Yes-associated protein regulation of adaptive liver enlargement and hepatocellular carcinoma development in mice.

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ABSTRACT

The Hippo kinase cascade, a growth-suppressive pathway that ultimately antagonizes the transcriptional coactivator Yes-associated protein (YAP), has been shown in transgenic animals to orchestrate organ size regulation. The purpose of this study was to determine whether in non–genetically modified mice (1) the Hippo pathway is involved in the regulation of adaptive liver enlargement caused by the mitogen 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), an agonist of constitutive androstane receptor and (2) a dysregulation of this pathway occurs during the development of chemically induced hepatocellular carcinoma (HCC). We show that liver enlargement caused by TCPOBOP was associated with an increase of YAP protein levels that paralleled the increase in 2-bromodeoxyuridine incorporation. Interestingly, when a second dose of TCPOBOP was given to mice with enlarged livers, no further increases in liver mass or YAP protein levels were observed, suggesting that the Hippo pathway prevents further growth of the hyperplastic liver. Viral-mediated exogenous expression of active YAP in mouse livers was able to partially overcome the block of hepatocyte proliferation. We also show that HCCs developed in mice given diethylnitrosamine and then subjected to repeated treatments with TCPOBOP had increased levels of YAP that were associated with down-regulation of microRNA 375, which is known to control YAP expression, and with enhanced levels of alpha-fetoprotein and connective tissue growth factor, two target genes of YAP.

ARTICLE

How organ growth is regulated and ceases when a tissue has reached its correct size is currently not understood. Notably, although growth of a mammalian organism is for the most part irreversible and the final size reached by an organism can be affected only during development, adaptive enlargement of organs appears to be completely reversible. The liver, for example, remains in a quiescent state in adult organisms but, under certain conditions, shows a remarkable regenerative capacity. Indeed, following a two-thirds surgical resection, a burst of proliferation occurs, and most of the liver size is regained within 3 to 4 days.1, 2 After the initial growth, no further enlargement of the liver is observed, suggesting the existence of pathways leading to termination of liver regeneration. Although some studies have initially proposed transforming growth factor β as the terminator of regeneration,3 no clear evidence has been reported. Even more impressive is the capacity of the liver to modify its size in response to physiological stimuli (such as hepatic

enlargement during pregnancy) or in response to xenobiotics with mitogenic potency. Under the latter condition, the liver can double its size in a few days, but undergoes a rapid regression following mitogen withdrawal, indicating that the liver recognizes its correct size and rapidly regresses to it, activating an apoptotic program.[4,5] Notably, even in spite of the continuous presence of growth inducers, the liver of non–genetically modified rodents never exceeds doubling of its mass, indicating that a precise regulation of tissue size must exist to prevent its further growth, likely incompatible with the survival of the organism.

Many xenobiotics able to induce liver enlargement are ligands of nuclear receptors of the steroid/thyroid receptor superfamily[6] and, interestingly, are also liver nongenotoxic carcinogens.[7–9] In spite of several studies, the key molecular events that govern the tumoral potency of ligands of nuclear receptors are still unclear. The breakthrough that many compounds with liver tumor–promoting ability are also potent inducers of hepatocyte proliferation led to the hypothesis that the mechanisms by which these agents cause liver neoplasia are a consequence of their mitogenic capacity that ultimately results in an increased rate of mutation.[10] However, this hypothesis has been questioned by the findings that the proliferative response of the liver to these mitogens is lost very shortly,[11, 12] suggesting that the hyperplastic liver becomes refractory to further mitogenic stimuli. These findings also suggest that the tumors arising in these enlarged livers may be the consequence of the escape of genetically damaged cells from the regulatory mechanisms governing the size of the organ. Thus, the identification of the molecular mechanisms responsible for the refractoriness of the enlarged liver to further mitogenic stimuli is critical for improving our knowledge of the control of organ size, and also for determining whether dysregulation of these pathways is a possible mechanism for the clonal expansion of resistant hepatocytes and their progression to hepatocellular carcinoma (HCC).

Recent studies in both *Drosophila* and mammals have implicated the Hippo signaling pathway as a potent regulator of organ size and tissue homeostasis.[13, 14] The mammalian Hippo cascade inactivates its primary effector Yes-associated protein (YAP) by promoting its cytoplasmic localization in an S127 phosphorylation-dependent manner, whereas loss of Hippo signaling leads to nuclear accumulation and therefore increased activity of YAP, which binds to transcription factors and regulates transcription of target genes involved in cell growth, proliferation, and survival. Using a conditional YAP transgenic mouse model, it was shown that overexpression of YAP in mice leads to HCC development, suggesting a direct link between dysregulation of the Hippo size-control pathway and liver tumorigenesis.[15–17] Moreover, recent work demonstrated the role of Mst1/2 kinases as tumor suppressors by showing that combined deficiency of *Mst1/2* kinases leads to loss of the inactivating phosphorylation of YAP, massive liver overgrowth, and development of HCC.[18–20] Interestingly, in humans, amplification of the chromosomal region containing the YAP gene (11q22) has been reported in several tumor types.[21, 22]

On the basis of these findings, we investigated whether (1) the Hippo pathway plays a critical role in the termination of the xenobiotic-induced liver overgrowth and (2) this pathway is defective in cancer cells arising in hyperplastic livers.

Abbreviations

AFP, alpha-fetoprotein; BrdU, 2-bromodeoxyuridine; CAR, constitutive androstane receptor; cDNA, complementary DNA; CTGF, connective tissue growth factor; Cyp2b10, cytochrome 2b10; DENA, diethylnitrosamine; HCC, hepatocellular carcinoma; miR-375, microRNA 375; mYAP, YAP mutated in Ser^{127} and Ser^{381}; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; TCPOBOP, 1,4-bis(2-(3,5-dichloropyridyloxy)benzene; YAP, Yes-associated protein.

MATERIALS AND METHODS

Animals.

C3H or CD-1 female mice (8 weeks old) were obtained from Charles River (Milano, Italy). All experiments were performed in accordance with the Universities Federation for Animal Welfare Handbook on the Care and Management of Laboratory Animals and the guidelines of the animal ethics committee of the University of Cagliari.

Induction of Liver Cell Proliferation.

In experimental protocol 1 (Fig. 1A), hepatocyte proliferation was induced by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (3 mg/kg body weight, dissolved in dimethyl sulphoxide–corn oil solution; Sigma-Aldrich, Milan, Italy). Controls received an equivalent amount of the vehicle. To determine the proliferative response of the liver to TCPOBOP, mice were given 2-bromodeoxyuridine (BrdU) dissolved in drinking water (1 mg/mL; Sigma-Aldrich, Milan, Italy) and sacrificed 1 week later. In experimental protocol 2 (Fig. 2A), mice were treated as in protocol 1 except that they were sacrificed 24 hours, 36 hours, and 1 week after one dose or 24 and 36 hours after two doses of TCPOBOP. BrdU dissolved in drinking water was given as shown in Fig. 2A.

Generation of Lentiviral Vectors, Lentivirus Production, and Mice Injection.

The p2xFlag CMV-YAP vector (a kind gift from M. Sudol) was digested with *EcoRI*, blunted, and the entire YAP complementary DNA (cDNA) was moved in the *EcoRV* site of the lentiviral vector p156RRLsin.PPTh CMV.MCS.pre. Lentiviruses were produced as described.23 The concentration of viral p24 antigen was assessed using the HIV-1 p24 core profile enzyme-linked immunosorbent assay kit (NEN Life Science Products) according to the manufacturer's instructions. For *in vivo* studies, viral particles of mutated YAP (mYAP) (Ser^{127}Ala and Ser^{381}Ala) and control vector were purified by way of ultracentrifugation and suspended in sterile, endotoxin-free phosphate-buffered saline. Viral particles (20 μg of purified p24/mice in 400 μL phosphate-buffered saline)

were injected into the tail vein of CD-1 mice 3 days after the first and 4 days prior to the second TCPOBOP administration (Fig. 3A).

Chemically Induced Liver Carcinogenesis Protocol.

A single intraperitoneal dose of diethylnitrosamine (DENA) (90 mg/kg body weight; Sigma) was injected, followed 1 week later by TCPOBOP administration (3 mg/kg) once a week for 27 weeks. Two control groups, one receiving DENA alone, the other treated with TCPOBOP alone for 27 weeks, were also included. All mice received BrdU in drinking water for 3 days before being sacrificed (Fig. 4A).

Immunohistochemistry

BrdU Staining.

BrdU was stained with a mouse antibody from Becton Dickinson (San Jose, CA) as described.24 Labeling index was expressed as the number of BrdU-positive hepatocyte nuclei per 100 nuclei. Results are expressed as the mean ± SD. At least 2,500 hepatocyte nuclei for each liver were scored.

YAP Staining.

Tissue sections were subjected to Target Retrieval Solution (Dako, Glostrup, Denmark) and exposed to four cycles at 700 W in a microwave oven. After washing with Dako Wash Buffer, endogenous peroxidase was blocked with Dako Blocking Buffer for 5 minutes at room temperature. The sections were incubated with the polyclonal antibody anti-YAP (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for 60 minutes at a dilution of 1:100. The final reaction was visualized using 3,3'-diaminobenzidine.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Analysis of MicroRNA-375.

Total RNA was extracted from frozen liver samples using Trizol Reagent (Invitrogen). cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit. Quantitative reverse-transcription polymerase chain reaction (PCR) amplification was performed with the reverse-transcription product TaqMan 2X Universal PCR Master Mix, No AmpErase UNG, mmu–microRNA 375 (miR-375) primers, and probe mix (Applied Biosystems). The endogenous control snoRNA202 was used to normalize microRNA expression levels.

Analysis of alpha-Fetoprotein, Birc5, Cytochrome 2b10, and Connective Tissue Growth Factor.

Two micrograms of total RNA, extracted with an RNeasy Plus Mini Kit (Qiagen), was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA

together with TaqMan Gene Expression Master Mix, alpha-fetoprotein (AFP), Birc5, cytochrome 2b10 (Cyp2b10), connective tissue growth factor (CTGF) primers, and probe mix (Applied Biosystems) were used to perform quantitative reverse-transcription PCR amplification. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous normalizer.

Western Blot Analysis

Total cell and nuclear extracts were prepared from frozen livers as described.24 For immunoblot analysis, equal amounts (100 to 150 μg/lane) of protein were electrophoresed on 12% or 8% sodium dodecyl sulfate–polyacrylamide gels. Membranes were incubated with primary antibodies and then with either anti-mouse or anti-rabbit horseradish peroxidase–conjugated immunoglobulin G (Santa Cruz Biotechnology). Immunoreactive bands were identified with chemiluminescence detection systems (Supersignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL).

Antibodies

For immunoblotting experiments, mouse monoclonal antibodies directed against actin (AC40) (Sigma-Aldrich), cyclin D1(72-13G), and proliferating cell nuclear antigen (PCNA) (PC-10) (Santa Cruz Biotechnology) were used. Rabbit polyclonal antibodies against YAP and phosphorylated YAP (Ser^{127}) were purchased from Cell Signaling Technology (Beverly, MA).

Statistical Analysis

Comparison between the treated and control group was performed with a nonparametric Mann-Whitney test.

RESULTS

Enlarged Livers Become Rapidly Refractory to Further Growth Stimuli.

As schematically represented in Fig.1A, mice were injected with a single dose of TCPOBOP or oil and sacrificed 1 week later. As shown in Fig. 1B, a single dose of TCPOBOP elicited a massive enlargement of the liver that doubled 7 days after mitogen administration (liver weight/body weight 10.96% versus 5.31% in controls). Liver enlargement was due, at least in part, to hepatocyte proliferation, as shown by the striking increase in BrdU incorporation (labeling index 43.95% versus 3.83% in controls) (Fig. 1C). Administration of a second dose of TCPOBOP 1 week after the first treatment (Fig. 1A, bottom), a time when the liver was twice the size of the normal adult liver, did not cause any further enlargement of the organ (liver weight/body weight 11.39% versus 10.96%) (Fig. 1B). Accordingly, no increase in BrdU incorporation was observed in this group (5.91% versus 43.95% of mice treated with only one dose; 3.83% in controls) (Fig. 1C). Because most of the effects of TCPOBOP are mediated by binding and activation of constitutive androstane receptor (CAR), we considered the possibility that the lack of proliferative response of the enlarged liver could be due to down-regulation or functional inactivation of CAR. We thus evaluated the

expression of Cyp2b10, a specific CAR target gene (Fig. 1D), and found that Cyp2b10 expression was increased almost 30-fold over control values both after the first as well as the second dose of TCPOBOP, thus showing that CAR was active in both the conditions.

These results suggest that the refractoriness of the enlarged liver to a second mitogenic stimulus is not due to lack of CAR transcriptional activity, but likely to the ability of the liver to sense its oversize and to trigger pathways aimed at inhibiting further growth.

YAP Levels Are Increased in Mouse Hepatocytes Treated with 1 Dose of TCPOBOP.

To determine whether dysregulation of the Hippo pathway was involved in the initial mitogenic response elicited by TCPOBOP, we treated the animals with either oil or TCPOBOP and sacrificed them 24 and 36 hours or 1 week after one dose, and 24 and 36 hours after two doses of TCPOBOP (Fig. 2A). As shown in Fig. 2B and 2C, BrdU incorporation was significantly increased 24 and 36 hours after a single dose of TCPOBOP, and returned to basal values 1 week after treatment. The increased labelling index was associated with increased protein levels of cyclin D1, cyclin A, and PCNA (Fig. 2D). Notably, YAP levels were also increased at these time points, indicating a dysregulation of the Hippo pathway during TCPOBOP-induced hepatocyte proliferation; the return of YAP levels to control values 1 week after treatment suggests reactivation of the Hippo pathway, leading to block of hepatocyte proliferation. Notably, when a second dose of TCPOBOP was administered 1 week after the first dose, namely at a time when the size of the liver was twice that of controls (Fig. 2A, lower part), only a very slight increase of BrdU incorporation (Fig. 2B,C) and YAP expression (Fig. 2D) was observed between 24 or 36 hours after mitogen administration. Accordingly, coactivating activity of YAP evaluated by measuring the expression levels of Birc-5/survivin, a target gene of YAP,17 revealed a 20-fold increase in the expression of this gene in the livers of mice sacrificed 24 hours after the first dose, but only a two-fold increase after the second dose of TCPOBOP (Fig. 2E). These findings suggest that YAP transitions from an active to an inactive state and is involved in the refractoriness of enlarged livers to a second mitogenic challenge. In this context, it is noteworthy that almost no residual hepatocyte proliferation occurs in mice exposed to the second dose of TCPOBOP after the first 36 hours, as also indicated in Fig. 1C.

Infection with an Active Form of YAP Overrides the Termination of Liver Growth.

To further investigate the role of the Hippo pathway in mitogen-induced liver enlargement, we cloned in a lentiviral vector an active form of YAP ($Ser^{127-381}$Ala, mYAP)25, 26 lacking the phosphorylation sites needed for its sequestration in the cytoplasm. Infection of mouse livers with mYAP 4 days before the second dose of TCPOBOP (Fig. 3A,B) did override the physiological termination of liver growth. Indeed, whereas no proliferation was observed after the second dose of TCPOBOP in mice transduced with control vector, a significant increase of cell proliferation was

observed when TCPOBOP was given to mice infected with the active YAP (6.54% of BrdU-positive nuclei versus 16.31%; $P < 0.01$) (Fig. 3,C,D). To investigate whether injection of mYAP could *per se* lead to hepatocyte proliferation regardless of TCPOBOP administration, we injected untreated mice with YAP lentiviruses and compared the proliferative response of hepatocytes with that of mice receiving two administrations of TCPOBOP, in the presence or absence of exogenous YAP (Fig. 3E). A further experimental group was treated with control lentiviruses. As shown in Fig. 3F, transduction of hepatocytes with YAP alone induced only a slight and not statistically significant increase of BrdU incorporation over control values. On the other hand, liver transduction with YAP of mice treated with TCPOBOP led to a highly significant increase in proliferation compared with TCPOBOP-treated mice transduced with control virus. These experiments strongly suggest that the Hippo pathway is involved in the refractoriness of enlarged liver to further mitogenic stimuli.

The Hippo Pathway Is Dysregulated in HCC.

Overexpression of YAP has been observed in many human tumors, enough that it is considered a candidate oncogene.22, 27 Despite the fact that TCPOBOP-induced enlarged livers do not grow further once they reach a certain size, they nevertheless develop HCC upon an initiating dose of a chemical carcinogen followed by repeated treatments with the mitogen.8, 28 We thus hypothesized that carcinogen-induced mutations could favor the escape of resistant cells from the growth-suppressive environment regulating normal hepatocytes, thus allowing them to proliferate and progress to HCC. To determine the status of the Hippo pathway in HCC, we adopted an experimental protocol wherein mice were given an initiating dose of DENA, followed by repeated injections of TCPOBOP for 27 weeks (Fig. 4A). Control mice were given DENA or TCPOBOP alone. As shown, the livers of mice treated with 27 injections of TCPOBOP were only twice that of controls, confirming the existence of a strict regulation of liver size (Fig 4B,C). Whereas at the time of sacrifice, control mice and animals treated with TCPOBOP alone were completely devoid of tumors, livers from all mice exposed to DENA+TCPOBOP exhibited multiple tumors (Fig. 4C), which on histological examination showed nuclear atypia, cellular pleomorphism, and increased trabecular size and were therefore classified as medium- to high-grade HCCs (Fig. 4D). All tumors showed a high proliferative rate as detected by way of BrdU immunohistochemistry (Fig. 4E); conversely, only a negligible proliferative activity was observed in nontumoral areas of the liver or in the liver from mice treated with TCPOBOP or DENA alone (Fig. 4E).

Western blot analysis on total cellular lysates (Fig. 5A) of 21 HCCs, revealed in most of the tumors a significant increase in the levels of YAP compared with those of mice treated with TCPOBOP alone or DENA alone. Notably, a remarkable increase of YAP levels was observed in the nuclear fraction of randomly selected HCCs (5B, top). Accordingly, immunohistochemical staining revealed the presence of several YAP-positive cells in the tumors (Fig. 5C), whereas no positive hepatocytes were observed in the livers of mice treated with DENA or TCPOBOP (data not

shown). Notably, YAP was localized mainly in the nucleus of tumoral hepatocytes, although a cytoplasmic localization was also observed. No major changes of phosphorylated YAP were detected in the cytosolic fractions between tumors and normal or hyperplastic livers (Fig. 5B, bottom).

To prove that YAP was indeed more active in HCCs, we evaluated the level of expression of two other genes that are direct transcriptional targets of YAP, namely AFP and CTGF.[15, 17] As shown in Fig. 6A,B, we found that the expression of these two genes was up-regulated in HCCs, 100% of the tumors showing increased expression of AFP and 60% exhibiting increased levels of CTGF.

miR-375 Is Down-regulated in HCC.

It was shown recently that miR-375 regulates the expression of YAP and is down-regulated in human HCC.[29] To verify whether down-regulation of miR-375 is associated with increased YAP expression in mouse HCC, we performed a real-time PCR analysis of miR-375 expression in 21 HCCs developed in DENA+TCPOBOP–treated mice and in livers from animals treated with DENA or TCPOBOP alone. Fig.6C shows that miR-375 was significantly down-regulated in HCC (17/21) ($P < 0.01$) and was inversely correlated with the protein levels of YAP (Fig. 5A). These findings support the notion that YAP is a direct target of miR-375, and suggests a role for miR-375 in the increased expression of YAP in HCCs.

DISCUSSION

This study shows that the Hippo pathway is involved in the control of direct hyperplasia induced by the CAR ligand TCPOBOP and is impaired in chemically induced HCC. The most significant findings are: (1) YAP activation is associated with CAR-induced hepatomegaly; (2) increased expression and nuclear translocation of YAP occurs in HCC; and (3) enhanced expression of YAP is associated with down-regulation of miR-375.

Recent studies, both in *Drosophila* and mammals, have implicated the Hippo signaling pathway as a potent regulator of organ size and tissue homeostasis. Moreover, recent studies have shown that overexpression of YAP[15] and combined *Mst1/2* deficiency[15–17] lead to massive liver overgrowth and development of HCC. These studies employed genetically modified animal models in which dysregulation of the transcriptional control of the Hippo pathway occurs in all hepatocytes.

The first question we asked was whether in nontransgenic mice, the Hippo pathway is involved in the adaptive liver enlargement that follows treatment with the CAR agonist, TCPOBOP, a well-known inducer of hepatocyte proliferation. Our study demonstrates that liver enlargement caused by TCPOBOP is associated with a temporary inactivation of the suppressive action of the Hippo pathway; indeed, increased levels of YAP paralleled the enhanced hepatocyte proliferation. Notably, a return to basal levels of YAP occurred 1 week after TCPOBOP treatment, a time when

proliferation had ceased; this suggests that the Hippo pathway is reactivated once the organ has reached a mass that is twice that of control liver.

We showed that a second treatment with TCPOBOP did not lead to further increase of the liver, demonstrating that this organ rapidly senses its oversize and activates mechanisms to inhibit additional growth. This was not due to lack of CAR activation by TCPOBOP, because the expression of the CAR target gene Cyp2b10 was significantly increased after the second dose of TCPOBOP, when proliferation was not observed. An important role in establishing the refractoriness of enlarged livers to further mitogenic stimuli might be played by the Hippo pathway. Indeed, the lack of proliferative response was associated with no increase of total YAP protein levels and, consequently, of no activation of YAP, as shown by the lack of increase of survivin mRNA levels; moreover, transduction of enlarged livers with activated YAP (mutated in $Ser^{127,381}$) partially reverted the proliferative block observed after the second dose of TCPOBOP and allowed hepatocyte proliferation.

Accumulating evidence of YAP up-regulation in diverse tumor types[27] suggests that the inactivation of the Hippo pathway allows cancer cells to evade the intrinsic size control mechanisms that normally maintain tissue homeostasis. The present study was aimed at investigating whether a dysregulation of the Hippo/YAP circuit occurs during the development of chemically induced mouse HCC. The rationale for this study stemmed from the finding that following the first treatment with TCPOBOP (which caused the doubling of the liver size), no further liver enlargement was observed after 2-27 injections of the drug (compare the relative liver weight in Fig. 1B and 4B). This finding clearly indicates that a growth-suppressive environment was generated in hyperplastic livers, preventing their further growth. On the other hand, the development of HCC in all mice given the genotoxic agent DENA prior to TCPOBOP suggests that initiated/mutated cells have escaped the growth-suppressive signals, thus clonally expanding to develop HCC. Our finding of increased YAP protein expression and its nuclear translocation in HCC cells suggests that dysregulation of the Hippo pathway may contribute to the escape from the environmental growth-suppressive constraint; it is noteworthy that a strong and increased nuclear YAP staining has been observed in human tumors, including HCC.[27, 30] In this study, we show that YAP nuclear translocation is accompanied by its increased activity because, in the same tumors, up-regulation of AFP and CTGF, two YAP target genes,[15, 17] was observed. Taken together, our findings suggest that YAP dysregulation could be involved in the development of DENA+TCPOBOP-induced HCC.

MicroRNAs have recently emerged as important modulators of gene expression in cancer,[31] including human HCC.[32] Very recently, Liu et al.[29] reported that miR-375 is a negative regulator of YAP; indeed, they found down-regulation of miR-375 in tumor tissues of HCC patients, which was accompanied by increased YAP levels. Moreover, they showed that miRNA-375 re-expression caused a severe decrease of YAP protein levels. In accordance with these results, we

found a decrease of miR-375 and an increase in YAP content in approximately 70% of mouse HCCs. Our data thus provide a possible mechanism underlying the increase of YAP in chemically induced HCCs. Whether down-regulation of miR-375 is due to epigenetic modifications is presently unknown and warrants further investigation, because modulation of this microRNA could be therapeutically targeted to reactivate the growth-suppressive effect of the Hippo pathway.

A better understanding of growth regulatory mechanisms may represent an important approach from a therapeutic point of view. HCC, the fifth most common malignant neoplasm and the third most frequent cause of cancer-related death worldwide, represents a major health problem.33, 34 A better definition of the molecular pathogenesis of HCC could have a significant impact on the development of new treatment strategies. The Hippo kinase cascade might have clear pathogenic implications in hepatocarcinogenesis, and its drivers might represent novel targets for molecular therapies. Although our present data do not establish a causal relationship between YAP activation and HCC development, the outcome of this study suggests nevertheless that the Hippo pathway can play an important role in HCC development also in non–genetically manipulated animals; moreover, it further supports the notion that pathways governing tissue overgrowth should be deeply explored as potential therapeutic targets in human HCC.

ACKNOWLEDGEMENTS

We thank M. Sudol for providing the YAP cDNA.

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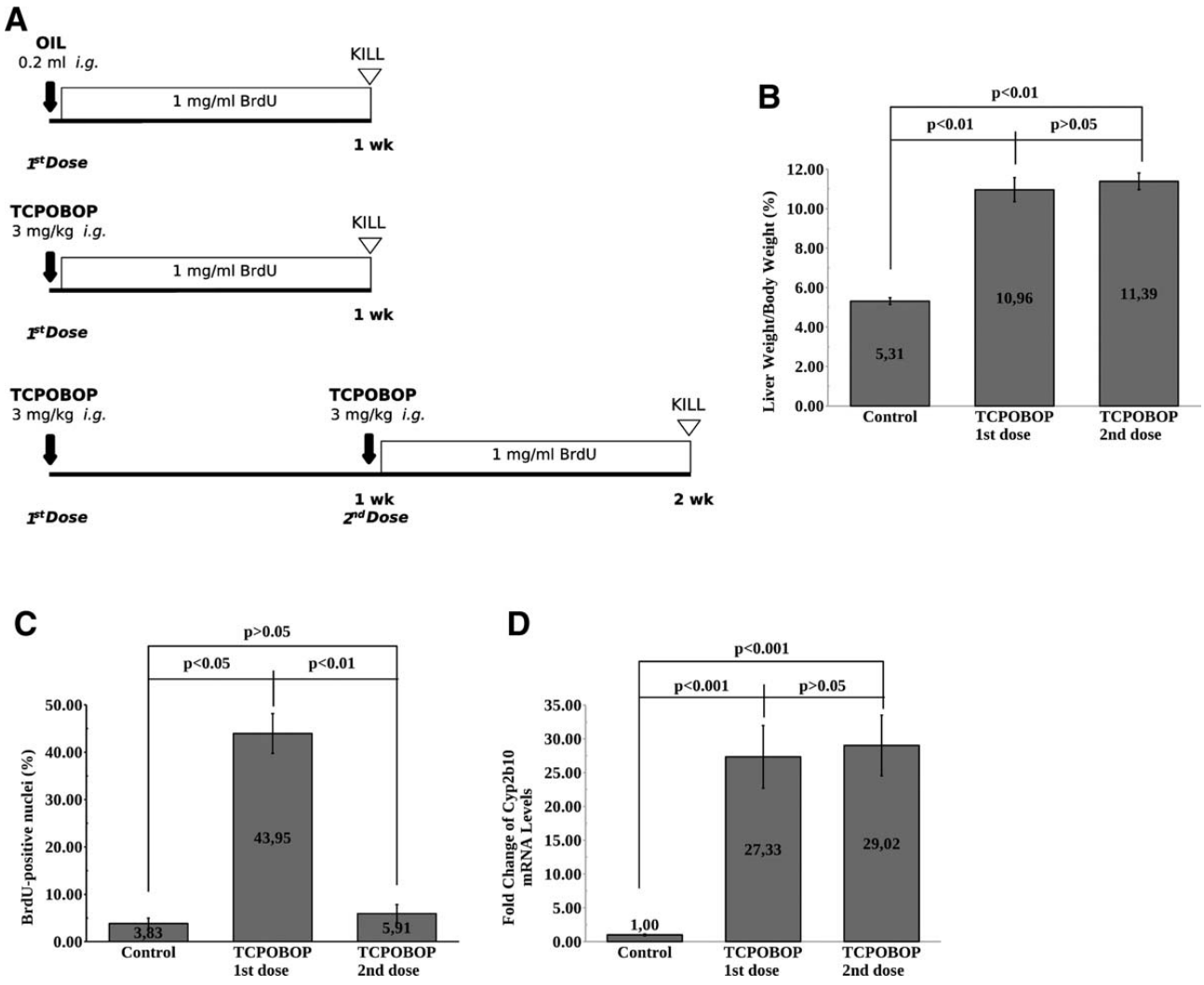


Fig. 1. (A) Experimental protocol. (B) Effect of one or two doses of TCPOBOP on relative liver weight (liver weight/body weight%) (C) and labeling index of hepatocytes . BrdU was given continuously in drinking water for 1 week after the first or second dose of TCPOBOP. Results are expressed as the mean 6 SD of four to five mice per group. (D) Real-time PCR analysis of Cyp2b10 in livers of mice sacrificed 24 hours after one or two doses of TCPOBOP. Results are expressed as the mean 6 SD of five mice per group.

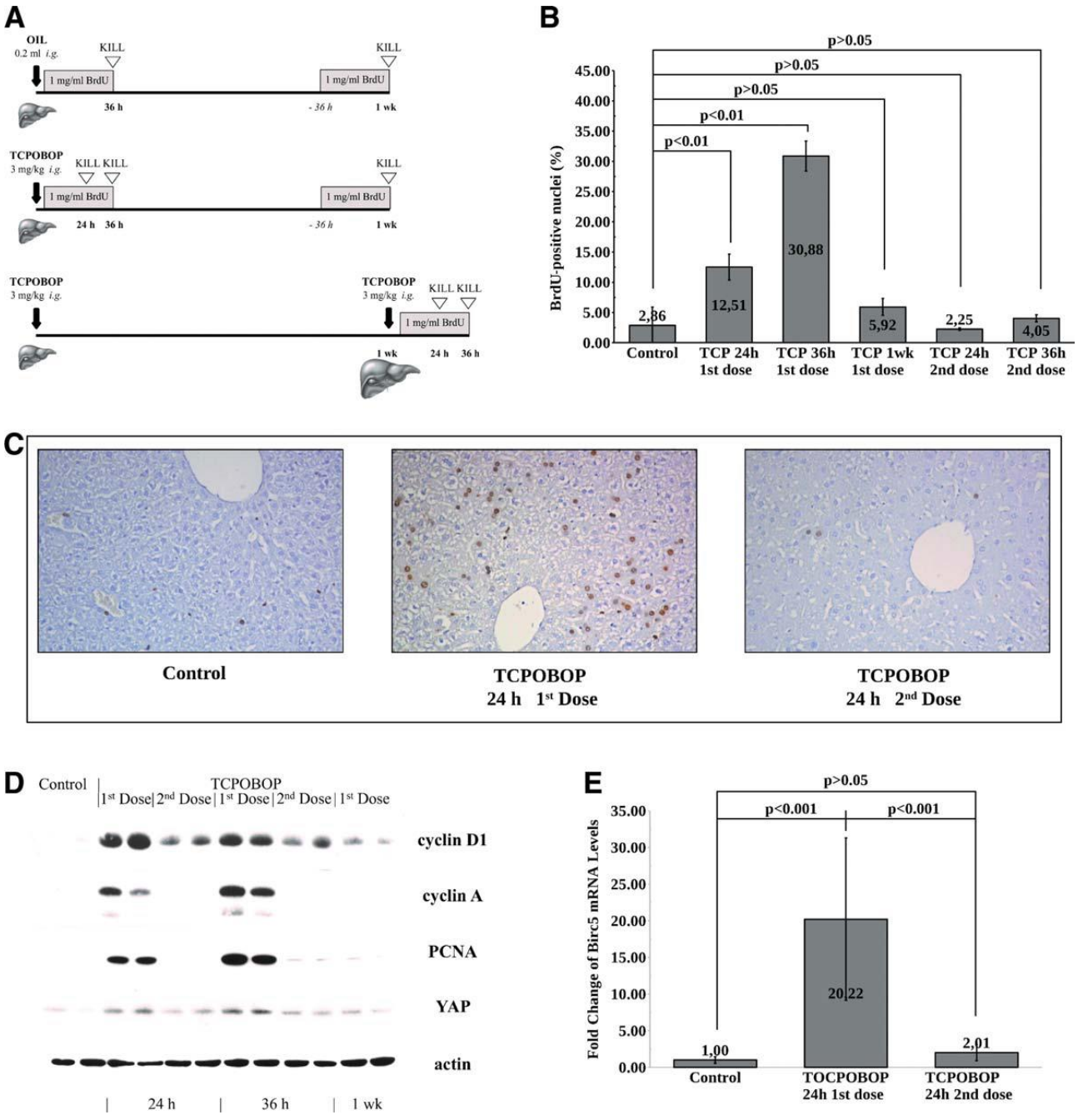


Fig. 2. (A) Experimental protocol. (B) Labeling index of hepatocytes from mice sacrificed 24 hours, 36 hours, and 1 week after one dose of TCPOBOP, or 24 and 36 hours after two doses. Results are expressed as the mean 6 SD of four to five mice per group. (C) Representative photomicrographs illustrating the presence of BrdU-positive hepatocytes 24 hours after one or two doses of TCPOBOP (original magnification _20). (D) Western blot analysis of cyclin D1, cyclin A, PCNA, and YAP in mice treated with one or two doses of TCPOBOP and sacrificed as indicated. Actin probing was used as a loading control. (E) Real-time PCR analysis of Birc-5/survivin in livers of mice sacrificed 24 hours after one or two doses of TCPOBOP.

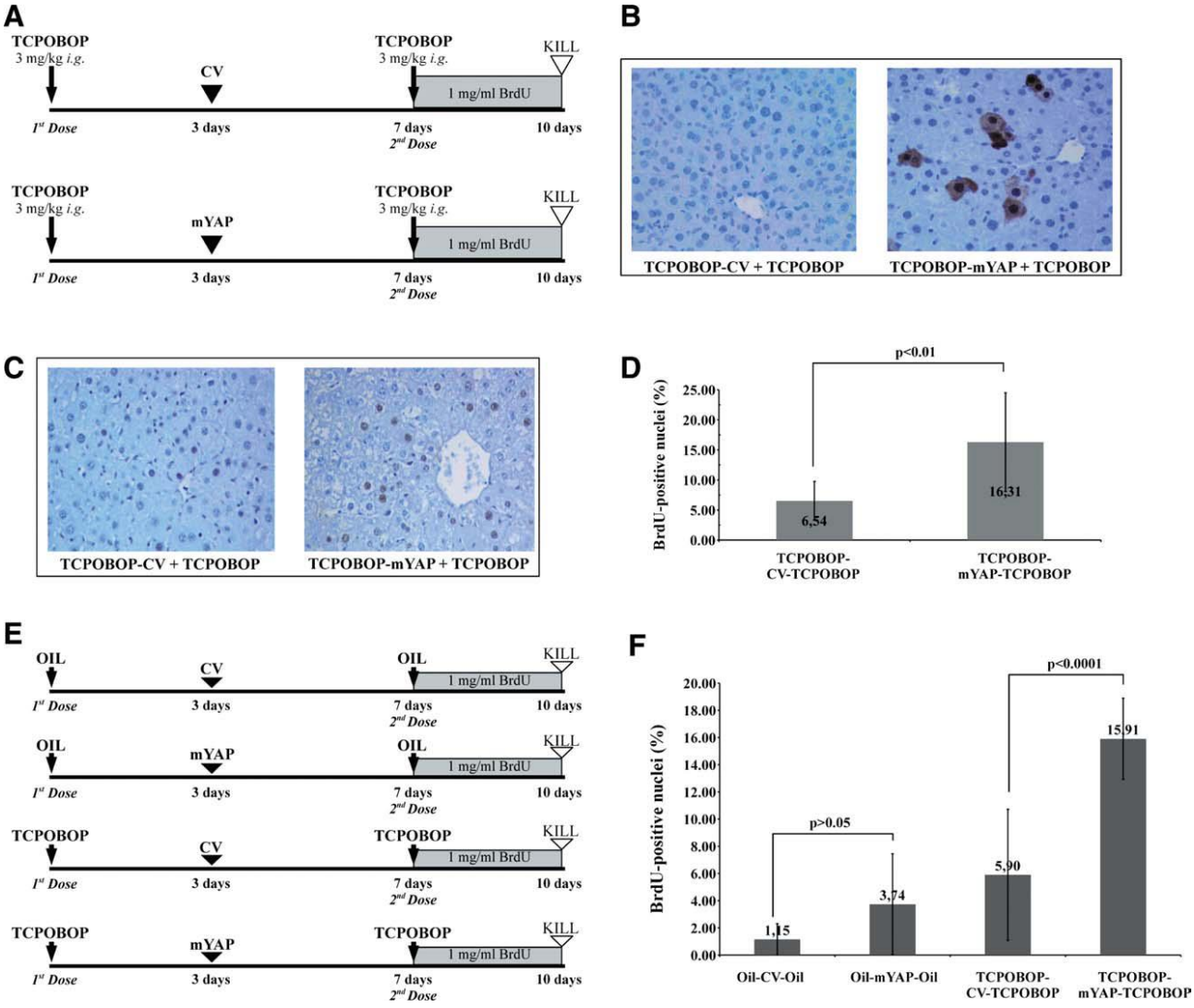
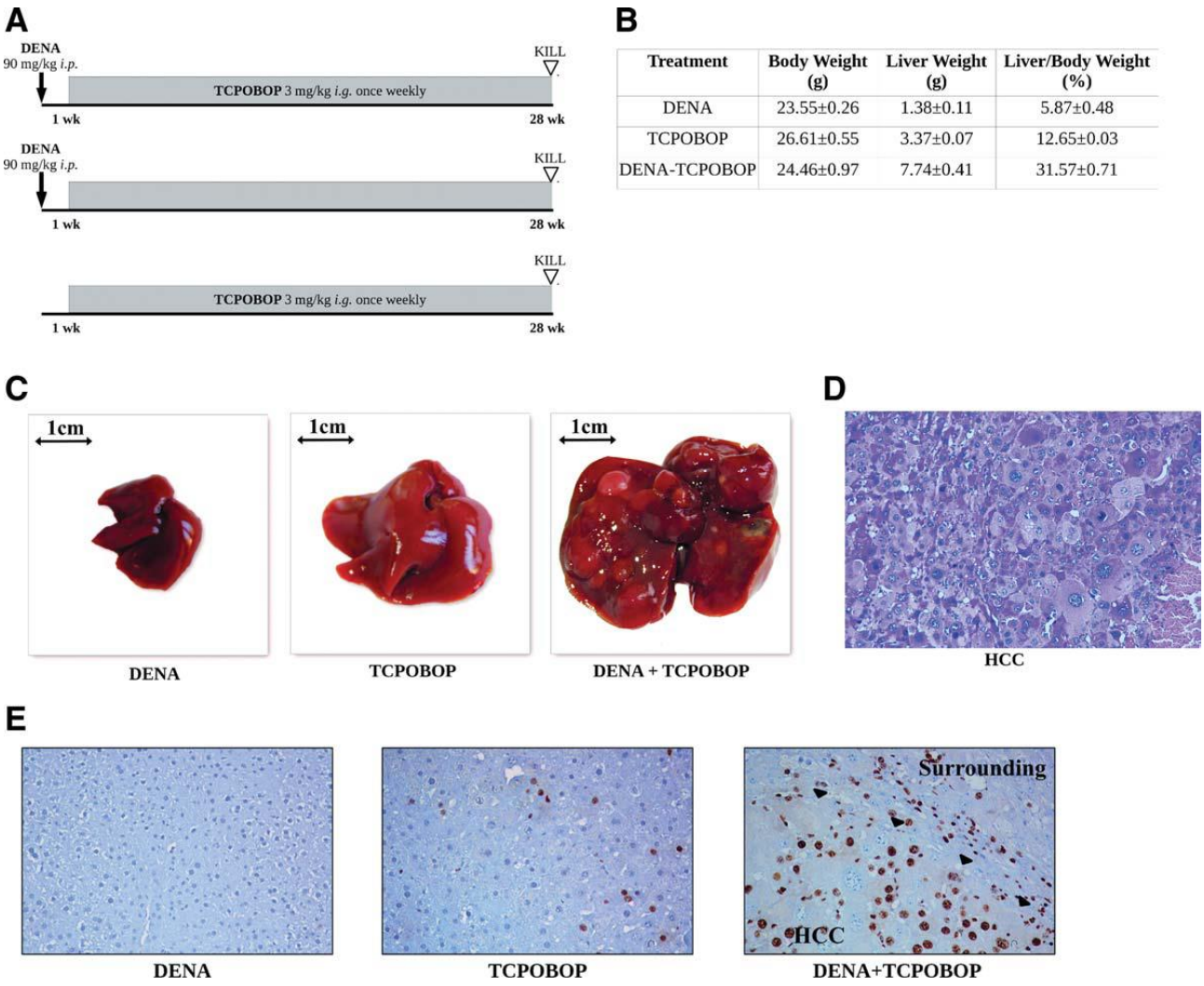


Fig. 3. (A) Experimental protocol 1. Mice were infected with lentiviruses carrying mYAP 4 days prior to and sacrificed 3 days after the second dose of TCPOBOP. (B) Representative photomicrographs illustrating the presence of transduced hepatocytes expressing mYAP (original magnification_20). (C) BrdU-positive hepatocytes 3 days after the second dose of TCPOBOP (original magnification _20). BrdU (1 mg/mL) dissolved in drinking water was given for 3 days after the second dose of TCPOBOP. (D) Labeling index. Results are expressed as the mean 6 SD of four mice per group. CV, control vector. (E) Experimental protocol 2. The experiment was performed as in (A) with the presence of two additional groups of mice not treated with TCPOBOP and injected with mYAP or control virus. (F) Labeling index. The graph shows the proliferative response observed in the livers. Results are expressed as the mean 6 SD of six mice per group. CV, control vector.



Treatment	Body Weight (g)	Liver Weight (g)	Liver/Body Weight (%)
DENA	23.55±0.26	1.38±0.11	5.87±0.48
TCPOBOP	26.61±0.55	3.37±0.07	12.65±0.03
DENA-TCPOBOP	24.46±0.97	7.74±0.41	31.57±0.71

Fig. 4. (A) Experimental protocol. Mice were given a single dose of DENA followed by treatment with TCPOBOP once a week for 27 weeks. (B) Body weight, liver weight, and liver weight/body weight ratio of mice treated as described in (A). (C) Livers of mice 28 weeks after DENA alone, TCPOBOP alone, or DENAþTCPOBOP. Several tumors are macroscopically evident. (D) Adenocarcinoma showing neoplastic cells arranged in thick layers, increased nuclear/cytoplasmic ratio, frequent pleomorphism including bizarre giant cells, and aberrant mitoses (hematoxylin and eosin stain; original magnification _20). (E) BrdU-positive hepatocytes in HCC (original magnification _20). Very few BrdU-positive hepatocytes are observed in the livers of mice given DENA or TCPOBOP alone and in the livers surrounding HCC.

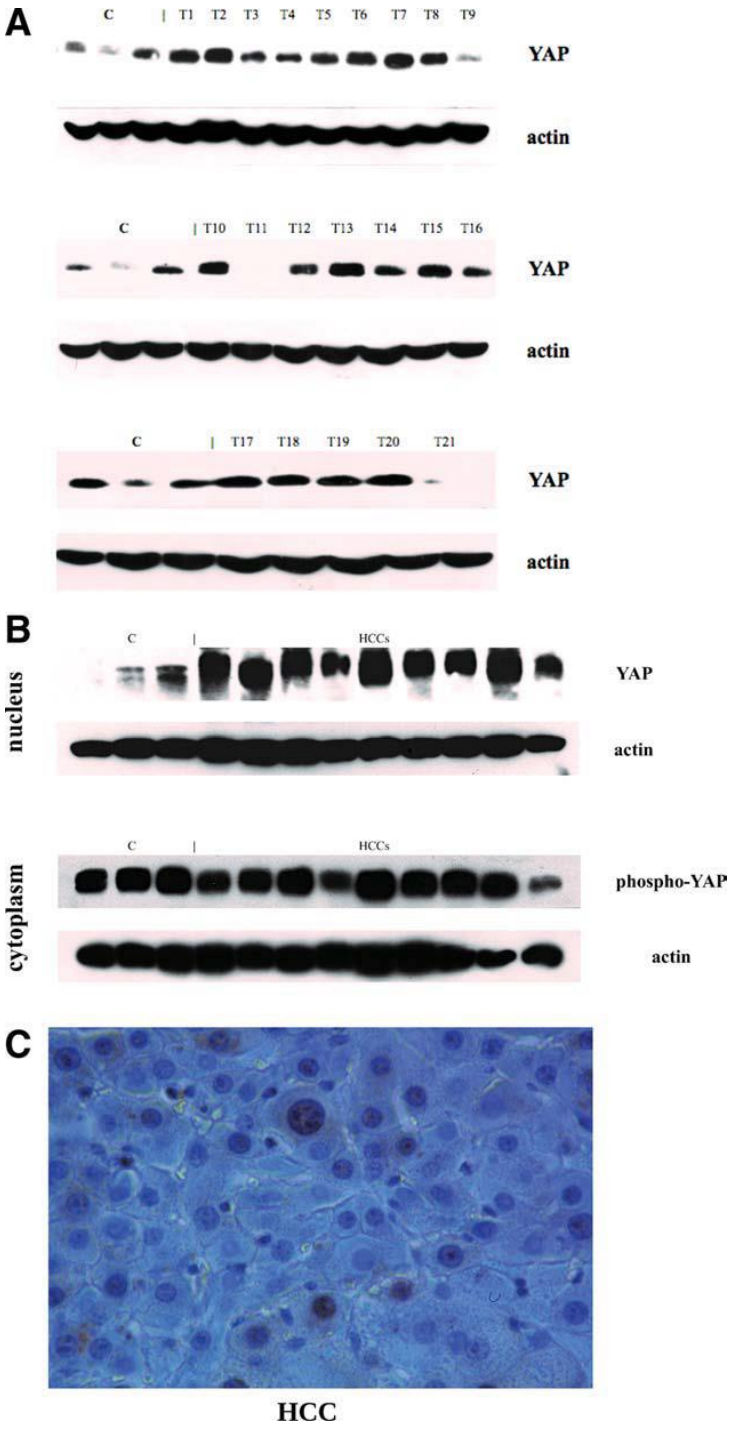


Fig. 5. (A) Western blot analysis of total YAP in HCC developed in mice treated with DENAþTCPOBOP. C, control livers (livers from DENA alone or TCPOBOP alone). (B) Western blot analysis of nuclear YAP and cytoplasmic phosphorylated YAP in HCCs. (C) Immunohistochemistry showing several nuclei positive for YAP in DENAþTCPOBOP-induced HCC.

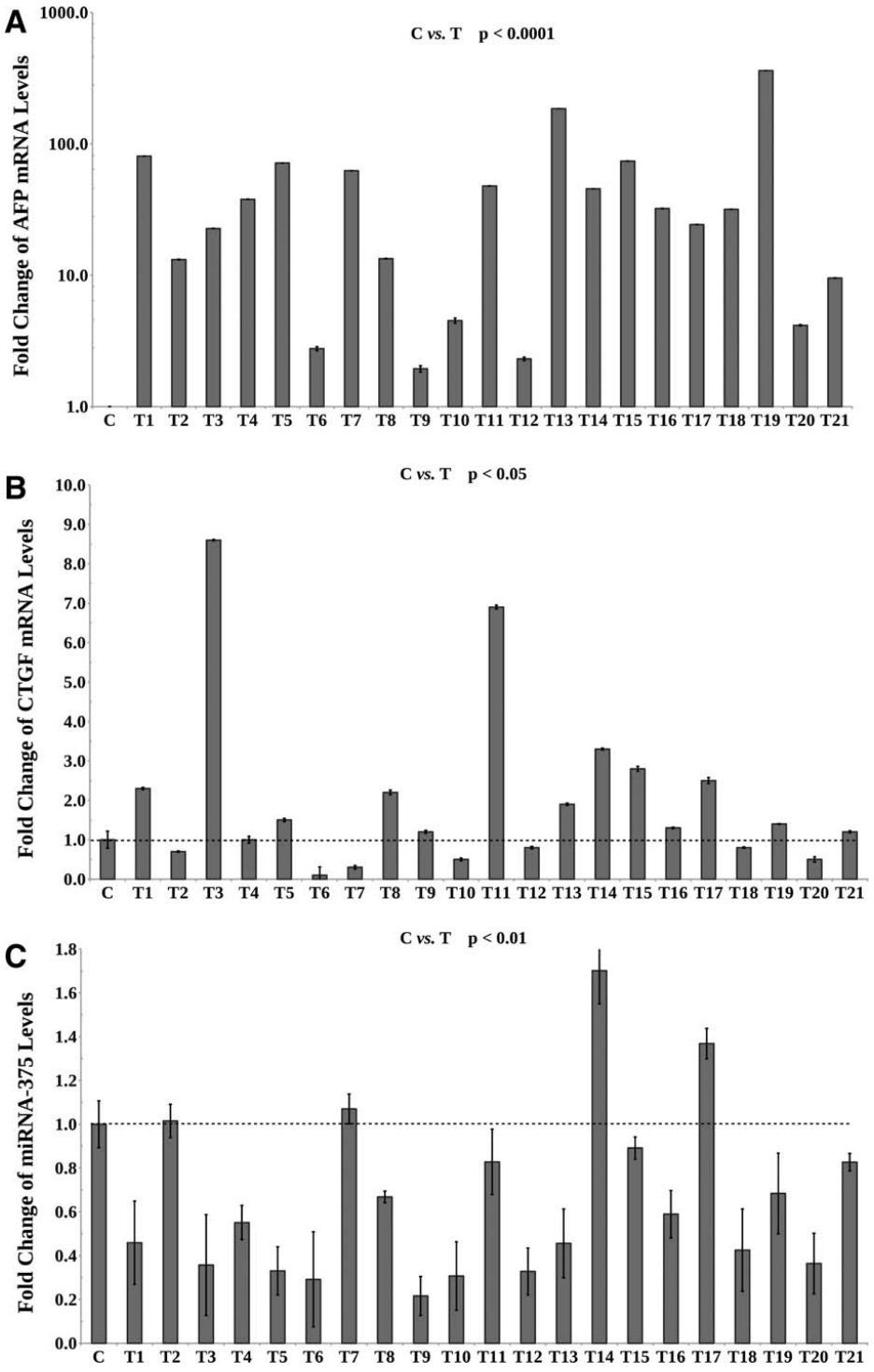


Fig. 6. (A,B) Real-time PCR analysis of AFP (A) and CTGF (B) in HCC or in the livers of mice given DENA or TCPOBOP alone. The values of AFP are reported as log fold change of HCC/control liver. (C) Expression levels of miR-375 were evaluated by way of TaqMan reverse-transcription PCR in mouse HCC and compared with control livers (C). The snoRNA202 was used as endogenous control. miR-375 was significantly down-regulated in HCC ($P < 0.01$).