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## TAZ is indispensable for c-MYC-induced hepatocarcinogenesis

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### Abstract

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Authors' contributions

Haichuan Wang, Shanshan Zhang, Yi Zhang, Jiaoyuan Jia, Jingxiao Wang, Xianqiong Liu, Jie Zhang, Xinhua Song, Silvia Ribback, Antonio Cigliano, and Binyong Liang acquired experimental data. Matthias Evert and Antonio Cigliano provided administrative, technical, or material support. Haichuan Wang, Jingxiao Wang, and Shanshan Zhang analyzed the data. Haichuan Wang drafted the manuscript. Diego F. Calvisi, Yong Zeng, and Xin Chen were involved in study design, drafting of the manuscript, study supervision, and obtaining funding.

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICME disclosure forms for further details.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2021.08.021>.

**Background & Aims:** Mounting evidence implicates the Hippo downstream effectors Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) in hepatocellular carcinoma (HCC). We investigated the functional contribution of YAP and/or TAZ to c-MYC-induced liver tumor development.

**Methods:** The requirement for YAP and/or TAZ in c-Myc-driven hepatocarcinogenesis was analyzed using conditional *Yap*, *Taz*, and *Yap; Taz* knockout (KO) mice. An hepatocyte-specific inducible TTR-CreER<sup>T2</sup> KO system was applied to evaluate the role of YAP and TAZ during tumor progression. Expression patterns of YAP, TAZ, c-MYC, and BCL2L12 were analyzed in human HCC samples.

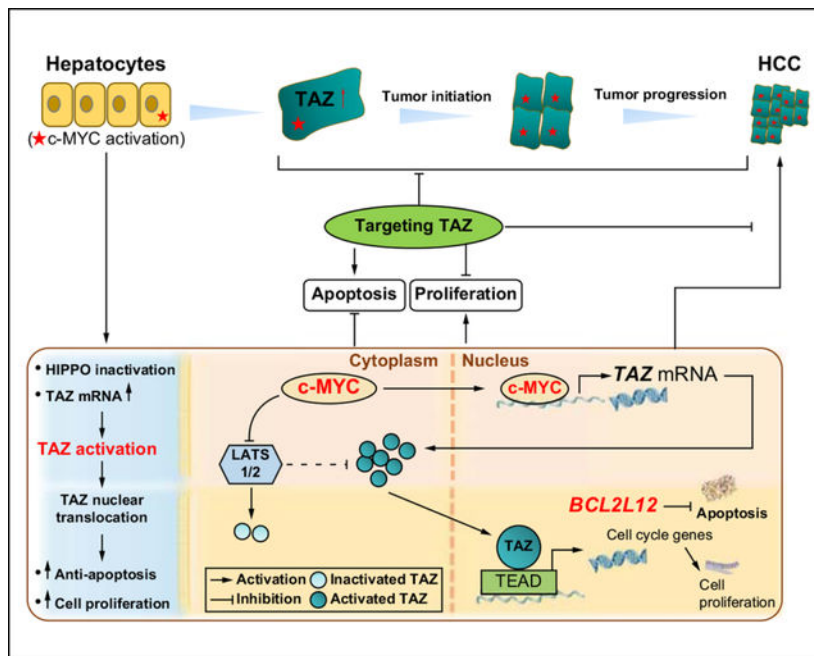
**Results:** We found that the Hippo cascade is inactivated in c-Myc-induced mouse HCC. Intriguingly, *TAZ* mRNA levels and activation status correlated with c-MYC activity in human and mouse HCC, but *YAP* mRNA levels did not. We demonstrated that TAZ is a direct transcriptional target of c-MYC. In c-Myc induced murine HCCs, ablation of *Taz*, but not *Yap*, completely prevented tumor development. Mechanistically, TAZ was required to avoid c-Myc-induced hepatocyte apoptosis during tumor initiation. The anti-apoptotic *BCL2L12* gene was identified as a novel target regulated specifically by YAP/TAZ, whose silencing strongly suppressed c-Myc-driven murine hepatocarcinogenesis. In c-Myc murine HCC lesions, conditional knockout of *Taz*, but not *Yap*, led to tumor regression, supporting the requirement of TAZ for c-Myc-driven HCC progression.

**Conclusions:** TAZ is a pivotal player at the crossroad between the c-MYC and Hippo pathways in HCC. Targeting TAZ might be beneficial for the treatment of patients with HCC and c-MYC activation.

### Lay summary:

The identification of novel treatment targets and approaches for patients with hepatocellular carcinoma is crucial to improve survival outcomes. We identified TAZ as a transcriptional target of c-MYC which plays a critical role in c-MYC-dependent hepatocarcinogenesis. TAZ could potentially be targeted for the treatment of patients with c-MYC-driven hepatocellular carcinoma.

### Graphical abstract



## Keywords

YAP; TAZ; c-MYC; Hepatocellular carcinoma; BCL2L12

## Introduction

Hepatocellular carcinoma (HCC) is one of the major cancer entities worldwide. Most HCCs are diagnosed at an advanced stage when therapeutic options are limited. Recently, the combination of the checkpoint inhibitor atezolizumab and the angiogenesis inhibitor bevacizumab demonstrated a significant therapeutic benefit and is now the first-line therapy for advanced HCC. However, a substantial portion of patients still progress under this regimen.<sup>1</sup> Further investigation into the mechanisms of hepatocarcinogenesis are needed to identify novel treatments for HCC.

c-MYC is a multifunctional transcription factor, controlling the hallmark expression programs responsible for cancer development.<sup>2</sup> c-MYC amplification is a relevant molecular event in human hepatocarcinogenesis, occurring in ~18–25% of human HCC samples.<sup>3</sup> The oncogenic potential of c-MYC has been proven *in vivo* by the finding that overexpression of c-Myc in the mouse liver induces HCC formation.<sup>4</sup> Since c-MYC overexpression alone induces elevated apoptosis in hepatocytes, anti-apoptotic signals are concomitantly activated to counterbalance these death stimuli during hepatocarcinogenesis. Indeed, loss of *TP53* or *MCL1* amplification, leading to apoptosis suppression, often co-occur with c-MYC amplification in human HCC patients (Fig. S1).<sup>3</sup>

Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are the transcriptional co-activators downstream of the Hippo tumor suppressor pathway. Activated YAP/TAZ translocate into the nucleus and function mainly by interacting

with the DNA binding proteins transcriptional enhanced associate domain (TEAD) to induce downstream target gene expression.<sup>5</sup> Once activated, YAP and TAZ contribute to HCC development by coordinately regulating cell cycle-related genes.<sup>6</sup> However, the 2 paralogs also show distinct functions under specific oncogenic stimuli.<sup>7,8</sup>

Herein, using human HCC samples combined with *in vitro* and *in vivo* approaches, we investigated the functional contributions and the underlying mechanisms by which YAP and/or TAZ modulate c-Myc-driven hepatocarcinogenesis.

## Materials and methods

### Mouse treatment and hydrodynamic injection

Wild-type *FVB/N* mice were from Charles River (Wilmington, MA, USA). *Yap<sup>flox/flox</sup>* and *Taz<sup>flox/flox</sup>* mice, kindly provided by Dr. Eric Olson from the University of Texas Southwestern Medical Center (Dallas, TX), were backcrossed to *FVB/N* mice for at least 6 generations before using for the following experiments. *Yap<sup>flox/flox</sup>; Taz<sup>flox/flox</sup>* mice were then generated by crossing *Yap<sup>flox/flox</sup>* mice with *Taz<sup>flox/flox</sup>* mice, followed by genotyping for validation. Hydrodynamic injections were performed as described.<sup>9</sup> The dosage of each plasmid in the various tumor models is depicted in Table S1. Mice were monitored by abdominal palpation and euthanized when they developed a high liver tumor burden. Mice were housed, fed, and monitored under protocols approved by the Committee for Animal Research at the University of California, San Francisco.

### Statistical analysis

The Prism 7.0 software (GraphPad, San Diego, CA) was used to analyze the data presented as means  $\pm$  SD. Comparisons between 2 groups were performed with a 2-tailed unpaired *t* test or Mann-Whitney test. Welch correction was applied when necessary. *p* values <0.05 were considered statistically significant. Kaplan-Meier survival data were evaluated using a Log-rank (Mantel-Cox) test.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

## Results

### Hippo cascade inactivation contributes to c-MYC-driven hepatocarcinogenesis

To investigate the role of Hippo signaling in c-MYC-driven hepatocarcinogenesis, we first determined the status of the main components of the Hippo pathway in liver tumor samples (Fig. 1A and Fig. S2A). Notably, the protein levels (Fig. S2A) and the kinase activity (Fig. S2B) of Hippo signaling kinases, LATS1 and LATS2, were significantly downregulated in the tumor lesions. In contrast, YAP/TAZ targets, such as *Ctgf* and *Cyr61*, were upregulated in c-Myc-driven HCC (hereafter referred to simply as c-Myc HCC [Fig. 1B]). These data suggest the inactivation of the Hippo cascade in c-Myc lesions.

Next, we determined whether the inactivation of the Hippo pathway contributes to c-Myc-induced hepatocarcinogenesis. Thus, we co-expressed c-Myc with *Lats2* (c-Myc/*Lats2*) to

activate the Hippo pathway, or the dominant-negative form of Tead2 (c-Myc/dnTead2) to block YAP/TAZ transcriptional activity. Additional mice were injected with c-Myc together with the pT3-EF1 $\alpha$  empty vector (c-Myc/pT3) as a control (Fig. S3A). In c-Myc/Lats2 and c-Myc/dnTead2 mice, tumor development was significantly delayed (Fig. S3B and S4), along with the decreased expression of *Ctgf* and *Cyr61* (Fig. S3C, D). Tumor cell proliferation, assessed by Ki-67 immunohistochemistry, was significantly inhibited. In addition, tumor cell apoptosis, quantified by cleaved-caspase 3 staining, was strongly induced (Fig. S3E–G). Following these results *in vivo*, we overexpressed Lats2 and dnTead2 in 2 c-Myc mouse HCC-derived cell lines, namely HCC3–4 and HCC4–4 cells.<sup>10</sup> Consistent with the mouse data, Lats2 and dnTead2 overexpression strongly inhibited c-Myc HCC cell growth *in vitro* (Fig. S5).

Overall, the present findings indicate that the impairment of Hippo signaling contributes to c-Myc-dependent hepatocarcinogenesis.

### Upregulation and activation of TAZ in c-MYC murine and human HCCs

The relative contribution of Hippo effectors, YAP and TAZ, was determined in c-Myc HCC. Intriguingly, we observed TAZ but not YAP elevation at the protein level in c-Myc HCC lesions (Fig. 1A). Furthermore, the nuclear accumulation of TAZ was much more pronounced than that of YAP in the same lesions, as assessed by western blotting (Fig. 1C, D) and immunohistochemistry (Fig. 1F). As c-MYC is a well-characterized transcriptional regulator, we asked whether this observation is due to, at least partially, the increased mRNA expression of *Wwtr1*, which encodes TAZ. Thus, we retrieved the microarray data from c-Myc HCC samples.<sup>11</sup> Notably, mRNA levels of *Wwtr1*, but not those of *Yap*, were significantly higher in c-Myc liver tumors than normal liver tissues (Fig. 1E). Similar results were obtained in liver tumors developed in *Alb/c-Myc*<sup>12</sup> and conditional doxycycline-regulated *LT2/MYC* transgenic mice<sup>13</sup> (Fig. S6).

Subsequently, we explored whether a similar differential regulation of YAP and TAZ by c-MYC occurs in human HCC samples. To test this hypothesis, we measured c-MYC activity in human HCC specimens (n = 64). A significant upregulation of c-MYC activity was detected in HCCs (Fig. 2A), especially in clinically aggressive tumors (<3 years' survival following partial liver resection [HCCP]), when compared with those with a better survival/prognosis (3 years' survival following partial liver resection [HCCB]; Fig. 2B). *WWTR1* mRNA levels, but not *YAP* expression, displayed a strong positive correlation with c-MYC activity (Fig. 2C, D). Next, we determined the nuclear localization status of YAP, TAZ, and c-MYC, a typical readout of activation of these genes, in these HCC samples, using immunohistochemistry. Nuclear immunoreactivity for TAZ, YAP, and c-MYC was detected in 36, 41, and 19 HCC specimens, respectively (56.3%; 64.1%; and 29.7%, respectively) (Fig. 2E). Of note, 16 of 19 (84.2%) c-MYC-positive tumors displayed concomitant TAZ nuclear staining, whereas only 8 of 19 (42.1%) c-MYC-positive tumors exhibited YAP nuclear accumulation (Fig. 2F). Among them, 7/8 HCCs were also positive for TAZ nuclear staining, indicating that c-MYC-activated human HCCs are frequently associated with TAZ activation.

Altogether, the data indicate increased *TAZ* mRNA expression and TAZ activation in c-MYC-driven murine and human HCC.

### TAZ is a c-MYC transcriptional target

Based on these data, we hypothesized that TAZ might be a direct target of c-MYC. To test this hypothesis, the inducible c-MYC estrogen receptor (MYC-ER) fusion protein system<sup>14</sup> was applied. We transfected human HCC cell lines (SNU449 and Focus) with MYC-ER or EGFP as a control. Activation of c-MYC was subsequently induced by 4-hydroxytamoxifen (4-OHT) administration. In both HCC cell lines, *WWTR1*, and the canonical c-MYC target genes, but not YAP mRNA, were increased upon 4-OHT treatment (Fig. 3A, B). Consistently, c-MYC activation also upregulated TAZ, but not YAP, protein levels (Fig. S7)

Next, we discovered a putative c-MYC binding site within the *WWTR1* promoter region (Fig. S8). Chromatin immunoprecipitation (ChIP) confirmed the binding of c-MYC to the identified sequence in the human HCC cell lines (Fig. 3C, D) and mouse c-Myc HCC samples (Fig. S9). Using a dual-luciferase reporter assay, we confirmed that c-MYC induces the activation of the full *WWTR1* promoter, but not the construct where the c-MYC binding site was deleted (Fig. 3E, F).

Altogether, the present findings indicate that TAZ, but not YAP, is a transcriptional target of c-MYC in HCC.

### Ablation of *Taz* completely prevents c-Myc-dependent hepatocarcinogenesis in mice

The present data suggest the differential activation of TAZ in c-Myc HCC. We hypothesized that c-Myc-induced hepatocarcinogenesis might depend on TAZ. To test this hypothesis, we investigated the precise functional contributions of YAP and TAZ to c-Myc-driven liver tumor development. Thus, *Yap*<sup>flox/flox</sup>, *Taz*<sup>flox/flox</sup>, and *Yap*<sup>flox/flox</sup>; *Taz*<sup>flox/flox</sup> mice were hydrodynamically injected together with c-Myc and pCMV-Cre plasmids (c-Myc/Cre). Additional mice were injected with c-Myc and pCMV empty vector (c-Myc/pCMV) as a control (Fig. 4A). Of note, Cre alone does not affect liver carcinogenesis in wild-type mice.<sup>15</sup> Deletion of *Yap* mildly delayed c-Myc HCC formation (Fig. 4B). Effective depletion of YAP and downregulation of its target genes *Ctgf* and *Cyr61* were detected in c-Myc/Cre *Yap*<sup>flox/flox</sup> liver tumor samples (Fig. 4C, D). In addition, inhibition of tumor cell proliferation and upregulation of apoptosis were observed (Fig. 4E and Fig. S10A). In striking contrast, loss of *Taz* strongly suppressed c-Myc-driven tumor development (Fig. 4F). Specifically, 7/9 c-Myc/Cre-injected *Taz*<sup>flox/flox</sup> mice did not exhibit any liver tumor even when harvested 21 weeks post-injection (w.p.i), while 2/9 mice each had 1 tumor nodule at the late stage (Fig. S10B). However, TAZ was only partially knocked down in these 2 nodules (Fig. 4G), suggesting that these tumors were, in fact, “escapers”. Consistently, concomitant deletion of *Yap* and *Taz* completely blocked c-Myc-driven hepatocarcinogenesis (Fig. 4H).

Altogether, the data demonstrate that intact TAZ is required for, whereas YAP partially contributes to, c-Myc-induced hepatocarcinogenesis.

## TAZ is necessary to prevent c-Myc-induced hepatocyte apoptosis during tumor initiation

Previous studies have shown that c-MYC induces cell proliferation while also promoting robust apoptosis. The extensive cell death induced by c-MYC overexpression is either abolished or profoundly reduced during carcinogenesis.<sup>16</sup> We hypothesized that TAZ might be required to prevent c-Myc-induced hepatocyte apoptosis during tumor initiation. To test this hypothesis, we analyzed the c-Myc(+) cells by immunohistochemistry in c-Myc/Cre and c-Myc/pCMV-injected *Taz<sup>flox/flox</sup>* mice at 4, 14, 21, and 28 days post-injection. c-Myc (+) cells were readily detected 4 days post-injection in livers from both mouse cohorts (Fig. S11). However, c-Myc (+) cells gradually diminished over time and became undetectable 21 days post-injection in c-Myc/Cre *Taz<sup>flox/flox</sup>* livers. In contrast, in c-Myc/pCMV-injected *Taz<sup>flox/flox</sup>* mice, sporadic c-Myc (+) cells were present at each timepoint analyzed, and eventually, some c-Myc (+) cells evolved into HCC (Fig. S12). The data are consistent with the hypothesis that TAZ suppresses c-Myc-induced hepatocyte death during tumor initiation.

To substantiate our findings *in vitro*, the HCC3-4 and HCC4-4 mouse c-Myc HCC cell lines were transfected with specific small-interfering (si)RNAs against *Yap* (si *Yap*) and *Taz* (si *Taz*), either alone or in combination. Notably, si *Taz* more significantly induced cell apoptosis when compared to si *Yap*, while the decrease in proliferation was only slightly more pronounced in *Taz*-silenced cells than in si *Yap* cells. Furthermore, *Taz* and *Yap*'s combined silencing did not provide additional anti-growth effects on the cell lines in terms of proliferation and cell death, confirming the predominance of TAZ over YAP in c-Myc-dependent growth (Fig. S13). Furthermore, we discovered that *Taz* and/or *Yap* silencing led to decreased expression of PCNA and increased expression of cleaved-caspase 3 (Fig. S14). Equivalent results were obtained in human HCC cell lines with high c-MYC expression (Fig. S15).

To further investigate this hypothesis, we tested whether co-expression of an anti-apoptosis gene could prevent the growth inhibitory effect induced by loss of TAZ. For this purpose, we chose to use Mcl-1, a well-characterized anti-apoptosis molecule. Overexpression of Mcl-1 is unable to induce HCC (Fig. S16). We injected c-Myc/Mcl-1 together with pCMV or pCMV-Cre plasmid in *Yap<sup>flox/flox</sup>*, *Taz<sup>flox/flox</sup>*, and *Yap<sup>flox/flox</sup>; Taz<sup>flox/flox</sup>* mice (Fig. S17A). All c-Myc/Mcl-1/pCMV-injected mice rapidly developed a lethal burden of HCC starting from 4.7 w.p.i. As described in the c-Myc HCC model, deletion of *Yap* mildly delayed c-Myc/Mcl-1 HCC. Strikingly, in contrast to the c-Myc HCC model, in which ablation of *Taz* completely prevented HCC formation, deletion of *Taz* alone or co-deletion of *Yap/Taz* could not abolish tumor formation driven by c-Myc/Mcl-1 co-expression and only slightly prolonged mouse survival (Fig. S17). Thus, Mcl-1 overexpression hampers tumor cell death induced by loss of *Taz* and/or *Yap* during c-Myc-dependent hepatocarcinogenesis, and it cooperates with c-Myc to promote HCC formation in the absence of TAZ and/or YAP.

Finally, we applied *C57BL/6J* mice because, unlike the *FVB/N* strain, c-Myc alone cannot consistently induce HCC formation in the *C57BL/6J* background due to massive hepatocyte apoptosis induced by c-Myc.<sup>17</sup> An additional oncogenic event is required to induce HCC in these mice.<sup>18</sup> We co-expressed c-Myc together with a constitutively activated form of TAZ (TAZS89A)<sup>19</sup> into *C57BL/6J* mice (Fig. 5A). Notably, c-Myc/TAZS89A rapidly induced HCC formation in these mice (Fig. 5B–G).



Overall, our findings indicate that TAZ is required to prevent c-Myc-induced hepatocyte apoptosis during tumor initiation.

### TAZ functions via TEAD-mediated transcriptional activation during c-Myc hepatocarcinogenesis

Our data show that TAZ, but not YAP, is indispensable for c-Myc-driven hepatocarcinogenesis. There are a couple of possible explanations for this. The first possibility is that TAZ and YAP have distinct functions, with only TAZ preventing c-Myc-induced apoptosis. The second possibility is that c-Myc specifically induces TAZ expression. In the second scenario, YAP, if activated, can rescue the tumor inhibition phenotype caused by TAZ loss. First, we determined whether activated YAP (YAPS127A) can rescue c-Myc-induced HCC formation in *Yap;Taz* double knockout (DKO) mice. Thus, we injected c-Myc/YAPS127A/Cre into *Yap<sup>fllox/fllox</sup>;Taz<sup>fllox/fllox</sup>* mice, with c-Myc/pT3EF1 $\alpha$ /Cre as a control (Fig. S18A). Overexpression of YAPS127A in *Yap;Taz* DKO mice promoted aggressive hepatocarcinogenesis, and mice required euthanasia by 6 w.p.i. In contrast, consistent with our previous results (Fig. 4H), no lesions were detected until 30 w.p.i in the c-Myc/pT3EF1 $\alpha$ /Cre-injected *Yap<sup>fllox/fllox</sup>;Taz<sup>fllox/fllox</sup>* group (Fig. S18B). We also co-injected c-Myc/YAPS127A plasmids into *C57BL/6J* mice (Fig. 5A). The co-transfection of c-Myc/YAPS127A plasmids rapidly induced liver tumor formation at the same latency and efficacy as in c-Myc/TAZS89A mice (Fig. 5B, C). Of note, YAPS127A<sup>20</sup> and/or TAZS89A<sup>21</sup> are unable to induce hepatocarcinogenesis (Fig. S19). Altogether, the data indicate that, if activated, both YAP and TAZ can significantly hinder c-Myc-induced apoptosis and synergize with c-Myc to initiate HCC formation *in vivo*.

As YAP and TAZ may function as transcriptional co-activators or via other non-transcriptional activities, we determined whether Tead-mediated transcriptional regulation is necessary for TAZ-dependent c-Myc-driven HCC formation. TEAD2-VP16 recapitulates YAP/TAZ/TEAD-mediated transcriptional properties faithfully, but it does not recapitulate the TEAD-independent functions of TAZ or YAP.<sup>22</sup> We co-expressed c-Myc, TEAD2-VP16, and pCMV-Cre plasmids in *Yap<sup>fllox/fllox</sup>;Taz<sup>fllox/fllox</sup>* mice (Fig. S18A). Similar to our observations following YAPS127A overexpression, TEAD2-VP16 synergized with c-Myc to induce rapid hepatocarcinogenesis in *Yap;Taz* DKO mice. All tumor lesions demonstrated equivalent rates of proliferation and apoptosis (Fig. S18B–E). Similarly, in *C57BL/6J* mice (Fig. 5A), c-Myc/TEAD2-VP16 co-expression promptly induced HCC, as observed in c-Myc/TAZS89A and c-Myc/YAPS127A mice. As expected, YAP/TAZ targets, including *Ctgf* and *Cyr61*, were upregulated in c-Myc/TEAD2-VP16 mouse liver tumors (Fig. 5D, E). Tumor cell proliferation was induced in all experimental groups with no statistical differences (Fig. 5C, F), while apoptosis was more pronounced in c-Myc/TEAD2-VP16 lesions (Fig. 5C, G).

Overall, the present findings demonstrate that activated forms of YAP/TAZ hamper c-Myc-induced hepatocyte apoptosis during tumor initiation via TEAD-mediated transcriptional regulation. In c-Myc HCC, TAZ is indispensable because TAZ, but not YAP, is preferentially induced by c-Myc at the transcriptional level.

## **BCL2L12 is a novel YAP/TAZ target promoting c-Myc HCC**

Next, we specifically searched for YAP/TAZ targets mediating the anti-apoptotic effects of TAZ along with c-MYC-driven hepatocarcinogenesis. The anti-apoptosis gene list<sup>23</sup> was retrieved from our previous RNA-sequencing data on human HCC cells subjected to *YAP* and/or *TAZ* silencing.<sup>6</sup> The previously identified YAP/TAZ targets with survival functions, such as *MCL1*<sup>24</sup> and *BCL2L1*,<sup>25</sup> did not demonstrate consistent downregulation following si *YAP* and/or si *TAZ* across the 4 HCC cell lines tested (Fig. S20). Instead, BCL2 like 12 (*BCL2L12*), which encodes a member of a family of proteins containing a Bcl-2 homology domain 2 (BH2), was consistently downregulated in the 4 cell lines after transfection with si *YAP* and/or si *TAZ* (Fig. 6A), suggesting that *BCL2L12* is a crucial target of YAP/TAZ in HCC. This hypothesis was first validated in c-Myc/Mcl-1 mice (Fig. 6B) deprived of both *Yap* and *Taz* genes. Also, combined overexpression of TAZS89A and c-Myc in *C57BL/6J* mice significantly upregulated *Bcl2l12* levels (Fig. 6C). Subsequently, we identified a candidate TEAD binding site<sup>26</sup> in the *BCL2L12* promoter (Fig. S21). A ChIP-PCR assay confirmed the binding of YAP or TAZ to the TEAD binding motif within the *BCL2L12* promoter in SNU449 and Focus HCC cells (Fig. 6D and 6E). These data demonstrate that the YAP/TAZ/TEAD axis directly regulates *BCL2L12* in HCC cells.

To further investigate the functional role of *BCL2L12* as a YAP/TAZ target gene during c-MYC hepatocarcinogenesis, we overexpressed *Bcl2l12* together with c-Myc/Cre plasmids in the *Yap<sup>flox/flox</sup>; Taz<sup>flox/flox</sup>* mice. Additional *Yap<sup>flox/flox</sup>; Taz<sup>flox/flox</sup>* mice were injected with c-Myc/Cre/pT3 as a negative control; and injected with c-Myc/*Bcl2l12*/pCMV as a positive control (Fig. 6F and Fig. S22). Consistent with our previous observations (Fig. 4H), c-Myc/Cre/pT3 injection did not lead to HCC formation in *Yap<sup>flox/flox</sup>; Taz<sup>flox/flox</sup>* mice. Strikingly, *Myc/Bcl2l12/Cre*-injected *Yap<sup>flox/flox</sup>; Taz<sup>flox/flox</sup>* mice rapidly developed liver tumors (Fig. 6G–I), similar to those observed in c-Myc/*Bcl2l12*/pCMV-injected *Yap<sup>flox/flox</sup>; Taz<sup>flox/flox</sup>* mice (Fig. S22). Conversely, *Bcl2l12* silencing significantly delayed c-Myc-driven hepatocarcinogenesis in mice, leading to decreased cell proliferation and increased apoptosis indices (Fig. S23).

In human HCC samples, levels of *BCL2L12* mRNA were significantly higher in HCC specimens compared with surrounding liver tissues (Fig. 7A). Analysis of the TCGA LIHC dataset revealed similar results (Fig. S24). Moreover, augmented *BCL2L12* mRNA expression was more evident in the HCCP group when compared with the HCCB cohort (HCCB; Fig. 7B). A positive correlation of *BCL2L12* mRNA levels with either *TAZ* or *YAP* mRNA expression was also found in the same subset of human HCC specimens (Fig. 7C, D). Our human data showed a ubiquitous activation of TAZ and/or YAP among human HCCs (61/64 [95.3%]; Fig. 2E). Therefore, we anticipate that most HCC samples are also positive for *BCL2L12*. By immunohistochemistry, concomitant nuclear staining for TAZ or YAP with intense cytoplasmic immunolabeling for *BCL2L12* was detected in 97.2% and 82.9% cases, respectively (Fig. 7E, F).

Altogether, the present findings indicate that *BCL2L12* is a key anti-apoptotic target of *YAP* and *TAZ* during hepatocarcinogenesis.

## Targeting TAZ as an effective treatment for c-Myc-induced murine HCC

Finally, we explored whether YAP and TAZ are necessary for c-Myc mouse HCC progression. Thus, we cloned a CreER<sup>T2</sup> plasmid into the pT3 vector under the hepatocyte-specific TTR promoter (pT3-TTRpro-CreER<sup>T2</sup>). c-Myc and pT3-TTRpro-CreER<sup>T2</sup> plasmids were injected into *Yap*<sup>fllox/fllox</sup> or *Taz*<sup>fllox/fllox</sup> mice. Five weeks post-injection, a cohort of mice was harvested as a “pretreatment” group. All mice developed liver tumors. Tamoxifen (or corn oil as a control) was introduced at 5 w.p.i to activate Cre in tumor liver cells (Fig. 8A). This strategy allowed us to delete YAP or TAZ in already formed c-Myc HCC lesions.

In the *Yap*<sup>fllox/fllox</sup> background, a similar survival length was observed in corn oil- and tamoxifen-treated mice (Fig. 8B). All mice developed a high tumor burden around 3 to 8 weeks under corn oil or tamoxifen treatment. Tumor cells were highly proliferative (Fig. S25A, B). Western blotting and immunohistochemistry confirmed the effective deletion of YAP (Fig. S25A, C). The results indicate that YAP is dispensable for c-Myc-driven HCC progression.

In the *Taz*<sup>fllox/fllox</sup> background, tamoxifen-mediated *Taz* deletion in c-Myc lesions significantly improved mouse survival (Fig. 8C). Indeed, all corn oil-treated mice developed a high tumor burden within 3 to 5 weeks post corn oil treatment. In striking contrast, all tamoxifen-treated mice were healthy, even when harvested at 28 w.p.i (Fig. 8C and Table S2). Grossly, the liver of tamoxifen-treated mice appeared smooth with few and small nodules (Fig. 8E), implying the regression of most tumor nodules upon *Taz* deletion.

To further elucidate the mechanism whereby TAZ ablation leads to regression of c-Myc HCC, we harvested tamoxifen-treated mice 2 weeks after the first dose of tamoxifen. At this time point, the liver surface revealed numerous tumor nodules (Fig. 8E). Tumor cells were positive for c-Myc but did not express TAZ (Fig. 8D). Notably, most tumor cells underwent apoptosis (Fig. 8E), with a profound decrease of *Bcl2l12* mRNA expression (Fig. S26). The data suggest that TAZ loss triggers robust cell death in c-Myc HCC cells, leading to tumor regression. The results also indicate that targeting TAZ might be an effective strategy for treating human HCCs with c-MYC activation/amplification.

## Discussion

Here, we specifically investigated the functional contribution of Hippo effectors YAP and/or TAZ in c-MYC HCC. Our studies uncover the unique role of TAZ/BCL2L12 pathway, which is required for c-MYC HCC initiation and progression. The data also indicate that *Yap* deletion mildly affects the initiation and progression of c-Myc HCCs. One possibility is that YAP regulates cell cycle-related genes,<sup>6</sup> and loss of *Yap* inhibits tumor growth due to the suppression of cell proliferation (Fig. 4E). Our data add to the current literature suggesting the distinct and overlapping roles of YAP and TAZ during hepatocarcinogenesis.<sup>6</sup> Additional studies, such as using steatosis or diethylnitrosamine-induced hepatocarcinogenesis models in combination with conditional *Yap* or *Taz* KO mice will provide further information about this critical issue.

Recently, Moya *et al.* showed that peritumoral activation of YAP and TAZ suppresses liver carcinogenesis.<sup>27</sup> Mechanistically, the tumor elimination is due to the non-cell autonomous tumor suppression mediated by YAP and TAZ, whereby the normal tissue surrounding liver tumors suppresses tumor growth, leading to tumor regression. Thus, using whole liver KO of *Yap* and/or *Taz* may be complicated by the opposing roles of these molecules in tumor cells *vs.* peritumoral hepatocytes. In this manuscript, we applied the hydrodynamic co-transfection approach, which allowed us to activate oncogenes while deleting *Yap* and/or *Taz* in the same, yet a small set of mouse hepatocytes, without affecting YAP/TAZ expression in the vast majority of surrounding liver cells. This allowed us to specifically investigate the tumor-intrinsic roles of YAP/TAZ in tumor development.

Our study has significant translational implications for the treatment of HCC with c-MYC activation. Aberrant c-MYC activation occurs in ~20% of human HCCs,<sup>4</sup> and those patients may be readily identified in clinics. Using an inducible Cre system, we demonstrate that deletion of *Taz* led to profound tumor cell apoptosis, and eventually, tumor regression. Targeting the Hippo/YAP/TAZ cascade has been proposed for HCC treatment (Fig. S27). However, as this pathway has a critical role in regulating normal organ homeostasis,<sup>28</sup> such a targeting strategy might be highly toxic, limiting therapeutic efficacy. Targeting YAP or TAZ individually, for instance, using siRNA-based gene silencing or CRISPR-Cas9-based gene deletion in HCC cells, may assist in avoiding this potential toxicity. In this regard, the present report's preclinical data indicate that targeting TAZ might be helpful for HCC with activated c-MYC. However, if these HCCs harbor additional genetic events preventing cell apoptosis, such as *TP53* mutation or *MCL1* amplification, targeting TAZ might be insufficient. Administration of TAZ inhibitors combined with apoptosis-inducing drugs might be a successful strategy for treating these patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgment

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## Data availability statement

The data that support the findings of this study are included within the article and its supplementary materials.

## Abbreviations

4-OHT                      4-hydroxytamoxifen

<b>ChIP</b>	chromatin immunoprecipitation
<b>DKO</b>	double knockout
<b>HCC</b>	hepatocellular carcinoma
<b>HCCB</b>	HCC with better prognosis
<b>HCCP</b>	HCC with poorer prognosis
<b>MYC-ER</b>	c-MYC estrogen receptor fusion protein
<b>siRNA</b>	small-interfering RNA
<b>TAZ</b>	transcriptional co-activator with PDZ-binding motif
<b>TEAD</b>	transcriptional enhanced associate domain
<b>w.p.i</b>	week(s) post-injection
<b>WWTR1</b>	WW domain containing transcriptional regulator 1
<b>YAP</b>	Yes-associated protein

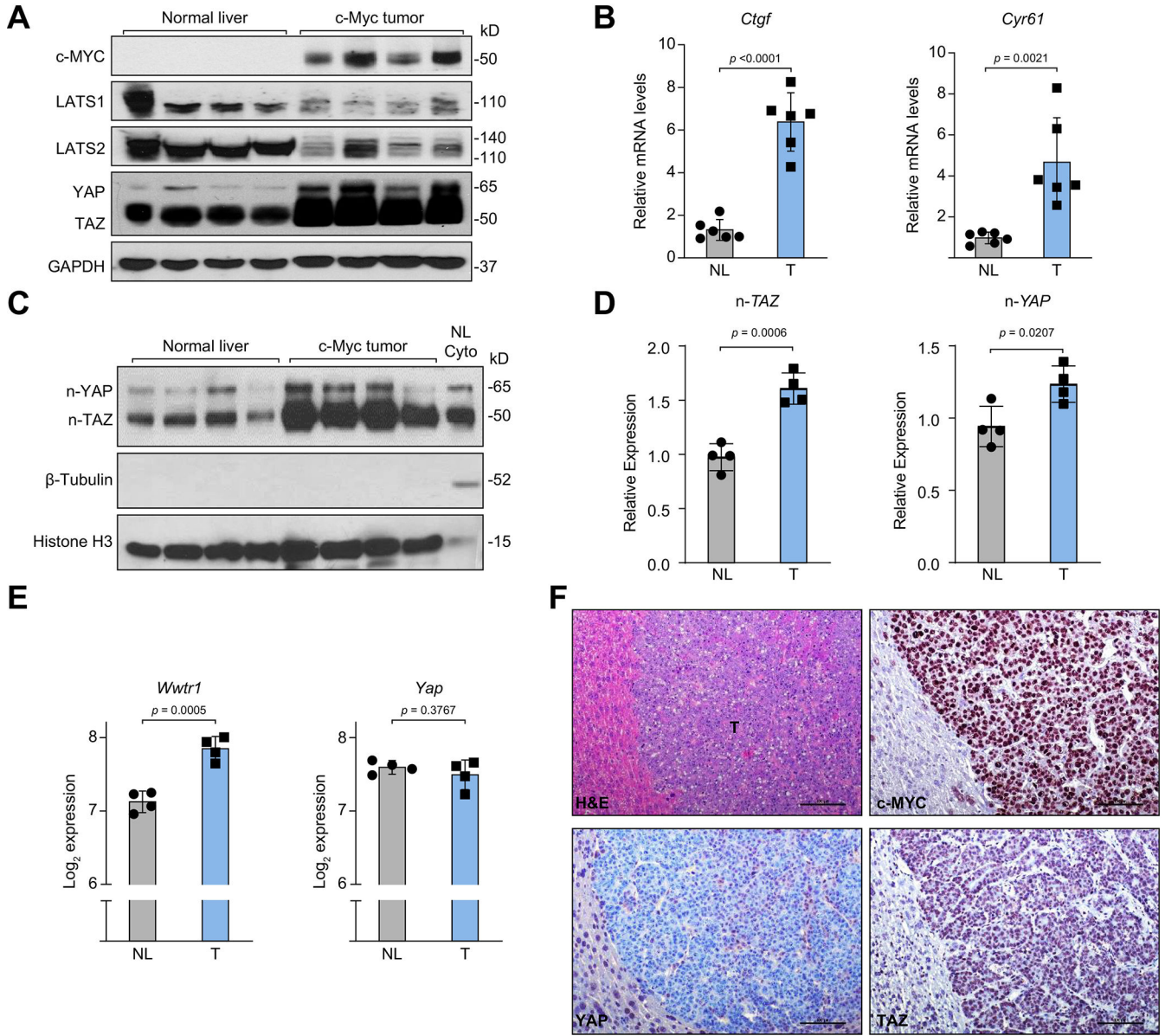
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**Highlights**

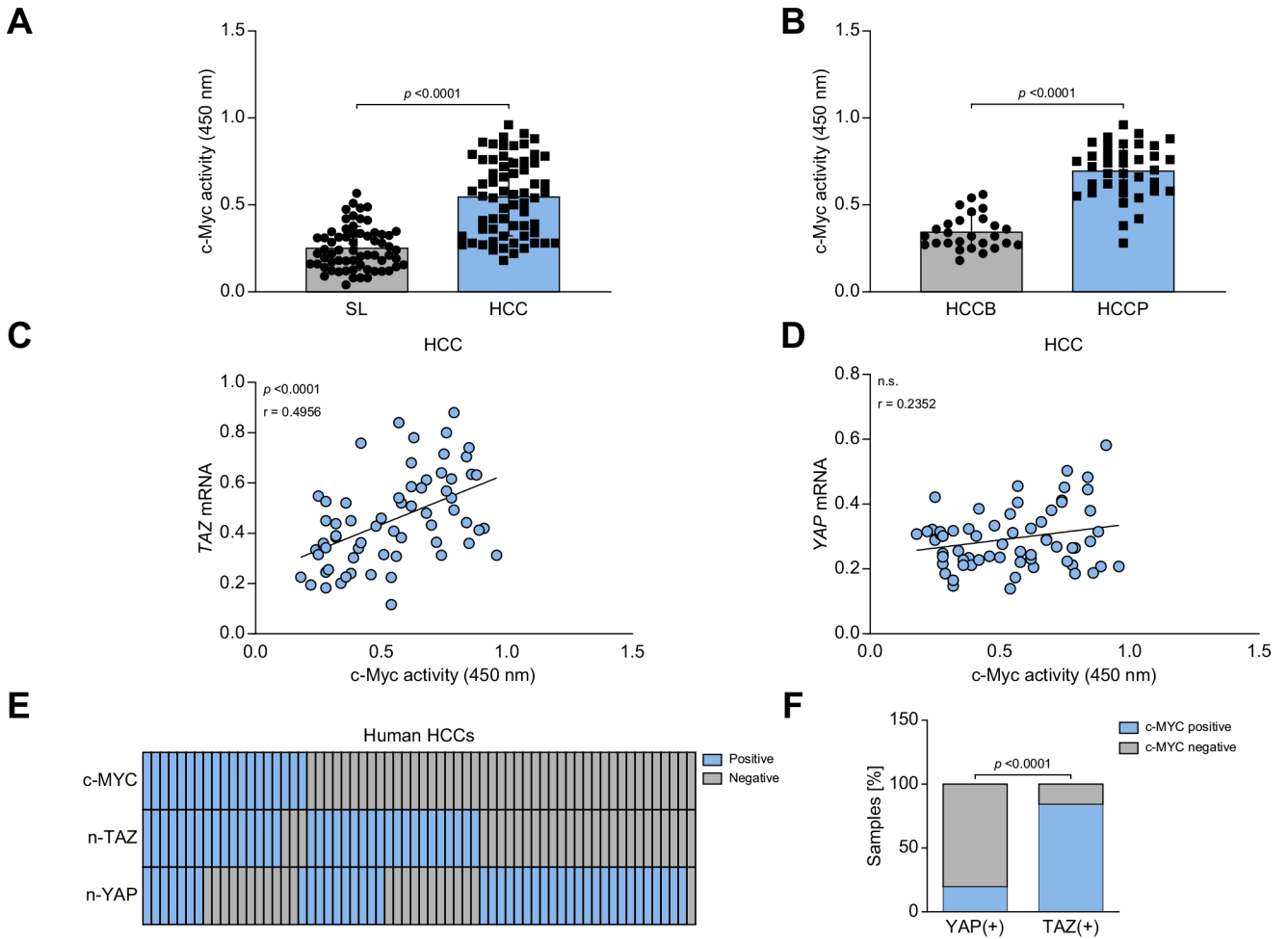
- TAZ is a downstream transcriptional target of c-MYC during hepatocarcinogenesis.
- Genetic ablation of *Taz* prevents c-MYC-initiated HCC.
- Activated TAZ/YAP regulate the BCL2L12 anti-apoptotic gene to promote c-MYC-induced hepatocarcinogenesis.
- Targeting TAZ leads to regression of c-MYC-induced HCC.



**Fig. 1. Activation of the YAP/TAZ cascade in c-Myc driven HCC in mice.**

(A) Western blotting results of LATS1, LATS2, and YAP/TAZ expression in the NL and c-Myc HCC tissues (T). (B) mRNA expression of *Ctgf* and *Cyr61*. (C) Western blotting results of nuclear YAP (n-YAP) and nuclear TAZ (n-TAZ) levels. Histone H3 and  $\beta$ -Tubulin were used as nuclear and cytoplasmic (Cyto) protein loading controls, respectively. (D) Quantification of nuclear YAP (n-YAP) and nuclear Taz (n-TAZ) levels. (E) *Yap* and *Taz* (*Wwtr1*) mRNA relative expression in NL and T tissues. N = 4 biological replicates for each group. (F) Representative immunohistochemistry of a c-Myc mouse tumor lesion (T) exhibiting robust nuclear immunoreactivity for c-MYC and TAZ, whereas only a few tumor cells display nuclear staining for YAP. Scale bar: 100  $\mu$ m. (B, D, E) Mean  $\pm$  SD; Unpaired *t* test, Welch's *t* test or Mann-Whitney test. HCC, hepatocellular carcinoma; NL, normal liver; T, tumor.





**Fig. 2. Evaluation of the relation between TAZ and YAP mRNA with c-MYC activity.**

(A) The activity of c-MYC in HCC (n = 64) and corresponding non-tumorous SL (n = 64).

(B) The activity of c-MYC in HCCB (n = 27) and HCCP (n = 37).

(C, D) Correlation between *WWTR1* (C) or *YAP* (D) mRNA expression and c-Myc activity.

$p$  values and correlation  $r$  values were calculated by Pearson correlation analysis.

(E) Activation status of c-MYC, YAP, and TAZ in human HCCs as determined by IHC staining.

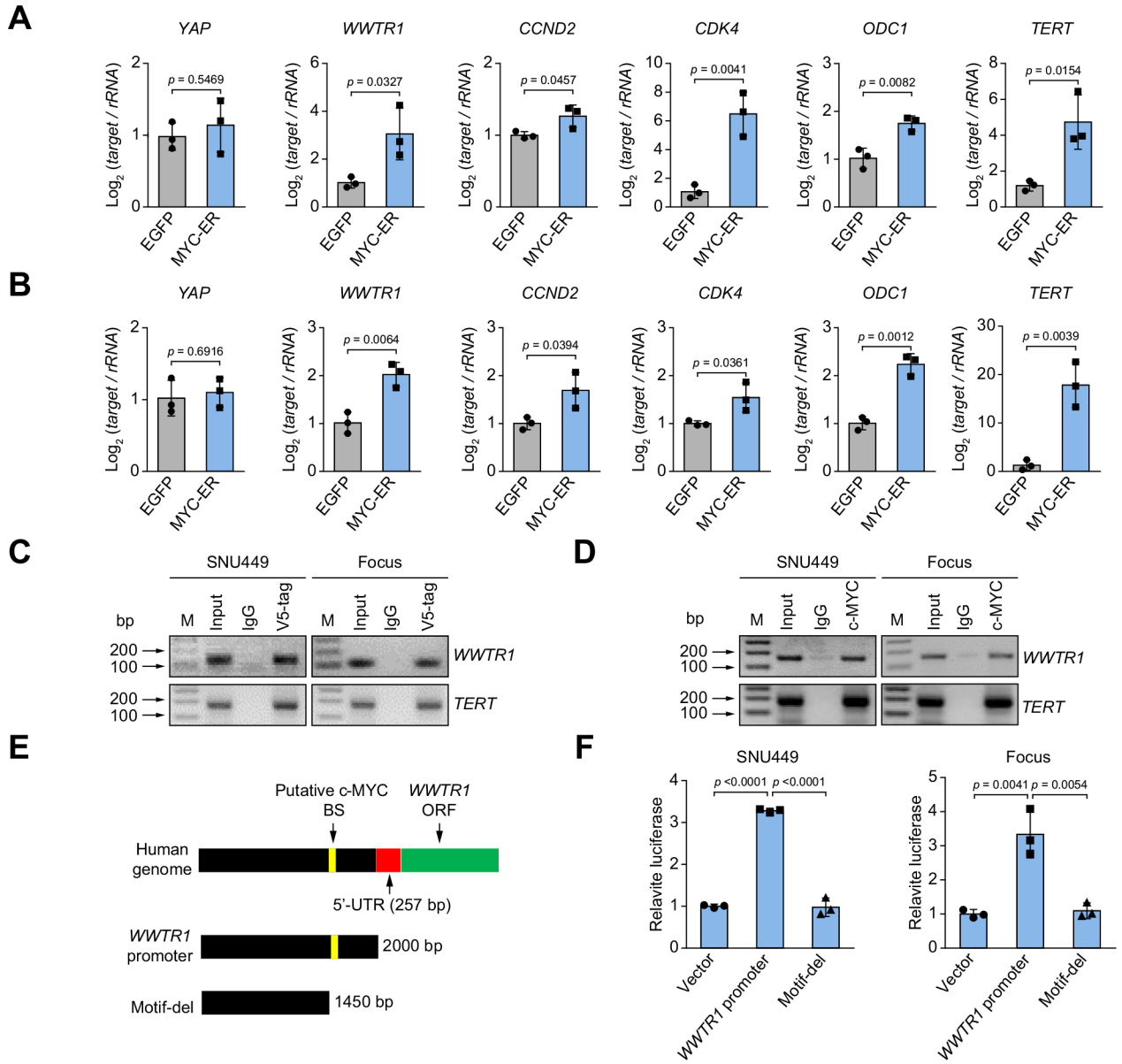
(F) Comparison of c-MYC positive and negative immunoreactivity frequency between YAP-positive (YAP

[+]) and TAZ-positive (TAZ [+]) human HCC samples.

(A, B) Mean  $\pm$  SD; Unpaired  $t$  test;

(C, D) Pearson correlation coefficient;

(F) Fisher exact test. HCC, hepatocellular carcinoma; HCCB, HCC with better prognosis; HCCP, HCC with poorer prognosis; IHC, immunohistochemistry; n.s., not significant; SL, surrounding liver.



**Fig. 3. c-MYC directly binds to the TAZ promoter to induce its transcriptional activation.** (A, B) qPCR analysis of mRNA levels of YAP, WWTR1, and c-MYC targets in the SNU449 (A) and Focus (B) cell lines transfected with 4-OHT inducible EGFP and MYC-ER plasmid. (C, D) Direct binding of c-MYC to WWTR1 promoter in SNU449 and Focus cells. The anti-V5-tag primary antibody was applied to immunoprecipitate exogenous transfected V5-tagged c-MYC (C), and the anti-c-MYC antibody was used to immunoprecipitate endogenous c-MYC (D). TERT promoter was applied as a positive control. (E) Schematic overview of the genetic structure of *WWTR1* in the human genome, pGL3-WWTR1-Promoter, and pGL3-Motif-del constructs. (F) Dual-luciferase assay with wild-type (*WWTR1* Promoter) or depleted c-MYC binding site (Motif-del) in the *WWTR1* promoter region, Vector was used as a negative control. (A, B) Mean ± SD; Mann-Whitney test. (F) Mean ± SD; One-way ANOVA test. 4-OHT, 4-hydroxytamoxifen; BS, binding site;

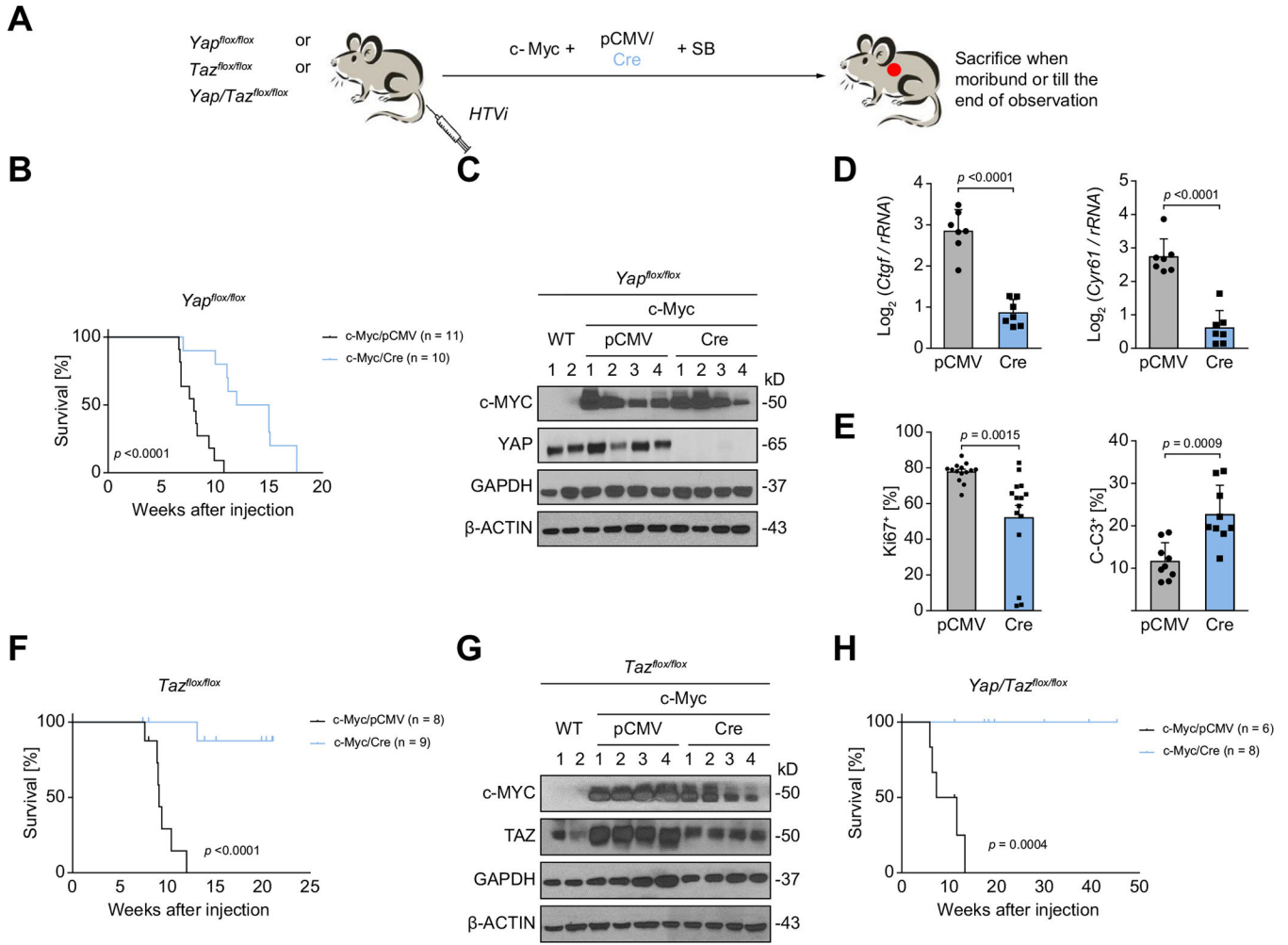
ORF, open reading frame; qPCR, quantitative real time PCR; UTR, untranslated region.  
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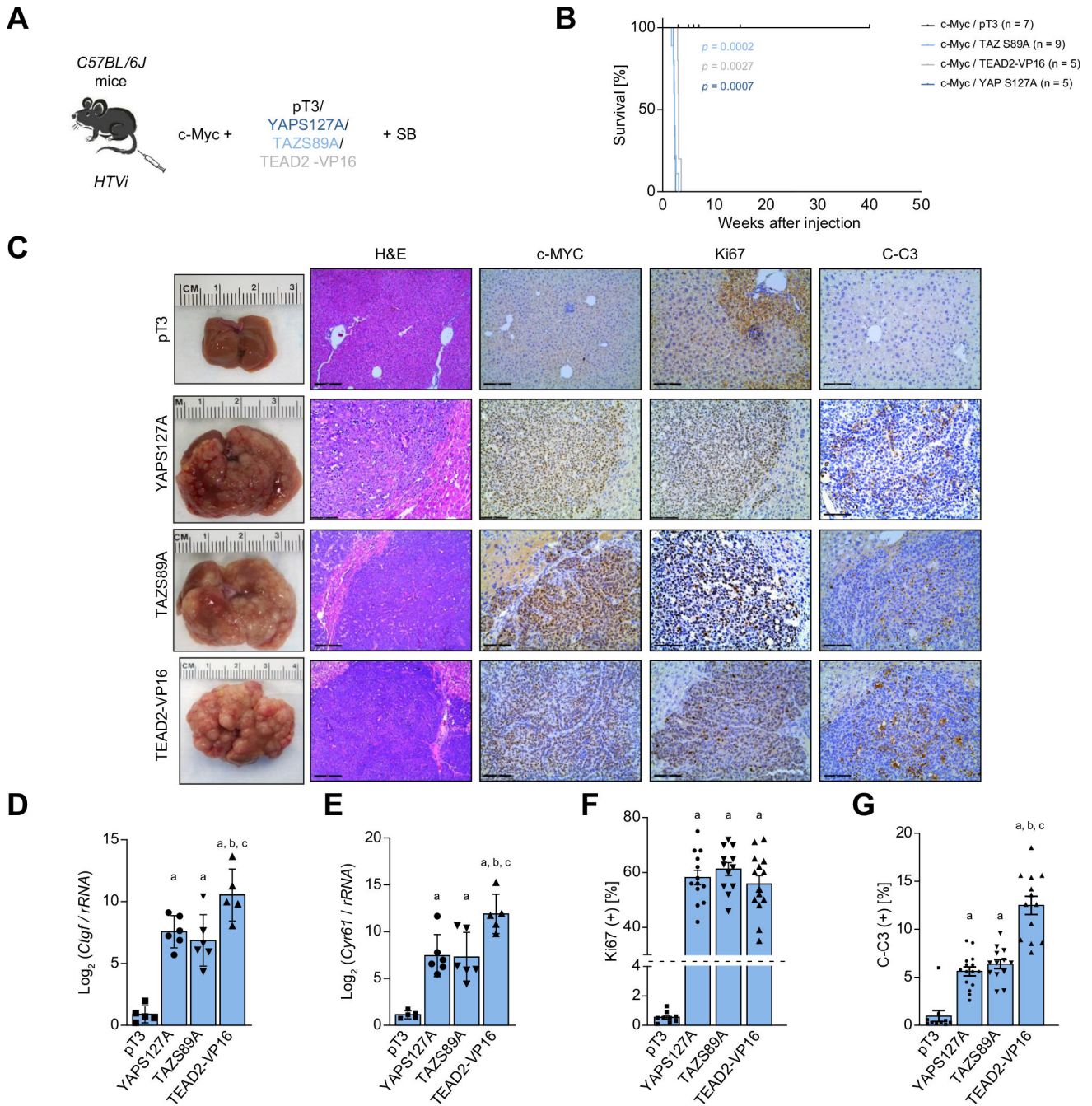
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**Fig. 4. TAZ is indispensable for c-Myc-dependent HCC development in mice.**

(A) Study design. *Yap<sup>flox/flox</sup>*, *Taz<sup>flox/flox</sup>*, *Yap/Taz<sup>flox/flox</sup>* conditional knockout mice were injected with c-Myc/pCMV or c-Myc/Cre plasmids. (B) Survival curves of *Yap<sup>flox/flox</sup>* mice injected with c-Myc. (C) Western blotting showing the expression level of c-MYC and depletion of YAP in *Yap*-depleted livers. WT refers to normal uninjected liver tissues from *Yap<sup>flox/flox</sup>* mice; whereas all the rest were from HCC lesions. GAPDH and  $\beta$ -ACTIN were used as a loading control. (D) mRNA expression of *Ctgf* and *Cyr61*. n = 6 samples for each group. (E) Analysis of Ki67<sup>+</sup> and C-C3<sup>+</sup> positive cells in control (pCMV) and *Yap*-depleted livers. (F) Survival curves of *Taz<sup>flox/flox</sup>* mice injected with c-Myc. (G) Western blotting showing the expression level of c-MYC and the partial depletion of TAZ in *Taz*-depleted livers. The liver tissues from the Cre group were harvested from 2 mice with single small tumor nodules. GAPDH and  $\beta$ -ACTIN were used as a loading control. (H) Survival curves of *Yap/Taz<sup>flox/flox</sup>* mice injected with c-Myc. (B, F, H) Log-rank (Mantel-Cox) test; (D, E) Mean  $\pm$  SD; Mann-Whitney test. C-C3, cleaved-caspase 3; HCC, hepatocellular carcinoma; HTVi, hydrodynamic tail vein injection; WT, wild-type. (This figure appears in color on the web.)



**Fig. 5. TAZ supports c-Myc oncogenic function in the absence of survival factors.** (A) Study design. *C57BL/6J* mice were injected with c-Myc/pT3 (n = 7), c-Myc/YAPS127A (n = 5), c-Myc/TAZS89A (n = 9), c-Myc/TEAD2-VP16 (n = 5). Mice were sacrificed when moribund. (B) Survival curve showing that either YAP or TAZ overexpression or TEAD transcriptional activation cooperates with c-Myc to induce HCC in mice.  $p$  values were calculated by Log-rank (Mantel-Cox) test. (C) Macroscopy, H&E, and immunostaining (c-MYC, Ki67, C-C3) of liver tissues 3 weeks post-injection. Scale bar: 100x, 200  $\mu$ m; 200x, 100  $\mu$ m. (D, E) mRNA expression of *Ctgf* and *Cyr61*. (F, G) Analysis of Ki67- and C-C3-positive cells in control and liver tumors.  $P < 0.005$  (a) vs. YAPS127A; (b) vs.

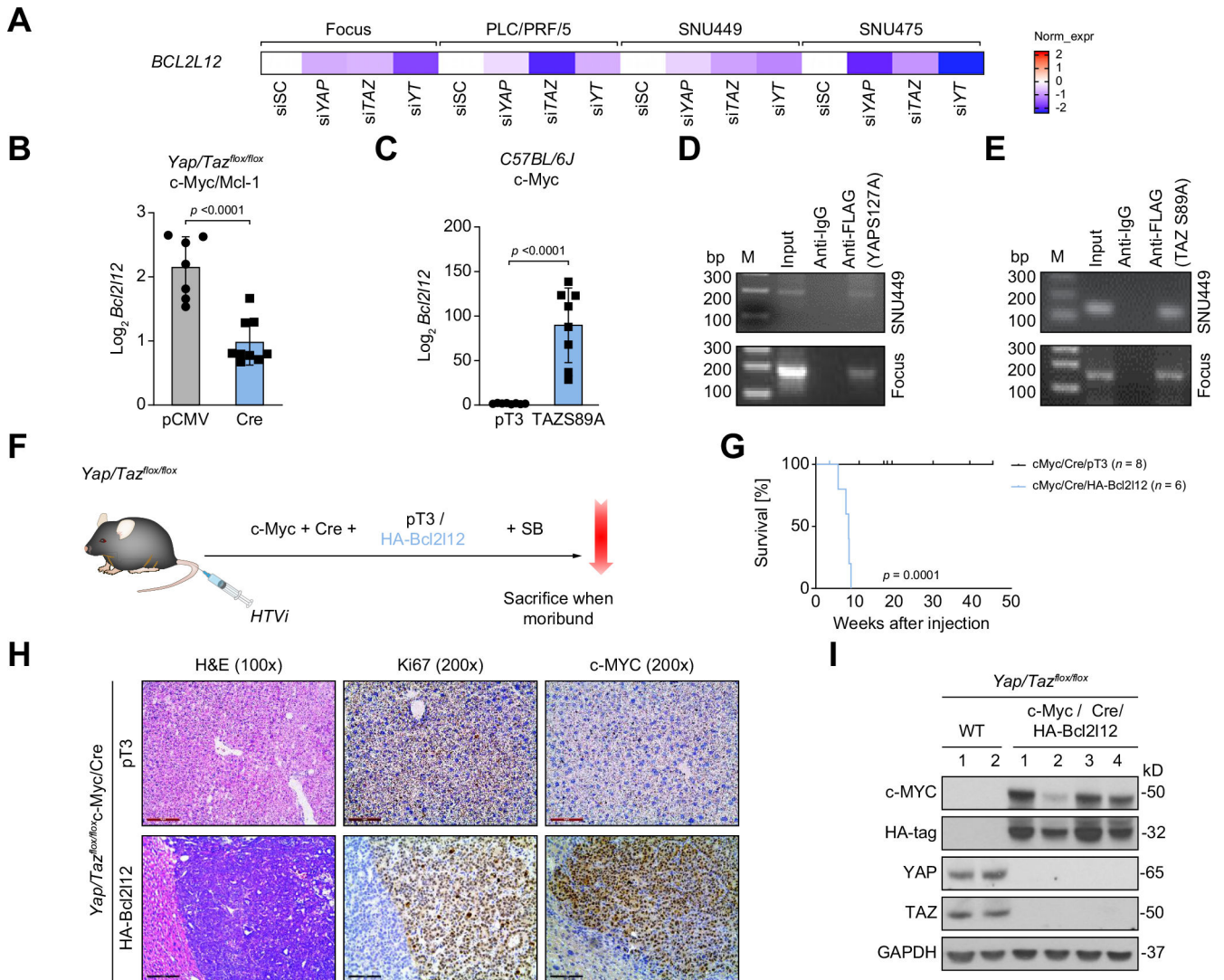
TAZS89A; (c) vs. TEAD2-VP16. (D, E, F, G) Mean  $\pm$  SD; One-way ANOVA test. C-C3, cleaved-caspase 3; HCC, hepatocellular carcinoma; HTVi, hydrodynamic tail vein injection.

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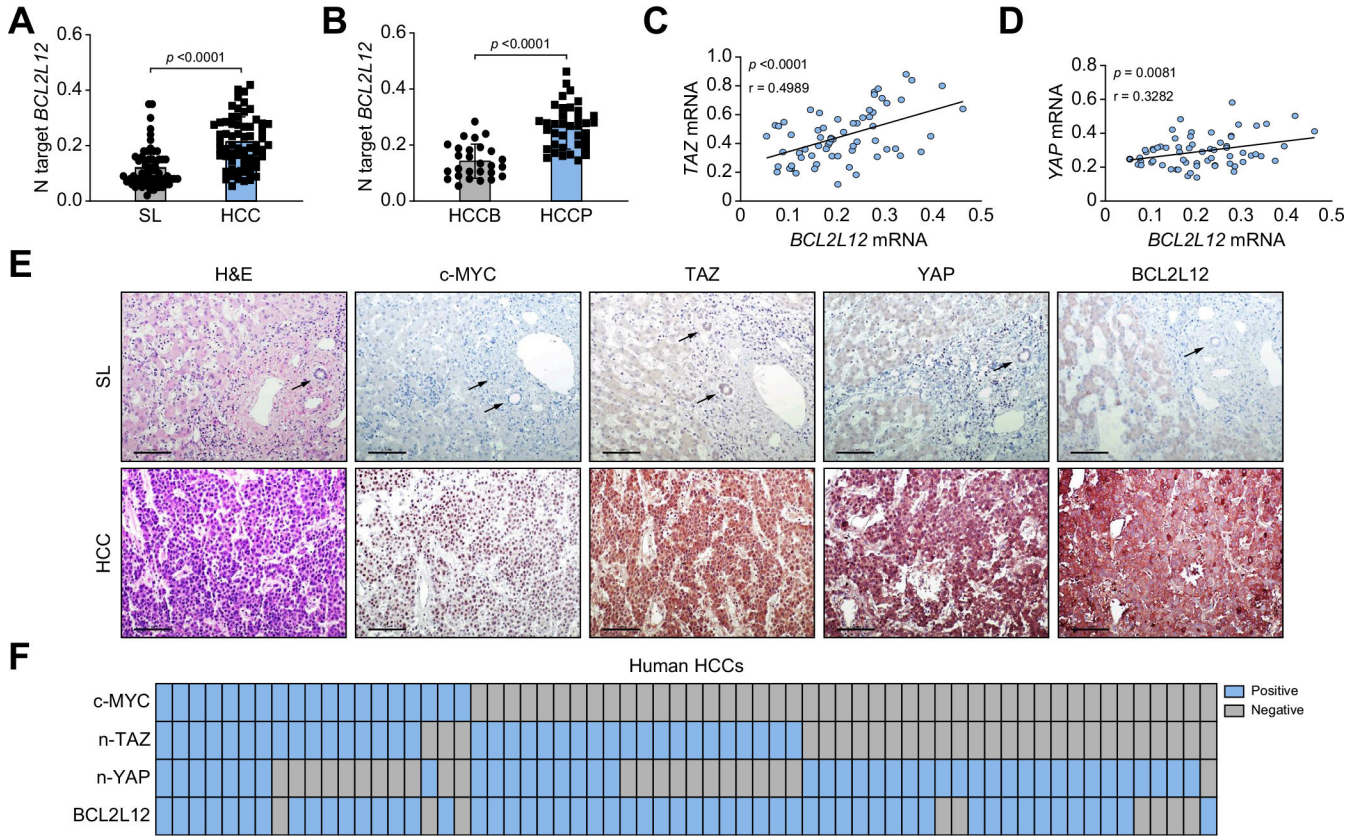
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**Fig. 6. BCL2L12 is a target regulated by YAP/TAZ during oncogene-driven liver tumor development.**

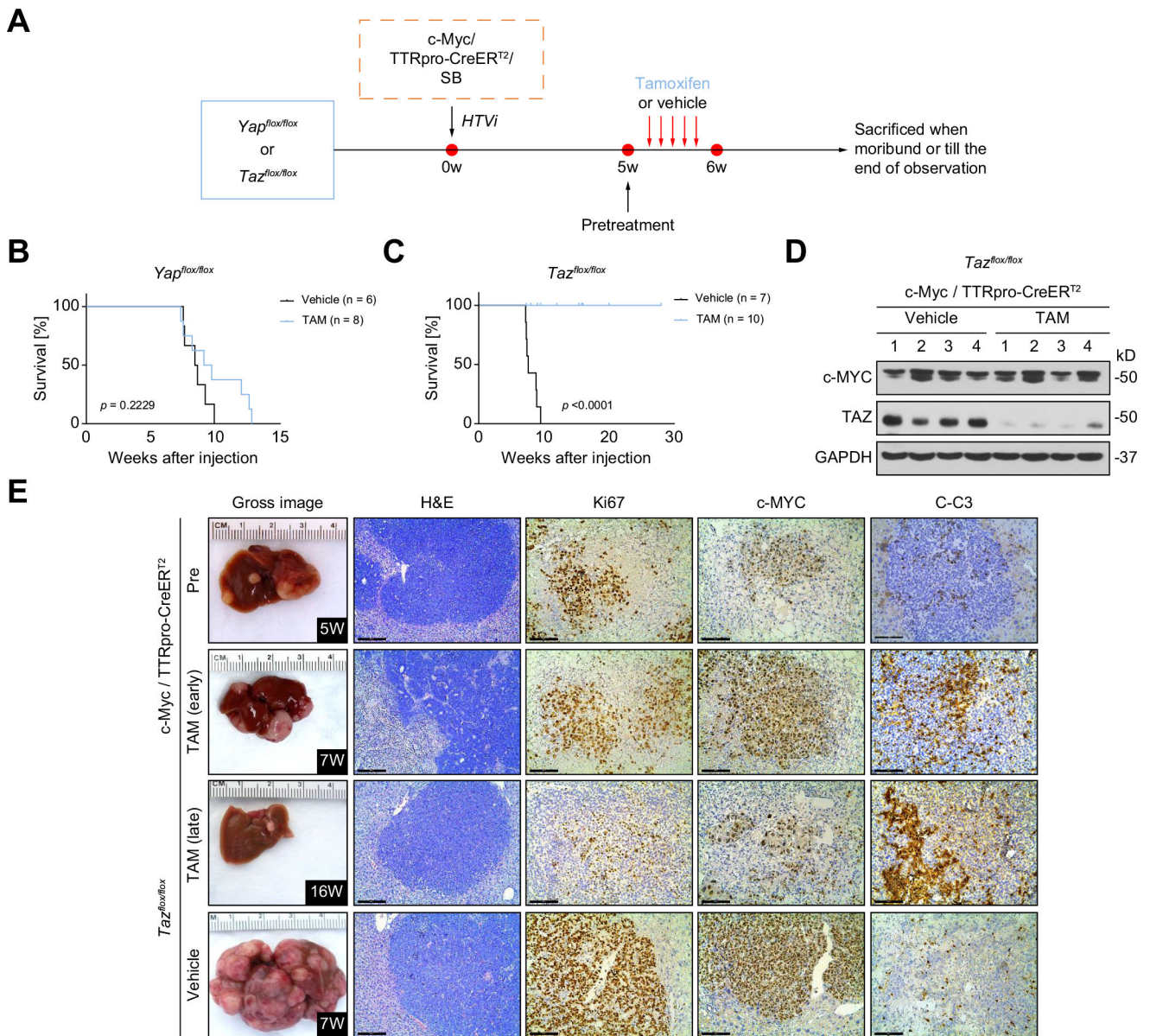
(A) Heatmap of anti-apoptosis gene signature in human HCC cell lines after *YAP* or/and *TAZ* depletion, showing *BCL2L12* as a potential target. (B) mRNA expression of *Bcl2l12* was significantly downregulated when deleting *Yap/Taz* in c-Myc/Mcl-1 mice.  $n = 9$  samples in each group. (C) Overexpression of TAZS89A in c-Myc induced tumors upregulated *Bcl2l12* expression in *C57BL/6J* mouse livers.  $n = 7$  samples in each group. (D, E) Direct binding of TEAD to *BCL2L12* promoter in SNU449 (D) and Focus (E) cells. Predicted ChIP-PCR product bands were at 100 bp-200 bp. (F) Study design. *Yap/Taz<sup>flox/flox</sup>* mice were injected with c-Myc/pCMV-Cre/pT3 (control,  $n = 8$ ) or c-Myc/Cre/HA-Bcl2l12 ( $n = 6$ ) plasmids. (G) Survival curves of *Yap/Taz<sup>flox/flox</sup>* mice injected with c-Myc/HA-Bcl2l12. (H) Histology analysis of liver tissue from both groups. Scale bar: 200  $\mu\text{m}$  for 100x, 100  $\mu\text{m}$  for 200x. (I) Western blotting results of c-MYC, HA-tagged BCL2L12, YAP, and TAZ expression. GAPDH was used as a loading control. (B, C) Mean  $\pm$  SD; Mann-Whitney test; (G) Log-rank (Mantel-Cox) test. ChIP, chromatin immunoprecipitation; HCC, hepatocellular carcinoma; HTVi, hydrodynamic tail vein injection; WT, wild-type.



**Fig. 7. *BCL2L12* expression in human HCC samples.**

(A) qPCR analysis of *BCL2L12* mRNA levels in HCC (n = 64) and corresponding non-tumorous SL (n = 64). (B) qPCR analysis of *BCL2L12* mRNA levels in HCCB (n = 27) and HCCP (n = 37). (C, D) Evaluation of the relation between *TAZ* and *YAP* mRNA with *BCL2L12* mRNA in HCC samples. *p* values and correlation *r* values were calculated by Pearson correlation analysis. (E) Representative immunohistochemical patterns of *BCL2L12* in human non-tumorous and neoplastic liver specimens. Scale bar: 100  $\mu$ m. (F) Scheme summarizing the distribution of positive and negative HCC samples for c-MYC, TAZ, YAP, and *BCL2L12* staining. (A, B) Mean  $\pm$  SD; U paired *t* test; (C, D) Pearson correlation coefficient. HCC, hepatocellular carcinoma; HCCB, HCC with better prognosis; HCCP, HCC with poorer prognosis; qPCR, quantitative real time PCR; SL, surrounding liver.





**Fig. 8. Targeting TAZ as an effective therapeutic strategy for c-Myc-induced hepatocarcinogenesis.**

(A) Study design. *Yap<sup>flox/flox</sup>* and *Taz<sup>flox/flox</sup>* mice were injected with c-Myc/TTRpro-CreER<sup>T2</sup> plasmids. Mice (n = 6) were sacrificed at 5 w.p.i as a pretreatment group, while the remaining mice were injected with TAM or vehicle. All mice were sacrificed when moribund or at the end of observation. (B, C) Survival curves of *Yap<sup>flox/flox</sup>* (B) and *Taz<sup>flox/flox</sup>* (C) mice in vehicle- or TAM-treated groups. (D) Western blotting showing the expression of c-MYC and TAZ in *Taz<sup>flox/flox</sup>* mice. GAPDH was used as a loading control. (E) Representative images of *Taz<sup>flox/flox</sup>* murine liver tumors, H&E staining, and IHC staining of Ki67, c-MYC, and C-C3 in the 4 groups. (Top panel) Pretreatment group (Pre, 5 w.p.i); (second panel) TAM-treated groups harvested at the early stage (TAM early; 7 w.p.i); (third panel) TAM-treated groups harvested at a late stage (TAM late; 16

w.p.i); (Bottom panel) Vehicle-treated group (7 w.p.i). (B, C) Log-rank (Mantel-Cox) test. Scale bar: 200  $\mu$ m. C-C3, cleaved-caspase 3; HTVi, hydrodynamic tail vein injection; IHC, immunohistochemistry; TAM, tamoxifen; w.p.i., weeks post-injection.

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