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Chromatin, Gene, and RNA Regulation

Molecular Cancer Research

A Protein Interaction between β-Catenin and Dnmt1 Regulates Wnt Signaling and DNA Methylation in Colorectal Cancer Cells

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Abstract

Aberrant activation of the Wnt signaling pathway is an important step in the initiation and progression of tumor development in diverse cancers. The central effector of canonical Wnt signaling, β-catenin (CTNNB1), is a multifunctional protein, and has been extensively studied with respect to its roles in cell–cell adhesion and in regulation of Wnt-driven transcription. Here, a novel mass spectrometry–based proteomics technique in colorectal cancer cells expressing stabilized β-catenin, was used to identify a protein–protein interaction between β-catenin and DNA methyltransferase I (Dnmt1) protein, the primary regulator of DNA methylation patterns in mammalian cells. Dnmt1 and β-catenin strongly colocalized in the nuclei of colorectal cancer cells, and the interaction is mediated by the central domain of the Dnmt1 protein. Dnmt1 protein abundance is dependent upon the levels of β-catenin, and is increased in cells expressing stabilized mutant β-catenin. Conversely, the Dnmt1 regulates the levels of nuclear β-catenin and β-catenin/TCF–driven transcription. In addition, lysine-specific demethylase 1 (LSD1/KDM1A), a regulator of DNMT1 stability, was identified as a component of the Dnmt1–β-catenin protein complex and perturbation of the Dnmt1–β-catenin interaction altered DNA methylation. In summary, a functional protein–protein interaction was identified between two critically important oncoproteins, in turn revealing a link between Wnt signaling and downstream nuclear functions mediated by Dnmt1.

Implications: Two critical oncoproteins, Dnmt1 and β-catenin, mutually regulate one each other's levels and activities in colorectal cancer cells. *Mol Cancer Res; 13(6); 969–81. ©2015 AACR.*

Introduction

Cancer cells typically exhibit complex, multilayered perturbations of signaling pathways and regulatory mechanisms (1). Dysregulation of the Wnt signaling pathway is a major factor in the initiation and progression of colorectal cancer. Canonical or β-catenin-dependent Wnt signaling is the best-defined branch of Wnt signaling, and is activated by binding of a Wnt ligand with specific cell surface receptor complexes. The subsequent signaling cascade leads to accumulation and nuclear translocation of β-catenin, the central effecter of canonical Wnt signaling. Activating mutations of β-catenin, which stabilize the protein, or loss of function of tumor suppressors such as adenomatous polyposis coli (APC), are causal events in the initiation of colorectal cancer (2, 3). β-Catenin is one of the best studied oncoproteins and diverse β-catenin protein–protein interactions have been identified. Recent studies, for example, have revealed the association of β-catenin with chromatin and epigenetic modifying complexes (4, 5), and it is clear that our knowledge of β-catenin protein function is quite incomplete.

In this study, we identify a protein–protein interaction between β-catenin and Dnmt1, the primary maintenance DNA methyltransferase in mammalian cells. Dnmt1 (Dnmt1 refers to the protein product of the DNMT1 gene) expression is altered in many different tumors (6–10), although somatic mutations of DNMT1 are relatively rare events in human cancers. Although best known for its role in DNA methylation, other functions have been defined for Dnmt1 protein, including the formation of transcriptional repressor complexes with HDAC and DMAP1 proteins (11) and with the LSD1 histone demethylase (12). In addition, many protein–protein interactions between Dnmt1 and transcription factors have been identified, principally mediated via the N-terminal regulatory region of the protein (13). DNMT1 itself is subject to multiple layers of regulation, including transcriptional (14) and posttranslational regulation through control of Dnmt1 protein stability (13, 15, 16). How these different mechanisms contribute to regulation of Dnmt1 (and DNA methyltransferase activities in general) in cancer cells is only partially understood. In addition, in many cancer cells aberrant expression of DNA methyltransferases occurs alongside dysregulated signal transduction pathways, but the extent to which signaling pathways regulate Dnmt1 and associated functions remains to be determined.

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Here, we use mass spectrometry, coimmunoprecipitation (co-IP), and confocal microscopy to identify and characterize the Dnmt1–β-catenin interaction. We show that the interaction is mediated by the central portion of the Dnmt1 protein and that Dnmt1 and β-catenin protein levels are mutually dependent. We show that Dnmt1 protein levels are responsive to exogenous Wnt activation that the response is not mediated via transcriptional mechanisms. Finally, to investigate the functional consequences of the Dnmt1–β-catenin interaction, we show that the interaction with Dnmt1 protein regulates both the level of β-catenin and β-catenin/TCF–driven transcriptional activity, and that CpG methylation of an imprinted locus is significantly reduced in cells lacking β-catenin. In summary, our study identifies a novel mechanism by which the levels of these two important oncoproteins are regulated in cancer cells and points to a regulatory link between Wnt signaling and epigenetic functions of Dnmt1.

Materials and Methods

Cell culture

Colorectal cancer cell lines RKO and HCT116 were maintained in McCoy-5A media (Life Technologies, 16600-108) containing 10% FBS (Life Technologies, 10438-026) and 1% streptomycin–penicillin (Life Technologies, 15140-148) at 37°C in CO_2 incubator (5% CO_2, 100% H_2O). The human embryonic kidney cell line HEK293T was maintained in DMEM media (Life Technologies, 11965-092) containing 10% FBS and 1% streptomycin–penicillin under the same conditions. For Wnt activation, media were removed and the cells were washed twice with serum-free McCoy5A media, and 1 mL serum-free McCoy5A media, added in each well of 6-well cell culture plate with purified Wnt3a protein (R&D Systems, Inc. 5036-WNP-010/CF) at the required final concentration of 30 ng/mL (17), and the cells were cultured for an additional 0.5, 1, 2, 3, 6, 12, or 24 hours before harvesting. Cells were harvested by scraping the cells off plates and then washed with cold PBS twice for immediate use or storage (−80°C). Knockout cell lines were provided by the respective laboratories in which they were generated and cultured under the same conditions as the parent cell lines (18, 19).

Protein extraction and quantification

Harvested cells were lysed in buffer (25 mmol/L Tris-HCl, pH7.4, 1 mmol/L EDTA, 150mmol/L NaCl, 1% NP-40, 50% glycerol, Protease inhibitor cocktail) by homogenization and incubated on ice for 30 minutes followed by centrifugation at 13,000 rpm for 30 minutes. Benzonase nuclease (Sigma E1014) was added to the lysis buffer as required and the supernatant (soluble fraction) kept for further analysis. Proteins were quantified by Bio-Rad protein assay dye (Bio-Rad 500-0006) at 595 nm.

RNA extraction and RT-PCR

Total RNA was extracted from Wnt3a stimulated HEK293T time course cells using the RNeasy Mini Kit (Qiagen, 74104) and 1 μg RNA was used in one step RT-PCRs using SuperScript One-Step RT-PCR with Platinum Taq (Life Technologies 10928-034) on DNMT1 with forward primer 5′-GTGGGGGACTGTGTCTC-TGT-3′and reverse primer 5′-TGCTGCCTTTGATGTAGTCG-3′, CTNNB1 with forward primer 5′-AAGCCTCTCGGTCTGTGG-3′ and reverse primer 5′-TGATGGTTCAGCCAAACGCT-3′, and GAPDH with forward primer 5′-CCGTCTAGAAAAACCTGCC -3′ and reverse primer 5′-GCCAAATTCGTTGTCATACC-3′ as loading control. One-step RT-PCR conditions were set according to the manufactory's protocol (Life Technologies 10928-034) with annealing temperature at 55°C. The amplified DNA products were then electrophoreed on 1.5% agarose gel containing SYBR Safe DNA gel stain (Life Technologies, S33102).

Flow-cytometry analysis

HEK293T cells at different time points (0, 0.5, 1, 2, 3, 6, 12, and 24 hours) after stimulation were collected, washed twice with ice-cold PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na_2HPO_4, 1.46 mmol/L KH_2PO_4), resuspended in 50 μL PBS buffer and fixed with 450 μL 100% Methanol in −20°C for at least 20 minutes. The cells were then treated with RNase (Life Technologies, 12091, Carlsbad, CA) in for 30 minutes before stained with 100 μg/mL propidium iodide and subsequently subjected to flow-cytometry analyzer (Beckman Coulter Epics XL flow cytometer). A minimum of 10,000 cells within the gated region were analyzed and data were captured and presented by Expo32 ADC Cytometry List Mode Data Acquisition & Analysis Software.

SDSPAGE and immunoblotting

Equal amounts (20 μg) of proteins from different samples were loaded on precast 4% and 12% Bis-Tris gel (Life Technologies NP-0335). Following electrophoresis, gels were either stained with Coomassie Brilliant Blue (Pierce 20278) or transferred to nitrocellulose membrane (Whatman 10402594). Western blotting was used to detect the protein with super signal ELISA Pico chemiluminescent substrate. Primary antibodies anti–β-catenin (Cell Signaling Technology 9581), anti–β-catenin (active; Cell Signaling Technology 8814), anti-Dnmt1 (Cell Signaling Technology 5119), and anti–α-tubulin (Cell Signaling Technology, Inc.) as loading control were applied at 1:1,000 and secondary antibodies horseradish peroxidase (HRP)–conjugated anti-mouse (Promega W4011) and HRP-conjugated anti-rabbit (Cell Signaling Technology 7074) were added at 1:20,000. Chemiluminescence detection using SuperSignal® ELISA Pico Chemiluminescent Substrate (Thermo Scientific PI-37070) was applied to all westerns. Bands were quantified by ImageJ (http://rsbweb.nih.gov/ij/; refs. 18, 19) and the mean values and SDs computed for three replicates.

Proteomic analysis

Standard in-gel tryptic digestion was performed according to the published method (20). The combined elution fractions were lyophilized in a SpeedVac Concentrator (Thermo Electron Corporation), resuspended in 100 μL of 0.1% formic acid and further cleaned up by reverse phase chromatography using C18 column (Harvard). The final volume was reduced to 10 μL by vacuum centrifugation and addition of 0.1% formic acid. Tryptic peptides were separated by online reverse phase nanoscale capillary liquid chromatography (nano-LC, Dionex Ultimate 3000 series HPLC system) coupled to electoral spray injection (ESI) tandem mass spectrometer (MS/MS) with octopole collision cell (Thermo-Finnegan LTQ Orbitrap). Loaded peptides were eluted on nano-LC with 90 minutes gradients ranging from 6% to 73% acetonitrile in 0.5% formic acid with a flow rate of 300 nL/min.

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Data-dependent acquisition was performed on the LTQ-Orbitrap using Xcalibur software (version2.0.6; Thermo Fisher Scientific) in the positive ion mode with a resolution of 60,000 at m/z range of 325.0 to 1,800.0, and using 35% normalized collision energy, up to five most intensive multiple charged ions were sequentially isolated, fragmented and further analyzed.

Mass spectrometry data processing

Raw LC-MS/MS data were processed using Mascot version 2.2.0 (Matrix Science). The sequence database was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 15 PPM. The raw data were searched against the human International Protein Index database (released in 2009 and containing 74,017 protein sequences) with fixed modification carbamidomethyl (C) and variable modification oxidation (M). The five fractions for each sample were combined as a single search in Mascot. Peptides were filtered at a significance threshold of $P < 0.05$ (Mascot). Scaffold (Proteome Software Inc.; version 3.00.04) was used to analyze LC-MS/MS–based peptide and protein identifications (21). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (22). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides (22).

Immunofluorescence

Cells were grown to 50% confluence on cover slip slides (12-545-80, Fisherbrand Cover Glasses) overnight and fixed first with 4% formaldehyde in PBS (phosphate Buffered Saline) for 15 minutes at room temperature. Fixed cells were blocked with blocking buffer composed of 1× PBS with 5% goat serum (Cell Signaling Technology 5425) and 0.3% Triton X-100 for 60 minutes at room temperature. Cells were then probed with an anti-FLAG (Sigma F1804) sera at dilution of 1:200 and β-catenin (D10A8) XP Rabbit antibody (Cell Signaling Technology 8480) at dilution of 1:100 for 2 hours at room temperature, washed with PBS buffer three times of 5 minutes each, then incubated with secondary Alexa Fluor 594 Goat Anti-Mouse IgG, highly cross-adsorbed antibody (Life Technologies A31624) and Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L), highly cross-adsorbed antibody (Life Technologies A11034) at a dilution of 1:1,000 for 30 minutes at room temperature in dark. Nuclei were stained with DAPI (Cell Signaling Technology 8961). Next, the Slides were rinsed with PBS and Prolong Gold Anti-Fade Reagent (Cell Signaling Technology 9071) was applied. Slides were then analyzed and images were taken on Leica TCS SP2 AOBS filter-free UV/spectral confocal laser scanner on an inverted DM IRE2 microscope.

RNAi

HCT116 and RKO cells were transfected with targeted siRNAs against DNMT1 and CTNNB1 using Lipofectamine 2000 transfection reagent (Life Technologies, cat no. 11668) and the cells were then incubated at 37ºC in CO_2 incubator and harvested 48 hours after transfection. Three gene unique 27mer siRNAs for each target were synthesized by Origene. The three target sequences of DNMT1are ACCAAAUUACGUAAAGAAGAAUUAT (SR301244A), AGCACAGAAGUCAACCCAAAGAUCT (SR301244B) and UGAGUGGAAAUUAAGACUUUAUGTA (SR301244C). The three target sequences of CTNNB1 are GGAUCACAAGAUGGAAUUUAUCAAA (SR301063A), CGCAUGGAAGAAAUAGUUGAAGGTT (SR301063B), and AGAAUUGAGUAAUGGUGUAGAACAC (SR301063C). Control siRNA (SR30004) is designed and provided by Origene.

LEF/TCF reporter assays

Targeted cells (HCT116, RKO, DNMT1^{KO}-HCT116, and CTNNB1^{KO}-HCT116) were seeded onto multiwall plates one day before transfection and grown to 70% and 90% confluence. Vectors of LEF/TCF reporter, negative control, and positive control were then individually premixed with transfection reagent Lipofectamine2000 (Life Technologies 11668) and Opti-MEM serum-free culture medium and incubated at room temperature for 20 minutes. For cotransfection with siRNA, three target sequences of either DNMT1 or CTNNB1 (see previous description in RNAi) were added into the premix and incubated at room temperature for 20 minutes before use. Luciferase assay was performed using Dual-Luciferase reporter assay system (Promega 1910) 48 hours after transfection. The cells were lysed in plate using passive lysis buffer (Promega 1941) for 15 minutes at room temperature with gentle shaking. Cell lysates were then transferred into a 96-well plate and development substrates were added according to the instructions (Promega 1910, Madison, WI). Firefly luciferase and Renilla luciferase activity were measure by microplate reader premium Quad4 Monochromators (Tecan Group Inc. Infinite M1000 pro). The promoter activity values were expressed as arbitrary units using a Renilla reporter for internal normalization according to the manufactory's protocol (Promega 1910). For experiments using TOPFlash/FOPFlash plasmids used were M50 and M51 Super 8x TopFLASH (Addgene; ref. 23). Experiments were done in triplicates for all biologic cell cultures/transfections and luminescent measurements. Mean (bar height) of relative luciferase units for each sample plus SD (error bar) were then calculated and plotted.

Quantification of protein half-life

HCT116, DNMT1^{KO}-HCT116, and CTNNB1^{KO}-HCT116 were grown in separate 6-well plates to log phase. Cycloheximide (Cell Signaling Technology 2112) was added to a final concentration of 50 μg/mL to terminate protein synthesis. After cycloheximide treatment, equal numbers of cells were collected at 0, 3, and 6 hours, and cell lysates were prepared as described. The lysates were analyzed by SDSPAGE and Western blotting. Bands corresponding to each protein were detected by using chemiluminescence and the intensity of bands was quantified using ImageJ. Half-life ($T_{1/2}$) was calculated as previously described (24).

Methylation analysis of H19 CpG islands

Genomic DNA was extracted with the QIAamp DNA Mini Kit (Qiagen 51804). Bisulfite treatment of the genomic DNA samples was carried out with the Qiagen EpiTect Kit (Qiagen 59104) according to the manufacturer's instructions, followed by PCR amplification with specific primers for H19 promoter region (forward: 5′-GGTCCCA/ideoxyU/ATGTAAGATTTTGGTGGAATAT-3′; reverse: 5′-GGCATAG/ideoxyU/ACAAACTCACACATCACAACC-3′). The PCR products were gel-purified, inserted into USER cloning vector zw102 (courtesy of Wang laboratory), and sequenced with T3 universal primer.

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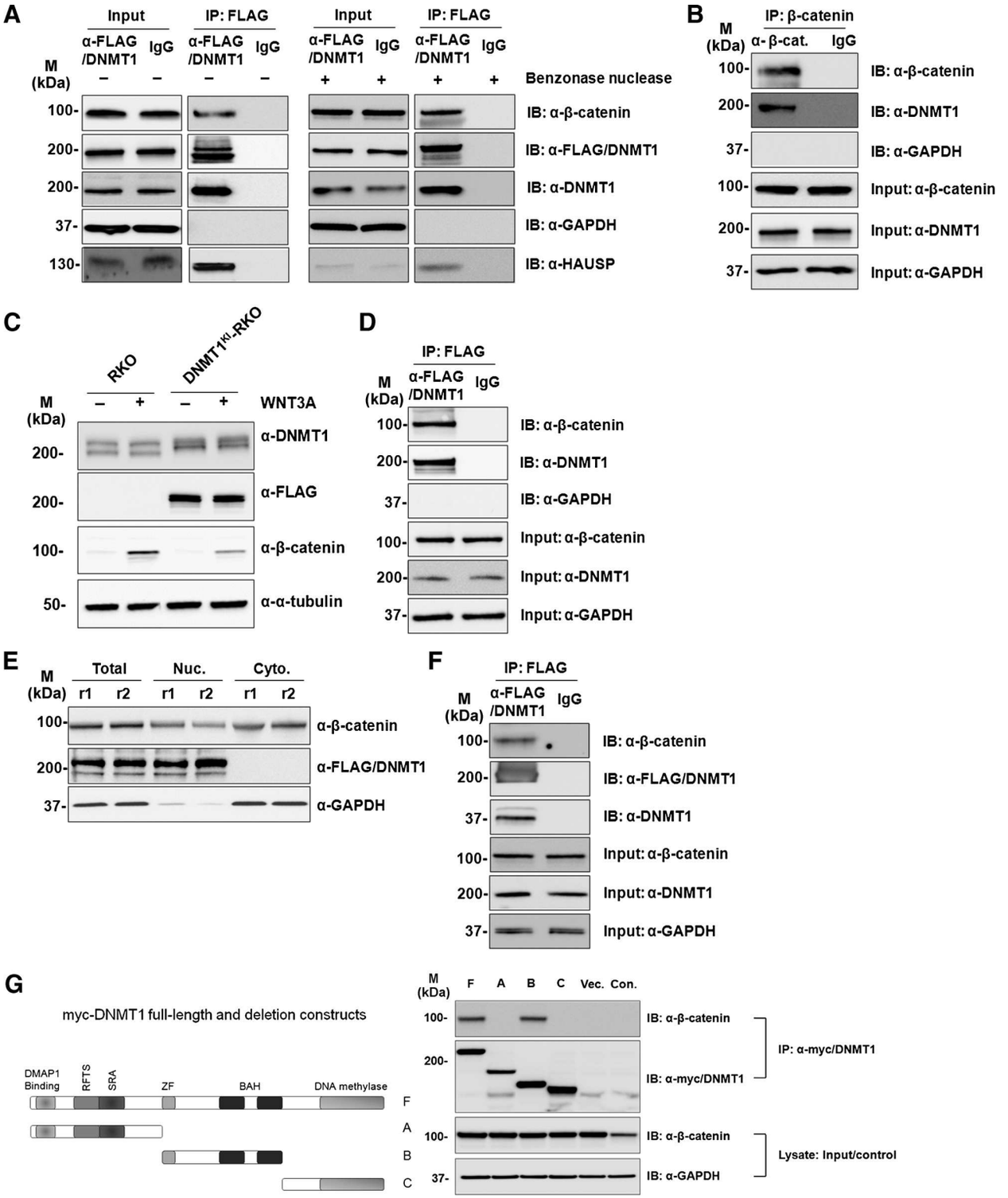

Figure 1.

Dnmt1 protein interacts with β-catenin in colorectal cancer cells. A, DNMT1 (FLAG) immunoprecipitates β-catenin in HCT116 cells. Total protein lysate from HCT116 with Benzonase untreated (−) or treated (+) was used in the immunoprecipitation and elution fractions were loaded on gel and blotted. B, β-catenin immunoprecipitates DNMT1 in HCT116 cells. C, β-catenin is induced by Wnt3a stimulation in both RKO and DNMT1^{KI}-RKO cells. D, Dnmt1 (anti-FLAG) immunoprecipitates β-catenin in DNMT1^{KI}-RKO cells. E, subcellular fractionation and protein expression levels inDNMT1^{KI}-HCT116 cells. F, Dnmt1 (anti-FLAG) immunoprecipitates β-catenin in nuclear fraction of DNMT1^{KI}-HCT116 cells. Twenty micrograms of proteins from each fraction was loaded on SDSPAGE followed by Western blot analyses with anti-Dnmt1, anti-FLAG, anti–β-catenin, anti-HAUSP, anti–α-tubulin, and anti-GAPDH. (*Continued on the following page.*)

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Results

A Dnmt1–β-catenin protein–protein interaction

We previously performed a large-scale proteomics study to map the Wnt-responsive proteome (17). Proteins were identified whose expression levels responded to exogenous Wnt3A stimulation and we observed that Dnmt1 protein levels exhibited a robust response to Wnt activation in HEK293T cells and in the colorectal cancer cell-lines, RKO, and HCT116. In this study, we used a novel affinity purification mass spectrometry (AP-MS) technique previously applied by us to identify Dnmt1 protein–protein interactions (15, 25). We introduced a 3xFLAG tag into the DNMT1 locus in HCT116 cells (DNMT1^{KI}-HCT116), expressing a stabilized mutant β-catenin (i.e., with constitutive activation of Wnt signaling). The DNMT1^{KI}-HCT116 cells were then used in AP-MS experiments as previously described (25). We filtered the dataset to remove contaminants and nonspecific binding proteins (26, 27, and then analyzed the identified peptides and proteins (Supplementary Table S1). Peptides corresponding to β-catenin (2 distinct peptides) were identified suggesting that Dnmt1 protein and β-catenin interact in HCT116 cells.

To validate the association between β-catenin and Dnmt1 proteins, co-IP experiments were performed. First, anti-FLAG immunoprecipitates from DNMT1^{KI}-HCT116 cells were analyzed as shown in Fig. 1A. To assess whether the β-catenin–Dnmt1 interaction is chromatin-dependent, we performed the co-IP experiments with and without the Benzonase nuclease (Supplementary Fig. S2). No differences were observed in the Dnmt1 signal indicating a soluble β-catenin–Dnmt1 protein complex. Western blots were also analyzed with anti-HAUSP/USP7 antibodies as a positive control, because USP7 has been shown previously to be associated with Dnmt1 protein complexes (15, 28). A co-IP experiment using native anti-Dnmt1 antibodies (instead of anti-FLAG) in parent HCT116 cells was also performed, showing the same result (Supplementary Fig. S3) as the anti-FLAG experiments. We next performed the reciprocal co-IP experiment from DNMT1^{KI}-HCT116 cells using native anti–β-catenin antibodies and observed Dnmt1 proteins in these samples (Fig. 1B), confirming the association of β-catenin and Dnmt1.

We previously performed AP-MS experiments on RKO cells with FLAG-tagged DNMT1 (DNMT1^{KI}-RKO; refs. 15, 25), but did not identify β-catenin peptides in these experiments. We reasoned, however, that because endogenous β-catenin levels are significantly lower in RKO cells than in HCT116 cells, exogenous activation of Wnt signaling in RKO cells might allow detection of the β-catenin–Dnmt1 interaction in RKO cells. We therefore treated DNMT1^{KI}-RKO cells with Wnt3a before Western and co-IP analysis as shown in Fig. 1C and D. Wnt3a treatment of DNMT1^{KI}-RKO cells increases β-catenin levels and β-catenin is detected in anti-FLAG immunoprecipitates from DNMT1^{KI}-RKO cells. We also analyzed nuclear and cytosolic subcellular fractions from DNMT1^{KI}-HCT116 cells. Dnmt1 protein is strongly localized to the nucleus of DNMT1^{KI}-HCT116 cells (Fig. 1E) and anti-FLAG IP from nuclear fractions identifies β-catenin (Fig. 1F).

To map which regions of Dnmt1 protein are necessary for the interaction with β-catenin, Myc-tagged deletion constructs of Dnmt1 protein were constructed as shown in Fig. 1G. These were expressed in HEK293 cells and immunoprecipitated for Western analysis with anti–β-catenin antibodies. As shown in Fig. 1G, β-catenin is only detected in association with full-length Dnmt1 protein or with the central region of the protein encompassing the zinc finger and two BAH (Bromo-adjacent homology) domains. Thus, neither the N-terminal region nor the C-terminal DNA methylase catalytic domains are necessary for the interaction of Dnmt1 protein with β-catenin.

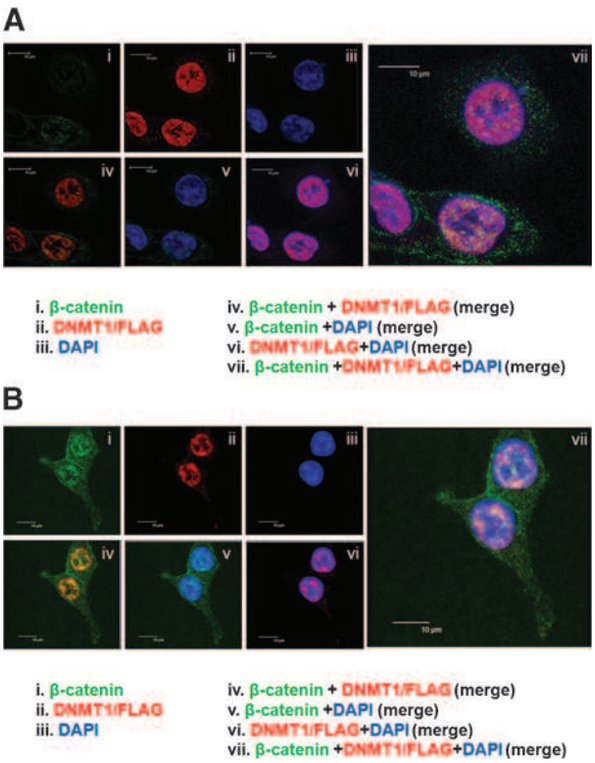

Figure 2.
Nuclear colocalization of Dnmt1 and β-catenin proteins. A, Dnmt1 protein colocalizes with β-catenin protein in HCT116 cells. B, Dnmt1 protein colocalizes with β-catenin protein in Wnt3a stimulated HCT116 cells. Images shown were captured by confocal microscopy. Dnmt1 is shown in red (anti–mouse-AlexaFluor594 secondary antibody); β-catenin is shown in green (anti–rabbit-AlexaFluor488 secondary antibody) and colocalization of Dnmt1 and β-catenin yellow (overlay images iv show all three colors). DAPI was used for cell nuclear staining (Blue); scale bar, 10 μm.

(*Continued.*) (M, standard protein marker; r, biologic replicate; Nuc, nuclear fractions; Cyto, cytoplasmic fractions; IP, immunoprecipitation; IB, immunoblotting; Input indicates equal loading for IP experiments). G, the central portion of Dnmt1 interacts with β-catenin. HEK293 cells were transfected with plasmids expressing full-length Myc-tagged Dnmt1 (F), three Myc-tagged Dnmt1 deletion constructs (A–C) as shown in the diagram of Dnmt1 protein as well as empty plasmid vector (Vec.). Cell lysates were immunoprecipitated with anti-Myc antibodies and cell lysate from nontransfected HEK293 cells (Con.) were used as immunoprecipitation control. Elution fractions were then loaded on SDSPAGE followed by Western blot analyses with anti–β-catenin antibodies. M refers to standard protein marker.

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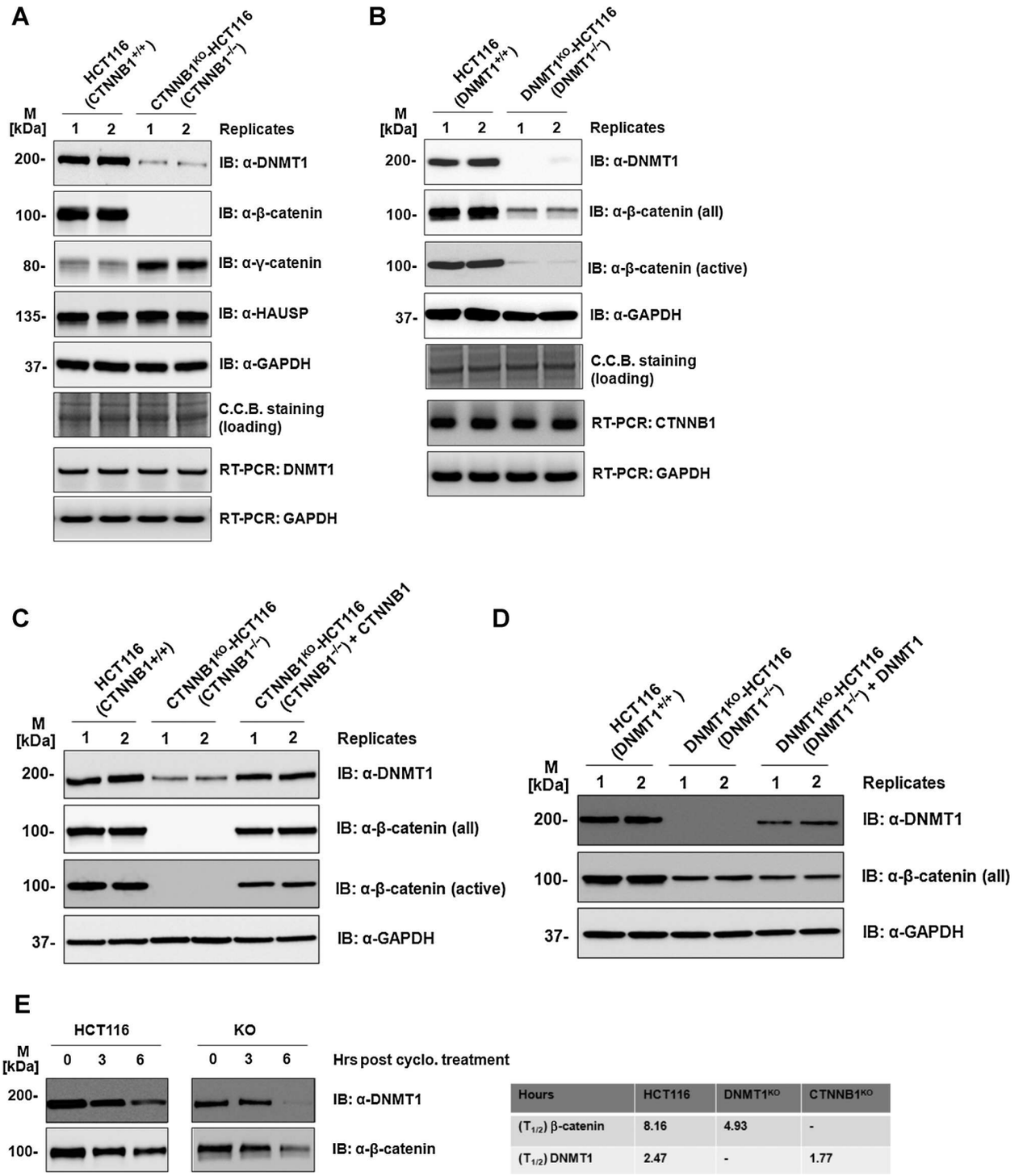

Hours	HCT116	DNMT1KO	CTNNB1KO
($T_{1/2}$) β-catenin	8.16	4.93	-
($T_{1/2}$) DNMT1	2.47	-	1.77

Figure 3.

The Dnmt1–β-catenin association is mutually stabilizing. A, knockout of CTNNB1 leads to reduced abundance of endogenous Dnmt1. Twenty micrograms of total protein lysate from CTNNB1^{KO}-HCT116 or HCT116 cells was loaded on SDSPAGE followed by Western blot analyses with anti-Dnmt1, anti–β-catenin, anti–γ-catenin, anti-HAUSP, and anti-GAPDH. One step RT-PCR was performed on the total RNA extracted from both HCT116 and CTNNB1^{KO}-HCT116 cells with specific primer pairs for Dnmt1 and GAPDH (as loading control). B, knockout of DNMT1 leads to reduced abundance of endogenous β-catenin. Twenty micrograms of total protein lysate from DNMT1^{KO}-HCT116or HCT116 cells was loaded on SDSPAGE followed by Western blot analyses with anti-Dnmt1, anti–β-catenin, and anti-GAPDH. One step RT-PCR was performed on the total RNA extracted from both HCT116 and DNMT1^{KO}-HCT116 cells with specific primer pairs for CTNNB1 and GAPDH (as loading control). M refers to standard protein marker. C, Dnmt1 protein expression is rescued in CTNNB1^{KO}-HCT116 cells by addition of β-catenin. β-Catenin protein expression level was restored by making transient transfection in CTNNB1^{KO}-HCT116 cells with plasmid vector containing full-length β-catenin. (*Continued on the following page.*)

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We next studied the subcellular localization of β-catenin–Dnmt1 association using confocal microscopy and immunofluorescence to visualize the proteins *in vivo* (Fig. 2). DNMT1^{KI}-HCT116 cells were either untreated (Fig. 2A) or treated with Wnt3a (Fig. 2B), and analyzed using anti–β-catenin and anti-FLAG antibodies. As is clearly shown, β-catenin signal is detected in both the cytosolic and nuclear compartments, whereas Dnmt1 signal is confined to the nucleus. The merged β-catenin and Dnmt1 signal shows strong colocalization of the two proteins in the nucleus, and this is most evident in cells treated with Wnt3a (Fig. 2B, panel iv).

In summary, mass spectrometry, co-IP, and immunofluorescence results indicate that β-catenin and Dnmt1 proteins are co-complexed in the nucleus, that this interaction increases in response to Wnt3a, and that the interaction occurs in multiple different cell lines.

Levels of Dnmt1 and β-catenin proteins are mutually dependent

We next determined how the association between Dnmt1 protein and β-catenin affects the levels of these two proteins. Two previously generated knockout cell lines, DNMT1$^{-/-}$ (DNMT1^{KO}-HCT116; ref. 29), CTNNB1$^{-/-}$ (CTNNB1^{KO}-HCT116; ref. 30), and were compared with parent HCT116 cells. Fig. 3A shows Western and RT-PCR analysis of parent HCT116 and CTNNB1^{KO}-HCT116 cells. The levels of Dnmt1 protein are substantially reduced in the CTNNB1^{KO}-HCT116 cells as compared with HCT116 parent cells. Notably, however, RT-PCR analysis reveals no difference between DNMT1 transcript levels in CTNNB1^{KO}-HCT116 and HCT116 cell lines, indicating that the lack of β-catenin does not affect DNMT1 transcript levels. We also immunoblotted these samples using anti–γ-catenin (plakoglobin) antibodies, and showed that the levels of plakoglobin are elevated in CTNNB1$^{-/-}$ cells, consistent with the previously described observations that plakoglobin can independently promote Wnt/TCF signaling in β-catenin–deficient cells (31). Figure 3B shows similar analysis in DNMT1$^{-/-}$ (DNMT1^{KO}-HCT116) cells. β-catenin levels are substantially reduced in DNMT1^{KO}-HCT116 as compared with HCT116 cells, although no difference in CTNNB1 transcript levels is apparent. We re-introduced β-catenin into CTNNB1^{KO}-HCT116 by transient transfection of a full-length β-catenin expression construct (Fig. 3C). As shown clearly in the Western analysis of these cells, re-expression of β-catenin rescues Dnmt1 protein expression in the CTNNB1$^{-/-}$cells, indicating the dependence of Dnmt1 protein levels on β-catenin, although in the reciprocal experiment (Fig. 3D) in which Dnmt1 was expressed in DNMT1^{KO}-HCT116 cells, significant restoration of β-catenin protein levels was not observed.

To investigate how Dnmt1 and β-catenin affect one another's stability, we measured protein half lives in the presence or absence of each protein. CTNNB1^{KO}-HCT116 and DNMT1^{KO}-HCT116 cells were treated with cycloheximide to block translation and then the protein degradation profiles observed. As shown in Fig. 3E, we found that Dnmt1 has a significantly shorter half-life than β-catenin, and that in CTNNB1^{KO}-HCT116 cells the half-life is reduced by approximately 30%. β-Catenin has a longer half-life that is reduced in the absence of Dnmt1. In DNMT1^{KO}-HCT116 cells, β-catenin half-life is reduced by approximately 40% as compared with parent HCT116 cells. DNMT1^{KO}-HCT116 and CTNNB1^{KO}-HCT116 cells were also treated with MG-132 proteasome inhibitor and levels of β-catenin and Dnmt1 analyzed by Western blot analysis (Supplementary Fig. S6). In DNMT1^{KO}-HCT116 cells, levels of β-catenin are markedly increased by the addition of MG-132 whereas in CTNNB1^{KO}-HCT116 cells, levels of Dnmt1 increase in response to MG-132, suggesting that the destabilization of Dnmt1 and β-catenin is mediated via the proteasome and can be inhibited through inhibition of proteasomal activity.

To further study the interdependence of β-catenin and Dnmt1 protein levels, we performed siRNA-mediated knockdown of DNMT1 or CTNNB1 in HCT116 and RKO cells. Knockdown of CTNNB1 in HCT116 (Fig. 4A) and in RKO cells (Fig. 4B) shows decreased levels of Dnmt1 protein in both cell lines. The effect is more marked in RKO cells than in HCT116 cells, and we noted that in HCT116 cells, siRNA-mediated CTNNB1 knockdown only partially reduced the levels of CTNNB1 (Fig. 4A), possibly due to the substantially higher levels of endogenous β-catenin in HCT116 cells. Similarly, knockdown of DNMT1 in HCT116 (Fig. 4C) and RKO (Fig. 4D) reduced levels of β-catenin proteins. The effect is also more marked in RKO cells, where the siRNA-mediated knockdown of DNMT1 dramatically reduced levels of Dnmt1 and β-catenin.

Regulatory mechanisms of the Dnmt1–β-catenin protein–protein interaction

We next tested two HCT116 derivative cell lines in which either the wild-type CTNNB1 allele (CTNNB1$^{-/\Delta 45}$-HCT116) or mutant CTNNB1 allele (CTNNB1$^{WT/-}$-HCT116) was disrupted (30). Using two different clones for each cell-line, we analyzed expression of β-catenin and Dnmt1. As shown in Fig. 5A, the levels of Dnmt1 protein are decreased in CTNNB1$^{WT/-}$-HCT116 cells, in line with the decreased levels of β-catenin in CTNNB1$^{WT/-}$-HCT116 as compared with CTNNB1$^{-/\Delta 45}$-HCT116 cells. Nuclear and cytosolic subcellular fractionations show a substantial reduction of Dnmt1 protein in the nucleus of the cells expressing only wild-type β-catenin (Fig. 5B). We next stimulated each of these cell lines with exogenous Wnt3A (Fig. 5C and D). The levels of β-catenin show a more robust response in the wild-type β-catenin cells as compared with the mutant β-catenin cells, and this response is

(*Continued.*) Total protein lysate (20 μg) from HCT116, CTNNB1^{KO}-HCT116, or CTNNB1^{KO}-HCT116 with ectopic expressed β-catenin cells was loaded on SDSPAGE followed by Western blot analyses with anti-Dnmt1, anti–β-catenin, anti–β-catenin (active), and anti-GAPDH (loading control). M refers to standard protein marker. D, β-catenin expression is not rescued in DNMT1^{KO}-HCT116 cells by addition of Dnmt1 protein.Transient transfection of DNMT1^{KO}-HCT116 cells with plasmid vector containing full-length Dnmt1 does not restore β-catenin expression. Total protein lysate (20 μg) from HCT116, DNMT1^{KO}-HCT116, or DNMT1^{KO}-HCT116 with ectopically expressed Dnmt1 was loaded on SDSPAGE followed by Western blot analyses with anti-Dnmt1, anti–β-catenin, and anti-GAPDH (loading control). M refers to standard protein marker. E, degradation profiles for β-catenin and Dnmt1 in HCT116, DNMT1^{KO}-HCT116, or CTNNB1^{KO}-HCT116 cells following cycloheximide treatment. Degradation rate constants were quantified by measuring the relative intensity of each protein by quantitative Western blotting at 0, 3, and 6 hours after cycloheximide treatment. The intensity data were fit to a first-order decay function to estimate the degradation rate constant, which then was used to calculate the half-life.

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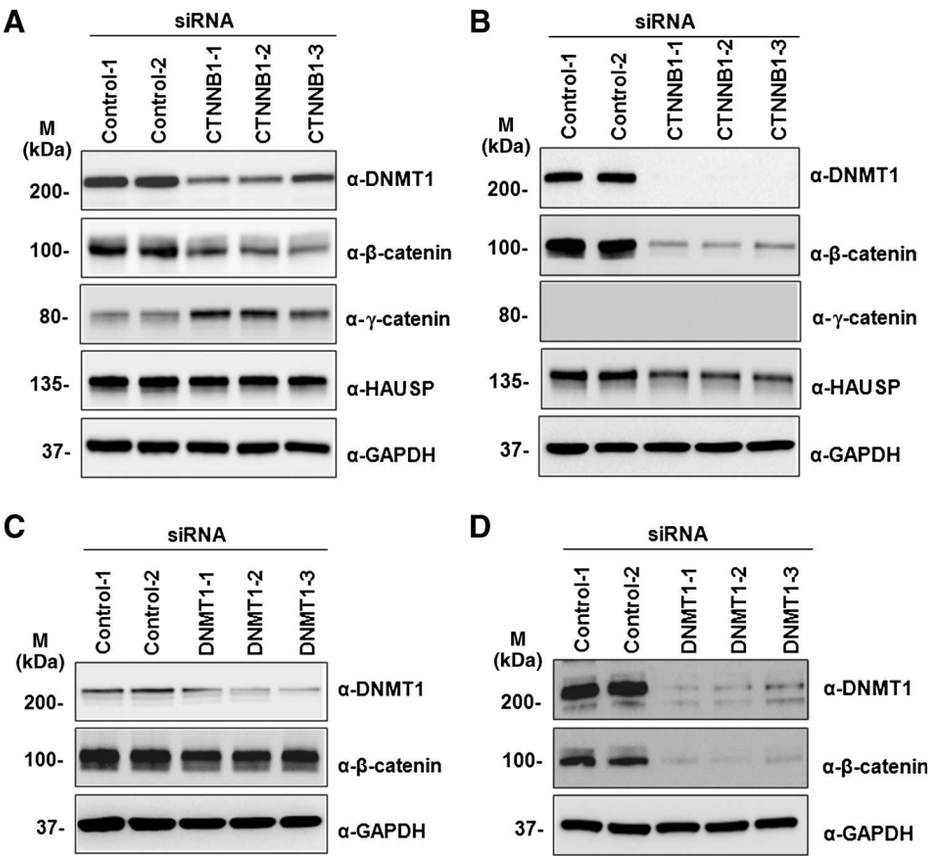

Figure 4.
siRNA analysis of the Dnmt1–β-catenin association. Knockdown of CTNNB1 leads to reduced abundance of endogenous Dnmt1 in HCT116 (A) and RKO (B) cells. HCT116 or RKO cells were transfected with control siRNA or three independent siRNAs against CTNNB1. Immunoblots were used to quantify Dnmt1, β-catenin, γ-catenin, and HAUSP with anti-Dnmt1, anti–β-catenin, anti–γ-catenin, anti-HAUSP sera. GAPDH was used as loading control. Knockdown of Dnmt1 leads to reduced abundance of endogenous CTNNB1 in HCT116 (C) and RKO (D) cells. HCT116 or RKO cells were transfected with control siRNA or three independent siRNAs against Dnmt1. Immunoblots were used to quantify Dnmt1and β-catenin with antibodies against Dnmt1 and β-catenin, respectively. GAPDH was used as loading control. M refers to standard protein marker.

reflected in the levels of Dnmt1 protein, which show a more marked increase in response to Wnt3A in the wild-type β-catenin cells than in the mutant β-catenin cells. We also tested whether the Dnmt1 response was responsive to exogenous Wnt stimulation in CTNNB1^{KO}-HCT116 cells. HCT116 and CTNNB1^{KO}-HCT116 cells were treated with Wnt3a and Western analysis performed (Supplementary Fig. S4). As expected, Wnt3a only stabilizes Dnmt1 in the HCT116 cells, showing that the stabilization of Dnmt1 in response to Wnt3A requires β-catenin. Indeed, in these experiments, we observed a slight decrease of Dnmt1 abundance in CTNNB1^{KO}-HCT116 cells treated with Wnt3A when compared with CTNNB1^{KO}-HCT116 cells not treated with Wnt3A, suggesting that a β-catenin–independent Wnt-mediated mechanism may also regulate Dnmt1 expression.

Because Dnmt1 protein plays a central role in DNA methylation during cell division and DNA replication (32, 33), we also tested whether Wnt-driven modulation of Dnmt1 protein levels might be attributed to cell-cycle regulation following Wnt3a stimulation. Flow cytometry was performed on cells harvested across the 24 hours time course following Wnt3a treatment. No gross alterations of relative numbers of cells at each time point were apparent (Supplementary Fig. S1), and we therefore concluded that the response of Dnmt1 protein levels to Wnt3a treatment is not cell-cycle related.

To investigate mechanisms by which β-catenin regulates Dnmt1 protein levels, we analyzed the expression of several Dnmt1 interacting proteins, including proteins that regulate Dnmt1 stability through methylation (SET7, LSD1; refs. 16, 34). Immunoblots of these proteins were performed on lysates from HCT116 and CTNNB1^{KO}-HCT116 cells as shown in Fig. 6A and B. We were particularly interested by the reduction in levels of LSD1 protein in CTNNB1^{KO}-HCT116 cells. LSD1 (KDM1A) encodes a lysine-specific demethylase, which has been shown to stabilize Dnmt1 through demethylation (35). As a negative control, we also immunoblotted a related lysine-specific demethylase, KDM3B, and found no difference in the levels of KDM3B protein between HCT116 and CTNNB1^{KO}-HCT116 cells (Fig. 6A). It may be that the reduction in LSD1 protein levels is attributable to destabilization of Dnmt1–LSD1 protein complexes by the reduction of Dnmt1 in and CTNNB1^{KO}-HCT116 cells. We next tested whether LSD1 protein was present in anti–β-catenin immunoprecipitates. Co-IP using anti–β-catenin showed that both Dnmt1 and LSD1 proteins were present (Fig. 6B), indicating that LSD1 is present in Dnmt1–β-catenin protein complexes, and indicating that LSD1 may function in the β-catenin–dependent regulation of Dnmt1.

Functional consequences of the Dnmt1–β-catenin protein–protein interaction

Our results predict that Wnt/β-catenin signaling should be altered according to Dnmt1-dependent regulation of β-catenin protein levels. We first compared TCF-reporter (TOPFlash) activity in cells transfected with DNMT1 expression vector in HCT116 and CTNNB1$^{-/\Delta 45}$-HCT116 (Fig. 7A). Next, we compared β-catenin/TCF signaling in DNMT1^{KO}-HCT116 and HCT116 cells treated with siRNA to reduce Dnmt1 levels as shown in Fig. 7B.

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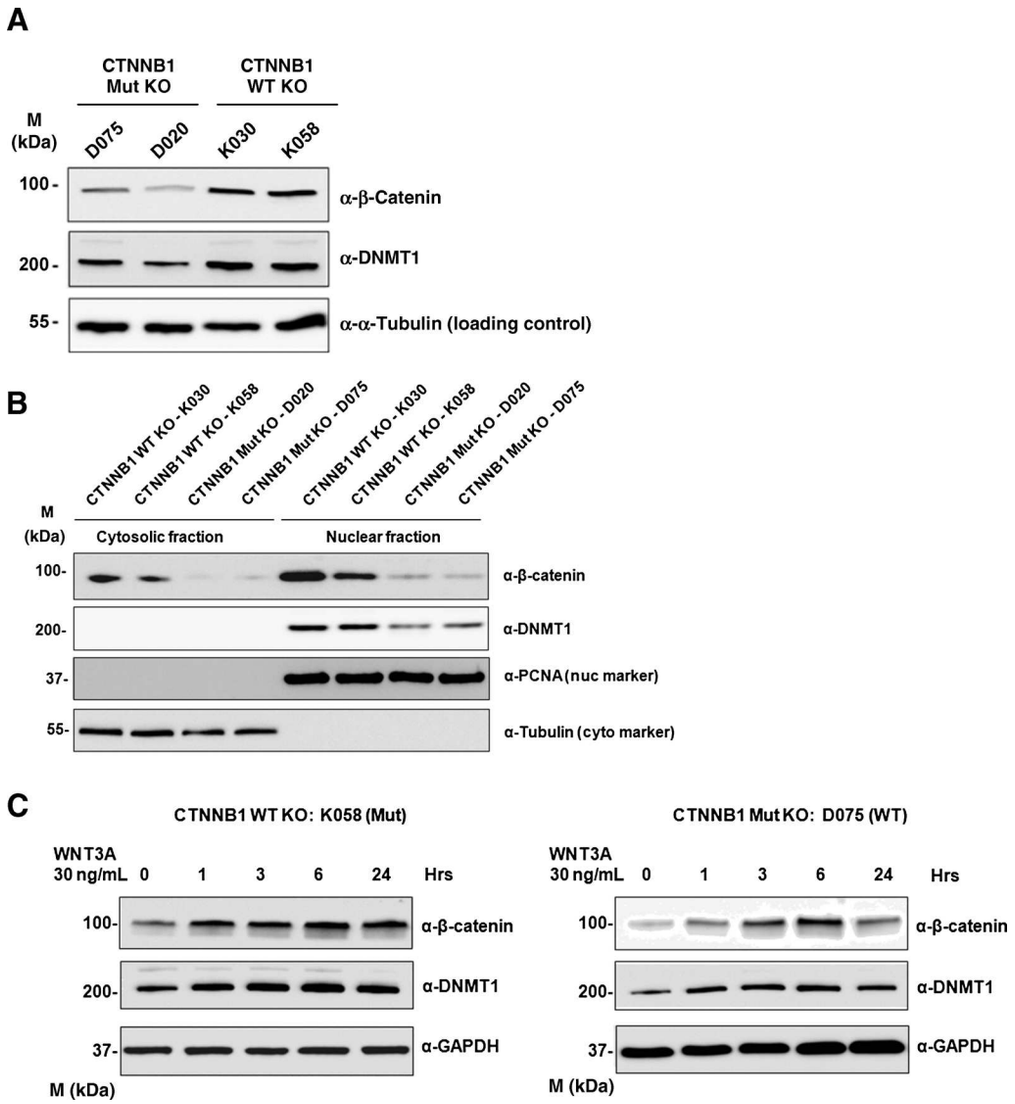

Figure 5.
Immunoblot analysis of Dnmt1 expression in cells expressing mutant or wild-type β-catenin. A, immunoblot analysis of cells expressing wild-type β-catenin (CTNNB1$^{WT/-}$-HCT116) and mutant β-catenin (CTNNB1$^{-/\Delta 45}$-HCT116). Total soluble proteins were extracted and compared in two separate clones for each cell-line, 20 μg of total protein was loaded on SDSPAGE and Western blots were performed using α–β-catenin, α-Dnmt1, and α–α-tubulin (loading control). B, immunoblot analysis of subcellular fractions of cells expressing wild-type β-catenin (CTNNB1$^{WT/-}$-HCT116) and mutant β-catenin (CTNNB1$^{-/\Delta 45}$-HCT116; two clones for each cell type). For each fraction, a total protein of 20 μg was loaded on SDSPAGE and Western blots were performed using α–β-catenin and α-Dnmt1. C, Wnt3A time course stimulation of CTNNB1$^{WT/-}$-HCT116 (clone D075) and CTNNB1$^{-/\Delta 45}$-HCT116 cells (clone K058). Cells were collected at 0, 1, 3, 6, and 24 hours post Wnt3A (30 ng/mL) stimulation on each cell line followed by total protein extraction. Total protein of 20 μg was loaded on SDSPAGE and Western blots were performed using α–β-catenin and α-Dnmt1.

Overexpression of DNMT1 increases TCF reporter activity approximately 2-fold and targeting of DNMT1 via siRNA or knockout reduces β-catenin signaling (~2- and ~4-fold, respectively; all $P < 0.05$ Student t test). In RKO cells and CTNNB1$^{-/WT}$-HCT116 cells, overall levels of TCF reporter activity are much lower (~10-fold), as expected (lower β-catenin levels) and statistically significant changes in TCF reporter were not detected in either overexpression or RNAi experiments (supplementary Information). Although we cannot rule out that knockdown or knock-out of DNMT1 affects TCF signaling through a β-catenin–independent mechanism, these results suggest that Dnmt1 protein regulates Wnt/TCF signaling by stabilizing β-catenin in the nucleus.

Finally, because DNMT1 is the primary mammalian maintenance DNA methyltransferase, we sought to determine whether destabilization of Dnmt1 in the absence of β-catenin also alters CpG methylation patterns. The H19 imprinted locus encodes a non–protein-coding RNA and functions as a tumor suppressor (35). We also previously found that the H19 imprinted locus was a sensitive marker for DNMT1 methylation activity in HCT116 cells (15). We therefore compared the CpG methylation status of approximately 20 clones each of HCT116 and

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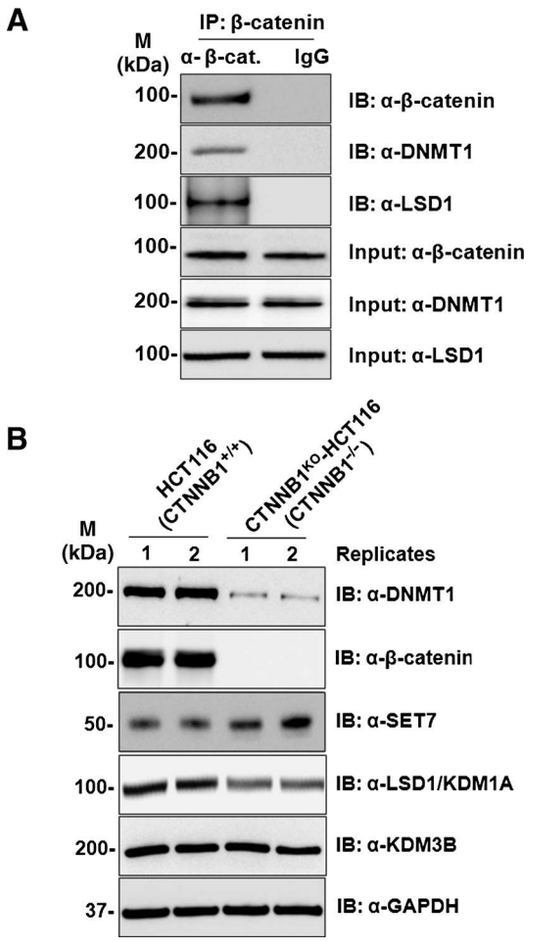

Figure 6

Analysis of known Dnmt1 interacting and regulatory proteins. A, anti–β-catenin immunoprecipitates both Dnmt1 and LSD1 proteins in HCT116 cells. Total protein lysate from HCT116 or immunoprecipitates was blotted with β-catenin, Dnmt1, and LSD1 antibodies. B, immunoblot analysis of HCT116 and CTNNB1 knockout (CTNNB1^{KO}-HCT116) cells (two replicates each) of Dnmt1 interacting and regulatory proteins. Total soluble proteins were extracted and compared in two separate clones for each cell-line (Clones are indicated as 1 and 2), 20 μg of total protein was loaded on SDSPAGE and Western blots were performed using anti-SET7, anti-LSD1 (lysine demethylase regulating Dnmt1 stability) antibody, and related lysine demethylase anti-KDM3B.

CTNNB1^{KO}-HCT116 cells. As shown in Fig. 7C, there is significantly reduced methylation in the CTNNB1^{KO}-HCT116 cells at several CpG loci, indicating that the lower levels of Dnmt1 protein in CTNNB1^{KO}-HCT116 cells has a knockon effect on DNA methylation activity. In concordance with this finding, we also observed increased levels of H19 mRNA transcripts in CTNNB1^{KO}-HCT116 as compared with HCT116 cells (Supplementary Fig. S7).

Discussion

Here, we analyze an interaction between Dnmt1 and β-catenin proteins that mutually regulates the levels of each protein in colorectal cancer cells. The regulation is not mediated via transcriptional activation of DNMT1, but instead through the interaction of Dnmt1 protein with β-catenin in the nucleus. Given the critical roles of both β-catenin and Dnmt1 cells in normal as well as cancer cells, our results point to an important mechanism by which the two proteins cross-regulate. We found that the lysine demethylase, LSD1, which regulates Dnmt1 stability, is present in Dnmt1–β-catenin protein complexes, suggesting that the stabilization of Dnmt1 in these protein complexes is mediated via lysine methylation. We investigated the downstream functional consequences of the β-catenin–Dnmt1 association, and showed that Dnmt1 protein levels regulate β-catenin/TCF signaling and that the absence of β-catenin impacts CpG methylation status, pointing to a regulatory link between Wnt/β-catenin signaling and the downstream functions of Dnmt1.

Although protein stability of Dnmt1 and β-catenin is an important outcome of the interaction between the proteins, the precise molecular mechanisms that result in increased β-catenin/TCF signaling activity remain to be determined. Although β-catenin expression rescues Dnmt1 expression in CTNNB1^{KO}-HCT116 cells, we did not observe rescue of β-catenin protein levels in DNMT1^{KO}-HCT116 cells following expression of Dnmt1 (Fig. 3). This may imply that the increased β-catenin/TCF signaling activity induced by Dnmt1 is not due to stabilization and increased levels of β-catenin, but from an alternative mechanism by which Dnmt1 increases β-catenin transcriptional activity. Other studies have shown that Dnmt1 can regulate transcription through protein interactions, in a methylation-independent manner. The Dnmt1 N-terminus was shown to mediate the interaction of Dnmt1 with HDAC and DMAP1 proteins, to form transcriptional repressor complexes (11), and many interactions between Dnmt1 and transcription factors have been identified (13). E-cadherin expression (in HCT116 cells) is regulated by an interaction between Dnmt1 and the transcriptional repressor Snail1, and in the same study that loss of the Dnmt1 N-terminal domains promoted β-catenin translocation to the nucleus (36). Stabilization of Dnmt1 protein, but not alterations of DNMT1 transcript levels are the cause of DNMT1 dysregulation in human mammary epithelial cells, and this stabilization is mediated via the Dnmt1 protein N-terminus (37). How LSD1 functions in the Dnmt1–β-catenin interaction remains to be determined, because LSD1 is known to regulate Dnmt1 stability via demethylation (34). Other modifying proteins may also be important; interactions of Dnmt1 protein with HAUSP/USP7, UHRF1 and Tip60 proteins regulate Dnmt1 by ubiquitination and acetylation (15, 38). Tip60 interacts with both Dnmt1 and β-catenin (39), and further mass spectrometry analysis will determine whether Tip60 is present in β-catenin–Dnmt1 protein complexes. Although we previously showed that Tip60 acetylates Dnmt1 protein, which promotes Dnmt1 degradation (15), the significance of the β-catenin–Tip60 interaction is as yet undetermined.

Our study points to a mechanism of cross-talk between Wnt signaling and DNA methylation mediated via a protein–protein interaction between β-catenin and Dnmt1. How this cross-talk manifests itself in tumors is as yet unknown, although in mouse, hypomorphic Dnmt1 alleles in the Apc Min background substantially reduce the number of polyps observed (40). Although the suppression of polyp formation was attributed to decreased CpG island methylation in the Dnmt1 hypomorphic mice, reduced Dnmt1 protein levels may also suppress polyp formation by destabilizing β-catenin and reducing Wnt/β-catenin–driven transcriptional activity via the mechanism that we have described.

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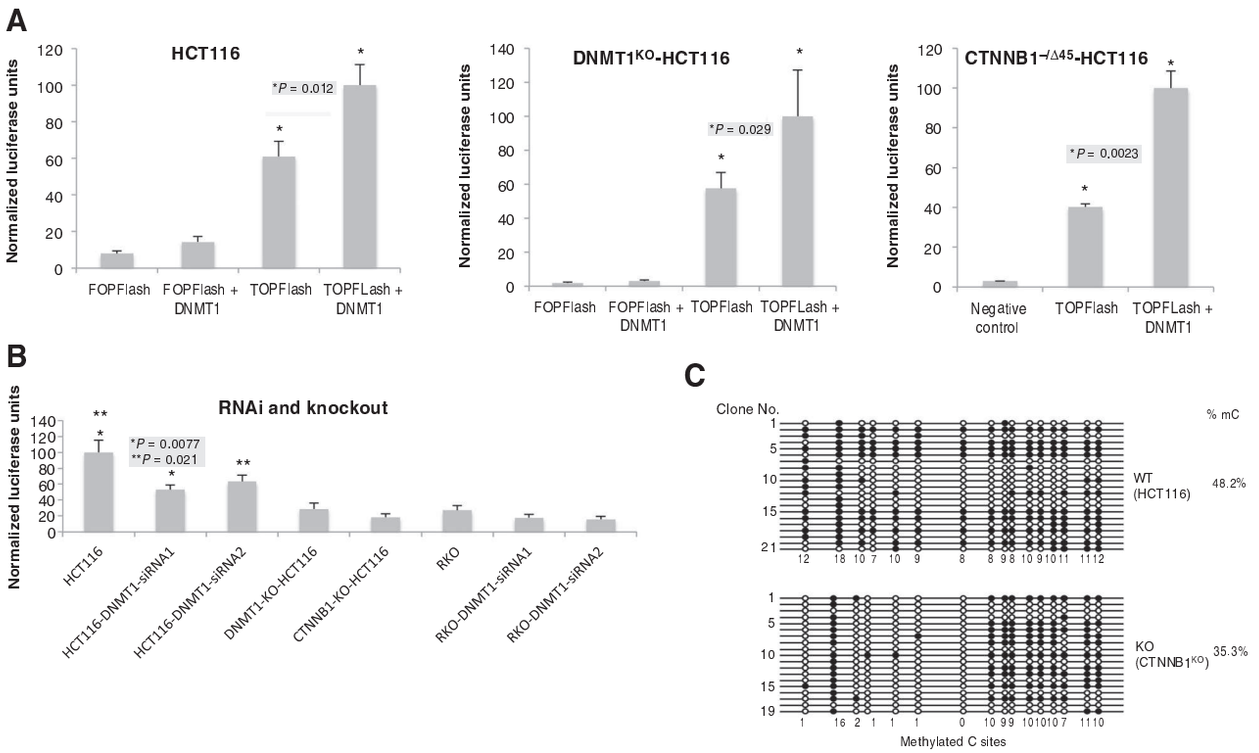

Figure 7.
Functional consequences of the Dnmt1–β-catenin interaction. A, TCF (luciferase) reporter activity in HCT116 and CTNNB1$^{-/\Delta 45}$-HCT116 (mutant β-catenin) cells (B) TCF (luciferase) reporter activity in HCT116 and RKO cells (treated with DNMT1 siRNAs) as well as DNMT1^{KO}-HCT116 and CTNNB1^{KO}-HCT116 cells. All cells were transfected with LEF/TCF reporter. Negative and positive controls were also used for signal normalization and transfection efficiency monitoring. Dual Luciferase assay was performed 48 hours after transfection and promoter activity values were expressed as arbitrary units using a Renilla reporter for internal normalization. Experiments were done in triplicates for biologic cell cultures/transfections and luminescent measurements. Average numbers (bar height) of relative luciferase units for each sample plus standard deviation (error bar) were plotted relative to maximum. C, methylated CpG sites are decreased in CTNNB1 KO cells at the H19 locus, especially on the first six (1, 3, 4, 5, 6, and 7) of seven CpG sites. Genomic DNAs from wild-type HCT116 (WT) and CTNNB1 knockout HCT116 (KO) clones were bisulfite treated. The CpG island of H19 locus was PCR amplified, cloned, and sequenced with the Sanger sequencing method. Twenty one and 19 clones were sequenced for WT and KO, respectively (methylated CpG sites are black spots, nonmethylated CpG sites are white spots and DNA sequences are represented as black lines).

Transcriptional regulation of DNMT1 by Wnt has also been shown (41). Using colorectal HT-29 cells (expressing truncated APC) the authors showed that transfection of full-length APC reduces DNMT1 mRNA and DNMT1 promoter–reporter activity. In addition, the authors also inhibited TCF-driven transcription using a dominant-negative TCF and found that this also reduces DNMT1 mRNA levels, indicating transcriptional control of DNMT1 expression. In our study, we do not observe transcriptional effects of Wnt/β-catenin on DNMT1 (as measured by steady-state RNA levels), but instead observed dramatic effects of Wnt and β-catenin on Dnmt1 protein levels. These differences may imply that activation of Wnt/β-catenin by APC mutations is not entirely functionally equivalent to activation by stabilizing CTNNB1/β-catenin mutations. We also note, as did the authors of the previous study, that there are no apparent TCF-binding sites in the DNMT1 promoter, and so the transcriptional activation of DNMT1 is unlikely to be mediated via direct binding of β-catenin/TCF in the DNMT1 promoter. Taken together, our study and these previous studies show that multilayered communication exists between DNMT1 and Wnt/β-catenin signaling. Although these and previous studies have been performed in cancer cell models, future work should establish the possible importance of cross-talk between DNMT1 and Wnt/β-catenin signaling in clinical samples. In addition, although our study has identified that an interaction between Wnt/β-catenin and Dnmt1 may alter DNA methylation patterns on a specific locus, it would be interesting to measure global patterns of DNA methylation to see how widespread this effect is.

Interestingly, several studies have demonstrated methylation-independent functions for Dnmt1. In HCT116 cells, Dnmt1 proteins lacking the C-terminal catalytic domain function as transcriptional repressors at specific loci (12). The authors speculated that Dnmt1 serves as a scaffold for recruitment of transcriptional repressive complexes, including interaction with the LSD1 histone demethylase. Intriguingly, knockdown of Dnmt1 protein was shown to regulate gene expression via a methylation-independent and histone deacetylation–independent mechanism (42). Our analysis showed that knockdown of Dnmt1 protein can regulate TCF/β-catenin transcriptional activity. Future studies will establish whether Dnmt1 protein levels also regulate endogenous Wnt/β-catenin targets. The role played by chromatin should also be considered. The interplay between soluble nuclear

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Dnmt1 proteins and chromatin-associated Dnmt1 is complex. For example, Dnmt1 protein forms soluble complexes with USP7 that then form trimeric complexes with chromatin-bound UHRF1 (28). In addition, in contrast with DNMT3A/B, a substantial portion of Dnmt1 protein appears to exist as a soluble pool in the nucleus rather than being associated with chromatin (43, 44). Finally, although the primary nuclear function of β-catenin is to regulate transcription via association with TCF transcription factors, our study shows that regulation of other proteins via stabilizing protein–protein interactions may also be an important function of nuclear β-catenin.

In summary, our work identifies a novel mechanism by which the levels of two key oncoproteins, β-catenin and Dnmt1 are regulated in cancer cells. The β-catenin–Dnmt1 interaction stabilizes each protein, and in turn regulates downstream β-catenin and Dnmt1 functions. Our study indicates a possible cancer-relevant mechanism of cross-regulation between Wnt signaling and DNA methylation. Given that the Wnt pathway plays a critical role in development and tissue differentiation, the mechanism revealed in this study could also explain how Wnt signaling drives tissue differentiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Song, Z. Wang, R.M. Ewing
Development of methodology: J. Song, Z. Du, R.M. Ewing
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Song, Z. Du, M. Ravasz, B. Dong, Z. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Song, M. Ravasz, Z. Wang, R.M. Ewing
Writing, review, and/or revision of the manuscript: J. Song, Z. Wang, R.M. Ewing
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Song, M. Ravasz, R.M. Ewing
Study supervision: J. Song, R.M. Ewing

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