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# BIRC6 promotes hepatocellular carcinogenesis: Interaction of BIRC6 with p53 facilitating p53 degradation

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**The genes that encode inhibitor of apoptosis proteins (IAPs) are frequently overexpressed in human cancers. However, the expression pattern and clinical significance of BIRC6, a member of IAPs, in hepatocellular carcinoma (HCC) remains unclear. Here we investigated the role of BIRC6 in hepatocellular carcinogenesis. We used immunoblot and immunochemical analyses to determine the levels of BIRC6 in 7 hepatoma cell lines and 160 HCC specimens. We evaluated the proognostic value of BIRC6 expression and its association with clinical parameters. A lentivirus-mediated silencing method was used to knockdown BIRC6, and the biological consequences of BIRC6 silencing in three hepatoma cell lines were investigated *in vitro* and *in vivo*. We found that BIRC6 overexpression was significantly correlated with serum ALT level and HCC vascular invasion. Patients with positive BIRC6 expression in tumor tissue had a poor survival and a high rate of recurrence. BIRC6 knockdown remarkably suppressed cell proliferation, caused G1/S arrest and sensitized hepatoma cells to sorafenib-induced apoptosis in hepatoma cells, which was partly reversed by RNA interference targeting p53. The mechanistic study revealed that BIRC6 interacted with p53 and facilitated its degradation. The *in vivo* study showed that BIRC6 knockdown inhibited xenograft tumor growth and increased the sensitivity of tumor cells to sorafenib in nude mice. Taken together, these findings demonstate that BIRC6 overexpression in HCC specimens is indicative of poor prognosis and that its interaction with p53 facilitates the degradation of p53, leading to carcinogenesis and an anti-apoptotic status.**



Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer, and ranks the third as a cause of cancer-related death worldwide.[1] Although genetic changes and their biological consequences are thought to be involved,[2] the molecular mechanisms of hepatocarcinogenesis have not been fully elucidated. Thus, it is necessary and valuable to explore the underlying pathogenesis.

Baculoviral inhibition of apoptosis protein repeat containing 6 (BIRC6) is a giant protein with an N-terminal single

**Key words:** apoptosis, G1/S arrest, growth inhibition, tumorigenesis, ubiquitination

**Abbreviations:** AFP: alpha fetoprotein; ALT: alanine aminotransferase; BCLC: Barcelona Clinic Liver Cancer; BIR: baculovirus inhibition of apoptosis protein repeat; BIRC6: baculoviral IAP repeat containing 6; DMSO: dimethyl sulfoxide; FITC: fluorescein isothiocyanate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HBsAg: hepatitis B virus surface antigen; HCC: hepatocellular carcinoma; IAPs: inhibition of apoptosis proteins; IHC: immunohistochemistry; K group: knockdown group; OS: overall survival; PDGFR: platelet-derived growth factor receptor; PI: propidium iodide; RT-PCR: reverse transcription polymerase chain reaction; SEM: standard error of the means; siRNA: short interfering RNA; SSCP-PCR: single strand conformational polymorphism-polymerase chain reaction; TTP: time to progression; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling staining; UBC: ubiquitin-conjugating; VEGF: vascular endothelial growth factor.

Additional Supporting Information may be found in the online version of this article.

W.T., R.X. and S.W. contributed equally to this work.

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**What's new?**

**Inhibition of apoptosis proteins (IAPs) are involved in carcinogenesis, but for some, such as BIRC6, an IAP that facilitates proteasomal degradation of pro-apoptotic proteins, the mechanisms by which they facilitate tumor development remain unclear. This report sheds new light on the effect of BIRC6 on hepatocellular carcinoma (HCC) development. BIRC6 was found to influence carcinogenesis via modulation of the cell cycle, cell proliferation, and apoptosis—activities that were dependent on its interaction with p53. In addition, BIRC6 expression levels were correlated with HCC prognosis. The findings suggest that BIRC6 is a promising prognostic marker and therapeutic target in HCC.**



baculovirus inhibition of apoptosis protein repeat (BIR) domain and a C-terminal ubiquitin-conjugating (UBC) enzyme domain.[3] *Via* the UBC domain, BIRC6 facilitates proteasomal degradation of proapoptotic proteins caspase-9 and Smac, thus playing a role in antiapoptosis.[4,5] BIRC6 is also a critical regulator of cytokinesis and midbody ring formation, which implies an important role of BIRC6 in cell proliferation.[6] BIRC6 belongs to the family of inhibition of apoptosis proteins (IAPs), a group of structurally and functionally similar proteins which are the key regulators of apoptosis and signal transduction.[7]

Another crucial regulator of cell proliferation and apoptosis, p53, has been reported to be involved in carcinogenesis.[2] Abnormalities in the tumor suppressor p53 are among the most common mechanisms of cancer progression.[8] The p53 pathway is aberrant in most human tumors, either by abnormal regulation of p53 expression or function or by mutation/deletion in the p53 gene.[8] p53 activity, expression and localization are mainly regulated by posttranslational modifications, such as phosphorylation, acetylation and ubiquitination.[9] Notably, previous studies have found that p53 is a key downstream effector of BIRC6.[10,11] However, it is unclear whether BIRC6 can interact with p53, and in which way BIRC6 regulates p53. Meanwhile, the role of such association in HCC carcinogenesis remains unknown.

Ample evidence indicated that BIRC6 is abnormally overexpressed in malignant tumors including brain, prostate, breast, colon, ovarian and nonsmall-cell lung cancer, childhood acute leukemia and melanoma.[10,12–18] BIRC6 was identified as a novel intervention target for neuroblastoma therapy.[19] Importantly, excessive expression of BIRC6 is significantly correlated with unfavorable clinical features at diagnosis, pathological classification, high cellular proliferation and poor prognosis in cancers.[12,16,17,20] However, the expression pattern, clinical significance and biological function of BIRC6 in hepatic carcinogenesis have not been examined. Our group has identified BIRC6 as a novel HCC biomarker by proteomic profiling of serum from HCC patients and healthy patients. BIRC6 levels were elevated in the serum of HCC patients. The area under the ROC curve (AUC) of BIRC6 is 0.768, indicating high specificity and sensitivity of BIRC6 in HCC diagnosis.[21] But whether BIRC6 protein correlates with clinical prognosis and the role of BIRC6 in HCC's progression needs further investigation. In the present study, we explored the prognostic significance and biologic features of BIRC6 in HCC, and the interaction between BIRC6 and p53. Our data demonstrated that BIRC6 upregulation in HCC predicted poor prognosis of patients and engaged in carcinogenesis. Furthermore, BIRC6 bind to p53 and promoted its degradation *via* the ubiquitin/proteasome pathway. Finally, we demonstrated that BIRC6 knockdown inhibited tumor growth of xenografts and increased the sensitivity of tumor cells to sorafenib in animal experiments.

## Material and Methods

### Patients, tissue specimens and clinical follow-up

Tumor specimens were obtained from 160 consecutive patients with HCC who underwent curative resection at the Liver Cancer Institute, Zhongshan Hospital, Fudan University between 2006 and 2008. Thirty fresh resection HCC tissues and the paired adjacent nontumorous tissues were collected for Western blot analysis. Ethical approval for human subjects was obtained from the Research Ethics Committee of Zhongshan Hospital, and informed consent was obtained from each patient.

All subjects were contacted every 3 months during the first postoperative year, and for at least 6 months afterward for survival and recurrence inquiry until death, contact failure, or until the end of the investigation, *i.e.*, May 31, 2012, with the median follow-up duration being 22 months (range 3–63 months). All patients were monitored prospectively by serum AFP and ultrasound examination every 3–6 months, according to the postoperative time. A diagnosis of recurrence was determined based on a typical imaging appearance in ultrasound or liver biopsy.

### Immunohistochemical staining and evaluation

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series diluted in distilled water. Following antigen retrieval with a 10 m*M* citrate buffer, HCC sections were incubated overnight at 4°C with primary antibody. Following 30 min incubation with secondary antibody, sections were developed in 3,3′-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. The primary antibodies are listed in the Supplementary Information.

The sections were observed under a light microscopy, for a histological review, to examine tumor microheterogeneity in antigen distribution. Five randomized microscopic views of 400-fold magnification of each section were observed and

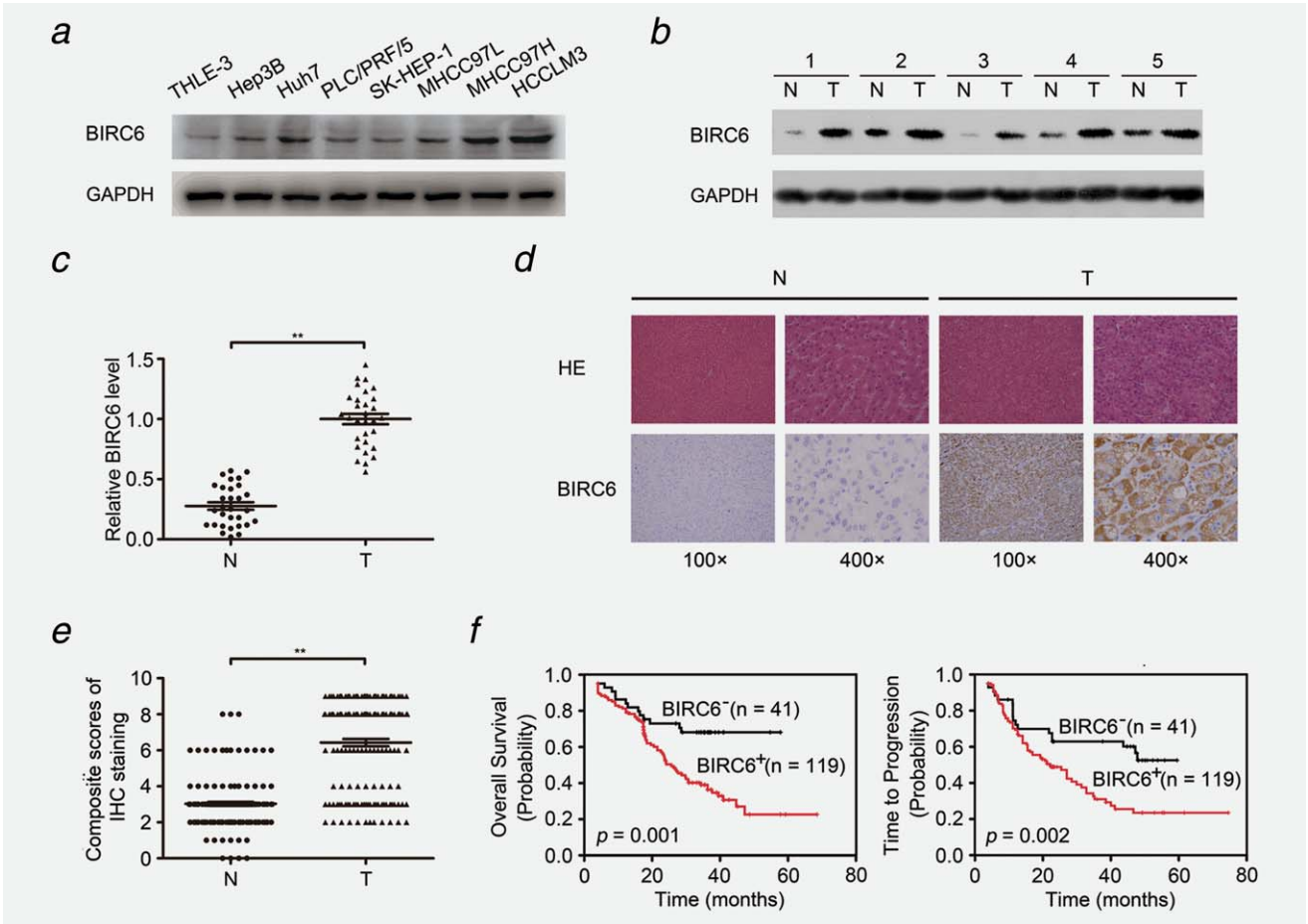


**Figure 1.** Overexpression of BIRC6 was associated with poor survival in hepatocellular carcinoma. (*a*) Expression of BIRC6 in an immortalized normal human liver epithelial cell (THLE-3) and seven hepatoma cell lines. (*b*) Typical patterns of BIRC6 expression in paired liver tissue samples. N, adjacent nontumorous tissues; T, tumor tissues. (*c*) Relative intensity of BIRC6 normalized to GAPDH was calculated in 30 cases. (*d*) Typical patterns of BIRC6 staining in tumor tissues and self-paired adjacent nontumorous liver tissues. (*e*) Scores of immunochemistry staining of BIRC6 in 160 cases. (*f*) Kaplan–Meier analysis of overall survival (OS) and time to progression (TTP) for BIRC6 expression. $^{**}p < 0.01$.

scored. A semiquantitative scoring system was based on both the staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and the percentage of positive cells (0, 0% positive cells; 1, 1–10% positive cells; 2, 11–50% positive cells; 3, >50% positive cells). The final score of each sample was obtained by multiplying the scores of staining intensity and percentage of positive cells. Samples were classified as negative when the final scores were 0–3 and positive when 4–9 (Ref. [22]). The evaluation of staining was carried out by two independent pathologists who were unaware of the patient outcomes.

### Establishment of stable BIRC6-knockdown clones

BIRC6 knockdown stable clones were established *via* lentiviral transduction. A panel of lentiviral particles with different BIRC6 target sequences (TRCN0000041-57-61) and pLKO.1-puro nonmammalian shRNA control transduction particles were purchased from MISSION$^{TM}$ Packing (Sigma Aldrich, St Louis, MO). Huh-7, SK-HEP-1 and MHCC97H cells were seeded at $5 \times 10^5$ cells per well in 6-well plates and allowed to reach 50% confluence on the day of transfection. Cells were transfected at a MOI of 1 for 24 hr, selected in 1 μg/ml puromycin for 1 week and then testified by Western blot analysis.

### Tumor-bearing mouse model and treatment

All animal work was performed in accordance with protocols approved by the Shanghai Medical Experimental Animal Care Commission. Ethical approval was obtained from the Research Ethics Committee of Zhongshan Hospital.

Seventy-two male Balb/c nude mice (4 weeks of age, 12–14 g) from Experimental Animals Center of Shanghai Institute of Life Science (Shanghai, China) were raised under specific pathogen-free conditions. For each cell line, mice were assigned at random to one of the following four groups with 6 mice per group: (*i*) mice of the cell control (CS) group were administered Cremophor EL/ethanol as an oral instillation daily; (*ii*) mice of the CS group were administered sorafenib tosylate; (*iii*) mice of the BIRC6 knockdown (KD) group

Table 1. Multivariate analyses of factors associated with OS and TTP

| | Hazard ratio (95% CI) | *p*-values |
|---|---|---|
| OS | | |
| BCLC stage (A *vs*. B/C) | 1.816 (1.064–3.101) | **0.029** |
| BIRC6 expression in tumor tissue (negative *vs*. positive) | 2.824 (1.528–5.221) | **0.012** |
| TTP[2] | | |
| BCLC stage (A *vs*. B/C) | 1.504 (0.845–2.678) | 0.166 |
| Tumor size, cm ($\leq$5 *vs*. >5) | 1.515 (0.927–2.478) | 0.098 |
| Vascular invasion (no *vs*. yes) | 1.533 (0.987–2.380) | 0.057 |
| BIRC6 expression in tumor tissue (negative vs. positive) | 2.200 (1.266–3.825) | **0.005** |

Variables were adopted for their prognostic significance by univariate analysis ($p < 0.05$).
Bold *p*-values less than 0.05 indicate statistic significance.
Abbreviations: BCLC, Barcelona Clinic Liver Cancer; OS, overall survival; TTP, time to progression.



administered Cremophor EL/ethanol; and (*iv*) mice of the KD group were administered sorafenib tosylate. Tumors were generated by harvesting cells from mid-log phase cultures using trypsin-EDTA. Cells were then pelleted and resuspended in a 50% mixture of Matrigel (BD Biosciences, San Jose, CA) in DMEM to a final cell count of $1 \times 10^8$/ml. A volume of 0.1 ml of the cell suspension was injected in the right flank of each mouse. When all animals in the study had established tumors averaging from 120 to 180 $mm^3$, sorafenib or Cremophor EL/ethanol was used. Sorafenib tosylate was administered gavage once daily for 21 days at dose levels of 30 mg/kg body weight. Tumor sizes were recorded twice a week. Mice were sacrificed at 4 weeks post-injection; tumors were excised and weighed. Tumor volume was calculated by the formula: $0.5 \times L \times W^2$ (L = length of tumor; W = width of tumor).

### Statistical analysis

GraphPad Prism 5.0 software (GraphPad-Prism Software, San Diego, CA) was used for statistical analyses. Categorical variables were compared by $\chi^2$ test. Continuous variables were compared using independent two sample *t*-test. Univariate and multivariate analyses were performed by the Cox proportional hazard model. Survival curves were done by the Kaplan–Meier method (the log-rank test). All data are presented as mean $\pm$ standard error of the mean (SEM) from three independent experiments. Pearson correlation analysis was used to evaluate the association between the expression of BIRC6 and p53. All the tests were two-tailed and a *p*-value $<0.05$ was considered statistically significant.

Additional materials and methods are shown in Supporting Information.

## Results

### Overexpression of BIRC6 in hepatoma cells and HCC tissues

In a panel of hepatoma cell lines, we observed that BIRC6 protein was highly expressed in Hep3B, Huh-7, PLC/PRF/5, SK-HEP-1, MHCC97L, MHCC97H and HCCLM3, compared with an immortalized normal human liver epithelial cell (THLE-3) (Fig. 1*a*). The Western blot data indicated that BIRC6 expression was upregulated in HCC tissues as compared with the paired adjacent normal tissues (Figs. 1*b* and 1*c*). These findings were supported by immunohistochemical analysis. Very faint BIRC6 staining was detected in the normal liver; whereas significant BIRC6 staining was observed in HCC tissues (Fig. 1*d*). The results of semi-quantitative scores of BIRC6 positive staining also indicated that BIRC6 was significantly overexpressed in HCC tissues as compared to paired adjacent normal tissues (Fig. 1*e*).

### Correlation between BIRC6 expression and clinicopathologic features of HCC

The cohort of the subjects was composed of 160 HCC patients. Postoperative cumulative recurrence and survival rates (in brackets) at 1- and 3-years were 41.3% (78.1%) and 55.6% (49.4%), respectively. Eighty nine patients were confirmed as tumor recurrence and 83 patients died at the end of follow-up. Patient clinical characteristics are listed in Supporting Information Table S1. Clinicopathologic analysis showed that BIRC6 overexpression was significantly correlated with vascular invasion ($p = 0.003$). However, the expression failed to correlate to other clinical pathological factors (Supporting Information Table S2).

### Effects of BIRC6 expression on prognosis of HCC

For semi-quantitative scoring analysis, all 160 cases were divided into BIRC6 positive group and negative group. Kaplan–Meier curves analysis showed that HCC patients with positive BIRC6 expression in tumor tissue had a poorer survival and higher risk of recurrence than those with negative expression. BIRC6 expression in tumor tissue was positively correlated to shorter overall survival (OS) and time to progression (TTP) ($p = 0.001$ and $p = 0.002$, respectively) (Fig. 1*f*).

Univariate analysis indicated that elevated BIRC6 protein levels were significantly associated with both OS ($p = 0.001$) and TTP ($p = 0.003$). Moreover, other factor which is indicative of shorter OS was BCLC stage. The factors which are associated with TTP included BCLC stage, tumor size and vascular invasion (Supporting Information Table S3).

To further investigate whether BIRC6 expression functions as a relatively independent factor for prognosis, we performed the multivariate analyses. Only variables that had presented with a significant prognostic value in univariate analyses were included into the multivariate models of survival. Our results showed that BIRC6 expression was significantly correlated with OS ($p = 0.012$) and TTP ($p = 0.005$); and that death and relapse were much more likely to occur in BIRC6-positive patients than in BIRC6-negative ones (Table 1). Therefore, BIRC6 expression in the tumor tissue could be considered as a relatively independent factor in the predicting prognosis of HCC patients.

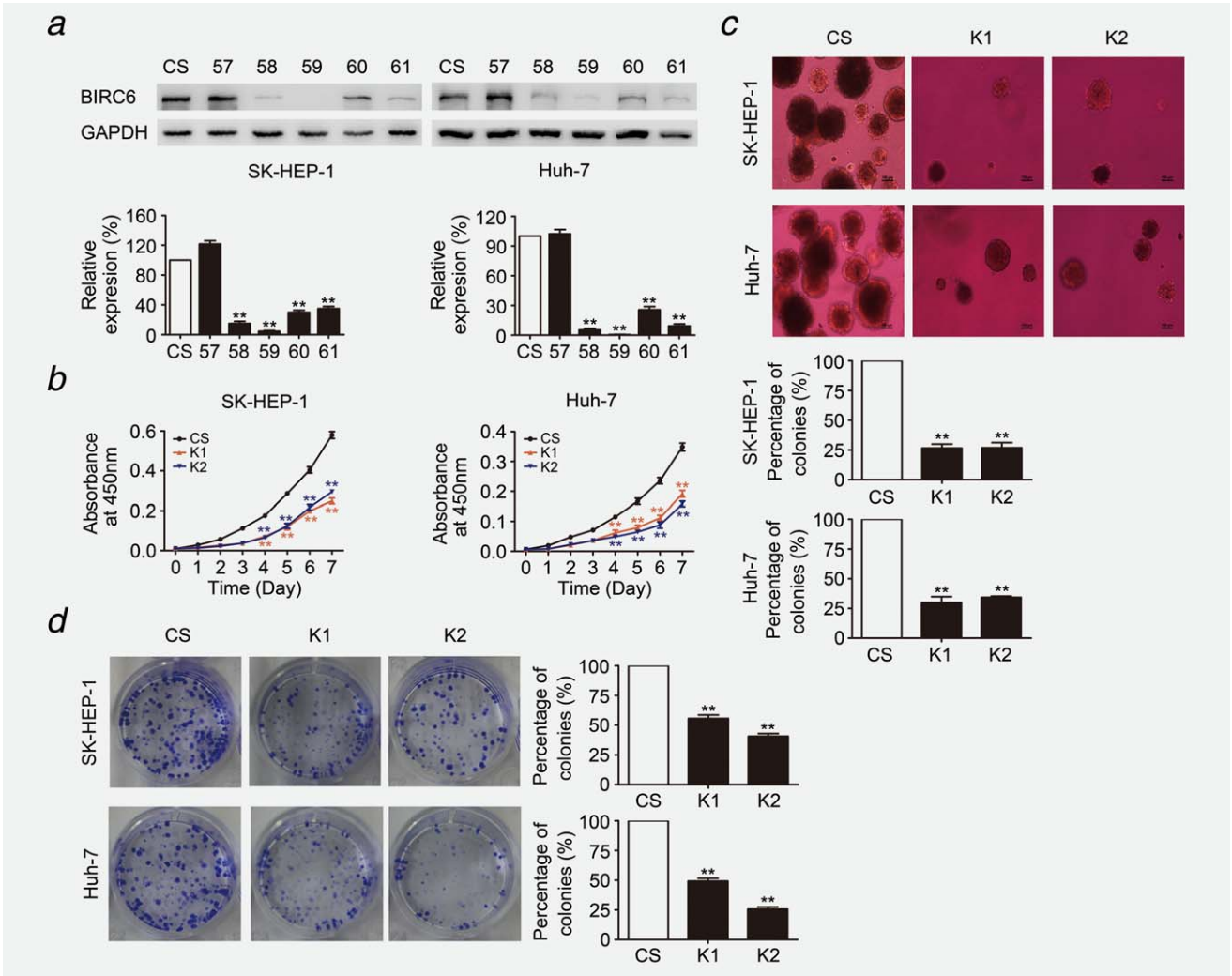


**Figure 2.** BIRC6 knockdown prevented hepatoma cell proliferation, interrupted cell cycle progression and sensitized hepatoma cells to sorafenib-induced apoptosis *in vitro*. (*a*) BIRC6 knockdown efficiency was testified by Western blot. Upper panel: representative Western blot pattern of BIRC6 in the control (CS) with five knockdown clones (57–61). Lower panel: relative intensity of BIRC6 normalized to GAPDH was calculated. (*b*) Cell-growth curves of CS and knockdown K1 and K2 clones. (*c*) Soft agar colony formation assay. Upper panel: typical pattern of colony formation. Lower: relative levels of colonies in knockdown clones normalized to the control. (*d*) Plate colony formation assay. Left panel: typical pattern of colony formation. Right panel: relative levels of colonies in knockdown clones normalized to the control. (*e*) Cell cycle distribution analysis performed by flow cytometry. Left panel: typical pattern of cell cycle assessed by PI staining. Right panel: quantification of the cell cycle distribution in all groups. (*f*) Cell apoptosis analysis performed by flow cytometry in the control and BIRC6 knockdown cells treated with sorafenib (5 μ*M*) for 48 hr or not. Upper panel: typical pattern of apoptosis assessed by Annexin V/PI staining. Lower panel: quantification of the apoptosis levels. *BIRC6 knockdown *versus* the control, $^{**}p < 0.01$.

### The effect of BIRC6 knockdown on hepatoma cell proliferation, cell cycle progression and chemosensitivity

Since the cDNA of BIRC6 extends for 14.5 kb, it is extremely difficult to manipulate the full-length cDNA to overexpress BIRC6 in a cell line. Instead, to examine the function of BIRC6 in HCC, we established stable BIRC6-knockdown clones in three hepatoma cell lines that differed in their status of p53: SK-HEP-1 cells expressing wild-type p53 whereas Huh-7 and MHCC97H cells producing mutant p53. As shown by Western blot analysis, the suppressed BIRC6 expression was confirmed in two BIRC6-knockdown cell lines (58 and 59) of each hepatoma line (Fig. 2*a* and Supporting Information Fig. S1*a*). Therefore, 58 and 59 clones, annotated as K1 and K2 clones, were used in the subsequent assays.

Stably suppressed BIRC6 expression significantly inhibited cell growth in a time-dependent manner *versus* the control (Fig. 2*b* and Supporting Information Fig. S1*b*), which suggested that BIRC6 was required for hepatoma cell proliferation. Anchorage-independent growth is vitally important in the formation of the tumor. In this study, BIRC6 knockdown significantly diminished the capacity of hepatoma cells to form colonies. The suppression of BIRC6 expression resulted in a decrease in colony formation in agar in two knockdown clones (Fig. 2*c* and Supporting Information Fig. S1*c*). Similarly, colony formation in plates was significantly reduced in K1 and K2 clones (Fig. 2*d* and Supporting Information Fig. S1*d*). Cell cycle analysis showed that knockdown of BIRC6 led to a reduced percentage of S phase and an increased

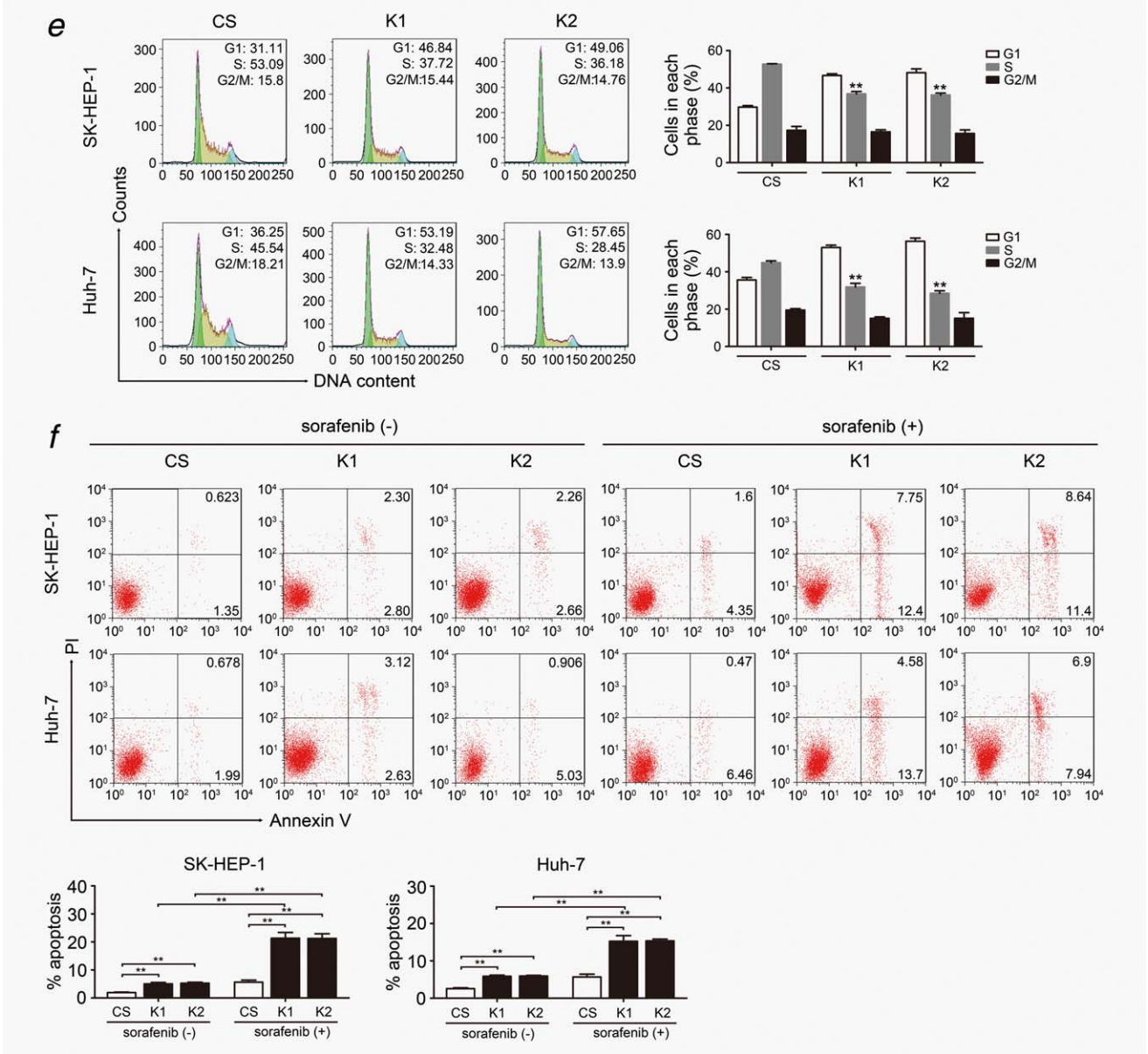


Figure 2. Continued.

percentage of G1 phase as compared to the controls (Fig. 2*e* and Supporting Information Fig. S1*e*). Annexin V/PI dye binding assay revealed that BIRC6 knockdown sensitized hepatoma cells of three lines to sorafenib-induced cell apoptosis (Fig. 2*f* and Supporting Information Fig. S1*f*).

### BIRC6 interacted with p53 and facilitated its degradation by ubiquitination

The findings shown above demonstrated that BIRC6 knockdown gave rise to the proapoptosis and reduced proliferation in hepatoma cells while the underlying mechanisms remained unknown. Given that p53, a critical molecular in regulating tumor proliferation and apoptosis, is closely related to IAPs.[10,11] We determined the p53 expression in these cells. It is evident that BIRC6 knockdown significantly increased the protein levels of endogenous p53, but did not change the mRNA levels of endogenous p53 (Figs. 3*a*, 3*b*, Supporting Information Figs. S2*a*, and S2*b*). Whereas, BIRC6 knockdown did not affect the expression of other antiapoptosis IAPs (Supporting Information Fig. S3). In the presence of proteasome inhibitor MG132, BIRC6 knockdown did not result in any changes in p53 expression between the control and knockdown cells, indicating that BIRC6 knockdown did not affect p53 translation (Fig. 3*c*, Supporting Information Fig. S2*c*). Interestingly, BIRC6 knockdown led to the increased p53 stability in cycloheximide-treated hepatoma cells (Fig. 3*d,* Supporting Information Fig. S2*d*), indicating that BIRC6 could regulate the degradation of p53 rather than synthesis. Since BIRC6 is an UBC enzymes/ubiquitin-ligase, which could ubiquitinate substrates,[23] we speculated that BIRC6

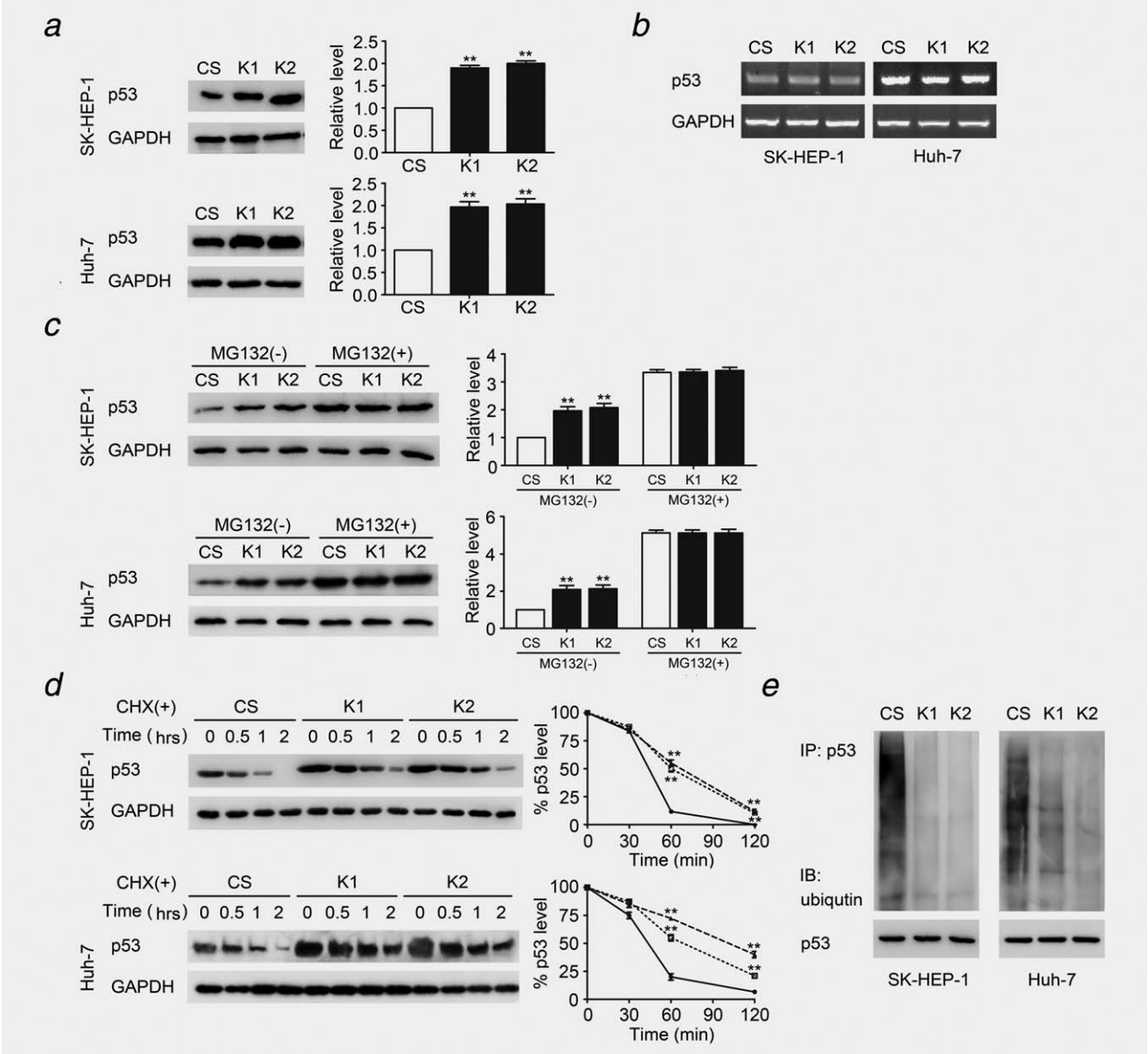


**Figure 3.** BIRC6 interacted with p53 and facilitated its degradation by ubiquitination *in vitro*. (*a*) p53 expression at translation level of hepatoma cells stably expressing BIRC6 knockdown or control. Left panel: typical pattern of Western blot. Right panel: quantification of the p53 protein levels relative to GAPDH. (*b*) p53 expression at transcription level of hepatoma cells by RT-PCR. (*c*) Cells were treated with or without MG132 (20 μ*M*, 12 hr) before harvest. Left panel: typical pattern of immunoblots. Right panel: Relative intensity of p53 protein levels normalized to the control without MG132 treatment. (*d*) Cells were treated with cycloheximide (20 μ*M*), and harvested at indicated time points. Left panel: immunoblots of p53. Right panel: Relative intensity of p53 protein levels. (*e*) Ubiquitination of p53 *in vivo* by BIRC6. Cell lysates were immunoprecipitated with anti-p53 antibody and immunoblotted by antiubiquitin antibody. (*f*) Immunofluorescence of BIRC6 expression in hepatoma cells using specific antibodies against BIRC6 (green) and p53 (red), respectively. (*g*) Hepatoma cells were subjected to coimmunoprecipitation with control IgG, anti-p53 and anti-BIRC6. Loading controls are also indicated. (*h*) Typical immunoblots of important proapoptosis and antiapoptosis participants when hepatoma cells were subjected to sorafenib treatment or not. $^{**}p < 0.01$.

may function to destabilize p53 by ubiquitination. Thus we performed *in vivo* ubiquitination assays and verified that BIRC6 knockdown significantly promoted the degradation of p53 by ubiquitination (Fig. 3*e* and Supporting Information Fig. S2*e*). Furthermore, we determined whether BIRC6 could directly interact with p53. Immunofluorescence experiment showed that BIRC6 and p53 are colocalized in the cytoplasm by confocal microscopy (Fig. 3*f* and Supporting Information Fig. S2*f*). Then we were able to coimmunoprecipitated BIRC6 with p53 in the three hepatoma cell lines regardless of their p53 mutation or deletion (Fig. 3*g* and Supporting Information Fig. S2*g*). Since Mdm2 is an important negative regulator of p53 and regulates p53 degradation by ubiqutination,[24] we investigated whether BIRC6 had influence on Mdm2. Mdm2

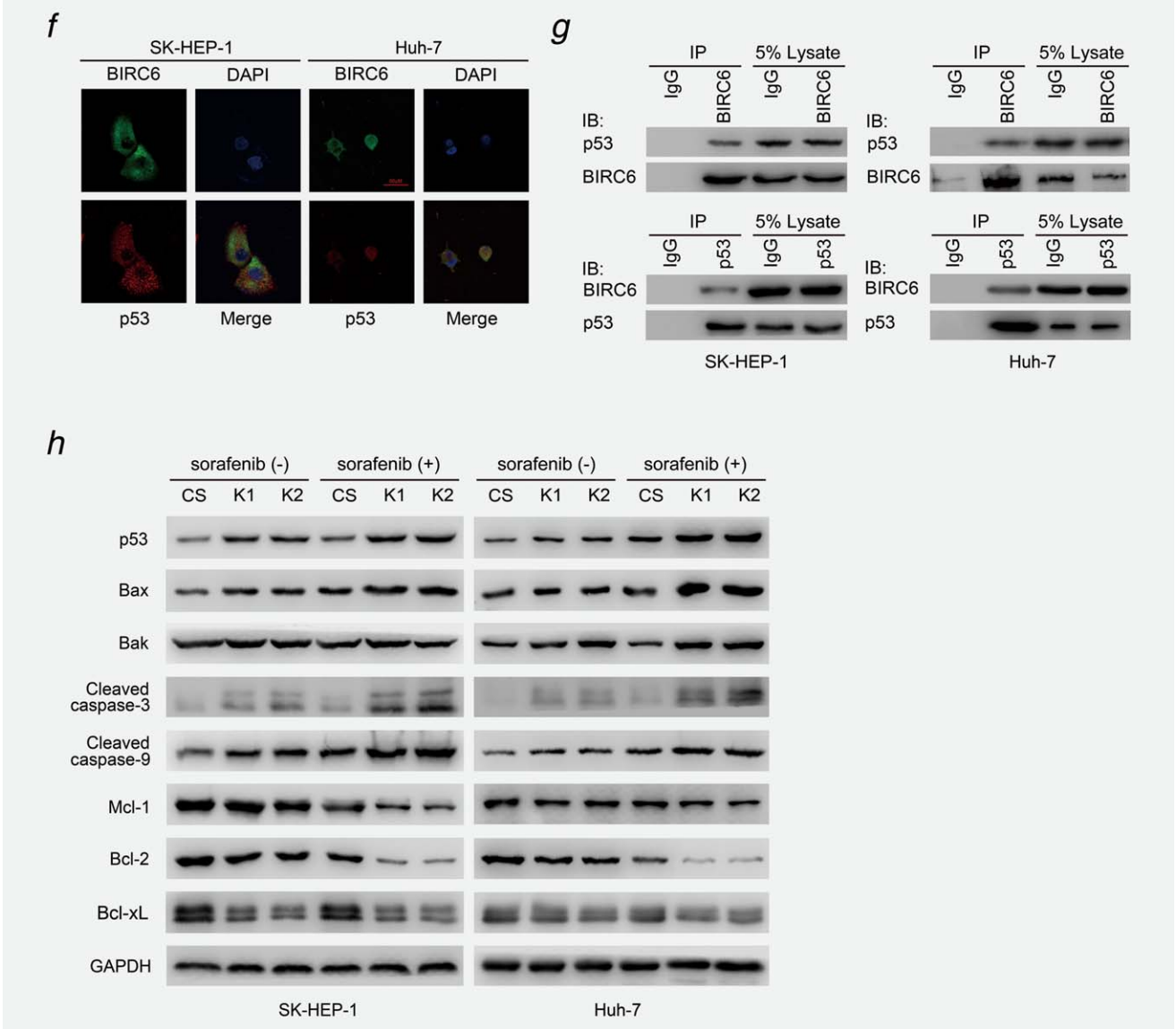


**Figure 3.** continued

expression was obviously decreased in BIRC6 knockdown groups (Supporting Information Fig. S4*a*). We conducted *in vivo* ubiquitination assays with the treatment of Nutlin-3, a small molecule inhibitor of the MDM2/p53 interaction, and found that BIRC6 knockdown still facilitated the degradation of p53 by ubiquitination independent of Mdm2 (Supporting Information Fig. S4b). The present study indicated that BIRC6 mediated p53 ubiquitination was not totally, but may be partially dependent on Mdm2. To study the downstream signal pathway of BIRC6, we assessed the effects of BIRC6 knockdown and sorafenib treatment on the expression levels of cell apoptosis-related proteins in SK-HEP-1 (p53 wild-type) and Huh-7 (p53 mutated) (Fig. 3*h*). The results indicated that BIRC6 knockdown strikingly enhanced the expression of proapoptotic proteins p53 and its downstream proapoptosis signal proteins including Bax,[25] Bak,[26] cleaved caspase-3 (Ref. [27] and −9 (Ref. [27), whereas, the knockdown reduced the expression of p53 downstream prosurvival Bcl-2 family proteins including Bcl-2 (Ref. [25), Mcl-1 (Ref. [28) and Bcl-xL[28] as compared with the control. Moreover, BIRC6 knockdown and sorafenib treatment synergistically enhanced the expression of p53, Bax, cleaved caspase-3 and −9, and attenuated the expression of Mcl-1, Bcl-2. However, the combination therapy had little effect on expression of Bak and Bcl-xL. In addition, we checked BIRC6 expression in the three hepatoma cells with sorafenib treatment and found that sorafenib had no significant effect on BIRC6 expression (Supporting Information Fig. S4*c*).

### p53 inhibition largely rescued the suppression of BIRC6 knockdown on cell proliferation, cell cycle and apoptosis

To further investigate the correlation between BIRC6 and p53, we silenced p53 expression in HCC cells with specific siRNA for p53 (si-p53). Surprisingly, we observed that the

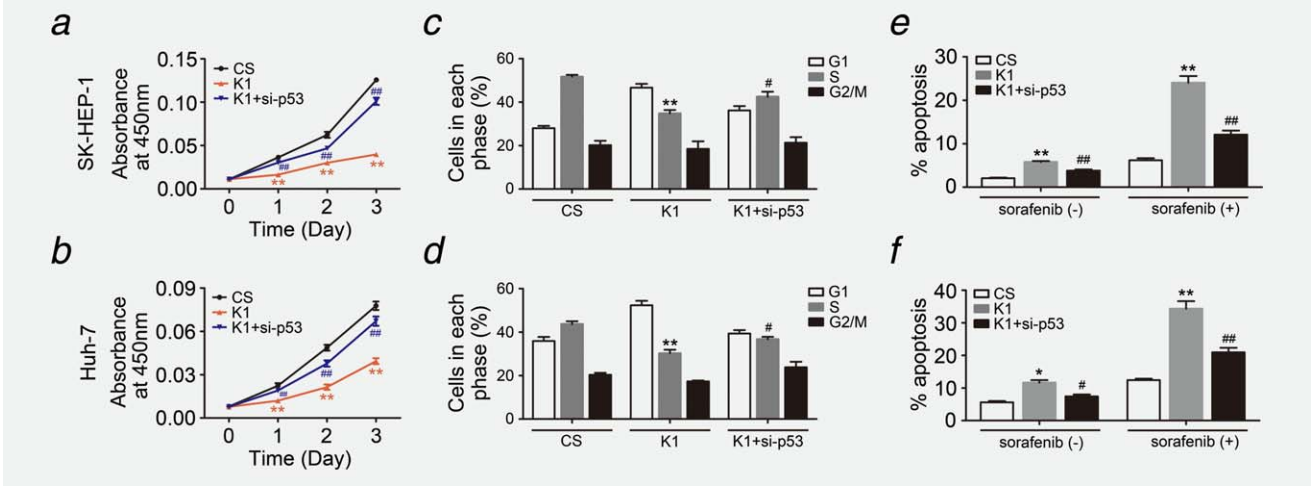


**Figure 4.** p53 inhibition partially rescued the suppression of BIRC6 knockdown on cell proliferation, cell cycle and promotion effect on apoptosis *in vitro*. (*a, b*) p53 inhibition partially rescued the suppression effect of BIRC6 knockdown on cell proliferation in SK-HEP-1 and Huh-7. (*c, d*) p53 inhibition partially rescued the suppression effect of BIRC6 knockdown on cell cycle in SK-HEP-1 and Huh-7. (*e, f*) p53 inhibition partially rescued the promotion effect of BIRC6 knockdown on cell apoptosis in SK-HEP-1 and Huh-7. *BIRC6 knockdown *versus* the control, $*p < 0.05$, $**p < 0.01$; $^{\#}$si-p53 *versus* BIRC6 knockdown, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

suppression effect of BIRC6 knockdown on cell proliferation, cell cycle and promotion effect on apoptosis was largely reversed, indicating that these effects partly depended on p53 (Figs. 4*a*–4*f* and Supporting Information Figs. S5*a*–S5*c*).

### BIRC6 knockdown inhibited xenograft tumor growth and increased the sensitivity of tumor cells to sorafenib therapy in nude mice

The finding that BIRC6 knockdown inhibited cell growth and sensitized hepatoma cells to sorafenib induced apoptosis *in vitro* prompted us to determine whether it exerts a similar effect *in vivo*. Control or BIRC6 stable knockdown clones of three hepatoma cell lines were subcutaneously inoculated into nude mice. Sorafenib tosylate was administered when all animals in the study had established tumors averaging from 120 to 180 $mm^3$. Notably, BIRC6 knockdown significantly inhibited tumor growth, as assessed by tumor volume and weight (Figs. 5*a*–5*c*). Furthermore, combination of BIRC6 knockdown and sorafenib treatment significantly inhibited the tumor growth compared to either BIRC6 knockdown or sorafenib treatment alone (Figs. 5*a*–5*c* and Supporting Information Figs. S6*a*–S6*c*). Histological analysis revealed that the endogenous expression of BIRC6 was apparently decreased in BIRC6 knockdown groups compared to control groups. In accordance with the studies *in vitro*, BIRC6 knockdown increased the level of p53, cleaved caspase-3, and cleaved caspase-9 *in vivo*. In addition, more TUNEL-positive cells were observed in BIRC6 knockdown clones as compared to control groups. Interestingly, as indicated by immunohistochemical staining, less expression of CD31 in BIRC6 knockdown clones demonstrated reduced vessel formation *versus* control groups in SK-HEP-1, Huh-7 and MHCC97H cells. Most importantly, combination of BIRC6 knockdown with sorafenib treatment resulted in a remarkable increase in cellular apoptosis as determined by cleaved caspase-3, cleaved caspase-9 and TUNEL staining, and reduced vessel formation as indicated by CD31 staining (Fig. 5*d* and Supporting Information Fig. S6*d*). To verify the angiogenesis inhibition effect of BIRC6 knockdown and sorafenib, we detected several important angiogenic factors by immunohistochemical staining. The result showed that the expression of VEGF, phosphor-VEGFR-2 and phosphor-PDGFR-β was significantly decreased in BIRC6-knockdown cells and sorafenib-treated cells alone, and the decreased expression was most obvious in BIRC6-knockdown cells with sorafenib treatment, which was in accordance with our previous finding of decreased expression of CD31, suggesting the synergistical inhibitory activity of BIRC6-knockdown and sorafenib on angiogenesis (Supporting Information Fig. S7). Taken together, it appears that increased growth inhibition, angiogenesis inhibition and aggravated apoptosis promotion by BIRC6 knockdown may contributed to enhanced sensitivity to sorafenib therapy *in vivo*.

## Discussion

Recent progresses in elucidation of molecular mechanisms underlying HCC have brought to the clinic the new therapy targets such as Met, vascular endothelial growth factor and so on.[29,30] Transfer of genetic material to target organs, *i.e.*, inhibition of oncogenes by monoclonal antibodies, small molecules and antisense drugs, has a positive impact in therapy for unresectable cancer. One example is the approval of sorafenib in the treatment of advanced HCC, which is an oral multikinase inhibitor of the vascular endothelial growth factor receptor and Ras kinase.[31]

BIRC6 is currently attracting considerable attention as a new target for anticancer therapies.[14,19,32] BIRC6 has been

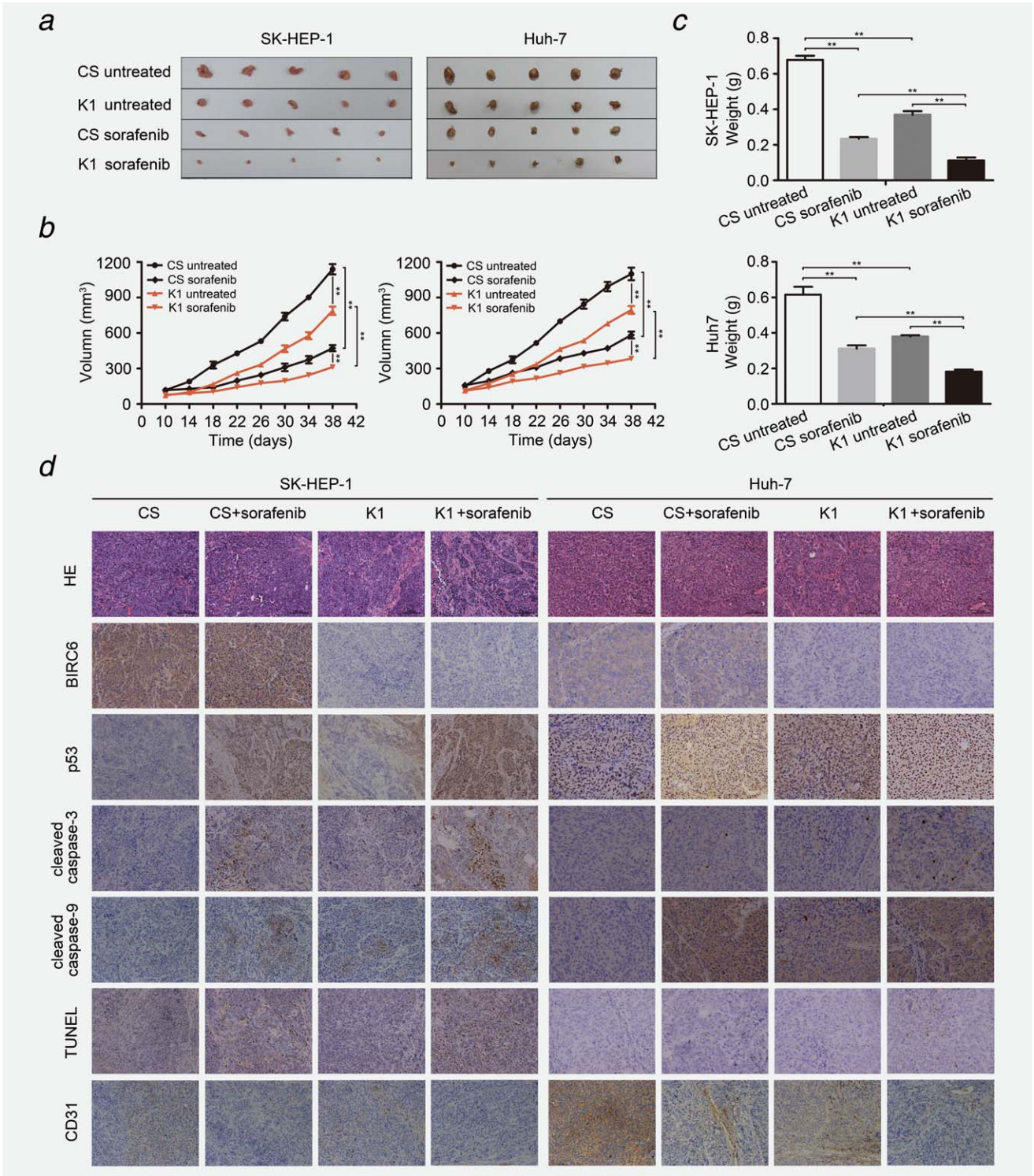


**Figure 5.** BIRC6 knockdown inhibited xenograft tumor growth and increased the sensitivity of tumor cells to sorafenib therapy in nude mice injected with SK-HEP-1 and Huh-7 cells. (*a*) Representative images of harvested tumors of each group. (*b*) *In vivo* subcutaneous tumor growth curves. (*c*) Total tumor weight of each group of mice. (*d*) Histological analysis of each group including eosin staining, BIRC6, p53, cleaved caspase-3, cleaved caspase-9, CD31 and TUNEL staining in tumor-bearing mouse models. $^{**}p < 0.01$.

reported to correlate with a variety of cancers. High BIRC6-expressing cells exhibit resistance to apoptosis, and the knockdown of BIRC6 sensitized the cells to chemotherapy-induced apoptosis and suppressed cell growth in glioma, breast cancer, colon cancer and nonsmall-cell lung cancer.[3,10,13,16] cDNA microarray and qRT-PCR analysis has demonstrated that BIRC6 is one of the most commonly up-regulated genes in human colorectal cancer,[14] which was in accordance with the result of proteomics analysis of colon cancer stem cells and differentiated tumor cells.[13] Furthermore, overexpression of BIRC6 correlates with poor prognosis in childhood *de novo* acute myeloid leukemia,[12] nonsmall-cell lung cancers[16] and ovarian cancer.[17] In our previous study, BIRC6 was identified as a novel HCC biomarker by proteomic profiling of serum from HCC patients and healthy patients.[21] In the present work, for the first time, we investigated the role of BIRC6 in HCC carcinogenesis *in vitro* and *in vivo*. We observed that overexpression of BIRC6 in the tumor tissue correlated with a poor survival and high risk of HCC recurrence. On the basis of these observations, we hypothesized that BIRC6 plays a potential role in HCC progression and further explored the underlying molecular mechanisms.

Escape of apoptosis is one of critical mechanisms for cancer cell to survive through (*i*) disrupted balance of proapoptotic and antiapoptotic proteins; (*ii*) reduced caspase activation, and (*iii*) impaired death receptor signaling.[33] Several pivotal genes in regulating apoptosis have been identified. The p53 protein, one of the best known tumor suppressor proteins, plays a key role in cell cycle regulation, development, differentiation, DNA recombination, chromosomal segregation and cellular senescence, stimulates apoptosis and suppresses tumorigenesis.[34] Under normal conditions, p53 is a short-lived protein with low levels of abundance and transcriptional activity in cells; however, upon external and internal stress, p53 is up-regulated by stabilization or transcriptional activation and accumulates in the nucleus to activate gene expression and promote apoptosis.[35] Accordingly, IAPs, endogenous inhibitors of capases promoting degradation of active caspases, are important antiapoptotic proteins.[7] As a member of IAPs, BIRC6 was an upstream regulator of p53, and BIRC6 silence initiates the mitochondrial pathway of apoptosis.[11] Given that p53 is a critical factor controlling the balance between carcinogenesis and apoptosis, we chose three hepatoma cell lines with differed p53 status, *i.e.*, SK-HEP-1(wild-type p53),[36] Huh-7 (Y220C),[36] MHCC97H (R249S)[37] and suppressed BIRC6 expression by an RNA interference approach in these cells in order to delineate the role and mechanism of BIRC6 in oncogenicity. The results showed that BIRC6 knockdown significantly inhibited proliferation, altered cell cycle distribution and promoted sorafenib-induced apoptosis *in vitro,* which could be partly reversed by p53 interference. Then the *in vivo* assay further demonstrated that BIRC6 knockdown suppressed tumor formation and promoted sorafenib-induced apoptosis in xenograft mice model.

In addition, we found that p53 expression was significantly increased in the stable BIRC6 knockdown cells as compared to the control regardless of its mutations. We further verified that BIRC6 negatively regulated p53 protein levels by facilitating its degradation *via* ubiquitination. BIRC6 colocalized and interacted with p53 in the cytoplasm. Notably, the inhibition effect of BIRC6 knockdown on cell proliferation and cell cycle, and the promotion effect on apoptosis was largely rescued by p53 interference, indicating the effect of BIRC6 on carcinogenesis was partially dependent on p53. Moreover, our evidence demonstrated that BIRC6 knockdown inhibited carcinogenesis and promoted apoptosis in the three HCC cell lines with differed p53 status. Regarding why BIRC6 knockdown still has biologic effects in HCC cells with mutated p53, we speculate that the following two scenarios may contribute to this effect. On the one hand, the effect caused by BIRC6 knockdown might be related to p53 functional status rather than p53 genotype change. In hepatoma cells, the mutated p53 still exhibits its transactivation activity as indicated by fact that the tumor suppressor p53 in Huh-7 cells is functionally intact despite the Y220C mutation.[38] Huh-7 cells are known to maintain an effective p53 transactivation activity comparable to that of Chang liver cells and HepG2 cells, both of which contain wild-type p53.[39,40] On the other hand, besides transcription activities the mutant p53 also executes nontranscription activities.[41] Mutant p53 still caused growth arrest and mediated apoptosis independent of the transcriptional activation function.[42,43] It has been reported that the p53-mediated apoptosis could occur in the presence of p53-DNA binding inhibitors, like PIASγ, suggesting that sequence-specific transactivation is not essential for p53-dependent apoptosis.[44] In our work, both wild-type p53 and mutant p53 showed antiproliferation and proapoptotic effect after BIRC6 knockdown. A similar study by Lu et al.[45] has demonstrated that knockdown of mortalin, a member of the heat shock protein 70 family, promoted apoptosis in MHCC97H and other p53 mutant cells, by activation of mutant p53 functions, independent of the wild type p53 transactivation. Therefore, we assumed that mutant p53 may have an intact transactivation activity, or be still functional even if the transcriptional activation ability was disturbed, which needs further study. This is molecularly significant due to the fact that more than half of the cancers possess mutant p53; and the maintenance of antiproliferation and proapoptotic effect of mutant p53 in these cancer cells[42,43] may well explain why BIRC6 knockdown still has biologic effects in HCC cells with mutated p53.

Recently, Lopergolo et al.,[10] put forward that BIRC6 knockdown stabilized only wild-type p53, but not mutant p53 (R280K) in MDA-MB-231, which inspired us to ascertain whether p53-BIRC6 interaction differed in various p53 mutations. We conducted coimmunoprecipitat assay in MDA-MB-231 cells. Unexpectedly, BIRC6 did not interact with the mutant p53 (R280K) (Supporting Information Fig. S8*a*). To verify our founding, we exogenously expressed

mutant p53 (R280K mutation) in Hep3B (p53 null) cells and performed coimmunoprecipitat assay (Supporting Information Fig. S8*b*). The result showed that there was still no significant interaction between BIRC6 and p53 in the Hep3B cells (Supporting Information Fig. S8*c*). Moreover, to explore why different mutations (R249S, Y220C and R280K) exerted diverse effect on the interaction between p53 and BIRC6, we constructed a computational model of p53-BIRC6 complex to analyze the interaction of p53 variants with BIRC6 and the binding affinity. The result showed that the Y220C and R249S mutation sites are far always from complex interface and can only address quite modest effects on p53 structure profile; thus, they could be recognized as neutral mutations that do not influence p53-BIRC6 interaction substantially (Supporting Information Figs. S9*b* and S9*c*). In contrast, the R280K mutation in p53 DNA-recognizing helix is located nearby the interface that may impose direct or indirect effect on the interaction, causing a large motion of its vicinal loop 114–123 away from the helix (Supporting Information Fig. S9*d*). Consequently, an obvious region of atomic collusions around the loop 114–123 is emerged, largely destabilizing the p53-BIRC6 system. In addition, the binding free energies of neutral Y220C and R249S variants to BIRC6 were estimated to be −29.1 and −23.9 kcal/mol, respectively, which are very close to that (−26.7 kcal/mol) of wild-type p53 interaction with BIRC6. However, the destabilized mutation R280K causes a considerable decrease in system's affinity, that is, $\Delta G$ changes from −26.7 to −15.2 kcal/mol upon this mutation. Therefore, the various structures of p53 mutants and corresponding binding affinity may explain the differences of p53 variants with BIRC6. Moreover, we analyzed the p53 status of the 160 tumor tissues by single strand conformational polymorphism-polymerase chain reaction (SSCP-PCR) and direct sequencing analysis. Of the 160 cases with HCC, 117 (73.1%) were found to have point mutations in exons 5 to 8 of the p53 gene of the tumor samples. Most of the mutations were the mutation of codon 249 (Arg → Ser, 47%, Group 1) and 220 (Tyr → Cys, 23%, Group 2) (Supporting Information Table S4). We further investigated the correlation between BIRC6 and p53 expression in tumor tissues by immunochemistry staining in Group 1 and 2 cases. The immunochemistry staining showed strong expression of BIRC6 and faint staining of p53 in tumor tissues (Supporting Information Fig. S10*a*). Pearson correlation analysis verified that BIRC6 expression levels negatively correlated with p53 expression ($R = -0.433$, $p = 0.001$ in Group 1 and $R = -0.512$, $p = 0.006$ in Group 2, Supporting Information Fig. S10*b*).

Our *in vitro* studies demonstrated that knockdown of BIRC6 is accompanied with up-regulation of the tumor suppressor p53 and activation of apoptosis through p53-mediated mitochondrial apoptotic pathways,[27] which includes up-regulation of proapoptotic factors such as Bax and Bak, down-regulation of Mcl-1, Bcl-2 and Bcl-xL, and activation of caspase-9 and caspase-3. In addition, knockdown of BIRC6 sensitized hepatoma cells to sorafenib-induced apoptosis *in vitro* and *in vivo*. Sorafenib, a multikinase inhibitor frequently used for treating advanced HCC patients, blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in HCC.[46] Interestingly, combination of BIRC6 knockdown and sorafenib treatment synergistically changed the levels of Bcl-2 family protein, including up-regulation of Bax and down-regulation of Mcl-1, Bcl-2 *in vitro*. Moreover, combination of BIRC6 knockdown and sorafenib treatment synergistically activated caspase-3 and 9 and induced apoptosis *in vitro* and *in vivo*. Indeed, the combination of BIRC6 knockdown and sorafenib treatment resulted in significant suppression on tumor growth and vessel formation in nude mouse as compared to BIRC6 knockdown and sorafenib treatment alone. These data strongly suggests a additive effect of BIRC6 knockdown-mediated mitochondria apoptosis pathway activation and sorafenib treatment-mediated RAF/MEK/ERK pathway inhibition on HCC carcinogenesis.

In summary, our study highlights that BIRC6 exerts multiple effect, *i.e.*, modulation of cell proliferation, cell cycle and apoptosis on HCC carcinogenesis, which is highly dependent on p53. p53 activation by BIRC6 knockdown and RAF/MEK/ERK pathway inhibition by sorafenib treatment could produce additive therapy effect. Therefore, BIRC6 may have therapeutic potential in the treatment of human HCC.

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