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LATS2 Suppresses Oncogenic Wnt Signaling by Disrupting β-Catenin/BCL9 Interaction

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SUMMARY

Abnormal activation of Wnt/β-catenin-mediated transcription is associated with a variety of human cancers. Here, we report that LATS2 inhibits oncogenic Wnt/β-catenin-mediated transcription by disrupting the β-catenin/BCL9 interaction. LATS2 directly interacts with β-catenin and is present on Wnt target gene promoters. Mechanistically, LATS2 inhibits the interaction between BCL9 and β-catenin and subsequent recruitment of BCL9, independent of LATS2 kinase activity. LATS2 is downregulated and inversely correlated with the levels of Wnt target genes in human colorectal cancers. Moreover, nocodazole, an antimicrotubule drug, potently induces LATS2 to suppress tumor growth in vivo by targeting β-catenin/BCL9. Our results suggest that LATS2 is not only a key tumor suppressor in human cancer but may also be an important target for anticancer therapy.

INTRODUCTION

The Hippo signaling pathway plays a critical role in oncogenesis by regulating cell proliferation, epithelial-mesenchymal transition, and apoptosis (Zhao et al., 2008, 2010; Wu et al., 2003; Harvey et al., 2003). Mammalian LATS (large tumor suppressor) 1 and 2, the homologs of Wts in *Drosophila*, are serine/threonine kinases and key components of the Hippo signaling pathway (Dong et al., 2007; Xu et al., 1995; Justice et al., 1995; Yabuta et al., 2000). In canonical Hippo signaling, LATS 1 and 2 phosphorylate YAP and promote YAP cytoplasmic retention and degradation, resulting in inhibition of cell proliferation and oncogenesis (Huang et al., 2005; Zhao et al., 2008). Although most studies have focused on canonical Hippo signaling, some studies suggest that individual components of the Hippo signaling pathway can regulate cell survival, oncogenesis, and cytokinesis independent of the Hippo-YAP cascade (Zhao et al., 2010). For example, LATS1 was found to regulate cytokinesis by inhibiting LIMK1 (Yang et al., 2004), and LATS2 bound to Mdm2 to activate p53, serving as a novel checkpoint for the maintenance of proper chromosome number (Aylon et al., 2006). Moreover, genetic studies showed that *Lats1* and *Lats2* may have unique functions in mouse development and oncogenesis. Although *Lats1*-deficient mice developed ovarian tumors and soft tissue sarcomas (St John et al., 1999), the deletion of *Lats2* in mice resulted in embryonic lethality (McPherson et al., 2004). Interestingly, *Lats2*-deficient mouse embryo fibroblasts (MEFs) lost contact inhibition and displayed centrosome amplification and genomic instability (McPherson et al., 2004).

The Wnt/β-catenin signaling pathway plays critical roles in development, stem cell self-renewal, and oncogenesis (MacDonald et al., 2009). The constitutive activation of Wnt/β-catenin signaling has been found to be associated with a variety of human cancers such as colorectal cancer, prostate cancer, and squamous cell carcinoma (SCC) (Morin et al., 1997; Korinek et al., 1997; You et al., 2002; Chen et al., 2001; Li and Wang, 2008). Wnt/β-catenin signaling promotes cell proliferation, survival, and invasive growth through β-catenin/Tcf-mediated transcription (Morin et al., 1997; Korinek et al., 1997). In the absence of Wnt/β-catenin signaling, Wnt target genes are silenced by Tcf/Lef family proteins and corepressors of Groucho/TLE1 and histone deacetylase 1 (HDAC1). Wnt signaling leads to an accumulation of cytosolic and nuclear β-catenin. Nuclear β-catenin stimulates gene transcription by recruiting chromatin-remodeling complexes and coactivators (MacDonald et al., 2009). Several transcription complexes or coactivators, including BCL9 and Pygopus (PYGO), polymerase-associated factor 1

(PAF1), and SET1 (trithorax), have been identified to be recruited by β-catenin (Parker et al., 2002; Mosimann et al., 2006; Kramps et al., 2002). Importantly, BCL9 is highly expressed in human tumor tissues, and the β-catenin/BCL9 complex has been identified as an important target for cancer therapy (Takada et al., 2012). β-Catenin-mediated transcription is tightly regulated by various signaling molecules and pathways. Because there is a crosstalk between Hippo and Wnt/β-catenin signaling (Zecca and Struhl, 2010; Varelas et al., 2010), we initially set out to explore how the Hippo signaling pathway regulates Wnt/β-catenin-mediated transcription. Unexpectedly, we found that LATS2 was capable of directly inhibiting β-catenin/BCL9-mediated transcription independent of the canonical Hippo-YAP signaling cascade and its associated kinase activity. LATS2 downregulation was found to be associated with the development and metastasis of human colorectal cancer and poor prognosis. Intriguingly, we found that nocodazole, a chemotherapeutic drug that inhibits the polymerization of microtubule, potently inhibited β-catenin/BCL9-mediated transcription and tumor growth by inducing LATS2. Our results suggest that LATS2 may suppress oncogenesis by targeting β-catenin/BCL9 in addition to phosphorylating YAP.

RESULTS

LATS2 Inhibits Wnt/β-Catenin-Mediated Transcription

To explore whether LATS2 modulated Wnt/β-catenin-mediated transcription, we utilized a SuperTopFlash (STopflash) reporter assay that measures β-catenin/Tcf-mediated transcription (Li and Wang, 2008). Overexpression of LATS2 significantly inhibited β-catenin/Tcf-mediated transcription in human embryonic kidney (HEK) 293T cells induced by LiCl, an inhibitor of GSK-3β (Figure 1A). Similarly, overexpression of LATS2 also inhibited β-catenin/Tcf-mediated transcription induced by Wnt-3a (Figure 1B). Canonical Wnt ligands bind to Frizzled receptors and LRP5/LRP6 receptors to stabilize cytosolic β-catenin, which is involved in multiple signaling cascades. The increased β-catenin translocates to the nucleus to activate gene transcription (MacDonald et al., 2009; Townsley et al., 2004). Recently, Varelas et al. (2010) showed that Hippo signaling inhibited Wnt/β-catenin signaling by promoting an interaction between TAZ and DVL2, which is the upstream of β-catenin. To determine the Wnt signaling step that is inhibited by LATS2, we examined whether LATS2 could directly inhibit STopflash reporter activity induced by overexpression of β-catenin-S4A (β-Cat), which carries four alanine substitutions in the GSK3β recognition site. Unexpectedly, overexpression of LATS2 also significantly inhibited β-Cat-induced transcription, suggesting that LATS2 could act on or downstream of β-catenin (Figure 1C). In contrast, overexpression of LATS1 did not inhibit β-Cat-induced transcription (Figures S1A and S1B). Moreover, overexpression of LATS2 did not affect NF-κB or AP-1 reporter activities (Figure S1C). To test whether endogenous LATS2 regulated β-catenin/Tcf-mediated transcription, we utilized siRNA to knock down LATS2. Western blot analysis confirmed that LATS2, but not LATS1, was efficiently depleted (Figure 1D). The knockdown of LATS2 significantly enhanced β-catenin/Tcf-mediated transcription induced by Wnt-3a (Figure 1E). To confirm the specificity of LATS2 siRNA, additional siRNA targeting different LATS2 sequence showed similar effects (Figures S1D and S1E). The restoration of LATS2 expression abolished enhanced β-catenin/Tcf-mediated transcription induced by LATS2 siRNA (Figure S1F). Moreover, we found that the knockdown of LATS2 also enhanced the expression of *AXIN2* and *DKK1*, two well-known Wnt target genes, induced by Wnt-3a (Figure 1F). To determine whether LATS2 kinase activity was required for the inhibition of Wnt/β-catenin signaling, we examined whether the kinase-dead LATS2 mutant (LATS2-KD) could inhibit β-catenin/Tcf-mediated transcription. Unexpectedly, overexpression of LATS2-KD also potently inhibited β-Cat-induced transcription. To further confirm our results, we deleted the entire kinase domain (amino acids 626–1,042) of LATS2 and found that the N-terminal fragment (amino acids 1–625) of LATS2 (LATS2N) also strongly inhibited β-Cat-induced transcription (Figure 1G). Because TAZ had been found to inhibit Wnt/β-catenin signaling (Varelas et al., 2010), we also examined whether the inhibition of β-catenin-mediated transcription by LATS2 was dependent on TAZ. However, the knockdown of TAZ did not affect LATS2-mediated inhibition of β-Cat-induced transcription (Figures 1H and 1I). Similarly, also the knockdown of YAP did not modulate LATS2 inhibition (Figures 1H and 1I). Moreover, we found that overexpression of YAP or TAZ did not affect inhibition of β-catenin-mediated transcription (Figures S1G and S1H). Taken together, our results suggest that LATS2 is capable of inhibiting Wnt/β-catenin-mediated transcription independent of canonical Hippo-YAP signaling.

LATS2 Directly Interacts with β-Catenin

Because LATS2 potently inhibited β-catenin-mediated transcription, we first performed coimmunoprecipitation (coIP) to examine whether LATS2 interacted with β-catenin. Flag-β-catenin and HA-LATS2 were expressed either individually or in combination in HK293T cells. The nuclear extracts were prepared and immunoprecipitated with HA antibodies. Flag-β-catenin could be detected in the immunoprecipitates with HA antibodies or vice versa (Figure 2A). However, the interaction between LATS2 and β-catenin was much weaker or could not be detected in the cytoplasm (Figure S2A). Because the kinase domain deletion mutant LATS2N retained inhibition of β-catenin-mediated transcription, we examined whether LATS2N could bind to β-catenin. CoIP revealed that HA-LATS2N, but not the C-terminal domain (amino acids 626–1,042) of LATS2 (HA-LATS2C), interacted with β-catenin (Figure 2B). Further mapping revealed that amino acids 250–575 of LATS2 were sufficient for interaction with β-catenin (Figures 2C and 2D). Interestingly, unlike their kinase domains, the sequence similarity between LATS1 and LATS2 in that region is extremely low (Yabuta et al., 2000). β-Catenin contains the C-terminal transactivation domain and multiple arm repeats that play critical roles in interacting with and recruiting other transcription components (MacDonald et al., 2009). We found that the LATS2 did not interact with the transactivation domain of β-catenin (data not shown). To further map the amino acid region of β-catenin that was required for interaction, we generated three fragments of β-catenin and found that β-catenin-133–694 containing arm repeats was able to pull down LATS2 (Figure 2E) or vice versa.

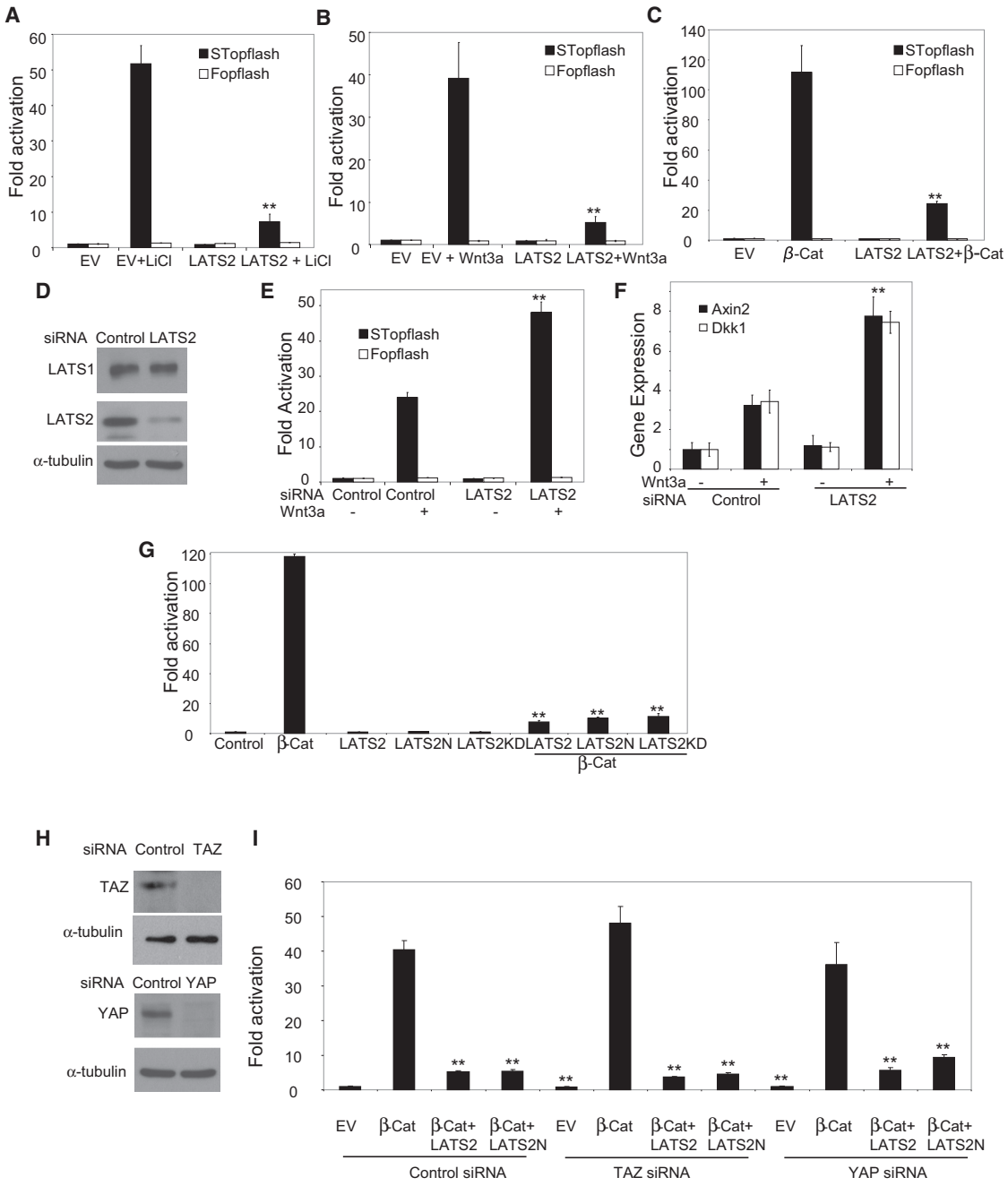

Figure 1. LATS2 Inhibits Wnt/β-Catenin-Mediated Transcription Independent of Hippo-YAP Signaling

(A) LATS2 inhibited STopflash reporter activities induced by LiCl in 293T cells. Experiments were performed at least three times. EV, empty vector.

(B) LATS2 inhibited STopflash reporter activities induced by Wnt-3a in 293T cells.

(C) LATS2 inhibited STopflash reporter activities induced by β-Cat in 293T cells.

(D) LATS2, but not LATS1, was knocked down by siRNA in 293T cells.

(E) LATS2 knockdown enhanced STopflash reporter activities induced by Wnt-3a.

(F) LATS2 knockdown enhanced the expression of *AXIN-2* and *DKK1* induced by Wnt-3a.

(G) LATS2 inhibited β-catenin-mediated transcription independent of its kinase activity.

(H) SiRNA knocked down TAZ and YAP.

(I) TAZ or YAP knockdown did not affect LATS2 inhibition of β-catenin-mediated transcription.

Values are mean ± SD for triplicate samples from a representative experiment. **$p < 0.01$, Student's t test.

See also Figure S1.

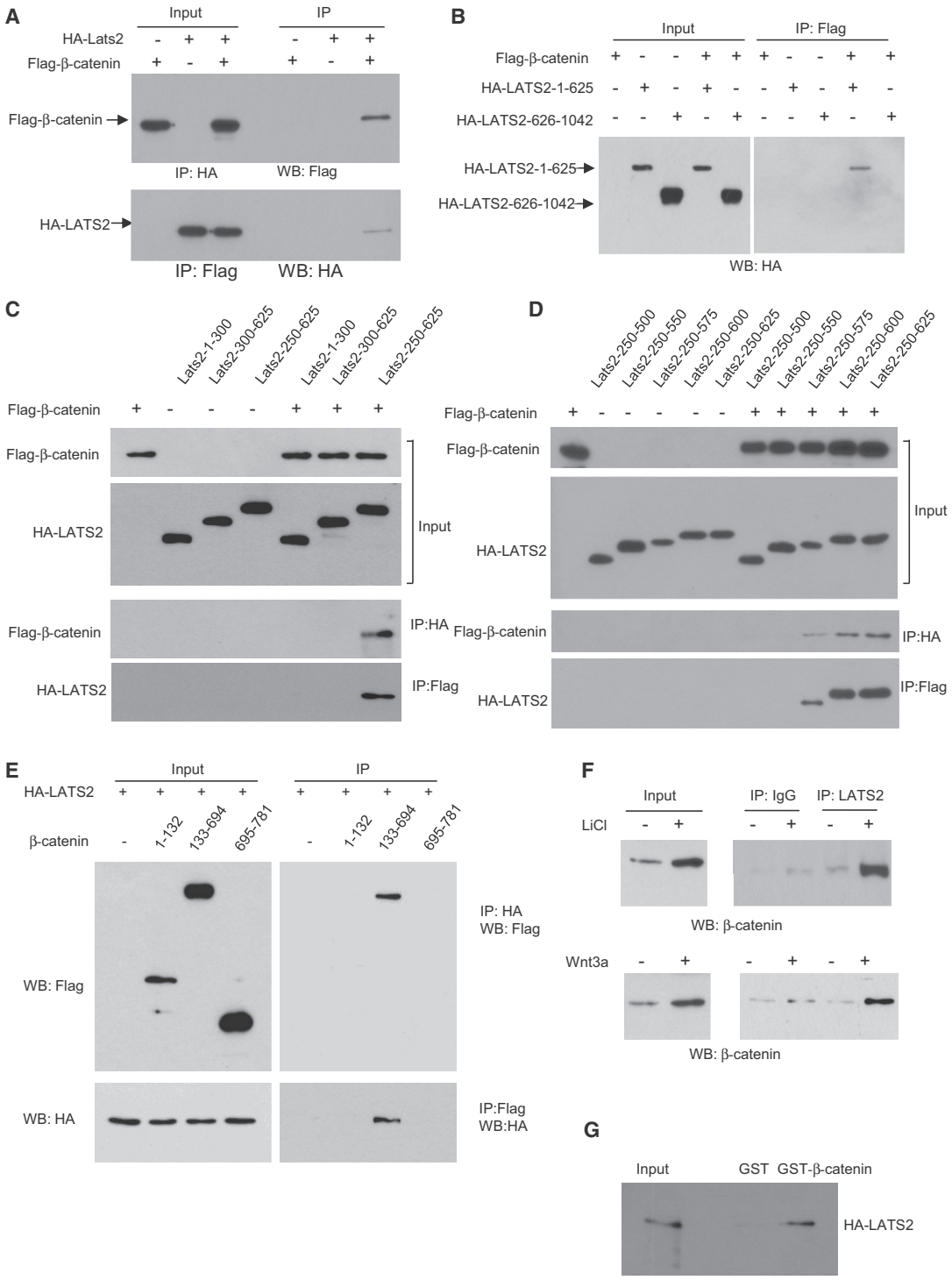

Figure 2. LATS2 Interacts with β-Catenin

(A) LATS2 and β-catenin interacted when overexpressed in 293T cells. IP, immunoprecipitation; WB, western blot.

(B) The kinase domain of LATS2 was not required for the interaction between β-catenin and LATS.

(C and D) The amino acids 250–575 of LATS2 were required for interaction with β-catenin.

(E) β-Catenin-133–694 containing arm repeats interacted with LATS2. 293T cells were cotransfected with A-LATS2 and a variety of β-catenin deletion mutants.

(F) Endogenous β-catenin interacted with LATS2 upon LiCl or Wnt-3a stimulation.

(G) GST-β-catenin fusion protein pulled down HA-LATS2 protein in vitro.

See also Figure S2.

The D162 and D164 residues of β-catenin are critical for the interaction between β-catenin and BCL9. However, the mutation of these two sites did not affect β-catenin interaction with LATS2 (Figure S2B). To further determine whether endogenous LATS2 and β-catenin interact, we treated 293T cells with LiCl and isolated the nuclear extracts from these cells. As shown in Figure 2F, whereas LATS2 antibodies negligibly pulled down β-catenin without stimulation, a significant amount of β-catenin was immunoprecipitated by LATS2 antibodies, as compared with IgG, upon LiCl stimulation. Similarly, we found that Wnt-3a also induced the complex formation between β-catenin and LATS2. To further confirm our results and determine whether β-catenin directly interacted with LATS2, we generated the recombinant glutathione S-transferase (GST)-β-catenin fusion protein and in-vitro-translated HA-LATS2 proteins. Our GST pull-down assay revealed that β-catenin could directly bind to LATS2 in vitro (Figure 2G). Taken together, our results suggest that LATS2 may inhibit β-catenin-mediated transcription by directly interacting with β-catenin.

LATS2 Is Downregulated and Inversely Correlated with β-Catenin-Mediated Transcription in Human Colorectal Cancers

To examine whether LATS2 played an inhibitory role in human colorectal cancer development, we strictly compared LATS2 expression in human colorectal cancer tissues with matched adjacent normal colorectal tissues using human Colon Cancer Screen-13 tissue microarray consisting of 50 samples. The intensity of immunostaining was qualitatively measured using Image-Pro Plus 6.0 image analysis software. Immunostaining revealed that LATS2 expression was significantly decreased in human colorectal cancer tissues compared to matched adjacent normal colorectal tissues (Figures 3A–3C). To examine whether LATS2 expression was inversely correlated with human colorectal progression, we stained LATS2 in human primary colorectal tumors and metastatic colorectal tumors grown in the liver or lymph node. LATS2 was nearly undetectable in human colorectal cancer metastasis from liver and lymph node compared with normal and primary colorectal cancer tissues (Figures 3D–3H and S3).

To further determine whether LATS2 is an important tumor suppressor gene in human colorectal cancer, we examined whether LATS2 downregulation was associated with patient prognosis using the colon cancer data set (GSE17537) in Gene Expression Omnibus (GEO). Patients were dichotomized into two groups as above or below median for LATS2 expression value. Kaplan-Meier survival plot revealed that *LATS2* expression was inversely associated with the survival times of patients with colorectal cancers using the log rank test ($p < 0.001$; Figure 3I). Interestingly, patients with high LATS2 expression also had longer recurrence-free survival times than those with low LATS2 expression ($p < 0.043$; Figure 3J).

To determine LATS2 as a negative regulator of Wnt/β-catenin-mediated transcription in human cancers, we immunostained the expression of LATS2, β-catenin, AXIN2, and MMP7 in human colorectal cancer tissue microarray consisting of 150 samples. Pearson correlation analysis showed that LATS2 was found to be inversely associated with tumor grade (Table S1). Using β-catenin as a control variable, LATS2 was also inversely associated with tumor grade (Table S2). Consistent with other studies, we found that over 85% of TMA samples were positively stained for cytoplasmic and nuclear β-catenin. Pearson correlation analysis showed that β-catenin expression was correlated with the levels of AXIN2 ($R^2 = 0.383$; $p < 0.001$) and MMP7 ($R^2 = 0.333$; $p < 0.001$) (Table S3). To determine whether LATS2 negatively regulated the expression of AXIN2 and MMP7 in vivo, partial correlation analysis was performed using β-catenin as a control variable. We found that LATS2 expression was inversely correlated with the levels of AXIN2 ($R^2 = -0.311$; $p < 0.01$) and MMP7 ($R^2 = -0.259$; $p < 0.01$) (Table S4). Taken together, our results suggest that LATS2 is a negative regulator of oncogenic β-catenin-mediated transcription in human colorectal cancers.

LATS2 Inhibits BCL9/BCL9L Recruitment by β-Catenin

To explore how LATS2 inhibited β-catenin-mediated transcription, we performed chromatin immunoprecipitation (ChIP) assays to determine whether LATS2 would affect the recruitment of β-catenin to the Wnt-regulated enhancer (WRE). As a strict negative control, a region located in the open reading frame (ORF) was also examined. We chose *AXIN2* and *DKK1* for our ChIP assays because their promoters are tightly regulated by Wnt/β-catenin. As shown in Figure 4A, overexpression of LAST2 did not inhibit the level of β-catenin on the *AXIN2* and *DKK1* promoters by overexpression of β-Cat. Conversely, the knockdown of LATS2 did not affect β-catenin binding to the *AXIN2* and *DKK1* promoters induced by LiCl (Figure 4B). Similarly, the overexpression or knockdown of LATS2 did not modulate β-catenin binding to Axin-2 and DKK1 promoters induced by Wnt-3a (Figures 4C and 4D). Interestingly, ChIP assays revealed that LATS2, but not LATS1, was present on the *AXIN2* and *DKK1* promoters. The knockdown of LATS2, but not LATS1, reduced its binding on the *AXIN2* and *DKK1* promoters (Figure 4E). To activate transcription, β-catenin recruits an array of transcription coactivators, including BCL9 and PYGO, p300/CBP, and the PAF1 complex, to the Wnt target gene promoter (Parker et al., 2002; Mosimann et al., 2006; Kramps et al., 2002; Townsley et al., 2004; Gu et al., 2009). Thus, we explored whether LATS2 might interfere with β-catenin-mediated recruitment of transcription coactivators to the Wnt target gene promoter. Overexpression or knockdown of LATS2 did not affect the recruitment of CBP/p300 to the *AXIN2* and *DKK1* promoters induced by Wnt-3a (data not shown). Importantly, overexpression of LATS2 inhibited recruitment of BCL9 to the *AXIN2* and *DKK1* promoters induced by Wnt-3a (Figure 4F); the knockdown of LATS2 enhanced BCL9 binding to the *AXIN2* and *DKK1* promoters upon Wnt-3a or LiCl stimulation (Figures 4G and 4H).

To further confirm that LATS2 inhibited BCL9 recruitment by β-catenin, we also performed coIP experiments to determine whether LATS2 interfered with the interaction between β-catenin and BCL9. As shown in Figure 5A, upon LiCl stimulation, a significant amount of β-catenin was pulled down by BCL9 or PYGO2 antibodies. BCL9L, a homolog of BCL9, was also found to interact with β-catenin to promote Wnt target gene transcription. We found that LiCl could also induce the interaction between β-catenin and BCL9L. However, the β-catenin levels pulled

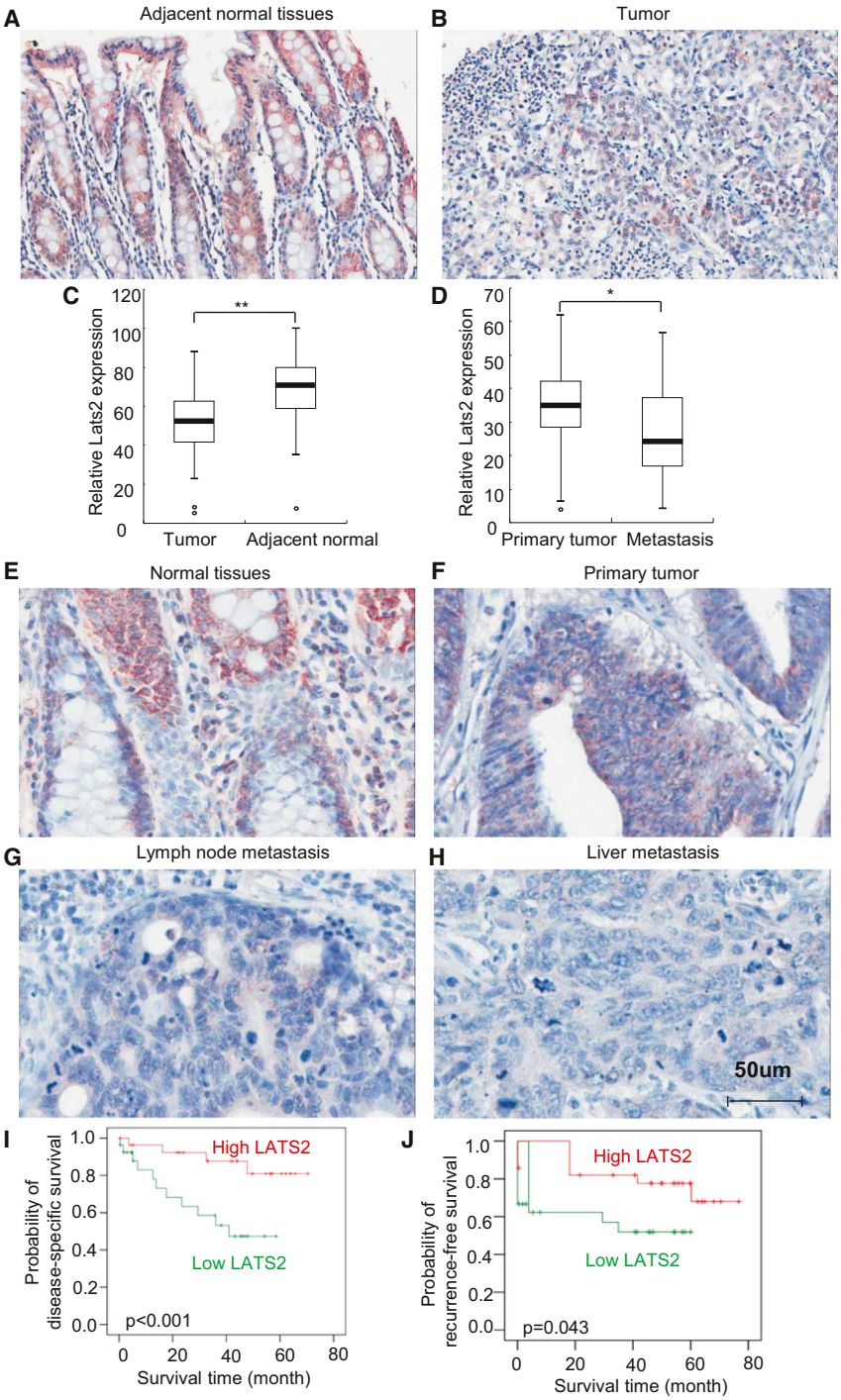

Figure 3. LATS2 Expression Is Inversely Associated with Human Colorectal Cancer Development and Prognosis

(A and B) LATS2 expression was decreased in human colorectal tumor tissues (B) compared with adjacent normal tissues (A). TMA was stained with LATS2 polyclonal antibodies.

(C) Quantitative measurement of LATS2 in human colorectal tumor tissues and adjacent normal tissues. The intensities of immunostaining were quantitatively measured using Image-Pro Plus 6.0 image analysis software. $^{**}p < 0.01$ (n = 50).

(D) Quantitative measurement of LATS2 in human primary colorectal tumors and metastatic tumors from the lymph node and liver. $^{*}p < 0.05$ (n = 30).

(E–H) LATS expression was lost in human metastatic colorectal tumors from the lymph node or liver. TMA consisting of adjacent normal tissues (E), primary tumors (F) and metastatic tumors from lymph node (G) and liver (H) was stained with LATS2 polyclonal antibodies.

(I) LATS2 expression was inversely associated with the survival times of patients with colorectal cancer. The published colon cancer data set (GSE17537) containing patient follow-up information was used. Kaplan-Meier survival plot was performed for disease-specific survival, and the log rank test was applied to test the survival differences using SPSS 17.0 software.

(J) LATS2 expression was inversely associated with the recurrence-free survival times of patients with colorectal cancer.

See also Tables S1–S4 and Figure S3.

down by BCL9, BLC9L, or PYGO2 antibodies were reduced when LATS2 was overexpressed. Similarly, LAT2 also inhibited the interaction between the active form of β-catenin, BCL9, BCL9L, and PYGO2 as determined using antiactive form of β-catenin. Overexpression of LATS2 inhibited the interaction between β-catenin, BCL9, BCL9L, and PYGO2 induced by Wnt-3a (Figure 5B). In contrast, the knockdown of LATS2 enhanced β-catenin-BCL9/BCL9L complex formation upon LiCl or Wnt-3a stimulation (Figures 5C and 5D). As a control, overexpression or knockdown of LATS2 did not affect the interaction between β-catenin and parafibromin 1 (Figures 5A–5D). Finally, we performed in vitro binding assays by using human GST-β-catenin fusion proteins and in-vitro-translated full-length LATS2, BCL9, and BCL9L. Although GST-β-catenin, but not GST, formed a complex with BCL9, the addition of LATS2 disrupted the BCL9 and β-catenin complex in a dose-dependent fashion (Figure 5E). Similarly, LATS2 also interfered with the BCL9L and β-catenin complex in a dose-dependent fashion (Figure 5F). We found that BCL9 and BCL9L were weakly expressed in HT29 cells compared with other colorectal cell lines, including SW620, HCT116, and SW480 (Figure S4A). Consistent with our findings, overexpression of LATS2N could not inhibit STopflash reporter activities in HT29 cells (Figure S4B). Of note, it was probably due to the induction of apoptosis that we were able to overexpress the full-length LATS2 in HT29 cells. In contrast, overexpression of LATS2N or LATS2 significantly inhibited STopflash reporter activities in SW620 and SW480

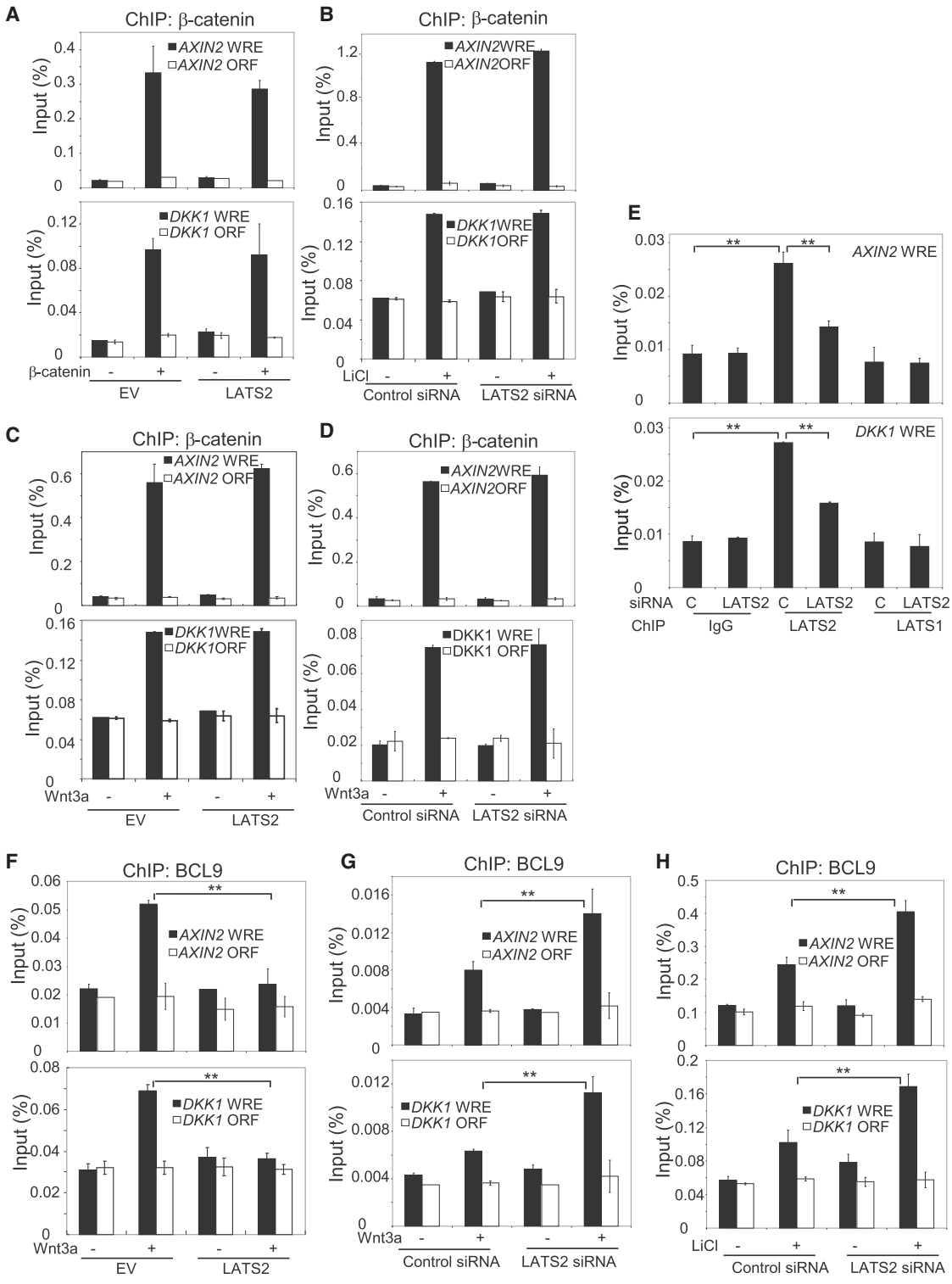

Figure 4. LATS2 Inhibits β-Catenin-Mediated Recruitment of BCL9 to the Wnt Target Gene Promoter

(A) 293T cells were cotransfected with β-catenin and LATS2 as indicated. The ChIP-enriched DNAs were quantitatively measured using real-time PCR with *AXIN-2*- and *DKK1*-specific primers. A region located in the ORF was utilized as a negative control.

(B) Cells were transfected with LATS2 or control siRNA for 48 hr and then treated with LiCl for 2 hr. ChIP assays were performed as described in (A).

(C) Cells were transfected with LATS2 for 24 hr and then treated with Wnt-3a. ChIP assays were performed as described in (A).

(legend continued on next page)

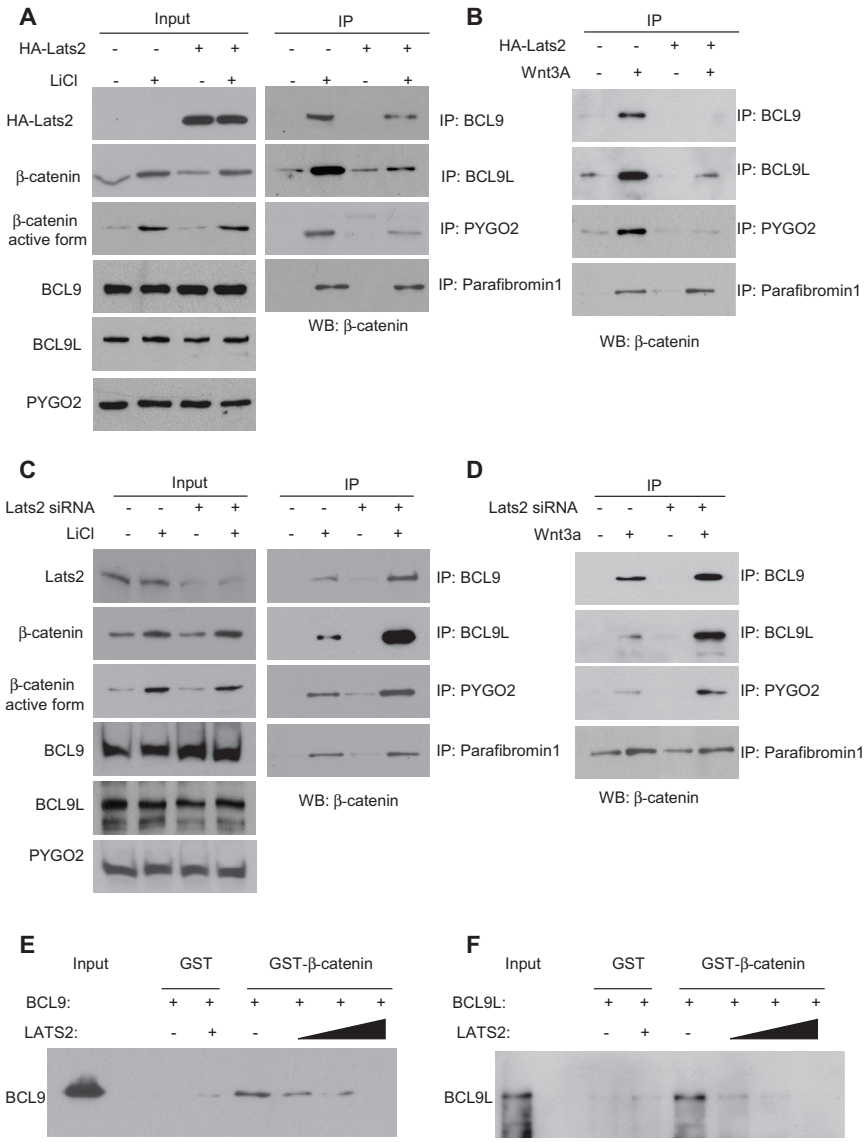

Figure 5. LATS2 Inhibits the Interaction between β-Catenin and BCL9

(A and B) LATS2 inhibited the interaction between β-catenin and BCL9, BCL9L or PYGO2 induced by LiCl (A) and Wnt-3a (B).

(C and D) LATS2 knockdown enhanced the interaction between β-catenin and BCL9, BCL9L or PYGO2 induced by LiCl (C) and Wnt-3a (D).

(E) LATS2 inhibited the direct interaction between β-catenin and BCL9 in vitro.

(F) LATS2 inhibited the direct interaction between β-catenin and BCL9L in vitro.

See also Figure S4.

and HCT116 cells (Figures S4C and S4D). LATS2 and LATS2N were unable to inhibit β-catenin-mediated transcription in HCT116 cells and 293T cells in which BCL9 and BCL9L were depleted (Figures S4E and S4F). Moreover, overexpression of LATS2 and LATS2N inhibited STopflash reporter activities in both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells, indicating that their inhibition is independent of p53 (Figure S4G). LATS2N also significantly inhibited the expression of Wnt target genes in SW480 cells, but not in HT29 cells (Figures S4H–S4J).

The knockout of *Lats2* in mice results in embryonic lethality (McPherson et al., 2004). To further confirm the essential role of LATS2 in the inhibition of β-catenin-mediated transcription, we utilized $Lats2^{-/-}$MEFs (Figure 6A). We found that the basal levels of DKK2 were increased in $Lats2^{-/-}$MEFs compared with $Lats2^{+/+}$MEFs (data not shown). To rule out the nonspecific effects of cell immortalization, we attempted to reintroduce *Lats2* back in $Lats2^{-/-}$MEFs. Unfortunately, we were unable to obtain stable clones, probably due to growth suppression mediated by overexpression of Lats2 through Hippo signaling. However, we were able to stably express LATS2N in $Lats2^{-/-}$MEFs. Interestingly, western blot analysis revealed that most LATS2N protein was detected in nuclear fractions in $Lats2^{-/-}$MEFs expressing LATS2N ($Lats2^{-/-}$MEF/LATS2N) (Figure 6B). STopflash report assays showed that LATS2N significantly inhibited β-catenin/Tcf-mediated transcription induced by Wnt-3a (Figure 6C). Importantly, the expressions of *Axin2* and *Dkk1* induced by Wnt-3a were significantly inhibited in $Lats2^{-/-}$MEF/LATS2N cells as compared to control $Lats2^{-/-}$MEF cells transduced with empty vector ($Lats2^{-/-}$MEF/V) (Figure 6D). ChIP assays using LATS2 antibodies revealed that LATS2N was detected in the *Axin2* promoter (Figure 6E). Although LATS2N did not impair β-catenin binding to the *Axin2* promoter induced by Wnt-3a, the recruitment of BCL9 and PYGO2 to the *Axin2* promoters induced by Wnt-3a was significantly reduced in $Lats2^{-/-}$MEF/LATS2N as compared with $Lats2^{-/-}$MEF/V (Figure 6G). Moreover, we found that ectopic expression of LATS2N also inhibited human colorectal cell tumor growth in vivo by inhibiting the β-catenin/BCL9 interaction (data not shown).

(D) Cells were transfected with LATS2 or control siRNA and then treated with Wnt-3a. ChIP assays were performed as described in (A).

(E) Cells were transfected with LATS2 or control siRNA and chromatin immunoprecipitated with anti-LATS2, anti-LATS1, or IgG.

(F) Cells were transfected with LATS2 or vector control and then treated with Wnt-3a. ChIP assays were performed using BCL9 antibodies.

(G and H) Cells were transfected with LATS2 or control siRNA for 48 hr and then treated with Wnt-3a or LiCl. ChIP assays were performed using BCL9 antibodies.

Values are mean ± SD for triplicate samples from a representative experiment. $^{**}p < 0.01$, Student's t test.

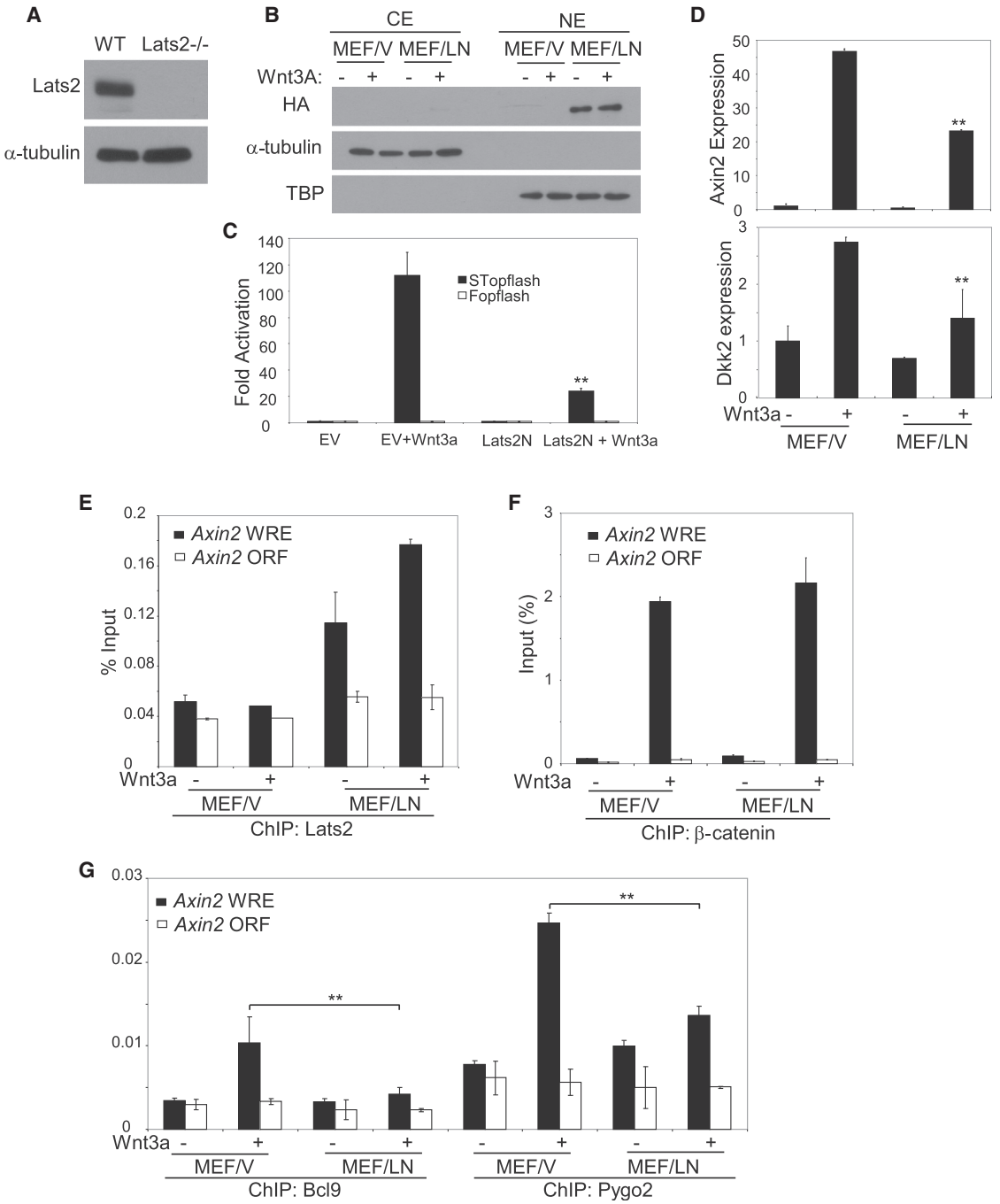

Figure 6. LATS2 Kinase Domain Deletion Mutant Inhibits Wnt/β-Catenin-Mediated Transcription *Lats2*$^{-/-}$MEFs

(A) Confirmation of LATS2 deletion in LATS2$^{-/-}$MEFs by western blot.

(B) *Lats2*$^{-/-}$MEFs were stably expressed with HA-LATS2N. Nuclear proteins were isolated from *Lats2*$^{-/-}$MEFs expressing HA-LATS2N (MEF/LN) or control vector (MEF/V) and probed with HA antibodies.

(C) LATS2N inhibited STopflash reporter activities induced by Wnt-3a.

(D) LATS2N inhibited the expression of *Axin2* and *Dkk1* induced by Wnt-3a.

(E and F) Both *Lats2*$^{-/-}$MEF/V and *Lats2*$^{-/-}$MEF/LATS2N cells were treated with Wnt-3a and chromatin immunoprecipitated with anti-LATS2 (E) or anti-β-catenin (F). The ChIP-enriched DNA was measured using real-time PCR with the *Axin-2*-specific primers.

(G) LATS2N inhibited the recruitment of BCL9 and PYGO2 to the *Axin* promoter induced by Wnt-3a.

Values are mean ± SD for triplicate samples from a representative experiment. **p < 0.01, Student's t test.

LATS2 Is Required for Nocodazole-Mediated Inhibition of Tumor Growth by Targeting β-Catenin/BCL9

Nocodazole is a chemotherapeutic drug that exerts its effect in tumor cells by inhibiting the polymerization of microtubules. Elegant studies by Aylon et al. (2006) showed that nocodazole potently induced LATS2 expression and inhibited cell growth. Based on all the above-mentioned findings, we hypothesized that nocodazole could exert its antitumor effect by inhibiting Wnt/β-catenin signaling via the induction of LATS2 in addition to targeting microtubules. As shown in Figure 7A, nocodazole potently induced LATS2 expression in HEK293T cells. Consistently, nocodazole significantly inhibited β-catenin-mediated transcription induced by LiCl or β-Cat overexpression (Figure 7B). We also found that nocodazole induced LATS2, but not LATS1, in a time-dependent manner in HCT116 cells (Figure 7C) and SW480 cells (Figure S5A). To test whether LATS2 was required for nocodazole-mediated inhibition of β-catenin-mediated transcription, we utilized shRNA to knock down LATS2. Western blot confirmed that shRNA efficiently knocked down nuclear LATS2 proteins in HCT116 cells, whereas there were no effects on nuclear β-catenin (Figure 7D). Although nocodazole significantly suppressed the expression of *AXIN2* and *MMP7* in HCT116 cells expressing scramble shRNA (HCT116/Scrsh), it could not affect the expression of *AXIN2* and *MMP7* in HCT116 cells expressing LATS2 shRNA (HCT116/LATS2sh) (Figure 7E). The restoration of LATS2 or LATS2N in HCT116/LATS2sh cells suppressed the expression of AXIN2 and MMP7, confirming the specificity of LATS2 shRNA (Figure 7F). Moreover, ChIP assays found that nocodazole induced LATS2 binding to the promoter of *AXIN2* or *MMP7* in HCT116/Scrsh cells, but not in HCT116/LATS2sh cells (Figure 7G). Consistently, we found that the occupancy of BCL9 on the promoter of *AXIN2* or *MMP7* was reduced in HCT116/Scrch cells, but not in HCT116/LATS2sh cells, upon nocodazole treatment (Figure 7H). In contrast, nocodazole did not interfere with β-catenin presence on the promoter of *AXIN2* or *MMP7* (Figure 7I). The constitutive activation of Wnt/β-catenin plays a critical role in human colorectal tumor growth. Because nocodazole inhibited Wnt/β-catenin by inducing LATS2, we further examined whether LATS2 was required for nocodazole-mediated antitumor growth in vivo. Both HCT116/Scrsh and HCT116/LATS2sh cells were inoculated into nude mice that were subsequently treated with nocodazole. Without nocodazole treatment, we found that the tumors generated from HCT116/LATS2sh cells in nude mice grew faster than those from HCT116/Scrsh cells. Although nocodazole significantly suppressed tumor growth of HCT116/Scrsh cells implanted in nude mice, it was unable to inhibit tumor growth of HCT116/LATS2sh cells (Figures 7J and 7K), implicating that nocodazole could target oncogenic β-catenin/BCL9 transcription in addition to other antitumor effects.

DISCUSSION

Given the critical roles of Wnt/β-catenin in development, stem cell function, and oncogenesis, Wnt/β-catenin signaling is regulated by a variety of signaling pathways and molecules (MacDonald et al., 2009). LATS2 as tumor suppressor has been found to be mutated or downregulated in several human cancers (Takahashi et al., 2005; Lee et al., 2009; Li et al., 2011). However, the molecular mechanism by which LATS2 suppresses oncogenesis is not fully understood. Recent studies suggest that LATS2 can inhibit oncogenesis by phosphorylating the transcription factors YAP and TAZ in the canonical Hippo signaling pathway. Our results reveal that LATS2 also is a nuclear repressor of β-catenin-mediated transcription by disrupting interaction between β-catenin and BCL9/PYGO2. Intriguingly, the antimicrotubule compound nocodazole can inhibit tumor growth by targeting β-catenin/BCL9 via the induction of LATS2, providing insights into nocodazole-mediated antitumor effects. Our results suggest that LATS2 may be an important target for anticancer therapies.

Wnt/β-catenin signaling is abnormally activated in a variety of human cancers. β-Catenin/TCF-mediated transcription plays important roles in tumor development and progression. There are several negative regulators that have been found to inhibit β-catenin/TCF-mediated transcription. For example, the nemo-like kinase (NLK) has been found to inhibit β-catenin/TCF binding to DNA by phosphorylating LEF1 and TCF4 (Ishitani et al., 1999). Here, we identified LATS2 as a nuclear repressor that targets the interaction between β-catenin and the coactivator BCL9. Intriguingly, unlike NLK, LATS2 kinase activity was not required for the inhibition of β-catenin/TCF-mediated transcription. Biochemical and ChIP assays revealed that LATS2 inhibited the recruitment of BCL9/PYGO2, but not β-catenin, to the Wnt target gene promoters. BCL9 is the coactivator of β-catenin that has been found to bind to the N-terminal arm repeats, whereas most of the transcription coactivators interact with the C-terminal domain of β-catenin (Kramps et al., 2002; Fiedler et al., 2008). It is possible that LATS2 might interfere with BCL9 recruitment by interacting with β-catenin. Supporting this notion, our in vitro binding assay demonstrated that LATS2 could disrupt the BCL9 and β-catenin complex.

Canonical Hippo signaling has been found to inhibit Wnt/β-catenin signaling through TAZ, which interacts with DVL2 (Varelas et al., 2010). Both TAZ and YAP are phosphorylated by LATS1 and LATS2. However, we found that knockdown of TAZ did not affect LATS2 inhibition of β-catenin-mediated transcription. Importantly, we found that LATS2 directly interacted with β-catenin and occupied on the Wnt target gene promoters. Our results suggest that nuclear LATS2 may play a direct role in the suppression of oncogenesis. Although recent studies focus on the phosphorylation of TAZ and YAP by LATS1 and LATS2 in the cytoplasm, earlier studies have shown that LATS2 was strongly detected in the nucleus (Yabuta et al., 2000). LATS2 has been found to inhibit androgen-regulated gene expression by interacting with the androgen receptor in the nucleus (Powzaniuk et al., 2004). Moreover, oncogenic H-Ras activation has been found to induce LATS2 accumulation in the nucleus. The inhibition of LATS2 expression overcomes a p53-dependent stress checkpoint and apoptosis and promotes H-Ras-mediated transformation (Aylon et al., 2009, 2010). Although the underlying mechanisms that control nuclear accumulation of LATS2 are unknown, it is possible that a variety of oncogenic signaling pathways may affect the subcellular localization of LATS2. In fact, we found that LATS2 can be detected in both the cytoplasm and nucleus of various tumor cell lines (data not shown). Although

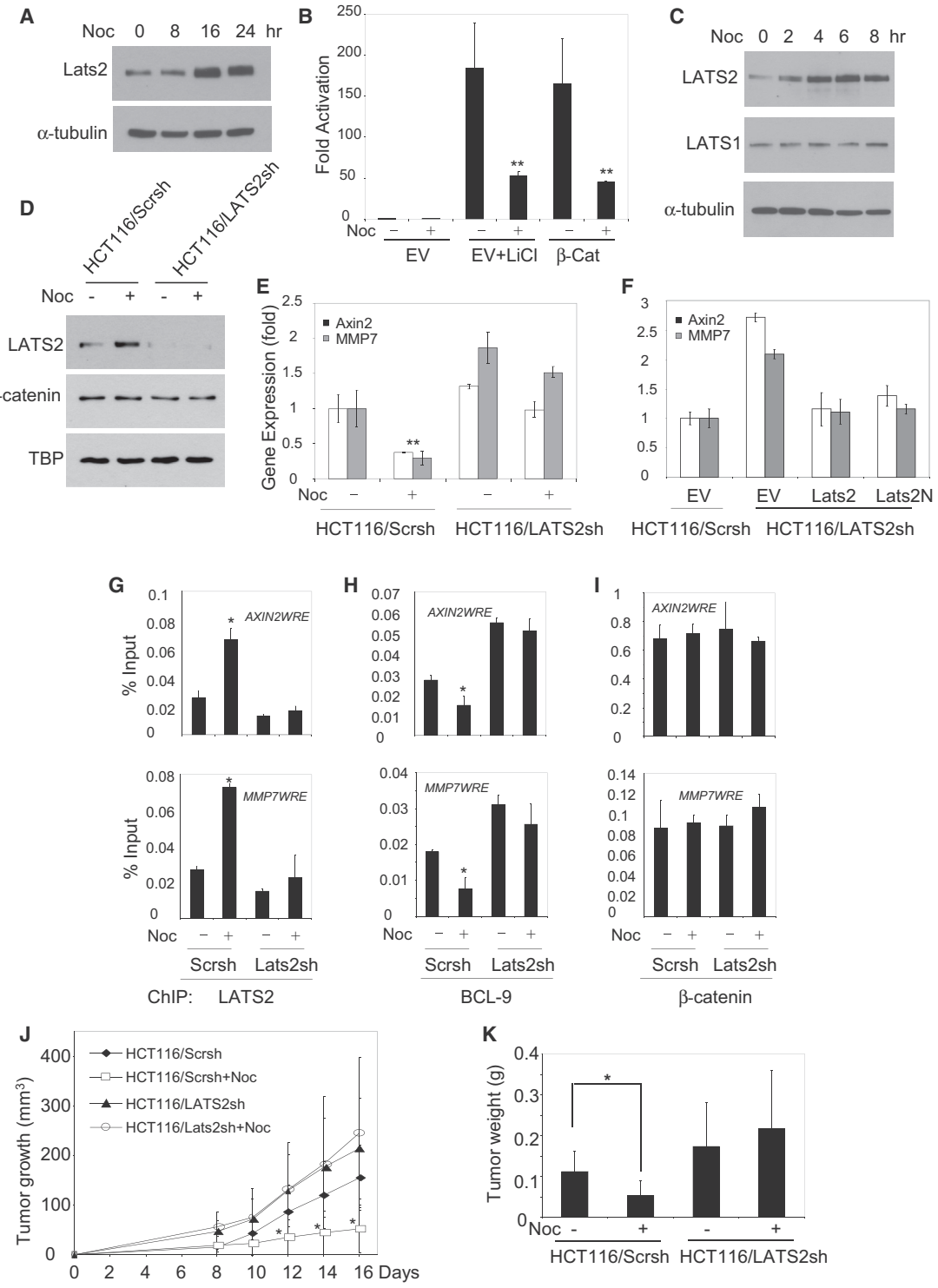

Figure 7. Nocodazole Induces LATS2 to Block β-Catenin/BCL9-Mediated Transcription and Inhibits Tumor Growth In Vivo

(A) Nocodazole (Noc) induced LATS2 in HEK293 T cells by western blot.

(B) Nocodazole significantly suppressed β-catenin/Tcf-mediated transcription induced by LiCl or β-Cat overexpression. **$p < 0.01$.

(C) Nocodazole induced LATS2, but not LATS1, in a time-dependent manner in HCT116 cells.

(D) The knockdown of nocodazole-induced LATS2 by shRNA. Nuclear proteins from HCT116 cells were isolated and probed with LATS2 antibodies.

(E) Nocodazole suppressed the expression of *AXIN2* and *MMP7* dependent of LATS2. Both HCT116/Scrsh and HCT116/LATS2sh cells were treated with nocodazole, and the expression of *AXIN2* and *MMP7* was determined by real-time RT-PCR. **$p < 0.01$.

(legend continued on next page)

the nuclear localization signal (NLS) is not identified in LATS2, interestingly, the kinase domain deletion mutant LATS2N was predominantly detected in the nucleus, suggesting that NLS may be present at the N terminal of LATS2. Alternatively, it is possible that the kinase domain of LATS2 may promote the cytoplasmic localization of LATS2. Consistent with the nuclear function of LATS2, our in vivo tumor growth model demonstrated that the LATS2N could potently suppress tumor growth by inhibiting β-catenin-dependent tumor growth.

Nocodazole is an antitumor agent that inhibits the polymerization of microtubule and disrupts microtubule cytoskeleton. Prolonged treatment with nocodazole induces cell-cycle arrest and/or apoptosis. Nocodazole has been found to induce LATS2 to translocate to the nucleus where LATS2 binds to Mdm2 and stabilizes p53, resulting in the activation of p53-mediated transcription (Aylon et al., 2006). By identifying the crosstalk between Wnt/β-catenin and LATS2, we found that nocodazole was a potent inhibitor of Wnt/β-catenin signaling in addition to targeting microtubule cytoskeleton, providing insights into the molecular mechanisms of nocodazole-mediated antitumor effect. In human colorectal cancer, the tumor suppressor gene adenomatous polyposis coli (APC) that plays an essential role in β-catenin degradation is most commonly mutated. If APC is normally expressed, β-catenin is often found to be mutated. The outcome of APC or β-catenin mutations results in the constitutive activation of β-catenin/Tcf-mediated transcription. As a therapeutic strategy for treating human colorectal cancer, it is important to target β-catenin-mediated transcription instead of the upstream sites of the Wnt/β-catenin signaling pathway. Intriguingly, we found that nocodazole inhibited oncogenic Wnt/β-catenin signaling in a LATS2-dependent manner by disrupting the β-catenin/BCL9 complex. Supporting this notion, the depletion of LATS2 significantly compromised the inhibition of tumor growth by nocodazole in vivo. Taken together, our results suggest that LATS2 is not only an important tumor suppressor in the development and metastasis of human colorectal cancer, but may also be an important target for anticancer therapy.

EXPERIMENTAL PROCEDURES

Plasmids, Oligonucleotides, and Antibodies

HA-LATS2, HA-LATS2KD, and YAP expression vectors were described previously (Zhao et al., 2008). LATS2 deletion mutant constructs were prepared using the pcDNA3.1 (Invitrogen) or retroviral expression vector pQCXIP (Clontech) by standard PCR subcloning. LATS1 expression vector was purchased from Addgene. The plasmid STopflash reporters, pActin-β-galactosidase, and CMV-β-galactosidase were used as previously described (Li and Wang, 2008). Primary antibodies were obtained from the following sources: β-catenin (BD); anti-Flag and α-tubulin (Sigma-Aldrich); anti-HA (Covance); anti-TBP, anti-BCL9 and anti-BCL9L (Abcam); anti-PYGO2 and anti-YAP (Santa Cruz Biotechnology); anti-LATS1 and anti-LATS2 (Bethyl Laboratories); and anti-LATS2 for immunostaining (McPherson et al., 2004). RT-PCR primers were purchased from Sigma-Aldrich: human *Dkk1* (5′-agcctcttaactccttggca-3′ and 5′-tccatgtcactgggttccta-3′); mouse *Axin2* (5′-CTGGCTCCAGAAGATCAC AA-3′ and 5′-AGGTGACAACCAGCTCACTG-3′); and mouse *Dkk1* (5′-GGT GATGCAGGCACTAGAGA-3′ and 5′-AAGCCTGCACAAATGACAAG-3′). The sequences of ChIP primers were 5′-GGCTGCGCTTTGATAAGGTCCT-3′ and 5′-ATCGCGAACGGCTGCTTATTTT-3′ (mouse *Axin2* WRE) and 5′-TTAC TGGCCAACGTGATGAT-3′ and 5′-GCTGTCAGGCACAGAGAGAG-3′ (mouse *Axin2* negative control). The siRNA oligonucleotides of *LATS2* (On-TargetPlus siRNA) and *YAP* (On-TargetPlus SMARTpool siRNA) were purchased from Thermo Fisher Scientific. The shRNA sequence for *LATS2* knockdown is 5′-GC ACGCATTTTACGAATTC-3′. All other sequences of RT-PCR and ChIP primers were as previously described (Li and Wang, 2008).

Cell Culture, Transfection, and Luciferase Reporter Assay

HEK293T, HCT116, SW480, and SCC-1 cells were cultured in DMEM containing 10% FBS and antibiotics (streptomycin and penicillin) at 37°C in a 5% CO_2/95% air atmosphere. MEF cells were maintained in DMEM supplemented with 15% FBS and NE-AAs (nonessential amino acids) at 37°C, 5% CO_2/95% air atmosphere in a humidified incubator. For transient transfections, 5×10^5 cells were seeded into 12-well plates for 12 hr and then transfected using Lipofectamine 2000 reagents according to the manufacturer's protocol (Invitrogen). The total amount of DNA in each individual well was kept constant by adding pcDNA3.1 empty vectors. Luciferase and β-galactosidase activity of total cell lysates were determined using Luc-Screen and Galacto-Star kits (Tropix), respectively.

For transfection of siRNA, 293T cells were plated at 40%–50% confluence and transfected with various amounts of siRNA using Oligofectamine reagents (Invitrogen). For siRNA combined with luciferase reporter experiments, 100 ng of STopflash and 50 ng of CMV-β-galactosidase constructs were transfected using Lipofectamine 2000 24 hr after the transfection of siRNA. To knock down *LATS2* in HCT116 and SW480 cells, *LATS2* shRNA or control shRNA was constructed in pLKO.1 vector (Addgene). To overexpress LATS2 or LATS2 deletion mutants in SW480 and MEF cells, LATS2 was subcloned into retroviral expression vector pQCXIP (Clontech) by standard PCR. To stably knock down or overexpress LATS2, the cells were seeded in 6-well plates and incubated overnight. The next day, respective viral particles were added evenly to the plates. After 24 hr, the medium was replaced with fresh medium and incubated overnight. From the next day onward, the cells were selected using puromycin (2 μg/ml) for at least 3–4 days. The knockdown efficiency was determined using immunoblot analysis.

Western Blot Analysis, Real-Time RT-PCR, CoIP, and GST Pull-Down

For each sample, 5×10^6 293T cells were lysed in lysis buffer for 10 min on ice. After centrifugation at 10,000 × *g* at 4°C, the supernatants were incubated with antibodies at 4°C for 2 hr, followed by incubation with protein A or protein G-Sepharose (GE Healthcare) for 1 hr. For nuclear coIP, the nuclear extracts were prepared from 1.5×10^6 293T cells, as described previously. Immunoprecipitates were washed three times with PBS and 0.1% NP40 buffer at 4°C. For each GST pull-down assay, 2 μg of GST or GST fusion β-catenin protein was incubated with glutathione beads (GE Healthcare) at 4°C for 1 hr, followed by PBS wash. The precipitate beads were then incubated in 1 ml PBS with various amounts of in-vitro-translated BCL9 or LATS2 at 4°C

(F) The restoration of LATS2 or LATS2N suppressed the expression of *AXIN2* and *MMP7* in HCT116/LATS2sh cells.
(G) Nocodazole induced LATS2 binding to the promoter of *AXIN2* or *MMP7* by ChIP assays. Both HCT116/Scrsh and HCT116/LATS2sh cells were treated with nocodazole, and the promoter of *AXIN2* or *MMP7* was chromatin immunoprecipitated with LATS2 antibodies. *$p < 0.05$.
(H) Nocodazole inhibited the occupancy of BCL9 on the promoter of *AXIN2* or *MMP7* via the induction of LATS2. Both HCT116/Scrch and HCT116/LATS2sh cells were treated with nocodazole, and the promoter of *AXIN2* or *BCL9* was chromatin immunoprecipitated with BCL9 antibodies. *$p < 0.05$.
(I) Nocodazole did not affect β-catenin binding to the promoter of *AXIN2* or *MMP7*.
(J) LATS2 was required for nocodazole-mediated inhibition of tumor growth in nude mice ($n = 7–8$). *$p < 0.05$.
(K) Comparisons of actual tumor weights at the end of nocodazole treatment. At the end of experiments, tumors from nude mice were dissected and weighted. *$p < 0.05$.
See also Figure S5.

overnight, followed by washing buffer for five times. Proteins bound to the beads were eluted with SDS-loading buffer at 98°C for 2 min and then subjected to SDS-PAGE. Western blot analysis was performed as described previously (Li and Wang, 2008).

Total RNA from each sample cell was purified using TRIzol reagents, and cDNA was synthesized with random primers using SuperScript III (Invitrogen). Quantitative RT-PCR analysis was carried out with iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ real-time PCR detection system (Bio-Rad). GAPDH levels were used as a loading control for real-time PCR.

ChIP Assays

ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's protocol. Cells were incubated with a dimethyl 3,3′-dithiobispropionimidate-HCl (DTBP; Pierce) solution (5 mmol) for 30 min on ice before formaldehyde treatment. For each ChIP reaction, 2×10^6 cells were used. All resulting precipitated DNA samples were quantified by real-time PCR. Data are expressed as the percentage of input DNA. The primer sequences used for real-time PCR were as previously described (Li and Wang, 2008).

In Vivo Tumor Growth and Immunostaining

In vivo tumor growth in nude mice was performed as described previously (Li and Wang, 2008). For nocodazole treatment, nude mice (seven to eight mice per group) were inoculated with HCT116/Scrsh or HCT116/LATS2sh cells for 6 days and then treated with nocodazole (10 mg/kg) every 2 days for 10 days. The animal protocol was approved by the Animal Research Committee at the University of California, Los Angeles.

TMAs were purchased from LifeSpan Biosciences. Colon Cancer Screen-12 TMA contains 140 colon adenocarcinoma cores with stage/grade and 10 normal colon cores. Colon Cancer Screen-13 TMA contains 50 colon adenocarcinoma specimens with matched adjacent normal tissues. Colon Cancer Screen-88 TMA contains 30 cases of adenocarcinoma, 30 metastasis, and 9 adjacent normal tissues. For immunostaining, antigen retrieval was performed by pressure cooking in a Decloaking Chamber (Biocare Medical) in citrate buffer (2.1 g/l citric acid [pH 6.0]) at 120°C for 20 min). TMA was stained with monoclonal antibodies against β-catenin (BD Biosciences), polyclonal antibodies against LATS2, polyclonal antibodies against AXIN2, and polyclonal antibodies against MMP7 at 4°C overnight. We then incubated sections with horseradish peroxidase-labeled polymer for 60 min, detected the immunocomplexes with AEC^+ chromogen (Dako EnVision System), and counterstained with hematoxylin. The intensity of immunostaining was measured by Image-Pro Plus 6.0 image analysis software (Media Cybernetics). The intensity of each image was calculated by normalizing the average integrated optical density (IOD) with the total selected area of interest (AOI). Correlation between the expressions of different genes was calculated using SPSS 17.0 software. For patient survival analysis, a colon cancer data set (GSE17537) containing patient follow-up information was used (Smith et al., 2010). Patients were dichotomized into two groups as above or below median for LATS2 expression value. Kaplan-Meier survival plot was performed for both disease-specific and recurrence-free survival, and the log rank test was applied to test the survival differences and calculate the p value using SPSS 17.0 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.037.

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