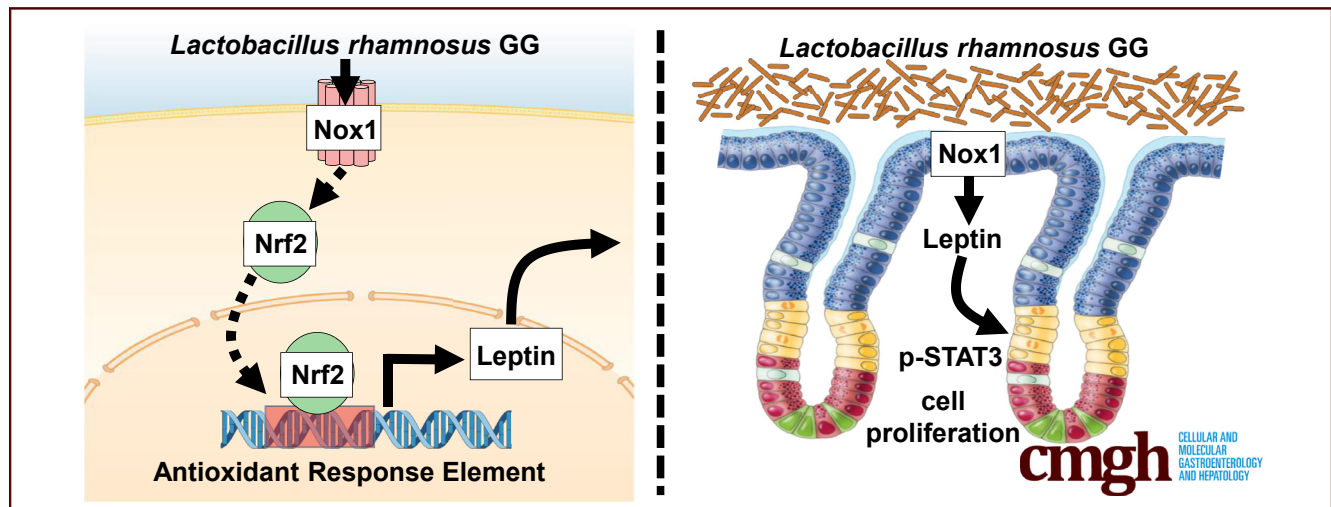


ORIGINAL RESEARCH

***Lactobacillus rhamnosus* GG–induced Expression of Leptin in the Intestine Orchestrates Epithelial Cell Proliferation**Trevor M. Darby,¹ Crystal R. Naudin,¹ Liping Luo,¹ and Rheinallt M. Jones^{1,2}¹Division of Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia; ²Emory Microbiome Research Center, Emory University School of Medicine, Atlanta, Georgia

SUMMARY

The signaling pathways that mediate the positive influences of *Lactobacillus rhamnosus* GG are largely unknown. We show that *L rhamnosus* GG–induced leptin expression orchestrates cell proliferation in the colon, thereby describing a mechanism whereby *L rhamnosus* GG in the gut lumen transduces signals to the gut epithelium.

BACKGROUND & AIMS: Identifying the functional elements that mediate efficient gut epithelial growth and homeostasis is essential for understanding intestinal health and disease. Many of these processes involve the *Lactobacillus*-induced generation of reactive oxygen species by NADPH oxidase (Nox1). However, the downstream signaling pathways that respond to Nox1-generated reactive oxygen species and mediate these events have not been described.

METHODS: Wild-type and knockout mice were fed *Lactobacillus rhamnosus* GG and the transcriptional and cell signaling pathway responses in the colon measured. Corroboration of data generated in mice was done using in organoid tissue culture and in vivo gut injury models.

RESULTS: Ingestion of *L rhamnosus* GG induces elevated levels of leptin in the gut epithelia, which as well as functioning in the context of metabolism, has pleiotropic activity as a chemokine that triggers cell proliferation. Consistently, using gut epithelial-specific knockout mice, we show that *L rhamnosus* GG–induced

elevated levels of leptin is dependent on a functional Nox1 protein in the colonic epithelium, and that *L rhamnosus* GG–induced cell proliferation is dependent on Nox1, leptin, and leptin receptor. We also show that *L rhamnosus* GG induces the JAK-STAT signaling pathway in the gut in a Nox1, leptin, and leptin receptor–dependent manner.

CONCLUSIONS: These results demonstrate a novel role for leptin in the response to colonization by lactobacilli, where leptin functions in the transduction of signals from symbiotic bacteria to subepithelial compartments, where it modulates intestinal growth and homeostasis. (*Cell Mol Gastroenterol Hepatol* 2020;9:627–639; <https://doi.org/10.1016/j.jcmgh.2019.12.004>)

Keywords: Leptin; Nox1; Probiotic; *Lactobacillus*; Probiotics.

The intestinal epithelium is a dynamic barrier that separates the potential antigenic load of the gut luminal content away from the gut epithelium. Therefore, the molecular events that mediate efficient gut epithelial homeostasis and renewal are of intense interest. Homeostasis in the intestine is known to be controlled by cell signaling pathways activated by growth factors and other small molecules.¹ Examples of small molecules that modulate tissue growth are reactive oxygen species (ROS), which may be generated as natural by-products of the normal metabolism of oxygen, or may be deliberately generated by the catalytic activity of enzymes, such as NADPH oxidase

(Nox).² ROS have been shown to have critical functions in mediating intracellular signaling by the rapid and reversible oxidative modification of target regulatory proteins.^{3,4} These include demonstrated functions in stem cell self-renewal in mouse spermatogonia⁵ in the regeneration of an amputated *Xenopus* tadpole tail,^{6,7} the control of the transition from proliferation to differentiation in the plant root,^{8,9} and the control of cellular proliferation and differentiation of *Drosophila* hematopoietic progenitors.^{10,11}

The presence of a gut microbiome is essential for normal tissue development. For example, germ-free mice have abnormally long intestinal villi, altered gastrointestinal motility, and exhibit slower crypt-to-tip transit of epithelial cells during epithelial turnover.^{12–15} We reported that some species of lactobacilli, which are commonly used probiotics and initial colonizers of the mammalian intestinal tract following natural birth, have evolved the property of being able to stimulate the generation of ROS in epithelial cells.^{16–18} This lactobacilli-induced generation of ROS also resulted in intestinal epithelial cell proliferation by processes requiring the catalytic action of Nox1.¹⁶ In further investigations, feeding of the same lactobacilli species was shown to enhance wound healing and facilitate intestinal epithelium restitution following inflicted mechanical injury, again by mechanisms that were formyl peptide receptor, ROS, and Nox1-dependent.^{19–23} However, little is known about the downstream cell signaling pathways that respond to lactobacilli-induced ROS in the intestinal epithelium, and mediate the transduction of bacterial-initiated signals to subepithelial compartments.

In a previous report, we conducted an RNAseq analysis to measure transcript enrichment in the colonic intestinal epithelium after colonization of germ-free mice with *Lactobacillus rhamnosus* GG (LGG).²⁴ One enriched transcript in the colon in response to LGG ingestion is the gene that codes for leptin. The function of leptin has been widely investigated in the context of its role as a peptide hormone and inflammatory cytokine involved in regulating food intake, metabolism, body fat, energy expenditure, and neuroendocrine function.²⁵ In addition, and considerably less well studied, is leptin's biologic activities as a mitogenic factor, which also suggests that leptin may also function in tissue homeostasis.^{26–30} Mechanistically, leptin signals via leptin receptor (LEP-R), which belongs to a family of class I cytokine receptors that are located on cell surface membrane in a variety of tissues. Binding of leptin to its receptor has been shown to activate many cell signaling pathways, including PI3K/AKT, ERK, and JAK/STAT.^{31–34} Intriguingly, the functional homologue of leptin in *Drosophila* is Upd2.³⁵ We recently reported that Upd2 in *Drosophila* is upregulated in gut epithelial cells in response to contact of with lactobacilli, with the subsequent activation of JAK/STAT signaling within intestinal stem cells adjacent to the basement membrane.¹¹ These observations prompted us to investigate the extent to which leptin functions in signaling events that transduce the proliferative influence of LGG in the gut epithelium.

Herein, we show that LGG upregulates *leptin* gene expression, and protein levels in colonic tissue and in the circulating serum. In addition, using gut epithelial-specific


knockout mice, we show that LGG-induced elevated levels of leptin is dependent on functional Nox1 protein in the gut. Furthermore, we show that as well as being dependent on a functional Nox1,¹⁶ LGG-induced cell proliferation in the colon is also blunted in leptin null *ob⁻/ob⁻*, and in LEP-R-deficient mice. Analysis of signaling pathways that are upregulated in response to LGG contact revealed robust phosphorylation of STAT3 within the colonic epithelia. The LGG-induced STAT3 phosphorylation was not detected in Nox1, leptin, nor LEP-R-deficient mice. Furthermore, LGG did not elicit cytoprotection in leptin null *ob⁻/ob⁻* mice, as previously demonstrated by our group²⁴ and others.³⁶ Finally, supplementation of growth media of intestinal organoids with leptin significantly increased the growth rate of colonoids. Together, these data imply that LGG elevates leptin levels in the gut epithelium, which then mediates the induction of signaling events, including JAK-STAT signaling, that induce cell proliferation by a paracrine noncell autonomous mechanism. These results highlight a previously unreported role for leptin in the host cell response to colonization by symbiotic lactobacilli, where leptin functions in the transduction of signals induced by lactobacilli in the colonic epithelia, which initiate activation of downstream proliferative signaling responses.

Results

LGG Induces Nox1-dependent Leptin Expression and Cell Proliferation in Intestinal Tissues

Transcript enrichment of *leptin* in the colonic epithelium was detected by RNAseq analysis in C57BL/6 germ-free mice at 4 hours following oral administration of LGG.²⁴ We confirm this observation using quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) analysis, both in the colonic epithelial scrapings, and in serum collected from C57BL/6 conventionally raised mice at 4 hours following oral administration of LGG (Figure 1A–C). Because we previously demonstrated a role for Nox1 in the transduction of LGG-initiated proliferative events in the colonic epithelium,¹⁶ we tested the extent to which Nox1 functions in LGG-induced leptin generation. Indeed, intestinal epithelial-specific knockout of Nox1 (B6.No^{x1}^{ΔIEC}) rendered the colonic epithelium unable to induce *leptin* gene transcription or leptin generation in enterocytes (Figure 1A–C). We previously demonstrated that Nox1 is required for LGG-induced cell proliferation in the colonic epithelium.¹⁶ Intriguingly, leptin has been implicated as an inducer of cell proliferation in many tissues.^{26–29} We thus tested if B6.Cg-*Lep^{ob}*/J mice (referred

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; LEP-R, leptin receptor; LGG, *Lactobacillus rhamnosus* GG; Nox, NADPH oxidase; PBS, phosphate buffered saline; PCR, polymerase chain reaction; ROS, reactive oxygen species; WT, wild-type.

 Most current article

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to hereafter as *ob^{-/-}*) that are deficient in leptin have an impaired response to LGG within the colonic epithelium. We show that as well as in our B6.No^{x1}^{ΔIEC} control mice, LGG

does not induce elevated numbers of phospho-histone H3-positive (Figure 1D and E), nor elevated numbers of Ki-67-positive cells (Figure 1F and G) in the colonic

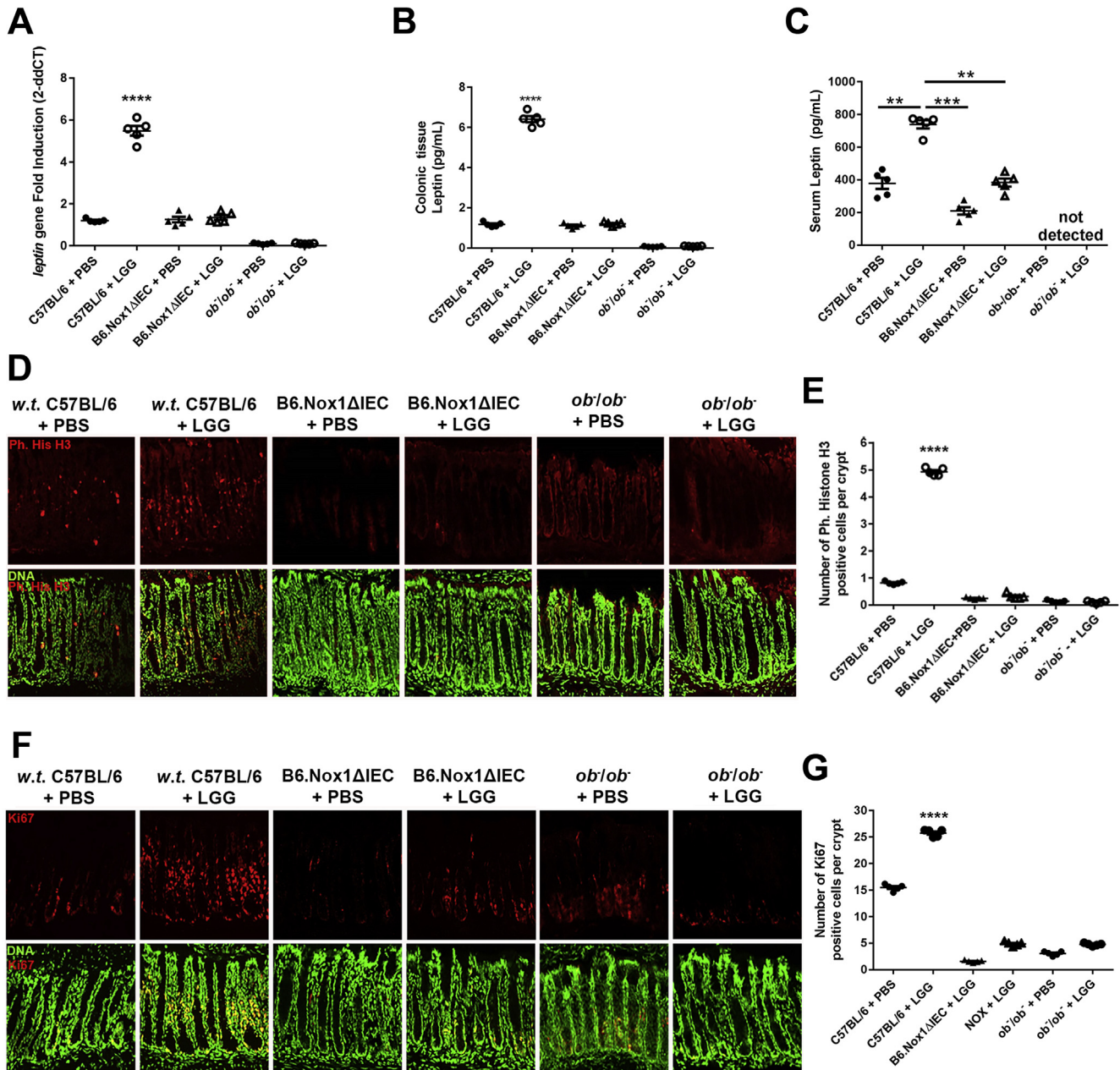


Figure 1. *Lactobacillus rhamnosus* GG induces Nox1-dependent leptin expression and cell proliferation in intestinal tissues. (A) Quantitative PCR analysis for the detection of *leptin* transcript enrichment in colonic tissue of WT C57BL/6, B6.No^{x1}^{ΔIEC}, or B6.Cg-*Lep^{ob/ob}*/J mice (*ob^{-/-}*) at 4 h following oral gavage feeding of Hank's balanced salt solution (HBSS) or 1×10^8 CFU *L rhamnosus* GG. (B) ELISA analysis for the detection of leptin levels in the colonic tissue of WT, B6.No^{x1}^{ΔIEC}, or *ob^{-/-}* mice at 4 h following oral gavage feeding of 1×10^8 CFU of HBSS or *L rhamnosus* GG. (C) ELISA analysis for the detection of leptin levels in the serum of WT, B6.No^{x1}^{ΔIEC}, or *ob^{-/-}* mice at 4 h following oral gavage feeding of 1×10^8 CFU of HBSS or *L rhamnosus* GG. (D) Detection of phospho-Histone H3 in cells within the colon of 6-wk-old WT or *ob^{-/-}* mice at 4 h following oral gavage feeding of 1×10^8 CFU of HBSS or *L rhamnosus* GG. (E) Numeration of phospho-Histone H3-positive cells in C. Twenty $\times 20$ fields were counted in 3 mice for each treatment. (F) Detection of Ki-67-positive cells in cells within the small intestine of 6-wk-old WT or *ob^{-/-}* mice at 4 h following oral gavage feeding of 1×10^8 CFU of HBSS or *L rhamnosus* GG. (G) Numeration of Ki-67-positive cells in Figure 2C. Twenty $\times 20$ fields were counted in 3 mice for each treatment. *** $P < .001$. Data were expressed as mean \pm standard error of the mean. Statistical tests (A–G): 1-way analysis of variance with post hoc Tukey multiple pairwise comparison. ** $P < .01$, *** $P < .001$, and **** $P < .0001$, $n = 5$.

epithelium of *ob⁻/ob⁻* mice, whereas significantly increased proliferation was detectable in wild-type (WT) C57BL/6 mice. Similar to our previous report,¹⁶ we noticed a trend toward fewer overall proliferating cells in the colon of B6.No^{x1}^{ΔIEC}, especially evident in Ki-67 staining (Figure 1F and G). There was a trend toward an increase in the number of Ki-67-positive cells in B6.No^{x1}^{ΔIEC} following LGG treatment, although to a much smaller extent than in WT mice. This may be caused by limited penetrance of the villin-Cre in the colon where the Cre recombinase may not be expressed in every colonic crypt. Together, these data suggest that leptin may be a candidate functional element that mediates lactobacilli-induced stimulation of cell proliferation within the gut epithelium.

LGG Induces Nox1 and Leptin-dependent JAK/STAT Signaling in Intestinal Epithelium

Binding of leptin to LEP-R activates several cell signaling events, including those with known functions in cell proliferation and differentiation, such as the JAK/STAT pathway.^{31–34} Gene Set Enrichment Analysis and Database for Annotation, Visualization and Integrated Discovery analysis of previously published RNAseq data accessible through Gene Expression Omnibus Series accession number GSE70715,²⁴ from an experiment where transcript enrichment in colonic tissue of germ-free mice treated with LGG for 4 hours was measured, revealed upregulation of genes that function in JAK/STAT pathway signaling (Figure 2A and B). In the colon, the state of JAK-STAT signaling is typically detected by the phosphorylation of STAT3, which are events necessary for pathway activation.^{37,38} We show that oral ingestion of LGG induces the phosphorylation of STAT3 in the colonic epithelium (Figure 2C and D). Importantly, LGG-induced phosphorylation of STAT3 was completely abolished in B6.No^{x1}^{ΔIEC} and *ob⁻/ob⁻* mice (Figure 2C and D). In addition, LGG induced the activation of the Cyclin family of proteins that control the progression of cells through the cell cycle, a response that was not detected in B6.No^{x1}^{ΔIEC} and *ob⁻/ob⁻* mice (Figure 2E). Together, these data show that LGG ingestion can induce JAK-STAT pathway, and downstream activation of proteins that control the progression of cells through the cell cycle in the colonic epithelium in a Nox1 and leptin-dependent manner.

LGG Induces Leptin Expression, but Not Cell Proliferation or STAT3 Phosphorylation in LEP-R Null Mice

Leptin is a hormone predominantly produced by adipose cells and enterocytes. Once generated, it is released from cells to exert influence on other cell types. Leptin is sensed by LEP-R, which is a single-transmembrane-domain type I cytokine receptor found on a wide range of cell types. To test if LGG-induced cell proliferation occurs in the gut via the sensing of leptin secreted by enterocytes and sensed by other cell types in the gut mucosa, we used LEP-R null mice. We detected an increase in leptin levels in the serum and gut tissue of LEP-R null mice in response to LGG oral administration, in a similar manner to WT mice (Figure 3A and B).

Interestingly, we found that LEP-R null mice had markedly higher levels of leptin compared with WT in noninduced conditions (Figure 3A and B). This may be caused by the dysregulation of negative feedback loops involved in the controlling tonic levels of cellular leptin generation. Importantly, despite LGG-induced elevated leptin levels, we did not detect an increase in number of p-STAT3-positive cells (Figure 3C and D), nor in the number of proliferative Ki-67-positive cells in the colon of LEP-R null mice in response to LGG oral administration (Figure 3E and F). These data indicate that LGG-induced STAT3 phosphorylation and phosphorylation is dependent on a functional LEP-R, and that these responses occur in cells of the gut epithelium by a paracrine or noncell autonomous mechanism.

Leptin Influences Colonic Organoids Complexity and Budding

We assessed the extent to which leptin directly influences cell proliferation and epithelial growth in cultured colonic organoids derived from primary colonic tissue. We categorize organoid growth in clearly identifiable structures including columnar spheres, cystic spheres, and colonoids (Figure 4A), and as described in previous reports.^{39–41} We show that organoids derived from colonic tissue (Figure 4B), or small intestine tissue (Figure 4C) of leptin-deficient *ob⁻/ob⁻* mice develop slower and form fewer complex structures up to 7 days of growth.

Importantly, structural complexity was restored following supplementation of the culture media with leptin (Figure 4D and E). It has been previously shown that leptin potentially induces proliferation in neural tissues.⁴² We show here by immunostaining with the proliferative marker phospho-Histone H3, that leptin also induces cell proliferation in colonic organoids. Whereas lower numbers of phospho-Histone H3-positive cells were detected in unsupplemented cultured organoids (as normalized to the DNA staining), supplementation of the culture media with leptin significantly increased the number of phospho-Histone H3-positive cells indicating that leptin directly induces cell proliferation in gut tissue (Figure 4F and G).

LGG Fails to Protect Leptin-deficient Mice From Acute Radiologic Intestinal Injury

Beneficial bacteria have been reported to have protective influences against acute intestinal injury. For example, we previously showed that LGG induced enhanced survival rates and limited tissue damage in response to radiologic insult in a Nox1-dependent mechanism.^{24,36} We assessed the extent to which leptin functioned in LGG-induced cytoprotection in the intestine. We first corroborate previous data by showing that mice fed LGG have significantly enhanced survival, are protected against weight loss, and have fewer apoptotic cells at within-colonic crypts following irradiation (Figure 5A–F). However, the cytoprotective influences of LGG against irradiation were lost in *ob⁻/ob⁻* mice. Although compared with WT mice, *ob⁻/ob⁻* mice exhibited marginally lower overall susceptibility to irradiation with

regards to weight loss, probably because *ob⁻/ob⁻* mice were heavier and had more body mass at the time of irradiation, *ob⁻/ob⁻* mice did not exhibit increased

radioprotection following the ingestion of LGG (Figure 5A and D). Furthermore, *ob⁻/ob⁻* phosphate buffered saline (PBS) and LGG-fed mice exhibited similar number of

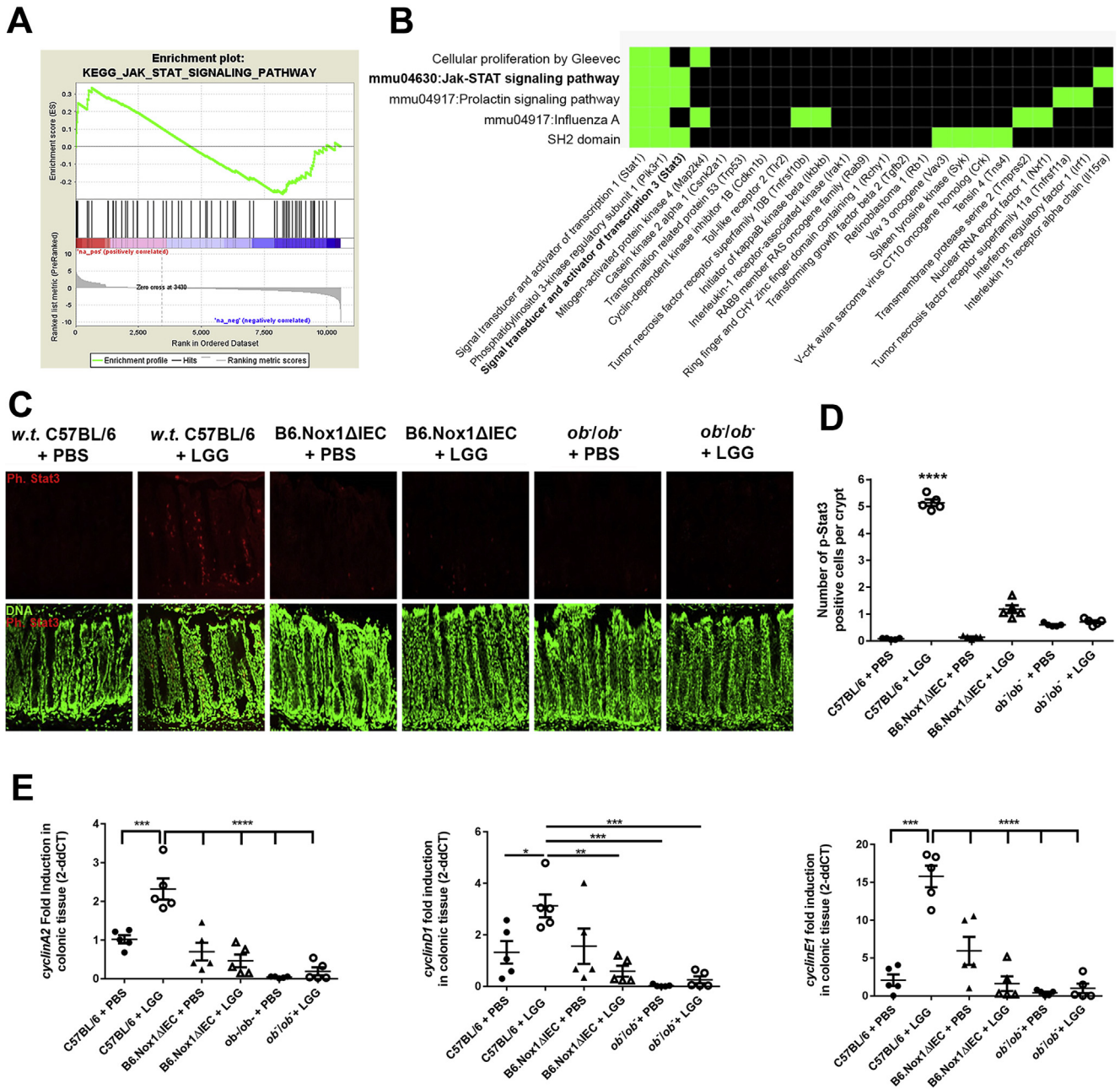
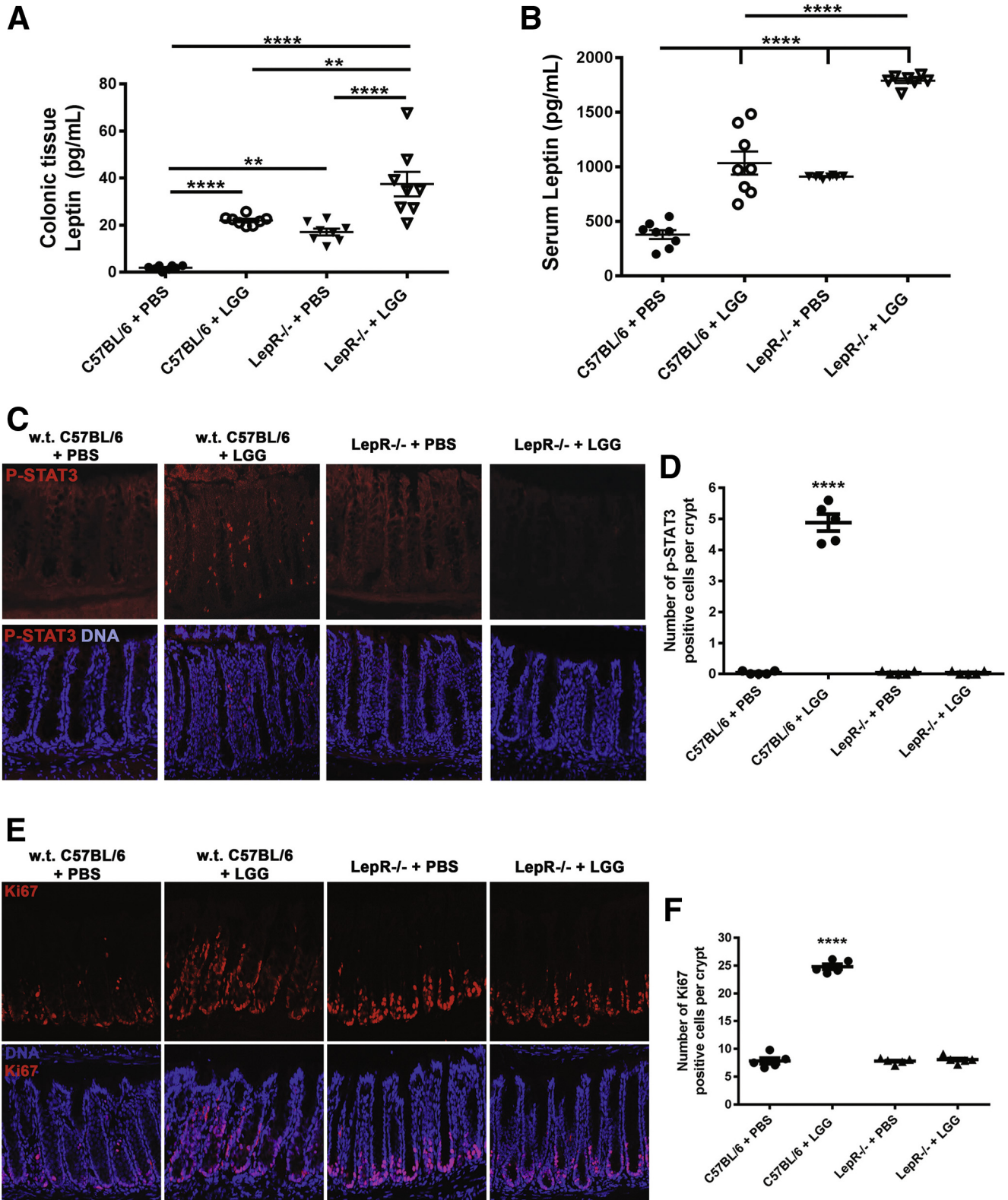


Figure 2. LGG induces Nox1, and leptin-dependent JAK/STAT signaling in intestinal epithelium. (A) Analysis of RNAseq data set from Gene Expression Omnibus Series accession number GSE70715 using Gene Set Enrichment Analysis pathway analysis enrichment chart showing overall positive enrichment of JAK-STAT signaling pathway genes in mice treated with LGG compared with control mice. (B) Analysis of RNAseq data set in A using Database for Annotation, Visualization and Integrated Discovery gene enrichment results highlighting key genes that are enriched in mice treated with LGG compared with control mice in JAK-STAT and related pathways (*Stat1*, *Pik3r1*, *Stat3*, and *IL15ra*). (C) Immunofluorescence analysis for the detection of phospho-STAT3 levels in the colonic tissue of WT, B6.No^{x1} Δ IEC, and B6.Cg-*Lep^{ob}*/J mice (*ob⁻/ob⁻*) at 4 h following oral gavage feeding of Hank's balanced salt solution (HBSS) or of 1×10^8 CFU or LGG. (D) Numeration of phospho-STAT3-positive cells in C. Twenty $\times 20$ fields were counted in 3 mice for each treatment. (E) Quantitative PCR analysis for the detection of *cyclin A2*, *cyclin D1*, and *cyclin E1* transcript enrichment in colonic tissue of WT, B6.No^{x1} Δ IEC, or B6.Cg-*Lep^{ob}*/J mice (referred to as *ob⁻/ob⁻*) at 4 h following oral gavage feeding of HBSS or 1×10^8 CFU LGG. Data are presented as mean \pm standard error of the mean. Statistical analysis (D and E): 1-way analysis of variance with post hoc Tukey multiple pairwise comparison. ***P* < .01, ****P* < .001, and *****P* < .0001; n = 5.

apoptotic cells at 6 hours after irradiation (Figure 5E and F). We conclude that as well as Nox1, LGG-induced leptin is a factor that contributes to gut cytoprotection following acute radiologic challenge.

Discussion

A considerable and ever expanding body of evidence substantiates the notion that symbiotic bacteria within the gut beneficially influences organismal health by promoting



intestinal homeostasis. However, only a few of the molecular elements that mediate this host cell and microbe interaction have been identified. In this study, we identified leptin and subsequent activation of the JAK-STAT signaling pathway as novel functional elements that respond to and mediate the host cellular response to symbiotic lactobacilli. Ingestion of LGG induced leptin generation and triggered JAK-STAT signaling, with both events being dependent on a functional *nox1* gene in the colonic epithelium. In addition, LGG induced activation of JAK-STAT signaling, and induced cell proliferation in the intestinal epithelium in a leptin-dependent manner, because this did not occur in *ob/ob* mice. Furthermore, we detected elevated levels of leptin in gut tissue of LEP-R null mice, but do not detect activation of JAK-STAT signaling or induced cell proliferation in LEP-R mice indicating paracrine mechanisms of leptin influence on cells within the gut epithelium. The cytoprotective influences of LGG against radiologic-induced tissue damage was abrogated in *leptin* null *ob/ob* mice, and supplementation of leptin on cultured colonic organoids increased growth. Altogether, these data form a cogent body of evidence that leptin and JAK-STAT signaling function intestinal epithelial response to contact with LGG.

Lactobacilli are primary colonizers of the mammalian neonatal gut and are dominant taxa for the first 2 years of life in humans.⁴³ We previously demonstrated specific functions of lactobacilli consistent with the physiological changes that occur in neonates following colonization. These responses include induced cell proliferation in the intestinal epithelium,^{16,19} cytoprotection,^{24,36} and tightening of gut epithelial permeability.^{44,45} Furthermore, these responses seem to involve the attachment of LGG to the intestinal mucus layer.⁴⁶ Therefore, it is apparent that taxa within the lactobacilli genus have evolved specific elements that induce host gene regulatory events within the colonic epithelium. Importantly, the host cellular response to contact with lactobacilli described in this report are consistent with physiological changes that occur in the intestine following colonization. We show evidence that leptin and JAK-STAT pathway are a conduit to lactobacilli-induced physiological responses in the intestinal epithelium similar to responses that occur during initial colonization by lactobacilli.

The JAK-STAT pathway signals to the nucleus inducing the expression of genes that regulate proliferation, differentiation, apoptosis, and immunity. The pathway can be activated by various ligands, including cytokines, such as

interferon and interleukin, and growth factors that bind to JAK receptor proteins at the cell surface.⁴⁷ Cell proliferation and differentiation events occur in cells that are at the base of the colonic crypt. Because symbiotic bacteria, such as LGG, that induce cell proliferation are only associated with the mucus layer, and do not come into direct contact with proliferative cells at the base of the crypt, signals must therefore be transduced from the apical end of epithelial cells, to lower compartments within the crypt, likely by noncell autonomous signaling involving cytokines. Although leptin has been primarily studied in relation to its function as a peptide hormone involved in controlling food consumption and metabolism, recent reports have demonstrated that it is an activator of JAK-STAT signaling.³³ Following LGG ingestion, we detected elevated leptin by ELISA and quantitative PCR within colonic epithelial tissue. This observation points to the possibility that leptin may act as a chemokine produced by enterocytes that activates JAK-STAT signaling within proliferating and differentiating cells at the base of the crypt.

Although we identify leptin as a factor that is responsive to LGG by a Nox1-dependent mechanism, the nuclear factor that is sensitive to Nox1-generated ROS, and induces *leptin* gene transcription remains enigmatic. One possible factor may involve Nrf2 cell signaling pathway.^{48,49} We recently reported that lactobacilli-induced ROS generation in epithelial cells activates the Nrf2 cell signaling pathway, and also elicited cytoprotection against exogenous insults to intestinal tissues.^{3,24} Intriguingly, there is also an emerging literature describing the role of the Nrf2 pathway in cell proliferation and differentiation in mice and *Drosophila*.^{11,50} In our previous investigation, we identified candidate genes that may be under the transcriptional control of Nrf2, because of the presence of a conserved antioxidant response element within their promoter region.²⁴ Leptin was identified as a gene with a putative antioxidant response element, and may be transcriptionally regulated by Nrf2. Identifying the extent to which Nrf2 functions in LGG-induced leptin generation is the focus of intense investigation within our research group.

Methods

Mice

All experiments were done using 6-week-old C57BL/6, in B6.Cg-*Lep^{ob}/J* mice (referred to as *ob/ob*), and

Figure 3. (See previous page). *Lactobacillus rhamnosus* GG induces leptin expression, but not cell proliferation or STAT3 phosphorylation in LEP-R null mice. (A) ELISA analysis for the detection of leptin levels in the colonic tissue of WT C57BL/6, or B6.BKS(D)-*Lep^{ob}/J* (*LEP-R^{-/-}*) mice at 4 h following oral gavage feeding of 1×10^8 CFU of PBS or *L. rhamnosus* GG. (B) ELISA analysis for the detection of leptin levels in the serum of WT, B6.No^{x1}^{ΔIEC}, or B6.BKS(D)-*Lep^{ob}/J* (*LEP-R^{-/-}*) mice at 4 h following oral gavage feeding of 1×10^8 CFU of PBS or *L. rhamnosus* GG. (C) Immunofluorescence analysis for the detection of phospho-STAT3 levels in the colonic tissue of WT C57BL/6 or B6.BKS(D)-*Lep^{ob}/J* (*LEP-R^{-/-}*) mice at 4 h following oral gavage feeding of PBS or of 1×10^8 CFU or *L. rhamnosus* GG. (D) Numeration of phospho-STAT3-positive cells in C. Twenty $\times 20$ fields were counted in 3 mice for each treatment. (E) Detection of phospho-Histone H3 in cells within the colon of 6-wk-old WT C57BL/6, or B6.BKS(D)-*Lep^{ob}/J* (*LEP-R^{-/-}*) mice at 4 h following oral gavage feeding of 1×10^8 CFU of PBS or *L. rhamnosus* GG. (F) Numeration of phospho-Histone H3-positive cells in Figure 1C. Twenty $\times 20$ fields were counted in 3 mice for each treatment. Data were expressed as mean \pm standard error of the mean. Statistical tests (A–F): 1-way analysis of variance with post hoc Tukey multiple pairwise comparison. ***P* < .01 and *****P* < .0001; n = 5.

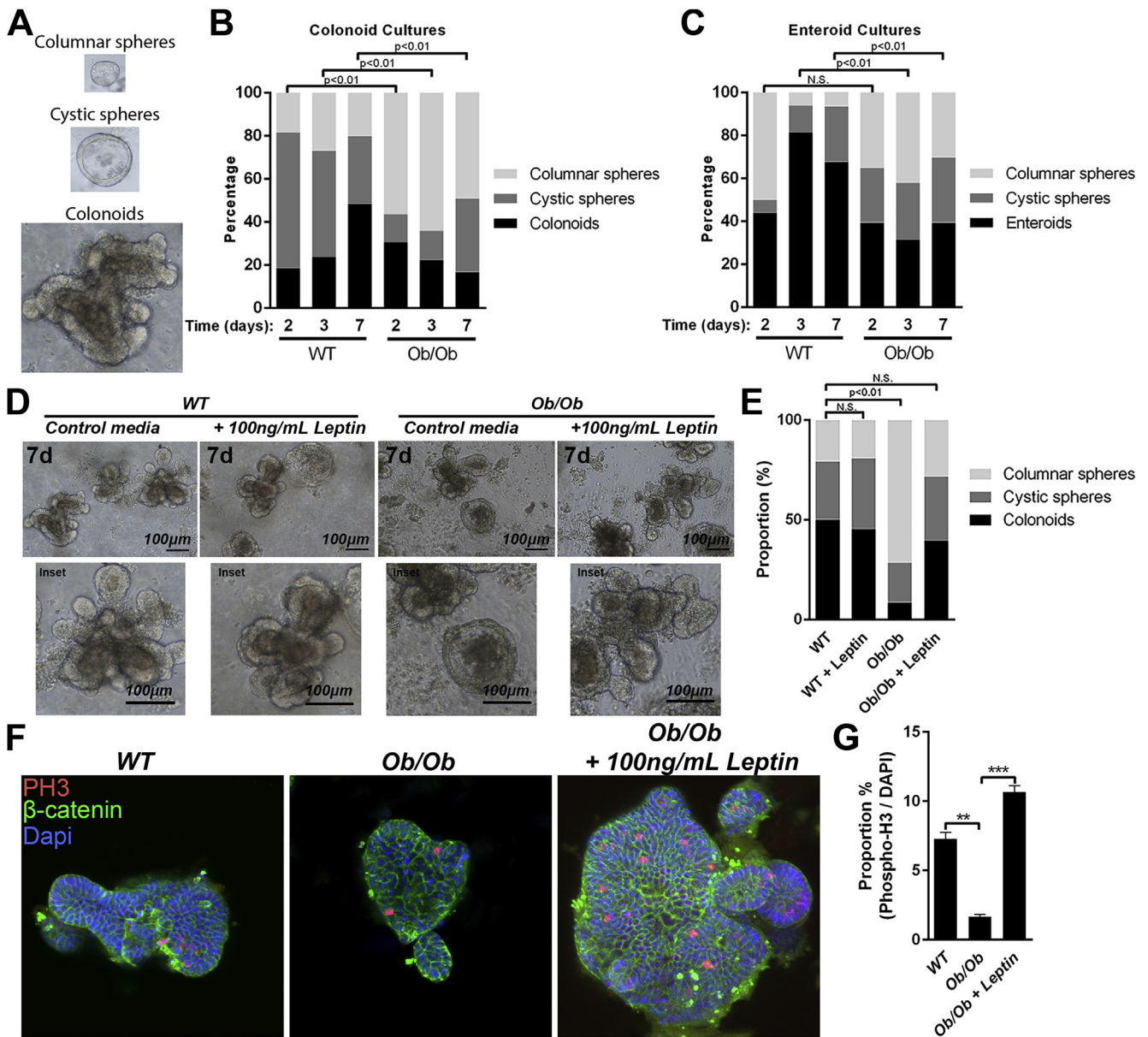


Figure 4. Leptin-deficient mice influence colonic organoids complexity and budding. (A) Representative images of morphologic structures quantified in enteroid or colonoid cultures. (B) Quantification of the developmental stages of colonoids derived from colonic tissue of WT C57BL/6, or B6.Cg-*Lep^{ob}/J* (*ob⁻/ob⁻*) age-matched mice at 2, 3, and 7 d post-plating. (C) Quantification of the developmental stages of small intestine-derived enteroids from WT C57BL/6, or B6.Cg-*Lep^{ob}/J* (*ob⁻/ob⁻*) age-matched mice at 2, 3, and 7 d post-plating. (D) Images of colonoids derived from colonic tissue of WT C57BL/6, or B6.Cg-*Lep^{ob}/J* mice (*ob⁻/ob⁻*). Postpassaging, some colonoids in culture media were supplemented with 100 ng/mL of PEGylated-leptin supplemented to the media 3 times (Day 1, Day 3, and Day 5). Colonoids were imaged at 7 d postpassaging per group, with the representative images shown. Scale bar represents 100 µm. (E) Quantification of the developmental stages of colonoids described in C at Day 7 postpassaging. (F) Immunofluorescent images of colonoids described in C and D at Day 7 postpassaging using antibodies against β-catenin (green), phospho-Histone H3 (red), and counterstained with DAPI for DNA (blue). (G) Quantification of the proportion of phospho-Histone 3–positive nuclei (relative to total nuclei) within colonoid cultures described in E. Phospho-Histone 3–positive nuclei were numerated using the 3-dimensional structures of each representative colonoid. For B, C, and E, 90–110 total structures were counted per experimental group. Data are presented as chi-square analyses performed in a series of 2 × 3 matrices for each time point in B and C, and in a 4 × 3 matrix in D, giving degrees of freedom of 2 and 6, respectively. The cutoff for statistical significance was $P = .05$. For F, data are presented as mean ± standard error of the mean. One-way analysis of variance with post hoc Tukey multiple pairwise comparison. ** $P < 0.01$ and *** $P < .001$; $n = 5$.

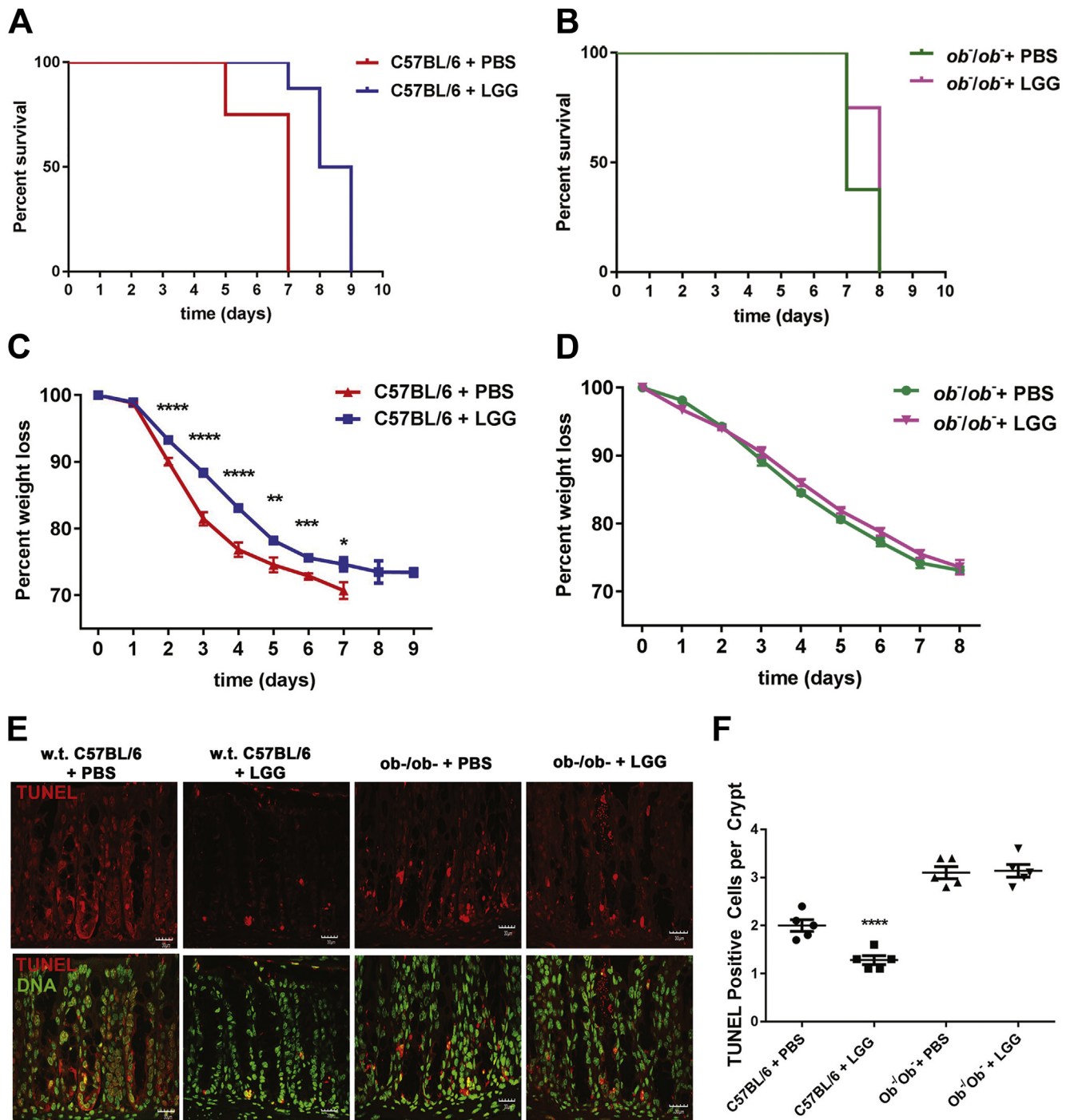


Figure 5. Colonization of the murine intestine with LGG induces leptin-dependent cytoprotection. (A) Survival of 6-wk-old WT mice fed LGG following 12-Gy irradiation insult. Note significantly increased survival of irradiated WT mice fed LGG in response to irradiation (log-rank test for WT vs WT + LGG; $P = .0013$; $n = 8$). (B) Survival of 6-wk-old B6.Cg-Lep^{ob}/J mice (ob⁻/ob⁻) fed LGG following 12-Gy irradiation insult. Note no significant increase in survival of ob⁻/ob⁻ mice fed LGG compared with unfed WT (log-rank test for WT vs ob⁻/ob⁻ + LGG; $P = .2150$; $n = 8$). Also note significantly decreased survival rate of Ob⁻/ob⁻ mice fed LGG compared with WT mice fed LGG represented in A (log-rank test for WT + LGG vs ob⁻/ob⁻ + LGG; $P = .0142$; $n = 8$), and no significant increase in survival of ob⁻/ob⁻ mice fed LGG compared with unfed WT (represented in A) (log-rank test for WT vs ob⁻/ob⁻ + LGG; $P = .2150$; $n = 8$). (C) Percent body weight loss of mice described in A. Statistical analysis represents comparison of WT vs WT + LGG on each respective day. Nonparametric unpaired Student t test * $P < .05$, ** $P < .01$, $n = 8$. (D) Percent body weight loss of mice described in B. Statistical analysis represents comparison of ob⁻/ob⁻ vs ob⁻/ob⁻ mice + LGG on each respective day. $n = 8$. (E) Detection by immunofluorescent staining of TUNEL-positive cells within colonic tissues harvested from 6-wk-old WT or ob⁻/ob⁻ mice fed PBS or LGG at 24 hours following 12-Gy irradiation insult. (F) Quantification of TUNEL-positive cells in (c). Data were expressed as mean \pm standard error of the mean. One-way analysis of variance with post hoc Tukey multiple pairwise comparison. *** $P < .001$ and **** $P < 0.0001$, $n = 5$.

B6.BKS(D)-*Lepr^{db}/J* (referred to as LEP-R^{-/-}) (Jackson Laboratories, Bar Harbor, ME) and in B6Nox1ΔIEC¹⁶ mice. Animal procedures were approved by the Institutional Animal Care and Use Committee of Emory University.

Bacterial Strains and Culture Preparation

The following bacteria were purchased from the American Type Culture Collection (Manassas, VA): LGG American Type Culture Collection 53103. All media were propagated according to instructions provided by the American Type Culture Collection.

ELISA for the Detection of Leptin Levels in Murine Tissue and Serum

Leptin from colonic tissue of 6-week-old bacterial (2×10^8 CFU/mL) fed mice was measured by ELISA (RayBiotech Life Ltd, Norcross, GA).

Quantitative PCR for the Detection of Transcript Enrichment in Murine Tissue

RNA from colonic tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and cleaned using RNeasy kit (Qiagen, Hilden, Germany) total RNA clean up protocol. The total mRNA (1 μg) was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (cat # 205311, Qiagen). Relative quantification of mRNA expression was performed using QuantiFast SYBR Green PCR Kit (cat # 204054, Qiagen) on a MyiQ Real time PCR system (Biorad, Hercules, CA). Delta-delta Ct analysis ($\Delta\Delta$ CT) method was used to quantify relative gene expression compared with actin control subjects, using the following primers: Cyclin A2-F,5' GGCTGACACTCTTCCG -3', Cyclin A2-R,5' CTGGTAGCAAGAATTAGAGCA T -3', Cyclin B1-F,5' GCAAGCCCA AGGTATCGA -3', Cyclin B1,5' GACTTTAGATGCTCTACG GA -3', Cyclin D1-F,5' GCTGTGCTATGCTGGTATCT -3', Cyclin D1,5' AGGGCATCTGTAAATACACT -3', Cyclin E1-F,5' TGACCAGTTTGCTTATGTT -3', Cyclin E1,5' CCGTGTCGTTGACATAGG -3', leptin-F,5' GTAGCTGCGACAGTACTTGGT -3', leptin-R,5' AGTAA-GAGGCTTTGGCGTT -3', β -Actin-F,5'-AATGTGGCTGAG-GACTTTGT-3', β -Actin, 5'-GGGACTTCTGTAAACCACTTATT-3'. The data generated by quantitative PCR assays were normalized using the average value of the PBS treatment control group.

Murine Subjects and γ -Irradiation

C57BL/6 mice were purchased from Jackson Laboratory and maintained by the Emory University Department of Animal Resources. For irradiation insults, whole bodies were subjected to 12 Gy of γ -radiation using a γ -Cell 40 137Cs irradiator at a dose rate of 75 rad/min. Pure cultures of beneficial bacteria (1×10^8 CFU total) were administered by oral gavage daily for 3 days before irradiation, and body weights and mortality were monitored. Animal experiments were approved by the Emory University Institutional Ethical Committee and performed according to the legal requirements. Histologic sections of the colon were prepared from 5 irradiated animals per treatment. Sections were

assessed by in situ fluorescein TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay (Millipore, Burlington, MA). Immunofluorescent TUNEL-positive cells were counted and the average number of positive cells in 40 field views $\times 20$ magnification per treated animal was determined.

Detection of Ki67, Phospho-Histone H3, and JAK-STAT Signaling in Murine Colonic Tissue

For the detection of cell proliferation and JAK-STAT signaling in murine tissues, 6-week-old mice were fed 100 μL of 2×10^8 CFU/mL of bacteria for 4 hours. Frozen sections of the distal colonic tissue were stained by immunofluorescence using the anti-phospho-Histone H3 antibody, anti-phospho-STAT3, and anti-Ki67 antibodies (Cell Signaling, Danvers, MA) before detection of fluorescence by Confocal microscopy. At least 5 mice were included in each experimental point, and 40 field views at $\times 20$ magnification of each intestinal preparation were numerated for phosphor-Histone H3, Ki67, and phosphor Stat3 positive cells.

Colonoid and Enteroid Cultures

After washing the mouse colons and intestines (?15 cm of jejunum) 15 times with cold PBS without Ca²⁺/Mg²⁺, the isolated intestinal tissue was cut into approximately 3- to 5-mm pieces and incubated in Gentle Cell Dissociation Reagent (Stem Cell Technologies, Vancouver, Canada). After removal of the Gentle Cell Dissociation Reagent, and resuspension with PBS with 0.1% bovine serum albumin, the villi and mucus were removed with a 70-μm cell strainer (BD Biosciences, East Rutherford, NJ). After being centrifuged at 8°C at $290 \times g$ for 5 minutes, the collected crypts were resuspended in cold Dulbecco's modified Eagle medium/F12 with 15 mM HEPES and counted. A total of 150 enteroid or colonoid crypts were plated in each well in fresh Matrigel at a ratio of 1:1. Passage of enteroids or colonoids derived from single cells was performed every 7–8 days. Post-passaging, half of the colonoids were stimulated with 100 ng/mL of PEGylated leptin (Peptides International, Louisville, KY) added to media 3 times weekly. Light microscopy was performed on cultures 2 days postpassaging, with representative images selected after counting 6 images at $\times 100$ magnification from each group. Organoid counts were performed 2, 3, and 7 days prepassaging, and at 7 days postpassaging, with small spheres consisting of enclosed sphere structures, large spheres having a defined lumen, budding spheres having 1 bud from the main structure, and enteroids or colonoids having multiple buds.

Preparation and Staining of 3-Dimensional Whole Mount Colonoids

Colonoids from 2 wells were resuspended with ice-cold sterile PBS and transferred to a 96 well v-bottomed plate. After being centrifuged at 8°C at $500 \times g$, the colonoids were gently washed with PBS and then fixed in acetone for

10 minutes. After washing a further 3 times with PBS, tissues were blocked for 30 minutes in 10% goat serum in working buffer (PBS with 1% bovine serum albumin and 0.1% Tween 20). Colonoids were then incubated in primary antibodies for 1 hour in working buffer: phospho-Histone H3 (Cell Signaling Technologies) and β -catenin (BD Biosciences), washed 3 times in PBS. Colonoids were then incubated in secondary antibodies (AlexaFluor-conjugated [Molecular Probes, Eugene, OR]) for 1 hour; DAPI was added at 1:10,000 for the final 5 minutes. Colonoids were then washed 3 times in PBS and mounted into an 8-chamber Ibidi slide (Ibidi, Martinsried, Germany) in Prolong Diamond (ThermoFischer Scientific, Waltham, MA) and cured overnight before imaging. Two-dimensional and 3-dimensional images were taken by confocal microscopy and Z-stack; postimaging the videos were produced in Imaris software (Bitplane, Belfast, UK).

Statistical Analysis

Data were evaluated using GraphPad Prism version 7.0 (San Diego, CA). Groups were compared using either Student unpaired *t* test or analysis of variance, as appropriate. Unless otherwise specified, data are presented as mean \pm standard error of the mean. Where multiple comparisons were made, post hoc testing used Bonferroni or Turkey correction. Log-rank (Mantel-Cox) test was used to plot Kaplan-Meier survival curves to compare diabetes-free survival. A *P* value of $\leq .05$ was considered significant.

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Correspondence

Address correspondence to: Rheinallt M. Jones, PhD, Division of Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics,

Emory University School of Medicine, 615 Michael Street, Atlanta, Georgia 30322. e-mail: rjones5@emory.edu; fax: (404) 727-8538.

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Rheinallt M. Jones and Trevor M. Darby conceived and designed the experiments. Trevor M. Darby and Liping Luo performed experiments on the murine animal model. Crystal R. Naudin performed experiments on enteroids. Rheinallt M. Jones and Trevor M. Darby wrote the manuscript.

Conflicts of interest

The authors disclose no conflicts.

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