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Fusobacterium nucleatum Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating TLR4 Signaling to NFκB, Upregulating Expression of microRNA-21

Yongzhi Yang, MD and PhD[1,2,3,#], **Wenhao Weng, MD and PhD**[4,5,#], **Junjie Peng, MD and PhD**[1,2,#], **Leiming Hong**[3,#], **Lei Yang**[3], **Yuji Toiyama, MD and PhD**[6], **Renyuan Gao**[3], **Minfeng Liu**[3], **Mingming Yin**[3], **Cheng Pan**[3], **Hao Li**[3], **Bomin Guo, MD and PhD**[7], **Qingchao Zhu, MD and PhD**[7], **Qing Wei, MD and PhD**[8], **Mary-Pat Moyer, PhD**[9], **Ping Wang, PhD**[10], **Sanjun Cai, MD and PhD**[1,2,*], **Ajay Goel, PhD**[4,*], **Huanlong Qin, MD and PhD**[3,*], and **Yanlei Ma, MD and PhD**[1,2,*]

[1]Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, Shanghai, 200032, China

[2]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, 200032, China

[3]Department of GI Surgery, Shanghai Tenth People's Hospital Affiliated to Tongji University, Shanghai, China

[4]Center for Gastrointestinal Research, Center for Translational Genomics and Oncology, Baylor Scott &White Research Institute and Charles A. Sammons Cancer Center, Texas, USA

[5]Department of Clinical Laboratory, Shanghai Tenth People's Hospital Affiliated to Tongji University, Shanghai, China

[6]Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Mie, Japan

[*]**Co-corresponding author:** Yanlei Ma, Huanlong Qin, Ajay Goel, and Sanjun Cai. Yanlei Ma, MD, PhD, Department of Colorectal Surgery, Fudan University Shanghai Cancer Center; Department of Oncology, Shanghai Medical College, Fudan University, 270 Dong'an Road, Shanghai, 200032, China, Tel.: +86 21 64175590; Fax: +86 21 54175590, yanleima@live.cn.; Huanlong Qin, MD, PhD, Department of GI Surgery, Shanghai Tenth People's Hospital Affiliated to Tongji University, 301 Yanchang Road, Shanghai 200072, China. Tel: +86 21 66300588; Fax: +86 21 66303643 hl-qin@hotmail.com.; Ajay Goel, PhD, Center for Gastrointestinal Research, Center for Translational Genomics and Oncology, Baylor Scott &White Research Institute and Charles A. Sammons Cancer Center, 3410 Worth Street, Suite 610, Dallas, TX 75246, USA. Tel: 214-820-2603; Fax: 214-818-9292, Ajay.Goel@BSWHealth.org.; Sanjun Cai, MD, PhD, Department of Colorectal Surgery, Fudan University Shanghai Cancer Center; Department of Oncology, Shanghai Medical College, Fudan University, 270 Dong'an Road, Shanghai, 200032, China, Tel.: +86 21 64175590; Fax: +86 21 54175590, sanjuncai@hotmail.com.

[#]These authors contributed equally to this work.

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[7]Department of Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

[8]Department of Pathology, Shanghai Tenth People's Hospital Affiliated to Tongji University

[9]INCELL Corporation, San Antonio, Texas, USA

[10]Department of Central Laboratory, Shanghai Tenth People's Hospital of Tongji University, School of Life Science and Technology, Tongji University, Shanghai 200072, China

Abstract

Background & Aims—Nearly 20% of the global cancer burden can be linked to infectious agents. *Fusobacterium nucleatum* promotes tumor formation by epithelial cells via unclear mechanisms. We aimed to identify microRNAs (miRNAs) induced by *F nucleatum* and evaluate their ability to promote colorectal carcinogenesis in mice.

Methods—Colorectal cancer (CRC) cell lines were incubated with *F nucleatum* or control reagents and analyzed in proliferation and would healing assays. HCT116, HT29, LoVo, and SW480 CRC cell lines were incubated with F nucleatum or phosphate buffer saline (PBS control) and analyzed for miRNA expression patterns and in chromatin immunoprecipitation assays. Cells were incubated with miRNAs mimics, control sequences, or small interfering (si) RNAs; expression of reporter constructs was measured in luciferase assays. CRC cells were incubated with *F nucleatum* or PBS and injected into BALB/C nude mice; growth of xenograft tumors was measured. C57BL $APC^{min/+}$, C57BL $miR21a^{-/-}$, and C57BL mice with full-length miR21a (controls) were given *F nucleatum* by gavage; some mice were given azoxymethane (AOM) and dextran sodium sulfate (DSS) to induce colitis and colon tumors. Intestinal tissues were collected and tumors were counted. Serum samples from mice were analyzed for cytokine levels by ELISAs. We performed in situ hybridization analyses to detect enrichment of *F nucleatum* in CRC cells. *F nucleatum* DNA in 90 tumor and matched non-tumor tissues from patients in China were explored for the expression correlation analysis; levels in 125 tumor tissues from patients in Japan were compared with their survival times.

Results—*F nucleatum* increased proliferation and invasive activities of CRC cell lines, compared with control cells. CRC cell lines infected with *F nucleatum* formed larger tumors, more rapidly, in nude mice than uninfected cells. $APC^{min/+}$ mice gavaged with *F nucleatum* developed significantly more colorectal tumors than mice given PBS and had shorter survival times. We found several inflammatory factors to be significantly increased in serum from mice given *F nucleatum* (interleukin 17F [IL17F], IL21, IL22, and MIP3A). We found 50 miRNAs to be significantly upregulated and 52 miRNAs to be significantly downregulated in CRCs incubated with *F nucleatum* vs PBS; levels of miR21 increased by the greatest amount (more than 4-fold). Inhibitors of miR21 prevented *F nucleatum* from inducing cell proliferation and invasion in culture. $miR21a^{-/-}$ mice had a later appearance of fecal blood and diarrhea after administration of AOM and DSS, and had longer survival times, compared with control mice. The colorectum of $miR21a^{-/-}$ mice had fewer tumors, of smaller size, and the $miR21a^{-/-}$ mice survived longer than control mice. We found RASA1, which encodes a RAS GTPase, to be one of the target genes consistently downregulated in cells that overexpressed miR21 and upregulated in cells exposed to miR21 inhibitors. Infection of cells with *F nucleatum* increased expression of miR21 by activating

TLR4 signaling to MYD88, leading to activation of the nuclear factor NFκB. Levels of *F nucleatum* DNA and miR21 were increased in tumor tissues (and even more so in advanced tumor tissues), compared with non-tumor colon tissues from patients. Patients whose tumors had high amounts of *F nucleatum* DNA and miR21 had shorter survival times than patients whose tumors had lower amounts.

Conclusions—We found infection of CRC cells with *F nucleatum* to increase their proliferation, invasive activity, and ability to form xenograft tumors in mice. *F nucleatum* activates TLR4 signaling to MYD88, leading to activation of the nuclear factor NFκB and increased expression of miR21; this miRNA reduces levels of the RAS GTPase RASA1. Patients with both high amount of tissue *F nucleatum* DNA and miR21 demonstrated a higher risk for poor outcomes.

Keywords

microbe; signal transduction; gene regulation; carcinogenesis

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most frequent cause of death following lung, liver and stomach cancer[1]. Surprisingly, the incidence of CRC in Asian countries, which were previously considered to have lower rates, has increased dramatically during past decade[2]. Unfortunately, the underlying mechanism(s) for the initiation and development of this global malignancy have not been yet fully elucidated.

It has been estimated that approximately 20% of the global cancer burden can be linked to infectious agents[3]. Human papillomaviruses and helicobacter pylori, for example, are well-known pathogens that cause cervical and gastric cancer. Considering almost 1.5 kilograms of microbiota inhabit the human gut, it is not surprising that certain viruses and/or bacteria might be related to human cancers[4]. However, the carcinogenic pathogens which cause CRC remain incompletely explored[5].

A growing body of evidence suggests a potential link between the *Fusobacterium nucleatum* (*F nucleatum*) and colorectal carcinogenesis. Several studies have demonstrated that *F nucleatum* is enriched in human colorectal adenomas and carcinomas compared with adjacent normal tissue[6, 7]. Furthermore, a higher amount of tissue *F nucleatum* DNA has been indicated to be associated with advanced tumor stage and poor prognosis in CRC[8–10]. Recent studies showed *F nucleatum* adheres to and invades endothelial and epithelial cells via its virulence factors such as adhesin A (FadA), fusobacterium autotransporter protein 2 (Fap2) and fusobacterial outer membrane protein A (FomA)[11–14]. Nevertheless, few studies have by far revealed downstream events of *F nucleatum* infection that can trigger colonic inflammation and carcinogenesis.

Our previous study demonstrated that microRNA21 (miR21) plays a pathogenic role in chronic inflammatory processes and the development of colitis-associated colon cancer[15]. Furthermore, data from our group and others have highlighted that specific miRNAs contribute to colorectal carcinogenesis, and several of these can serve as biomarkers for

diagnosis, prognosis, and metastasis prediction in CRC patients[15–20]. Therefore, we hypothesized that *F nucleatum* infection may regulate several cancer-specific miRNAs to promote the development of CRC. To the best of our knowledge, the impact of *F nucleatum*-infection and its impact on downstream effects on miRNA regulation have not been interrogated to date.

In the present study, we aimed to identify *F nucleatum*-induced miRNAs, decipher the mechanism(s) for the regulation of miRNAs by *F nucleatum*, and demonstrate its biological and clinical significance in CRC. Our study attempts to use miRNA regulation as novel angle to clarify the carcinogenic role of *F nucleatum* in CRC, and provide a novel evidence that *F nucleatum* could serve as a potential prognostic and therapeutic target in patients with this malignancy.

MATERIALS AND METHODS

Experimental methods (including statistical analysis) are described in detail in the Supplementary Information.

RESULTS

F nucleatum promotes CRC cell proliferation and invasion in cell lines and a xenograft animal model

Compared to the untreated cells or those treated with *E coli* DH5α, *F nucleatum* significantly promoted cell growth in HCT116 and LoVo cells after treatment at 48 hr ($P < 0.05$ and $P < 0.001$ respectively, Figure 1A and Supplementary Figure 1A). Consistently, we observed *F nucleatum* treatment significantly induced S-phase accumulation in LoVo cells ($P < 0.01$) but less marked increase in HCT116 cells (Figure 1B and Supplementary Figure 1B). Furthermore, *F nucleatum* treatment remarkably enhanced cell invasion in both HCT116 and LoVo cells ($P < 0.01$ in both cell lines after *F nucleatum* treatment at 48 hr, Figure 1C and Supplementary Figure 2).

To confirm these *in vitro* results, we subcutaneously injected *F nucleatum*-treated HCT116 cells into male BALB/C nude mice. In concordance with our previous findings, *F nucleatum* treated cells reported an accelerated tumor growth and heavier tumor weight compared to untreated or *DH5α* treated cells ($P < 0.01$). Moreover, we observed enhanced staining for the proliferation marker Ki-67 in *F nucleatum*-treated xenograft tissues (Figure 1D). Similarly, we generated a xenograft model derived from *F nucleatum*-treated LoVo cells and successfully validated above results (Supplementary Figure 3). However, *F nucleatum* treatment did not affect cell apoptosis in both HCT116 and LoVo cells (Supplementary Figure 4). Together, our results highlight that *F nucleatum* exerts oncogenic function in CRC.

F nucleatum promotes tumorigenicity in $APC^{Min/+}$ mouse

The classic Vogelgram multistep model for CRC indicates tumor suppressor gene, adenomatous polyposis coli (APC) gene acts as a gatekeeper and alterations in the APC/Wnt-signaling trigger adenoma–carcinoma sequence[21]. The $APC^{Min/+}$ mice carry APC

mutations, which demonstrates a consequent predisposition to multiple intestinal neoplasia[22]. Therefore, we hypothesized that chronic *F nucleatum* infection in $APC^{Min/+}$ mice may induce synergistically oncogenic effect by enhancing the aberrant epithelial cell growth, and consequently leading to the initiation of CRC.

To confirm our hypothesis, we administrated *F nucleatum* or phosphate buffer saline (PBS as negative control, NC) into 5–6 weeks old $APC^{Min/+}$ mice, which received antibiotics by gavage administration for 3 days beforehand (Figure 2A). Surprisingly, 20 weeks after *F nucleatum* treatment, $APC^{Min/+}$ mice presented serious complications including ascites (60% versus 30%, $P = 0.178$), bloody diarrhea (80% versus 20%, $P = 0.0246$), gut dilatation (60% versus 30%, $P = 0.178$) and splenomegaly (80% versus 40%, $P = 0.0679$) when compared with the PBS group (Figure 2B). Notably, we further found that the colorectum showed significant increase in tumor numbers (3.10 ± 3.38 versus 0.67 ± 1.00, $P = 0.042$), tumor size (7% versus 0% for tumors with size > 3mm) and tumor load (6.40 ± 6.64 versus 1.27 ± 1.90, $P = 0.0161$; Figure 2C). The small intestine demonstrated mild increase in the tumor numbers, size and tumor load as well (Sumpplementary Figure 5). In particular, *F nucleatum*-treated $APC^{Min/+}$ mice had a shorter survival period compared to control group (median survival of 164.5 days for *F nucleatum*-treated mice and 200 days for NC mice, P=0.0129, Figure 2D). In addition, we found colorectal tumors from *F nucleatum* treated mice showed high expression levels of proliferative marker PCNA compared to negative control, suggesting that *F nucleatum* could effectively stimulate cell proliferation (Figure 2E).

Accumulating evidence demonstrates that inflammatory cells secrete a variety of pro-inflammatory or growth factors during tumorigenesis and these factors contribute to cancer develpoment[23]. To examine whether these factors could also be induced by *F nucleatum* infection, we measured serum levels of a panel of inflammatory factors. Interestingly, we found several inflammatory factors were significantly up-regulated in *F nucleatum*-treated mice including IL17F ($P = 0.0022$), IL21 ($P = 0.0152$), IL22 ($P = 0.0411$) and MIP3a ($P = 0.0087$, Figure 2F). Collectively, our results suggest that *F nucleatum* could stimulate immune response, increase serum inflammatory factors and promote tumorigenicity in $APC^{Min/+}$ mice.

MiR21 is a downstream target of F nucleatum

Accumulating evidence have shown deregulation of miRNA expression in CRC[24]. Given the importance role of miRNAs in cell homeostasis and colorectal carcinogenesis, it is no surprise that *F nucleatum* might take advantage of miRNA-mediated regulation of gene expression to promote tumorigenesis.

To determine whether *F nucleatum* affected miRNA expression, we first treated HCT116, HT29, SW480 and LoVo cells with *F nucleatum* or PBS and subsequently performed microarray analysis to identify differentially expressed miRNAs. Impressively, among the 657 miRNAs, 50 miRNAs were significantly up-regulated ($P < 0.05$) while 52 miRNAs were significantly down-regulated ($P < 0.05$, Figure 3A and Supplementary Table 1). To obtain more accurate results, we used several criteria (signal intensity ≥ 500, $P < 0.001$ and $\log_2$ Fold change ≥ 2) to screen robust miRNA candidates. Only 6 miRNAs (let-7f-5p,

let-7i-5p, miR-23a-3p, let-7a-5p, miR-23b-3p, and miR-21-5p) were found to be dramatically over-expressed. We further performed independent experiments, in which we treated multiple CRC cell lines with *F nucleatum*, to validate our microarray results (Figure 3A and Supplementary Figure 6). Strikingly, miR21 consistently showed to be the most up-regulated miRNA induced by *F nucleatum* infection, with a nearly ≥ 4-fold increase, compared to other miRNAs, suggesting miR21 is probably a direct target of *F nucleatum*.

To determine whether *F nucleatum* exerts oncogenic function in a miR21 dependent manner, we performed miR21 loss of function assays. Since HCT116 has the highest level of miR21 (Supplementary Figure 7A), we treated HCT116 with miR21 inhibitors alone or together with *F nucleatum*. As expected, *F nucleatum* treatment significantly promoted tumor growth and cell invasion, while inhibition of miR21 showed remarkable tumor suppressive effects (Figure 3B–3C). However, when we treated cell lines with both *F nucleatum* and miR21 inhibitors, inhibition of miR21 completely abolished *F nucleatum*'s effect on cell growth and invasion, highlighting the important role of miR21 in sustaining *F nucleatum*'s functions (Figure 3B–3C).

To further confirm our assumption that *F nucleatum* promotes colorectal progression through miR21, we next employed the miR21a knockout (KO, miR21a$^{-/-}$) mice (Figure 3D). Azoxymethane (AOM) is a recognized procarcinogen with organotropism for the colon and AOM induced tumorigenesis is significantly enhanced by the inflammatory agent dextran sodium sulfate (DSS). Thus, both miR21a wild-type (WT) and KO mice were initially administrated *F nucleatum* and then subjected to AOM/DSS treatment. WT mice showed an earlier appearance of fecal blood and diarrhea than miR21a$^{-/-}$ mice (Figure 3E). As shown in Figures 3F, the colorectum in miR21a$^{-/-}$ mice had fewer tumor numbers (2.14 ± 1.21 versus 3.86 ± 1.21, $P = 0.0309$), smaller tumor size (7% versus 15% for > 5mm tumors) and less tumor load (7.86 ± 4.06 versus 15.14 ± 5.01, $P = 0.0093$) compared to WT mice. Likewise, but a non-significant trend was observed in small intestine in miR21a$^{-/-}$ mice (Supplementary Figure 7B–C). Furthermore, miR21a$^{-/-}$ mice had longer survival than WT mice (median survival of 228.5 days for miR21a$^{-/-}$ mice and 163 days for WT mice, $P = 0.0288$, Figure 3F).

Taken together, we for the first time report that miR21 is a direct target of *F nucleatum* and miR21 knockdown/knockout can alleviate the oncogenic function of *F nucleatum* during colorectal carcinogenesis.

F nucleatum regulates expression of miR21 novel target RASA1 and activates MAPK signaling pathway

Since *F nucleatum* promoted tumorigenesis through miR21, we therefore questioned whether *F nucleatum* is able to regulate miR21 targets as well. Several already established targets of miR21 (PTEN, RECK, SPRY1 and RHOB) were tested in HCT116 and LoVo cells. After *F nucleatum* treatment, all of these miR21 downstream targets were downregulated in *F nucleatum*-treated cells when compared to *F nucleatum*-untreated group (Supplementary Figure 8), suggesting that *F nucleatum* infection affects CRC development likely through miR21. To identify novel miR21 target genes, we began to query the different public prediction database including DIANA-microT-CDSv5.0, miRanda-rel2010,

miRDB4.0, miRWalk, PicTar5, PITA, RNA22v2, RNAhybrid2.1 and Targetscan6.2[25]. Interestingly, we identified several novel potential candidates (KCNMB2, LUM, MSH2, TRPM7, CHD7, RASA1, and C17orf39), which were predicted in at least 8 databases (Figure 4A and Supplementary Table 2). We next overexpressed or inhibited miR21 in HCT116 and LoVo cells by using miR21 mimics or inhibitors. We found RASA1 was the only gene that was consistently downregulated in miR21 overexpressing cells while up-regulated in miR21 inhibition cells, suggesting RASA1 was a putative target gene of miR21 (Supplementary Figures 9–10 and Figure 4B). Moreover, we found RASA1 expression was downregulated in cancer vs. normal tissues in CRC patients, indicating the up-regulation of miR21 may lead to the reduced expression of RASA1 in CRC (Figure 4C).

To support our hypothesis that miR21 directly regulates RASA1 expression through its 3′-UTR, we generated luciferase reporter plasmids which harbored either wild type (WT) or mutated (MT) miR21 binding sites within the 3′-UTR of RASA1 (Figure 4D). HCT116 and LoVo were transiently transfected with luciferase constructs along with miR21 mimics, inhibitors or negative controls. In line with our previous results, miR21 overexpression or inhibition significantly suppressed or increased luciferase activity of the reporter genes containing WT 3′-UTR of RASA1, but no inhibitory effects were observed in mutated cell lines (Figure 4E). Collectively, these results indicated that miR21 suppressed expression of RASA1 through direct binding within the putative 3′-UTR binding sites of RASA1.

RASA1 is a member of RAS GTPase activating proteins (RAS-GAP) family. The well-known oncoprotein RAS can be inactivated through binding to the RAS-GAP members[26]. Several studies have shown that RASA1 mutation or loss of function in CRC results in the activation of RAS-MAPK cascade[27–29]. Therefore, we assumed that *F nucleatum* infection may lead to the reduced expression of RASA1 expression through miR21, and result in the activation of MAPK signaling pathway. As shown in Figure 4F, the expression of RASA1 was significantly down-regulated in *F nucleatum*-infected CRC cells. On the contrary, *F nucleatum* infections failed to increase RASA1 expression in normal intestinal epithelial cells NCM460 and IEC cells probably due to their low expression of miR21. Likewise, compared to miR21a$^{-/-}$ mice, the expression of RASA1 and another known miR21 target PDCD4 were significantly reduced, but the phosphorylation of extracellular signal-regulated kinase (P-ERK) was overexpressed in *F nucleatum*-infected miR21 WT mice tissues (Figure 4F). Taken together, we identified novel miR21 target RASA1 in CRC, and that *F nucleatum* can regulate RASA1 expression and activate MAPK pathway in a miR21 dependent manner.

TLR4/MYD88/NFκB pathway is activated by F nucleatum infection

Although *F nucleatum* infection can induce miR21 expression in CRC, the exact mechanisms for regulation of miR21 upon *F nucleatum* infection remain unclear. Hence, we aimed to find certain pathways that are affected by *F nucleatum* and identify the upstream regulators that could potentially control expression levels of miR21. We treated HCT116 cells with *F nucleatum* or PBS and then performed gene expression microarray analysis. The pathway analysis (KEGG and Reactome pathways) implicated *F nucleatum* infection can significantly stimulate TLR4/MYD88/NFκB pathway in CRC cells (Figure 5A). To validate this result, we incubated HCT116 cells with *F nucleatum*, *DH5α* or PBS for 6h, 12h and

24h. The phosphorylation levels of NFκB subunit p65 and p50 were substantially up-regulated, while NFκB inhibitor IκB-α was down-regulated by *F nucleatum* or DH5α treatment compared to PBS treatment, suggesting that activation of the NFκB pathway is a frequent event that occurs following bacterial infection (Figure 5B). Furthermore, silencing of NFκB significantly impaired the *F nucleatum*'s oncogenic effect on cell proliferation and cell invasion (Figure 5C and Supplementary Figure 11). Of note, we found CRC tissues with high amount of *F nucleatum* showed hyperactivity of NFκB, suggesting that *F nucleatum* requires NF- κB activation to affect CRC cells phenotype (Figure 5D).

We next investigated the specific surface receptors which can bind to *F nucleatum* and transmit signaling to NFκB pathway. As shown in Figure 5A, our microarray data showed that toll-like receptor (TLRs)/MYD88 cascade was substantially activated upon *F nucleatum* infection. We further validated that *F nucleatum* treatment induced the TLR4, TLR2, MYD88 expression in HCT116 cells (Figure 5E). Recently, several studies have reported that lipopolysaccharide (LPS) of *F nucleatum* can be recognized by TLR4[30, 31]. Also, it is widely acknowledged that NFκB is the key downstream effector of TLR4 signaling[32, 33]. Thus, we conclude that *F nucleatum* stimulates NFκB pathway through activation of TLR4/MYD88 cascade. We performed knockdown of either TLR4 or MYD88 in HCT116 cells and subsequently incubated HCT116 with *F nucleatum* or PBS. The results showed that silencing of TLR4/MYD88 cascade prevents NFκB activation from *F nucleatum* infection (Figures 5F). Collectively, we herein identified TLR4/MYD88/NFκB as a direct signaling pathway that is stimulated by *F nucleatum* in CRC cells.

F nucleatum regulates miR21 expression through TLR4/MYD88/NFκB pathway

Since *F nucleatum* is able to activate TLR4/MYD88/NFκB pathway, we wondered whether this pathway could be involved in the regulation of miR21 by *F nucleatum*. Because NFκB is a transcription factor, we assumed TLR4/MYD88/NFκB pathway may regulate miR21 expression in a promoter dependent manner. Therefore, we exploited bioinformatic analysis to decipher potential NFκB binding sites in upstream of transcription start site (TSS) of miR21. Interestingly, we identified the consensus binding sequence (5′GGAGGACTCC3′) for p65 in the promoter region of miR21. To determine whether NFκB can bind to this region and regulate miR21 expression, we first knocked down p65 in HCT116 and LoVo and observed significant down-regulation of miR21 expression (Figure 6A and 6B). Further, we generated luciferase reporter plasmids containing either wild type (WT) or mutant (MT) p65 binding sites in miR21 promoter region to examine the direct interaction between NFκB and miR21 promoter (Figure 6C). We transfected p65 shRNA or luciferase reporter constructs (pGL3-WT and pGL3-MT) in HEK293T cells. The luciferase reporter assay showed that co-transfection of pGL3-WT and p65 shRNA resulted in a significant decrease in the promoter activity of miR21, while p65 knockdown had no effect on mutant miR21 promoter activity (Figure 6C), supporting that NFκB regulates miR21 promoter activity in CRC. To further support our hypothesis that *F nucleatum* enhanced the binding of NFκB to miR21 promoter, we treated HCT116 and LoVo cells with *F nucleatum* or PBS following Chromatin immunoprecipitation (ChIP) assay. Consistently, qPCR results showed a more than 5 fold enrichment of miR21 promoter in p65 pulled-down DNA samples compared to IgG samples

(Figure 6D and 6E). Our results clearly showed that *F nucleatum* induce miR21 expression through NFκB.

More interestingly, we observed that *F nucleatum* failed to up-regulate miR21 expression when TLR4 or MYD88 were silenced in HCT116 and LoVo cells, highlighting the importance of TLR4/MYD88/NFκB pathway in the regulation of miR21 by *F nucleatum* (Figure 6F and 6G). Furthermore, CRC tissues from *F nucleatum* treated $APC^{min/+}$ mice showed hyperactivity of miR21 upstream regulator: NFκB, as well as activation of miR21 downstream targets: RASA1, PDCD4 and MAPK pathway (Figure 6H). Together, our results strongly suggest that *F nucleatum* regulates miR21 expression through TLR4/MYD88/NFκB pathway in CRC.

Overabundance of F nucleatum correlates with high expression of miR21 in human CRC tissues and indicates poor clinical outcome

Although we have interrogated the molecular regulation of miR21 by *F nucleatum*, the clinical significance of this regulation remains unknown. We used fluorescence in situ hybridization (FISH) assays to image the *F nucleatum* infection as well as miR21 expression in CRC tissues and adjacent normal tissues. Compared to normal mucosa, *F nucleatum* was found to invade CRC tissues, often accompanied by high levels of miR21 (Figure 7A). To further demonstrate the expression correlation between *F nucleatum* and miR21, we measured *F nucleatum* at both DNA level and miR21 transcript expression in 90 matched fresh frozen CRC and normal tissues. Strikingly, both *F nucleatum* DNA and miR21 levels were higher in cancer tissues ($P = 0.0083$ for *F nucleatum*, $P < 0.001$ for miR21, Figure 7B). Furthermore, the *F nucleatum* DNA and miR21 expression was specifically much higher in advanced tumors (Stage III/IV, Figure 7C). Notably, the overabundance of *F nucleatum* was associated with advanced T stage ($P = 0.027$), proliferation marker Ki-67 expression ($P = 0.017$) and lymphatic invasion ($P = 0.031$). The up-regulation of miR21 was associated with distant metastasis ($P=0.006$), and TNM stage ($P=0.008$), suggesting that *F nucleatum* and miR21 have the same oncogenic role in cell proliferation and invasion, and promotion of CRC development (Supplementary Table 3). Consistently, we found high *F nucleatum* levels were robustly associated with high expression of miR21 in cancer tissues, suggesting that *F nucleatum* infection might have led to the induction of miR21 in invaded tumor tissues ($r = 0.4537$, $P < 0.001$, Figure 7D).

To investigate the prognostic impact of regulation of miR21 by *F nucleatum* in CRC patients, we used another independent cohort of CRC patients, with complete follow-up information (fresh frozen tissues, n = 125). As shown in Figure 7E, high amount of *F nucleatum* correlated with remarkably poor overall survival in CRC patients, compared to the patients with low or undetectable amount of *F nucleatum*. More importantly, when we segregated patients into 4 groups based on *F nucleatum* levels and miR21 expression, we discovered that the group with both high *F nucleatum* and high miR21 expression showed worst prognosis compared to other 3 groups (Figure 7E).

Taken together, our results showed that *F nucleatum* is heavily enriched in cancer tissues and more so in the advanced tumors, leading to the high expression of miR21 in locally-advanced tumors. Furthermore, a subgroup of CRC patients with both high *F nucleatum*

levels and miR21 expression has a higher risk for poor clinical outcomes, which has important implications for their clinical management.

DISCUSSION

Identification of specific carcinogenic microorganisms remain an important area of research for many cancers, particularly CRC[34]. Among the recently identified and published CRC-related microbes, the recently-discovered *F nucleatum* has garnered most attention. Herein, we for the first time have identified that miR21 is a downstream target of *F nucleatum* in the colon. Furthermore we have unraveled the underlying molecular mechanism(s) that TLR4/MYD88/NFκB signaling pathway is activated upon the *F nucleatum* infection, and hyperactive NFκB subsequently binds to the promoter of miR21 and initiates an oncogenic cascade in CRC (Figure 7F). Consistent with these results, expression levels of *F nucleatum* and miR21 are heavily enriched in cancer tissues and particularly in advanced tumors. The *F nucleatum* levels were positively associated with miR21 in cancer tissues, suggesting that *F nucleatum* infection leads to the induction of miR21 in tumor tissues, and such patients often have higher risk and worse clinical outcomes.

Although recent studies have implicated overabundance of *F nucleatum* in association with colorectal adenomas and cancer, it remains uncertain whether *F nucleatum* is a causative pathogen for CRC or only an opportunistic pathogen or simply a commensal bacteria associated with the CRC microenvironment[35, 36]. Our data reveal that *F nucleatum* infection increase tumor burden in $APC^{Min/+}$ mice and stimulate the increase of serum inflammatory factors, suggesting that *F nucleatum* plays a driver role, instead of being simple a passenger, in intestinal tumorigenesis. Furthermore, we also found that *F nucleatum* infection promotes the proliferation and invasion both *in vitro* and *in vivo*, and *F nucleatum* infection is a more frequent phenomenon in advanced tumors; suggesting *F nucleatum* infections not only contribute to the initiation of CRC but also to the development of CRC.

To understand how *F nucleatum* promotes CRC, several groups have previously characterized the interaction between *F nucleatum* and intratumoral immune cells, and have shown that *F nucleatum* promotes inflammation and tumorigenesis by modulating the tumor immune microenvironment via expansion of myeloid derived immune cells[6, 37]. Interestingly, we noticed that a recent study provided novel mechanistic insights that *F nucleatum* can invade epithelial cells via its virulence factors FadA and activate pro-oncogenic signals to promote CRC, suggesting that *F nucleatum* may not only regulate immune cells but also targets tumor cell itself[12]. Since we have previously reported the role of several miRNAs in CRC, we hypothesized that *F nucleatum* infection may have a potential impact on miRNA expression during the progression of CRC. Surprisingly, we discovered that a large number of miRNAs were differentially expressed upon *F nucleatum* infection and miR21 was among the most robustly induced by *F nucleatum* infection. Our previous studies have demonstrated that miR21 plays a pathogenic role in chronic inflammatory processes and the development of colitis-associated colon cancer[15]. It is conceivable that the presence of *F nucleatum* drives oncogenic signaling through induction of oncogenic miR21. Nevertheless, our results for the first time have established a crosstalk between *F nucleatum* infection and epithelial cells miRNA genes. Interestingly, a recent

review published by Nosho et al. in 2016[38] predicted the underlying association of *F nucleatum* with miR21 in CRC, which happens to be consistent with our present results. It is worth noting that a recent study showed that host fecal miRNAs modulated genes expression in *F nucleatum* and therefore affected bacterial growth, suggesting that miRNAs may bridge the communication gap between *F nucleatum* and its host[39].

Our data also firstly illustrated that *F nucleatum* can downregulate the expression of miR21 downstream targets such as RASA1. The MAPK signaling pathway is a major miR21 regulated network that empowers cells with an aggressive phenotype, and is often inappropriately activated in CRC[40, 41]. Despite the presence of KRAS mutations, recent studies indicate that CRC are deficient in GAP due to its collaborative action with onco-miRs[29]. To this end, *F nucleatum* could be a promising therapeutic target which can help alleviate inhibition of RASA1 from miR21 and prevent activation of the MAPK pathway.

In this study, we attempted to reveal the mechanism for the regulation of miR21 by *F nucleatum*. We found *F nucleatum*-mediated infection leads to a strong induction of TLR4/MYD88 and results in the sustained activation of NFκB. The hyperactive NFκB binds to the promoter of miR21 and increases its transcription level. However, the modulation of miR21 by *F nucleatum* seems to be more complicated. Despite the fact that *F nucleatum* activated TLR4/MYD88/NFκB, we also noticed that DH5α could activate this pathway. However, we did not notice up-regulation of miR21 following DH5α infection, suggesting that *F nucleatum* adopts other collaborative mechanisms to enhance the effect of TLR4/MYD88/NFκB on miR21 expression. Notwithstanding the above, TLR4/MYD88/NFκB could, at least in part, explain how *F nucleatum* infection stimulates the overexpression of miR21 in CRC.

From a clinical viewpoint, we further propose that in addition to its functional and therapeutic role, using *F nucleatum* and miR21 levels offer an added value as integrated biomarkers for better prognosis assessment of CRC, considering that a subgroup of CRC patients with their high levels associate with a higher risk and worse clinical outcome.

In conclusion, our current findings provide critical insights into the molecular mechanisms for the regulation of miR21 by *F nucleatum*. The clinical results presented here also highlight the emergence of both *F nucleatum* and miR21 as risk factors for survival outcomes in CRC patients. Our data provides previously unrecognized and novel evidence for a pathogenic role of *F nucleatum* in CRC, which opens new horizons for targeting microbiota as well as miRNAs alterations for colorectal cancer prevention and treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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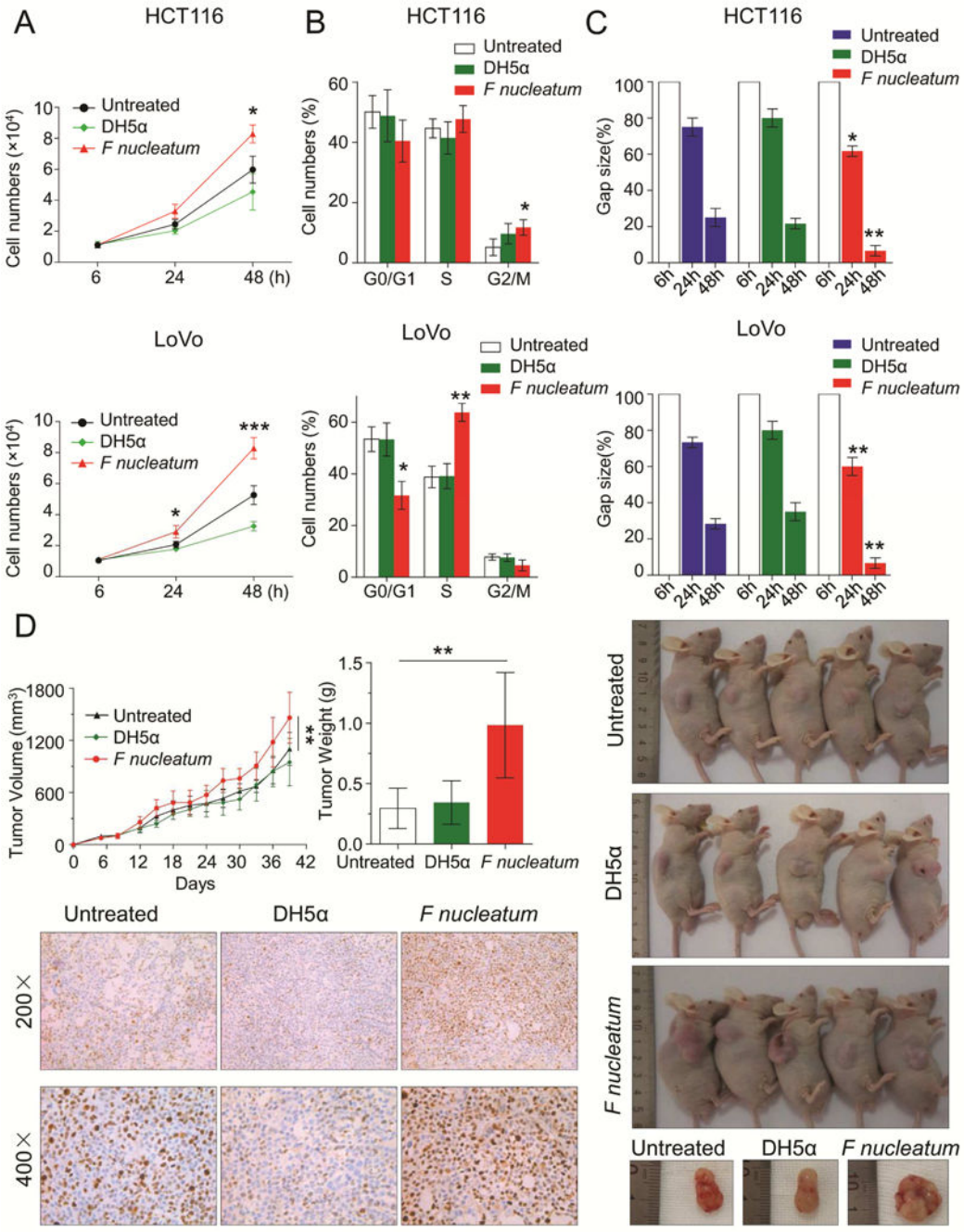

Figure 1. *F nucleatum* promotes CRC cell proliferation and invasion *in vitro* and *in vivo*

A) HCT116 and LoVo cells were incubated with PBS, DH5α *E coli* or *F nucleatum*. The cell proliferation rates were evaluated by cell number counting after treatment at 6, 24 and 48 hr (*$P < 0.05$, ***$P < 0.001$, unpaired Student's *t* test). **B)** The cell cycle distribution of treated or control cells was determined by flow cytometry-based assay after 48 hr (*$P < 0.05$, **$P < 0.01$, unpaired Student's *t* test). **C)** The scratch wound healing assay was performed to evaluate the invasive capability of treated or control cells (*F nucleatum* treated cells vs control cells, *$P < 0.05$, **$P < 0.01$, unpaired Student's *t* test). **D)** HCT116 cells

treated with PBS, *DH5α*, or *F nucleatum* were subcutaneously injected into male BALB/C nude mice to produce xenograft tumors in animals (n=5 per group). The upper left panel of figures illustrate tumor growth curves (** $P < 0.01$ by one-way analysis of variance [ANOVA] and Bonferroni's multiple comparison test) and tumor weight (** $P < 0.01$ by one-way ANOVA,). The right panel of figure shows the representative images of xenograft mice. The bottom left figures depict representative immunostaining (200× and 400×) for Ki-67 in xenograft tumor tissues. These results are representative of at least three independent experiments. Bars represent standard deviation (SD).

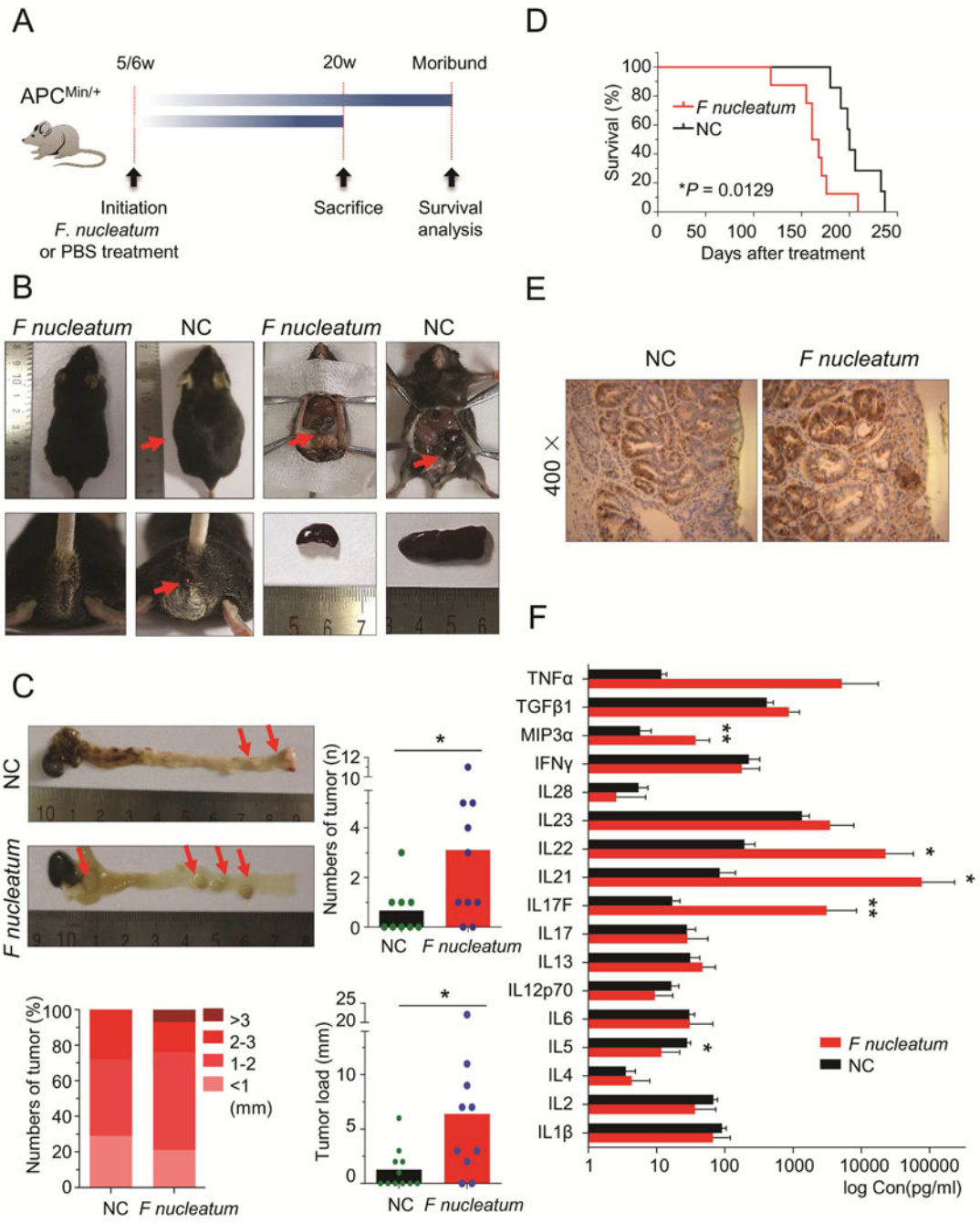

Figure 2. *F nucleatum* promotes tumorigenicity and CRC progression in Apc$^{Min/+}$ mice

A) APC$^{Min/+}$ mice were administrated with *F nucleatum* or PBS (negative control, NC) and sacrificed after treatment at 20 weeks. For the survival analysis, we compared survival time between *F nucleatum* treated group and PBS group. **B)** On 20 weeks, *F nucleatum*-treated APC$^{Min/+}$ mice presented with ascites, bloody diarrhea, gut dilatation and splenomegaly. **C)** The left upper figure illustrates a representative image of a colon of APC$^{Min/+}$ mice with or without *F nucleatum* treatment. The upper right figure and the bottom figure shows the administration of *F nucleatum* for 20 weeks which resulted in significant increase in tumor

numbers, size and tumor load in the colorectum (n = 10 per group,*P<0.05, Mann-Whitney U test). **D)** The Kaplan-Meier survival curves show *F nucleatum* treatment reduced the survival rates of $APC^{min/+}$ mice (n = 8 per group, Log-rank (Mantel-Cox) test). **E)** Representative image of an immunostaining for PCNA in *F nucleatum*-treated or *F nucleatum*-untreated CRC tissues. **F)** The serum level of inflammatory factors in $APC^{min/+}$ mice with or without treatment of *F nucleatum*. (n = 10 per group, *P < 0.05, **P < 0.01, Mann-Whitney U test). Results represent means ± SD. The red arrows indicated the positive location.

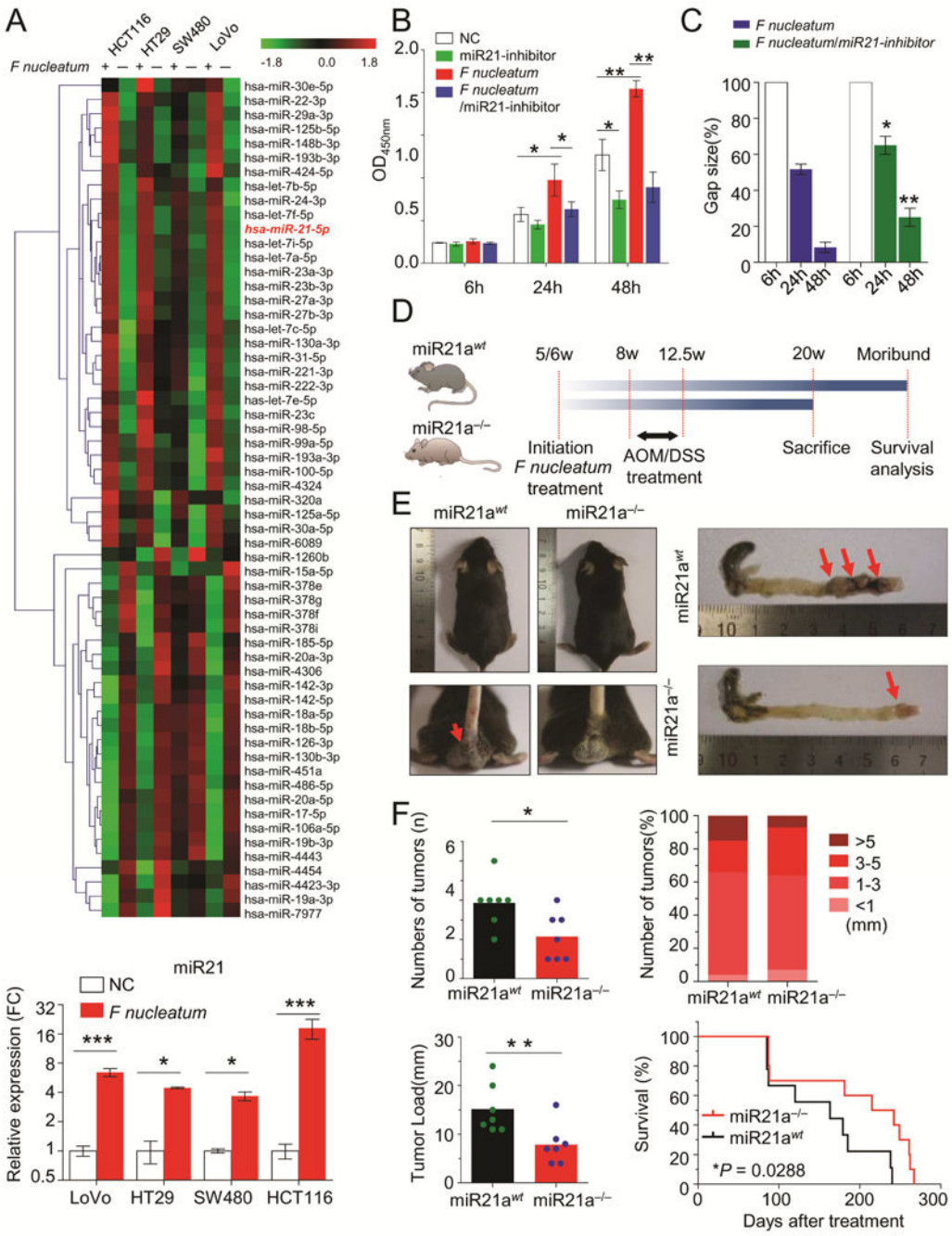

Figure 3. *F nucleatum* regulates miR21 expression in CRC

A) Microarrays were performed to identify the differentially expressed miRNA between *F nucleatum*-treated and control cells. The miR21 expression was consistently up-regulated in different CRC cells after *F nucleatum* treatment (*$P < 0.05$, ***$P < 0.001$, unpaired Student's *t* test). **B and C)** CCK-8 cell viability assay and wound healing assays illustrated that inhibition of miR21 completely abolished *F nucleatum*'s effect on cell growth and invasion(*$P < 0.05$, **$P < 0.01$ by unpaired Student's *t* test). **D)** Both miR21 WT and knockout (KO) mice were initially administrated *F nucleatum* and then subjected to

AOM/DSS treatment. At 20 weeks, mice were sacrificed for various experiments. **E)** The left representative image showed miR21 KO decreased the bloody diarrhea rates during the *F nucleatum* infection (n = 7 per group), and the right image showed a representative colon of the miR21 KO or wild type mice. **F)** The colorectum in miR21 KO mice had fewer tumor numbers, smaller size and less tumor load compared to WT mice (n = 7 per group,* $P<0.05$, Mann-Whitney U test). Furthermore, miR21 KO mice had longer survival rates than WT mice (n = 10 per group by log-rank (Mantel-Cox) test). These results are representative of at least three independent experiments. Results represent means values ± SD. The red arrows indicated the positively stained cells.

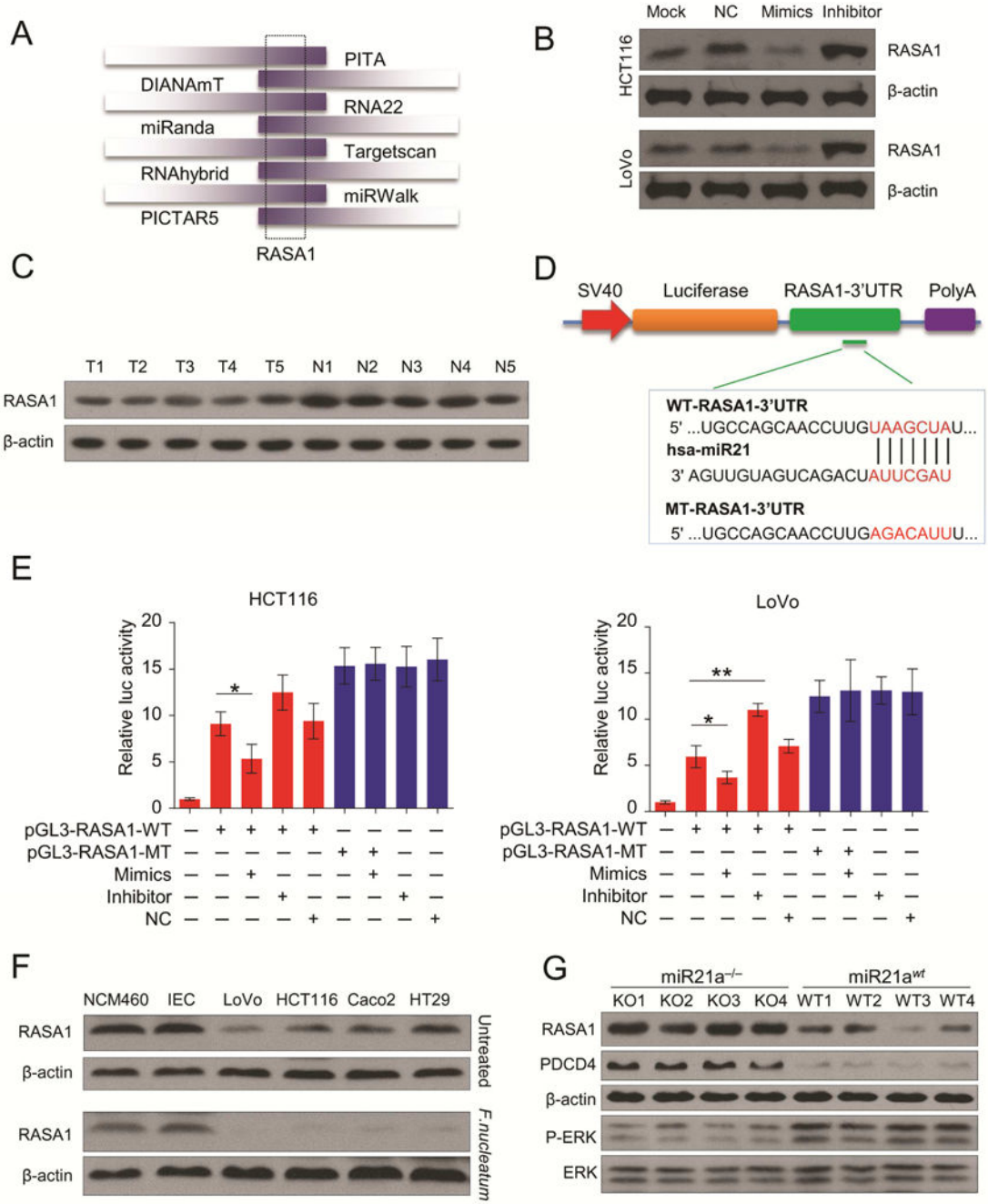

Figure 4. *F nucleatum* regulates expression of miR21 target gene RASA1 and activates MAPK signaling pathway

A) RASA1 was predicted as a putative miR21 target through analysis of 8 public prediction databases. **B)** The representative images of Western blot show overexpression or inhibiton of miR21 downregulated or upregulated RASA1 expression in HCT116 cells (n = 3 per group) and LoVo cells (n = 3 per group). **C)** Western blot showed RASA1 expression was lower in cancer tissues compared to matched normal tissues from CRC patients (n = 5 per group). **D)** We generated luciferase reporter plasmids which harbor either wild type (WT) or mutant

(MT) miR21 binding sites in 3′-URT of RASA1. **E)** HCT116 and LoVo were transiently transfected with luciferase constructs along with miR21 mimics, inhibitors or negative controls. After 48hr, the luciferase activity was measured. All bars represent the mean values ± s.d. of three independent experiments. (*$P < 0.05$, **$P < 0.01$ by unpaired Student's *t* test) **F)** The protein expression levels of RASA1 were significantly down-regulated in *F nucleatum*-infected CRC cells, while *F nucleatum* infections failed to increase RASA1 expression in normal intestinal epithelial cells NCM460 and IEC cells (n = 3). Compared to miR21 KO mice, the expression of RASA1 and another known miR21 target PDCD4 was significantly reduced, but P-ERK overexpressed in *F nucleatum*-infected miR21 WT mice tissues (n = 4 mice per group).

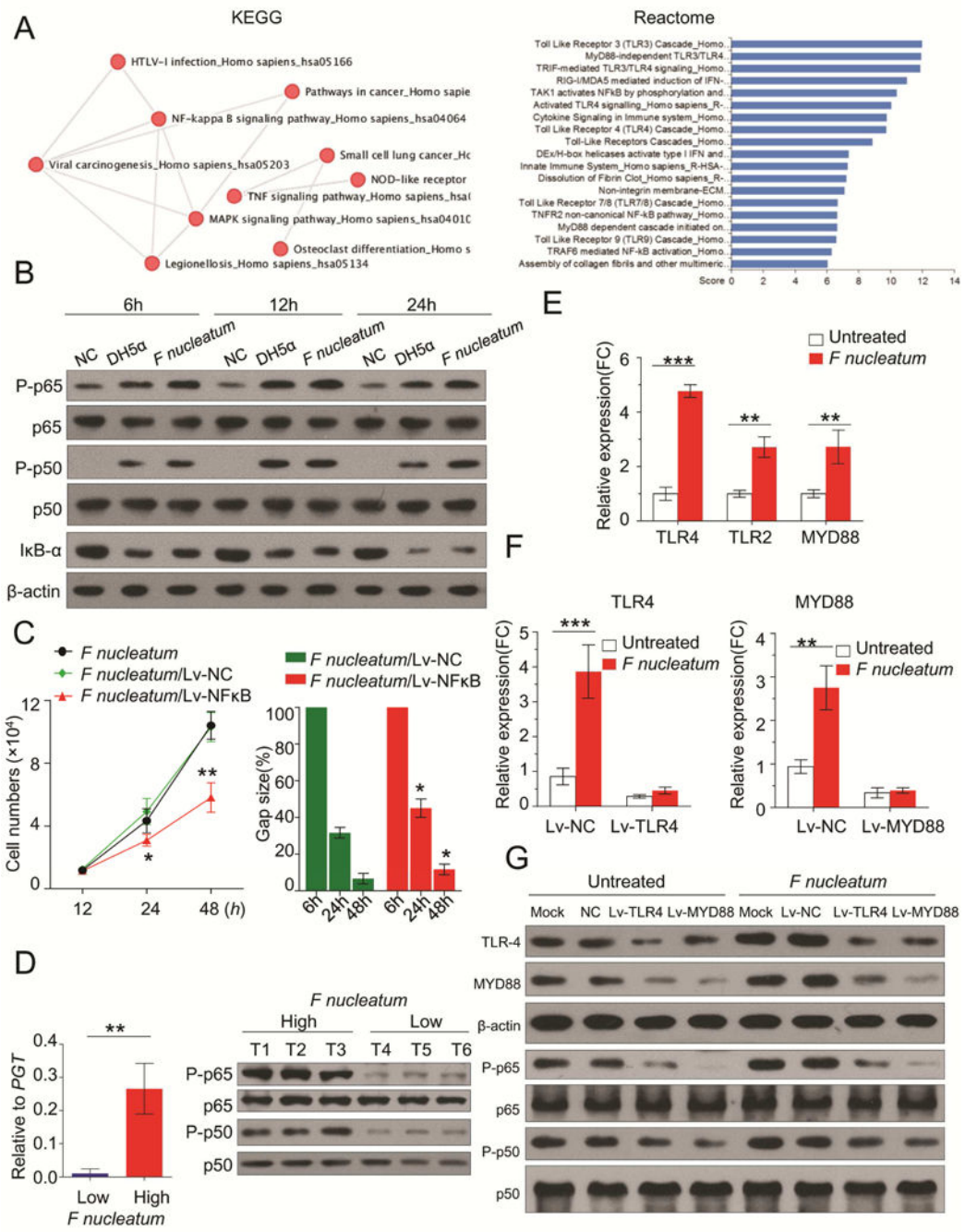

Figure 5. TLR4/MYD88/NFκB pathway is activated by *F nucleatum* infection

A) We treated HCT116 cells with *F nucleatum* or PBS (n = 3 per group) and then performed gene expression microarray analysis. The pathway analysis (KEGG and Reactome pathways) implicates *F nucleatum* infection can significantly stimulate TLR4/MYD88/NFκB pathway in CRC cells. **B)** HCT116 cells were incubated with *F nucleatum*, *DH5α* or PBS for 6h, 12h and 24h. NFκB subunit p65 and p50, phosphorylation level of p65 and p50 (P-p65 and P-p50), and inhibitor IκB-α were measured by Western blot. **C)** Silencing of NFκB significantly impaired the *F nucleatum*'s oncogenic effect on cell proliferation

(proliferation assay) and cell invasion (wound healing assay) (*$P < 0.05$, **$P < 0.01$ by unpaired Student's t-test. Bars represent SD of three experiments). **D)** qPCR results of *F nucleatum* DNA level in the low or high group (left figure, n = 6). Western blots analysis indicated that CRC tissues with high burden of *F nucleatum* showed activation of NFκB (right figure, n = 3). **E)** *F nucleatum* treatment induced the TLR4, TLR2, MYD88 expression in HCT116 (**$P < 0.01$, ***$P < 0.001$ by unpaired Student's t-test. Bars represent SD of three experiments). **F)** qRT-PCR showed successfully silencing of TLR4 or MYD88 by Lv-TLR4 or Lv-MYD88 (**$P < 0.01$, ***$P < 0.001$ by unpaired Student's t-test. Bars represent SD of three experiments). Western blots illustrated either TLR4 or MYD88 knockdown prevented NFκB activation from *F nucleatum* infection.

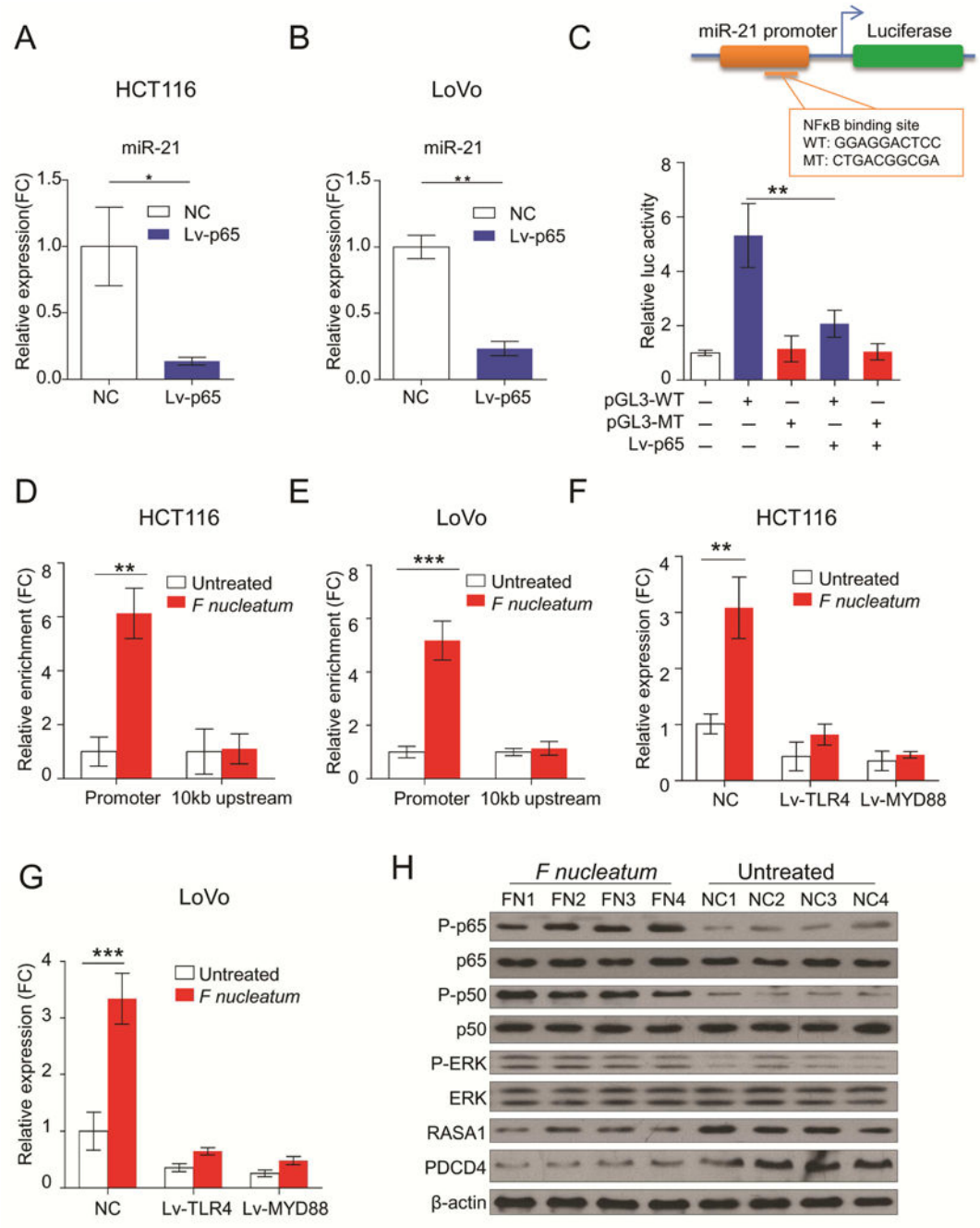

Figure 6. *F nucleatum* regulates miR21 expression through TLR4/MYD88/NFκB pathway

A and B) Knockdown of p65 in HCT116 or LoVo significantly down-regulated miR21 expression (** $P < 0.01$ by unpaired Student's *t* test. Bars represent SD of three experiments). **C)** We generated luciferase reporter plasmids containing either wild type (WT) or mutant type (MT) of p65 binding sites in miR21 promoter. HEK293T cells were transfected with p65 shRNA or luciferase constructs (pGL3-WT and pGL3-MT) (** $P < 0.01$ by unpaired Student's *t* test). **D and E)** HCT116 cells and LoVo were treated with *F nucleatum* or PBS following ChIP assay. qPCR results showed a more than 5 fold

enrichment of miR21 promoter in p65 pulled-down DNA samples compared to IgG samples (**$P < 0.01$ by unpaired Student's *t* test). **F and G)** The qRT-PCR assay indicated that *F nucleatum* failed to up-regulate miR21 expression when TLR4 or MYD88 was silenced in HCT116 and LoVo (**$P < 0.01$ by unpaired Student's *t* test). **H)** Western blot analysis showed that CRC tissues from *F nucleatum*-treated $APC^{min/+}$ mice showed activation of NFκB, as well as other members including RASA1, PDCD4 and MAPK pathway (n = 4 mice per group).

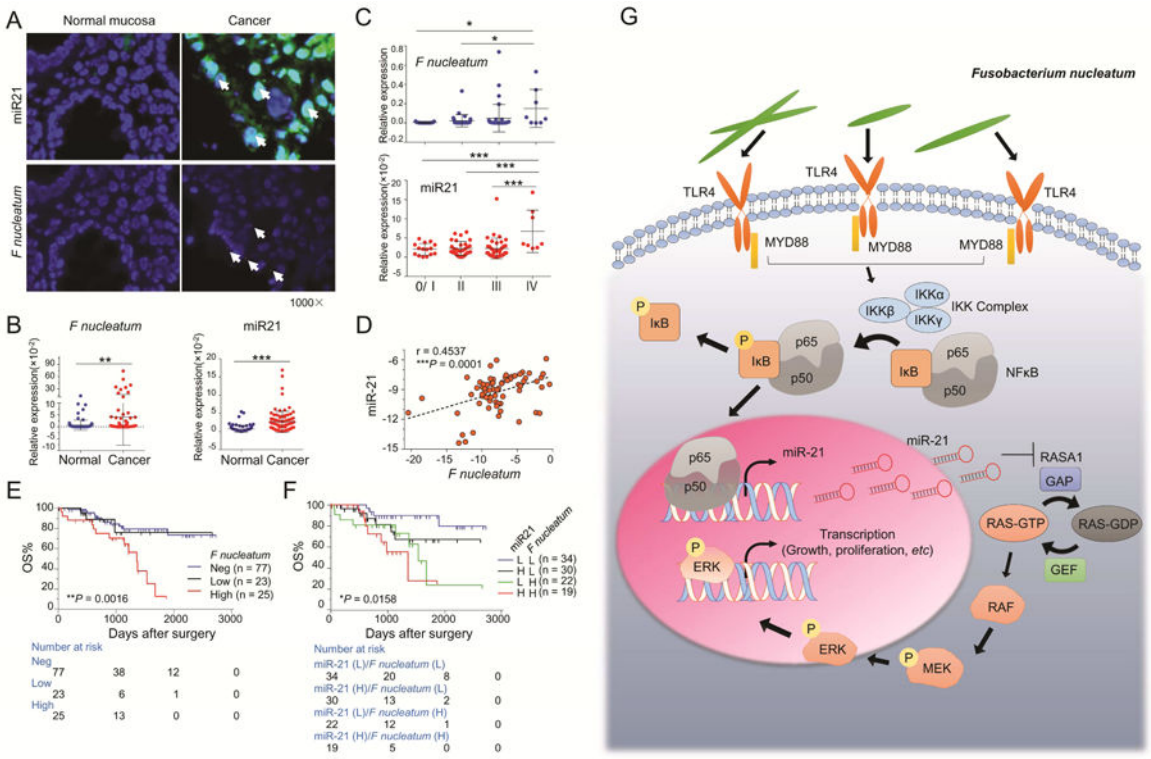

Figure 7. Overabundance of *F nucleatum* correlates with high expression of miR21 in CRC and indicates poor clinical outcome

A) FISH assay showed *F nucleatum* (1000×) was enriched in the mucosa of CRC tissues and accompanied by high level of miR21 (1000×). The white arrows indicate positive staining. **B)** The qRT-PCR analysis showed that both the expression levels of *F nucleatum* and miR21 were higher in the cancer tissues than in the adjacent normal tissues (n = 90. $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired Student's *t* test. Bars represent SD). **C)** The qRT-PCR analysis showed *F nucleatum* and miR21 was overexpressed in cancer tissues in a stage dependent manner (n = 90. $^{*}P < 0.05$, $^{***}P < 0.001$ by one way ANOVA and Bonferroni's multiple comparison test. Bars represent SD). **D)** The amount of *F nucleatum* DNA was positively associated with miR21 expression in cancer tissues (n = 90. $^{***}P < 0.001$ by two-tailed nonparametric Spearman correlation.). **E)** Kaplan–Meier survival curve for 125 clinical specimens showed high amount of *F nucleatum* were associated with remarkably poor overall survival (OS) in CRC patients (log-rank (Mantel-Cox) test). Furthermore, subgroup of CRC patients with both high *F nucleatum* DNA level and miR21 expression has a higher risk clinical outcome (log-rank (Mantel-Cox) test). Neg, negative; L, low; H, high. **F)** The illustration of the hypothetical mechanism by which *F nucleatum* regulates miR21 expression through TLR4/MYD88/NFκB pathway in CRC.