Mediator Is a Transducer of Wnt/β-Catenin Signaling*

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Seokjoong Kim‡, Xuan Xu‡, Andreas Hecht‡, and Thomas G. Boyer††

From the ‡Department of Molecular Medicine and the Institute of Biotechnology, University of Texas Health Science Center, San Antonio, Texas 78245-3207 and the †Institut für Molekular Medizin und Zellforschung, Universität Freiburg, D-79104 Freiburg, Germany

Signal transduction within the canonical Wnt/β-catenin pathway drives development and carcinogenesis through programmed or unprogrammed changes in gene transcription. Although the upstream events linked to signal-induced activation of β-catenin in the cytoplasm have been deciphered in considerable detail, much less is known regarding the mechanism by which β-catenin stimulates target gene transcription in the nucleus. Here, we show that β-catenin physically and functionally targets the MED12 subunit in Mediator to activate transcription. The β-catenin transactivation domain bound directly to isolated MED12 and intact Mediator both in vitro and in vivo, and Mediator was recruited to Wnt-responsive genes in a β-catenin-dependent manner. Disruption of the β-catenin/MED12 interaction through dominant-negative interference- or RNA interference-mediated MED12 suppression inhibited β-catenin transactivation in response to Wnt signaling. This study thus identifies the MED12 interface within Mediator as a new component and a potential therapeutic target in the Wnt/β-catenin pathway.

Members of the Wnt family of secreted glycoproteins regulate a plethora of mammalian cell fate and behavioral decisions during embryogenesis and stem cell homeostasis (1, 2). These pleiotropic effects derive from distinct signal transduction pathways, each initiated by engagement of Wnt ligands with members of the seven-pass transmembrane receptors of the Frizzled family. The first and best characterized of these pathways, the canonical Wnt pathway, culminates in programmed changes in target gene transcription through the action of a key nuclear effector called β-catenin (3–5). In the absence of Wnt signaling, the steady-state level of cytoplasmic β-catenin is suppressed by a protein complex composed of Axin, the APC (adenomatous polyposis coli) tumor suppressor protein, and glycogen synthase kinase-3β, the latter of which phosphorylates β-catenin, marking it for destruction by the ubiquitin/proteasome pathway (6–10). Binding of Wnt ligand to Frizzled and its coreceptor LRP5/6 leads to inactivation of the Axin complex and stabilization of β-catenin. Stabilized β-catenin then translocates into the nucleus, whereupon its interaction with members of the T cell factor (TCF)/lymphoid enhancer factor (LEF) family of DNA-binding proteins positions β-catenin to activate transcription from the promoters of Wnt target genes that function in developmental regulation, cell proliferation, and cell/cell as well as cell/matrix interactions (11–18). Dysregulation of Wnt/β-catenin signaling leading to unprogrammed transcription of Wnt target genes has been proposed to be a driving force in the development of many cancers, including colorectal cancers (19–21).

Although the upstream events linked to signal-induced accumulation of β-catenin in the cytoplasm have been deciphered in considerable detail, the underlying mechanism(s) by which β-catenin activates transcription in the nucleus remains comparatively poorly understood. Thus, although β-catenin has been proposed to overcome nucleosome-mediated promoter repression and/or to promote transcription preinitiation complex assembly through functional interactions with the catalytic subunit (BRG1) of the SNF/SWI chromatin remodeling complex, the histone acetyltransferases p300/CBP, and the TATA box-binding protein (22–26), these interactions are unlikely to wholly account for the unique regulatory specificity inherent to β-catenin-directed transcription. In this regard, recent genetic studies in Caenorhabditis elegans and Drosophila melanogaster have revealed that mutations in Mediator subunits MED1 (SOP-3, TRAP220), MED6, MED12 (DPY-22/SOP-1, KTO), MED13 (LET-19, SKD), and MED23 (SUR2) variously affect developmental processes regulated by Wnt signaling (27–31). However, whether these observations reflect a direct or indirect requirement for Mediator in Wnt signaling has heretofore remained unknown.

Mediator is an evolutionarily conserved multiprotein interface between gene-specific transcription factors and the RNA polymerase II general transcription machinery (32–39). In this capacity, Mediator serves to promote the assembly, activation, and regeneration of transcription complexes on core promoters during the initiation and re-initiation phases of transcription (40–49). Because of its direct association with both signal-activated transcription factors and the RNA polymerase II transcription machinery, Mediator has been proposed to function as a general conduit and integrator of regulatory signals that converge on the promoters of protein-coding genes (50). Consistent with this idea, several Mediator subunits are functionally required for activated transcription in response to diverse cell signaling pathways. These include MED1 (TRAP220, ARC/DRIP205) for nuclear receptor, MED14 (TRAP170, ARC/DRIP/CRSP150) for coactivator (required for Sp1 function)) for interferon-γ, MED23 (TRAP150β, ARC/DRIP/CRSP130, human SUR2) for Ras/mitogen-activated protein kinase (MAPK), and MED15 (ARC105, PCQAP) for TGF-β signaling pathways (51–54). Note that, in this study, we utilize the unified nomenclature suggested by Bourbon et al. (55) to identify Mediator subunits. Where first introduced, however, each Mediator subunit will also be identified by its original name to facilitate recognition.

The role of Mediator as an established signal transducer coupled with its genetic links to the Wnt pathway prompted us to examine the requirement for Mediator in β-catenin-driven transcription. Herein, we identify a direct physical interaction between β-catenin and the MED12...
subunit in Mediator that is functionally required for activated transcription in response to Wnt signaling. Our study thus identifies MED12 as a new component in the Wnt/β-catenin pathway and further implicates Mediator in a broad range of developmental and pathological processes driven by canonical Wnt signal transduction.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The antibodies used for immunoblotting, affinity purification, and co-immunoprecipitation analyses were as follows. Anti-MED1 (catalog no. sc-8998), anti-MED6 (catalog no. sc-9433), anti-MED12 (catalog no. sc-5372), anti-MED16 (catalog no. sc-5366), anti-MED17 (catalog no. sc-12453), anti-CDK8 (catalog no. sc-13155, sc-1521), anti-HDAC1 (catalog no. sc-8410), and anti-transcription factor IIH p89 (catalog no. sc-293) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-MED23 (catalog no. 551175) and anti-β-catenin (catalog no. 610154) antibodies were purchased from Pharmingen. Anti-MED1 antibody M2 was purchased from Sigma. To generate rabbit anti-MED30 and anti-MED4 polyclonal antibodies, recombinant FLAG-His6-MED30 or FLAG-His6-MED4 was expressed in *Escherichia coli* strain BL21(DH3), purified under denaturing conditions by nickel-nitrioltriacetic acid fast flow chromatography (Qiagen Inc.), and used to immunize rabbits (Covance, Berkeley, CA). Specific antibodies were purified from rabbit serum by passage over an antigen-cross-linked affinity column and were used directly for immunoblot analysis and chromatin immunoprecipitation (ChIP) assay or were first cross-linked to protein A-Sepharose (GE Healthcare) using dimethyl pimelimidate (Sigma) for immunoprecipitation assays.

**Expression and Reporter Plasmids**—All expression vectors for β-catenin, Xenopus TCF3, and MED23, luciferase/β-galactosidase reporters have been described (24, 26, 36, 57). pRC/CMV-MED12, a multipurpose mammalian MED12 expression plasmid, was a kind gift from Dr. Leonard P. Freedman and used for *in vitro* transcription/translation of full-length MED12. pCMV-3xFLAG-MED12, a mammalian MED12 expression vector coding MED12 N-terminally tagged with three tandem FLAG epitopes, was constructed by transferring the MED12 cDNA from pRC/CMV-MED12 to p3xFLAG-CMV (Sigma) through a multi-step subcloning procedure. pRC/CMV-MED12–(1–1058) for *in vitro* transcription/translation of a MED12 N-terminal fragment was constructed by restriction digestion of pRC/CMV-MED12 with XbaI and subsequent plasmid religation. pET28-MED12-(626–1749) for *in vitro* transcription/translation of a MED12 internal fragment was constructed by subcloning an EcoRI-Xhol fragment of MED12 into pET28 (Novagen). pGBKTT7-MED12–(1749–2231) for *in vitro* transcription/translation of a MED12 C-terminal fragment was constructed by subcloning a Xhol-Not1 fragment of MED12 into pGBKTT7 (Clontech). pCS2+MED12-PQL-His6-FLAG and pCS2+MED12-OPA-His6-FLAG for *in vitro* transcription/translation and mammalian expression of Flag and His6 epitope-tagged MED12 PQL and OPAL domain, respectively, were constructed by subcloning PCR-amplified fragments encoding the MED12 PQL (amino acids 1651–2086) and OPAL (amino acids 2087–2212) domains into pCS2+His6-FLAG engineered to contain the His6 and FLAG epitope tag sequences in the multicloning site of pCS2+. pCS2+HA-MED15 and pET28-CyC were constructed by PCR-based subcloning of cDNAs encoding MED15 (cDNA clone MGC:20267); American Type Culture Collection) and cyclin C (cDNA clone MGC:19502; Open Biosystems, Huntsville, AL) into pCS2+ and pET28, respectively, and used for *in vitro* transcription/translation of full-length MED15 and cyclin C.

**Cell Culture, Transfections, RNA Interference, and Reporter Assays**—293 and HeLa cells were obtained from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium with 10% bovine growth serum (HyClone). 293 Top cells were a generous gift of Dr. Harold E. Varmus and were cultured in Dulbecco’s modified Eagle’s medium with 10% bovine growth serum and 400 ng/ml G418. T-REx HeLa cells (Invitrogen) were cultured in minimum Eagle’s medium with 10% bovine growth serum and 5 μg/ml blasticidin (Invitrogen). Parental and Wnt3α-expressing L cells (American Type Culture Collection) were cultured and used for preparation of Wnt-conditioned medium as described previously (58). For transient reporter assays, cells were seeded into 24- or 12-well cell culture plates 24 h prior to transfection. Cells (30–40% confluent) were transfected with FuGENE 6 (Roche Applied Science) following the manufacturer’s instructions. 48 h post-transfection, transfected cells were harvested and assayed for luciferase (Promega Corp.) and β-galactosidase (Applied Biosystems, Foster City, CA) activity as described previously (59). For MED12 RNA interference (RNAi) experiments, cells were transfected with MED12-specific (catalog no. M-009092-00) or control (catalog no. D-001210-01-05) small interfering RNA (siRNA; Dharmacon, Chicago, IL) using TransIT-siQUEST transfection reagent (Mirus Bio Corp., Madison, WI) 48 h prior to transfection of β-catenin expression and reporter plasmids using FuGENE 6 as described above. Reporter assay was performed 24 h post-transfection. Each transfection was repeated a minimum of three times in duplicate.

**Glutathione S-Transferase (GST) Pulldown Assays**—Fusion proteins of GST with full-length β-catenin (referred to as GST-βcat-FL), an N-terminal β-catenin fragment spanning amino acids 1–284 (referred to as GST-βcat-N), and a C-terminal β-catenin fragment spanning amino acids 630–781 and therefore including the transactivation domain (referred to as GST-βcat-C) were overexpressed in *E. coli* strain BL21(DE3), and soluble lysates were prepared in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, and 0.2% Nonidet P-40 by sonication. HeLa/S3 nuclear extracts were prepared as described (60). Recombinant Mediator subunits and fragments were expressed and radiolabeled with [35S]methionine by translation in *vitro* (TNT SP6/T7 quick coupled transcription/translation system, Promega Corp.).

For GST pulldown assays using HeLa/S3 nuclear extract, 20 μg of each GST-β-catenin derivative was immobilized on glutathione-Sepharose beads (GE Healthcare) and washed extensively. Immobilized beads were subsequently incubated with 1 μg of HeLa/S3 nuclear extract, dialyzed previously against 0.1 M KCl buffer D (20 mM HEPES (pH 7.9), 0.2 mM EDTA, and 20% glycerol). After overnight incubation at 4 °C, beads were washed five times with 0.15 M KCl buffer D containing 0.2% Nonidet P-40. Bound proteins were eluted in Laemmli sample buffer. 10% of each eluate was resolved by SDS-12% PAGE and analyzed by Coomassie Brilliant Blue staining to ensure that equivalent amounts of GST-β-catenin fusion proteins were immobilized. The remaining 90% of each eluate was resolved by SDS-10% PAGE and processed for immunoblot analysis using antibodies specific for Mediator subunits. GST pulldown assays using radiolabeled recombinant Mediator subunits were performed similarly except that bovine serum albumin was added to the binding mixture at a final concentration of 1.5 μg/μl as a blocking reagent, and 0.2 M KCl buffer D containing 0.1% Nonidet P-40 was used for washes. Eluates were resolved by SDS-12% PAGE and visualized by PhosphorImager analysis (GE Healthcare).

For Mediator purification, a soluble nuclear extract was prepared from MED6-HA/S3, a HeLa/S3-derived clonal cell line engineered by retrovirus-mediated gene transfer to express a hemagglutinin epitope-tagged Mediator MED6 subunit. Nuclear extract was first fractionated by phosphocellulose chromatography, and a 0.3–0.5 M KCl step fraction was subjected to 12CA5 (anti-hemagglutinin) affinity purification.
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Peptide eluates were dialyzed against 0.1 M KCl buffer D and used in GST pulldown assays.

Dual GST pulldown/protein cross-linking assays were performed as described previously (61) with minor modifications. Briefly, glutathione-Sepharose-immobilized GST and GST-β-catenin derivatives were incubated with HeLa/S3 nuclear extract and washed as described above. Subsequently, beads were equilibrated and resuspended in cross-linking buffer (20 mM HEPES (pH 7.9) and 100 mM KCl), followed by incubation with variable concentrations of dithiobis(succinimidyl) propionate (DSP; Pierce) to introduce reversible cross-links between directly interacting proteins. After incubation for 10 min at room temperature on a rocking platform, the cross-linking reaction was immediately quenched by the addition of 1 M Tris (pH 7.5) and subsequently terminated by the addition of quenching buffer (30 mM Tris (pH 7.5) and 100 mM KCl) for 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature. Uncross-linked proteins were removed with urea wash buffer (30 mM Tris (pH 7.5) and 6 M urea); 15 min at room temperature.

RESULTS

The β-Catenin Transactivation Domain Binds Directly to Mediator—To investigate a possible physical interaction between β-catenin and Mediator, we initially examined the abilities of several GST-β-catenin derivatives to bind Mediator present in HeLa nuclear extract using a GST pulldown assay and immunoblot analysis using antibodies specific for Mediator subunits MED1, MED6 (ARC/DRIP33), MED12 (TRAP230, ARC/DRIP240), MED16 (TRAP95, DRIP92), MED17 (TRAP80, ARC/DRIP/CRSP77), MED23, and CDK8 (human SRB10). Both GST-fused full-length β-catenin (GST-βcat-FL) and its transactivation domain (GST-βcat-C) bind intact Mediator, as scored by the presence in bound eluates of all Mediator subunits examined (Fig. 1B). By contrast, GST alone as well as fused to the β-catenin N terminus (GST-βcat-N), which is principally involved in the regulation of β-catenin stability, failed to interact with Mediator (Fig. 1B). Interestingly, a weak interaction between the β-catenin N terminus and CDK8 free of Mediator was consistently observed in repeated binding assays (Fig. 1B), although the significance of this interaction is unclear at present.

To determine whether β-catenin binds intact Mediator directly, we examined the ability of GST-βcat-FL and GST-βcat-C to bind Mediator purified from a HeLa/S3-derived cell line expressing a hemagglutinin epitope-tagged Mediator MED6 subunit. This analysis revealed that both full-length β-catenin and its isolated transactivation domain bound intact purified Mediator (Fig. 1C), demonstrating that the interaction between β-catenin and Mediator is indeed direct.

To examine the physical interaction between endogenous β-catenin and Mediator, we employed co-immunoprecipitation analysis using nuclear extracts from human HeLa cervical carcinoma and BG-1 ovarian carcinoma cells treated with LiCl. LiCl is an inhibitor of glycogen synthase kinase-3β that normally phosphorlates β-catenin in the absence of Wnt signaling, thereby marking it for destruction by the ubiquitin/proteasome pathway; thus, analogous to the Wnt ligand, LiCl promotes the nuclear accumulation of stabilized β-catenin (64). Immunoprecipitation of intact Mediator using MED30-specific antibody resulted in the specific and efficient co-immunoprecipitation of β-catenin (Fig. 1D), confirming the association of β-catenin with Mediator in LiCl-stimulated cells. Collectively, these binding studies reveal that β-catenin interacts with Mediator both in vitro and in vivo.

β-Catenin Recruits Mediator to Wnt-responsive Genes—To begin to explore a possible functional interaction between β-catenin and Mediator, we initially asked whether Mediator is recruited to β-catenin target genes in response to Wnt signaling. To answer this question, we employed ChIP analysis to monitor Wnt-induced occupancy of TCF-binding elements (TBEs) within the axin2 and DKK1 genes by both...
\(\beta\)-catenin and Mediator in 293Top cells (14). For this purpose, 293Top cells were treated with control or Wnt3α-conditioned medium for 1 h prior to ChIP analysis. We first confirmed, by quantitative RT-PCR analysis, that the axin2 and DKK1 genes are targets of Wnt-dependent regulation in 293Top cells; relative to control medium, treatment with Wnt3α-conditioned medium stimulated axin2 and DKK1 expression by 5.5- and 4-fold, respectively (Fig. 2A). As expected, \(\beta\)-catenin was detected by ChIP in association with axin2 and DKK1 TBEs only in Wnt-treated cells (Fig. 2B), an observation concordant with the established \(\beta\)-catenin-dependent regulation of these genes (14, 57, 65–70). Notably, occupancy of axin2 and DKK1 TBEs by Mediator was also observed only in Wnt-responsive gene elements in a \(\beta\)-catenin-dependent manner.

To determine more specifically whether \(\beta\)-catenin recruits Mediator to Wnt-responsive genes, we used ChIP to compare the resident status of both \(\beta\)-catenin and Mediator on axin2 TBEs in 293Top/\(\beta\)cat and 293Top/\(\beta\)cat cells, consistent with \(\beta\)-catenin-dependent regulation of this Wnt-responsive gene (Fig. 2D). Notably, occupancy of axin2 TBEs by Mediator was also observed only in the \(\beta\)-catenin-expressing 293Top/\(\beta\)cat cells (Fig. 2D). Taken together, the results of these ChIP analyses demonstrate that Mediator is recruited to Wnt-responsive gene elements in a \(\beta\)-catenin-dependent manner.

**\(\beta\)-Catenin Targets the MED12 Subunit in Mediator—**To identify the target subunit of \(\beta\)-catenin within Mediator, we employed a dual GST pulldown/protein cross-linking assay (61, 71). Briefly, glutathione-Sepharose-immobilized GST-\(\beta\)cat-C (transactivation domain) was used to capture Mediator present in HeLa nuclear extract, and the GST-\(\beta\)cat-C-Mediator complex was treated with increasing concentrations of the heterobifunctional cross-linking agent DSP. Following denaturing washes to remove uncleaved proteins, Laemmli sample buffer was used to reverse DSP-induced cross-links and to elute proteins from the glutathione-Sepharose beads. Subsequently, eluates were resolved by SDS-PAGE and processed by immunoblot analysis for the presence of individual Mediator subunits and HDAC1 as a negative control as indicated. Input corresponds to 5% of the total nuclear extract used in immunoprecipitation reactions.

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**FIGURE 1.** \(\beta\)-Catenin binds to Mediator both in vitro and in vivo. A, a schematic diagram of full-length \(\beta\)-catenin is shown. The N- and C-terminal (transactivation domain) fragments used in binding reactions are demarcated. As, amino acids. B and C, immobilized GST-\(\beta\)-catenin derivatives were incubated with HeLa/S3 nuclear extract or purified Mediator, respectively. Following extensive washing, bound proteins were eluted in Laemmli sample buffer, resolved by SDS-10% PAGE, and analyzed by Western blotting (WB) for the presence of individual Mediator subunits as indicated. Input represents 5% of the total nuclear extract (B) or 30% of the purified Mediator (C) used in binding reactions. In B, 10% of the eluate was resolved by SDS-12% PAGE and visualized by Coomassie Brilliant Blue staining to ensure that equivalent amounts of GST-\(\beta\)-catenin derivatives were used in binding reactions. In C, purified Mediator used in binding reactions was resolved by SDS-4–12% gradient PAGE and visualized by silver staining. Note that, in B and C, the apparent reduction in the MED23 immunoblot signal derived from co-migration during SDS-PAGE of MED23 and GST-\(\beta\)cat is reflected in the MED23 signal size, and GST-\(\beta\)cat is rendered the MED23 signal diffuse. Because the amount of GST-\(\beta\)cat far exceeded that of MED23 in the gel, co-migration of the two proteins rendered the MED23 signal diffuse. HA, hemagglutinin. D, nuclear extracts derived from HeLa and BG-1 cells treated with 30 mM LiCl for 6 h were subjected to immunoprecipitation (IP) using rabbit anti-MED30 polyclonal antibody conjugated to protein A-Sepharose. Preimmune rabbit IgG conjugated to protein A-Sepharose was used as a negative control. Following extensive washing, immunoprecipitates were resolved by SDS-11% PAGE and visualized by silver staining for the presence of individual Mediator subunits and HDAC1 as a negative control as indicated. Input corresponds to 5% of the total nuclear extract used in immunoprecipitation reactions.

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**FIGURE 2.** A schematic diagram of axin2 and DKK1 TBEs only in 293Top/\(\beta\)cat cells. Relative to Wnt-responsive gene elements in a \(\beta\)-catenin-dependent manner. To identify the target subunit of \(\beta\)-catenin within Mediator, we employed a dual GST pulldown/protein cross-linking assay (61, 71). Briefly, glutathione-Sepharose-immobilized GST-\(\beta\)cat-C (transactivation domain) was used to capture Mediator present in HeLa nuclear extract, and the GST-\(\beta\)cat-C-Mediator complex was treated with increasing concentrations of the heterobifunctional cross-linking agent DSP. Following denaturing washes to remove uncleaved proteins, Laemmli sample buffer was used to reverse DSP-induced cross-links and to elute proteins from the glutathione-Sepharose beads. Subsequently, eluates were resolved by SDS-PAGE and processed by immunoblot analysis for the presence of individual Mediator subunits and HDAC1 as a negative control as indicated. Input corresponds to 5% of the total nuclear extract used in immunoprecipitation reactions.
FIGURE 2. Mediator is recruited to Wnt-responsive genes in a β-catenin-dependent manner. A and B, 293Top cells were treated with control conditioned medium from parental L cells (CM) or, alternatively, conditioned medium from a Wnt3α-expressing L cell clone (WCM) as indicated. A, total RNA recovered from 293Top cells treated for 4 h with control or Wnt3α-conditioned medium was processed by quantitative RT-PCR analysis for the levels of axin2, DKK1, and glyceraldehyde-3-phosphate dehydrogenase mRNAs. The mRNA levels of axin2 and DKK1 were normalized to those of glyceraldehyde-3-phosphate dehydrogenase and are expressed as the ratio of normalized axin2 and DKK1 mRNA levels in 293Top cells treated with control conditioned medium. B, soluble chromatin prepared from 293Top cells treated for 1 h with control or Wnt3α-conditioned medium was subjected to immunoprecipitation with rabbit polyclonal antibody specific for estrogen receptor-α (ERα) as a negative control, β-catenin, or the integral Mediator subunit MED6 to mark Mediator. Immunoprecipitated DNA was PCR-amplified using primers that span functionally critical TCF-binding sites within the axin2 (positions 1651–2086) and DKK1 (positions 794 to 215) genes. Input corresponds to direct PCR amplification of 0.25% of the soluble chromatin that was used in immunoprecipitation reactions. C, total RNA recovered from 293Top or 293Top/βcat cells as indicated was processed by quantitative RT-PCR analysis for the levels of axin2 and glyceraldehyde-3-phosphate dehydrogenase mRNAs. The mRNA level of axin2 was normalized to that of glyceraldehyde-3-phosphate dehydrogenase and is expressed as the ratio of the normalized axin2 mRNA level in 293Top cells. D, soluble chromatin prepared from 293Top or 293Top/βcat cells as indicated was subjected to immunoprecipitation and PCR amplification with axin2-specific primers as described for B. Input corresponds to direct PCR amplification of 0.25% of the soluble chromatin that was used in immunoprecipitation reactions.

ally the same results were observed when the cross-linking assay was repeated using GST-βcat-FL (data not shown).

On the basis of the results of these cross-linking assays, we next examined the ability of β-catenin to bind directly to radiolabeled recombinant MED12 and MED23 using a standard GST pulldown assay. We also included MED15 as a negative control in these binding experiments. It was reported previously that inhibition of MED15 function does not alter the expression of Wnt target genes during Xenopus embryonic development, indicating that MED15 is unlikely to represent a relevant transcriptional target of β-catenin in Mediator (52). These binding experiments revealed that MED12, but not MED23 or MED15, could interact efficiently with the β-catenin transactivation domain (Fig. 3B). Furthermore, MED23-deficient mouse embryonic stem cells support efficient β-catenin-directed transcriptional activation, providing definitive evidence that MED23 is neither a physical nor a functional target of β-catenin.

To confirm that β-catenin can interact with MED12 in vivo, we performed co-immunoprecipitation analyses using LiCl-treated 293 cells transfected with or without FLAG epitope-tagged MED12. This analysis revealed that endogenous β-catenin could be precipitated by FLAG-specific antibody only in the presence, but not in the absence, of FLAG-tagged MED12 (Fig. 3C), indicating that the two proteins interact in vivo. On the basis of these collective findings, we conclude that MED12 is a direct physical target of β-catenin within Mediator.

To more narrowly define the β-catenin interaction domain on MED12, we tested the ability of GST-βcat-C to bind to a series of truncated MED12 fragments radiolabeled by translation in vitro. The β-catenin transactivation domain bound most efficiently to a fragment of MED12 corresponding to amino acids 1651–2086 (Fig. 3D). Interestingly, this region (previously designated the PQL domain due to its high content of Pro, Gln, and Leu residues) has been shown to mediate the interaction of MED12 with transcriptional activators SOX9 and Rta (61, 72).

Disruption of the β-Catenin/MED12 Interaction Inhibits β-Catenin Transcriptional Activity—Should β-catenin target Mediator for a functional interaction through its MED12 interface, the β-catenin-binding domain on MED12 (PQL domain) might be expected to inhibit the transactivation function of β-catenin through dominant-negative interference. To test this possibility, we initially examined, in 293 cells, the effect of MED12 PQL domain overexpression on the transactivation activity of a constitutively stabilized β-catenin derivative carrying four alanine substitutions in the glycogen synthase kinase-3β recognition site (26) using a transient reporter assay. This analysis revealed that ectopic expression of the MED12 PQL domain, but not the neighboring MED12 OPA domain, to which β-catenin does not bind, efficiently inhibited the transactivation activity of β-catenin in a dose-dependent manner in both a synthetic (TOPflash) and a natural (axin2) β-catenin-

3 A. J. Berk and J. L. Stevens, personal communication.
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response promoter (Fig. 4, A–C). By contrast, MED12 PQL domain overexpression did not influence the activity of the adenovirus E1A transactivation domain (Fig. 4D), the established target of which in Mediator is MED23 (36, 53). Immunoblot analysis of transfected whole cell extracts revealed that MED12 PQL domain overexpression did not affect the expression level of β-catenin, indicating that the dominant-negative influence of the MED12 PQL domain on β-catenin transactivation is direct (Fig. 4, A and B). Furthermore, MED12 PQL-domain-mediated dominant-negative inhibition of β-catenin transcriptional activity was also observed in HeLa cells, indicating that this effect is not cell type-specific (Fig. 4E).

To determine whether endogenous β-catenin is similarly susceptible to dominant-negative inhibition by the MED12 PQL domain, we examined the influence of MED12 PQL domain overexpression on TOPflash reporter activity in 293 cells treated with LiCl or Wnt3a-conditioned medium. As expected, ectopic expression of the MED12 PQL domain inhibited the transcriptional activity of endogenous β-catenin stabilized by pharmacological or physiological activation of the Wnt pathway (Fig. 5, A and B). Taken together, these findings support the hypothesis that Mediator transduces Wnt signals through a functional interaction between β-catenin and its Mediator interface.

To more rigorously examine the functional interaction between β-catenin and MED12, we monitored the influence of RNAi-mediated suppression on β-catenin-directed transactivation. In HeLa cells, MED12-specific siRNA reduced the steady-state level of MED12 protein by ∼75% and inhibited the transcriptional activity of β-catenin in the TOPflash reporter by ∼60% (Fig. 6). To confirm that this effect is mediated through the β-catenin transactivation domain, we also monitored the influence of MED12 knockdown on the activity of the isolated β-catenin transactivation domain tethered to the Gal4 DNA-binding domain. This analysis revealed that RNAi-mediated MED12 depletion (∼75%) inhibited Gal4-β-catenin transactivation domain activity by ∼80% (Fig. 6). Collectively, these findings provide further evidence for a functional interaction between β-catenin and the MED12 interface within Mediator.

MED12 Is a Principal Target of β-Catenin within a MED12/CDK8/CycC-containing Mediator Submodule—MED12 in association with MED13, CDK8, and CycC (cyclin C; human SRB11) is thought to compose a discrete submodule within metazoan Mediator based on several lines of reasoning. First, MED12, MED13, CDK8, and CycC homologs in Schizosaccharomyces cerevisiae and Schizosaccharomyces pombe compose a subcomplex that is both biochemically dissoociable from and stable independently of yeast Mediator (73). Second, two functionally distinct forms of yeast and metazoan Mediator complexes with or without MED12, MED13, CDK8, and CycC can be resolved biochemically (33). Thus, should MED12 function to anchor and/or stabilize a MED12/
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FIGURE 4. The MED12 PQL domain inhibits the transactivation function of β-catenin in a dominant-negative manner. 293 cells (A–D) and HeLa cells (E) were transfected with (i) the TOPflash or FOPflash reporter plasmid harboring four consensus or mutant TCF/LEF-binding sites, respectively, upstream of the minimal c-fos promoter (A, C, and E), pAxin2-Luc harboring the β-catenin-responsive murine axin2 promoter (B), and pG5-E1B-Luc harboring five tandem Gal4 DNA-binding sites upstream of the minimal adenovirus E1B promoter (D); (ii) pCS2+βCatS33A expressing a constitutively stabilized β-catenin derivative (A–C and E) and pGal4-E1A expressing a Gal4 DNA-binding domain-E1A activation domain chimera (D); (iii) an internal control plasmid (pCH110) expressing β-galactosidase; and (iv) the indicated nanogram quantities of pCS2+MED12-PQL-His6-FLAG and pCS2+MED12-OPA-His6-FLAG plasmids expressing the FLAG epitope-tagged MED12 PQL and OPA domains, respectively. In E, HeLa cells were transfected with an additional plasmid, pCS2+xTCF3 expressing Xenopus TCF3. 48 h post-transfection, transfected whole cell lysates were assayed for luciferase activity. Luciferase activities were normalized against β-galactosidase values and are expressed relative to the level of luciferase activity obtained in cells transfected without activator, arbitrarily assigned a value of 1. For experiments using the TOPflash and FOPflash reporter systems (A, C, and E), the ratio of TOPflash to FOPflash reporter activity for each independent transfection in a series was first calculated and used to establish the relative luciferase activity. Error bars represent the mean ± S.D. of at least three independent transfections performed in duplicate. In A and B, a portion of the whole cell lysate used in luciferase assays was resolved by SDS-10% PAGE and processed by Western blot analysis for the expression levels of MED12 and its subunit, the latter of which served as an internal loading control.

FIGURE 5. The MED12 PQL domain inhibits the transactivation function of endogenous β-catenin stabilized by Wnt signaling. A and B, 293 cells were transfected with the TOPflash or FOPflash reporter plasmid, an internal control β-galactosidase expression plasmid (pCH110), and the indicated nanogram quantities of pCS2+MED12-PQL-His6-FLAG and pCS2+MED12-OPA-His6-FLAG expressing the FLAG epitope-tagged MED12 PQL and OPA domains, respectively. Luciferase activities were first normalized to β-galactosidase values and calculated as the ratio of TOPflash to FOPflash reporter activity and are expressed relative to the level of luciferase activity obtained in control medium-treated cells transfected with no PQL domain, arbitrarily assigned a value of 1. Error bars represent the mean ± S.D. of at least three independent transfections performed in duplicate. In A, transfected cells were treated for 24 h prior to harvest with control conditioned medium derived from parental L cells (−) or, alternatively, conditioned medium derived from a Wnt3a-expressing L cell clone (WNT3a CM: +) as indicated. Harvested whole cell lysates were assayed for luciferase activity. Luciferase activities were first normalized to β-galactosidase values and calculated as the ratio of TOPflash to FOPflash reporter activity and are expressed relative to the level of luciferase activity obtained in control medium-treated cells transfected with no PQL domain, arbitrarily assigned a value of 1. Error bars represent the mean ± S.D. of at least three independent transfections performed in duplicate.

MED13/CDK8/CycC submodule within Mediator, the functional consequence of MED12 depletion on β-catenin-directed transactivation could conceivably derive from concomitant depletion of one or more additional submodule components. To address this issue, we examined, by immunoblot analysis, the influence of MED12 knockdown in HeLa cells on the steady-state levels of CDK8 and CycC proteins in nuclear extracts as well as the stable incorporation of CDK8 into Mediator. We observed that RNAi-mediated MED12 depletion resulted in reduced...
steady-state protein levels of CDK8 and CycC and a concordant reduction in CDK8 in immunoprecipitated Mediator (Fig. 7, A and B). These results suggest that MED12 is critical to ensure the integrity of a MED12/CDK8/CycC-containing Mediator submodule and provide further support for a conserved architectural organization between yeast and metazoan Mediator complexes.

To exclude the possibility that CDK8 and CycC reduction accompanying MED12 knockdown depletes an additional physical target(s) of β-catenin within Mediator, we examined the ability of β-catenin to bind directly to radiolabeled recombinant CDK8 and CycC in vitro. These binding experiments revealed that MED12, but not CDK8 or CycC, could interact efficiently with the β-catenin transactivation domain (Fig. 7C). Thus, neither CDK8 nor CycC is likely to represent a direct physical target of β-catenin within Mediator. These results suggest that depletion of CDK8 and CycC accompanying MED12 knockdown is unlikely to account for the reduced transcriptional activity of β-catenin. Thus, although we cannot entirely exclude the possibility that β-catenin targets an additional subunit within a MED12/CDK8/CycC-containing submodule, our results nonetheless provide compelling evidence that MED12 is a principal physical and functional target of β-catenin within Mediator.

**DISCUSSION**

The physiological and pathological manifestations of Wnt/β-catenin signaling derive from programmed and unprogrammed changes in target gene transcription through poorly defined mechanisms. Herein, we have identified and characterized a physical interaction between β-catenin and the MED12 interface in Mediator that, based upon the following criteria, appears to be functionally important for transduction of Wnt signals through β-catenin to RNA polymerase II. First, the β-catenin transactivation domain binds specifically and directly to isolated MED12 and intact Mediator in vitro. Second, endogenous β-catenin binds MED12 and intact Mediator in vivo, and Mediator is recruited to Wnt-responsive genes in a β-catenin-dependent manner. Third, the β-catenin-binding domain on MED12 (PQI domain) inhibits the transcriptional activity of β-catenin activated by Wnt signaling in a dominant-negative manner. Fourth, RNAi-mediated MED12 suppression inhibits the transcriptional activity of β-catenin. These findings thus...
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identify MED12 as a direct and functionally important transducer of canonical Wnt/β-catenin signaling.

Previous studies revealing repressive as well as activating functions for MED12 suggest a complex regulatory role for this Mediator subunit in gene transcription. For example, MED12 and Mediator subunits MED13, CDK8, and CycC mark a transcriptionally repressive form of Mediator (ARC-L) that is biochemically and structurally distinguishable from an activating form of Mediator (CRSP) lacking this submodule and additionally containing MED26 (CRSP70) (74). However, MED12 is also target of the SOX9 and viral Rta activation domains, supporting a role for MED12 in activator-dependent transcription (61, 72). Finally, targeted disruption of Srb8, the presumptive MED12 homolog in S. pombe, leads to both up- and down-regulation of gene expression, indicating a context-dependent role for Srb8 in the both repression and activation of gene transcription (75). With respect to control of Wnt-regulated transcriptional programs, previous genetic studies in C. elegans have implicated MED12 in the suppression of β-catenin-dependent transcription (27, 31). Our finding herein that MED12 mediates β-catenin-activated transcription supports a dual role for MED12 in Wnt-regulated transcription.

In this regard, it is perhaps notable that CBP has also been proposed to both positively and negatively regulate Wnt signaling (22, 23, 26, 76). For example, in addition to its documented role as a transcriptional coactivator of β-catenin in response to Wnt signaling, genetic studies have also implicated CBP in suppression of Wnt signaling through effects on TCF/LEF. In the absence of Wnt signaling, TCF/LEF factors are known to recruit Groucho and C-terminal binding protein corepressors to inhibit β-catenin target gene transcription (77–80). Because Mediator containing the MED12/MED13/CDK8/CycC submodule has been implicated previously in reversible repression of transcriptionally induced genes (81), it is possible that a dual function for Mediator in Wnt-regulated transcription could reflect its participation in TCF/LEF-mediated default repression in the absence of Wnt signaling as well as its role as a coactivator of β-catenin in response to Wnt signaling. Further characterization of the functional interaction between β-catenin and MED12 will be required to clarify the basis for its context-dependent role in both repression and activation of Wnt target gene transcription.

Our identification of MED12 as a direct target of the β-catenin transactivation domain was based initially on the results of protein cross-linking analyses indicating their close proximity in the GST-β-catenin-Mediator complex. However, this approach is inherently limited by a fixed number of currently available Mediator subunit-specific antibodies. Thus, although MED12 has nonetheless been validated by further physical and functional analyses as a bona fide direct target of the β-catenin transactivation domain, we cannot exclude the possibility that an additional target subunit(s) of β-catenin exists within Mediator. In this regard, we note that RNAi-mediated MED12 suppression reduced the transcriptional activity of β-catenin in a TopFlash reporter and of Gal4-β-catenin in a Gal4-responsive reporter by ~60 and ~80%, respectively. This partial reduction in β-catenin transactivation activity could reflect incomplete MED12 knockdown (>70%) or, alternatively, a β-catenin requirement for an additional coactivator target(s), possibly p300/CBP, BRG1, or TATA box-binding protein, or even a second target in Mediator. In this regard, other activators have been proposed to target more than one Mediator subunit, although the functional and mechanistic implications of dual targets within Mediator remain to be defined (82, 83).

The functional interaction between β-catenin and MED12 established herein reveals a novel molecular interface of potential therapeutic value in colorectal cancer. Because many colorectal cancers involve mutagenic activation of the Wnt/β-catenin pathway, attenuation of β-catenin transcriptional activity has been proposed as a rational approach to reverse the malignant phenotype (84). Thus far, small molecule inhibitors of TCF/β-catenin and CBP/β-catenin interactions have shown promise as inhibitors of β-catenin-directed transcription and colorectal cancer cell growth (85, 86). We speculate that the β-catenin/MED12 interface might similarly represent an attractive target for therapeutic intervention by small molecule drugs. Further characterization of the physical and functional interaction between β-catenin and MED12 should clarify this issue and further reveal the contribution of Mediator to the entire spectrum of developmental and pathological processes driven by Wnt signaling.

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