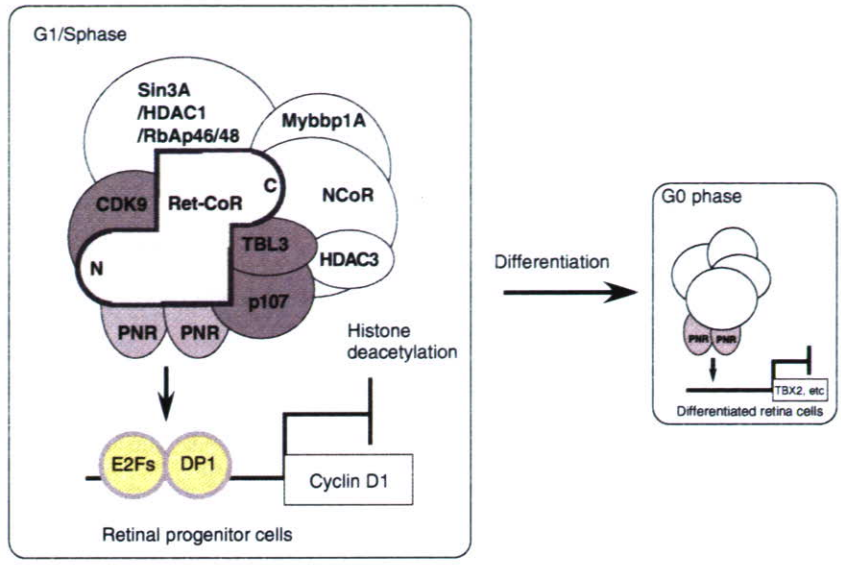


**Figure 7** Schematic diagrams of PNR and Ret-CoR complex function. In the proliferative stage cells such as retinal progenitor cells, PNR is recruited together with a Ret-CoR containing complex on their target gene promoters at G1/S phase and inhibitory for cell proliferation. After cell differentiation, PNR with the other co-repressor complex(es) may control the target genes at G0 phase.

gene expresses in developing, but not in differentiated retina, Ret-CoR appears physiologically important for the PNR suppressor function for cell cycle regulation in retinal progenitor cells (Figure 7). Through the PNR-mediated transcriptional controls, Ret-CoR may serve as a suppressor for S-cone cell proliferation. Given the facts that Ret-CoR expression was also seen in brain, it is also possible to speculate that Ret-CoR co-represses the function of the other sequence-specific regulators, which determine cell fate of neurons through transcriptional control. In this respect, it is interesting to examine functional interaction of Ret-CoR with TLX and its relevance to physiological events in brain by comparing with the reported co-repressors (Perissi and Rosenfeld, 2005), although in the tested promoters of PNR and TLX target genes, Ret-CoR appeared unlikely to functionally associate with TLX.

### *Multiple co-repressor complexes facilitate the transrepressive function of PNR?*

Transcriptional regulation by sequence-specific regulators upon the target gene promoters is believed to couple with histone modification and chromatin remodeling (McKenna and O'Malley, 2002; Belandia and Parker, 2003; Kitagawa *et al*, 2003; Perissi and Rosenfeld, 2005). Reflecting the biological events on the chromatin, a number of co-regulator complexes for NRs has been recently suggested to support the complicated but sequential cycling events for transcriptional events (Metivier *et al*, 2003). In this regard, it is reasonable to speculate that PNR requires multiple co-repressor complexes for its transrepressive function.

In retinal progenitor cells, we presume that the Ret-CoR HDAC complex co-represses PNR as a major co-repressor complex. However, from the findings that clear recruitment of Ret-CoR to the TBX2 gene promoter was undetectable, irrespective of the PNR-transrepressive function, in the differentiated retina of mice (Figure 6C), PNR appears to associate with the other HDAC complex(es) in differentiated retina cells. In fact, by a ChIP analysis of the TBX2 gene promoter in Y79 cells, a recruitment of HDAC1 without Ret-CoR was seen (Figure 5F). Together with the observations that Ret-CoR was absent on the TBX2 promoter in mouse retina (Figure 6C), PNR may associate with the other co-repressor complex(es), at least, in differentiated retina cells (Figure 7).

### *The cell cycle-dependent appearance of Ret-CoR is regulated through protein degradation?*

Our findings suggested that the Ret-CoR complex acts as a negative regulatory complex of the cell cycle through inhibition of cell cycle-related factor gene expression via increased Ret-CoR protein levels during the G1/S transition stage. Supporting the cell cycle-dependent appearance of Ret-CoR protein, in the Ret-CoR harbors a destruction box R (1142)-R-A-L-G-R-M-V-E (1150) (compared to the consensus R-X-X-L-X-X-X-X-N/D/E) (Glotzer *et al*, 1991) identified in cyclin B as an interacting domain for E3 ligase and for cell cycle-regulated protein degradation (Wei *et al*, 2004). Consistently, Ret-CoR was highly susceptible to ubiquitination (Figure 3C), perhaps appearance of Ret-CoR protein is, at least in part, attributed to regulated protein degradation through the ubiquitin-dependent proteasomal pathway (Vodermaier, 2004; Peters, 2006). As endogenous Ret-CoR protein expression appears to be tightly regulated via the cell cycle, Ret-CoR function in the developing retina may be specific to late G1 stage or the G1/S transition stage, presumably in retinal progenitor cells. It is possible that through the co-regulatory function of such cell cycle-dependent co-repressor complexes, retina cell differentiation can be tightly regulated by PNR, along with other classes of sequence-specific regulators such as TRβ2 (Ng *et al*, 2001) and Crx (Furukawa *et al*, 1997), which together may constitute a photoreceptor-specific transcription factor cascade (Kobayashi *et al*, 1999; Gerber *et al*, 2000; Haider *et al*, 2000; Yanagi *et al*, 2002). In tissues that do not express PNR, such as the brain, cell cycle-dependent Ret-CoR complexes may also serve as co-repressor complexes for other sequence-specific regulators such as TLX. Although it remains unclear whether the functions of other known

 

co-regulator complexes are also cell cycle-dependent, our findings suggest a model in which the level of expression of a co-regulator complex is regulated in a cell cycle-dependent manner.

## Materials and methods

### *Purification and characterization of the Ret-CoR complex*

Nuclear extracts (Yanagisawa *et al*, 2002; Kitagawa *et al*, 2003) from 293F cells transformed with Ret-CoR and Y79 cells with PNR were loaded onto an anti-FLAG M2 affinity resin column, and washed extensively with washing buffer (20 mM Tris–HCl (pH 8.0), 300 mM KCl, 0.2 mM EDTA, 0.05% NP40, 10% glycerol, 0.5 mM PMSF and 1 mM DTT). Bound proteins were eluted from the column by incubation with 133 μg/ml FLAG peptide in washing buffer for 30 min at room temperature. Next, the eluted solution was applied on the Protino column (MACHEREY-NAGEL) for His-tag binding and washed with a buffer His (40 mM HEPES pH 7.4, 300 mM KCl, 0.05% NP40, 10% glycerol). Ret-CoR complex was eluted by 250 mM Imidazole buffer His. For fractionation on glycerol gradients, elutants were layered on top of 13 ml linear 10–40% glycerol gradients in washing buffer and centrifuged for 16 h at 4°C at 40 000 r.p.m. in a SW-40 rotor (Beckman, CA). After getting each fraction, Re-IP was carried out with anti-RbAp46 antibody for fraction 10–12. Protein standards used were ovalbumin (44 kDa), β-globulin (158 kDa) and thyroglobulin (667 kDa). Each samples were applied on NuPAGE Bis–Tris 4–12% gradient gel (Invitrogen). Identification of each component was performed following our past papers (Kitagawa *et al*, 2002, 2003; Yanagisawa *et al*, 2002) using Voyager DE-STR (Perspective Biosystems).

### *Cell-cycle analysis*

Y79 cells were synchronized at G1/S phase (with thymidine) and at G2/M phase (with demecolcine), essentially as described in our previous report (Kitagawa *et al*, 2003). Briefly, for the G1/S arrest, cells were exposed for 24 h with 2.5 mM thymidine in RPMI medium supplemented with 3% serum. Nine hours after the drug release, cells were cultured again for 16 h in the presence of 2.5 mM thymidine. Cells were yielded approximately 70% G1/S population. For the G2/M phase arrest, cells synchronized at G1/S phase by thymidine were released by incubation for 9 h with RPMI medium with 10% serum, and were treated with 0.015 μg/ml demecolcine for 8 h, which yielded approximately 75% G2/M population.

### *In vitro transcription*

*In vitro* transcription with chromatin template was performed according to the previous report (Kitagawa *et al*, 2003; An and Roeder, 2004) using histone octamers from HeLa cell (Fujiki *et al*, 2005). Template DNA $pG_5ML$ was kindly provided by Dr Robert G Roeder. Recombinant GAL-PNR LBD was expressed by pET system (Novagen), and purified by Protino Ni column under denaturing condition. Then the protein was refolded in a native buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF). Co-repressor activity of the purified Ret-CoR complex for PNR was attenuated in the presence of 500 nM TSA.

### *HDAC assay*

HDAC assay was performed using HDAC Fluorescent Activity Assay/Drug Discovery Kit (AK-500, BIOMOL) according to the manufacturer's instructions. Briefly, cell extracts prepared from Y79 cells in 24-well plate were immunoprecipitaed with antibodies indicated and incubated with the substrate at 30°C for 30 min. After incubation, the reaction was stopped and the fluorescence was analyzed by microplate reading fluorimeter (Perkin Elmer).

### *Retroviral production and infection*

shPNR, shRet-CoR and shControl expressing retroviruses were produced using pSIREN and 293gp-2 cells (Clontech). shPNR (corresponded to nucleotides 757–781), shRet-CoR (corresponding to nucleotides 2384–2408) and shControl (LacZ from *Escherichia Coli*: corresponding to nucleotides 291–311) was inserted in the pSIREN vector. Retina primary culture cells were infected by incubating them with the virus and 5 μg/ml hexadimethrine bromide (Sigma) following the manufacture's protocol (Clontech).

### *Retinal culture and cell proliferation assay*

Neural retinas were dissected from postnatal day 0 mice or 3-month-old mice and cultured as pellets. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium (with Glutamax) and Ham's F12 (Gibco), supplemented with 10% FBS, insulin (10 μg/ml) and transferine (100 μg/ml). The cells were cultured 3 days by replacing half of the medium in the dish with fresh medium and used in cell proliferation assay.

The cell proliferation assay by incorporation of BrdU was performed using BrdU labelling and detection kit III (Roche) according to the instruction manual. All values are mean ± s.d. of at least three independent experiments.

### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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# Vitamin K Induces Osteoblast Differentiation through Pregnane X Receptor-Mediated Transcriptional Control of the Msx2 Gene[▽]

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**Vitamin K is a fat-soluble vitamin that serves as a coenzyme for vitamin K-dependent carboxylase. Besides its canonical action, vitamin K binds to the steroid and xenobiotic receptor (SXR)/pregnane X receptor (PXR) and modulates gene transcription. To determine if the osteoprotective action of vitamin K is the result of the PXR/SXR pathway, we screened by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis the PXR/SXR target genes in an osteoblastic cell line (MC3T3-E1) treated with a vitamin K2 (menaquinone 4 [MK4]). Osteoblastic differentiation of MC3T3-E1 cells was induced by MK4. Msx2, an osteoblastogenic transcription factor, was identified as an MK4-induced gene. Functional analysis of the Msx2 gene promoter mapped a vitamin K-responsive element (PXR-responsive element [PXRE]) that was directly bound by a PXR/retinoid X receptor α heterodimer. In a chromatin immunoprecipitation analysis, PXR was recruited together with a coactivator, p300, to the PXRE in the Msx2 promoter. MK4-bound PXR cooperated with estrogen-bound estrogen receptor α to control transcription at the Msx2 promoter. Knockdown of either PXR or Msx2 attenuated the effect of MK4 on osteoblastic differentiation. Thus, the present study suggests that Msx2 is a target gene for PXR activated by vitamin K and suggests that the osteoprotective action of MK4 in the human mediates, at least in part, a genomic pathway of vitamin K signaling.**

The K vitamins are a group of fat-soluble vitamins that occur in two natural forms: phyloquinones (K1) and menaquinones (K2). Vitamin K (VK) has classically been associated with blood coagulation (31). In its canonical role, VK serves as a coenzyme for VK-dependent carboxylase. This enzyme converts glutamate residues into γ-carboxyglutamate (Gla) residues in VK-dependent proteins, such as prothrombin, and factors IX and X (6, 10, 29). Such VK-induced protein modification also occurs in osteocalcin (7, 21) and matrix Gla protein (MGP) (22). Thus, VK may exert beneficial effects on bone formation and remodeling. In fact, animal studies suggest that VK deficiency results in a reduction in bone mass together with hypocarboxylation of osteocalcin (25).

Clinically, the most common form of K2, menaquinone 4 (MK4), has been shown to prevent bone fractures (3). This osteoprotective effect is more pronounced in K2 than in K1, and hence MK4 has been used to treat osteoporotic patients in Japan (9, 11, 28). However, the bone phenotypic abnormalities of mice deficient in osteocalcin and MGP do not fully support the classical view that the osteoprotective action of VK is the result of the modification of skeletal proteins. These mice, which are genetically deficient for osteocalcin or MGP, exhibited bone mass increases instead of losses (4). This suggests that the osteoprotective action of VK is mediated by another pathway.

MK4 recently has been shown to act as a ligand for the steroid and xenobiotic receptor (SXR) in human osteoblastic cells (33). It transcriptionally regulates gene expression and represents a new pathway of VK action. The SXR and its mouse homolog, the pregnane X receptor (PXR), respond to xenobiotics and pregnenones. PXR and SXR are members of the nuclear receptor (NR) gene superfamily and bind to specific DNA elements (PXR-responsive elements [PXRE]) as heterodimers with one of the retinoid X receptor (RXR) subtypes (α, β, and γ) (1, 2, 15, 16, 18). Like the other NR members, ligand binding to PXR/SXR induces dissociation of corepressors and recruitment of coactivators for ligand-induced transactivation in the target gene promoters (17). Thus, these findings suggest that it is feasible that the osteoprotective VK action mediates its transcriptional control of the VK target genes via PXR/SXR. In fact, several PXR/SXR genes recently have been shown to transcriptionally respond to VK (8).

To test this idea, we screened VK target genes in an osteoblastic cell line (MC3T3-E1) treated with MK4 with two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE). A prime osteoblastogenic factor, Msx2, was identified, and a PXRE was located in its gene promoter. MK4 interacted with the PXRE via PXR/RXRα binding in vivo and in vitro. Osteoblast genesis in MC3T3-E1 cells was induced by MK4, but knockdown of Msx2 by RNA interference (RNAi) abrogated the MK4 effect. The present study suggests that Msx2 is a target gene for VK-activated PXR/SXR. It implies that the osteoprotective VK action takes place, at least in part, on a genomic level by stimulating osteoblast differentiation through Msx2 gene induction.

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## MATERIALS AND METHODS

**Plasmids.** The full-length cDNA for the mouse PXR (15) was subcloned into a pcDNA3 expression vector (Invitrogen) tagged with the hemagglutinin epitope

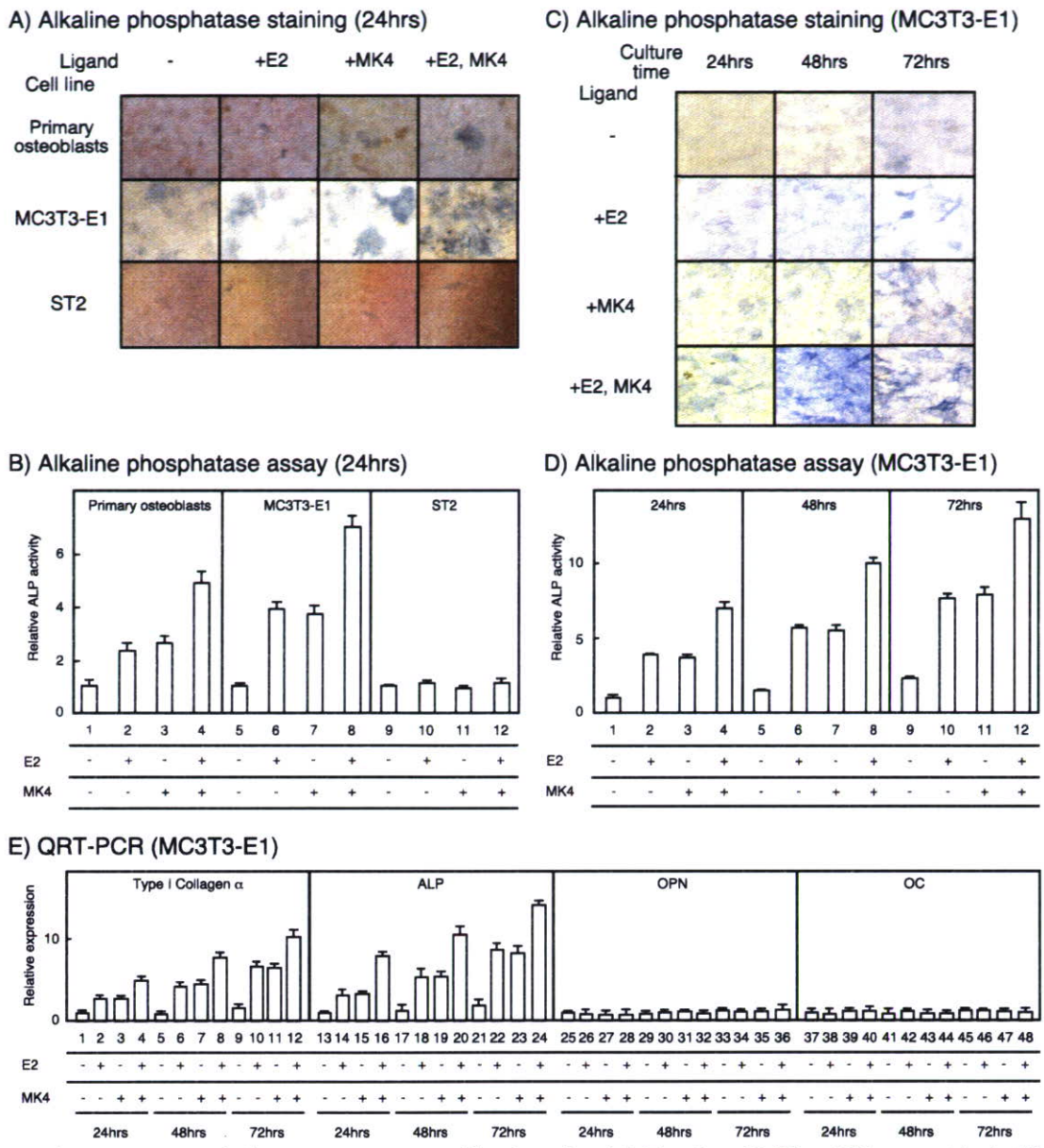


FIG. 1. VK and estrogen cooperatively promote mature osteoblast formation. (A) The effect of MK4 and E2 on osteoblastic differentiation in the presence or absence of MK4 (1 μM) and E2 (1 nM). After 24 h, osteoblast differentiation was detected by ALP staining. Upper panel, primary osteoblasts from mouse calvaria; middle panel, MC3T3-E1 cells; bottom panel, ST2 cells. (B) The effect of MK4 and E2 on osteoblastic differentiation in the presence or absence of MK4 (10 μM) and E2 (10 nM). After 24 h, ALP activity was measured using the optical density at 405 nm. All values are means ± standard deviations for at least three independent experiments. (C) The effect of MK4 and E2 in the early stage of osteoblastic differentiation in MC3T3-E1 cells. After 24-, 48-, and 72-h treatments, ALP staining was performed. (D) The effect of MK4 and E2 in the early stage of osteoblastic differentiation in MC3T3-E1 cells. After 24-, 48-, and 72-h treatments, ALP activity was measured. (E) Relative expression levels of the marker genes of osteoblastic differentiation. The total RNA for the qRT-PCR was extracted using the ISOGEN kit (Nippon Gene) from MC3T3-E1 cells treated with MK4 and E2 or left untreated. OPN, osteopontin; OC, osteocalcin.

at the N terminus. The mouse Msx2 promoters, the sequences of which were derived from the Ensembl genome browser (http://www.ensembl.org/index.html), were subcloned into a pGL3-Basic vector (Promega).

**Animals.** Estrogen receptor α knockout (ERαKO) mice were kindly provided by P. Chambon (19). Genotyping for ERαKO mice was routinely performed on DNA isolated from tail snips by a PCR procedure (19).

**Cell culture, transient transfection, and luciferase assay.** MC3T3-E1 and ST2 cells were cultured with alpha minimum essential medium (α-MEM) (GIBCO) containing 10% fetal bovine serum (FBS) (GIBCO) at 37°C and 5% $CO_2$ (31). HEK 293T cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (GIBCO) at 37°C and 5% $CO_2$. For transfection, cells were plated in the corresponding medium supplemented with 10% charcoal-stripped FBS in 12-well plates 1 day before transfection. Transfection was performed with Lipofectamine (Invitrogen) with Plus reagent (Invitrogen) or Polyfect (QIAGEN) as directed by the manufacturer's protocol. After 3 h, 17β-estradiol (E2) (10 nM) and MK4 (10 μM) were added to α-MEM containing 2% FBS, and the cells were incubated continuously at 37°C for 24 h. As a reference to normalize transfection efficiency, 2.5 ng/well of pRL-CMV plasmid (Promega) was cotransfected in all experiments. Luciferase activity was determined using the luciferase assay system (Promega) (5).

**Osteoblast primary culture.** Calvaria of newborn mice were digested for 80 min at 37°C in phosphate-buffered saline (PBS) containing 0.1% collagenase A

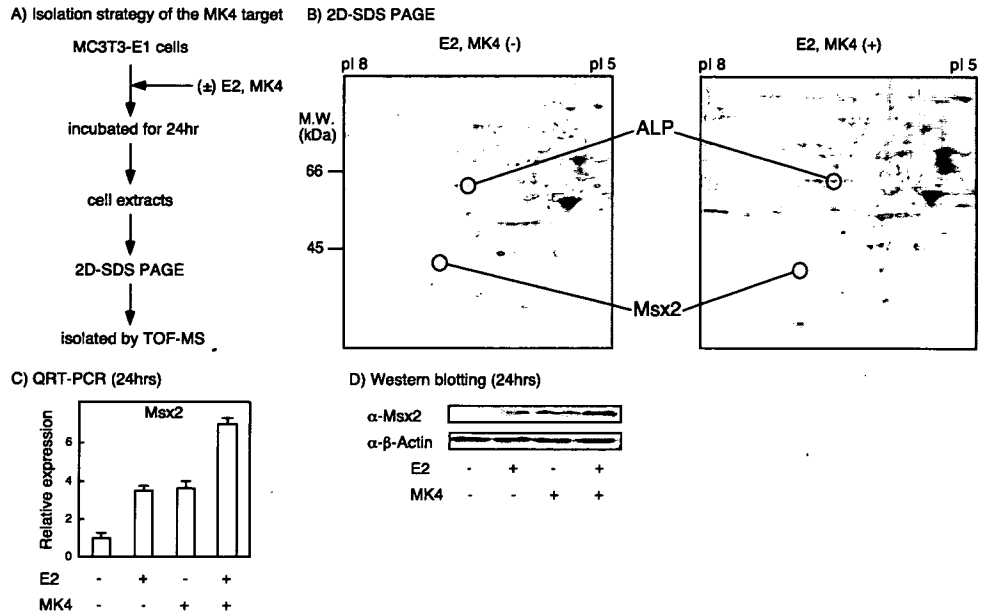


FIG. 2. Identification of Msx2 as a regulatory target of VK and estrogen in MC3T3-E1 cells. (A) Strategy for isolating MK4 targets. MC3T3-E1 cells were incubated for 24 h in the presence or absence of MK4 (1 μM) and E2 (1 nM). Total cell lysates then were separated by 2D SDS-PAGE, and the bands were examined with matrix-assisted laser desorption ionization–time-of-flight mass spectroscopy (labeled TOF-MS) for protein identification. (B) Comparison of protein expression by 2D SDS-PAGE in the presence or absence of MK4 and E2. M.W., molecular size. (C) The effect of MK4 and E2 on Msx2 gene expression. Msx2 mRNA expression was analyzed by qRT-PCR. (D) The effect of MK4 and E2 on Msx2 protein expression. Msx2 protein levels were determined by immunoblotting. β-Actin was used as a control.

(Roche). Cells were plated with α-MEM containing 10% FBS. Media were changed every 3 days (32).

**ALP staining.** For alkaline phosphatase (ALP) staining, primary osteoblasts, MC3T3-E1 and ST2 cells, were washed twice with PBS, fixed in 3.7% formaldehyde, and stained with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.6 mg/ml fast-blue BB salt (Sigma), 2 mM $MgCl_2$, 5 μl/ml *N,N*-dimethylforamide (Wako), and 100 mM Tris-HCl (pH 8.8) buffer at 37°C for 5 to 10 min. When the cells turned blue, the cells were washed twice with PBS (32).

**ALP assay.** The ALP assay was performed using the LabAssay ALP kit (Wako) according to the manufacturer's instructions. In brief, primary osteoblasts, MC3T3-E1 and ST2 cells, were washed twice with ice-cold PBS and then were solubilized with lysis buffer (20 mM Tris-HCl [pH 7.9], 1% NP-40, 1 mM EDTA, 150 mM NaCl, 2.5 mM $MgCl_2$, 5% glycerol) containing protease inhibitors, followed by determination of the ALP activity in lysates. The protein concentration was determined with a Bio-Rad protein assay kit.

**qRT-PCR.** For quantitative real-time reverse transcription-PCR (qRT-PCR), 1 μg of total RNA from each sample was reverse transcribed into first-strand cDNA with random hexamers using Superscript III reverse transcriptase (Invitrogen). Primer sets for all genes were purchased from Takara Bio Inc. (Tokyo, Japan). Real-time RT-PCR was performed using SYBR premix EX *Taq* (Takara) with the thermal cycler Dice RealTime System TP800 (Takara) according to the manufacturer's instructions. Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. To correct for variability in RNA recovery and the efficiency of reverse transcription, glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified and quantified in each cDNA preparation. Normalization and calculation steps were performed as reported previously (34).

**2D SDS-PAGE analysis.** For 2D SDS-PAGE analysis, MC3T3-E1 cells were washed with ice-cold PBS, collected by centrifugation at 2,000 × *g*, resuspended in 50 μl lysis buffer (20 mM Tris-HCl [pH 7.9], 1% NP-40, 1 mM EDTA, 150 mM NaCl, 2.5 mM $MgCl_2$, 5% glycerol) containing protease inhibitors, incubated on ice for 30 min, and then centrifuged for 30 min at 12,000 × *g*. After centrifugation, the supernatants were cleaned with a 2D-Clean-Up kit (Amersham). These samples were separated with the 2D SDS-PAGE system (Bio-Rad). The 2D SDS-PAGE gels were visualized with the SilverQuest silver staining kit (Invitrogen).

**ABCD assay.** For the avidin-biotin complex DNA (ABCD) assay, sense and antisense oligonucleotide DNAs that were biotinylated at the 3′ terminus were incubated at 100°C for DNA annealing and then were cooled slowly at room temperature. To prepare the beads-DNA complex, biotin-conjugated double-stranded oligonucleotide DNA was mixed with a 50% slurry of avidin beads (Tetralink tetrameric avidin resin; Promega). Cells were lysed with lysis buffer (10 mM Tris-Cl [pH 7.8], 1 mM EDTA, 150 mM NaCl, 0.1% NP-40) containing protease inhibitors for 1 h at 4°C. Whole-cell lysates were clarified by centrifugation, mixed with the 50% slurry of avidin beads, and pelleted at 3,000 × *g*. Supernatants were added to the beads-DNA complex and rotated for 1 h at 4°C to mix. The beads were collected by centrifugation at 3,000 × *g* and were washed successively in the lysis buffer. Proteins were resolved with SDS-PAGE, and Western blotting was performed with the corresponding antibody (12).

**ChIP analysis.** Chromatin immunoprecipitation (ChIP) analysis was performed using the ChIP assay kit (Upstate Biotechnology Inc.) (13, 38) according to the manufacturer's instructions. MC3T3-E1 cells were cultured in the presence or absence of MK4 and E2. Soluble chromatin prepared from $1 \times 10^6$ cells was immunoprecipitated with antibodies against the indicated proteins.

**Generation of adenovirus.** Recombinant adenoviruses carrying RNAi of Msx2 and PXR were constructed using the RNAi-Ready pSIREN-Shuttle vector (BD Biosciences Clontech) first and then were moved to the Adeno-X vector (Clontech) by being spliced into the I-CeuI and PI-SceI site. The parental virus genomes in HEK 293 cells (ATCC) were constructed according to the manufacturer's protocol (20). MC3T3-E1 cells then were infected by incubation with the recombinant adenovirus.

## RESULTS

**Vitamin K2 promotes osteoblast differentiation.** We explored the osteoprotective effects of K2 on a molecular level to better understand how K2 clinically prevents osteoporosis. We first tested the effects of MK4, a K2. The effect of MK4 was measured by ALP assay of primary cultured mice osteoblastic cells, derived from calvaria, to detect osteoblastic differentiation. As shown in the lower panels of Fig. 1 A and B (lanes 1 to 4), ALP activity was induced in the primary cultured osteoblastic cells treated with MK4 for 24 h. Likewise, E2 was stimulatory, and the actions of MK4 and E2 were additive. To clarify the stages of differentiation at which MK4 and E2 act,

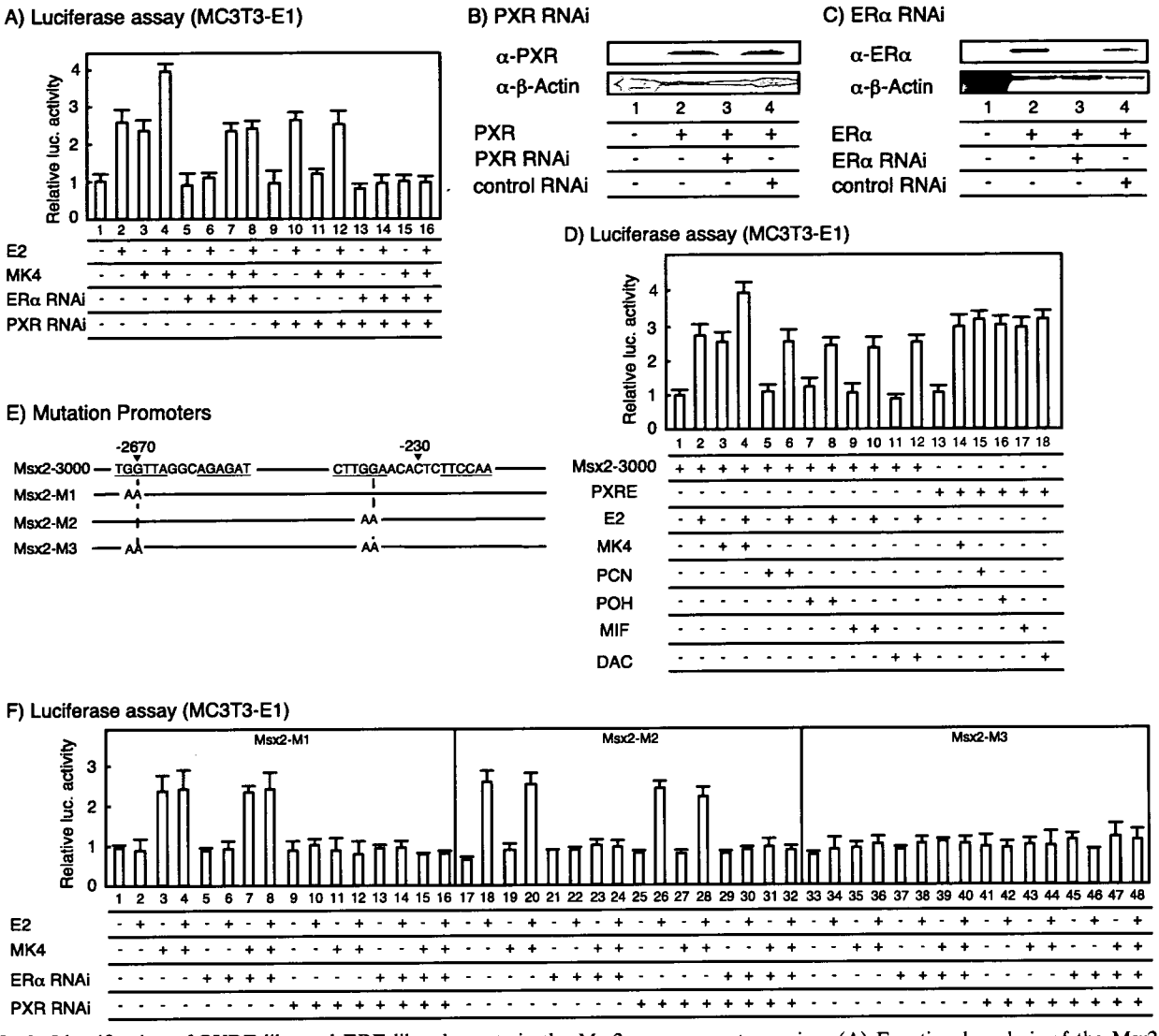


FIG. 3. Identification of PXRE-like and ERE-like elements in the Msx2 gene promoter region. (A) Functional analysis of the Msx2 gene promoter. Plasmid constructs pSIREN-Shuttle-PXR (100 ng) and pSIREN-Shuttle-ERα (100 ng), together with the Msx2-3000 reporter (400 ng), were transfected in MC3T3-E1 cells, and the transfected cells were cultured for 24 h. Promoter activity was measured by luciferase (luc.) activity. All values are means ± standard deviations for at least three independent experiments. (B and C) Gene-specific knockdown using PXR (B) and ERα (C) RNAi vectors. The RNAi vectors were transfected into MC3T3-E1 cells for 48 h, and the protein levels were determined by immunoblotting. β-Actin was used as a control. (D) Regulation of the Msx2-3000 reporter by known PXR ligands, such as pregnenolone 16α-carbonitrile (PCN), 17α-hydroxypregnenolone (POH), mifepristone (MIF), and dexamethasone 21-acetate (DAC), in MC3T3-E1 cells. The amounts of each transfected plasmid are listed above for panel A. (E) Mutation of the Msx2 gene promoters. These mutant promoters were generated with a mutagenesis kit (Stratagene). (F) Analysis of the mutated Msx2 gene promoters by MK4 and E2 using endogenous expression receptors in MC3T3-E1 cells. The amounts of each transfected plasmid are described above for panel A.

we tested ST2 cells and MC3T3-E1 cells. ST2 cells are bipotential cells derived from mouse bone marrow mesenchymal stem cells that differentiate into either osteoblasts or adipocytes under the proper conditions (32). MC3T3-E1 cells are derived from mouse calvaria and are preosteoblastic cells that can be induced to differentiate into mature osteoblasts (30). As shown in the middle panels of Fig. 1A and B (lanes 5 to 8), ALP induction following treatment with MK4 and/or E2 for 24 h was observed in MC3T3-E1 cells but not in ST2 cells, as shown in the bottom panels of Fig. 1A and B (lanes 9 to 12). The additive action of MK4 and E2 in MC3T3-E1 cells was more pronounced following a longer culture period (Fig. 1C and D). Our results suggest that MK4 and E2 act from the stage at which preosteoblasts differentiate into mature osteoblasts but not at the earlier stage of the cell fate decision. To confirm this, we measured the expression of osteoblast genesis marker genes in the MC3T3-E1 cells treated with MK4 and E2 by qRT-PCR. Early osteoblast genesis marker genes (encoding type I collagen α and ALP) were induced by both MK4 and E2 treatments, while mRNA levels of late osteoblast genesis marker genes (encoding osteopontin and osteocalcin) were unaltered following 24 h of treatment (Fig. 1E).

**Msx2 is a target gene for MK4 and E2 in MC3T3-E1 cells.** To elucidate the molecular pathway through which MK4 and

E2 induce osteoblast genesis, we screened for MK4 target proteins in untreated and treated MC3T3-E1 cells with 2D SDS-PAGE (Fig. 2A and B). E2 was used for cotreatment, since it potentiated MK4 action in osteoblastic differentiation. As shown in Fig. 2B, expression levels of several candidate proteins were up-regulated or down-regulated by the treatment, and we tried to identify them by matrix-assisted laser desorption ionization–time-of-flight mass spectroscopy analysis. One of these was Msx2, a protein that was shown to be a critical factor for inducing mesenchymal stem cells to differentiate into preosteoblasts (26). We reasoned that Msx2 mediates MK4 action as an MK4 target gene. Indeed, induction of Msx2 gene expression by MK4 alone was visible, and the additive induction with E2 was at both the transcriptional level (qRT-PCR) and the translational level (Western blotting) (Fig. 2C and D).

**Msx2 promoter confers responsiveness to MK4 and E2 through cognate NRs.** To test the idea that MK4 acts as a PXR ligand in the transcriptional control of the mouse Msx2 gene, we performed a promoter analysis of the Msx2 gene by using a luciferase reporter assay on MC3T3-E1 cells. The responsiveness to MK4 and E2 was tested with a luciferase gene reporter driven by the mouse Msx2 gene promoter region (−1 to −3000) (designated Msx2-3000). Either MK4 or E2 was sufficient to activate transcription alone, and synergy of MK4 with E2 was observed (Fig. 3A). Knockdown of PXR and ERα by RNAi (Fig. 3B and C) abolished the MK4- and E2-induced transactivation of the Msx2 gene promoter (Fig. 3A, lanes 5 to 16). Other known PXR ligands also were tested with this promoter. Though MK4 was effective in stimulating the transcription of the Msx2 gene promoter, none of the other tested ligands (pregnenolone 16α-carbonitrile, 17α-hydroxypregnenolone, mifepristone, and dexamethazone 21-acetate) stimulated transcription alone. Additionally, none demonstrated synergy with E2 (Fig. 3D). We used a series of promoter deletion mutants to map an MK4-responsive element in the proximal promoter (−1 to −390) (data not shown) that contained a consensus PXR/RXR binding-related element (designated Msx2-PXRE) at around bp −230 (Fig. 3E). Additionally, a consensus element (designated Msx2-ERE) related to the ER-responsive element (ERE) was found in the distal promoter region (−2672 to −2658) (see Fig. 3E and 4A). Short elements containing either Msx2-PXRE or Msx2-ERE conferred the expected response to the cognate NR ligand (data not shown). To verify the impact of the response elements, we introduced two point mutations into each of the elements (Fig. 3E). As shown in Fig. 3F, Msx2-M1, which was mutated at Msx2-ERE, lost the E2 response; likewise, the MK4 response was abolished by the mutation (Msx2-M2) in Msx2-PXRE. As expected, no NR ligand response was seen in the mutant (Msx2-M3) that was mutated at both Msx2-PXRE and Msx2-ERE (Fig. 3F).

**Direct DNA binding of PXR on the Msx2 promoter.** To determine whether PXR bound to the proposed PXRE (Fig. 4A), we examined the DNA binding ability of PXR and ERα on the Msx2 gene promoter with an ABCD assay. We tested whole-cell extracts of MC3T3-E1 cells transiently overexpressing PXR, RXRα, or ERα. PXR/RXRα bound to Msx2-PXRE, while ERα binding was not seen for Msx2-PXRE (Fig. 4B). Conversely, ERα bound, as expected, to Msx2-ERE (Fig. 4A and B). Moreover, in this assay, p300 also associated with the receptors. To verify if the observed DNA binding of PXR and ERα in vitro reflected the physiological events in the Msx2 promoter, we performed a ChIP analysis on the endogenous promoter of the Msx2 gene. E2-induced recruitment of the ERα, but not PXR, was observed in the upstream region of Msx2-ERE. In contrast, the ligand-independent association of PXR took place in the region of the distal promoter containing Msx2-PXRE (Fig. 4C). Consistent with the type of recruited receptors, the known NR coactivator p300 also was recruited to the promoter (Fig. 4C).

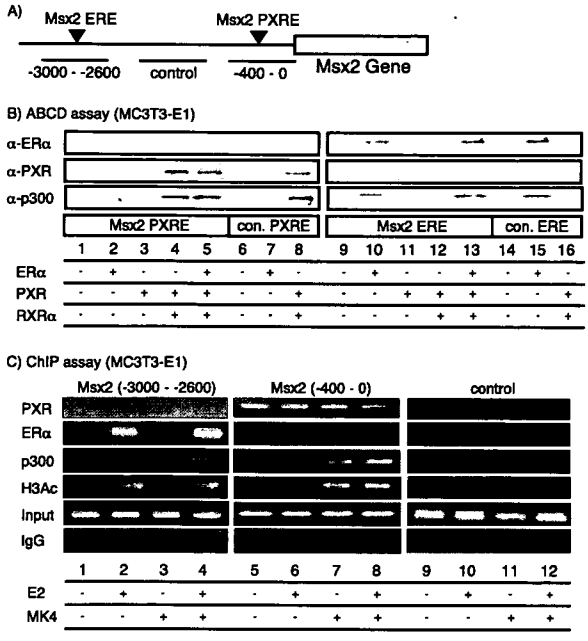


FIG. 4. Direct recruitment of PXR/RXRα to Msx2-PXRE in the Msx2 gene promoter. (A) The position of Msx2-PXRE and Msx2-ERE in the Msx2 gene promoter. (B) The recruitment of PXR/RXRα and ERα to Msx2-PXRE and Msx2-ERE in the Msx2 gene promoter in vitro. The ABCD assay was performed with two copies of Msx2-PXRE and Msx2-ERE that were conjugated to the beads and incubated with whole-cell extracts from HEK 293T cells that overexpressed PXR, RXRα, and ERα together with the respective ligands for the NRs. The proteins interacting with DNA-conjugated beads were detected by immunoblotting. α-ER, anti-ER antibody; α-PXR, anti-PXR antibody; α-p300, anti-p300 antibody, con., consensus. (C) The recruitment of PXR/RXRα and ERα to the Msx2 gene promoter in vivo. ChIP analysis was performed with soluble chromatin prepared from MC3T3-E1 cells treated with MK4 and E2 for 1 h and immunoprecipitated with the indicated antibodies. Extracted DNA samples were amplified using primer pairs to detect the regions containing either Msx2-PXRE or Msx2-ERE. IgG, immunoglobulin G.

**MK4-induced osteoblast genesis is mediated by PXR.** To confirm the physiological impact of PXR function on VK-induced osteoblast genesis, we knocked down either Msx2 or PXR in MC3T3-E1 cells by RNAi with adenovirus (Fig. 5A and B). Expression of endogenous Msx2 and PXR was significantly attenuated by RNAi (Fig. 5A and B). As expected, osteoblast genesis induced by MK4 was attenuated when cells were infected with Msx2 RNAi adenovirus (the middle panels of Fig. 5C and D [lanes 5 to 8]). Moreover, in primary osteoblasts derived from ERαKO mice, osteoblast genesis induced by E2 also was attenuated (Fig. 5E). The induction of marker

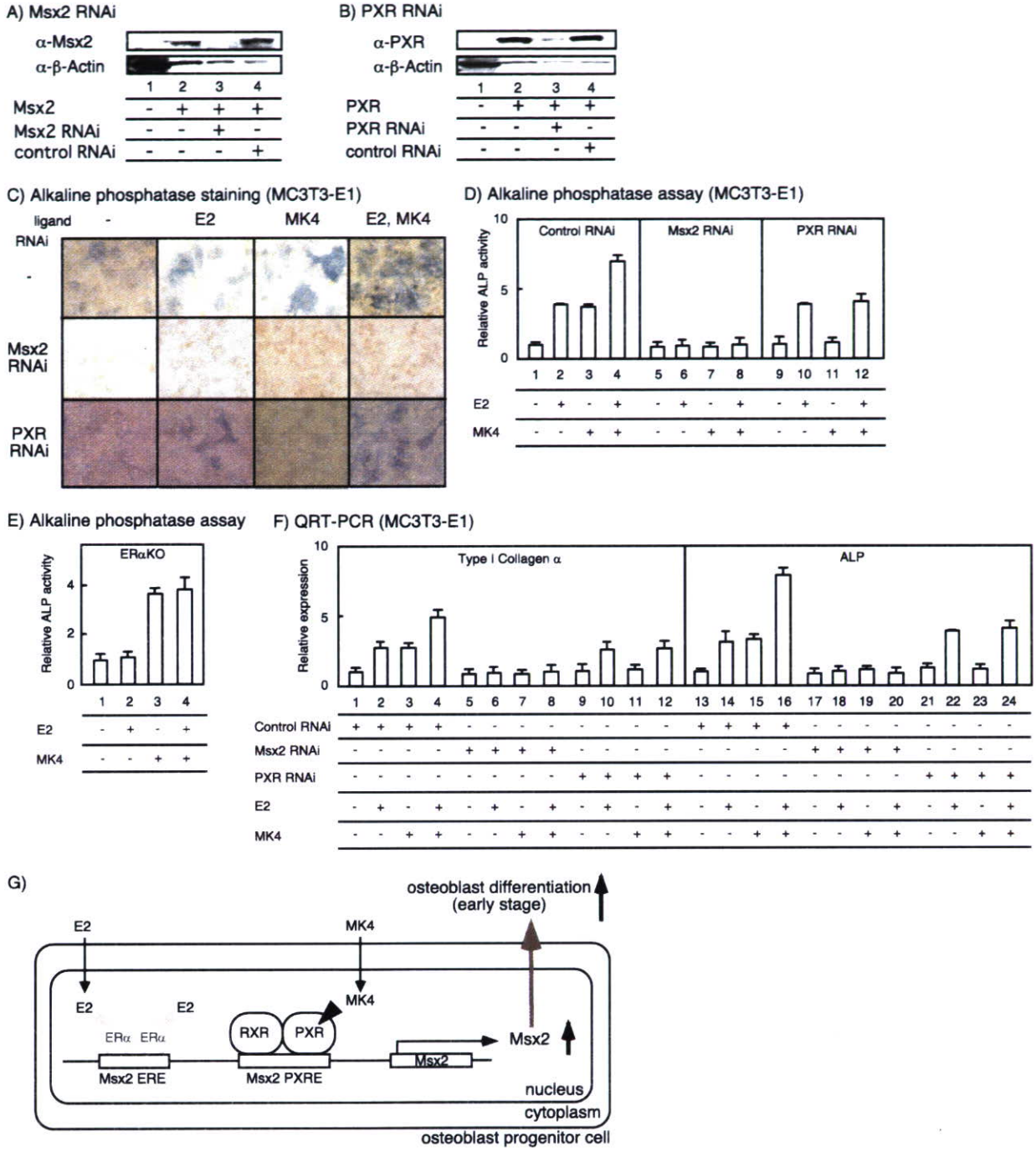


FIG. 5. VK and estrogen cooperatively promote osteoblast differentiation through Msx2 gene induction. (A and B) Gene-specific knockdown using Msx2 (A) and PXR (B) RNAi adenovirus. MC3T3-E1 cells were cultured with the RNAi adenovirus for 48 h, and the protein levels were determined by immunoblotting with each antibody. β-Actin antibody (α-β-actin) was used as a control. α-Msx2, anti-Msx2 antibody; α-PXR, anti-PXR antibody. (C) Msx2 mediates the effect of MK4 and E2 in osteoblast differentiation. MC3T3-E1 cells infected with the RNAi adenovirus for 48 h were incubated with MK4 and E2 for 24 h. Osteoblast differentiation was detected by ALP staining. (D) Msx2 mediates the effect of MK4 and E2 in osteoblast differentiation. MC3T3-E1 cells infected with the RNAi adenovirus for 48 h were incubated with MK4 and E2 for 24 h, and then ALP activity was measured. (E) The effect of E2 and MK4 in primary ERαKO osteoblast differentiation. Primary osteoblasts derived from ERαKO mice were incubated with MK4 and E2 for 24 h. The osteoblast differentiation was measured by ALP assay. (F) The expression levels of osteoblast differentiation marker genes induced by MK4 and E2 with Msx2 or PXR RNAi. MC3T3-E1 cells were infected with the RNAi (control, Msx2, or PXR) adenovirus for 48 h, followed by either no treatment or treatment with MK4 and E2 for 24 h. The total RNA then was extracted and used for qRT-PCR. (G) A schematic view of the VK and E2 actions at the Msx2 gene promoter.

genes (encoding type I collagen α and ALP) was, as expected, also abrogated with Msx2 RNAi adenovirus (Fig. 5F, lanes 5 to 8 and 17 to 20). Moreover, PXR RNAi also inhibited MK4-dependent osteoblast genesis, as shown in the bottom panels of Fig. 5C, D (lanes 9 to 12), and F (lanes 9 to 12 and 21 to 24).

## DISCUSSION

This study demonstrates that Msx2 contains a PXRE in its gene promoter and that it is a direct target gene for MK4-bound PXR. Reflecting the similar effects of E2 and MK4, an ERE also was mapped in the Msx2 gene promoter. Although each of the response elements alone was not remarkable, the combined effects of MK4 and E2 were evident in transactivation. In osteoblastic differentiation induced by MK4, E2, or both, the knockdown of endogenous Msx2 abrogated the effects of the NR ligands. Thus, Msx2 likely mediates, at least in part, the effects of both MK4 and E2 on osteoblastic differentiation. Msx2 is one of the prime osteoblastogenic factors in an intact animal (26). Our observation that osteoblast genesis is induced by MK4 in cultured preosteoblastic cells suggests that MK4 exerts its osteoprotective action by upregulating Msx2 gene expression and increasing osteoblast genesis (Fig. 5G). Since extracellular matrix Tsukushi genes also are VK target genes (8), the osteoprotective effects of VK in the human are mediated both by the classical pathway of protein γ-carboxylation and through genomic action via PXR/SXR.

PXR/SXR is activated by a number of endogenous and exogenous ligands (23). PXR/SXR plays a prominent role in the detoxification of chemicals (36). PXR/SXR is considered a global sensor for low-molecular-mass fat-soluble drugs that also is responsible for the timely degradation of these compounds (14). Thus, in contrast to steroid hormone receptors, PXR/SXR responds to a variety of ligands with unrelated structures. The three-dimensional structure of the PXR/SXR ligand binding domain (LBD) is known (35). The LBD is flexible, with a wider cave than those of steroid receptors (37). This permits PXR/SXR to capture a variety of ligands, including MK4. Shifting of the C-terminal transactivation helix H12 in the ERα LBD alters the conformation of the receptor, depending on whether an agonist or antagonist is bound. This plasticity enables ERα to respond to a variety of different ligands (27). It is possible that MK4 binding induces an H12 shift that is distinct from those induced by the other ligands, since the transactivation of PXR in the Msx2 promoters was induced by MK4 but not by the other known PXR/SXR ligands in the osteoblastic cell lines. Thus, the MK4-induced angle in the H12 shifting of PXR might be preferential to recruit coactivator/coactivator complexes to the Msx2 gene promoter in preosteoblasts. In fact, the MK4-induced association of PXR with the well-characterized coactivator (p300) was detectable in both an ABCD assay and a ChIP analysis of the Msx2 gene promoter. Our observations are a starting point for investigating coregulators (17, 24) associating with MK4-bound PXR in osteoblastic cells. The identification of PXR coregulators will enhance the understanding of the osteoprotective effects of VK at a molecular level.

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# A Regulatory Circuit Mediating Convergence between Nurr1 Transcriptional Regulation and Wnt Signaling[▽]

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**The orphan nuclear receptor Nurr1 is essential for the development and maintenance of midbrain dopaminergic neurons, the cells that degenerate during Parkinson's disease, by promoting the transcription of genes involved in dopaminergic neurotransmission. Since Nurr1 lacks a classical ligand-binding pocket, it is not clear which factors regulate its activity and how these factors are affected during disease pathogenesis. Since Wnt signaling via β-catenin promotes the differentiation of Nurr1$^+$ dopaminergic precursors in vitro, we tested for functional interactions between these systems. We found that β-catenin and Nurr1 functionally interact at multiple levels. In the absence of β-catenin, Nurr1 is associated with Lef-1 in corepressor complexes. β-Catenin binds Nurr1 and disrupts these corepressor complexes, leading to coactivator recruitment and induction of Wnt- and Nurr1-responsive genes. We then identified KCNIP4/calsenilin-like protein as being responsive to concurrent activation by Nurr1 and β-catenin. Since KCNIP4 interacts with presenilins, the Alzheimer's disease-associated proteins that promote β-catenin degradation, we tested the possibility that KCNIP4 induction regulates β-catenin signaling. KCNIP4 induction limited β-catenin activity in a presenilin-dependent manner, thereby serving as a negative feedback loop; furthermore, Nurr1 inhibition of β-catenin activity was absent in PS1$^{-/-}$ cells or in the presence of small interfering RNAs specific to KCNIP4. These data describe regulatory convergence between Nurr1 and β-catenin, providing a mechanism by which Nurr1 could be regulated by Wnt signaling.**

Expression and maintenance of the dopaminergic phenotype in the ventral midbrain (VM) require the orphan nuclear receptor (NR) Nurr1 (NR4A2) (48, 59). Genetic ablation of *Nurr1* produces embryonic lethality due to a nearly complete absence specifically of mesencephalic dopaminergic neurons, which are critical for motor function. Nurr1 regulates both the differentiation and the maintenance of these dopaminergic cells, as *Nurr1*$^{+/-}$ mice appear normal at birth but develop motor deficits resulting from reduced numbers of dopaminergic neurons and lower dopamine levels in the striatum (22). At the molecular level, Nurr1 binds specific response elements in the promoters of genes involved in dopaminergic neurotransmission, such as the genes encoding tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase, and the dopamine transporter (20, 26, 44, 45). In Parkinson's disease, mesencephalic dopaminergic neurons degenerate, ultimately leading to severe motor deficits; correspondingly, Nurr1 levels appear to be reduced (10, 11). Therapeutic strategies that promote Nurr1 function in Parkinson's disease might therefore restore dopaminergic function or even increase the number of dopaminergic neurons. However, Nurr1 has a closed ligand-binding pocket and thus appears to be regulated by ligand-independent mechanisms (55). These mechanisms include changes in the expression of its RNA and protein (36, 47, 54) and changes by second messenger signaling systems and coactivators that modify its transcriptional activity (15, 21, 23, 27, 43). Identifying factors that govern Nurr1 activity is thus important for understanding the development and pathophysiology of VM dopaminergic neurons.

Among the potential factors regulating Nurr1 function, a strong candidate is signaling downstream of the Wnt family of secreted glycoproteins. Wnt signaling is required for the establishment of the midbrain/hindbrain region of the developing nervous system, including VM dopaminergic neurons (reviewed in reference 5), in part by promoting the expression of transcription factors that specify regional identity, such as engrailed (13). In the mouse VM region, the Wnt signaling molecule β-catenin is highly expressed and active in Nurr1$^+$ precursor cells, as evidenced by local expression of TOPGAL, a β-catenin-responsive reporter (7). Specific Wnt molecules also promote the proliferation and differentiation of Nurr1$^+$ dopaminergic precursor cells cultured from the VM (7), and Wnt-5a is expressed in VM glial cells, potentially explaining their ability to induce the dopaminergic phenotype in vivo (6). Thus, there is clear evidence that Wnt signaling via β-catenin regulates the development of Nurr1$^+$ precursors in vivo, but the molecular mechanisms underlying this effect are unknown.

Canonical Wnt signaling is triggered by binding of a secreted Wnt family member to the membrane receptor complex of

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low-density lipoprotein-related protein 5 (LRP-5) or LRP-6 and frizzled, leading to the accumulation of cellular β-catenin protein through inhibition of protein degradation of β-catenin (reviewed in reference 4). β-Catenin is constitutively degraded through sequential phosphorylation events promoted within at least two distinct molecular complexes. The major complex consists of axin and its associated proteins, and the second complex involves presenilin-1 (PS1) and PS2, the membrane proteins responsible for the intramembranous processing of the β-amyloid precursor protein in Alzheimer's disease (24, 25, 39, 51, 57). β-Catenin phosphorylation is inhibited by Wnt activation of frizzled/LRP receptors, leading to protein accumulation and translocation to the nucleus, where it activates TCF/LEF transcriptional complexes in the promoters of target genes. Additionally, β-catenin can act as a coactivator for several NRs and modify transcription in that manner (29, 32, 50, 52; reviewed in reference 35).

In the present study, we examined the potential relationship between Nurr1 and Wnt signaling. We found that Nurr1 is present in corepressor complexes on TCF/LEF elements prior to Wnt signaling, after which β-catenin binds to Nurr1 and acts as a transcriptional cofactor. Furthermore, we identified KCNIP4 as a β-catenin/Nurr1 target that modulates Wnt signaling by interacting with the PS complex.

## MATERIALS AND METHODS

**Reagents.** Full-length human Nurr1, human β-catenin, and rat LEF-1 cDNAs were cloned into pcDNA3.1 (Invitrogen) and, for Nurr1, also inserted into FLAG- and hemagglutinin (HA)-tagged pIRES2-EGFP Q-vector (Clontech). β-Catenin S33A and mutant promoter vectors were made with a site-directed mutagenesis kit (Stratagene). For glutathione *S*-transferase (GST) pull-down assays, each deletion mutant was inserted into pGEX4-1 5-2 vector (Amersham Biosciences). TOPFLASH was purchased from Upstate Biotechnology. The 2×-NBRE (AAAGGTCA) and promoter regions of cyclin D1 (positions −872 to +8), TH (positions −1110 to 0), and KCNIP4 (positions −1043 to −2) were cloned from a human genomic library (Clontech) and inserted into the PGL3-basic vector containing thymidine kinase to generate luciferase reporters.

The following commercially available antibodies were used: anti-Nurr1, anti-C-terminal binding protein (anti-CtBP), anti-transducin-like enhancer (anti-TLE), anti-protein inhibitor of activated STATy (anti-PIASy), and anti-axin from Santa Cruz Biotechnology; anti-PS1 from Chemicon; anti-CREB-binding protein (anti-CBP) and anti-LEF-1 from Upstate Biotechnology; anti-histone deacetylase 1 (anti-HDAC-1) and anti-HDAC-3 from Affinity Bioreagents; and anti-β-catenin, anti-p45 β-catenin, and anti-p33/37/41 β-catenin from Cell Signaling. Anti-KCNIP4 antibody was obtained from Takeshi Iwatsubo (University of Tokyo).

The following small interfering RNA (siRNA) pools (SMARTpool) were purchased from Dharmacon: Nurr1, M-003427-00; CtBP1, M-008609-01; TLE1, M-015528-00; HDAC-1, M-003493-02; HDAC-3, M-003496-00; PIASy, M-006445-00; β-catenin, M-003482-00; CBP, M-003477-01; KCNIP4, M-021472-00; PS1, M-004998-01; and nonspecific control, D-001210-02-05.

**Cell culture.** PS1$^{-/-}$ murine embryonic fibroblasts were kindly given by Bart de Strooper (Leuven, Belgium). All cells were routinely maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. To establish stable transformants, 293F cells were infected with retrovirus made with Amphopac 293F cells as the packaging cell line. The packaging cell line was made by transfection of pQCXIN retroviral vectors and was cultured for 2 weeks with 750 μg/ml G418 for transformant selection. For large-scale purification, 293F cells were cultured in 293F SFM (Life Technologies) supplemented with Glutamax (Life Technolgies) in a bioreactor.

**Nuclear extraction and purification of Nurr1 interactants.** Nuclear extracts (28) from 293F stable transformants expressing tagged Nurr1 and from SK-N-MC cells were loaded onto an anti-FLAG M2 affinity resin column and washed extensively (20 mM Tris-HCl [pH 8.0], 300 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Bound proteins were eluted from the column by incubation with 133 g/ml FLAG peptide in washing buffer for 30 min at room temperature. The eluted solution was similarly applied to an HA resin column and washed, and Nurr1 complexes were eluted with the HA peptide.

**Immunoprecipitation and GST pull-down assay.** Cells were transfected with 5 μg of each expression vector and immunoprecipitated with anti-FLAG antibody (Sigma) for Western blotting with specific antibodies (28). For the GST pull-down assay, full-length human Nurr1, LEF-1, and β-catenin were translated in vitro and incubated with GST-fused mutants of Nurr1, LEF-1, and β-catenin immobilized on glutathione-Sepharose beads prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Chromatin immunoprecipitation (ChIP).** Soluble chromatin from 293F or SK-N-MC cells was prepared with an acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology) and immunoprecipitated with antibodies against the indicated proteins in the presence and absence of LiCl (28). LiCl stimulation was started 45 min before fixing the cells with formaldehyde (49). Specific primer pairs were designed to amplify the promoter region of cyclin D1 (5′-GGCTCCAGGACTTTGCAACTTC-3′ and 5′-GGCGCCTCAGGGATG G-3′), TH (5′-GCTGTCTCAGCCCCCC-3′ and 5′-CTGGGTCCCCCACCTTC C-3′), the KCNIP4 TCF/LEF site (5′-GTATTCTGCACCTCGGCCCT-3′ and 5′-TACTGCTGCACAAAGTTAGGCTGAG-3′), and the KCNIP4 Nurr-responsive element (NBRE) (5′-CAGCCATAGGGAAGGCAAATAG-3′ and 5′-AGAAGTCAAAATTAAAATGCAGATTTCTGTGTCC-3′) from human genomic DNA. PCR conditions were optimized to allow semiquantitative measurement, and PCR products were visualized on 2% agarose–Tris-acetate-EDTA gels.

**Microarray and quantitative RT-PCR analysis.** Microarray and quantitative reverse transcription-PCR (RT-PCR) were performed as previously described (37, 42). Gene-specific primers and probes for human KCNIP4 and TH were purchased from Applied Biosystems. For cyclin D1, the following primers and probe were used: probe, 5′-AAGGAGACCATCCCCCTGACGGC-3′; forward primer, 5′-GCATGTTCGTGGCCTCTAAGA-3′; and reverse primer, 5′-CGG TGTAGATGCACAGCTTCTC-3′.

**Immunofluorescence.** SK-N-MC cells were seeded (40,000/cm$^2$) on glass coverslips in six-well plates. Twenty-four hours after LiCl treatment, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 45 min at 4°C. Primary antibodies were used at a dilution of 1:100 to 1:250 with blocking reagent (Roche) and were incubated for 1 h at room temperature. After four PBS washes (5 min each), secondary antibodies coupled to fluorescein isothiocyanate, tetramethyl rhodamine isocyanate (both from Sigma), or Alexa Fluor488 (Molecular Probes) at a 1:100 dilution were incubated with the cells for 30 min. Cell nuclei were stained with Hoechst 33342 (Molecular Probes), and coverslips were mounted using Slow-fade reagent (Molecular Probes). Immunofluorescence signals were evaluated with a Nikon T300 microscope.

**Transfection and luciferase assay.** Human 293F cells and SK-N-MC cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and, at 40 to 50% confluence, were transfected with plasmids, using Lipofectamine-Plus reagents (Invitrogen) in 12-well dishes. Total amounts of DNA were adjusted by supplementation with up to 1.0 μg of empty vector. Luciferase activities were determined using a luciferase assay system (Promega). As a reference plasmid to normalize transfection efficiency, 2 ng pRL-CMV plasmid (Promega) was cotransfected in all experiments (28, 58). All values are means ± standard deviations for at least three independent experiments. For RNA interference (RNAi), two-step transfection was performed with Trans IT-TKO and Trans IT-NTI transfection reagents (Mirus) following the manufacturer's recommendations. All siRNAs were evaluated for efficacy by immunoblotting (data not shown).

## RESULTS

**Transcriptional cross talk between Nurr1 and Wnt signal transduction.** We first determined if Wnt signaling regulates the function of Nurr1. Either Wnt-1 or LiCl was used to activate β-catenin-mediated canonical Wnt signaling, and effects on Nurr1 transcriptional activity were measured. 293F cells stably overexpressing Nurr1 or parental control cells, which do not express detectable Nurr1, were transiently transfected with a luciferase reporter plasmid containing a consensus DNA binding site for Nurr1 (NBRE) (17). Overexpression of Nurr1 increased the activity of this reporter, as expected (Fig. 1A). Nurr1 transactivation function was enhanced when Wnt signaling was activated by either Wnt-1, LiCl, or a constitutively

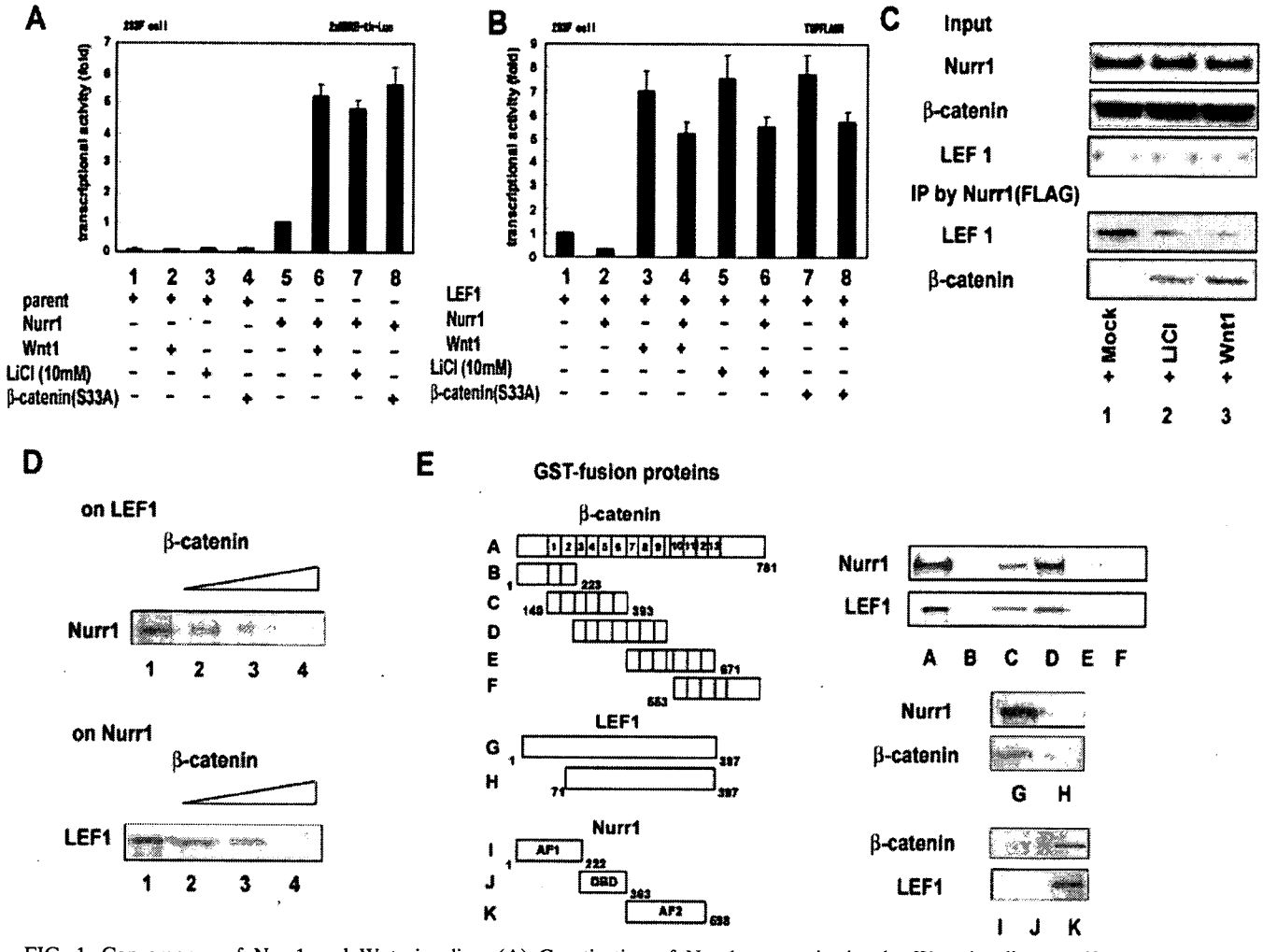


FIG. 1. Convergence of Nurr1 and Wnt signaling. (A) Coactivation of Nurr1 transactivation by Wnt signaling. Luciferase assays were performed with 293F cells transfected with a consensus 2×-NBRE-containing luciferase reporter plasmid (2× NBRE-tk-luc) (400 ng), with or without 200 ng of the indicated expression vector (Nurr1, a constitutively active form of β-catenin [β-catenin S33A], or Wnt-1), in the presence or absence of LiCl (10 mM) or Wnt-1. (B) Nurr1 repression of TCF/LEF transactivation. Luciferase assays were performed with 293F cells transfected with a TOPFLASH luciferase reporter plasmid (containing a consensus TCF/LEF binding site) (400 ng), with or without the indicated expression vectors, in the presence or absence of LiCl (10 mM) or Wnt-1. (C) Signaling-dependent interaction between FLAG-Nurr1, LEF-1, and β-catenin. Exogenous proteins were expressed in 293F cells, which were treated with vehicle, LiCl, or Wnt-1 for 24 h. Transfected cell extracts were subjected to IP with mouse anti-FLAG antibody and then immunoblotted. (D) Competitive binding between β-catenin and Nurr1 for LEF-1. GST-tagged LEF-1 (top) or Nurr1 (bottom) was mixed with in vitro-translated Nurr1 (top) or LEF-1 (bottom) in the presence of increasing levels of unlabeled, in vitro-translated β-catenin. Following GST pull-down, in vitro-translated Nurr1 and LEF-1 were visualized by immunoblotting. (E) Physical interaction and mapping of interaction domains of Nurr1, β-catenin, and LEF-1. Associations of GST-fused, in vitro-translated Nurr1, LEF-1, and β-catenin proteins with the indicated deletions were tested in a GST pull-down assay.

active mutant (S33A) of β-catenin (41) (Fig. 1A). Similar results were observed in other clones from the same transfection (data not shown). We then tested for the opposite modulation by using a reporter construct harboring a consensus DNA binding site for TCF/LEF (TOPFLASH), which is a target of canonical Wnt signaling. Nurr1 overexpression produced a modest but reproducible inhibition of TOPFLASH activity in either the absence or presence of Wnt-1, LiCl, or S33A β-catenin (Fig. 1B). These data suggested that Wnt signaling, specifically through β-catenin, modulates Nurr1 activity and, conversely, that Nurr1 influences β-catenin activity.

**Direct physical interaction of Nurr1 with β-catenin and Lef-1.** To determine the molecular basis of this mutual coregulation, the physical interaction of Nurr1 with β-catenin and LEF-1 was tested by coimmunoprecipitation of 293F cells overexpressing all three proteins (Fig. 1C). Lef-1, but not β-catenin, was coimmunoprecipitated with FLAG epitope-tagged Nurr1 in nuclear extracts from unstimulated 293F cells. Following stimulation with Wnt-1 or LiCl, β-catenin was recovered with anti-FLAG antibodies, while the amount of coprecipitating Lef-1 was reduced, suggesting that β-catenin competes with Nurr1 for Lef-1 binding (Fig. 1C). GST pull-down experiments showed that this competition is direct. GST–Lef-1 and in vitro-translated β-catenin were aliquoted into four assay tubes with increasing levels of recombinant β-catenin protein. In the absence of recombinant β-catenin,

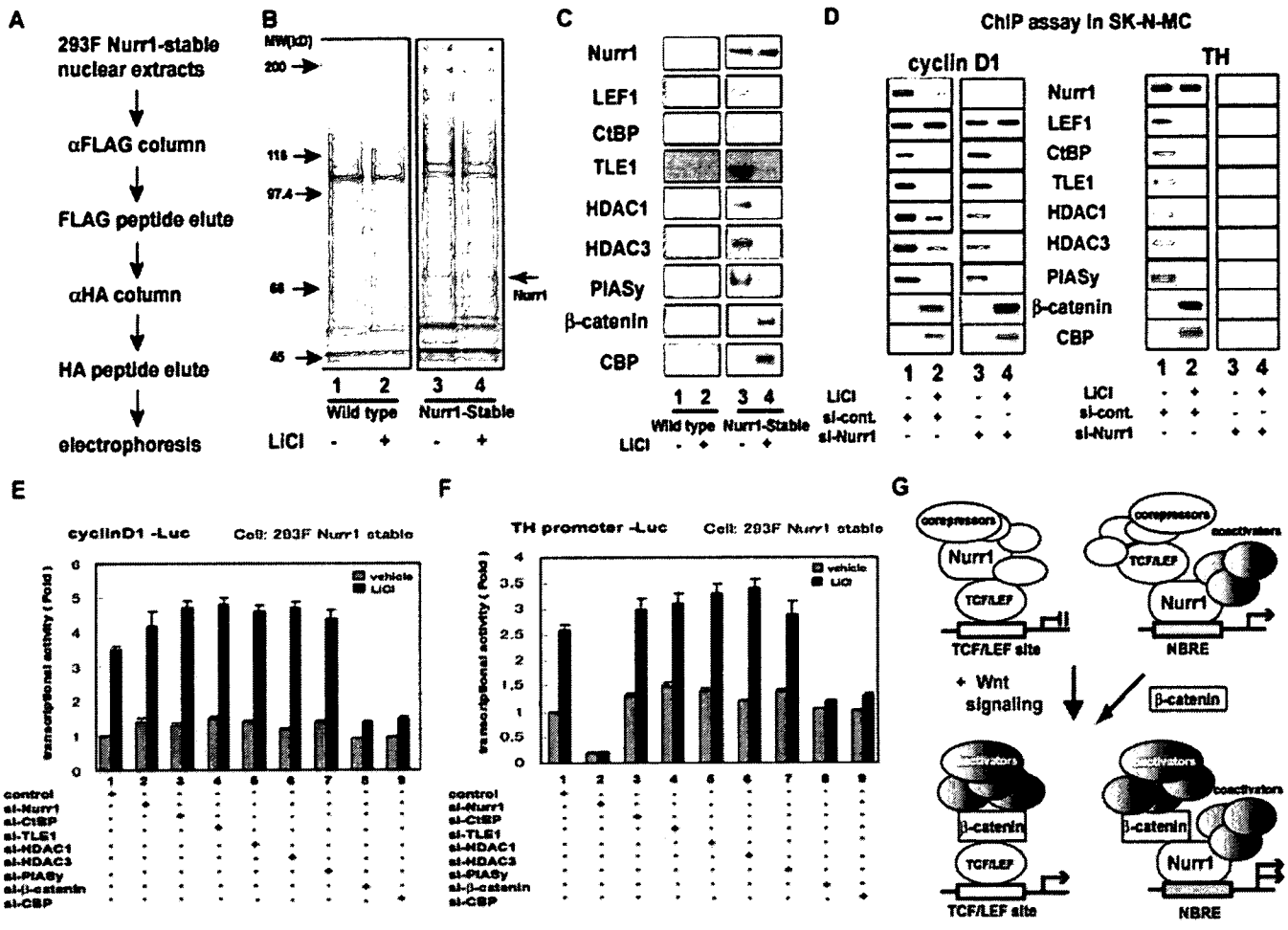


FIG. 2. Functional interplay between Nurr1, β-catenin, and LEF-1. (A) Schematic diagram of the biochemical purification procedure of FLAG/HA-Nurr1 interactants from a 293F cell stable transformant treated with and without LiCl. (B) Fractions eluted from anti-HA resin were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining. The arrow on the right indicates the Nurr1 protein. (C) Nurr1 and potential interacting proteins were detected by immunoblotting in eluates from parental and Nurr1-expressing cells treated with or without LiCl. (D) ChIP assays examining the association of the genomic regions containing the TCF/LEF site in cyclin D1 (left) and the NBRE site in TH (right) with Nurr1 and interacting proteins (indicated in the middle) in SK-N-MC cells. siRNAs (100 nM) were transfected prior to a 24-h treatment with LiCl or vehicle. Following cross-linking, sonication, and immunoprecipitation, genomic regions recovered with the indicated antibodies were detected by semiquantitative RT-PCR. (E) Effects of siRNAs on transcriptional regulation of the cyclin D1 promoter in Nurr1-expressing 293F cells. Each siRNA (100 nM) was transfected with a luciferase reporter containing the cyclin D1 promoter (cyclin D1-Luc) in the presence and absence of LiCl. (F) Effects of siRNAs on transcriptional regulation of the TH promoter in Nurr1-expressing 293F cells. siRNAs were transfected as described above with a luciferase reporter containing the TH promoter linked to luciferase (TH promoter-Luc) in the presence and absence of LiCl. (G) Schematic representation of the relationships among Nurr1, LEF-1, and β-catenin under the control of activated canonical Wnt signaling at the TCF/LEF site (cyclin D1) and the NBRE (TH).

GST–Lef-1 pull-down recovered in vitro-translated Nurr1; the addition of β-catenin protein to the reaction mix dose-dependently reduced the amount of Nurr1 recovered. Identical results were seen when GST-Nurr1 was used as the bait for Lef-1 (Fig. 1C).

To determine which of the protein-protein interaction sites in β-catenin possess an affinity for Nurr1, the interacting domains were mapped by GST pull-down assay (Fig. 1E). A series of β-catenin deletion mutants fused to GST were produced, diluted to equal concentrations, and incubated with either in vitro-translated Nurr1 or Lef-1. Constructs containing armadillo repeats 3 to 10 retained the binding affinity for both Nurr1 and Lef-1, suggesting that both proteins interact with β-catenin in this region. Deletion of the Lef-1 N-terminal 70 amino acids caused a loss of binding of Lef-1 to both Nurr1 and β-catenin. Finally, only amino acids 363 to 598 of Nurr1, encompassing the closed ligand-binding domain and AF2, interacted with Lef-1 or β-catenin. These data suggest that the C terminus of Nurr1 binds to the N terminus of Lef-1 and that, in the presence of β-catenin, these interaction sites compete for binding to the armadillo domains of β-catenin.

**Characterization of the Nurr1/β-catenin complex.** To further characterize the molecular properties of the Nurr1/β-catenin complex, Nurr1 was purified from nuclear extracts of 293F cells stably expressing FLAG/HA-tagged Nurr1 in the presence of LiCl, with untransfected wild-type cells serving as a control (Fig. 2A). Endogenous proteins interacting with Nurr1 were purified through anti-FLAG and then anti-HA

affinity columns, visualized by silver staining (Fig. 2B), and characterized by Western blotting for transcriptional cofactors (Fig. 2C). Silver staining showed that immunocapture of FLAG/HA-Nurr1 resulted in the recovery of Nurr1, at approximately 70 kDa (validated by mass spectrometry [data not shown]), as well as the copurification of many proteins, most of which were equally recovered in the presence or absence of LiCl. The protein mixture was then analyzed by Western blotting for cofactors selected from the literature. In the absence of LiCl, Lef-1 as well as the corepressors CtBP, TLE-1, HDAC-1, HDAC-3, and PIASy (18) were purified with Nurr1 but were not present in controls. LiCl treatment reduced the amounts of these proteins associated with Nurr1 and instead led to the recovery of endogenous β-catenin and CBP, a key component of histone acetyltransferase complexes (9) (Fig. 2C). Thus, β-catenin interaction with Nurr1 coincides with a switch from Nurr1 association with Lef-1 and corepressors to complex formation with CBP.

**Identification and function of endogenous Nurr1/β-catenin complexes.** The proposed assembly of these factors on endogenous target promoters for TCF/LEF-1 (cyclin D1) and Nurr1 (TH) was then tested by ChIP with SK-N-MC cells, which express endogenous Nurr1 (26) (Fig. 2D). Cross-linked chromatin fragments averaging 250 bp in length were generated from SK-N-MC cells treated with nonsilencing control siRNA or Nurr1 siRNA and were immunoprecipitated with antibodies to Nurr1, Lef-1, and the cofactors identified above as being associated with Nurr1 in 293F cells. Semiquantitative PCR was used to detect the presence of the genomic region containing the TCF/LEF element in the cyclin D1 promoter. As expected, LEF-1 and its corepressors were associated with the cyclin D1 promoter in the absence of LiCl. Nurr1 was also associated with the TCF/LEF region in the cyclin D1 promoter in unstimulated cells. LiCl treatment reduced the amount of Nurr1 associated with the TCF/LEF region as well as the amounts of corepressor proteins, while β-catenin and CBP association increased. These data suggest that the LiCl-mediated cofactor switch observed during purification of overexpressed Nurr1 in 293F cells also occurs on native promoters with endogenous proteins. Since Nurr1 overexpression modestly inhibits TCF/LEF responsiveness to Wnt-1 and LiCl (Fig. 1A), the same analysis was performed with cells transfected with siRNA targeting Nurr1. Nurr1 siRNA treatment caused a loss of Nurr1 association with the TCF/LEF element but did not alter the association of Lef-1 with the cyclin D1 promoter, indicating that Nurr1 is not essential for Lef-1 binding to TCF/LEF elements, as expected. Nurr1 siRNA slightly reduced the amounts of HDAC-1, HDAC-3, and PIASy associated with the TCF/LEF region under unstimulated conditions, but did not abolish them, consistent with Nurr1 modulating TCF/LEF repression. Following LiCl treatment, β-catenin and CBP again were associated with the TCF/LEF element, with more β-catenin association detected than that with control siRNA-treated cells. Like the case under basal conditions, less association between the TCF/LEF region and the corepressors was observed in the presence of Nurr1 siRNA. Thus, Nurr1 siRNA appears to enhance the association of β-catenin with the TCF/LEF region of the cyclin D1 promoter and to reduce the amounts of associated corepressors.

Similar experiments were then performed to analyze endogenous Nurr1 association with the genomic region containing the NBRE in the TH promoter. In the absence of LiCl, Nurr1, Lef-1, and the corepressors were associated with the NBRE-containing region. LiCl promoted the association of β-catenin and CBP and the loss of corepressors without altering the amount of associated Nurr1. Consistent with the requirement for Nurr1 for binding the NBRE, siRNAs targeting Nurr1 abolished Nurr1, corepressor, CBP, and β-catenin association with the NBRE in the TH promoter. Thus, β-catenin interaction with Nurr1 at the NBRE in the TH promoter is associated with a loss of corepressors and recruitment of CBP.

To determine the functional relevance of this β-catenin-associated change in cofactor recruitment, promoter-reporter assays were performed to assess the effects of siRNAs targeting Nurr1, β-catenin, and the cofactors on the response of the cyclin D1 and TH promoters in 293F Nurr1 stable transformants (Fig. 2E and F). siRNAs targeting Nurr1 and the corepressors caused a modest but reproducible increase in cyclin D1 promoter responsiveness to LiCl, consistent with the data obtained using the TOPFLASH reporter (Fig. 1B). Knockdown of β-catenin or CBP nearly completely abolished LiCl responsiveness, confirming the known role for these factors in Wnt-stimulated cyclin D1 expression. As shown in Fig. 2F, TH promoter activity was stimulated by LiCl, similar to the effect observed using the 2×-NBRE reporter construct (Fig. 1A). siRNAs targeting Nurr1 inhibited promoter activity approximately fivefold and abolished LiCl responsiveness, while siRNAs targeting the corepressors tended to cause a small increase in activity of the TH promoter. In contrast, siRNAs targeting β-catenin and CBP abrogated the LiCl responsiveness of the TH promoter. Together, these data confirm the observations that Nurr1 modestly inhibits the cyclin D1 promoter and that the TH promoter is stimulated by LiCl in a process requiring Nurr1, β-catenin, and CBP. These data are summarized graphically in Fig. 2G.

**Identification of KCNIP4 gene as a target gene coregulated by Nurr1 and β-catenin.** In order to determine if genes other than those for cyclin D1 and TH are mutually regulated by Nurr1/β-catenin, we used microarrays to screen for genes that respond to both Nurr1 overexpression and Wnt-1 stimulation, particularly when both are present. Two clones of 293F cells stably expressing Nurr1 or wild-type parental cells were treated with LiCl or Wnt-1, and resulting changes in RNA levels were scrutinized for genes that responded to Nurr1 and LiCl treatment conditions more robustly than to either condition alone; in parallel, the same experiment was performed following LiCl or Wnt-1 stimulation of SK-N-MC cells transfected with Nurr1 or control vector (data not shown). One candidate gene, that encoding KCNIP4, was selected for further analysis based on these criteria and because we hypothesized it could be regulating Wnt signaling (see below). When analyzed by quantitative real-time RT-PCR, KCNIP4 RNA was approximately twofold lower in Nurr1$^+$ 293F stable transformants than in parental cells, suggesting Nurr1 repression of KCNIP4 expression (Fig. 3A). Following LiCl treatment, KNCIP4 RNA was induced 2.5-fold more in the Nurr1 stable transformants than in controls by 24 h. In SK-N-MC cells, LiCl induced the expression of cyclin D1 RNA, as expected, but also significantly induced TH (2-fold) and KCNIP4 (5.5-fold) RNAs. Similar results were obtained using Wnt-1 (data not shown). Thus,

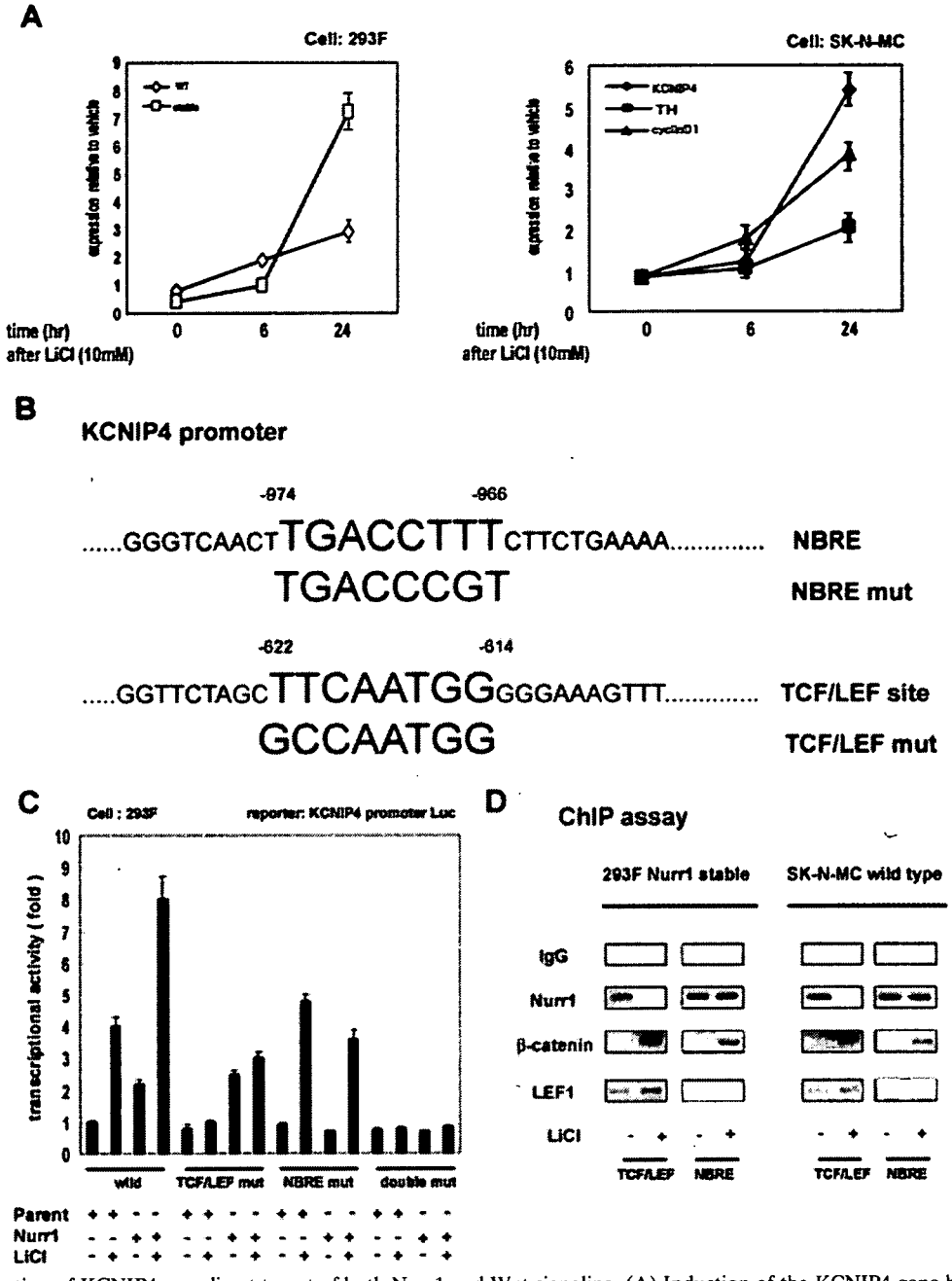


FIG. 3. Identification of KCNIP4 as a direct target of both Nurr1 and Wnt signaling. (A) Induction of the KCNIP4 gene by LiCl in 293F cells and SK-N-MC cells. The gene expression levels were measured in triplicate by quantitative RT-PCR and normalized to those of vehicle-treated parental cells. (B) Schematic presentation of putative binding sites for Nurr1 and TCF/LEF in the KCNIP4 promoter. Binding sites for Nurr1 (NBRE) and TCF/LEF/LY12 (TCF/LEF site) are shown. Two point mutations (NBRE mut and TCF/LEF mut) are displayed for reference. (C) The promoter region of the KCNIP4 gene is regulated by Nurr1 and TCF/LEF elements. Luciferase assays were performed with 293F cells transfected with reporters containing the KCNIP4 promoter or mutants (400 ng), with or without the indicated expression vectors (200 ng) (full-length Nurr1 or parent vector), in the presence and absence of LiCl (10 mM). Data were normalized to values for unstimulated parental cells expressing the wild-type KCNIP4-luciferase construct (first bar). (D) Recruitment of Nurr1 and TCF/LEF to the endogenous KCNIP4 promoter. ChIP analyses were performed with SK-N-MC cells, using specific antibodies for the indicated factors following 10 mM LiCl or vehicle treatment for 24 h. Genomic regions were tested for association with the indicated proteins by semiquantitative PCR.

KNCIP4 gene expression is regulated by Wnt-1/LiCl, and its expression is influenced by Nurr1.

To determine if the mechanism underlying these regulations involved direct transcriptional induction, the putative promoter region for KCNIP4 was searched, revealing potential binding sites for Nurr1 and β-catenin/LEF-1 (Fig. 3B). In a promoter-reporter assay with 293F cells, the promoter region of the KCNIP4 gene (positions −1043 to −2) was responsive to both LiCl treatment and Nurr1 overexpression and, as with the native RNA, showed a greater response when both were

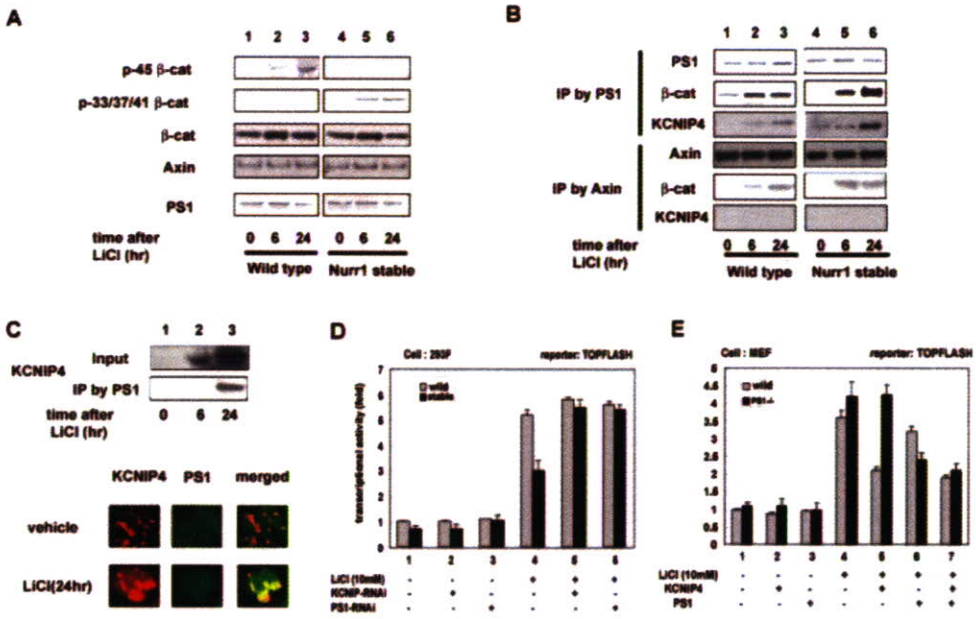


FIG. 4. Modulation of Wnt signaling by KCNIP4 depends on PS1. (A) Nurr1 regulation of the phosphorylation state of β-catenin. Nurr1-expressing or parental 293F cells were treated with 10 mM LiCl and subjected to Western blotting with specific antibodies for the indicated proteins. (B) LiCl-induced assembly of KCNIP4 with PS1 and β-catenin. Coimmunoprecipitation was performed with parental 293F cells or Nurr1 stable transformants treated with 10 mM LiCl. (C) Intracellular colocalization of PS1 with KCNIP4. Native coimmunoprecipitation was performed with SK-N-MC cells before and after LiCl stimulation (upper panels). Immunofluorescence was also performed with antibodies against KCNIP4 and PS1 (lower panels). (D) KCNIP4 and PS1 coregulate transcriptional activity of TCF/LEF in 293F cells. Luciferase assays were performed with 293F parental or Nurr1-expressing cells transfected with TOPFLASH (400 ng) and with the indicated siRNA (100 nM) for 24 h prior to treatment with 10 mM LiCl. (E) Effect of KCNIP4 on TCF/LEF-mediated transcription requires PS1. Luciferase assays were performed with wild-type or $PS1^{-/-}$ mouse embryonic fibroblasts (MEF) transfected with TOPFLASH (400 ng) and with the indicated expression vector (200 ng) in the presence or absence of LiCl (10 mM).

present. Mutational analysis of the KCNIP4 promoter revealed that the putative TCF/LEF and NBRE are required for induction by LiCl and Nurr1, respectively (Fig. 3C). Mutation of the TCF/LEF-like sequence significantly inhibited the response to LiCl, whereas mutation of the potential NBRE inhibited LiCl responsiveness only in the Nurr1 stably transformed cells. Importantly, mutating both sites abolished the response to both LiCl and Nurr1 expression, suggesting that together these sites account for Nurr1 and LiCl sensitivity of the KCNIP4 promoter.

The TCF/LEF and NBRE regions of the KCNIP4 gene promoter were then analyzed by ChIP assays with both 293F and SK-N-MC cells (Fig. 3D). In both cell types, Nurr1 was associated with the genomic regions containing the TCF/LEF and NBRE sequences in the absence of LiCl. β-Catenin was not associated with either sequence, and Lef-1 was associated only with the TCF/LEF-containing region, confirming that the assay distinguished between these elements, which are situated 344 bp apart. As observed with the cyclin D1 promoter, LiCl caused β-catenin to associate with and Nurr1 to dissociate from the TCF/LEF region, while Lef-1 association remained constant. The response of the putative NBRE was similar to that observed with the TH promoter: LiCl caused a recruitment of β-catenin with no change in Nurr1 association. These data, together with the promoter-reporter assay data, suggest that KCNIP4 gene expression is regulated by Nurr1 and β-catenin interaction at or near sequences resembling TCF/LEF and NBRE sites.

**PS1-mediated degradation of β-catenin is regulated by KCNIP4.** KCNIP4 is a protein that has been reported to interact with PS1 (34), a polytopic integral membrane protein that regulates β-catenin degradation in the cytoplasm (24). To determine if KCNIP4 induction by Nurr1/β-catenin influences PS1-mediated β-catenin phosphorylation, we first examined the casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK3β) phosphorylation sites of β-catenin (p45 and p33/37/41, respectively) in the presence and absence of Nurr1. CKIα phosphorylation precedes GSK3β phosphorylation, which in turn triggers β-catenin degradation; PS1 regulates the GSK3β phosphorylation steps specifically (24). When cells were treated with the GSK3β inhibitor LiCl in the absence of Nurr1, β-catenin phosphorylated at the CKIα p45 site accumulated, whereas no accumulation of p33/37/41 was observed (Fig. 4A). However, in the presence of Nurr1 cDNA, p33/37/41 forms of β-catenin accumulated and p45 forms did not. Thus, Nurr1 expression alters the GSK3β-dependent phosphorylation status of β-catenin.

Next, coimmunoprecipitation experiments were used to examine the effects of Nurr1 expression and LiCl stimulation on the assembly of cytoplasmic β-catenin complexes (Fig. 4B). 293F Nurr1 stable transformants or parental control cells were treated with LiCl, and the two major regulators of β-catenin degradation, PS1 and axin, were collected by immunoprecipitation. In parental cells, LiCl caused the expected increase in β-catenin coimmunoprecipitated with PS1 and axin. In contrast, the KCNIP4 protein displayed a time-dependent associ-

ation specifically with PS1 but not with axin. Nurr1 expression increased the relative abundance of β-catenin and KCNIP4 associated with PS1 but had no detectable effect on the axin/β-catenin complex. Further evidence for LiCl-mediated KCNIP4 interaction with PS1 was obtained by examining the intracellular localization of KCNIP4 protein in SK-N-MC cells. LiCl treatment induced KCNIP4 protein expression, as seen by Western blotting and immunofluorescence (Fig. 4C). After 24 h, the KCNIP4 protein was coimmunoprecipitated with PS1 and appeared to be colocalized partially with PS1, as seen in the merged images of the KCNIP4 and PS1 fluorescent signals. Thus, Nurr1/β-catenin induction of KCNIP4 is associated with remodeling of the PS1 signaling complex that regulates β-catenin phosphorylation.

**PS-dependent Nurr1 repression of β-catenin signaling.** The significance of PS1-KCNIP4 interaction was tested in 293F cells by RNAi and in mouse embryonic fibroblasts derived from PS1 knockout mice ($PS1^{-/-}$) (3). As shown above, Nurr1 overexpression modestly reduced TOPFLASH reporter activity in 293F cells (Fig. 4D). siRNA targeting either PS1 or KCNIP4 restored TOPFLASH activity to that seen in parental cells not overexpressing Nurr1, suggesting that KCNIP4 and PS1 are required for Nurr1-mediated TOPFLASH repression. This hypothesis was then tested in $PS1^{-/-}$ fibroblasts transfected with KCNIP4 or PS1 cDNA (Fig. 4E). Transfection with either plasmid had no effect on TOPFLASH activity in unstimulated wild-type or $PS1^{-/-}$ cells. In wild-type fibroblasts, as in 293F cells, KCNIP4 overexpression repressed LiCl-induced TOPFLASH activity, confirming that KCNIP4 inhibits β-catenin signaling. In $PS1^{-/-}$ embryonic fibroblasts, cells showed a slightly greater TOPFLASH response to LiCl than did wild-type cells, as reported previously (24), reflecting the relatively minor but biologically significant (57) contribution of PS1 to inhibiting β-catenin activity. Interestingly, the inhibitory effect of KCNIP4 cDNA was absent in $PS1^{-/-}$ cells, suggesting that KCNIP4 repression of β-catenin signaling requires PS1. Consistent with this hypothesis, PS1 cDNA cotransfection into $PS1^{-/-}$ cells inhibited TOPFLASH activity and restored the inhibitory effect of KCNIP4. These data together indicate that Nurr1/β-catenin induction of KCNIP4 serves to inhibit β-catenin signaling in a PS1-dependent feedback loop.

**Nurr1 regulation of nuclear β-catenin levels.** A prediction of the above model is that Nurr1 regulates the accumulation of β-catenin in the nucleus. To test this possibility, nuclei were prepared from cells treated with LiCl, with or without Nurr1 expression. In SK-N-MC cells (Fig. 5A, top two panels), LiCl caused a transient increase in nuclear β-catenin; cotransfection of siRNA directed towards Nurr1 elevated the amount and duration of nuclear β-catenin. Conversely, transfection of Nurr1 cDNA into 293F parental cells (Fig. 5A, bottom five panels) inhibited nuclear β-catenin accumulation. Interestingly, functional human variants of Nurr1 that possess reduced transcriptional activity (31) also alter the amount of nuclear β-catenin. We then tested the effect of these mutations on the Wnt signaling pathway by transiently transfecting Nurr1 cDNAs into 293F parental cells. As expected, the mutant forms of Nurr1 were less able to activate the NBRE-luciferase reporter than was wild-type Nurr1, but all were responsive to the additional stimulatory effects of LiCl (Fig. 5A). Nurr1 mutants had a generally reduced ability to inhibit LiCl-mediated stimulation of TOPFLASH activity, such that those most impaired in transactivation were also less able to suppress TOPFLASH activity. In contrast, the mutations did not affect KCNIP4 promoter activity in a consistent manner. Thus, human mutations in Nurr1 appear to affect not only transcriptional activation from an NBRE but also Nurr1 regulation of Wnt signaling pathways in a complex manner.

## DISCUSSION

Nurr1 is an orphan NR best known for its essential role in the development and maintenance of the midbrain dopaminergic neurons that regulate motor control and degenerate during Parkinson's disease. Here we tested the possibility that Wnt signaling regulates Nurr1, as Wnt is critical for the regional specification of the midbrain (5) and influences the proliferation and differentiation of $Nurr1^{+}$ neuronal precursors in cell culture (6, 7). We found that β-catenin acts as a transcriptional cofactor for Nurr1, most likely by direct physical association (although other possibilities, including interaction of β-catenin with RXR or other Nurr1-associated factors, are not excluded by the current data). This interaction changes the transcriptional machinery associated with Nurr1 from a corepressor to a coactivator complex. Furthermore, we found that Nurr1 regulates Wnt signaling and propose that the major mechanism of inhibition is by inducing KCNIP4, a Nurr1/β-catenin target that functions as a regulatory subunit of the PS1 complex promoting β-catenin degradation. These findings are summarized in a model that incorporates these data (Fig. 6). It is important that the contribution of this proposed signaling system to dopaminergic cell differentiation and maintenance remains to be explored in vivo. It will be interesting to analyze mice that have a dopaminergic cell-specific deletion of β-catenin, PS1, or KCNIP4.

Several extracellular signals have been reported to modulate the transcriptional function of NRs through exchanging coregulators and cointegrators (14, 40). In this study, we show that Nurr1 can exist in corepressor complexes that are remodeled to CBP-containing coactivator complexes by β-catenin. This finding extends the role that β-catenin plays in NR signaling, as synergistic interactions with the androgen receptor and several other NRs have been described (35).

In vitro β-catenin interacts predominantly with the C-terminal domain of Nurr1 (Fig. 1), which is significant considering that Nurr1, as well as the other members of the NR4A group, rely heavily on the N-terminal AF1 domain for transcriptional regulation, unlike many NRs (33, 38, 56). The Nurr1 C-terminal domain possesses a cell type-specific transactivation function that does not normally interact with common NR coactivators, such as SRC-1 (8), as it lacks the common NR coactivator binding site (12, 55). This C-terminal activity was proposed to be regulated instead by tissue-specific ligands or cofactors (8). Our data suggest that β-catenin is one of these factors and that it acts somewhat similarly to an endogenous ligand in that it activates by interacting with the C-terminal domain. Given the unique function and structure of the Nurr1 C-terminal domain, it would be interesting to determine the structural changes in Nurr1 brought about by β-catenin binding. The precise binding site for β-catenin within the Nurr1 C-terminal domain has not been determined, but the recent

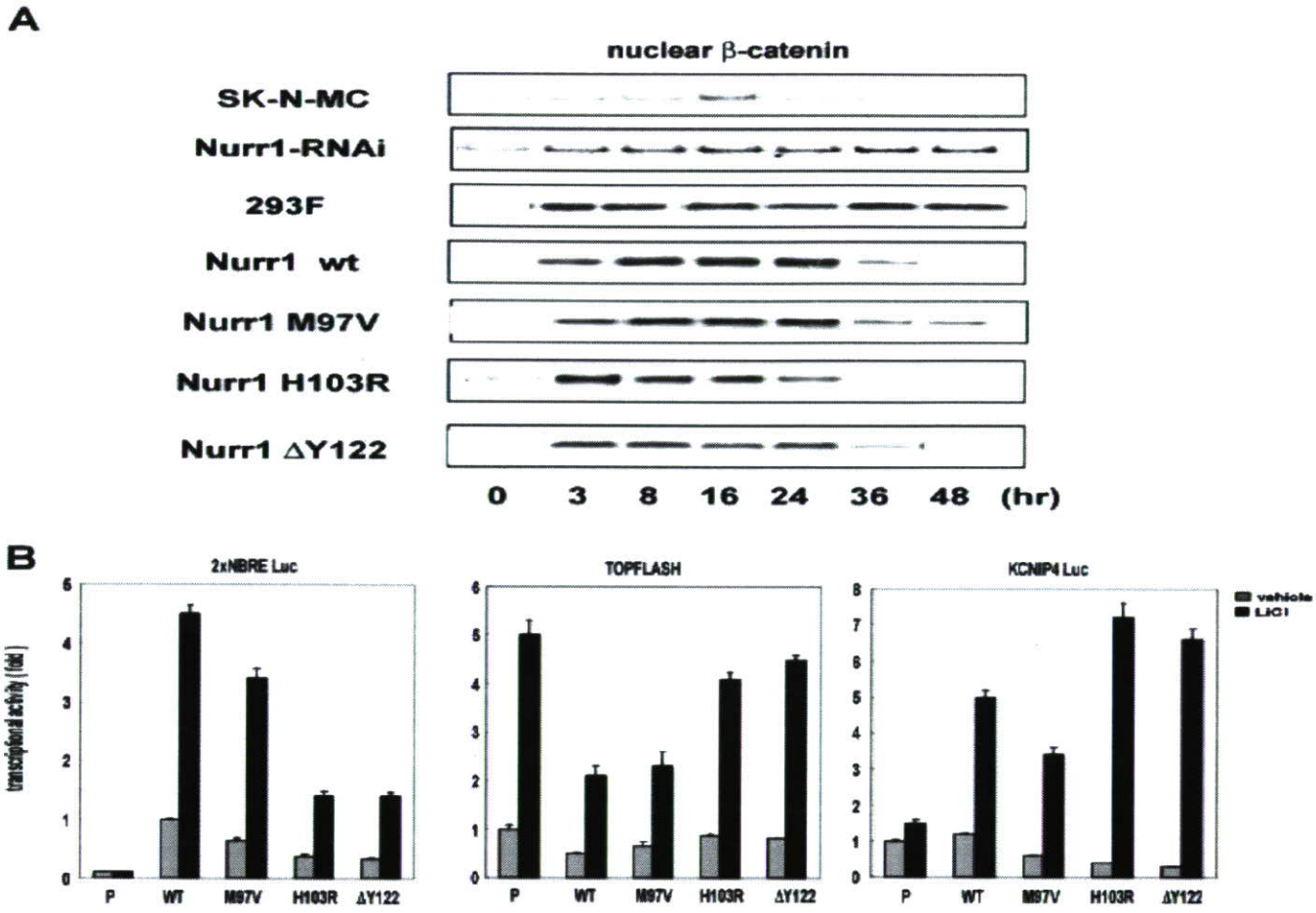


FIG. 5. Nurr1 modulates the degradation of β-catenin. (A) Duration of nuclear accumulation of β-catenin in SK-N-MC cells transfected with control or Nurr1 siRNA and in 293F cells stably expressing wild-type or mutant Nurr1. Nuclear extracts were subjected to Western blotting at each time point after 10 mM LiCl stimulation. (B) Transcriptional properties of each Nurr1 mutant on NBRE, TOPFLASH, and KCNIP4 promoter. Luciferase assays were performed with 293F cells transfected with the indicated reporters (400 ng) and stably expressing wild-type or mutated Nurr1 in the presence or absence of LiCl (10 mM).

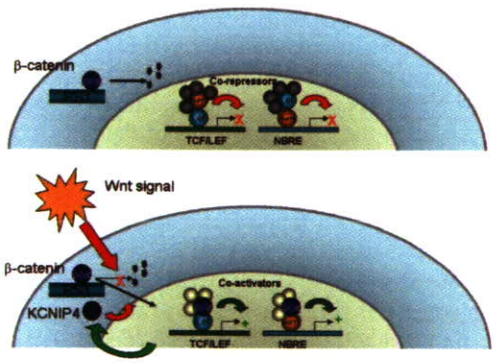


FIG. 6. Model for Nurr1 and Wnt cross-regulation. In the absence of Wnt signaling (top), Nurr1 is associated with corepressors in genes containing TCF/LEF binding sites, and Lef-1 is associated with Nurr1 on Nurr-responsive elements. Following Wnt-mediated β-catenin accumulation in the nucleus (bottom), TCF/LEF genes become derepressed and β-catenin switches Nurr1-associated proteins from corepressors to coactivators. Both β-catenin and Nurr1 induce KCNIP4, which associates with a membrane-associated PS1 complex and promotes β-catenin degradation in a negative feedback loop.

discovery of a large, functionally important hydrophobic pocket opposite the place where the classic coactivator binding site would normally be (53) raises this location as a possibility.

Using microarrays to identify an endogenous gene controlled by this convergent signaling pathway, we found the KCNIP4 gene. The KCNIP4 promoter contains apparent Lef-1 and Nurr1 response elements, and interestingly, the KCNIP4 protein interacts with the cytoplasmic domain of PS1, which acts as a scaffold for β-catenin signaling and degradation (24, 25, 39, 51, 57). We found that KCNIP4 promotes PS-mediated degradation of β-catenin and thus appears to be a regulatory subunit for the PS complex. Transcriptional induction of KCNIP4 appears to be a primary mechanism by which Nurr1 inhibits β-catenin activity, since the absence of KCNIP4 or PS1 abrogated the ability of Nurr1 to inhibit TOPFLASH. Although, as shown in Fig. 2, Nurr1 expression increases and Nurr1 siRNA decreases the levels of corepressors associated with the TCF/LEF region of the cyclin D1 promoter, these effects could also be indirect through the modulation of β-catenin cytoplasmic stability via KCNIP4. Further experiments will be required to determine if Nurr1 plays a significant role in