Genome-scale CRISPR-Cas9 screen of Wnt/β-catenin signaling identifies therapeutic targets for colorectal cancer

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Aberrant activation of Wnt/β-catenin pathway is a key driver of colorectal cancer (CRC) growth and of great therapeutic importance. In this study, we performed comprehensive CRISPR screens to interrogate the regulatory network of Wnt/β-catenin signaling in CRC cells. We found marked discrepancies between the artificial TOP reporter activity and β-catenin–mediated endogenous transcription and redundant roles of T cell factor/lymphoid enhancer factor transcription factors in transducing β-catenin signaling. Compiled functional genomic screens and network analysis revealed unique epigenetic regulators of β-catenin transcriptional output, including the histone lysine methyltransferase 2A oncoprotein (KMT2A/Mll1). Using an integrative epigenomic and transcriptional profiling approach, we show that KMT2A loss diminishes the binding of β-catenin to consensus DNA motifs and the transcription of β-catenin targets in CRC. These results suggest that KMT2A may be a promising target for CRCs and highlight the broader potential for exploiting epigenetic modulation as a therapeutic strategy for β-catenin–driven malignancies.

INTRODUCTION

Colorectal cancer (CRC) represents one of the major malignancies and a leading cause of cancer-related death worldwide. Aberrant Wnt/β-catenin pathway plays a pivotal role in colon carcinogenesis (1). Cytoplasmic β-catenin is phosphorylated by a protein complex containing adenosomatous polyposis coli (APC), AXIN1 or AXIN2, casein kinase 1α (CK1α), and glycogen synthase kinase-3β (GSK3β), leading to β-catenin destruction through ubiquitin-proteasome system. Wnt binding to the LDL receptor related protein 5/6 (LRP5/6)–frizzled receptors results in the disassembly of the β-catenin–destruction complex and consequent accumulation of β-catenin. β-Catenin then enters into the nucleus and binds to T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to initiate the transcription of β-catenin downstream targets (2).

Nearly all colorectal tumors (CRC) harbor genetic mutations that lead to the hyperactivation of β-catenin signaling (3). For example, germline or spontaneous mutations in tumor suppressor APC may cause constitutive activation of β-catenin in colon stem cells and the development of colonic polyps, which may eventually evolve into colorectal carcinomas (4). Hyperactivated β-catenin initiates the expression of various downstream targets through binding to the promoter regions via TCF/LEF transcription factors. Studies using transcriptomic approaches have characterized various β-catenin–responsive targets, such as cMYC, AXIN2, ASCL2, LGR5, and CD44 (5). Collectively, these targets promote proliferation (e.g., cMYC) and maintain a stem cell state (e.g., LGR5 and ASCL2), highlighting the potential value of developing treatments that target β-catenin signaling in cancer. However, β-catenin itself is an intractable drug target (6, 7). Moreover, the mechanisms underlying β-catenin–driven transcription remain largely elusive, underscoring the need to identify therapeutically tractable components of β-catenin transcriptional output.

Over the past decade, a number of functional genomic screens targeting the Wnt/β-catenin pathway have been performed using focused gene libraries or at whole-genome scale (8, 9). Aside from β-catenin itself, little overlap in screen hits has been reported among these different screens (8–12). This may be attributed to cell lineage/ type effects, the high rate of off-target effects observed with RNA interference (RNAi), and the use of artificial reporter systems (e.g., TOPFLASH) that may not recapitulate the physiological chromatin environment necessary for β-catenin transcriptional output. In the past few years, CRISPR-Cas9 technology has been repurposed for functional genomic screens and, based on its lower rate of off-target effects, has largely replaced RNAi technology (13). Moreover, CRISPR-Cas9 has permitted the facile and expedient generation of knock-in cell lines (14).

Here, we used CRISPR-Cas9–based, functional genomic approaches to generating colon cancer cell lines harboring β-catenin reporters at their endogenous genomic loci. We performed a genome-scale CRISPR-Cas9–mediated loss-of-function screening using an artificial TOP reporter and two β-catenin endogenous reporters. We identified notable differences in the transcriptional regulation among artificial and endogenous reporters. In particular, the transcription of endogenous reporters requires various epigenetic regulators that are dispensable for the artificial TOP reporter. We show that the use of endogenous reporters uncovered new targets of high relevance to the oncogenic property of β-catenin, compared with an artificial reporter system. We identified multiple hits from endogenous reporters and validated their ability to regulate oncogenic...
β-catenin transcriptional output. We validated the histone methyltransferase KMT2A/MLL1 (histone lysine methyltransferase 2A oncprotein/mixed-lineage leukemia 1) as a regulator of β-catenin chromatin occupancy and transcriptional output and demonstrated its selective dependence for growth in β-catenin–active CRC cells. Targeting KMT2A using KMT2A-menin inhibitors selectively reduced the viability of β-catenin–active cells and CRC organoids, but not β-catenin–inactive cells and normal organoids. Last, we show that KMT2A expression is associated with malignant CRC growth in vivo and shortened survival in patients with CRC. Our study provides critical insights into the mechanisms underlying β-catenin–mediated transcription and demonstrates the therapeutic promise of targeting epigenetic regulators in controlling oncogenic β-catenin activity.

RESULTS

Genome-wide CRISPR-Cas9 loss-of-function screening using an artificial TOP-dGFP reporter system

In the past decade, functional genomic strategies have been exploited in a number of studies to identify and investigate new Wnt/β-catenin regulators. We conducted a meta-analysis of five independent studies that exploited RNAi or gene-trap technologies to screen for regulators. We conducted a meta-analysis of five independent studies to identify and investigate new Wnt/β-catenin regulators. We hypothesized that bona fide negative regulator of the DLD1 TOP-GC reporter, as previously described using RNAi-based approaches (9). Contrary to their critical roles in DLD1 TOP-GC reporter activity, we unexpectedly found that ablation of TCF/LEF proteins, LEF1 and BCL9L, had no proliferation effect, while loss of TCF7L2 produced weak growth effects (Fig. 1F and fig. S1E). Cumulatively, these results reveal a shortcoming of the artificial TOP-based reporter in reflecting transcriptional activity limited to specific TCF/LEF1 family members.

Previous studies have yielded controversial results regarding the role of TCF4 in colon tumorigenesis (19, 20). To address this issue, we investigated the involvement of TCF/LEF transcription factors in the transcription of β-catenin endogenous targets. Ablation of any single TCF/LEF transcription factor did not markedly diminish the expression of well-documented β-catenin targets, such as cMYC and AXIN2 (Fig. 1G). However, when overexpressing a domain-negative TCF4 (dTCF4) that could not bind to β-catenin, the levels of β-catenin targets were strongly diminished, suggesting a redundancy among TCF/LEF factors in the transcription of β-catenin endogenous targets (fig. S2, A and B) (21). Hence, since TOP reporter and β-catenin endogenous targets are differentially regulated by TCF/LEF transcription factors, we suggest that β-catenin may drive the transcription of endogenous targets in a manner distinct from TOP reporter activity. These findings underscore the disconnection between artificial reporter readouts and biological output and highlight the need to use tools that accurately reflect physiologically relevant β-catenin transcriptional output.

Development and utilization of endogenous β-catenin reporters to identify transcriptional mediators of the Wnt/β-catenin pathway

We then set out to establish endogenous reporters of β-catenin by knock-in of cassettes expressing dGFP and red fluorescent protein (RFP) designated as GFP-low and GFP-high, respectively. Genes required for reporter activity were identified by analyzing sgRNA enrichment in the GFP-low and GFP-high sorted cells compared to the unsorted DLD1 TOP-GC samples. In parallel, to identify those genes that were also required for DLD1 growth, we continued culturing the other half of the DLD1 TOP-GC cells, up to 21 days after infection. For both FACS (7-day) and cell viability (21-day) analyses, we used the maximum likelihood estimation (MLE) algorithm in the MAGeCK-VISPR package to systematically analyze genes whose sgRNAs affected reporter and proliferation activity, respectively (table S1) (17). Notably, multiple known Wnt/β-catenin components, including β-catenin, AXIN2, TCF4 (encoded by the tcf7l2 gene), Lymphoid enhancer factor-1 (LEF1), B-cell CLL/lymphoma 9-like (BCL9L) and casein kinase 1 α (encoded by csnk1a1 gene), were identified as high-confidence hits in the DLD1 TOP-GC reporter cells (Fig. 1D). Likewise, similar results obtained from GFP-high samples also showed multiple core components of the Wnt/β-catenin pathway as top hits (fig. S1B). Overall, these screens identified 1097 putative regulators of β-catenin–dependent reporter activity, in which 497 genes were also required for DLD1 proliferation (Fig. 1E). sgRNAs targeting mediator complex subunits were enriched in the GFP-low population, consistent with previous findings showing a role for mediator complex subunit 12 (MED12) and cyclin-dependent kinase 8 (CDK8) in β-catenin–mediated transcription (fig. S1C) (8, 18). The ability of TCF/LEF transcription factors to regulate transcription from the DLD1 TOP-GC reporter was validated using FACS analysis (fig. S1D). TCF7L2, a well-documented TCF/LEF transcription factor, was identified as a bona fide negative regulator of the DLD1 TOP-GC reporter, as previously described using RNAi-based approaches (9).

We validated the histone methyltransferase KMT2A/MLL1 (histone lysine methyltransferase 2A oncprotein/mixed-lineage leukemia 1) as a regulator of β-catenin chromatin occupancy and transcriptional output and demonstrated its selective dependence for growth in β-catenin–active CRC cells. Targeting KMT2A using KMT2A-menin inhibitors selectively reduced the viability of β-catenin–active cells and CRC organoids, but not β-catenin–inactive cells and normal organoids. Last, we show that KMT2A expression is associated with malignant CRC growth in vivo and shortened survival in patients with CRC. Our study provides critical insights into the mechanisms underlying β-catenin–mediated transcription and demonstrates the therapeutic promise of targeting epigenetic regulators in controlling oncogenic β-catenin activity.

Development and utilization of endogenous β-catenin reporters to identify transcriptional mediators of the Wnt/β-catenin pathway

We then set out to establish endogenous reporters of β-catenin by knock-in of cassettes expressing dGFP and red fluorescent protein (RFP)
in frame with the terminal coding exons of two well-characterized direct β-catenin target genes, cMYC and AXIN2 at their endogenous loci (Fig. 2A). In these endogenous reporters, dGFP mRNA is serially transcribed with β-catenin endogenous targets, whereas RFP is constitutively expressed under the elongation factor 1α (EF1α) promoter. dGFP coding sequence is demarcated from the endogenous genetic sequence by a Thosea asigna virus 2A (T2A) cleavage peptide to ensure that the expression and function of endogenous proteins are not perturbed. Treating these reporter cell lines (DLD-1cMYC-R and DLD1 AXIN2-R) with β-catenin sgRNA confirmed that reporter activity was coupled with the expression of the gene of interest (Fig. 2B). Last, to permit the identification of target gene expression specifically in the context of β-catenin activity, we knocked-in the dGFP cassette into the cMYC locus in RKO cell line. RKOcMYC-R were validated using the bromodomain and extraterminal domain (BET) inhibitor, JQ-1, which has been previously demonstrated to inhibit
cMYC transcription in this cell line (22). As expected, RKO<sup>cMYC-R</sup> showed apparent and specific reduction in GFP levels after JQ-1 treatment (Fig. 2B).

We then used these three endogenous reporter cell lines (DLD1<sup>cMYC-R</sup>, DLD1<sup>AXIN2-R</sup>, and RKO<sup>cMYC-R</sup>) to perform a whole-genome CRISPR screen and identify genes required for both β-catenin target transcriptional output and growth. The CRISPR screen and computational analyses were conducted as described above for the TOP reporter (table S1). Confirming the validity of our approach, β-catenin scored as a top hit in both DLD1<sup>cMYC-R</sup> and DLD1<sup>AXIN2-R</sup>, but not in the RKO<sup>cMYC-R</sup> reporter (Fig. 2, C to E, and fig. S3A). In contrast, knockout (KO) of individual TCF/LEF family protein did not abrogate the activity of the endogenous reporters in both our CRISPR screens (Fig. 2, C and D) and single-gene KO analyses (fig. S3B). These findings provide a notable demonstration that β-catenin–mediated transcription of endogenous targets does not exclusively rely on individual TCF/LEF transcription factor.

To identify genes required specifically for target gene expression in the context of β-catenin activity, we performed a subtractive analysis of genetic regulators found in the context of the DLD1 (β-catenin–active) and RKO (β-catenin–inactive) reporters. We found 476 gene hits specific to the endogenous reporters in β-catenin–active cells, as compared to the β-catenin–inactive cells, RKO<sup>cMYC-R</sup> reporter. Of these, 72% (346 of 476) were also identified as proliferation hits at
day 21 of our screen and followed up for further analysis (Fig. 2F). We further compared the gene hits identified with the endogenous reporter with those found when using the artificial TOP-GC reporter system. Gene ontology pathway analysis showed that genes identified uniquely from the endogenous reporter systems, in contrast with the artificial specific reporter, were particularly enriched for epigenetic regulators, cell cycle, and G protein–coupled receptor pathway genes (fig. S3C). We also found that specific multicomponent complexes, such as transcriptional regulators, protein complexes involved in vesicular trafficking, and G2-M cell cycle transition, were exclusively enriched in endogenous reporters, but not in the DLD1TOP-GC reporter (Fig. 2G).

A number of genes, which comprised these enriched signatures, including E1A binding protein p300 (EP300), Polo-like kinase 1 (PLK1), CDC20, and Vacular H+–adenosine triphosphatases, have been previously implicated in the modulation of β-catenin transcriptional activity (23–26). This work highlights the unique genetic repertoire β-catenin–mediated transcription of endogenous targets work in a manner distinctly from the artificial TOP-GC reporter.

Development and screening of an epigenetic focused CRISPR library using artificial and endogenous β-catenin reporters

Epigenetic reprogramming plays an instrumental role in β-catenin–mediated transcription (27). We hypothesized that the endogenous reporter system would be particularly useful for interrogating epigenetic pathways and chromatin regulators that would not be found using the artificial reporter systems. As described above, our whole-genome CRISPR screen was enriched in genes involved in epigenetic regulation. To pursue this observation in a systematic and unbiased manner, we generated a deep coverage epigenetic-focused CRISPR KO library (EpiCK) containing 1251 genes with ~9 sgRNAs per gene (table S2). We performed and analyzed CRISPR screens on the reporters using the EpiCK library following the same approach as the whole-genome CRISPR screens, as described above (table S2). We found 55 genes that were necessary for β-catenin–mediated endogenous transcription. Notably, we found that the two endogenous reporters (DLD1cMYC-R and DLD1AXIN2-R) had clearly higher overlap with each other (39 hits exclusively identified) than with the TOP-GC reporter (1 and 3 hits for DLD1cMYC-R and DLD1AXIN2-R, respectively) (Fig. 3A). Similar results were found from our whole-genome CRISPR screen analysis, where gene hits overlapped tightly between the DLD1cMYC-R and DLD1AXIN2-R reporters (261 overlapping genes) compared with the TOP-GC reporter (48 and 23 overlapping genes for DLD1cMYC-R and DLD1AXIN2-R, respectively) (Fig. 3B).

The endogenous reporters in DLD1 cells displayed substantially more overlapping genetic hits with each other than other reporters across both the EpiCK and Brunello library screens (Fig. 3, A and B). This high similarity between DLD1AXIN2-R and DLD1cMYC-R reporters suggests that a number of transcription regulators that drive β-catenin endogenous targets are uniquely identified from endogenous reporter–specific hits. These findings imply that artificial and endogenous reporters, for the same pathway, are largely driven by different genetic regulators.

Validation of candidate Wnt/β-catenin pathway regulators identify KMT2A as a key player of β-catenin–mediated transcription

Candidate Wnt/β-catenin regulator genes from our whole-genome CRISPR and EpiCK screens were pursued for further validation. Inclusion criteria for selection were the ability to (i) regulate both endogenous reporters in DLD1, but not RKO cells, and (ii) dependence for proliferation in DLD1 cells. Further, we excluded genes that exhibited pan-essentiality in cell lines [using Broad Institute, Dependency Map (Depmap.org)] and genes that exhibited minimal to no expression [RNA-seq by Expectation Maximization (RSEM), <1] in colon cancer. Using these criteria, we first investigated the overlaps of epigenetic regulators identified from the Brunello and EpiCK libraries. Using this approach, four genes, including CTNNBI, KM-T2A, LAS1L, and SATB2, were identified as common hits in both the EpiCK and Brunello CRISPR screens (Fig. 3C). In addition, the section criteria enabled us to obtain a total of 39 candidate genes from the Brunello and EpiCK libraries, of which 22 were only identified in the endogenous reporters (fig. S4A). Candidate genes were validated, first, by testing their ability to regulate the two endogenous β-catenin reporter activities in DLD1 cells using two independent sgRNAs targeting each gene. Using this approach, we identified 26 genes that, when ablated, led to markedly decreased activities of both endogenous reporters [using a cutoff of 1.5-fold enrichment compared to nontargeting control (NTC)] (Fig. 3D and table S3). Candidate genetic regulators were then grouped into TOP-GC–dependent and TOP-GC–independent hits (fig. S4B). Notably, 70% of confirmed β-catenin transcriptional regulators were characterized as TOP-GC–independent hits. In contrast, TOP-GC–dependent hits were generally found to be nonspecific transcriptional/translational factors, such as ribosome and nucleolar proteins. We then filtered general factors from the candidate list and tested the ability of our top 11 candidates to directly regulate the transcription of β-catenin endogenous targets in two CRC cell lines, DLD1 and SW480. Gene expression analysis confirmed that ablation of most of the 11 candidate genes caused impaired expression of β-catenin target genes in both β-catenin–dependent CRC cells (Fig. 3E).

Notably, one of these genes, KMT2A (also known as Mll1), an oncogene in a subset of acute lymphoblastic leukemias, was identified as a consistent hit from both the epigenetic-focused and whole-genome CRISPR screens only when endogenous reporters were used (fig. S5, A and B). KMT2A is a histone lysine methyltransferase critical for the development and maintenance of hematopoietic stem cells. KMT2A is an oncogene in a subset of acute lymphoblastic leukemias marked by chromosomal rearrangements that lead to expression of a KMT2A fusion oncogene (28). To test more broadly the ability of KMT2A to regulate β-catenin–mediated proliferation and transcription, we ablated KMT2A expression in a panel of three β-catenin–active cell lines (DLD1, SW480, and SW480) and three β-catenin–inactive cell lines (RKO, A549, and MIA PaCa-2). Remarkably, we found that ablation of KMT2A selectively impaired the proliferation of β-catenin–active cells (Fig. 4A). Similarly, KMT2A depletion was found to inhibit DLD1 growth in a soft-agar colony formation assay. Restoration of KMT2A by ectopic expression rescued KMT2A sgRNA–mediated growth inhibition, highlighting that these effects are specific to KMT2A loss (Fig. 4B and fig. S5C). Consistent with the selective growth effects in β-catenin–active cell lines, the mRNA levels of β-catenin targets were uniquely down-regulated in β-catenin–active cells following KMT2A KO (Fig. 4C). Western blot analysis confirmed that ablation of KMT2A reduced cMYC expression in DLD1 and SW480 cells, but not in RKO cells (Fig. 4D). These findings explicitly demonstrate that KMT2A plays a crucial role in β-catenin–mediated transcription in CRC cells.
KMT2A ablation causes crypt outgrowth and proliferative impairment in neoplastic intestinal organoids

We further used intestinal organoids derived from Villin-Cre-ERT2;Apc\textsuperscript{fl/fl} mice to determine the role of KMT2A in Wnt-driven intestinal malignancy. We generated intestinal organoid lines isogenic for the presence of APC by tamoxifen [4-hydroxy-tamoxifen (4-OHT)]-induced, Cre-mediated deletion of the floxed Apc alleles. As expected, loss of Apc led to the transformation of enteroid-like normal organoids into spheroid-like tumorigenic organoids (fig. S6). To determine the consequence of KMT2A loss on normal and malignant intestinal homeostasis, β-catenin and KMT2A were ablated using CRISPR-Cas9 in the isogenic Apc\textsuperscript{WT} and Apc\textsuperscript{KO} intestinal organoids. Consistent with previous studies, loss of β-catenin resulted in a complete block in organoid growth. Depletion of KMT2A also led to growth effects in normal intestinal organoids, albeit not to the same severity as seen upon β-catenin KO (fig. S6, A and B). In contrast, deletion of Kmt2a in Apc\textsuperscript{KO} organoids led to a notable morphological metamorphosis characterized by de novo crypt formation, cytostasis, and architectural changes reflective of a prodifferentiation effect (fig. S6, C to E). These changes were also found in the Apc\textsuperscript{KO} upon β-catenin loss, suggesting that both Kmt2a and β-catenin are necessary for maintaining a dedifferentiated tumorigenic state in CRC. These findings show that loss of KMT2A phenocopies β-catenin in regulating properties of both stemness and growth in tumorigenic intestinal organoids.

Chromatin immunoprecipitation sequencing and RNA sequencing experiments confirm that ablation of KMT2A impairs the transcriptional activity of β-catenin in CRC cells

Previous studies have provided some evidence linking KMT2A to CRC proliferation and invasion, but the mechanism is not well defined...
We hypothesized that KMT2A may directly affect β-catenin binding or activity at promoter and enhancer elements. To test this possibility, we performed RNA sequencing (RNA-seq) in KMT2A-WT and KMT2A-KO CRC cells. In parallel, we used chromatin immunoprecipitation sequencing (ChIP-seq) to assess β-catenin binding and H3K4 trimethylation (H3K4me3) in the presence or absence of KMT2A. As expected, β-catenin binding overlapped with the H3K4me3 mark on the promoters of β-catenin targets (Fig. 5A and fig. S7A). Consistent with previous reports in other lineages, the level of H3K4me3 itself was largely unaffected by loss of KMT2A (Fig. 5A and fig. S7B) (30, 31). In line with these data, we found that overexpression of full-length KMT2A or its SET domain–deleted form (KMT2A-ΔSET) could both rescue the expression of β-catenin targets in KMT2A-KO cells, suggesting a dispensable role of KMT2A H3K4 methyltransferase activity in β-catenin–mediated transcription (fig. S7, C and D). H3K4me3 enrichment in transcription start site...
(TSS) regions has been shown to mark active promoters (32). Hence, we clustered β-catenin–binding peaks at −5 to 5 kb around TSS regions into H3K4me3-overlapping peaks and H3K4me3-nonoverlapping peaks. Notably, loss of KMT2A led to apparent loss of β-catenin binding at H3K4me3-overlapping peaks. In contrast, β-catenin–binding to H3K4me3-nonoverlapping peaks were not obviously perturbed following KMT2A depletion (Fig. 5B). Consistent with this, H3K4me3-overlapping β-catenin peaks have dramatically

Fig. 5. ChIP-seq and RNA-seq analyses indicated a critical involvement of KMT2A in β-catenin–mediated transcription. (A) Normalized ChIP-seq tracks of β-catenin and H3K4me3 at the loci of cMYC and ASCL2 in WT and KMT2A-KO CRC cells. (B) ChIP-seq profiles of β-catenin in WT and KMT2A-KO CRC cells around TSSs clustered by their overlapping with H3K4me3 peaks. (C) The charts show the proportion of up-regulated and down-regulated β-catenin peaks in KMT2A-KO groups in comparison to the NTC groups in DLD1 and SW480 cells. The differences were determined using a cutoff of 1.5-fold change of average peak value. (D) Volcano plot shows differential gene expression of KMT2A-KO cells compared with the NTC group. (E) Gene Set Enrichment Analysis (GSEA) analysis of down-regulated and up-regulated β-catenin target signatures in KMT2A-KO DLD1 cells. FDR, false discovery rate; NES, normalized enrichment score.
higher proportion of down-regulated peaks than the up-regulated ones, whereas H5K4me3-nonoverlapping peaks did not show this trend (Fig. 5C). In addition, we analyzed whether KMT2A may directly bind to the promoter regions of β-catenin targets. To this end, we performed ChIP–quantitative polymerase chain reaction (qPCR) to investigate the enrichments of β-catenin and KMT2A on cMYC promoter. We observed apparent and overlapping β-catenin and KMT2A binding across a number of sites proximal to the cMYC promoter. These data suggest that β-catenin and KMT2A localize to the same promoter regions to drive gene transcription, suggesting that KMT2A may directly participate in enabling the appropriate localization and/or binding of β-catenin to chromatin. In sum, these findings indicate that KMT2A plays an important role in facilitating β-catenin occupancy at H3K3me3-occupied active promoters.

To determine whether these effects were directly related to transcriptional outputs from the affected genomic targets, we assessed gene expression in CRC cells in the presence or absence of KMT2A by whole transcriptomic profiling (RNA-seq). Transcriptomic analysis showed down-regulation of a number of key β-catenin target genes, such as cMYC, LGR5, and ASCL2 in KMT2A-ablated CRC cells (Fig. 5D). Gene Set Enrichment Analysis (GSEA) using a previously published dataset of a β-catenin–regulated gene signatures (131 up-regulated and 204 down-regulated targets) in CRC revealed a significant enrichment, in both directions, with KMT2A-regulated gene signatures (Fig. 5E) (5). Consistent with a role of KMT2A in maintaining intestinal spheroid stemness, volcano plot and GSEA analysis both suggested that KMT2A deletion caused apparent down-regulation of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)α stemness signature and the up-regulation of Keratin 20 (KRT20)α differentiation signature (Fig. 5D and fig. S7F) (33). These confirm a critical role of KMT2A in regulating β-catenin targets in CRC cells and further implicate KMT2A as an important factor for the proper localization of β-catenin to proximal regulatory elements necessary to drive gene expression.

KMT2A inhibition selectively suppresses the malignant potential of β-catenin–active CRC cells

KMT2A regulates transcription as part of large macromolecular protein complex called COMPASS (complex of proteins associated with set 1). Within this context, KMT2A binds to menin that recruits KMT2A to its target genes. This interaction has been found to be critical for KMT2A-driven tumorigenesis in MLL fusion-driven leukemias (34, 35) and in the NPM1–mutated acute myeloid leukemia (AML) (36). To clarify the role of KMT2A-menin association in β-catenin–mediated transcription, we used a KMT2A N-terminal fragment (KMT2A-N) that has been reported to specifically block KMT2A-menin binding (37). Real-time (RT)–qPCR analysis revealed that overexpression of KMT2A-N impaired the expression of β-catenin targets in CRC cells (Fig. 6A). Western blot results confirmed that overexpression of KMT2A-N caused the down-regulation of cMYC protein, whereas β-catenin level was unchanged (Fig. 6B). Accordingly, KMT2A-N overexpression decreased the proliferation of CRC cells (Fig. 6C). These data support the notion that targeting the KMT2A-menin association may reduce β-catenin transcriptional activity and CRC proliferation.

Recently, a variety of potent chemical inhibitors have been developed that disrupt the KMT2A-menin interactions (38–40). We speculated that targeting KMT2A-menin association using chemical inhibitors may diminish β-catenin–driven transcription and CRC malignancy. To test this, we investigated whether KMT2A-menin inhibitors may selectively impair the growth of β-catenin–active CRC. We first used KMT2A-menin inhibitor MI-463 to assess its antitumoral effects on CRC cells. MI-463 exhibited significantly higher cytotoxic effects on β-catenin–active CRC cells, compared with β-catenin–inactive cells (Fig. 6D). RT-PCR analysis revealed that exposure to MI-436 significantly reduced the expression of cMYC in β-catenin–active CRC cells (Fig. 6E). We also found that MI-463 specifically suppressed the colony-forming capacities of β-catenin–active CRC cells (Fig. 6F). We further examined whether MI-463 may affect the growth of patient-derived colon cancer organoids harboring clinically relevant CRC-driver mutations (fig. S8). As predicted, exposure to MI-463 led to markedly reduced organoid formation (Fig. 6G). We also tested a newly developed KMT2A-menin inhibitor MI-1481, which shows one of the most potent inhibitory activities so far (40). Notably, CRC organoids were highly sensitive to MI-1481 exposure with median effective concentration (EC50) markedly lower as compared to a normal human colonic organoid model (Fig. 6, H and I). These findings suggest that the KMT2A-menin interaction is critical for β-catenin transcriptional output and highlight the therapeutic potential for KMT2A-menin inhibitors in β-catenin–active CRC.

KMT2A expression is associated with colon tumor growth in vivo and shortened survival of patients with CRC

KMT2A has been tightly implicated as an oncogene in human leukemia, but its role in maintaining solid-tumor growth in vivo has not been characterized. To address this, we used a doxycycline (Dox)–inducible short hairpin–mediated RNA (shRNA) system to knockdown KMT2A expression in established xenografted tumor models in the mouse. DLD1, SW480, and RKO cell lines carrying the Dox-inducible KMT2A shRNA were established and validated for knockdown of KMT2A and its cognate downstream target homeobox A9 (HOXA9) (fig. S9, A to C). Consistent with CRISPR-mediated KO effects, we found that RNAi-mediated depletion of KMT2A in DLD1 and SW480 cells, but not RKO cells, led to reduced proliferation (fig. S9D). To determine the effect of KMT2A knockdown on CRC tumor growth in vivo, we depleted KMT2A in established xenograft tumor models of β-catenin–dependent (DLD1 and SW480) and β-catenin–independent CRC cell lines (RKO cells). Consistent with our in vitro data, KMT2A depletion significantly retarded growth only in β-catenin–active CRC tumor models, but not β-catenin–inactive tumors (Fig. 7A). Consistent with this, the survival curves also showed that interference of KMT2A selectively extended the survival time of mice harboring β-catenin–active CRC tumor models (fig. S9E). Equivalent level of KMT2A depletion was observed in all three models by immunohistochemistry, confirming that differential activity observed was due to biological effects of KMT2A depletion (Fig. 7B). Collectively, these findings indicate that KMT2A is necessary for β-catenin–dependent tumor growth in vivo.

Last, we investigated KMT2A expression in CRC tumor specimens. Using a well-annotated tissue microarray of 128 patients with CRC, we scored KMT2A expression by immunohistochemical staining. We found that KMT2A was abundantly expressed in a large proportion of CRC tissues, whereas KMT2A expression was largely absent in normal intestinal epithelial cells (Fig. 7C). No significant difference of KMT2A expression was observed across different tumor stages (Fig. 7D). Moreover, high KMT2A expression correlated with worsened survival (Fig. 7E). Together, these findings demonstrate that KMT2A is abundantly expressed in...
the neoplastic colonic epithelium and may promote CRC growth in vivo.

**DISCUSSION**

Aberrant Wnt/β-catenin signaling plays a central role in the development of CRC (1). Although vigorous efforts have been made to interrogate the mechanisms whereby β-catenin drives colon tumorigenesis, much remains unclear with regard to its contribution to CRC initiation and progression. In particular, studies assessing multiple transcriptional cofactors of β-catenin have led to controversial results (20, 41). Here, through genome-scale screens using the artificial TOP-GC reporter and newly generated β-catenin endogenous reporters, we found notable differences in the transcriptional mechanisms among the artificial and endogenous reporters in CRC cells. Ablation of TCF/LEF transcriptional factors marginally affects β-catenin–mediated transcription of endogenous targets, despite their critical roles in the regulation of the artificial TOP-GC reporter. We identified multiple genetic hits that are relevant to β-catenin physiological function and characterized targeting KMT2A as a therapeutic strategy for β-catenin–driven CRC.

The artificial TOP reporter system is a widely used tool in the investigation of Wnt/β-catenin signaling, including genomic and drug screens (42, 43). However, the relevance of this reporter to the transcription of β-catenin endogenous targets remains to be determined. Several CRC cells harboring Wnt/β-catenin mutations and assumed to be β-catenin active, including HT29, LOVO, and LS411N, displayed TOP reporter activities comparable to β-catenin–inactive cells (44, 45). Our screening data and functional results both indicated that targeting TCF/LEF transcription factors only marginally affected the expression of most β-catenin targets and the proliferation of CRC cells. Notably, we found that a variety of genes with tumor-suppressive function in CRC, such as ARID1A and HUWE1, are specifically required for the TOP-GC reporter, but not endogenous
reporters. Moreover, our CRISPR screens identified a unique requirement of epigenetic regulation in β-catenin–mediated endogenous transcription. These findings indicate marked discrepancies between TOP-GC reporter activity and β-catenin oncogenic function. Overall, we speculate that the activity of TOP reporter system may be regulated distinctly from the transcription of β-catenin endogenous targets.

Because our investigation has explicitly indicated that β-catenin activates TOP reporter through recruiting transcription cofactors distinctly from those initiating the transcription of endogenous targets, we used β-catenin endogenous targets MYC and AXIN2 as reporters to perform genome-wide screen. This strategy enables us to identify multiple β-catenin transcription coactivators that have not been identified previously. For instance, we identified multiple subunits [activating signal cointegrator 1 complex subunit 3 (ASCC3), ASCC2 and thyroid hormone receptor interactor-4 (TRIP4)] of the activating signal cointegrator 1 (ASC-1) complex as the hits of the endogenous reporters. In addition, another hit, ring finger protein 113A (RNF113A), has been reportedly involved in the activation of this complex (46). ASC-1 complex was initially identified as a transcription coactivator of nuclear receptors (47). This complex functions as a DNA helicase, whose activity may be indispensable to gene

Fig. 7. KMT2A overexpression is associated with malignant tumor growth in vivo and shortened survival in patients with CRC. (A) Growth curves of shNTC and shKMT2A#1 DLD1, SW480, and RKO xenograft. Statistical analysis was conducted using two-way ANOVA test with Sidak adjustment for multiple comparisons. (B) Histological examination of CRC xenograft tumors using hematoxylin and eosin (H&E) staining and KMT2A immunohistochemistry. (C) Representative immunohistochemical (IHC) staining for KMT2A in nontumor colon and CRC specimens. The 0 to 3 scores represent negative, weak, modest, and strong expression. (D) The expression profile of KMT2A across different CRC stages. Difference was assessed using one-way ANOVA and Tukey’s post hoc test. (E) Kaplan-Meier survival curves using KMT2A H score demonstrate a worsened survival of patients with CRC with high KMT2A expression. Median KMT2A H score (49.02) was used as a cutoff to designate high- and low-expressing patients. Log-rank Mantel-Cox test was used to determine statistical difference.
transcription (48). In addition to ASC-1 complex, our screen also identified other novel β-catenin regulators, such as zinc finger CCHC-type containing 14 (ZCCHC14) and DEAH-box helicase 38 (DHX38). Another genomic screen identified ZCCHC14 as a critical regulator of hepatitis B surface antigen expression, suggesting a broad biology function of this protein (49). Some hits in our screen, such as special AT-rich sequence-binding protein 2 (SATB2) and ATPase H+ transporting accessory protein 1 (ATP6AP1), have been implicated in the transduction of Wnt/β-catenin pathway in previous studies (26, 50). Further work is required to explore the translational potential of these hits in CRC therapy.

Despite the importance of Wnt/β-catenin in CRC development, targeting β-catenin remains far from clinical success. Although hundreds of β-catenin–interacting partners have been characterized in the past several decades, the investigation of therapeutic targets selectively required for β-catenin–active CRC cells remains unsatisfactory. Many regulators of β-catenin, such as PLK1 and CK1α, are pan-essential for cancer cell lines. Our screening study identified a group of noncommonly essential genes that are specifically required for β-catenin–mediated endogenous transcription. In addition, we validated that targeting KMT2A may selectively impair β-catenin–mediated endogenous transcription and the growth of β-catenin–active cells. In addition to our study, recent work has implicated KMT2A not only in Wnt-driven tumor progression but also in tumor initiation (51, 52). KMT2A is a well-documented oncogene and therapeutic target in acute leukemia. Of great intrigue, studies also established a pivotal role of Wnt/β-catenin signaling in KMT2A/MII1-driven leukemias in terms of the establishment and maintenance of leukemic stem cells, suggesting a close interplay between β-catenin and KMT2A in tumorigenesis (53). Our work showing that KMT2A and β-catenin spatially colocalize at distinct chromatin sites and that loss of KMT2A hampers β-catenin binding at target sites suggests a direct role of KMT2A in mediating β-catenin transcriptional activity. Future proteomic analysis using proximity-labeling techniques (e.g., dCas9-APEX2 proximity labeling) would be of great interest to comprehensively interrogate the β-catenin interactome at the chromatin level of individual target gene loci.

Last, the ability of β-catenin and KMT2A to impinge on cancer stemness is of particular interest and highlights a genetic interaction between the two proteins. Deletion of KMT2A causes the loss of stem cell properties of tumorigenic organoids, characterized by the differentiation phenotype and reduced expression of LGR5, as confirmed by recent work (52). The transcriptional profile of KMT2A-ablated CRC cells also indicates a marked down-regulation of stem cell markers [LGR5 and achaete scute-like 2 (ASCL2)] and a concurrent up-regulation of epithelial differentiation markers (KRT20, KRT19, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and CDKN1A). Thus, KMT2A may serve as a druggable target against CRC stemness, whose elimination is of pivotal importance in preventing tumor recurrence and metastasis (54).

In summary, we performed genome-scale CRISPR-Cas9 screening of Wnt/β-catenin pathway in CRC and unexpectedly uncovered an apparent discrepancy between TOP reporter activity and β-catenin–mediated CRC progression. Given the widespread use of artificial signaling reporters, this finding has potentially broad implications in the field of cell biology. We also characterized KMT2A as a promising therapeutic target of β-catenin–active CRC and provide compelling evidence highlighting an important role for KMT2A in the context of CRC. At a translational level, our work showing that KMT2A is highly expressed across all stages of CRC is important. While our patient cohort size is limited to making a direct inference on the prognostic value of KMT2A expression in CRC, our findings are consistent with recent studies showing an unfavorable survival of patients with CRC with high KMT2A expression. While complete deletion of KMT2A in both normal and malignant intestinal organoids, we find that specific disruption of the KMT2A–menin interaction has distinct effects on the growth of human CRC organoids compared to normal colonic lines. This may imply that, unlike complete loss of KMT2A, inhibiting the KMT2A–menin interaction may be a therapeutically tractable strategy. Further studies will be required to translate these findings into targeted therapy in CRC.

**MATERIALS AND METHODS**

**Cell lines and organoid culture**

DLD1, SW480, RKO, SW48, MIA PaCa-2, A549, and 293T cells were obtained from American Type Culture Collection and cultured with Dulbecco’s modified Eagle medium (DMEM; Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Bovogen, Melbourne, Australia), 2 mM l-glutamine, and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37°C in a 5% CO2 humidified incubator. Cas9-expressing cells were generated by infection of Cas9-expressing lentivirus prepared with lentiCas9-Blast (Addgene, #52962).

Normal colon and CRC organoids were isolated from surgically resected tissues of patients diagnosed with stage II/III CRC, as described by Sato et al. (55). All samples were obtained from the Monash Medical Center (Clayton) with informed patient consents in accordance with the Declaration of Helsinki and approval by the Monash Health Human Research Ethics Committee (no. HREC/17/ MonH/119). The normal colon organoids were maintained in 500 μl of the IntestiCult Organoid Growth Medium (STEMCELL Technologies, Vancouver, Canada) supplemented with Primocin (200 μg/ml; InvivoGen, San Diego, CA, USA). CRC organoids was cultured with DMEM/F12 (Thermo Fisher Scientific) supplemented with B27 (Thermo Fisher Scientific, #17500-048) and N2 (Thermo Fisher Scientific, #17502-048) supplements, Primocin (100 μg/ml; InvivoGen, #ant-pm-1), recombinant human epidermal growth factor (EGF) (50 ng/μl; PeproTech, NJ, USA, #AF-100-15), 10 nM gastrin (Sigma-Aldrich, #G9145), 500 nM A83-01 (Tocris Bioscience, Bristol, UK, #2939), 1.25 mM N-acetylcysteine (Sigma-Aldrich, #A9165), 10 mM nicotinamide (Sigma-Aldrich, #N0636), 20% Noggin-conditioned medium, 10% R-Spondin1–conditioned medium, and 10% Wnt3A-conditioned medium. The conditioned medium was obtained as previously described (56). Three hundred organoids were seeded into 24-well plates and treated with KMT2A-menin inhibitors 24 hours later. The organoids were passaged weekly at a ratio of 1:4. After incubation for 21 days, organoid viability was assessed using the alamarBlue HS Kit (Thermo Fisher Scientific, #A50101).

**Generation of reporter cell lines**

A lentiviral 7× TOP-dGFP.mCherry vector (designated as TOP-GC; Addgene, #35491) was coinfected with pSAX2 (Addgene, #12260) and pMD2.G (Addgene, #12259) helper vectors into 293T cells to produce lentivirus (15). The viral juice was collected and used to infect Cas9-expressing DLD1 cells to establish DLD1TOP-GC reporter cells. After lentiviral infection, monoclones with both GFP and cas9 expression were selected using puromycin (500 μg/ml).

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mCherry fluorescence were selected and examined by their reporter responsiveness to β-catenin depletion using flow cytometry analysis.

Endogenous reporter vectors were constructed as follows. First, the targeting vector HR130PA-1 (System Biosciences) was modified to express a dGFP by incorporating the mouse ornithine decarboxylase PEST sequence. Homology arms were then designed, generated (gene sequencing, GENEWIZ), and cloned into the targeting vector such that they flank the terminal exon of the cMYC and AXIN2 genes to generate a gene-T2A-dGFP knock-in. gRNAs were designed to generate a cut site within 10 base pairs (bp) of the integration site and cloned into the lentiGuide-Puro plasmid (Addgene, #52963). The primers for constructing the reporters are shown in table S4. Colon cancer cell lines were transfected with a cocktail of three plasmids (targeting vector, gRNA plasmid, and Cas9 plasmid) at a 1:1:1 ratio using Lipofectamine 2000. After transfection, cells were briefly subjected to puromycin selection for 3 days and cultured for 1 week before sorting and clonal selection of GFP/RFP+ cells. Homologous recombination at the integration site was tested in single-cell clones by PCR at both the 5’ and 3’ integration sites and further confirmed by Sanger sequencing for both the cMYC and AXIN2 genes. Cell lines harboring homozygous integration were selected for further use. Before pooled CRISPR library screening, the reporter cell lines were infected with a lentivirus-expressing Cas9 (lentiCas9-Blast) and selected using blasticidin (5 μg/ml) for 10 days. Reporter cell lines were further treated with CRISPR RNAs (crRNAs): trans-activating crRNA (tracrRNA) duplexes (Integrated DNA Technologies, Singapore) targeting the puromycin-resistance gene embedded in the knocked-in targeting cassette. Clones with conferred puromycin sensitivity were pooled and expanded in preparation for pooled CRISPR screen. Before screening, the biological fidelity of the endogenous reporter cell lines was validated by ablation of β-catenin (using β-catenin–targeted sgRNA expressing lentiGuide-Puro lentiviruses) and testing reporter activity by flow cytometric analysis of GFP levels.

Epigenetic library design

Each gene in epigenetic library was selected on the basis of gene ontology including proteins involved directly or indirectly in chromatin modification and DNA methylation. Additional proteins involved in DNA damage response and repair were also included in the list. Together, 1251 genes were selected for targeting. For each gene, the corresponding sgRNA sequences were obtained from the sgRNA library constructed by Doench et al. (16). To further increase sgRNA coverage, additional sequences were also obtained from the sgRNA library constructed by Sanjana et al. (57), assuring that each sequence did not overlap with each other and did not contain Bsm BI restriction site. Pooled oligos for these sequences were ordered and synthesized from CustomArray (Bothell, WA, USA) with the following final sequences: 5′-AGGCACTGTGCTAGACGCCGTCTCAGACCGNG31GTITTCGAGACGTAAAGTGC-CCGGCCCCACAT-3′, where N20 represent sgRNA sequences. The pooled oligos were PCR-amplified, digested with Esp3I, and cloned into lentiGuide-Puro vector. Overall, our resulting epigenetic library contained 11,227 unique sgRNA sequences with 9 to 13 sgRNAs per gene.

CRISPR-Cas9 screen

Library amplification, lentiviral package, titration, and transduction were performed according to a standard protocol (58). Briefly, for Brunello library screening, 1.4 × 10^8 CRC reporter cells were infected with lentiviral sgRNA library at a multiplicity of infection of 0.3. Infected cells were selected with puromycin (2 μg/ml) at 24 hours after infection. At day 7, at least 4 × 10^7 cells were frozen as unsorted parental cells, and another 4 × 10^7 cells were subjected to FACS-sorted using a FACSAria Fusion sorter (BD Biosciences, San Jose, CA). A total of 4 × 10^7 cells were also collected for proliferation analysis at day 21 after infection.

Unsorted and sorted cells were subjected to genomic DNA extraction using the DNeasy Blood and Tissue Kit (QIAGEN, #69506). PCR amplification of DNA samples were carried out using a NEBNext high-fidelity 2× PCR master mix (New England Biolabs, #M0541L) as described previously. We amplified at least 253 and 68 μg of genomic DNA for unsorted Brunello and EpiCK cell samples, respectively. The DNA samples of all sorted samples were loaded for PCR amplification. The PCR products were separated and subjected to gel purification using the QIAquick Gel Extraction Kit (QIAGEN, #28704). Different barcoded samples were combined and sequenced using the Illumina NextSeq500 System. In the screening procedure, a representation of at least 500 and 1000 per sgRNA is achieved for Brunello library (4 × 10^7 representation) and EpiCK library (1.12 × 10^7 representation), respectively. Each screen has two independent replicates.

The sgRNA sequences were aligned to the library sequences using Bowtie. MAGeCK-VISPR MLE algorithm was used to systematically analyze the genes in both sgRNA enrichment data and proliferation data. The selection criteria for FACS validation are MAGeCK P < 0.1 in DLD1 endogenous reporters and DLD1 proliferation, MAGeCK P ≥ 0.1 in RKO-cMYC reporter, DepMap noncommon essential, and Cancer Cell Line Encyclopedia (CCLE) RSEM expression of >1. Common essential gene lists and CCLE expression data were downloaded in July 2019 from DepMap (https://depmap.org/portal/download/).

CRISPR-Cas9 KO, shRNA, and overexpression

The sgRNA constructs are obtained from Sanger Whole-Genome CRISPR-Arrayed Libraries, unless indicated. The sgRNA sequences are summarized in table S4. Plasmids were extracted and purified using the HP 96-Well Plasmid Miniprep Kit (Sigma-Aldrich, #N9604). sgRNA lentiviruses were generated by infecting 2.2 × 10^5 293T cells with 100 ng of sgRNA plasmid, 100 ng of psPAX2, and 10 ng of pMD2G using Lipofectamine 2000. Thirty-six hours after transfection, viral juice was collected and stored at −80°C. CRC cells (4 × 10^5) were plated into 6-cm dishes and added with 30 μl of viral juice in the presence of polybrene (5 μg/ml). Twenty-four hours after the infection, cells were selected with puromycin-containing medium (2 μg/ml).

KMT2A shRNA oligos (shKMT2A#1, 5′-GATTCGAACACCCAGTTATTC-3′; shKMT2A#2, 5′-TGCCAAGCACTGTGCAAATTA-3′) were subcloned into the pHUSH vector. The β-catenin shRNA construct was obtained from Addgene (#18803). The shRNA constructs (2 μg) were cotransfected with psPAX2 (1.5 μg) and pMD2.G (0.5 μg) helper vectors into a six-well 80 to 90% confluent 293T to produce shRNA-expressing viruses. CRC cells were infected with the viruses in the presence of polybrene (5 μg/ml) and subjected to puromycin selection to obtain stable shRNAexpressing cells.

The pLNCX-KMT2A and empty vectors were gifts from A. Yokoyama (National Cancer Center, Tsuruoka, Japan) (34). Control and KMT2A-KO CRC cells were transfected with empty pLNCX, pLNCX-KMT2A

helper vectors into 293T cells, followed by virus concentration using N. Subcloned into lentiGuide-Puro plasmid. sgRNA viruses were preconditioned medium [DMEM/F12 medium supplemented with 2% FBS and propidium iodide (PI) (1 μg/ml)]. The samples were analyzed using an LSRFortessa X20 cytometer (BD Biosciences) in 96-well plate high-throughput mode. The data were analyzed using FlowJo software (FlowJo, Ashland, OR). Cell debris, doublets, and PI+ cells are removed through gating. Cells are divided into GFP-high and GFP-low proportions through a threshold of 5% GFP-low in parental reporter cells. The sgRNAs targeting two genes (TRIM64B and OR5D16) that are not expressed in CRC cells served as negative controls.

Western blot analysis
Cell samples were lysed using radioimmunoprecipitation assay lysis buffer [20 mM Heps (pH 7.4), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and 1 mM EDTA] supplemented with 1× protease inhibitor cocktail (Roche), 10 mM NaF, 1 mM Na3 VO4, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, #23225). Protein samples were separated using NuPAGE 4 to 12% bis-tris (Thermo Fisher Scientific, #NP0321PK2) gel or 3 to 8% tris-aceitate (for KMT2A; Thermo Fisher Scientific, #EA0375PK2) gel and transferred into immobilon-FL polyvinylidene difluoride membranes. Membranes were blocked using insect-protcting blocking buffer (LI-COR) for 1 hour and incubated with the following primary antibodies: KMT2A (Santa Cruz Biotechnology, sc-374392), β-catenin (Cell Signaling Technology, #8480S), cMYC (Cell Signaling Technology, #5605S), and β-actin (Thermo Fisher Scientific, #MA5-15739-D800), overnight at 4°C. After incubation with dye-labeled Odyssey secondary antibodies for 1 hour, protein bands were visualized using the Odyssey Imaging System (LI-COR, Lincoln, NE).

Mouse organoid isogenic model
Mouse small intestinal organoids were isolated from Villin-CreERT2;APCfl/fl (APC5806 flox) mice. The organoids were maintained in organoid culture medium [DMEM/F12 medium supplemented with GlutaMAX, 20 mM Heps, 1% penicillin-streptomycin, N2 and B27 supplements, recombinant human EGF (50 ng/μl), 1.25 mM N-acetylcysteine, 10% Noggin-conditioned medium, and 5% R-Spondin1–conditioned medium] and passaged every 3 to 5 days.

For viral infection, sgRNA sequences (shown in table S4) were subcloned into lentIGuide-Puro plasmid. sgRNA viruses were prepared by cotransfecting sgRNA plasmid with psPAX2 and pMD2.G helper vectors into 293T cells, followed by virus concentration using the Lenti-X Concentrator (TaKaRa, #631231). Organoids were pretreated with 10 mM nicotinamide, 20 μM Rho kinase (ROCK) inhibitor Y-27632, and 10 μM GSK3β inhibitor CHIR99021 for 3 days. Thereafter, organoids were collected, briefly digested using TrypLE for 3 min at 37°C, and resuspended using organoid culture medium containing concentrated lentiviruses in 24-well plates. Following centrifugation at 800g for 90 min, the organoids were placed at 37°C for 4 hours, replated into Matrigel, and cultured in organoid culture medium supplemented with 10 μM Y-27632 and 10 μM CHIR99021 for 4 days. Twenty-four hours after infection, the organoids were selected using the corresponding antibodies for at least 7 days [blasticidin (8 μg/ml) for Cas9-blast and puromycin (1.6 μg/ml) for sgRNA viruses]. The organoids were first infected with Cas9-blast virus and then treated with or without 100 nM 4-OHT for 24 hours to establish APC-WT and APC-KO isogenic organoids. Following sgRNA lentivirus infection, the organoids were passaged, examined using a digital microscope, and subjected to 5-bromo-2′-deoxyuridine (Brdu) incorporation assay.

Brdu incubation assay
Brdu incorporation assay was performed using Abcam Brdu reagent (#ab142567) and anti-Brdu antibody (#ab6326) in accordance with the manufacturer’s protocol. Briefly, organoids were incubated with 10 μM Brdu in organoid culture medium for 4 hours at 37°C and subsequently fixed using 4% formaldehyde for 30 min. DNA hydrolysis was conducted through 40-min incubation with 1 M HCl, followed by 10-min incubation with 0.1 M sodium borate (pH 8.5). The organoids were then subjected to immunostaining using standard immunofluorescence protocol. The Brdu signals were stained using Alexa Fluor 488–conjugated goat anti-mouse immunoglobulin G (IgG; Thermo Fisher Scientific, #A28175), and cell nuclei were stained using 4′,6-diamidino-2-phenylindole (10 μg/ml). The immunostaining was examined using a DeltaVision Ultra widefield microscope (Applied Precision). Six random nonoverlapping fields were analyzed in each group.

Design and expression of KMT2A-N–truncated form
Codon-optimized sequence expressing 2 to 167 amino acids of KMT2A fragment (KMT2A-N), in conjunction with three repeat cMYC nuclear localization signal (NLS) sequence (PAAKRKVLK), Myc, and Flag epitopes, was custom-synthesized by Integrated DNA Technologies (Singapore) and subcloned into a pLVX-IRE-Neo vector. Lentiviruses were prepared by cotransfecting empty or KMT2A-N–expressing pLVX-IRE-Neo vectors with psPAX2 and pMD2.G helper vectors into 293T cells and subjected to CRC cell infection. Following G418 selection (800 μg/ml), cell samples were harvested and subjected to Western blot analysis at day 14 after infection. At day 9 after infection, cells were subjected to RNA isolation and RT-qPCR examination of β-catenin targets.

 Colony formation and soft agar assays
CRC cells were plated into six-well plates at a density of 500 cells per well. Twenty-four hours later, cells were incubated with KMT2A-menin inhibitor, and medium was changed for every 2 to 3 days. Two weeks later, cells were washed twice with phosphate-buffered saline, fixed for 30 min using methanol, and stained with 0.5% crystal violet. Colonies with at least 50 cells were counted.

DLD1 cells (1 × 104 per well) were seeded into a 12-well plate in DMEM supplemented with 3% Noble agar (BD Biosciences) and
RT-qPCR analysis
Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer’s protocol. A total of 100 ng of RNA was subjected to RT-qPCR analysis with the GoTaq 1-Step RT-qPCR System (Promega, #A6020). Alternatively, 1 μg of total RNA was reverse-transcribed into complementary DNA using SuperScript III (Thermo Fisher Scientific, #18080-085) and subjected to quantitative analysis using SYBR Green PCR Master Mix (Thermo Fisher Scientific, #4309155). All reactions were performed in triplicate using the Applied Biosystems QuantStudio 7 Flex Real-Time PCR System in a 384-well plate format. The primers are presented in table S4.

ChIP-seq, ChIP-qPCR, and RNA-seq
Cells were fixed with 1% formaldehyde for 10 min and quenched with 0.125 M glycine. Chromatin was sonicated using a Covaris ultra sonication device (Covaris, Woburn, MA, USA) to an average length of 500 bp. Precleared chromatin was immunoprecipitated overnight at 4°C with the following antibodies: rabbit anti–β-catenin (Cell Signaling Technology, #9581) and rabbit anti–H3K4me3 (Active Motif, #39159). Slurry of Magna ChIP Protein A+G Magnetic Beads (Merck) was subsequently added to the immunoprecipitated lysates and incubated for 2 hours at 4°C. Complexes were then washed and eluted from beads with SDS buffer. ChIP and input samples were subjected to ribonuclease and proteinase K treatment. Cross-links were reversed by incubation at 65°C, and DNA was purified. Illumina sequencing libraries were prepared from the ChIP and input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and input DNAs by the standard consecutive enzymatic steps of

Statistical analysis
Statistical analysis was conducted using GraphPad Prism 8. The respective statistical tests are disclosed in figure legends. All data are presented as means ± SD. Statistical differences are indicated as follows: ns (no significant) \( P > 0.05 \), *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \).


Acknowledgments: We thank H. Abud and J. Rosenbluh of Monash Biomedicine Discovery Institute, W. Herath, and M. Docanto for critical advices and technical assistance. We also appreciate A. Yokoyama of National Cancer Center, Tsrusoka for providing us with KMT2A constructs. Funding: This study was supported by NHMRC grant APP1144969, DOD Peer Reviewed Cancer Research Program (PRCRP) grant W81XWH-17-1-0553, the Victorian Government Operational Infrastructure Support Scheme, and ACS Mission Boost grant MBG-19-095-01-COLN. Author contributions: C.W., S.M., and R.F. conceived the study, performed experiments, analyzed data, and wrote the manuscript. A.D., H.K.C., D.X., Y.L., D.Z., and D.S. performed experiments and analyzed data. C.S. and J.B. analyzed the screen data and provided bioinformatic supports. T.C. and J.G. provided KMT2A-menin inhibitors and analyzed the data. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplemental Materials. Human CRC organoids, R-Spond1, Wnt3A, and Noggin-producing cells are available from R.F. under a material transfer agreement with Hudson Institute of Medical Research. ChiP-seq and RNA-seq data were deposited in NCBI GEO database under the accession number (GSE156083). The output of MAGeCK-VISPR and R codes to generate volcano plots can be found in https://github.com/ronfiresteinlab/ Brunello, BCAT. Additional data related to this paper may be requested from the authors.

Submitted 14 October 2020
Accepted 29 March 2021
Published 19 May 2021
10.1126/sciadv.abf2567

Genome-scale CRISPR-Cas9 screen of Wnt/β-catenin signaling identifies therapeutic targets for colorectal cancer
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Sci Adv 7 (21), eabf2567.
DOI: 10.1126/sciadv.abf2567

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