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Mutual interaction between YAP and c-Myc is critical for carcinogenesis in liver cancer

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ABSTRACT

Yes-associated protein (YAP), the downstream effector of Hippo signaling pathway as well as c-Myc has been linked to hepatocarcinogenesis. However, little is known about whether and how YAP and c-Myc interacts with each other. In this study, we find YAP–c-Myc interaction is critical for liver cancer cell both in vitro and in vivo. Moreover, both c-Myc and YAP proteins are closely correlated in human liver cancer samples. Mechanistically, YAP promotes c-Myc transcriptional output through c-Abl. By contrast, c-Myc enhances protein expression independent of transcription. Taken together, our study uncovers a novel positive auto-regulatory feedback loop underlying the interaction between YAP and c-Myc in liver cancer, suggesting YAP and c-Myc links Hippo/YAP and c-Myc pathways, and thus may be helpful in the development of effective diagnosis and treatment strategies against liver cancer.

1. Introduction

Liver cancer is the fifth most common cancer worldwide and the third leading cause of cancer death [1]. The treatment options for these hepatic malignancies are extremely limited mainly because the mechanisms of pathogenesis are not clearly known. Recently, dysfunctional YAP signaling pathway has been linked to hepatocarcinogenesis [2]. Transgenic mice with liver-targeted YAP overexpression demonstrated a dramatic increase in liver size and eventually developed tumors [3]. In addition, clinical studies revealed that YAP was an independent predictor associated with poor disease free survival and overall survival in liver cancer [4]. In view of the vital roles of YAP playing in the development of liver cancer, it was extremely important to understand how YAP exerts its carcinogenic property in liver cancer.

The role of c-Myc in hepatocarcinogenesis has been extensively investigated. Chromosome gains at c-Myc locus are among the most frequently reported genetic abnormalities in advanced human liver cancers [5]. In animal models, c-Myc also proved to be a key contributor to hepatic carcinogenesis [5]. Inactivation of c-Myc in invasive liver cancers led to sustained tumor regression with concomitant proliferation arrest, differentiation, and apoptosis of tumor cells [6]. Using a comparative functional genomic approach, Kaposi-Novak et al. [7] implicated c-Myc as a central mediator of human hepatocarcinogenesis and its activation is required for maintenance and expansion of transformed cells.

In the present study, we highlight the role of mutual interaction between YAP and c-Myc in liver tumorigenesis, thus providing a positive feedback loop to promote cellular YAP and c-Myc output. Our data also showed that the two proteins were closely correlated in tumor samples, suggesting the important role of their feedback loop in liver cancer. Collectively, this work summarizes a potential mechanism for liver tumorigenesis.

2. Materials and methods

2.1. Cell culture and vectors

HepG2, Bel-7402 and SMMC-7721 cells were cultured in DMEM. shRNAs against YAP, c-Myc and c-Abl were cloned into pLKO.1 lentiviral vectors. The cDNA fragments encoding human c-Myc were cloned into pGIPZ-based lentiviral [8] and pcDNA3.1 (+) vector. The primers used in this study were listed in Supplemental Table S1.

Abbreviations: HCC, hepatocellular carcinoma; IF, immunofluorescence; IHC, immunohistochemistry; TMA, tissue microarray; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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2.2. Immunohistochemistry (IHC), immunofluorescence (IF), and Western blotting (WB)

For IHC, human liver cancer tissue microarray (TMA) slides were purchased from U.S. Biomax (Rockville, MD, USA). Slides were incubated in primary antibodies against c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA, #SC-789) and YAP65 (Epitomics, Burlingame, CA, USA, #2060), respectively.

For IF, cells were incubated in primary antibodies against YAP (Santa Cruz Biotechnology, #SC-101199), c-Abl (Cell Signaling Technology, Boston, MA, USA, #2862) or c-Myc (Cell Signaling Technology, #5605).

For WB, WB was performed as described previously [9]. Primary antibodies used were: Flag (Cell Signaling Technology, #2368), HA (Cell Signaling Technology, #3724), YAP (Epitomics, #2060), c-Myc (Cell Signaling Technology, #5605 or Epitomics, #1472), c-Abl (Cell Signaling Technology, #2862), Cleaved caspase 3 (Epitomics, #1476) and GAPDH (Cell Signaling Technology, #5174).

2.3. Cell proliferation, soft-agar assays and quantitative RT-PCR

Cell proliferation was measure by a MTT-based proliferation assay, anchorage-independent soft-agar growth assay and quantitative RT-PCR was performed as previously described [10]. Primers for qPCR are available in Supplemental Table S1.

2.4. Immunoprecipitation

Cell lysates were incubated with indicated antibodies and protein A/G beads (Life technologies) overnight. The immunoprecipitates were washed five times, and then subjected to Standard Western blotting analysis.

2.5. Luciferase reporter analysis

The c-Myc promoter region was PCR amplified using primers listed in Supplemental Table S1 and cloned into pGL3/Basic vector. The c-Myc reporter containing c-Myc response element was purchased from Beyotime (Beyotime, Haimen, China). The luciferase reporter constructs were stably co-transfected with a Renila luciferase expression plasmid into cells. Luciferase activities were analyzed using a dual-luciferase reporter kit (Promega, Madison, WI, USA).

2.6. Xenograft mouse model

5×10^6 Bel-7402 cells expressing shRNA or protein as indicated were subcutaneously injected into the athymic nude mice (Bikai, Shanghai, China). Tumor size was measured every six days using a caliper, and the tumor volume was calculated as $0.5 \times L \times W^2$, with L indicating length and W indicating width. The mice were euthanized at 60 days after injection.

3. Results

3.1. YAP and c-Myc are critical for liver cancer cells

We examined if YAP and c-Myc are important for liver cancer cells. YAP or c-Myc-specific shRNAs with high knockdown efficiency (Fig. 1A and B) were used to silence protein expression in both Bel-7402 and SMMC-7721 cells. We found that inhibition of either YAP or c-Myc decreased cell proliferation compared to control, as measured by a MTT-based assay (Fig. 1C). Furthermore, we found that either YAP or c-Myc knockdown impaired the ability of these cells to form colonies in soft agar (Fig. 1D), whereas markedly increased apoptosis, as shown by increased caspase 3 cleavage (Fig. 1E). Also, reduced tumor volume was detected by knockdown of either YAP or c-Myc in Bel-7402 cells (Fig. 1F). These data indicate that both YAP and c-Myc are important for human liver cancer cell growth and survival, and the two proteins have similar function.

3.2. YAP promotes c-Myc via interaction with c-Abl

An interesting question arising from our data is whether and how YAP and c-Myc regulates with each other. We observed that c-Myc protein was reduced when YAP was knocked down as measured by Western blotting analysis (Fig. 2A). We also found that in SMMC-7721 cells with YAP knocked down, transcription of c-Myc as well as YAP target gene, CTGF [11] was significantly inhibited (Fig. 2B), suggesting YAP regulates c-Myc transcriptional activity. Then, we used a c-Myc luciferase reporter system to confirm our hypothesis that YAP regulates c-Myc activity. We found that luciferase activity from this reporter was greatly inhibited by two independent YAP shRNAs (Fig. 2C). Collectively, these experiments demonstrate that YAP may play an important role in maintaining c-Myc transcription activity.

It was reported that YAP recruits c-Abl and protects downstream target protein from ubiquitin E3 ligase mediated degradation [12]. Interestingly, c-Abl was also found as a regulator to c-Myc [13]. We hypothesized that YAP regulates c-Myc may via its interaction with c-Abl. To determine whether YAP and c-Abl co-localize in the liver cancer cells, we immunostained for endogenous YAP and c-Abl proteins. Both YAP and c-Abl gave strong signals, and co-localized mainly in the nucleus (Fig. 2D). To address how c-Abl works on c-Myc expression, c-Myc was detected in Bel-7402 and SMMC-7721 cells with c-Abl knocked down. We found that c-Myc was down-regulated by knockdown of c-Abl (Fig. 2E), suggesting c-Abl is a positive regulator to c-Myc. Furthermore, we found that both c-Myc mRNA expression levels (Fig. 2B and F) and its promoter activity (Fig. 2G) could be significantly reduced by either knockdown of YAP or c-Abl, demonstrating that YAP regulates c-Myc via c-Abl primarily at transcriptional level.3.3. c-Myc promotes YAP independent of transcription

Then, we tested whether c-Myc also regulates YAP expression. We found that cells with c-Myc knocked down had a much lower level of YAP as compared to control cells (Fig. 3A). Also, we found that c-Myc knockdown did not significantly affect YAP mRNA levels, whereas greatly inhibited its target, hTERT expression [14] (Fig. 3B), thus ruled out the possibility that c-Myc regulates YAP transcription. To determine whether YAP and c-Myc co-localize in the cells, we immunostained for endogenous YAP and c-Myc proteins. Both YAP and c-Myc co-localized in liver cancer cells (Fig. 3C). To further confirm the relationship between YAP and c-Myc, we performed IHC using TMA on 395 human liver cancer samples. We found that both c-Myc and YAP proteins are closely correlated with each other (Fig. 3D). Next, we performed co-IP experiments and found that exogenous YAP-Flag could be readily pulled down by c-Myc-HA. Taken together, these experiments establish a close relationship between YAP and c-Myc in liver cancers, and c-Myc regulates YAP independent of transcription.

3.4. The interaction between YAP and c-Myc promotes liver cancer tumor growth

On the basis of the roles of the interaction between YAP and c-Myc, we investigated the growth of Bel-7402 clones after injection into athymic mice. Compared to the control cells, Bel-7402 cells stably expressing either YAP or c-Myc shRNA effectively prevented tumor growth, and overexpressing of c-Myc could rescue YAP

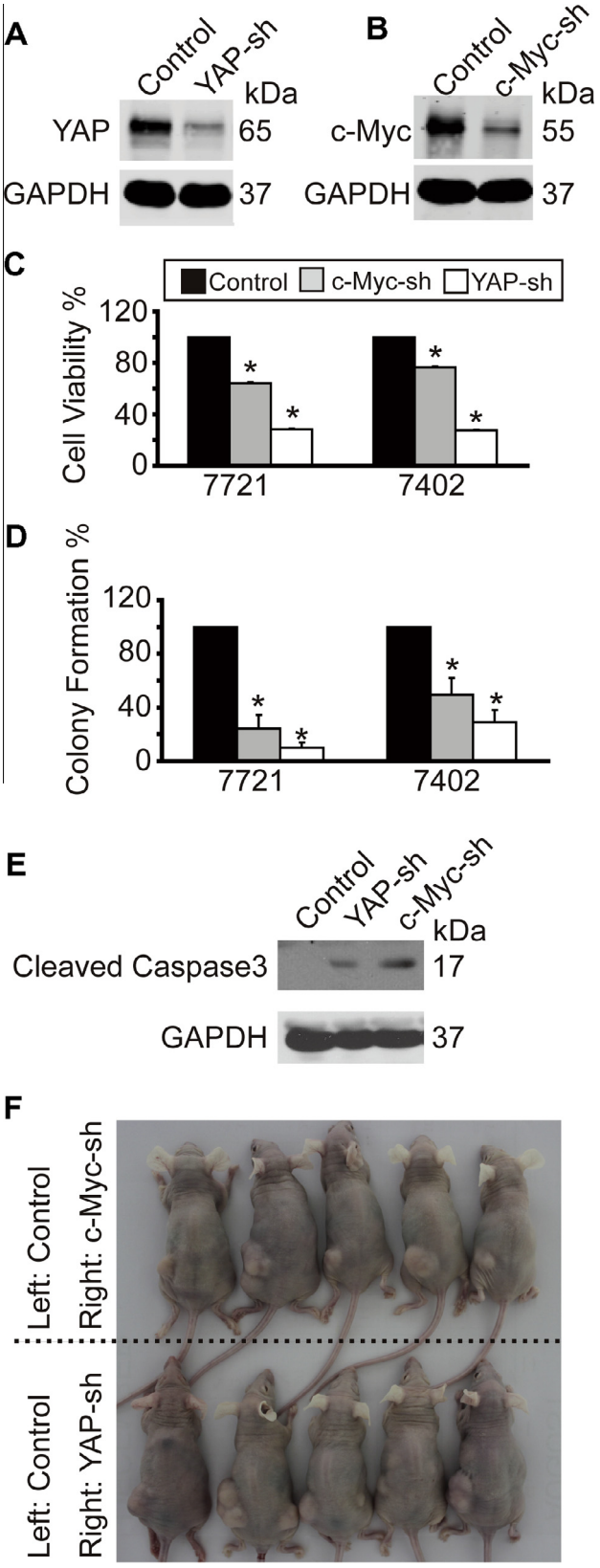


Fig. 1. Both YAP and c-Myc are critical for liver cancer cells. (A and B) Validation of knockdown efficiency of shRNAs. Western blots of YAP (A) and c-Myc (B) protein in SMMC-7721 cells infected with shRNAs targeting GFP (control), YAP or c-Myc (sh#1 + #2) as indicated. (C) Knockdown of YAP or c-Myc reduces cell proliferation as measured by MTT assays in control (against GFP) and Bel-7402 or SMMC-7721 cells with either YAP or c-Myc (sh#1 + #2) knocked down. $^{*}p < 0.01$ analyzed by Student t test. (D) Transformation activities were evaluated by anchorage-independent soft agar colony formation assays in control (infected with shRNA targeting GFP) and Bel-7402 or SMMC-7721 cells with either YAP or c-Myc (sh#1 + #2) knocked down. $^{*}p < 0.01$ analyzed by Student t test. (E) Knockdown of YAP or c-Myc (sh#1 + #2) induces apoptosis, as measured by Western blotting for cleaved form of Caspase 3 in SMMC-7721 cells. (F) Silencing of YAP or c-Myc inhibits xenograft tumorigenesis. Pictures were captured 60 days after subcutaneous injection of Bel-7402 cells expressing shRNA against GFP (control), YAP or c-Myc (sh#1 + #2). $n = 5$ per group.

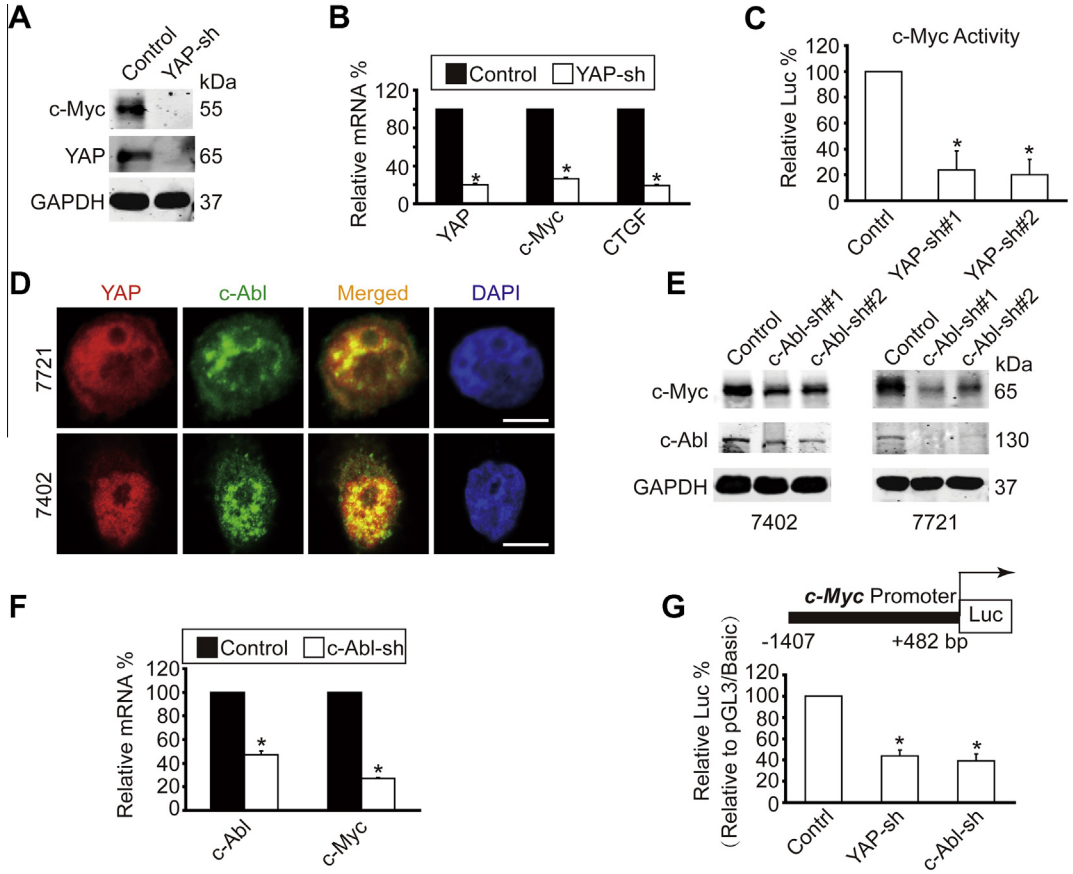


Fig. 2. YAP promotes c-Myc via c-Abl. (A) Knockdown of YAP inhibits c-Myc. Western blots of c-Myc and YAP as indicated in control (infected with shRNA targeting GFP) and SMMC-7721 with YAP knocked known. (B) Knockdown of YAP reduces c-Myc transcription. c-Myc mRNA expression tested by qPCR in control and SMMC-7721 cells with YAP knocked down. GAPDH mRNA was treated as a loading control. $^{*}p < 0.01$ versus control analyzed using Student *t* test. (C) YAP affects c-Myc activity. Luciferase activities from a c-Myc reporter were measured in control (infected with shRNA against GFP) and SMMC-7721 cells with YAP knocked down. $^{*}p < 0.01$ versus control analyzed using Student *t* test. (D) Colocalization of YAP and c-Abl in liver cancer cell lines. Cells were harvested for immunofluorescence (IF) analysis by both anti-YAP and anti-c-Abl antibodies. Scale bar, 15 μM. (E) c-Abl promotes c-Myc. Western blots of c-Myc in control (infected with shRNA against GFP) and Bel-7402 or SMMC-7721 cells with c-Abl knocked down. (F and G) Knockdown of c-Abl reduces c-Myc transcription and promoter activity. c-Myc mRNA expression tested by qPCR in control and SMMC-7721 cells with c-Abl (sh#1 + #2) knocked down. $^{*}p < 0.01$ versus control analyzed using Student *t* test. (F). And Luciferase activities from c-Myc promoter compassing −1407 to +482 relative to the transcription start site were measured in control (infected with shRNA against GFP) and SMMC-7721 cells with c-Abl (sh#1 + #2) or YAP knocked down (G).

shRNA induced phenotype in a nude mouse model (Fig. 4A). Thus confirmed such close relationship in vivo.

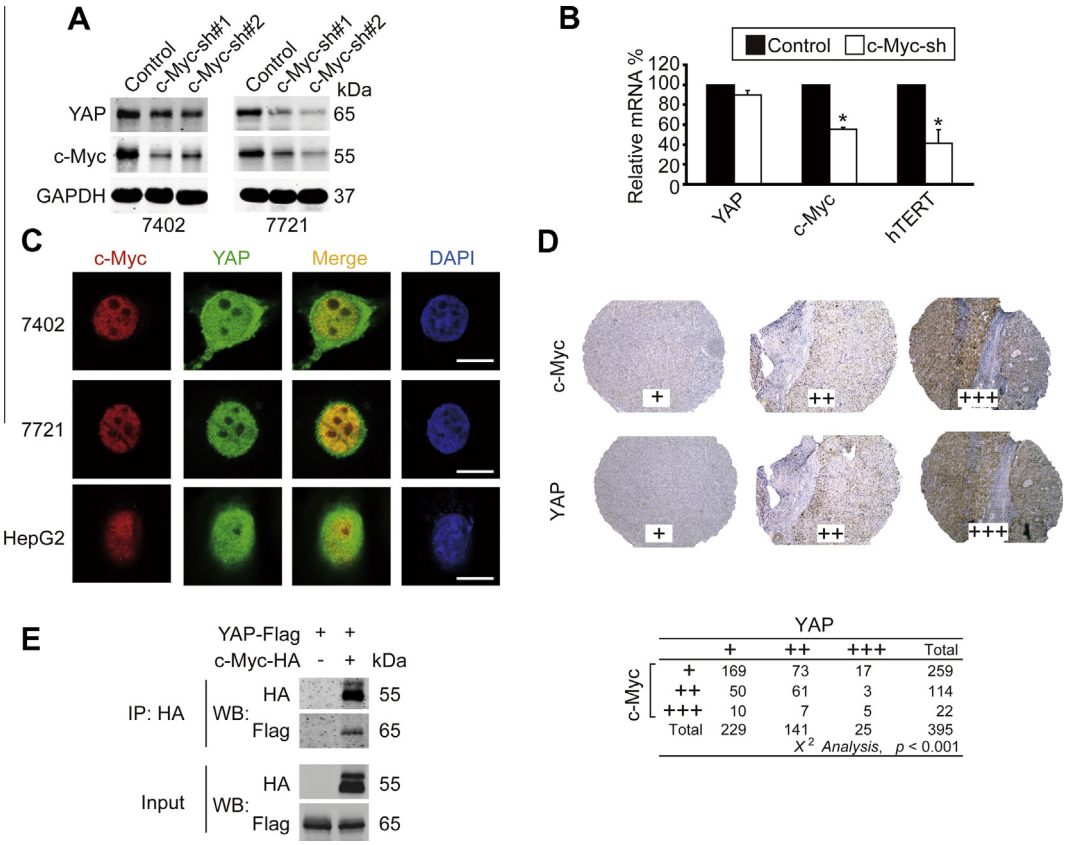
4. Discussion

Although we have reported that the mutual interaction between YAP and CREB promotes tumorigenesis in liver cancer [15], the crosstalk between YAP and other proteins is still poorly understood. As overexpression of CREB only partly rescued inhibitory effects induced by knockdown of YAP [15], leading us to explore potential proteins contributing to YAP carcinogenic property. Here we show that YAP–c-Myc interaction is also critical for liver cancer cell both in vitro and in vivo, via a positive autoregulatory feedback loop. Moreover, both c-Myc and YAP proteins are closely correlated, suggesting an important role of this feedback loop in liver cancers.

Myc is a widely conserved transcriptional regulator that controls many aspects of cell biology, and its deregulation is a key mediator of numerous cancers. Oncogenomic approaches in mouse transgenic models of liver cancers identified YAP as a tumor accelerant that can synergize with c-Myc deregulation [16]. Coexpression of Yki (Drosophila Homolog of Yap) and dMyc significantly enhances clonal growth over that stimulated by Yki or dMyc alone in Drosophila [17], which is a striking parallel to our finding that interaction between YAP and c-Myc is important for liver cancer. In this study, we found YAP is a stimulator to c-Myc transcription, which is also consistent to the finding in Drosophila [17]. However, contrary effects were detected as c-Myc plays different roles on YAP in Drosophila and human liver cancer cells. In Drosophila, high levels of Myc repress Yki expression through both transcriptional and posttranscriptional mechanisms, whereas in liver cancer cells, c-Myc promotes YAP expression at protein level. Because different cell types or research models are known to express distinct levels and types of certain functions, it is expected that different results may be obtained for liver cancer cells and Drosophila development model.

The human immunodeficiency virus matrix protein p17 increases phosphorylation and the DNA-binding activity of CREB and c-Myc through the time- and dose-dependent activation of the cAMP/PKA and MEK/ERK signaling pathways. Interestingly, both signaling pathways are synergistically activated upon costimulation [18]. Recently, we reported the role of the interaction between YAP and CREB in liver tumorigenesis and reveal a novel link between two major onco-proteins [15]. On the basis of our and other's studies we hypothesize CREB, YAP and c-Myc may involved in the same complex, which is critical for liver cancer tumorigenesis.

In conclusion, we found that both YAP and c-Myc are critical for cell survival and maintenance of transformative phenotype. We further found a positive feedback for YAP and c-Myc in liver



c-Myc \ YAP	+	++	+++	Total
+	169	73	17	259
++	50	61	3	114
+++	10	7	5	22
Total	229	141	25	395

X^2 Analysis, $p < 0.001$

Fig. 3. c-Myc promotes YAP independent of transcription. (A) Knockdown of c-Myc reduces YAP expression. Western blots of YAP in control (infected with shRNA against GFP) and Bel-7402 or SMMC-7721 cells with c-Myc knocked down. (B) c-Myc does not affect YAP mRNA. YAP mRNA expression tested by qPCR in control and SMMC-7721 cells with c-Myc (sh#1 + #2) knocked down. (C) Colocalization of YAP and c-Myc in liver cancer cell lines. Cells were harvested for immunofluorescence (IF) analysis by both anti-YAP and anti-c-Myc antibodies. Scale bar, 15 μM. (D) c-Myc correlates with YAP. Representative IHC images of c-Myc and YAP staining from the TMA analysis (upper panel). Statistical analysis by Chi-square test of the TMA data is shown in the lower panel. (E) c-Myc binds to YAP. c-Myc-HA was co-transfected with YAP-Flag into Bel-7402 cells as indicated. YAP and c-Myc associations were examined by co-IP assays as indicated.

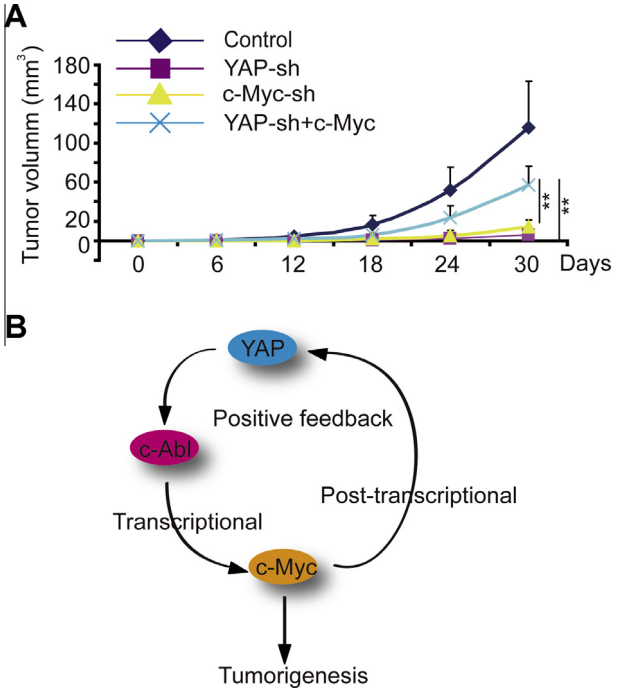


Fig. 4. Promotion of YAP by c-Myc in xenograft model and possible mechanism involved. (A) Ectopic expression of c-Myc rescues silencing of YAP in vivo. Tumor volumes were measured for 30 days after subcutaneous injection. $n = 5$ per group. $^{**}p < 0.001$ versus control analyzed using Student t test. (B) Possible mechanism underlying interaction between YAP and c-Myc in liver cancer cells.

cancers. We showed YAP drives c-Myc transcription via interaction with c-Abl. The up-regulation of c-Myc protects and enhances YAP protein expression. Accumulation of YAP in turn promotes transcription of c-Myc (Fig. 4B). To our knowledge, our results establish a new signaling mechanism by which the interaction between YAP and c-Myc promotes liver cancer growth.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.071.

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