

# Histone acetyltransferase PCAF accelerates apoptosis by repressing a GLI1/BCL2/BAX axis in hepatocellular carcinoma

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P300/CBP-associated factor (PCAF), a histone acetyltransferase (HAT), has been found to regulate numerous cell signaling pathways controlling cell fate by acetylating both histone and non-histone proteins. We previously reported that PCAF upregulates cell apoptosis by inactivating Serine/Threonine Protein Kinase 1 (AKT1) signaling and consequently inhibits hepatocellular carcinoma (HCC) cell growth. Here, we show that PCAF can directly acetylate cytoplasmic GLI1 protein at lysine 518, preventing its nuclear translocation and promoter occupancy, and consequently suppressing Hedgehog (Hh) signaling in HCC. Further, our results show that GLI1 can increase Bcl-2 expression and downregulate BAX. Interestingly, forced expression of PCAF reduced Bcl-2 expression, upregulated BAX and repressed cell apoptosis. Further, we provide evidence that knockdown of GLI1 abrogates the inhibitory effect of PCAF on the growth of HCC *in vitro*. PCAF was also found to sensitize HCC cells to 5-fluorouracil (5-FU) treatment by regulating GLI1/Bcl-2/BAX axis-dependent apoptosis. *In vivo* experiments also confirmed the regulatory effect of PCAF on the GLI1/Bcl-2/BAX axis and its synergistic antitumor effects with 5-FU. Gene expression microarray studies showed that PCAF was downregulated in HCC tissues compared with adjacent liver tissues and that PCAF expression was significantly associated with longer overall survival and recurrence-free survival after surgery. Together, these results show that PCAF can induce cell apoptosis by modulating a GLI1/Bcl-2/BAX axis that in turn suppresses HCC progression, and suggest that 5-FU may exert a stronger anti-tumor effect in patients with PCAF expression in HCC tumors.

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Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the second most common cause of cancer-related death (Globocan 2012, IARC).<sup>1</sup> Curative treatments such as local ablation, surgical resection and liver transplantation improve the prognosis of HCC patients.<sup>2</sup> However, owing to the shortage of donor livers, liver resection and local ablation remain the mainstays of curative therapy for HCC in high incidence Asian countries.<sup>3</sup> Unfortunately, radical hepatic resection can only be applied to the minority of HCC patients who present with early stage disease and is associated with a high incidence of postsurgical recurrence,<sup>4</sup> due in part to the presence of preoperative subclinical liver metastases. Thus, there is an urgent need to identify predictive markers for HCC outcomes after hepatic resection, determine the molecular mechanisms of HCC progression and develop novel therapeutics.

P300/CBP-associated factor (PCAF) is a member of the GNAT (GCN5-related N-acetyltransferase) acetyltransferase family which was originally found to repress cellular transformation as a factor displaced from p300/CBP complexes by oncoprotein E1A.<sup>5</sup> Recent studies have demonstrated that PCAF modulates the activities of several oncogenes and tumour repressors through acetylation of either histones or transcription factors, consequently impacting cancer progression. Our preliminary data showed that PCAF induced HCC

cell apoptosis by acetylating histone H4 protein and activating AKT signaling.<sup>6</sup> However, the underlying molecular mechanism of PCAF-induced cell apoptosis in HCC is still unclear.

Hedgehog (Hh) signalling was initially described when the Hh mutant phenotype was reported in a fly model in 1980.<sup>7</sup> Since its vertebrate counterparts were isolated in the early 1990s, remarkable progress has been made in investigating the function of Hh signalling as well as the Hh signalling response network.<sup>8</sup> It has been found that Hh signalling plays an important part in the development of several body structures by controlling the fate of the progenitor cells giving rise to these structures.<sup>9</sup> When aberrantly activated, Hh signalling mediates carcinogenesis and induces aggressive cancer phenotypes, enhancing recurrence, metastasis and chemotherapy resistance.<sup>10</sup> Glioma-associated oncogene 1 (GLI1), a transcription factor, is a Hh-transcriptional target gene that also functions as the final mediator of Hh transcriptional regulation. GLI1 upregulates its own expression and consequently auto-enhances Hh signal activation.<sup>11</sup> In previous studies, we found that GLI1 was aberrantly overexpressed in HCC and predisposed to poor prognosis after liver resection by inducing the epithelial–mesenchymal transition phenotype in a SNAI1-dependent manner.<sup>12</sup>

In this study, we show that PCAF protein binds to GLI1 protein in the cytoplasm and directly acetylates it at lysine 518,

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**Abbreviations:** PCAF, P300/CBP-associated factor; HCC, Hepatocellular carcinoma; GLI1, GLI family zinc finger 1; HATs, Histone acetyltransferases

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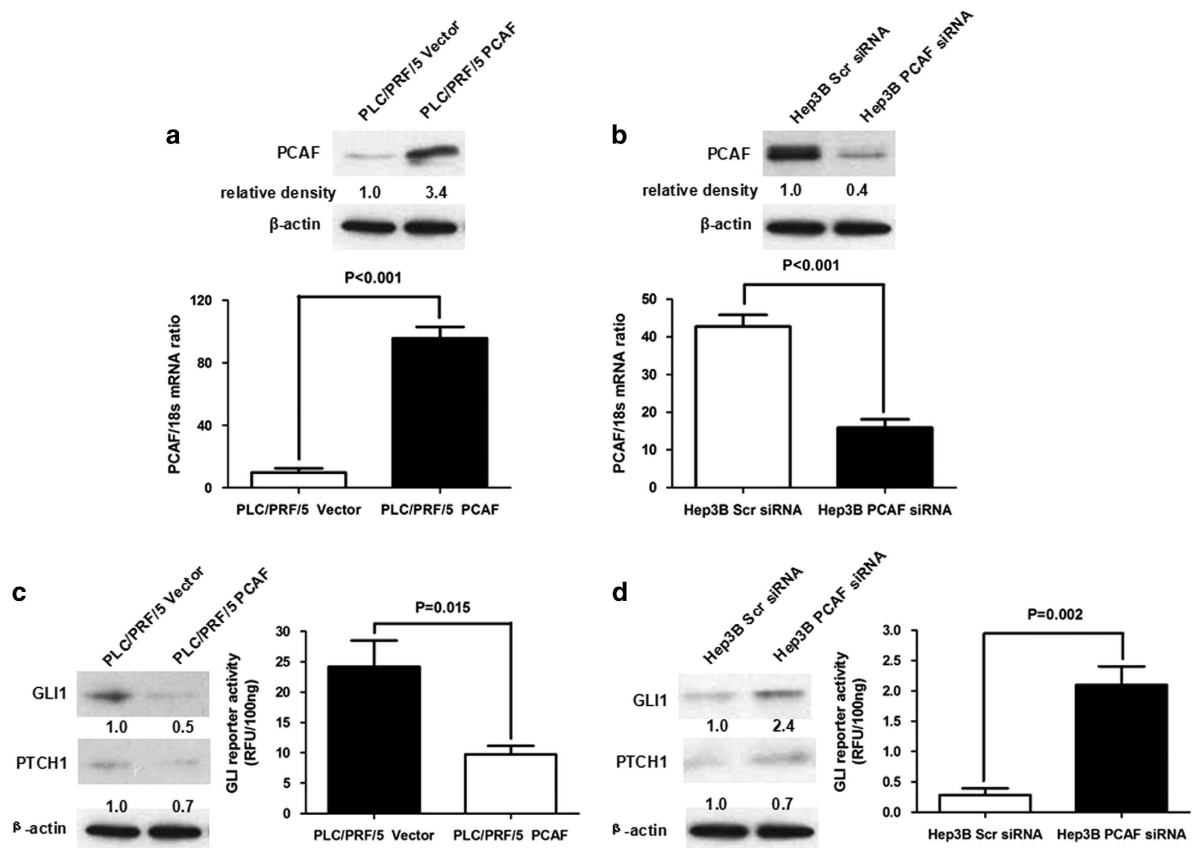
preventing the nuclear shuttling of GLI1 protein and in turn suppressing Hh signalling. Consequently, downregulation of PCAF in HCC results in the hyperactivation of Hh signalling and GLI1 overexpression. We also show that PCAF suppresses Bcl-2 expression, increases BAX expression and consequently accelerates HCC cell apoptosis. Thus, our data suggest that GLI1 plays a critical role in a PCAF/Bcl-2/BAX driven cascade of cellular apoptosis.

## Results

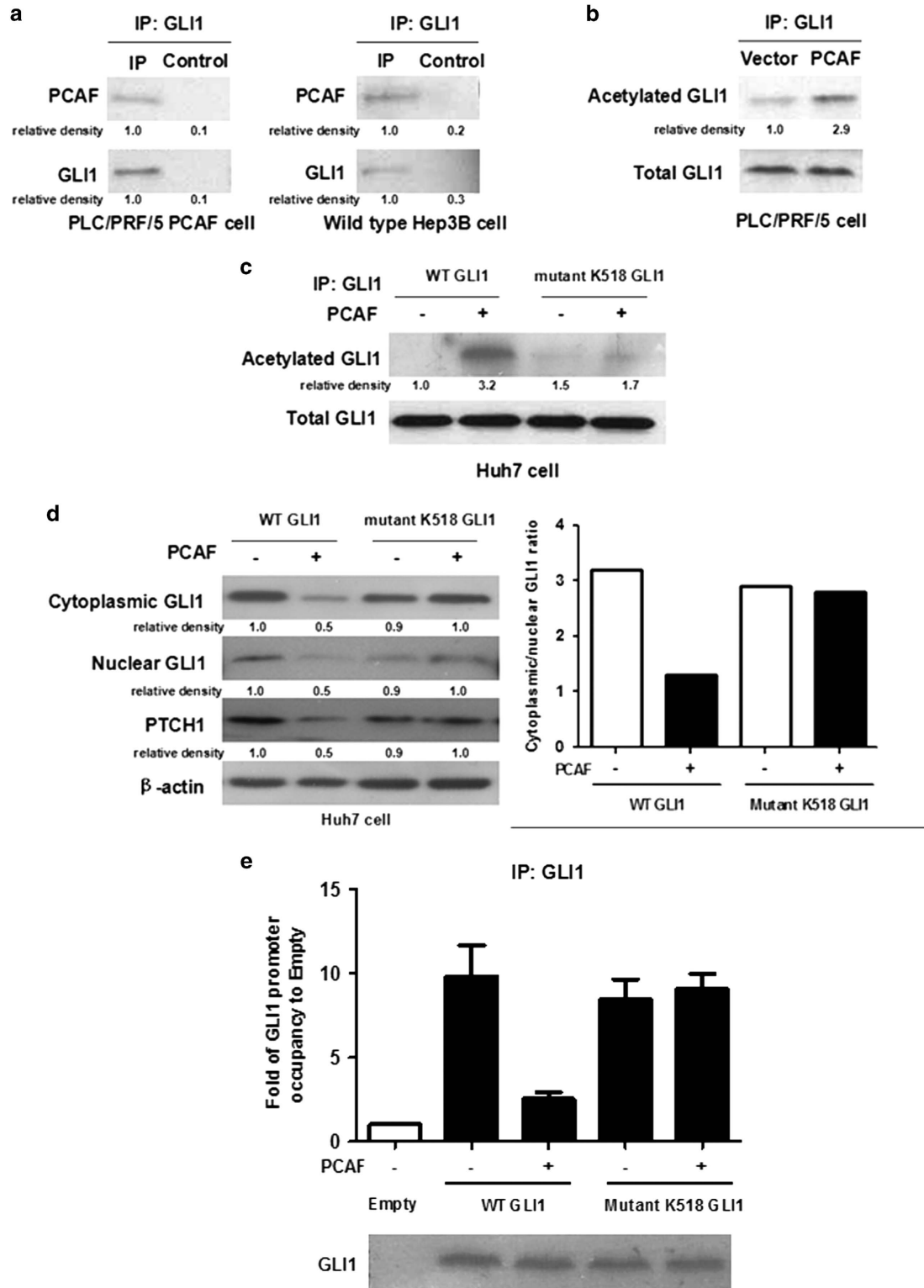
**PCAF exerts a negative effect on Hh signalling in HCC cells.** To determine whether PCAF regulates activation of the Hh pathway in HCC, we enhanced PCAF expression in the PLC/PRF/5 HCC cell line by transfecting a PCAF-expressing plasmid (Figure 1a) and decreased expression of PCAF in the Hep3B HCC cell line by transfecting siRNA targeting PCAF (Figure 1b).<sup>6</sup> We measured the effect of PCAF on Hh pathway activity by measuring the expression of both GLI1 and Patched (PTCH1), which is used as a read-out for GLI activity, and also by measuring GLI-dependent reporter activity. PCAF overexpression reduced the expression of both GLI1 and PTCH1 and inhibited GLI-dependent reporter

activity in PLC/PRF/5 cells (Figure 1c). The opposite results were obtained in Hep3B cells after knockdown of PCAF (Figure 1d). These data suggest that PCAF negatively regulates Hh signalling in HCC cells.

**PCAF prevents cytoplasmic-to-nuclear shuttling and promoter occupancy of GLI1 protein by modulating its acetylation.** PCAF has intrinsic HAT activity which has been found to modulate transcriptional activation by acetylating both histones and non-histone proteins.<sup>13</sup> We therefore examined whether PCAF represses Hh signalling by acetylating GLI1. As shown in Figure 2a, PCAF co-immunoprecipitated with cytoplasmic GLI1 protein in cytoplasmic extracts from PLC/PRF/5 cells transfected with PCAF-expressing plasmid. Comparable results were also obtained for wild-type Hep3B cells (Figure 2a). Thus, these data, which show that both endogenous and exogenous PCAF protein co-immunoprecipitate with cytoplasmic GLI1 protein, suggest that PCAF protein binds directly to GLI1 in the HCC cell cytoplasm. Next, we purified cytoplasmic GLI1 protein by co-immunoprecipitation (Co-IP) and performed western immunoblotting with an anti-acetyl lysine antibody. As shown in Figure 2b, there was more acetylated



**Figure 1** PCAF repressed the activation of Hh signalling in HCC cell. (a) Plasmids expressing the full-length PCAF mRNA or the control empty were stably transfected into PLC/PRF/5 cells. Compared with PLC/PRF/5 Vector cells, PLC/PRF/5 PCAF cells had significantly higher PCAF expression at the levels of both mRNA and protein. (b) siRNA sequences against PCAF downregulated the mRNA and protein expression of PCAF apparently in Hep3B cells. (c) Enhanced PCAF expression lead to the decrease of expression of both GLI1 and PTCH1 and suppressed GLI-dependent reporter activity in PLC/PRF/5 cells, which indicated that PCAF expression inactivated Hh pathway in HCC cells. (d) Knockdown of PCAF resulted in the upregulation of GLI1 and PTCH1 expression and increased GLI-dependent reporter activity in Hep3B cells. These data demonstrated that aberrant silencing of PCAF lead to the hyperactivation of Hh pathway



**Figure 2** PCAF abolished cytoplasm-to-nucleus translocation and promoter occupancy of GLI1 protein via modulating its acetylation. (a) PCAF was found to be co-immunoprecipitated with cytoplasmic GLI1 protein in both PLC/PRF/5 PCAF and wild-type Hep3B cells. (b) As assessed by Co-IP and western immunoblotting assays, there was more acetylated GLI1 in PLC/PRF/5 PCAF cells than that in PLC/PRF/5 Vector cells. (c) Enforced expression of PCAF also upregulated acetylation of GLI1 protein in cytoplasm of Huh7 cells, however, mutation of K518 abolished the capacity of GLI1 to be acetylated by PCAF. (d) PCAF overexpression resulted in the decreased expression of GLI1 and PTCH1 and higher ratio of cytoplasmic GLI1 to nuclear GLI1. However, it did not influence expression of mutant GLI1 and PTCH1 and the ratio of cytoplasmic to nuclear mutant GLI1. (e) As assessed by ChIP (IP: GLI1 protein) and qRT-PCR assay, PCAF overexpression was found to abrogate promoter occupancy of GLI1, whereas mutation at the K518 residue abolished this effect of PCAF. The Control group refers to Huh7 cells transfected with control vectors for both GLI1-expressing and PCAF-expressing plasmids

cytoplasmic GLI1 protein in PLC/PRF/5 cells transfected with PCAF-expressing plasmid (PLC/PRF/5 PCAF) than in PLC/PRF/5 cells transfected with control plasmid (PLC/PRF/5 Vector). These results strongly suggest that PCAF acetylates cytoplasmic GLI1 in HCC cells. We therefore assessed whether cytoplasmic GLI1 protein is acetylated by PCAF at the potentially acetylable lysine residue K518. We engineered a mutant GLI1 K518 plasmid via site-directed mutagenesis of the wild-type GLI1-expressing plasmid and transfected either the mutant GLI1 plasmid or the wild-type GLI1 plasmid into Huh7 cells expressing both PCAF and GLI1 at low levels. As assessed by Co-IP assay, forced expression of PCAF upregulated acetylation of wild-type GLI1 protein in the cytoplasm of Huh7 cells, whereas mutation of K518 abolished the capacity of GLI1 to be acetylated by PCAF (Figure 2c).

To determine the effect of acetylation of GLI1 by PCAF on Hh signalling, we examined the ratio of cytoplasmic to nuclear GLI1 in the previously described Huh7 cell model. As shown in Figure 2d, PCAF overexpression led to the decreased expression of GLI1 and PTCH1 and a higher ratio of cytoplasmic to nuclear GLI1. Interestingly, enhanced expression of PCAF did not affect the expression of mutant GLI1 and PTCH1 or the ratio of cytoplasmic to nuclear mutant GLI1 (Figure 2d). These data prompted us to investigate the possibility that acetylation could interfere with the promoter occupancy of GLI1. Therefore, we analyzed GLI1 recruitment to the GLI-responsive element of the PTCH1 promoter by a chromatin immunoprecipitation assay using a primary antibody targeting GLI1 protein. PCAF overexpression abrogated promoter occupancy of GLI1, whereas mutation at the K518 residue abolished this effect of PCAF, as shown in Figure 2e. Thus, the data support the hypothesis that PCAF negatively regulates Hh signalling by acetylating cytoplasmic GLI1 and preventing its translocation into the nucleus.

**PCAF represses Bcl-2 expression and upregulates BAX via negative regulation of GLI1 and consequently induces cell apoptosis.** To identify the role of GLI1 in PCAF-induced cell apoptosis, we first examined the impact of GLI1 on the anti-apoptotic factor Bcl-2 and the pro-apoptotic factor BAX. As shown in Figure 3a, the overexpression of GLI1 led to upregulation of Bcl-2 and downregulation of BAX in Hep3B cells. By contrast, knockdown of GLI1 decreased Bcl-2 expression and increased BAX expression in PLC/PRF/5 cells (Figure 3b). Owing to the negative regulatory effect of PCAF on GLI1 in HCC mentioned above, we determined whether the GLI1/Bcl-2/BAX axis mediates PCAF-driven cell apoptosis in HCC. As shown in Figures 4a and b, forced expression of PCAF in PLC/PRF/5 cells promoted cell apoptosis and inhibited cell growth, which is consistent with our previous results.<sup>6</sup> Further, forced expression of PCAF induced both downregulation of Bcl-2 and upregulation of BAX (Figure 4c). In addition, silencing GLI1 using siRNAs abolished the effect of PCAF overexpression on cell apoptosis, cell growth and expression of both Bcl-2 and BAX, showing that the effect of PCAF is mediated through GLI1 (Supplementary Figures A–C).

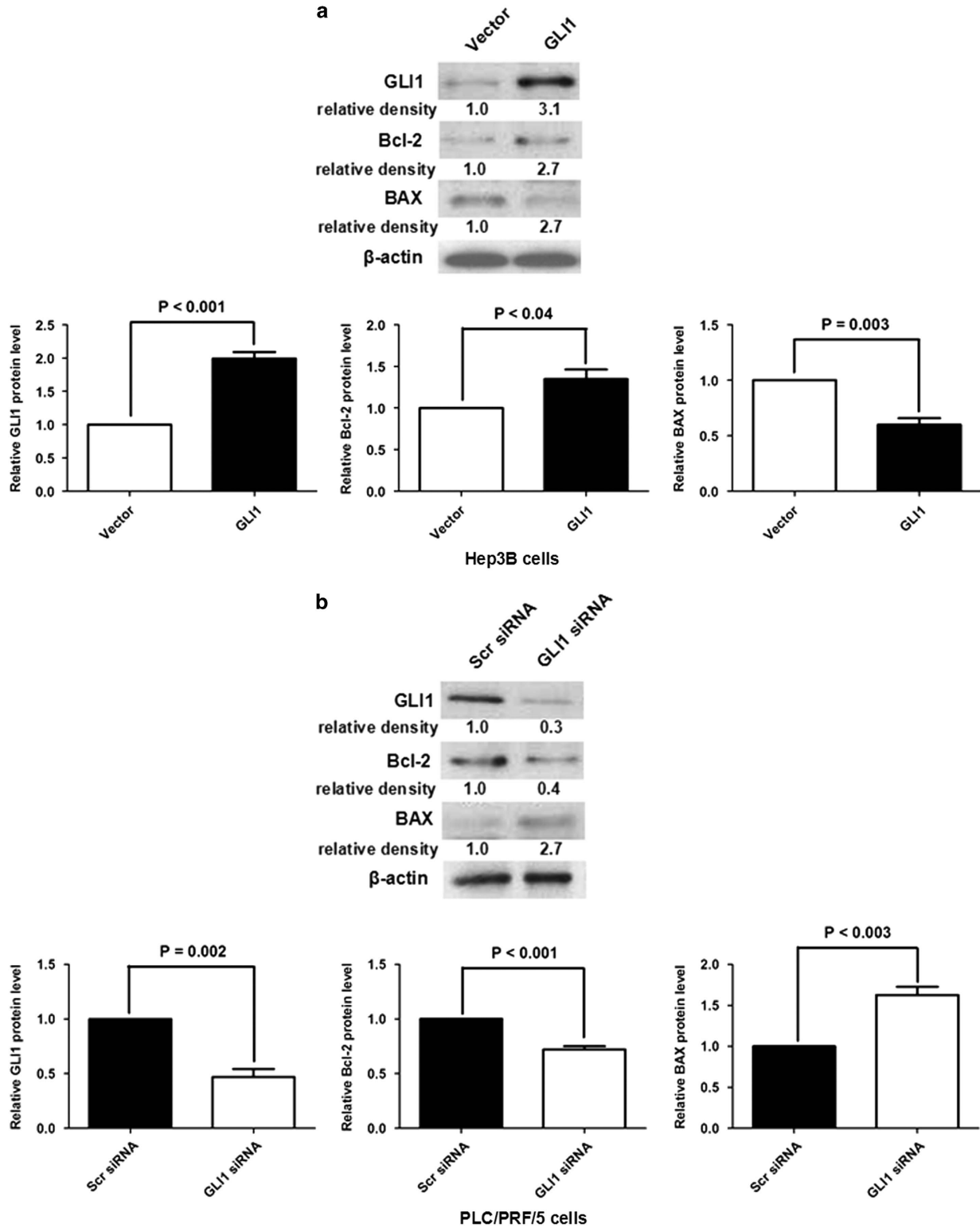
**PCAF sensitizes HCC cells to 5-fluorouracil (5-FU) treatment by regulating GLI1/Bcl-2/BAX axis-dependent apoptosis.** 5-FU has been used for both systemic and locoregional chemotherapy of HCC. Unfortunately, owing to the rapid development of acquired resistance, 5-FU is of limited benefit in treatment of patients with advanced HCC.<sup>14</sup> Resistance to 5-FU-based chemotherapy in cancer cells is partly induced by activation of the Bcl2/BAX anti-apoptotic pathway.<sup>15</sup> Therefore, we hypothesized that PCAF would sensitize HCC cells to 5-FU based chemotherapy through effects on the GLI1/Bcl-2/BAX axis. To identify the optimal 5-FU concentration for the viability assays, we treated PLC/PRF/5 cells with different concentrations of 5-FU for 48 h. PLC/PRF/5 cells displayed a dose-dependent decrease in cell viability following 5-FU treatment as assessed by MTT assay (Figure 5a). The viability of PLC/PRF/5 cells was inhibited at concentrations from 20 to 80  $\mu$ M. Next, both PLC/PRF/5 PCAF cells and PLC/PRF/5 Vector cells were treated with 5-FU at a concentration of 20  $\mu$ M for 48 h and apoptosis was assessed by annexin V-FITC and PI labeling. As shown in Figure 5b, while apoptosis was enhanced in both groups by 5-FU treatment, the percent apoptosis of PLC/PRF/5 PCAF cells was increased 32.3% by 5-FU treatment, whereas apoptosis was only increased by 14.7% in PLC/PRF/5 Vector cells. Consistent with our hypothesis, the MTT assay showed that 5-FU exerted a greater inhibitory effect on cell viability in PLC/PRF/5 PCAF cells (Figure 5c). Thus, PCAF sensitizes PLC/PRF/5 cells to 5-FU treatment. To determine whether the Bcl-2/BAX apoptosis pathway was involved in the enhancement of the 5-FU-related pro-apoptotic effect on HCC cells mediated by PCAF, we examined the impact of 5-FU on the expression of Bcl-2 and BAX in both PLC/PRF/5 PCAF cells and PLC/PRF/5 Vector cells by western immunoblotting. As shown in Figures 5d, 5-FU treatment further decreased Bcl2 expression and enhanced BAX expression in PLC/PRF/5 PCAF cells compared with PLC/PRF/5 Vector cells, consistent with the demonstrated effects on cell apoptosis and viability.

**PCAF is downregulated in HCC and predicts better survival and lower recurrence after resection.** To assess the expression of PCAF in HCC tissues and the association of PCAF expression with HCC survival, we analyzed PCAF mRNA expression levels in a published gene expression microarray study of 139 HCC patients and examined the association of PCAF expression with patient survival. PCAF mRNA was detectable in 138 of the 139 HCC patients (99.3%). PCAF expression was decreased in tumor compared with adjacent benign liver tissue in 100 (72.5%) of the 138 HCCs (Figure 6a).

Follow-up survival information was available for 113 of the 138 HCC patients. These 113 HCC patients were classified into low PCAF and high PCAF groups. The low PCAF group included patients with lower PCAF expression in tumor than in adjacent benign tissues, whereas the high PCAF group included patients with higher PCAF expression in tumor than in adjacent benign tissues. As shown in Table 1, there were no significant differences in the demographic and clinical characteristics of the two groups. Comparison of Kaplan–Meier overall survival curves showed significantly longer

postsurgical survival in the high PCAF group (HR = 0.515; 95% CI: 0.315, 0.843;  $P=0.008$ ; Figure 6b). The median overall survival was 70.5 months in the high PCAF group

compared with 23.5 months in the low PCAF group. The 3-year survival rate in the high PCAF group was 60%, which was significantly higher than the 40% 3-year survival in the low



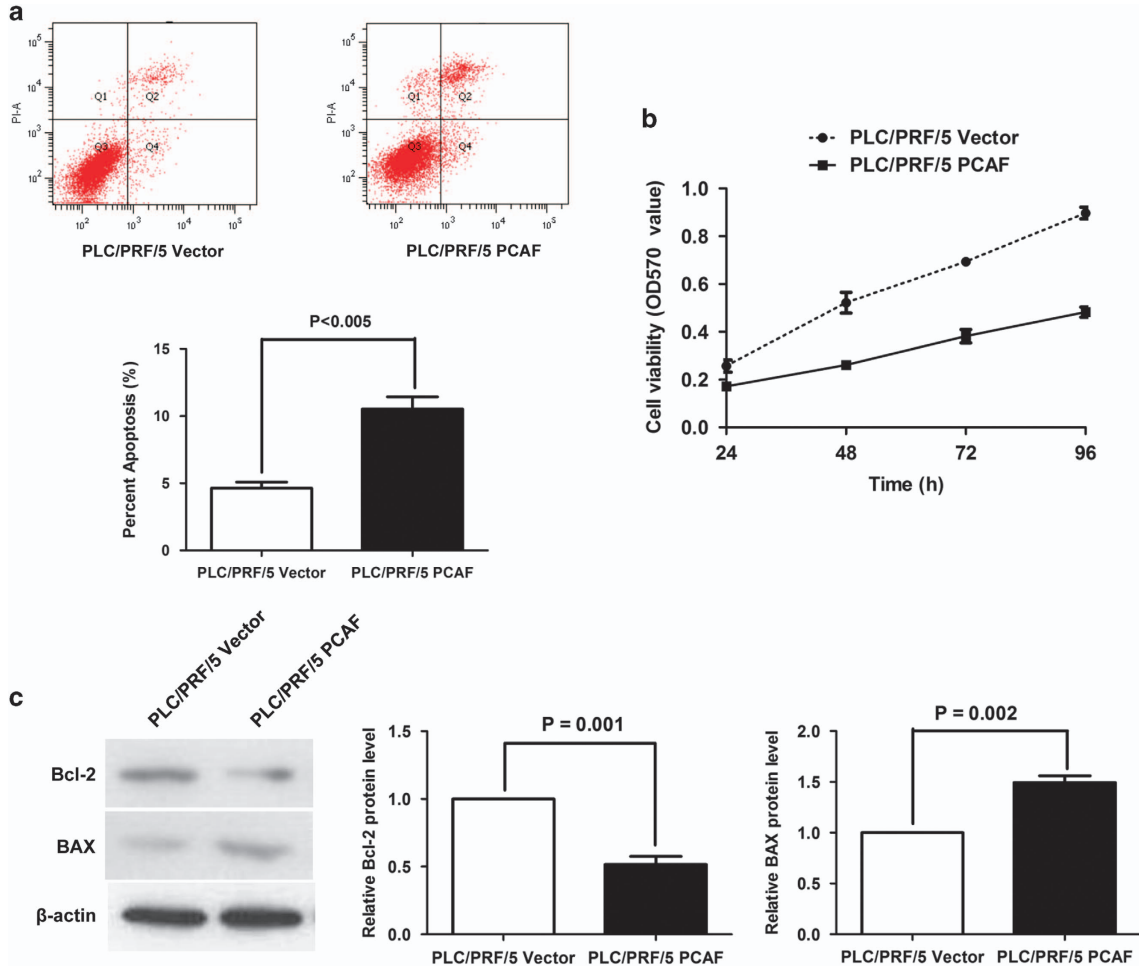
**Figure 3** GLI1 upregulated Bcl-2 expression and downregulated expression of BAX in HCC cells. (a) GLI1 overexpression after transfecting GLI1-expressing plasmid was confirmed by western immunoblotting in Hep3B cells. Western immunoblotting assay showed that GLI1 overexpression induced upregulation of Bcl-2 and downregulation of BAX in Hep3B cells. (b) After silencing GLI1 expression, the expression of Bcl-2 was decreased and BAX expression was increased in PLC/PRF/5 cells



PCAF group. Analysis of 5-year survival rates was similar; the high PCAF group had a 5-year survival of 55% versus 24% in the low PCAF group. Further, patients in the high PCAF group also had significantly lower rates of recurrence after surgical resection (HR = 0.419; 95% CI: 0.225, 0.780;  $P=0.006$ ; Figure 6c). The median recurrence-free survival in the high PCAF group was 85.3 months compared with 34.4 months in the low PCAF group. Consistent with this observation, the 3-year recurrence-free survival rate in the high PCAF was 73% compared with 47% in the low PCAF group. Similarly, the 5-year recurrence-free survival rate in the high PCAF group

was 62% compared with 25% in the low PCAF group. These clinical data further support the tumor suppressor effect of PCAF on HCC progression and suggest the utility of PCAF levels as a potential biomarker for predicting the postsurgical prognosis of HCCs.

**PCAF suppresses tumor growth and enhances the therapeutic efficacy of 5-FU in an *in vivo* mouse model.** The critical roles of PCAF in cell apoptosis, synergistic antitumor effects of PCAF and 5-FU, and the significant correlation of PCAF with postsurgical survival of HCCs

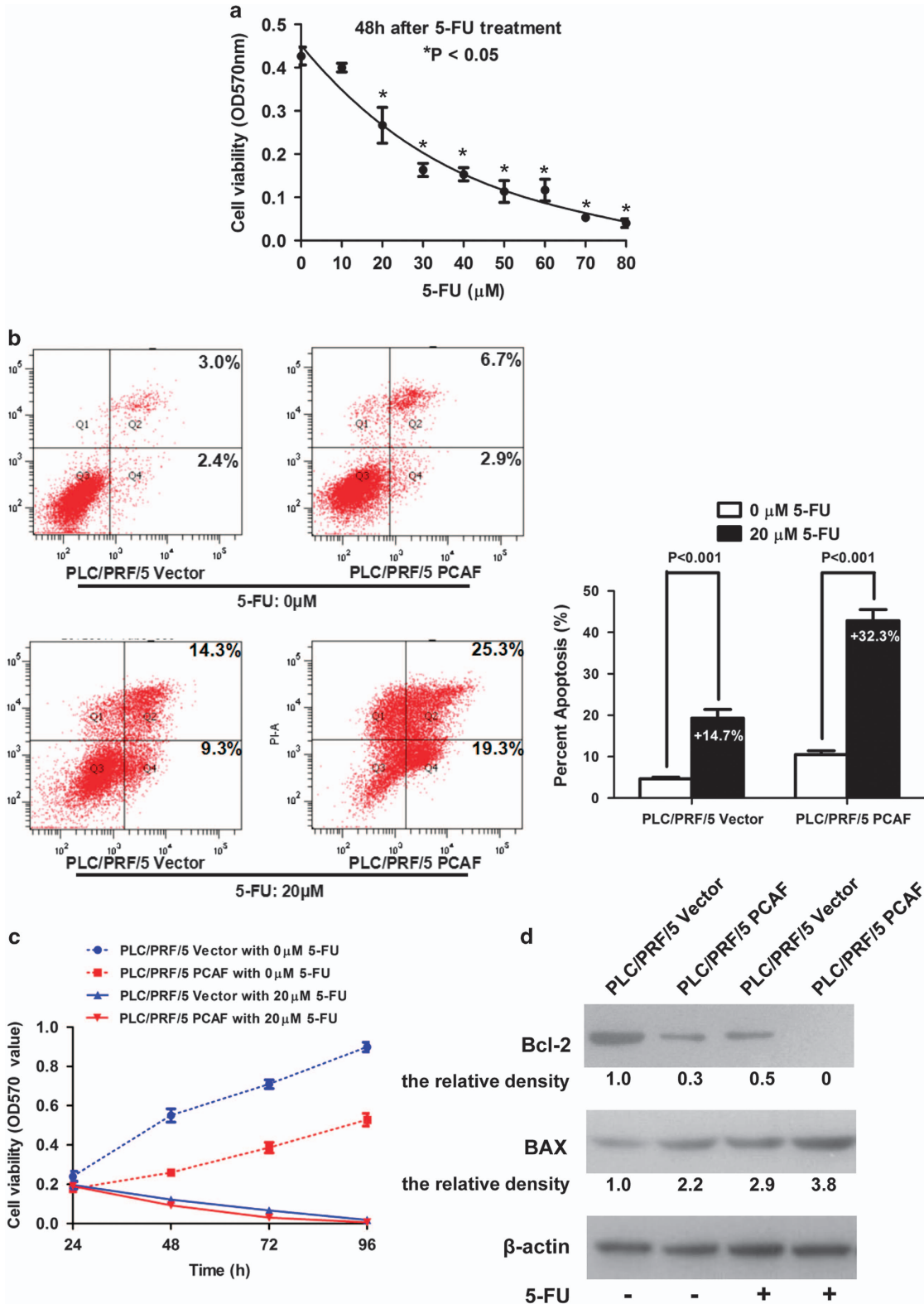


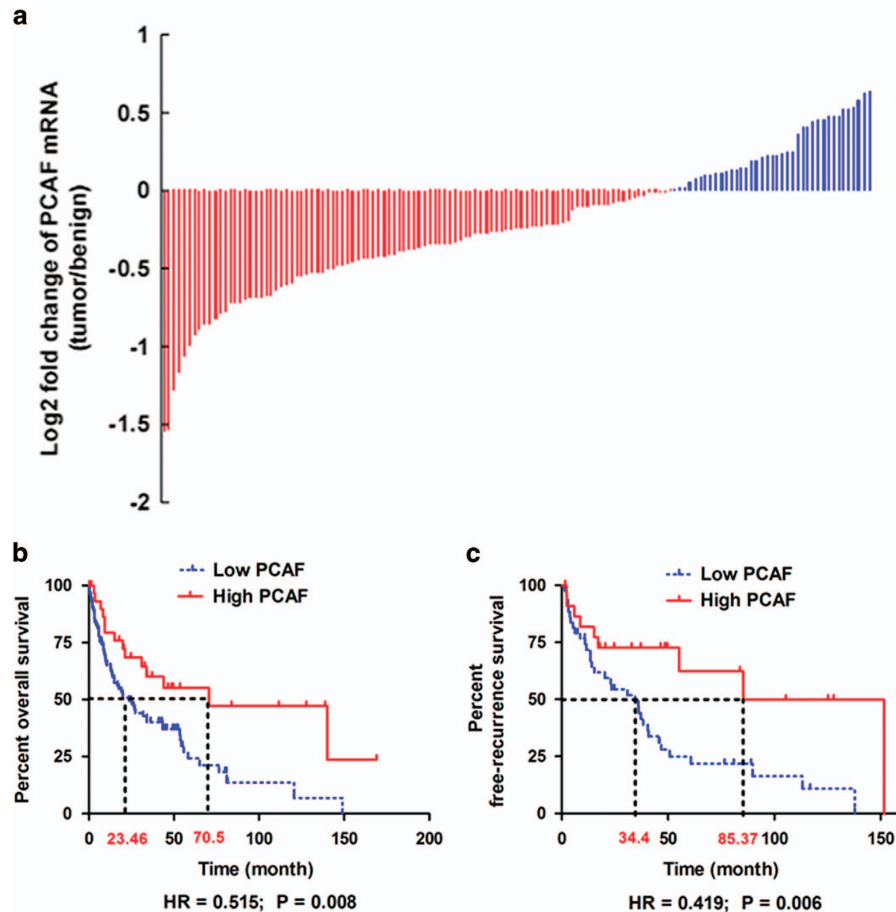
**Figure 4** PCAF decreased the Bcl-2/BAX ratio and induced cellular apoptosis. (a) Annexin V-FITC/PI labeling showed that forced expression of PCAF resulted in an approximately twofold increase in apoptosis of PLC/PRF/5 cells. The percentages of cells in Quadrants 2 and 4 were calculated as the percentage of apoptosis in the quantitative graph. (b) As assessed by MTT assay, PCAF overexpression suppressed cell viability of PLC/PRF/5 cells at 24, 48, 72 and 96 h. (c) Western immunoblotting assay showed that enhanced expression of PCAF repressed Bcl-2 expression and increased BAX expression in PLC/PRF/5 cells

**Figure 5** PCAF sensitizes HCC cells to 5-FU via modulating GLI1/Bcl-2/BAX axis. (a) As assessed by MTT assay, PLC/PRF/5 cells displayed a dose-dependent decrease in cell viability following 5-FU treatment, and cell viability was repressed significantly at concentrations from 20 to 80  $\mu\text{M}$ . We compared the results of MTT assay between control group (0  $\mu\text{M}$  5-FU) and eight other concentrations of 5-FU (range: 10–80  $\mu\text{M}$ ) by Mann-Whitney test and the difference with  $P < 0.05$  was set as statistically significant. (b) As assessed by Annexin V-FITC/PI labeling assay, cell apoptosis was enhanced after 5-FU treatment at the concentration of 20  $\mu\text{M}$  in both groups and the apoptosis percentage of PLC/PRF/5 PCAF cells was increased about 32.3% by 5-FU treatment, whereas it resulted in only 14.7% increase in PLC/PRF/5 Vector cells. (c) MTT assay displayed that 5-FU exerted stronger inhibitory effect on cell viability in PLC/PRF/5 PCAF cells. In consistent with the data shown in Figure 4b, MTT assay showed that OD570 values in PLC/PRF/5 Vector cells with 0  $\mu\text{M}$  5-FU were magnificently higher than those in PLC/PRF/5 PCAF cells with 0  $\mu\text{M}$  5-FU at 48, 72 and 96 h, respectively (all  $P$  value  $< 0.05$ ). Moreover, it was found that OD570 values in PLC/PRF/5 Vector cells with 20  $\mu\text{M}$  5-FU was significantly higher than those in PLC/PRF/5 PCAF cells with 20  $\mu\text{M}$  5-FU at both 48 and 72 h, respectively (all  $P$  value  $< 0.05$ ). Mann-Whitney test was used here. (d) Western immunoblotting assay showed that 5-FU treatment lead to least Bcl2 expression and most BAX expression in PLC/PRF/5 PCAF cells compared with the other three groups

strongly suggest that PCAF may have utility as a predictor of outcome of HCCs after surgery and also that 5-FU treatment may have a better therapeutic effect in HCC patients with high tumor PCAF expression. PCAF expression significantly

inhibited tumor growth, as shown in Figures 7a and b. To verify the PCAF-induced changes in the GLI1/Bcl-2/BAX axis *in vivo*, we performed immunohistochemical staining using antibodies against PCAF, GLI1, Bcl-2 and BAX in





**Figure 6** PCAF is downregulated in HCC and predicts better survival. (a) PCAF mRNA was found in 138 of 139 HCC patients (99.3%) and there was decreased PCAF expression in tumor compared with adjacent liver tissues in 100 (72.5%) of the HCCs. (b) Comparison of Kaplan–Meier overall survival curves displayed that the high PCAF group had a obviously longer postsurgical survival time. (c) After analyzing the Kaplan–Meier recurrence-free survival curves, there was a slower rate of tumor recurrence after surgery found in the high PCAF group

consecutive sections of xenografts derived from PLC/PRF/5 Vector and PLC/PRF/5 PCAF cells. Consistent with our *in vitro* results, PCAF-driven downregulation of GLI1 occurred *in vivo* (Figure 7c). Further, PCAF expression also resulted in the downregulation of Bcl-2 and upregulation of BAX *in vivo* (Figure 7c). As shown in Figure 7a, there was a much stronger anti-tumor effect of 5-FU in mice implanted with PCAF-expressing cells compared with Vector control cells, confirming that PCAF expression sensitized HCC xenografts to the anti-tumor effect of 5-FU *in vivo*.

## Discussion

Programmed cell death exerts a key role in tissue development and homeostasis. Aberrant cell apoptosis contributes to carcinogenesis and also enhances resistance to chemotherapy. The rate of HCC recurrence after surgery remains high and there is currently no effective anti-HCC therapy in the clinic that prevents postsurgical recurrence and prolongs survival. Therefore, it is important to uncover the mechanisms of aberrant HCC cell apoptosis and exploit the therapeutic options they provide.

The histone acetyltransferase PCAF has been shown to be involved in the modulation of differentiation, angiogenesis, cell cycle progression, gluconeogenesis and carcinogenesis.<sup>6,16–18</sup> In HCC, PCAF promotes cell apoptosis and inhibits tumor growth by regulating acetylation of histone H4.<sup>6</sup> Previous studies revealed that although initially characterized as HATs, acetyltransferase enzymes also acetylate non-histone proteins.<sup>19</sup> Since the first report of p53 as a non-histone target of HATs,<sup>20</sup> there has been a rapid increase in the description of new non-histone targets of HATs, including importin- $\alpha$  adaptor,<sup>21</sup> E1A viral oncoprotein<sup>22</sup> and FOXP3.<sup>23</sup> Thus, we investigated whether PCAF modulates HCC cell apoptosis via acetylating non-histone protein and explored the underlying signaling pathways mediating these effects. Co-IP assays revealed that PCAF directly bound to GLI1 protein and acetylated it in the cytoplasm. Western immunoblotting and reporter luciferase assays showed that the cytoplasmic-to-nuclear shuttling of GLI1 protein was abrogated by PCAF-driven acetylation of GLI1, thus abolishing activation of Hh signaling in HCC.

Since Bcl-2 was initially discovered as an oncoprotein in follicular lymphoma because of its repression of cellular



**Table 1** Demographic information and clinical features of patients with HCCs expressing low versus high levels of PCAF compared with the adjacent benign tissue

	Low PCAF group (number; %)	High PCAF group	$\chi^2$	P
<i>Gender</i>				
Male	60 (69.8%)	20	0.500	0.479
Female	26 (30.2%)	6		
<i>Age (years)</i>				
≥ 50	46 (54.8%)	17	2.735	0.098
< 50	38 (45.2%)	6		
<i>APF (ng/dl)</i>				
≥ 400	36 (50%)	14	0.347	0.556
< 400	36 (50%)	18		
<i>HBsAg</i>				
Positive	40 (81.6%)	8	0.015	0.904
Negative	9 (18.4%)	2		
<i>Etiology</i>				
HBV or HCV infection	53 (67.1%)	20	0.213	0.644
Other	26 (32.9%)	12		
<i>Tumor size (cm)</i>				
≥ 5	48 (68.6%)	19	1.193	0.274
< 5	22 (31.4%)	14		
<i>Liver cirrhosis</i>				
Yes	37 (43%)	11	0.004	0.949
No	49 (57%)	15		
<i>TNM staging</i>				
I+II	14 (30.4%)	3	3.289	0.070
III+IV	32 (69.6%)	23		

apoptosis,<sup>24</sup> it has been considered a critical mediator which controls the intrinsic apoptosis pathway (the Caspase-9/Caspase-3/Caspase-6/Caspase-7 cascade).<sup>25</sup> BAX is another member of the Bcl-2 family which has been established as a pro-apoptotic factor. BAX can release cytochrome c after the formation of both BAX/Bak hetero-oligomers and BAX/BAX homodimers, and induce cell apoptosis. Bcl-2 can interact with BAX, promote the formation of Bcl-2/BAX heterodimer, and decrease the amount of both BAX/Bak hetero-oligomer and BAX/BAX homodimer. Consequently, the ratio of Bcl-2 to BAX controls cell apoptosis.<sup>26,27</sup> Here, we found that PCAF simultaneously repressed Bcl-2 expression and upregulated BAX in HCC cells, both *in vitro* and *in vivo*. Further, we found that the suppression of GLI1 expression abrogated the changes in the Bcl-2/BAX ratio driven by PCAF in HCC cells. GLI1 has recently been shown to bind directly to the Bcl-2 promoter.<sup>28</sup> Therefore, these data strongly supported the concept that GLI1 mediates PCAF-induced HCC cell apoptosis by regulating the Bcl-2/BAX ratio.

Most chemotherapy drugs exert their anti-tumor function through activation of the 'mitochondrial' or intrinsic pathway of apoptosis. The Bcl-2/BAX axis is the key trigger activating this apoptosis pathway. In this study, we found that 5-FU had a stronger anti-HCC effect in cells with higher PCAF expression both *in vitro* and *in vivo*. Further analyses showed that 5-FU treatment induced a larger decrease in the Bcl-2/BAX ratio in HCC cells with high PCAF expression. These results from both

*in vitro* and *in vivo* experiments suggest that PCAF expression sensitizes HCC cells to treatment with 5-FU by regulating GLI1/Bcl-2/BAX axis-dependent apoptosis.

In our previous study, we detected the expression of PCAF protein in 35 HCC patients from a medical center in China by IHC staining and found that PCAF expression in HCC tissues was significantly associated with better long-term survival after surgery.<sup>29</sup> We examined PCAF mRNA expression in 139 HCCs from Mayo Clinic, the Cancer Institute of the Chinese Academy of Medical Sciences, the University of Leuven and the US National Cancer Institute. The results showed that PCAF was downregulated in most HCC tissues and that high PCAF expression in HCC tissues was significantly associated with longer overall survival and recurrence-free survival after surgery, which is consistent with our previous findings. Hence, PCAF could potentially serve as a predictive biomarker for the outcome of patients with HCCs after surgical resection.

In summary, we have provided the first evidence that PCAF acetylates cytoplasmic GLI1 protein and prevents its cytoplasmic-to-nuclear shuttling. This inhibits activation of Hh signaling, leading to a decrease in the Bcl-2/BAX ratio, and ultimately inducing cellular apoptosis. We also confirmed that HCC patients with high tumor PCAF expression had better postsurgical prognosis and were potentially more sensitive to 5-FU-based chemotherapy regimens.

#### Materials and Methods

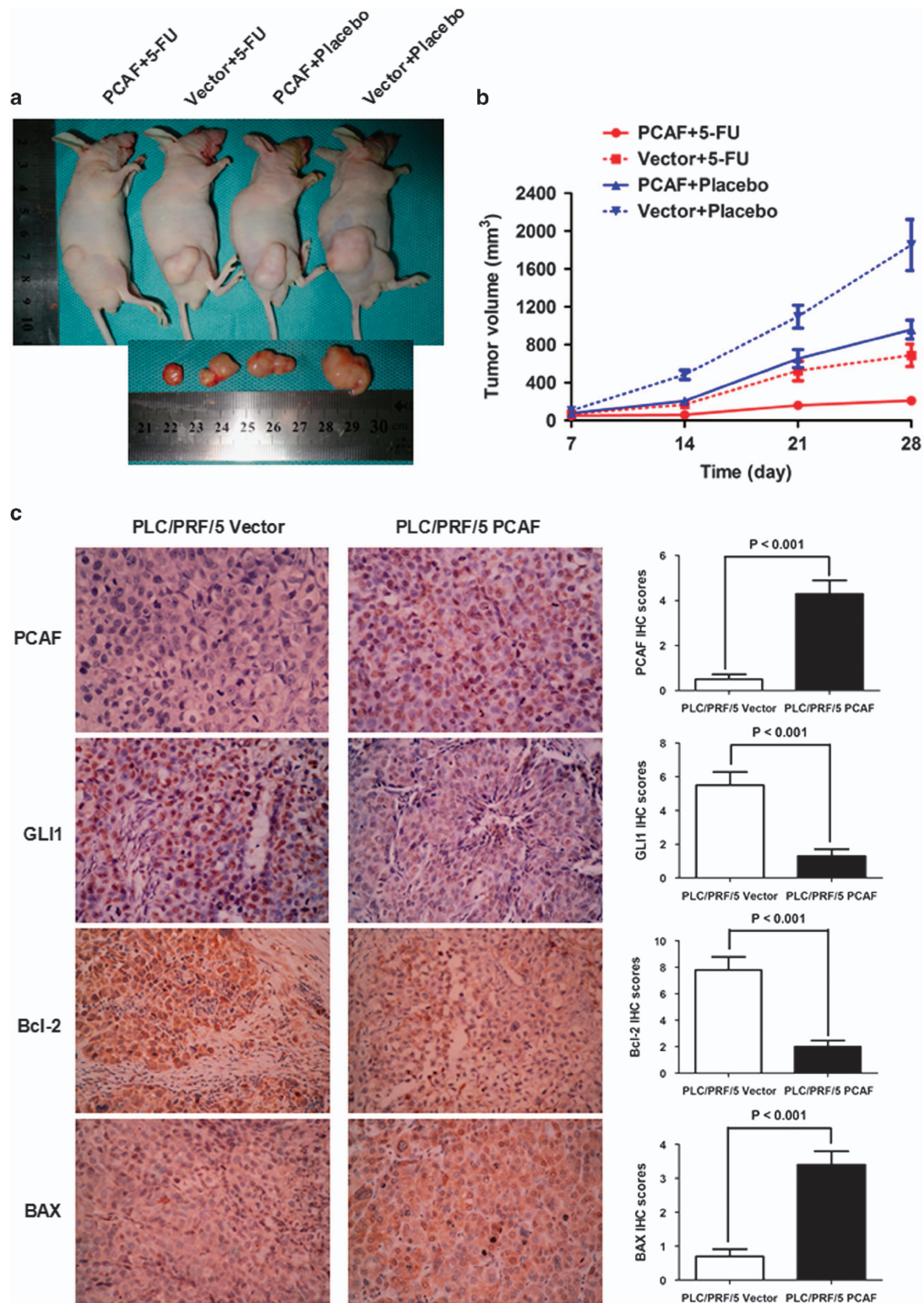
**Cell culture and reagents.** PLC/PRF/5 and Hep3B cells were grown in complete MEM medium with 10% FBS. 5-FU was obtained from Sigma-Aldrich (St. Louis, MO, USA). The PCAF-expressing plasmid and its empty plasmid pCMV6-Entry were both from Origene Technologies Inc. (Rockville, MD, USA). The cDNA of GLI1 was cloned into the pCMV-Tag2B vector from Stratagene (Santa Clara, CA, USA) as GLI1-expressing plasmid. The rabbit monoclonal PCAF antibody, mouse monoclonal GLI1 antibody, mouse monoclonal PTCH1 antibody, rabbit monoclonal Bcl-2 antibody and rabbit monoclonal BAX antibody were from Cell Signaling (Danvers, MA, USA). The rabbit polyclonal anti-acetyl lysine antibody was obtained from Abcam (Cambridge, MA, USA). The mouse monoclonal  $\beta$ -actin antibody was from Boster Biotechnology (Wuhan, China). The IHC detection kit (Catalog No.: SP-9001) was purchased from ZSGB Bio. (Beijing, China). Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit was from Invitrogen (Carlsbad, CA, USA).

**Site-directed mutagenesis.** Mutant GLI1 K518 plasmid was produced by site-directed mutagenesis of the wild-type GLI1-expressing plasmid (Quikchange II XL Site-directed mutagenesis kit, Stratagene) using the following primers: Forward: TAGGGACCCGGGGTCTCATGCTGCCAGCTTGT; Reverse: TGGGACAAGCTGGGCA GCATGAGACCCCGGGT. Mutant sequences were confirmed by PCR assay.

**Establishment of PCAF stable transfectant clones.** PCAF-expressing plasmid and pCMV6-Entry plasmid were transfected into PLC/PRF/5 cells using FuGENE6 Transfection Reagent from Promega (Madison, WI, USA). Stable transfection was achieved as described previously.<sup>6</sup>

**RNAi-mediated knockdown.** The siRNAs used in this investigation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): PCAF siRNA (Catalog No. sc-36198), GLI1 siRNA (Catalog No. sc-37911) and scrambled siRNA (Catalog No. sc-37007). Transfection was performed as described previously.<sup>5</sup>

**Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).** Total RNA from HCC cells was isolated with the Rneasy kit from Qiagen Co. (Valencia, CA, USA). cDNA synthesis and qRT-PCR analysis were done as described.<sup>12</sup> The ABI TaqMan probes (Applied Biosystems, Carlsbad, CA, USA) targeting PCAF (Hs00187332\_m1) and 18 s rRNA (Hs99999901\_s1)



**Figure 7** PCAF suppresses growth of HCC xenografts and enhances the therapeutic efficacy of 5-FU in nude mice *in vivo*. (a) At 28 days after inoculation of HCC cells, HCC xenografts from mice inoculated with PLC/PRF/5 Vector cells and receiving placebo treatment (Vector+Placebo) were the largest among the four groups, whereas xenografts from mice inoculated with PLC/PRF/5 PCAF cells and receiving 5-FU (PCAF+5-Fu) were the smallest. (b) PCAF expression significantly repressed tumour growth *in vivo*. Xenografts from the Vector+Placebo group were significantly larger than xenografts from each of the other three groups at days 14, 21 and 28 after HCC cell inoculation (all  $P$  values  $< 0.05$ , Student's  $t$  test). On the other hand, xenografts from the PCAF+5-FU group were smaller than xenografts from each of the other three groups at days 14, 21 and 28 (all  $P$  values  $< 0.05$ ). (c) As assessed by IHC staining, PCAF expression inhibited GLI1 expression *in vivo*. HCC xenografts from PLC/PRF/5 PCAF cells also showed downregulation of Bcl-2 and upregulation of BAX



were used here. There were six replicates in each measurement and the assessment was repeated three times.

**Co-IP assay and western immunoblotting.** A Co-IP assay was performed to test the interaction between PCAF protein and GLI1 protein in PLC/PRF/5 PCAF cells. Total protein lysate was prepared in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 2  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1 mM PMSF, 1 mM sodium vanadate and 10 mM sodium fluoride). The lysate was precleared with protein A/G-agarose beads and the total protein concentration was measured using the BCA method. Total protein was diluted to 1  $\mu$ g/ $\mu$ l with PBS and mixed with primary antibodies against PCAF and GLI1 or IgG. The mixtures were shaken on a rotating shaker at 4 °C overnight. The supernatant was collected and used for immunoblotting. Western immunoblotting was carried out as described previously<sup>6</sup> with the following anti-human primary antibodies: GLI1 antibody (1 : 1000 dilution), GLI1 antibody (1 : 1000 dilution), PTCH1 antibody (1 : 1000 dilution), Bcl-2 antibody (1 : 1000 dilution), BAX antibody (1 : 1000 dilution), anti-acetyl lysine antibody (1 : 1000 dilution) and  $\beta$ -actin antibody (1 : 100 dilution).

**Luciferase reporter assay.** The GLI-Luciferase reporter plasmid containing eight consecutive GLI-binding sites downstream of the luciferase gene (8XGL1) was a gift from Prof. Qingyong Ma (Department of Hepatobiliary Surgery, the First Affiliated Hospital of Xi'an Jiaotong University). The luciferase reporter assay was conducted as described previously.<sup>12</sup> There were three replicates in each experiment, and the assessment was repeated six times.

**Cell viability assay and cell apoptosis detection.** The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure cell viability at 24, 48, 72 and 96 h. Briefly, HCC cells were cultured in 96-well plates at the concentration of  $1 \times 10^4$  and stained with 100  $\mu$ l MTT (0.5 mg/ml) for 4 h at 37 °C. After that, the culture medium was removed and 150  $\mu$ l dimethyl sulfoxide from Sigma-Aldrich was added to each well. The absorbance was assessed at 570 nm. Six replicates were assessed for each time point. Cell apoptosis was assessed by flow cytometry assay using annexin V-FITC and PI labeling, according to the manufacturer's recommendations (Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit, Invitrogen). FACS analysis was conducted using a FACSCalibur flow cytometry system (Becton Dickinson, San Jose, CA, USA). All assessments (MTT assay and cell apoptosis detection) were repeated at least six times.

**Microarray gene expression profiling.** Tumor and adjacent benign tissues were obtained from 139 HCC patients undergoing surgical resection for HCC at the Mayo Clinic, the Cancer Institute of the Chinese Academy of Medical Sciences, the University of Leuven and the US National Cancer Institute. The study was approved by the Institutional Review Boards of the four medical centers. These HCC samples are different from those in our preliminary investigation.<sup>29</sup> The demographic information, clinical features and follow-up information on the patients have been reported in Supplementary Table 2 of Lee et al.<sup>30</sup> RNAs extracted from 139 HCCs were analyzed with the Human Array-Ready Oligo Set (version 2.0) from Qiagen in the US National Cancer Institute, which contains 70-mer probes for 21 329 genes. The details of microarray gene expression profiling have also been previously described.<sup>30</sup> The data have been deposited at Gene Expression Omnibus (GEO/GPL1528, GEO/GPL1529, GEO/GSE1897, GEO/GSE1898 and GEO/GSE4024).

**In vivo experiments.** Forty 4–6-week-old male nude mice were randomly assigned into four groups: PCAF+5-FU, Vector+5-FU, PCAF+Placebo and Vector+Placebo group. Briefly, we inoculated  $5 \times 10^5$  PLC/PRF/5 Vector and PLC/PRF/5 PCAF cells subcutaneously into 10 nude mice each and observed the growth of the tumors over time. To confirm the synergistic antitumor effects of PCAF with 5-FU, 5-FU at a dose of 8 mg/kg was injected intraperitoneally for five consecutive days each week for 4 weeks. Control mice received dimethyl sulfoxide diluent alone as placebo. Mice were killed on the twenty-eighth day after cell injection. IHC staining was performed in xenograft tissues to detect the expression of PCAF, GLI1, Bcl-2 and BAX as described previously.<sup>31</sup> The staining intensity was scored as four grades: 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). The percentage of positive cells was also expressed into five categories, in which a score of 0 was given for 0–5%, 1 for 6–25%, 2 for 26–50%, 3 for 51–75% and 4 for > 75%. The staining intensity and average percentage of positive cells

were measured for 10 independent high magnification ( $\times 400$ ) fields. The ultimate staining score was obtained by multiplying the staining intensity and the percentage of positive cells. All experimental protocols were approved by the Institutional Animal Care and Use Committee of our hospital.

**Statistical analysis.** All data represent at least three independent experiments and are expressed as means and standard errors of the mean. Differences between the Kaplan–Meier curves of HCCs with upregulated or downregulated PCAF expression in the tumour tissue in comparison with the adjacent benign tissue were analyzed using the log rank test. Demographic information of patients from the low PCAF and high PCAF groups was compared by  $\chi^2$  test. Differences between groups were compared with an unpaired two-tailed *t* test or Mann-Whitney test.

### Conflict of Interest

The authors declare no conflict of interest.

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