YAP–IL-6ST autoregulatory loop activated on APC loss controls colonic tumorigenesis

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Loss of tumor suppressor adenomatous polyposis coli (APC) activates β-catenin to initiate colorectal tumorigenesis. However, β-catenin (*CTNNB1*) activating mutations rarely occur in human colorectal cancer (CRC). We found that APC loss also results in up-regulation of IL-6 signal transducer (IL-6ST/gp130), thereby activating Src family kinases (SFKs), YAP, and STAT3, which are simultaneously up-regulated in the majority of human CRC. Although, initial YAP activation, which stimulates *IL6ST* gene transcription, may be caused by reduced serine phosphorylation, sustained YAP activation depends on tyrosine phosphorylation by SFKs, whose inhibition, along with STAT3-activating JAK kinases, causes regression of established colorectal tumors. These results explain why *APC* loss is a more potent initiating event than the mere activation of *CTNNB1*.

colorectal cancer | adenomatous polyposis coli | IL-6ST/gp130 | YAP | STAT3

Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths in males and third in females (1). Although early CRC (stages I and II) can be controlled by surgical resection accompanied by chemotherapy, advanced CRC (stages III and IV) is associated with high mortality rates (2). In such patients, targeted therapies, including EGF receptor and angiogenesis inhibitors, prolong survival only by several months (3). Furthermore, only a small fraction of CRC patients, whose tumors are mismatch repair-deficient, respond positively to immunotherapy (4). Undoubtedly, the future of CRC therapy depends on identification of novel and ubiquitously acting oncogenic mediators whose targeting will cause tumor regression in most patients.

CRC pathogenesis often follows a well-defined multistep genetic pathway that leads to sequential activation of several key signal transducers and transcription factors (5, 6). The most frequent tumor-initiating event is inactivation of the *adenomatous polyposis coli* (*APC*) tumor suppressor, resulting in stabilization and irreversible activation of β-catenin (*CTNNB1*) (7). The much higher frequency of *APC* loss-of-function mutations relative to *CTNNB1* gain-of-function mutations (8) suggests that APC loss leads to activation of at least one more oncogenic pathway that remains to be identified. Subsequent mutations that disrupt the tumor suppressive p53 and TGF-β pathway and activate Ras-MAP kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling mediate malignant progression (9). So far, however, MAPK and PI3K inhibition had only a marginal impact on survival in advanced CRC patients (10) and restoration of APC, p53, and TGF-β tumor suppressor activity remains an elusive goal.

CRC pathogenesis is enhanced by inflammation (11). In the case of inflammatory bowel diseases, which greatly increase CRC risk, inflammation is caused by autoimmunity (12). However, even sporadic CRC, initiated by APC loss, depends on "tumor-elicited inflammation," which originates from localized loss of the intestinal epithelial barrier (13). Barrier disruption results in invasion of early benign tumors (adenomas) by components of the colonic microbiota, which activate IL-23–synthesizing myeloid cells and expand tumor-resident IL-17–producing T lymphocytes (13).

Significance

Current therapy for advanced colorectal cancer (CRC) is unsatisfactory and CRC remains a major cause of cancer-related deaths. Thus, novel and ubiquitously acting oncogenic mediators that are amenable to pharmacological targeting need to be identified. We found that loss of *adenomatous polyposis coli* (*APC*), which is mutated in the majority of human CRC, results in up-regulation of the signaling protein IL-6ST/gp130. This results in activation of Src family kinases (SFKs), YAP, Notch, and STAT3, which are simultaneously activated in 64% of human CRC. In addition to better explaining how APC loss initiates colorectal tumorigenesis, we show that combined treatment with SFK and JAK inhibitors results in regression of established colorectal tumors in mice.

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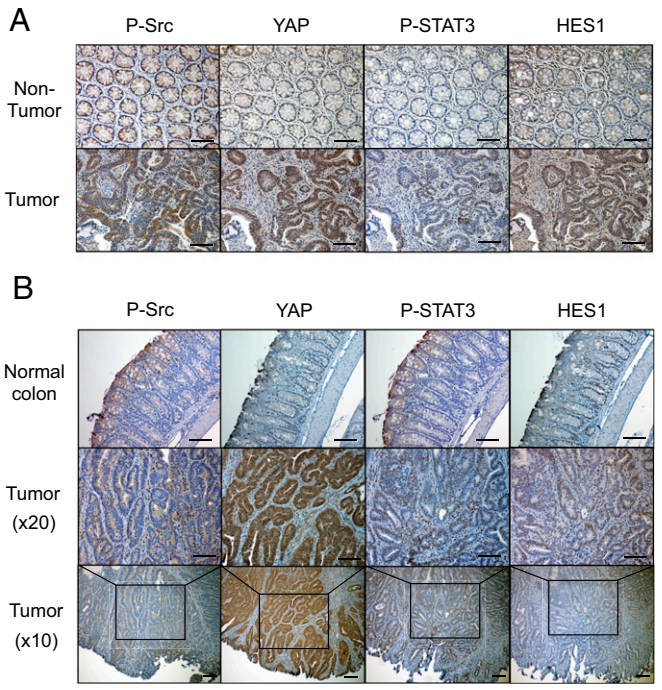

Fig. 1. Multiple gp130-responsive signaling pathways are activated in human and mouse colorectal cancer. (*A*) Paraffin-embedded sections of surgically removed human CRC ($n = 17$) and matched normal colon tissues ($n = 7$) were stained with P-Src, YAP, P-STAT3, or HES1 antibodies. (*B*) Paraffin-embedded colon sections from tumor-bearing CPC-APC mice ($n = 3$) and WT controls ($n = 3$) were stained as above. (Scale bars, 100 μm.)

Subsequent activation of IL-17 receptor A (IL-17RA) stimulates proliferation of early tumor progenitors and causes adenoma growth (14). Consistent with these experimental findings, epidemiological studies revealed that elevated IL-23 and IL-17 expression in low-grade human CRC predicts rapid progression to fatal metastatic disease (15).

The normal function of the Wnt–β-catenin pathway is to control the proliferation and differentiation of crypt-localized gastrointestinal epithelial stem cells (16). By activating ERK and NF-κB, engagement of IL-17RA augments epithelial proliferation and regeneration after injury (14). Other signaling pathways responsible for epithelial survival and injury repair rely on the key transcriptional regulators STAT3 and YAP (16, 17). Although the role of STAT3 in regeneration and colorectal tumorigenesis is unequivocal (18, 19), it is still debated whether YAP is a tumor suppressor (20) or an oncogenic driver (21). Here we show that Src, YAP, STAT3, and Notch are coordinately activated in mouse APC-deficient intestinal organoids and colonic tumors and in 64% of human CRC specimens. These pathways respond to the dramatic up-regulation of the IL-6 signal transducer (IL-6ST or gp130), a protein that serves as a co-receptor for IL-6, IL-11, and related cytokines. Constitutive gp130 activation in mouse intestinal epithelial cells (IEC) accelerates colorectal tumorigenesis initiated by APC loss. Conversely, inhibition of Src family kinases (SFKs) and JAK tyrosine kinases that maintain YAP and STAT3 activation results in death of CRC progenitors and regression of established tumors.

Results

Concomitant Src, YAP, Notch, and STAT3 Activation in Human CRC. SFKs, YAP, Notch, and STAT3 are critical mediators of inflammation-driven mucosal regeneration and are activated in inflammatory bowel diseases (16, 17), which increase CRC risk (12). To query their involvement in colorectal tumorigenesis, we stained a collection of human CRC surgical specimens ($n = 17$) with antibodies to phosphorylated Src and STAT3, YAP, and HES1, a Notch target. Strikingly, 59% of the tumors exhibited concomitant activation and up-regulation of all four signaling molecules relative to nontumor tissue (Fig. 1*A*, Fig. S1*A*, and Table S1). Analysis of mouse colon tumors induced by APC loss revealed an identical scenario: concomitant Src, STAT3, YAP, and Notch activation in tumor specimens (Fig. 1*B*). We further confirmed and extended the human data using another cohort of human colon tissue microarrays (TMA). Approximately 67% of these CRC specimens ($n = 27$) were positive for all four markers, none of which were strongly expressed in normal tissue (Fig. S1 *B* and *C* and Table S2). Positivity of all four markers tended to be higher at advanced disease stages (Fig. S1*D*).

APC Loss Results in Src, YAP, Notch, and STAT3 Activation. APC inactivation is the most common initiating event in human CRC development (22), which in addition to β-catenin stabilization was found to activate YAP through an ill-defined mechanism (23, 24). To examine whether APC loss is responsible for STAT3, SFK, YAP, and Notch activation, we established WT and $Apc^{-/-}$ mouse small intestinal (SI) organoids (enteroids) by transducing $Apc^{F/F}$ organoids with Adeno-Cre virus. APC-null enteroids exhibited increased STAT3 and Src tyrosine (Y) phosphorylation (Fig. 2*A*). YAP expression and Y phosphorylation were also up-regulated, along with mRNAs encoding YAP targets, connective tissue growth factor (*Ctgf*) and *Cyr61*, and the Notch ligand *Jag1* (Fig. 2 *A* and *B*). $Apc^{-/-}$ organoids exhibited balloon-like morphology and up-regulation of mRNAs encoding the stem cell markers *Lgr5* and *Bmi1* (Fig. S2 *A* and *B*). Treatment with SFK inhibitors, PP2 and AZD0530, suppressed YAP expression and Y phosphorylation in $Apc^{-/-}$enteroids, but had

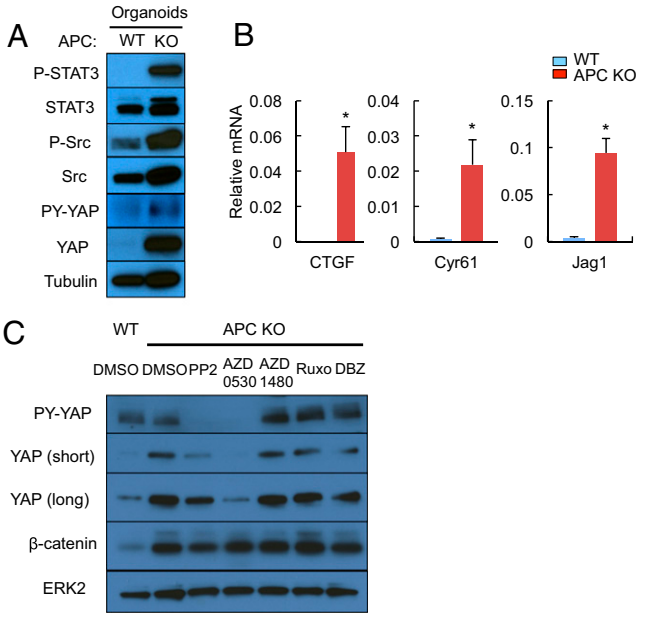

Fig. 2. APC ablation results in SFK-dependent YAP activation in intestinal organoids. (*A*) WT and APC-null SI organoids were lysed and analyzed for expression and phosphorylation of the indicated proteins by immunoblotting (IB). (*B*) RNAs from WT and APC-null enteroids and indicated transcripts were analyzed by quantitative RT-PCR (qRT-PCR). Results are means ± SEM ($n = 3$). *$P < 0.05$. (*C*) WT and APC-null enteroids were treated with the indicated inhibitors (PP2, AZD0530, and DBZ at 10 μM, and AZD1480 and Ruxolitinib at 3 μM) or vehicle (DMSO) for 24 h. Total lysates were IB-analyzed with the indicated antibodies.

a minimal effect on β-catenin activation (Fig. 2*C*). The Src inhibitor PP2 also inhibited YAP and Notch activation in human CRC cell lines (Fig. S2*C*). The JAK1/2 inhibitors, AZD1480 and Ruxolitinib, or the Notch/γ-secretase inhibitor dibenzazepine (DBZ), did not affect YAP or β-catenin activation (Fig. 2*C*).

APC Loss Induces gp130 and IL-11R Up-Regulation. To determine how APC loss activates STAT3, SFKs, YAP, and Notch, we examined the expression status of gp130-related molecules known to activate all of the aforementioned signaling pathways (17). APC loss led to dramatic up-regulation of *Il6st* (gp130) and *Il6r* mRNAs and a more modest increase in *Il11r* mRNA (Fig. 3*A*). Substantial gp130 and IL-11R up-regulation was also observed at the protein level, whereas IL-6R expression was only modestly increased (Fig. 3*B*). Addition of exogenous IL-6, IL-11, or soluble IL-6R (sIL-6R) plus IL-6 to $Apc^{-/-}$ enteroids led to a further increase in STAT3 phosphorylation without affecting gp130 expression (Fig. 3*C*). APC-deficient enteroids also exhibited elevated *Il6* and *Lif* mRNAs (Fig. S3*A*) and IL-6 neutralization attenuated STAT3, Src, and YAP activation (Fig. S3*B*). APC-deficient enteroids exhibited moderately elevated expression of *Il17ra* and *Il22ra* mRNAs, but lower expression of *Il17rc* mRNA (Fig. S3*C*). Time-course analysis using $Apc^{F/F}$ organoids that express 4-hydroxytamoxifen (4-OHT)–regulated Cre recombinase revealed strong induction of *Axin2*, a direct β-catenin target (7), peaking 2 d after 4-OHT addition (Fig. S3*D*). *Il6st* (gp130) mRNA was also up-regulated, but its induction peaked on day 4 and paralleled induction of the classic YAP targets *Ctgf* and *Cyr61* (Fig. S3*D*).

We also prepared WT and $Apc^{-/-}$ colon enteroids whose morphological features paralleled those of small intestinal enteroids (Fig. S4*A*). $Apc^{-/-}$ colon enteroids showed YAP and STAT3 activation and up-regulation of mRNAs encoding CTGF, Cyr61, Jag1, Hes1, Lgr5, Bmi1, gp130, IL-6R, and IL-11R (Fig. S4 *B* and *C*). Human colon organoids rendered APC deficient also showed YAP up-regulation (Fig. 3*D* and Fig. S4*D*). Of note, epithelial gp130 expression was markedly increased in both human CRC specimens and mouse colon tumors (Fig. 3 *E* and *F*). Stromal expression of gp130 remained low.

A Positive Autoregulatory Loop Controls gp130 and YAP Expression. To determine the role of gp130 in activation of the signaling pathways described above, we silenced its expression in $Apc^{-/-}$ organoids with CRISPR/Cas9 technology. The gp130 deficiency

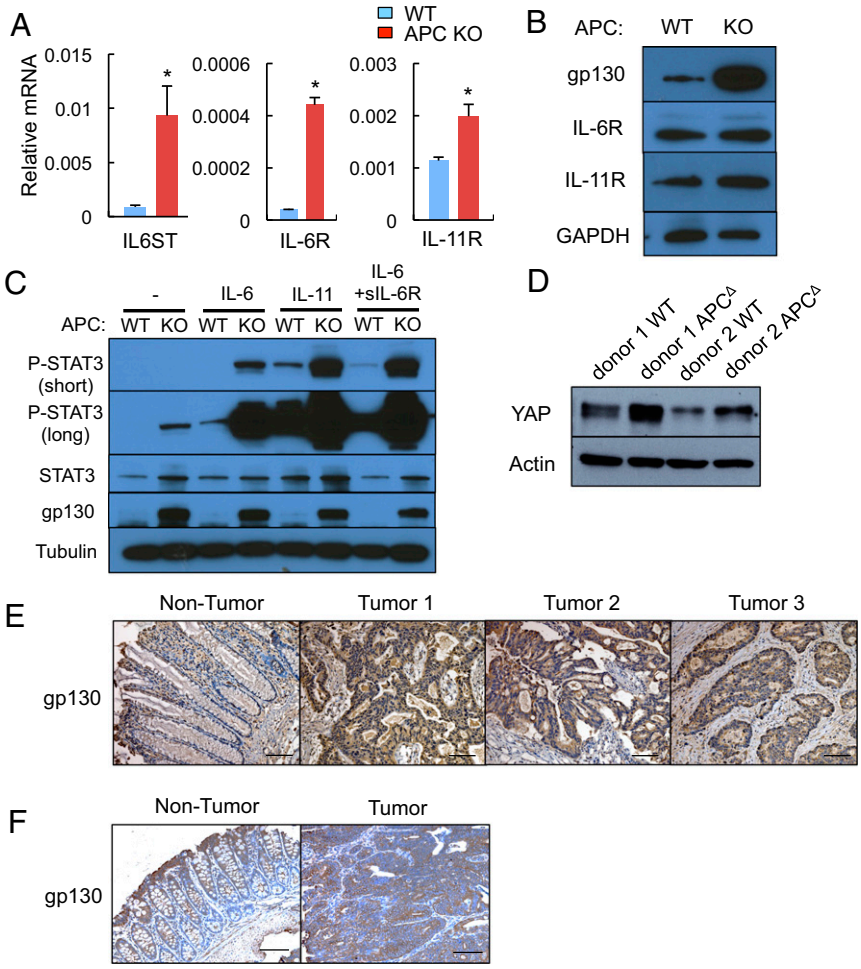

Fig. 3. APC ablation up-regulates gp130, IL-6R, and IL-11R expression. (*A*) WT and APC-null SI enteroids were analyzed for expression of the indicated mRNAs by qRT-PCR. Results are means ± SEM ($n = 3$). *$P < 0.05$. (*B*) WT and APC-null SI enteroids were lysed and IB-analyzed for the indicated proteins. (*C*) WT and $Apc^{-/-}$ SI enteroids were stimulated with IL-6 (100 ng/mL), IL-11 (100 ng/mL), or IL-6 (100 ng/mL) + sIL-6R (100 ng/mL) for 30 min, lysed, and IB-analyzed for protein expression and phosphorylation. (*D*) WT and APC-deficient human colon organoids were lysed and IB-analyzed for the indicated proteins. (*E*) Paraffin-embedded sections of surgically removed human CRC ($n = 17$) and matched normal colon sections ($n = 7$) were stained with a gp130 antibody; 65% of the cancer samples (11 of 17) showed similar gp130 positivity to the three examples depicted here. (*F*) Paraffin-embedded colon sections from tumor-bearing CPC-APC mice were stained with a gp130 antibody. (Scale bars, 100 μm.)

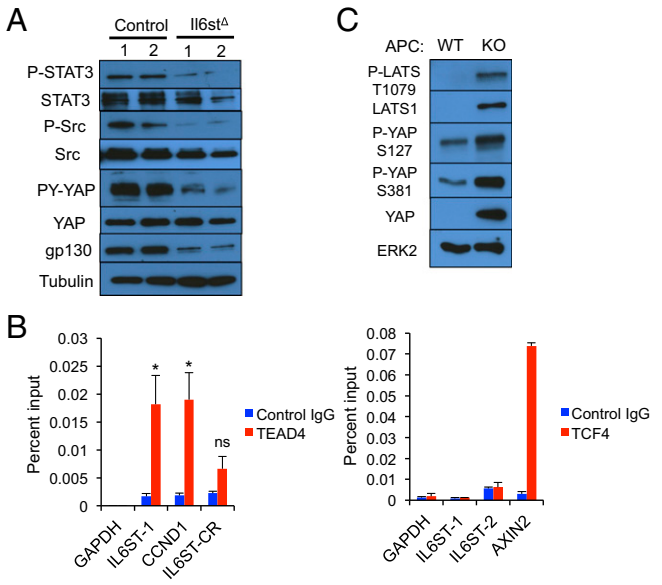

Fig. 4. A YAP-IL-6ST autoregulatory loop. (*A*) *Apc*$^{-/-}$ and *Il6st*-ablated *Apc*$^{-/-}$ enteroids were lysed and analyzed for expression and phosphorylation of the indicated proteins. (*B*) DLD-1 cells were analyzed by ChIP using TEAD4 (*Left*) and TCF4 (*Right*) antibodies and control IgG for occupancy of the *IL6ST* regulatory region. The *GAPDH*, *CCND1*, and *AXIN2* promoters served as negative and positive controls. DLD-1 cells were stimulated with 10% (vol/vol) FBS before ChIP analysis. The precipitated DNA was quantitated by real-time PCR with primers specific for the promoter regions or a control region (CR) of the indicated genes. (*Left*) Data are means ± SEM of three independent experiments. *P* values were determined using one-way ANOVA test followed by Tukey's multiple comparison test, $*P < 0.05$; ns, not significant ($P > 0.05$). (*Right*) Data are means ± SEM of duplicates from a representative experiment. (*C*) WT and APC-null enteroids were lysed and analyzed for expression and phosphorylation of the indicated proteins.

inhibited STAT3, SFKs and the activating Y phosphorylation of YAP (Fig. 4*A*), suggesting that gp130 acts upstream to STAT3, SFKs, and YAP. Next, we examined the mechanisms responsible for gp130 up-regulation in APC ablated organoids. Scanning of the *IL6ST* gene regulatory region revealed putative binding sites for transcription factor 4 (TCF4) and TEA domain transcription factor 4 (TEAD4), which are binding partners for β-catenin and YAP, respectively, and STAT3 (Fig. S5*A*). To determine which of these binding sites are occupied in human CRC, we performed ChIP experiments on DLD-1 CRC cells. The only transcription factor found to occupy the *IL6ST* regulatory region was TEAD4 (Fig. 4*B*). No significant binding of TCF4 or STAT3 was observed, even after incubation of DLD-1 cells with IL-6 (Fig. 4*B* and Fig. S5*B*). To validate the importance of these results, we silenced expression of YAP and its paralog TAZ, whose expression is up-regulated in the absence of YAP (25), in different human CRC cell lines. Down-regulation of YAP and TAZ resulted in decreased *IL6ST* mRNA expression in these cells (Fig. S5*C*).

Given these results, which implicate YAP in gp130 induction and gp130 in YAP activation, we examined in closer detail whether Hippo pathway activity, which negatively regulates YAP, is perturbed by APC ablation. Immunoblot analysis indicated that APC ablation actually led to up-regulation of large tumor suppressor 1 (LATS1), a component of the mammalian Hippo pathway, which was phosphorylated at T1079 (Fig. 4*C*). Paralleling the increase in LATS1 expression, APC ablation increased YAP phosphorylation at the LATS phospho-acceptor sites S127 and S381, whose phosphorylation inhibits YAP nuclear translocation and promotes its proteasomal degradation (Fig. 4*C*). These results further support the importance of SFK-mediated Y phosphorylation, which results in YAP activation even in cells with intact Hippo signaling (17), and show that Hippo pathway inactivation cannot fully account for YAP up-regulation. However, we found that YAP was already phosphorylated on the inhibitory serines in WT enteroids and that the fold-increase in its S phosphorylation seemed lower than the overall increase in its expression (Fig. 4*C*). To further examine this point, we separated YAP phospho-isoforms on Phos-tag SDS/PAGE gels, which revealed that the relative ratio of underphosphorylated YAP to total YAP in APC-deficient enteroids was higher than in WT enteroids (Fig. S5*D*). These results are consistent with the previous report that APC ablation results in decreased LATS1 activity (23), and suggest that this may only give rise to the initial surge in YAP activity that induces *Il6st* transcription.

gp130 Activation Accelerates CRC Development, Which Depends on SFK and JAK Activities. Expression of constitutively active gp130 (gp130^{Act}) in IEC using the *Villin* promoter activates STAT3, SFK, YAP, and Notch signaling (17). When mice expressing gp130^{Act} in IEC reached 12–15 mo of age, 30% of them developed SI tumors (Fig. S6*A*). However, introduction of the *Villin-gp130*^{Act} transgene into *Apc*$^{+/\Delta IEC}$ mice, in which loss of WT *Apc* initiates intestinal tumorigenesis (26), augmented and accelerated formation of small intestinal and colonic adenomas harboring activated β-catenin (Fig. 5 and Fig. S6 *B–D*). Enhanced tumor development was dependent on YAP and STAT3, as ablation of either molecule inhibited tumorigenesis in

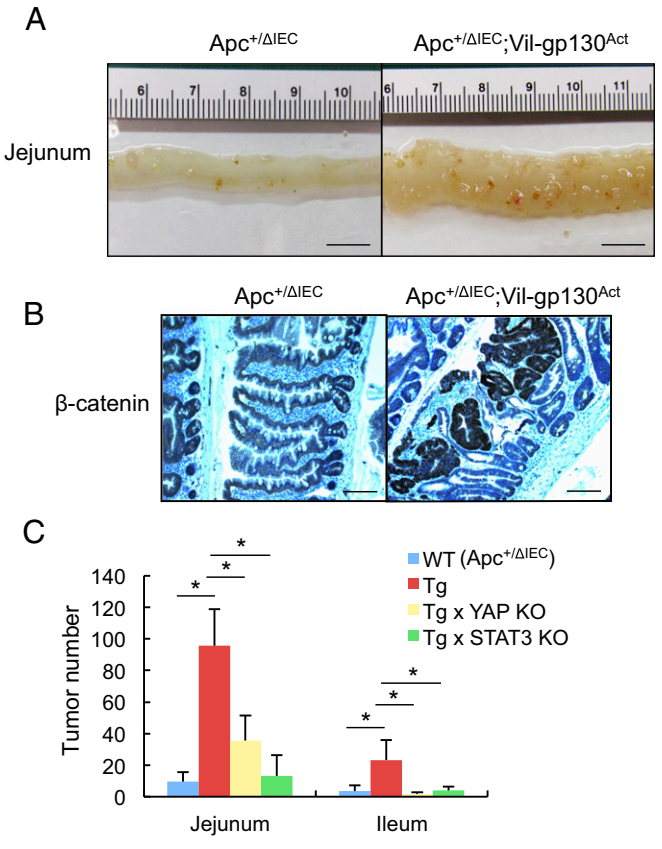

Fig. 5. YAP or STAT3 ablation inhibits intestinal tumorigenesis. (*A*) Representative images of *Apc*$^{+/\Delta IEC}$ and *Apc*$^{+/\Delta IEC}$;*Vil-gp130*^{Act} SIs. (Scale bars, 10 mm.) (*B*) β-Catenin staining of paraffin-embedded SI sections from *Apc*$^{+/\Delta IEC}$ and *Apc*$^{+/\Delta IEC}$;*Vil-gp130*^{Act} mice. (Scale bars, 100 μm.) (*C*) Tumor numbers in the jejunum and ileum of the indicated mouse strains ($n = 5–8$ per group). Results are means ± SEM; $*P < 0.05$.

$Apc^{+/\Delta IEC}$;*Vil-gp130*^{Act} mice (Fig. 5*C* and Fig. S6*E*). To determine the effect of simultaneous SFK and JAK inhibition on CRC development, we treated $Apc^{-/-}$ enteroids and organoids established from a spontaneous SI tumor in *Vil-gp130*^{Act} mice with SFK and/or JAK inhibitors. Whereas SFK inhibition reduced organoid size and survival, JAK1/2 inhibition had a rather marginal effect (Fig. 6*A* and Fig. S7). However, together the SFK and JAK inhibitors led to extensive organoid death. To extend these results to our in vivo colon tumor system, we treated 3.5-mo-old tumor-bearing CPC-APC (*Cdx2-Cre* × $Apc^{F/+}$) mice with PP2 and/or Ruxolitinib for 1.5 mo. Whereas each inhibitor alone led to modest tumor regression, the two inhibitors together resulted in a reduction in tumor load, although the difference between the Ruxolitinib and Ruxolitinib + PP2 groups was not statistically significant (Fig. 6*B*). No obvious damage to nontumor tissue was observed.

Discussion

The results described above shed new light on the mechanism of CRC initiation and identify novel and broadly important targets for its treatment. CRC is rather unique in its almost exclusive dependence on initiating *APC* loss-of-function mutations (7, 9). Although APC inactivation results in β-catenin activation, *CTNNB1* gain-of-function mutations, which occur in 40% of hepatocellular carcinomas (27), are rare in CRC (8). Our results demonstrate that *APC* loss, as opposed to *CTNNB1* activation, provides additional selective advantages to CRC-initiating cells because it makes them highly responsive to local inflammatory signals provided by members of the IL-6 cytokine family. By up-regulating gp130 expression, APC loss results in heightened sensitivity to IL-6, IL-11, and sIL-6R, which act as tumor promoters in mouse models (28, 29) and are up-regulated in human CRC (18). Whereas IL-6 is induced upon activation of IL-17RA signaling (14), IL-11 is provided by cancer-associated fibroblasts (30). The discrepancy between high IL-6R mRNA and hardly elevated cell surface protein expression might be a result of increased IL-6R shedding. Indeed, the finding that IL-6 + sIL-6R elicits a stronger response than IL-6 alone suggests more gpl30 on the cell surface than IL-6R.

In addition to activation of STAT3, a transcription factor that is of great importance in CRC establishment (18, 29), IL-6, sIL-6R, and IL-11 activate SFKs that support Hippo-independent YAP activation and thereby induce Notch receptors and ligands (17). Curiously, however, the initial signal that leads to YAP activation in APC-deficient cells and consequent induction of IL-6ST/gp130 expression seems to be loss of LATS1 activity (23), a serine/threonine kinase that promotes YAP nuclear exclusion and degradation (31). Given the small decrease in LATS activity (23), the majority of YAP activation in mouse colorectal tumors and human CRC cell lines is propagated by SFK-mediated tyrosine phosphorylation, which induces YAP nuclear translocation, even in cells that harbor active LATS1. Treatment of APC-deficient organoids or human CRC cells with SFK inhibitors results in a marked decrease in YAP expression as well as inhibition of Notch activation. Of note, an independent and unbiased screen for compounds that inhibit YAP activation in breast cancer has netted the clinically approved SFK and c-Abl inhibitor, Dasatinib (32). Thus, the initial spike in YAP activity, which was proposed to take place upon disruption of a complex between APC and LATS1 (23), gives rise to much greater Hippo-independent and SFK-dependent YAP activation through the induction of *IL6ST* gene transcription and up-regulation of gp130 expression (Fig. S8). Up-regulation of gp130 connects YAP and its downstream targets to localized inflammatory signals that are provided by IL-6 family members. Our findings re-enforce the important oncogenic function of YAP (21) and explain the basis for its frequent activation in CRC and possibly other epithelial tumors that are devoid of mutations that disrupt Hippo signaling. Indeed, the SFK–YAP module also functions in skin cancer (33). YAP up-regulation in epithelial cancers is also known to account for acquired drug resistance (21), but the underlying mechanisms were not reported. Our results indicate that at least in CRC, the major mechanism responsible for sustained YAP activation is the YAP–IL-6ST–SFK autoregulatory loop described above. Given the strong correlation between YAP and SFK activation in 60–70% of human CRC, this amplification loop, which links APC loss to YAP and STAT3 activation, is of great clinical relevance. Indeed, a combined treatment with SFK and JAK1/2 inhibitors that block YAP and STAT3 activation, respectively, results in substantial regression of colorectal tumors that were initiated by *Apc* ablation. These findings strongly support the merit of testing such compounds, several of which have been clinically approved, in advanced human CRC, which currently accounts for 700,000 yearly deaths worldwide (1).

Of further interest is the observation that two different cohorts of CRC patients, one collected in China and the other in Austria, who differ in their ethnicity and dietary habits, exhibit nearly identical rates of SFK, STAT3, YAP, and Notch activation. These findings suggest that none of these parameters are strongly influenced by the gut microbiota. Indeed, *APC* ablation in vivo (i.e., in the presence of microbiota) had the same effect on these signaling pathways as its ablation in germ-free organoids.

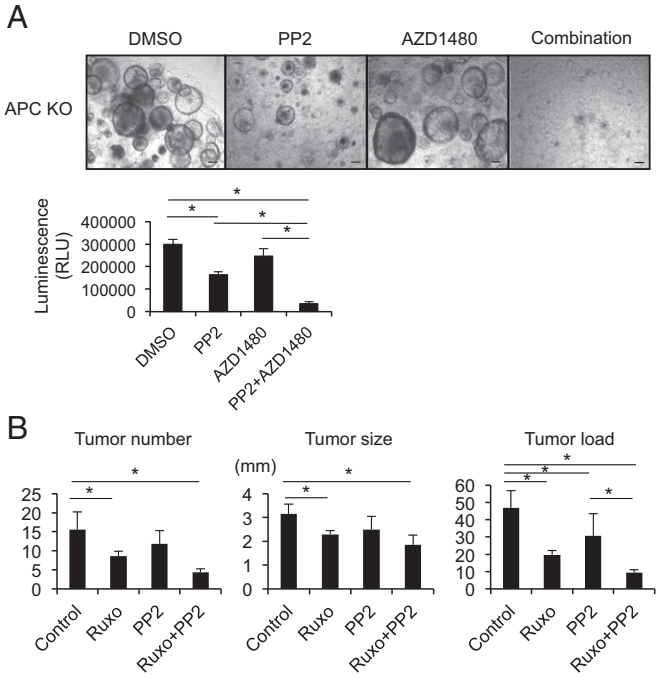

Fig. 6. Combined treatment with SFK and JAK inhibitors causes regression of colorectal tumors. (*A*) Representative images of $Apc^{-/-}$ enteroids treated with the indicated inhibitors (PP2, a SFK inhibitor at 10 μM; and AZD1480, a JAK inhibitor at 3 μM) for 3 d. Cell viability was measured using a CellTiter-Glo assay. Results are means ± SEM; *$P < 0.05$. (Scale bars, 100 μm.) (*B*) Tumor number, size, and load in CPC-APC mice treated with the indicated kinase inhibitors for 1.5 mo starting at 3.5 mo of age (n = 5–6 per group). Results are means ± SEM; *$P < 0.05$.

Methods

Human Colon Samples. Human colorectal cancer tissues were collected through a study that was approved by the Institutional Review Board of Elitehealth Institute, Guangzhou, China. Written informed consent was obtained from all study participants. Tissue samples were collected and processed at the time of surgery, and were used for staining. Additional tissue

specimens were obtained with informed consent from CRC patients at the Medical University of Graz, with approval from the local ethics committee and used to construct TMAs, as described in *SI Methods*.

Mice. *Villin-Cre* mice were obtained from the Jackson Laboratory. *Villin-gp130^{Act}* transgenic (Tg), *Stat3$^{F/F}$*, *Yap$^{F/F}$*, and *Villin-Cre^{ERT2}* mice have been described previously (17, 34–36). *Apc$^{F/F}$*, *Cdx2-Cre* (CPC), and *Cdx2-Cre^{ERT}* mice were described previously (26, 37). All mice were on the C57BL/6 background and were maintained in filter-topped cages on autoclaved food and water at the University of California, San Diego (UCSD) according to NIH guidelines. All experimental procedures were reviewed and approved by the UCSD Institutional Animal Care and Use Committee and all experiments were performed in accordance with UCSD and NIH guidelines and regulations.

Detailed information about experimental procedures, human samples, mice, reagents, and statistical analyses can be found in *SI Methods*.

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1. Torre LA, et al. (2015) Global cancer statistics, 2012. *CA Cancer J Clin* 65(2):87–108.
2. Brenner H, Kloor M, Pox CP (2014) Colorectal cancer. *Lancet* 383(9927):1490–1502.
3. Pohl M, Schmiegel W (2016) Therapeutic strategies in diseases of the digestive tract—2015 and beyond targeted therapies in colon cancer today and tomorrow. *Dig Dis* 34(5):574–579.
4. Le DT, et al. (2015) PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med* 372(26):2509–2520.
5. Vaiopoulos AG, Kostakis ID, Koutsilieris M, Papavassiliou AG (2012) Colorectal cancer stem cells. *Stem Cells* 30(3):363–371.
6. Markowitz SD, Bertagnolli MM (2009) Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 361(25):2449–2460.
7. Fearon ER (2011) Molecular genetics of colorectal cancer. *Annu Rev Pathol* 6:479–507.
8. Cancer Genome Atlas N; Cancer Genome Atlas Network (2012) Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 487(7407):330–337.
9. Armaghany T, Wilson JD, Chu Q, Mills G (2012) Genetic alterations in colorectal cancer. *Gastrointest Cancer Res* 5(1):19–27.
10. Shimizu T, et al. (2012) The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer. *Clin Cancer Res* 18(8):2316–2325.
11. Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. *Cell* 140(6):883–899.
12. Terzic J, Grivennikov S, Karin E, & Karin M (2010) Inflammation and colon cancer. *Gastroenterology* 138(6):2101–2114 e2105.
13. Grivennikov SI, et al. (2012) Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* 491(7423):254–258.
14. Wang K, et al. (2014) Interleukin-17 receptor a signaling in transformed enterocytes promotes early colorectal tumorigenesis. *Immunity* 41(6):1052–1063.
15. Tosolini M, et al. (2011) Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* 71(4):1263–1271.
16. Karin M, Clevers H (2016) Reparative inflammation takes charge of tissue regeneration. *Nature* 529(7586):307–315.
17. Taniguchi K, et al. (2015) A gp130-Src-YAP module links inflammation to epithelial regeneration. *Nature* 519(7541):57–62.
18. Taniguchi K, Karin M (2014) IL-6 and related cytokines as the critical lynchpins between inflammation and cancer. *Semin Immunol* 26(1):54–74.
19. Grivennikov S, et al. (2009) IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* 15(2):103–113.
20. Barry ER, et al. (2013) Restriction of intestinal stem cell expansion and the regenerative response by YAP. *Nature* 493(7430):106–110.
21. Zanconato F, Cordenonsi M, Piccolo S (2016) YAP/TAZ at the roots of cancer. *Cancer Cell* 29(6):783–803.
22. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* 61(5):759–767.
23. Cai J, Maitra A, Anders RA, Taketo MM, Pan D (2015) β-Catenin destruction complex-independent regulation of Hippo-YAP signaling by APC in intestinal tumorigenesis. *Genes Dev* 29(14):1493–1506.
24. Azzolin L, et al. (2014) YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the Wnt response. *Cell* 158(1):157–170.
25. Moroishi T, et al. (2015) A YAP/TAZ-induced feedback mechanism regulates Hippo pathway homeostasis. *Genes Dev* 29(12):1271–1284.
26. Hinoi T, et al. (2007) Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res* 67(20):9721–9730.
27. Totoki Y, et al. (2014) Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. *Nat Genet* 46(12):1267–1273.
28. Putoczki TL, et al. (2013) Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically. *Cancer Cell* 24(2):257–271.
29. Grivennikov SI (2013) IL-11: a prominent pro-tumorigenic member of the IL-6 family. *Cancer Cell* 24(2):145–147.
30. Calon A, et al. (2012) Dependency of colorectal cancer on a TGF-β-driven program in stromal cells for metastasis initiation. *Cancer Cell* 22(5):571–584.
31. Zhao B, Li L, Tumaneng K, Wang CY, Guan KL (2010) A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev* 24(1):72–85.
32. Oku Y, et al. (2015) Small molecules inhibiting the nuclear localization of YAP/TAZ for chemotherapeutics and chemosensitizers against breast cancers. *FEBS Open Bio* 5:542–549.
33. Li P, et al. (2016) αE-catenin inhibits a Src-YAP1 oncogenic module that couples tyrosine kinases and the effector of Hippo signaling pathway. *Genes Dev* 30(7):798–811.
34. Takeda K, et al. (1999) Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10(1):39–49.
35. Zhang N, et al. (2010) The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. *Dev Cell* 19(1):27–38.
36. el Marjou F, et al. (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* 39(3):186–193.
37. Feng Y, et al. (2013) Sox9 induction, ectopic Paneth cells, and mitotic spindle axis defects in mouse colon adenomatous epithelium arising from conditional biallelic Apc inactivation. *Am J Pathol* 183(2):493–503.
38. Nam JS, Ino Y, Sakamoto M, Hirohashi S (2002) Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis. *Clin Cancer Res* 8(7):2430–2436.
39. Ootani A, et al. (2009) Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat Med* 15(6):701–706.
40. Zhao B, et al. (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* 22(14):1962–1971.
41. Sato T, et al. (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459(7244):262–265.
42. Sato T, et al. (2011) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 141(5):1762–1772.
43. Koo BK, et al. (2011) Controlled gene expression in primary Lgr5 organoid cultures. *Nat Methods* 9(1):81–83.
44. Wang N, et al. (2014) Adenovirus-mediated efficient gene transfer into cultured three-dimensional organoids. *PLoS One* 9(4):e93608.
45. Sanjana NE, Shalem O, Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11(8):783–784.
46. Schwank G, et al. (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* 13(6):653–658.
47. Koo BK, Sasselli V, Clevers H (2013) Retroviral gene expression control in primary organoid cultures. *Curr Protoc Stem Cell Bio* 27:Unit 5A.6.