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TRIB2 acts downstream of Wnt/TCF in liver cancer cells to regulate YAP and C/EBPα function

Jiayi Wang[1,2,*], **Joo-Seop Park**[3], **Yingying Wei**[4], **Mihir Rajurkar**[1], **Jennifer L. Cotton**[1], **Qishi Fan**[2], **Brian C. Lewis**[5], **Hongkai Ji**[4], and **Junhao Mao**[1,6]

[1]Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605

[2]Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiaotong University, Shanghai, China 200025

[3]Divisions of Pediatric Urology and Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229

[4]Department of Biostatistics, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205

[5]Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, MA 01605

SUMMARY

Dysregulation of Wnt signaling is closely associated with human liver tumorigenesis. However, liver cancer-specific Wnt transcriptional programs and downstream effectors remain poorly understood. Here, we identify tribbles homolog 2 (TRIB2) as a direct target of Wnt/TCF in liver cancer, and demonstrate that transcription of Wnt target genes, including TRIB2, is coordinated by the TCF and FoxA transcription factors in liver cancer cells. We show that Wnt-TRIB2 activation is critical for cancer cell survival and transformation. Mechanistically, TRIB2 promotes protein stabilization of the YAP transcription coactivator through interaction with the βTrCP ubiquitin ligase. Furthermore, we find that TRIB2 relieves the liver tumor suppressor protein C/EBPα-mediated inhibition of YAP/TEAD transcriptional activation in liver cancer cells. Altogether, our study uncovers a novel regulatory mechanism underlying liver cancer-specific Wnt transcriptional output, and suggests that TRIB2 functions as a signaling nexus to integrate the Wnt/βCatenin, Hippo/YAP and C/EBPα pathways in cancer cells.

INTRODUCTION

The pathogenesis of the gastrointestinal cancers, including liver and colon cancers, is associated with aberrant Wnt signaling (Nejak-Bowen and Monga, 2011; Schepers and

[6]Correspondence should be addressed to JM (junhao.mao@umassmed.edu).
[*]Current address: Department of Clinical Laboratory Medicine, Shanghai Tenth People's Hospital, Tongji University, Shanghai, China, 200072

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Clevers, 2012; White et al., 2012). In mammalian cells, the canonical Wnt pathway is mediated by regulation of cytosolic βCatenin levels. When βCatenin is stabilized in the cytoplasm, it translocates to the nucleus and interacts with the T cell factor (TCF) family of transcription factors to activate downstream gene expression (Clevers and Nusse, 2012). In colorectal carcinoma (CRC), adenomatous polyposis coli (APC) and βCatenin mutations have been reported in 80% of cases (Schepers and Clevers, 2012; White et al., 2012). In hepatocellular carcinoma (HCC), the most common liver cancer, mutations of βCatenin and Axin1 have been reported in 30-40% of cases (de La Coste et al., 1998; Miyoshi et al., 1998; Satoh et al., 2000). In hepatoblastoma, a common pediatric liver cancer, 80-90% of tumors harbor genetic mutations related to Wnt signaling (Armengol et al., 2011; Cairo et al., 2008), indicating broad involvement of the pathway in liver tumorigenesis.

Recent studies suggest that Wnt signaling may utilize distinct downstream programs in colon and liver cancers. Wnt/TCF-dependent gene transcription has been extensively studied in human CRC cells (Sabates-Bellver et al., 2007; van de Wetering et al., 2002; Van der Flier et al., 2007). Among these, c-Myc has been identified as a direct target of βCatenin/TCF and a critical Wnt effector in CRC (Sansom et al., 2007; van de Wetering et al., 2002; Yochum et al., 2008). However, genetic deletion of c-Myc in mice rescues the proliferative phenotype of APC deficiency in the intestine, but not in the liver (Reed et al., 2008). Furthermore, large-scale expression profiling analyses of human HCC samples classify Wnt and Myc activation as different HCC subclasses with distinct clinical characterization (Hoshida et al., 2009), highlighting the idea of a context-dependent Wnt transcriptional program in liver cancer.

The Hippo and CCAAT-enhancer-binding protein (C/EBP) pathways are two tumor-suppressive pathways that have been implicated in hepatic carcinogenesis (Avruch et al., 2011; Koschmieder et al., 2009; Pan, 2010). The Hippo pathway regulates a cytosolic Mst-Lats kinase cascade to phosphorylate and inhibit the activity of a key transcription coactivator, Yes-associated protein (YAP). YAP, in turn, interacts with downstream transcription factors, including the TEAD proteins, to regulate gene expression (Halder and Johnson, 2011; Zhao et al., 2011). Although amplification of the YAP locus is restricted to only 5-10% of human HCC (Zender et al., 2006), it has been reported that YAP protein expression is up-regulated in greater than 50% of tumors(Dong et al., 2007). Further, dysregulation of the Hippo pathway causing YAP up-regulation induces HCC formation in mice(Dong et al., 2007; Lu et al., 2010; Song et al., 2010; Zhou et al., 2009). C/EBPα belongs to a family of basic region leucine zipper transcription factors, and its growth inhibitory activity is critical for maintenance of hepatic cell quiescence and suppression of HCC formation (Iakova et al., 2003; Wang et al., 2001). Despite their importance in liver cancer, the molecular mechanisms governing the activity of these critical pathways are not well understood.

In this study, we identified TRIB2, a pseudokinase protein, as an important Wnt downstream effector in liver cancer. By intersecting genome-wide TCF4 and FoxA1/2 ChIP-seq and DNase-seq analyses, we found that TCF4 cooperates with the chromatin pioneering factors FoxA1/2 in liver cancer cells to regulate transcription of Wnt target genes, including TRIB2. In addition, our results demonstrated that TRIB2 activity is critical for HCC cell survival

and transformation, and suggest that it functions as a signaling nexus to couple Hippo/YAP and C/EBPα to Wnt-induced liver tumorigenesis.

RESULTS

TRIB2 is a downstream Wnt/TCF target gene in liver cancer cells

To elucidate the Wnt/TCF-controlled oncogenic program in liver cancers, we performed genome-wide TCF4 ChIP-seq and transcriptional profiling analyses in liver cancer cells, followed by interrogating a catalog of Wnt/βCatenin-associated genes identified in human liver cancer samples. HepG2 is a human liver cancer cell line that carries an active mutation of βCatenin (de La Coste et al., 1998). To block Wnt activation, a truncated repressor form of TCF4, dominant negative (DN)-TCF4 (van de Wetering et al., 2002), was introduced into these cells. We found that DN-TCF4 expression blocked Wnt activation, induced apoptosis and inhibited the ability of HepG2 cells to undergo anchorage-independent growth (Figure S1). These results highlight the functional importance of Wnt/TCF activation in liver cancer cells.

CisGenome (Ji et al., 2008) analysis of TCF4 ChIP-seq in the HepG2 genome revealed 36713 genomic regions of TCF4 interaction, indicating an active cis-regulatory network controlled by TCF4 in these cells (Table S1). TCF4 binding sites in several well-known Wnt target genes, such as LGR5, ZNRF, SP5 and Axin2, are also among the top-ranked peaks (Table S1). Next, we intersected the TCF4 ChIP-seq dataset with the transcriptional profiling data from DN-TCF4-expressing HepG2 cells (Table S2), as well as a list of 784 genes associated with Wnt/βCatenin activation identified by previous comprehensive transcriptome analyses conducted in human liver cancers (Table S3) (Boyault et al., 2007; Cairo et al., 2008; Hoshida et al., 2009; Stahl et al., 2005). From these analyses, we identified 63 genes that carry unique TCF4 binding regions located inside or nearby their genomic loci and depend on Wnt/TCF input for expression in liver cancer cells (Figure 1A).

To examine whether these genes are specific Wnt targets in liver cancer cells, we compared DN-TCF4 down-regulated genes in HepG2 and LS174T cells (Table S2 and S4). LS174T is a βCatenin mutated CRC cell line (van de Wetering et al., 2002). In addition, we performed qPCR analysis of mRNA expression of selected genes carrying top-ranked TCF4 peaks, together with Axin2 and c-Myc (Figure 1B). Expression of Axin2, a generic Wnt target involved in negative feedback regulation of the pathway, was down-regulated by DN-TCF4 in both cell lines (Figure 1B). Interestingly, we found that DN-TCF4 markedly inhibited c-Myc expression in LS174T cells, but not in HepG2 cells (Figure 1B). Moreover, our ChIP-seq analysis did not detect TCF4 interaction in the genomic regions within or nearby the c-Myc locus in HepG2 cells (Table S1), further supporting the notion for distinct transcriptional programs employed by Wnt/TCF in HCC and CRC cells. Together, these analyses showed that expression of 42 out of these 63 genes was specifically regulated by DN-TCF4 in HepG2 cells (Figure 1B, Table S2, S4). Among them, the TRIB2 gene has a TCF4 cis-regulatory region among the highest enriched in both TCF4 ChIP-seq (Table S1) and ChIP-qPCR (Figure S2) assays; therefore we decided to focus our current analysis on TRIB2.

TRIB2 is a member of the tribbles family pseudokinases involved in both hematopoietic and non-hematopoietic malignancies (Hegedus et al., 2007; Yokoyama and Nakamura, 2011), although there was no prior information regarding its function in liver tumorigenesis. To further explore Wnt regulation of TRIB2, we performed immunohistochemistry (IHC) of βCatenin and TRIB2 using tissue microarray analysis (TMA) on both human HCC and CRC samples. TRIB2 protein was highly expressed in a subset of HCCs and its expression was closely correlated with βCatenin cytoplasmic/nuclear accumulation in HCC (Figure 1C), but not in CRC samples (Figure S3). Analysis of human HCC cell lines also revealed a correlation between Wnt pathway activation and TRIB2 expression (Figure 1D-H). TRIB2 had higher expression in βCatenin mutated HepG2 cells than βCatenin wild-type Huh7 and Hep3B cells (Figure 1D and Figure S1). Moreover, exogenous expression of the Wnt1 ligand induced βCatenin nuclear accumulation and TRIB2 up-regulation in Huh7 cells (Figure 1H). Dominant active βCatenin (DA-βCatenin) expression increased TRIB2 expression in both Huh7 and HL7702 cells, a non-transformed human hepatocyte cell line (Wang et al., 2010) (Figure 1F, G). Taken together, these experiments establish TRIB2 as a Wnt specific target gene in HCC.

FoxA factors regulate Wnt/TCF-mediated transcription in liver cancer cells

An interesting question arising from our analyses is how differential regulation of Wnt target gene expression is achieved in liver cancer cells. Therefore, we performed de novo motif analysis of the TCF4 binding regions identified in our ChIP-seq assay. Interestingly, we found that, in addition to the core TCF motif, the FoxA motif was one of the two enriched sequences within the TCF4 peaks (p-value $< 10^{-20}$) (Figure 2A). The FoxA family transcription factors (FoxA1, 2, 3) are critical for mammalian liver development (Le Lay and Kaestner, 2010). In addition, FoxA1 has been shown to cooperate with estrogen receptor (ER) or androgen receptor (AR) to establish lineage-specific transcriptional programs in breast and prostate cancer cells (Hurtado et al., 2011; Robinson and Carroll, 2012). Thus, we hypothesized that FoxA factors may be involved in transcriptional regulation of liver cancer-specific Wnt target genes.

To test this idea, we performed both FoxA1 and FoxA2 ChIP-seq analyses in the HepG2 genome. By intersecting with the TCF4 ChIP-seq data, we found that a subset of TCF4 binding regions overlapped with the FoxA-occupied sites (Figure 2B). Of 36,713 TCF4 peaks, 2040 overlapped with the FoxA1 peaks and 1942 overlapped with the FoxA2 peak (Figure 2B). More strikingly, more than half of the top-1000 ranked TCF4 sites had overlapping FoxA1 or FoxA2 peaks in HepG2 cells (Figure 2B), suggesting that TCF4-regulated transcriptional output is likely susceptible to FoxA input in liver cancer cells.

We next examined the expression levels of FoxA factors in HCC and CRC. IHC showed that FoxA1 was highly expressed in more than 90% of human HCC samples (Figure 2C, D). Of the three FoxA factors, both FoxA1 and FoxA2 exhibited significantly higher expression in HepG2 cells than two Wnt-activated CRC cell lines, LS174T and HCT116 (Figure 2E, F, and Figure S4). To explore potential FoxA regulation of Wnt/TCF-mediated transcription, we compared the transcriptional profiles of HepG2 and LS174 cells expressing DN-TCF4 or shRNAs against FoxA1/2 (Figure 2G-I, Table S2, S4-6). Consistent with the idea of

differential regulation of Wnt transcriptional outputs, our analyses showed that DN-TCF4-regulated genes differed significantly between these two cell types (Figure 2G). Furthermore, we found that expression of more than 20% of DN-TCF4-downregulated genes in HepG2 cells was influenced by FoxA1/2 knockdown, while only 5% of them were co-regulated by FoxA1/2 in LS174T cells (Figure 2H). Together, these data support for a role of FoxA1/2 in controlling expression of a subset of Wnt/TCF target genes in liver cancer cells

FoxA factors regulate euchromatic conditions at distal enhancers

Next we explored the nature of TCF-FoxA interaction in HCC cells. We found that TCF4 did not appear to physically interact with FoxA1 in HepG2 cells (Figure S5A), suggesting the involvement of other mechanism. FoxA factors have been reported to exhibit pioneering function to maintain euchromatic conditions at specific cis-regulatory regions (Robinson and Carroll, 2012; Zaret and Carroll, 2011). It is unknown whether FoxA1 and FoxA2 act as pioneer factors to regulate transcription in liver cancer cells.

To address this question, we performed the DNase I hypersensitive sites sequencing (DNase-seq) analysis in HepG2 cells to map genome-wide nucleosome-free open chromatin regions, and intersected with FoxA1/2 ChIP-seq data (Figure 3A, B). We found substantial overlapping between these datasets. More than 40% of FoxA1 and FoxA2 binding regions overlapped with DNase-seq regions, supporting the idea that FoxA1 and FoxA2 have pioneering function in HepG2 cells (Figure 3A). In contrast, only 5% of the TCF4 ChIP-seq peaks were found to overlap with the DNase-seq regions (Figure 3B). However, when we examined the regulatory elements co-occupied by both TCF4 and FoxA1/2, more than 80% of them were identified as the nucleosome-free euchromatin regions by DNase-seq (Figure 3B). These data strongly suggest that FoxA1/2 factors are involved in regulating euchromatin conditions, thereby contributing to TCF transcriptional output in HCC cells.

To further characterize TCF-FoxA cooperation, we examined in detail a TCF4 distal enhancer region identified in the intron region of the TRIB2 gene, which contains three TCF binding sites and one FoxA binding site (Figure 3C and S6). Our ChIP-PCR data showed that both TCF4 and FoxA1 were able to bind to this enhancer region in HepG2 cells (Figure 3C). The co-occupancy of TCF4 and FoxA1 was further confirmed by sequential re-ChIP experiments (Figure 3D). We also generated the TRIB2 enhancer-driven luciferase reporter (TRIB2-BS-Luc) constructs with a wild-type or mutated FoxA binding site (Figure S5B). In HepG2 cells transiently transfected with TRIB2-BS-Luc or a generic Wnt luciferase reporter, TOP-Flash, DN-TCF4 significantly inhibited the activity of both reporter constructs (Figure 3E). Furthermore, in stably-transfected HepG2 cells, the reporter with the mutated FoxA site (Del-FoxA) exhibited much lower activity as compared to the wild-type construct (Figure 3F). In addition, FoxA1/2 knockdown in these cells inhibited the TRIB2-BS-Luc activity and blocked TCF4 binding to the TRIB2 enhancer (Figure 3F, G), while DN-TCF4 ectopic expression or βCatenin knock-down did not significantly affect FoxA1 binding to this region (Figure 3H, and S5C), suggesting that the pioneering function of FoxA1/2 does not depend on Wnt/TCF input, although it is critical for TCF4 binding to the enhancer in HepG2 cells. Given that Foxa1/2 proteins have lower expression in CRC cells

than HCC cells (Figure 2C-F) , we set out to test whether increased FoxA1 expression in LS174T cells may affect TCF4 binding. We found that the enhancer was not normally occupied by TCF4 in LS174T cells (Figure 3I); however, FoxA1 over-expression in these cells resulted in TCF4 binding and TRIB2 up-regulation (Figure 3I-K). Together, these data highlight the involvement of FoxA factors in TCF-controlled TRIB2 transcription in liver cancer cells.

TRIB2 is critical for liver cancer cell survival and transformation

Next, we examined the functional importance of Wnt/TCF-dependent TRIB2 expression. Two different shRNA constructs were used to silence TRIB2 expression, and a combination of both constructs effectively depleted TRIB2 protein in HepG2 cells (Figure 4A). We found that inhibition of TRIB2 expression decreased cell numbers and cell proliferation (Figure 4B, E). TRIB2 knockdown also markedly increased apoptosis in HepG2 cells, as shown by caspase 3 cleavage and increased caspase 3/7 activity (Figure 4C, D). In contrast, TRIB2 knockdown did not affect proliferation and apoptosis in HL7702 hepatocytes (Figure 4D, E). Furthermore, we found that TRIB2 knockdown impaired the ability of HepG2 cells to form colonies in soft agar in vitro and generate tumors in xenograft models in vivo (Figure 4F-J), suggesting that TRIB2 activity is critical for human HCC cell survival and maintenance of their transformative phenotype.

To examine the effect of TRIB2 on Wnt oncogenic activity, we co-expressed DN-TCF4 and TRIB2 in HepG2 cells. TRIB2 partially rescued DN-TCF4-induced apoptotic and colony formation phenotypes (Figure 4F, K). In addition, we found that TRIB2 knockdown inhibited DA-βCatenin-mediated protection from Doxorubicin-induced apoptosis in Huh7 cells (Figure 4L), further suggesting that TRIB2 plays an important role in Wnt-dependent cell survival. Taken together, these results suggest that TRIB2 is a critical albeit not the sole Wnt downstream effector during liver tumorigenesis.

Wnt and TRIB2 regulate YAP in liver cancer cells

It is currently unclear whether Wnt interacts with Hippo signaling in liver cancers. Here, we found that expression of YAP, the intracellular transducer of Hippo signaling, was significantly down-regulated by DN-TCF4 in HepG2 cells and up-regulated by DA-βCatenin or Wnt1 ligand in HL7702 and Huh7 cells (Figure 5A, B and Figure S6 A-D), suggesting that Wnt regulates Hippo/YAP activity in liver cancer cells.

Because TRIB2 was identified as a Wnt/TCF effector in HCC, we investigated whether TRIB2 is involved in regulation of YAP expression. TRIB2 knockdown in HepG2 cells inhibited transcription of two known YAP target genes, CTGF and ANKRD1 (Dupont et al., 2011; Zhao et al., 2008) (Figure 5C). Interestingly, we found that the YAP mRNA level was not significantly affected, although TRIB knockdown did markedly decrease YAP protein level (Figure 5C, D). We also tested whether TRIB2 affects YAP phosphorylation and cellular localization. We found that the cells expressing shTRIB2 had a lower level of YAP phosphorylation (p-YAP) (Figure 5D); however, the ratio of p-YAP to total YAP was similar in cells with or without TRIB2 knockdown (Figure 5E). Further, YAP nuclear localization was not affected by TRIB2 knockdown (Figure 5F).

TRIB2 regulates YAP protein stabilization

TRIB2 has been reported to function as an adaptor protein that interacts with E3 ubiquitin ligases and thereby modulates protein stability of downstream effectors (Grandinetti et al., 2011; Keeshan et al., 2006). A recent study shows that YAP protein is regulated by ubiquitination and proteasomal degradation (Zhao et al., 2010). Therefore, we assessed whether TRIB2 regulates YAP expression in liver cancer cells via ubiquitin-mediated protein degradation.

In HepG2 cells with TRIB2 knockdown, we detected accumulation of ubiquitinated YAP (Figure 5G). When HepG2 cells were treated with the protein synthesis inhibitor cycloheximide (CHX), YAP protein was unstable with a half-life of approximately 2 hours (Figure 5H). We found that TRIB2 knockdown further accelerated YAP degradation (Figure 5J); however, YAP was stabilized when TRIB2 was expressed in HepG2 cells or DA-βCatenin was expressed in HL7702 hepatocytes (Figure 5H-J, and Figure S6E), indicating that the Wnt/TRIB2 axis regulates YAP protein stability. βTrCP is the substrate binding subunit of the SKP1-CUL1-F-box ($SCF^{\beta TrCP}$) E3 ubiquitin ligase involved in YAP proteasomal degradation (Zhao et al., 2010). Thus, we examined whether TRIB2 regulates YAP degradation through βTrCP. We found that shTRIB2-induced YAP down-regulation was rescued by simultaneous βTrCP knockdown in HepG2 cells (Figure 5K). Furthermore, TRIB2 expression reversed the CUL1 destabilization effect of YAP in a dose-dependent manner (Figure 5L), suggesting that TRIB2-regulated YAP stabilization is mediated by the $SCF^{\beta TrCP}$ complex. Next, we examined whether TRIB2 directly interacts with βTrCP. The coimunoprecipitation (co-IP) experiment using endogenous TRIB2 and βTrCP proteins from HepG2 cells in vivo or GST-purified proteins in vitro showed that these proteins readily co-immunoprecipitated (Figure 5M, and Figure S7A). In addition, consistent with previous reports that the C-terminal domain of TRIB proteins is important for ubiquitin ligase interaction (Grandinetti et al., 2011; Keeshan et al., 2006), we found that TRIB2 C-terminal deletion abolished its ability to bind to βTrCP (Figure 5N). Together, these results suggest that TRIB2 promotes YAP stabilization in liver cancer cells through direct interaction with the βTrCP ubiquitin complex.

C/EBPα regulates YAP transcriptional activity

To further investigate TRIB2 oncogenic activity in liver cancer cells, we examined other possible downstream mechanisms regulated by TRIB2. TRIB proteins have been reported to bind to MEK and MKK4/7 and regulate MAPK and JNK pathway activity (Hegedus et al., 2007; Yokoyama and Nakamura, 2011). However, we did not detect significant changes of the activity of these pathways in HepG2 cells when TRIB2 was knocked down (data not shown), suggesting that the MAPK and JNK pathways are not direct targets of TRIB2 in liver cancer cells.

TRIB2 interaction with the E3 ligases COP1 or TRIM21 induces C/EBPα degradation in leukemia and lung cancer cells (Grandinetti et al., 2011; Keeshan et al., 2006). It is unknown whether TRIB2 regulates C/EBPα in HCC. We found that TRIB2 knockdown in HepG2 cells markedly increased endogenous C/EBPα levels (Figure 6A). Ectopic expression of C/EBPα also strongly induced apoptosis, inhibited proliferation and colony formation in

HepG2 cells (Figure 6B-D). These phenotypes were similar to those observed in the cells with TRIB2 or YAP knocked down (Figure 4 and Figure 6B-D), suggesting that YAP and C/EBPα are two potential downstream effectors of TRIB2 in liver cancer.

TRIB2-mediated C/EBPα regulation in HepG2 cells is likely independent of βTrCP. C/EBPα up-regulation induced by TRIB2 knockdown was not affected by βTrCP down-regulation (Figure 6E). Further, co-IP experiments showed that deletion of the COP1-interacting domain of TRIB2 did not totally abolish TRIB2-βTrCP interaction (Figure S7B), suggesting that distinct protein interactions are involved in TRIB2 regulation of different downstream E3 ligases. Consistent with this idea, C/EBPα overexpression or knockdown did not change YAP protein levels (Figure 6F). However, we found that C/EBPα inhibited the activity of a YAP-dependent TEAD-luciferase reporter (Zhao et al., 2008) in a dose-dependent manner in HepG2 cells (Figure 6G). In addition, qPCR and immunoblot analyses showed that expression of the YAP target, CTGF, was downregulated by C/EBPα overexpression (Figure 6H, I). YAP ChIP analysis also revealed that C/EBPα decreased YAP occupancy on the CTGF promoter region in HepG2 cells (Figure 6J). Further supporting the notion that C/EBPα acts downstream of Wnt/TRIB2 to regulate YAP, we found that DA-βCatenin expression was able to decrease C/EBPα expression in HL7702 hepatocytes (Figure 6K), and C/EBPα knock-down in these cells increased CTGF transcription (Figure 6L). These results raised an intriguing possibility that C/EBPα may inhibit YAP-mediated downstream transcription during liver tumorigenesis.

C/EBPα inhibits YAP/TEAD interaction

Protein-protein interaction of YAP through its WW domains, which recognize proline-rich motifs, most commonly PPxY, is important for its function (Sudol, 2010). Interestingly, we identified a PPxY (PPGY) motif located in the known N-terminal protein interacting regions of C/EBPα (Figure 7A). Thus, we hypothesized that C/EBPα may directly bind YAP and subsequently regulate YAP transcriptional activation.

To test this hypothesis, we performed co-IP of C/EBPα and YAP, and found that IP of endogenous C/EBPα pulled down endogenous YAP in HepG2 cells (Figure 7B). Next, we examined whether their interaction is mediated by the PPGY motif of C/EBPα and the WW domains of YAP. We utilized a C/EBPα mutant construct that lacks the PPGY motif (C/EBPα-ΔPPGY) and a WW-mutated form of YAP (YAP-mWW) and performed co-IP in transfected HEK293T cells (Figure 7C, D). The results showed that the WW and PPGY motifs in the YAP and C/EBPα proteins are important for their interaction (Figure 7C, D).

The TEAD transcription factors play a critical role in mediating YAP-dependent downstream transcriptional activation (Pan, 2010; Zhao et al., 2011). We examined whether C/EBPα affects YAP-TEAD interaction. We found that C/EBPα overexpression impaired the ability of YAP to bind to TEAD4 (Figure 7E), and C/EBPα-ΔPPGY exhibited less ability to inhibit YAP/TEAD-induced gene transcription, measured by the TEAD4 luciferase reporter activity (Figure 7F). C/EBPα-ΔPPGY was also less potent than wild-type C/EBPα to induce apoptosis and inhibit proliferation and anchorage-independent colony formation in HepG2 cells (Figure 7G-K). Further, we found the inhibitory effect of C/EBPα-ΔPPGY was largely resistant to the rescue by YAP expression (Figure 7G, H), suggesting the

involvement of YAP inhibition in the growth suppressing activity of C/EBPα. Taken together, these data suggest a possible functional interaction between YAP and C/EBPα acting downstream of Wnt/TRIB2 in liver tumorigenesis (Figure 7L).

DISCUSSION

Growing evidence suggests that Wnt oncogenic activity in liver and colon cancers is mediated by distinct downstream transcriptional programs. TCF4 is the major TCF factor mediating Wnt signaling in intestinal development, homeostasis and tumorignesis (Korinek et al., 1998; van Es et al., 2012). Our results suggest that TCF4 may also contribute to Wnt-dependent liver tumorigenesis and act in concert with the FoxA factors to regulate HCC-specific Wnt target gene expression. FoxA proteins are known to function as chromatin pioneer factors (Zaret and Carroll, 2011), and co-occupancy of FoxA1 with ER or AR at cis-regulatory regions is believed to be essential for establishing differential gene expression controlled by the steroid hormones in breast and prostate cancer cells (Hurtado et al., 2011; Robinson and Carroll, 2012). In the context of Wnt-dependent transcription in gastrointestinal cancers, we propose a model that preferentially high expression of both FoxA1 and FoxA2 in HCC provides additional regulatory input into βCatenin/TCF-mediated transcription and thereby controls expression of a subset of liver cancer-specific Wnt targets, including TRIB2. Interestingly, a recent report suggests that FoxA interactions with ER and AR play a role in establishing sexual dimorphism of HCC (Li et al., 2012). It is currently unknown whether Wnt/TCF activation is linked to HCC sexual dimorphism; however, it is likely that the complex cis-regulatory networks employed by TCF-FoxA and FoxA-ER/AR are critical for liver cancer development.

Our results are the first to link the function of TRIB pseudokinases to liver cancer. Due to the lack of consensus sequences in their kinase domains, it is believed that TRIB proteins do not possess kinase activity but rather, act as molecular adaptors regulating protein-protein interaction (Hegedus et al., 2007). Indeed, it appears that their ability to modulate ubiquitin ligase activity is critical for their oncogenic function (Grandinetti et al., 2011; Keeshan et al., 2006). In this study, we found that TRIB2 directly binds to the E3 ligase βTrCP leading to inhibition of proteasomal-dependent degradation of YAP, a new effector of TRIB2. Interestingly, the interaction between TRIB2 and other E3 ligases such as COP1 and TRIM21 causes C/EBPα degradation (Grandinetti et al., 2011; Keeshan et al., 2006), suggesting that TRIB2-mediated regulation of protein stability is dependent on its binding specificity of downstream ubiquitin ligases.

Our study points to TRIB2 as an important integrating point to connect the Wnt, Hippo/YAP, and C/EBPα pathways during tumorigenesis (Figure 7L). Our data provide evidence for Wnt-mediated downstream regulation of Hippo signaling in HCC. The functional interactions between Wnt and Hippo/YAP in development and cancers are likely complex and context-dependent (Barry et al., 2013; Heallen et al., 2011; Rosenbluh et al., 2012; Varelas and Wrana, 2012). Our results suggest that Wnt modulates YAP activity in HCC cells through at least two different mechanisms, transcriptional control of gene expression and TRIB2-mediated post-translational regulation of protein stability. Our study also reveals a previously unappreciated crosstalk between C/EBPα and YAP. It suggests that blocking

YAP degradation and simultaneously relieving C/EBPα-mediated inhibition of YAP/TEAD activation may serve as a critical downstream mechanism underlying TRIB2 tumorigenic activity in liver cancer cells. C/EBPα-induced growth arrest also involves interaction with CDK2/4 (Wang et al., 2001). Interestingly, a recent report shows that cyclin D2/CDK4 activity plays an important role in Wnt/APC-dependent tumorigenesis (Cole et al., 2010). Further elucidation of the interplay among these critical signaling pathways may lead to design of more effective therapeutic strategies for human gastrointestinal cancers.

EXPEMENTAL PROCEDURES

Cell culture, Transfection and Lentiviral infection

HepG2, LS174T, HCT116, Hep3B, Huh7, HEK293T, NIH-3T3 and HL7702 cells were cultured in DMEM supplemented with 10% FBS. Detailed protocols of cell transfection and lentiviral infection, including the information of lentiviral expression vectors, shRNA constructs and luciferase reporter constructs, are described in Supplemental Experimental Procedures.

Immunohistochemistry, Immunofluorescence and Immunoblotting

Human HCC and CRC tissue microarray slides were purchased from Biomax, Genvelop or UMass Cancer Center Tissue Bank. Immunohistochemistry, immunofluorescence and immunoblot analyses were performed as previously described (Rajurkar et al., 2012), and detailed antibody information is shown in Supplemental Experimental Procedures

Cell proliferation, Caspase3/7 activity and Colony-formation assay

Cell proliferation was measure by CellTiter proliferation assay kit (Promega) and Caspase 3/7 activity was determined using Caspase-Glo assay system (Promega), in accordance with the manufacturer's instructions. Detailed protocol for anchorage-independent soft-agar colony formation assay is described in Supplemental Experimental Procedures.

Quantitative RT-PCR

cDNA synthesis was conducted using Invitrogen Superscript II kit. GAPDH was used as an internal control and the sequences of primers used are listed in Supplemental Experimental Procedures. qPCR assays were conducted in triplicates and standard deviation was used to calculate error bars.

ChIP-seq, DNase-seq and de novo motif analysis

HepG2 TCF4 (TCF7L2), FoxA1 (sc101058) and FoxA2 ChIP-seq and DNase-seq data were obtained from the ENCODE project (http://genome.ucsc.edu/ENCODE/downloads.html) and analyzed using CisGenome. Detailed information is described in Supplemental Experimental Procedures.

Microarray Analysis

HepG2 or LS174T cells were transfected with a DN-TCF4 GFP expression vector, and cells with high-level GFP expression were isolated 48 hours post transfection using flow

cytometry. For FoxA1/2 knockdown, cells infected with lentivirus expressing shRNAs against FoxA1/2 were undergone puromycin selection for 4 days before RNA isolation. RNA was labeled and hybridized to human Gene ST1.0 chips (Affymetrix), in accordance with the manufacturer's instructions. Statistical analyses were performed using dChip (http://www.hsph.harvard.edu/cli/complab/dchip/).

Xenograft Mouse Model

1×10^7 HepG2 cells expressing DN-TCF4 or shRNAs against GFP or TRIB2 were subcutaneously injected into the right and left flanks of athymic nude mice (Taconic). Tumor size was measured every three days using a caliper, and 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 45 days after injection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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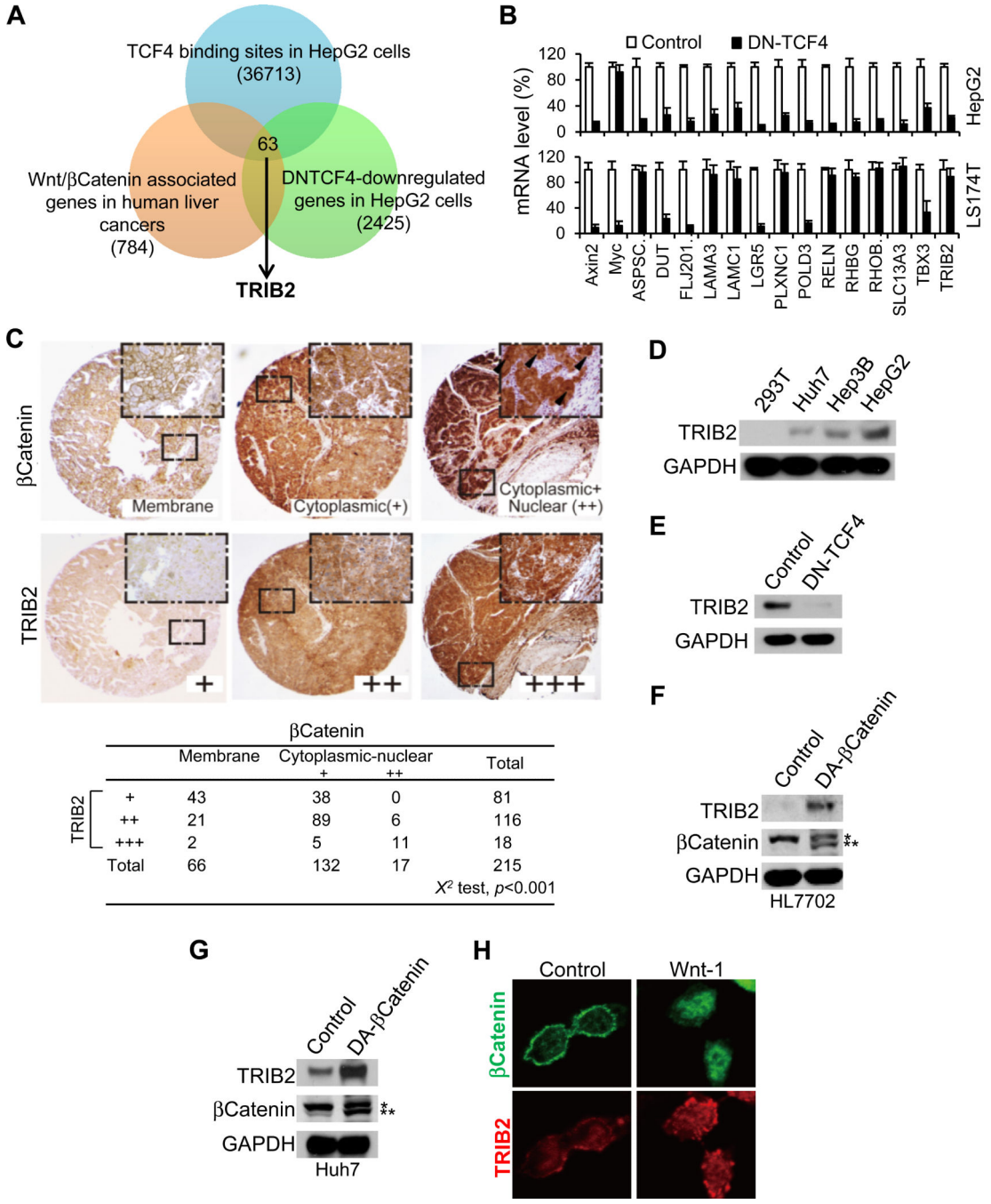

Fig. 1. TRIB2 acts downstream of Wnt-βCatenin signaling in HCC

(**A**) Venn diagram showing overlapping of Wnt-TCF associated genes carrying unique TCF4 binding sites identified in HepG2 cells and human liver cancer samples. (**B**) qPCR analysis of selected Wnt target genes in HepG2 and LS174T cells with and without DN-TCF4 expression. Error bars indicate mean ± s.d. (**C**) Representative IHC images of βCatenin and TRIB2 staining showing HCC samples with membrane, cytoplasmic or nuclear βCatenin expression and the correlated levels of TRIB2 expression. Arrows point to tumor cells with nuclear βCatenin staining. Statistical analysis of the TMA data is shown in the bottom panel. (**D**) TRIB2 expression in HEK293T cells, Huh7, Hep3B and HepG2 cells. (**E**) Immunoblot analysis of TRIB2 expression in control and DN-TCF4-expressing HepG2 cells. (**F-H**) Wnt/βCatenin activation induces TRIB2 expression.

Immunoblot analysis of TRIB2 in HL7702 (F) and Huh7 (G) cells with or without expression of DA-βCatenin. *: endogenous βCatenin; **: DA-βCatenin. Immunofluorecence staining of βCatenin and TRIB2 in Huh7 cells with or without Wnt1 over-expression (H). See also Figure S1, S2, S3 and Table S1, S2, S3.

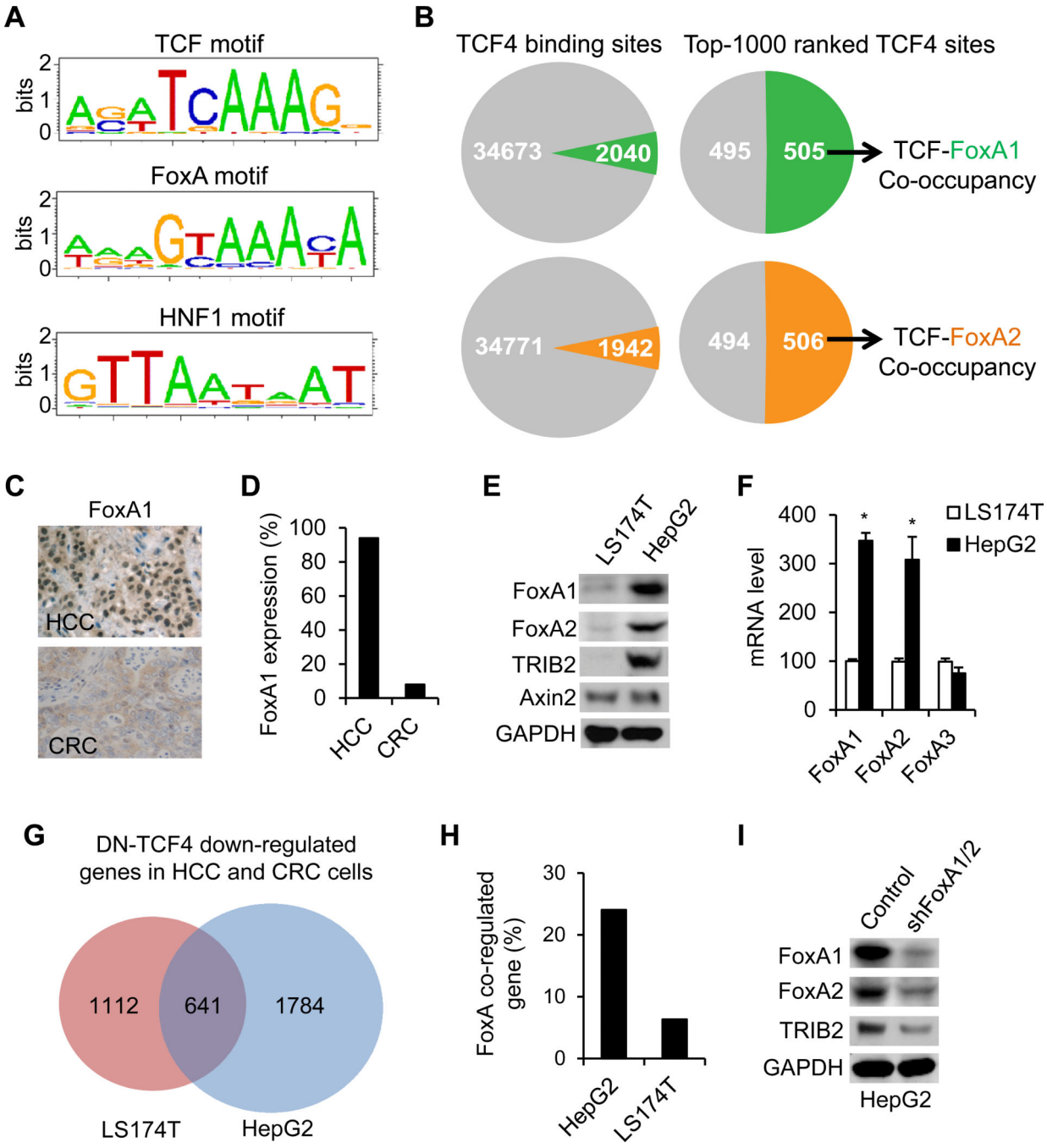

Fig. 2. TCF4 and FoxA control Wnt transcriptional output in liver cancer cells

(**A**) De novo motif analysis of TCF4 binding sites revealed the presence of the enriched TCF, FoxA and hepatocyte nuclear factor 1 (HNF1) motifs. (**B**) TCF4 and FoxA1/2 ChIP-seq analyses in the HepG2 genome revealed co-occupancy of TCF4 and FoxA1/2 on cis-regulatory regions. (**C-F**) FoxA expression in HCC and CRC. Representative IHC images of FoxA1 staining in HCC and CRC (C). The percentile of high FoxA1 expression in HCC (n=59) and CRC (n=48) samples (D). Immunoblot analysis of FoxA1, FoxA2, TRIB2 and Axin2 expression in LS174T and HepG2 cells (E). Relative mRNA levels of FoxA1, FoxA2 and FoxA3 in LS174T and HepG2 cells (F). **P*<0.01, error bars indicates mean ± s.d. (**G**) Venn diagram showing distinct TCF downstream transcriptional programs in LS174T and HepG2 cells. (**H**) The percentile of DN-TCF4 and FoxA1/2-

knockdown co-regulated genes in HepG2 and LS174T cells. (**I**) Immunoblot analysis of FoxA1, FoxA2 and TRIB2 in HepG2 cells with or without FoxA1/2 knockdown. See also Figure S4 and Table S4, S5, S6.

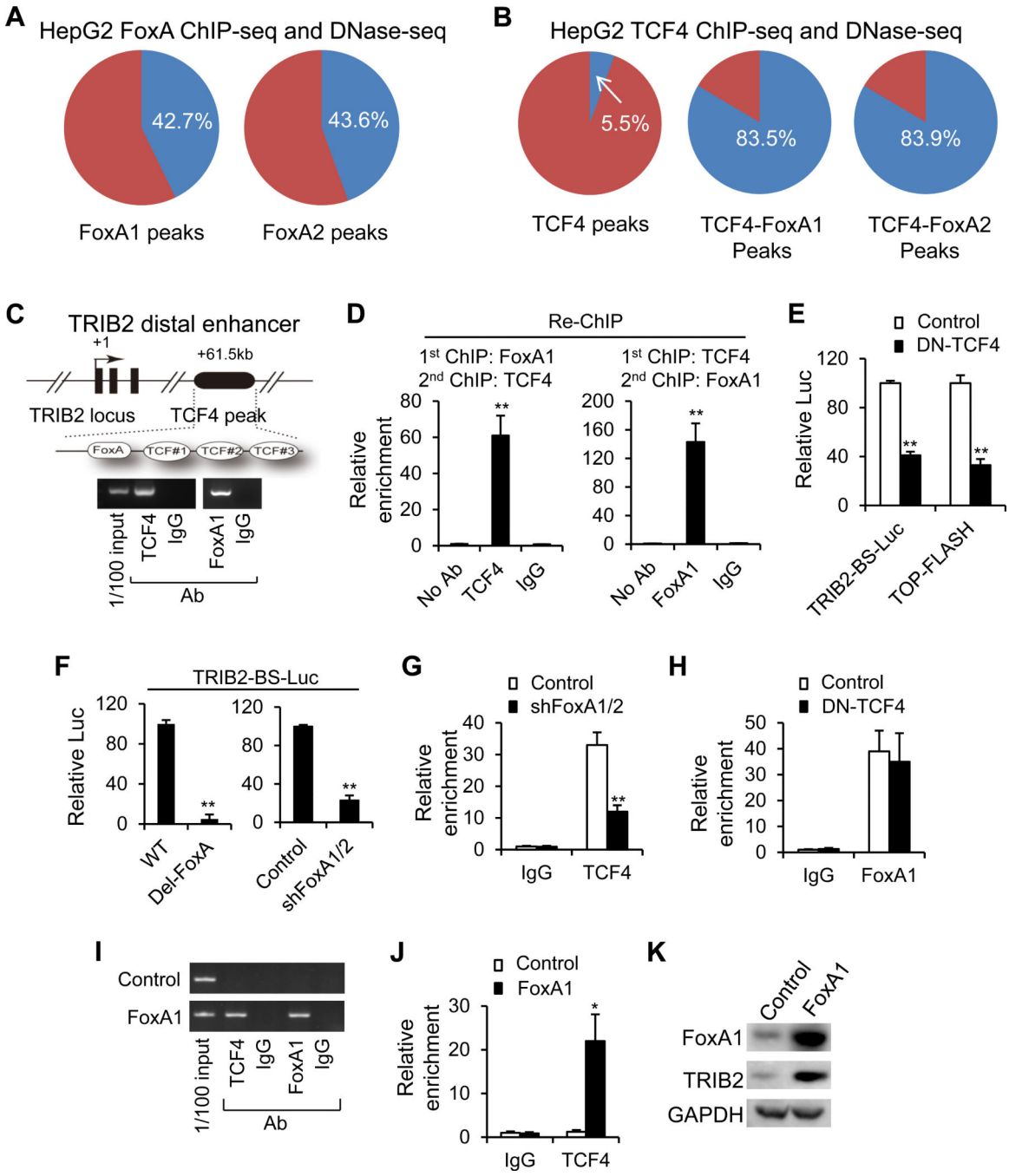

Fig. 3. FoxA pioneering factors regulate TCF4 binding at distal enhancer

(**A, B**) Intersection of DNase-seq, FoxA1/2 and TCF4 ChIP-seq. 41315 FoxA1 peaks (42.7%) and 31285 FoxA2 peaks (43.6%) overlapped with DNase-seq peaks (A). 5.5% of TCF4 peaks and over than 80% of TCF4-FoxA1/2 co-occupied peaks overlapped with DNase-seq peaks (B). (**C**) Diagram showing a TCF4-occupied enhancer in the TRIB2 locus located 61.5kb downstream of the transcriptional starting site (TSS, +1). ChIP-PCR in HepG2 cells was performed with control IgG, TCF4 or FoxA1 antibody (ab) as indicated. (**D**) Sequential ChIP with antibody against TCF4 followed by antibody against FoxA1 (Re-ChIP) and vice versa. (**E**) The activity of the TRIB2-enhancer luciferase reporter (TRIB2-BS-Luc) and TOP-Flash reporter in HepG2 cells transfected with DN-TCF4. (**F**) The activity of the luciferase reporters driven by the wild-type (WT) or FoxA site-

deleted (Del-FoxA) enhancer in stably-transfected HepG2 cells. (**G, H**) FoxA1/2 are required for TCF4 binding to the TRIB2 enhancer. TCF4 (G) and FoxA1 (H) ChIP-qPCR in HepG2 cells with FoxA1/2 knockdown or DN-TCF4 expression. Enrichment is calculated based upon qPCR relative to IgG control. (**I, J**) ChIP from control or FoxA1-overexpressing LS174T cells was performed with TCF4 or FoxA1 antibody as indicated. The presence of the enhancer was detected by PCR (I) and enrichment was measured by qPCR (J). (**K**) Immunoblot analysis of FoxA1 and TRIB2 expression in LS174T cells with or without FoxA1 overexpression. **P*<0.05, ***P*<0.01, error bars indicate mean ± s.d. See also Figure S5.

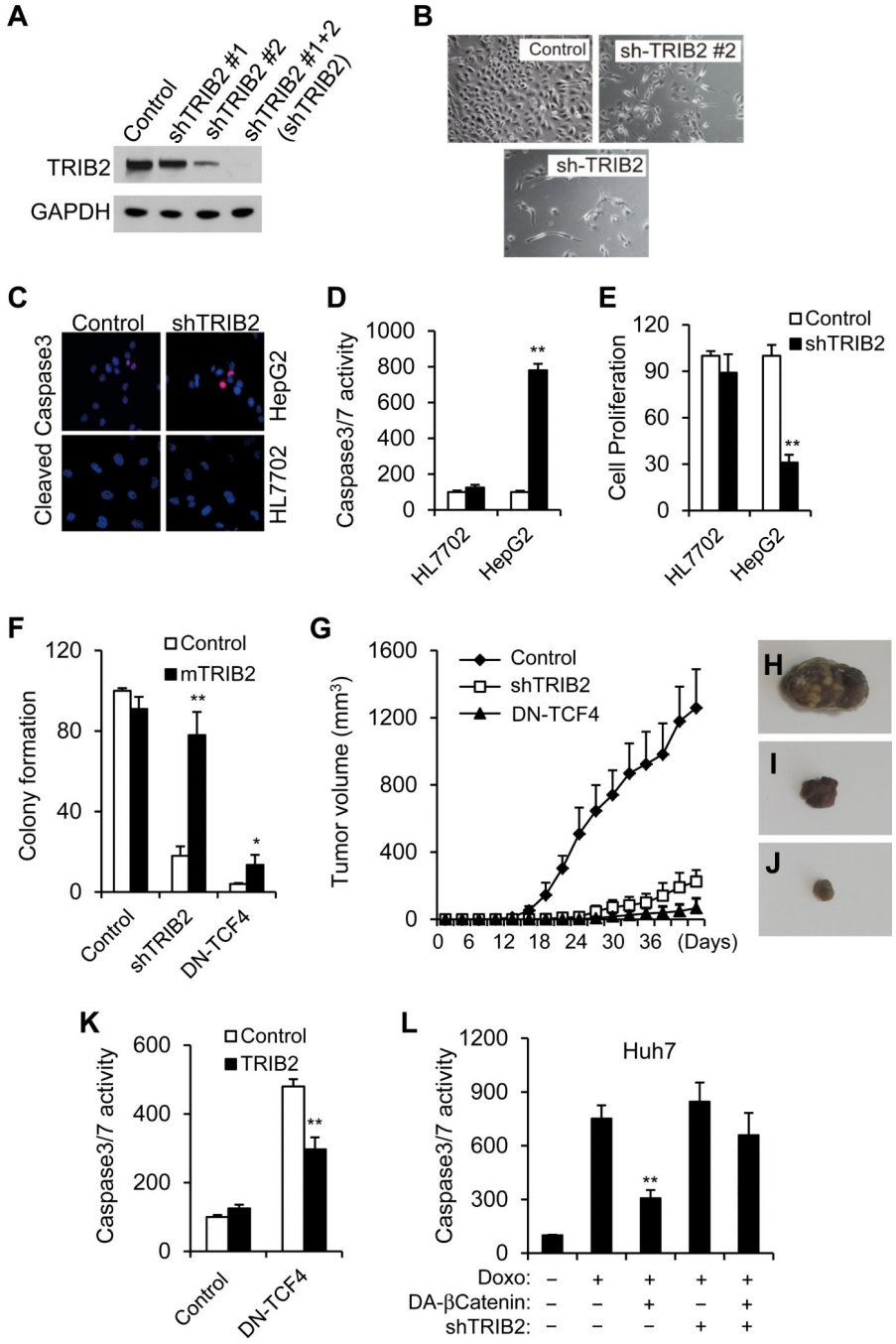

Fig. 4. TRIB2 is required for live cancer cell survival and transformation

(**A**) Immunoblot analysis of TRIB2 levels in HepG2 cells expressing shRNA constructs against GFP (control) or TRIB2 (shTRIB2 #1, shTRIB2 #2, or a combination of shTRIB2 #1+#2, referred as shTRIB2). (**B**) Phase-contrast images of HepG2 cells expressing shRNA against GFP or TRIB2. Cells were plated at a density of 5000 per well and imaged six days later. (**C-E**) TRIB2 knockdown induces apoptosis and blocks proliferation in HepG2 cells, but not in HL7702 cells, measured by cleaved caspase 3 staining (C), caspase 3/7 activity (D) and MTT-based proliferation assay (E). (**F**) Anchorage-independent soft-agar colony formation in HepG2 cells with TRIB2 knockdown or DN-TCF4 expression, in the absence or presence of a mouse TRIB2 expression vector that is resistant to shRNA targeting human TRIB2. (**G-J**) Silencing TRIB2 expression in HepG2 cells

inhibits tumor growth in vivo. Images of the tumors formed in nude mice induced by HepG2 cells expressing shGFP (H), shTRIB2 (I) or DN-TCF4 (J) are shown. Tumor volumes were measured for 45 days after injection (G) (n=5 mice per group). (**K**) Caspase 3/7 activity in DN-TCF4-expressing HepG2 cells with and without TRIB2 expression. (**L**) Caspase 3/7 activity in Huh7 cells expressing DA-βCatenin or shRNA against TRIB2, with or without Doxorubicin (Doxo) treatment. **P*<0.05, ***P*<0.01, error bars indicate mean ± s.d.

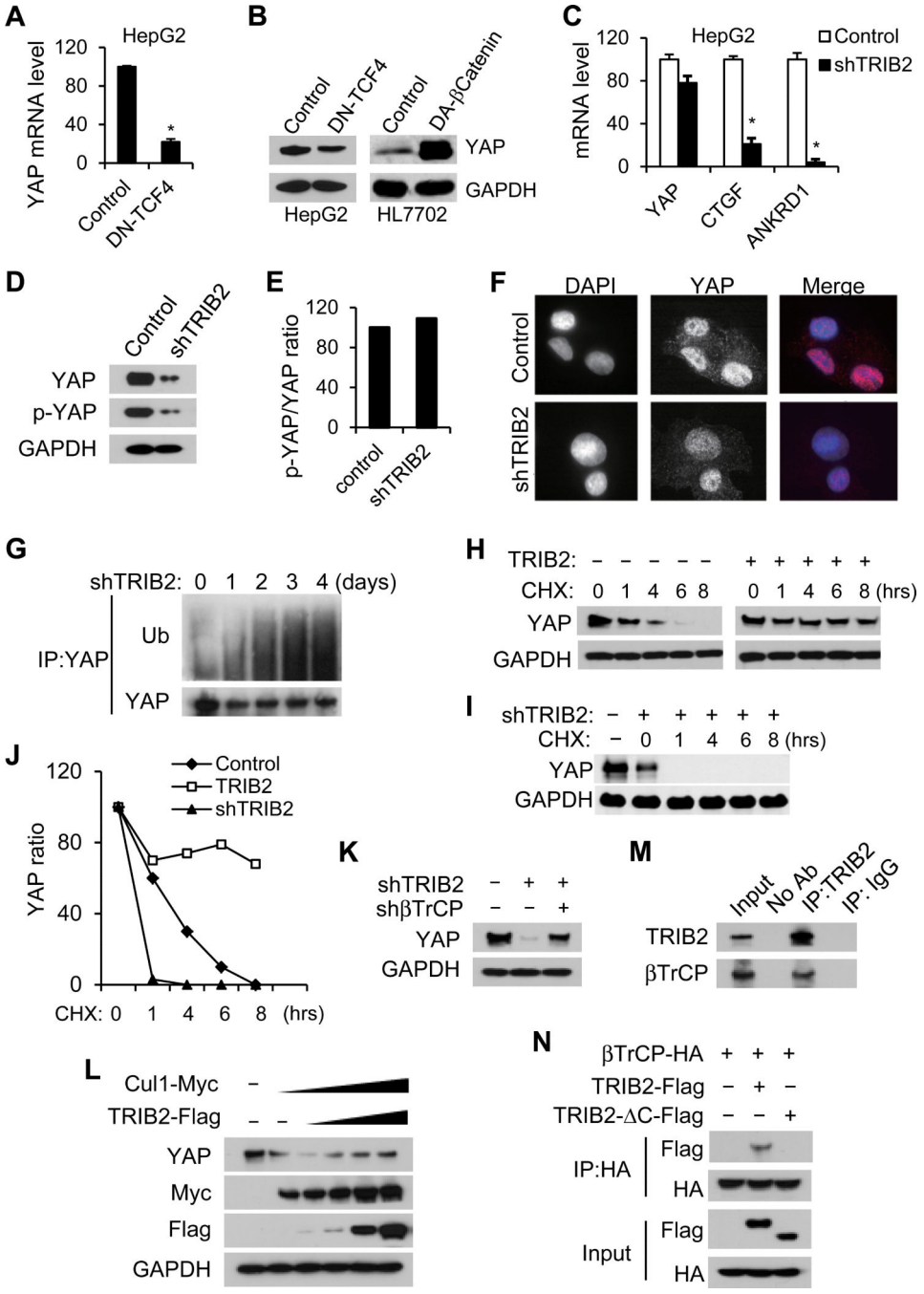

Fig. 5. TRIB2 protects YAP from βTrCP-mediated degradation

(**A-B**) Analysis of YAP mRNA (A) and protein (B) levels upon DN-TCF4 expression in HepG2 cells. **P*<0.01, error bars indicates mean ± s.d. Immunoblot analysis of YAP in HL7702 cells with or without DA-βCatenin expression (B). (**C**) qPCR analysis of YAP, CTGF and ANKRD1 expression in HepG2 cells. **P*<0.01, error bars indicates mean ± s.d. (**D**) Immunoblot analysis of YAP and phosphorylated YAP (p-YAP) in HepG2 cells with or without TRIB2 knockdown. (**E**) Quantification of p-YAP/total YAP ratio. (**F**) YAP intracellular localization in HepG2 cells with or without TRIB2 knockdown. (**G**) Ubiquitination of YAP in HepG2 cells with TRIB2 knockdown. (**H-J**) TRIB2 stabilizes YAP. In HepG2 cells expressing TRIB2 (H) or shRNA against TRIB2 (I), protein synthesis was blocked by treatment of CHX for the indicated time. Relative YAP protein levels were

quantified by YAP/GAPDH ratio (J). (**K**) Immunoblot analysis of endogenous YAP in HepG2 cells with TRIB2 or βTrCP knockdown. (**L**) TRIB2 blocks Cul1-induced YAP degradation in transfected HEK293T cells. (**M**) TRIB2 binds to endogenous βTrCP in HepG2 cells. (**N**) TRIB2 C-terminal domain is responsible for βTrCP interaction. Immunoblot analysis shows that full length TRIB2, but not C-terminal deletion mutant (TRIB2ΔC), binds to βTrCP. See also Figure S6, S7.

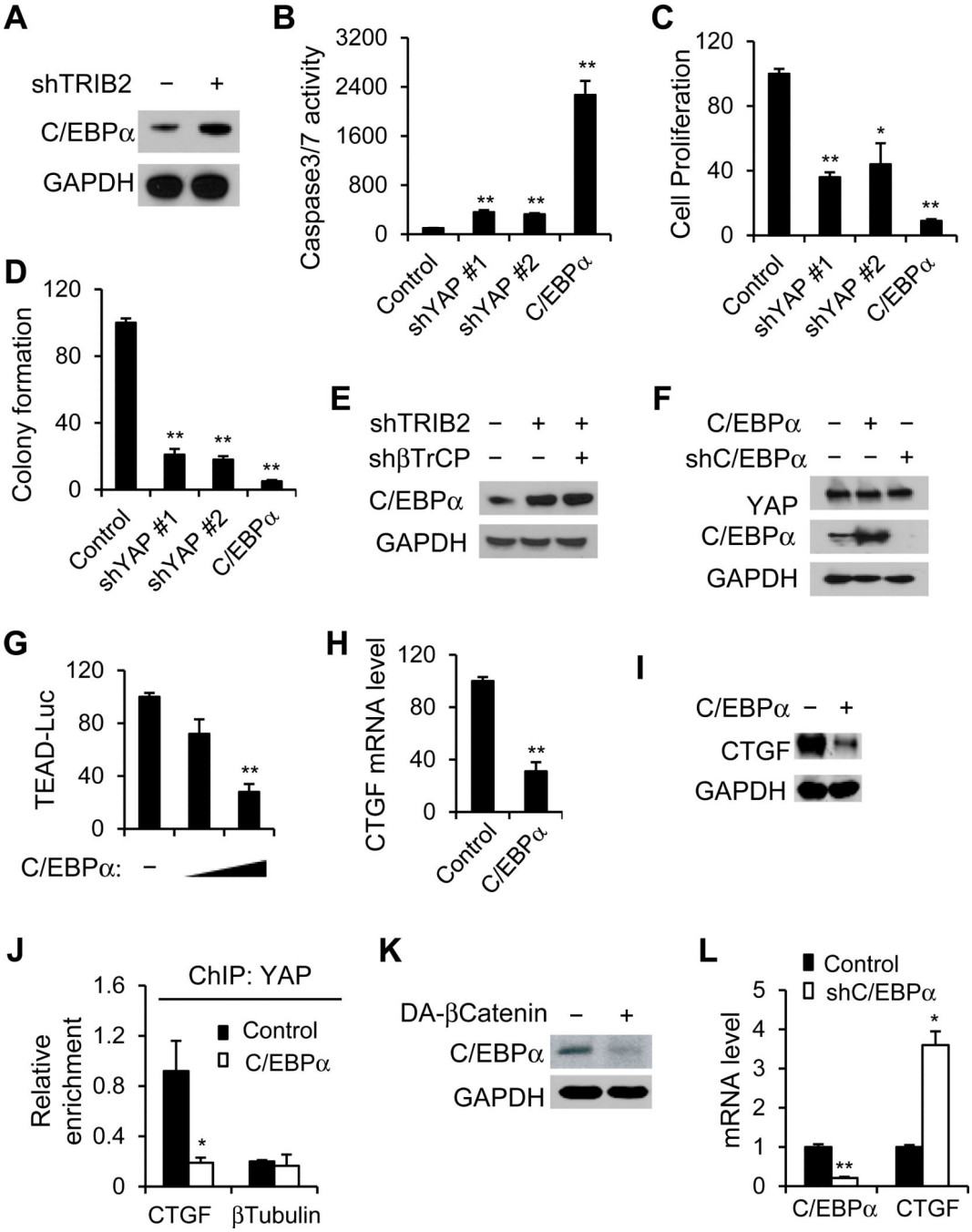

Fig. 6. C/EBPα regulates YAP transcriptional activity

(**A**) Immunoblot analysis of C/EBPα in HepG2 cells with or without TRIB2 shRNAs. (**B-D**) Caspase-3/7 activity (B), cell proliferation (C), and colony formation (D) in HepG2 cells with C/EBPα over-expression or YAP knockdown (shYAP#1 and shYAP#2). (**E**) Immunoblot analysis of C/EBPα in HepG2 cells expressing shRNAs against TRIB2 or β-TrCP. (**F**) Immunoblot analysis of YAP and C/EBPα in HepG2 cells overexpressing C/EBPα or shRNA against C/EBPα. (**G**) C/EBPα inhibits YAP/TEAD-induced gene transcription, measured by a Gal4-TEAD4/UAS-Luciferase reporter in HepG2 cells. qPCR (**H**) and immunoblot (**I**) analyses of CTGF expression in HepG2 cells with or without C/EBPα expression. (**J**) C/EBPα blocks YAP binding to the CTGF promoter. ChIP using anti-YAP antibody was performed in HepG2 cells with or without C/EBPα

overexpression. The βTubulin genomic region was used as a negative control. (**K**) Immunoblot analysis of C/EBPα in HL7702 cells with or without DA-βCatenin expression. (**L**) qPCR analysis of C/EBPα and CTGF mRNA in HL7702 cells with or without C/EBPα knock-down. **P*<0.05, ***P*<0.01, error bars indicate mean ± s.d.

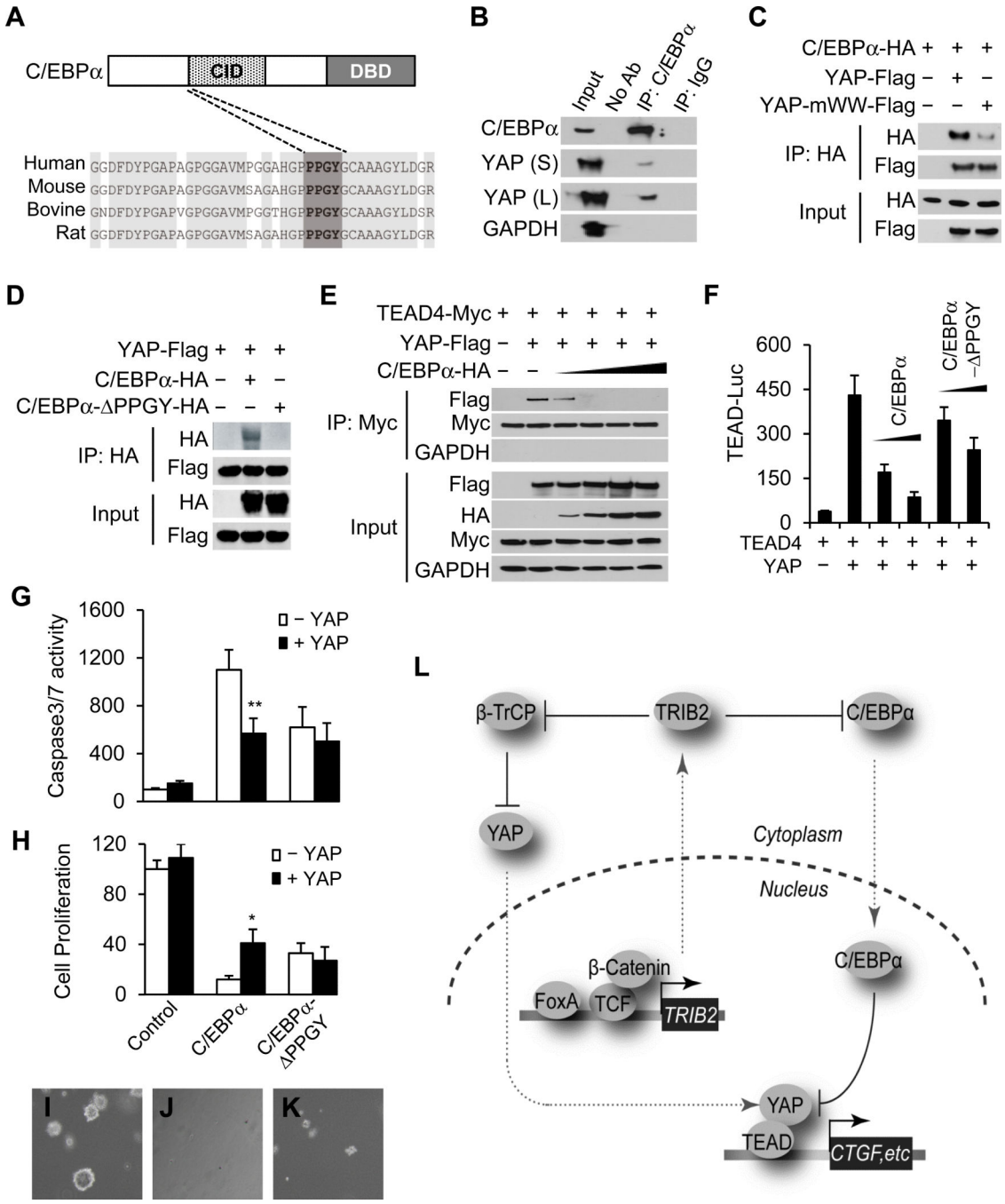

Fig. 7. C/EBPα inhibits YAP/TEAD interaction

(**A**) Diagram showing the PPGY motif of C/EBPα, adjacent to the CDK interacting domain (CID). DBD: DNA binding domain. (**B**) C/EBPα binds to endogenous YAP. (S) shorter exposure; (L) longer exposure. (**C-D**) PPGY and WW domains mediate C/EBPα and YAP interaction. Co-IP of wild-type or mutated YAP and C/EBPα proteins in transfected HEK293T cells. (**E**) C/EBPα blocks YAP/TEAD4 interaction. In transfected HEK293 cells, TEAD4 was immunoprecipitated with an anti-Myc antibody, and immunoblot analysis of YAP was done by an anti-Flag antibody. (**F**) The inhibitory activity of C/EBPα and C/EBPα-ΔPPGY on YAP-mediated transcription, measured by a TEAD4-luciferase reporter in HepG2 cells. (**G**, **H**) Caspase-3/7 activity (G) and proliferation (H) of HepG2 cells expressing C/EBPα, C/EBPα-ΔPPGY and YAP. **P*<0.05, ***P*<0.01, error

bars indicate mean ± s.d. (**I**-**K**) Representative images of soft-agar colony formation in control (I), C/EBPα-expressing (J), or C/EBPα-ΔPPGY-expressing (K) HepG2 cells. (**L**) Model showing a role for TRIB2 in controlling HCC cell survival and transformation by interlinking the Wnt/TCF, Hippo/YAP and C/EBPα pathways.