Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

HHS Public Access
Author manuscript
Science. Author manuscript; available in PMC 2018 December 15.

Published in final edited form as:
Science. 2017 December 15; 358(6369): 1443–1448. doi:10.1126/science.aal5240.

Analysis of *Fusobacterium* persistence and antibiotic response in colorectal cancer

Susan Bullman[1,2], **Chandra S. Pedamallu**[1,2], **Ewa Sicinska**[1], **Thomas E. Clancy**[3], **Xiaoyang Zhang**[1,2], **Diana Cai**[1,2], **Donna Neuberg**[1], **Katherine Huang**[2], **Fatima Guevara**[1], **Timothy Nelson**[1], **Otari Chipashvili**[1], **Timothy Hagan**[1], **Mark Walker**[2], **Aruna Ramachandran**[1,2], **Begoña Diosdado**[1,2], **Garazi Serna**[4], **Nuria Mulet**[4], **Stefania Landolfi**[4], **Santiago Ramon y Cajal**[4], **Roberta Fasani**[4], **Andrew J. Aguirre**[1,2,3], **Kimmie Ng**[1], **Elena Élez**[4], **Shuji Ogino**[1,3,5], **Josep Tabernero**[4], **Charles S. Fuchs**[6], **William C. Hahn**[1,2,3], **Paolo Nuciforo**[4], and **Matthew Meyerson**[1,2,3,*]

[1]Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA

[2]Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts, USA

[3]Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115, USA

[4]Vall d'Hebron University Hospital, Vall d'Hebron Institute of Oncology, Barcelona, CIBERONC, Universitat Autònoma de Barcelona, Spain

[5]Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

[6]Yale Cancer Center, Yale School of Medicine, New Haven, Connecticut, USA

Abstract

Colorectal cancers comprise a complex mixture of malignant cells, non-transformed cells, and microorganisms. *Fusobacterium nucleatum* is among the most prevalent bacterial species in colorectal cancer tissues. Here we show that colonization of human colorectal cancers with *Fusobacterium* and its associated microbiome, —including *Bacteroides*, *Selenomonas*, and *Prevotella* species, —is maintained in distal metastases, demonstrating microbiome stability between paired primary and -metastatic tumors. In situ hybridization analysis revealed that *Fusobacterium* is predominantly associated with cancer cells in the metastatic lesions. Mouse xenografts of human primary colorectal adenocarcinomas were found to retain viable *Fusobacterium* and its associated microbiome through successive passages. Treatment of mice bearing a colon cancer xenograft with the antibiotic metronidazole reduced *Fusobacterium* load, cancer cell proliferation, and overall tumor growth. These observations argue for further investigation of antimicrobial interventions as a potential treatment for patients with *Fusobacterium*-associated colorectal cancer.

[*]Correspondence to: matthew_meyerson@dfci.harvard.edu.

Supplementary Materials
Materials and Methods
Figs. S1 to S15 Tables S1 to S9

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

The cancer-associated microbiota is known to influence cancer development and progression, most notably for colorectal cancer (1–5). Unbiased genomic analyses have revealed an enrichment of *Fusobacterium nucleatum* in human colon cancers and adenomas relative to non-cancerous colon tissues (6, 7). These observations have been confirmed in studies of multiple colon cancer patient cohorts from around the world (8–12). Increased tumor levels of *F. nucleatum* have been correlated with lower T-cell infiltration (13); with advanced disease stage and poorer patient survival (10, 11, 14); and with clinical and molecular characteristics such as right-sided anatomic location, BRAF mutation, and hypermutation with microsatellite instability (9, 12, 15).

Studies in diverse experimental models have suggested a pro-tumorigenic role for *Fusobacterium*. Feeding mice with *Fusobacterium* (16–18), infection of colorectal cancer cell lines with *Fusobacterium* (19–21), and generation of xenografts derived from *Fusobacterium*-infected colorectal cancer cell lines (17) were all observed to potentiate tumor cell growth. Suggested mechanisms have ranged from enhanced tumor cell adhesion and invasion (17, 19, 22), to modulation of the host immune response (16, 23), to activation of the Toll-like receptor 4 pathway (17, 20, 21). However, not all animal or cellular studies of *Fusobacterium* have demonstrated a cancer-promoting effect (24). A recent editorial has highlighted the importance of studying *Fusobacterium* infection in colon cancer as a component of the diverse microbiota within the native tumor microenvironment (25).

To investigate the role of *Fusobacterium* and its associated microbiota in native human colorectal cancers, we analyzed five independent cohorts of patient-derived colorectal cancers for *Fusobacterium* and microbiome RNA and/or DNA. Where technically possible, we performed *Fusobacterium* culture and tested the effect of antibiotic treatment upon the growth of propagated patient-derived colon cancer xenografts. These cohorts (table S1) include: (i) 11 fresh-frozen primary colorectal cancers and paired liver metastases (frozen paired cohort); (ii) 77 fresh-frozen primary colorectal cancers with detailed recurrence information (frozen primary cohort); (iii) published data from 430 resected fresh-frozen colon carcinomas from The Cancer Genome Atlas (26) (TCGA cohort), together with data from 201 resected fresh-frozen hepatocellular carcinomas from TCGA (27); (iv) 101 formalin-fixed paraffin-embedded colorectal carcinomas and paired liver metastases (FFPE paired cohort); and (v) 13 fresh primary colorectal cancers used for patient-derived xenograft studies (xenograft cohort).

Using the frozen paired cohort, we tested whether we could culture viable *Fusobacterium* from primary colorectal carcinomas and corresponding liver metastases. Quantitative polymerase chain reaction (qPCR) studies showed that 9 of 11 (82%) snap-frozen primary tumors (table S2) were positive for *Fusobacterium* in the primary tumor [patients one through nine (P1 through P9)]; we could isolate *Fusobacterium* from 73% of these tumors (n = 8 of 11 tumors; P1 through P8) (Fig. 1A). In addition, we cultured *Fusobacterium* from two liver metastases (P1 and P2) from *Fusobacterium*-positive primary tumors. Five metastatic specimens had inadequate amounts of tissue for culture but were positive for *Fusobacterium* by qPCR (P3 through P7), for a total of seven primary-metastatic tumor pairs (64%) testing positive for *Fusobacterium* by qPCR (Fig. 1A). This finding extends previous results showing the presence of *Fusobacterium* nucleic acids in hepatic and lymph node

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

metastases of colon cancer (7, 22, 28) to now demonstrate that viable *Fusobacterium* are present in distant metastases.

To address whether the same *Fusobacterium* is present in primary cancers and metastases, we performed whole-genome sequencing of pure *Fusobacterium* isolates from primary and metastatic tumors from two patients (P1 and P2). For both patients, the primary-metastatic tumor pairs harbored highly similar strains of *Fusobacterium*, with >99.9% average nucleotide identity, despite the tissue being collected months (P2) or even years (P1) apart (Fig. 1B and fig. S1). We cultured *Fusobacterium. necrophorum* subsp. *funduliforme* from the primary colorectal tumor and liver metastasis of P1 and *F. nucleatum* subsp. *animalis* from the primary tumor and metastasis of P2. We also cultured other anaerobes, including *Bacteroides* species, from the primary-metastasis pairs (table S3). Our finding of nearly identical, viable *Fusobacterium* strains in matched primary and metastatic colorectal cancers confirms the persistence of viable *Fusobacterium* through the metastatic process and suggests that *Fusobacterium* may migrate with the colorectal cancer cells to the metastatic site.

To quantitate the relative abundance (RA) of *Fusobacterium* and to evaluate the overall microbiome in the paired primary and metastatic tumors, we performed RNA sequencing of 10 primary cancers and their matched liver metastases from the frozen paired cohort (P1 to P6 and P8 to -P11). PathSeq analysis (29) of the RNA sequencing data showed that the same *Fusobacterium* species were present, at a similar relative abundance, in the paired primary-metastatic tumors (Fig. 1C, samples P1 to P6) and that the overall dominant microbiome was also qualitatively similar. In addition to *F. nucleatum* and *F. necrophorum*, primary cancer microbes that persisted in the liver metastases included *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, and several typically oral anaerobes such as *Prevotella intermedia* and *Selenomonas sputigena* (Fig. 1C). In contrast, there was little similarity between bacterial sequences in the primary colorectal cancer and liver metastasis in the lone sample where *Fusobacterium* was present in the primary cancer but not detected in the metastasis (Fig. 1C, sample P8) or in the three samples with low or undetectable levels of *Fusobacterium* in the primary cancer (Fig. 1C, samples P9 to -P11). Jaccard index analysis revealed a high correlation between the dominant bacterial genera in the primary tumor and metastasis for *Fusobacterium*-positive pairs, but a low correlation between bacterial genera in the primary tumor and metastasis for *Fusobacterium*-negative pairs (Fig. 1D and fig. S2).

Targeted bacterial 16S ribosomal RNA (rRNA) gene sequencing on DNA from the 11 frozen paired samples confirmed that (i) *Fusobacterium* species are present in paired primary-metastatic tumors, (ii) the relative abundance of *Fusobacterium* is correlated between primary tumors and metastases, and (iii) the dominant microbial genera in the liver metastases correspond to those in the primary tumors, demonstrating microbiome stability between paired *Fusobacterium*-positive primary-metastatic tumors (P= 0.01), (fig. S3).

To investigate the relationship between *Fusobacterium* and cancer recurrence, we performed microbial culture and bacterial 16S rRNA gene sequencing in a blinded fashion on the "frozen primary cohort" of 77 snap-frozen colorectal cancers lacking paired metastases (n = 21 with recurrence, n = 56 without recurrence) (table S4), discovered that 44 of 77 tumors

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(57%) had cultivable *Fusobacterium* species and 45 of 77 had >1% *Fusobacterium* relative abundance. We found no correlation between *Fusobacterium* load or culture with either recurrence or stable disease, in this cohort (fig. S4). To assess *Fusobacterium* persistence and its correlation with clinical parameters, we analyzed the 101 primary-metastasis pairs from the FFPE paired cohort (table S5). We found that 43% (n = 44 of 101) of primary colorectal cancers tested positive for *Fusobacterium* by qPCR and 45% (n = 20 of 44) of liver metastases arising from these primary tumors were *Fusobacterium*-positive (fig. S5A). To determine the spatial distribution of *Fusobacterium* in these tumors, *Fusobacterium* RNA *in situ* hybridization (ISH) analysis was performed on five qPCR-positive primary-metastasis pairs from this cohort (table S6, Fig. 2, and fig. S6). Both biofilm and invasive *F. nucleatum* were observed in primary colorectal cancer (Fig. 2, A to D). Invasive *F. nucleatum* distribution was highly heterogeneous and focal, found in isolated or small groups of cells with morphology consistent with that of malignant cells, and located close to the lumen and ulcerated regions. *F. nucleatum* was also observed in glandular structures present in the tumor center and invasive margins, but to a lesser extent. In adjacent normal mucosa (when present), *F. nucleatum* was exclusively located in the biofilm. In liver metastasis, *F. nucleatum* was predominantly localized in isolated cells whose histomorphology is consistent with colon cancer cells (Fig. 2, E to H), although occasional stromal *F. nucleatum* could be observed as well. No *F. nucleatum* was detected in the adjacent residual liver parenchyma.

Notably, none of the 57 *Fusobacterium*-negative primary colorectal tumors were associated with a Fusobacterium-positive liver metastasis (n = 0 of 57; P = 0) (fig. S5A). Consistent with previous reports (15), the presence of *Fusobacterium* in paired primary tumors and corresponding metastases was enriched in metastatic cancers of the cecum and ascending colon cancers (n = 10 of 20 *Fusobacterium*-positive primary-metastasis pairs, P = 0.002), (fig. S5B), whereas cancers that were *Fusobacterium*-negative in both primary and metastatic lesions were more likely to be rectal cancers (n = 29 of 57 of the *Fusobacterium*-negative primary-metastasis pairs, P = 0.016), (fig. S5B). To assess the relationship between patient survival and *Fusobacterium* presence in the primary cecum and ascending colon, we carried out PathSeq (29) analysis on RNA sequencing data from the 430 primary colon adenocarcinomas in the TCGA cohort. Patients with cancer of the cecum and ascending colon exhibited worse overall survival than patients with non-cecum ascending colon cancer (P = 0.01) (fig. S5C). Among patients with cecum and ascending colon tumors, we observed poorer overall survival in correlation with tumor *Fusobacterium* load (fig. S5D), (P = 0.004).

To determine whether *Fusobacterium* is associated with primary liver hepatocellular carcinoma, we performed PathSeq analysis (29) of RNA sequencing data from 201 primary liver tumors from the TCGA cohort. This analysis demonstrated that *Fusobacterium* is rare in primary liver carcinomas and that the relative abundance of *Fusobacterium* is significantly enriched in liver metastases arising from colorectal cancers compared with primary liver cancers (P = 0.008) (Fig. 1E). PathSeq analysis of data from the TCGA cohort also confirmed that the microbes present in liver metastases of *Fusobacterium*-positive colorectal carcinomas are similar to those associated with *Fusobacterium* in primary colorectal carcinoma. *Selenomonas*, *Bacteroides*, and *Prevotella* genera were shared between primary

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and metastatic colorectal cancers and also correlated with *Fusobacterium* abundance in primary colon adenocarcinoma (Fig. 1F, fig. S7, and table S7).

Given that metastatic colorectal carcinomas harbored cultivable *Fusobacterium*, we wondered whether viable *Fusobacterium* could persist in xenografts from human colorectal cancers, which would provide a valuable model system for evaluating the effects of microbiota modulation on cancer growth. In a double-blinded approach, 13 fresh human primary colorectal tumors from the xenograft cohort were evaluated, by culture or qPCR, for the presence of *Fusobacterium*. In parallel, these tumors were implanted subcutaneously, by an independent investigator, into Nu/Nu mice to establish patient-derived xenografts (PDXs) (table S8). All five *Fusobacterium*–culture positive tumors resulted in successful xenografts (fig. S8), one of four qPCR-positive but culture-negative tumors gave rise to a successful xenograft, and none of the four *Fusobacterium*-negative tumors generated successful xenografts (P = 0.003). Tumor grade did not appear to significantly influence successful xenograft formation (P = 0.1) (fig. S9A), although we noted a modest association between *Fusobacterium*-cultivability and high-grade tumors in this cohort (n = 4 of 5 tumors, P = 0.03) (fig. S9B).

Next, we sought to determine whether *Fusobacterium* would remain viable and stably associated with a xenograft. A PDX derived from an *F. nucleatum* culture–positive colon cancer (COCA36) was passaged to F8, over 29 weeks, and tested for *F. nucleatum*. We cultured *F. nucleatum* from this PDX for up to four generations and 124 days *in vivo*. All xenograft generations, from F1 through F8, were positive for *Fusobacterium* by qPCR (Fig. 3A). Additionally, we cultured other anaerobic bacteria, including *B. fragilis* and B. thetaiotaomicron, from both the primary tumor and PDXs. We further cultured *Fusobacterium* from PDXs generated from two additional patient tumors (table S9). qPCR and microbiome analysis of fecal pellets and oral swabs from the PDX-bearing animals were negative for *Fusobacterium* species (fig. S10), arguing against the possibility of *Fusobacterium* arising from the endogenous murine microbiota.

To evaluate the overall microbiome stability and to identify bacteria that are persistently associated with the primary colorectal tumor and derived xenografts, we carried out unbiased total RNA sequencing followed by PathSeq analysis, which revealed that *F. nucleatum* and other Gram-negative anaerobes, including *B. fragilis* and *S. sputigena*, persist in these PDX models for multiple generations (Fig. 3B). The bacteria that persist within the PDX include the genera that we report to persist in distant-site metastases to the liver (Fig. 1C) and that are enriched in *Fusobacterium*-associated colorectal cancer from analysis of TCGA data (Fig. 1F). Bacterial 16S rRNA gene sequencing further confirmed the persistence of *Fusobacterium* and co-occurring anaerobes in these primary colorectal tumors and derived xenografts (fig. S11).

Transmission electron microscopy showed that *F. nucleatum* isolates from both the primary colon carcinoma and PDX were invasive when incubated with human colon cancer cell lines HT-29 and HCT-116. Upon infection with *F. nucleatum*, we saw evidence of bacterial cells within vesicle-like structures in the cancer cell (fig. S12, A to C). We also observed evidence of bacterial adhesion and invasion in the respective patient xenograft tissue (fig. S12D).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Finally, we asked whether treatment of *Fusobacterium*-positive colon cancer xenografts with either (i) an antibiotic to which *Fusobacterium* is resistant or (ii) an antibiotic to which *Fusobacterium* is sensitive would affect tumor growth. We chose erythromycin as a resistant antibiotic because the *F. nucleatum* clinical isolates were resistant to high concentrations of erythromycin (minimum inhibitory concentration >25 μg/ml) (fig. S13A). After oral gavage of the *Fusobacterium*-harboring PDX COCA36, with erythromycin, we observed a slight decrease in tumor volume compared with mice treated with the vehicle control. However, erythromycin did not significantly affect the trajectory of tumor growth ($P = 0.073$) (fig. S13B), *Fusobacterium* tumor load ($P = 0.98$) (fig. S13C), or tumor cell proliferation ($P = 0.3$) (fig. S13D).

For a *Fusobacterium*-killing antibiotic, we chose metronidazole because fusobacteria are known to be highly sensitive to this drug (30). We then confirmed sensitivity of the *F. nucleatum* isolate from PDX COCA36 (minimum inhibitory concentration < 0.01 μg/ml) (fig. S14). Because PDXs could not be generated from *Fusobacterium*-negative primary tumors, we treated *Fusobacterium*-free xenografts derived from HT-29 colon adenocarcinoma cells with metronidazole to assess whether metronidazole inhibits the growth of *Fusobacterium*-negative colorectal carcinomas. This experiment revealed no significant change in tumor growth ($P = 0.88$) (Fig. 4A).

Finally, oral administration of metronidazole to mice bearing *Fusobacterium*-positive PDXs resulted in a statistically significant decrease in the trajectory of tumor growth, compared with PDXs in mice treated with vehicle ($P = 0.0005$) (Fig. 4A). Treatment with metronidazole was associated with a significant decrease in *Fusobacterium* load in the tumor tissue ($P = 0.002$) (Fig. 4B), as well as a significant reduction in tumor cell proliferation ($P = 0.002$) (Fig. 4C and fig. S15).

We have shown that (i) *Fusobacterium* is persistently associated with distant metastases from primary human colorectal cancers; (ii) invasive *Fusobacterium* can be detected in liver metastases by ISH; (iii) *Fusobacterium* co-occurs with other Gram-negative anaerobes in primary and matched metastatic tumors; (iv) *Fusobacterium* survives in colorectal cancer PDXs through multiple generations; and (v) treatment of a *Fusobacterium*-harboring PDX model with the antibiotic metronidazole decreases *Fusobacterium* load, cancer cell proliferation, and tumor growth. The persistence of *Fusobacterium* and its associated microbiome in both metastasis and PDXs, as well as the ability of antibiotic treatment to reduce PDX growth, point to the potential of *Fusobacterium*, and its associated microbiota, to contribute to colorectal cancer growth and metastasis. On the basis of our observation that the dominant microbiome is highly similar in primary-metastatic pairs and the concordance of *Fusobacterium* strains found in primary tumors and paired metastases, we hypothesize that *Fusobacterium* travels with the primary tumor cells to distant sites, as part of metastatic tissue colonization. This suggests that the tumor microbiota are intrinsic and essential components of the cancer microenvironment.

Our results highlight the need for further studies on microbiota modulation as a potential treatment for *Fusobacterium*-associated colorectal carcinomas. One concern is the negative effect of broad spectrum antibiotics on the healthy intestinal microbiota. Given that

Author Manuscript

metronidazole targets a range of anaerobic bacteria, including co-occurring anaerobes that persist with *Fusobacterium*, one would ideally want to develop a *Fusobacterium*-specific antimicrobial agent to assess the effect of selective targeting of *Fusobacterium* on tumor growth. Important questions raised by our findings are whether conventional chemotherapeutic regimens for colorectal cancer will affect the colon cancer microbiota and whether the microbiota will modulate the response to such therapies. A recent study, reporting that colorectal tumors with a high *Fusobacterium* load are more likely to develop recurrence (21), supports the concept that *Fusobacterium*-positive tumors may benefit from anti-fusobacterial therapy. Our results provide a strong foundation for pursuing targeted approaches for colorectal cancer treatment directed against *Fusobacterium* and other key constituents of the cancer microbiota.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants (R35 CA197568 to M.M., R35 CA197735 to S.O., K07 CA148894 to K.N., and R01 CA118553 and R01 CA169141 to C.S.F.) and Dana-Farber/Harvard Cancer Center GI SPORE P50 grant CA 127003 to C.S.F. (this grant also supports S.B., A.J.A., W.C.H., K.N., and S.O.). In addition, M.M. is supported by an American Cancer Society Research Professorship; W.C.H. is supported by the Hale Family Center for Pancreatic Cancer; C.S.F. is supported by the Project P Fund for Colorectal Cancer Research, Stand-up-to-Cancer (Colorectal Cancer Dream Team), the Chambers Family Fund for Colorectal Cancer Research, the Team Perry Fund, and the Clark Family Fund for GI Cancer Research; S.B. is supported by a Prevent Cancer Foundation Figdor Family Fellowship; G.S., N.M., E.E., P.N., and J.T. are supported by the Cellex Private Foundation; and P.N. is supported by the Banco Bilbao Vizcaya Argentaria Foundation. M.M. is on the Scientific Advisory Board of and holds stock in OrigiMed, a biotechnology company that provides sequencing information for cancer diagnostics. C.S.F. is on the Board of CytomX Therapeutics, a biotechnology company that is developing therapeutic antibodies for cancer, and is a paid consultant for Eli Lilly, Genentech, Merck, Sanofi, Five Prime Therapeutics, Merrimack Pharmaceuticals, Bayer, Agios Pharmaceuticals, Taiho Oncology, and KEW Group. M.M. and S.B. are inventors on U.S. Provisional Patent Application no. 62/534,672, submitted by the Broad Institute and Dana-Farber Cancer Institute, that covers targeting of Fusobacterium for treatment of colorectal cancer. All raw sequencing data from this study can be accessed at the National Center for Biotechnology Information (NCBI) under the bioproject PRJNA362951. Bacterial whole-genome sequences have been deposited at DNA Data Bank of Japan/European Nucleotide Archive/GenBank, with the following NCBI accession, GenBank assembly accession, and BioSample numbers, respectively: *F. necrophorum* subsp. *funduliforme* P1_CP patient P1 primary colorectal tumor (NPNF00000000, GCA_002761995.1, and SAMN07448029), *F. necrophorum* subsp. *funduliforme* P1_LM patient P1 liver metastasis (NPNE00000000, GCA_002762025.1, and SAMN07448030), *F. nucleatum* subsp. *animalis* P2_CP patient P2 primary colorectal tumor (NPND00000000, GCA_002762005.1, and SAMN07448031), and *F. nucleatum* subsp. *animalis* P2_LM patient P2 liver metastasis (NPNC00000000, GCA_002762015.1, and SAMN07448032).

References and Notes

1. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan TJ, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science. 2012; 338:120–123. DOI: 10.1126/science.1224820 [PubMed: 22903521]
2. Hope ME, Hold GL, Kain R, El-Omar EM. Sporadic colorectal cancer – role of the commensal microbiota. FEMS Microbiol Lett. 2005; 244:1–7. DOI: 10.1016/j.femsle.2005.01.029 [PubMed: 15727814]
3. Rowland IR. The role of the gastrointestinal microbiota in colorectal cancer. Curr Pharm Des. 2009; 15:1524–1527. DOI: 10.2174/138161209788168191 [PubMed: 19442169]
4. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, Sears CL. A human colonic commensal promotes colon

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009; 15:1016–1022. DOI: 10.1038/nm.2015 [PubMed: 19701202]

5. Yang L, Pei Z. Bacteria, inflammation, and colon cancer. World J Gastroenterol. 2006; 12:6741–6746. DOI: 10.3748/wjg.v12.i42.6741 [PubMed: 17106919]
6. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res. 2012; 22:299–306. DOI: 10.1101/gr.126516.111 [PubMed: 22009989]
7. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, Ojesina AI, Jung J, Bass AJ, Tabernero J, Baselga J, Liu C, Shivdasani RA, Ogino S, Birren BW, Huttenhower C, Garrett WS, Meyerson M. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Res. 2012; 22:292–298. DOI: 10.1101/gr.126573.111 [PubMed: 22009990]
8. McCoy AN, Araújo-Pérez F, Azcárate-Peril A, Yeh JJ, Sandler RS, Keku TO. Fusobacterium is associated with colorectal adenomas. PLOS ONE. 2013; 8:e53653.doi: 10.1371/journal.pone.0053653 [PubMed: 23335968]
9. Tahara T, Yamamoto E, Suzuki H, Maruyama R, Chung W, Garriga J, Jelinek J, Yamano HO, Sugai T, An B, Shureiqi I, Toyota M, Kondo Y, Estécio MRH, Issa J-PJ. Fusobacterium in colonic flora and molecular features of colorectal carcinoma. Cancer Res. 2014; 74:1311–1318. DOI: 10.1158/0008-5472.CAN-13-1865 [PubMed: 24385213]
10. Flanagan L, Schmid J, Ebert M, Soucek P, Kunicka T, Liska V, Bruha J, Neary P, Dezeeuw N, Tommasino M, Jenab M, Prehn JHM, Hughes DJ. Fusobacterium nucleatum associates with stages of colorectal neoplasia development, colorectal cancer and disease outcome. Eur J Clin Microbiol Infect Dis. 2014; 33:1381–1390. DOI: 10.1007/s10096-014-2081-3 [PubMed: 24599709]
11. Ito M, Kanno S, Nosho K, Sukawa Y, Mitsuhashi K, Kurihara H, Igarashi H, Takahashi T, Tachibana M, Takahashi H, Yoshii S, Takenouchi T, Hasegawa T, Okita K, Hirata K, Maruyama R, Suzuki H, Imai K, Yamamoto H, Shinomura Y. Association of Fusobacterium nucleatum with clinical and molecular features in colorectal serrated pathway. Int J Cancer. 2015; 137:1258–1268. DOI: 10.1002/ijc.29488 [PubMed: 25703934]
12. Li YY, Ge Q-X, Cao J, Zhou Y-J, Du Y-L, Shen B, Wan Y-JY, Nie Y-Q. Association of Fusobacterium nucleatum infection with colorectal cancer in Chinese patients. World J Gastroenterol. 2016; 22:3227–3233. DOI: 10.3748/wjg.v22.i11.3227 [PubMed: 27004000]
13. Mima K, Sukawa Y, Nishihara R, Qian ZR, Yamauchi M, Inamura K, Kim SA, Masuda A, Nowak JA, Nosho K, Kostic AD, Giannakis M, Watanabe H, Bullman S, Milner DA, Harris CC, Giovannucci E, Garraway LA, Freeman GJ, Dranoff G, Chan AT, Garrett WS, Huttenhower C, Fuchs CS, Ogino S. Fusobacterium nucleatum and T cells in colorectal carcinoma. JAMA Oncol. 2015; 1:653–661. DOI: 10.1001/jamaoncol.2015.1377 [PubMed: 26181352]
14. Mima K, Nishihara R, Qian ZR, Cao Y, Sukawa Y, Nowak JA, Yang J, Dou R, Masugi Y, Song M, Kostic AD, Giannakis M, Bullman S, Milner DA, Baba H, Giovannucci EL, Garraway LA, Freeman GJ, Dranoff G, Garrett WS, Huttenhower C, Meyerson M, Meyerhardt JA, Chan AT, Fuchs CS, Ogino S. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. Gut. 2016; 65:1973–1980. DOI: 10.1136/gutjnl-2015-310101 [PubMed: 26311717]
15. Mima K, Cao Y, Chan AT, Qian ZR, Nowak JA, Masugi Y, Shi Y, Song M, da Silva A, Gu M, Li W, Hamada T, Kosumi K, Hanyuda A, Liu L, Kostic AD, Giannakis M, Bullman S, Brennan CA, Milner DA, Baba H, Garraway LA, Meyerhardt JA, Garrett WS, Huttenhower C, Meyerson M, Giovannucci EL, Fuchs CS, Nishihara R, Ogino S. Fusobacterium nucleatum in colorectal carcinoma tissue according to tumor location. Clin Transl Gastroenterol. 2016; 7:e200.doi: 10.1038/ctg.2016.53 [PubMed: 27811909]
16. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, Chung DC, Lochhead P, Hold GL, El-Omar EM, Brenner D, Fuchs CS, Meyerson M, Garrett WS. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013; 14:207–215. DOI: 10.1016/j.chom.2013.07.007 [PubMed: 23954159]
17. Yang Y, Weng W, Peng J, Hong L, Yang L, Toiyama Y, Gao R, Liu M, Yin M, Pan C, Li H, Guo B, Zhu Q, Wei Q, Moyer M-P, Wang P, Cai S, Goel A, Qin H, Ma Y. Fusobacterium nucleatum increases proliferation of colorectal cancer cells and tumor development in mice by activating Toll-

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

like receptor 4 signaling to nuclear factor-κB, and up-regulating expression of microRNA-21. Gastroenterology. 2017; 152:851–866.e24. DOI: 10.1053/j.gastro.2016.11.018 [PubMed: 27876571]

18. Yu YN, Yu T-C, Zhao H-J, Sun T-T, Chen H-M, Chen H-Y, An H-F, Weng Y-R, Yu J, Li M, Qin W-X, Ma X, Shen N, Hong J, Fang J-Y. Berberine may rescue Fusobacterium nucleatum-induced colorectal tumorigenesis by modulating the tumor microenvironment. Oncotarget. 2015; 6:32013–32026. DOI: 10.18632/oncotarget.5166 [PubMed: 26397137]
19. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. Cell Host Microbe. 2013; 14:195–206. DOI: 10.1016/j.chom.2013.07.012 [PubMed: 23954158]
20. Chen Y, Peng Y, Yu J, Chen T, Wu Y, Shi L, Li Q, Wu J, Fu X. Invasive Fusobacterium nucleatum activates beta-catenin signaling in colorectal cancer via a TLR4/P-PAK1 cascade. Oncotarget. 2017; 8:31802–31814. [PubMed: 28423670]
21. Yu T, Guo F, Yu Y, Sun T, Ma D, Han J, Qian Y, Kryczek I, Sun D, Nagarsheth N, Chen Y, Chen H, Hong J, Zou W, Fang J-Y. Fusobacterium nucleatum promotes chemoresistance to colorectal cancer by modulating autophagy. Cell. 2017; 170:548–563.e16. DOI: 10.1016/j.cell.2017.07.008 [PubMed: 28753429]
22. Abed J, Emgård JEM, Zamir G, Faroja M, Almogy G, Grenov A, Sol A, Naor R, Pikarsky E, Atlan KA, Mellul A, Chaushu S, Manson AL, Earl AM, Ou N, Brennan CA, Garrett WS, Bachrach G. Fap2 mediates Fusobacterium nucleatum colorectal adenocarcinoma enrichment by binding to tumor-expressed Gal-GalNAc. Cell Host Microbe. 2016; 20:215–225. DOI: 10.1016/j.chom.2016.07.006 [PubMed: 27512904]
23. Gur C, Ibrahim Y, Isaacson B, Yamin R, Abed J, Gamliel M, Enk J, Bar-On Y, Stanietsky-Kaynan N, Coppenhagen-Glazer S, Shussman N, Almogy G, Cuapio A, Hofer E, Mevorach D, Tabib A, Ortenberg R, Markel G, Mikli K, Jonjic S, Brennan CA, Garrett WS, Bachrach G, Mandelboim O. Binding of the Fap2 protein of Fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack. Immunity. 2015; 42:344–355. DOI: 10.1016/j.immuni.2015.01.010 [PubMed: 25680274]
24. Tomkovich S, Yang Y, Winglee K, Gauthier J, Mühlbauer M, Sun X, Mohamadzadeh M, Liu X, Martin P, Wang GP, Oswald E, Fodor AA, Jobin C. Locoregional effects of microbiota in a preclinical model of colon carcinogenesis. Cancer Res. 2017; 77:2620–2632. DOI: 10.1158/0008-5472.CAN-16-3472 [PubMed: 28416491]
25. Holt RA, Cochrane K. Tumor potentiating mechanisms of Fusobacterium nucleatum, a multifaceted microbe. Gastroenterology. 2017; 152:694–696. DOI: 10.1053/j.gastro.2017.01.024 [PubMed: 28143770]
26. The Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012; 487:330–337. DOI: 10.1038/nature11252 [PubMed: 22810696]
27. The Cancer Genome Atlas Research Network. Comprehensive and integrative genomic characterization of hepatocellular carcinoma. Cell. 2017; 169:1327–1341.e23. DOI: 10.1016/j.cell.2017.05.046 [PubMed: 28622513]
28. Yu J, Chen Y, Fu X, Zhou X, Peng Y, Shi L, Chen T, Wu Y. Invasive Fusobacterium nucleatum may play a role in the carcinogenesis of proximal colon cancer through the serrated neoplasia pathway. Int J Cancer. 2016; 139:1318–1326. DOI: 10.1002/ijc.30168 [PubMed: 27130618]
29. Kostic AD, Ojesina AI, Pedamallu CS, Jung J, Verhaak RGW, Getz G, Meyerson M. PathSeq: Software to identify or discover microbes by deep sequencing of human tissue. Nat Biotechnol. 2011; 29:393–396. DOI: 10.1038/nbt.1868 [PubMed: 21552235]
30. Löfmark S, Edlund C, Nord CE. Metronidazole is still the drug of choice for treatment of anaerobic infections. Clin Infect Dis. 2010; 50(suppl 1):S16–S23. [PubMed: 20067388]
31. Martin FE, Nadkarni MA, Jacques NA, Hunter N. Quantitative microbiological study of human carious dentine by culture and real-time PCR: Association of anaerobes with histopathological changes in chronic pulpitis. J Clin Microbiol. 2002; 40:1698–1704. DOI: 10.1128/JCM.40.5.1698-1704.2002 [PubMed: 11980945]
32. Ludwig W. Nucleic acid techniques in bacterial systematics and identification. Int J Food Microbiol. 2007; 120:225–236. DOI: 10.1016/j.ijfoodmicro.2007.06.023 [PubMed: 17961780]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

33. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. Jun 23.2014 arXiv:1406.5823 [stat.CO].

34. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012; 19:455–477. DOI: 10.1089/cmb.2012.0021 [PubMed: 22506599]

35. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics. 2008; 9:75.doi: 10.1186/1471-2164-9-75 [PubMed: 18261238]

36. Markowitz VM, Mavromatis K, Ivanova NN, Chen I-MA, Chu K, Kyrpides NC. IMG ER: A system for microbial genome annotation expert review and curation. Bioinformatics. 2009; 25:2271–2278. DOI: 10.1093/bioinformatics/btp393 [PubMed: 19561336]

37. Grant JR, Stothard P. The CGView Server: A comparative genomics tool for circular genomes. Nucleic Acids Res. 2008; 36(suppl 2):W181–W184. DOI: 10.1093/nar/gkn179 [PubMed: 18411202]

38. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009; 25:1754–1760. DOI: 10.1093/bioinformatics/btp324 [PubMed: 19451168]

39. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 1997; 25:3389–3402. DOI: 10.1093/nar/25.17.3389 [PubMed: 9254694]

40. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol. 2011; 12:R60.doi: 10.1186/gb-2011-12-6-r60 [PubMed: 21702898]

41. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30:2114–2120. DOI: 10.1093/bioinformatics/btu170 [PubMed: 24695404]

42. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011; 27:2194–2200. DOI: 10.1093/bioinformatics/btr381 [PubMed: 21700674]

43. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010; 7:335–336. DOI: 10.1038/nmeth.f.303 [PubMed: 20383131]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

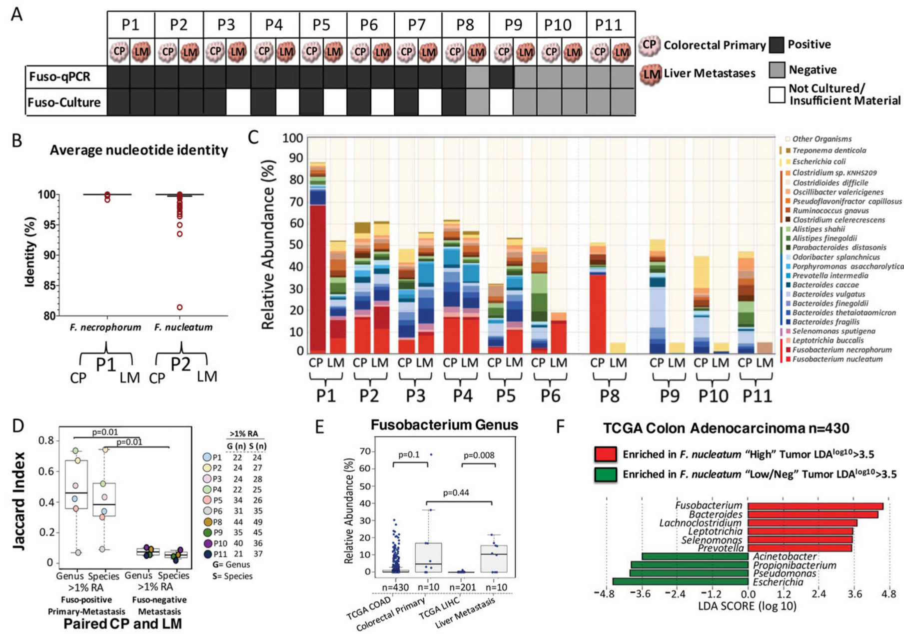

Fig. 1. *Fusobacterium* colonizes liver metastases of *Fusobacterium* associated colorectal primary tumors

(A) Schematic of *Fusobacterium* culture and *Fusobacterium*-targeted qPCR status of paired snap-frozen colorectal primary tumors and liver metastases from 11 patients (P1 to P11) from the frozen paired cohort. (B) Aligned dot plot representing the average nucleotide identity (ANI) of whole-genome sequencing data from *F. necrophorum* isolated from paired primary colorectal tumor (CP) and liver metastasis (LM) of P1 and *F. nucleatum* isolate cultured from paired primary tumors and liver metastasis of P2. *F. necrophorum* P1 two-way ANI: 100% (SD: 0.01%) from 10,220 fragments; *F. nucleatum* P2 two-way ANI: 99.99% (SD: 0.23%) from 7334 fragments. (C) Species-level microbial composition of paired colorectal primary tumors and liver metastases (frozen paired cohort), assayed by RNA sequencing followed by PathSeq analysis for microbial identification. For simplicity, only organisms with >2% relative abundance (RA) in at least one tumor are shown. The colors correspond to bacterial taxonomic class. Red, Fusobacteriia; pink, Negativicutes; blue/green, Bacteroidia; orange, Clostridia; yellow, Gamma-proteobacteria; dark brown, Spirochaetes. The samples are separated into three groups: *Fusobacterium*-positive primary tumor and metastases (n = 6 pairs), *Fusobacterium*-positive primary tumor and *Fusobacterium*-negative metastases (n = 1 pair), and *Fusobacterium*-negative primary tumor and metastases (n = 3 pairs). P7 had insufficient tissue for RNA sequencing analysis. (D) Box plots represent the Jaccard index (proportion of shared genera or species) between paired colorectal primary tumors and liver metastases at both the genus and species level at 1% RA. The box represents the first and third quartiles, and error bars indicate the 95% confidence level of the median. Paired samples that were positive for *Fusobacterium* in both the primary tumor and metastasis were compared with paired samples where the metastasis was *Fusobacterium*-negative. P values were determined using Welch's two-sample t test. (E) Box plots of *Fusobacterium* RA in primary colon adenocarcinoma (COAD) (n = 430) and primary liver hepatocellular carcinoma (LIHC) (n = 201) from TCGA (TCGA cohort) and primary-metastasis pairs from 10 patients. The box represents the first and third quartiles,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and error bars indicate the 95% confidence level of the median. P values were determined using Welch's two-sample t test with correction for unequal variances. (F) Identification of bacteria that co-occur with *Fusobacterium* in primary COAD (TCGA cohort). Primary COAD tumors were subset into two groups: *Fusobacterium* "High" if *Fusobacterium* RA was >1% (n = 110, median RA = 5%, mean RA = 7.4%) and *Fusobacterium* "Low/Neg" if RA was <1% (n = 320, median RA = 0.06%, mean RA = 0.16%). The bar plot illustrates genera enriched (red) and depleted (green) in COAD with >1% *Fusobacterium* RA. LDA, linear discriminant analysis.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

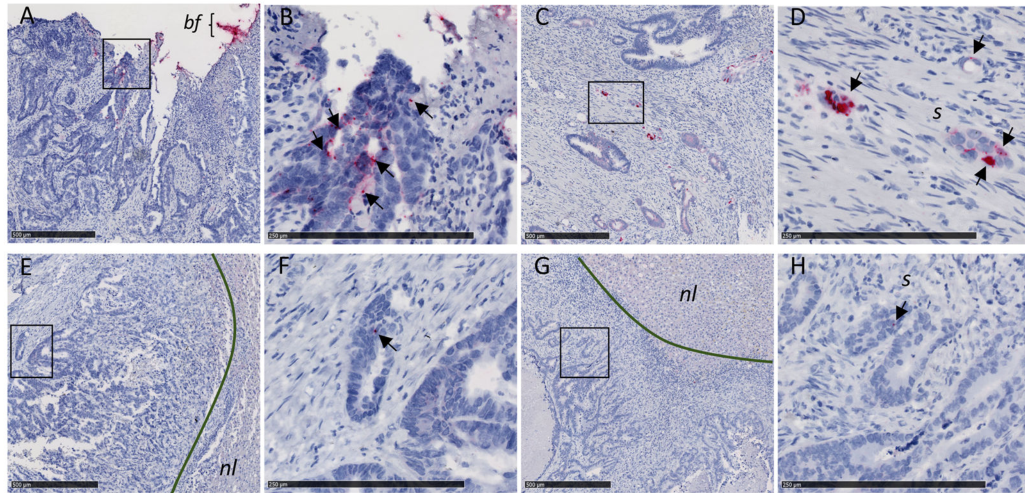

Fig. 2. *F. nucleatum* RNA ISH analysis of matched primary colorectal tumors and liver metastases

Representative images of *F. nucleatum* spatial distribution in paired samples from P187 primary colorectal tumor (A and B) and liver metastasis (E and F) and P188 primary colorectal tumor (C and D) and liver metastasis (G and H) from the FFPE paired cohort are shown. Arrows indicate cells with histomorphology consistent with that of colon cancer cells infected by invasive *F. nucleatum* (red dots) in both primary colorectal tumors (B and D) and matched liver metastases (F and H). *Fusobacterium*-containing biofilm (bf) is highlighted in the colorectal tumor of P187 (A). *Fusobacterium* was not detected in normal liver (nl) tissue [(E) and (F)]. s, stroma. Panels (B), (D), (F), and (H) show magnification of the boxed areas in (A), (C), (E), and (G), respectively. Scale bars: 500 mm in (A), (C), (E), and (G); 250 mm in (B), (D), (F), and (H).

Author Manuscript

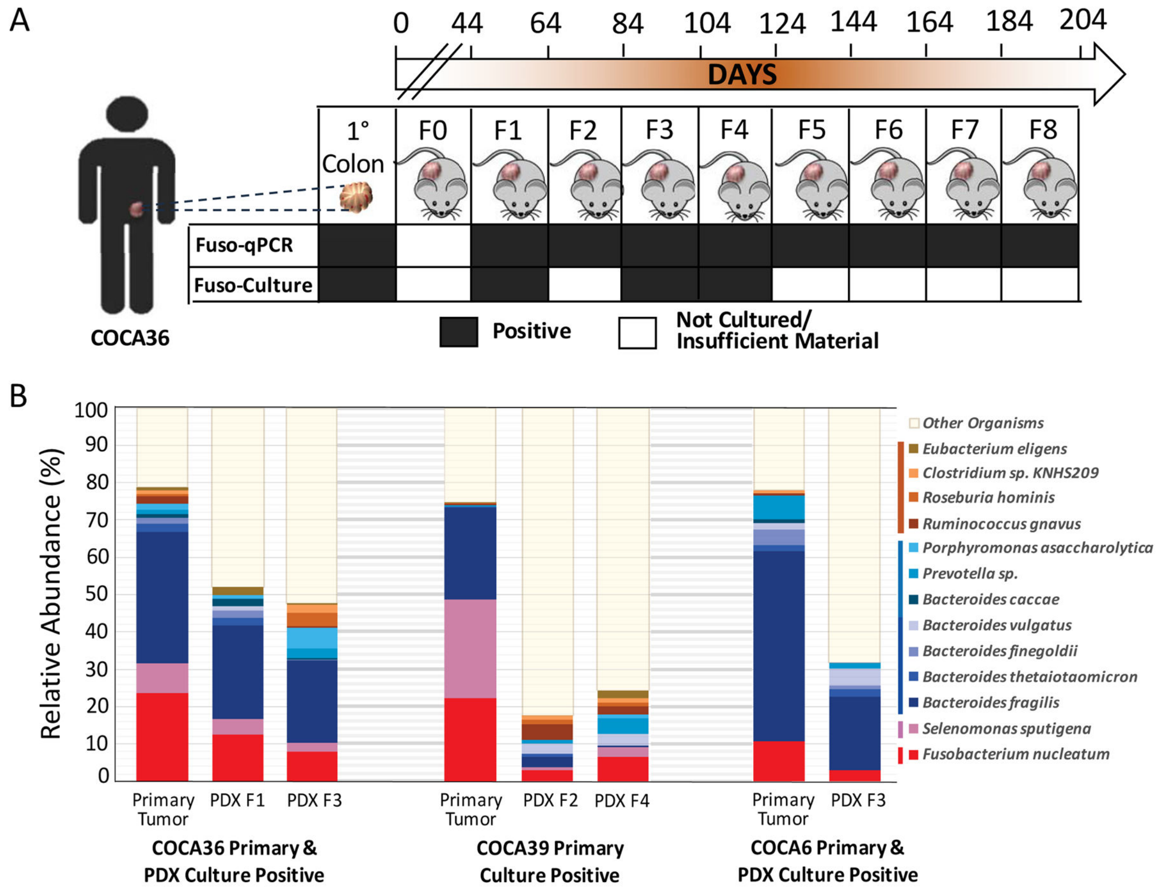

Fig. 3. *Fusobacterium* and co-occurring anaerobes persist in colon adenocarcinoma PDXs

(A) Assessment of *Fusobacterium* persistence in PDX COCA36 over a period of 204 days. *Fusobacterium* persistence was determined via microbial culture and *Fusobacterium*-targeted qPCR. F0 denotes the first implantation of the tumor into mice; F1 to F8 represent sequential xenograft passages after F0. (B) Species-level microbial composition of three patient primary colon adenocarcinomas (COCA36, COCA39, and COCA6) and subsequent PDXs. Total RNA sequencing was carried out, followed by PathSeq analysis for microbial identification. For simplicity, selected species with >1% relative abundance in the primary tumor and either corresponding PDX are shown. The colors correspond to bacterial taxonomic class. Red, Fusobacteriia; pink, Negativicutes; blue/green, Bacteroidia; orange, Clostridia.

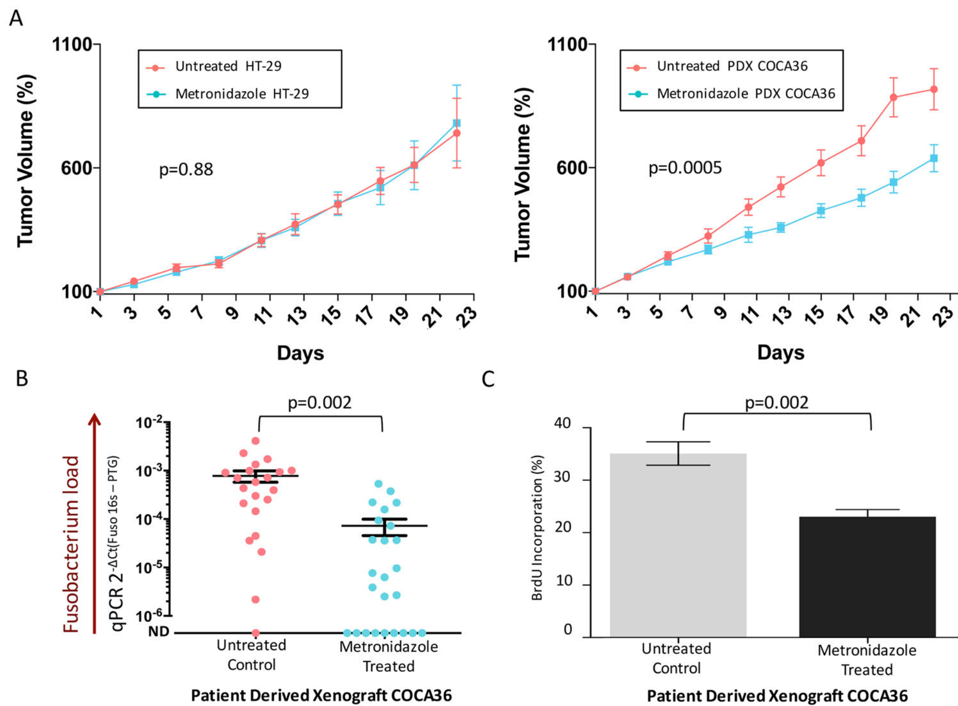

Fig. 4. Treatment of *Fusobacterium*-colonized PDXs with metronidazole reduces tumor growth *in vivo*

(A) (Left) Tumor volume percentage of *Fusobacterium*-free xenografts derived from HT-29 cells treated with metronidazole (treated; 19 animals) or with vehicle (untreated; 20 animals). (Right) Tumor volume percentage of *Fusobacterium*-positive PDX tumors (COCA36) treated with metronidazole (treated; 25 animals) or with vehicle (untreated; 22 animals). P values were determined by the Wald test. Tumors were measured in a blinded fashion on Mondays, Wednesdays, and Fridays each week. Error bars represent mean ± SEM. The remaining number of HT-29-derived xenografts and PDX-implanted animals at each time point is included in the supplementary materials. (B) Assessment of *Fusobacterium* tissue load. *Fusobacterium*-targeted qPCR on PDX tissue (COCA36) after treatment with metronidazole (treated) or with vehicle (untreated). ND, not detected. The center bar represents the mean; error bars indicate SEM. P values were determined using Welch's two-sample t test. DCt, delta cycle threshold; PTG, prostaglandin transporter. (C) Bromodeoxyuridine (BrdU) immunohistochemistry of PDX tumors to assess cell proliferation. The bar plot represents the percentage of cells with BrdU incorporation in treated and untreated PDXs (n = 6 animals). per arm); error bars denote mean ± SEM. P values were determined using the Welch's two-sample t test.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript