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(54) ANTI-CANCER DRUG SCREENING METHOD USING ROR

(57) The present invention relates to a method for screening an anticancer agent using RORα, the method comprising the steps of: culturing cells; bringing a potential substance into contact with the cells; determining whether the phosphorylation level of RORα in the cells increases as compared to that in control cells (not brought into contact with the potential substance); and selecting the potential substance as an anticancer agent if the phosphorylation level of RORα in the cells increases.

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Description

Technical Field

[0001] The Wnt genes encode a large family of cysteine-rich secreted polypeptides that mediate diverse signalling processes. Aberrant activation of-Wnt signaling plays important roles as a major driving force linked to developmental defects and tumorigenesis (Klaus and Birchmeier, 2008; Korinek et al., 1997; Morin et al., 1997; Willert et al., 2003). 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; Liang et al., 2007; Liu et al., 2005). In the absence of Wnt activation, the level of β-catnbin in the cytoplasm remains low due to the degradation of β-catenin by 26S proteasome after paired phosphorylation through casein kinase I (CKI) and glycogen synthase kinase-3β (GSK-3β (Orford et al., 1997; Salic et al., 2000). 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; Giles et al., 2003; Molenaar et al., 1996; Moon et al., 2002).

[0002] Noncanonical Wnt signaling pathways affected by Wnt ligands such as Wnt5a have divers and occasionally opposing roles (Slusarski et al., 1997; Torres et al., 1996). Noncanonical Wnts are both antagonistic and synergistic to canonical Wnt signalling 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 Wnd/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.

Background Art

[0003] A mouse model of spontaneous intestinal tumorigenesis, designated $APC^{min/+}$ is widely used to explore Wnt/β-catenin signalling (Fodde et al., 1994; Shibata et al., 1997; Su et al., 1992). The genetic basis of familial associated polyposis (FAP) was mapped to the adenomatous polyposis coli (*APC*) gene, and germline and sporadic mutations in *APC* occur in most of FAP (Groden et al.,1991; Kinzler et al., 1991). $APC^{min/+}$ mice have a mutation in the *APC* gene causing hyperactivation of Wnt/β-catenin signaling and die within 6 months from severe intestinal tumor development. 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 Wnd/β-catenin signalling by promoting ubiquitination and degradation of β-catenin (Major et al., 2007). Recently, CDK8, a cyclin-dependent kinase member of the mediator complex, has been shown to be necessary for β-catenin-dtiven 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.

[0004] Members of the orphan nuclear receptor family play various roles in signal integration, including modulation of neurogenesis, homeostasis, and disease by regulating subsets of gene expression both positively and negatively (Blumberg and Evans, 1998; 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 et al., 1994; Gold et al., 2003; Lau et al., 1994). *Staggerer* (*sg*) is a classical mutation of the *RORα* gene that blocks Purkinje cell differentiation, resulting in cerebellar hypoplasia and congenital ataxia (Hamilton et al., 1996). *Sg* mice exhibits phenotypes regarding lipid metabolism, bone metabolism, hyperinflammatory responses, and mainly cerebellar development and approximately 50% of the mice die shortly after weaning, which makes studying RORα function with *Sg* mice very difficult (Doulazmi et al., 2006).

[0005] 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 (Peifer and Polakis, 2000). This interaction might elicit changes in oncogenesis and cellular adhesion (Polakis, 2000; van de Wetering et al., 2002). 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.

[0006] Thus, the present inventors identified a critical role of RORα at the crossroads between - the canonical and the noncanonical Wnt signalling pathways in attenuating β-catenin transcriptional activity in a phosphorylation-dependent manner in colon cancer, based on cell culture, colorectal carcinoma tissues, and mouse cancel model studies. Biochemical purification of RORα containing complex identifies β-catenin as a component, providing a novel link between RORα and Wnt signaling pathway. Analysis of RORα interactions with β-catenin reveals that the RORα-mediated inhibition of Wnt/β-catenin signalling requires Wnt5a/PKCα induced phosphorylation on serine residue 35 of RORα, and the binding of RORα to β-catenin is triggered and enhanced by phosphorylation of RORα. Intriguingly, reduction of phosphorylation of RORα concomitant with downregulation of PKC is correlated with activation of Wnt target genes and tumor progression in colorectal carcinoma tissues. The present inventors invented the present invention by revealing of role of RORα in transrepression of the Wnt/β-catenin signalling pathway, thereby regulating cell proliferation and tumor progression in a pathophysiological model.

SUMMARY OF THE INVENTION

[0007] It is an object of the present invention to provide a method for effectively screening an anticancer agent

[0008] Another object of the present invention is to provide a useful method for diagnosing cancer.

[0009] The present invention provides a method for effectively screening an anticancer agent using RORα.

[0010] The present invention provides a useful method for diagnosing cancer using RORα.

[0011] The inventive method for screening a substance inducing the pohsphorylation of serine in the N-terminal region of RORα is useful for identifying a cancer therapeutic agent. Particularly, the method of the present invention may be applied to any cancer caused by the Wnt/β-catenin signal, in which the cancer is preferably colorectal cancer.

[0012] The present invention can be advantageously used to diagnose cancer by measuring the amount of RORα *in vivo.*

BRIEF DESCRIPTION OF THE DRAWINGS

[0013]

Figure 1. Purification of RORα-containing complex and identification of β-catenim as a binding partner

(A) RORα-containing complex was purified from extracts obtained from HEK293 cells stably expressing Flag-tagged RORα. As a negative control, a mock purification from HEK293 cells stably expressing an empty vector was performed. The bound proteins were resolved by SDS-PAGE and prepared for LC-MS/MS analysis. (B) Peptide sequences of RORα-associated polypeptides obtained from LC-MS/MS analysis revealed that GRIP1 and β-catenin are components of the RORα-containing complex. (C) β-catenin and GRIP1 were detected from the elutes by immunoblot analysis. (D) Coimmunoprecipitation of endogenous RORα with β-catenin in HEK293 cells. (E) Ni^+-NTA-agarose pulldown assay was performed with plasmids expressing each GFP-tagged β-catenin deletion construct and HisMax-RORα. (F) Illustration of the structure of various β-catenin deletion constructs and binding to RORα.

Figure 2. Requirement of RORα in mediating transcriptional repression of β-catenin transcriptional activity

(A) Real-time quantitative RT-PCR analysis of the *cyclin D1, c-myc* and *c-jun* transcripts in HCT116 cells in the presence of either shRNA against β-catenin or dominant negative TCF.
(B) Measurement of *cyclin D1* and *c-myc* transcripts after transfection of RORα in HCT116 colon cancer cells. (C and D) Overexpression of RORα inhibited the TCF/β-catenin-mediated activation of the TOPFLASH reporter (C) and *cyclin D1* promoter reporter (D). (E) Measurement of mRNA abundance of *cyclin D1* after knockdown of RORα by two independent shRNAs in HCT116 colon cancer cells. (F and G) Introduction of shRNAs against RORα increased the transcriptional activation of the TOPFLASH reporter (F) or the *cyclin D1* promoter reporter (G). Data are represented as mean±s.d. for three independent experiments. (H) Two-step ChIP assay to determine whether RORα and β-catenin are assembled on the same promoter. (I) HCT116 cells with or without exogenous expression of RORα were coimmunoprecipitated with anti-TCF antibody and the immunoprecipitated materials were subject to immunoblotting against anti-β-catenin antibody. Interaction between TCF and β-catenin was not changed with RORα overexpression. (J) ChIP assay on the *cyclin D1* promoter in HCT116 cells with or without overexpression of RORα. Occupancy of the *cyclin D1* promoter by RORα, β-catenin, TCF and RNA polymerase II is indicated. (K) The shRNA-coupled ChIP assay was performed on *cyclin D1* and *c-jun* promoters in HCT116 cells. Knockdown of β-catenin resulted in decreased histone acetylation as well as decreased recruitment of RORα on the promoters, indicating that RORα binds through β-catenin.

Figure 3. RORα is phosphorylated by PKCα on serine 35

(A) Schematic representation of RORα showing the location of the N-terminal domain (NTD), the DNA-binding domain (DBD), the hinge region, the ligand-binding domain (LBD), and the AF-2 domain. (B) Ni^+-NTA-agarose pulldown 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 pulldown materials (right panel) were analyzed by immunoblotting against anti-β-caknin IgG or anti-Xpress IgG. (C) Illustration of the structure of deletion fragments of RORα. (D) Interaction of each RORα deletion construct with HisMax-β-catenin was assessed by Ni^+-NTA-agarose pulldown assay. (E) Coimmunoprecipitation of RORα with various PKC isoforms. (F) Synthesized peptides of RORα (NQESARKSE) 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 (caPCKα) or kinase deficient form of PKCα (kdPCKα) immunoprecipitated from cell lysates as the kinase and purified GST-RORα N-temminal wild-type (WT) or S35A proteins as substrates were performed. The reaction samples were subjected to 12 % SDSPAGE, and 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 phophorylated RORα on S35 site as assessed by immunoprecipitation with anti-phospho-RORαS35 antibody. (C) Immunoblot against anti-phospho-RORαS35 antibody indicated that caPKCα increased phosphorylation of RORα, whereas kdPKCα failed to phosphorylate RORα. (D) Treatment of Go6976, a PKCα inhibitor, abolished TPA-dependent phosphorylation of RORα as assessed by immonoblotting with anti-phospho-RORαS35 antibody. (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 RORαWT or S35A in HCT116 cells after treatment with TPA in the absence or presence of Go6976. (G) ChIP analysis on the *cyclin D1* promoter after transfection with Flag-RORαWT, S35A, or S35D in HCT116 cells. (H) ChIP analysis on the *cyclin D7* promoter in HCT116 cells after TPA treatment for indicated time. (I) ChIP assay was performed on the *cyclin* D7 promoter in the absence or presence of siRNA against PKCα with treatment of TPA for 90 min. (J) Real-time quantitative RT-PCR analysis of the *cyclin D7* transcript in HCT116 cells in the presence of TPA for indicated time. (K) Knockdown effect of siRNA against RORα or PKCα was assessed in the expression of the *cyclin D1* transcript in HCT116 cells.

Figure 5. Wnt5a antagonizes the canonical Wnt signaling by transrepression function of RORα

(A) Treatment of Wnt5a increased phosphorylated RORα and PKCα but decreased cyclin D1 expression as assessed by immunoblot analysis against each antibody.

(B) *Cyclin D1* transcript was measured after introducing siRNAs against RORα or PKCα in the presence of Wnt5a in HCT116 cells. (C) Real-time quantitative RTPCR analysis of the *cyclin D1* transcript in HCT116 cells after introducing RORα, S35A, or S35D in the presence of Wnt5a. (D) ChIP assay on the *cyclin D1* promoter with treatment of either TPA or Wnt5a in HCT116 cells. Occupancy of the *cyclin D1* promoter by phosphorylated RORα, β-catenin RNA polymerase II, H3K9me2, H3K4me2, CBP, pCAF, p300, or SMRT was indicated. (E) Transwell cell migration assay for RORα, RORαS35A, or RORαS35D-expressing HCT116 cells with treatment of Wnt5a. Values are represented as mean ± s.d. for three independent experiments. (F) 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 6 place wells. Colonies were counted in 10 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 and characterization of tumors in $APC^{min/+}$ mice with or without RORα

(A) Immunoblot analysis against anti-phospho-RORαS35, and anti-phospho-PKCα antibodies in human colorectal tumor tissue samples (T) along with matched normal tissue samples (N). (B) Effects of RORα on mortality in APC$^{min/+}$mice. (C) The number of visible polyps (>1.0 mm) in the small intestine was counted by stereoscopic microscopy in age (20 to 24 weeks old)-and sex-matched APC$^{min/+}$ mice and APC$^{min/+}$ RORα transgenic mice. (D) Schematic model of downregulation of canonical Wnt signalling 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-catenm target genes, such as *cyclin D1*, *c-myc,* and *c-jun,* 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. This crosstalk modulates the invasive activity of tumor cells by inhibiting Wnt target genes that are involved in tumor progression, proliferation, and growth.

Figure 7 shows validation of shRNAs against RORα Knockdown of RORα by two different types of shRNAs was validated by immunoblotting analysis.
Figure 8 shows mass spectrometifc analysis of RORαS35A peptide.
Synthesized peptides of RORαS35A (NQEAARKSE) were used as substrates in the kinase assay with purified PKCα enzyme. The phosphorylated peptide samples were analyzed by mass spectrometric analysis.
Figure 9 shows the transcriptional activation function and DNA binding activity of RORα is not required for the repressive function on Wnt target genes

(A) HCT116 cells were transfected with Flag-RORα WT, DBD mutant (C90A), or Δ AF2 mutant, and the cell extracts were immunoprecipitated with anti-Flag antibody followed by immunoblotting against anti-β-catenin antibody. (B) Introduction of RORα WT, DBD mutant (C90A), or Δ AF2 mutant inhibited the TCF/β-catenin mediated activation of the *cyclin D1* promoter reporter. Data are represented as mean ±s.d. for three independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

[0014] To achieve the above objects, in a first aspect, the present invention provides a method for screening an anticancer agent using RORα. More specifically, the present invention provides a method for screening an anticancer agent, the method comprising the steps of: culturing cells; bringing a potential substance into contact with the cells; determining whether the phosphorylation level of RORα in the cells increases as compared to that in control cells (not brought into contact with the potential substance); and selecting the potential substance as an anticancer agent if the phosphorylation level of RORα increases.

[0015] The phosphorylation level of RORα may be measured by, but not limited to, electrophoresis, fluorescence spectrometry, mass spectrometry, an immunoassay or a PCR assay. The immunoassay may be an immunoblot assay.

[0016] The phosphorylation level of RORα may be measured by analyzing the expression level of a Wnt target gene. The Wnt target gene may be any one selected from the group consisting of cyclin D1, c-myc, and c-jun. The expression level of the Wnt target gene may be analyzed by any of electrophoresis, fluorescence spectrometry, mass spectrometry, an immunoassay and a PCR assay. Preferably, the expression level of the Wnt target gene may be analyzed by an RT-PCR assay. The method for screening the anticancer agent may be applied to any cancer.

[0017] The cancer cells that are used to screen the anticancer agent are preferably cells of the same origin, but are not limited thereto. For example, cells that are used to screen an agent for treating colorectal cancer are preferably colorectal cells.

[0018] In a second aspect, the present invention provides a method of diagnosing cancer by measuring the amount of RORα *in vivo.* More preferably, the present invention provides a method for diagnosing cancer, the method comprising the steps of: collecting cells from a subject; measuring the phosphorylation level of RORα in the cells collected from the subject; and determining that cancer is highly likely to develop if the phosphorylation level of RORα in the cells is lower than in that in cells of a normal person.

[0019] The phosphorylation level of RORα may be measured by, but not limited to, electrophoresis, fluorescence spectrometry, mass spectrometry, an immunoassay or a PCR assay. The immunoassay may be an immunoblot assay.

[0020] In a third aspect, the present invention provides a RORα-overexpressed APC$^{min/+}$ RORα mouse. The mouse is useful for studies on anticancer agents.

[0021] Hereinafter, the present invention will now be described in detail with reference to examples However, these examples are not intended to limit the scope of the present invention as defined in the appended claims.

Example 1: Materials and Reagents

[0022] 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-acetyl-histone H3, acetyl-histone H4, dimethyl-H3K9, and dimethyl-H3K4 antibodies (Upstate Biotechnology), anti-FLAG (Sigma), anti-RNA polymerase II (Berkeley Antibody Company), anti-Xpress (Invitrogen), and anti-phospho-Ser antibodies (Alexis). Anti-phospho-RORαS35 antibody was generated by Abmat PKCα enzyme was purchased from Cell Signaling.

Example 2:Mouse strains and generation of RORα transgenic mice

[0023] A pair of APC$^{min/+}$ mice were purchased from the Jackson Laboratory and housed in the animal facility of the Seoul National University according to standards of the Association for Assessment and Accreditation of Laboratory Animal Care. To construct RORα transgenic mice, full-length human RORα cDNA fused in frame with Myc tag was subcloned into the pCAGGS expression vector under the control of human CMV immediate early enhancer linked to the chicken β-actin promoter. To derive RORα transgenic mice, the pCAGGS-RORα SalI/HindIII fragment was microinjected into fertilized eggs derived from C57BL/6J mice. Integration of the transgene into the offspring genome was assessed by PCR analysis. The experiments were carried out with approval of the Institutional Animal Care and Ethics Committee.

Example 3: Human colon cancer tissue specimens

[0024] 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.

Example 4: Purification of RORα-containing complex

[0025] RORα-containing complex was purified from extracts obtained from HEK293 cells stably expressing Flag-tagged RORα. As a negative control, a mock purification from HEK293 cells stably expressing an empty vector was performed. The RORα-containing complex was immunoprecipitated using anti-Flag antibody-conjugated agarose beads from extracts that were washed to remove non-specific contaminants, and the bound materials were eluted by competition with the Flag peptide (0.1 mg/ml). The bound proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and prepared for LCMS/MS analysis.

Example 5: *In vitro* kinase assay

[0026] *In vitro* kinase assays using PKCα immunoprecipitated from HEK293 cell lysates or purified PKCα enzyme as the kinase and purified GST-RORα proteins 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 dithiothreitol (DTT), and 10 μCi of [γ-^{32}P]ATP. The reactions were terminated by adding 5X Laemmli sample buffer and by boiling for 10 min. Samples were subjected to 12% SDS-PAGE, and phosphorylated RORα was detected by autoradiography.

Example 6: Liquid Chromatography-Mass Spectrometry

[0027] Small quantities (100μM) of synthetic peptides (RORα or RORαS35A) were used as substrates in the kinase assay with PKCα enzyme; 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 Institutes

Example 7: Reporter assays

[0028] Using a luciferase system (Promega), the luciferase activity was measured using a luminometer 48 hr after transfection and normalized by β-galactosidase expression. Values are expressed as means ± standard deviations for at least three independent experiments.

Example 8: ChIP, two-step ChIP, and shRNA-coupled ChIP assays.

[0029] The ChIP, two-step ChIP, and shRNA-coupled ChIP assays were conducted as previously described (Baek et al., 2002; Kim et al., 2005).

Example 9: Construction of plasmids and shRNAs

[0030] RORα DBD mutant (C90A) was generated by site-directed mutagenesis, and the cysteine at Cys90 was replaced by alanine. RORαΔ AF2 was generated as follows: *EcoRI* at the 5' ends and *BamHI* site at the 3' ends of RORα (amino acids 1 to 505) was amplified by PCR and the fragment was subcloned into the *EcoRI*/*BamHI* sites of 3X Flag tagged-CMV 10 vector. The constructs were verified by DNA sequencing. The target sequences of shRNA against RORα, β-catenin, and non-specific (NS) shRNAs are as follows: shRORα-1, 5'-CGGUGCGCAGACAGAGCUAUU-3' (Sequence number 1); shRORα-2, 5'-GAGGUAUCUCAGUAACGAAGA-3' (Sequence number 2); shβ-catenin, 5'-GUCCUGUAUGAGUGGGAAC-3' (Sequence number 3) (Kim et al., 2005), and shNS, 5'-CUGGACUUCCAGAAGAACAUC-3' (Sequence number 4). Oligonucleotide of siPKCα duplex sequence is follows: 5'-GAUCCGCGUCCUGUUGUAUGAAAUUUCAAGAGAA-3' (Sequence number 5)(Hsieh et al., 2007).

Example 10: Real-Time quantitative RT-PCR

[0031] The abundance of mRNA was detected by an ABI prism 7300 system with SYBR Green (molecular probes). Primer pairs were designed to amplify 90-150 bp mRNA specific fragments, and confirmed to be unique products by melting curve analysis. The PCR conditions were 95°C (5 min) and 40 cycles of 95°C (30 s), 57°C (30 s), and 72°C (30 s).

[0032] The quantity of mRNA was calculated using the ΔΔCt method and normalized by using primers to detect β-actin or HPRT. All reactions were performed in triplicates.

Example 11: Indirect immunofluorescence analysis

[0033] HCT116 cells were grown on coverslips, and were washed three times with phosphate-buffered saline (PBS) 12 hrs after transfection. The cells were then fixed with 2% paraformaldehyde in PBS for 30 min at room temperature, washed in PBS, and permeabilized with 0.5% Triton X-100 in PBS (PBS-T) for 30 min at room temperature. Blocking was performed with 3% horse serum and 10% gelatin in PBS-T for 30 min. For staining, cells were incubated with affinity-purified anti-Flag IgG for 1 hr, followed by three washes in PBS-T. The stained cells were incubated for 1 hr with fluorescein isothiocyanate-conjugated secondary antibodies (Jackson Immuno Research Lab.), followed by three washes in PBS-T.

Example 12: Transwell cell migration assay

[0034] HCT116 cells stably expressing RORα, RORαS35A, or RORαS35D were used in Transwell cell migration assays along with control cells. Transwell cell migration assay was conducted as previously described (Kim et al., 2006). Cultured cells were pretreated with Wnt5a (100ng/m1) for 2 hr, and 2.5 x 10^4 HCT116 cells were loaded onto the top of a 24-well Transwell chamber assay plate (BD Biosciences). Conditioned McCoy's 5A medium containing 15% fetal bovine serum was added to the bottom chamber as a chemoattractant. After 22 hr incubation, the cells that had migrated to the lower chamber of the filter were fixed with 100% methanol, stained with DAPI, and quantified by counting the total number of cells in four different fields. All experimental studies were performed according to the manufacturer's protocols. Values are expressed as means± standard deviations for at least three independent experiments.

Example 13: Anchorage-independent growth assay

[0035] Anchorage-independent growth of HCT116 cells containing RORα, RORαS35A, or RORαS35D was determined by analyzing cellular growth in semisolid medium. 10^5 cells were placed in McCoy's 5A media containing 0.4% noble agar containing 10% FCS. Cells were allowed to grow for 3 weeks in 5% CO_2, and the formation of colonies containing >50 cells was analysed.

Example 14: Statistical analysis

[0036] Statistical differences in test and control samples were determined by student's t-test using the Statview package (Abacus Concepts, Inc., Berkeley, CA).

Example 15: Purification of RORα-containing complex and identification of β-catenin as a binding partner

[0037] To investigate the as-yet-unidentified functional modules of RORα, we used a Flag epitope-tag strategy to purify RORα-containing complexes. We generated cell lines that stably express Flag-tagged RORα, and incubated the extracts in anti-Flag M2 affinity gel. After washing with buffer containing increasing salt concentrations of up to 500 mM, the proteins retained on the affinity chromatography column were eluted with buffer containing Flag peptide. We employed liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) to identify proteins in the RORα-containing complex purified from the Flag M2 affinity column. Transcriptional coactivators such as glucocorticoid receptor-interacting protein 1 (GRIP1) and β-catenin were copurified with Flag-RORα (Figures 1A and 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 1C and 1D). 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 (Figures 1E and 1F). These data suggest the possibility that RORα might function as a coregulator for Wnt/β-catenin signaling. Taken together, the identification of β-catenin from RORα-containing complex suggested the probable functional link between the RORα and the Wnt/β-catenin signaling pathways.

Example 16: Attenuation of β-catenin transcriptional activity by RORα

[0038] We used the HCT116 colorectal cancer cell line in which Wnt/β-catenin signaling pathway is constitutively active to examine whether RORα is directly involved in the modulation of Wnt target genes in well-established Wnt signaling-dependent colon cancer cells. In addition to TOPFLASH reporter that has TCF/LEF binding site, *cyclin D1* or *c-rnyc* gene transcripts are used as readout for Wnt signaling activation. Knockdown of β-catenin with shRNA or introduction of dominant negative form of TCF attenuated induction of *cyclin D1* or *c-myc* gene transcripts (Figure 2A), suggesting that increased *cyclin D1* or *c-myc* transcript is related to Wnt signaling activation in HCT116 colon cancer cells. Introduction of RORα suppressed the induction of *cyclin D1* and *c-myc* transcripts (Figure 2B). The overexpression of RORα almost entirely repressed the TCF/β-catenin-mediated activation of TOPFLASH and the *cyclin D1* promoter-luciferase reporters (Figures 2C and 2D). We silenced the expression of endogenous RORα by using shRNAs and validated the functional knockdown effects of two independent shRNAs on Wnt target genes by immunoblotting analysis (Figure 7). In contrast to RORα overexpression, silencing of endogenous RORα by specific shRNAs caused further activation of Wnt target gene (Figure 2E) as well as the TOPFLASH and the *cyclin D1* promoter-luciferase reporters (Figures 2F and 2G). These data suggest that RORα is involved in the attenuation of β-catenin-mediated transcriptional activation and the overexpression of RORα has an opposing effect on the expression of Wnt target genes.

[0039] These unexpected findings led us to explore the molecular mechanism of the RORα mediated transcriptional repression of Wnt target genes in detail. The repression of β-catenin-mediated transcriptional activation by RORα can be postulated by two mechanisms. First, RORα directly interacts with β-eatenin and sequesters it away from its transcription factor, TCF in a DNA binding-independent manner. Second, RORα transrepresses β-catenin-mediated transcription by directly binding to β-mtenin and possibly inhibiting the recruitment of other coactivators to the Wnt target promoters for transcriptional repression. In order to examine whether a sequestering or a transrepression process is involved in the repression of β-catenin-mediated transcriptional activation by RORα, we performed a two-step chromatin immunoprecipitation (ChIP) assay on the *cyclin D1* promoter (Figure 2H). Soluble chromatins were divided into two aliquots. One of these aliquots was immunoprecipitated with anti-RORα antibodies followed by release of the immune complexes and reimmunoprecipitated with anti-β-catenin antibodies. The other aliquot was first immunoprecipitated with anti-β-catenin antibodies followed by reimmunoprecipitation with anti-RORα antibodies. The two-step ChIP assay revealed that both RORα and β-catenin were simultaneously detected on the promoter (Figure 2H). This supports a model in which RORα transrepresses β-catenin-mediated transcriptional activation by directly binding to β-catenin on the same promoter. To exclude the possibility that RORα completely displace TCF from β-catenin, which may lead to the inhibition of TOPFLASH reporter, coimmunoprecipitation assay was performed to examine whether the direct simultaneous interaction of β-catemn with RORα and TCF occurs. Increased expression of RORα failed to affect the interaction of β-catenin with TCF, supporting our model of simultaneous binding of β-catenin to RORα and TCF (Figure 2I).

[0040] Additional ChIP assays on *cyclin D1* promoter revealed that the overexpression of RORα significantly repressed the β-catenin-mediated transcriptional activation with a concomitant increase in RORα binding and decrease in the recruitment ofRNA polymerase II (Figure 2J). Recruitment of β-catenin and TCF was not affected by RORα overexpression. In parallel, we performed shRNA-coupled ChIP assay on *cyclin D1* and *c-jun* promoters by employing shRNA against RORα or β-catenin (Figure 2K). Knockdown of β-catenin resulted in the failure of histone acetylation and exhibited diminished RORα recruitment, suggesting that the recruitment of RORα to the promoter is through β-catenin. Consistent with these data, the knockdown of RORα did not change the recruitment of β-catenin; however, it induced a significant increase in the histone acetylation levels (Figure 2K). These data suggest that the binding of RORα on the *cyclin D1* and *c-jun* promoters is mediated through β-catenin and the binding of RORα to β-catenin confers a repressive effect on Wnt target genes.

Example 17: RORα is phosphorylated by protein kinase Cα on serine 35

[0041] 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 pulldown assay indicated that the N-terminal domain of RORα is responsible for β-catenin binding (Figure 3B). We further prepared serial deletion mutants of the N-terminal domain of RORα and wished to search for molecular determinants of β-catenin binding (Figure 3C). Fine deletion mapping revealed that N-tenninal fragment of RORα spanning amino acids 32 to 41 is indispensable for β-catenin binding (Figure 3D). Since phosphorylation is a dynamic process and the removal and addition of phosphate can change the protein binding affinity, we searched for putative phosphorylation sites. Identification of a PKC consensus site (S/TX$_{0-2}$R/K$_{1-3}$) in the region of RORα spanning amino acids 32 to 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 ofPKCs, including PKCβ2, PKCχ, PKCδ, PKCε, PKCι, PKCη, and PKCζ failed to bind RORα (Figure 3E).

[0042] The selective binding of PKCα to RORα led us to investigate whether PKCα is directly responsible for RORα phosphorylation. Mass spectrometric analysis of RORα peptide after the PKCα kinase assay revealed that RORα is phosphorylated by PKCα (Figure 3F). The calculated molecular mass of the RORα peptide is 1620.8 Da, and addition of a phosphate group increases the mass by 113 Da The phosphorylated RORα peptide had its main peak at 1734.1 Da Treatment of a PKC activator, 12-O-tetradecanoylphorbol-13acetate (TPA) increased phosphorylation of endogenous RORα (Figure 3G). RORαS35A mutant, in which serine residue is mutated to alanine, was not phosphorylated in the presence of TPA, whereas either wild-type (WT) or S39A mutant exhibited TPA-induced phosphorylation (Figure 3H). Mass spectrometric analysis confirmed that no peaks corresponding to phosphorylated forms of the RORαS35A peptide after PKCα kinase assay were detected (Figure 8). These data demonstrate the site-specific phosphorylation on the S35 site of RORα by PKCα.

[0043] 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 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 glutathione-S-transferase (GST)RORαWT 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).

Example 18: Phosphorylation of RORα by PKCα is crucial for downregulation of Wnt/β-catenin target genes

[0044] The antibody raised against phosphorylated RORαS35 peptide specifically recognized the phosphorylated peptide as assessed by dot blot analysis (Figure 4A). Immunoprecipitation analysis by specific, purified anti-phospho-RORαS35 IgG revealed that the wild-type, but not S35A of RORα was subject to phosphorylation 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.

[0045] 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, confirming that phosphorylation of the S35 site of RORα is crucial for the binding to β-catenin. These data clearly indicate that TPA/PKCα dependent phosphorylation of RORα modulates binding affinity of RORα toward β-catenin.

[0046] 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, whereas RORαS35D resulted in increased recruitment to the promoter (Figure 4G). Both RORαDAF2 mutant that has impaired transcriptional activation function and RORαC90A mutant that has impaired DNA binding activity exhibited similar binding affinity to β-catenin (Figure 9A) and repressive functions on TCF/β-catenin-mediated activation (Figure 9B), indicating that neither the transcriptional activity nor DNA binding activity of RORα is required for the repressive function on Wnt target genes. TPA treatment increased the recruitment of phosphorylated RORα as assessed by anti-phospho-RORαS35 IgG on the *cyclin D1* promoter along with concomitant decrease in RNA polymerase II recruitment, whereas the recruitment of β-catenin on the promoter was not altered by TPA treatment (Figure 4H).

[0047] Further, knockdown of PKCα diminished the recruitment of RORα on the *cyclin D1* promoter, confirming that increased binding of RORα on the promoter is due to the PKC-dependent phosphorylation of RORα (Figure 4I). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis indicated that TPA treatment resulted in the downregulation of the *cyclin D1* transcript in HCT116 cells (Figure 4J). In support of the phosphorylation-triggered transrepression mechanism of RORα on Wnt target genes, the knockdown of either PKCα or RORα abolished TPA-mediated downregulation of Wnt target gene expression (Figure 4K). 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 this increased binding is directly responsible for the downregulation of Wnt target genes.

[0048] Example 19: Wnt5a antagonizes the canonical Wnt signalling by transrepression function of RORα.

[0049] Since the noncanonical Wnt signalling pathway triggered by the Wnt5a, a noncanonical Wnt ligand, activates downstream PKCs and CaMKII (Jonsson et al., 1998; Weeraratna et al., 2002), 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 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). Consistent with these data, Wnt5a treatment reduced the expression of *cyclin D1* transcript, and the knockdown of PKCα or RORα by each shRNA abolished Wnt5a-dependent downregulation *of cyclin D1* transcript (Figure 5B). These data confirm that the downregulation of Wnt target genes by Wnt5a is indeed mediated by PKCα activation. Thereafter, we examined whether Wnt5a/PKCα-dependent RORα phosphorylation is capable of suppressing β-catenin-mediated activation of Wnt target genes. Quantitative RT-PCR analysis revealed that RORαS35D, not RORαS35A, induced the downregulation of the *cyclin D1* transcript (Figure 5C). 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.

[0050] Given that RORα exerts its repressive effect by directly binding to β-catenin on the promoter and the binding site for RORα on β-catenin resides in the armadillo repeat domains of β-catenin that demonstrate overlap with the binding sites of a subset of coactivators (Figure 1E), the transrepression mechanism ofRORα on β-caenin 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 5D). 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 5D). 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.

[0051] As upregulation of cyclin D1, c-myc, 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 non-treated cells and RORαS35D-expressing cells exhibited a significant decrease of cell migration in the presence of Wnt5a (Figure 5E and data not shown). 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.

[0052] 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 growth, we asked whether RORαS35D, but not RORαS35A could suppress the colony-forming ability of HCT116 cells in soft agar. Consistent with the anti-proliferative properties of RORαS35D, HCT116 cells expressing RORαS35D grew significantly slower than control cells (Figure 5F and data not shown). Furthermore, the size of the colonies formed by RORαS35Dexpressing 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 phosphorylation-dependent manner.

Example 20: Reduction of RORα phosphorylation is frequent in human colorectal cancers

[0053] To find the clinical relevance of our findings, we examined the expression of phosphorylated RORα and PKCα 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 (>73%) cases, and of these 22 cases, 14 cases of them (>46%) exhibited the reduction of phosphorylation of PKCα (Table 1 and Figure 6A). We further investigated the expression of Wnt target genes in these normal and tumor samples by quantitative RT-PCR All of the cases in which reduction of RORα phosphorylation and PKCα inactivation exhibited the increased expression of Wnt target genes (Table 1). Wnt5a expression has been reported to be down-regulated in multiple tumors including colon, breast, and prostate, whereas it is upregulated in brain, stomach, kidney, and skin tumors (Blanc et al., 2005; Iozzo et al., 1995; Kremenevskaja et al., 2005). Further quantitative RT-PCR analysis supported the idea that downregulation of Wnt5a in colon tumor correlates with inactivation of PKCα and reduction of RORα phosphorylation (Table 1). These data suggest that reduction of RORα phosphorylation along with inactivation of PKCα and downregulation of Wnt5a is frequent event in colorectal cancer.

Table 1

Fold (Tumor/Normal tissues)

				Wnt targets		
No.	phospho-RORα	phospho-PKCα	*Wnt5a*	*CyclinD1*	*c-Myc*	*c-Jun*
	1 △	△	+42△	-1.4▼	+56△	+18△
2	NC	△	+S6△	+42△	+12△	+56△.
3	NC	△	+160△	+6.0△	+49△	+58△
4	▼	△	+16△	-69▼	+11△	+2.3△
5	▼	▼	-42▼	+120△	+25△	NC
6	▼	▼	-66▼	+13△	+16△	+4.4△
7	▼	▼	-6.3▼	+8.4△	+132△	+20△
8	NC	△	+32△	+45△	+50△	NC
9	▼	▼	-18▼	+9.1△	+430△	NC
10	▼	▼	-20▼	+1.4△	+1.8△	+20△
11	▼	▼	-24▼	+6.7△	+1.3△	+4.9△
12	NC	NC	+13△	-2.5▼	-1.8▼	-1.7▼
13	▼	▼	-11▼	+1.3△	+8.4△	+5.1△
14	▼	▼	-13▼	NC	+7.4△	+3.3△
15	NC	△	+16△	+4.5△	+6.1△	+11△
16	▼	NC	+190△	-1.8▼	+9.4△	+10△
17	▼	NC	+49△	+3.2△	+14△	NC
18	▼	▼	NC	+3.5△	+5.4△	+1.7△
19	▼	▼	-23▼	+3.0△	+9.4△	+1.8△
20	NC	▼	+47△	NC	NC	NC
21	△	▼	+38△	+3.4△	+2.9△	+32△
22	▼	NC	+62△	-1.3▼	+8.7△	+6.8△
23	▼	▼	-4.5▼	+1.7△	+1.2△	NC
24	▼	NC	-4.0▼	NC	+5.1△	+2.5△
25	▼	▼	-1.2▼	+20△	+6.3△	+33△
26	▼	△	NC	-1.8△	-1.8▼	NC
27	▼	NC	-99▼	-1.7▼	+3.7△	+34△
28	▼	▼	-3.5▼	+1.8△	+1.5△	+13△
29		NC	NC	+8.5△	NC	+8.1△
▼ 30	▼	▼	-3.7▼	+2.1△	+2.0△	+6.5△

Example 21: The reduced polyp development in APC$^{min/+}$RORα transgenic mouse compared with those in APC$^{min/+}$ mouse

[0054] To examine whether tumor suppressive function of RORα is applied to spontaneous intestinal tumorigenesis of mouse model, we generated APC$^{min/+}$ mice crossed with RORα transgenic mice and analyzed sex-and age-matched mice. We generated transgenic mice expressing a human RORα cDNA, and confirmed the increase of RORα expression in colon and intestine tissues (data not shown). APC$^{min/+}$ mice die within 24 weeks of age, whereas mortality of APC$^{min/+}$ RORα transgenic mice was decreased compared with APC$^{min/+}$ littermate controls (Figure 6B). We quantified the number of polyps by stereoscopic microscopy, and found the decrease of the number of visible polyps (>1.0 mm in diameter) in the intestines or colon of $APC^{min/+}$ RORα transgenic mice compared with that in $APC^{min/+}$ mice (Figure 6C). These data support the notion that RORα influences the Wnt signaling mediated tumor formation and growth by suppressive function of Wnt signaling. Taken together, RORα affects modulation of cell and tumor growth in $APC^{min/+}$ mouse model of intestinal tumorigenesis.

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[0055]

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```
<110>   SNU Research and Development Business Foundation

<120>   Screening methods for anticancer agents by using ROR alpha.

<160>   5

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<213>   Artificial Sequence

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cuggacuucc agaagaacau c                                                21


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<220>
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```

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```

Claims

1. A method for screening an anticancer agent, the method comprising the steps of:

   culturing cells;
   bringing a potential substance into contact with the cells;
   determining whether the phosphorylation level of RORα in the cells increases as compared to that in control cells (not brought into contact with the potential substance); and
   selecting the potential substance as an anticancer agent if the phosphorylation level of RORα in the cells increases.

2. The method of claim 1, wherein the phosphorylation level of RORα is measured by any one selected from the group consisting of electrophoresis, fluorescence spectrometry, mass spectrometry, an immunoassay or a PCR assay.

3. The method of claim 2, wherein the immunoassay is an immunoblot assay for anti-phospho-RORαS35IgG.

4. The method of claim 1, wherein the phosphorylation level of RORα is measured by analyzing the expression level of a Wnt target gene.

5. The method of claim 4, wherein the expression level of the Wnt target gene is analyzed by an immunoblot assay.

6. The method of claim 4, wherein the expression level of the Wnt target gene is analyzed by a RT-PCR assay.

7. The method of claim 2, wherein the cells are colorectal cancer cells.

8. A method of diagnosing cancer, comprising the steps of:

   collecting cells from a subject;
   measuring the phosphorylation level of RORα in the cells collected from the subject; and
   determining that cancer is highly likely to develop if the phosphorylation level of RORα in the cells is lower than in that in cells of a normal person.

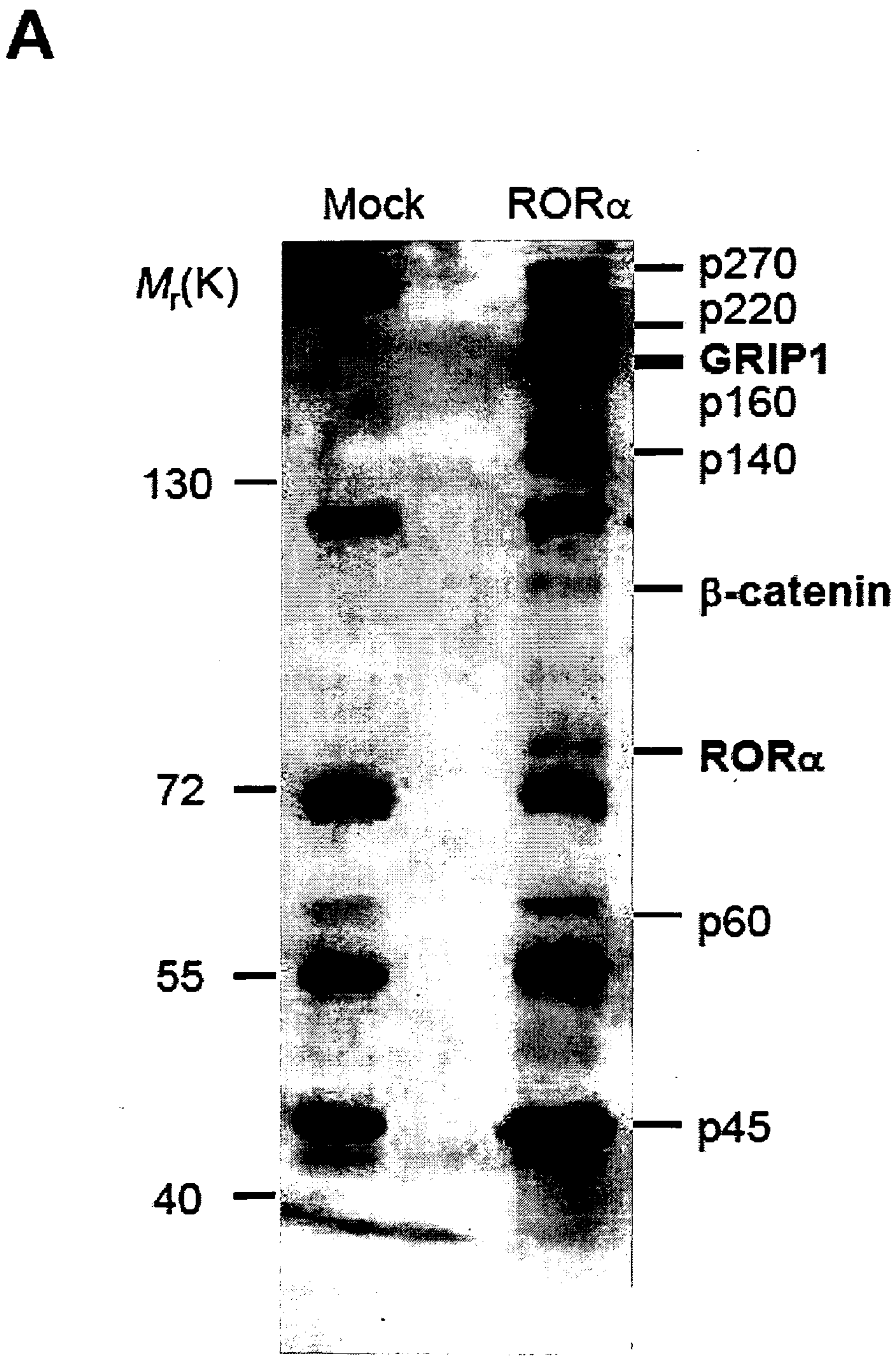
A
Mock
RORα
M_r(K)
130
72
55
40
p270
p220
GRIP1
p160
p140
β-catenin
RORα
p60
p45

Fig. 1 A

B

Protein ID	Peptide sequence for RORα
GRIP1	PVLPSSESFTTRTLMMR
	GQGLNMTPSMVAPSGMPATMSNPR
	NLLPKSIVNGGSWSGEPPR
	NSHTFNCRMLVK
	SIVNGGSWSGEPPR
	LHDSK
β-catenin	SVENCVCIMR
	SLEGEKTGSRDVIPMDALGPDGYSTVDR
	YQEAEPGPLGSAVGSQR
	RRPECGR

Fig. 1B

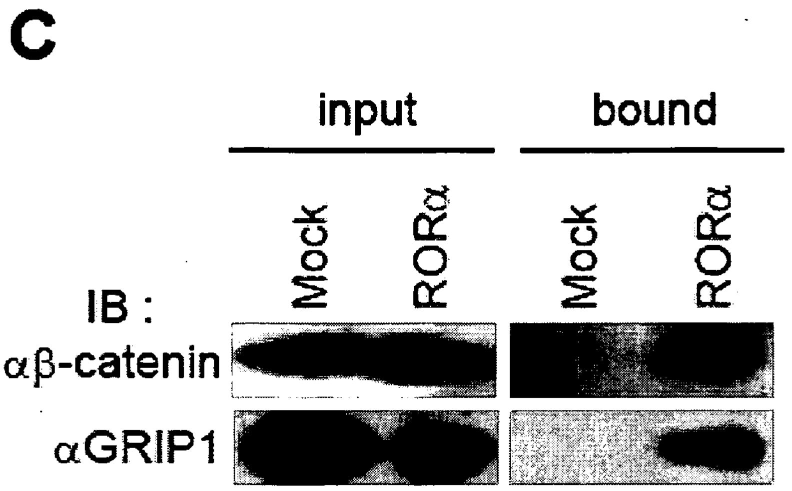
C
input
bound
Mock
RORα
Mock
RORα
IB :
αβ-catenin
αGRIP1

Fig. 1C

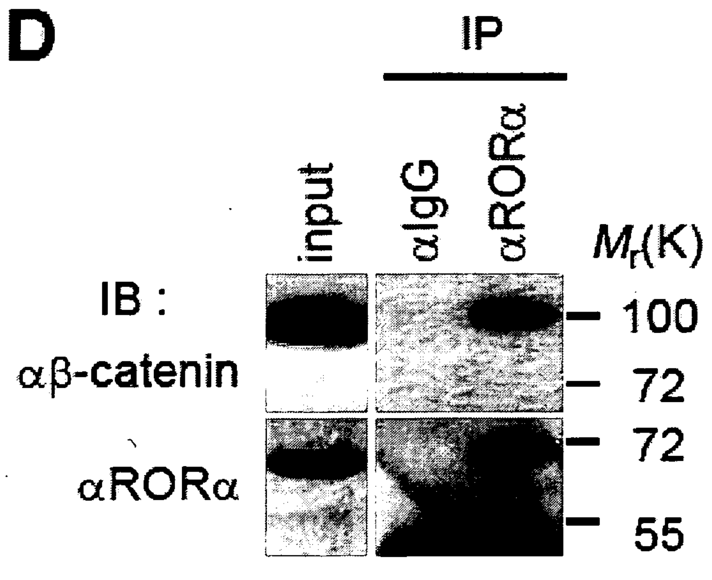
D
IP
input
αIgG
αRORα
M_r(K)
IB :
αβ-catenin
αRORα
100
72
72
55

Fig. 1D

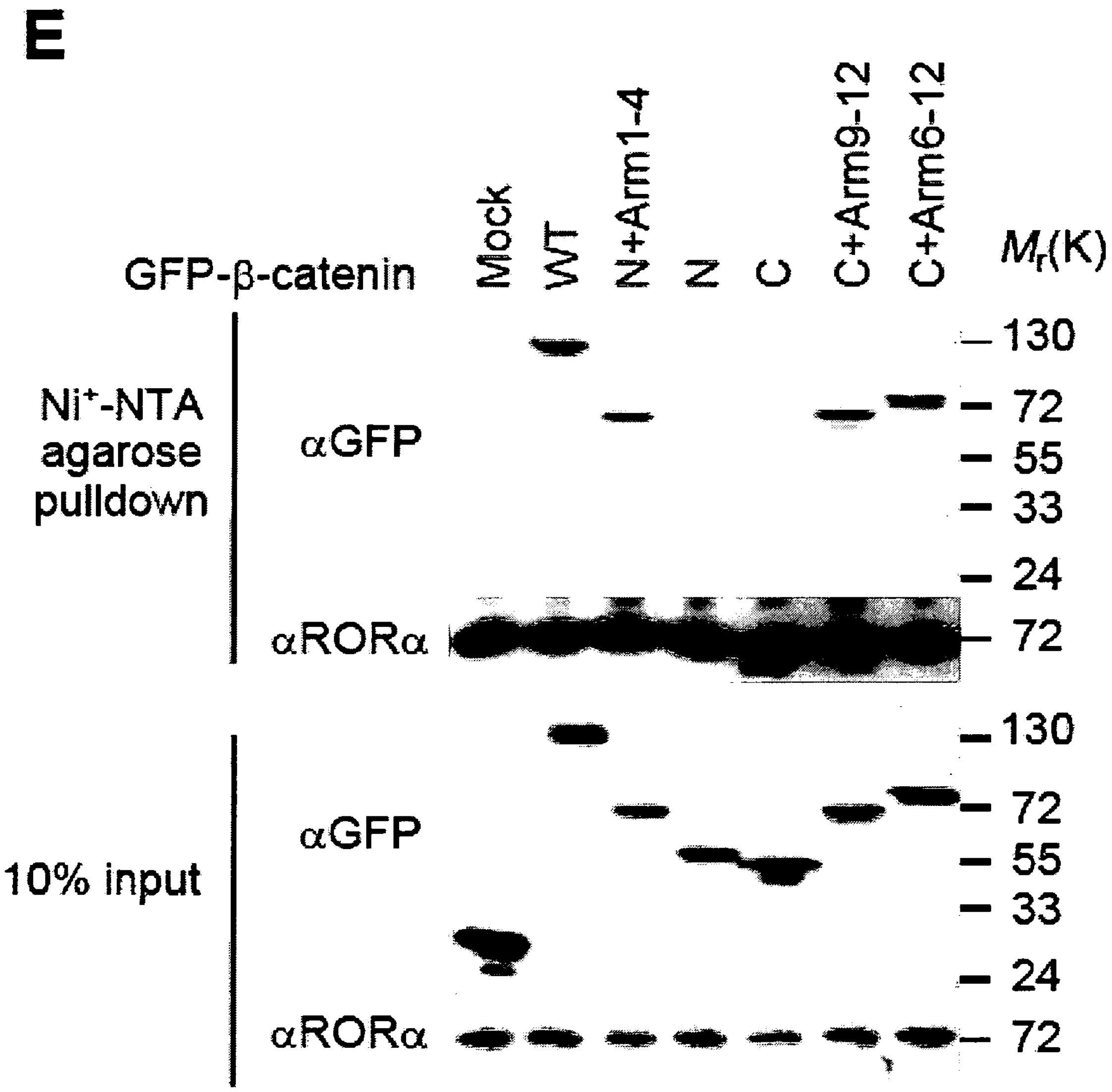
E
GFP-β-catenin
Mock
WT
N+Arm1-4
N
C
C+Arm9-12
C+Arm6-12
M_r(K)
Ni⁺-NTA agarose pulldown
αGFP
αRORα
10% input
αGFP
αRORα
130
72
55
33
24
72
130
72
55
33
24
72

Fig. 1E

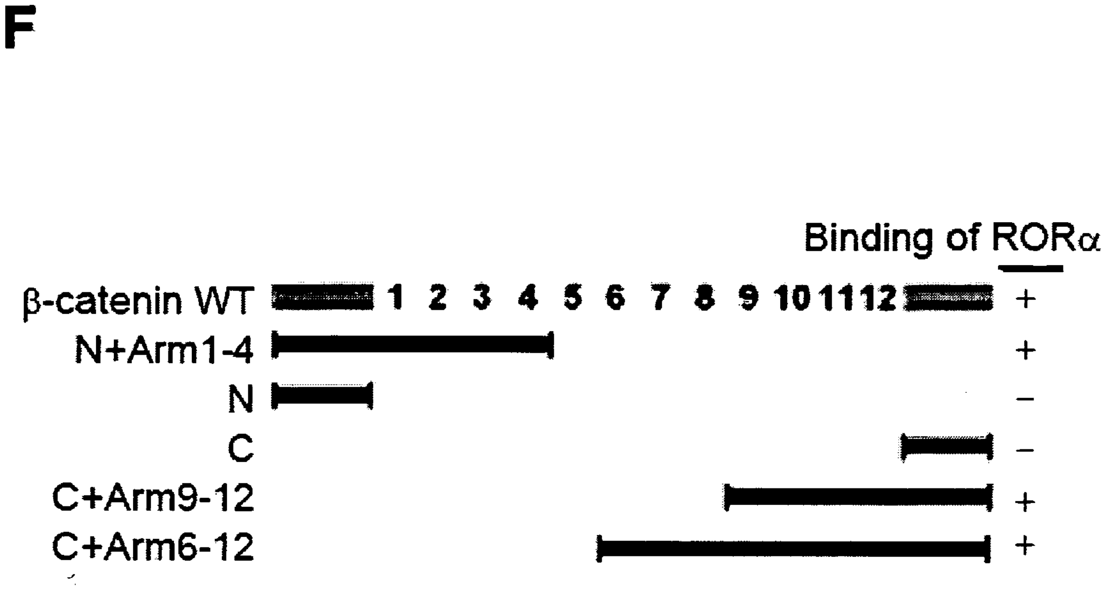
F
Binding of RORα
β-catenin WT
1 2 3 4 5 6 7 8 9 10 11 12
+
N+Arm1-4
+
N
−
C
−
C+Arm9-12
+
C+Arm6-12
+

Fig. 1F

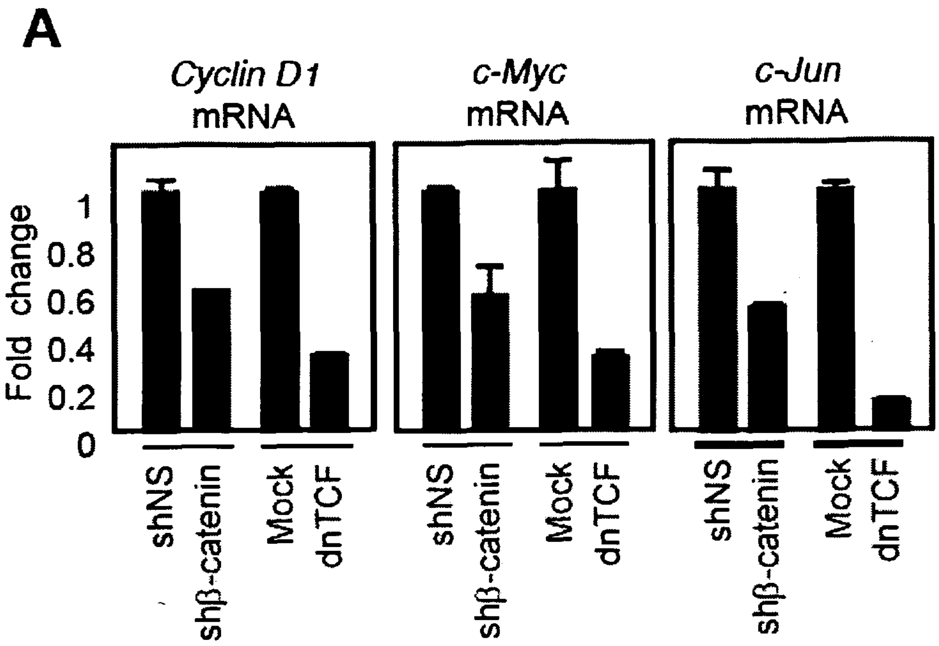
A
Cyclin D1 mRNA
c-Myc mRNA
c-Jun mRNA
Fold change
1
0.8
0.6
0.4
0.2
0
shNS
shβ-catenin
Mock
dnTCF

Fig. 2A

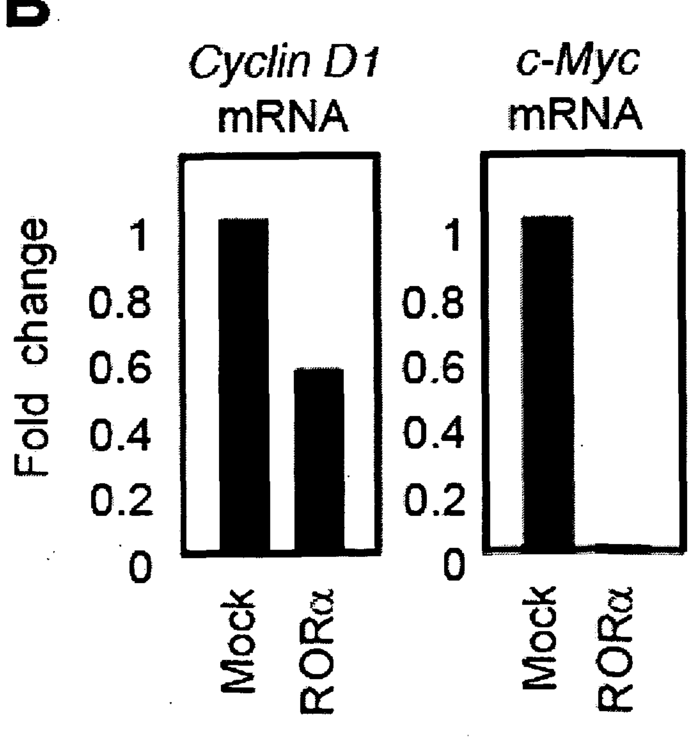
B
Cyclin D1 mRNA
c-Myc mRNA
Fold change
1
0.8
0.6
0.4
0.2
0
Mock
RORα

Fig. 2B

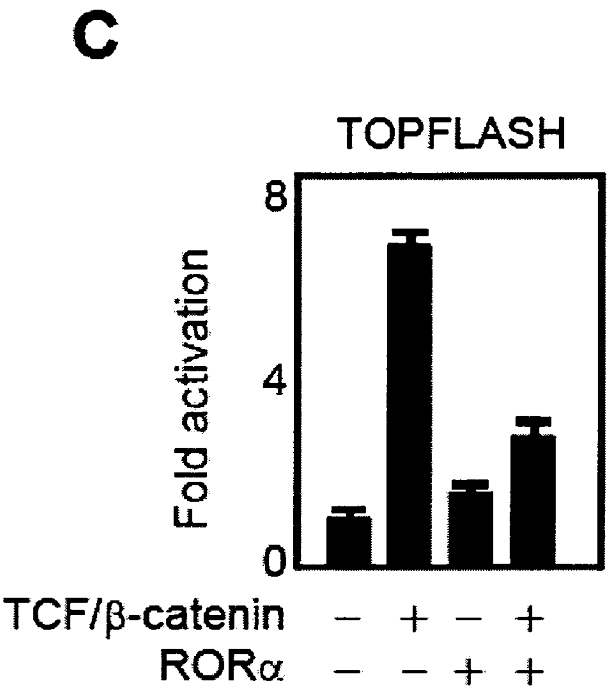
C
TOPFLASH
Fold activation
8
4
0
TCF/β-catenin − + − +
RORα − − + +

Fig. 2C

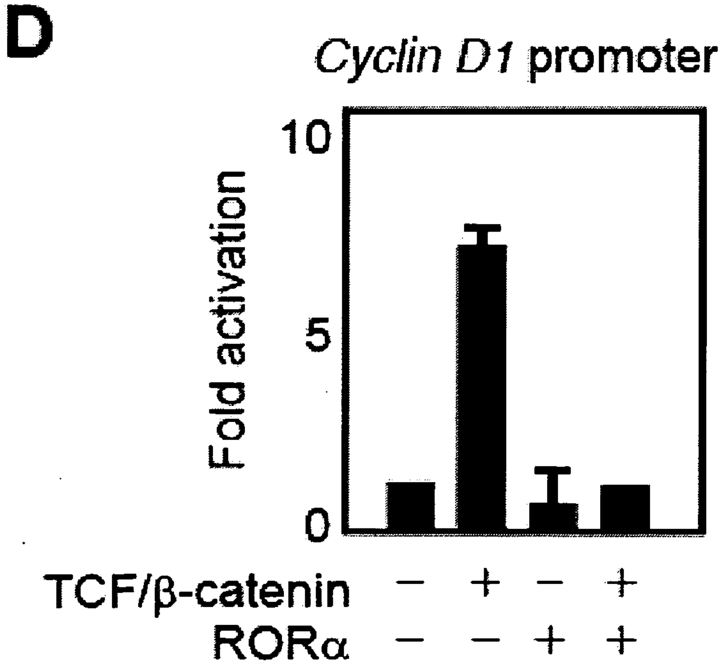
D
Cyclin D1 promoter
Fold activation
10
5
0
TCF/β-catenin − + − +
RORα − − + +

Fig. 2D

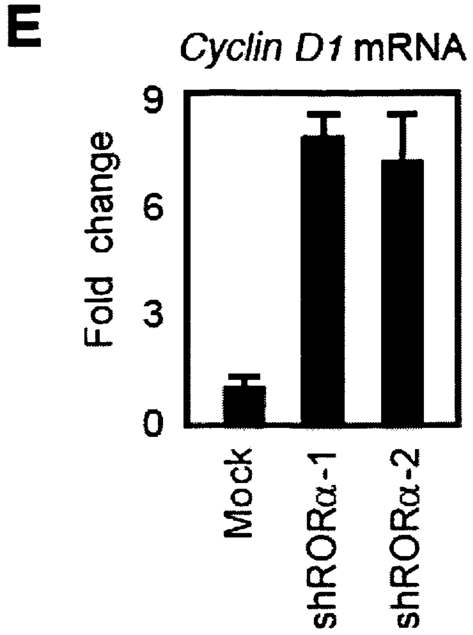
E
Cyclin D1 mRNA
Fold change
9
6
3
0
Mock
shRORα-1
shRORα-2

Fig. 2E

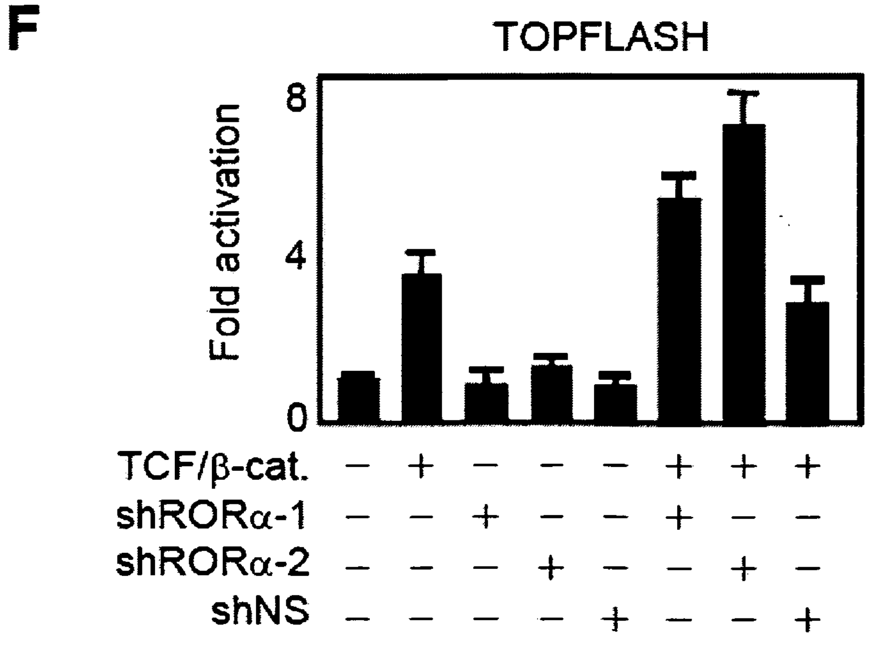
F
TOPFLASH
Fold activation
8
4
0
TCF/β-cat. − + − − − + + +
shRORα-1 − − + − − + − −
shRORα-2 − − − + − − + −
shNS − − − − + − − +

Fig. 2F

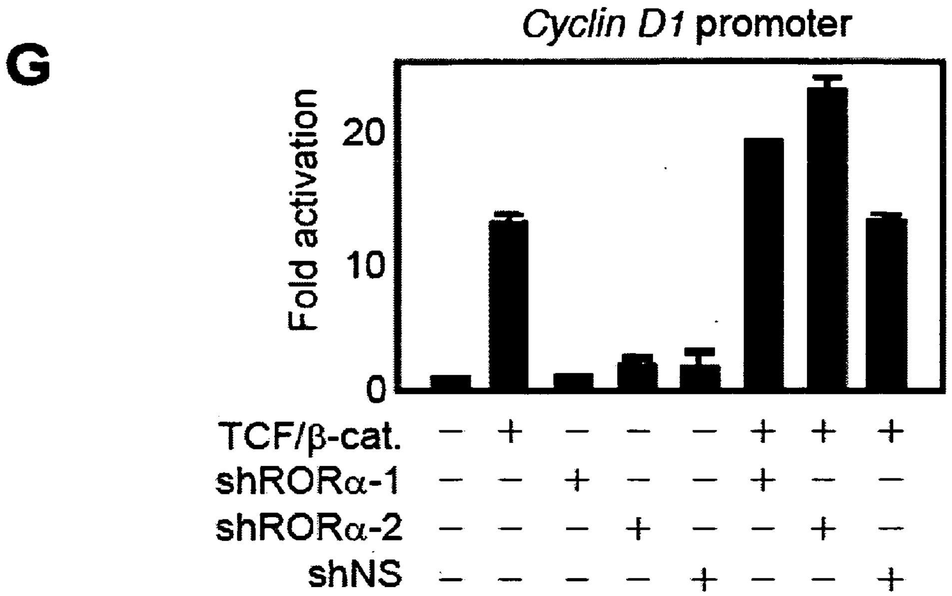
G
Cyclin D1 promoter
Fold activation
0
10
20
TCF/β-cat. − + − − − + + +
shRORα-1 − − + − − + − −
shRORα-2 − − − + − − + −
shNS − − − − + − − +

Fig. 2G

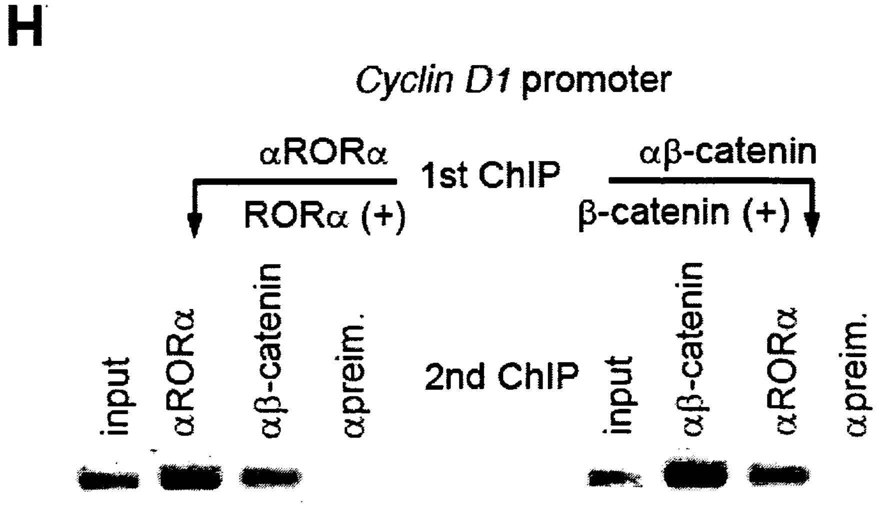
H
Cyclin D1 promoter
αRORα
RORα (+)
1st ChIP
αβ-catenin
β-catenin (+)
input
αRORα
αβ-catenin
αpreim.
2nd ChIP
input
αβ-catenin
αRORα
αpreim.

Fig. 2H

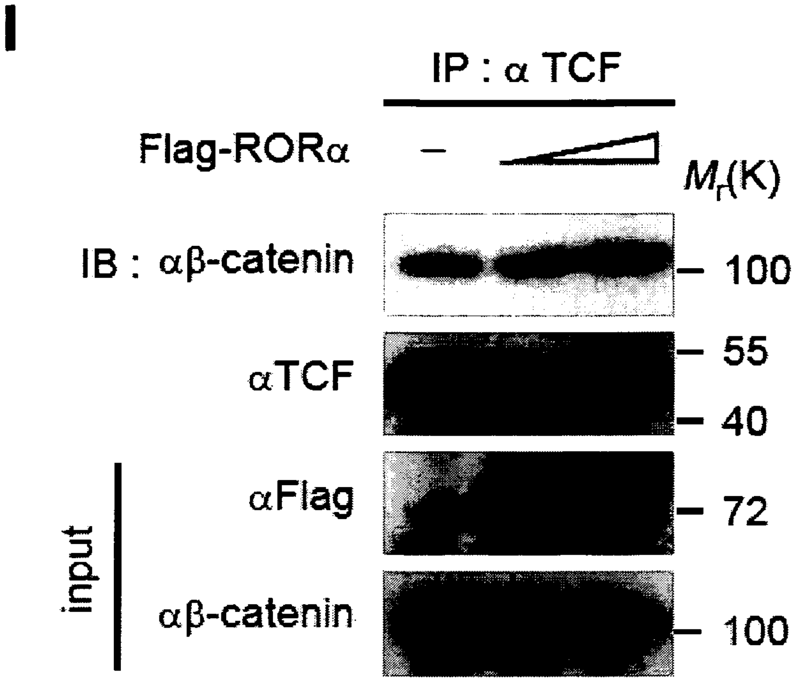
I
IP : α TCF
Flag-RORα
–
M_r(K)
IB : αβ-catenin
100
αTCF
55
40
input
αFlag
72
αβ-catenin
100

Fig. 2I

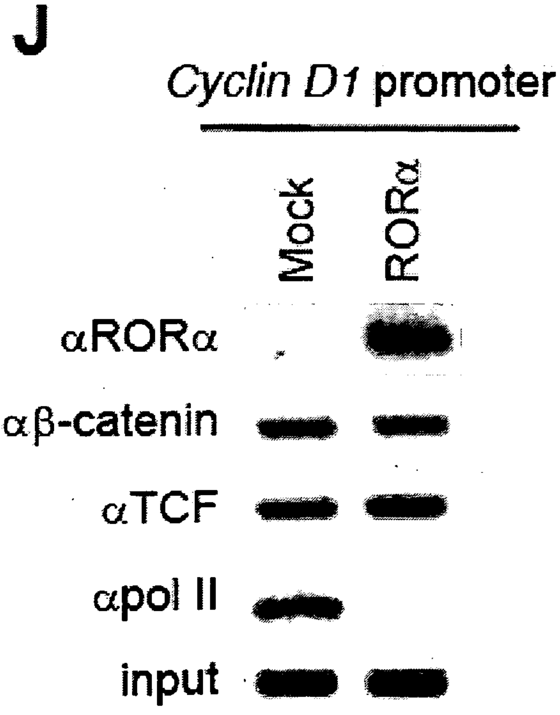
J
Cyclin D1 promoter
Mock
RORα
αRORα
αβ-catenin
αTCF
αpol II
input

Fig. 2J

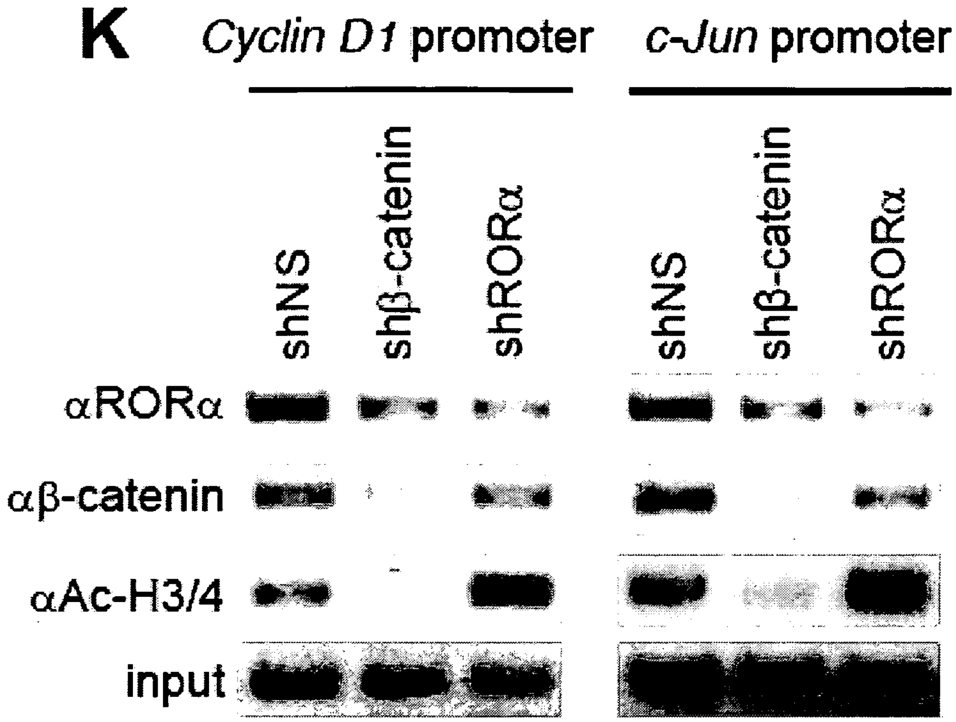
K
Cyclin D1 promoter
c-Jun promoter
shNS
shβ-catenin
shRORα
shNS
shβ-catenin
shRORα
αRORα
αβ-catenin
αAc-H3/4
input

Fig. 2K

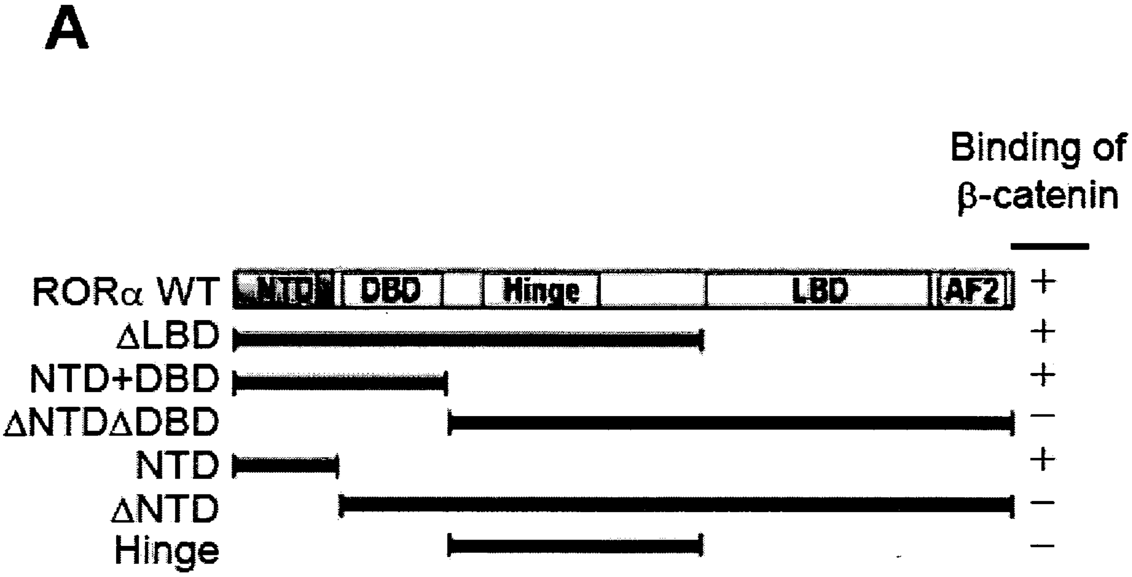
A
Binding of β-catenin
RORα WT
NTD
DBD
Hinge
LBD
AF2
+
ΔLBD
+
NTD+DBD
+
ΔNTDΔDBD
−
NTD
+
ΔNTD
−
Hinge
−

Fig. 3A

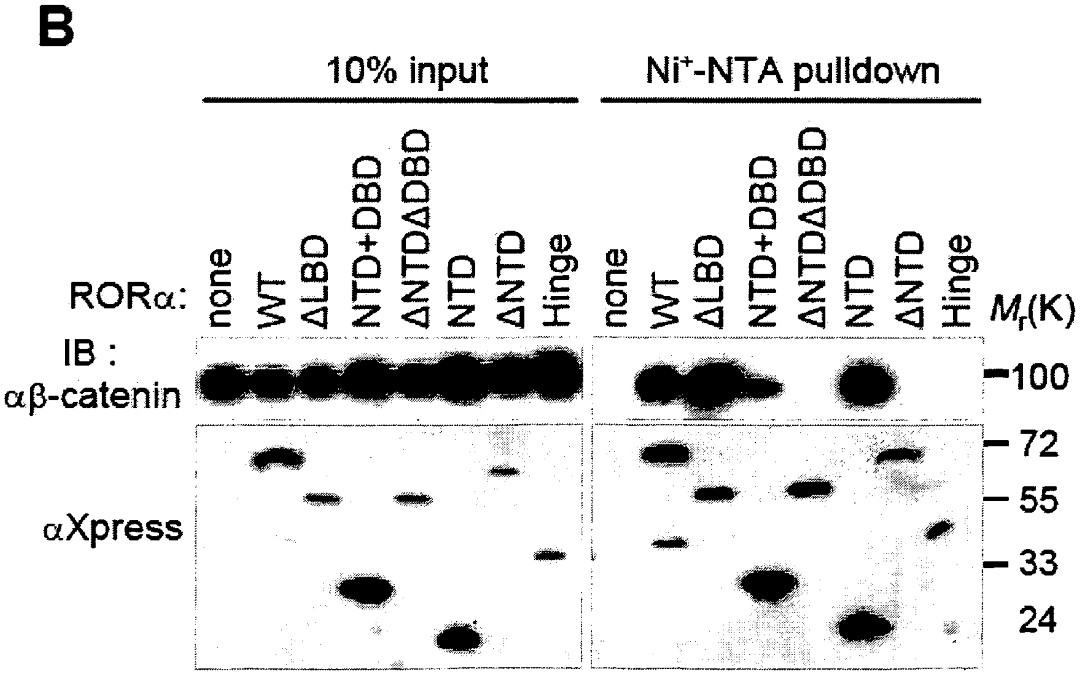
B
10% input
Ni⁺-NTA pulldown
RORα:
none
WT
ΔLBD
NTD+DBD
ΔNTDΔDBD
NTD
ΔNTD
Hinge
none
WT
ΔLBD
NTD+DBD
ΔNTDΔDBD
NTD
ΔNTD
Hinge
Mr(K)
IB :
αβ-catenin
αXpress
100
72
55
33
24

Fig. 3B

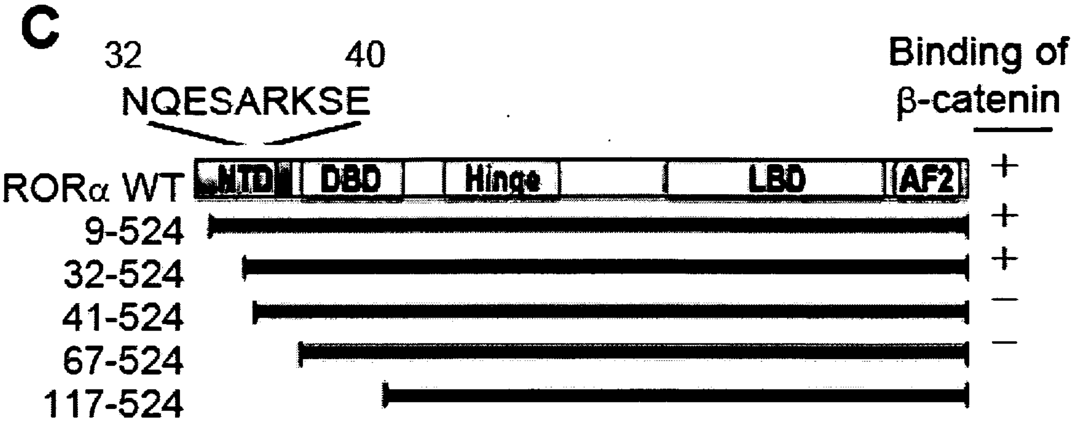
C
32
40
NQESARKSE
Binding of
β-catenin
RORα WT
NTD
DBD
Hinge
LBD
AF2
9-524
32-524
41-524
67-524
117-524
+
+
+
−
−

Fig. 3C

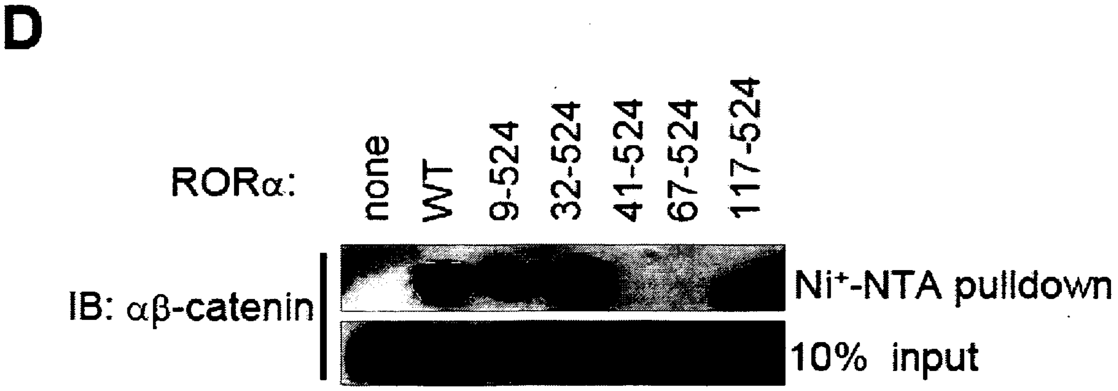
D
RORα:
none
WT
9-524
32-524
41-524
67-524
117-524
IB: αβ-catenin
Ni+-NTA pulldown
10% input

Fig. 3D

E

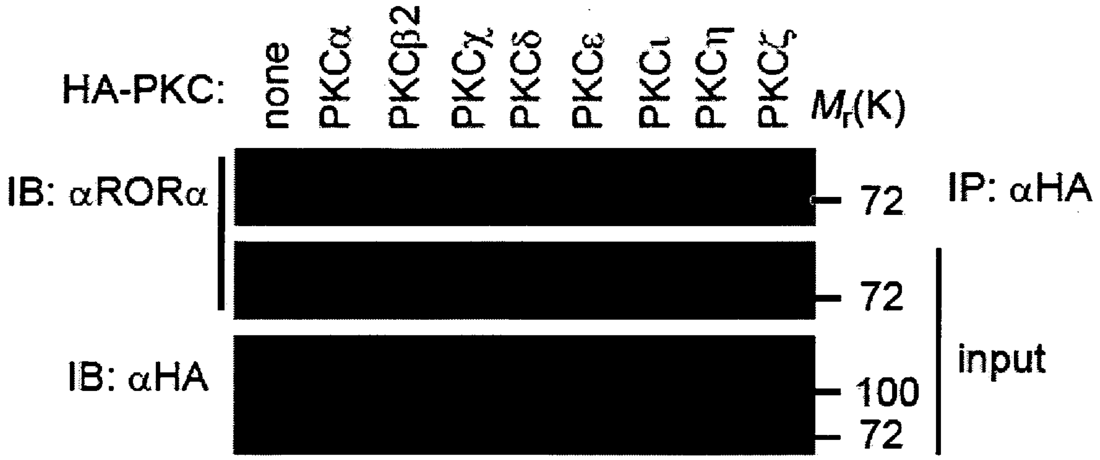
HA-PKC:
none
PKCα
PKCβ2
PKCχ
PKCδ
PKCε
PKCι
PKCη
PKCζ
M_r(K)
IB: αRORα
IB: αHA
72
72
100
72
IP: αHA
input

Fig. 3E

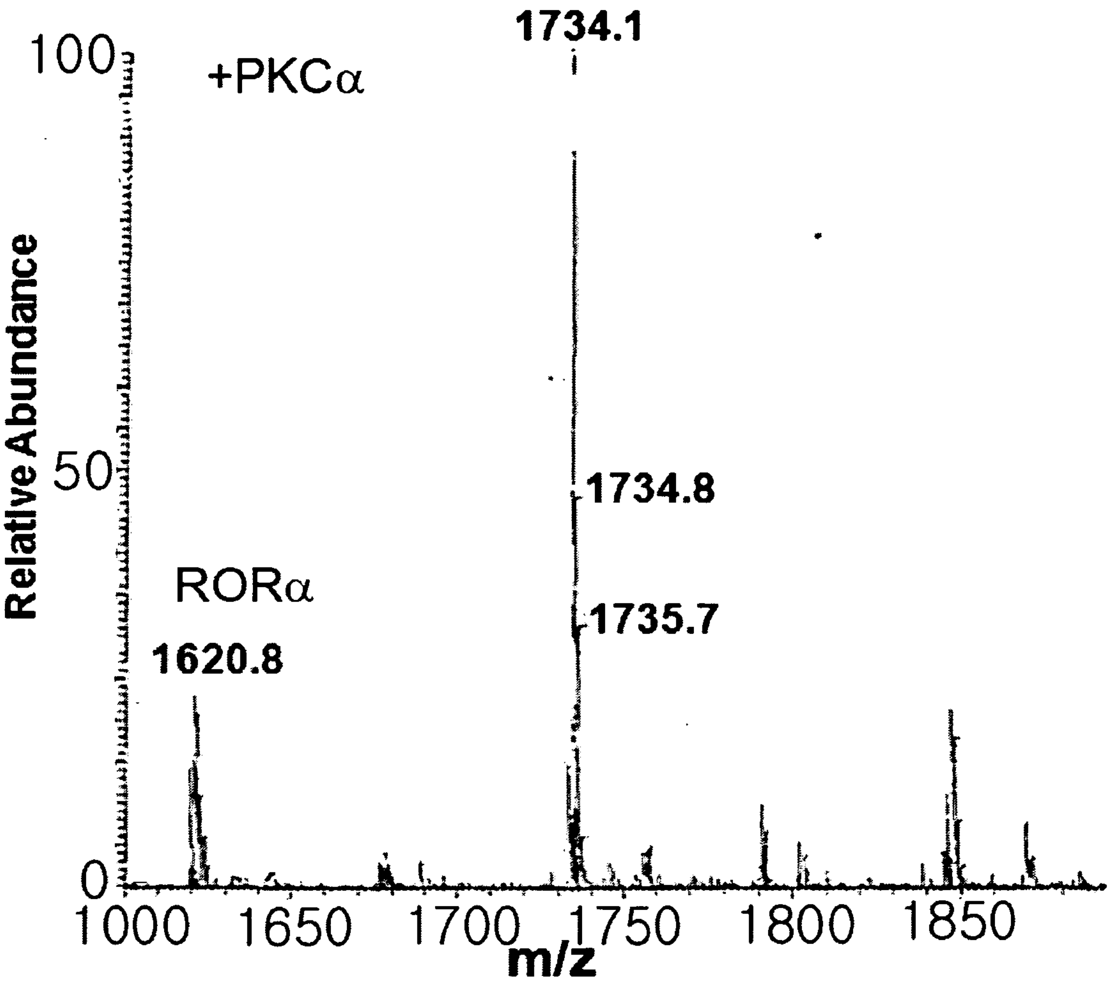
1734.1
100
+PKCα
Relative Abundance
50
1734.8
RORα
1735.7
1620.8
0
1000
1650
1700
1750
1800
1850
m/z

Fig. 3F

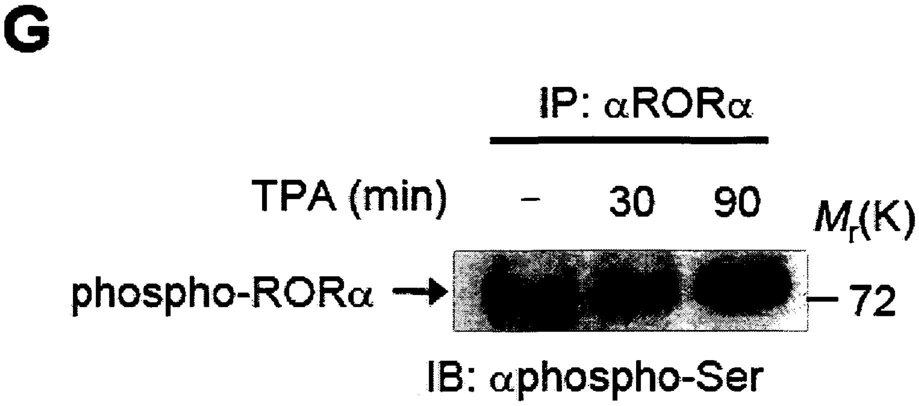
G
IP: αRORα
TPA (min)
-
30
90
M_r(K)
phospho-RORα
72
IB: αphospho-Ser

Fig. 3G

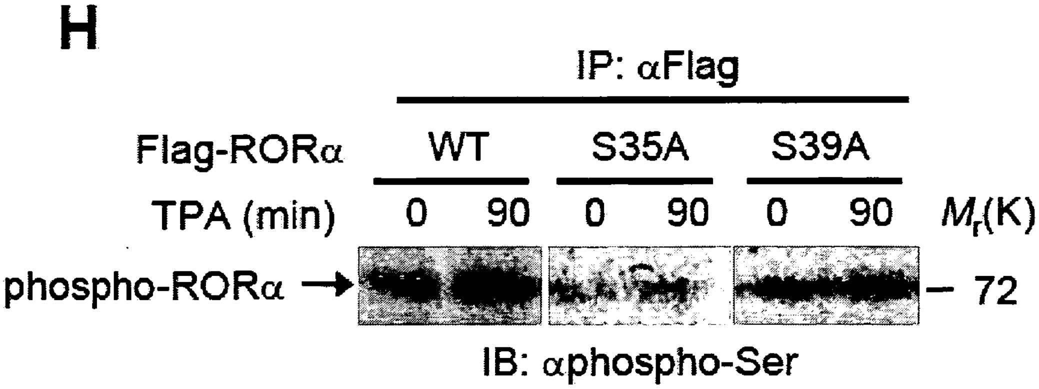
H
IP: αFlag
Flag-RORα
WT
S35A
S39A
TPA (min)
0
90
0
90
0
90
M_r(K)
phospho-RORα
72
IB: αphospho-Ser

Fig. 3H

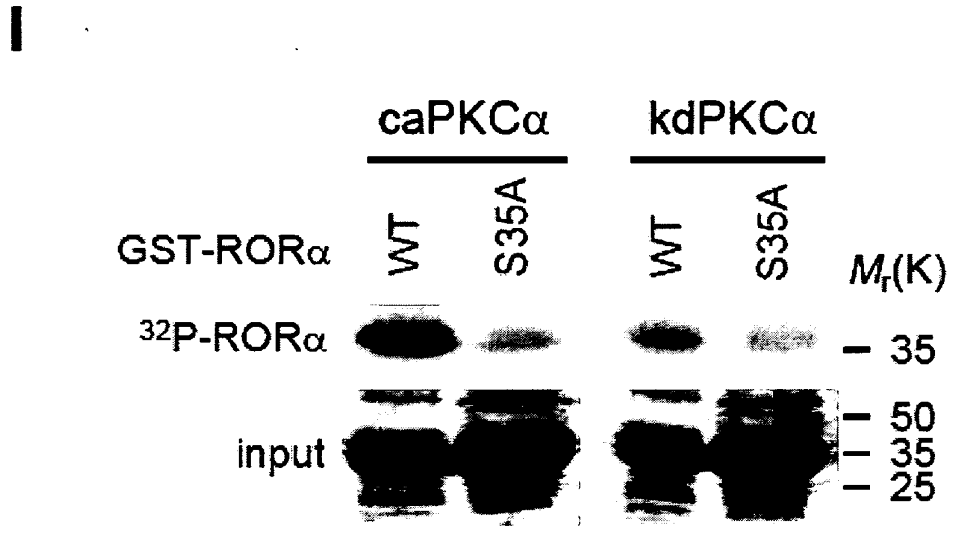
I
caPKCα
kdPKCα
GST-RORα
WT
S35A
WT
S35A
M_r(K)
^{32}P-RORα
35
input
50
35
25

Fig. 3I

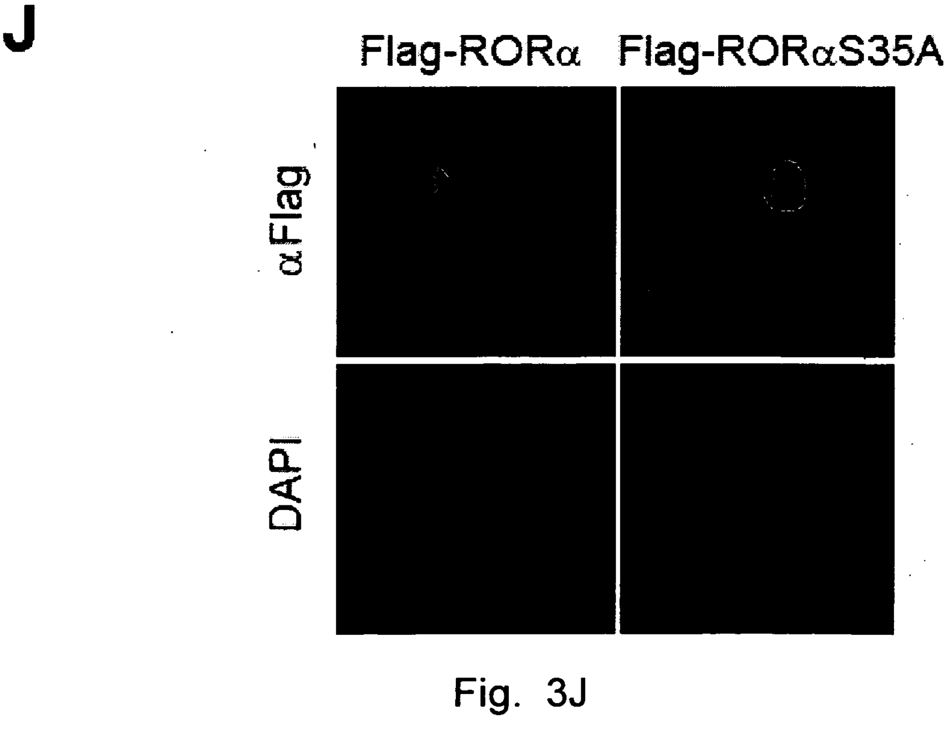
J
Flag-RORα
Flag-RORαS35A
αFlag
DAPI

Fig. 3J

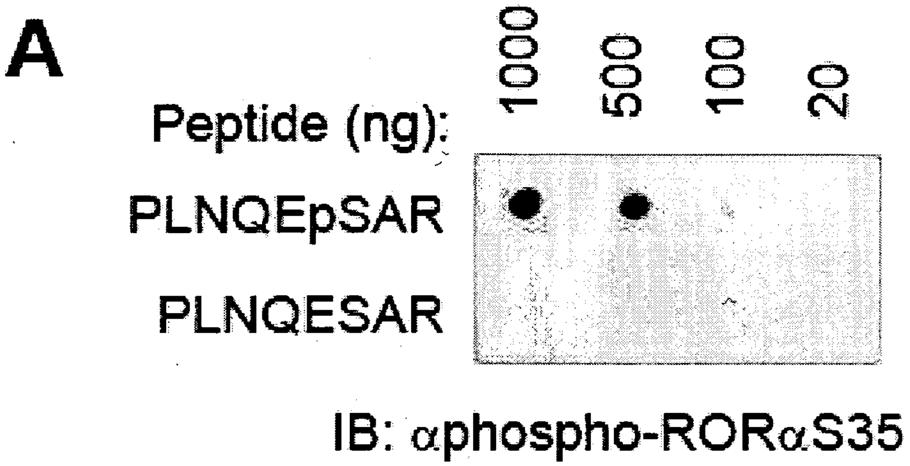
A
Peptide (ng):
1000
500
100
20
PLNQEpSAR
PLNQESAR
IB: αphospho-RORαS35

Fig. 4A

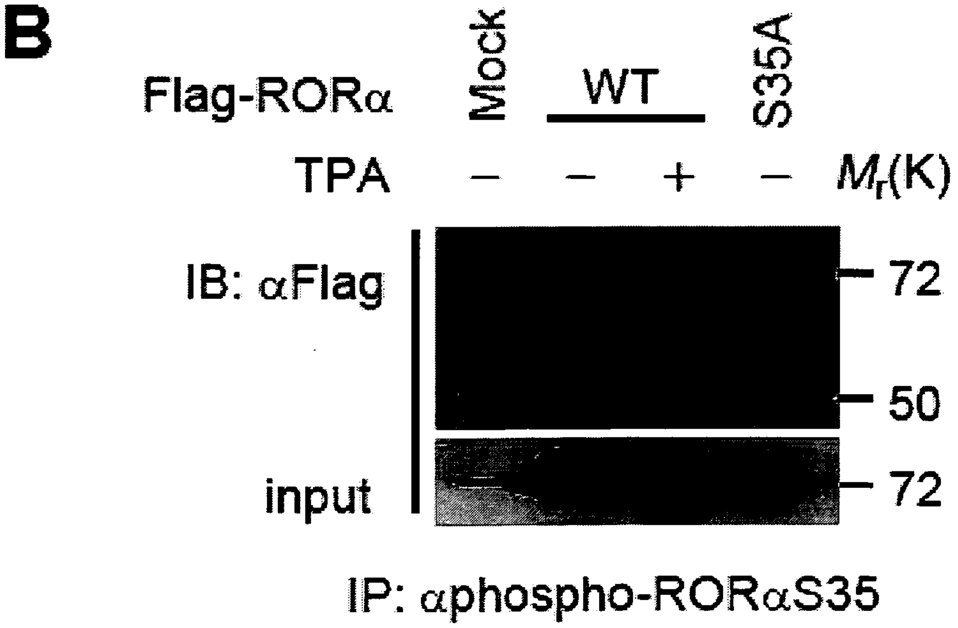
B
Flag-RORα
Mock
WT
S35A
TPA
–
–
+
–
M_r(K)
IB: αFlag
72
50
input
72
IP: αphospho-RORαS35

Fig. 4B

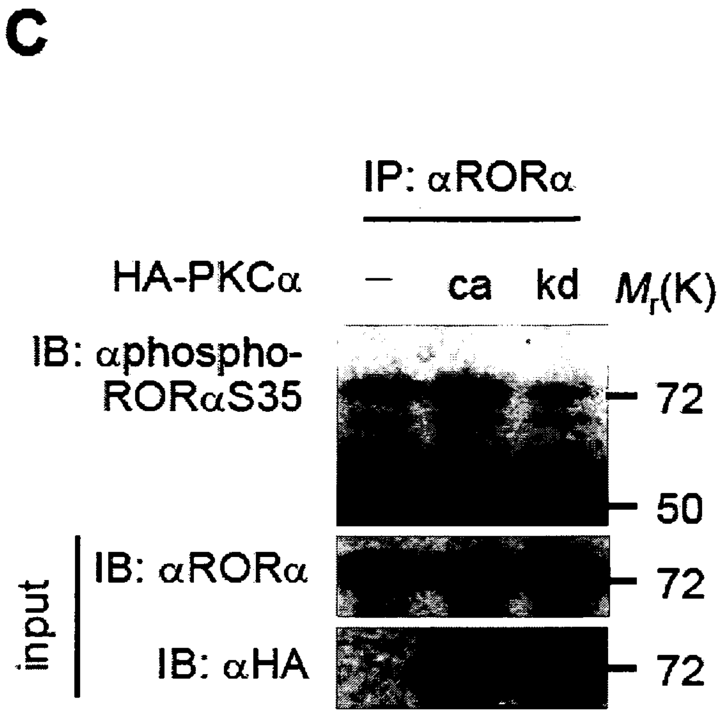
C
IP: αRORα
HA-PKCα
–
ca
kd
M_r(K)
IB: αphospho-RORαS35
72
50
input
IB: αRORα
72
IB: αHA
72

Fig. 4C

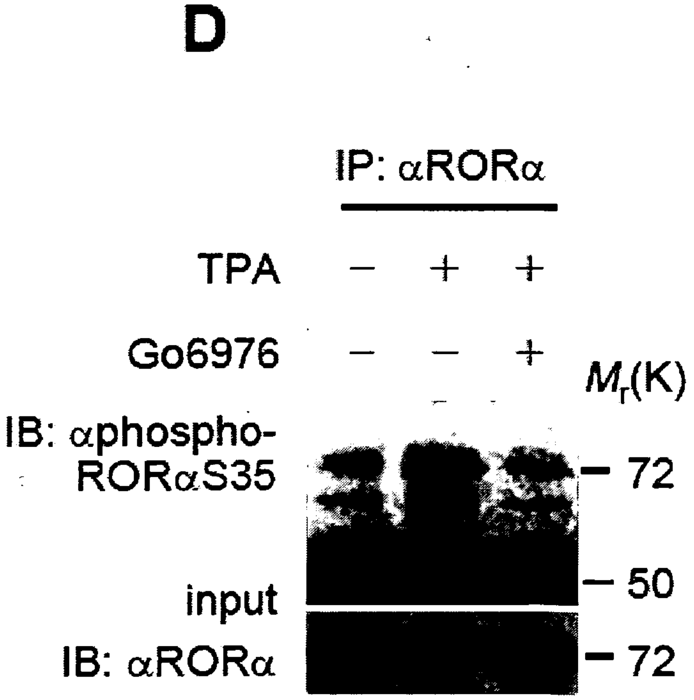
D
IP: αRORα
TPA
– + +
Go6976
– – +
M_r(K)
IB: αphospho-RORαS35
72
50
input
IB: αRORα
72

Fig. 4D


E
IP: αFlag
Flag-RORα
Mock
WT
S35D
S35A
M_r(K)
IB: αβ-catenin
100
input
100

Fig. 4E

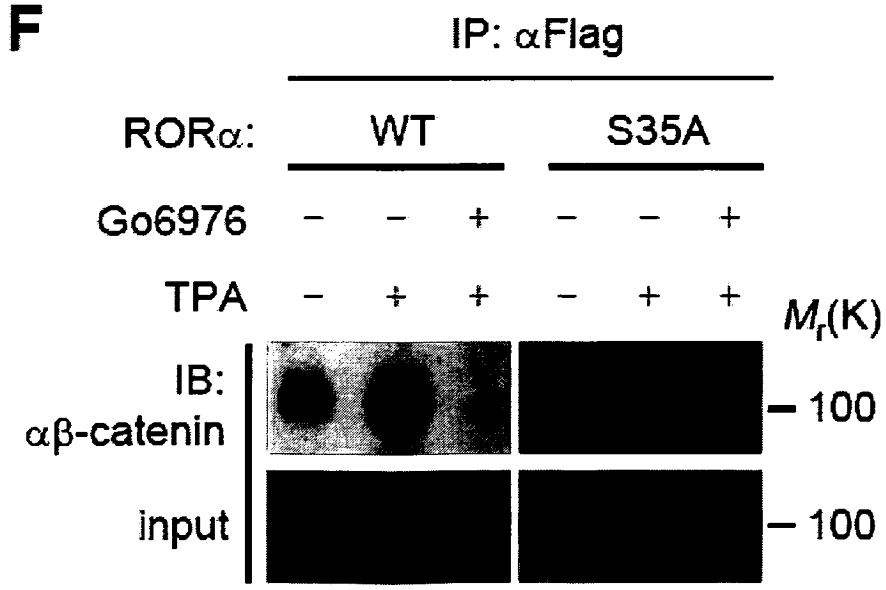
F
IP: αFlag
RORα:
WT
S35A
Go6976
– – + – – +
TPA
– + + – + +
M_r(K)
IB: αβ-catenin
100
input
100

Fig. 4F

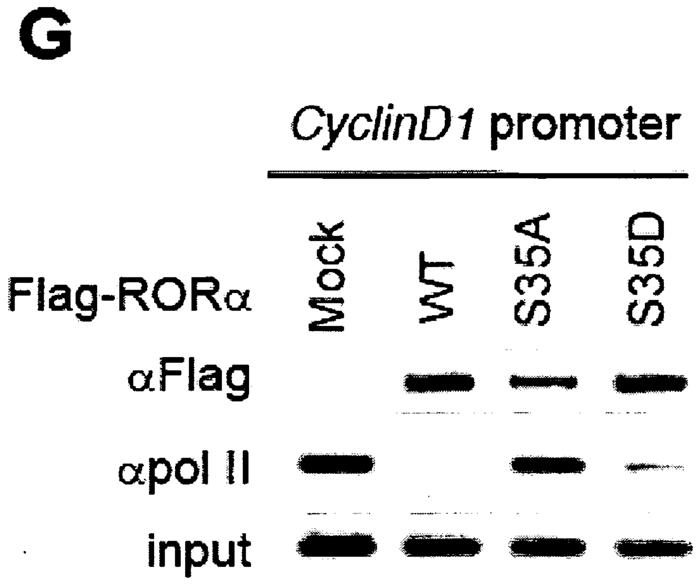
G
CyclinD1 promoter
Flag-RORα
Mock
WT
S35A
S35D
αFlag
αpol II
input

Fig. 4G

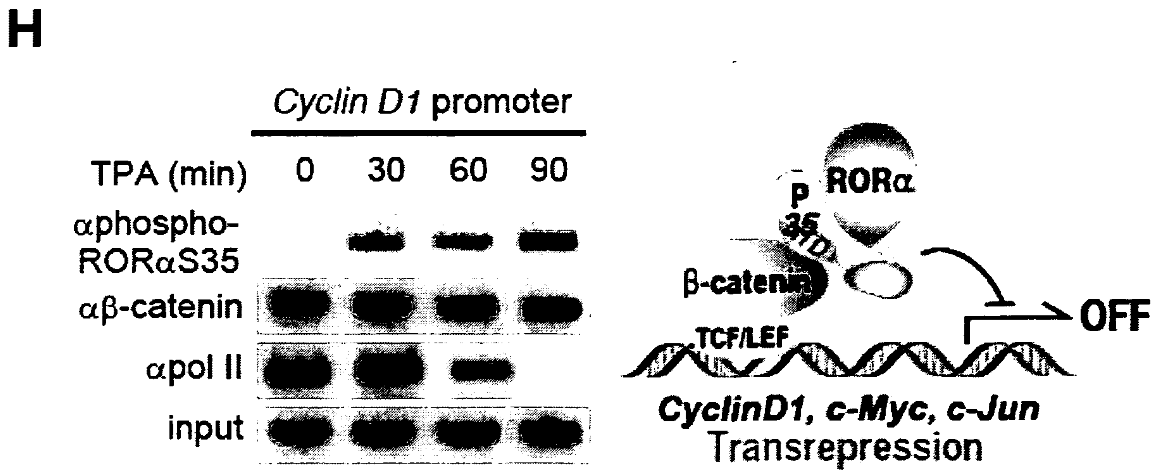
H
Cyclin D1 promoter
TPA (min)
0
30
60
90
αphospho-RORαS35
αβ-catenin
αpol II
input
P
35
RORα
β-catenin
TCF/LEF
OFF
CyclinD1, c-Myc, c-Jun
Transrepression

Fig. 4H

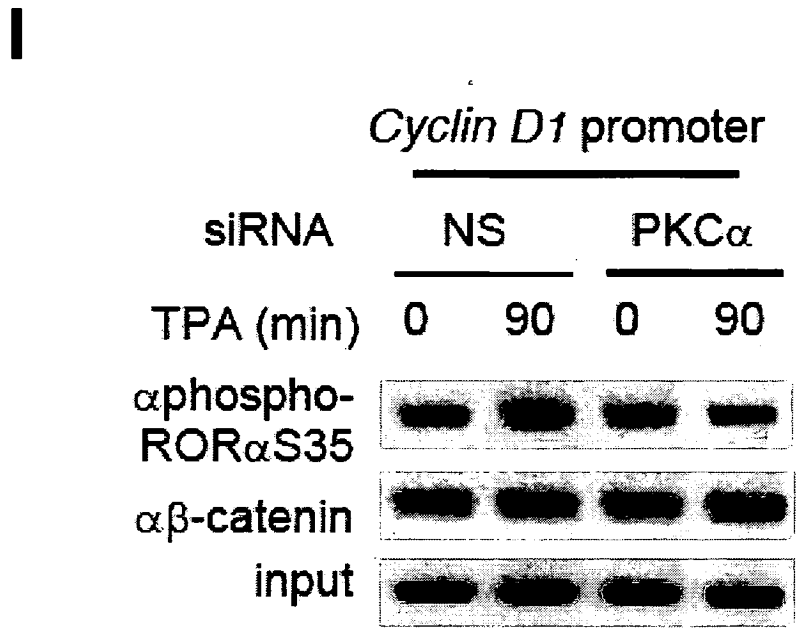
I
Cyclin D1 promoter
siRNA
NS
PKCα
TPA (min)
0
90
0
90
αphospho-RORαS35
αβ-catenin
input

Fig. 4I

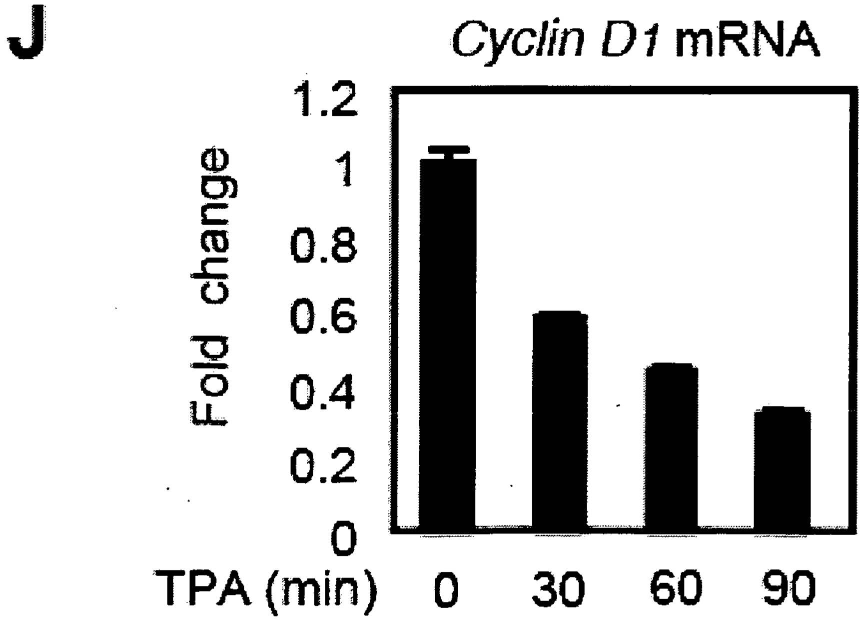
J
Cyclin D1 mRNA
Fold change
1.2
1
0.8
0.6
0.4
0.2
0
TPA (min)
0
30
60
90

Fig. 4J

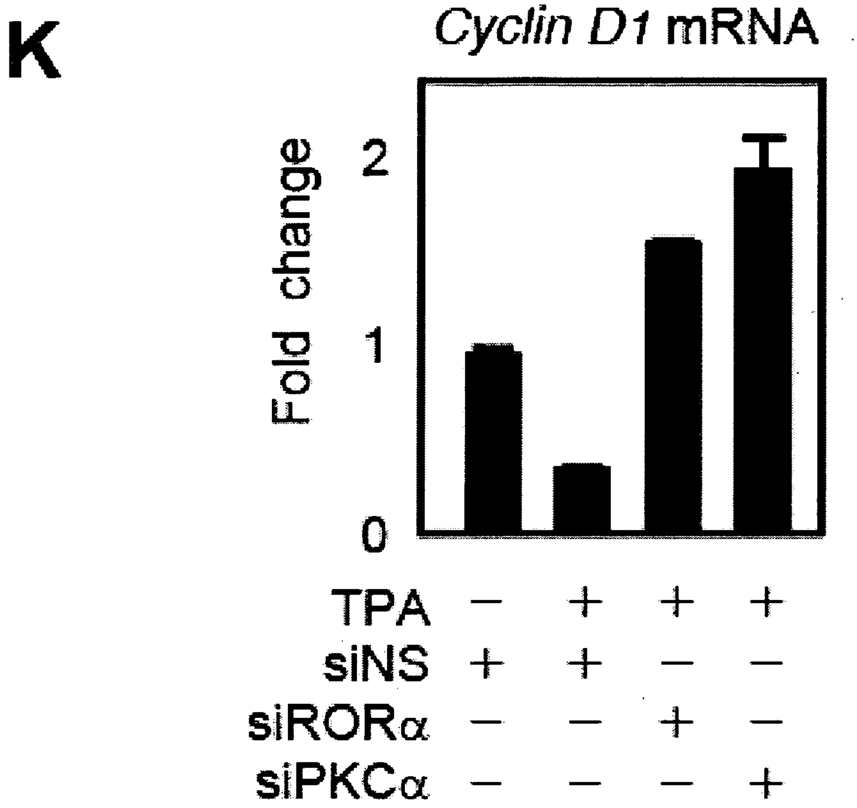

Fig. 4K

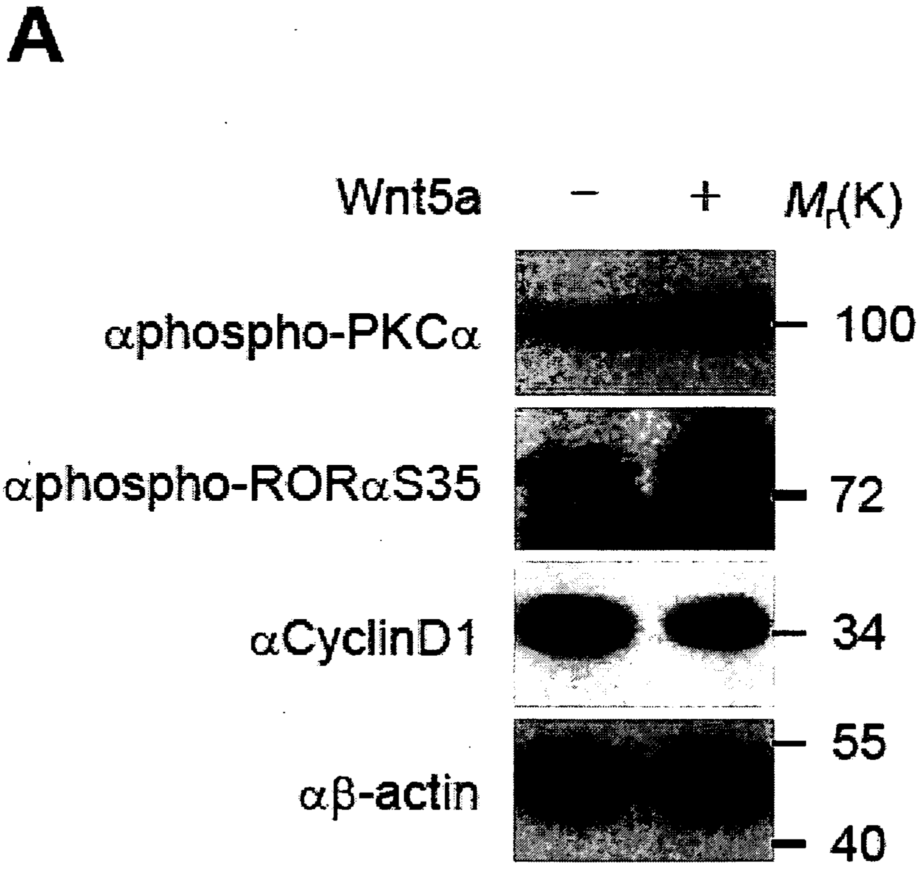
A
Wnt5a
−
+
M_r(K)
αphospho-PKCα
100
αphospho-RORαS35
72
αCyclinD1
34
αβ-actin
55
40

Fig. 5A

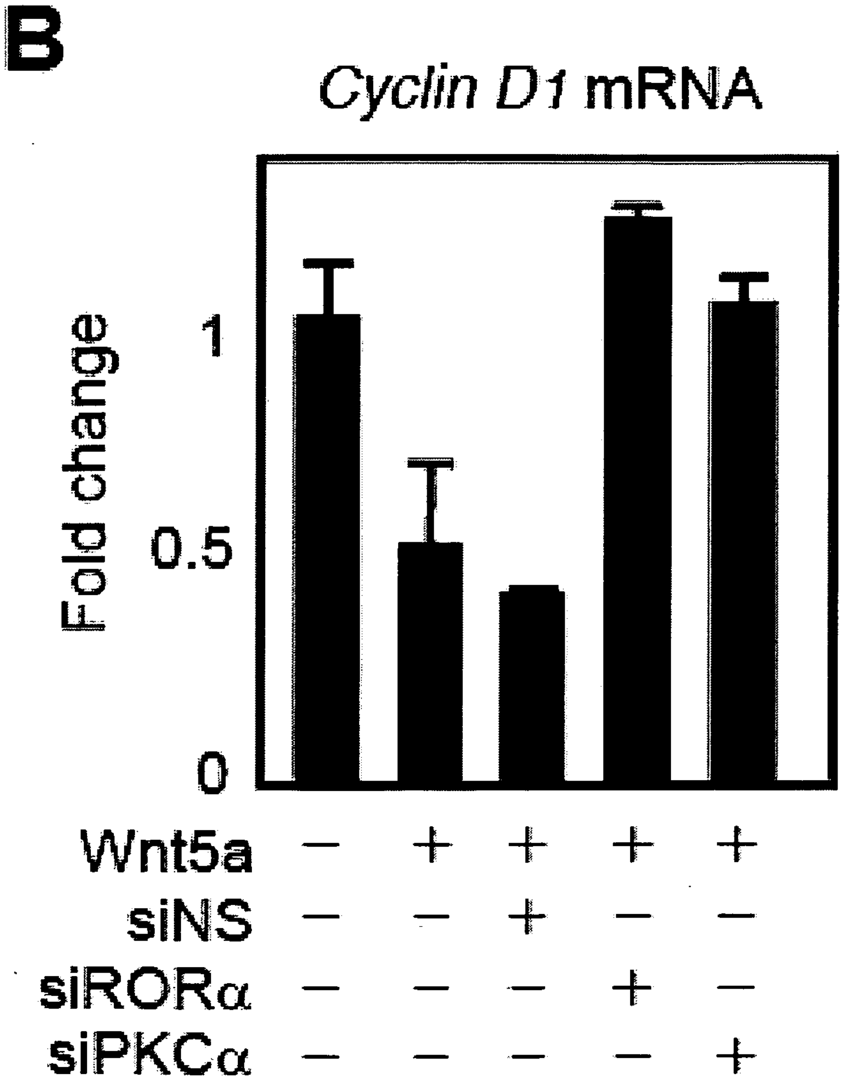

Fig. 5B

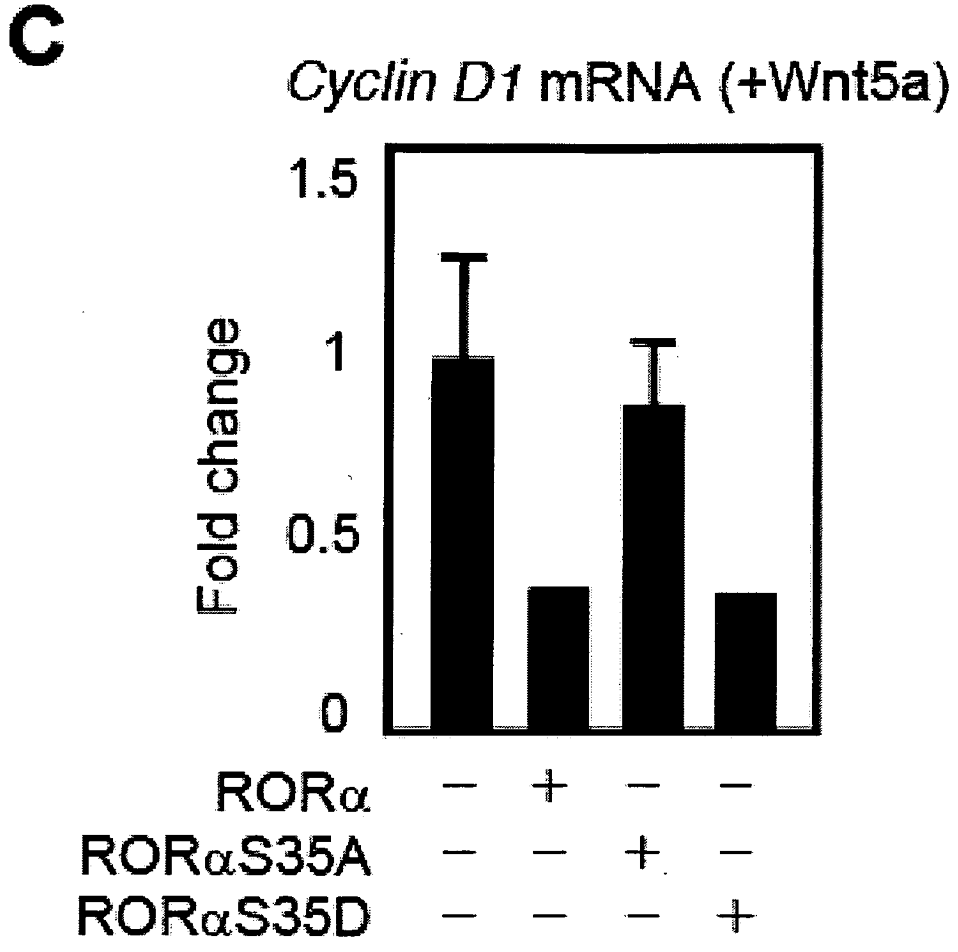
C
Cyclin D1 mRNA (+Wnt5a)
Fold change
1.5
1
0.5
0
RORα − + − −
RORαS35A − − + −
RORαS35D − − − +

Fig. 5C

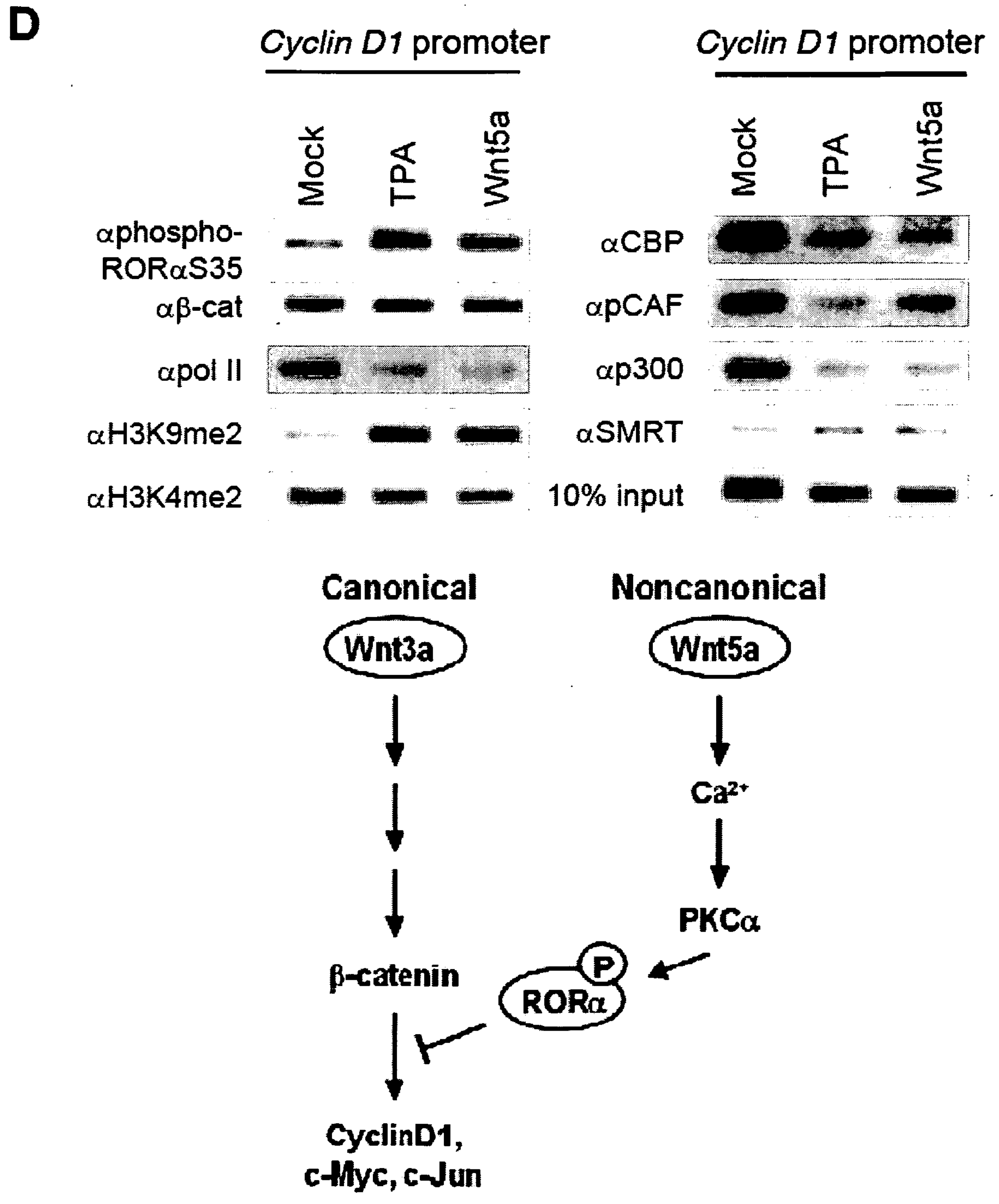
D
Cyclin D1 promoter
Mock
TPA
Wnt5a
αphospho-RORαS35
αβ-cat
αpol II
αH3K9me2
αH3K4me2
Cyclin D1 promoter
Mock
TPA
Wnt5a
αCBP
αpCAF
αp300
αSMRT
10% input
Canonical
Wnt3a
β-catenin
CyclinD1, c-Myc, c-Jun
Noncanonical
Wnt5a
Ca2+
PKCα
P
RORα

Fig. 5D

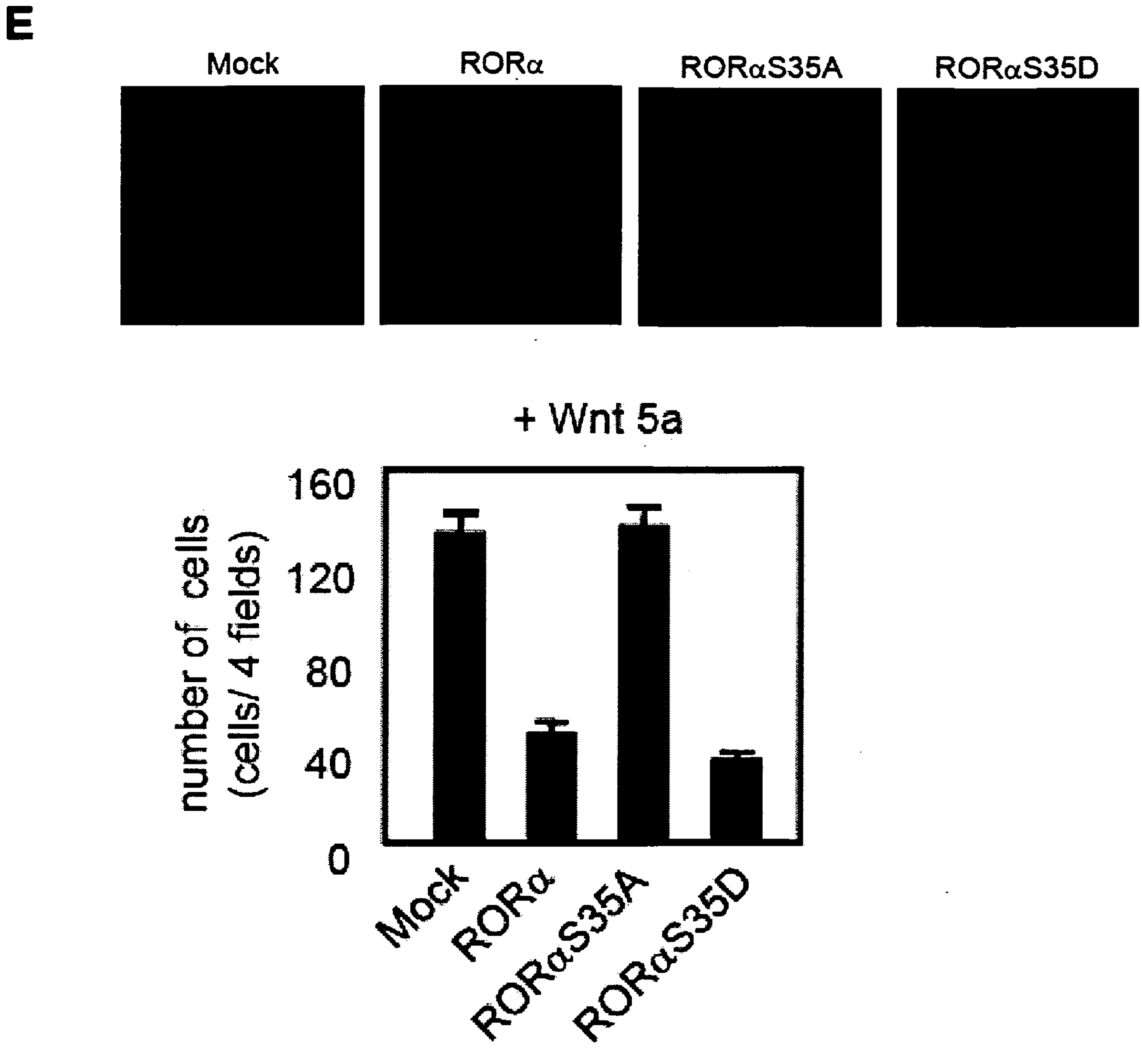
E
Mock
RORα
RORαS35A
RORαS35D
+ Wnt 5a
number of cells
(cells/ 4 fields)
160
120
80
40
0
Mock
RORα
RORαS35A
RORαS35D

Fig. 5E

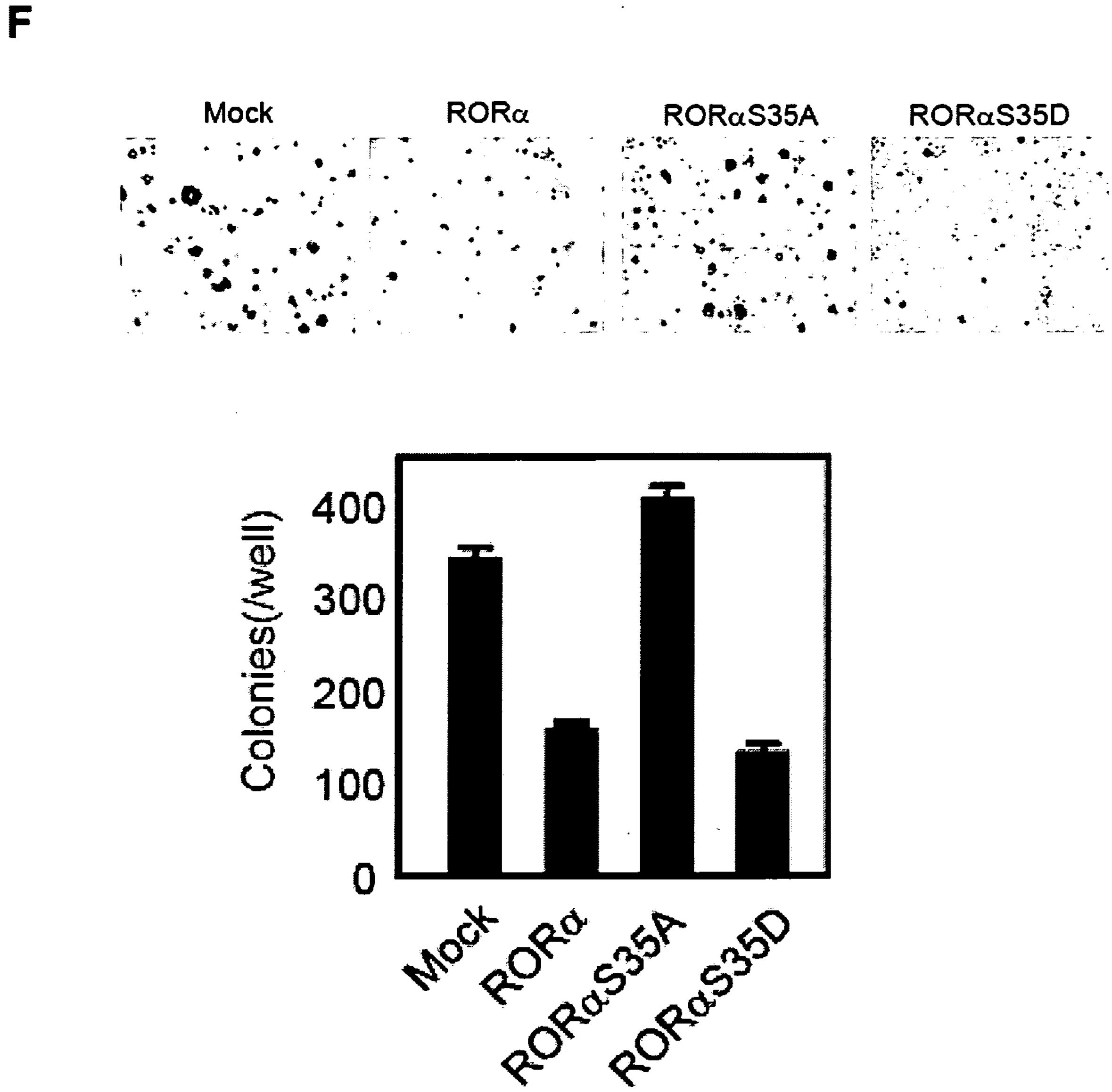
F
Mock
RORα
RORαS35A
RORαS35D
Colonies(/well)
400
300
200
100
0
Mock
RORα
RORαS35A
RORαS35D

Fig. 5F

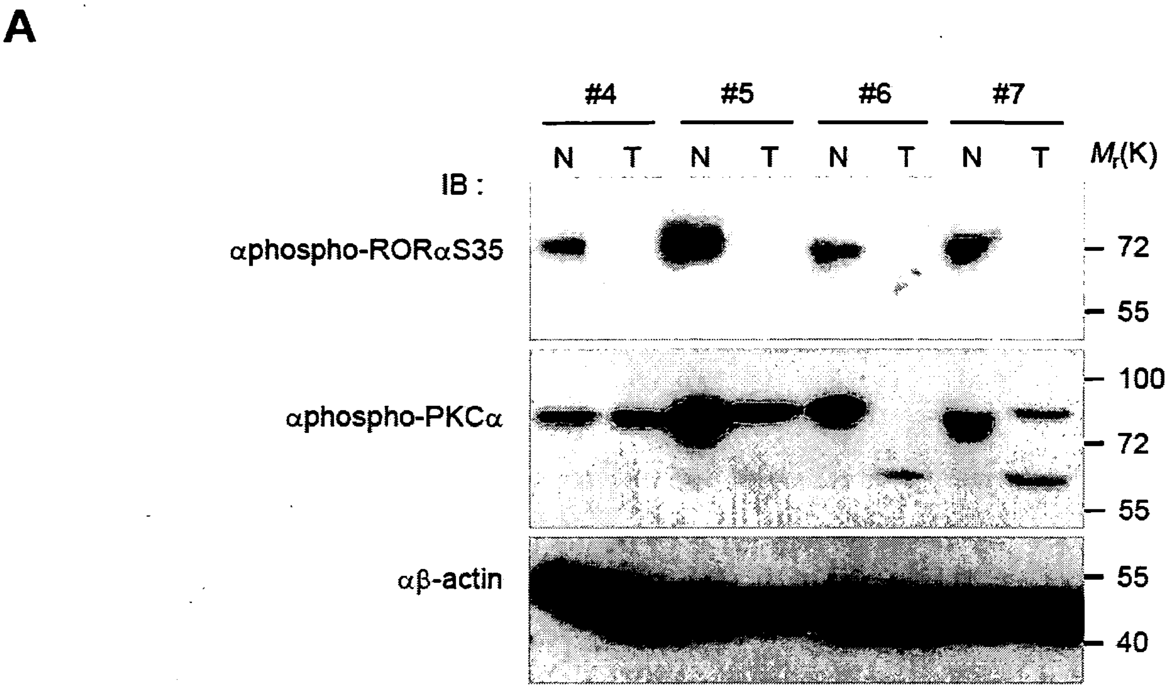
A
#4
#5
#6
#7
N T N T N T N T
M_r(K)
IB :
αphospho-RORαS35
αphospho-PKCα
αβ-actin
72
55
100
72
55
55
40

Fig. 6A

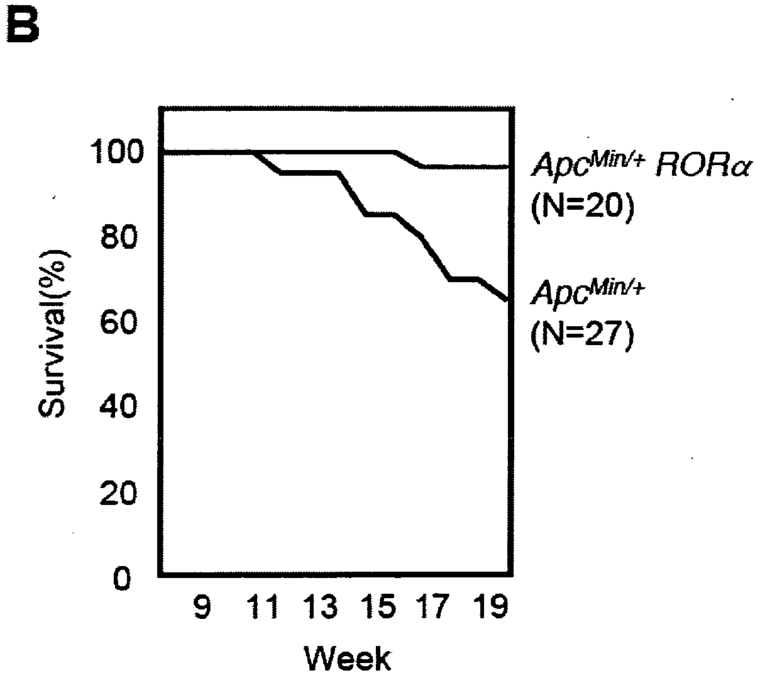
B
Survival(%)
100
80
60
40
20
0
9
11
13
15
17
19
Week
$Apc^{Min/+}$ RORα
(N=20)
$Apc^{Min/+}$
(N=27)

Fig. 6B

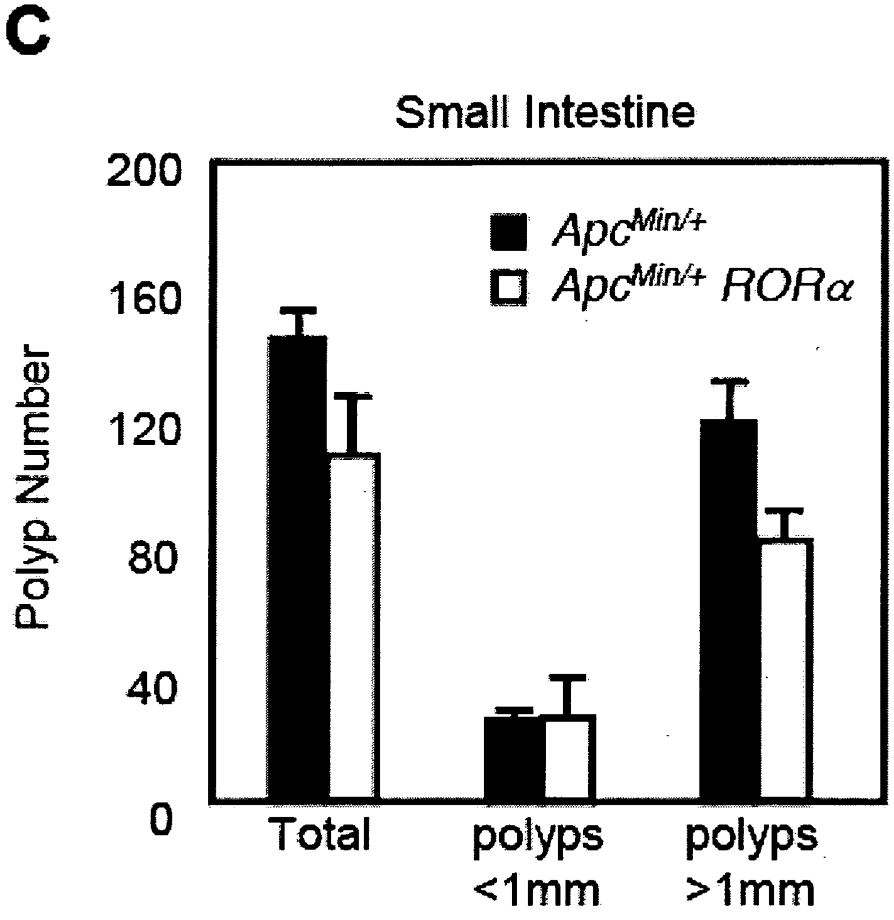
C
Small Intestine
■ $Apc^{Min/+}$
□ $Apc^{Min/+}$ RORα
Polyp Number
200
160
120
80
40
0
Total
polyps <1mm
polyps >1mm

Fig. 6C

D

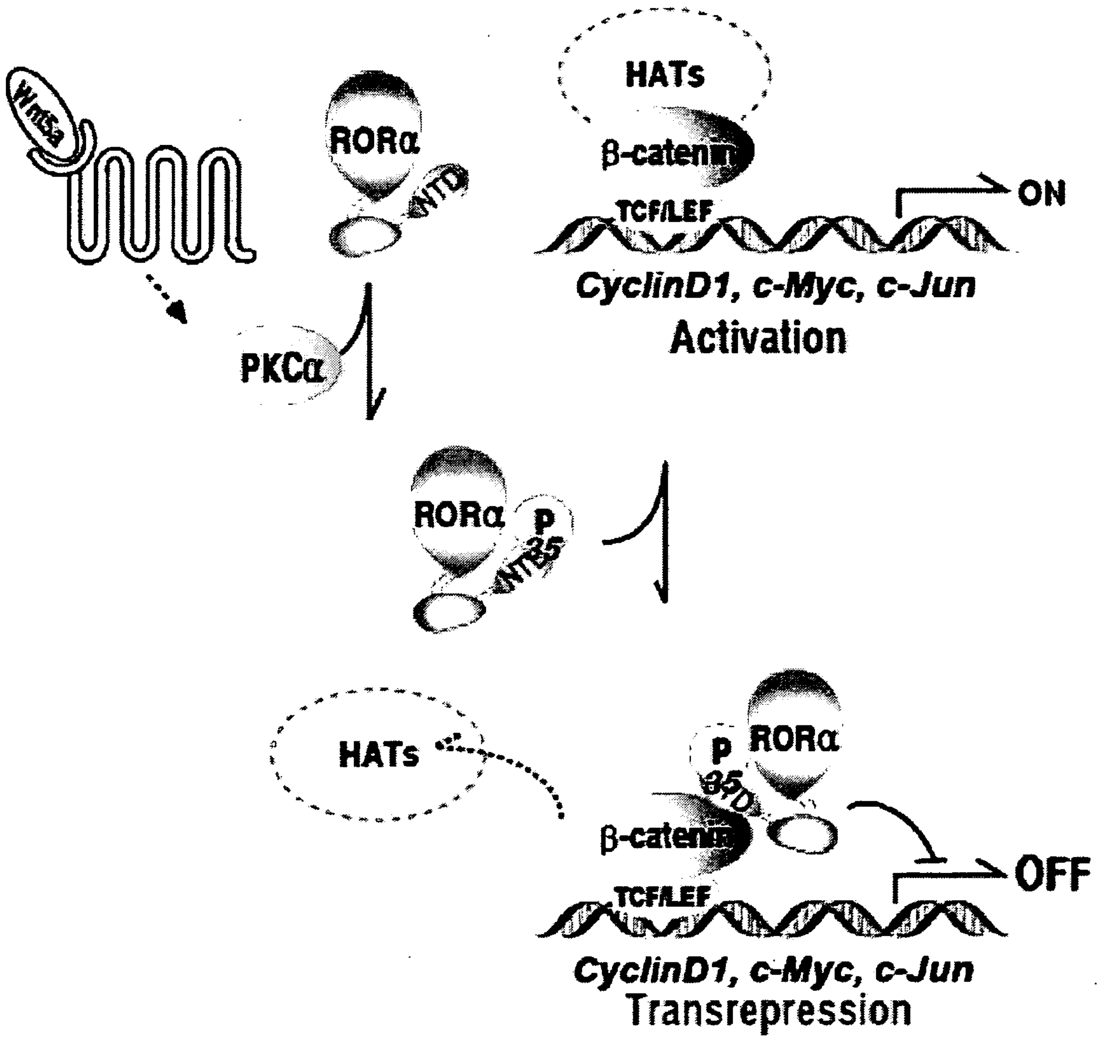
Wnt5a
RORα
NTD
HATs
β-catenin
TCF/LEF
ON
CyclinD1, c-Myc, c-Jun
Activation
PKCα
RORα
P
35
NTD
HATs
P
RORα
35D
β-catenin
TCF/LEF
OFF
CyclinD1, c-Myc, c-Jun
Transrepression

Fig. 6D

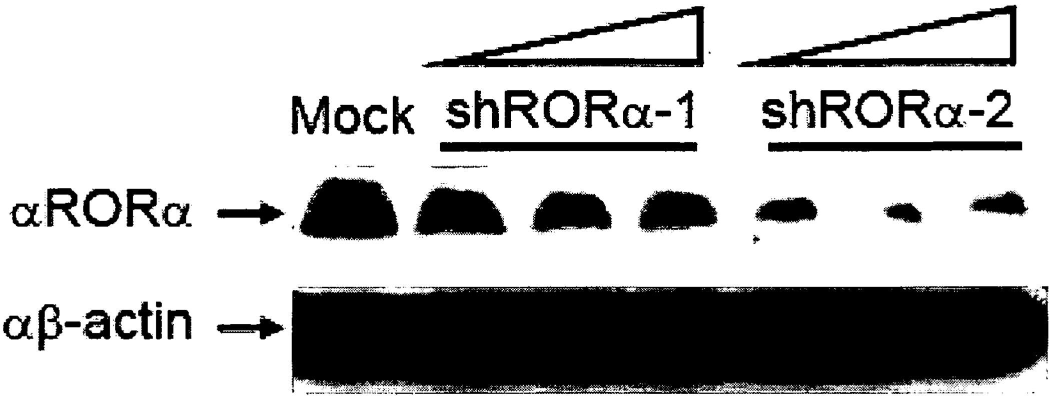

Fig. 7

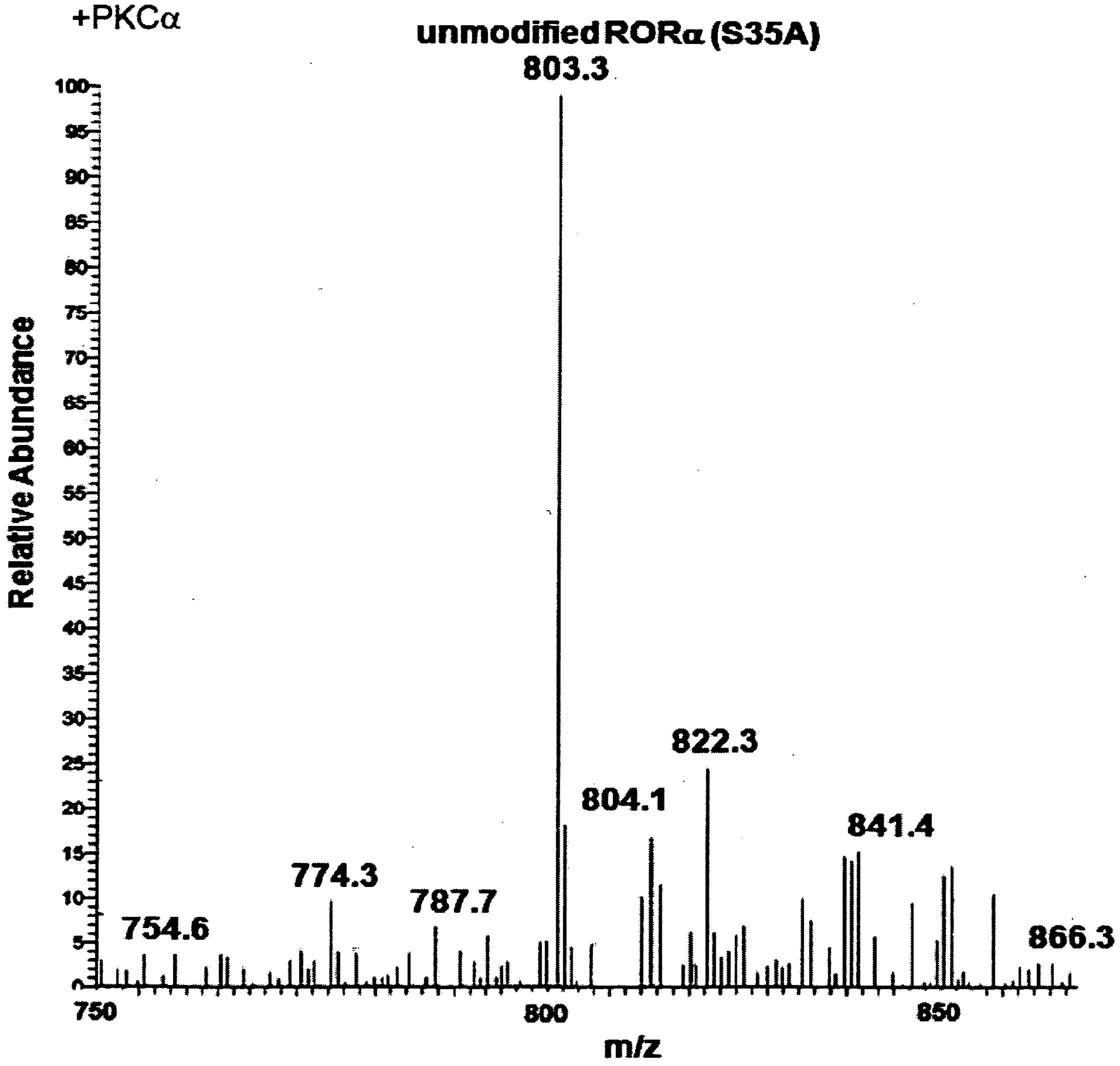
+PKCα
unmodified RORα (S35A)
803.3
822.3
804.1
841.4
774.3
787.7
754.6
866.3
Relative Abundance
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
750
800
850
m/z

Fig. 8

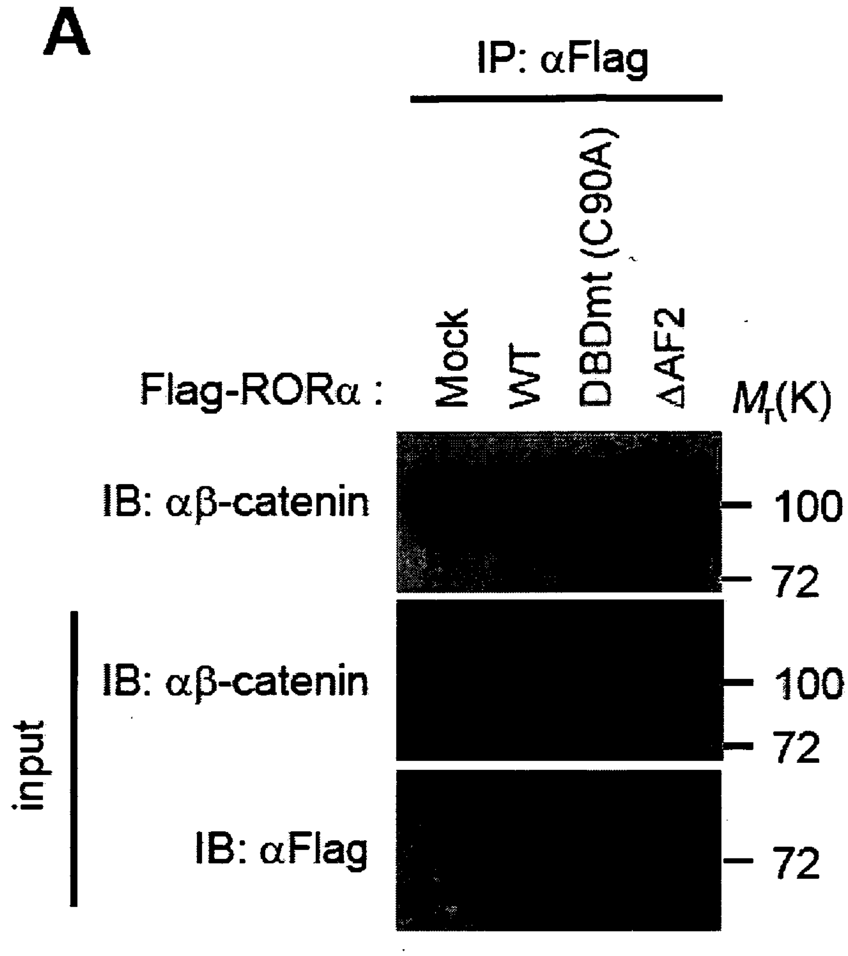
A
IP: αFlag
Flag-RORα :
Mock
WT
DBDmt (C90A)
ΔAF2
M_r(K)
IB: αβ-catenin
100
72
input
IB: αβ-catenin
100
72
IB: αFlag
72

Fig. 9A

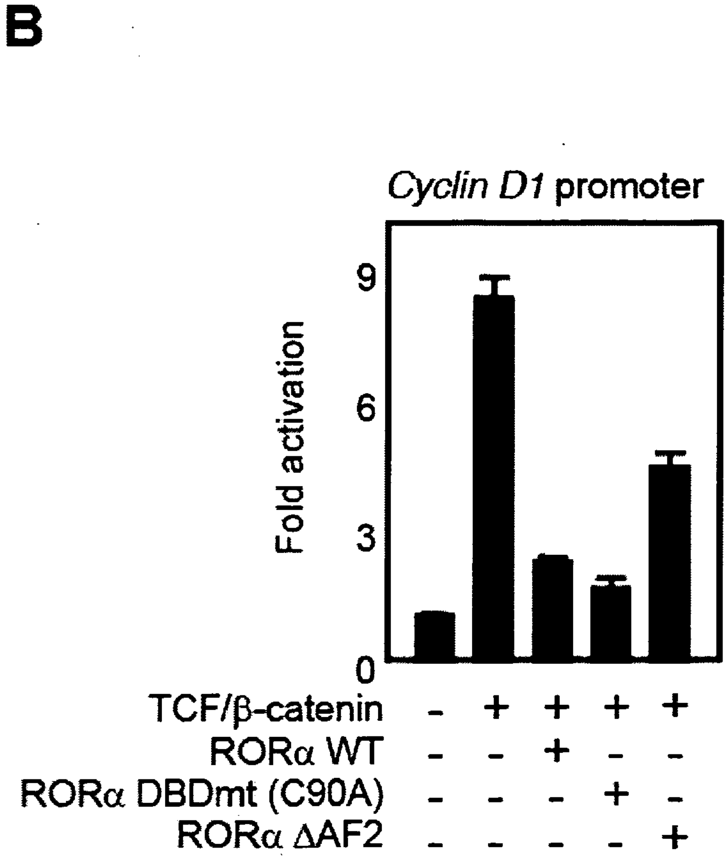
B
Cyclin D1 promoter
Fold activation
9
6
3
0
TCF/β-catenin - + + + +
RORα WT - - + - -
RORα DBDmt (C90A) - - - + -
RORα ΔAF2 - - - - +

Fig. 9B

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专利名称(译)	使用ror的抗癌药物筛选方法		
公开(公告)号	EP2431482A4	公开(公告)日	2012-06-13
申请号	EP2010729336	申请日	2010-01-08
[标]申请(专利权)人(译)	首尔大学校产学协力团		
申请(专利权)人(译)	首尔国立大学的产业基础		
当前申请(专利权)人(译)	首尔国立大学的产业基础		
[标]发明人	KIM KEUN IL LEE JI MIN BAEK SUNG HEE		
发明人	KIM, KEUN IL LEE, JI MIN BAEK, SUNG HEE		
IPC分类号	C12Q1/68 G01N33/574 G01N33/53 C12N5/071 G01N33/50 G01N33/573		
CPC分类号	G01N33/57419 G01N33/5011 G01N33/5041 G01N33/573		
代理机构(译)	BOTTERO，CLAUDIO		
优先权	1020090001519 2009-01-08 KR		
其他公开文献	EP2431482A2 EP2431482B1		
外部链接	Espacenet		

摘要(译)

本发明涉及使用RORα筛选抗癌剂的方法，该方法包括以下步骤：培养细胞;使潜在的物质与细胞接触;确定细胞中RORα的磷酸化水平是否与对照细胞（未与潜在物质接触）相比增加;如果细胞中RORα的磷酸化水平增加，则选择潜在物质作为抗癌剂。