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Supplemental Information

Fap2 Mediates *Fusobacterium nucleatum* Colorectal Adenocarcinoma Enrichment by Binding to Tumor-Expressed Gal-GalNAc

Jawad Abed, Johanna E.M. Emgård, Gideon Zamir, Mouhammad Faroja, Gideon Almogy, Amalie Grenov, Asaf Sol, Ronit Naor, Eli Pikarsky, Karine A. Atlan, Anna Mellul, Stella Chaushu, Abigail L. Manson, Ashlee M. Earl, Nora Ou, Caitlin A. Brennan, Wendy S. Garrett, and Gilad Bachrach

Supplemental Information

Inventory of Supplemental Information

1. **Supplemental data:**
   contains supplemental Figures S1-S3 and Table S1:
   - **Figure S1 (related to Figure 3, Figure 4, Figure 5)**
   - **Figure S2 (related to Figure 6)**
   - **Figure S3 (related to Figure 6)**
   - **Table S1 (related to Figure 5)**
2. **Supplemental Experimental Procedures.**

Supplemental Data

Fn	Bacterial 2 fold dilution → GalNAc (-)	Bacterial 2 fold dilution → GalNAc (+)	Fap2 Present	Sub Species	Source
ATCC 23726			Yes	*nucleatum*	ATCC
MUT K50			No		Coppenhagen-Glazer Set al. 2015
MUT D22			No		Coppenhagen-Glazer S et al. 2015
ATCC 10953			Yes	*polymorphum*	ATCC
PK1594			Yes	*ND*	Kolenbrander and Andersen 1989
CTI1			Yes	*animalis*	BEI Repository
CTI2			Yes	*nucleatum*	Gur et al. 2015
CTI3			No	*animalis*	BEI Repository
CTI5			No	*animalis*	BEI Repository
CTI6			Yes	*polymorphum*	BEI Repository
CTI7			No	*vincentii*	Gur et al. 2015
EAVG_002			Yes	*animalis*	Strauss J et al. 2007

Figure S1. Related to Figure 2, Figure 3 and Figure 4. Summary of the hemagglutination activity (indicating Fap2 presence), presence of Fap2 in the strain's genome, sub-species designation, and source and/or reference for the fusobacterial strains used in this study.

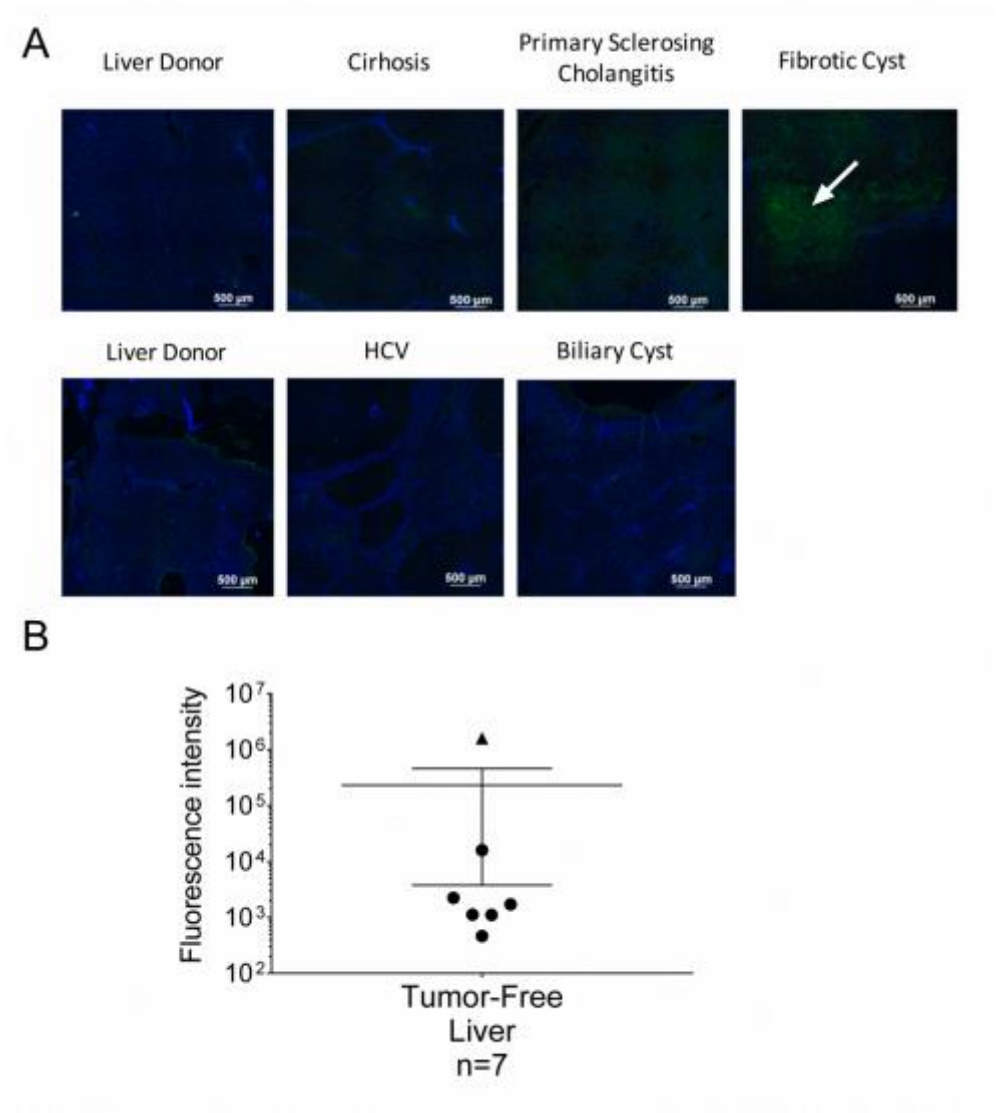

Figure S2. Related to Figure 6. Fusobacterial occurrence in tumor-free liver biopsy samples correlates with high Gal-GalNAc expression levels. Representative images (A), and quantitative analysis (B) of Gal-GalNAc stained with FITC-PNA (green) in the tumor-free liver biopsy samples presented in Figure 5 stained with Hoechst (blue). High expression levels of Gal-GalNAc (indicated by a white arrow) are found in the sample with a fibrotic cyst where fusobacterial gDNA was detected.

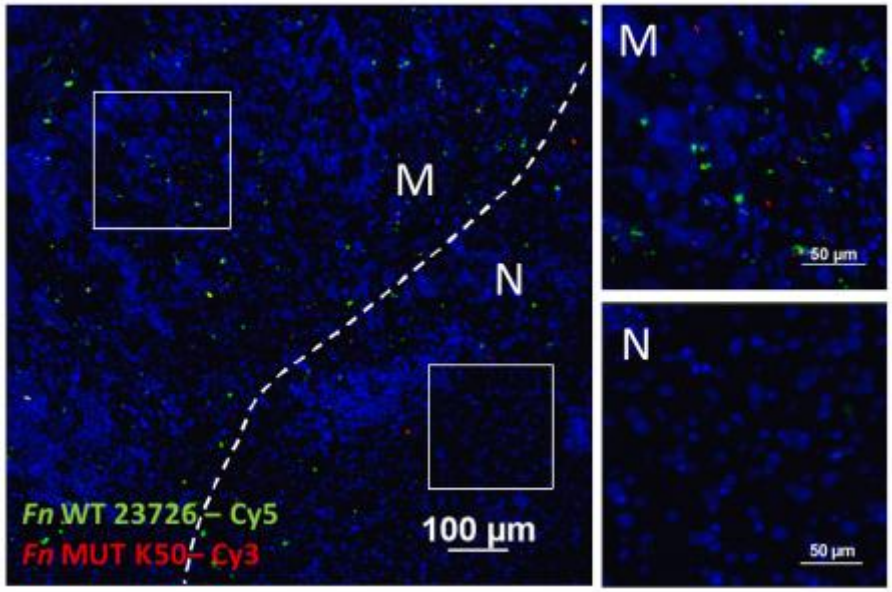

Figure S3. Related to Figure 6. Attachment of Cy5-labeled (green) *Fn* and of its Cy3-labeled (red) Fap2-inactivated mutant K50 to a representative Hoechst-stained (blue) human CRC liver metastasis section. Bacterial staining of the experiment presented in Figure 5 were reversed to allay concerns of bias from the dye used. M = metastatic deposit. N = normal tissue. Right sided images are enlargements from inset boxes in the left sided image.

Table S1. Post-inoculation *F. nucleatum* detection in small intestinal tissues of *ApcMin+/-* and wild-type mice.

Fusobacterial strain	**ATCC 23726**		**K50**	
Mouse strain	***C57BL/6 APC Min/+***	**C57BL/6**	**C57BL/6 APC Min+/**	**C57BL/6**
Level of Detection Relative fusobacterial abundance 2^-(Ct mGAPDH - Ct Fusobacteria)	0.000624895	At-Below Limit of Detection	0.002795797	At-Below Limit of Detection
	0.001861324	At-Below Limit of Detection	0.000998605	At-Below Limit of Detection
	0.001802023	At-Below Limit of Detection	0.01044503	At-Below Limit of Detection
	0.001108379	At-Below Limit of Detection	At-Below Limit of Detection	At-Below Limit of Detection
	0.001625388	At-Below Limit of Detection	At-Below Limit of Detection	At-Below Limit of Detection
	At-Below Limit of Detection	At-Below Limit of Detection	At-Below Limit of Detection	At-Below Limit of Detection
	0.002203775		0.000211801	
	0.000452238		5.11467E-05	
	0.002810542		0.00121229	
	0.005141536		0.000333728	
	0.002173139		0.000569232	
	0.04484091		At-Below Limit of Detection	
			At-Below Limit of Detection	
			At-Below Limit of Detection	
			0.000260758	
			At-Below Limit of Detection	
# of samples with detectable *F. nucleatum*	**11/12**	**0/6**	**9/16**	**0/6**

Table S1. Related to Figure 5. Post-inoculation *F. nucleatum* detection in small intestinal tissues of $Apc^{Min+/-}$ and wild type mice.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

F. nucleatum strains ATCC 23726, K50, D22, ATCC 10953, PK 1594, CTI-1, CTI-2, CTI-3, CTI-5, CTI-6, CTI-7, EAVG_002 (Coppenhagen-Glazer et al., 2015; Gur et al., 2015) and *P. gingivalis* ATCC 33277 were grown in Wilkins Chalgren broth (Oxoid, UK) or on Columbia agar plates (Oxoid, UK) supplemented with 5% defibrinated sheep blood (Novamed, Israel) in an anaerobic chamber (Bactron I-II Shellab, USA) with an atmosphere of 90% N_2, 5% CO_2 and 5% H_2 at 37°C. Plates were supplemented with 5 μg/ml thiamphenicol (Sigma-Aldrich, Israel) and broth with 2.5 μg/ml thiamphenicol for mutants K50 and D22.

Determination of tumor size

Real time imaging using a CCD camera was used to validate the development of CRC. Luciferin 5mg/ml was injected intraperitoneally 15 minutes before image capture. A caliper was used to assess the volume of the tumors and experiments were conducted when tumor volumes were around 2500 mm^3 calculated as a 3 dimension sphere's volume according to the equation: $V = 4/3(\pi r^3)$.

Quantification of bacteria using plating and qPCR

Tissue samples were homogenized using a Fastprep (MP Biomedicals, USA), serially diluted, and plated on Columbia agar plates supplemented with 0.15% (final concentration) crystal violet and 5% (final concentration) defibrinated sheep blood (Van Tassell et al., 2012). Plates were incubated in an anaerobic chamber at 37°C for 6 days, and then colonies were enumerated.
DNA extraction from fresh tissues was performed using the DNeasy Blood & Tissue Kit (Qiagen, Germany), samples were incubated at 56°C overnight. DNA extraction from FFPE tissues was performed using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany). A custom TaqMan primer and probe set was used to amplify *F. nucleatum* DNA. The cycle threshold (Ct) values for *F. nucleatum* and of *P. gingivalis* were normalized to the amount of murine gDNA in each reaction by using a primer and probe set for the reference gene (*Gapdh*). The fold difference ($2^{-\Delta Ct}$) in *F. nucleatum* abundance in tumor versus normal tissue was calculated as described before (Castellarin et al., 2012). Each reaction contained 1ng of DNA and was assayed in triplicate in 20 μL reactions containing 2× qPCRBIO Lo-ROX Probe Mix as appropriate for individual qPCR machines. Reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The primers and probe sequences for each assay were as follows: *F. nucleatum nusG* forward primer, 5' CAACCATTACTTTAACTCTACCATGTTCA 3'; Fusobacteria reverse primer, 5' ATTGACTTTACTGAGGGAGATTATGTAAAAATC 3'; Fusobacteria FAM probe, 5'- /56-FAM/TCAGCAACT/ZEN/TGTCCTTCTTGATCTTTAAATGAACC/3IABkFQ/ -3'; *catP* (for amplifying *F. nucleatum* mutant - K50) forward primer, 5'-GAAGGTTGACCACGGTATCAT-3'; *catP* reverse primer, 5'-CGCAACGGTATGGAAACAATC-3'; *catP* FAM probe, 5'-/56-FAM/ATGGAAGGA/ZEN/AAGCCAAATGCTCCG /3IABkFQ/-3'; Mouse *Gapdh* forward primer, 5'-AATGGTGAAGGTCGGTGTG-3'; Mouse *Gapdh* reverse primer, 5'-GTGGAGTCATACTGGAACATGTAG-3'; Mouse *Gapdh* FAM probe, 5'-/56-

FAM/TGCAAATGG/ZEN/CAGCCCTGGTG/3IABkFQ/-3'; *P. gingivalis* primer/probe set used was ISPg1 described before (Hajishengallis et al., 2011). Human *GAPDH* forward primer, 5'-TGAGTGTGGCAGGGACT-3'; Human *GAPDH* reverse primer, 5'-AGGGTGGTGGACCTCAT-3'; Human *GAPDH* FAM probe, 5'-/56FAM/CAGCAAGAG/ZEN/CACAAGAGGAAGAGAGA/3IABkFQ/-3'.

Flow cytometry and competition assays

F. nucleatum (10^9 CFU/ml) was labeled with fluorescein isothiocyanate (FITC, 0.1 mg/ml in PBS; Sigma-Aldrich) for 30 min at room temperature and washed three times in PBS. FITC-labeled bacteria were used at a multiplicity of infection of 10. Bacteria were incubated with cells in 96 well plates, for 30 min at room temperature and washed twice prior to flow cytometry (Accuri, C6 flow cytometer, BD, USA). Analysis was performed using Flowjo 10.0.8 software (Tree Star, Ashland, OR, USA). FITC-labeled PNA lectin (Sigma-Aldrich) was incubated at a final concentration of 140 mM per 2.5×10^5 cells per well. For competition experiments, bacteria or PNA were incubated with GalNAc (concentration range: 0, 50, 100 and 300 mM) for 30 min prior to incubation with cells.

Histology and immunofluorescence

FFPE sections were stained with hematoxylin and eosin or for immunofluorescence microscopy as described below. For PNA binding, sections were blocked with PBS supplemented with 10% BSA, 10% FBS and 0.5% Triton X-100 for 2h at room temperature followed by incubation with FITC-labeled PNA (50 µg/ml in PBS) overnight at 4ºC. For fusobacterial binding, bacteria were labeled with FITC, or with Cy3 – (PA23001 Life sciences GE) or Cy5 (PA25001 Life sciences GE) solution diluted 1:10 as described above. Sections were blocked with TBS (0.05 M Tris-HCl [pH 7.8], 0.1 M NaCl) supplemented with 20% BSA, 20% FBS and 5% Triton X-100 for 7h at room temperature, followed by incubation with the labeled bacteria (3×10^7 bacteria / ml blocking solution) overnight at 4ºC. The slides were then washed once with PBS + 0.5% Tween 20 followed by 2 washes with PBS for 10 min each, and then incubated with Hoechst 33258 diluted 1:5000 for 15 min at room temperature. Gal-GalNAc removal was performed by incubating human sections with 12 mU/section O-glycanase (Endo-a-N-acetylgalactosaminidase, Sigma-Aldrich) in 50mM sodium-phosphate buffer (pH = 5), overnight at 37Cº. Fluorescence intensity FITC-labeled PNA was evaluated using the ImagePro Analyzer 7.0 software (Cybernetics, USA). Tumor and normal sections were prepared for each patient or mouse. The region of interest was determined by defining the tumor and normal regions in H&E stained sections. Three arbitrary fields of each region were selected as representative and converted to 8 bit images. The sum of fluorescent intensities that were above threshold (minimum=30) were determined.

Bacteria were counted by two independent examiners for a total area of $1600\mu m^2$ (each field) and converted to bacteria/mm^2.