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TITLE: Oral GUCY2C Ligand Blocks Colorectal Tumor Progression in Patients

PRINCIPAL INVESTIGATOR: Scott Waldman

CONTRACTING ORGANIZATION: Thomas Jefferson University
Philadelphia PA 19107

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Fort Detrick, Maryland 21702-5012

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Scott Waldman

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1020 Walnut St Ste 1
Philadelphia PA 19107-5567

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The overall objective of this proposal is to develop a prevention strategy for colorectal cancer by exploiting the role of GUCY2C signaling in inhibiting colorectal epithelial transformation. The translational hypothesis suggests that oral linaclotide restores GUCY2C signaling, repairs epithelial dysfunction, and blocks progression of tumors in patients. The linked mechanistic hypothesis suggests that linaclotide prevents tumor progression by blocking β-catenin accumulation through proteosomal degradation. In the Translational Aim, we will conduct a "window of opportunity" trial in patients with either established adenomas or carcinomas that will define the impact of 7 days of oral linaclotide on epithelial dysfunction and hyperproliferation compared to placebo. In the Mechanistic Aim, we will test the ability of linaclotide to activate GUCY2C signaling and induce proteosomal degradation of β-catenin, reversing mutant APC signaling, in organoids derived from human adenomas and carcinomas identified in Aim 1. We have received regulatory approval to initiate the trial at Jefferson and await that approval at Fox Chase Cancer Center and the VA Puget Sound. We will begin enrolling patients at Jefferson in November 2019.

15. SUBJECT TERMS
Adenomas, Adenomatous polyposis coli gene (APC), Beta-catenin, Chemoprevention, Clinical trial, Colectomy, Colorectal cancer, Cyclic GMP, DNA damage, Endoscopy, Guanylin, Guanylyl cyclase C, Linaclotide, Metabolism, Proliferation, Therapy, Signaling,Vasodilator-stimulated phosphoprotein (VASP)

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1. INTRODUCTION

This project addresses the ***FY17 PRCRP Topic Area*** of "colorectal cancer" and the ***FY17 PRCRP Military Relevance Focus Area*** involving "gaps in cancer prevention, early detection/diagnosis, prognosis, treatment and/or survivorship that may affect the general population but have a particularly profound impact on the health and well-being of active duty Service members, Veterans, and their beneficiaries". It explores mechanisms in colorectal cancer that can be leveraged for novel disease prevention strategies. In ≥90% of colorectal cancers, mutations in APC drive β-catenin accumulation and nuclear translocation that activate transcriptional programs driving tumorigenesis. GUCY2C is the intestinal receptor for the hormone guanylin expressed in the colorectum. In health, the guanylin-GUCY2C axis opposes epithelial cell β-catenin accumulation. Guanylin is universally lost in APC-β-catenin-driven tumorigenesis, mediated by mutant APC-β-catenin signaling. Guanylin loss is required for tumorigenesis because GUCY2C stimulation reciprocally blocks the β-catenin accumulation required for transformation. We have demonstrated that transgenic guanylin ***eliminates*** intestinal tumorigenesis in all mouse models examined. Moreover, our NCI phase I trial revealed that the oral GUCY2C ligand ***linaclotide*** regulated colorectal cell proliferation in healthy volunteers, suggesting that reconstitution of normal GUCY2C signaling with oral linaclotide could prevent or inhibit colorectal tumor progression. The ***overall objective*** is to develop a prevention strategy for colorectal cancer by exploiting the role of GUCY2C signaling in inhibiting colorectal epithelial malignant transformation. The ***translational hypothesis*** suggests that oral linaclotide restores GUCY2C signaling, repairs epithelial dysfunction, and blocks progression of adenomas or carcinomas in patients. The linked ***mechanistic hypothesis*** suggests that linaclotide prevents tumor progression by blocking β-catenin accumulation through proteosomal degradation. **The specific aims of proposed studies includes:** the ***Translational Aim***, in which we will conduct a "window of opportunity" trial in patients with either established adenomas or carcinomas that will define the impact of 7 days of oral linaclotide on epithelial dysfunction and hyperproliferation compared to placebo; and a ***Mechanistic Aim***, in which we will test the ability of linaclotide to activate GUCY2C signaling and induce proteosomal degradation of β-catenin, reversing mutant APC signaling, in organoids derived from human adenomas and carcinomas identified in Aim 1. The military relevance of this project can best be appreciated by considering that colorectal cancer is the 4th leading cause of cancer, and the 2nd leading cause of cancer mortality, with a ~50% death rate, in military and civilian populations. In that context, military populations are burdened by the increasing rate of disease in younger patients (<50 yo) who present with advanced disease which is more likely to recur. The impact of prevention strategies for colorectal cancer on the military health system can best be appreciated by considering that in 2015 medical care for new cases of colorectal cancer will cost the VA Health System ~$400M annually, while the economic impact of each year of life lost is ~$170M annually.

2. KEYWORDS

- Adenomas
- Adenomatous polyposis coli gene (APC)
- Beta-catenin
- Chemoprevention
- Clinical trial
- Colectomy
- Colorectal cancer
- Cyclic GMP
- DNA damage
- Endoscopy
- Guanylin
- Guanylyl cyclase C
- Linaclotide
- Metabolism
- Proliferation
- Therapy
- Signaling
- Vasodilator-stimulated phosphoprotein (VASP)

3. ACCOMPLISHMENTS

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

- **What were the major goals of the project?**
  - Major Task 1. Clinical trial of oral linaclotide in patients with colorectal adenomas and carcinomas (Months 1-30).
  - Major Task 2: Oral linaclotide re-establishes GUCY2C signaling in adenomas and carcinomas (Months 6-36).
  - Major Task 3: Oral linaclotide reconstitutes guanylin expression in adenomas and carcinomas (Months 6-36).
  - Major Task 4: Oral linaclotide will repair mutant APC-β-catenin signaling in adenomas and carcinomas (Months 6-36).
  - Major Task 5: Linaclotide reverses epithelial dysfunction (Months 6-36).

- **What was accomplished under these goals?**
  - Major Activities
    - Finalize study protocol;
    - Submit finalized protocol to IRB at Thomas Jefferson University for approval for conduct at Jefferson and Fox Chase Cancer Center;
    - Submit study protocol for approval by HRPO.
    - Received IRB approval for study protocol at Thomas Jefferson University for both Thomas Jefferson University and Fox Chase Cancer Center.
    - Received HRPO approval for the study protocol at Thomas Jefferson University.
    - Arrange for drug delivery by Ironwood to Thomas Jefferson University and Fox Chase Cancer Center for mid-April 2019.
    - Finalized the Pharmacy Manual for the study.
    - Held Site Initiation Visit (SIV) at Thomas Jefferson University for both Thomas Jefferson University and Fox Chase Cancer Center on Friday, February 22, 2019.
    - Study protocol revised according to feedback from SIV (v3.2), submitted and received IRB approval at Thomas Jefferson University and Fox Chase Cancer Center.
    - Fox Chase Cancer Center initial HRPO submission is undergoing review.
    - Fox Chase Cancer Center completed the database creation to support data collection and entry for the three participating sites.
    - VA Puget Sound received contingent IRB approval for the amended study protocol.
    - Drug delivery by Ironwood to Thomas Jefferson University and Fox Chase Cancer Center
    - Fox Chase Cancer Center and VA Puget Sound submitted to HRPO.
    - VA Puget Sound received final IRB approval for the amended study protocol.
    - VA Puget Sound received initial HRPO approval
    - Received final approval for final data/sample sharing agreements between Thomas Jefferson University and Fox Chase Cancer Center.
    - Received final approvals to initiate patient recruitment at Thomas Jefferson University and Fox Chase Cancer Center.
  - Specific Objectives
    - Obtained local and central approval of study protocol to permit study initiation in first quarter of 2019.

- Obtained local and central approval of study protocol to permit study initiation in second quarter of 2019.
- Obtain central approval of study protocol and final data/sample sharing agreement to permit study initiation in third quarter of 2019.
- Received final local approval for final data/sample sharing agreements between Thomas Jefferson University and Fox Chase Cancer Center that permit initiation of patient enrollment in October, 2019.

- Key Outcomes
  - Study protocol was finalized;
  - Study protocol was submitted to Thomas Jefferson University IRB for approval for conduct at Jefferson and Fox Chase Cancer Center.
  - Study protocol was approved by IRB at Thomas Jefferson University for both Thomas Jefferson University and Fox Chase Cancer Center.
  - Study protocol was submitted to VA Puget Sound for approval for conduct.
  - Study protocol was submitted to HRPO for approval at Thomas Jefferson University.
  - Study protocol was approved by IRB at VA Puget Sound.
  - Study protocol v3.2 was submitted to HRPO and approved at Thomas Jefferson University.
  - SIV for study initiation at Thomas Jefferson University and Fox Chase Cancer Center was held.
  - All legal agreements (data/sample sharing agreements) between Thomas Jefferson University and Fox Chase Cancer Center were completed to enable initiating patient enrollment in October 2019.

- **What opportunities for training and professional development has the project provided?**
  - Ellen Caparosa, MD, and Alicja Zalewski, MD are surgical residents at Thomas Jefferson University. As part of their clinical training program, they take 1-2 years off from clinical activities to engage in research. During those research years, they enrolled in the NIH-supported T32 Postdoctoral Training Program in Clinical Pharmacology as full-time postdoctoral fellows. This is a training program that integrates research, experiential rotations (e.g., FDA) and formal didactic coursework to prepare physicians for academic careers as clinician investigators. Drs. Caparosa and Zalewski are the primary investigators spearheading the organization of this trial at Thomas Jefferson University. They lead the efforts to obtain local regulatory and legal approval for the trial, and they are leading efforts to recruit patients, enroll them in the trial, place them on study drug, collect samples and analyze endpoints. As part of their training program, they are learning about how to organize, initiate, conduct and complete clinical trials, including regulatory and legal frameworks. They work one-on-one with the PI of the project for this training experience. At the completion of the program, they receive certification of completion, and are eligible to sit for the national certifying exam in Clinical Pharmacology.

- **How were the results disseminated to communities of interest?**
  - Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Complete data/sample sharing agreements to add VA Puget Sound to the existing agreement between Thomas Jefferson University and Fox Chase Cancer Center to enable opening enrollment at VA Puget Sound.

- Conduct “mini” site initiation visit (“refresher” SIV) for Thomas Jefferson University on October 17, 2019.
- Begin enrolling subjects at Thomas Jefferson University and Fox Chase Cancer Center immediately after the mini-SIV on October 17, 2019.
- Conduct a Site Initiation visit between VA Puget Sound and Thomas Jefferson University and Fox Chase Cancer Center once data/sample sharing agreements have been completed.
- Begin molecular analyses of samples to establish whether oral linaclotide:
  - Restores the GUCY2C signaling axis in tumors;
  - Reconstitutes guanylin expression in tumors;
  - Repairs mutant APC-beta-catenin signaling in tumors;
  - Reverses epithelial dysfunction in tumors.

4. IMPACT

- *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
- **What was the impact on the development of the principal discipline(s) of the project?**
    - We have spent the last period obtaining local and national regulatory and legal approvals to conduct this multi-institutional trial. We are positioned now to begin enrolling patients into the trial and initiating analyses. As such, at this time there is Nothing to Report."
- **What was the impact on other disciplines?**
    - See above. There is "Nothing to Report."
- **What was the impact on technology transfer?**
    - See above. There is "Nothing to Report."
- **What was the impact on society beyond science and technology?**
    - See above. There is "Nothing to Report."

5. CHANGES/PROBLEMS

The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

- **Changes in approach and reasons for change**
  - Nothing to report.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - We anticipated initiating enrollment by September, 2019. However, there was a delay in obtaining legal approval for the data/sample sharing agreements across institutions. Those agreements have been finalized between Thomas Jefferson University and Fox Chase Cancer Center. We are initiating patient enrollment in October, 2019. Also, we are working to finalize data/sample sharing agreements that include VA Puget Sound and we anticipate that they will begin enrollment in the fourth quarter of 2019.
- **Changes that had a significant impact on expenditures**
  - Nothing to report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - **Significant changes in use or care of human subjects**
    - As above, we anticipated initiating enrollment by September, 2019. However, there was a delay in obtaining legal approval for the data/sample sharing agreements across institutions. Those agreements have been finalized between Thomas Jefferson University and Fox Chase Cancer Center. We are initiating patient enrollment in October, 2019. Also, we are working to finalize data/sample sharing agreements that include VA Puget Sound and we anticipate that they will begin enrollment in the fourth quarter of 2019.
- **Significant changes in use or care of vertebrate animals.**
    - Nothing to report.
- **Significant changes in use of biohazards and/or select agents**
    - Nothing to report.

6. PRODUCTS, INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES

- *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*
- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.
  - **Journal publications.**
    - Caparosa, E.M., Stem, J., Snook, A.E., and Waldman, S.A. (2019) Biomarker targeting of colorectal cancer stem cells. Biomarkers in Medicine. 13:891-894. Acknowledges federal support.
    - Blomain, E.S, Rappaport, J., Pattison, A.M., Bashir, B., Snook, A.E., and Waldman, S.A. (2019) APC-β-catenin silences the intestinal guanylin-GUCY2C tumor suppressor axis. (Submitted). Acknowledges federal support.
  - **Books or other non-periodical, one-time publications.**
    - None
  - **Other publications, conference papers, and presentations.**
    - None
- **Website(s) or other Internet site(s)**
    - None
- **Technologies or techniques**
    - None
- **Inventions, patent applications, and/or licenses**
    - None
- **Other Products**
    - None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Scott Waldman
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Dr. Waldman is the Principal Investigator of this project
Funding Support:	NA

Name:	Angela Pallotto
Project Role:	Coordinator CRU
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2
Contribution to Project:	Angela Pallotto has performed work in the area of implementation of the clinical trial, including obtaining institutional approvals and communicating with participating sites.
Funding Support:	NA

Name:	Elinor Leong
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	5
Contribution to Project:	Elinor Leong has performed work in the area of sample analysis, setting up lab, ordering and tracking supplies needed for this study.
Funding Support:	NA

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Waldman, Scott
Active Other support:

Below Active Other Support end date has been updated:

Waldman, (PI)

Title: Novel gene delivery systems for colorectal cancer

Funding Agency: Targeted Diagnostics and Therapeutics, Inc.

Goals of Project: The major goal of this project is to define the utility of ST and GCC for delivering gene replacement candidates to treat colorectal tumors.

Specific aims/tasks: see above

Estimated start and end date: 11/1/94-**6/30/20**

Level of Funding: $16,025 (current directs)

Level (%) of effort in the project: 0.12 calendar/1% effort

Point of Contact at the funding agency: Harry Arena

OVERLAP

None

Below Active Other Support end date and current directs has been updated:

Waldman (PI)

Title: Novel gene delivery systems for colorectal cancer

Funding Agency: Targeted Diagnostics and Therapeutics, Inc.

Goals of Project: This project will cover various clinical trials and is a supplement to the above award listed.

Specific aims/tasks: see above

Supplement Project 1 (A14703)

Estimated start and end date: 01/01/17-12/30/19

Level of Funding: $576,923 (current period Directs)

Level (%) of effort in the project: .6 calendar/5% effort

Point of Contact at the funding agency: Harry Arena

The below Active Support is new support.

Waldman (PI)
Title: Novel gene delivery systems for colorectal cancer/Targeting colorectal tumor with heat-stable exterotoxin
Funding Agency: Targeted Diagnostics and Therapeutics, Inc.
Total Costs: $975,000* currently advanced account/budget in review.
Goals of Project: This project will cover various clinical trials and is a supplement to the above award listed.

Specific aims/tasks: The specific aim of this project is to further define the utility of guanylyl cyclase C as a novel vaccine target to prevent disease recurrence in GI cancers in a Phase I/II clinical trials.
Project #2 (A14704)
Estimated start and end date: 07/01/19-12/31/19* currently advanced account
Level (%) of effort in the project: .6 calendar/5% effort
Point of Contact at the funding agency: Harry A. Arena, CEO
OVERLAP
None

Below Active Other Support end date and current directs have been updated:

Knudsen (PI)

Title: Translational research in cancer

Funding Agency: NIH/NCI 5 P30 CA56036

Goals of Project: Institutional Cancer Center Core Grant.

This is the Kimmel Cancer Center Core Grant.

Specific aims/tasks: see above

Estimated start and end date: 6/1/10-5/31/**19**

Level of Funding: **$23,691** (current period Directs/Salary and Fringe only)

Level (%) of effort in the project: 1.2 calendar/10% effort

Point of Contact at the funding agency: Barbara A. Fisher, Grants Management Officer

OVERLAP

None

Below Active Other Support current directs have been updated:

Waldman (PI)

Title: (PQ1) GUCY2C hormone loss translates APC-Beta-catenin mutations into epithelial transformation

Funding Agency: NIH/NCI 1R01CA206026-02

Goals of Project: This study explores a new mechanism required for the transformation of normal intestinal cells into premalignant intestinal cells.

Specific aims/tasks:

Aim 1. Guanylin loss drives formation of the pre-malignant field (Pathophysiological Aim).

Aim 2. Regulation of guanylin-GUCY2C signaling by APC-β-catenin is central to formation of the pre-malignant field (Mechanistic Aim).

Aim 3. Guanylin replacement eliminates transformation (Chemoprevention Aim).

Estimated start and end date: 05/09/16-04/30/21

Level of Funding: **$221,887** (current period Directs)

Level (%) of effort in the project: 2.4 calendar/20% effort

Point of Contact at the funding agency: Joy Kearse, Grants Management Specialist

OVERLAP

None

Below Active Other Support has ended:

Waldman (PI)

Title: Plecanatide and tumorigenesis in obese mice

Funding Agency: Synergy

Goals of Project: This project specific aim is to establish that plecanatide can prevent colorectal tumorigenesis in obesity.

Specific aims/tasks: See above goal

Estimated start and end date: 07/07/16-06/01/**19**

Level (%) of effort in the project: .6 calendar/5% effort

Point of Contact at the funding agency: Gary S. Jacob 212-297-2127

<u>OVERLAP</u>

None

Below Active Other Support current directs have been updated:

Waldman (PI)

Title: Guanylin-GUCY2C axis in colorectal cancer initiation

Funding Agency: NIH/NCI 5 R01 CA204481-03

Total Cost

Goals of Project: The overall goal of this proposal is to test the hypothesis that mutations in the APC-β-catenin axis initiate colorectal cancer by inhibiting the expression of the intestinal hormone guanylin, reversibly silencing the GUCY2C tumor suppressor.

Specific aims/tasks:

Aim 1. Mutant APC signaling blocks guanylin transcription as an obligatory step in tumor initiation (Pathophysiological Aim).

Aim 2. The guanylin-GUCY2C axis reciprocally suppresses mutant APC signaling (Mechanistic Aim).

Aim 3. Colorectal cancer is a disease of hormone loss that can be prevented by hormone replacement (Prevention Aim).

Estimated start and end date: 01/01/17-12/31/21

Level of Funding: **$235,135** (current period Directs)

Level (%) of effort in the project: 2.4 calendar/20% effort

Point of Contact at the funding agency: Aida Vasquez, Grants Management Specialist

<u>OVERLAP</u>

None

- **What other organizations were involved as partners?**

  Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
  - Not applicable
- **QUAD CHARTS:**
  - Not applicable

9. APPENDICES

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

1. Caparosa, E.M., Stem, J., Snook, A.E., and Waldman, S.A. (2019) Biomarker targeting of colorectal cancer stem cells. Biomarkers in Medicine. 13:891-894. Acknowledges federal support.
2. Blomain, E.S, Rappaport, J., Pattison, A.M., Bashir, B., Snook, A.E., and Waldman, S.A. (2019) APC-β-catenin silences the intestinal guanylin-GUCY2C tumor suppressor axis. (Submitted). Acknowledges federal support.

Commentary

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Biomarkers in Medicine

Biomarker targeting of colorectal cancer stem cells

Ellen M Caparosa[1,2], Jonathan Stem[1], Adam E Snook[2] & Scott A Waldman*,[2]
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"CRC continues to be a major public health burden. Given this, new therapeutic approaches must be developed that aim to eliminate the origin of tumorigenesis, target recurrence and drug resistance, and combat metastases. Therapy directed against CSCs would not only address this goal, but also would allow more targeted therapy with less systemic toxicity"

First draft submitted: 28 May 2019; Accepted for publication: 3 June 2019; Published online: 6 August 2019

Keywords: biomarkers • cancer stem cells • colorectal cancer • immunotherapy

Colorectal cancer (CRC) is a significant public health burden. It is the third most commonly diagnosed cancer, and the second highest cause of cancer related deaths when men and women are combined in USA. Overall, the lifetime risk of developing CRC is approximately 1 in 22 for men and 1 in 24 for women, and it is expected that over 51,000 deaths will occur due to CRC in USA in 2019. Metastatic disease remains the principle cause of death, with patient survival rates significantly declining with both local and distant metastases [1].

Stem cells are characterized by their capacity for self-renewal and the ability to give rise to diverse, specialized cell types. Cancer stem cells (CSCs) are a subpopulation of stem-like cells within a heterogeneous tumor that also exhibit capacity for self-renewal and differentiation. There is mounting evidence that CSCs can be considered the origin of tumorigenesis, metastases, drug resistance and relapse [2].

CSCs were first described in 1994 in human acute myeloid leukemia. An acute myeloid leukemia-initiating cell population enriched with cells expressing the cell surface marker CD34, but not the surface marker CD38 ($CD34^{+}/CD38^{-}$), was transplanted into severe combined immune-deficient mice and found to induce leukemia in contrast to nonenriched tumor cells [3]. In the early 2000s, CSCs were identified in solid tumors. Selective targeting of biomarkers either enriched in, or specific to, CSCs will allow for the administration of highly-potent cytotoxic agents with limited systemic toxicity, in contrast to conventional chemotherapeutics that target all rapidly-dividing cells.

Current recommendations for adjuvant treatment of Stage III and select patients with Stage II colon cancer includes adjuvant chemotherapy. Combined radiation and chemotherapy also are recommended for patients with stage II and III rectal cancer [4]. These existing therapeutic approaches treat tumors as if all of their heterogeneous cancer cells have unlimited proliferative potential and can acquire the ability to metastasize. Unfortunately, disease recurrence and progression occur despite these treatments. CSCs may be responsible for this treatment evasion, with increased ability to survive due to increased DNA repair, redox capacity, drug efflux and maintenance of dormancy. Molecules involved in these mechanisms are provocative targets for development of new therapies.

CSCs have increased capacity for DNA repair via multiple mechanisms. Cells incur DNA damage from radiation when reactive oxygen species (ROS) exceed the antioxidant capacity of the cell. Compared with other cells, CSCs highly express ROS scavengers, such as glutathione (GSH). This confers resistance to ROS-mediated DNA damage, and studies have shown that pharmacologic reduction of GSH results in greater radio-sensitivity in CSCs. CD44 has been widely used as a gastrointestinal (GI) CSC marker. Its variant isoforms (CD44v) also are highly expressed in GI cancers, and are involved in regulating GSH. Specifically, a recent study demonstrated that CD44v stabilizes a glutamine-cysteine transporter, xCT, to control the intracellular level of GSH in CRC cells. Additionally, they have increased resistance to multiple chemotherapeutic agents such as cisplatin and docetaxel [5]. Thus, one therapeutic

Future Medicine

approach to disarm CSC resistance mechanisms is through increasing susceptibility to oxidative stress. GSH and CD44v are clear potential targets. These studies demonstrate that targeting cellular defense against ROS can impact cell viability.

Another major factor in CSC resistance to standard therapy includes superior drug efflux mechanisms. Cell membrane transport proteins, such as the ATP-binding cassette (ABC) transporters, drive the export of toxic chemicals and drugs. Hydrophobic chemotherapeutic drugs are eliminated from the cytosol into the extracellular space via these transport proteins. In colorectal CSCs, there is increased expression of the MDR1 transport protein, which is a member of the ABC transporter family. This increase confers resistance in these cells to treatment with multiple drugs [6]. Several mechanisms can be utilized to disrupt these transporters in order to sensitize CSCs to chemotherapy. ABC transporter allosteric inhibitors have shown promise as potential therapy against multidrug resistance. Another approach targets the transcriptional regulation of ABC transporters, though this requires further development [7].

Differentiation of CSCs results in a loss of self-renewal capabilities, and also moves the cells out of a relatively senescent state. Both of these characteristics confer resistance to standard therapies. One of the best known examples of induced CSC differentiation is the use of all-trans retinoic acid, a retinoid and chemopreventative agent that was first used in the treatment of acute promyelocytic leukemia [8]. All-trans retinoic acid also induces differentiation in colon cancer cell lines [9]. Similarly, PPAR-γ agonists induce stem cell differentiation. Studies show that there is increased PPAR-γ expression in colonic adenocarcinoma, and that the PPAR-γ agonist troglitazone induces colon cancer cell differentiation [10].

Beyond therapies that induce differentiation, CSCs can be targeted specifically for elimination. CSCs have unique cell surface antigens that distinguish them from differentiated cells. These surface markers used for identification and isolation of CSCs are important targets for therapy. Well known colorectal CSC surface antigens include CD24, CD44, CD133, CD166 and aldehyde dehydrogenase [11].

Cancer immunotherapy is a treatment approach that utilizes the body's immune system to induce antitumor response. Immune cells exert cytotoxic effects on target cells in an antigen-specific manner, which allows specific targeting of cells of interest and can confer long-term memory responses, which may be of benefit in cancer relapse. Additionally, the toxicity of conventional treatments such as chemotherapy and radiation is partly dependent on cell cycle phase. In comparison to non-CSCs, CSCs are relatively quiescent [12]. Since immunotherapies target CSCs independent of proliferative status, this bypasses an important mechanism of CSC resilience.

T cells can be engineered to target tumors through expression of a CAR. Briefly, CAR T cells are engineered to express an artificial receptor composed of a targeting domain derived from an antibody connected to intracellular T-cell receptor signaling domains. CAR T cells have been remarkably successful in treating hematologic malignancies. In light of their success and the recent US FDA approval of tisagenlecleucel and axicabtagene, there is increased impetus to expand CAR T-cell therapy to the field of solid organ tumors. A recent Phase I clinical trial in patients with hepatocellular, pancreatic and CRC demonstrated safety and efficacy of treatment with CAR T cells directed against CD133, which is a surface marker expressed by various CSCs of epithelial origin [13]. Another Phase I clinical trial tested CAR T cells directed against carcinoembryonic antigen in ten patients with metastatic CRC. Of ten patients, seven with progressive disease stablized after CAR T-cell therapy, with two patients exhibiting tumor shrinkage by positon emission tomography [14]. Additional clinical trials testing CAR T cells engineered against other biomarkers in colon cancer are underway (NCT03013712, NCT03851146).

Cancer vaccines can treat existing cancer, or prevent their development. Several studies have assessed anti-CSC vaccines. An important proof of concept study demonstrated that CSC-enriched populations, identified by high expression of aldehyde dehydrogenade (ALDH^{high}), generated CSC-specific antibody and T-cell responses *in vivo*. In this study, dendritic cells pulsed with CSC-lysate were more effective in inducing antitumor immunity, and ultimately resulted in slower tumor growth and decreased metastases compared with dendritic cells pulsed with whole cell lysate [15]. This suggests that that autologous tumor cell vaccines targeting CSCs may yield greater antitumor effects. Phase I/II clinical trials administering CSC vaccines are being conducted in patients with liver (NCT02089919), colorectal (NCT02176746) or pancreatic (NCT02074046) cancers.

CSCs also can be targeted via innate immunity. For example, CSCs have significantly increased susceptibility to natural killer (NK) cells when compared with non-CSCs in colorectal tumors. This increased susceptibility was associated with increased expression of NK activating ligands NKp30 and NKp44, in addition to decreased expression of inhibitory ligands such as MHC class I on CSC surfaces. γδ T cells also exhibit anti-CSC activity in

CRC, in addition to other malignancies [16]. Ultimately, NK and γδ T cells may be of significant clinical benefit in the treatment of colon cancer.

Targeting cell surface markers to deliver cytotoxic agents represents another class of potential interventions. Aptamers are single stranded oligonucleotide or peptide molecules that are nearly 20-times smaller than antibodies. Due to their small size, they have excellent tissue penetration and thus an advantage over antibody therapy. Aptamers bind with high affinity to a specific target and are internalized by cells [17]. Aptamers directed against CSCs were first engineered against epithelial cell adhesion molecule, a membrane protein that is overexpressed in most solid cancers and also has been identified as a CSC marker. This epithelial cell adhesion molecule aptamer bound to multiple cancer cell lines, including the colon adenocarcinoma cell lines HT-29 and SW480, and was internalized by these cell lines [18]. An RNA aptamer targeting CD133 also was internalized by several GI cancer cell lines, including HT-29 cells. Further, the CD133 aptamer displayed superior tumor penetration when compared with a traditional CD133 antibody [19].

Antibody–drug conjugates (ADCs) are comprised of a monoclonal antibody that is linked to a small, cytotoxic agent, which creates a selective and cytotoxic therapeutic agent. ADCs are internalized by cells, and subsequently undergo lysosomal processing and cleavage in order to activate the cytotoxic agent. Thus, the therapy is delivered only to cells that display the antigen. An ADC was recently developed against the adult stem call marker LGR5, which is a leucine-rich repeat-containing G protein coupled receptor that is highly expressed in GI cancers, including colon cancers. Mice were subcutaneously inoculated with a human colon metastases cell line (LoVo). Once tumors reached a standard size, mice were treated with either phosphate-buffered saline vehicle, an unconjugated anti-LGR5 monoclonal antibody, or the LGR5 ADC. All mice treated with the LGR5 ADC had either a significant reduction in tumor size or a complete response without a detectable tumor [20].

Conclusion

CRC continues to be a major public health burden. Given this, new therapeutic approaches must be developed that aim to eliminate the origin of tumorigenesis, target recurrence and drug resistance, and combat metastases. Therapy directed against CSCs would not only address this goal, but also would allow more targeted therapy with less systemic toxicity.

Despite promising laboratory advances in CSC targeted therapy, there are still major barriers. For example, some of the therapies directed at differentiation biomarkers are inherent to stem cells in general, and not just CSCs. Specifically targeting cell surface biomarkers is a unique opportunity to directly address the CSC population. Thus, drug carrier systems, vaccines, and CAR T-cell therapies have promising potential. However, this is not without barriers; CSC surface markers may vary across different patients, some surface markers also are expressed on noncancerous stem cells, and it is possible that subsets of CSCs do not express known markers. Despite this, biomarker targeting of CRC stem cells remains a promising field with the potential to revolutionize cancer treatments.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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APC-β-catenin-TCF signaling silences the intestinal guanylin-GUCY2C tumor suppressor axis

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Abstract:	Sporadic colorectal cancer initiates with mutations in APC or its degradation target β-catenin, producing TCF-dependent nuclear transcription driving tumorigenesis. The intestinal epithelial receptor, GUCY2C, with its canonical paracrine hormone guanylin, regulates homeostatic signaling along the crypt-surface axis opposing tumorigenesis. Here, we reveal that expression of the guanylin hormone, but not the GUCY2C receptor, is lost at the earliest stages of transformation in APC-dependent tumors in humans and mice. Hormone loss, which silences GUCY2C signaling, reflects transcriptional repression mediated by mutant APC-β-catenin-TCF programs in the nucleus. These studies support a pathophysiological model of intestinal tumorigenesis in which mutant APC-β-catenin-TCF transcriptional regulation eliminates guanylin expression at tumor initiation, silencing GUCY2C signaling which, in turn, dysregulates intestinal homeostatic mechanisms contributing to tumor progression. They expand the mechanistic paradigm for colorectal cancer from a disease of irreversible mutations in APC and β-catenin to one of guanylin hormone loss whose replacement, and reconstitution of GUCY2C signaling, could prevent tumorigenesis.
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APC-β-catenin-TCF signaling silences the intestinal guanylin-GUCY2C tumor suppressor axis

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Abstract

Sporadic colorectal cancer initiates with mutations in APC or its degradation target β-catenin, producing TCF-dependent nuclear transcription driving tumorigenesis. The intestinal epithelial receptor, GUCY2C, with its canonical paracrine hormone guanylin, regulates homeostatic signaling along the crypt-surface axis opposing tumorigenesis. Here, we reveal that expression of the guanylin hormone, but not the GUCY2C receptor, is lost at the earliest stages of transformation in APC-dependent tumors in humans and mice. Hormone loss, which silences GUCY2C signaling, reflects transcriptional repression mediated by mutant APC-β-catenin-TCF programs in the nucleus. These studies support a pathophysiological model of intestinal tumorigenesis in which mutant APC-β-catenin-TCF transcriptional regulation eliminates guanylin expression at tumor initiation, silencing GUCY2C signaling which, in turn, dysregulates intestinal homeostatic mechanisms contributing to tumor progression. They expand the mechanistic paradigm for colorectal cancer from a disease of irreversible mutations in APC and β-catenin to one of guanylin hormone loss whose replacement, and reconstitution of GUCY2C signaling, could prevent tumorigenesis.

INTRODUCTION

There is a well-established relationship between mutations in the APC tumor suppressor and colorectal cancer[1-5], the 4th most common cancer, and the 2nd leading cause of cancer death.[6] The current paradigm suggests that monoallelic inactivation creates *APC* heterozygosity and a potential vulnerability for tumorigenesis.[1-5, 7]. This potential is realized by a second event resulting in functional loss of heterozygosity (LOH), eliminating APC from a destruction complex which targets β-catenin for ubiquitination and proteasomal degradation.[1-5, 7] As a consequence, cytosolic β-catenin accumulates and translocates to the nucleus, and with its binding partner TCF, re-programs transcription to drive transformation.[8-11] Indeed, ≥90% of sporadic colorectal cancers initiate with inactivating mutations in APC (~85%) or mutations in β-catenin that block degradation (~5%).[1-5] However, specific molecular mechanisms coupling these mutations to tumorigenesis continue to be refined.

GUCY2C is the membrane-bound receptor guanylate cyclase expressed by intestinal epithelial cells.[12, 13] Canonical GUCY2C ligands include uroguanylin produced in small intestine, guanylin produced in colorectum, and bacterial heat-stable enterotoxins (STs), all of which increase epithelial cell cyclic guanosine monophosate (cGMP).[12, 13] The GUCY2C-cGMP signaling axis modulates intestinal secretion, one mechanism by which bacteria induce diarrhea.[14-16] Linaclotide (*Linzess™*) and plecanatide (*Trulance™*) are orally available GUCY2C ligands that are congeners of ST and uroguanylin, respectively. These peptides increase intestinal secretion and are FDA-approved to treat chronic constipation syndromes.[13, 17]

The architecture of the intestinal epithelium is maintained by continuous regenerative cycles of proliferation, migration, differentiation, and apoptosis.[18-23] In turn, these are the processes that

are canonically disrupted in cancer.[24] Beyond secretion, the paracrine hormone-GUCY2C signaling axis regulates these normal homeostatic processes.[18-23] In that context, silencing GUCY2C produces crypt hyperplasia, increasing proliferating progenitor cells and accelerating their cell cycle.[18, 19, 21-23] Conversely, GUCY2C signaling inhibits proliferation by decreasing β-catenin and its transcriptional targets cyclin D and Myc.[18, 19, 21, 22] Also, there is a metabolic gradient along the crypt-surface axis, where proliferating crypt cells depend on glycolysis, while differentiated surface cells depend on oxidative phosphorylation.[19] Silencing GUCY2C imposes an aberrant phenotype along that axis, characterized by reduced mitochondria, increased glycolysis, and decreased oxygen consumption, recapitulating the Warburg metabolic phenotype in tumors.[19] Moreover, silencing GUCY2C increases DNA oxidation and double strand breaks, mutagenesis induced by alkylating agents, and chromosomal instability[18, 19]

Guanylin is reduced [25-28], while GUCY2C is conserved [29-31], in colorectal adenomas and carcinomas in humans and mice. In that context, GUCY2C agonists reduce epithelial transformation in genetic, carcinogen, and inflammatory mouse models of intestinal tumorigenesis.[26, 32-35] These observations suggest a pathophysiological model in which transformation reduces guanylin expression, driving tumorigenesis by suppressing GUCY2C-cGMP signaling. Here, we reveal that guanylin hormone expression is eliminated, and GUCY2C-cGMP signaling is silenced, as an immediate downstream consequence of mutant APC-β-catenin-TCF nuclear transcriptional re-programming.

RESULTS

GUANYLIN HORMONE, BUT NOT THE GUCY2C RECEPTOR, IS LOST IN HUMAN COLORECTAL TUMORIGENESIS

Guanylin protein (Figure 1A-C) and mRNA (Figure 1D) are eliminated in human colorectal adenocarcinomas compared to normal mucosa. These results are consistent with those obtained with samples from the TCGA[36], which revealed a median reduction of ~250-fold in GUCA2A mRNA in 339 tumors with mutations in the APC-β-catenin signaling pathway compared to 51 normal tissues (Figure 1E). Loss of hormone expression is an early event in transformation, and guanylin protein (Figure 1A-C) and mRNA (Figure 1F) are absent in tubular adenomas, which arise primarily from mutations in APC-β-catenin-TCF signaling[1-5, 7, 37], compared to normal tissue in patients. Similar results were obtained with tissues in a GEO dataset (accession number GSE8671)[38-40], which revealed a median reduction of ~250-fold in GUCA2A mRNA in 32 adenomas with mutations in the APC-β-catenin signaling pathway compared to matched normal adjacent tissues (Figure 1G). Further, guanylin hormone expression is absent in tumors from patients with familial adenomatous polyposis (FAP), a hereditary colorectal cancer syndrome in which patients are heterozygous for one mutant allele of *APC* (Figure 1A-B, H; Supplementary Figure 1). Hormone loss associated with reductions in mRNA in transformed tissue does not reflect gene mutations or changes in chromosomal structure and there were only 2 tumors with missense mutations in the *GUCA2A* gene from a total of 1378 colorectal adenocarcinomas with mutations in *APC* or *CTNNB1* (β-catenin) in the cBioPortal database.[41, 42]

In contrast to the hormone guanylin, expression of GUCY2C protein and mRNA are maintained in adenocarcinomas (Figure 2A-D) and tubular adenomas (Figure 2A-E; see Figure 1A for H&E micrographs). These results agree with those obtained with samples from the TCGA[36], in which expression of GUCY2C mRNA was preserved in 339 tumors with mutations in the APC-β-catenin signaling pathway, compared to 51 normal tissues (Figure 2F). Similarly, they agree with results

obtained with samples from a GEO dataset (accession number GSE8671)[38-40], in which expression of GUCY2C mRNA was preserved in 32 adenomas with mutations in the APC-β-catenin signaling pathway, compared to matched normal adjacent tissues (Figure 2G). Further, GUCY2C protein (Figure 2A-B) and mRNA (Figure 2H) expression was maintained in adenomas from patients with FAP (see Figure 1A for H&E micrographs). Moreover, the GUCY2C gene is a "cold spot" and there were 14 tumors with missense mutations in, and 1 tumor with a deletion of, the GUCY2C gene from a total of 1378 colorectal adenocarcinomas with mutations in *APC* or *CTNNB1* in the cBioPortal database.[41, 42] Taken together, these observations reveal that guanylin hormone mRNA and protein expression is lost, but expression of the GUCY2C receptor is maintained, in the earliest stages of APC-driven transformation in patients.

MUTATIONS IN APC DRIVE THE LOSS OF GUANYLIN, BUT NOT GUCY2C, IN MICE

Loss of guanylin at the earliest stages of tumorigenesis in patients supports a mechanism in which mutations in APC signaling disrupt hormone expression. In that context, $Apc^{CKO/CKO}$ mice in which biallelic *Apc* loss can be conditionally induced in a temporal- and tissue-specific fashion, recapitulating the initiating event in intestinal tumorigenesis[1-5, 7, 37], provide a unique opportunity to test this hypothesis. Tamoxifen-induced biallelic loss of *Apc* in intestinal epithelial cells of *vil-Cre-*ER^{T2}-$Apc^{CKO/CKO}$ mice activated canonical Wnt signaling, with accumulation of β-catenin and its downstream transcriptional targets c-Myc and Axin2 (Figure 3A-B). This dysregulated Wnt signaling disrupted normal epithelia architecture, with attenuation of villus structures, crypt hyperplasia, and extension of $PCNA^{+}$ proliferating cells, normally confined to the crypt base, along the crypt-surface axis (Figure 3C). Biallelic *Apc* loss eliminated guanylin, but not GUCY2C, protein and mRNA expression (Figure 3D-F). Loss of guanylin hormone silenced the retained GUCY2C

receptor (Figure 3D, F), reducing phosphorylation of vasodilator-stimulated phosphoprotein (VASP), an immediate downstream target of cGMP signaling in intestinal epithelial cells (Figure 3D)[34]

APC-β-CATENIN-TCF SIGNALING SUPPRESSES GUANYLIN EXPRESSION IN HUMAN COLORECTAL CANCER CELLS

The foregoing suggests that oncogenic signaling produced by mutations in APC that drive intestinal tumorigenesis silence the expression of the hormone guanylin. Loss-of-function mutations in APC, or gain-of-function mutations in β-catenin, produce accumulation and nuclear translocation of β-catenin, where it binds to the TCF4 transcription factor to re-program gene expression driving transformation.[8-11] In that context, we directly tested the individual roles of APC, β-catenin, and TCF4 in the regulation of guanylin expression. HT29 human colon cancer cells express mutant APC and are devoid of guanylin mRNA and protein expression (Figure 4A).[43] Induced expression of a wild type APC transgene in these cells restores normal Wnt signaling (Figure 4B, Supplementary Figure 2) and interrupts expression of β-catenin downstream transcriptional targets (Figure 4C).[43] Further, restoring the expression of wild type APC reconstitutes the expression of guanylin mRNA (Figure 4D) and protein (Figure 4E). Similarly, LS174T human colon cancer cells express mutant β-catenin, and also are devoid of guanylin mRNA and protein expression (Figure 4F).[10, 44] Induced suppression of mutant β-catenin expression with siRNA in these cells (Figure 4G) interrupts the expression of its downstream transcriptional targets (Figure 4H). Suppression of mutant β-catenin in these cells re-establishes the expression of guanylin mRNA and protein (Figure 4I-J). Finally, DLD1 human colon cancer cells express mutant APC and wild type β-catenin and are devoid of guanylin mRNA and protein expression (Figure 4K).[10, 44] Induced expression of a dominant negative form of TCF4 in these cells

(Figure 4K-L) interrupts the expression of its downstream transcriptional targets (Figure 4M). Moreover, the dominant negative form of TCF4 restores the expression of guanylin mRNA and protein in these cells (Figure 4N-O). Dominant negative TCF similarly reconstituted guanylin mRNA and protein expression in LS174T cells (Supplementary Figure 3). Moreover, hormone expression is temporally linked to alterations in APC-β-catenin signaling, and guanylin mRNA recovers within hours of DNTCF protein expression (Supplementary Figure 4). Taken together, these observations demonstrate that guanylin mRNA and protein expression is suppressed by APC-β-catenin-TCF signaling.

APC-β-CATENIN-TCF SIGNALING REGULATES GUANYLIN NUCLEAR TRANSCRIPTION

Loss of guanylin in adenomas and tumors is associated with reduced expression of the mRNA for the hormone. Also, guanylin loss in epithelial cells following dysregulation of APC-β-catenin-TCF signaling is associated with loss of the transcript for the hormone. Moreover, APC-β-catenin-TCF signaling, which regulates guanylin expression, induces re-programming of nuclear transcription circuits which drives transformation.[8-11] Here, we explored the contribution of nuclear transcription of new mRNA to the regulation of guanylin expression by APC-β-catenin-TCF signaling. Nascent guanylin mRNA was labeled with ethynyl-uridine and biotin-azide click-chemistry, followed by affinity purification and quantification by qRT-PCR analysis (Figure 5A).[45, 46] In HT29 human colorectal cancer cells, induction of transgenic wild type APC (Figure 5B-C) reconstitutes the generation of nascent guanylin mRNA (Figure 5D). Similarly, in LS174T human colorectal cancer cells, suppression of mutant β-catenin with siRNA (Figure 5E-F) restores guanylin mRNA synthesis (Figure 5G). Finally, in DLD1 human colorectal cancer cells, induced expression of DNTCF (Figure 5H-I) re-establishes nascent guanylin mRNA synthesis (Figure 5J).

These observations demonstrate that dysregulated APC-β-catenin-TCF signaling mediates guanylin hormone loss as part of its canonical re-programming of nuclear transcription.[8-11]

Discussion

Mutations in APC that constitutively activate Wnt signaling initiate more than 80% of sporadic, and 100% of FAP-associated, colorectal tumors.[1-5, 7, 37] Mutations in APC typically result in loss of function, inactivating the multimeric protein complex targeting β-catenin for proteasomal degradation.[1, 2, 4, 7, 8] In turn, β-catenin accumulates in the cytosol, translocates to the nucleus and binds to TCF, re-programming transcription that drives transformation.[9, 10] While a role for APC in intestinal tumorigenesis is well established, mechanistic steps leading from dysregulated Wnt signaling to transformation continue to be defined. Here, we reveal that loss of the hormone guanylin, which silences the GUCY2C receptor, is mediated by mutant APC-β-catenin signaling. In that context, guanylin loss, and GUCY2C silencing, is universal in tubular adenomas and adenocarcinomas initiated by mutations in APC signaling in patients. It is one of the earliest events in tumorigenesis and is tightly linked temporally with mutant APC-β-catenin signaling. Guanylin loss is highly conserved across species in which APC mutations drive tumorigenesis, including mice and humans. Moreover, loss-of-function mutations in APC, or gain-of-function mutations in β-catenin, produce a TCF-dependent block of nuclear guanylin mRNA transcription that eliminates hormone production in mouse intestine in vivo and in human colorectal cancer cells in vitro. Together, these observations demonstrate that transcriptional repression of guanylin hormone production which silences the GUCY2C receptor is an immediate downstream consequence of dysregulated APC-β-catenin signaling that canonically drives intestinal transformation.

APC-β-catenin-TCF signaling regulates guanylin hormone production by eliminating the expression of newly synthesized transcripts. In that context, the precise mechanisms by which APC-β-catenin-TCF signaling regulates guanylin transcription remain to be defined. While APC and β-catenin signal through transcriptional regulation of gene expression[9, 10], chromatin immunoprecipitation and mRNA sequencing (ChIP-seq) analysis reveals that they do not bind directly to the guanylin promoter in murine intestinal crypt cells and human colorectal cancer cell lines in which guanylin expression is suppressed (Supplementary Figure 5). In that context, guanylin expression in intestinal cells requires association of the hepatocyte nuclear factor-1α (HNF-1α) transcription factor to a consensus nucleotide binding site located in the immediate 5'-flanking region of the promoter.[47] However, the expression of HNF-1α is not reduced in tumors nor regulated by APC-β-catenin-TCF signaling (Supplementary Figure 6).[11] Beyond HNF-1α, transcription factors regulating the expression of guanylin remain undefined. In silico mapping of the guanylin promoter (Encode: UCSC Genome Browser database)[48] reveals consensus binding sequences for transcription factors, including those regulated by APC-β-catenin-TCF signaling. These observations are consistent with a hypothesis, currently being explored, in which APC-β-catenin-TCF signaling regulates the expression of the guanylin gene through its downstream canonical transcriptional regulatory network that drives oncogenesis.[11]

Early universal loss of guanylin, silencing GUCY2C, in a mechanism conserved across species, suggests that the paracrine hormone-GUCY2C-cGMP axis opposes intestinal transformation induced by mutant APC-β-catenin signaling. Indeed, silencing GUCY2C amplifies intestinal tumorigenesis produced by mutations in *Apc* or by the carcinogen azoxymethane (AOM) in mice.[18, 19, 34] Conversely, luminal replacement of GUCY2C agonists reduces the number and size

of tumors driven by *Apc* mutations in mice.[26, 32, 33, 35] These observations support the hypothesis that guanylin loss and silencing of the GUCY2C axis is one molecular mechanism contributing to the progression of tumorigenesis initiated by mutant APC-β-catenin-TCF signaling.

The precise mechanisms by which guanylin-GUCY2C-cGMP signaling opposes intestinal tumorigenesis remain to be defined. For example, GUCY2C signaling activates p53, reduces DNA damage, and amplifies DNA damage repair opposing spontaneous and carcinogen-induced mutational events, which could block *Apc* LOH in cells heterozygous for this tumor suppressor.[18-20, 49] Alternatively, GUCY2C inhibition of cell cycle drivers, like cyclin D, and activation of cell cycle inhibitors, like p21, which restricts proliferation and the size of the crypt compartment could prevent tumor progression.[18, 19, 21, 22, 34, 35, 50] Beyond these canonical homeostatic mechanisms that are essential in organizing the crypt-surface axis, and are disrupted in tumorigenesis, GUCY2C might directly oppose APC-β-catenin signaling. The guanylin-GUCY2C-cGMP axis regulates intracellular concentrations of β-catenin, and its downstream transcriptional targets like c-Myc and axin, in intestinal cells.[18, 19, 34, 50] This regulation may involve suppression of β-catenin mRNA transcription through a mechanism mediated by cGMP-dependent protein kinase.[51, 52] In that context, it is tempting to speculate that there may be a reciprocal negative feedback loop between guanylin-GUCY2C and APC-β-catenin signaling systems organizing the crypt-surface axis.[18, 19, 34, 51, 52] In this paradigm, Wnt signaling at the base of the crypt suppresses the guanylin-GUCY2C-cGMP axis, favoring regeneration and proliferation. Conversely, guanylin-GUCY2C signaling at the surface suppresses APC-β-catenin signaling, favoring cellular differentiation and maturation. Tumorigenesis co-opts this regulatory mechanism, and mutant

Wnt signaling suppresses guanylin expression because cGMP signaling blocks nuclear β-catenin accumulation required for transformation.

The present observations demonstrate that guanylin expression is transcriptionally repressed by APC-β-catenin-TCF signaling in intestinal epithelial cells. In turn, suppression of guanylin expression, which silences the GUCY2C signaling axis, appears to contribute to APC-β-catenin-driven colorectal tumorigenesis. These studies reveal for the first time a novel molecular step leading from mutations in APC that constitutively activate Wnt signaling to transformation of intestinal epithelial cells. Surprisingly, silencing of the GUCY2C tumor suppressor occurs through a previously unanticipated mechanism of hormone loss, rather than through genetic inactivation of the receptor by a mutational event. In turn, loss of guanylin hormone, but retention of the GUCY2C receptor, expands the prevailing paradigm for colorectal tumorigenesis from an irreversible oncogenomic disease of mutational APC inactivation to a reversible functional disease of hormone insufficiency. Indeed, guanylin loss induced by mutant APC-β-catenin signaling creates a unique disease-specific vulnerability that can be leveraged to eliminate tumor initiation by oral GUCY2C hormone replacement.[31] The potential for immediate translation of these observations is underscored by the availability of the oral GUCY2C agonists linaclotide (*Linzess™*) and plecanatide (*Trulance™*) to treat chronic constipation syndromes.[13, 17, 31]

MATERIALS AND METHODS

HUMAN SAMPLES

The study was approved by the local Institutional Review Board (control #14D.376). For these studies, specimens were provided in a de-identified fashion by the Department of Pathology at Thomas Jefferson University Hospital and Cooperative Human Tissue Network (CHTN:

https://www.chtneast.org). All tumors underwent routine clinical profiling in the Department of Pathology to ensure that they originated through the conventional pathway (Apc-β-catenin) by demonstrating that they were wild type with respect to BRAF (eliminating tumors arising from the serrated pathway) and mismatch repair proficient, with intact MLH1, MSH2, PMS2, and MSH6 (eliminating tumors arising from the microsattelite instability pathway).[53]

ANIMAL MODELS

All animal protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. *Apc*^{CKO} mice contain a conditional knockout allele of APC with loxP sites flanking exon 14, producing a truncated APC protein in the context of Cre-mediated recombination (NCI Mouse Repository, #01XAA).[54] *Apc*^{CKO} mice were crossed with our previously characterized *vil-Cre-ER*^{T2} mice to induce biallelic APC inactivation in intestinal epithelial cells (*vil-Cre-ER*^{T2}-*Apc*$^{CKO/CKO}$).[20] Mice were bred onto the ROSA$^{mT/mG}$ background, a fluorescent Cre-reporter model that expresses membrane-targeted green fluorescent protein in recombined cells.[55] Breeding strategies used to generate the appropriate models have been described.[20] Conditional mouse models were induced with intraperitoneal administration of tamoxifen.[20] All mice were genotyped by sequencing DNA from tail-clips.

CELL CULTURE REAGENTS

McCoy's 5A and Dulbecco’s Modified Eagle Medium-F12 (DMEM-F12) were obtained from Thermo Fisher Scientific (Waltham, MA). Hygromycin B, zeocin, blasticidin hydrochloride, and doxycycline hydrochloride also were from Thermo Fisher Scientific. HyClone fetal bovine serum was obtained from GE Healthcare Life Sciences (Pittsburgh, PA). Zinc chloride was obtained from Sigma (St. Louis, MO).

CELLS

LS174T and DLD1 cells containing either the Tet-inducible siRNA to β-catenin or dominant-negative TCF4 (DNTCF4) were obtained from Dr. H. Clevers in November 2013.[10, 44] LS174T and DLD1 cells were cultured in DMEM-F12 containing 10% FBS, Zeocin (500 µg/mL), and blasticidin (10 µg/mL). Conditional cell lines were induced with 1 µg/mL doxycycline. HT29 cells containing the zinc-inducible APC construct were received from Dr. B. Vogelstein in September 2013.[43] HT29 cells were cultured in McCoy's 5A containing 10% FBS and Hygromycin (600 µg/mL), and were induced with 300 µM zinc chloride. Conditional cell lines were authenticated at each use by testing their genetic inducibility. Cell lines were confirmed to be free of mycoplasma semiannually.

IMMUNOFLUORESCENCE

Tissues were fixed in 4% paraformaldehyde, processed and embedded in paraffin. Antigens were retrieved (Dako target retrieval buffer pH 9.0 or sodium citrate buffer pH 6.0 depending on application) and stained. Antibody to β-catenin was from Santa Cruz Biotechnology (Dallas, TX), Anti-PCNA (#29, 1:1000) was from Abcam (Cambridge, MA), and anti-GUCA2A for human guanylin (#HPA018215, 1:500) was from Sigma. Guanylin antisera (#2538, 1:100) used for mouse tissue staining was a gift from Dr. M. Goy.[56] Monoclonal antibodies to GUCY2C (1:2,000) were previously validated.[34] Background autofluorescence was reduced with sudan black (0.5% solution in 70% ethanol). Secondary antibodies were from Thermo Fisher Scientific. GUCY2C and GUCA2A were detected using tyramide signal amplification.[33] Fluorescence images were captured with an EVOS FL auto cell imaging system (Thermo Fischer Scientific). To quantify GUCA2A and GUCY2C protein expression, a single in-focus plane was acquired. Using ImageJ

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software, quantification in defined regions of interest (ROIs) was performed by calculating corrected total cell fluorescence (CTCF), using the following equation: [CTCF = integrated density – (area of selected cell X mean fluorescence of background readings)]. CTCF for each tissue section was calculated relative to control ROI.

IMMUNOBLOTS

Protein was extracted from intestinal mucosa dissected from mouse intestine (20-50 µg protein per lane) or total lysates from cells (10^6/well, 6-well plates) from *in vitro* experiments. Tissue or cell lysates were extracted in T-Per or M-Per (Thermo Fisher Scientific), respectively, supplemented with protease and phosphatase inhibitors (Roche, St. Louis, MO). Protein was quantified by immunoblot analysis employing antibodies to: GFP (cat. #13970, 1:1000), Axin2 (cat. #32197, 1:1000), c-Myc (cat. #32072, 1:1000) from Abcam; GAPDH (cat. #2118, 1:5000), β-catenin (cat. #8480, 1:1000), human APC (cat. #2504, 1:1000), TCF4 (cat. #2569, 1:1000), β-Actin (cat. #4967, 1:3000), phosphorylated VASP-ser239 (cat. #3114, 1:1000) from Cell Signaling Technology (Danvers, MA); mouse APC (cat. #896, 1:1000) from Santa Cruz Biotechnology (Dallas, TX); GUCA2A (cat. # HPA018215, 1:100) from Sigma or guanylin antisera (#2538, 1:100)[56]; and GUCY2C (1:500)[34]. Immunoblot images were captured on the BioRad ChemiDoc MP imaging station and bands were quantified by densitometry normalized to that of GAPDH or β-actin using ImageJ. Average relative intensity reflects at least two independent experiments each with at least three biological replicates.

MESSENGER RNA ANALYSIS

Tissue samples were flash frozen in liquid nitrogen and stored at -80°C until use; cell samples were lysed directly and used fresh. RNA was extracted and purified using the RNeasy kit (Qiagen,

Germantown, MD). Following isolation, RNA concentration and purity were measured using the Nanodrop 1000 (Thermo Fisher Scientific) and two-step quantitative (q)RT-PCR used to interrogate gene expression. Complementary DNA was produced using the Taqman RT-PCR kit (Life Technologies, Carlsbad, CA) according to the manufacturer's specifications and then quantified by PCR (Applied Biosystems, Foster City, CA) using Taqman primer probes (Life Technologies).

NEW RNA SYNTHESIS

Newly synthesized RNA was prepared using ethynyl-uridine (EU) "click" chemistry, with the Click-iT nascent RNA capture kit (cat. #C10365, Thermo Fisher Scientific). Briefly, inducible cancer cell lines were cultured for two to three days in the presence of inducing agent (doxycycline or zinc), followed by EU (500 nM) for three hours, which is incorporated into newly synthesized RNA. Total cell RNA was extracted as above using the Qiagen RNeasy kit and stored at -80°C until further use. EU-RNA was isolated from total RNA by a copper-catalyzed "click" reaction between the reactive alkyne moiety and biotin azide, followed by capture with magnetic streptavidin-coated beads. Captured EU-RNA was immediately reverse transcribed to produce complementary DNA and quantified by PCR, as above.

STATISTICAL ANALYSES

Statistical significance was determined by either one-way ANOVA or two-tailed Student's *t* test, where appropriate, for analyses of immunoblot and immunoflouresence intensity, mRNA fold change determinations, and biochemical assays. For animal studies, minimum cohort sizes were computed using a power of 80% and a significance level of 0.05 (2-tailed test) employing a priori predictions of effect size and variance established by preliminary studies or literature review.

Operators were blinded to sample identities for analyses. Comparisons between two groups at single time points were analyzed by Student's *t* test, or by the Mann-Whitney test for measures not satisfying normality assumptions. All statistical tests were calculated using GraphPad Prism (La Jolla, CA). Analyses represent mean $\pm$ SEM of n=3, unless otherwise indicated, and * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****, $p<0.0001$.

CONFLICTS OF INTEREST

SAW is a member of the Board and Chair of the Scientific Advisory Board of, and AES is a consultant for, Targeted Diagnostics & Therapeutics, Inc. which provided research funding that, in part, supported this work and has a license to commercialize inventions related to this work.

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FIGURE LEGENDS

Figure 1. Loss of GUCA2A expression in tumors in human colorectum. (A-C) GUCA2A protein quantified by (A-B) immunofluorescence or (B) immunoblot in adenocarcinomas (sporadic and FAP) and adenomas and normal mucosa. Where appropriate, matched normal adjacent tissues (NAT) and tumors from the same patient are highlighted with a connecting line. Corresponding H&E images (A) highlight histological changes associated with transformation. (D) GUCA2A mRNA quantified by RT-PCR in adenocarcinomas and matched normal mucosa (n=17). (E) GUCA2A mRNA expression quantified by RNASeq in adenocarcinomas (n=339) and normal mucosa (n=51) from human colorectum from the TCGA database.[36] (F) GUCA2A mRNA quantified by RT-PCR in tubular adenomas (9) and normal mucosa (n=8). (G) GUCA2A mRNA expression quantified by RNASeq in adenomas and matched normal mucosa (n=32) from the human colorectum from a GEO dataset (accession number GSE8671).[38-40] (H) GUCA2A mRNA quantified by RT-PCR analysis in FAP adenomas (n=5) and normal mucosa (n=6). Green, GUCA2A; blue, DAPI. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****, $p<0.0001$. Scale bar=100 µm.

Figure 2. Retention of GUCY2C expression in tumors in human colorectum. (A-C) GUCY2C protein quantified by (A-B) immunofluorescence or (C) immunoblot (canonical doublet at 140-160 kDa) in adenocarcinomas (sporadic and FAP) and adenomas and normal mucosa. Where appropriate, matched normal adjacent tissues (NAT) and tumors from the same patient are highlighted with a connecting line. (D) GUCY2C mRNA quantified by RT-PCR in adenocarcinomas and matched normal mucosa (n=17). (E) GUCY2C mRNA quantified by RT-PCR analysis in tubular adenomas (n=8) and normal mucosa (n=9). (F) GUCY2C mRNA expression quantified by RNASeq in carcinomas (n=339) and normal mucosa (n=51) from human colorectum from the TCGA

database.[36] (G) GUCY2C mRNA expression quantified by RNASeq in adenomas and matched normal mucosa (n=32) from the human colorectum from a GEO dataset (accession number GSE8671).[38-40] (H) GUCY2C mRNA quantified by RT-PCR analysis in FAP adenomas (n=5) and normal mucosa (n=6). Red, GUCY2C; blue, DAPI. ns, not significant. Scale bar=100 μm.

Figure 3. GUCA2A and GUCY2C in colonocytes following biallelic *Apc* in-activation in mice. Wildtype (WT) and *vil-Cre-ER*^{T2}-*Apc*$^{CKO/CKO}$ (CKO) mice received 100 mg/kg tamoxifen IP and colons with crypts were harvested 5 days later. (A) Immunblot analysis of APC, β-catenin and its downstream transcriptional targets. (B) Quantification of c-MYC and Axin2 mRNA by RT-PCR. (C) H&E and PCNA$^+$ cell staining of colons from WT and CKO mice. (D) Immunoblot analysis of GUCA2A, GUCY2C, and their downstream signaling target, phosphorylated (P-)VASP. (E) Quantification of GUCA2A and GUCY2C mRNA by RT-PCR. (F) Immunofluorescence of GUCY2C (magenta), GUCA2A (green) and DAPI (blue) in colons from WT and CKO mice. ns, not significant; *, $p<0.05$; ****, $p<0.0001$. Scale bar= 100 μm.

Figure 4. Regulation of GUCA2A expression by APC-β-catenin-TCF signaling in human colon cancer cells. (A-B) HT29 cells carrying a transgene containing wildtype APC under a zinc-inducible promoter[43] and treated with 300 μM zinc express wild type APC, with an associated loss of β-catenin, in a time-dependent fashion. (C) Induction of wildtype APC reduced (red) downstream transcriptional targets upregulated by β-catenin-TCF signaling and increased (green) transcriptional targets downregulated by β-catenin-TCF signaling. (D-E) Wildtype APC induced expression of GUCA2A (D) mRNA and (E) protein. (F-G) A transgene containing an siRNA to β-catenin under the control of a doxycycline-inducible promoter in LS174T cells[10, 44] suppressed the accumulation of β-catenin when treated with 1 μg/mL doxycycline. (H) Suppression of β-catenin

reduced (red) downstream transcriptional targets upregulated by β-catenin-TCF signaling and increased (green) transcriptional targets downregulated by β-catenin-TCF signaling. (I-J) Suppression of β-catenin induced expression of GUCA2A (I) mRNA and (J) protein. (K-L) A transgene containing DNTCF under the control of a doxycycline-inducible promoter in DLD1 cells[10, 44] inhibited TCF activity when treated with 1 μg/mL doxycycline. (M) Induction of DNTCF reduced (red) downstream transcriptional targets upregulated by β-catenin-TCF signaling and increased (green) transcriptional targets downregulated by β-catenin-TCF signaling. (N-O) DNTCF induced expression of GUCA2A (N) mRNA and (O) protein. (C, H, M) Gene expression normalized to non-induced cells and transformed to a $\log_2$ scale. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Figure 5. APC-β-catenin-TCF signaling suppresses the expression of new guanylin mRNA transcripts. (A) Schematic shows EU-incorporation into newly synthesized RNA, biotinylation by click-chemistry, and isolation with magnetic streptavidin beads. (B-D) Treatment with 300 μM zinc of HT29 cells carrying a transgene containing wild type APC under a zinc-inducible promoter[43] induced the accumulation of nascent guanylin mRNA transcripts labeled with EU. (E-G) Treatment with 1 μg/mL doxycycline of LS174T cells carrying a transgene containing an siRNA to β-catenin under the control of a doxycycline-inducible promoter[10, 44] induced the accumulation of nascent guanylin mRNA transcripts labeled with EU. (H-I) Treatment with 1 μg/mL doxycycline of DLD1 cells carrying a transgene containing DNTCF under the control of a doxycycline-inducible promoter[10, 44] induced the accumulation of nascent guanylin mRNA transcripts labeled with EU. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Figure 1

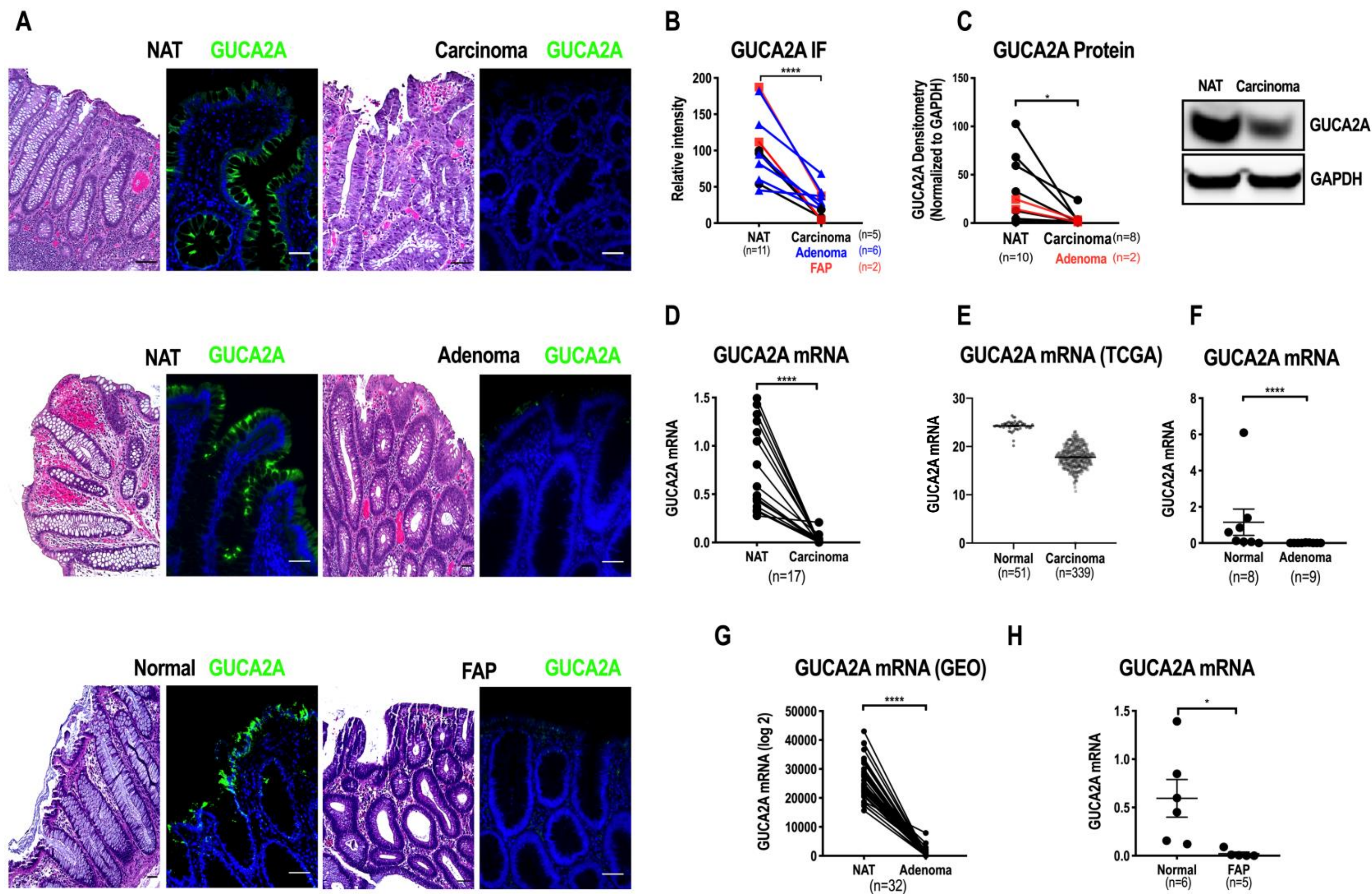


Figure 2

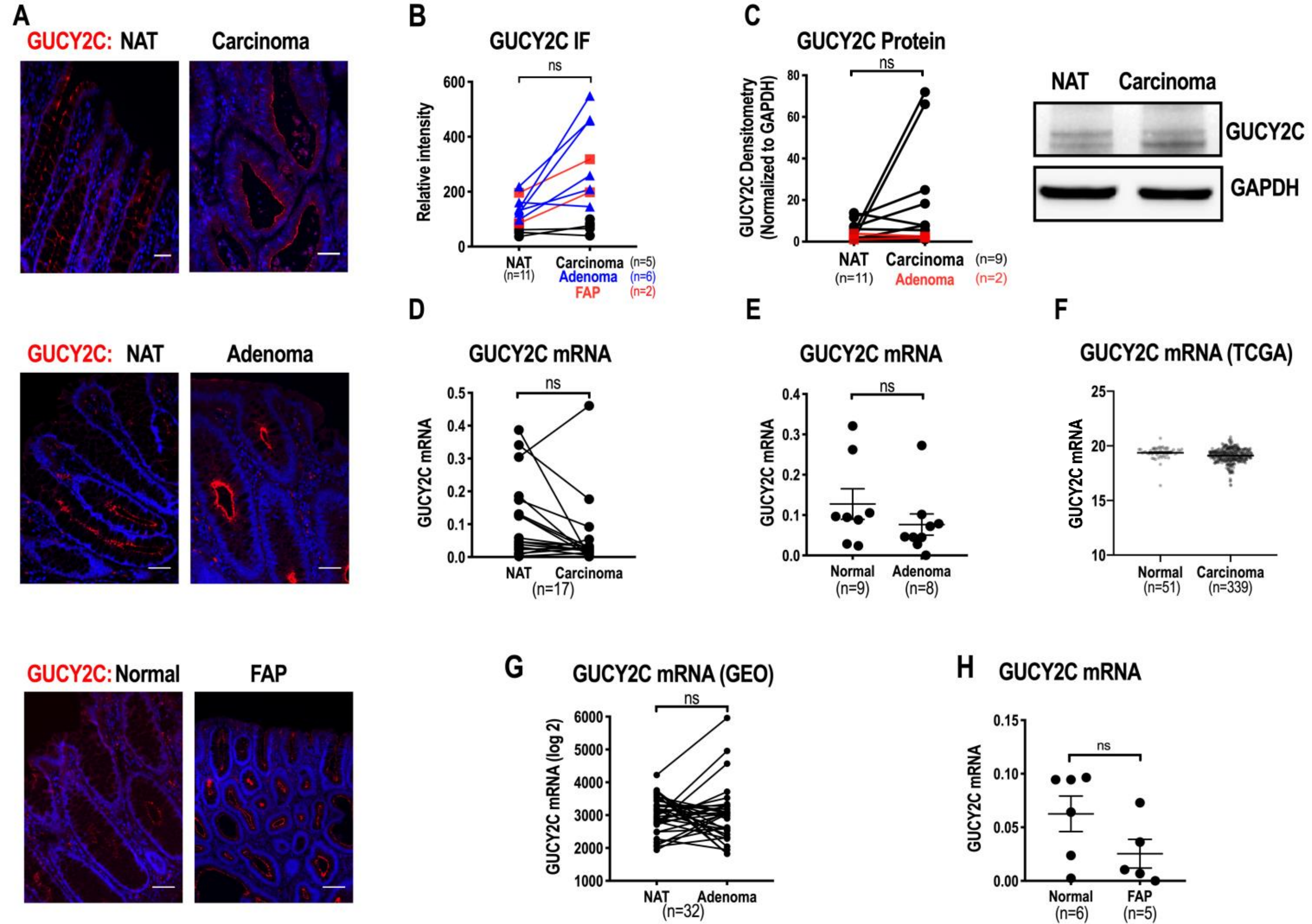


Figure 3

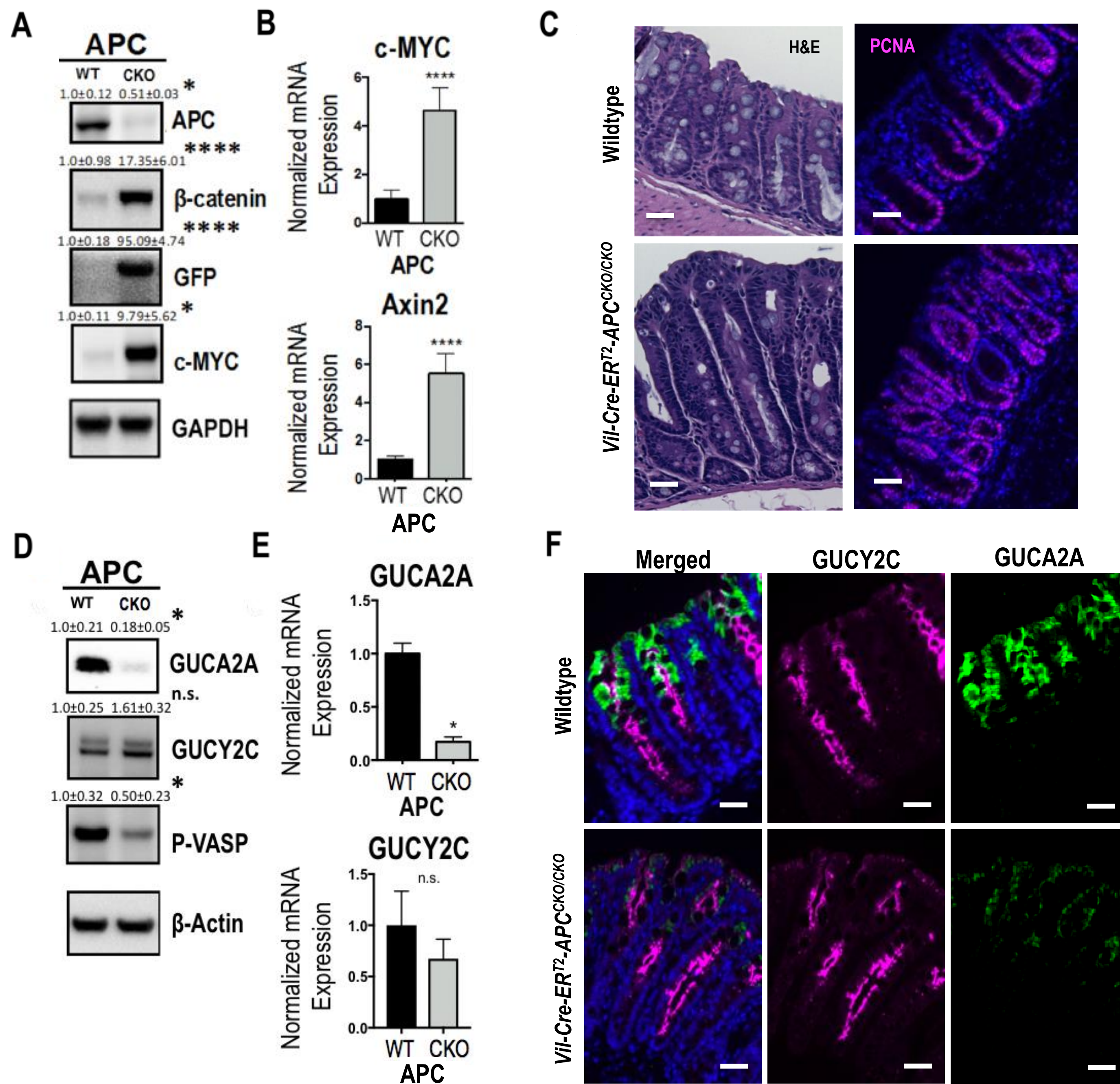


Figure 4

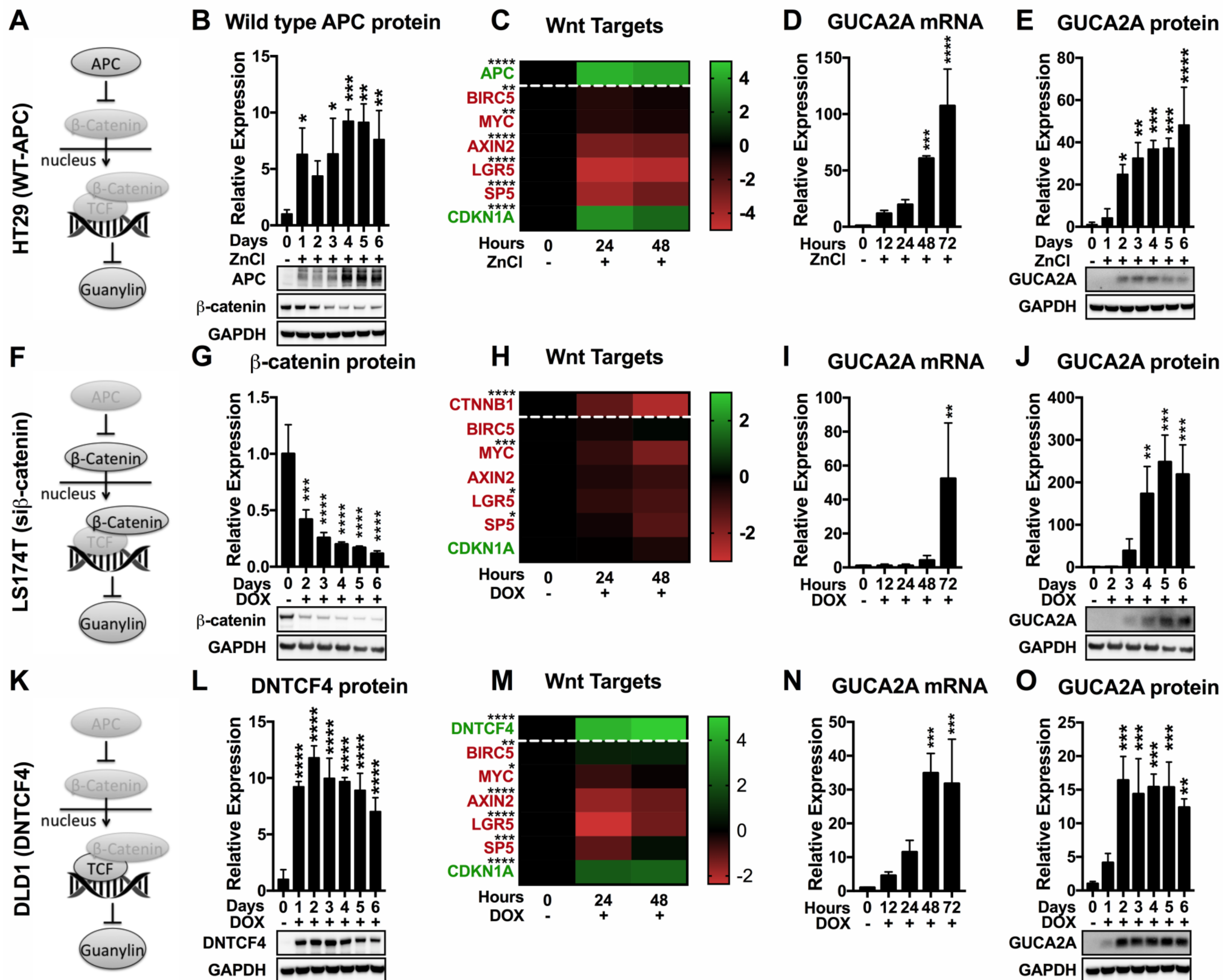
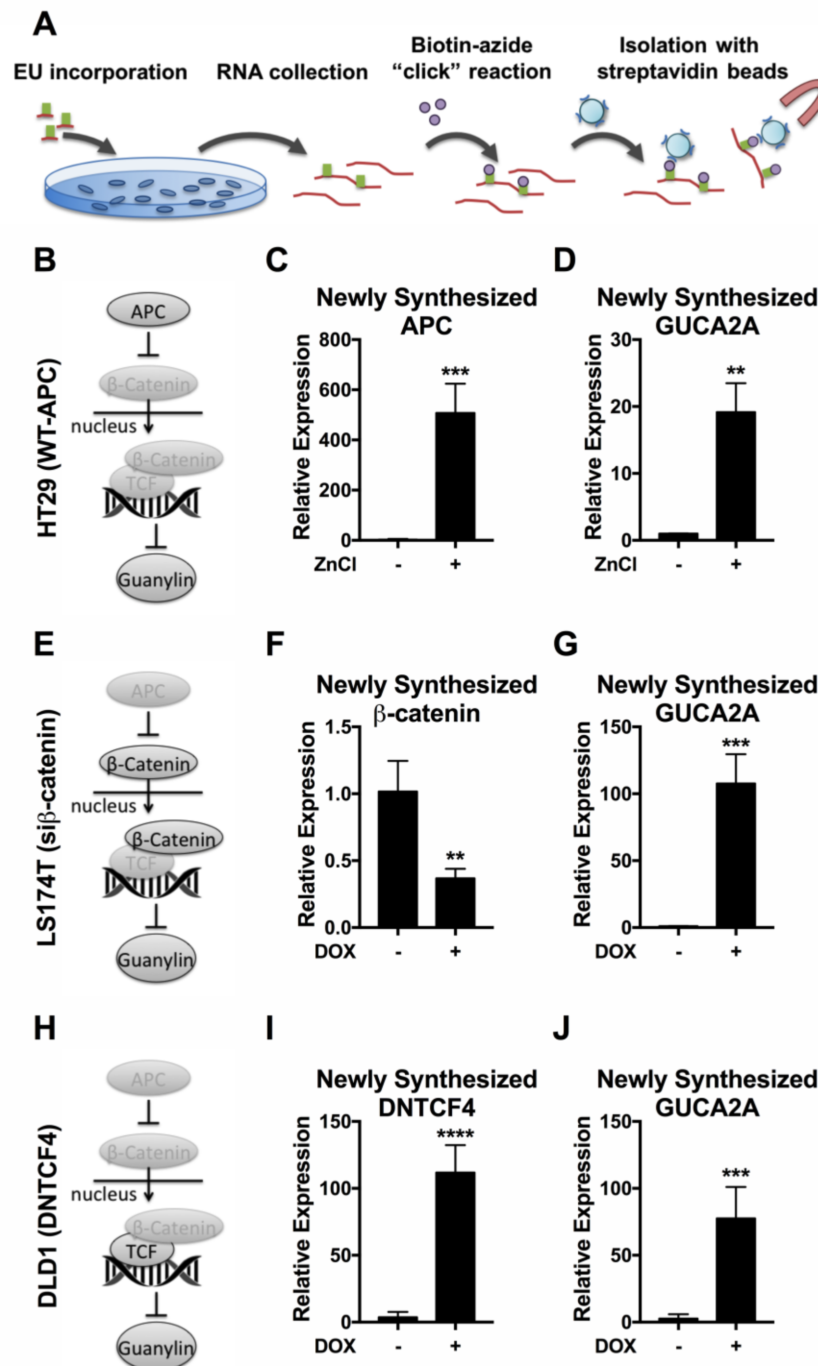


Figure 5



Supplementary Figures Legends

Supplementary Figure 1. GUCA2A expression in FAP patients. GUCA2A protein quantified by immunohistochemistry in FAP adenomas and matched normal adjacent mucosa (n=6).

Supplementary Figure 2. Time course of β-catenin degradation induced by wild type APC in HT29 cells. Treatment with 300 μM zinc of HT29 cells carrying a transgene containing wild type APC under the control of a zinc-inducible promoter induced degradation of β-catenin. **, p<0.01; ****, P<0.0001.

Supplementary Figure 3. Regulation of guanylin expression by DNTCF signaling in LS174T cells. (A-B) A transgene containing DNTCF under the control of a doxycycline-inducible promoter in LS174T cells[1,2] suppressed the accumulation of β-catenin when treated with 1 μg/mL doxycycline. (C) Induction of DNTCF reduced (red) downstream transcriptional targets upregulated by β-catenin-TCF signaling and increased (green) transcriptional targets downregulated by β-catenin-TCF signaling. Gene expression is normalized to non-induced cells and transformed to a log_2 scale. (D-E) DNTCF induced expression of GUCA2A (D) mRNA and (E) protein. **, p<0.01; ***, p<0.001; ****, P<0.0001.

Supplementary Figure 4. Time course of guanylin expression induced by DNTCF signaling in LS174T cells. Treatment with 1 μg/mL doxycycline of LS174T cells carrying a transgene containing DNTCF under the control of a doxycycline-inducible promoter[1,2] induced expression of DNTCF which preceded induction of GUCA2A mRNA expression. **, p<0.01; ****, P<0.0001.

Supplementary Figure 5. TCF4 and β-catenin binding is absent in the GUCA2A locus. (A) UCSC genome browser plots of SP5 (a canonical β-catenin/TCF4 target gene) and GUCA2A reveal

enrichment of β-catenin and TCF4 by ChIP-seq at the proximal promoter of SP5, and not GUCA2A, in wild type mouse intestinal crypts, similar to tissues explored herein (see Figure 3). Genome build mm9; GEO dataset GSE31939.[3,4] (B) In human colorectal cell lines, ChIP-seq of β-catenin in SW480 cells and TCF4 in HCT-116 cells reveals enrichment in the SP5, but not the GUCA2A, locus. Genome build hg19; GEO dataset GSE53927 (SW480 cells) and ENCODE transcription factor binding track (HCT-116 cells).[5,6] (C) Similarly, in LS180 human colon cancer cells, β-catenin and TCF4 are enriched at the SP5, but not the GUCA2A, locus. Genome build hg18; GEO dataset GSE31939.[7,8]

Supplementary Figure 6. β-catenin-TCF-dependent transformation is associated with loss of guanylin, but not the transcription factor HNF1α. (A) HNF1α mRNA expression quantified by RNASeq in adenocarcinomas (n=212) and normal mucosa (n=40) from human colorectum from the TCGA database. (B-C) Treatment with 1 μg/mL doxycycline of LS174T cells carrying a transgene containing an siRNA to β-catenin or DNTCF under the control of a doxycycline-inducible promoter induced the accumulation of guanylin protein, but did not affect levels of HNF1α. ****, $P<0.0001$.

Supplementary Figure Legend References

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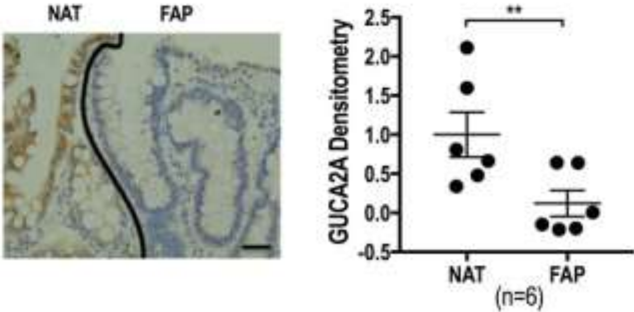
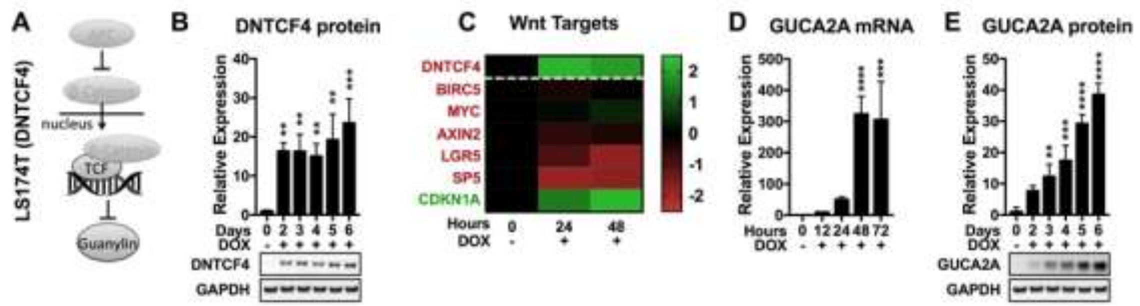
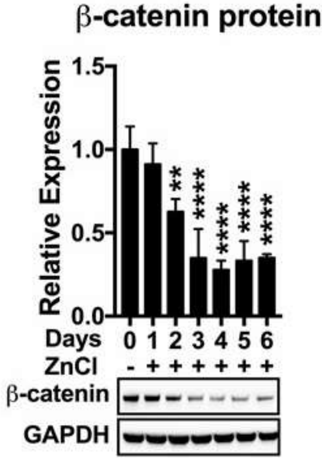
Supplementary Figure 1



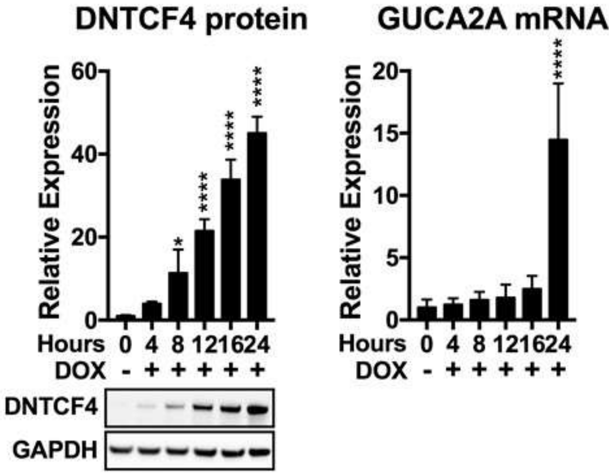
Supplementary Figure 2



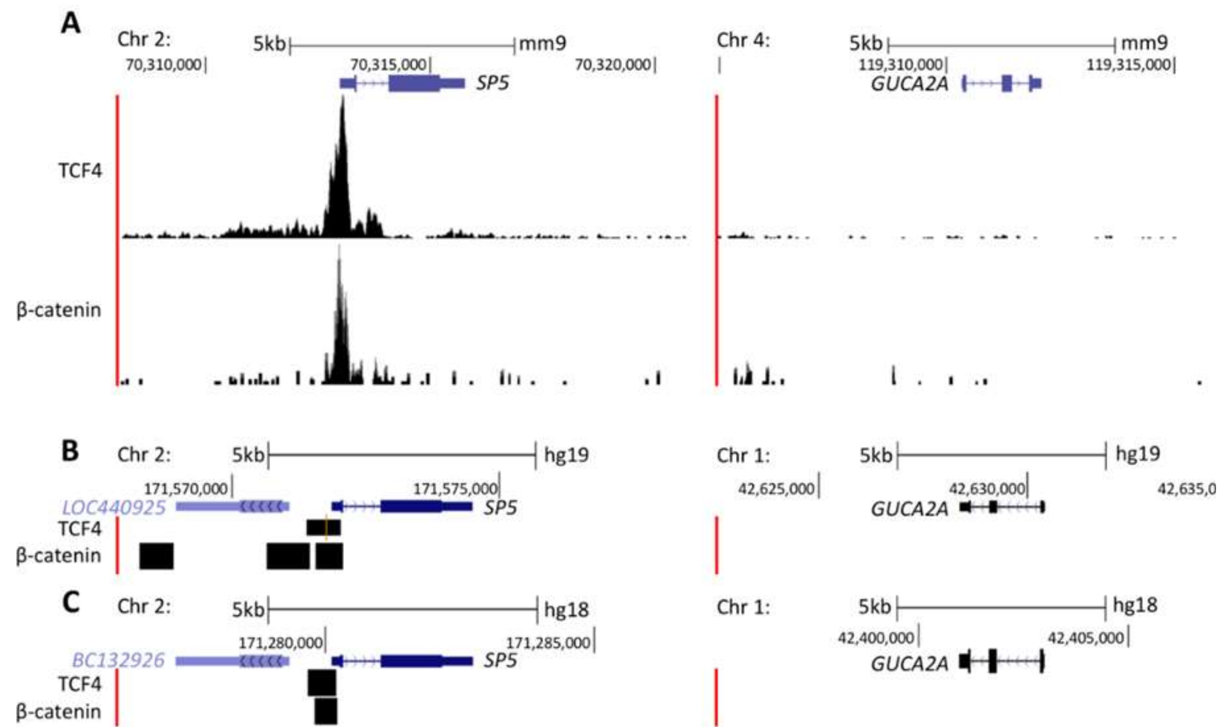
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6