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FoxM1 Promotes β-Catenin Nuclear Localization and Controls Wnt Target-Gene Expression and Glioma Tumorigenesis

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SUMMARY

Wnt/β-catenin signaling is essential for stem cell regulation and tumorigenesis, but its molecular mechanisms are not fully understood. Here, we report that FoxM1 is a downstream component of Wnt signaling and is critical for β-catenin transcriptional function in tumor cells. Wnt3a increases the level and nuclear translocation of FoxM1, which binds directly to β-catenin and enhances β-catenin nuclear localization and transcriptional activity. Genetic deletion of FoxM1 in immortalized neural stem cells abolishes β-catenin nuclear localization. FoxM1 mutations that disrupt the FoxM1–β-catenin interaction or FoxM1 nuclear import prevent β-catenin nuclear accumulation in tumor cells. FoxM1–β-catenin interaction controls Wnt target gene expression, is required for glioma formation, and represents a mechanism for canonical Wnt signaling during tumorigenesis.

INTRODUCTION

The canonical Wnt signal transduction pathway is a primary signaling system in stem/progenitor cells and cancer cells (Clevers, 2006; Huang and He, 2008; Moon et al., 2004; Nusse, 2008). Wnt binding to cell surface receptors, including a Frizzled (Fz) protein and LDL receptor-related protein 6 (LRP6), initiates a transduction cascade leading to stabilization of the transcription coactivator β-catenin, which then enters the nucleus to form a transcriptional complex with T cell factor (TCF) or lymphoid enhancer factor (LEF) to activate the expression of Wnt target genes, such as *c-Myc* and *cyclin D1* (Clevers, 2006; Moon et al., 2004). However, the molecular mechanisms that regulate β-catenin nuclear localization and transcriptional activation are not well understood.

Wnt/β-catenin signaling plays a critical role in cancer formation, including regulation of transformation, cell proliferation, and invasion. Persistent activation of β-catenin has been implicated in a variety of human cancers, including glioblastoma multiforme (GBM), the most malignant form of glioma (Zheng et al., 2010). Unlike colorectal cancer, in which high levels of β-catenin are frequently found as a result of mutational loss of

Significance

The Wnt/β-catenin signaling pathway is aberrantly activated in human cancers and is critical for cancer formation. A key feature of Wnt signaling activation is β-catenin nuclear localization, for which the underlying molecular mechanisms remain elusive. Here we show that FoxM1, which is also activated in most human tumors, is a critical component of Wnt signaling and plays an essential role in regulating β-catenin activation. FoxM1 directly interacts with β-catenin and is necessary and sufficient for its nuclear localization and transcriptional activation in tumor cells. The FoxM1 and β-catenin interaction is a mechanism for controlling canonical Wnt signaling and is required for glioma formation. This interaction provides insights into carcinogenesis and strategies for therapeutic intervention of this important pathway.

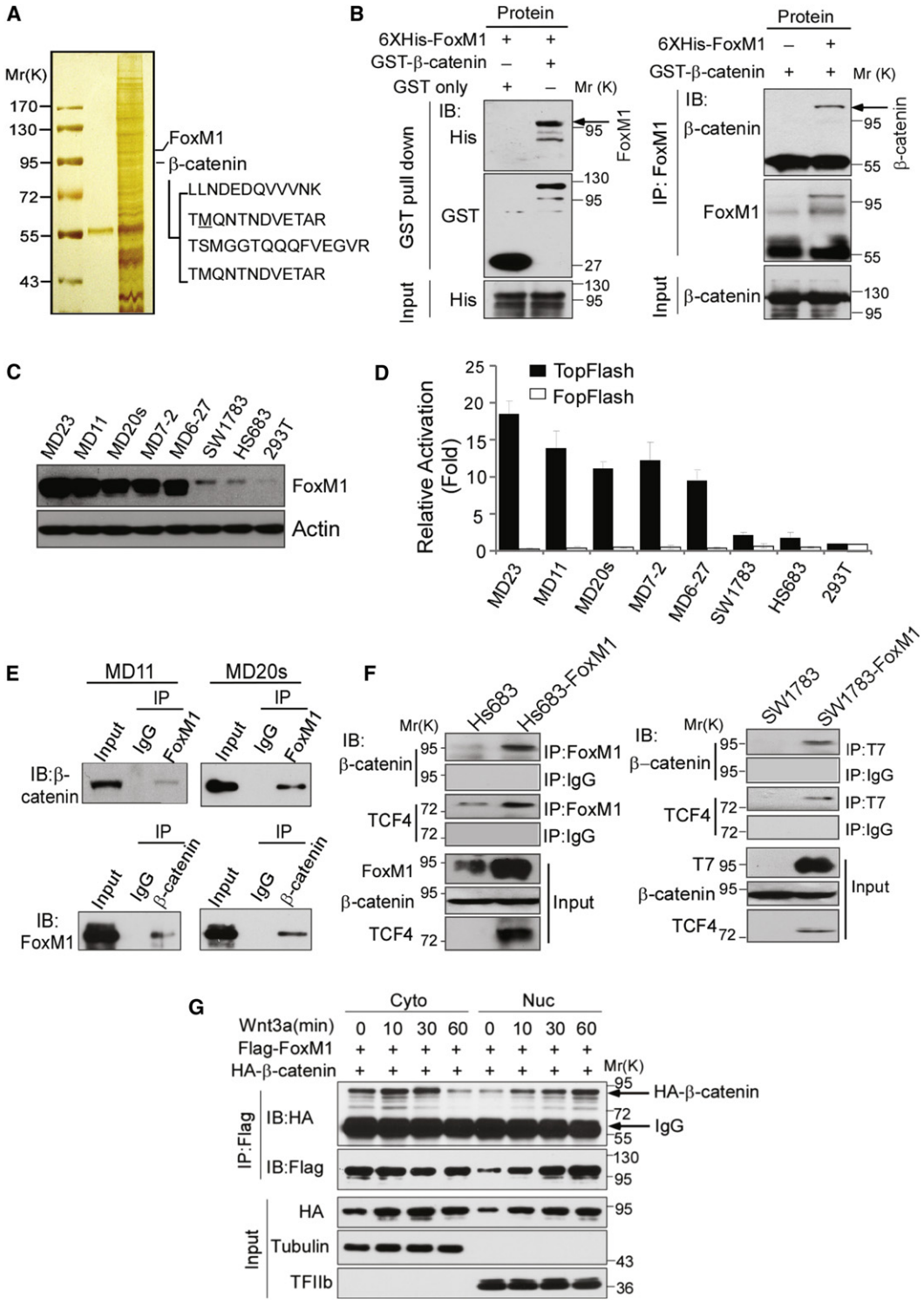

Figure 1. FoxM1 and β-Catenin Directly Interact In Vitro and In Vivo

(A) 293T cells were transfected with Flag-FoxM1 or a control plasmid. Cell extracts were subjected to IP using an anti-Flag antibody. The IP protein complex was subjected to LC-MS/MS analysis. Four identified peptide sequences of β-catenin are shown.

(B) GST pull-down was performed using purified GST-β-catenin and 6xHis-FoxM1, followed by IB with an anti-His antibody (left panel). IP was performed using purified 6xHis-FoxM1 and GST-β-catenin with an anti-FoxM1 antibody, followed by IB with anti-FoxM1 and anti-β-catenin antibodies (right panel).

(C) Western blotting of the FoxM1 protein in eight cell types, including five established GICs and two glioma cell lines.

(D) Cells were transfected with the TOP-Flash or control FOP-Flash reporter to determine reporter activities 48 hr after transfection. Promoter activation (fold) was calculated relative to that in 293T cells. Values are mean ± SD for triplicate samples.

the *adenomatous polyposis coli* (*APC*) gene or stabilizing mutations in the *CTNNB1* (*β-catenin*) gene itself (Polakis, 2000), in sporadic gliomas, the frequency of *APC* loss or *CTNNB1* mutations appears to be low (Paraf et al., 1997). This suggests that genetic mutations may not be the major driving force leading to elevated β-catenin nuclear accumulation and, hence, activation in GBM. Importantly, the Wnt/β-catenin pathway is known to critically regulate self-renewal and differentiation of neural stem/progenitor cells (Chenn and Walsh, 2002; Nusse, 2008). These findings prompted us to examine the molecular mechanism of Wnt/β-catenin signaling activation and its potential role in tumorigenicity of GBM-initiating cells (GICs).

Like β-catenin, the forkhead box M1 (FoxM1) transcription factor is an important regulator of animal development and cell proliferation (Korver et al., 1998; Ye et al., 1997). Furthermore, overexpression of FoxM1 is common in human tumors (Pilarsky et al., 2004), including glioma (Liu et al., 2006). Thus, gene expression analyses by the Cancer Genome Atlas (TCGA) found FoxM1 to be overexpressed in GBM clinical specimens compared with nontumor controls (Hodgson et al., 2009). Because overactivation of FoxM1 and β-catenin occurs in many human cancers, we are interested in potential functional connections between these two intensively studied oncogenic pathways. There is evidence that FoxM1 and β-catenin may have related or common functions in tumor. For example, gut-specific deletion of FoxM1 suggests its key role in gastrointestinal tumorigenesis and potential regulation of β-catenin/TCF4 signaling (Yoshida et al., 2007). However, because FoxM1 and β-catenin both have pleiotropic roles, it is unclear whether a common molecular mechanism underlies these related phenotypes. In the current study, we explored the possibility of FoxM1 as a key component in mediating β-catenin nuclear accumulation in Wnt signaling and determined the biological consequence of FoxM1–β-catenin interaction in brain tumor formation.

RESULTS

FoxM1 Interacts Directly with β-Catenin In Vitro and In Vivo

A Flag-tag affinity procedure was used to purify a FoxM1-containing complex, which was subjected to LC-MS/MS analysis. The peptide sequences of β-catenin found in the complex suggested that β-catenin is a potential binding partner of FoxM1 (Figure 1A and Figures S1A and S1B, which are available with this article online). The interaction between β-catenin and FoxM1 was confirmed by immunoprecipitation (IP) analysis of 293T cells transfected with HA-tagged β-catenin and Flag-FoxM1 (Figure S1C). The physical interactions between β-catenin and FoxM1 were further analyzed in vitro using recombinant GST-β-catenin and His-FoxM1. In a GST pull-down assay, purified His-FoxM1 bound directly to GST-β-catenin (Figure 1B, left). Reciprocally, in an IP assay using an anti-FoxM1 antibody, GST-β-catenin also bound to His-FoxM1 (Figure 1B, right). Thus, β-catenin binds to FoxM1 directly in vitro.

We detected endogenous FoxM1 expression in 293T cells, human glioma cell lines Hs683 and SW1783 (grade III), and a panel of GIC cell lines (MD 11, 20 s, 23, 7-2, and 6-27). These GICs are primary neurosphere-derived cells isolated from fresh surgical specimens of GBM and have an enriched GIC population (Figures S1D–S1G). FoxM1B is the predominant FoxM1 isoform in 293T cells and in the human glioma cells, including the GICs (Figures S1H–S1J). Moreover, GICs expressed substantially higher levels of FoxM1 than did Hs683 and SW1783 cells (Figure 1C). Furthermore, Wnt activity was substantially higher in GICs than in Hs683 and SW1783 cells, as determined using the TOP-Flash reporter (Figure 1D), indicating that Wnt activity is deregulated and correlated with the FoxM1 levels in the cells.

We next determined whether β-catenin and FoxM1 interact in glioma cells. In MD11 and MD20s cells, endogenous FoxM1 bound to endogenous β-catenin, as determined by co-IP assays (Figure 1E). When Hs683 and SW1783 cells overexpressed T7-tagged FoxM1, endogenous β-catenin bound to FoxM1 (Figure 1F, left) and T7-FoxM1 (Figure 1F, right). Moreover, endogenous TCF4 protein, a well-known binding partner and transcriptional target of β-catenin, could be detected in the FoxM1 and T7-FoxM1 precipitates, and its level was increased upon FoxM1 overexpression (Figure 1F). Thus, β-catenin and FoxM1 interact in tumor cells. We also examined the specificity of FoxM1 isoforms interacting with β-catenin and found that the FoxM1B binding ability is higher than that of FoxM1C (Figures S1K and S1L).

Wnt Promotes Nuclear Translocation of Both FoxM1 and β-Catenin

Because Wnt3a is known to stabilize β-catenin and increase its nuclear accumulation, we examined the interaction of FoxM1 with β-catenin upon Wnt3a stimulation in 293T cells that had been cotransfected with HA-β-catenin and Flag-FoxM1. The amount of β-catenin that bound to FoxM1 in the nucleus increased substantially with Wnt3a treatment in a time-dependent manner (Figure 1G). Surprisingly, the amount of Flag-FoxM1 that translocated to the nucleus also increased with Wnt3a treatment in a time-dependent manner (Figure 1G). Thus, β-catenin interacts with FoxM1 in both the cytoplasm and the nucleus, and Wnt activation increases the translocation of both proteins to the nucleus.

To further investigate the nuclear translocation of FoxM1 and β-catenin during Wnt activation, we cotransfected 293T cells

(E) Nuclear extracts of MD11 and MD20s cells were subjected to IP using FoxM1 antibody or control IgG, followed by IB with β-catenin antibody (upper panels). Reciprocal IP was done using β-catenin antibody or control IgG, followed by IB with the FoxM1 antibody (lower panels).
(F) Lysates of Hs683 and SW1783 cells that stably express T7-FoxM1 or a control vector were subjected to IP using anti-FoxM1 or anti-T7 antibodies, followed by IB with anti-β-catenin or anti-TCF4 antibodies.
(G) 293T cells were cotransfected with Flag-FoxM1 and HA-β-catenin plasmids. The cells were treated with 20 ng/ml Wnt3a, and cytoplasmic or nuclear extracts were prepared at the indicated time points and subjected to IP and IB analyses with the indicated antibodies. Tubulin and TFIIb were used as controls for cytoplasmic and nuclear fractions, respectively.
See also Figure S1.

with DsRed2-N1-FoxM1 and CFP-β-catenin and monitored the fluorescence intensities by time-lapse live imaging of the cells treated with Wnt3a every 10 s for 10 min. We reasoned that, during this early time window of Wnt treatment, we would observe primarily β-catenin translocation rather than stabilization of β-catenin that was already present as a result of overexpression. FoxM1 and β-catenin were colocalized in both the cytoplasm and nucleus, and Wnt3a increased the levels and nuclear translocation of both proteins (Figure 2A and Figure S2A). The fluorescence intensities of both proteins increased with time in the nuclei of Wnt3a-treated cells (Figure 2B). Note that the nuclear translocation of both proteins was probably underestimated, considering the effect of photo-bleaching during live imaging (Figure S2B). Indeed, Wnt3a promoted the nuclear translocation of endogenous FoxM1 and β-catenin (Figure 2C) and increased FoxM1 levels (Figure 2D) in 293T cells, as determined by immunoblotting (IB) analysis. Wnt3a also promoted the nuclear translocation of endogenous FoxM1 and β-catenin and increased FoxM1 levels in MD11 cells, as determined by immunofluorescence (IF) analysis (Figure 2E). Thus, Wnt activation increases the level and nuclear translocation of FoxM1, indicating that FoxM1 is a downstream component of Wnt signaling. However, FoxM1 mRNA levels did not increase upon Wnt3a treatments in MD11 cells (Figure S2C). Next, we inhibited new protein synthesis in the cells by cycloheximide and found that Wnt3a treatment resulted in a decrease in the endogenous FoxM1 degradation compared with control treatment (Figure 2F). These results indicated that Wnt3a increased FoxM1 levels through, at least in part, inhibition of FoxM1 protein degradation.

FoxM1 Is Required for β-Catenin Nuclear Localization in Tumor Cells

FoxM1 contains a functional nuclear localization signal (NLS) domain and shuttles between the cytoplasm and the nucleus (Ma et al., 2005). Because the amount of β-catenin that bound to FoxM1 was directly proportional to the amount of FoxM1 in the nucleus (Figure 1G), we examined whether FoxM1 plays a role in β-catenin nuclear localization. Expression levels of the endogenous FoxM1 and β-catenin increased in the cytoplasm and nucleus of Wnt3a-treated cells (Figure 3A), consistent with the findings of the IP assay (Figure 1G), of live imaging of the Wnt3a-treated 293T cells (Figures 2A and 2B), and of the IF assay of the Wnt3a-treated MD11 cells (Figure 2E). Overexpression of Flag-FoxM1 was sufficient to increase nuclear β-catenin while concomitantly decreasing cytoplasmic β-catenin (Figure 3A). These data suggest that the FoxM1 expression level is increased upon Wnt signaling and that increased expression of FoxM1 directly increases β-catenin nuclear accumulation.

To ascertain the role of FoxM1 in β-catenin nuclear accumulation, we examined β-catenin nuclear localization in FoxM1 null cells. Deletion of FoxM1 in $FoxM1^{fl/fl}$ immortalized neural stem cells (NSCs) did not change the total level of β-catenin expression but abolished β-catenin nuclear localization (Figures 3B and 3C). Moreover, β-catenin nuclear localization induced by Wnt3a was abolished in FoxM1 null immortalized MEFs derived from $FoxM1^{-/-}$ mice (Figure 3D). These results suggest that FoxM1 is critical for β-catenin nuclear localization.

Consistently, knockdown of FoxM1 by a FoxM1-siRNA, or via two independent FoxM1-shRNAs (Liu et al., 2006), in MD11 and MD20s cells substantially decreased the levels of nuclear β-catenin as determined by IB and IF analyses (Figures 3E–3G). In contrast, when FoxM1 expression was restored in sh-FoxM1 MD11 cells, β-catenin nuclear localization was restored (Figure 3G).

We also analyzed the effect of reducing FoxM1 on Wnt3a-induced β-catenin nuclear localization in glioma cells. Wnt3a increased β-catenin nuclear accumulation in MD11 and MD20s cells expressing the control shRNA (Figure 3H). In contrast, expression of either of the two shRNAs against FoxM1 abolished Wnt3a-induced β-catenin nuclear but not cytoplasmic accumulation (Figure 3H). Conversely, overexpression of FoxM1 counteracted the inhibitory effect of Wnt antagonist DKK1 on β-catenin nuclear accumulation (Figure S2D). Thus, these data indicate that FoxM1 does not affect β-catenin level but is required for β-catenin nuclear accumulation in tumor cells.

FoxM1 Nuclear Translocation and Binding to β-Catenin Are Required for FoxM1-Mediated β-Catenin Nuclear Accumulation in Tumor Cells

β-Catenin consists of an NH_2-terminal domain, a central armadillo (Arm) repeat domain (residues 141–664) composed of 12 Arm repeats, and a COOH-terminal domain (residues 664–782) (Figure 4A, left). Using a series of bacterially expressed GST-β-catenin deletion mutant proteins, we found that Arm repeats 11–12 of β-catenin interacted with FoxM1 (Figure 4A, right). FoxM1 consists of an NH_2-terminal domain, a conserved forkhead box domain (residues 232–332), an NLS domain (residues 350–366), and a COOH-terminal domain (Figure 4B, left). We found that the forkhead box domain of FoxM1 interacted with β-catenin (Figure 4B, right). Also, co-IP experiments in 293T cells revealed that β-catenin bound to FoxM1 mutants harboring the forkhead box domain but not to mutants without it (Figure 4C). An artificial fusion protein between the forkhead box domain and the SV40 NLS (235-347+NLS) was sufficient for binding to β-catenin (Figure 4C).

Next, we determine the effect of FoxM1 mutants on β-catenin nuclear accumulation. β-Catenin accumulated in the nuclei of cells expressing full-length FoxM1 or the mutant with both the forkhead box and NLS domains (Figure 4D). In contrast, β-catenin failed to accumulate in the nuclei of cells expressing FoxM1 mutants lacking the forkhead box domain. The FoxM1 mutant without the NLS domain impaired the nuclear accumulation of both FoxM1 and β-catenin. Importantly, the fusion fragment 235-347+NLS promoted the nuclear accumulation of both the FoxM1 mutant and β-catenin (Figure 4D). Thus, the nuclear localization of β-catenin depends on the interaction of β-catenin with FoxM1 and on FoxM1 nuclear translocation.

FoxM1–β-Catenin Interaction Is Required for β-Catenin–TCF-Mediated Transcription and for the Expression of β-Catenin Target Genes in Tumor Cells

It is well established that nuclear β-catenin associates with members of the TCF/LEF family of DNA-bound transcription factors on TCF-binding elements (TBEs), also called Wnt-responsive elements (WREs), to mediate Wnt target gene expression (Clevers, 2006; Moon et al., 2004). We used the

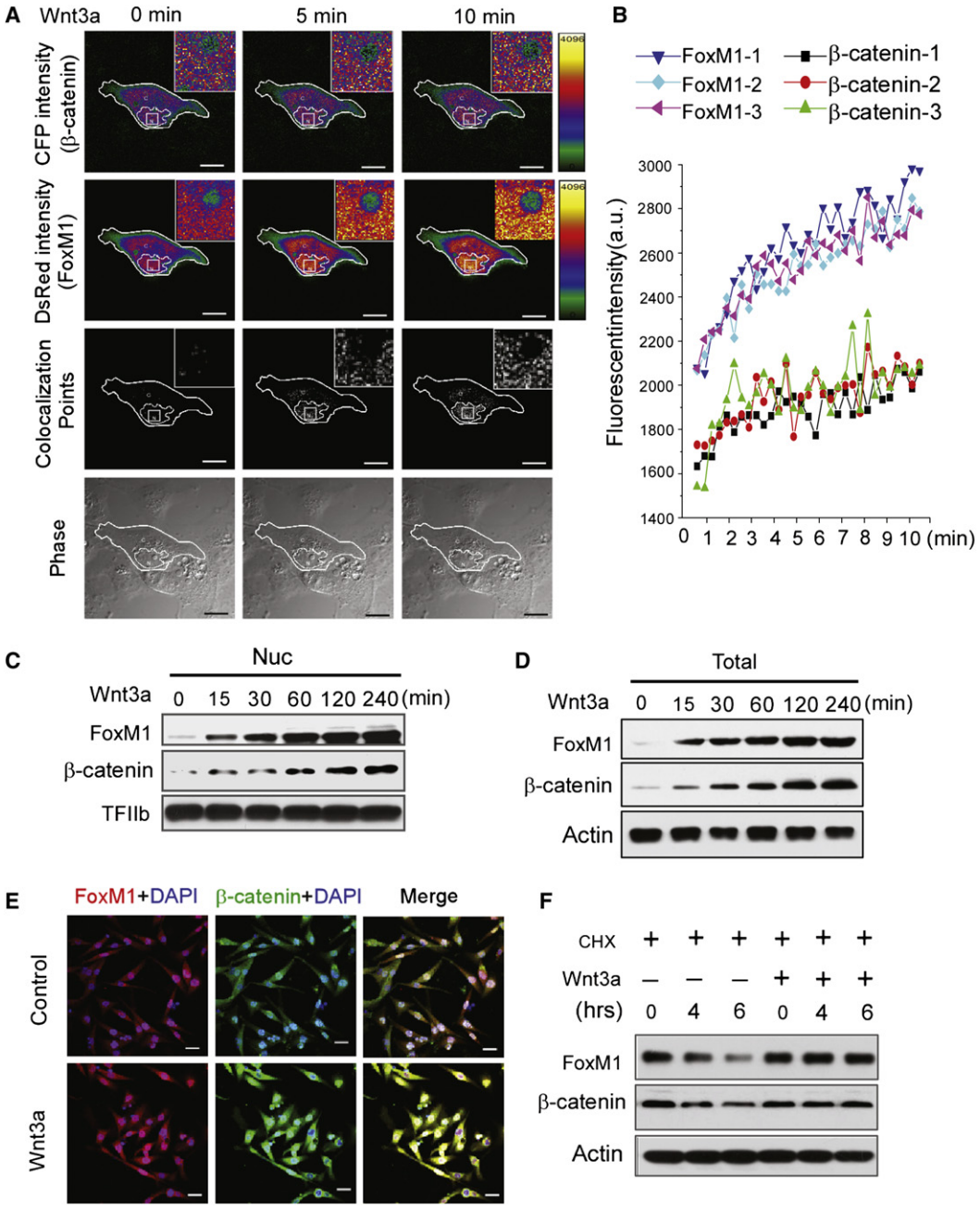

Figure 2. FoxM1 Promotes β-Catenin Nuclear Accumulation

(A, B) 293T cells were transfected with DsRed2-N1-FoxM1 plus CFP-β-catenin for 48 hr and then treated with 20 ng/ml Wnt3a. Sequential live images of FoxM1 and β-catenin nuclear localization/colocalization were taken every 10 s after Wnt3a treatment using a confocal imaging system.

(A) DsRed or CFP fluorescence intensities of the cells at each time point (0, 5, and 10 min) are shown as pseudocolor images. The white spots (colocalization points) of merged DsRed and CFP images indicate that FoxM1 and β-catenin colocalized in the cell (scale bar, 10 μm). The boxed area in the nucleus is shown at higher magnification in the upper right corner.

(B) Three positions in the nucleus were randomly chosen, and fluorescence intensities at these positions were calculated for each time point.

(C) Nuclear levels of β-catenin and FoxM1 in 293T cells treated with 20 ng/ml Wnt3a for indicated times were analyzed by IB.

(D) Total levels of β-catenin and FoxM1 in 293T cells treated with 20 ng/ml Wnt3a for indicated times were analyzed by IB.

(E) MD11 spheres were dissociated and plated on coverslips precoated with poly-L-ornithine and fibronectin in serum-free media with EGF and bFGF for 48 hr. The cells were then treated with control PBS or Wnt3a (20 ng/ml) for 4 hr. Cytoplasmic and nuclear levels of FoxM1 and β-catenin in the cells were analyzed by triple IF staining (scale bar, 40 μm).

(F) MD11 cells were treated with cycloheximide (CHX, 50 mg/ml) and with or without Wnt3a (20 ng/ml) at the indicated time points. Cell extracts were prepared and analyzed by IB.

See also Figure S2.

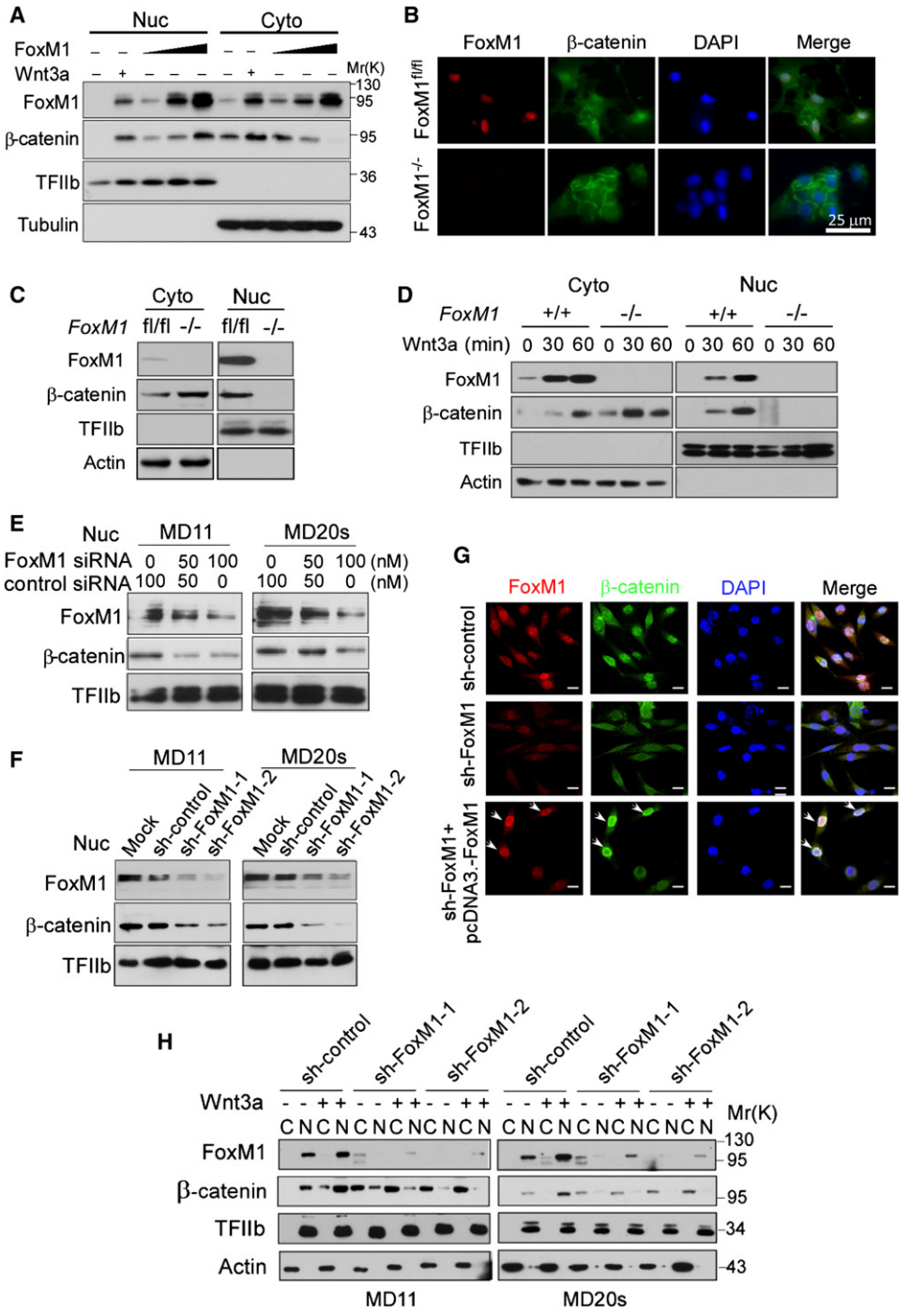

Figure 3. FoxM1 Is Required for Constitutive and Wnt3a-Induced β-Catenin Nuclear Accumulation

(A) Cytoplasmic and nuclear levels of FoxM1 and β-catenin in 293T cells transfected with increasing amounts of the Flag-FoxM1 expression plasmid were analyzed by IB. The cells treated with 20 ng/ml Wnt3a for 60 min were used as a positive control.

(B and C) FoxM1$^{-/-}$ NSCs were generated by isolation of primary NSCs (from FoxM1$^{fl/fl}$ mice), which were immortalized with the SV-40 large-T antigen and then infected with Ad-Cre to delete the floxed alleles of FoxM1. Cytoplasmic and nuclear levels of FoxM1 and β-catenin in immortalized FoxM1$^{fl/fl}$ and FoxM1$^{-/-}$ NSCs were analyzed by IF (B) and IB (C).

(D) FoxM1$^{-/-}$ or FoxM1$^{+/+}$ MEFs were immortalized with the SV-40 large-T antigen. They were then treated with Wnt3a (20 ng/ml) for the indicated times. Cytoplasmic and nuclear levels of FoxM1 and β-catenin were analyzed by IB.

(E) Nuclear levels of FoxM1 and β-catenin were examined in MD11 and MD20s cells that were treated with two different amounts of the FoxM1 or control siRNA.

(F) Nuclear levels of FoxM1 and β-catenin were examined in MD11 and MD20s cells that stably express two different shRNAs for FoxM1 (sh-FoxM1-1 or sh-FoxM1-2) or a control shRNA (sh-control).

TOP-Flash reporter to ascertain whether FoxM1 and its mutants affect β-catenin–TCF/LEF transcriptional activity. Interestingly, overexpression of full-length FoxM1 alone strongly activated the TOP-Flash reporter, and thus the transcriptional activity of β-catenin–TCF (Figure 4E). This TOP-Flash activation was probably mediated by engaging endogenous TCF and β-catenin, because FoxM1 overexpression increased levels of TCF4 (Figure 1F) and nuclear β-catenin (Figure 2F). A FoxM1 mutant containing the forkhead box and NLS domains stimulated TOP-Flash, whereas FoxM1 mutants lacking the forkhead box domain or the NLS domain did not (Figure 4E). Importantly, the FoxM1 fragment 235-347+SV40NLS also stimulated TOP-Flash activity (Figure 4E). Thus, the ability of FoxM1 to activate the β-catenin–TCF transcriptional function correlates fully with FoxM1-mediated β-catenin nuclear translocation.

To distinguish the action of FoxM1 as a β-catenin partner from the possibility that FoxM1 acts as a DNA-binding transcription factor, we generated the FoxM1 R286A/H287A mutant, which is incapable of DNA binding, as predicted from crystal structure studies (Littler et al., 2010). Indeed, this mutant dramatically decreased the ability of FoxM1 to activate a reporter from multimerized FoxM1-binding elements (Figure S3A) as well as decreased the ability to bind to FoxM1 response elements in the c-Myc promoter (Figure S3C). However, this mutant maintained the same ability to promote β-catenin nuclear localization (Figure 5A), bind to the β-catenin–TCF-binding elements in the c-Myc promoter (Figure S3D), and activate the β-catenin–TCF reporter (Figure S3B), indicating that the effect of FoxM1 on β-catenin–TCF transcriptional activity depends on interaction with β-catenin but not on FoxM1's own DNA-binding property.

We next determined whether FoxM1 is required for β-catenin–TCF-mediated transcription. As with FoxM1 overexpression, a constitutively stabilized mutant β-catenin (S33Y) activated TOP-Flash, which was inhibited by the FoxM1 siRNA in 293T cells (Figure 5B), suggesting that FoxM1 is required for β-catenin signaling. Conversely, dominant-negative TCF4 (DN-TCF4), which cannot bind to β-catenin, abolished the activation of TOP-Flash by FoxM1 (Figure 5B), suggesting that the effect of FoxM1 is mediated by binding of β-catenin to TCF4. Knocking down FoxM1 also suppressed the activation of the TOP-Flash reporter in MD11 and MD20s cells, likely as a result of endogenous β-catenin–TCF signaling (Figure 5C).

We further examined several endogenous and prototypic Wnt/TCF/β-catenin target genes. Expression levels of Axin2, LEF-1, c-Myc, and cyclin D1 were increased in MD20s cells that overexpressed FoxM1 (Figure 5D) and were decreased in MD11 and MD20s cells with knocking down of FoxM1 (Figure 5E) and in NSCs with genetic deletion of FoxM1 (Figure 5F). Furthermore, overexpression of the FoxM1 R286A/H287A mutant increased the expression of Axin2 in 293T cells (Figure 5G), indicating that the effect of FoxM1 on Wnt target gene expression depends on interaction with β-catenin.

Mutual Recruitment of FoxM1 and β-Catenin to Wnt Target-Gene Promoters

Because FoxM1 is required for activation of Wnt target genes and can form a complex with nuclear β-catenin and TCF4, FoxM1 might be recruited to TBEs/WREs in chromatin. We first determined whether FoxM1 regulates the *LEF-1* promoter, which contains three WREs (Figure 6A). Overexpression of FoxM1 in 293T cells increased the activity of the wild-type *LEF-1* promoter but not the mutant *LEF-1* promoter, which harbors three mutated WREs (Figure 6A), suggesting that FoxM1 activates the promoter via the WREs. Moreover, FoxM1, β-catenin, and TCF4 bound to the WRE region of *LEF-1* promoter in MD11 cells (Figure 6B). Furthermore, Wnt3a stimulation caused a dramatic increase in FoxM1 and β-catenin binding to the *LEF-1* promoter, which was occupied by TCF4 constitutively in 293T cells (Figure 6C).

Axin2 and *c-Myc* are also well-known Wnt target genes (through characterized TBEs/WREs in their promoters) (Clevers, 2006; Moon et al., 2004). We found that in MD11 cells, FoxM1, β-catenin, and TCF4 were recruited to the endogenous *Axin2* promoter (Figure 6D) and also to a transfected *c-Myc* promoter harboring wild-type TBEs (TBE1/2) but not to a *c-Myc* promoter harboring mutant TBEs (TBE1m/2m; Figure 6E). Moreover, FoxM1 associated with the WREs in the *LEF-1* promoter that had been immunoprecipitated by the anti-β-catenin or anti-TCF4 antibody (Figure 6F). Together, these results indicate that FoxM1, β-catenin, and TCF4 are recruited to WREs in Wnt target-gene promoters as a DNA-binding complex.

Next, we determined whether nuclear FoxM1 plays a role in assembly of the β-catenin–TCF transcription activation complex. We observed that association of β-catenin with the *LEF-1* promoter was diminished upon FoxM1 depletion (Figure 6G). Next, we examined the effect of FoxM1 depletion on the binding between β-catenin and TCF4 using exogenous Myc-TCF4 (because *TCF4* is a target gene of FoxM1). We found that the binding between β-catenin and TCF4 was decreased upon FoxM1 depletion (Figure 6I). Thus, the decreased association of β-catenin with the *LEF-1* promoter upon FoxM1 depletion was probably because both the nuclear β-catenin level (Figure 3E) and the binding between β-catenin and TCF4 were decreased (Figure 6I). These results indicate that FoxM1 is important for assembly of the β-catenin–TCF transcription activation complex.

Given the binding between β-catenin and FoxM1, we also examined whether β-catenin is required for FoxM1 binding to the Wnt target-gene promoter. Depletion of β-catenin diminished the binding of FoxM1 to the LEF-1 promoter (Figure 6H). Moreover, depletion of β-catenin by a siRNA or genetic deletion reduced or abolished the association between FoxM1 and TCF4 (Figures 6J and 6K), indicating that the association of FoxM1 with TCF4 is mediated by β-catenin. Collectively, the above results suggested that FoxM1 and β-catenin mutually depend on each other for recruitment to WREs occupied by TCF4 in Wnt target-gene promoters.

(G) Triple IF staining for FoxM1 (red), β-catenin (green), and nuclei (DAPI, blue) was performed in sh-FoxM1 and sh-control MD11 cells and in sh-FoxM1 MD11 cells that were transiently transfected with a pcDNA3.1-FoxM1 plasmid. Note that restoration of FoxM1 expression in sh-FoxM1 MD11 cells restores β-catenin nuclear localization (scale bar, 25 μm).
(H) MD11 and MD20s cells that stably express sh-FoxM1 or sh-control were treated with 50 ng/ml Wnt3a for 2 hr. IB was performed on cytoplasmic or nuclear extracts.

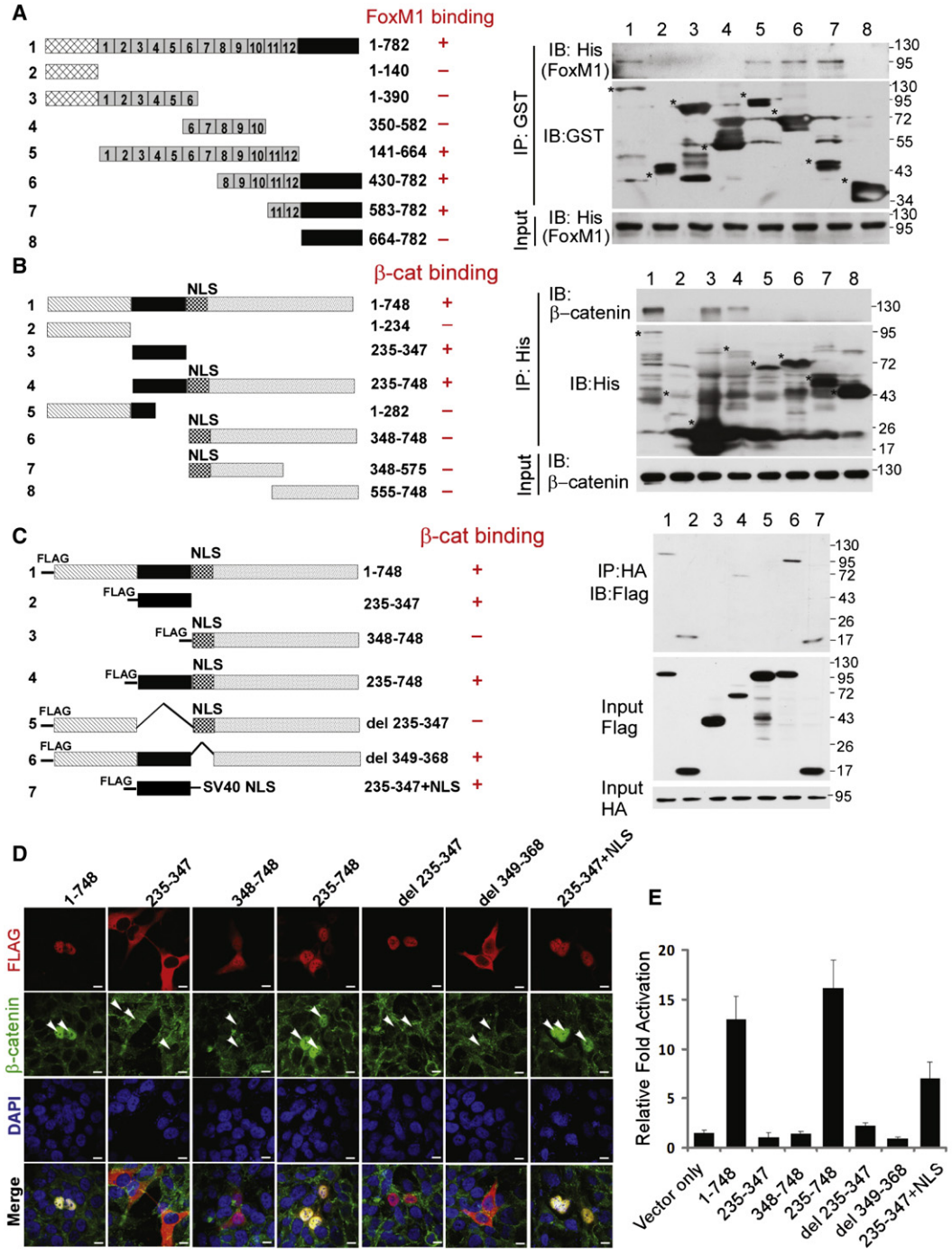

Figure 4. FoxM1 Nuclear Translocation and Binding to β-Catenin Are Required for FoxM1-Mediated β-Catenin Nuclear Accumulation

(A) Schematic illustration of β-catenin deletion mutants (left panel) and a GST pull-down experiment using bacterially expressed and purified wild-type and mutant β-catenin as GST-fusion proteins and His-FoxM1 (right panel).

(B) Schematic illustration of FoxM1 deletion mutants (left panel) and IP using bacterially expressed and purified His-tagged wild-type and mutant FoxM1 proteins and GST-β-catenin (right panel).

(C) Schematic illustration of Flag-FoxM1 deletion mutants in a mammalian expression system (left panel). Lysates from 293T cells expressing the Flag-FoxM1 mutants and HA-β-catenin were subjected to IP (right panel).

(D) 293T cells expressing Flag-FoxM1 or mutants were processed for triple IF staining for Flag-FoxM1 (red), β-catenin (green), and nuclei (DAPI, blue). White arrows indicate colocalization of FoxM1 and β-catenin in nuclei (scale bar, 10 μm).

(E) 293T cells were cotransfected with Flag-FoxM1 or mutants plus the TOP-Flash or control FOP-Flash luciferase reporter. The ratios between TOP-Flash and FOP-Flash were determined 48 hr after transfection, and promoter activation (fold) was calculated relative to that in cells transfected with the control vector. Values are mean ± SD for triplicate samples from a representative experiment.

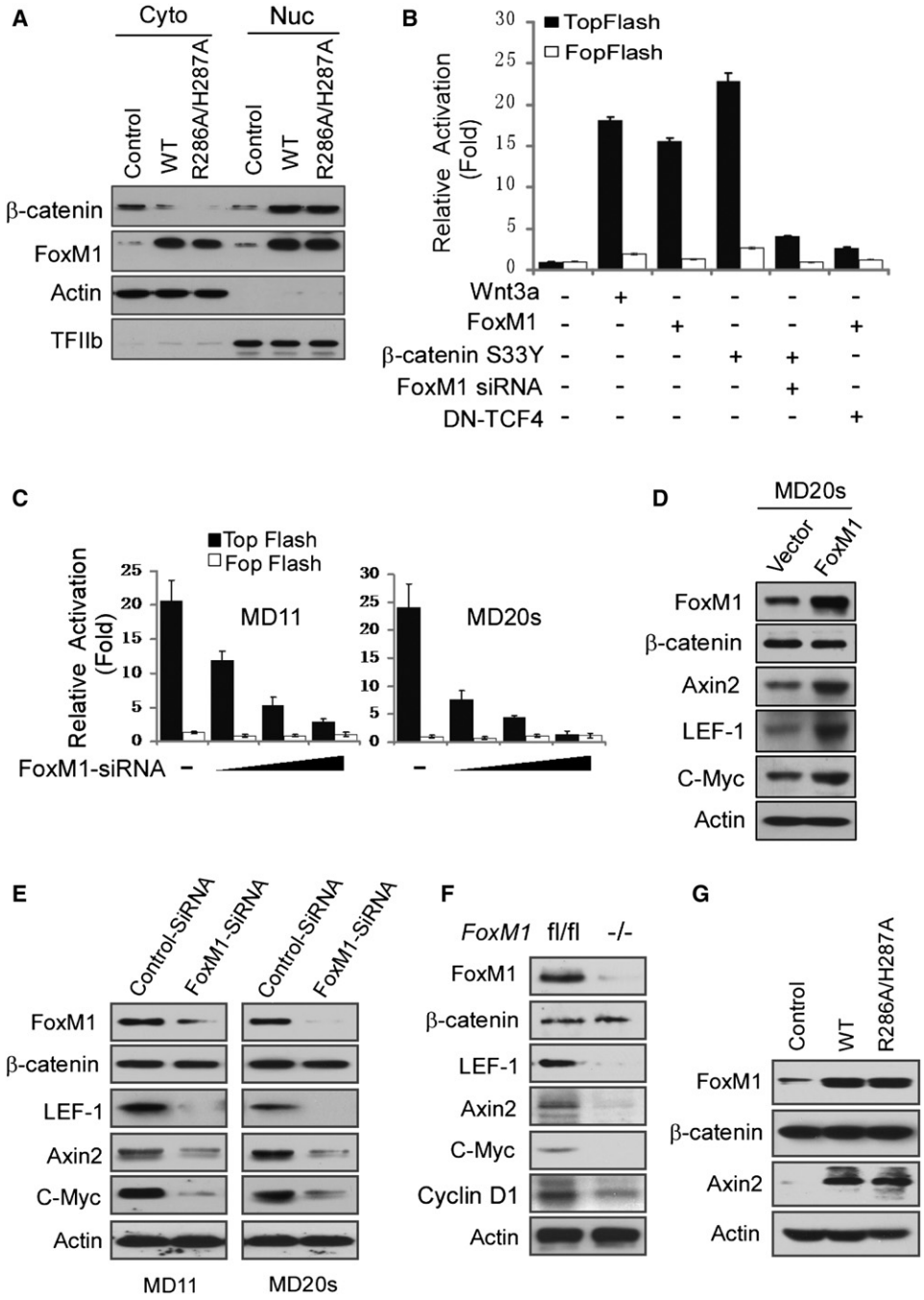

Figure 5. FoxM1 Activates Wnt/β-Catenin Signaling

(A) IB analysis of cytoplasmic and nuclear levels of β-catenin and FoxM1 in 293T cells that were transfected with a vector (Control), FoxM1 wild-type (WT), or FoxM1 with mutations at Arg-286 and His-287 (R286A/H287A) plasmid.

(B) Mutual dependence of FoxM1 and β-catenin in activating the TOP-Flash reporter. FOP-Flash was used as negative control. 293T cells were cotransfected with Flag-FoxM1, β-catenin S33Y, DN-TCF4 expression plasmid, FoxM1-siRNA, or combinations as indicated. Values are mean ± SD for triplicate samples. Cells treated with 20 ng/ml Wnt3a for 12 hr were used as a positive control.

(C) Activities of TOP-Flash or FOP-Flash in MD11 and MD20s cells cotransfected with an increasing amount of FoxM1-siRNA. Values are mean ± SD for triplicate samples.

(D) Cellular levels of FoxM1, β-catenin, Axin2, LEF-1, and c-Myc in MD20s cells transfected with a vector or FoxM1 expression plasmid were analyzed by IB.

(E) Cellular levels of FoxM1, β-catenin, Axin2, LEF-1, and c-Myc in MD11 or MD20s cells transfected with control-siRNA or FoxM1-siRNA were examined by IB.

(F) Cellular levels of FoxM1, β-catenin, Axin2, LEF-1, c-Myc, and cyclin D1 in FoxM1-knockout NSCs were examined by IB.

(G) Cellular levels of FoxM1, β-catenin, and Axin2 in 293T cells transfected with a vector (control), FoxM1 (WT), or FoxM1 R286A/H287A plasmid.

See also Figure S3.

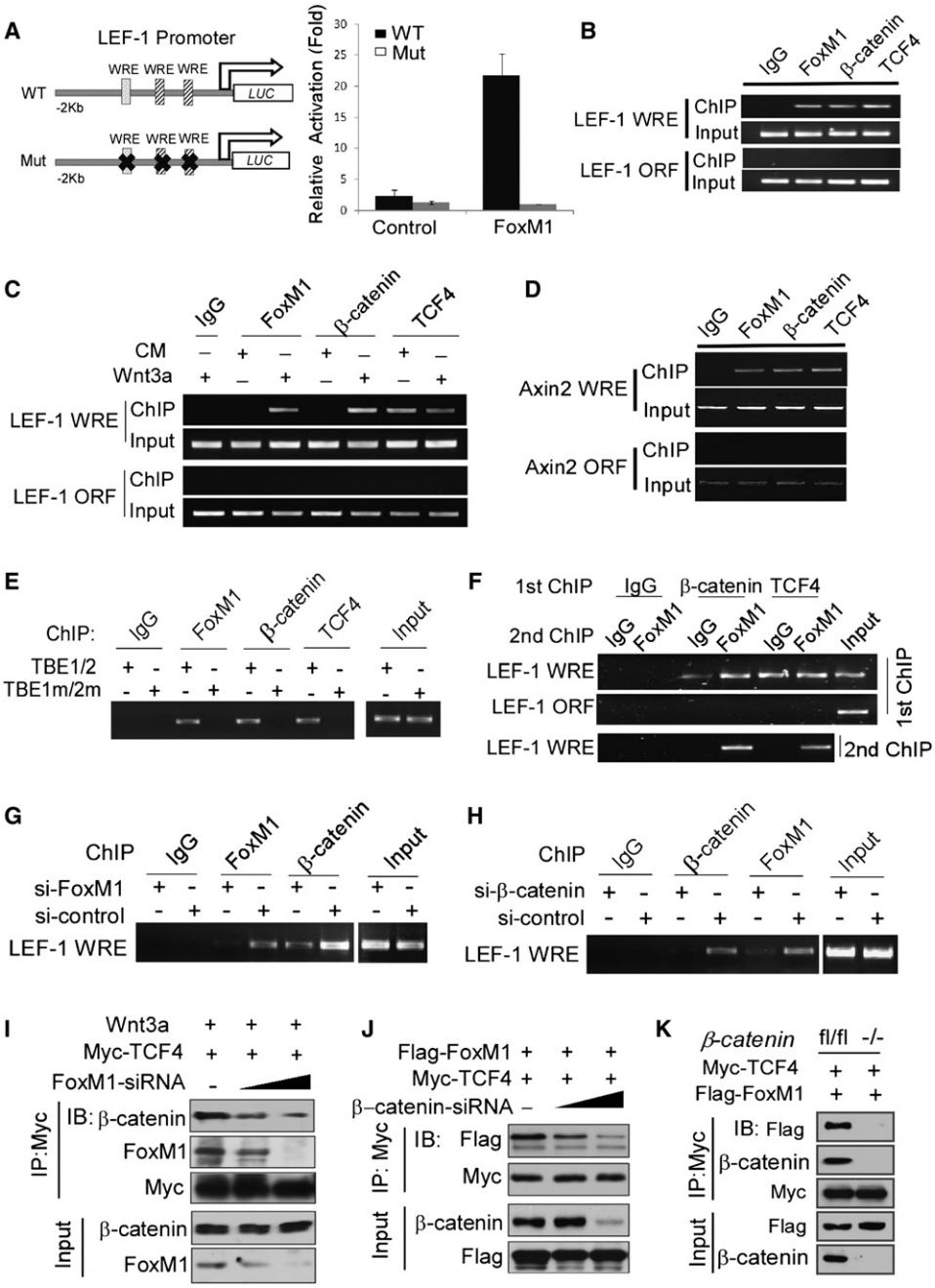

Figure 6. Mutual Recruitment of FoxM1 and β-Catenin to Wnt Target-Gene Promoters

(A) FoxM1 activates the *LEF-1* promoter via the WREs. The three WREs of the *LEF-1* promoter were mutated to generate the mutant *LEF-1* promoter. The relative luciferase activity of wild-type (WT) or mutant (Mut) *LEF-1* promoters was determined in 293T cells transfected with the FoxM1 or control vector. Values are mean ± SD for triplicate samples.

(B and C) ChIP assays on WREs of *LEF-1* promoter or the ORF region of *LEF-1* gene were performed in MD11 cells (B) or 293T cells with or without Wnt3a treatment (20 ng/ml) for 6 hr (C).

(D) ChIP assays on WREs of *Axin2* promoter or the ORF region of *Axin2* gene were performed in MD11 cells.

(E) A luciferase reporter driven by the *c-Myc* promoter with wild-type TBEs (pBV-TBE1/2-luc) or mutant TBEs (pBV-TBE1m/2 m-luc) was transfected into MD11 cells. Then ChIP assays were performed using PCR primer pairs flanking the *c-Myc* promoter (on the pBV vector).

(F) FoxM1 and β-catenin co-occupied the *LEF-1* promoter in MD11 cells.

(G and H) ChIP assays were performed in MD11 cells that were transfected with FoxM1-siRNA, β-catenin-siRNA, or control siRNA.

(I) Co-IP analyses of β-catenin–TCF4 interaction in 293T cells that were cotransfected with Myc-TCF4 and increasing amounts of FoxM1-siRNA and treated with 20 ng/ml Wnt3a for 6 hr.

(J and K) Co-IP analyses of the association of FoxM1 with TCF4 in 293T cells that were cotransfected with Flag-FoxM1, Myc-TCF4, and β-catenin-siRNA (J) or in β-catenin$^{-/-}$ MEFs (K).

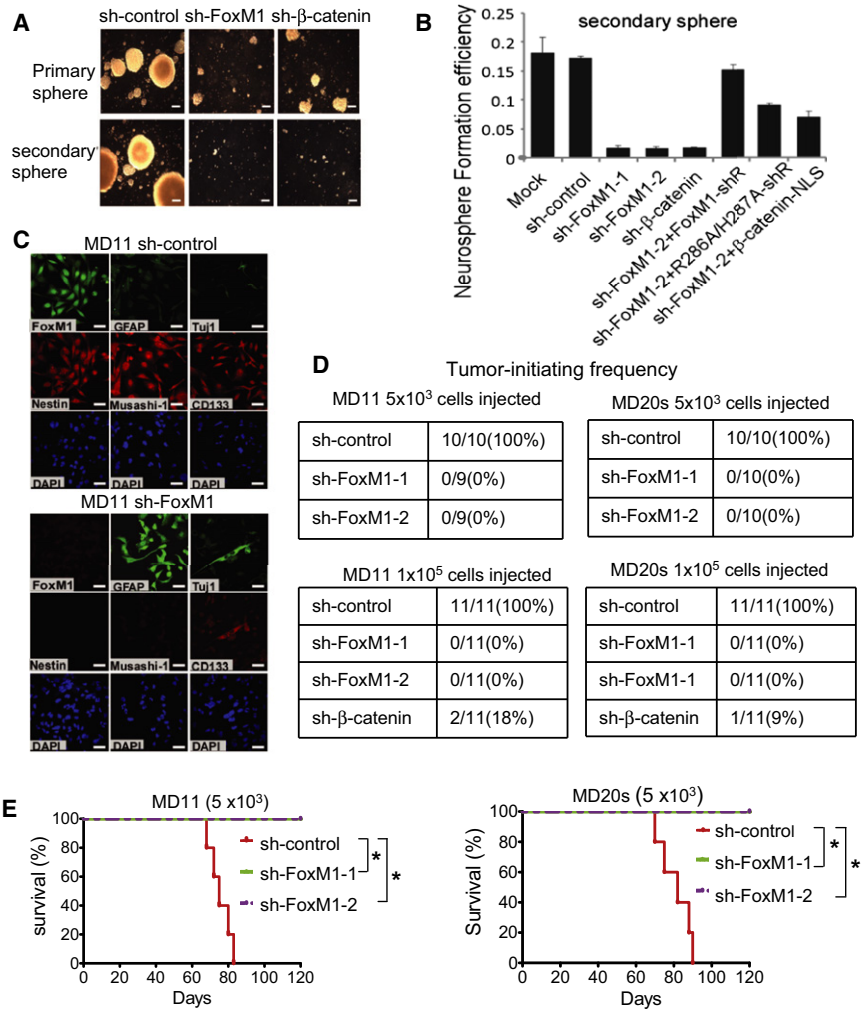

Figure 7. FoxM1 and β-Catenin Maintain GIC Self-Renewal and Glioma Formation

(A) Photographs of primary and secondary neurosphere formation of MD11 cells that express shRNAs for control, FoxM1, or β-catenin. Scale bar, 100 μm.

(B) The primary and secondary neurosphere formation efficiency (spheres/cells plated) of the cells in (A). Values are mean ± SD for triplicate samples. shR: shRNA-resistant FoxM1 (or its mutant).

(C) Dissociated MD11 cells from spheres were plated on coverslips precoated with poly-L-ornithine and fibronectin, and the indicated markers were examined via IF (scale bar, 40 μm).

(D and E) MD11 or MD20s cells (5×10^3 or 1×10^5 cells/mouse) that express the indicated shRNAs were implanted intracranially in nude mice. Mice were killed when they were moribund or 120 days after implantation. Tumor formation was determined by histology. Tumor-initiating frequency was then calculated (D). Survival of mice (n = 5) was evaluated by Kaplan-Meier analysis (E). *p < 0.001.

(F) ShRNA-resistant R286A/H287A mutant or β-catenin-NLS rescued the tumor-initiating capacity of GICs that express the FoxM1-shRNA. MD11 or MD20s-shFoxM1 cells (1×10^5 cells/mouse) that express shRNA-resistant FoxM1 wt, shRNA-resistant R286A/H287A, or β-catenin-NLS were implanted intracranially in nude mice. Mice were killed when they were moribund or 70 days after implantation. Tumor-initiating frequency was then calculated.

See also Figure S4.

Interaction of FoxM1 and β-Catenin Plays a Critical Role in GIC Self-Renewal and Differentiation

To study the effect of FoxM1 and β-catenin on the self-renewal of GICs, we generated primary cultures of tumor cells (PCTCs) in the presence of serum, and in parallel, neurospheres from human GBM samples. The neurospheres showed enrichment for GICs, because, first, they maintained neurosphere formation ability and thus exhibited self-renewal ability (Lee et al., 2006; Singh et al., 2003) (Figure S4A); second, they expressed high levels of the neuroprogenitor markers Nestin, CD133, and SSEA-1 (Figure S4D); and third, they could undergo multilineage differentiation, acquiring the expression of GFAP (astrocytic marker) and Tuj-1 (neuronal marker) (Figures S4B and S4C). Importantly, high levels of FoxM1 (Figure S4D) and Wnt reporter activity (Figure S4E) were present in these GICs compared with the PCTCs. Thus, we investigated whether FoxM1 and β-catenin were required for GIC self-renewal. Knockdown of FoxM1 or β-catenin in MD11 and MD20s cells substantially decreased the size and number of spheres formed in primary and secondary sphere formation assays (Figures 7A and 7B). Next, the cells were assayed for neural colony-forming ability, a more stringent test for the presence of self-renewing cells (Louis et al., 2008). Knockdown of FoxM1 or β-catenin substantially reduced the efficiency of neural colony formation in GICs (Figures S4F). Moreover, the inhibitory effect of sh-FoxM1 on self-renewal of GICs was rescued by shRNA-resistant R286A/H287A, which binds to β-catenin but not to FoxM1-response elements or β-catenin-NLS (which translocates into the nucleus constitutively) (Figure 7B and Figure S4F).

We investigated whether FoxM1 and β-catenin also affect GIC differentiation. FoxM1 knockdown substantially inhibited the expression of CD133, Nestin, Sox2, and Musashi-1 and up-regulated the differentiation markers of Tuj1 and GFAP in GICs (Figure 7C and Figure S4G). Depletion of β-catenin in

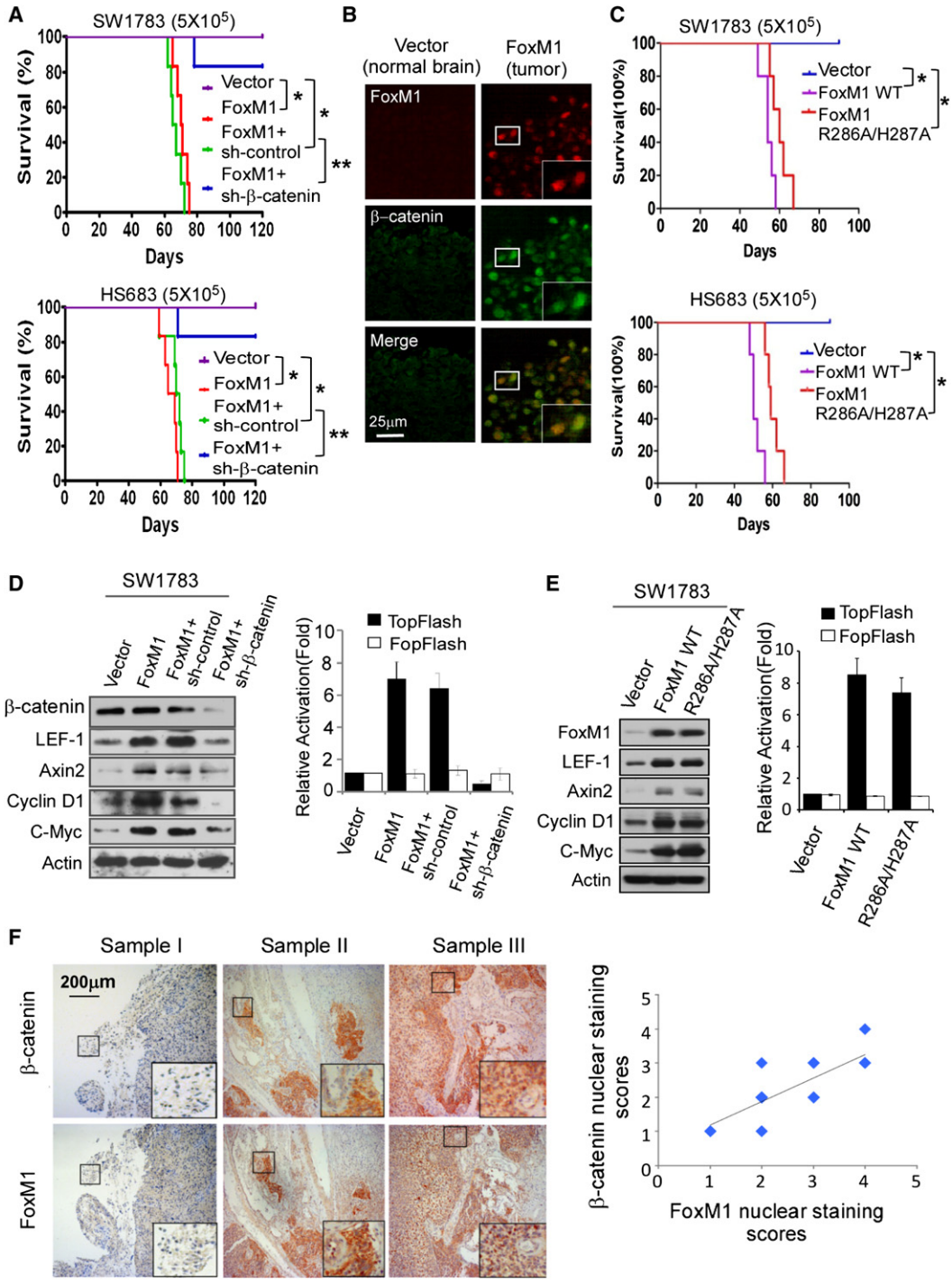

Figure 8. Interaction of FoxM1 and β-Catenin Controls Glioma Formation

(A) The tumorigenicity of SW1783 or Hs683 cells that stably express vector, FoxM1, FoxM1+sh-control, or FoxM1+sh-β-catenin and the survival of the mice were evaluated as described in Figure 7D. *$p < 0.001$ and **$p < 0.01$.

(B) Sections of tumors produced by SW1783-FoxM1 cells or brain tissue from mice injected with SW1783-vector cells were IF-stained with FoxM1 and β-catenin antibodies, followed by confocal microscopic analysis.

(C) The tumorigenicity of SW1783 or Hs683 cells that stably express vector, FoxM1, or FoxM1 R286A/H287A and the survival of the mice. *$p < 0.001$.

(D) Cellular levels of β-catenin, LEF-1, Axin2, c-Myc, and cyclin D1 (left panel) and activities of TOP-Flash or FOP-Flash (right panel) in SW1783 cells that stably express vector, FoxM1, FoxM1+sh-control, or FoxM1+sh-β-catenin. Values are mean ± SD for triplicate samples.

(E) Cellular levels of β-catenin, LEF-1, Axin2, c-Myc, and cyclin D1 (left panel) and activities of TOP-Flash or FOP-Flash (right panel) in SW1783 cells that stably express vector, FoxM1, or FoxM1 R286A/H287A. Values are mean ± SD for triplicate samples.

GICs produced almost identical results (Figure S4H). Therefore, FoxM1 perturbed the balance between progenitor cell renewal and the commitment to differentiation. Moreover, these effects of sh-FoxM1 were rescued by shRNA-resistant R286A/H287A or β-catenin-NLS (Figure S4I), supporting that FoxM1–β-catenin interaction controls the self-renewal of GICs.

Next, we examined the cell-cycle progression of the above cell lines. An endogenous FoxM1-β-catenin complex and Wnt signal activity were observed in asynchronous GICs and in GICs in cell-cycle phases G0/G1, S, and G2/M, with a relatively higher level seen in G2/M GICs (Figures S4M–S4O). Comparison of cell-cycle profiles of the above cell lines revealed a similar cell-cycle distribution under asynchronous conditions (Figure S4P). However, when synchronized and released from G1/S transition, sh-FoxM1 GICs exhibited a delay in mitotic entry (Figure S4P). Depletion of β-catenin in GICs also resulted in G2/M arrest (Figure S4P). Expression of shRNA-resistant R286A/H287A or β-catenin-NLS in sh-FoxM1 cells partially restored normal cell-cycle progression. Therefore, these data indicated that the FoxM1/β-catenin interaction is required for G2/M transition and proper mitotic progression.

Interaction of FoxM1 and β-Catenin Controls Tumor Formation by Glioma Cells

Next, we examined the effect of FoxM1 or β-catenin depletion and their interaction on the tumor-initiating ability of GICs in an animal model. All mice injected with sh-control MD11 and MD20s cells displayed brain tumors that showed characteristic GBM features. In contrast, mice bearing sh-FoxM1 cells did not develop brain tumors. Therefore, the tumor-initiating ability was substantially reduced in cells with suppressed FoxM1 expression (100% decrease, Figure 7D). The tumor-initiating ability was also substantially reduced by suppressed β-catenin expression (Figure 7D). In addition, the mice bearing sh-FoxM1 or sh-β-catenin cells survived significantly longer than controls (Figure 7E and Figure S4J). Moreover, FoxM1, β-catenin, and Wnt signal activity levels were highly elevated in the brain tumor cells (Figures S4K, S4L, and S4Q), and the proteins colocalized in tumor cell nuclei (Figure S4K). Furthermore, the inhibitory effect of sh-FoxM1 on tumorigenicity of GICs was rescued by shRNA-resistant R286A/H287A or β-catenin-NLS (Figures 7F). These results support a role for the FoxM1–β-catenin complex in the induction of GBM in vivo.

To further ascertain that β-catenin mediates the tumorigenic effect of FoxM1, we examined the tumorigenicity of glioma cells that overexpress FoxM1 but are deficient in β-catenin. First, overexpression of FoxM1 promoted SW1783 cells to exhibit GIC characteristics, because SW1783-FoxM1 cells (but not control SW1783) were able to form neurospheres and expressed SSEA-1 and Nestin (Figure S5A). Second, SW1783 and Hs683 cells did not form brain tumors in nude mice, but SW1783-FoxM1 and Hs683-FoxM1 cells did (Figure 8A), indicating that FoxM1 overexpression is responsible for tumor formation. However, β-catenin knockdown in SW1783-FoxM1 or Hs683-FoxM1 cells diminished their tumorigenicity (Figure 8A), indicating that FoxM1 tumor promotion depends on β-catenin. Moreover, in the SW1783-FoxM1 brain tumors, FoxM1 levels were highly elevated in tumor cell (Figure S5B), β-catenin mostly localized in the cell nuclei, and it colocalized with FoxM1 (Figure 8B). Furthermore, overexpression of FoxM1 R286A/H287A was able to induce tumor formation (Figures 8C). Consistently, SW1783-FoxM1 and SW1783-R286A/H287A cells exhibited higher levels of TOP-Flash reporter and Axin2, LEF-1, c-Myc, and cyclin D1 expression than SW1783 control cells did (Figures 8D and 8E). In contrast, knockdown of β-catenin in SW1783-FoxM1 cells abolished elevated expression of the reporter and the Wnt target genes (Figures 8D). Finally, FoxM1 regulates the nuclear β-catenin level and thus tumor cell growth in mouse high-grade glioma models (Figures S5G–S5I). Together, these results show that the FoxM1–β-catenin interaction controls the expression of Wnt/β-catenin target genes and tumorigenesis of glioma cells.

Nuclear β-Catenin Expression in Human GBM Correlates with Levels of Nuclear FoxM1

We analyzed the significance of FoxM1-mediated β-catenin activation in human GBM using a panel of 40 GBM samples. FoxM1 was moderately expressed in 14 samples and was highly expressed in 18 samples. The expression levels of nuclear FoxM1 directly correlated with those of nuclear β-catenin (Figures 8F), further supporting the critical role of FoxM1 in β-catenin nuclear accumulation in human GBM. Also, the expression levels of Wnt targets Axin2 and LEF-1 directly correlated with the levels of FoxM1 (Figures S5C and S5D).

Next, we performed IF staining on eight frozen GBM tumor samples that were FoxM1 positive. Sections from the same tumor tissues were used for costaining for FoxM1 and β-catenin and for Nestin and GFAP. Nuclear FoxM1 colocalized with nuclear β-catenin in tumor cells (Figures S5E and S5F). Moreover, FoxM1 expression correlated directly with Nestin expression but inversely with GFAP expression in tumors (Figure S5F). Together, our data suggest that, in GBM tumors that express high levels of FoxM1, β-catenin and FoxM1 probably promote GIC self-renewal, as evidenced by the increase in the number of cells expressing neuroprogenitor cell markers.

DISCUSSION

Here, we demonstrate that FoxM1 is required for β-catenin nuclear accumulation in tumor cells, that nuclear FoxM1 and β-catenin form a functional complex with TCF4 on Wnt target-gene promoters, and that the functional interaction between FoxM1 and β-catenin promotes GIC self-renewal and tumorigenesis. Therefore, the FoxM1–β-catenin interaction represents a critical mechanism for controlling canonical Wnt signaling and GIC self-renewal, proliferation and tumorigenesis.

(F) Expression of FoxM1 and β-catenin was examined via IHC staining in 40 GBM specimens. Left: Representative β-catenin and FoxM1 expression levels are shown in three GBM tumor sections. Right: Staining of nuclear FoxM1 or β-catenin was scored as 1 to 4. The correlation was significant as determined by Pearson's correlation test ($r = 0.682$; $p < 0.001$).
See also Figure S5.

FoxM1 and Wnt/β-Catenin Signaling

Aberrant activation of the Wnt/β-catenin signaling pathway is widespread in human cancers. In some cancers, such as colorectal carcinoma, Wnt/β-catenin pathway activation is caused by mutations in genes encoding Wnt signaling components, such as mutational loss of *APC* or *CTNNB1* "gain-of-function" mutations that increase the stability of β-catenin. In other cancers, including glioma, which do not exhibit these prototypic mutations, other mechanisms are likely to be involved in Wnt/β-catenin activation. Interestingly, FoxM1 is overexpressed in many human cancers, including glioma. Our results show that FoxM1 is an unexpected but key mediator of Wnt/β-catenin signaling and acts by binding to β-catenin and enhancing β-catenin nuclear localization and transcriptional activity.

FoxM1 appears to govern two critical and related aspects of β-catenin nuclear function: nuclear accumulation and assembly of a transcription activation complex. The molecular mechanisms for β-catenin nuclear activation during Wnt signaling remain enigmatic, although several mechanisms have been proposed. Some studies suggest that β-catenin shuttles from the cytoplasm to the nucleus by interacting directly with the nuclear pore complex (Henderson and Fagotto, 2002). Others suggest that TCF/LEF and β-catenin coactivators such as Pygopus/Bcl9 mediate β-catenin nuclear import or retention/anchoring (Townsley et al., 2004). We show that FoxM1 is necessary and sufficient for β-catenin nuclear accumulation. FoxM1 interacts with Arm repeats 10–12 of β-catenin and enhances β-catenin nuclear localization, whereas depletion of FoxM1 inhibits both constitutive and Wnt3a-induced β-catenin nuclear localization. Importantly, deletion of the FoxM1 gene in NSCs abolishes β-catenin nuclear localization. We identified the minimal FoxM1 domains required for β-catenin nuclear accumulation. The forkhead box domain of FoxM1, which mediates the binding of β-catenin and FoxM1, and the NLS domain of FoxM1, which mediates FoxM1 nuclear translocation, are together necessary and sufficient for β-catenin nuclear accumulation. The simplest interpretation is that FoxM1 provides a functional NLS domain for nuclear import of the FoxM1–β-catenin complex. Alternatively, FoxM1 may provide a nuclear anchor for β-catenin to counteract nuclear export of β-catenin by other factors, such as APC (Henderson and Fagotto, 2002). We note a recent study suggesting that EGFR/RAS signaling promotes β-catenin nuclear accumulation (Phelps et al., 2009). Interestingly, EGFR/RAS signaling leads to FoxM1 activation and nuclear translocation (Ma et al., 2005; Park et al., 2009). The FoxM1–β-catenin signaling axis may underlie these observations.

In unstimulated cells, β-catenin was found as both membrane-bound and cytoplasmic protein. Recently, it was suggested that FoxM1 regulates the level of membrane-bound β-catenin in lung endothelial cells by mediating the transcription of β-catenin mRNA (Mirza et al., 2010). However, this regulation seems to be restricted to endothelial cells but not fibroblasts (Mirza et al., 2010). Consistent with this, we observed that FoxM1 did not affect the total level of β-catenin expression in MEFs, NSCs, 293T cells, or glioma cells. The cytoplasmic pool, but not the membrane-bound pool, of β-catenin rapidly turns over in unstimulated cells, and Wnt stimulation stabilizes the cytoplasmic pool and induces β-catenin nuclear accumulation (Clevers, 2006; Moon et al., 2004). Our results indicate that FoxM1 is important for promoting β-catenin nuclear accumulation.

In the nucleus, β-catenin is recruited to promoters of Wnt target genes through its interaction with TCF/LEF proteins, and the β-catenin–TCF/LEF complex is essential for transcriptional activation of Wnt target genes. Strikingly, we also found that nuclear FoxM1 and β-catenin form a tripartite functional complex with TCF/LEF on Wnt target-gene promoters. FoxM1 and TCF/LEF bind to nonoverlapping Arm repeats of β-catenin, which mediates the association between FoxM1 and TCF/LEF. Interestingly, FoxM1 overexpression can activate the TOP-Flash reporter in a β-catenin–TCF/LEF-dependent manner. This transactivation ability is independent of the DNA-binding activity of FoxM1, because a FoxM1 mutant that is incapable of DNA binding can still activate the TOP-Flash reporter and regulate Wnt-target gene expression. Furthermore, we found that association of β-catenin with WREs in chromatin and the binding between β-catenin and TCF4 were decreased upon FoxM1 depletion. Together, these findings suggest that FoxM1 promotes not only β-catenin nuclear accumulation but also the assembly of a FoxM1–β-catenin–TCF/LEF transcription complex on WREs in chromatin, thereby playing a central role in Wnt/β-catenin-dependent transcriptional programs.

FoxM1 has been shown to regulate the cell cycle by binding to promoters of key cell-cycle genes (Kalinichenko et al., 2004; Ye et al., 1997). However, because the majority of GICs are in the G1 phase under asynchronous condition and because most of the experiments for the effect of FoxM1 on β-catenin nuclear accumulation and activity were performed in these asynchronous cells, we believe it unlikely that the effect of FoxM1 on Wnt/β-catenin signaling is the secondary consequence of the role of FoxM1 in cell cycle. Furthermore, because both FoxM1 and β-catenin knockdowns resulted in a similar G2/M block in GICs when they are synchronized at the G1/S transition, and the FoxM1 DNA-binding mutant and a nuclear-localized β-catenin were each capable of partially rescuing this G2/M block, we further infer that the effect of FoxM1 on the cell cycle is, in part, mediated by its association with β-catenin. This is consistent with the fact that many target genes of β-catenin signaling have cell-cycle regulatory roles.

FoxM1 Regulation and Wnt Signaling

Recent studies show that FoxM1 is aberrantly activated in many human cancers and plays an essential role in tumorigenesis (Kalinichenko et al., 2004; Pilarsky et al., 2004; Yoshida et al., 2007). Although our findings link FoxM1 to the Wnt/β-catenin tumorigenic pathway, the mechanism of FoxM1 overexpression in tumors, including GBM, remains unclear. We showed that Wnt3a increases both cytoplasmic and nuclear FoxM1 levels and promotes FoxM1 nuclear accumulation in GBM cells. Others have shown that GBM cells overexpress the Wnt ligands and receptors, such as Wnt3, Wnt6, and Fz9 (Zheng et al., 2010). Thus, FoxM1 behaves similarly to TCF4 and LEF-1, which not only mediate but also are targets of Wnt signaling, setting up positive regulatory loops involved in carcinogenesis (MacDonald et al., 2009).

FoxM1-Mediated Wnt/β-Catenin Signaling in GIC Self-Renewal and Glioma Formation

The Wnt signaling pathway is known to be essential for the maintenance of tissue-specific stem cells in some organs, including NSCs (Clevers, 2006; Moon et al., 2004; Nusse, 2008; Zheng et al., 2010). Accordingly, GICs may possess some properties of normal NSCs and be responsible for the initiation of glioma (Lee et al., 2006; Park and Rich, 2009; Singh et al., 2003). We found that FoxM1 regulates β-catenin activation in both NSCs and GICs. Moreover, β-catenin activation is required for GIC self-renewal and tumorigenicity, and FoxM1 plays an essential role in Wnt/β-catenin signaling in the maintenance of GIC self-renewal and glioma formation. These results were supported further by our finding of a strong correlation between elevated nuclear β-catenin and FoxM1 levels in human primary GBM. The role of FoxM1 in Wnt/β-catenin signaling is unlikely to be limited to glioma, because FoxM1 is also essential for intestinal tumorigenesis (Yoshida et al., 2007), which has a well-established association with overactivation of β-catenin signaling (Clevers, 2006).

In summary, our study has revealed that FoxM1 is a critical regulator of Wnt/β-catenin signaling and that FoxM1 overexpression contributes to the up-regulation of β-catenin signaling in tumors, such as glioma, in which *APC* or *CTNNB1* mutations are uncommon. Given the importance of FoxM1 and the Wnt/β-catenin signaling pathway in human cancers in general, our findings not only provide an improved understanding of the molecular mechanisms underlying β-catenin activation and tumorigenesis but also suggest additional targets for therapeutic intervention.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions

The human glioma cell lines (Hs683 and SW1783) and the human embryonic kidney cell line (293T) were obtained from the American Type Culture Collection and cultured in DMEM with 10% FBS. The GIC lines were established by isolating neurosphere-forming cells from surgical specimens of human GBM using a method described previously (Lee et al., 2006). The study was approved by the Institutional Review Board of M. D. Anderson Cancer Center, and informed consent was obtained from all subjects. These GIC lines were cultured as GBM neurospheres in DMEM/F12 medium containing B27 supplement (Invitrogen) and bFGF and EGF (20 ng/ml each).

Time-Lapse Imaging with Confocal Laser-Scanning Microscopes

293T cells were transiently cotransfected with pDsRed-FoxM1 and pCFP-β-catenin. The localization and intensity of FoxM1 and β-catenin were imaged with a confocal imaging system (Olympus FluoView FV1000), with the DsRed and CFP fluorescent proteins activated by laser at 543 and 457 nm, respectively. Fluorescence intensity was quantified with FluoView platform software. Time-lapse plots of relative nuclear fluorescence intensity were produced from the cells treated with Wnt3a.

IF Staining and Confocal Microscopy

GICs were grown on chamber slides precoated with poly-L-ornithine and fibronectin. Cells were fixed with 4% paraformaldehyde, permeabilized for 5 min with PBS containing 0.1% Triton X-100 (PBS-T), quenched with 50 mM NH_4Cl in PBS-T, and blocked with 1% BSA in PBS-T. Immunostaining was performed using the appropriate primary and secondary antibodies, and images were acquired using an Olympus FluoView FV1000 confocal microscope.

Intracranial Tumor Assay

All mouse experiments were approved by the Institutional Animal Care and Use Committee of M. D. Anderson. Male athymic BALB/c nude mice were purchased from NCI. Glioma cells were injected intracranially into nude mice as described previously (Liu et al., 2006). Animals were killed when they were moribund; the remaining animals were killed 120 days after glioma-cell injection. Each mouse's brain was harvested, fixed in 4% formaldehyde, and embedded in paraffin. Tumor formation was determined by histologic analysis of H&E-stained sections.

Statistical Analysis

We determined the significance of differences in the human GBM data using Pearson's correlation test, in the in vitro data using Student's t test (two-tailed), and in the in vivo data using the Mann-Whitney *U* test. $p < 0.05$ was considered to be significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and may be found with this article online at doi:10.1016/j.ccr.2011.08.016.

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