GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Phosphorylated Ezrin (Thr567) Regulates Hippo Pathway and Yes-Associated Protein (Yap) in Liver

Yuhua Xue, Bharat Bhushan, Wendy M. Mars, William Bowen, Junyan Tao, Anne Orr, John Stoops, Yanping Yu, Jianhua Luo, Andrew W. Duncan, and George K. Michalopoulos

From the Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania

Accepted for publication
March 26, 2020.

Address correspondence to
George K. Michalopoulos,
M.D., Ph.D., 200 Lothrop St.,
BST S410, Pittsburgh,
PA 15261. E-mail:
michalopoulosgk@upmc.edu.

The activation of CD81 [the portal of entry of hepatitis C virus (HCV)] by agonistic antibody results in phosphorylation of Ezrin via Syk kinase and is associated with inactivation of the Hippo pathway and increase in yes-associated protein (Yap1). The opposite occurs when glypican-3 or E2 protein of HCV binds to CD81. Hepatocyte-specific glypican-3 transgenic mice have decreased levels of phosphorylated (p)-Ezrin (Thr567) and Yap, increased Hippo activity, and suppressed liver regeneration. The role of Ezrin in these processes has been speculated, but not proved. We show that Ezrin has a direct role in the regulation of Hippo pathway and Yap. Forced expression of plasmids expressing mutant Ezrin (T567D) that mimics p-Ezrin (Thr567) suppressed Hippo activity and activated Yap signaling in hepatocytes *in vivo* and enhanced activation of pathways of β -catenin and leucine rich repeat containing G protein-coupled receptor 4 (LGR4) and LGR5 receptors. Hepatoma cell lines JM1 and JM2 have decreased CD81 expression and Hippo activity and up-regulated p-Ezrin (T567). NSC668394, a p-Ezrin (Thr567) antagonist, significantly decreased hepatoma cell proliferation. We additionally show that p-Ezrin (T567) is controlled by epidermal growth factor receptor and MET. Ezrin phosphorylation, mediated by CD81-associated Syk kinase, is directly involved in regulation of Hippo pathway, Yap levels, and growth of normal and neoplastic hepatocytes. The finding has mechanistic and potentially therapeutic applications in hepatocyte growth biology, hepatocellular carcinoma, and HCV pathogenesis. (*Am J Pathol* 2020, 190: 1427–1437; <https://doi.org/10.1016/j.ajpath.2020.03.014>)

Yes-associated protein (Yap1) plays a pivotal role in liver physiology, including maintaining liver size,¹ deciding cell fate,^{2–4} mediating liver repair after injury,^{5,6} and regulating energy utilization.^{7,8} High Yap expression has also been reported in 50% hepatocellular carcinomas (HCCs).⁹ Understanding of the underlying mechanism by which neoplastic cells bypass normal signals and maintain higher levels of Yap is crucial for developing new HCC therapies¹⁰ involving Yap and Hippo pathways. In normal cells, Hippo pathway and Yap levels and phosphorylation are heavily regulated, among others, by physical force¹¹ transmitted dynamically by cytoskeleton proteins,¹² via modulated conformation of key proteins adjacent to plasma membrane. One such protein connecting plasma membrane to cytoskeleton is Ezrin, a member of the Ezrin-Radixin-Moesin

family. Members of the Ezrin-Radixin-Moesin family often compensate in physiological function for each other.^{13–16} Among them, Ezrin is expressed abundantly¹⁷ in HCC¹⁸ and other types of tumors,^{19–25} and has been shown to be interacting with epidermal growth factor receptor (EGFR)^{26,27} and c-Met.^{25,28} It is involved in mediating the malignant behavior in multiple cancers,^{29–32} and has been targeted as potential therapeutic target³³ in cancer progression^{27,34–37} and metastasis.^{38–40} Phosphorylation of Ezrin at conserved residue Thr657 in C-terminal disrupts dormant head-to-tail structure and allows Ezrin to bind with

Supported by the Menten Endowment Foundation of the University of Pittsburgh and the Cleveland Foundation.

Disclosures: None declared.

actin,^{17,41} thus linking actin with membrane receptors.⁴² Previous studies have shown that phosphorylated (p)-Ezrin (Thr567) specifically correlates with Yap expression.⁴³ p-Ezrin (Thr567) assumes active conformation and is distributed in patterns with time and spatial constraints.^{41,44} Other studies have shown that p-Ezrin (Thr567) inhibitor can reverse malignant behavior,⁴⁵ however, the underlying molecular mechanism is not fully understood.

Herein, we investigated whether persistent p-Ezrin (Thr567) expression in hepatocytes would affect components of the Hippo pathway and lead to increased Yap activity. To perform these studies, we employed direct transfection of mutant Ezrin in which threonine 567 is replaced by aspartic acid. This mutant Ezrin has been shown to mimic p-Ezrin (T567).¹⁷ Our study demonstrated that forced expression of mutant Ezrin (T567D) induces higher Yap activity, and that the physiological p-Ezrin (Thr567)–Yap axis is modulated not only by glypican-3 (GPC3)/CD81/Syk kinase⁴³ but also EGFR and MET receptor tyrosine kinases, which control hepatocyte proliferation.⁴⁶

Materials and Methods

Animal Protocols and Handling

Details are provided in our previous studies.⁴³ All animals studies were performed under University of Pittsburgh (Pittsburgh, PA) Institutional Animal Care and Use Committee protocol 14053713. Animals were cared for any pain and suffering and sacrificed under NIH and Association for Assessment and Accreditation of Laboratory Animal Care International guidelines.

Details of generation of the MET^{ff/ff};TamCre^{+/+} mice, inhibition of EGFR signaling, and studies for their effects on liver regeneration after partial hepatectomy have been previously described.^{46,47}

Constructs and Reagents

Wild-type (WT; pHJ421; plasmid number 20680) and constitutive activated Ezrin (EzrinT567D; pHJ423; plasmid number 20680) were purchased from Addgene (Cambridge, MA), and cDNA sequences were subcloned into a PT3EF5 α vector. WT Ezrin, activated mutant (EzrinT567D), and pCMV-SB constructs have been previously described.^{48,49}

Hydrodynamic Tail Vein Injection

Wild-type FVB/N mice (aged 6 to 8 weeks; Charles River, Wilmington, MA) were used. For hydrodynamic tail vein injection, empty PT3EF5 α vector, WT Ezrin or EzrinT567D (10 μ g), and pCMV/SB (0.8 μ g) were diluted into 2 mL 0.9% NaCl solution, sterile filtered with 0.2- μ m filters, and then injected into mouse lateral tail vein within 5 to 7 seconds, as described.⁴⁹ Mice were sacrificed at the time ranging from 1 to 12 weeks after injection. Parts of livers

were used for histopathologic analysis, and parts were fresh frozen for protein isolation. Green fluorescent protein (GFP) was used as lineage tracing marker to detect plasmids recipient cells.

Immunohistochemistry

The immunohistochemistry protocols were described previously.⁵⁰ Rabbit anti-human Ezrin monoclonal antibody, rabbit anti-human GFP antibody (Cell Signal Tech, Danvers, MA), and rabbit anti-human proliferating cell nuclear antigen (PCNA) antibody (Abcam, Cambridge, UK) were used to detect the expression of Ezrin, GFP, and PCNA, respectively.

Hepatoma Cell Line Cultures, Transfection, and Detection of Proteins by Western Blot Analysis

Technical details regarding maintenance of cell lines HepaRG, JM1, and JM2 and conditions of transfection, isolation of proteins, and Western blot analysis have been described previously.⁴³ The following primary antibodies were used: anti-Yap, p-Yap, Mst1, p-Mst1, β -actin, Lamin B1, Ezrin, or anti-p-Ezrin (Thr567; Cell Signal Tech), and anti-CD81 (GeneTex, Irvine, CA).

Isolation of GFP⁺ Cells Using an FACS Sorter

Isolation of primary hepatocytes from liver was performed by a well-established two-step collagenase digestion.⁵¹ All fluorescence-activated cell sorting (FACS) analyses were performed with an FACS Aria II-SORP (BD Biosciences, San Jose, CA) equipped with a 130- μ m nozzle and running DiVa software version 8.0.2 (BD Biosciences). FACS plots were generated with FlowJo software version 9.9.6 (TreeStar, Ashland, OR). Hepatocytes were separated on the basis of size (forward scatter) and granularity (side scatter), and live cells were selected by propidium iodide exclusion; cell doublets and clumps were excluded. GFP⁺ and GFP[−] hepatocytes were sorted directly into Trizol (Invitrogen, Carlsbad, CA) or lysis buffer.

RNA Isolation and Microarray Expression Profiling

Total RNA was extracted from sorted cells using Trizol and RNeasy mini-kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions for small sample quantities. For comparison between primary GFP⁺ and GFP[−] cells, the Ovation Pico WTA System V2 (NuGEN, Redwood, CA; P/N 3302-60) was used in the library preparation. Briefly, total RNAs were extracted from the sorted cells with Trizol and purified through RNeasy column (Qiagen); details are as in the manufacturer's protocol. With Ovation Pico WTA System V2, total RNA, 50 ng per sample with primer and enzyme, was incubated in the thermocycler at 25°C for 30 minutes, then 42°C for 15 minutes and 70°C for 15 minutes

to synthesize the first strand cDNA. Then, the sample with additional reagents for second strand cDNA was heated for one cycle of 25°C, 10 minutes, 50°C, 30 minutes, and 80°C, 20 minutes. Purified, the sample was heated in a mixture of single primer isothermal amplification and enzyme for one cycle of 4°C, 1 minute, 47°C, 75 minutes, and 95°C, 5 minutes. The single primer isothermal amplification cDNA was then fragmented and labeled with biotin in the reaction using ENCORE Biotin Module kit (NuGEN; P/N 4200-60). Biotinylated cDNA (5 µg) in the cocktail was hybridized to the Affymetrix GeneChip mouse genome 430 2.0 array (Affymetrix, Santa Clara, CA) at 45°C, rotated for 16 hours. The processes of washing and streptavidin phycoerythrin staining were accomplished on the Affymetrix Fluidic Station 450. And the arrays were scanned in Affymetrix scanner 7G, and signals were acquired with GCOS software version 1.4 (Affymetrix). The data obtained from the arrays were submitted to the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE145572).

Statistical Analysis

Details on statistical analyses have been previously described.⁴³ Briefly, tissue array data were analyzed using GraphPad Prism5 software (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

EzrinT567D Enhances Yap Activity and Cell Proliferation in HepaRG Cells

To investigate the effect of p-Ezrin (Thr567) on Hippo pathway and Yap regulation, three plasmids were first constructed, including PT3EF5α empty vector control, WT Ezrin, and mutant EzrinT567D. As an immortalized human cell line, HepaRG cells (Thermo Fisher Scientific,

Pittsburgh, PA) keep many of the features of normal human hepatocytes as well as some properties of cholangiocytes.^{52,53} This cell line was used to study the effect of forced EzrinT567D expression on Hippo pathway and Yap. After 24 hours of transient transfection, a significant increase of exogenous Ezrin expression (Figure 1A) was observed. Endogenous Ezrin was also present and its expression was not affected after injection of the plasmids. The relative proportions of Yap and p-Yap as percentage of total Yap protein (Figure 1C) were determined from the Yap and p-Yap expression data of Figure 1B. The data show that mutant Ezrin led to a strong decrease of p-Yap as a percentage of the total Yap protein, indicating suppression of the Hippo pathway. This is consistent with the finding that administration of the EzrinT567D vector caused a decrease in cleaved and activated p-MST1 and p-MOB1 (Figure 1A). HepaRG cells transfected with EzrinT567D also entered into enhanced proliferation compared with control WT Ezrin and simple GFP plasmids (Figure 1D).

Hydrodynamic Injection of EzrinT567D Plasmid Leads to Increased Yap and Cell Proliferation in Mouse Liver

The plasmids described above (PT3EF5α empty vector control, WT Ezrin, and mutant EzrinT567D) were each separately delivered by hydrodynamic injection to the mouse liver. Overexpression of any plasmid did not lead to any tumor formation in mice 3 months after injection. All three plasmids were equally effective in transfection of hepatocytes, and the overall percentage of hepatocytes receiving each plasmid did not exceed 15%. Immunoreactivity of GFP and Ezrin was detected in hepatocytes of both WT Ezrin and EzrinT567D 1 week after injection (Figure 2A). There was predominantly cell membrane immunoreactivity in EzrinT567D, whereas in hepatocytes receiving WT Ezrin, immunoreactivity was seen in whole cytoplasm. The overall percentage of PCNA-positive hepatocytes in random optical fields was assessed in each of

Figure 1 Overexpression of mutant Ezrin in human hepatocytic cell line (HepaRG) cells results in lower Hippo pathway activity and enhanced cell proliferation. **A** and **B**: HepaRG was analyzed for protein expression of Ezrin, phosphorylated (p)-MST1, and p-MOB1 (**A**) and Yap/Taz and p-Yap (**B**), after transfection for 48 hours by control (green fluorescent protein backbone), wild-type Ezrin, and mutant Ezrin plasmids. **C**: Quantification of expression of Yap and p-Yap from the data shown in **B**. **D**: The effects of transfection with wild-type and mutant Ezrin on cell proliferation of HepaRG cells [24 hours of transfection, followed by 24 hours of bromodeoxyuridine (BrdU) incorporation]. * $P < 0.05$.

Figure 2 Forced expression of wild-type (W) Ezrin and mutant (M) Ezrin in liver via hydrodynamic tail vein (HDTV) injection. All immunohistochemistry stains were performed in livers at 1 week after HDTV. **A:** Immunohistochemistry staining for green fluorescent protein (GFP) and Ezrin. **B:** Increased percentage of proliferating cell nuclear antigen (PCNA)—positive hepatocytes in normal livers following transfection with W and M Ezrin, indicated by histology and quantification in the adjacent bar graph. There is increase in PCNA-positive hepatocytes in transfection with mutant over wild-type Ezrin. **C** and **D:** Enhanced focal proliferation and formation of clonal hepatocyte clusters of mutant Ezrin transfected hepatocytes during liver regeneration after partial hepatectomy (for details, see [Results](#)). * $P < 0.05$. Original magnification: $\times 400$ (**A**); $\times 200$ (**B**); $\times 100$ (**C** and **D**). G, control normal liver, injected only with GFP containing plasmid.

the three groups ([Figure 2B](#)). There was small but statistically significant increase in percentage of PCNA-positive hepatocytes with transfection of the mutant Ezrin compared with the transfection with WT Ezrin. Either of these two groups, however, had significantly higher percentage of PCNA-positive hepatocytes compared with transfection with the control GFP plasmid. It was also examined if EzrinT567D positive cells have a proliferative advantage during liver regeneration after partial hepatectomy ([Figure 2, C and D](#)). The Ezrin-positive hepatocytes were counted in the different groups (control, WT Ezrin, and mutant Ezrin) before and 10 days after partial hepatectomy. Lobes of liver resected at the time of hepatectomy were used as the control, zero time point. Most of the Ezrin-positive hepatocytes at that time were present as single cells.

At 10 days after partial hepatectomy, these single cells expanded into small cell clusters. The distribution of the size (number of cells) for each of the Ezrin-positive clusters was assessed at day 10 after partial hepatectomy, and the results are shown in [Figure 2, C and D](#). In livers transfected with the mutant Ezrin, there were significantly higher numbers of hepatocytes in two, three, four, and five cell clusters compared with transfection with WT Ezrin.

Isolation of GFP⁺ Hepatocytes from Whole Hepatocyte Suspension 1 Week after EzrinT567D Hydrodynamic Vein Injection

FACS analysis was performed to enrich GFP⁺ cells, using primary hepatocytes from mice transfected (hydrodynamic

Figure 3 **A:** Fluorescence-activated cell sorting was used to separate green fluorescent protein—positive (GFP⁺) hepatocytes (successfully transfected cells) from GFP⁻ hepatocytes (non-transfected cells) at 1 week following hydrodynamic injection of plasmid containing GFP and mutated Ezrin (T567D). **B:** Gene expression microarray was used to assess gene expression in the two cell types. mRNA expression level between GFP⁺ (transfected mutant Ezrin) cells and GFP⁻ (non-transfected) cells. Yap downstream gene *Ctgf*, *Cyr61*, and *glypican-3* (*Gpc3*) expression values were assessed from the microarray. FSC, forward scatter.

injection) with the GFP mutant (T567D Ezrin plasmid). The GFP⁺ cell number was approximately 3.5% of hepatocytes (Figure 3A). To determine whether EzrinT567D efficiently modulated Yap signaling, GFP⁺ and GFP⁻ cells were sorted, total mRNA was harvested, and expression profiling analysis by microarray was performed. GFP⁺ cells demonstrated elevated expression of Yap transcriptional targets, such as connective tissue growth factor (*Ctgf*), cysteine rich angiogenic inducer 61 (*Cyr61*), and *Gpc3*, at mRNA level, indirectly demonstrating the increase in Yap protein that occurred as a result of transfection with the GFP-Ezrin (T567D) plasmid (Figure 3B). The material collected from the GFP⁺ hepatocytes was insufficient for performing Western blot analysis.

Global Analysis of Gene Expression Changes in Hepatocytes Associated with Uptake of the Mutant Ezrin (T567D)

To determine the downstream molecular events after EzrinT567D overexpression in hepatocytes, our microarray data was analyzed to investigate changes in the global gene expression profile. To filter out genes relevant to EzrinT567D overexpression, we specifically looked at genes that were significantly expressed and altered (either up-regulated or down-regulated at least 1.75-fold) in GFP⁺ cells compared with GFP⁻ cells. On the basis of these criteria, 1508 genes were found to be altered by EzrinT567D overexpression. Of these genes, 1019 were up-regulated and 489 were down-regulated (top 50 up-regulated and down-regulated genes) (Supplemental Tables S1 and S2). This specific set of genes was further analyzed using Ingenuity Pathway Analysis (IPA). The top activated and inhibited upstream regulating protein signals leading to the observed gene array changes are shown in Table 1. On the basis of the directionality and intensity of changes in downstream gene expression, Yap was predicted to be activated in GFP⁺ cells. In fact, upstream regulator analysis using IPA predicted Yap among top 10 upstream

regulators altered by EzrinT567D overexpression based on *P* value of overlap (Table 1). *P* value of overlap of Yap downstream gene network altered by EzrinT567D overexpression with all known Yap downstream genes in reference genome was 6.67×10^{-6} .

In addition to Yap signaling network, IPA analysis of gene expression profile of downstream gene signatures predicted activation by EzrinT567 of several other important upstream regulators relevant for cell proliferation and cell cycle progression. These include β -catenin, leucine rich repeat containing G protein-coupled receptor 5 (LGR5), and LGR4 as top three activated upstream regulators based on activation Z-score (Figure 4). Altered gene networks downstream of Yap, Lgr4, Lgr5, and β -catenin are shown in Figure 4. Further analysis of these genes for altered diseases or functions using IPA revealed significant activation of cell proliferation, cell viability, and cell survival, and significant inhibition of necrosis and apoptosis by EzrinT567D overexpression. Furthermore, IPA predicted several relevant canonical pathways, including growth hormone signaling, cell cycle control of chromosomal replication, and P53 signaling, to be significantly altered by EzrinT567D overexpression. These data are shown in Supplemental Tables S1 and S2.

MET and EGFR Also Modulate p-Ezrin (Thr567) in the Mouse Liver

The role of signaling of mitogenic receptors EGFR and MET in regulation of p-Ezrin was also examined. It has been previously shown that inhibition of either EGFR or MET signaling pathways has small effects on liver regeneration, whereas combined inhibition abolishes liver regeneration.⁴⁶ The expression of p-Ezrin (T567) in mice under these conditions was examined. In the combined Δ MET + EGFR-inhibited mice, p-Ezrin (Thr567) expression was completely abolished at days 7 and 10, whereas Δ MET or EGFR-inhibited treatment alone caused no significant changes (Figure 5).

Table 1 Top Upstream Regulators Altered Based on *P* Value

Upstream regulator	Expression fold change	Predicted activation state	Activation z-score	<i>P</i> value of overlap
<i>LGR5</i>	3.458	Activated	3.988	8.54×10^{-16}
<i>LGR4</i>	1.59	Activated	3.889	8.76×10^{-13}
<i>RORA</i>	1.71	Not activated	0.498	3.59×10^{-9}
<i>NR1I3</i>	1.524	Not activated	1.816	0.00000742
<i>RORC</i>	−1.139	Not activated		0.00000901
<i>FOXO3</i>	1.135	Not activated	1.093	0.0000031
<i>WT1</i>		Not activated	−0.342	0.00000563
<i>YAP1</i>	−1.063	Not activated	0.942	0.00000667
<i>TGFB1</i>		Not activated	1.477	0.0000067
<i>ZBTB20</i>	1.732	Not activated	−1.155	0.0000154
<i>SRC</i>		Activated	2.244	0.0000157
Top activated upstream regulators based on z-score				
Upstream regulator	Expression fold change	Predicted activation state	Activation z-score	<i>P</i> value of overlap
<i>CTNNB1</i>	1.222	Activated	4.299	0.0138
<i>LGR5</i>	3.458	Activated	3.988	8.54×10^{-16}
<i>LGR4</i>	1.59	Activated	3.889	8.76×10^{-13}
<i>PDX1</i>		Activated	3.576	0.0577
<i>TCF7L2</i>	1.58	Activated	3.21	0.00642
<i>IL2</i>		Activated	2.953	0.0234
<i>PDGF BB</i>		Activated	2.923	0.000178
<i>SP3</i>	−1.011	Activated	2.915	0.212
<i>IGF2</i>		Activated	2.912	0.000162
<i>GATA1</i>		Activated	2.892	0.293
Top inhibited upstream regulators based on z-score				
Upstream regulator	Expression fold change	Predicted activation state	Activation z-score	<i>P</i> value of overlap
<i>miR-30c-5p</i>		Inhibited	−3.088	0.138
<i>let-7</i>	2.063	Inhibited	−3.017	0.033
<i>miR-124-3p</i>		Inhibited	−2.892	0.431
<i>mir-17</i>		Inhibited	−2.748	0.0146
<i>ZMPSTE24</i>	−1.378	Inhibited	−2.537	0.000403
<i>miR-291a-3p</i>		Inhibited	−2.414	0.0871
<i>miR-17-5p</i>		Inhibited	−2.3	0.0111
<i>CST5</i>		Inhibited	−2.294	0.0494
<i>RB1</i>		Inhibited	−2.26	0.297
<i>TCF7L1</i>	−1.543	Inhibited	−2.236	0.0379

p-Ezrin (Thr567) Inhibitor Treatment Induces Significant Growth Reduction in HCC Cells

Ezrin, p-Ezrin (Thr567), and nuclear Yap expression were assessed by Western blot analysis in two established rat hepatoma cell lines and found to be significantly increased in comparison to primary rat hepatocytes (Figure 6A). Among those two cell lines, JM2 had relatively higher rate of cell proliferation. Both p-Ezrin (Thr567) and Yap were up-regulated in the two cell lines. The compound NSC668394 (Millipore, Billerica, MA) acts as inhibitor of the effects of p-Ezrin (Thr567). It has been shown in previous studies to reduce leukocyte recruitment¹⁵ and hepatocyte growth factor (HGF)/Met-mediated growth of melanoma cells.²⁸ When the p-Ezrin (Thr567) inhibitor

NSC668394 was added to the cultures, it caused significant decrease in growth in both HCC cell lines (Figure 6B).

Discussion

Our present study demonstrates a direct involvement of Ezrin phosphorylation in Thr567 in regulation of Hippo-Yap within hepatocytes. Hippo-Yap is controlled with multiple regulators, including receptor tyrosine kinases, Wnt signaling, and integrins.⁵⁴ We have concentrated on the role of GPC3 in regulation of the Hippo pathway because we first noticed that Yap expression is decreased in GPC3 transgenic mice concomitant with suppression of liver regeneration.¹ It has since been established that GPC3 binds

Predicted Activation of YAP Downstream Network

Predicted Activation of β -catenin Downstream Network

Predicted Activation of LGR4 Downstream Network (Highly Significant)

Predicted Activation of LGR5 Downstream Network (Highly Significant)

Figure 4 Upstream regulators predicted to be altered by the mutant Ezrin (T567D) overexpression. Ingenuity Pathway Analysis of microarray data showing predicted activation of Yap, Lgr5, Lgr4, and β -catenin by overexpression of Ezrin (T567D) based on downstream gene expression profile. Positive z-score represents predicted activation of upstream regulator based on expression profile of downstream genes. *P* values signify extent of overlap between the set of downstream target genes of an upstream regulator in data set compared with all known downstream target genes of that upstream regulator in the reference genome. The symbols in the right side of the figure indicate the following: red shapes, up-regulated genes; green shapes, down-regulated genes, with intensity of color reflecting extent of up-regulation or down-regulation. **Asterisks** indicate genes with multiple probes present in gene array, and only highest expression probe was considered for this analysis.

with tetraspanin CD81 in the cell membrane surface⁵⁵ and that this association causes inhibition of the CD81-associated Syk, a serine-threonine kinase.⁴³ Previous studies by other investigators have shown that treatment of lymphocytes with a CD81 agonistic antibody resulted uniquely in phosphorylation of Ezrin in Thr567 site⁵⁶ and that this phosphorylation was mediated by Syk kinase.⁵⁶ In previous studies, there was a consistent correlation between Ezrin phosphorylation, enhanced expression of Yap, and decrease in p-Yap.⁴³ These findings suggested that Ezrin phosphorylation potentially participates in Hippo modulation and consequent increase in the Yap/p-Yap ratio. There was no direct evidence, however, to substantiate this involvement. Such evidence is provided by the current study. The mutated Ezrin, in which threonine 567 is

replaced by aspartic acid, was employed. This mutated Ezrin mimics the configuration of p-Ezrin (T567) in that, similar to threonine 567 phosphorylation, it converts Ezrin from a folded to a linear form.¹⁷ The latter structural change allows Ezrin to connect intracytoplasmic domains of cell membrane receptors with cytoskeletons.

The direct effect of mutated Ezrin in decreased activation of Hippo component kinase MST1 and Mob1 (a protein associated with LATS kinase, also a Hippo component), and dramatic increase in the Yap/p-Yap ratio (a sign of Hippo pathway inhibition), is shown using the hepatocellular carcinoma line HepaRG in Figure 1. This cell line is a relatively well-differentiated hepatocellular carcinoma, although it also has features of cholangiocyte differentiation.⁵³ This is also consistent with the data shown in Figure 1A, demonstrating

Figure 5 Glypican-3/CD81, Met, and epidermal growth factor receptor (EGFR) are involved in p-Ezrin (Thr567) modulation. Western blot analysis of the expression of p-Ezrin (T567) in livers following partial hepatectomy and subjected to inhibition of EGFR (EGFRi), Met, or both. The tissues were obtained from our previous study.⁴⁶

relatively high levels of endogenous Ezrin in the HepaRG cells. Ezrin is highly expressed in cholangiocytes compared with hepatocytes, and a decrease in Ezrin is associated with cholestatic disorders.⁵⁷ It was observed that all HepaRG cells in our culture expressed cytokeratin 19, a well-characterized biomarker for cholangiocytes (data not shown). The transfection of HepaRG cells with mutant Ezrin not only decreased activation of Mob1 and MST1 components of the Hippo pathway but also resulted in decrease of phosphorylated Yap (the product of the kinase cascade of the Hippo pathway) and enhanced HepaRG cell proliferation. Increased levels of active Yap are associated with many hepatocellular carcinomas, and the results shown in Figure 1D connect this change with enhanced cell proliferation.

The effect of the mutated Ezrin was then demonstrated by transfection of normal hepatocytes via a hydrodynamic injection of a plasmid expressing GFP and the cDNA for normal or mutated Ezrin. The results of Figure 2 demonstrate that the expression sites of the two forms of Ezrin in hepatocytes differ, with the normal Ezrin expressed throughout the cytoplasm, whereas the mutated Ezrin is firmly attached to the plasma membrane, as expected from the Ezrin-related literature documenting that phosphorylated Ezrin changes from folded (curvilinear) to unfolded (rectilinear) form and becomes associated with plasma membrane.¹⁷

The correlation between Ezrin phosphorylation and Yap functionality was also demonstrated by the analysis of gene expression patterns of hepatocytes expressing mutant Ezrin (isolated by cell sorting for GFP). Increased expression of genes *Ctgf*, *Cyr61*, and *Gpc3*, well known to be regulated by Yap from previous studies, was found.^{58–60} The combined data shown in Figures 1, 2, and 3 document the direct role of Ezrin (T567) phosphorylation in regulation of the Hippo pathway and Yap/p-Yap ratio.

The effect of Ezrin phosphorylation on hepatocyte proliferation was also shown by the findings of Figure 2, C and D, in which Ezrin-transfected hepatocytes were more capable of forming multicell clusters during liver

regeneration compared with the control (GFP only) plasmids. Noticeably, hepatocytes transfected with mutant Ezrin formed larger clusters compared with hepatocytes transfected with WT Ezrin. All types of transfected hepatocytes were present as mostly single cells at the time in which partial hepatectomy was performed to initiate liver regeneration.

Despite the currently presented evidence that Ezrin (T567) phosphorylation plays a direct role in regulation of the Hippo pathway and Yap in hepatocytes, the mechanism by which this occurs is not clear. As mentioned above, p-Ezrin (T567) changes the configuration of the protein from curvilinear to rectilinear, resulting in direct association with the plasma membrane, facilitating interaction between plasma membrane components and intracellular events. p-Ezrin (T567) is known to interact with EGFR and MET, and may thus provide association between EGFR and MET and Hippo activity. Such interaction is shown in Figure 5. Both of these receptor tyrosine kinases are known to interact with Hippo pathway via Merlin.^{61,62} Figure 5 does indicate that p-Ezrin (T567) is under some control by the two kinases. Although separate inhibition of the signaling in each one receptor had minor effects, combined inhibition eliminated p-Ezrin (T567) at days 7 and 10 after MET/EGFR double knockout. The data indicate that it is possible that each of the two kinases may play a compensatory role for loss of signaling of the other, in relation to p-Ezrin (T567) expression. Surprisingly, p-Ezrin (T567) at day 2 after partial hepatectomy is increased as a result of combined inhibition of signaling of both MET and EGFR. It is not clear why this is occurring, although it may represent a compensatory mechanism, which should be further investigated. Of interest is also the activation of pathways related to *Lgr4/5*, shown in Table 1. These receptors for R-spondins promote the canonical Wnt/ β -catenin pathway by interacting with the Frizzled receptors, and this finding explains the enhanced pathway controlled by β -catenin (Table 1) that we also found in this study (Figure 4). The precise mechanism by which activation of so many growth-related pathways by

Figure 6 Cell growth inhibition due to p-Ezrin (Thr567) antagonist treatment in rat hepatocellular carcinoma cells. **A:** The JM1 and JM2 rat hepatoma cell lines were analyzed for protein expression of CD81, p-Ezrin, and total Ezrin, compared with freshly isolated rat hepatocytes (rat PHC). **B:** Both cell lines express p-Ezrin (T567), total Ezrin, and nuclear Yap more than the freshly isolated hepatocytes. CD81 is absent, as shown previously.⁴⁵ Treatment of the cell lines with the Ezrin (T567) antagonist NSC668394 causes suppression of cell proliferation. Cell proliferation was assessed by bromodeoxyuridine (BrdU) immunohistochemistry. The y axis indicates number of BrdU-positive cells per $\times 200$ optic field. Statistical significance of the findings is indicated. * $P < 0.05$. DMSO, dimethyl sulfoxide.

T567 phosphorylation of Ezrin is effectuated needs to better understood. These mechanisms have relevance not only to liver regeneration but also to zonation in lobules and hepatocyte differentiation.⁶³

The data presented in this article are also important for two additional reasons, with potential translational implications.

HCV and Hippo Regulation via CD81

CD81 is the portal of hepatitis C virus (HCV)⁶⁴ to infect hepatocytes. A recent study showed that addition of the HCV E2 protein to cultures of HepaRG and primary human hepatocytes resulted in activation of Hippo pathway, with activation of kinases MST1 and LATS1, decrease in p-Ezrin (T567), and decrease in Yap and Taz.⁴³ The findings are identical to the results of interaction of GPC3 with CD81, and the p-Ezrin (T567) role in Hippo/Yap regulation appears to be involved in both the HCV and GPC3 effects.⁴³ There is currently no literature other than our recent work on the role of Hippo pathway activation in the pathology associated with HCV.⁴³ Given the data presented in the current article, regulation of p-Ezrin (T567) expression (down-regulated in HCV infection) may have translational therapeutic applications and/or provide a better understanding of the mechanisms by which HCV acts on human hepatocytes. Hepatocyte necrosis and apoptosis occurs in HCV infection, and it is well documented that Yap down-regulation is associated with enhanced apoptosis in normal and neoplastic cells.⁶⁵

Enhanced Yap Expression in Hepatocellular Carcinomas

Numerous studies have documented enhanced expression of Yap and decreased p-Yap in hepatocellular carcinomas. Accumulating evidence indicates enhanced activation of Yap in HCC could be from dysregulation of upstream receptors, such as c-Met, EGFR,⁶⁶ and GPC3/CD81.⁴³ This may appear paradoxical, in view of the fact that most HCCs also express high levels of GPC3, a protein that we have shown to be associated with Yap down-regulation. However, most HCCs (78%) do not express CD81 on their plasma membrane.⁴³ Given the fact that GPC3 down-regulates Yap by binding to CD81 and that CD81 is absent in 78% of human HCCs, increased expression of GPC3 cannot have an effect on Yap expression in these tumors. In addition, GPC3 transcriptional level is modulated by Yap.⁶⁰ Thus, this absence of CD81 generates a vicious cycle, in which GPC3 cannot inhibit Yap and Yap increase causes more GPC3 expression. The data shown in Figure 6, however, using two rat hepatoma cell lines developed in our laboratory, document that p-Ezrin (T567) is nonetheless increased, and that addition of an inhibitor of p-Ezrin (T567) (NSC668394) inhibits the growth of the two cell lines. The inhibition was more pronounced in JM2 cells, which have less endogenous p-Ezrin (T56) compared with JM1 cells, suggesting that the concentration of the antagonist NSC668394 may not have been sufficient for JM1 cells to deliver a stronger inhibition of cell proliferation. The p-Ezrin (T567) inhibitor NSC668394 is well characterized,^{15,28} and the effects shown in this study point to the possibility of a pharmacology in which the expression of Yap, a signaling protein associated with enhanced cell growth, may be down-regulated by p-Ezrin (T567)

antagonism, leading to the possibility of new therapeutics for HCC. The results of the current study establish a specific correlation between aberrant persistent p-Ezrin (Thr567) and oncogenic Yap in HCC, and its potential translational significance.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.03.014>.

References

- Liu B, Bell AW, Paranjpe S, Bowen WC, Khillan JS, Luo JH, Mars WM, Michalopoulos GK: Suppression of liver regeneration and hepatocyte proliferation in hepatocyte-targeted glypican 3 transgenic mice. *Hepatology* 2010, 52:1060–1067
- Yimlamai D, Christodoulou C, Galli GG, Yanger K, Pepe-Mooney B, Gurung B, Shrestha K, Cahan P, Stanger BZ, Camargo FD: Hippo pathway activity influences liver cell fate. *Cell* 2014, 157:1324–1338
- Chen YA, Lu CY, Cheng TY, Pan SH, Chen HF, Chang NS: WW domain-containing proteins YAP and TAZ in the hippo pathway as key regulators in stemness maintenance, tissue homeostasis, and tumorigenesis. *Front Oncol* 2019, 9:60
- Benhamouche S, Curto M, Saotome I, Gladden AB, Liu CH, Giovannini M, McClatchey AI: Nf2/Merlin controls progenitor homeostasis and tumorigenesis in the liver. *Genes Dev* 2010, 24:1718–1730
- Tharehalli U, Svinarenko M, Kraus JM, Kuhlwein SD, Szekeley R, Kiesle U, Scheffold A, Barth TFE, Kleger A, Schirmbeck R, Kestler HA, Seufferlein T, Oswald F, Katz SF, Lechel A: YAP activation drives liver regeneration after cholestatic damage induced by Rbpj deletion. *Int J Mol Sci* 2018, 19:3801
- Planas-Paz L, Sun T, Pikirolek M, Cochran NR, Bergling S, Orsini V, et al: YAP, but not RSPO-LGR4/5, signaling in biliary epithelial cells promotes a ductular reaction in response to liver injury. *Cell Stem Cell* 2019, 25:39–53
- Cox AG, Tsomides A, Yimlamai D, Hwang KL, Miesfeld J, Galli GG, Fowl BH, Fort M, Ma KY, Sullivan MR, Hosios AM, Snay E, Yuan M, Brown KK, Lien EC, Chhangawala S, Steinhauser ML, Asara JM, Houvras Y, Link B, Vander Heiden MG, Camargo FD, Goessling W: Yap regulates glucose utilization and sustains nucleotide synthesis to enable organ growth. *EMBO J* 2018, 37:e100294
- Santinon G, Brian I, Pocaterra A, Romani P, Franzolin E, Rampazzo C, Biccato S, Dupont S: dNTP metabolism links mechanical cues and YAP/TAZ to cell growth and oncogene-induced senescence. *EMBO J* 2018, 37:e97780
- Johnson R, Halder G: The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. *Nat Rev Drug Discov* 2014, 13:63–79
- Guo L, Teng L: YAP/TAZ for cancer therapy: opportunities and challenges (review). *Int J Oncol* 2015, 46:1444–1452
- Irvine KD, Shraiman BI: Mechanical control of growth: ideas, facts and challenges. *Development* 2017, 144:4238–4248
- Sun S, Irvine KD: Cellular organization and cytoskeletal regulation of the Hippo signaling network. *Trends Cell Biol* 2016, 26:694–704
- Chambers DN, Bretscher A: Ezrin mutants affecting dimerization and activation. *Biochemistry* 2005, 44:3926–3932
- Michie KA, Bermeister A, Robertson NO, Goodchild SC, Curmi PMG: Two sides of the coin: Ezrin/Radixin/Moesin and merlin control membrane structure and contact inhibition. *Int J Mol Sci* 2019, 20:1996
- Rey-Gallardo A, Tomlins H, Joachim J, Rahman I, Kitscha P, Frudd K, Parsons M, Ivetic A: Sequential binding of ezrin and moesin to L-selectin regulates monocyte protrusive behaviour during trans-endothelial migration. *J Cell Sci* 2018, 131:jcs215541
- McClatchey AI, Fehon RG: Merlin and the ERM proteins—regulators of receptor distribution and signaling at the cell cortex. *Trends Cell Biol* 2009, 19:198–206
- Tsai FC, Bertin A, Bousquet H, Manzi J, Senju Y, Tsai MC, Picas L, Miserey-Lenkei S, Lappalainen P, Lemichez E, Coudrier E, Bassereau P: Ezrin enrichment on curved membranes requires a specific conformation or interaction with a curvature-sensitive partner. *Elife* 2018, 7:e37262
- Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS: A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006, 12:410–416
- Akisawa N, Nishimori I, Iwamura T, Onishi S, Hollingsworth MA: High levels of ezrin expressed by human pancreatic adenocarcinoma cell lines with high metastatic potential. *Biochem Biophys Res Commun* 1999, 258:395–400
- Ohtani K, Sakamoto H, Rutherford T, Chen Z, Kikuchi A, Yamamoto T, Satoh K, Naftolin F: Ezrin, a membrane-cytoskeletal linking protein, is highly expressed in atypical endometrial hyperplasia and uterine endometrioid adenocarcinoma. *Cancer Lett* 2002, 179:79–86
- Song J, Fadiel A, Edusa V, Chen Z, So J, Sakamoto H, Fishman DA, Naftolin F: Estradiol-induced ezrin overexpression in ovarian cancer: a new signaling domain for estrogen. *Cancer Lett* 2005, 220:57–65
- Kobel M, Langhammer T, Hüttelmaier S, Schmitt WD, Krieser K, Dittmer J, Strauss HG, Thomssen C, Hauptmann S: Ezrin expression is related to poor prognosis in FIGO stage I endometrioid carcinomas. *Mod Pathol* 2006, 19:581–587
- Wei YC, Li CF, Yu SC, Chou FF, Fang FM, Eng HL, Uen YH, Tian YF, Wu JM, Li SH, Huang WW, Li WM, Huang HY: Ezrin overexpression in gastrointestinal stromal tumors: an independent adverse prognosticator associated with the non-gastric location. *Mod Pathol* 2009, 22:1351–1360
- Abdou AG, Maraee AH, El-Sayed EM, Elnaidany NF: Immunohistochemical expression of ezrin in cutaneous basal and squamous cell carcinomas. *Ann Diagn Pathol* 2011, 15:394–401
- Zhao J, Zhang X, Xin Y: Up-regulated expression of Ezrin and c-Met proteins are related to the metastasis and prognosis of gastric carcinomas. *Histol Histopathol* 2011, 26:1111–1120
- Saygideger-Kont Y, Minas TZ, Jones H, Hour S, Celik H, Temel I, Han J, Atabay N, Erkizan HV, Toretzky JA, Uren A: Ezrin enhances EGFR signaling and modulates erlotinib sensitivity in non-small cell lung cancer cells. *Neoplasia* 2016, 18:111–120
- Jorgren F, Nilbert M, Rambeck E, Bendahl PO, Lindmark G: Ezrin expression in rectal cancer predicts time to development of local recurrence. *Int J Colorectal Dis* 2012, 27:893–899
- Huang L, Qin Y, Zuo Q, Bhatnagar K, Xiong J, Merlino G, Yu Y: Ezrin mediates both HGF/Met autocrine and non-autocrine signaling-induced metastasis in melanoma. *Int J Cancer* 2018, 142:1652–1663
- Geiger KD, Stoldt P, Schlote W, Derouiche A: Ezrin immunoreactivity is associated with increasing malignancy of astrocytic tumors but is absent in oligodendrogliomas. *Am J Pathol* 2000, 157:1785–1793
- Elzagheid A, Korkeila E, Bendardaf R, Buhmeida A, Heikkilä S, Vaheri A, Syrjänen K, Pyrhonen S, Carpen O: Intense cytoplasmic ezrin immunoreactivity predicts poor survival in colorectal cancer. *Hum Pathol* 2008, 39:1737–1743
- Li Q, Gao H, Xu H, Wang X, Pan Y, Hao F, Qiu X, Stoecker M, Wang E, Wang E: Expression of ezrin correlates with malignant phenotype of lung cancer, and in vitro knockdown of ezrin reverses the aggressive biological behavior of lung cancer cells. *Tumour Biol* 2012, 33:1493–1504
- Tan J, Zhang C, Qian J: Expression and significance of Six1 and Ezrin in cervical cancer tissue. *Tumour Biol* 2011, 32:1241–1247

33. Monni R, Haddaoui L, Naba A, Gallais I, Arpin M, Mayeux P, Moreau-Gachelin F: Ezrin is a target for oncogenic Kit mutants in murine erythroleukemia. *Blood* 2008, 111:3163–3172
34. Makitie T, Carpen O, Vaheri A, Kivela T: Ezrin as a prognostic indicator and its relationship to tumor characteristics in uveal malignant melanoma. *Invest Ophthalmol Vis Sci* 2001, 42:2442–2449
35. Li L, Wang YY, Zhao ZS, Ma J: Ezrin is associated with gastric cancer progression and prognosis. *Pathol Oncol Res* 2011, 17: 909–915
36. Patara M, Santos EM, Coudry Rde A, Soares FA, Ferreira FO, Rossi BM: Ezrin expression as a prognostic marker in colorectal adenocarcinoma. *Pathol Oncol Res* 2011, 17:827–833
37. Xie JJ, Xu LY, Wu ZY, Zhao Q, Xu XE, Wu JY, Huang Q, Li EM: Prognostic implication of ezrin expression in esophageal squamous cell carcinoma. *J Surg Oncol* 2011, 104:538–543
38. Bruce B, Khanna G, Ren L, Landberg G, Jirstrom K, Powell C, Borczuk A, Keller ET, Wojno KJ, Meltzer P, Baird K, McClatchey A, Bretscher A, Hewitt SM, Khanna C: Expression of the cytoskeleton linker protein ezrin in human cancers. *Clin Exp Metastasis* 2007, 24: 69–78
39. Brambilla D, Fais S: The Janus-faced role of ezrin in “linking” cells to either normal or metastatic phenotype. *Int J Cancer* 2009, 125: 2239–2245
40. Carneiro A, Bendahl PO, Akerman M, Domanski HA, Rydholm A, Engellau J, Nilbert M: Ezrin expression predicts local recurrence and development of metastases in soft tissue sarcomas. *J Clin Pathol* 2011, 64:689–694
41. Gautreau A, Louvard D, Arpin M: Morphogenic effects of ezrin require a phosphorylation-induced transition from oligomers to monomers at the plasma membrane. *J Cell Biol* 2000, 150:193–203
42. Asp N, Kvalvaag A, Sandvig K, Pust S: Regulation of ErbB2 localization and function in breast cancer cells by ERM proteins. *Oncotarget* 2016, 7:25443–25460
43. Xue Y, Mars WM, Bowen W, Singhi AD, Stoops J, Michalopoulos GK: Hepatitis C virus mimics effects of glypican-3 on CD81 and promotes development of hepatocellular carcinomas via activation of Hippo pathway in hepatocytes. *Am J Pathol* 2018, 188: 1469–1477
44. Parameswaran N, Enyindah-Asonye G, Bagheri N, Shah NB, Gupta N: Spatial coupling of JNK activation to the B cell antigen receptor by tyrosine-phosphorylated ezrin. *J Immunol* 2013, 190: 2017–2026
45. Celik H, Bulut G, Han J, Graham GT, Minas TZ, Conn EJ, Hong SH, Pauly GT, Hayran M, Li X, Ozdemirli M, Ayhan A, Rudek MA, Toretzky JA, Uren A: Ezrin inhibition up-regulates stress response gene expression. *J Biol Chem* 2016, 291:13257–13270
46. Paranjpe S, Bowen WC, Mars WM, Orr A, Haynes MM, DeFrances MC, Liu S, Tseng GC, Tsagianni A, Michalopoulos GK: Combined systemic elimination of MET and epidermal growth factor receptor signaling completely abolishes liver regeneration and leads to liver decompensation. *Hepatology* 2016, 64:1711–1724
47. Tsagianni A, Mars WM, Bhushan B, Bowen WC, Orr A, Stoops J, Paranjpe S, Tseng GC, Liu S, Michalopoulos GK: Combined systemic disruption of MET and epidermal growth factor receptor signaling causes liver failure in normal mice. *Am J Pathol* 2018, 188: 2223–2235
48. Hao JJ, Liu Y, Kruhlak M, Debell KE, Rellahan BL, Shaw S: Phospholipase C-mediated hydrolysis of PIP2 releases ERM proteins from lymphocyte membrane. *J Cell Biol* 2009, 184:451–462
49. Tao J, Calvisi DF, Ranganathan S, Cigliano A, Zhou L, Singh S, Jiang L, Fan B, Terracciano L, Armeanu-Ebinger S, Ribback S, Dombrowski F, Evert M, Chen X, Monga SPS: Activation of beta-catenin and Yap1 in human hepatoblastoma and induction of hepatocarcinogenesis in mice. *Gastroenterology* 2014, 147:690–701
50. Koral K, Paranjpe S, Bowen WC, Mars W, Luo J, Michalopoulos GK: Leukocyte-specific protein 1: a novel regulator of hepatocellular proliferation and migration deleted in human hepatocellular carcinoma. *Hepatology* 2015, 61:537–547
51. Bhawe VS, Paranjpe S, Bowen WC, Donthamsetty S, Bell AW, Khillan JS, Michalopoulos GK: Genes inducing iPS phenotype play a role in hepatocyte survival and proliferation in vitro and liver regeneration in vivo. *Hepatology* 2011, 54:1360–1370
52. McGill MR, Yan HM, Ramachandran A, Murray GJ, Rollins DE, Jaeschke H: HepaRG cells: a human model to study mechanisms of acetaminophen hepatotoxicity. *Hepatology* 2011, 53:974–982
53. Dianat N, Dubois-Pot-Schneider H, Steichen C, Desterke C, Leclerc P, Raveux A, Combettes L, Weber A, Corlu A, Dubart-Kupperschmitt A: Generation of functional cholangiocyte-like cells from human pluripotent stem cells and HepaRG cells. *Hepatology* 2014, 60:700–714
54. Elbediwy A, Vincent-Mistiaen ZI, Spencer-Dene B, Stone RK, Boeing S, Wculek SK, Cordero J, Tan EH, Ridgway R, Brunton VG, Sahai E, Gerhardt H, Behrens A, Malanchi I, Sansom OJ, Thompson BJ: Integrin signalling regulates YAP and TAZ to control skin homeostasis. *Development* 2016, 143:1674–1687
55. Liu B, Paranjpe S, Bowen WC, Bell AW, Luo JH, Yu YP, Mars WM, Michalopoulos GK: Investigation of the role of glypican 3 in liver regeneration and hepatocyte proliferation. *Am J Pathol* 2009, 175: 717–724
56. Coffey GP, Rajapaksa R, Liu R, Sharpe O, Kuo CC, Krauss SW, Sagi Y, Davis RE, Staudt LM, Sharman JP, Robinson WH, Levy S: Engagement of CD81 induces ezrin tyrosine phosphorylation and its cellular redistribution with filamentous actin. *J Cell Sci* 2009, 122:3137–3144
57. Hatano R, Akiyama K, Tamura A, Hosogi S, Marunaka Y, Caplan MJ, Ueno Y, Tsukita S, Asano S: Knockdown of ezrin causes intrahepatic cholestasis by the dysregulation of bile fluidity in the bile duct epithelium in mice. *Hepatology* 2015, 61:1660–1671
58. Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang CY, Chinnaiyan AM, Lai ZC, Guan KL: TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* 2008, 22:1962–1971
59. Shimomura T, Miyamura N, Hata S, Miura R, Hirayama J, Nishina H: The PDZ-binding motif of Yes-associated protein is required for its co-activation of TEAD-mediated CTGF transcription and oncogenic cell transforming activity. *Biochem Biophys Res Commun* 2014, 443:917–923
60. Lu L, Li Y, Kim SM, Bossuyt W, Liu P, Qiu Q, Wang Y, Halder G, Finegold MJ, Lee JS, Johnson RL: Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. *Proc Natl Acad Sci U S A* 2010, 107:1437–1442
61. Guerrero PA, Yin W, Camacho L, Marchetti D: Oncogenic role of Merlin/NF2 in glioblastoma. *Oncogene* 2015, 34:2621–2630
62. Shrestha Y, Schafer EJ, Boehm JS, Thomas SR, He F, Du J, Wang S, Barretina J, Weir BA, Zhao JJ, Polyak K, Golub TR, Beroukhi R, Hahn WC: PAK1 is a breast cancer oncogene that coordinately activates MAPK and MET signaling. *Oncogene* 2012, 31:3397–3408
63. Planas-Paz L, Orsini V, Boulter L, Calabrese D, Pikiolk M, Nigsch F, Xie Y, Roma G, Donovan A, Marti P, Beckmann N, Dill MT, Carbone W, Bergling S, Isken A, Mueller M, Kinzel B, Yang Y, Mao X, Nicholson TB, Zamponi R, Capodiceci P, Valdez R, Rivera D, Loew A, Ukoumadu C, Terracciano LM, Bouwmeester T, Cong F, Heim MH, Forbes SJ, Ruffner H, Tchorz JS: The RSP0-LGR4/5-ZNRF3/RNF43 module controls liver zonation and size. *Nat Cell Biol* 2016, 18:467–479
64. Kim CW, Chang KM: Hepatitis C virus: virology and life cycle. *Clin Mol Hepatol* 2013, 19:17–25
65. Xu X, Chen Y, Wang X, Mu X: Role of Hippo/YAP signaling in irradiation-induced glioma cell apoptosis. *Cancer Manag Res* 2019, 11:7577–7585
66. Thomas MB, Chadha R, Glover K, Wang X, Morris J, Brown T, Rashid A, Dancy J, Abbruzzese JL: Phase 2 study of erlotinib in patients with unresectable hepatocellular carcinoma. *Cancer* 2007, 110:1059–1067