

AWARD NUMBER: W81XWH-18-1-0297

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

PRINCIPAL INVESTIGATOR: Rory Smoot

**CONTRACTING ORGANIZATION: Mayo Clinic
ROCHESTER, MN**

REPORT DATE: AUGUST 2020

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Fort Detrick, Maryland 21702-5012**

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14. ABSTRACT During the reporting period significant progress has been made on understanding the regulatory mechanisms of YAP tyrosine phosphorylation as well as the impact of YAP tyrosine phosphorylation on both YAP transcriptional activity, as well as oncogenic fate, and sensitivity to a standard of care chemotherapy. Specifically we have now demonstrated the the tyrosine phosphatase SHP2 can directly regulate YAP activity and chemosensitivity through down regulation of YAP tyrosine phosphorylation. These data have just been accepted for publication in <i>Molecular Cancer Research</i> . Furthermore we have now observed that downregulation of YAP tyrosine phosphorylation is associated with a phenotype switch from cholangiocarcinoma to more predominantly hepatocellular carcinoma.					
15. SUBJECT TERMS Src family kinase, dasatinib					
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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 for period number 2 were:

Major Task 2: Determine role of tyrosine phosphorylation on YAP localization

Major Task 3: Identify YAP binding partners regulated by tyrosine phosphorylation

These major tasks have been completed during this reporting 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 second reporting period have been achieved.

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

Data obtained during this reporting period has now been accepted for publication in Molecular Cancer Research.

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 reporting period 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:

Society of Surgical Oncology Annual Conference

American Association for Cancer Research Annual Conference

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.

Abstracts were submitted to AACR annual meeting, International Liver Cancer Association meeting, and the Cholangiocarcinoma Foundation meeting. The meetings have been either postponed or altered to virtual meetings only.

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.

During the next reporting period we will pursue several areas of inquiry to complete the major tasks set forth in the SOW. First we will continue to evaluate the transcriptional program of YAP in the setting of tyrosine phosphorylation utilizing the tissue we have obtained from the tumor models. Next, we will continue to work on LCK knockdown in our murine CCA model, and continue to pursue the alternative strategies of testing HuCCT1-LCK knockout cells in a NOD/SCID model and pursuing LCK pharmacologic inhibitors.

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.

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).*

Buckarma E, Werneburg N, Conboy C, Kabashima A, O’Brien D, Wang C, Rizvi S, **Smoot RL**. The YAP-Interacting Phosphatase SHP2 Can Regulate Transcriptional Co-activity and Modulate Sensitivity to Chemotherapy in Cholangiocarcinoma [Accepted] *Mol Cancer Res.* ;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

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

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

EeeLN Buckarma MD

Post-doctoral fellow

11 person months

Dr. Buckarma has performed in vitro experiments, data analysis, and presentation of the data

Funding for Dr. Buckarma is through a T32 training grant

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.*

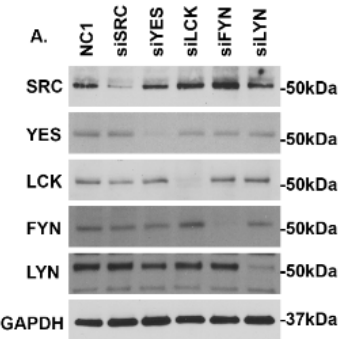
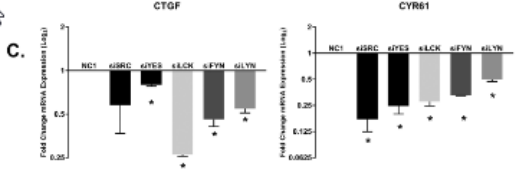
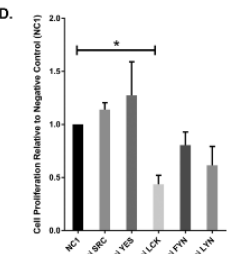
QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

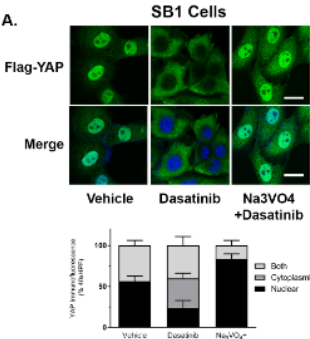
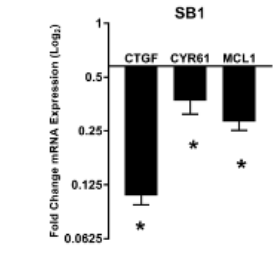
9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

**STATEMENT OF WORK –
Sep 18, 2017 PROPOSED
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 A.  NC1 siSRC siYES siLCK siFYN siLYN SRC 50kDa YES 50kDa LCK 50kDa FYN 50kDa LYN 50kDa GAPDH 37kDa	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.  CTGF CYR61 NC1 siSRC siYES siLCK siFYN siLYN pYAP^{Y387} 75kDa YAP 75kDa GAPDH 37kDa	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.  NC1 siSRC siYES siLCK siFYN siLYN	5-6

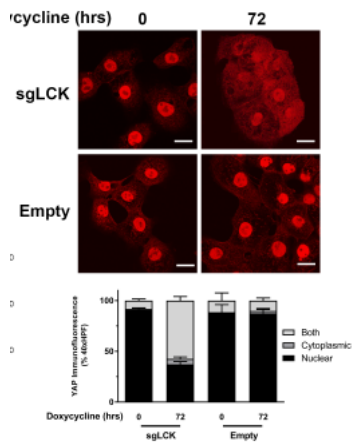
Milestone(s) Achieved: Identification of specific SFK responsible for tyrosine phosphorylation of YAP	6
Major Task 2: Determine role of tyrosine phosphorylation on YAP localization	
Subtask 1: Evaluate sub-cellular localization of S127-YAP +/- SFK inhibition and tyrosine phosphatase inhibition Cell lines 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 	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. 	9-10

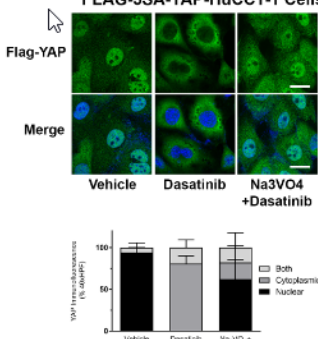
Subtask 3: Downregulate SFK activity via exogenous expression of dominant negative CSK and evaluate YAP sub-cellular localization and Y357 phosphorylation in S127A-YAP cell line (SB1)

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.



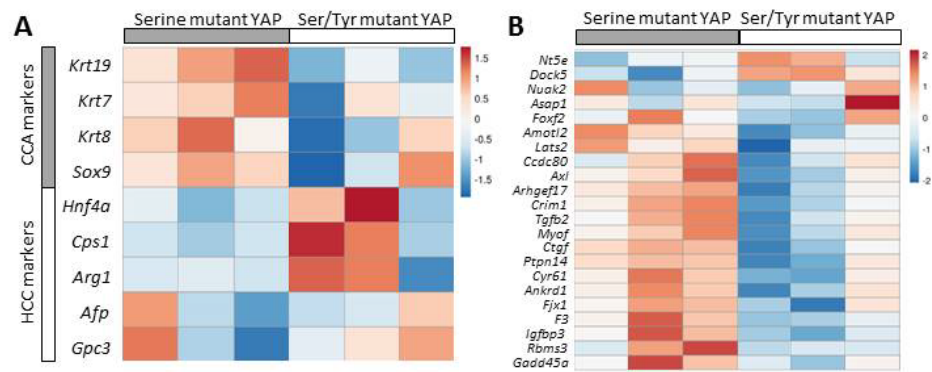
Subtask 4: Downregulate LATS1/2 activity via shRNA and evaluate YAP sub-cellular localization and transcriptional activity +/- SFK inhibition Cell lines used: HuCCT-1 shRNA constructs: shLATS1/2 [Dharmacon] Progress: Completed. We adjusted our strategy given the difficulty getting complete knockdown 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  Flag-YAP Merge Vehicle Dasatinib Na3VO4 + Dasatinib YAP Translocation (% cells) Legend: Both (white), Cytoplasmic (grey), Nuclear (black) <table><tr><th>Condition</th><th>Nuclear (%)</th><th>Cytoplasmic (%)</th><th>Both (%)</th></tr><tr><td>Vehicle</td><td>~100</td><td>~0</td><td>~0</td></tr><tr><td>Dasatinib</td><td>~100</td><td>~0</td><td>~0</td></tr><tr><td>Na3VO4 + Dasatinib</td><td>~100</td><td>~0</td><td>~0</td></tr></table>	Condition	Nuclear (%)	Cytoplasmic (%)	Both (%)	Vehicle	~100	~0	~0	Dasatinib	~100	~0	~0	Na3VO4 + Dasatinib	~100	~0	~0	12-15
Condition	Nuclear (%)	Cytoplasmic (%)	Both (%)														
Vehicle	~100	~0	~0														
Dasatinib	~100	~0	~0														
Na3VO4 + Dasatinib	~100	~0	~0														
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.

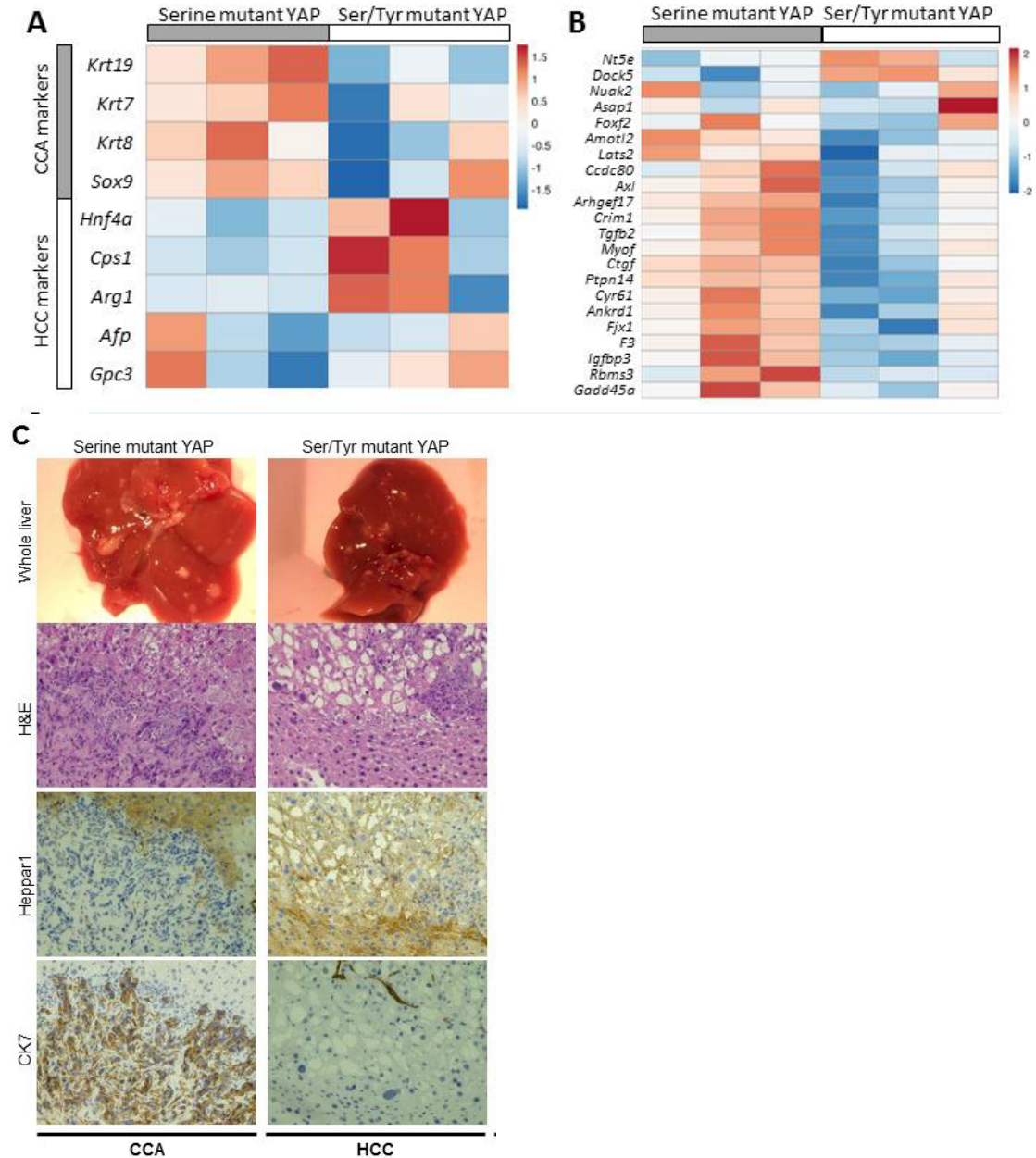
Subtask 2: Validation of expression of genes identified in Subtask 1 by immunoblot +/- SFK inhibition. Cell lines used: SB-1 and HuCCT-1 Progress: In progress. We have demonstrated significant downregulation of YAP target genes with SFK inhibition in both SB1 and HuCCT1 cells. We continue to evaluate additional gene targets. Experimental details published in PMID:29903769. B. <table><caption>Approximate data from Figure B</caption><thead><tr><th>Cell Line</th><th>Gene</th><th>Fold Change mRNA Expression (Log₂)</th></tr></thead><tbody><tr><td rowspan="3">HuCCT-1</td><td>CTGF</td><td>~0.5</td></tr><tr><td>CYR61</td><td>~0.3</td></tr><tr><td>MCL1</td><td>~0.3</td></tr><tr><td rowspan="3">SB1</td><td>CTGF</td><td>~0.1</td></tr><tr><td>CYR61</td><td>~0.4</td></tr><tr><td>MCL1</td><td>~0.3</td></tr></tbody></table> RT-PCR for YAP target genes in HuCCT1 and SB1 cell lines following incubation with the pan-Src kinase inhibitor dasatinib (1μM, 2hrs). <table><caption>Immunoblot Data</caption><thead><tr><th>Protein</th><th>Dasatinib (-)</th><th>Dasatinib (+)</th><th>Molecular Weight</th></tr></thead><tbody><tr><td>MCL-1</td><td>Strong band</td><td>Weak band</td><td>~37kDA</td></tr><tr><td>GAPDH</td><td>Strong band</td><td>Strong band</td><td>~37kDA</td></tr></tbody></table> Cell lysates from HuCCT1 cells +/- dasatinib (1μM, 2hrs) were subjected to immunoblot for MCL1. GAPDH was used as a loading control.	Cell Line	Gene	Fold Change mRNA Expression (Log ₂)	HuCCT-1	CTGF	~0.5	CYR61	~0.3	MCL1	~0.3	SB1	CTGF	~0.1	CYR61	~0.4	MCL1	~0.3	Protein	Dasatinib (-)	Dasatinib (+)	Molecular Weight	MCL-1	Strong band	Weak band	~37kDA	GAPDH	Strong band	Strong band	~37kDA	28-29
Cell Line	Gene	Fold Change mRNA Expression (Log ₂)																												
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Protein	Dasatinib (-)	Dasatinib (+)	Molecular Weight																											
MCL-1	Strong band	Weak band	~37kDA																											
GAPDH	Strong band	Strong band	~37kDA																											
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: In progress. 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. We are continuing the analysis and evaluating the effects of downregulation on chemosensitivity.	29-32																													
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: In progress. We have started developing the knockout SB1 cell lines. We have expressed the Cas9 and sgRNA constructs and have sorted cells based on fluorophore positivity. We are awaiting further selection and validation of the knockouts. Stable knockout has not been achieved in the SB1 cell line. We continue to attempt this knockout with different sgRNA constructs, however as an alternative we have also utilized our HuCCT1 LCK knockout cells and have an IACUC approval for implantation in NOD/SCID mice for tumor growth studies and therapeutic trials. These alternative approach experiments are <u>NOT</u> funded by this DOD grant.	32-36

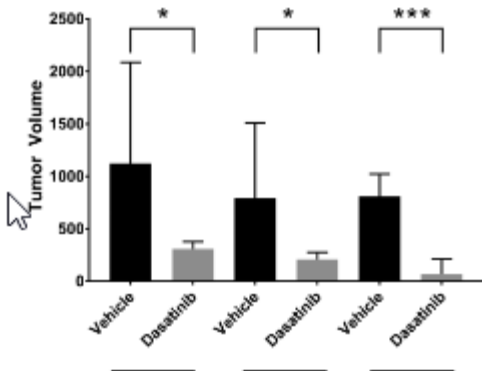
Subtask 3: Biliary instillation of S127A-YAP or S127A-Y357F-YAP in c57/bl6 mice and evaluation for tumor burden, morphology, and YAP localization.

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

Progress: In progress. 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. 	32-36
Milestone(s) Achieved: Preclinical data demonstrating efficacy of SFK inhibition in CCA	36

The Hippo Pathway and YAP Signaling: Emerging Concepts in Regulation, Signaling, and Experimental Targeting Strategies with Implications for Hepatobiliary Malignancies

Nathan Werneburg¹, Gregory J. Gores¹, Rory L. Smoot²

¹Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine and Science, Rochester, MN, USA

²Division of Hepatobiliary and Pancreas Surgery, Mayo Clinic College of Medicine and Science, Rochester, MN, USA

Address for correspondence: Gregory J. Gores, MD

Professor of Medicine and Physiology

Mayo Clinic

200 First Street SW, Rochester, Minnesota 55905

Tel: 507 284 0686; Fax: 507 284 0762

E-mail: gores.gregory@mayo.edu

or

Rory L. Smoot, MD

Assistant Professor of Surgery

Mayo Clinic

200 First Street SW, Rochester, Minnesota 55905

Tel: 507 284 1529; Fax

E-mail: smoot.rory@mayo.edu

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Abstract

The Hippo pathway, and its effector protein YAP (a transcriptional co-activator) have been identified as important in the biology of both hepatocellular carcinoma and cholangiocarcinoma. First identified as a tumor suppressor pathway in *Drosophila*, the understanding of the mammalian YAP signaling and its regulation continues to expand. In its “on” function the canonical regulatory Hippo pathway, a well described serine-threonine kinase module, regulates YAP function by restricting its subcellular localization to the cytoplasm. In contrast, when the Hippo pathway is “off”, YAP translocates to the nucleus and drives co-transcriptional activity. Given the role of Hippo/YAP signaling in hepatic malignancies investigators have sought to target these molecules; however, standard approaches have not been successful based on the pathway's negative regulatory role. More recently additional regulatory mechanisms, such as tyrosine phosphorylation, of YAP have been described. These represent positive regulatory events that may be targetable. Additionally, several groups have identified potentiating feed-forward signaling for YAP in multiple contexts; suggesting other experimental therapeutic approaches to interrupt these signaling loops. Herein, we explore the current data supporting alternative YAP regulatory pathways, review the described feed-forward signaling cascades that are YAP dependent, and explore targeting strategies that have been employed in pre-clinical models of hepatic malignancies.

Introduction

Yes-associated protein (YAP) and its ortholog transcriptional coactivator with a PDZ-binding domain (TAZ) function canonically as transcriptional co-activators, and are the effector proteins of the Hippo pathway. The Hippo pathway is serine/threonine kinase module that phosphorylates YAP on serine residues, especially serine 127; the consequence of which is sequestration of YAP in the cytoplasm, limiting its co-activating transcriptional function. In this manner, the Hippo pathway acts as a regulator of YAP function, with the components and function of the pathway well described.¹⁻⁹(Fig. 1) The recognition that the pathway acts as a tumor suppressor, with mutations/deletions in the pathway associated with tissue overgrowth and oncogenesis (including well documented association with development of hepatocellular carcinoma, cholangiocarcinoma, and mixed hepatocellular-cholangiocarcinoma tumors) has led many investigators to seek strategies to target the pathway therapeutically. Given its negative regulatory function this has proven difficult with standard approaches. More recently novel, non-Hippo, regulation of YAP has been described. This regulation has focused on tyrosine phosphorylation of YAP, which activates YAP, independent of the Hippo pathway. This regulation has been described in both malignant and benign conditions. Moreover, additional evolving concepts in YAP signaling have recently been reported. Namely the concept of feed-forward oncogenic signaling pathways whereby YAP can drive the transcription of target genes involved in “activating” YAP. Herein, we will review briefly the canonical regulatory pathway, focus on evolving concepts of YAP regulation, and review YAP in hepatic malignancies. We also discuss experimental approaches for targeting YAP in cancer.

Hippo Pathway and YAP/TAZ Regulation by Serine Phosphorylation (Figure 1)

Examination of YAP/TAZ regulation typically begins with the canonical regulatory entity the Hippo pathway and its core components, a series of serine/threonine kinases and other associated proteins. The Hippo pathway acts as a brake, restraining YAP activity, such that “activation” of the pathway culminates in serine phosphorylation of YAP and its cytoplasmic sequestration. This canonical regulatory pathway was first described in *Drosophila*. Mutagenesis screens, examining potential tumor suppressor genes in mosaic flies, led to recognition of a fly with overgrowth of the imaginal disc. Several components of the Hippo pathway were identified and cloned, including Wts (human ortholog Large Tumor Suppressor 1/2), Hpo (human ortholog Mammalian Sterile Twenty-like 1/2), and Sav (human ortholog Salvador).^{2,3,8} The fly and human proteins share sufficient homology that expression of the human counterparts of the Hippo pathway compensate for their loss in *Drosophila*.^{3,10} Since the discovery of the Hippo pathway the understanding of its complexity and cross-talk with other pathways has advanced considerably. The Hippo pathway does not have a dedicated cell surface receptor nor ligand; instead a variety of signaling pathways can interact with and regulate Hippo pathway activation. Hence, YAP integrates multiple signaling pathways. Several of these outside regulatory inputs have been investigated but many have remained unstudied, however irrespective of the signal engaging the Hippo pathway, frequently it’s initiation leads to activation of mammalian STe20-like kinase 1/2 (MST 1/2) which phosphorylates and subsequently activates large tumor suppressor 1/2 (LATS 1/2); followed by phosphorylation of YAP on serine residue 127 by LATS1/2.¹¹ Phosphorylation at Ser127 on YAP results in its cytosolic retention through recognition and binding by the acidic adapter protein 14-3-3 essentially restricting YAP from entering the nucleus and preventing its transcriptional activity.¹² Although serine 127 has been identified as the canonical “Hippo” phosphorylation site,

additional work has demonstrated that mutant YAP with all 5 serine residues changed to alanine (5SA-YAP) enhances YAP activity in stimulating cell proliferation and oncogenic transformation as compared to wild-type YAP.¹³ Subsequent work has suggested that serine 127 and 381 are the most important residues for oncogenic transformation;¹³ however, taken together these results suggest that additional serine residues other than S127 may serve as unique regulatory sites in YAP. For example, the other HXRXXS motif that has been shown to be phosphorylated *in vivo* by LATS is Ser381 (in isoform 2) / Ser397 (in isoform 1). Phosphorylation of this motif leads to additional phosphorylation of YAP by Casein kinase 1 epsilon (CK1δ/ε) and subsequent ubiquitination and degradation.⁹ The specificity of LATS for an individual site, and/or the cellular context regulating this specificity have yet to be defined. Further complicating the understanding of the regulatory mechanisms for YAP are the observations that other serine kinases such as Akt and Nemo-like Kinase (NLK) can also engage these regulatory sites.^{12,14} Since mutations in the Hippo pathway components are rare much of the recent work performed to date on the Hippo pathway and YAP regulation examines the regulation of Hippo component activity through outside factors influencing Hippo signaling, including YAP target genes.

Feed-Forward YAP Signaling (Figure 2)

The model in which YAP transcriptional targets may regulate either the Hippo pathway directly, or YAP directly, has been proposed in several recent studies. Most recently a siRNA-based automated kinome screen for targets that may regulate YAP localization was reported.¹⁵ This work identified NUA2, a member of the AMP kinase family, as a positive regulator of YAP activity. Further investigation noted that LATS was required for the activity, indicating that NUA2 was acting on the Hippo pathway as a negative regulator. Interestingly the

investigators also noted that NUA2 itself was a YAP target gene, as confirmed by chromatin immunoprecipitation assays identifying YAP/TAZ, TEAD, and JUN binding to a known NUA2 enhancer. This finding was further supported by work, published simultaneously, that identified NUA2 as a conserved YAP transcriptional target comparing transcriptomes from the human cholangiocarcinoma cell line HUCCT-1 and a murine model in which S127A-YAP is overexpressed in the mouse liver.¹⁶ Thus NUA2 was confirmed as a YAP target gene, in several tumor cell lines/models, and was also shown to negatively regulate the Hippo pathway (at LATS) leading to YAP activation. Additionally, oncogenic cross-talk leading to feed-forward signal propagation has been observed in embryonal rhabdomyosarcoma models between the Notch pathway and YAP.¹⁷ In these studies Notch signaling was observed to upregulate YAP at a transcriptional level; with YAP then upregulating Notch ligands and the Notch transcription factor RBPJ. Another recent study demonstrated a novel mechanism of YAP activation in metastatic lymph nodes with an autocrine YAP signaling signature.¹⁸ Upregulation of YAP via bile acid signaling in lymph nodes harboring metastatic melanoma was noted, in conjunction with upregulation of bile acid production in these nodes; supporting the concept of a potentiating feed-forward signal. These data strengthened this “feed-forward” concept of YAP signaling that has also been previously demonstrated for both G-protein linked receptors and receptor tyrosine kinases. The first demonstration of this phenomenon was in ovarian cancer cell lines. These investigators demonstrated that the EGF receptors EGFR and ERBB3 as well as the agonists NRG1 and HBEGF were upregulated by YAP and subsequently demonstrated that the agonists could activate YAP in an autocrine feed-forward loop, promoting oncogenesis.¹⁹ Similarly, evaluating the role of prostaglandin E2 (PGE2) signaling in colon cancer cell lines and a chemically induced murine colitis model, PGE2 upregulation of YAP mRNA levels and

transcriptional activity was identified. Subsequently these investigators demonstrated that increased YAP transcriptional activity was associated with upregulation of the PGE2-producing enzyme, prostaglandin-endoperoxide synthase 2, and the PGE2 receptor, prostaglandin E receptor 4 via a YAP-TEAD4 mechanism. The end result of which was a feed-forward PGE2 signaling loop mediated through YAP activity.²⁰ Additionally, gp130 was identified as a YAP-TEAD4 target gene in colorectal cancer cells. In this model, gp130 augmented activation of YAP via tyrosine phosphorylation by Src family kinases (SFK).²¹ This feed-forward loop was initiated by APC loss. Signaling through receptor tyrosine kinases leading to feed-forward YAP signaling has been demonstrated in cholangiocarcinoma models.^{22,23} Specifically, activation of the fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) pathways have been associated with YAP activation and subsequent ligand or receptor upregulation.^{22,23} Furthermore this line of investigation was notable for the observation that the regulation of YAP activity by PDGF signaling appeared to be regulated via SFKs, independent of the Hippo pathway itself, unlike what was demonstrated for the target NUAK2.¹⁶ Specifically, tyrosine phosphorylation of YAP on the tyrosine 357 residue appeared to be a nuclear retention signal in CCA.

Tyrosine Phosphorylation Regulating YAP (Figure 3)

While the canonical regulation of YAP activity is via the Hippo pathway, accumulating evidence suggests a central role for tyrosine phosphorylation in regulating YAP subcellular localization, and co-transcriptional activity, at least in certain contexts. The role of Src family kinases in facilitating YAP phosphorylation has been observed in several studies, and is gaining increasing recognition within the field as an important regulator of YAP activity. For example, the Src family kinase YES, regulates embryonic cell self-renewal programs via a YAP-TEAD2

pathway.²⁴ In these studies, leukemia inhibitory factor (LIF), an IL-6 family cytokine, activated YES, which subsequently led to increased tyrosine phosphorylation of YAP. These investigators demonstrated both direct interaction of the SH2 domain of YES with the LIF receptor (gp130 subunit) and also demonstrated direct interaction of YES with YAP via co-immunoprecipitation. Subsequently, others evaluated beta-catenin active colon cancer cell lines and suggested a YAP-TBX5 complex was required for proliferation.²⁵ Evaluation of YAP regulatory factors demonstrated that YAP and YES interacted in the beta-catenin active colon cancer cell line SW480 and that both YES and SRC may phosphorylate YAP on the tyrosine 357 residue. Knockdown studies suggested that while SRC could phosphorylate YAP, only knockdown of YES was associated with inhibition of proliferation. Finally, these investigators rescued YAP knockdown with both wild-type and a tyrosine mutant (Y357F-YAP) and noted that phosphorylation of the Y357 residue was necessary to rescue proliferation. A role for SFK mediated YAP activation in epithelial regeneration in the setting of inflammation, utilizing multiple models of inflammation and proliferation has also been reported.²⁶ In a transgenic model in which gp130 was activated, intestinal epithelial cells (IECs) demonstrated an increase in serine phosphorylated YAP (Hippo-regulated YAP), even though the cells demonstrated increased YAP transcriptional activity. Further evaluation of alternative YAP regulatory mechanisms suggested that IECs had increased levels of active SFK, and stimulation with IL-6 was associated with increased levels of Y357-YAP phosphorylation. This was also evident when evaluating the tyrosine phosphorylation status of YAP in mouse hepatocytes following partial hepatectomy. These effects could be blocked utilizing a semi-selective SFK inhibitor PP2. Similar to previous results, these investigators found that gp130 interacted directly with YES (based on co-immunoprecipitation) and also that SRC co-immunoprecipitated with gp130.

Based on these previous observations our group also explored the role of SFKs in regulating YAP subcellular localization and transcriptional co-activity in several models of cholangiocarcinoma^{23,27}. In these models, inhibition of PDGF with a small molecule inhibitor was associated with relocation of YAP from the nucleus to the cytoplasm and a decrease in YAP target gene transcription; however, the levels of S127-YAP phosphorylation were static or even slightly decreased suggesting the Hippo pathway was not central to the observed effects on YAP. Subsequently, we noted that Y357-YAP phosphorylation was markedly decreased following PDGF inhibition. Focusing on Y357-YAP phosphorylation, and the SFKs as the most likely mediator of YAP tyrosine phosphorylation, we performed siRNA and CRISPR/Cas9 mediated downregulation of individual SFK and observed a central role for the SFK family member LCK in regulating YAP tyrosine phosphorylation and nuclear retention in cholangiocarcinoma.²⁷ Evaluation of LATS activation in these models did not identify any change in activity following SFK inhibition, further suggesting tyrosine phosphorylation represented a Hippo-independent mechanism of YAP regulation. These data were supported by recent work utilizing photobleaching combined with mathematical modeling to examine nuclear export rate for YAP; the export rate could be regulated specifically by tyrosine phosphorylation via Src family kinases, and was noted to be the major determinant of YAP subcellular distribution.²⁸

YAP in Hepatic Malignancies

Hepatocellular Carcinoma (HCC)

The role of the Hippo pathway and YAP signaling in HCC is well documented. The seminal paper, clearly defining a kinase cascade in mammals controlling cellular proliferation and organ size, confirmed in vitro findings by over-expressing YAP in murine livers.¹

Overexpression of YAP in the short term lead to massive overgrowth of the liver, by hyperplasia, and longer term over-expression of YAP lead to tumor formation consistent with HCC. Similarly MST1/2 and Sav1 conditional knockout mice as well as Mob1a/1b deficient mice develop tumors, which have demonstrated either HCC, CCA, or mixed histology.^{4,5,7,29} Demonstrating the importance of Hippo signaling to human HCC, evaluation of a gene signature associated with “silencing of the hippo pathway” was developed from MST1/2 and Sav1 mice, and when applied to human HCC patients was a significant negative prognosticator.³⁰ Importantly, this signature predicted outcome in both western and eastern patient cohorts. Other novel YAP/TAZ related regulatory mechanisms associated with HCC include non-coding RNAs. While YAP has previously been demonstrated to sequester and limit the activity of p72 a subunit of the microprocessing machinery, there are also multiple specific miRNAs that have been described targeting either YAP/TAZ or components of the Hippo pathway, and associated with HCC.³¹⁻³³

Furthermore, Hippo pathway signaling (and the effectors YAP/TAZ) have been associated with conditions predisposing to HCC development, such as non-alcoholic steatohepatitis (NASH) and fibrosis. The role of Hippo signaling in the progression from steatosis to NASH and development of HCC was recently explored in the background of hyperactive AKT signaling, a known model of NASH and HCC development. Livers-specific PTEN knockout (the negative regulator of AKT) was associated with high levels of p-AKT, steatosis, progression to NASH, and eventually HCC (typically at 50 to 60 weeks of age). PTEN and Sav1 double knockout mice were observed to have a markedly accelerated progression of fatty liver disease and tumor formation (by 15 weeks of age). Mechanistic studies on these models suggested that YAP/TAZ activity lead to upregulation of the insulin receptor substrate

IRS2, which subsequently further increased the activation of AKT. In these models downregulation of this signaling circuit via an AKT inhibitor was effective in reducing TAZ expression as well as overall liver size and the size of liver tumors.³⁴ TAZ has previously been associated with liver inflammation and progression to fibrosis, with silencing associated with decreases in inflammation and fibrosis, but without any difference in steatosis.³⁵ These data suggest a protective role of the Hippo pathway, by restraining YAP/TAZ activity, and specific to AKT signaling this protection likely requires Skp2, suppressing cell ploidy.³⁶ The role of the Hippo pathway in limiting progression of NASH to HCC is further supported by the finding that the obesity-associated protein, JCAD, was able to inhibit LATS2 by directly binding the catalytic domain, inhibiting the Hippo kinase module, leading to YAP activation, and progression of NASH to HCC.³⁷ Upregulation of JCAD was observed in the setting of high free-fatty acids. The role of YAP/Hippo signaling in regulation of liver fibrosis has been explored in the setting of hepatic stellate cell (HSC) activation. YAP signaling has been demonstrated to be integral to both HSC activation directly as well as the ability of HSCs to upregulate metabolic pathways necessary for survival and proliferation, specifically glutaminolysis.^{38,39} Finally, the end result of steatohepatitis and liver fibrosis is increasing extracellular matrix (ECM) stiffness, which can lead to changes in YAP/Hippo regulation due to mechanotransduction. The ability of mechanotransduction to regulate YAP has been well covered in previous reviews⁴⁰⁻⁴³, however specific to hepatocarcinogenesis recent work has linked the ECM proteoglycan Agrin to YAP activation and hepatocarcinogenesis.⁴⁴ Increasing ECM stiffness was associated with a dramatic increase in Agrin levels. Increased Agrin was associated with decreased activity of the Hippo kinase cascade, and subsequently an increase YAP signaling activity.

Cholangiocarcinoma (CCA)

The importance of YAP to cholangiocarcinoma biology was first suggested in MST1/2 and Sav1 conditional knockout mice.^{4,5,7} All mice in both models developed tumors with the MST1/2 demonstrating more HCC tumors but intrahepatic cholangiocarcinoma tumors were also noted, and the Sav1 knockout mice demonstrating mixed histology tumors. Other Hippo pathway knockout animals have also demonstrated either mixed histology or intrahepatic cholangiocarcinoma tumors, while activated YAP models have been developed that also demonstrate either mixed histology or intrahepatic cholangiocarcinoma.^{29,45} For example biliary instillation of a sleeping beauty based set of transposons expressing myristolated AKT and S127A-YAP (YAP that cannot be negatively regulated by the Hippo pathway) leads to reproducible development of tumors that are histologically and immunophenotypically consistent with intrahepatic cholangiocarcinoma.⁴⁵ Development of these tumors is markedly more efficient when administering IL-33, a known biliary mitogen, to the animals, and interestingly IL-6 can be substituted for IL-33. Multiple investigators have evaluated YAP staining in clinical specimens of human cholangiocarcinoma.⁴⁶⁻⁵⁰ In these series, the majority of specimens have nuclear localized YAP, a hallmark of YAP activity. Strikingly, although YAP appears to be “activated” in a majority of human cholangiocarcinoma specimens, mutations in Hippo pathway components are uncommon in human tumors. For example, in the TCGA patient cohort only 3 patients (8%) had a mutation in Hippo pathway related genes (2 patients with a Salvador1 mutation and 1 patient with an NF2 mutation).⁵¹ As such, regulation of YAP via cross-talk from other signaling pathways has been of significant interest with demonstration of receptor tyrosine kinase activation of YAP (via Src family kinases) in cholangiocarcinoma, independent of the Hippo pathway. Further delineation of other potential regulatory mechanisms remains an active area of research.

Experimental Targeting Strategies for YAP/Hippo in Hepatobiliary Cancer Models

While no YAP-specific targeting strategies are currently being evaluated in clinical trials, pre-clinical data is available in various tumor models demonstrating efficacy of novel approaches, including in cholangiocarcinoma and HCC. The majority of these strategies have focused on interrupting feed-forward signaling cascades, disrupting the YAP-TEAD interaction, or targeting novel regulatory mechanisms such as non-coding RNAs. We have previously demonstrated efficacy of the pan-SFK inhibitor dasatinib in patient derived xenograft cholangiocarcinoma models.²⁷ In these studies we noted a decrease in tyrosine phosphorylated YAP, decrease in YAP transcriptional activity, an increase in TUNEL positive cells, and a markedly decreased tumor size as compared to control animals. Interestingly, others have previously demonstrated activity of dasatinib in IDH-mutated intrahepatic cholangiocarcinoma, including xenograft models.⁵² This study developed from a screen of small molecule inhibitors against a large panel of cancer cell lines. The most significant effect was noted for dasatinib treatment of IDH-mutant cholangiocarcinoma, however, these investigators did not evaluate the role of the Hippo pathway or YAP signaling specifically in their studies. Yuan et. al. explored the efficacy of targeting NIAK2 as a means of targeting hepatic YAP activity in a murine knock-in model employing S127A-YAP.¹⁶ These mice develop hepatomegaly, and employing a semi-specific inhibitor in these animals, the investigators demonstrated a significant decrease in hepatomegaly and proliferating hepatocytes. Additionally, the investigators evaluated efficacy of their novel inhibitor in nude mice bearing CCA cell line xenografts. Prolonged treatment (30days) was well tolerated and demonstrated efficacy manifest as significantly reduced tumor growth rates as compared to vehicle treated controls. The tolerance of therapy is important to note, as our group has previously observed therapy-limiting toxicity when evaluating verteporfin

in murine models of CCA.²² Verteporfin, used clinically currently as a photosensitizer for photodynamic therapy, is known to interrupt TEAD-YAP binding; however, it may be limited therapeutically given off-target effects/toxicity. In hepatocellular carcinoma, targeting of the poly ADP-ribose polymerase (PARP) family members tankyrase 1 and 2 has been explored for therapeutic efficacy. Cell line based studies have demonstrated therapeutic effect, which was associated with upregulation of the negative YAP regulators angiomin-like 1 and 2 (AMOTL1/2) as well as decreased YAP protein levels and downregulation of YAP co-transcriptional activity.^{53,54} Additional studies in HCC models have explored inhibition of Aurora kinases as a therapeutic strategy. Efficacy was noted, with evidence supporting downregulation of YAP downstream of the kinases as contributing to effects observed.⁵⁵

Conclusions

The understanding of the roles and regulation of YAP, either directly or via the Hippo pathway, continue to expand. Multiple examples of feed-forward signaling in a variety of cancer models have been identified. Further delving into the regulation of YAP in these models has led to accumulating data supporting a role for SFK in regulating YAP directly via tyrosine phosphorylation, an interesting finding with potential therapeutic implications; although strategies targeting specific SFKs are presently limited by the significant similarities between family members and the lack of specific inhibitors. Finally, the importance of YAP/Hippo pathway in the biology of hepatobiliary malignancies has been well described and underscores the continued interest in novel therapeutic targeting strategies, especially for these tumor types; strategies that are currently in pre-clinical testing phases.

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

EeeLN Buckarma¹, Nathan Werneburg², Caitlin Conboy³, Ayano Kabashima², Daniel O'Brien⁴,
Chen Wang⁴, Sumera Rizvi², Rory Smoot¹

¹Department of Surgery, ²Division of Gastroenterology and Hepatology, ³Department of Oncology, ⁴Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, Minnesota

Address for correspondence: Rory L. Smoot MD

Assistant Professor of Surgery

Mayo Clinic

200 First Street SW, Rochester, Minnesota 55905

Tel: 507 284 1529; Fax: 507 284 5196

E-mail: smoot.rory@mayo.edu

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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 (CCA). 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 CCA. A screen for YAP interacting tyrosine phosphatases identified SHP2, and characterization of CCA cell lines demonstrated an inverse relationship between SHP2 levels and pYAP^{Y357}. Human sequencing data demonstrated lower SHP2 levels in CCA tumors as compared to normal liver. Cell lines with low SHP2 expression and higher levels of pYAP^{Y357} were resistant to gemcitabine and cisplatin. In CCA 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 anti-apoptotic 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 utilizing an MCL1 inhibitor.

Significance: 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 (CCA), 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 CCA 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 CCA oncogenesis and progression [5-12]. Therefore, we have continued to evaluate YAP signaling in CCA, and more specifically its phospho-regulation. Our group and others have noted that a significant proportion of CCA specimens have YAP localized to the nucleus, presumably representing a co-transcriptionally active molecule[5-7, 10, 11]. Importantly, we have further observed that nuclear YAP is tyrosine phosphorylated in CCA, which may have significant functional and targeting implications [9].

The canonical phospho-regulatory 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), that 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 CCA 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 CCA than serine phosphorylation [9]. We have linked the SRC-family kinase LCK to YAP tyrosine phosphorylation, which corroborates other reports of SRC family kinases (SFK) 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 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 CCA and modulating CCA sensitivity to gemcitabine and cisplatin.

Herein, we examine tyrosine phosphatase activity regulating YAP activation in CCA 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 CCA 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 co-activity. The observed increases in YAP activity were accompanied by increases in cellular proliferation and expression of the pro-survival 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 CCA cell line SB1, and the human pancreatic adenocarcinoma cell line (PANC1) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.2% primocin under standard conditions. Due to cell density and serum regulation of the Hippo pathway, cell culture experiments were performed at near confluence (~80%). For authentication of the HuCCT-1 and KMCH cell lines, short tandem repeat (STR) 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. All cell lines were maintained at 37 degrees Celsius in the presence of 5% CO₂.

Antibodies and reagents. SHP099 (Selleckchem), NSC87877 (Cayman), Doxycycline hyclate (Sigma), S63845 (APExBIO), Vertoporfin and Gemcitabine and Cisplatin (Cayman) was added to cells at final concentrations from 0.05-100 µM per experimental design. The following primary antibodies were used for immunoblot analysis: actin (C-11 Santa Cruz) phospho YAP^{Y357} (ab62751 abcam) total YAP (63.7 Santa Cruz, 4912 CST), GAPDH (MAB374 Millipore), phospho SRC^{Y416} (2101 Cell Signaling), SRC (L4A1 Cell Signaling), SHP2 (33975 Cell Signaling) and MCL-1 (ac32087 abcam). The following primary monoclonal antibody were used for immunofluorescence and/or immunohistochemistry: total YAP (63.7 Santa Cruz),

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 TCGA CHOL primary tumor samples (n=36) and compared them to GTEx normal liver samples (n=110) [33-35]. The data was 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 RNASeq experiments utilizing similar bioinformatics methods. A t-test was conducted and a box plot was constructed comparing the CHOL samples to the normal liver samples.

Immunoprecipitation and Immunoblot analyses. Whole-cell lysates were collected by adding ice cold lysis buffer (Cell Signaling Technologies) containing protease inhibitors (Sigma-Aldrich), phosphatase inhibitors (Roche Diagnostics) and 1 mM PMSF. Tissue sample lysates were homogenized with a glass dounce and ice cold lysis buffer containing protease inhibitors, phosphatase inhibitors and 1 mM PMSF. Samples were vortexed and lysed on ice for approximately 20 minutes. The lysed tissue was then centrifuged at 12,000g 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 manufacturer's instructions. To conduct

immunoblot analysis, proteins were then resolved by SDS-PAGE electrophoresis 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 1:1000 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) were added to membrane at a concentration of 1:5000 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. The fixed cells were next incubated at room temperature with blocking buffer containing 5.0% bovine serum albumin (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 fluorescent 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 $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ method as previously described.[37] Data are reported as fold expression compared to 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 biologic replicates completed for each condition/cell line. The primers used are listed in Supplemental Table 1.

TEAD reporter assay. Global TEAD transcriptional activity was assessed in HuCCT1 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 NSC or SHP099 at 10 uM for 24 hours prior to measurement of firefly luciferase activity with a luciferase assay system (BPS Bioscience).

CRISPR/Cas9 doxycycline induced knockout. The sequences 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, Cambridge, MA, USA). To produce lentiviruses, FgH1tSHP2 or FUCas9Cherry plasmids were transiently co-transfected 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, Madison, WI, USA). After 48 and 72 hours, supernatant containing lentiviruses was harvested and passed through a 0.45- μ M filter. Target KMCH cells were transduced with the lentiviruses in the

presence of 8 µg/mL polybrene for 24 hours. After an additional 3 days in culture, 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, Mendota Heights, MN, USA) was added to culture medium at a final concentration of 7.5 µg/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 3000, and 4000 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 µM) or S63845 (5 µM) 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, 100 µM) 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, Madison, WI, USA).

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 μ L) 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 mm³. Combination gemcitabine (15mg/kg IP) and cisplatin (1mg/kg IP), gemcitabine (15mg/kg IP), cisplatin (1mg/kg IP) and S63845 (25 mg/kg IV) 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; tumor volume was calculated using the formula: Volume (mm³) = (length X width²)/2. Animals were sacrificed and tumors extracted 15 days after the 1st treatment administration. Extracted tumor specimen volume and weights were collected.

Statistics. 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 since 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 CCA 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 CCA cell line, followed by mass spectroscopy, we noted an association of tyrosine-protein phosphatase non-receptor types 1, 11, 22, 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 non-receptor 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, *PTPRK*) and then confirming levels by immunoblot in the CCA cell lines HuCCT1 and KMCH (Fig 1a-b). These two CCA cell lines were utilized as they have distinct pYAP^{Y357} status and YAP cellular compartmentation (nucleus vs. cytoplasm); with HuCCT1 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-c). In the CCA 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* gene) and pYAP^{Y357}; suggesting that these phosphatases may be in fact regulators of phosphorylation status in CCA (Fig 1a-b). Furthermore, these data indicated that the HuCCT1 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 HuCCT1 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 CCA cell lines. We next evaluated publically available RNA sequencing databases and found that *PTPN11* (SHP2) expression specifically was lower in cholangiocarcinoma specimens as compared to normal liver, further supporting a potential role for decreased SHP2 levels in cholangiocarcinoma biology (Fig. 1d). Indeed, in The Cancer Genome Atlas (TCGA)-CHOL dataset, five of the included CCA samples have a deletion in *PTPN11* (SHP2) and gene expression is correlated with copy number (p=0.003). While SHP2 has been demonstrated to be an oncogene based on its role in RAS signal transduction in multiple tumor types, notably it has previously been demonstrated as a tumor suppressor in hepato-carcinogenesis, and based on this we further explored the possibility that SHP2 phosphatase activity could directly regulate YAP activity, thus behaving as a tumor suppressor in CCA [39-42].

SHP2 can regulate YAP activity and subcellular localization in CCA. As an initial step we confirmed the findings of our immunoprecipitation-mass spectroscopy screen by performing co-immunoprecipitation experiments which demonstrated that SHP2 directly interacts with YAP in CCA 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 CCA utilizing a pharmacologic approach with attention to the effects of SHP2 inhibition on YAP phosphorylation status, subcellular localization, and transcriptional activity. In both HuCCT1 and KMCH cells, exposure to the SHP2 inhibitor NSC87877 (NSC) 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). NSC has activity against SHP1 as well as SHP2, with SHP1 a known

negative regulator of SFK activity by limiting its autophosphorylation [44]. In order to evaluate the possibility that increased pYAP^{Y357} levels may be due to 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 (Supplemental Fig. 1a). Furthermore, we evaluated the levels of active SRC (pSRC^{Y416}) by immunoblot and did not observe an increase in pSRC levels following NSC exposure (Supplemental Fig. 1b). Taken together these data suggested 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 co-transcriptional 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 (Supplemental Fig. 1c), 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 NSC, 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, co-transcriptional activity was also increased. Evaluation of YAP-TEAD transcription broadly utilizing a luciferase reporter construct demonstrated significant increases after both NSC and SHP099 exposure and specific expression of the YAP target genes *CTGF* and *CYR61* were increased in both HuCCT1 and KMCH cells (Figs. 2d-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 to the HuCCT1 cells, even though significant increases in YAP target gene levels were also noted in HUCCT1 cells. We next explored whether SHP2 inhibition by NSC may also shift YAP from a cytoplasmic to nuclear compartment in a non-CCA 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 NSC shifted YAP to the nuclear compartment (Supplemental Fig. 2a). These findings supported a role for SHP2 regulating YAP tyrosine phosphorylation, subcellular compartmentalization and co-transcriptional 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 (Supplemental Fig. 3a). 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 and Supplemental Fig. 3b). The increase in pYAP^{Y357} levels was associated with a shift of YAP to the nuclear compartment and increased YAP co-transcriptional activity as measured by expression of cognate YAP target genes, similar to pharmacologic SHP2 inhibition (Fig. 3b-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 CCA.

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 HuCCT1 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-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} levels, YAP baseline localization, or YAP co-transcriptional activity (Fig. 4a-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 pYAP357 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 CCA.

Tyrosine phosphatase activity is a critical determinant of CCA proliferation and in vitro sensitivity to standard of care chemotherapy. Given the increases in YAP tyrosine phosphorylation and transcriptional co-activity observed with SHP2 downregulation, we examined the effect of SHP2 modulation on cellular proliferation. In both KMCH and HuCCT1 cells, incubation with NSC increased cellular proliferation compared to controls (Fig. 5a). We

next examined sgSHP2-KMCH cells compared to 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 HuCCT1 cells, enforced expression of wildtype 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 CCA cell lines to gemcitabine and cisplatin, the current standard of care combinatorial therapy for CCA. 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 to 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). In order 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 pro-survival 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 NSC 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 MCL-1 in cell lines that demonstrate high pYAP^{Y357} and concomitant resistance to gemcitabine and cisplatin therapy may restore chemosensitivity. Indeed, both HuCCT1 and sgSHP2-KMCH cells treated with the MCL1 inhibitor S63845 (S6) demonstrated sensitivity to gemcitabine and cisplatin while S6 alone had no effect (Fig. 5h). We then asked whether or not sensitivity to gemcitabine and cisplatin could similarly be restored in the HuCCT1 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 S6 sensitized the HuCCT1 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 CCA cells to standard of care chemotherapy via an MCL1-dependent mechanism.

Deletion of SHP2 reduces in vivo sensitivity of CCA xenografts to standard of care chemotherapy, which can be overcome by the addition of MCL1 inhibition. Based on the findings in the *in vitro* experiments we explored the effects of phosphatase deletion on the sensitivity of CCA 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 to 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-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). These observations were congruent with our *in vitro* findings, and further supported a role for SHP2 in modulating the sensitivity of CCA 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: (1) the tyrosine phosphatase SHP2 can regulate tyrosine phosphorylation of YAP, (2) phosphatase activity specifically is required for regulation of YAP, and (3) 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 CCA. Tsutsumi et. al 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 co-immunoprecipitation experiments, and shRNA mediated knockdown of SHP2 in these cells did not appear to effect YAP localization. These findings lead the investigators to conclude that SHP2 was not regulating YAP/TAZ subcellular localization in this model, but that 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 CCA 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 CCA 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 et. al 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 et. al we saw a decrease in nuclear localized YAP with enforced over expression 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 reconstitution experiments; re-expressing wild type SHP2 in CCA 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 CCA. We did not specifically note PTPN14 as a YAP associated phosphatase in evaluation of our CCA 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 CCA 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 hepatocellular carcinoma 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 anti-cancer therapeutics [47]. Our observations would suggest that this may be counterproductive in some tumor types, such as CCA, 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 hyper-activated 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 CCA 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 CCA, 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. Additionally, other than genetic alterations, 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 CCA 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 CCA and may select patients who will benefit from MCL1 directed combinatorial therapy.

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FIGURE LEGENDS

Figure 1. Tyrosine phosphatase levels are altered in CCA and have an inverse relationship to YAP phosphorylation.

(A) mRNA expression of YAP associated tyrosine phosphatases in HuCCT-1 and KMCH cell lines. *, $p < 0.05$. (B) Cell lysates from the HuCCT-1, and KMCH cell lines were subjected to immunoblot 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 40x. Scale bar = 10 microns. (D) *PTPN11* (SHP2) differential expression analysis of public TCGA CCA samples compared to public GTEx normal liver samples. **, $p < 0.01$.

Figure 2. SHP2 can regulate YAP activity and subcellular localization in CCA.

(A) 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. (B) Cell lysates from the HuCCT-1, and KMCH cell lines treated with NSC87877 (NSC, 10 μ M, 3 hours) were subjected to immunoblot 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 +/- NSC (10 μ M, 3 hours) with quantification of YAP localization. Original magnification 40x. Scale bar = 10 microns. (D) mRNA expression of CTGF and CYR61 in HuCCT-1 and KMCH +/- tyrosine phosphatase inhibitor NSC (10 μ M, 3 hours). Fold change relative to vehicle. *, $p < 0.05$. (E) Global TEAD transcriptional activity in HuCCT1 and KMCH cells using a luciferase-based TEAD reporter. Cells were treated with NSC or SHP099 at 10 μ M 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 for pERK, pYAP^{Y357}, pYAP^{S127} and SHP2. Total YAP and actin

were used as a loading control. (B) KMCH cell lines transfected with a doxycycline 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 40x. Scale bar = 10 microns. (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 non-induced control. *, $p < 0.05$.

Figure 4. The Phosphatase Activity of SHP2 is required for YAP regulation.

(A) Cell lysates from the HuCCT-1 cell line were transfected with WT SHP2 or C459S SHP2 (phosphate dead construct) and subjected to immunoblot for pYAP^{Y357} and SHP2. Total YAP and actin were used as loading controls. (B) HuCCT-1, HuCCT-1 + WT-SHP2, and HuCCT-1 + C4592-SHP2 underwent immunofluorescence for YAP and representative images are displayed with quantification of YAP localization. Original magnification 40x. Scale bar = 10 microns. (C) mRNA expression of CTGF and CYR61 in HuCCT-1, HuCCT-1 + WT-SHP2, and HuCCT-1 + C4592-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 µg/mL) for 48 hours. The sgSHP2-KMCH cell lines were then washed and transfected with lenti virus over expressing WTSHP2 or C459S SHP2 constructs for 48 hours. Cell lysates were subjected to immunoblot 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 40x. Scale bar = 10 microns. (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 CCA proliferation and in vitro sensitivity to standard of care chemotherapy.

(A) HuCCT-1, and KMCH cell lines +/- NSC (10 μ M, 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 zero 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 μ g/mL) for 48 hours. 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 zero 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 zero 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 nM, 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 (100 nM, 72 hours). ***, $p < 0.001$. (F) Cell lysates from the KMCH cell lines treated with NSC (10 μ M, 3 hours) or transfected with empty and SHP2 targeting CRISPR sgRNA were subjected to immunoblot for MCL-1. Actin was used as a loading control. . (G) Cell lysates from the KMCH cell line treated with Verteporfin (10 μ M, 3 hour) followed by NSC (10 μ M, 3 hours) were subjected to immunoblot for MCL-1. sgEmpty-KMCH and sgSHP2-KMCH cells treated with Verteporfin (10 μ M, 3 hour) were subjected to immunoblot 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 +/- S63845 (100 nM and 5 μ M, 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 +/- S63845 (100 nM and 5 μ M, 72 hours). ***, $p < 0.001$.

Figure 6. Deletion of SHP2 reduces in vivo sensitivity of CCA 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 q3d x 2 weeks intraperitoneal injection of either combination gemcitabine (15mg/kg) and cisplatin (1mg/kg) or normal saline. **, $p < 0.01$. (D) Cell lysates from xenografts of sgEmpty-KMCH and sgSHP2-KMCH were subjected to immunoblot for pYAP^{Y357}. Total YAP and actin were used as a loading control. (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 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 q3d x 2 weeks treatment. Treatments included S6 (25 mg/kg), combination gemcitabine (15mg/kg) cisplatin (1mg/kg), combination gemcitabine (15mg/kg) cisplatin (1mg/kg) and S6 (25 mg/kg), or normal saline. *, $p < 0.05$, **, $p < 0.01$.

Figure 7. Summary of the Model. SHP2 modulates proliferation and chemoresistance by regulating the tyrosine phosphorylation (activation) of YAP.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. SHP1 does not appear to regulate pYAP^{Y357} levels.

(A) Cell lysates from the KMCH cell line +/- SHP099 (10 μ M, 3 hours) were subjected to immunoblot for phosphorylated pYAP^{Y357}. Total YAP and actin were used as loading controls. (B) Cell lysates from the KMCH cell line +/- NSC (10 μ M, 3 hours) were subjected to immunoblot for phosphorylated pSRC. Total SRC and GAPDH were used as loading controls. (C) Representative images of SHP2 immunofluorescence in KMCH. Original magnification 40x. Scale bar = 10 microns.

Supplemental Figure 2. SHP2 inhibition by NSC also shifts YAP from a cytoplasmic to nuclear compartment in a non-CCA cell line.

(A) PANC1 cell lines incubated with NSC (10 μ M, 3hours). Immunofluorescence for YAP was performed and representative images with quantification of YAP localization are displayed. Original magnification 40x. Scale bar = 10 microns.

Supplemental Figure 3. SHP2 mediated deletion following incubation with doxycycline.

(A) The KMCH cell line transfected with a doxycycline inducible CRISPR/Cas9 construct targeting SHP2 were incubated with doxycycline (7.5 μ g/mL) for the noted time. Cell lysates were subjected to immunoblot for SHP2. Actin was used as a loading control. (B) KMCH cells incubated with NSC (10 μ M, 3hours). Cell lysates were subjected to immunoblot for pERK and ERK. Actin was used as a loading control.

Supplemental Table 1. RT-PCR primers

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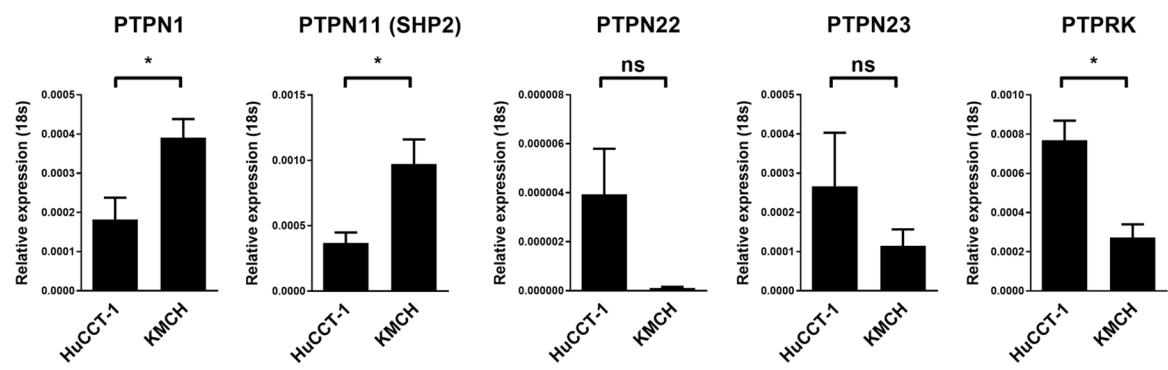
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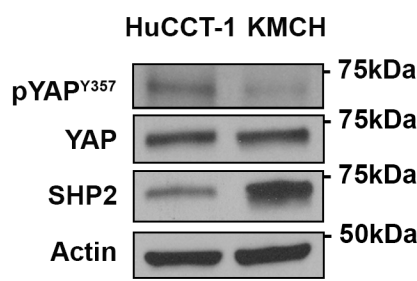
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FIGURE 1.

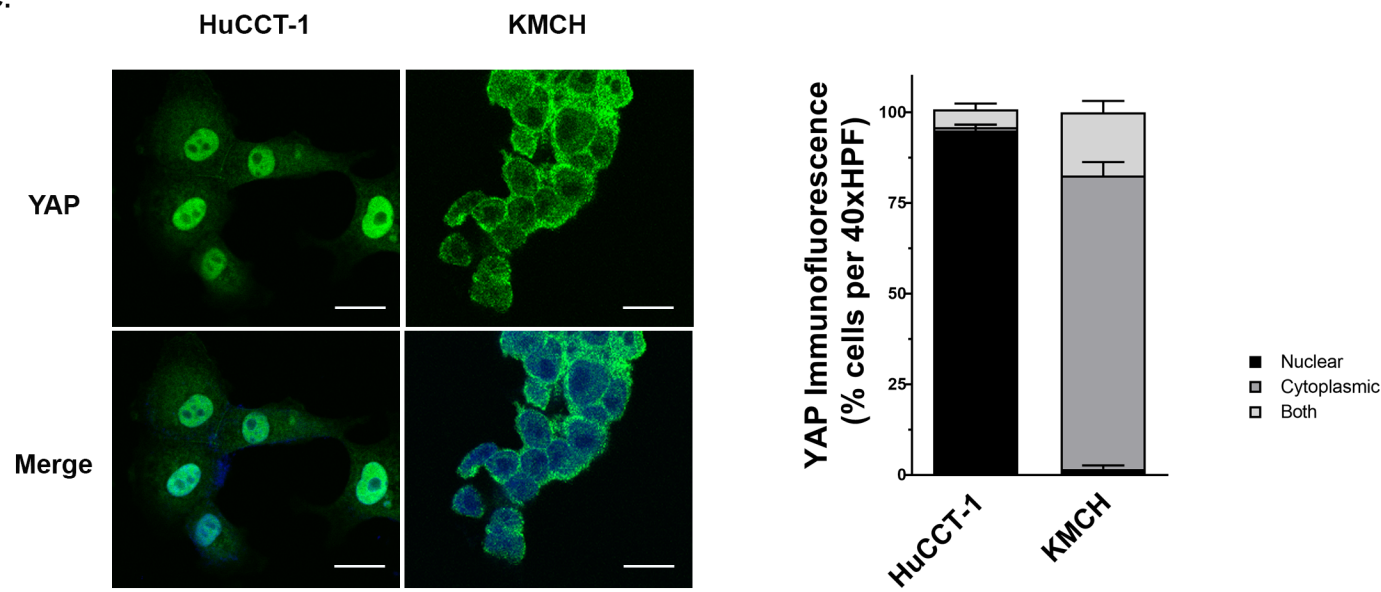
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B.



C.



D.

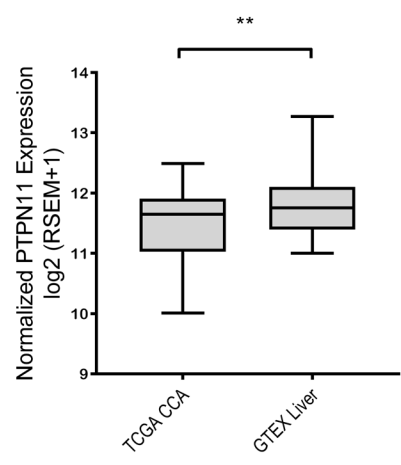


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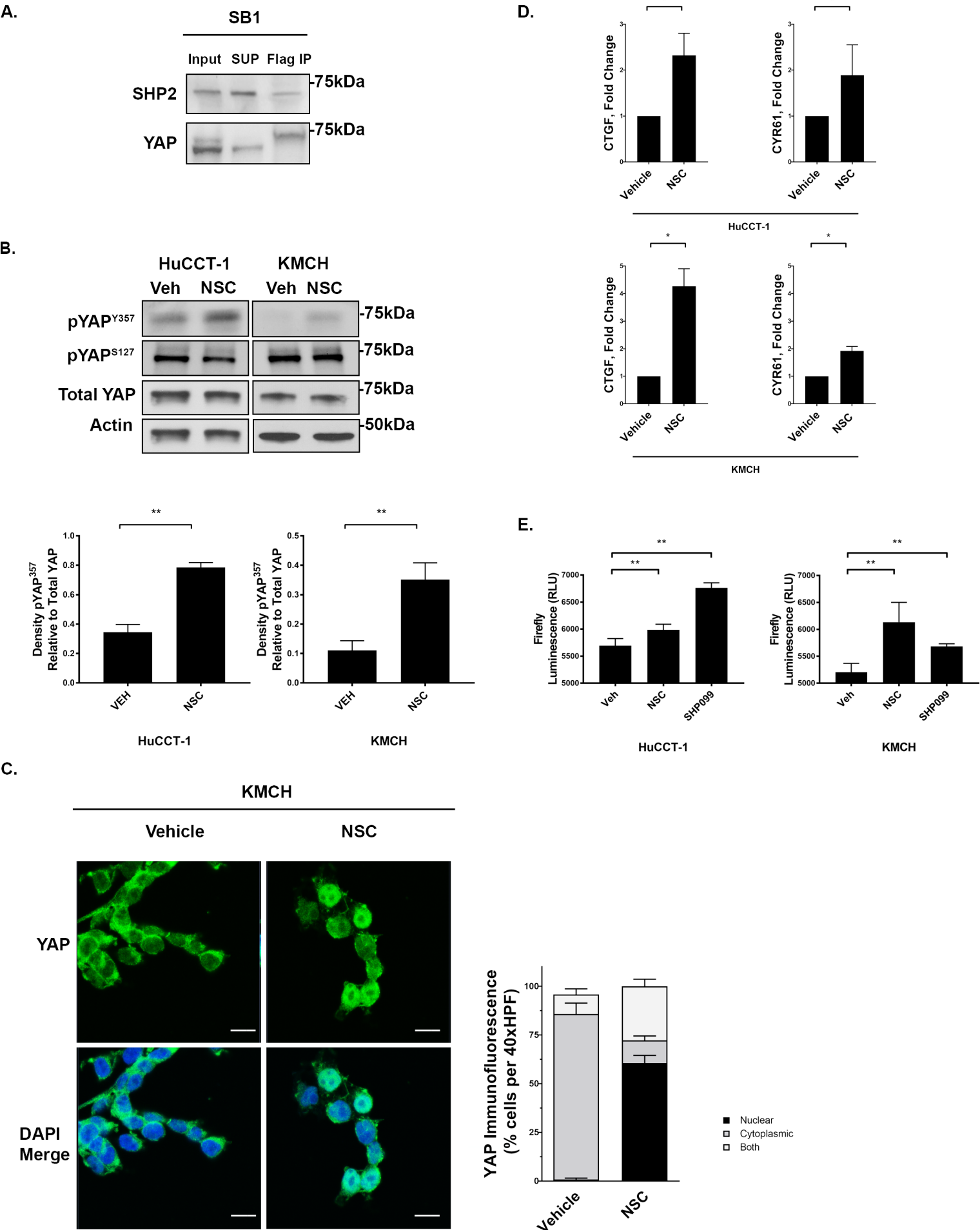
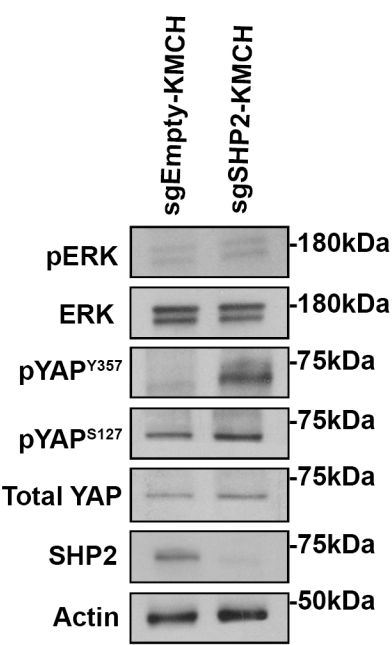
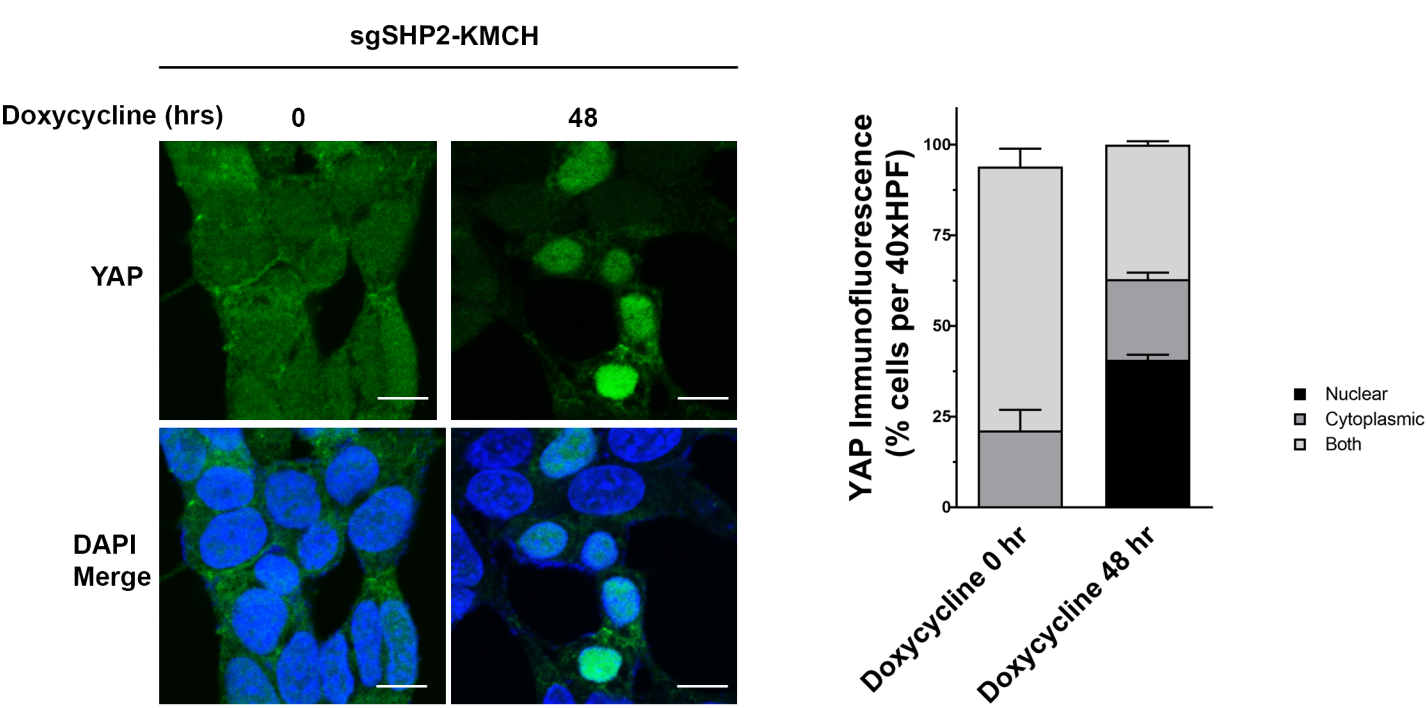


FIGURE 3.

A.



B.



C.

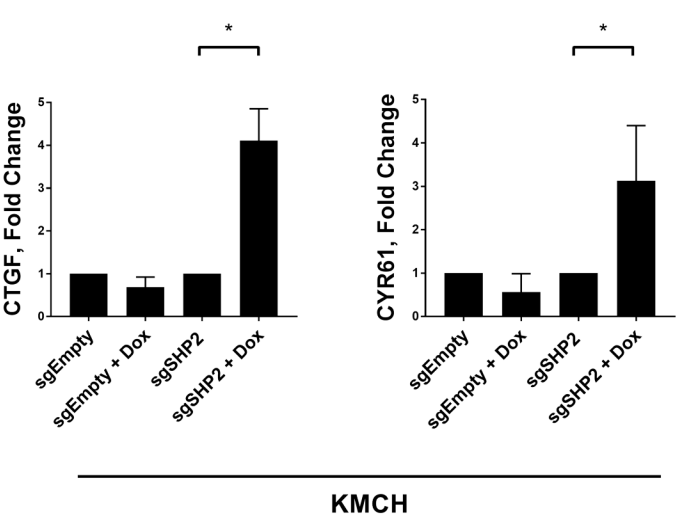
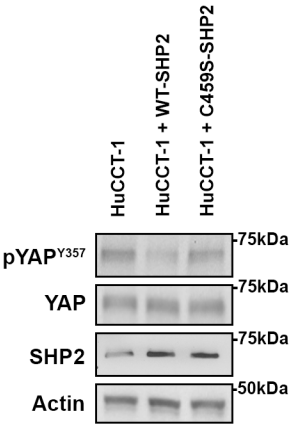
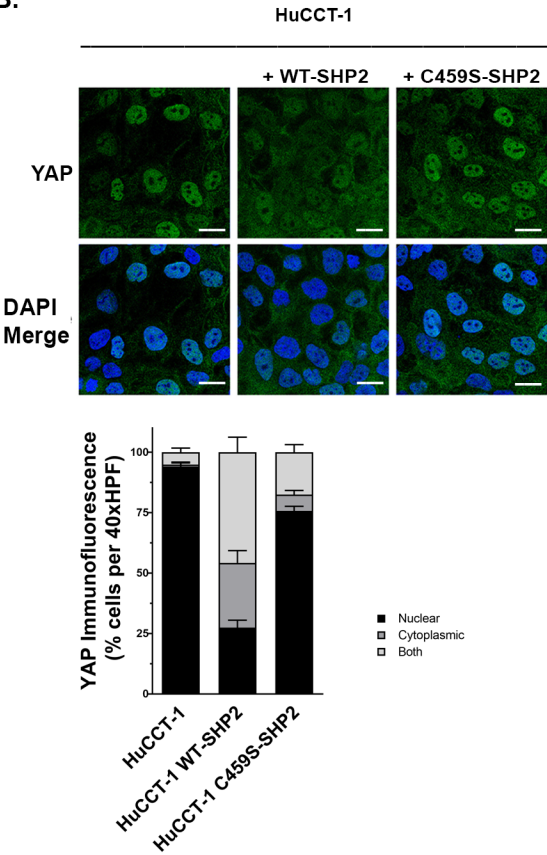


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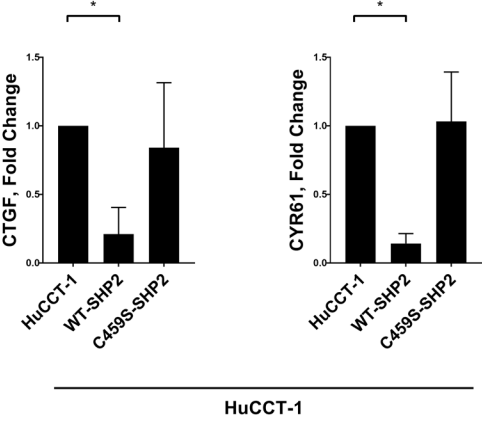
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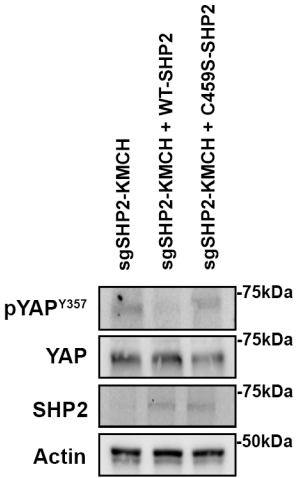
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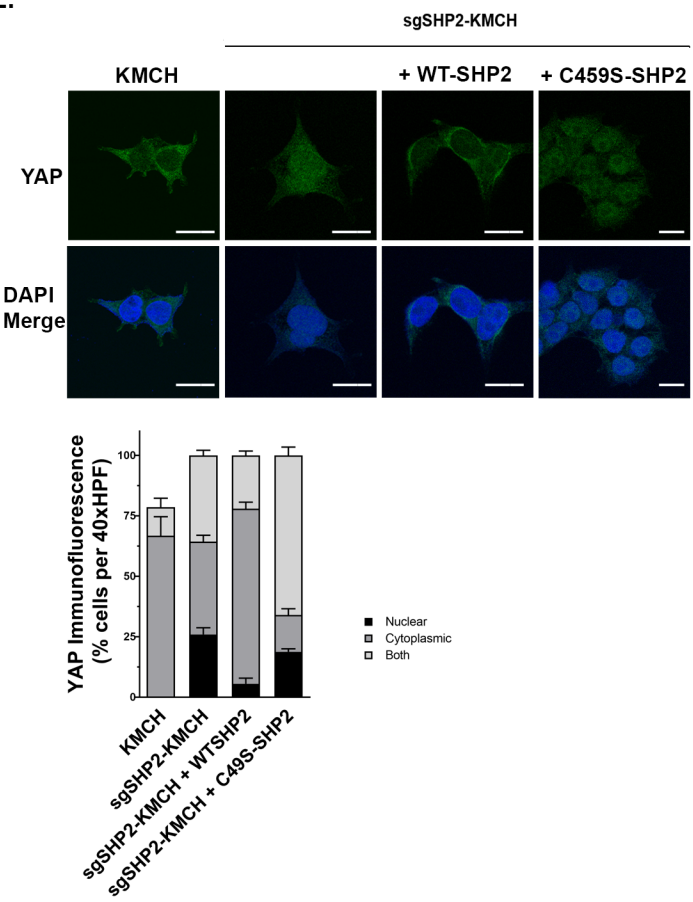
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D.



E.



F.

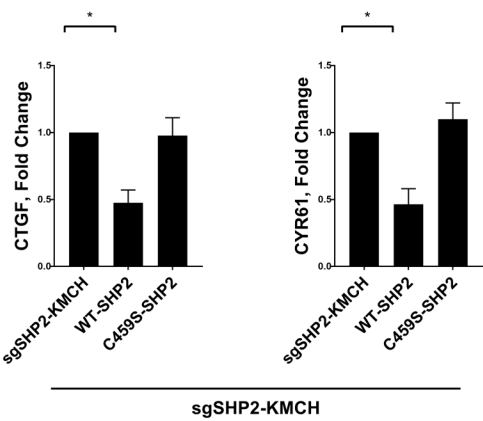


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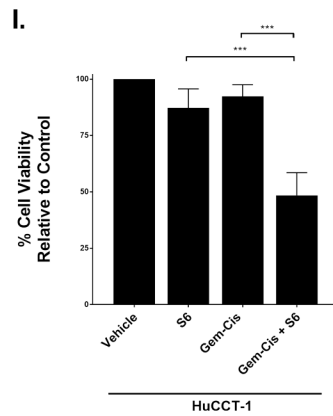
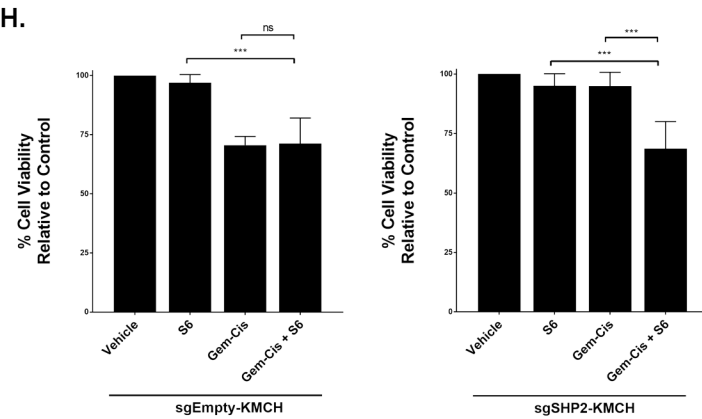
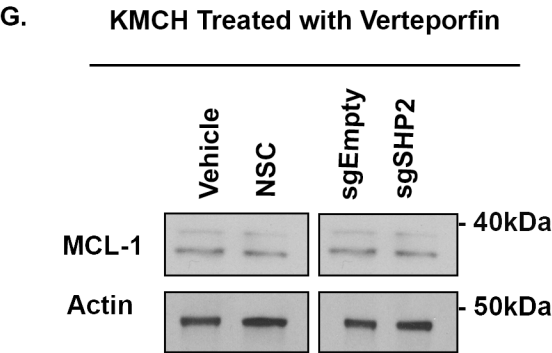
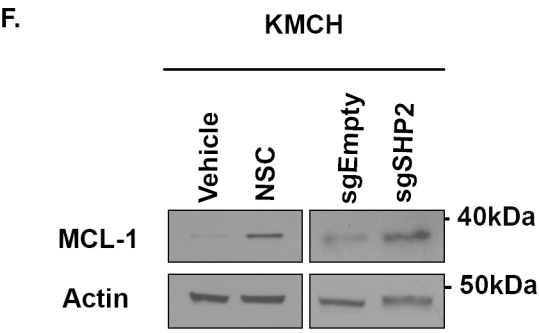
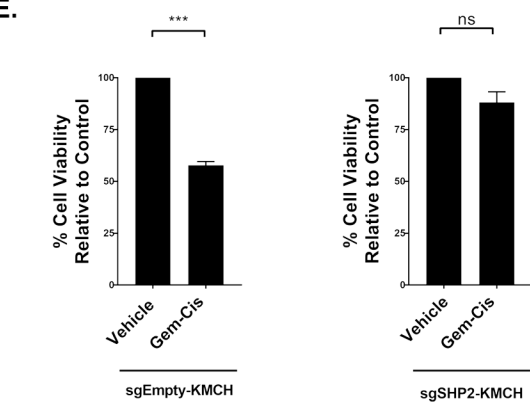
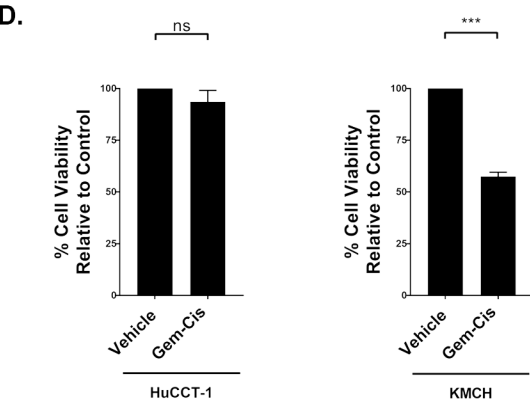
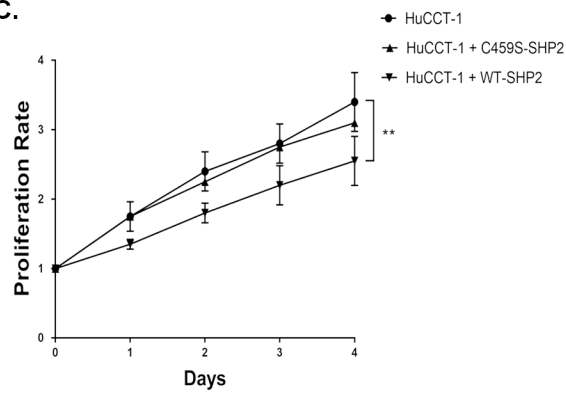
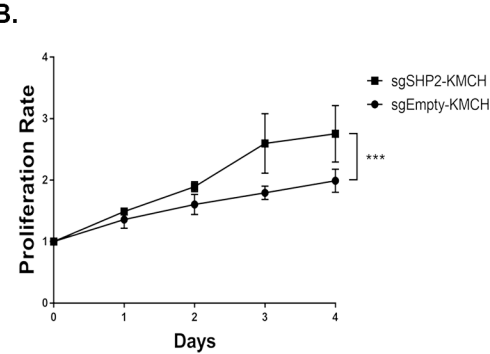
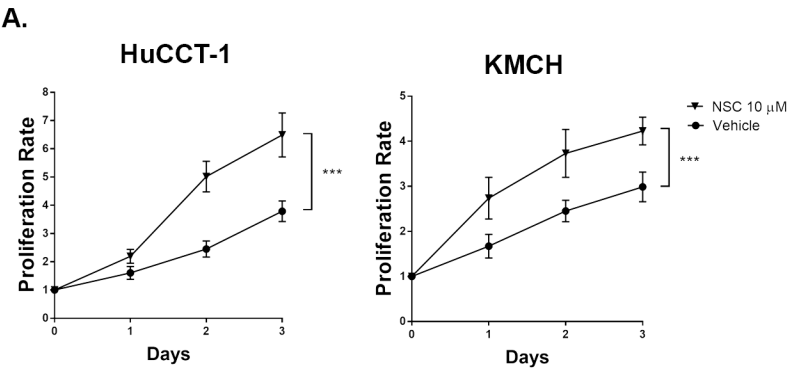


FIGURE 6.

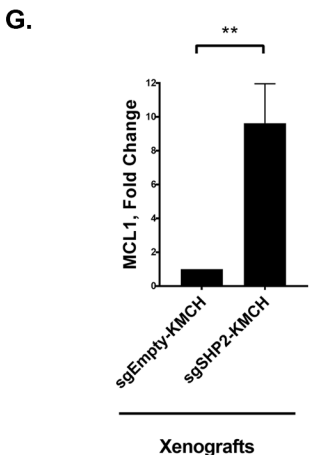
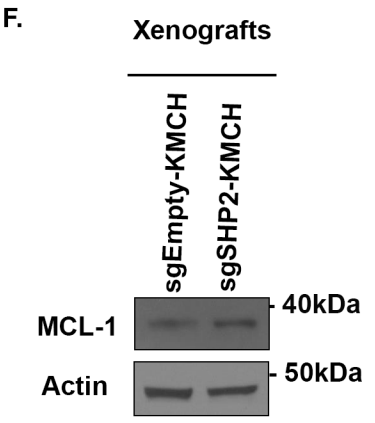
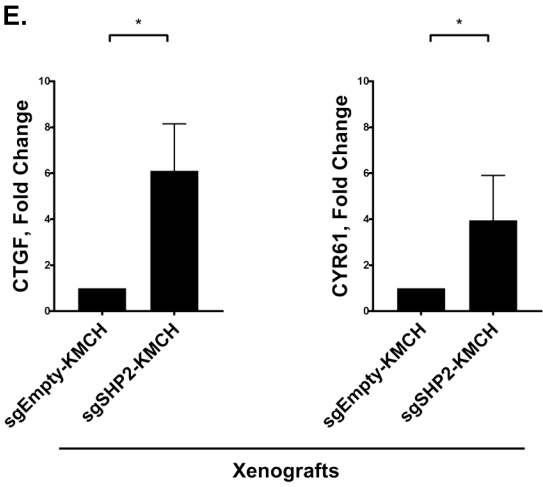
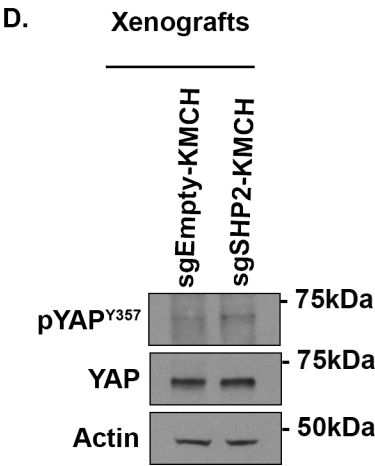
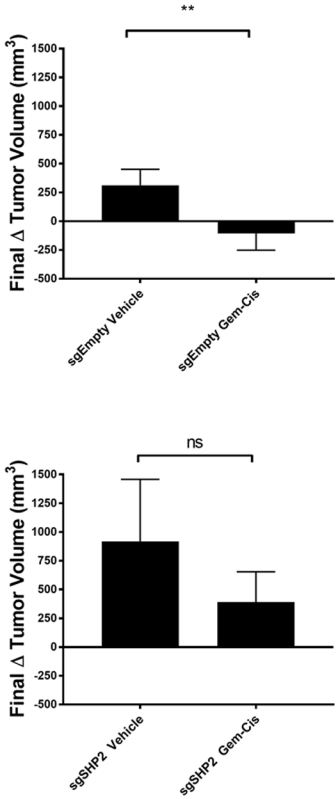
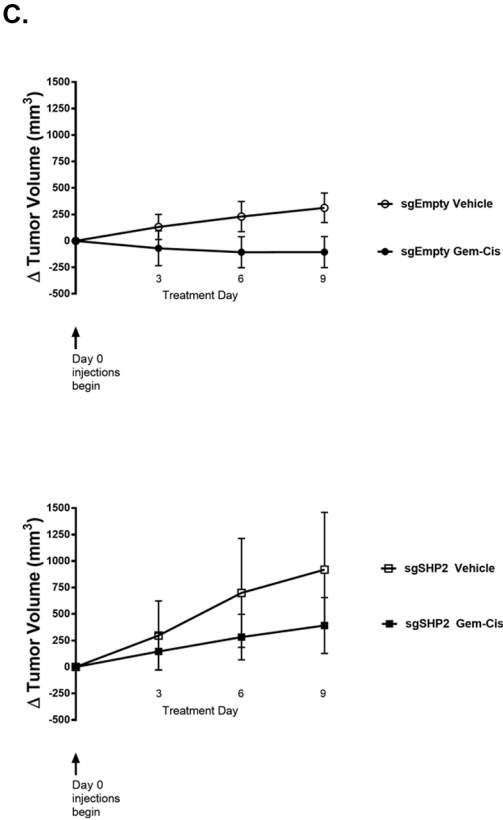
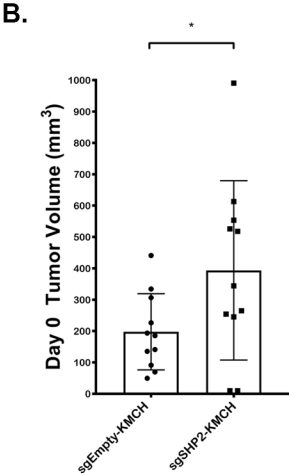
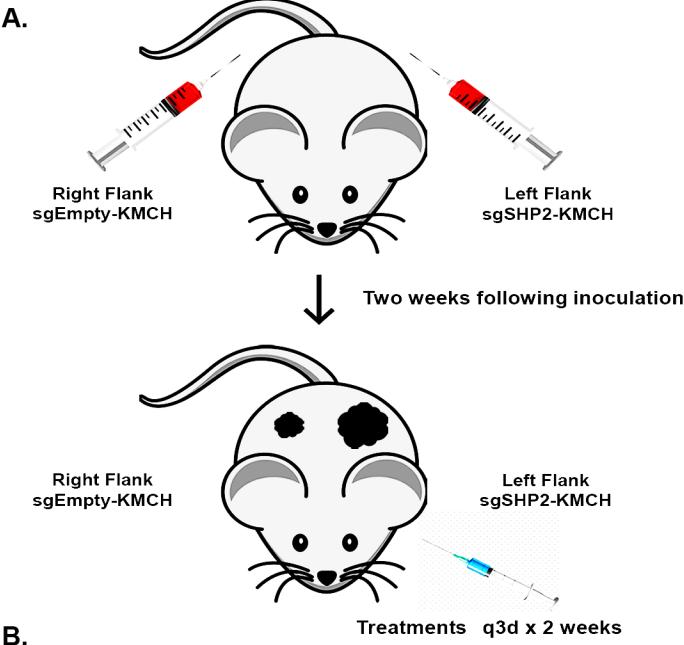


FIGURE 6.

H.

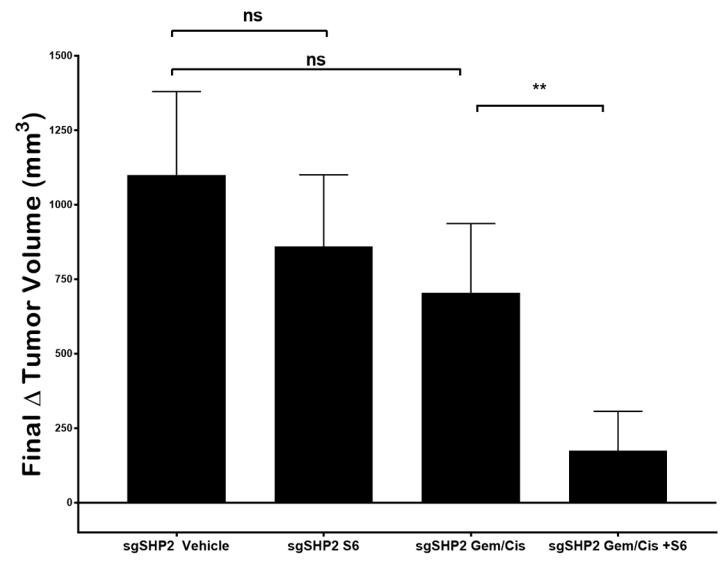
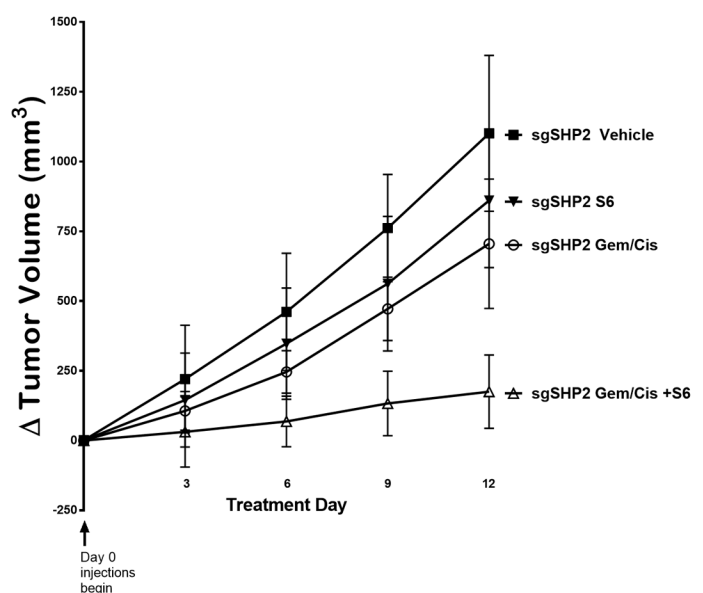
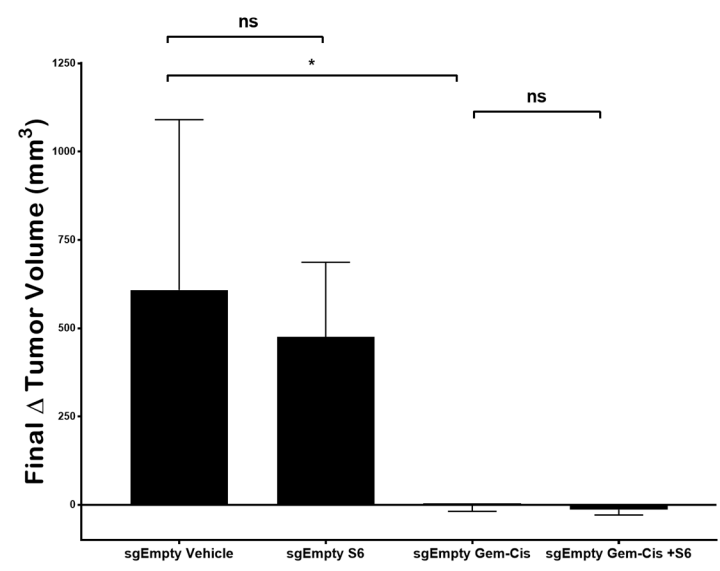
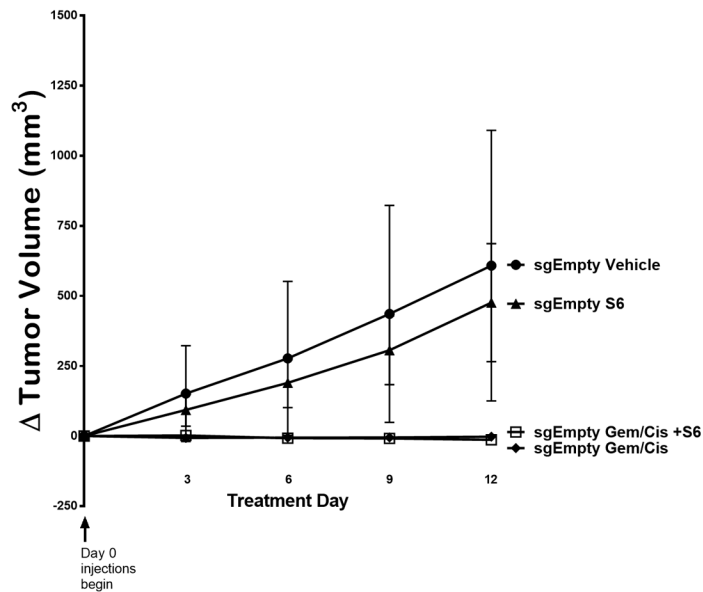
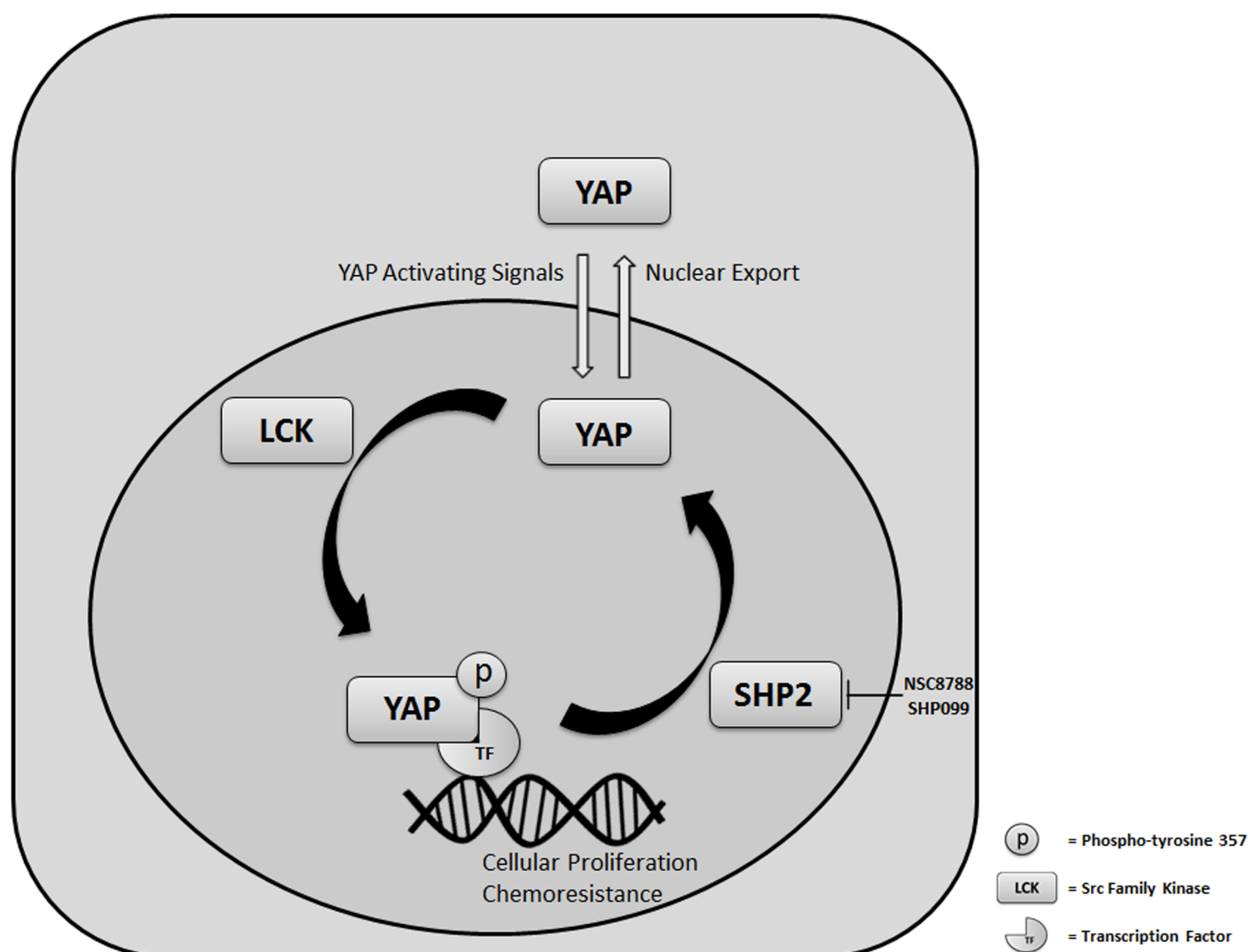
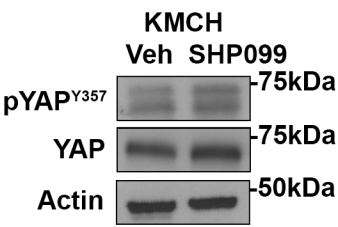


FIGURE 7.

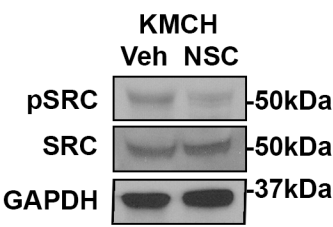


SUPPLEMENTAL FIGURE 1.

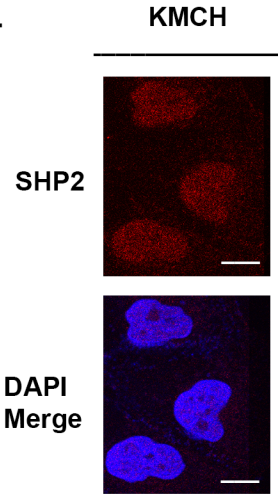
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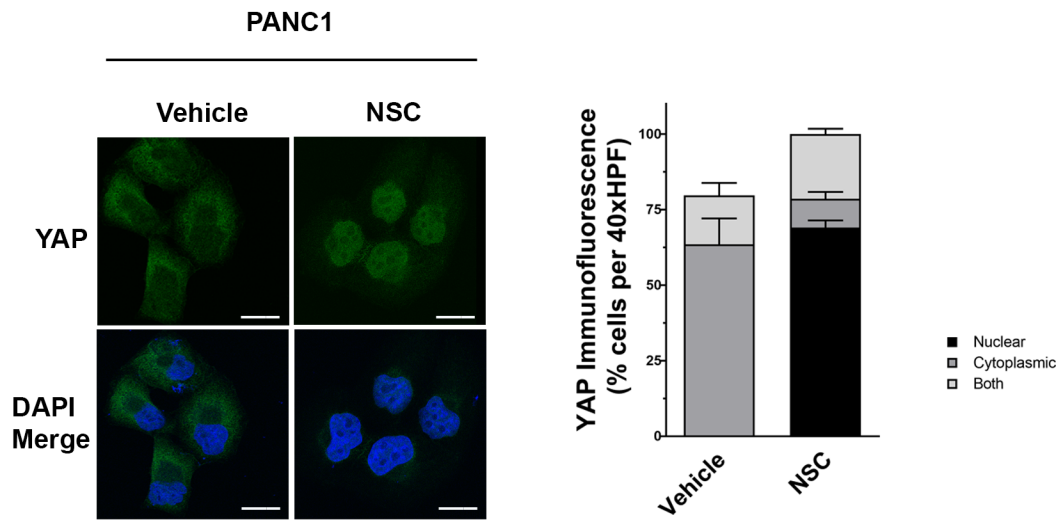


C.

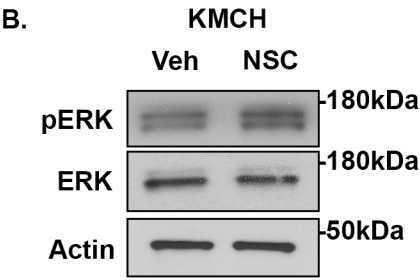
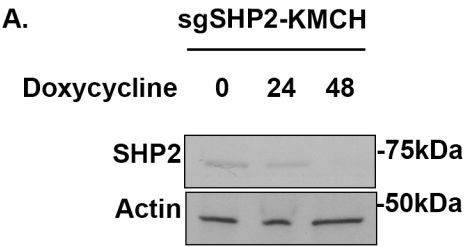


SUPPLEMENTAL FIGURE 2.

A.



SUPPLEMENTAL FIGURE 3.



SUPPLEMENTAL TABLE 1.

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
<i>Human</i>		
<i>CTGF</i>	CAGTGTCTGACTTCGACAACGC	CCATCGGCGTGTTTGGAGTA
<i>CYR61</i>	GAGTGGGTCTGTGACGAGGAT	GGTTGTATAGGATGCGAGGCT
<i>MCL-1</i>	GAGGGCGACTTTTGGCTAC	GTACCCGTCCAGCTCCTCTT
<i>Mouse</i>		
<i>Ctgf</i>	CACTCTGCCAGTGGAGTTCA	AAGATGTCATTGTCCCCAGG
<i>Cyr61</i>	GAACCGCGAGTTCTTTTCAA	AGGACGCACTTCACAGATCC
<i>Housekeeping</i>		
<i>18S</i>	CGCTTCCTTACCTGGTTGAT	GAGCGACCAAAGGAACCATA