

## Identification, Isolation, and Characterization of Human LGR5-positive Colon Adenoma Cells

Michael K. Dame<sup>1\*</sup>, Durga Attili<sup>1\*</sup>, Shannon D. McClintock<sup>1</sup>, Priya H. Dedhia<sup>2</sup>, Peter Ouillette<sup>1</sup>, Olaf Hardt<sup>3</sup>, Alana M Chin<sup>4</sup>, Xiang Xue<sup>5</sup>, Julie Laliberte<sup>6</sup>, Erica L Katz<sup>1</sup>, Gina M Newsome<sup>1</sup>, David Hill<sup>7</sup>, Alyssa Miller<sup>7,8</sup>, David Agorku<sup>3</sup>, Christopher H Althelm<sup>7</sup>, Andreas Bosio<sup>3</sup>, Becky Simon<sup>9</sup>, Linda C Samuelson<sup>5,7</sup>, Jay A Stoerker<sup>6</sup>, Henry D Appelman<sup>1</sup>, James Varani<sup>1</sup>, Max S. Wicha<sup>9</sup>, Dean E. Brenner<sup>9,10</sup>, Yatrik M. Shah<sup>5,7</sup>, Jason R Spence<sup>4,7</sup>, Justin A. Colacino<sup>11,12</sup>

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<sup>1</sup>Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan

<sup>2</sup>Department of Surgery, University of Michigan, Ann Arbor, Michigan

<sup>3</sup>Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

<sup>4</sup>Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, Michigan

<sup>5</sup>Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan

<sup>6</sup>Progenity, Inc., Ann Arbor, Michigan

<sup>7</sup>Department of Internal Medicine, Division of Gastroenterology, University of Michigan Medical School, Ann Arbor, Michigan

<sup>8</sup>Cellular and Molecular Biology Program, University of Michigan, Ann Arbor, Michigan

<sup>9</sup>Department of Internal Medicine, Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, Michigan

<sup>10</sup>Department of Pharmacology, University of Michigan, Ann Arbor, Michigan

<sup>11</sup>Department of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, Michigan

<sup>12</sup>Department of Nutritional Sciences, University of Michigan School of Public Health, Ann Arbor, Michigan

\*- These authors contributed equally to this work

**Short title:** Isolation of human LGR5(+) colon adenoma cells

**Correspondence:** Justin Colacino, PhD  
Department of Environmental Health Sciences  
6651 SPH1, 1415 Washington Heights  
Ann Arbor, MI, USA 48109  
Email: colacino@umich.edu  
Tel: 734-647-4347

1 **Abbreviations:** DKK4, dickkopf WNT signaling pathway inhibitor 4; FGF20, fibroblast growth factor 20;  
2 FFPE, formalin fixed paraffin embedded; ISC, intestinal stem cell; ISH, *in situ* hybridization; LGR5,  
3 leucine-rich repeat-containing g-protein coupled receptor 5; MACS, magnetic-activated cell sorting;  
4 OLFM4, olfactomedin 4; TCGA, The Cancer Genome Atlas

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## ABSTRACT

30 The intestine is maintained by stem cells, marked by LGR5 expression, located at the base of crypts.  
31 Genetically engineered mouse models have provided information about marker genes and stem cell  
32 pathways. Less is known about human intestinal stem cells due to difficulty detecting and isolating these  
33 cells. We established an organoid repository from patient-derived adenomas, adenocarcinomas, and normal  
34 colon, which we analyzed for variants in 71 colorectal cancer (CRC) associated genes. Normal and  
35 neoplastic colon tissue organoids were analyzed for LGR5 expression by immunohistochemistry. LGR5-  
36 positive cells were isolated from 4 adenoma organoid lines and analyzed by RNA-sequencing. LGR5  
37 expression in epithelium and stroma was associated with tumor stage. Integrating functional experiments  
38 with RNA-seq data from LGR5-positive adenoma organoid cells and normal colon, we associated expression  
39 of CRC-specific genes, including *DKK4*, with LGR5 expression. This system can be used to study LGR5-  
40 expressing cells in human tissue homeostasis and carcinogenesis.

41 **Key words:** biobank, organoid, stem cell, stroma, *DKK4*, enteroid, *OLFM4*, Wnt signaling, differentiation,  
42 regeneration, development

## INTRODUCTION

43 In adult mammals, the intestine is a site of rapid cellular turnover, mediated by a population of  
44 intestinal stem cells (ISCs) that reside at the base of intestinal crypts (Barker, van de Wetering, and Clevers  
45 2008). These stem cells are identified by expression of several genetic markers in the mouse (van der Flier et  
46 al. 2009, Montgomery et al. 2011, Powell et al. 2012, Yan et al. 2012). Among these, *Lgr5* (leucine-rich  
47 repeat-containing g-protein coupled receptor 5) is the best characterized, and genetically modified mice  
48 provide a robust toolbox for isolating and manipulating these ISCs (Barker et al. 2007, Snippert et al. 2010,  
49 Sato et al. 2009). The LGR family of proteins (LGR4, 5, 6) code for receptors of the secreted R-spondin  
50 proteins (Rspo1- 4). Together, *Lgr/Rspo* acts to potentiate Wnt pathway signaling (Carmon et al. 2012, de  
51 Lau et al. 2011). Lineage tracing experiments have demonstrated that differentiated cell lineages in mouse  
52 small intestine and colonic crypts are clonally derived from *Lgr5*(+) ISCs (Barker et al. 2007). Additionally,  
53 lineage tracing studies have revealed that *Lgr5* marks a population of stem-like cells within precancerous  
54 adenoma tissue that drive adenoma growth (Schepers et al. 2012), and human colorectal cancers overexpress  
55 *LGR5* (Junttila et al. 2015). Previous efforts to expand, isolate, and experimentally characterize primary  
56 human *LGR5* cells have been hampered by two distinct issues: (1) The difficulty in obtaining cultures highly  
57 enriched for epithelial stem cells (Wang, Yamamoto, et al. 2015), and (2) a paucity of specific reagents to  
58 detect and isolate live *LGR5*(+) human cells (Barker 2014). To this effect, previous studies have reported  
59 varied localization of *LGR5* within the normal crypt or did not assay normal tissue with antibody-based  
60 methods (Becker, Huang, and Mashimo 2008, Kleist et al. 2011, Fan et al. 2010, Takahashi et al. 2011,  
61 Kobayashi et al. 2012, Kemper et al. 2012). Recent efforts have utilized alternative strategies for the  
62 identification of *LGR5*(+) human colon cells, including *in situ* RNA hybridization (Jang, Lee, and Kim 2013,  
63 Baker et al. 2015). Due to these difficulties in the identification and isolation of human colon stem cells, the  
64 vast majority of knowledge about the role of *LGR5*(+) cells in colon tissue homeostasis and carcinogenesis is  
65 inferred from animal studies.

66 Intestinal organoid culture provides a method to culture tissue-derived cells that maintains the cellular  
67 heterogeneity in the intestinal epithelium (Sato, Stange, et al. 2011, Dedhia et al. 2016). Here we establish an

68 organoid biobank of precancerous human adenoma tissues, as well as normal colon and colon  
69 adenocarcinoma. We report detailed and robust methods for the culture, identification, and isolation of  
70 human colon LGR5(+) cells from primary adenomas growing in long-term organoid culture using  
71 commercially available antibodies. Using these methods, we quantify LGR5 protein expression in human  
72 intestinal tissues, including a colon adenocarcinoma tissue microarray (TMA). Using magnetic bead and  
73 fluorescent activated cell sorting (FACS) to enrich and isolate LGR5(+) and LGR5(-) cells from organoids,  
74 we conducted RNA sequencing and defined the expression profile of human LGR5(+) adenoma cells. Here,  
75 we were able to make correlations between *LGR5* mRNA expression and the expression of *DKK4*, a gene not  
76 detectable in normal colon tissue but associated with colorectal cancer. We further extended the cell  
77 isolation methods to show they are robust, allowing for isolation of LGR5(+) cells from fresh normal human  
78 intestinal tissues. Collectively, we have identified novel associations between the ISC marker, LGR5, and  
79 cancer progression. Moreover, the methods and datasets presented provide powerful tools for basic  
80 biological studies of the role of LGR5(+) cells in human colon homeostasis, as well as translational studies in  
81 chemoprevention and precision medicine designed to target LGR5(+) cell populations.

## MATERIALS AND METHODS

82 *Establishment of organoid cultures from human colonic adenomas.*

83 Isolation of human colonic crypts and adenomas; and culture and maintenance of adenoma organoid  
84 cultures, were performed using our previously described protocol (Dame et al. 2014). Adenoma tissue was  
85 acquired by endoscopy and adenocarcinoma tissue was collected from colonic resections according to  
86 protocols approved by the University of Michigan Institutional Review Board (IRB;  
87 HUM00064405/0038437/00030020). Normal colonic tissues were collected from deceased donors through  
88 the Gift of Life, Michigan (HUM00105750). De-identified human fetal intestinal tissue was obtained from  
89 the University of Washington Laboratory of Developmental Biology and approved by University of  
90 Michigan IRB (HUM00093465). Growth media used for organoid cultures included KGM-Gold™ (Lonza,  
91 Walkersville, MD; KGMG) (Dame et al. 2014), a serum-free epithelial medium containing epidermal growth  
92 factor (EGF) and pituitary extract; and L-WRN conditioned medium (Miyoshi and Stappenbeck 2013),

93 containing high levels of Wnt3a, R-spondin-3 and Noggin, with added 10mM Nicotinamide (Sigma-Aldrich,  
94 St. Louis, Missouri). To drive organoids from a budding to cyst morphology, cultures were switched from  
95 the reduced medium, KGMG, to the stem cell support medium, L-WRN, for 3-4 weeks. Consistent with  
96 previous reports, organoids formed cystic structures in the presence of Wnt ligand provided by L-WRN  
97 medium (Sato, van Es, et al. 2011, Farin, Van Es, and Clevers 2012, Matano et al. 2015, Drost et al. 2015,  
98 Onuma et al. 2013). Cultures were propagated in Matrigel (Corning; Bedford, MA) which was made to  
99 8mg/mL in growth media, in 6-well tissue culture plates. Cultures were passaged every 4-7 days by digesting  
100 Matrigel in cold 2mM EDTA and plated on the first day with 10 $\mu$ M Y27632 (Miltenyi Biotec; Bergisch  
101 Gladbach, Germany), a Rho-associated protein kinase (ROCK) inhibitor.

#### 102 *Single Cell Isolation and Magnetic separation for LGR5(+) and LGR5(-) cells*

103 Single cell suspensions of adenoma organoids or normal intact colon tissue were generated using the  
104 Tumor Dissociation Kit (human; Miltenyi Biotec) in combination with the gentleMACS Dissociator  
105 (Miltenyi Biotec) with protocol modifications. The enzymes were prepared in HBSS modified to 0.13mM  
106 calcium and 0.9mM magnesium (10% standard HBSS concentrations) to minimize differentiation of the  
107 epithelial cells while supporting enzymatic activity. All plasticware, including cell strainers and columns,  
108 were 0.1% BSA-coated, and buffers contained 5-10 $\mu$ M Y27632. Cells were labeled with anti-LGR5  
109 MicroBeads (human; Miltenyi Biotec) and the LGR5(+) cells were enriched by Magnetic-Activated Cell  
110 Sorting (MACS). Cells were applied through a cold BSA-coated 20 $\mu$ m cell strainer (CellTrics of Sysmex  
111 Europe GmbH; Norderstedt, Germany) to LS Columns (Miltenyi Biotec) in the above HBSS buffer  
112 containing 200 Kunitz units/mL DNase (Sigma-Aldrich), 0.5% BSA in DPBS (Sigma-Aldrich), and 5 $\mu$ M  
113 Y27632. The magnet-bound positive and flow-through negative fractions were analyzed and isolated by  
114 FACS.

#### 115 *LGR5 Immunohistochemistry*

116 Formalin fixed, paraffin sections were cut at 5-6 microns and rehydrated to water. Heat induced  
117 epitope retrieval was performed with FLEX TRS High pH Retrieval buffer (pH 9.01; Agilent Technologies,

118 154 #K8004; Santa Clara, CA) for 20 minutes (Figure 1A, C and Figure 3A). After peroxidase blocking, the  
119 antibody LGR5 rabbit monoclonal clone STE-1-89-11.5 (Miltenyi Biotec, #130-104-945) was applied at a  
120 dilution of 1:50 (Figure 1A, C ) or 1:100 (Figure 3A) at room temperature for 60 minutes. The FLEX HRP  
121 EnVision System (Agilent Technologies) was used for detection with a 10 minute DAB chromagen  
122 application. Note, sections freshly cut were compared to those that were stored at room temperature for 4  
123 weeks, and showed more robust LGR5 staining (data not shown). The colon cancer tissue microarray (Figure  
124 3A; 2 normal, 3 adenoma, 70 adenocarcinoma; 2 core samples per specimen) was freshly cut and provided  
125 by BioChain Institute, Inc. (Newark, CA; Z7020032, lot B508131).

### 126 *LGR5 Immunohistochemistry scoring for staining intensity in the epithelium and in the stroma*

127 The TMA, along with five additional FFPE normal colon samples from warm cadaveric colon  
128 resections, were scored for staining intensity in both the epithelium and then separately in the stroma (Figure  
129 3). Scoring was conducted by two independent viewers on blinded samples at 8X and 20X magnification.  
130 Scoring key: 0 = non-specific or < 1%; 1 = 1-10% or only evident at 20X magnification; 2 = 10-50% or light  
131 diffuse staining >50%; 3 = >50%. Stage T2 (n=25) and T3 (n=44) tumors were compared to normal colon  
132 and adenoma (termed Stage T0; n=10). TMA cancers with grades I & I-II were grouped, termed “Grade I”,  
133 and cancers grade II-III & III were grouped, termed “Grade III” for further analyses. LGR5 stromal and  
134 epithelial staining for adenomas (n=3), cancer Grade I (n=20), II (n=38), and III (n=10) were compared to  
135 normal colon tissue (n=7).

### 136 *Statistical Analyses*

137 For IHC analyses, LGR5 stromal or epithelial staining intensity categories were plotted by tumor  
138 stage and grade using boxplots. Differences in LGR5 staining in the stroma or epithelium by stage or grade  
139 were quantified by linear regression, treating epithelial or stroma staining intensity (0, 1, 2, or 3) as a  
140 continuous dependent variable and either tumor stage or grade as a categorical independent variable, setting  
141 normal tissue as the reference group. For qPCR analyses, differences between biological replicates across  
142 experimental conditions were assessed using t-test. For both IHC and qPCR analyses, differences were

143 considered statistically significant at  $p < 0.05$ . Statistical methodology for genomic variant characterization  
144 and RNA-sequencing differential expression analyses can be found in the Supplemental Information.

145 Detailed methods for organoid culture, genomic variant characterization, histological procedures and  
146 scoring, ISH, western analysis, LGR5(+) cell isolation and culture, flow cytometry, DKK4 analysis, RNA  
147 sequencing, and data analysis are provided in the Supplemental Information. All authors had access to the  
148 study data and reviewed and approved the final manuscript.

149 **Data Access:** The RNA-seq and genomic variant raw data are publically available at ArrayExpress under  
150 accession number E-MTAB-4698.

## 151 **RESULTS**

### 152 *Isolation, Culture and genomic characterization of human adenoma organoids*

153 We have developed an ongoing repository (Table 1) of organoids from patient-derived adenomas  
154 (n=17; including 2 high-risk sessile serrated adenomas), adenocarcinomas (n=4; including 1 colitis-  
155 associated cancer), and normal colon (n=9). All have been cryopreserved at early passage, reestablished from  
156 frozen stock, are mycoplasma-free, and have demonstrated long-term culture (i.e., > 6 months in continuous  
157 culture). The neoplasm-derived organoids have been genomically characterized for variants in a panel of  
158 common CRC driver mutations across 71 genes (Table 1).

### 159 *LGR5 immunochemical specificity in human colon and intestine.*

160 Two antibody clones for human LGR5 were used in this study: a rabbit monoclonal antibody  
161 generated against a peptide sequence of LGR5 (clone STE-1-89-11.5) and a rat monoclonal antibody  
162 generated to a full-length LGR5 protein (clone 22H2.8). At the onset of this study these antibodies were in  
163 development by Miltenyi Biotec, but now both antibodies are commercially available (Miltenyi Biotec  
164 GmbH, Bergisch Gladbach, Germany; see Methods and Supplemental Methods). Details of their  
165 development have been presented previously (Agorku et al. 2014, 2013), including the use of human *LGR4*  
166 and *LGR6* stable transfectants to demonstrate lack of cross-reactivity with these close homologues.

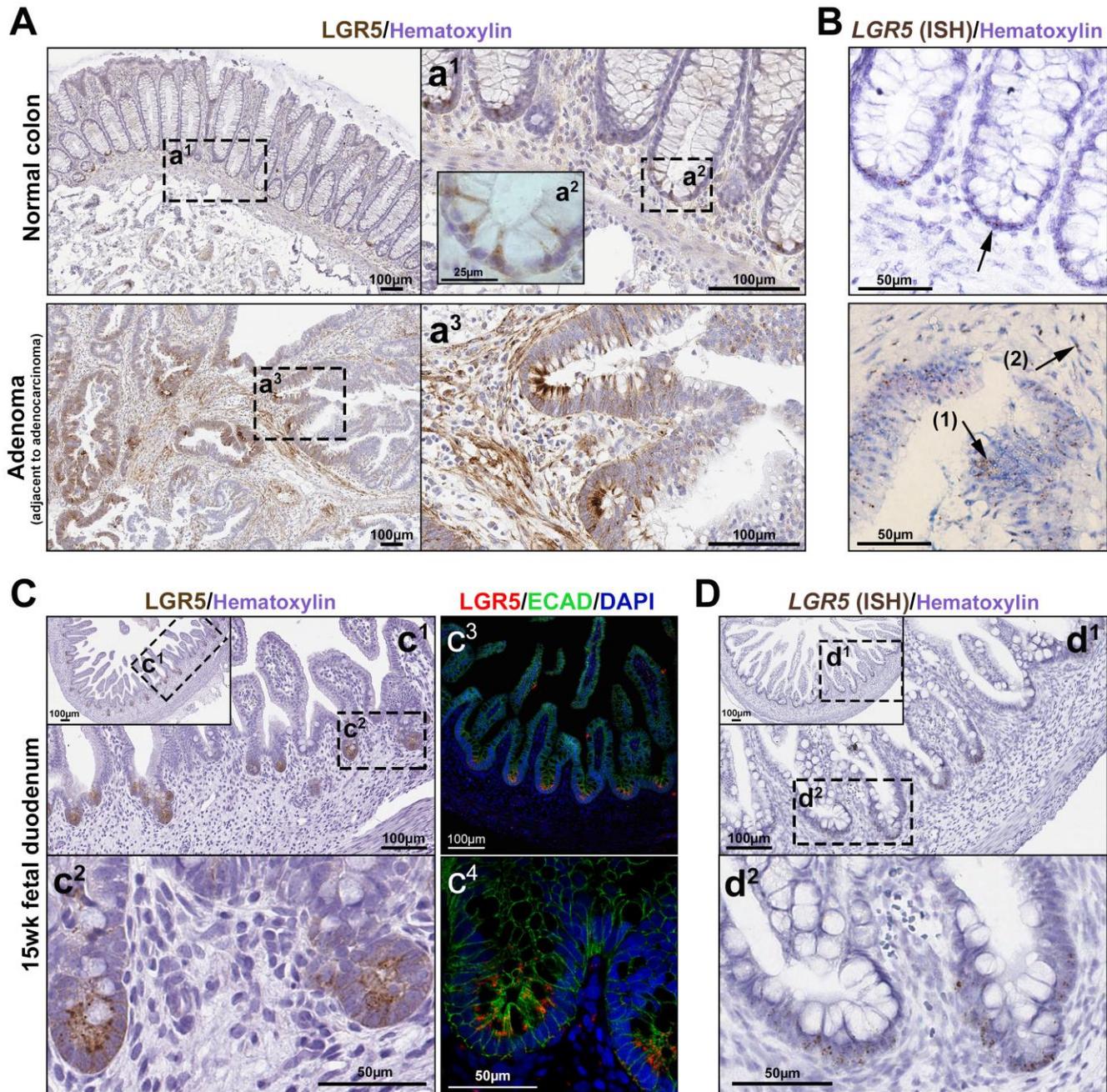
Neoplasm	ID	Gender & Age		Location	Variants Detected
Adenoma (45mm)	282	F	66	ascending	APC Ala759fs, APC Gln1429*, ATM Arg337His, ATM Lys926Glu, EP300 Pro1802Pro, MSH2 R909Q
Adenoma (10mm)	300	M	59	transverse	APC Ser985fs, APC Arg1450*, BRCA1 Leu954Leu, BRAF Thr599dup, APC E1317Q
Adenoma (60mm)	469	M	86	cecum, ascending	APC Gln1378*, APC Glu536*, TCF7L2 Ser35fs, KRAS Gly12Asp, KRAS Gly12Ser
Adenoma (≥10mm)	569	F	18	sigmoid	APC Ser1355fs, ATM Phe858Leu, ATM Pro1054Arg, KRAS Ala146Thr, KRAS Gly12Val.
Adenoma (15mm)	574	F	69	hepatic flexure, proximal transverse	APC Arg876*, APC Glu1464*
Adenoma (30mm)	575	M	73	ascending	APC Arg1450*, APC His864fs, DMD Lys34Asn, KRAS Gln61His
Adenoma (20mm)	584	M	61	ascending	APC Thr1556fs, KRAS Ala146Val, MET Arg988Cys, PMS2 Gly29Ala, TP53 Arg267Trp, EP300 Asp1579Asn
Adenoma (35mm)	590	F	58	ascending	BUB1B Arg550*, FLCN His429fs, MLH1 Lys443fs, MSH3 Lys381fs, PALB2 Met296fs, TCERG1 Arg889fs, CTNNA1 Met826Thr, CTNNB1 Ser45Phe, MAP2K4 Val127Ala, MLH3 Pro564Ser, PIK3R1 Arg188Cys
Adenoma (15mm)	726	M	51	ascending	APC Ser837*, APC Arg1450* and AXIN2 Phe791Cys
Adenoma (25mm)	735	M	72	ascending	APC Leu665*, APC Arg1450*, CTNNB1 Ala126Ser, KRAS Gly12Asp
Adenoma (20mm)	772	F	79	rectum	APC Arg216*, APC Glu225*, AKT1 Gly393Gly, PIK3R1 Ile82fs
Adenoma (associated with adenocarcinoma)	14881	M	46	rectum	TP53 Pro151Ser, KRAS Gly12Asp, APC Pro1443fs
Adenoma: FAP (2 mm)	236	F	26	ascending	APC Thr1556fs, APC Leu143fs, MLH3 E624Q
Adenoma: Lynch	610	F	59	ascending	MSH3 Lys383fs, POLE Pro441Leu, DMD Val2498Ala, APC Ser1321Ser, KRAS Gln61His, PIK3CA Glu545Lys, ERBB2 Tyr411Cys, CDH1 His121Tyr, BLM Asn515fs, ERBB2 Pro1207His, MLH3 Asn674fs, APC Arg976fs, FBXW7 Ser668fs, AXIN2 Gly665fs, APC Ser1465fs, TCERG1 Arg958fs, BRCA2 Val1852Ile, GPC6 Gly243Trp, BRCA2 Lys1691fs, BRCA2 Thr3033fs, MUTYH Pro65fs, TCF7L2 Lys485fs, SLC9A9 Ala268Val, ERBB2 Arg47His, MSH2 R909Q
Adenoma (2-5mm)	664	M	52	cecum	MSH6 A20V
Adenoma: Sessile serrated (20mm)	245	F	54	ascending	BRAF Val600Glu, WBSCR17 Ser432Ser
Adenoma: Sessile serrated (23mm)	708	F	76	cecum	BRAF Val600Glu, DCC Asn472fs
Adenocarcinoma	781	F	52	descending	PIK3CA Glu545Lys, TP53 Phe113delPhe, APC Arg232*
Adenocarcinoma	815	F	74	cecum	KRAS Gly12Asp, APC Glu1309*, APC Glu1379*, MLH3 Asp1131Gly, EGFR Val323Ile, AKT1 Thr197Thr
Adenocarcinoma	861	F	76	ascending	SMAD4 Arg361His, PMS2 Thr9Ser, BRAF Val600Glu, APC Thr1556fs, APC Leu749fs, SLC9A9 Ala445Thr, BRCA1 Ser1517Pro, CDC27 Ala15Thr, WBSCR17 Ala577Val, PIK3CA Asn345Lys, DMD Arg550His, SLC9A9 Ile397fs, PMS2 M62I
Adenocarcinoma: IBD-associated	21	M	57	ascending	APC Gly1312*, TP53 Gly266Arg, PIK3CA Glu726Lys, KRAS Ala146Thr, BRAF Gly469Ala, PIK3CA Arg38Cys
Normal colon	78, 80, 81, 83, 84, 85, 87, 88, 89			F (ages 29, 33, 45, 49, 52, 56, 62), M (ages 21, 55); ascending	

167 **Table 1.** Repository of patient-derived colon adenoma and adenocarcinoma organoids. A targeted colorectal cancer  
168 DNA sequencing panel was used to determine the presence of variants for 71 different oncogenes and tumor  
169 suppressor genes often mutated in colorectal cancers. Stop codon (\*); frame shift (SS).

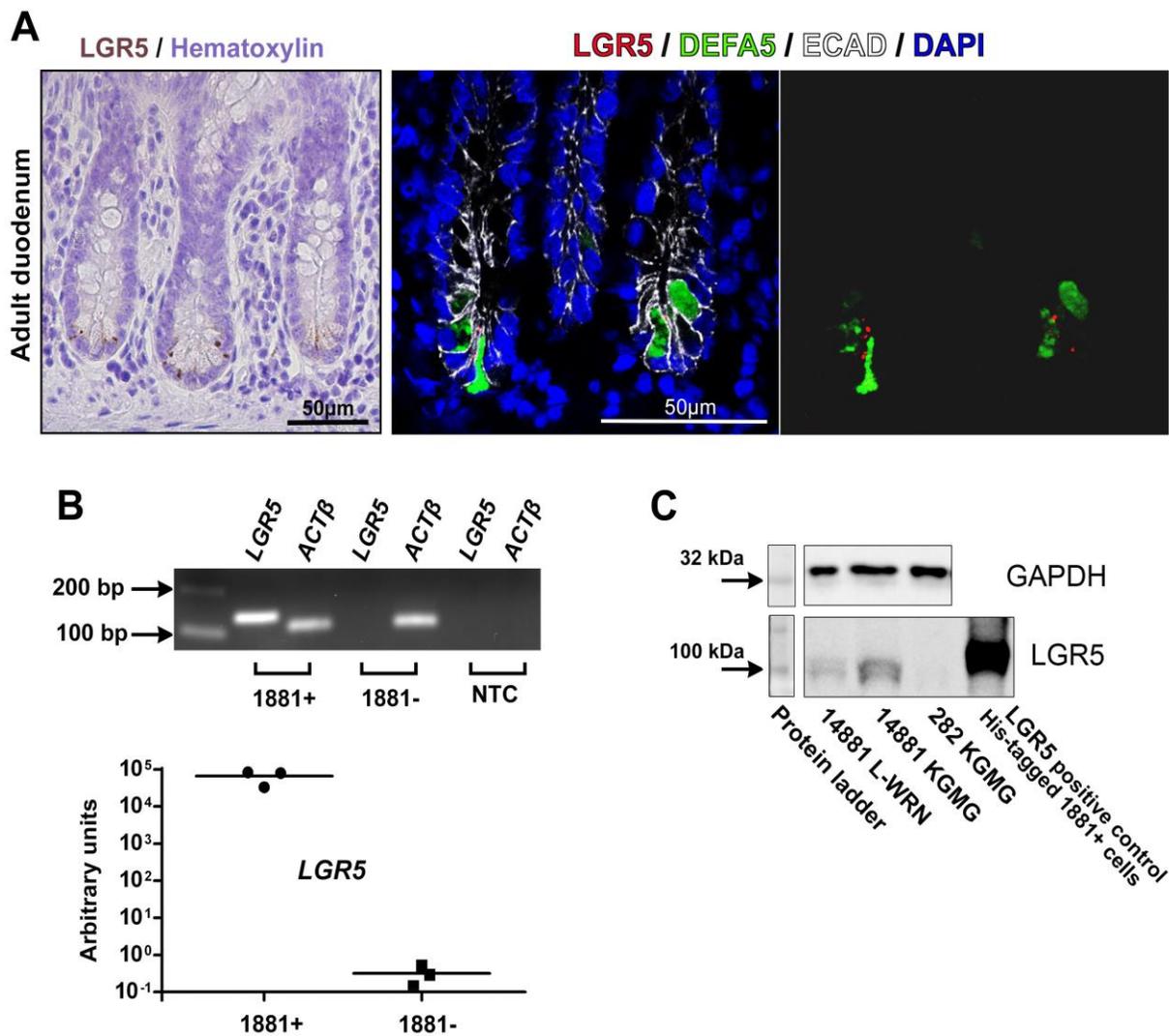
170 LGR5 immunohistochemical (IHC) expression was localized with clone STE-1-89-11.5 to the crypt  
171 base columnar (CBC) cells, with rare staining in the stromal *lamina propria* in normal formalin fixed  
172 paraffin embedded (FFPE) colon (Figure 1a<sup>1</sup>). At high magnification this staining pattern was shown to mark  
173 thin cells (Figure 1a<sup>2</sup>), consistent with the morphology of CBC cells. From the same patient, an adenoma,  
174 adjacent to adenocarcinoma, showed intensified staining at the dysplastic crypt bases (Figure 1a<sup>3</sup>) and  
175 sporadic focal staining throughout the more disorganized epithelial component. In contrast to the matched  
176 normal tissue, stromal staining was pronounced in the adenoma associated with cancerous tissue (Figure  
177 1a<sup>3</sup>). Supportive *LGR5 in situ* hybridization (ISH) staining was observed in the normal CBC cells (Figure 1B  
178 top panel); in the dysplastic epithelium (Figure 1B, arrow-1) and in the associated stroma (arrow-2).

179 The human fetal small intestine has been shown to express high levels of *LGR5* mRNA relative to  
180 adult by RNA-sequencing (Finkbeiner et al. 2015). Consistent with this, robust and specific LGR5 protein  
181 staining by IHC and immunofluorescence (Figure 1C), in conjunction with *LGR5* ISH (Figure 1D), was  
182 observed in the proliferative zone of the 15-week fetal gut. In contrast, IHC and IF staining in adult  
183 duodenum (Figure 2A) showed weak punctate LGR5(+) staining in cells present between Paneth cells  
184 marked by DefensinA5 (DEFA5), consistent with published ISH and RNA-sequencing data (Finkbeiner et al.  
185 2015).

186 Clone STE-1-89-11.5 was further demonstrated to be specific for human LGR5 by Western blotting.  
187 Mouse 1881 lymphoma cells that were previously transfected with human *LGR5* served as a positive control  
188 [1881(+); provided by Miltenyi Biotec]. Transfection stability was confirmed by mRNA expression analysis  
189 (Figure 2B). The antibody showed strong reactivity against the human LGR5 1881(+) cell line, as well as  
190 measurable activity against one adenoma organoid (specimen #14881) (Figure 2C).



191 **Figure 1. LGR5 immunochemical localization in human colon, colonic adenoma and duodenum.**  
 192 (A) LGR5 IHC staining in normal human colon (one of five representative patients) at low (a1) and high (a2)  
 193 magnification, as well as adenoma (high-grade dysplasia associated with an adenocarcinoma; specimen  
 194 14881) from the same patient (a3).  
 195 (B) LGR5 expression by in situ hybridization provides a conventional reference for the LGR5 staining in  
 196 normal crypts (upper panel) and in the adenoma (bottom panel); glandular LGR5 (arrow-1) and stromal  
 197 expression (arrow-2) in adenoma;  
 198 (C) LGR5 IHC (c1, c2) and IF staining (c3, c4) in fetal duodenum, and  
 199 (D) ISH expression in the same duodenum specimen.



200 **Figure 2. Further specificity analysis of human LGR5 antibody**

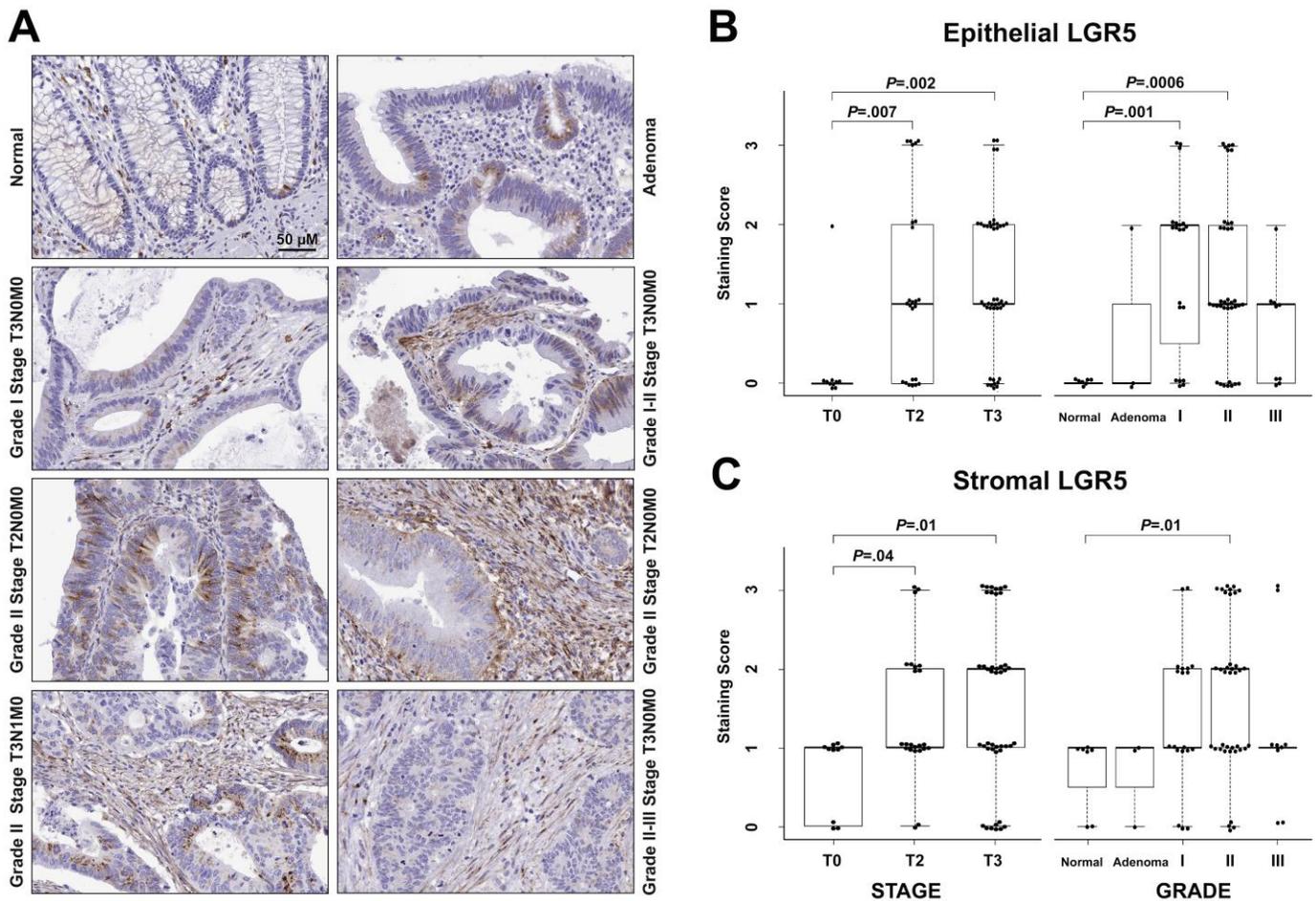
201 (A) LGR5 immunohistochemical and immunofluorescent staining in adult duodenum showed weak punctate  
 202 LGR5 expression, while adjacent cells stained with the paneth cell marker DefensinA5 (DEFA5) (middle  
 203 and right panel).

204 (B) Mouse 1881 lymphoma cells were previously transfected with human *LGR5* [1881(+); Miltenyi Biotec].  
 205 To confirm transfection stability, the 1881 LGR5(+) and 1881 LGR5(-) cells were assayed for *LGR5*  
 206 expression (n=3 biological replicates), and visualized with agarose gel analysis of qRT-PCR amplified  
 207 products.

208 (C) Measurement of human LGR5 protein expression by Western blotting with the rabbit monoclonal anti-  
 209 human LGR5 antibody clone STE-1-89-11.5 in 1881(+) cells, 282 adenoma organoids cultured in KGMG,  
 210 and 14881 adenoma organoids cultured in KGMG or L-WRN.

211 LGR5 immunohistochemical expression in human colonic adenoma and adenocarcinoma; levels of  
 212 glandular and stromal reactivity.

213 LGR5 IHC staining was performed in a FFPE TMA, which included 2 normals, 3 adenoma, and 70  
 214 adenocarcinoma, with staining of 5 additional normal colon autopsy samples obtained for our studies (Figure  
 215 3). These were scored for staining intensity in the glandular epithelium and separately in the stroma. Unlike  
 216 normal colon, which showed staining in select cells at the base of the crypt, the adenomas stained intensely  
 217 in distinct zones of epithelium or for the entire dysplastic crypt (Figure 3A and Figure 4C, left column).



218 **Figure 3. LGR5 immunohistochemical staining intensity in the epithelium and stroma across a range**  
 219 **of colorectal adenocarcinoma stages and grades.**

220 (A) Representative images of LGR5 staining in the epithelial and stromal components in normal colon (n=5  
 221 normal autopsy samples) and from a CRC tissue microarray (n=2 normals, 65-68 neoplasm).

222 (B) Association between epithelial or

223 (C) stromal staining intensity, and stage/grade of the tissue.

224 Neoplastic tissue that retained crypt-like architecture, often showed pronounced LGR5 staining segregated to  
225 the base (Figure 1a<sup>3</sup>). Relative to normal tissue, the adenocarcinomas showed increased LGR5 staining in  
226 both the epithelium and in the stroma, with the greatest significance at higher cancer stages (Figure 3B, C;  
227 stage T3; epithelium  $P=.002$ ; stroma  $P=.01$ ) and grades (Grade II; epithelium  $P=.0006$ ; stroma  $P=.01$ ).

228 *Strategy for the isolation of LGR5(+) cells from patient-derived adenoma organoids.*

229 Figure 4A outlines the approach developed for isolation and analysis of LGR5(+) cells from human  
230 adenoma. Adenoma organoids were established in culture (approx. 2 months) and genomically characterized  
231 (Figure 4B lists mutations associated with individual specimens; see Table 1 for full organoid bank). The  
232 organoid cultures used in this study (14881, 282, 584, 590) were established in ‘reduced medium’, KGM-  
233 Gold™ (KGMG), a serum-free epithelial medium containing epidermal growth factor (EGF) and pituitary  
234 extract (Dame et al. 2014). When these organoid cultures were transferred to L-WRN medium (Miyoshi and  
235 Stappenbeck 2013) (which includes Wnt3a, R-spondin-3, Noggin, Nicotinamide, and EGF), they transitioned  
236 from budding structures (Figure 4C, middle column) to thin-walled spherical cysts (Figure 4C, right  
237 column), with the exception of organoid specimen 14881, which maintained a mix of thick-walled cysts and  
238 budding structures in L-WRN (not shown). Accompanying this shift in morphology was an increase in *LGR5*  
239 mRNA expression in L-WRN-cultured organoids compared to KGMG-cultured organoids, assessed by qRT-  
240 PCR (Figure 7A, left graph). Flow cytometric analysis revealed that L-WRN-cultured organoids had an  
241 increase in the number of LGR5 positive cells (Figure 4D, comparing “stain” in the first and second row;  
242 Figure S1). ImageStream analysis visually established the specificity of the fluorescently tagged LGR5  
243 antibody to viable cells, rather than to cellular debris (Figure 4E). Even with L-WRN-enhanced LGR5, a low  
244 percentage of LGR5(+) cells was detected by flow cytometry, and therefore magnetic bead positive  
245 enrichment was used prior to FACS to increase the number of cells obtained.

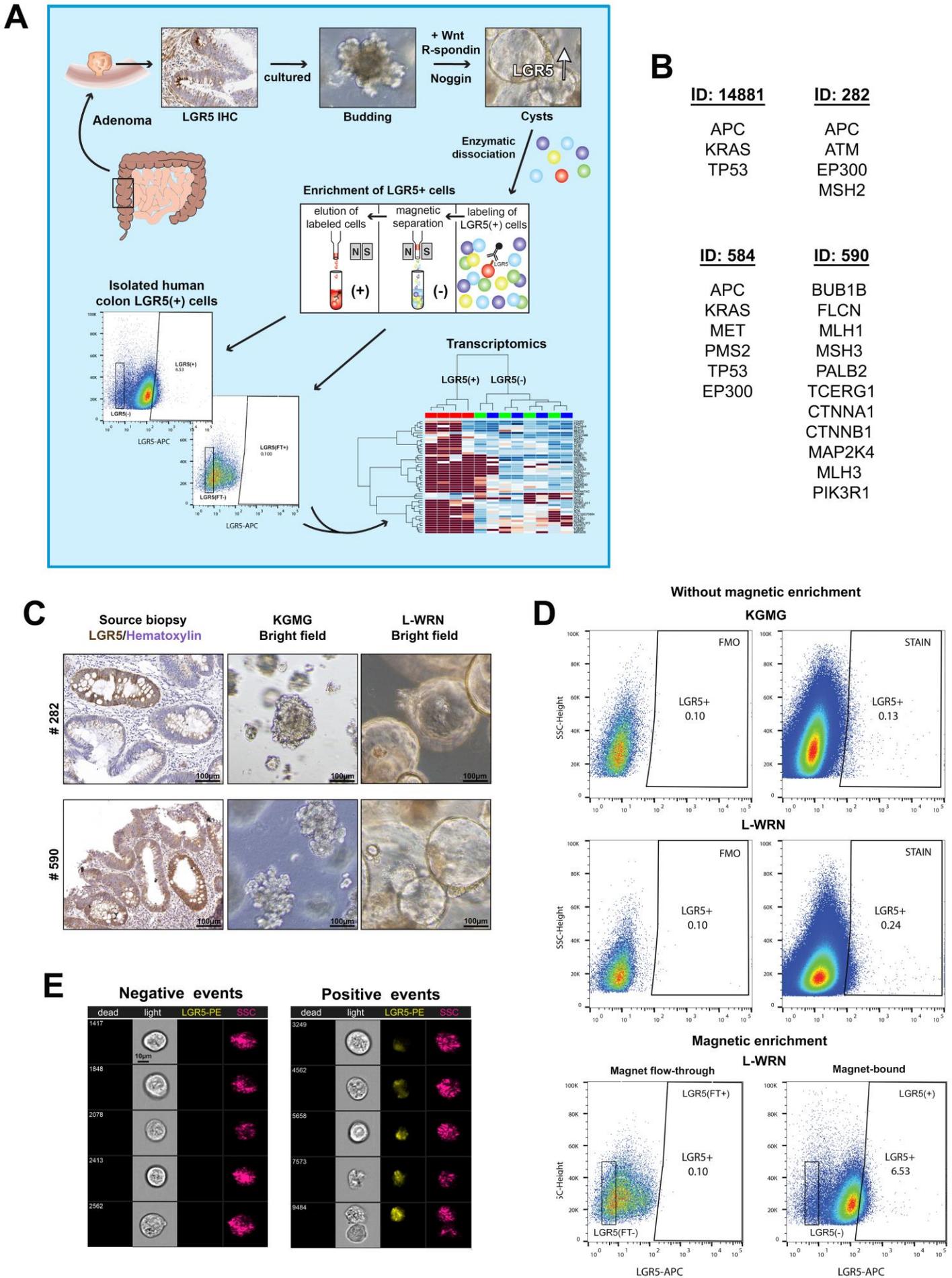


Figure 4

246 **Figure 4. Strategy for the isolation of LGR5(+) cells from patient-derived colon adenoma.**

247 (A) Graphical representation of complete procedure.

248 (B) Establishment of an organoid culture repository from human colonic adenomas; genomic  
249 characterization of organoids (see Table 1 for full organoid bank).

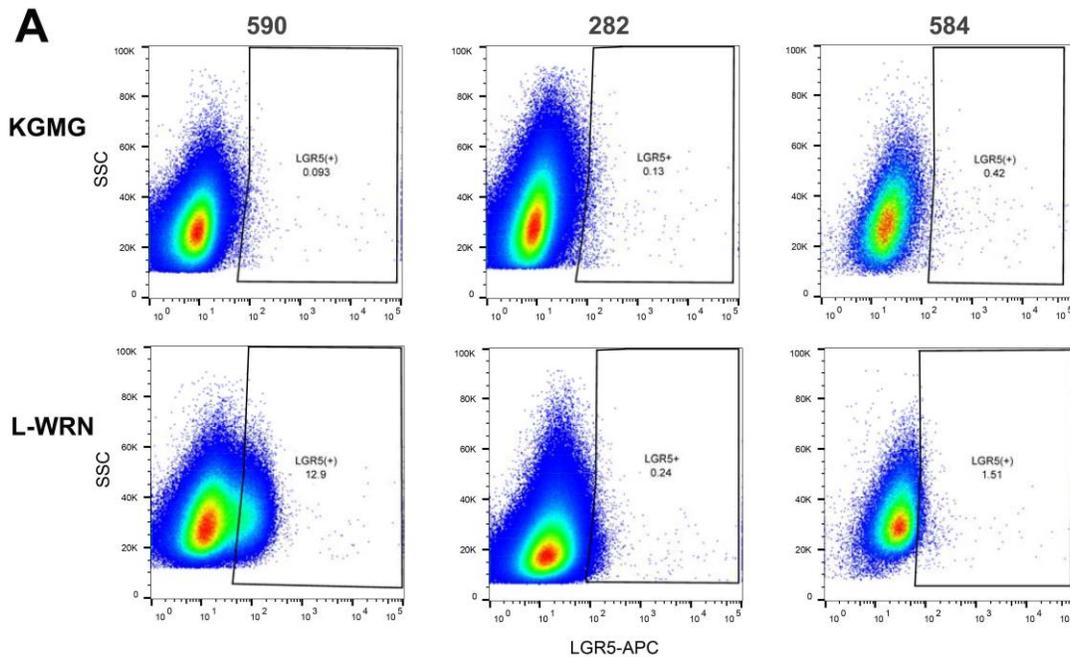
250 (C) Culture of adenoma organoids for LGR5 enrichment. LGR5 IHC staining (left column) in biopsied large  
251 adenoma (> 10mm; two representative specimens are shown). Organoids with budding morphology (middle  
252 column) derived from these specimens cultured in the reduced medium KGMG. The same cultures with  
253 cystic morphology (right column) after being transferred for 3-4 weeks to L-WRN medium.

254 (D) Representative scatterplots of LGR5 expression in organoids cultured in KGMG or L-WRN (specimen  
255 282). Live, LGR5(+) human adenoma cells were obtained after LGR5-magnetic bead enrichment by  
256 isolating DAPI(-) and LGR5(+) cellular populations.

257 (E) ImageStream analysis shows the cellular identity and positive antibody staining of sorted LGR5(+)  
258 events.

259 The 1881 LGR5(+) cells were used to validate the magnetic bead positive separation protocol and the  
260 high specificity of antibody clone 22H2.8. LGR5 expressing and non-expressing cells were mixed in varying  
261 proportions, magnetically separated, and analyzed by flow cytometry (Figure S1B). The initial ratio of 1881  
262 LGR5(+) to 1881 LGR5(-) cells was predicted as shown by the pre-magnet flow histograms (Figure S1B,  
263 left column). The post-magnetic separation shows almost complete separation of 1881 LGR5(+) cells in the  
264 positive fraction (Figure S1B, right column) from 1881 LGR5(-) cells in the negative fraction (middle  
265 column).

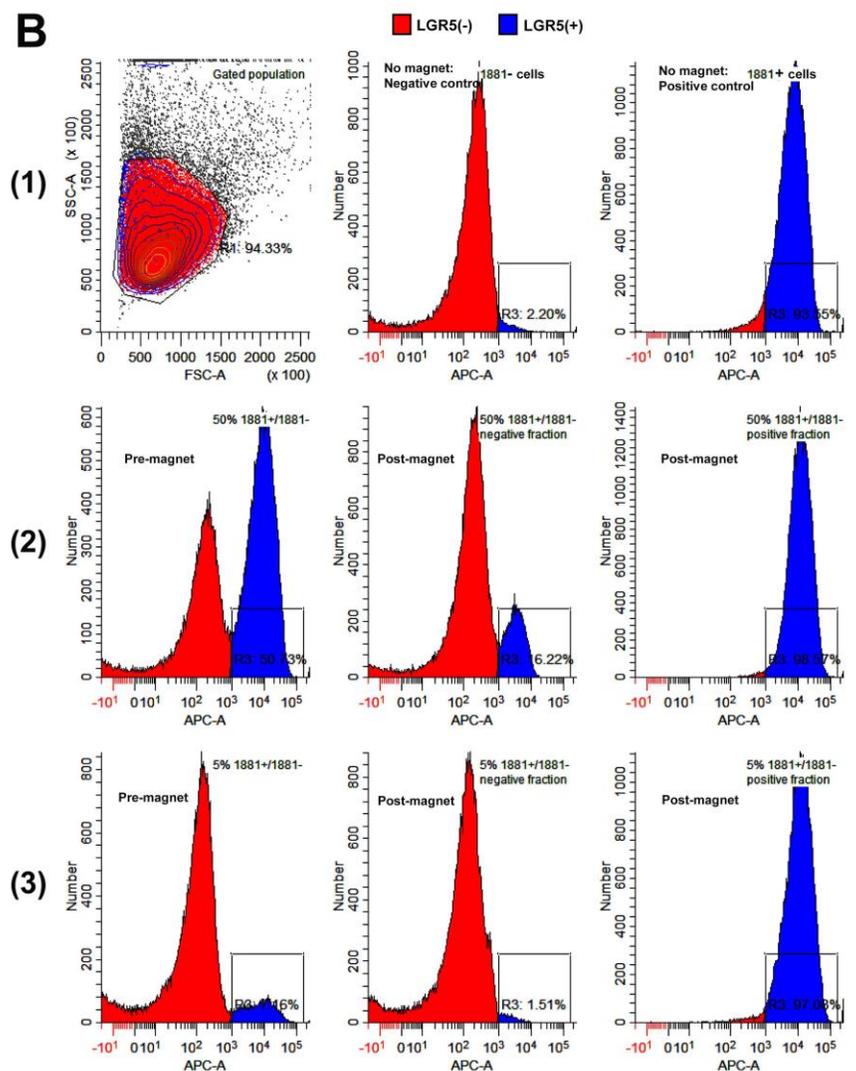
266 After magnetic bead based enrichment of the adenoma organoid cells, the unbound flow-through  
267 negative population was used to set the LGR5 FACS gate (Figure 4D bottom row). Magnetic bead-bound  
268 LGR5(+) cells were isolated via FACS, and importantly, we observed that while only a small percentage of  
269 cells bound to beads were LGR5(+) (Figure 4D bottom row), on average, magnetic bead pulldowns of  
270 LGR5(+) cells led to an 9.2-fold enrichment in the number of LGR5(+) cells obtained compared to samples  
271 without magnetic enrichment, although this did vary with specimen (specimens 282, 584, 590; SE=6.8; n=4  
272 sorts no magnet, n=8 sorts with magnet).



273 **Figure S1 (related to Figure 4).**  
 274 **Development of procedures for**  
 275 **enrichment of LGR5(+) cells; L-WRN**  
 276 **culture and magnetic-activated cell**  
 277 **sorting (MACS).**

278 (A) Flow cytometric analysis for  
 279 enhanced number and fluorescent  
 280 intensity of LGR5(+) cells resulting  
 281 from organoids cultured in the reduced  
 282 medium KGMG and then transferred for  
 283 3-4 weeks to L-WRN medium.

284 (B) Flow cytometry spike-in  
 285 experiments to confirm LGR5 antibody  
 286 specificity and to validate the magnetic  
 287 bead enrichment strategy. (1) Pure  
 288 populations of 1881 LGR5(+) and 1881  
 289 LGR5(-) cells were analyzed by flow to  
 290 establish gating strategy. 1881 LGR5(+)  
 291 and 1881 LGR5(-) cells were mixed in  
 292 the proportions, (2) 50/50 and (3) 5/95;  
 293 and analyzed by flow cytometry before (left column) and after magnetic separation  
 294 (middle/right columns) with rat monoclonal anti-human LGR5 antibody clone 22H2.8.



295 *Transcriptomic profiling of LGR5(+) adenoma cells*

296 The gene expression signature of human LGR5(+) intestinal cells is essentially uncharacterized,  
297 which has significantly limited our understanding of the role of these cells in human intestinal stem cell  
298 biology and colorectal cancer progression. Using the methods described above, we isolated LGR5(+) cells  
299 from four different adenoma-derived organoid lines and conducted RNA-seq on three sorted cellular  
300 populations from each (Figure 5, Figure S2): (1) magnetic column flow-through cells, termed LGR5(FT-);  
301 (2) cells bound to the magnet, but LGR5 negative based on FACS, termed LGR5(-); and (3) cells bound to  
302 the magnet and LGR5 positive based on FACS, termed LGR5(+). Genomic variant analysis showed that  
303 each of these patient specimens has functionally significant mutations within commonly mutated colon  
304 cancer genes, confirming the adenoma, and not normal, identity of these organoids grown in L-WRN (Table  
305 1). Expression profiles of the LGR5(-) and LGR5(FT-) were found to be largely similar at both the gene  
306 expression and pathway level (Figure S2C, D). The transcriptome-wide similarity between these two LGR5-  
307 negative populations suggests that magnetic bead enrichment alone is not sufficient to isolate LGR5(+) cells.  
308 Due to transcriptional similarity between negative populations, our analysis focused on comparisons between  
309 the magnetic bead-bound cells deemed to be LGR5(+) and LGR5(-) by FACS (Figure 5).

310 Multidimensional scaling on the top 500 most variably expressed genes revealed that samples  
311 clustered distinctly by the patient of origin, not by the expression of LGR5 (Figure 5A, Figure S2A). Despite  
312 this, we identified 519 differentially expressed genes (FDR  $P$ -value  $< .05$ ) between the two cell fractions  
313 across the four genetically diverse adenomas (Figure 5B and Table S1). Expression values for all  
314 differentially expressed genes for each specimen, as well comparisons between LGR5(+) cells and  
315 LGR5(FT-) cells, are reported in Table S1. We determined that *LGR5* had the highest level of statistical  
316 enrichment in the LGR5(+) cells (FDR=3.8E-21) and was expressed an average of 5.5 fold higher compared  
317 to LGR5(-) cells, lending confidence to the specificity of the LGR5 antibody and the separation procedure.  
318 Unsupervised hierarchical clustering analysis using the top 50 most differentially expressed genes showed  
319 clear separation between the LGR5(+) and LGR5(-) samples from three of the four enteroid lines (Figure  
320 5C), suggesting both commonalities and heterogeneity in the LGR5(+) cell gene expression signature

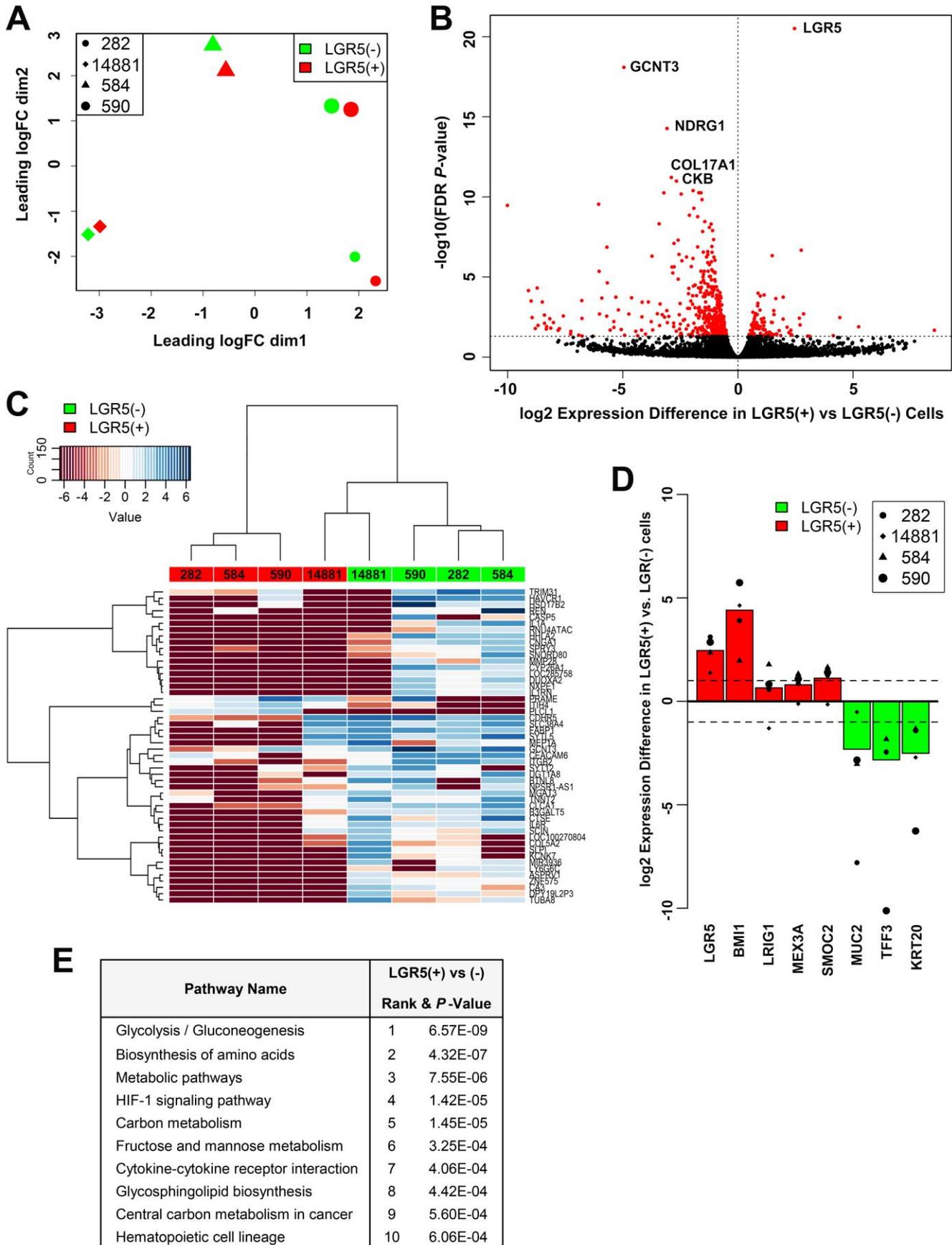


Figure 5

321 **Figure 5. Transcriptomic profiling of human LGR5(+) and LGR5(-) primary adenoma cells isolated**  
322 **from four patient-derived organoid cultures.**

323 (A) Multidimensional scaling plot of the LGR5(+) and LGR5(-) cells, based on the top 500 most variable  
324 genes. Patient identifiers #14881, 228, 584, and 590.

325 (B) False discovery rate (FDR) volcano plot of the log(2) ratio of gene expression between the LGR5(+) and  
326 LGR5(-) cells.

327 (C) Unsupervised hierarchical clustering heatmap of the 50 most differentially expressed genes by fold  
328 change between the LGR5(+) (red) and LGR5(-) (green) populations.

329 (D) Log(2) fold change in gene expression between LGR5(+) and LGR5(-) cells for known markers of colon  
330 stem (red) and differentiated (green) cells.

331 (E) The top 10 most enriched KEGG pathways for differentially expressed genes between the LGR5(+) and  
332 LGR5(-) cells.

333 **Figure S2 (Related to Figure 5). Transcriptomic profiling of human LGR5(+) primary adenoma cells;**  
334 **comparisons between sorted MACS magnet-bound LGR5(-) and sorted MACS flow-through**  
335 **LGR5(FT-) cells.**

336 (A) Multidimensional scaling plot of the LGR5(+), sorted MACS magnet-bound LGR5(-) cells, and sorted  
337 MACS flow-through LGR5(FT-) cells based on the top 500 most variable genes.

338 (B) Unsupervised hierarchical clustering heatmap of the 50 most variable genes between the LGR5(+) (red),  
339 LGR5(-) (green), and LGR5(FT-) (blue) populations.

340 (C) The top 10 most enriched KEGG pathways for differentially expressed genes between the LGR5(+) and  
341 LGR5(-); and LGR5(+) and LGR5(FT-) cells.

342 (D) Gene expression overlap between the [LGR5(+) vs. LGR5(-)], [LGR5(+) vs. LGR5(FT-)], and [LGR5(-)  
343 vs. LGR5(FT-)]

344 between specimens. When the expression signature of LGR5(+) cells were clustered with those from both  
345 the LGR5(-) and LGR5(FT-) cells, however, there was clear separation of the LGR5(+) cells across all four  
346 organoid lines (Figure S2B). Stem cell markers associated with the colon as well as other tissue-specific stem  
347 cells including *BMII*, *MEX3A*, and *SMOC2* were upregulated in LGR5(+) cells, while known markers of  
348 colonic differentiation, including *MUC2*, *TFF3*, and *KRT20* were down-regulated (Figure 5D). Pathway  
349 analyses revealed differential expression in LGR5(+) cells for genes involved in metabolism, including the  
350 pathways, *glycolysis and gluconeogenesis*, *biosynthesis of amino acids*, *metabolic pathways*, *carbon*  
351 *metabolism*, *fructose/mannose metabolism*, *glycosphingolipid biosynthesis*, and *central carbon metabolism*

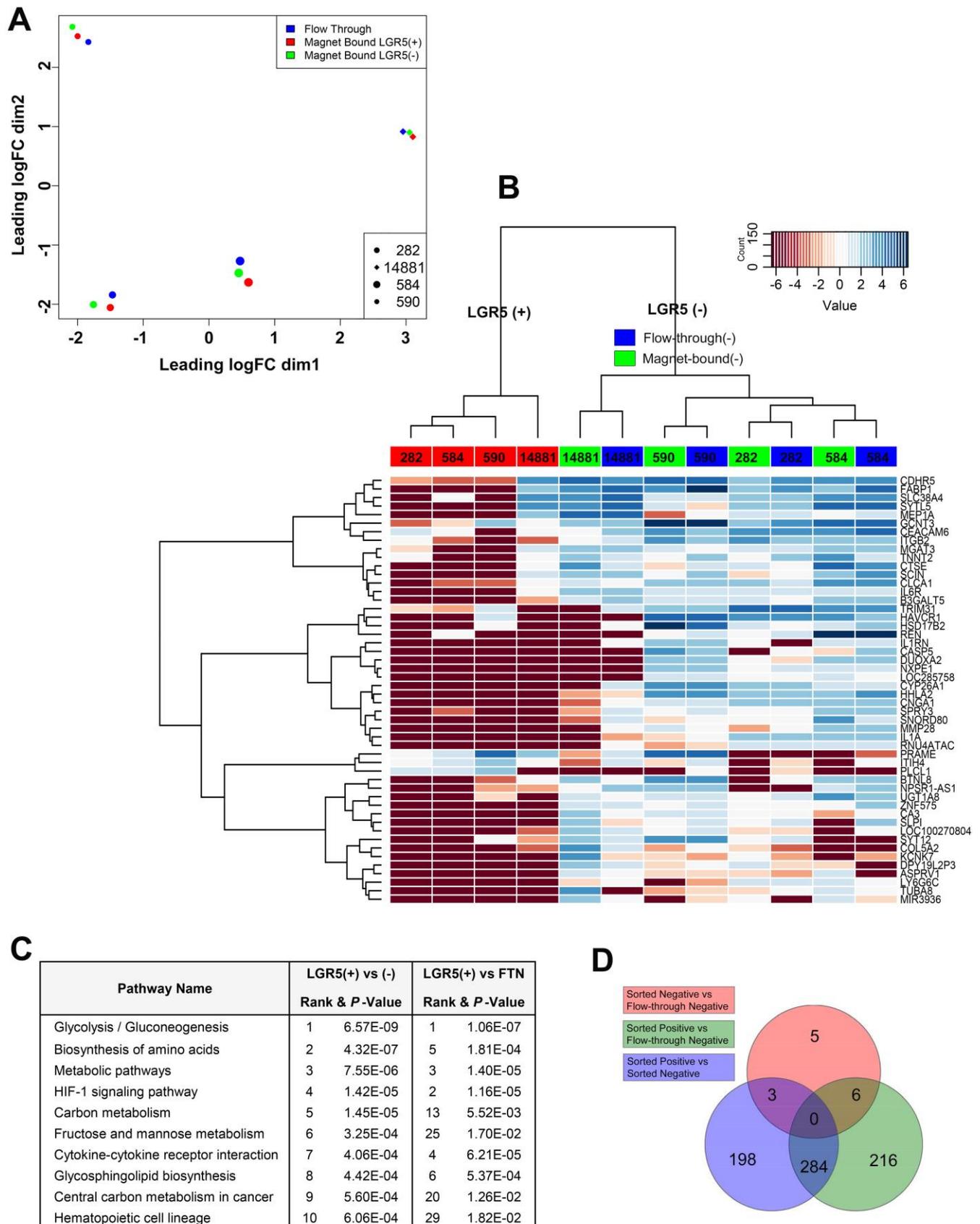


Figure S2

352 *in cancer* (Figure 5E; Figure S2C). Additionally, LGR5(+) cells had significantly differentially expressed  
 353 genes involved in *HIF-1 signaling, cytokine-cytokine receptor interaction, hematopoietic cell lineage,*  
 354 *MAPK signaling, PI3K/AKT signaling, and pathways in cancer* (Table S1; Figure 5E, Figure S2C, Figure  
 355 S3-8).

356 We also identified a high level of  
 357 concordance between LGR5(+) cells and a  
 358 previously reported set of genes associated  
 359 with high levels of Wnt signaling in mouse  
 360 cancer stem cells (based on TOP-GFP Wnt  
 361 reporter activity (Vermeulen et al. 2010)  
 362 (Figure 6A). In order to identify a “human  
 363 adenoma LGR5(+) cell gene signature” with  
 364 more stringent parameters, we compared  
 365 genes upregulated in LGR5(+) cells to both  
 366 LGR5(-) and to LGR5(FT-) cells to which we  
 367 identified 33 genes (Table 2). A comparison  
 368 between these 33 genes in the “human  
 369 adenoma LGR5(+) cell gene signature” and  
 370 the previously published “murine intestinal  
 371 stem cell signature,” generated from isolated  
 372 Lgr5<sup>HI</sup> ISCs (Munoz et al. 2012), identified 4  
 373 overlapping genes that were enriched in both  
 374 populations, including the canonical ISC  
 375 markers *LGR5, SMOC2, and CDCA7* (Figure 6B). This analysis also revealed a large number of genes in the  
 376 human adenoma LGR5(+) cell signature that did not overlap with the mouse Lgr5 stem cell signature.

Gene	(+ vs. (-))		(+ vs. (FT-))	
	logFC	FDR	logFC	FDR
LGR5	2.456	3.1E-21	2.896	9.9E-30
PLA2G2A	2.746	2.1E-07	1.990	1.9E-04
STARD9	1.487	4.7E-07	0.942	0.005
STK38L	0.874	1.0E-04	0.669	0.008
TBC1D1	0.971	1.5E-04	1.044	4.0E-05
MYRIP	1.963	1.8E-04	1.801	0.001
ITPR2	0.866	4.4E-04	0.735	0.005
ZAK	0.854	0.001	0.785	0.002
INHBB	1.555	0.001	1.990	8.6E-06
APBB2	0.647	0.002	0.529	0.019
RHEB	0.770	0.002	0.613	0.027
PRSS56	1.566	0.002	2.242	8.0E-07
PTPRD	0.740	0.003	0.743	0.003
PRAME	4.424	0.003	3.403	0.028
SMOC2	1.121	0.004	1.329	2.9E-04
SLC39A6	0.709	0.004	0.643	0.013
MAP1A	1.152	0.006	1.182	0.005
HNRPD	0.589	0.009	0.550	0.020
ICK	0.858	0.010	0.774	0.027
KREMEN1	0.740	0.013	0.936	0.001
SCRN1	0.932	0.017	0.880	0.027
TMEM19	1.097	0.018	1.047	0.028
ABCB1	0.645	0.019	0.701	0.008
TGFB3	1.181	0.020	1.274	0.009
ZNF219	1.106	0.020	1.113	0.021
GLT25D2	1.365	0.027	1.601	0.004
CTNNB1	0.632	0.030	0.798	0.002
GINS2	1.231	0.030	1.210	0.032
IL17RD	0.653	0.036	0.759	0.007
CDCA7	0.623	0.038	0.612	0.042
CFTR	0.484	0.038	0.493	0.031
LRP4	0.677	0.047	0.896	0.002
HIP1	0.852	0.049	0.870	0.039

**Table 2.** Genes upregulated in human adenoma LGR5(+) cells when compared to both LGR5(-) and to LGR5(FT-)

377           Given that our transcriptional analysis compared cells, LGR5(+) vs. LGR5(-), isolated from  
378 adenoma-derived organoid cultures (as opposed to intact tissue biopsies), we reasoned that it was possible  
379 that LGR5(+) enriched genes might be masked by comparing fractions which in whole were cultured in  
380 medium that promotes high levels of WNT signaling (L-WRN). Therefore, we compared LGR5(+) cells with  
381 previously published RNA-seq data from whole-thickness normal human colon (Uhlen et al. 2015) (Figure  
382 6C, D). When we compared the 33 genes from our “human adenoma LGR5(+) cell gene signature” (Table 2)  
383 to normal colon, we observed a strong enrichment for a majority of the genes (Figure 6C). In an unbiased  
384 comparison of all genes (Figure 6D), two of the most over-expressed genes by overall fold change in  
385 LGR5(+) adenoma cells compared to normal colon were the Wnt pathway inhibitor *DKK4* (dickkopf WNT  
386 signaling pathway inhibitor 4) and *FGF20* (fibroblast growth factor 20). Both were virtually undetectable in  
387 the normal colon (Table S1). An analysis of TCGA colorectal cancer gene expression data (The Cancer  
388 Genome Atlas 2012) revealed that both *DKK4* and *FGF20* are significantly upregulated in colorectal tumors  
389 compared to normal tissue (Figure 6E).

390 **Figure 6. Comparisons between genes in the human adenoma LGR5(+) cell gene signature and**  
391 **previously published murine and human signatures.**

392 (A) Concordance of adenoma LGR5(+) cell gene expression with gene expression associated with Wnt  
393 signaling in mouse cancer stem cells, based on TOP-GFP Wnt reporter activity (Vermeulen et al. 2010).

394 (B) Gene expression overlap between genes upregulated in LGR5(+) vs. LGR5(-) cells and the *Lgr5* mouse  
395 intestinal stem cell signature reported in Muñoz et al (Munoz et al. 2012).

396 (C) Comparison of gene expression between LGR5(+) adenoma cells and previously published RNA-seq  
397 data from normal human colon (Uhlen et al. 2015) for genes in the "human adenoma LGR5+ cell gene  
398 signature.

399 (D) The top ten most differentially expressed genes by magnitude between LGR5(+) adenoma cells and  
400 normal human colon (all genes; unbiased analysis) (Uhlen et al. 2015).

401 (E) An analysis of TCGA colorectal cancer gene expression data through OncoPrint™ of *DKK4* and *FGF20*  
402 comparing colorectal adenocarcinoma expression with normal colon and rectum.

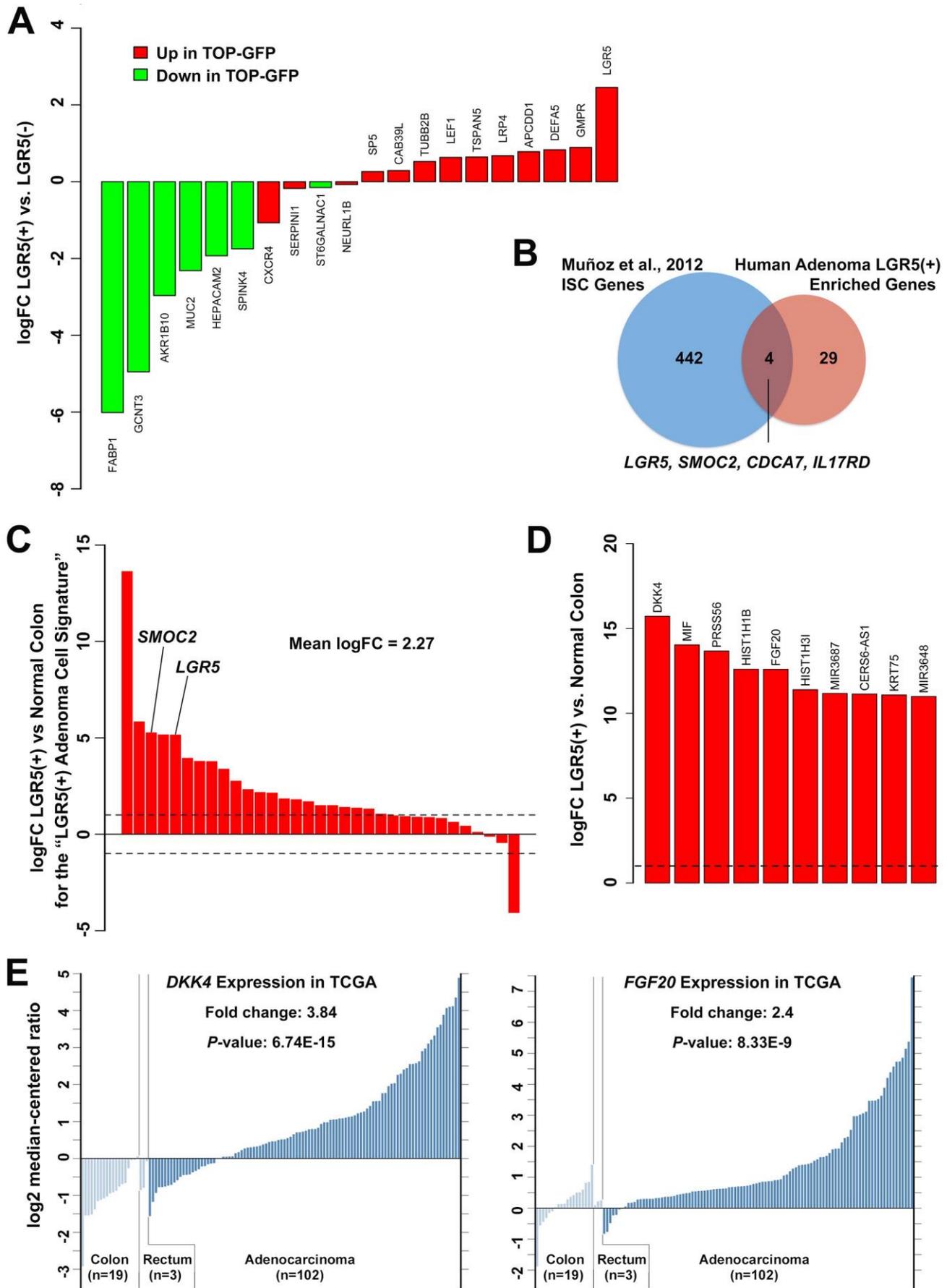
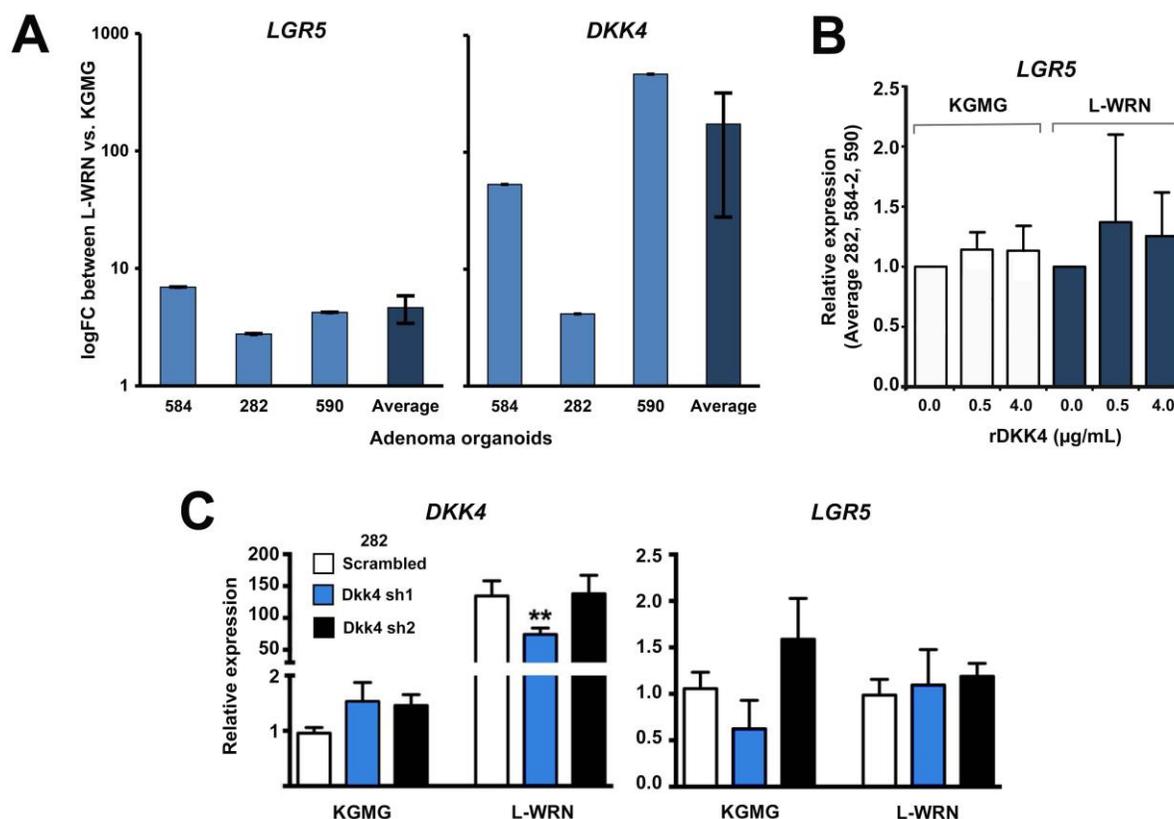


Figure 6

403 We further assessed *DKK4* in 3 of the 4 of adenoma organoids (282, 584, 590) in which *DKK4* was  
 404 expressed at higher levels in the *LGR5*(+), relative to *LGR5*(-) cells (4-fold, 1.9E-06 FDR; Table S1), by  
 405 comparing expression when cultured in L-WRN vs KGMG. Both *LGR5* (average fold change  $4.6 \pm 1.2$ SE)  
 406 and *DKK4* (average fold change  $174.3 \pm 146$ SE) show significantly enhanced expression in organoids when  
 407 cultured in L-WRN (Figure 7B). When these organoids were treated with recombinant human *DKK4* for  
 408 72hrs, there was no significant change in *LGR5* expression in either KGMG or L-WRN (Figure 7C). With a  
 409 *DKK4* stable organoid knockdown (specimen 282), LWRN-enhanced *DKK4* expression was significantly  
 410 attenuated, but LWRN-enhanced *LGR5* expression did not change (Figure 7D). Taken together, these data  
 411 suggest that *DKK4* may not play a significant role in *LGR5*(+) stem cell maintenance, but that *DKK4* could  
 412 be a novel marker associated with *LGR5* expression in neoplastic tissue.



413 **Figure 7. *DKK4* (dickkopf WNT signaling pathway inhibitor-4) comparisons to *LGR5* expression.**  
 414 (A) Relative *LGR5* and *DKK4* mRNA expression in L-WRN vs KGMG cultures. Individual specimen  
 415 standard error as technical replicates, n=6; with average and SE values of the three biological replicates.  
 416 (B) *LGR5* mRNA expression levels in adenoma organoids after treatment for 72 hrs with recombinant *DKK4*  
 417 in KGMG and L-WRN. (C) *DKK4* and *LGR5* expression levels in stable adenoma organoids with two *DKK4*  
 418 knockdowns (*Dkk4* sh1, *Dkk4* sh2) and scramble small hairpin RNA (scramble).  
 419

## 420 Identification and Isolation of Normal Colon LGR5(+) Cells

421 Due to the enrichment of LGR5(+) cells grown in media promoting high-WNT signaling levels, we  
422 also wanted to isolate LGR5(+) cells directly from normal colon tissue to demonstrate robustness of the  
423 isolation and purification methods. Using the same dissociation methods as were employed for the adenoma  
424 organoid cultures, dissociated single cells from 3 independent normal cadaveric colon specimens were sorted  
425 for EpCAM positivity and LGR5 expression. LGR5(+) cells comprised 2.35%, 0.63%, and 0.76% of the  
426 EpCAM(+) cells in independent experiments. A representative distribution of LGR5(+) staining from a  
427 normal colon, demonstrates that fluorescent intensity for LGR5(+) events were 2-3 orders of magnitude  
428 above baseline (Figure 8A). LGR5(+) cells were collected and subsequently analyzed by qPCR for *LGR5*  
429 and the intestinal stem cell marker *OLFM4*, which was recently shown to be expressed in normal human  
430 colon, and in adenoma (Jang et al. 2016). We identified an average of 7.5-fold increase in *LGR5* expression  
431 and 2.3-fold increase in *OLFM4* mRNA expression in the sorted LGR5(+) normal colon cells relative to the  
432 LGR5(-) cells (Figure 8B). Further, protein expression of OLFM4 was confirmed by IF, which showed  
433 staining at the bottom 1/3<sup>rd</sup> of the normal colon crypts (Figure 8C).

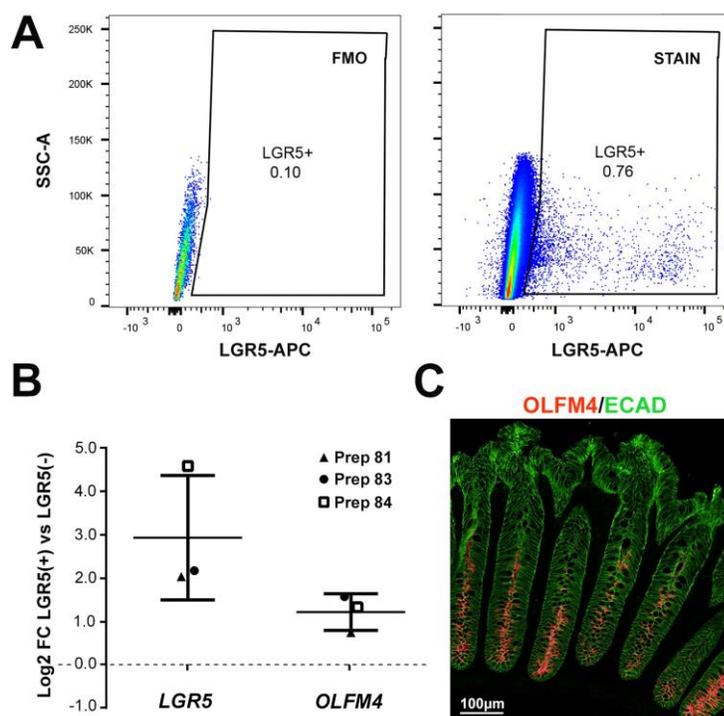
### 434 Figure 8. Identification, isolation and culture 435 of patient-derived normal colon LGR5(+) cells.

436 cells. (A) Flow cytometry gating strategy for  
437 LGR5(+) cells isolated directly from warm  
438 autopsy normal colon tissue (representative  
439 scatter plots of 3 specimens; specimen 84).

440 Includes EpCAM(+) inclusion criteria, using a  
441 phycoerythrin (PE)-conjugated antibody (not  
442 shown). To set the LGR5-APC(+) gating, either  
443 magnet flow-through (specimen 81 & 83), as  
444 done with organoid sorts, or rigorous so-called  
445 fluorescence-minus-one controls [FMO; DAPI(-),  
446 EpCAM(+);specimen 84] were used.

447 (B) mRNA expression by qPCR of *LGR5* and

448 *OLFM4* across all 3 patient samples. (C) OLFM4 immunofluorescent staining of normal colon crypts.



## DISCUSSION

449 LGR5 has been identified as one of the few cell surface markers available for the identification and  
450 isolation of cycling stem cells in the colonic crypt, although efforts to generate effective antibodies to isolate  
451 these cells have largely been unsuccessful.(Barker 2014). Recently antibody drug conjugates (ADCs) have  
452 been developed to target cells expressing LGR5, demonstrating therapeutic potential (Gong et al. 2016,  
453 Junttila et al. 2015), but as part of these applications primary human cell isolation and characterization was  
454 limited (Hirsch and Ried 2016). Thus, the role of LGR5(+) stem cells in human intestinal development,  
455 homeostasis, and carcinogenesis remains poorly understood. The identification and isolation of human  
456 LGR5(+) adenoma cells, as well as the establishment of methods for the long term culture and propagation  
457 of organoids enriched for these rare cells, as described in this study, provides some of the first insight into  
458 the characteristics of these cells in humans. Further, we confirm that our methods are robust for the  
459 identification and isolation of LGR5(+) cells from normal human colon tissues. Ideally, methods and data  
460 sets presented in this work will be widely utilized to overcome a major hurdle in the field of colorectal  
461 cancer stem cell biology.

462 Human primary intestinal organoid culture systems represent an unparalleled tool to study colorectal  
463 cancer biology (Matano et al. 2015) and to develop precision medicine platforms for targeted therapeutics  
464 (van de Wetering et al. 2015). Here, we report a living biobank of human normal colon, adenoma, and  
465 adenocarcinoma tissues. We used this biobank to study and isolate LGR5(+) cells from four genetically  
466 diverse adenoma-derived organoids. We found that LGR5 is expressed at higher levels in organoids in  
467 response to Wnt3a, Rspodin-3, and Noggin, and that this is associated with a cystic phenotype, consistent  
468 with other reports (Sato, van Es, et al. 2011, Farin, Van Es, and Clevers 2012, Matano et al. 2015, Drost et al.  
469 2015, Onuma et al. 2013). While this report was in preparation, a study eloquently demonstrated that *Mex3a*  
470 marks a subset of *Lgr5*(+) slow cycling intestinal cells (Barriga et al. 2017). Indeed, we show that *MEX3A* is  
471 among the upregulated genes in isolated human colon LGR5(+) cells. Interestingly, while gene expression  
472 associated with LGR5 expression in human adenoma significantly overlapped with *Lgr5*<sup>HI</sup> stem cells in the  
473 mouse (Munoz et al. 2012), there were several interesting examples where human expression patterns were

474 opposite to that of the mouse. For example, *SEMA3B* and *PLCE1* are enriched in mouse *Lgr5<sup>HI</sup>* cells, but  
475 were downregulated (FDR=0.04 and 0.002 respectively) in human LGR5(+) adenoma cells. Determining  
476 whether differential expression of these genes reflects differences in human compared to mouse biology or  
477 adenoma compared to normal human colon biology represents an important future direction for this research.

478         Histological approaches that compared LGR5(+) cells from adenoma and adenocarcinoma samples  
479 with normal colon revealed a number of unexpected biological findings. For example, we identified the  
480 presence of stromal LGR5 staining, and confirmed the specificity of this signal with *LGR5* mRNA ISH. We  
481 did not however identify obvious stromal staining in normal tissue. We identified that stromal staining of  
482 LGR5 was associated with cancer stage based on a cohort of independent samples on a tissue microarray.  
483 Others have identified the presence of multipotent *Lgr5*(+) stromal cells in the oral mucosa and tongue of  
484 adult mice expressing the *Lgr5*-EGFP reporter with a phenotype resembling neural crest cells (Boddupally et  
485 al. 2016). Our finding is likely of high relevance in light of the identification of stromal gene expression  
486 having a strong correlation with colorectal cancer subtypes (Calon et al. 2015). Future work should focus on  
487 understanding the role of these putative LGR5 expressing stromal cells in colorectal carcinogenesis.

488         Transcriptional comparisons of LGR5(+) adenoma stem cells with published normal colonic RNA-  
489 seq data (Uhlen et al. 2015), identified genes strongly upregulated in LGR5(+) adenoma cells that were  
490 virtually unexpressed in the normal human colon: *DKK4* and *FGF20*. *DKK4* is a negative regulator of Wnt  
491 signaling that has been reported as both upregulated (Matsui et al. 2009, Pendas-Franco et al. 2008) and  
492 downregulated in CRC (Baehs et al. 2009). A recent study showed that *DKK4* acts selectively to inhibit a  
493 subset of WNT ligands, but is proteolytically inactivated (Sima et al. 2016). Elevated *DKK4* expression is  
494 linked with CRC chemoresistance (Ebert et al. 2012) and metastasis (Chen et al. 2015), both processes  
495 associated with cancer stem cells. *DKK4* expression has also been correlated with *FGF20* expression (Matsui  
496 et al. 2009), and *FGF20* is a known Wnt target gene (Kato and Kato 2007), although the role that *FGF20*  
497 plays in colorectal carcinogenesis is not well defined. We identified that *DKK4* and *FGF20* are significantly  
498 upregulated in colorectal cancers, compared to normal colon, in data from TCGA. The knockdown of *DKK4*,  
499 or treatment of organoids with recombinant *DKK4*, did not modulate *LGR5* expression, calling into question

500 whether there is a functional role for DKK4 in LGR5(+) cells. However, the association with *LGR5*  
501 expression suggests that DKK4 could be a novel biomarker of colon adenoma stem cells or cells with high  
502 Wnt activity, and may prove useful for colon cancer prevention or treatment efforts.

503 Notably, our experiments used a two-step enrichment methodology to obtain purified cells (magnet  
504 followed by FACS), yet we still encountered a relatively small population of LGR5(+) cells. This could be  
505 explained, in part, by work showing rapid internalization of LGR5 from the plasma membrane to the trans-  
506 golgi network (Snyder et al. 2017). Thus, it is possible that our antibody-based methods will only recognize  
507 rare cells expressing the highest levels of LGR5 protein on the cell surface. On one hand, this may preclude  
508 us from isolating cells that express *LGR5* mRNA but that do not have abundant protein localized to the cell  
509 surface. On the other hand, this same caveat would enhance specificity for the sorted population of cells, as  
510 supported by our gene expression profiling.

511 Our study presents a blueprint for the identification, *in vitro* culture, isolation, and experimental  
512 characterization of human LGR5(+) cells. We anticipate our methods can be readily adapted for the isolation  
513 and characterization of the LGR5(+) cell populations in other human tissues including stomach (Barker et al.  
514 2010, Simon et al. 2012), hair (Jaks et al. 2008), mammary gland (de Visser et al. 2012), prostate (Wang,  
515 Wang, et al. 2015), and kidney (Barker et al. 2012). Additionally, we expect that the organoid culture  
516 methods described here will provide an experimental platform for the development of novel  
517 chemopreventive and chemotherapeutic agents that target LGR5(+) stem cells.

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## Supplemental Experimental Procedures and Figures

### 1 *Establishment of Organoid Cultures from Human Colonic Adenomas.*

2           The isolation and culture procedure was based on recently described methodology for establishing  
3 and maintaining unlimited growth of human colonic adenoma tissue in organoid culture (Dame et al. 2014)  
4 founded in the original work of Sato *et al* (Sato, Stange, et al. 2011). Use of this procedure has generated a  
5 working cryorepository of adenoma organoids (Table S1). Tissue was collected from subjects recruited  
6 through the Molecular Pathology and Biosample Core (MPBC) as part of the GI SPORE at the University of  
7 Michigan. Colonic tissue was acquired by endoscopy (adenomas) or from colonic resections  
8 (adenocarcinomas) and according to protocols approved by the University of Michigan Institutional Review  
9 Board (IRB; HUM00064405/0038437/00030020). Adult small intestinal and colonic tissue was collected  
10 from deceased donors through the Gift of Life, Michigan (University of Michigan IRB HUM0010148810).  
11 Fetal small intestinal tissue was obtained from the University of Washington Laboratory of Developmental  
12 Biology and approved by University of Michigan under IRB approval (HUM00093465).

13           Two growth media were used. The patient-derived adenoma organoid specimens selected for this  
14 study were maintained in continuous culture in a reduced medium (KGM-Gold, 195769; Lonza,  
15 Walkersville, MD); a serum-free epithelial medium containing epidermal growth factor (EGF) and pituitary  
16 extract, with the antimicrobials 25µg/mL gentamicin (Gibco, #15750060; Grand Island, NY) and 50µg/mL  
17 Primocin (InvivoGen, #ant-pm-1; San Diego, CA) and made to 0.15mM calcium with CaCl<sub>2</sub> (PromoCell  
18 GmbH; # 34006; Heidelberg, Germany). The second medium (L-WRN medium) was employed to drive the  
19 budding organoids into a cystic morphology; high in Wnt3a, R-spondin-3 and Noggin, all provided by a  
20 conditioned medium from stably transfected support L-cells as described by Miyoshi (Miyoshi and  
21 Stappenbeck 2013). The complete L-WRN medium contained Advanced DMEM/F-12 (Gibco, 12634028),  
22 2mM GlutaMax (Gibco, #35050-061), 10mM HEPES (Gibco, #15630080), N-2 media supplement (Gibco;  
23 #17502048), B-27 supplement minus vitamin A (Gibco; 12587010), 50 units/mL penicillin, 0.05 mg/mL  
24 streptomycin (Gibco, #15070063), 50µg/ml Primocin (InvivoGen; San Diego, CA), 1mM N-Acetyl-L-  
25 cysteine (Sigma-Aldrich, A9165; St. Louis, MO), and 100ng EGF/mL (R&D Systems, Inc., 236-EG;

26 Minneapolis, MN), as well as the addition of 10mM Nicotinamide (Sigma-Aldrich, N0636). To drive the  
27 cultures to a cyst morphology, cultures were gradually switched from KGMG to L-WRN for three to four  
28 weeks (3 days KGMG post-passage, 5 days 50/50 L-WRN; 5 days 75/25; 100% L-WRN until predominately  
29 cystic). Consistent with previous reports, organoids formed cystic structures in the presence of Wnt ligand  
30 provided by L-WRN medium (Onuma et al. 2013, Drost et al. 2015, Sato, van Es, et al. 2011, Farin, Van Es,  
31 and Clevers 2012, Matano et al. 2015).

32 Growth of normal colon organoids and some adenoma specimens required the above L-WRN  
33 medium with additional growth factors: 10 $\mu$ M SB202190 (Sigma-Aldrich; S7067) and 500nM A83-01  
34 (R&D Tocris, #2939) (Sato, Stange, et al. 2011). Further, a subset of these adenoma showed inhibition of  
35 growth with the p38 MAP kinase inhibitor, SB202190 (Sigma-Aldrich; #S7067), as described by Fujii et al  
36 (Fujii et al. 2016). 10mM Nicotinamide was used at the initial establishment of the specimens in organoid  
37 culture, and then removed for continued expansion.

38 Cultures were propagated in Matrigel (Corning, #354234; Bedford, MA) which was diluted to  
39 8mg/mL in growth media on 6-well tissue culture plates (USA Scientific CytoOne, #CC7682-7506; Ocala,  
40 FL). All plasticware, excluding culture plates, were coated in 0.1% bovine serum albumen in DPBS (Sigma-  
41 Aldrich; #A8806) to prevent tissue/organoids/cells from adhering to surfaces. Cultures were passaged every  
42 4-7 days by digesting Matrigel in cold 2mM EDTA in DPBS and plated the first day with the Rho-associated  
43 protein kinase (*ROCK*) inhibitor, 10 $\mu$ M Y27632 (Miltenyi Biotec GmbH, #130-104-169; Bergisch Gladbach,  
44 Germany). For normal colon and select adenoma grown in L-WRN, 2.5 $\mu$ M CHIR99021 (Miltenyi Biotec,  
45 #130-103-926) was also added at passaging.

#### 46 *Genomic Characterization of Organoid Cryorepository Specimens.*

47 DNA was extracted from Allprotect Tissue Reagent (Qiagen, #76405; Germantown, MD) preserved  
48 samples or directly from fresh organoid preparations with the All prep DNA/RNA/protein mini kit (Qiagen,  
49 #80004). For specimens 574 and 584, DNA was isolated from FFPE source tumor with the QIAamp DNA  
50 FFPE Tissue Kit, (Qiagen, #56404).

51 The QIAseq Targeted DNA colorectal cancer panel (Qiagen, #DHS-002Z) was used to determine the  
52 presence of somatic and germline variants (Figure 4B, Table 1). The panel is compatible with Illumina  
53 sequencing technology and covers 71 different oncogenes and tumor suppressor genes often mutated in  
54 colorectal cancers. The assay was performed using 80ng of purified gDNA following the vendor's manual.  
55 The libraries were sequenced on a HiSEQ 2500 using V2 chemistry (Illumina Inc; San Diego, CA). The  
56 sequencing data was demultiplexed and the Fastq files generated using Casava. The fastq files were imported  
57 into the QIAseq DNA enrichment portal for alignment and variant analysis. The targets were covered at an  
58 average read depth of 2000x with a coverage uniformity at 20% of the mean over 99%. Somatic variants  
59 were manually inspected in the integrative genomics viewer (IGV, Broad Institute). The variants of low  
60 quality or with an allele frequency below 5% were filtered out.

61 Whole exome sequencing was performed for specimens 590 and 14881. Briefly, DNA was  
62 fragmented to 250bp using standard Covaris sonication. Fragmented DNA was prepared as a standard  
63 Illumina gDNA library using IntegenX reagents. Then the samples were PCR amplified, libraries were  
64 checked for quality and quantity. Samples then underwent exome capture (using the Roche Nimblegen  
65 SeqCap EZ v3.0 according to the manufacturer's protocols (Roche Nimblegen, Indianapolis, IN). The  
66 SeqCap EZ Human Exome Library v3.0 covers 64 Mb of coding exons and miRNAs. Captured libraries  
67 were sequenced on a HiSeq 2500 using 100 Cycle paired end reads. The exome sequencing data was  
68 analyzed by the variant calling pipeline developed by the University of Michigan Bioinformatics Core. For  
69 each of the samples, paired-end reads were aligned to the hg19 reference genome using BWA v0.7.8 (Li and  
70 Durbin 2009), followed by removal of sequence duplicates using PicardTools v1.79  
71 (<http://picard.sourceforge.net>), local realignment around INDELS and base quality score recalibration using  
72 GATK v3.2-2 (DePristo et al. 2011). Read coverage on exome capture target regions was calculated using  
73 BEDTools v2.20.1 (Quinlan and Hall 2010). Normal-Tumor paired alignment files were submitted to  
74 MuTect v1.1.4 (Cibulskis et al. 2013), Strelka v1.0.14 (Saunders et al. 2012), and VarScan v2.3.7 (with its  
75 false-positive filter) (Koboldt et al. 2012) for the detection of somatic and germline SNPs and INDELS.  
76 Candidate variant calls across all samples and patients were merged using Jacquard (Gates et al. 2015) into a

77 single VCF file that included all variant loci whose filter field passed in MuTect or Strelka or VarScan  
78 (VarScan calls were limited to somatic variants confirmed in false-positive filter). Variants were annotated  
79 using SnpEff v4.0/hg19 (Cingolani et al. 2012), dbNSFP v2.4 (Liu, Jian, and Boerwinkle 2011, 2013),  
80 dbSNP v138, and 1000 Genomes v3 (Abecasis et al. 2012). For variants associated with multiple effects or  
81 multiple transcripts, a single “top effect” annotation was nominated based on annotation confidence,  
82 predicted impact, gene region, and transcript length. Common variants (at or above 5% overall population  
83 allele frequency as reported by 1000 Genomes) were excluded. Subsequent variant calling was as follows:  
84 rejected Impact Ranks low & modifier and accepted high & moderate; rejected Population Category  
85 common; excluded dbNSFP when damaging was equal to zero for all nine data bases, and selected allelic  
86 frequency  $\geq 0.15$ .

87 *Further Specificity Studies of LGR5 Antibodies and Development of Procedures for Enrichment of LGR5(+)*  
88 *cells by Magnetic-activated Cell Sorting (MACS).*

89 Mouse pre-B cell lymphoma 1881 cells transfected with his-tagged *LGR5* [1881(+)] or wild type  
90 [1881(-)] (provided by Miltenyi Biotec) were cultured in suspension in RPMI (Gibco, #21870-076)  
91 containing 20mM HEPES, 2mM L-glutamine, 10% FBS (Hyclone characterized, GE Healthcare Life  
92 Sciences, #SH30071.03; Marlborough, MA), 50 units/mL penicillin, 0.05 mg/mL streptomycin, 1mM  
93 sodium pyruvate (Gibco, #11360070), and 50 $\mu$ M  $\beta$ -mercaptoethanol (Gibco, #21985023). Transfected cells  
94 were selected using 15  $\mu$ g/ml Blasticidin S (Gibco, #R210-01).

#### 95 *LGR5* quantitative real-time PCR of 1881 *LGR5*(+) and 1881 *LGR5*(-) cells

96 To confirm transfection stability, the 1881 *LGR5*(+) and 1881 *LGR5*(-) cells were assayed for *LGR5*,  
97 and visualized with agarose gel analysis of qRT-PCR amplified products. mRNA expression of 1881  
98 *LGR5*(+) and 1881 *LGR5*(-) cells was quantified by qRT-PCR (three biological replicates). Total RNA was  
99 extracted using TRIzol Reagent (Invitrogen, #15596-026; Carlsbad, CA) according to the manufacturer’s  
100 recommendations. Complementary DNA was generated using TaqMan Reverse Transcription Reagents  
101 (Applied Biosystems, N808024; Carlsbad, CA). Quantitative real-time PCR was performed using Brilliant II

102 SYBR Green Master Mix (Agilent technologies, #600828; Santa Clara, CA) on a StepOnePlus Real-Time  
103 PCR System (Applied Biosystems). All reactions were run with 40 cycles of 95°C for 10 minutes, 94°C for  
104 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, followed by a melt curve of 95°C for 10 seconds,  
105 60°C for 1 minute and then increasing at 0.5 degree increments to 95°C. Gene expression analysis was  
106 normalized to the housekeeping gene *ACTB* and values were plotted as arbitrary units. The qRT-PCR  
107 amplified products (Figure 2B) were analyzed by running on a 1.5% Agarose gel (Bio-Rad Laboratories,  
108 #1620137; Hercules, California) to confirm the target amplicons size and to indicate that there were no non-  
109 specific amplification products. Primer sequences were as follows: *hLGR5* (Finkbeiner and Spence 2013):  
110 CAGCGTCTTCACCTCCTACC (Forward primer); TGGGAATGTATGTCAGAGCG (Reverse primer);  
111 126 bp band size. *mACTB* (Riera et al. 2014): CTAAGGCCAACCGTGAAAAG (Forward primer);  
112 CCAGAGGCATACAGGGACA (Reverse primer); 104 bp band size.

### 113 Flow cytometry spike-in experiments

114 Cells were analyzed on a LSRII cytometer (BD Biosciences) to confirm LGR5 antibody specificity  
115 and validate the magnetic bead enrichment strategy (magnetic-activated cell sorting, MACS) (Figure S1B).  
116 1881 LGR5(+) and wild type 1881 LGR5(-) cells were mixed at varying proportions and analyzed by flow  
117 cytometry before and after magnetic separation with rat monoclonal antibody anti-human LGR5 clone  
118 22H2.8 conjugated to magnetic beads (Miltenyi Biotec, #130-104-072), with the allophycocyanin (APC)  
119 anti-bead check reagent conjugate to recognize the bound magnetic beads (Miltenyi Biotec, #130-098-892).  
120 Detailed flow cytometry/MACS methods are as described in the below methods section, “Single Cell  
121 Isolation and Magnetic separation for LGR5(+) and LGR5(-) cells”.

### 122 *ImageStream Analysis*

123 Cells were dissociated from adenoma organoid specimen 574 and stained with rat monoclonal  
124 antibody anti-human LGR5 clone 22H2.8 conjugated to phycoerythrin (PE) (2µg/mL; Miltenyi Biotec, #130-  
125 100-848). Dead cells were excluded using the Live/Dead Fixable Violet Dead Cell Stain Kit (405 nm  
126 excitation; Molecular Probes, #L34955; Eugene, Oregon). Gating strategy set the PE-positive cell population

127 at 0.04% of the viable FMO-PE control with Rat IgG2b-PE (Thermo Fisher Scientific Ebioscience,  
128 #NC9634273; Waltham, MA). Cells were analyzed and imaged with the Amnis ImageStreamX Mark II  
129 (EMD Millipore; Kankakee, IL) for brightfield, SSC, and fluorescence. Analysis strategy follows: gradient  
130 RMS for focus, to intensity of MC 1 (Live-Dead) for viability, to intensity of MC 3 (PE) for rough positivity,  
131 to area channel 2 (fsc) vs. intensity channel 6 (ssc) for sizing to eliminate debris, to finally refined PE  
132 histogram. Samples were analyzed offline in the Amnis IDEAS software platform to generate images at 60x  
133 magnification (Figure 4E).

### 134 *Western analysis*

135 Whole-cell extracts were isolated, separated and transferred to nitrocellulose as previously described  
136 (Xue et al.). Cell lysates were prepared with the following: 150mM NaCl, 50mM Tris/ HCl pH8, 1% NP-40,  
137 0.5% Triton-X 100, Protease inhibitor complete (Roche Life Science, # 04 693 116 001; Penzberg,  
138 Germany), fresh 2µg/mL aprotinin, fresh 10µg/mL leupeptin, fresh 100µg/mL PMSF, at 200µl buffer-mix  
139 per  $1 \times 10^7$  cells and incubated for 30 minutes on ice. Samples were centrifuged at 13,000xg for 15min at 4C  
140 and supernatant transferred to a fresh tube to determine the protein concentration (Bradford-assay). Before  
141 loading, samples were boiled for 5min at 95C. 15µg of sample protein was loaded to a Tris-Glycine 4-20%  
142 gradient gel (using Tris Glycine SDS running buffer). Protein was transferred to a nitrocellulose membrane  
143 and the membrane was blocked in TBS/Tween/milk powder (150mM NaCl; 50mM Tris pH 7,0; 0.1% Tween  
144 20; 5% skim milk powder) for 1h at room temperature. The membrane was incubated with primary antibody  
145 0.1µg/mL rabbit anti human LGR5 STE-1-89-11.5 (Miltenyi Biotec, #130-104-945) in 10mL  
146 TBS/Tween/milk powder overnight (shaking slightly) at 4°C, followed by 3X washes for 5min with  
147 TBS/Tween (150mM NaCl; 50mM Tris pH 7,0; 0.1% Tween 20) (shaking slightly). The membrane was  
148 incubated with secondary antibody (HRP goat anti rabbit IgG, 1:2000; Cell Signaling Technology; Boston,  
149 MA), diluted in 10mL TBS/Tween/milk powder for 1h at room temperature (shaking slightly), followed by  
150 3X washes for 5min with TBS/Tween, 1X wash with deionized water for 5 minutes. The chemiluminescent  
151 signal was detected using Immobilon™ Western Kit (EMD Millipore) (Figure 2C).

152 *LGR5 Immunohistochemistry*

153 Formalin fixed, paraffin sections were cut at 5-6 microns and rehydrated to water. Heat induced  
154 epitope retrieval was performed with FLEX TRS High pH Retrieval buffer (pH 9.01; Agilent Technologies,  
155 #K8004; Santa Clara, CA) for 20 minutes (Figure 1A, C and Figure 3A). After peroxidase blocking, the  
156 antibody LGR5 rabbit monoclonal clone STE-1-89-11.5 (Miltenyi Biotec, #130-104-945) was applied at a  
157 dilution of 1:50 (Figure 1A, C) or 1:100 (Figure 3A) at room temperature for 60 minutes. The FLEX HRP  
158 EnVision System (Agilent Technologies) was used for detection with a 10 minute DAB chromagen  
159 application. Slides were counterstained with Harris Hematoxylin for 5 seconds and then dehydrated and  
160 coverslipped (University of Michigan Comprehensive Cancer Center Tissue Core Research Histology and  
161 IHC Laboratory). Note, sections freshly cut were compared to those that were stored at room temperature for  
162 4 weeks, and showed more robust LGR5 staining (data not shown). The colon cancer tissue microarray  
163 (TMA) (Figure 3A; 2 normal, 3 adenoma, 70 adenocarcinoma, 2 cores per specimen) was freshly cut and  
164 provided by BioChain Institute, Inc. (Newark, CA; Z7020032, lot B508131). Alternatively, retrieval was  
165 performed with R-Buffer B (pH 8.5; Electron Microscopy Sciences; Hatfield, PA) in a pressurized Retriever  
166 2100 (Electron Microscopy Sciences) overnight (Figure 2A, Figure 4C). After peroxidase blocking, the  
167 antibody was applied at a dilution of 1:1000 at 4C overnight. ImmPACT DAB kit (Vector Labs, SK-4105;  
168 Burlingame, CA) was used, with DAB chromagen applied for 40-45 seconds.

169 IHC images were taken and scanned on an Aperio AT2 instrument by the University of Michigan  
170 Pathology departmental Slide Scanning Service at 40X (Figure 1A, B top panel, c<sup>1</sup>, c<sup>2</sup>, and D; Figure 3A)  
171 and on an IHC Olympus SZX16 at 8x zoom (Figure 1B bottom panel). 100X insets were captured on a Zeiss  
172 Axio M1 (Figure 1a<sup>2</sup>). Brightfield images were captured on an Olympus IX70, with a DP71 digital camera  
173 (Figure 4A, C).

174 *LGR5 Immunohistochemistry scoring for staining intensity in the epithelium and in the stroma*

175 The TMA, along with 5 additional FFPE normal colon samples from autopsies, were scored for  
176 staining intensity in both the epithelium and then separately in the stroma (Figure 3). Two FFPE biopsied

177 large (> 10mm) adenomas, used to derive organoids for this study, were also assessed for reactivity  
178 (specimen 282 and 590), but these were not simultaneously scored since they were stained under different  
179 conditions (Figure 4C left column). Scoring was conducted by two independent viewers on blinded samples  
180 at 8X and 20X magnification. Scoring key: 0 = non-specific or < 1%; 1 = 1-10% or only evident at 20X  
181 magnification; 2 = 10-50% or light diffuse staining >50%; 5 = >50%. A standard pathology peer review  
182 method was used to resolve discrepancies in scoring between two independent scorers, with a third party  
183 reviewer intervening when necessary (Morton et al. 2010). The vast majority of samples were in  
184 concordance between the two viewers. Stage T2 (n=25) and T3 (n=44) tumors were compared to normal  
185 colon and adenoma (termed Stage T0; n=10). TMA cancers with grades I and I-II were grouped (termed  
186 “Grade I”; n=20) and cancers grade II-III and III were grouped (termed “Grade III”; n=10) for further  
187 analyses. LGR5 stromal and epithelial staining for adenomas (n=3), and Grade I, II (n=38), and III cancers  
188 were compared to normal colon tissue (n=7). Differences in LGR5 staining in the stroma or epithelium by  
189 stage or grade were quantified by linear regression.

### 190 *LGR5 Immunofluorescence*

191 Rehydrated sections were retrieved with R-Buffer B (pH 8.5; Electron Microscopy Sciences) in a  
192 pressurized Retriever 2100 (Electron Microscopy Sciences) overnight. After 3X PBS washing, slides were  
193 blocked for 1 hour with 0.5% Triton X-100 and 5% donkey serum in PBS. Slides were incubated overnight  
194 at 4°C with the following antibodies in 0.05% Tween 20 and 5% donkey serum: LGR5 clone 89.11 at 1:500  
195 (Figure 1c<sup>3</sup>c<sup>4</sup>); anti-OLFM4 antibody at 1:500 (Abcam, #ab85046; Cambridge, MA)(Figure 8C); anti-  
196 defensin5a at 1:500 (Abcam, #ab90802)(Figure 2A); anti-E-cadherin at 1:500 (BD Biosciences, # 610181,  
197 San Jose, CA) (Figure 8C); and anti-E-cadherin at 1:500 (R&D, #AF748)(Figure 2A). Slides were washed  
198 3X for 5 minutes each, followed by 1 hour room temperature incubation with the following secondaries  
199 (1:1000): LGR5 plus DAPI (Figure 1c<sup>3</sup>/c<sup>4</sup>; Figure 2A) and OLFM4 (Figure 8C) secondaries (biotinylated  
200 secondary donkey anti-rabbit; Jackson Immuno Research Labs, #711-065-152; West Grove, PA); defensin5a  
201 (Figure 2A) and E-cadherin secondary (Figure 8C)(donkey anti-mouse 488; Jackson Immuno Research Labs,  
202 #715-545-150); E-cadherin secondary (Figure 2A) (donkey anti-goat 647; Jackson Immuno Research Labs,

203 #705-605-147). Slides were washed 3X. LGR5 and OLFM4 were amplified with the SK4105 TSA Kit with  
204 Alexa 594 tyramide (Invitrogen, #T20935). All slides were mounted with Prolong Gold without DAPI  
205 (Molecular Probes, #P36930). Images were taken on a Nikon A1 confocal microscope.

## 206 *LGR5 in Situ Hybridization*

207 Formalin fixed, paraffin sections were cut at 5-6 microns and rehydrated to water. *In situ*  
208 hybridization was performed using the RNAscope 2.0 HD detection kit [Advanced Cell Diagnostics (ADC),  
209 #310035; Newark, CA] according to the standard provided protocol (Figure 1B, D). The human LGR5 probe  
210 was designed by ADC using the NCBI LGR5 sequence (NCBI Reference Sequence: NM\_003667.2) and  
211 targets the region between 560-1589 base pairs (HS-LGR5, 311021, lot 15022A). All incubations were  
212 performed at 40°C in the ADC HyBEZ hybridization system oven (#310010). Images were taken and  
213 scanned on an Aperio AT2 instrument by the University of Michigan Pathology departmental Slide Scanning  
214 Service.

## 215 *Single Cell Isolation and by Magnetic-Activated Cell Sorting for LGR5(+) and LGR5(-) cells (Figure 4)*

### 216 Organoid dissociation into single cells

217 On the day of sorting, the cultures were treated with 10 $\mu$ M Y27632 for 2.5 hrs prior to harvest.  
218 Matrigel was digested for 45 minutes with cold 2mM EDTA in DPBS and organoids were washed 3X with  
219 cold DPBS (100xg at 4 °C). Structures were dissociated into single cells using the Tumor Dissociation Kit  
220 (human) (Miltenyi Biotec, #130-095-929) with the protocol modifications described as follows. The  
221 enzymes were prepared in warm HBSS modified to 0.13mM calcium and 0.9mM magnesium [10% HBSS,  
222 calcium, magnesium, no phenol red (Gibco, #14025092) with 90% HBSS, no calcium, no magnesium  
223 (Gibco, #14170112)] to minimize differentiation of the epithelial cells while supporting enzymatic activity.  
224 The organoids were suspended in 20mL of enzyme solution containing 5 $\mu$ M Y27632, then transferred to two  
225 BSA-coated C-tubes (Miltenyi Biotec, #130-096-334) and dissociated with a gentleMACS Dissociator  
226 (Miltenyi Biotec, #130-093-235) using the program *h\_tumor\_01*, once at the onset and every 15 minutes for  
227 1 hour, and finishing with two program runs. Between runs the suspension was slowly rotated at 37°C. The

228 cell suspension was then poured over a succession of cold BSA-coated cell strainers into 0.5% BSA-2mM  
229 EDTA-PBS: 100 $\mu$ m (Corning, #DL 352360) to 40 $\mu$ m (Corning, #DL 352340) to 20 $\mu$ m (CellTrics of Sysmex  
230 Europe GmbH, #04-0042-2315; Norderstedt, Germany). The cells were washed 3X in the 0.5% BSA-2mM  
231 EDTA-PBS and centrifuged for 5 minutes at 500xg. Cells were counted under trypan blue exclusion to  
232 estimate live cell number using a Countess Automated Cell Counter (Invitrogen, #C10227).

### 233 Magnetic bead-LGR5 antibody labeling

234 Single cells were resuspended and processed in cold 0.5% BSA-2mM EDTA-PBS containing 5 $\mu$ M  
235 Y27632 in a BSA-coated tube (using BSA-coated tips for mixing) pelleted at 500xg, and otherwise stained  
236 according to the product instructions (Miltenyi Biotec: Anti-human LGR5 MicroBeads #130-104-072; clone  
237 22H2.8; APC-Check Reagent #130-098-892).

### 238 Magnetic separation

239 Stained cells were resuspended at 10<sup>7</sup> cells/mL in a DNase Buffer containing HBSS (1:10  
240 Ca<sup>2+</sup>Mg<sup>2+</sup>), 0.5% BSA, 5 $\mu$ M Y27632, 200 Kunitz units/mL DNase (Sigma-Aldrich, #D5025). Cells were  
241 applied over the columns (Miltenyi Biotec, LS Columns , # 130-042-401) through a cold BSA-coated 20 $\mu$ m  
242 cell strainer at 1mL per column. Columns were prepped by coating in the DNase Buffer for 45 minutes at  
243 4°C prior to use. All washes were performed with the DNase Buffer. After removing the column from the  
244 magnet, 2mL DNase Buffer was applied to the column to flush out the magnetically-bound cells into a  
245 15mL BSA-coated tube. Unlabeled flow-through cells (magnet intact) and labeled magnet-bound cells were  
246 pelleted at 500xg for 5 minutes and resuspended in 0.1% BSA-PBS-2mM EDTA with 10 $\mu$ M Y27632.

### 247 *Flow Cytometry*

248 Cells were analyzed on a LSRII cytometer (BD Biosciences) and sorted on a MoFlo Astrios  
249 (Beckman Coulter; Brea, California) instrument at the University of Michigan Cancer Center Flow  
250 Cytometry core facility. Events first passed through a routine light-scatter and doublet discrimination gate,  
251 followed by exclusion of dead cells using 4',6-diamidino-2-phenylindole (1 $\mu$ M DAPI dilactate; Molecular  
252 Probes, # D3571). Gating strategy set the APC-positive cell population at 0.05-0.1% of the viable MACS

253 flow-through cells, LGR5(FT+) (Figure 4D; Figure S1A; Figure 8A, specimen 81, 83). Gating strategy set  
254 the APC-positive cell population at 0.05-0.1% of the viable FMO-APC check reagent control for the analysis  
255 of samples that were not MACS processed (Figure 4D top and middle rows; Figure S1A). For RNA  
256 expression analyses, cell fractions were sorted into RLT lysis buffer (Qiagen). Flow cytometric data analysis  
257 was performed using Winlist 3D software (Verity) and FlowJo vX.0.7 (Tree Star). For each specimen, three  
258 cell fractions were FACS and collected (Figure 4A; Figure S2): MACS magnet-bound positives, LGR5(+);  
259 MACS magnet-bound negatives, LGR5(-); and MACS unbound flow-through, LGR5(FT-).

### 260 *Normal Colonic Crypt Isolation from Tissue, Single Cell Dissociation, Magnetic Separation and Flow* 261 *Cytometry*

262 Crypts were isolated from three warm autopsy normal colon specimens (6 cm<sup>2</sup>) and processed as  
263 previously described (Dame et al. 2014), a modification of Sato et al (Sato, Stange, et al. 2011). Crypts were  
264 dissociated into single cells, as described above for the adenoma organoids, and a final 1mL of a dense crypt  
265 pellet was processed per C-tube. Magnetic separation and flow cytometry (Becton-Dickinson FACS Aria III  
266 sorter ) were as above, with the added gating/analysis for EpCAM expression using a phycoerythrin (PE)-  
267 conjugated antibody (2µg /mL buffer/≤ 10<sup>6</sup> cells; BioLegend, #324205; San Diego, CA) and an EpCAM  
268 isotype control (2µg /mL buffer/≤ 10<sup>6</sup> cells; BioLegend, #400311). EpCAM-PE(+)/DAPI(-) cells were  
269 interrogated for LGR5 expression as done above with MAC processed samples (Figure 8A, specimen 81,83)  
270 or with rigorous so-called fluorescence-minus-one controls [FMO; DAPI(-), EpCAM(+)] for sorting of pre-  
271 magnet cells (Figure 8A, specimen 84).

### 272 *High Throughput RNA Sequencing (RNA-seq)*

273 RNA was extracted from sorted cells using the RNeasy Micro Kit (Qiagen, #74004) with on column  
274 DNase digestion. RNA concentration and quality were determined using a Nanodrop (Thermo Scientific)  
275 and Bioanalyzer (Agilent). Due to the small number of cells following FACS and corresponding low level of  
276 input RNA, we depleted ribosomal RNAs with RiboGone (Takara Clontech, #634846; Mountain View, CA)  
277 and prepared sequencing libraries utilizing the SMARTer Stranded RNA-Seq kit (Takara Clontech, #

278 634839) following the manufacturer's recommended protocol. Libraries were multiplexed over 2 lanes and  
279 sequenced using paired end 75 cycle reads (sample 14881) and 125 cycle reads (samples 282, 584, and 590)  
280 on a HiSeq sequencer (Illumina) at the University of Michigan DNA Sequencing Core Facility (Figure 5;  
281 Figure S2).

## 282 *RNA-Seq Data Analysis*

### 283 Alignment and quality control

284 Computational analyses were performed using the Flux high-performance computer cluster hosted by  
285 Advanced Research Computing (ARC) at the University of Michigan. Raw sequencing read quality was  
286 assessed utilizing FastQC. The first five nucleotides of the both reads in each read pair were trimmed with  
287 SeqTK due to the presence of adapter sequence and high nucleotide redundancy. A splice junction aware  
288 build of the human genome (GRCh37) was built using the genomeGenerate function from STAR 2.3.0  
289 (Dobin et al. 2013). Read pairs were aligned to the genome using STAR, using the options  
290 “outFilterMultimapNmax 10” and “sjdbScore 2”.

### 291 Differential expression testing

292 The aligned reads were assigned to genomic features (GRCh37 genes) using HTSeq-count, with the  
293 set mode “union”. We conducted differential expression testing on the assigned read counts per gene  
294 utilizing edgeR (Robinson, McCarthy, and Smyth 2010). Analysis were conducted to compare expression of  
295 LGR5(+), LGR5(-), and LGR5(FT-) cells, adjusting for study subject as a covariate using glmLRT. To  
296 reduce the dispersion of the dataset due to lowly expressed genes, genes with a mean aligned read count less  
297 than five across all samples were excluded from analysis. Normalized counts per million were estimated  
298 utilizing the “cpm” function in edgeR. Genes were considered differentially expressed between conditions at  
299 a false discovery rate adjusted p-value < 0.05 (Figure 5; Figure S2; Table S1).(Benjamini and Hochberg  
300 1995).

## 301 Comparison to previously published datasets

302 RNA-seq data from normal colon (samples ERR315348, ERR315357, ERR315400, ERR315403,  
303 ERR315462, ERR315484 from Tissue Based Map of the Human Proteome Science REF) were processed as  
304 described above, and differential gene expression between the LGR5(+) cells and normal colon were  
305 calculated as described above (Figure 6C, D). Overlap between genes overexpressed in LGR5(+) cells with  
306 the previously reported *Lgr5*+ ISC gene signature (Munoz et al. 2012) was calculated by identifying the  
307 overlap between genes present in both the Muñoz et al ISC gene expression signature and genes identified as  
308 upregulated in LGR5(+) adenoma cells (Figure 6B; Table S2). Statistical significance of the overlap between  
309 these gene signatures was calculated using the hypergeometric distribution. Genes in the Muñoz et al ISC  
310 signature that were not present at detectable levels in our dataset were excluded from this calculation.

## 311 Pathway analyses

312 Differentially expressed pathways were identified utilizing iPathways (Advaita; Plymouth, MI). A  
313 directional analysis was conducted on all genes by including *P*-value of the differential expression test as a  
314 measure of effect size and log2 fold difference in expression as a measure of effect direction. KEGG  
315 biological pathways and Gene Ontology biological processes were considered differentially expressed at a *P*-  
316 value <0.05 (Figure S3-8; Table S1).

317 *DKK4* (*Dickkopf WNT Signaling Pathway Inhibitor-4*) Interrogation; Comparisons to LGR5 Expression.

## 318 *DKK4* and *LGR5* quantitative real-time PCR of adenoma organoids grown in L-WRN vs. KGMG

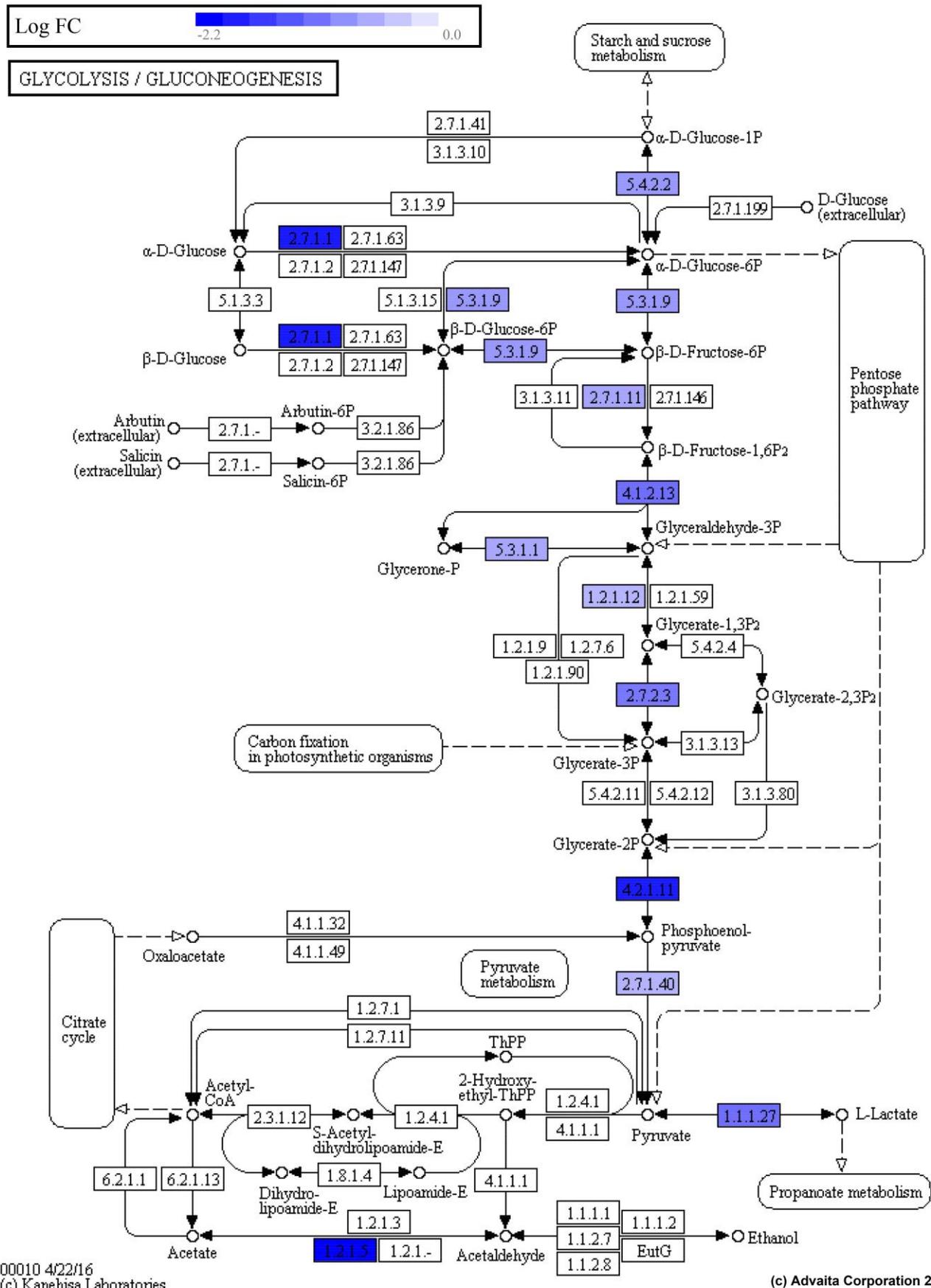
319 Cultured organoids from three adenomas, 282, 584, and 590, were transitioned from KGMG to L-  
320 WRN, with the concomitant shift from budding to cystic structures. *LGR5* and *DKK4* mRNA expression  
321 were then assessed as previously described above and presented as increased expression in L-WRN relative  
322 to KGMG (Figure 7A). *DKK4* primer sequences were as follows: *hDKK4* (Cui, Taub, and Gardner 2007):  
323 GGAGCTCTGGTCCTGGACTT (Forward primer); TCTGGTATTGCAGTCCGTGT (Reverse primer); 102  
324 bp band size.

325 Recombinant human DKK4 protein treatment of adenoma organoids

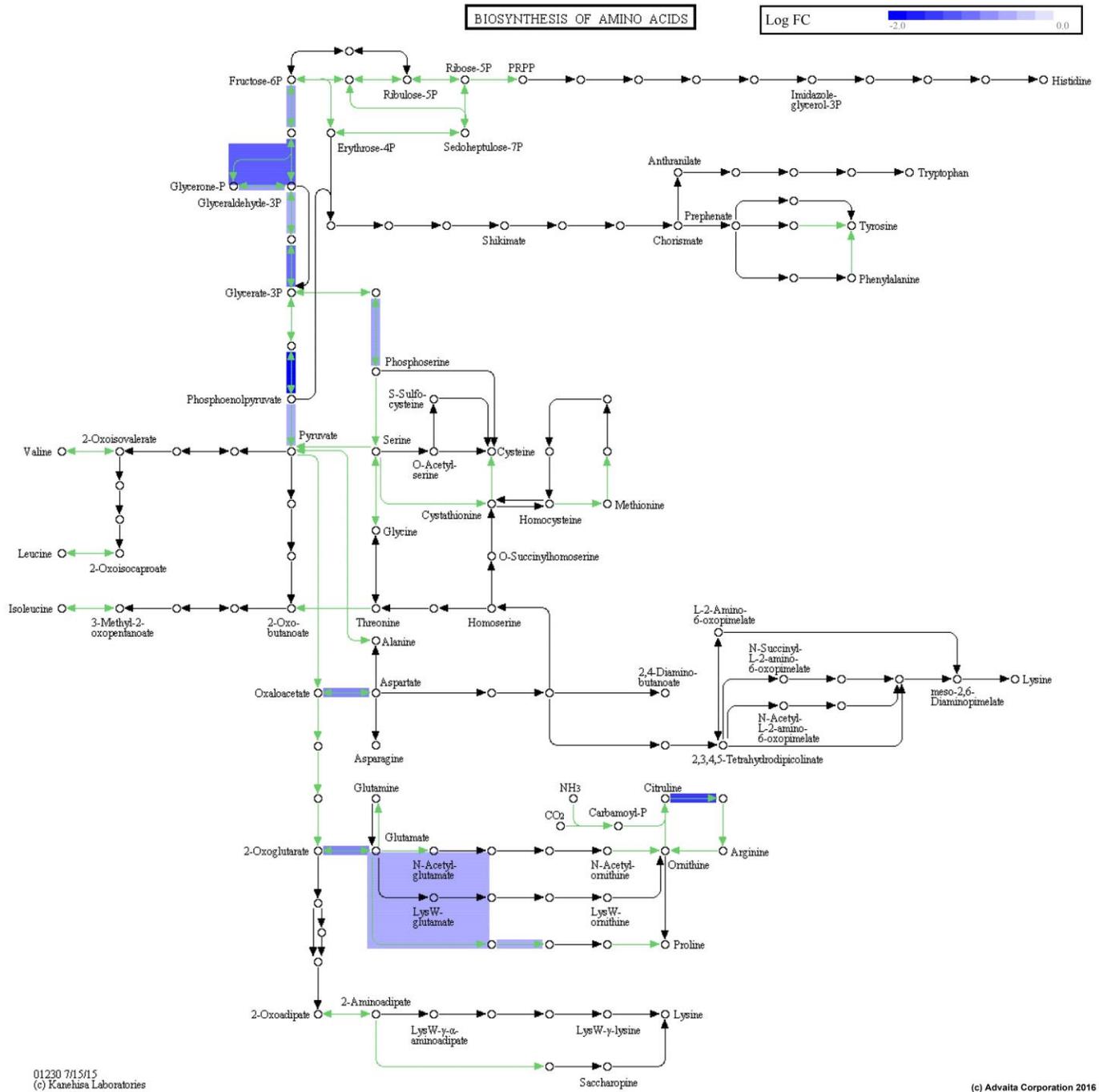
326 Three organoid specimens (282, 584, 590) were prepared for treatment as previously described (Xue  
327 et al.). Organoids were removed from Matrigel and mechanically dissociated into uniform structures between  
328 20-100  $\mu\text{m}$  in diameter with the gentleMACS (Miltenyi Biotec) using programs *h\_Tumor\_01.01* followed by  
329 *m\_Lung\_01.01*, and then by passing through a BSA-coated 100 $\mu\text{m}$  cell strainer. Organoids were then seeded  
330 at 1:1 split ratio at approximately 3000 small organoids/50  $\mu\text{l}$  Matrigel pad/well/24-well plates. After 24 hrs  
331 of plating, the cultures were treated with 0.5 and 4.0 $\mu\text{g}/\text{mL}$  (350 $\mu\text{L}/\text{well}$ ) of recombinant DKK4 (R&D  
332 Systems, #1269-DK) for 72 hrs in either KGMG or L-WRN. Treated organoids were harvested and assessed  
333 for changes in LGR5 mRNA by qPCR (Figure S10C).

334 DKK4 knockdown organoids

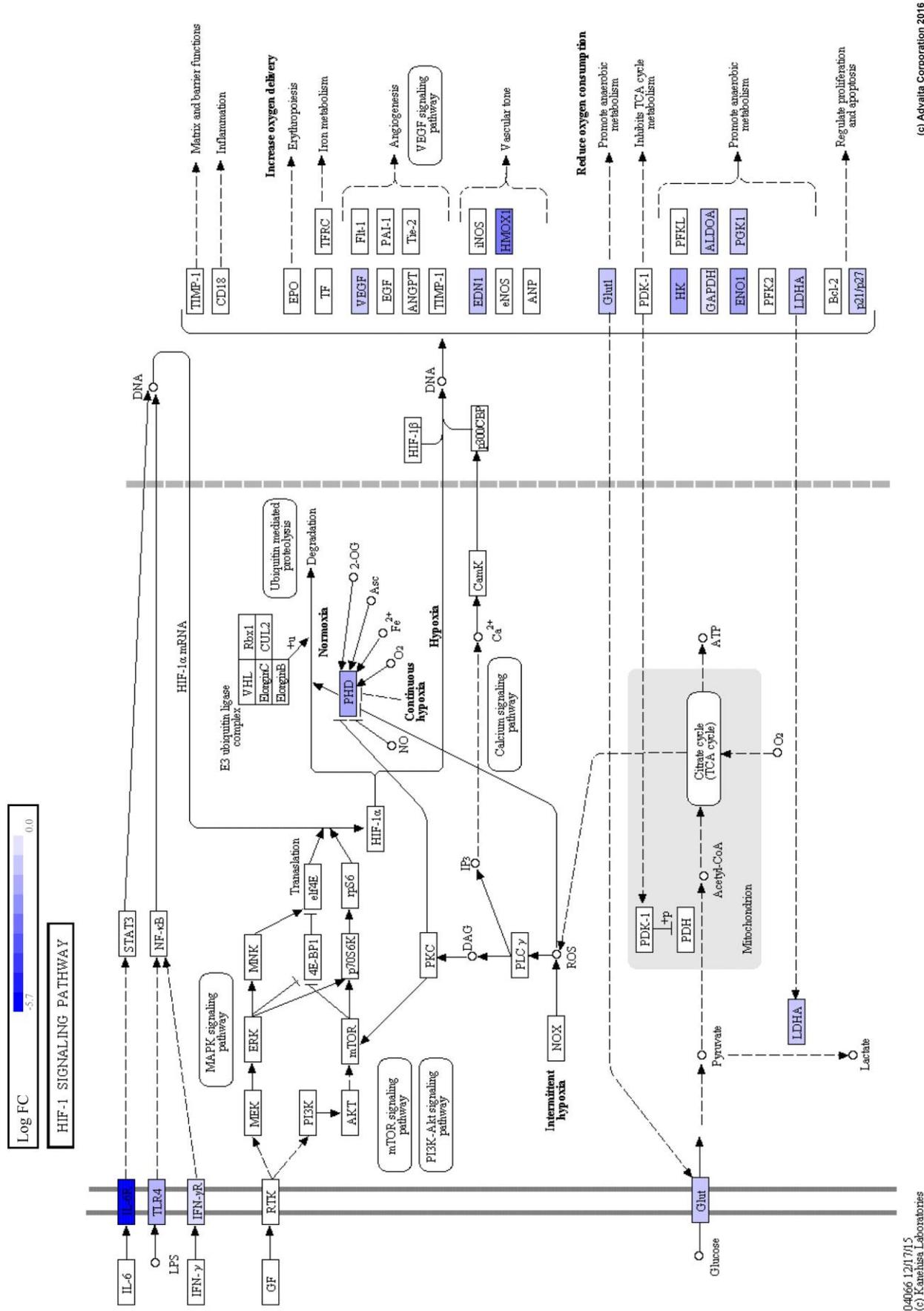
335 Stable adenoma organoid *DKK4* knockdowns and scramble small hairpin RNA were generated with  
336 lentivirus infection of adenoma organoid specimen 282 and selected with 1 $\mu\text{g}/\text{mL}$  puromycin (Xue et al.).  
337 The GIPZ lentiviral shRNA vectors (Open Biosystems of GE Dharmacon; Lafayette, CO) for human *DKK4*  
338 sh1DKK4 (Clone ID: V2LHS\_204025) and sh2DKK4 (Clone ID: V2LHS\_197942) have bicistronic  
339 expression of GFP and the puromycin selection marker to allow visualized by GFP fluorescence. To assess  
340 transduction efficiency, and the effect of *DKK4* knockdown on *LGR5*, knockdowns grown in both KGMG  
341 and L-WRN were analyzed for changes in mRNA by qPCR (Figure 7D).



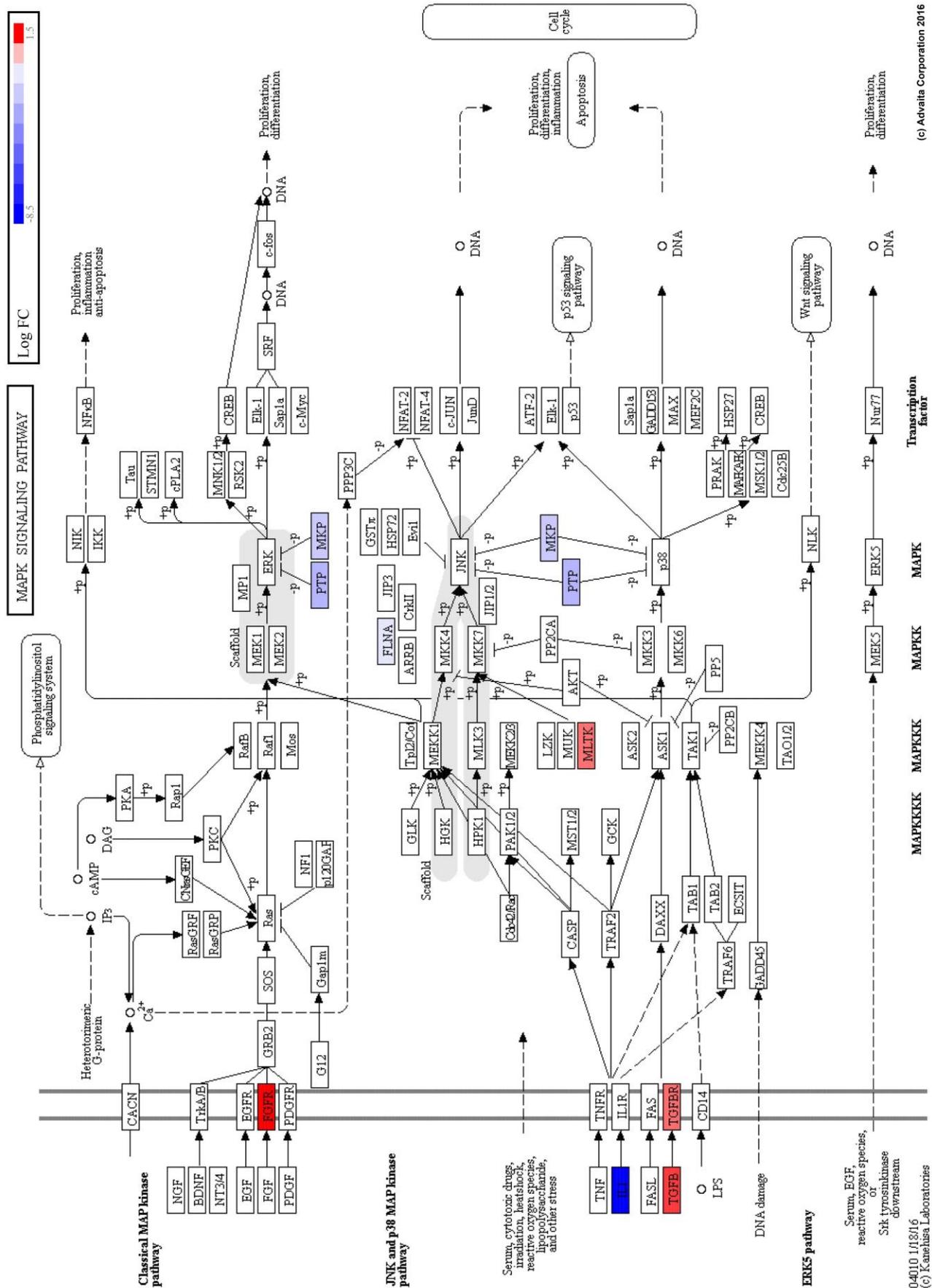
**Figure S3 (Related to Figure 5):** Relative expression of genes involved in the KEGG pathway “Glycolysis and Gluconeogenesis” between LGR5(+) and LGR5(-) human adenoma cells ( $P$ -value=6.57E-09).



**Figure S4 (Related to Figure 5):** Relative expression of genes involved in the KEGG pathway “Biosynthesis of Amino Acids Pathway” between LGR5(+) and LGR5(-) human adenoma cells ( $P$ -value=4.32E-07).

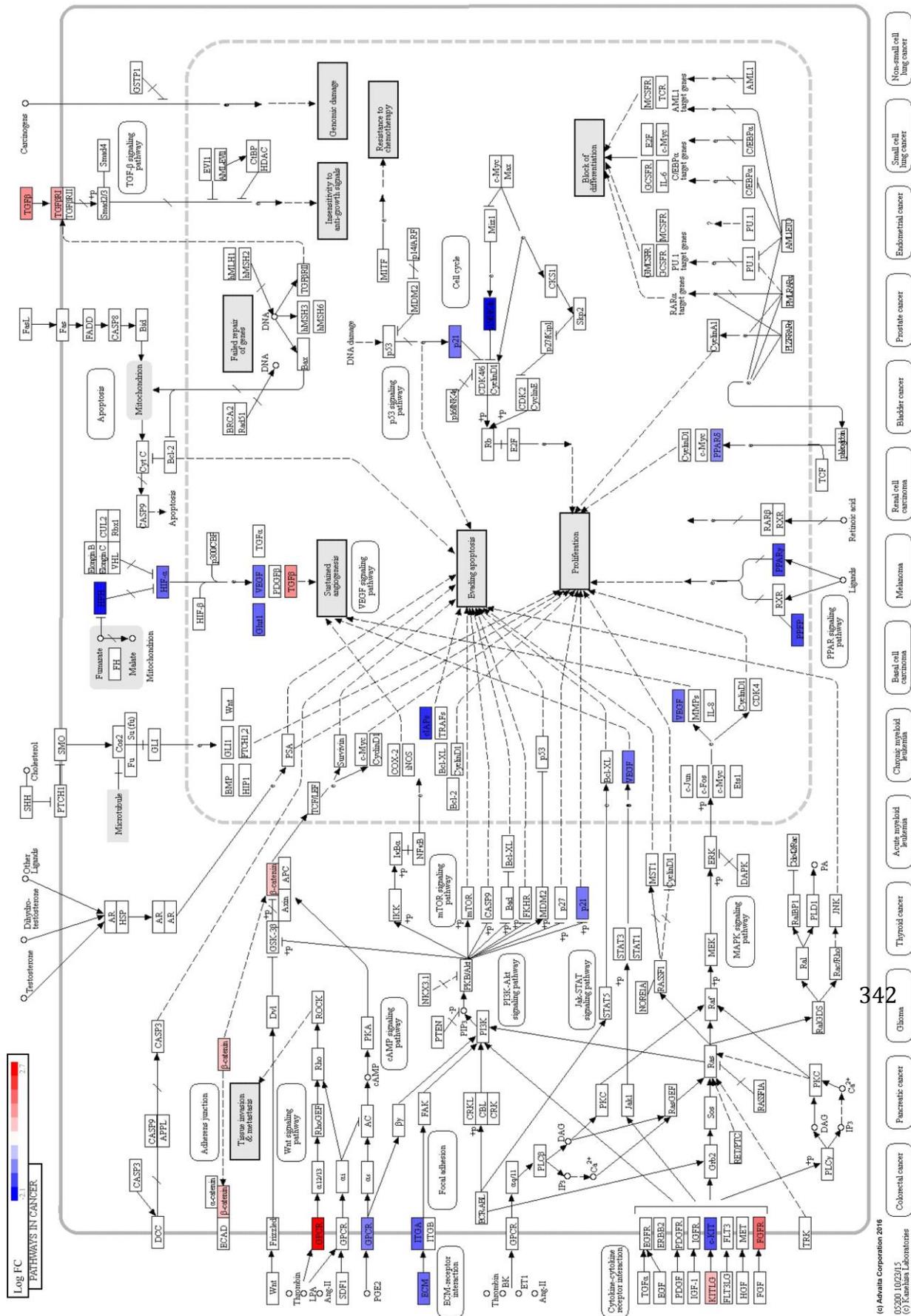


**Figure S5 (Related to Figure 5):** Relative expression of genes involved in the KEGG pathway “HIF-1 Signaling Pathway” between LGR5(+) and LGR5(-) human adenoma cells ( $P$ -value=1.42E-05).



**Figure S6 (Related to Figure 5):** Relative expression of genes involved in the KEGG pathway “MAPK Signaling Pathway” between LGR5(+) and LGR5(-) human adenoma cells ( $P$ -value=.0105).





**Figure S8 (Related to Figure 5):** Relative expression of genes involved in the KEGG pathway “Pathways in Cancer” between LGR5(+) and LGR5(-) human adenoma cells ( $P$ -value=.0133)

343 **Table S1 (Related to Figure 5, Figure 6 and Figure S2-8): LGR5(+) Adenoma RNA-seq analyses**

344 Tab 1: LGR5(+) vs. LGR5(-) significant genes

345 Tab 2: LGR5(+) vs. LGR5(-) all genes

346 Tab 3: LGR5(+) vs. LGR5(FT-) significant genes

347 Tab 4: LGR5(+) vs. LGR5(FT-) all genes

348 Tab 5: LGR5(+) gene signature

349 Tab 6: LGR5(+) vs. LGR5(-) significant KEGG pathways

350 Tab 7: LGR5(+) vs. LGR5(FT-) significant KEGG pathways

351 Tab 8: LGR5(+) adenoma cell vs. normal colon gene expression for genes in the “LGR5+ adenoma  
352 stem cell gene expression signature”

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