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β-Catenin-Driven Cancers Require a YAP1 Transcriptional Complex for Survival and Tumorigenesis

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SUMMARY

Wnt/β-catenin signaling plays a key role in the pathogenesis of colon and other cancers; emerging evidence indicates that oncogenic β-catenin regulates several biological processes essential for cancer initiation and progression. To decipher the role of β-catenin in transformation, we classified β-catenin activity in 85 cancer cell lines in which we performed genome-scale loss-of-function screens and found that β-catenin active cancers are dependent on a signaling pathway involving the transcriptional regulator YAP1. Specifically, we found that YAP1 and the transcription factor TBX5 form a complex with β-catenin. Phosphorylation of YAP1 by the tyrosine kinase YES1 leads to localization of this complex to the promoters of antiapoptotic genes, including *BCL2L1* and *BIRC5*. A small-molecule inhibitor of YES1 impeded the proliferation of β-catenin-dependent cancers in both cell lines and animal models. These observations define a β-catenin-YAP1-TBX5 complex essential to the transformation and survival of β-catenin-driven cancers.

INTRODUCTION

β-catenin signaling plays a key role in colon development and cancer (Clevers, 2006). The destruction complex composed of AXIN1, GSK3β, and adenomatous polyposis coli (APC) phosphorylates serine residues in β-catenin, which leads to its proteasomal degradation (Clevers, 2006). Binding of Wnts to the LPR6-Frizzled receptor inactivates this complex, leading to accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin forms a complex with TCF4 that drives the transcription of genes that contribute to cell proliferation (Klaus and Birchmeier, 2008). Individuals carrying *APC* germline mutations (familial adenomatous polyposis) develop colonic polyps that progress to colon cancer (Kinzler and Vogelstein, 1996), and mutations in the tumor suppressor *APC* or the oncogene β-catenin have been found in the majority of spontaneously arising colon cancers (Cancer Genome Atlas Network, 2012).

β-catenin is a component of the adherent junctions (Baum and Georgiou, 2011) and, in the nucleus, binds to TCF4 and several transcriptional regulators. For example, when cancer cell lines are cultured under hypoxic conditions, β-catenin forms a complex with HIF-1, leading to hypoxia adaptation (Kaidi et al., 2007), and in prostate cancer cells, a β-catenin-androgen receptor (AR) complex increases the transcription of AR (Mulholland et al., 2002). β-catenin and YAP1 also coregulate genes that are essential for cardiac development (Heallen et al., 2011). These observations suggest that, through interactions with different partners, β-catenin regulates many biological processes.

Yes-associated protein 1 (YAP1) is a transcriptional modulator that has been implicated in stem cell differentiation and the control of organ size (Pan, 2010). YAP1 regulates several context-specific transcriptional programs (Badouel et al., 2009) and promotes proliferation and tumor growth (Overholtzer et al., 2006; Zhao et al., 2008). Indeed, YAP1 is recurrently amplified in hepatocellular cancer, in which YAP1 is essential for survival of tumors that harbor *YAP1* amplifications (Zender et al., 2006). Furthermore, inducible transgenic expression of

a stabilized YAP1 mutant (S127A) in mice induced liver hyperplasia and colonic adenomas (Camargo et al., 2007).

YAP1 transcriptional activity is regulated by several mechanisms. In quiescent cells, Hippo pathway-mediated serine phosphorylation of YAP1 inhibits nuclear import and promotes its degradation (Zhao et al., 2012). In contrast, YES1-mediated phosphorylation of YAP1 activates YAP1 in embryonic stem cell self-renewal (Tamm et al., 2011), and ABL-mediated phosphorylation of YAP1 in response to DNA damage results in transcription of proapoptotic genes (Levy et al., 2008). Recent work suggests that YAP1 also plays a role in mechanotransduction in a Hippo-independent manner (Dupont et al., 2011).

Although stabilization and localization of β-catenin contribute to adenoma formation, our understanding of β-catenin regulation and function in cancer remains incomplete. For example, Rac1-mediated phosphorylation of β-catenin has been shown to affect β-catenin activation and localization (Wu et al., 2008). Moreover, in zebrafish and some human cell lines, *APC* loss alone resulted in impaired differentiation but failed to induce nuclear localization of β-catenin and transformation (Phelps et al., 2009). To gain insights into β-catenin activity in malignant transformation, we classified β-catenin activity in a panel of human cancer cell lines in which we have systematically characterized genetic alterations, gene expression, and gene essentiality. Here, we report the identification of an alternative transcriptional regulatory complex required for the β-catenin-driven transformation and tumor maintenance.

RESULTS

Identification of Essential Genes in β-Catenin-Active Cancer Cell Lines

To identify genes whose expression is essential in cell lines that exhibit β-catenin activity, we used a β-catenin/TCF4 reporter (Fuerer and Nusse, 2010) to classify β-catenin activity in 85 cancer cell lines in which we had previously performed genome-scale loss-of-function screens (Cheung et al., 2011), transcriptional profiling, and global copy number analyses (Barretina et al., 2012) (Figure 1A). To evaluate the specificity of this reporter, we used colon cancer cell lines (DLD1, Colo205, and HCT116) that harbor mutations in components of the Wnt/β-catenin pathway. Expression of two distinct β-catenin-specific short hairpin RNAs (shRNAs) suppressed β-catenin expression (Figure 1B) and inhibited β-catenin/TCF4 reporter activity (Figure 1C) in these cell lines. Of the 85 cell lines, 19 showed reporter activity that was at least 10-fold above background (Figure 1D and Tables S1 and S2 available online). We note that two colon cancer cell lines that harbor *APC* mutations (HT29 and LS411N) exhibited little β-catenin activity and were classified as reporter inactive.

We applied a two-class permutation analysis (see Extended Experimental Procedures) to cell lines classified as either β-catenin active or inactive and identified genes whose expression was essential for the survival/proliferation of β-catenin-active cells (Table S3). We identified β-catenin as the top candidate and found 49 other genes that scored as significantly essential for the proliferation/survival of β-catenin-active cells (q value < 0.25).

We tested whether suppressing each of the top 250 genes that scored in the above analysis affected β-catenin/TCF4 reporter activity in an APC-mutated cell line (Figure S1A). Suppression of *APC* or *CSNK1A1* induced increased reporter activity, and suppression of β-catenin or BCL9L inhibited reporter activity (Figure S1B). Although suppression of 44 genes inhibited β-catenin/TCF4 reporter activity in the DLD1 cell line, >80% of the genes that scored as selectively essential in β-catenin-active cell lines did not regulate the this reporter, suggesting that the majority of these essential genes represent codependencies not directly related to β-catenin/TCF4-regulated gene regulation.

β-catenin binds TCF/LEF family transcription factors to regulate gene expression (Klaus and Birchmeier, 2008). Because we failed to identify TCF4 as required for the survival of β-catenin-active cell lines, we manipulated the expression of TCF4 by using TCF4-specific shRNAs (Figure 1E) or perturbed TCF4 function by expressing a dominantly interfering allele of *TCF4* (TCF4 DN; Korinek et al., 1997). Expression of the TCF4 DN allele inhibited β-catenin/TCF4 reporter activity (Figure 1F). In contrast, suppression of TCF4 increased the activity of the β-catenin/TCF4 reporter (Figure 1F), as recently reported (Tang et al., 2008). We then assessed the consequences of suppressing β-catenin or *TCF4* on the proliferation and anchorage-independent (AI) growth of β-catenin-active (HuTu80, DLD1, and HCT116) or -inactive (RKO) cells. Suppression of β-catenin or TCF4 failed to affect the proliferation (Figure 1G) or AI growth (Figure 1H) of RKO cells (β-catenin reporter inactive) but inhibited these phenotypes in β-catenin-active cell lines. In contrast, depletion of TCF4 only partially inhibited (30%–40%) the proliferation and AI growth of β-catenin-active cell lines (Figures 1G and 1H). Although we cannot exclude the possibility that residual TCF4 remains in these cells or that other TCF family members compensate for TCF4 suppression, these observations suggest that β-catenin acts in part in a TCF4-independent manner.

Based on these observations, we examined the list of the top 50 scoring genes (q < 0.25) that were essential for the proliferation of β-catenin-active cell lines and identified a striking enrichment for proteins related to the transcriptional regulator YAP1 (Table S4). When we classified the cell lines based on the mutational status of oncogenes commonly found in colon cancers, such as *KRAS*, *BRAF*, or *PIK3CA* (Wood et al., 2007), we failed to find any enrichment for genes related to YAP1 (Table S4). These observations suggested that these essential genes are specific for β-catenin-active cells.

YAP1 Is Essential for the Transforming Properties of β-Catenin-Active Cell Lines

YAP1 is a transcriptional coactivator that scored as essential for the proliferation/survival of β-catenin-active cells (rank 32, q value = 0.24, Table S4). To confirm the observed dependency on YAP1 in β-catenin-active cell lines, we introduced two independent *YAP1*-specific shRNAs into a panel of colon cancer cell lines with high or undetectable β-catenin activity (Table S1). We found that YAP1 expression was selectively required for the proliferation and AI growth of β-catenin-active cells (Figures 2A, 2B, and S2A). When we examined the two cell lines that harbored *APC*-inactivating mutations but did not exhibit β-catenin/TCF4

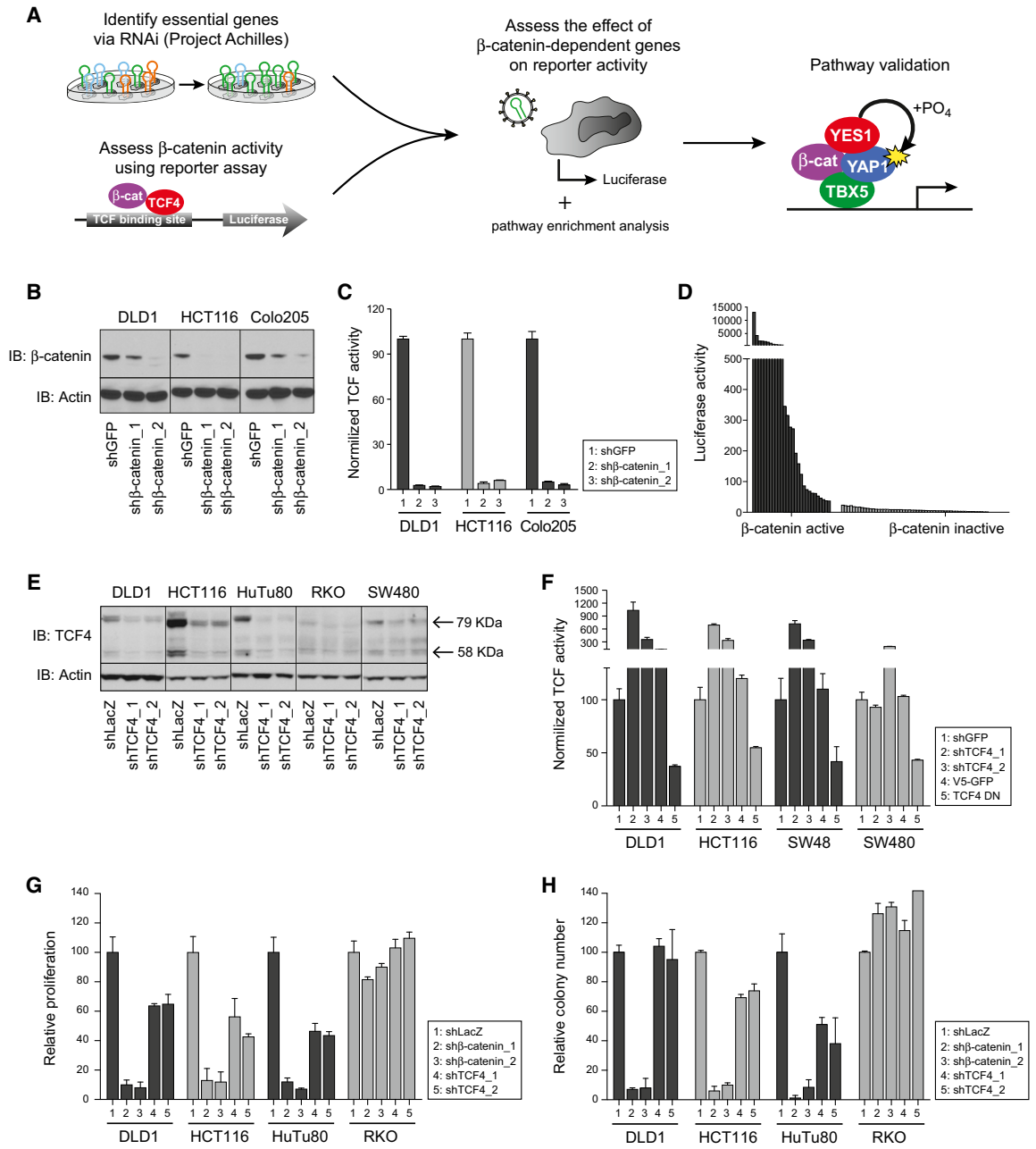

Figure 1. Identification of Genes Essential for β-Catenin-Active Cell Lines

(A) Strategy to identify genes required in β-catenin-active cell lines.

(B) β-catenin expression in cells expressing β-catenin-specific shRNAs. IB indicates immunoblotting.

(C) Activity of the β-catenin/TCF4 reporter following suppression of β-catenin. LacZ was used for normalization.

(D) β-catenin/TCF4 reporter activity in 85 cell lines.

(E) TCF4 expression following introduction of *TCF4*-specific shRNAs.

(F) β-catenin/TCF4 activity following suppression of TCF4 or expression of DN-TCF4.

(G and H) (G) Proliferation or (H) AI growth following suppression of TCF4.

Data are presented as mean ±SD for three independent experiments. See also Figure S1 and Tables S1, S2, and S3.

reporter activity (Table S1), we found that these cell lines (HT29 and LS411N) were dependent on *YAP1* and β-catenin (Figures S2B and S2C). To eliminate the possibility that the observed effects were due to off-target effects, we expressed LacZ or YAP1 in parallel cultures of HuTu80 cells expressing control shRNAs or the two *YAP1*-specific shRNAs, one of which (shYAP1 2) targets the *YAP1* 3′ untranslated region (UTR). We found that forced expression of YAP1 rescued the proliferation

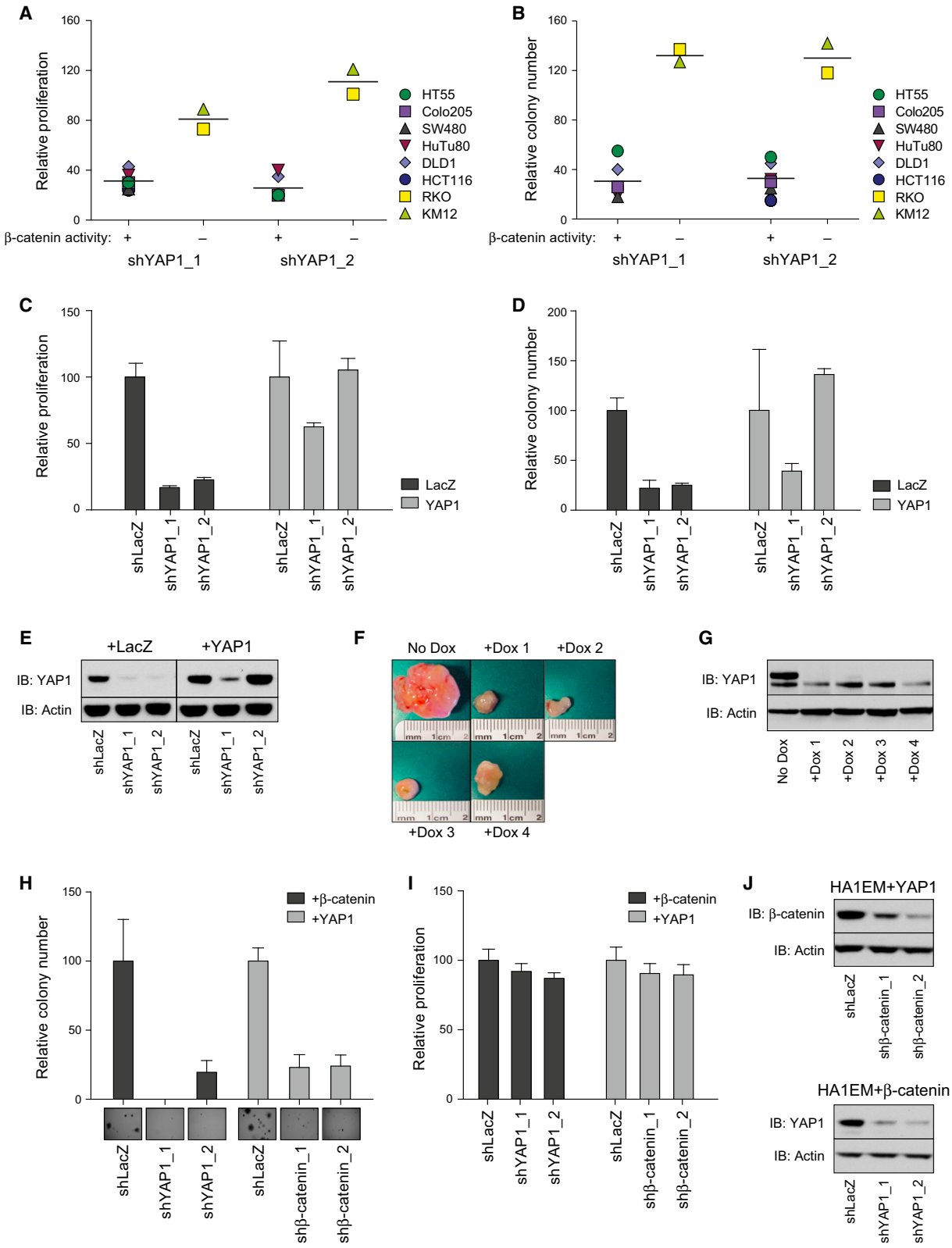

Figure 2. YAP1 Is Essential for Tumorigenicity of β-Catenin-Dependent Cancer Cell Lines

(A and B) (A) Proliferation and (B) Al growth of the indicated cancer cell lines following suppression of *YAP1*. Classification of β-catenin activity in each cell line is noted.

(legend continued on next page)

and AI growth of HuTu80 cells in which YAP1 was suppressed (Figures 2C–2E).

The YAP1-related protein TAZ has been reported to bind to YAP1 and also to regulate Wnt signaling by inhibiting DVL1 (Varelas et al., 2010). TAZ did not score as essential for β-catenin-active cell lines, and when we suppressed the expression of TAZ in β-catenin-active cells (Figure S2D), we failed to observe an effect on proliferation (Figure S2E). These observations suggest that TAZ is not required in cells that exhibit β-catenin activity.

We found that suppression of *YAP1* failed to affect the activity of the β-catenin/TCF4 reporter (Figure S1A). Because YAP1 was reported to affect reporter activity in SW480 cells (Zhou et al., 2011), we suppressed *YAP1* in four additional colon cancer lines that harbor mutations that activate the Wnt/β-catenin pathway and failed to detect decreased reporter activity (Figure S2F) or alterations in the transcription of known β-catenin/TCF4 target genes such as c-Myc, AXIN2, and SOX4 (Figure S2G) (He et al., 1998; Yan et al., 2001). Moreover, suppression of *YAP1* failed to affect the stability of β-catenin (Figure S2H).

To determine whether YAP1 is required for tumorigenicity, we developed an orthotopic colon cancer model in which subcutaneous tumor xenografts derived from an established colon cancer cell line are implanted into the cecum of a second host. Orthotopic implantation of these tumors resulted in infiltration of the colon and liver metastases (Figure S3A). We used this model to determine whether β-catenin or YAP1 were required for tumor growth. Specifically, we developed vectors that harbored doxycycline-inducible shRNAs targeting either β-catenin or YAP1 and introduced these vectors into HCT116 cells. shRNA expression was induced after cecal implantation with doxycycline. We found that tumors expressing the inducible β-catenin-specific shRNAs showed diminished expression of β-catenin and were substantially smaller (Figures S3B and S3C). When we analyzed tumors expressing an inducible *YAP1*-specific shRNA, we found that suppression of YAP1 also inhibited tumor growth by 80%–90% (Figures 2F and 2G), indicating that YAP1 was essential for tumorigenic growth.

These observations confirmed that YAP1 expression is required for the tumorigenicity of β-catenin-active cells. To determine whether YAP1 contributes to cell transformation, we expressed a stabilized form of β-catenin (S33A, S37A, T41A, and S45A) that cannot be phosphorylated (Morin et al., 1997) or YAP1 (Zhao et al., 2010) in HA1EM cells, a nontumorigenic immortalized kidney epithelial cell line that is rendered tumorigenic by the forced expression of myristoylated AKT1 (Boehm et al., 2007). Expression of stabilized β-catenin or YAP1 sufficed to promote AI growth (Figure 2H), indicating that expression of either YAP1 or activated β-catenin transforms these cells. These immortalized cells were not dependent on stabilized β-catenin or YAP1 for proliferation (Figure 2I). However, suppression of β-catenin inhibited the AI growth of cells expressing stabilized YAP1, and suppression of YAP1 reduced the AI growth of cells expressing stabilized β-catenin (Figures 2H and 2J). Together, these observations implicate YAP1 as an essential gene in β-catenin-mediated transformation and suggest that YAP1 and β-catenin cooperate to induce transformation.

YAP1, β-Catenin, and TBX5 Form a Complex

YAP1 and β-catenin have recently been shown to coregulate genes critical for cardiac development (Heallen et al., 2011). By using SW480 and HuTu80 cells, we found that endogenous YAP1 and β-catenin interact. Specifically, we found that β-catenin-specific, but not control immunoglobulin, immune complexes contained endogenous YAP1 (Figure 3A). Moreover, when we isolated YAP1 immune complexes, we detected endogenous β-catenin (Figure 6A).

Both YAP1 and β-catenin are transcriptional coregulators (Kaidi et al., 2007; Zhao et al., 2008). When we re-examined the list of genes that were required for proliferation/viability of β-catenin-active cells, we failed to find the known β-catenin partner TCF4 or the TEAD transcription factor family, a known YAP1 partner (Zhao et al., 2008), but noted that the transcription factor TBX5 was highly ranked in this analysis (rank 8, q value = 0.1, Table S4). To confirm that TBX5 was required in β-catenin-active cells, we expressed *TBX5*-specific shRNAs in β-catenin-active cells (Figure 3B) and found that *TBX5* suppression induced a 60%–80% decrease in proliferation and AI growth (Figures 3C and 3D). Although suppression of *TBX5* expression also inhibited the proliferation of β-catenin-inactive cell lines to some degree (Figure 3C), these observations indicate that β-catenin-active cell lines are also dependent on *TBX5* expression.

To determine whether TBX5 interacts with YAP1 and β-catenin, we isolated TBX5-specific immune complexes from 293T or DLD1 cells and found that TBX5 binds to both β-catenin and YAP1 (Figures 3E and 3F). These findings corroborate a prior report showing that TBX5 and YAP1 interact in 293T cells when overexpressed (Murakami et al., 2005) and identify a complex composed of YAP1, β-catenin, and TBX5.

BCL2L1 and *BIRC5* Are Transcriptional Targets of the β-Catenin-YAP1-TBX5 Complex

Among the genes that we identified as required in β-catenin-active cell lines were *BIRC5* (survivin) and *BCL2L1* (Table S4). By using *BIRC5*- or *BCL2L1*-specific shRNAs (Figures S4A and S4B), we found that depletion of *BIRC5* or *BCL2L1* impaired the proliferation and AI growth of β-catenin-dependent cell lines (Figures 4A and 4B). These observations corroborate recent observations that cancer cell lines that harbor activating

(C–E) (C) Proliferation, (D) AI growth, and (E) expression of YAP1 in HuTu80 cell lines overexpressing WT YAP1 and the indicated *YAP1*-specific or control (shLacZ) shRNAs.

(F) Effects of suppressing *YAP1* on orthotopic colon tumors.

(G) YAP1 expression in tumors shown in (F).

(H–J) HA1EM cells expressing β-catenin or YAP1 and YAP1- or β-catenin-specific shRNAs. (H) AI growth, (I) proliferation, or (J) protein levels. In (H), inset shows representative images from AI growth assay.

Data are presented as mean ±SD for three independent experiments. See also Figures S2 and S3 and Table S4.

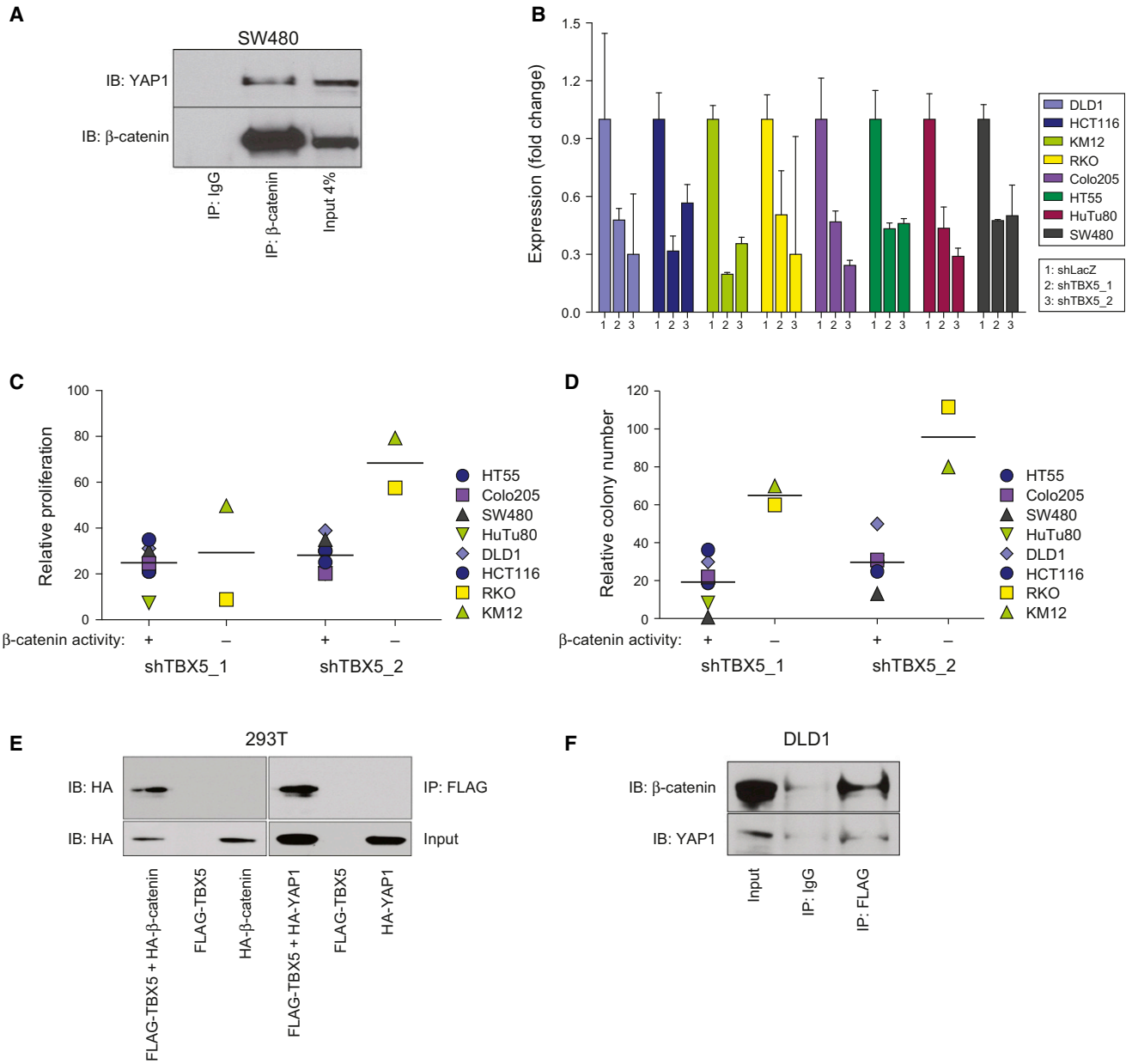

Figure 3. YAP1, β-Catenin, and TBX5 Interact

(A) β-catenin or control IgG immune complexes were isolated from SW480 lysates, and the indicated proteins were analyzed by immunoblotting.

(B) *TBX5* mRNA levels measured by quantitative PCR in cells expressing *TBX5*-specific or control (shLacZ) shRNAs. Data are presented as mean ±SD for three independent experiments.

(C and D) (C) Proliferation or (D) AI growth of the indicated cells following suppression of *TBX5*.

(E) The indicated expression vectors were introduced into 293T cells, and FLAG immune complexes were isolated and analyzed by immunoblotting with an anti-HA antibody.

(F) FLAG-immune complexes were isolated from DLD1 cells stably expressing a FLAG-epitope-tagged TBX5 protein and analyzed by immunoblotting with the indicated antibodies.

mutations in β-catenin are particularly sensitive to navitoclax, an inhibitor of BCL2 family members, including BCL-XL (S. Schreiber, personal communication).

To test whether *BIRC5* and *BCL2L1* are transcriptional targets of the β-catenin-YAP1-TBX5 complex, we examined the messenger RNA (mRNA) levels of *BIRC5* and *BCL2L1* in cell lines (HT55 and HCT116) in which we had suppressed either YAP1 or β-catenin. We found that the expression of both *BIRC5* and *BCL2L1* was dependent on the presence of β-catenin and YAP1 (Figures 4C and 4D), suggesting that the

β-catenin-YAP1-TBX5 complex is involved in the transcriptional regulation of these genes.

To determine whether β-catenin and YAP1 directly regulate *BCL2L1* and *BIRC5* expression, we performed a chromatin immunoprecipitation (ChIP) assay focused on sites in the *BCL2L1* and *BIRC5* promoters identified by β-catenin-specific ChIP sequencing (ChIP-seq) and found that both β-catenin and YAP1 were bound to these promoters (Figure 4E). Furthermore, suppression of *TBX5* expression in HuTu80 cells abrogated the binding of β-catenin (Figure 4F) or YAP1 (Figure 4G) to these promoters. Similar to what we found when we suppressed YAP1, YES1, or β-catenin (Figures 4C and 4D), suppression of TBX5 expression resulted in decreased expression of *BIRC5* and *BCL2L1* (Figure 4H).

To investigate whether BCL2L1 and BIRC5 contribute to the proliferation arrest that is observed following suppression of either β-catenin or YAP1 in β-catenin-active cancer cell lines, we stably expressed the antiapoptotic isoform of BCL2L1 (BCL-XL) or BIRC5 in HuTu80 (β-catenin active). Following overexpression of these genes, we expressed *YAP1*- or β-catenin-specific shRNAs. Ectopic expression of BCL-XL or BIRC5 rendered the levels of these proteins independent of β-catenin or YAP1 (Figure 4I) and partially restored the proliferation of cell lines in which we suppressed either β-catenin or YAP1 (Figure 4J), suggesting that these genes are targets of the β-catenin-YAP-TBX5 complex.

YES1 Kinase Activity Is Essential for the Transforming Properties of β-Catenin-Dependent Cancers

YAP1 was originally identified as a YES1-associated protein (Sudol et al., 1995). We found that the SRC family tyrosine kinase YES1 was essential for the growth of β-catenin-active cell lines (rank 30, q value = 0.24, Table S4). As we observed for YAP1, suppression of *YES1* inhibited the proliferation, AI growth, and tumor formation of β-catenin-active cell lines (Figures 5A–5D and S5A). Furthermore, when we suppressed *YES1* expression, we also found reduced levels of *BIRC5* and *BCL2L1* (Figures 4C and 4D). We confirmed that *YES1*-specific shRNAs did not alter the expression of the closely related kinase SRC (Figures S5A and S5B). These observations confirmed that YES1 expression was required in β-catenin-active cell lines.

YAP1 binds to YES1 and is phosphorylated by SRC family kinases in embryonic stem cells (Tamm et al., 2011). We confirmed that YES1 and YAP1 interact in the β-catenin-active colon cancer cell line SW480 (Figure 5E). Previous studies have shown that YAP1 is able to bind to other SRC family members such as SRC in HeLa cells (Zaidi et al., 2004). However, in colon cancer cell lines, we failed to detect an interaction between YAP1 and SRC or FYN (Figure 5E).

To determine whether YES1 or SRC phosphorylates YAP1, we expressed YAP1 in 293T cells and assessed YAP1 tyrosine phosphorylation when coexpressed with YES1 or SRC (Figure 5F). We detected phosphorylated YAP1 only when YAP1 was coexpressed with SRC or with activated mutant version of YES1 (Y537F). We failed to detect phosphorylation of YAP1 when coexpressed with wild-type (WT) YES1, indicating that YAP1 phosphorylation requires the active form of YES1 (Figure 5F). Although both YES1 and SRC phosphorylated YAP1, suppression of *SRC* failed to inhibit the proliferation and AI growth of β-catenin-active cell lines (Figures S5C–S5E). Thus, we concluded that both YES1 and SRC are able to phosphorylate YAP1, but only YES1 is essential for the survival of β-catenin-active cell lines.

In 293T cells, we did not detect phosphorylated YAP1 when expressed alone (Figure 5F). In contrast, we readily detected YAP1 tyrosine phosphorylation in HuTu80 or SW480 cells expressing WT YAP1 (Figure 5H). Furthermore, treatment of colon cancer cells expressing WT YAP1 with the tyrosine kinase inhibitor dasatinib (Lombardo et al., 2004) inhibited the tyrosine phosphorylation of YAP1 (Figure 5I). These results confirm reported observations that demonstrated that YES1 is activated in colon cancer cell lines and tumors (Peña et al., 1995).

Prior work has demonstrated that SRC family members phosphorylate tyrosine residues contained with the sequence motif YXXP (Levy et al., 2008). YAP1 harbors one tyrosine residue with this motif (tyrosine 357). Under conditions in which active YES1 phosphorylated WT YAP1 in 293T cells, we failed to detect tyrosine phosphorylation of the YAP1 Y357F mutant in either 293T or colon cancer cell lines (Figures 5G and 5H). These observations confirm that YES1 phosphorylates YAP1 at tyrosine 357.

We then tested whether phosphorylation of YAP tyrosine 357 was essential for YAP1 function. Specifically, when we expressed WT or mutant Y357F YAP1 in HuTu80 cells expressing *YAP1*-specific shRNAs, we found that WT, but not Y357F, YAP1 was able to rescue the antiproliferative and AI growth effects of the *YAP1*-specific shRNA (compare Figures 2C and 2D to S5F and S5G) when expressed at equivalent levels (Figures 2E and S5H). Together, these observations confirm that YES1 is essential for the tumorigenicity of β-catenin-dependent cell lines and suggest that YES1-mediated phosphorylation of tyrosine 357 regulates YAP1 activity.

To assess the relationship between YES1 and YAP1 in vivo, we examined the effect of suppressing these genes on zebrafish development. Microinjection of zebrafish embryos with a high concentration (200 μM) of *YAP1*- or *YES1*-specific morpholinos resulted in severe developmental phenotypes (Figure S5I). Specifically, the *YAP1* morphants developed craniofacial abnormalities and cardiac edema, whereas the *YES1* morphants exhibited craniofacial abnormalities associated with pharyngeal defects (Figure S5I). These phenotypes resemble defects observed when high concentrations of β-catenin-specific morpholinos were injected (Zhang et al., 2012) and confirm previous reports showing that *YAP1* and *YES1* are essential for early embryonic development in zebrafish (Jiang et al., 2009; Tsai et al., 2005).

Microinjection of *YAP1* or *YES1* morpholinos at lower doses (50 μM) avoided global toxicity but impaired gut development (Figure S5I). Intestinal fatty-acid-binding protein (IFABP and FAPB2) is expressed in intestinal epithelial cells, where it plays a key role in gut metabolism and is used as a marker of gut development (Goessling et al., 2008). Morpholino-mediated suppression of *YAP1* or *YES1* expression dramatically inhibited gut formation as determined by both fluorescence microscopy of Tg(fabp2:RFP)as200 gut reporter embryos and by examination of *IFABP* expression by in situ hybridization (Figure 5J).

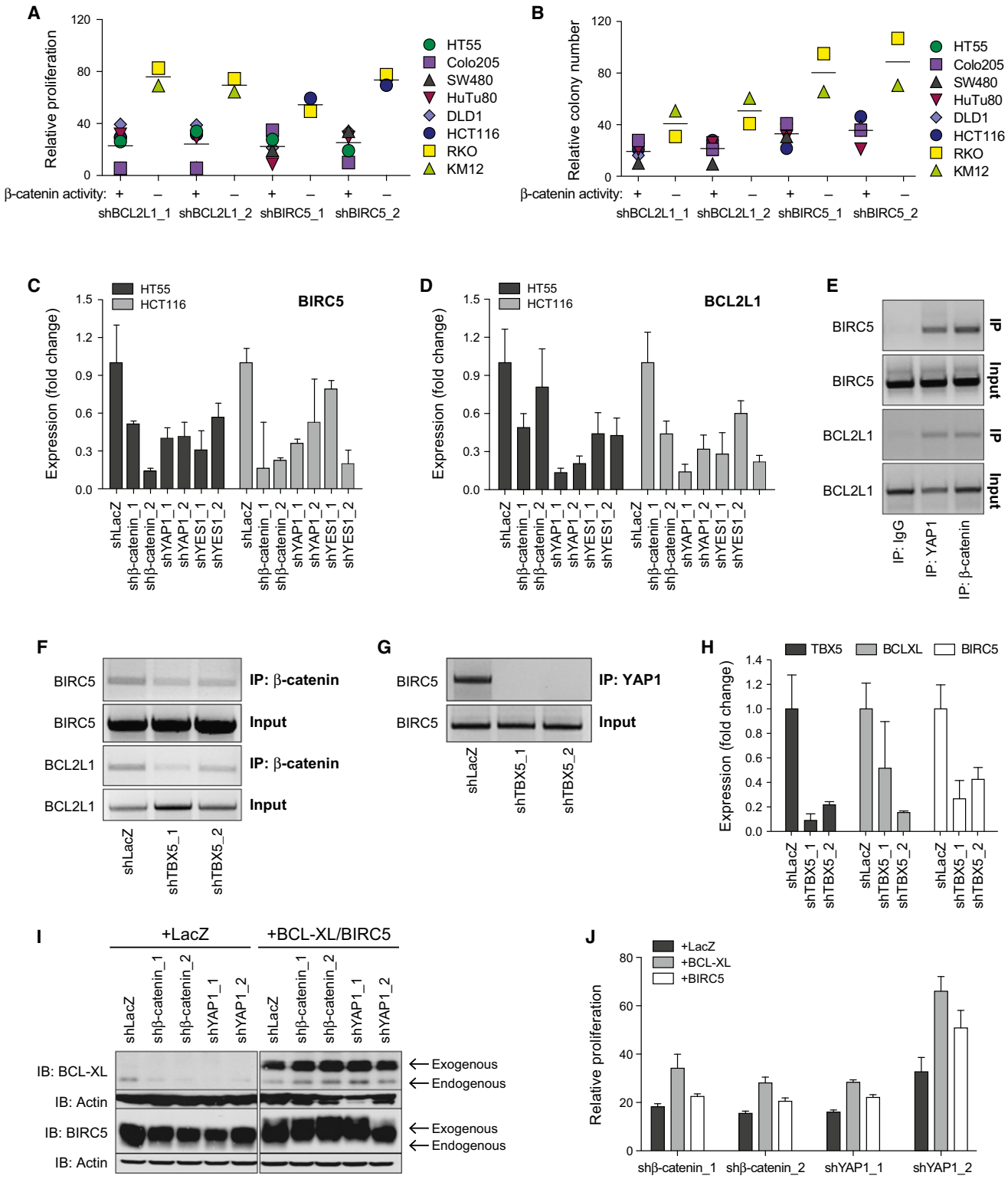

Figure 4. *BCL2L1* and *BIRC5* Are Transcriptional Targets of the β-Catenin-YAP1-TBX5 Complex

(A and B) (A) Proliferation or (B) AI growth following suppression of *BCL2L1* or *BIRC5* in the indicated cell lines.

(C and D) mRNA levels of *BCL2L1* and *BIRC5* in HT55 or HCT116 cells expressing β-catenin-specific, *YAP1*-specific, or control shRNAs.

(E) β-catenin, YAP1, or control immune complexes were isolated from HuTu80 cells and were subjected to ChIP analysis with primers derived from the promoter regions of *BCL2L1* (1–1,000 bp) and *BIRC5* (−952–0 bp).

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Furthermore, treatment of zebrafish embryos postfertilization (dpf) with 2 μM dasatinib inhibited gut formation to a similar extent as the *YAP1*- or *YES1*-specific morpholinos (Figure 5K), indicating that YES1 kinase activity is essential for zebrafish gut development. Because the Wnt/β-catenin pathway has been shown to be crucial for gut development in zebrafish (Cheesman et al., 2011), we concluded that phosphorylation of YAP1 by YES1 is essential for developmental and malignant processes that are dependent on the function of β-catenin.

Previous studies have shown that, in response to cell contact inhibition, activation of the Hippo pathway induces serine 127 phosphorylation and cytosolic accumulation of YAP1 (Zhao et al., 2012). By using immunofluorescence, we found that both YAP1 and β-catenin were constitutively localized in the nucleus in colon cancer cell lines regardless of cell density or β-catenin activity (Figure S6A) and that suppression of β-catenin failed to alter YAP1 localization (Figure S6B). Collectively, these observations suggest that, in contrast to nontransformed cell lines (Zhao et al., 2007), culture density does not regulate YAP localization in colon cancer cell lines.

YES1 Kinase Activity Regulates the Activity of the YAP1-β-Catenin-TBX5 Complex

To determine whether the interaction between β-catenin and YAP1 was regulated by YES1, we expressed two distinct *YES1*-specific shRNAs in HuTu80 cells and found that suppression of *YES1* expression abrogated the formation of the β-catenin-YAP1 complex (Figure 6A).

Treatment of zebrafish embryos with dasatinib, which inhibits YES1, resulted in a similar phenotype to that of suppressing *YES1* expression (Figures 5J and 5K). Thus, we used dasatinib to test whether YES1 kinase activity was essential for the β-catenin-YAP1 interaction. In contrast to what we observed when we suppressed *YES1* expression, treatment of the SW480 colon cancer cell line with dasatinib increased the interaction between β-catenin and YAP1, indicating that YES1 kinase activity is not required for formation of the β-catenin-YAP1 complex (Figure 6B). The dasatinib-induced increase in β-catenin-YAP1 interaction was reversed by expression of a dasatinib-resistant form of YES1 or SRC (Figure 6C). Furthermore, we found that the YAP1 mutant (YAP1 Y357F), which cannot be tyrosine phosphorylated, interacted with β-catenin when expressed in 293T cells or in colon cancer cell lines (Figures 6D and 6E). Thus, the interaction of YES1 with YAP1 and β-catenin is essential for formation of the β-catenin-YAP1 complex in a manner independent of YES1 kinase activity.

Because YES1 suppression disrupted the activity of the β-catenin-YAP1-TBX5 complex, we tested whether YES1 kinase activity was required for binding of the β-catenin-YAP1-TBX5 complex to specific target promoters. Treatment of HCT116 cells with dasatinib inhibited the binding of β-catenin and YAP1 to the *BCL2L1* and *BIRC5* promoters (Figure 6F). Moreover, treatment of HCT116 or HuTu80 with dasatinib resulted in downregulation of *BCL2L1* and *BIRC5* expression, which was reversed by expression of a dasatinib-resistant form of YES1 (Figure 6G). These observations suggest that phosphorylation of YAP1 by YES1 is required for the activity of the β-catenin-YAP1-TBX5 complex.

β-Catenin-Active Cancers Are Sensitive to SRC Family Inhibitors

The observation that the β-catenin-YAP1-TBX5 complex is required for the survival of β-catenin-active cells suggests that disrupting the activity of this complex may selectively affect β-catenin-active cancers. To test this hypothesis, we exposed β-catenin-active and -inactive cell lines to a wide range of dasatinib concentrations. Indeed, we found that β-catenin-active cell lines were 6.4–16 times more sensitive to dasatinib than cells that lack β-catenin activity (Figure 7A and Table S5).

Because dasatinib inhibits a broad range of tyrosine kinases (Lombardo et al., 2004), we tested whether the observed effects on cell proliferation were due to its effects on SRC family members. Specifically, we expressed dasatinib-resistant YES1 or SRC mutants (Du et al., 2009) in HuTu80 or HCT116 cells and then tested the sensitivity of these cells to dasatinib. We found that expression of either of these mutants rescued the proliferation arrest induced by dasatinib (Figures 7B and 7C). These observations confirm that the tyrosine kinase activity of YES1 is required for the proliferation of β-catenin-active cell lines.

To corroborate these findings, we investigated the effects of inhibiting YES1 in colonic organoids and zebrafish. Primary colon organoids can be propagated in vitro as explants in air-liquid interface cultures (Ootani et al., 2009). Under these conditions, colon organoids recapitulate multilineage differentiation and $Lgr5^{+}$ intestinal stem cells. We cultured colon organoids from $Apc^{flox/flox}$; villin-CreER mice, which were exposed to tamoxifen in vitro to delete *Apc*. These WT or *Apc* null organoids were then treated with dasatinib (1–100 nM). We found that *Apc* null organoids were ($p < 0.005$) more sensitive to dasatinib than WT organoids (Figures 7D and 7E). Specifically, we observed a 70% decrease in growth of APC null organoids treated with 50 nM of dasatinib compared to a 5% growth inhibition of WT organoids treated with dasatinib (Figure 7E). These observations demonstrate that inhibition of YES1 kinase activity in *APC* null epithelium reverses the hyperproliferation induced by *APC* loss.

Stabilization of β-catenin in AXIN1 temperature-sensitive mutant zebrafish (Masterblind) induces a β-catenin-dependent hyperproliferation of intestinal epithelial cells (Cheesman et al., 2011). By using this model, we treated WT or AXIN1 mutant zebrafish at 6 dpf with 2 μM dasatinib for 48 hr, which suppressed

(F and G) (F) β-catenin or (G) YAP1 immune complexes derived from HuTu80 cells expressing *TBX5*-specific shRNAs were subjected to ChIP analysis using primers for *BIRC5*.

(H) mRNA levels of *BCL2L1* and *BIRC5* in HCT116 cells expressing *TBX5*-specific shRNAs.

(I) Immunoblot analysis of BCL-XL or BIRC5 in HuTu80 cells overexpressing BCL-XL, BIRC5, or LacZ and YAP1-specific, β-catenin-specific, or control shRNAs.

(J) Proliferation of the cell lines described in (I).

Data are presented as mean ±SD for three independent experiments. See also Figure S4.

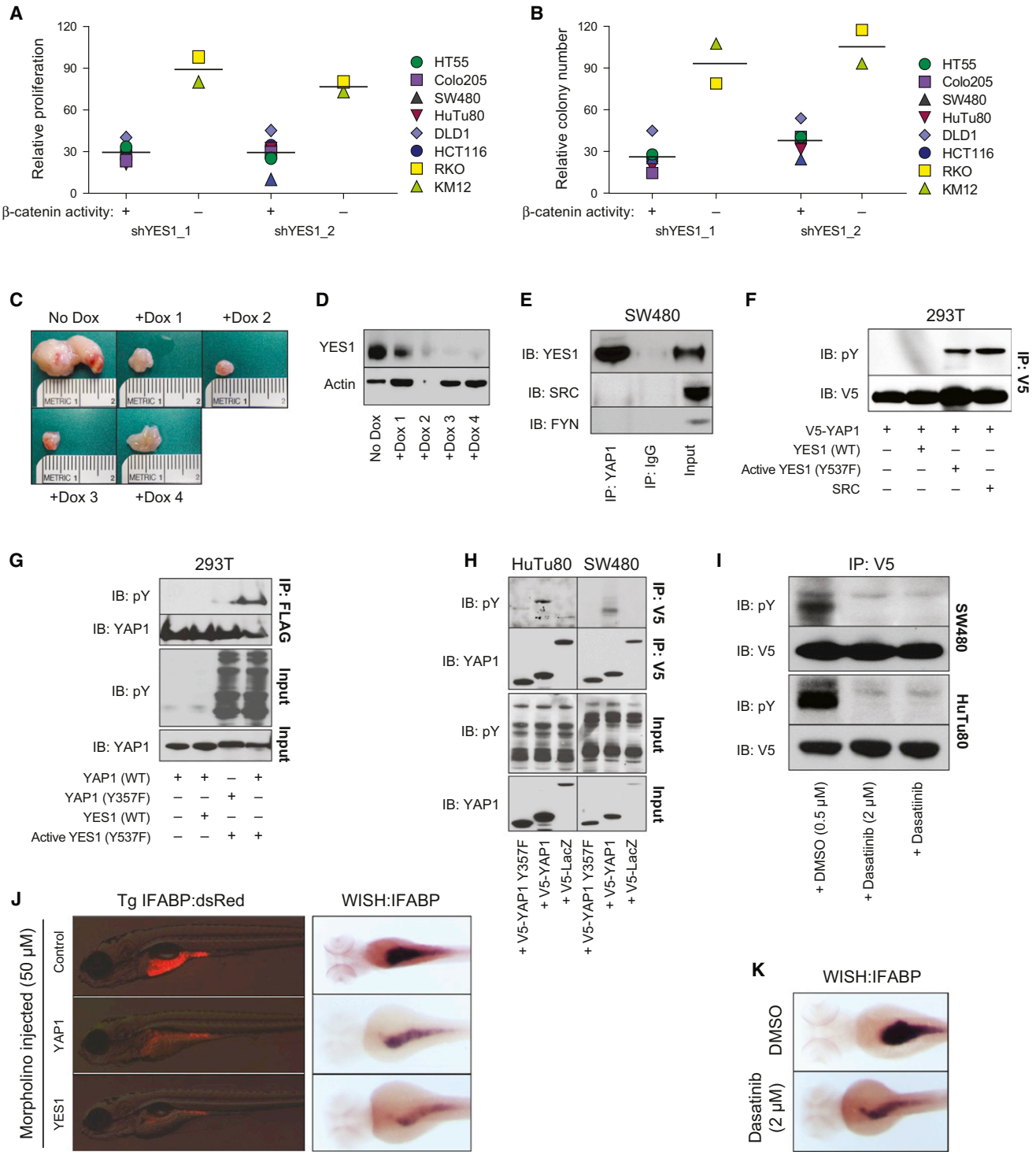

Figure 5. YES1 Is Essential for Tumorigenicity of β-Catenin-Active Cells

(A and B) (A) AI growth or (B) proliferation following suppression of *YES1* expression.

(C) Effects of suppressing *YES1* on orthotopic HCT116 colon tumors.

(D) YES1 expression in tumors shown in (C).

(E) YAP1 immune complexes were isolated from SW480 cells, and the indicated proteins were analyzed by immunoblotting.

(F) V5-epitope-tagged YAP1 and WT or activated (Y537F) YES1 or SRC expression constructs were introduced into 293T cells. V5 immune complexes were analyzed by immunoblotting with the PY99 phosphotyrosine-specific antibody.

(legend continued on next page)

intestinal hyperplasia in the AXIN1 mutants as assessed by morphology (Figure 7F) or hematoxylin and eosin staining (H&E) (Figure 7G). Furthermore, the number of epithelial cells was significantly ($p < 0.0001$) decreased in AXIN1 mutant zebrafish treated with dasatinib (Figure 7H). In contrast, we failed to observe changes in the intestinal structure or cell number in WT zebrafish treated with dasatinib, indicating that the effect of dasatinib was specific to AXIN1 mutant animals (Figures 7G and 7H). We concluded that inhibition of YES1 kinase activity inhibits the β-catenin-dependent proliferation in cultured human cancer cells, in colon epithelial organoids, and in a zebrafish model of intestinal hyperplasia.

DISCUSSION

Identification of Codependent Genes in Cells that Exhibit Active β-Catenin Signaling

By using loss-of-function data derived from Project Achilles (Cheung et al., 2011), we identified 50 genes whose expression was preferentially required for the proliferation of β-catenin-active cell lines. The use of a large number of cell lines allowed us to perform permutation analyses to ensure that the genes identified by this approach robustly distinguished the two groups (FDR $q < 0.25$). Indeed, when we arbitrarily assigned cell lines to two classes, we failed to identify genes that distinguished these groups.

This approach allowed us to identify a set of proteins related to the transcriptional regulator YAP1 that is essential for the proliferation/survival of β-catenin-active cell lines. β-catenin forms a complex with YAP1 and TBX5, which promotes colon cancer cell survival and contributes to malignant transformation. These observations reveal hitherto unidentified components of the β-catenin pathway that play key roles in survival of β-catenin-active cells.

Cancer Cell Lines that Exhibit β-Catenin Activity Require YAP1

YAP1 is an effector of the Hippo pathway (Zhao et al., 2011), an oncogene in hepatocellular cancers (Zender et al., 2006), and a protein involved in mechanotransduction (Dupont et al., 2011). In hepatocellular carcinomas that harbor 11q22 amplifications, YAP1 cooperates with a coamplified gene *CIAP1* to accelerate tumor formation (Zender et al., 2006). Genetically engineered mice that express a stabilized YAP1 mutant (S127A) develop colonic adenomas and liver hyperplasia (Camargo et al., 2007). YAP1 has also been reported to be overexpressed in many epithelial cancers (Steinhardt et al., 2008). However, when we analyzed YAP1 expression in 807 cancer cell lines (Barretina et al., 2012), we found that YAP1 was expressed in most epithelial cell lines (Figure S7), and we failed to identify a correlation between YAP1 dependence and YAP1 copy number or expression. Based on the strong correlation between YAP1 and β-catenin dependency, we conclude that YAP1 induces transformation in β-catenin-active cancers primarily through its interactions with β-catenin.

The Hippo pathway controls organ size by regulation of YAP1. Specifically, upon cell contact inhibition, the Hippo pathway is triggered, leading to a cascade of phosphorylation events resulting in activation of the MST1/2 kinases. Activated Mst1/2 phosphorylate LATS1/2, which, in turn, phosphorylate YAP1 on serine 127, leading to inactivation of YAP1 by proteasomal degradation and cytosolic retention of YAP1 (Zhao et al., 2011). Inactivation of the Hippo pathway by tissue-specific germline deletion of Mst1/2 induced hepatocellular cancers (Zhou et al., 2009) or colonic hyperplasia and adenomas (Zhou et al., 2011). However, recurrent deletions or loss of heterozygosity involving these genes have not been identified in human cancers. Here, we failed to find a correlation between YAP1 nuclear localization or expression and YAP1 dependency, corroborating prior work (Zhou et al., 2011). Because YAP1 is regulated by Hippo-dependent and -independent mechanisms (Dupont et al., 2011), further studies are necessary to determine the role of Hippo signaling in colon cancer pathogenesis.

β-catenin regulates YAP1 expression by binding to the YAP1 promoter (Konsavage et al., 2012). Specifically, both β-catenin and TCF4 bind to sequences upstream of *YAP1*, and suppression of β-catenin expression resulted in decreased *YAP1* mRNA levels. However, we found comparable levels of YAP1 across a large number of cell lines, including β-catenin-inactive colon cancer cell lines (Figures S2A and S7), suggesting that β-catenin is not the primary driver of YAP1 sensitivity in these cell lines. However, it remains possible that the β-catenin-TCF4 complex participates in a feedback loop that enhances YAP1 expression.

The β-Catenin-YAP1-TBX5 Complex

TBX5, a member of the T-box family of transcription factors, plays key roles in cardiac muscle development and limb identity (Rodriguez-Esteban et al., 1999). Germline mutations in TBX5 occur in the Holt-Oram syndrome (Mori and Bruneau, 2004). TBX5 has also been shown to form a complex with TAZ and YAP1 that induces transcription of atrial natriuretic factor (Murakami et al., 2005). Here, we found that TBX5 forms a complex with β-catenin and YAP1 that is found at the *BCL2L1* and *BIRC5* promoters. These observations extend prior work that

(G) FLAG-epitope-tagged WT or mutated (Y357F) YAP1 were transfected into 293T cells together with activated (Y537F) YES1. FLAG-epitope immune complexes were isolated and analyzed by immunoblotting with a phosphotyrosine antibody.

(H) V5 immune complexes were isolated from HuTu80 or SW480 cells stably expressing WT or Y357F V5-epitope-tagged YAP1 or control V5-LacZ. Immune complexes were analyzed by immunoblotting with a phosphotyrosine antibody.

(I) HuTu80 or SW480 cells stably expressing V5-epitope-tagged WT YAP1 were treated for 6 hr with 0.5 or 2 μM of dasatinib. V5 immune complexes were analyzed by immunoblotting with a phosphotyrosine antibody.

(J) Transgenic IFABP:RFP zebrafish were injected with 50 μM of *YAP1*- or *YES1*-specific morpholinos. Red fluorescence was assessed 4 dpf, or *IFABP* expression was assessed 3 dpf by using whole-mount in situ hybridization with an *IFABP*-specific probe.

(K) Embryos were exposed to 2 μM of dasatinib at 2 dpf, and IFABP expression was assessed after 24 hr by using whole-mount in situ hybridization.

See also Figures S5, S6, and S7.

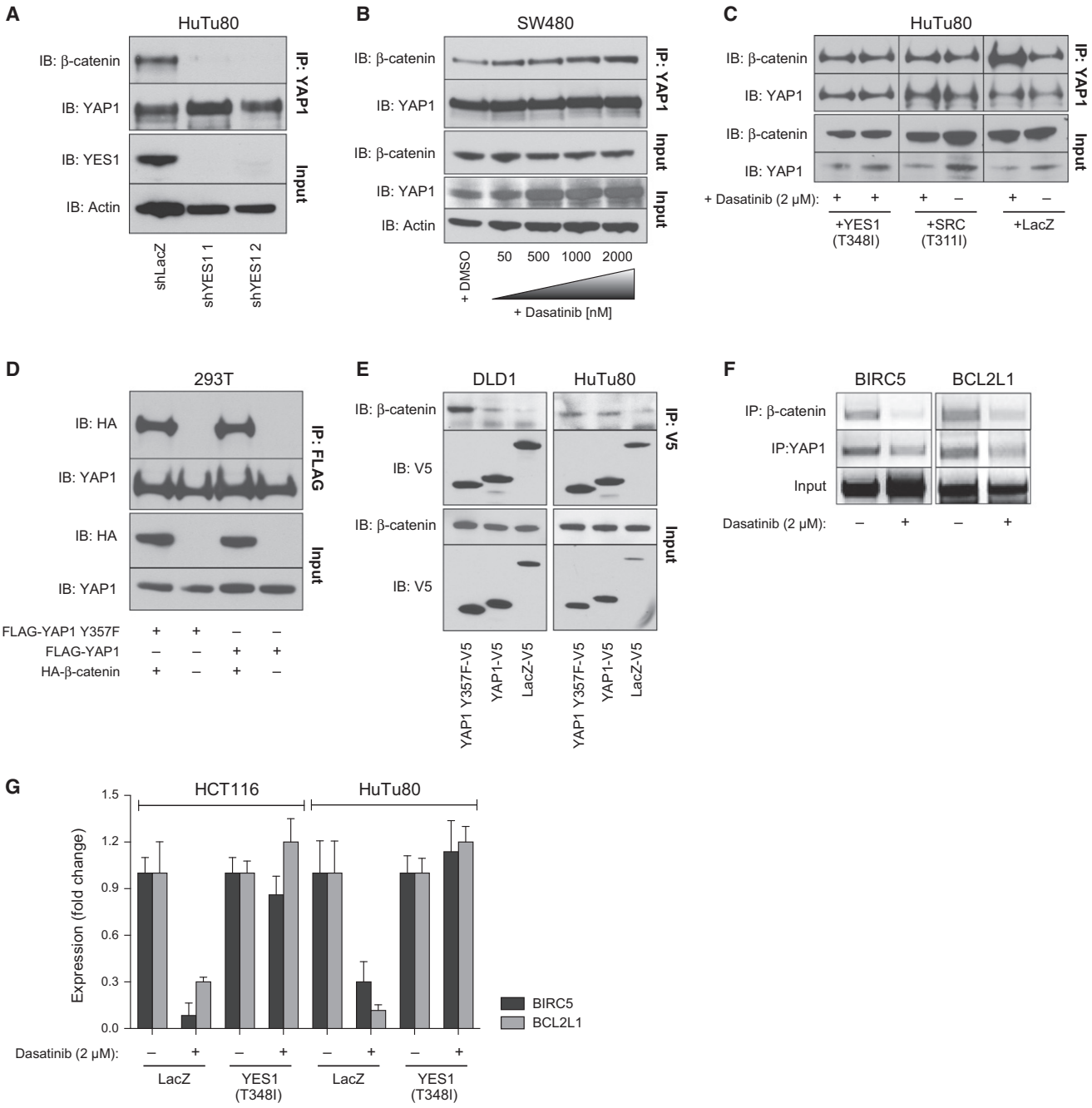

Figure 6. Expression of YES1 Is Essential for Formation of the YAP1-β-Catenin-TBX5 Complex

(A) YAP1 immune complexes were isolated from HuTu80 cells expressing *YES1*-specific or control shRNAs, and β-catenin abundance was analyzed by immunoblotting.

(B) SW480 cells were treated for 6 hr with increasing concentrations of dasatinib, and β-catenin-YAP1 complexes were assessed as in (A).

(C) HuTu80 cells expressing dasatinib-resistant YES1 or SRC mutants were treated with 2 μM of dasatinib for 6 hr, and the β-catenin-YAP1 interaction was assessed as in (A).

(D) 293T cells were transfected with FLAG-epitope-tagged WT or Y357F YAP1 (5 μg) with or without HA-epitope-tagged β-catenin. FLAG immune complexes were assessed for the presence of HA-tagged proteins.

(E) V5 immune complexes were isolated from DLD1 or HuTu80 colon cancer cell lines stably expressing V5-epitope-tagged WT or Y357F YAP1 or control LacZ, and the presence of β-catenin was assessed by immunoblotting.

(F) β-catenin or YAP1 immune complexes from HCT116 cells treated with 2 μM of dasatinib or vehicle (DMSO) were subjected to ChIP analysis.

(G) mRNA levels of *BCL2L1* and *BIRC5* in HCT116 or HuTu80 cells treated for 1 hr with 2 μM of dasatinib. Error bars represent mean ±SD.

showed that TBX5 binds the *BIRC5* and *BCL2L1* promoters when overexpressed in 293T cells (He et al., 2011).

YAP1 interacts with the TEAD family of transcriptional factors to regulate both developmental and cancer-associated phenotypes (Zhao et al., 2008). We did not find that TEAD family members were differentially required for proliferation of β-catenin-active cells. Although the YAP1-TEAD complex regulates other cancer phenotypes (Lamar et al., 2012), our observations implicate TBX5 as a key transcription factor target of the β-catenin-YAP1 complex. Moreover, because β-catenin interacts with different transcription factors such as the AR (Mulholland et al., 2002) and HIF-1 (Kaidi et al., 2007), these observations suggest that both YAP1 and β-catenin regulate several transcriptional programs through interactions with distinct transcription factors.

TCF4 Dependency in β-Catenin-Dependent Cancers

Although the β-catenin-TCF4 complex plays an important role in colon adenoma initiation, several lines of evidence suggest that β-catenin may also contribute to cancer in a TCF4-independent manner. Specifically, although expression of a dominantly interfering allele of TCF4 inhibits the β-catenin/TCF4 reporter activity in β-catenin-dependent colon cancer cell lines (Korinek et al., 1997; Figure 1H), suppression of TCF4 induces a substantial increase in β-catenin/TCF4 reporter activity and only partially affects the proliferation and AI growth of β-catenin-dependent cell lines (Figures 1I and 1J) (Tang et al., 2008). Moreover, the HT29 and LS411N colon cancer cell lines harbor *APC* mutations and depend on β-catenin expression for survival yet failed to exhibit detectable β-catenin/TCF4 reporter activity and were dependent on YAP1 for survival.

Furthermore, compound genetically engineered mice that express the APC^{min} allele and lack one *TCF4* allele develop aggressive, metastatic colon cancers (Angus-Hill et al., 2011). Whole-genome sequencing of colon cancer genomes has revealed recurrent *TCF4-VTI1A* translocations that create a dominantly interfering allele of *TCF4* (Bass et al., 2011) as well as inactivating mutations and copy number loss involving TCF4 (Cancer Genome Atlas Network, 2012). In aggregate, these observations suggest that TCF4 may contribute initially to adenoma formation but then is mutated or lost to foster malignant transformation. We cannot exclude the possibility that a residual amount of TCF4 remains in the experiments presented herein, and these observations do not exclude the possibility that other TCF or LEF proteins may be essential for β-catenin-mediated transformation.

YES1 Regulates the Formation and Activity of the β-Catenin-YAP1-TBX5 Complex

We found that the SRC family member YES1 is also specifically essential for the proliferation and transformed phenotype of β-catenin-active cells both because YES1 is necessary for the formation of the β-catenin-YAP1-TBX5 complex and because phosphorylation of YAP1 on Y357 by YES1 is required for the localization and activity of this complex.

Several tyrosine kinases, including YES1 (Tamm et al., 2011), SRC (Zaidi et al., 2004), and ABL (Levy et al., 2008), phosphorylate YAP1. We found that suppression of SRC or ABL failed to affect the proliferation/survival of β-catenin-active cells, demonstrating that, in this context, YES1 plays a dominant role in regulating the β-catenin-YAP1-TBX5 complex. Moreover, treatment of β-catenin-active cells with dasatinib inhibited the activity of the β-catenin-YAP1-TBX5 complex and the survival of β-catenin-active cancer cell lines in a manner that is rescued by expression of dasatinib-resistant YES1 or SRC mutants. Extending these findings, we found that dasatinib induces an antiproliferative effect in both murine and fish experimental models of APC loss/WNT pathway activation. These findings corroborate a recent report that showed that treatment of APC^{min} mice with dasatinib decreases intestinal adenomas (Nautiyal et al., 2011). Together, these observations support a role for YES1 phosphorylation in β-catenin-driven cancers.

Conclusions

We have identified a β-catenin-YAP1-TBX5 complex required for the survival and transformation of β-catenin-active cancer cell lines. YES1 regulates the formation of this complex and localization to specific promoters, which is dispensable for β-catenin/TCF4 activity, but which regulates transcription of prosurvival genes. These observations demonstrate that deregulated β-catenin stability and function drive malignant transformation through interactions with at least two distinct transcriptional complexes (β-catenin-YAP1-TBX5 and β-catenin-TCF4). Although further work is necessary to decipher the specific roles of each of these complexes in tumorigenesis, these observations provide a framework to explain recent observations that loss of TCF4 activity is associated with tumor progression (Angus-Hill et al., 2011).

Although no specific inhibitors of YES1 exist, the sensitivity of β-catenin-active cancer cell lines and animal models to dasatinib suggests that YES1 is an attractive target in β-catenin-driven cancers. Moreover, we found that suppression of *BCL2L1* and *BIRC5* also inhibited the proliferation/survival of β-catenin-active cell lines. Because small-molecule inhibitors of BCL2L1 and BIRC5 are currently under investigation, these molecules may also prove useful for targeting of β-catenin-active cancers.

EXPERIMENTAL PROCEDURES

In Vivo Orthotopic Tumor Model

4×10^6 HCT116 cells were injected into the flanks of immunodeficient mice (NCr Nude, Taconic). Tumors were extracted, cut into 1 mm^3 cubes, and implanted into a pouch created in the cecum of a second mouse. For experiments with inducible shRNAs, the mice were fed a doxycycline diet 2 days after cecal implantation. Tumors were examined 3 weeks postimplantation.

Three-Dimensional Primary Intestinal Organoid Culture

Colons from $Apc^{flox/flox}$; villin-CreER mice were dissected lengthwise and washed in cold PBS. 0.5–1 cm segment per dish was minced extensively on ice and embedded in a 3D collagen gel by using a double-dish culture system (Ootani et al., 2009). Tamoxifen (Sigma, 2 μM in ethanol) or vehicle (ethanol) was applied on the day of initial plating for 7 days to generate APC null or WT organoids. Organoids were recovered from collagen gel by collagenase IV incubation followed by 0.05% trypsin/EDTA incubation to dissociate organoids into single cells. 5,000 cells per well were seeded into 96 well transwell plates (Fisher Scientific). Organoids were treated with the indicated concentration of dasatinib (in DMSO) for 7 days and were quantified by using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

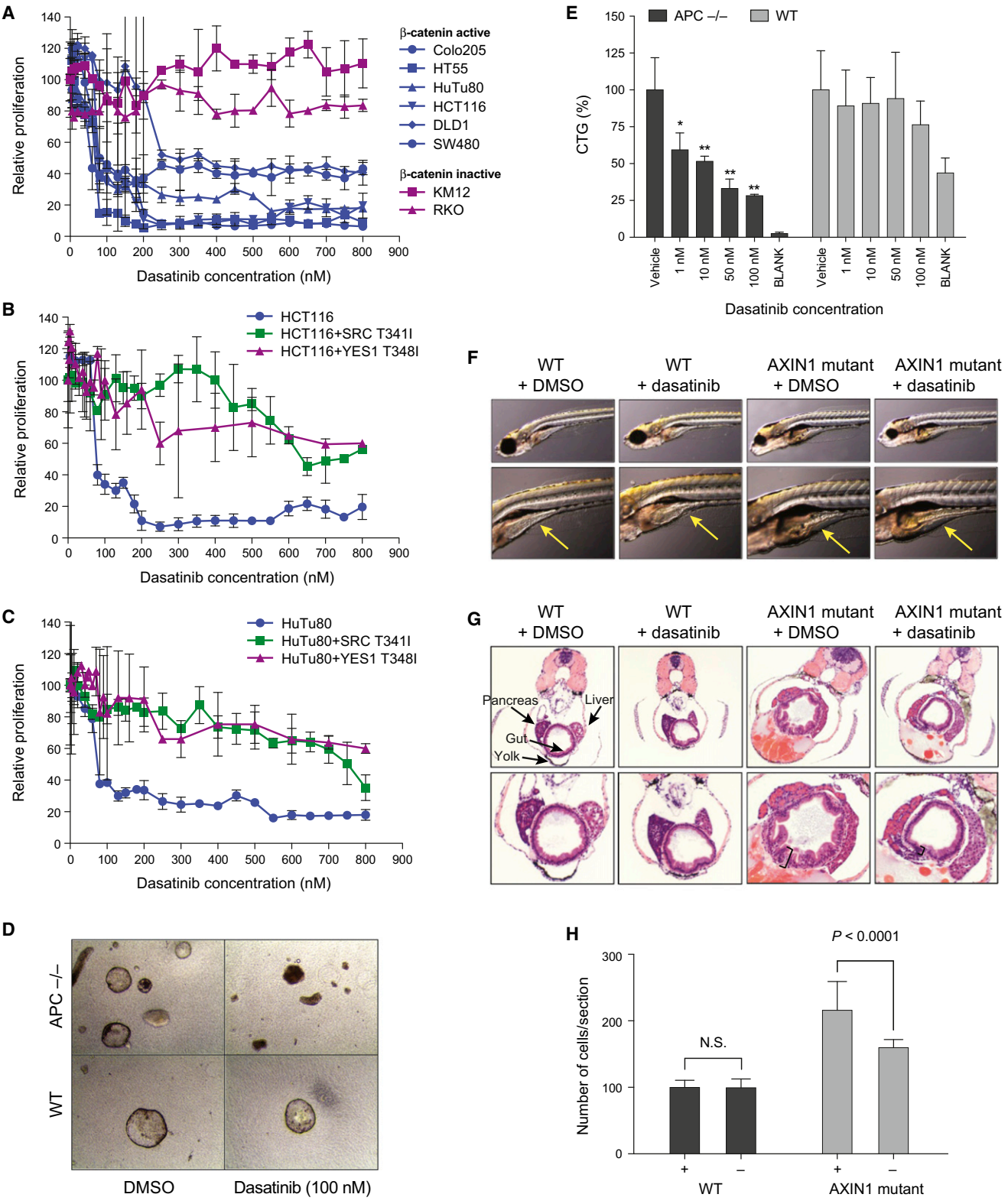

Figure 7. Dasatinib Impairs the Proliferation of β-Catenin-Active Cell Lines

(A) Proliferation (7 days) following dasatinib treatment.

(B and C) Proliferation of (B) HCT116 or (C) HuTu80 cells following dasatinib treatment of cells stably expressing dasatinib-resistant YES1 (T348I) or SRC (T341I) mutants. Data are presented as mean ±SD for four independent experiments.

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Zebrafish Experiments

Zebrafish were maintained according to institutional animal care and use committee (IACUC-BIDMC) protocols. Validated morpholinos (MO) (GeneTools, PhiloMath, OR) designed against the ATG site of YES1 (5′-CCTCTTTACTCTTGACACAGCCCAT-3′) (Jopling and Hertog, 2007) or YAP1 (5′-AGCAACATTAACAACTCACTTTAGG-3′) (Skouloudaki et al., 2009) were injected into WT or gut reporter (*Tg(fabp2:RFP)as200*) zebrafish at the one-cell stage. At 4 dpf, the gut morphology of intestinal reporter embryos was imaged by fluorescent microscopy (Discovery, Carl Zeiss). Whole-mount in situ hybridization experiments were conducted by using standard zebrafish protocols (http://zfin.org), and the gut tissue was visualized by using the established marker IFABP (Mudumana et al., 2004). The axin1^{tm213} mutant line Masterblind was reared at 30°C (temperature required for homozygote phenotype to be fully penetrant). Larvae were fixed overnight in 4% PFA, processed and embedded in JB-4 resin, cut into 7 μM sections, and stained with Hematoxylin and Eosin (Sullivan-Brown et al., 2011). The total number of intestinal epithelial cells and intestinal wall thickness was quantified in sections at a location of the intestinal bulb that had a comparable amount of pancreatic and liver tissue (20 sections quantified represent four sections of five animals).

Sequences of shRNAs and Primers

The target sequences of the shRNAs used are listed in Table S6. The sequences of the primers used for RT-PCR and CHIP are listed in Table S7. For further details, see Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.11.026.

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(D) Representative images of colon organoids derived from WT or APC null mice treated for 6 days with 100 nM of dasatinib.
(E) Quantification of results in (D). Error bars represent SD from four replicates. Blank denotes wells where no organoids were added.
(F) WT or AXIN1 mutant (Masterblind) zebrafish were treated with 2 μM of dasatinib from 6–8 dpf. Arrow indicates developing gut.
(G) H&E staining of zebrafish in (F). Width of epithelium is noted by bars.
(H) The number of epithelial cells/section was measured in WT or AXIN1 mutant zebrafish treated with 2 μM of dasatinib or DMSO. Error bars represent the SD from 20 different sections from five treated fish. p value was calculated by using Student's t test.
See also Table S5.

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