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Novel Regulatory Mechanism of Canonical Wnt Signaling by Dopamine D₂ Receptor through Direct Interaction with β-catenin

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Nonstandard Abbreviations:

Quinpirole, (4a*R*,8a*R*)-5-propyl-4,4a,5,6,7,8,8a,9-octahydro-1*H*-pyrazolo[3,4-*g*]quinolone
Wnt, Wingless/int; GPCR, G protein-coupled receptor; Dvl, deshevelled; GSK3β, glycogen
synthase kinase 3β; D$_2$R, dopamine D$_2$ receptor; LEF-1, lymphoid enhancing factor-1; TCF,
T cell factor; TOP-FLASH, Tcf optimal promoter-flash reporter gene; FZD, frizzled; LRP5/6,
low density lipoprotein receptor-related protein 5/6; HA, hemagglutinin; DAPI, 4',6-
diamidino-2-phenylindole; GST, glutathione-S-transferase; PTX, pertussis toxin.

Abstract

Classical G protein-coupled receptors (GPCRs) and canonical Wnt pathways were believed to use distinct signaling pathways. However, recent studies have shown that these two pathways interacts each other by sharing several intermediate signaling components. Recent *in vivo* studies showed that anti-psychotic drugs, which block dopamine D2-like receptors, increase the cellular levels of downstream signaling components of canonical Wnt pathway, such as dishevelled (Dvl), glycogen synthase kinase 3β (GSK3β), and β-catenin. These results suggest that some functional interactions might exist between Wnt pathway and D2-like receptors. In this study, we show that among five different dopamine receptor subtypes, D_2 receptor (D_2R) selectively inhibited the Wnt signaling, which was measured by lymphoid enhancing factor-1 (LEF-1)-dependent transcriptional activities. D_2R-mediated inhibition of Wnt signaling was agonist- & G protein-independent, and did not require receptor phosphorylation or endocytosis. D_2R inhibited the LEF-1-dependent transcriptional activities and this inhibitory activity was not affected by the inhibition of GSK-3β, suggesting that D_2R inhibited the Wnt signaling by acting on the downstream of GSK3β. D_2R directly interacted with β-catenin through the 2nd and 3rd loops, leading to a reduction of β-catenin distribution in the nucleus, resulting in an inhibition of LEF-1-dependent transcription. This is a novel mechanism for the regulation of canonical Wnt signaling by GPCRs, in which receptor proteins recruit β-catenin from cytosol to the plasma membrane, resulting in the decrement of the β-catenin/LEF-1-dependent transcription in the nucleus.

Introduction

Seven transmembrane receptors are divided into classical and atypical family. The former represents GPCRs and the latter includes Frizzled (FZD). FZD is the receptor for Wnt, a family of highly conserved secreted glycoproteins (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). Until recently, it has been believed that these two receptor systems use distinct downstream signaling pathways, however, some of recent studies have shown that two pathways might interact each other by sharing intermediate signaling components such as G proteins, GRKs (GPCR kinases), β-arrestins, Axins (axis inhibitor I), or scaffolding proteins such as Dvl family proteins (Force et al., 2007).

The canonical Wnt pathway is activated when Wnt proteins bind to FZD and low density lipoprotein receptor-related protein 5/6 (LRP5/6) located on the plasma membrane, causing the receptors to activate Dvl family proteins which are cytoplasmic signaling proteins (Moon, 2005). These series of events ultimately alter the level of β-catenin in the cytosol, which enters the nucleus and acts as a transcriptional co-activator.

The cellular level of β-catenin is tightly controlled by a multi-protein complex, called a destruction complex, which is composed of GSK3β, casein kinase Iα (CKIα), Axin, and adenomatous polyposis coli (APC) (Kimelman and Xu, 2006; Liu et al., 2002). In the absence of Wnt, β-catenin is phosphorylated by CKIα and GSK-3β in the destruction complex, and undergoes ubiquitin-mediated proteasomal degradation (Cadigan and Liu, 2006). In the presence of Wnt, Dvl, which is activated through FZD and LRP5/6, causes the dissociation of destruction complex and inhibition of GSK3β, leading to an accumulation of β-catenin in the cytosol. Accumulated β-catenin moves into the nucleus where it binds to transcription factor, lymphoid enhancing factor/T-cell factor (LEF/TCF), and activates the

transcription (Brunner et al., 1997; Cadigan and Liu, 2006).   Cyclin D1 and c-myc are typical target genes of Wnt (Cadigan and Nusse, 1997; Moon, 2005).

Dopamine is involved in the control and coordination of the movement, cognition/emotion, and the hormone release from the pituitary via three major dopaminergic pathways; nigrostriatal, mesocorticolimbic, and tuberoinfundibular, respectively.   The importance of proper dopaminergic function is evident when any of these systems becomes compromised as in Parkinson's disease (Lee et al., 1978), schizophrenia (Seeman, 1987), or hyperprolactinemia (Cunnah and Besser, 1991).   The dopamine receptors differ in their pharmacological profiles and tissue distributions.   Based on their pharmacological and functional characteristics, the dopamine receptors are classified into two subfamilies, D1- and D2-like receptors (Brown and Makman, 1972; De Camilli et al., 1979; Kebabian and Calne, 1979).   The D1-like receptors have been subdivided into the D_1 and D_5 receptors (D_1R, D_5R) (Dearry et al., 1990; Sunahara et al., 1991; Sunahara et al., 1990), and the D2-like receptors have been subdivided into the D_2, D_3, and D_4 receptors (D_2R, D_3R, D_4R) (Bunzow et al., 1988; Sokoloff et al., 1990; Van Tol et al., 1991). D_1R and D_5R are positively coupled to adenylyl cyclase by the $G\alpha s$, whereas the D_2R, D_3R, and D_4R inhibit this enzyme through coupling to $G\alpha i/o$ (Missale et al., 1998).

Functional interactions between dopaminergic nervous system and Wnt have not been reported at cellular or molecular level.   Interestingly, recent studies *in vivo* showed an increase in cellular levels of Dvl, GSK3β, and β-catenin after long-term treatment with antipsychotic drugs which are the blockers of D2-like receptors (Alimohamad et al., 2005a; Alimohamad et al., 2005b).   In addition, roles of the Wnt signaling pathways in schizophrenia and antipsychotic drug action are recently being emphasized (Freyberg et al.,

2010).   These results suggest that there could be functional interaction between Wnt

pathway and D_2R/D_3R, which are the major targets of currently used neuroleptics.

In this study, roles and molecular mechanisms of dopamine receptors on the Wnt

signaling were determined.   Our results show that among five subtypes of dopamine

receptors (D_1R-D_5R), only D_2R inhibits canonical Wnt signaling. D_2R interacted with β-

catenin through the 2nd and 3rd intracellular loops, and inhibited the entry of β-catenin into the

nucleus, leading to an inhibition of the LEF-1-dependent transcription.   These findings

demonstrate that the functional regulation of Wnt signaling by GPCRs could occur through

direct interaction with β-catenin independently of the upstream signaling components.   It is

expected that these results could be extended to other GPCRs to establish common paradigms

for their functional interactions.   In addition, these results could provide a new

understanding for the etiology of dopamine- and Wnt-related disorders, and novel strategies

for their treatments.

Materials and methods

Materials.   (-)Quinpirole, forskolin, anti-FLAG antibodies-conjugated agarose beads,

pertussin toxin (PTX), haloperidol, clozapine, antibodies to GFP and FLAG, and lithium

chloride (LiCl), were purchased from Sigma/Aldrich Chemical Co. (St Louis, MO, USA).

Alexa 594-labeled anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA,

USA).   Antibodies to β-catenin, lamin B1, actin, and HRP-labeled secondary antibodies,

were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).   Antibodies to

caveolin-1 were from BD biosciences (San Jose, CA, USA).   Antibodies to β-arrestin and

GRK2 were kindly provided by Dr. Robert Lefkowitz and Premont, respectively (Duke University, NC, USA).

Plasmid constructs. Human dopamine D_1-D_5 receptors (D_1R-D_5R) in pCMV5 are described in a previous study (Cho et al., 2006). Receptor expression levels of D1-like and D2-like receptors were determined as described previously (Kim et al., 2001; Kim et al., 2004). D_2R and D_3R tagged with M2-FLAG epitope at the N-terminus tail or with green fluorescent protein (GFP) at the C-terminus tail are described in previous reports (Kim et al., 2005; Kim et al., 2001). Chimeric receptors between D_2R and D_3R in which the 2nd and 3rd intracellular loops are exchanged, are described in previous studies (Kim et al., 2001; Robinson and Caron, 1996). A phosphorylation-deficient D_2R mutant, D_2R-IC2/3, is described in a previous study (Cho et al., 2010). In this mutant, all the serine and threonine residues located within the 2nd and 3rd intracellular loops were altered to alanine and valine residues, respectively. GFP-tagged and various deletion mutants of β-catenin constructs are described in a previous study (Kim et al., 2000). N-terminal–deleted β-catenin (β-catenin ΔN) lacks the first 86 amino acids, and Arm β-catenin lacks both the N-terminal 86 amino acids and the C-terminal 123 amino acids. Kinase dead form of GSK3β and 14-3-3 were provided by K.Y. Lee (Chonnam National University, Korea). FLAG-tagged β-catenin was provided by K.Y.Choi (Yonsei University, Korea). Small hairpin RNAs (shRNAs) for GFP, β-arrestin2, and GRK2 are described in a previous study (Cho et al., 2010). shRNA for β-catenin was obtained from Addgene (Cambridge, MA, USA).

Cell culture and transfection. Human embryonic kidney cells (HEK-293), SH-SY5Y cells, and Wnt3a-producing L929 cells, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell culture reagents were obtained from either Cellgro (Herndon, VA, USA) or Invitrogen. HEK-293 cells were cultured in minimal essential medium (MEM)

supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamicin in a humidified

atmosphere containing 5% CO_2.   Human neuroblastoma SH-SY5Y cells and L929 cells

were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS.

Primary brain cell culture and immunocytochemistry.   Primary cortical neuronal

cultures are described in previous study (Kwon et al.), and the cells were transfected using

lipofectamin2000 (Invitrogen).   For immunocytochemistry, cells were fixed with ice-cold

4% paraformaldehyde in PBS, pH 7.4 for 1 hr.   Cells were incubated with PBS containing

with 3 % FBS and 1% BSA for 1h, then, incubated with FLAG antibody overnight at 4 °C.

After three washes, cells were incubated with Alexa 594 conjugated secondary antibody for 2

h at room temperature.   After 3 washes with washing buffer, the cells were mounted in

Vectashield containing with DAPI (Vector Laboratories, Burlingame, CA, USA) and viewed

with a fluorescence microscope (Olympus, Japan).

Preparation of stable cell lines.   To establish stable RNAi cell lines, HEK-293 cells were

transfected with GFP (control), β-arrestin2, GRK2, or β-catenin RNAi plasmid.   After 2

days, cells were selected with 500 µg/ml G418 (GFP, β-arrestin2, and GRK2 RNAi) or 1

µg/ml puromycin (β-catenin RNAi).   For D_2R-expressing cell lines, HEK-293 cells were

transfected with pRC/CMV vector or D_2R-pRC/CMV, and then selected with 500 µg/ml

G418.   Knockdown of target protein was confirmed by immunoblotting and the receptor

expression was determined by ^{3}H-sulpiride binding.

Determination of Wnt signaling.   Cells were transfected with TOP-FLASH reporter gene

or its control FOP-FLASH reporter gene along with combinations of pRL/TK control vector.

Mouse Wnt1 and Dvl-1constructs were co-transfected or cells were treated with the

conditioned medium containing Wnt3a for 16h for the activation of TOP-FLASH.   Reporter

gene activity was determined using dual luciferase assay kit (Promega, Madison, WI, USA).

Total amount of DNA transfected in each experimental group was adjusted to be equal.

Immunoprecipitation.   Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH

7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), centrifuged at 12,000xg for 30 min

at 4°C.   Supernatants were incubated with 35 μl of FLAG-agarose beads for 2 h 30 min on

the rotation wheel, and beads were washed with washing buffer (50 mM Tris, pH 7.4, 150

mM NaCl, 10% glycerol, 1% NP-40) three times for 5 min each.   Immunoprecipitates were

analyzed on the SDS-PAGE gel and immunoblotted.

In vitro **binding studies**.   The 3rd cytoplasmic loop of D$_2$R was bacterially expressed as a

fusion protein with glutathione-S-transferase (GST).   Since the whole 3rd loop of D$_2$R was

not expressed as soluble protein (data not shown), the 3rd loop of D$_2$R was divided into two

regions (I3D$_2$-N and I3D$_2$-C).   I3D$_2$-N covers R-227 to I-304 and I3D$_2$-C covers from E-250

to K-342.   GST fusion protein with the 3rd loop of rat D$_3$R (I3D$_3$) is described in a previous

study (Cho et al., 2003).   BL21 bacterial cells were treated with 0.5 mM IPTG for 2 h, lysed,

centrifuged, and the resulting supernatant was aliquoted and stored at -70℃ until use.

Lysates of HEK-293 cells expressing wild-type or deletion mutants of GFP-β-catenin were

incubated with the glutathione agarose beads which had been bound to GST fusion proteins.

Agarose beads were washed and retained proteins were eluted with SDS sample buffer, and

the eluents were analyzed with SDS-PAGE gel and immunoblotted with antibodies to GFP.

Immunocytochemistry and confocal microscopy in HEK-293 cells.   One day after

transfection with FLAG-β-catenin and GFP-D$_2$R, the cells were seeded onto 35-mm dishes

containing a centered, 1-cm well that was formed from a glass coverslip sealed hole in plastic

(confocal dishes) and allowed to recover for one day.   Next day, the cells were fixed with

4% paraformaldehyde, for 20 min at room temperature and permeabilized with 0.1% Triton

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X-100.   Cells were labeled with M2 FLAG antibodies (Sigma) at the 1:500 dilutions and

Alexa 594-conjugated anti-mouse antibodies at 1:250 dilutions.   Cells were examined by

Zeiss laser scanning confocal microscopy.

Preparation of Wnt3a conditioned medium. The Wnt3a-producing L929 cells were

cultured with DMEM containing 10% fetal bovine serum for 24 h.   The medium was

changed to serum-free DMEM and the cells were cultured for 36 h.   The culture medium

(Wnt3a conditioned medium, Wnt3a-CM) was then harvested, centrifuged at 1000xg for 10

min, and filtered through a nitrocellulose membrane with 0.22 μm pore size.   The activity of

Wnt3a-CM was assayed on the normal L929 cells by examining the β-catenin level.

Subcellular fractionation.   Cell lysates were fractionated into cytosolic, membrane, and

nuclear fraction according to a previous report (Pan et al., 2005).   Briefly, cells were

incubated with buffer-1 (10 mM HEPES/KOH pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM

DTT, 0.2 mM PMSF, 1 mM Na_3VO_4) for 20 min, and centrifuged at 2,000xg.   The

supernatants were centrifuged for another 10 min at 15,000xg, and the supernatant was saved

as cytosolic fraction.   The pellets were lysised with RIPA buffer to constitute the membrane

fraction.   The pellet from the first centrifugation step was washed with buffer-1 for 15min,

centrifuged at 15,000xg for 10 min, and the resulting pellet was incubated with buffer-2 (20

mM HEPES/KOH pH 7.8, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5

mM DTT, 0.2 mM PMSF, 1 mM Na_3VO_4) for 20 min.   After centrifugation at 24,000xg for

10 min, the supernatant was collected as nuclear extract.

Statistics.   All of the results are expressed as mean ± SEM. Comparisons between

experimental groups were performed by student's *t* test.

Results

D_2R constitutively inhibits canonical Wnt signaling pathways

The Wnt signaling was measured by TOP-FLASH assay, which measures LEF-1-dependent gene transcription. FOP-FLASH was used as negative control. When Wnt1 and Dvl-1 were co-expressed in HEK-293 cells, the LEF-1-dependent gene transcription increased around 15 times above Mock transfected group. Among five different dopamine receptor subtypes tested, Wnt signaling was selectively inhibited in cells expressing the D_2R (Fig.1A). There are two different isoforms of alternatively spliced D_2R, short and long form in which 29 extra amino acids are inserted in middle of the 3rd cytoplasmic loop (Bunzow et al., 1988; Giros et al., 1989). Wnt signaling was inhibited to the similar extent by short and long alternatively spliced forms of D_2R (Supplemental Fig.1), suggesting that 29 amino acid insert in the 3rd cytoplasmic loop does not contribute on the inhibition of Wnt signaling. It is noticeable that D_3R which is closely related to D_2R did not have any effect on the Wnt signaling. Virtually the same results were obtained when the LEF-1-dependent transcriptional activity was induced by treating the cells with Wnt3a-CM (Fig.1B). The selectivity of D_2R on the Wnt signaling was also confirmed in SH-SY5Y cells, dopaminergic human neuroblastoma cells (Fig.1C). Effect of D_2R in Wnt signaling was agonist-independent. Treatment with increasing concentrations of quinpirole (0.01-100 nM), a D_2R agonist, did not alter the magnitude of the inhibitory effect of D_2R on Wnt signaling compared with vehicle group (Fig.1D). Although previous *in vivo* results had shown that antipsychotic drugs elevated cellular levels of Wnt signaling components, our results show that haloperidol or clozapine do not have any effect on the inhibition of Wnt signaling by D_2R (Fig.1E).

Characterization of D_2R-mediated inhibition of Wnt signaling

Next we examined whether D_2R-mediated regulation of Wnt signaling occurs through G protein coupling or receptor endocytosis. When cells were treated with 100 ng/ml PTX at which the coupling between D_2R and $G\alpha i/o$ proteins is completely abolished (Kim et al., 1995), the inhibitory activity of D_2R on the Wnt signaling was not affected (Fig.2A). These results were corroborated by using a point mutant of D_2R in which the arginine or aspartic acid residue of DRY (Asp-Arg-Tyr) motif was altered to histidine (R132H-D_2R) or asparagine (D131N-D_2R). R132H-D_2R lacks both G protein coupling and agonist-induced endocytosis but shows intact ligand binding properties (Kim and Caron, 2008) while D131N-D_2R shows intact G protein coupling and ligand binding properties but reveals 4 fold increase of agonist-induced endocytosis (Supplemental Fig.2A)(Kim et al., 2008). As shown in Fig.2B&C, R132H-D_2R and D131N-D_2R showed the similar extent of inhibitory activities on Wnt signaling, suggesting that D_2R exerts constitutive inhibitory activity toward Wnt signaling without the involvement of G protein coupling or receptor endocytosis.

Next, we tested the involvement of receptor phosphorylation on the D_2R-mediated inhibition of Wnt signaling. For this, a mutant of D_2R in which all the possible phoshorylation sites located within the 2nd and 3rd intracellular loops were mutated, D_2R-IC2/3, was used. As shown in Fig.2D, WT-D_2R and D_2R-IC2/3 showed similar extent of inhibition of Wnt signaling, suggesting that receptor phosphorylation is not a critical cellular event for the inhibition of Wnt signaling by D_2R.

It was recently reported that GRK2 inhibits the canonical Wnt signaling (Wang et al., 2009). The involvement of GRK2 in the D_2R-mediated inhibition of Wnt signaling was tested using GRK2 knockdown cells. Wnt signaling was moderately ($p < 0.05$) increased in

GRK2 knockdown cells, however, the inhibitory activities of D_2R on Wnt signaling were similar in control and GRK2 knockdown cells (Supplemental Fig.2B).

β-arrestin and Akt are reported to be involved in the regulation of canonical Wnt pathway (Bryja et al., 2007; Chen et al., 2001; Fukumoto et al., 2001).   A recent study has shown that D_2R forms a signaling complex, β-arrestin2/Akt/PP2A, and regulates Akt/GSK3β signaling in a G protein-independent and β arrestin2-dependent manner (Beaulieu et al., 2005).   These results suggest that β-arrestin might play some roles in the D_2R-mediated inhibition of Wnt signaling.   Wnt1/Dvl1-induced (Fig.2E) but not β-catenin-induced (Fig.2F) increase in TOP-FLASH activity was reduced when endogenous β-arrestin2 was lowered (Supplemental Fig.2C).   However, in both cases the inhibitory activities of D_2R on TOP-FLASH activity were similar.   These results suggest that D_2R inhibits Wnt signaling independently of β-arrestin and confirm a previous study, which showed that β-arrestins synergistically activates Wnt signaling through interaction with Dvl (Chen et al., 2001).

The 2nd and 3rd intracellular loops of D_2R are involved in the inhibition of Wnt signaling

The D_2R and D_3R possess high homology, with the two sharing 46% overall amino acid homology and 78% identity in the transmembrane domains (Giros et al., 1990). Similarly, D_2R and D_3R share many signaling and regulatory properties when they are expressed in mammalian cells (Kim et al., 2001).   In this sense, it was unexpected that only D_2R but not D_3R selectively inhibited the Wnt signaling.   To determine the receptor regions of D_2R involved in the regulation of Wnt signaling, chimeric receptors between D_2R and D_3R in which the 2nd and 3rd cytoplasmic loops were exchanged (Kim et al., 2001; Robinson and Caron, 1996) were used.   Schematic diagrams of the chimeric receptors between D_2R and D_3R are shown in Fig3.A.   As shown in Fig.3B, the inhibitory activities of D_2R decreased as

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the 2nd and 3rd intracellular loops were replaced with those of D$_3$R.   Also, sequential

replacement of the 2nd and 3rd loop of D$_3$R with those of D$_2$R changed the phenotype of D$_2$R

to that of D$_3$R (Fig.3C), suggesting that both the 2nd and 3rd loops are involved in the

inhibition of Wnt signaling.

D$_2$R regulates canonical Wnt signaling at the level of β-catenin

The point of the involvement of D$_2$R for the regulation of Wnt signaling was determined

by testing the inhibitory activity of D$_2$R for the canonical Wnt signaling which was initiated

at different levels of Wnt cascade.   D$_2$R inhibited Wnt- or Dvl-1-induced LEF-1-dependent

transcriptional activities (Fig.4A), suggesting that D$_2$R acts at the downstream of FZD and

LRP5/6.   D$_2$R also inhibited the TOP-FLASH activity which was induced by β-catenin

(Fig.4B), regardless of the blockade of GSK3β (Fig.4C), suggesting that D$_2$R act at the level

of β-catenin or downstream.   In accordance with this, D$_2$R also inhibited the TOP-FLASH

activity which was raised by treatment with LiCl, an inhibitor of GSK3β (Fig.4D).   D$_3$R

which was employed as negative control did not have any effect.   Cellular levels of β-

catenin were elevated by co-expression of Wnt1/Dvl-1 (Fig.4E) or by treatment with lithium

(Fig.4F), however, D$_2$R did not affect the total cellular amount of β-catenin, suggesting that

D$_2$R does not affect the transcription/translation or the stability of endogenous β-catenin.

Interaction between D$_2$R and β-catenin mediates the inhibition of Wnt signaling

To understand the molecular mechanisms involved in the D$_2$R-mediated inhibition of

Wnt signaling, cellular components which are involved in the Wnt signaling, such as GSK3β,

β-catenin, and 14-3-3, were tested for the interactions with D$_2$R.   Along with Chibby, an

antagonist of β-catenin, 14-3-3 is known to form a tri-molecular complex with β-catenin (Li

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et al., 2008) to control the subcellular localization and stabilization of β-catenin. Among the

proteins tested, only β-catenin interacted with D_2R (Supplemental Fig.3A). In accordance

with TOP-FLASH assay, interaction between D_2R and β-catenin did not change after agonist

(quinpirole) or antagonist (haloperidol) treatment (Supplemental Fig.3B).

Since the 2nd and 3rd intracellular loops of D_2R were responsible for the inhibition of Wnt

signaling (Fig.3) and D_2R interacts with β-catenin (Supplemental Fig.3A&B), we tested

whether β-catenin interacts with this receptor region. Both wild-type D_2R and a chimeric

D_3R which contains the 2nd and 3rd intracellular loops of D_2R, D_3R-(D_2-IC23) interacted with

endogenous β-catenin (Fig.5A), suggesting that the inhibition of Wnt signaling by D_2R is

mediated through the interaction between β-catenin and the 2nd and 3rd cytoplasmic loops.

In contrast, D_3R which was employed as a negative control did not interacted with β-cateinin.

Involvement of the intracellular loops of D_2R for the interaction with β-catenin was further

confirmed by testing the interaction between β-catenin and the 3rd intracellular loop of D_2R

fused to GFP (GFP-I3D_2). As shown in Fig.5B&C, GFP-I3D_2 interacted with β-catenin,

and co-expression of GFP-I3D_2 significantly inhibited the β-catenin-induced increase in

TOP-FLASH activity. Inhibitory activity of D_2R on the β-catenin-induced increase in TOP-

FLASH activity, was confirmed by confocal microscopic studies in HEK-293 cells which

stably express D_2R. Co-expression of D_2R prevented the entry of β-catenin into the nucleus

(Fig.5D). As expected, co-expression of β-catenin inhibited the D_2R-mediated decrement of

Wnt signaling in a dose-dependent manner (Fig5E).

Since D_2R interacts with β-catenin and inhibits Wnt signaling, we were curious whether

stimulation of Wnt pathway reciprocally regulates D_2R signaling. Stimulation of Wnt

pathway by co-expression of Wnt1/Dvl-1 (Supplemental Fig.4A) or β-catenin (Supplemental

Fig.4B) did not have any effect on the signaling of D_2R. Similar results were obtained by ERK activation assay (Supplemental Fig.4C). In addition, knockdown of endogenous β-catenin did not alter the signaling (Supplemental Fig.4D) and internalization (Supplemental Fig.4E) of D_2R. These results together suggest that D_2R-mediated regulation of Wnt signaling is unidirectional and the interaction with β-catenin did not affect G protein coupling efficacy of D_2R.

The armadillo repeat domain of β-catenin interacts with the N-terminal part of the 3rd intracellular loop of D_2R

Interaction between D_2R and β-catenin was further characterized by glutathione-S-transferase (GST) pull down assay. For this, the 3rd intracellular loops of D_2R and D_3R were expressed as fusion proteins with GST. As shown in Fig.6A, only the N-terminus part of the intracellular 3rd loop of D_2R (GST-I3D$_2$-N) interacted with β-catenin. On the other hand, GST, GST-I3D$_2$-C, or GST-I3D$_3$ (the whole 3rd intracellular loop of D_3R) failed to bind β-catenin. In order to determine the domain of β-catenin that binds to the 3rd intracellular loop of D_2R, the GST pull down assay was performed for three deletion constructs of β-catenin (Fig. 6B). The central part of β-catenin, the armadillo repeat domain, seems to be the region responsible for the interaction with the 3rd intracellular loop of D_2R. As shown in Fig.6C, both the N-terminus deletion mutant of β-catenin (ΔN-β-catenin) or the armadillo repeat domain itself was enough to bind to the 3rd intracellular loop of D_2R.

D_2R alters the subcellular distribution of β-catenin to prevent the nuclear translocation

Since D_2R binds to β-catenin (Fig.6) and inhibits LEF-1-dependent transcriptional activity by acting at downstream of GSK3β (Fig.4), we determined whether D_2R inhibits the TOP-

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FLASH activity by altering the subcellular localization of β-catenin.   The cellular levels of

β-catenin were raised by transfecting with Wnt1/Dvl-1 because the cellular content of β-

catenin was too low to manipulate.   Cell lysates from different experimental groups were

fractionated into cytosolic, plasma membrane, and nuclear fraction by employing actin,

caveolin-1, and lamin B1, respectively, as internal control.   As shown in Fig.7A, D_2R alone

increased β-catenin level in the membrane and cytosolic faction by 2.1 and 1.6 times

compared with the Mock group, respectively (compare lane-1 and lane-3).   Co-expression of

Wnt1/Dvl-1 resulted in the increases of β-catenin levels in all the fractions tested (compare

lane-1 and lane-2).   Compared with Mock group, 8.6, 1.7, 1.7, and 1.5 times increased in β-

catenin levels were observed for total lysate, membrane fraction, cytosolic fraction, and

nuclear fraction, respectively.   When D_2R was additionally expressed with Wnt1/Dvl-1, the

Wnt1/Dvl-1-induced elevation of the cellular β-catenin levels in the nuclear (Fig.7A4) and

cytosolic fraction (Fig.7A3) reverted to the basal levels (compared lane-2 and lane-4).   On

the other hand, β-catenin levels in the membrane fraction (Fig.7A2) and total cell lysate

(Fig.7A1) rather increased or remained the same, respectively.   These results suggest that

D_2R on the plasma membrane binds to β-catenin, shifting its distribution to the plasma

membrane, and prevents the β-catenin/LEF-1 complex from moving into the nucleus as

shown in Fig.5D.   These results were corroborated by immunocytochemical studies.   β-

catenin was largely distributed in the cytosolic compartment and to a less extent in the

nuclear fraction (Fig.5D).   When D_2R was co-expressed, β-catenin and D_2R co-localized on

the plasma membrane and in the cytosol of HEK-293 cells (Fig.7B, upper panel).   Similar

results were obtained from primary cultured brain cortical neurons (Fig.7B, lower panel).

These results suggest that the interaction between two proteins could be the major cellular

mechanism for the regulation of Wnt signaling.

Discussion

This study is the first that answers whether and how the functional interactions between dopaminergic nervous system and Wnt signaling occur. Among five dopamine receptor subtypes characterized, only D_2R inhibits the canonical Wnt signaling (Fig.1A). The mode of regulation is one-directional; D_2R inhibits Wnt signaling but not *vice versa*. Our study also shows how D_2R controls canonical Wnt signaling. D_2R does not crosstalk with FZD or LRP5/6 on the plasma membrane. A direct and selective regulation of canonical Wnt signaling through interaction with β-catenin is the focus of this study.

In the absence of Wnt signaling, β-catenin is included as a member of the destruction complex which is composed of APC, Axin, CKIα, GSK3β. Under these conditions, β-catenin is phosphorylated by CKIα and GSK-3β, and then undergoes ubiquitination-dependent proteasomal degradation. When Wnt signal is initiated, this destruction complex is dissembled and the degradation of β-catenin is inhibited, facilitating its entry to the nucleus. Most of the previous studies have shown that the crosstalk of the signal transduction pathways between Wnt and classical GPCR occurs at GSK3 level (Force et al., 2007; Shevtsov et al., 2006). Results from this study show that the interaction between certain GPCRs and β-catenin could also control the canonical Wnt signaling pathway independently of the destruction complex. Considering that β-catenin is a well known multifunctional protein, the interaction between D_2R and β-catenin might exert additional functional roles other than D_2R-mediated inhibition of Wnt signaling. For example, β-catenin is usually bound to Cadherin, a cell adhesion molecule, and is involved in the formation of adherins junctions or assembly of synaptic vesicle (Bamji et al., 2003; Xu and Kimelman, 2007). Therefore, it is possible that β-catenin, through interaction with D_2R, might play certain roles in the control of dopaminergic synaptic transmission in which D_2R acts as an autoreceptor.

Wnt signaling is reported to play important roles in the dopamine-related adult brain functions, such as Parkinson' disease (Inestrosa and Arenas, 2010), a neurodegenerative disease in the dopaminergic nervous system, and schizophrenia (Inestrosa and Arenas, 2010), an affective disorder which is caused by an over-activation of dopaminergic transmission. However, the functional relationship between Wnt and dopaminergic nervous system has not been conducted except couple of indirect *in vivo* studies. For example, it was reported that chronic treatment with antipsychotic drugs results in the elevation of intermediate components involved in the Wnt signaling such as Dvl-3, GSK3β, and β-catenin (Alimohamad et al., 2005a; Alimohamad et al., 2005b; Sutton et al., 2007). Results from our study could provide mechanistic explanation for these *in vivo* results as follows. Chronic treatment with antipsychotics blocks dopaminergic neurotransmission by blocking D2-like receptors. A decrease in dopaminergic transmission results in an elevation of cellular levels of D2-like receptors through negative feedback mechanism. Axin 2, a target gene of β-catenin, acts as a central player in the negative feedback loop of Wnt cascade by decreasing the stability of β-catenin (Jho et al., 2002). Since the β-catenin activity will be constitutively inhibited when the cellular levels of D2-like receptors are elevated, chronic treatment with antipsychotics will increase the Wnt signaling by removing the negative feedback imposed on it by Axin2, resulting in an accumulation of intermediate signaling components such as Dvl-3, GSK3β, and β-catenin. In accordance with this, it was reported that Wnt pathway is activated in schizophrenic patients (Miyaoka et al., 1999). Therefore, it is expected that elucidation of the functional interactions between Wnt and GPCRs such as dopamine receptors, will provide the fundamentals for the pathophysiological basis of the related diseases.

This study is the first to show that classical GPCRs regulate Wnt signaling through direct interaction with β-catenin without involvement of upstream components of Wnt signaling pathway.   More detailed studies, for example, identification of characteristic protein motif involved for their interactions, is going to make it possible to apply these findings to other GPCRs.

Authorship Contributions

Participated in research design: KM Kim, KS Kim, CY Shin

Conducted experiments: CC Min, DI Cho, KJ Kwon

Performed data analysis: CC Min, KM Kim

Wrote or contributed to the writing of the manuscript: CC Min, KM Kim

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Footnotes

Figure legends

Fig.1. Effects of dopamine receptor subtypes on the Wnt signaling. (*A*) Effects of dopamine receptor subtypes on the Wnt signaling were determined by TOP-FLASH as described in *Experimental Methods*. Cells were transfected with Wnt1 in pCDNA3.0 (1 μg) and Dvl-1 in pRC/CMV (1 μg) together with 2 μg of each dopamine receptor subtypes in pCMV5 for 100 mm culture dishes. Mock groups were transfected with only reporter gene/pRL-TK control vector. D_2R represents the short form of alternatively spliced D_2R unless specified otherwise. Receptor expression levels were maintained between 1.7-1.9 pmol/mg membrane proteins. ***:$p<0.001$ compared with Wnt1/Dvl-1 group. (*B*) Effect of D_2R and D_3R on the Wnt3a-induced TOP-FLASH activity. HEK-293 cells expressing D_2R or D_3R were treated with control-CM or Wnt3a-containing CM for 16h. ***:$p<0.001$ compared with pCMV5 group. (*C*) Effect of D_2R and D_3R on the Wnt signaling was confirmed in SH-SY5Y human neuroblastoma cell lines. ***:$p<0.001$ compared with pCMV5 group. (*D*) Effects of overnight treatment with quinpirole, a selective agonist of D_2R, were tested on the D_2R-mediated inhibition of Wnt signaling. ***:$p<0.001$ compared with Wnt1/Dvl-1 group. *(E)* Effects of the antagonists of D2-like receptors on the D_2R-mediated inhibition of Wnt signaling. Cells were treated with 10 μM haloperidol or clozapine for 16h before reporter gene assay. ***:$p<0.001$ compared with Wnt1/Dvl-1/pCMV5 group.

Fig.2. Characterization of D_2R-mediated inhibition of Wnt signaling. (*A*) Involvement of D_2R signaling through G protein in the regulation of Wnt signaling was determined by inhibiting the coupling between D_2R and $G\alpha i/o$. Cells were treated overnight with 100 ng/ml pertussis toxin and the TOP-FLASH activity was determined. ***:$p<0.001$ compared

with pCMV5 group.   (*B*) Involvement of receptor signaling and endocytosis in the inhibition of Wnt signaling were tested using a point mutant of D_2R in which 132th Arg residue located within the Asp-Arg-Tyr motif was changed to His.   ***:$p<0.001$ compared with pCMV5 group.   (*C*) The relationship between the endocytosis of D_2R and the inhibitory activity on the Wnt signaling was tested using D131N-D_2R.   Cells were treated with 100 nM quinpirole for 6 h to induce receptor endocytosis before reporter gene assay.   ***:$p<0.001$ compared with pCMV5 group.   (*D*) Relationship between receptor phosphorylation and inhibition of Wnt signaling were tested using a mutant of D_2R, D_2R-IC2/3 in which all the serine and threonine residues located within the 2nd and 3rd intracellular loops were mutated to alanine and threonine residues, respectively.   ***:$p<0.001$ compared with pCMV5 group.   (*E,F*) Roles of β-arrestin on the D_2R-mediated inhibition of Wnt signaling were studied using HEK-293 cells which stably express either control RNAi plasmid or β-arrestin2 RNAi plasmid.   Wnt signaling was stimulated by co-expressing either Wnt1/Dvl-1 (*D*) or β-catenin (*E*).   ***:$p<0.001$ compared with pCMV5 group;   ###:$p<0.001$ compared with control RNAi group.

Fig.3. Receptor regions responsible for the D_2R-mediated inhibition of Wnt signaling were determined using chimeric receptors between D_2R and D_3R.   (*A*) Scheme for chimeric receptors between D_2R and D_3R.   Either the 2nd intracellular loop or the 3rd intracellular loop, or both loops were switched between D_2R and D_3R.   (*B*) TOP-FLASH activity was determined in cells which express wild-type or chimeric D_2Rs in which the 2nd and 3rd intracellular loops were switched with those of D_3R.   (*C*) TOP-FLASH activity was determined in cells which express wild-type or chimeric D_3Rs in which the 2nd and 3rd intracellular loops were switched with those of D_2R (*C*).   Receptor expression levels were

maintained between 1.7-1.9 pmol/mg proteins.   **: $p<0.01$, ***: $p<0.001$ compared with

pCMV5 group.   ###: $p<0.001$ when D_2R-(D_3- IC23) group was compared with D_2R group, or

when D_3R-(D_2-IC23) group was compared with D_3R group.

Fig.4. Determination of the molecular target of D_2R for the inhibition of Wnt signaling.

Reported gene assay was conducted as in Fig.1A.   (*A*) Cells were transfected with Wnt1

or/and Dvl-1 along with pCMV5 or D_2R. **:$p<0.01$, ***:$p<0.001$ compared with pCMV5

group.   (*B*) Cells were transfected with GFP-β-catenin along with pCMV5, D_2R, or D_3R.

***:$p<0.001$ compared with β-catenin/pCMV5 group.   (*C*) Cells were transfected with

kinase-dead form of GSK3β (GSK3β-KD) along with Mock plasmid or D_2R.   ***:$p<0.001$

compared with β-catenin only group.   ###:$p<0.001$ compared with β-catenin/GSK3β-KD

group. $$$: $p<0.001$ when β-catenin/GSK3β-KD group compared with β-catenin group.   (*D*)

Cells transfected with pCMV5 or D_2R were treated with vehicle or 40 mM LiCl overnight.

***:$p<0.001$ compared with pCMV5 group.   (*E*) Cells were transfected with Wnt1/Dvl-1

along with Mock plasmid, D_2R, or D_3R.   (*F*) Cells transfected with pCMV5 or D_2R, were

treated with 40 mM LiCl overnight.   Cell lysates were analyzed with SDS-PAGE gel and

immunoblotted with antibodies for β-catenin and actin.   Experiments in Fig.4E&F were

conducted three independent times, and representative results are shown.

Fig.5. Specific and functional interaction between D_2R and β-catenin.   (*A*) Identification of

the 2nd and/or 3rd intracellular loops of D_2R as the interacting regions with β-catenin.   Cells

transfected with FLAG-D_2R, -D_3R, or –D_3R-(D_2-IC23), were treated with 40 mM LiCl

overnight to elevate cellular levels of β-catenin.   Cell lysates were immunoprecipitated with

anti-FLAG beads, analyzed on the SDS-PAGE gel, and immunoblotted with antibodies to β-

2 8

catenin.   Experiments were conducted three independent times, and representative results are shown.   (*B*) Interaction between β-catenin and isolated fragment of the 3rd intracellular loop of D$_2$R (I3D$_2$).   Cells were transfected with FLAG-β-catenin and GFP-I3D$_2$.   Cell lysates were immunoprecipitated with anti-FLAG beads, analyzed on the SDS-PAGE gel, and immunoblotted with antibodies to GFP or FLAG. Experiments were conducted three independent times, and representative results are shown.   (*C*) Effect of isolated fragment of the 3rd intracellular loop of D$_2$R on the TOP-FLASH activity.   Reporter gene assay was conducted in cells transfected with D$_2$R or GFP-I3D$_2$.   ***:$p<0.001$ compared with β-catenin/GFP group.   (*D*) Effects of D$_2$R on the subcellular distribution of β-catenin.   HEK-293 cells which were stably transfected either with pRC/CMV or D$_2$R-pRC/CMV were transiently transfected with GFP-β-catenin.   (*E*) Effects of over-expression of β-catenin on the inhibitory activities of D$_2$R on Wnt signaling.   Increasing amounts of β-catenin was co-expressed along with D$_2$R. ***, ###:$p<0.001$ compared with pCMV5 group and Mock/D$_2$R group, respectively.

Fig.6. Determination of interaction between D$_2$R and β-catenin by GST pull-down assay. (*A*) Bacterial lysates containing the GST fusion proteins of the 3rd intracellular loops of D$_2$R (I$_2$D$_2$-N, the N-terminus part; I$_2$D$_2$-C, the C-terminus part) or D$_3$R (I3D$_3$) were mixed with the cell lysates of HEK-293 cells transfected with Mock plasmid or GFP-β-catenin.   After three washes, GST beads were incubated with SDS sample buffer.   The eluents were analyzed with SDS-PAGE gel, and blotted with antibodies to β-catenin (upper figure, GST pull-down part).   Blot from HEK-293 cell lysates is shown in the input part.   The figure in the bottom shows the SDS-PAGE gel which contains lysates of HEK-293 cells (HEK-293 lysates) and 'after-wash' of bacterial cell lysates.   (*B*) Schematic representation of the β-catenin

constructs. FL indicates the construct containing full length β-catenin. ΔN indicates the construct containing arm repeats and C-terminal domain (amino acids 87–781). Arm indicates the construct containing only 12 arm repeats domain (amino acids 87–658). (*C*) Bacterial lysates containing the GST-I$_3$D$_2$-N was mixed with lysates of HEK-293 cells containing the full length, N-terminus-deleted (ΔN), the armadillo repeat domain of β-catenin (Arm). GST pull-down was conducted as in Fig.6A except that it was blotted with antibodies to GFP. The figure in the bottom of each blot shows the SDS-PAGE gel which contains 'after-wash' of bacterial cell lysates. All experiments were conducted three independent times, and representative results are shown. Asterisk represents the main band of β-catenin.

Fig.7. Effects of D$_2$R on the subcellular distribution of β-catenin. (*A*) Cells were divided into four different groups and were transfected with corresponding constructs. Cell lysates were prepared from each experimental group, and fractionated into membrane, cytosolic, and nuclear fraction, as described in Materials and methods. After analysis on the SDS-PAGE gel, they were blotted with antibodies to β-catenin. Actin was used as reference proteins for total cellular extract and cytosol fraction; caveolin-1 and lamin B1 were employed as reference proteins for plasma membrane and nuclear fraction, respectively. All experiments were conducted three independent times, and representative results are shown. (*B*) Co-localization between β-catenin and D$_2$R was determined by confocal microscopy. (Upper panel) HEK-293 cells were transfected with FLAG-β-catenin along with GFP-D$_2$R. Cells were labeled with FLAG-β-catenin and with Alexa 594-conjugated anti-mouse secondary antibodies. DAPI (4',6-diamidino-2-phenylindole) was included in the mounting solution to stain nuclear regions (shown blue). (Lower panel) Primary cortical neurons were plated at 5

x 10^5 cells per well.   After two days, cells were transiently transfected with FLAG-β-catenin and D_2R-GFP using Lipofectamine 2000 (Invitrogen).   Immunocytochemistry was conducted as described in Materials and Methods.
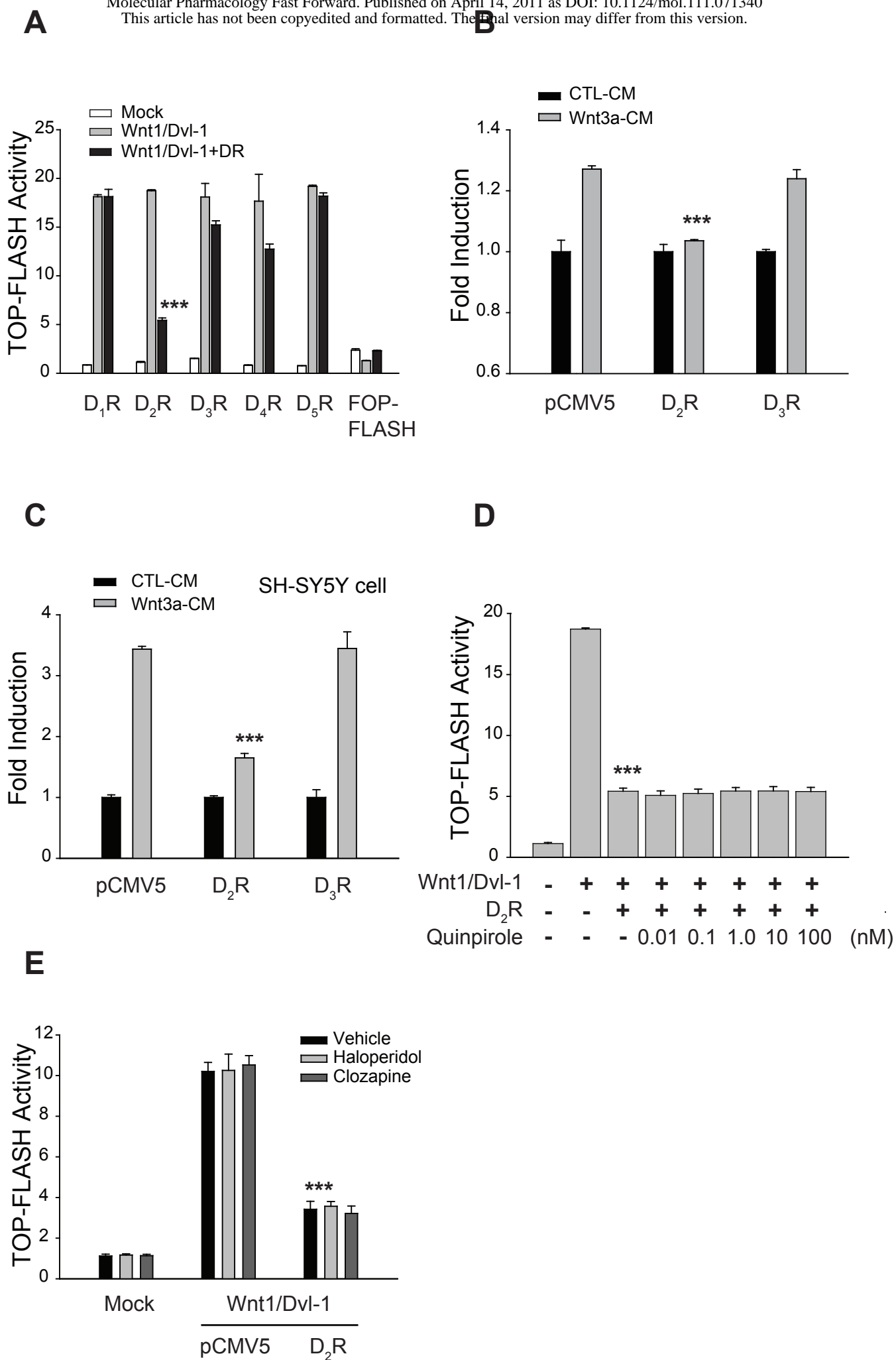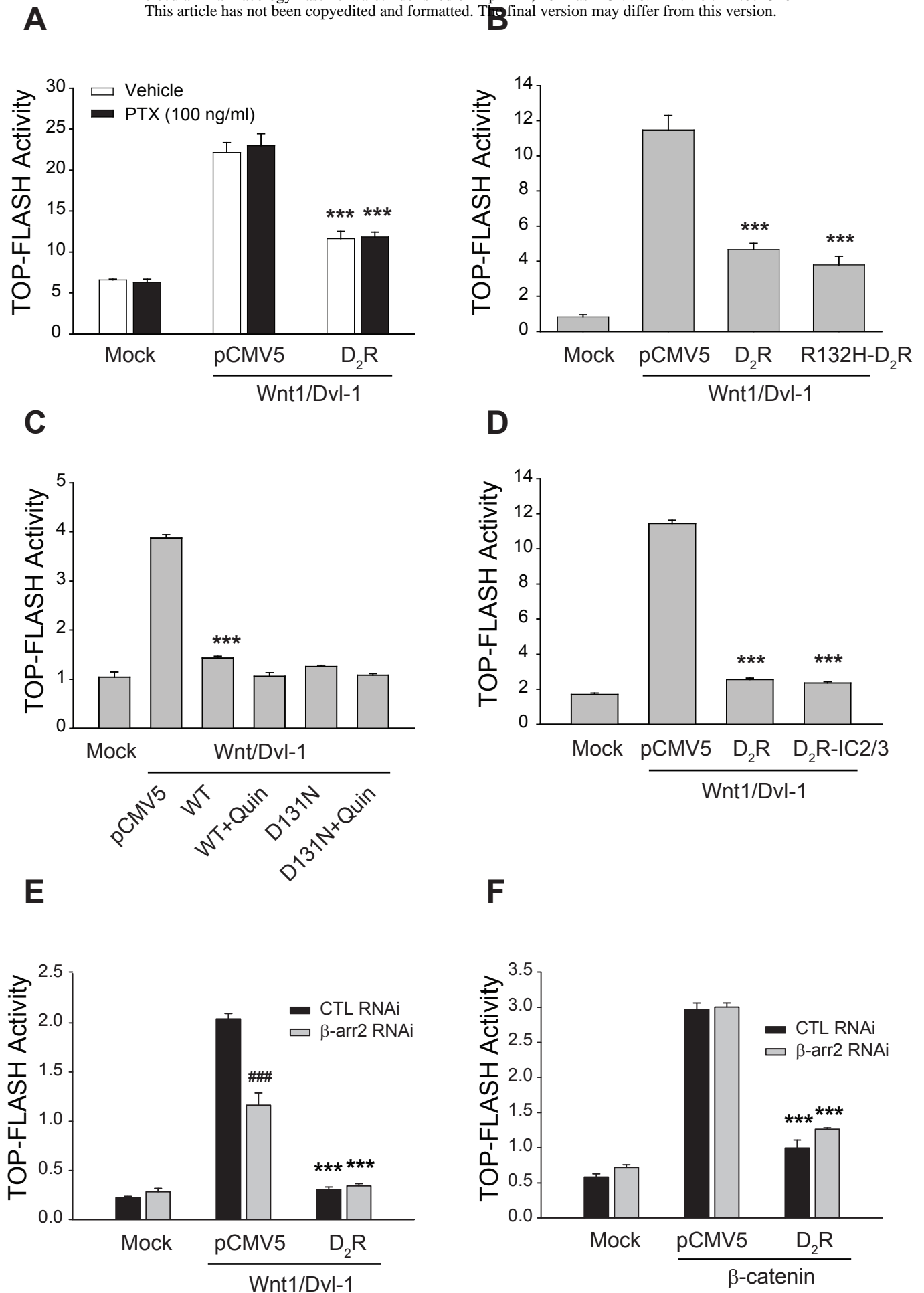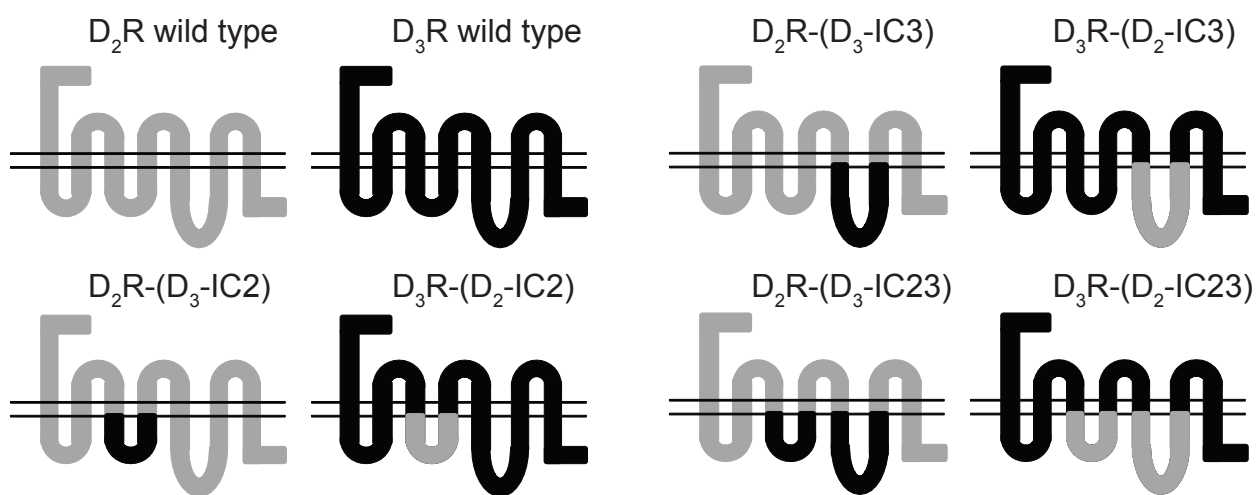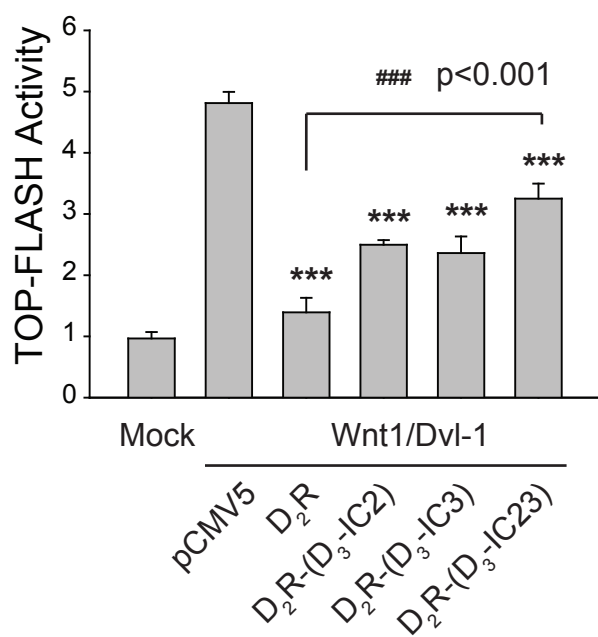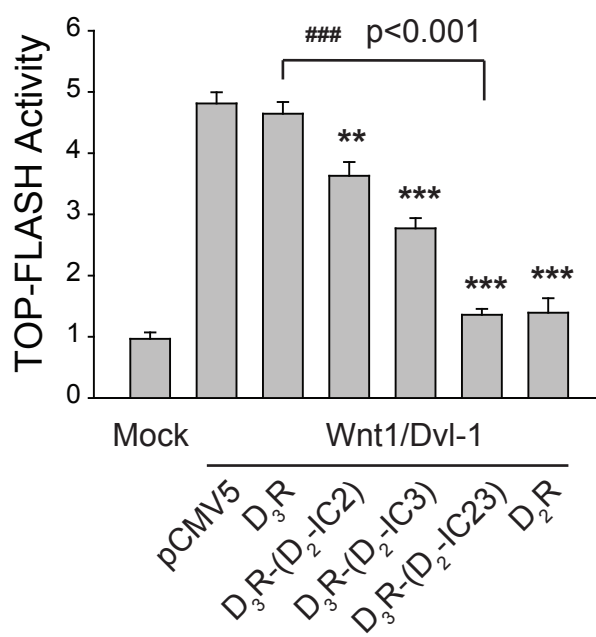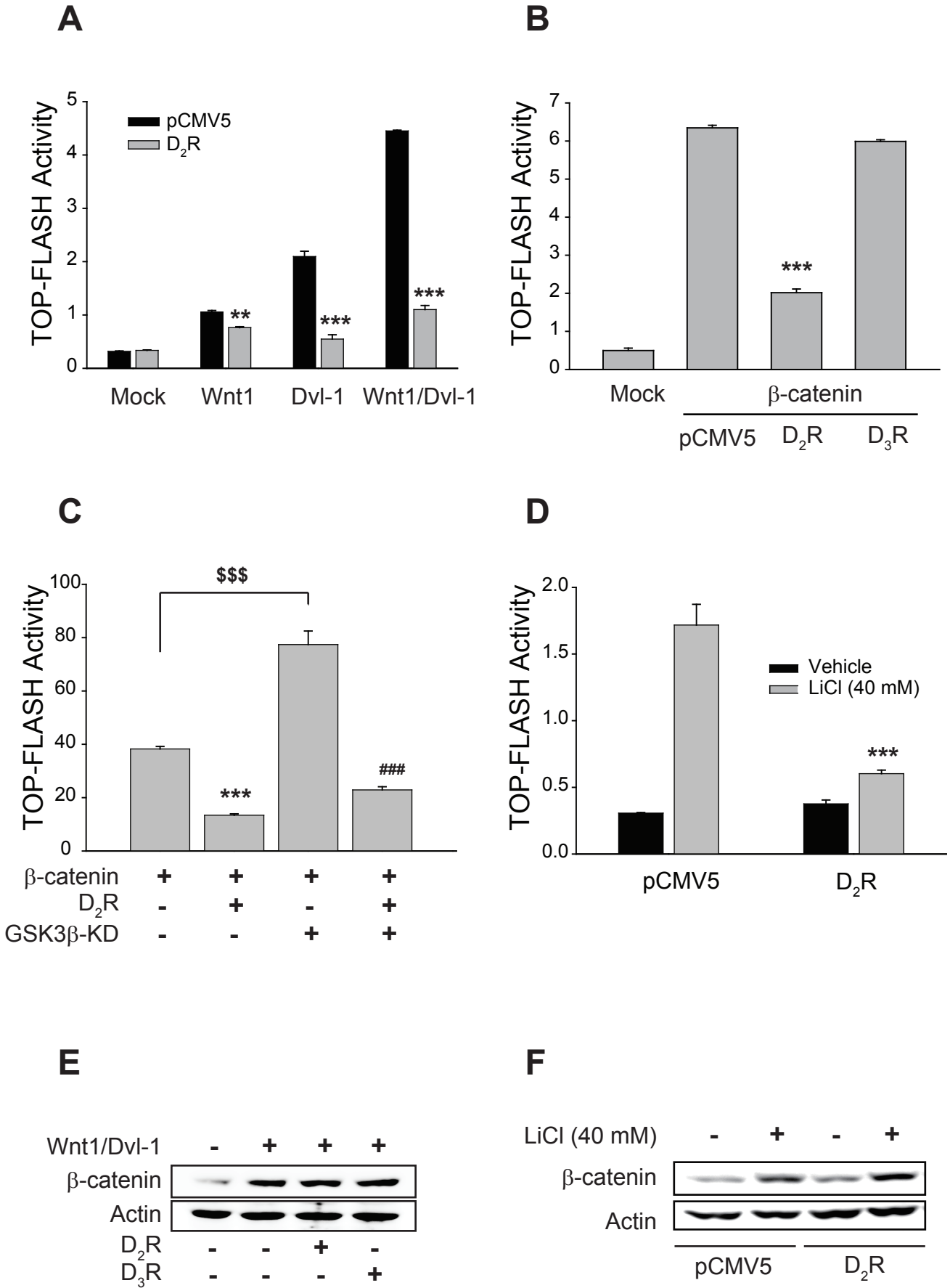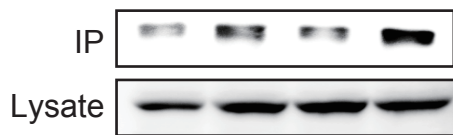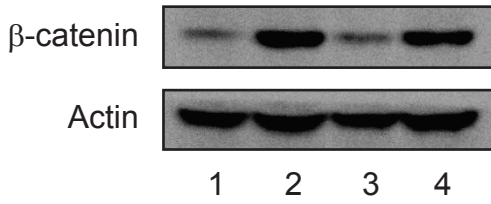
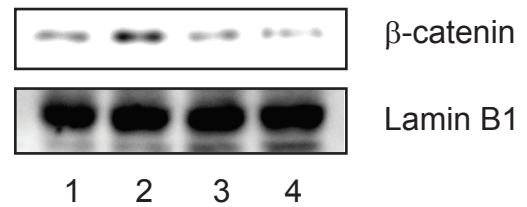Fig.1

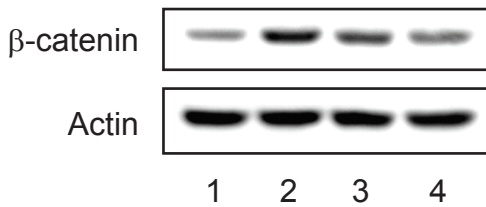Fig.2

Fig.3
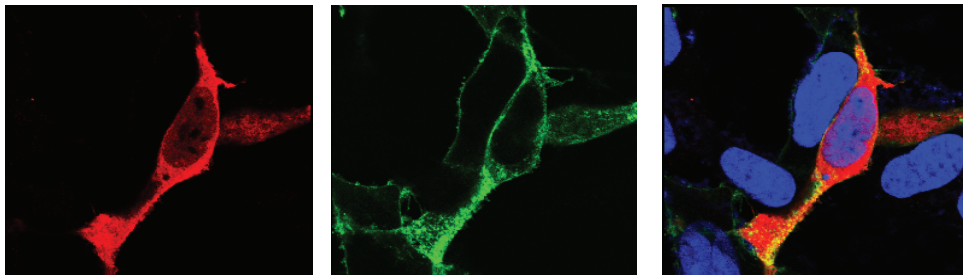
Fig.4

Fig.5

Fig.6

Fig.7
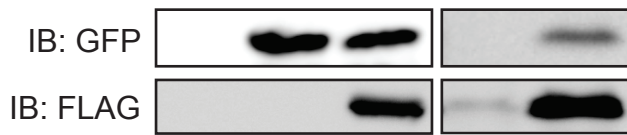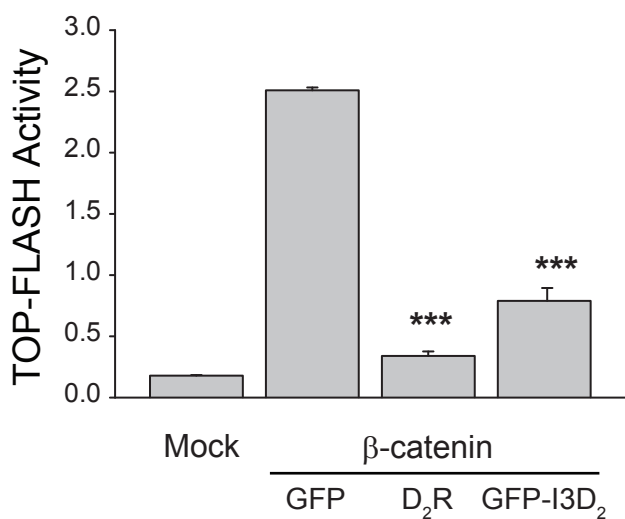
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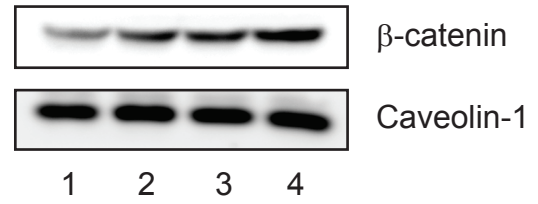
1. Mock   2. Wnt1/Dvl
3. D$_2$R   4. Wnt1/Dvl+D$_2$R

A3. Cytosolic fraction   A4. Nuclear fraction

B

FLAG–β-catenin   GFP-D$_2$R   Merged

HEK-293

Primary cortical neuron
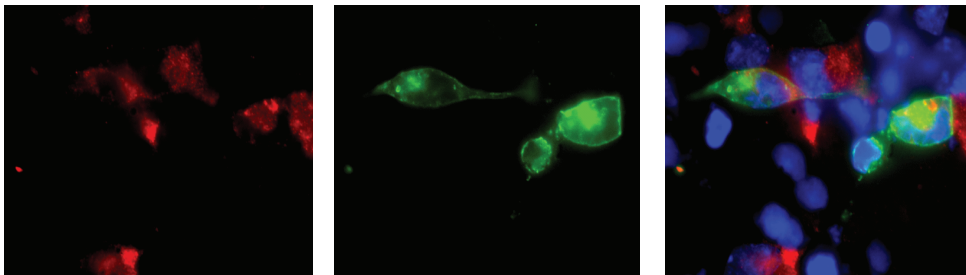
SUPPLEMENTAL DATA

Title: Novel Regulatory Mechanism of Canonical Wnt Signaling by Dopamine D_2 Receptor through Direct Interaction with β-catenin

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Figure Legends for Supplemental Data

Supplemental Figure 1. Selectivity between the short form and long form of D_2R on the Wnt signaling.   Experiments were conducted as described in Fig.1A.   Receptor expression levels were around 1.7 pmol/mg protein.

Supplemental Figure 2. Establishment of experimental resources needed to study the roles of receptor endocytosis, GRK2, and β-arrestin2 in the D_2R-mediated inhibition of Wnt signaling. (*A*) Comparative characterization of endocytic properties of wild-type and D131N-D_2R. Agonist-induced endocytosis of D2R was determined by utilizing the hydrophilic properties of ^{3}H-sulpiride.   (*B*) Roles of GRK2 on the D_2R-mediated inhibition of Wnt signaling.   HEK-293 cells which stably express either GFP-RNAi plasmid (control) or GRK2-RNAi plasmid were used.   Cell lysates were blotted with antibodies to GRK2 or actin.   Wnt signaling was induced by co-expressing Wnt1/Dvl-1.   ***:$p<0.001$ compared with Wnt1/Dvl-1 group; [#]:$p<0.05$ compared with control RNAi group.   (*C*) Establishment of β-arrestin2 knockdown cells.

HEK-293 cells were stably transfected either with control shRNA plasmid or β-arrestin2 shRNA plasmid. Cell lysates were blotted with antibodies to β-arrestin2 or actin.

Supplemental Figure 3. Characterization of the interaction between D_2R and β-catenin. *(A)* Specificity of the interaction between D_2R and β-catenin. Cell lysates were prepared from the HEK-293 cells which were transfected with FLAG-D_2R together with β-catenin or HA-14-3-3ζ or HA-GSK3β. Cell lysates were immunoprecipitated with anti-FLAG beads, analyzed on the SDS-PAGE gel, and immunoblotted with antibodies to β-catenin or HA. Experiments were conducted three independent times, and representative results are shown. *(B)* Effects of dopamine or haloperidol on the interaction between D_2R and β-catenin. Cells were treated with 10 μM dopamine or haloperidol for 30 min before immunoprecipitation as described in Fig.S3A.

Supplemental Figure 4. Effects of Wnt signaling on D_2R signaling and internalization. (*A*) Cells were transfected with D_2R along with Wnt1 or Dvl-1 or both Wnt1/Dvl-1. (*B-D*) Effects of β-catenin on the signaling of D_2R. (*B*) Cells were transfected with D_2R along with GFP vector or GFP-β-catenin. Receptor expression levels were between 1.7-1.9 pmol/mg protein (A-D). Signaling of D_2R was measured by determining the agonist-induced inhibition of cAMP production (*A,B,D*) or ERK activation (*C*). (*C*) Cells expressing GFP-ERK along with D_2R or D_2R/FLAG-β-catenin were treated with serum-free media containing vehicle or 100 ng/ml pertussis toxin (PTX) overnight, treated with 10 μM DA for 5 min. Cell lysates were blotted with phosphor-ERK antibodies or ERK antibodies or anti-FLAG antibodies. (*D*) Expression of

endogenous β-catenin was inhibited by stably expressing shRNAi of β-catenin.   Cell lysates were analyzed on the SDS-PAGE gel, and immunoblotted with antibodies to β-catenin and actin. (*E*) Effects of β-catenin on the internalization of D_2R.   Cells were transfected with D_2R-pCMV5 (~1.3 pmol/mg protein) were treated with 10 µM dopamine (DA) or 1 µM phorbol 12-myristate 13-acetate (PMA) for 1h.

Cellular cAMP was measured as described previously by an indirect method which had been used for the determination of D_2R and D_3R signaling (Cho et al.; Cho et al., 2007).   A reporter plasmid containing the firefly luciferase gene under the control of multiple cAMP responsive elements (CRE) and with pRL-TK control vector was utilized to measure the whole cell cAMP levels.

The internalization of D_2R was measured based on the hydrophilic properties of ^3H-sulpiride (Kim et al., 2001).   HEK-293 cells expressing D_2R were seeded 1 day after transfection at a density of 1.5×10^5 cells/well in 24-well plates.   The following day, cells were rinsed once and pre-incubated for 15 min with 0.5 ml of pre-warmed serum-free medium containing 10 mM HEPES, pH 7.4, at 37°C.   Cells were stimulated with 10 µM DA or 1 µM PMA for 1h.   Cells were incubated with 250 µl of [^3H]-sulpiride (final concentration 2.2 nM) at 4°C for 150 min in the absence and presence of unlabeled competitive inhibitor (10 µM haloperidol).   Cells were washed three times with the same medium and 1% Triton X-100 was added.   Samples were mixed with 5 ml Lefkofluor scintillation fluid and counted on a liquid scintillation analyzer.

D_2R-mediated ERK activation was measured as described previously (Beom et al., 2004).   After transfection, cells were cultured in 25-mm well plates, and then starved overnight
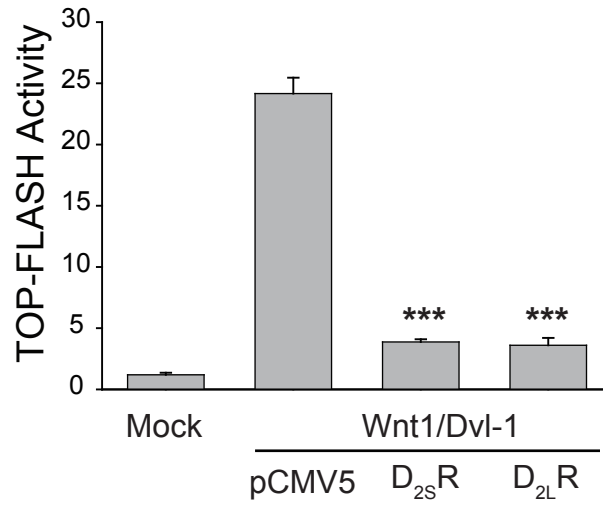
in serum-free culture medium containing 0.1% BSA. Cells were treated with 10 μM DA

dissolved in 10 μM ascorbic acid for 5 min, the medium was aspirated, and SDS sample buffer

was added directly to culture wells.   After incubating 20 min at 65 ^{o}C, samples were sonicated

to shear the genomic DNA.   Proteins were separated by SDS–PAGE and transferred onto

nitrocellulose membranes, and blotted with antibodies for phospho-ERK or ERK at 1,000
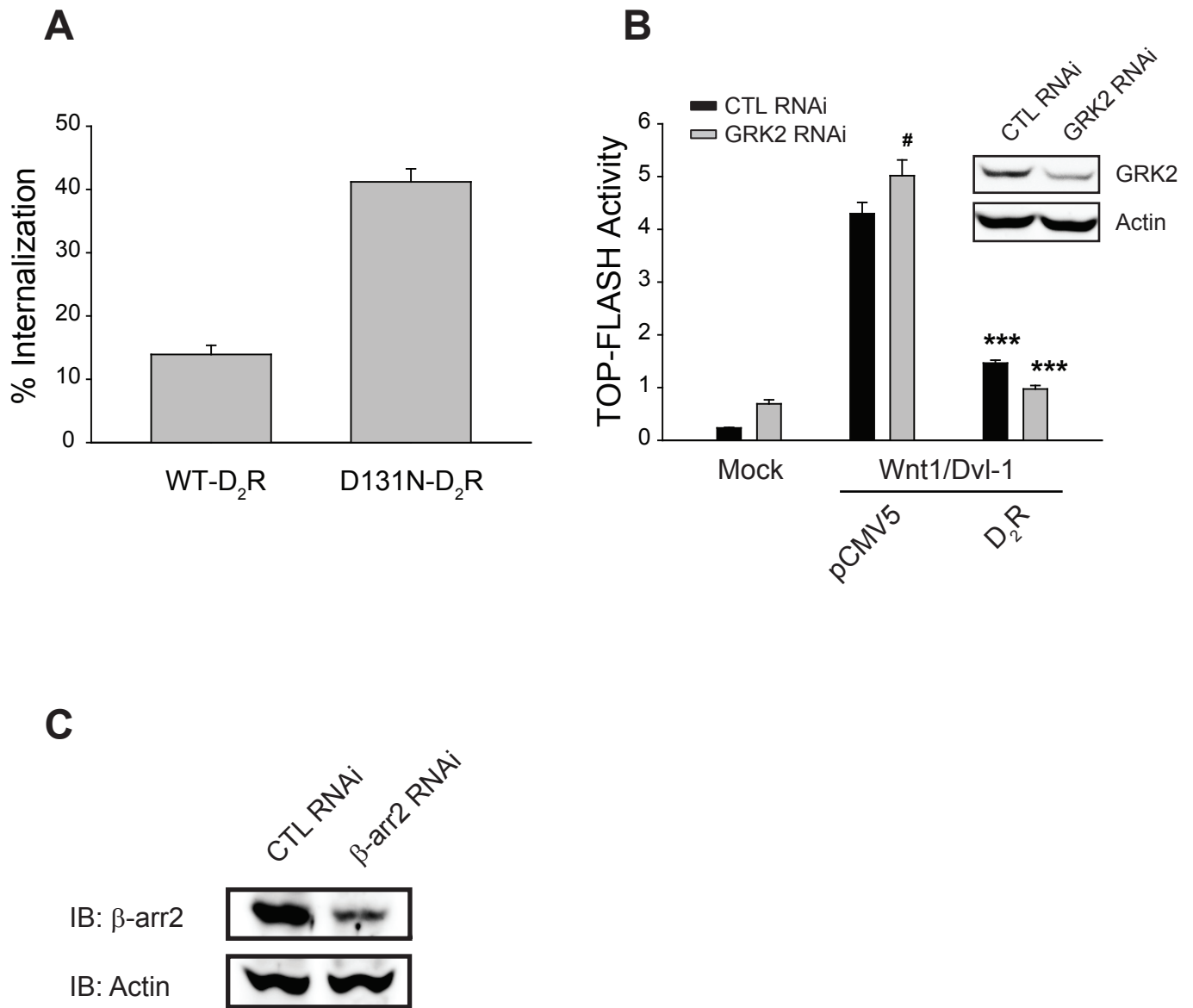
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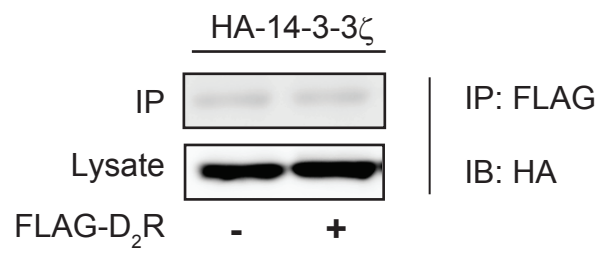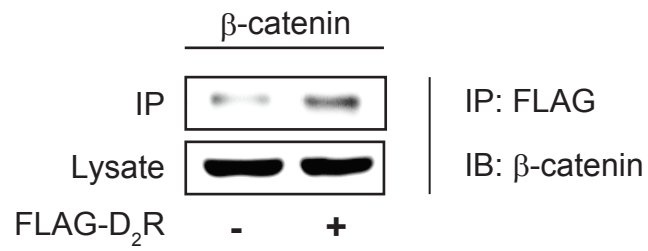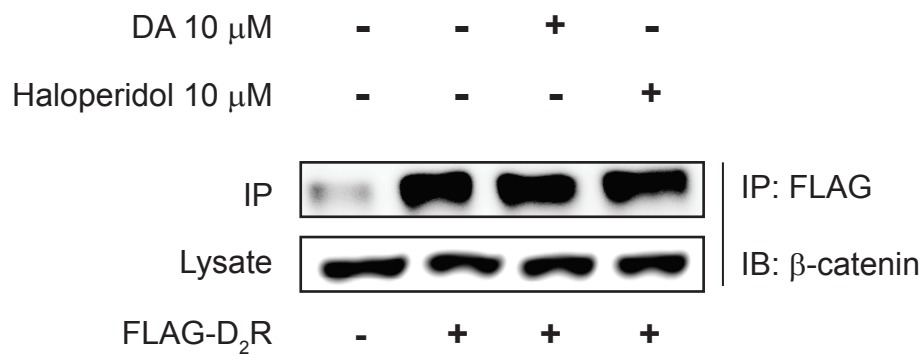
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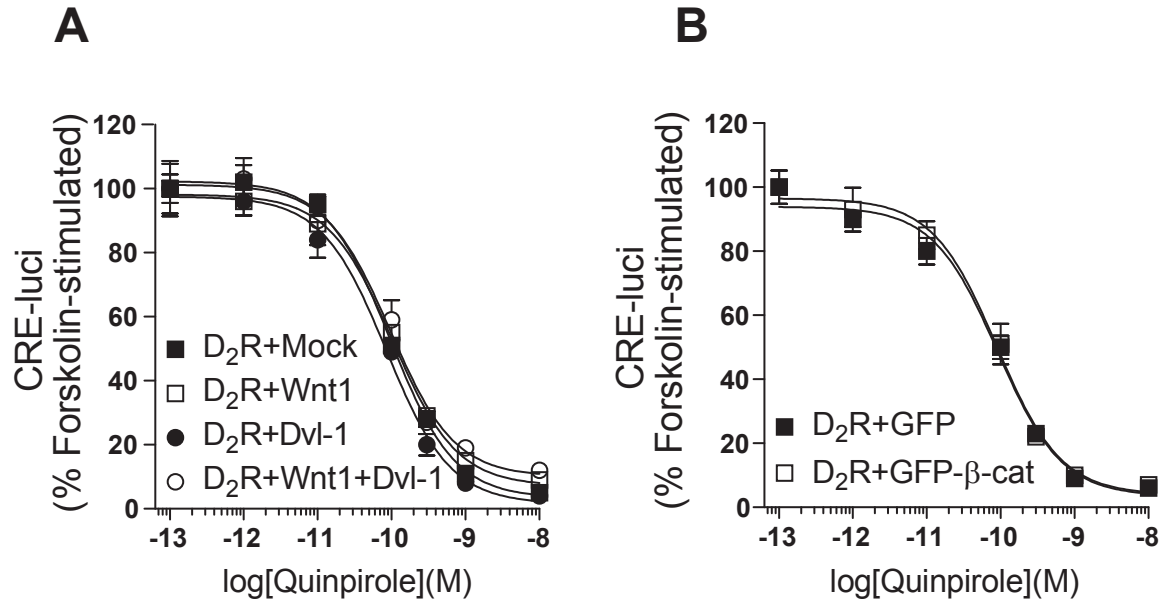
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A

B

C

A

B