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STRAP is a Critical Mediator of APC Mutation-Induced Intestinal Tumorigenesis through a Feed-Forward Mechanism

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Abstract

BACKGROUND AND AIMS: Inactivation of the *Apc* gene is a critical early event in the development of sporadic colorectal cancer (CRC). The expression of serine-threonine kinase receptor-associated protein (STRAP) is elevated in CRCs and is associated with poor outcomes. We investigated the role of STRAP in Apc mutation-induced intestinal tumor initiation and progression.

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Author contributions:

T.V. conceived the study, conducted the experiments, analyzed the data and wrote the manuscript; A.D., G.Y., and B.Z. assisted with Strap mice experiments; C.B. and P.B. assisted with human organoid experiments; L.J. assisted with ChIP experiments and RNA-seq analyses; K.M. performed immunofluorescence microscopy experiments; I.E. performed histological analysis, R.S.W. analyzed the scRNA data; S.B. assisted with bioinformatics analyses; T.S. and U.M. helped to conduct the study; P.K.D. conceived and supervised the study, wrote the manuscript, and provided financial support. All authors read and approved the final manuscript.

Disclosures:

The authors declare no competing interests.

Transcript Profiling:

All original RNAseq data were deposited in the Gene Expression Omnibus (GEO) at NCBI (GSE160428).

Access link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE160428

METHODS: We generated *Strap* intestinal epithelial knockout mice (*Strap*$^{\Delta IEC}$) by crossing mice containing floxed alleles of *Strap* (*Strap*$^{fl/fl}$) with *Villin*-Cre mice. Then, we generated *Apc*$^{Min/+}$;*Strap*$^{fl/fl}$;*Vill*-Cre (*Apc*$^{Min/+}$;*Strap*$^{\Delta IEC}$) mice for RNAseq analyses to determine the mechanism of function of STRAP. We used human colon cancer cell lines (DLD1, SW480 and HT29) and human and mouse colon tumor-derived organoids for STRAP knockdown/knockout and overexpression experiments.

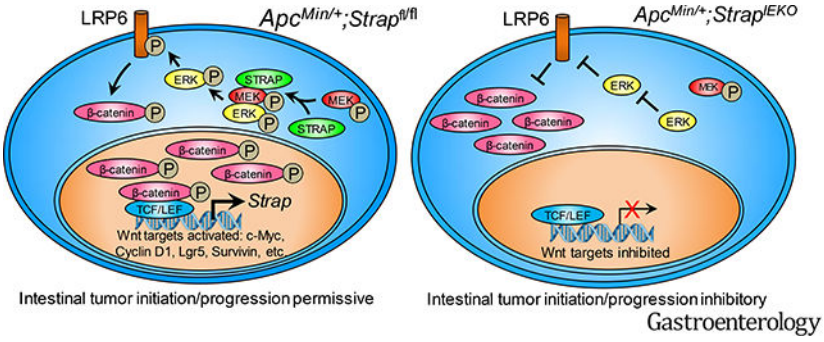
RESULTS: Strap deficiency extended the average survival of *Apc*$^{Min/+}$ mice by 80 days and decreased the formation of intestinal adenomas. Expression profiling revealed that the intestinal stem cell (ISC) signature, the Wnt/β-catenin signaling, and the MEK/ERK pathway are downregulated in Strap-deficient adenomas and intestinal organoids. Correlation studies suggest that these STRAP-associated oncogenic signatures are conserved across murine and human colon cancer. STRAP associates with MEK1/2, promotes binding between MEK1/2 and ERK1/2, and subsequently induces the phosphorylation of ERK1/2. STRAP activated Wnt/β-catenin signaling through MEK/ERK-induced phosphorylation of LRP6. STRAP was identified as a target of mutated Apc and Wnt/β-catenin signaling as ChIP and luciferase assays revealed putative binding sites of the β-catenin/TCF4 complex on the *Strap* promoter.

CONCLUSION: Therefore, STRAP is a target of and is required in Apc mutation/deletion-induced intestinal tumorigenesis through a novel feed-forward STRAP/MEK-ERK/Wnt-β-catenin/STRAP regulatory axis.

Lay summary:

The authors identified a cellular protein that mediates intestinal tumor initiation and progression and that can be targeted for anti-tumor response.

Graphical Abstract



Keywords

Intestinal cancer; tumorigenesis; mouse models

INTRODUCTION

Aberrant activation of Wnt/β-catenin signaling results in the formation and progression of intestinal tumors.[1] In about 80% of sporadic colon cancers, APC deletions/mutations are involved in the regulation of Wnt/β-catenin signaling.[2] APC inactivation leads to β-catenin

stabilization, and consequently, to deregulation of the Wnt pathway through activation of TCF/LEF targets such as c-Myc, Survivin, and Cyclin D1.[3] Additionally, MEK/ERK signaling is involved in intestinal tumorigenesis. Activated ERK1/2 are translocated into the nucleus, where they phosphorylate and activate various nuclear transcription factors and enhance gene transcription.[4] The oncogenic activation of MEK/ERK signaling in colon cancer activates the Wnt/β-catenin pathway.[5] Therefore, it is appropriate to examine the link between these two signaling pathways and the mechanism for their upstream regulation in colon cancers.

STRAP (Serine-Threonine Kinase Receptor-Associated Protein) is a member of the family of WD-40 repeat proteins. STRAP, first identified in our laboratory as an inhibitor of TGF-β signaling, interacts with Smad7 and synergizes with it in suppression of the canonical TGF-β signaling. We and others showed the upregulation of STRAP in human cancers, including breast cancer,[6] lung cancer,[7] and colon cancer.[8] Recently, we found that STRAP epigenetically activates the Notch pathway and maintains the stem-like properties of colon cancer cells. Mechanistically, STRAP disassembles the PRC2 complex by disrupting the interaction between SUZ12 and EZH2.[9]

STRAP is upregulated in more than 50% of colon adenomas,[10] and the expression of STRAP is much higher in stage I human colon cancers relative to other stages.[9] Therefore, it is essential to determine the unknown function of STRAP in the initiation of intestinal tumor formation. Here, we demonstrate that STRAP is required for inactivated APC-induced tumor development through activation of Wnt/β-catenin signaling and that, in intestinal cancers, it functions as an upstream regulator of the link between MEK/ERK and Wnt/β-catenin signaling. Conditional deletion of *Strap* results in a dramatic decrease in intestinal tumorigenesis mediated by APC deletion. STRAP knockdown in human colon cancer cells diminishes β-catenin/TCF4 transcriptional activity. STRAP regulates Wnt/β-catenin signaling by activating the MEK/ERK pathway. Furthermore, STRAP is a target of TCF4/β-catenin signaling and is required for inactivated APC- and Wnt/β-catenin-induced stemness and tumorigenicity through a complex feed-forward loop.

MATERIALS AND METHODS

Generation of *Strap* Conditional Knockout (KO) Mice

We generated *Strap*$^{fl/fl}$ transgenic mice in a C57BL/6 genetic background using ZFN targeting technology.[11] LoxP1 and loxP2 sites were integrated into intron 2 and intron 4 of the *Strap* gene, respectively. STRAP ZFN target site/cleavage sites are CAC AGC CTT ATG TAC ttggag TGG GGA GAC GGC TGG for Intron 2 and TTC CTA CCA GTA GTT ACT accaat AGT GAC TTT GAC AAA GGT C for Intron 4 (NCBI Ref Seq: NC_000072.6). ZFN pairs were microinjected into C57BL/6 zygotes.

Animal experiments

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham, in accordance with international guidelines for biomedical research involving animals. All mice were housed in

a pathogen-free barrier environment for the duration of the study. A detailed description of animal experiments is provided in the Supplementary Material.

Culture of organoids derived from colon tumors of patients

Organoids derived from patient colon tumors P07132016T, F131T, F114T, and F130T were generated as described before.[12] The organoids were cultured in human intestinal stem cell (HISC) medium supplemented with 10 μM ROCK inhibitor.

Additional Materials and Methods

Additional details regarding the materials and methods can be found in the Supplementary Materials.

RESULTS

Characterization of mice with conditional knockout of the *Strap* gene in the intestinal epithelium

We have shown that homozygous deletion of STRAP causes lethality at embryonic day E9.5.[13] Therefore, we generated *Strap* conditional knockout mice ($Strap^{\Delta IEC}$) by crossing a mouse line containing floxed alleles of *Strap* ($Strap^{fl/fl}$) with another mouse line expressing Cre-recombinase under control of the *villin* promoter (*Vill*-cre)[14] (Figure 1A–C, Supplementary Figure 1A–G). Despite a substantial reduction in STRAP levels in the epithelium, there was no histologic change or difference in Ki67 staining in either the small intestine (Supplementary Figure 2A) or colon (Supplementary Figure 2B). The length of the small intestine and villi, and the width of crypts remained unchanged (Supplementary Figure 2C). Similarly, the length of the colon and the widths of colonic crypts were unchanged (Supplementary Figure 2D).

Conditional deletion of *Strap* reduces $Apc^{Min/+}$ mediated tumorigenesis

To investigate the importance of STRAP for intestinal tumor formation and survival, we crossed $Apc^{Min/+}$;$Strap^{fl/fl}$ mice with $Strap^{fl/+}$;*Vill*-Cre mice to generate $Apc^{Min/+}$;$Strap^{fl/+}$;*Vill*-Cre ($Apc^{Min/+}$;$Strap^{\Delta IEC/+}$) and $Apc^{Min/+}$;$Strap^{fl/fl}$;*Vill*-Cre ($Apc^{Min/+}$;$Strap^{\Delta IEC}$) mice. We established a cohort containing $Apc^{Min/+}$;$Strap^{fl/fl}$ (n=33), $Apc^{Min/+}$;$Strap^{\Delta IEC/+}$(n=29), and $Apc^{Min/+}$;$Strap^{\Delta IEC}$ (n=20), which were maintained until they showed signs of intestinal illness. Deletion of STRAP increased the survival of $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice as compared with $Apc^{Min/+}$;$Strap^{fl/fl}$ mice with heterozygous mice showing an intermediate delay of intestinal cancer-related death (Figure 1D).

To ensure that the longer life span was due to decreased tumorigenicity, we aged a second cohort of mice with same backgrounds to 110 days. Compared to $Apc^{Min/+}$;$Strap^{fl/fl}$ mice, $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice displayed a reduction in the numbers of tumors (Figure 1F) and in tumor size (Figure 1G) in both small intestines and colons. This suggests that STRAP deficiency suppressed both tumor initiation and progression. Heterozygous deletion of *Strap* also decreased total numbers of tumors (Figure 1F) and average tumor size (Figure 1G). These results indicate that the longer life span was due to decreased tumorigenicity.

Additionally, the effects of STRAP loss were independent of gender (Supplementary Figure 3A and B).

Deletion of *Strap* decreases stemness in adenomas and tumor organoids by downregulating Wnt/β-catenin signaling

To identify pathways mediating the effects of STRAP in the development of intestinal cancers, we established the mRNA expression profiles of intestinal adenomas formed in $Apc^{Min/+}$ mice with or without *Strap* deletion. By applying next-generation sequencing (NGS), we identified 1275 downregulated mRNAs and 838 upregulated mRNAs in *Strap*-deficient $Apc^{Min/+}$ mice compared to $Apc^{Min/+}$ mice (Supplementary Figure 4A). Hallmark gene set (mSigDB) and KEGG analyses showed that genes related to different oncogenic signaling pathways such as Wnt, MAPK, PI3K-Akt, TNFα, and IL-2/STAT5 are downregulated in $Apc^{Min/+}$;$Strap^{\Delta IEC}$ adenomas. Among them, the Wnt/β-catenin pathway is mostly enriched in both data sets (Supplementary Figure 4B and C). Then, we performed gene set enrichment analysis (GSEA) on differentially expressed genes using gene sets from the mSigDB Collections and from previous studies to determine which signatures and pathways were being affected by Strap deletion. GSEA showed that mRNAs characteristic of ISCs, Wnt/β-catenin, MEK/ERK signaling, colon adenomas, and EMT are downregulated upon deletion of Strap (Figure 2A and Supplementary Figure 4D). Strap deletion decreased expression of target genes of the Wnt/β-catenin and MEK/ERK pathways (Figure 2B). These results imply that STRAP is important for maintenance of ISCs and for activation of Wnt/β-catenin and MEK/ERK signaling, which could be involved in colon cancer initiation mediated by APC mutation.

To study the role of STRAP in the regulation of intestinal stem cells, intestinal crypts were isolated from small intestine of $Apc^{Min/+}$;$Strap^{\Delta IEC}$ and $Apc^{Min/+}$;$Strap^{fl/fl}$ and cultured in ENR medium to form enteroids. Enteroids are a type of organoid obtained from the small intestine and the formation of enteroids requires cells from the intestinal crypt stem cell niche.[15] APC mutations accompanied by elevated Wnt signaling promote spheroid-shaped enteroids, as indicated by the absence of crypt buds.[16] Organoids isolated from $Apc^{Min/+}$;$Strap^{fl/fl}$ mice were mostly spheroids consistent with high Wnt activity and maintenance of the stem cell phenotype. In contrast, organoids prepared from $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice exhibited more budding and generated more enteroids by day 8 (Figure 2C and D), which represents more of a differentiated phenotype. Furthermore, we collected organoids at day 8 and analyzed the expression of markers for stemness and differentiation by qRT-PCR. Organoids generated from $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice showed upregulation of several differentiation markers (*Lyz2, Chga, Si, Slc2a,* and *Lzp*) and downregulation of stemness markers (*Lgr5*, *EphB3*) and spheroid markers (*Lgr4, Trop1, Spp1*) compared to those in organoids derived from $Apc^{Min/+}$;$Strap^{fl/fl}$ mice (Supplementary Figure 6A).

To determine if STRAP activates Wnt/β-catenin signaling in vivo, we assessed the nuclear translocation of β-catenin, expression of Survivin, a major target of Wnt/β-catenin; and KI67 in colon tumors collected from 110-day-old $Apc^{Min/+}$;$Strap^{fl/fl}$ and $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice. Colon tumors from $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice showed less β-

catenin nuclear localization, less expression of Survivin, and slower rates of proliferation (decreases in Ki67-positive cells) (Figure 2E, Supplementary Figure 5A–D). Western blot analyses confirmed the downregulation of c-Myc and Survivin in $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice compared to that in $Apc^{Min/+}$;$Strap^{fl/fl}$ mice (Supplementary Figure 6C). QRT-PCR analyses showed that the mRNA levels of several major WNT target genes were downregulated in tumors of $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice compared to those in $Apc^{Min/+}$;$Strap^{fl/fl}$ (Figure 2F). Notably, there was no change in β-catenin at either mRNA or protein levels. Strap-deficient mouse organoids showed downregulation of c-Myc and Survivin at both mRNA and protein levels (Supplementary Figure 6A and B). These results show that *Strap* deletion attenuates the Wnt/β-catenin signaling induced by APC mutation.

STRAP knockout in human colon cancer cell lines reduces activity of the β-catenin/TCF complex.

To determine whether these functions of STRAP in regulating Wnt/β-catenin signaling can be translated into human cell models, we used two human colon cancer cell lines, DLD1 and SW480, which contain APC truncations. For these experiments, we generated STRAP knockdown cell lines using lentivirus and STRAP-KO derivatives employing CRISPR/Cas9 technology. STRAP deletion decreased proliferation of colon cancer cell lines (Supplementary Figure 7A and B). Deletion of *Strap* decreased the expressions of c-Myc, Cyclin D1, and Survivin, three major targets of Wnt/β-catenin signaling in SW480 (Figure 3A) and DLD1 (Figure 3B) cells. We also overexpressed STRAP in colon cancer cells by using adenovirus containing a STRAP expression plasmid and the exogenous protein levels of STRAP were detected by western blots (Supplementary Figure 8A and B).[7] Overexpression of STRAP increased the proliferation of STRAP wild-type and knockout SW480 and DLD1 cells (Supplementary Figure 8C and D). Overexpression of STRAP increased the expression of c-Myc in both cell lines (Supplementary Figure 8E and F). qRT-PCR analyses showed that, in SW480 (Figure 3C) and DLD1 (Figure 3D) cells, *Strap* knockout decreased mRNAs levels of major Wnt/β-catenin target genes, indicating that STRAP regulates the expression of these targets. These results were confirmed in STRAP-overexpressing cells, as STRAP upregulation increased the expression of Wnt target genes (Supplementary Figure 8G and H). Of note, there was no effect on the protein and mRNA levels of β-catenin with alterations in STRAP expression.

We then tested β-catenin/TCF4 complex activity using its signaling reporter TOP-FLASH (containing three copies of β-catenin/TCF4 binding sites) with the negative control FOP-FLASH. The results showed that STRAP knockdown decreased TOP-FLASH activity in both DLD1 (Figure 3E) and SW480 (Figure 3F) cells, but overexpression of STRAP increased TOP-FLASH activity in both cell lines (Supplementary Figure 8I and J). As downregulation of STRAP did not affect β-catenin levels, we analyzed the nuclear accumulation of β-catenin in STRAP knockdown cells by performing Western blot analyses of nuclear and cytoplasmic fractions. STRAP knockdown decreased nuclear β-catenin but increased cytoplasmic levels (Figure 3G and H). To confirm reduction of nuclear β-catenin in intact cells with reduced STRAP functionality, we performed immunofluorescence assays in DLD1 STRAP knockdown cells and SW480 *Strap* knockout cells with their respective controls. Indeed, the nuclear staining of β-catenin (quantitation) was 2 to 3-fold reduced in

STRAP knockdown or knockout cells (Figure 3I and J) (Supplementary Figure 7C and D). Co-immunoprecipitation and Western blot assays further demonstrated that the β-catenin/TCF4 association was reduced in *Strap* KO clones (Figure 3K). Altogether, these data suggest that STRAP promotes the transcription of c-Myc, Cyclin D1, and Survivin through enhancing Wnt/β-catenin signaling in human colon cancer cell lines.

The crosstalk between Wnt/β-catenin and TGFβ signaling has been previously described[17,18] and STRAP has been known to inhibit TGFβ signaling.[19] To determine the role of STRAP in the crosstalk between Wnt/β-catenin and TGF-β signaling, we performed luciferase assays in wild-type and *Strap* knockout DLD1 and SW480 clones using TOP-FLASH reporter. Treatment of these cells with SB431542, a TGF-β receptor kinase inhibitor, had no differential effect on the reporter activity (Supplementary Figure 9A *and* B). These results indicate that STRAP has no effect in the crosstalk between TGF-ß and Wnt/ß-catenin signaling. Conversely, we also analyzed whether STRAP-mediated Wnt/β-catenin signaling has any effect on TGF-β signaling. We transfected the wild-type and *Strap* knockout DLD1 clones with TGF-β/Smad responsive p3TP-Lux reporter, treated with TGF-β in the presence or absence of Wnt/β-catenin signaling inhibitor iCRT3, and performed luciferase assays. There was no difference in the TGF-ß-induced reporter activity either in STRAP KO clones compared to control clones or in iCRT3 treated cells (Supplementary Figure 9C). These results have been confirmed in $Apc^{Min/+};Strap^{\Delta IEC}$ mice as there were no differences in the mRNA levels of TGF-β target genes in adenomas collected from $Apc^{Min/+};Strap^{\Delta IEC}$ mice compared to control mice (Supplementary Figure 9D). Conclusively, *Strap* knockout has no effect on the crosstalk between TGF-β and Wnt/β-catenin signaling.

Strap deletion deactivates the MEK/ERK pathway in an APC-mutated background.

To determine the mechanisms by which STRAP regulates Wnt/β-catenin signaling in $Apc^{Min/+};Strap^{\Delta IEC}$ mice, we utilized reverse-phase protein array (RPPA), a functional proteomic analysis to get a comprehensive overview of signaling pathways. The expression levels of 246 proteins and phosphorylated forms were assessed in intestinal adenomas (Figure 4A). A total of 68 proteins displayed significant enrichment or suppression ($P<0.05$) in $Apc^{Min/+};Strap^{\Delta IEC}$ mice compared to control $Apc^{Min/+};Strap^{fl/fl}$ mice (data not shown). Of these, 21 proteins reached higher significance thresholds ($P < .01$), signifying a high degree of confidence for their involvement in STRAP-mediated tumor development. Phosphorylated forms of ERK1/2, BRAF, SRC, and MTOR were decreased in *Strap*-deficient adenomas. Decreased phosphorylation of ERK1/2 results in reduced activity of MEK/ERK signaling (Figure 4B). This observation is consistent with our RNAseq analysis, which showed reduced expression of target genes in the MEK/ERK pathway in *Strap*-deficient adenomas (Figure 2A). Western blot analysis confirmed that the phosphorylation of ERK1/2 was decreased in intestinal epithelial cells collected from $Apc^{Min/+};Strap^{\Delta IEC}$ mice compared to those from control $Apc^{Min/+};Strap^{fl/fl}$ mice (Figure 4C). Importantly, phosphorylated ERK1/2 levels were lower in both colon tumors and adjacent normal intestinal tissues in $Apc^{Min/+};Strap^{\Delta IEC}$ mice (Figure 4D). Together, these results indicate that STRAP activates MEK/ERK signaling in $Apc^{Min/+}$ mice.

STRAP-mediated activation of MEK/ERK signaling induces transcriptional activity of the β-catenin/TCF4 complex

The oncogenic activation of MEK/ERK signaling in colon cancers activates the canonical Wnt/β-catenin pathway by inducing phosphorylation of LRP6, a co-receptor of Wnt/β-catenin signaling.[5] We hypothesized that, in colon cancers, STRAP activates MEK/ERK signaling, which in turn mediates β-catenin/TCF4-induced signaling. Western blot analysis showed that phosphorylation of ERK1/2 was decreased in STRAP KO colon cancer cells, but there was no change in the phosphorylation of MEK1/2 (Figure 5A and B), suggesting that the effect of STRAP occurred at the step in which MEK1/2 phosphorylates ERK1/2. The total protein levels of MEK1/2 and ERK1/2 were unchanged, indicating that the effect of STRAP KO is posttranslational. We also checked the phosphorylation of LRP6, a target of MEK/ERK signaling. STRAP KO lowered the phosphorylation of LRP6 (Figure 5A and B). These results were confirmed using DLD1 and SW480 knockdown cell lines (Supplementary Figure 10A and B). Therefore, STRAP induces Wnt/β-catenin signaling by phosphorylating and activating LRP6 through ERK1/2 signaling.

STRAP exerts its functions through its scaffolding functions and interaction with other proteins.[19,20] To further study the mechanism by which STRAP regulates MEK/ERK signaling, we performed immunoprecipitation and Western blot assays to assess whether STRAP interacts with components of the MEK/ERK signaling pathway. We transfected 293T cells with MEK1-Flag and STRAP-HA plasmids. Co-immunoprecipitation of STRAP-HA was detected in the anti-Flag immunoprecipitates. Reciprocally, MEK1-Flag was detected in anti-HA immunoprecipitates (Figure 5C). In contrast, ERK2-Flag was not detected in the STRAP immunoprecipitates and vice versa (Figure 5D). Furthermore, endogenous MEK1/2 was co-immunoprecipitated by anti-STRAP antibodies from DLD1 and SW480 cells, and STRAP was co-immunoprecipitated by anti-MEK antibodies (Figure 5E). In contrast, we did not observe any endogenous interaction of STRAP with ERK1/2 (Figure 5F). Thus, the data indicate that STRAP selectively interacts with MEK1/2, and not with ERK1/2.

To test whether STRAP facilitates the association between MEK and ERK, we co-transfected Flag-tagged MEK and ERK together with increasing concentrations of HA-tagged STRAP. As expected, MEK was co-immunoprecipitated by the anti-ERK antibodies. Increasing STRAP overexpression enhanced the interaction between MEK1/2 and ERK1/2 with no change in their protein levels (Figure 5G). Then we performed immunoprecipitation assays with antibodies targeting MEK1/2 and accomplished Western blots for ERK1/2 in STRAP knockout cells to confirm whether endogenous STRAP has any effect on the association between MEK1/2 and ERK1/2. STRAP deletion decreased the endogenous interaction between MEK1/2 and ERK1/2 in SW480 and DLD1 KO cells (Figure 5H and I). These observations provide an important mechanism of how STRAP is involved in the MEK/ERK interaction, which is required for the phosphorylation of ERK1/2 by MEK1/2.

To determine if STRAP regulates target genes of the Wnt/β-catenin pathway specifically through activating the MEK/ERK pathway, we overexpressed STRAP in colon cancer cells and treated them with the MEK/ERK inhibitor U0126[21] and the β-catenin inhibitor iCRT3.[22] Western blot analyses showed that, in DLD1 (Figure 5J) and SW480

(Supplementary Figure 11A) cells, STRAP overexpression by adenoviruses induced levels of Survivin and c-Myc, which was reversed by U0126 treatment. Treatment with iCRT3 decreased the expression of c-Myc and Survivin to a similar extent. Quantitative RT-PCR analyses also indicated that mRNAs of several target genes of Wnt/β-catenin signaling were upregulated in STRAP-overexpressing DLD1 and SW480 cells (Supplementary Figure 11B and C); these effects were reduced by U0126 and iCRT3 treatments. To confirm this at the transcriptional level, we transfected cells with a TOPFLASH reporter and performed luciferase assays. β-Catenin/TCF4 transcriptional activity in DLD1 (Figure 5K) and SW480 cells (Supplementary Figure 11D) was enhanced by STRAP, but these effects were abolished by U0126 and iCRT3 treatments. These data indicate that, in colon cancers, STRAP-regulated transcriptional activity of the nuclear β-catenin/TCF complex is mediated through MEK/ERK signaling.

STRAP knockdown suppresses the proliferation of human colon tumor organoids

We evaluated the above-mentioned functions of STRAP in the growth of human colon tumor organoids in 3D culture. To this end, we generated and cultured several organoid lines derived from human colon tumors.[12] We knocked down STRAP in these organoids using lentivirus and puromycin selection.[23] Organoid-forming assay (Supplementary Figure 12A) showed that STRAP downregulation decreased the proliferation of two organoid lines P07132016T (Figure 6A and Supplementary Figure 12B) and F131T (Figure 6B and Supplementary Figure 12C). STRAP knockdown cells formed smaller organoids (Supplementary Figure 12D and E) compared to parental cells. We subcutaneously injected shSTRAP and shCtrl single cells derived from tumor organoids into immunodeficient mice. STRAP knockdown significantly decreased the tumor growth in the nude mice (Figure 6C and D). Western blot analyses showed that STRAP knockdown decreased the protein expression of Wnt targets such as Survivin, Cyclin D1, and c-Myc in both P07132016T (Figure 6E) and F131T (Figure 6F) organoids. Importantly, we also observed that STRAP knockdown decreased phosphorylation of ERK1/2. In addition, STRAP downregulation by shRNAs in these two organoid lines as well as other organoid lines, F114T and F130T, decreased the mRNA expression of Wnt/β-catenin target genes (Supplementary Figure 13A–D). To test the specificity of these effects of STRAP, we overexpressed it in these organoids by using adenovirus expressing STRAP. Overexpression of STRAP significantly increased the proliferation of P07132016T and F131T organoid lines (Supplementary Figure 14A and B). Western blot analyses showed that overexpression of STRAP induced the expression of Cyclin D1, c-Myc, and Survivin, confirming the regulatory role of STRAP on Wnt/β-catenin targets in these organoids (Figure 6G and H). This is further supported by STRAP-induced mRNA expression of Wnt/β-catenin target genes (Supplementary Figure 14C and D).

To corroborate our murine data with human, we used normalized RNA-seq gene expression data from the Cancer Genome Atlas (TCGA) project of Colon Adenocarcinoma (COAD) downloaded from the UCSC Xena web portal. Linear regression analyses show that STRAP expression positively correlates with mRNA expressions of Wnt/β-catenin target genes (Supplementary Figure 15A). Furthermore, samples are grouped into STRAP-high and -low expression with the median as the cutoff, and 3309 differentially expressed genes (DGE) are identified. Hallmark gene set (mSigDB) and KEGG analyses of genes upregulated

in the STRAP-high expression group show that genes in Wnt/β-catenin, mTOR, TNFα, and IL-2/STAT5 pathways are significantly enriched (Supplementary Figure 15B and C). Furthermore, statistical significance of overlapping genes (169) between human and mouse data sets is confirmed by a hypergeometric test (Supplementary Figure 16A). GSEA of DGEs shows that the intestinal stem cells, Wnt/β-catenin, and colon adenoma signatures are highly enriched in STRAP-high group (Supplementary Figure 16B). To explore the heterogeneity of STRAP expression across single-cell transcriptome profiles, we utilized the scRNA-seq data of 29 primary colorectal tumors.[24] k-Means clustering and tSNE projections were used to visualize these data for unique clustering. STRAP-high and - low/negative-expression were used to categorize these clusters. The top 10% of Strap-expressing cells were classified as the high group, while the bottom 10% were considered the low/negative group (Supplementary Figure 17A). Differential gene expression analysis was then performed, and we identified 924 upregulated mRNAs in STRAP-high cells (fold change >2, $p<0.01$). Importantly, 97 genes upregulated in STRAP-high cells were found to be downregulated in adenomas collected from $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice. Major Wnt target genes were found in the overlapping genes (Supplementary Figure 17B). Hallmark gene set (mSigDB) and KEGG analysis of overlapping genes showed that genes are significantly enriched in Wnt/β-catenin, PI3K, MAPK, MTORC1, and TNFα signaling pathways (Supplementary Figure 17C and D). Taken together, these results indicate that STRAP-associated gene signatures related to Wnt/β-catenin signaling are conserved across murine and human colon cancer.

Inactivated APC and activated Wnt/β-catenin signaling upregulate STRAP expression

Using GEO2R of PubMed (http://www.ncbi.nlm.nih.gov/geo/geo2r/), we analyzed changes in mRNA levels of *Strap* from Gene Expression Omnibus datasets of colon tumors isolated from $Apc^{Min/+}$ and azoxymethane (AOM)-treated mice (GSE accession No. GSE5204).[25] The search results showed that *Strap* mRNA levels were elevated in tumors of $Apc^{Min/+}$ and AOM-induced mice compared to normal mouse colon tissues (Supplementary Figure 18A) with corresponding hyperactivated Wnt signaling. These data provided the first evidence that STRAP expression is associated with aberrant activation of Wnt/β-catenin signaling in colon tumors. Similarly, we observed upregulation of *Strap* mRNA in colon tumors collected from $Apc^{Min/+}$ mice compared to epithelial cells collected from WT Apc mice (Supplementary Figure 18B). The upregulation of STRAP protein in the tumors of three $Apc^{Min/+}$ mice was confirmed by Western blot analyses (Figure 7A). To determine if STRAP is a target of Wnt/β-catenin signaling, we transfected human colon cancer cells (RKO) with a *Strap*-promoter luciferase reporter (−2087 to +93) and treated them with Wnt3A conditioned medium. Wnt3A increased activity of the *Strap* promoter (Figure 7B). Additionally, treatment of cells with Wnt3A increased the expression of Strap in both mRNA (Supplementary Figure 18C) and protein levels (Figure 7C). These results raised the possibility that the β-catenin/TCF4 complex could bind to the *Strap* promoter. Genomatix software suite (www.genomatix.de) revealed several putative TCF/LEF binding sites within the *Strap* promoter. We performed ChIP analysis using antibodies to β-catenin with primers amplifying four regions (ST1–4) of the promoter between 1920 and −752 bp upstream from the transcriptional start site. ChIP analyses identified three functional TCF/LEF binding sites from −1916 to −1186 bp of the *Strap* promoter (Figure 7D and E). To confirm

the involvement of these sites in β-catenin/TCF4 complex-mediated induction in *Strap* promoter activity, we generated luciferase constructs containing the Strap promoter with deletions at these TCF/LEF binding sites. We transfected RKO cells with these WT and mutant constructs and performed luciferase assays. Deletions at all three major sites strongly decreased the STRAP promoter activity induced by Wnt3A (Figure 7F), whereas deletion of each individual site had intermediate effects. These results have been confirmed with DLD1 and SW480 cell lines (Supplementary Figure 19A and B).

To determine whether inhibition of Wnt/β-catenin signaling suppresses the expression of STRAP, we treated colon cancer cells with iCRT3 and ICG001, two β-catenin/TCF4 inhibitors,[22] and analyzed the expression of STRAP. In two cell lines, iCRT3 treatment reduced the expression of STRAP at both protein (Figure 7G and H, *top*) and mRNA levels (Supplementary Figure 18D). Similar observations were made with the other inhibitor, ICG001 (Figure 7G and H, *bottom* and Supplementary Figure 18E). To test whether this occurs at the transcriptional level, we transfected human colon cancer cells (RKO) with a *Strap* promoter reporter and treated them with iCRT3 and ICG001. The β-catenin inhibitors decreased reporter activity (Supplementary Figure 18E and F). To investigate the effects of β-catenin inhibitors on known targets of STRAP based on our previous study,[9] we analyzed the mRNA levels of HES1 and JAG2 in iCRT3-treated CRC cells. The results showed that iCRT3 treatment significantly decreased the expression of these genes. To confirm that these effects are through STRAP, we overexpressed STRAP in iCRT3 treated cells by adenovirus. Restoring the expression of STRAP in inhibitor-treated cells significantly rescued the expression of these genes (Supplementary Figure 19C *and* D). Altogether, our findings reveal that STRAP is a novel target of Wnt/β-catenin signaling and it is important for inactivated APC- and Wnt/β-catenin-induced stemness and tumor initiation through a complex feed-forward loop.

DISCUSSION

Here, we have shown that STRAP is required for efficient cell proliferation and tumorigenesis following APC mutation. Conditional deletion of Strap expression in the intestines of $Apc^{Min/+}$ mice extended their survival by 80 days and decreased the development and formation of intestinal adenomas. As APC mutations were found in approximately 80% of sporadic colon tumors, and STRAP is upregulated in more than 50% adenomas, these results contribute to an important understanding of intestinal cancer development and progression. Based on GSEA analysis using RNA-seq data in intestinal adenomas collected from $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice, we found that Strap deletion resulted in regulation of the intestinal stem-like gene signature, colon adenoma and Wnt/β-catenin signaling target gene signature. Previous studies suggested that ISCs represent the cells of origin for colon cancer.[26] Therefore, the effects of *Strap* deletion are presumably due to the reduced ISC stemness. Deletion of *Strap* leads to downregulation of target genes of Wnt/β-catenin signaling with crucial functions. These include ISC markers such as *Lgr5, Foxq1, Tnfrsf19, EphB3,* etc. The reduced ISC signature observed in *Strap*-deficient adenomas contribute to the maintenance of stemness and suppression of differentiation in ISCs leading to the onset of intestinal tumorigenesis. Importantly, analysis of human datasets showed that STRAP-related signaling pathways and gene signatures are conserved

between humans and mice, emphasizing the clinical correlation of data collected from *Strap* conditional knockout mice model. The results from this study explain why upregulation of STRAP is associated with worse survival following adjuvant therapy and why patients with low STRAP expression benefit from the treatment.[27] This study also suggests that this preclinical mouse model could be used for future drug development and targeting of STRAP- specific signatures and pathways.

STRAP binds with GSK-3β and reduces the phosphorylation, ubiquitination, and degradation of β-catenin through preventing its binding to GSK-3β in the destruction complex containing WT APC. Therefore, the effect of STRAP on β-catenin stabilization is lost in colon cancer cell lines with APC truncation/mutation.[28] Here, we found that STRAP promotes Wnt signaling in the context of APC mutation through a different mechanism, which is independent of β-catenin stabilization. Colon tumors from $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice displayed a decrease in β-catenin nuclear localization. The expressions of targets of Wnt/β-catenin were lower in $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice, but there was no change in β-catenin expression levels. The results from these experiments with mice are verified by our findings with human colon cancer cell lines and organoids that downregulation of STRAP impaired Wnt/β-catenin activity and decreased the expression of β-catenin downstream target genes.

Another novel observation is that STRAP regulates Wnt/β-catenin signaling through activation of the MEK/ERK pathway in an Apc-mutated background. A recent report showed that the oncogenic activation of MEK/ERK signaling in colon cancers increases LRP6 (a co-receptor of Wnt/β-catenin signaling) phosphorylation that results in nuclear localization and transcriptional activity of β-catenin without affecting its expression.[5] Functional proteomic analyses of intestinal epithelial cells from $Apc^{Min/+}$;$Strap^{\Delta IEC}$ and control mice showed that the level of phosphorylation of ERK1/2, an effector of MEK/ERK signaling, was lower upon *Strap* deletion. Importantly, immunohistochemistry and Western blot analyses indicated that the levels of phosphorylated ERK1/2 were decreased in both colon tumors and adjacent tissues in $Apc^{Min/+}$;$Strap^{\Delta IEC}$ mice. We confirmed these effects of STRAP deficiency on the MEK/ERK pathway in human cell lines and organoids derived from colon tumors of patients. STRAP deletion in colon cancer cells also decreased the phosphorylation of LRP6. Mechanistically, STRAP recruits MEK1/2 and increases the binding between MEK1/2 and ERK1/2 for phosphorylation of ERK1/2. *Strap* knockout in human colon cancer cell lines impairs the interaction between MEK1/2 and ERK1/2, resulting in a decrease in the phosphorylation of ERK1/2. Thus, the results show that STRAP induces Wnt/β-catenin signaling by activating the MEK/ERK pathway and inducing LRP6 phosphorylation.

Our results also show that conditional knockout of *Strap* in $Apc^{Min/+}$ mice and in human cell lines and organoids downregulates the expression of Wnt signaling targets including c-Myc, Survivin, Cyclin D1, Lgr5, and Ephb3. Previous studies established c-Myc as the mediator of early stages of neoplasia following APC loss as c-Myc deletion rescues all the phenotypes following APC loss and thus nearly blocks tumor formation.[29] Therefore, it is possible that STRAP-mediated regulation of c-Myc and other Wnt/β-catenin targets is involved in APC mutation-induced tumor initiation. Although previous results suggest that c-Myc is a target for the treatment of cancers with APC mutations, given the difficulty in targeting a

transcription factor such as c-Myc, it may be more effective to inhibit upstream regulators of c-Myc and other Wnt targets. In this respect, it is necessary to identify inhibitors that block the functions of STRAP and abolish its effects on the expression of c-Myc and other Wnt/β-catenin targets. Although deletion of *Strap* suppresses tumor initiation and development following APC mutation, it does not show any toxic effects on the normal intestinal tissue. Altogether, these findings suggest that STRAP is a potential therapeutic target for the treatment of colon cancer, especially for cancers with APC mutation.

Although STRAP is involved in oncogenic signaling, it remains unknown how it is regulated in cancers. Here, we have established a new mechanism that STRAP is a target of the Wnt/β-catenin pathway. Activation of Wnt/β-catenin signaling and APC inactivation increased expression of STRAP. ChIP assays identified TCF/LEF binding sites in the *Strap* promoter through which Wnt signaling promoted *Strap* gene expression. Treatment of cells with inhibitors of TCF/β-catenin complex downregulated the expression of STRAP. Thus, our findings reveal that STRAP is a target of Wnt/β-catenin signaling and that it has crosslinks between the MEK/ERK and Wnt/β-catenin pathways. Therefore, the contribution of STRAP to colon carcinogenesis is linked to the activities of an altered Wnt signaling and the MEK/ERK pathway. In this manner, the two signaling pathways maintain the stemness phenotype induced by APC mutation through a feed-forward mechanism STRAP/MEK-ERK/Wnt-β-catenin/STRAP in which STRAP is a critical factor.

In conclusion, this study reveals an unknown function of STRAP in mouse and human models in mediating mutated APC-induced intestinal tumor initiation and progression by activating the Wnt/β-catenin pathway. Another novel observation is that STRAP-induced activation of the MEK/ERK pathway is involved in promoting β-catenin signaling and its target gene expression. We also showed that inactivation of APC and activation Wnt/β-catenin signaling increased the expression of STRAP transcriptionally. Therefore, future studies will determine whether STRAP is a molecular target for the treatment of colon cancers of patients with APC mutation and Wnt/β-catenin activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

CRC	colorectal cancer

ISC	intestinal stem cell
ΔIEC	intestinal epithelial knockout
IACUC	Institutional Animal Care and Use Committee
RPPA	reverse-phase protein array
GSEA	gene set enrichment analysis
MSigDB	molecular signatures database
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
shRNA	short hairpin RNA
TCGA	The Cancer Genome Atlas

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"Author names in bold designate shared co-first authorship"

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What you need to know:

Background and Context:

Mutations in the *Apc* gene is a critical early event in intestinal tumor development. We investigate how STRAP is involved in Apc mutation-induced intestinal tumor initiation and progression using mouse and human models.

New findings:

This study identified a novel feed-forward loop between STRAP and Wnt/β-catenin signaling through activating the MEK/ERK pathway to promote intestinal tumorigenesis. STRAP is a target of TCF4/β-catenin signaling and conditional deletion of *Strap* results in a dramatic decrease in intestinal tumorigenesis mediated by APC deletion.

Limitations:

The authors didn't evaluate the effect of STRAP deficiency in patient-derived xenografts (PDX) of intestinal cancer, which might provide more insight into the application of targeting STRAP in patients.

Impacts:

These in vitro and in vivo studies provide a preclinical proof-of-principle that STRAP is a potent molecular target for the treatment of colon cancer of patients with APC mutation and Wnt/ß-catenin activation.

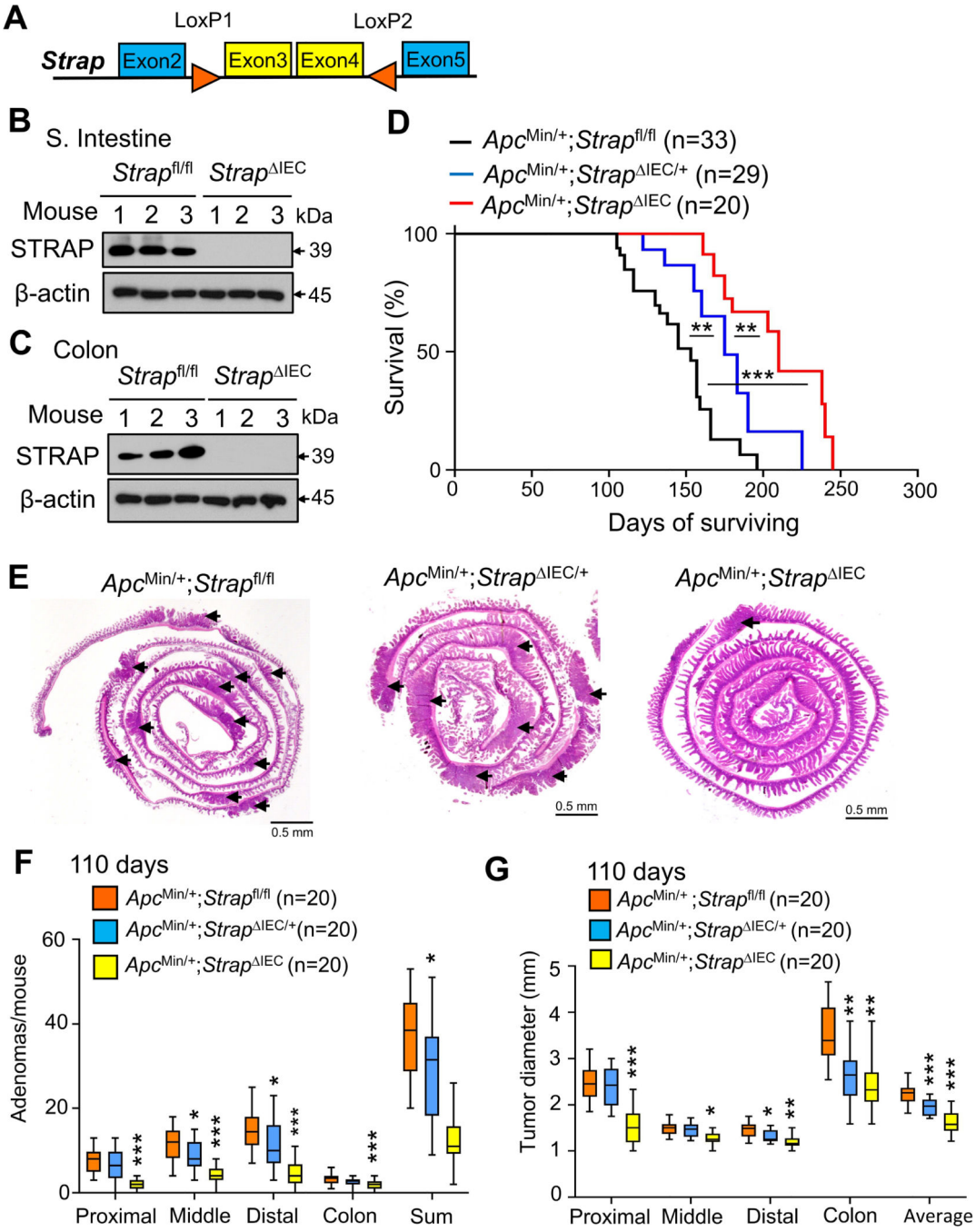

Figure 1. Deletion of *Strap* in *Apc*$^{Min/+}$ mice prolongs survival and decreases the frequency of adenomas.

(*A*) Scheme for *Strap* gene exons 3 and 4 floxed with loxP1 and loxP2 sites. (*B, C*) The small intestine (*B*) and colon (*C*) tissues from the *Strap*$^{fl/fl}$ and *Strap*$^{\Delta IEC}$ mice were tested for STRAP expression by Western blot analyses with β-actin as a loading control. (*D*) Kaplan–Meier survival analysis of *Apc*$^{Min/+}$ mice with indicated genotypes and numbers of mice. The results were subjected to a log-rank test. (*E*) Representative sections through rolls of the small intestine (jejunum) stained with hematoxylin and eosin (HE). Scale bar, 500 μm; Arrows for polyps. (*F, G*) Quantification of adenoma numbers/mouse (*F*) and tumor

diameters (*G*) in the intestines of 10 male and 10 female 120-day-old *Apc*$^{Min/+}$ mice per indicated genotype are shown. The box plot shows tumor number and size by the location. Differences were analyzed by two-sided Kruskal-Wallis test followed by Mann–Whitney test. **P* < .05, ***P* < .01, ****P* < .001.

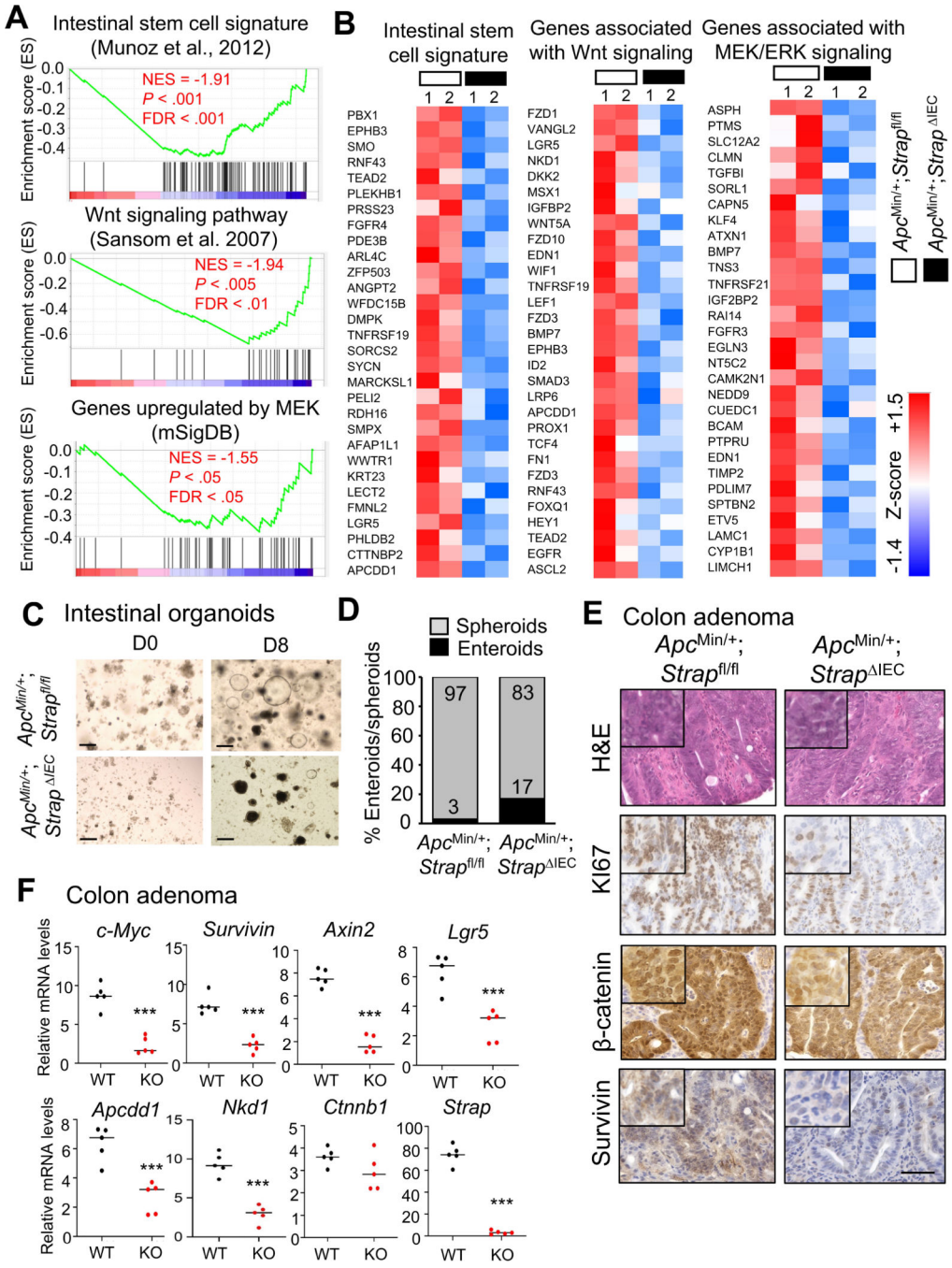

Figure 2. *Strap* deficiency decreases intestinal stem cells by impairing Wnt/β-catenin signaling.

(*A*) GSEA comparing gene expression profiles from *Apc*$^{Min/+}$;*Strap*$^{fl/fl}$ and *Apc*$^{Min/+}$;*Strap*$^{\Delta IEC}$ adenomas from 120-day-old mice with Lgr5-positive stem cells, Wnt/β-catenin signaling, and a gene signature of over-activation of MEK/ERK signaling. NES: normalized enrichment score, FDR: false discovery rate. (*B*) Heatmaps of selected, differentially expressed mRNAs ($P < .05$) from intestinal stem cell gene signatures, Wnt/β-catenin signaling and MEK/ERK signaling gene signatures analyzed in (*A*). Colors indicate relative expression values from minimum (green) to maximum (red) for each

RNA sample per differentially regulated mRNA. (*C*) Phase contrast micrographs from 8 day-cultures after plating crypts isolated from indicated mice. Scale bar, 100 μm. (*D*) Graph showing the percentage of enteroids (black) versus spheroids (gray) at day 8 cultured in triplicate from three mice per genotype. (*E*) Adenomas from $Apc^{Min/+};Strap^{fl/fl}$ and $Apc^{Min/+};Strap^{\Delta IEC}$ littermates were immunostained to detect β-catenin, Survivin, and KI67 by H&E. Magnification, 20X, and 40X for the insets. Scale bar, 100 μm. Data are representative of three mice per group. (*F*) Colon tumor tissues collected from $Apc^{Min/+};Strap^{fl/fl}$ (n=5) and $Apc^{Min/+};Strap^{\Delta IEC}$ (n=5) mice were used for qRT-PCR analyses with primers for various Wnt target genes. Results were subjected to an unpaired, two-tailed Student's t-test. ***$P < .001$.

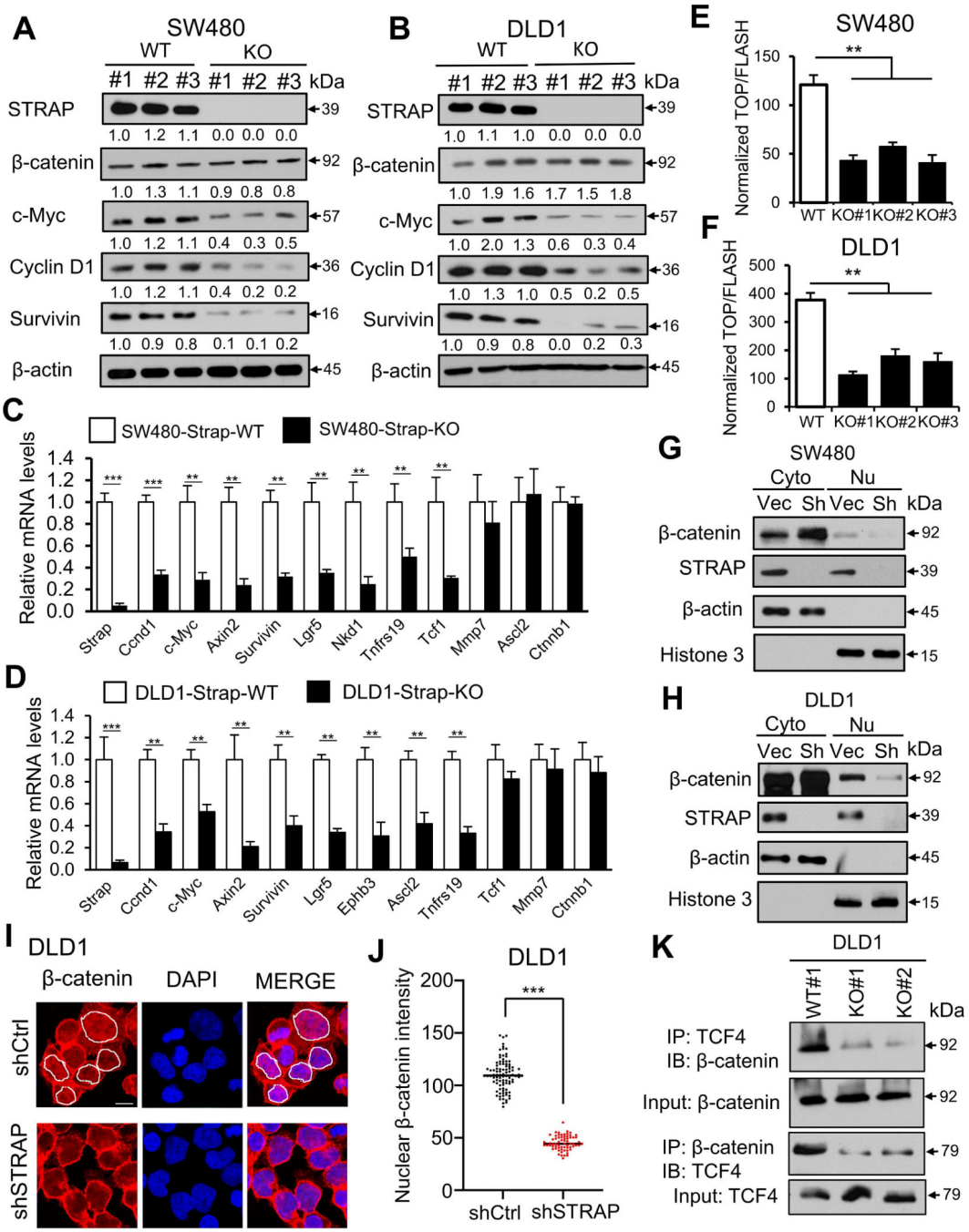

Figure 3: *Strap* knockout in CRC cells reduces the activity of Wnt/β-catenin signaling.

(*A, B*) Expression levels of indicated proteins in STRAP knockout (KO) and control (WT) clones derived from SW480 (*A*) and DLD1 (*B*) cell lines were analyzed by Western blot analyses. (*C, D*) mRNA levels of Wnt target genes in *Strap* knockout (STRAP-KO, n=3) and control (STRAP-WT, n=3) clones derived from SW480 (*C*) and DLD1 (*D*) cells as indicated were determined by qRT-PCR and shown as relative fold changes using GAPDH as a loading control. Significance levels were determined by Student's t test. $^{**}P < .01$, $^{***}P < .001$. (*E, F*) TOPFLASH/FOPFLASH luciferase reporter constructs were transfected into

SW480 (*E*) or DLD1 (*F*) control and *Strap* KO cells. Values were normalized to FOPFLASH luciferase activity and plotted. (*G, H*) Western blot analyses of β-catenin in nuclear and cytoplasmic fractions extracted from control and STRAP knockdown SW480 (G) and DLD1 (H) cells were performed. Complete separation of proteins in two compartments was verified by assessment of histone 3 (nucleus) and β-actin (cytoplasm). (*I*) STRAP knockdown and control DLD1 cells were fixed for immunofluorescence and stained for β-catenin protein (red) and DNA (blue). Scale bar 20 μm. (*J*) The background corrected nuclear signal for each cell was quantified by drawing regions of interest around nuclei (identified by DAPI) on maximum intensity projection of 3 consecutive optical sections of 1.5 micron each which was done by Zen Desk 3.2 imaging software. Five random fields were counted. (*H)* TCF-4 or β-catenin was immunoprecipitated from DLD1 cells with anti-TCF-4 or anti-β-catenin antibodies. Immune complexes were then analyzed by immunoblotting with antibodies against β-catenin or TCF-4 proteins. IgG was used as a negative control. All experiments were repeated at least 3 times independently. The relative densities of bands are shown under immunoblots after normalization to the levels of β-actin.

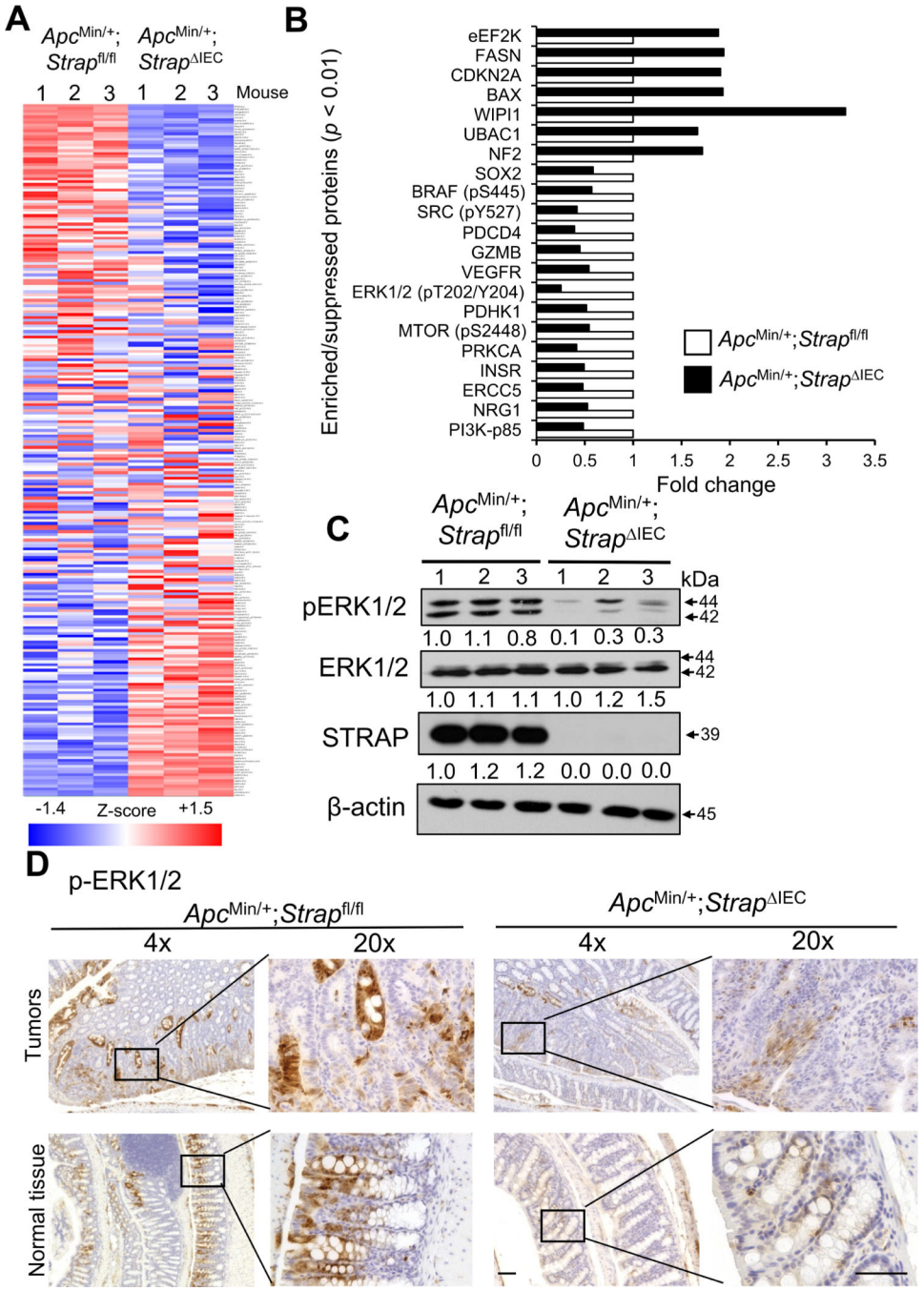

Figure 4. Functional proteomic analysis of signaling pathways regulated by STRAP.

(*A*) Heatmap of a Reverse Phase Protein Array (RPPA) comparing expression levels of 246 proteins in adenomas collected from *Apc*$^{Min/+}$;*Strap*$^{\Delta IEC}$ mice with those from *Apc*$^{Min/+}$;*Strap*$^{fl/fl}$ mice. (*B*) Proteins displaying highly significant enrichment or suppression ($P < .01$) in adenomas collected from *Apc*$^{Min/+}$;*Strap*$^{\Delta IEC}$ mice compared with those from *Apc*$^{Min/+}$;*Strap*$^{fl/fl}$ mice. (*C*) Proteins were analyzed by Western blotting for the phosphorylation of ERK1/2 and for total ERK1/2, and STRAP levels in adenomas collected from *Apc*$^{Min/+}$;*Strap*$^{\Delta IEC}$ mice were compared with those from *Apc*$^{Min/+}$;*Strap*$^{fl/fl}$ mice. (*D*)

Adenomas and normal tissues collected from $Apc^{Min/+};Strap^{fl/fl}$ and $Apc^{Min/+;}Strap^{\Delta IEC}$ mice were immunostained to detect phosphorylated ERK1/2. Low-magnification (4x) images and partial high-magnification (20X) images are shown; scale bar, 100 μm. Data are representative of three mice per group.

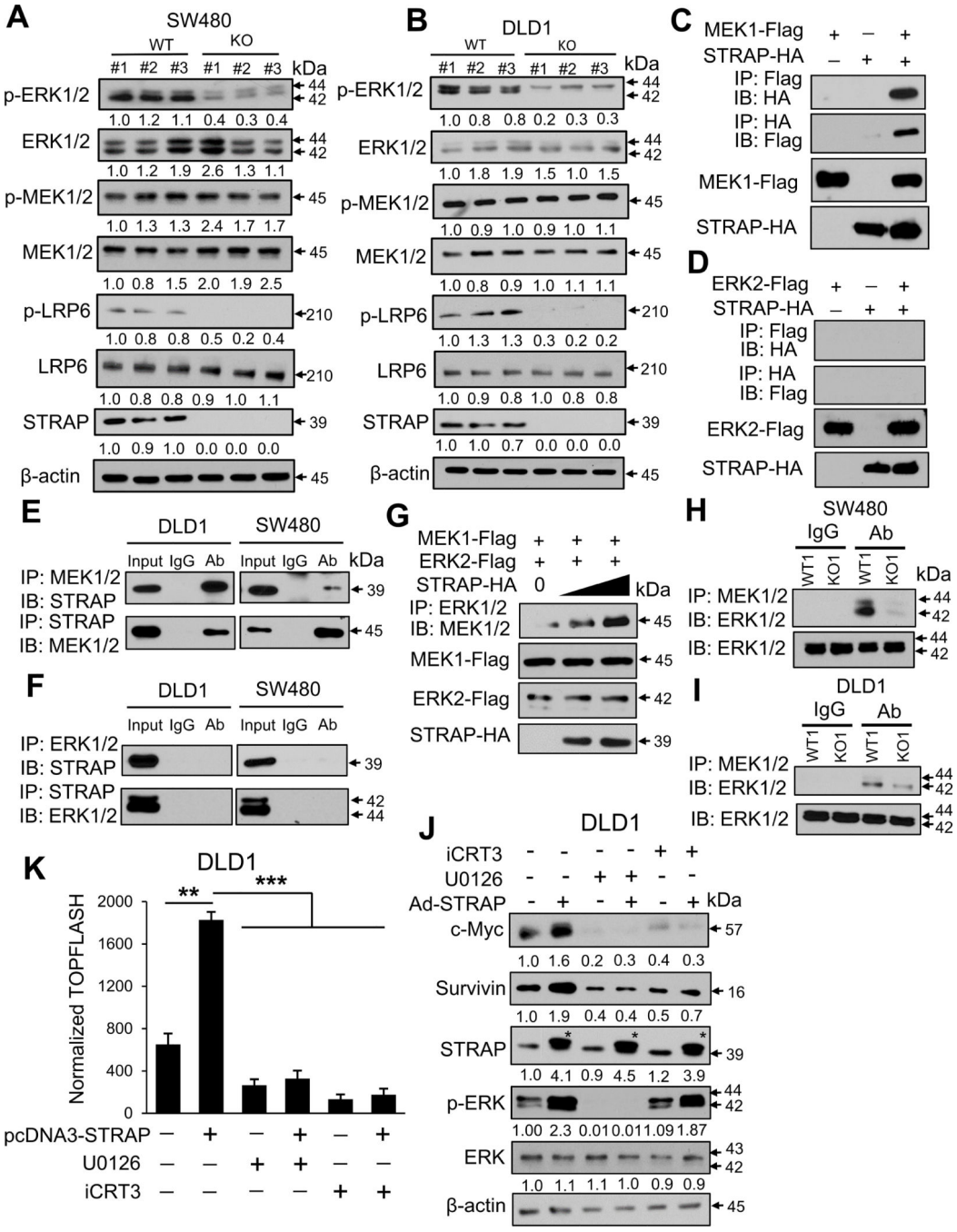

Figure 5. STRAP-induced MEK/ERK signaling promotes β-catenin transcriptional activity.

(*A, B*) Proteins were analyzed by Western blotting for the expression of ERK1/2, MEK1/2, LRP6, and their phosphorylated forms as well as for STRAP in *Strap* knockout (KO) and control (WT) clones derived from SW480 (*A*) and DLD1 (*B*) cell lines. (*C, D*) 293T cells were co-transfected with MEK1-Flag and STRAP-HA plasmids (*C*) and ERK2-Flag and STRAP-HA (*D*) as indicated. Lysates were subjected to reciprocal immunoprecipitation with either anti-Flag or anti-HA antibodies and analyzed by Western blotting with anti-HA or anti-Flag antibodies, respectively. (*E, F*) MEK (*E*) or ERK1/2

(*F*) was immunoprecipitated with anti-MEK antibodies from lysates of indicated cells. Immune complexes were then analyzed by immunoblotting with anti-STRAP antibodies (top panels). STRAP was immunoprecipitated with anti-STRAP antibodies from lysates of indicated cell lines. Immune complexes were then analyzed by immunoblotting with anti-MEK antibodies (*E*) and anti-ERK antibodies (*F*) (bottom panels). (*G*) 293T cells were co-transfected with Flag-tagged MEK1 and ERK2 together with increasing concentrations of STRAP-HA plasmid. Cell lysates were used for immunoprecipitation with anti-ERK1/2 antibodies. Co-immunoprecipitated MEK1 was detected by Western blotting. (*H and I*) MEK was immunoprecipitated with anti-MEK antibodies from lysates of SW480 (H) or DLD1 (I) cells. Immune complexes were then analyzed by Western blotting with anti-ERK1/2 antibodies. (*J*) STRAP-overexpressing cells with adenovirus were treated with 20 μM U0126 or 20 μM iCRT3 for 24 hours, and indicated proteins were analyzed by Western blotting. *, exogenous STRAP. The relative intensities of bands normalized to β-actin are shown. (*K*) Cells were co-transfected with full-length STRAP plasmid and TOPFLASH/FOPFLASH luciferase reporter constructs. After 24 hours of transfection, cells were treated with U0126 or iCRT3 for another 24 hours. TOPFLASH reporter activity normalized to FOPFLASH activity is plotted. Significance levels were determined by Student's t test. ** $P < .05$, *** $P < .01$.

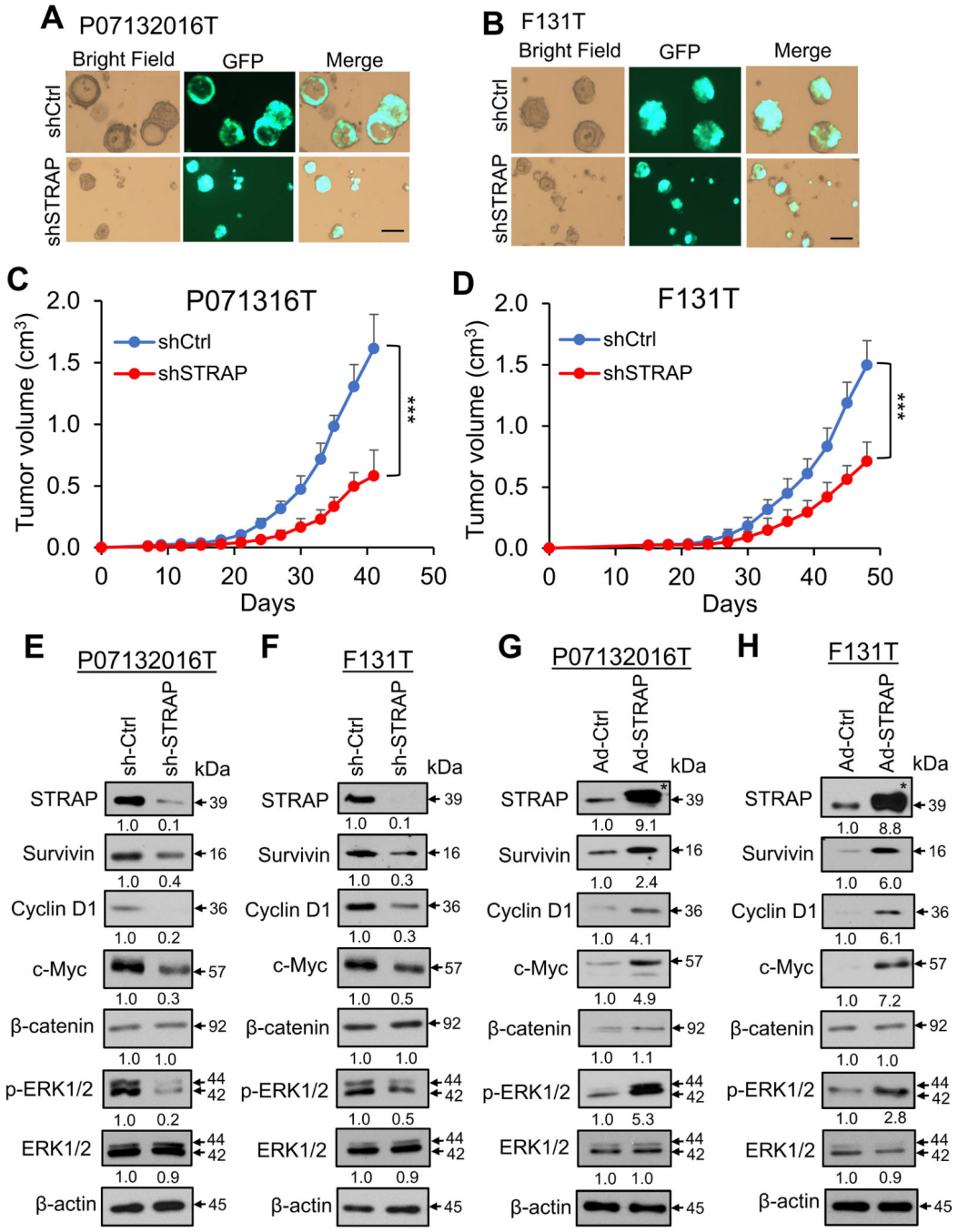

Figure 6. STRAP knockdown inhibits the growth of human colon tumor organoids.

(*A*, *B)* Representative images of organoids, P07132016T (*A*) and F113T (*B*) derived from colon tumors of patients are shown. STRAP knockdown organoids were plated in Matrigel at a density of 500 cells per well in 24-well plates and were cultured for 14 days. Magnification, 20X; Scale bar, 200 μm. (*C*, *D*) STRAP knockdown and control organoids were dissociated using TrypLE into single cells and subcutaneously injected to the flank regions of nude mice. ***$P < .01$. (*E-H*) Expression levels of indicated proteins in STRAP knockdown and control organoids (*E* and *F*) or STRAP overexpressed and control organoids

(*G* and *H*) were analyzed by Western blot analyses. *, exogenous STRAP. All experiments were repeated twice independently. Relative intensities of bands normalized to β-actin are shown.

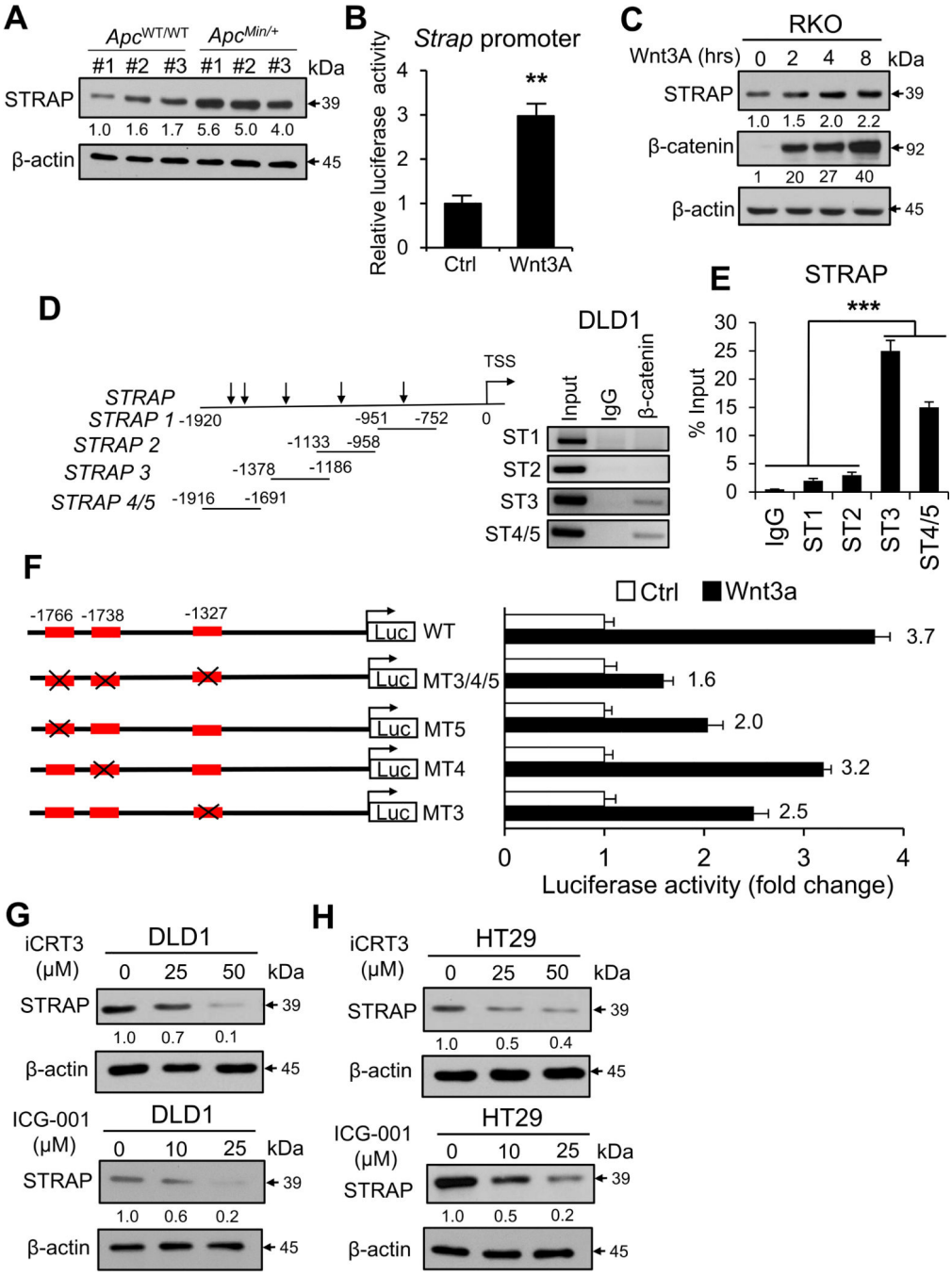

Figure 7: Activation of Wnt/β-catenin signaling induces STRAP expression.

(*A*) Western blot analyses for STRAP in three mice in each group with β-actin as control are shown. (*B*) The *Strap* promoter luciferase reporter (−2087 to +93) was transfected into RKO cells. Cells were treated with Wnt3A containing conditioned media for 24 hours, and normalized luciferase activity was analyzed. (*C*) Western blot analyses of STRAP and β-catenin proteins from RKO cells treated with Wnt3A conditioned media for indicated times. (*D*) β-Catenin binds to the *Strap* promoter. PCR primers were used to amplify four regions containing 5 TCF/LEF binding sites of the promoter between −1920 and −752

bp upstream of the transcription start site (TSS) following ChIP with β-catenin antibody. PCR products were analyzed on a 1% agarose gel. (*E*) PCR products precipitated with the antibodies were analyzed by qPCR as a percentage of the input. Results are the means ±s.d. of at least three separate experiments. $^{**}P < .01$, $^{***}P < .001$. (*F*) Luciferase assay in RKO cells treated with control medium or Wnt3A-conditioned medium using plasmid constructs containing the upstream promoter region of *Strap* with either the wild-type or progressively deleted TCF/LEF binding sites (3–5). (*G, H*) DLD1 (G) or HT29 cells (H) were treated with various concentrations of iCRT3 and ICG-001 for 24 hours, and downregulation of STRAP at the protein level was demonstrated by Western blotting. The relative intensities of bands normalized with β-actin are shown. All experiments were repeated at least 3 times independently.