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Slap restricts oncogenic Src-family kinase signaling to maintain colonic epithelial homeostasis

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eLife Assessment

This is an **important** study on the role of Slap in restricting Src activity and proliferation of colonic cells in vivo. The authors present **solid** evidence that an EPHB2-SRC signaling axis stimulates the proliferation of colon precursors and is controlled by the Src-binding protein SLAP, whose loss promotes tumorigenesis.

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Abstract

Src-family kinases (SFKs) regulate proliferation in colonic epithelial cells (CECs), but the mechanisms that restrain their activity remain poorly defined. We identify Src-like adaptor protein (SLAP), a negative regulator of receptor tyrosine kinase signaling, as a key suppressor of SFK activity in the colon. Constitutive and inducible epithelial-specific Slap deletion using a villin-CreERT2 model increases CEC proliferation and accelerates tumorigenesis in the azoxymethane/dextran sodium sulfate (AOM/DSS) model. Slap deficiency also enhances SFK-dependent expansion of normal and tumor-derived colonic organoids. Mechanistically, we identify the receptor tyrosine kinase EPHB2 as a critical upstream activator of SFKs and a direct target of SLAP-mediated regulation. Loss of Slap increased EphB2 protein abundance and tyrosine phosphorylation and enhanced its association with active SRC. Pharmacological inhibition of EPHB2 suppressed SRC activation and reversed the hyperproliferative phenotype induced by Slap deficiency. Together, these findings uncover a non-genetic mechanism driving SFK activation during colonic transformation and establish SLAP as a tumor suppressor that constrains oncogenic EPHB2–SFK signaling in the colonic epithelium.

Introduction

The membrane-anchored tyrosine kinase (TK) SRC plays important roles in intestinal homeostasis and tumorigenesis. In particular, SRC and other members of its family (i.e., FYN and YES) drive intestinal stem/progenitor cell proliferation, tissue regeneration and tumorigenesis in Drosophila and mouse models (Cordero et al., 2014; Imada et al., 2016; Kohlmaier et al., 2015; Sirvent et al., 2020). SRC is also an oncogene in human colorectal cancer (CRC). Aberrant SRC expression and activity in CRC (50% of CRC patients) is a marker of poor clinical prognosis, a promoter of therapeutic resistance and a potent driver of metastasis (Sirvent et al., 2012; Summy and Gallick, 2003; Yang et al., 2022). This tumour activity is associated with cancer stem cell and

epithelial-to-mesenchymal transition features. However, SRC is rarely mutated in CRC and the mechanisms underlying its oncogenic function remain elusive (Irby et al., 1999; Sirvent et al., 2012; Summy and Gallick, 2003). Consistently, SRC inhibitors developed for the clinic have failed in CRC due to ineffective signalling inhibition and inadequate patient selection (Daud et al., 2012; Parseghian et al., 2017; Reddy et al., 2015). Elucidating the mechanisms underlying this persistent signalling may reveal essential non-genetic mechanisms by which TKs promote tumorigenesis and allow the development of effective TK-based therapies in CRC.

During evolution, TK activities have come under the tight control of small adaptor proteins that exert negative regulatory functions (Naudin et al., 2016). The best example is the SOCS regulation of JAK/STAT inflammatory pathways. We discovered that SRC signalling is under the control of such an inhibitory mechanism through the membrane-anchored adaptor SLAP (Src-Like Adaptor Protein), which display high sequence homology with SRC N-terminus (Manes et al., 2000; Pandey et al., 1995; Roche et al., 1998; Sirvent et al., 2008). Mechanistically, SLAP promotes degradation or prevents SRC binding to upstream receptors, resulting in inhibition of SRC signaling (Dragone et al., 2006a, 2006b; Kazi and Rönnstrand, 2012; Myers et al., 2006, 2005; Roche et al., 1998; Sirvent et al., 2008; Wybenga-Groot and McGlade, 2015). Slap-deficient mice revealed an essential function for this adaptor in controlling lymphocyte development and activation, where it is highly expressed (Sosinowski et al., 2001). We additionally reported an unsuspected tumour suppressor function of SLAP in CRC (Naudin et al., 2014). Notably, SLAP was found to be abundantly expressed in the intestinal epithelium. SLAP levels were reduced in 50% of CRC samples analysed, and SLAP silencing in experimental CRC models promoted tumour formation and liver metastasis. Mechanistically, SLAP promotes degradation of SRC signalling RTK EPHA2 (Naudin et al., 2014) and destabilization of the mTORC2 complex, resulting in AKT signaling inhibition (Mevizou et al, 2025). Although this observation suggests an important mechanism in the control of SRC function in the colon, genetic evidence for this mechanism is lacking. Here, we developed Slap-deficient mice models showing that Slap regulates SFK colonic epithelial function during homeostasis and tumorigenesis.

Materials and methods

Generation of genetically modified mouse models

All mouse experiments were conducted in strict accordance with the European Community guidelines (86/609/EEC) and the French National Committee for the care and use of laboratory animals (87/848), complied with the ARRIVE guidelines, and were approved by the French Ministry of Higher Education, Research and Innovation (APAFIS#2022031511382008). All procedures were performed in the institutional animal facility (agreement #F3417216). Mice were housed in temperature-controlled ventilated cages (20–22 °C) under a 12 h light/dark cycle, with relative humidity maintained between 45% and 55%, and were kept under pathogen-free conditions. *Slap*$^{tm1a(KOMP)}$ mice were generated from JM8A1.N3 embryonic stem cells (C57BL/6N genetic background) produced by the trans-NIH KnockOut Mouse Project (KOMP) and obtained from the KOMP Repository (*www.komp.org*). Genotyping was performed by PCR analysis of genomic DNA isolated from mouse tail biopsies. The Slap $^{tm1a(KOMP)}$, Tg(CAG-flpo)1Afst, and Tg(Vil1-cre/ERT2)23Syr mouse lines, as well as compound lines, were maintained on a C57BL/6 background and bred in a specific opportunistic pathogen-free (SOPF) facility, and experiments were conducted in a specific pathogen-free (SPF) facility.

Slap-inactivated mice (*Slap -/-*) were generated by insertion of a *LacZ* reporter cassette into the Slap locus, resulting in functional gene inactivation. The *LacZ* cassette was flanked by FRT sites, allowing recombination upon crossing with FlpO deleter mice. FlpO-mediated recombination generated the recombined *Slap* allele, whereas FlpO-negative littermates retaining the unrecombined *LacZ* allele were used as controls.

Inducible *Slap* knockout mice were generated by crossing Slap floxed mice with CreERT2 transgenic mice. Tamoxifen treatment induced Cre-mediated excision of the floxed Slap allele, resulting in gene inactivation. To activate CreERT2, control and experimental mice received two

intraperitoneal injections of tamoxifen (2 mg per injection).

Mice genotyping

Mice tail biopsies underwent alkaline lysis and incubation at 92 °C for 20 minutes, followed by reaction neutralization. PCR amplification was conducted with the ready mix MyTAQRed mix (meridian). Resulting products were run on a 2% ethidium-bromide agarose gel (Sigma–Aldrich), using a 1 kb DNA ladder (Thermo Scientific), and imaged with a ChemiDoc MP system (Bio-Rad). The following primers were used: CSD-lacF: GCTACCATTACCAGTTGGTCTGGTGTC; CSD-neoF: GGGA TCTCATGCTGGAGTTCTTCG; CSD-loxF: GAGATGGCGCAACGCAATTAATG; CSD-Sla-R: TCTCTGAGATGC CCCATTTTACCCC; CSD-Sla-ttR: GTTTCTTGGCACTGACTAGAGCAGG; CSD-Sla-F: GTTAACAACACACCTA CCCCTTGGC.

Chemical induction of tumorigenesis by AOM/DSS

Inducible epithelial SLAP knockout mouse model was treated with tamoxifen to induce Slap depletion. DSS and AOM/DSS treatment was performed essentially as described in (Nguyen et al., 2025; Paul et al., 2022). Briefly, 10 days following tamoxifen treatment, a first AOM injection was performed. Shortly after the first round of DSS at 2% was given to the mice for 7 consecutive days. A week later another AOM injection was performed followed by 2 other rounds of DSS treatments two weeks apart. The mice were then sacrificed at day 80. Colons or tumors were then excised for tumoroids formation, cryopreservation or fixation followed with IHC analysis.

β-galactosidase activity

To visualize the activity of the Slap promoter driving lacZ expression, we used 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal, Sigma-Aldrich) as described in (Nguyen et al., 2025). Organs were cryopreserved and 10 µm tissue sections were prepared. Sections were treated with 0.5% glutaraldehyde for 10 min at room temperature followed by overnight incubation at 37°C in a staining solution containing 1 mg/ml X-gal, 5 mM potassium ferricyanide (K3Fe(CN)6), 5 mM potassium ferrocyanide (K4Fe(CN)6) and 2 mM MgCl2, 0.1% Triton in PBS-1X. The synthetic substrate X-gal gives an insoluble blue precipitate when cleaved by β-galactosidase. Samples were washed in PBS-1X for 5 min and rinsed briefly with dH2O before mounting with aqueous mounting medium (Sigma-Aldrich).

Immunohistochemistry of paraffin embedded tissues

Fluorescent and bright-field immunohistochemistry on paraffin-embedded tissue Intestinal tissues were fixed in neutral-buffered formalin for 24 hours at RT, embedded in paraffin and tissue sections (4 µm) were prepared with a microtome (MICROM). Sections were incubated in successive baths of xylene and ethanol and antigen retrieval was performed by boiling the sections with 10 mM sodium citrate (pH 6.4) or Tris-EDTA buffer (pH 9) for 20 minutes. Tissues were treated with TBS-1 × 2% serum 0.1% Triton to block non-specific binding. Samples were then incubated with primary antibodies overnight at 4 °C. In the case of Bright-field immunohistochemistry, sections were treated with BIOXALL (Vector laboratories) for 10 minutes before blocking and incubation with primary antibodies. Secondary reagent staining was revealed using DAB (Vector Laboratories), and sections were counterstained with hematoxylin (Sigma-Aldrich) and then mounted with aqueous mounting medium (Sigma-Aldrich). As for fluorescence immunohistochemistry, sections were incubated in the presence of secondary antibodies conjugated to Alexa-488 or cyanin3 (Jackson ImmunoResearch Laboratories) and Hoechst (2 µg/mL, Sigma–Aldrich) and mounted with aqueous mounting medium (Sigma-Aldrich). For histological analysis, the tissue sections were deparaffinized and stained with hematoxylin, eosin, and Alcian blue.

RNA in situ hybridization

ISH for Lgr5 was performed with the RNAscope FFPE assay kit (Bio-techne) according to the manufacturer's instructions. Briefly, 4 mm formalin-fixed, paraffin embedded tissue sections were pretreated with heat and protease digestion and then hybridized with a target probe for Lgr5 (Probe - Hs-LGR5-C2, Biotechne). Thereafter, a fluorophore signal amplification system was hybridized to the target probe.

RNA extraction and qPCR

Total RNA was extracted using RNeasy Plus Mini Kit columns (Qiagen). First-strand cDNA synthesis was performed with 1 µg of purified RNA using SuperScript VILO cDNA synthesis KIT (Thermo Fisher Scientific) according to the manufacturer's instructions. qRT-PCR experiments were performed using LightCycler 480 SYBR Green I Master (Roche Diagnostics) on the LightCycler 480 system using the following primers EHPB2_F 5'-CGGCTGCATGTCCCTCATC-3', EHPB2_R 5'-GTCCCCG TTACAGTAGAGCTT-3'; GAPDH_F 5'-TCTCCTCTGACTTCAACAGCGAC-3' & GAPDH_R 5'-CCCTGTTGCTG TAGCCAAATTC-3. Relative expression was determined using the threshold cycle relative quantification method. Data were normalized to the expression levels of GAPDH.

ALDH analysis

The ALDH assay was performed as described in (Nguyen et al., 2025) and according to the protocol of the ALDEFLUORTM kit (#01700, Stemcell). FACS analysis of was preformed through the Novocyte ACEA 2 fluorescence-activated cell sorting flow cytometer and data were analyzed using NovoExpress software.

Antibodies and reagents

Antibodies used in for biochemistry: anti-Myc (#2276S, CST), anti-EphB2 D2X2I (#83029, CST), anti-EphB2 (for IHC, # AF467 R&D Systems), anti-FLAG (M2 antibody, Sigma-Aldrich), anti-HA (Invitrogen, # 26183), anti-Actin (Sigma-Aldrich, #A2228), anti-pTyr 4G10 (gift from P. Mangeat, CRBM, Montpellier, France), anti-SLAP clone C-19 (Santa Cruz Biotechnology, sc-1215). SFK activity was assessed using an anti-phospho-SRC antibody (Invitrogen, PA5-97366 for WB, and #2101 from CST for IHC), which also cross-reacts with other p-SFKs, revealed increased SFK activity. All antibodies utilized in IHC were used according to supplier protocols. SFK activities were revealed with the help of SignalStain® Boost IHC Detection Reagent. Inhibitors used in this study: SRCi eCF506 (MedChemExpress, #HY-112096), EPHB2 inhibitor ALW-II-49-7 (EPHB2i, MedChemExpress, #HY-18833), Pan Eph inhibitor ALW-II-41-27 (pan-EPHi, MedCehmExpress, # HY-18007). SLAP-FLAG (WT and SH3*SH2* mutant, mut) constructs were described in (Naudin et al., 2014). EPHB2-myc construct was from (Narayanan et al., 2023). EPHB2 Y594F/Y602F mutant (YF mutant) was obtained by QuickChange Site-Directed Mutagenesis Kit (Agilent) using the following oligonucleotides: 5'-gaccccaggcatgaagatctTcatcgatcctttcacctTcgaggaccccaacgaggcag-3' and 5'-ctgcctc gttggggtcctcgAaggtgaaaggatcgatgAagatcttcatgcctggggtc-3'. Other reagents: Advanced DMEM/F12 (Gibco), B27 supplement (ThermoFischer Scientific), N2 supplement (ThermoFischer Scientific), GlutaMAX (ThermoFischer Scientific), Hepes (ThermoFischer Scientific), N-acetyl cysteine (Sigma-Aldrich), CHIR-99021 (Tebu-BIO), Recombinant human R-spondin-1 (Peprotech). Tamoxifen (Merck group), Azoxymlethane (Sigma-Aldrich).

Cell culture and transfections

HT29, SW620 and HEK293T cell lines were obtained from ATCC (Rockville, MD). HT29 and SW620 cells stably expressing SLAP (or mock as a control) were described in (Mevizou et al, 2025; Naudin et al., 2014). Cells were cultured at 37°C and 5% CO2 in a humidified incubator in Dulbecco's Modified Eagle's Medium (DMEM) GlutaMAX (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 µg/ml of streptomycin, and were routinely tested (once a

week) for Mycoplasma using MycoAlert Mycoplasma detection kit (Lonza, #LT07-318). Transient plasmid transfections in HEK293T cells were performed with the jetPEI reagent (Polyplus-transfection) according to the manufacturer's instructions.

Biochemistry

Co-immunoprecipitation and Western Blotting (WB) were described in (Mevizou et al, 2025; Naudin et al., 2014). Cells or isolated colonic crypts were lysed on ice using lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 6 mM β-octylglucoside), supplemented with complete mini EDTA-free protease and phosphatase inhibitor cocktail tablets (Roche). Protein lysates (15-35 µg) were resolved by SDS-PAGE, transferred to LF/PVDF membranes using the Trans-Blot® Turbo™ system (Bio-Rad), and incubated overnight at 4 °C with the appropriate primary antibody (1:1000). Membranes were then incubated for 45 minutes with the HRP-conjugated secondary antibody (Cell Signaling, 1:4000). Signals were detected using ECL Plus (Amersham Biosciences) and imaged with the Amersham Imager 600 (GE Healthcare Life Sciences). Signal quantification and analysis were carried out using ImageJ software. For SLAP-EPHB2 association experiments, HEK293T cells were co-transfected for 48 h with the indicated FLAG-tagged and EPHB2 constructs either WT or the YF EPHB2 mutant. Endogenous EPHB2 association experiments were performed in HT29 cells stably expressing SLAP-FLAG. Cell-lysates were subjected to SLAP-FLAG immunoprecipitation using anti-FLAG magnetic beads (#A3697, Pierce). EPHB2-myc was immunoprecipitated using MYC-Tag (9B11) mouse monoclonal antibody (#2276, CST) and protein G sepharose beads (Thermo Fisher Scientific).

Organoids derived from colonic crypts

Inducible epithelial Slap knockout models were sacrificed 10 days after tamoxifen induction. Organoids were performed as described in (Nguyen et al., 2025). Briefly, the colons were collected and washed with PBS containing antibiotics. They were then cut open and further dissected into small pieces then washed with a wash buffer containing PBS 1x, penicillin streptomycin (Gibco), and Fungin (Invivo Gen) until supernatant is clear. These pieces were then incubated in a crypt isolation buffer on ice for 30 minutes (Wash buffer, 1% BSA fraction and 25 mM EDTA). Crypt fractions were then isolated after thorough pipetting, centrifuged at 300g, 4°C for 5 minutes. 500 crypts were resuspended in M1 medium mixture (Advanced DMEM/F12 supplemented with 1% Penstrep, 1% Fungin, 10 mM Hepes, 1% GlutaMAX, N-2, B-27, 1 Mm N-acetyl cysteine and Matrigel (Corning) (ratio 1:2) The mixture was plated in a 24 well plate. After Matrigel polymerization, 500 µL of M2 medium (M1 media supplemented with EGF (50 ng/mL, Biotechne), Noggin (100 ng/mL, Stem cell technologies), R-spondin1 (500 ng/mL, Stem cell technologies), Y27 (10 µM, Sigma-Aldrich), CHIR-99021 (3 µM, Tebu-Bio) and WNT3a (50 n/ mL, Thermo Fisher Scientific) was added to each well containing or not the SRC inhibitor eCF506 (100 nM, Medchem express) and in other cases the EphB inhibitors EPHB2i (ALW-II-49-7, 200 nM) and the pan-EPH inhibitor pan-EPHi (ALW-II-41-27, 100 nM, Medchem express). For organoids derived from AOM/DSS transformed CECs, tumors were excised from mice treated with AOM/DSS at day 80. They were dissociated using the Tumor Dissociation Kit mouse (Miltenyi Biotec). The dissociation was the stopped by DMEM medium containing 10% SVF. The mixture was then centrifuged the pellet was resuspended in DMEM F12 without factors. Cells were counted and plated at almost 10,000 cells per well in a 24 well plate in a mixture containing 3D Matrigel. After 30 minutes of incubation a complete F12 medium was added to the solidified domes containing or not the SRC inhibitor eCF506 (100 nM) (Fraser *et al*, 2016).

Tumoroids-derived from CRC cells

2 000 - 5 000 human CRC cells were resuspended in Advanced DMEM/F12 supplemented with 1% Penstrep, 2mM L-glutamine, N-2 and Matrigel (Corning) (1:2 ratio) prior to plating in 24-well plates. After polymerization of Matrigel, 0.5 mL Advanced DMEM/F12 supplemented with EGF (20 ng/ml, Biotechne) and FGF (10 ng/ml, Biotechne) was added. Tumoroids were cultured at 37°C and 5% CO2 in a humidified incubator.

Microscopy and imaging

Histological slides were digitized using a Nanozoomer scanner (Hamamatsu) with a 40X objective and viewed with NDP.view2 software (Hamamatsu). Fluorescence images were captured using an AxioImager Z2 microscope (Zeiss) operated with Zen software. Image processing was performed in ImageJ.

TCGA COAD analysis

Gene expression and clinical data from the TCGA colon adenocarcinoma (COAD) cohort were analyzed using GEPIA2 (*http://gepia2.cancer-pku.cn/*). Kaplan–Meier disease-free survival analyses were performed using the GEPIA2 Survival Analysis module, with patients stratified into high-and low-expression groups based on the median expression value. Stem cell-like signature scores were calculated using the GEPIA2 Signature Score module (Merlos-Suárez et al., 2011) from the indicated gene set, and associations with disease-free survival were assessed by log-rank testing.

Statistical analysis

All analysis was performed using GraphPad Prism (9.3.1). Data are presented as the mean ± SEM. the Mann-Whitney test was used. The statistical significance level is illustrated with p values: *p≤0.05, **p≤0.01, ***p≤0.005, **** p≤0.0051(t-test).

Results

Constitutive *Slap* inactivation increases CEC proliferation

To investigate SLAP function in the colon, we generated constitutive Slap-deficient mice by inserting a *LacZ* cassette into the Slap gene (*Slap-/-* mice) (Fig. 1A). β-galactosidase activity staining revealed a gradient of Slap expression toward the top of the crypts throughout the colonic epithelium (Fig. S1A). This expression pattern was confirmed at the protein level by immunofluorescence staining using a Slap-specific antibody (Fig. 1A). Loss of Slap led to increased colon thickness in 3-month-old mice, characterized by elongated crypts (Fig. 1A). This epithelial hyperplasia was accompanied by elevated colonic epithelial cell (CEC) proliferation (Fig. 1A) and an increased number of goblet cells (Fig. S1B), indicating that Slap regulates both CEC proliferation and differentiation.

RNAscope analysis of Lgr5, a marker of colonic epithelial stem cells (CSCs) (Barker et al., 2007), showed increased expression at crypt bases in Slap-deficient mice (Fig. 1A), suggesting enhanced CSC traits. Given the role of SFKs in regulating colonic stem/progenitor proliferation (Cordero et al., 2014; Imada et al., 2016; Kohlmaier et al., 2015; Sirvent et al., 2020), we assessed whether Slap modulates SFK activity. Immunohistochemical analysis of an antibody detecting the activated form of Src (pSRC), which also cross-reacts with other p-SFKs, revealed increased SFK activity throughout the crypts of Slap-deficient mice (Fig. S1B). This suggests that SLAP plays a role in regulating intestinal epithelial homeostasis, in addition to its well-established function in lymphocyte development (Sosinowski et al., 2001).

Cell-autonomous role of Slap in CEC proliferation

To determine if Slap functions in a cell-autonomous manner, we generated an inducible, intestinal-specific Slap-deficient mouse model by crossing *Slap flox/flox* mice with *villin-CreERT2* mice (*Slap $^{f/f}$ Villin Cre^{ERT2}*). Tamoxifen treatment for 10 days efficiently deleted Slap in the epithelium, as confirmed by a marked reduction in Slap immunofluorescence staining in the intestinal epithelium, while resident immune cells retained Slap expression (Fig. S1B). Colonic Slap depletion recapitulated the phenotype of constitutive Slap deficient mice, including increased colon thickness, enhanced CEC proliferation (Fig. 1B), elevated goblet cell numbers (Fig. S1C), and increased CSC activity (Fig. 1B). To further investigate the cell-autonomous role of Slap in

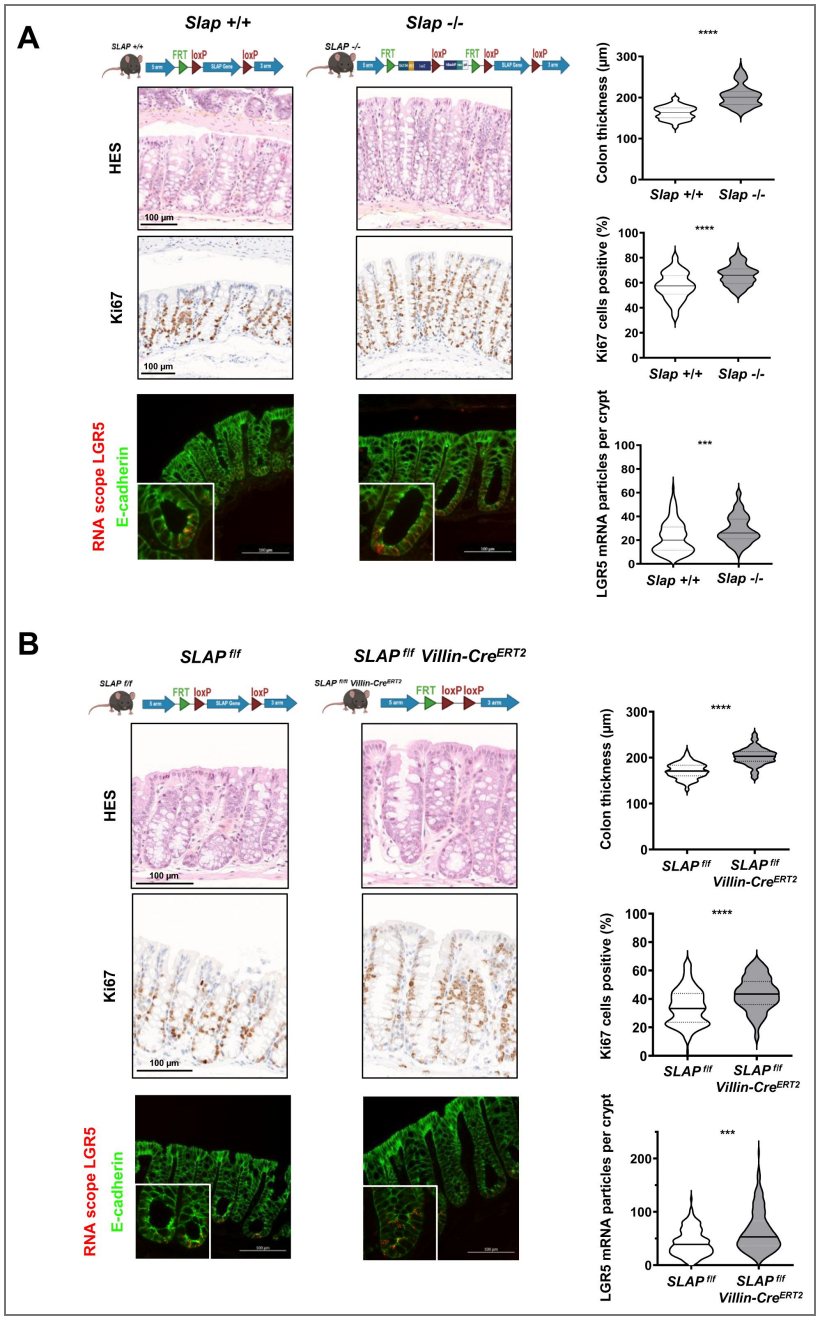

Figure 1. Murine Slap inactivation induces colonic epithelial hyperplasia.

(A) Constitutive Slap deletion. Top: Hematoxylin and eosin (H&E) staining of transverse colon sections. Crypt thickness was measured and quantified as mean ± SEM from 25-40 crypts per mouse (n = 6 female mice per group). Middle: Immunohistochemistry (IHC) for the proliferation marker Ki67, with quantification of Ki67-positive colonic epithelial cells (CECs). (mean ± SEM from 20-40 crypts per mouse; n = 5-6 mice per group). Bottom: RNAscope analysis of *Lgr5* to identify colonic stem cells, combined with E-cadherin immunofluorescence to delineate crypt architecture. Inset, higher (4X) magnification highlighting *Lgr5* expression. *Lgr5* RNA particles were quantified and are shown as mean ± SEM from 20-40 crypts per mouse (n = 5-6 mice per group). ***p<0.001, ****p<0.0001 Mann–Whitney test. **(B)** Inducible epithelial-specific Slap deletion (villin-CreERT2). Top: H&E staining of transverse colon sections with quantification of crypt thickness (mean ± SEM from 25-40 crypts per mouse, n = 6 mice/group). Middle: IHC for Ki67 with quantification of Ki67-positive CECs (mean ± SEM from 20-40 crypts per mouse; n = 5-6 mice per group). Bottom: RNAscope analysis of Lgr5 combined with E-cadherin immunofluorescence; Inset, higher (4X) magnification highlighting *Lgr5* expression. Lgr5 RNA particles were quantified (mean ± SEM from 20-40 crypts per mouse, n = 5-7 mice per group). Mice were analyzed at 3 months of age, 10 days after tamoxifen induction. ***p<0.001, ****p<0.0001 Mann–Whitney test.

regulating CEC proliferation, we performed ex vivo analyses using isolated colonic crypts. Organoids derived from Slap-deficient epithelium were more numerous and larger than those from controls (Fig. 2A).

Consistent with a role for Slap in restraining SFK signaling, Slap-deficient colonic crypts exhibited a 2-3-fold increase in SFK activity and global cellular protein tyrosine phosphorylation (pTyr) levels (Fig. 2B). An increase in protein tyrosine phosphorylation was also observed in organoids derived from Slap-deficient CECs and was reduced following treatment with the selective SFK inhibitor eCF506 (SRCi) (Fraser et al., 2016). Consistently, SRCi treatment decreased pSRC levels, confirming effective SFK inhibition (Fig. S2). Functionally, the enhanced growth of Slap-deficient organoids was abolished by eCF506 treatment, demonstrating that the proliferative phenotype was SFK-dependent (Fig. 2C). Together, these findings establish SLAP as a cell-autonomous suppressor of SFK-driven CEC proliferation.

Inducible Slap inactivation in the colon promotes colon tumorigenesis

We next evaluated Slap's role in colon tumorigenesis using the azoxymethane/dextran sulfate sodium (AOM/DSS) model of colitis-associated cancer (CAC), in which a single injection of the carcinogen AOM is followed by repeated cycles of DSS-induced colonic inflammation, leading to the development of colorectal tumors that recapitulate key features of inflammation-driven CRC (De Robertis et al., 2011) (Fig. 3A). In the inducible Slap knockout model, Slap deletion was initiated by tamoxifen (TAM) administration 10 days before AOM treatment and maintained by a second TAM administration at day 45 to ensure sustained Slap inactivation throughout tumor development (Nguyen et al., 2025). Under these conditions, Slap-deficient mice developed significantly more tumors and exhibited increased SFK activity within tumors compared with control animals (Fig. 3B). Furthermore, tumoroids generated from these mice also showed enhanced growth, which was abolished by SFK kinase inhibition (Fig. 3C), further supporting an SFK-dependent oncogenic mechanism. Interestingly, pharmacological SFK inhibition had no effect on tumoroids derived from control AOM/DSS-treated mice, consistent with previous reports that AOM/DSS-induced carcinogenesis involves oncogenic RAS mutations (Takahashi & Wakabayashi, 2004) and chronic inflammation. These findings suggest that while RAS-driven transformation can occur independently of SFK activity, Slap loss unleashes a parallel, SFK-dependent oncogenic pathway. The human relevance of this SLAP anti-oncogenic function was confirmed by modulating SLAP expression in CRC cells. In human SLAP-low HT29 and SW620 cell-lines, SLAP overexpression inhibited aldehyde dehydrogenase (ALDH) activity (Fig. S3A), a well-established marker of CRC stem cells, as well as tumoroid growth (Fig. S3B). Together, these results establish SLAP as a critical suppressor of SFK-driven colon tumorigenesis and cancer stem cell activity.

Slap target EphB2 to restrict SFK proliferative function in CEC

To investigate the Slap-dependent mechanism driving this phenotype, we hypothesized that Slap targets an upstream SFK activator within the RTK family that regulates colonic stem cells and CEC proliferation. Among RTKs expressed in colonic crypts, EphB2 and EphB3 are highly enriched in the CSC compartment and promote kinase-dependent CEC proliferation (Batlle et al., 2002; Genander et al., 2009; Holmberg et al., 2006). SFKs have also been reported as interactors and downstream effectors of EPHB2 signaling (Chang et al., 2024; Georgakopoulos et al., 2006; Leroy et al., 2009; Pasquale, 2024; Zisch et al., 1998). Supporting this model, Western Blotting analysis revealed elevated EphB2 protein levels in isolated Slap-deficient colonic crypts (Fig. 4A), which was further confirmed by IHC analysis showing increased EphB2 expression throughout the colonic crypts of Slap-deficient mice (Fig. 4B). This effect was recapitulated in CRC cells where enforced SLAP expression in SLAP-low HT29 cells decreased EPHB2 protein abundance. Interestingly, SLAP overexpression did not alter EPHB2 mRNA levels (Fig. S3A), in accordance with a posttranslational mechanism involved in this process, as reported for EPHA2 (Naudin et al., 2014). Co-expression experiments in HEK293T cells revealed that EPHB2 associates with SLAP (Fig. 4C), and this interaction required the SLAP SH2 and SH3 domains, as

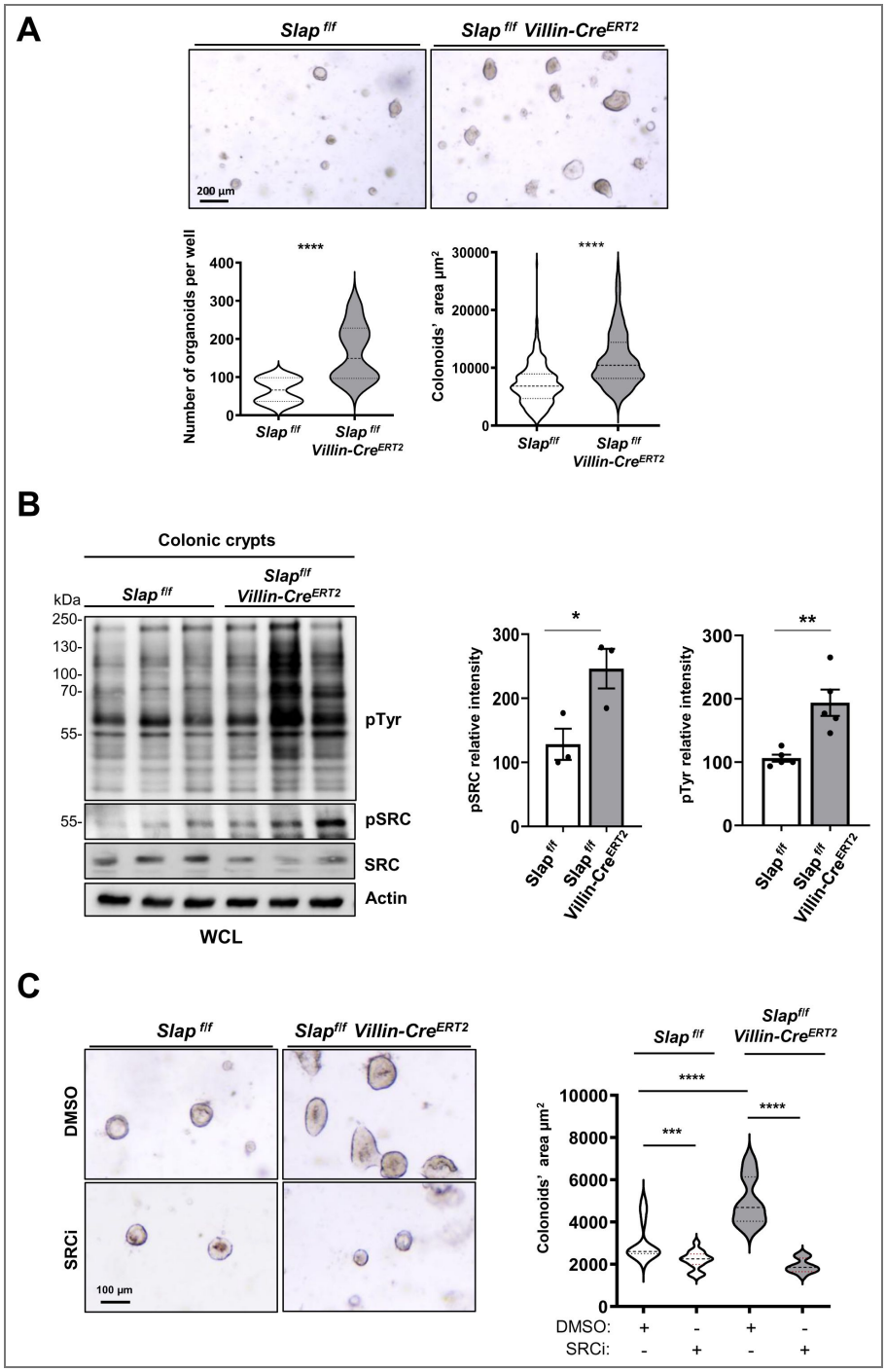

Figure 2. Intestinal Slap inactivation enhances colonic organoid development through SFK activation.

(A) Slap-dependent colonic organoid development. Representative images of colon-derived organoids from *Slap* $^{f/f}$ and *Slap* $^{f/f}$ *Villin-Cre*^{ERT2} mice cultured in Matrigel for 2 days. Quantification of organoid number and area is shown as mean ± SEM from 100-200 organoids per mouse (n = 8 mice per group). ****p<0.0001 Mann–Whitney test. **(B)** Intestinal Slap deletion increases SFK activity and global protein tyrosine phosphorylation in colonic crypts. Representative immunoblots (right) and quantification (left) of phospho-SFK (pSRC) and total phospho-tyrosine levels in lysates from isolated colonic crypts of the indicated genotypes. Data are presented as mean ± SEM from n = 4-5 mice per group. *p<0.05; **p<0.001 t test. **(C)** Slap-dependent colonic organoid expansion requires SFK activity. Representative images and quantification of colon-derived organoids from *Slap* $^{f/f}$ and *Slap* $^{f/f}$ *Villin-Cre*^{ERT2} mice cultured for 2 days in the presence or absence of the SRC-family kinase inhibitor eCF506 (100 nM). Data are shown as mean ± SEM from 100-200 organoids per mouse, n = 4 mice per group. ***p<0.001, ****p<0.0001 Mann–Whitney test.

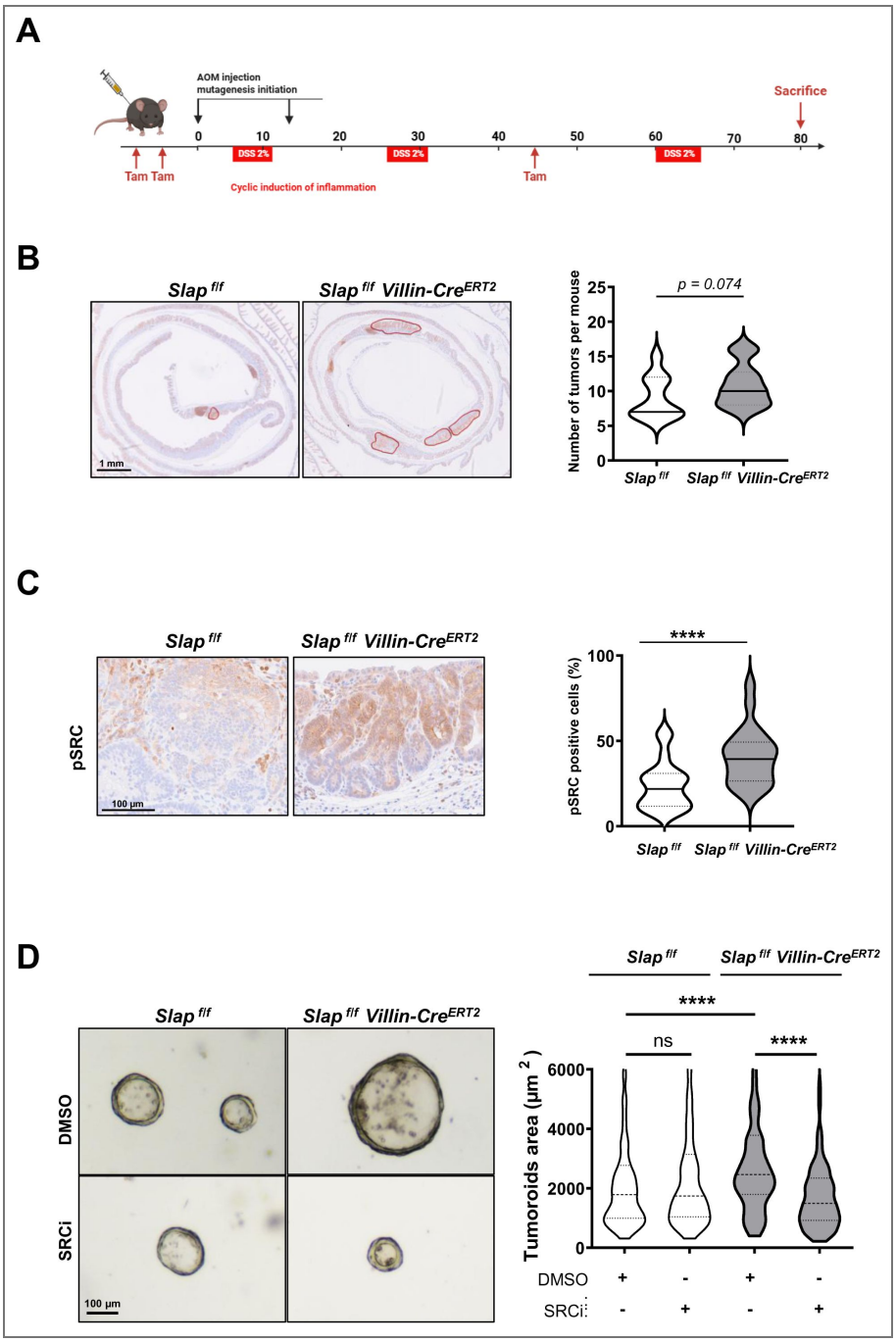

Figure 3. Inducible Slap intestinal inactivation drives colon tumorigenesis and promotes SFK-dependent tumoroid growth.

(A) Experimental workflow of AOM/DSS-induced colon tumorigenesis. Mice received tamoxifen, followed 10 days later by a single azoxymethane (AOM) injection to induce mutagenesis and one cycle of dextran sodium sulfate (DSS) administered for 7 consecutive days. A second AOM injection was then performed, followed by two additional DSS cycles (7 days each). Mice were sacrificed at day 80. **(B)** Epithelial Slap loss accelerates colon tumor development. Left, representative H&E-stained sections showing transformed regions. Right, quantification of epithelial lesion area (mean ± SEM; n = 11-15 mice per group. Statistical analysis is shown (Mann–Whitney test). **(C)** Slap depletion increases SFK activation in colonic tumors. Left, representative immunohistochemistry for phosphorylated SFK (pSRC) in transformed epithelium. Right, quantification of p-SFK–positive cells per mm² (mean ± SEM; n = 7-9 mice per group; ****p < 0.0001, Mann–Whitney test). **(D)** Tumoroids derived from *Slap* $^{f/f}$ and *Slap* $^{f/f}$ *Villin-Cre*^{ERT2} mice were cultured in 3D Matrigel in the presence or absence of SRC inhibitor (SRCi) and imaged at day 3. Tumoroid area (µm²) was quantified following SRCi treatment (mean ± SEM; 150–250 organoids per mouse, n = 4 mice per group; ns: p>0.05; ****p < 0.0001, Mann–Whitney test).

it was reduced with a SLAP mutant harboring inactive point mutations in the SH2 and SH3 domains (SLAP-mut) (Fig. 4C)(Mevizou et al., 2026). The interaction also depended on phosphorylation of the juxta-membrane Tyr596 and Tyr602, which mediates SRC-SH2 binding (Zisch et al., 1998), because mutation of these residues to phenylalanine (YF EPHB2) diminished SLAP-EPHB2 binding (Fig. 4C). Endogenous EPHB2 similarly interacted with SLAP in SLAP-expressing HT29 cells, confirming this association in CRC cells (Fig. S4B). Consistent with enhanced EphB2 signaling, isolated Slap-deficient colonic crypts displayed increased EphB2 tyrosine phosphorylation together with elevated levels of associated phosphorylated SFKs (Fig. 4A). Collectively, these findings identify EPHB2 as a novel SLAP interactor and target, supporting a role for SLAP in the regulation of EPHB2/SFK signaling.

We next asked whether EphB2 contributes to the Slap-dependent regulation of colonic organoid growth by inhibition of EphB2 kinase activity with the selective inhibitor ALW-II-49-7 (EPHB2i) (Choi et al., 2009; Noberini et al., 2012; Zhang et al., 2025). While EPHB2i had no effect on the growth of wild-type organoids, it markedly suppressed the enhanced growth observed in Slap-deficient organoids (Fig. 4C). Treatment with the pan-EPH kinase inhibitor ALW-II-41-27 (pan-EPHi) (Amato et al., 2014; Choi et al., 2009; Ferrao Blanco et al., 2024), produced a similar reduction (Fig. 4C), supporting a role for EphB2 kinase activity in the Slap-dependent phenotype. Of note, EPHB2i did not alter the kinase activity of SRC expressed in HEK293T cells in contrast to EPHB2, arguing against a direct off-target effect on SFK (Fig. S3B). Moreover, pEPHi reduced both the elevated phosphotyrosine levels and SFK activation induced by Slap deletion in colonic organoids (Fig. S2), demonstrating that SFK hyperactivation in Slap-deficient CECs is dependent upon EphB2 signaling. Together, these data show that Slap limits colonic organoid growth by restricting EphB2-dependent activation of Src and reveal an EphB2-SFK signaling axis as a key driver of the hyperproliferative phenotype associated with Slap-deficiency. (Fig. S4C). Notably, a similar mechanism was observed in human CRC cells. Pharmacological inhibition of EPHB2 reduced tumoroid growth in a CRC model expressing low levels of SLAP, whereas this inhibitory effect was largely abolished upon SLAP overexpression. Importantly, a SLAP mutant (SLAP mut) that is unable to interact with EPHB2 fails to suppress tumoroid growth and restores sensitivity to EPHB2 inhibition, indicating that the tumor-suppressive activity of SLAP depends on its interaction with EPHB2 (Fig. S5A-C). Collectively, these findings indicate that SLAP restrains the tumor-promoting activity of EPHB2 and suggest that the SLAP–EPHB2–SRC signaling axis is conserved between normal CECs and CRC.

Finally, we sought additional evidence supporting SLAP-EPHB2 signaling in CRC patients. We observed a weak but significant inverse correlation between SLAP expression and the colorectal CSC-like activity score in TCGA tumors (Fig. S5A). In addition, although expression of EPHB2, SLAP, or the SLAP-related gene SLA2 alone was not associated with disease-free relapse in microsatellite-stable (MSS) colorectal cancer patients (Fig S5B), co-expression of SLAP and SLAP2 with EPHB2 was associated with improved prognosis (Fig 5D). Notably, this association was specific to MSS tumors, which are characterized by low immune infiltration, and was not observed in microsatellite instability (MSI) CRC, which display high immune infiltration (Fig. 5D). Together, these findings suggest that SLAP expression in tumor cells may contribute to the regulation of EPHB2-dependent CSC signaling in CRC.

Discussion

SFKs are potent drivers of intestinal stem cell proliferation and CRC, yet how their activity is restrained in normal epithelium remains unclear. Here, we identify the adaptor protein SLAP as a key epithelial-intrinsic regulator of SFK signaling in the colon. Epithelial loss of Slap increases proliferation, expands the stem cell compartment, and elevates SFK activation. Complementary organoid studies demonstrate that this requirement is cell autonomous. Importantly, pharmacologic SFK inhibition fully rescues the hyperproliferative phenotype of Slap-deficient organoids and tumoroids, revealing a crypt-intrinsic proliferative program that is SFK-dependent and normally constrained by Slap.

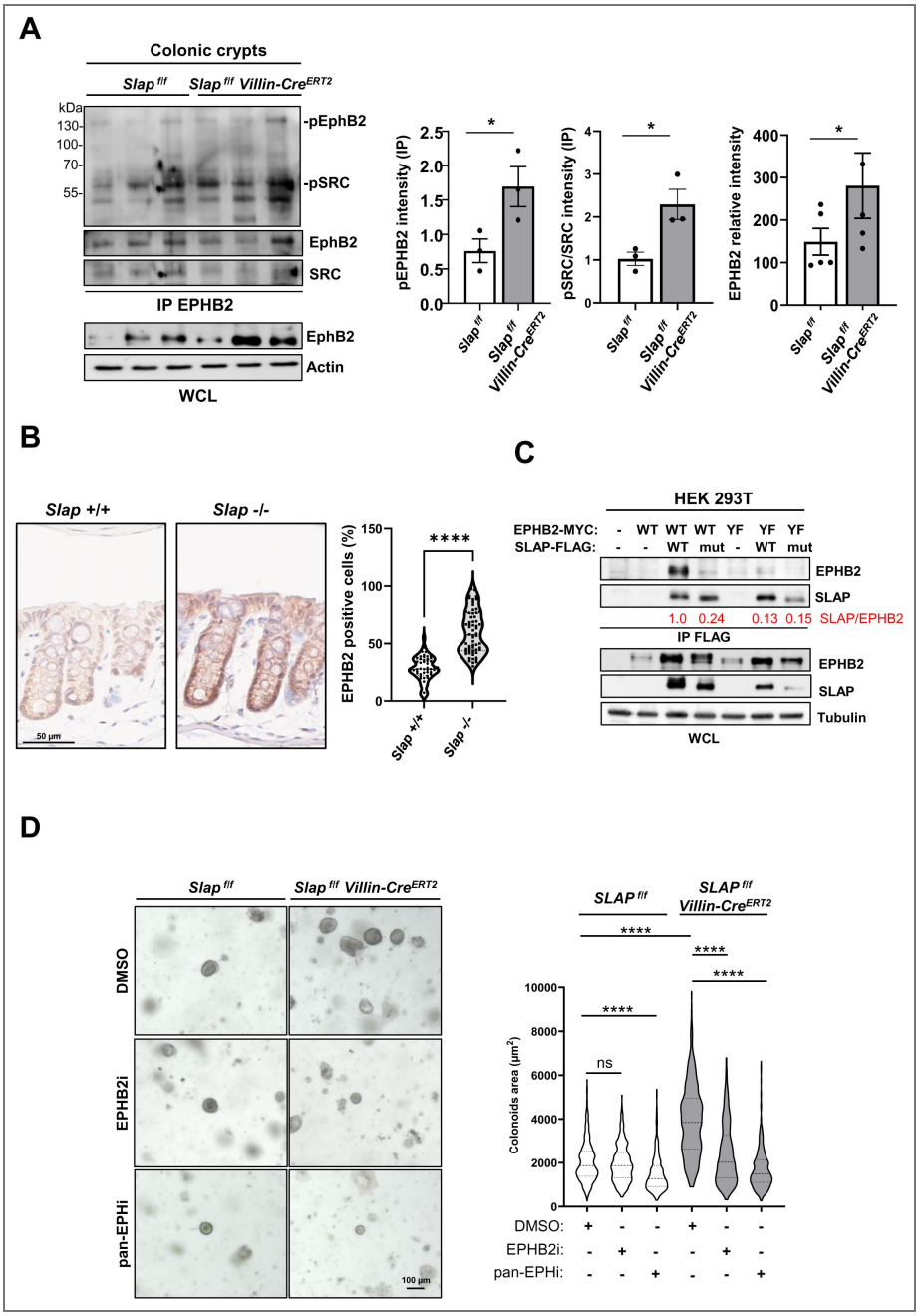

Figure 4. Slap targets EphB2 to restrict SFK proliferative signaling in CEC.

(A) Intestinal Slap deletion increases EphB2 expression, EphB2 tyrosine phosphorylation, and its association with active SRC (pSRC) in colonic crypts. Left, quantification; right, representative immunoblots of EphB2 levels, EphB2 tyrosine phosphorylation, and associated pSRC following EphB2 immunoprecipitation from isolated colonic crypt lysates of the indicated mouse strains. Mean ± SEM; n = 3–5 mice per group; *p < 0.05, Student's t-test. **(B)** IHC analysis of EphB2 expression in colonic crypts from control and Slap-deficient mice. Left, representative images; right, quantification (mean ± SEM; n = 3 mice per group; ****p < 0.0001, Mann–Whitney test). **(C)** SLAP–EPHB2 interaction in HEK293T cells. Co-immunoprecipitation of the indicated SLAP-FLAG constructs (wild-type, WT; or SH3*SH2* mutant, SLAP mut) and EPHB2-MYC constructs (wild-type, WT; or Y596F/Y602F mutant, YF) transfected into HEK293T cells. Expression levels of SLAP and EPHB2 and relative quantification of SLAP-EPHB2 interaction are shown (representative example of three independent experiments). **(C)** Slap-dependent colon organoid expansion requires EphB2 activity. Representative images and quantification of colon-derived organoids from *Slap* $^{f/f}$ and *Slap* $^{f/f}$ *Villin-Cre*^{ERT2} mice treated with the indicated EPH inhibitors (EPHB2i: 200 nM, pan-EPH2i: 100 nM, or DMSO control) and analyzed at day 2 (mean ± SEM; 150-200 organoids per mouse; n = 4 mice per group; ns: p>0.05; ****p < 0.0001, Mann–Whitney test).

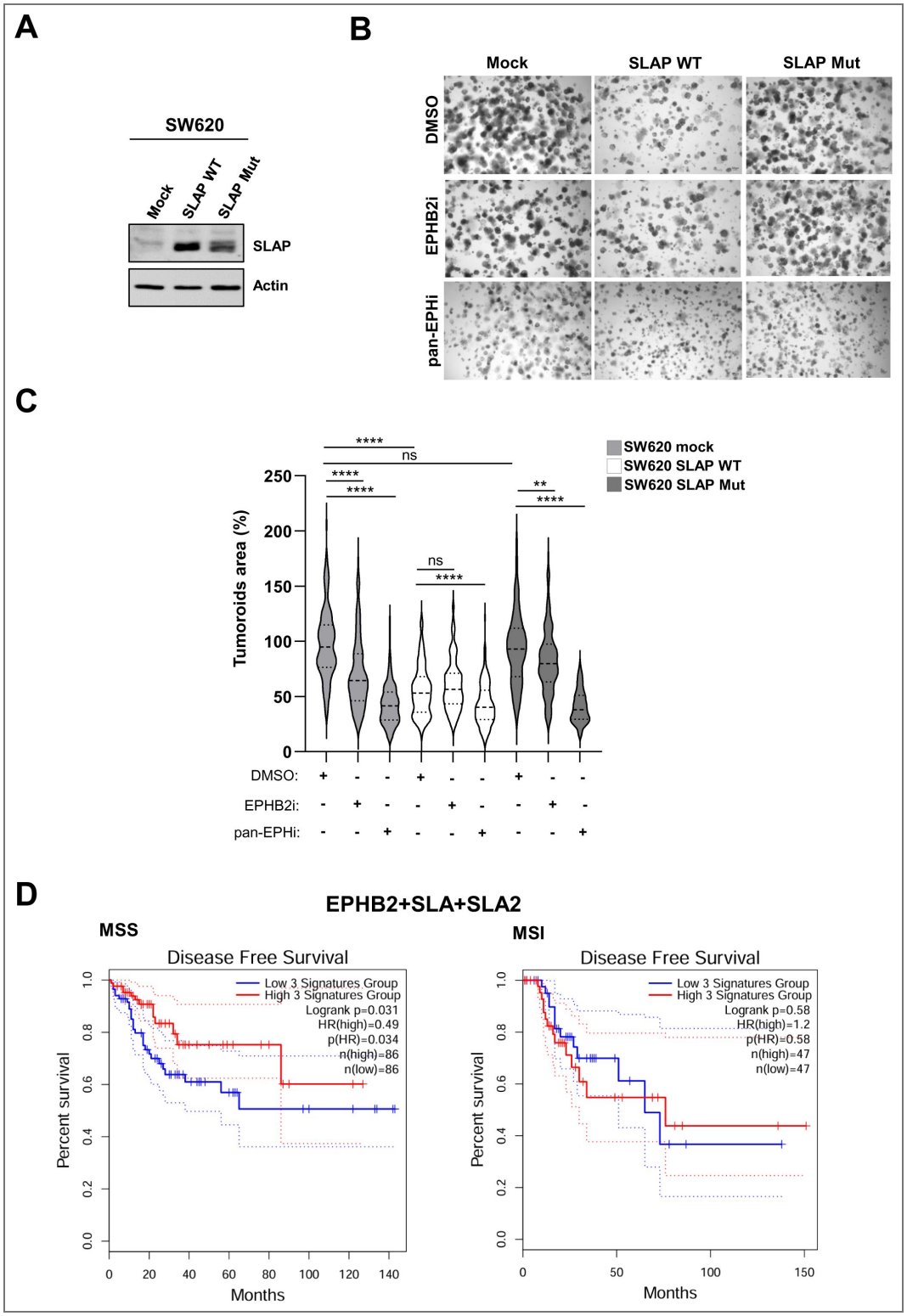

Figure 5. SLAP suppresses EPHB2-dependent tumoroid development of CRC cells and is associated with improved prognosis in MSS CRC.

(A) SLAP expression in SW620 CRC cells transduced with empty vector (mock), wild-type SLAP, or an EPHB2-binding-defective SLAP mutant (SLAP Mut). **(B)** Representative tumoroid images from SW620 cells expressing mock, SLAP, or SLAP m and treated with indicated EPHB2 inhibitors (EPHB2i: 200 nM, pan-EPH2i: 100 nM, or DMSO control). **(C)** Quantification of tumoroid size from (B) (mean ± SEM; 150-200 tumoroids; n = 3; ns: p>0.05; **p<0.001; ***p<0.0005; ****p < 0.0001, Mann–Whitney test). **(D)** Disease-free relapse analysis of TCGA MSS and MSI CRC patients stratified according to combined EPHB2, SLAP, and SLA2 expression. High SLAP/SLA2 and EPHB2 co-expression is associated with improved prognosis in MSS patients.

Mechanistically, our findings identify EPHB2 as a critical upstream activator of SRC signaling that is negatively regulated by SLAP. EPHB2 is highly expressed in intestinal stem and progenitor cells, where it contributes to crypt homeostasis and colorectal tumorigenesis (Batlle et al., 2005; Genander et al., 2009; Holmberg et al., 2006). We show that genetic loss of Slap results in elevated EPHB2 expression, increased SRC activation, and enhanced epithelial proliferation, whereas pharmacological inhibition of EPHB2 suppresses SRC activation and completely reverses the growth advantage of Slap-deficient organoids. Furthermore, EPHB2 inhibition selectively impairs the growth of CRC tumoroids expressing low levels of SLAP, supporting the existence of a conserved SLAP-EPHB2-SRC signaling axis in both normal and transformed intestinal epithelium.

Although the precise molecular mechanism remains to be established, SLAP may restrain EPHB2 signaling through several non-mutually exclusive mechanisms. SLAP has been shown to promote the ubiquitin-dependent downregulation of multiple RTKs through interactions with E3 ubiquitin ligases, including CBL family proteins, UBE4A, and UBE3C (Mevizou et al., 2025; Myers et al., 2006; Naudin et al., 2014; Sirvent et al., 2008; Zutshi et al., 2024). Alternatively, SLAP may limit productive SRC recruitment and activation downstream of EPHB2 through its SH2 and SH3 domains (Roche et al., 1998). Future studies will be required to determine how SLAP controls EPHB2 abundance and signaling output in intestinal stem cells. In addition, other RTKs known to activate SFKs, including members of the EGFR family (Cordero et al., 2014; Wong et al., 2012), may also contribute to the phenotype associated with SLAP loss. Moreover, Slap loss may affect EphB2's role in cell positioning (Genander et al., 2009; Holmberg et al., 2006), contributing further to excessive proliferation.

Together, these findings establish SLAP as an epithelial tumor suppressor that limits SFK-driven stem cell proliferation and reveal a non-genetic mechanism of SRC oncogenic activation in CRC involving RTK hyperactivation. (Leroy et al., 2009). More broadly, they suggest that SLAP loss may define CRC subsets with heightened dependence on SFK signaling, offering opportunities for therapeutic stratification and refinement of SRC-targeted interventions.

Figure supplements

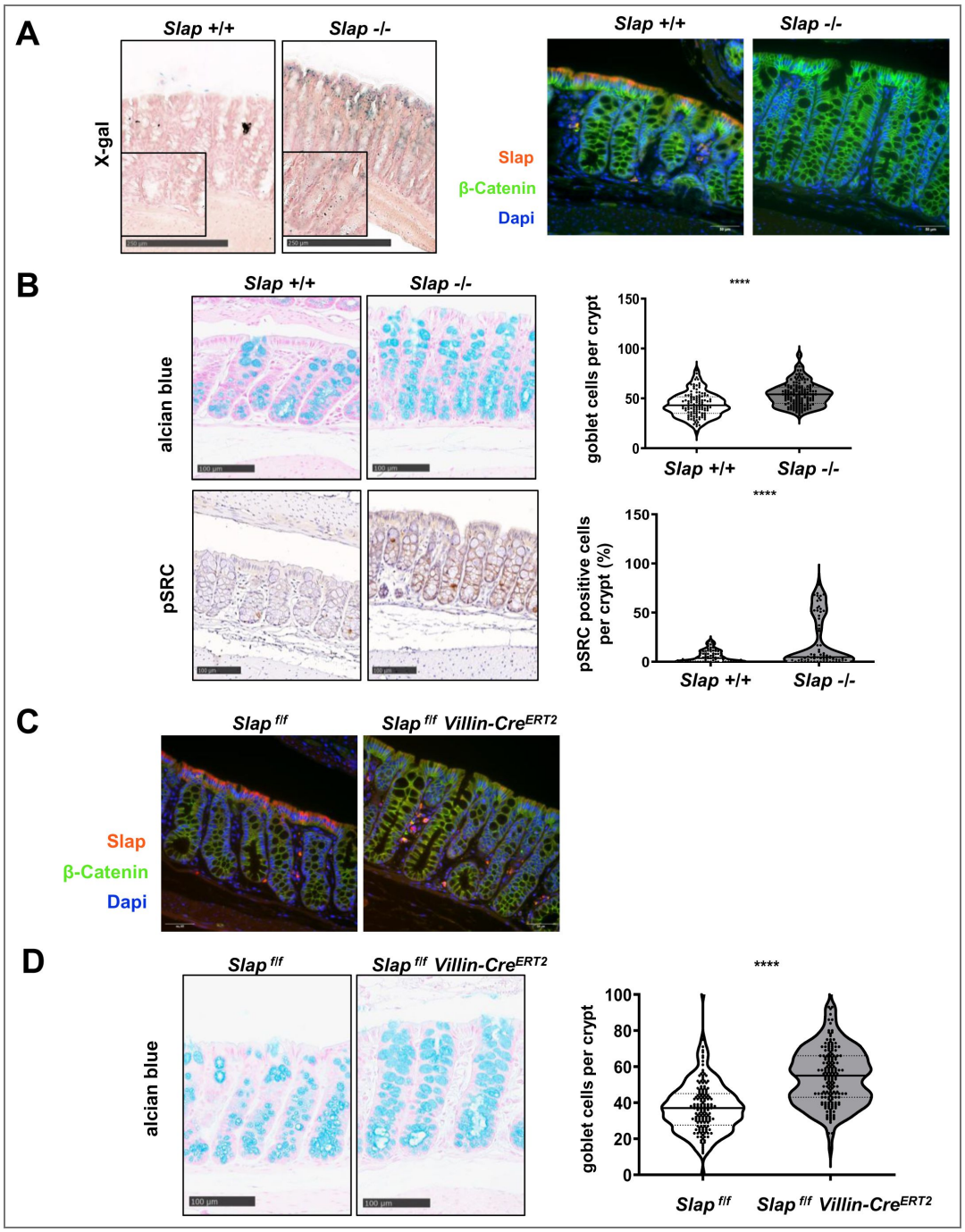

Figure S1. Slap inactivation increases goblet cell numbers and SFK activity in the colon. (**A**) Left: X-Gal staining of Slap expression. Insert: 4X magnification highlighting Slap expression at the base of colonic crypts. Right: IF staining for Slap (red), nuclei (blue), and β-catenin (green). (**B**) Slap inactivation increases goblet cell numbers and SFK activity. Top: Alcian blue staining of goblet cells with quantification. Bottom: IHC for p-SRC and its quantification.(**C**) Inducible Slap inactivation increases goblet cell numbers. Top: IF staining of Slap (red), nuclei (blue), and β-catenin (green) in the colon after 10 days of tamoxifen treatment. Bottom: Alcian blue staining of goblet cells and quantification.

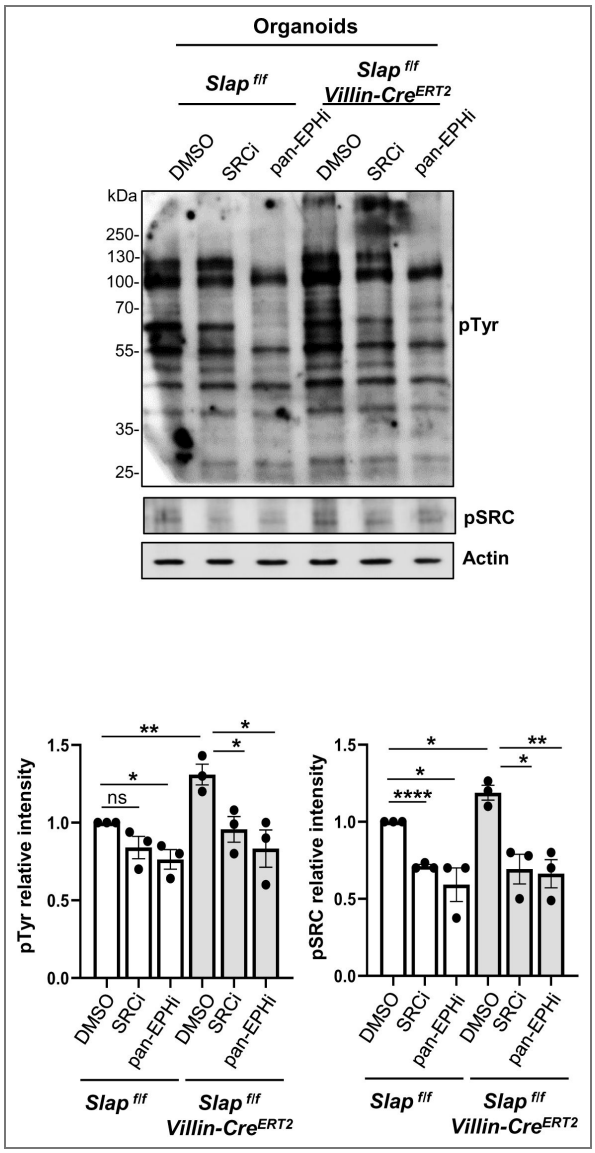

Figure S2. EPH-dependent phosphotyrosine accumulation and SFK activation in Slap-deficient colonic organoids.

pTyr and activated SFKs (pSRC) levels in WT and Slap-deficient colonic organoids under the indicated conditions (EPHi and SRCi: 100nM for 3hrs); top: presentative example; bottom: quantification (mean ± SEM n=3; ns: P>0.05; *P < 0.05, **P < 0.01, ****P < 0.0001).

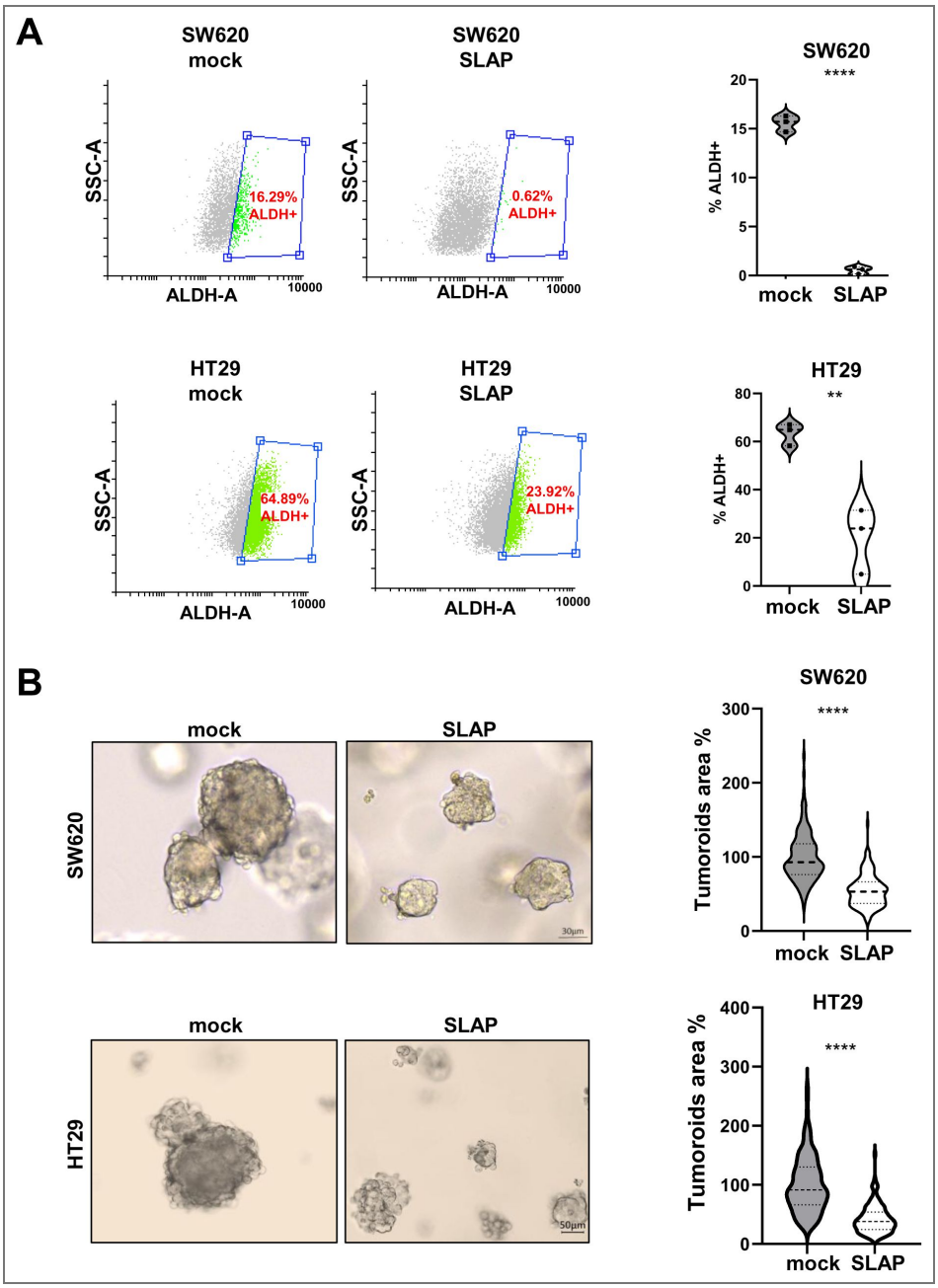

Figure S3. SLAP suppresses human CRC stem cell properties.

(A) SLAP overexpression reduces ALDH activity in SLAP-low HT29 and SW620 cells (representative FACS plots and quantification; mean ± SEM; n=3; **p<0.01, ****p<0.0001; unpaired t-test).**(B)** SLAP overexpression decreases tumoroid formation in HT29 and SW620 cells (representative images and quantification 25-50 organoids/condition; mean ± SEM; n=3; ****p<0.0001; Mann–Whitney).

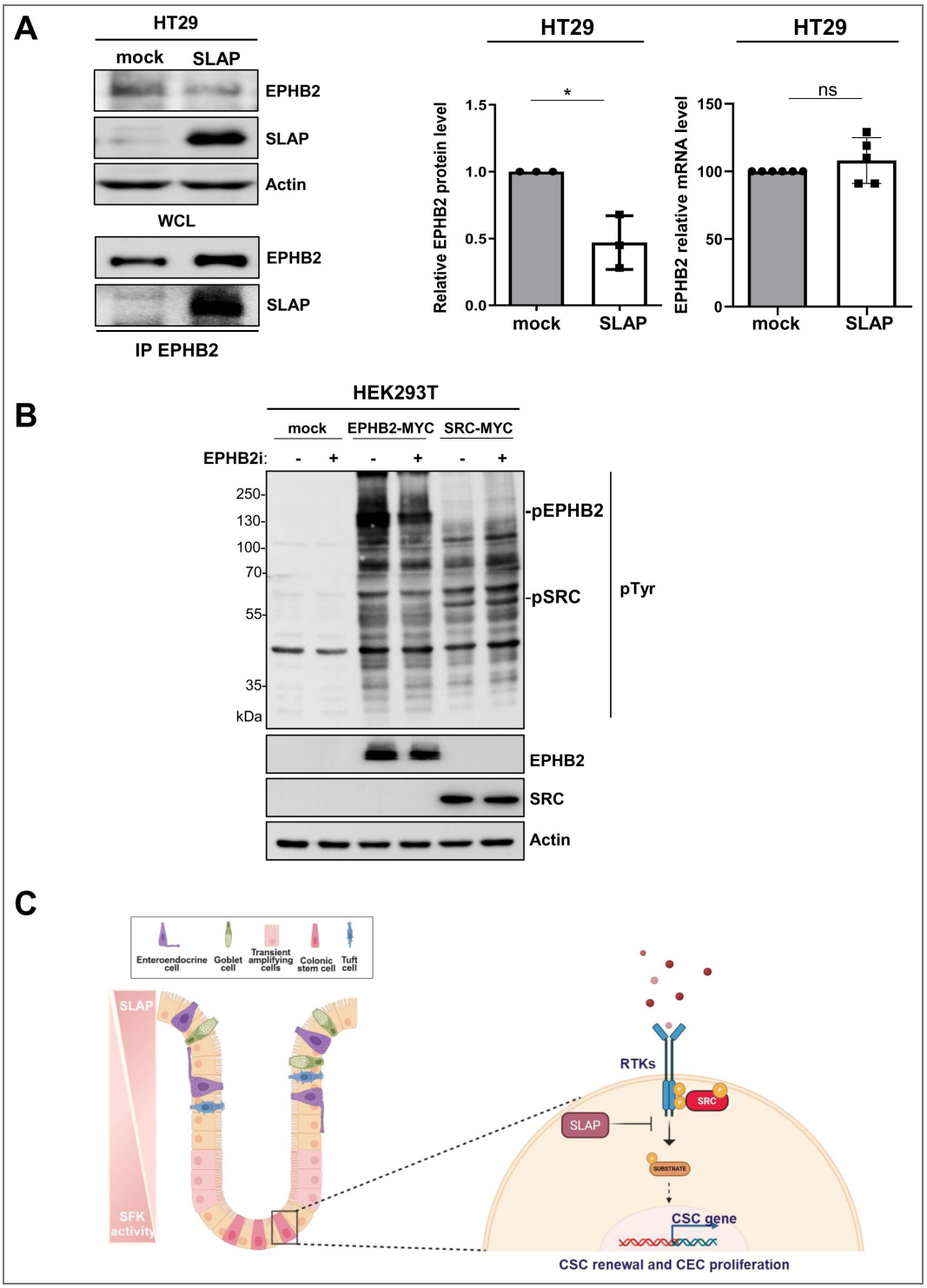

Figure S4. SLAP interacts with EPHB2 and reduces its levels in HT29 cells.

(A) EPHB2 expression and association with SLAP in CRC cells (transcript and protein; mean ± SEM; n=3-5; ns>0.05; *p<0.05; t-test). **(B)** EPHB2i (400 nM) reduces EPHB2 activity (i.e. pTyr level) in HEK293T cells. **(C)** Model of SLAP regulation of SFK signaling in CSCs to maintain colonic epithelial homeostasis by limiting RTK activity.

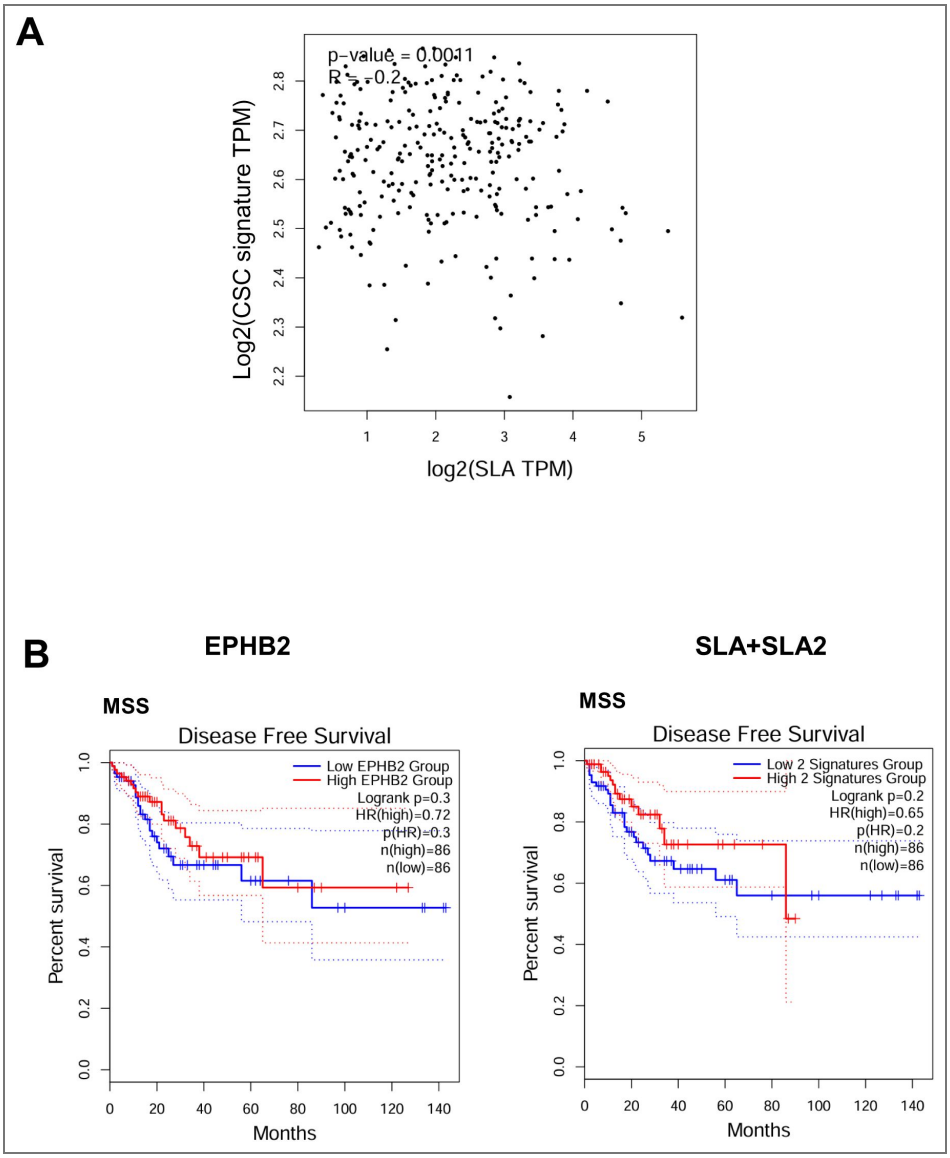

Figure S5. Contribution of SLAP expression to the regulation of EPHB2-dependent CSC signaling in CRC patients.

(A) Negative correlation between SLAP expression and curated CSC/progenitor gene signature (15 genes including LGR5, ASCL2, OLFM4, BMI1, PROM1, CD44, MKI67, EPCAM, SOX9, SMOC2, TNFRSF19, AXIN2, CYP26A1, ITGA6, PCNA) in TCGA COAD samples (R = −0.2, p = 0.0011). **(B)** EPHB2, or SLA and the SLAP-related gene SLA2 expression do not correlate with disease-free survival in TCGA MSS COAD samples.

Data availability

The data are available upon reasonable request.

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Additional information

Authors contribution

All authors contributed extensively to the work presented in this paper. Experimental analysis and Data acquisition: DN, ZH, FC, VS, KE, YB, ZH, MM, JN, JP and AS. Genetical modified mouse models: AFL, AS, DN, CP and MH. Project supervision: SR and AS. Funding acquisition and Writing of the paper: SR.

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Peer reviews

Reviewer #1 (Public review):

Naim et al., use genetically engineered mouse models and tissue culture cell lines to investigate the role of the SLAP adaptor protein in colonic epithelium and colon tumour formation. The SLAP adaptor protein is known to be a negative regulator of tyrosine kinase signaling in hematopoietic cells but its role outside the immune system is less well defined. Here the authors use genetically engineered SLAP deficient mice, tissue specific SLAP KO, and colonic organoids to demonstrate that SLAP is expressed in cells of the colonic epithelium where it acts as a cell autonomous regulator of proliferation and differentiation. In addition, they provide biochemical evidence that loss of SLAP expression in cultured colonic organoids results in increased Src family kinase activity and global tyrosine phosphorylation, consistent with its known role as a suppressor of tyrosine kinase activity in immune cells. Consistently, treatment with a SRC kinase inhibitor inhibited growth of SLAP deficient organoids. These data provide solid evidence of a cell autonomous role of SLAP in the colonic epithelium.

Using a chemically induced model of colitis-associated cancer the authors demonstrate that inactivation of SLAP shows a trend toward increased tumor formation as well as significantly increased Src family kinase activity within tumors. Tumor spheres from SLAP deficient animals showed enhanced growth that was suppressed by treatment with a Src family kinase

inhibitor. Of note, the latter effect was specific to SLAP deficient tumor spheres. These observations are convincing and support the authors conclusion that SLAP has a tumor suppressor role in CRC through inhibition of SFK signaling.

Mechanistically, elevated expression of the EPHB2 receptor tyrosine kinase was detected in immunoblots and by IHC of SLAP KO colonic crypts. In addition, in SLAP deficient crypts, levels of phosphorylated EPHB2 are increased and associated with activated SRC family kinases. Using an EPHB2 inhibitor, the role of EPHB2 in the growth of SLAP deficient colonic organoids, and downstream SRC phosphorylation was demonstrated. The authors also show that low expression of SLAP in human CRC cell line organoids sensitizes to the growth inhibitory effects EPH inhibition which can be reversed by SLAP over expression but not expression of a SH2/SH3 mutant form of SLAP.

Overall, this work provides evidence of SLAP adaptor function in restricting EPH tyrosine kinase signaling the colonic epithelium and suggests that loss of SLAP expression promotes tumorigenesis in this context.

https://doi.org/10.7554/eLife.110324.2.sa2

Reviewer #2 (Public review):

Summary:

Protein tyrosine kinases are submitted to diverse regulatory mechanisms controling their activity in normal situation. The authors previously identified SLAP (Src-like adaptor protein), a negative regulator of receptor tyrosine kinase (RTK) signaling, as a key suppressor of the cytoplasmic tyrosine kinase SRC in the normal colon and demonstrated that SLAP is downregulated in a majority of colorectal cancers (CRCs).

In this study, the authors further explored slap functions in mouse models using constitutive and inducible epithelial-specific Slap deletion (villin-CreERT2 model). They found that loss of slap augments colonic epithelial cell proliferation and that induction of tumorigenesis by the AOM/DSS protocol mimicking CRC leads to more aggressive tumors in the absence of slap. This effect is apparently cell-autonomous as growth of normal and tumoral colonic organoids is SLAP-dependent in in vitro settings. Finally, the authors define that, in colon, SLAP represses EphB2, an RTK lying upstream of SRC, and show that inhibitors of EphB2 can partially limit tumorigenic development in vitro.

Strengths:

The manuscript is clearly and concisely written, making it easy to follow. Data obtained in the mouse models are very convincing.

Weaknesses:

Direct evidence that EphB2 is activated/phosphorylated in the absence of SLAP is lacking as conclusions are only based on results obtained with inhibitors. Some other issues have to be addressed before acceptance, in particular the relevance of the findings in CRC patients.

Comments on revised version.

The authors have satisfactorily addressed my concerns.

https://doi.org/10.7554/eLife.110324.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Naim et al. use genetically engineered mouse models and tissue culture cell lines to investigate the role of the SLAP adaptor protein in colonic epithelium and colon tumour formation. The SLAP adaptor protein is known to be a negative regulator of tyrosine kinase signaling in hematopoietic cells, but its role outside the immune system is less well defined. Here, the authors use genetically engineered SLAP-deficient mice, tissue-specific SLAP KO, and colonic organoids to demonstrate that SLAP is expressed in cells of the colonic epithelium, where it acts as a cell-autonomous regulator of proliferation and differentiation. In addition, they provide biochemical evidence that loss of SLAP expression in cultured colonic organoids results in increased Src family kinase activity and global tyrosine phosphorylation, consistent with its known role as a suppressor of tyrosine kinase activity in immune cells. Consistently, treatment with an SRC kinase inhibitor inhibited the growth of SLAP-deficient organoids. These data provide solid evidence of a cell-autonomous role of SLAP in the colonic epithelium.*
>
> *This work would be improved by further description and interpretation of the SLAP expression pattern shown in the constitutive and tissue-specific KO to further support the conclusions made. In Supplementary Figure 1, magnification of the colon epithelium areas with SLAP expression shown by b-gal and anti-SLAP staining, highlighting regions of interest, would better support the conclusions regarding SLAP expression in specific regions of the colon epithelium. In Supplementary Figure 1B, the authors should indicate that the SLAP staining referred to is epithelial and in resident immune cells, as is mentioned in the text. Also, magnification of the boxed area of LRG5 staining in Figure 1 would improve this figure.*

We thank the reviewer for their positive and constructive evaluation of our work.

We have revised Fig 1 and S1 to better highlight SLAP expression patterns. Specifically, we have included higher-magnification images of the colonic epithelial regions, with clearly indicated regions of interest (new Figure S1). We have also clarified in the legend that SLAP staining is observed in both epithelial and resident immune cells, as described in the text. Additionally, we have provided a magnified view of the boxed area showing LGR5 staining in Figure 1 to improve clarity.

> *Using a chemically induced model of colitis-associated cancer, the authors demonstrate that inactivation of SLAP shows a trend toward increased tumor formation (though this did not reach significance) as well as increased Src family kinase activity within tumors. Tumor spheres from SLAP-deficient animals showed enhanced growth that was suppressed by treatment with a Src family kinase inhibitor. Of note, the latter effect was specific to SLAP-deficient tumor spheres. These observations are convincing and support the authors' conclusion that SLAP has a tumor suppressor role in CRC through inhibition of SFK signaling.*
>
> *Mechanistically, elevated expression of the RTK, EphB2, was detected in immunoblots of SLAP KO colonic crypts, while overexpression of SLAP in CRC cell lines downregulated EphB2 protein levels. Using an EPHB2 inhibitor, the role of EPHB2 in the growth of SLAP-deficient colonic organoids was demonstrated. While these data generally support the authors' conclusion that SLAP limits colonic organoid growth by downregulating RTKS such as EphB2 and downstream Src family kinase activity, they do not show which cell*

> *types/regions in the colonic epithelium have increased EPHB2 protein and how this relates to SLAP and phospho-SRC expression, as shown in Figure 1 and Figure S1 immunocytochemistry. The expression of EphB2 and its role in colonic tumorsphere growth were not investigated.*
>
> *Overall, this work provides evidence of SLAP adaptor function in restricting tyrosine kinase signaling in the colonic epithelium, and suggests that loss of SLAP expression could promote tumorigenesis in this context.*

We thank the reviewer for their positive assessment of our tumour studies and for recognizing the evidence supporting a tumor suppressor role for SLAP through inhibition of SFK signaling.

To address the reviewer's mechanistic concerns, we performed additional experiments that are now included in the revised manuscript. We confirmed that loss of Slap is associated with increased EPHB2 expression in colonic crypts by IHC (new Figure 4B) and directly tested the role of EPHB2 in the Slap-deficient phenotype: EPH inhibition reduced both pTyr levels and SRC activation in Slap-deficient organoids (new Figure S2), demonstrating that SFK hyperactivation depends on upstream EPHB2 signaling. Consistent with this mechanism, we also observed increased EPHB2 tyrosine phosphorylation and active SRC (pSRC) association in isolated colonic epithelial cells following Slap deletion (new Figure 4A).

To extend these findings to the tumour context, we examined the effect of EPH inhibition in human CRC tumoroids (new Figure 5). Pharmacological EPHB2 inhibition reduced tumoroid growth in CRC cells expressing low levels of SLAP, whereas this effect was largely lost upon SLAP overexpression. An SH2- or SH3-inactivating point SLAP mutant failed to suppress tumoroid growth and restored sensitivity to EPHB2 inhibition, further supporting EPHB2 as a critical target of SLAP-mediated tumour suppression. Together, these new data identify EPHB2 as a critical upstream activator of SRC that is negatively regulated by SLAP and strengthen our conclusion that deregulated EPHB2-SRC signaling drives the hyperproliferative phenotype associated with SLAP loss.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Protein tyrosine kinases are subject to diverse regulatory mechanisms controlling their activity in normal situations. The authors previously identified SLAP (Src-like adaptor protein), a negative regulator of receptor tyrosine kinase (RTK) signaling, as a key suppressor of the cytoplasmic tyrosine kinase SRC in the normal colon and demonstrated that SLAP is downregulated in a majority of colorectal cancers (CRCs).*
>
> *In this study, the authors further explored SLAP functions in mouse models using constitutive and inducible epithelial-specific Slap deletion (villin-CreERT2 model). They found that loss of SLAP augments colonic epithelial cell proliferation and that induction of tumorigenesis by the AOM/DSS protocol mimicking CRC leads to more aggressive tumors in the absence of SLAP. This effect is apparently cell-autonomous as growth of normal and tumoral colonic organoids is SLAP-dependent in in vitro settings. Finally, the authors define that, in colon, SLAP represses EphB2, an RTK lying upstream of SRC, and show that inhibitors of EphB2 can partially limit tumorigenic development in vitro.*
>
> *Strengths:*
>
> *The manuscript is clearly and concisely written, making it easy to follow. The data obtained in the mouse models are very convincing.*
>
> *Weaknesses:*

> *Direct evidence that EphB2 is activated/phosphorylated in the absence of SLAP is lacking, as conclusions are only based on results obtained with inhibitors. Some other issues have to be addressed before acceptance, in particular, the relevance of the findings in CRC patients.*

We thank the reviewer for their positive and constructive evaluation of our work.

We agree that direct evidence linking SLAP loss to activation of the EPHB2-SRC pathway would strengthen the study. To address this point, we performed additional experiments that are now included in the revised manuscript. In addition to demonstrating increased EPHB2 expression upon Slap deletion, we found that loss of Slap enhances the EPHB2 tyrosine phosphorylation (an index of EPHB2 activity) and association between EPHB2 and pSRC in isolated colonic epithelial cells, supporting increased signaling through this pathway (new Figure 4A). Furthermore, pharmacological inhibition of EPHB2 reduced both SRC activation and the hyperproliferative phenotype observed in Slap-deficient organoids (new Figure S2). Together, these findings provide functional and biochemical evidence that deregulated EPHB2 signaling contributes to SRC activation in the absence of SLAP. We also examined the effect of EPHB2 inhibition in human CRC tumoroids (new Figure 5).

Pharmacological EPHB2 inhibition reduced tumoroid growth in CRC cells expressing low levels of SLAP, whereas this effect was largely lost upon SLAP overexpression. Together, these new data identify EPHB2 as a critical upstream activator of SRC that is negatively regulated by SLAP and strengthen our conclusion that deregulated EPHB2-SRC signaling drives the hyperproliferative phenotype associated with SLAP loss.

To address the relevance of our findings in CRC patients, we also extended our analyses to human datasets (new Figure 5D). We observed a significant inverse correlation between SLAP expression and a colorectal cancer stem cell-like activity score in TCGA tumours. In addition, co-expression of SLAP and SLAP2 with EPHB2 was associated with improved disease-free survival in microsatellite-stable (MSS) CRC patients, whereas no such association was observed in microsatellite instability (MSI) tumours. These findings support the clinical relevance of the SLAP-EPHB2 signaling axis and are consistent with a role for SLAP in restraining EPHB2-dependent CSC signaling in CRC.

> ***Recommendations for the authors:***
>
> ***Reviewing Editor Comments:***
>
> *Both reviewers have reported that the study of the EPHB2-SLAP-Src axis is not very developed, as most results are derived from using inhibitors. A key question is whether EPHB2 is activated by SLAP depletion and whether it is critical to SRC activation. Addressing these questions would greatly improve the paper.*

We thank the Reviewing Editor for this important suggestion. We would be happy for the editors to assess the revised version without involving the reviewers again. In the revised manuscript, we have substantially strengthened the mechanistic link between SLAP loss, EPHB2 activation, and SRC signaling. We show that Slap deletion increases both EPHB2 expression and tyrosine phosphorylation, enhances EPHB2-pSRC association in colonic epithelial cells. Importantly, pharmacological inhibition of EPHB2 suppresses SRC activation and rescues the hyperproliferative phenotype of Slap-deficient organoids. We further demonstrate that EPHB2 inhibition selectively impairs growth of CRC tumoroids with low SLAP expression, whereas this effect is largely abolished upon SLAP overexpression. An SH2- or SH3-inactivating point SLAP mutant failed to suppress tumoroid growth and restored sensitivity to EPHB2 inhibition, further supporting EPHB2 as a critical target of SLAP-mediated tumour suppression. Together, these new biochemical and functional data establish

EPHB2 as a critical upstream activator of SRC that is negatively regulated by SLAP and significantly reinforce the central conclusions of the study.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Evidence of SLAP expression in the colon is an important basis for these studies and could be moved to the main Figure 1 rather than being in the supplementary material.*

We thank the reviewer for this suggestion. While we agree that documenting SLAP expression in the colon is important, we have retained these data in Figure S1 to maintain a concise main figure set, consistent with the recommended format for Short Reports.

> *(2) Define AOM/DSS and briefly describe the model at first mention. In addition, the model in 3A includes TAM treatment at 45 days, but this is not mentioned in the text. Why is this done?*

We have better defined the AOM/DSS protocol at first mention in the revised manuscript and specified the rationale for tamoxifen administration at day 45, which is required to maintain efficient SLAP deletion throughout the duration of the experiment (90 days).

> *(3) Evidence that the SRC inhibitor decreased phospho-tyrosine levels in addition to inhibiting the growth of organoids should be included.*

We included data showing the inhibitory effect of the used SRC inhibitor on global phospho-tyrosine levels in organoids in the revised manuscript.

> *(4) Further experiments investigating the involvement of EphB2 in colonic tumor formation are of interest and would increase the significance of this work.*

We included data showing that SLAP modulation affects the response of tumoroids derived from cell lines to EphB2 inhibition, providing complementary mechanistic insights.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) The authors should confront their findings with data obtained in normal and pathological tissues: are SLAP, SRC, and EphB2 co-expressed, at the single cell level, in normal colon and CRC? In which cell populations? Is loss of SLAP associated with poor prognosis in CRC patients?*

We thank the reviewer for this important suggestion. We agree that assessing the relevance of the SLAP-EPHB2-SRC axis in human CRC is important. However, transcriptomic datasets have inherent limitations in this context, as SLAP primarily regulates signaling at the post-transcriptional level and SRC activity cannot be reliably inferred from mRNA expression.

To address the clinical relevance of our findings, we performed additional analyses of CRC patient datasets. We found a significant inverse correlation between SLAP expression and a colorectal cancer stem cell-like activity score in TCGA tumours. Furthermore, co-expression of SLAP and SLAP2 with EPHB2 was associated with improved disease-free survival in microsatellite-stable (MSS) CRC patients. These findings are consistent with a role for SLAP in restraining EPHB2-dependent signaling in CRC. Finally, while our data identify EPHB2 as a critical upstream regulator of SRC signaling controlled by SLAP, we do not exclude the possibility that additional receptor tyrosine kinases contribute to the effects of SLAP loss during colorectal tumorigenesis.

> *(2) In Figure 4A, total EphB2 levels are increased in the absence of SLAP in colonic crypts. However, the level of EphB2 phosphorylation is not shown. This is an important point to address. Which ligand(s) may activate EphB2?*

We agree that assessing EPHB2 activation is important. To address this point, we have included new data in the revised manuscript showing that Slap deletion increases EPHB2 tyrosine phosphorylation in isolated colonic epithelial cells, providing direct evidence that EPHB2 signaling is enhanced in the absence of SLAP. In addition, we show that loss of Slap increases EPHB2-pSRC association and that pharmacological inhibition of EPHB2 reduces SRC activation and suppresses the hyperproliferative phenotype of Slap-deficient organoids. Together, these findings establish EPHB2 as a critical upstream regulator of SRC signaling following SLAP loss.

Regarding EPHB2 activation, previous studies have shown that EPHB2 is primarily activated by ephrin-B ligands expressed within the intestinal crypt compartment (Batlle et al., 2002). EPHB2 signaling may also be reinforced through cooperation with other Eph receptors, particularly EPHB3, which is highly expressed in intestinal stem and progenitor cells (Holmberg et al., 2006; Genander et al., 2009). In addition, SRC has been reported to phosphorylate EPH receptors, raising the possibility of bidirectional signaling that could further amplify EPHB2-SRC pathway activity (Leroy et al., 2009; Hochgräfe et al., 2010).

> *(3) In the absence of SLAP, inhibitors of EphB2 should also decrease SRC activity as EphB2 lies upstream of SRC (Figure S3C). Does this occur in organoids?*

We now show that EphB2 inhibition reduces SRC activity in SLAP-deficient organoids.

> *(4) What is the status of EphB2 and SRC (total, phosphorylated) in SW620 and HT29 CRC cells in the absence of SLAP?*

SW620 and HT29 cells are SLAP-low CRC models. Given that SLAP expression is already minimal in these cells, further depletion is unlikely to provide meaningful additional insight.

> *(5) Expression of SLAP is associated with a decrease in the stem cell compartment in CRC cell lines (Figure S2). Is there a stem cell signature associated with low SLAP levels in CRC?*

We analyzed TCGA colorectal cancer datasets using a published colorectal cancer stem cell (CSC) signature. We found a low but significant inverse correlation between SLAP expression and the CSC-like activity score, supporting our experimental observations that SLAP restrains stem cell properties in CRC cells and organoids.

> *(6) Does overexpression of the mutant form of SLAP (SLAPmut) limit SLAP effects in SW620 and HT29 CRC cells in Figure S2?*

We have now performed the requested experiments and found that, unlike wild-type SLAP, SLAPmut failed to inhibit tumoroid growth in CRC cells. These results are consistent with our previous findings showing that SLAPmut lacks tumour suppressor activity in CRC cells (Naudin et al., Nat Commun, 2014) and further support the requirement of SLAP signaling functions for the regulation of CRC stem-like properties.

> *(7) Total SRC is missing in Figure 2B*

Total SRC levels are now included in the revised figure.

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