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RORα Attenuates Wnt/β-Catenin Signaling by PKCα-Dependent Phosphorylation in Colon Cancer

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SUMMARY

Wnt family members play diverse roles in development and disease. Noncanonical Wnt ligands can inhibit canonical Wnt signaling depending on the cellular context; however, the underlying mechanism of this antagonism remains poorly understood. Here we identify a specific mechanism of orphan nuclear receptor RORα-mediated inhibition of canonical Wnt signaling in colon cancer. Wnt5a/PKCα-dependent phosphorylation on serine residue 35 of RORα is crucial to link RORα to Wnt/β-catenin signaling, which exerts inhibitory function of the expression of Wnt/β-catenin target genes. Intriguingly, there is a significant correlation of reduction of RORα phosphorylation in colorectal tumor cases compared to their normal counterpart, providing the clinical relevance of the findings. Our data provide evidence for a role of RORα, functioning at the crossroads between the canonical and the noncanonical Wnt signaling pathways, in mediating transrepression of the Wnt/β-catenin target genes, thereby providing new approaches for the development of therapeutic agents for human cancers.

INTRODUCTION

The Wnt genes encode a large family of cysteine-rich secreted polypeptides that mediate diverse signaling processes. Aberrant activation of Wnt signaling plays an important role as a major driving force linked to developmental defects and tumorigenesis (Klaus and Birchmeier, 2008). Wnt signaling pathways have been divided into two categories: one is the canonical Wnt/β-catenin signaling pathway, and the other is the noncanonical Wnt/Ca^{2+} signaling pathway (Kühl et al., 2000; Veeman et al., 2003). In the canonical Wnt/β-catenin signaling pathway, the canonical Wnts bind to the Frizzled (Frz) family proteins and low-density lipoprotein receptor-related (LRP) 5 or 6, and this binding activates dishevelled (Dvl) and inhibits the activity of GSK-3β; this inhibition results in the stabilization and subsequent translocation of β-catenin to the nucleus for the regulation of target gene expression with T cell factor (TCF)/lymphoid enhancer factor (LEF) (Behrens et al., 1996; Molenaar et al., 1996).

Noncanonical Wnt signaling pathways affected by Wnt ligands such as Wnt5a have diverse and occasionally opposing roles (McDonald and Silver, 2009). Noncanonical Wnts are either antagonistic or synergistic to canonical Wnt signaling pathway, depending on their receptor context. *Wnt5a*-deficient mice show increased β-catenin signaling in the distal limb, indicating that Wnt5a is involved in the negative regulation of the Wnt/β-catenin signaling pathway (Nemeth et al., 2007). In contrast, Wnt5a has been shown to activate Wnt/β-catenin signaling in the presence of Frz4 and LRP5 (Mikels and Nusse, 2006). Given that the activation of the noncanonical Wnt signaling pathway results in intracellular Ca^{2+} release and activation of Ca^{2+} sensitive enzymes such as Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC), the noncanonical Wnt pathways are apparently different from the canonical Wnt pathway.

As Wnt/β-catenin signaling is crucial for the maintenance of cellular homeostasis, a variety of positive and negative cellular regulators have been identified using genetic, proteomic, and RNA interference-based screening approaches. Runx3 forms a ternary complex with TCF4/β-catenin and suppresses the DNA-binding activity of TCF4/β-catenin (Ito et al., 2008). Wilms tumor suppressor WTX antagonizes Wnt/β-catenin signaling by promoting ubiquitination and degradation of β-catenin (Major et al., 2007). Receptor tyrosine kinase-like orphan receptor (Ror) 2 is a Ror family of receptor tyrosine kinases and has been shown to act as a receptor or coreceptor for Wnt5a (Liu et al., 2008). A cyclin-dependent kinase member of the mediator complex, CDK8 has been shown to be necessary for

β-catenin-driven transcriptional activation (Firestein et al., 2008). Given that dysregulated transcriptional activity of β-catenin is crucial for colorectal tumorigenesis and progression, identification of genes that are responsible for genetic perturbations is important to explore complex malignant processes.

Members of the orphan nuclear receptor family play various roles in signal integration, including modulation of neurogenesis, homeostasis, and disease by positively or negatively regulating subsets of gene expression (Giguère, 1999; Mangelsdorf et al., 1995). The retinoic acid-related orphan nuclear receptor (ROR) α is a member of the orphan nuclear receptor family for which no cognate ligands have been identified thus far (Giguère, 1999). Given that nuclear receptors function as potent regulators of normal physiology as well as pathologies such as cancer, the orphan nuclear receptors can functionally interact with potent oncogenic systems, for example, the Wnt and PKC signaling pathways. This interaction might elicit changes in oncogenesis and cellular adhesion (Polakis, 2000). Compared to other classes of nuclear receptors, the function and related signaling pathways for the orphan nuclear receptor RORα have not yet been studied extensively.

Here we identify a critical role of RORα at the crossroads between the canonical and the noncanonical Wnt signaling pathways in attenuating β-catenin transcriptional activity in a phosphorylation-dependent manner in colon cancer, based on cell culture and colorectal carcinoma tissue studies. Our data demonstrate a so-far-unrevealed role of RORα in transrepression of the Wnt/β-catenin signaling pathway, thereby regulating cell proliferation and tumor progression.

RESULTS

Identification of β-Catenin as a Binding Protein for RORα

To investigate the as-yet-unidentified functional modules of RORα, we employed a Flag epitope-tag strategy and attempted to purify binding proteins for RORα (Figure 1A). Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) identified binding proteins for RORα purified from the Flag M2 affinity column (Figure 1A and see Table S1 available online). Transcriptional coactivators such as glucocorticoid receptor-interacting protein 1 (GRIP1) and β-catenin were copurified with Flag-RORα (Figure 1B). The presence of GRIP1 coactivator, a well-known binding partner for RORα (Atkins et al., 1999), confirms and delineates the functional link between these molecules.

The binding of β-catenin to RORα was confirmed by both immunoblotting analysis of elutes and an endogenous coimmunoprecipitation assay (Figures 1B and 1C). The binding site mapping for RORα on β-catenin indicated that RORα interacted with the armadillo repeat domains of β-catenin and not with the N- and C-terminal domains, which overlap with the binding sites for a subset of coactivators (Figure 1D). These data suggest that RORα might function as a coregulator for Wnt/β-catenin signaling. Together, the affinity purification of RORα-binding proteins and the identification of β-catenin as a binding protein for RORα suggest the probable functional link between the RORα and the Wnt/β-catenin signaling pathways.

We used the HCT116 colorectal cancer cell line in which Wnt/β-catenin signaling pathway is constitutively active and examined whether RORα is directly involved in the modulation of Wnt target genes. In addition to TOPFLASH reporter, which has a TCF/LEF binding site, Wnt target gene transcripts are used as a readout for Wnt signaling activation. Knockdown of β-catenin with shRNA or introduction of dominant-negative TCF that has impaired binding to β-catenin reduced the expression of Wnt/β-catenin target gene transcripts including *cyclin D1*, c-*Myc*, c-*Jun*, or *Axin* (Figure 1E) but had little or no effect on expression of non-Wnt/β-catenin target genes (Figure 1F), suggesting that the increased expression of Wnt/β-catenin target gene transcripts is related to specific Wnt signaling activation in HCT116 colon cancer cells.

We silenced the expression of endogenous RORα by using two different shRNAs and validated the functional knockdown effect of shRNAs by immunoblotting analysis (Figure 1G). Silencing of endogenous RORα by specific shRNAs caused further increase of Wnt target gene transcripts such as *cyclin D1*, *Axin*, or *LEF1* (Figure 1H). Consistent with the data, knockdown of RORα further activated the TOPFLASH and *cyclin D1* promoter-luciferase reporters (Figures 1I and 1J). These data suggest that RORα is involved in the attenuation of β-catenin-mediated transcriptional activation.

The RORα-Mediated Transcriptional Repression of Wnt/β-Catenin Target Genes

These unexpected findings that RORα attenuates β-catenin-mediated transcriptional activation led us to explore the molecular mechanism of the RORα-mediated transcriptional repression of Wnt/β-catenin target genes in detail. First, we examined whether RORα binds to TCF directly. Glutathione S-transferase (GST) pull-down assay revealed that TCF exhibited strong binding to β-catenin as reported (Miravet et al., 2002) but failed to bind RORα directly (Figure 2A). These data allowed us to exclude the possibility that RORα competitively displaces β-catenin from TCF for transcriptional repression. To examine whether RORα interacts with β-catenin and sequesters it away from TCF in a DNA-binding-independent manner or RORα transrepresses β-catenin-mediated transcription, we performed a two-step chromatin immunoprecipitation (ChIP) assay for RORα and β-catenin on the *cyclin D1* or c-*myc* promoters (Figure 2B). The two-step ChIP assay revealed that both RORα and β-catenin were simultaneously detected on the promoters. Since RORα failed to bind TCF directly, the recruitment of RORα to the Wnt/β-catenin target promoters appears to be mediated by β-catenin.

To further examine whether TCF is responsible for tethering RORα and β-catenin to the Wnt/β-catenin target promoters, we examined their binding by ChIP to TOPFLASH reporter containing the TCF-binding sites (Figure 2C). We found that, depending on the TCF DNA-binding sites on TOPFLASH reporter, TCF was recruited along with β-catenin and RORα, whereas FOPFLASH reporter with mutant TCF binding sites failed to recruit TCF, β-catenin, and RORα (Figure 2C). Consistent with the data, mutant *cyclin D1* promoter containing impaired TCF-binding site failed to recruit TCF, β-catenin, and RORα (Figure 2C). These data support the model that RORα is recruited to the TCF-binding

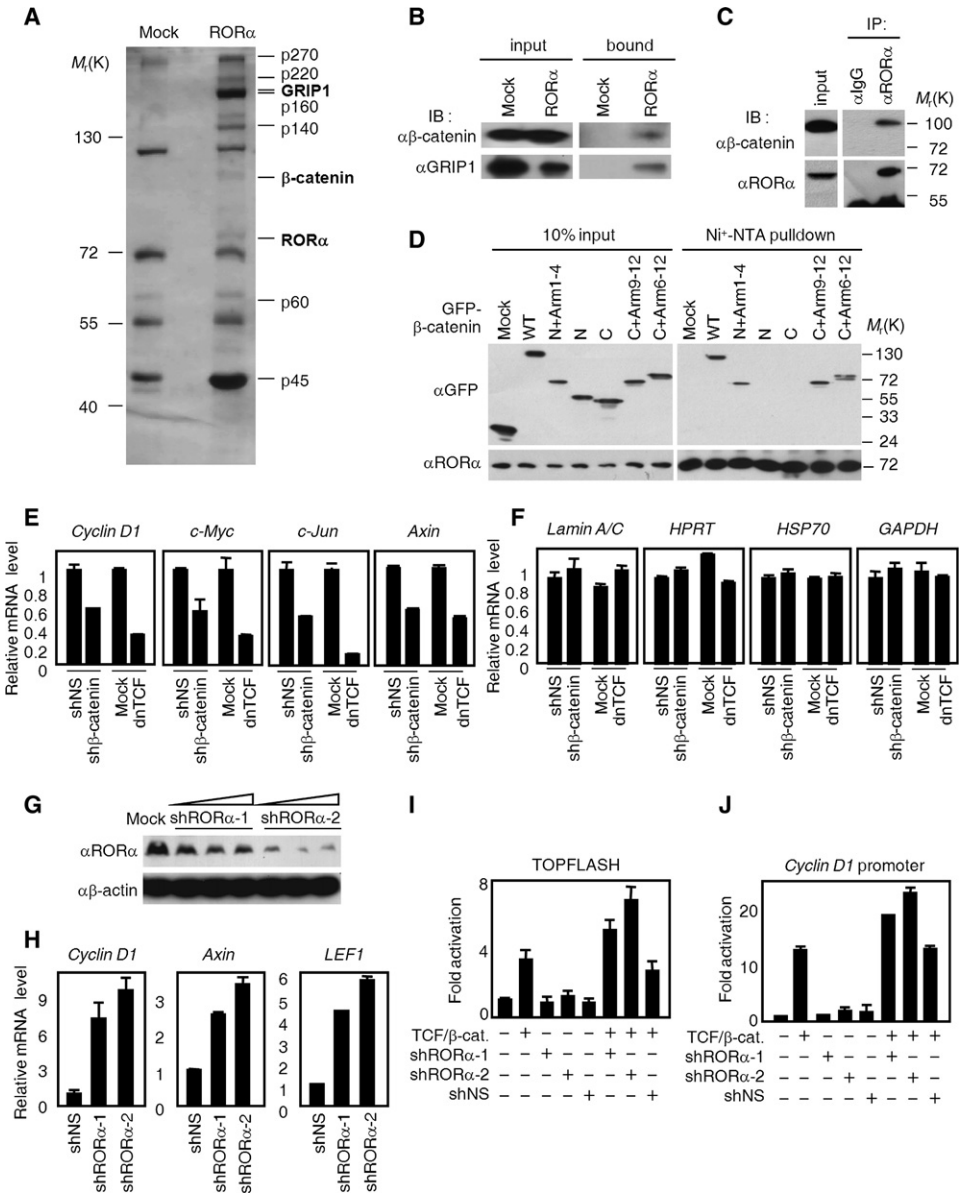

Figure 1. Purification of RORα-Binding Proteins and Attenuation of β-Catenin Transcriptional Activity by RORα

(A) RORα-binding proteins were purified from extracts obtained from HEK293 cells stably expressing Flag-tagged RORα. The bound proteins were resolved by SDS-PAGE and prepared for LC-MS/MS analysis.

(B) β-Catenin and GRIP1 were detected from the elutes by immunoblot analysis.

(C) Coimmunoprecipitation of endogenous RORα with β-catenin in HEK293 cells.

(D) Ni^{+}-NTA-agarose pull-down assay was performed with plasmids expressing each GFP-tagged β-catenin deletion construct and HisMax-tagged RORα. Each β-catenin construct spans the following amino acids; WT (aa 1–781), N+Arm1-4 (aa 1–381), N (aa 1–150), C (aa 667–781), C+Arm9-12 (aa 489–781), and C+arm6-12 (aa 361–781).

(E and F) Real-time quantitative RT-PCR analysis of the Wnt/β-catenin target gene transcripts (E) or the non-β-catenin target gene transcripts (F) in HCT116 cells in the presence of shRNA against β-catenin or dominant negative TCF. The quantity of mRNA was normalized by β-actin. Data are represented as mean ± SD for three independent experiments.

(G) Knockdown of RORα by two different shRNAs was validated by immunoblotting analysis 48 hr after transfection with each shRNA.

(H) Measurement of mRNA abundance of *cyclin D1*, *Axin*, and *LEF1* after knockdown of RORα by shRNAs in HCT116 colon cancer cells. Data are represented as mean ± SD for three independent experiments.

(I and J) Introduction of shRNA against RORα increased the transcriptional activation of the TOPFLASH reporter (I) or *cyclin D1* promoter-luciferase reporter (J). Data are represented as mean ± SD for three independent experiments.

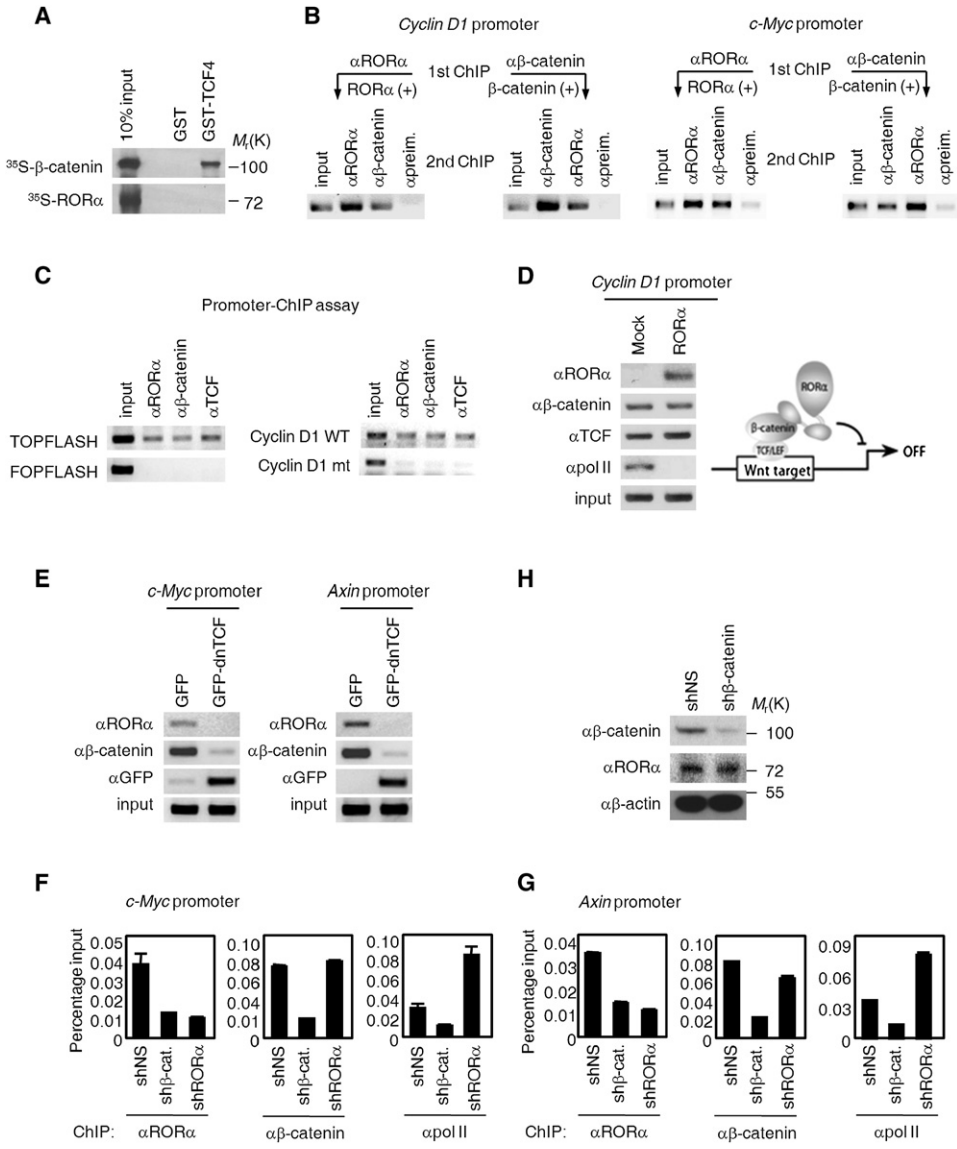

Figure 2. The Molecular Mechanism of the RORα-Mediated Transcriptional Repression of Wnt/β-Catenin Target Genes

(A) GST pull-down assay was conducted using in vitro-translated ^{35}S-methionine-labeled RORα or β-catenin with GST-TCF4 protein, revealing no direct interaction with RORα.

(B) Two-step ChIP assay was performed to determine whether RORα and β-catenin are assembled on the same promoter.

(C) Ability of TCF to recruit β-catenin and RORα by promoter ChIP assay in HCT116 cells. The binding of RORα, β-catenin, and TCF on the promoter was examined by ChIP assay to either TOPFLASH reporter containing the TCF-binding site or FOPFLASH reporter containing mutant TCF-binding site. ChIP assay was also conducted to either *cyclin D1* promoter encompassing TCF-binding region or mutant *cyclin D1* promoter containing impaired TCF-binding site.

(D) ChIP assay on *cyclin D1* promoter in HCT116 cells with overexpression of RORα. Occupancy of the promoter by β-catenin, TCF, and RNA polymerase II is indicated.

(E) ChIP assay was conducted on the c-*Myc* and *Axin* promoters from HCT116 cells transfected with either GFP or GFP-tagged dominant-negative TCF (GFP-dnTCF). Occupancy of the c-*Myc* and *Axin* promoters by β-catenin and RORα is indicated.

(F and G) The shRNA-coupled ChIP assay was performed on *cyclin D1* promoter (F) and *Axin* promoter (G) in HCT116 cells. Cells were harvested 48 hr after transfection with shRORα-2. Data are represented as mean ± SD for three independent experiments.

(H) Immunoblot analysis of RORα and β-catenin expression was performed 48 hr after transfection of shRNA against β-catenin.

site through β-catenin. Overexpression of RORα had little or no effect on the recruitment of TCF and β-catenin on the promoter but attenuated recruitment of RNA polymerase II and increased transcriptional repression (Figure 2D). Introduction of dominant-negative TCF, which has impaired binding to β-catenin, almost completely abolished recruitment of both β-catenin and RORα to the c-*Myc* and *Axin* promoters, suggesting that both β-catenin and RORα bind to the promoter via TCF (Figure 2E).

Further, we performed shRNA-coupled ChIP assay on c-*Myc* and *Axin* promoters by employing shRNAs against RORα or β-catenin (Figures 2F and 2G). Knockdown of β-catenin resulted in the diminished RORα recruitment along with decreased RNA polymerase II recruitment, suggesting that the recruitment of RORα to the promoter is through β-catenin. On the other hand, the knockdown of RORα did not change the recruitment of β-catenin; however, it induced a significant increase in the recruitment of RNA polymerase II (Figures 2F and 2G). Total protein levels of RORα were not affected by knockdown of β-catenin (Figure 2H). These data clearly suggest that the binding of RORα on the Wnt/β-catenin target promoters is mediated through β-catenin and that the binding of RORα to β-catenin confers a repressive effect on the expression of Wnt/β-catenin target genes.

RORα Is Phosphorylated by Protein Kinase Cα on Serine 35

To investigate the domains of RORα that are capable of direct physical interactions with β-catenin, we prepared various RORα deletion mutants (Figure 3A). Ni^{+}-NTA-agarose pull-down assay indicated that the N-terminal domain (NTD) of RORα is responsible for direct β-catenin binding (Figure 3B). In order to test the possibility of whether β-catenin colocalizes with RORα as a coregulator for RORα target gene promoters as well as Wnt/β-catenin target gene promoters, ChIP assay was performed on the *Pcp4* and *Shh* promoters (Figure S1A). Expression of RORαΔNTD, which has deleted NTD, failed to recruit β-catenin on the *Pcp4* and *Shh* promoters. The luciferase assay performed on RORE-luciferase reporter revealed that RORαΔNTD exhibited diminished transcriptional activation (Figure S1B). These data confirm that the binding of RORα to β-catenin is mediated by NTD of RORα. We further prepared serial deletion mutants of the NTD of RORα and wished to search for molecular determinants of β-catenin binding (Figure 3C). Fine deletion mapping revealed that the N-terminal fragment of RORα spanning amino acids 32–41 is indispensable for direct β-catenin binding (Figure 3D). Identification of a PKC consensus site (S/T-X_{0-2}R/K_{1-3}) in the region of RORα spanning amino acids 32–41 permitted us to perform a coimmunoprecipitation assay with various PKCs to investigate whether RORα binds to specific PKC isoforms. In vivo coimmunoprecipitation assays indicated that RORα specifically bound to PKCα, whereas other isoforms of PKCs including PKCβ2, PKCχ, PKCδ, and PKCη failed to bind RORα (Figure 3E and data not shown).

The selective binding of PKCα to RORα led us to investigate whether PKCα is directly responsible for RORα phosphorylation. Mass spectrometric analysis of wild-type (WT) RORα after the PKCα kinase assay revealed that RORα is phosphorylated by PKCα (Figure 3F). Treatment of a PKC activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), increased phosphorylation of endogenous RORα after about 60 min (Figure 3G and data not shown). RORαS35A mutant, in which serine residue is mutated to alanine, failed to be phosphorylated by TPA treatment, whereas either WT or S39A mutant exhibited TPA-induced phosphorylation (Figure 3H).

To further examine whether PKCα directly phosphorylates RORα on S35, we performed an in vitro kinase assay using the constitutive active form of PKCα (caPKCα) or the kinase-deficient mutant form of PKCα (kdPKCα) immunoprecipitated from cell lysates with anti-Flag antibodies. The immunoprecipitated materials from either caPKCα or kdPKCα were incubated with bacterially expressed and purified GST-RORα WT or S35A proteins. Indeed, caPKCα phosphorylated purified RORα proteins, whereas kdPKCα failed to phosphorylate RORα proteins (Figure 3I). As expected, the RORαS35A proteins failed to be phosphorylated by caPKCα, thus confirming the S35 site-specific phosphorylation of RORα by PKCα. Immunohistochemical studies revealed that both RORα and RORαS35A exhibited an almost exclusive nuclear staining pattern in HCT116 cells, indicating that phosphorylation of RORα did not alter the nuclear localization (Figure 3J).

Phosphorylation of RORα by PKCα Is Crucial for Downregulation of Wnt/β-Catenin Target Genes

The antibody raised against phosphorylated RORαS35 peptide recognized the phosphorylated peptide exclusively, as assessed by dot blot analysis (Figure 4A). Immunoprecipitation analysis by purified anti-phospho-RORαS35 IgG revealed that RORα was subject to phosphorylation on S35 site by TPA treatment (Figure 4B). Consistent with these data, introduction of caPKCα, not kdPKCα, increased phosphorylation of RORα, whereas treatment of Go6976, a specific PKCα inhibitor, abolished the TPA-induced phosphorylation of RORα, as assessed by immunoblotting against anti-phospho-RORαS35 IgG (Figures 4C and 4D). These data demonstrate that TPA-dependent activation of PKCα is responsible for the phosphorylation of RORα on the S35 site.

Since protein phosphorylation alters the binding affinity of proteins, we examined whether the phosphorylation of RORα affected its binding affinity toward β-catenin. RORαS35D, which mimics constitutive phosphorylation of RORα, exhibited strong binding to β-catenin, whereas RORαS35A exhibited little or no binding to β-catenin (Figure 4E). Consistent with these data, the binding of RORα to β-catenin was significantly increased by TPA treatment, and TPA-induced increased binding was almost completely abolished by treatment with Go6976 (Figure 4F). Failure of TPA-induced phosphorylation of RORαS35A abrogated the binding of RORα to β-catenin, whereas S35D was insensitive to Go6976 treatment, confirming that phosphorylation of the S35 site of RORα is crucial for the binding to β-catenin (Figure 4F). These data clearly demonstrate that TPA/PKCα-dependent phosphorylation of RORα modulates the binding affinity of RORα toward β-catenin.

To further examine whether the RORα-mediated downregulation of Wnt target genes is affected by RORα phosphorylation that leads to increased binding to β-catenin, we performed a ChIP assay on *cyclin D1* promoter with the introduction of either RORαS35A or RORαS35D. As expected, RORαS35A exhibited diminished recruitment to the promoter along with increased RNA polymerase II recruitment, whereas RORαS35D resulted in increased recruitment to the promoter with diminished recruitment of RNA polymerase II (Figure 4G). TPA treatment for 90 min increased the recruitment of phosphorylated RORα on the *cyclin D1* promoter without altering β-catenin recruitment (Figure 4H). Further, knockdown of PKCα diminished the recruitment of RORα on the *cyclin D1* promoter, confirming

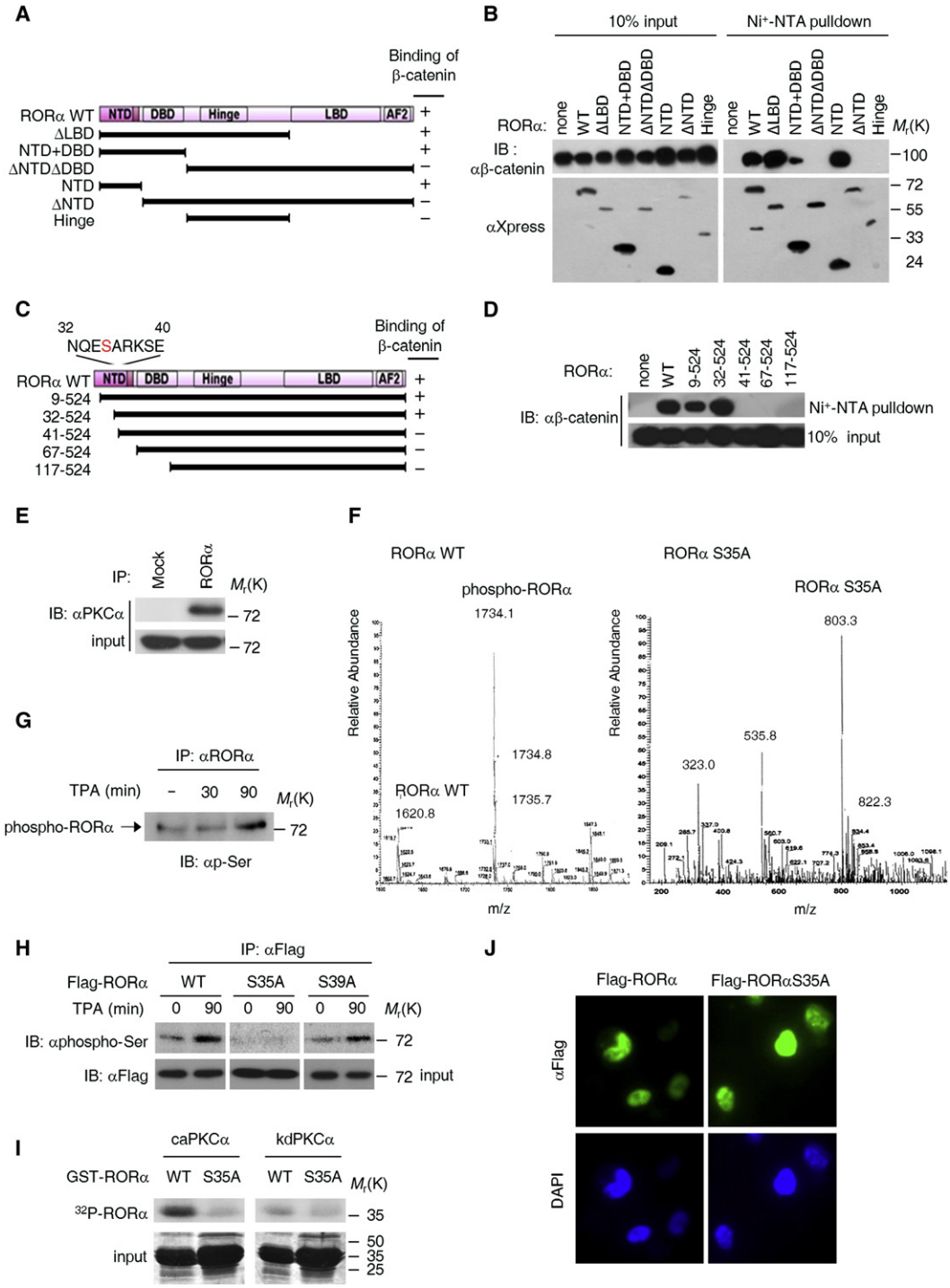

Figure 3. RORα Is Phosphorylated by PKCα on Serine 35

(A) Schematic representation of RORα showing the location of the NTD, the DNA-binding domain (DBD), the hinge region, the ligand-binding domain (LBD), and the AF-2 domain.

(B) Ni$^+$-NTA-agarose pull-down assay revealed that RORα segments that span amino acids 1–65 corresponding to NTD are sufficient to bind β-catenin. HEK293 cells were cotransfected with plasmids expressing each His-tagged RORα deletion construct and β-catenin. Whole-cell extracts (left panel) and Ni$^+$-NTA-agarose pull-down materials (right panel) were analyzed by immunoblotting against anti-β-catenin IgG or anti-Xpress IgG.

(C) Illustration of the structure of deletion fragments of RORα.

(D) Interaction of each RORα deletion construct with HisMax-tagged β-catenin was assessed by Ni$^+$-NTA-agarose pull-down assay.

(E) Coimmunoprecipitation of RORα with PKCα.

that the PKCα-dependent phosphorylation of RORα is responsible for increased binding of RORα on the promoter (Figure 4H). The functional knockdown effect of siRNA against PKCα was validated by immunoblotting analysis (Figure 4I).

To investigate whether PKCα-dependent phosphorylation of RORα and increased binding of phosphorylated RORα on the promoter can be extended to other Wnt target gene promoters, we have treated cells with TPA in the absence or presence of Go6976 on *Axin* promoter. ChIP assay on *Axin* promoter revealed that the TPA-induced enhanced recruitment of phosphorylated RORα on the promoter was reversed by Go6976 treatment (Figure 4J). To demonstrate that the recruitment of phosphorylated RORα on *cyclin D1* promoter is not limited to HCT116 colon cancer cell line, we also performed ChIP assays in other β-catenin-expressing colon cancer cell lines including SW620 and DLD-1. Either introduction of caPKCα or TPA treatment increased the recruitment of phosphorylated RORα on the promoter along with diminished RNA polymerase II in SW620 and DLD-1 cells as well as HCT116 colon cancer cells (Figures 4K and 4L).

We have treated cells with Wnt3a to induce transcriptional activation of canonical Wnt target genes and measured the TPA-mediated transcriptional repression in a more quantitative way in both ChIP assay and qRT-PCR (Figures 4M and 4N). ChIP assay revealed that TPA treatment increased recruitment of phosphorylated RORα on the *cyclin D1* promoter concomitant with decreased recruitment of RNA polymerase II, and qRT-PCR analysis confirmed that TPA treatment antagonized Wnt3a-induced transcriptional activation of the *Axin* and *cyclin D1* transcripts in HCT116 cells (Figures 4M and 4N). In accordance to the phosphorylation-triggered transrepression mechanism of RORα on Wnt target genes, TPA treatment antagonized Wnt3a-induced Wnt target gene expression, and the knockdown of either PKCα or RORα abolished TPA-mediated downregulation of Wnt target gene expression (Figures 4O and 4P). Taken together, these data strongly demonstrate that PKCα-dependent phosphorylation of RORα triggers increased binding of RORα to the target promoters through β-catenin and that this increased binding is directly responsible for the downregulation of Wnt target genes.

Wnt5a Antagonizes the Canonical Wnt Signaling by Transrepression Function of RORα

Since the noncanonical Wnt signaling pathway triggered by the Wnt5a activates downstream PKCs and CaMKII (Kühl et al., 2000), we examined whether Wnt5a induces PKCα activation leading to the following RORα phosphorylation and downregulation of Wnt target genes in colon cancer cells. Wnt5a treatment increased phosphorylation of PKCα as assessed by immunoblotting against anti-phospho-PKCα antibody that recognizes an active form of PKCα (Figure 5A). Further immunoblotting analysis against anti-phospho-RORαS35 IgG revealed that treatment of Wnt5a increased phosphorylation of RORα concomitant with downregulation of cyclin D1 expression in colon cancer cells (Figure 5A).

Next, we examined whether suppressive function of RORα mediated by Wnt5a can be extended to noncanonical Wnt5a/PKC downstream targets. It has been reported that Wnt5a/PKC pathway increases Snail expression and decreases E-cadherin expression independent of canonical Wnt/β-catenin signaling (Dissanayake et al., 2007). Immunoblotting analysis revealed that Wnt5a-induced phosphorylated RORα failed to suppress noncanonial Wnt5a/PKC downstream targets exemplified by Snail, and knockdown of RORα had little or no effect on expression of Snail (Figure 5B). In contrast, expression of cyclin D1 expression was diminished by Wnt5a treatment, and knockdown of RORα almost completely abolished the Wnt5a-dependent decreased expression of cyclin D1 (Figure 5B). Although we cannot exclude the possibility that phosphorylated RORα can suppress specific Wnt5a/PKC downstream targets in a similar way to canonical Wnt/β-catenin target genes, at least RORα failed to exert suppressive function on Wnt5a-mediated expression of Snail and E-cadherin in colon cancer cells.

Consistent with these data, Wnt5a treatment reduced Wnt3a-induced expression of *cyclin D1* and c-*myc* transcripts, and the knockdown of RORα or PKCα by each shRNA abolished Wnt5a-dependent downregulation of *cyclin D1* and c-*Myc* transcripts (Figures 5C and 5D). These data confirmed that the downregulation of Wnt target genes by Wnt5a is mediated by PKCα activation, and the antagonistic function of Wnt5a in Wnt3a-induced transcriptional activation of Wnt/β-catenin target genes is indeed mediated by RORα. Thereafter, we examined whether Wnt5a-dependent RORα phosphorylation is capable of suppressing β-catenin-mediated increase of *Axin* transcript. Quantitative RT-PCR analysis revealed that RORα WT, not RORαS35A, induced the Wnt5a-mediated downregulation of the *Axin* transcript (Figure 5E). These data suggest that RORα mediates Wnt5a-dependent suppressive effects on the canonical Wnt signaling pathway in a phosphorylation-dependent manner in colon cancer cells and establish RORα as an essential downstream of Wnt5a inhibiting the induction of Wnt/β-catenin target genes.

Given that RORα exerts its repressive effect by directly binding to β-catenin on the promoter and that the binding site for RORα on β-catenin resides in the armadillo repeat domains of β-catenin that overlap with the binding sites of a subset of coactivators

(F) Synthesized peptides of RORα WT (NQESARKSE) and RORαS35A (NQEAARKSE) were used as substrates in the kinase assay with PKCα enzyme. The phosphorylated peptide samples were analyzed by LC-MS analysis.
(G) HCT116 cells were treated with TPA at an indicated time period, and cell lysates were immunoprecipitated with anti-RORα antibody, followed by immunoblotting analysis against anti-phospho-Ser antibody indicating phosphorylated RORα at endogenous level.
(H) HCT116 cells were treated with TPA one day after transfection with Flag-RORα WT, S35A, or S39A. Immunoprecipitation assay was conducted with anti-Flag antibody, and the phosphorylated RORα was detected by immunoblot analysis with anti-phospho-Ser antibody.
(I) In vitro kinase assays using either constitutive active form (caPKCα) or kinase-deficient form of PKCα (kdPCKα) immunoprecipitated from cell lysates as the kinase and purified GST-RORα N-terminal WT or S35A proteins as substrates were performed. Phosphorylated RORα was detected by autoradiography.
(J) HCT116 cells were transfected with Flag-RORα WT or S35A and stained with antibodies directed against Flag epitope. The fluorescence-conjugated secondary antibody was visualized using fluorescence microscopy, and nuclear staining with DAPI was shown.

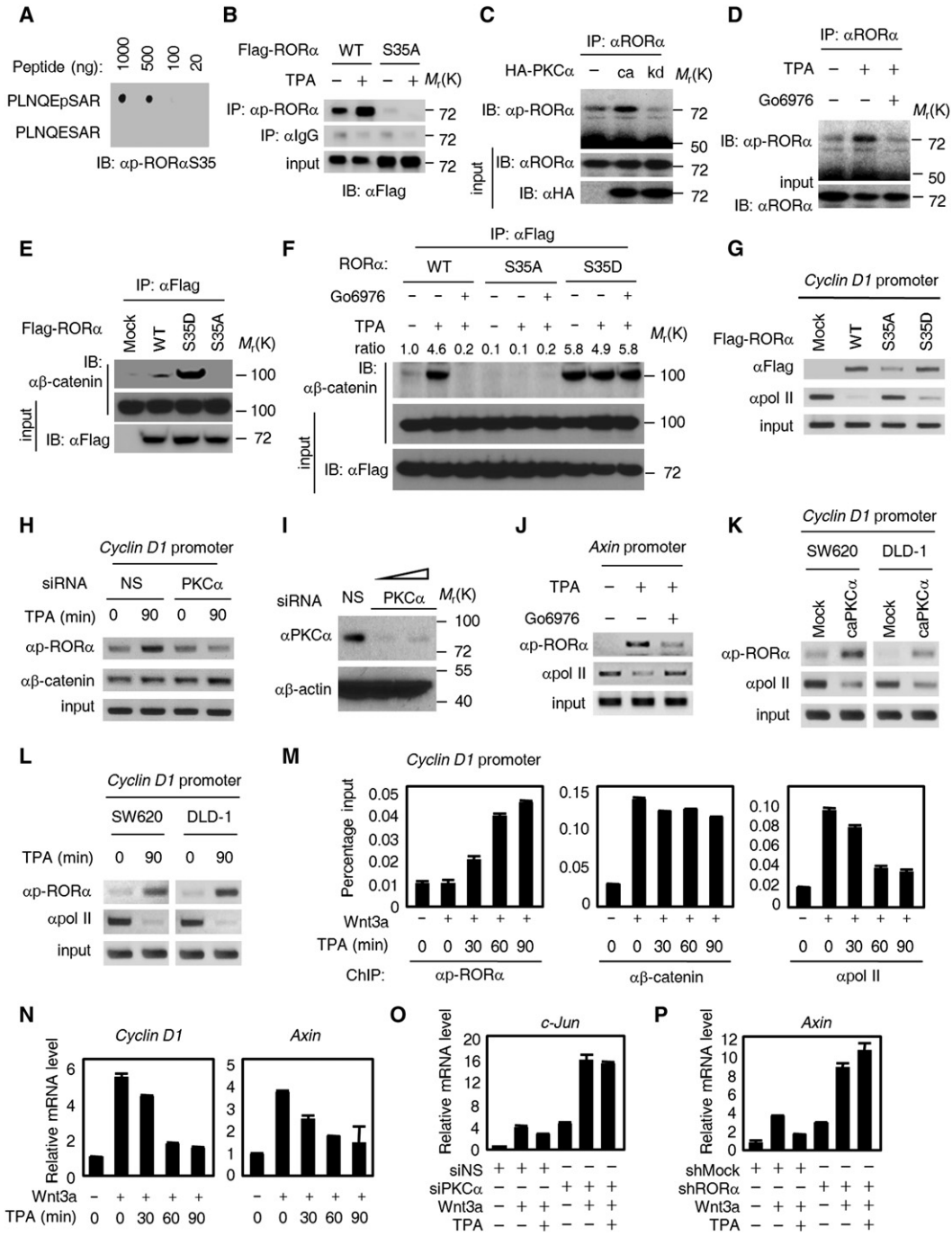

Figure 4. PKCα-Dependent Phosphorylation of RORα Is Crucial for Downregulation of Wnt/β-Catenin Target Genes

(A) The specificity of the antibody raised against phosphorylated RORαS35 peptide was assessed by dot blot analysis.

(B) Treatment of TPA increased phosphorylated RORα on S35 site as assessed by immunoprecipitation with anti-phospho-RORαS35 antibody.

(C) HCT116 cells were transfected with either HA-caPKCα or kdPKCα, and the cell extracts were subject to immunoblot analysis against anti-phospho-RORαS35 antibody.

(D) Treatment of Go6976, a PKCα inhibitor, abolished TPA-dependent phosphorylation of RORα.

(E) HCT116 cells were transfected with Flag-RORα WT, S35D, or S35A, and the cell extracts were immunoprecipitated with anti-Flag antibody followed by immunoblotting against anti-β-catenin antibody.

(F) Coimmunoprecipitation assay of β-catenin with either RORα WT, S35A, of S35D in HCT116 cells after treatment with TPA for 90 min in the absence or presence of Go6976.

(G) ChIP analysis on the *cyclin D1* promoter after transfection with Flag-RORα WT, S35A, or S35D in HCT116 cells.

(Figure 1D), the transrepression mechanism of RORα on β-catenin might be achieved by competition for β-catenin binding with a subset of coactivators. ChIP assay on the *cyclin D1* promoter revealed that treatment with Wnt5a or TPA increased the recruitment of phosphorylated RORα to the promoter, whereas the recruitment of CBP, p300, and pCAF coactivators to the promoter was significantly decreased (Figure 5F). Indeed, the TPA- or Wnt5a-dependent phosphorylation of RORα attenuated the β-catenin-dependent transcriptional activation, leading to the increased methylation of histone H3K9 and decreased RNA polymerase II recruitment (Figure 5F). These results indicate that the downregulation of Wnt target genes is a direct consequence of RORα binding triggered by phosphorylation, and the transrepression mechanism of RORα on β-catenin is achieved, at least in part, by competition with a subset of coactivators for β-catenin binding and possibly recruitment of histone lysine methyltransferases for transcriptional repression.

As upregulation of cyclin D1 or c-jun is correlated with cell proliferation and migration, we next examined whether phosphorylation of RORα could inhibit cellular migration. Transwell cell migration assay that measured the increase in cell number for RORα, RORαS35A, or RORαS35D-expressing HCT116 colon cancer cells revealed that Wnt5a treatment attenuated migration of HCT116 colon cancer cells compared to nontreated cells and that RORαS35D-expressing cells exhibited a significant decrease of cell migration (Figure 5G). These results suggest that a mechanism underlying RORα-mediated inhibition of cell migration is, at least in part, through the inhibition of Wnt target genes in a phosphorylation-dependent manner. We then considered other properties known to be important for cell and tumor growth. As anchorage-independent growth is an important property of tumor cell, we asked whether RORαS35D, but not RORαS35A, could suppress the colony-forming ability of HCT116 cells in soft agar. Consistent with the antiproliferative properties of RORαS35D, HCT116 cells expressing RORαS35D grew significantly slower than control cells, whereas RORαS35A-expressing cells failed to exhibit suppression of anchorage-independent growth (Figure 5H). Furthermore, the size of the colonies formed by RORαS35D-expressing cells was much smaller than those formed by the control cells. These data suggest that RORα has a significant role in regulating cellular growth in a phosphorylation-dependent manner.

Reduction of RORα Phosphorylation Is Frequent in Human Colorectal Cancers

To find the clinical relevance of our findings, we examined the expression of phosphorylated RORα in the 30 colorectal cancer tissues and matched normal mucosa specimens. Immunoblot analysis against anti-phospho-RORαS35 IgG revealed the reduction of RORα phosphorylation in 22 out of 30 (>70%) colorectal tumor cases compared to their normal counterpart, while the expression of pan-RORα remained almost constant (Figure 6A and Table S2). Consistent with the previous report (McGarrity and Peiffer, 1994), PKCα activity is reduced in the tumor samples, and immunoblotting analysis against anti-phospho-PKCα IgG confirmed the reduction of PKCα phosphorylation in 16 out of 30 (>50%) colorectal tumor cases compared to their normal counterpart (Figure 6A and Table S3). Statistical analysis confirmed that there is a significant correlation of reduction of RORα phosphorylation and PKCα phosphorylation in tumor tissue specimens compared to normal tissue specimens (Figures 6B and 6C). These data demonstrate that reduction of RORα phosphorylation is a frequent event in human colorectal cancer.

DISCUSSION

We identified the critical roles of RORα at the crossroads of the canonical and the noncanonical Wnt signaling pathways in colon cancer cells and human colorectal tumor tissues. RORα confers a transrepression function to the β-catenin-mediated transcriptional activation of Wnt target genes involved in tumor progression and growth, by the enhanced binding to β-catenin via the Wnt5a/PKCα-dependent phosphorylation on serine 35 of RORα and possibly by competing with other coactivators for binding to β-catenin (Figure 6D). These data provide a possible mechanistic explanation for the tumor suppressive activity of RORα and suggest that RORα might play important biological roles in cellular processes such as cell proliferation and tumor development, which are controlled by the Wnt and PKC pathways; this implicates the presence of higher levels of regulation that integrate different signaling pathways in human disease.

The canonical Wnt/β-catenin pathway regulates fundamental cellular functions including cell proliferation and tumorigenesis, largely by modulating gene transcription. Some members of the Wnt ligand family such as Wnt5a can activate noncanonical Wnt signaling other than the canonical Wnt signaling, depending on the types of the cell surface receptors, and Wnt5a overexpression has been reported to increase PKCs while inhibiting β-catenin activity (Davis et al., 2008; Mikels and Nusse, 2006). Although several lines of studies have investigated the link between the canonical and the noncanonical Wnt signaling pathways, the mechanisms of Wnt5a-mediated inhibition of the canonical Wnt pathway are unclear (Nemeth et al., 2007; Westfall et al., 2003). Our study shed light on the function of RORα as a critical modulator of Wnt signaling by providing the

(H) ChIP assay was performed on the *cyclin D1* promoter in the absence or presence of siRNA against PKCα with treatment of TPA for 90 min.
(I) Knockdown of PKCα by shRNA was validated by immunoblotting analysis.
(J) Chip assay was conducted on *Axin* promoter in HCT116 cells after treatment with TPA for 90 min in the absence or presence of Go6976 for 12 hr. Occupancy of the promoter by phospho-RORαS35 and RNA polymerase II is indicated.
(K and L) ChIP analysis on the *cyclin D1* promoter in SW620 and DLD-1 cells with either introduction of caPKCα (K) or TPA treatment for 90 min (L).
(M and N) ChIP analysis on the *cyclin D1* promoter (M) and real-time quantitative RT-PCR analysis of the *cyclin D1* transcript (N) in HCT116 cells after TPA treatment for indicated times after Wnt3a (100 ng/ml) stimulation for 12 hr. Data are represented as mean ± SD for three independent experiments.
(O and P) The knockdown of either PKCα (O) or RORα (P) abolished TPA-mediated downregulation of *Axin* or *cyclin D1* transcript after Wnt3a stimulation for 12 hr. RORα-2 shRNA was used in the experiment. Data are represented as mean ± SD for three independent experiments.

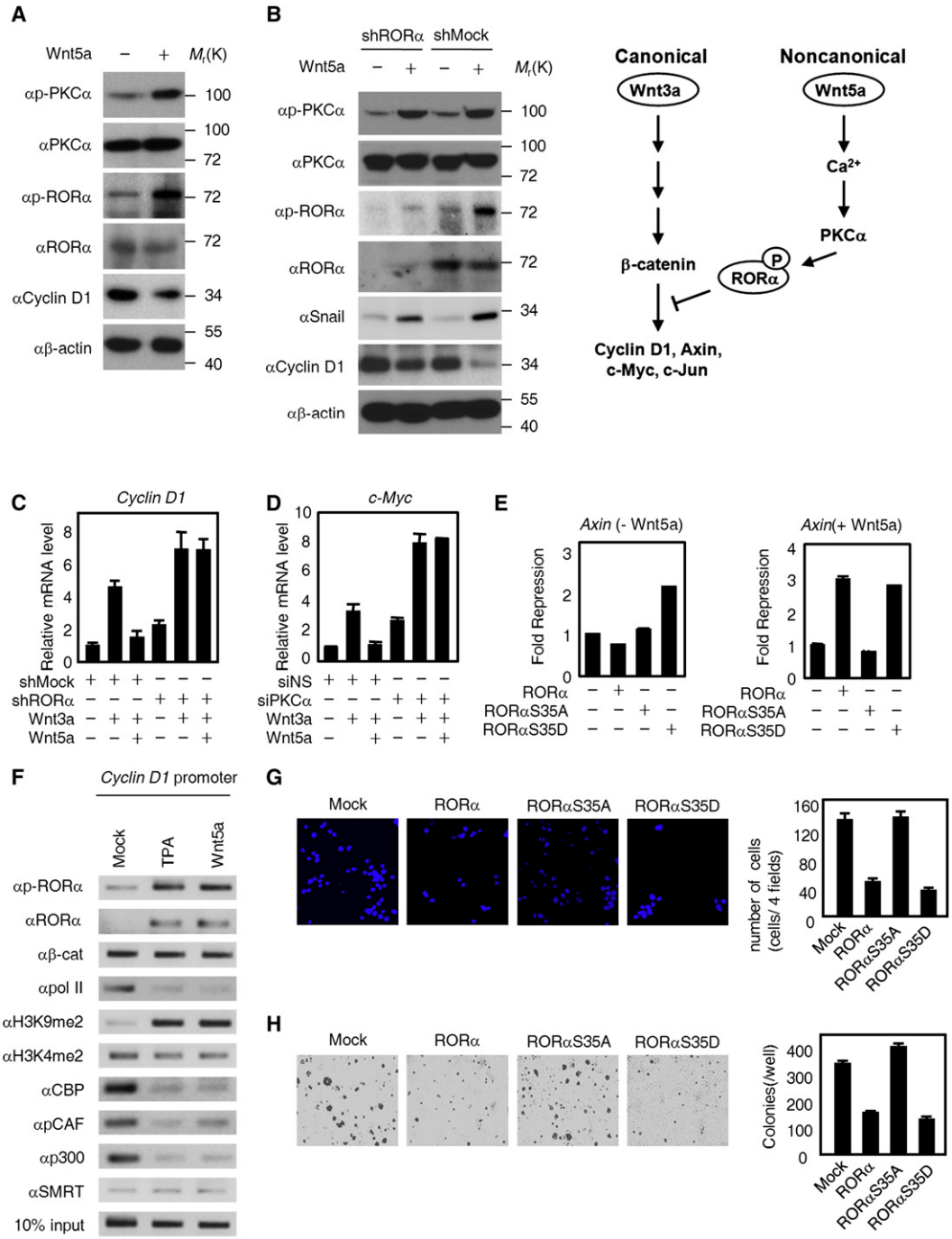

Figure 5. Wnt5a Antagonizes the Canonical Wnt Signaling by Transrepression Function of RORα

(A) Treatment of Wnt5a (100 ng/ml) for 12 hr increased phosphorylated RORα and PKCα but decreased cyclin D1 expression as assessed by immunoblot analysis against each antibody in HCT116 cells.

(B) Immunoblot analysis was conducted in DLD-1 cells in the absence or presence of shRORα after Wnt5a treatment for 12 hr. Expression of cyclin D1, Snail, and E-cadherin was assessed by each antibody.

(C and D) *Cyclin D1* transcript was measured after introducing shRNAs against RORα (C) or siRNA against PKCα (D) in the absence or presence of Wnt5a after Wnt3a treatment for 12 hr in HCT116 cells. Cells were pretreated with 100 ng/ml of Wnt3a for 12 hr and then treated with 100 ng/ml of Wnt5a for 12 hr. Data are represented as mean ± SD for three independent experiments.

(E) Real-time quantitative RT-PCR analysis of the *Axin* transcript in HCT116 cells after introducing RORα, RORαS35A, or RORαS35D with treatment of Wnt5a for 12 hr. Data are represented as mean ± SD for three independent experiments.

(F) ChIP assay on the *cyclin D1* promoter with treatment of either TPA for 90 min or Wnt5a for 12 hr in HCT116 cells.

molecular basis for the Wnt5a-mediated suppression mechanism of canonical Wnt signaling pathways in colon cancer. This antagonism between the canonical and the noncanonical Wnt signaling pathways may be an important mechanism for controlling oncogenesis and developmental processes. Thus, identification of key molecules that are responsible for genetic perturbations of canonincal Wnt signaling is important to understand complicated malignant processes in colorectal cancer. Further, the observation that loss of or reduced expression of Wnt5a in about 50% of Dukes B colon tumor supports clinical relevance of this signal axis (Dejmek et al., 2005). Our data provide a signaling axis in colon cancer that may be prototypic of cell- and context-specific strategies in certain types of cancers and developmental processes.

Staggerer (*Sg*) mice have a spontaneous loss-of-function phenotype of the *RORα* gene and block Purkinje cell differentiation, resulting in cerebellar hypoplasia and congenital ataxia (Hamilton et al., 1996; Steinmayr et al., 1998). Thus far, many studies have focused mainly on the analysis of cerebellar defects (Gold et al., 2003). Approximately 50% of the *Sg* mice die shortly after weaning, which makes studying RORα function with *Sg* mice very difficult (Doulazmi et al., 2006). Our studies have widened the spectrum of the roles of RORα to the negative regulation of tumor progression and proliferation in colon cancer using a combination of a proteomic approach and molecular mechanism approach in cell culture model and in clinical tissue samples of patients with colorectal carcinoma. RORα might be involved in the fine-tuning of physiological processes by integrating systematic regulatory crosstalk in order to coordinate attenuated transcriptional responses to tumor progression. Alternatively, it is conceivable that modulation of the phosphorylation status of RORα in certain physiological settings might be explained, at least in part, by the regulatory mechanism of the Wnt signaling pathways that influence diverse physiological phenomena.

The present study suggests a delicate and potentially complementary strategy to the possible therapeutic effects of RORα. Since we found that the N-terminal region of RORα including serine 35 can specifically block the β-catenin transcriptional activity depending on phosphorylation status, we suggest that this region may serve as a prototype for identifying antagonists that might act to prevent diseases possibly mediating the Wnt/β-catenin signaling pathway. It is tempting to suggest that the development of therapeutic reagents that can increase phosphorylation levels of RORα would be efficient for treating specific human cancers. Defining the cross-regulation of RORα and the Wnt/β-catenin signaling axis represents a powerful strategy in integrating transcriptional responses with specific signaling pathways, particularly those that are physiologically and pathologically relevant, and may provide additional strategies to modify therapeutic approaches for treatment of specific human cancers.

EXPERIMENTAL PROCEDURES

Materials and Reagents

The following antibodies were purchased from Santa Cruz Biotechnology: anti-β-catenin, cyclin D1, phospho-PKCα, and RORα. The following commercially available antibodies were used: anti-acetylated histone H3, acetyl-H4, dimethyl-H3K9, and dimethyl-H3K4 antibodies (Upstate Biotechnology); anti-Flag (Sigma); anti-RNA Polymerase II (Berkeley Antibody Company); anti-Xpress (Invitrogen); anti-phospho-Ser antibodies (Alexis); and anti-Snail (Cell Signaling). Anti-phospho-RORαS35 antibody was generated by Abmart (China). PKCα enzyme was purchased from Cell Signaling (catalog number 7578).

In Vitro Kinase Assay

In vitro kinase assays using PKCα immunoprecipitated from HEK293 cell lysates or purified PKCα enzyme as the kinase and purified GST-RORα proteins or small quantities (100 μM) of synthetic peptides (RORαWT or RORαS35A) as substrates were performed at 30°C for 30 min in kinase assay buffer containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 10 μCi of [γ-^{32}P]ATP. The reactions were terminated by adding 5× Laemmli sample buffer and by boiling for 10 min. Samples were subjected to 12% SDS-PAGE, and phosphorylated RORα was detected by autoradiography. For LC-MS analysis, the reaction was stopped by 10% TCA precipitation for 10 min at 4°C. After removing the precipitates by centrifugation, the supernatants were retrieved, and phosphorylated peptides in the supernatants were analyzed by LC-MS at the Korea Basic Science Institute.

RNA Interference by shRNA

The target sequences of shRNA against RORα, β-catenin, and nonspecific (NS) shRNA are as follows: shRORα-1, 5′-CGGUGCGCAGACAGAGCUAUU-3′; shRORα-2, 5′-GAGGUAUCUCAGUAACGAAGA-3′; shβ-catenin, 5′-GUCCUGUAUGAGUGGGAAC-3′ (Kim et al., 2005); and shNS, 5′-CUGGACUUCCAGAAGAACAUC-3′. Oligonucleotide of siPKCα duplex sequence is follows: 5′-GAUCCGCGUCCUGUUGUAUGAAAUUUCA AGAGAA-3′ (Hsieh et al., 2007).

Immunoblotting and Image Analysis

Images were acquired using a LSD-4000 mini chemiluminescence imager (FUJIFILM), and bands were quantified by densitometry with the Multi Gauge software (FUJIFILM) according to the manufacturer's instructions.

Human Colon Cancer Tissue Specimens

For the analysis of phosphorylated RORα and PKCα expression in human tissue samples, 30 paired fresh frozen colon cancer tissues and matched normal mucosa tissues were selected. The frozen fresh human tissue specimens were supplied from the Liver Cancer Specimen Bank supported by National Research Resource Bank Program of the Korea Science and Engineering Foundation in the Ministry of Science and Technology. The consents to use the tissue specimens for research purposes were obtained from patients, and the utilization of the specimens for this research was authorized by the Institutional Review Board of College of Medicine, Yonsei University.

Statistical Analysis

Statistical differences in test and control samples were determined by Student's t test using the Statview package (Abacus Concepts, Inc., Berkeley, CA).

(G) Transwell cell migration assay for RORα, RORαS35A, or RORαS35D-expressing HCT116 cells with treatment of Wnt5a. Values are represented as mean ± SD for three independent experiments.

(H) The anchorage-independent growth of HCT116 cells expressing RORα, RORαS35A, or RORαS35D in soft agar. Values are expressed as mean ± SEM for two experiments in six place wells. Colonies were counted in ten different fields, and total colony number/well was calculated. Representative image is shown for each group.

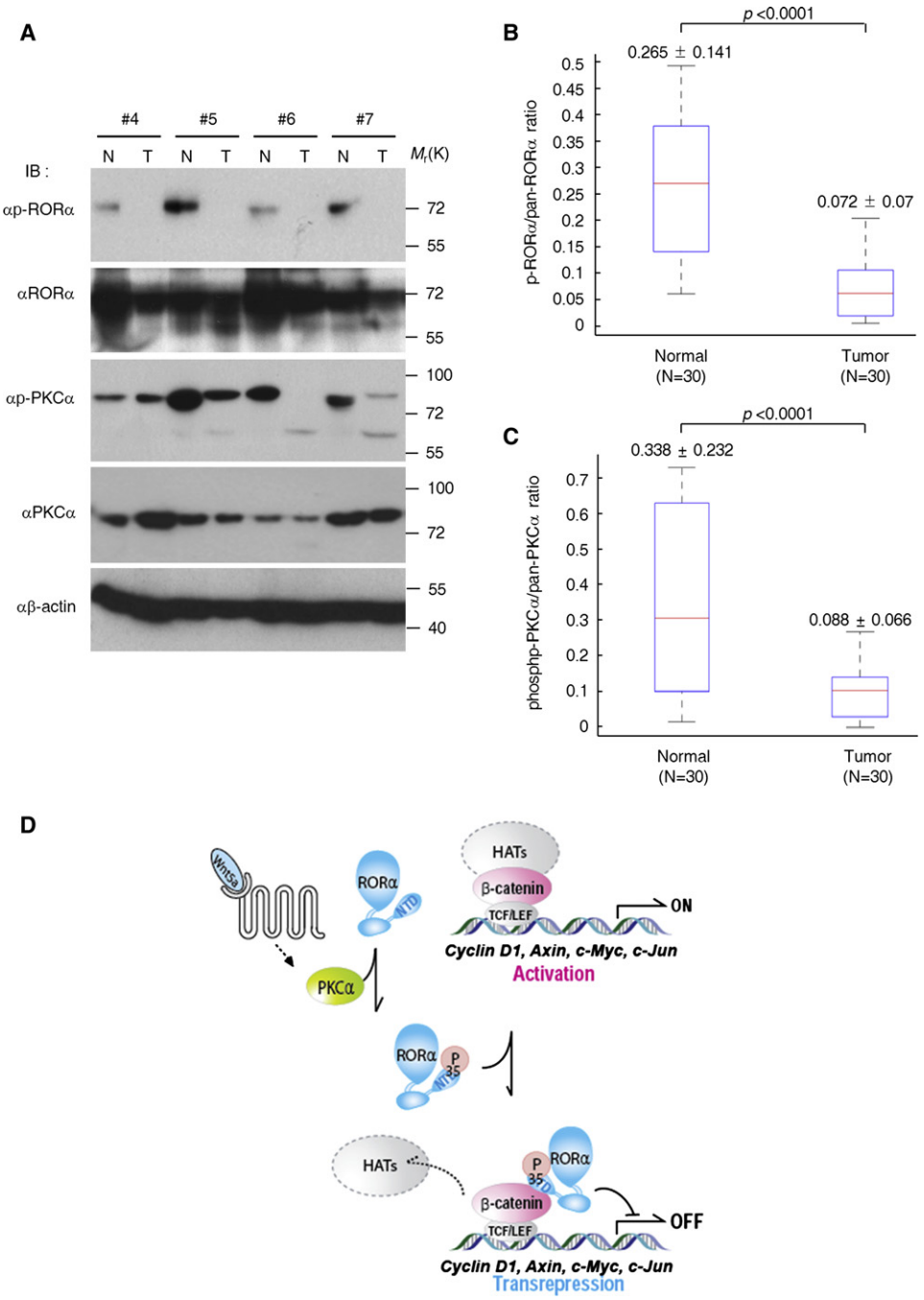

Figure 6. Reduction of RORα Phosphorylation in Human Colorectal Tissues

(A) Immunoblot analysis against anti-phospho-RORαS35, anti-pan-RORα, anti-phospho-PKCα, and anti-pan-PCKα antibodies in human colorectal tumor tissue samples (T) along with matched normal tissue samples (N).

(B and C) Statistical analysis of either the ratio of phosphorylated RORα to pan-RORα (B) or the ration of phosphorylated PKCα to pan-PKCα (C) in the 30 human colorectal tissues compared to normal specimen calculated using paired t test. Data are presented as bar and whisker graphs, showing the median and the distribution of 50% (bar) and 99.3% (whisker) of all specimen examined. $p < 0.0001$ as compared tumor tissues to normal tissues.

(D) Schematic model of downregulation of canonical Wnt signaling by Wnt5a/PKCα-dependent phosphorylation of RORα in colon cancer. RORα confers a transrepression function to the β-catenin-mediated transcriptional activation of Wnt/β-catenin target genes by the enhanced binding to β-catenin via the phosphorylation on serine 35 residue of RORα and possibly by competing with other coactivators for binding to β-catenin.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2009.12.022.

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