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Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin

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SUMMARY

Fusobacterium nucleatum (*Fn*) has been associated with colorectal cancer (CRC), but causality and underlying mechanisms remain to be established. We demonstrate that *Fn* adheres to, invades and induces oncogenic and inflammatory responses to stimulate growth of CRC cells through its unique FadA adhesin. FadA binds to E-cadherin, activates β-catenin signaling, and differentially regulates the inflammatory and oncogenic responses. The FadA-binding site on E-cadherin is mapped to an 11 amino acid region. A synthetic peptide derived from this region of E-cadherin abolishes FadA-induced CRC cell growth, and oncogenic and inflammatory responses. FadA levels in the colon tissue from patients with adenomas and adenocarcinomas is >10–100 times higher compared to normal individuals. The increased FadA expression in CRC correlates with increased expression of oncogenic and inflammatory genes. This study unveils a mechanism by which *Fn* can drive CRC and identifies FadA as a potential diagnostic and therapeutic target for CRC.

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AUTHOR CONTRIBUTIONS Y.W.H conceived, designed, and supervised the study. W.L. provided clinical specimens. X.W., M.R., Y.H. and G.C. performed the experiments. Y.W.H., X.W., M.R. and W. L. interpreted the data and wrote the manuscript.

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INTRODUCTION

The human intestinal microbiome contains >1000 different species totaling 10^{14} microorganisms and plays an extremely important role in the maintenance of the normal physiology of the gut, including energetic metabolism, proliferation and survival of epithelial cells, and protection against pathogens (Tremaroli and Backhed, 2012). The microbiota exerts both beneficial and detrimental effects on host contributing to healthy or disease (Sekirov et al., 2010). Recently, two research teams simultaneously reported overabundance of a specific microorganism, *Fusobacterium nucleatum* (*Fn*), in colorectal carcinoma tissues (Castellarin et al., 2012; Kostic et al., 2012). However, it was unknown if *Fn* was a cause or a consequence of CRC.

Fn is an opportunistic commensal anaerobe in the oral cavity, implicated in various forms of periodontal diseases. Outside the oral cavity, it is one of the most prevalent species in extra-oral infections (Han, 2011). It is highly prevalent in intrauterine infections associated with pregnancy complications such as preterm birth, stillbirth, and neonatal sepsis (Han et al., 2009; 2010; Wang XW et al., 2012). *Fn* adheres to and invades endothelial and epithelial cells, a likely mechanism utilized for its systemic dissemination (Han et al., 2000; 2004). The attachment and invasion take place via adhesin FadA, a virulence factor identified from *Fn* (Han et al., 2005; Xu et al., 2007). Immunofluorescent staining of non-permeabilized *Fn* using anti-FadA monoclonal antibodies showed that FadA was expressed on the bacterial surface (Ikegami et al., 2009).

FadA is highly conserved among *Fn* (Han et al., 2005). It exists in two forms, the non-secreted intact pre-FadA consisting of 129 amino-acid (aa) residues, and the secreted mature FadA (mFadA) consisting of 111 aa without the 18-aa signal sequence (Han et al., 2005). The mFadA monomer is predominantly alpha-helical and forms a “hairpin” like structure (Nithianantham et al., 2009). The monomers are linked together in a head-to-tail pattern to form uniformly long and thin filaments, with no binding activity (Temoin et al., 2012). When mFadA is mixed with pre-FadA, they form activity complexes, FadAc, consisting of heterogeneous filaments, presumably due to varying degrees of filament bundling (Xu et al., 2007; Temoin et al., 2012). We have recently demonstrated that the vascular endothelial (VE) cadherin (CDH5), a member of the cadherin superfamily, is a receptor for FadA, required for *Fn* to attach and invade endothelial cells (Fardini et al., 2011). The receptor for FadA on epithelial cells was not identified.

In the present study, we demonstrate that FadA binds to E-cadherin on CRC and non-CRC cells, mediating *Fn* attachment of and invasion into the cells. FadA modulates E-cadherin and activates β-catenin signaling, leading to increased expression of transcription factors, oncogenes, Wnt genes, and inflammatory genes, as well as growth stimulation of CRC cells. Further, we show that while FadA binding to CRC cell is sufficient to turn on the Wnt and oncogenes, its internalization mediated by clathrin is needed to activate the inflammatory genes. This study reveals a mechanism by which *Fn* contributes to CRC and identifies FadA as a potential diagnostic and therapeutic target for CRC.

RESULTS

Fn stimulates human CRC cell proliferation

Wild-type *Fn* 12230 significantly stimulated proliferation of human colon cancer cells HCT116, DLD1, SW480, and HT29, but only weakly stimulated RKO. It did not stimulate the non-CRC cells HEK293. Compared to the untreated cells or those incubated with *E. coli* DH5α, the growth stimulation increased by approximately 100% for HCT116, DLD1, SW480, and HT29, but only 18% for RKO, after 72 hours (Fig. 1a). The *fadA*-deletion

mutant US1 weakly stimulated the growth of all cancer cell lines. The *fadA*-complementing clone, USF81, restored proliferation of HCT116, DLD1, SW480, and HT29 to the wild-type level. Furthermore, HCT116 growth was enhanced by purified FadAc in a dose-dependent manner, with the maximum stimulation observed at 1 mg/ml, while mFadA exhibited no stimulatory effect (Fig. 1b). Neither FadAc or mFadA stimulated growth of RKO. These results indicate that stimulation of CRC cell by *Fn* is FadA-dependent.

FadA binds to E-cadherin on CRC cells

To investigate the mechanism by which FadA stimulates CRC cell growth, we set out to identify CRC cell receptors for FadA. It was previously shown that FadA binds to VE-cadherin on endothelial cells (Fardini et al., 2011). Cadherins are a large family of calcium-dependent cell adhesion glycoproteins, each composed of five extracellular repeat domains (EC1-EC5), a transmembrane domain, and a highly conserved cytoplasmic tail that binds other cytoplasmic components including β-catenin (Gumbiner, 2005) (Fig. 2a). Given the 33.5% similarity between VE- and E-cadherins, we speculated if FadA also bound to E-cadherin. E-cadherin is present on epithelial cells, including the non-cancerous HEK293, as well as the CRC cells, except RKO (Fig. 2b). FadA binding to E-cadherin was directly tested by co-immunoprecipitation. FadAc co-precipitated with E-cadherin, while mFadA did not (Fig. 2c). Using the GST pull-down assay, we determined that FadAc bound specifically to EC5 of E-cadherin, but not to EC1-4, the transmembrane, or the cytoplasmic domains (Fig. 2d). Deletions of various regions in EC5 showed that region 3 was responsible for FadA binding (Fig. S2).

FadA promotes *Fn* attachment and invasion of E-cadherin-expressing cells

Attachment and invasion are hallmarks of *Fn*. Thus, we tested the role of FadA binding to E-cadherin in these processes. *Fn* attachment and invasion of non-CRC cells HEK293 was inhibited by mouse monoclonal antibody HECD-1 raised against the extracellular domain of E-cadherin (Fig. S3a). Deletion of *fadA* (US1) severely impaired the ability of *Fn* to bind and invade HEK293, whereas the *fadA*-complemented clone, USF81, restored the activities. Down-regulation of E-cadherin expression by siRNA in HEK293 significantly inhibited attachment and invasion by wild-type *Fn* and USF81 (Fig. S3b). These results indicate that *Fn* attachment and invasion of HEK293 requires FadA and E-cadherin.

Similar observations were made with CRC cells HCT116 expressing E-cadherin. US1 ($fadA^-$) was defective in attachment and invasion of HCT116, compared to wild-type *Fn* or USF81 ($fadA^+$) (Fig. 3a). Inhibition E-cadherin expression by siRNA reduced attachment and invasion (Fig. 3a). In contrast, no difference was observed among *Fn* 12230, US1 and USF81 in their weak binding and invasion of the non-E-cadherin-expressing RKO cells (Fig. 3b). Transfection of RKO with the full-length E-cadherin led to increased binding and invasion by *Fn* 12230 and USF81 to levels comparable to those observed in HCT116 (Fig. 3b). These results indicate that FadA mediates *Fn* attachment and invasion of CRC cells via E-cadherin.

E-cadherin can be internalized via clathrin (Bryant and Stow, 2004). Pitstop2, a clathrin inhibitor (von Kleist et al., 2011), prevented *Fn* invasion of HCT116, without affecting attachment (Fig. 3c). *Fn* stimulated expression of the inflammatory genes including NF-kappaB and cytokines IL-6, 8, and 18 from HCT116 (Fig. 3d). Such stimulation was abolished in the presence of the clathrin inhibitor, indicating that invasion was required for the stimulation of inflammation (Fig. 3d).

Identification of inhibitory peptides to prevent *Fn* attachment and invasion

Since FadA bound to the EC5 domain of E-cadherin, we tested the role of EC5 in *Fn* attachment and invasion. *Fn* attachment and invasion of HCT116 was inhibited by purified GST-EC5 fusion protein in a dose-dependent manner, with maximum inhibition observed at 0.1 μM (Fig. 4a & 4b). Synthetic peptides derived from different regions of EC5 were then tested for their ability to inhibit *Fn* attachment and invasion (Fig. 4a & 4c). Peptide 3, corresponding to region 3, exhibited similar inhibitory effect as EC5 (compare Fig 4b & 4c). To determine the minimal sequences required for inhibition, sequential deletions of peptide 3 were generated. The results showed that the 11-aa peptide (ASANWTIQYND) was the minimum required, and was designated as the "inhibitory peptide" (IP) (Fig. 4d).

FadA promotes CRC cell proliferation via E-cadherin

Stimulation of HCT116 growth by *Fn* was inhibited by the CDH1-specific siRNA, EC5, and the inhibitory peptide (Fig. 1c). In the non-E-cadherin-expressing RKO cells, transfection of the full-length E-cadherin resulted in growth stimulation by *Fn* (Fig. 1d). As in HCT116, such stimulation was diminished by EC5 and the inhibitory peptide (Fig. 1d). Similar observations were made using purified FadAc instead of *Fn* (Fig. S1). These results elucidate the critical role of E-cadherin in promoting *Fn*-driven CRC cell growth.

FadAc activates E-cadherin-mediated cellular signaling

To investigate the downstream events subsequent to FadA binding to E-cadherin, HCT116 were fractionated following incubation with purified FadA. FadAc, but not mFadA, bound to the HCT116 membranes within five minutes of incubation, leading to E-cadherin phosphorylation on the membrane and internalization (Fig. 5a). This was accompanied by decreased phosphorylation of β-catenin, β-catenin accumulation in the cytoplasma and translocation into the nucleus, resulting in activation of β-catenin-regulated transcription (CRT), as evidenced by increased expression of transcription factors lymphoid enhancer factor (LEF)/T-cell factor (TCF), NF kappaB, and oncogenes Myc and Cyclin D1 (Fig. 5a). It was previously shown that protein tyrosine kinase plays a crucial role in E-cadherin endocytosis and recycling (Le et al., 2002). Interestingly, the protein tyrosine kinase (PTK) inhibitor, Genistein, not only prevented E-cadherin phosphorylation and internalization, but also abolished FadA binding to the membranes and its internalization, as well as the above-described CRT activation (Fig. 5a). These results suggest that phosphorylation of E-cadherin or other cellular components are required for activation of CRT. FadA may bind to phosphorylated E-cadherin, or it may bind to non-phosphorylated E-cadherin, which is then phosphorylated leading to positive feedback. The central role of β-catenin in regulating the cellular responses was confirmed using HCT116 β-catenin$^{-/-}$ cells, which did not affect FadA binding to E-cadherin, phosphorylation of E-cadherin, or the internalization, but prevented all gene activations tested in the nuclei (Fig 5a).

The clathrin inhibitor, although did not affect FadA binding to or E-cadherin phosphorylation on the membranes, inhibited FadA and E-cadherin internalization. A surprise consequence was the divergent responses observed in the nuclei. Translocation of β-catenin and expression of LEF/TCF, Wnt and oncogenes were unaffected. In contrast, no NF kappaB activation was observed (Fig. 5a). These results indicate that tumor growth and inflammatory responses, although both requiring β-catenin, are differentially regulated.

The Western blot analysis of protein levels were corroborated with real-time quantitative PCR (qPCR) analysis of the mRNA levels (Fig 5b–5e). While PTK inhibitor and CDH1-specific siRNA inhibited activation of tumor growth and inflammatory genes, the clathrin inhibitor only inhibited the inflammatory genes, but not the Wnt or oncogenes.

To further confirm the role of FadA in CRT activation, we performed confocal microscopy analysis and observed nuclei translocation of β-catenin in HCT116 in response to wild-type *Fn*, but not to US1 (*fadA*$^{-}$) (Fig. 5f). In addition, wild-type *Fn* and USF81 (*fadA*$^{+}$), but not US1 (*fadA*$^{-}$), activated the luciferase reporter gene in TOPFlash carrying the β-catenin-response promoter, but not in FOPFlash carrying β-catenin-non-response promoter (Fig 5g).

FadA promotes E-cadherin-mediated CRC tumor growth and induction of pro-inflammatory cytokines in xenograft mice

To examine the effects of FadA and *Fn* on CRC cell growth in vivo, HCT116 were inoculated into nude mice, followed by treatment with either purified protein or bacteria. Tumor growth was increased by 20% after 3 weeks of treatment by FadAc, compared to those treated with mFadA or BSA (Fig. 6a and 6d). No increase was detected in the presence of 0.01 mmol inhibitory peptide, but not the control peptide, indicating the role of FadA binding to E-cadherin in tumor growth (Fig. 6b and 6d). FadAc had no stimulatory effect on the non-E-cadherin-expressing RKO (Fig. 6c).

When wild-type *Fn* was injected into HCT116 xenografts, abscess formation was observed within 3–5 days (data not shown). Immunohistochemical analysis using anti-*Fn* antibodies showed that wild-type *Fn* invaded into the tumor tissues while US1 (*fadA*$^{-}$) did not (Fig. 6e). Nor did *E. coli* DH5α (data not shown). *Fn* invasion was prevented by the inhibitory peptide, but not by the control peptide (Fig. 6e). FadAc (Fig. S4) and wild-type *Fn* (Fig. 6f) stimulated the tumor growth genes and the inflammatory genes to same extent, which were inhibited by the inhibitory peptide, but not the control peptide, consistent with the observations in vitro.

Patients with CRC and precancerous adenomas have elevated FadA gene and expression levels compared to normal individuals

We examined FadA gene and expression levels in human colon specimens from the following 5 groups: (1) normal non-cancerous individuals (n=14), (2) normal tissues from patients with precancerous adenomas (n=16); (3) precancerous adenomas (n=16); (4) normal tissues from patients with adenocarcinomas (n=19); and (5) adenocarcinomas (n=19). A step-wise increase of FadA gene copies was observed from Group 1 to Group 2–4, and to Group 5, with >1 log difference between each step (Fig. 7a). The biggest difference was observed between the non-cancerous controls and CRC, with > 2 logs difference (Fig. 7a). The FadA mRNA levels in the colon tissues, when normalized to GAPDH, also showed a stepwise increase correlating with the FadA gene copy numbers (data not shown). When the FadA mRNA levels were normalized to *Fn* 16S RNA to reflect FadA expression in *Fn*, a significance increase was only observed in the carcinoma tissues (Group 5), indicating *Fn* exhibits increased virulence in CRC, compared to the normal and precancerous tissues (Fig. 7b). Consistent with the increase of FadA, expression of a representative Wnt gene, Wnt7b, and a representative inflammatory gene, NF kappa b2, were also significantly increased in CRC, corroborating with the results obtained in vitro and in xenograft mice.

DISCUSSION

CRC is the second leading cause of cancer death in men and women combined in the U.S. (ACS, 2012). Worldwide, over one million new cases are diagnosed each year (Bartlett and Chu, 2012). Understanding the mechanisms of CRC and identifying risk factors are important to human health. Increasing evidence supports a relationship between infective agents and various human malignancies (Plottel and Blaser, 2011). Previous studies have provided mechanistic insights into the microbial involvement in the development of CRC (Arthur et al., 2012; Cuevas-Ramos et al., 2010; Lee et al., 2010; Wu et al., 2009). It was

reported that *Bacteroides fagilis* enterotoxin BFT cleaves E-cadherin to activate the β-catenin signaling (Wu et al., 1998; 2003). Here, we report a mechanism by which *Fn* modulates E-cadherin/β-catenin signaling.

Fn significantly stimulates proliferation of E-cadherin-expressing CRC cells, but not those without E-cadherin. The stimulation requires FadA, an E-cadherin ligand. The *fadA*-deletion mutant US1 has weak stimulatory activity. These observations indicate that the interactions between FadA and E-cadherin play a primary role in promoting CRC tumor growth, while the FadA-independent pathway(s) play a minor role.

E-cadherin is a tumor suppressor, which functions through β-catenin (Bryant and Stow, 2004; Peifer and Polakis, 2000). It has been previously reported that E-cadherin is present in 100% of the normal mucosa of CRC patients and in 75% of cancer specimens (Mohri, 1997). Loss or heterogeneous expression of E-cadherin correlated with advanced stages of CRC and poor prognosis (Mohri, 1997; Dorudi et al., 1993). FadA binding to E-cadherin inhibits its tumor suppressor activity, resulting in increased CRT. This phenotype is consistent with the adenomatous polyposis coli (APC) mutation, the most common mutation in CRC (Kinzler and Vogelstein, 2002). The four human colon cancer cell lines stimulated by FadA, HCT116, DLD1, SW480, and HT29, carry either an APC mutation (DLD1, SW480, and HT29) (Yang et al., 2006) or a β-catenin mutation (HCT116) (Morin et al., 1997), thus have increased CRT background (Chan et al., 2002). FadA, both in purified form and in *Fn*, further enhanced CRT and stimulated their growth, attesting to its carcinogenic potency. Interestingly, FadA does not stimulate the growth of the non-cancerous HEK293, suggesting that it promotes oncogenesis after a mutation occurs.

FadA is the major component mediating *Fn* attachment and invasion (Xu et al., 2007). It binds to an 11-aa region in the EC5 domain on E-cadherin, and is internalized with E-cadherin. The 11-aa inhibitory peptide inhibits *Fn* from binding and invading, and abolishes all subsequent host responses, including tumor growth and inflammatory responses. Thus, this inhibitory peptide not only prevents *Fn* colonization but also inhibits *Fn*-mediated oncogenesis.

Inflammation plays a key role in CRC oncogenesis (Baron and Sandler, 2000; Ekbom et al., 1990;). In this issue of Cell Host and Microbe, Kostic et al showed that Fn generates a pro-inflammatory microenvironment that is conducive for colorectal neoplasia progression (Kostic et al., 2013). Both the observations herein and by Kostic et al support that inflammatory stimulation by *Fn* further reinforces the oncogenic potential of this microorganism. As shown in the xenografts, FadA alone was able to stimulate the inflammatory responses to the same extent as *Fn*, indicating FadA is the major stimulant of inflammation.

Our results reveal that tumor growth and inflammation are differentially regulated, although both require β-catenin, the master regulator. It is often difficult to distinguish the roles of adherence and invasion as these two processes are tightly correlated (Martin et al., 2004; Swidsinski et al., 1998), with the former being a prerequisite for the latter. Using the clathrin inhibitor, we are able to functionally separate *Fn* attachment from invasion and demonstrate that although invasion is not required for tumor growth, it is essential for the induction of inflammation, which is consistent with a previous report that the invasive potential of *Fn* correlates with the host IBD status (Strauss et al., 2011).

The mechanistic investigation is corroborated with studies in humans. Previous studies have shown that *Fn* is readily detected in the tissues, but not in the stools (Chen et al., 2012). This may be due to its adherent and invasive properties. Hence, we examined the presence of *Fn* in colon tissue specimens, rather than stools. The results unveil a stepwise increase of FadA

gene copies from the baseline of normal individuals to adenomas and normal tissues adjacent to adenomas and adenocarcinomas, and to adenocarcinomas. The "normal" tissues from patients with adenomas and adenocarcinomas are thus "pseudo-normal", compared to the non-cancerous controls. This is consistent with the findings by McCoy et al (McCoy et al., 2013). Given the fact that FadA mediates *Fn* binding to both CRC and non CRC cells, and the fact that the normal tissues also express E-cadherin, it is not surprising that *Fn* can colonize both tumor and non-tumor sites. Elevated *Fn* colonization in the normal tissues may predispose the host to the development of adenomas and/or adenocarcinoma, with carcinogenesis being accelerated when a mutation occurs. The fact that FadA expression levels in *Fn*, as well as the host inflammatory and oncogenic responses, are increased only in the cancerous tissues, but not in the precancerous state, further supports this notion.

The finding of elevated FadA levels in the precancerous state implies translational potential. Compared to the metagenomic approach in the previous studies (Castellarin et al., 2012; Kostic et al., 2012), qPCR is significantly less time consuming and more cost effective, suitable for clinical purposes. FadA is unique to *Fn* (Han et al., 2005), hence, is an ideal potential diagnostic marker to identify individuals at risk for developing adenomas and/or adenocarcinomas. Based on the results shown in Fig 7, it is possible that diagnostic criteria may be developed to define healthy, precancerous, and cancerous states according to FadA gene copy levels. One limitation of the current study is the small sample size. The potential use of FadA as a diagnostic marker needs to be evaluated in a much larger prospective cohort. Future studies are also warranted to examine the use of the inhibitory peptide and/or its derivatives to eradicate *Fn*, either as a preventive therapy for individuals with increased risk, or as a complementary therapy for those with CRC.

In conclusion, we have elucidated an oncogenic mechanism of CRC. Findings from this study will significantly impact our understanding, diagnosis, prevention and treatment of CRC.

EXPERIMENTAL PROCEDURES

Bacterial strains, cell cultures, construction of plasmids, protein purification and GST pull-down assays

Bacteria and human cell lines were cultured as previously described (Fardini et al., 2011; Han, 2006; Zhang et al., 2012). EC domains of E-cadherin were amplified by PCR from pcDNA3-E-cadherin (AddGene, MA) vector using primers listed in Table S1. Protein expression and purification, GST pull-down assays were all performed as previously described (Fardini et al., 2011; Xu et al., 2007).

Tissue culture attachment and invasion assays

These assays were performed as previously described (Han et al., 2000). Briefly, cells were seeded in 24-well or 96-well plates at 8×10^4 cells or 2.5×10^4 cells per well in the growth medium and grown to 100% confluent. Bacteria were added to the cells at a multiplicity of infection (MOI) of 50. Following 1 hr incubation at 37°C in 5% CO_2, the monolayers were washed 3 times with D-PBS, pH 7.1, supplemented with Ca^{2+} and Mg^{2+}. Cells were lyzed with water for 20 min at 37°C. Serial dilutions of the lysates were plated onto blood agar plates to enumerate the total cell-associated bacteria. For invasion assays, the bacteria were incubated with the monolayers at 37°C for 4 hrs, followed by washes with PBS. Fresh media containing 300 μg/ml gentamicin and 200 μg/ml metronidazole were added to the monolayers and incubated for an additional hour to kill extracellular bacteria. The cells were then washed and lyzed with water as described above. The levels of attachment and invasion were expressed as the percentage of bacteria recovered following cell lysis relative to the

total number of bacteria initially added. Each experiment was performed in triplicates and repeated at least twice.

Antibodies, peptides, and Western blot analysis

The following antibodies were used: anti-FadA monoclonal antibody (mAb) 5G11-3G8 (Xu et al., 2007), mAb anti-CDH1 (Abcam or Cell Signaling Tech), polyclonal anti-phospho-CDH1 (ECM Biosciences), mAb anti-GAPDH (Invitrogen), mAb anti-β-catenin (R&D systems), polyclonal anti-phospho-β-catenin (Cell Signaling Tech), mAb anti-LEF/TCF1 (Invitrogen), mAb anti-NFkB (Invitrogen), mAb anti-Myc (Cell Signaling Tech), mAb anti-cyclin D1 (Cell Signaling Tech), mAb anti-EGFR (Cell Signaling Tech), mAb anti-GAPDH (Cell Signaling Tech), mAb anti-PCNA (Cell Signaling Tech), polyclonal goat-anti-mouse or goat-anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (Pierce Biotechnology). Peptides were synthesized by Neo Group, Inc (Cambridge, MA). Western blot analyses were performed as previously described (Fardini et al., 2011).

Co-immunoprecipitation (Co-IP) assay

500 μg of HEK293 cell lysate prepared with CO-IP Lysis/Wash Buffer (Pierce) were mixed with 100 μg of BSA or *E. coli* lysate expressing FadAc or mFadA, followed by the addition of mouse monoclonal anti-CDH1 antibodies. The protein complex was captured by agarose A/G beads (Santa Cruz) as previously described (Chan et al., 2002), followed by elution and Western blot analysis. An equal volume of the elutes were loaded onto SDS-PAGE.

DNA and siRNA transfection

CDH1 transfections were performed as previously described (Lebreton et al., 2011). siRNA assays were performed using the FlexiTube siRNA anti-CDH1 or anti-beta-catenin reagent and All Stars FlexiTube Control siRNA from Qiagen (Valencia, CA) according the manufacturer's instructions.

Cell proliferation assay

CRC cells were seeded in 24-well plates at 1×10^4 cells per well in the growth medium. Cells were untreated, or incubated with FadAc or peptides at indicated concentration, or with bacteria at a multiplicity of infection (MOI) of 1000:1. Cell numbers were counted at 24-hour intervals using a hemocytometer. Each experiment was performed in triplicates and repeated at least twice.

Preparation of subcellular fractions

Cells were incubated with 1 mg/ml of FadAc or mFadA followed by the extraction of subcellular fractions. When indicated, 50 μM of the corresponding protein tyrosine kinase inhibitor (Genistein) and clathrin inhibitor (Pitstop2) were pre-incubated with cells for 1 hour. Membrane, cytosolic and nuclei fractions were prepared using the Compartmental Protein Extraction kit (Millipore) according to the manufacturer's instructions.

Luciferase reporter assay

HCT116 cells were seeded in 96-well plates at 2.5×10^4 cells per well in the growth medium and grown to 100% confluent. Cells were transfected with 0.2 μg of TOPFlash, a luciferase reporter vector carrying TCF promoter upstream of the luciferase gene and can be activated by β-catenin, or FOPFlash (with mutations rendering insensitivity to β-catenin activation), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. On the following day, cells were treated with bacteria at a multiplicity of infection (MOI) of 1000:1 for 2 hours. Reporter assays were performed using the luciferase reporter system (Promega, Madison, WI). The experiment was performed in triplicates and repeated twice.

Immunofluorescent and immunohistochemical (IHC) staining

Immunofluorescent staining of cells was performed as previously described. Mouse anti-β-catenin mAb and Alexa Fluor 634-conjugated goat anti-mouse polyclonal antibodies were used (Fardini et al., 2011). IHC analysis of xenograft tumors were performed as previously described, using rabbit anti-*Fn* polyclonal antibodies (Han et al., 2004). Pre-immune serum from the same rabbit was used as control.

Xenografts

The animal protocol was approved by the Case Western Reserve University Institutional Animal Care and Use Committee. An inoculum of 5×10^6 cells were injected s.c. and bilaterally into 4- to 6-week-old female nude mice (5 per group) as previously described (Zhang et al., 2012). The mice were randomized to receive one of the following: FadAc or mFadA (each at 80 μg), BSA (0.01 μmol, i.e. 660 μg), or peptide (0.01 μmol), or bacteria at 1×10^7 cfu, at each inoculation site. Xenograft volumes were calculated as previously described (Zhang et al., 2012).

Clinical specimens

This study was approved by the University Hospitals of Cleveland Institutional Review Board. A total of 19 cases diagnosed with colonic adenocarcinoma and 16 cases of adenomas were retrieved from files at the Department of Pathology, University Hospitals Case Medical Center, Cleveland, Ohio. H&E slides were reviewed to confirm the presence of adenocarcinoma (or adenoma). A representative block of colon adenocarcinoma (or adenoma) and a block of normal colon from the same patient were used. In addition, normal colon tissues were derived from 14 individuals undergoing resection for benign colon pathology or resection of adjacent organs. Exclusion criteria were history of gastrointestinal malignancies, presence of prominent inflammation or abscess, and history of inflammatory bowel disease. All cases were from within the last 12 months. Genomic DNA was extracted from formalin-fixed paraffin-embedded tissue samples as previously described (Tang et al., 2009). RNA was extracted using PureLink FFPE Total RNA Isolation Kit (Invitrogen) or RNeasy FFPE Kit (Qiagen).

Real-time Quantitative PCR (qPCR)

Total RNA was extracted from CRC cells, xenografts, or clinical specimens. cDNA synthesis and RT-PCR were performed as previously described, using primers listed in Table S1 (Lebreton et al., 2011). Data were analyzed by the ΔΔCt method (Livak and Schmittgen, 2001) and normalized to the GAPDH or *Fn*-specific 16S rRNA. To quantify the *fadA* gene copies, plasmid carrying *fadA* was serially diluted to 10^2–10^8 *fadA* copies/μl and used to generate standard curves for Ct values. The *fadA* gene copies in the clinical samples were calculated based on the standard curves. Each experiment was performed in triplicates and repeated at least three times.

Statistical analysis

The differences between groups were examined by two-tailed one-way analysis of variance (*ANOVA*) followed by Student-Newman-Keuls (SNK) test. For the clinical specimens the Kruskal–Wallis non-parametric test was performed followed by Conover test. $p<0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- *F. nucleatum* (*Fn*) adhesin FadA binds E-cadherin and promotes CRC cell proliferation
- A peptide from the FadA-binding region of E-cadherin prevents *Fn* mediated carcinogenesis
- FadA promotes inflammation and E-cadherin-mediated CRC tumor growth in xenograft mice
- Tissue from human adenomas and adenocarcinomas have elevated FadA expression levels

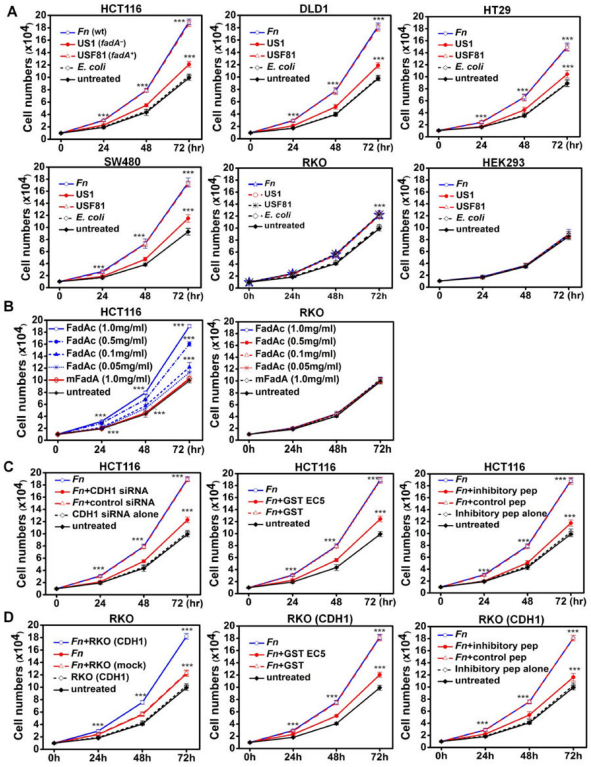

Figure 1. *Fn* and FadA stimulate proliferation of human colon cancer cells via E-cadherin

A. Wild type *Fn* (*Fn*) and the *fadA*-complementing USF81 (*fadA*$^{+}$) stimulated proliferation of human CRC cells HCT116, DLD1, SW480, and HT29, compared to untreated cells or those incubated with *E. coli*. US1 (*fadA*$^{-}$) only weakly stimulated their growth. *Fn*, USF81 and US1 all weakly stimulated the growth of CRC cells RKO, but not the non-CRC cells HEK293. B. Purified FadAc stimulated HCT116 cell growth in a dose-dependent manner, while mFadA did not. Neither FadAc nor mFadA stimulated RKO cell growth. C. Suppression of *Fn*-stimulated cell growth by inhibiting E-cadherin. *Fn*-stimulated HCT116 growth was inhibited by siRNA specific for CDH1, GST-EC5 fusion protein, and the inhibitory peptide (IP), but not by non-specific siRNA, GST, or the control peptide (CP). D. *Fn* stimulated the growth of RKO cells transfected with CDH1, but not mock-transfected RKO. Growth stimulation of CDH1-transfected RKO cells was suppressed by GST-EC5 and the inhibitory peptide, but not by GST or the control peptide. The results are presented as mean±SD. ***p<0.001. See also Figure S1.

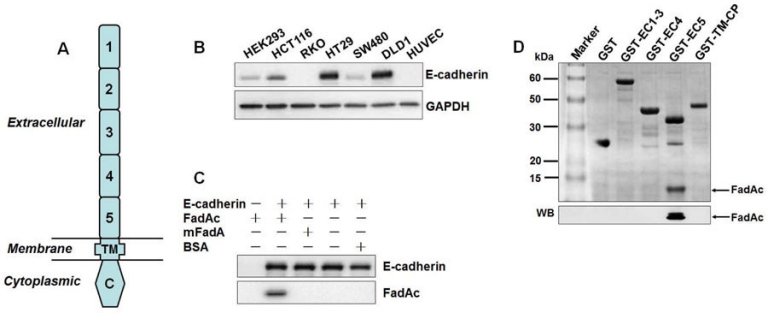

Figure 2. E-cadherin is FadA receptor

A. Schematic representation of the E-cadherin (CDH1) structure. E-cadherin has five extracellular cadherin (ECs) repeats, numbered EC1–5 starting from N-terminal. TM, transmembrane domain; C, cytoplasmic domain. B. E-cadherin is expressed in epithelial cell HEK293 and most CRC cells. E-cadherin in HEK293 and human CRC cell lines HCT116, RKO, HT29, SW480 and DLD1 was examined by Western blot. Human umbilical vein endothelial cells (HUVEC) were included as a negative control. The endogenous GAPDH was used as a loading control. C. E-cadherin co-immunoprecipitates with FadAc. HEK293 cell lysate expressing E-cadherin was mixed with *E. coli* lysates expressing FadAc, or mFadA, or BSA, followed by incubation with mouse anti-CDH1 monoclonal antibodies (mAb) and captured with agarose A/G beads. E-cadherin and FadA in the bead elutes were detected by Western blot. D. FadA binds to EC5. Purified GST or GST-fusion proteins carrying EC1–3, EC4, or EC5 were incubated with *E. coli* lysates expressing FadAc, followed by capture with GST resin. The eluted components were subjected to SDS-PAGE, followed by Coommassie blue staining (top panel) and Western blot (WB) using anti-FadA mAb 5G11-3G8 (bottom panel). See also Figure S2.

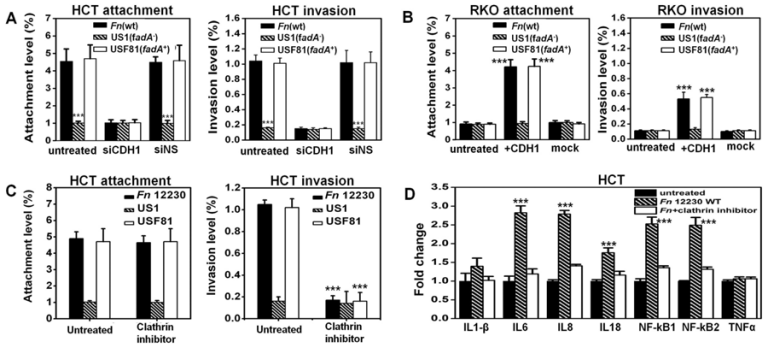

Figure 3. *Fn* adheres to and invades E-cadherin-expressing CRC cells

A. *Fn* adheres to and invades the E-cadherin-expressing HCT116 via FadA and E-cadherin. The *fadA*-deletion mutant US1 (*fadA*$^{-}$) was defective for attachment and invasion, compared to wild type *Fn* and the *fadA*-complementing clone USF81 (*fadA*$^{+}$). Transfection with siRNA to inhibit E-cadherin expression (siCDH1) reduced attachment and invasion, while the non-specific siRNA (siNS) did not. B. Wild-type *Fn*, US1, and USF81, were defective for attachment and invasion of the non-E-cadherin-expressing RKO cells. Transfection of full-length CDH1 into RKO enhanced attachment and invasion by wild type *Fn* and USF81 (*fadA*$^{+}$), but not by US1 (*fadA*$^{-}$). C. The clathrin inhibitor, Pitstop2, inhibits *Fn* and USF81 (*fadA*$^{+}$) invasion of HCT116, without affecting their attachment. D. Wild type *Fn* stimulates expression of NF-kappaB and pro-inflammatory cytokines IL-6, 8, and 18 in HCT116, which was inhibited by the clathrin inhibitor. Expression levels in untreated HCT116 were designated as "1". For A, B, and D, the attachment and invasion levels were expressed as percent bacteria recovered from the host cells relative to the initial inoculum. For wild type *Fn*, these levels reflect recovering approximately 9000 CFU per well (in a 96-well plate) from the attachment assay and approximately 2000 CFU from the invasion assay. The invasion level of *E. coli* DH5a into HCT116 was <0.01%, i.e. < 20 CFU recovered per well (data not shown). For C, the original attachment (4.4±0.8%) and invasion (1.3±0.1%) levels without inhibition were designated as "100%", and the relative inhibition values were shown. The results are presented as the mean±SD. ***p<0.001. See also Figure S3.

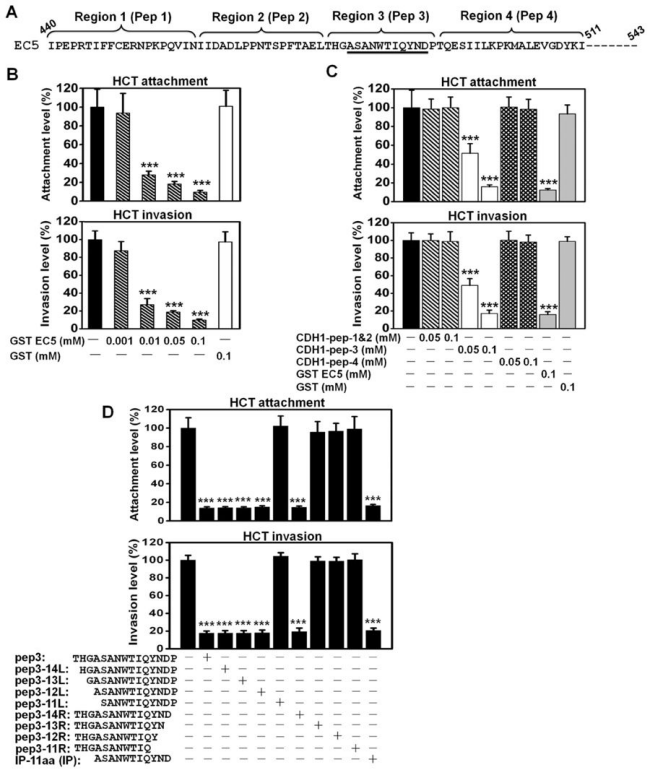

Figure 4. Identification of synthetic peptides to inhibit *Fn* attachment and invasion of HCT116 cells

A. Partial amino-acid sequence of the EC5 domain. The regions and the corresponding peptides (pep) are shown above the sequence. The sequences corresponding to the inhibitory peptide (IP, see below) are underlined. Peptide 4 was the control peptide (CP) in all studies. B. Purified GST-EC5 fusion protein inhibits wild type *Fn* attachment and invasion of HCT116 in a dose-dependent manner. C. *Fn* attachment and invasion of HCT116 cells were inhibited by a synthetic peptide corresponding to region 3 (pep 3) on the EC5 domain, not by peptides corresponding to regions1&2, or 4. D. The inhibitory effects of synthetic oligopeptides carrying sequential deletions from the N- and C-termini of region 3 on *Fn* attachment and invasion. Deletion of 3 residues from N-terminal and 1 residue form C-terminal did not affect the inhibitory function. An 11-aa peptide (ASANWTIQYND) was found as the minimum sequence required for inhibition of *Fn* attachment and invasion. All values were expressed as relative to those without inhibition, which were designated as "100%". The actual attachment and invasion levels were 6.3±1.4% and 1.6±0.1%, respectively, for B, and 5.9±0.7% and 1.4±0.1%, respectively, for C. The results are presented as mean±SD. ***$p<0.001$.

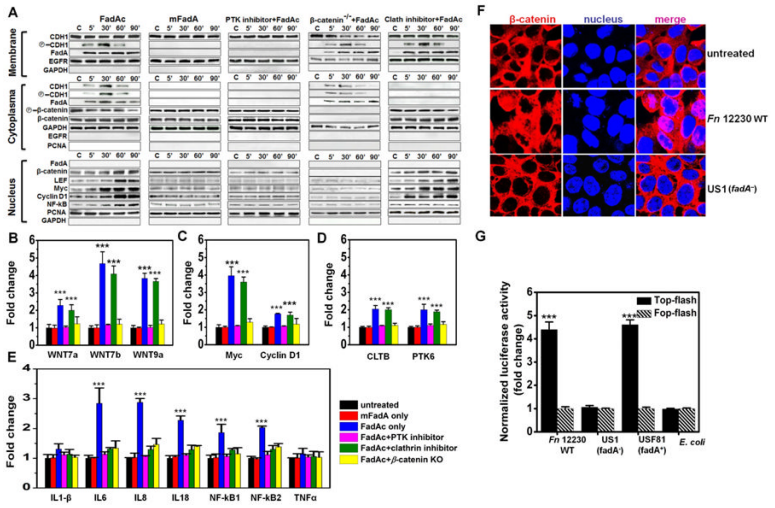

Figure 5. FadAc activates E-cadherin-mediated cellular signaling

A. FadAc, but not mFadA, binds to the membranes of HCT116, accompanied by phosphorylation of E-cadherin on the membrane; internalization of E-cadherin and FadA, reduced phosphorylation of β-catenin, and accumulation of β-catenin in the cytoplasma; and translocation of β-catenin and activation of transcription factors lymphoid enhancer factor (LEF)/T-cell factor (TCF), NF kappaB, and oncogenes Myc and Cyclin D1 in the nuclei; all as detected by Western blot. Protein tyrosine kinase (PTK) inhibitor, Genistein, inhibits all FadAc-activated functions. No gene activation was detected in the HCT116 β-catenin$^{-/-}$ cells, despite binding and internalization of FadA and E-cadherin. The clathrin inhibitor, Pitstop2, prevented E-cadherin and FadA internalization and activation of NF kappaB, but did not affect nuclei translocation of β-catenin or expression of LEF/TCF, Myc or Cyclin D1. The epidermal growth factor receptor (EGFR), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and proliferating cell nuclear antigen (PCNA) were used as loading controls for membrane, cytoplasmic, and nucleus, respectively, and for examination for cross-contamination between the subcellular fractions. B–E. FadAc, but not mFadA or BSA, activates expression of Wnt signaling genes 7a, 7b, 9a (B), oncogenes Myc and Cyclin D1 (C), clathrin (cltb) and protein tyrosine kinase genes (ptk6) (D), and NF-kappaB and pro-inflammatory cytokines IL-6, 8, and 18 (E) in wild-type or β-catenin knockout HCT116 cells following 2 hrs incubation as determined by qPCR. The clathrin inhibitor inhibited expression of the inflammatory genes but not the Wnt or oncogenes, while inhibition of β-catenin by siRNA suppressed expression of all genes. Expression levels in untreated HCT116 were designated as “1”. F. Wild type *Fn* (*Fn*), but not US1 (*fadA*$^{-}$), induces nuclei translocation of β-catenin following 2 hrs incubation as observed by confocal microscopy. β-catenin was stained with Alex 634 (red) and the nuclei with 4',6-diamidino-2-phenylindole (DAPI) (blue). The purple color in the merged images indicates translocation of β-catenin into the nucleus. G. Luciferase reporter gene expression following HCT116 transfection with TOPFlash (activated by β-catenin) or FOPFlash (insensitive to β-catenin activation). *Fn* was incubated with the transfected cells at a MOI of 1,000:1 for 2 hours, followed by measurement of the luciferase activity. Values obtained with FOPFlash were designated as “1” and those obtained with TOPFlash were expressed as fold changes. Data are presented as mean fold changes ±SD of two independent experiments, each in triplicate. ***p<0.001.

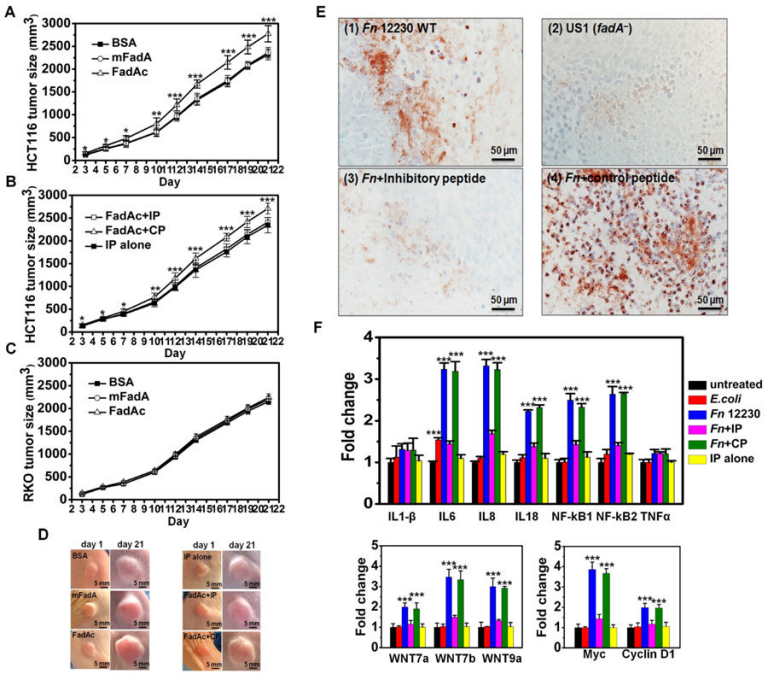

Figure 6. FadA promotes E-cadherin-mediated CRC tumor growth and induction of pro-inflammatory cytokines in xenograft mice

HCT116 or RKO were injected subcutaneously and bilaterally into female nude mice, which were then randomized (5 per group) to receive treatments. A–C. FadAc stimulates HCT116 but not RKO xenograft growth. Purified FadAc, mFadA, or BSA were injected into xenografts of HCT116 either alone (A), or along with the inhibitory (IP) or control (CP) peptides (B), or RKO alone (C) in nude mice. IP alone is injected into HCT116 as a negative control (B). D. Representative tumors from A and B are shown. The first day of protein injection was designated as “day 1”. All tumors look the same on day 1. Notice the size increase of the tumor treated with FadAc and FadAc+CP on day 21, compared to other tumors on the same day. E. Immunohistochemical staining of xenografts infected with wild-type *Fn* (*Fn*), alone and with the inhibitory or control peptides, and the *fadA*-deletion mutant US1 (*fadA*⁻) using rabbit anti-*Fn* polyclonal antibodies. For controls, xenografts infected with wild-type *Fn* was stained with pre-serum, and xenografts infected with *E. coli* DH5α were stained with anti-*Fn* antibodies (data not shown). F. Wild-type *Fn* induces expression of NF-kappaB and pro-inflammatory cytokines IL-6, 8, and 18; Wnt 7a, 7b, and 9a; and Myc and Cyclin D1 in HCT116 xenografts, as determined by qPCR. The inductions were inhibited by IP, but not by CP. *E. coli* only weakly induced IL-6. The results are presented as mean±SD. ***$p<0.001$. See also Figure S4.

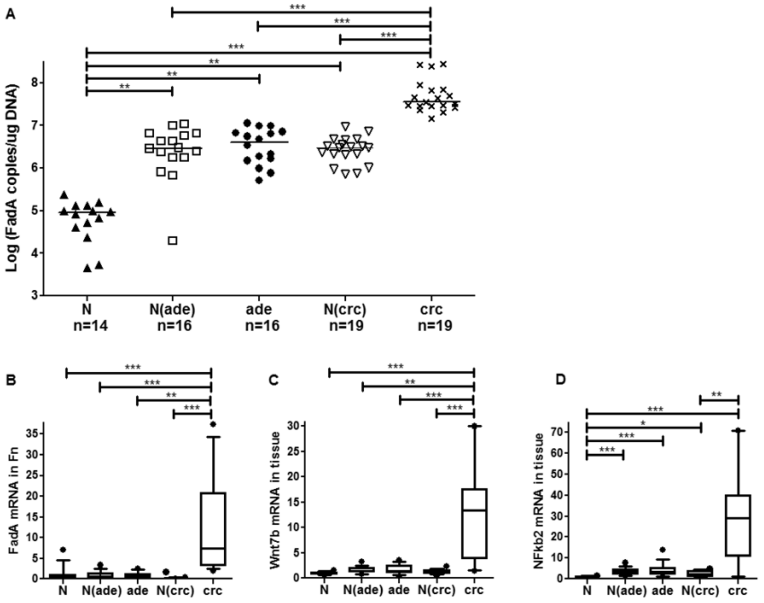

Figure 7. Quantification of *fadA* gene copies and FadA, Wnt7b and NFkB2 expression in health, precancerous adenomas, and carcinomas

DNA and RNA were extracted from full-thickness colon specimens from the following 5 groups: (1) normal non-cancerous controls (N; n=14); (2) Normal tissues from patients with precancerous adenomas [N(ade); n=16]; (3) Precancerous adenomas (ade; n=16); (4) Normal tissues from patients with carcinomas [N(crc); n=19]; and (5) Carcinomas (crc; n=19). Gene copy numbers of *fadA* (A) were measured using DNA and determined using the standard curves. FadA mRNA levels in *Fn* were normalized to *Fn* 16 rRNA (B), and Wnt7b (C) and NFkb2 (D) mRNA levels were each normalized to the endogenous GAPDH. The average value of Group 1 (N) was designated “1”, and the fold changes of the other groups were determined by comparing to Group 1. The horizontal bars in a represent the median values. For B–D, the boxes show the 25/75 percentiles and the lines within the boxes the median values. Whiskers show the 10/90 percentiles. *p<0.05, **p<0.01 and *** p<0.001.