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EXTRA VIEW

NLRC3 regulates cellular proliferation and apoptosis to attenuate the development of colorectal cancer

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ABSTRACT

Nucleotide-binding domain, leucine-rich-repeat–containing proteins (NLRs) are intracellular innate immune sensors of pathogen-associated and damage-associated molecular patterns. NLRs regulate diverse biologic processes such as inflammatory responses, cell proliferation and death, and gut microbiota to attenuate tumorigenesis. In a recent publication in *Nature*, we identified NLRC3 as a negative regulator of PI3K–mTOR signaling and characterized its potential tumor suppressor function. Enterocytes lacking NLRC3 cannot control cellular proliferation because they are unable to suppress activation of PI3K–mTOR signaling pathways. In this Extra-View, we explore possible mechanisms through which NLRC3 regulates cellular proliferation and cell death. Besides interacting with PI3K, NLRC3 associates with TRAF6 and mTOR, confirming our recent finding that NLRC3 negatively regulates the PI3K–mTOR axis. Herein, we show that NLRC3 suppresses c-Myc expression and activation of PI3K–AKT targets FoxO3a and FoxO1 in the colon of *Nlrc3*$^{-/-}$ mice, suggesting that additional signaling pathways contribute to increased cellular proliferation. Moreover, NLRC3 suppresses colorectal tumorigenesis by promoting cellular apoptosis. Genes encoding intestinal stem cell markers BMI1 and OLFM4 are upregulated in the colon of *Nlrc3*$^{-/-}$ mice. Herein, we discuss recent findings and explore mechanisms through which NLRC3 regulates PI3K–mTOR signaling. Our studies highlight the therapeutic potential of modulating NLRC3 to prevent and treat cancer.

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Introduction

Nucleotide-binding domain (NOD), leucine-rich-repeat (LRR)–containing proteins (NLRs) are a large family of cytoplasmic sensors that regulate an extraordinarily diverse range of biologic functions. Deregulation of the functional activity of NLRs leads to the development of inflammatory diseases, autoimmunity and reproductive diseases.[1,2] Certain NLRs play a role in the initiation of inflammation, whereas others mediate the suppression of inflammation. Therefore, the coordinated activation and suppression of NLRs is essential to overcome infection and physiologic aberration and to restore homeostasis. NLRP3, NLRP6, NLRP12, NLRC4, and the DNA sensor AIM2 have central roles in preventing intestinal inflammation and colorectal cancer (CRC).[3-11]

NLRC3 (also known as CLR16.2 or NOD3) is a poorly characterized member of the NLR family.[1] It was identified in a genomic screen of genes encoding proteins bearing LRRs and nucleotide-binding domains.[12] NLRC3 consists of an N-terminal caspase activation and recruitment domain, a central nucleotide-binding domain, and a C-terminal LRR domain.[13,14] NLRC3 is highly expressed in human and mouse immune cells.[14] Luciferase reporter assays show that overexpression of NLRC3 in 293T and Jurkat T cells impairs the activation of NF-κB.[14] NLRC3 functions as a negative regulator of NF-κB activation downstream of Toll-like receptors (TLRs)[13] and type I interferon production downstream of the stimulator of interferon genes (STING), a DNA sensor.[15] A meta-analysis recently identified a potential link between NLRC3 and cancer.[16] An analysis of 10 databases revealed lower expression of the gene encoding NLRC3 in tumors of patients with CRC than of healthy controls,[16] highlighting a potential role for NLRC3 in the development of CRC. Our recent study shows that NLRC3 is critical in restricting tumorigenesis in both a colitis-associated CRC model and the *Apc*$^{Min/+}$ model, a spontaneous mouse model of intestinal cancer. Further, enterocytes lacking NLRC3 cannot control cellular proliferation because of a failure in suppressing the activation of phosphatidylinositol-3-kinase (PI3K) – mechanistic target of rapamycin (mTOR) signaling axis.[17] NLRC3 inhibits association of the PI3K p85 subunit with the PI3K p110α catalytic subunit, thereby preventing the activation of PI3K and its downstream targets PDK1, AKT, and mTOR.[17] Our new finding of the direct association of NLRC3 with TRAF6 and mTOR provides an additional molecular mechanism by which NLRC3 regulates mTOR modulation. Apart from mTOR activation, NLRC3 also regulated cellular proliferation by suppressing c-Myc expression and reducing the activation of PI3K–AKT targets, the forkhead box O (FoxO) proteins FoxO3a and FoxO1. Increased expression of genes encoding

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BMI1 and OLFM4 in the colon of mice lacking NLRC3 suggests that NLRC3 regulates proliferative signaling pathways involved in stem cell proliferation, maintenance, and differentiation. Moreover, the reduced activation of caspase-8, caspase-7 and caspase-3 in colon of *Nlrc3*$^{-/-}$ mice suggests that the tumor suppressor function of NLRC3 is also attributed to its ability to promote apoptosis. From the clinical standpoint, understanding the complex network through which NLRC3 promotes intestinal homeostasis can lead to the development of new therapeutic approaches to prevent and treat cancer.

Results and discussion

NLRC3 sequesters TRAF6 to modulate mTOR signaling

In our recent study, we reported that deletion of NLRC3 results in the co-localization of mTOR with lysosomal-associated membrane protein 1, leading to auto-activation of mTOR, which subsequently targets S6 and 4E-BP1.[17] The interaction of NLRC3 with PI3K subunits, but not with AKT and PDK1, inhibits activation of the PI3K–AKT–mTOR axis.[17] NLRC3 contains binding sites for tumor necrosis factor receptor–associated factors (TRAFs) within the nucleotide-binding domain, which allow its association with the TLR signaling molecule TRAF6.[13] Association with NLRC3 leads to auto-ubiquitylation and degradation of TRAF6, thereby tempering TLR-dependent activation of the NF-κB signaling pathway.[13] Similarly, the interaction of TRAF6 with p62 is required for translocation of mTOR to the lysosome for its subsequent activation.[18] One possibility is that NLRC3 regulates mTOR activation by directly associating with TRAF6 and mTOR. To investigate this, we overexpressed NLRC3, TRAF6, and mTOR in 293T cells and co-immunoprecipitated with TRAF6. We observed that NLRC3, TRAF6, and mTOR were in the same complex (Fig. 1a). Consistent with this, NLRC3-based immunoprecipitation also demonstrated the interaction of NLRC3 with mTOR, further establishing that NLRC3 plays a role in the mTOR signaling pathway (Fig. 1b). Given that NLRC3 interacts with TRAF6 and leads to its degradation,[13] loss of NLRC3 provides the TRAF6–p62 complex a platform to translocate mTOR to the lysosome for its subsequent activation. In line with our recent observation that loss of NLRC3 results in higher cellular proliferation owing to increased activation of mTOR signaling,[17] another study reported that loss of TRAF6 impairs division in cancer cells[18] due to defective activation of mTOR.

NLRC3 does not regulate inflammasome activation

Certain members of the NLR family can form inflammasome complexes.[19] Inflammasome-mediated caspase-1 activation during dextran sulfate sodium (DSS)–induced colitis is protective against colitis and colitis-associated tumorigenesis, particularly through the production of IL-18.[3,4,9,20] However, we found that caspase-1 was similarly activated in wild-type (WT) and *Nlrc3*$^{-/-}$ mice (Fig. 2a), which is in line with our previous finding that IL-18 production is similar in colons of WT and *Nlrc3*$^{-/-}$ mice 14 d after azoxymethane (AOM) injection.[17]

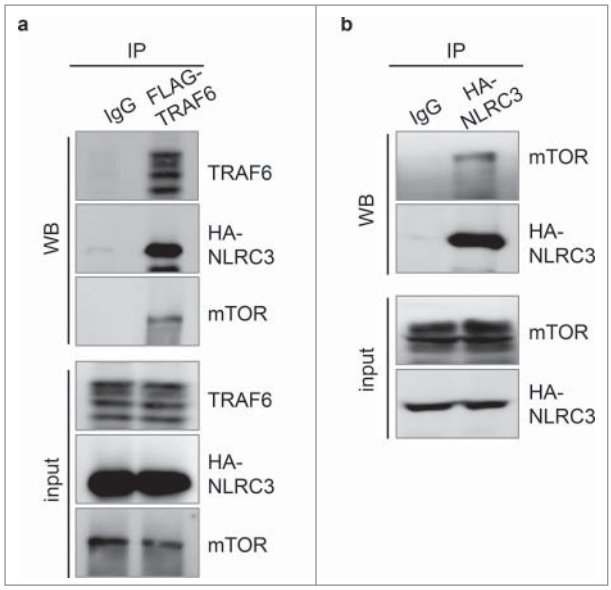

Figure 1. NLRC3 sequesters mTOR-TRAF6 complex. (A) Human embryonic kidney (HEK) 293T cells were transfected with the FLAG-TRAF6, HA-NLRC3, and mTOR plasmids. TRAF6 immunoprecipitates were analyzed for TRAF6, HA, and mTOR expression by western blot. (B) 293T cells were transfected with HA-NLRC3 and mTOR plasmids. HA immunoprecipitates were analyzed for mTOR and HA expression. Data represent 1 experiment representative of 2 independent experiments.

NLRC3 regulates cellular proliferation and apoptosis

Alterations in the physiologic equilibrium between epithelial proliferation and apoptosis in the colonic mucosa are associated with increased risk of CRC. The loss of equilibrium occurs in the very early phases of tumorigenesis.[21] Since alteration in the signaling molecules associated with epithelial proliferation and apoptosis in response to AOM/DSS peaks in colitic phase, which happens 14 d after AOM injection,[17] we used this time point for further analyses. Mice lacking NLRC3 have increased numbers of Ki67^{+} and PCNA^{+} cells in the intestinal crypt, suggesting increased proliferation of the colonic epithelium.[17] Multiple signaling pathways can regulate cellular proliferation. Apart from the regulation of mTOR targets, negative regulation by NLRC3 at the PI3K–AKT–mTOR axis can affect the expression or regulation of various molecules critical in cellular proliferation. Emerging evidences suggest that PI3K–AKT–mTOR signaling regulates transcriptional activity of c-Myc,[22,23] which is frequently deregulated in various cancers, including gastrointestinal cancers.[24] We found that mice lacking NLRC3 had increased expression of the oncoprotein c-Myc in the colon 14 d after AOM injection (Fig. 2b). c-Myc is required for the transcription of many cell cycle–related proto-oncogenes such as cyclin D1,[25] and cyclin D1 is overexpressed in various human cancers, including colon cancer.[26] We identified cyclin D1^{+} cells in the intestinal epithelium by immunohistochemical analysis. Before exposure to AOM and DSS, numbers of cyclin D1^{+} cells were similar in the intestinal epithelium of WT mice and *Nlrc3*$^{-/-}$ mice (Fig. 2c). However, 14 d after AOM injection, numbers of cyclin D1^{+} cells per intestinal crypt were significantly higher in *Nlrc3*$^{-/-}$ mice than in WT mice (Fig. 2c).

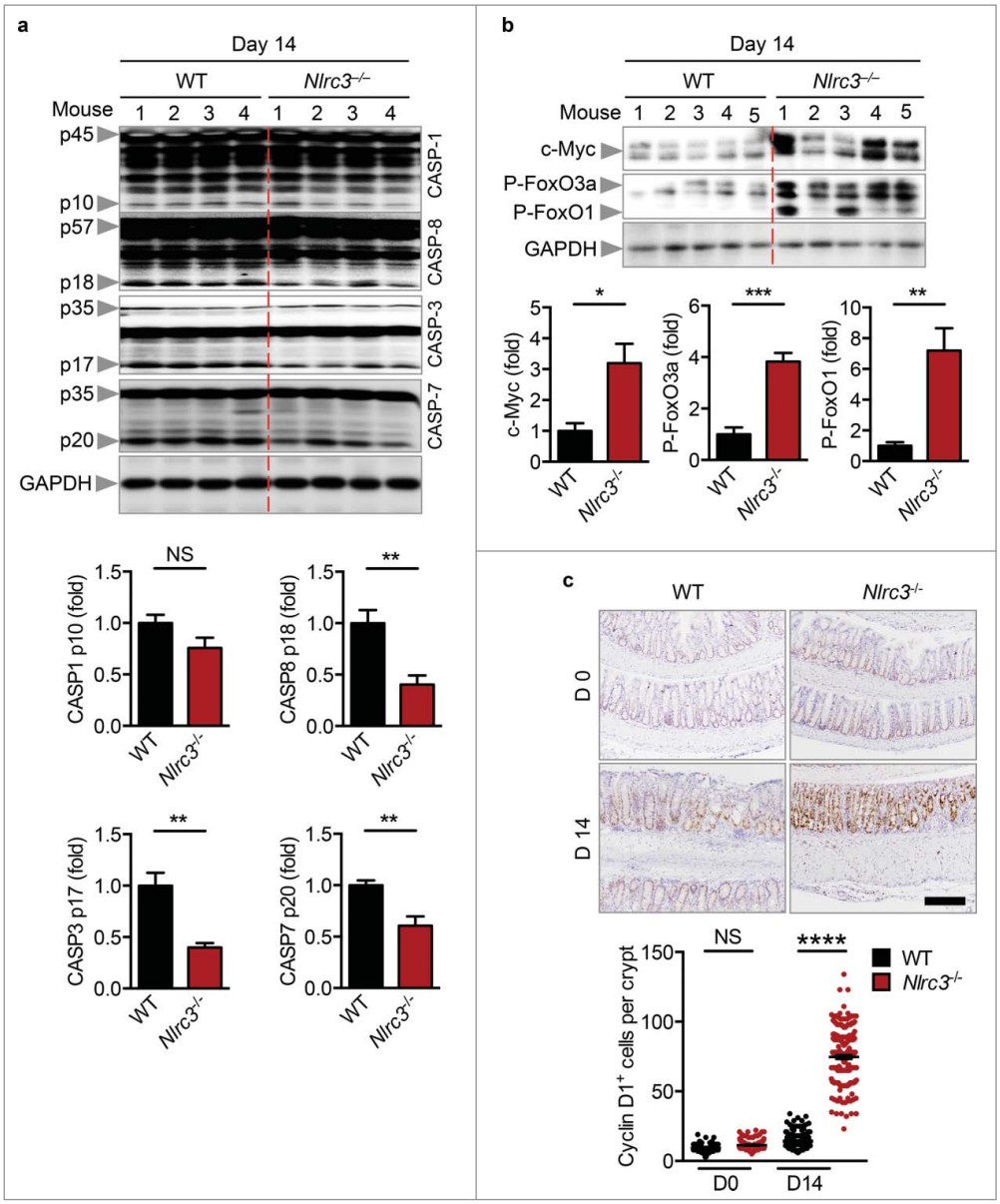

Figure 2. NLRC3 regulates cellular proliferation and apoptosis. (A) Immunoblot analysis of caspase-1 (CASP-1), caspase-8 (CASP-8), caspase-3 (CASP-3), and caspase-7 (CASP-7) activation and GAPDH (loading control) in the colon of WT and *Nlrc3*$^{-/-}$ mice 14 d after AOM injection. (B) Immunoblot analysis of c-Myc, phosphorylated FoxO3a and FoxO1 (P-FoxO3a and P-FoxO1), and GAPDH (loading control) in the colon of WT and *Nlrc3*$^{-/-}$ mice 14 d after AOM injection. Protein band intensity was normalized to the loading control and expressed relative to that of the WT, set at 1. (C) Images and quantification of the number of cyclin D1^{+} cells in each crypt of WT (day 0, $n = 5$; day 14, $n = 8$) and *Nlrc3*$^{-/-}$ (day 0, $n = 5$; day 14, $n = 8$) mice (left). Scale bar, 200 μm (C). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; NS, not statistically significant [2-tailed *t*-test (B and C)]. Data are from 1 experiment representative of 2 (mean and s.e.m. in A and C).

Besides activating mTOR, the PI3K–AKT signaling axis phosphorylates FoxO proteins FoxO3a and FoxO1, which are considered bona fide tumor suppressors because they regulate cell proliferation, apoptosis, metabolism, and survival.[27] Deregulation of genes encoding FoxO3a and FoxO1 is associated with the progression of several types of tumors, including CRC.[28-30] AKT-mediated phosphorylation leads to sequestration of FoxO3a and FoxO1 in the cytosol in an inactive state and ubiquitin-mediated degradation.[31] Because NLRC3 regulates the PI3K–AKT signaling axis, we determined the phosphorylation status of FoxO3a and FoxO1 in colons of WT and *Nlrc3*$^{-/-}$ mice 14 d after AOM injection. Consistent with increased activation of AKT, we observed higher phosphorylation of FoxO3a and FoxO1 in the colon of *Nlrc3*$^{-/-}$ mice than of WT mice (Fig. 2b). These results support that FoxO3a and FoxO1 are potential targets of NLRC3 for regulation of tumorigenesis.

Reduced apoptosis can also contribute to increased tumor burden. Therefore, we determined caspase-8, caspase-3, and caspase-7 activation in colons of WT and *Nlrc3*$^{-/-}$ mice 14 d after AOM injection. Caspase-8, caspase-3, and caspase-7 activation was lower in the colon of *Nlrc3*$^{-/-}$ mice than of WT mice (Fig. 2a), suggesting that reduced apoptosis during the early phase in the absence of NLRC3 contributes to increased tumorigenesis. However, the mechanism by which NLRC3 promotes cell death remains to be determined.

NLRC3 regulates colonic stem cells and sensitivity to tumorigenesis

Stem cells are considered to be the cells of origin of CRC because they can undergo self-renewal, unlimited proliferation, and differentiation.[32] Several markers, such as LRR-containing G-protein–coupled receptor 5 (LGR5), sex-determining region y-box 9 (SOX9), olfactomedin-4 (OLFM4), and Musashi1 (MSI1), have been used to identify stem cells and quantify their stemness.[32,33] Colonic stem cells harvested from mice lacking NLRC3 develop more readily into organoids than do those isolated from WT mice.[17] Expression of genes encoding stem cell markers LGR5,[17] BMI1, MSI1, OLFM4, and SOX9 was similar in colon tissues from WT and *Nlrc3*$^{-/-}$ mice (Fig. 3) before AOM injection, suggesting that the increased number and size of intestinal organoids derived from the colonic epithelium of *Nlrc3*$^{-/-}$ mice were because of increased colony-forming capacity rather than differences in the numbers of starting intestinal stem cells. There was no change in the expression of *Lgr5, Msi1*, and *Sox9* in the colons of WT and *Nlrc3*$^{-/-}$ mice 14 d after AOM injection (ref. 17 and Fig. 3). However, the expression of genes encoding BMI1 and OLFM4 was significantly higher in the colon of *Nlrc3*$^{-/-}$ mice than in WT mice 14 d after AOM injection (Fig. 3). Aberrant expression of BMI1 has been associated with increased risk of colon cancer, and BMI1 inhibition by small molecules reduces tumor burden in primary human colorectal tumor xenograft models.[34] Unlike Lgr5^{+} cells, the Bmi1^{+} population is relatively quiescent, radiation resistant, and can regenerate in response to injury or ablation of Lgr5-expressing cells.[35] Given that the PI3K–AKT signaling axis mediates the phosphorylation of BMI1 and enhances its ability to promote prostate carcinogenesis,[36] it is possible that NLRC3 suppresses PI3K-AKT–mediated BMI1 phosphorylation to inhibit colorectal tumorigenesis.

Similarly, expression of the gene encoding OLFM4 is upregulated in human inflammatory bowel disease[37] and early-stage colon cancer.[38] Transcription of the gene encoding OLFM4 is regulated by NF-kB,[39] Notch,[40] and PU.1.[41] The negative regulatory role of NLRC3 in NF-κB signaling downstream of TLRs can at least explain the increased expression of *Olfm4* in the colon of *Nlrc3*$^{-/-}$ mice. Whether NLRC3 also regulates Notch and PU.1 remains to be established. Moreover, inhibition of PI3K activation is sufficient to suppress *OLFM4* expression,[39] which could explain decreased *Olfm4* expression in the colon of *Nlrc3*$^{-/-}$ mice. OLFM4 interacts with NLRC1 and NLRC2,[42] which are intracellular bacterial sensors associated with Crohn disease.[43,44] Future studies are needed to investigate the interaction between NLRC3 and OLFM4.

Since activation of the PI3K–AKT pathway positively correlates with upregulation of *Bmi1* and *Olfm4* expression in the colon of *Nlrc3*$^{-/-}$ mice, studying the post-translational modifications in BMI1 and OLFM4 is likely to provide mechanistic insights into how their oncogenic potential can be manipulated.

Colorectal cancer in Nlrc3$^{-/-}$ mice is not driven by microbiota

The human gut is colonized by a diverse microbial population that includes bacteria, fungi and viruses,[45] which play a pivotal role in maintaining homeostasis in the gut. Breakdown of the homeostasis by dysbiosis or deregulation of immune responses results in various pathological conditions such as inflammatory bowel diseases and CRC.[46] To reduce

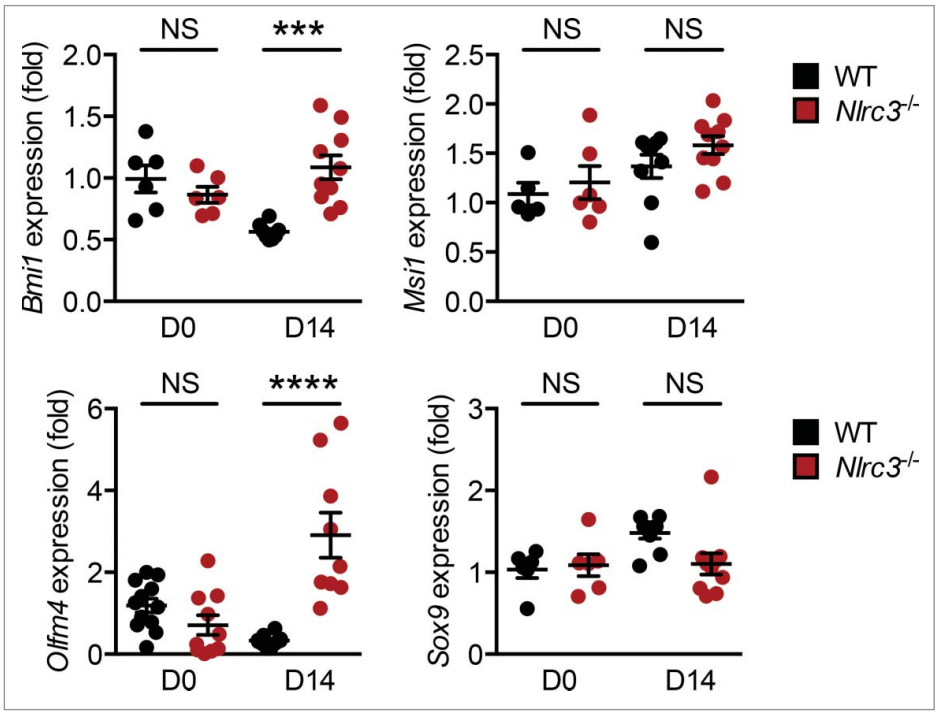

Figure 3. NLRC3 regulates stem cell markers. Relative expression of genes encoding BMI1, MSI1, OLFM4, and SOX9 in colon tissues of untreated WT and *Nlrc3*$^{-/-}$ mice or in WT and *Nlrc3*$^{-/-}$ mice 14 d after AOM injection. Each symbol represents 1 mouse. $^{***}P < 0.001$; $^{****}P < 0.0001$; NS, not statistically significant (2-tailed *t*-test). Data represent 2 independent experiments (mean and s.e.m.).

the occurrence of such pathological conditions, the gut has several innate immune sensors belonging to the NLR family that are critical for modulating microbial ecology to protect against tumor development.[47] Intestinal cell proliferation and progression of CRC is regulated by the composition of gut microbiota. To investigate whether an altered gut microbiota increases the susceptibility of *Nlrc3*$^{-/-}$ mice to CRC, we analyzed the levels of major bacterial species of gut microbiota in stool samples of separately housed (single housed) WT, *Nlrc3*$^{+/-}$, and *Nlrc3*$^{-/-}$ mice. Real-time quantitative PCR of 11 major bacterial populations revealed that WT, *Nlrc3*$^{+/-}$, and *Nlrc3*$^{-/-}$ mice harbored similar levels of *Bacteroides, Lactobacillus*, mouse intestinal *Bacteroides* (MIB), Prevotellaceae, *Prevotella, Paraprevotella, Bifidobacterium, Eubacterium rectale, Clostridium*, and *Akkermansia*. Compared with WT mice, *Nlrc3*$^{-/-}$ mice had elevated levels of segmented filamentous bacteria (SFB) (Fig. 4a), which are associated with IL-17– and IL-22–mediated chronic inflammation.[48] Interestingly, co-housing equilibrated the relative abundance of SFB in WT and *Nlrc3*$^{-/-}$ mice (Fig. 4b). However, *Nlrc3*$^{-/-}$ mice co-housed with WT mice were as susceptible to tumorigenesis as were separately housed *Nlrc3*$^{-/-}$ mice.[17] These findings suggest that differences in these bacterial populations do not contribute to the protective role of NLRC3 during the development of CRC.

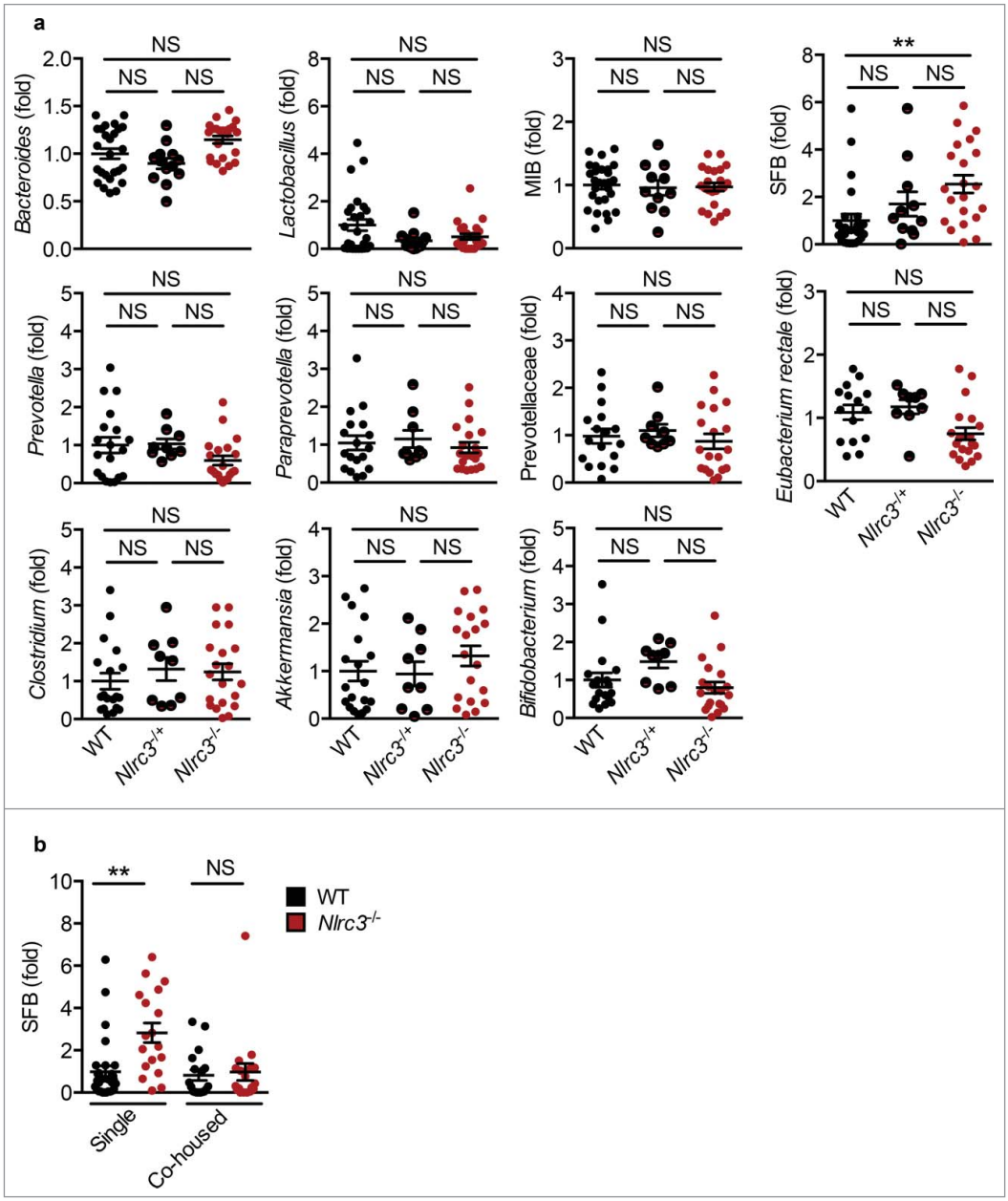

Figure 4. Landscape of gut microbiota in *Nlrc3*$^{-/-}$ mice. (A) Levels of *Bacteroides, Lactobacillus*, mouse intestinal *Bacteroides* (MIB), segmented filamentous bacteria (SFB), *Prevotella, Paraprevotella*, Prevotellaceae, *Eubacterium rectale, Clostridium, Akkermansia*, and *Bifidobacterium* in untreated WT, *Nlrc3*$^{-/+}$ and *Nlrc3*$^{-/-}$ mice. (B) Levels of SFB in separately housed (single housed) WT and *Nlrc3*$^{-/-}$ mice and co-housed WT and *Nlrc3*$^{-/-}$ mice. The level of genes was expressed relative to that of the WT, set at 1. Each symbol represents 1 mouse. $^{**}P < 0.01$; NS, not statistically significant (2-tailed *t*-test). Data represent 2 independent experiments (mean and s.e.m.).

Future directions

Our current and previously published studies have established that NLRC3 negatively regulates PI3K–mTOR pathways, which integrate upstream signals from growth factors, nutrients, and cellular energy to regulate various biologic processes involved in cell metabolism, growth, proliferation, and survival (Fig. 5).[17,49] Still, the role of NLRC3 in various other signaling pathways that drive cellular proliferation remains to be explored. The Wnt signaling pathway, which has been well studied in enterocyte proliferation, activates mTOR,[50] suggesting a link between NLRC3 and the Wnt signaling pathway. However, the exact mechanism by which NLRC3 modulates Wnt signaling needs further investigation. Changes in metabolite levels can contribute to tumorigenesis.[51] Thus, it is possible that NLRC3 acts as a nutrient sensor for the mTOR signaling pathway. Metabolic sensors such as AMP-activated protein kinase act as tumor suppressors by regulating mTOR activity and thus inhibiting the translation of many proteins required for rapid cell growth.[52] Hence, it is possible that there is crosstalk between these 2 metabolic sensors to regulate mTOR activity and suppress tumorigenesis. NLRC3-interacting partners mTOR and TRAF6 inhibit autophagy, a catabolic machinery to generate nutrients and energy required for cellular activities upon nutrient starvation,[53,54] but the exact mechanism of this regulation remains to be elucidated. The role of NLRC3 is not confined to cellular proliferation. Given the contribution of mTOR signaling to metabolic diseases such as diabetes, obesity, and atherosclerosis, and other inflammatory disorders such as arthritis,[55,56] a protective role of NLRC3 can be speculated in the treatment and prevention of these diseases. Our study and those by others demonstrated that NLRC3 contributes to the negative regulation of inflammation.[13,15,17] Moreover, the expression of NLRC3 is substantially downregulated in the nasal mucosa of patients with the autoimmune disease Wegener granulomatosis compared with that in healthy individuals.[57] Unraveling the molecular and metabolic responses modulated by NLRC3 could provide precious insights into developing therapeutics for infectious disease, autoinflammation, and cancer.

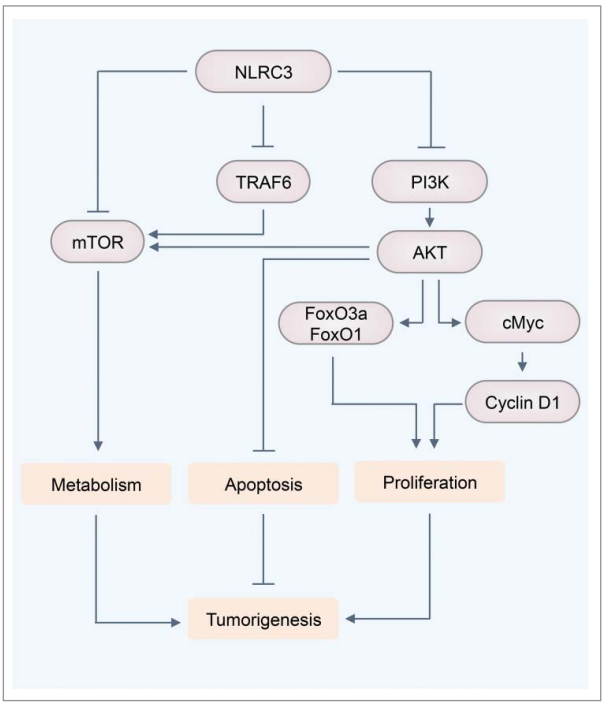

Figure 5. NLRC3 negatively regulates PI3K-mTOR signaling. NLRC3 interacts with PI3K to inhibit activation of downstream molecule AKT, which regulates mTOR and FoxO3a/O1 phosphorylation, and cMyc expression. NLRC3 interacts with mTOR or TRAF6 to regulate mTOR activity.

Methods

Cell culture

The embryonic kidney epithelial cell line HEK293T (ATCC#3216, American Type Culture Collection) was cultured in DMEM (11995073, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (TMS-013-B, Millipore) and 1% penicillin and streptomycin (15070–063, ThermoFisher Scientific). Cells were maintained at 37°C with 5% CO_2.

Mice

WT (C57BL/6) and *Nlrc3*$^{-/-}$ [17] mice were bred and maintained under specific pathogen-free conditions at St. Jude Children's Research Hospital (St. Jude), Memphis, TN. Animal study protocols were approved by the St. Jude Animal Care and Use Committee.

AOM-DSS model of colorectal tumorigenesis

Previously established protocols were followed to induce colitis-associated CRC in mice.[17]

Western blotting

Proteins from the colon were extracted using RIPA lysis buffer supplemented with proteinase and phosphatase inhibitors (Roche). Western blotting was performed as described previously.[58] Primary antibodies were caspase-1 p10 (1:500 dilution, sc-515, Santa Cruz Biotechnology), c-Myc (1:1,000, #5605, Cell Signaling Technology), caspase-3 (1:1,000, #9662, Cell Signaling Technology), cleaved caspase-3 (1:1,000, #9661, Cell Signaling), caspase-7 (1:1,000, #9492, Cell Signaling Technology), cleaved caspase-7 (1:1,000, #9491, Cell Signaling), caspase-8 (1:1,000, #9746, Cell Signaling Technology), cleaved caspase-8 (1:1,000, #8592, Cell Signaling Technology), Phospho-FoxO1 and FoxO3a (1:1,000, #9464, Cell Signaling Technology), mTOR (1:1,000, 2972, Cell Signaling Technology), TRAF6 (1:1,000, #8028, Cell Signaling Technology), and GAPDH (1:10,000; #5174, Cell Signaling Technology). Immunoblots were quantified using Image J.

Histology and microscopy

Colons were processed as described previously.[58] Cyclin D1 expression in the intestinal epithelium was detected by immunoperoxidase staining for cyclin D1 (#241R-15, Cell Marque). The number of cyclin D1^{+} cells per crypt in each animal was counted (at least 18–20 crypts per mouse).

Quantitative real-time PCR

RNA was isolated from the colon by using Trizol (15596026, ThermoFisher Scientific) and reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems). For PCR analysis of intestinal bacteria, fecal DNA was extracted as described previously.[59] Gene expression was assessed using the 2 × SYBR Green Master Mix (4368706, Applied Biosystems) according to the manufacturer's instructions. Sequences for qRT-PCR primers were as follows: *Bmi1*-F 5′-GTT CGA TGC ATT TCT GCT TG-3′; *Bmi1*-R 5-′TGG CTC GCA TTC ATT TTA TG-3′; *Sox9*-F 5′-TCC ACG AAG GGT CTC TTC TC-3′; *Sox9*-R 5′-AGG AAG CTG GCA GAC CAG TA-3′; *Msi1*-F 5′-AAT TCG GGG AAC TGG TAG GT-3′; *Msi1*-R 5′-GAT GCC TTC ATG CTG GGT AT-3′; *Olmf4*-F 5′-CAG CCA CTT TCC AAT TTC ACT G-3′; *Olmf4*-R 5′-GCT GGA CAT ACT CCT TCA CCT TA-3′; *β-actin*-F 5′-CAG CTT CTT TGC AGC TCC TT-3′, and *β-actin*-R 5′-CAC GAT GGA GGG GAA TAC AG-3′; *Eubacteria* (Universal)-F 5′-ACT CCT ACG GGA GGC AGC AGT-3′; *Eubacteria* (Universal)-R 5′-ATT ACC GCG GCT GCT GGC-3′; Prevotellaceae-F 5′-CCA GCC AAG TAG CGT GCA-3′; Prevotellaceae-R 5′-TGG ACC TTC CGT ATT ACC-3′; *Bacteroides*-F 5′-GGT TCT GAG AGG AGG TCC C-3′; *Bacteroides*-R 5′-GCT GCC TCC CGT AGG AGT-3′; MIB-F 5′-CCA GCA GCC GCG GTA ATA-3′; MIB-R 5′-CGC ATT CCG CT ACT TCT C-3′; *SFB*-F 5′-GAC GCT GAG GCA TGA GAG CT-3′; SFB-R 5′-GAC GGC ACG GAT TGT TAT TCA-3′; *Lactobacillus*-F 5′-GGA AAC AGA TGC TAA TAC CG-3′; *Lactobacillus*-R 5′-CAC CGC TAC ACA TGG AG-3′; *Prevotella*-F 5′-CAC GGT AAA CGA TGG ATG CC-3′; *Prevotella*-R 5′-GGT CGG GTT GCA GAC C-3′; *Paraprevotella*-F 5′-AGG GGC AGC ATG GAC CC-3′; *Paraprevotella*-R 5′-CCT TTC AGG AGA CTA TCC CGG A-3′; *Clostridium*-F 5′-CTC AAC TTG GGT GCT GCA TTT-3′; *Clostridium*-R ATT GTA GTA CGT GTG TAG CCC-5′; *Akkermansia*-F 5′-CAG CAC GTG AAG GTG GGG AC-3′; *Akkermansia*-R 5′-CCT TGC GGT TGG CTT CAG AT-3′; *Eubacterium rectale*-F 5′-GCT TCT TAG TCA GGT ACC GTC A-3′; *Eubacterium rectale*-R 5′-ACT CCT ACG GGA GGC AGC-3′; *Bifidobacterium*-F 5′-TCG CGT CYG GTG TGA AAG-3′; *Bifidobacterium*-R 5′-CCA CT CCA GCR TCC AC-3′. Levels of *Bmi1, Msi1, Olfm4*, and *Sox9* expression were normalized to *β-actin*. Levels of the 16S rRNA gene from each bacterial population were normalized to the 16S rRNA gene of *Eubacteria*.

Co-immunoprecipitation assays

Co-immunoprecipitation assays were performed as described previously.[17]

Statistical analyses

GraphPad Prism 6.0 software was used for data analysis. Data are shown as mean ± standard error of the mean (s.e.m.). Statistical significance was determined by *t* tests (2-tailed) for 2 groups or one-way ANOVA (with multiple comparisons tests) for 3 or more groups. $P < 0.05$ was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

R.K. and T.D.K. conceptualized the study; R.K. and R.K.S.M. designed the methodology; R.K., R.K.S.M., and Q.Z. performed the experiments; R.K., R.K.S.M., and Q.Z. conducted the analysis; R.K. and T.D.K. wrote the original draft of the manuscript; R.K.S.M. and Q.Z. reviewed and edited the manuscript; and T.D.K. provided the resources and supervised the study.

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