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ORIGINAL ARTICLE

Transcriptional regulation of NDUFA4L2 by NFIB induces sorafenib resistance by decreasing reactive oxygen species in hepatocellular carcinoma

Li Zhou[1] | Lin-Hong Mao[1,2] | Xia Li[1] | Qing-Liang Wang[3] | Si-Yuan Chen[1] | Zhi-Ji Chen[1] | Jing Lei[1] | Hong-Tao Liu[1] | Si-Qi Liao[1] | Tao Ran[1] | Xiao-Qin Li[1] | Zhi-Hang Zhou[1] | Song He[1]

[1]Department of Gastroenterology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

[2]Department of Gastroenterology, Chengdu Second People's Hospital, Sichuan, China

[3]Department of Pathology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

Correspondence
Zhi-Hang Zhou and Song He, Department of Gastroenterology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China.
Email: zhouzhihang@cqmu.edu.cn and hedoctor65@cqmu.edu.cn

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Abstract

Sorafenib is one a first-line therapeutic drugs for advanced hepatocellular carcinoma (HCC). However, only 30% of patients benefit from sorafenib due to drug resistance. We and other groups have revealed that nuclear factor I B (NFIB) regulates liver regeneration and carcinogenesis, but its role in drug resistance is poorly known. We found that NFIB was more upregulated in sorafenib-resistant SMMC-7721 cells compared to parental cells. NFIB knockdown not only sensitized drug-resistant cells to sorafenib but also inhibited the proliferation and invasion of these cells. Meanwhile, NFIB promoted the proliferation and invasion of HCC cells in vitro and facilitated tumor growth and metastasis in vivo. Knocking down NFIB synergetically inhibited tumor growth with sorafenib. Mechanically, gene expression profiling and subsequent verification experiments proved that NFIB could bind with the promoter region of a complex I inhibitor NDUFA4L2 and promote its transcription. Transcriptional upregulation of NDUFA4L2 by NFIB could thus inhibit the sorafenib-induced reactive oxygen species accumulation. Finally, we found that NFIB was highly expressed in HCC tissues, and high NFIB expression level was associated with macrovascular invasion, advanced tumor stage, and poor prognosis of HCC patients ($n = 156$). In summary, we demonstrated that NFIB could transcriptionally upregulate NDUFA4L2 to enhance both intrinsic and acquired sorafenib resistance of HCC cells by reducing reactive oxygen species induction.

KEYWORDS
HCC, NDUFA4L2, NFIB, ROS, sorafenib

Abbreviations: ChIP, chromatin immunoprecipitation; **ChIP-Seq**, chromatin immunoprecipitation sequencing; **DEGs**, differentially expressed genes; **ETC**, electron transport chain; **H&E**, hematoxylin and eosin; **HCC**, hepatocellular carcinoma; **IC50**, 50% inhibitory concentration; **IHC**, immunohistochemical staining; **NAC**, N-acetyl-L-cysteine; **NDUFA4L2**, NADH dehydrogenase 1 alpha subcomplex subunit 4-like 2; **NFIB**, nuclear factor I B; **ROS**, reactive oxygen species.

Li Zhou and Lin-Hong Mao contributed equally to this work.

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth most common reason for cancer-related death worldwide, causing nearly 745,000 deaths yearly.[1] Higher rates of HCC are observed in South-Eastern Asia and Africa. Although HCC diagnosis and treatment technology have continued to improve in recent decades, the survival time of HCC patients remains among the shortest for cancers.[2] This fact is largely due to the complicated pathogenesis. In addition, many HCC patients are unable to undergo surgery because of the advanced stage of diagnosis. Sorafenib is among the first-line therapies for advanced HCC.[3] It is a multi-kinase inhibitor, exerting both anti-angiogenesis and anti-proliferative effects by inhibiting RAF kinase, vascular endothelial growth factor receptor, and platelet-derived growth factor receptor-β tyrosine kinase.[4] Sorafenib can only extend the survival period of patients by 2–3 months. Intrinsic or acquired drug resistance is the main reason for treatment failure. The objective remission rate is only approximately 20%, and acquired resistance occurs soon after the initial response.[4] At present, the resistance mechanism of sorafenib is not fully understood, including the activation of EGFR and c-Jun, the activation of PI3K/AKT signaling pathway, and the increase of tumor cell stemness.[5] Therefore, it is essential to further elucidate the resistance mechanism of sorafenib.

Nuclear factor I (NFI) family members play important roles in DNA replication and gene expression.[6] The family consists of four genes (NFIA, NFIB, NFIC, and NFIX) encoding proteins that interact with DNA as homologous dimers or heterodimers. It has been reported that NFIB could promote liver cell regeneration[7] and also maintain the skin

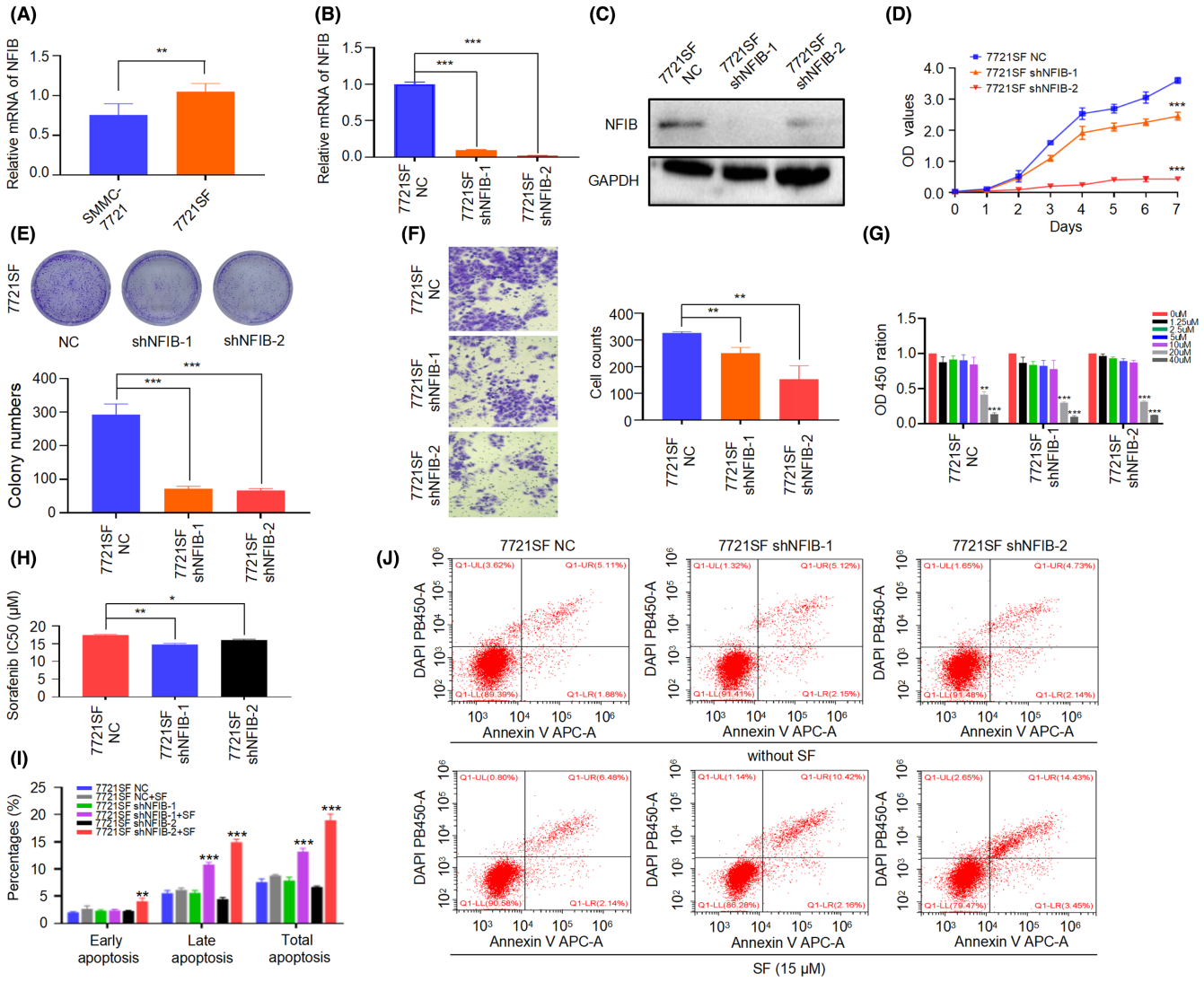

FIGURE 1 NFIB enhances proliferation, invasion,n and resistance to sorafenib-resistant HCC cells. (A) qRT-PCR was used to detect the expression of NFIB in sorafenib-resistant SMMC-7721 cells (7721SF) and SMMC-7721 cells. (B, C) Knockdown of NIFB in 7721SF cells was validated in mRNA and protein level. (D–F) Cell proliferation, colony formation and cell invasion were inhibited in 7721SF after knocking down NFIB. (G) Knocking down NFIB dramatically decreases cell viability after sorafenib treatment. (H) Knocking down NFIB dramatically decreases the IC50 of sorafenib to 7721SF cells. (I, J) Knocking down NFIB significantly increases sorafenib-induced cell apoptosis in 7721SF cells. NC, normal control; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

stem cell fate.[8] The role of NFIB in tumors is tissue specific. On the one hand, NFIB promoted the proliferation of triple-negative breast cancer, myeloproliferative tumors, colorectal cancer, bladder cancer cells, and laryngeal squamous cell carcinoma but inhibited cell apoptosis.[9,10] NFIB was also reported to reduce the apoptosis of HepG2 cells.[11] On the other hand, it could inhibit malignant phenotype of osteosarcoma, cutaneous squamous cell carcinoma, and lung adenocarcinoma.[9] NFIB was also relevant to chemotherapy sensitivity of tumors. It could not only reduce the sensitivity of p53-mutated triple-negative breast cancer cells and ovarian cancer cells to paclitaxel[12,13] but also decrease the sensitivity of colorectal cancer cells to 5-fluorouracil and oxaliplatin.[14,15] Our recent study showed that hepatocyte-specific knockout of NFIB facilitated the occurrence of hepatocellular carcinoma.[16] Further study is needed to reveal the role of NFIB in HCC drug resistance.

NADH dehydrogenase 1 alpha subcomplex subunit 4-like 2 (NDUFA4L2) was located in the mitochondria and was an inhibitory subunit of the electron transport chain (ETC) complex I.[17] Complex I was the first step of oxidative phosphorylation and the main site of reactive oxygen species (ROS) generation.[18,19] Therefore, NDUFA4L2 could inhibit ROS production.[20] Studies have reported that NDUFA4L2 was highly expressed in non-small cell lung cancer,[20] human chondrosarcoma cells,[21] renal clear cell carcinoma,[22] colorectal cancer,[23] HCC,[18,24] and glioblastoma.[25] Accordingly, high NDUFA4L2 expression is closely related with the poor prognosis of these cancer patients,[20] and NDUFA4L2 is a novel significant stage-specific differentially expressed genes in HCC.[24] Recent studies showed that NDUFA4L2 renders breast cancer cells resistant to trastuzumab by suppressing ROS production,[26] promotes osteosarcoma cell metastasis,[27] and augments glioma proliferation.[25]

In the present study, we found that transcription factor NFIB was highly expressed in sorafenib-resistant HCC cells compared to parental cells, and knockdown of NFIB reduced sorafenib resistance,

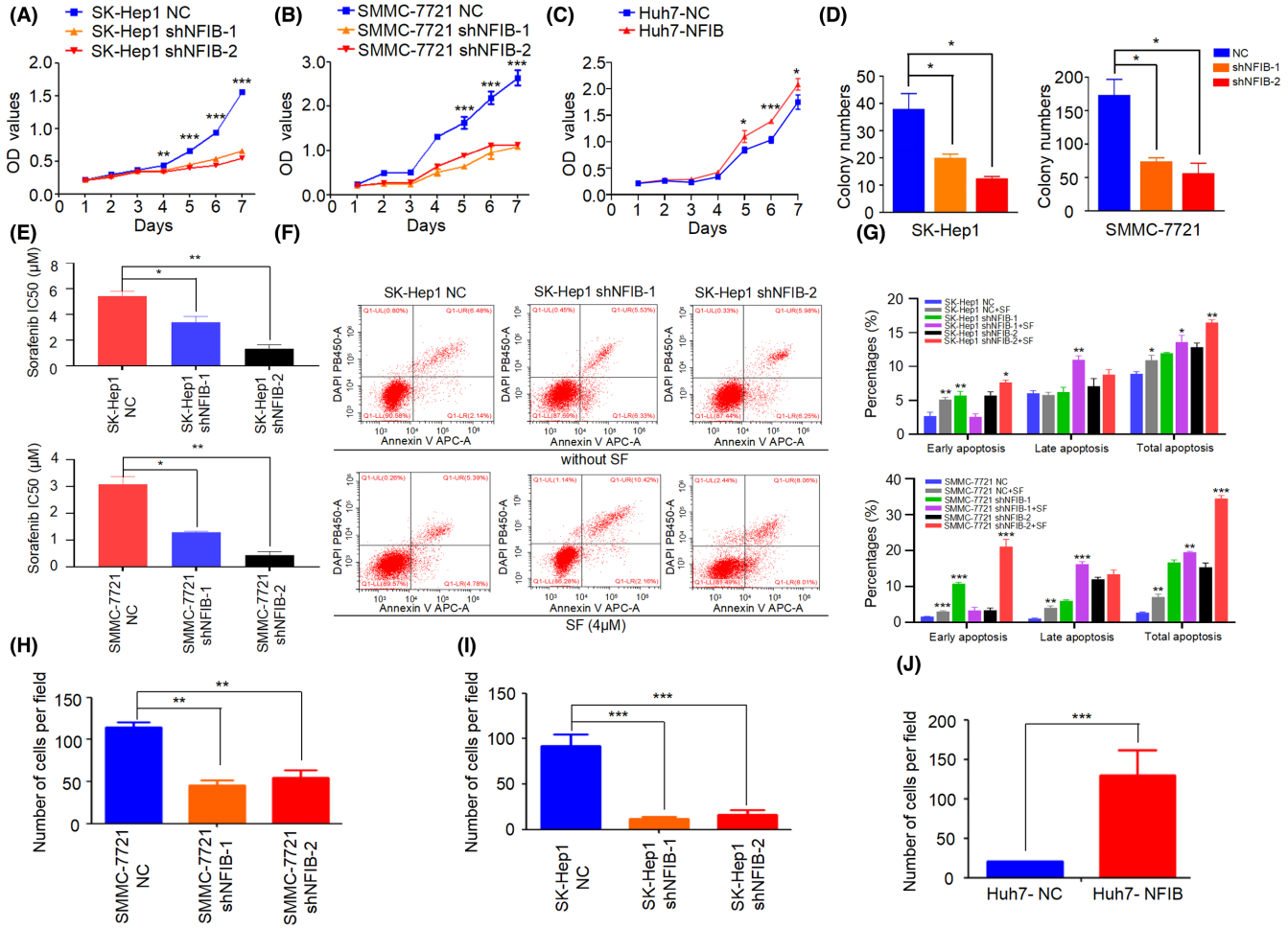

FIGURE 2 NFIB enhances resistance to sorafenib and proliferation of HCC cells. (A) Knockdown of NFIB in SK-Hep1 cells significantly abrogates cell proliferation. (B) Knockdown of NFIB in SMMC-7721 cells significantly abrogates cell proliferation. (C) Overexpression of NFIB promotes Huh7 cell proliferation. (D) Knockdown of NFIB inhibits colony formation in both SK-Hep1 and SMMC-7721 cells. (E) Knocking down NFIB dramatically decreases the IC50 of sorafenib to SK-Hep1 and SMMC-7721 cells. (F) Flow cytometry analysis of the apoptosis of SK-Hep1 cells with NFIB or with NFIB knockdown before and after sorafenib exposure. (G) The statistical results of the cell apoptosis assay. Knocking down NFIB significantly increases sorafenib-induced cell apoptosis in SK-Hep1 (upper panel) and SMMC-7721 (lower panel) cells. Knocking down NFIB abrogates cell invasion in SMMC-7721 (H) and SK-Hep1 (I) cells while overexpressing NFIB augments cell invasion (J). NC, normal control; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

proliferation, and invasion of those cells. Additionally, NFIB promoted in vivo tumor growth and metastasis of HCC cells. To further study the mechanism by which NFIB regulated drug resistance, gene expression profiling was performed on HCC cell lines after NFIB knockdown. The results showed that ROS inhibitory factor NDUFA4L2 expression was significantly increased. We revealed that NFIB could bind with the promoter region of NDUFA4L2 and promote its transcription. Transcriptional upregulation of NDUFA4L2 by NFIB could, thus, inhibit the sorafenib-induced ROS accumulation. Finally, the clinical significance of NFIB was elucidated in clinical samples. High expression of NFIB was associated with macrovascular invasion, advanced cancer stage, and poor prognosis of HCC patients. Our results implied that NFIB might be a novel target to predict response to sorafenib or overcome sorafenib resistance.

2 | MATERIALS AND METHODS

Detailed Materials and Methods are shown in Appendix S1.

3 | RESULTS

3.1 | NFIB was upregulated in sorafenib-resistant hepatocellular carcinoma cells to render the resistance

We first detected the expression of NFIB in sorafenib-resistant SMMC-7721 cells (7721SF) and the parental SMMC-7721 cells. The expression of NFIB in 7721SF increased in mRNA level

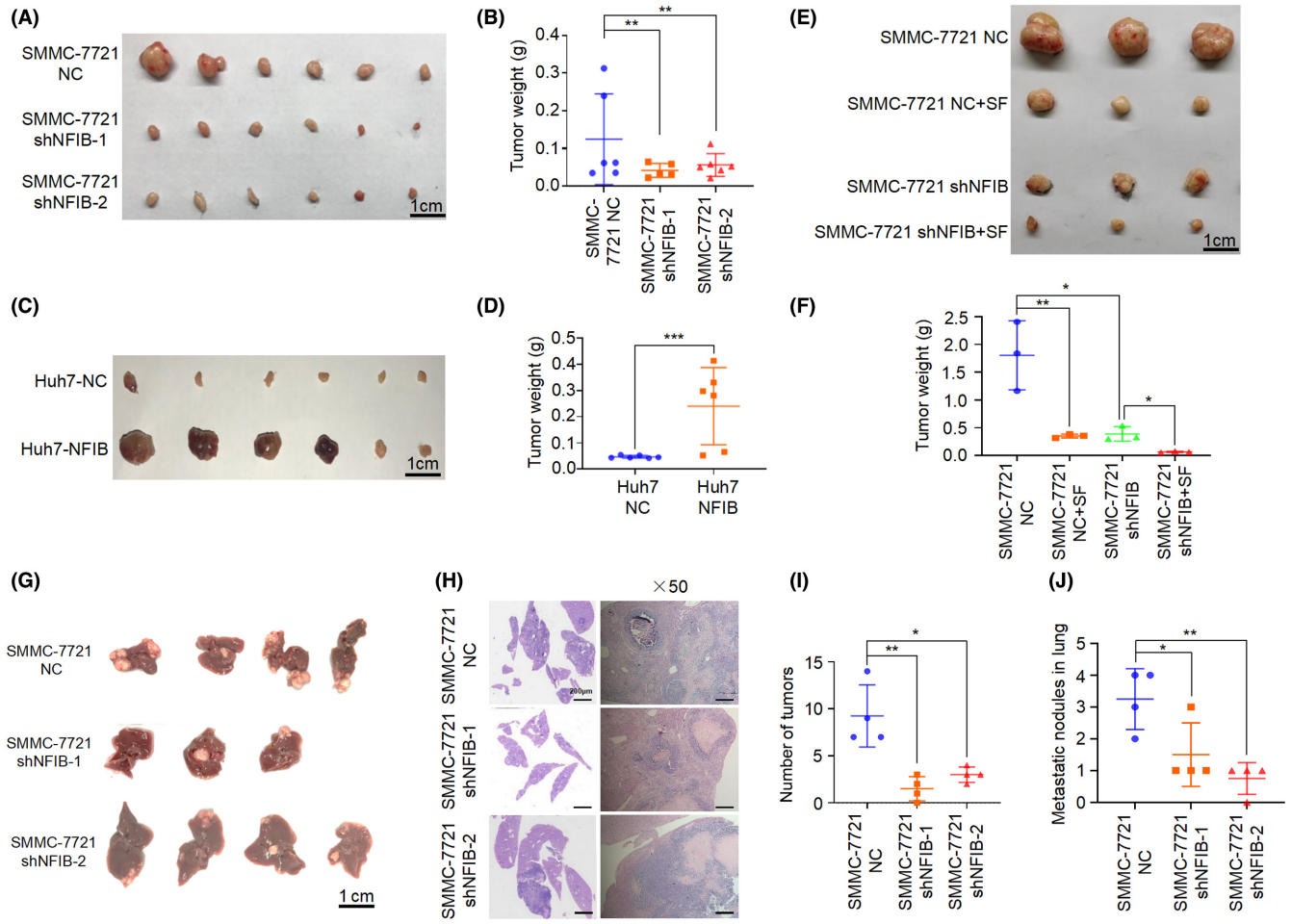

FIGURE 3 NFIB promotes tumor growth and metastasis and reduces sensitivity to sorafenib of HCC cells in vivo. (A) Gross picture of subcutaneous transplants originating from NFIB-knockdown SMMC-7721 cells and control cells. (B) Tumor weight originating from NFIB-knockdown SMMC-7721 cells is significantly lower than control. (C) Gross picture of subcutaneous transplants originating from NFIB-overexpression Huh7 cells and control cells. (D) Tumor weight originating from NFIB-overexpression Huh7 cells is significantly larger than that of control. (E) Gross picture of subcutaneous transplants originating from NFIB-knockdown SMMC-7721 cells and control cells with or without sorafenib treatment. (F) The inhibitory effect of sorafenib on tumor weight are augmented in NFIB-knockdown SMMC-7721 cells in comparison to the control group. (G) Gross picture of orthotopic transplants originating from NFIB-knockdown SMMC-7721 cells and control cells. (H) H&E staining of liver section to show the primary tumors in the liver. (I) The number of tumor nodules in the liver is reduced in NFIB-knockdown SMMC-7721 cells. (J) The number of metastatic foci in the lung is significantly reduced after NFIB knockdown. NC, normal control; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

(Figure 1A). Then, we used lentiviruses to knock down NFIB to reveal its function. Knockdown efficiency of NFIB was verified by qRT-PCR and western blot experiments (Figure 1B,C). Specific human primers were synthesized by Sangon Biotech (listed in Table S1). CCK-8 assay (Figure 1D), colony formation (Figure 1E), and transwell assay (Figure 1F) showed that compared with the control group, the proliferation, colony formation and invasion ability of NFIB-knockdown cells were significantly reduced. Moreover, knocking down NFIB increased the toxicity of sorafenib to the resistant cells (Figure 1G), which was further reflected by decreased IC50 (Figure 1H). Knocking down NFIB also increased sorafenib-induced apoptosis (Figure 1I,J). Taken together, we found that NFIB was upregulated in sorafenib-resistant HCC cells to render the resistance.

3.2 | NFIB increases both intrinsic and acquired resistance to sorafenib of hepatocellular carcinoma cells

After revealing the role of NFIB in acquired sorafenib resistance, we further explored the function of NFIB in intrinsic resistance and other malignant behaviors. We examined the expression of NFIB in HCC cells, including HepG2, SK-Hep1, SMMC-7721, Huh7, PLC/PRF/5, and MHCC-97H cells. The qRT-PCR and western blot analysis revealed significantly higher NFIB expression in Sk-Hep1 and SMMC-7721 cells and lower expression in HepG2 and Huh7 cells (Figure S1A,B). NFIB was then overexpressed in Huh7 cells (Figure S1C,D) and depleted in Sk-Hep1 and SMMC-7721 cells (Figure S1E-H). We found that knockdown of NFIB significantly

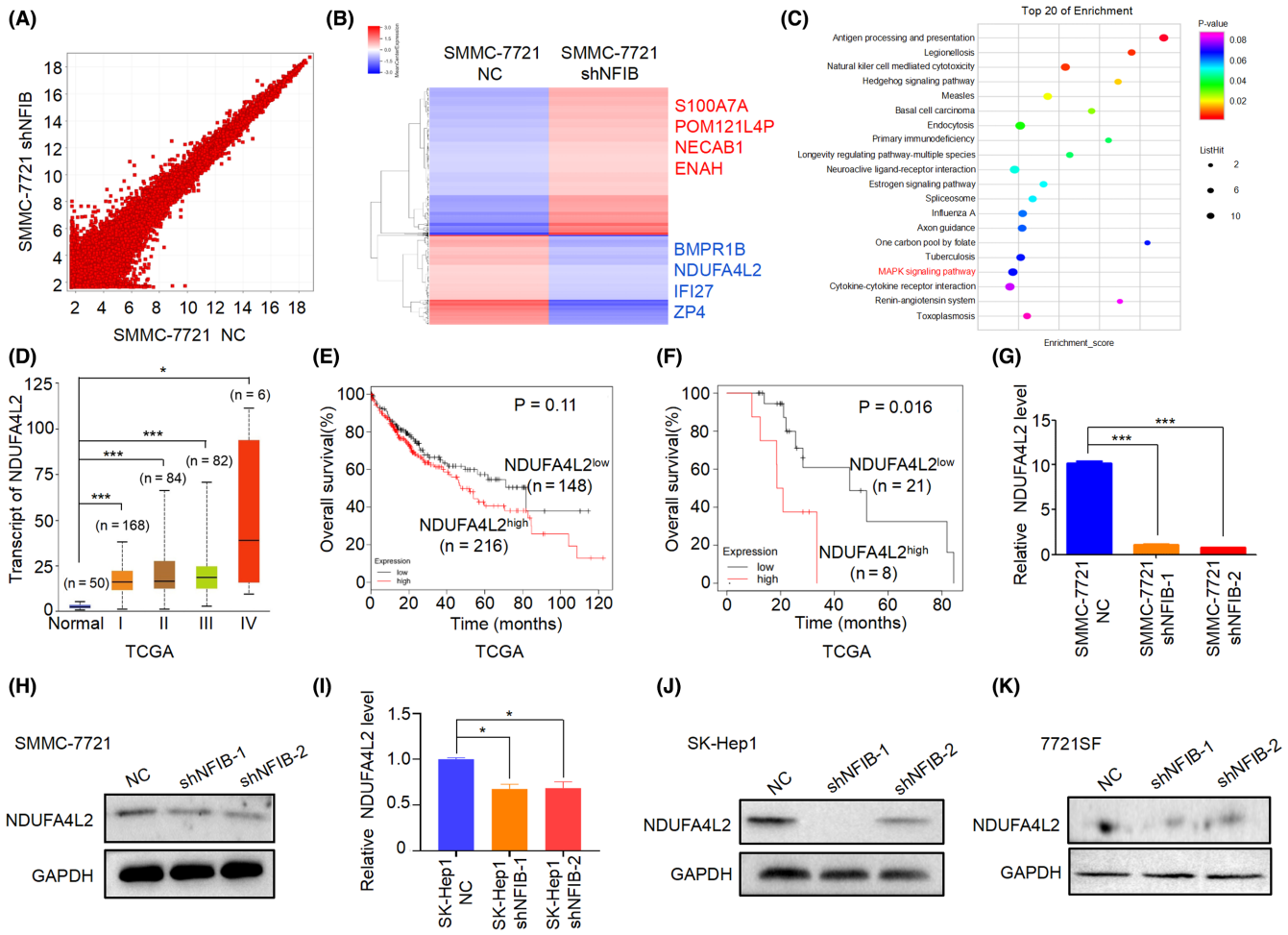

FIGURE 4 NFIB upregulates the complex I inhibitor NDUFA4L2 in HCC cells. (A) Scatter plot of the DEGs after knocking down NFIB; (B) The heatmap showing the significantly changed genes after knocking down NFIB. Upregulated genes are in red color, and downregulated genes are in blue color. (C) KEGG enrichment analysis results of the DEGs. (D) TCGA data showing that NDUFA4L2 expression level increases with the tumor stage. (E) NDUFA4L2 expression level is negatively associated with the overall survival time of HCC patients from the TCGA database. (F) The negative prognostic value of NDUFA4L2 in HCC patients with sorafenib treatment. Both the mRNA level (G–J) and protein abundance are decreased in NFIB-knockdown SMMC-7721 cells and SK-Hep1 cells. (K) The NDUFA4L2 protein abundance is reduced by NFIB knockdown in sorafenib-resistant cells. DEGs, differentially expressed genes. Scale bar: 200 μm. NC, normal control; $^{*}p < 0.05$; $^{***}p < 0.001$

inhibited the proliferation (Figure 2A,B), while overexpression of NFIB promoted cell proliferation (Figure 2C). Consistently, knockdown of NFIB inhibited the colony formation ability of HCC cells (Figure 2D and Figure S2A). In addition, knocking down NFIB significantly reduced the IC50 of HCC cells to sorafenib (Figure 2E and Figure S2C,D) and sorafenib-induced cell apoptosis (Figure 2F,G and Figure S2E). We further showed that knockdown of NFIB significantly inhibited cell invasion (Figure 2H,I and Figure S2B) and vice versa (Figure 2J). Altogether NFIB renders intrinsic resistance to sorafenib of HCC cells and also promotes proliferation and invasion.

3.3 | Knockdown of NFIB shows synergistic effect with sorafenib in vivo

We then examined the in vivo function of NFIB in HCC tumor growth and drug sensitivity. Subcutaneous transplantation showed that knockdown of NFIB also significantly decreased weight (Figure 3A,B). NFIB overexpression facilitated tumor growth (Figure 3C,D). More importantly, we found that knockdown of NFIB combined with sorafenib could further reduce tumor weight in vivo (Figure 3E,F). Further, a liver in situ injection experiment was used to evaluate the effect of NFIB on the growth and metastasis of HCC cells. The results showed that the number of tumors originating from NFIB-knockdown cells was significantly lower than that of control cells (Figure 3G–I). The metastatic nodules in the lung of mice injected with NFIB-knockdown cells were significantly fewer than that of the control group (Figure 3J). In summary, these in vivo results demonstrated that NFIB promoted sorafenib resistance, tumor growth, and metastasis of HCC cells.

3.4 | NFIB upregulates the Complex I inhibitor NDUFA4L2

To explore the mechanism by which NFIB promotes HCC progression, gene microarray profiling was applied to identify the differentially

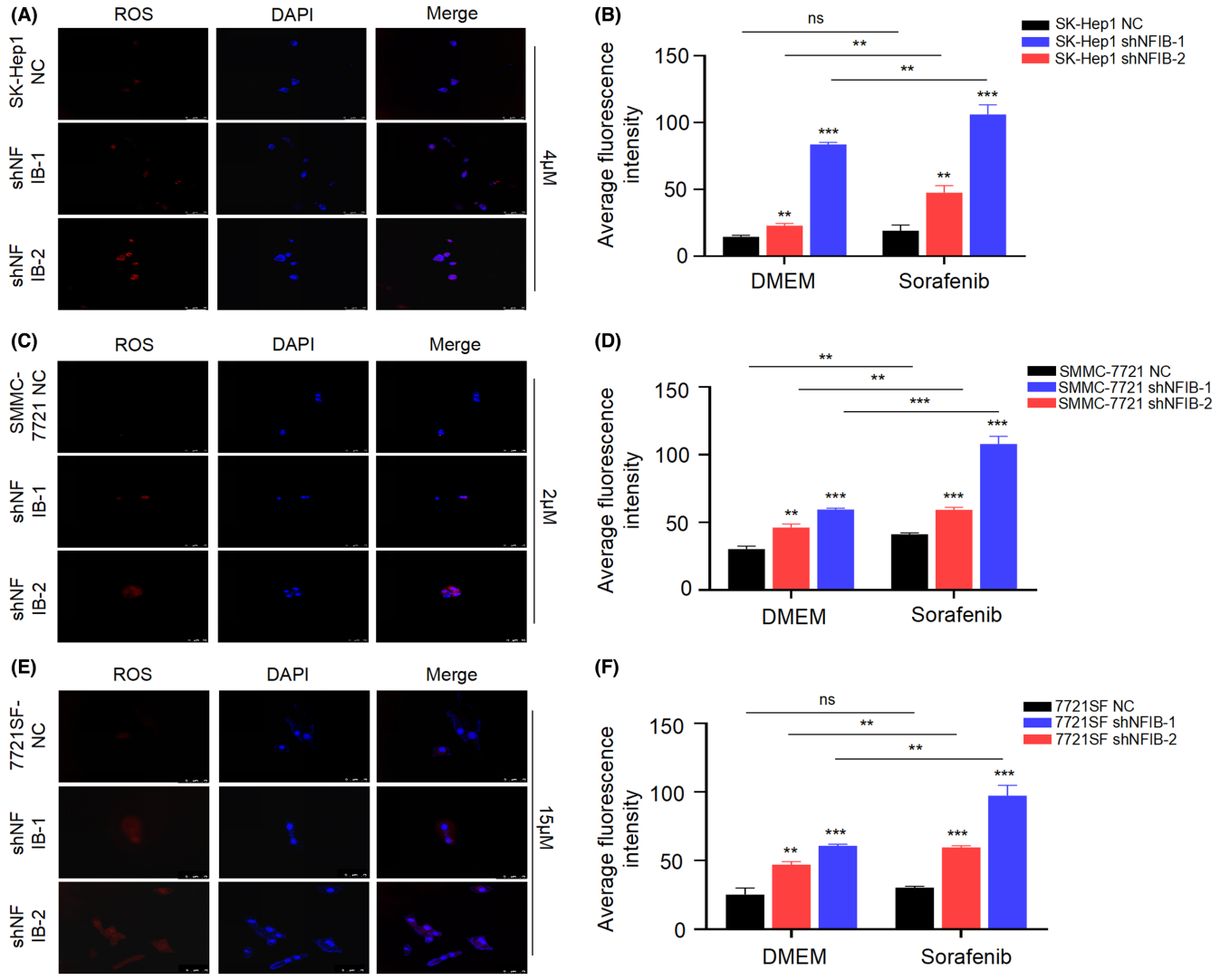

FIGURE 5 NFIB reduces reactive oxygen species (ROS) load in sorafenib-treated HCC cells. The intracellular ROS was determined in SMMC-7721 cells, SK-Hep1 cells and 7721SF cells. (A, B) Increased ROS after knocking down NFIB in SK-Hep1 cells, which is further increased in after sorafenib treatment. Similar results are found in SMMC-7721 cells (C, D) and 7721SF cells (E, F). 7721SF, sorafenib-resistant SMMC-7721 cells. NC, normal control; $^{**}p<0.01$; $^{***}p<0.001$

expressed genes after knocking down NFIB in SMMC-7721 cells. The results showed that 1819 genes were significantly upregulated and 1106 genes were significantly downregulated (Figure 4A,B). We found that the expression of NDUFA4L2 was significantly downregulated after NFIB knockdown. The differentially expressed genes (DEGs) are listed in Table S2. KEGG analysis showed that the differentially expressed genes were enriched in MAPK signaling pathways (Figure 4C). Therefore, the western blot confirmed that knockdown of NFIB dramatically reduced the phosphorylation of P38 (Figure S3A,B). A qRT-PCR experiment was used to verify the top 19 DEGs in SMMC-7721 cells, and NDUFA4L2 was the most significantly downregulated gene (Figure S3C). NDUFA4L2, an inhibitory component of Complex I in the electron transfer chain, reduces ROS production[17] and has been reported to promote cancer progression.[25] Bioinformatic analysis of the TCGA HCC data showed that the NDUFA4LA expression level was dramatically augmented, and its expression increased with cancer stage (Figure 4D). High NDUFA4L2 expression is associated with shorter overall survival time in HCC patients (Figure 4E). Interestingly, its high expression level also correlated with poor prognosis in HCC patients using sorafenib (Figure 4F). We further confirmed that the expression of NDUFA4L2 was reduced in both SMMC-7721 and SK-Hep1 cells after NFIB-knockdown (Figure 4G–J). Knockdown of NFIB could consistently result in reduced NDUFA4L2 expression in sorafenib-resistant SMMC-7721 cells (Figure 4K).

Reactive oxygen species assay experiments showed that the intracellular ROS levels were significantly increased after knocking down NFIB in SK-Hep1 (Figure 5A,B, Figure S4A), SMMC-7721 cells (Figure 5C,D, Figure S4B), and sorafenib-resistant SMMC-7721 cells (Figure 5E,F, Figure S4C), which was further elevated after sorafenib exposure (Figure 5 and Figure S4). These results revealed that NFIB mitigates the toxicity of sorafenib by upregulating NDUFA4L2 to reduce intracellular ROS accumulation. To further verify that NDUFA4L2 mediated the ROS production after depressing NFIB, we silenced NDUFA4L2 expression using small

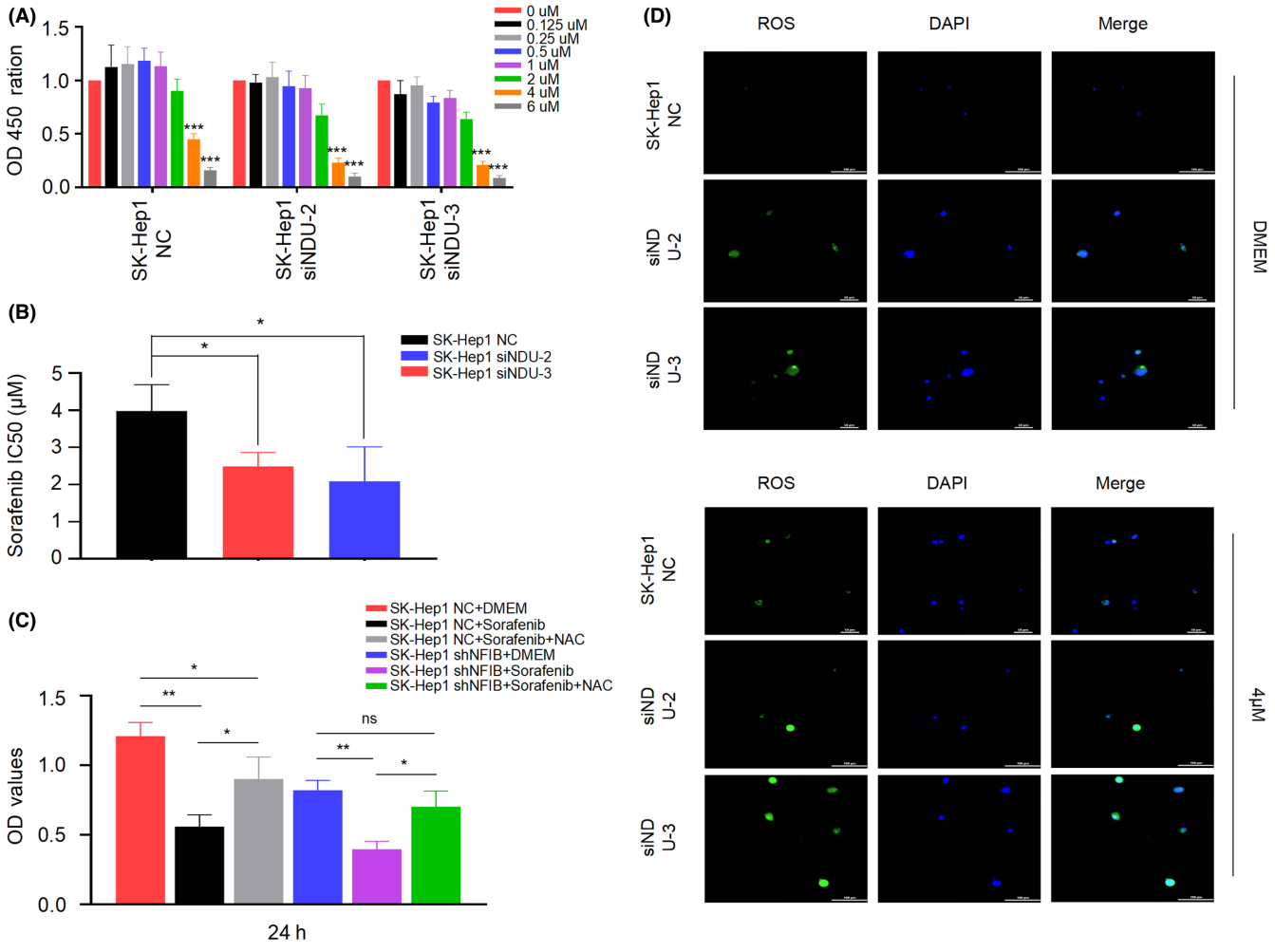

FIGURE 6 NDUFA4L2 increases the resistance of HCC cells to sorafenib by reducing the intracellular ROS level. (A) Knocking down NDUFA4L2 decreases cell viability after sorafenib treatment. (B) Knocking down NDUFA4L2 decreases the IC50 of sorafenib to SK-Hep1 cells. (C) reactive oxygen species (ROS) scavenger NAC (1:500) was added to detect intracellular ROS levels. (D) The intracellular ROS was determined in NDUFA4L2 knockdown SK-Hep1 cells. Increased ROS after knocking down NDUFA4L2 in SK-Hep1 cells, which is further increased in after sorafenib treatment. NAC, N-acetyl-L-cysteine; NC, normal control; shNFIB, knockdown NFIB groups. $^{**}p<0.01$; $^{***}p<0.001$

interfering RNA (siRNA) sequences (siNDU-1, siNDU-2, and siNDU-3) in SK-Hep1 cells. The knockdown efficiency of mRNA and protein levels was verified (Figure S5A,B). Subsequently, CCK-8 (Figure S5C), colony formation (Figure S5D), and apoptosis assays (Figure S5E) were used to reveal that the proliferation of SK-Hep1 cells was decreased and apoptosis was increased after NDUFA4L2 knockdown. Further, it was found that SK-Hep1 cells were more sensitive to sorafenib after knockdown of NDUFA4L2 (Figure 6A,B). We also found that the intracellular ROS accumulated after NDUFA4L2 knockdown, which was further increased after sorafenib treatment (Figure 6D). The above results are consistent with previous research.[18] To further verify that this effect was directly caused by ROS overloading, we showed that the cell viability of NFIB-knockdown HCC cells was partially restored after the addition of ROS scavenger N-acetyl-L-cysteine (NAC) (Figure 6C). In conclusion, these results indicated that NFIB promoted cell proliferation and resistance to sorafenib of HCC cells by upregulating NDUFA4L2 to avoid ROS overloading.

3.5 | NFIB promotes the transcription of NDUFA4L2

NFIB could act as a transcription factor to regulate gene transcription, such as p21 and PINK1.[6] ChIP-Seq experiment was used to determine the binding sites of NFIB protein in HCC cells. The results showed that the binding sites were scattered among the whole genome (Figure 7A). Most NFIB binding sites are in the distant intergenic (38.98%), intron (45.039%) and promoter regions (8.9%) (Figure 7B). The motif analysis revealed that NFIB can bind with different kinds of motifs, including the canonical NFIB binding sequencing (CAAAGTGC) (Figure 7C). KEGG enrichment analysis showed the NFIB binding genes were associated with metabolic pathways, cellular response to stress and others (Figure 7D). Interestingly, there is a binding site in the promoter region of NDUFA4L2 gene (Figure 7E). The dual-luciferase reporter gene experiment showed that NFIB could promote the transcription of NDUFA4L2 in both SMMC-7721 and SK-Hep1 cells (Figure 7F). Altogether, these results

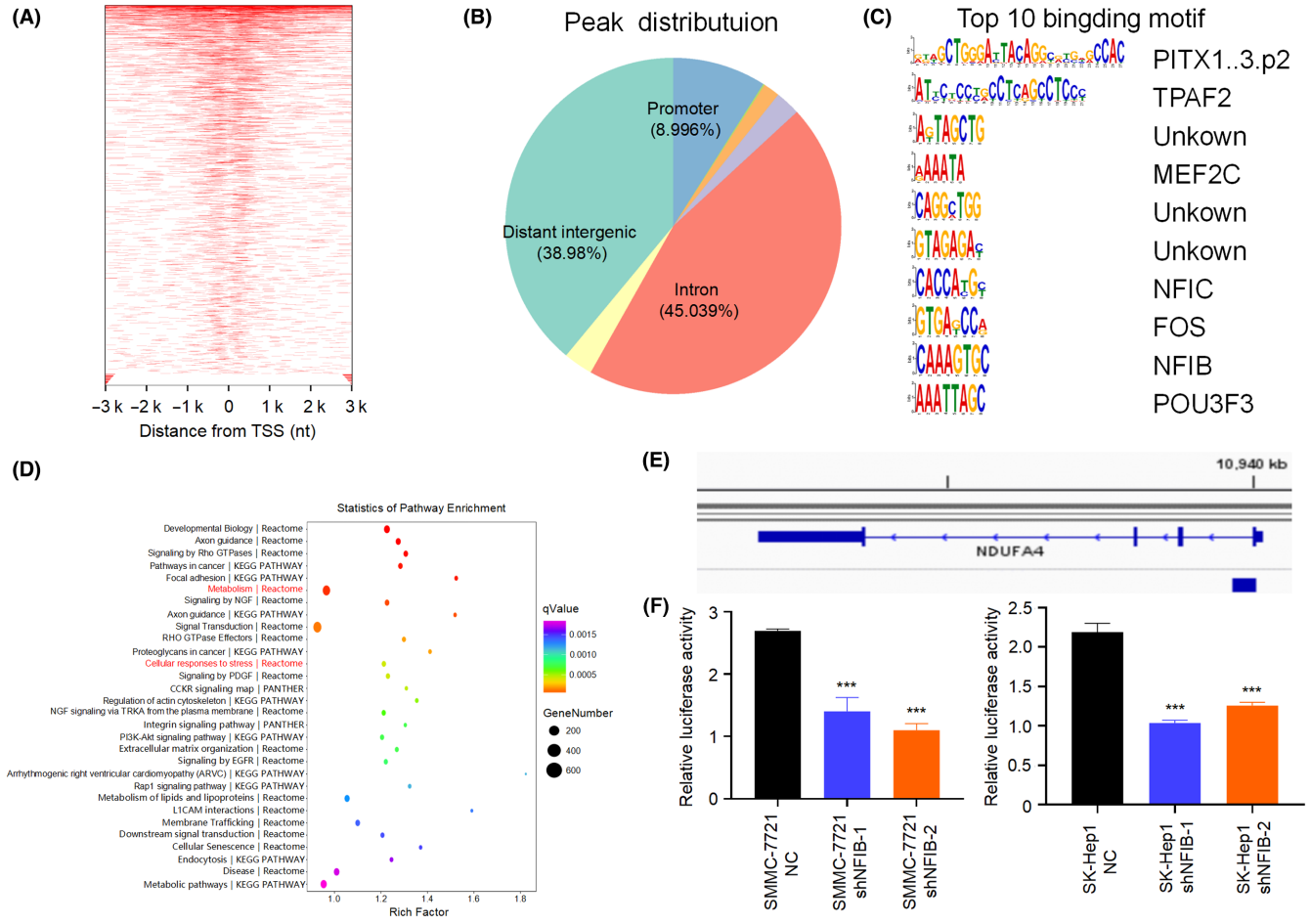

FIGURE 7 NFIB promotes the transcription of NDUFA4L2 in HCC cells. ChIP-Seq experiment was used to determine the binding sites of NFIB protein in HCC cells. (A) The results showed that the binding sites were scattered among the whole genome. (B) Most NFIB binding sites are in the distant intergenic (38.98%), intron (45.039%) and promoter regions (8.9%). (C) The motif analysis revealed that NFIB can bind with different kinds of motifs, including the canonical NFIB binding sequencing (CAAAGTGC). (D) KEGG enrichment analysis showed the NFIB binding genes were associated with metabolic pathways and cell response to stress. (E) There is a binding site in the promoter region of NDUFA4L2 gene. (F) The dual-luciferase reporter gene experiment showed that NFIB could promote the transcription of NDUFA4L2. NC, normal control; ***$p < 0.001$

demonstrated that NFIB could bind with the NDUFA4L2 promoter region to enhance its transcription.

3.6 | High NFIB expression correlates with macrovascular invasion, advanced stage, and poor prognosis of hepatocellular carcinoma patients

Finally, we aimed to elucidate the clinical significance of NFIB in HCC. We examined the expression of NFIB in HCC tissue using the public TCGA database and found that the NFIB mRNA level was increased in HCC tissues (Figure S6A). This result was confirmed in fresh HCC tissues from 38 patients, including 28 male and 10 female patients (Figure 8A, $p = 0.0154$). Then we detected the protein expression level of NFIB in HCC samples from 156 patients by immunohistochemical staining (Figure 8B). A χ^2 test revealed that positive NFIB expression significantly correlated with macrovascular invasion ($p = 0.014$) and advanced Barcelona Clinic Liver Cancer (BCLC) stage ($p = 0.001$) (Figure 8C,D, Table 1). In analyzing the follow-up information from 132 patients, high NFIB expression was also associated with shorter disease-free survival (Figure 8E) and overall survival (Figure 8F). Univariate Cox proportional hazards regression analysis revealed that the NFIB expression level was associated with a shorter overall survival time (Table S3). Accordingly, high NFIB expression correlated with shortened recurrence-free survival (Figure S6B) and progression-free survival (Figure S6C) using the public KMPlotter database. NFIB has prognostic value in hepatitis virus-related HCC patients (Figure S6D) and the macrovascular invasion-free subgroup (Figure S6E). These results indicate that NFIB is associated with progression and poor prognosis of HCC.

4 | DISCUSSION

Although great progress has been made in the diagnosis and treatment of HCC in the past few decades, the prognosis of HCC patients is still poor.[28] It is critical to better understand its

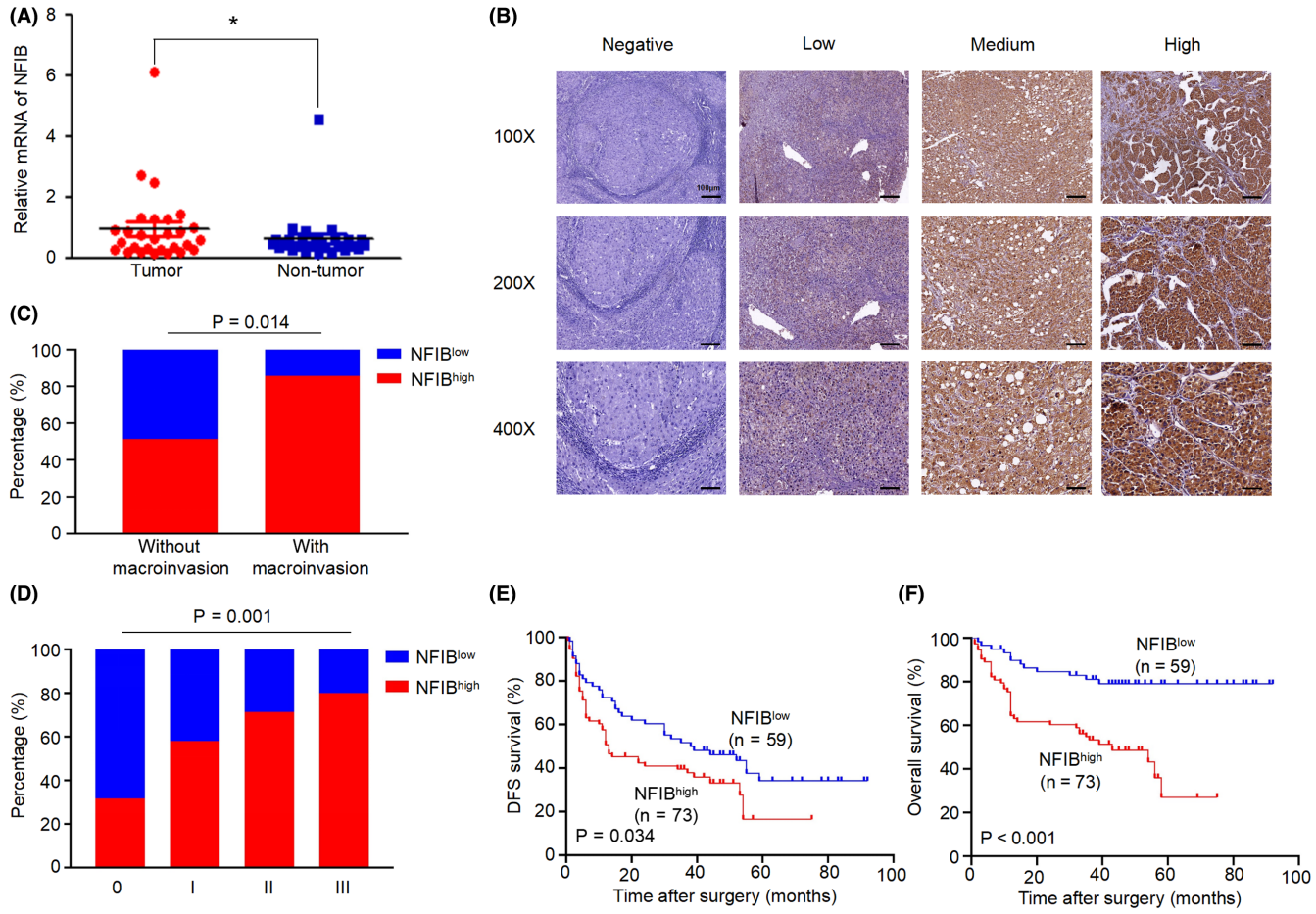

FIGURE 8 NFIB expression is associated with poor prognosis of hepatocellular carcinoma (HCC) patients. (A) The higher expression level of NFIB in HCC tissues in comparison with adjacent tissues ($n = 38$). (B) Representative immunohistochemistry images for NFIB in human HCC tissue, 85 cases for high expression, and 71 cases for low expression. (C) Expression of NFIB was significantly increased in HCC tissues with macrovascular invasion. (D) The high expression of NFIB increased with the increase in tumor stage. (E) NFIB expression level is significantly associated with short disease-free survival time. (F) NFIB expression level is significantly associated with short overall survival time. Scale bar: 100 μm. $^*p < 0.05$

TABLE 1 The correlation between NFIB expression and clinicopathological parameters

Features	Number of patients (%)	NFIB expression status		p
		Low ($n = 71$) No.patient (%)	High ($n = 85$) No.patient (%)	
Gender				
Male	134 (85.9%)	60 (44.8%)	74 (55.2%)	0.648
Female	22 (14.1%)	11 (50.0%)	11 (50.0%)	
Age				
≤52	84 (53.8%)	45 (53.6%)	39 (46.4%)	0.029
>52	72 (46.2%)	26 (36.1%)	46 (63.9%)	
Number of nodules				
1	141 (90.4%)	65 (46.1%)	76 (53.9%)	0.917
2	10 (6.4%)	4 (40.0%)	6 (60.0%)	
3	5 (3.2%)	2 (40.0%)	3 (60.0%)	
Tumor size (cm)				
≤3	73 (46.8%)	35 (47.9%)	38 (52.1%)	0.567
>3	83 (53.2%)	36 (43.4%)	47 (56.6%)	
Tumor size (cm)				
≤5	109 (69.9%)	47 (43.1%)	62 (56.9%)	0.361
>5	47 (30.1%)	24 (51.1%)	23 (48.9%)	
Macrovascular invasion				
0	142 (91.0%)	69 (48.6%)	73 (51.4%)	0.014
1	14 (9.0%)	2 (14.3%)	12 (85.7%)	
Distant metastasis				
0	147 (94.2%)	69 (46.9%)	78 (53.1%)	0.183
1	9 (5.8%)	2 (22.2%)	7 (77.8%)	
Child				
1	141 (90.4%)	66 (46.8%)	75 (53.2%)	0.319
2	15 (9.6%)	5 (33.3%)	10 (66.7%)	
BCLC stage				
0	41 (26.3%)	28 (68.3%)	13 (31.7%)	0.001
1	88 (56.4%)	37 (42.0%)	51 (58.0%)	
2	7 (4.5%)	2 (28.6%)	5 (71.4%)	
3	20 (12.8%)	4 (20.0%)	16 (80.0%)	
Differentiation degree				
Poor	23 (14.7%)	13 (56.5%)	10 (43.5%)	0.261
Moderate	107 (68.6%)	44 (41.1%)	63 (58.9%)	
Well	26 (16.7%)	14 (53.8%)	12 (46.2%)	
Microvascular invasion				
Negative	152 (97.4%)	70 (46.1%)	82 (53.9%)	0.626
Positive	4 (2.6%)	1 (25.0%)	3 (75.0%)	
Ki67 20%				
Low	101 (64.7%)	47 (46.5%)	54 (53.5%)	0.728
High	55 (35.3%)	24 (43.6%)	31 (56.4%)	
ECOG PS score				
0	122 (78.2%)	56 (45.9%)	66 (54.1%)	0.895
1	27 (17.3%)	13 (48.1%)	14 (51.9%)	
2	6 (3.8%)	2 (33.3%)	4 (66.7%)	
3	1 (0.6%)	0 (0.0%)	1 (100.0%)	

pathological and molecular mechanismsand provide novel targets for the diagnosis and treatment of HCC. Sorafenib targets multiple tyrosine kinases, including RAF, VEGFR, and PDGFR, to suppress their downstream proliferation and survival signaling pathways.[29] However, the clinical efficacy of Sorafenib treatment in HCC is modest, and it can only extend patients' median overall survival by 3 months.[30] The existence of intrinsic or acquired drug resistance is considered a major obstacle contributing to the failure of sorafenib treatment in HCC patients.[31,32] Previous studies have shown that sorafenib treatment restrained tumor growth partly through suppression of tumor angiogenesis. However, sorafenib treatment can lead to the activation of HIF-1α or HIF-2α in cancer cells, which in turn induce the expression of VEGF and other proangiogenic factors to confer HCC resistance to sorafenib treatment.[33,34] The major mechanism of sorafenib-mediated antiproliferative action is through downregulation of the RAF/MEK/ERK pathway. For example, downregulating EGFR expression or inhibiting EGFR kinase activity can increase the sensitivity of cells to sorafenib.[35] The expression of c-Jun increased in HCC cells treated with sorafenib, and inhibition of c-Jun expression could promote the sensitivity of HCC cells to sorafenib.[36] $CD133^+$ HCC stem cells are resistant to sorafenib by preferentially activating Akt and Bcl-2.[37] Decreasing CD44 can also increase the sensitivity of HCC cells to sorafenib.[38] Recent studies demonstrated that deregulated NFIB is associated with the clinicopathological features of some tumors.[9] NFIB promotes cell proliferation, apoptosis, and autophagy of HCC through regulating gene expression and cancer-related signaling pathways.[11] One recent study showed that NFIB reduces apoptosis of hepatocytes and promotes their regeneration,[7] and our previous study found that NFIB can affect the urea cycle to prevent the occurrence of HCC.[7] Our present study found that NFIB can inhibit the proliferation and invasion of sorafenib-resistant HCC cells and increase their resistance to sorafenib. It should be noted that NFIB not only promoted growth and invasion but also reduced the intrinsic and acquired sensitivity to sorafenib of HCC cells in vitro and in vivo. Moreover, we demonstrated that positive NFIB was associated with macrovascular invasion and BCLC stage.

Reactive oxygen species is a general term for unstable, reactive, and partially reduced oxygen derivatives.[39] The production of ROS in a variety of cancers has been proven to be a "double-edged sword." Low levels of ROS can promote the survival and metastasis of cancer cells, while excessive ROS accumulation inhibits cancer cells by inducing G2/M cell cycle arrest and apoptosis to inhibit the growth of cells.[40,41] Sorafenib can induce excessive accumulation of ROS in HCC cells, leading to the release of cytochrome C from mitochondria into the cytoplasm, triggering programmed cell death and thereby killing HCC cells.[40,42] ROS scavengers can reduce the killing effect of sorafenib on HCC cells,[43,44] which shows that sorafenib is at least partly dependent on ROS production to kill HCC cells. We found that NFIB could reduce the accumulation of ROS in HCC cells caused by sorafenib. Conversely, knockdown of NFIB could increase ROS accumulation. Therefore, we concluded that NFIB may reduce the sensitivity of HCC cells to sorafenib by reducing the excessive accumulation of ROS, but the mechanism of NFIB regulating ROS needs to be further explored.

Nuclear factor I B can directly or indirectly regulate gene transcription. Studies have shown that NFIB can directly promote the transcription of EZH2,[45] PINK1[6], RIP2,[46] and ERO1A[47] or inhibit the transcription of p21,[12] CDK6, and CDK4.[48] However, it can also regulate gene expression by regulating chromatin accessibility.[49] One latest study showed that NFIB could regulate circular RNA circMAP7D1 expression in gastric cancer.[50] In the present study, we found that NFIB can bind to the promoter region of NDUFA4L2 and promote its transcription. NDUFA4L2 is an inhibitory component of Complex I, which is the main source

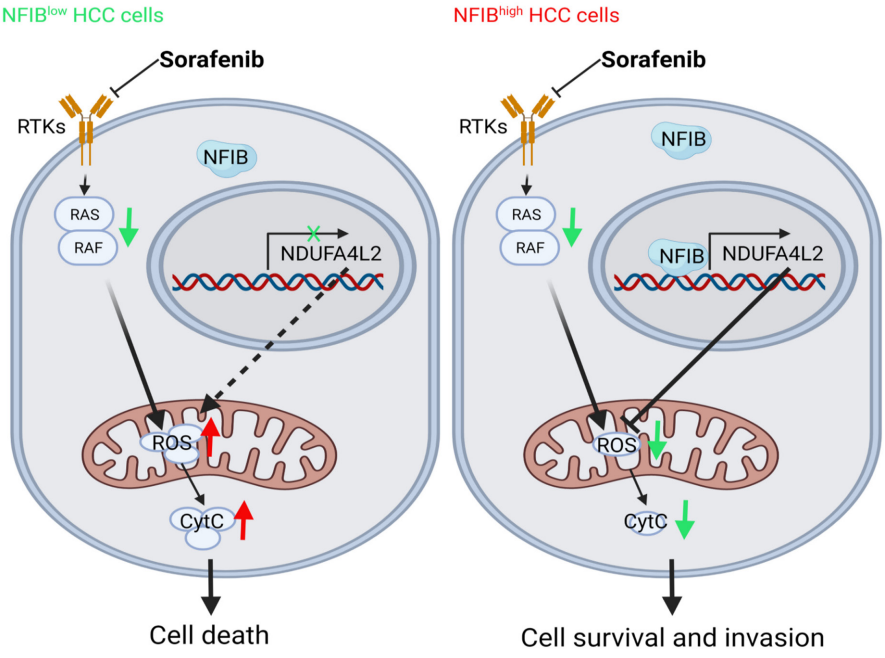

FIGURE 9 Schematic diagram of the NFIB/NDUFA4L2/ROS pathway in regulation of sorafenib-resistant HCC survival and invasion. High expression of NFIB in HCC cells promotes the transcription of NDUFA4L2 to prevent sorafenib-caused excessive ROS accumulation, thus augmenting cell viability and invasion.

of intracellular ROS. HIF1α is currently the only transcriptional regulator of NDUFA4L2,[17,50] and our result would enlarge its regulatory mechanism.

In conclusion, we revealed that NFIB induces the resistance of HCC cells to sorafenib by promoting the transcription of NDUFA4L2 to decrease the ROS level (Figure 9). High NFIB expression correlates with advanced tumor stage and poor prognosis. These results suggest that NFIB might be a novel target for the diagnosis and treatment of HCC.

AUTHOR CONTRIBUTIONS

Conception and design: Zhi-Hang Zhou and Song He. Acquisition of data: Xia Li, Qing-Liang Wang, Tao Ran, and Zhi-Ji Chen. Analysis and interpretation of data: Li Zhou, Lin-Hong Mao, Si-Yuan Chen, Jing Lei, Hong-Tao Liu, and Si-Qi Liao. Data curation: Li Zhou, Lin-Hong Mao, and Zhi-Hang Zhou. Writing original draft: Li Zhou, Lin-Hong Mao, and Zhi-Hang Zhou.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ETHICAL APPROVAL

Approval of the research protocol by an Institutional Review Board of Chongqing Medical University (No. 2019–133).

INFORMED CONSENT

All informed consent was obtained from the subject(s) and/or guardian(s).

ANIMAL STUDIES

The use and care of animals were approved by the Institutional Animal Care and Use Committee at Chongqing Medical University.

ORCID

Zhi-Hang Zhou https://orcid.org/0000-0003-1356-9872

REFERENCES

1. Villanueva A. Hepatocellular Carcinoma. *N Engl J Med*. 2019;380:1450-1462.
2. Llovet JM, Kelley RK, Villanueva A, et al. Hepatocellular carcinoma. *Nat Rev Dis Primers*. 2021;7:6.
3. Su GL, Altayar O, O'Shea R, et al. AGA clinical practice guideline on systemic therapy for hepatocellular carcinoma. *Gastroenterology*. 2022;162:920-934.
4. Niu L, Liu L, Yang S, Ren J, Lai PBS, Chen GG. New insights into sorafenib resistance in hepatocellular carcinoma: responsible mechanisms and promising strategies. *Biochim Biophys Acta Rev Cancer*. 2017;1868:564-570.
5. Tang W, Chen Z, Zhang W, et al. The mechanisms of sorafenib resistance in hepatocellular carcinoma: theoretical basis and therapeutic aspects. *Signal Transduct Target Ther*. 2020;5:87.
6. Wang N, Yuan J, Liu F, et al. NFIB promotes the migration and progression of kidney renal clear cell carcinoma by regulating PINK1 transcription. *PeerJ*. 2021;9:e10848.
7. Roy S, Bantel H, Wandrer F, et al. miR-1224 inhibits cell proliferation in acute liver failure by targeting the antiapoptotic gene Nfib. *J Hepatol*. 2017;67:966-978.
8. Adam RC, Yang H, Ge Y, et al. NFI transcription factors provide chromatin access to maintain stem cell identity while preventing unintended lineage fate choices. *Nat Cell Biol*. 2020;22:640-650.
9. Becker-Santos DD, Lonergan KM, Gronostajski RM, Lam WL. Nuclear factor I/B: a master regulator of cell differentiation with paradoxical roles in cancer. *EBioMedicine*. 2017;22:2-9.
10. Tang T, Zeng F. NFIB-mediated lncRNA PVT1 aggravates laryngeal squamous cell carcinoma progression via the miR-1301-3p/MBNL1 Axis. *J Immunol Res*. 2021;2021:8675123-8675117.
11. Zhang Q, Cao LY, Cheng SJ, Zhang AM, Jin XS, Li Y. p53-induced microRNA-1246 inhibits the cell growth of human hepatocellular carcinoma cells by targeting NFIB. *Oncol Rep*. 2015;33:1335-1341.
12. Liu RZ, Vo TM, Jain S, et al. NFIB promotes cell survival by directly suppressing p21 transcription in TP53-mutated triple-negative breast cancer. *J Pathol*. 2019;247:186-198.
13. Ying H, Zhao R, Yu Q, Zhang K, Deng Q. CircATL2 enhances paclitaxel resistance of ovarian cancer via impacting miR-506-3p/NFIB axis. *Drug Dev Res*. 2022;83:512-524.
14. Kashiwagi E, Izumi H, Yasuniwa Y, et al. Enhanced expression of nuclear factor I/B in oxaliplatin-resistant human cancer cell lines. *Cancer Sci*. 2011;102:382-386.
15. Liu Z, Chen J, Yuan W, et al. Nuclear factor I/B promotes colorectal cancer cell proliferation, epithelial-mesenchymal transition and 5-fluorouracil resistance. *Cancer Sci*. 2019;110:86-98.
16. Zhou L, Wang QL, Mao LH, et al. Hepatocyte-specific Knock-out of Nfib aggravates hepatocellular tumorigenesis via enhancing urea cycle. *Front Mol Biosci*. 2022;9:875324.
17. Tello D, Balsa E, Acosta-Iborra B, et al. Induction of the mitochondrial NDUFA4L2 protein by HIF-1alpha decreases oxygen consumption by inhibiting complex I activity. *Cell Metab*. 2011;14:768-779.
18. Lai RK, Xu IM, Chiu DK, et al. NDUFA4L2 fine-tunes oxidative stress in hepatocellular carcinoma. *Clin Cancer Res*. 2016;22:3105-3117.
19. Jiang L, Yin X, Chen YH, et al. Proteomic analysis reveals ginsenoside Rb1 attenuates myocardial ischemia/reperfusion injury through inhibiting ROS production from mitochondrial complex I. *Theranostics*. 2021;11:1703-1720.
20. Meng L, Yang X, Xie X, Wang M. Mitochondrial NDUFA4L2 protein promotes the vitality of lung cancer cells by repressing oxidative stress. *Thorac Cancer*. 2019;10:676-685.
21. Andreas K, Haupl T, Lubke C, et al. Antirheumatic drug response signatures in human chondrocytes: potential molecular targets to stimulate cartilage regeneration. *Arthritis Res Ther*. 2009;11:R15.
22. Minton DR, Fu L, Mongan NP, Shevchuk MM, Nanus DM, Gudas LJ. Role of NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 in clear cell renal cell carcinoma. *Clin Cancer Res*. 2016;22:2791-2801.
23. Lv Y, Nie SL, Zhou JM, et al. Overexpression of NDUFA4L2 is associated with poor prognosis in patients with colorectal cancer. *ANZ J Surg*. 2017;87:E251-E255.
24. Sarathi A, Palaniappan A. Novel significant stage-specific differentially expressed genes in hepatocellular carcinoma. *BMC Cancer*. 2019;19:663.
25. Chen Z, Wei X, Wang X, et al. NDUFA4L2 promotes glioblastoma progression, is associated with poor survival, and can be effectively targeted by apatinib. *Cell Death Dis*. 2021;12:377.

26. Yuan Y, Gao H, Zhuang Y, et al. NDUFA4L2 promotes trastuzumab resistance in HER2-positive breast cancer. *Ther Adv Med Oncol*. 2021;13:17588359211027836.
27. Xu WN, Yang RZ, Zheng HL, Jiang LS, Jiang SD. NDUFA4L2 regulated by HIF-1alpha promotes metastasis and epithelial-mesenchymal transition of osteosarcoma cells through inhibiting ROS production. *Front Cell Dev Biol*. 2020;8:515051.
28. Yan T, Yu L, Zhang N, et al. The advanced development of molecular targeted therapy for hepatocellular carcinoma. *Cancer Biol Med*. 2022;19:1-16.
29. Liu L, Cao Y, Chen C, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res*. 2006;66:11851-11858.
30. Bruix J, Raoul JL, Sherman M, et al. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *J Hepatol*. 2012;57:821-829.
31. Chen X, Yang G, Guo X, et al. DJ-1/FGFR-1 signaling pathway contributes to sorafenib resistance in hepatocellular carcinoma. *Oxid Med Cell Longev*. 2022;2022:2543220.
32. Li L, Yu S, Chen J, Quan M, Gao Y, Li Y. miR-15a and miR-20b sensitize hepatocellular carcinoma cells to sorafenib through repressing CDC37L1 and consequent PPIA downregulation. *Cell Death Discov*. 2022;8:297.
33. Liang Y, Zheng T, Song R, et al. Hypoxia-mediated sorafenib resistance can be overcome by EF24 through Von Hippel-Lindau tumor suppressor-dependent HIF-1alpha inhibition in hepatocellular carcinoma. *Hepatology*. 2013;57:1847-1857.
34. Zhao D, Zhai B, He C, et al. Upregulation of HIF-2alpha induced by sorafenib contributes to the resistance by activating the TGF-alpha/EGFR pathway in hepatocellular carcinoma cells. *Cell Signal*. 2014;26:1030-1039.
35. Ezzoukhry Z, Louandre C, Trecherel E, et al. EGFR activation is a potential determinant of primary resistance of hepatocellular carcinoma cells to sorafenib. *Int J Cancer*. 2012;131:2961-2969.
36. Chen W, Xiao W, Zhang K, et al. Activation of c-Jun predicts a poor response to sorafenib in hepatocellular carcinoma: preliminary clinical evidence. *Sci Rep*. 2016;6:22976.
37. Kim BH, Park JW, Kim JS, Lee SK, Hong EK. Stem cell markers predict the response to sorafenib in patients with hepatocellular carcinoma. *Gut Liver*. 2019;13:342-348.
38. Fernando J, Malfettone A, Cepeda EB, et al. A mesenchymal-like phenotype and expression of CD44 predict lack of apoptotic response to sorafenib in liver tumor cells. *Int J Cancer*. 2015;136:E161-E172.
39. Yang H, Villani RM, Wang H, et al. The role of cellular reactive oxygen species in cancer chemotherapy. *J Exp Clin Cancer Res*. 2018;37:266.
40. Tuy K, Rickenbacker L, Hjelmeland AB. Reactive oxygen species produced by altered tumor metabolism impacts cancer stem cell maintenance. *Redox Biol*. 2021;44:101953.
41. Zhou X, Xiao Q, Fu D, et al. Efficacy of rigosertib, a small molecular RAS signaling disrupter for the treatment of KRAS-mutant colorectal cancer. *Cancer Biol Med*. 2021;18:213-228.
42. Vishnoi K, Ke R, Viswakarma N, et al. Ets1 mediates sorafenib resistance by regulating mitochondrial ROS pathway in hepatocellular carcinoma. *Cell Death Dis*. 2022;13:581.
43. Coriat R, Nicco C, Chereau C, et al. Sorafenib-induced hepatocellular carcinoma cell death depends on reactive oxygen species production in vitro and in vivo. *Mol Cancer Ther*. 2012;11:2284-2293.
44. Gao L, Wang X, Tang Y, Huang S, Hu CA, Teng Y. FGF19/FGFR4 signaling contributes to the resistance of hepatocellular carcinoma to sorafenib. *J Exp Clin Cancer Res*. 2017;36:8.
45. Fane ME, Chhabra Y, Hollingsworth DEJ, et al. NFIB mediates BRN2 driven melanoma cell migration and invasion through regulation of EZH2 and MITF. *EBioMedicine*. 2017;16:63-75.
46. Sun H, Li N, Tan J, et al. Transcriptional regulation of RIP2 gene by NFIB is associated with cellular immune and inflammatory response to APEC infection. *Int J Mol Sci*. 2022;23:3814.
47. Zilli F, Marques Ramos P, Auf der Maur P, et al. The NFIB-ERO1A axis promotes breast cancer metastatic colonization of disseminated tumour cells. *EMBO Mol Med*. 2021;13:e13162.
48. Zhou L, Wang Y, Ou C, et al. microRNA-365-targeted nuclear factor I/B transcriptionally represses cyclin-dependent kinase 6 and 4 to inhibit the progression of cutaneous squamous cell carcinoma. *Int J Biochem Cell Biol*. 2015;65:182-191.
49. Denny SK, Yang D, Chuang CH, et al. Nfib promotes metastasis through a widespread increase in chromatin accessibility. *Cell*. 2016;166:328-342.
50. Yang H, Wu Z, Liu X, Chen M, Zhang X, Jiang Y. NFIB promotes the progression of gastric cancer by upregulating circMAP7D1 to stabilize HER2 mRNA. *Mol Med Rep*. 2021;23:269.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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