Mastermind-like 1 Is a Specific Coactivator of β-Catenin Transcription Activation and Is Essential for Colon Carcinoma Cell Survival

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Abstract

Misregulation of the Wnt signaling pathway has been linked to many human cancers including colon carcinoma and melanoma. The primary mediator of the oncogenic effects of the Wnt signaling pathway is β-catenin. Accumulation of nuclear β-catenin and transcription activation of lymphoid enhancer factor 1 (LEF1)/T-cell factor (TCF) target genes underlie the oncogenic activity. However, the mechanism of β-catenin–mediated transcriptional activation remains poorly understood. In this study, we identified Mastermind-like 1 (Maml1), which is thought to be a specific coactivator for the Notch pathway, as a coactivator for β-catenin. We found that Maml1 participates in the Wnt signaling by modulating the β-catenin/TCF activity. We show *in vivo* that Maml1 is recruited by β-catenin on the cyclin D1 and c-Myc promoters. Importantly, we show that Maml1 functions in the Wnt/β-catenin pathway independently of Notch signaling. Finally, we show that the knockdown of Mastermind-like family proteins in colonic carcinoma cells results in cell death by affecting β-catenin–induced expression of cyclin D1 and c-Myc. This is the first demonstration of a role for the Mastermind-like family in another signaling pathway and that the knockdown of Mastermind-like family function leads to tumor cell death. [Cancer Res 2007;67(18):8690–8]

Introduction

The Wnt/β-catenin signaling pathway is vital for proper development of organisms from fly to human. Wnt signaling affects cellular decisions by modulating distinct processes such as differentiation and proliferation. For example, Wnt signaling regulates development of human stem cells of the intestine and epidermis by controlling cell fate along crypt-villus axis and hair formation (1). In addition to a vital role in development, misregulation of the Wnt signaling pathway has been linked to many human cancers including colon carcinoma and melanoma (2, 3).

A general scheme for Wnt/β-catenin signaling has been established. The binding of Wnt to Frizzled and LDL cell-surface receptors initiates a cascade of signaling events, including the recruitment of Dishevelled and the subsequent inactivation of glycogen synthase kinase 3β (GSK-3β). The inhibition of GSK-3β phosphorylation of β-catenin leads to the stabilization of cytoplasmic β-catenin and its translocation into the nucleus (4, 5). In the nucleus, β-catenin interacts with the T-cell factor (TCF) family of transcription factors [TCF1, lymphoid enhancer factor (LEF), TCF3, and TCF4], converting them from repressors to activators (6). It does this, in part, by recruiting nuclear factors such Bcl9/Legless coactivator via the central armadillo (ARM) repeats, thus permitting an association with Pygopus. The role of Pygopus/Legless in β-catenin transcription activation is not clear; however, they seem to function by recruiting chromatin remodeling factors or by transporting β-catenin in the nucleus (7).

In the absence of Wnt signaling, β-catenin is sequestrated in the cytoplasm by a destruction complex containing adenomatous polyposis coli (APC), protein phosphatase 2, and axin. In this complex, β-catenin is phosphorylated by casein kinase Iα and GSK-3β. Phosphorylation of β-catenin by these kinases leads to ubiquination by β-TrCP and degradation by the proteasome. This mechanism has evolved to tightly regulate the levels of β-catenin activity in the nucleus.

A grave consequence of aberrant Wnt signaling is constitutive transcriptional activation of β-catenin/TCF target genes, associated with various important processes of tumorigenesis such as sustained cellular proliferation in the absence of growth signals (1, 2, 8). Because TCF is critical for tumorigenesis, identification of modulators of its activity is critically important for development of novel therapeutics. This concept is further supported by experiments using a TCF-VP16 chimera that constitutively activates β-catenin target genes such as *cyclin D1*, a critical gene in G_1 progression, and causes cellular transformation (9, 10).

Mastermind-like 1 (Maml1) is an integral component of the Notch pathway whose function is poorly understood. *Maml1* encodes a nuclear coactivator protein that binds to the ankyrin repeat domain of Notch proteins. It forms a trimeric complex with the intracellular domain of Notch and the DNA binding protein, CSL (11). It is thought that Maml1 functions, at least in part, by recruiting histone acetyltransferases such as p300 (12).

Here, we report that Maml1 acts as a specific coactivator of β-catenin/TCF. We show *in vivo* that Maml1 is recruited by β-catenin to a TCF site on the *cyclin D1* promoter. Importantly, we show that Maml1 functions in the Wnt/β-catenin pathway independently of Notch signaling. Finally, we show that deletion of the Mastermind-like family in colon carcinoma cells results in cell death due to a failure to sustain β-catenin–mediated expression of cyclin D1 and c-Myc. These data support a new role for Maml1 as an integral component of the Wnt pathway and it is essential in β-catenin–mediated tumorigenesis.

Materials and Methods

Cell culture and transient transfections. Human HeLa, H1299, 293T, and SW480 cells and rat RKE cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Inc.).

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Transfections were carried out with Lipofectamine 2000 (293T and RKE), Fugene (H1299 and SW480), and Lipofectamine (HeLa) according to the manufacturer's instructions.

Spodoptera frugiperda IPLB-Sf21 (Sf21 cells) were maintained in Sf-900 II SFM medium (Life Technologies) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies).

Baculovirus generation and purification. For recombinant baculovirus production, Bacmids containing cDNAs of *Maml1* and *β-catenin* were generated following the Bac-to-Bac baculovirus expression system (Invitrogen). Bacmids were transfected into Sf21 cells using Cellfectin reagent (Invitrogen). Recombinant baculovirus was amplified and titered. The expression of the recombinant proteins was confirmed by Western blot analysis of cell lysates. Recombinant Flag-tagged baculovirus-expressed proteins were purified using Flag M2 agarose beads (Sigma) and eluted with Flag peptide. Recombinant His-tagged baculovirus-expressed proteins were purified using Ni-NTA agarose beads (Qiagen). His-tagged proteins were eluted with elution buffer containing 250 mmol/L imidazole. All baculovirus-expressed proteins were dialyzed in storage buffer [100 mmol/L KCl, 20 mmol/L HEPES (pH 7.9), 20% glycerol, and 1 mmol/L DTT].

Antibodies. The following antibodies were purchased from commercial sources: mouse anti-Flag antibody (clone M2, Sigma), mouse anti–β-catenin antibody (BD Transduction Laboratories), and horseradish peroxidase–coupled donkey anti-mouse antibody and anti-rabbit antibodies (Jackson ImmunoResearch Laboratories).

Western blotting. Cell lysates were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked and incubated with anti-Flag antibody (Sigma), anti–β-catenin, or anti-Maml1 antibodies followed by incubation with the antimouse or antirabbit antibody conjugated with horseradish peroxidase. For detection, enhanced chemiluminescence reaction (Amersham Biosciences) was done according to the manufacturer's specification.

Reporter luciferase assays. HeLa, H1299, and SW480 cells were seeded on six-well plates at 100,000 per well 1 day before transfection and then transfected with various combinations of expression plasmid DNA corresponding to a final amount of 2 μg of DNA. The total amount of plasmids was maintained constant by adding appropriate amounts of empty vectors without inserts. The transfected cells were harvested at 48 h posttransfection and luciferase activities were measured with the luciferase reporter assay system (Promega). Luciferase values were corrected for transfection efficiency by normalizing to β-galactosidase activity.

Coimmunoprecipitation assays. Forty-eight hours after transfection, cells were lysed for 30 min at 4°C using NP40 lysis buffer [150 mmol/L NaCl, 50 mmol/L HEPES (pH 7.4), 1.5 mmol/L EDTA, 10% glycerol, 1% NP40, supplemented with 0.5 mmol/L DTT, 0.2 mmol/L Pefabloc, 1 μg/mL leupeptin, 1 μg/mL aprotinin (Roche), 50 mmol/L NaF, and 0.5 mmol/L vanadate]. After centrifugation, supernatants were incubated overnight at 4°C with anti–β-catenin antibody, anti-Maml1 antibody, or Flag M2 beads. The immunocomplexes were washed extensively with lysis buffer, and the precipitates were boiled in Laemmli buffer and assayed by Western blot.

For purified proteins, 100 to 200 ng of Maml1-Flag and β-catenin-His were mixed together and incubated in *in vitro* complex buffer [250 mmol/L NaCl, 100 mmol/L KCl, 20 mmol/L HEPES (pH 7.9), 20% glycerol, 1% NP40, supplemented with 0.5 mmol/L DTT, 0.2 mmol/L Pefabloc, 1 μg/mL leupeptin, 1 μg/mL aprotinin (Roche)]. Proteins were immunoprecipitated with anti-Flag or anti–β-catenin antibodies or immunoglobulin G (IgG) for 4 h followed by the addition of protein G beads for 30 min. Samples were extensively washed with *in vitro* complex buffer and analyzed by Western blot.

Chromatin immunoprecipitation experiments. Three million cells were plated 24 h before cross-linking. The chromatin immunoprecipitation assay followed the protocol provided by Upstate Biotechnology. Immunoprecipitated DNA was analyzed by PCR. PCR primers amplified regions specific to the TCF binding site of human *cyclin D1* promoter gene (forward, 5′-GAGCGCATGCTAAGCTGAAA-3′; reverse, 5′-GGACAGACGGCCAAAGAATC-3′). PCR products were analyzed on 2% agarose/Tris-borate EDTA gels with ethidium bromide staining. PCR reactions using input DNA before immunoprecipitation were used as controls. PCR primers amplifying ALU repeat region were used as negative controls (forward, 5′-CGACTTCAAGACAATCATTGCTGTG-3′; reverse, 5′-GGTGGTTAAATAAAGAAGCCAGCC-3′).

Small interfering RNA transfections. hMaml1, hMaml2, hMaml3, and human β-catenin small interfering RNAs (siRNA) were purchased from Dharmacon, Inc. (siGenome SMARTpool M-013417, M-013568, M-013813, and M-003482). The control GL3 siRNA was purchased from Dharmacon (Luciferase GL3 duplex D-001400-01). siRNA transfections were done with SW480 cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions, using a final concentration of 120 nmol/L for each siRNA in the culture medium. Two transfections were assayed successively with time interval of 24 h. After 72 h, the number of cells per well was counted and the results were presented as a percentage of the control siRNA.

Results

Maml1 acts with β-catenin to enhance cyclin D1 transcription. Considering the function of Maml1 in the formation of an active Notch transcription complex, we reasoned that Maml1 might play a more general role in transcriptional regulation. We sought to examine its role in activation of other pathways. One such pathway, Wnt/β-catenin, was investigated because both β-catenin and Notch are involved in converting a transcriptional repressor into a transcriptional activator. Therefore, we asked whether Maml1 could modulate β-catenin target gene activity. We used a *TOP-FLASH* luciferase reporter that contains multiple copies of an optimal TCF-binding site (13). Transfection of β-catenin in HeLa cells increased TCF-directed luciferase activity 2-fold compared with pcDNA alone (Fig. 1*A*). In contrast, there is a dramatic increase (30×) in activity when Maml1 is cotransfected with β-catenin (Fig. 1*A*). β-Catenin and Maml1 had no effect on the *TOP-FLASH* luciferase reporter, which contains multiple copies of mutant form of TCF binding sites, indicating that Maml1 has a TCF-specific effect. To examine if this coactivation was specific to Maml1, we tested the other Mastermind-like family members. Maml2 and Maml3 were also able to potentiate the β-catenin activity at approximately the same level as Maml1, suggesting that all the Mastermind-like family members could be involved in the Wnt pathway.

Because β-catenin protein stability is a major factor governing its capacity to transactivate target gene promoters, we examined whether β-catenin levels altered the ability of Maml1 to potentiate activation. The phosphorylation in the NH_2-terminal region influences the stability of β-catenin. The Ser/Thr residues are phosphorylated by GSK-3β and targeted by β-TrCP for ubiquination and degradation, thereby mutations of these phosphorylation sites stabilize the protein. To test if Maml1 acts as a coactivator in the presence of high levels of β-catenin, we expressed a mutant form of the β-catenin protein in which Ser^{37} has been changed to alanine. This mutation renders β-catenin resistant to GSK-3β phosphorylation and targeting to the proteasome, which leads to constitutive activation of β-catenin-TCF target genes (14). When β-catenin S37A and Maml1 were expressed simultaneously, reporter gene activity increased in a dose-dependent manner up to an additional 12-fold level compared with β-catenin S37A alone (10× versus 120× activity; Fig. 1*B*). The presence of Maml1 did not affect the stability of the β-catenin S37A protein (Fig. 1*B*). These results indicate that Maml1 does not act to stabilize β-catenin but acts to regulate the activity of the promoter.

To evaluate the physiologic relevance of the β-catenin coactivation by Maml1, we chose to test an authentic target of

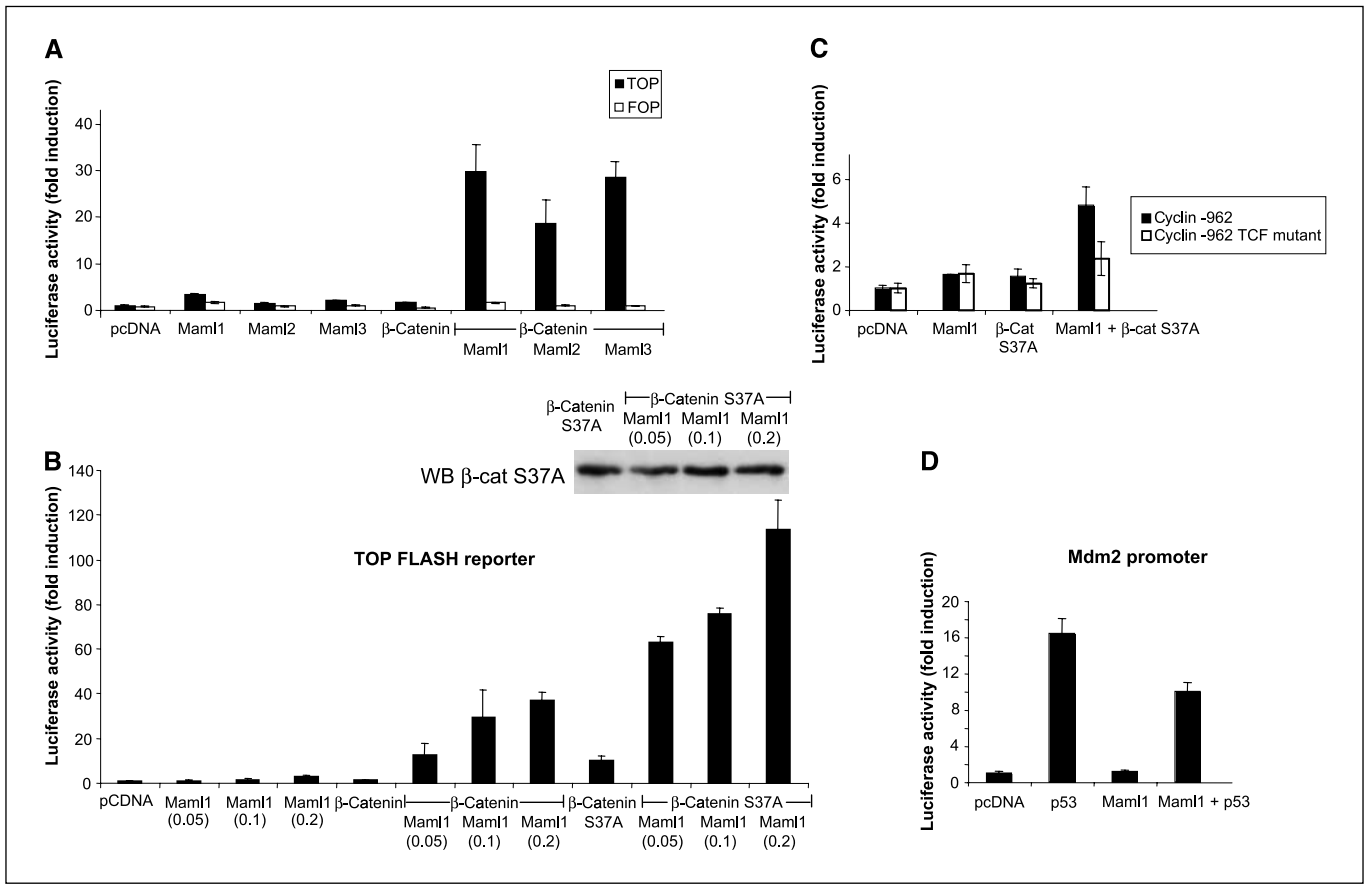

Figure 1. Maml1 acts with β-catenin to enhance cyclin D1 transcription. *A,* HeLa cells were cotransfected with 0.4 μg of TOP-FLASH luciferase reporter (*black*) or FLOP luciferase reporter (*white*), 0.4 μg of β-catenin, and 0.2 μg of Maml1, Maml2, or Maml3. Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing vector pcDNA alone. *B,* HeLa cells were cotransfected with 0.4 μg of TOP-FLASH luciferase reporter, different amounts of Maml1 (0.05–0.2 μg), 0.4 μg of β-catenin or β-catenin S37A, and 0.2 μg of Maml1, Maml2, or Maml3. Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing vector pcDNA alone. *C,* HeLa cells were cotransfected with 0.4 μg of cyclin D1 (−962) luciferase reporter (*black*) or cyclin D1 (−962) TCF mutant luciferase reporter, 0.2 μg of Maml1, and 0.4 μg of Flag-tagged β-catenin S37A. Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing pcDNA vector alone. The expression of Flag-tagged β-catenin S37A was detected by Western blot analysis with anti-Flag antibody. *D,* HeLa cells were cotransfected with 0.4 μg of Mdm2 luciferase reporter, 0.2 μg of Maml1, and 0.4 μg of p53. Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing pcDNA vector alone. The experiments were done in triplicate each time and were repeated at least twice. *Columns,* mean; *bars,* SE.

β-catenin, the *cyclin D1* promoter. The *cyclin D1* promoter fragment used encompasses sequences from −973 to +134 relative to the transcriptional start site and includes the β-catenin TCF response element around position −75 (10). We show that Maml1 potentiates the activity of β-catenin to activate the *cyclin D1* transcription (Fig. 1*C*). Figure 1*C* shows that deletion of TCF binding site −75, which prevented β-catenin–mediated activation, affected Maml1-mediated enhancement in the −962 cyclin D1 reporter. Thus, we confirmed the specificity of the activation due to TCF. Importantly, the effects of β-catenin and Maml1 on the *cyclin D1* reporter gene activity were strictly dependent on the presence of optimal TCF-binding sites within the *cyclin D1* promoter, indicating that binding of TCF to target sites mediates recruitment of both proteins (Fig. 1*C*).

To ensure the specificity of the transcriptional activation on the *cyclin D1* promoter, we tested the activity of Maml1 on an *Mdm2*-luciferase reporter. The expression of p53 was able to activate transcription of *Mdm2*, whereas Maml1 alone did not modify *Mdm2* transcription. *Mdm2* reporter activity did not increase in the presence of Maml1 or β-catenin either alone or in combination (Fig. 1*D*). Therefore, Maml1 shows promoter and transcription factor specificity and is not simply affecting transcription in a general manner.

The COOH-terminal region of Maml1 is critical to the activity in Notch and Wnt pathways. To determine which region of Maml1 is important for the activity of the *cyclin D1* promoter in the β-catenin pathway, we generated Maml1 truncation mutants and tested their signaling activities in H1299 cells (Fig. 2*A* and *B*). We compared the effects of the *Maml1* truncations on both the Notch and β-catenin pathways to determine if coactivation of Nic and β-catenin requires similar domains of Maml1. First, we verified by Western blot that the mutants were expressed at similar levels (Fig. 2*B*). We then cotransfected H1299 cells with the combination of *β-catenin*, *Maml1*, and either the −1,748 *cyclin D1* or *8xCSL* reporter construct. Truncating the COOH-terminal amino acid residues (844–1,016) of Maml1 decreased its transcriptional β-catenin signaling activity by ∼55% (Fig. 2*C, lane 4*). The 1–640 and 1–305 mutant forms of Maml1 do not efficiently coactivate with β-catenin the *cyclin D1* reporter gene (Fig. 2*C, lanes 5* and *6*). Therefore, the COOH-terminal region (640–1,016) contains a domain responsible for most of the transcriptional activation, for both β-catenin and Notch activity (Fig. 2*D, lanes 4–6*). Thus,

we identified a critical region at 640–1,016 amino acids for the activity of Maml1 in both pathways. The mutant 1–305 is a dominant negative in the Notch pathway and, indeed, we observed less transcription activation by this mutant than with Notch intracellular active form (Nic) alone. Interestingly, when the sequence 1–305 is fused to the COOH-terminal part (640–1,016), we restored the stimulatory effect of Maml1 on *cyclin D1* and *8xCSL* reporters (Fig. 2*C* and *D, lane 8*). This finding is consistent with the notion that the COOH-terminal region of Maml1 functions as the major transcriptional activation domain.

We conclude from these assays that a functional consequence of Maml1 expression is enhanced transcriptional activity of TCF/β-catenin complexes on target gene promoters. An efficient transactivation is dependent on an intact domain between amino acids 640 and 840 of Maml1.

β-Catenin and Maml1 interact *in vitro* and *in vivo*. The observation that Maml1 promotes transcription of *cyclin D1* via β-catenin suggests that a physical interaction could exist between β-catenin and Maml1. We asked whether Maml1 interacts directly *in vitro* with β-catenin by coimmunoprecipitation experiments. His-tagged β-catenin protein and Maml1-Flag protein were purified independently from infected Sf21 cells and incubated together to assess association. With an anti-Maml1 antibody, we coimmunoprecipitated β-catenin and Maml1-Flag proteins (Fig. 3*A, lane 2*); Maml1 is coimmunoprecipitated with β-catenin using an anti–β-catenin antibody (Fig. 3*A, lane 3*). As a control, neither Maml1 nor β-catenin immunoprecipitated with IgG (Fig. 3*A, lane 1*). Therefore, we show that Maml1 is stably associated with β-catenin *in vitro*.

To determine whether the interaction we observed between β-catenin and Maml1 in the *in vitro* complex system also occurs *in vivo*, we examined the interaction in a β-catenin transformation assay. β-Catenin has been shown to transform RKE cells. Therefore, we produced transformed RKE cells by transfection with a Flag-tagged β-catenin S37A and transformation was confirmed by colony formation in soft-agar assays (Fig. 3*B*). A coimmunoprecipation experiment was then used to show an association between

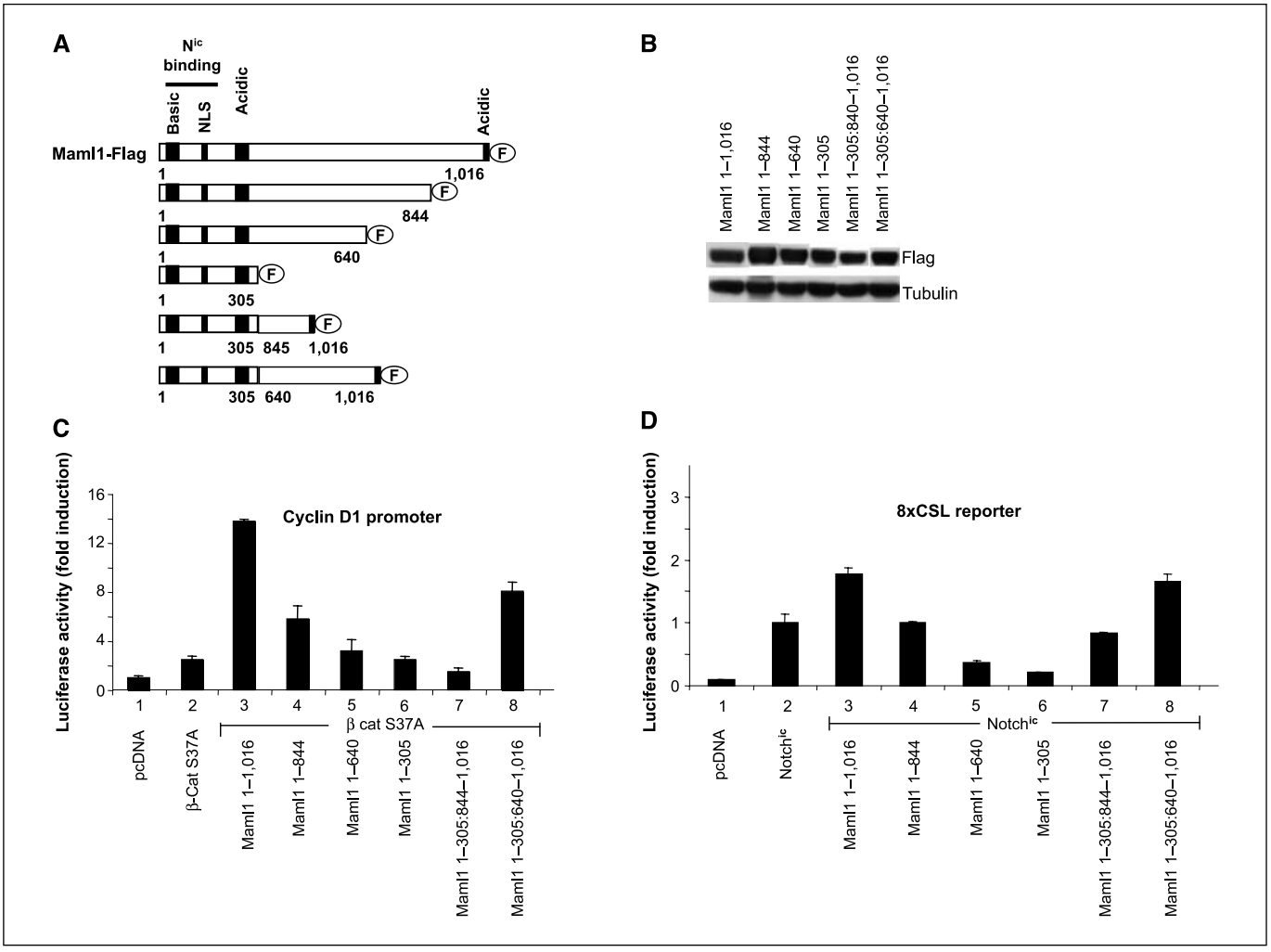

Figure 2. The COOH-terminal region of Maml1 is critical to the activity in Notch and Wnt pathways. *A,* schematic diagram of full-length and truncated Flag-tagged Maml1 constructs used in this study. *B,* equal expression of different truncations of Maml1 was confirmed by Western blot with anti-Flag antibody. *C,* H1299 cells were cotransfected with 0.4 μg of cyclin D1 (−1,745) luciferase reporter, 0.2 μg of truncated Maml1 plasmids, and 0.3 μg of β-catenin S37A. Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing pcDNA vector alone. The experiments were done in triplicate each time and were repeated at least twice. *Columns,* mean; *bars,* SE. *D,* H1299 cells were cotransfected with 0.4 μg of 8xCSL luciferase reporter, 0.2 μg of truncated Maml1 plasmids, and 0.3 μg of Notch intracellular (Notch^{ic}). Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing Notch intracellular alone.

Maml1 and β-catenin. Coimmunopecipitation of endogenous Maml1 with anti-Maml1 antibody followed by immunoblotting with anti–β-catenin antibody revealed a 92-kDa band corresponding to β-catenin (Fig. 3*C*). The observed interaction between Maml1 and β-catenin led us to consider the possibility that Maml1 is targeted specifically to TCF target genes to facilitate efficient transcriptional activation and transformation by β-catenin.

Maml1 is a potent activator of Wnt signaling in SW480 cells independently of Notch activity. To further explore the potential requirement for Maml1 activity in TCF/β-catenin transcription, we cotransfected Maml1 and *TOP-FLASH* reporter gene into the human colon adenocarcinoma cell line SW480. An APC mutation in this cell line abolishes the β-catenin control mechanism (15). This leads to constitutive transcriptional activation by endogenous β-catenin expression. Cotransfection of Maml1 markedly enhanced the activity of the *TOP-FLASH* reporter in a dose-dependent manner (Fig. 4*A*). Interestingly, we did not observe direct activation by Maml1 on the *8xCSL* reporter, indicating that there is no Notch activity in this cell line. We asked whether the increase of transcriptional activity of the β-catenin pathway is due to Maml1 only and not due to a cross-talk with the Notch pathway. We investigated the activity of Maml1 in the presence of γ-secretase inhibitor (GSI), a potent inhibitor of the Notch pathway. GSI prevents the cleavage of Notch and its release from the membrane. Subsequently, GSI blocks nuclear Notch transactivation on target genes. In the presence of the inhibitor, the combination Notch and Maml1 does not stimulate the *8xCSL* reporter, indicating that Notch activity is blocked (Fig. 4*B*). In contrast, GSI has no effect on the activation of the TOP-flash reporter or *cyclin D1* induced by Maml1 (Fig. 4*B*), indicating that Maml1 coactivation on β-catenin/TCF genes is independent of Notch activity.

A coimmunoprecipation experiment was used to show an association between endogenous Maml1 and β-catenin in the SW480 cell line. Coimmunopecipitation of endogenous Maml1 with anti-Maml1 antibody followed by immunoblotting with anti–β-catenin antibody revealed a 92-kDa band corresponding to β-catenin (Fig. 4*C*). TCF immunoprecipitation was used as a positive control of ability to coimmunoprecipitate β-catenin. Therefore, these data show that endogenous Maml and β-catenin interact in SW480 cells.

To examine if Maml1 is localized to the *cyclin D1* promoter, chromatin immunoprecipitation experiments were carried out. One set of primers was designed to encompass TCF element within the *cyclin D1* promoter. A PCR signal is observed only when we coimmunoprecipitated with Maml1 and β-catenin antibodies. In SW480 cells, Maml1 and β-catenin bound to the *cyclin D1* promoter at the TCF binding site (Fig. 4*D*). To ensure the specificity of the binding of Maml1 and β-catenin on the *cyclin D1* promoter, we verified that we are unable to amplify nonspecific regions using primers designed for ALU repeats. In the presence of GSI, Maml1 still localizes to the *cyclin D1* promoter, indicating that β-catenin recruits Maml1 independently of Notch activity (Fig. 4*D*).

Maml1 is essential for colon carcinoma cell survival. To ask whether Maml1 plays an essential role in the β-catenin pathway, and therefore in SW480 cell survival, we used siRNA to deplete hMaml1. We transfected Maml1 siRNA mix into SW480, followed by Western blot assay to test its efficiency. In the presence of control siRNA (LuciGL3), the Maml1 protein is expressed in SW480; in contrast, in the presence of Maml1 siRNA, Maml1 protein was knocked down in SW480 (Fig. 5*A*). We then determined the percentage of cell survival after Maml1 depletion in SW480. When

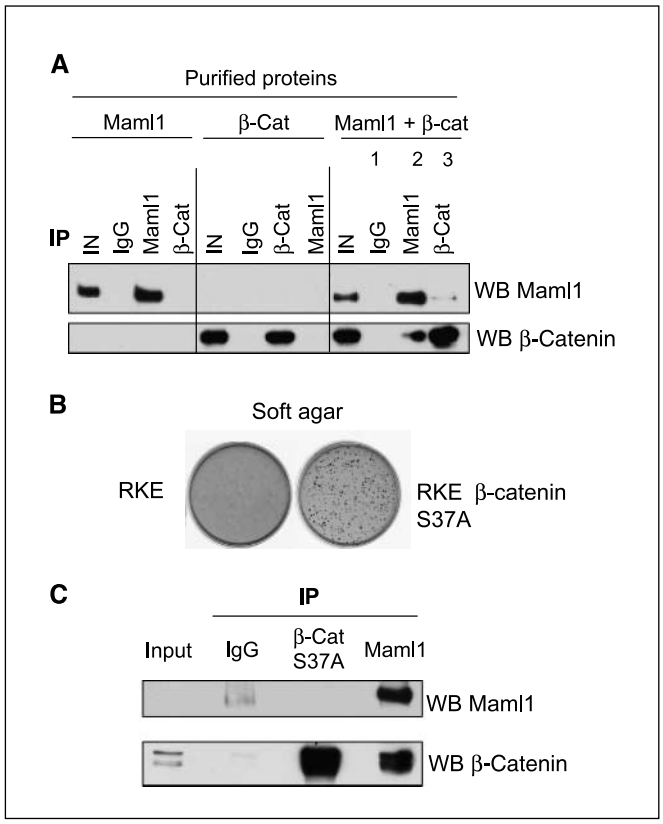

Figure 3. β-Catenin and Maml1 interact *in vitro* and *in vivo*. *A,* Flag-tagged purified Maml1 protein and His-tagged purified β-catenin protein were tested for an interaction in a coimmunoprecipitation assay. Maml1 and β-catenin were detected by Western blot with specific anti-Maml1 and anti–β-catenin antibodies, respectively. The input (*IN*) represents 10% of the proteins used in each binding reaction. The negative control is IgG. *B,* RKE clones expressing β-catenin S37A were tested in soft-agar assay. Crystal violet staining was used to detect foci formation. *C,* coimmunoprecipitation of β-catenin and Maml1 was analyzed by immunoblot using lysates derived from RKE-β-catenin S37A cells. The input β-catenin and Maml1 are shown in lane *Input*. Maml1 was immunoprecipitated using an antibody specific to Maml1 and any associated β-catenin was detected by immunoblot with anti–β-catenin antibody. A control anti-IgG serum was used to ensure the specificity of the immunoprecipitation.

the cells were treated with 120 nmol/L Maml1 siRNA, we observed 53% cell survival compared with control after 72 h of transfection (Fig. 5*A* and *B*). Because we obtained 47% cell death and not complete death, we investigated whether the Maml family members were expressed in this cell line. Maml2 and Maml3 expression was visualized by reverse transcription-PCR (RT-PCR) assay (data not shown). We did not observe a significant difference in survival between cells transfected with control siRNA and cells transfected with hMaml2 siRNA or hMaml3 siRNA (data not shown). In contrast, the combined knockdown of hMaml1 and hMaml2 by siRNA affects SW480 cell survival; only 37% of the cells survived compared with the control (Fig. 5*B*). The same decrease of number of cells was observed when hMaml1 siRNA and hMaml3 siRNA were mixed (data not shown). Interestingly, Maml2- and Maml3-associated knockdown did not induce cell death, indicating a central role of Maml1 in SW480 cell survival.

Maml1 is required for β-catenin–mediated target gene transcription *in vivo*. To assess whether Maml1 is required for β-catenin transcription activity *in vivo*, levels of hMaml1 and hMaml2 proteins in SW480 cells were reduced by targeted siRNAs, and the effects on c-Myc and cyclin D1 mRNA levels were

examined. As shown in Fig. 6*A* and *B*, endogenous c-Myc mRNA levels declined substantially (5-fold) with β-catenin siRNA compared with control siRNA. Similarly, cyclin D1 mRNA was decreased (2.5-fold) in β-catenin siRNA–treated cells compared with control siRNA–treated cells (Fig. 6*B*). Importantly, knockdown of Maml proteins in SW480 also significantly decreased c-Myc and cyclin D1 mRNA levels (3-fold; Fig. 6*A* and *B*). Furthermore, a combined knockdown of β-catenin, Maml1, and Maml2 had a cooperative affect in the repression of c-Myc and cyclin D1 mRNA levels. Under these conditions, the level of cyclin D1 mRNA was strongly reduced (4-fold; Fig. 6*B*). These data strongly indicate that Maml1 is a key component of β-catenin–mediated transcription of c-Myc and cyclin D1.

We next used chromatin immunoprecipitation experiments to determine whether Maml is recruited to Wnt target promoters *in vivo* by β-catenin. As shown in Fig. 6*B*, cyclin D1 RNA levels decreased on the addition of β-catenin siRNA, indicating that β-catenin is not present in the *cyclin D1* enhancer. We used this same condition to ask whether Maml1 is recruited to the *cyclin D1* enhancer by β-catenin. When β-catenin expression was knocked down by β-catenin siRNA, Maml1 did not bind to the *cyclin D1* enhancer (Fig. 6*C*) as determined by chromatin immunoprecipitation. The chromatin immunoprecipitation conditions used here were specific because Maml1 was not detected at an ALU repeat region (Fig. 6*C*), nor was *cyclin D1* promoter DNA recovered in immunoprecipitation with control IgG (Fig. 6*C*). The chromatin immunoprecipitation data support the conclusion that Maml1 is recruited by β-catenin to form a transcriptional activation complex on the *cyclin D1* promoter in SW480 cells.

Discussion

Previous studies have shown that β-catenin is an essential nuclear effector of Wnt signals. It is agreed that the stability and amount of cytoplasmic β-catenin are important steps in the signaling event (16). The free cytosolic pool of β-catenin is then under tight control by a destruction complex assembled on APC, axin, and GSK-3β, which are main targets of Wnt signaling (17). However, high levels of cytoplasmic β-catenin are not sufficient to promote Wnt signaling (18). How β-catenin accomplishes target gene activation is unclear. β-Catenin interacts with TCF factors and may alter promoter architecture and displace Groucho or C+BP (19). In this study, we have identified Maml1 as an important coactivator of β-catenin. Using both *in vitro* and *in vivo* approaches, we found that Maml1 participates in Wnt signaling by modulating β-catenin/TCF activity.

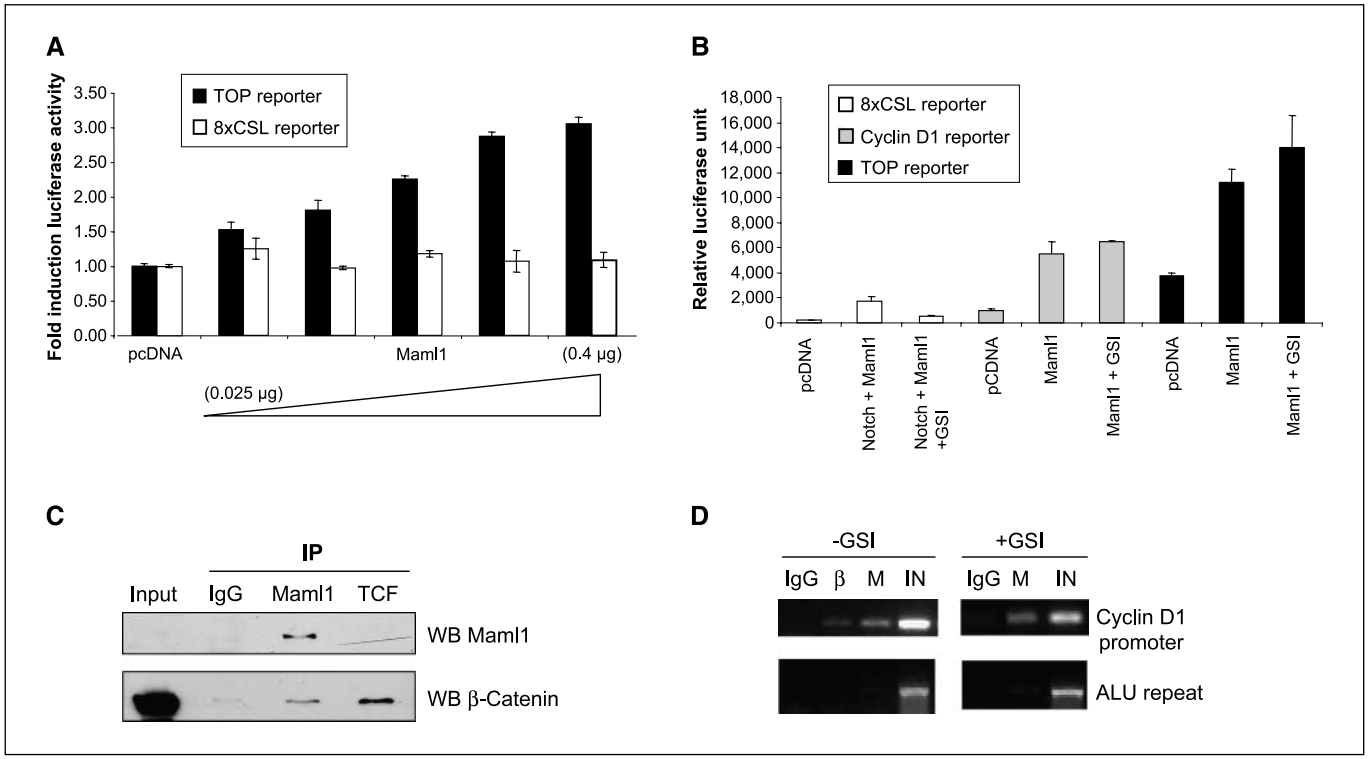

Figure 4. Maml1 is a potent activator of Wnt pathway in SW480 cells independently of Notch. *A,* SW480 cells were cotransfected with 0.4 μg of TOP-FLASH luciferase reporter (*black*) or 8xCSL luciferase reporter (28) and increasing amounts of expression plasmid encoding Maml1. Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing pcDNA vector alone. *B,* SW480 cells were cotransfected with 0.4 μg of 8xCSL luciferase reporter (28), 0.3 μg of Notch intracellular, and 0.2 μg of Maml1. SW480 cells were cotransfected with 0.4 μg of cyclin D1 (−1,745) luciferase reporter (*gray*) or TOP-FLASH luciferase reporter (*black*) and 0.2 μg of Maml1 plasmid. The cells were treated with 1 μmol/L GSI 24 h before the luciferase assay. Luciferase assays were done 48 h after transfection. Luciferase activity, corrected for β-galactosidase activity, is expressed as fold induction relative to cells expressing pcDNA vector alone. The experiments were done in triplicate each time and were repeated at least twice. *Columns,* mean; *bars,* SE. *C,* coimmunoprecipitation of TCF and Maml1 was analyzed by immunoblot using lysates derived from SW480 cells. The input β-catenin and Maml1 are shown in lane *Input*. Maml1 was immunoprecipitated with an antibody specific to Maml1 and any associated β-catenin was detected by immunoblot with anti–β-catenin antibody. A control anti-IgG serum was used to ensure the specificity of the immunoprecipitation. *D,* chromatin immunoprecipitation analysis by RT-PCR of β-catenin induction of cyclin D1 transcription in SW480 cells in the absence and presence of GSI. Precipitated chromatin, using the anti–β-catenin or anti-Maml1 antibody, was amplified by PCR using primers specific for the cyclin D1 promoter or ALU repeats.

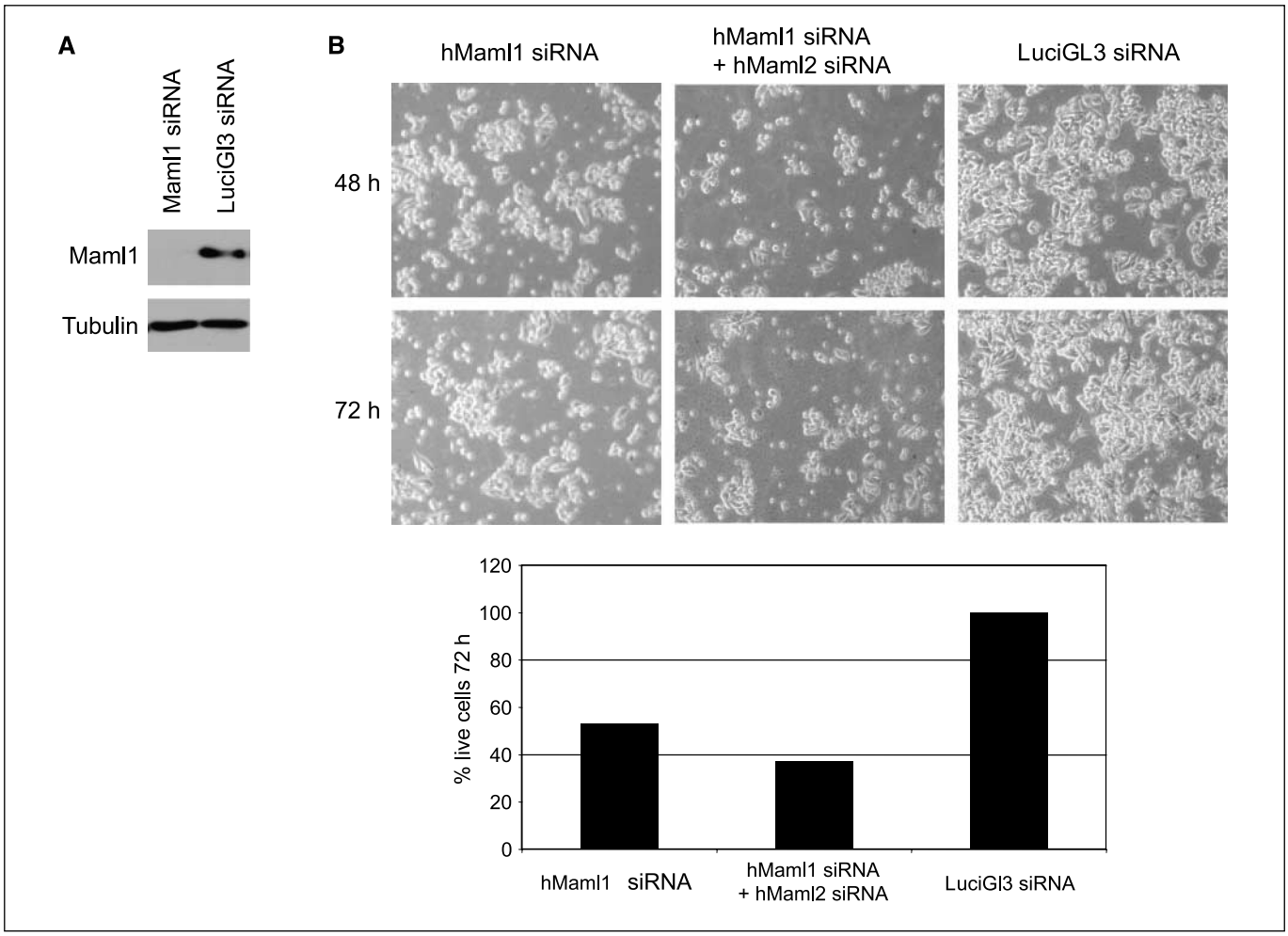

Figure 5. Maml1 is essential for colon carcinoma cell survival. *A,* SW480 cells were transfected with 120 nmol/L hMaml1 siRNA or 120 nmol/L luciferase GL3 (*LuciGl3*) siRNA. Two transfections were assayed successively with a time interval of 24 h. The expression of Maml1 was detected by Western blot analysis with anti-Maml1 antibody. *B,* SW480 cells were transfected with 120 nmol/L Maml1 siRNA, Maml1 plus Maml2 siRNAs, luciferase GL3 siRNA. Photographs of transfected SW480 were taken 48 and 72 h after transfection. After 72 h, the number of cells per well was counted and the results are presented as a percentage of the control siRNA.

Cotransfection of Maml1 and β-catenin or β-catenin S37A into HeLa cells resulted in a considerable activation of the TCF-responsive *TOP-FLASH* promoter. In fact, whereas mutant β-catenin induced transcription maximally up to 10-fold, coexpression of Maml1 results in a marked dose-dependent induction of transcription up to 70-fold (Fig. 1*B*). These results show a clearly robust synergistic association between Maml1 activity and β-catenin to drive downstream transcriptional events in the Wnt signaling cascade. Most regulators of the canonical Wnt pathway, like APC and axin, are examples of proteins that modify the half life of β-catenin and thereby alter β-catenin/TCF cotranscriptional activity (20, 21). Maml1 had no effect on the stabilization of β-catenin and, therefore, Maml1 must play a direct role in the β-catenin transcription activity of Wnt target genes (Fig. 1*B*).

It has previously been shown that cyclin D1 is induced by activation of the Wnt signaling pathway through a TCF binding site in the *cyclin D1* promoter (10). We sought to determine if Maml1 could modify the activity of *cyclin D1* promoter. By transient promoter assays, we have shown that the concomitant expression of Maml1 and β-catenin increases the activity of *cyclin D1* promoter in HeLa cells (Fig. 1*C*). Cyclin D1 protein abundance is elevated in human adenocarcinomas and adenomatous polyps of the colon (22, 23). Expression of Maml1 in colon cancer cell line SW480 increased β-catenin/TCF–mediated transcription (Fig. 4). Furthermore, by *in vivo* chromatin immunoprecipitation studies, we were able to immunoprecipitate endogenous Maml1 on a specific TCF binding site in the human *cyclin D1* promoter and it is recruited to the promoter by β-catenin (Fig. 4*D*). Maml1 regulates cyclin D1 expression independently of Notch pathway (Fig. 4*B* and *D*) and therefore is a potent effector in tumorigenic processes.

To understand how Maml1 could modify the target gene expression of TCF, we looked for the assembly of a complex formed by Maml1 and β-catenin. We show that Maml1 is able to associate with β-catenin *in vitro* and *in vivo* (Figs. 3 *A* and *D* and 4*C*). What is the role of Maml1 in the β-catenin pathway? β-Catenin interacts with proteins known to be involved in chromatin remodeling including Brg1 (24) and CREB binding protein/p300 (25). The association of β-catenin with Maml1, therefore, may facilitate the recruitment of the transcription machinery to target gene promoters. It is possible that the interaction with Maml1 is part of a mechanism by which β-catenin alters chromatin structure

at target gene promoters by linking TCF proteins to specific chromatin remodeling complexes.

In the Notch pathway, Maml1 seems to function by coordinating assembly of the transcriptional activation complex and recruitment of additional coactivators. Perhaps this is a general mechanism by which Maml1 functions (Fig. 6*D*). That is, Maml1 may coordinate β-catenin transcriptional activation complexes by fostering assembly of β-catenin with TCF and recruitment of additional coactivators such as Legless and Pygopus. These coactivators were implicated in the nuclear localization of β-catenin (4, 7) as well as in transcription (26, 27). However, Maml1 also plays a role in the turnover of Notch transcriptional activation. It was reported that Maml1 recruits cyclin C:cyclin-dependent kinase 8, which phosphorylate Nic, resulting in rapid destruction of Notch transcriptional activation complex (28). It is also possible that this mechanism is conserved, and Maml1 plays a role in the turnover of β-catenin transcription.

The deregulation of Wnt/β-catenin signaling pathway is an important factor in colon carcinomas. The key effectors downstream of β-catenin in colon carcinogenesis are not completely understood. It is generally thought that c-Myc is an important effector of β-catenin (22). In addition, in a series of reports, it was shown that cyclin D1 is also a critical target of β-catenin (22). Furthermore, expression of cyclin D1 antisense in SW480 cells reduced their tumorigenic potential in Nude mice (22), indicating that sustained cyclin D1 expression by β-catenin is required for colon tumorigenesis. To determine whether Maml1 is a key determinant in Wnt/β-catenin–dependent tumorigenesis, we tested the effect of Maml1 by siRNA in SW480 colon carcinoma cells (Fig. 5). We found that the expression of

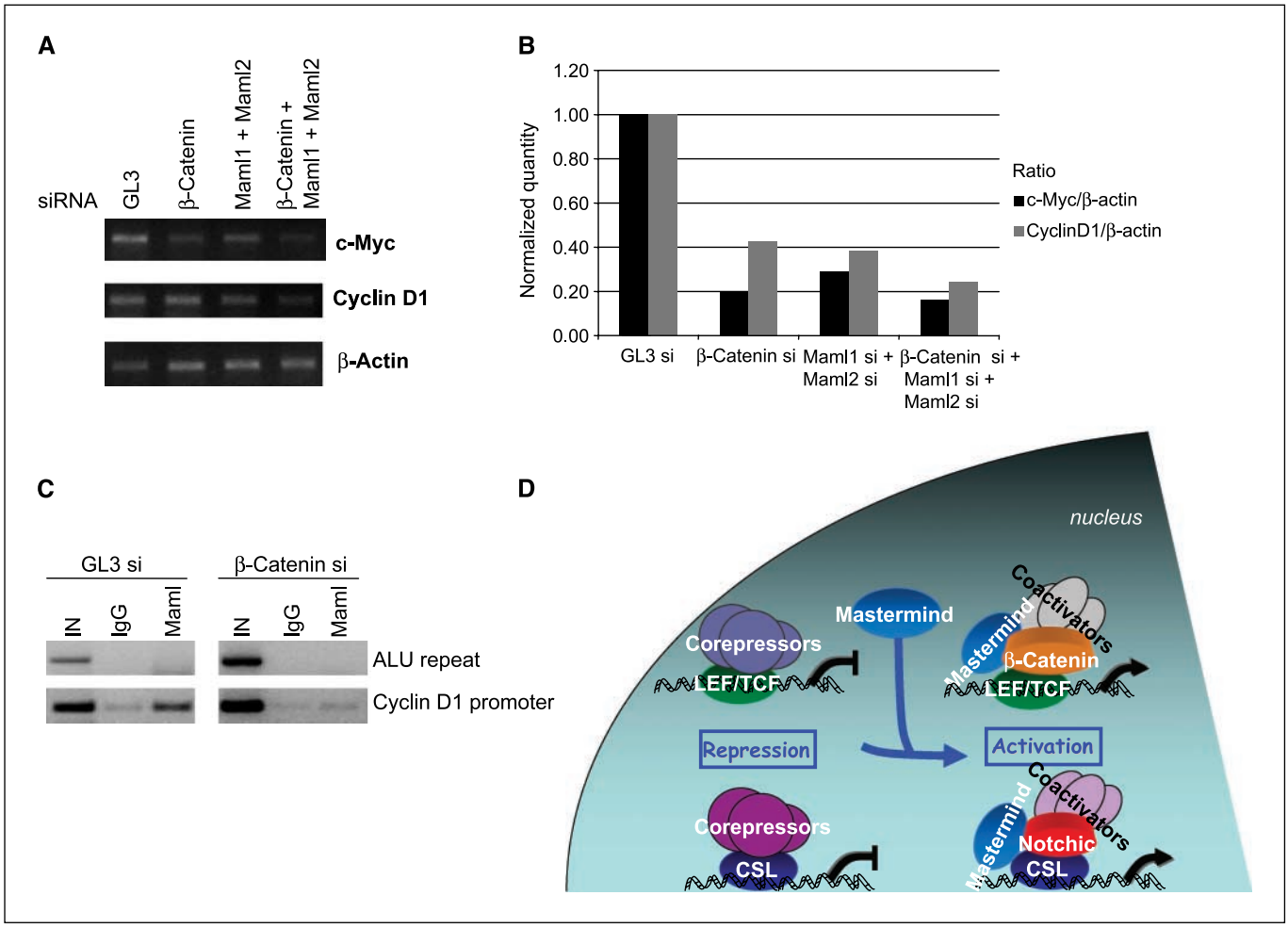

Figure 6. Maml1 is required for β-catenin–mediated target gene transcription *in vivo*. *A,* SW480 cells were transfected with 120 nmol/L luciferase GL3 siRNA; 40 nmol/L hMaml1 siRNA + 40 nmol/L hMaml2 siRNA + 40 nmol/L luciferase GL3 siRNA; 40 nmol/L β-catenin siRNA + 80 nmol/L luciferase GL3 siRNA; or 40 nmol/L hMaml1 siRNA + 40 nmol/L hMaml2 siRNA + 40 nmol/L β-catenin siRNA. Two transfections were assayed successively with a time interval of 24 h. Five hours after the second transfection, the cells were put in 0.1% FBS-DMEM. After 72 h, RNA was extracted and RT-PCR was assayed for c-Myc, cyclin D1, and β-actin levels. *B,* quantification of RT-PCR results corrected by β-actin quantity. To capture the signal corresponding to the different target genes, we use the charge-coupled device camera of the Bio-Rad analyzer, and quantification was achieved using the Quantity One 4.5 software. We verified that the PCR was done in the linear range and the experiments were reproduced at least thrice. *C,* chromatin immunoprecipitation analysis by RT-PCR of β-catenin knockdown by siRNA treatment on cyclin D1 transcription in SW480 cells. Precipitated chromatin with anti-hMaml1 antibody was amplified by PCR using primers specific for the cyclin D1 promoter or ALU repeats. *D,* Maml1 acts as a key integrator of signaling pathways of transcription. This schematic model depicts the role of Maml1 as a key factor in remodeling of transcriptional repressors into activators. Both the Notch and β-catenin pathways share a common theme in transcriptional regulation. In both pathways, the DNA binding component of the activation complex (LEF/TCF or CSL) exists in a repressor complex in the absence of signal. Signaling by Wnt or Notch results in the conversion of the repressor form into a transcriptional activator. We propose that this step in the mechanism is mediated by Maml1. In an analogous manner to the Notch pathway, we propose that Maml1 forms a complex with TCF and β-catenin to recruit specific coactivators.

Mastermind-like family proteins in SW480 is required for sustained expression of cyclin D1 and c-Myc and is critical for cell survival (Figs. 5 and 6). These data indicate that Mastermind-like family is required for the survival of colon tumor cells likely through its action with β-catenin to activate *cyclin D1* transcription.

The results presented in this study show that Maml1 is an essential coactivator of β-catenin and reveal a potential role for Maml1 as a key integrator of signaling pathways of transcription (Fig. 6*D*). In summary, we have shown that Maml1 provides a modulatory effect on the activity of β-catenin/TCF. Reduction of Maml1 expression could be an alternative target for cancer therapy regulating both Notch and β-catenin–mediated transcription of target genes such as *c-myc* and *cyclin D1*.

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