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RESEARCH PAPER

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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GUCA2A; guanylyl cyclase C; colorectal cancer; transcriptional regulation; chemoprevention

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.

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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. Residual guanylin mRNA or protein in some tumors reflects incomplete dissection of associated normal mucosa from pathology specimens. 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,[1-5,7,40] compared to normal tissue in patients. Similar results were obtained with tissues in a GEO dataset (accession number GSE8671),[37-39] 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

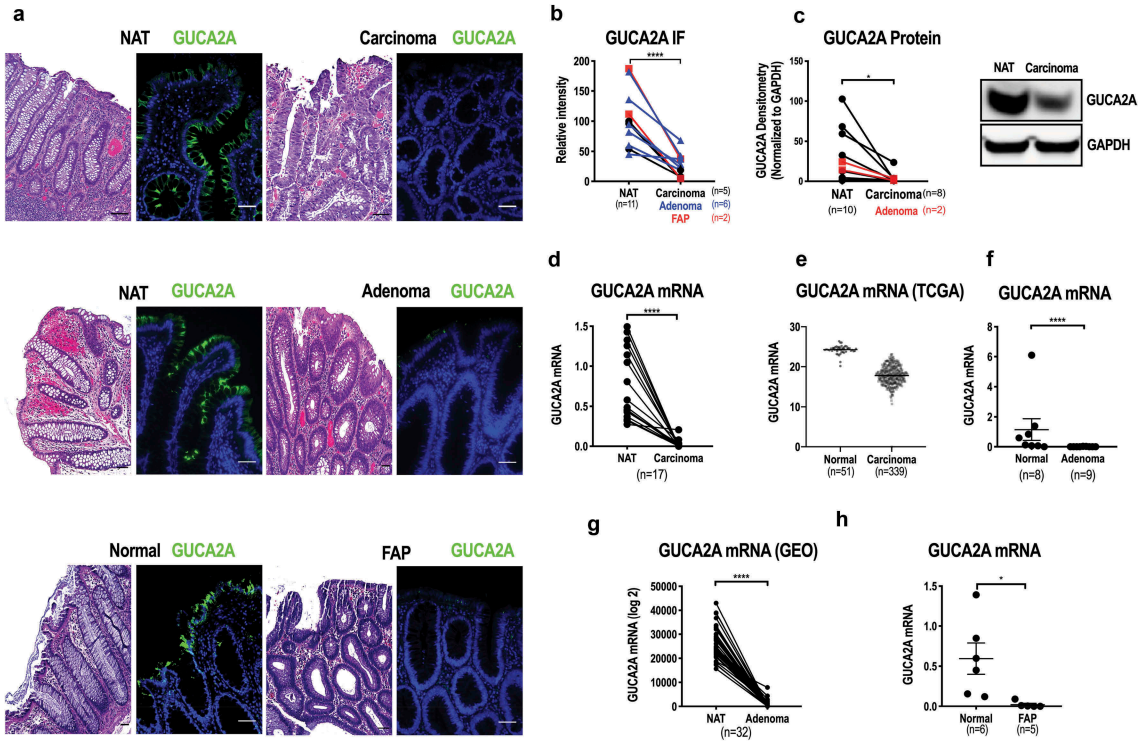

Figure 1. Loss of GUCA2A expression in tumors in human colorectum.

(a-c) GUCA2A protein quantified by (a-b) immunofluorescence or (c) immunoblot (a representative immunoblot is displayed) in adenocarcinomas (sporadic and FAP), 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).[37-39] (h) GUCA2A mRNA quantified by RT-PCR analysis in FAP adenomas (n = 5) and normal mucosa (n = 6). Green, GUCA2A; blue, DAPI. *, $p < .05$; **, $p < .01$; ***, $p < .001$, ****, $p < .0001$. Scale bar = 100 μm.

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),[37-39] 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,[1-5,7,40] 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

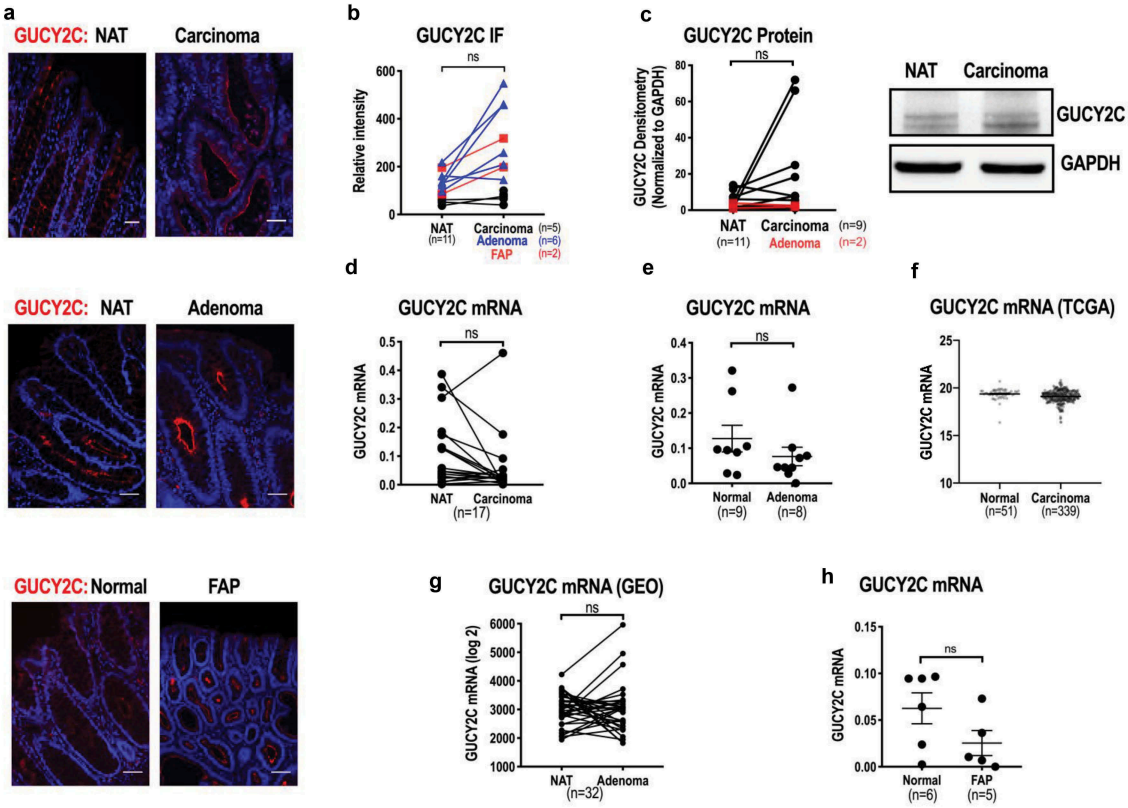

Figure 2. Retention of GUCY2C expression in tumors in human colorectum.

(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).[37-39] (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.

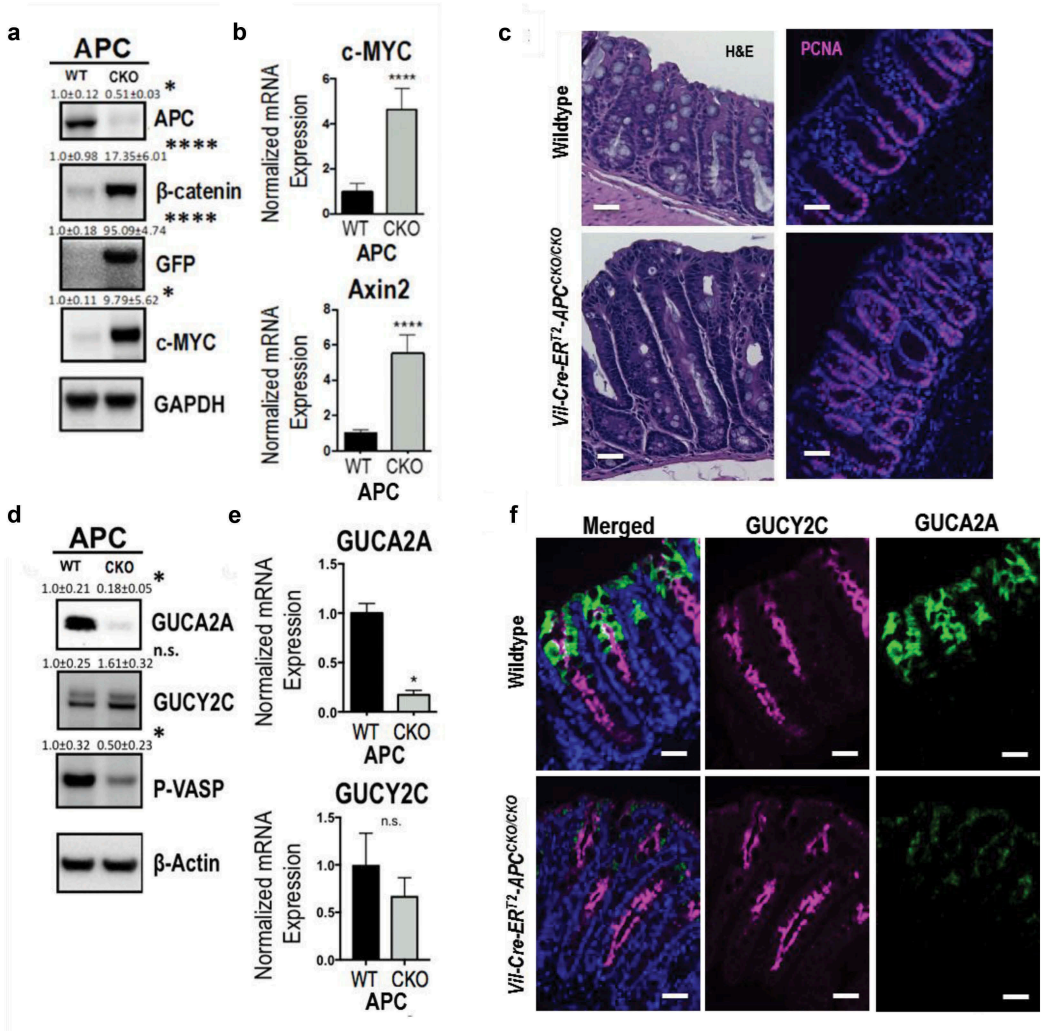

Figure 3. GUCA2A and GUCY2C in colonocytes following biallelic *Apc* in-activation in mice.

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; *, $p < .05$; ****, $p < .0001$. Scale bar = 100 μm.

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 reestablishes 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.

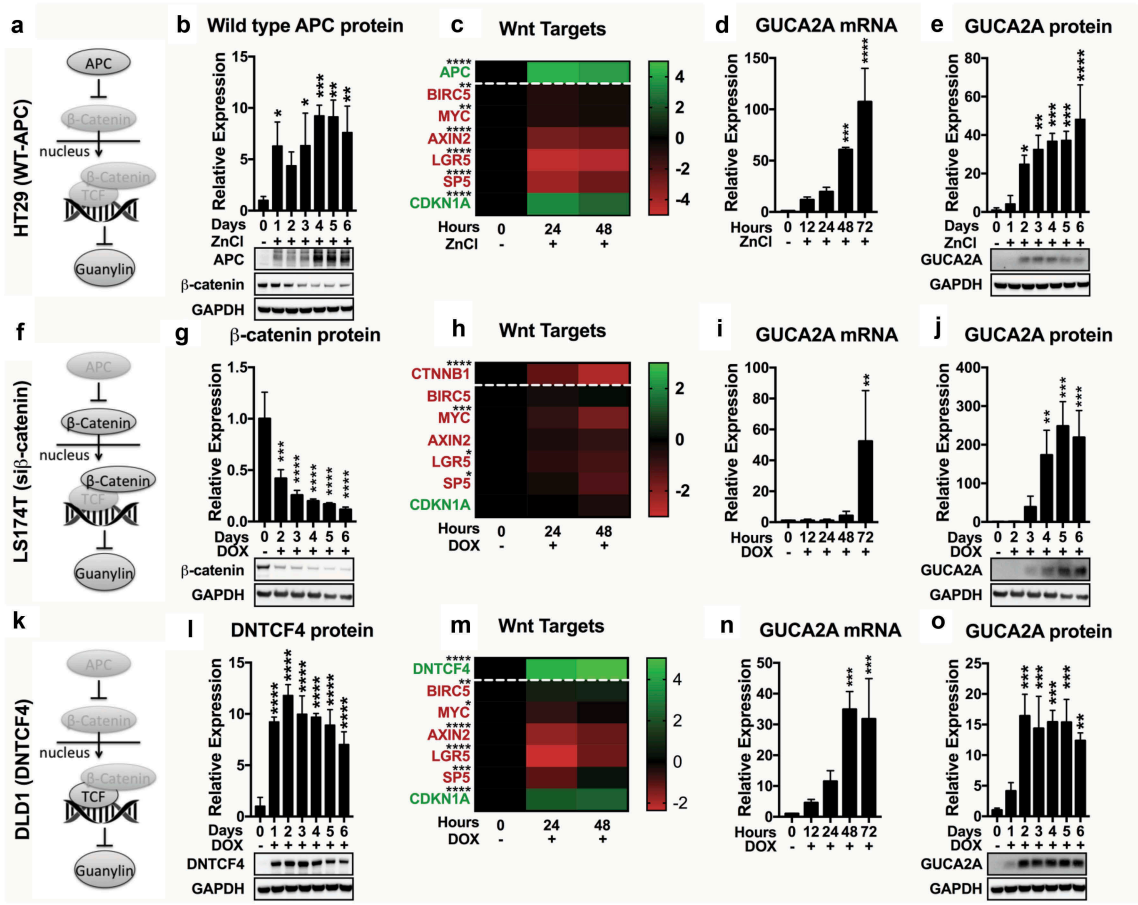

Figure 4. Regulation of GUCA2A expression by APC-β-catenin-TCF signaling in human colon cancer cells.

(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. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$.

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) reestablishes 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.[1-5,7,40] Mutations in APC typically result in loss of function, inactivating the multimeric protein complex targeting β-catenin for proteasomal

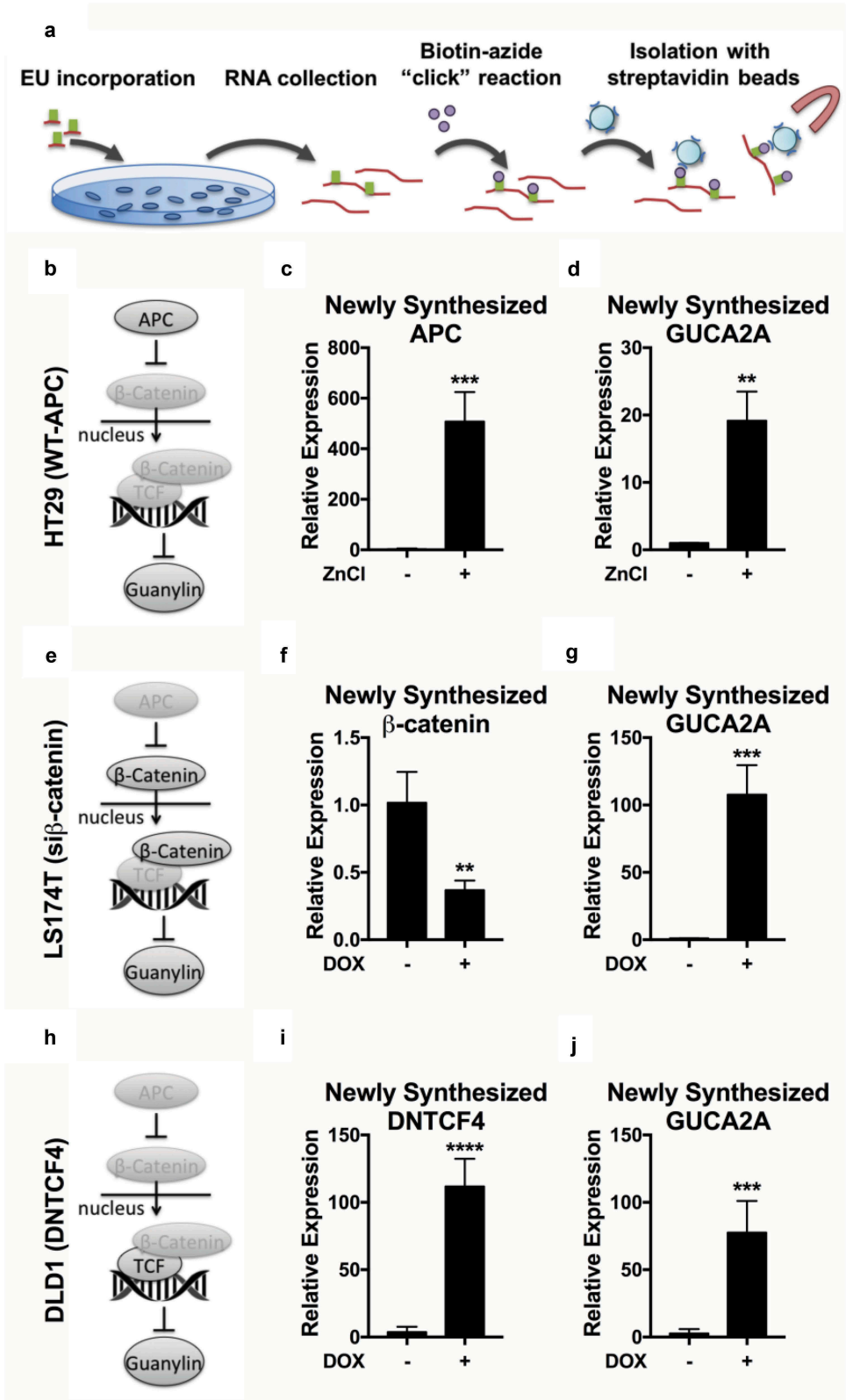

Figure 5. APC-β-catenin-TCF signaling suppresses the expression of new guanylin mRNA transcripts.

(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. **, $p < .01$; ***, $p < .001$; ****, $p < .0001$.

degradation.[1,2,4,7,8] In turn, β-catenin accumulates in the cytosol, translocates to the nucleus and binds to TCF, reprogramming 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 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 ± SEM of n = 3, unless otherwise indicated, and * $p < .05$, ** $p < .01$, *** $p < .001$, ****, $p < .0001$.

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Disclosure of Potential Conflicts of Interest

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