

## ORIGINAL ARTICLE

# E2F1-mediated AUF1 upregulation promotes HCC development and enhances drug resistance via stabilization of AKR1B10

Ting Zhang<sup>1</sup> | Guiwen Guan<sup>1</sup> | Jing Zhang<sup>1</sup> | Huiling Zheng<sup>1</sup> | Deyao Li<sup>1</sup> |  
Wengong Wang<sup>2</sup> | Fengmin Lu<sup>1</sup> | Xiangmei Chen<sup>1</sup> 

<sup>1</sup>Department of Microbiology and Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China

<sup>2</sup>Beijing Key Laboratory of Protein Posttranslational Modifications and Cell Function, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China

## Correspondence

Xiangmei Chen and Fengmin Lu, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Haidian District, Beijing 100191, China.  
Emails: xm\_chen6176@bjmu.edu.cn (XC); lu.fengmin@hsc.pku.edu.cn (FL)

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## Abstract

The AU-rich binding factor 1 (AUF1) is one of the well known adenylate-uridylylate-rich element (ARE)-specific RNA-binding proteins (ARE-BPs) for which dysregulation has been reported in various human cancers. However, the involvement of AUF1 in the initiation and progression of hepatocellular carcinoma (HCC) is still elusive. In this study, we aimed at exploring the clinical significance, function, and mechanism of the abnormal expression of AUF1 in HCC. Using a bioinformatics analysis of The Cancer Genome Atlas (TCGA) and Liver Cancer Institute (LCI) database, we identified that AUF1 was abnormally highly expressed in HCC tissues and that the high expression of AUF1 was correlated with poor prognosis in patients with HCC. We also confirmed the increased AUF1 expression and its prognostic value in our HBV-related HCC cohorts. AUF1 overexpression in hepatoma cells promoted cell proliferation and increased the resistance of hepatoma cells toward doxorubicin, whereas knockdown of AUF1 exerted the opposite effects. Mechanistically, we demonstrated that AKR1B10 was a critical target of AUF1 and was essential for sustaining the AUF1-induced proliferation and drug resistance of hepatoma cells. AUF1 increased AKR1B10 expression by binding to the 3'UTR region of AKR1B10 mRNA and stabilizing AKR1B10 mRNA. Additionally, we demonstrated that E2F1 enhanced AUF1 expression in HCC at the transcription level. Our study revealed a novel role of AUF1 in promoting the development and drug resistance of HCC via the post-transcriptional regulation of AKR1B10 expression. The E2F1/AUF1/AKR1B10 axis can serve as a potential therapeutic target in HCC.

## KEYWORDS

AKR1B10, AUF1, E2F1, hepatocellular carcinoma, RNA-binding protein

**Abbreviations:** ARE-BPs, ARE-binding proteins; AREs, adenine/uridine-rich elements; AUF1, AU-rich binding factor 1; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; RA, retinoic acid; RBPs, RNA-binding proteins.

Ting Zhang, Guiwen Guan, and Jing Zhang contributed equally to this work.

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## 1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most aggressive human cancers and currently ranks as the third leading cause of cancer-related deaths worldwide.<sup>1</sup> Despite recent progress in clinical treatment, the 5-y overall patient survival rate for HCC is less than 15%.<sup>2</sup> The poor prognosis of patients with HCC is mainly because of delayed diagnosis, limited therapeutic options, frequent cancer metastasis, and high cancer recurrence rates.<sup>3,4</sup> Therefore, a better understanding of the molecular mechanisms underlying the development of HCC is crucial for the development of novel therapeutic drugs.

Recent transcriptomic studies have revealed that transcriptome dysregulation plays driving roles during the development and progression of HCC,<sup>5,6</sup> and that abnormal post-transcriptional control of mRNA metabolism is related to the significant transcriptomic imbalance in HCC tumors.<sup>7-9</sup>

The post-transcriptional regulation of mRNA is mediated by the so-called RBPs. Among RBPs, the ARE-BPs are the best known regulators of post-transcription in eukaryotes.<sup>10,11</sup> Typically, AREs are short linear motifs with a high content of complementary A and U nucleotides located in the 3' untranslated region (3'UTR) of mRNAs. ARE-BPs can recognize and bind to hundreds of cellular mRNA through interactions with the ARE motif and regulate the mRNA metabolism of their targets, including mRNA processing, turn-over, localization, and translational control.<sup>12,13</sup> Numerous studies have demonstrated that mRNAs of many oncogenes are regulated by ARE-BPs and that dysregulated ARE-BP expression plays important roles in the development and progression of human cancers.<sup>14,15</sup>

AU-rich binding factor 1 (AUF1), also called heterogeneous nuclear ribonucleoprotein D (hnRNP D), is one of the well known ARE-BPs that is predominantly associated with mRNA-destabilizing activity. The target genes of AUF1 are involved in many physiological processes such as cell proliferation,<sup>15</sup> apoptosis,<sup>16</sup> senescence and metastasis,<sup>17</sup> and dysregulation of AUF1 has been reported in breast, skin, thyroid, and liver cancers. Recent studies have reported that AUF1 can also stabilize mRNA,<sup>15,17,18</sup> suggesting that AUF1 plays a critical and complex role in tumorigenesis. However, the involvement of AUF1 in HCC initiation and progression is still elusive.

In the present study, we investigated the expression pattern and role of AUF1 in HCC. We found that AUF1 was significantly upregulated in tumor tissues, and the higher expression level of AUF1 was associated with poor prognoses in patients with HCC. Interestingly, we demonstrated that AUF1 promoted HCC development and drug resistance by upregulating AKR1B10, an independent risk factor for HCC.<sup>19,20</sup> Moreover, we identified that AUF1 was a direct transcriptional target of E2F1. Collectively, these data support a model in which the E2F1/AUF1/AKR1B10 axis plays an important role in the development of HCC and can serve as a potential therapeutic target in HCC.

## 2 | MATERIALS AND METHODS

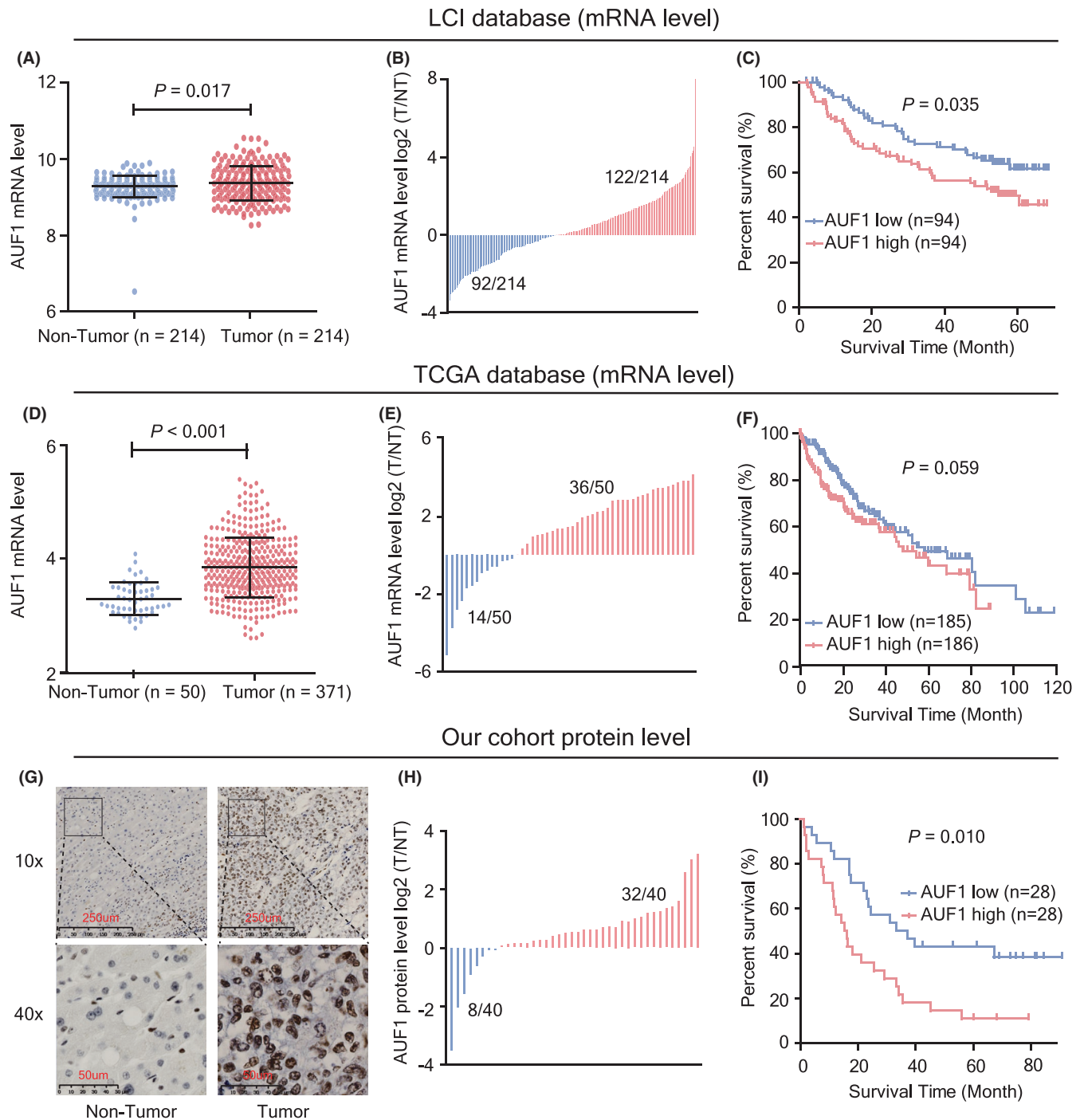
Detailed Materials and Methods are shown in Supporting information.

## 3 | RESULTS

### 3.1 | AUF1 is highly expressed in HCC and predicts poor prognosis

To evaluate the importance of ARE-BPs in HCC, we explored the expression profiles of 8 ARE-BPs in 214 patients with HBV-related HCC from the LCI database; these included AUF1, HUR, ZFP36, KSRP, TIA1, BRF1, HUD, and Hsp70, which have been frequently dysregulated in tumors and shown in previous studies to affect the occurrence and development of tumors.<sup>7,13,21</sup> The results showed that expression of *ZFP36*, *BRF1*, and *HUD* was downregulated, while other ARE-BPs were upregulated in HCC tissues compared with that in the paired non-tumor tissues (Figures 1A,B and S1A). Survival analysis of patients showed that only AUF1 expression level in tumor tissues was negatively associated with the overall survival time of patients with HCC ( $P = .035$ ; Figures 1C and S1B). The upregulation of AUF1 in HCC tissues was also observed in TCGA RNA-seq data set that enrolled 371 patients with HCC ( $P < .001$ ; Figure 1D,E). The survival analysis in this cohort also showed that patients with high AUF1 expression had a worse prognosis, although the difference was not statistically significant ( $P = .059$ ; Figure 1F). It is well known that elevated Alpha fetoprotein (AFP) levels are associated with the poor prognosis of patients with HCC.<sup>22</sup> In line with this, the AUF1 expression levels in patients with serum AFP levels  $\geq 300$  ng/mL were significantly higher than for patients with serum AFP levels  $< 300$  ng/mL in both the LCI ( $P < .001$ ; Figure S1C) and TCGA databases ( $P < .001$ ; Figure S1D). Additionally, the expression levels of AUF1 mRNA were significantly higher in the tumor tissues derived from patients with HCC who had Barcelona Clinic Liver Cancer (BCLC) stage C or larger tumors than that from patients who had BCLC stage A ( $P = .011$ ; Figure S1E) or smaller tumors ( $P = .047$ ; Figure S1F).

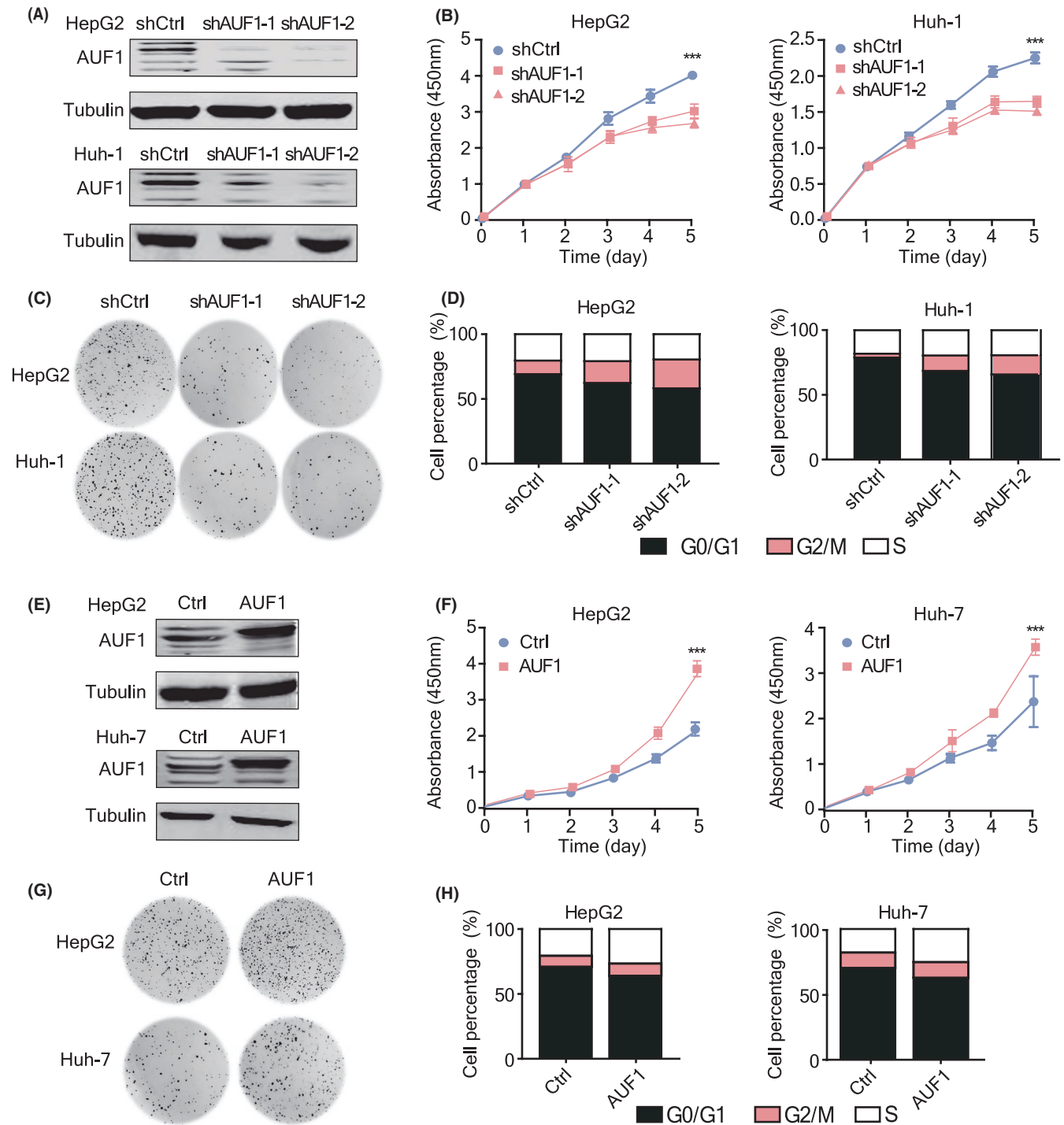
To further confirm the expression and the prognostic value of AUF1, an immunohistochemistry (IHC) assay was performed to determine the expression of AUF1 at the protein level in our 66 HBV-related HCC samples. Overall, we observed positive staining of AUF1 protein in 95.5% (63/66) of HCC tumor tissues, including 22 tissues with very strong AUF1 staining (IHC score  $\geq 3$ ), 27 with moderate staining ( $2 \leq$  IHC score  $< 3$ ), and 14 with weak positive staining (IHC score = 1). In contrast, AUF1 staining in the adjacent non-tumor liver tissues was very weak (Figure 1G and Table S1). Consistent with the IHC results, western blot assay showed that 72% (32/40) of tumor tissues had marked AUF1 upregulation (Figures 1H and S2A). Further survival analysis confirmed the poor prognosis of patients in this HBV-HCC cohort who had higher



**FIGURE 1** Expression and prognostic value of AUF1 in patients with HCC. A, AUF1 mRNA levels in HCC tissues and matched non-tumor tissues from the LCI database (n = 214). B, Abnormal expression of AUF1 mRNA in 214 HCC tissues from the LCI database. T/NT indicates the expression level ratio of tumor tissues and their corresponding non-tumor tissues. C, Kaplan-Meier analysis of AUF1 mRNA and overall survival from 188 HBV-HCC tissues from the LCI database. D, AUF1 mRNA level in 371 HCC tissues and 50 non-tumor tissues from TCGA database. E, Abnormal expression of AUF1 mRNA in 50 HCC tissues from TCGA database. F, Kaplan-Meier analysis of AUF1 mRNA and overall survival from 371 HCC tissues from TCGA database. G, H, AUF1 protein levels in paired HBV-HCC tissues detected using immunohistochemistry (IHC) staining (n = 66) and western blot assay (n = 40). I, Kaplan-Meier analysis of AUF1 IHC score and overall survival from 56 HBV-HCC tissues

AUF1 expression in tumor tissues ( $P = .010$ ; Figure 1I). In addition, the upregulation of AUF1 was also observed in multiple human hepatoma cell lines (Figure S2B). These data demonstrated that

the expression of AUF1 was frequently upregulated in HCC tumor tissues and higher AUF1 expression always predicted a poor prognosis for patients with HCC.



**FIGURE 2** AUF1 promotes the growth of HCC cells in vitro. A, AUF1 stable knockdown cell models were established. The knockdown efficiency of 2 shRNA were verified using western blot assay. B-D, Cell proliferation rate (B), colony formation ability (C), and cell cycle (D) were detected in AUF1-knockdown HCC cell lines. E, AUF1 stable overexpression cell models were established. Protein levels of AUF1 were measured using western blot assay. F-H, Cell proliferation rate (F), colony formation ability (G), and cell cycle (H) were detected in AUF1-overexpression HCC cell lines. \*\*\* $P < .001$

### 3.2 | AUF1 promotes the growth of hepatoma cells in vitro

The above results suggested that AUF1 might promote HCC progress. To address this issue, we established stable AUF1 knockdown models in HepG2 and Huh-1 cells using 2 independent shRNA expression

plasmids (Figure 2A). Silencing of AUF1 inhibited the proliferation rates of both HepG2 and Huh-1 cells (Figure 2B). Correspondingly, AUF1 knockdown significantly repressed the colony formation capability of HepG2 and Huh-1 cells (Figure 2C) and induced a G2/M cell cycle arrest in hepatoma cells (Figure 2D). We further performed a gain-of-function study (Figure 2E) and investigated the influence of

AUF1 overexpression on cell proliferation. When trying to construct AUF1 overexpression cell lines, we selected HepG2 and Huh-7 cells due to their higher infection efficiency for lentivirus. As anticipated, stable overexpression of AUF1 in both HepG2 and Huh-7 cells significantly promoted cell proliferation (Figure 2F) and colony formation (Figure 2G), and triggered more cells to enter S phase (Figure 2H). These results demonstrate that AUF1 could promote the growth of hepatoma cells in vitro.

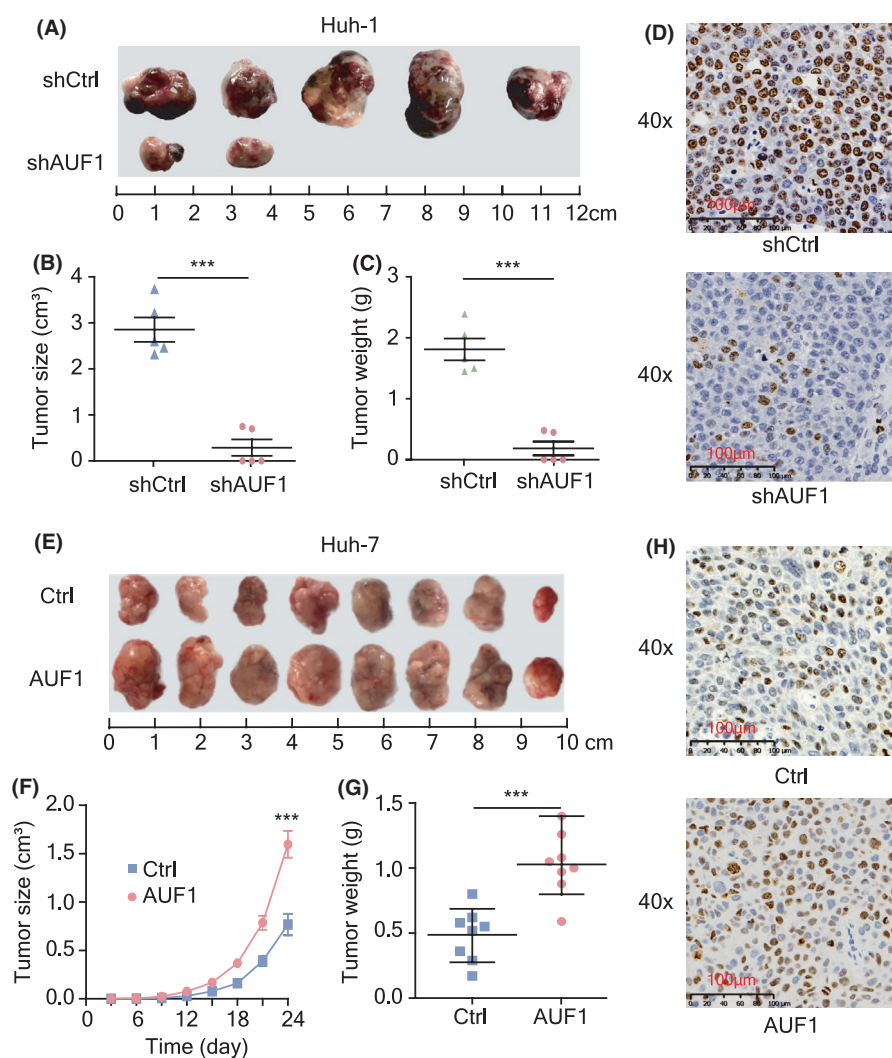
### 3.3 | AUF1 promotes the development of hepatoma cells in vivo

To further validate the involvement of AUF1 in the progression of HCC, Huh-1 cells with stable knockdown of AUF1, or control cells, were utilized for tumor formation in NPG/Vst mice. Silencing of AUF1 expression resulted in a decreased tumor size and tumor weight in NPG/Vst mice (Figure 3A-C). Ki-67 staining in tumors subjected to AUF1 knockdown was also weaker than that in the control tumors (Figure 3D). In contrast, overexpression of AUF1 in Huh-7

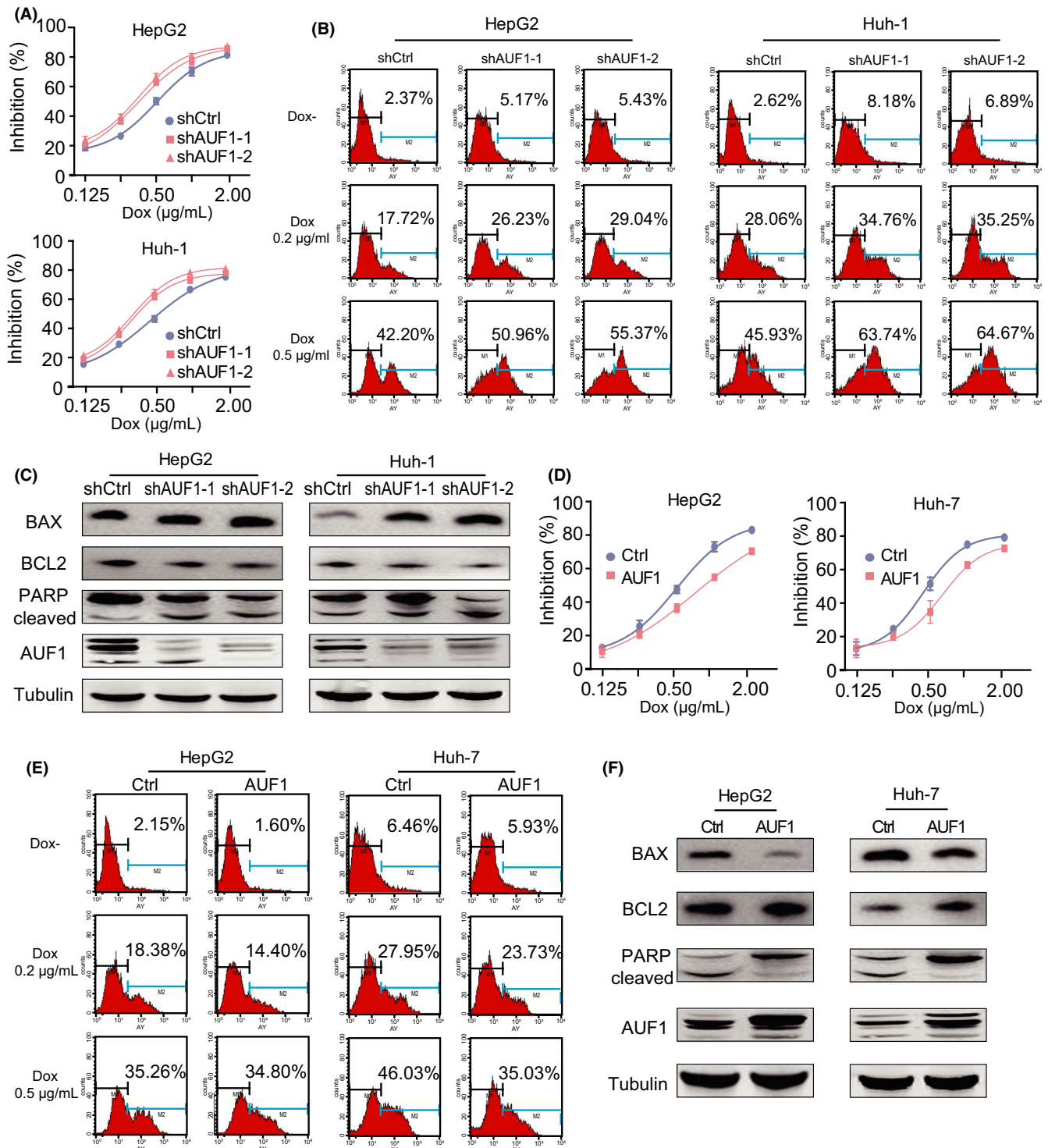
cells resulted in a significant increase in tumor growth (Figure 3E-G) and Ki-67-positive cells in tumor tissues (Figure 3H). Taken together, these data further confirmed that AUF1 markedly promoted HCC development.

### 3.4 | AUF1 enhances the chemotherapeutic drug resistance of hepatoma cells

To evaluate whether AUF1 elevates the chemotherapeutic drug resistance of hepatoma cells, hepatoma cells with AUF1 silencing or overexpression were treated with different doses of doxorubicin (Dox) and the cell viability was detected. The results showed that AUF1 silencing led to more susceptible cells to Dox than the control cells in a drug concentration-dependent manner in HepG2 and Huh-1 cells (Figure 4A). Moreover, silencing of AUF1 expression significantly increased Dox-induced apoptosis in HCC cells in a dose-dependent manner (Figure 4B), which was also demonstrated by increased expression level of Bax, cleaved-PARP proteins and decreased expression level of Bcl2 (Figure 4C). In contrast, AUF1



**FIGURE 3** AUF1 promotes HCC tumorigenicity in vivo. A-C, Subcutaneous injection of Huh-1 cells with stable knockdown of AUF1 or control cells into NPG/Vst mice ( $n = 5$ ). Images of neoplasms from each group of NPG/Vst mice (A), measurement of tumor volumes (B), and tumor weight (C). D, Immunohistochemistry staining of proliferation marker Ki-67 in tumor tissues. Scale bars, 100  $\mu\text{m}$ . E-G, Subcutaneous tumor model of Huh-7 cells with AUF1 overexpression or control cells injected into NPG/Vst mice ( $n = 8$ ). Images of neoplasms from each group of NPG/Vst mice (E), measurement of tumor volumes (F), and tumor weight (G). H, Immunohistochemistry staining images of Ki67 in tumor tissues. Scale bars, 100  $\mu\text{m}$ . \*\*\* $P < .001$



**FIGURE 4** AUF1 expression in hepatoma cells with Dox resistance. A, Inhibitory effects of Dox toward AUF1-knockdown HCC cell lines. B, Percentage of annexin V-positive cells in AUF1 knockdown cells after Dox treatment for 48 h. C, Western blot analysis shows the apoptotic cascade protein expression levels upon AUF1 knockdown in hepatoma cells after Dox (0.5 μg/mL) treatment for 48 h. D, E, Incidence of resistance (D) and apoptosis (E) were detected in AUF1-overexpressed HCC cell lines. F, Western blot analysis showed the apoptotic cascade protein expression levels upon AUF1 overexpression in hepatoma cells after Dox (0.5 μg/mL) treatment for 48 h

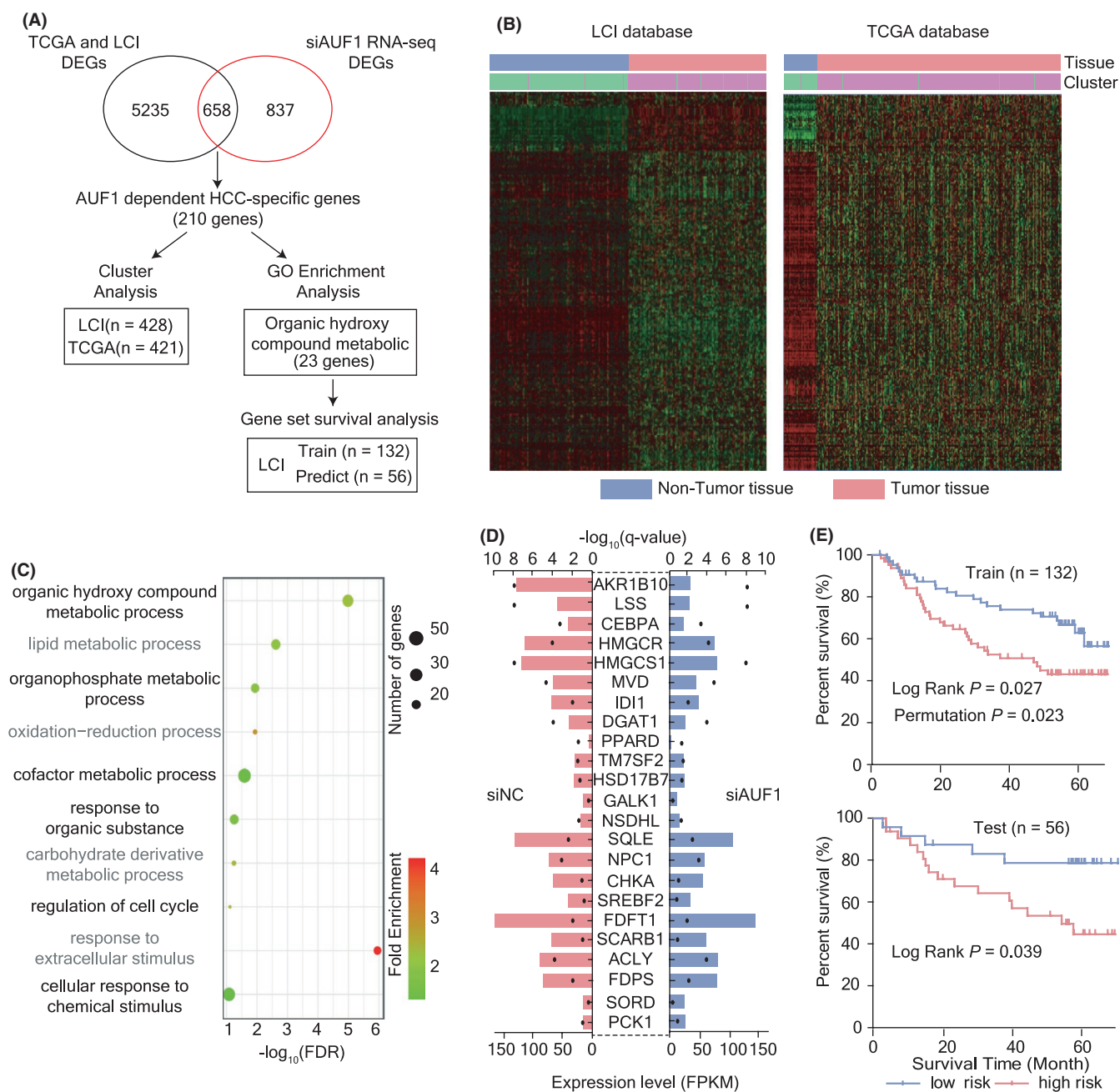
overexpression significantly reduced the cell susceptibility to Dox and the Dox-induced apoptosis in Dox-treated HepG2 and Huh-7 cells (Figure 4D-F). These data demonstrated that AUF1 protected hepatoma cells against Dox-induced apoptosis and enhanced the chemotherapeutic drug resistance.

### 3.5 | AUF1 preferentially affects the expression of metabolism-related genes in HCC

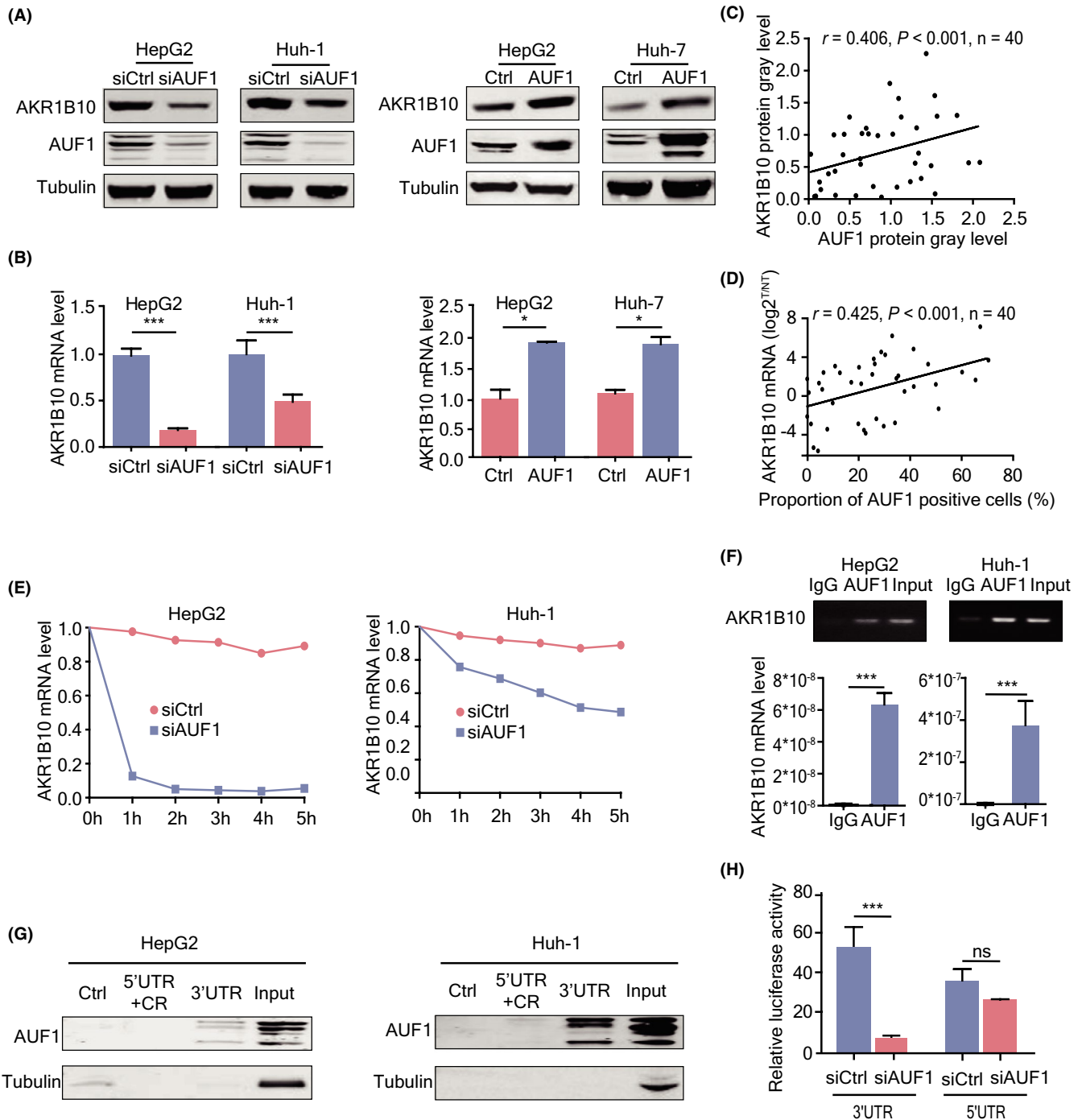
To gain insights into the molecular mechanism underlying the pro-tumorigenic role of AUF1, RNA-seq assay was performed to

investigate the transcriptome changes caused by AUF1 silencing in HepG2 cells. In total, 1495 transcripts that were significantly altered upon AUF1 knockdown were identified (Table S2), consistent with the notion that AUF1 is an ARE-BP that can lead to transcriptomic imbalance. Among these genes, 658 transcripts were found to be

differentially expressed between HCC and non-tumor samples in both the LCI and TCGA data sets (Figure 5A). As AUF1 was upregulated in HCC tissues, theoretically, the downregulated genes after AUF1 knockdown should be upregulated in HCC, or vice versa. Accordingly, 210 genes expressed in a reciprocal pattern were identified among



**FIGURE 5** Screening of AUF1-dependent HCC-related genes. A, Analysis flow chart. DEGs indicates differentially expressed genes. B, Cluster analysis of HCC tissues and non-tumor tissues using 210 AUF1-dependent HCC-specific genes for the LCI and TCGA HCC samples. C, Gene Ontology enrichment analysis of 210 AUF1-dependent HCC-specific genes. The color of the dot represents the fold enrichment, and the size of the dot represents the number of genes enriched in the pathway. D, Differential expression analysis of 23 AUF1-dependent organic hydroxy compound metabolism genes in siAUF1 RNA-seq. The length of bar represents fold change, and the black dot is used to indicate the q-value. E, Survival gene set analysis of 23 AUF1-dependent organic hydroxy compound metabolism genes for HCC in the LCI database. Log-Rank indicates the log-rank test *P*-value, and permutation indicates the permutation test *P*-value; in total 1000 random trials were conducted



**FIGURE 6** AUF1-enhanced AKR1B10 expression through post-transcriptional regulation. A, B, Western blot and RT-qPCR analyses of AKR1B10 protein (A) and mRNA (B) in hepatoma cells with AUF1 overexpression or knockdown. C, Expression levels of AUF1 protein were positively correlated with AKR1B10 in 40 pairs of human HCC samples determined by western blot. Data were digitalized using ImageJ software statistical analysis. D, mRNA expression levels of AKR1B10 in 40 pairs of HCC samples were positively correlated with protein expression of AUF1. E, Half-life of AKR1B10 mRNA in HepG2 and Huh-1 cells with AUF1 knocked down. The levels of AKR1B10 mRNA was tested using qRT-PCR at the indicated time followed by actinomycin D treatment (5 mg/mL). F, Binding of AUF1 to AKR1B10 mRNA detected using ribonucleoprotein immunoprecipitation (RNP-IP) assays. AKR1B10 mRNA abundance in immune precipitates was tested using RT-PCR and qRT-PCR. G, Motif in AKR1B10 mRNA interacted with AUF1 identified using RNA pulldown assay. Ctrl indicates the negative control, and Input indicates total RNA extracted. 5'UTR, CR, and 3'UTR, respectively, indicate the corresponding regions of AUF1 mRNA. H, Luciferase activity of the reporter containing the 3'UTR or 5'UTR of the AKR1B10 transcript detected using luciferase activity assays. \* $P < .05$ ; \*\*\* $P < .001$

these 658 HCC-specific genes (Table S3). We found that these 210 AUF1-dependent HCC-specific genes could be used to discriminate accurately the tumor from non-tumor, with accuracies of more than

97.4% in TCGA and 97.7% in the LCI data sets (Figure 5B). Therefore, we reasoned that these 210 transcripts might accurately represent AUF1-dependent transcripts that acted as key drivers of HCC.



To further analyze the functional importance of the AUF1-dependent HCC-specific genes, we performed Gene Ontology (GO) analysis. The results showed that the functions of these genes were mainly enriched in metabolism-associated signatures including organic hydroxy compound metabolism, lipid metabolism, organophosphate metabolism, and oxidation-reduction processes, of which organic hydroxy compound metabolism was ranked as the most enriched pathway (Figure 5C and Table S4). We named a total of 23 genes as AUF1-dependent organic hydroxy compound metabolism genes (Figure 5D). To evaluate the prognosis predictive value of these 23 genes for HCC, we conducted a gene set survival analysis. In total, the results for 188 patients with HCC in the LCI database with BCLC staging information available were selected and randomly divided into training set and test set with a ratio of 7:3, and analyzed using BRB-array tools. We found that in the training set, the log-rank *P*-value and permutation *P*-value of the survival risk prediction model constructed by these 23 genes reached .027 and .023, respectively. In the test set, the log-rank *P*-value also reached .039. The above results suggested that AUF1 preferentially affected the expression of metabolism-related genes and that AUF1-dependent organic hydroxy compound metabolism genes are important prognostic risk factors for HCC (Figure 5E).

### 3.6 | AUF1 is physically associated with AKR1B10 in HCC and upregulates AKR1B10 expression by increasing its mRNA stability

Among the genes affected by AUF1 knockdown, *AKR1B10* is the top one with the most significant difference. As our previous studies and other studies have proved that dysregulation of *AKR1B10* expression is involved in HCC development,<sup>20</sup> we considered that *AKR1B10* might be a target gene of AUF1. To further confirm the regulation of AUF1 on *AKR1B10* expression, we detected *AKR1B10* expression at mRNA and protein levels in hepatoma cells with AUF1 overexpression or knockdown. As shown in Figure 6A,B, AUF1 knockdown significantly decreased *AKR1B10* expression at both the protein and mRNA levels, while AUF1 overexpression increased *AKR1B10* expression. Next, we analyzed the correlation between *AKR1B10* and AUF1 levels in 40 pairs of HCC tissue specimens in which both *AKR1B10* and AUF1 protein expression had been detected using western blot assays. We found that *AKR1B10* expression was up-regulated in 71.0% (27/40) of HCC tumor tissues, and the protein levels of *AKR1B10* and AUF1 were correlated with each other in these 40 HCC tissues ( $r = .406$ ,  $P < .001$ ; Figure 6C). Additionally,

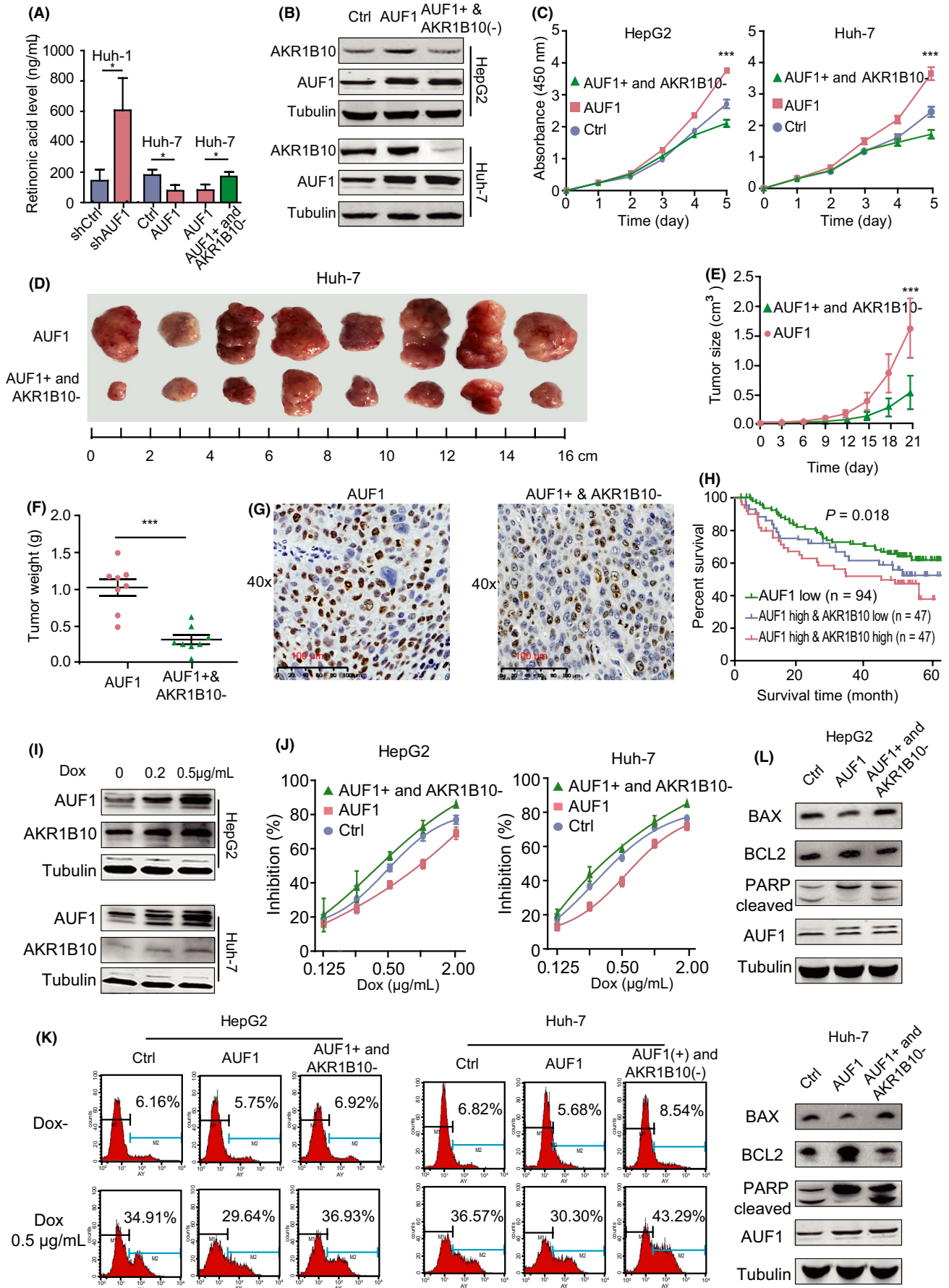
co-localization analysis of the IHC results showed that, in adjacent sections of HCC tissues, most regions that stained for AUF1 were also positive for *AKR1B10* and vice versa (Figure S3). Furthermore, using another 40 HCC tissues in which AUF1 protein had been determined through IHC and *AKR1B10* mRNA was tested using a qRT-PCR assay, we also observed a marked positive correlation between *AKR1B10* mRNA levels and the AUF1 protein level ( $r = .425$ ,  $P < .001$ ; Figure 6D).

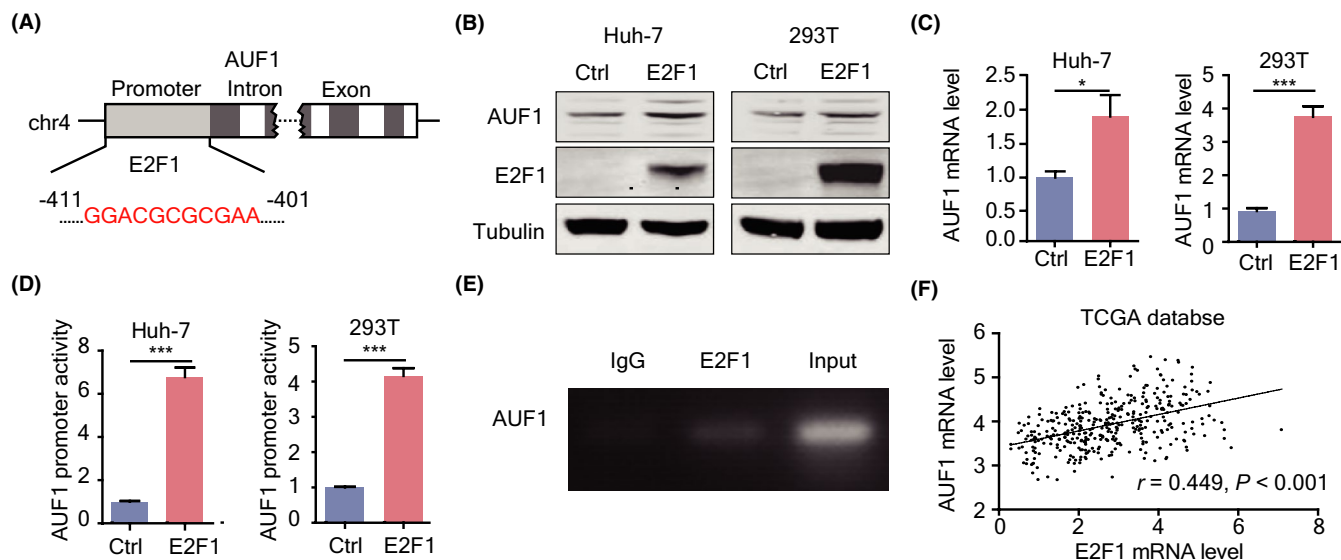
Next, we analyzed whether there existed potential AREs in *AKR1B10* mRNA. Using AREsite we identified conserved AU-rich element clusters in the 3'UTR of *AKR1B10* mRNA (Figure S4). RNA turn-over assay showed that the half-life of *AKR1B10* mRNA was significantly shorter in AUF1 knockdown cells than that in control cells (Figure 6E). RNA immunoprecipitation (RIP) assays showed that AUF1 could strongly bind to *AKR1B10* mRNA (Figure 6F). Further RNA pulldown assay demonstrated that only the *AKR1B10* 3'UTR region could combine with AUF1 (Figure 6G). Consistent with this result, knockdown of AUF1 significantly decreased luciferase activity of the reporter containing the 3'UTR region of the *AKR1B10* transcript, but had no effect on the 5'UTR region of the *AKR1B10* transcript (Figure 6H). Taken together, these data indicated that AUF1 enhanced *AKR1B10* expression by increasing its mRNA stability following binding to its 3'UTR region.

### 3.7 | AUF1-promoted HCC development and drug resistance are AKR1B10 dependent

It has been reported that *AKR1B10* functions as an efficient retinal reductase that may reduce RA levels and therefore promoting cell proliferation.<sup>23,24</sup> Among the differentially expressed genes described in the AUF1 knockdown RNA-seq data, only 2 RA metabolism-related genes, *AKR1B10* and *AKR1C2*, were downregulated in AUF1 knockdown cells. Compared with *AKR1C2*, *AKR1B10* had a higher activity to metabolize RA and more significant down-regulation of expression in the AUF1 knockdown cells. Therefore, we speculated that AUF1 could promote the expression of *AKR1B10*, thereby promoting RA metabolism and HCC development. As expected, RA levels were significantly higher in tumors from mice bearing AUF1-knockdown cells, but decreased in tumors from mice bearing AUF1-overexpression cells. In addition, the increase of RA levels induced by AUF1 overexpression was almost completely reversed by *AKR1B10* knockout (Figure 7A). These results suggested that the tumor-promoting function of AUF1 in HCC was at least partially mediated by the catalytic efficiency of *AKR1B10*.

**FIGURE 7** *AKR1B10* contributed to the oncogenic function of AUF1. A, RA levels in tumors from the indicated tumor-bearing mice were measured. B, *AKR1B10* stable knockout cell models were established based on AUF1-overexpression cells. Knockout efficiency was verified using western blot assay. C, *AKR1B10* knockout rescued the AUF1 overexpression-induced malignant phenotypes of HCC cells in vitro. D-F, *AKR1B10* knockout rescued the AUF1-enhanced tumor formation in vivo. G, Representative micrographs of immunohistochemistry staining of proliferation marker Ki-67 in tumors. Scale bars, 100  $\mu$ m. H, Kaplan-Meier analysis of the LCI cohorts using the expression levels of AUF1 and *AKR1B10*. I, Western blot analysis shows AUF1 and *AKR1B10* protein expression in HCC cells after Dox treatment. J-L, *AKR1B10* knockout rescues AUF1 overexpression-induced incidence of resistance and apoptosis. \* $P < .05$ ; \*\*\* $P < .001$





**FIGURE 8** Transcriptional regulation of *AUF1* by *E2F1* in HCC. A, Scheme of putative *E2F1* binding sites in the *AUF1* promoter region. B, C, *E2F1*-regulated *AUF1* expression in Huh-7 and 293T cells at both protein and mRNA levels. D, *E2F1* increases the transcriptional activity of the *AUF1* promoter. E, CHIP-qPCR was performed using chromatin from Huh-7 cells and anti-*E2F1* antibody. Non-specific IgG antibody was used as the control. F, Correlation analysis between the expression levels of *AUF1* and *E2F1* in HCC tissues from TCGA database

Next, we set out to determine whether *AUF1* exerted its functions depending on *AKR1B10* in HCC. For this purpose, we established stable HepG2 and Huh-7 cells expressing the *AUF1*, as well as a CRISPR/Cas9 system that targeted *AKR1B10* (Figure 7B). As anticipated, silencing of *AKR1B10* expression rescued cell proliferation (Figure 7C) and colony formation (Figure S5A), and induced a G2/M cell cycle arrest in HepG2 and Huh-7 cells that overexpressed *AUF1* (Figure S5B). Furthermore, our results also showed that *AUF1*-enhanced tumor formations of Huh-7 cells in NPG/Vst mice were attenuated by *AKR1B10* knockout, both in the weight and volume of the tumor ( $P < .001$ ; Figure 7D-F). Simultaneously, the percentage of Ki-67-positive cells in tumors from *AUF1*-overexpressing cells was decreased significantly by *AKR1B10* knockout (Figure 7G). In addition, we jointly analyzed the power of *AUF1* and *AKR1B10* mRNA expression levels on the prognosis of HCC. The results showed that the survival rates of patients with HCC with only *AUF1* low expression showed the best overall survival, while patients with both high *AUF1* and *AKR1B10* expression was the worst of those in the LCI database ( $P = .018$ ; Figure 7H), which suggested that high levels of *AKR1B10* worsened the poor prognosis of patients with HCC who exhibited higher *AUF1* expression. These data altogether indicated that *AKR1B10* is a major target of *AUF1* in HCC and that the upregulation of *AKR1B10* by *AUF1* is vital for maintaining the oncogenic phenotype of HCC.

It has been reported that *AKR1B10* participates in the cellular metabolism of Dox and idarubicin, resulting in drug resistance. In light of our observation that *AUF1* enhanced the drug resistance to Dox in hepatoma cells, we reasoned that *AKR1B10* might be required for *AUF1*-induced drug resistance. To test this hypothesis, we firstly evaluated the effects of Dox on the expression of *AUF1* and *AKR1B10*. The results showed that, upon Dox treatment, both *AUF1* and *AKR1B10* protein expression in HepG2 and Huh-7 cells was significantly

increased (Figure 7I). Furthermore, we found that the *AKR1B10* deficiency completely abrogated the *AUF1*-mediated changes in cell viability, cell apoptosis, and apoptosis markers, such as Bax, Bcl2, and cleaved PARP. (Figure 7J-L). These data indicated that *AUF1*-induced drug resistance to Dox also relied on the upregulation of *AKR1B10*.

### 3.8 | *E2F1* triggers the transcriptional activation of *AUF1* in HCC

Transcriptional regulation is responsible for the upregulation of many oncogenes. In an effort to better understand the upstream regulation mechanism of *AUF1* in HCC, we analyzed the *cis*-elements in the *AUF1* promoter region using the LASAGNA database. Notably, a potential *E2F1* binding site was identified in *AUF1* promoter region (Figure 8A). We then evaluated whether *E2F1* could regulate *AUF1* expression in HCC. To start with, an *E2F1* expressing plasmid was transiently transfected into Huh-7 and HEK 293T cells due to their relatively higher transfection efficiency and the expression of *AUF1* was detected. The results showed that *E2F1* significantly increased both mRNA and protein levels of *AUF1* in these cells (Figure 8B,C). Consistently, overexpression of *E2F1* increased the promoter activity of the *AUF1* gene (Figure 8D). Next, as shown in Figure 8E, CHIP-PCR assay showed that *E2F1* was able to specifically bind to the *AUF1* promoter region. Furthermore, the expression of *E2F1* was upregulated in HCC tissues from TCGA data sets and the expression levels of *AUF1* and *E2F1* were positively correlated ( $r = .449$ ,  $P < .001$ ; Figure 8F). Taken together, these data suggested that *E2F1* is an important transcription factor of the *AUF1* gene and that overexpression of *E2F1* may contribute to the *AUF1* upregulation in HCC.

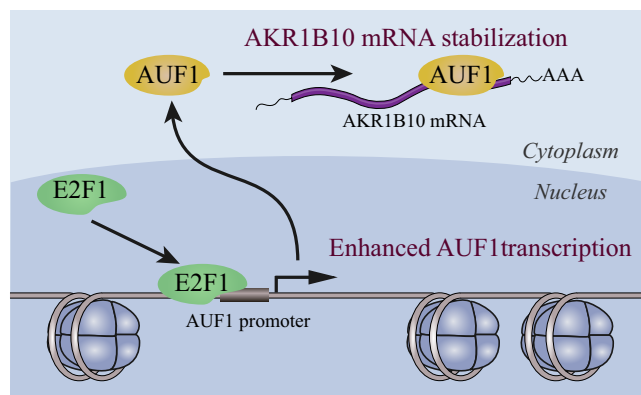


FIGURE 9 Scheme of the E2F1/AUF1/AKR1B10 axis

## 4 | DISCUSSION

Dysregulation of AUF1 has been proposed to have a role in various type of cancers during cancer initiation, progression, and chemoresistance.<sup>15,25</sup> However, functions and mechanisms of AUF1 in the development and progression of HCC are still obscure. In this study, we demonstrated that the expression of AUF1 was significantly increased in HCC and correlated with the poor prognosis of patients with HCC. By regulating the mRNA stability of its functional target *AKR1B10*, AUF1 contributed to HCC development and chemoresistance. Moreover, this is also the first publication to report that E2F1 regulates *AUF1* transcription and increases *AUF1* expression (Figure 9).

To date, the limited reports addressing AUF1 expression in HCC revealed that AUF1 was highly expressed in the tumor tissues and AUF1 high expression was closely associated with hepatitis B surface antigen status.<sup>26,27</sup> However, its abundance and biological activity in HCC are completely unknown. In this study, we demonstrated that AUF1 is frequently overexpressed in HCC tissues, not only from the LCI and TCGA databases, but also in our HBV-related HCC cohorts. AUF1 promoted cell growth, colony formation, and tumor formation of hepatoma cells, and enhanced the Dox resistance of these cells. Furthermore, we demonstrated that AUF1 expression levels in HCC tissues were closely associated with the poor survival of patients with HCC in both the LCI database, TCGA database, and our HCC cohort. Therefore, we concluded that AUF1 may play an important role in promoting HCC progression.

Changes in metabolic processes represent key drivers of tumorigenesis and malignancy in the liver.<sup>28</sup> However, whether AUF1 could regulate liver metabolism favoring HCC progression is still poorly understood. In this study, we demonstrated that organic hydroxy compound metabolism was the most significantly enriched pathway altered by AUF1 ablation, which enriched 23 AUF1-dependent genes. Most importantly, expression of these organic hydroxy compound metabolism genes was associated with the overall survival of patients with HCC. Therefore, the abnormal organic hydroxy compound metabolism induced by AUF1 overexpression in HCC may be associated with HCC aggressiveness.

AKR1B10 is a NADPH-dependent reductase that metabolizes a variety of endogenous compounds such as aromatic and aliphatic aldehydes and dicarbonyl compounds, and some drug ketones.<sup>29</sup> Increasing evidence has demonstrated that AKR1B10 is highly expressed in several solid tumors including HCC and is considered as a relevant biomarker for the development of HCC.<sup>23</sup> In addition, AKR1B10 has been recently reported to be significantly upregulated in some cancer cell lines acquiring resistance toward chemotherapeutic agents, suggesting the validity of the enzyme as a chemoresistance marker.<sup>30</sup> Our data here revealed that *AKR1B10* is the one of the most significant genes among the 23 AUF1-dependent organic hydroxy compound metabolism genes. We demonstrated that expression of *AKR1B10* and *AUF1* was positively correlated in an expanded cohort of clinical HCC samples. AUF1 could bind to and stabilize *AKR1B10* transcripts by interacting with its 3'UTR region. In addition, *AKR1B10* silencing was sufficient to revert to the oncogenic phenotype and chemoresistance induced by AUF1. Therefore, we concluded that *AKR1B10* is indispensable for the regulation of HCC development and drug sensitivity by AUF1 in HCC.

It has been reported that AUF1 upregulation can be driven by activation of the ERK pathway.<sup>31</sup> However, mechanisms of AUF1 upregulation in HCC are still obscure. Through analysis of the published comparative genomic hybridization (aCGH) data and the MEXPRESS database from TCGA, we found that no copy number increase of AUF1 was identified in HCC tissues. In addition, we did not find any methylation sites significantly related to the expression levels of *AUF1* (data not shown). These analyses suggested that the abnormal expression of AUF1 may be caused by upregulation of *AUF1* transcription. E2F1 is a transcription factor that contributes to the regulation of many cellular processes including cell proliferation.<sup>32-34</sup> Because a growing body of evidence has indicated that the upregulation of E2F1 expression is intimately associated with HCC and that there are potential E2F1 binding sites in the *AUF1* promoter region, it is not surprising to find that E2F1 promotes *AUF1* transcription, thereby upregulating AUF1 expression. Interestingly, E2F1 levels also affected growth factor-induced ERK phosphorylation.<sup>35,36</sup> Whether the ERK pathway is involved in E2F1-induced AUF1 upregulation deserves further exploration.

In summary, we propose a model that explains the possible mechanism for AUF1 in HCC development. In the model, E2F1 promotes the expression of *AUF1* by upregulating its transcription. Then, AUF1 binds to and stabilizes *AKR1B10* mRNA, leading to upregulation of *AKR1B10* expression. *AKR1B10* is required for the promoting role of AUF1 in HCC development and chemoresistance. Therefore, specifically targeting hepatocyte AUF1 or the E2F1/AUF1/AKR1B10 axis may be a potential therapeutic strategy for HCC treatment in the future.

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## DISCLOSURE

The authors have declared that no competing interest exists.

## AUTHOR CONTRIBUTIONS

Xiangmei Chen conceived and supervised the study. Ting Zhang, Guiwen Guan, and Jing Zhang performed experiments and analyzed data. Huiling Zheng, Deyao Li, and Wengong Wang provided scientific suggestions. Xiangmei Chen, Fengmin Lu, Ting Zhang, and Guiwen Guan wrote the manuscript. All authors discussed the results and commented on the manuscript.

## DATA AVAILABILITY STATEMENT

Complete RNA-seq files and gene expression values have been deposited in NCBI's Gene Expression Omnibus and are accessible through Gene Expression Omnibus (GEO) Series accession number GSE162706.

## ORCID

Xiangmei Chen  <https://orcid.org/0000-0003-0302-6866>

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#### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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