Published in final edited form as:

Mol Cancer Res. 2011 May ; 9(5): 660–670. doi:10.1158/1541-7786.MCR-10-0223.

A coactivator role of CARM1 in the dysregulation of β -catenin activity in colorectal cancer cell growth and gene expression

Chen-Yin Ou¹, Melissa J. LaBonte², Philipp C. Manegold³, Alex Yick-Lun So^{4,5}, Irina Ianculescu¹, Daniel S. Gerke¹, Keith R. Yamamoto^{4,5}, Robert D. Ladner², Michael Kahn^{1,3}, Jeong Hoon Kim⁶, and Michael R. Stallcup¹

¹ Department of Biochemistry and Molecular Biology, University of Southern California, Los Angeles, California 90089-9176, USA

² Department of Pathology, University of Southern California, Los Angeles, California 90089-9176, USA

³ Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine, University of Southern California, Los Angeles, California 90089-9176, USA

⁴ Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94518-2517, USA

⁵ Chemistry and Chemical Biology Graduate Program, University of California, San Francisco, CA 94518-2517, USA

⁶ Samsung Medical Center and Center for Clinical Research, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea

Abstract

Aberrant activation of Wnt/ β -catenin signaling, resulting in the expression of Wnt regulated oncogenes, is recognized as a critical factor in the etiology of colorectal cancer. Occupancy of β -catenin at promoters of Wnt target genes drives transcription, but the mechanism of β -catenin action remains poorly understood. Here, we show that CARM1 (coactivator associated protein arginine methyltransferase 1) interacts with β -catenin and positively modulates β -catenin-mediated gene expression. In colorectal cancer cells with constitutively high Wnt/ β -catenin activity, depletion of CARM1 inhibits expression of endogenous Wnt/ β -catenin target genes and suppresses clonal survival and anchorage-independent growth. We also identified a colorectal cancer cell line (RKO) with a low basal level of β -catenin, which is dramatically elevated by treatment with Wnt3a. Wnt3a also increased expression of a subset of endogenous Wnt target genes, and CARM1 was required for the Wnt-induced expression of these target genes and the accompanying dimethylation of arginine 17 of histone H3. Depletion of β -catenin from RKO cells diminished the Wnt-induced occupancy of CARM1 on a Wnt target gene, indicating that CARM1 is recruited to Wnt target genes through its interaction with β -catenin and contributes to transcriptional activation by mediating events (including histone H3 methylation) which are downstream from the actions of β -catenin. Therefore, CARM1 is an important positive modulator of Wnt/ β -catenin transcription and neoplastic transformation, and may thereby represent a novel target for therapeutic intervention in cancers involving aberrantly activated Wnt/ β -catenin signaling.

Requests for reprints: Michael R. Stallcup, Department of Biochemistry and Molecular Biology, USC/Norris Comprehensive Cancer Center, 1441 Eastlake Avenue, NOR 6316, University of Southern California, Los Angeles, CA 90089-9176, USA. Phone: (323) 865-3852. Fax: (323) 865-3866. stallcup@usc.edu.

Conflicts of interest: none

Keywords

CARM1; Wnt/ β -catenin; coactivators; transcriptional regulation; colorectal cancers

Introduction

Wnt/ β -catenin signaling is indispensable for the development of the gastrointestinal system, and aberrant activation of this pathway is implicated in disease, notably in colorectal cancer (1–3). A hallmark of Wnt pathway activation is the elevation of β -catenin protein levels. In the absence of Wnt signaling, β -catenin protein is targeted to a multisubunit degradation complex, which is composed of the scaffolding proteins adenomatous polyposis coli (APC) and Axin, and the protein kinases casein kinase 1 α (CK1 α) and glycogen synthase kinase 3 β (GSK3 β). Phosphorylation of β -catenin within this complex targets it for poly-ubiquitination by β TrCP and subsequently for proteasome-mediated proteolysis (3). Binding of Wnt ligands to transmembrane frizzled receptors disrupts the destruction complex, and thereby stabilizes and causes accumulation of β -catenin protein. β -catenin then translocates into the nucleus, where it binds to various transcription factors, including members of the lymphoid enhancer factor (LEF)/T cell factor (TCF) family, and functions as a transcription coactivator to activate expression of Wnt target genes (3).

Various mutations in components of the Wnt signaling pathway, which are frequently observed in colorectal cancer, result in an abnormally elevated β -catenin protein level; this leads to constitutive formation of LEF/TCF- β -catenin complexes and alters expression of β -catenin-regulated genes, including well known proto-oncogenes. Proteins encoded by β -catenin-controlled genes likely cooperate in neoplastic transformation. In support of this notion, analysis of Wnt pathway component mutations in mice and identification of mutations in human clinical specimens has shown that abnormal stabilization of β -catenin leads to colon tumorigenesis (4). Wnt/ β -catenin target genes include known pro-proliferative genes (e.g. c-myc (5)), anti-apoptosis genes (e.g. survivin (6, 7)) and genes involved in metastasis (e.g. S100A4 (8)). GPR49, a newly identified Wnt target gene, encodes an orphan G protein-coupled receptor and was proposed as a potential marker of tumor-initiating colorectal cancer stem cells (9). In addition, GPR49 was found overexpressed in colon tumors compared with normal colon tissues; and knockdown of GPR49 in colon cancer cells induced apoptosis (10). Thereby, GPR49, in concert with other Wnt/ β -catenin targets, promotes growth and/or survival of cancers with defects in β -catenin regulation.

When bound to target genes through its interaction with LEF/TCF transcription factors, β -catenin functions as a primary coactivator by recruiting additional coactivators, each of which makes specific contributions to the remodeling of chromatin conformation and/or the assembly of an active transcription complex. Therefore, it is crucial to identify and characterize the additional coactivators that modulate or mediate the transcriptional activity of β -catenin. Coactivator CARM1 (coactivator associated protein arginine methyltransferase 1) belongs to the protein arginine methyltransferase family, which methylate arginine residues in proteins. CARM1 methylates a subset of proteins that play crucial roles in transcription. For instance, CARM1 methylates arginine 17 of histone H3 (H3R17) at promoters, and this event is associated with transcriptional activation (11, 12). CARM1 functions as a transcriptional coactivator for many different DNA-binding transcriptional activator proteins, including estrogen receptor, androgen receptor, c-Fos, peroxisome proliferator-activated receptor γ , and NF κ B (11). It was previously shown that CARM1 binds to β -catenin *in vivo* and functions in synergy with β -catenin as a coactivator for LEF1-mediated expression of artificial and transiently transfected reporter plasmids, suggesting the role of CARM1 in β -catenin signaling (13).

Here, we report that CARM1 is over-expressed in human colon cancer cell lines. Importantly, we report that CARM1 plays a critical role in clonal survival and anchorage-independent growth of colorectal cancers by mediating Wnt/ β -catenin signaling. Wnt3a signaling induced CARM1 occupancy at the promoter of an endogenous Wnt target gene in its native chromosomal location; CARM1 occupancy correlated with arginine dimethylation of histone H3 (H3R17me₂). Indeed, we showed that endogenous CARM1 is required for both Wnt3a-dependent gene activation and H3 arginine dimethylation at a target gene promoter in colon cancer cells. Moreover, we examined the mechanism by which CARM1 directs Wnt/ β -catenin signaling. We demonstrated that the methyltransferase domain of CARM1 specifically interacts with β -catenin; β -catenin recruits CARM1 to the promoter, and regulated transcription is dependent on CARM1. Interestingly, CARM1 synergized with the coactivator p300 to further enhance Wnt/ β -catenin-activity. Collectively, our studies suggest CARM1 plays an essential role in oncogenic growth of colon cancers through the positive regulation of Wnt/ β -catenin oncogenes, and is thereby a potential therapeutic target in cancers involving abnormally activated Wnt/ β -catenin signaling.

Materials and methods

For detailed description of Materials and Methods, please refer to Supplementary Data. Briefly, plasmids, cell culture, luciferase reporter gene assay, GST-pull down, chromatin immunoprecipitation, quantitative reverse-transcriptase PCR (qRT-PCR), cell proliferation assay and colony formation assay in soft agar were done as described previously (14). CARM1 and β -catenin were stably depleted using short hairpin RNA (shRNA)-expressing lentiviruses. Colony formation assay was performed as described previously (15).

Results

CARM1 interacts with β -catenin, but not LEF1

Previous studies in our lab have demonstrated the *in vivo* interaction between CARM1 and β -catenin (13). To define the precise domain(s) by which CARM1 interacts with β -catenin, HA-tagged CARM1 and selected fragments were synthesized *in vitro* and incubated with GST- β -catenin fusion protein bound to glutathione-Sepharose beads. The associated bound proteins were purified and analyzed by immunoblot. Full length HA-CARM1 was bound by GST- β -catenin, but not by GST (Fig. 1A upper panel); and conversely β -catenin synthesized *in vitro* also bound specifically to full length GST-CARM1 (lower panel). Various domains of CARM1 were synthesized *in vitro* and tested for binding to GST- β -catenin (Fig. 1B). The N-terminal and C-terminal domains of CARM1 (amino acids 3–200 and 461–608, respectively) did not interact with β -catenin, whereas five overlapping fragments of CARM1 (amino acids 3–460, 3–500, 100–460, 121–608, and 241–608) bound specifically to GST- β -catenin, indicating that the methyltransferase domain (which resides within amino acids 150–480) is responsible for β -catenin binding. Since the methyltransferase domain is conserved among members of the protein arginine methyltransferase (PRMT) family (11), β -catenin may also interact with other PRMTs besides CARM1. Since β -catenin functions as a coactivator for the LEF/TCF family of DNA-binding transcriptional activator proteins, we also tested whether there is an interaction between CARM1 and LEF1; however, no interaction was detected by GST pull-down assay (Fig. 1C), indicating that CARM1 interaction with β -catenin is specific and may play a functional role in Wnt-dependent gene induction.

CARM1 and β -catenin cooperate as transcriptional coactivators for LEF1

To address whether the binding of CARM1 to β -catenin could modulate β -catenin-mediated transcription, we monitored the influence of CARM1 overexpression on the transcriptional

activity of β -catenin tethered to the Gal4 DNA-binding domain (DBD) in transient reporter gene assays. Compared with Gal4 DBD alone, Gal4 DBD fused to β -catenin strongly activated expression of a luciferase gene controlled by Gal4 response elements, and ectopic expression of CARM1 further enhanced the transcription in a dose-dependent manner (Fig. 2A). Overexpression of CARM1 had minimal or no effect on the activity of Gal4 DBD alone or Gal4 DBD fused to LEF1, highlighting the specificity of CARM1 on β -catenin mediated transcription.

The LEF/TCF family of DNA-binding transcriptional activator proteins are known to recruit β -catenin to their target genes, where β -catenin functions as a powerful coactivator. We therefore examined the ability of CARM1 and β -catenin to enhance LEF1-mediated expression of a luciferase reporter plasmid pGL3OT, which is driven by multiple LEF/TCF-binding elements. As shown previously (13), CARM1 overexpression dramatically enhanced reporter gene activation by LEF1 and β -catenin, and this synergy disappeared when β -catenin was omitted (Fig. 2B). Given that CARM1 does not interact with LEF1 directly (Fig. 1C) and that coactivator function of CARM1 depends on the presence of β -catenin, we conclude that CARM1 functions as a secondary coactivator in the transcriptional activation of genes controlled by LEF1, i.e. it is recruited to the LEF1 target gene by interaction with β -catenin, not with LEF1. The methyltransferase activity of CARM1 was not required for its cooperative coactivator function with β -catenin, since the point mutation E267Q, which eliminates CARM1 methyltransferase activity, did not diminish CARM1 coactivator function in this transient reporter gene assay (Supplementary Fig. S1).

Since histone acetyltransferase p300 was shown previously to be a coactivator for β -catenin-mediated transcription (16, 17), we tested whether p300 and CARM1 could cooperate to regulate LEF1/ β -catenin mediated reporters. We observed synergistic cooperation of CARM1 and p300 with β -catenin and LEF1 to enhance reporter gene expression, and this synergy was completely dependent on the presence of β -catenin (Fig. 2C). These results agree with the currently-accepted model that β -catenin associates with DNA-bound LEF1 and functions as a scaffold protein to recruit additional coactivators, such as CARM1 and p300, each of which makes a unique and specific contribution to the highly complex transcriptional initiation process.

CARM1 protein expression in colon cancer cell lines

Aberrant activation of Wnt/ β -catenin signaling has been implicated in the development of cancers, most notably colorectal cancers. Our reporter gene data suggest that CARM1 acts as a positive regulator for β -catenin activity. To conduct further studies of CARM1 in a physiologically relevant model system, we chose human colon cancer cell lines for subsequent experiments. When examined by western blot, nine different colon cancer cell lines were found to express CARM1 protein at varying levels, which were much higher than the expression in the FHC cell line, a model for normal human colon cells (Fig. 3A and Supplementary Fig. S2A). Thus, CARM1 appears to be overexpressed during the development or progression of colorectal carcinoma.

CARM1 mediates expression of endogenous β -catenin target genes

The HT29 human colorectal carcinoma cell line contains truncated APC protein, which is unable to mediate degradation of β -catenin (4). Abundant β -catenin then occupies Wnt-responsive elements (WREs) in the nucleus and abnormally over-expresses Wnt/ β -catenin target genes. Reintroduction of wild type APC to HT29 cells reduces β -catenin levels, dissociates β -catenin from the promoter and subsequently abrogates transcriptional activation (5, 6, 18). Several Wnt/ β -catenin target genes (e.g. c-myc (5), survivin (6) and TCF-1 (18)) were identified by the introduction of wild-type APC into HT29 cells. To

investigate a possible physiological role of CARM1 in Wnt/ β -catenin signaling, we initially used chromatin immunoprecipitation (ChIP) to test whether CARM1 associates with the endogenous WRE of a well known Wnt/ β -catenin target gene, the Axin2 gene, in its native chromosomal location. ChIP performed with antibodies directed against β -catenin or CARM1 produced a higher recruitment signal at the Axin2 WRE than at the 3' UTR, which lacks WRE sequences; in contrast, two control IgG samples gave equal low background signals for both locations (Fig. 3B). Therefore, β -catenin and CARM1 specifically occupy the WRE of the Axin2 promoter.

To test whether CARM1 is important for expression of endogenous Wnt/ β -catenin target genes, HT29 cells were infected with lentiviral vectors encoding short-hairpin RNA (shRNA) against CARM1 or β -catenin to specifically reduce the levels of those proteins. Immunoblot analysis of infected cell populations showed that the protein levels of CARM1 or β -catenin were specifically decreased by their respective shRNAs, when compared with control nonspecific shRNA (Fig. 3C and Supplementary Fig. S2B). Consistent with previous reports, reduction of β -catenin levels had a moderate to dramatic effect on the expression of protein products from four Wnt target genes: c-myc, Axin2, S100A4 and GPR49. While β -catenin appeared to play a major role in the expression of Axin2 and GPR49, the expression of c-myc and S100A4 only partially depended on β -catenin and thereby presumably is controlled by other transcription factor(s) besides LEF1/ β -catenin (Fig. 3D). Importantly, depletion of CARM1 levels also had a substantial impact on the expression of these four target genes, thus suggesting that CARM1 is required for constitutive expression of endogenous Wnt target genes controlled by β -catenin. Similar results were obtained with a second shRNA which is targeted against a different sequence in the CARM1 mRNA from the one shown in Fig. 3D (Supplementary Fig. S3).

CARM1 plays a critical role in β -catenin-mediated oncogenesis

CARM1 is essential for expression of Wnt/ β -catenin target genes that are involved in oncogenic growth and cellular transformation (e.g. c-myc, GPR49 and S100A4) (Fig. 3D). Therefore, we next examined phenotypic consequences of CARM1 depletion in the highly proliferative human colorectal carcinoma HT29 cells. We first compared the cellular proliferation rates of HT29 cell populations stably infected with lentiviral vectors encoding shRNAs against a nonspecific sequence (NS) or CARM1. As compared with HT29 shNS cells, the depletion of CARM1 had little or no effect on cellular proliferation (Fig. 4A). However, when the same lentiviral vector-infected HT29 cell populations were tested for their ability to form colonies in standard tissue culture dishes, we found that depletion of CARM1 resulted in lower numbers of expanding colonies (Fig. 4B). Interestingly, the approximate sizes of colonies (by visual inspection) were unaffected by CARM1 reduction. Similar results were obtained with a second shRNA directed against a different target sequence in CARM1 mRNA (Supplementary Fig. S4). These results indicate that CARM1 is important for clonogenic cell survival in low cell density conditions, but not for proliferation rate in standard cell culture conditions. Soft agar conditions were used next to measure anchorage-independent cell survival, colony formation, and growth; these traits typically correlate with a tumorigenic phenotype *in vivo*. We demonstrated previously that depletion of β -catenin causes a dramatic decrease in colony formation in soft agar (14). Here we observed that the HT29 cell population expressing the non-specific shRNA formed significantly more and larger colonies in soft agar than the HT29 cell population expressing shRNA against CARM1 (Fig. 4C). Thus, reductions in CARM1 protein level significantly compromised important cell traits associated with the transformed phenotype of colorectal carcinoma cells.

To test whether the above results in HT29 cells can be observed in other colon cancer cell lines with constitutively elevated levels of β -catenin, we examined the commonly used colon

cancer cell line HCT116. As in HT29 cells, depletion of CARM1 from HCT116 cells with either of two different shRNAs (Supplementary Fig. S5A) caused reduced expression of several Wnt target genes: Axin2, S100A4, and c-myc (Supplementary Fig. S5B). Expression of GPR49 mRNA (another Wnt target gene which required CARM1 in HT29 cells (Fig. 3D)) was too low to be quantified in HCT116 cells (data not shown). Colony formation of HCT116 cells plated at low density on standard tissue culture dishes was also substantially reduced by depletion of CARM1 by either of the two shRNAs against CARM1 (Supplementary Fig. S5C). Thus, the requirement of CARM1 for expression of Wnt/ β -catenin target genes and for colony formation is characteristic in multiple colon cancer cell lines with constitutively high β -catenin levels.

Wnt3a ligand induces recruitment of β -catenin and CARM1 to WREs associated with endogenous Wnt target genes

CARM1 depletion reduced expression of Wnt/ β -catenin target genes in HT29 and HCT116 cells, indicating that CARM1 is involved in the transcriptional activation process directed by β -catenin. However, crosstalk between the Wnt/ β -catenin cascade and other signaling cascade(s) which converge on the regulation of the same genes has been reported, and thus it is possible that the action of CARM1 on Wnt/ β -catenin target gene expression is not the result of direct interaction of CARM1 with β -catenin at LEF/TCF binding sites, but through interaction of CARM1 with other transcription factors associated with the same promoters. Therefore, we identified a cell system which allowed us to test the role of CARM1 in Wnt-inducible expression of endogenous target genes.

RKO human colon carcinoma cells have low cellular levels of β -catenin protein; however, after 1 hour of stimulation with purified Wnt3a ligand, we observed both nuclear and cytosolic accumulation of β -catenin (Fig. 5A). In addition, mRNA levels of two Wnt target genes, Axin2 and GPR49, were induced more than three fold after a seven hour exposure to Wnt3a ligand (Fig. 5B). Equivalent results were obtained with either purified Wnt3a or Wnt3a contained in conditioned medium produced by L cells stably expressing Wnt3a. Treatment of RKO cells with conditioned medium containing Wnt3a caused a similar elevation of β -catenin protein levels and mRNA levels of Axin2 and GPR49 (data not shown). The mRNAs from many other known target genes of Wnt (including c-myc, c-jun, Fra-1, Survivin, and BMP4) were expressed in RKO cells but were further elevated very little or not at all by treatment with Wnt3a (data not shown), presumably because their expression is already elevated by deregulation of other signaling pathways and is therefore not dependent on Wnt in these cells.

In ChIP experiments on cells grown for 1 hour in Wnt3a conditioned medium or control conditioned medium, β -catenin was recruited to the endogenous WRE of the Axin2 gene in the native chromosomal site in response to Wnt3a (Fig. 5C). Additionally, we detected the association of endogenous CARM1 with the WRE in a Wnt3a-dependent manner, and the nucleosomes located near the native Axin2-WRE acquired CARM1-dependent dimethylation on histone H3 Arginine 17 (H3R17me₂). For the other Wnt-inducible target gene in RKO cells, GPR49, the WRE responsible for its Wnt-inducible expression has not been identified. However, although the expression of other classical Wnt target genes is not upregulated by Wnt in these cells, it is still possible that β -catenin is recruited to the WREs associated with these genes in response to Wnt. Therefore, we examined occupancy of the WRE associated with the Survivin gene, a classical Wnt target gene which is expressed in RKO cells but is not further induced by Wnt3a (data not shown). We observed that, as with the Axin2 WRE, Wnt3a treatment increased occupancy of the Survivin WRE by both β -catenin and CARM1 (Supplementary Fig. S6). Thus, Wnt-inducible occupancy by β -catenin and CARM1 is observed even when Wnt does not cause increased expression of a classical

Wnt target gene. Our results demonstrate that RKO cells represent a novel and convenient system for studying the mechanism of Wnt regulated gene expression.

CARM1 is required for Wnt3a-dependent transcriptional activation

To assess the requirement for CARM1 in Wnt3a-dependent activation of the Axin2 and GPR49 genes, CARM1 expression was efficiently and specifically silenced in RKO cells by infection with lentiviral vectors encoding shRNA against CARM1 (Fig. 6A). Upon CARM1 silencing, the Wnt3a-induced increase in Axin2 and GPR49 was almost eliminated, while normal induction of expression of these genes was observed in cells expressing a non-specific shRNA (Fig. 6B). However, depletion of CARM1 did not interfere with the Wnt3a-induced accumulation of β -catenin (Fig. 6A). Thus, the influence of CARM1 in Wnt3a/ β -catenin signaling is not due to an effect on β -catenin protein accumulation but rather events downstream.

Wnt3a-dependent recruitment of CARM1 to Axin2 WRE requires β -catenin

In order to examine the mechanism of CARM1 recruitment to WREs and the contributions of CARM1 to Wnt3a-dependent target gene activation, ChIP assays were performed for the Wnt response elements in the Axin2 promoter in RKO cells expressing shRNA against CARM1, β -catenin, or a non-specific sequence. Immunoblots confirmed the efficient and specific depletion of CARM1 as well as the expected increase in β -catenin protein levels in response to conditioned medium containing Wnt3a (Fig. 6C). ChIP assays demonstrated that depletion of CARM1 essentially eliminated the Wnt3a-induced dimethylation of arginine 17 of histone H3 in nucleosomes associated with the Axin2 WRE; but CARM1 depletion had no effect on the Wnt3a-regulated recruitment of β -catenin to the Axin2 WRE (Fig. 6D). In contrast, reduction of cellular levels of β -catenin by either of two different shRNAs (Fig. 6E) diminished the recruitment of CARM1 to the Axin2 WRE (Fig. 6F). Thus, β -catenin recruitment does not depend on the recruitment of CARM1; instead β -catenin recruitment is required for the recruitment of CARM1. This shows that CARM1 is required for events downstream from the recruitment of β -catenin and CARM1, including the dimethylation of arginine 17 of histone H3. These results are consistent with the results of transient reporter gene assays, which showed that CARM1 action as a coactivator for LEF1 is dependent on the presence of β -catenin (Fig. 2).

Discussion

The Wnt/ β -catenin signaling cascade is highly conserved during evolution and plays a crucial role in the embryonic development of all animal species. Wnt signals regulate cellular processes such as cellular proliferation, survival and differentiation; misregulation of this signaling pathway is heavily implicated in the development of cancers, most notably colorectal cancers (3, 4). Uncontrolled Wnt signaling leads to constitutive activation of many oncogenes which are tightly regulated and only activated transiently during normal development. The pivotal mediator which responds to upstream Wnt signals and executes the downstream gene activation program is β -catenin. Stimulatory Wnt ligands induce elevated levels of β -catenin, which binds to transcription factors associated with the promoters of Wnt target genes; β -catenin recruits other transcriptional coactivators which activate transcription of the Wnt target genes (3, 19). Identification and functional investigation of proteins that bind β -catenin and cooperate in regulation of Wnt target genes will thus provide novel and exciting insights into the highly complicated mechanism of Wnt target gene activation. β -catenin-associated coactivators may also represent novel targets for therapeutic intervention against cancers with dysregulated Wnt signaling.

CARM1 is a member of the protein arginine methyltransferase family, and it methylates a large number of cellular substrates including histones as well as non-histone proteins involved in transcription, RNA processing, and other cellular functions. Deregulation of CARM1 expression is implicated in the pathogenesis of cancers, such that CARM1 expression has been correlated with tumor staging (20). CARM1 functions as a transcriptional coactivator for several different types of DNA-binding transcriptional activator proteins, and thus altered CARM1 expression likely affects many transcriptional programs which target genes that control proliferation rate or other oncogenic properties. In fact, studies from the MCF7 breast cancer cell line showed that CARM1 is a positive regulator of estrogen receptor-responsive genes and is essential for estrogen-stimulated growth and proliferation of breast carcinomas (21). Therefore, to further explore the mechanistic knowledge of how deregulated CARM1 might transform cells, we tested whether CARM1 is transcriptionally involved in oncogenic activation of the Wnt/ β -catenin signaling cascade in human colorectal carcinomas.

We showed that CARM1 interacts with β -catenin (Fig. 1) and cooperates with β -catenin at the transcriptional level in transient reporter gene assays (Fig. 2). In a more physiologically relevant model, CARM1 was essential for expression of several Wnt target genes (including known oncogenes) in the HT29 and HCT116 colorectal cancer cell lines (Fig. 3D and Supplementary Fig. S5), which have aberrant activation of Wnt/ β -catenin signaling and thus constitutive β -catenin regulated transcription. In HT29 cells the relative requirement for CARM1 versus β -catenin varied on the four different Wnt target genes tested: Expression of GPR49 and Axin2 was affected more strongly by depletion of β -catenin than by depletion of CARM1, while expression of S100A4 and c-Myc was affected to approximately equal extents by depletion of each of the two coactivators (Fig. 3D). These results suggest that the Wnt/ β -catenin pathway plays a more dominant role in controlling expression of Axin2 and GPR49 than c-Myc and S100A4. In fact, Wnt/ β -catenin signaling is only one of several signaling cascades regulating expression of c-Myc and S100A4; c-Myc expression is known to be regulated by many transcription factors, including nuclear receptors and AP1 (22, 23), and the S100A4 gene is reported to be under the control of transcription factor NFAT5 (nuclear factor of activated T-cells 5) (24).

Since CARM1 works as a coactivator for multiple transcription factors, and since Wnt target genes are controlled by Wnt/ β -catenin signaling and by transcription factors regulated by other signaling cascades (13, 25), it is difficult to assess whether the requirement of CARM1 for constitutively high expression of Wnt target genes in cells with deregulated Wnt/ β -catenin signaling is due to CARM1 actions through LEF1/ β -catenin or through other transcription factors that bind to the same promoter. To address this problem we identified a cell line in which we could observe Wnt-inducible gene expression. After screening a number of colon cancer cell lines we identified RKO as a colon cancer cell line which has normal Wnt/ β -catenin signaling. Importantly, in RKO cells the induction of Axin2 and GPR49 transcription by Wnt3a ligands was blocked by CARM1 depletion (Fig. 6B), and Wnt3a ligand caused recruitment of CARM1 to the WRE of the Axin2 promoter, resulting in enhanced methylation of Arg 17 of histone H3 in nucleosomes associated with the WRE (Fig. 5C). Together, these results indicate that CARM1 acts directly at the Axin2 promoter to mediate the actions of LEF1/ β -catenin in response to Wnt signaling. Therefore, CARM1 is a bona fide coactivator for Wnt/ β -catenin signaling.

Our results also have begun to dissect the mechanism of CARM1 action. Depletion of β -catenin from RKO cells diminished Wnt-induced occupancy of the Axin2 WRE by CARM1, thus demonstrating that CARM1 recruitment in response to Wnt3a depends upon the presence of β -catenin. In contrast, depletion of CARM1 levels did not diminish Wnt-induced accumulation of β -catenin in the cells or the recruitment of β -catenin to the Wnt target gene;

instead, CARM1 depletion reduced methylation of Arg 17 on histone H3 at the WRE (Fig. 6D). These findings indicate that mechanistically, CARM1 acts downstream from β -catenin and facilitates transcription complex formation by facilitating steps that are downstream from the recruitment of β -catenin and CARM1 to the promoter. In the LEF1/ β -catenin reporter gene system, methyltransferase activity of CARM1 was not required for its transcriptional coactivator activity (Fig. S1). The C-terminal activation domain of CARM1 may be responsible for the coactivator function of CARM1 in this case (26). In addition, the importance of CARM1 enzymatic activity in the expression of endogenous Wnt target genes should be tested, given that 1) plasmid-based reporter gene studies may omit the contribution of core histone and its tail to the regulation of transcriptional activation, and 2) previous reports have indicated that arginine-specific histone methylation by CARM1 is a significant part of the transcriptional activation process for some genes.

Abnormal expression of CARM1 has been linked to human prostate (20, 27), breast (28), and colorectal cancers (29). In this report, we showed that CARM1 protein levels are higher in a panel of colorectal cancer cell lines than in a cell line that more closely resembles normal colon epithelial cells (Fig. 3A). Moreover, CARM1 silencing adversely affected clonal survival and anchorage-independent growth of colorectal cancer cell lines with constitutively high levels of β -catenin (Fig. 4 and Supplementary Figure S5). These CARM1-dependent phenotypes are presumably due to the fact that CARM1 depletion causes reduced expression of genes involved in Wnt-driven tumorigenesis (e.g. c-myc (5)), metastasis (e.g. S100A4 (8)) and prevention of apoptosis (e.g. GPR49 (10)). Thus, CARM1 plays a key role in Wnt signaling through its role as a transcriptional coactivator that mediates the actions of β -catenin on Wnt target genes; some of the Wnt target genes that require CARM1 are critical for maintaining important aspects of the transformed phenotype of colon cancer cell lines. Therefore, selective inhibition of CARM1 methyltransferase activity or CARM1 binding to β -catenin may be a potential strategy for therapeutic treatment of abnormally activated Wnt/ β -catenin signaling in colorectal cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Grants DK055274 to MRS and CA20535 to KRY from the National Institutes of Health.

We thank Ms. Kelly Chang for expert technical assistance.

References

1. Polakis P. Wnt signaling and cancer. *Genes Dev.* 2000; 14:1837–51. [PubMed: 10921899]
2. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature.* 2005; 434:843–50. [PubMed: 15829953]
3. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell.* 2009; 17:9–26. [PubMed: 19619488]
4. Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer.* 2008; 8:387–98. [PubMed: 18432252]
5. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. *Science.* 1998; 281:1509–12. [PubMed: 9727977]
6. Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. *Cancer Res.* 2001; 61:8664–7. [PubMed: 11751382]

7. Kim PJ, Plescia J, Clevers H, Fearon ER, Altieri DC. Survivin and molecular pathogenesis of colorectal cancer. *Lancet*. 2003; 362:205–9. [PubMed: 12885482]
8. Stein U, Arlt F, Walther W, et al. The metastasis-associated gene S100A4 is a novel target of beta-catenin/T-cell factor signaling in colon cancer. *Gastroenterology*. 2006; 131:1486–500. [PubMed: 17101323]
9. Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature*. 2007; 449:1003–7. [PubMed: 17934449]
10. McClanahan T, Koseoglu S, Smith K, et al. Identification of overexpression of orphan G protein-coupled receptor GPR49 in human colon and ovarian primary tumors. *Cancer Biol Ther*. 2006; 5:419–26. [PubMed: 16575208]
11. Bedford MT, Clarke SG. Protein arginine methylation in mammals: who, what, and why. *Mol Cell*. 2009; 33:1–13. [PubMed: 19150423]
12. Lee YH, Stallcup MR. Minireview: protein arginine methylation of nonhistone proteins in transcriptional regulation. *Mol Endocrinol*. 2009; 23:425–33. [PubMed: 19164444]
13. Koh SS, Li H, Lee YH, Wideltz RB, Chuong CM, Stallcup MR. Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators. *J Biol Chem*. 2002; 277:26031–5. [PubMed: 11983685]
14. Ou CY, Kim JH, Yang CK, Stallcup MR. Requirement of cell cycle and apoptosis regulator 1 for target gene activation by Wnt and beta-catenin and for anchorage-independent growth of human colon carcinoma cells. *J Biol Chem*. 2009; 284:20629–37. [PubMed: 19520846]
15. LaBonte MJ, Manegold PC, Wilson PM, et al. The dual EGFR/HER-2 tyrosine kinase inhibitor lapatinib sensitizes colon and gastric cancer cells to the irinotecan active metabolite SN-38. *Int J Cancer*. 2009; 125:2957–69. [PubMed: 19536776]
16. Hecht A, Vlemminckx K, Stemmler MP, van Roy F, Kemler R. The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J*. 2000; 19:1839–50. [PubMed: 10775268]
17. Sun Y, Kolligs FT, Hottiger MO, Mosavin R, Fearon ER, Nabel GJ. Regulation of beta -catenin transformation by the p300 transcriptional coactivator. *Proc Natl Acad Sci U S A*. 2000; 97:12613–8. [PubMed: 11050151]
18. Roose J, Huls G, van Beest M, et al. Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science*. 1999; 285:1923–6. [PubMed: 10489374]
19. Mosimann C, Hausmann G, Basler K. Beta-catenin hits chromatin: regulation of Wnt target gene activation. *Nat Rev Mol Cell Biol*. 2009; 10:276–86. [PubMed: 19305417]
20. Hong H, Kao C, Jeng MH, et al. Aberrant expression of CARM1, a transcriptional coactivator of androgen receptor, in the development of prostate carcinoma and androgen-independent status. *Cancer*. 2004; 101:83–9. [PubMed: 15221992]
21. Frietze S, Lupien M, Silver PA, Brown M. CARM1 regulates estrogen-stimulated breast cancer growth through up-regulation of E2F1. *Cancer Res*. 2008; 68:301–6. [PubMed: 18172323]
22. Iavarone C, Catania A, Marinissen MJ, et al. The platelet-derived growth factor controls c-myc expression through a JNK- and AP-1-dependent signaling pathway. *J Biol Chem*. 2003; 278:50024–30. [PubMed: 14523011]
23. Dubik D, Shiu RP. Transcriptional regulation of c-myc oncogene expression by estrogen in hormone-responsive human breast cancer cells. *J Biol Chem*. 1988; 263:12705–8. [PubMed: 3045126]
24. Chen M, Sinha M, Luxon BA, Bresnick AR, O'Connor KL. Integrin alpha6beta4 controls the expression of genes associated with cell motility, invasion, and metastasis, including S100A4/metastasin. *J Biol Chem*. 2009; 284:1484–94. [PubMed: 19011242]
25. Chesnutt C, Burrus LW, Brown AM, Niswander L. Coordinate regulation of neural tube patterning and proliferation by TGFbeta and WNT activity. *Dev Biol*. 2004; 274:334–47. [PubMed: 15385163]
26. Teyssier C, Chen D, Stallcup MR. Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. *J Biol Chem*. 2002; 277:46066–72. [PubMed: 12351636]

27. Majumder S, Liu Y, Ford OH 3rd, Mohler JL, Whang YE. Involvement of arginine methyltransferase CARM1 in androgen receptor function and prostate cancer cell viability. *Prostate*. 2006; 66:1292–301. [PubMed: 16705743]
28. El Messaoudi S, Fabbizio E, Rodriguez C, et al. Coactivator-associated arginine methyltransferase 1 (CARM1) is a positive regulator of the Cyclin E1 gene. *Proc Natl Acad Sci U S A*. 2006; 103:13351–6. [PubMed: 16938873]
29. Kim YR, Lee BK, Park RY, et al. Differential CARM1 expression in prostate and colorectal cancers. *BMC Cancer*. 10:197. [PubMed: 20462455]

Fig. 1. Interaction between CARM1 and β-catenin

GST pulldown assays were performed as described in Materials and Methods, using *in vitro* translated HA-tagged proteins and bacterially generated GST fusion protein bound to glutathione-Sepharose beads. Bound proteins were detected by immunoblot analysis using antibodies against HA tag. The diagram shows the central methyltransferase domain and N-terminal and C-terminal domains of CARM1.

Fig. 2. Cooperation of CARM1 and β -catenin as coactivators for transcriptional activation by LEF1

(A) CV-1 cells were transfected in 12-well plates with luciferase reporter plasmid GK1-Luc (300 ng) containing multiple Gal4-binding elements, pM plasmids expressing Gal4-DBD alone or Gal4-DBD fused to LEF1 or β -catenin (100 ng), and pSG5.HA-CARM1 (100, 200 or 600 ng). Luciferase assays were performed on cell extracts as described under Materials and Methods. Results shown are from a single experiment and are representative of five independent experiments. (B) CV-1 cells were transfected with luciferase reporter plasmid pGL3OT (200 ng) controlled by LEF1-binding elements, pSG5.HA-LEF1 (0.5 ng), pSG5.HA- β -catenin (200 ng), and pSG5.HA-CARM1 (100, 200 or 300 ng). Results are from a single experiment, which is representative of three independent experiments. (C) As

in B, plasmids used for reporter gene assays were pGL3OT (200 ng), pSG5.HA-LEF1 (0.5 ng), pSG5.HA- β -catenin (200 ng), pSG5.HA-CARM1 (200 ng), and pCMV.p300 (200 ng).

Fig. 3. CARM1 regulates expression of endogenous target genes of β -catenin in colon cancer cell lines

(A) CARM1 expression in colon cancer cell lines. Total cell lysates from the indicated cell lines were analyzed by immunoblot with the indicated antibodies. (B) ChIP assays were done with HT29 cell chromatin, using antibodies against β -catenin or CARM1, or with normal rabbit IgG (IgG_1, Santa Cruz Biotechnology; IgG_2, Bethyl Laboratory). Immunoprecipitated DNA was analyzed by qPCR with primers for the WRE of the Axin2 gene or with primers for the 3'-untranslated region (3' UTR) of the Axin2 gene. Results shown are mean and range of variation of duplicate PCR reactions for one experiment and are representative of two independent experiments. (C) HT29 cells were infected with lentivirus encoding shRNA against CARM1, β -catenin, or a nonspecific sequence (NS), and populations of infected cells were analyzed by immunoblot with the indicated antibodies. (D) Total RNA from the lentivirus-infected cells in C was examined by qRT-PCR analysis, with primers specific for the indicated mRNAs. Results shown are normalized to the level of GAPDH mRNA, are the mean and range of variation for duplicate PCR reactions from a single experiment, and are representative of at least five independent experiments. p values from paired, two-tailed t tests for qRT-PCR analyses from n independent experiments comparing Wnt target gene mRNA levels in cells expressing shCARM1 and shNS were as follows: for Axin2, $p=0.041$ ($n=7$); for c-myc, $p=0.021$ ($n=7$); for GPR49, $p=0.016$ ($n=6$); for S100A4, $p=0.035$ ($n=5$).

Fig. 4. Role of CARM1 in HT29 cell proliferation, survival, and colony formation

(A) Populations of HT29 cells infected with the indicated lentivirus were plated in cell culture dishes, and cell proliferation was monitored on the indicated days by MTS assay as described in Materials and Methods. (B) Colony formation assays were performed in 6-well cell culture plates with the lentivirus-infected HT29 cell populations from A. Viable colonies were fixed and stained with crystal violet. After washing, the stain absorbed by the cells was dissolved in 10% SDS solution and quantified by absorbance at 595 nm. Results shown are mean $\pm$ S.D. for 4 wells and are representative of two independent experiments. (C) To determine colony formation efficiency in soft agar, lentivirus-infected HT29 cell populations from A were mixed with agar and plated in 35-mm Petri dishes. Microscope images are shown with a scale bar representing 100 μ m. Colonies in each dish were counted by Molecular Imager Gel Doc XR system (Bio-Rad). Results shown are mean $\pm$ S.D. from triplicate culture dishes from a single experiment and are representative of two independent experiments.

Fig. 5. Expression of Wnt target genes and recruitment of coactivators to target gene promoters in response to Wnt3a

(A) RKO cells were treated with purified Wnt3a ligand for 1 hr. Cells were then harvested, and nuclear and cytosolic fractions were analyzed by immunoblot with the indicated antibodies. (B) RKO cells were treated with purified Wnt3a or untreated and harvested at the indicated times for qRT-PCR analysis of the indicated mRNAs. Results shown are normalized to the level of GAPDH mRNA, are the mean and range of variation for duplicate PCR reactions from a single experiment, and are representative of at least two independent experiments. (C) Sheared chromatin from RKO cells grown with Wnt3a conditioned medium (Wnt3a-CM) or Control conditioned medium (1 hr treatment) was immunoprecipitated with the indicated antibodies. Immunoprecipitated DNA was analyzed by qPCR with primers for the WRE of the Axin2 gene. ChIP results shown are from a single experiment, which is representative of two independent experiments.

Fig. 6. CARM1 is required for expression of Wnt target genes in response to Wnt3a

(A–B) RKO cells were infected with lentivirus encoding shRNA against CARM1 or a nonspecific sequence (NS). Infected cell populations grown in the absence or presence of purified Wnt3a for 7 hr were then harvested for immunoblots (A) or for qRT-PCR (B). The levels of *GPR49* and *Axin2* were normalized to that of *GAPDH* mRNA. Results shown in the bar graphs are the mean and range of variation for duplicate PCR reactions from a single experiment, and are representative of three independent experiments. *p* values were determined by paired, two-tailed *t* tests from the three independent experiments comparing the fold induction of Wnt target gene expression by Wnt3a in cells expressing shCARM1 and shNS (for *GPR49*, *p*=0.011; for *Axin2*, *p*=0.031) (C–D), Lentivirus-infected cell populations from A–B were grown for 1 hr in conditioned medium containing (Wnt3a CM) or lacking Wnt3a; cells were then harvested for either immunoblots using the indicated

antibodies (C) or ChIP assays with the indicated antibodies to examine occupancy of the WRE in the Axin2 promoter (D). (E & F) Populations of RKO cells infected with lentivirus encoding shNS or sh β -catenin were treated with Wnt CM or control CM for 1 hr. Treated cells were subjected to either immunoblot analysis using the indicated antibodies (E) or ChIP analysis with the indicated antibodies and primers for the Axin2 WRE as described in Materials and Methods (F). Results shown for all ChIP assays in Fig. 6 are the mean and range of variation for duplicate PCR reactions from a single experiment, and are representative of two independent experiments.