

AWARD NUMBER: W81XWH-18-1-0297

**TITLE: Mechanisms of Oncogenesis in the Primary Liver Cell Cancer
Cholangiocarcinoma**

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CONTRACTING ORGANIZATION: Mayo Clinic

REPORT DATE: NOVEMBER 2021

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE NOVEMBER 2021		2. REPORT TYPE FINAL		3. DATES COVERED 8/1/18- 7/31/2021	
4. TITLE AND SUBTITLE Mechanisms of Oncogenesis in the Primary Liver Cell Cancer				5a. CONTRACT NUMBER W81XWH1810297	
				5b. GRANT NUMBER W81XWH-18-1-0297	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Rory Smoot E-Mail:smoot.rory@mayo.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) MAYO CLINIC 200 1ST ST SW ROCHESTER MN 55905-0002				8. PERFORMING ORGANIZATION REPORT	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This is a final report for this award. During year 3 and over the course of the award significant progress was made and the study objectives were completed. At the completion of year 3 a final and third manuscript was submitted focusing on the effects of therapeutic targeting of the SRC family kinase LCK in CCA cells and patient derived xenografts. Over the course of the award three full manuscripts were completed with one under review and two others already published and reported in previous annual progress reports. The work supported by this award has served as the preliminary data for an R01 application that will be submitted in the October 2021 cycle and LCK as a target as identified by this work was part of a successful DOD IDEA award for novel targeting approaches that is undergoing activation currently.					
15. SUBJECT TERMS Src family kinase, LCK					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The study goals are to further define the molecular mechanisms of cholangiocarcinoma oncogenesis and assess susceptibility of cholangiocarcinoma to targeted therapies.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Src family kinase, LCK, dasatinib, cholangiocarcinoma, Hippo pathway, tyrosine phosphorylation, SHP2, PTPN11

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

See attached statement of work.

Major goals of the project:

Specific Aim 1: Identify Src-family kinase (SFK) responsible for Yes-associated protein (YAP) tyrosine phosphorylation and evaluate role in YAP localization/activation

Specific Aim 2: Identify YAP binding partners regulated by tyrosine phosphorylation and transcriptional program

Specific Aim 3: Evaluate therapeutic efficacy of SFK inhibition in two animal models of cholangiocarcinoma (CCA)

The major tasks under each specific aim were completed during the award period.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

The major tasks proposed for the award have been achieved.

Please see attached statement of work for specific progress on each subtask and detailed discussion of data obtained.

Two previously published papers from this award were attached in prior annual progress reports and have been attached again to this final report. A third manuscript has been submitted to *Signal Transduction and Targeted Therapy*.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The opportunities for training during this course of this award have included attendance at the apoptosis journal club, which is a graduate level course.

The opportunities for professional development have included attendance at the following conferences:

Americas Hepato-Pancreato-Biliary Association Annual Conference

The Cholangiocarcinoma Foundation Annual Conference

American Association for Cancer Research: The Hippo Pathway Signaling, Cancer, and Beyond

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

During the course of the award abstracts were submitted to AACR annual meeting, International Liver Cancer Association meeting, the Cholangiocarcinoma Foundation meeting, and Americas Hepato Pancreato Biliary Association.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to report

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The impact of the findings to date includes additional support that Src family kinases can regulate YAP which can drive cholangiocarcinoma growth and survival. We have now demonstrated that tyrosine phosphatases play an important role in regulating YAP activity in cholangiocarcinoma, and that these enzymes can regulate the sensitivity to chemotherapy by modulating YAP target genes. Translationally we demonstrated therapeutic efficacy of targeting LCK specifically in multiple models of CCA including cell lines and patient derived xenografts.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Sugihara T, Werneburg NW, Hernandez MC, Yang L, Kabashima A, Hirsova P, Yohanathan L, Sosa C, Truty MJ, Vasmatzis G, Gores GJ, Smoot RL. YAP Tyrosine Phosphorylation and Nuclear Localization in Cholangiocarcinoma Cells Are Regulated by LCK and Independent of LATS Activity. *Mol Cancer Res.* 2018 Oct;16(10):1556-1567. doi: 10.1158/1541-7786.MCR-18-0158. Epub 2018 Jun 14. PMID: 29903769; PMCID: PMC6170676. Acknowledgement of federal support YES

Buckarma EH, Werneburg NW, Conboy CB, Kabashima A, O'Brien DR, Wang C, Rizvi S, **Smoot RL**. The YAP-Interacting Phosphatase SHP2 Can Regulate Transcriptional Coactivity and Modulate Sensitivity to Chemotherapy in Cholangiocarcinoma. *Mol Cancer Res.* 2020 Oct;18(10):1574-1588. doi: 10.1158/1541-7786.MCR-20-0165. Epub 2020 Jul 9. PMID: 32646966; PMCID: PMC7541657. acknowledgement of federal support YES

Werneburg N, Gores GJ, **Smoot RL**. The Hippo Pathway and YAP Signaling: Emerging Concepts in Regulation, Signaling, and Experimental Targeting Strategies With Implications for Hepatobiliary Malignancies. *Gene Expr.* 2020;20(1):67-74. doi:10.3727/105221619X15617324583639; acknowledgement of federal support YES

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Podium presentation: Americas Hepato-Pancreato-Biliary Association Annual Meeting.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*

- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
 Project Role: Graduate Student
 Researcher Identifier (e.g. ORCID ID): 1234567
 Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Rory Smoot MD
 No change

Gregory Gores MD
 No Change

Nathan Werneburg MA
 Technician
 6 person months
 Mr. Werneburg has performed in vitro experiments on cell lines.
 Funding for Mr. Werneburg is provided through the Mayo Clinic Department of Surgery

Caitlin Conboy, MD PhD
 Post-doctoral fellow
 12 person months
 Dr. Conboy has performed in vitro experiments and data analysis.
 Funding for Dr. Conboy is through the Mayo Clinic Clinician Investigator Program

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

The PI Dr. Rory Smoot is funded as a Co-I on the Mayo Clinic SPORE in Hepatobiliary Disease for an additional 13% FTE.

The PI Dr. Rory Smoot is funded as a Co-PI by a State of Minnesota Regenerative Medicine Grant for an additional 2% FTE.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

- 9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

YAP Tyrosine Phosphorylation and Nuclear Localization in Cholangiocarcinoma Cells Are Regulated by LCK and Independent of LATS Activity



Takaaki Sugihara¹, Nathan W. Werneburg¹, Matthew C. Hernandez², Lin Yang³, Ayano Kabashima¹, Petra Hirsova^{1,4}, Lavanya Yohanathan², Carlos Sosa⁵, Mark J. Truty², George Vasmatazis⁶, Gregory J. Gores¹, and Rory L. Smoot²

Abstract

The Hippo pathway effector, Yes-associated protein (YAP), is a transcriptional coactivator implicated in cholangiocarcinoma (CCA) pathogenesis. YAP is known to be regulated by a serine/threonine kinase relay module (MST1/2–LATS1/2) culminating in phosphorylation of YAP at Serine 127 and cytoplasmic sequestration. However, YAP also undergoes tyrosine phosphorylation, and the role of tyrosine phosphorylation in YAP regulation remains unclear. Herein, YAP regulation by tyrosine phosphorylation was examined in human and mouse CCA cells, as well as patient-derived xenograft (PDX) models. YAP was phosphorylated on tyrosine 357 (Y357) in CCA cell lines and PDX models. SRC family kinase (SFK) inhibition with dasatinib resulted in loss of YAPY357 phosphorylation, promoted its translocation from the nucleus to the cyto-

plasm, and reduced YAP target gene expression, including cell lines expressing a LATS1/2-resistant YAP mutant in which all serine residues were mutated to alanine. Consistent with these observations, precluding YAPY357 phosphorylation by site-directed mutagenesis (YAPY357F) excluded YAP from the nucleus. Targeted siRNA experiments identified LCK as the SFK that most potently mediated YAPY357 phosphorylation. Likewise, inducible CRISPR/Cas9-targeted LCK deletion decreased YAPY357 phosphorylation and its nuclear localization. The importance of LCK in CCA biology was demonstrated by clinical observations suggesting LCK expression levels were associated with early tumor recurrence following resection of CCA. Finally, dasatinib displayed therapeutic efficacy in PDX models. *Mol Cancer Res*; 16(10); 1556–67. ©2018 AACR.

Introduction

Cholangiocarcinoma (CCA) is a tumor with features of biliary epithelial differentiation and extremely limited treatment options. The underlying molecular mechanisms of both tumorigenesis and progression remain incompletely understood; however, accumulating data implicates the Hippo pathway and its effector Yes-associated protein (YAP) in CCA pathogenesis (1–3).

The Hippo pathway is a developmental pathway important for organ size control (4). Alterations in this pathway have been

implicated in the pathogenesis of multiple types of cancer, including CCA, although mutations in the pathway components themselves are rare (5–9). The paucity of specific Hippo pathway mutations and the lack of a dedicated cell-surface receptor have increased interest in understanding the role of signaling cross-talk between the Hippo and other signal transduction pathways. Indeed, cross-regulatory networks have been demonstrated for a wide variety of cell-surface receptors including G-protein-linked receptors and more recently receptor tyrosine kinases such as FGF, EGF, and platelet-derived growth factor (PDGF) in Hippo regulation (2, 8, 10). The Hippo pathway itself consists of an upstream serine kinase relay module (comprised of MST1/2 and LATS1/2) which regulates the cotranscriptional activators YAP and TAZ (4). Although YAP and TAZ are both regulated by the Hippo pathway, YAP has been more strongly implicated in CCA biology (1, 2, 8). Hippo pathway activation acts as a "brake" to transcription. This serine phosphorylation of YAP restrains the protein in the cytosol through interactions with protein 14-3-3, limiting its function as a transcriptional coactivator. The role of serine phosphorylation regulating YAP in the context of developmental processes is well described; however, emerging data suggest that tyrosine phosphorylation of YAP may also play a role in regulating YAP localization and transcriptional activity, especially in cancer (2, 11).

We recently described cross-talk between the PDGF and Hippo signaling pathways in CCA (2). We demonstrated regulation

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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doi: 10.1158/1541-7786.MCR-18-0158

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of YAP localization by tyrosine phosphorylation in a manner independent of the core Hippo serine/threonine kinase relay module. Inhibition of PDGF receptors induced a redistribution of YAP from the nucleus (transcriptionally active) to the cytosol (transcriptionally inactive) even when YAP was mutated at the canonical LATS 1/2 phosphorylation site, serine 127 (S127). The baseline nuclear localization of YAP appeared to be mediated by SRC family kinases (SFK), acting by phosphorylation of tyrosine 357 (Y357) on YAP. However, the specific SFK regulating YAP^{Y357} phosphorylation was not examined in these studies. Neither was the potential therapeutic role of inhibiting SFK in CCA explored. Given the lack of therapeutic options for CCA and the role of YAP in its pathogenesis, these questions merit further consideration.

Herein, we examine tyrosine phospho-regulation of YAP cellular compartmentation and target gene expression in CCA cells and tissue. The results demonstrate enhanced YAP tyrosine phosphorylation in CCA as compared with normal human cholangiocytes. More importantly, the SFK inhibitor dasatinib blocks YAP tyrosine phosphorylation, induces YAP redistribution from the nucleus to the cytosol, and downregulates expression of YAP target genes. siRNA-targeted knockdown studies suggest a pivotal role for the SFK member LCK in mediating YAP^{Y357} phosphorylation, a previously unrecognized function for LCK. Most significantly, this tyrosine phospho-regulation of YAP subcellular localization was independent of serine phospho-regulatory mechanisms, suggesting separate cytosolic and nuclear retention signals for YAP, a novel concept in YAP regulation. The efficacy of YAP downregulation by SFK inhibition translated therapeutically to increases in CCA cell death both *in vitro* and *in vivo*, providing further evidence supporting the evaluation of SFK inhibition as a therapeutic strategy in CCA.

Materials and Methods

Cell culture

The human CCA cell line HuCCT-1, mouse CCA cell line SB1, and normal human cholangiocyte cell line (12) were cultured in DMEM supplemented with 10% FBS and 0.2% primocin under standard conditions. The murine SB1 cell line was isolated from our murine model of YAP-associated CCA and has been described in detail (13). This cell line expresses flag-tagged YAPS127A and myr-AKT. Due to cell density and serum regulation of the Hippo pathway, cell culture experiments were performed at near confluence (~80%) in serum-starved cells. Serum-starved cells were cultured overnight in media without serum. The media were replaced again 3 hours prior to initiation of experimental conditions. For authentication of the HuCCT-1 cell line, short tandem repeat analysis was performed by the Genome Analysis Core of the Medical Genome Facility (Mayo Clinic, Rochester, MN). All cell lines underwent *Mycoplasma* contamination testing periodically using Plasmotest-Mycoplasma Detection kit (InvivoGen). Cell lines were used within 40 passages of reanimation.

Antibodies and reagents

Dasatinib (Selleckchem), KW-2449 (Selleckchem), sodium orthovanadate (Sigma), and/or latrunculin A (Abcam) were added to cells at final concentrations from 0.1 to 10 μ mol/L per experimental design. The following primary antisera were used for immunoblot analysis: actin (C-11; Santa Cruz Biotechnology), phospho YAP^{Y357} (ab62751 Abcam), phospho YAP^{S127} (4911;

Cell Signaling Technology), total YAP (63.7; Santa Cruz Biotechnology; 4912 CST), lamin B1 (D8P3U; Cell Signaling Technology), GAPDH (MAB374; Millipore), phospho SRC^{Y416} (2101; Cell Signaling Technology), SRC (L4A1; Cell Signaling Technology), YES (3201; Cell Signaling Technology), LCK (2657; Cell Signaling Technology), FYN (4023; Cell Signaling Technology), LYN (44; Santa Cruz Biotechnology), LATS1 (C66B; Cell Signaling Technology), phospho LATS1 (S909; Cell Signaling Technology), and MCL-1 (S-19; Santa Cruz Biotechnology). The following primary monoclonal antibodies were used for immunofluorescence and/or immunohistochemistry: total YAP (63.7; Santa Cruz Biotechnology), phospho YAP^{Y357} (ab62751; Abcam), SOX9 (AB5535; Millipore), and anti-FLAG M2 (F1804; Sigma-Aldrich). ProLong Antifade with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) was used for nuclear staining.

Immunoprecipitation and immunoblot analyses

Whole-cell lysates were collected by adding ice-cold lysis buffer (Cell Signaling Technology) containing protease inhibitors (Sigma-Aldrich), phosphatase inhibitors (Roche Diagnostics), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Cells were then scraped to collect cells, vortexed, and lysed on ice for approximately 20 minutes. The lysed cells were then centrifuged at 12,000 $\times$ g for 15 minutes to remove cellular debris. Supernatant was transferred to a clean tube and protein concentration determined by the Bradford (Sigma-Aldrich) protein assay. For immunoprecipitation, whole-cell lysates of SB1 cells that contain the FLAG-YAPS127A were incubated with equilibrated anti-FLAG M2 magnetic beads (Sigma-Aldrich) overnight at 4°C. The FLAG-YAPS127A was then eluted from the beads with FLAG peptide following the manufacturer's instructions and evaluated by mass spectroscopy (Supplementary Methods). For immunoblot, proteins were then resolved by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Membranes were blotted with primary antibodies at 4°C overnight in 5% BSA-TBS Tween. The primary antibody dilution was 1:1,000 unless otherwise indicated. After overnight incubation, membranes were washed for 30 minutes in TBS Tween and then horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit, and goat (Santa Cruz Biotechnology) were added to membrane at a concentration of 1:5,000 and incubated for 1 hour at room temperature. Immunoblots were visualized with enhanced chemiluminescence (ECL) or ECL prime (GE Healthcare Life Sciences). For experiments in which multiple proteins were evaluated, membranes were stripped and reblotted.

Immunofluorescence

Cells were grown on glass coverslips, and once the cells reached desired confluency, they were treated per experimental conditions. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The fixed cells were then incubated at room temperature with blocking buffer containing 5.0% BSA and 0.1% glycine. After blocking, the cells were incubated with primary antibody in blocking buffer overnight at 4°C. After washing, the cells were incubated with the corresponding secondary antibodies diluted in blocking buffer for 1 hour at room temperature in the dark. After secondary incubation, the coverslips were again washed and mounted onto slides using ProLong Antifade (Invitrogen-Molecular Probes) containing DAPI. The slides were analyzed by fluorescent confocal microscopy (LSM 780, Zeiss).

Immunohistochemistry

Liver sections were fixed in 10% buffered formaldehyde and embedded in paraffin. Blocks were sectioned into 3.5 $\mu\text{mol/L}$ slices. Sections were deparaffinized, hydrated, and incubated with primary antibody overnight at 4° C. Sections were stained with antibody for SOX9 (1:1000), YAP (1:50), and phospho YAP^{Y357} (1:50). Bound antibody was detected with biotin-conjugated secondary antibody and diaminobenzidine as a substrate.

Quantitative and qualitative PCR

mRNA was isolated from cells using TRIzol Reagent (Ambion). Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase and random primers (Life Technologies). After reverse transcription, cDNA was analyzed using real-time PCR (Light Cycler 480 II; Roche Diagnostics) for the quantitation of the target genes; SYBR Green (Roche Diagnostics) was used as the fluorophore. Expression was normalized to either 18 S or *GAPDH* and relative quantification performed according to the $2^{-\Delta\text{CT}}$ or $2^{-\Delta\Delta\text{CT}}$ method as previously described (14). Data are reported as fold expression compared with calibrator as geometric mean and geometric standard deviation of expression relative to calibrator. Technical replicates were completed for each run, and a minimum of three biological replicates completed for each condition/cell line. The primers used are listed in Supplementary Table S1.

In vitro kinase assay

The LCK kinase enzyme system and ADP-Glo kinase assay (Promega) were used to assess LCKs' ability to phosphorylate YAP *in vitro*. The protocol for the LCK kinase assay was followed as described by the manufacturer. Briefly for luminescent assay, 50 $\mu\text{mol/L}$ DTT, 2 mmol/L MnCl_2 , 10 mmol/L ultrapure ATP, and 0.1 μg of LCK enzyme were added to wells containing 1x reaction buffer. Recombinant YAP (Abcam) 1 μg or Poly (Glu4, Tyr1) peptide 1 μg were added, and enzyme reaction was incubated at room temperature (RT) for 60 minutes. Note that 25 μL of ADP-Glo reagent was added to well and incubated at RT for 40 minutes, and 50 μL of kinase detection reagent was then added and incubated at RT for 60 minutes. Luminescence was then measured on Synergy H1 (BioTek) plate reader. For immunoblot detection, 50 $\mu\text{mol/L}$ DTT, 2 mmol/L MnCl_2 , 10 mmol/L ultrapure ATP, and 90 ng or recombinant YAP protein were incubated for 1 hour with and without the presence of 0.1 μg of LCK enzyme. After enzyme reaction incubation, 4x Laemmli sample buffer was added, samples were boiled for 10 minutes and then resolved on 10% gel for transfer and immunoblotting.

RNA interference and transfection

SRC, *YES*, *LCK*, *FYN*, and *LYN* were transiently knocked down in the HuCCT-1 cell line with validated siRNAs (Dharmacon). Cells were grown in 6-well plates and transfected with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. After 48 hours of incubation, they were used for indicated studies. The efficiency and specificity of knockdown were assayed by immunoblot and RT-PCR.

CRISPR/Cas9 knockdown and doxycycline induction

The sequence of single guide RNA (sgRNA) for LCK was designed using online CRISPR design tool (<http://crispr.mit.edu/>) and was 5'-TCCCCGACCACTGGTACCTACGA-3'. DNA oligonucleotides for the sgRNA were synthesized and cloned into

the FgH1tUTG plasmid, a kind gift of Dr. Marco Herold (15). To produce lentiviruses, FgH1tLCK or FUCas9Cherry plasmids were transiently cotransfected into HEK293T cells with the packaging plasmids pRSV-Rev, pMDLg/pRRE, and pCMV-VSV-G (all from Addgene) using FuGENE HD Transfection Reagent (Promega Corporation). After 48 and 72 hours, supernatant containing lentiviruses was harvested and passed through a 0.45- μm filter. Target HuCCT-1 cells were transduced with the lentiviruses in the presence of 8 $\mu\text{g/mL}$ polybrene for 24 hours. After the additional 3-day culturing, m-cherry (Cas9) and GFP (inducible sgRNA) dual-positive cells were sorted on a single-cell basis into a 96-well plate by flow cytometry. To induce sgRNA expression, doxycycline hyclate (SIGMA) was added to culture medium at a final concentration of 7.5 $\mu\text{g/mL}$. The efficacy of CRISPR/Cas9-mediated gene deletion was determined by immunoblot analysis.

YAP serine mutant generation

pCMV-Flag-YAP-5SA/S94A was a gift from Kunliang Guan (Addgene plasmid # 33103). HuCCT-1 cells were transfected using FugeneHD (Promega) following the manufacturer's instructions. Following 48-hour transfection, the cells were used for subsequent experiments.

YAP tyrosine mutant generation

To construct pEGFP-YAP, pENTR-YAP (a generous gift from Dr. Xin Chen, UCSF, San Francisco, CA) was digested with *Bgl*II and *Eco*RI, and purified YAP fragment was ligated into a fragment of *Bgl*II-*Eco*RI pEGFP-C3 vector. For site-directed mutagenesis of tyrosine residues, primers were designed using NEBaseChanger on-line tool, and PCR was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). To make pEGFP-YAP Y357F, PCR was performed with pENTR-YAP as a template using oligonucleotides 5'-ATGAGCAGCTTCAGTGTCCCTC-3' and 5'-GCCTAGTCCACTGTCTGTAC-3' as primers (the complementary sequences underlined). Triple mutant pEGFP-YAP Y341,357,394F was prepared in a similar way by consecutive mutations of pENTR-YAP Y357F using primers 5'-AGTGG-CACCTTTCACCTCTCGAG-3' and 5'-GTTAAGGAAAGGATCTGAGC-3', followed by TCCCCAGACTTCCTGAAGCCATTC and 5'-ACGGTTCTGCTGTGAGGG-3'. Finally, pENTR plasmids with mutated YAP were digested with *Bgl*II and *Eco*RI, and YAP fragments were inserted into a *Bgl*II-*Eco*RI pEGFP-C3 vector. All constructs of YAP contain a FLAG epitope at the amino-terminus.

RNA sequencing and bioinformatics

Following Institutional Review Board (IRB) approval, patient-derived xenografts (PDX) of resected intrahepatic CCA tumors were created as described previously (2). RNA sequencing was undertaken in 7 resected ICC PDX. Clinicopathologic features and recurrence-free and overall survival were abstracted for the patients. Differentially expressed genes were identified between early recurrence (<10 months) and late recurrence with a $\log_2$ threshold of 1.5 with a *P* value of <0.01. For this cohort of patients, spliced aligned was carried out using TopHat v2.1.0 (TopHat2) with two conditions, one corresponding to early recurrence (L10 group) and the second condition corresponding to late recurrence (G10 group) with 4 and 3 replicates, respectively (16). The reference genome and the annotation transcriptome utilized in this work for all the alignments were based on the iGenome package provided by Illumina. TopHat2 package utilized Bowtie v2.2.3.0 as the underlying read-alignment software (17). For

downstream analysis, we utilized cufflinks v2.2.1 to assemble transcripts for all the samples (18–21). These programs rely on the accepted_hits.bam generated after running TopHat2. Finally, Cuffdiff v2.2.1 was used to generate differential gene expression, and the results were analyzed via the cuffdiff package v2.10.0 (22). Differential gene expression was measured using fragments per kilobase of transcript per million fragments mapped (FPKM). We calculated the fragment per kilobase of transcript per million (FPKM) of each transcript and gene using statistical significance in Cuffdiff, which reports statistical significance based on whether the *P* value is greater than the FDR after applying the Benjamini–Hochberg correction (22). The Biomarker Discovery RNA-seq (BMD_RNA-seq) pipeline workflow was utilized to carry out alignment and differential gene expression (23).

Quantification of cell death/proliferation

Cell death and proliferation were quantified by counting using Celigo (Nexcelom) after staining with propidium iodide (Sigma) and Hoechst 33342 (Invitrogen), respectively.

PDX generation and treatment

Mayo Clinic, Rochester, has an ongoing IRB-approved protocol for collection and xenotransplantation of resected biliary tract cancers. In accordance with this protocol and an established Institutional Animal Care and Use Committee protocol, human CCA tumors were implanted in NOD/SCID mice as previously described by us (8). Once the tumors had reached palpable size (~125 mm³), size was recorded and treatment with dasatinib (15 mg/kg) or vehicle begun daily for 3 days or 2 weeks via gavage as previously described (24). At the end of the treatment period, all mice were sacrificed and tumor tissue obtained for further studies.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining

The fluorescent terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (*in situ* cell death detection kit; Roche) was utilized on tissue sections. Sections were paraformaldehyde-fixed and hydrated. The TUNEL assay was then performed using the manufacturer's protocol. Slides were mounted with ProLong Antifade (Invitrogen-Molecular Probes) containing DAPI. The slides were analyzed by fluorescent confocal microscopy (LSM 780; Zeiss). Dead cells were quantified by counting TUNEL-positive nuclei in 20 random microscopic fields (20X).

Methods regarding mass spectrometry, protein identification, and statistical methods are located in the Supplementary Methods.

Results

Tyrosine 357 phosphorylation is increased in CCA cells and tissue

Initially, we examined the phosphorylation status of YAP tyrosine 357 (YAP^{Y357}) at baseline in multiple models of CCA as compared with normal human cholangiocytes. YAP^{Y357} phosphorylation was readily identified in HuCCT-1 and SB1 cells as compared with normal human cholangiocytes (Fig. 1A). The YAP^{Y357} phosphorylation observed in these cell lines was consistent with the YAP^{Y357} phosphorylation observed in both CCA PDX (Fig. 1B) and parental tumors from which the SB1 cell line was derived (Fig. 1C).

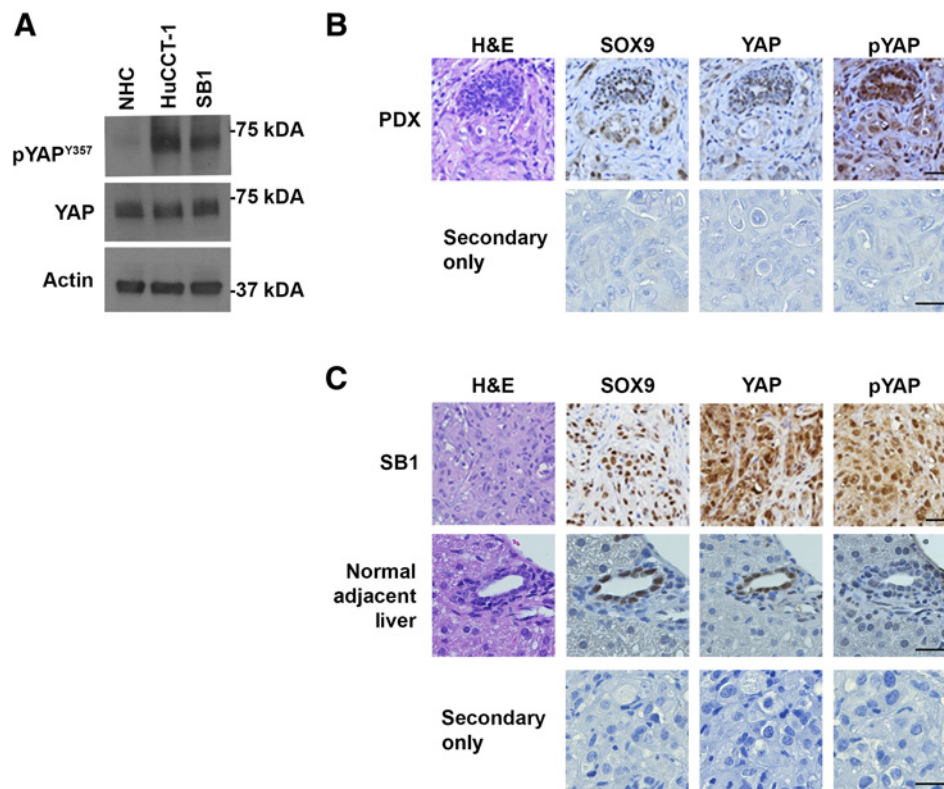
Similar to our previous observations that phosphorylated YAP^{Y357} was enriched in the nucleus of HuCCT-1 cells, immunohistochemistry also demonstrated significant enrichment of the phosphorylated YAP^{Y357} in the nuclei of tumors from the PDX and our YAP-driven murine model of CCA (Fig. 1B and C). Consistent with the minimal phosphorylated YAP^{Y357} noted in normal human cholangiocytes by immunoblot analysis, immunohistochemistry of normal adjacent liver from SB1 tumor-bearing mice also revealed minimal nuclear phosphorylated YAP^{Y357} in cholangiocytes despite the presence of unphosphorylated YAP in this cell type (Fig. 1C). These data indicate that although nuclear YAP is present in normal cholangiocytes, the YAP is not Y357 phosphorylated. In contrast, in CCA a significant proportion of YAP localized to the nucleus is phosphorylated at Y357.

Multiple SFK are expressed in CCA

We next evaluated the expression pattern of SFK in the SB1 cell line and multiple CCA PDX. We have previously demonstrated that five SFK are expressed in the HuCCT-1 cell line similar to the normal human cholangiocyte cell line. These five include the ubiquitously expressed SRC, YES, and FYN, as well as LCK and LYN. Evaluation of the SB1 cell line demonstrated expression of these five SFK as well (Fig. 2A). We then profiled CCA PDX from multiple unique patients. These intrahepatic CCA xenografts all expressed these five SFK similar to the CCA cell lines (Fig. 2A; Supplementary Fig. S1). We next evaluated the effect of SFK activity on YAP target gene expression. Incubation of both the HuCCT-1 and SB1 cell lines with the SFK inhibitor dasatinib (clinically utilized as a BCR-ABL inhibitor but with nanomolar affinity for SRC family kinases) led to downregulation of the YAP target genes CTGF, CYR61, and MCL-1 (Fig. 2B). Similar to our previous observations in HuCCT-1 cells, incubation of the SB1 cell line with dasatinib also blocked the activating phosphorylation of SFK at tyrosine 419 (SFK^{Y419}; Fig. 2C). The positive autoregulatory catalytic domain is highly conserved across the SFK members precluding definitive identification of individual members by immunoblot; however, inhibition of SFK tyrosine phosphorylation was accompanied by a parallel decrease in YAP^{Y357} phosphorylation (Fig. 2D). Given that c-Abl kinase inhibition could also account for the observations with dasatinib treatment, we also examined YAP^{Y357} phosphorylation and YAP subcellular localization following treatment with KW-2449, a structurally dissimilar c-Abl kinase inhibitor. This more specific c-Abl kinase inhibitor displayed minimal effect on YAP^{Y357} phosphorylation and no effect on YAP subcellular localization (Supplementary Fig. S2). We next evaluated the effects of SFK inhibition by dasatinib *in vivo*, in a CCA PDX model following a short course of treatment (3 days). Similar on-target effects were noted in the PDX including reduced SFK^{Y419} phosphorylation, YAP^{Y357} phosphorylation, and YAP target gene expression (Fig. 2B–D). Importantly, a significant decrease in YAP^{Y357} phosphorylation was also observed by immunohistochemistry (Fig. 2E). These data strongly suggest that in CCA, SFK activity is linked to YAP^{Y357} phosphorylation, and pharmacologic inhibition can decrease YAP^{Y357} phosphorylation and YAP target gene expression both *in vitro* and *in vivo*.

The SFK member LCK selectively regulates YAP tyrosine phosphorylation and target gene expression, and LCK expression levels are associated with tumor recurrence

Given the multiple members of the SFK expressed in CCA, we next sought to determine which member was responsible for

**Figure 1.**

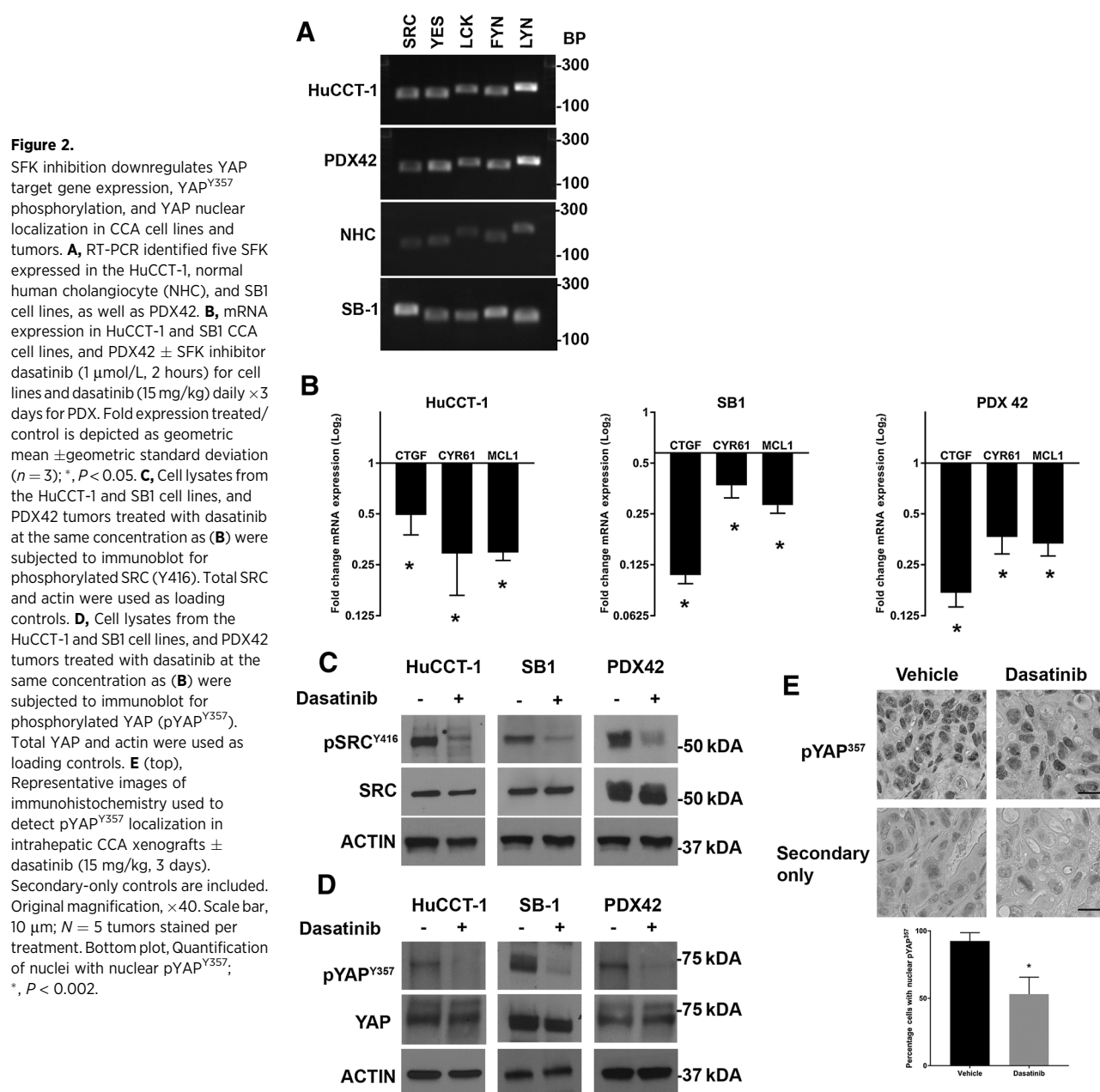
YAP^{Y357} is phosphorylated in CCA cell lines and tumors. **A**, Cell lysates from the normal human cholangiocyte (NHC), HuCCT-1, and SB1 were subjected to immunoblot for phosphorylated YAP tyrosine 357 (pYAP^{Y357}). Total YAP and actin were used as loading controls. **B** and **C**, Representative images of immunohistochemistry used to detect SOX9, YAP, and phosphorylated YAP (pYAP^{Y357}) expression in CCA PDX (**B**), parental tumor from which the SB1 cell line was derived (**C**), and from normal adjacent liver (**C**). Secondary-only controls are included. *N* = 5 tumors/livers were stained. Original magnification, $\times 40$. Abbreviation: H&E, hematoxylin and eosin. Scale bar, 50 μ m.

YAP^{Y357} phosphorylation. Each previously identified family member was targeted by siRNA in HuCCT-1 cells. Targeted knockdown was verified for each member by immunoblot analysis and RT-PCR (Fig. 3A; Supplementary Fig. S3). Only knockdown of *LCK* was associated with a significant decrease in YAP^{Y357} phosphorylation (Fig. 3B). Utilizing the YAP target genes *CTGF* and *CYR61* as markers of YAP cotranscriptional activity, we demonstrated downregulation of cotranscriptional activity with knockdown of multiple SFK; however, the most dramatic downregulation of YAP cotranscriptional activity was noted in the siLCK-HuCCT1 cells as compared with a nontargeted control (Fig. 3C). Cell proliferation was evaluated by cell counting following transfection with siSFK or a nontargeting siRNA control (NC-1) in HuCCT-1 cells. Only siLCK-HuCCT-1 cells demonstrated a significant downregulation of proliferation as compared with controls (Fig. 3D). In order to overcome the possible effect of residual LCK expression following siRNA knockdown, we next evaluated YAP^{Y357} phosphorylation and nuclear localization in HuCCT-1 cells utilizing an inducible CRISPR/Cas9 system. Incubation of the sgLCK-Cas9-HuCCT-1 cell line with doxycycline leads to a more complete loss of LCK (Fig. 3E). Genetic deletion of *LCK* was paralleled by a near complete repression of YAP^{Y357} phosphorylation, and subsequent redistribution of nuclear YAP to the cytosol, with undetectable levels at 72 hours (Fig. 3E and F). To confirm our findings implicating LCK as a direct mediator of YAP tyrosine phosphorylation, we next examined the ability of LCK to phosphorylate YAP directly *in vitro*. Utilizing recombinant YAP as a phospho-substrate with LCK in a cell-free assay, we were able to directly demonstrate efficient YAP Y357 phosphorylation by LCK (Fig. 3G). Finally, the role of SFK expression in patients with resected intrahepatic CCA was examined by comparing RNA sequencing profiles in PDX from patients who had undergone

curative-intent resection of their tumors. Differential expression was evaluated for patients with early tumor recurrence (less than 10 months) as compared with patients with late tumor recurrence (greater than 10 months) and identified 200 genes. The patient cohort with early recurrence had a markedly upregulated expression of *LCK*, log₂ fold change 7.77, *P* = 0.00005, representing the third highest upregulated gene in this patient group (Supplementary Table 2). No other SFK were noted to be differentially expressed. Interestingly, tumor specimens from patients with early recurrence also displayed upregulation of ARID3a, a known binding partner of the YAP-TEAD4 complex (Fig. 3H). Taken together, these data suggest a role for LCK in regulating YAP^{Y357} phosphorylation and YAP cotranscriptional activity in CCA.

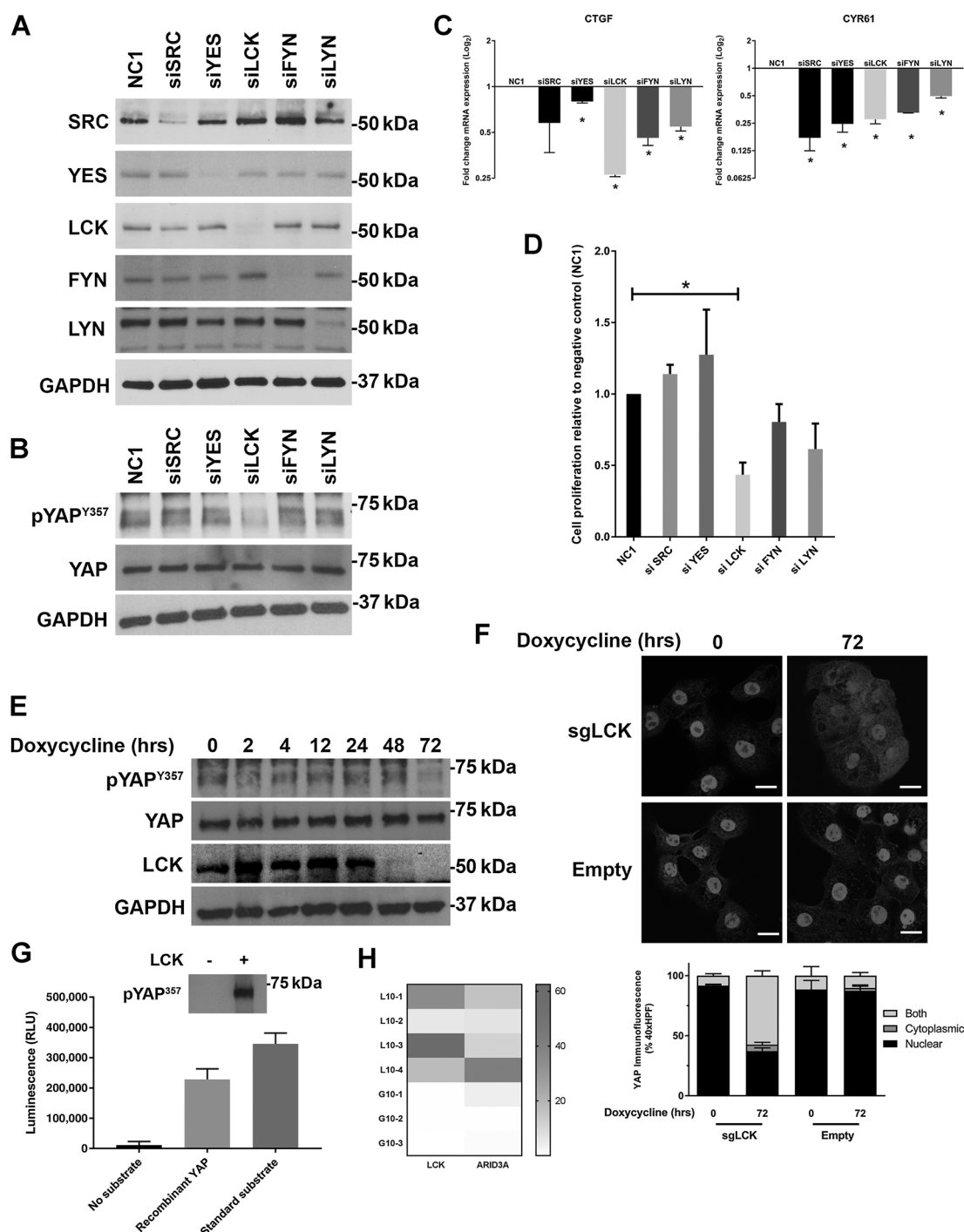
YAP tyrosine phosphorylation regulates nuclear retention, independent of S127 serine/threonine phosphorylation

We next sought to confirm the role of tyrosine phosphorylation in regulating YAP subcellular localization. The SB1 cells express an epitope-tagged YAP containing the S127A mutation, rendering S127A-YAP insensitive to serine/threonine kinase phosphorylation, providing a useful reagent to determine if YAP subcellular localization may be controlled independent of the canonical serine/threonine kinase cascades. We examined S127A-YAP subcellular localization in SB1 cells at baseline and following incubation with dasatinib. Following treatment with dasatinib, S127A-YAP translocated from the nucleus to the cytoplasm (Fig. 4A). Next, we examined potential S127A-YAP-binding partners in the presence and absence of dasatinib by immunoprecipitation of the FLAG epitope followed by mass spectroscopy. We identified multiple tyrosine phosphatases associated with S127A-YAP, and this association increased following incubation with dasatinib (Table 1). In keeping with these findings, we observed that



incubation of HuCCT-1 cells with the tyrosine phosphatase inhibitor sodium orthovanadate (Na_3VO_4) abolished the dasatinib-induced nuclear to cytoplasmic redistribution of YAP (Fig. 4B). Similarly, S127A-YAP cellular localization in the SB1 cells incubated with Na_3VO_4 and dasatinib remained nuclear (Fig. 4A). These data suggest that YAP nuclear retention in CCA cells is likely independent of S127 phosphorylation by serine/threonine kinases and that YAP nuclear retention is due, in part, to a balance between tyrosine kinase and tyrosine phosphatase activity. To further validate this nuclear retention pathway and rule out the possibility that the Hippo kinase complex could be regulating YAP localization through phosphorylation of serine residues other than S127, we next overexpressed a FLAG-tagged YAP in which eight serine residues are mutated to alanine (FLAG-

5SA-YAP), in HuCCT-1 cells. The localization of this 5SA-YAP as determined by immunofluorescence of the epitope-tag was examined following dasatinib incubation. Confirming our previous findings, 5SA-YAP also redistributed from the nucleus to the cytosol in dasatinib-treated cells, and this redistribution was abrogated by preincubation with Na_3VO_4 (Fig. 4C). To exclude an effect of dasatinib on the actin cytoskeleton, which is known to regulate YAP cellular localization, we also examined wild-type YAP localization after pretreatment with the actin inhibitor latrunculin-A. Following incubation with latrunculin-A, the cells rounded as expected, however YAP was still localized to the nucleus and subsequently redistributed to the cytosol only after dasatinib treatment (Fig. 4D). Recent work has also suggested a role for tyrosine phosphorylation in regulating LATS1 activity

**Figure 3.**

The SFK member LCK regulates YAP tyrosine phosphorylation and transcriptional activity in CCA. **A**, Cell lysates from HuCCT-1 cell lines transfected with targeting and nontargeting siRNA were subjected to immunoblot for SRC, YES, LCK, FYN, and LYN. GAPDH was used as a loading control. **B**, Cell lysates from HuCCT-1 cell lines transfected with nontargeting siRNA and siRNA for five SFK were subjected to immunoblot for phosphorylated YAP (pYAP^{Y357}). Total YAP and GAPDH were used as loading controls. **C**, *CTGF* and *CYR61* mRNA expression in HuCCT-1 cell line transfected with nontargeting siRNA (NC1) and siRNA for five SFK. Fold expression targeting/nontargeting is depicted as geometric mean $\pm$ geometric standard deviation ($n = 3$); *, $P < 0.05$. **D**, Relative cell proliferation in HuCCT-1 cell lines transfected with targeting and nontargeting siRNA for five SFK, quantitation; *, $P < 0.05$. **E**, HuCCT-1 cell lines transfected with a doxycycline-inducible CRISPR/Cas9 construct targeting LCK were incubated with doxycycline (7.5 μ g/mL) for the noted time. Cell lysates were subjected to immunoblot for pYAP^{Y357} (total YAP as loading control) and LCK (GAPDH as loading control). **F**, HuCCT-1 cell lines transfected with a doxycycline-inducible CRISPR/Cas9 construct targeting LCK or empty vector were incubated with doxycycline (7.5 μ g/mL) for the noted time. Immunofluorescence for YAP was undertaken, and representative images are displayed (top) with quantification of YAP localization (bottom). Original magnification, $\times 40$. Scale bar, 10 μ m. **G**, Phosphorylation of recombinant YAP in a cell-free system by recombinant LCK was evaluated by luminescence and immunoblot (inset). **H**, Heat map representation of FPKM for *LCK* and *ARID3a* from PDX derived from patients undergoing curative intent liver resection with recurrence in less than 10 months (L10) versus recurrence in greater than 10 months (G10).

Table 1. Tyrosine phosphatases associated with YAP in murine SB1 cells: ratio of total spectrum count (TSC) in dasatinib-treated/-untreated controls

Phosphatase	Ratio TSC treated/control
PTPN11	3.5
PTPRK	3.0
PTPN1	2.0
PTPN23	2.0

(25). In order to exclude an effect of dasatinib on LATS1 activity as a mechanism of regulating YAP subcellular compartmentation in CCA cells, we examined the phosphorylation status of serine 909 on LATS1 (LATS1^{S909}) in the presence and absence of dasatinib. LATS1^{S909} phosphorylation signifies an enzymatically active LATS1. Incubation of HuCCT-1 cells with dasatinib led to the expected decrease in YAP^{Y357} phosphorylation with no appreciable effect on LATS1^{S909} phosphorylation (Fig. 4E). Previous work has demonstrated that SFK may also phosphorylate YAP on tyrosines 341, 357, and 394 (26). Therefore, we also examined the role of these tyrosine phospho sites in regulating YAP cellular compartmentation by expressing an eGFP-fusion protein in which these three tyrosine residues were mutated to phenylalanine (Y341F, Y357F, Y394F-YAP-eGFP). Compared with the wild-type YAP-eGFP, which was located in both the nucleus and cytosol, Y341,357,394F-YAP-eGFP was enriched in the cytosol and was excluded from the nucleus (Fig. 4F). We next examined whether mutation of the Y357 site was sufficient to limit YAP localization to the cytosol. Indeed, Y357F-YAP-eGFP was also excluded from the nucleus, suggesting that phosphorylation of the Y357 residue was sufficient to regulate nuclear retention of YAP in CCA cells (Fig. 4F). Importantly, incubation of the wild-type YAP-eGFP HuCCT-1 cells with dasatinib leads to rapid redistribution of the YAP to the cytosol, suggesting transport kinetics for the GFP-tagged protein were similar to untagged protein (Fig. 4G). Taken together, these data suggest a nuclear retention pathway for YAP regulated by tyrosine phosphorylation of Y357 and independent of YAP serine phosphorylation and therefore LATS1 activity.

The SFK inhibitor dasatinib is therapeutic *in vitro* and *in vivo*

Incubation of HuCCT1 and SB1 cells with dasatinib leads to an increased cell death (Fig. 5A) and loss of cell number in a concentration-dependent manner with significant effects noted at clinically achievable concentrations ($\leq 1 \mu\text{mol/L}$; Fig. 5B). Consistent with prior observations that the prosurvival protein MCL-1 is a YAP target gene, dasatinib treatment of the CCA cells leads to a decrease in MCL-1 protein and gene expression (Fig. 2B and C). Next, we examined dasatinib therapeutic efficacy in NOD/SCID mice bearing CCA PDX. PDX from 3 different patients (PDX 42, 115, 135) were implanted bilaterally in the mice and tumor-bearing animals treated with daily gavage of dasatinib (15 mg/kg) or vehicle; initiated once the tumors were palpable ($\sim 125 \text{ mm}^3$). Following 14 days of treatment, there was a dramatic difference in the size of tumors from dasatinib-treated animals as compared with vehicle treated (Fig. 5D). This was accompanied by a marked increase in cell death as indicated by TUNEL staining (Fig. 5E). The increase in cell death was an early process and presented even after short course treatment (3 days). Collectively, SFK inhibition with dasatinib was tumor suppressive *in vitro* and *in vivo*, suggesting the LCK-mediated YAP tyrosine phosphorylation pathway in CCA is potentially therapeutically targetable.

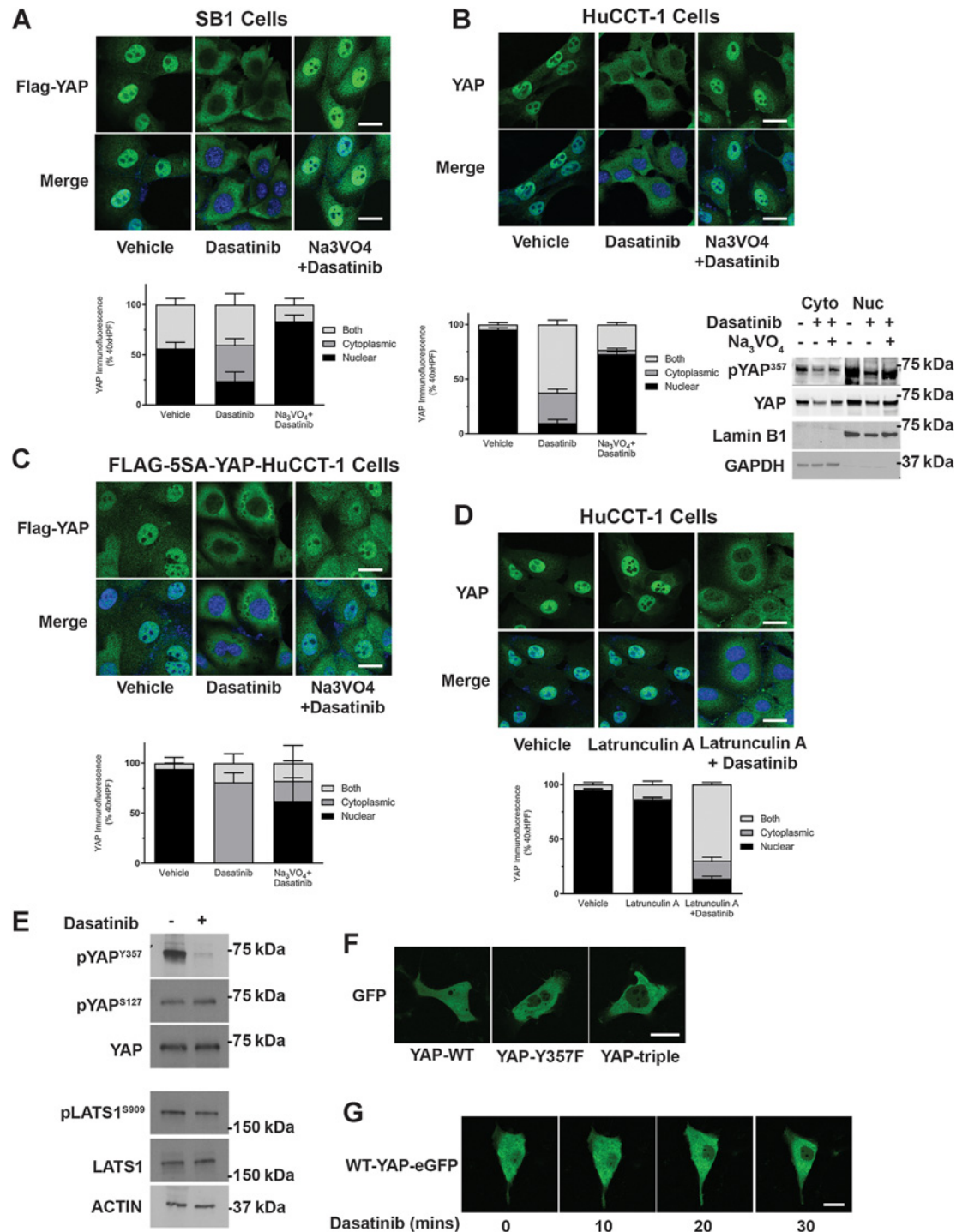
Discussion

This study delineates a regulatory role for YAP tyrosine phosphorylation in CCA cell lines and PDX (Fig. 6). These data indicate that (1) tyrosine phosphorylation regulates YAP nuclear retention independent of the serine/threonine cytosolic retention pathway, (2) the SRC-family kinase LCK mediates YAP^{Y357} phosphorylation and subsequent activity, and (3) SFK inhibition is therapeutic *in vitro* and *in vivo*. These findings are discussed in greater detail below.

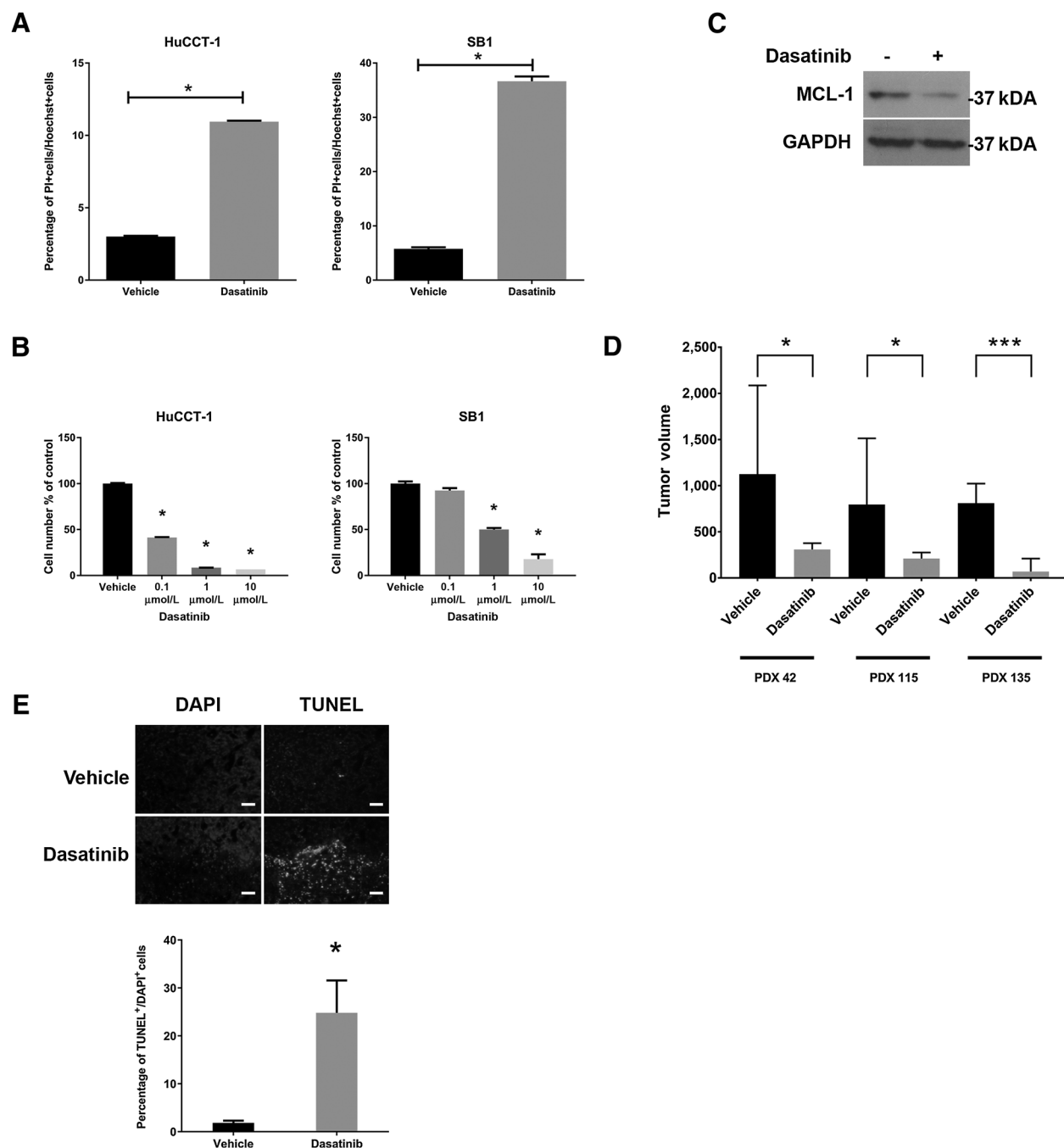
The canonical regulatory pathway described for YAP involves a core Hippo serine/threonine kinase relay module involving MST1/2 and LATS1/2. This serine/threonine kinase relay culminates in phosphorylation of regulatory serine residues on YAP; regulatory serine phosphorylation promotes YAP cytosolic sequestration and/or proteasomal degradation (4, 27). This pathway, which we have termed the cytosolic retention pathway, restrains the activity of YAP; and consistent with these concepts, we and others have demonstrated complete exclusion of phosphorylated YAP^{S127} from the nucleus (2, 28). However, accumulating evidence also implicates receptor tyrosine kinases in the regulation of YAP cellular compartmentation (2, 8). In the current study, we further examined the role of tyrosine phosphorylation in regulating YAP nuclear localization, a so-called nuclear retention pathway. We were able to separate the serine/threonine and tyrosine phospho-regulation effects by utilizing our CCA cell line expressing an epitope-tagged S127A-YAP and subsequently by enforced expression of an epitope-tagged 5SA-YAP in a human CCA cell line. S127A-YAP is predominantly compartmentalized to the nucleus. Utilizing an inhibitory paradigm to inform the regulatory pathways, we noted redistribution of S127A-YAP from the nucleus into the cytosol following SFK inhibition. This redistribution of S127A-YAP out of the nucleus was accompanied by its association with tyrosine phosphatases and was blocked by a tyrosine phosphatase inhibitor. These data suggest a regulatory process whereby YAP tyrosine phosphorylation promotes nuclear retention. Conversely, dephosphorylation, either through decreased tyrosine kinase activity or via tyrosine phosphatase activity, enhances transport of YAP out of the nucleus. Although S127 is the most comprehensively studied serine residue regulating YAP cellular compartmentation, other potential regulatory serine phosphorylation sites exist in YAP, and one possibility is that the Hippo kinase module could be regulating the subcellular localization by phosphorylation at serines other than S127. Moreover, LATS1 tyrosine phosphorylation may also negatively regulate LATS1 serine kinase activity (25). In this scenario, SFK inhibition may increase LATS1 activity and subsequent YAP phosphorylation on multiple serine residues. Our data argue against this mechanism in CCA cells as YAP^{S127} phosphorylation did not increase in HuCCT-1 cells treated with dasatinib, the 5SA-YAP (in which 8 serine residues have been mutated to alanine) still redistributed to the cytosol with dasatinib treatment, and p-LATS1^{S909} was not altered. Additional studies will be required to fully understand the interplay between the cytosolic and nuclear retention pathways and potential cross-regulation; however, our data suggest functions for both serine and tyrosine phospho-regulation of YAP and its cellular compartmentation.

Based on our previous observations linking SFK activity to PDGF regulation of YAP activity and localization, we sought to

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**Figure 4.**

Phosphorylation of YAP^{Y357} regulates nuclear retention in CCA. **A**, Representative images of FLAG immunofluorescence in the SB1 CCA cell line incubated with vehicle, dasatinib (100 nmol/L, 1 hour), or dasatinib (100 nmol/L, 1 hour) and sodium orthovanadate (10 mmol/L, 1 hour; top plot) and quantification of YAP nuclear localization (bottom plot). **B**, Representative images of YAP immunofluorescence in HuCCT-1 cells incubated with vehicle, dasatinib (100 nmol/L, 1 hour), or dasatinib (100 nmol/L, 1 hour) and sodium orthovanadate (10 mmol/L, 1 hour; top plot), quantification of YAP nuclear localization (bottom left plot), and cell fractionation evaluated by immunoblot (bottom right plot). Lamin B1 and GAPDH were used as nuclear and cytoplasmic markers, respectively. **C**, Representative images of FLAG immunofluorescence in HuCCT-1 cells transfected with FLAG-5SA-YAP incubated with vehicle, dasatinib (100 nmol/L, 1 hour) and sodium orthovanadate (10 mmol/L, 1 hour; top plot) and quantification of YAP nuclear localization (bottom plot). **D**, Representative images of YAP immunofluorescence in HuCCT-1 cells incubated with vehicle, latrunculin A (1 μmol/L, 1 hour), or latrunculin A (1 μmol/L, 1 hour) and dasatinib (100 nmol/L, 1 hour; top plot) and quantification of YAP nuclear localization (bottom plot). **E**, Cell lysates from HuCCT-1 cells treated with dasatinib (1 μmol/L, 2 hours) were subjected to immunoblot for pYAP^{Y357}, pYAP^{S127} (total YAP as a loading control), and pLATS1^{S909} (total LATS1 as a loading control). Actin was used as a loading control. **F**, Representative images of fluorescent microscopy in HuCCT-1 cells expressing wild-type or mutated YAP-eGFP. **G**, Representative images of time lapse fluorescent microscopy in HuCCT-1 cells expressing wild-type YAP-eGFP. Original magnification, ×40. Scale bar, 10 μm for all images.

**Figure 5.**

The SFK inhibitor dasatinib is therapeutic both *in vitro* and *in vivo*. **A**, Cell death was evaluated after staining with propidium iodide in HuCCT1 and SB1 cells treated with dasatinib (1 $\mu\text{mol/L}$, 24 hours). *, $P < 0.05$. **B**, HuCCT-1 and SB1 cells were incubated with vehicle or the indicated concentration of dasatinib for 3 days. Cell proliferation was evaluated by cell counting after staining with Hoechst 33342 and was normalized to vehicle treatment. *, $P < 0.01$. **C**, Cell lysates from HuCCT-1 cell lines incubated $\pm$ dasatinib (1 $\mu\text{mol/L}$, 2 hours) were subjected to immunoblot for MCL-1. GAPDH was used as a loading control. **D**, Tumor-bearing NOD/SCID mice from PDX42, PDX115, and PDX 135 were treated with daily gavage of vehicle or dasatinib (15 mg/kg, 14 days; $n = 7/\text{treatment}$) and tumor volumes recorded daily. Day 14 tumor volumes are displayed for all three PDX. *, $P < 0.05$ and ***, $P < 0.001$. **E**, Representative images following TUNEL staining in tumors from vehicle- and dasatinib-treated animals (top plot), with quantification of TUNEL-positive cells (bottom plot). Original magnification, $\times 10$. Scale bar, 50 μm .

determine more precisely which SFK may be involved in this pathway. In IDH1/2-mutated CCA, Saha and colleagues demonstrated a central role for SRC itself (29). In our studies, we did not find a significant effect on YAP tyrosine phosphorylation with

downregulation of SRC levels. We did however note a decrease in YAP tyrosine phosphorylation and transcriptional activity with siRNA-targeted knockdown of LCK. This association between LCK and YAP phosphorylation has not been previously reported. In

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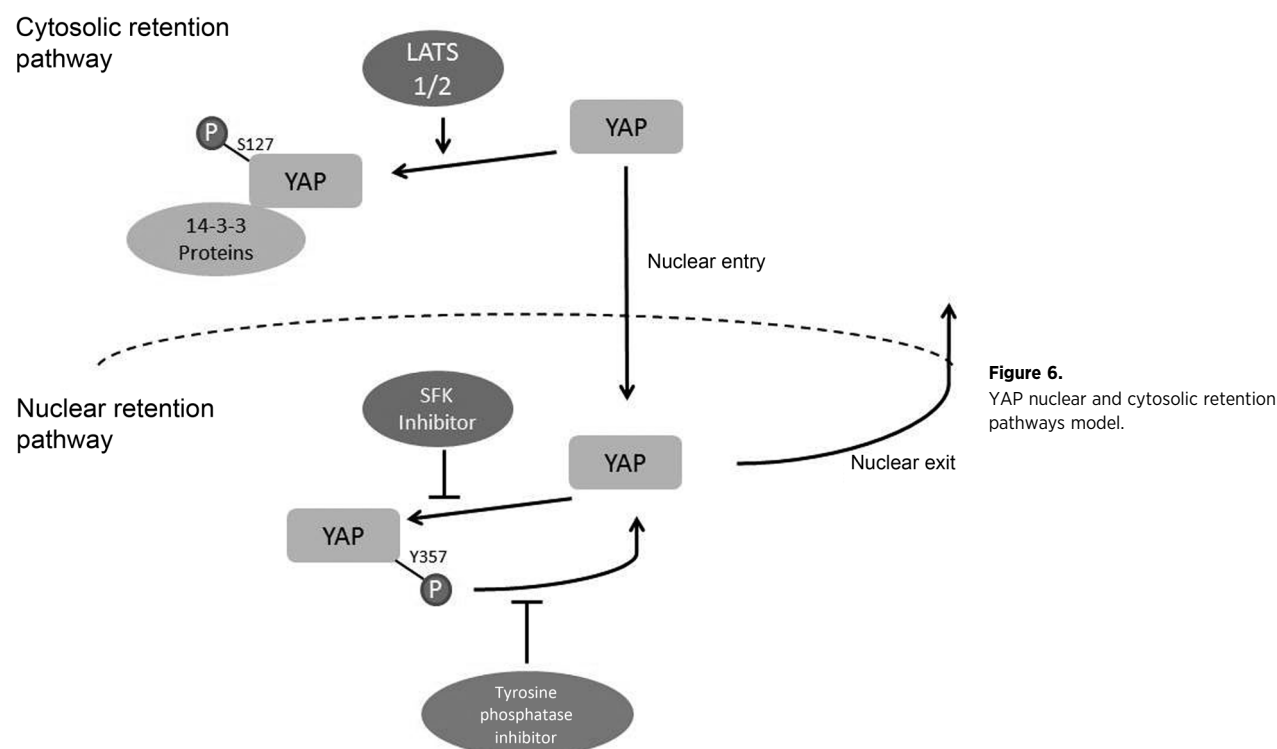


Figure 6.
YAP nuclear and cytosolic retention pathways model.

fact, LCK is most comprehensively described in its role in signal transduction from the T-cell receptor to downstream intracellular signaling molecules (30). We found LCK is expressed in not only normal human cholangiocytes, but both of the CCA cell lines we utilized, and the CCA PDX tumors examined. Interestingly, when dasatinib was undergoing development as a pan-SRC kinase inhibitor, it was LCK that was utilized as the binding partner for the compound screen and subsequent optimization strategies (31). It is possible that other SFK are involved in YAP regulation in a redundant fashion, but that thresholds for expression may be different, such that more complete knockdown of the other expressed family members may be necessary to see effects on YAP phosphorylation and localization. The degree of homology between the family members makes pharmacologic inhibition of individual members challenging; however, other strategies such as CRISPR/Cas9 constructs would further delineate the roles of the individual SFK.

The tyrosine phosphorylation of YAP is an attractive therapeutic target as it represents an activating phosphorylation (vs. the inhibitory serine phosphorylation). SFK inhibition can be achieved with the pan-SRC inhibitor dasatinib, and efficacy has previously been demonstrated in IDH-mutated intrahepatic CCA models (29). We found dramatic sensitivity to dasatinib in all of our models, cell lines, and PDX. The effect appears to be, at least partially, due to downregulation of the prosurvival YAP target gene MCL-1. Further studies are underway to determine if targeted downregulation of LCK has a similar effect on tumor survival; however, the accumulated preclinical data suggest SFK inhibition as a therapeutic strategy in intrahepatic CCA.

Overall, our study contributes to the understanding of YAP function and regulation in cancer. Our observations extend the concept of phospho-regulation of YAP to include a role

for tyrosine phosphorylation independent of LATS 1/2 serine phosphorylation and a separation of the cytosolic and nuclear retention signals. In addition, we have identified a potentially novel role for LCK in the regulation of YAP in CCA. Finally, we have generated additional preclinical data supporting the role of SFK inhibition in the treatment of intrahepatic CCA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

This work was supported by NIH grants R01DK59427 (to G.J. Gores), Optical Microscopy Core services and a Pilot and Feasibility Award (to R.L. Smoot) provided through the Mayo Clinic Center for Cell Signaling in Gastroenterology (P30DK084567), the Mayo Clinic Center for Biomedical Discovery Pilot Award (to R.L. Smoot), Department of Defense CDMRP Career Development Award CA171017 (to R.L. Smoot), the Mayo Clinic Medical Genome Facility - Proteomics Core, a supplement for liver cancer infrastructure

(NCI Cancer Center Support Grant 5P30 CA15083-43C1), MH CZ - DRO (UHHK, 00179906; to P. Hirsova), and by the Mayo Clinic.

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Received February 14, 2018; revised April 18, 2018; accepted May 30, 2018; published first June 14, 2018.

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Molecular Cancer Research

YAP Tyrosine Phosphorylation and Nuclear Localization in Cholangiocarcinoma Cells Are Regulated by LCK and Independent of LAT5 Activity

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Mol Cancer Res 2018;16:1556-1567. Published OnlineFirst June 14, 2018.

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The YAP-Interacting Phosphatase SHP2 Can Regulate Transcriptional Coactivity and Modulate Sensitivity to Chemotherapy in Cholangiocarcinoma

EeeLN H. Buckarma¹, Nathan W. Werneburg², Caitlin B. Conboy³, Ayano Kabashima², Daniel R. O'Brien⁴, Chen Wang⁴, Sumera Rizvi², and Rory L. Smoot¹



ABSTRACT

The Hippo pathway effector Yes-associated protein (YAP) is localized to the nucleus and transcriptionally active in a number of tumor types, including a majority of human cholangiocarcinomas. YAP activity has been linked to chemotherapy resistance and has been shown to rescue KRAS and BRAF inhibition in RAS/RAF-driven cancers; however, the underlying mechanisms of YAP-mediated chemoresistance have yet to be elucidated. Herein, we report that the tyrosine phosphatase SHP2 directly regulates the activity of YAP by dephosphorylating pYAP^{Y357} even in the setting of RAS/RAF mutations, and that diminished SHP2 phosphatase activity is associated with chemoresistance in cholangiocarcinomas. A screen for YAP-interacting tyrosine phosphatases identified SHP2, and characterization of cholangiocarcinomas cell lines demonstrated an inverse relationship between SHP2 levels and pYAP^{Y357}. Human sequencing data demonstrated lower SHP2

levels in cholangiocarcinomas tumors as compared with normal liver. Cell lines with low SHP2 expression and higher levels of pYAP^{Y357} were resistant to gemcitabine and cisplatin. In cholangiocarcinomas cells with high levels of SHP2, pharmacologic inhibition or genetic deletion of SHP2 increased YAP^{Y357} phosphorylation and expression of YAP target genes, including the antiapoptotic regulator MCL1, imparting resistance to gemcitabine and cisplatin. *In vivo* evaluation of chemotherapy sensitivity demonstrated significant resistance in xenografts with genetic deletion of SHP2, which could be overcome by utilizing an MCL1 inhibitor.

Implications: These findings demonstrate a role for SHP2 in regulating YAP activity and chemosensitivity, and suggest that decreased phosphatase activity may be a mechanism of chemoresistance in cholangiocarcinoma via a MCL1-mediated mechanism.

Introduction

Cancer of the biliary tract, cholangiocarcinoma, is increasing in worldwide incidence (1–3). Unfortunately, treatment options remain limited, with standard chemotherapy regimens providing only modest increases in patient survival. The median overall survival with the current standard-of-care systemic chemotherapy regimen of gemcitabine and cisplatin is <1 year (4). Therapeutic advances for cholangiocarcinomas will require an understanding of the oncogenic signaling pathways driving this malignancy and response to therapy. The Hippo pathway effector, Yes-associated protein (YAP), has been implicated in cholangiocarcinomas oncogenesis and progression (5–12). Therefore, we have continued to evaluate YAP signaling in cholangiocarcinomas, and more specifically its phosphoregulation. Our group and others have noted that a significant proportion of cholangiocarcinomas specimens have YAP localized to the nucleus, presumably representing a cotranscriptionally active molecule (5–7, 10, 11). Importantly, we have further observed that

nuclear YAP is tyrosine phosphorylated in cholangiocarcinomas, which may have significant functional and targeting implications (9).

The canonical phosphoregulatory pathway for YAP, the Hippo pathway, is a kinase module of serine/threonine kinases (MST1/2 and LATS1/2) and various scaffolding proteins (MOB1 and SAV), which when active, culminate in serine phosphorylation of YAP leading to its cytoplasmic sequestration and/or ubiquitination and degradation, such that when the Hippo pathway is active, YAP activity is restrained (13). Therapeutic targeting of YAP via the Hippo pathway is difficult due to the lack of a dedicated cell surface receptor for the pathway, and the fact that the pathway acts as a negative regulator, as such traditional therapeutic strategies are challenging. The interest in targeting Hippo/YAP activity stems from not only an observed upregulation in multiple cancer types, but also the observations that YAP activity can modulate sensitivity to both traditional DNA damaging agents and targeted therapies, and that YAP can rescue directed inhibition of KRAS and BRAF in RAS/RAF-driven cancers (14–25). However, the precise mechanisms of chemoresistance tied to YAP activity have yet to be fully delineated.

In continuing to explore YAP activity in cholangiocarcinomas and its effects on proliferation and therapeutic response, we have recently demonstrated that the tyrosine phosphorylation status of YAP is more important in determining its subcellular localization and transcriptional activity in cholangiocarcinomas than serine phosphorylation (9). We have linked the SRC family kinase (SFK) LCK to YAP tyrosine phosphorylation, which corroborates other reports of SFKs' ability to regulate YAP activity (26–30). Consistent with a role for LCK in regulating YAP nuclear compartmentation, tyrosine phosphatases were also implicated in YAP regulation as kinase inhibition was ineffective in regulating YAP subcellular localization in the presence of a pan-tyrosine phosphatase inhibitor (9). Tyrosine phosphatases are known to interact with YAP, for example, previous work has

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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Mol Cancer Res 2020;18:1574–88

doi: 10.1158/1541-7786.MCR-20-0165

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demonstrated a regulatory role of PTPN14 in regulating YAP activity, but this regulation was independent of the tyrosine phosphatase activity, and PTPN11 (SHP2) has been shown to interact with YAP, but it was suggested that YAP regulated SHP2 localization; however, phosphatase regulation of YAP was not explored in detail (31, 32). Given our preliminary observations, we were interested in exploring the role of tyrosine phosphatases in regulating YAP activity in cholangiocarcinomas and modulating cholangiocarcinomas sensitivity to gemcitabine and cisplatin.

Herein, we examine tyrosine phosphatase activity regulating YAP activation in cholangiocarcinomas and its effects on sensitivity to therapy. The results demonstrate an inverse relationship between levels of the tyrosine phosphatase SHP2 and levels of pYAP^{Y357} in cholangiocarcinomas cell lines. Furthermore, downregulation of SHP2 pharmacologically or utilizing CRISPR/Cas9 was associated with increased levels of YAP tyrosine phosphorylation, and consequently increased YAP nuclear localization and transcriptional coactivity. The observed increases in YAP activity were accompanied by increases in cellular proliferation and expression of the prosurvival BCL2 family member MCL1, culminating in resistance to gemcitabine and cisplatin both *in vitro* and *in vivo*.

Materials and Methods

Cell culture

The human cholangiocarcinoma cell lines HuCCT-1 and KMCH, the murine cholangiocarcinomas cell line SB1, and the human pancreatic adenocarcinoma cell line PANC1 were cultured in DMEM supplemented with 10% FBS and 0.2% primocin under standard conditions. Because of cell density and serum regulation of the Hippo pathway, cell culture experiments were performed at near confluence (~80%). Human HuCCT-1, KMCH, and PANC1 cell lines were obtained from a long-term laboratory stock that was authenticated by short tandem repeat analysis performed by the Genome Analysis Core of the Medical Genome Facility (Mayo Clinic, Rochester, MN). All cell lines underwent *Mycoplasma* contamination testing periodically using Plasmotest Mycoplasma Detection Kit (InvivoGen). Cell lines were used within 40 passages of reanimation. All cell lines were maintained at 37°C in the presence of 5% CO₂.

Antibodies and reagents

SHP099 (Selleckchem), NSC87877 (Cayman), Doxycycline Hyclate (Sigma), S63845 (APEX-BIO), Verteporfin, and Gemcitabine and Cisplatin (Cayman) were added to cells at final concentrations from 0.05 to 100 µmol/L per experimental design. The following primary antibodies were used for immunoblot analysis: actin (C-11, Santa Cruz Biotechnology), phospho YAP^{Y357} (ab62751, Abcam), total YAP (63.7, Santa Cruz Biotechnology, 4912 CST), GAPDH (MAB374, Millipore), phospho SRC^{Y416} (2101, Cell Signaling Technology), SRC (L4A1, Cell Signaling Technology), SHP2 (33975, Cell Signaling Technology), and MCL-1 (ac32087, Abcam). The following primary mAbs were used for immunofluorescence and/or IHC: total YAP (63.7, Santa Cruz Biotechnology) and phospho YAP^{Y357} (ab62751, Abcam). ProLong Antifade with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies) was used for nuclear staining.

SHP2 differential expression analysis

We examined the SHP2 (*PTPN11*) RSEM transformed normalized gene expression values from The Cancer Genome Atlas (TCGA) CHOL primary tumor samples ($n = 36$) and compared them with GTEx normal liver samples ($n = 110$; refs. 33–35). The data were downloaded

from the University of California Santa Cruz's Xenabrowser (36). The source of the TCGA CHOL normalized expression values came from the University of North Carolina TCGA genome characterization center, and the source of the GTEx normalized expression values came from the University of California Santa Cruz Computational Genomics Lab. These gene expression values were derived from similar RNA-sequencing experiments utilizing similar bioinformatics methods. A *t* test was conducted and a box plot was constructed comparing the CHOL samples with the normal liver samples.

Immunoprecipitation and immunoblot analyses

Whole-cell lysates were collected by adding ice-cold lysis buffer (Cell Signaling Technology) containing protease inhibitors (Sigma-Aldrich), phosphatase inhibitors (Roche Diagnostics), and 1 mmol/L PMSF. Tissue sample lysates were homogenized with a glass Dounce and ice-cold lysis buffer containing protease inhibitors, phosphatase inhibitors, and 1 mmol/L PMSF. Samples were vortexed and lysed on ice for approximately 20 minutes. The lysed tissue was then centrifuged at 12,000 × *g* for 15 minutes to remove cellular debris. Supernatant was transferred to a clean tube and protein concentration determined by the Bradford (Sigma-Aldrich) Protein Assay. For immunoprecipitation, whole-cell lysates of SB1 cells that contain the FLAG-YAPS127A were incubated with equilibrated anti-FLAG M2 Magnetic Beads (Sigma-Aldrich) overnight at 4°C. The FLAG-YAPS127A was then eluted from the beads with FLAG peptide following the manufacturer's instructions. To conduct immunoblot analysis, proteins were then resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies at 4°C overnight in 5% BSA-TBS Tween. The primary antibody dilution was 1:1,000 unless otherwise indicated. After incubation, membranes were washed for 30 minutes in TBS Tween and then horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit, and goat (Santa Cruz Biotechnology) were added to membrane at a concentration of 1:5,000 and incubated for 1 hour at room temperature. Immunoblots were visualized with enhanced chemiluminescence (ECL) or ECL Prime (GE Healthcare Life Sciences). For experiments in which multiple proteins were evaluated, membranes were stripped and reblotted.

Immunofluorescence

Cells were grown on glass coverslips, once the cells reached desired confluency they were treated per experimental conditions. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Next, the fixed cells were incubated at room temperature with blocking buffer containing 5.0% BSA and 0.1% glycine. Following blocking, the cells were incubated with primary antibody in blocking buffer overnight at 4°C. After washing, the cells were incubated with secondary antibodies diluted in blocking buffer for 1 hour at room temperature in the dark. Following secondary incubation, the coverslips were again washed and mounted onto slides using ProLong Antifade (Invitrogen-Molecular Probes) containing DAPI. The slides were analyzed by fluorescence confocal microscopy (LSM 780, Zeiss).

Quantitative and qualitative PCR

mRNA was isolated from cells and tissues using TRIzol Reagent (Ambion). Reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase and random primers (Life Technologies). After reverse transcription, cDNA was analyzed using real-time PCR (Light Cycler 480 II, Roche Diagnostics) for the quantitation of the target genes, SYBR Green (Roche Diagnostics) was used as the fluorophore. Expression was normalized to 18 S and

relative quantification performed according to the $2^{-\Delta C_t}$ or $2^{-\Delta\Delta C_t}$ method as described previously (37). Data are reported as fold expression compared with calibrator as geometric mean and geometric SD of expression relative to calibrator. Technical replicates were completed for each run and a minimum of three biologic replicates completed for each condition/cell line. The primers used are listed in Supplementary Table S1.

TEAD reporter assay

Global TEAD transcriptional activity was assessed in HuCCT-1 and KMCH cells using a luciferase-based TEAD reporter. Cells were transduced with lentivirus encoding a reporter construct with the TEAD response elements and a minimal TATA promoter driving expression of firefly luciferase (BPS Bioscience, #79833). Transduced cells were selected with puromycin. Cells were treated with NSC87877 or SHP099 at 10 $\mu\text{mol/L}$ for 24 hours prior to measurement of firefly luciferase activity with a Luciferase Assay System (BPS Bioscience).

CRISPR/Cas9 doxycycline-induced knockout

The sequence of single-guide RNA (sgRNA) for SHP2 was designed using online CRISPR design tool (<http://crispr.mit.edu/>) and was 5'-GGTGATTACTATGACCTGTA-3'. DNA oligonucleotides for the sgRNA were synthesized and cloned into the FgH1tUTG Plasmid (Addgene). To produce lentiviruses, FgH1tSHP2 or FUCas9Cherry plasmids were transiently cotransfected into HEK293T cells with the packaging plasmids pRSV-Rev, pMDLg/pRRE, and pCMV-VSV-G (all from Addgene) using FuGENE HD Transfection Reagent (Promega Corporation). After 48 and 72 hours, supernatant containing lentiviruses was harvested and passed through a 0.45- μm filter. Target KMCH cells were transduced with the lentiviruses in the presence of 8 $\mu\text{g/mL}$ polybrene for 24 hours. After an additional 3 days in culture, mCherry (Cas9) and GFP (inducible sgRNA) dual-positive cells were sorted on a single cell basis into a 96-well plate by flow cytometry. To induce sgRNA expression, Doxycycline Hyclate (Sigma) was added to culture medium at a final concentration of 7.5 $\mu\text{g/mL}$. The efficacy of CRISPR/Cas9-mediated gene deletion was determined by immunoblot analysis.

SHP2 wild-type/phosphatase mutants

Following the manufacturer's instructions, HuCCT-1 cells were transfected using SHP2(WT)@pLKO-Trex-SBP, a gift from Darrin Stuart (Addgene, plasmid # 85457; <http://n2t.net/addgene:85457>; RRID:Addgene_85457) and SHP2(C459S)@pLKO-Trex-HA, a gift from Darrin Stuart (Addgene, plasmid # 85459; <http://n2t.net/addgene:85459>; RID:Addgene_85459). Following 48-hour transfection, the cells were used for subsequent experiments.

Cell viability

HuCCT-1, KMCH, and sgSHP2-KMCH cells were plated in triplicate in 96-well plates at a density of 3,000 and 4,000 cells per well, respectively, according to the growth characteristics of each cell line and treated with various compounds at a range of concentrations. After 24 hours, cells were treated with NSC87877 (10 $\mu\text{mol/L}$) or S63845 (5 $\mu\text{mol/L}$) for 24, 48, and 72 hours. For combination experiments, cells were incubated for 24 hours and treated with gemcitabine and cisplatin (0.05, 0.1, 1, 10, and 100 $\mu\text{mol/L}$) for 24, 48, and 72 hours. Cell viability following treatment was assessed using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and CellTiter-Glo Luminescent Cell Viability Assay (Promega).

In vivo studies

The study protocol was approved by the Institutional Animal Care and Use Committee at Mayo Clinic (A00004673-19). Experiments were carried out utilizing 8-week-old male NOD/SCID mice. Xenografts were developed by separate subcutaneous injections of sgEmpty-KMCH and sgSHP2-KMCH cells (5×10^5 cells suspended in 40% DMEM and 60% Matrigel, totaling 100 μL) into the flank area of each mouse. In each mouse, the right flank was utilized for control tumor (sgEmpty-KMCH) and left flank utilized for experimental tumor (sgSHP2-KMCH). Tumor volume and body weight were recorded every 3 days. Treatment was initiated 2 weeks after tumor implantation when tumors had reached a volume of approximately 200 mm^3 . Combination gemcitabine (15 mg/kg i.p.) and cisplatin (1 mg/kg i.p.), gemcitabine (15 mg/kg i.p.), cisplatin (1 mg/kg i.p.), and S63845 (25 mg/kg i.v.) or normal saline was administered for 2 weeks (on days 0, 3, 6, 9, and 12). Administration of gemcitabine was 11 hours after light onset (HALO) followed by cisplatin 15 HALO (38). Tumor progression was monitored on treatment days (0, 3, 6, 9, and 12) by measuring the width and length of the palpable mass, and tumor volume was calculated using the formula: volume (mm^3) = (length $\times$ width²)/2. Animals were sacrificed and tumors extracted 15 days after the first treatment administration. Extracted tumor specimen volume and weights were collected.

Statistical analysis

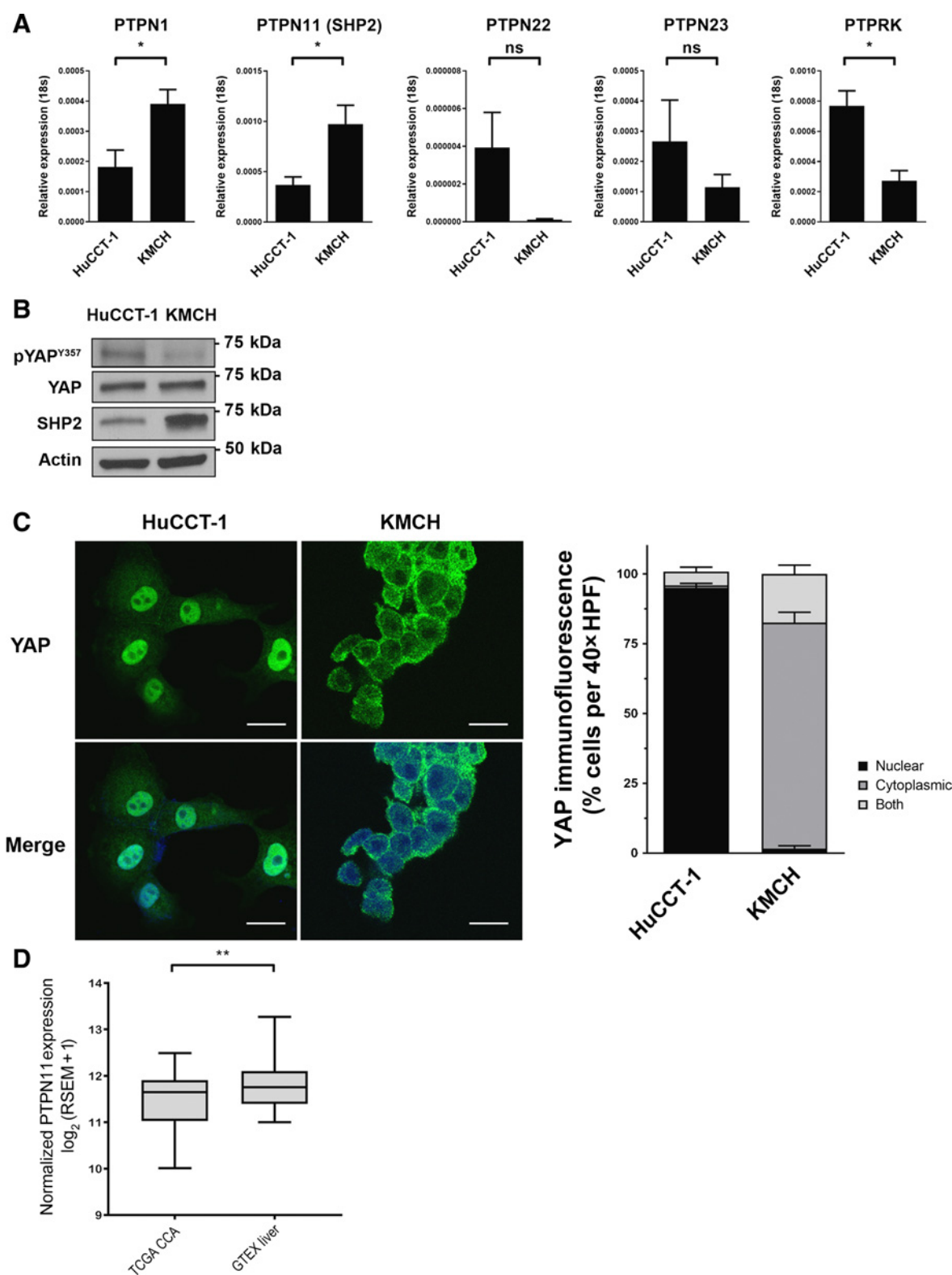
Statistical analyses were performed using two-tailed Student *t* test or Mann-Whitney U test and GraphPad Prism 8 Software (GraphPad Software Inc.). Paired *t* test was used because data were normally distributed. All the data are presented as mean $\pm$ SEM and *P* value less than 0.05 was considered significant.

Results

Tyrosine phosphatase levels are altered in cholangiocarcinomas and have an inverse relationship to YAP phosphorylation

We have previously observed an association of a number of tyrosine phosphatases with YAP at baseline and dependent on its tyrosine phosphorylation status. In this previous screen, utilizing immunoprecipitation of an epitope-tagged YAP in a murine cholangiocarcinomas cell line, followed by mass spectroscopy, we noted an association of tyrosine-protein phosphatase nonreceptor types 1, 11, 22, and 23 as well as receptor-type tyrosine-protein phosphatase kappa with YAP, which increased following incubation with the SFK inhibitor, dasatinib (9). Tyrosine-protein phosphatase nonreceptor type 11 (also known as SHP2) has previously been identified as a YAP binding protein; and it has been proposed that YAP functions as a shuttle for SHP2 as part of confluency sensing machinery (31). The remaining phosphatases had not previously been identified as YAP-interacting phosphatases. We initiated our evaluation by assessing levels of these candidate YAP-interacting tyrosine phosphatases by RT-PCR (*PTPN1*, 11, 22, 23, and *PTPRK*) and then confirming levels by immunoblot analysis in the cholangiocarcinomas cell lines HuCCT-1 and KMCH (Fig. 1A and B). These two cholangiocarcinomas cell lines were utilized as they have distinct pYAP^{Y357} status and YAP cellular compartmentation (nucleus vs. cytoplasm), with HuCCT-1 cells demonstrating higher levels of pYAP^{Y357} and nuclear localized YAP, whereas KMCH cells demonstrate lower levels of pYAP^{Y357} and cytoplasmic localized YAP at baseline (Fig. 1B and C). In the cholangiocarcinomas cell lines, we noted an inverse relationship between the levels of the YAP-interacting tyrosine phosphatases SHP2 (encoded by the *PTPN11* gene) and PTP1b (encoded by the *PTPN1*

SHP2 Modulates YAP Conferring Resistance to Chemotherapy

**Figure 1.**

Tyrosine phosphatase levels are altered in cholangiocarcinomas and have an inverse relationship to YAP phosphorylation. **A**, mRNA expression of YAP-associated tyrosine phosphatases in HuCCT-1 and KMCH cell lines. *, $P < 0.05$; ns, not significant. **B**, Cell lysates from the HuCCT-1 and KMCH cell lines were subjected to immunoblot analysis for phosphorylated YAP tyrosine 357 (pYAP^{Y357}) and SHP2. Total YAP and actin were used as loading controls. **C**, Representative images of YAP immunofluorescence in HuCCT-1 and KMCH cell lines with quantification of YAP localization. Original magnification, 40 $\times$. Scale bar, 10 μ m. **D**, *PTPN11* (SHP2) differential expression analysis of public TCGA cholangiocarcinomas samples compared with public GTEx normal liver samples (**, $P < 0.01$).

gene) and pYAP^{Y357}, suggesting that these phosphatases may be in fact regulators of phosphorylation status in cholangiocarcinomas (Fig. 1A and B). Furthermore, these data indicated that the HuCCT-1 and KMCH cells have not only unique pYAP^{Y357} profiles, but also have unique phosphatase profiles making them informative models to further dissect the role of tyrosine phosphatases in YAP regulation. Importantly, the HuCCT-1 cell line carries an activating KRAS^{G12D} mutation and the KMCH cell line carries an activating BRAF^{L485W} mutation allowing us to evaluate the SHP2 regulation of YAP independent of direct effects on the MAP kinase signaling pathway (as SHP2 functions in signal transduction upstream of RAS activation). Expression levels of *PTPN22*, *23*, and *PTPRK* did not correlate with pYAP^{Y357} levels in these two cholangiocarcinomas cell lines. We next evaluated publicly available RNA-sequencing databases and found that *PTPN11* (SHP2) expression specifically was lower in cholangiocarcinoma specimens as compared with normal liver, further supporting a potential role for decreased SHP2 levels in cholangiocarcinoma biology (Fig. 1D). Indeed, in TCGA CHOL dataset, five of the included cholangiocarcinomas samples had a deletion in *PTPN11* (SHP2) and gene expression was correlated with copy number ($P = 0.003$). While SHP2 has been demonstrated to be an oncogene on the basis of its role in RAS signal transduction in multiple tumor types, notably it has previously been demonstrated as a tumor suppressor in hepato-carcinogenesis, and on the basis of this we further explored the possibility that SHP2 phosphatase activity could directly regulate YAP activity, thus behaving as a tumor suppressor in cholangiocarcinomas (39–42).

SHP2 can regulate YAP activity and subcellular localization in cholangiocarcinomas

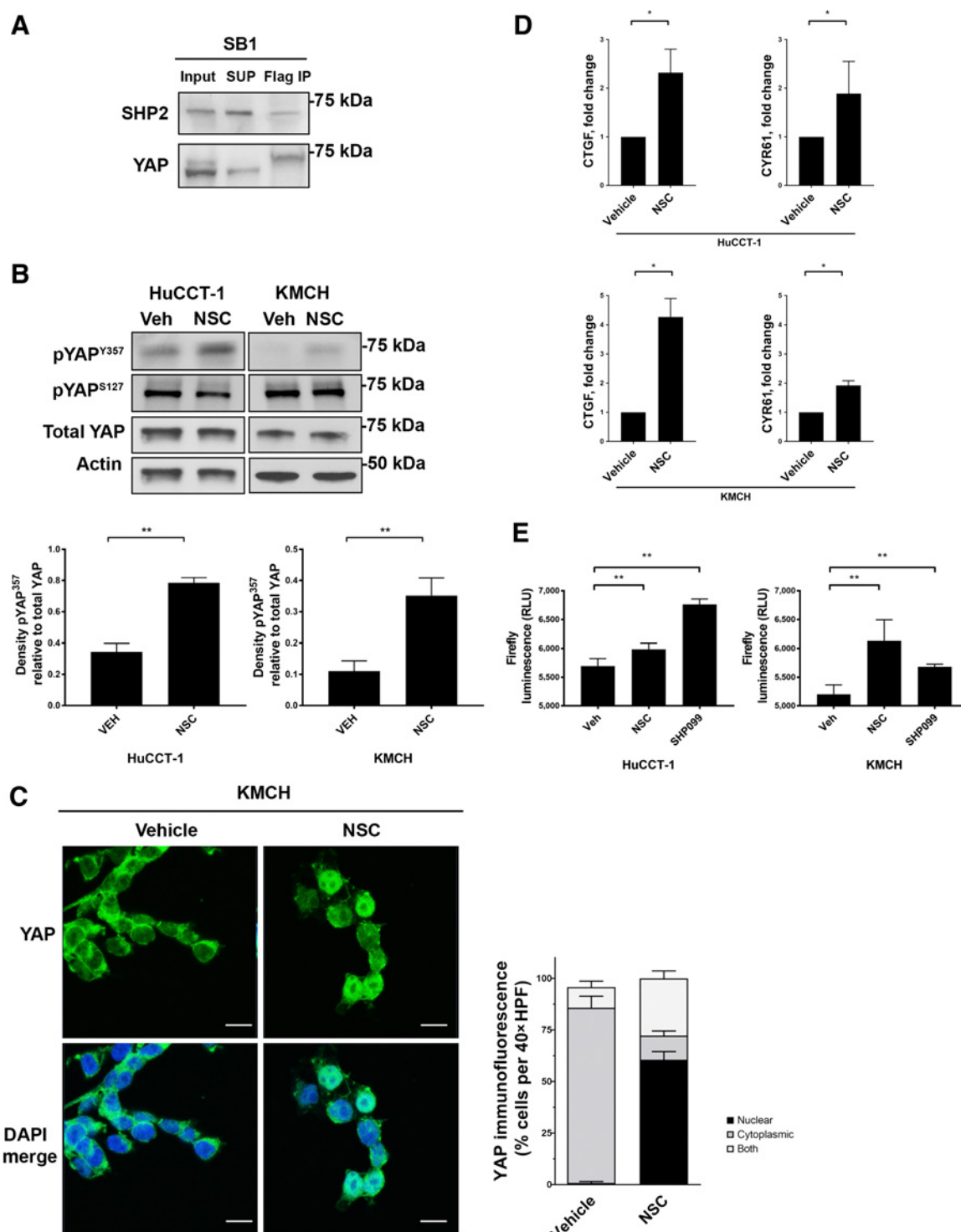
As an initial step, we confirmed the findings of our immunoprecipitation–mass spectrometry screen by performing coimmunoprecipitation experiments, which demonstrated that SHP2 directly interacts with YAP in cholangiocarcinomas cells (Fig. 2A). As SHP2 has previously been demonstrated to dephosphorylate the Y357 residue of YAP (43), we then explored the SHP2–YAP interaction in cholangiocarcinomas utilizing a pharmacologic approach with attention to the effects of SHP2 inhibition on YAP phosphorylation status, subcellular localization, and transcriptional activity. In both HuCCT-1 and KMCH cells, exposure to the SHP2 inhibitor, NSC87877 was associated with increases in pYAP^{Y357} abundance, and consistent with effects on tyrosine phosphorylation independent of the serine/threonine Hippo kinase cascade, no change was noted in pYAP^{S127} abundance (Fig. 2B). NSC87877 has activity against SHP1 as well as SHP2, with SHP1 as a known negative regulator of SFK activity by limiting its autophosphorylation (44). To evaluate the possibility that increased pYAP^{Y357} levels may be because of reduced SHP1 activity (leading to increased SFK activity) we utilized a second, structurally dissimilar SHP2 inhibitor, SHP099, and found this inhibitor also increased pYAP^{Y357} levels (Supplementary Fig. S1A). Furthermore, we evaluated the levels of active SRC (pSRC^{Y416}) by immunoblot analysis and did not observe an increase in pSRC levels following NSC87877 exposure (Supplementary Fig. S1B). Taken together, these data suggest that SHP1 was not involved in regulating pYAP^{Y357} levels and that there was baseline tonic activity of SHP2 on YAP regulating its Y357 phosphorylation status. We next sought to evaluate the consequence of SHP2 inhibition in relation to YAP subcellular localization and cotranscriptional activity. Shuttling of YAP into and out of the nucleus has been shown to be regulated by tyrosine phosphorylation limiting the interaction of YAP with exportin proteins, leading to nuclear accumulation of YAP (26). As such we evaluated the subcellular

localization of SHP2 in KMCH cells to determine whether SHP2 would be localized in the nucleus, and noted at baseline, SHP2 was indeed enriched in the nucleus (Supplementary Fig. S1C), consistent with the possibility that the low levels of nuclear YAP in KMCH cells were secondary to SHP2 phosphatase activity. Furthermore, exposure of KMCH cells (in which YAP is localized to the cytoplasm at baseline) to NSC87877, was associated with a shift of YAP to the nuclear compartment (Fig. 2C). Consistent with the increases in pYAP^{Y357} abundance and the anticipated activation of YAP, cotranscriptional activity was also increased. Evaluation of YAP-TEAD transcription, broadly utilizing a luciferase reporter construct, demonstrated significant increases after both NSC87877 and SHP099 exposure and specific expression of the YAP target genes *CTGF* and *CYR61* were increased in both HuCCT-1 and KMCH cells (Figs. 2D and E). In keeping with the profiling data suggesting that KMCH cells had lower pYAP^{Y357} levels and higher SHP2 levels, we noted larger fold increases in YAP cognate target gene expression in KMCH cells compared with the HuCCT-1 cells, even though significant increases in YAP target gene levels were also noted in HuCCT-1 cells. We next explored whether SHP2 inhibition by NSC87877 may also shift YAP from a cytoplasmic to nuclear compartment in a non-cholangiocarcinomas cell line. We utilized the PANC1 pancreas cancer cell line in which, like KMCH cells, at baseline YAP is enriched in the cytoplasm. Similar to KMCH cells, incubation of the PANC1 cells with NSC87877 shifted YAP to the nuclear compartment (Supplementary Fig. S2A). These findings supported a role for SHP2 regulating YAP tyrosine phosphorylation, subcellular compartmentalization, and cotranscriptional activity; therefore, we sought to further explore the specificity of these findings utilizing a genetic paradigm. We utilized an inducible CRISPR/Cas9-mediated deletion of *PTPN11* (SHP2). Deletion of SHP2 in the KMCH cell line was achieved after 48 hours of treatment with doxycycline (Supplementary Fig. S3A). In the KMCH cells, which at baseline have high SHP2 levels, deletion of SHP2 was associated with an increase in pYAP^{Y357} levels (Fig. 3A). In addition, we assessed the Hippo and MAP kinase pathways following SHP2 deletion and observed no significant change in either pYAP^{S127} or pERK levels (Fig. 3A), similar to pharmacologic SHP2 inhibition (Fig. 2B; Supplementary Fig. S3B). The increase in pYAP^{Y357} levels was associated with a shift of YAP to the nuclear compartment and increased YAP cotranscriptional activity as measured by expression of cognate YAP target genes, similar to pharmacologic SHP2 inhibition (Fig. 3B and C). These data suggest that SHP2 can directly regulate YAP activity by regulating the abundance of pYAP^{Y357} independent of the Hippo pathway and distinct from SHP2-mediated effects on the MAP kinase pathway, and thus may indeed behave as a tumor suppressor in cholangiocarcinomas.

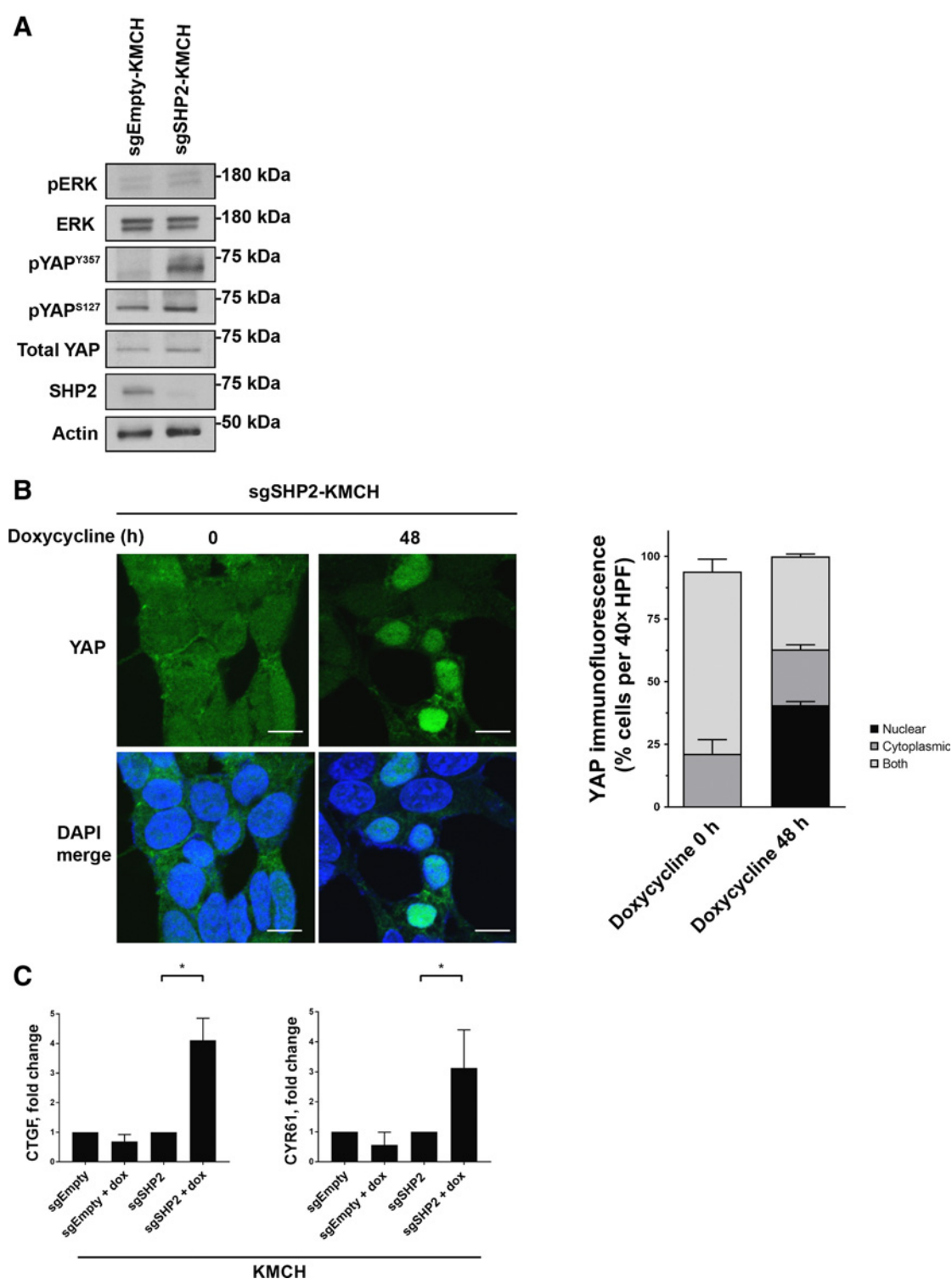
The phosphatase activity of SHP2 is required for YAP regulation

Tyrosine phosphatases may regulate YAP activity via mechanisms unrelated to their phosphatase activity due to scaffolding functions (31, 32). Given this, we next examined the role of phosphatase activity specifically in SHP2-mediated YAP regulation. We initiated these studies by exploring YAP localization and activity following enforced expression of wild-type SHP2 in HuCCT-1 cells (which have low baseline SHP2 levels). In HuCCT-1 cells overexpressing wild-type SHP2, pYAP^{Y357} levels were decreased and the localization of YAP was shifted from the nucleus to the cytoplasmic compartment. YAP transcriptional activity was also decreased (Fig. 4A and C). To extend these findings, we expressed a SHP2 construct in which the phosphatase domain was mutated (C459S-SHP2). Expression of this phosphatase-dead construct had no demonstrable effect on pYAP^{Y357}

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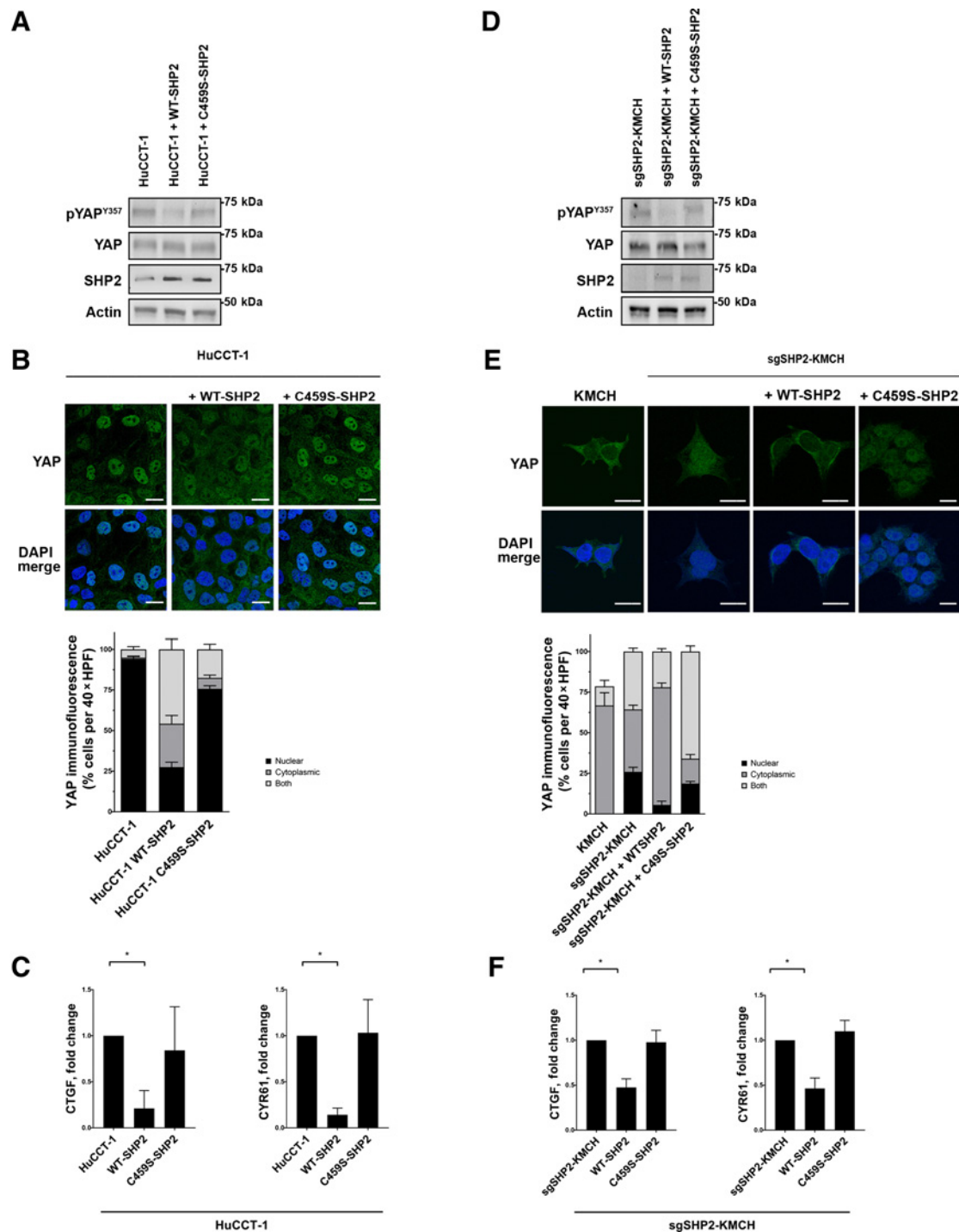
**Figure 2.**

SHP2 can regulate YAP activity and subcellular localization in cholangiocarcinomas. **A**, Cell lysates of SB1 cells that contain FLAG-YAP^{S127A} were subjected to anti-Flag immunoprecipitation and Western blot analysis of endogenous SHP2. Total YAP served as a loading control in inputs. **B**, Cell lysates from the HuCCT-1 and KMCH cell lines treated with NSC87877 (NSC, 10 μ mol/L, 3 hours) were subjected to immunoblot analysis for phosphorylated pYAP^{Y357} and pYAP^{S127} with representative densitometry of pYAP^{Y357} relative to total YAP. **, $P < 0.01$. Total YAP and actin were used as loading controls. **C**, Representative images of YAP immunofluorescence in KMCH cell lines $\pm$ NSC87877 (10 μ mol/L, 3 hours) with quantification of YAP localization. Original magnification, 40 $\times$. Scale bar, 10 μ m. **D**, mRNA expression of CTGF and CYR61 in HuCCT-1 and KMCH $\pm$ tyrosine phosphatase inhibitor, NSC87877 (10 μ mol/L, 3 hours). Fold change relative to vehicle (veh). *, $P < 0.05$. **E**, Global TEAD transcriptional activity in HuCCT-1 and KMCH cells using a luciferase-based TEAD reporter. Cells were treated with NSC87877 or SHP099 at 10 μ mol/L for 24 hours prior to measurement of firefly luciferase activity (**, $P < 0.01$).

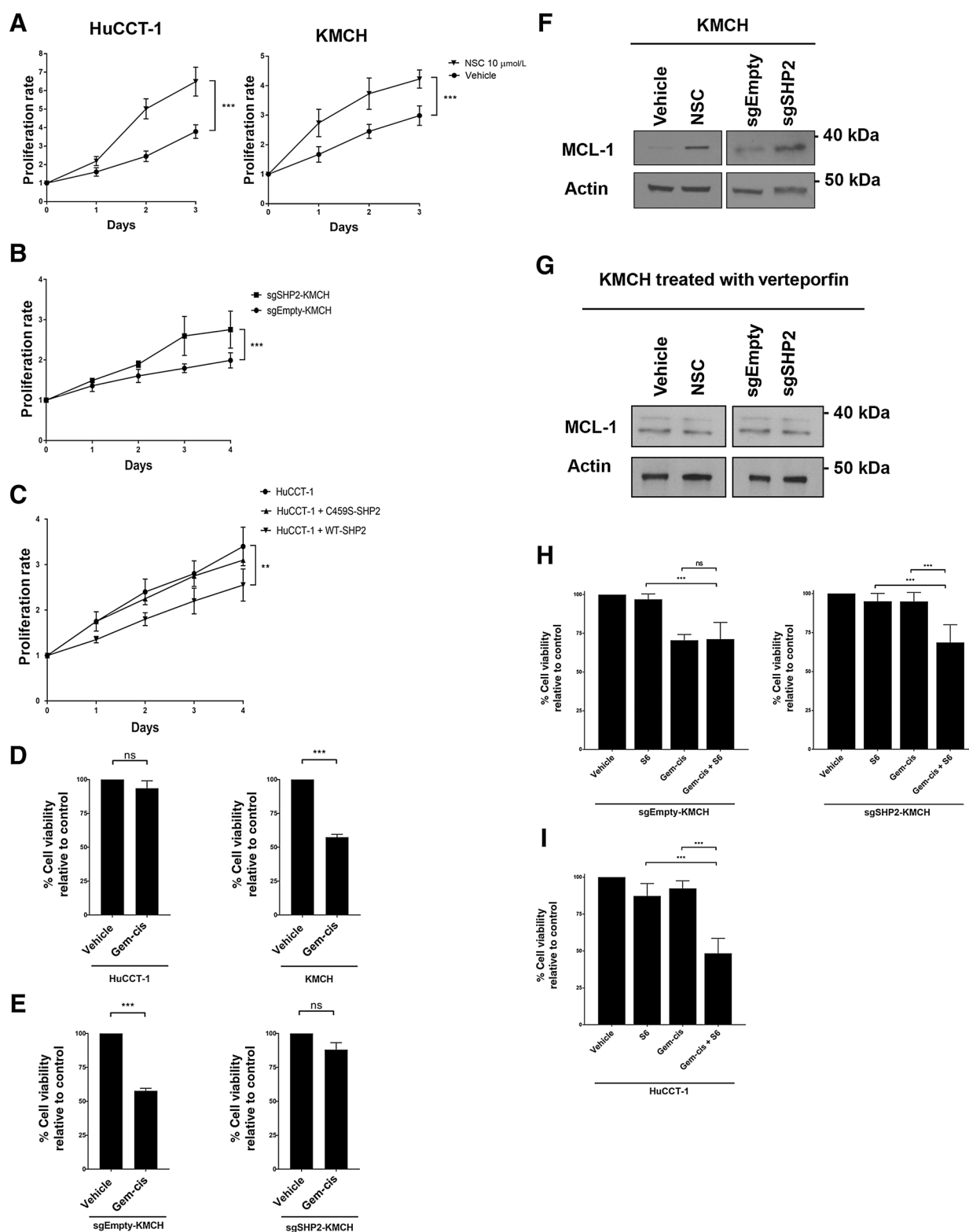
**Figure 3.**

SHP2 deletion increases YAP nuclear localization and transcriptional activity. **A**, Cell lysates from KMCH cell lines transfected with empty or SHP2-targeting CRISPR sgRNA were subjected to immunoblot analysis for pERK, pYAP^{Y357}, pYAP^{S127}, and SHP2. Total YAP and actin were used as loading controls. **B**, KMCH cell lines transfected with a doxycycline (dox)-inducible CRISPR/Cas9 construct targeting SHP2 were incubated with doxycycline (7.5 μg/mL) for the noted time. Immunofluorescence for YAP was undertaken and representative images with quantification of YAP localization are displayed. Original magnification, 40×. Scale bar, 10 μm. **C**, CTGF and CYR61 mRNA expression in KMCH cell line transfected with sgEmpty and sgSHP2 ± induction with doxycycline (7.5 μg/mL). Fold change relative to noninduced control (*, $P < 0.05$).

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**Figure 4.**

The phosphatase activity of SHP2 is required for YAP regulation. **A**, Cell lysates from the HuCCT-1 cell line were transfected with wild-type (WT) SHP2 or C459S SHP2 (phosphate dead construct) and subjected to immunoblot analysis for pYAP^{Y357} and SHP2. Total YAP and actin were used as loading controls. **B**, HuCCT-1, HuCCT-1 + WT-SHP2, and HuCCT-1 + C459S-SHP2 underwent immunofluorescence for YAP and representative images are displayed with quantification of YAP localization. Original magnification, 40 $\times$. Scale bar, 10 μ m. **C**, mRNA expression of CTGF and CYR61 in HuCCT-1, HuCCT-1 + WT-SHP2, and HuCCT-1 + C459S-SHP2. Fold change relative to parental cell line control. *, $P < 0.05$. **D**, KMCH cell line transfected with a doxycycline-inducible CRISPR/Cas9 construct targeting SHP2 (sgSHP2-KMCH) were incubated with doxycycline (7.5 μ g/mL) for 48 hours. The sgSHP2-KMCH cell lines were then washed and transfected with lentivirus-overexpressing WT-SHP2 or C459S SHP2 constructs for 48 hours. Cell lysates were subjected to immunoblot analysis for pYAP^{Y357} and SHP2. Total YAP and actin were used as loading controls. **E**, Immunofluorescence for YAP in KMCH, sgSHP2-KMCH, sgSHP2-KMCH + WT-SHP2, and sgSHP2-KMCH + C459S-SHP2 was performed and representative images with quantification of YAP localization are displayed. Original magnification, 40 $\times$. Scale bar, 10 μ m. **F**, mRNA expression of CTGF and CYR61 in sgSHP2-KMCH, sgSHP2-KMCH + WT-SHP2, and sgSHP2-KMCH + C459S-SHP2. Fold change relative to parental sgSHP2-KMCH cell line (*, $P < 0.05$).

**Figure 5.**

Tyrosine phosphatase activity is a critical determinant of cholangiocarcinomas proliferation and *in vitro* sensitivity to standard-of-care chemotherapy. **A**, HuCCT-1 and KMCH cell lines $\pm$ NSC87877 (NSC, 10 $\mu\text{mol/L}$, 3 days) were subjected to MTS assay daily for 3 days. The proliferation rate was calculated by the daily absorbance value relative to the respective day 0 value. ***, $P < 0.001$. **B**, KMCH cell lines transfected with a doxycycline-inducible CRISPR/Cas9 construct targeting SHP2 (sgSHP2-KMCH) were incubated with doxycycline (7.5 $\mu\text{g/mL}$) for 48 hours. (Continued on the following page.)

levels, YAP baseline localization, or YAP cotranscriptional activity (Fig. 4A and C). To further explore these observations, we pursued a deletion-reconstitution approach utilizing our inducible sgSHP2-KMCH cells. Following SHP2 deletion, doxycycline was withdrawn, and SHP2 levels were then reconstituted with either wild-type or phosphatase-dead constructs (Fig. 4D). Reconstitutions with wild-type SHP2, but not phosphatase-dead SHP2, was associated with a decrease in pYAP^{Y357} levels, YAP baseline nuclear localization, and decreased YAP transcriptional activity (Fig. 4D–F). These observations suggest that the phosphatase activity of SHP2 is integral in regulating YAP in cholangiocarcinomas.

Tyrosine phosphatase activity is a critical determinant of cholangiocarcinomas proliferation and *in vitro* sensitivity to standard-of-care chemotherapy

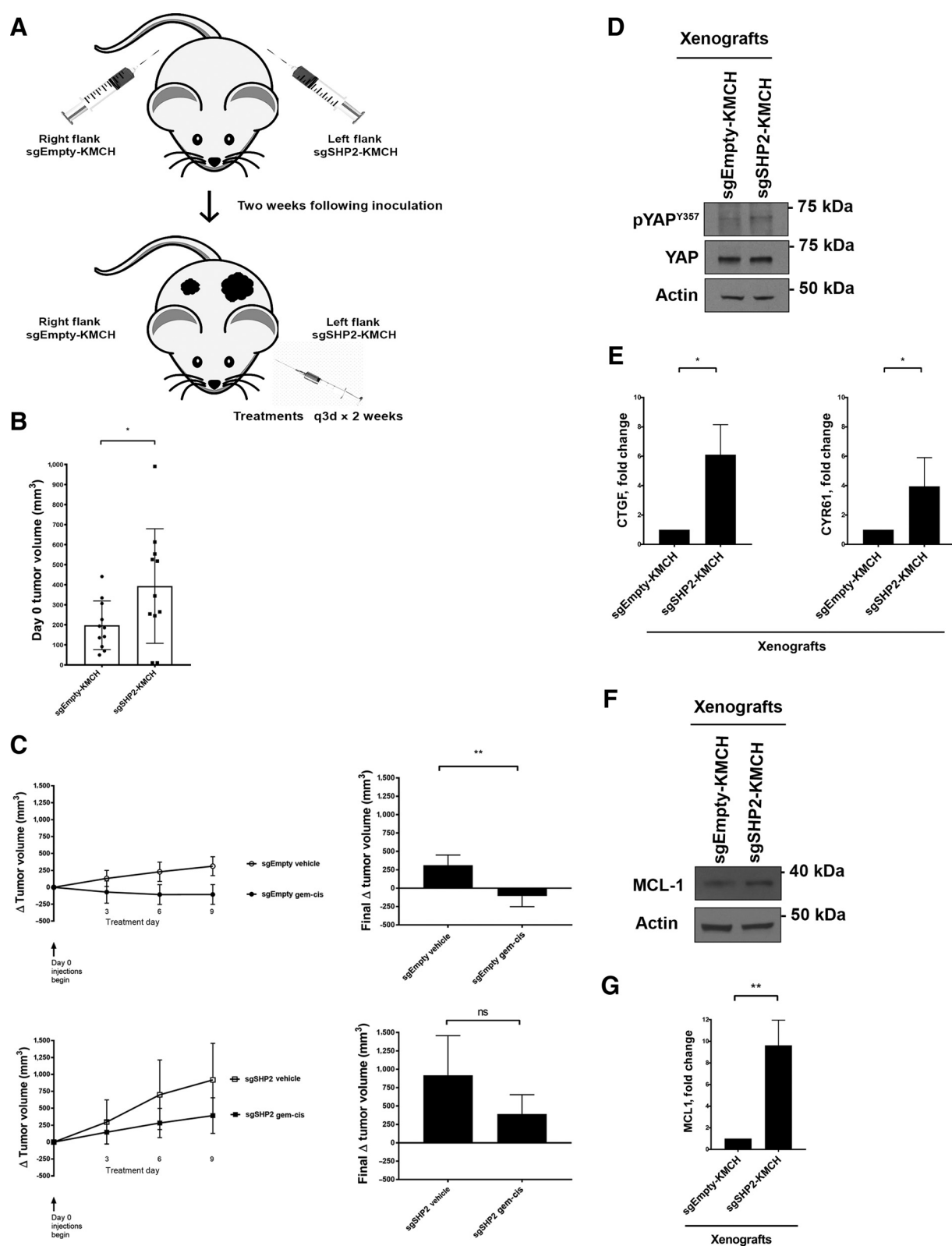
Given the increases in YAP tyrosine phosphorylation and transcriptional coactivity observed with SHP2 downregulation, we examined the effect of SHP2 modulation on cellular proliferation. In both KMCH and HuCCT-1 cells, incubation with NSC87877 increased cellular proliferation compared with controls (Fig. 5A). We next examined sgSHP2-KMCH cells compared with sgEmpty-KMCH control cells after induction with doxycycline. Congruent with the pharmacologic observation, knockdown of SHP2 expression was associated with an increase in cellular proliferation (Fig. 5B). Correspondingly, in HuCCT-1 cells, enforced expression of wild-type SHP2 was associated with a decrease in cellular proliferation, while C459S-SHP2 had no demonstrable effect (Fig. 5C). Increased YAP activity has been associated with chemoresistance (14–24), and given this we next examined the possibility that regulation of pYAP^{Y357} by tyrosine phosphatases may also alter the sensitivity of cholangiocarcinomas cell lines to gemcitabine and cisplatin, the current standard-of-care combinatorial therapy for cholangiocarcinomas. We first explored this possibility utilizing our HuCCT-1 and KMCH cell lines. We observed decreased cell death in HuCCT-1 cells (low SHP2 levels) as compared with KMCH cells (high SHP2 levels) incubated with gemcitabine and cisplatin consistent with the possibility that YAP activation status, as denoted by pYAP^{Y357} levels, modulates chemotherapeutic sensitivity (Fig. 5D). To further evaluate this possibility, we next incubated the sgEmpty-KMCH and sgSHP2-KMCH cell lines with gemcitabine and cisplatin. In-line with our previous observations demonstrating increased YAP^{Y357} phosphorylation and activity following SHP2 deletion, the sgSHP2-KMCH cells were resistant to the combination of gemcitabine and cisplatin (Fig. 5E). As we have previously demonstrated the prosurvival BCL2 family protein, MCL1, to be a YAP target gene (45), we contemplated whether SHP2 inhibition and/or deletion could increase MCL1 levels as a mechanism of resistance to therapy. Indeed, following either NSC87877 exposure or CRISPR/Cas9-mediated deletion of SHP2, we observed dramatic increases in

MCL1 levels (Fig. 5F). We assessed the YAP dependence of MCL1 accumulation by repeating the studies in the presence of the YAP-TEAD inhibitor, verteporfin. Verteporfin inhibited the MCL1 accumulation, supporting the concept that SHP2 inhibition/deletion increases MCL1 via a YAP-dependent mechanism (Fig. 5G). We then hypothesized that targeting MCL1 in cell lines that demonstrate high pYAP^{Y357} and concomitant resistance to gemcitabine and cisplatin therapy may restore chemosensitivity. Indeed, both HuCCT-1 and sgSHP2-KMCH cells treated with the MCL1 inhibitor, S63845 demonstrated sensitivity to gemcitabine and cisplatin, while S63845 alone had no effect (Fig. 5H). We then asked whether or not sensitivity to gemcitabine and cisplatin could similarly be restored in the HuCCT-1 cell line, which like the sgSHP2-KMCH have high levels of pYAP^{Y357}, low baseline levels of SHP2, and are resistant. Indeed, the addition of S63845 sensitized the HuCCT-1 cells to gemcitabine and cisplatin (Fig. 5I). These findings suggest that high pYAP^{Y357} levels, which can be driven by loss of SHP2 activity, can reduce the sensitivity of cholangiocarcinomas cells to standard-of-care chemotherapy via an MCL1-dependent mechanism.

Deletion of SHP2 reduces *in vivo* sensitivity of cholangiocarcinomas xenografts to standard-of-care chemotherapy, which can be overcome by the addition of MCL1 inhibition

On the basis of the findings in the *in vitro* experiments, we explored the effects of phosphatase deletion on the sensitivity of cholangiocarcinomas cell line xenografts to gemcitabine plus cisplatin in a flank model. The sgEmpty-bearing KMCH cells were placed in one flank and the sgSHP2-KMCH in the other flank of NOD/SCID mice, ensuring equal chemotherapy dosing of each tumor within each animal (Fig. 6A). In keeping with the observations noted *in vitro*, the SHP2 phosphatase-knockout cell line xenografts (sgSHP2-KMCH) developed tumors with faster growth (Fig. 6B). When treated with gemcitabine plus cisplatin, the sgEmpty-KMCH tumors stabilized and decreased in size, while the sgSHP2-KMCH continued to grow with the same kinetics as the vehicle-treated sgEmpty-KMCH tumors. The vehicle-treated SHP2-deleted tumors displayed the fastest growth rates (Fig. 6C). Immunoblot evaluation of YAP from these tumors demonstrated increased levels of pYAP^{Y357} in the sgSHP2-KMCH-bearing tumors as compared with the sgEmpty-KMCH xenografts, consistent with the *in vitro* results (Fig. 6D). Accordingly, YAP cognate target gene expression was higher in the SHP2-knockout xenografts (Fig. 6E). Furthermore, immunoblot and gene expression evaluation of MCL1 levels were also noted to be higher in the SHP2-knockout xenografts (Fig. 6F and G). We then asked whether the addition of the MCL1 inhibitor, S63845, could restore chemosensitivity *in vivo* (46). Indeed, the addition of S63845 to gemcitabine and cisplatin partially restored sensitivity in sgSHP2-KMCH cell line xenografts (Fig. 6H).

(Continued.) The KMCH and sgSHP2-KMCH cells were evaluated with MTS assay daily for 4 days. The proliferation rate was calculated by the daily absorbance value relative to the respective day 0 value. ***, $P < 0.001$. C, HuCCT-1 cell lines were transfected with WT SHP2 and C459S SHP2 constructs for 48 hours. HuCCT-1, HuCCT-1 + WT-SHP2, and HuCCT-1 + C459S SHP2 were assessed with MTS assay for 4 days. The proliferation rate was calculated by the daily absorbance value relative to the respective day 0 value. **, $P < 0.01$. D, Percent cell viability (relative to control) of HuCCT-1 and KMCH cell lines measured by CellTiter-Glo assay following incubation with gemcitabine/cisplatin (100 nmol/L, 72 hours). ***, $P < 0.001$. E, Percent cell viability (relative to control) of KMCH and sgSHP2-KMCH cell lines measured by CellTiter-Glo assay following incubation with gemcitabine/cisplatin (gem-cis, 100 nmol/L, 72 hours). ***, $P < 0.001$. F, Cell lysates from the KMCH cell lines treated with NSC87877 (10 μ mol/L, 3 hours) or transfected with empty and SHP2-targeting CRISPR sgRNA were subjected to immunoblot analysis for MCL-1. Actin was used as a loading control. G, Cell lysates from the KMCH cell line treated with verteporfin (10 μ mol/L, 3 hour) followed by NSC87877 (10 μ mol/L, 3 hours) were subjected to immunoblot analysis for MCL-1. sgEmpty-KMCH and sgSHP2-KMCH cells treated with verteporfin (10 μ mol/L, 3 hour) were subjected to immunoblot analysis for MCL-1. Actin was used as a loading control. H, Percent cell viability (relative to control) of sgEmpty-KMCH and sgSHP2-KMCH cells measured by CellTiter-Glo assay following incubation with gemcitabine/cisplatin $\pm$ S63845 (S6, 100 nmol/L and 5 μ mol/L, 72 hours). ***, $P < 0.001$. I, Percent cell viability (relative to control) of HuCCT-1 cells measured by CellTiter-Glo assay following incubation with gemcitabine/cisplatin $\pm$ S63845 (100 nmol/L and 5 μ mol/L, 72 hours; ***, $P < 0.001$). ns, not significant; Veh, vehicle.

**Figure 6.**

Deletion of SHP2 reduces *in vivo* sensitivity of cholangiocarcinomas xenografts to standard-of-care chemotherapy. **A**, *In vivo* study design. **B**, Tumor volume of mice (sgEmpty-KMCH and sgSHP2-KMCH) on treatment day 0. *, $P < 0.05$. **C**, Δ Tumor volume of mice (sgEmpty-KMCH and sgSHP2-KMCH) with every 3 days (q3d) x 2 weeks intraperitoneal injection of either combination of gemcitabine (gem, 15 mg/kg) and cisplatin (cis, 1 mg/kg) or normal saline. **, $P < 0.01$. (Continued on the following page.)

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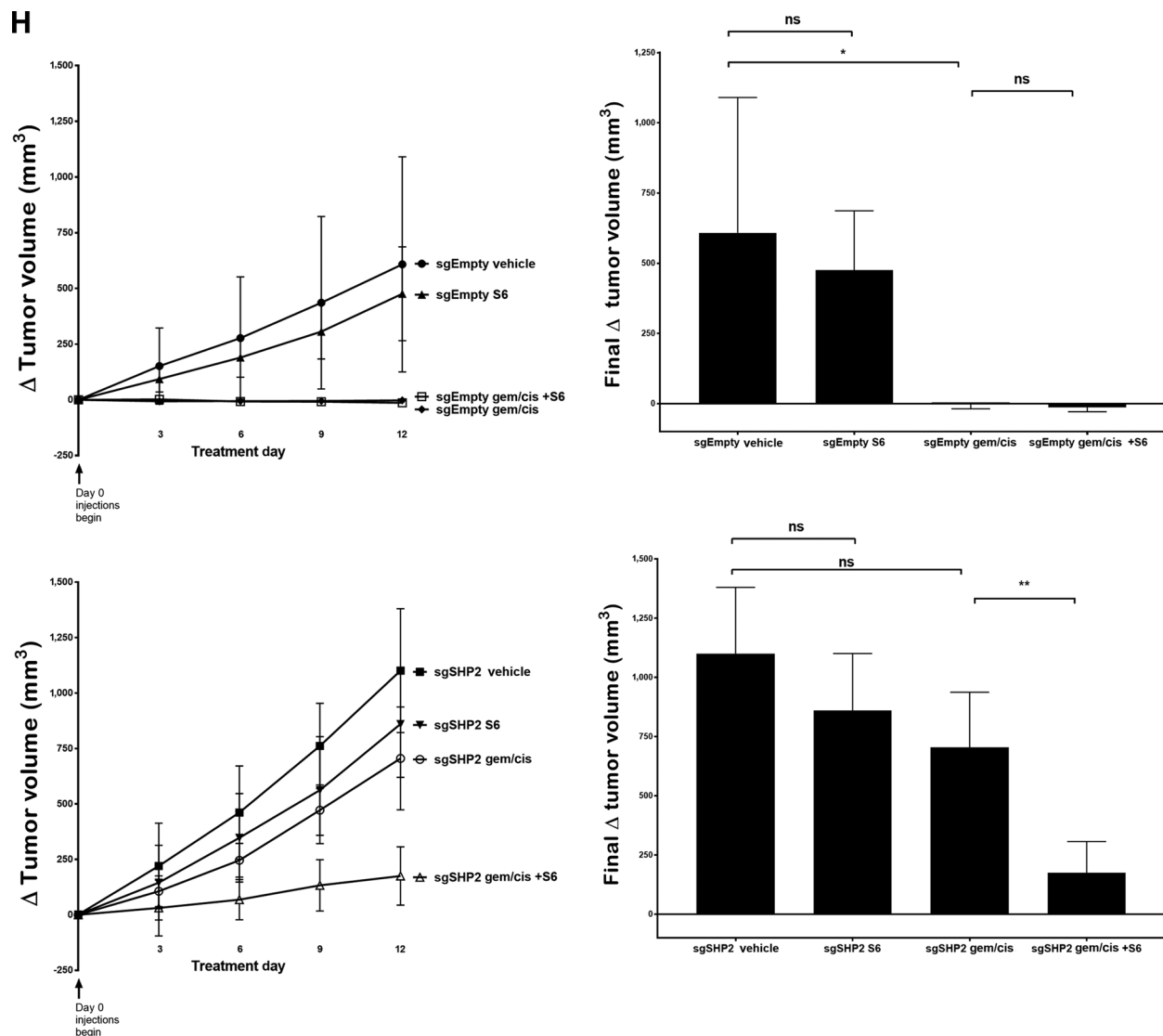


Figure 6.

(Continued.) **D**, Cell lysates from xenografts of sgEmpty-KMCH and sgSHP2-KMCH were subjected to immunoblot analysis for pYAP^{Y357}. Total YAP and actin were used as loading controls. **E**, CTGF and CYR61 mRNA expression of sgEmpty-KMCH and sgSHP2-KMCH xenografts. Fold change relative to sgEmpty-KMCH control. *, $P < 0.05$. **F**, Cell lysates of sgEmpty-KMCH and sgSHP2-KMCH xenografts were subjected to immunoblot analysis for MCL-1. Actin was used as a loading control. **G**, MCL-1 mRNA expression of sgEmpty-KMCH and sgSHP2-KMCH xenografts. Fold change relative to sgEmpty-KMCH control. **, $P < 0.01$. **H**, Δ Tumor volume of mice (sgEmpty-KMCH and sgSHP2-KMCH) with every 3 days $\times$ 2 weeks treatment. Treatments included S63845 (25 mg/kg), combination of gemcitabine (15 mg/kg) and cisplatin (1 mg/kg), combination of gemcitabine (15 mg/kg), cisplatin (1 mg/kg), and S63845 (25 mg/kg), or normal saline (*, $P < 0.05$; **, $P < 0.01$). ns, not significant.

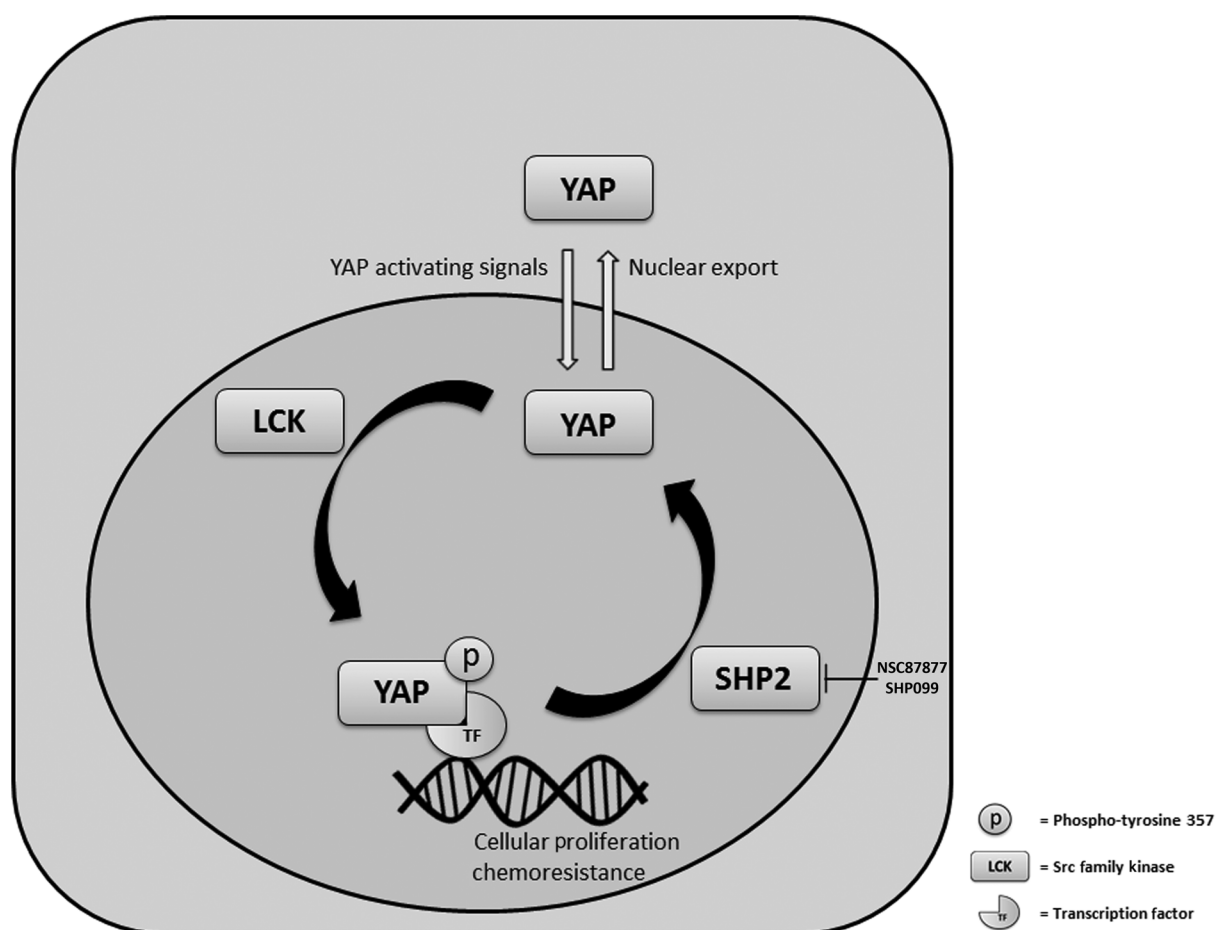
These observations were congruent with our *in vitro* findings, and further supported a role for SHP2 in modulating the sensitivity of cholangiocarcinomas to gemcitabine/cisplatin combinatorial therapy via a YAP-MCL1 mechanism.

Discussion

This study supports a role for the tyrosine phosphatase SHP2 in regulating YAP tyrosine phosphorylation and sensitivity to chemotherapy in cholangiocarcinoma (Fig. 7). These data indicate that: (i) the tyrosine phosphatase SHP2 can regulate tyrosine phosphorylation of YAP, (ii) phosphatase activity specifically is required for regulation

of YAP, and (iii) deletion/inhibition of SHP2 increases cellular proliferation and decreases sensitivity to standard-of-care chemotherapy via YAP- and MCL1-dependent mechanisms. These findings are discussed in greater detail below.

We identified the tyrosine phosphatase SHP2 as regulating YAP in cholangiocarcinomas. Tsutsumi and colleagues previously identified SHP2 as a YAP/TAZ-interacting protein following the observation that SHP2 subcellular localization was regulated by cell density in human gastric epithelial cells, and this regulation could be disrupted by the serine/threonine kinase inhibitor, staurosporine (31). Direct interaction of SHP2 and YAP/TAZ was demonstrated by coimmunoprecipitation experiments, and short hairpin RNA (shRNA)-mediated

**Figure 7.**

Summary of the model. SHP2 modulates proliferation and chemoresistance by regulating the tyrosine phosphorylation (activation) of YAP.

knockdown of SHP2 in these cells did not appear to effect YAP localization. These findings led the investigators to conclude that SHP2 was not regulating YAP/TAZ subcellular localization in this model, but YAP/TAZ were regulating SHP2 localization. Of note, YAP/TAZ were highly enriched in the nucleus in these cells at baseline and based on our observations, and proposed mechanism of SHP2 regulation of YAP, we would not have expected to see any significant effects on YAP localization with SHP2 downregulation in that model, just as they observed. The ability of SHP2 to regulate YAP, and in turn the proliferation and chemosensitivity of cholangiocarcinomas is initially striking, given the previously described function of SHP2 as an oncogene in multiple tumor types through its positive effect on receptor tyrosine kinase–RAS signaling (39–41). In fact, receptor tyrosine kinase–driven malignancies have been demonstrated to be sensitive to SHP2 inhibition (47). However, SHP2 has also been shown to be a tumor suppressor, specifically in hepatocellular carcinogenesis (42). In these previous studies, deletion of SHP2 was accompanied by increased inflammatory signaling through STAT3 and eventually development of hepatocellular carcinoma (HCC) in mice (42). These effects were magnified in an injury-inflammation model of HCC. Taken together these findings suggest that the oncogenic potential of SHP2 signaling is likely tissue and context dependent, such that it can act both as an oncogene through effects on MAP kinase signaling, as

well as a tumor suppressor through effects on YAP activity. Importantly, we assessed the function of SHP2 in cholangiocarcinomas cell lines that were RAS/RAF mutated, and as such could assess the YAP-specific effects of SHP2 modulation without any obscuring effects on MAP kinase signaling.

Other studies have demonstrated a potential role for tyrosine phosphatases in the regulation of YAP. Wang and colleagues described a role for the protein tyrosine phosphatase PTPN14 in regulating YAP subcellular localization in the breast cancer cell line MCF10A (32). These investigators noted that overexpression of PTPN14 led to a dramatic shift of YAP from the nucleus to the cytoplasm and conversely, downregulation by shRNA led to redistribution of YAP to the nucleus. The pYAP^{Y357} levels were not evaluated; however, these investigators did overexpress a phosphatase-dead construct, which also was associated with YAP cytoplasmic translocation in the MCF10A cells, suggesting that the phosphatase activity of PTPN14 was not required for YAP regulation in these cells. Given these previous findings, we specifically evaluated the role of the phosphatase function of SHP2 in regulating YAP. Similar to Wang and colleagues we saw a decrease in nuclear localized YAP with enforced overexpression of wild-type SHP2, however, we also noted a decrease in pYAP^{Y357} levels, and neither of these were observed with the catalytically inactive C459S-SHP2 construct. These data were further supported by the

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reconstitution experiments, reexpressing wild-type SHP2 in cholangiocarcinomas cells, in which endogenous *PTPN11* (SHP2) was deleted via a CRISPR/Cas9 approach. These data suggest that, unlike PTPN14 in breast cancer cells, the phosphatase activity of SHP2 is important for the regulation of YAP in cholangiocarcinomas. We did not specifically note PTPN14 as a YAP-associated phosphatase in evaluation of our cholangiocarcinomas cells and it is possible that there are tissue/cell type specific differences both in the phosphatases associating/regulating YAP, and the specific mechanisms of action of those interactions.

Our finding that SHP2 levels/activity can modulate the sensitivity of cholangiocarcinomas cells to the standard-of-care combinatorial therapy gemcitabine and cisplatin is consistent with previous data in multiple tumor types suggesting that increased YAP levels/activity can decrease the sensitivity to cytotoxic chemotherapy (15–17, 19, 21–23). For example, decreased YAP activities through genetic silencing or pharmacologic approaches (utilizing the YAP-TEAD inhibitor, verteporfin) have been associated with increased chemotherapy sensitivity to Taxol in colorectal cancer models and cisplatin in HCC models (21–23). Furthermore, YAP activation has been associated with decreased efficacy of other DNA damaging agents such as radiation (16–18). Even the efficacy of targeted agents has been observed to be affected by YAP activation, whereby resistance is associated with elevated YAP activity/levels (16, 20, 24). In RAS/RAF-driven cancers, YAP signaling has been shown to overcome directed inhibition, suggesting that YAP activation can act as a parallel survival pathway (20, 25). The activation of YAP we observed in our models with SHP2 deletion and pharmacologic inhibition is of significant relevance given the evaluation of SHP2 inhibitors as anticancer therapeutics (47). Our observations would suggest that this may be counterproductive in some tumor types, such as cholangiocarcinomas, which may be dependent on the balance of RAS/RAF/YAP activation. In contrast, our finding that MCL1 was markedly upregulated downstream of YAP activation, and that MCL1 inhibition could restore sensitivity to gemcitabine and cisplatin in the resistant cell lines indicates that targeting MCL1 may be a viable therapeutic approach, especially in tumors with hyperactivated YAP.

The role of tyrosine phosphatases as a regulator of YAP activity and by extension chemotherapeutic sensitivity is an intriguing paradigm. YAP has been observed to be “activated” in a number of tumor types; however, an underlying genetic mechanism for this activation is not often apparent when examining canonical regulators, such as Hippo pathway components. For example, a small minority of patients with cholangiocarcinomas have an identifiable mutation in a canonical regulator (NF2 and Salvador) in the TCGA analysis (48). Thus, additional regulatory pathways continue to be of considerable interest

in understanding YAP biology and function in oncogenesis and response to therapy. In cholangiocarcinomas, tyrosine phosphatases as a gene family, are often mutated (49); however, whether or not all these are direct YAP-interacting proteins and whether or not these derangements affect tumor progression or response to therapy has yet to be defined, and is an active area of investigation in our group. In addition, other than genetic alterations, and other possible mechanisms of regulating phosphatase activity and by extension YAP activity, warrant further investigation.

Overall, our study contributes to the expanding knowledge regarding mechanisms of YAP regulation, independent of the Hippo pathway. Our observations provide further support for the model of tyrosine phosphorylation of YAP as a key regulator of function and a nuclear retention signal, and suggest a novel role for SHP2 tyrosine phosphatase activity in modulating this signal. These observations may have implications for further understanding YAP regulation in cholangiocarcinomas specifically, and response to therapy broadly. We speculate that pYAP^{Y357} phosphorylation and or SHP2 abundance may be biomarkers for response to standard-of-care chemotherapy in cholangiocarcinomas and may select patients who will benefit from MCL1-directed combinatorial therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

E.H. Buckarma: Data curation, writing-original draft, writing-review and editing. **N.W. Werneburg:** Data curation. **C.B. Conboy:** Data curation. **A. Kabashima:** Writing-original draft. **D.R. O'Brien:** Data curation. **C. Wang:** Data curation. **S. Rizvi:** Writing-review and editing. **R.L. Smoot:** Writing-original draft, writing-review and editing.

Acknowledgments

This work was supported by a Department of Defense Career Development Grant W81XWH-18-1-0297 (to R.L. Smoot), the National Institute of Diabetes and Digestive and Kidney Diseases of the NIH under award number T32DK07198 (to E.H. Buckarma), K08CA236874 from the National Cancer Institute (to S. Rizvi), Optical Microscopy Core services provided through the Mayo Clinic Center for Cell Signaling in Gastroenterology (P30DK084567), the Mayo Clinic Medical Genome Facility - Proteomics Core, the Mayo Clinic Hepatobiliary SPORE (P50 CA210964) from the NCI, the Mayo Clinic Department of Surgery, and Mayo Clinic.

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Received February 19, 2020; revised May 15, 2020; accepted July 2, 2020; published first July 9, 2020.

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Molecular Cancer Research

The YAP-Interacting Phosphatase SHP2 Can Regulate Transcriptional Coactivity and Modulate Sensitivity to Chemotherapy in Cholangiocarcinoma

EeeLN H. Buckarma, Nathan W. Werneburg, Caitlin B. Conboy, et al.

Mol Cancer Res 2020;18:1574-1588. Published OnlineFirst July 9, 2020.

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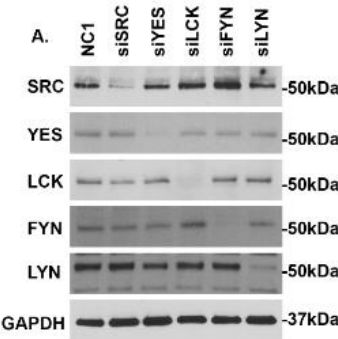
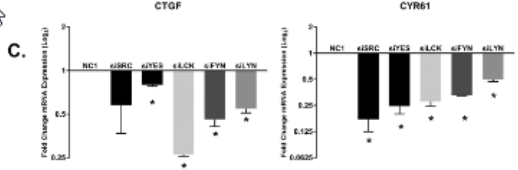
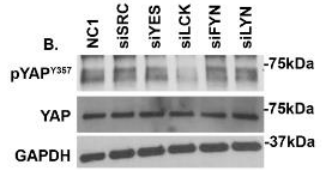
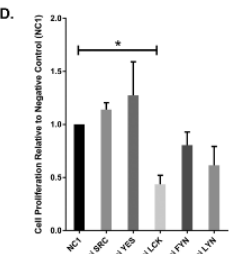
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**STATEMENT OF WORK –
START DATE Aug 1, 2018**

Site 1: Mayo Clinic
200 1st ST SW
Rochester, MN 55905
PI: Rory L. Smoot, MD
Designated Mentor: Gregory J. Gores, MD

Specific Aim 1: Identify Src-family kinase (SFK) responsible for Yes-associated protein (YAP) tyrosine phosphorylation and evaluate role in YAP localization/activation	Timeline
Major Task 1: Identify which Src family kinase mediates YAP tyrosine phosphorylation	Months
Subtask 1: Development of Flag-tagged YAP1 cell line by transfecting HuCCT-1 cells. Cell lines used: HuCCT-1 (Commerically available, but validated line present in our lab) Construct: Flag-tagged YAP1 [Addgene] Progress: Completed. We have developed a cell line from our mouse model of cholangiocarcinoma (SB1 cell line) containing the flag-tagged YAP. This cell line has been validated in the following publication: YAP-associated chromosomal instability and cholangiocarcinoma in mice. PMID 29464042	1-2
Subtask 2: Perform co-immunoprecipitation experiments in the presence or absence of PDGFR stimulation to detect direct binding between YAP and members of the SFK including Src, Yes, Fyn, Lck, and Lyn. Cell lines used: HuCCT-1-Flag-YAP Construct: Flag-Tagged YAP1 [Addgene] Progress: Completed. We performed immunoprecipitation followed by Mass spec in the SB1 cell line pulling down YAP in the presence and absence of dasatinib, a Src Kinase Inhibitor. We utilized this inhibitor approach rather than a stimulatory approach with PDGF because the cholangiocarcinoma cell lines have activated YAP at baseline. The src family kinases were not found to be associated specifically with YAP by IP and mass spec suggesting the interaction may be too transient to detect or the interaction may not be direct. Addendum: Re-evaluation of these experiments noting YAP interacting proteins demonstrated association of several tyrosine phosphatases with YAP which was modulated by the tyrosine phosphorylation status. The phosphatase SHP2 was observed to directly regulated YAP activity by modulating the tyrosine phosphorylation status. These data have been accepted for publication in <i>Molecular Cancer Research</i> pending final formatting submission.	1-3

Subtask 3: Downregulate individual SFK identified in Subtask 2 by siRNA Cell lines used: HuCCT-1 Progress: Completed. Achieved good on-target downregulation with the siRNA approach. Experimental details published in PMID:29903769. Figure 3 	4-6 -6
Subtask 4: Evaluation of YAP phosphorylation and YAP target gene expression (CTGF, Cyr61, and MCL-1) following SFK knockdown using qRT-PCR and immunoblot Cell lines used: si(SFK)-HuCCT-1 Progress: Completed. Downregulation of LCK demonstrated the most significant effects on YAP phosphorylation and the most consistent effects on YAP target gene expression. Experimental details published in PMID:29903769. C.  B. 	5-6
Subtask 5: Evaluate cell death/proliferation following SFK knockdown using Caspase 3/7 assay and Cell counting assay Cell lines used: si(SFK)-HuCCT-1 Progress: Completed. Downregulation of LCK had the most dramatic effect on proliferation based on cell counting. Experimental details published in PMID:29903769. D. 	5-6

Milestone(s) Achieved: Identification of specific SFK responsible for tyrosine phosphorylation of YAP	6
Major Task 2: Determine role of tyrosine phosphorylation on YAP localization	
Subtask 1: Evaluate sub-cellular localization of S127-YAP +/- SFK inhibition and tyrosine phosphatase inhibition Cell lines used: SB-1 Inhibitors: SFK inhibitor - Dasatinib [Selleckchem] Tyrosine phosphatase inhibitor - Sodium orthovanadate [Sigma-Aldrich] Progress: Completed. YAP localization modulated by dasatinib exposure which could be eliminated by pretreatment with sodium orthovanadate. Experimental details published in PMID:29903769. A. SB1 Cells Flag-YAP Merge Vehicle Dasatinib Na3VO4 + Dasatinib YAP Fractional Localization (% Nucleus) Vehicle Dasatinib Na3VO4 + Dasatinib Legend: Both, Cytoplasmic, Nucleus	6-9
Subtask 2: Evaluate S127-YAP transcriptional activity +/- SFK inhibition by comparing expression levels of YAP target genes Cell lines used: SB-1 Progress: Completed. We noted significant downregulation of YAP target gene expression following exposure of cells to dasatinib. Experimental details published in PMID:29903769. SB1 CTGF CYR61 MCL1 Fold Change mRNA Expression (Log2)	9-10

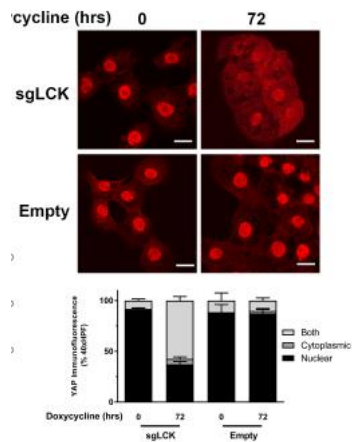
Subtask 3: Downregulate SFK activity via exogenous expression of dominant negative CSK and evaluate YAP sub-cellular localization and Y357 phosphorylation in S127A-YAP cell line (SB1)

Cell lines used: SB-1

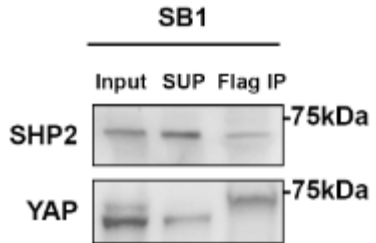
Construct: CSK (c-Src kinase) [Addgene]

Progress: In progress. Instead of downregulating all Src family members with overexpression of CSK we have focused now on downregulating the specific SFK, namely LCK, that is regulating YAP. We have produced an LCK knockout cell line in the HuCCT-1 cells that is doxycycline inducible utilizing CRISPR/Cas9 and are now working towards developing the SB1 LCK knockout cell line (which could then be used in our in vivo model). Experimental details published in PMID:29903769.

9-12



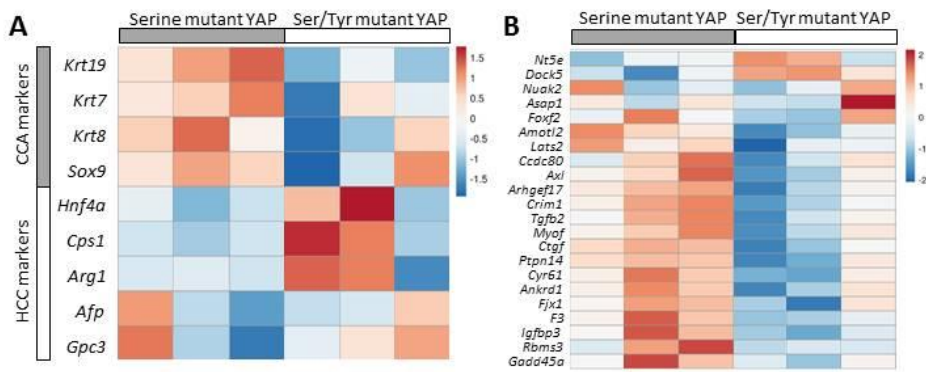
Subtask 4: Downregulate LATS1/2 activity via shRNA and evaluate YAP sub-cellular localization and transcriptional activity +/- SFK inhibition Cell lines used: HuCCT-1 shRNA constructs: shLATS1/2 [Dharmacon] Progress: Completed. We adjusted our strategy given the difficulty getting complete knockdown of LATS1/2 activity with shRNA approach and the off-target effects on the cells. Alternatively we overexpressed an epitope-tagged YAP molecule in which all serine sites had been mutated to alanine which effectively kills the phosphorylation sites and ensures that the mutated molecule can not be regulated by LATS1/2. This mutated molecule still could be regulated by dasatinib exposure, indicating tyrosine phosphorylation was driving its regulation, not serine. Experimental details published in PMID:29903769. C. FLAG-5SA-YAP-HuCCT-1 Cells <table><caption>YAP Translocation Data (Estimated from Graph)</caption><thead><tr><th>Condition</th><th>Nuclear (%)</th><th>Cytosolic (%)</th><th>Both (%)</th></tr></thead><tbody><tr><td>Vehicle</td><td>~10</td><td>~10</td><td>~80</td></tr><tr><td>Dasatinib</td><td>~10</td><td>~10</td><td>~80</td></tr><tr><td>Na3VO4 + Dasatinib</td><td>~10</td><td>~10</td><td>~80</td></tr></tbody></table>	Condition	Nuclear (%)	Cytosolic (%)	Both (%)	Vehicle	~10	~10	~80	Dasatinib	~10	~10	~80	Na3VO4 + Dasatinib	~10	~10	~80	12-15
Condition	Nuclear (%)	Cytosolic (%)	Both (%)														
Vehicle	~10	~10	~80														
Dasatinib	~10	~10	~80														
Na3VO4 + Dasatinib	~10	~10	~80														
Milestone(s) Achieved: Determination of role of tyrosine phosphorylation of YAP in regulating subcellular localization	15																
Specific Aim 2: Identify YAP binding partners regulated by tyrosine phosphorylation and transcriptional program																	
Major Task 3: Identify YAP binding partners regulated by tyrosine phosphorylation																	
Subtask 1: Immunoprecipitation of S127A- YAP +/- SFK inhibition followed by mass spectroscopy as a screen for differentially YAP binding proteins Cell lines used: SB-1 Inhibitor: SFK inhibitor –Dasatinib [Selleckchem] Progress: Completed. Evaluation of this data demonstrated significant modulation of the tyrosine phosphatase SHP2 interaction with YAP based on tyrosine phosphorylation status. The initial experimental details published in PMID:29903769. This subsequently led to exploration of SHP2 as a YAP regulating molecule. This data has now been accepted for publication in <i>Molecular Cancer Research</i>. Final submitted version is attached to the report as addendum.	16-19																

Subtask 2: Confirmatory co-immunoprecipitation of candidate YAP binding proteins identified by mass spectroscopy in Subtask 1 +/- SFK inhibition Cell lines used: SB-1 Progress: Completed. We have now demonstrated that SHP2 co-immunoprecipitates with YAP utilizing a flag-tagged YAP construct in SB1 cells, see attached figure. Experimental details listed in attached accepted manuscript.  Cell lysates of SB1 cells that contain FLAG-YAPS127A were subjected to anti-Flag immunoprecipitation and western blot analysis of endogenous SHP2. Total YAP serves as a loading control in inputs.	20-22
Subtask 3: Establish Y357A-YAP expressing HuCCT-1 cells in which binding of previously identified YAP binding genes regulated by tyrosine phosphorylation (Subtask 1 and 2) will be evaluated by co-immunoprecipitation. Cell lines used: HuCCT-1 Progress: In progress. Expression of the Y357A-YAP construct has been possible in a transient fashion in the HuCCT1 cells and work continues to develop a stable cell line for evaluation. As an alternative strategy we have isolated cells from our <i>in vivo</i> model in which transposons bearing the Y357A-YAP construct are injected into the bile duct of a mouse (Major task 5, Subtask 3). Cells from these tumors (akin to the SB1 cell line) have been isolated and are currently undergoing additional evaluation and validation. Briefly the cells are isolated from intact tumors utilizing mechanical dissociation. Cell suspensions are then cultured in DMEM with 10%FBS and pen-strep antibiotics. Cells are passaged until a uniform cell population is obtained. These cells have then been subjected to RT-PCR analysis for cholangiocarcinoma and hepatocellular carcinoma associated genes. We will plan to repeat FLAG-tag based immunoprecipitation in these cells as well as the SB1 cells (Y357A wildtype) to get an unbiased evaluation of YAP binding proteins dependent on the tyrosine phosphorylation status. Identified proteins will be confirmed by direct co-immunoprecipitation in these cell lines.	22-25
Milestone(s) Achieved: Identification of YAP binding proteins altered by tyrosine phosphorylation	25
Major Task 4: Evaluate transcriptional program +/- SFK inhibition	

Subtask 1: RNAsequencing +/- SFK inhibition in YAP wild type (HuCCT-1) and S127A-YAP (SB-1) cell lines to identify differentially expressed YAP target genes regulated by tyrosine phosphorylation of YAP

Cell lines used: SB-1 and HuCCT-1

Progress: Completed. The sequencing in both the HuCCT1 and SB1 has been completed. Bioinformatic analysis demonstrated significant downregulation of YAP target genes specifically CTGF and CYR61 with SFK inhibition. In addition we have now extended these experiments to include evaluation of tumors with either wild-type YAP tyrosine sites or bearing the Y357A defunctioning mutation (Major task 5, Subtask 3). The sequencing of these tumors has also demonstrated significant YAP signature alterations. Further bioinformatics analysis is ongoing. Unpublished data.



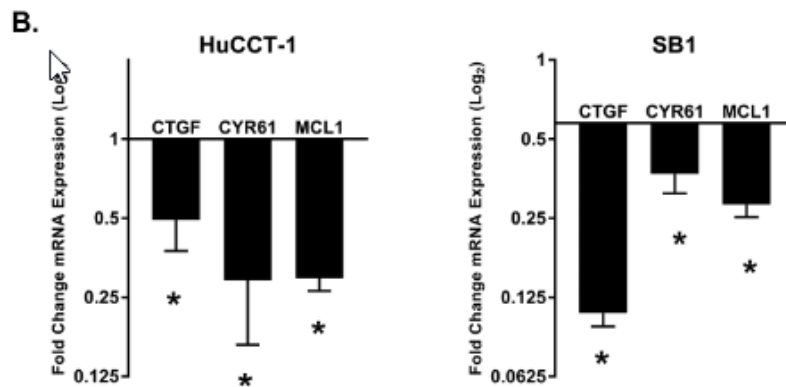
Tumors from mice initiated by either S127A-YAP or S127A-Y357F-YAP biliary instillation (n=3 each) were subjected to RNA sequencing. Evaluation of signatures associated with hepatocellular and cholangiocarcinoma (Panel A) and YAP target gene expression (Panel B) demonstrated that the compound mutation including defunctioning Y357F-YAP was associated with increased expression of hepatocellular carcinoma markers and downregulation of cognate YAP target genes.

Subtask 2: Validation of expression of genes identified in Subtask 1 by immunoblot +/- SFK inhibition.

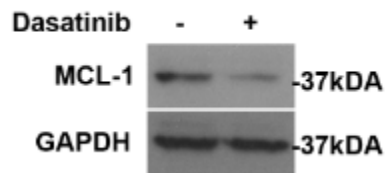
Cell lines used: SB-1 and HuCCT-1

Progress: Completed. We have demonstrated significant downregulation of YAP target genes with SFK inhibition in both SB1 and HuCCT1 cells. Experimental details published in PMID:29903769.

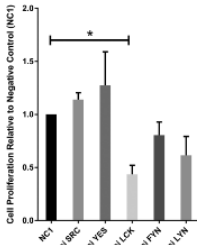
28-29



RT-PCR for YAP target genes in HuCCT1 and SB1 cell lines following incubation with the pan-Src kinase inhibitor dasatinib (1 μ M, 2hrs).



Cell lysates from HuCCT1 cells +/- dasatinib (1 μ M, 2hrs) were subjected to immunoblot for MCL1. GAPDH was used as a loading control.

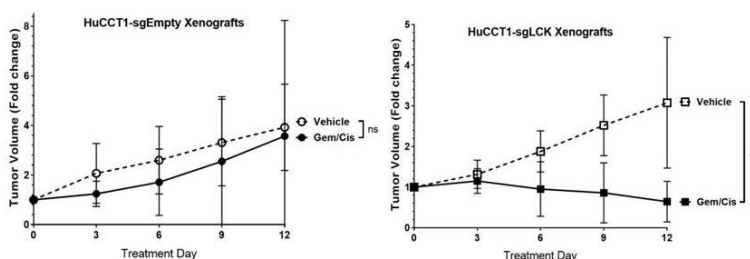
Subtask 3: For cytoprotective genes identified and validated in Subtasks 1 and 2 and SFK identified in major task #1, overexpressing and knockout HuCCT-1 cell lines will be established to evaluate the effect on cell survival and proliferation (by cell counting) +/- SFK inhibition. Cell lines used: HuCCT-1 and SB-1 Progress: Completed. We have downregulated LCK in HuCCT1 cells and have demonstrated decreased YAP activity in those cells specifically. We have noted decreased proliferation in LCK knockout cells and D.  <table border="1"> <caption>Cell Proliferation Relative to Negative Control (NC1)</caption> <thead> <tr> <th>Condition</th> <th>Relative Proliferation</th> </tr> </thead> <tbody> <tr> <td>NC1</td> <td>1.0</td> </tr> <tr> <td>siYAP</td> <td>~1.2</td> </tr> <tr> <td>siYAP</td> <td>~1.3</td> </tr> <tr> <td>siLCK</td> <td>~0.4</td> </tr> <tr> <td>siPTN</td> <td>~0.8</td> </tr> <tr> <td>siLIM</td> <td>~0.6</td> </tr> </tbody> </table>	Condition	Relative Proliferation	NC1	1.0	siYAP	~1.2	siYAP	~1.3	siLCK	~0.4	siPTN	~0.8	siLIM	~0.6	29-32
Condition	Relative Proliferation														
NC1	1.0														
siYAP	~1.2														
siYAP	~1.3														
siLCK	~0.4														
siPTN	~0.8														
siLIM	~0.6														
Milestone(s) Achieved: Identification of transcriptional signature affected by YAP tyrosine phosphorylation	32														
Specific Aim 3: Evaluate therapeutic efficacy of SFK inhibition in two animal models of cholangiocarcinoma (CCA)															
Major Task 5: Test efficacy of genetic silencing of SFK in murine model of CCA															
Subtask 1: Complete IACUC submission and submit documents for ACURO approvals Progress: Completed.	24														

Subtask 2: Implantation of doxycycline-inducible SFK CRISPR/Cas9 knockout SB-1 stable cell lines produced in task 4.2 orthotopically in the liver of syngeneic c57/bl6 mice and following tumor growth, treatment with doxycycline (n=10) versus vehicle (n=10)

[20 mice per group x 3 unique SFK knockdown cell lines (may need to be adjusted based on identification of SFK in major task #1) =60 mice total]

Progress: Completed via alternative approach. We were able to demonstrate that inducible sgLCK-HuCCT1 cells were more sensitive to chemotherapy than wildtype cells in vivo. LCK knockout SB1 cells were not able to be successfully technically created. These studies were completed via alternative approach and as such were NOT FUNDED by this award but results are reported here to demonstrate completion.

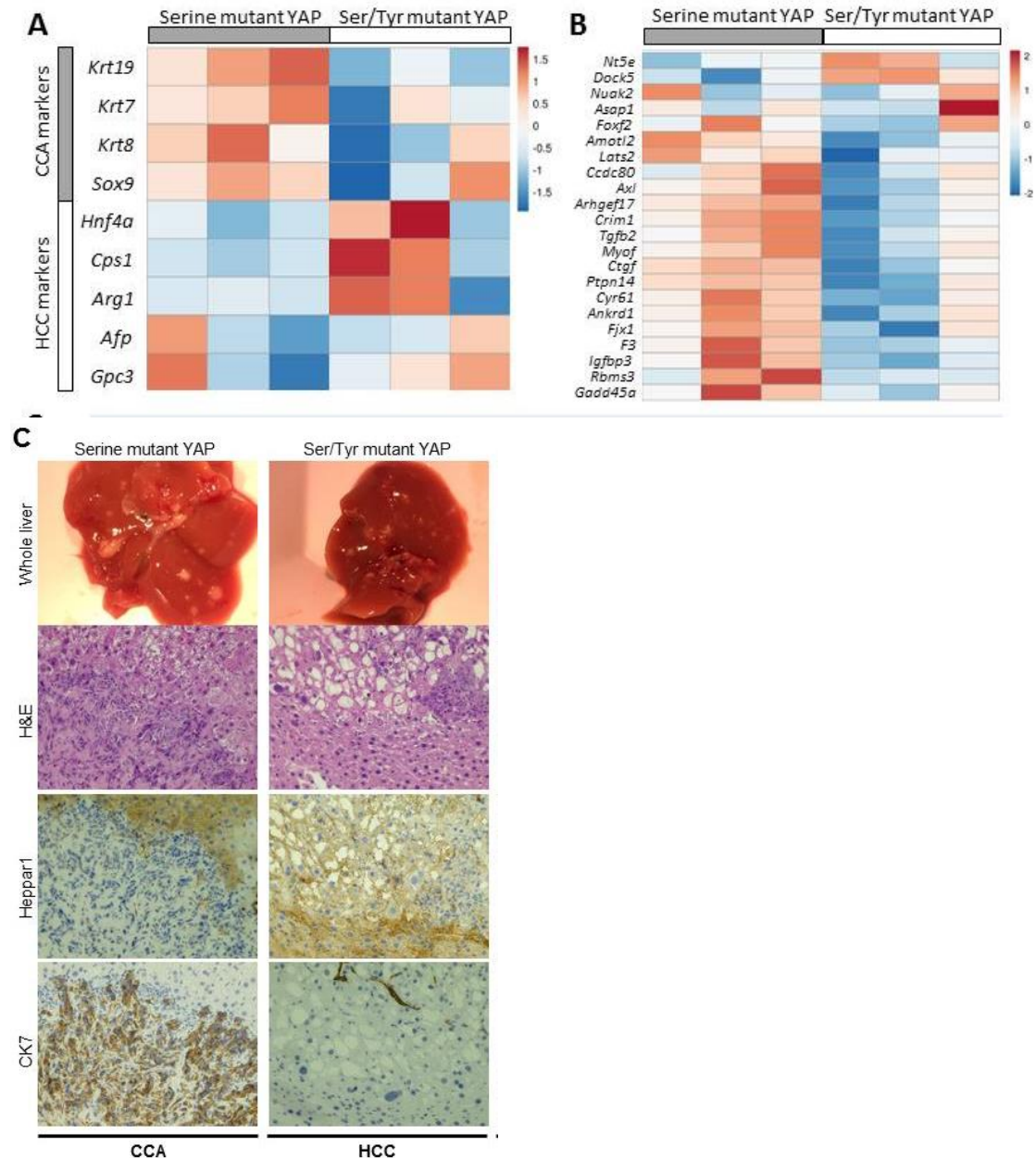
Inducible sgLCK cells or sgEMPTY control cells were induced with doxycycline and placed into the bilateral flanks of NOD/SCID mice. Once palpable tumors developed mice were then treated with vehicle or gemcitabine and cisplatin every three days and tumor size measured.



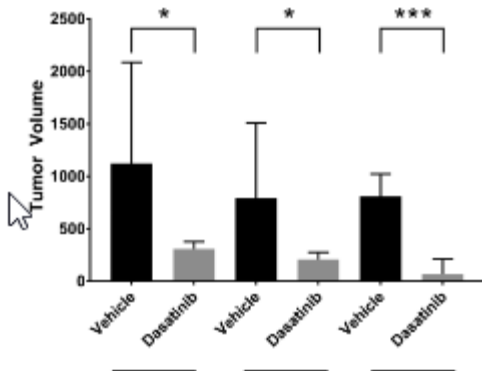
Subtask 3: Biliary instillation of S127A-YAP or S127A-Y357F-YAP in c57/bl6 mice and evaluation for tumor burden, morphology, and YAP localization.

[10 mice per group x 2 groups =20 mice total]

Progress: Completed. We have performed these experiments and are not evaluating the tissue to signaling and phenotype. Interestingly the Y357F mutation appears to shift the predominant morphology of the tumors to hepatocellular carcinomas. Unpublished data.



Tumors from mice initiated by either S127A-YAP or S127A-Y357F-YAP biliary instillation (n=3 each) were subjected to RNA sequencing. Evaluation of signatures associated with hepatocellular and cholangiocarcinoma (Panel A) and YAP target gene expression (Panel B). Morphology and immunohistochemistry staining of tumors (Panel C)

Milestone(s) Achieved: Preclinical data demonstrating efficacy of SFK knockdown in CCA	36																		
Major Task 6: Test efficacy of pharmacologic SFK inhibition in murine model of CCA																			
Subtask 1: Submit documents for IACUC, IRB, HRPO, and ACURO approvals Progress: Completed.	24																		
Subtask 2: Expansion of 3 unique patient-derived tumors into flanks of NOD/SCID mice and treatment with the SFK inhibitor dasatinib followed by evaluation of tumor growth, YAP localization, and YAP phosphorylation status. [20 mice per group x 3 groups (unique PDX) = 60mice total] Progress: Completed Treatment with Dasatinib. Have access to novel LCK specific inhibitor and will submit modification to treat PDX with this inhibitor given in vitro responses. D.  <table><caption>Approximate Tumor Volume Data from Graph D</caption><thead><tr><th>Group</th><th>Treatment</th><th>Approximate Tumor Volume</th></tr></thead><tbody><tr><td rowspan="2">Group 1</td><td>Vehicle</td><td>~1100</td></tr><tr><td>Dasatinib</td><td>~300</td></tr><tr><td rowspan="2">Group 2</td><td>Vehicle</td><td>~800</td></tr><tr><td>Dasatinib</td><td>~200</td></tr><tr><td rowspan="2">Group 3</td><td>Vehicle</td><td>~800</td></tr><tr><td>Dasatinib</td><td>~100</td></tr></tbody></table>	Group	Treatment	Approximate Tumor Volume	Group 1	Vehicle	~1100	Dasatinib	~300	Group 2	Vehicle	~800	Dasatinib	~200	Group 3	Vehicle	~800	Dasatinib	~100	32-36
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Milestone(s) Achieved: Preclinical data demonstrating efficacy of SFK inhibition in CCA	36																		