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Endosomal Adaptor Proteins APPL1 and APPL2 Are Novel Activators of β-Catenin/TCF-mediated Transcription*

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Canonical Wnt signaling regulates many aspects of cellular physiology and tissue homeostasis during development and in adult organisms. In molecular terms, stimulation by Wnt ligands leads to the stabilization of β-catenin, its translocation to the nucleus, and stimulation of TCF (T-cell factor)-dependent transcription of target genes. This process is controlled at various stages by a number of regulatory proteins, including transcriptional activators and repressors. Here we demonstrate that the endosomal proteins APPL1 and APPL2 are novel activators of β-catenin/TCF-mediated transcription. APPL proteins are multifunctional adaptors and effectors of the small GTPase Rab5, which localize to a subpopulation of early endosomes but are also capable of nucleocytoplasmic shuttling. Overexpression of APPL1 or APPL2 protein stimulates the activity of β-catenin/TCF-dependent reporter construct, whereas silencing of APPL1 reduces it. Both APPL proteins interact directly with Reptin, a transcriptional repressor binding to β-catenin and HDAC1 (histone deacetylase 1), and this interaction was mapped to the pleckstrin homology domain of APPL1. Moreover, APPL proteins are present in an endogenous complex containing Reptin, β-catenin, HDAC1, and HDAC2. Overexpression of either APPL protein relieves Reptin-dependent transcriptional repression and correlates with the reduced amounts of HDACs and β-catenin associated with Reptin as well as with the lower levels of Reptin and HDAC1 on the promoters of β-catenin target genes. We propose that APPL proteins exert their stimulatory effects on β-catenin/TCF-dependent transcription by decreasing the activity of a Reptin-containing repressive complex.

Wnt/β-catenin signaling is implicated in a variety of cellular processes, including proliferation, differentiation, survival, and apoptosis (1–3). The principle regulatory mechanism that controls nuclear accumulation of β-catenin is its escape from the proteasomal degradation in the cytoplasm (4). In unstimulated cells, β-catenin is phosphorylated, ubiquitinated, and targeted for degradation in a process mediated by a destruction complex containing GSK3β (glycogen synthase kinase 3β), casein kinase 1ϵ, Axin, and the tumor suppressor APC (adenomatous polyposis coli) protein (5–8). Upon binding of Wnt ligands to their cognate receptors Frizzled and LRP5/6 (9, 10), the destruction complex is inactivated, which leads to the accumulation of cytosolic β-catenin and its relocation to the nucleus. The nuclear bipartite complex between β-catenin and TCF/Lef (T-cell factor/lymphoid enhancer factor) proteins, in which TCF/Lef proteins provide a DNA-binding domain and β-catenin presents a transactivation domain, plays a key role as a transcription factor for the target genes of the wingless/Wnt pathway (11, 12).

The function of β-catenin in transcriptional regulation is modulated by various protein complexes and reflects the fact that β-catenin itself interacts with several proteins (2, 3). Among them, Pontin (also known as RuvBL1, Rvb1, TIP49a, and TIP49) and Reptin (also known as RuvBL2, TIP49b, and TIP48) constitute a pair of antagonistic regulators, with Pontin acting as activator and Reptin as repressor of β-catenin-mediated transcription (13–15). Pontin and Reptin are highly conserved proteins related to the helicase subset of the AAA+ family of ATPases (16) with a broad range of functions in DNA replication, DNA repair, transcription, and chromatin remodeling, thus controlling cell growth, proliferation, and carcinogenesis (17, 18). Pontin and Reptin are constituents of several chromatin-remodeling or transcriptional complexes and can form hexamers or double hexamers (19), although they also function independently and even antagonistically with respect to each other. For example, when bound to the promoter of the metastasis suppressor gene KAI1, Reptin in complex with β-catenin acts as a repressor due to the concomitant recruitment of histone deacetylase 1 (HDAC1) via its direct interactions with Reptin (20). In contrast, Pontin associated with the Tip60 complex on the KAI1 promoter acts as a transcriptional activator. Moreover, Reptin was shown to act in a complex with the corepressor TLE1 (Groucho), HDAC1, HDAC2, and β-catenin to silence the expression of β-catenin target genes Hesx1 and Pit1 (21), thus further underscoring the role of Reptin in mediating β-catenin-dependent transcriptional repression via interactions with HDACs.[3]

Recently, an increasing number of proteins with their primary roles in endocytosis have been reported to undergo nucleocytoplasmic shuttling and participate in transcriptional regulation or chromatin remodeling (22). Among them are the two homologous

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[3] The abbreviations used are: HDAC, histone deacetylase; GST, glutathione *S*-transferase; siRNA, small interfering RNA; esiRNA, endoribonuclease-prepared siRNA; BAR, Bin1/amphiphysin/Rvs167; PH, pleckstrin homology; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NTA, nitrilotriacetic acid; aa, amino acid(s); GFP, green fluorescent protein; EGFP, enhanced GFP; 2D BN/SDS-PAGE, two-dimensional Blue Native/SDS-PAGE; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; HA, hemagglutinin.

adaptor proteins, APPL1 and APPL2 (adaptor proteins containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif), which are effectors of the small GTPase Rab5, a key regulator of early steps of endocytosis (23). We have previously shown that APPL proteins localize predominantly in the cytoplasm on the surface of a distinct subpopulation of early endosomes and to a lower degree in the cell nucleus (23). They interact with a number of transmembrane receptors (TrkA, DCC, and receptors for follicle-stimulating hormone and adiponectin) (24–27), signaling molecules (Akt, GIPC, phosphatidylinositol 3-kinase, and OCRL) (24, 27–30), and nuclear proteins (NuRD (nucleosome remodeling and histone deacetylase complex)) (23). Consequently, APPL proteins not only participate in endosomal transport but appear also to be located at the crossroads of various signaling pathways regulating cell metabolism, proliferation, survival, or apoptosis.

Here we report that APPL1 and APPL2 proteins are novel positive regulators of β-catenin/TCF-dependent transcription. They interact directly with Reptin via their PH domains and relieve Reptin-mediated repression of β-catenin/TCF target genes by modulating the interactions within the β-catenin-Reptin-HDAC complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HeLa cells were grown in modified Eagle's medium supplemented as above. The BCA protein concentration measuring kit was from Pierce. Glutathione-Sepharose beads and pGEX-6P vectors were from GE Healthcare. Wnt3a-expressing mouse L cells and parental L cells were obtained from ATCC (CRL-2647 and CRL-2648, respectively), and conditioned media from these cell lines were obtained as recommended by the supplier. Oligonucleotides were custom-synthesized by the Institute of Biochemistry and Biophysics (Warsaw, Poland). The following antibodies were used: anti-Reptin (catalog number ab36569; Abcam), anti-HDAC1 (for immunoblotting (catalog number ab19845) and for chromatin immunoprecipitation (catalog number ab46985); Abcam), anti-Myc (catalog number 05-419; 9E10; Upstate Biotechnology, Inc.), anti-β-catenin (catalog number 610154; BD Bioscience), anti-HA (catalog number sc-805; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GAPDH (catalog number sc-25778; Santa Cruz Biotechnology), anti-His (catalog number 34660; Qiagen), anti-GST (catalog number 27-4577; GE Healthcare), and anti-HDAC2 (catalog number 05-814; Upstate Biotechnology). Polyclonal antibodies against recombinant APPL1 and APPL2 proteins were generated by immunization of rabbits according to standard procedures (23). Alexa-conjugated secondary antibodies used for immunofluorescence were from Invitrogen.

Plasmids—Full-length human Reptin cDNA was amplified by PCR using the specific oligonucleotides 5′-AGCGGATCCCCATCATGGCAACCGTT-3′ (forward primer) and 5′-AACGCGGCCGCTTCAGGAGGTGTCCATGG-3′ (reverse primer) and the expressed sequence tag clone (accession number BC004531, obtained from the German Resource Center for Genome Research) as a template. The amplified ~1.4-kb PCR product was cloned into pcDNA3.1 (Invitrogen), pGEX-6P (GE Healthcare), and pET28 (Novagen) plasmids. APPL1, APPL2, and Rab5-Q79L encoding plasmids were previously described (23, 31). Human Pontin was amplified from the expressed sequence tag clone (accession number BC002993, obtained from the German Resource Center for Genome Research) by PCR using the oligonucleotides 5′-GTTACTCGAGCCGCGTCTGCAAAATGAAGATT-3′ (forward primer) and 5′-GGGGCGGCCGCTCGGAGTCTCTTACTGCTGAAA-3′ (reverse primer), followed by subsequent cloning into the BamHI and NotI restriction sites of the pcDNA3.1 vector (Invitrogen). All constructs were verified by sequencing.

GST Pull-down and in Vitro Binding Assays—The full-length or truncated GST-APPL1 and GST-APPL2 fusion proteins were expressed and purified according to the manufacturer's instructions (GE Healthcare). Isopropyl-1-thio-β-D-galactopyranoside (Sigma) at a concentration of 0.5 mM was used to induce the expression. The purified GST-APPL proteins bound to the glutathione-Sepharose beads were incubated overnight at 4 °C with lysates of HEK293 cells transfected with HA-Reptin. After washing with 150 mM NaCl, 10 mM Tris, pH 8.0, 5 mM EDTA, 5 mM dithiothreitol, and 0.1% Triton X-100, the beads were collected and tested for the bound proteins by immunoblotting with anti-HA-specific antibodies.

In vitro translation reaction was carried out in a TNT coupled reticulocyte lysate using the Transcend non-radioactive translation detection system (Promega) according to the manufacturer's recommendations. For the *in vitro* binding reaction, 20 μl of purified bead-bound GST or GST-APPL1 fragments were incubated for 4 h at 4 °C in binding buffer 1 (20 mM Tris pH 8.0, 150 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 0.2% Nonidet P-40, 10% glycerol) with 3.5 μl of *in vitro* translated Reptin. The samples were then washed three times in binding buffer 1. The bound proteins were liberated by boiling in Laemmli sample buffer and were analyzed by SDS-PAGE and immunoblotting with antibodies against Reptin.

Ni^{2+}-nitrilotriacetic acid (NTA) pull-down was performed as described (32). Briefly, 2–5 μg of purified His_6-Reptin protein were mixed with equal quantities of GST-APPL1 (aa 1–428) and GST-APPL2 (aa 1–377) proteins (cleaved by PreScission protease) in 300 μl of binding buffer 2 (20 mM HEPES, pH 7.5, 20 mM imidazole, 120 mM potassium acetate, and 0.1% Triton X-100) and incubated for 1 h at 4 °C. Beads were washed three times, and bound proteins were eluted by Laemmli sample buffer. Products were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific antibodies.

Production and Purification of Short Double-stranded RNA Duplexes—Optimal endoribonuclease-prepared siRNA (esiRNA) target regions with a length of 300–500 bp were selected using the DEQOR Web server. In brief, T7 promoter sequence was added to the selected regions of Reptin or APPL1 cDNAs by two PCRs. The first PCR reaction was carried out by using gene-specific primer pairs that were tagged at 5′ ends with a part of the T7 promoter (underlined). During the second PCR, primers specific to T7 promoter were used to amplify the whole T7 sequence. The sequences for these primers are as follows: for APPL1–1, 5′-TCACTATAGGGAGAGGCCATG-

ATGTTCCCCATTAC-3′ (forward primer) and 5′-TCACTATAGGGAGACGGGATCACTGGCTACTTCCA-3′ (reverse primer); for APPL1–2, 5′-TCACTATAGGGAGAGGATTCTCTTGTTGCCCCAGA-3′ (forward primer) and 5′-TCACTATAGGGAGACTCCCCCTCATTGTTTGACTC-3′ (reverse primer); for Reptin, 5′-TCACTATAGGGAGAGCCTGACACGCCATTCACA-3′ (forward primer) and 5′-TCACTATAGGGAGACCCCAGCTTGGAGATCTTGC-3′ (reverse primer); and for T7 promoter, 5′-GCTAATACGACTCACTATAGGGAGAG-3′ (forward primer) and 5′-GCTAATACGACTCACTATAGGGAGAC-3′ (reverse primer). For control EGFP esiRNA, the PCRs were performed in one step using the following primers (T7 sequence underlined): 5′-GCTAATACGACTCACTATAGGGAGAGGTGAGCAAGGGCGAGGA-3′ (forward primer) and 5′-GCTAATACGACTCACTATAGGGAGACTACAGCTCGTCCATGCCGA-3′ (reverse primer). Further esiRNA synthesis was carried out as described previously (33). The concentration of esiRNA was determined by measuring $A_{260\ nm}$.

Transient Transfection and Luciferase Assays—SuperTOPflash and SuperFOPflash luciferase reporters and plasmids encoding *Renilla* (pRL-SV40, pRL-TK) and β-catenin (pCIneo-β-catenin) were kind gifts from Dr. Vladimir Korinek (Institute of Molecular Genetics, Prague). Typically, HEK293 cells were transfected at 50–60% confluence with the appropriate plasmids using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). In silencing experiments, esiRNA against Reptin, APPL1, or EGFP (at a final concentration of 33 nM) were cotransfected with reporter plasmids by using Lipofectamine 2000 (Invitrogen) or SureFECT (SABiosciences). Transfected cells were collected and lysed with passive lysis buffer (Promega) after 48–72 h. The resulting cell lysates were assayed using the dual luciferase assay kit according to the manufacturer's instructions (Promega). The firefly luciferase activity derived from the TCF-responsive reporter SuperTOPflash was normalized to its respective *Renilla* luciferase activity driven by the SV40 or TK promoters as a control for the transfection efficiency. For Wnt3a experiments, 30 h post-transfection, cells were treated with control conditioned or Wnt3a-conditioned medium and incubated for a further 18 h. Reporter gene activity was measured 48 h after the transfection.

Immunofluorescence—HeLa cells were plated on 12-mm coverslips in 24-well plates in 500 μl of modified Eagle's medium with 10% of serum. At about 80% confluence, cells were transfected with Myc-Reptin, pEGFP-C3-Rab5-Q79L, and pCMV-Rab5-Q79L plasmids using FuGENE 6 (Roche Applied Science). 24 h post-transfection, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and processed for immunofluorescence with mouse monoclonal anti-Myc and rabbit anti-APPL1 antibodies, followed by appropriate secondary antibodies. Afterward, cells were examined with a laser-scanning confocal microscope (Leica TCS SP2 AOBS) using a ×63/1.4 numerical aperture oil immersion objective. Three confocal *z*-sections (8-bit, 1024 × 1024-pixel resolution) per image were taken and processed with Leica software as maximal projections. The presented figure was assembled using Adobe Photoshop CS2.

Immunoprecipitation—HEK293 cells were homogenized in immunoprecipitation lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, and 1% Nonidet P-40. Homogenates were incubated on ice for 15 min, and insoluble fractions were removed by centrifugation at 10,000 × *g* for 10 min. Immunoprecipitation assays were performed using goat anti-mouse IgG or sheep anti-rabbit IgG Dynabeads (Invitrogen) according to the manufacturer's instructions. Collected beads were boiled in 50 μl of Laemmli sample buffer for 10 min, and the precipitated proteins were analyzed by immunoblotting. Protein identification by mass spectrometry in APPL1 immunoprecipitates from HeLa cells was performed as described previously (23).

Assay for HDAC Activity—The assay was performed using the Fluor de Lys kit (Fluorogenic Histone Deacetylase Lysyl Substrate/Developer) according to the manufacturer's instructions (Biomol). Briefly, the Dynabeads containing Reptin immunocomplex were incubated with 150 μl of 100 μmol/liter acetylated substrates for a maximum of 30 min with gentle rocking at room temperature. As negative controls, the same amounts of rabbit IgG cross-linked Dynabeads undergoing immunoprecipitation assays were incubated with the substrates. Subsequently, equal aliquots from each condition were mixed with developer solution (100 μM) and incubated for 30 min at room temperature. After excitation at 360 nm, emitted light was detected at 460 nm in a fluorometric reader (BMG Labtech). The fluorescence intensity (measured in relative fluorescence units) derived from the assay buffer was subtracted from each experimental sample.

Two-dimensional Blue Native/SDS-PAGE (2D BN/SDS-PAGE) Analysis—Blue Native/PAGE was performed according to a modification of the protocol by Schägger (34). All buffers were adjusted to pH 7.0 and filtered through 0.2-μm filters. A 6–13% gradient gel with a 3.5% stacking gel was poured in the glass plates using 1-mm spacers. The cathode buffer (15 mM BisTris/HCl, 50 mM Tricine) containing 0.02% (w/v) Coomassie Brilliant Blue G250 and the anode buffer (50 mM BisTris/HCl) were chilled to 4 °C before loading. Electrophoresis was performed at 10 mA at 4 °C for 1.5 h. Later, cathode buffer was replaced by the same buffer containing 0.005% Coomassie Brilliant Blue G250, and the electrophoresis was continued overnight at 8 mA. Since the dye front had run off of the gel, the lanes were cut out and incubated with 2% (w/v) SDS and 1% β-mercaptoethanol for 10 min at room temperature. For performing the second dimension electrophoresis, an 8% glycine-SDS gel with a 4% stacking gel was poured in glass plates (1.5-mm spacers). The excised lanes were then inserted between the glass plate assemblies and sealed with agarose (0.7% (w/v) agarose). The solution containing 1% SDS and 150 mM β-mercaptoethanol was added on top of the sealed strips and allowed to diffuse into the gel for 10 min. Gel plates were then placed in the electrophoretic tank and overlaid with 500 μl of 2× Laemmli buffer with 5% β-mercaptoethanol. Electrophoresis was performed at 20 mA for 1 h and then at 50 mA for 6 h.

RNA Extraction and Reverse Transcription—Total RNA was isolated from HEK293 cells using RNeasy plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA integrity was checked by running on a paraformaldehyde gel, and subsequently RNA concentrations were quantified by measuring $A_{260\ nm}$. First strand cDNA was prepared by SuperScript-III Reverse Tran-

scriptase system (Invitrogen) according to the manufacturer's protocol by using the oligo(dT)$_{20}$ primer. The yield of cDNA was measured according to the PCR signal generated from the β-actin amplified from 30 cycles. Semiquantitative reverse transcription-PCR was performed to measure the β-catenin/TCF target gene (c-*jun*, c-*myc*, and cyclin D1) expression. Primer sequences used are as follows: for GAPDH, 5′-TGGGCTACACTGAGCACCAG-3′ (forward primer) and 5′-CAGCGTCAAAGGTGGAGGAG-3′ (reverse primer); for β-actin, 5′-CAGGTCATCACCATTGGCAAT-3′ (forward primer) and 5′-TCTTTGCGGATGTCCACGT-3′ (reverse primer); for c-*jun*, 5′-GCATGAGGAACCGCATCGCTGCCTCCAAGT-3′ (forward primer) and 5′-GCGACCAAGTCCTTCCCACTCGTGCACAC-T-3′ (reverse primer); for c-*myc*, 5′-CGTCTCCACACACATCAGAGCAA-3′ (forward primer) and 5′-TCTTGGCAGCAGGATAGTCCTT-3′ (reverse primer), and for cyclin D1, 5′-CCGTCCATGGGGAAGATC-3′ (forward primer) and 5′-ATGGCCAGCGGGAAGAC-3′ (reverse primer). PCRs were performed on a Mastercycler PCR machine (Eppendorf) using ~1000 ng of cDNA, 5 pmol of each primer, a 200 μM concentration of each dNTP, and 1 unit of Taq polymerase (Fermentas) in a total volume of 50 μl. The PCR program started with a 95 °C denaturation for 5 min, followed by 32 cycles of 95 °C/30 s, 55 °C/30 s, and 72 °C/40 s, and ended with 10 min of elongation at 72 °C. The PCR samples were electrophoresed on 2% agarose gel in TBE buffer (89 mM Tris-base, pH 7.6, 89 mM boric acid, 2 mM EDTA). The gel was stained with ethidium bromide and photographed on top of UV light box.

Chromatin Immunoprecipitation Assay—HEK293 cells (10-cm confluent plate) treated with Wnt3a-conditioned medium were cross-linked by 1% paraformaldehyde for 10 min at 37 °C. After removal of medium and washing with phosphate-buffered saline, extraction of chromatin was performed in lysis buffer I (5 mM Hepes, pH 7.5, 85 mM KCl, 0.5% Nonidet P-40, protease inhibitors), followed by incubation on ice for 20 min. After centrifugation at 1200 rpm for 5 min, the pellet was resuspended in 3 ml of lysis buffer II (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS) and incubated on ice for 10 min. Fragmentation of chromatin was performed on ice by 10 rounds of sonication (10 s each) in a Sonics Vibra Cell sonicator with power setting at 50–60%. 20 μl of the sample was digested by proteinase K for 2–3 h and loaded on agarose gel for checking the chromatin fragment size, which was ~500 bp. Immunoprecipitation assays were performed using Dynabeads (Invitrogen) according to the manufacturer's instructions. To elute precipitated chromatin from the beads, 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) was added to the beads, and the mixture was incubated at room temperature for 15 min. Reversal of cross-link was made by adding 20 μl of 5 M NaCl, 10 μl of 500 mM EDTA, 20 μl of 1 M Tris, pH 6.5, and incubating at 65 °C overnight. Proteins in the DNA sample were removed by incubation with 2 μl of proteinase K solution (20 mg/ml) for 2 h at 37 °C. The sample was then extracted by PCR filter columns (A&A Biotech), and the DNA was dissolved in 30 μl of TE buffer containing 10 μg of DNase-free RNase A (Sigma), followed by incubation for 2 h at 37 °C. PCR was performed from these immunoprecipitates, from chromatin processed in the absence of antibody or in the presence of unspecific IgG, as well as from input chromatin using the primer pair covering the β-catenin binding site of Wnt target gene promoters (cyclin D1 and Axin2). Primer sequences used are as follows: for cyclin D1, 5′-GCTTTCCATTCAGAGGTGTG-3′ (forward primer) and 5′-CCGAAAATTCCAGCAGCAGC-3′ (reverse primer) and for Axin2, 5′-CTGGAGCCGGCTGCGCTTTGATAA-3′ (forward primer) and 5′-CGGCCCCGAAATCCATCGCTCTGA-3′ (reverse primer). PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide.

RESULTS

Reptin Is Detected in Complex with APPL1 and APPL2—In search of proteins interacting with APPL proteins, we performed immunoprecipitation assays from lysates of HeLa cells followed by mass spectrometry analyses. Peptides corresponding to Reptin were detected in the APPL1 immunoprecipitates, along with components of the NuRD corepressor complex (23). In order to confirm the association between Reptin and APPL proteins, we performed coimmunoprecipitation experiments using lysates of HEK293 cells transiently expressing HA-Reptin together with APPL1-Myc. Reptin was readily detectable in the anti-Myc immunoprecipitate using anti-HA antibodies (Fig. 1*A*). Moreover, in the lysates of HEK293 cells transiently transfected with HA-Reptin and APPL2-Myc, anti-HA antibodies coimmunoprecipitated the overexpressed APPL2 (Fig. 1*B*). Furthermore, we detected endogenous APPL1 in immunoprecipitates obtained by a specific anti-Reptin antibody from lysates of untransfected HEK293 cells, arguing that the interaction of Reptin and APPL1 occurs also between endogenously expressed proteins (Fig. 1*C*). We could not detect the binding of endogenous APPL2 with Reptin due to the very low abundance of APPL2 protein in HEK293 cells.

In order to further confirm the specificity of interactions between Reptin and APPL1/2 proteins by an independent method, *in vitro* GST pull-down experiments were performed (Fig. 1*D*). Purified GST and full-length GST-APPL1 and GST-APPL2 fusion proteins were immobilized by binding to glutathione-Sepharose beads and incubated with equal amounts of extracts from HEK293 cells transfected with HA-Reptin. Bound proteins were analyzed by Western blotting with anti-HA antibodies. HA-Reptin was specifically retained by GST-APPL1 and GST-APPL2 but not by GST alone. Together, the results of immunoprecipitation and pull-down experiments demonstrate that Reptin is found in complex with both APPL1 and APPL2 proteins.

Given that Pontin and Reptin can act together in several processes as a double hexamer complex (19), we next sought to assess the binding of Pontin and APPL1. Accordingly, coimmunoprecipitation experiments were carried out by using lysates from transiently transfected HEK293 cells expressing Myc-Pontin and HA-APPL1. Although Reptin was readily found in the anti-Myc immunoprecipitates of Pontin, no APPL1 protein was detected (Fig. 1*E*), indicating that APPL1 does not bind to Pontin. This further suggested that APPL proteins might regulate a function of Reptin independent of its association with Pontin.

Mapping the Reptin Interaction Domain on APPL1—To define the region of APPL1 protein responsible for the interaction with Reptin, GST pull-down assays were performed using a series of GST-APPL1 deletion constructs encompassing the various functional domains (23, 35) (Fig. 2*A*). The well established interaction between Reptin and Pontin was used as a positive control (Fig. 2*B*,

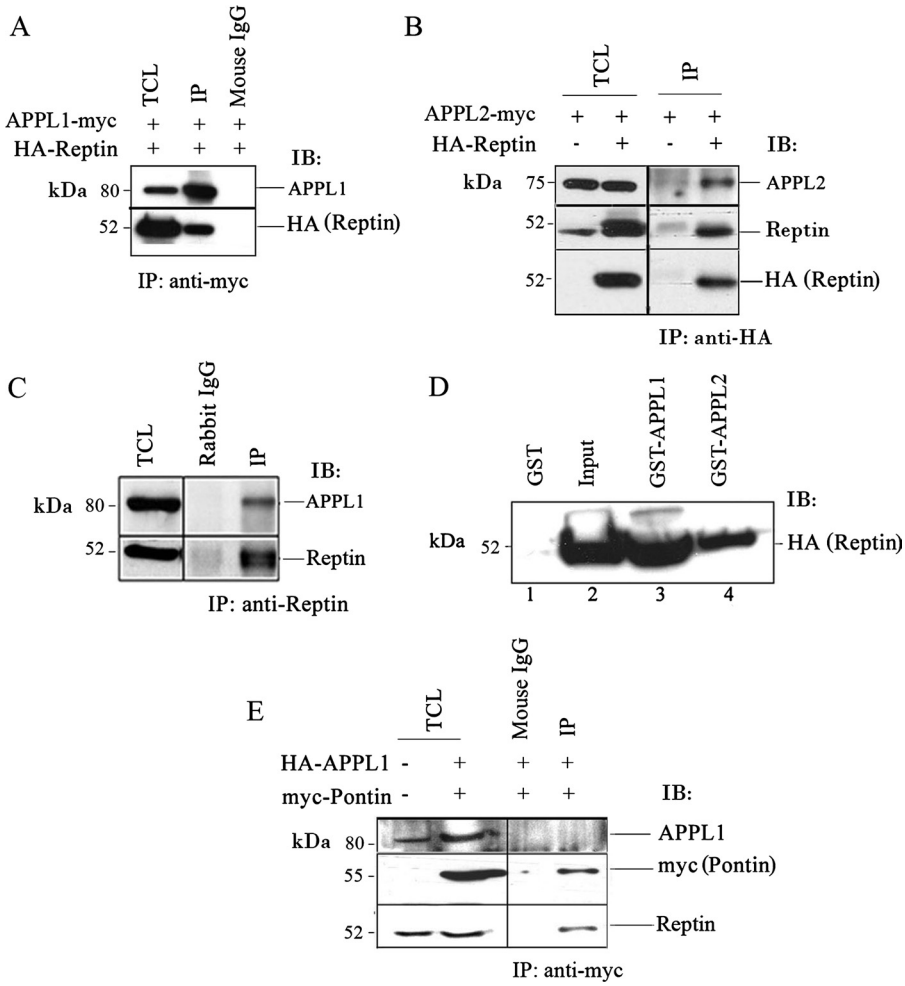


FIGURE 1. **Both APPL1 and APPL2 proteins interact with Reptin.** *A*, HEK293 cells were transiently transfected with HA-tagged Reptin and APPL1-Myc. Forty-eight hours post-transfection, cell extracts were prepared, and coimmunoprecipitation was performed with mouse monoclonal anti-Myc antibody. The association of Reptin was analyzed by Western blotting with anti-HA antibodies. No nonspecific binding was detected with control rabbit IgG. *B*, lysates from HA-Reptin- and APPL2-Myc-transfected HEK293 cells were subjected to coimmunoprecipitation with anti-HA antibodies, and Western blot detection was performed with polyclonal anti-APPL2 antibodies. In the cell lysates without HA-Reptin transfection, no nonspecific binding of APPL2 was detected. *C*, interaction between endogenous Reptin and APPL1 proteins was detected by coimmunoprecipitation from HEK293 cell lysates using rabbit polyclonal anti-Reptin antibodies and probing with anti-APPL1 antibodies. *D*, GST (*lane 1*), full-length GST-APPL1 (*lane 3*), and GST-APPL2 (*lane 4*) fusion proteins were incubated with lysates of HEK293 cells expressing HA-Reptin. After washing the resins, bound proteins were eluted by Laemmli buffer and analyzed by Western blotting with anti-HA antibodies. Input (10% of total) of the lysate is shown in *lane 2*. *E*, HEK293 cells were transiently cotransfected with Myc-Pontin and HA-APPL1. After lysis, coimmunoprecipitation was performed with anti-Myc antibody and the association of APPL1 or endogenous Reptin was analyzed by probing with the corresponding antibodies. *IP*, immunoprecipitation; *IB*, immunoblotting; *TCL*, total cell lysate.

lane 3). Immunoblotting of the bound material revealed that Reptin was retained only by the N terminus of APPL1 (1–428 aa containing BAR and PH domains; Fig. 2*B*, *lane 4*). However, it did not bind to the C terminus comprising the PTB domain (429–709 aa; *lane 5*). By further mapping, the minimal Reptin-binding region was identified to lie between aa 273 and 428, encompassing the PH domain (Fig. 2*B lane 6*), indicating that this region of APPL1 is sufficient to bind with Reptin.

Reptin Interacts Directly with Both APPL Proteins—The results of immunoprecipitation and pull-down experiments established that Reptin and APPL proteins are found in a complex; however, these interactions could occur either directly or via other proteins present in the cell lysates. In order to distinguish between these possibilities, we performed two types of experiments. First, we produced Reptin by *in vitro* translation and incubated it with the 273–428 aa fragment of APPL1 immobilized on glutathione-Sepharose beads. After extensive washing, the material retained on beads was analyzed by Western blot with Reptin-specific antibodies. Reptin was detected bound to GST-APPL1-(273–428) beads, whereas no background binding of Reptin was visible in the case of GST beads (Fig. 3*A*), indicating that Reptin binds directly to APPL1 via a region encompassing the PH domain. Second, we performed *in vitro* binding assays of bacterially expressed, recombinant Reptin and APPL proteins as the most stringent tests for direct protein-protein interactions. To this end, we produced His_6-tagged Reptin immobilized on Ni^{2+} NTA beads. Reptin-containing or empty Ni^{2+} NTA beads were subsequently incubated with the recombinant N termini of APPL proteins (1–428 aa for APPL1 and 1–377 aa for APPL2, both fragments comprising BAR and PH domains), which were purified as GST fusion proteins, followed by the cleavage of GST (Fig. 3*B*). Both N-terminal fragments of APPL1 and APPL2 were specifically retained on Reptin-containing Ni^{2+} NTA beads, whereas no unspecific binding to empty beads was observed. Taken together, these data confirm the direct physical interactions of Reptin and APPL1 as well as Reptin and APPL2.

Reptin Is Not Detected on APPL-positive Early Endosomes—Having established that Reptin and APPL proteins bind directly, we next sought to determine a possible intracellular location of these interactions. APPL proteins are known to colocalize with each other on a distinct subpopulation of early endosomes, bearing the small GTPase Rab5 but largely devoid of other markers of canonical early endosomes, such as EEA1 (23). In addition to the membrane-bound endosomal pool, a fraction of APPL proteins is also found soluble in the cytosol or in the nucleus (23). Similarly, both nuclear and cytoplasmic localization have been reported for Reptin (17, 36). Since available commercial antibodies do not detect endogenous Reptin in immunofluorescence applications, we therefore analyzed the distribution of Myc-tagged overexpressed Reptin with respect to the localization of APPL1 in HeLa cells (similarly to HEK293 cells, APPL2 is expressed at low levels in HeLa cells used in this study).

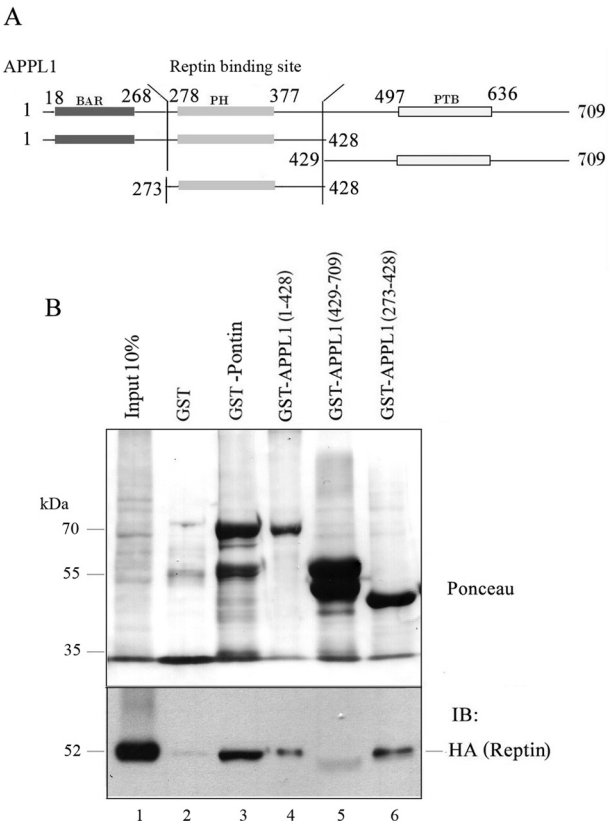


FIGURE 2. **Mapping of Reptin-binding region in APPL1 protein.** *A*, schematic representation of APPL1 protein with its modular organization and the deletion fragments used in the pull-down assays. *PTB*, phosphotyrosine binding. *B*, GST pull-down assays performed by incubation of APPL1 fragments and HEK293 cell lysates overexpressing HA-tagged Reptin. *Top*, the amounts of GST-APPL1 fragments and GST-Pontin shown by Ponceau staining. *Bottom*, bound Reptin was visualized by immunoblotting (*IB*) with anti-HA antibodies. Input (10% of total) of the HEK293 cell lysate is shown in *lane 1*.

Immunostaining of cells revealed that overexpressed Myc-Reptin is mainly detected both in the cell nuclei and cytoplasm (Fig. 4*A*). However, we could not detect any enrichment of Reptin on endosomes marked by APPL1 (Fig. 4, *A–C*). In order to visualize better the endosomal compartments, cells were cotransfected with Myc-Reptin and the Rab5-Q79L constitutively active mutant, which causes increased fusion and thus enlarged endosomes (31). No recruitment of Myc-Reptin was observed on endosomes that were mildly enlarged by expressing low levels of GFP-Rab5-Q79L (Fig. 4, *D–F*). In cells overexpressing high amounts of Rab5-Q79L and thus exhibiting giant endosomes (Fig. 4, *G–I*), Myc-Reptin was occasionally detected on the confined domains of endosomal membranes, which were also highly enriched in APPL1. These data suggest that a small pool of Reptin might be localized to endosomes, although normally the colocalization with APPL1 in this compartment is undetectable. Overall, immunofluorescence analyses demonstrated that, in agreement with the literature, both proteins are present in cytoplasmic and nuclear pools, thus permitting their interaction *in vivo*.

APPL1 Is a Part of a β-Catenin-Reptin-HDAC Ternary Complex—In order to elucidate the functional importance of the interactions between APPL proteins and Reptin, we focused on β-catenin-dependent transcription as one of the processes in which Reptin does not act in complex with Pontin (14). It was previously reported that Reptin-mediated repression activity at the KAI1 promoter requires β-catenin for binding the promoter and HDAC1 providing deacetylase activity (20). The ability of Reptin to bind both β-catenin and HDAC1 further suggested the formation of a β-catenin-Reptin-HDAC1 complex at the promoter. In order to analyze whether APPL1 bound to Reptin could also be detected in complex containing β-catenin and HDAC1, we performed coimmunoprecipitation with anti-Reptin antibody using HEK293 cell lysates (Fig. 5*A*). Interestingly, endogenous Reptin specifically retained APPL1 along with β-catenin, HDAC1, and HDAC2, suggesting that APPL proteins could potentially be a part of the ternary complex. However, in this conventional immunoprecipitation assay, we could not exclude the possibility that Reptin forms independent subcomplexes with APPL1 or with β-catenin-HDAC1.

In order to address this question directly, 2D BN/SDS-PAGE analysis was performed (34). In 2D BN/SDS-PAGE, the positions of protein spots migrating in one vertical lane are indicative of their presence in the same complex. We separated the endogenous multiprotein complexes from HEK293 cell lysates under native conditions by 2D BN/SDS-PAGE, followed by immunoblotting for Reptin, APPL1, β-catenin, HDAC1, and HDAC2. These analyses revealed two distinct complexes containing APPL1 protein (Fig. 5*B*), one of which included Reptin, β-catenin, HDAC1, and HDAC2. Interestingly, the majority of APPL1 protein appeared to be involved in another independent complex also containing HDAC1/2. Cumulatively, the results of coimmunoprecipitation and 2D BN/SDS-PAGE analyses indicate that APPL1 is present in a ternary complex comprising Reptin, β-catenin, and HDAC1/2.

APPL Proteins Are Positive Regulators of β-Catenin/TCF-dependent Transcription—We next wished to determine the potential functions underlying the interaction of APPL proteins with Reptin as well as with β-catenin and HDACs. Based on the evidence that Reptin binds to β-catenin in protein complexes that regulate the transcription of β-catenin/TCF target genes, luciferase reporter assays were performed to test whether APPL1 and APPL2 proteins affect β-catenin/TCF-dependent transcriptional activity. HEK293 cells were transiently transfected with expression vectors encoding APPL1 or APPL2 in different amounts (Fig. 6*A*). The luciferase activities derived from the cotransfected TCF-responsive reporter (SuperTOPflash, which contains optimal TCF-binding sites upstream of a minimal promoter element) (37) or the control reporter construct (SuperFOPflash having mutated TCF-binding sites) were assayed 48 h after transfection. As shown previously, ectopic expression of β-catenin activated TCF-dependent transcription (38, 39) (Fig. 6*A*). Importantly, overexpression of either APPL1 or APPL2 increased the levels of β-catenin-stimulated reporter activity in a dose-dependent manner (Fig. 6*A*). No modulation of the SuperFOPflash reporter activity by either APPL protein was detected. A similar dose-dependent increase in the reporter activity was observed upon APPL overexpression when Wnt3a-conditioned medium was used for stimulation of cells instead of β-catenin co-transfection (Fig. 6*C*). In both cases, however, the stimulatory effect of APPL2 was consistently lower than that of APPL1. Moreover, silencing of

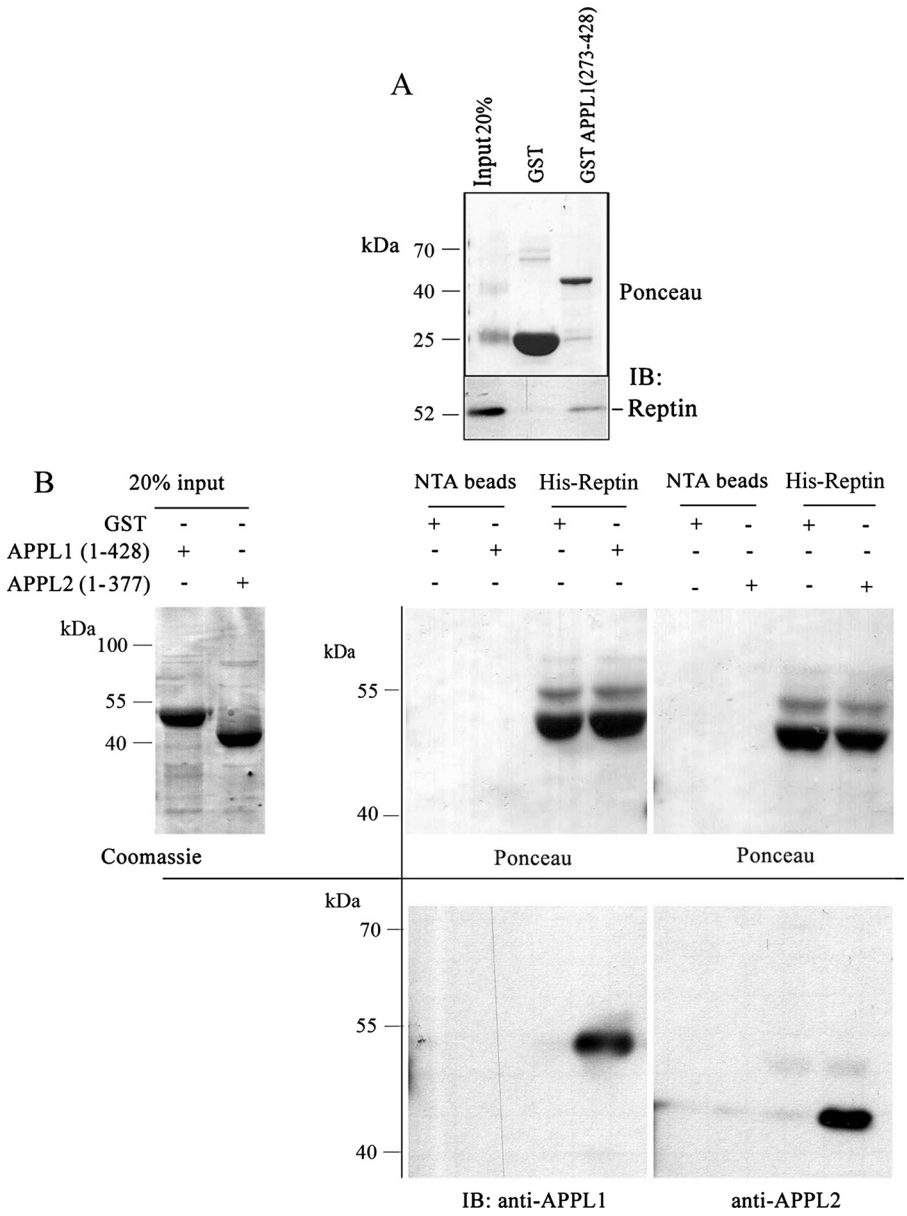


FIGURE 3. **Reptin binds directly with APPL proteins.** *A*, Reptin was *in vitro* translated and subjected to the pull-down assay by incubating with GST only or the GST-APPL1-(273–428) fragment, followed by immunoblotting (*IB*) with anti-Reptin antibody. Ponceau staining of fusion proteins shows the amounts used. *B*, *in vitro* binding assay of bacterially expressed, recombinant Reptin and APPL proteins. N termini of APPL1 (aa 1–428) and APPL2 (aa 1–377) were purified as GST fusion proteins, followed by tag cleavage with PreScission protease. They were incubated either with empty Ni^{2+}-NTA beads or the Ni^{2+}-NTA beads containing purified His_6-tagged Reptin. After extensive washing of the resins, bound proteins were resolved by SDS-PAGE followed by blotting with antibodies against APPL1 or APPL2 proteins.

APPL1 in HEK293 cells by two independent esiRNAs targeting different regions of the gene (Fig. 6*E*) resulted in the inhibition of the reporter activity, both in β-catenin- and Wnt3a-stimulated cells (Fig. 6, *B* and *D*). Since APPL2 is practically undetectable in HEK293 cells, we could not test the effects of its knockdown on transcription. Cumulatively, these results provided a first indication that APPL proteins can act as positive regulators of β-catenin/TCF-dependent transcription.

We also tested the effects of APPL overexpression in HEK293 cells without any stimulation. In this case, overexpression of APPL1 moderately increased the activity of SuperTOPflash reporter, whereas overexpression of APPL2 had no effect (Fig. 6*F*). At present, we cannot provide an explanation for the observed differences between the two proteins. In HEK293 cells APPL1 is a predominant form, so it is plausible that only the endogenously present protein is active in driving the reporter stimulation.

We further verified these observations by checking the expression of known β-catenin/TCF target genes, such as cyclin D1 (40, 41), c-*myc* (42), and c-*jun* (43), upon overexpression of APPL1 or APPL2 in unstimulated or Wnt3a-treated cells by semiquantitative reverse transcription-PCR. As shown in Fig. 6*G*, overexpression of APPL1 but not of APPL2 increased the transcript levels of c-*jun*, c-*myc*, and cyclin D1 in unstimulated cells, thus correlating with the results of the reporter assay (Fig. 6*F*). Instead, in cells treated with Wnt3a-conditioned medium, overexpression of either APPL1 or APPL2 proteins caused higher expression of the Wnt target genes (Fig. 6*G*), again in agreement with the luciferase assay (Fig. 6*C*). These data indicate that the positive effects of APPL proteins on transcription are not limited to the reporter construct but are also relevant *in vivo* for β-catenin/TCF target genes.

APPL Proteins Relieve Reptin-mediated Transcriptional Repression—We next investigated the effects of the simultaneous overexpression of APPL and Reptin on β-catenin/TCF-mediated transcription. Reptin overexpression inhibited the activity of the β-catenin-stimulated reporter, as previously reported (14), and this repression was relieved in a dose-dependent manner by adding increasing amounts of APPL1 or APPL2 (Fig. 7*A*). In order to further examine the interplay between Reptin and APPL proteins in the regulation of transcription, we decided to reduce the levels of Reptin by esiRNA transfection and to measure the effects of APPL1 or APPL2 overexpression under such conditions. As shown in Fig. 7*B*, esiRNA against Reptin (esi-Reptin) transfected in HEK293 cells reduced the levels of the protein as compared with the control (esi-EGFP). As expected, the knockdown of Reptin stimulated reporter activity above basal levels (Fig. 7*C*). Introduction of nonspecific esiRNA against EGFP together with increasing amounts of APPL1 protein resulted in dose-dependent stimulation of β-catenin/TCF-

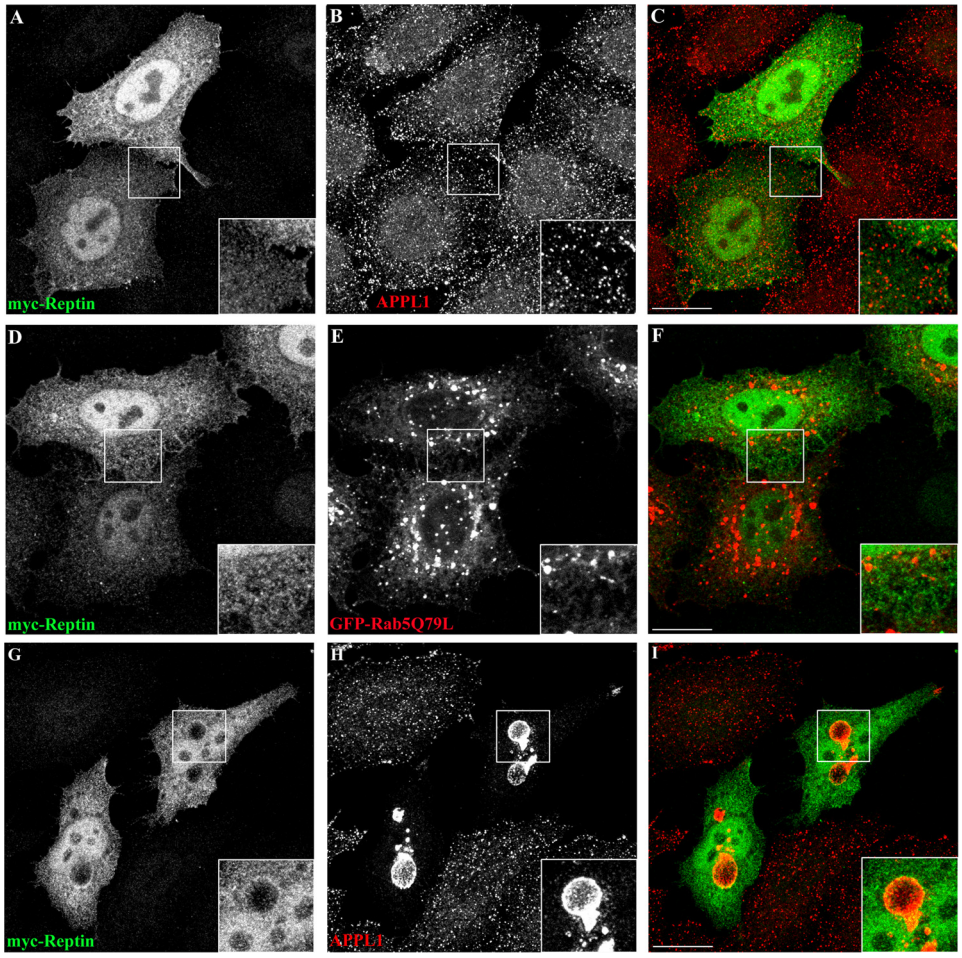


FIGURE 4. **Colocalization analysis of Reptin and APPL1 proteins.** HeLa cells grown on coverslips were transiently transfected with Myc-Reptin (*A–I*), pEGFP-Rab5-Q79L (*D–F*), and pCMV-Rab5-Q79L (*G–I*). After 24 h, cells were fixed and stained with anti-Myc (*A*, *D*, and *G*) and anti-APPL1 (*B* and *H*) antibodies. GFP staining of Rab5-Q79L is shown in *E*. *C*, *F*, and *I* represent overlays. All images are *z*-stack maximal projections. *Scale bar*, 15 μm.

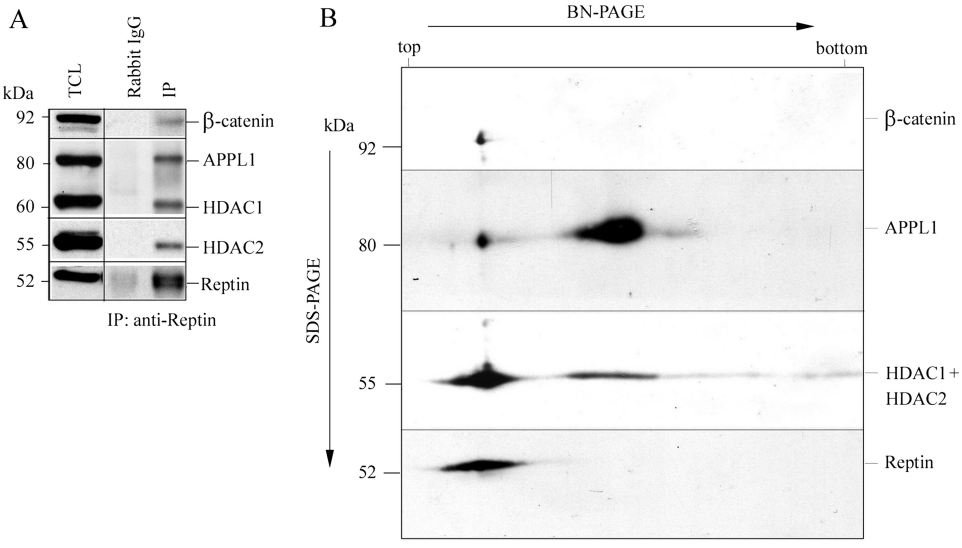


FIGURE 5. **APPL1 is a part of the β-catenin-Reptin-HDAC1/2 complex.** *A*, HEK293 cell lysates were subjected to a coimmunoprecipitation assay (*IP*) using anti-Reptin or control IgG, and the resultant precipitates were subjected to immunoblotting (*IB*) with the indicated antibodies. *TCL*, total cell lysate. *B*, a 2D BN/SDS-PAGE analysis of HEK293 cell extracts. Multiprotein complexes were resolved on 6–13% acrylamide gradient gel, followed by Tris-glycine-SDS-PAGE (8% gel). Immunoblotting was performed with specific antibodies recognizing the indicated proteins.

mediated transcription identical to that of the APPL1 proteins alone (compare Figs. 6*A* and 7*C*). This control demonstrated that the transfection with esiRNA *per se* did not affect the activity of the β-catenin/TCF reporter. The addition of lower amounts of APPL1 plasmid (either 0.25 or 0.5 μg) on top of esi-Reptin (0.25 μg) did not stimulate transcription above levels observed for esi-Reptin alone (Fig. 7*C*). Surprisingly, upon higher overexpression of APPL1 (1 μg of plasmid), β-catenin/TCF reporter activity was significantly increased (Fig. 7*C*). These data point out that a balance between the amounts of Reptin and APPL proteins appears to be important for regulating β-catenin/TCF-dependent transcription. We further verified this conclusion in the experiments in which we simultaneously overexpressed high doses of APPL1 (1 μg) together with decreasing amounts of esi-Reptin (0.25–0.1 μg). Cotransfection of 1 μg of APPL1 together with 0.25 μg of esi-Reptin caused the maximal 3-fold increase in the activity of the β-catenin-stimulated reporter, which was decreased dose-dependently upon lowering the amounts of esi-Reptin (Fig. 7*D*). Taken together, these data establish that APPL proteins modulate β-catenin/TCF-dependent transcription along with Reptin. As positive regulators of the pathway, APPL proteins can relieve Reptin-mediated repression of transcription, and the relative abundance of both Reptin and APPL proteins determines the final level of β-catenin/TCF-dependent transcriptional activity.

APPL1/APPL2 Proteins Reduce the Association between β-Catenin, Reptin, and HDACs and Affect Their Recruitment to the Promoters of Wnt Target Genes—The observed functional cooperation between APPL proteins and Reptin in the regulation of transcription prompted us to investigate its possible molecular mechanisms. We hypothesized that in view of their direct physical interactions with Reptin, APPL proteins could modulate the composition

and thus repressive activity of the Reptin-containing complex. Considering that APPL1 is also present in a Reptin-independent complex containing HDACs (Fig. 5*B*), overexpression of APPL proteins (which relieved Reptin-mediated transcriptional repression) could potentially interfere with the binding of HDACs to the Reptin-repressive complex. To investigate such a possibility, we assessed the composition of Reptin-containing complexes upon APPL1 or APPL2 overexpression. We performed immunoprecipitation of endogenous Reptin from lysates of HEK293 cells transfected with APPL1-Myc- or APPL2-Myc-expressing plasmids in comparison with cells transfected with empty vector (Fig. 8*A*). Interestingly, overexpression of either APPL protein clearly reduced the levels of HDAC1 and HDAC2 associated with Reptin. We further verified this effect in a converse experiment in which we analyzed immunoprecipitates of endogenous HDAC2 (Fig. 8*B*). Although endogenous Reptin is found in complex with HDAC2 in mock-transfected cells, its levels are reduced in the complex isolated from cells overexpressing YFP-APPL1. These results indicate that one potential mechanism underlying the observed transcriptional stimulation by APPL proteins, both without and upon Reptin overexpression, could involve the reduction of HDAC1/2 binding with Reptin. Intriguingly, β-catenin was also decreased in the Reptin immunoprecipitates of lysates containing overexpressed APPL1 or APPL2 proteins (Fig. 8*A*), although the total levels of β-catenin in cells were not changed. This indicates that an increase of β-catenin/TCF-dependent transcription via APPL proteins probably depends also on the relative abundance of β-catenin as well as HDAC1/2 in the Reptin complex.

We next tested whether the reduction of HDAC1/2 binding to Reptin upon APPL overproduction correlates with the changes in Reptin-associated deacetylase activity. We isolated Reptin-bound immunocomplexes from HEK293 cells transfected with APPL1-Myc, APPL2-Myc, or empty plasmid and measured the deacetylase activity (Fig. 8*C*). Compared with basal levels in mock-transfected cells, overexpressed APPL proteins reduced the levels of Reptin-associated HDAC activity. These results suggest that lowering the amounts of HDACs in the Reptin-containing complex upon APPL overproduction corresponds to the decreased enzymatic activity present in this complex.

To further verify the effects of APPL overexpression upon binding of β-catenin and Reptin, we performed β-catenin immunoprecipitation from Wnt3a-stimulated HEK293 cells. We could observe that overexpression of either APPL protein reduced the amounts of Reptin bound to β-catenin as compared with mock-transfected cells (Fig. 9*A*). Finally, we tested whether the levels of APPL proteins may affect the binding of β-catenin, Reptin, and HDAC1 to the promoters of Wnt target genes in Wnt3a-stimulated HEK293 cells. In chromatin immunoprecipitation experiments, overexpression of either APPL protein caused an increased association of β-catenin with the cyclin D1 and Axin2 promoters with respect to mock-transfected cells (Fig. 9*B*). In contrast, the levels of Reptin and HDAC1 on the same promoters were diminished (Fig. 9*B*), arguing that APPL overexpression affects the recruitment of repressive complexes to the Wnt target genes.

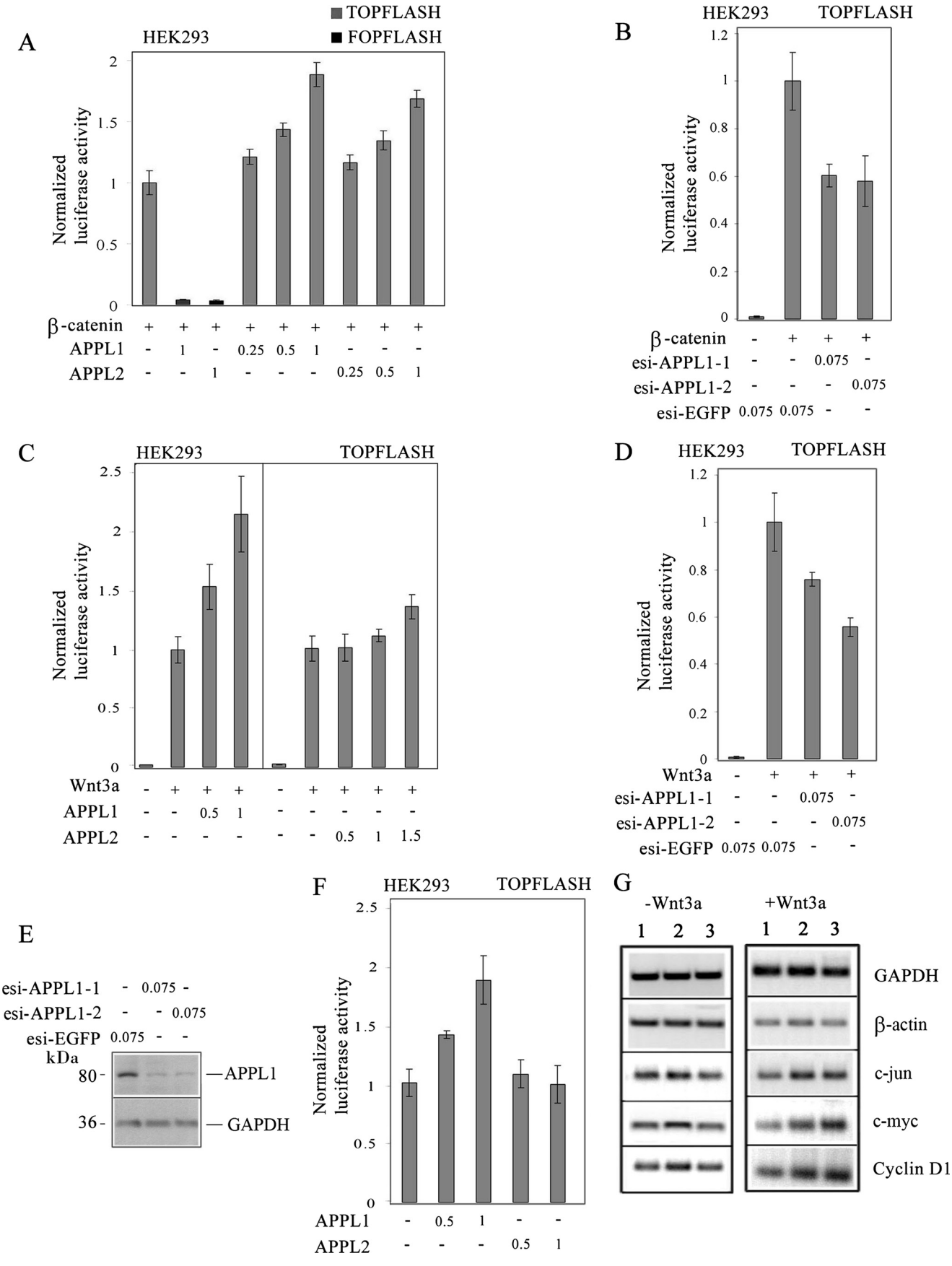
DISCUSSION

Here we report that the endosomal adaptor proteins APPL1 and APPL2 are novel activators of β-catenin/TCF-dependent transcription. Both APPL proteins exhibit direct interactions with the transcriptional repressor Reptin and can counter its inhibitory effects on transcription. Furthermore, APPL proteins are components of a complex containing Reptin, HDAC1, HDAC2, and β-catenin. Upon overproduction, APPL proteins affect the composition of this complex, reducing the amounts of HDACs and β-catenin associated with Reptin. Moreover, they increase the recruitment of β-catenin and reduce the amounts of Reptin and HDAC1 at the promoter of Wnt target genes. We propose that APPL proteins exert their stimulatory effects on β-catenin/TCF-dependent transcription by decreasing the activity of Reptin-containing repressive complexes.

APPL Proteins as Activators of β-Catenin/TCF-dependent Transcription—APPL proteins are multifunctional adaptors primarily localized to a subpopulation of early endosomes. However, a pool of APPL proteins is also capable of undergoing nuclear translocation. In this respect, they resemble several components of the Wnt/β-catenin pathway, which, although initially characterized for their cytoplasmic functions, do undergo nucleocytoplasmic shuttling. This is the case for Dishevelled, Dapper1, APC, Axin, GSK3β, Bcl-9/Legless, and βTrCP in addition to β-catenin itself (44–50). It has long been known that the levels of nuclear β-catenin are pivotal for its effects on transcription. However, more recent data argue that the ratios between the amounts of β-catenin-interacting proteins and regulators in the nucleus may be of equal importance (3).

We demonstrate that APPL proteins interact directly with Reptin, a transcriptional repressor in the Wnt/β-catenin pathway. Reptin is known to bind β-catenin (14) and HDAC1 (20), and the latter interaction is crucial for providing the repressive activity of the Reptin-containing complex, as shown for the KAI1 promoter (20). We present evidence that APPL proteins are present in a complex containing Reptin, β-catenin, HDAC1, and HDAC2. Moreover, they are capable of modulating the composition and thus probably the activity of this complex. Based on our data, we envisage that increased levels of either APPL protein sequester Reptin and HDACs away from β-catenin-containing transcriptional complexes. Further studies are required to delineate the exact order of events and the possible interactions of APPL proteins within this complex. They may be of intricate nature, particularly because APPL proteins also bind the NuRD corepressor complex containing class I HDACs (23), and our data indicate that APPL1 may weakly interact *in vitro* with HDAC1 and HDAC2.[4] Taking into account a large number of interactions exhibited by Reptin (17), it is likely that the Reptin-associated repressive complex could involve additional components, which may contribute to its activity via interactions with other key regulatory proteins. Importantly, in a recent genome-wide RNA interference screen for the regulators of the Wnt/β-catenin pathway, APPL2 scored as a positive regulator (51). This result, based on SuperTOPflash reporter activity in

[4] M. Banach-Orlowska, I. Pilecka, A. Torun, B. Pyrzynska, and M. Miaczynska, submitted for publication.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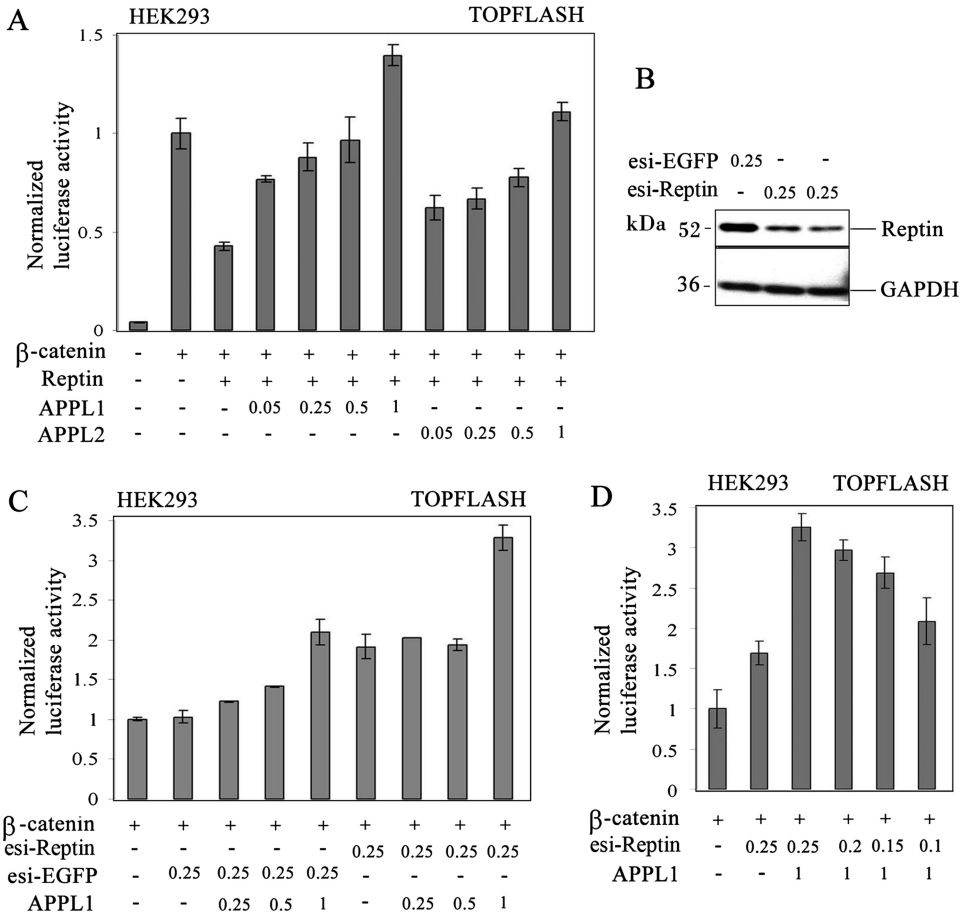

FIGURE 7. **Overexpression of either APPL protein reduces Reptin-mediated repression of β-catenin/TCF-dependent transcriptional activity.** Luciferase reporter assays were performed in HEK293 cells transiently transfected with the different combinations of plasmids, as indicated. In all assays, the amount of DNA transfected was kept constant by cotransfection with empty pcDNA3.1 vector. Values are mean ± S.D. from two or three independent transfections performed in parallel and are representative of at least three experiments. Data are normalized to the luciferase activity stimulated by β-catenin alone, which was arbitrarily set to 1 unit of relative activity. *A*, plasmids encoding SuperTOPflash reporter (0.1 μg), *Renilla* luciferase (0.1 μg), β-catenin (0.3 μg), Reptin (0.5 μg), or APPL1 or APPL2 (0.05, 0.25, 0.5, and 1 μg) were cotransfected into HEK293 cells, as indicated. After 48 h, the transfected cells were lysed, and the lysates were subjected to the luciferase activity assay. *B*, the knockdown of Reptin by esiRNA (0.25 μg, corresponding to 33 nM in a 24-well plate) in HEK293 cells was analyzed by Western blot. Nonspecific esiRNA against EGFP was used as a control. GAPDH levels indicate equal amounts of lysates loaded in each lane. *C*, reporter gene activity was measured in HEK293 cells 72 h after transient transfection of the increasing amounts of APPL1 protein (0.25, 0.5, and 1 μg) together with 0.25 μg of esiRNA against EGFP or Reptin. *D*, APPL1 (1 μg) was cotransfected together with decreasing amounts of either esi-Reptin or esi-EGFP (0.25, 0.2, 0.15, and 0.1 μg). HEK293 cells were lysed 72 h after transient transfection, and reporter gene activity was measured.

response to Wnt3a stimulation, represents an independent confirmation of our data that identify APPL proteins as activators of β-catenin/TCF-dependent transcription.

In general, the mechanisms of regulating the activity of transcriptional repressors by modulating the amounts of associated HDACs, as we propose for APPL proteins, appear to be common in the Wnt/β-catenin pathway. Dapper1, originally identified as a protein binding to Dishevelled (52), can associate with HDAC1 and enhance the interaction of Lef1 with HDAC1 (at the expense of Lef1 binding to β-catenin), thus maintaining Lef1 in the repressive state (47), as previously proposed (53). A transcriptional corepressor TIS7 inhibits the expression of β-catenin target genes, such as c-*myc* or osteopontin, by inducing the interaction of β-catenin with enzymatically active HDACs (54). These reports indicate that the balance between the stimulatory and inhibitory transcriptional activities of complexes containing β-catenin can be modulated by changing the amounts of associated repressive HDACs. This could be achieved by altering the local concentration and/or availability of β-catenin or HDAC-interacting partners and could result from the activity of various signaling pathways.

APPL Proteins as Dual Function Adaptors Acting in Endocytosis and Signaling—It is becoming clear that endocytic proteins may affect signal transduction processes at multiple

FIGURE 6. **APPL proteins are positive regulators of β-catenin/TCF-dependent transcription.** Luciferase reporter assays were performed in HEK293 cells transiently transfected with the different combinations of plasmids and/or esiRNA, as indicated (all values are in μg of DNA or esiRNA). In all assays, the amount of DNA transfected was kept constant by cotransfection with empty pcDNA3.1 vector. Values are mean ± S.D. from two or three independent transfections performed in parallel and are representative of at least three experiments. *A*, SuperTOPflash reporter having TCF-binding sites (0.1 μg) or SuperFOPflash with mutated TCF-binding sites (0.1 μg) were cotransfected with plasmids encoding *Renilla* luciferase (0.1 μg), β-catenin (0.3 μg), or APPL1 or APPL2 (0.25, 0.5, and 1 μg), as indicated. After 48 h, the transfected cells were lysed, and the lysates were subjected to the luciferase activity assay. Data are normalized to the luciferase activity stimulated by β-catenin alone, which was arbitrarily set to 1 unit of relative activity. *B*, reporter gene activity was measured in β-catenin-stimulated HEK293 cells 72 h after transient transfection of two different esiRNA (0.075 μg, corresponding to 33 nM in a 96-well plate) against APPL1 (esi-APPL1–1 and esi-APPL1–2). Nonspecific esiRNA against EGFP (esi-EGFP) was used as a control. Values are normalized to the luciferase activity derived from esi-EGFP-treated cells stimulated by β-catenin. *C*, HEK293 cells transfected with the indicated plasmids were stimulated by Wnt3a-conditioned medium for 18 h, and reporter gene activity was measured 48 h after transfection. Data are normalized to the luciferase activity stimulated by Wnt3a, which was arbitrarily set to 1 unit of relative activity. *D*, HEK293 cells transfected with two independent esiRNA against APPL1 (esi-APPL1–1 and esi-APPL1–2) were stimulated by Wnt3a-conditioned medium for 18 h, and reporter gene activity was measured 72 h after transfection. Nonspecific esiRNA against EGFP (esi-EGFP) was used as a control. Values are normalized to the luciferase activity derived from esi-EGFP-treated, Wnt3a-stimulated cells. *E*, the efficiency of APPL1 knockdown was tested by Western blotting (0.075 μg of esiRNA, corresponding to 33 nM in a 96-well plate). GAPDH levels indicate equal amounts of lysates loaded in each lane. *F*, APPL proteins were overexpressed in HEK293 cells, and reporter gene activity was measured 48 h after transfection without any stimulation. Data are normalized to the luciferase activity in unstimulated cells transfected with an empty pcDNA3.1 plasmid, which was arbitrarily set to 1 unit of relative activity. *G*, HEK293 cells transiently transfected with either empty plasmid (pcDNA 3.1; *lane 1*) or plasmids expressing APPL1 (*lane 2*) and APPL2 (*lane 3*) were either left untreated (−*Wnt3a panel*) or stimulated with Wnt3a-conditioned medium for 8 h (+*Wnt3a panel*). Thirty-eight hours post-transfection, cells were trypsinized, total RNA was isolated, and semiquantitative reverse transcription-PCR was performed with gene-specific primers, as indicated. Amplified samples were loaded onto 2% agarose gel and stained with ethidium bromide.

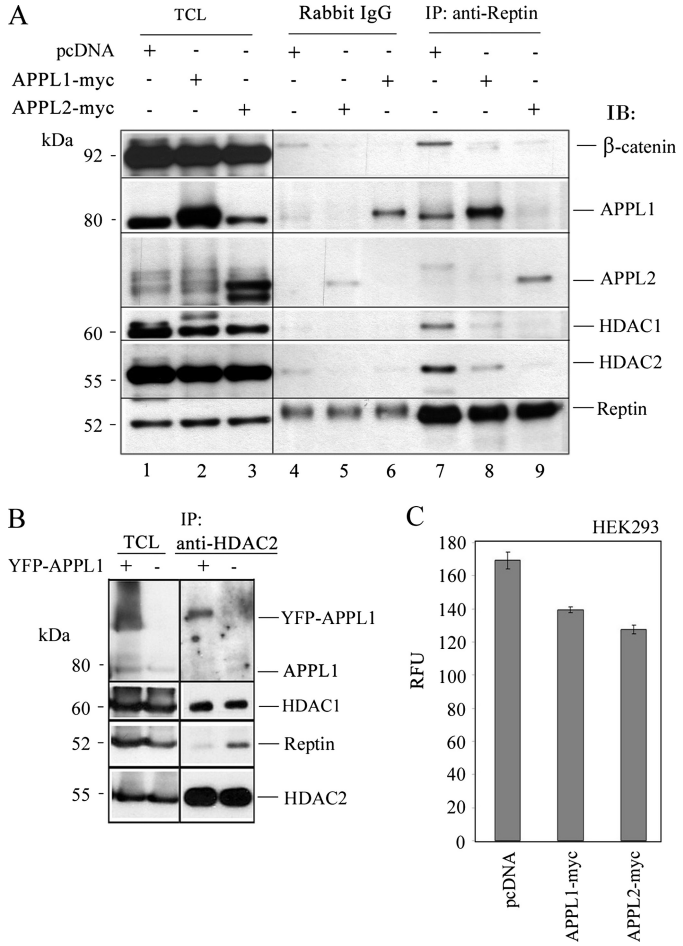

FIGURE 8. **Overexpression of APPL proteins reduces the amounts of HDACs bound to Reptin.** *A*, HEK293 cells were transiently transfected with APPL1-Myc and APPL2-Myc. Forty-eight hours post-transfection, cell extracts were prepared, and a coimmunoprecipitation assay (*IP*) was performed with either anti-Reptin or control IgG. The resultant precipitates were analyzed by immunoblotting (*IB*) against the indicated antibodies (*A*) or were subjected to fluorometric assays for measuring the respective HDAC activities (*C*). Each HDAC activity assay was performed in duplicate, and relative fluorescence unit (*RFU*) values shown are the averages ± S.D. from which the RFU values of assay buffer were subtracted. *B*, HEK293 cells were cotransfected with plasmids encoding YFP-tagged APPL1 or empty vector. Cell extracts were prepared and immunoprecipitated using anti-HDAC2. Bound proteins were separated and visualized by immunoblotting using anti-HDAC2, anti-HDAC1, anti-Reptin, and anti-APPL1 antibodies.

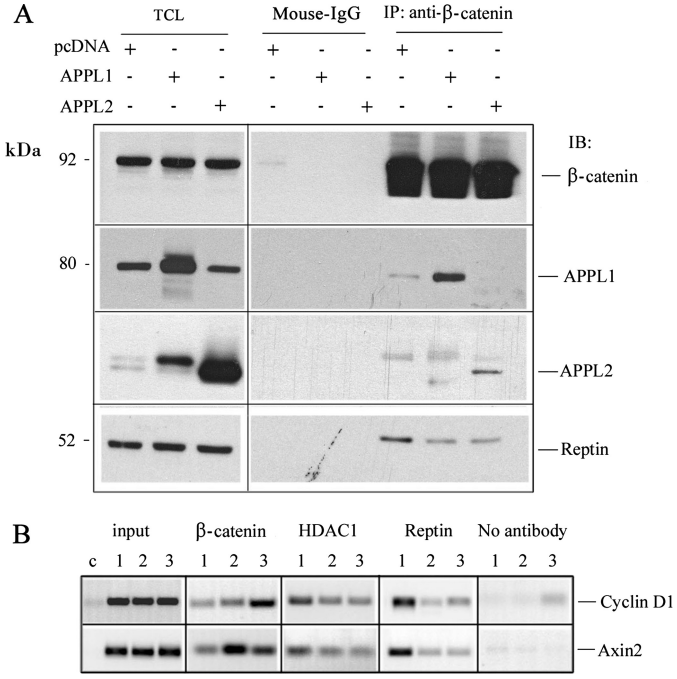

FIGURE 9. **Recruitment of β-catenin, Reptin, and HDAC1 to the promoters of Wnt target genes is affected upon overexpression of APPL proteins.** *A*, overexpression of APPL proteins reduces the amount of Reptin bound to β-catenin. HEK293 cells were transiently transfected with APPL1 or APPL2. Thirty-six hours post-transfection, cells were treated with Wnt3a-conditioned medium for 4 h. Subsequently, cell extracts were prepared, and a coimmunoprecipitation assay (*IP*) was performed with anti-β-catenin or control IgG. The resulting precipitates were analyzed by immunoblotting (*IB*) with the indicated antibodies. *B*, APPL proteins increase the recruitment of β-catenin and reduce the amounts of Reptin and HDAC1 at the Wnt target gene promoters. HEK293 cells were transfected with either empty pcDNA 3.1 (*lane 1*) or plasmids expressing APPL1 (*lane 2*) and APPL2 (*lane 3*). Thirty-six hours post-transfection, cells were treated with Wnt3a-conditioned medium for 4 h and then subjected to chromatin immunoprecipitation using anti-β-catenin, anti-HDAC1, and anti-Reptin antibodies. PCR was performed from these immunoprecipitates by using the primer pair covering the β-catenin binding sites at the promoters of cyclin D1 and Axin2. PCR products were resolved by agarose gel and stained with ethidium bromide. *Lane c*, PCR mixture without template.

levels (55, 56). Endocytosis has long been known to regulate the levels of plasma membrane receptors and to contribute to the down-regulation of signaling. More recently, however, it has become evident that in several cases signaling initiated at the plasma membrane can continue intracellularly from receptors internalized into the endosomal compartments (57–59). Endosomes can thus serve as platforms for active signal propagation, and many endocytic proteins appear to be bifunctional, providing a coordinated control between membrane transport and signaling events (58, 60). Intriguingly, several endocytic proteins, including APPL1/2, β-arrestins, HIP1, Dab1/2, Eps15, Epsin1, CALM, and the components of ESCRTs (endosomal sorting complexes required for transport) shuttle to the nucleus, where they may interact with nuclear partners and modulate the levels and specificity of gene transcription (22). In most cases, it is not clear whether these proteins act as messengers transmitting signals from the cytoplasm into the nucleus or whether they "moonlight" and perform independent functions.

APPL proteins are effectors of the small GTPase Rab5, which is a master regulator of early steps in endocytosis (23). Interestingly, Rab5 itself seems to affect β-catenin/TCF-dependent transcription, although the results of two reports on this issue are conflicting (61, 62). Here we tested whether APPL1 localized to endosomes might be involved in binding to Reptin; however, the observed lack of significant recruitment of Reptin to APPL-positive early endosomes argues against this possibility. We have previously shown that the binding of APPL1 to the endosomal membranes is strictly dependent on its interaction with Rab5, which occurs via the BAR and PH domains of APPL1 (23). In the present study, the association between APPL1 and Reptin has been mapped to the PH domain of APPL1. Interestingly, a majority of the known proteins interact with APPL1 at its C-terminal PTB domain (63), and so far, only Rab5 and Reptin appear to bind via its PH domain. This may imply that the association of APPL proteins with Reptin and Rab5 could be mutually exclusive. It is thus possible that the functions of

APPL proteins in Wnt signaling and in endocytosis are independent of each other; however, finding out whether the proper distribution of APPL proteins between endosomes and the nucleus is important for Wnt signaling will await more detailed studies. Moreover, a precise determination of internalization routes and transport kinetics of the Wnt/Frizzled/LRP5/6 ligand-receptor complex, as well as the identification of molecules governing these endocytic processes will be instrumental in understanding the relationship between endocytosis, endocytic proteins, and Wnt signaling.

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