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MAD2B, a Novel TCF4-binding Protein, Modulates TCF4-mediated Epithelial-Mesenchymal Transdifferentiation*[S]

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Chun-Fu Hong[‡§], **Yu-Ting Chou**[§], **Young-Sun Lin**[¶], **and Cheng-Wen Wu**[§‖1]

From the [‡]*Graduate Institute of Life Science, National Defense Medical Center, Taipei 11490, the* [§]*Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, the* [¶]*Development Center for Biotechnology, Taipei 22180, and the* [‖]*National Yang-Ming University, Taipei 11221, Taiwan*

T cell factor 4 (TCF4) interacts with β-catenin in the WNT signaling pathway and transactivates downstream target genes involved in cancer progression. To identify proteins that regulate TCF4-mediated biological responses, we performed a yeast two-hybrid screen to search for a TCF4-binding protein(s) and found that MAD2B interacts with TCF4. We confirmed that MAD2B is a TCF4-binding protein by co-immunoprecipitation. Using the TOPFLASH reporter assay, we found that MAD2B blocks TCF4-mediated transactivation. The MAD2B binding regions of TCF4 were identified by TCF4 deletion mapping and electrophoretic mobility shift assay analysis. TCF4 and MAD2B interactions abolished the DNA binding ability of TCF4. Knockdown of MAD2B in SW480 colorectal cancer cells led to the conversion of epithelial cells to a mesenchymal fibroblastoid phenotype (epithelial-mesenchymal transdifferentiation). An E-cadherin promoter reporter analysis showed that MAD2B modulates TCF4-mediated E-cadherin expression. MAD2B knockdown blocked E-cadherin expression and significantly induced mesenchymal markers, such as N-cadherin and vimentin. Mesenchymal induction was accompanied by F-actin redistribution and the appearance of a fibroblastoid phenotype. MAD2B knockdown also increased both mRNA and protein levels of Slug, a known TCF4-induced E-cadherin transcriptional repressor. A chromatin immunoprecipitation assay showed that MAD2B silencing enhances the ability of TCF4 to bind the Slug promoter. Thus, MAD2B is a novel TCF4-interacting protein. This study provides the first evidence for the involvement of MAD2B in TCF4-mediated epithelial-mesenchymal transdifferentiation.

The WNT signaling cascade is a crucial cell development and growth regulatory pathway (1–3). The key event of WNT signaling is the activation of the T cell factor (TCF)[2]-β-catenin complex. WNT binding to transmembrane Frizzled receptors is followed by phosphorylation and functional deactivation of glycogen synthase kinase-3β. Then membrane-associated β-catenin is released and stabilized in the cytoplasm. Accumulated β-catenin associates with TCFs and translocates into the nucleus, leading to transcriptional activation of downstream target genes (3, 4). Inappropriate activation of the WNT signaling pathway, such as by mutation of adenomatous polyposis coli and glycogen synthase kinase-3β, is often involved in early premalignant lesions of the intestine and tumorigenesis of several cell types (5). Human germ line mutations in the adenomatous polyposis coli result in familial adenomatous polyposis, which is characterized by multiple intestinal adenomas (6). Up to 80% of colorectal cancers (CRCs) show mutations in both adenomatous polyposis coli alleles (7). Accumulation of β-catenin protein and transactivation of a certain set of TCF4 target genes by accumulated β-catenin are crucial for colorectal carcinogenesis (8).

Within the TCF family proteins, TCF4, which is encoded by the Tcf7L2 gene, is predominantly expressed in CRC cells. TCF4 binds directly to DNA through its high mobility group domains and transactivates target genes by coupling with β-catenin, a binding partner that provides transcriptional activation domains. TCF4-β-catenin complex-mediated gene transactivation of CRC cells by dominant-negative TCF4 switches off genes involved in cell proliferation and turns on genes involved in cell differentiation (9).

Epithelial-mesenchymal transdifferentiation (EMT), a dedifferentiation program, is critical for the oncogenic and invasive properties of tumor cells of epithelial origin. TCF/β-catenin transcription factor up-regulates mesenchymal genes and participates in EMT (10, 11). E-cadherin, a well characterized cell-cell adhesion molecule responsible for the maintenance of epithelial cell shape (12), is down-regulated by WNT signaling directly and indirectly (13–15). The TCFs-β-catenin complex binds directly to the promoter region of E-cadherin and cooperates with bone morphogenetic protein-Smad signaling to down-regulate E-cadherin expression (14). WNT signaling also up-regulates E-cadherin transcriptional repressors, such as the Snail family proteins Snail and Slug (12). Loss of E-cadherin expression is associated with the onset of EMT (16). EMT mediates cancer metastasis processes, such as disassembly of cadherin-mediated cell-cell adhesion, acquisition of fibroblast phenotypes, and increased invasiveness. Disruption of E-cadherin-mediated cell-cell adhesion induced by EMT are considered key steps for the progression of metastasis (17, 18).

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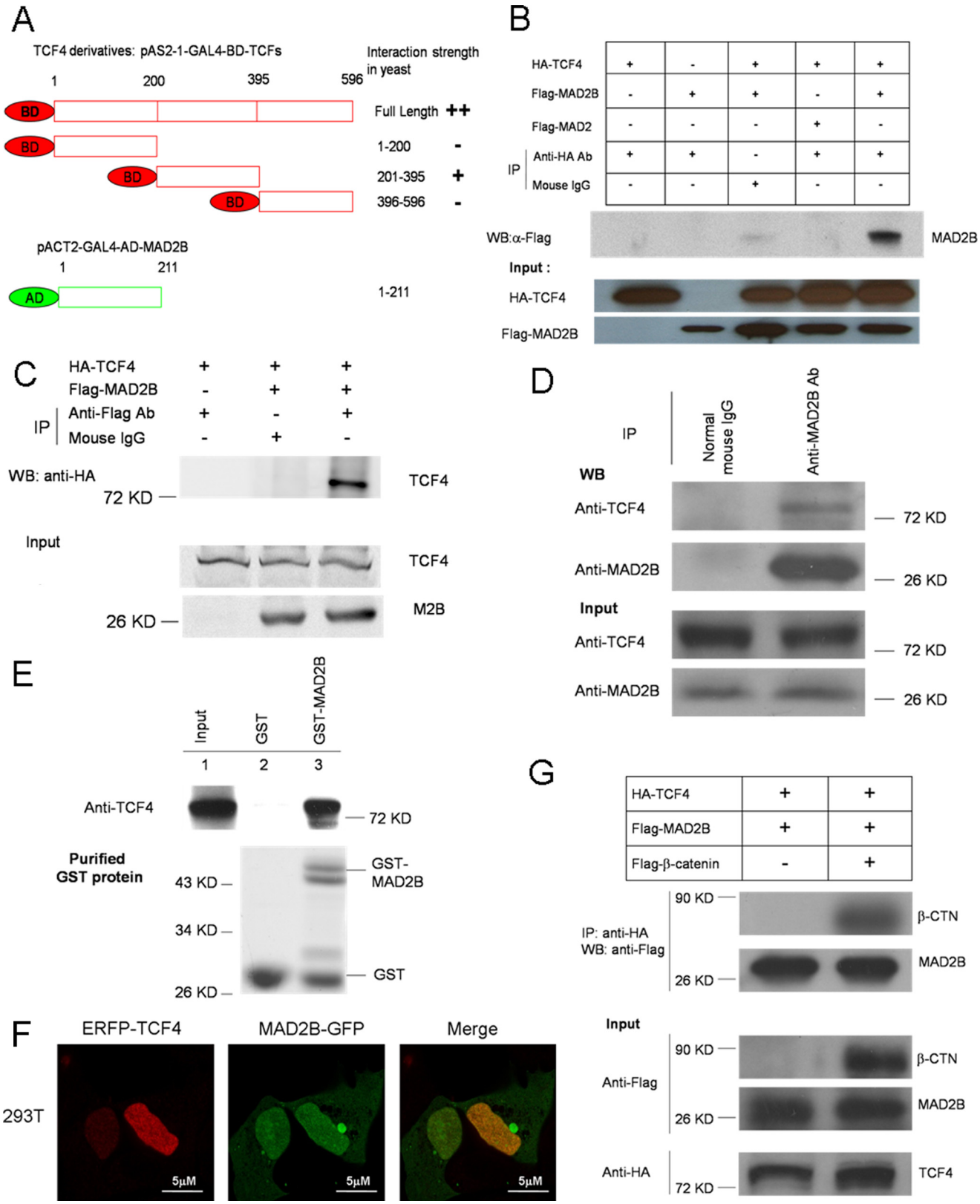
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] To whom correspondence should be addressed: National Yang-Ming University, No. 155, Sec. 2, Linong St., Taipei 11221, Taiwan. Tel.: 886-2-2826-7919; Fax: 886-2-2823-6518; E-mail: cwwu@ym.edu.tw.

[2] The abbreviations used are: TCF, T cell factor; HEK cells, human embryonic kidney cells; ChIP, chromatin immunoprecipitation; GST, glutathione *S*-transferase; EMT, Epithelial-mesenchymal transdifferentiation; TBS, TCF-binding site; CRC, colorectal cancer; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; ERFP, enhanced red fluorescent protein; APC, anaphase-promoting complex.

Interaction between MAD2B and TCF4

MAD2B was identified in a yeast two-hybrid screen for TCF4-interacting partners. Human MAD2B (also known as MAD2L2 and hRev7) is the human homolog of yeast Rev7 and shares 53% similarity with human MAD2 (also known as MAD2L1), a key mitotic checkpoint protein (19). Although MAD2B shares structure similarity with MAD2, they have different preferences for associated proteins. MAD2B, for example, prefers to bind Cdh1, but MAD2 forms a complex with cdc20 to inhibit anaphase-promoting complex (20). Similar to MAD2, MAD2B is thought to play multiple roles in the regula-



tion of cell physiology (21–24). In this study we determined whether MAD2B regulates TCF4-mediated biological processes. We found that MAD2B functions as a negative modulator of TCF4-induced EMT.

EXPERIMENTAL PROCEDURES

Plasmid Construction—For the yeast two-hybrid assay, TCF4 and its derivatives were cloned by inserting PCR-amplified gene fragments into the EcoRI and BamHI sites of pAS2–1 (Clontech). MAD2B was cloned into pACT2 between the EcoRI and XhoI sites. For mammalian expression, the TCF4 gene was cloned into pcDNA3.0-HA (Invitrogen) between the BamHI and XhoI sites. The human MAD2B gene was cloned into pRK5-FLAG (BD Biosciences) between the EcoRI and XbaI sites. The pDsRed-TCF4 plasmid was constructed by inserting the TCF4 gene into the pDsRed-C1 plasmid (Clontech) between the EcoRI and BamHI/blunt-end sites. The EGFP-MAD2B plasmid was constructed by inserting the MAD2B gene into the EcoRI and SmaI sites of EGFP-N3 (Clontech). The *Escherichia coli* expression plasmid pET29b-MAD2B was constructed by inserting the MAD2B gene into the EcoRI and XhoI sites of the pET29b plasmid (Novagen).

The MAD2B knockdown lentiviral expression vectors were constructed by inserting two pairs of short hairpin double-stranded oligos, clone 1 (sense, 5′-CCGGGATGCAGCTTTACGTGGAAGACTCGAGTCTTCCACGTAAAGCTGCATCTTTTTG-3′; antisense, 5′-AATTCAAAAAGATGCAGCTTTACGTGGAAGACTCGAGTCTTCCACGTAAAGCTGCATC-3′) and clone 2 (sense, 5′-CCGGACGACAAGACCTCAACTTTGGCTCGAGCCAAAGTTGAGGTCTTGTCGTTTTTG-3′; antisense, 5′-AATTCAAAAAACGACAAGACCTCAACTTTGGCTCGAGCCAAAGTTGAGGTCTTGTCGT-3′) into the EcoRI and AgeI sites of pLKO.1. si-MAD2B is from clone-1 oligos; siMAD2B-2 is from clone-2 oligos. The pGL3-cdh1 promoter plasmid was constructed by inserting the human E-cadherin promoter region (−481 to +127) into the SmaI and HindIII sites of the pGL3-basic plasmid (Promega).

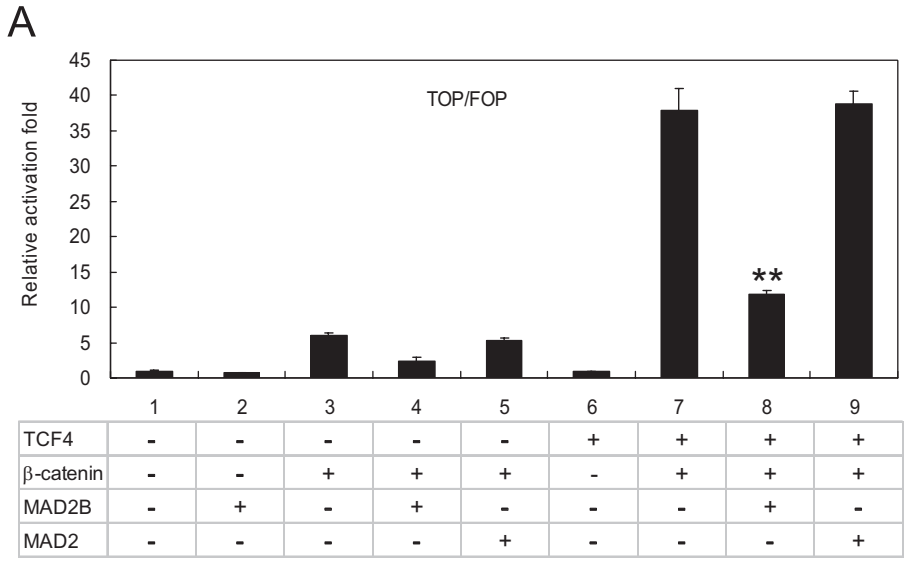
Yeast Two-hybrid Screen—pAS2–1-TCF4 with Gal4DB was used to screen a mixture of cDNA libraries from mammary gland and fetal brain (Clontech), in which all cDNAs were cloned into the pACT2 plasmid. Approximately 2×10^6 transformants of the Y190 strain were screened according to the manufacturer's protocol. Several cDNA clones from the activation domain library encoded proteins that interacted with TCF4. These clones were isolated and sequenced. One of them was a full-length MAD2B.

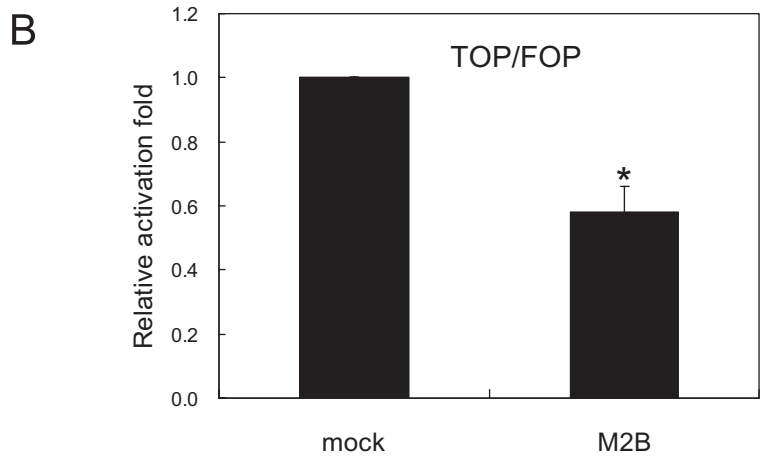
Cell Culture—HEK293T cells (human embryonic kidney cells) and SW480 human colon cancer cells (with mutant adenomatous polyposis coli) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone) in a 37 °C, 5% CO_2 incubator. Human colon cancer cells HCT116 (with mutant β-catenin) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum in a 37 °C, 5% CO_2 incubator. For puromycin selection, lentiviral-infected HCT116 cells were incubated in RPMI 1640 containing 1.5 μg/ml puromycin, and SW480 cells were selected in Dulbecco's modified Eagle's medium containing 2 μg/ml puromycin.

Co-immunoprecipitation and Western Blot Analysis—HEK293T cells (2×10^6) were seeded on 100-mm dishes 24 h before transfection. The pCDNA3-HA-TCF4 and pRK5-FLAG-MAD2B plasmids were transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were lysed in 1 ml if radioimmune precipitation assay lysis buffer (50 mM Tris-Cl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_3VO_4, 50 mM NaF) 48 h after transfection. Co-immunoprecipitation was performed with 1 μg of each antibody, and Western blots were performed as described (25). The following monoclonal antibodies were used: anti-HA (Berkeley Antibody Co., Inc.), anti-FLAG (Sigma), anti-TCF4 (Upstate Biotechnology, Inc.,), anti-MAD2B (BD Biosciences), anti-E-cadherin (BD Biosciences), anti-N-cadherin (BD Biosciences), anti-vimentin (BD Biosciences), and anti-mouse IgG (Santa Cruz Biotechnology). Anti-β-catenin was rabbit polyclonal antibody (Cell Signaling).

Immunofluorescence Staining—Cells were seeded on glass coverslips at ~30% confluence 24 h before transfection. Forty-eight hours after transfection, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 10 min. After blocking with 3% skim milk in phosphate-buffered saline (PBS), cells were incubated with primary antibody diluted in PBS overnight at 4 °C and then incubated with secondary antibody for 2 h at room temperature. Cells were mounted with Prolong Gold antifade reagent (Invitrogen) and imaged with a confocal microscope.

FIGURE 1. **MAD2B interacts with TCF4 in yeast and mammalian cells.** *A*, TCF4 interacts with MAD2B in yeast. Yeast two-hybrid assay results for Y190 yeast cells that were co-transformed with pACT2-MAD2B and pAS2–1 containing full-length TCF4 (amino acids 1–596) or TCF4 derivatives (amino acids 1–200, 201–395, 396–596). Binding activity was scored: + for interaction, and – for no interaction. ++ stands for stronger interaction. *B*, TCF4 interacts with MAD2B in mammalian cells. HA-tagged TCF4 and FLAG-tagged MAD2B or FLAG-tagged MAD2 plasmids were co-transfected into HEK293T cells for 48 h. Cell lysate was collected and immunoprecipitated (*IP*) with anti-HA followed by Western blot (*WB*) analysis with an antibody (*Ab*) against FLAG or HA. *C*, cells co-transfected with HA-TCF4 and FLAG-MAD2B as in *B* were collected and immunoprecipitated with anti-FLAG followed by Western blot analysis with an antibody against FLAG or HA. *D*, MAD2B interacts with endogenous TCF4 in colon cancer cells. Whole-cell lysate was collected from HCT116 colon cancer cells and subjected to co-immunoprecipitation with anti-MAD2B followed by Western blot analysis with an antibody against TCF4 or MAD2B. *E*, MAD2B interacts with TCF4 *in vitro*. His-TCF4 was purified from HEK293T cells. His-TCF4 was incubated with GST-MAD2B or GST purified from bacteria. The MAD2B-TCF4 protein complex was immunoblotted with anti-TCF4. The *top panel* shows the binding of TCF4 to GST-MAD2B. An input and pulldown products were separated by SDS-PAGE and detected by Western blot analysis with antibodies specific for TCF4. The *bottom panel* is Coomassie Blue-stained gel showing relative amounts of GST and GST-MAD2B. *F*, MAD2B and TCF4 both localize in the nucleus. Plasmids expressing EGFP-MAD2B and ERFP-TCF4 fluorescent fusion proteins were co-transfected into HEK293T cells. Fluorescence images were analyzed by confocal microscopy. *G*, overexpression of stabilized β-catenin does not affect the interaction between MAD2B and TCF4. HA-tagged TCF4 and FLAG-tagged MAD2B were co-transfected into HEK293T together with FLAG-tagged β-catenin (*β-CTN*) or empty pRK5-FLAG vector. 48 h after transfection cell lysate was collected and immunoprecipitated with anti-HA followed by Western blot analysis with an anti-FLAG antibody.





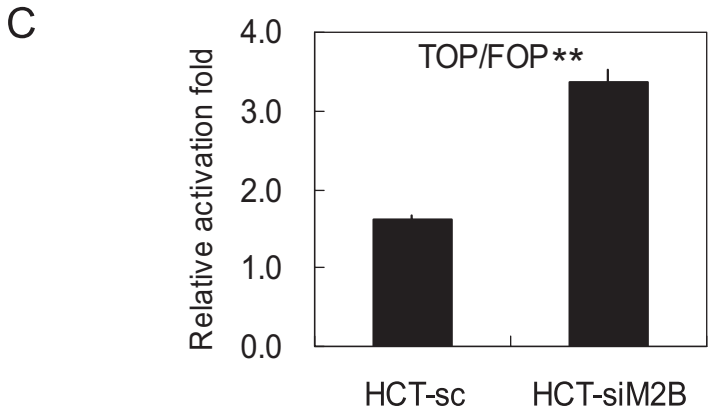


FIGURE 2. **MAD2B represses the transcriptional activity of TCF4.** *A*, MAD2B represses the transcriptional activity of TCF4 in HEK293T cells. HEK293T cells in 6-well plates were co-transfected with 0.5 μg of TCF4, 0.5 μg of β-catenin, and/or 2 μg of MAD2B (or 2 μg of MAD2) plasmids as indicated. TOPFLASH (*TOP*) or FOPFLASH (*FOP*) plasmids (0.5 μg) were used for the reporter assay. Cell lysate was collected 48 h after transfection, and relative luciferase activity was measured according to a standard dual-luciferase assay protocol. The results are presented as the TOP/FOP ratio. *B*, MAD2B represses the transcriptional activity of TCF4 in HCT116 colon cancer cells. HCT116 cells in 6-well plates were transfected with 0.5 μg of TOPFLASH or FOPFLASH plasmid together with 2 μg of MAD2B (*M2B*) plasmid as indicated. Relative luciferase activity was measured. *C*, silencing MAD2B in HCT116 cells releases the repression of TCF4 transcriptional activity. TOPFLASH or FOPFLASH plasmid (0.5 μg) was transfected into HCT116-scramble (*HCT-sc*) and HCT116-siMAD2B (*HCT-siM2B*) cells followed by the dual-luciferase assay. pRL-TK (50 ng) was co-transfected in the above experiments as an internal control. Data represent the mean $\pm$ S.D. of triplicate determinations. *, $p < 0.05$ compared with control; **, $p < 0.01$ compared with control.

Luciferase Reporter Assay—TOPFLASH (with three repeats of the TCF-binding site) or FOPFLASH (with three repeats of a mutated TCF-binding site) plasmid was transfected into HEK293T cells or HCT116 (scramble and siMAD2B) cells together with the indicated plasmids. Renina luciferase plasmid (pRL-TK) was co-transfected as an internal control. Cell lysate was collected 48 h after transfection, and the dual-luciferase assay was performed according to the manufacturer's protocol. The pGL3-cdh1 promoter or the pGL3-Slug promoter plasmid was transfected, and the same luciferase reporter assay was carried out.

Electrophoretic Mobility Shift Assay—pcDNA3.0-His-TCF4 plasmid was transfected into HEK293T cells, and His-TCF4 fusion protein was purified 48 h after transfection following a standard His-tag protein purification protocol. His-MAD2B fusion protein was purified following a standard protocol after induction of a pET29b-MAD2B-transformed BL21 (DE3) *E. coli* strain with 1 mM isopropyl 1-thio-β-D-galactopyranoside overnight at 25 °C. For the electrophoretic mobility shift assay, 10 ng of purified His-TCF4 and 0.01 pmol of P^{32}-labeled probe containing TCF-binding sites (TBSs, CCCTTTGATCTTACC) was incubated in 40 μl of Buffer D (20 mM HEPES, pH 8.0, 20% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA) and 1 μg of poly(dI-dC)) for 30 min at room temperature. Electrophoresis was then performed at 4 °C in a 4% nondenaturing polyacrylamide gel containing 0.5× Tris-buffered EDTA (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0).

Lentiviral Infection—Twenty-four hours before transfection, 2.2×10^6 HEK293T cells were seeded onto a 100-mm culture dish. Using a standard calcium phosphate transfection protocol, we co-transfected 10 μg of the pLKO.1-based lentiviral vector, 9 μg of Δ8.9 plasmid, and 2.5 μg of vesicular stomatitis virus G protein (VSVG) plasmid into HEK293T

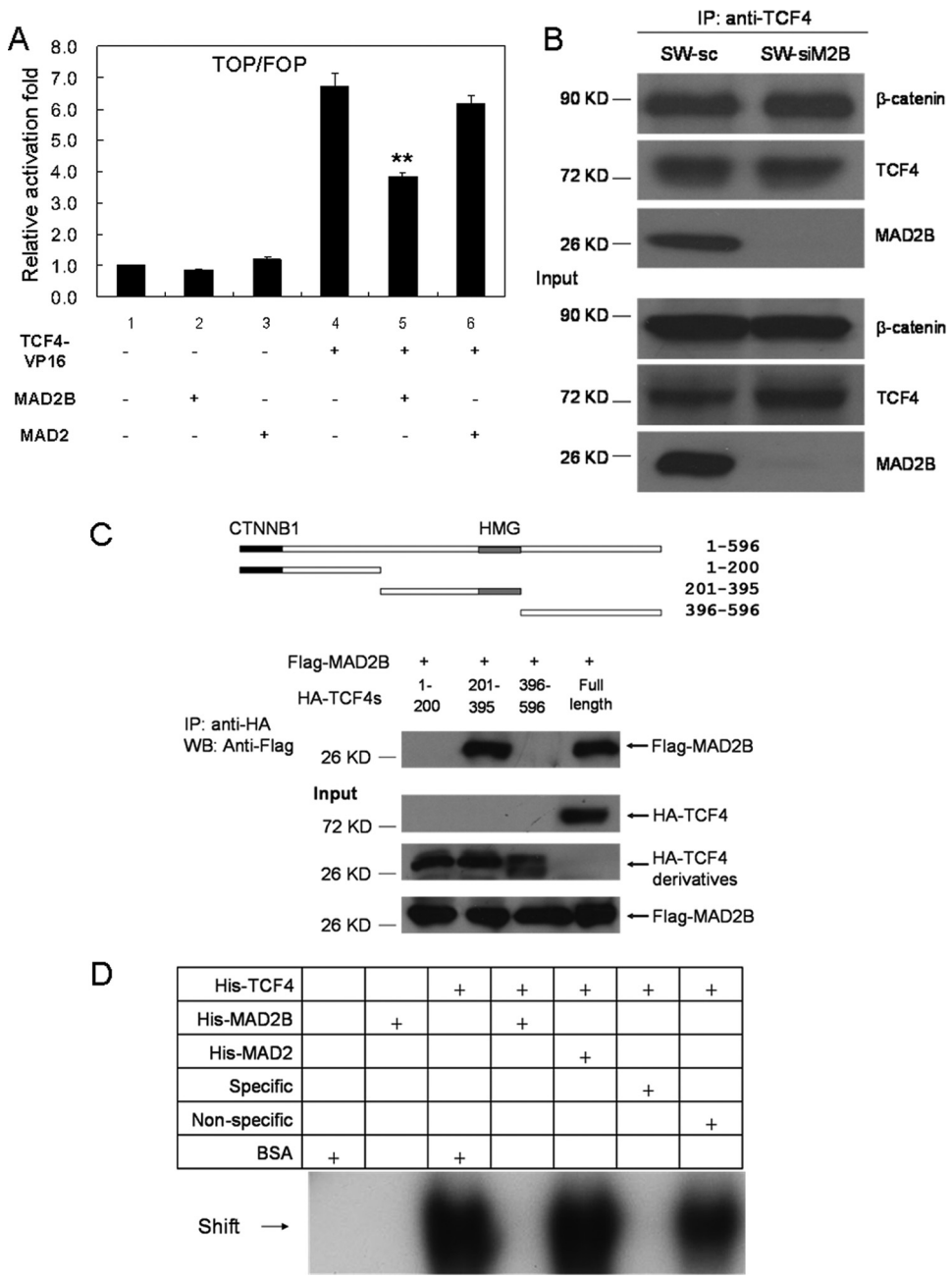


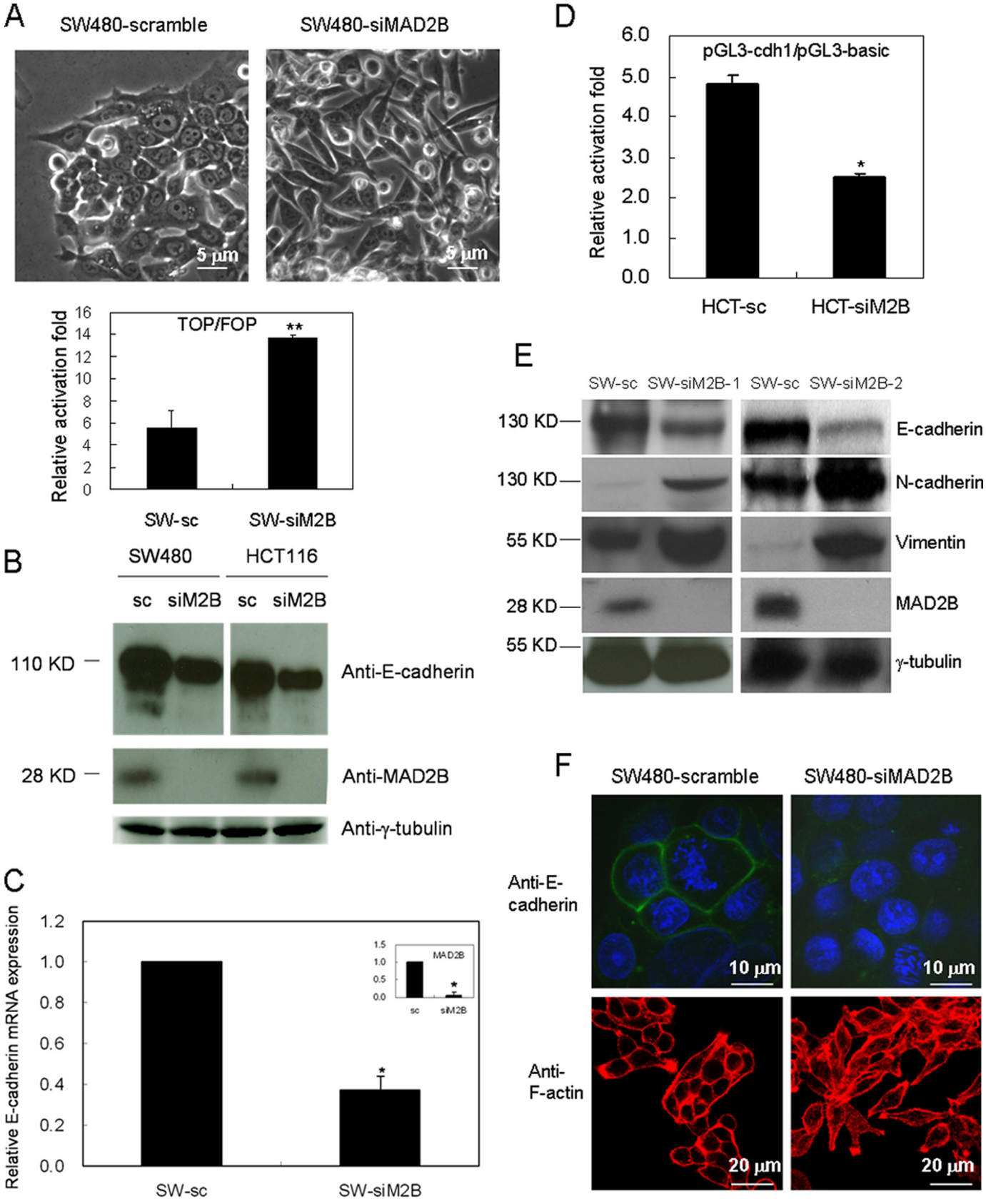
FIGURE 3. **MAD2B suppresses TCF4-mediated transcription by inhibiting the DNA binding activity of TCF4.** *A*, MAD2B suppresses TCF4-VP16-mediated transcription. TOPFLASH or FOPFLASH (0.2 μg) were co-transfected into HEK293T cells in 24-well plates with 0.2 μg of TCF4-VP16AD and/or 0.4 μg of MAD2B (or 0.4 μg of MAD2) plasmids as indicated. Relative luciferase activity was determined 48 h after transfection and is shown as the TOP/FOP ratio. pRL-TK (10 ng) was cotransfected as an internal control. Data represent the mean ± S.D. of triplicate determinations. **, $p < 0.01$ compared with control. *B*, silencing of MAD2B does not interfere with TCF4-β-catenin complex formation. SW480-scramble (*SW-sc*) and SW480-siMAD2B (*SW-siM2B*) cells were lysed 48 h after plating onto a 100-mm culture dish. Whole cell lysate was collected and immunoprecipitated (*IP*) with anti-TCF4 followed by Western blot analysis with an anti-β-catenin, anti-TCF4, and anti-MAD2B antibody. *C*, the middle region of TCF4 interacts with MAD2B. Functional domains of TCF4 and the corresponding deletion mutants are shown. HA-TCF4 derivatives expressing plasmids (amino acids 1–200, 201–395, or 396–596) were co-transfected with a FLAG-MAD2B plasmid into HEK293T cells. Whole cell lysate was collected 48 h after transfection and immunoprecipitated with anti-HA followed by Western blot (*WB*) analysis with anti-FLAG or anti-HA. *CTNNB1*, catenin-binding site; *HMG*, high mobility group. *D*, MAD2B blocks the DNA binding activity of TCF4. His-tagged TCF4 protein was purified from HEK293T cells transfected with the His-TCF4-expressing plasmid. His-tagged MAD2B and His-tagged MAD2 proteins were extracted from *E. coli* (BL21DE3). Electrophoretic mobility shift assay experiments were performed by incubating a ^{32}P-labeled TBS probe with purified His-TCF4, His-MAD2B, His-MAD2, bovine serum albumin (*BSA*), a cold specific TBS, and/or nonspecific probe as indicated. The band shift is indicated by the *arrowhead*.

cells. The medium was replaced with fresh normal culture medium 24 h later. Virus-containing supernatants were collected 48 h after transfection. Lentiviral infection was performed by adding virus solution to cells at the desired multiplicity of infection in the presence of 8 ng/ml Polybrene. Fresh normal culture medium containing a suitable puromycin concentration was added to cells 24 h after infection; cells remained under selection until all of the mock-transfected cells died. Surviving cells were pooled and cultured for further analysis.

Quantitative Real-time PCR—RNA was extracted from cultured cells using TRIzol (Invitrogen) according to the manufacturer's protocol. Extracted total RNA was then reverse-transcribed into cDNA using the oligo(dT)12–18 primer with Superscript III reverse transcriptase (Invitrogen). Twenty-fold dilutions of each cDNA were prepared for subsequent PCR amplification with the SYBR Green I Master kit using Lightcycler480 instrument (Roche Applied Science). Primer sequences were designed to detect specific genes as follows: MAD2B forward, 5′-TGCATCTCATCCTCTACGTG-3′, and reverse, 5′-TCCTGGATATACTGATTCAGC-3′; E-cadherin forward, 5′-ATCCAAAGCCTCAGGTCATAA-3′, and reverse, 5′-TTCTTGGGTTGGGTCGT-3′; Slug forward, 5′-TTCGGACCCACACATTACCT-3′, and reverse, 5′-TTGGAGCAGTTTTTGCACTG-3′; glyceraldehyde-3-phosphate dehydrogenase forward, 5′-CGACCACTTTGTCAAGCTCA-3′, and reverse, 5′-AGGGGTCTACATGGCAACTG-3′.

Chromatin Immunoprecipitation (ChIP)—The ChIP assay was performed with an EZ CHIP chromatin immunoprecipitation kit according to standard protocols (Upstate). Briefly, cells were maintained in 100-mm cell culture plates and then were fixed with formaldehyde for 10 min. Cells were lysed in SDS lysis buffer, and the chromatin DNA was extracted and sonicated into fragments 200–1000 bp in length. Immunoprecipitation was performed with

1 μg of anti-TCF4 (Upstate) or mouse normal IgG as a negative control. DNA fragments precipitated in the immunoprecipitation product were amplified and quantified with the Lightcycler480 SYBR Green I Master kit. The Slug primer sequences for the ChIP assay were: forward, 5′-TCTTCCAGTTCTTCCGATCA-3′, and reverse, 5′-GCCGCGTGCAAATTAAGTA-3′.



RESULTS

Identification and Characterization of MAD2B as a TCF4-interacting Protein—To identify TCF4 interaction partners, we used Gal4-AD-TCF4 as bait to perform a yeast two-hybrid screen in the Y190 yeast strain. MAD2B was identified as a TCF4-binding protein. The binding affinity of MAD2B for different TCF4 functional domains was tested in a yeast two-hybrid system. The middle region (amino acids 201–395) of TCF4, but not the N terminus (amino acids 1–200) or the C terminus (amino acids 396–596), interacted with MAD2B (Fig. 1*A*). To confirm whether TCF4 interacts with MAD2B or its homolog MAD2 in mammalian cells, plasmids encoding HA-tagged TCF4 and FLAG-tagged MAD2B (or FLAG-tagged MAD2) were co-transfected into HEK293T cells. Anti-HA antibody was used to precipitate the HA-TCF4-interacting complex. We found that FLAG-MAD2B, but not FLAG-MAD2, co-precipitated with HA-TCF4, suggesting that the interaction between TCF4 and MAD2B is specific (Fig. 1*B*). Conversely, when anti-FLAG antibody was used to precipitate the FLAG-MAD2B-interacting complex, HA-TCF4 was detected in the FLAG-MAD2B complex (Fig. 1*C*). We examined whether the interaction between MAD2B and TCF4 could be detected endogenously in HCT116 cells, a cell line with high endogenous expression of TCF4. Co-immunoprecipitation with mouse monoclonal antibodies against MAD2B yielded endogenous TCF4 (Fig. 1*D*). To further confirm the interaction between TCF4 and MAD2B, a glutathione *S*-transferase (GST) pulldown assay was performed. Purified TCF4 interacted with GST-MAD2B fusion protein but not GST alone (Fig. 1*E*). These data suggest that MAD2B interacts with TCF4 in both yeast and mammalian cells.

To determine the cellular localization of MAD2B and TCF4, EGFP-MAD2B and ERFP-TCF4 were co-transfected into HEK293T cells. Confocal microscopy showed that MAD2B and TCF4 both localize in the nucleus (Fig. 1*F*). During the activation of the WNT signaling, accumulated β-catenin associates with TCFs and translocates into the nucleus, leading to transcriptional activation of downstream target genes. To further examine the effect of accumulated β-catenin on interaction between MAD2B and TCF4, MAD2B and TCF4 were co-transfected into HEK293T cells together with non-degradable-β-catenin (S33Y) (Fig. 1*G*). A co-immunoprecipitation assay further demonstrated that overexpressed β-catenin did not affect the interaction between TCF4 and MAD2B, suggesting that MAD2B interaction with a TCF4 does not respond to accumulated β-catenin upon WNT signal activation.

MAD2B Suppresses TCF4-mediated Transcription—To understand the functional role(s) of the MAD2B and TCF4 interaction, we evaluated the effect of MAD2B on TCF4-mediated transactivation of the TOPFLASH plasmid, a common-used TK-based promoter reporter plasmid that contains three copies of TBS. When FLAG-MAD2B, HA-TCF4, and FLAG-β-catenin (S33Y), an active form of β-catenin, were co-transfected into HEK293T cells, MAD2B significantly attenuated TCF4-β-catenin-mediated transactivation. MAD2, the non-TCF4-interacting MAD family protein, did not, however, suppress TCF4-β-catenin-mediated transcription (Fig. 2*A*).

Deregulated TCF4-β-catenin signaling is important for the occurrence of CRC (2). For example, HCT116 cells contain mutated β-catenin, which sensitizes TCF4-mediated transcription. To evaluate the effects of MAD2B expression on TCF4-mediated transactivation in CRC, we transfected HCT116 cells with the TOPFLASH reporter with or without MAD2B expression plasmids. Co-transfection of HCT116 cells with a MAD2B expression plasmid significantly attenuated endogenous TCF4-mediated transactivation (Fig. 2*B*). To evaluate the effect of endogenous MAD2B on TCF4-β-catenin signaling, we examined TOPFLASH reporter activity in MAD2B-silenced HCT116 or control cells. MAD2B silencing promoted a 2-fold increase in TOPFLASH activity (Fig. 2*C*), thus suggesting that MAD2B negatively modulates TCF4-mediated transactivation.

Interaction of TCF4 with MAD2B Inhibits the DNA Binding Activity of TCF4—TCF4 does not contain a transactivation domain. It can, however, transactivate genes by forming a transcription factor complex in which TCF4 provides the DNA binding moiety and β-catenin (or another protein) contributes a transactivation domain. To evaluate whether MAD2B-mediated suppression of TCF4 activity is because of the blocked formation of TCF4 transcription factor complexes, we fused the VP16 transactivation domain with TCF4 and carried out the TOPFLASH reporter assay. VP16-TCF4 transactivated the TOPFLASH reporter (Fig. 3*A*). Co-transfection of HEK293T cells with MAD2B attenuated VP16-TCF4-mediated transactivation. We performed an endogenous co-immunoprecipitation assay with anti-TCF4 in control and MAD2B-silenced SW480 cells, and the result showed that MAD2B silencing did not

FIGURE 4. **MAD2B silencing induces fibroblastoid morphology and reduces E-cadherin expression.** *A*, knockdown of MAD2B induces fibroblastoid morphology. SW480 colon cancer cells were infected with lentiviral short hairpin RNA pLKO.1-siMAD2B to knock down MAD2B or with control vector (pLKO.1-scramble). Cells were pooled 48 h after puromycin selection, and 8×10^5 cells were re-plated onto 100-mm dishes. Photographs were taken with a phase-contrast microscope 72 h after seeding. SW480 cells infected with pLKO.1-siMAD2B and pLKO.1-scramble were further applied to the TOPFLASH reporter assay as described in Fig. 2*C*. **, $p < 0.01$ compared with control. *B*, knockdown of MAD2B (*siM2B*) reduces E-cadherin protein expression. Whole-cell lysate from cells as in *A* was collected and subjected to Western blot analysis with antibodies against E-cadherin and MAD2B. *C*, knockdown of MAD2B reduces E-cadherin mRNA expression. Cells as in *A* were treated with Trizol. Total RNA was harvested and used for quantitative real-time PCR with E-cadherin and MAD2B primers. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. The *inset graph* showed that MAD2B mRNA level was knocked down in SW480 cells infected with pLKO.1-siMAD2B. Data represent the mean ± S.D., $n = 3$. *, $p < 0.05$ compared with control. *D*, MAD2B silencing down-regulates E-cadherin transcription. pGL3-cdh1-promoter reporter plasmid (0.25 μg) was transfected into HCT116-scramble and HCT116-siMAD2B cells in 6-well plates, and the relative luciferase activity was examined. pRL-TK (50 ng) was used as an internal control. Results are presented as the ratio of pGL3-cdh1 to pGL3-basic. Data represent the mean ± S.D., $n = 3$. *, $p < 0.05$ compared with control. *E*, MAD2B silencing in SW480 cells induces expression of the mesenchymal markers N-cadherin and vimentin. Two MAD2B-silenced SW480 cells (SW-siM2B and SW-siM2B-2) and SW480-scramble cells (1×10^6) were seeded onto 100-mm culture dishes, and cell lysate was collected 36 h later and used for Western blot analysis with antibodies against E-cadherin, N-cadherin, vimentin, MAD2B, and γ-tubulin. *F*, MAD2B silencing in SW480 cells attenuates E-cadherin expression and induces F-actin redistribution. Cells as in *A* were subjected to immunofluorescence staining. SW480-scramble and SW480-siMAD2B cells were stained for E-cadherin with mouse anti-E-cadherin (1:50) followed by fluorescein isothiocyanate-conjugated anti-mouse IgG (1:100). Nuclei were stained with 4′,6-diamidino-2-phenylindole (*blue*). F-actin staining was performed with Alexa-555-phalloidin (1:40).

interfere with the TCF4-β-catenin complex formation (Fig. 3*B*). These data suggest that MAD2B represses TCF4-mediated transcription via a mechanism that is independent of TCF4-β-catenin transcription complex. The yeast two-hybrid assay described above suggests that the middle region of TCF4, which harbors a DNA binding high mobility group domain, interacts with MAD2B. To determine whether the middle region of TCF4 is necessary for the MAD2B interaction in mammalian cells, HA-tagged TCF4-derived plasmids encoding the N-terminal (amino acids 1–200), middle (amino acids 201–395), and C-terminal (amino acids 396–596) domains as well as full-length TCF4 were co-transfected into HEK293T cells with FLAG-MAD2B. Anti-HA was used to immunoprecipitate the HA-TCF4 derivatives-interaction complex. The middle region of TCF4 as well as the full-length construct, but not the N terminus or C terminus, interacted with MAD2B (Fig. 3*C*). Because the middle region of TCF4 also harbors the high mobility group DNA binding domain, we determined whether interaction with MAD2B hinders the ability of TCF4 to bind DNA. An electrophoretic mobility shift assay revealed that TCF4 binds to a P^{32}-labeled TBS and a cold specific TBS competes for TCF4 binding, suggesting that the interaction between TCF4 and the TBS is specific (Fig. 3*D*). MAD2B, but not MAD2, abolished the TBS binding ability of TCF4, suggesting that MAD2B inhibits the DNA binding ability of TCF4.

MAD2B Modulates Mesenchymal-Epithelial Transdifferentiation—To understand the biological function of MAD2B in CRC, we knocked down MAD2B in SW480 cells using lentiviral-based short hairpin RNA. MAD2B knockdown in SW480 cells led to significant morphological changes, including a fibroblastoid phenotype (Fig. 4, *A* and *F*). TCFs-β-catenin signaling regulates expression of E-cadherin, which is responsible for the maintenance of epithelial cell shape (11, 15, 26). Because MAD2B negatively modulates TCF-4-β-catenin signaling, we examined whether MAD2B silencing-induced morphological changes result from altered E-cadherin expression. Knockdown of MAD2B in HCT116 and SW480 cells significantly reduced both protein and mRNA levels of E-cadherin (Fig. 4, *B* and *C*). E-cadherin is down-regulated by TCF4-β-catenin signaling (27). The promoter region of E-cadherin (nucleotides −481 to +127, transcription start site at +1), which contains major regulatory elements, was cloned into a pGL3-basic plasmid (referred to as pGL3-cdh1) to determine whether E-cadherin promoter activity is influenced by MAD2B silencing. MAD2B knockdown down-regulated E-cadherin promoter activity 2-fold (Fig. 4*D*), suggesting that MAD2B enhances E-cadherin expression.

TCFs-β-catenin transcription factor up-regulates mesenchymal genes and participates in EMT (11, 15, 26). Because MAD2B knockdown in SW480 cells led to morphological changes and enhanced TCFs-β-catenin signaling, we examined whether MAD2B modulates EMT expression. Western blotting showed that mesenchymal-associated markers, such as N-cadherin and vimentin, were up-regulated in MAD2B-silenced SW480 cells, and expression of the epithelial marker E-cadherin was decreased (Fig. 4*E*). E-cadherin was expressed at cell-cell junctions of SW480 cells, and MAD2B knockdown attenuated E-cadherin expression (Fig. 4*F*). Knockdown of MAD2B also led to F-actin re-distribution and loss of cell-cell junctions (Fig. 4*F*), thus suggesting that MAD2B modulates EMT expression.

MAD2B Regulates TCF4-mediated Slug Expression—Slug mediates TCFs-β-catenin-induced down-regulation of E-cadherin (26, 27). Because MAD2B up-regulates E-cadherin expression and antagonizes TCF-β-catenin signaling, we determined whether MAD2B silencing-induced EMT reflects altered Slug expression. Both quantitative real-time PCR and Western blot analysis showed that knockdown of MAD2B in SW480 cells significantly increased both protein and mRNA levels of Slug, suggesting that MAD2B suppresses Slug expression (Fig. 5, *A* and *B*).

TCFs bind directly to the promoter region of Slug and activate Slug expression upon WNT signaling (26, 27). A TCF consensus binding sequence (TACAAAGC) similar to the corresponding sequences in the mouse and *Xenopus* Slug promoters was identified within 1 kilobase upstream of the human Slug promoter (26). Because MAD2B antagonized TCF4-β-catenin signaling and blocked EMT expression, we hypothesized that MAD2B may antagonize TCF4-β-catenin-induced EMT by blocking the ability of TCF4 to bind the Slug promoter. After a ChIP assay with anti-TCF4 to immunoprecipitate the TCF4-DNA complex, real-time PCR was used to evaluate the ability of TCF4 to bind the Slug promoter. TCF4 bound the Slug promoter, and MAD2B silencing enhanced TCF4 binding to the Slug promoter (Fig. 5*C*), thus suggesting that MAD2B negatively modulates Slug expression by interfering with the DNA binding ability of TCF4.

DISCUSSION

The TCF4-β-catenin complex is the major transcriptional effector of WNT signaling in intestinal cells. Aberrant expression and abnormal activation of the TCF4-β-catenin complex is associated with several tumor progresses, especially in CRCs (2, 5). How the activity of TCF4-β-catenin is regulated under normal physiological conditions and during tumorigenic progression is an important issue. We have previously shown that Daxx, a human cell death-associated protein, interacts with the TCF4-β-catenin complex, leading to decreased expression of cyclinD1 and cell cycle arrest at the G_1 phase (28). In this study MAD2B was identified as a TCF4-interacting protein. MAD2B altered the DNA binding ability of TCF4 and, therefore, reduced TCF4 transcriptional activity. MAD2B also modulated expression of Slug, enhanced E-cadherin expression, and attenuated N-cadherin and vimentin expression. Hence, it is a negative regulator of EMT (Fig. 6).

MAD2B was first identified as an inhibitor of the anaphase-promoting complex (APC)-Cdh1 (20, 29). Through Cdh1 binding, MAD2B inhibits the activity of the Cdh1-APC complex and, thus, functions as a mitotic spindle assembly checkpoint protein, preventing the onset of anaphase until all chromosomes are properly aligned at the metaphase plate. We have found a novel role for MAD2B as an inhibitor of the TCF4-β-catenin complex, which thus prevents EMT in colon cancer cells. The TCF4-β-catenin complex also determines the balance between cell proliferation and differentiation (9). Because MAD2B functions both as a mitotic spindle assembly check-

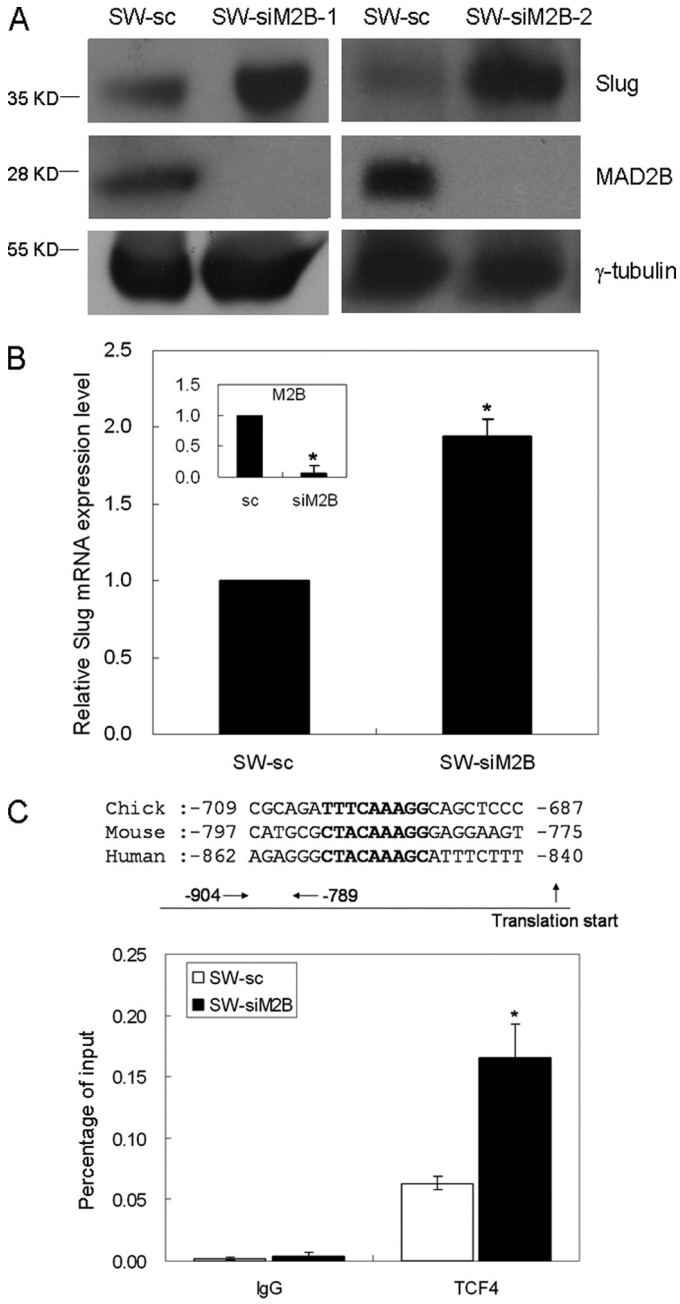


FIGURE 5. **MAD2B silencing induces Slug expression and promotes the Slug promoter binding activity of TCF4.** *A*, MAD2B silencing induces Slug protein expression. 1×10^6 of SW480-scramble and SW480-siMAD2B cells (*SW-siM2B* and *SW-siM2B-2*) were seeded onto 100-mm culture dishes, and total cell lysate was collected 36 h later and subjected to Western blot analysis with antibodies against Slug and MAD2B. *B*, MAD2B silencing induces Slug mRNA expression. Total RNA was harvested from SW480-scramble and SW480-siMAD2B cells as in *A* and used for quantitative real-time PCR with primers for Slug and MAD2B. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase. The *inset graph* showed that MAD2B mRNA level was knocked down in SW480-siMAD2B cells. Data represent the mean ± S.D., $n = 3$. *, $p < 0.05$ compared with control. *C*, MAD2B silencing enhances the ability of TCF4 to bind the Slug promoter. SW480-scramble and SW480-siMAD2B cells were treated with formaldehyde and subjected to a ChIP assay with TCF4 antibodies. Precipitated genomic DNA was used for quantitative real-time PCR with primers for the human Slug promoter region. The real-time PCR target contained the consensus TBS, which is highly homologous with the TBS in chick and mouse Slug promoters (*upper half* of *panel C*). Conservative TBS was *highlighted in bold*. Normal mouse IgG was used as an antibody control. Data are presented as the percentage of input control. Experiments were performed in duplicate. *, $p < 0.05$ compared with control.

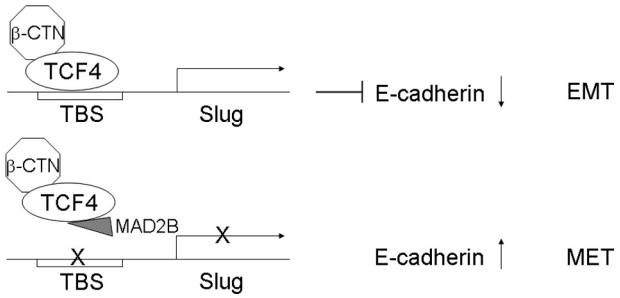


FIGURE 6. **Model of functional Interplay between MAD2B and TCF4 in modulation of epithelial-mesenchymal transdifferentiation.** TCF4-β-catenin complex binds the Slug promoter on TBS and transactivates Slug expression, which suppresses transcription of E-cadherin and induces EMT. Interaction of TCF4 with MAD2B inhibits the DNA binding ability of TCF4, which attenuates transactivation of Slug, thus releasing the suppression of E-cadherin and leading to mesenchymal-epithelial transdifferentiation (*MET*). *β-CTN*, β-catenin.

point protein and a negative regulator of EMT, it is possible that MAD2B plays a key role in the modulation of TCF4-β-catenin complex-mediated cell fate. Further studies are required to determine whether MAD2B is involved in coupling cell mitotic events with differentiation.

Recently, it has been shown that *Shigella* uses special tactics to target MAD2B to influence epithelial renewal and promote bacterial colonization of intestinal epithelium (30). IpaB, a *Shigella* effector, binds MAD2B, leading to the release of activated Cdh1-APC complex, which causes G_2/M phase arrest and the attenuation of gut epithelium self-renewal, thus allowing bacterial colonization. MAD2B could be a hijack target for diseases in which controlling deregulated cell growth and differentiation is desirable. The reverse process of EMT, mesenchymal-epithelial transition, is also critical for the processes of cancer metastasis, such as colonization (31). Our work indicates that MAD2B knockdown in SW480 cells leads to an increase in the E-cadherin transcriptional suppressor Slug, shifting the balance from EMT to mesenchymal-epithelial transition. Because MAD2B acts as a mitotic checkpoint protein and also participates in genomic stability and mesenchymal-epithelial transition, it is possible that MAD2B may be a direct hijack target during cancer progression. Indeed, MAD2B is underexpressed in chromophobe tumors of renal cell carcinoma (32). Although there are no reports of MAD2B knock-out or transgenic mice, MAD2 heterozygous mice develop lung cancer, and MAD2 transgenic mice develop lymphoma and lung cancer, suggesting that deregulation of the MAD family of proteins induces tumorigenesis (33, 34). MAD2B is overexpressed in both colon and breast cancer (35, 36). TCF4-β-catenin signaling plays important roles in various stages of cancer development. Our finding that MAD2B modulates TCF4-β-catenin-mediated EMT provides a novel mechanism for the involvement of MAD2B in cancer development.

In TCF4 downstream target genes we found c-Myc other than Slug was negatively regulated by MAD2B, supporting that MAD2B modulates cell proliferation and differentiation (supplemental Fig. 1). However, some TCF4 downstream target genes such as cyclin D1 and MMP7 were not significantly affected in MAD2B-silenced cells. Because there is thought to be some functional redundancy within the TCFs family (37, 38),

this observation raises the possibility whether other members of TCFs family may compensate for the inhibitory effect of MAD2B on TCF4-mediated transcription in a promoter-specific manner. In this study we found that MAD2B interacts with TCF4, but MAD2 does not. Members of the MAD family have different preferences for associated proteins. MAD2B, for example, prefers to bind Cdh1, but MAD2 forms a complex with cdc20 to inhibit APC activity (20). Whether MAD2B or MAD2 binds other members of the TCF family and modulates TCF/β-catenin complex-mediated transcription requires further study. p31 (comet) interacts with MAD2 and antagonizes the ability of MAD2 to inhibit the cdc20-APC complex (39). Thus, it will be important to identify proteins that antagonize the functions of MAD2B. In addition to TCF4 and Cdh1, HCCA2 and Elk-1 also interact with MAD2B and participate in cell-cycle regulation and the mitogen-activated protein kinase (MAPK) pathway, respectively (22, 40). We are currently addressing whether HCCA2 and Elk-1 can bind MAD2B and neutralize MAD2B-mediated suppression of the TCF4-β-catenin complex.

MAD2B modulates expression of Slug, a TCF4-β-catenin-inducible gene, and E-cadherin, a downstream target gene of Slug. Both sequence predictions and the ChIP assay indicated that TCF4 binds to the Slug promoter. Other cis-regulatory elements are also observed on the Slug promoter. In addition to WNT, transforming growth factor-β and bone morphogenetic protein-Smad signaling regulate Slug and subsequently elicit EMT (26). Thus, cooperation between MAD2B and other signaling pathways may be required for fine-tuning WNT-mediated regulation of EMT.

In summary, we have identified MAD2B as a novel TCF4 interaction partner that suppresses TCF4-mediated transactivation by interfering with its DNA binding activity. This report demonstrates that MAD2B affects TCF4-mediated EMT through the regulation of Slug expression and provides a novel mechanism for MAD2B-induced deregulation of cell growth and differentiation.

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