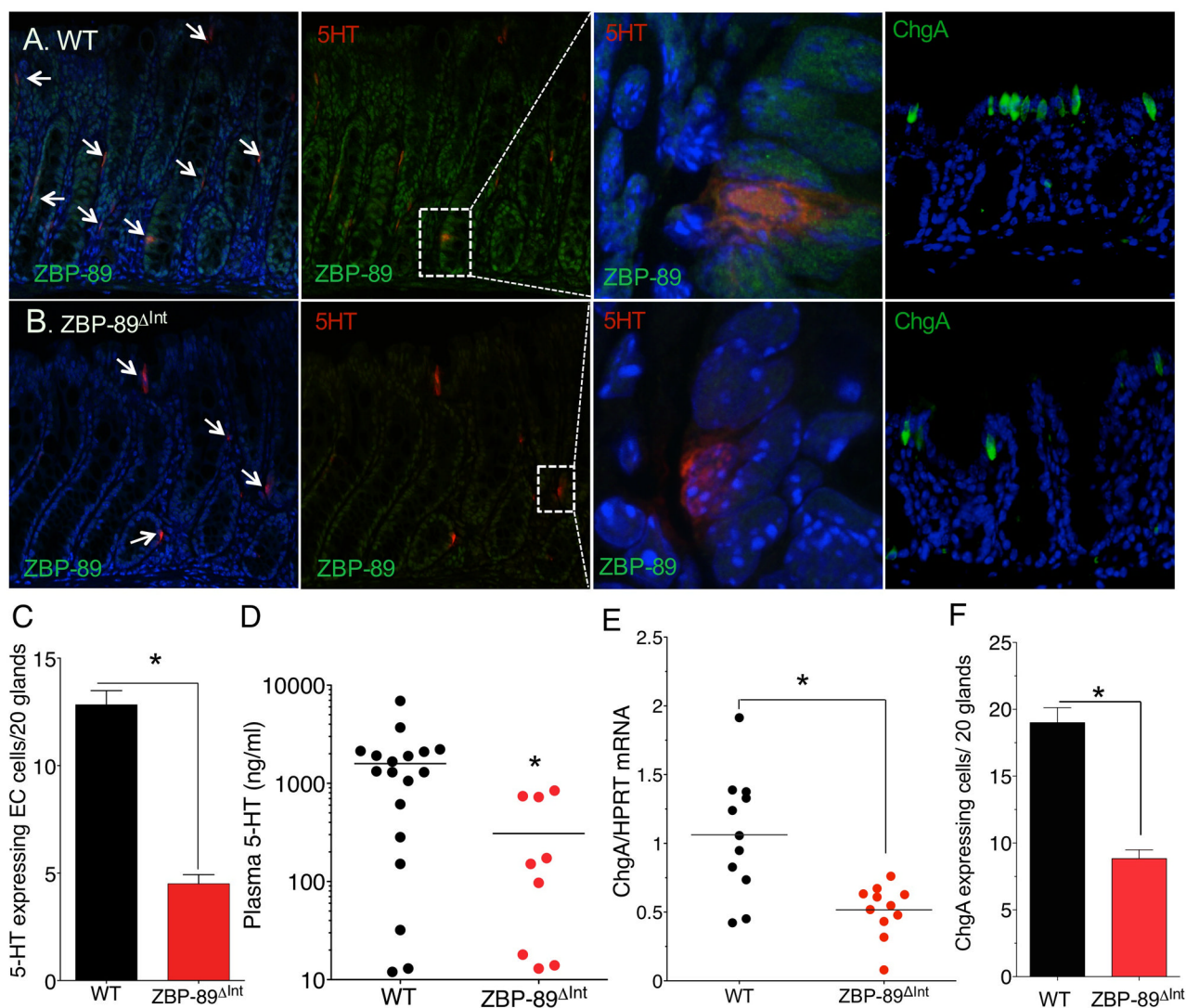
**Figure 1. Conditional deletion of locus encoding transcription factor *Zfp148***

(A) Schematic representation of mouse ZBP-89 locus (*Zfp148*) and strategy for gene targeting. Intestine specific deletion was achieved by breeding to the VillinCre line (ZBP-89<sup>ΔInt</sup>). (B) PCR Genotyping. Lane 1= DNA markers, lane 2 = wild-type (+/+) tail DNA, lane 3 = targeted locus, lanes 4–6, intestinal mucosa where Cre is active. ZBP-89 expression in the colon of WT and ZBP-89<sup>ΔInt</sup> mice detected by (C) Western blot of ZBP-89 normalized to β-actin from after colonic epithelium separated by chelation. (D) Immunofluorescence. FITC label = ZBP-89, DAPI = nuclei. Arrows indicate residual ZBP-89 nuclear staining in the lamina propria/smooth muscle layer.



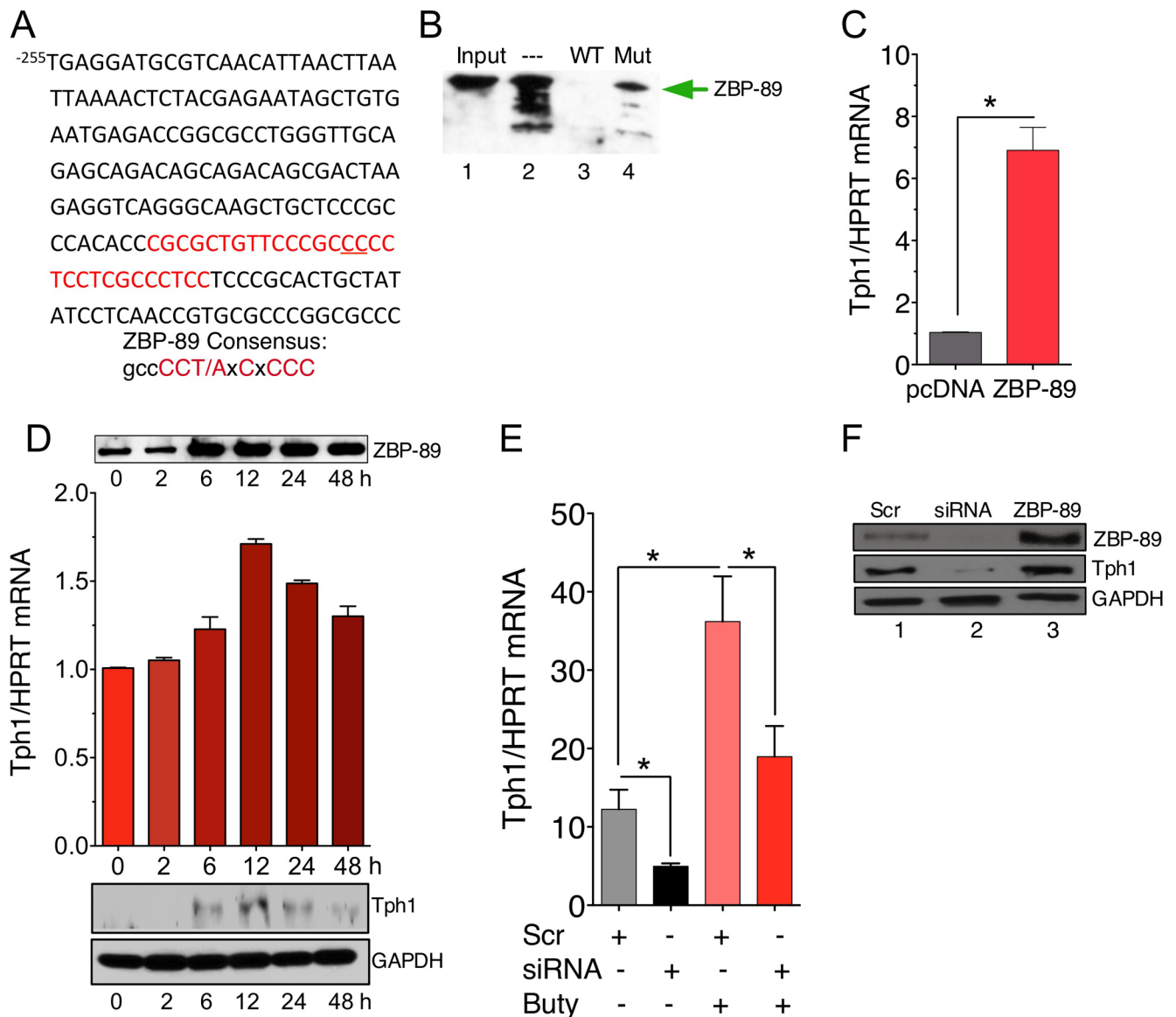
**Figure 2. Microarray analysis of ZBP-89 $\Delta$ Int mice**

(A) Mean relative expression (N=2 mice) of several genes in the ZBP-89 $\Delta$ Int versus WT mice. (B) RT-qPCR for *Tph1* mRNA and (C) for *Tph2* mRNA. (D) *Tph2* mRNA in isolated epithelium. (E) *Tph1* versus *Tph2* in isolated mesenchymal/smooth muscle layer. Shown is the mean  $\pm$  SEM for N = 5. \* $P$  < 0.05.



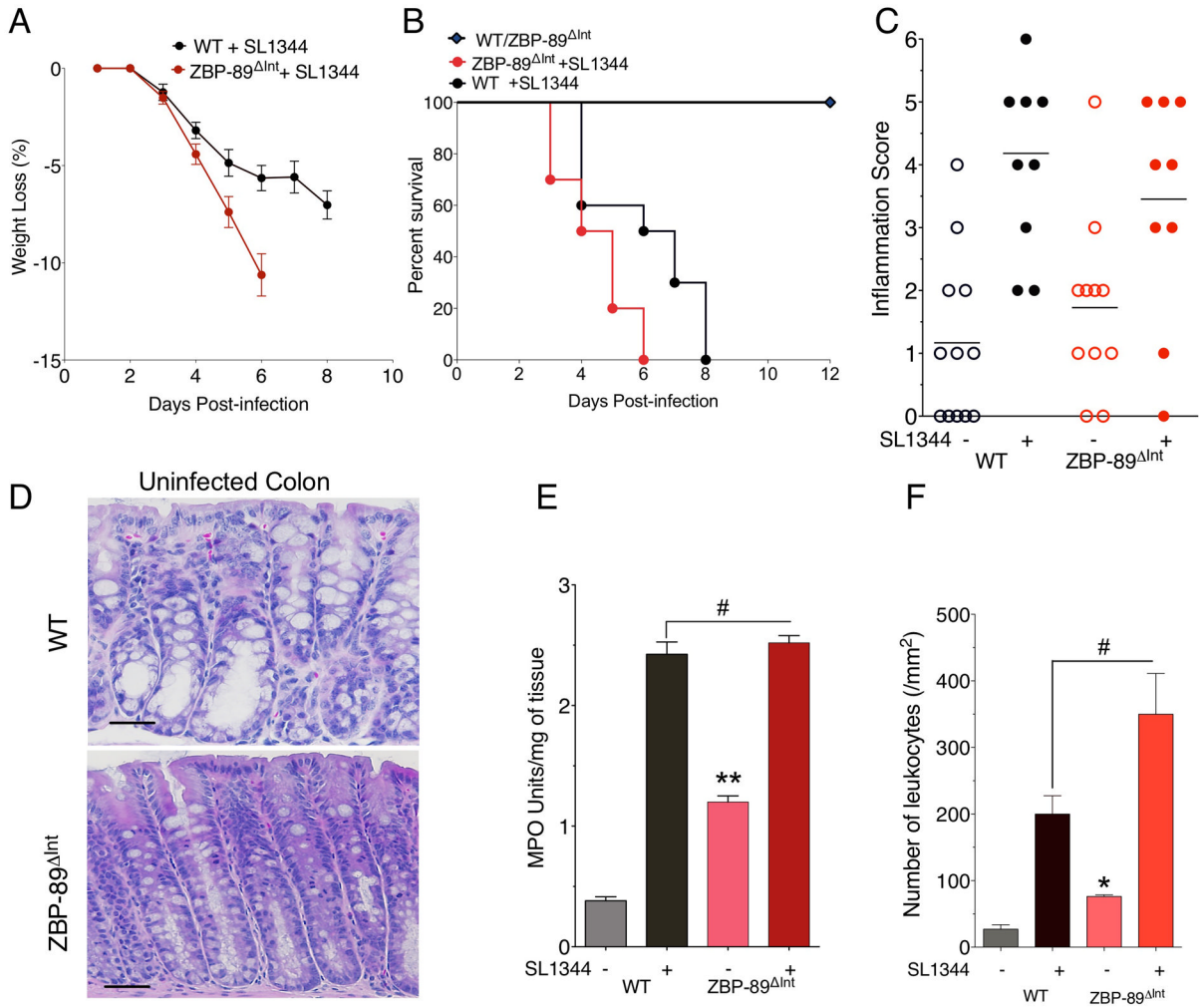
### Figure 3. Reduced 5HT+ cells in ZBP-89<sup>ΔInt</sup> mice

(A) Immunofluorescent staining for 5HT (red), and ZBP-89 (FITC, confocal) or ChgA (FITC) with DAPI (blue) in WT and (B) ZBP-89<sup>ΔInt</sup> mice. Low power merged views on left, (Scale bar, 100  $\mu$ m). High power view of ZBP-89 with 5HT (600x) (confocal image) or ChgA with DAPI (400x). (C) Morphometric analysis of 5HT-expressing cells, N = 12 mice; (D) Plasma 5HT (ng/ml); (E) *ChgA* mRNA and (F) Morphometric analysis of ChgA-expressing cells in WT and ZBP-89<sup>ΔInt</sup> mouse colons, N = 8 mice. \*P < 0.05.



**Figure 4. ZBP-89 binds and regulates mouse Tph1**

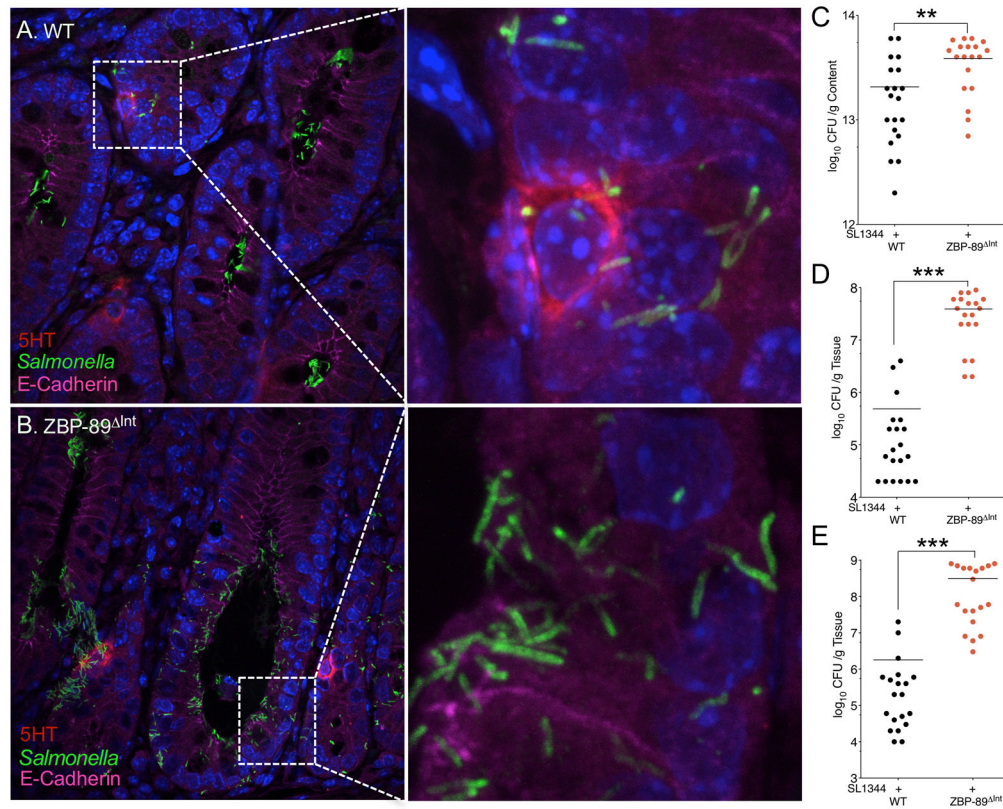
(A) *mTph1* promoter from  $-255$  to  $-55$  bp. ZBP-89 DNA element highlighted (red); two bp mutation underlined. (B) DAPA using a 30 bp biotinylated DNA element at  $-123$  bp. Lane 1: input, lane 2: probe alone with  $50 \mu\text{g}$  of STC-1 nuclear extract, lane 3: 20x unlabeled probe competitor, lane 4: 20x 2 bp mutation competitor. (C) RT-qPCR analysis of *mTph1* mRNA after ZBP-89 expression in STC-1 cells. (D) Butyrate ( $2.5 \text{ mM}$ ) induction of ZBP-89 protein, *Tph1* mRNA and protein in STC-1 cells,  $N=5$ . Both ZBP-89 and Tph1 western blots normalized to same GAPDH. (E) ZBP-89 siRNA versus scrambled siRNA oligos transfected into STC-1 cells  $\pm 2.5 \text{ mM}$  butyrate for 6h. *Tph1* mRNA levels determined by RT-qPCR.  $N=3$ ,  $*P < 0.05$ . (F) Western blot of ZBP-89 and mTph1 protein levels after transfection of scrambled (Scr) (lane 1) or siRNA oligos for mZBP-89 (lane 2) or a ZBP-89 expression vector (lane 3) into STC-1 cells.



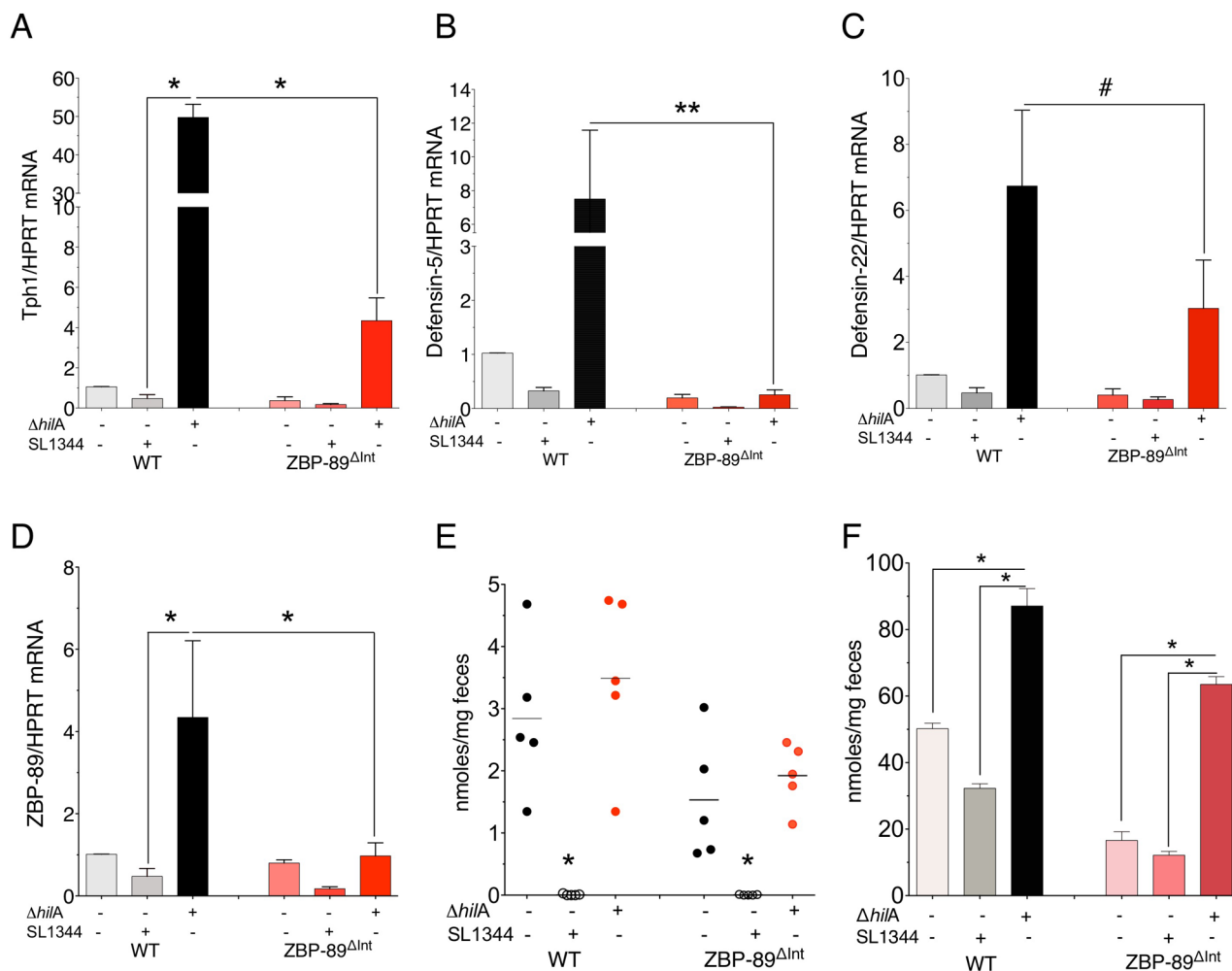
**Figure 5. Increased susceptibility to *S. typhimurium* in ZBP-89 $\Delta$ Int mice**

(A) Mouse body weight at euthanization. Percent survival of WT, N = 20 (black) versus ZBP-89 $\Delta$ Int N = 20 *SL1344* infected mice (red) from 4 independent experiments. Log-rank test demonstrated a statistically significant difference between the 2 groups ( $P = 0.0165$ ) (C) Colitis scores (D) H&E stains of colon from WT and ZBP-89 $\Delta$ Int mice prior to infection; (E) Myeloperoxidase (MPO) activity; (F) Leukocyte cell count from colons of WT versus ZBP-89 $\Delta$ Int mice before and after *SL1344* infection for 6 days. \* $P < 0.05$ .





**Figure 6. Salmonella-infected colonic glands in WT and ZBP-89 $\Delta$ Int mice**  
 (A) Confocal image of *Salmonella* with 5HT-expressing EC cells 4 day p.i. in wild type mice. (B) versus ZBP-89 $\Delta$ Int. Quantitative cultures of *Salmonella* SL1344 in (C) colon, (D) liver, (E) spleen for in WT versus ZBP-89 $\Delta$ Int mice at euthanization (CFU/g content, Log<sub>10</sub>) for two separate experiments. \*  $P < 0.05$ . \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .



**Figure 7. *S. typhimurium*  $\Delta$ hilA induces Tph1 and defensin mRNA levels** mRNA levels 4 days after gavage with  $\Delta$ hilA mutant for (A) *Tph1*; (B) *defensin 5*; (C) *defensin 22*; (D) ZBP-89. N = 6 mice per WT versus ZBP-89 $\Delta$ Int for two independent experiments. Mean  $\pm$  SEM. # $P$  = 0.42; \* $P$  < 0.05; \*\* $P$  < 0.01. (E) Fecal butyrate and (F) Fecal SCFA levels in the colon. \* $P$  < 0.05.