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The Ets transcription factor GABP is a novel component of the Hippo pathway essential for growth and antioxidant defense

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Summary

The transcriptional co-activator YAP plays an important role in organ size control and tumorigenesis. However, how *Yap* gene expression is regulated remains unknown. This study shows that the Ets family member GABP binds to the *Yap* promoter and activates YAP transcription. The depletion of GABP downregulates YAP, resulting in a G1/S cell cycle block and increased cell death, both of which are substantially rescued by reconstituting YAP. GABP can be inactivated by oxidative mechanisms, and acetaminophen-induced GSH depletion inhibits GABP transcriptional activity and depletes YAP. In contrast, activating YAP by deleting Mst1/Mst2 strongly protects acetaminophen-induced liver injury. Similar to its effects on YAP, the Hippo signaling inhibits GABP transcriptional activity through several mechanisms. In human liver cancers, enhanced YAP expression is correlated with increased nuclear expression of GABP. Therefore, we conclude that GABP is an activator of *Yap* gene expression and a potential therapeutic target for cancers driven by YAP.

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Supplemental Information
The supplemental information for this article includes one table, six figures, supplemental experimental procedures, and supplemental references.

Keywords

GABP; Hippo pathway; YAP; Glutathione depletion; Liver injury; liver cancer

Introduction

The Hippo pathway is an evolutionarily conserved protein kinase cascade that negatively regulates the oncogenic transcriptional coactivator Yes-associated protein (YAP) and its paralog TAZ(Pan, 2010; Zhao et al., 2011). In the canonical Hippo pathway, the kinase core consists of the Ste20-like kinases Mst1/Mst2 (Hippo in *Drosophila*), which in association with the WW-domain scaffolding protein WW45 (Salvador) phosphorylate the NDR family kinases Lats1/2 (Warts) and the noncatalytic protein Mob1A/B (Mats). Phospho-Mob1A/B then binds to and promotes the autophosphorylation and activation of Warts/Lats, which in turn phosphorylates YAP (yorkie; Yki), resulting in its binding to 14-3-3. This interaction promotes YAP/Yki nuclear exit, thereby inhibiting YAP/Yki function. Intranuclear YAP/Yki promotes cell proliferation and inhibits cell death through the Scalloped/TEAD transcription factor(s) (Lamar et al., 2012; Liu-Chittenden et al., 2012; Zhao et al., 2008).

The loss of any component of the kinase core results in a YAP-dependent increase in proliferation, resistance to apoptosis and massive organ overgrowth (Zhou et al., 2009; Zhou et al., 2011). Similarly, overexpression of a “Hippo-resistant” YAP mutant leads to the expansion of progenitor cells and cancer development in multiple organs (Camargo et al., 2007). Thus, the central function of the Hippo pathway is to inhibit the function of YAP/Yki (Sudol et al., 2012) to restrain organ overgrowth.

YAP is a candidate oncogene in humans because YAP protein expression and/or nuclear localization levels are elevated in many human cancers, and the 11q22 amplicon, which encompasses the *Yap* gene, is frequently observed in human cancers (Overholtzer et al., 2006; Zender et al., 2006). This and other findings indicate that regulating the YAP protein level is a very important aspect of its oncogenic function. Although numerous studies have investigated YAP phosphorylation, degradation and nuclear localization (Basu et al., 2003; Huang et al., 2005; Levy et al., 2008; Zhao et al., 2007), far fewer studies have addressed the regulation of YAP expression. Previous research has implicated c-Jun (Danovi et al., 2008), β-catenin (Konsavage et al., 2012) and microRNA-375 (Liu et al., 2010) in the regulation of *Yap* gene expression, but the timing, context and impact of their actions remain unclear. Thus, how *Yap* gene expression is regulated during normal development, organ size control and cancer development remains to be fully understood.

When examining which transcription factors affect the regulation of *Yap* gene expression under physiological conditions, we identified an Ets family transcription factor called GA-binding protein (GABP) (LaMarco and McKnight, 1989), also known as nuclear respiratory factor 2 (NRF-2) (Virbasius et al., 1993) or adenovirus E4 transcription factor 1 (E4TF-1) (Watanabe et al., 1993). GABP specifically binds to multiple Ets-binding sequences (GGAAG) that are present in the *Yap* promoter and activates it. Among the more than two dozen mammalian Ets factors in this family, GABP is the only obligate multimeric complex composed of two distinct and unrelated proteins, GABPα and GABPβ (LaMarco et al., 1991; Thompson et al., 1991). GABPα mediates DNA binding through its Ets domain but lacks transcriptional activity. GABPβ contains the transcription activation domain, a nuclear localization determinant and four ankyrin repeats that mediate its heterodimerization with GABPα. GABP is ubiquitously expressed and regulates lineage-restricted genes, ribosomal and mitochondrial genes and genes that control cellular growth (Yu et al., 2011; Yu et al., 2012).

As with *Yap* (Morin-Kensicki et al., 2006), homozygous deletion of the *Gabpa* gene in mice results in early embryonic lethality (Xue et al., 2008), indicating that GABPα is critically important for organ development. The transcriptional activity of the GABP complex is regulated by its redox state through the oxidation of one or more cysteine residues in the DNA-binding and dimerization domains of the GABPα subunit (Chinenov et al., 1998). Treating 3T3 cells with the glutathione (GSH)-depleting agent pro-oxidant diethyl maleate (DEM) almost completely inhibits GABPα DNA binding activity and the dimerization of GABPα/GABPβ in nuclear extracts. In contrast, the antioxidant N-acetylcysteine (NAC) substantially protects GABP DNA binding activity from DEM-mediated inhibition (Martin et al., 1996).

In addition, in vitro studies have demonstrated that both subunits of GABPα/β can be phosphorylated directly by MAP kinases (Flory et al., 1996). The threonine at position 280 of GABPα and the serine 170 and threonine 180 of GABPβ were identified as the major phosphorylation sites in vitro and in vivo (Fromm L, 2001). The activity of several Ets transcription factors is augmented by phosphorylation (Wasylyk et al., 1998), and the transcriptional activity of GABP may also regulated by phosphorylation. Nevertheless, the physiological context and functional effect of GABP phosphorylation on its transcriptional activity remain to be elucidated.

In this study, we demonstrate that the Ets family transcription factor GABPα/GABPβ is required for YAP expression in vitro and in vivo and that YAP is an important effector downstream of GABP for cell survival and cell cycle progression. Moreover, we show that YAP, through its ability to promote the expression of an antioxidant transcriptional program, exerts positive feedback regulation on GABP. Finally, we show that the Hippo pathway, in addition to directly inhibiting YAP, also inhibits GABP function through both phosphorylation and direct protein-protein interaction. Taken together, these results suggest that GABP is a central regulator of *Yap* gene transcription, and that similar to YAP, GABP is negatively regulated by the Hippo kinase pathway.

Results

GABP is a physiologic activator of the *Yap* promoter

To identify transcription factors that regulate the *Yap* promoter, we generated 5′-biotinylated 3.6-, 2.6-, 2-, 1- or 0.36-kb DNA fragments corresponding to *Yap* promoter sequences upstream of the ATG start codon (Figure S1A). Each of these fragments was incubated with mouse liver lysates, followed by pull-down with streptavidin-agarose beads. A protein band near 45 kD (Figure S1A) was identified as GABPβ by mass spectrometry. Although GAPBβ contains a transcriptional activation domain, it cannot directly bind to DNA and must form a heterodimer with the Ets-domain protein GABPα to do so. Therefore, we analyzed the *Yap* promoter pull-down samples for GABPα by immunoblotting and confirmed that both GABPβ and GABPα are present in the *Yap* promoter pull-down samples (Figure S1B). DNA sequence analysis of the *Yap* promoter showed that it contains 16 GGAAG sequences; this motif is an Ets family transcription factor binding site (EBS) and is bound by GABP (Figure 1A).

Next, we sought to determine whether GABPα/GABPβ binds to the *Yap* promoter. EMSA gel shift experiments were performed using HeLa cell nuclear extracts and biotin-labeled DNA probes corresponding to segments of the *Yap* promoter that contain the EBS. As visualized by EMSA, the addition of these probes to HeLa nuclear extracts resulted in a pair of upshifted bands, suggestive of GABPα/β dimers and α/β2 tetramers, and these bands were further upshifted upon the addition of the anti-GABPα antibody (Figure 1B). Chromatin immunoprecipitation assays in HeLa or primary mouse hepatocyte extracts using

the anti-GABPα antibody and PCR primers specific for the *Yap* promoter indicated that endogenous GABPα is associated with the *Yap* promoter in vivo (Figure 1C).

To determine whether the GABPα/GABPβ and EBS sequence are both required for the transcriptional activity of the *Yap* promoter, we constructed a *Yap* promoter truncation series containing different GABPα binding sites fused upstream of a luciferase reporter (Luc) and cotransfected these constructs into 293T cells with GABPα, GABPβ1L, GABPα + GABPβ1L or empty vector(Figure 1D). The *Yap* promoter-driven luciferase activity of the reporter plasmid was dramatically increased when cotransfected with GABPα + GABPβ1L compared with an empty vector. In contrast, only a slight increase in luciferase activity was observed when YAP-Luc was cotransfected with GABPα or GABPβ1L alone, which may reflect the heterodimerization of the transfected GABP subunit with the endogenous GABPβ or GABPα. Moreover, the level of luciferase induction correlated with the number of EBS sequences on the YAP-Luc reporter, although the luciferase activity of YAP3600-Luc was slightly lower than that of YAP2600-Luc (Figure 1D).

We also examined the effect of individual EBS sites on GABPα/GABPβ1L-driven luciferase expression (Figure S1C). Deletion of the EBS sites between −67 and +70 from the YAP2600-luciferase construct resulted in a modest decrease in luciferase activity, whereas deletion of the EBS site at +697 increased luciferase activity to comparable levels. Overall, no single site had a dominant contribution toward the reporter gene expression. The −67 and +70 sites, each of which contains two adjacent EBS sites, were equally important for luciferase expression from YAP362-luciferase. The deletion of either of the two adjacent EBS sites reduced luciferase activity by half, and the deletion of both resulted in total abolition of luciferase activity (Figure S1D). Therefore, GABP binds to multiple EBS sequences on the *Yap* promoter and upregulates YAP transcriptional activity.

The GABPα/β1L complex is the most effective Ets family transcription factor for the activation of the *Yap* promoter

Each of the three isoforms of the GABPβ subunit, GABPβ1L, β1S and β2, can associate with GABPα to form the functional heterodimeric transcription factor GABPα/β. The GABP β1L and β1S isoforms, both encoded by the *Gabpb1* gene, have identical 332 amino-terminal domains but differ in their C-terminal regions due to differential mRNA splicing. Both β1 isoforms heterodimerize with GABPα with similar affinities (Suzuki et al., 1998). GABPβ1L has a longer C-terminal tail (50 amino acids), which contains a leucine zipper-like domain that enables the formation of GABPβ1L homodimers and α2β2 GABP tetramers when two Ets motifs are adjacent or brought into proximity (Sawada J, 1994). In contrast, the C-terminus of GABPβ1S contains 15 amino acids, lacks the C-terminal leucine zipper-like structure of GABPβ1L and cannot form β-β dimers or α2β2 tetramers. GABPβ2, encoded by *Gabpb2*, shares an 87% identity with GABPβ1 and can form both GABPβ2 homodimers and heterodimers with GABPβ1L. Therefore, we compared the activity of YAP2600-Luc when coexpressed with GABPα alone or with each of the three isoforms of GABPβ (Figure 1E).

Each heterodimer resulted in stronger luciferase expression than GABPα alone. Among the heterodimers, GABPα+GABPβ1L resulted in the highest luciferase activity, GABPα +GABPβ1S showed the lowest activity and GABPα+GABPβ2 showed an intermediate level of activity (Figure 1E). Thus, GABPα+GABPβ1L, perhaps through its ability to form an α2β2 tetramer and bind to adjacent EBS sites (such as the −67 or +70 sites), enables the most robust activation of the *Yap* promoter. The overexpression of GABPα or GABPα +GABPβ1L greatly increased the expression of endogenous YAP in 293T cells, whereas GABPβ1L alone did not increase YAP expression (Figure 1F).

This result suggests that in 293T cells, endogenous GABPβ may be present in amounts sufficient to engage the transfected GABPα, thereby forming active heterodimers. GABP cooperates with other transcription factors, such as P300, Esrra, PGC1α, YY1 and C/EBPβ, to activate gene expression(Hock and Kralli, 2009). The coexpression of P300, Esrra, PGC1α, YY1 or C/EBPβ with YAP2600-Luc did not stimulate luciferase expression, and their coexpression with GABPα/β1L failed to enhance GABP-stimulated luciferase activity from YAP2600-Luc. Only C/EBPβ enhanced GABP-stimulated YAP2600-Luc luciferase activity by approximately 30% (Figure S1E).

The Ets family of proteins, identified by its highly conserved DNA binding domain, the ETS domain, is one of the largest families of transcription factors (Hollenhorst et al., 2011). Because there are 16 putative EBS sequence sites present upstream of the YAP coding sequences (Figure 1A), we investigated whether Ets family transcription factors other than GABP could stimulate *Yap* promoter activity. In addition to GABPα and GABPβ1L, we obtained cDNAs corresponding to 17 Ets family members (Figure S1F) and generated Myc-tagged expression constructs for each family member. The YAP2600-Luc construct was cotransfected with each of these cDNA constructs, and protein expression was verified by immunoblotting using an anti-Myc antibody (Figure S1G). The luciferase assay results showed that only GABPα/β1L resulted in a significant increase in YAP2600-Luc luciferase activity (approximately 10-fold), and none of the other Ets family members examined significantly enhanced the *Yap* promoter activity (Figure S1H). These results suggest that within the Ets family, GABPα/β1L exhibits considerable specificity as a regulator of *Yap* promoter activation and transcription.

GABP is required for the expression of YAP, and YAP is an important downstream effector of GABP

After serum starvation, GABPα can be induced in cells by the addition of serum (Yang et al., 2007). We demonstrated that the level of the GABPα, but not GABPβ, increases progressively after the re-addition of serum to serum-deprived MEFs, and the abundance of YAP increases in parallel (Figure S2A). qPCR assays demonstrated that the induction of GABPα and YAP proteins by serum was accompanied by an increase in their respective mRNA levels (Figure S2B). The expression of the E3 ligase protein Skp2, which is encoded by a known transcriptional target of GABP (Yang et al., 2007), was also enhanced by the re-addition of serum, but the expression of the cdk inhibitor protein P21, a substrate of Skp2, was concomitantly decreased (Figure S2A). To determine whether GABP is required for the expression of endogenous YAP, we examined the effect of the shRNA-induced depletion of GABPα or GABPβ on the abundance of endogenous YAP in HepG2 cells. Although the depletion of GABPα (Figure 2A, left) or GABPβ (Figure 2A, right) mRNAs did not alter the abundance of the other GABP mRNAs (Figure S2A), the depletion of either GABP subunit substantially reduced YAP and Skp2 mRNA (Figure 2B) and protein (Figure 2A) levels.

Previous studies have suggested that GABP is required for cell cycle progression and may regulate cell survival (Yang et al., 2007). Consistent with this view, the shRNA-induced depletion of GABPα in HepG2 cells resulted in an increased number of apoptotic cells (Figure 2C) and cells accumulating in G0/G1 but fewer cells in S phase (Figure 2D). The cotransfection of YAP cDNA and GABPα shRNA markedly reduced the number of apoptotic cells (Figure 2C) and partially rescued the G1/S block (Figure 2D). The inhibitory effect of GABPα or GABPβL1 depletion on colony formation was also significantly ameliorated by the coexpression of YAP (Figures S2C and D). These results provide further evidence that YAP is a downstream target of GABP and indicate that the positive effect of GABP on cell cycle progression and cell survival is achieved, at least in part, through YAP.

GABP may promote cell cycle progression, in part by increasing YAP and Skp2 expression, during post-hepatectomy liver regeneration. Within several days after a two-thirds hepatectomy, the remaining liver cells proliferate synchronously to restore liver mass and function. Liver cell proliferation, as indicated by an increase in the cell proliferation marker proliferating cell nuclear antigen (PCNA), was not evident at 24 hours but reached significant levels at 36 hours after resection (Figure 2E). In contrast, the expression levels of GABP and its targets, YAP and Skp2, increased within 24 hours after the hepatectomy, before the onset of hepatocyte proliferation, and remained elevated for 72 hours (Figures 2E and S2E). Furthermore, mice injected with an adenovirus expressing GABPα and β showed increased YAP expression (Figure 2F), enhanced hepatocyte proliferation (Figure S2F) and enlarged liver mass (Figure 2G). The ability of GABP overexpression to drive hepatocyte proliferation strongly supports the hypothesis that the upregulation of GABP (and YAP) expression that occurs early during post-hepatectomy liver regeneration contributes to hepatocyte proliferation and liver enlargement.

Glutathione depletion inhibits GABP-dependent YAP expression

The transcriptional activity of GABP is regulated by oxidation/reduction both in vitro and in vivo (Martin et al., 1996). Pro-oxidant conditions, such as treatment with the GSH-depleting agent DEM, do not change the protein level of GABP but inhibit its ability to bind DNA due to the oxidation of GABPα cysteine residues (Cys388 and Cys401). In addition, oxidation of GABPα Cys421 inhibits the heterodimerization of GABPα/GABPβ, thereby inhibiting GABP-dependent gene expression (Chinenov et al., 1998). To determine whether the GABP-driven activation of the *Yap* promoter driven is also affected by pro-oxidant treatment, HeLa cells that were transfected with YAP2600-Luc and either GABPα +GABPβ1L or control vector were treated with DEM with or without the antioxidant NAC, a precursor of GSH synthesis. Treatment with DEM alone resulted in a dramatic decrease in the GABP-stimulated YAP2600-Luc luciferase activity, which was partially restored by combined treatment with DEM plus NAC. However, NAC alone had minimal effect (Figure 3A).

We confirmed that DEM treatment causes a substantial decrease in the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) (Figure 3B). Treatment of HepG2 cells (Figure 3C) or primary mouse hepatocytes (Figure S3A) with DEM resulted in a progressive decrease in the protein levels of YAP, the transcriptional target of GABP, and Skp2 and cMyc, which are transcriptional targets of YAP. This effect was observed markedly at 24 hours, although the GABP protein subunit levels were not reduced in either cell type. Similar to the YAP2600-Luc transcriptional activity (Figure 3A), combined treatment with NAC plus DEM restored the protein levels of YAP, Skp2 and cMyc, in contrast to either DEM or NAC treatment alone (Figure 3C; Figure S3A). Real-time PCR analysis suggested that the reduced YAP and Skp2 protein levels in DEM-treated samples was accompanied by reduced mRNA levels. The mRNA level of another YAP transcriptional target, CTGF, (Zhao et al., 2008), was also reduced by DEM, whereas the mRNA levels of the GABP subunits were not significantly altered (Figure S3B).

Immunofluorescence staining of HeLa cells showed that under normal culture conditions, GABPα was localized exclusively in the nucleus, but upon DEM treatment, a portion translocates to the cytoplasm (Figure 3D). In contrast, GABPβ was found in both the cytoplasm (as bright dots) and the nucleus, and its distribution was not affected by DEM treatment (Figure 3D). DEM treatment resulted in cell cycle arrest and enhanced cell death, both of which could be partially rescued by NAC treatment or by increasing YAP expression (Figure 3E). The MTT cell proliferation assay further confirmed that YAP overexpression could reduce DEM-induced inhibition of cell proliferation (Figure 3F). In

summary, the oxidant-induced inhibition of GABP is accompanied by a downregulation of YAP, and the restoration of YAP substantially ameliorates oxidant-induced cell cycle arrest and apoptosis. These results strongly support the hypothesis that YAP is an important downstream effector of GABP.

The Mst1/Mst2 double knockout liver exhibits an increased expression of YAP

Inactivating the Hippo signaling pathway in the liver by the dual inactivation of Mst1 and Mst2 results in increased YAP protein levels (Zhou et al., 2009). Although this increase most likely reflects, in part, a reduction in YAP degradation (Zhao et al., 2010), qPCR analysis in this study showed that YAP mRNA is increased 2- to 3-fold in Mst1/Mst2 double knockout (DKO) livers (Figure 4A). We examined whether this increase in YAP mRNA involves the activation of GABP. Lysates prepared from the livers of 6-week-old and 4-month-old wild-type (WT) or Mst1/Mst2 DKO mice were analyzed by immunoblotting (Figure 4B). Livers from 6-week-old Mst1/Mst2 DKO mice were hyperplastic but nontumorous, whereas at livers from 4-month-old Mst1/Mst2 DKO mice contained multiple foci of hepatocellular carcinoma (HCC) and tumors of mixed HCC and cholangiocarcinoma cellularity (Zhou et al., 2009).

Phospho-Mob and phospho-YAP levels were previously demonstrated to be dramatically reduced in tissues from Mst1/Mst2 DKO mice (Zhou et al., 2009). The GABPα protein level was increased in all DKO samples, whereas the GABPβ level was increased only in 4-month-old DKO livers (Figure 4B). The increased expression of YAP and GABP subunits in Mst1/Mst2 DKO livers was confirmed by immunohistochemistry (Figure 4C). YAP expression in the WT liver was evident predominantly in the periportal area and in cells lining the bile ducts. GABPβ1 showed a similar distribution, whereas GABPα expression was more diffuse. In the Mst1/Mst2 DKO liver, the expression of all three proteins was widespread, greatly enhanced and strongly intranuclear.

In a reciprocal manner, the stable expression of Mst1 in an HCC cell line derived from the Mst1/Mst2 DKO liver resulted in a significant reduction in the expression of the YAP1 protein, but the levels of the GABP were not altered (Figure 4D). The transcriptional activity of the *Yap* promoter-driven luciferase reporter plasmids was also strongly reduced by the expression of Mst1 (Figure 4E). The effects of Mst1 restoration in the Mst1/2 DKO HCC cell line were the opposite of the effects of the dual deletion of liver Mst1 and Mst2, except that the restoration of Mst1 in the HCC cell reduced GABP transcriptional activity without altering the expression level of the GABP subunits. Nevertheless, we next investigated the mechanism by which Hippo signaling suppresses GABP activity.

Lats1 binds to and promotes the phosphorylation of GABPβ, inhibiting the homodimerization and nuclear localization of GABPβ

We performed co-immunoprecipitation assays to determine whether Flag-tagged plasmids that express components of the Hippo pathway, including Lats1, Mst2, Mob1, WW45 and YAP, can bind specifically to Myc-GABPα or -GABPβ. Indeed, both Flag-Lats1 and Flag-WW45 associates with Myc-GABPβ1L but not with Myc-GABPα (Figure 5A). In addition, endogenous Lats1 was coprecipitated with GABPβ1 from WT liver extracts (Figure 5B). Cell fractionation experiments showed that the transfection of Mst2, Lats1 or both into HeLa cells reduced the nuclear levels of endogenous GABP1α and GABP1β and increased their cytoplasmic levels (Figure S4A). This result was confirmed by immunofluorescence staining of GABPα and GABPβ (Figure 5C).

Interestingly, although Mst2 does not directly bind to GABPβ1L, the overexpression of Mst2 alone also resulted in the redistribution of GABPα/GABPβ1, suggesting that this redistribution may result from the activation of the kinase activity of Lats (Figure S4B). Furthermore, the overexpression of Mst2 or Lats1 resulted in the disappearance of the condensed bright cytoplasmic dots of GABPβ1 (Figures 5C and S4B). We speculate that this disappearance reflects an Mst2/Lats-induced disruption of GABPβ1 cytoplasmic homodimers, which are formed via the GABPβ1 C-terminal leucine zipper-like domain. Also, the overexpression of Mst2/Lats1 reduced the homodimerization of GABPβ1 but did not affect the association of GABPα with GABPβ1 (Figure 5D).

Expressed alone, GABPα does not exhibit preferential nuclear localization but relies on the GABPβ nuclear localization sequence (NLS, aa 243-319) for nuclear entry (Sawa C, 1996). Using GFP-tagged GABPβ fragments, we demonstrated that the binding site on GABPβ for Flag-Lats1 is located between amino acids 241 and 319, which contain the NLS (Figure S4C). Thus, the binding of Lats1 to GABPβ may directly interfere with the ability of the heterodimeric GABP to enter the nucleus.

Previous work has shown that Ser170 and Thr180 of GABPβ can be phosphorylated directly by MAP kinases (Flory et al., 1996; Fromm L, 2001). Therefore, we examined whether GABPβ phosphorylation regulates GABP nuclear localization. An in vitro kinase assay showed that Lats1 itself can phosphorylate GABPβ but not GABPα (Figure 5E). Among the Myc-tagged GABPβ fragments (1-166, 1-260 or 260-383) overexpressed in 293T cells, only GABPβ(1-260) resulted in an upshifted band (Figure 5F), suggesting that GABPβ(1-260) may be phosphorylated. Consistent with this idea, cotransfection of Myc-tagged GABPβ(1-260) with Mst2/Lats1 increased the relative abundance of the slower-migrating GABPβ(1-260) band (Figure 5G). To determine whether the phosphorylation of Ser170 and Thr180 or both are responsible for the upshifted GABPβ(1-260) band, S170A, T180A or S170A/T180A mutant GABPβ(1-260) expression plasmids were constructed and cotransfected with Mst2/Lats1. The S170A and S170A/S180A GABPβ(1-260) mutants, but not GABPβ(1-260) T180A, lost the slower-migrating GABPβ(1-260) band, indicating that the phosphorylation of Ser170 is responsible for the upshifted band of GABPβ(1-260) (Figure 5G).

Furthermore, cotransfection with Mst2/Lats1 not only enhanced the abundance of the upshifted band (Figure 5G) but also strongly stimulated the association of GABPβ with 14-3-3, which did not occur with the GABPβ Ser170A mutant (Figure 5H, second panel from bottom). Binding to 14-3-3 promotes the nuclear exit of the protein that is complexed with 14-3-3, as demonstrated for phospho-YAP (Zhao et al., 2007). Thus, Lats1 can bind to the NLS-encompassing region of GABPβ, interfering with GABPβ nuclear translocation. In addition, Lats1 can promote the phosphorylation of GABPβ Ser170, thereby promoting its association with 14-3-3 and favoring the nuclear exit of GABP. Finally, our results demonstrate that the coexpression of Mst2/Lats1 with GABPα/GABPβ1L strongly inhibits YAP2600-Luc luciferase activity and that this inhibition is completely abolished upon cotransfection of Mst2/Lats1 with a GABPα/GABPβ1L(S170A) mutant protein (Figure 5I). These results suggest that the Hippo signaling pathway suppresses GABP transcriptional activity via a mechanism that depends on the phosphorylation status of GABPβ Ser170.

Acetaminophen-induced hepatotoxicity involves GABP inactivation and YAP depletion

We found that the GSH/GSSG ratio is significantly increased in the Mst1/Mst2 DKO liver (Figure S5A). Consistent with this observation, the levels of several enzymes that promote the accumulation of GSH, such as glutathione reductase (GSR) and the modifying subunit

(GCLM) of the γ-glutamyl-cysteine ligase catalytic subunit (GCLC), and several antioxidant proteins, including NAD(P)H:quinone oxidoreductase (NQO1), cytosolic thioredoxin (Txn1) and mitochondrial thioredoxin (Txn2), are all increased in Mst1/Mst2-deficient liver tissue relative to the WT liver (Figure S5B). Thus, the observed GABP activation and increase in YAP mRNA levels in the Mst1/2 DKO liver may be partially due to the increased GSH/GSSG ratio and reduced GABP oxidation.

The administration of acetaminophen (APAP, 300 mg/kg) to C57Bl/6 mice depletes reduced GSH in the liver and results in hepatocellular necrosis, as indicated by the increased plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Henderson et al., 2000). After oral administration of APAP, the levels of YAP and Skp2 in the liver of WT mice were reduced within 6 hours and were barely detectable after 12 hours (Figure 6A), Although the GABPα and GABPβ mRNA levels were unaffected by APAP (Figure 6B), the GABPα and GABPβ protein levels were decreased by 12 hours after APAP treatment (Figure 6A). The YAP mRNA level was also dramatically reduced at this time point (Figure 6B). Interestingly, the livers of Mst1/Mst2 DKO mice, in which YAP is underphosphorylated and overexpressed, were protected from APAP-induced hepatotoxicity. Compared with WT, the APAP-induced increase in plasma ALT and AST (Figure 6C) was dramatically reduced in the livers of Mst1/2 DKO mice. Histological examination confirmed that the hepatic necrosis caused by APAP treatment in WT mice (Figure 6D, left) was virtually eliminated in the Mst1/2 DKO mice (Figure 6D, middle).

The importance of YAP overexpression in APAP resistance in Mst1/2 DKO mice was assessed using transgenic mice with constitutively nuclear expression of YAP(Ser127Ala) in their liver. These mice also exhibited substantial protection from APAP-induced hepatic necrosis, as shown by histology (Figure 6D, right) and plasma ALT/AST levels (Figure 6E). The hypothesis that both direct APAP-induced tissue damage and APAP-induced depletion of YAP contribute to APAP-hepatotoxicity is supported by the observation that liver-specific inactivation of YAP itself results in areas of spontaneous liver necrosis as early as 4 weeks of age (Figure 6F, top). These areas of necrosis became much more severe by 8 weeks (Figure 6F, bottom) and were accompanied by increased levels of plasma ALT and AST activity compared with that of WT littermates (Figure S5C). Interestingly, YAP knockout livers exhibited a decreased GSH/GSSG ratio that may contribute to the progressive cellular damage, whereas the GSH/GSSG ratio was increased in YAP transgenic livers (Figure S5D). Hepatic extracts of WT, $Yap^{+/-}$ and $Yap^{-/-}$ mice were immunoblotted for a variety of antioxidant regulators and proteins involved in mitochondrial biogenesis.

The results demonstrated (Figure S5E) that YAP deletion significantly reduced the expression of ATP1β1, TYMS, COX5b, Tfam2, TXN2, Sp1, SOD2, SOD3, PRDX1, NQO1 and GSR. Conversely, YAP overexpression in HepG2 cells greatly increased the luciferase activity driven by promoters of the antioxidant *Txn2* or *Nqo-1* genes, whereas Mst2/Lats1 modestly suppresses the expression of these reporters (Figures S5F). Thus, YAP deficiency reduces the expression of a variety of genes that encode mitochondrial proteins and proteins with antioxidant properties, resulting in increased cellular ROS and a diminished GSH/GSSG ratio.

We determined the survival of WT, Mst1/2 liver DKO, YAP liver-null and YAP liver-transgenic mice treated with a near-lethal dose of APAP (300 mg/kg) (Figure 6G). Consistent with previous observations, approximately 50% of WT mice died within 9 hours, and another 30% by 15 hours after the oral administration of APAP. All mice with liver-specific deletions of YAP were dead within 7 hours. In contrast, Mst1/2 liver DKO mice were completely resistant to APAP-induced death, and only 20% of YAP liver-transgenic mice died within 15 hours of APAP treatment (Figure 6G). We also treated mice that had

received adenovirus-encoded GFP or GFP-GABPα+β with APAP (300 mg/kg). The mice overexpressing GABPα+β showed modest protection from APAP-induced death (Figure 6H). Thus, GABPα+β or YAP overexpression, via anti-apoptotic and antioxidant defense mechanisms, protects against APAP-induced liver damage. We conclude that the decrease in YAP levels after APAP treatment is partly due to APAP-induced inhibition of GABP and is an important contributor to APAP-induced hepatic necrosis.

Loss of Hippo signaling is correlated with the increased nuclear localization of GABP and YAP in human liver cancers

Increased YAP expression and signaling is the essential precursor for the development of HCC in Mst1/2 liver DKO mice (Zhou et al., 2009). Our results demonstrate that enhanced GABP expression/activity contributes to the increased YAP levels observed in this model. Previous studies have shown that Hippo signaling is commonly lost in human HCCs, as shown by the loss of the cleaved active Mst1 catalytic fragments and decreased levels of pYAP and pMob1 (Zhou et al., 2009), and our current results demonstrate that Hippo signaling inhibits GABP activity (Figure 5). Therefore, we inquired whether GABP activity or expression is upregulated in human HCC.

We examined liver-derived tumorous and nontumorous tissues from approximately 50 Chinese liver cancer patients. Immunohistochemistry of the nontumorous regions of human liver showed that staining for YAP, GABPα and GABPβ was most intense in the cells surrounding the bile ducts. In the HCCs, the total YAP staining was enhanced, and YAP nuclear staining was more prevalent than in nontumorous regions. In normal livers, GABPα showed a more widespread distribution than YAP, whereas GABPβ staining showed periportal and nuclear localization similar to YAP. In human HCCs, GABPα and GABPβ staining was greatly intensified in the nucleus compared with that observed in the adjacent nontumorous liver. The expression of YAP, GABPα or GABPβ was also estimated by immunoblotting analysis and real-time PCR. The relative expression levels of YAP, GABPα or GABPβ were all significantly higher in human HCCs compared with nontumorous livers (Figures 7D–F and S6). Consistent with a previous result (Zhou et al., 2009), the inactivation of the Hippo signaling pathway was also evident in most liver cancer specimens, as indicated by the diminished levels of pMob (T35) and pLats1 (S909) (Figures 7D, 7E and S6). These results suggest that the enhanced expression of YAP in human HCC results in part from the activation of GABP, and that similar to the Mst1/2 DKO mouse liver, the increased YAP expression is due in part to the inactivation of the Hippo signaling pathway.

Discussion

GABP is a heteromeric transcription factor that binds to a GA-rich Ets binding site (GGAAG) in DNA and comprises two unrelated subunits: GABPα, a member of the Ets family, and GABPβ, a Notch-Ankyrin repeat protein (Rosmarin et al., 2004). The *Yap* promoter region (-3300 bp to + 207 ATG site) contains 16 EBS sites. However, among the 18 Ets family proteins studied, only the GABP heterodimer significantly activated transcription from the YAP promoter. These observations, together with the presence of GABP on the *Yap* promoter in HeLa cells and primary mouse hepatocytes, strongly support the physiological relevance of GABP regulation of YAP transcription.

The GABP transcription factor has been linked to the regulation of diverse functional classes of genes, including many genes that encode key cell cycle control proteins (Yang et al., 2007). The depletion of either the GABPα or GABPβ subunits results in a reduction of YAP mRNA, G1/S cell cycle blocking and increased cell death. These cell fate outcomes are

substantially rescued by restoring YAP expression. Thus, GABP is required for the expression of YAP, and YAP is an important downstream effector of GABP for cell proliferation and survival. The ability of adenovirus-encoded GABP to promote hepatocyte proliferation in vivo together with the increased abundance of GABP and YAP within 24 hours after a partial hepatectomy support the idea that GABP-induced YAP expression contributes to post-hepatectomy liver regeneration.

In addition to identifying GABP as a critical regulator of YAP expression, we provide evidence that the transcriptional activities of YAP and GABP are negatively regulated by the Hippo signaling pathway. As a result, the deletion of Mst1 and Mst2 from the mouse liver is accompanied by an increase in the YAP mRNA level. Conversely, the reconstitution of Mst1 expression in an HCC cell line derived from the Mst1/2 DKO liver strongly suppresses the GABP-dependent transcriptional activity of the *Yap* promoter without altering GABPα/β expression.

Various mechanisms appear to be involved in the Mst1/Mst2-mediated inhibition of GABP activity. Lats1, the inhibitory YAP kinase of the canonical Hippo pathway, can directly bind to GABPβ1 at a segment contiguous with the GABPβ1 NLS domain, thereby interfering with GABP nuclear translocation. Lats1 binding to GABPβ1 also disrupts GABPβ1 homodimerization, thereby inhibiting the tetramerization of the GABPα/GABPβ dimers, which is important for optimal GABP transcriptional activity. Whether Lats1 binding modulates the interaction of GABP with its cotranscriptional modulators remains unknown. The Hippo pathway also inhibits GABP through GABPβ1 phosphorylation. The overexpression of Mst2/Lats1 stimulates the phosphorylation of GABPβ1(Ser170), GABPβ1 binding to 14-3-3 and GABP nuclear exit.

Previous studies have shown that both subunits of GABP (Thr280 of GABPα, Ser170 and Thr180 of GABPβ) can be directly phosphorylated by the MAP kinases ERK (Flory et al., 1996) and SAPK/JNK (Hoffmeyer et al., 1998) in response to exposure to serum, active phorbol esters, UV light and methyl methane sulfonate, which strongly induce the SAPK/JNK and p38 kinases (Wasylyk et al., 1998). The overexpression of Mst2 can activate SAPK/JNK (Ura et al., 2007), and TAO kinase, which was recently shown to operate as a direct upstream activator of Hippo kinase, can activate the SAPK/JNK and p38 kinases in certain situations (Boggiano et al., 2011; Poon et al., 2011). The identity of the GABPβ (Ser170) protein kinases that are regulated by the Hippo pathway in vivo remain to be elucidated. We observed that Lats1 itself can phosphorylate GABPβ but not GABPα in vitro, but the functional significance of this modification is not yet known.

A third mechanism by which Hippo signaling can inhibit the GABP transcriptional activity is through the ability of Mst1/Mst2 to modify the GSH/GSSG ratio in a manner that is unfavorable to GABP transcriptional activity. The elimination of Mst1/Mst2 from the liver is accompanied by an increase in the GSH/GSSH ratio, due in part to the increased expression of a cohort of enzymes that promote GSH synthesis and the scavenging of oxidants. The restoration of Mst1 reverses this response and reduces the GSH/GSSG ratio. Thus, Hippo signaling may promote the oxidative inactivation of GABP. The increase in GSH/GSSG in the Mst1/Mst2 DKO livers appears to be mediated by the increased YAP activity (Figure 6J). Similarly, *Yki* was recently reported to reduce ROS in *Drosophila* by upregulating mitochondrial function and enhancing antioxidant expression (Nagaraj et al., 2012). However, the results of studies on the effects Hippo signaling on ROS production (Abdollahpour et al., 2012; Choi et al., 2009) and the effects of ROS on Hippo signaling (Lehtinen et al., 2006; Ohsawa et al., 2012) are conflicting and do not allow a general conclusion.

In contrast, the importance of GSH depletion on the hepatic toxicity of acetaminophen (Tylenol) is very well established. Overdose of acetaminophen causes severe GSH depletion, ROS generation and serious liver injury, and it may even result in death (Henderson et al., 2000). In this study, we have shown that an acetaminophen overdose inhibits GABP transcriptional output and leads to a profound depletion of YAP within 12 hours of treatment. Moreover, the YAP deficiency greatly sensitizes mice to acetaminophen hepatotoxicity due to a marked decrease in the expression of mitochondrial and antioxidant genes, and the restoration of active YAP markedly ameliorates acetaminophen hepatotoxicity. However, harnessing the proliferative and anti-apoptotic functions of YAP for the treatment of acetaminophen overdose-induced liver failure is a daunting challenge, in view of the oncogenic function of YAP in human liver cancers.

As shown by the decreased levels of phospho-YAP and Mob1, Hippo signaling is frequently lost in human HCCs (Zhou et al., 2009). In view of the current data that the loss of Hippo signaling restores GABP transcriptional activity and enhances YAP mRNA abundance, GABP emerges as a potential therapeutic target in human HCCs and in other cancers driven by YAP.

Experimental Procedures

Animals

Mst1, *Mst2*, or *Yap* gene conditional knockout or transgenic mice have been previously described (Camargo et al., 2007; Zhou et al., 2009). Wild-type C57BL/6 mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained under specific pathogen-free conditions at Xiamen University Laboratory Animal Center (XMULAC). Mouse work was conducted with the approval of the Institutional Animal Care and Use Committee and in strict accord with good animal practice as defined by XMULAC.

APAP-induced Hepatotoxicity

Mice were administered acetaminophen (*N*-acetyl-*p*-aminophenol; APAP; Sigma, St. Louis, MO, USA) via oral gavage after 12 hours of starvation. APAP was used at a concentration of 300 mg/kg in phosphate-buffered saline (PBS). To assess the injurious effects of APAP on liver histology and function, serum was collected from mice via cardiac puncture. Serum samples were taken at 3, 6 and 12 hours after gavage. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined in the blood specimen using an ALT/AST assay kit (20030106, NJJCBio, China). For histopathology, the dissected liver tissues were fixed in buffered formalin, embedded in paraffin, and then processed for tissue-section staining with hematoxylin and eosin.

Human Liver and HCC Samples

Human samples were obtained under informed consent from the Human Tissue Banks of Xiamen Hospital of Traditional Chinese Medicine and Zhongshan Hospital of Xiamen University. All experiments were performed under Xiamen University Review Board approval. Snap-frozen biopsies from specimens of normal liver tissue (distant from the tumor) and HCC were collected. The diagnosis of HCC and normal liver was confirmed based on histological findings by independent pathologists.

Statistical Analyses

Data are representative of at least three independent experiments. Error bars represent SD; n≥3. Student's *t*-test (two-tailed) was used to assess the differences between means for all data analyzed. Tests with a *p*-value less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The heterodimeric Ets transcriptional factor GABP is required for YAP expression.
2. YAP is an important downstream effector of GABP for cell survival and proliferation.
3. The Hippo pathway regulates the activity of GABP by phosphorylation.
4. YAP is essential for cellular and tissue defenses against oxidative stress.

Yap is an important downstream effector of the Hippo pathway. However, how the *Yap* gene expression is regulated remains elusive. In this study, Zhou, Chen and colleagues demonstrated that Ets family member GABP is critical for *Yap* gene expression, and the GABP activity is regulated by the Hippo pathway. GABP mediated YAP expression protects Acetaminophen-induced liver injury. Both GABP and YAP expressions are enhanced in human liver cancers. Therefore, GABP is a potential therapeutic target for liver diseases.

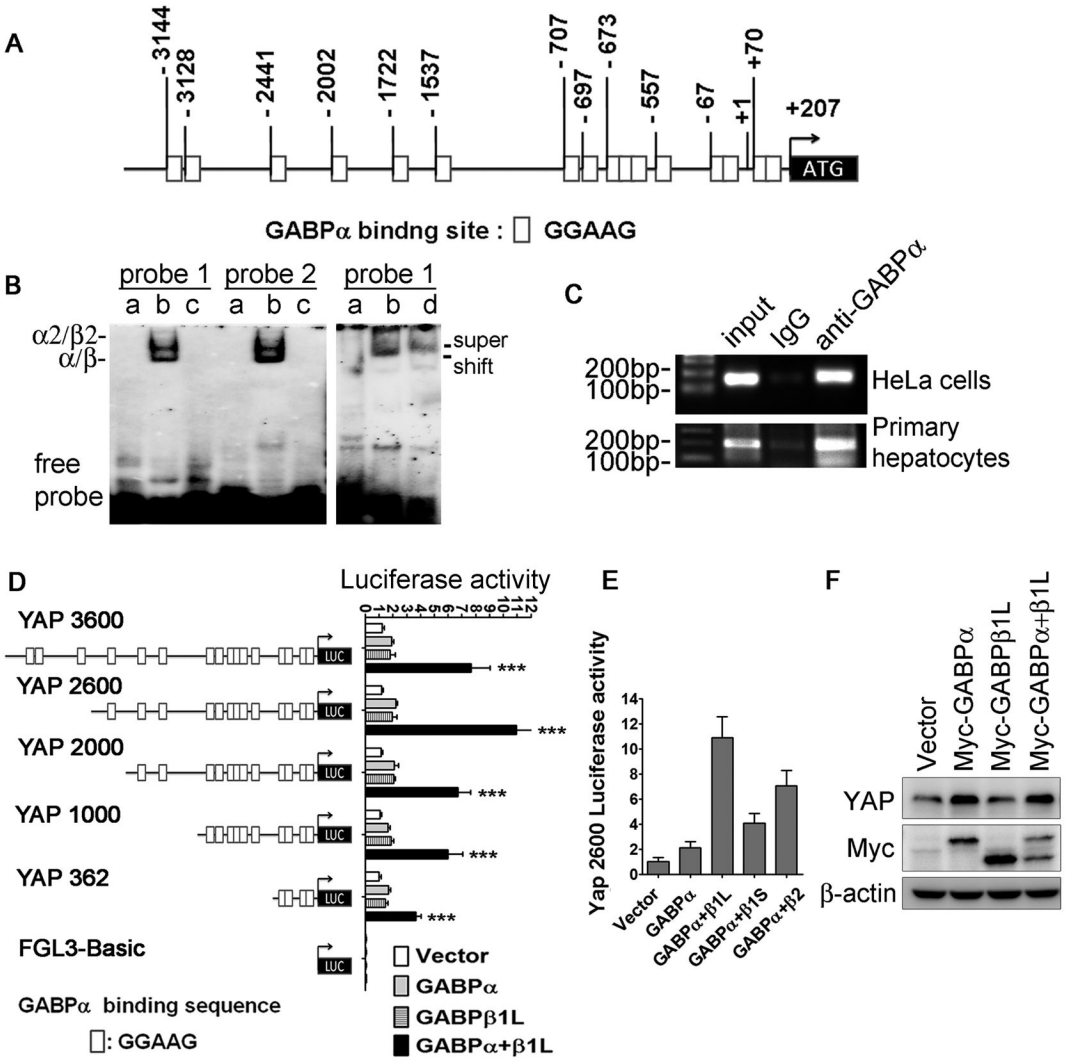

Figure 1. GABP acts on the mouse *Yap* promoter in vitro and in vivo

(A) Multiple Ets binding sites (GGAAG) are found in the *Yap* promoter region and are notably present in tandem repeats.

(B) Electrophoretic mobility shift and supershift analysis of the binding specificities of GABPα on the EBS of the *Yap* promoter. EMSA experiments were performed using two different biotinylated probes (the sequences are presented in Table S1) with no nuclear extracts (a), HeLa cell nuclear extracts (b) or HeLa cell nuclear extracts that were pre-depleted with anti- GABPα antibodies (c). The α2β2 tetramers and α/β dimers are indicated on the left. The supershift assay was performed by directly adding anti- GABPα antibodies to the assay (d). The supershifted bands are indicated on the right side.

(C) The chromatin immunoprecipitation assay shows that GABPα binds to the *Yap* promoter in HeLa cells and in primary mouse hepatocytes.

(D) GABPα/β1 enhances the luciferase activity driven by the 5′ flanking regions of the mouse *Yap* gene. The firefly luciferase activity was normalized against the Renilla luciferase activity and is presented as the level relative to the normalized activity obtained with pGL3-YAP362-Luc cotransfected with an empty vector.

(E) In contrast to GABPβ1S or GABPβ2, GABPβ1L cooperates with GABPα, resulting in the highest *Yap* promoter-driven luciferase activity.

(F) Overexpression of GABPα increases endogenous YAP expression in 293T cells. The data are representative of at least three independent experiments. Error bars represent the SD; n=3. *, $p<0.05$; ***, $p<0.001$. The p values refer to comparisons between samples transfected with GABPα+β and with control vector. See also Figure S1.

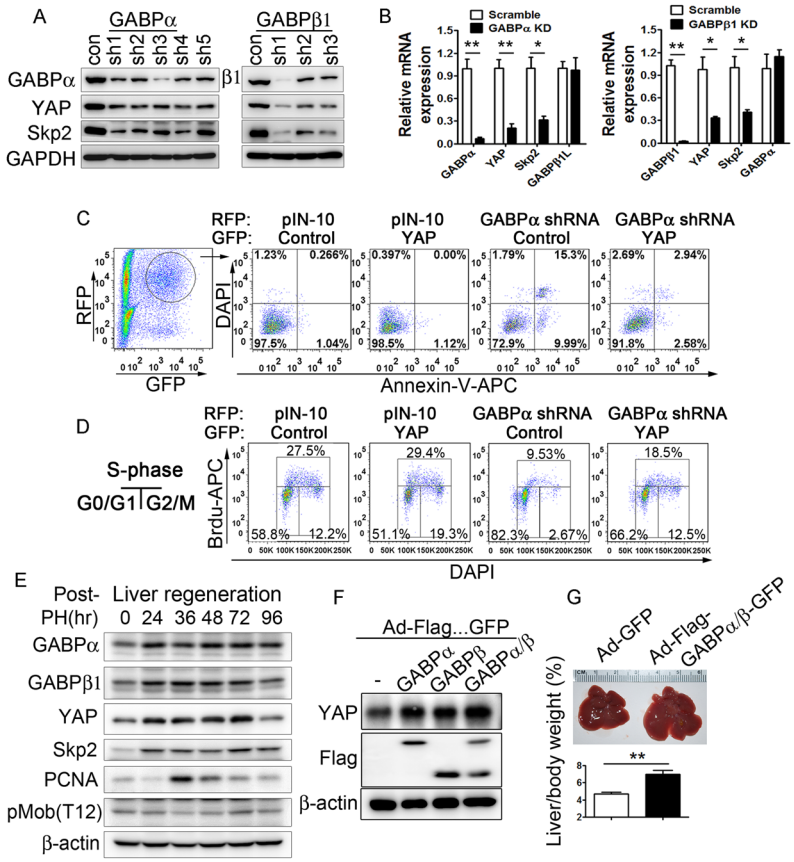

Figure 2. Cell cycle arrest and cell apoptosis resulting from the knockdown of GABP can be rescued or prevented by YAP overexpression

(A and B) Doxycyclin-induced knockdown of GABPα or β dramatically decreases the protein levels (A) and the transcript levels (B) of Skp2 and YAP. *, p<0.05; **, p<0.01. (C and D) HepG2 cells were cotransfected with either GABPα shRNA-RFP or control shRNAs and either GFP-YAP or the control vector. At 72 hours post-transfection flow cytometry analysis was performed with Annexin V/DAPI staining (C) or BrdU/DAPI staining (D). GFP^+RFP^+ cells were gated and plotted as indicated. The knockdown of GABPα enhances the early apoptosis ($AnnexinV^+DAPI^-$) and late apoptosis ($AnnexinV^+DAPI^+$) of HepG2 cells, whereas the overexpression of YAP restores cell survival to normal levels (C). The knockdown of GABPα induces an increase in the G1 phase (bottom left quadrant) and a decrease in S phase (top quadrant) cells, whereas the overexpression of GFP-YAP restores the cell cycle to normal levels (D).

(E) The levels of GABPα, β, YAP, Skp2, PCNA, pMob and β-actin expression in regenerating livers after hepatectomies were determined by immunoblotting with the indicated antibodies.

(F and G) Mice injected with an adenovirus expressing GABPα and β. YAP expression level was determined by immunoblotting the liver samples (F). The liver mass was weighed, and the results expressed as a bar graph (G).

The data are representative of at least three independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Error bars represent the SD; n=3. See also Figure S2.

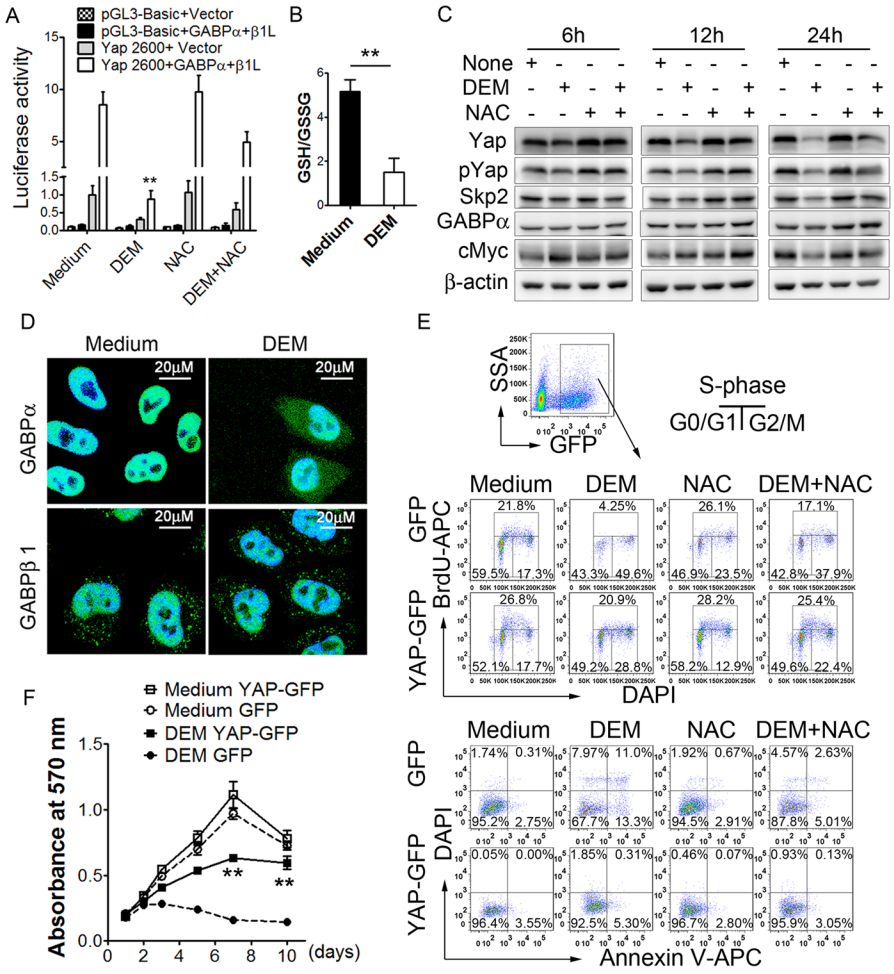

Figure 3. Glutathione depletion inhibits GABP-dependent YAP expression and its impact on cell proliferation and death

(A–B) GABP-mediated *Yap* promoter-driven luciferase activity (A) is inhibited by DEM treatment and restored by NAC addition. DEM treatment decreases glutathione levels (B). Error bars represent the SD; n=3. **, $p<0.01$. The *p* values refer to comparisons between treatments with and without DEM.

(C) The YAP, Skp2 and cMyc protein levels in HepG2 cells are inhibited by DEM treatment and restored by NAC addition.

(D) Immunofluorescence staining shows that DEM treatment increases the cytoplasmic retention of GABPα in HepG2 cells.

(E–F) Cell cycle arrest and cell apoptosis resulting from the DEM treatment can be rescued or prevented by the overexpression of YAP, as shown by flow cytometry analyses with Annexin V/DAPI staining or BrdU/DAPI staining (E) and MTT cell proliferation assays (F). Error bars represent the SD; n=3. **, $p<0.01$. The *p* values refer to comparisons between DEM plus YAP-GFP and DEM plus GFP transfections.

The data are representative of at least three independent experiments. See also Figure S3.

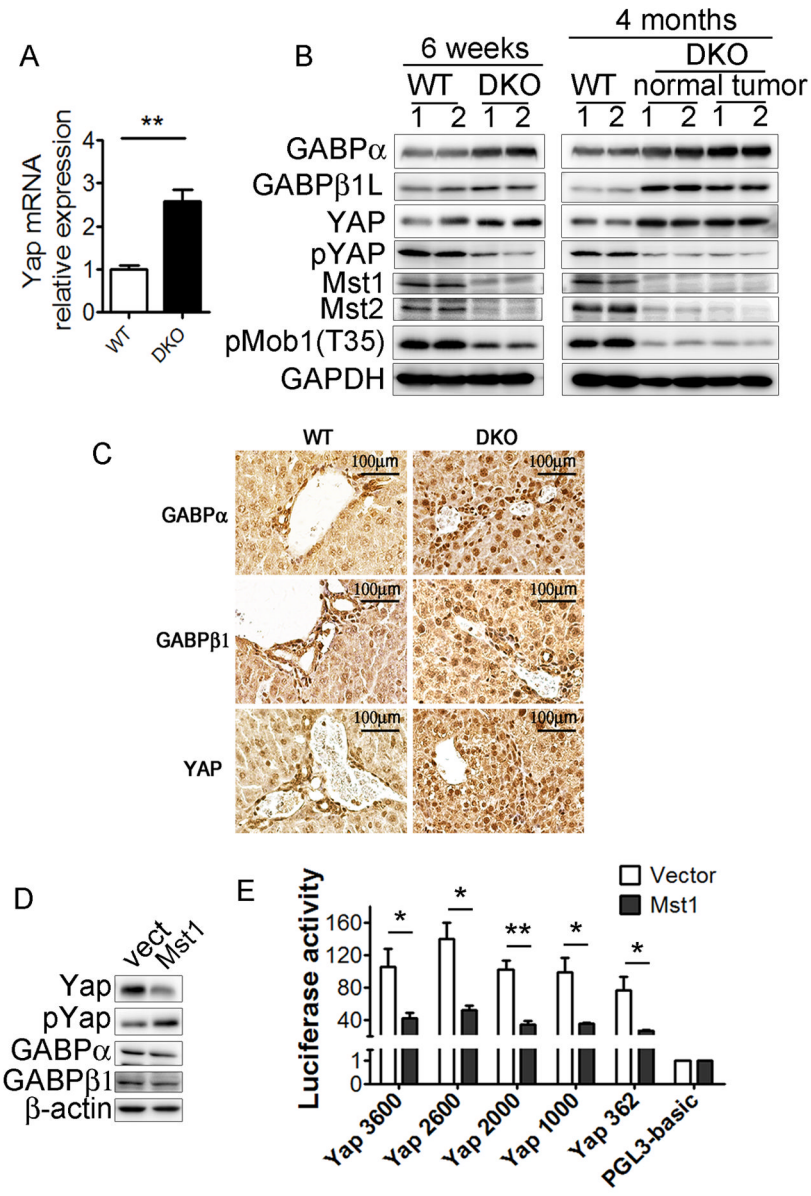

Figure 4. Mst1/Mst2 double knockout cells exhibit an enhanced expression of *Yap* gene and increased GABP activity

(A) qPCR analysis of YAP mRNA in WT and Mst1/Mst2 double knockout liver. Error bars represent the SD; n = 5. **, $p<0.01$.

(B) A panel of cell cycle regulators in liver tissues from WT and $Mst1^{-/-}Mst2^{F/F}$ *Alb-Cre* mice (normal or HCC tissues) were detected by immunoblotting.

(C) IHC analysis of GABP α, β1 or YAP expression in liver tissue from WT and $Mst1^{-/-}Mst2^{F/F}$ *Alb-Cre* mice. The deletion of Mst1/2 increases the expression levels of GABP α, β1 and YAP.

(D) The reconstitution of Mst1 reduces YAP expression in HCC1 cells.

(E) The reconstitution of Mst1 reduces *Yap* promoter-driven luciferase activity in HCC1 cells. Error bars represent the SD; n=3. *, $p<0.05$; **, $p<0.01$.

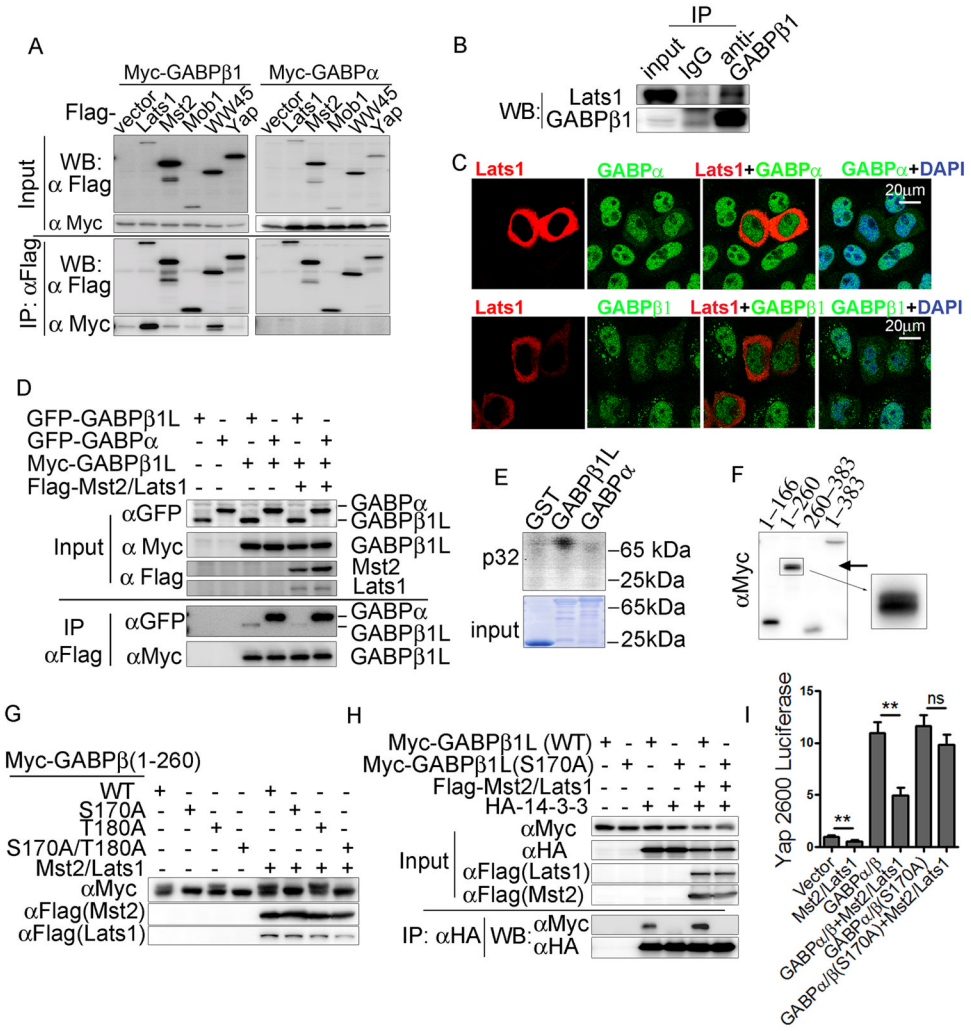

Figure 5. Lats1 phosphorylates GABPβ1 and regulates GABP cytoplasmic retention in HepG2 cells

(A) Lats1 and WW45 physically interact with GABPβ1 but not with GABPα, as shown by pull-down assays.

(B) Endogenous Lats1 was co-immunoprecipitated with GABPβ1 from wild-type liver lysates.

(C) The overexpression of Lats1 increases the cytoplasmic retention of GABPα or β1, as shown by immunofluorescence staining.

(D) Mst2 or Lats1 regulates GABPβ1L dimerization. 293T cells were transfected with GFP-GABPα, GFP-GABPβ1L, Myc-GABPβ1L or Myc-Mst2/Lats1 using the indicated combinations, and the dimerization of the GABP subunits was determined.

(E) Lats1 kinase phosphorylates GABPβ1 in vitro. An in vitro kinase assay was performed using GST-GABPα or β1L recombinant protein and Flag-Lats1 kinase.

(F and G) domain-deletion mutants of GABPβ1L were expressed in HepG2 cells. GABPβ1L (1-260) fragments exhibit two bands (F). Mst2/Lats1 enhances the upshifted band of WT GABPβ1L (1-260) fragments. The GABPβ1L mutant S170A, but not S180A, abolishes this upshift (G).

(H) Mst2/Lats1 promotes the interaction of GABPβ1 with 14-3-3 via the phosphorylation of Ser170. The mutant GABPβ1L (1-260) S170A abolishes this interaction.

(I) Mst2/Lats1 inhibits the GABPα/β-driven YAP2600-Luc luciferase activity. This inhibition is abolished with the mutant GABPβ1L(S170A). Error bars represent SD; n=3. **, $p<0.01$.

The data are representative of at least three independent experiments. See also Figure S4.

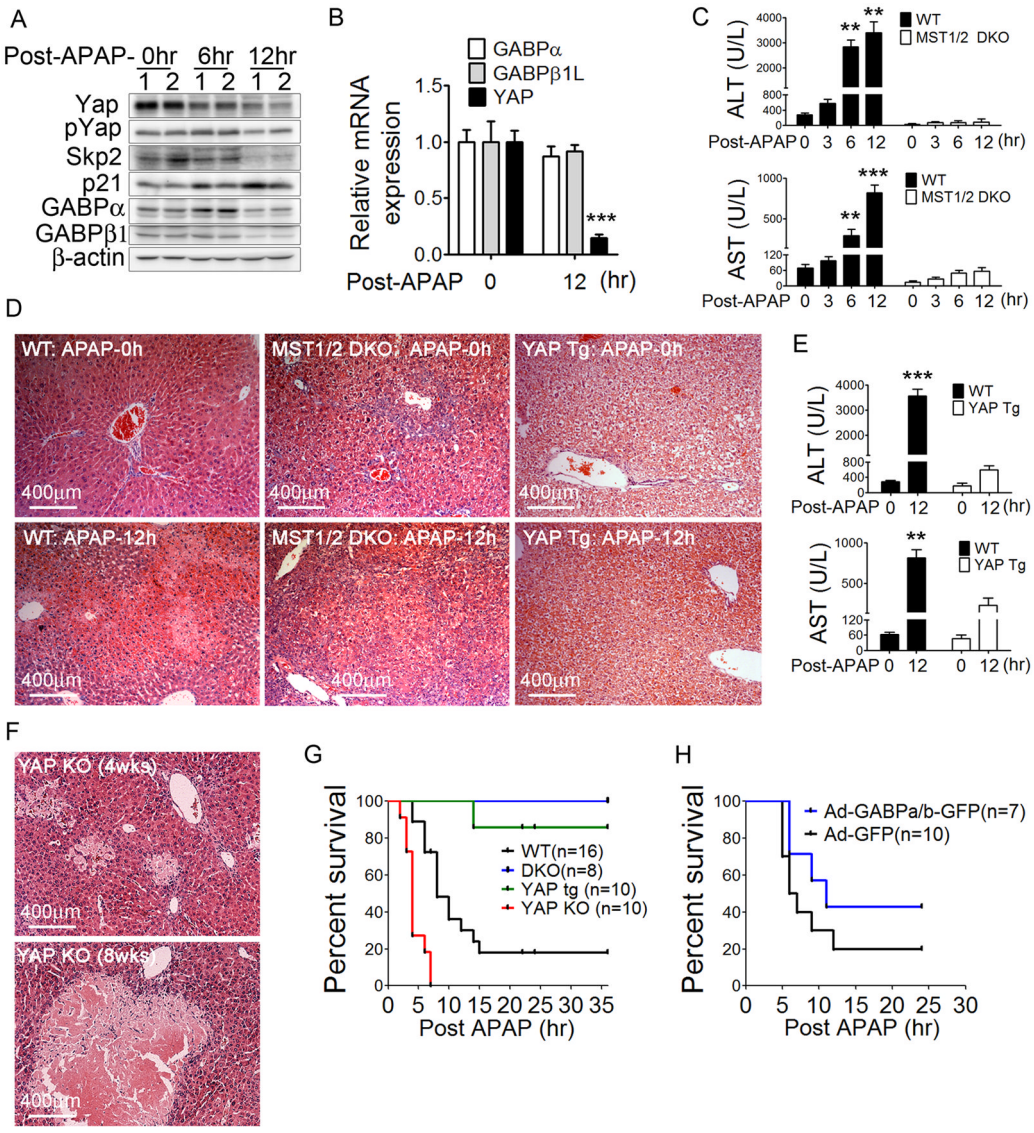

Figure 6. GABP-dependent YAP expression is responsible for APAP-induced hepatotoxicity

(A and B) APAP administration decreases YAP expression. At the indicated times after APAP administration (300 mg/kg), the levels of YAP, p-YAP, Skp2, P21, GABPα and β1 proteins in the liver were determined by immunoblotting with specific antibodies (A). The mRNA level of YAP, but not GABPα or β1, was decreased at 12 hours after APAP administration (B). Error bars represent the SD; n=3. ***, $p<0.001$. The p values refer to comparisons of YAP mRNA expression between 12 and 0 hours post-APAP treatment.

(C) The gradually increasing levels of the liver enzymes ALT and AST in the serum of WT mice after the administration of APAP are almost absent in $Mst1^{-/-}Mst2^{F/F}$ Alb-Cre mice. Error bars represent the SD, n=6. **, $p<0.01$; ***, $p<0.001$. The p values refer to comparisons between samples from WT and Mst1/Mst2 DKO mice.

(D) H&E staining shows that Mst1/2 liver DKO mice or liver-specific YAP transgenic mice exhibit less liver damage after APAP treatment compared with WT mice.

(E) Compared with WT mice, liver-specific YAP transgenic mice have lower levels of ALT and AST in the serum after APAP treatment (300 mg/kg). Error bars represent the SD; n=6.

**, $p<0.01$; ***, $p<0.001$. The p values refer to comparisons between samples of WT and YAP transgenic mice.

(F) Four-week-old (top) and 8-week-old (bottom) liver-specific YAP knockout mice spontaneously exhibit cell necrosis in livers.

(G) Kaplan-Meier survival curves for WT, Mst1/2 liver DKO, liver-specific YAP(S127A) transgenic or YAP liver knockout mice over 36 hours after a single toxic dose of APAP (300 mg/kg).

(H) Kaplan-Meier survival curves over 36 hours for WT mice injected with adenovirus-encoded GABPα and β or empty vector after a single toxic dose of APAP treatment (300 mg/kg).

See also Figure S5.

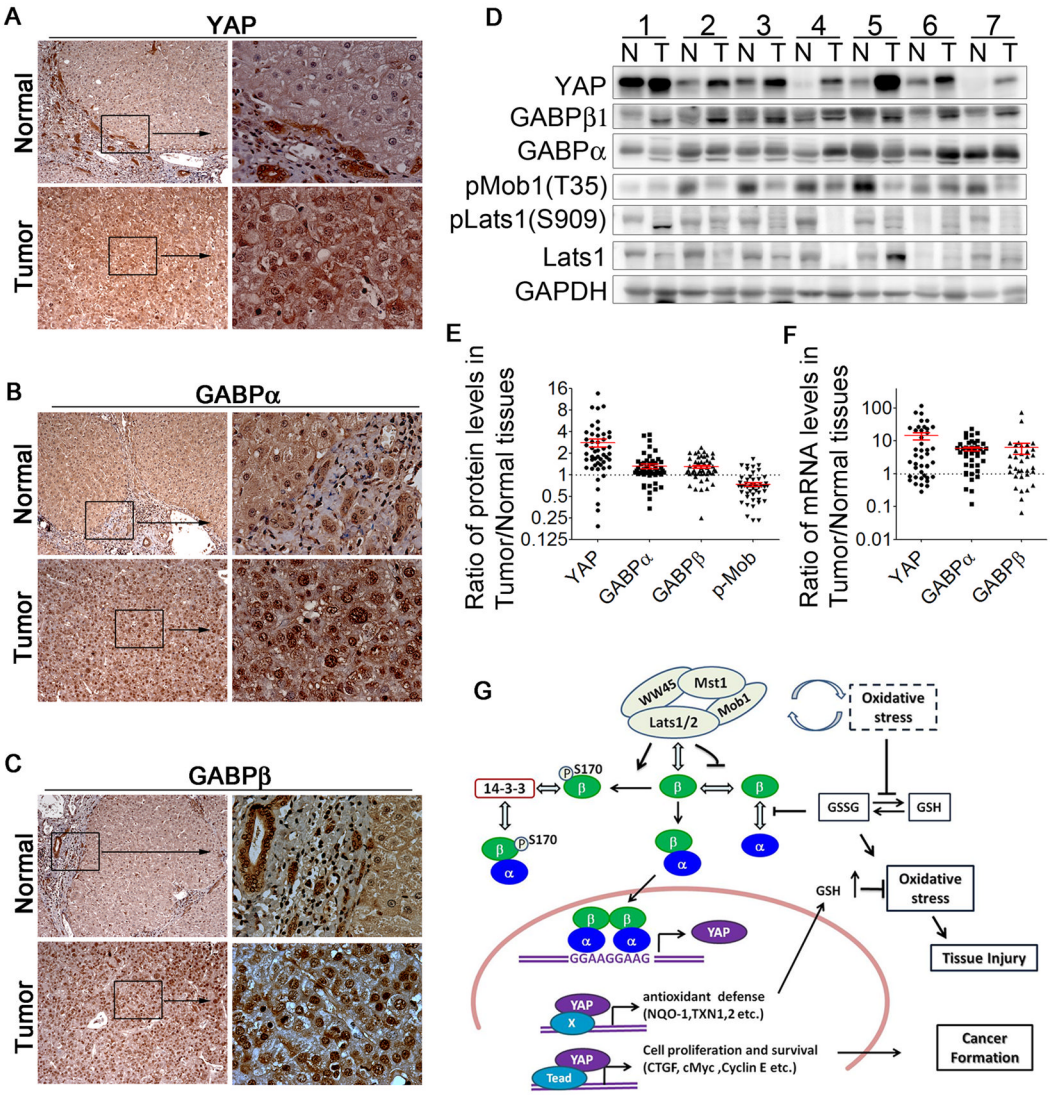

Figure 7. Loss of Hippo signaling is correlated with an increased nuclear localization of GABP and YAP in human liver cancers

(A to C) IHC analysis of YAP1 (A), GABPα (B) or β1 (C) in tissue sections of nontumorous livers (N) or liver cancers (T) isolated from the same patient. Greater expression levels of YAP1, GABPα and β1 are found in the biductal areas of normal livers and in all liver cancer cells.

(D and E) The expression levels of YAP, GABPα and GABPβ are significantly increased in liver cancer (T) compared with the nontumorous liver tissue (N) isolated from the same patient. Seven representative paired samples analyzed by immunoblotting with the indicated antibodies are shown (D). See also Figure S5 for the remaining 42 paired samples. Quantification of the intensities of immunoblot bands by Imagine gel software (E).

(F) The mRNA levels of GABPα, β1 and YAP were quantified by qPCR, and the ratio of the relative mRNA expression in nontumorous liver tissue (N) and liver cancer (T) from the same patient was plotted. Error bars represent the SD.

(G) A proposed working model for how the Hippo signaling pathway regulates cell growth and antioxidant defenses via modulation of GABP activity. The Hippo pathway kinase, Lats1, binds to GABPβ1 at the nuclear localization sequence (241-319 aa) and

phosphorylates GABPβ1 on Ser170, which disrupts the homodimerization of GABPβ1/β1 and promotes GABPβ1/14-3-3 association, resulting in the exit of GABP from the nucleus and termination of its transcriptional activation. The heterodimerization of GABPα/β1 can also be inhibited by depletion of glutathione (GSH). Hippo (Mst1/2)-Wts (Lats1/2) signaling reduces the GSH/GSSG ratio in the liver, thereby suppressing GABP activity. Upon the loss of Hippo signaling, GABP translocates to the nucleus, where it activates the expression of a set of genes, including YAP. YAP is essential for several cellular and tissue responses against oxidative stress, including the increase in NOQ-1, HO-1 and other antioxidant regulators. Overactive YAP can also cooperate with TEAD to promote organ growth and tumorigenesis, including the development of HCC.
See also Figure S6.